Aparici-Espert, MI.; García-Laínez, G.; Andreu Ros, MI.; Miranda Alonso, MA.; Lhiaubet, VL. (03-2). Oxidatively Generated Lesions as Internal Photosensitizers for Pyrimidine Dimerization in DNA. ACS Chemical Biology. 13(3):542-547.
https://doi.org/10.1021/acschembio.7b01097
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ACS Chem. Biol., Just Accepted Manuscript • DOI: 10.1021/acschembio.7b01097 • Publication Date (Web): 04 Jan 2018

Downloaded from http://pubs.acs.org on January 7, 2018

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Oxidatively Generated Lesions as Internal Photosensitizers for Pyrimidine Dimerization in DNA

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KEYWORDS: cyclobutane thymine dimers, DNA damage, energy transfer, photochemistry, Trojan horse
ABSTRACT

In this work, the attention is focused on UVA-photosensitized reactions triggered by a DNA chromophore-containing lesion, namely 5-formyluracil. This is a major oxidatively generated lesion that exhibits an enhanced light absorption in the UVB-UVA region. The mechanistic study combining photochemical and photobiological techniques shows that irradiation of 5-formyluracil leads to a triplet excited state capable of sensitizing formation of cyclobutane pyrimidine dimers in DNA via a triplet-triplet energy transfer. This demonstrates for the first time that an oxidatively generated DNA damage can behave as an intrinsic sensitizer and result in an important extension of the active fraction of the solar spectrum with photocarcinogenic potential. Overall, this raises the question of an aggravated photomutagenicity of the 5-formyluracil lesion.

TABLE OF CONTENTS

![Diagram showing the formation of cyclobutane pyrimidine dimers]
The DNA of all living organisms is continuously damaged by endogenous processes as well as by exogenous genotoxic chemicals and physical agents \textit{ie.} UV and ionizing radiation. The resulting chemical changes induce complex cell responses that can finally end in gene mutation and cancer development.\textsuperscript{1}

Oxidative processes are among the most frequently involved in DNA damage formation, 8-oxo-7,8-dihydroguanine being considered as the signature of such reactions. However, the importance of 5-formyluracil (ForU) should not be neglected, as it represents the major oxidation product of pyrimidine bases.\textsuperscript{2-3} Indeed, ForU is part of the most important lesions formed under \(\gamma\)-radiation, after those derived from guanine oxidation.\textsuperscript{2-4} ForU has also been obtained in significant yields as a result of UVA irradiation of DNA in the presence of Type I photosensitizers as menadione, benzophenone or a nitro substituted naphthalimide.\textsuperscript{3, 5} Interestingly, ForU is produced during endogenous processes as an intermediate of thymine oxidation catalyzed by thymine hydroxylase, an enzyme that belongs to the metabolic and catabolic dioxygenases found in a wide range of organisms including bacteria, yeast, plants, and humans.\textsuperscript{6}

The presence of ForU residues in the DNA molecule is not innocuous and induces miscoding during replication with a relatively high frequency.\textsuperscript{7-10} This has been attributed to the strong electron withdrawing formyl substituent at C5 that increases the acidity of N3 proton and thus affects the Watson-Crick interactions involved in base pairing.\textsuperscript{11} Moreover, ForU is able to form covalent cross-links with proteins through the particular chemistry of the aldehyde functional group with amino or thiol-containing amino acids.\textsuperscript{12-13}

From a photochemical point of view, the presence of formyl group at C5 might be relevant because it is expected to affect the distribution and nature of the excited
states by comparison with those of thymine having the unaltered C5-methyl group. This is of special interest in connection with our recent finding that the (6-4) photoproduc, obtained by direct DNA irradiation, is able to act as an intrinsic photosensitizer and to generate secondary photodamages in its neighborhood.\textsuperscript{14-15} In principle, other primary lesions could also play this role, but this would require that the initially formed damage fulfils the basic properties of efficient DNA photosensitizers, which include to exhibit a UVB/UVA absorption that extends to longer wavelength regions to allow selective excitation, and to generate a reasonably populated sufficiently energetic triplet excited state. With this background, we have investigated for the first time the potential of an oxidatively generated lesion, ForU, to behave as an internal DNA photodamaging agent. In this context, the photophysical study of ForU was run out focusing the attention on the two main relevant points for intrinsic photosensitizers, \textit{i.e.} extended UV absorption toward the UVA region and formation of a triplet excited state.

As shown in Figure 1A, ForU presents a main absorption band in phosphate buffer (pH 7.4) with maximum at \textit{ca.} 297 nm and a tail that reaches up to almost 350 nm. Thus, ForU fulfils the first important characteristic as its absorption is red shifted in comparison with the characteristic UVC absorption of DNA, allowing for an extended action spectrum together with a selective excitation in the UVB/UVA region.
Figure 1. (A) UV-Vis absorption spectrum of ForU in PBS (green line) and phosphorescence emission spectrum in EtOH at 77 K (red line). Inset: chemical structure of ForU. (B) Phosphorescence emission spectra of ForU in PBS alone (black line) or in the presence of calf thymus DNA (1 mM in bases, pink line).

As regards the issue dealing with the possible generation of the ForU triplet excited state (\(^3\)ForU), laser flash photolysis was performed in nitrogen flushed phosphate buffer (Nd:YAG, 266 nm). The obtained transient spectrum showed a broad signal from 320 to 560 nm with maximum at ca. 460 nm (Figure 2). This species was effectively quenched by oxygen (Figure 2, inset), with a lifetime of 1.75 µs under nitrogen atmosphere, 0.56 µs for air conditions and 0.15 µs when the solution was saturated with oxygen. The corresponding bimolecular rate constant \(k_{\text{q}}(O_2)\) obtained from the Stern Volmer plot was of ca. \(4 \times 10^9\) M\(^{-1}\) s\(^{-1}\). Thus, this transient was assigned to the triplet-triplet transition of ForU.
Figure 2. Transient absorption spectra of a N₂-bubbled solution of ForU (7.7 mM) in PBS obtained at different times after 266 nm laser excitation. Inset: Decays monitored at 460 nm under N₂ (black), air (red) and O₂ (blue) atmosphere.

In addition, formation of singlet oxygen (¹O₂) as a result of ³ForU quenching was studied by means of EPR experiments, using TEMP (2,2,6,6-tetramethylpiperidine) as specific spin trap. The well-established triplet signal of TEMPO free radical (g=2.006, aN= 17.3 G) was observed after irradiation of a ForU aqueous solution in the presence of TEMP (see Figure 1S in Supporting Information).¹⁶ Thus, ForU can act as a DNA oxidative agent through generation of reactive oxygen species able to induce formation of 8-oxo-7,8-dihydroguanine.

Next, the ³ForU energy was determined by phosphorescence experiments performed in EtOH at 77 K. The spectrum exhibits a large band centered at 416 nm (Figure 1A, red line). A triplet excited state energy (Eₜ) of ca. 314 kJ mol⁻¹ was obtained from the wavelength corresponding to the 20 % of the emission intensity. Upon complexation with DNA, a small blue-shift was observed in the phosphorescence band (see Figure 1B); this is consistent with the observations recently reported for other photosensitizers and points to a slight increase of the triplet energy within DNA.¹⁷ This range of Eₜ values is somewhat higher than that of isolated Thd in bulk solution (Eₜ of
ca. 310 kJ mol\(^{-1}\))\(^{18}\) but, it is more than 40 kJ mol\(^{-1}\) above that determined for Thy in DNA.\(^ {17, 19}\) Thereby, the high \(E_T\) value of \(^3\)ForU makes it a feasible energy donor for a triplet–triplet energy transfer to the thymine nucleobase, which once excited can react with a ground state counterpart to form the well-known cyclobutane thymine dimers (\(T<>T\)).

The occurrence of such a process was first investigated using a model dyad (Thy-Thy, Scheme 1) containing two covalently linked thymine units. The synthesis of this \(N\)-methylated thymine dyad was performed adapting the described protocol.\(^ {20-21}\) A solution containing Thy-Thy (8.7 mM) and ForU (5.3 mM) was monochromatically photolyzed at \(\lambda=320\) nm in a deaerated mixture of water:acetonitrile (1:1, v:v), and the kinetics of the reaction was followed by reverse phase HPLC. As shown in Figure 3, the irradiation gives rise to a clean photoreaction where the peak of Thy-Thy eluting at 22 min decreases with irradiation time while only one product eluting at 12 min is formed. The photoproduct was assigned to the \(cis\)-\(syn\) cyclobutane thymine dimer of the dyad (Thy<>Thy, Scheme 1) by comparison with the synthesized compound (see Supporting Information). It is noteworthy that little if any photodegradation of ForU occurred during the employed reaction time. Control experiments based on irradiation of Thy-Thy alone at the same wavelength showed a very slow degradation ruling out that significant formation of Thy<>Thy in the presence of ForU occurs by direct excitation (see Figure 2S of Supporting Information).
Scheme 1. Model system used to study pyrimidine cyclobutane dimer formation.

Figure 3. HPLC chromatograms obtained for ForU:Thy-Thy (5.3 mM: 8.7 mM) in H$_2$O:CH$_3$CN (1:1, v:v) irradiated from 0 to 3h30 at 320 nm.

The photoreaction was also followed by NMR spectroscopy in CD$_3$CN:D$_2$O (1:1, v:v) as solvent (Figure 4). Likewise, a clean process was observed with this technique. The [2+2] photocycloaddition reaction results in saturation of the C5-C6 double bond and provokes characteristic changes of the proton signals. Differences were observed not only for the protons of the nucleobase but also for those of the methylene bridge.
Concerning the thymine moiety, the diagnostic H6 proton moves from 7.75 to 4.35 ppm while the signal of the methyl group protons at C5 is shifted from 2.25 to 1.80 ppm (signals at 3.3 ppm, 2.2 ppm and 1.6 ppm correspond to the protons of the trimethylene bridge). The integral of both signals was used, as a double check, to monitor the reaction course, reaching after 7 h of irradiation a chemical yield for Thy<>Thy formation of ca. 60 %.
**Figure 4.** $^1$H NMR spectra in D$_2$O:CD$_3$CN (1:1, v:v) (A) pure Thy-Thy (red line) and Thy<>Thy (green line). Equimolar mixture of Thy-Thy and ForU (7x10$^{-3}$ M) irradiated 4h (B) or (C) 7 h.

In addition, a dyad containing two cytosines (Cyt-Cyt, Scheme 1) was also synthesized and irradiated monochromatically at 320 nm in the presence of ForU. The kinetics of the reaction, followed by HPLC (Figure 5), showed that Thy-Thy reacts much faster than Cyt-Cyt. These data are in agreement with the triplet excited state energy of the two bases, as $^3$Cyt lies ca. 20 kJ mol$^{-1}$ above $^3$Thy.$^{22}$ Formation of the uracil homodimers after deamination was revealed through UPLC-HRMS experiments (see Figure 3S in Supporting Information), which allowed detection of the exact mass $m/z$ 265.0929 corresponding to the formula C$_{11}$H$_{13}$N$_4$O$_4$. 
Figure 5. Time-dependent photodegradation of Thy-Thy (black) and Cyt-Cyt (red) in the presence of ForU ($\lambda_{irr}=320$ nm).

The obtained results revealed that ForU is able to photosensitize the dimerization of pyrimidines in bulk solution. Thus, the next step was to confirm the occurrence of this process when the whole DNA molecule is the target. For this purpose, experiments were carried out on a plasmid DNA using agarose gel electrophoresis. This technique allows a rapid detection of single strand breaks (ssb) that induce the conversion of native supercoiled form I into the circular form II. Quantitation of the cleaved proportion of DNA is performed by densitometry taking advantage of the different electrophoretic mobility of both forms. A limitation of this technique relies on the preferential detection of lesions, mainly resulting from an oxidative pathway, that end in ssb formation. However, treatment with selective enzymes, which specifically recognize the damage and cleave the DNA backbone at its site, brings a reliable solution to this problem. In this context, T4 endonuclease V was used to reveal the photosensitization of cis-syn cyclobutane pyrimidine dimers (Pyr<>Pyr) by ForU.

A first experiment consisted of the estimation of ssb obtained by UVA irradiation of ForU (25 or 50 $\mu$M) in the presence of plasmid DNA (pBR322, 38 $\mu$M in bp). As shown in Figure 6, the presence of ForU induced a concentration and dose dependent formation of ssb, reaching after 15 minutes of irradiation values of 30 and
50 % for 25 and 50 µM, respectively. These DNA backbone ruptures should result from sugar oxidation through formation of radicals derived from ForU excitation. Next, formation of Pyr<>Pyr was studied using a ForU concentration of 40 µM that provided a good balance between relevant Pyr<>Pyr formation and low ssb. The obtained results are shown in Figure 6B, which demonstrate that ForU can indeed act as a triplet energy donor to generate Pyr<>Pyr (mainly Thy<>Thy) in DNA.

Figure 6. (A) Agarose gel electrophoresis for single strand break (ssb) formation of UVA-irradiated samples of pBR322 (38 µM in bp) alone or in the presence of ForU (25 or 50 µM). (B) Quantitation of ssb formation for UVA-irradiated samples of pBR322 (38 µM in bp) alone or in the presence of ForU (25 or 50 µM in black and grey, respectively). Inset: Quantitation of cyclobutane pyrimidine dimer formation for UVA-irradiated samples of pBR322 (38 µM in bp) in the presence of ForU (40 µM).

In summary, the present work has demonstrated that 5-formyluracil can be...
excited in the UVB-UVA region, to give a highly energetic (>314 kJ mol\(^{-1}\)) triplet excited state. This species is characterized by its low temperature phosphorescence emission in solid matrix (\(\lambda_{\text{em}} \approx 415\ \text{nm}\)), as well as by laser flash photolysis in solution at room temperature (\(\lambda_{\text{max}} \approx 460\ \text{nm}\)). Triplet-triplet energy transfer from ForU to thymine, both in a model dyad and in isolated DNA, leads to formation of cyclobutane pyrimidine dimers. This constitutes the first example of an oxidatively generated lesion acting as internal photosensitizer for pyrimidine dimerization in DNA. In principle, the closely related 5-formylcytosine, derived from hydroxyl radical and one electron oxidation of the epigenetic mark precursor 5-methylcytosine, might also present remarkable photosensitizing properties; this possibility is currently being investigated.

METHODS

Reagents and solvents

5-Formyluracil (ForU), thymine (Thy), cytosine, TEMP, sodium hydride, iodomethane, 1,3-dibromopropene, agarose, phosphate-buffered saline (PBS) tablets, ethanol and acetonitrile were purchased from Carbosynth and Sigma-Aldrich, and used as received. Acetonitrile was dried with a SPS system. DNA pBR322 was obtained from Roche and T4 endonuclease V from Werfen.

Characterization

The \(^1\)H and \(^{13}\)C NMR spectra were measured with a 300 MHz instrument, and CDCl\(_3\) was used as solvent for all the spectra of synthesized compounds. The solvent signal was taken as the reference using a chemical shift (\(\delta\)) of \(\approx 7.26\ \text{ppm}\) and 77.16 ppm for \(^1\)H NMR and \(^{13}\)C NMR, respectively. Coupling constants (\(J\)) are given in Hz.
Exact mass values were determined by using a Waters ACQUITY™ XevoQToF spectrometer (Waters Corp.) connected to the UPLC system via an electrospray ionization (ESI) interface. The ESI source was operated in positive or negative ionization mode with the capillary voltage at 3.0 kV. Leucine-enkephalin was used as the lock mass generating an [M+H]+ ion (m/z 556.2771) or [M-H]- ion (m/z 554.2615) at a concentration of 500 pg/mL and a flow rate of 20 µL/min to ensure accuracy during the MS analysis. For Cyt-Cyt experiments, an Acquity UPLC HSS T3 column (150 mm × 2.1 mm, 1.8 µm) was employed with an injection volume of 1 µL. The mobile phase was increased from 95% water (acidified with 0.1% formic acid) and 5% acetonitrile (acidified with 0.1% formic acid) to 100 % of acetonitrile (acidified with 0.1% formic acid) in 10 min, during all the gradient a flow rate of 0.3 mL min⁻¹ was used.

**Photophysical instrumentation**

*UV-Vis absorption.* UV absorption spectra were registered on a Cary 50 spectrophotometer (Varian) using a quartz cuvette of 1 cm of optical path and 3 mL capacity.

*Steady-state photolysis.* Monochromatic irradiation (λ_{irr}= 320 nm) experiments were carried out with a Xenon lamp (150 W for HPLC experiments and 75 W for NMR) equipped with a monochromator from Photon Technology Instruments (model 101). The dimerization of the model dyad Thy-Thy was performed with Thy-Thy (8.7 mM) and ForU (5.3 mM) in a mixture (1:1, v:v) of milliQ water : acetonitrile.

For experiments with plasmid DNA, a multilamp Luzchem photoreactor equipped with 355 nm lamps was used as irradiation source. The UVA dose received for t = 5, 10 and 15 min was 2.3, 4.5 and 6.8 J/cm², respectively (ie. irradiance of 7.5 mW/cm²).
**EPR trapping measurements.** The measurements were performed in a Wildman Suprasil/aqueous quartz-ware flat cell (volume 150 µl, length 60 mm) with a Bruker EMX10/12 EPR spectrometer, using the following parameters: microwave power, 20 mW; modulation amplitude, 1.0 G; and modulation frequency, 100 kHz. Aerated water solutions of 10 mM TEMP containing a 0.35 mM of ForU were irradiated at 290 nm using the monochromatic system described above.

**Laser flash photolysis (LFP).** Experiments were carried out with a pulsed Nd:YAG (L52137 V LOTIS TII) laser system instrument setting 266 nm as excitation wavelength. The pulse duration was of ca. 10 ns and the energy was adjusted at 26 mJ pulse\(^{-1}\). The apparatus is composed of a pulsed laser, a Xe lamp, a 77250 Oriel monochromator and a photomultiplier. The output signal from a Tektronix oscilloscope was transferred to a personal computer. The transient spectra were recorded at room temperature employing quartz cells of 1 cm optical path length. Experiments were conducted in PBS solutions with a ForU concentration of 7.7 \times 10^{-5} \text{ M}.

**Phosphorescence emission.** The phosphorescence experiments were performed on solutions of ForU in ethanol with an absorbance of 0.8 at the excitation wavelength of 320 nm (value determined for a 1 cm optical path). Samples of ForU (\(A_{320}\) of ca. 0.8) in PBS in the absence or in the presence of calf thymus DNA (1 mM bases) were also prepared. Then, the solution was transferred to a quartz tube of 5 mm diameter. The emission was measured at 77 K, gate time 50 µs, delay 500 µs.

**HPLC analysis**

The irradiated mixtures were analyzed by reverse phase HPLC using a Varian ProStar instrument equipped with a diode array detector which covers a detection range from 200 to 400 nm. For Thy-Thy experiments, a Mediterranea Sea C18 column (250 mm ×
4.6 mm, 5 µm) was employed with an injection volume of 10 µL (removed from the cuvette at different irradiation times). The mobile phase was an isocratic mixture of 80 % water and 20 % acetonitrile and a flow rate of 1 mL min⁻¹. For Cyt-Cyt experiments, a Synergi Polar-RP 80 Å column (150 mm × 4.6 mm, 4 µm) was employed with an injection volume of 10 µL (removed from the cuvette at different irradiation times). The mobile phase was an isocratic mixture of 98 % aqueous ammonium acetate (20 mM) and 2 % acetonitrile and a flow rate of 1 mL min⁻¹. All the analysis were run with a monitoring wavelength λ=240 nm, which allows detection of all the compounds. Their respective concentrations were determined from calibration curves obtained from the pure compounds.

The samples were prepared using a mixture of H₂O:acetonitrile (1:1, v:v) as solvent, flushed with N₂ and irradiated with monochromatic light at 320 nm. Three experiments were carried out: 1) Thy-Thy dyad and ForU mixture (8.7 mM: 5.3 mM), 2) control experiment of Thy-Thy dyad irradiated alone (7.7 mM) and 3) control experiment of ForU irradiated alone (6.4 mM).

¹H NMR Kinetic study of thymine dimer formation from Thy-Thy model dyad.

A mixture of Thy-Thy model dyad and ForU (1:1) (7 mM) were dissolved in D₂O:CD₃CN in a 1:1 ratio (v:v) and monochromatically irradiated at 320 nm (with 75W Xe lamp system). The irradiation was followed by ¹H NMR spectroscopy after 0, 2, 4 and 7 hours. The signal of D₂O was used as reference with a chemical shift δ of ca. 4.79 ppm. The yield of formation was determined from comparing the integral of the singlet signal at 4.35 ppm that corresponds to two protons for the CPD product (Hdim) and the singlet at 7.75 ppm which integrated for 2 protons of the model dyad Thy-Thy (HThy-
Thy); as well as the integral of methyl group at C5 at δ 2.25 and 1.8 ppm for Thy-Thy and Thy<>Thy, respectively.

**Plasmid DNA damage - Agarose Gel Electrophoresis**

Samples containing 5 µL (9 nM, 38 µM in base pair) of supercoiled circular DNA (pBR322, 4361 base pairs) in absence or presence of ForU (from 25 to 50 µM) were employed in the electrophoresis experiments. The samples were irradiated using a multilamp photoreactor with fluorescent tubes emitting in the 300-400 nm range with a maximum at 355 nm. For measurement of pyrimidine dimer formation, the samples were next incubated for 1 h at 37 ºC with an excess of T4 endonuclease V. Finally, the samples were loaded on a 0.8 % agarose gel containing SYBR Safe. After electrophoresis, the relative abundance of supercoiled DNA (form I) and relaxed DNA (form II) was quantified by densitometry. The mean data were obtained from the results of three independent experiments.

**FUNDING SOURCES**

The present work was supported by Spanish Government (CTQ2015-70164-P, Severo Ochoa program/SEV-2012-0267, BES-2013-066566, CSIC 201680I007), Instituto de Salud Carlos III (RD16/0006/0030, FIS PI16/01877), Generalitat Valenciana (Prometeo/2017/075).

**SUPPORTING INFORMATION**

The Supporting Information is available free of charge on the ACS Publications website [http://pubs.acs.org](http://pubs.acs.org) at DOI: xxxxx
This includes the synthesis of the model dyads Thy-Thy, Thy<>Thy and Cyt-Cyt, the EPR spectra obtained in the presence of TEMP, the HPLC and UPLC analysis and, NMR spectra of the synthesized compounds.

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REFERENCES


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