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5-Carboxy-2′-deoxyuridine, a new photooxidation product of thymidine

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Abstract

In the present study, we have identified 5-carboxy-2′-deoxyuridine (CdUrd; **5**) as one of the main oxidation products of thymidine (**1**) upon menadione photosensitization to UVA radiation. Anion-exchange chromatography and capillary zone electrophoresis allowed the isolation of this modified nucleoside which represents 19% of the initial product, after 3 h of irradiation. Mechanistic studies showed that CdUrd **5** results from UVA photooxidation of 5-formyl-2′-deoxyuridine (FdUrd; **4**). The formation of **5** may be rationalized in terms of autoxidation of the aldehydic group of **4**, according to a well established pathway. Thus, it was shown that FdUrd **4** could be oxidized into CdUrd **5** in the presence of a peracid in aqueous solution. Moreover, CdUrd **5** was also detected by capillary electrophoresis upon far-UV photooxidation and γ-irradiation of thymidine (**1**) in aerated aqueous solution. The presence of **5** was confirmed by mass spectrometry analysis of the products of the photooxidized and γ-irradiated solutions. © 1997 Elsevier Science S.A.

Keywords: Photosensitation; Gamma radiolysis; Oxidized nucleoside; DNA damage

1. Introduction

It is well known that exposure of living organisms to the UV part of solar radiation may induce deleterious effects including lethality, mutagenesis and carcinogenesis [1,2]. These effects result, at least partly, from photoinduced modifications to genomic DNA including base damage, abasic sites, DNA strand breaks and DNA–protein cross-links [3–6].

The main deleterious effects of far-UV radiation are mostly accounted for by the formation of several classes of DNA base lesions, mostly dimeric pyrimidine photoproducts [4,6,7]. Moreover, it has been shown that 5-carboxyuracil is generated upon exposure of thymine to UVC radiation [8–10]. The formation of the latter photoproduct results from the oxidation of the exocyclic methyl group of thymine. It should be added that methyl oxidation products, including 5-hydroxymethyl-2′-deoxyuridine (**3**) and 5-formyl-2′-deoxyuridine (**4**) are formed predominantly upon UVA photosensitization of thymidine (**1**) in the presence of 2-methyl-1,4-naphthoquinone [11]. However, 5-carboxy-2′-deoxyuridine (CdUrd, compound **5**) was not characterized in the latter study.

It should be noted that CdUrd **5** is known to result from either hydrolysis of 5-trifluoromethyl-2′-deoxyuridine (TFMdUrd) under physiological conditions [12] or upon alkali treatment of 5-tribromomethyl-2′-deoxyuridine (TBrMdUrd) [13]. 5-Trifluoromethyl-2′-deoxyuridine or trifluridine is used in the treatment of the corneal endothelium as an antiviral against herpes [14]. Clough et al. [15], in their studies about the biological effects of TFMdUrd and its hydrolysis product, have shown that CdUrd **5** is an inhibitor of the de novo pyrimidine biosynthetic pathway involving orotate phosphoribosyl transferase and orotidine 5′-phosphate decarboxylase. In other respects, it was reported that the base moiety, 5-carboxyuracil (CU) is the main end product of the catalyzed oxidation of thymine by thymine hydroxylase, a non-heme iron protein [16,17].

We report herein the results of our studies on the formation of 5-carboxy-2′-deoxyuridine (**5**) in aerated aqueous solutions of thymidine (**1**) upon exposure to various oxidation conditions including photosensitization, photolysis and γ-irradiation.

2. Materials and methods

2.1. Chemicals

Thymidine (**1**) was purchased from Pharma Waldhöf (Pharma-Waldhöf GmbH, Düsseldorf, Germany). 2-

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Methyl-1,4-naphthoquinone (menadione) and boric acid were obtained from Merck (Darmstadt, Germany). *m*-Chloroperbenzoic acid was purchased from Sigma (St Louis, MO, USA). Triethylamine was obtained from Prolabo (Prolabo, Paris, France). Diethyl ether, acetic acid and HPLC-grade methanol were from Carlo Erba (Farmitalia Carlo Erba, Milan, Italy). NaOH was obtained from SDS (France). Ammonium formate was obtained from BDM Laboratory Supplies, Poole (UK). Buffers for high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) were prepared using water obtained from a Milli-Q system (Millipore, Milford, MA, USA).

2.2. HPLC analysis

HPLC was performed using a Spectra System P2000 Pump (Spectra Physics, Fremont, CA, USA) equipped with a Rheodyne 7125 (Berkeley, CA, USA) injector loop of 20 μl for the analytical experiments and 500 μl for the preparative separations. The detection was achieved with a Spectra System UV2000 and the data were recorded and processed using a Spectra System Chromjet Integrator. Different chromatographic systems were used. *System A*: reversed phase high performance liquid chromatography (RPLC); column, Merck LiChrocart LiChrospher 100RP-18e (5 μm, 125× 4 mm I.D.); eluent, 25 mM triethylammonium acetate buffer (pH 7; TEAA)–methanol (96:4, v:v) at a flow rate of 1 ml min^{-1}. *System B*: RPLC, a home-made semi-preparative Nucleosil octadecylsilyl silica gel column (300× 7.5 mm I.D.; mean particle size 10 μm) from Macherey–Nagel (Düren, Germany); eluent, H_2O–methanol (97.5:2.5, v:v) at a flow rate of 1 ml min^{-1}. *System C*: high performance anion-exchange chromatography (HPAEC); column, Pharmacia Ressource Q (1 ml; 15 μm); eluent, a linear gradient of 0–7% of a 1 M ammonium formate buffer (pH 7) in H_2O at a flow rate of 4 ml min^{-1} over a period of 15 min.

2.3. Capillary electrophoresis analysis

Capillary zone electrophoresis (CZE) analysis was performed on a Beckman CE-PACE 5500 capillary electrophoresis instrument (Beckman Instruments, Fullerton, CA, USA). An untreated fused silica capillary of 57 cm total length×75 μm I.D. was used (375 μm O.D.) with the window located at a distance of 50 cm. The column temperature was maintained at 25 °C during the analyses. The samples were injected under pressure for 1 s (≈5 nl) and separated using a 50 mM sodium borate buffer which pH was adjusted at either 8.0±0.1 (System D) or 10.0±0.1 (System E) with a 1 N NaOH solution. The instrument was set at a fixed voltage of 30 kV, leading to a constant current of 13 μA for System D and 73 μA for System E, respectively. UV data were collected by either a UV spectrometer or a Diode array detector set at 214 nm and at a rate of 10 points per second, prior to be processed with the Gold Software. Quantitative analyses of dThd **1**, FdUrd **4** and CdUrd **5** were obtained using the corrected peak area approach.

2.4. Spectroscopic measurements

Ultraviolet absorption spectra were obtained in water with a HP 8542A diode array spectrophotometer (Hewlett-Packard, Amsterdam, The Netherlands).

Fast atom bombardment (FAB) mass spectrometry analyses were carried out on a model ZAB 2-SEQ spectrometer (Fisons-V.G, Manchester, UK) operating both in the positive and negative modes. The modified nucleosides were dissolved in either a glycerol or a thioglycerol matrix.

The electrospray–mass spectrometric measurements of the nucleosides were carried out on a Perkin-Elmer Sciex API III triple quadrupole mass spectrometer (Perkin-Elmer Sciex) equipped with a nebulizer-assisted electrospray (ionspray) source. The instrument was tuned and calibrated in the positive mode and polarities were inverted for the negative-mode operations aimed at detecting anions [18,19]. Experiments were performed at a declustering potential (orifice voltage) of −60 V. Samples in ammonium acetate solution, were infused at a flow rate of 3 μl min^{-1} into the source through a 50 μm I.D. fused silica capillary connected to a Harvard 22 syringe pump and a Valco C6 W injector equipped with a 1 μl internal loop. To promote deprotonation of the nucleosides in the negative mode, the solvent contained 20% methanol and 0.1% triethylamine in water. Mass spectra were the average of five to ten scans obtained with a dwell time of 2 ms per 0.5 m/z step over a m/z range varying from 100 to 600.

^1H NMR and ^{13}C NMR spectra were recorded on either a AM400 Brüker spectrometer (Brüker, Wissemburg, France) or a U 500 Varian spectrometer (Varian Instrument Division, Palo Alto, CA, USA) operating in the Fourier transform mode. The ^1H and ^{13}C chemical shifts are expressed in ppm with respect to 3-(trimethylsilyl)propionic acid (TSP) used as the internal reference in 99.99% deuterium oxide (D_2O). Assignment of ^1H and ^{13}C signals was achieved by specific homo- and heteronuclear decoupling experiments and two-dimensional ^1H–^{13}C heteronuclear correlated NMR experiments (XHCORRC). The ^1H NMR spectra of CdUrd **5** in D_2O was computer simulated by using the iterative LAOCOON III and PANIC Brüker programs. The chemical shifts and coupling constants were obtained with a quadratic error of less than 0.05.

2.5. Irradiation sources

A Rayonet photochemical reactor (Southern New England Ultraviolet Company, Handem, CT) was used for the photoreactions (the lamps of the reactor were used at 70% of their maximum power). For the photosensitized and direct photolysis reactions involving UVA radiation, 16 black 350 nm lamps (approximately 4.5 W with about 90% of the energy in the 350 nm range for each lamp) in the Rayonet

photochemical reactor were utilized. For the UVC photolysis, 16 germicidal lamps (λ_{max} = 254 nm; approximately 8 W of mainly 254 nm radiation for each lamp) were used.

A ^{60}Co source with a dose rate of 50 Gy min^{-1} (overall dose = 18 kGy) was used for the γ-radiolysis experiments.

2.6. Irradiations procedures

Calibrated aqueous solutions of nucleosides (5 mM) were used. A continuous flow of air maintained the solutions saturated with oxygen during the irradiations performed at room temperature. In the different studies, 250 ml of the calibrated solution of thymidine (**1**) were irradiated. Aliquots of the reaction mixture were taken (100 μl) after 0, 10, 20, 40, 60, 90, 120, 150, 180 and 360 min of irradiation, respectively. 10 μl of the collected fractions were dissolved in the CZE running buffer. All the fractions were analyzed at 214 nm by CZE with system D (Tr(dThd) = 2.12 min; Tr(FdUrd) = 2.43 min; Tr(CdUrd) = 2.84 min) or system E (Tr(dThd) = 3.17 min; Tr(FdUrd) = 3.96 min; Tr(CdUrd) = 4.87 min). The analyses were made in triplicate. Then, the solution mixture was concentrated under reduced pressure and lyophilised at the end of the irradiation. The resulting residue was dissolved in 1 ml of the CZE running buffer and, then, analyzed again by CZE (see later) combined with UV photodiode array detection.

For the UVA photosensitized reactions, 0.1 equivalents of menadione (3.25 mg; 0.5 mM) were added to the previous nucleoside solution (250 ml; 5 mM). The resulting solution was exposed in a test tube to 350 nm light during 3 h for the solutions of thymidine (**1**) and 8 h for the solutions of FdUrd **4**. For the direct UVA photoreaction, the solution of FdUrd **4** was irradiated over a period of 8 h.

2.7. Isolation and characterization of 5-carboxy-2'-deoxyuridine (5)

250 ml of a 5 mM aqueous solution of thymidine (**1**; 302.5 mg) were exposed to 350 nm radiation in the presence of menadione (0.5 mM). Aliquots of the reaction mixture (50 μl) were taken after 0, 30, 60, 90, 120 and 180 min of irradiation, respectively. The samples were analyzed by RPLC (System B; k'(CdUrd) = 0.5; k'(FdUrd) = 12.6; k'(dThd) = 15.1) and HPAEC (System C; k'(CdUrd) = 6.37). After 3 h of irradiation, the solution mixture was concentrated under reduced pressure and purified by HPAEC (system C). The appropriate fractions (k' = 6.37) were pooled and then lyophilised.

UV (λ_{max}; H_2O): 272 nm [ε = 9714 M^{-1} cm^{-1}]

FAB-MS positive mode: $[M+H]^+ = 273 \pm 0.1$ Da; $[B+2H]^+ = 157 \pm 0.1$ Da; $[dR]^+ = 117 \pm 0.1$ Da.

FAB-MS negative mode: $[M-H]^- = 271 \pm 0.1$ Da; $[BH]^- = 155 \pm 0.1$ Da.

^1H NMR (499.838 MHz; D_2O) and *^{13}C NMR* (100.62 MHz; D_2O); see Tables 1 and 2.

2.8. Menadione-mediated photosensitization of 5-formyl-2'-deoxyuridine (4)

3.25 mg of menadione (0.5 mM) were added to 250 ml of the calibrated solution of FdUrd **4**. The resulting solution was exposed for 8 h to UVA radiation. Aliquots of the reaction mixture were taken (100 μl) after 0, 10, 20, 40, 60, 90, 120, 150, 180, 240 and 480 min of irradiation, respectively. 10 μl of the collected fractions were dissolved in 990 μl of the CZE running buffer. All the fractions were analyzed by CZE with the wavelength of the UV detector set up at 214 nm (system E; Tr(FdUrd) = 3.96 min; Tr(CdUrd) = 4.87 min). After 3 h of irradiation, 5 ml of the solution mixture were collected, concentrated under reduced pressure and then lyophilised. The resulting residue was dissolved in 1 ml of the CZE running buffer and then analyzed again by CZE (system E) with the UV photodiode array detector.

*ESI-MS in the negative mode of CdUrd **5** in the crude mixture*: $[M-H]^- = 271 \pm 0.1$ Da.

2.9. UVA photolysis of 5-formyl-2'-deoxyuridine (4)

250 ml of a 5 mM aqueous solution of FdUrd **4** were exposed over a period of 8 h to the 350 nm light of the Ray-

Table 1
499.83 MHz ^1H NMR chemical shifts of CdUrd **5** obtained in D_2O as inferred from computer iterative analysis (LAOCOON III), and 100.62 MHz ^{13}C NMR chemical shifts (ppm) obtained in D_2O

δ (ppm)	1'	2'	2''	3'	4'	5'	5''	2	4	5	6	COOH
^1H	6.41	2.51	2.55	4.60	4.17	3.96	3.89				8.78	
^{13}C	86.2	39.3		70.3	87.1	61.1		151.1	163.8	108.3	146.6	184.3

Table 2
(499.83 MHz) ^1H NMR coupling constants of CdUrd **5** obtained in D_2O as inferred from computer iterative analysis (LAOCOON III)

$^3J_{i-j}$ (Hz)	1'–2'	1'–2''	2'–2''	2'–3'	2''–3'	3'–4'	4'–5'	4'–5''	5'–5''
CdUrd	6.7	6.5	−14.2	6.7	4.1	3.8	3.5	5.1	−12.5

onet reactor. Typically for both RPLC and CZE analyses, aliquots of the reaction mixture (100 μl) were taken after 0, 10, 20, 40, 60, 90, 120, 180, 240 and 480 min, respectively. Then, the collected fractions were dissolved in the CZE running buffer and analyzed by CZE (system E) with the wavelength of the UV detector set up at 214 nm (Tr(FdUrd) = 3.96 min; Tr(CdUrd) = 4.87 min) and RPLC (System A; k'(CdUrd) = 0.82 and k'(FdUrd) = 5.15). Then, the solution which was irradiated for 8 h, was concentrated under reduced pressure and lyophilised. A fraction of the resulting residue was dissolved in 1 ml of the CZE running buffer and then analyzed again by CZE (system E) with the UV photodiode array detector. The second part of the residue was dissolved in H_2O and, subsequently, purified by RPLC (system A). Different fractions of the chromatogram were collected, lyophilised and then analyzed by ESI-MS. The expected pseudomolecular ion of the peracid intermediate was observed in the ESI-MS analyses of both the crude irradiated mixture and the first RPLC fraction ($0.06 < k' < 0.62$).

ESI-MS negative mode: CdUrd **5**:
$[M-H]^- = 271 \pm 0.1$ Da; Peracid intermediate:
$[M-H]^- = 287 \pm 0.1$ Da.

2.10. Reaction of 5-formyl-2'-deoxyuridine (4) with m-chloroperbenzoic acid

1 ml of a 1 M ethanol solution of *m*-chloroperbenzoic acid (MCPBA; 10 equivalents) was added to 20 ml of a 5 mM aqueous solution of 5-formyl-2'-deoxyuridine (**4**). The resulting solution was stirred in the dark at room temperature. After 24 h, 100 μl of the mixture were collected and analyzed by CZE with System E (Tr(FdUrd) = 3.94 min; Tr(CdUrd) = 4.82 min; Tr(MCPBA) = 5.00 min). Furthermore, the solution mixture was washed by 3 × 40 ml of diethyl ether in order to remove the excess of MCPBA. Then, the aqueous layer was evaporated to dryness. The resulting residue was dissolved in the CZE running buffer and then analyzed by CZE in System E with the UV detector and the UV photodiode array detectors. The aqueous layer was analyzed by ESI-MS.

ESI-MS in the negative mode of CdUrd 5 in the crude mixture: $[M-H]^- = 271 \pm 0.1$ Da.

2.11. UVA photosensitized reaction of thymidine (1)

The experimental conditions used for the UVA menadione photosensitization of a 5 mM aqueous solution of thymidine (**1**) are described above. The different aliquots collected were analyzed by CZE with system D (Tr(dThd) = 2.12 min; Tr(FdUrd) = 2.43 min; Tr(CdUrd) = 2.84 min).

ESI-MS in the negative mode of CdUrd 5 in the crude mixture: $[M-H]^- = 271 \pm 0.1$ Da.

2.12. Far UV photolysis and γ-radiolysis of aerated aqueous solutions of thymidine (1)

5 mM aqueous solutions of thymidine (**1**) were exposed during 6 h to UVC radiation and γ-rays, respectively, under the experimental conditions described above. The different aliquots collected were analyzed by CZE with system E (Tr(dThd) = 3.17 min; Tr(FdUrd) = 3.96 min; Tr(CdUrd) = 4.87 min).

ESI-MS in the negative mode of CdUrd 5 in the crude mixture: $[M-H]^- = 271 \pm 0.1$ Da.

3. Results and discussion

3.1. Identification and characterization of 5-carboxy-2'-deoxyuridine (5)

Menadione-mediated photosensitization of thymidine (**1**) [11] was recently reconsidered in order to prepare large amounts of 5-formyl-2'-deoxyuridine (FdUrd; compound **4**). Under these conditions, the photoreaction is reported to lead to the formation of FdUrd **4** as one of the major stable photoproducts. For preparative purpose, we have increased the irradiation time in order to achieve the complete degradation of thymidine (**1**). Surprisingly, the decomposition of **1** was not accompanied by an increase in the yield of FdUrd **4**. The purification of the main photoproduct which exhibited a low capacity factor (k'(CdUrd) = 0.5) was difficult to be achieved by RPLC (System B). On the other hand, HPAEC was shown to be suitable for resolving the crude mixture of the photosensitized products of thymidine (**1**). An example of both RPLC and HPAEC separations is given in Fig. 1.

The HPAEC elution profile obtained, using System C, shows the presence of a photoproduct with a high capacity factor (k' = 6.37). The latter compound was isolated and characterized by extensive spectroscopic measurements

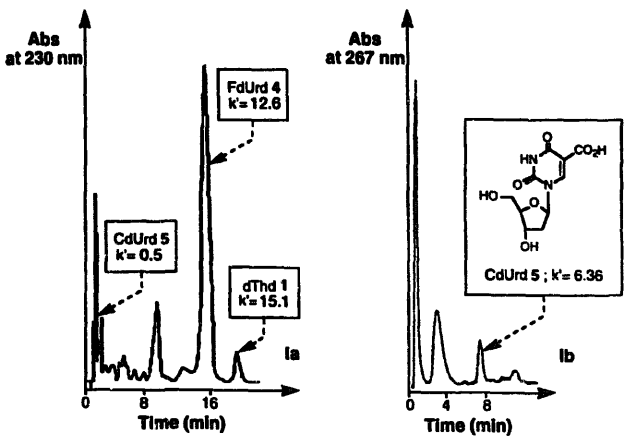

Fig. 1. RPLC (a; system B) and HAEPC (b; system C) elution profiles of an aerated aqueous solution of thymidine (**1**) exposed for 3 h to 350 nm radiation in the presence of menadione.

including (i): ^{1}H and ^{13}C NMR; (ii): FAB-MS in the positive and negative modes.

The ^{1}H NMR data of the photoproduct are reported in Tables 1 and 2. They revealed the presence in the low field region of the ^{1}H NMR spectrum of a singlet ($\delta = 8.78$ ppm) corresponding to pyrimidine H_6. In addition, the spectrum shows a set of signals characteristic of a 2-deoxyribose moiety. The pattern of the signal of the anomeric proton appears as a pseudo-triplet at 6.41 ppm. This is due to the occurrence of similar coupling constants between $H_{1'}$ and vicinal $H_{2'}$ and $H_{2''}$ ($^{3}J_{1'-2'} = 6.7$ Hz and $^{3}J_{1'-2''} = 6.5$ Hz), respectively. These features are in agreement with a β-furanosyl structure of the pyrimidine nucleoside photoproduct. Conformational features of the sugar moiety have been inferred from a detailed ^{1}H NMR analysis in D_2O. The relatively high magnitude of the $H_{1'}$, $H_{2'}$ *trans* coupling constant ($^{3}J_{1'-2'} = 6.7$ Hz) associated with low values for the other *trans* coupling constants ($^{3}J_{2''-3'} = 4.1$ Hz and $^{3}J_{3'-4'} = 3.8$ Hz) may be accounted for by a preferential C_2' *endo* puckered conformation [20]. In addition, we may note a predominance (53%) of the staggered *gg* rotamer about the $C_{4'}$–$C_{5'}$ bond. Information on the orientation of the base with respect to the sugar moiety was provided by considering the anisotropic effects of the pyrimidine ring on the chemical shifts of $H_{1''}$ and $H_{2''}$ protons [21,22]. In this respect, there is no significant downfield shift effect on the $H_{2'}$ signal. Therefore, this is indicative of a preferential *anti* conformation of the pyrimidine base. Furthermore, additional structural information was inferred from ^{13}C NMR chemical shifts analysis (Tables 1 and 2). These data confirm the presence of ten signals corresponding to the sugar moiety as well as to the base residue. Particularly relevant is the signal at 184.3 ppm which is characteristic of the carbonyl group of a carboxylic function. Thus, 5-carboxy-2′-deoxyuridine (**5**) appears to be the likely structure for this photoproduct.

The FAB-MS spectra recorded in the negative mode exhibits a notable pseudomolecular ion at m/z 271 ($[M-H]^-$). In addition, a predominant peak corresponding to the base moiety ($[B-2H]^-$) at m/z 155 is observed. Furthermore, the presence of the same fragments was noted in the positive FAB-MS of the modified nucleoside, namely the pseudomolecular peak ($[M+H]^+$) at m/z 273 and the base moiety fragment ($[B+2H]^+$) at m/z 157, respectively. This provided further support for the 5-carboxy-2′-deoxyuridine (**5**) structure. It should be added that unambiguous proof of structure was provided by X-ray crystallography determination of CdUrd **5** [23].

3.2. Mechanistic aspects of the formation of 5-carboxy-2′-deoxyuridine (5) upon photooxidation conditions

As described above, we have isolated CdUrd **5** from the 350 nm photolysis of a 5 mM aerated aqueous solution of thymidine (**1**) in the presence of 0.1 mM menadione [11]. Under these conditions, the major degradation pathway involves the oxidation of the exocyclic methyl group of **1**.

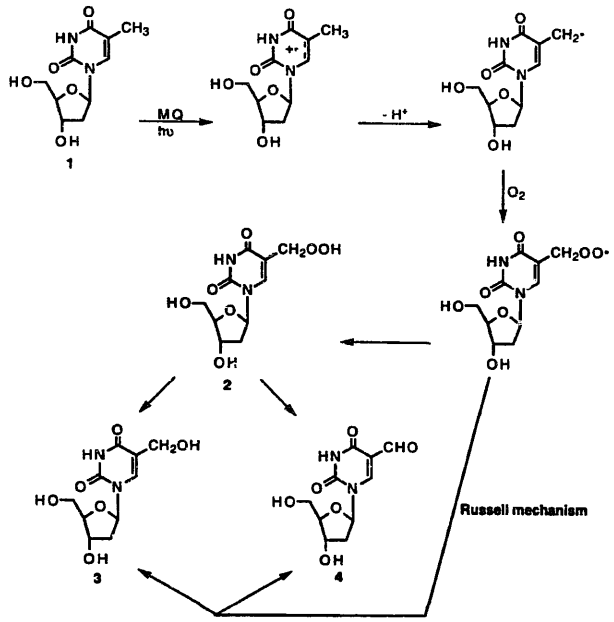

Fig. 2. Mechanism of photosensitized oxidation of the exocyclic methyl group of thymidine (**1**) at 350 nm in the presence of menadione [11,24].

This has been explained in terms of a charge transfer reaction from thymidine (**1**) to triplet excited menadione which leads to the generation of a pyrimidine radical cation (Fig. 2). The latter intermediate undergoes a fast deprotonation and the resulting neutral radical reacts with molecular oxygen to give rise to 5-hydroperoxymethyl-2′-deoxyuridine (**2**), after subsequent reduction and protonation reactions. Hydrolytic decomposition of **2** is expected to lead to the formation of the two main photoproducts of thymidine (**1**): 5-hydroxymethyl-2′-deoxyuridine (HMdUrd; compound **3**) and 5-formyl-2′-deoxyuridine (FdUrd; compound **4**). In addition, competitive dimerization of the transient peroxy radical involved in the formation of **2** may lead to **3** and **4** according to a Russell mechanism [24].

Preliminary experiments on menadione mediated photosensitization of thymidine (**1**) have shown that the yield of 5-carboxy-2′-deoxyuridine (**5**) increased with the time of exposure to UVA radiation. On the other hand, a concomitant decrease in the yield of 5-formyl-2′-deoxyuridine (**4**) was noted. This observation suggested that CdUrd **5** results from the oxidation of FdUrd **4**. A reasonable mechanism for the latter process would imply autoxidation of the aldehydic function [25,26].

In order to support this hypothesis, we have checked whether the formation of 5-carboxy-2′-deoxyuridine (**5**) results from the photoxidation of 5-formyl-2′-deoxyuridine (**4**). For this purpose, a 5 mM aerated aqueous solution of FdUrd **4** was exposed to 350 nm light either in the presence or in the absence of the photosensitizer. CdUrd **5** was detected in the crude mixtures by CZE (Tr(CdUrd) = 4.87 min) and mass spectrometry ($m/z = 271$; $[M-H]^-$) analyses. The related data collected by CZE are summarized in Fig. 3.

These data clearly show that CdUrd **5** is the major photoproduct of FdUrd **4**, irrespective of the presence of menadione

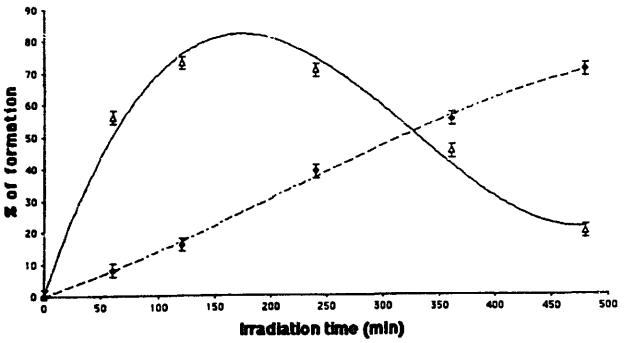

Fig. 3. Formation of CdUrd **5** by photooxidation of an aerated aqueous solution of FdUrd **4** at 350 nm either in the presence of (△) or in the absence of (◆) of menadione.

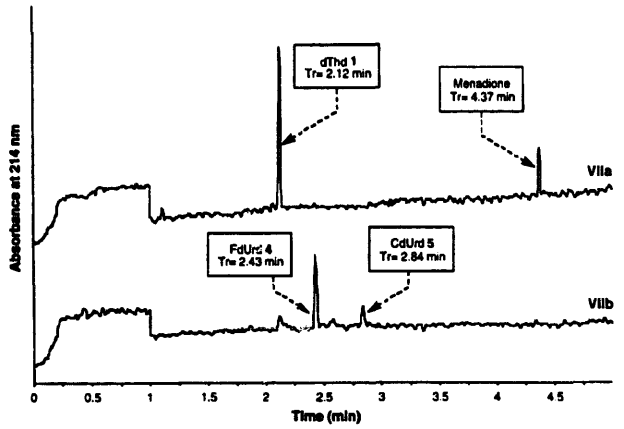

Fig. 4. Electrophoregrams of an aerated aqueous solution of thymidine (**1**) exposed to 350 nm in the presence of menadione at (a) $t=0$ h and (b) $t=$ 3 h, diluted in the running buffer (1/100), (system D).

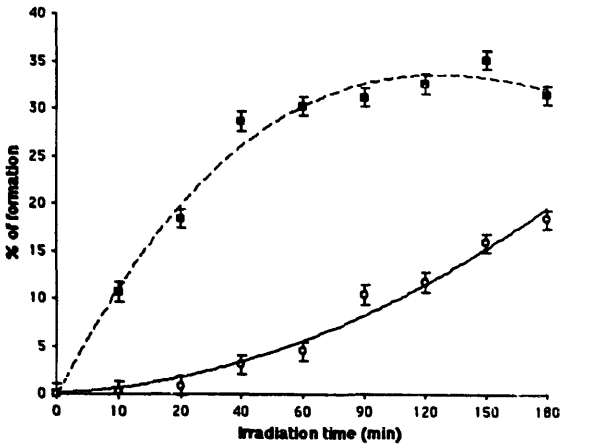

Fig. 5. Formation of FdUrd 4 (■) and CdUrd 5 (○) upon menadione mediated photosensitization of thymidine (**1**) in an aerated aqueous solution ($\lambda=350$ nm).

or not. The unexpected formation of CdUrd **5** in the absence of the photosensitizer could be explained by an overlap around 300 nm between the UV absorption of FdUrd **4** and the spectral energy distribution of the lamps used in the mini-reactor (data available on request). Furthermore, it should be noted that CdUrd **5** is also prone to photo-degradation. This fact was illustrated by the decrease in the amount of **5** while increasing the photoreaction time.

To confirm the mechanism of autoxidation of the aldehydic group of FdUrd **4**, we have looked for two intermediate steps, which are: (i) the photo-induced formation of a peracid upon irradiation of FdUrd **4**; (ii) the formation of CdUrd **5** by reaction of FdUrd **4** with a peracid.

In order to provide evidence for the peracid intermediate, ESI-MS analysis of the crude irradiation mixture of FdUrd **4** upon 350 nm photolysis was carried out. A pseudomolecular ion at $m/z=287$ which could be attributed to this intermediate, was observed (data available on request).

In order to confirm the possibility that CdUrd **5** may result from the oxidation of the aldehydic function of FdUrd **4** by a peracid, the reaction of **4** with 10 equivalents of *m*-chloroperbenzoic acid (MCPBA) was studied (data available on request). CdUrd **5** was detected in the aqueous layer of the crude mixture by CZE (system E) and electrospray ionization mass spectrometry (ESI-MS) analyses. Indeed, the ESI-MS spectrum exhibits the expected pseudomolecular ion at $m/z=271$ ($[M-H]^-$).

It should be added that the formation of FdUrd **4** and CdUrd **5** upon UVA menadione photosensitization of a 5 mM aerated aqueous solution of thymidine (**1**) was reinvestigated. For this purpose, capillary zone electrophoresis was used. An example of CZE separation (System D) is depicted in Fig. 4.

This electrophoregram (Fig. 4) corresponds to the photoreaction mixture of an aerated aqueous solution of thymidine (**1**) exposed to 350 nm light in the presence of menadione for 3 h. CdUrd **5** was detected on the basis of its retention time (Tr(CdUrd) = 2.84 min) and its UV spectrum obtained with an UV photodiode array detector. Furthermore, the presence of **5** was confirmed again by electrospray ionization mass spectrometry (ESI-MS) analysis in the negative mode of the concentrated crude photooxidation mixture. During the course of the photooxidation, aliquots of the reaction mixture were taken and analyzed by CZE (System D). The collected data corresponding to the formation of CdUrd **5** are summarized in Fig. 5. It should be noted that CdUrd **5** was detected after only 10 min of irradiation, representing 0.3% of the starting material. On the other hand, the amount of CdUrd **5** corresponds to 19% of the starting material (when FdUrd **4** represents 31%) after 3 h of photoreaction.

3.3. Formation of 5-carboxy-2'-deoxyuridine (5) upon different conditions of irradiation of thymidine

Using similar analytical conditions to those described above (see later), the formation of CdUrd **5** upon exposure of 5 mM thymidine (**1**) aerated aqueous solution to UVC radiation and to γ-rays was investigated.

CdUrd **5** was detected after 20 min of exposure of **1** to UVC radiation (data available on request). The presence of photoproduct **5** was confirmed by CZE (System E) on the basis of its retention time and UV spectrum obtained with an UV photodiode array detector. Furthermore, this received

further confirmation from the ESI-MS analysis in the negative mode of the concentrated crude photooxidation mixture ($[M-H]^-$ at m/z 271).

5-Formyl-2′-deoxyuridine (**4**) was found to be generated upon exposure of γ-rays of an aerated aqueous solution of thymidine (**1**) [27,28] and in DNA [29,30]. In the present work, we have searched for the possible formation of CdUrd **5** using the conditions set up for the analysis of the UVC photoproducts. It was found that CdUrd **5** may be also induced by $HO^{\bullet}$ mediated decomposition of thymidine (**1**) (data available on request). CdUrd **5** was detected (0.2% of the starting material) after exposure of thymidine (**1**) for 1 h in aerated aqueous solution to γ-rays (^{60}Co; 45 Gy min^{-1}). It should be noted that the obtained values are in agreement with the observations of Cadet and Téoule [31] who have shown that the oxidation of the exocyclic methyl group of thymidine is a relatively minor radiation-induced pathway.

4. Conclusion

5-Carboxy-2′-deoxyuridine (**5**) is a novel thymidine photoproduct resulting from the oxidation of the aldehydic group of 5-formyl-2′-deoxyuridine (**4**). The formation of **5** (19% of the starting material) upon menadione photosensitization of thymidine (**1**) at 350 nm is an important event. Moreover, the formation of **5** was detected upon exposure of an aerated aqueous solution of thymidine (**1**) to UVC and γ-rays.

5-Hydroxymethyl-2′-deoxyuridine (**3**) and 5-formyl-2′-deoxyuridine (**4**) were detected in DNA when exposed to ionizing radiation [29,30,32] and UVA [33,34]. Consequently, 5-carboxy-2′-deoxyuridine (**5**) is likely to be produced within DNA. Therefore, it would be of interest to study the formation of **5** in irradiated DNA and its possibility to be repaired. In addition, it would be also interesting to determine the misreading properties of CdUrd **5** residues in DNA during DNA replication and mutagenesis studies using DNA fragments that contain CdUrd **5** at specific sites [35].

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