The final publication is available at

https://doi.org/10.1016/j.dyepig.2018.08.024

Additional Information

Photochemical formation of a fluorescent thymidine-pterin adduct in DNA

Sandra Estébanez,[a] Carolina Lorente,[a] Maira Gaspar Tosato,[a] Miguel A. Miranda,[b] M. Luisa Marín,[b] Virginie Lhiaubet-Vallet,[b,*] Andrés H. Thomas[a,*]

[a] Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), CCT La Plata-CONICET. Diagonal 113 y 64, (1900) La Plata, Argentina.

[b] Instituto Universitario Mixto de Tecnología Química (UPV-CSIC), Universitat Politécnica de Valencia, Consejo Superior de Investigaciones Científicas, Avenida de los Naranjos, s/n, 46022, Valencia (Spain).

*Corresponding authors

Dr. Andrés H. Thomas
E-mail: athomas@inifta.unlp.edu.ar
Postal Address: C. C. 16, Sucursal 4, (B1904DPI), La Plata, Argentina
Phone: +54 221 4257430 int.189
Fax: +54 221 425464

Dr. Virginie Lhiaubet-Vallet
E-mail: lvirgini@itq.upv.es
Postal Address: Instituto de Tecnología Química (UPV-CSIC), Avda de los Naranjos, s/n, 46022, Valencia, Spain
Phone: 963877815, ext 77815
Fax: 963873409

ABSTRACT

The photochemistry of the DNA biomacromolecule is an issue of paramount importance as it is part of the etiology of skin cancer development, being ultraviolet sunlight radiation the most relevant environmental carcinogen. Herein, we demonstrate the potential of pterin, an endogenous compound, to form covalent adduct under UVA irradiation with a short thymine oligomer as well as with the whole DNA polymer. Our approach is based on the spectroscopic features of pterin, which allow, by monitoring specific absorption or emission wavelengths, the following-up of the covalent binding. The results are confirmed by HPLC coupled with mass spectrometry, revealing the attachment of one or two pterin units to the homothymine 5-mer oligonucleotide. Altogether the findings point toward the role of pterin as endogenous sensitizer and genotoxic compound.
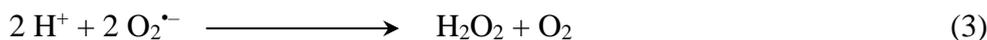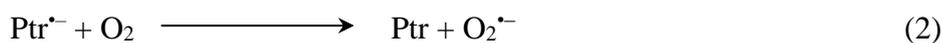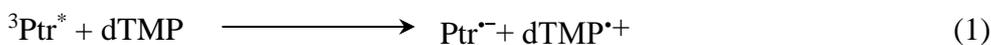
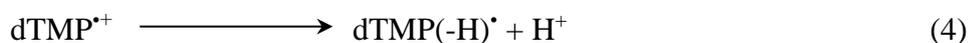Keywords: pterins, thymine, photosensitization

Introduction

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[1] and participates in the regulation of melanin biosynthesis,[2] the main pigment of skin.[3] Notably, in vitiligo[4] the H_4Bip metabolism is altered[5] producing aromatic pterin accumulation in the affected tissues, which is important given that this disease induces a depigmentation due to the lack of the melanin pigment. Thus, protection of UV fails while concentration of oxidized pterin, an established photosensitizer, increases. Indeed, it has been reported that under UV-A irradiation pterins are not only able to generate reactive oxygen species (ROS) such as $^1O_2$,[6,7,8] but they are also responsible for DNA damage formation.[9,10] Pterin (Ptr), the parent unsubstituted compound of oxidized pterins (Figure 1), and the vitiligo-related pterin derivatives (biopterin, formylpterin and carboxypterin) are efficient photosensitizers inducing the degradation of purine nucleotides.[11,12,13,14,15] In neutral and acidic media, the predominant mechanism involves an initial electron transfer (type I mechanism)[16] from the nucleotide to the triplet excited state of pterins. All the nucleobases may undergo one-electron oxidation, according to their corresponding ionization potentials that follow the order: guanine < adenine < cytosine ~ thymine (Thy).[17,18,19] It is accepted that most type I photosensitizers produce damage only at guanine base.[20]

Interestingly, Ptr is able to photoinduce the oxidation of Thy in the nucleotide thymidine 5'-monophosphate (dTMP, Figure 1) and the products formed depend on the presence of O_2 in the media.[21] After excitation of Ptr and formation of its triplet excited state ($^3Ptr^*$), the process is initiated with an electron transfer reaction from dTMP to $^3Ptr^*$, which leads to the formation of the Ptr radical anion ($Ptr^{\bullet-}$) and the dTMP radical cation

(dTMP$^{\bullet+}$) (Reaction 1). Electron transfer from Ptr$^{\bullet-}$ or its protonated form (PtrH$^{\bullet}$) to O$_2$ regenerates Ptr and yields O$_2^{\bullet-}$/HO$_2^{\bullet}$ (Reaction 2), which in turn disproportionates to H$_2$O$_2$ (Reaction 3).[22]

$$^3\text{Ptr}^* + \text{dTMP} \longrightarrow \text{Ptr}^{\bullet-} + \text{dTMP}^{\bullet+} \qquad (1)$$

$$\text{Ptr}^{\bullet-} + \text{O}_2 \longrightarrow \text{Ptr} + \text{O}_2^{\bullet-} \qquad (2)$$

$$2\,\text{H}^+ + 2\,\text{O}_2^{\bullet-} \longrightarrow \text{H}_2\text{O}_2 + \text{O}_2 \qquad (3)$$

In air equilibrated solutions, the oxidation products of dTMP formed by photosensitization with Ptr can be explained taking into account reactions of dTMP$^{\bullet+}$ generated using other photosensitizers.[23,24,25] The two main competitive reactions of dTMP$^{\bullet+}$ are deprotonation (Reaction 4) and hydration (Reaction 5). The former pathway leads mainly to the (5-uracilyl)-methyl radical, which traps oxygen on the methylene group and yields 5-formyl-2'-deoxyuridine 5'-monophosphate (5-FordUMP) and 5-(hydroxymethyl)-2'-uridine 5'-monophosphate (5-HmdUMP).[21,26,27] Hydration, in turn, leads predominantly to the 6-hydroxy-5,6-dihydrothymin-5-yl radical that traps oxygen on position 5 [23,28] to yield 1-(2-deoxy-beta-D-erythro-pentofuranosyl-5-phos-phate)-5-hydroxy-5-methylhydantoin (5-HO-5MHMP) and thymidine glycol 5'-monophosphate (dTMPGly).[21,23,29]

$$\text{dTMP}^{\bullet+} \longrightarrow \text{dTMP(-H)}^{\bullet} + \text{H}^+ \qquad (4)$$

$$\text{dTMP}^{\bullet+} + \text{HO}^- \longrightarrow \text{dTMP(OH)}^{\bullet} \qquad (5)$$

On the other hand, UV-A irradiation of dTMP in the presence of Ptr under anaerobic conditions shows a completely different behavior, not observed for other photosensitizers. In this case, coupling of the PtrH$^{\bullet}$ and dTMP(-H)$^{\bullet}$ radicals takes place yielding an adduct (Ptr-dTMP)(-2H) (Reaction 6), and this pathway competes with back

electron transfer to regenerate Ptr and dTMP (Reaction 7).[21] This reaction also occurs using 2'deoxythymidine (dT) as a substrate.[30] In both cases, the photogenerated adduct has the intact pterin moiety and retains its photophysical properties.[21,30]

$$Ptr^{•-}/PtrH^{•} + dTMP^{•+}/dTMP(-H)^{•} \longrightarrow \longrightarrow (Ptr-dTMP)(-2H) \qquad (6)$$

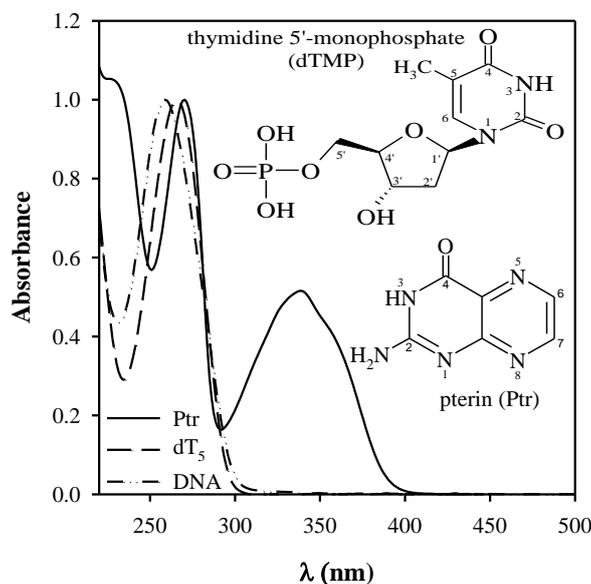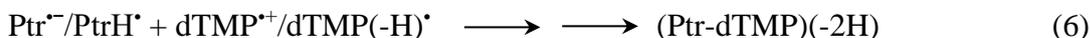$$Ptr^{•-}/PtrH^{•} + dTMP^{•+}/dTMP(-H)^{•} \longrightarrow \longrightarrow Ptr + dTMP \qquad (7)$$

Figure 1. Normalized absorption spectra of Ptr (pH 5.5), dT$_5$ (pH 5.5) and DNA (pH 6.5) in air-equilibrated aqueous solutions. Molecular structure of Ptr and dTMP.

The main objective of this work is to find out if Ptr is able to photodamage Thy when the nucleotide is part of the DNA polymer. Specifically, the attention is centered on demonstrating if formation of the adduct does also take place, and if so on comparing the efficiency of the process under different experimental conditions and the properties of the obtained photoproducts. Thus, we investigated first the photoreactivity undergone by a single stranded oligonucleotide with sequence 5´-d(TTTTT)-3´ (dT$_5$) in the presence of Ptr under UV-A radiation, and then, that taking place in the presence of the whole biomacromolecule, i.e. eukaryotic DNA. Under these conditions, the only light absorbing

species was Ptr since the nucleobases, which are the chromophores of DNA, do not absorb radiation in the UV-A region (Figure 1).

2. MATERIALS AND METHODS

2.1. General

Pterin (Ptr) was purchased from Schircks Laboratories (Jona, Switzerland) and used without further purification. DNA (type I, from calf thymus), single stranded oligonucleotide 5´-d(TTTTT)-3´ (dT$_5$), tris(hydroxymethyl)aminomethane (TRIS), ammonium acetate (NH$_4$Ac), 2´-deoxyguanosine 5´-monophosphate (dGMP), 2´-deoxyadenosine 5´-monophosphate (dAMP), 2´-deoxycytidine 5´-monophosphate (dCMP) and other chemicals were provided by Sigma-Aldrich and used without further purification. Sephadex G-25 columns were purchased from GE Healthcare and Acetonitrile (ACN) from J. T, Baker. 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) or pH-meter Mettler Toledo Seven Easy. The pH of the aqueous solutions was adjusted by adding very small aliquots (a few µL) of concentrated (0.1 - 2M) HCl or NaOH solutions using a micropipette.

2.2. Steady-state irradiation

Samples were irradiated in 1x1 cm fluorescence quartz cells at room temperature. Two different irradiation systems were employed. In system I, the sample was irradiated with a Rayonet RPR 3500 lamps (Southern N.E.Ultraviolet Co.) with emission centered at 350 nm [bandwidth (full width at half-maximum) of ∼20 nm]. System II consists in a

multilamp photoreactor equipped with 12 lamps (Osram Sylvania, F15T8/BLB) emitting from 310 to 410 nm with a maximal output (1 mW/cm^2) at *ca.* 360 nm.

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. Saturated Argon or Nitrogen solutions were obtained by bubbling for 20 min with these gases (Linde, purity 99.998%), previously saturated in water.

2.3. Analysis of irradiated solutions

2.3.1. UV–vis spectrophotometry

Electronic absorption spectra were recorded on a Shimadzu UV-1800 or Cary 50 (Varian) spectrophotometer, using quartz cells of 1 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 (HPLC)

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. In some cases, for further analysis, the products were isolated from HPLC runs (preparative HPLC), by collecting the mobile phase after passing through the detectors.

A BioSep-SEC-s2000 column (silica, 300 x 7.8 mm, 14.5 μm, Phenomenex) was used for DNA products separation. A solution of 20 mM TRIS (pH 7.0) was used as

mobile phase, with a flow rate of 1 mL/min. A Synergi Polar-RP column (ether-linked phenyl phase with polar endcapping, 150 x 4.6 μm, Phenomenex) was used for polydT products separation. Two solvents were employed for the separation: solvent A, 10 mM NH₄Ac (pH 7.0); solvent B, ACN. The flow rate was 1 mL/min. The elution profile was as follows: 0 min, 100% A; 5 min, 100% A; 20 min, 90% A, 10% B; 25 min, 90% A, 10% B; 30 min, 100% A; 45 min, 100% A.

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 x 50 mm) column (Waters), and isocratic elution with 8 mM NH₄AC (pH= 7.0) 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.

2.3.4. Fluorescence measurements

(I) 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.[31]

(II) Photon Technology International system (model L-201), including a 150 W xenon lamp coupled with a monochromator (model 101).

RESULTS

Photosensitization of dT₅

The first aim of this work was to find out whether the photobehavior previously observed for dTMP and dT in solution and in the presence of Ptr[21,30] is conserved when the nucleobase is included in an oligonucleotide chain. Therefore we used a single stranded homothymidine 5-mer oligonucleotide (dT₅) as a substrate. We exposed aqueous solutions containing dT₅ and pterin (Ptr) at pH 5.5 to UV-A radiation (350 nm). Under these conditions the thymine moiety is not ionized.[32] Ptr, in turn, presents a neutral and an anionic form that in water are in equilibrium with a pK$_a$ value around 8. It has been shown that the two acid-base forms have different photochemical properties.[8] At pH 5.5 more than 99 % of Ptr is in its neutral form that is the predominant one at physiological pH. The solubility of Ptr in neutral and acidic aqueous solutions is about 200 µM. In addition, under pathological conditions, in which pterins accumulate in tissues such as skin, the concentrations found have been reported to be in the micromolar order.[33] Taking into accounts these facts a concentration of 150 µM was chosen for Ptr in our study.

The irradiated samples were analyzed by spectrophotometry and chromatography: HPLC coupled to a photodiode array detector (HPLC-PDA), HPLC coupled to a fluorescence detector (HPLC-FL) and UPLC coupled to a mass spectrometry system (UPLC-QTof-MS) (see Experimental Section). Due to the chemical differences both reactants were well separated by chromatographic analysis, with short retention times (t_r) for Ptr and longer t_r values for the oligonucleotide (Figure 2).

When oxygen-free aqueous solutions containing dT₅ and Ptr were exposed to UV-A radiation (350 nm), significant changes were observed in the absorption spectra of the

solutions (Figure S1), thus indicating that a photochemical process takes place. HPLC-PDA analysis showed that the chromatographic peaks corresponding to both the dT_5 and Ptr decreased with irradiation time (Figure 2). The rate of Ptr consumption was higher than that registered in a control performed in the absence of dT_5 (Inset Figure 2). Chromatograms recorded at 340 nm showed that several products, with t_r close to that corresponding to the intact oligonucleotide, presented absorbance in the UV-A region (Figure 3a). Moreover, the absorption spectra of some of these products could be adequately registered and showed a band centered approximately at 340 nm, which is similar to the typical low-energy band of pterins (Figure 3a).
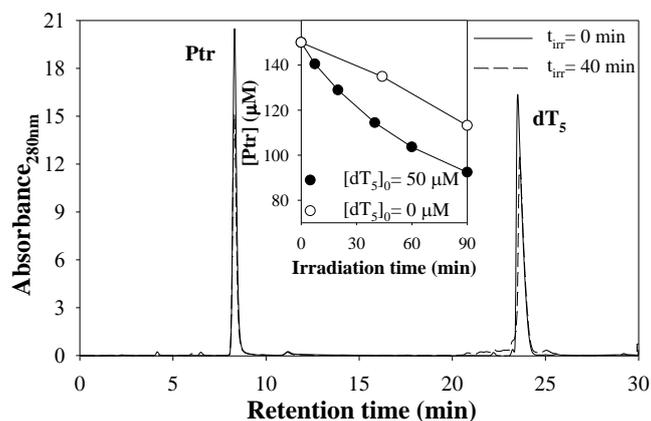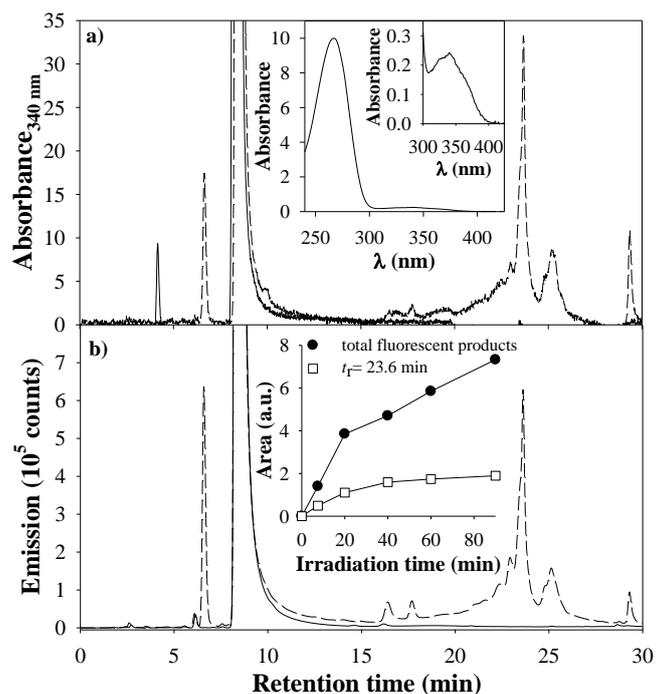

Figure 2. HPLC analysis of a solution containing dT_5 and Ptr before (solid line) and after 40 min (dashed line) of irradiation under anaerobic conditions. Chromatograms obtained using the PDA detector at 280 nm. Inset: evolution of the Ptr concentration as a function of irradiation time. $[Ptr]_0 = 150~\mu M$, (o) $[dT_5]_0 = 0~\mu M$, (●) $[dT_5]_0 = 50~\mu M$, pH = 5.5±0.1.

Figure 3. HPLC analysis of a solution containing dT$_5$ and Ptr before (solid line) and after 40 min (dashed line) of irradiation under anaerobic conditions. Chromatograms obtained using a) the PDA detector (340 nm), and b) the fluorescence detector (excitation at 350 nm, emission at 450 nm). Insets: a) absorption spectra of the products and detail in the range 300-420 nm; b) evolution of the area of the peaks corresponding to fluorescent products. [Ptr]$_0$ = 150 μM, [dT$_5$]$_0$ = 50 μM, pH = 5.5±0.1

In control experiments carried out irradiating oxygen-free dT$_5$ solutions in the absence of Ptr, no spectral changes and no consumption, monitored by HPLC, were observed as a function of irradiation time. These controls ruled out chemical changes generated by the direct excitation of the oligonucleotide. Besides, the emission spectra of Ptr in the presence and the absence of dT$_5$ were recorded by steady state fluorescence and compared. Results showed that the fluorescence spectrum of Ptr did not change in the presence of dT$_5$ in the range of concentrations used in this work (0–50 μM), thus suggesting that there is no interaction between the two molecules. Likewise, no change in the absorption spectrum of Ptr was observed in the presence of dT$_5$. Therefore, it can be assumed that the photochemical processes described in the previous paragraphs are

initiated by diffusion controlled reactions and that no association of the molecules is required before excitation.

HPLC-FL analysis of irradiated oxygen-free samples showed that several products were fluorescent and emitted at 450 nm when excited at 350 nm (Figure 3b), which is compatible with the fluorescence properties of pterins. The most intense peak presented a t_r value similar to that of the untreated oligonucleotide (Figure 3b). The area of this peak and the area of all the fluorescent products with peaks close to that of the intact dT_5 increased with irradiation time, indicating the accumulation of fluorescent products (Figure 3b). Taking also into account the decrease in the Ptr concentration, these results suggested that under anaerobic conditions the photochemical process leads to the binding of Ptr to the oligonucleotide to yield a set of fluorescent products. Finally, it is noteworthy that a peak with a t_r value lower than that of Ptr was also observed (Figure 3b) and could result from a product of the photolysis of Ptr.

To confirm that the photolysis under anaerobic conditions of dT_5 in the presence of Ptr leads to the covalent attachment of the photosensitizer to the oligonucleotide, a qualitative analysis of the photoproducts was carried out by means of UPLC-QTof-MS. Therefore, solutions containing dT_5 and Ptr were analyzed in negative ion mode (ESI$^-$) before and after irradiation. In the untreated sample, as expected, two chromatographic peaks were observed, with t_r values of 0.8 and 7.6 min. Mass spectrum of the former peak showed the signal corresponding to the intact molecular ion of Ptr as [Ptr–H]$^-$ species at m/z 162.04. The molecular formula of the dT_5 is $C_{50}H_{65}N_{10}O_{33}P_4$ and its molecular weight is 1458.27 Da. This value is an average of different weights due to the naturally occurring isotopes of carbon, although isotopes of nitrogen and oxygen also contribute. Therefore the registered mass spectrum of the dT_5 (chromatographic peak at t_r 7.6 min) consisted of groups of signals for the mono-charged ([dT_5–H]$^-$, m/z 1457.27), di-charged ([dT_5–2H]$^{2-}$

, m/z 728.13) and tri-charged ions ($[dT_5–3H]^{3-}$, m/z 485.09), the group of signals corresponding to the di-charged ion being much more intense than that corresponding to other ions (Figures 4a).
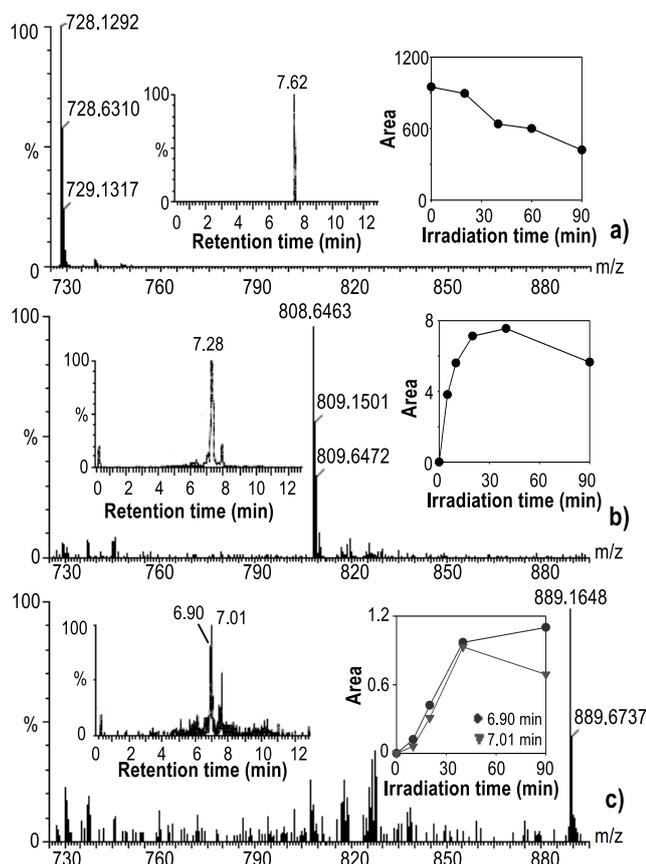

Figure 4. Mass spectrometry analysis of a solution containing dT$_5$ and Ptr after 40 min of irradiation under anaerobic conditions. MS spectra (ESI$^-$ mode) of the di-charged ions of: a) dT$_5$; b) the product with t_r at 7.3 min; c) the product with t_r at 6.9 min. Insets: mass chromatograms for the specific ion mass of a) [dT$_5$–2H]$^{2-}$; b) [dT$_5$+Ptr–4H]$^{2-}$; c) [dT$_5$+2Ptr–6H]$^{2-}$ and evolution of the area of the corresponding peaks as a function of irradiation time.

The chromatograms of solutions irradiated under anaerobic conditions showed, besides the peaks corresponding to the reactants, a main peak at t_r 7.3 min, which is very close to that of the intact nucleotide. Mass spectra of this product showed a set of signals with m/z 808.65 (Figure 4b), which correspond to a di-charged ion of a compound bearing both the photosensitizer and the oligonucleotide moieties ($[dT_5+Ptr–4H]^{2-}$). The mass

chromatograms of irradiated solutions were registered for the specific ion mass of $[dT_5+Ptr-4H]^{2-}$ (Inset Figure 4b) and the peaks were integrated. Data showed that the concentrations of this product increased as a function of irradiation time up to 40 min and then decreased (Inset Figure 4b).

The UPLC-QTof-MS equipment was used for tandem mass spectrometry (MS/MS) analysis. In the fragmentation of nucleotides using soft ionization MS methods, the loss of the base, via a 1,2-elimination, is a prominent reaction, yielding the base as a deprotonated anion ($[Thy-H]^-$).[34] The MS/MS spectrum of the ion with mass $[dT_5+Ptr-4H]^{2-}$ was recorded in the ESI$^-$ mode and compared to that corresponding to intact oligonucleotide ($[dT_5-2H]^{2-}$) (Figure 5). The MS/MS spectrum of the product showed a fragment at m/z 286 that was missing in the MS/MS spectrum of the discharged ion of the $[dT_5-2H]^{2-}$ (Figure 5). This fragment corresponds to the Thy linked to a Ptr ($[Thy+Ptr-3H]^-$). Interestingly, this peak at m/z 286 has already been observed in the MS/MS spectrum of the dT-Ptr and dTMP-Ptr adduct, resulting from irradiation of Ptr with the free nucleoside dT or nucleotide dTMP, respectively.[21,30] In this context, it was proposed that the Thy unit was linked through the CH_2 group to position 6 or 7 of the pterin moiety.[30] Therefore the results obtained in this work strongly suggest that, after the electron transfer step, the radicals ions (or neutral radicals formed by protonation/deprotonation) combine to yield an adduct where the pterinic moiety is covalently attached to the oligonucleotide. Although more experiments would be necessary to determine the specific way the two molecules are linked, the structure depicted in Scheme 1 can be proposed for the adduct formed in dT_5. Likewise, it is not possible to differentiate if there is a preferred position in the chain for the formation of the adduct (end or middle).
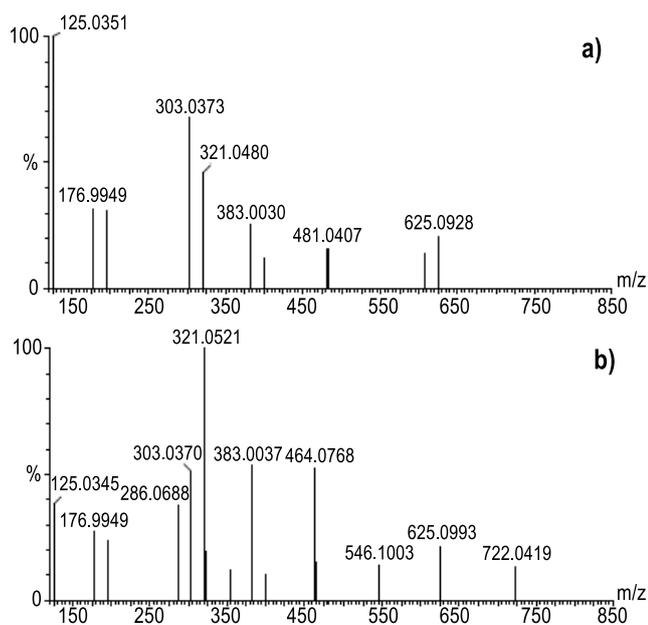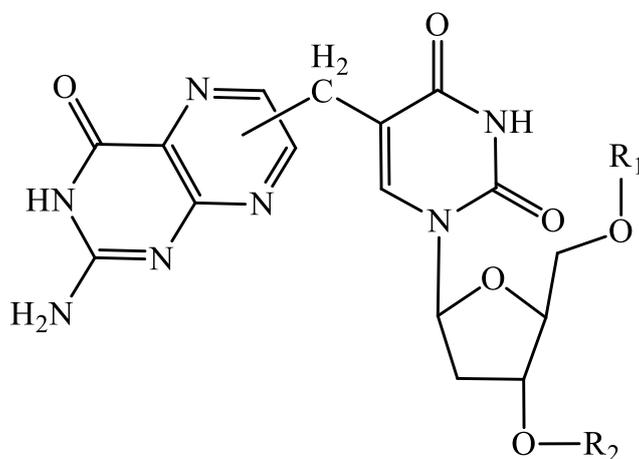
Figure 5. MS/MS spectra recorded in ESI⁻ mode of a) the di-charged ion of dT₅ ([dT₅–2H]²⁻ and b) the di-charged ion of the product with *t*r at 7.3 min ([dT₅+Ptr–4H]²⁻). Analysis of a solution containing dT₅ and Ptr after 40 min of irradiation under anaerobic conditions.

Scheme 1. Proposed molecular structure for the adduct formed when dT₅ is irradiated in the presence of Ptr under anaerobic conditions.

The mass spectra of solutions irradiated for long times under anaerobic conditions were recorded at different *t*r values to look for additional products. Mass spectra registered for *t*r values in the range 6.9 - 7.0 min showed a set of signals with *m/z* 889.16 (Figure 4c), which corresponds to a di-charged ion of an oligonucleotide bearing two molecules

of Ptr ($[dT_5+2Ptr–6H]^{2-}$). The mass chromatograms of irradiated solutions registered for the specific ion mass of $[dT_5+2Ptr–6H]^{2-}$ showed two peaks (Inset Figure 4c), suggesting the formation of at least two isomers with different t_r values. These peaks were integrated, and data showed that the concentrations of these products increased as a function of irradiation time (Inset Figure 4c). It is worth mentioning that the detection of two adducts in the same molecule indicates that the covalent binding of the first Ptr moiety does not prevent the addition of a second one due to steric hindrance.

UV-A irradiation of air-equilibrated aqueous solutions containing dT_5 and Ptr also led to significant spectral changes (Figure S1). Analysis by HPLC-PDA showed that the chromatographic peaks corresponding to both the dT_5 and Ptr also decreased with irradiation time (Figure 6). New peaks, with t_r values close to that of the intact oligonucleotide were detected in the irradiated samples (Figure 6), revealing the formation of several photoproducts. However, in contrast to experiments carried out under anaerobic conditions, the products did not show absorption in the UV-A (Insets Figures 6 and 7). Moreover, analysis by HPLC-FL showed that the photoproducts of the degradation of dT_5 (with t_r values close to that of the intact oligonucleotide) did not fluoresce upon excitation at 350 nm (Figure 7), thus suggesting that those products do not bear the pterin moiety.

The results showed that the photochemical process in the presence of oxygen does not lead to the binding of the pterin moiety to the oligonucleotide, which is in agreement with the behavior observed using dTMP as a substrate (*vide supra*). It is worth mentioning that the rate of Ptr consumption determined under aerobic conditions was equal within the experimental error to that registered in a control performed in the absence of dT_5. Therefore the consumption of Ptr corresponds, in this case, to the photolysis of the photosensitizer itself and not to the formation of an adduct with the oligonucleotide.
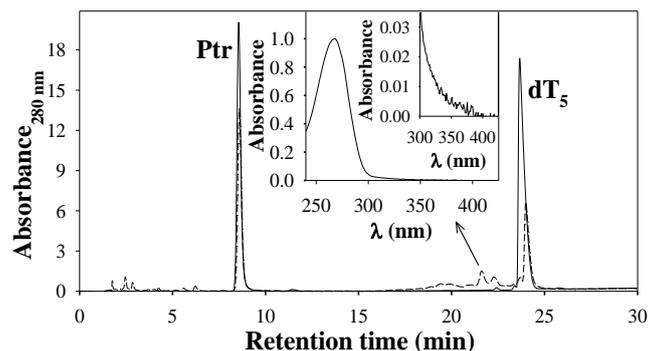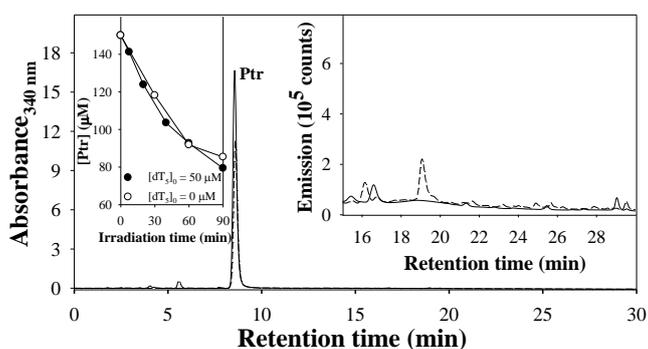
Figure 6. HPLC analysis of an air-equilibrated solution containing dT$_5$ and Ptr before (solid line) and after 40 min (dashed line) of irradiation. Chromatograms obtained using the PDA detector at 280 nm. Insets: absorption spectra of the products and detail in the range 300-420 nm. [Ptr]$_0$ = 150 μM, [dT$_5$]$_0$ = 50 μM, pH = 5.5$\pm$0.1.

Figure 7. HPLC analysis of an air-equilibrated solution containing dT$_5$ and Ptr before (solid line) and after 40 min (dashed line) of irradiation. Chromatograms obtained using the PDA detector at 340 nm. Left inset: evolution of the Ptr concentration as a function of irradiation time; right inset: chromatograms obtained using the fluorescence detector (excitation at 350 nm, emission at 450 nm); [Ptr]$_0$ = 150 μM, [dT$_5$]$_0$ = 50 μM, pH = 5.5$\pm$0.1.

In addition, mass spectrometry analysis was performed. The mass chromatograms of air-equilibrated solutions irradiated in the 5-60 min time range were registered for the specific ion masses corresponding to the products with one and two pterin residues attached to the oligonucleotide ([dT$_5$+Ptr–4H]$^{2-}$ and [dT$_5$+2Ptr–6H]$^{2-}$). None of these peaks were observed in the chromatograms confirming that the Ptr is not attached to the oligonucleotide in the presence of oxygen.

17

Photosensitization of eukaryotic DNA

After having investigated the interaction between Ptr and dT_5, the photoreactivity of Ptr and formation of an adduct were investigated in the presence of double stranded DNA. The concentration of calf thymus DNA was calculated in micromolar of base pairs (μM bp), using the corresponding molar absorption coefficient at 260 nm (ε_{260nm} = 13200 $M^{-1}cm^{-1}$).[35] The experiments were carried out in aqueous solution at pH condition 6.5 ± 0.1 in which DNA is found in the physiologically-relevant B-form conformation.[36,37] The samples were analyzed before and after irradiation by HPLC-PDA and HPLC-FL, using a size-exclusion column. Due to the differences in size, both reactants were well separated, with t_r values of 5.0 min and 12.9 min for the DNA and Ptr, respectively.

Before studying the damage to DNA photoinduced by Ptr, we set out to control the interaction between the two molecules. An aqueous solution containing Ptr (150 μM) and DNA (150 μM bp) was prepared and kept in the dark for 6 hours at room temperature. The solution was analyzed by HPLC, steady state fluorescence and spectrophotometry at different times after its preparation. The absorption and the emission spectra of Ptr did not change in the analyzed time window. In the same way, the area of the chromatographic peaks corresponding to DNA and Ptr did not vary either. In addition, in the HPLC-FL chromatograms no fluorescence compatible with Ptr properties was detected in the DNA peak. Finally, the DNA incubated with Ptr was separated with Sephadex columns and no emission was detected upon excitation at 350 nm. These controls allowed to discard the incorporation of the photosensitizer to the macromolecule in the dark, at least, for 6 hours. The interaction between Ptr and DNA is negligible or needs more time to occur. Consequently, the photolysis experiments were performed within this time window.

A solution containing DNA (150 μM bp) and Ptr (150 μM) was bubbled with Argon and then irradiated. HPLC-PDA analysis showed that besides the peak corresponding to the intact DNA molecule, a new peak at a t_r value close to that of the intact DNA (t_r = 5.4 min) appeared upon irradiation (Figure 8a), and its area increased with irradiation time. Chromatograms recorded at 340 mm showed only one peak with a maximum corresponding to the product detected in chromatograms recorded at 260 nm (Figure 8b). Moreover, the absorption spectra recorded for this peak showed a band in the UV-A region (Inset Figure 8a), indicating the presence of the pterin moiety. It is noteworthy that no absorbance at 340 nm was observed for the peak of the intact DNA. In control experiments, no changes in the chromatograms of aqueous solution of DNA were registered, even after irradiation for more than 120 min, thus excluding the possibility that spurious effects of direct light absorption by the nucleobases could lead to alterations in the macromolecule.

If we assume that the molar absorption coefficient of Ptr does not change significantly, the amount of Ptr attached to the macromolecule can be estimated, using the calibration curve of the free photosensitizer. As shown in Figure 8c, in 90 min of irradiation the concentration of Ptr attached to the DNA is 16.5 μM, which corresponds to a molecule of Ptr every 9 base pairs. The rate of incorporation of Ptr to the DNA molecule was lower than the rate of consumption of Ptr, which is logical taking into account that Ptr underwent photodegradation in the absence of DNA (Figure 8c). However, the rate of consumption of Ptr in the presence of DNA was higher than in its absence, and the difference corresponded, within the experimental error, to the rate of incorporation of Ptr to the DNA (Figure 8c).
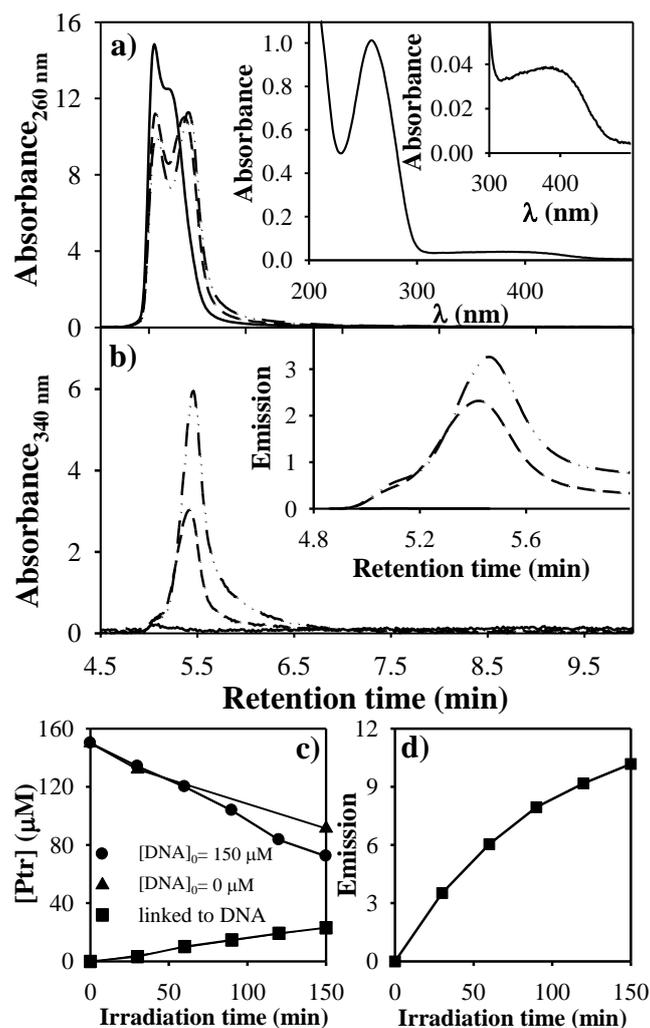
Figure 8. HPLC analysis of a solution containing DNA and Ptr before (solid line), after 60 (dashed line) and 120 min (dashed-dot line) of irradiation under anaerobic conditions. Chromatograms obtained using a) the PDA detector at 260 nm, b) the PDA detector at 340 nm, c) evolution of the free and attached Ptr concentrations as a function of irradiation time, d) evolution of the area of the peaks corresponding to fluorescent products. Insets: a) absorption spectrum recorded for the peaks registered with the solution irradiated 60 min and detail in the range 300-500 nm, b) chromatograms obtained using the fluorescence detector (excitation at 350 nm, emission at 450 nm). $[Ptr]_0 = 150$ μM, $[DNA]_0 = 150$ μM bp, pH = 6.5±0.1.

HPLC-FL analyses showed that, apart from the Ptr peak, only the peak corresponding to the DNA product presented fluorescence upon excitation at 350 nm (Inset Figure 8b), which is in agreement with HPLC-PDA analysis. The area of this peak

increased with irradiation time (Figure 8d). Therefore, it can be concluded that, upon irradiation under anaerobic conditions, Ptr is incorporated into the DNA structure, and the product formed retains some spectroscopic properties of the photosensitizer such as its absorbance in the UV-A region and its fluorescence.

In another set of experiments, irradiated air-equilibrated aqueous solutions containing DNA (150 μM bp) and Ptr (150 μM) were analyzed. The chromatograms registered at 260 nm showed that the intact DNA was consumed upon irradiation (Figure 9a). Simultaneously the area and the t_r value of the maximum of a broad peak corresponding to the products increased with irradiation time. This behavior indicated that the DNA molecule was randomly cut yielding fragments in a wide range of molecular weights. The fragmentation of DNA photoinduced by pterins in the presence of oxygen has been reported in the literature.[9,10]

The chromatograms registered at 340 nm presented peaks with shapes similar to those registered for the chromatograms at 260 nm (Figure 9b). The total area of the products with absorbance at chromatograms at 340 nm did not steadily increase with irradiation time, as in the case of the photolysis under anaerobic conditions (Figure 8), but it reached a plateau at about 60 min of irradiation (Inset Figure 9b). However, absorption spectra recorded at different t_r values and for different irradiation times did not show the typical absorption band of pterins (Inset Figure 9a). It is worth mentioning that several oxidation products of guanine present absorption bands in the UV-A region.[38,39] The area of the products at 340 nm as a function of the irradiation time showed a very fast increase during the first hour of photolysis and reached a plateau after 1.5 hours. This behavior may be explained by a fast oxidation of guanine residues. Moreover, HPLC-FL analysis showed a total fluorescence intensity lower than that observed in oxygen-free solutions, which can be attributed to the emission of some products of guanine.[38]
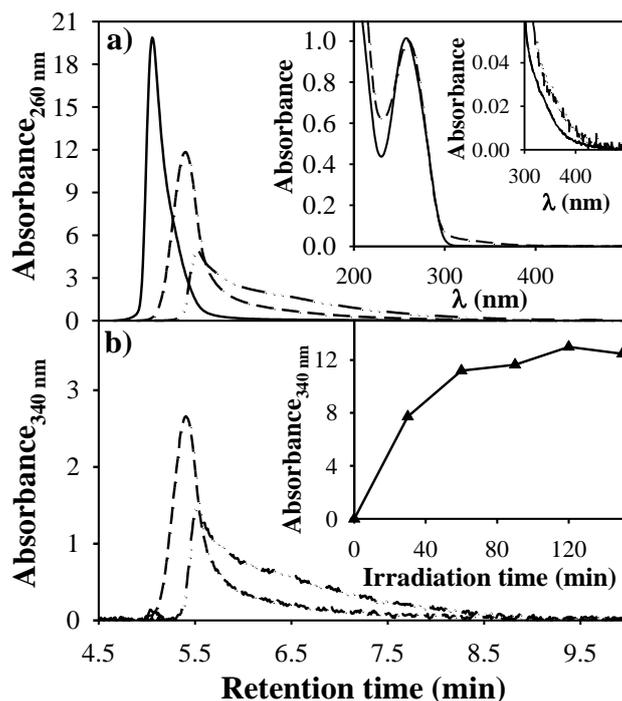
Figure 9. HPLC analysis of a solution containing DNA and Ptr before (solid line), after 60 (dashed line) and 120 min (dashed-dot line) of irradiation under aerobic conditions. Chromatograms obtained using a) the PDA detector at 260 nm and b) the PDA detector at 340 nm. Insets: a) absorption spectra recorded for the peaks registered with the solution before (solid line) and after 60 min (dashed line) of irradiation and detail in the range 300-500 nm; b) evolution of the area of the peaks of the chromatograms registered at 340 nm. [Ptr]$_0$ = 150 μM, [DNA]$_0$ = 150 μM bp, pH = 6.5±0.1.

Although Ptr-Thy adduct is formed in the anaerobic photochemical processes with free thymine nucleotide (dTMP) and dT$_5$, the formation of an equivalent adduct in DNA, a much more complex molecular system, is not straightforward. In this macromolecule the nucleobases are not completely exposed and the dynamic processes can be hindered. In addition, the other nucleobases might efficiently compete with thymines for the electron transfer to the triplet excited state of Ptr. In particular, purine nucleobases (guanine and adenine) are more reactive than Thy due to their lower ionization potentials.[19] It is also worth mentioning that the other pyrimidine nucleobase, cytosine (Cyt), has not been investigated in previous studies, and then the formation of a Ptr-Cyt

22

adduct cannot be discarded.

Therefore, to investigate the formation of Ptr adducts with other nucleobases different from Thy, solutions containing Ptr and a free nucleotide were irradiated in similar conditions as those used in the experiments with dT_5 (pH, energy, etc), which are, in turn, similar to those used previously for the synthesis of the Ptr-Thy adduct, using dTMP as a substrate (see Introduction).[21] In this way, the following nucleotides were used: 2´-deoxyguanosine 5´-monophosphate (dGMP), 2´-deoxyadenosine 5´-monophosphate (dAMP) and 2´-deoxycytidine 5´-monophosphate (dCMP). Thus each O_2-free solution containing Ptr (150 μM) and a given nucleotide (1 mM) was irradiated and then analyzed by HPLC. Additionally a control Ptr solution without any nucleotide was also irradiated under the same experimental conditions. For the three investigated nucleotides, no evidence of the formation of adducts was found, that is, i) no difference between consumption of Ptr in the presence and in the absence of nucleotide was observed; ii) no fluorescent products were detected. These results supported the hypothesis that the fluorescent product(s) formed when DNA was irradiated in the presence of Ptr under anaerobic conditions was(were) Ptr-Thy adduct(s).

On the other hand, when a solution containing Ptr and DNA were irradiated in the presence of oxygen the adduct was not formed and the prevalent effect on the macromolecule is the random cleavage, probably due to the oxidation at the guanine sites, as previously reported.[9,10] Considering the behavior observed for the Ptr-photosensitization of free nucleotides[13,21] (see Introduction), a similar mechanism can be assumed for the DNA. The oxidation of the Ptr radical anion by dissolved O_2, prevents the formation of the Ptr-Thy adduct.

In summary, the yields of the different chemical processes photoinduced by Ptr in the DNA molecule strongly depend on the presence of O_2: in air-equilibrated solution

random cleavage takes place, whereas in the absence of O_2 the Ptr-Thy adduct is formed. Indeed, the O_2 concentration value in biological systems is between these two limit concentration conditions. Since in some tissues the O_2 concentration can be very low and Ptr freely passes across biomembranes,[40] the formation of the Ptr-Thy adduct cannot be discarded in cellular DNA. In addition, the concentration of Ptr used in our studies is in the same order of magnitude as those determined for oxidized pterins in diseased skin cells; *e.g.* in human keratinocytes and cells cultures from suction blister roofs.[33]

Fluorescence properties of the Ptr-Thy adduct

As reported previously the Ptr-Thy adduct formed by reaction of dTMP is fluorescent, and its emission spectrum is similar to that of free Ptr.[21] To study the fluorescence properties of the products of the Ptr-photosensitization of DNA, the samples irradiated under anaerobic conditions were purified through a sephadex column to eliminate the free Ptr. The fluorescence spectra of the isolated DNA were recorded by excitation at 350 nm for different irradiation times (Figure 10). Under these conditions, the emission was negligible before irradiation, confirming that the free Ptr was efficiently eliminated, and increased with irradiation time, indicating that the photochemical process generates fluorescent products that accumulated during, at least, 4 hours. In addition, the intensity of the excitation spectra also increased with irradiation time (inset Figure 10). The emission and excitation spectra were similar to those reported for free Ptr.[8]
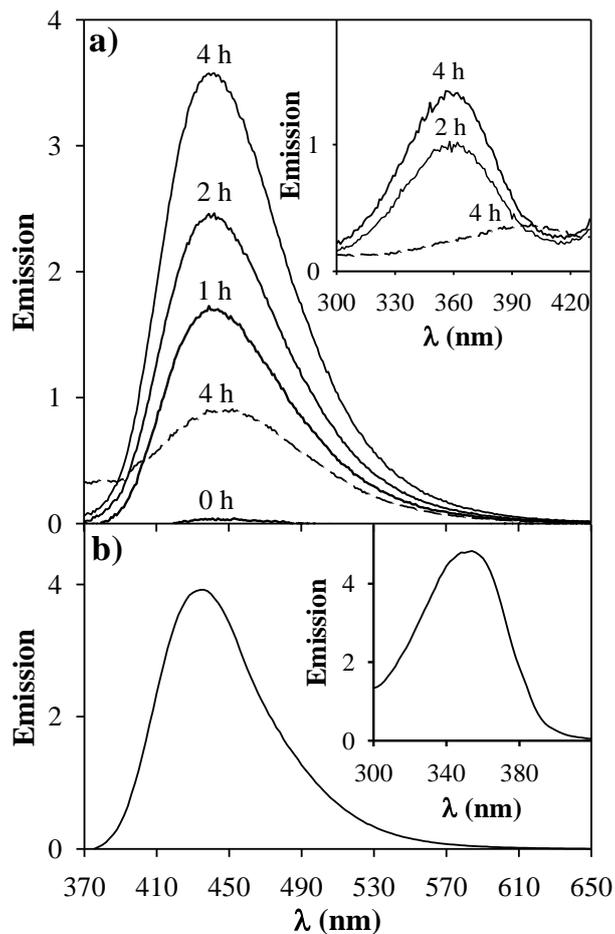
Figure 10. a) Emission spectra, obtained upon excitation at 350 nm, of DNA samples irradiated in the presence of Ptr under anaerobic conditions (solid line). For comparative purposes the spectrum of a sample irradiated in the presence of Ptr under aerobic conditions was included (dashed line). Inset: corresponding excitation spectra obtained at 440 nm. The irradiation time appears above each spectrum. The DNA was separated from Ptr by chromatography. $[Ptr]_0 = 150$ μM, $[DNA]_0 = 150$ μM bp, pH = 6.5±0.1. b) Emission spectrum, obtained upon excitation at 350 nm, of dT_5 samples irradiated 60 min in the presence of Ptr under anaerobic conditions. Inset: corresponding excitation spectrum obtained by fixing the emission wavelength at its maximum ca. 440 nm. The dT_5 was separated from Ptr by chromatography. $[Ptr]_0 = 150$ μM, $[dT_5]_0 = 50$ μM, pH = 5.5±0.1.

In another set of experiments samples irradiated under aerobic conditions were also purified through a sephadex column to eliminate the free Ptr and the fluorescence spectra of the isolated DNA were recorded under the same experimental conditions as those used for the analysis of the oxygen-free solutions. After four hours of irradiation,

the DNA irradiated in the presence of oxygen showed fluorescence (Figure 10a), but its intensity was lower than that registered for one hour of irradiation under anaerobic conditions. This weak fluorescence might be due to some Ptr attached to the macromolecule, which seems unlikely, taking into account the behavior observed for the dTMP and dT_5, and that the maximum of the corresponding excitation spectrum did not match to the maximum of the spectra recorded in the anaerobic experiments. Alternatively, the weak emission might be due to the emission of some fluorescent products of guanine, previously reported.[38]

The products of the reaction using dT_5 as a substrate were isolated from the HPLC runs (Experimental). In particular, the oxygen-free irradiated solutions of the oligonucleotide and Ptr were injected in the HPLC equipment and the fraction in the range of t_r between 22 and 23 min was collected (Figure 2). This fraction contained most of the products of dT_5 and the remaining intact oligonucleotide. Fluorescence analysis of this solution showed emission and excitation spectra similar to those registered for the DNA products (Figure 10).

Finally, time-resolved experiments were performed on the samples described in the previous paragraphs, that is, oxygen-free solutions containing Ptr and DNA or dT_5 were irradiated and then the photosensitizer was separated through sephadex column filtration or HPLC, respectively. The study was performed by excitation at 341 nm and the corresponding fluorescence decays were recorded at 450 nm (Figure 11). For both samples the emission decays were clearly bi-exponential with a main long-lived component and a minor short-lived component. The fluorescence lifetimes (τ_F) and the pre-exponential factors were similar for both samples (Figure 11). Moreover, the τ_F values of the long-lived components were in the same order that the τ_F reported for free Ptr in aqueous solution (7.6 ($\pm$0.4) ns).[8]

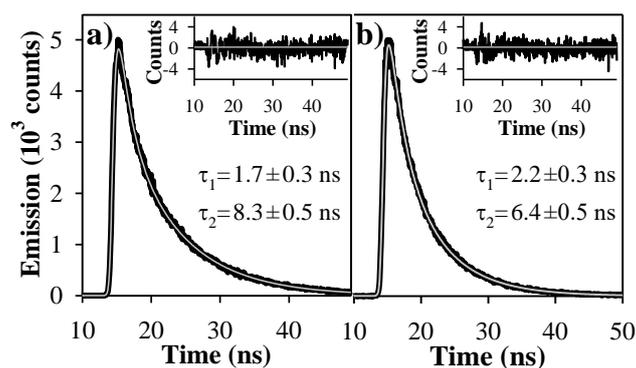**Figure 11. Emission decays recorded at 450 nm (λ_{exc} = 341 nm). Insets: residual analysis. a) DNA samples irradiated in the presence of Ptr under anaerobic conditions. [Ptr]$_0$ = 150 μM, [DNA]$_0$ = 150 μM bp, pH = 6.5±0.1. b) dT$_5$ samples irradiated 60 min in the presence of Ptr under anaerobic conditions. [Ptr]$_0$ = 150 μM, [dT$_5$]$_0$ = 50 μM, pH = 5.5±0.1.**

The results presented in this section showed that the photoproducts formed with both substrates are fluorescent with emission properties similar to that of Ptr. This fact is relevant because it means that Ptr retains its fluorescent properties when it is covalently linked to DNA. It is worth mentioning that the similarity in the results obtained for products of dT$_5$ and DNA suggests that the fluorophore(s) in both cases is(are) the same. Finally, the determination of two fluorescent components might be due to the formation of several products with the pterin moiety linked in different way to the substrates.

Conclusions

The photochemistry of the DNA biomacromolecule is an issue of paramount importance as it is part of the etiology of skin cancer development, being ultraviolet sunlight radiation the most relevant environmental carcinogen. Herein, we demonstrate

the potential of pterin, an endogenous compound, to form covalent adduct under UVA irradiation in the absence of oxygen.

Thus, in a first stage, UV-A irradiation (350 nm) of oxygen-free aqueous solutions (pH 5.5) containing the single stranded homothymidine 5-mer oligonucleotide (dT$_5$) and pterin (Ptr) showed the formation of a covalent adduct consisting of the pterinic moiety covalently attached to a thymine (Thy) base. Moreover, the oligonucleotide bearing two molecules of Ptr was also observed. In contrast, in the presence of oxygen the photochemical process does not lead to the binding of the pterin moiety to the oligonucleotide. Our studies were extended to eukaryotic DNA. The yields of the different chemical processes photoinduced by Ptr onto the DNA molecule strongly depend on the presence of oxygen: in air-equilibrated solution random cleavage takes place, whereas in the absence of oxygen the formation of covalent adduct is observed. The Ptr-Thy adduct formed with both biological substrates retain the spectroscopic properties of the photosensitizer; e.g. absorption band in the UV-A region and similar fluorescence emission properties.

Indeed, these results might be biologically relevant, and formation of the Ptr-Thy adduct cannot be discarded in cellular DNA in connection with the facts that (*i*) the O$_2$ concentration can be very low in some tissues, (*ii*) Ptr freely passes across biomembranes, and (*iii*) the concentration of Ptr used in our studies is in the same order of magnitude as those determined for oxidized pterins in diseased skin cells. Altogether the findings point toward the role of Ptr as endogenous sensitizer and genotoxic compound.

Acknowledgements

References

(1)   Ziegler, I *Med. Res. Rev*. **1990**, 10, 95–114.

(2)   Nichol, C. A.; Smith, G. K.; Duch, D. S. *Annu. Rev. Biochem*. **1985**, 54. 729–764.

(3)   Schallreuter, K. U.; Wood, J. M. Pittelkow, M. R.; Gütlich, M.; Lemke, K. R.; Rödl, W. et al. *Science* **1994**, 263, 1444–1446.

(4)   Glassman, S. J. *Clin. Sci.* **2011**, 120, 99–120.

(5)   Schallreuter, K. U.; Moore, J.; Wood, J. M.; Beazley, W. D.; Peters, E. M. J.; Marles, L. K. et al., *J. Invest. Dermatol.* **2001**, 116, 167–174.

(6)   Neverov, K.; Mironov, E. A.; Lyudnikova, T. A. *Biochemistry Moscow*. **1996**, 61, 1149–1155.

(7)   Egorov, S. Y.; Krasnovsky, A. A.; Bashtanov, M. Y.; Mironov, E. A.; Ludnikova, T. A.; Kritsky, M. S. *Biochemistry Moscow.***1999**, 64, 1117–1121.

(8)   Lorente, C.; Thomas, A. H. *Acc. Chem. Res.* **2006**, 39, 395–402.

(9)   Ito, K.; Kawanishi, S. *Biochemistry* **1997**, 36, 1774–1781.

(10) Hirakawa, K.; Suzuki, H.; Oikawa S.;Kawanishi, S. *Arch. Biochem. Biophys.*, **2003**, 410, 261–268.

(11) Petroselli, G.; Dántola, M. L.;Cabrerizo, F. M.; Capparelli, A. L.; Lorente, C.; Oliveros, E. Thomas, A. H. *J. Am. Chem. Soc.* **2008**, 130, 3001–3011.

(12) Serrano, M. P.; Lorente, C.; Vieyra, F. E. M.; Borsarelli, C. D.; Thomas, A. H. *Phys. Chem. Chem. Phys.* **2012**, 14, 11657–65.

(13) Serrano, M. P.; Lorente, C.; Borsarelli, C. D.; Thomas, A. H. *ChemPhysChem.* **2015**, 16, 2244–2252.

(14) Petroselli, G.; Erra-Balsells, R.; Cabrerizo, F. M.; Lorente, C.; Capparelli, A. L.; Braun, A. M.; Oliveros, E.; Thomas, A. H. *Org. Biomol. Chem.* **2007**, 5, 2792–2799.

(15) Serrano, M. P.; Lorente, C.; Borsarelli, C. D.; Thomas, A. H. *Photochem. Photobiol.* **2013**; 89, 1456–1462.

(16) Baptista, M. S.; Cadet, J.; Di Mascio, P.; Ghogare, A. A.; Greer, A.; Hamblin, M. R.; Lorente, C.; Nunez, S. C.; Ribeiro, M. S.; Thomas, A. H.; Vignoni, M.; Yoshimura, T. M. *Photochem. Photobiol.*, in press (DOI: 10.1111/php.12716).

(17) Steenken, S.; Jovanovic, S. V.; *J. Am. Chem. Soc.* **1997**, 119, 617–618.

(18) Yanagawa, H.; Ogawa, Y.; Ueno, M. *J. Biol. Chem.* **1992**, 267, 13320–13326.

(19) Burrows, C. J.; Muller, J. G.; *Chem. Rev.* **1998**, 98, 1109–1152.

(20) Cadet, J.; Wagner, J. R. *Cold Spring Harb. Perspect. Biol.* **2013**, 5:a12559.

(21) Serrano, M. P.; Vignoni, M.; Lorente, C.; Vicendo, P.; Oliveros, E.; Thomas, A. H.; *Free Radic. Biol. Med.* **2016**; 96, 418–431.

(22) Sawyer, D. T.; Valentine, J. S. *Acc. Chem. Res.* **1981**, 14, 393-400.

(23) Wagner, J. R.; van Lier, J. E.; Berger, M.; Cadet, J. *J. Am. Chem. Soc.* **1994**, 116, 2235–2242.

(24) Cadet, J.; Wagner, J. R.; Shafirovich, V. Geacintov, N. E. *Int. J. Radiat. Biol.* **2014**, 90, 423–432.

(25) Cadet, J.; Wagner, J. R.: Arch. Biochem. *Biophys.* **2014**, 557, 47–54.

(26) von Sonntag, C.; Schuchmann, H. P. *Angew. Chem. Int. Ed.* **1991**, 30, 1229–1253.

(27) von Sonntag, C.; Schuchmann, H.P. Peroxyl radicals in aqueous solutions. In Peroxyl Radicals, Z. B. Alfassi, Ed., John Wiley and Sons, New York, 1997 pp 173–234.

(28) Murali Krishna, C.; Decarroz, C.; Wagner, J. R.; Cadet, J.; Riesz, P. *Photochem. Photobiol.* **1987**, 46, 175–182.

(29) Ravanat, J. -L.; Douki, T.; Cadet, J. *J. Photochem. Photobiol. B Biol.* **2001**, 63, 88–102.

(30) Estébanez, S.; Thomas, A. H.; Lorente, C. *ChemPhysChem*, **2018**, 19, 300–306.

(31) Denofrio, M. P.; Ogilby, P. R.; Thomas, A. H. Lorente, C. *Photochem. Photobiol. Sci.* **2014**, 13, 1058-1065.

(32) Tajmir-Riahi, H. A.; Ahmad, R.; Naoui, M.; Diamantoglou, S. *Biopolymers* **1995**, 35, 493-501.

(33) Schallreuter, K. U.; Moore, J.; Wood, J. M.; Beazley, W. D.; Peters, E. M. J.; Marles, L. K.; Behrens-Williams, S. C.; Dummer, R.; Blau, N.; Thöny, B. J. *J. Invest. Dermatol.* **2001**, 116, 167–174.

(34) Mcluckey, S. A.; Berkel, G. J.; Glish, G. L. *J. Am. Soc. Mass Spectrom.* **1992**, 3, 60–70.

(35) Kumar, C. V.; Asuncion, E. H. *J. Am. Chem. Soc.* **1993**, 115, 8547-8553.

(36) O'Connor, T.; Mansy, S.; Bina, M.; McMillin, D. R.; Bruck, M. A.; Tobias, R. S. *Biophys. Chem.* **1981**, 15, 53-64.

(37) Tajrnir-Riahi, H. A.; Neault, J. F.; Naoui, M. *FEBS Lett.* **1995**, 370, 105-108.

(38) Torun, L.; Morrison, H. *Photochem. Photobiol.*, **2003**, 77, 370–375.

(39) Serrano, M. P.; Estébanez Ruiz, S.; Vignoni, M.; Lorente, C.; Vicendo, P.; Oliveros, E.; Thomas, A. H. *New J. Chem.* **2017**, 41, 7273–7282.

(40) Thomas, A. H.; Catalá, A.; Vignoni, M. *Biochim. Biophys. Acta* **2016**, 1858, 139−145.
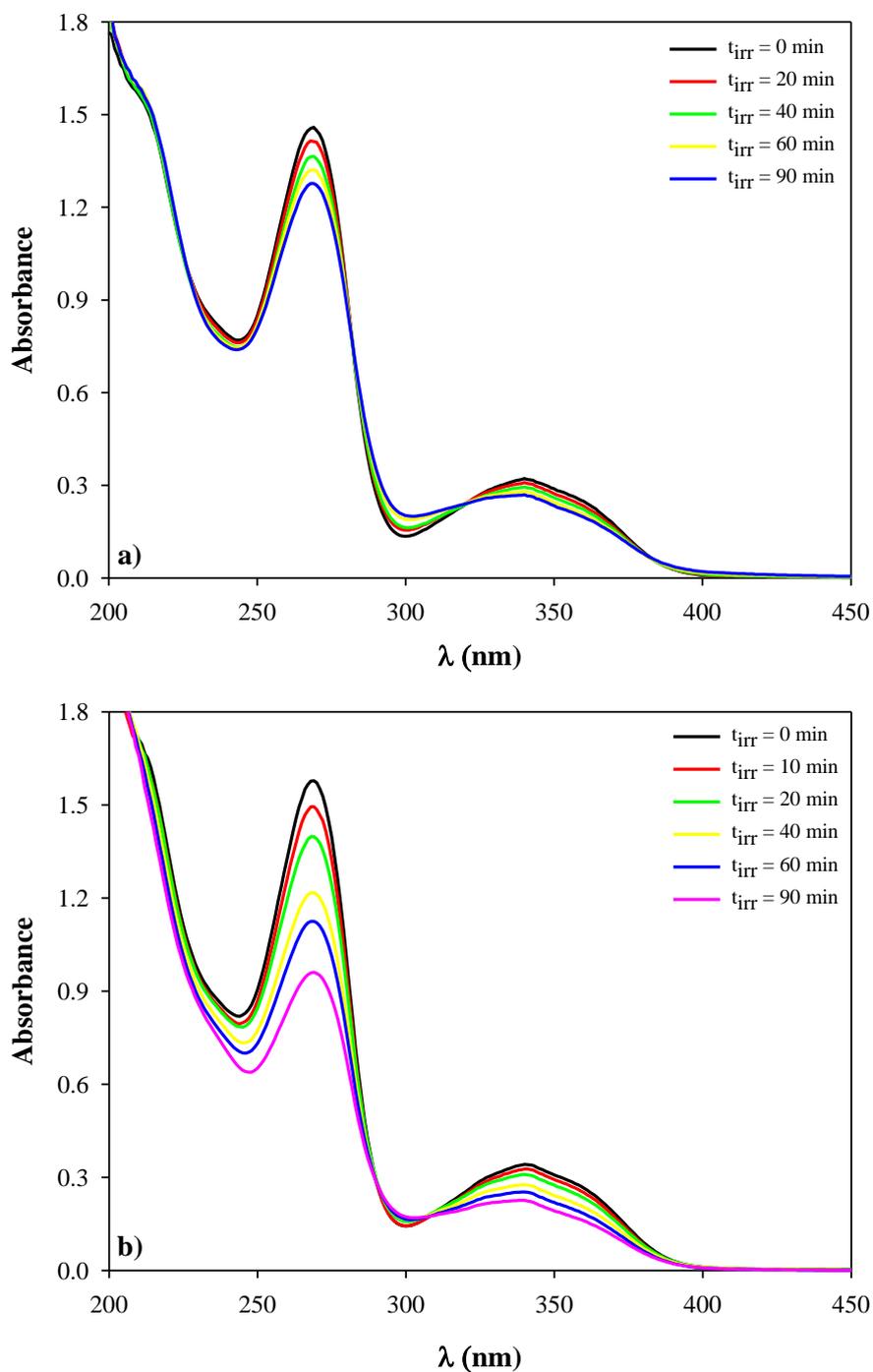
Figure S1. Time evolution of the absorption spectrum of a solution of dT_5 irradiated in the presence of Ptr. a) Experiment performed in the absence of oxygen; b) experiment performed in the presence of oxygen. Optical path length = 1.0 cm; $[Ptr]_0$ = 150 μM, $[dT_5]_0$ = 50 μM, pH = 5.5.