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Deoxythymidine-pterin fluorescent adduct formation by a photosensitized process.

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Abstract

A new fluorescent compound was isolated from UV-A irradiated aqueous solutions containing pterin (Ptr) and 2´deoxythymidine (dT) in anaerobic conditions. Pterins are widespread in the living systems in small amounts, but they are accumulated in some pathological situations. Under UV-A radiation, pterins are photochemically active, fluorescent and photosensitize the generation of singlet oxygen ($^1O_2(^1\Delta_g)$). The isolated compound was structurally characterized, by liquid chromatography coupled to tandem mass spectrometry, and its photophysical properties were studied by time correlated single photon counting technique. The molecular weight and the analysis of the fragmentation correspond to a molecule where the pterinic moiety is attached to the thymine nucleobase. The product exhibit photophysical properties similar to those of Ptr, including relatively high fluorescence and $^1O_2$ production quantum yields.

Introduction

Many modifications in the DNA induced by electromagnetic radiation have been extensively analyzed in the last decades, including those produced by direct absorption by the nucleobases, as well as others caused by photosensitized reactions, where another molecule, the photosensitizer, absorbs radiation. The photosensitized oxidation reactions may be divided into two main groups: type I, where radicals are formed from the photosensitizer triplet excited state, and type II, where singlet oxygen ($^1O_2$ $^1\Delta_g$, denoted throughout as $^1O_2$) is formed by energy transfer to the molecular oxygen.[1]

Thymine (Thy) is the DNA nucleobase with the lowest oxidation potential and, according to this property, it should be the less oxidable one. Nevertheless, damage on Thy were reported by direct UV absorption and by photosensitized reactions, in equal amounts of reaction that those occurring at guanine nucleobase.[2] During irradiation in the range 200-300 nm (UV-B range and UV-C), the major photoproducts found over Thy nucleosides are cyclobutane pyrimidine dimers (CPD), which are strongly correlated with mutagenesis and carcinogenesis processes.[3] However, the most abundant radiation reaching the earth surface is of a wavelength higher than 300 nm, and the damage over DNA is due to photosensitized reactions.[4] It was demonstrated that CPD lesions are also formed upon UV-A irradiation (310-400 nm) through photosensitized processes as a result of a triplet energy-transfer mechanism.[5] The photooxidation of the Thy moiety in different substrates via a type I mechanism has been proven using different sensitizers such as menadione[6], benzophenone[7] and pterin,[8] in the presence of oxygen. Oxidative damage of Thy may be explained by a proton coupled electron transfer (PCET) mechanism, with the formation of a Thy radical. The products formed from the Thy radical can be divided into those coming from the reaction at its methyl group and those from hydration at its 5,6-double bond.[2]

Pterins are biomolecules found in all living systems, participating in several photobiological processes and with important physiological functions, such as being an essential enzymatic cofactor for the hydroxylation of aromatic aminoacids[9] which is involved in the synthesis of melanin.[10] Aromatic or oxidized pterins are photochemically active in aqueous solution under UV-A excitation. Pterins present relatively high quantum yields of fluorescence, and during irradiation undergo photooxidation producing reactive oxygen species (ROS), including $^1O_2$.[11],[12],[13],[14] In the late 1990´s, it was shown that pterins are able to damage DNA,[15],[16] and then the photosensitizing properties of these compounds and the corresponding mechanisms became relevant. We have investigated the degradation of several nucletotides in the presence of pterins under UV-A irradiation, such as 2'-deoxyguanosine 5'-monophosphate (dGMP),[17],[18],[19],[20] 2'-deoxyadenosine 5'-monophosphate (dAMP),[21],[22] and 2'-deoxythymidine 5'-monophosphate (dTMP).[8]

When solutions containing the pyrimidine nucleotide dTMP and pterin (Ptr) are exposed to UV-A irradiation,[8] Ptr is excited to its triplet excited state ($^3Ptr^*$), which deactivates by several pathways, but in the presence of a suitable electron donor, such as dTMP, it undergoes electron-transfer to form the Ptr radical anion ($Ptr^{\bullet-}$) and the corresponding dTMP radical cation ($dTMP^{\bullet+}$).

Under aerobic conditions, the electron transfer from $Ptr^{\bullet-}$/$PtrH^\bullet$ to O_2 regenerates Ptr and yields $O_2^{\bullet-}$/$HO_2^\bullet$, which in turn disproportionate to H_2O_2. This process avoids the reaction between the radicals of the photosensitizer and the substrate. Structures for the dTMP oxidation products coming from the two main competitive reactions of $dTMP^{\bullet+}$ deprotonation (Reaction 1) and hydration (Reaction 2) were proposed.[8]

$$dTMP^{\bullet+} \longrightarrow dTMP(-H)^\bullet + H^+ \quad (1)$$

$$dTMP^{\bullet+} + OH^- \longrightarrow dTMP(OH)^\bullet \quad (2)$$

After deprotonation and hydration, several stable products were characterized by mass spectrometry: thymidine glycol 5′-monophophate, 1-(2-deoxy-β-D-erythro-pentofuranosyl-5-phosphate)-5-hydroxy-5-methyl hydantoin, 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine 5′-monophosphate, 5-formyl-2′-deoxyuridine 5′-monophosphate, 5-(hydroxymethyl)-2′-deoxyuridine5′-monophosphate and 5-(hydroperoxymethyl)-2′-deoxyuridine 5′-monophosphate.[8] Under anaerobic conditions, dTMP also reacts with Ptr upon UV-A irradiation, and a product with spectroscopic properties similar to Ptr was detected. This product was characterized as an adduct between Ptr and dTMP with a molecular mass of 483.08 Da corresponding to [Ptr – dTMP -2H].

In this work, we have characterized and adduct containing Ptr and 2´deoxythymidine (dT) and its photophysical properties were investigated (Figure 1). This adduct is formed during UV-A irradiation of O_2-free solutions containing Ptr and dT. The adduct presents a photophysical behavior different from the dT but close to pterins photophysical properties, including relatively high fluorescence quantum yields and singlet oxygen production. The potential incorporation of a fluorophore and a triplet excited states generator to DNA makes the adduct interesting from a biological and analytical point of view.

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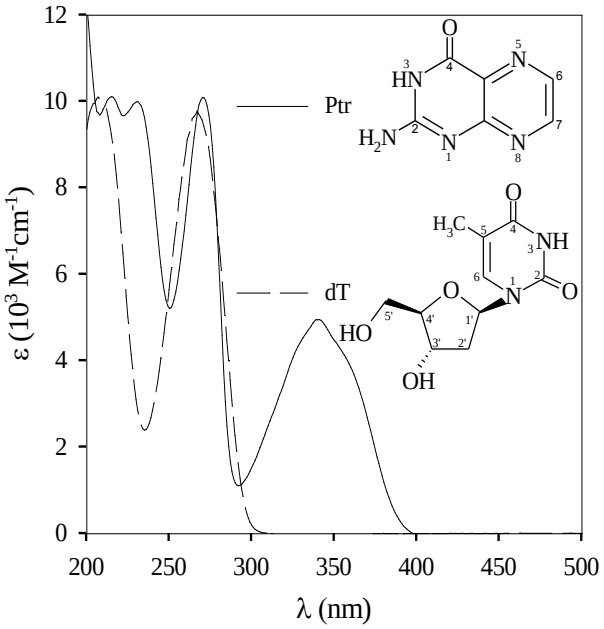

Figure 1. Absorption spectra of Ptr and dT in air-equilibrated aqueous solutions at pH 5.5. Molecular structure of Ptr and dT.

Results and discussion

Preparation of the deoxythymidine-pterin adduct

Aqueous solutions containing pterin (Ptr) and 2'-deoxythymidine (dT) were irradiated at 350 nm during 3 hours in anaerobic conditions. As can be inferred from the corresponding absorption spectra (Figure 1), under these experimental conditions, Ptr was the only absorbing species, whereas dT did not absorb radiation. Since Ptr presents a pK_a at 7.9,[11] and the acid and the basic forms show different photosensitizing properties, the experiments were performed at pH ~ 5.5, where Ptr is present at more than 99% in its acid form the predominant form in biological systems. The photochemical reactions were followed by UV-visible spectrophotometry and HPLC with spectrophotometric and fluorescence detection.

In control experiments deaerated aqueous solutions of both reactants were independently exposed to UV-A radiation. Neither of them was consumed, clearly indicating that both Ptr and dT are photostable. In contrast, in aqueous solution containing both Ptr and dT compounds (pH 5.5) significant changes in the absorption spectra were observed during irradiation in the absence of O_2. The irradiated solutions were analyzed by HPLC and the concentrations of Ptr and dT were determined. The profiles showed that both compound concentrations decreased upon irradiation, with a 1:1 molar stoichiometric ratio (Figure 2).

Chromatograms of irradiated solutions showed the formation of several photoproducts, one of them being predominant at a retention time (t_R) of 11.2 minutes, which is higher than the t_R values corresponding to both dT and Ptr. This product presented an absorption band similar to the typical low-energy band of pterins and emitted at 450 nm when excited at 340 nm (Figure 3), which is compatible with the fluorescence properties of pterins. In addition, the concentration of the main photoproducts increased with irradiation time (Figure 3c), although their rate of formation decreased with the consumption of Ptr, which is the limiting reactant in

the process. The other chromatographic peaks observed could correspond to minor products formed under anaerobic conditions or products of the aerobic photosensitization at very low concentrations due to traces of remaining O_2.

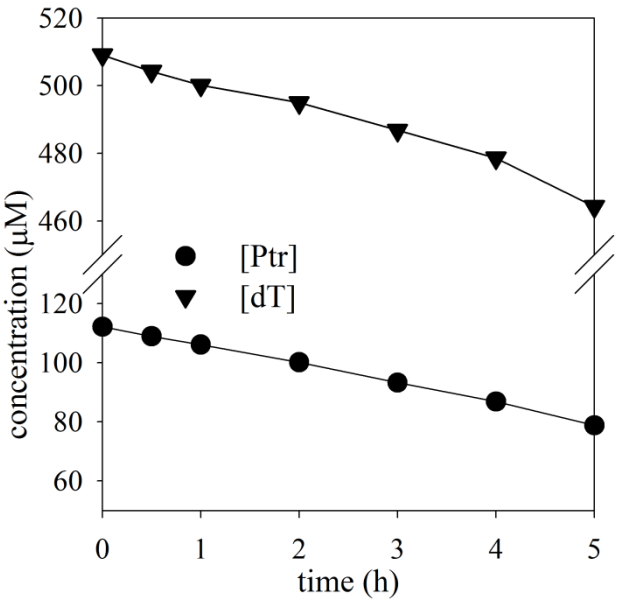

Figure 2. Evolution of the Ptr (●) and dT (▼) concentrations in oxygen free aqueous solutions under UV-A irradiation as a function of time, λ_{exc} = 350 nm, pH = 5.5; $[Ptr]_0$ ~ 100 μM, $[dT]_0$ ~ 500 μM. Errors on individual experimental points are ~ ± 4 μM.

HPLC analysis of air-equilibrated solutions exposed to UV-A irradiation showed the formation of different products, but the fluorescent product with t_R = 11.2 minutes, observed under anaerobic conditions, was not formed. Moreover, fluorescent products or products with the typical absorption band of pterins were not detected. This control confirmed that the fluorescent adduct is formed, only if O_2 is eliminated from the solution before irradiation.

The behavior explained in the previous paragraphs is similar to that observed for the irradiation of dTMP in the presence of Ptr[7] and suggests that, as in the case of the nucleotide,

the main product detected here, under anaerobic conditions, contains the Ptr moiety. This means that, as expected, the phosphate does not participates in the photochemical process that leads to formation of the adduct.

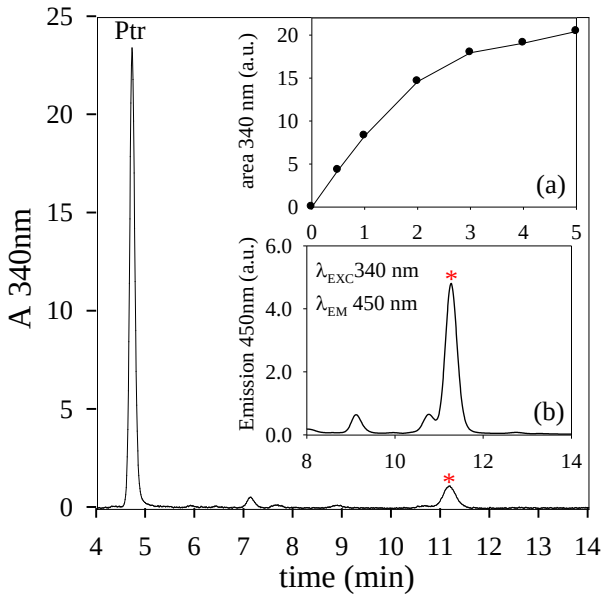

Figure 3. Chromatogram of an irradiated O_2-free aqueous solutions containing dT and Ptr, registered using the UV/vis detector (λ_{an}= 340 nm); t_{irr}= 4 h; $[dT]_0$=500 μM; $[Ptr]_0$=100 μM, pH= 5.5 Insets: a) evolution of the peak area (340 nm) of the photoproduct at t_R = 11.2 min (∗); b) Chromatogram obtained by HPLC-FL analysis (λ_{EXC} = 340 nm, λ_{EM} = 450 nm) after 4h of irradiation.

The experimental conditions were optimized to maximize the production of the adduct and then the compound was isolated collecting the corresponding fraction from HPLC chromatograms (see Experimental Section). The collected aqueous solutions were concentrated by passing a slow stream of nitrogen onto the surface of the solution or were completely dried and the solid was dissolved in H_2O or D_2O, according to specific experiments. After these treatments the purity of the resulting solutions was checked by HPLC. Aerated aqueous solutions of the isolated product were kept in the dark at 4°C in order to check its stability. During 10 days, no significant changes in the absorption spectrum of solution were observed.

In the same way, HPLC measurements showed that the product concentration did not decrease during this period of time.

Photophysical characterization of the adduct

The absorption spectrum of the isolated product presents the typical absorption band of the pterins, centered at ~ 340 nm (Table 1, Figure 4a) and is relatively close to the addition of the individual spectra of Ptr and dT. These results suggested that the adduct bears the intact chromophore of Ptr linked to the thymine moiety.

The emission spectrum (Figure 4b) and the fluorescence quantum yield (Φ_F, 0.29 ± 0.02) are similar to those corresponding to Ptr (Table 1). Fluorescence emission decay was fitted monoexponecially, and the lifetime was 7.2 ± 0.1 ns, which is very close to that reported for Ptr. Moreover, the fluorescence excitation spectrum of the adduct is very similar to the Ptr spectrum (Figure 4b). The fact that the fluorescence emission properties of the adduct are quite similar to those corresponding to free Ptr supports the hypothesis that the adduct bears the intact pterin moiety and indicates that the binding to the nucleobase does not significantly affect the singlet excited state of the Ptr.

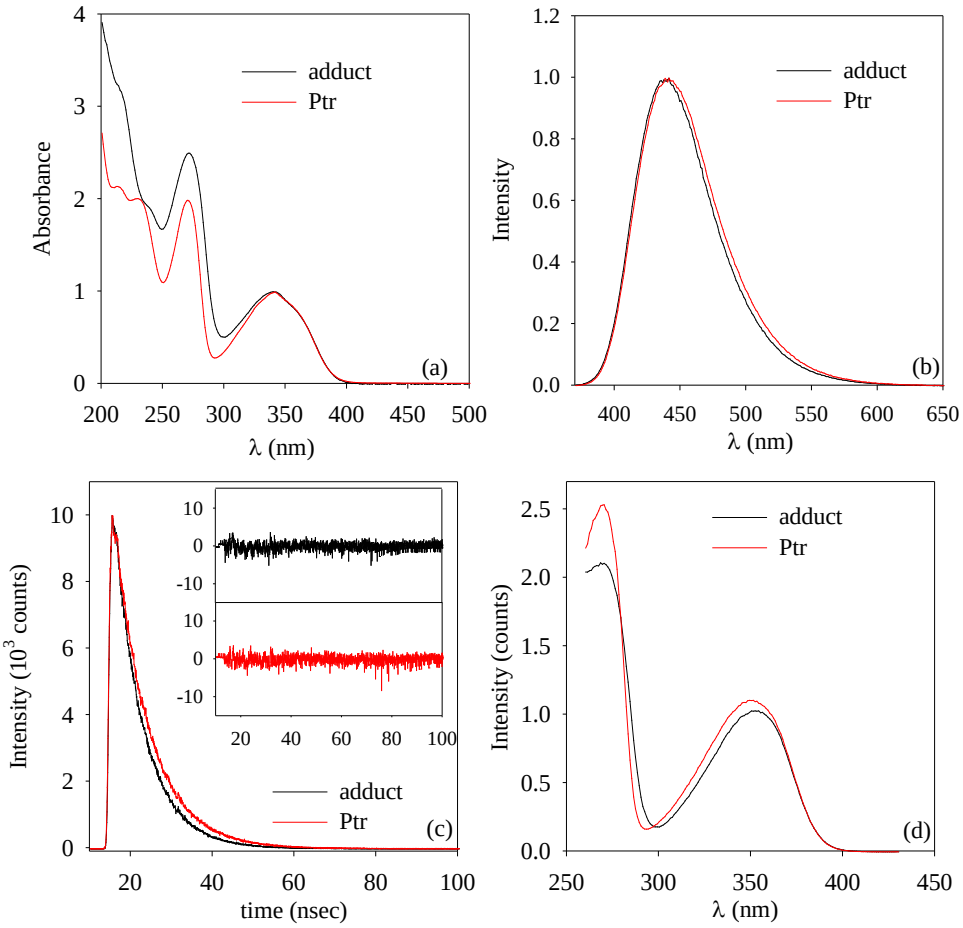

Figure 4: a) Normalized absorption spectra; b) Normalized emission spectra; c) Fluorescence decays (λ_{EM} = 450 nm);d) Excitation spectra (λ_{EM} = 440 nm)

	Ptr	**Adduct**
Absorption (λ_{MAX}, nm)	340	340
Emission (λ_{MAX}, nm)	440±1	437±1
Φ_F	0.32 ± 0.01	0.29 ± 0.02
τ_F (ns)	7.6 ± 0.4	7.2 ± 0.1
Φ_Δ	0.18 ± 0.02	0.07± 0.01

Table 1. Photophysical properties of Ptr and adduct

It was previously reported that Ptr is a good photosensitizer of singlet oxygen ($^1O_2$) under UV-A radiation,[23] with a quantum yield of $^1O_2$ production (Φ_Δ) of 0.18 ± 0.02 at pH

5.5. To find out if the adduct was able to generate $^1O_2$, an air-equilibrated D_2O solution (pD= 5.5) was irradiated at 350 nm and the typical infrared spectrum of $^1O_2$ was registered (Figure 5). The corresponding Φ_Δ for the adduct was found to be 0.07± 0.01, which is significantly lower than that reported for Ptr.

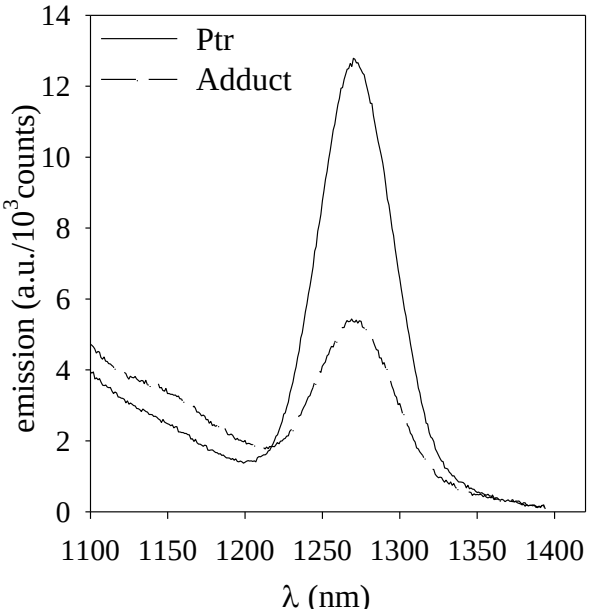

Figure 5. NIR emission spectra (aerated D_2O solutions, matched absorbance at λ_{EXC} = 350 nm, pD= 5.9). Solid lines, Ptr; dash lines, adduct.

Chemical structure of the adduct

Solutions containing Ptr and dT were analyzed by UPLC coupled to mass spectrometry (UPLC-QTof-MS, see Experimental Section), both in positive and negative ion modes (ESI^+ and ESI^-, respectively). Non irradiated solutions showed the signals corresponding to the intact molecular ions of Ptr and dT, as $[M + H]^+$ at *m/z* 164.0572 and 243.0981 in ESI^+ mode and as $[M - H]^-$ species at *m/z* 162.0415 and 241.0827 in ESI^- mode, respectively. The resolution was much better in ESI^- than in ESI^+ mode. Therefore all the results presented in this section correspond to mass spectrometry analysis carried out in the former mode.

Irradiated solutions were analyzed in the same way and the mass spectra corresponding to the chromatographic peaks of the reactants and photoproducts were registered. The main product showed a t_R value higher than those corresponding to the reactants and its mass spectrum showed a signal at *m/z* 402.1152, which may be assigned to a compound resulting from the addition of Ptr and dT ([Ptr + dT – 3H]$^-$ (Table 2).

To better study the chemical structure of the adduct the UPLC-QTof-MS equipment was used for tandem mass spectrometry (MS/MS) analysis. The MS/MS spectrum of the adduct in the ESI$^-$ mode (Figure 6, Table 2) showed an intense signal at *m*/*z* 286.0691, which can be attributed to a fragment bearing both the Ptr and the thymine base (Thy) moieties ([Ptr + Thy − 3H]$^-$). Additionally, signals at m/z 162.0419, 176.0593, 227.0577 and 241.0796 corresponding to the fragments of [Ptr − H]$^-$, ([Ptr + CH_3 − H]$^-$, [dT – CH_3]$^-$ and [dT – H]$^-$, respectively, were detected. According to this fragmentation pattern in the adduct the pterin moiety is linked to the methyl group of the thymine molecule.

Ion	Elemental composition	Observed m/z	Calculated m/z	Error (ppm)
Ptr-dT(–2H)				
[Ptr + dT – 3H]$^-$	$C_{16}H_{16}N_7O_6$	402.1152	402.1162	2.502
[Ptr + Thy − 3H]$^-$	$C_{11}H_8N_7O_3$	286.0691	286.0689	0.831
[Ptr − H]$^-$	$C_6H_4N_5O$	162.0419	162.0416	1.945
[Mep − H]$^-$	$C_7H_6N_5O$	176.0615	176.0572	24.226
[dT – CH_3 − H]$^-$	$C_9H_{11}N_2O_5$	227.0577	227.0668	40.061
[dT – H]$^-$	$C_{10}H_{13}N_2O_5$	241.0796	241.0824	11.807
MeP-dT(–2H)				
[Mep + dT – 3H]$^-$	C17H18N7O6	416.1298	416.1319	4.942
[MeP + Thy – 3H]$^-$	$C_{12}H_{10}N_7O_3$	300.0841	300.0845	1.374
[Mep – H]$^-$	$C_7H_6N_5O$	176.0538	176.0572	19.510
[Ptr – H]$^-$	$C_8H_8N_5O$	190.0727	190.0729	0.973
[dT – H]$^-$	$C_{10}H_{13}N_2O_5$	241.0740	241.0824	35.036

Table 2. Observed and calculated mass/charge ratios, empirical formula and mass errors of compounds and main fragments observed by MS/MS analysis

In another set of experiments 6-methylpterin (Mep) and 6,7-dimethylpterin (Dmp) were utilized as photosensitizers instead of Ptr. When Dmp was used, the adduct was not formed; whereas when Mep was used, the adduct was formed, but the yield was about a 56% of the yield obtained when Ptr was the photosensitizer. These results suggested that in the adduct the pterin moiety is linked to the nucleobase through positions 6 or 7.

Solutions irradiated using Mep as photosensitizer where analyzed by UPLC-QTof-MS. The main product with similar spectroscopic characteristics to Mep presents a signal at *m/z* 416.1298, which may be assigned to a compound resulting from the addition of Mep and dT ($[Mep + dT - 3H]^-$, Table 2). The MS/MS analysis (Figure 7, Table 2) showed an intense signal at *m*/*z* 300.0841, which can be attributed to a fragment bearing both the Mep and the Thy moieties ($[Mep + Thy - 3H]^-$). The fragments corresponding to Mep, Dmp and dT at *m*/*z* 176.0640, 190.0727 and 241.0740, respectively, were observed.

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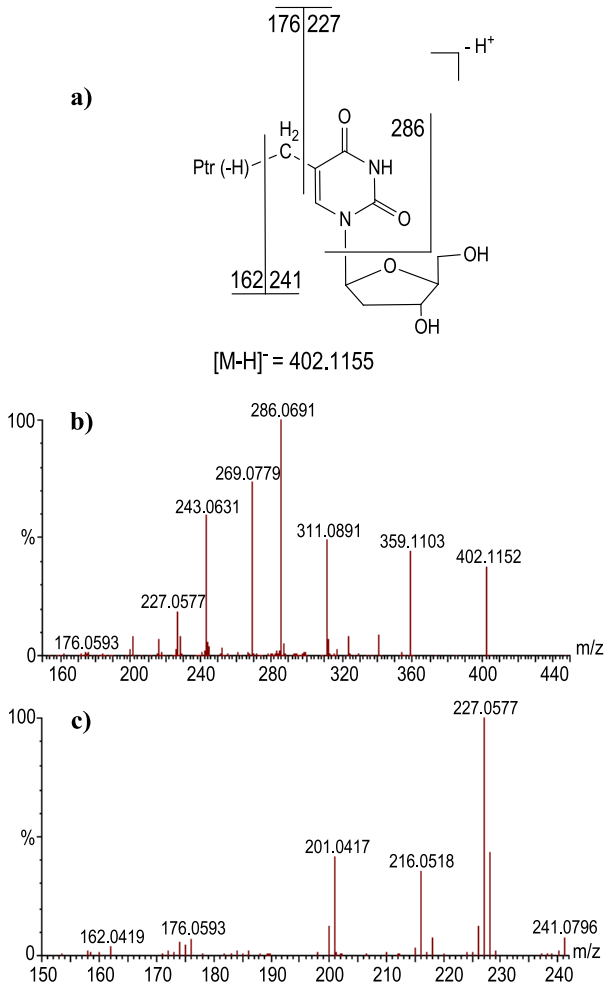


Figure 6. MS/MS analysis of the adduct formed by Ptr photosensitization of dT in the absence of O_2. a) Proposed chemical structure and fragmentation of the adduct; b) MS/MS spectra recorded in ESI^- mode in full scale (m/z between 150 and 450); c) detail of the MS/MS spectra (m/z between 150 and 243). Collision energy: 25 eV, $[dT]_0$ = 1 mM, [Ptr] = 150 μM, irradiation time = 4 h.

Taking into account that the photophysical studies indicated that the adduct has the intact chemical structure of Ptr and the mass spectrometry analysis, we can propose that the photochemical reaction between Ptr and dT under anaerobic conditions leads to the formation of two isomers, whose structures are depicted in Scheme 1.

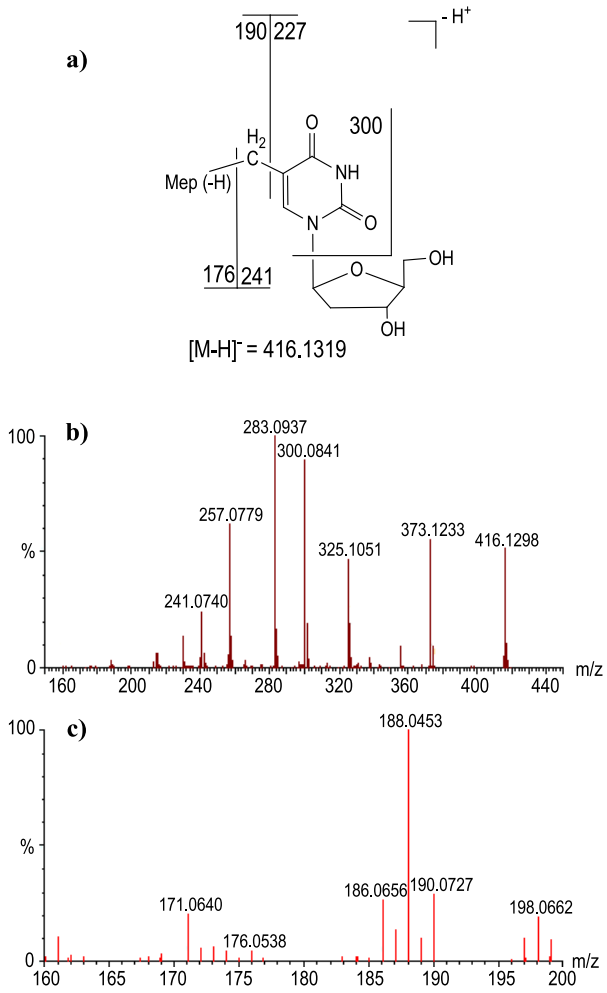
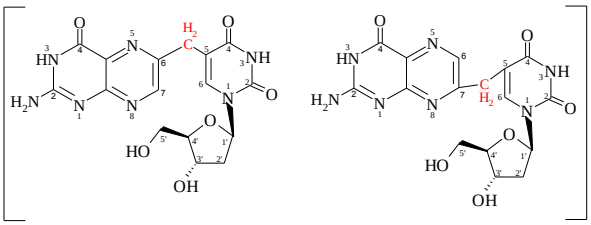


Figure 7. MS/MS analysis of the adduct formed by Mep photosensitization of dT in the absence of O_2. a) Proposed chemical structure and fragmentation of the adduct; b) MS/MS spectra recorded in ESI^- mode in full scale (m/z between 150 and 450); c) detail of the MS/MS spectra (m/z between 150 and 243). Collision energy: 25 eV. $[dT]_0$ = 1 mM, [Mep] = 100 μM, irradiation time = 4 h.

Conclusions

In this study we have identified novel compounds derived from the photoreaction between Ptr and dT. Considering mass spectrometry results we can propose that dT reacts with

Ptr to yield two structural isomers (Scheme 1). Summarizing, after absorption of UV-A radiation by Ptr, electron-transfer from dT to the Ptr triplet excited state (^{3}Ptr*) takes place to yield the corresponding radical pair, Ptr$^{\bullet-}$ and dT$^{\bullet+}$. In a second step, coupling of both radicals would yield a primary adduct containing a dihydropterin moiety (H_2Ptr – dT (-H)) where the dT unit is linked through the CH_2 group to position 6 or 7 of the 5,6-(or 7,8-)dihydropyrazine ring. Re-aromatization when the solution is exposed to air, would yield the observed fluorescent product Ptr-dT(-2H) where the aromatic structure of Ptr is regenerated.



Scheme 1. Proposed molecular structure for the product Ptr-dT(-2H)

The novel compounds, Ptr-dT(-2H), have a relative high fluorescence quantum yield (0.29) close to that of Ptr (0.32), indicating that Ptr moiety is undamaged. Moreover, Ptr-dT(-2H) photosensitizes the generation of $^1O_2$, with a Φ_Δ of 0.08. The present findings underscore the importance of the presence of fluorescent products that can be generated in environments with low oxygen concentration in thymidine-containing molecules including DNA.

Experimental Section

General

Pterin (Ptr) was purchased from Schircks Laboratories (Jona, Switzerland) and used without further purification. Deoxythymidine (dT), formic acid and other chemicals were

provided by Sigma-Aldrich and used without further purification. Acetonitrile was purchased 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). The pH of the aqueous solutions was adjusted by adding very small aliquots (few μL) of concentrated (0.1 – 2 M) HCl or NaOH solutions using a micropipette.

Steady-state irradiation

Aqueous solutions containing Ptr and dT were irradiated in fluorescence quartz cells (1 x 0.4 or 1 x 1 cm) at room temperature, using 3 Rayonet 3500RPR lamps emitting at 350 nm (bandwidth ≈ 20 nm) (Southern N. E. Ultraviolet Co.). The incident photon flux ($q_{p,0}$) was determined by actinometry using Aberchrome 540 as an actinometer[24],[25] ($q_{p,0}$ = 3.1(±0.1) x10^{-5} einstein L^{-1} cm^{-1}). Most of the experiments were performed in O_2 free solutions, obtained by bubbling for 20 min with Ar (Linde, purity > 99.998%), previously saturated in water.

Analysis of irradiated solutions

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

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

separation. For spectroscopic analysis, the adduct was isolated from HPLC runs (preparative HPLC), by collecting the mobile phase after passing through the PDA detector. In this case the solvent used was a solution of H_2O (pH = 3.3) and ACN (97:3).

Mass spectrometry analysis. The liquid chromatography equipment coupled to mass spectrometry (LC/MS) system was equipped with an UPLC chromatograph (ACQUITY UPLC from Waters), equipped with a UV/vis detector (Acquity TUV), coupled to a quadrupole time-of-flight mass spectrometer (Xevo G2-QTof-MS from Waters) (UPLC-QTof-MS), equipped with an electrospray ionization source (ESI). UPLC analyses were performed using an Acquity UPLC BEH C18 (1.7 μm; 2.1 × 50 mm) column (Waters), and isocratic elution with 25 mM formic acid (pH = 3.2) and ACN (97:3) at a flow rate of 0.5 mL min^{-1}. The mass spectrometer was operated in positive and negative ion modes. For high mass accuracy, the Q-Tof was calibrated using 0.1% phosphoric acid in 50:50 MeOH/H_2O (vol/ vol). The instrument drift was compensated by applying a lock mass correction. Therefore the samples were injected in the chromatograph, the components were separated and then the mass spectra were recorded for the 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 recorded using different scan ranges.

Emission studies

Fluorescence measurements. Steady-state and time-resolved fluorescence measurements were performed at room temperature using a single-photon-counting equipment FL3 TCSPC-SP (Horiba Jobin Yvon), described elsewhere.[26]

Singlet oxygen ($^1O_2$) detection. The experiments were carried out at room temperature using D_2O as a solvent since the lifetime of $^1O_2$ (τ_Δ) is much longer in D_2O than in H_2O.[27],[28] The sample solution (0.8 mL) in a quartz cell (1 cm x 0.4 cm) was irradiated was irradiated with a

CW 450W Xenon source. The luminescence in the near-infrared (NIR) region was detected at 90° with respect to the incident beam using the NIR PMT Module H10330-45 (Hamamatsu). Corrected emission spectra obtained by excitation at 340 nm were recorded between 950 and 1400 nm, and the total integrated $^1O_2$ phosphorescence intensities (IP) were calculated by integration of the emission band centered at ca. 1270 nm. The equipment was described in detail elsewhere.[19]

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Keywords: 2'-deoxythymidine • electron transfer • radicals • photosensitization • pterin

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