VIP DNA Photodamage

DOI: 10.1002/anie.201302176

Photosensitization of DNA by 5-Methyl-2-Pyrimidone Deoxyribonucleoside: (6-4) Photoproduct as a Possible Trojan Horse**

Victoria Vendrell-Criado, Gemma M. Rodríguez-Muñiz, M. Consuelo Cuquerella, Virginie Lhiaubet-Vallet, and Miguel A. Miranda**

Ultraviolet (UV) light is able to modify the chemical composition of natural DNA inducing changes in the structure of the nucleobases.[1] In spite of the efficiency of the DNA repair machinery,[2] some photodamage persists in the DNA and can result in mutagenesis, which is one origin of skin cancer.[1b,3] Within this context, pyrimidine (Pyr) dimers are among the most relevant DNA lesions caused by UV light exposure; they include cyclobutane pyrimidine dimers (CPDs) and the so called (6-4) photoproducts (6-4PPs).[4] CPDs arise from a formal [2+2] photocycloaddition between the C5-C6 double bonds of two adjacent Pyr nucleobases. 6-4PPs are formed by an initial Paternò-Büchi reaction between the C4 carbonyl (or imino) group of a 3′ Pyr and the C5-C6 double bond of another Pyr at the 5′ end, followed by oxetane (or azetidine) ring opening (see Figure 1).

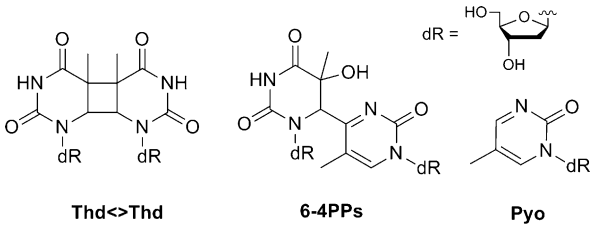


Figure 1. Formulas of thymidine cyclobutane dimers (Thd<>Thd), thymidine (6-4) photoproducts (6-4PPs) and 1-(β-D-2′-deoxyribosyl)-5-methyl-2-pyrimidone (Pyo).

The UV absorption of Pyr bases displays a characteristic band centered at 260 nm, which disappears in the CPDs as a result of cyclobutane ring formation. Conversely, 6-4PPs exhibit a new UV band appearing at 320 nm associated with the presence of a 5-methyl-2-pyrimidone chromophore. Selective irradiation of this moiety triggers a 4π electrocyclization that yields a Dewar valence isomer.[5]

With this background, it appears feasible that photogeneration of the pyrimidone chromophore within the double helix could actually constitute the insertion of a potential endogenous sensitizer absorbing in the UVA-UVB range. Thus, the 6-4PPs could act as Trojan horses, extending the active fraction of light and aggravating its harmful effects.

To test this concept, 1-(β-D-2′-deoxyribosyl)-5-methyl-2-pyrimidone (Pyo, Figure 1) was selected as a model of 6-4PPs, and its behavior as a photosensitizer was investigated. The Pyo was synthesized by a modification of a reported procedure (see Supporting Information).[6] Irradiation of Pyo was conducted upon addition of non-covalently bonded supercoiled circular DNA (pBR322), which is a useful method for detecting DNA damage. Native supercoiled form I is converted into circular form II after a single-strand break (SSB). The different electrophoretic mobility of both forms can be used to quantify the number of SSBs induced. Because CPDs cannot be directly observed as SSBs, a specific DNA repair enzyme must be added to reveal the damage. T4 endonuclease V cleaves the supercoiled DNA at the site of the dimers; this cleavage was used to monitor CPD formation mediated by Pyo (Figure 2). The agarose gel (inset) showed a consistent increase of DNA form II with irradiation time in the presence of Pyo. This trend was accentuated when the DNA and Pyo were incubated with T4 endonuclease V. SSB production without or with treatment with the enzyme were quantified by densitometry of the gel bands; the values after 30 minutes of irradiation were 20% and 45% of form II, respectively. Notably, the difference between these two values

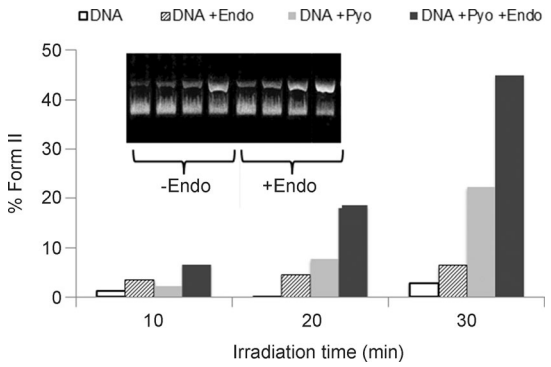


Figure 2. Formation of DNA form II upon irradiation of plasmid pBR322 in the presence or absence of Pyo (300 nm < λ_{irr} < 400 nm), treated or not with T4 endonuclease V (Endo). Inset: agarose gel of the irradiated mixtures of DNA + Pyo in the presence and in the absence of endonuclease.

[*] V. Vendrell-Criado, Dr. G. M. Rodríguez-Muñiz, Dr. M. C. Cuquerella, Dr. V. Lhiaubet-Vallet, Prof. M. A. Miranda
Instituto de Tecnología Química UPV-CSIC
Universitat Politècnica de València
Consejo Superior de Investigaciones Científicas
Avenida de los Naranjos s/n, 46022 Valencia (Spain)
E-mail: lvirgini@itq.upv.es
mmiranda@qim.upv.es

[**] We gratefully acknowledge support from theSpanish Government (CTQ2009-13699, CTQ2012-32621, RiRAAF RETICS RD12/0013/0009, Ramon y Cajal contract RyC2007-00476 to V.L.-V. and JAE-Predoc 2011-00740 to V.V.-C.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201302176.

corresponds to the amount of CPD formation. Overall, this experiment clearly demonstrates that Pyo indeed acts as a DNA photosensitizing agent.

Different mechanisms can be involved in photosensitized DNA damage; they include Pyr dimerization through triplet–triplet energy transfer (TTET) and purine oxidation mediated by reactive oxygen species (ROS).[7] In all cases, a detailed knowledge of the photophysical properties of an endogenous or exogenous agent is of paramount importance to evaluate its potential as a photosensitizer.

For this purpose, the photophysical properties of Pyo have been addressed. Thus, the absorption spectrum showed a UVB-UVA band centered at 310 nm in neutral aqueous solution, while the fluorescence spectrum displayed a band with a maximum at 380 nm (Figure 3). A singlet-state energy

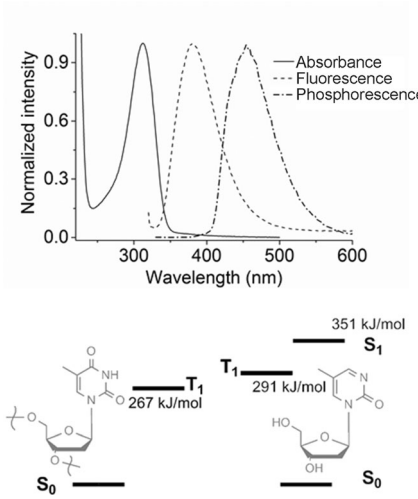


Figure 3. Top) Normalized absorbance and fluorescence spectra of Pyo in PBS. Phosphorescence spectrum in EtOH at 77 K. Bottom) Schematic representation of TTET from Pyo to Thd.

of 351 kJ mol^{-1} was determined from the intersection between the normalized fluorescence and excitation spectra. Moreover, a triplet-state energy of 291 kJ mol^{-1} was obtained from the phosphorescence spectrum recorded in ethanol at 77 K (Figure 3). Notably, this energy value is approximately 24 kJ mol^{-1} higher than that of thymine in DNA, determined by sensitization experiments.[7b,8]

Laser flash photolysis studies were conducted to establish the triplet excited-state behavior of Pyo. In this context, a transient absorption band at 420 nm, with a lifetime of 9.7 μs under a N_2O atmosphere (Figure 4), was observed following the laser pulse. The triplet nature of this transient species was confirmed by selective excitation of xanthone at 355 nm in the presence of Pyo. Under these conditions, the band corresponding to the xanthone triplet excited state (^{3}Xan*, λ_{max} = 620 nm) disappeared concomitantly with the appearance of the 420 nm band from the ^{3}Pyo* (see Supporting Information). Plotting the reciprocal of ^{3}Xan* lifetimes (τ) against Pyo concentration, a quenching rate constant (k_q) of $1.6\times10^9\,M^{-1}s^{-1}$ was obtained.

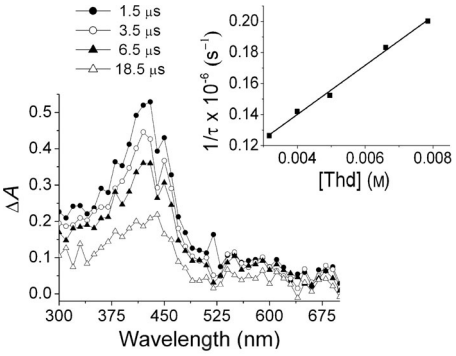


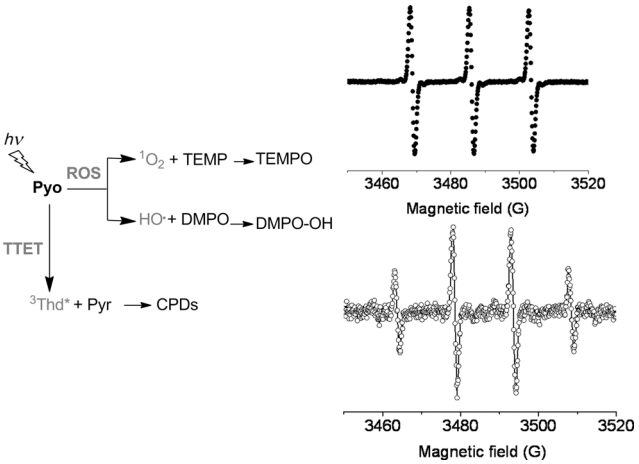
Figure 4. Laser flash photolysis of Pyo in PBS at different times after laser excitation under N_2O. Inset: Reciprocal of ^{3}Pyo* lifetimes in the presence of different Thd concentrations.

Following the photophysical characterization of ^{3}Pyo*, its involvement in a possible TTET to the Thd nucleoside was evaluated through quenching studies. In accordance with the small triplet–triplet energy gap between acceptor (Thd) and donor (Pyo), the k_q found from the Stern–Volmer plot (inset of Figure 4) was $1.6\times10^7\,M^{-1}s^{-1}$.

It is well established that TTET from a photosensitizer is at the origin of thymidine cyclobutane dimer (Thd<>Thd) formation.[7a,b] Irradiation of Thd/Pyo mixtures (1:1 molar ratio) in aqueous solution, ensuring selective excitation of Pyo with a monochromatic light source (λ_{exc} = 320 nm), was monitored by UV/Vis spectrophotometry through the decrease of the Thd absorption band with time. The reaction rate increased with the Thd/Pyo ratio. These photoreactions were also followed by HPLC-MS through detection of the corresponding Thd<>Thd sodium adduct ions at *m*/*z* 507. Analysis by UPLC coupled with MS/MS led to unambiguous identification of Thd<>Thd formation, based on the exact mass measurements (*m*/*z* 507.1693, obtained for $C_{20}H_{28}N_4O_{10}Na$) and on the fragmentation pattern.[9] Indeed, the main fragment ions were observed at *m*/*z* 391.1234 for $C_{15}H_{20}N_4O_7Na$ and *m*/*z* 275.0769 for $C_{10}H_{12}N_4O_4Na$, which correspond to the loss of one or two deoxyribose moieties, respectively (see Supporting Information).[9]

As mentioned above, oxidative processes mediated by ROS represent another relevant class of DNA damage. The possible generation of these reactive species was investigated by EPR using spin-trapping methods. In particular, singlet oxygen generation, anticipated from the oxygen quenching of ^{3}Pyo* observed in laser flash photolysis, was confirmed by irradiation of an aqueous solution of Pyo in the presence of 2,2,6,6-tetramethylpiperidine (TEMP), which gave rise to the corresponding stable free radical TEMPO.

This species became apparent in EPR as its characteristic triplet signal with a_N = 17.3 G and g = 2.0053 (see Scheme 1 and Supporting Information).[10] Moreover, an increase in the signal intensity was observed when water was replaced with D_2O, owing to the longer lifetime of $^1O_2$ in this solvent. The hydroxyl radical represents a highly reactive and biologically harmful ROS. Steady state photolysis of Pyo in the presence of the typical spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) showed the appearance of a quartet with $a_N = a_G =$



Scheme 1. Photochemical processes involved in DNA-damage mediated by Pyo. EPR spectra of TEMPO (top) and DMPO-OH (bottom) are shown on the right.

15.15 G, and $g = 2.0057$, assigned to formation of the DMPO-OH adduct (see Scheme 1 and Supporting Information).[11] Further evidence supporting HO$^\bullet$ production by Pyo upon absorption of UVB-UVA light was obtained by laser flash photolysis in the presence of terephthalic acid. This led to the growth of a signal absorbing at 350 nm that matched with the reported hydroxyl-terephthalic acid adduct (see Supporting Information).[12]

In summary, 5-methyl-2-pyrimidone is the main chromophore of the (6-4) photoproducts and behaves as a DNA photosensitizer. This has been clearly demonstrated by a combination of agarose gel electrophoresis and photochemical studies. Interestingly, the triplet excited state of a (6-4) lesion produced in the dinucleotide thymidylyl(3′→5′)thymidine, has been detected after 3.5 ns as a band centered at approximately 420 nm by ultrafast transient absorption spectroscopy.[5,13] As a consequence, the 6-4PPs can result in even more mutations than estimated, because they could be responsible for higher CPD formation and oxidative damage, acting as Trojan horses and extending the active fraction of light towards the UVA range.

Experimental Section

Triplet excited state of Pyo as sensitizer of Thd dimerization in DNA: Samples containing supercoiled circular DNA (pBR322; 5 μL; 20 μM of base-pairs) in the absence or presence of Pyo (0.7 mM) were used in the electrophoresis experiment. The samples were irradiated using a multilamp photoreactor with lamps emitting in the 300–400 nm range with a maximum at 355 nm. Next, they were incubated for 1 h at 37 °C with an excess of T4 endonuclease V. The samples were loaded on a 0.8% agarose gel containing ethidium bromide. After electrophoresis, the relative abundance of supercoiled DNA (form I) and relaxed DNA (form II) was quantified by densitometry.

Spectroscopic measurements: All the spectroscopic measurements were performed at room temperature using quartz cells of 1 cm optical path. The fluorescence of the samples was registered after adjusting their absorbance to approximately 0.1 at the excitation wavelength ($\lambda_{exc} = 313$ nm). Pyo was dissolved in ethanol, placed in a quartz tube and cooled at 77 K to obtain its phosphorescence spectrum.

Laser flash photolysis (LFP) was made using a pulsed Xe/HCl Excimer Laser ($\lambda_{exc} = 308$ nm) or a Nd:YAG laser ($\lambda_{exc} = 355$ nm). The single pulses were ca. 10 ns duration. A pulsed Xe lamp was employed as detecting light source. The LFP apparatus consisted of the pulsed laser, the Xe lamp, a monochromator, and a photomultiplier made up of a tube, housing, and power supply. The output signal from the oscilloscope was transferred to a personal computer.

The transient absorption spectrum of Pyo in PBS was obtained after direct excitation at 308 nm. The samples were purged with N_2O and their absorbance at the excitation wavelength adjusted to approximately 0.3.

Thymidine was used as a quencher for the triplet states of Pyo. The experiment was carried out adding increasing amounts of the nucleoside (from 1.3 mM to 7.9 mM) to a solution of Pyo and monitored at 420 nm.

Detection of HO$^\bullet$ generation was achieved by excitation of Pyo (8.5×10^{-2} mM) in the presence of terephthalic acid (1 mM) in PBS solutions purged with N_2. The decay of the terephthalic acid-OH adduct was monitored at 350 nm.

Moreover, quenching of ^{3}Xan* by Pyo was also performed using the Nd:YAG laser as the excitation source. With this purpose, Pyo (from 0 mM to 0.5 mM) was added to a water/acetonitrile 2:1 (*v*/*v*) Xan solution, and the traces at 420 and 520 nm were registered, corresponding to ^{3}Pyo* and ^{3}Xan*, respectively.

Steady-state photolysis: Thymidine (2.2×10^{-4} M) was added to a solution of Pyo (1.0×10^{-4} M) in PBS. The solution was placed in a quartz cuvette and de-aerated by bubbling N_2. Irradiation was performed with a Xe lamp equipped with a monochromator to ensure monochromatic light irradiation at 320 nm. The progress of the reaction was monitored by UV/Vis and UPLC MS/MS. A parallel experiment was performed using a 1:1 Pyo/Thd ratio under the same conditions.

UPLC MS/MS was performed on a XevoQToF spectrometer connected to the UPLC system through an electrospray ionization (ESI) interface. The ESI source was operated in positive ionization mode using leucine-enkephalin as the lock mass ($[M+H]^+$ ion *m*/*z* 556.2771). The separation was carried out on an ACQUITY UPLC HSS T3 C18 column (150 mm × 2.1 mm i.d., 1.8 μm). The column temperature was maintained at 40 °C. The analysis was done using a methanol/water mixture (containing 0.01% formic acid) 20:80 as mobile phase and a flow of 0.2 mL min^{-1}. More detailed information is provided in the Supporting Information.

Received: March 14, 2013
Published online: May 9, 2013

Keywords: cyclobutane pyrimidine dimers · DNA damage · photochemistry · pyrimidones · triplet excited states

[1] a) G. P. Pfeifer, A. Besaratinia, *Photochem. Photobiol. Sci.* **2012**, *11*, 90–97; b) O. D. Schärer, *Angew. Chem.* **2003**, *115*, 3052–3082; *Angew. Chem. Int. Ed.* **2003**, *42*, 2946–2974; c) A. T. Johnson, O. Wiest, *J. Phys. Chem. B* **2007**, *111*, 14398–14404.

[2] a) Z. Liu, X. Guo, C. Tan, J. Li, Y.-T. Kao, L. Wang, A. Sancar, D. Zhong, *J. Am. Chem. Soc.* **2012**, *134*, 8104–8114; b) J. Li, Z. Liu, C. Tan, X. Guo, L. Wang, A. Sancar, D. Zhong, *Nature* **2010**, *466*, 887–890; c) Z. Liu, C. Tan, X. Guo, Y.-T. Kao, J. Li, L. Wang, A. Sancar, D. Zhong, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 14831–14836; d) C. B. Harrison, L. L. O'Nei, O. Wiest, *J. Phys. Chem. A* **2005**, *109*, 7001–7012.

[3] a) E. Sage, P.-M. Girard, S. Francesconi, *Photochem. Photobiol. Sci.* **2012**, *11*, 74–80; b) H. Ikehata, T. Ono, *J. Radiat. Res.* **2011**, *52*, 115–125; c) C. Dessinioti, C. Antoniou, A. Katsambas, A. J. Stratigos, *Photochem. Photobiol.* **2010**, *86*, 481–491.

[4] a) J. Cadet, S. Mouret, J.-L. Ravanat, T. Douki, *Photochem. Photobiol.* **2012**, *88*, 1048 – 1065; b) K. Heil, D. Pearson, T. Carell, *Chem. Soc. Rev.* **2011**, *40*, 4271 – 4278.
[5] K. Haiser, B. P. Fingerhut, K. Heil, A. Glas, T. T. Herzog, B. M. Pilles, W. J. Schreier, W. Zinth, R. de Vivie-Riedle, T. Carell, *Angew. Chem.* **2012**, *124*, 421 – 424; *Angew. Chem. Int. Ed.* **2012**, *51*, 408 – 411.
[6] a) D. Cech, A. Holy, *Collect. Czech. Chem. Commun.* **1977**, *42*, 2246 – 2260; b) B. Gildea, L. W. McLaughlin, *Nucleic Acids Res.* **1989**, *17*, 2261 – 2281; c) R. Wightman, A. Holy, *Collect. Czech. Chem. Commun.* **1973**, *38*, 1381 – 1396.
[7] a) M. C. Cuquerella, V. Lhiaubet-Vallet, J. Cadet, M. A. Miranda, *Acc. Chem. Res.* **2012**, *45*, 1558 – 1570; b) M. C. Cuquerella, V. Lhiaubet-Vallet, F. Bosca, M. A. Miranda, *Chem. Sci.* **2011**, *2*, 1219 – 1232; c) G. M. Rodríguez-Muñiz, M. L. Marin, V. Lhiaubet-Vallet, M. A. Miranda, *Chem. Eur. J.* **2012**, *18*, 8024 – 8027.
[8] F. Bosca, V. Lhiaubet-Vallet, M. C. Cuquerella, J. V. Castell, M. A. Miranda, *J. Am. Chem. Soc.* **2006**, *128*, 6318 – 6319.
[9] T. Douki, M. Court, J. Cadet, *J. Photochem. Photobiol. B* **2000**, *54*, 145 – 154.
[10] Y. Lion, M. Delmelle, A. Van de Vorst, *Nature* **1976**, *263*, 442 – 446.
[11] A. G. Motten, G. R. Buettner, C. F. Chignell, *Photochem. Photobiol.* **1985**, *42*, 9 – 15.
[12] A. Samuni, S. Goldstein, A. Russo, J. B. Mitchell, M. C. Krishna, P. Neta, *J. Am. Chem. Soc.* **2002**, *124*, 8719 – 8724.
[13] B. P. Fingerhut, T. T. Herzog, G. Ryseck, K. Haiser, F. F. Graupner, K. Heil, P. Gilch, W. J. Schreier, T. Carell, R. de Vivie-Riedle, W. Zinth, *New J. Phys.* **2012**, *14*, 065006.

