Photosensitized Oxidation of 5-Methyl-2′-deoxycytidine by 2-Methyl-1,4-naphthoquinone: Characterization of 5-(Hydroperoxymethyl)-2′-deoxycytidine and Stable Methyl Group Oxidation Products§

Carine Bienvenu,† J. Richard Wagner,‡ and Jean Cadet*,†

Contribution from the Département de Recherche Fondamentale sur la Matière Condensée, SCIB/LAN, CEA-Grenoble, 17 avenue des Martyrs, F-38054 Grenoble Cedex 9, France, and Département de Médecine Nucléaire et Radiobiologie, Université de Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4

Received June 20, 1996⊗

Abstract: 5-Methylcytosine is a minor nucleobase of eukaryotic DNA which plays a central role in the regulation of gene expression. UV-A irradiation of an aerated aqueous solution of 5-methyl-2′-deoxycytidine in the presence of menadione (MQ) as a type I photosensitizer leads to the formation of several stable oxidation products. Emphasis was placed in this study on the isolation and the characterization of the major class of decomposition products whose formation involves the oxidation of the methyl group. These include 5-(hydroperoxymethyl)-2′-deoxycytidine and two stable decomposition products namely, 5-(hydroxymethyl)-2′-deoxycytidine and 5-formyl-2′-deoxycytidine. Structural assignment of the latter modified nucleosides was inferred from extensive spectroscopic measurements (^{1}H and ^{13}C NMR, UV spectroscopy, and mass spectrometry). In addition, conformational analysis of the oxidized nucleosides was inferred from detailed ^{1}H NMR analysis. All of the above photooxidation products appear to arise from the deprotonation of the 5-methyl-2′-deoxycytidine radical cation which is generated by menadione photosensitization.

Introduction

5-Methylcytosine is a minor base component of most eukaryotic DNA. Methylation of cytosine occurs largely at dinucleotide sequences that are involved in the regulation of gene expression.[1,2] 5-Methylcytosine residues of CpG islands are hot spots for mutations.[3] Indeed, it has recently been reported that the predominant mutation in the human p53 tumor suppressor gene is a C to T transition at codon 248, as the result of spontaneous hydrolytic deamination of 5-methylcytosine to thymine.[4] Exposure of DNA to photodynamic agents and ionizing radiation is responsible for the induction of various oxidative base lesions including 8-oxo-7,8-dihydroguanine and 5-formyluracil.[5−9] These and several other oxidative base modifications have been shown to be potentially mutagenic.[8−11] In the present study, near-UV photolysis of an aerated aqueous solution containing 5-methyl-2′-deoxycytidine and menadione allows efficient formation of methyl oxidation products of the pyrimidine moiety. It is likely that most of the DNA damage induced by exposure to UV-A (320−400 nm) light results from the action of endogenous and exogenous cellular photosensitizers rather than from direct absorption of photons by DNA bases.[12] 2-Methyl-1,4-naphthoquinone (menadione), a component of the vitamin K3, is an efficient type I photosensitizer toward pyrimidine bases.[7,13,14] Photoexcited MQ or related quinone derivatives have been found to give rise to DNA single as well as double strand breaks.[15] Efficient electron transfer reaction between pyrimidine bases and MQ in its triplet excited state has been shown to occur on the basis of flash photolysis and steady-state experiments.[16,17] The photochemistry of the major pyrimidine DNA bases and nucleosides has been studied to a great extent.[12,18−20] On the other hand, the photosensitized

* To whom correspondence should be addressed. Phone: (33) 476-88-49-87. Fax: (33) 76-88-50-90. E-mail: cadet@drfmc.ceng.cea.fr.

† SCIB/LAN.

‡ Université de Sherbrooke.

§ DMSO-d_6, deuteriated dimethyl sulfoxide; D_2O, deuterium oxide; for^{5}Cyt, 5-formylcytosine; for^{5}dCyd, 5-formyl-2′-deoxycytidine; for^{5}Ura, 5-formyluracil; for^{5}dUrd, 5-formyl-2′-deoxyuridine; hm^{5}dCyd, 5-(hydroxymethyl)-2′-deoxycytidine; hm^{5}dUrd, 5-(hydroxymethyl)-2′-deoxyuridine; HPLC, high performance liquid chromatography; hpm^{5}dCyd, 5-(hydroperoxymethyl)-2′-deoxycytidine; *k′*, capacity factor; m^{5}dCyd, 5-methyl-2′-deoxycytidine; MS (FAB$^+$), mass spectrometry (fast atom bombardment in the positive mode); MS (ES), mass spectrometry (electrospray); MQ, 2-methyl-1,4-naphthoquinone (menadione); NMR, nuclear magnetic resonance; 8-oxoGua, 8-oxo-7,8-dihydroguanine; TMS, tetramethylsilane; TSP, 3-(trimethylsilyl)-propionate-*2,2,3,3,-d*$_4$ sodium salt; UV-A, ultraviolet radiation (320−400 nm).

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

(1) Razin, A.; Riggs, A. *Science* **1980**, *210*, 604.
(2) Razin, A; Cedar, H. *Cell* **1991**, *64*, 1123.
(3) Rideout, W. M.; Coetzee, G. A.; Olumi, A. F.; Jones, P. A. *Science* **1990**, *249*, 1288.
(4) Magewu, A. N.; Jones, P. A. *Mol. Cell. Biol.* **1994**, *14*, 4225.
(5) Lindahl, T. *Nature* **1993**, *362*, 709.
(6) Cheng, K. C.; Cahill, D. S.; Kasai, H.; Nishimura, S.; Loeb, L. A. *J. Biol. Chem.* **1992**, *267*, 166.
(7) Decarroz, C; Wagner, J. R.; van Lier, J. E.; Murali Krishna, C.; Riesz, P.; Cadet, J. *Int. Radiat. Biol.* **1986**, *50*, 491.
(8) Kasai, H.; Iida, A., Yamaizumi, Z.; Nishimura, S.; Tanooka, H. *Mutat. Res.* **1990**, *243*, 249.
(9) Bjelland, S.; Birkeland, N.-K.; Benneche, T.; volden, G.; Seeberg, E. *J. Biol. Chem.* **1994**, *269*, 30489.
(10) Cadet, J.; Berger, M.; Douki, T; Ravanat, J.-L. *Reviews Physiol. Biochem. Pharmacol.* **1996** (in press).
(11) Grollman, A. P.; Moriya M. *Trends Genet.* **1993**, *9*, 246.
(12) Cadet, J.; Vigny, P. In *Bioorganic Photochemistry. Photochemistry and the Nucleic Acids*; Morrison, H., Ed.; John Wiley and Sons: New York, 1990; Vol. 1, pp 1−272.
(13) Murali Krishna, C.; Decarroz, C.; Wagner, J. R.; Cadet, J.; Riesz, P. *Photochem. Photobiol.* **1987**, *46*, 175.
(14) Wagner, J. R.; Berger, M.; Cadet, J.; van Lier, J. E. *J. Chromatogr.* **1990**, *504*, 191.
(15) Wagner, J. R.; Fisher, G. J.; Decarroz, C.; Cadet, J. *Photochem. Photobiol.* **1984**, *48s*.
(16) Koch, T.; Ropp, J. D.; Sligar, S. G.; Schuster, G. B. *Photochem. Photobiol.* **1993**, *58*, 554.
(17) Fisher, G. J.; Land, E. *Photochem. Photobiol.* **1983**, *37*, 27.

S0002-7863(96)02073-2 CCC: $12.00 © 1996 American Chemical Society

Table 1. ^{1}H NMR Chemical Shifts[a] (ppm) and Proton−Proton Coupling Constants[b] (Hz) of 5-Formyl-2′-deoxycytidine (**6**), 5-(Hydroxymethyl)-2′-deoxycytidine (**7**), and 5-(Hydroperoxymethyl)-2′-deoxycytidine (**5**) as Inferred from Computer Iterative Analysis (LAOCOON III Program) of 400.13 Mhz (hm⁵dcyd and for⁵dCyd) and 250.13 MHz (hpm⁵dcyd) ^{1}H NMR Spectra in D_2O

	H1′	H2′	H2″	H3′	H4′	H5′	H5″	C*H*$_2$	C*H*O	H6
hm⁵dCyd (**7**)	6.34	2.39	2.52	4.52	4.14	3.94	3.85	4.53		7.97
for⁵dCyd (**6**)	6.44	2.61	2.82	4.68	4.38	4.15	4.03		9.75	9.04
hpm⁵dCyd (**5**)	6.32	2.40	2.53	4.51	4.14	3.93	3.84	no[c]		8.07

	$J_{1'2'}$	$J_{1'2''}$	$J_{2'2''}$	$J_{2'3'}$	$J_{2''3'}$	$J_{3'4'}$	$J_{4'5'}$	$J_{4'5''}$	$J_{5'5''}$
hm⁵dCyd (**7**)	6.7	6.4	−14.1	6.6	4.2	4.1	3.4	5.1	−12.5
for⁵dCyd (**6**)	5.7	6.5	−14.2	6.5	4.8	4.4	3.4	5.0	−12.6
hpm⁵dCyd (**5**)	6.6	6.2	−14.2	6.5	4.6	4.1	3.5	5.0	−12.6

[a] The accuracy of the measurement is provided to ±0.01 ppm. [b] Accuracy of the measurement is provided to ±0.1 Hz. [c] Not observed (under the peak of HOD at 5 ppm).

Table 2. ^{1}H NMR Chemical Shifts (ppm) of hm⁵dCyd (**7**) and hpm⁵dCyd (**5**) Obtained at 250.13 MHz in DMSO-d_6

	H1′	H2′	H2″	H3′	H4′	O*H*3′	O*H*5′	CH$_2$OO*H*	C*H*$_2$	H6
hpm⁵dCyd (**5**)	6.24	2.06	2.24	3.89	3.68	5.33	5.14	11.78	4.71	8.00
hm⁵dCyd (**7**)	6.29	2.05	2.21	3.88	3.67	5.39	5.10	nd[a]	4.30	7.86

[a] Not determined.

Table 3. 100.7 MHz ^{13}C NMR Chemical Shifts (ppm) of for⁵dCyd (**6**) and hm⁵dCyd (**7**) Obtained in D_2O

	C1′	C2′	C3′	C4′	C5′	C2	C4	C5	C6	CHO	CH$_2$OH
for⁵dCyd (**6**)	87.3	40.2	69.9	87.6	60.8	155.3	162.8	105.8	154.7	190.5	
hm⁵dCyd (**7**)	86.2	39.4	70.4	86.7	61.1	nd[a]	nd[a]	nd[a]	140.5		57.8

[a] Not determined.

oxidation of 5-methyl-2′-deoxycytidine has not been addressed although there are been a number of studies with regard to direct far-UV photolysis.[21−23] Emphasis was placed in this work on the identification of the main final oxidation photoproducts that arise from the deprotonation of the menadione photosensitized formation of the 5-methyl-2′-deoxycytidine radical cation.

Experimental Section

Detection of Hydroperoxide by HPLC Post-Column Reaction. The specific detection of peroxides was carried out by using a modified HPLC assay that was initially described by Wagner *et al.*[14,23] The analytical system included two pumps equipped with pulse dampeners. The first pump, a Waters M45 (Mildford, MA), was connected to a semi-preparative ODS column. The second one, a Gilson 302 pump (Gilson, Middleton, WI), was used to deliver the peroxide reagent. The eluent and reagent flow-rates were 1.5 and 0.7 mL/min, respectively. The eluent and reagent were mixed with the aid of a right angle tee and passed through a reactor chamber (1.3 mL) immersed in a water bath thermostated at 70 °C. Then, the mixture was cooled by passing through a tube of 2 m length maintained at room temperature in a water bath. The absorption of the mixture was measured at 546 nm by using a Waters 484 UV-visible detector (Mildford, MA). The peroxide reagent consisted of 0.3 mM xylenol orange (tetrasodium salt), 0.75 mM ammonium ferrous sulfate, 0.3 M sorbitol and 75 mM H_2SO_4.[25]

Photosensitization. The photolysis of aerated aqueous solutions containing 5-methyl-2′-deoxycytidine and menadione was carried out with a rayonet photochemical reactor (Southern New England Ultraviolet Company, Handem, CT) with a bank of 16 black lamps that emit 90% of their light within the 350 ± 10 nm range. The concentration of oxygen was maintained at about 0.25 mM by continuously bubbling the solution with air during photolysis.

Table 4. Conformational Features of Methyl Oxidation Products of 5-methyl-2′-deoxycytidine (hm⁵dCyd, hpm⁵dCyd, and for⁵dCyd) and Thymidine (for⁵dUrd and hm⁵dUrd) in D_2O as Inferred from ^{1}H NMR Measurements

	% C2 *endo*[a]	% *gg*[b]
hm⁵dCyd (**7**)	62.0	53.6
hm⁵dUrd	62.0[c]	51.5[c]
hpm⁵dCyd (**5**)	61.7	53.6
for⁵dCyd (**6**)	56.4	54.6
for⁵dUrd	58.9[c]	59.8[c]

[a] Percentage of C2′ *endo* = $J(1'2')/[J(1'2') + J(3'4')]$.[32] [b] Percentage of rotamer *gg* = $[13.7 - (J(4'5') + J(4'5''))]100/9.7$.[32] [c] As inferred from ^{1}H NMR analysis.[26]

5-Formyl-2′-deoxycytidine (6). An aqueous solution (150 mL) of 3.6 mM 5-methyl-2′-deoxycytidine (130 mg) and 0.8 mM menadione (20 mg) was exposed to near-UV light for 1 h at ambient temperature (*vide supra*). Then, the solution was evaporated to dryness under reduced pressure and the resulting residue was resuspended in a minimum volume of water (≅ 500 μL) prior to purification by HPLC. The separation of the methyl group oxidation products resulting from the photosensitization of m⁵dCyd (**1**) was achieved on a semi-preparative HPLC system. The mobile phase consisted of a mixture of water and methanol (95:5) (v:v) at a flow-rate of 3 mL/min. The main eluting fraction ($k' = 4.5$) was found to contain 5-formyl-2′-deoxycytidine (**6**). Lyophilization of the combined fractions provided 18 mg (yield 13%) of **6**. The ^{1}H NMR chemical shifts and scalar proton-proton coupling constants of **6** obtained in D_2O are reported in Table 1. The ^{13}C NMR chemical shifts of for⁵dcyd **6** are listed in Table 3. ν_{max} (KBr)/cm^{-1} 1638 (broad band), 1504, 1238, 1089. MS (FAB$^+$) *m*/*z* 140.1 (100%, [base + 2H]$^+$); 256.1 (68%, [M + H]$^+$); 278.1 (42%, [M + Na]$^+$); 117.1 (26%, [sugar moiety]$^+$). HRMS calculated for $C_5H_6N_3O_2$ ([base + 2H]$^+$) 140.0460; found 140.0486. UV (H_2O, pH = 7) λ_{max} (nm) 284.

5-(Hydroxymethyl)-2′-deoxycytidine (7). Lyophilization of the combined HPLC fractions (k'=1.4) yielded 10 mg (yield 7.3%) of a white powder which was identified as 5-(hydroxymethyl)-2′-deoxycytidine (**7**). ^{1}H and ^{13}C NMR data of **7** are reported in Tables 1−3. ν_{max} (KBr)/cm^{-1} 1660, 1611, 1525, 1060. MS (FAB$^+$) *m*/*z* 258.1 (100%, [M + H]$^+$); 280.1 (29%, [M + Na]$^+$); 142.1 (93%, [base +

(18) Wagner, J. R.; van Lier, J. E.; Decarroz, C.; Berger, M.; Cadet, J. *Methods Enzymol*. **1990**, *186B*, 502.
(19) Douki, T.; Cadet J. In *Interface between Chemistry and Biochemistry*; Jolies, P., Jornvall, H., Eds.; Birkauser Verlag: Basel, Switzerland, 1995; pp 173−197.
(20) Taylor, J. S.; Nadji, S. *Tetrahedron* **1991**, *47*, 2579.
(21) Shaw, A. A.; Shetlar M. D. *Photochem. Photobiol*. **1989**, *49*, 267.
(22) Douki, T.; Cadet, J. *Biochemistry* **1994**, *33*, 11942.
(23) Celewicz, L.; Shetlar, M. *Photochem. Photobiol*. **1992**, *55*, 823.
(24) Wagner, J. R.; van Lier, J. E.; Decarroz, C.; Cadet, *J. Bioelectrochem. Bioenerg*. **1987**, *18*, 155.
(25) Wolff, S. P. *Methods Enzymol.* **1994**, *233*, 182.

2H]$^+$); 117.1 (44%, [sugar moiety]$^+$). HRMS calculated for $C_{10}H_{16}N_3O_5$ 258.1090; found 258.0985. UV (H_2O, pH = 7) λ_{max} (nm) 274.

5-(Hydroperoxymethyl)-2′-deoxycytidine (5). An aerated aqueous solution (10 mL) of 4.15 mM 5-methyl-2′-deoxycytidine (**1**) (10 mg) and 0.6 mM menadione (1 mg) was exposed to near-UV light for 25 min at ambient temperature (*vide supra*). Thus, the solution was concentrated to 2 mL by rotary evaporation (<30 °C). Rapid separation of the photoproducts was achieved by using a semipreparative HPLC system with bidistillated water (pH = 6.0) as the mobile phase at a flow-rate of 2 mL/min. The eluting fraction (k' = 6.8) was evaporated to dryness under reduced pressure (<30 °C) giving 0.8 mg of 5-hydroperoxymethyl-2′-deoxycytidine (**5**) (yield 7%). The dry residue was resuspended either in D_2O or DMSO-d_6 prior to NMR analysis (Tables 1 and 2). (ES/MS) *m*/*z* 158.2 (100%, [base + 2H]$^+$); 274.2 (30%, [M + H]$^+$); 140.2 (18%, [base + 2H − H_2O]$^+$); 547.1 (15%, [2M + H]$^+$). The presence of an hydroperoxide group in **5** was confirmed by HPLC analyses involving a peroxide specific postcolumn reaction detection.

Reduction of 5-Formyl-2′-deoxycytidine (6). 5-Formyl-2′-deoxycytidine (**6**) (8 mg, 31 mmol) and sodium borohydride (2 mg, 52 mmol) were dissolved in 2 mL of anhydrous methanol. The reaction was taken to completion after stirring overnight at room temperature. The resulting slightly alkaline solution (pH = 9) was acidified (pH = 6) by dropwise addition of acetic acid. Then, the mixture was evaporated to dryness under reduced pressure. The oily residue was dissolved in 10 mL of methanol, and the resulting solution was evaporated to dryness under diminished pressure. The latter operation was repeated until the methyl borate sodium salt was completely removed. The final residue was then dissolved in a minimum volume of water (≅ 200 μL) prior to HPLC analysis. This was achieved on the semipreparative HPLC system by using a (95:5) (v:v) mixture of water and methanol as the isocratic eluent at a flow-rate of 2 mL/min. The major fraction (k' = 1.4) was collected and lyophilized giving 6 mg of 5-(hydroxymethyl)-2′-deoxycytidine (**7**) (yield 74%).

Time Course Study of the Decomposition of 5-(Hydroperoxymethyl)-2′-deoxycytidine (5) in Aqueous Solution. The thermal decomposition of 5-hydroperoxymethyl-2′-deoxycytidine (**5**) was monitored by NMR analysis. hpm^{5}dCyd **5** was kept at 297 K in D_2O and ^{1}H spectra were recorded at increasing periods of time on a WR 250 Brüker spectrometer. The decay of hpm^{5}dCyd **5** was followed by monitoring the area of the H-6 singlet in the low field region of the spectrum (δ = 8.07 ppm). Under these conditions, the half-life of **5** was found to be 9.5 ± 0.5 h (Figure 1).

Photosensitization of 5-(Hydroxymethyl)-2′-deoxycytidine (7). Aerated aqueous solutions (5 mL) containing 1.16 mM of menadione (1 mg) and 3.6 mM of m^{5}dCyd (**1**) (4.3 mg) were exposed to near-UV light (λ_{max} = 350 nm) as described. The yield of **6** was determined by integrating its HPLC peak (k' = 1.4). For this purpose 50 μL aliquots were removed during the irradiation and 10-fold diluted. Then, 20 μL of the diluted solution were directly injected on the above analytical HPLC system. No decomposition of initial m^{5}dCyd (**7**) was detected when the photolysis was carried out in the absence of menadione.

Results and Discussion

Isolation of the Stable Photooxidation Products of 5-methyl-2′-deoxycytidine. Modified nucleosides **6** and **7** are the major photooxidation products of the menadione-photosensitized oxidation of m^{5}dCyd (**1**) in aerated aqueous solution. Nucleosides **6** and **7** were separated by HPLC on a ODS column using a mixture of water and methanol (95:5) v/v as the eluent. The early eluting HPLC photoproduct (k' = 1.4) was identified as 5-(hydroxymethyl)-2′-deoxycytidine (**7**). The second and later eluting photoproduct (k' = 4.5) was identified as 5-formyl-2′-deoxycytidine (**6**). 5-Hydroperoxymethyl-2′-deoxycytidine (**5**) was also separated on the ODS column (k' = 6.8) by using water as the mobile phase.

Characterization of 5-Formyl-2′-deoxycytidine (6). The FAB mass spectrum of **6** recorded in the positive mode exhibits a pseudomolecular peak at *m*/*z* = 256.1. In addition, an abundant quasi-molecular clustered ion was observed at *m*/*z* = 278.1. This which differs by 22 mass units from the [M + H]$^+$ peak, is characteristic of a quasi-molecular ion [M + Na]$^+$. The fragmentation pattern shows the occurrence of a significant cleavage of the *N*-glycosidic bond. This was inferred from the presence of two important fragments at *m*/*z* = 117.1 (sugar moiety) and *m*/*z* = 140.1 (base moiety + 2H), respectively. In addition, the exact mass measurement carried out on the ion [B + 2H]$^+$ (*m*/*z* = 140.0486) is consistent with an empirical formula of $C_5H_6N_3O_2$ for this fragment. On the basis of these findings, it is concluded that **6** is not deaminated. The structure of **6** was subsequently confirmed by detailed ^{1}H NMR spectroscopic analysis (Table 1). In particular, the ^{1}H signal that resonates as a singlet in the low field region (δ = 9.75 ppm) of the spectrum is strongly indicative of the presence of an aldehyde group in the structure. Moreover, the bathochromic shift of the UV absorption maximum (λ_{max} = 284 nm) may be rationalized in terms of conjugation of the latter carbonyl group with the C5−C6 double bond. In addition, the ^{1}H signal at δ = 7.97 ppm which is characteristic of a vinylic proton was assigned as those of pyrimidine H-6. Inspection of the ^{13}C NMR spectrum which exhibits ten resonance signals (Table 3) provides additional relevant structural information. Unambiguous assignment of the signals of the 2-deoxy-*erythro*-pentofuranose moiety, formyl group and C6 carbon was inferred from two-dimensional ^{1}H−^{13}C heteronuclear correlated NMR experiment (XHCORRC) (data not shown). The ^{13}C signal of the aldehyde group appears in the low field region of the spectrum (δ = 190.5 ppm) as expected from the conjugation of the carbonyl group with the pyrimidine double bond. This may be rationalized in terms of mesomeric acceptor effect of the carbonyl oxygen atom that enhances the partial positive charge on C6 and, therefore, promotes a downfield shift of the latter carbon.

Characterization of 5-(Hydroxymethyl)-2′-deoxycytidine (7). Compound **7** was obtained as one of the main menadione photosensitized oxidation products of 5-methyl-2′-deoxycytidine (**1**). The modified nucleoside **7** was characterized on the basis of extensive spectroscopic measurements. The positive fast atom bombardment spectrum of **7** exhibits two characteristic clustered ions at *m*/*z* = 258.1 and 280.1. These were assigned as the pseudo-molecular [M + H]$^+$ and quasi-molecular [M + Na]$^+$ ions, respectively. In addition, the cleavage of the N-glycosidic bond gave rise to two predominant ions at *m*/*z* = 142.1 [base + 2H]$^+$ and *m*/*z* = 117.1 (2-D-deoxy-*erythro*-pentose moiety). The exact mass measurement of the pseudo-molecular ion [M + H]$^+$ as inferred by high resolution FAB-MS analysis is 258.0985. This suggests an empirical formula of $C_{10}H_{15}N_3O_5$ for **7**. Further structural information was gained from ^{1}H and ^{13}C analyses. The 400 MHz ^{1}H NMR spectrum of **7** obtained in D_2O exhibits nine resonances which were completely assigned (Table 1). In particular, the H6 signal at δ = 7.97 ppm is strongly indicative of the unsaturation of the C5−C6 bond. This is also consistent with the strong UV absorption band at λ_{max} = 274 nm. The singlet at δ = 4.53 ppm was assigned as the resonance of the methylene protons of the 5-hydroxymethyl group which are magnetically equivalent. It should be noted that the resonance of the latter signal is downfield shifted by 0.13 ppm with respect to that of the corresponding methylene group of 5-(hydroxymethyl)-2′-deoxyuridine.[26] The signal of the latter methylene protons appears as a doublet (δ = 4.30 ppm) in the ^{1}H NMR spectrum of **7** recorded in DMSO-d_6 (Table 2). Interestingly, the doublet due to scalar coupling with the attached OH group (δ = ppm) collapses into a singlet upon addition of D_2O. Such a coupling is not observed, as expected, for hpm^{5}dCyd **5** (*vide infra*). The ^{13}C spectrum of **7** displays seven resonance signals (Table 3),

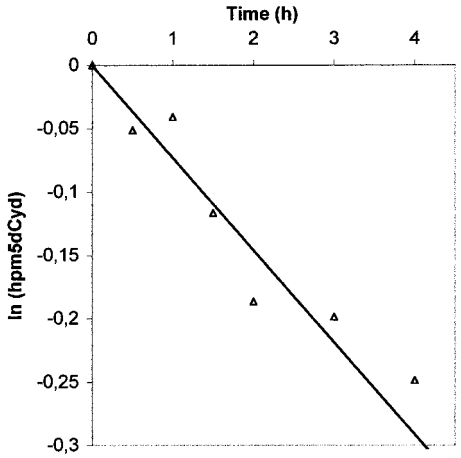

Figure 1. Decomposition rate of the 5-(hydroperoxymethyl)-2′-deoxycytidine (**5**) as inferred from NMR analysis performed in D_2O at 297 K.

the bulk of them was unambiguously assigned by two-dimensional $^1H-^{13}C$ correlated NMR analysis (XHCORRC) (data not shown). The C6 signal resonating in the down field region (δ = 140.5 ppm) of the ^{13}C NMR spectrum is of diagnostic of the unsaturation of the C5−C6 bond. Another relevant structural signal is those of 5-hydroxymethyl group at δ = 57.8 ppm in the vicinity of C5′ (δ = 61.1 ppm). Further support for the structure of for⁵dCyd (**6**) was provided by its quantitative conversion into **7** upon mild reduction with $NaBH_4$.

Characterization of 5-(Hydroperoxymethyl)-2′-deoxycytidine (5). The mild electrospray-MS technique was found to be particularly suitable for the analysis of unstable 5-(hydroperoxymethyl)-2′-deoxycytidine (5). The mass spectrum of **5** exhibits a major fragment at *m*/*z* 158.2 $[base + 2H]^+$ and a notable pseudomolecular ion at *m*/*z* 274.2 $[M + H]^+$. Relevant structural information on **5** was also provided by ^1H NMR analysis in D_2O and DMSO-d_6. The ^1H NMR features of **5** and **7** are very similar (Tables 1 and 2) with the exception of the chemical shift for the methylene protons of the hydroperoxymethyl group. It should be noted that the latter signal of **5** is not observable when D_2O was used as the NMR solvent since it is masked by the HOD signal. Further support for the structure of 5-(hydroperoxymethyl)-2′-deoxycytidine (**5**) was provided by the consideration of the ^1H NMR features obtained in DMSO-d_6 (Table 2). The main characteristic signal is a singlet whose resonance is in the low field region (δ = 11.78 ppm) of the spectrum. This, which is not observed in D_2O due to chemical exchange was assigned as the proton of the hydroperoxide group. In addition, the ^1H NMR spectrum of **5** in DMSO-d_6 exhibits a singlet at δ = 4.71 ppm. Integration of the latter resonance signal showed that it was twice as large as that of the H1′ signal. This signal was assigned as the methylene protons of the hydroperoxymethyl group. It should be noted that **5** was not enough stable to obtain a ^{13}C NMR spectrum. However, further confirmation of the assignment of **5** was given by a post-column reaction which confirmed that **5** has an hydroperoxide function.[14,24,25] The thermal decomposition of **5** was monitored by ^1H NMR measurements at 297 K in D_2O. The rate constant for the decay of **5** was determined to be $(7.3 \pm 0.4) \times 10^{-2}$ h^{-1} whereas the half-life time was found to be 9.5 ± 0.5 h (see Figure 1). This is approximately 200-fold smaller than that of 5-(hydroperoxymethyl)-2′-deoxyuridine.[27,28] In addition, it was shown that the main stable decomposition product of 5-(hydroperoxymethyl)-2′-deoxycytidine (**5**) in aqueous solution is 5-formyl-2′-deoxycytidine (**6**). This contrasts with the hydrolytic decomposition of 5-(hydroperoxymethyl)-2′-deoxyuridine which leads to the predominant formation of 5-(hydroxymethyl)-2′-deoxyuridine.[28,29] Therefore, the presence of an amino group adjacent to the hydroperoxymethyl group has a pronounced effect on the rate and mode of decomposition of the hydroperoxide **5** in aqueous solution.

Conformational Analysis. 5-Formyl-2′-deoxycytidine (**6**), 5-(hydroxymethyl)-2′-deoxycytidine (**7**) and 5-(hydroperoxymethyl)-2′-deoxycytidine (**5**) exhibit similar conformational features as inferred from detailed ^1H NMR analysis in D_2O (Table 3). This allows an estimation of the relative importance of the two main puckered forms of the sugar moiety which are in a dynamic equilibrium: C2′ *endo* ⇌ C3′ *endo*. The C2′ *endo* pucker form corresponds to the conformation in which the C2′ atom is displaced toward the C5′ from the mean plane of the other atoms of the 2-deoxy-β-D-*erythro*-pentofuranose ring. The percentage of C2′ *endo* may be inferred from the equation %C2′ *endo* $= 100J1'2'/(J1'2' + J3'4')$.[30,31] Accordingly, ^1H NMR analyses of products **5**, **6**, and **7** suggest that the conformation of the sugar moiety is similar for each nucleoside, with the population C2′ *endo* being predominant. The conformation of the 5′-exocyclic hydroxymethyl group is usually described in terms of a dynamic equilibrium between three main staggered rotamers, namely, *gauche-gauche* (*gg*), *trans-gauche* (*tg*), and *gauche-trans* (*gt*). Karplus equations are used to estimate the *gg* rotamer population.[32] It may be concluded that the population of *gg* rotamers predominates for any of the nucleosides **5**−**7**. The conformational features of **6** and **7** are similar to those observed for 5-formyl-2′-deoxyuridine (for⁵dUrd) and 5-(hydroxymethyl)-2′-deoxyuridine (hm⁵dUrd), respectively.[7,26] This indicates that the substitution of the keto group at C4 by an amino group induces only minor conformational changes in these nucleosides. On the other hand, 5-formylcytidine the riboside analog of **6** has been reported to be in a predominant C3′ *endo* conformation in aqueous solution.[33] This appears to be a general trend for pyrimidine ribonucleosides.[34] It should be added that the above results concerning the conformational properties of hm⁵dCyd (**7**) in solution are not consistent with those obtained by X-ray crystallography.[35] Curiously, the sugar ring of the crystal structure of hm⁵dCyd (**7**) preferentially adopts a C3′ *endo* and a *tg* conformation. This suggests that the solvent and hydrogen bonding play a major role in determining the conformation of hm⁵dCyd (**7**). Another interesting conformational feature of nucleosides is the orientation of the pyrimidine base with respect to the sugar moiety. A *syn* conformation implies that C2 lies over the sugar ring. This leads to anisotropic effects mediated by the 2-keto group on the chemical shifts of the sugar protons, mostly on the H2′ proton.[36,37] However, we may note a lack of any significant deshielding effects on the H2′ chemical shift of the three methyl oxidation products **5**−**7**

(26) Birnbaum, G. I.; Deslauriers, R.; Lin, T.-S.; Shiau, G. T.; Prusoff, W. H. *J. Am. Chem. Soc.* **1980**, *102*, 4236.
(27) Cadet, J.; Téoule, R. *Bull. Soc. Chim. Fr.* **1975**, 879.
(28) Wagner, R.; van Lier, J. E.; Berger, M.; Cadet, J. *J. Am. Chem. Soc.* **1994**, *116*, 2235.
(29) Tofigh, S.; Frenkel, K. *Free Rad. Biol. Med.* **1989**, *7*, 131.
(30) Altona, C.; Sundaralingham, M. *J. Am. Chem. Soc.* **1972**, *94*, 8205.
(31) Altona, C.; Sundaralingham, M. *J. Am. Chem. Soc.* **1973**, *95*, 2333.
(32) Ramaswany, H.; Sarma, R. H. In *Nucleic Acid Geometry and Dynamics*; Sarma, R. H., Ed.; University Park Press: Baltimore, 1974; pp 1−45.
(33) Kawai, G.; Yokogawa, T.; Nishikawa, K; Ueda, T.; Hashizume, T.; McCloskey J. A.; Yokoyama, S.; Watanabe, K. *Nucleosides Nucleotides* **1994**, *13*, 1189.
(34) Uhl, W.; Reiner, J.; Gassen H. G. *Nucleic Acids Res.* **1983**, *11*, 1167.
(35) Li, J.; Kumar, S. V.; Stuart, A. L.; Delbaere, L. T.; Gupta, S. V. *Acta Crystallogr.* **1994**, *C50*, 1837.
(36) Schweizer, M. P.; Banta, E. B.; Witkowski, J. T.; Robins, R. K. *J. Am. Chem. Soc.* **1973**, *95*, 3770.
(37) Cadet, J.; Taïeb, C.; Remin, M.; Niemczura, W. P., Hruska, F. E. *Biochim. Biophys. Acta* **1980**, *608*, 435.

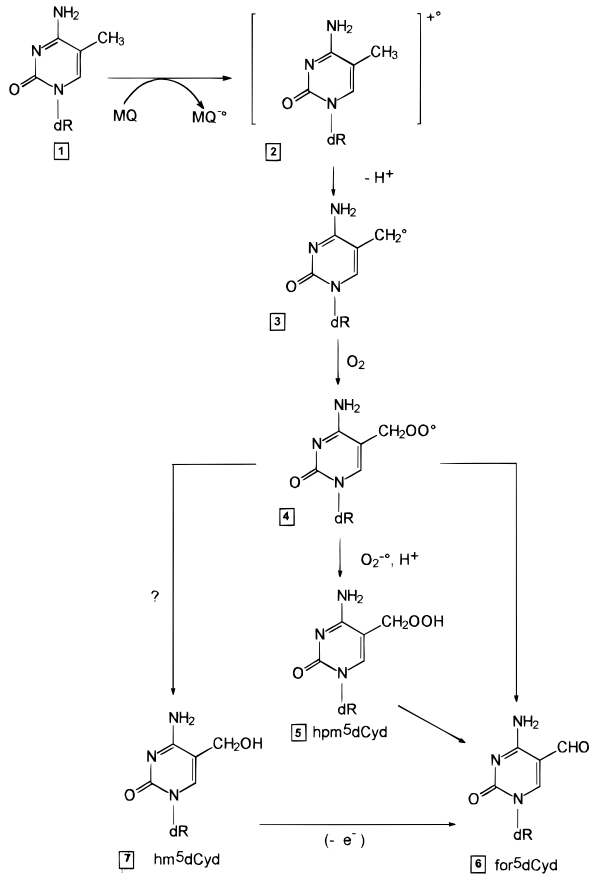

Figure 2. Mechanisms of the formation of the 5-formyl-2′-deoxycytidine and 5-(hydroxymethyl)-2′-deoxycytidine *via* deprotonation of the 5-methyl-2′-deoxycytidine radical cation.

of m^{5}dCyd (**1**). This strongly suggests that nucleosides **5**−**7** adopt a preferential *anti* conformation in aqueous solutions.

Mechanisms of Formation of the Final 5-Methyl-2′-deoxycytidine Photooxidation Products. Isolation of 5-(hydroperoxymethyl)-2′-deoxycytidine (**5**) combined with earlier studies on the sensitized photooxidation of pyrimidine nucleosides allows us to propose a mechanism for the formation of the methyl oxidized m^{5}dCyd photoproducts (Figure 2). It is well documented that the initial step of the menadione-mediated photooxidation of pyrimidine nucleosides in aerated aqueous solution consists of an activation of the sensitizer. The menadione is excited by photon absorption to a singlet state which is further converted into a triplet excited state species which has a longer lifetime. In a subsequent step, electron transfer from the pyrimidine to the triplet excited quinone sensitizer is expected to occur.[38,39] As a result, both the pyrimidine radical cation and the menadione radical anion are generated. It has been demonstrated that the menadione radical anion has a short turnover (half-life time = 3.5 μs) due to a fast reaction with molecular oxygen.[40] The latter reaction allows the regeneration of the menadione in its ground state leading to the formation of the radical superoxide ($O_2^{\bullet-}$). It has to be noted that $O_2^{\bullet-}$ does not exhibit any significant reactivity toward DNA bases, at least, in aqueous solution.[41] The formation of the menadione-mediated final photooxidation products of thymidine has been rationalized in terms of competitive hydration and deprotonation reactions of the initial pyrimidine radical cation.[7] The present study focused on the final products resulting from the deprotonation of the 5-methyl-2′-deoxycytidine radical cation (**2**) Deprotonation of the radical cation **2** which appears to be the predominant decomposition pathway (≅ 60%) gives rise to the formation of the methyl-centered radical **3** as recently shown by an EPR study.[42] It is reasonable to assume that in a subsequent step, 5-(2′-deoxycytidylyl)methyl neutral radical **3** reacts with molecular oxygen leading to the formation of the peroxyl radical **4**. Then, radical **4** may be converted into 5-(hydroperoxymethyl)-2′-deoxycytidine (**5**) upon reduction by $O_2^{\bullet-}$, followed by protonation as proposed for the menadione photosensitized formation of thymidine hydroperoxides.[40] It should be mentioned that the formation of 5-(hydroxymethyl)-2′-deoxycytidine (**7**) can be explained in terms of decomposition of the unstable peroxyl radical **4** through a Russell mechanism.[43,44] Moreover, it is reasonable to suggest that the latter reaction pathway may partly account for the formation of 5-formyl-2′-deoxycytidine (**6**). In addition, evidence was provided that **6** is produced, at least partly, by menadione sensitization of 5-(hydroxymethyl)-2′-deoxycytidine (**7**) to UV-A in aerated aqueous solution. This result suggests that hm^{5}dCyd (**7**), as m^{5}dCyd (**1**), is susceptible to lead to the formation of for^{5}dCyd (**6**) *via* a transient pyrimidine radical cation. It is important to note that the yield of **6** decreases after 60 min of irradiation. This observation clearly demonstrates that **6** is degraded by menadione-mediated photooxidation. It can be added that neither 5-formyl-2′-deoxyuridine nor 5-carboxy-2′-deoxycytidine was detected in the 5-methyl-2′-deoxycytidine (**1**) irradiated mixture.

Conclusion

The oxidation of the methyl group of m^{5}dCyd (**1**) has been previously investigated by using a chemical method.[45] In the present work, we reported that the 350 nm irradiation of an aerated aqueous solution of 5-methyl-2′-deoxycytidine in the presence of menadione leads to the formation of 5-formyl-2′-deoxycytidine (**6**) and 5-(hydroxymethyl)-2′-deoxycytidine (**7**) as the main final oxidized photoproducts. Moreover, the isolation of 5-(hydroperoxymethyl)-2′-deoxycytidine (**5**), an unstable intermediate provided further support for the mechanism of formation of the latter oxidation products. The structures of hm^{5}dCyd (**7**), for^{5}dCyd (**6**) and hpm^{5}dCyd (**5**) have been established on the basis of extensive spectroscopic measurements. It is well documented that 5-methylcytosine derivatives may be converted into related thymine compounds by an hydrolytic deamination reaction in aqueous solution. However, such a deamination reaction occurs more efficiently with C5−C6 saturated 5-methylcytosine derivatives.[46,47] In agreement with these observations, we has found that both for^{5}dCyd (**6**) and hm^{5}dCyd (**7**) do not deaminate and remain stable in aqueous solution. Our current purpose is to measure UV-A induced formation of 5-formylcytosine and 5-hydroxymethylcytosine in DNA. In a future stage, attempts will be made to study the enzymatic repair of such lesions. In this respect it should be interesting to determine if these two DNA

(38) Schenck, G. O. *Ind. Eng. Chem.* **1968**, 40.
(39) Foote, C. *Photochem. Photobiol.* **1991**, *54*, 659.
(40) Wagner, J. R.; van Lier, J. E.; Johnston, L. J. *Photochem. Photobiol.* **1978**, *28*, 661.
(41) Cadet, J.; Téoule, R. *Photochem. Photobiol.* **1978**, *28*, 661.
(42) Malone, M. E.; Cullis, P. M.; Symons M. C. R.; Parker, A. W. *J. Phys. Chem.* **1995**, *99*, 9299.
(43) Russell, G. A. *J. Am. Chem. Soc.* **1957**, *79*, 3871.
(44) Simic, M.; Hayon, E. *FEBS Lett.* **1974**, *44*, 334.
(45) Itahara, T.; Yoshitake, T.; Koga, S.; Nishino, A. *Bull. Chem. Soc. Jpn.* **1994**, *67*, 2257.
(46) Wyszynski, M.; Gabbara, S.; Ashok, S.; Bhagwat, A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 1574.
(47) Bienvenu, C.; Cadet, J. *J. Org. Chem.* **1996**, *61*, 2632.

lesions are substrates for the glycosylase action of the AlkA repair enzyme.[48]

Acknowledgment. We thank Colette Lebrun (SCIB/LRI, CEA-Grenoble) and Daniel Ruffieux (Biochimie C/CHRU Grenoble) for recording the mass spectra. We also acknowledge Maurice Berger and Cédric D'Ham (SCIB/LAN CEA-Grenoble) for their assistance in performing HPLC analyses. This work was partly supported by grants from NATO and the French Ministry of Science and Research (ACC-SV N°8-MESR 1995).

(48) Berdal, K. G.; Bjoras, M.; Bjelland, S.; Seeberg, E. *EMBO J.* **1990**, *9*, 4563.

Supporting Information Available: Copies of ^{1}H spectra of **6**, **7**, supporting figures, and experimental details concerning the spectroscopic measurements and HPLC analyses (10 pages). See any current masthead page for ordering and Internet access instructions.

JA962073H