Formation of Cyclobutane Dimers and (6–4) Photoproducts upon Far-UV Photolysis of 5-Methylcytosine-Containing Dinucleoside Monophosphates

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ABSTRACT: The far-UV photochemistry of 5-methylcytosine, a minor DNA base, was studied in three dinucleoside monophosphates, including m^{5}dCpT, Tpm^{5}dC, and m^{5}dCpdC. The model compounds were exposed to 254-nm radiation, and the resulting photoproducts were isolated by reverse-phase HPLC and characterized as cyclobutane dimers, (6–4) adducts, and the related Dewar valence isomers by UV, mass, and ^{1}H NMR spectroscopies. The rate of formation of the different photoproducts was compared with those obtained by photolysis of TpT and the corresponding cytosine dinucleoside monophosphates, including dCpT, TpdC, and dCpdC. The formation of deaminated m^{5}dC-containing photoproducts was observed in each of the far-UV irradiated solution of m^{5}dCpT, Tpm^{5}dC, and m^{5}dCpdC. They were shown to be generated mainly through a photochemical process since methylation of the C5 atom of the cytosine ring appeared to dramatically decrease the deamination rate of the C5–C6 saturated photoproducts.

Direct photochemical reactions of nucleobases are involved in the lethal and mutagenic effects of UV-B light, the part of the solar light spectrum mostly responsible for the apparition of skin cancer (Taylor, 1990). UV-C (230–290 nm) and UV-B (290–320 nm) radiations have been shown to induce the formation of dimeric photoproducts between adjacent pyrimidine bases in DNA (Figure 1). Three major types of photoproducts are generated, including cyclobutane dimers, pyrimidine (6–4) pyrimidone adducts, and their Dewar valence isomers (Cadet & Vigny, 1990; Cadet et al., 1992). The photochemistry of cytosine and thymine has been extensively studied by using either monomeric model compounds, including bases, nucleosides, and nucleotides (Beukers & Berends, 1960; Varghese & Wang, 1968; Varghese, 1971; Cadet et al., 1985), or dinucleoside monophosphates (Johns et al., 1964; Liu & Yang, 1978a,b; Franklin et al., 1985; Rycyna & Alderfer, 1985; Taylor & Cohrs, 1987; Kan et al., 1987, 1992; Taylor & Lu, 1990; Douki & Cadet, 1992; Lemaire & Ruzsicska, 1993a).

5-Methylcytosine is a minor pyrimidine nucleobase, endogenously generated by enzymatic methylation of cytosine, and is mainly observed at cytosine–guanine sequences (Ehrlich & Wang, 1981). The amount of 5-methylcytosine can be as high as 30% in some species, but the mean value is around 2–3% in eukaryotic cells. The biological role (Adams & Burdon, 1985) of this base is mostly related to the regulation of genetic expression and the stabilization of DNA structures such as the Z-conformation (van Lier et al., 1983). Therefore, exogenously induced modifications of 5-methylcytosine residues are likely to be biologically significant. However, only a little attention has been paid to the photochemistry of 5-methylcytosine.

The only few available data on the formation of cyclobutane dimers and (6–4) photoproducts involving 5-methylcytosine residues are based on indirect observations of the lesions. Most of these studies reported the lack of formation of these photoproducts upon far-UV[1] irradiation of either 5-methylcytosine DNA (Ehrlich et al., 1986a) or 5-methyl-2′-deoxycytidine (Ehrlich & Dove, 1983). Based on these early works, it was assumed that 5-methylcytosine does not undergo dimerization and/or formation of (6–4) photoproducts upon far-UV irradiation. For instance, this statement was used by Glickman et al. (1986) to indirectly prove the formation of the cytosine–cytosine pyrimidine (6–4) pyrimidone adduct in far-UV irradiated DNA, on the basis that methylation of cytosine led to a decrease in the number of alkali-labile sites at CC sequences. In constrast, a study carried out on oligonucleotides and naked DNA suggested the formation of cyclobutane dimers containing 5-methylcytosine, though the photoproducts were neither isolated nor unambiguously characterized (Barna et al., 1988). The only well-identified 5-methylcytosine far-UV photoproducts are pyrimidine ring-opening products obtained in aqueous and organic solutions (Shaw & Shetlar, 1989, 1990; Celewicz & Shetlar, 1992).

Since previous works did not provide clear evidence for either the absence or the formation of 5-methylcytosine-containing dimeric photoproducts, we have studied the photochemical reactions of this nucleobase when incorporated in bipyrimidine dinucleoside monophosphates. These molecules are better model compounds for the study of dimerization than monomeric DNA components. The formation of bipyrimidine photoproducts is an intramolecular reaction in dinucleoside monophosphates, like in DNA, and is more favored than the corresponding bimolecular reaction of monomeric model compounds. We, herein, report the isolation and the characterization of the photoproducts of three 5-methylcytosine-containing dinucleoside monophosphates (m^{5}dCpT, Tpm^{5}dC, and m^{5}dCpdC). A comparison of the

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[1] Abbreviations: dCpdC, 2′-deoxycytidylyl-(3′–5′)-2′-deoxycytidine; dCpT, 2′-deoxycytidylyl-(3′–5′)-thymidine; DNA, deoxyribonucleic acid; HPLC, high-performance liquid chromatography; m^{5}dCpdC, 5-methyl-2′-deoxycytidylyl-(3′–5′)-2′-deoxycytidine; m^{5}dCpT, 5-methyl-2′-deoxycytidylyl-(3′–5′)-thymidine; NMR, nuclear magnetic resonance; ODS, octadecylsilane column; TpdC, thymidylyl-(3′–5′)-2′-deoxycytidine; Tpm^{5}dC, thymidylyl-(3′–5′)-5-methyl-2′-deoxycytidine; TpT, thymidylyl-(3′–5′)-thymidine; TSP, potassium trimethylsilylpropionate; UV, ultraviolet light; FAB, fast atom bombardment.

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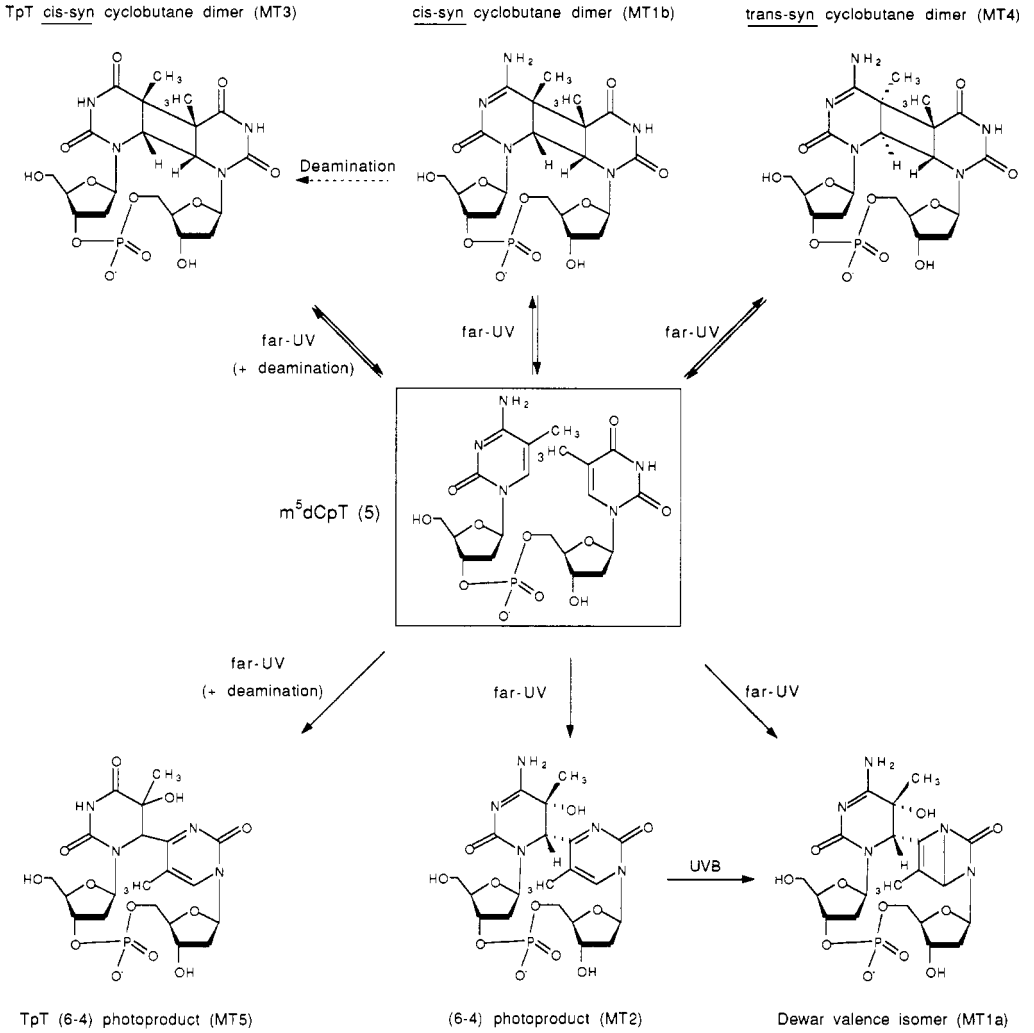


FIGURE 1: Far-UV photochemistry of m^{5}dCpT. MT3 was also obtained from MT1b by a slow deamination reaction in water.

rate of their formation with those of the related thymine- and cytosine-containing photoproducts of dinucleoside monophosphates, including TpT, TpdC, dCpT, and dCpdC, was achieved. Deamination of 5-methylcytosine photoproducts was also investigated and found to occur mainly through a photochemical process.

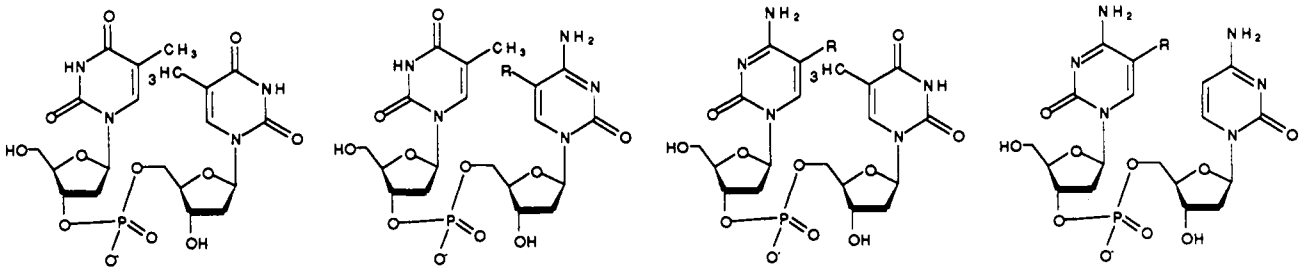
MATERIALS AND METHODS

Chemicals. 2′-Deoxycytidine and thymidine were obtained from Pharma-Waldhof (Düsseldorf, Germany). 5-Methyl-2′-deoxycytidine and chemicals used for the synthesis of the dinucleoside monophosphates, including benzoyl chloride, triazole, 4-chlorophenyl dichlorophosphate, 2,4,6-triisopropylbenzenesulfonyl chloride, and *N*-methylimidazole, were purchased from Aldrich-Chemie (Steinheim, Germany).

Spectroscopic Analysis. ^{1}H NMR measurements of 400.13 MHz were carried out on a AM 400 spectrometer (Brüker, Wissenbourg, France). The samples were solubilized in 99.96% D_2O (Eurisotop, Paris, France) after chemical exchange of the labile protons in 99.8% D_2O. Chemical shifts were inferred from first-order spectra analysis and expressed with respect to potassium trimethylsilylproprionate (TSP) used as an internal reference. FAB (fast atom bombardment) mass spectrum analyses were carried out in the negative mode ionization on a VG ZAB 2-EQ apparatus (Manchester, U.K.). The sample to be analyzed was dissolved in glycerol. The peaks corresponding to the glycerol matrix ions were predominant in most spectra. In addition, very low fragmentation was observed but the pseudo-molecular ions [M – H] were observed for all compounds. UV spectra was obtained on a DU-88 (Beckmann, Irvine, CA) spectrophotometer, with the sample dissolved in water.

High-Performance Liquid Chromatography Separations. Photoproducts were isolated by reverse-phase HPLC in the ion suppression mode. The chromatographic system consisted of a L 6200 Hitachi (Tokyo, Japan) intelligent pump equipped with a semipreparative (250 × 7 mm i.d., particle size 10 μm) Nucleosil 100–10 C_{18} octadecylsilyl silica gel column (Macherey-Nagel, Düren, Germany). The isocratic eluent was a 25 mM ammonium formate solution, and the flow rate was 3 mL min^{-1}. The void volume of the column was 7.5 mL. The detection was provided by a LKB-Bromma 2151 variable wavelength UV spectrometer (Pharmacia-LKB, Uppsala, Sweden) set at 230 nm. Fluorescence detection, specific for the (6–4) photoproducts, was achieved by using a F 1050 spectrofluorimeter (Hitachi, Tokyo, Japan) with the excitation and emission wavelengths set at 320 and 390 nm, respectively.

Synthesis of Dinucleoside Monophosphates. Dinucleoside monophosphates were synthesized by using a triester method in liquid phase. The phosphorylation was carried out according to Chattopadahyaya and Reese (1979), and the condensation of the second nucleoside was achieved by using the method reported by Miyoshi and Takura (1980). Seven compounds, including TpT, TpdC, dCpT, and dCpdC, and the related 5-methylcytosine derivatives, including Tpm^{5}dC, m^{5}dCpT, and m^{5}dCpdC, were prepared (Figure 2).



1 2 R=H 3 R=C H 3 4 R=H 5 R=C H 3 6 R=H 7 R=C H 3

FIGURE 2: Structure of the dinucleoside monophosphates used in the present study: (**1**) TpT, (**2**) TpdC, (**3**) Tpm^{5}dC, (**4**) dCpT, (**5**) m^{5}dCpT, (**6**) dCpdC, (**7**) m^{5}dCpdC.

Far-UV Irradiations. The dinucleoside monophosphates (50 mg) were dissolved in 25 mL of water, and the resulting solution was placed in a 15-cm diameter Petri dish. The solutions were irradiated for 1 h with a VL-215 G germidical lamp (Bioblock, Illkirch, France) emitting mainly at 254 nm. The overall UV dose was 1.5 kJ m^{-2}, as determined by the actinometric method based on the formation of 1,3-dimethyluracil photohydrate (Shaw & Shetlar, 1990). To prevent possible deamination of the cytosine or 5-methylcytosine-containing photoproducts, the solutions were immediately frozen after irradiation and freeze-dried. The samples were thawed immediately prior to HPLC purification. For the experiments aimed at comparing the photolysis yields, the samples were diluted to reach a UV absorption of 1 OD at the UV maximum of each dinucleoside monophosphate.

Photoproducts of TpT, TpdC, and dCpT. The photoproducts of TpT (**1**), TpdC (**2**), and dCpT (**4**) were obtained as described in the literature. The fractions collected were the following: TpT: TT1 (*cis–syn* cyclobutane dimer), TT2 ((6–4) photoproduct), and TT3 (*trans–syn* cyclobutane dimer) (Kan et al., 1988); dCpT: CT1 (Dewar valence isomer), CT2 (deaminated *cis–syn* cyclobutane dimer), and CT3 ((6–4) photoproduct) (Douki & Cadet, 1992; Douki et al., 1991); TpdC: TC1 (Dewar valence isomer) and TC2 ((6–4) photoproduct) (Franklin et al., 1985; Taylor & Lu, 1990).

Irradiation of dCpdC. The 254-nm irradiation of dCpdC (**6**) in aqueous solution provided one main compound, CC1 ($k' = 4$), in a yield that was 1 order of magnitude lower than that of the photoproducts of dCpT, TpT, or TpdC. It exhibited a maximum centered at 260 nm in its UV spectra. In addition, signals corresponding to the H5 and H6 protons of a cytosine moiety (two doublets with $\delta = 6.35$ and 7.95 ppm, $J = 8.5$ Hz) were observed in its ^{1}H NMR spectrum. It should be added that no fluorescent photoproduct was observed in the photolyzed mixture.

Isolation of Photoproducts of Tpm^{5}dC. Tpm^{5}dC (**3**) (50 mg) was irradiated for 1 h with the 30-W germicidal lamp. The sample was concentrated to 1 mL by evaporation *in vacuo* and injected on the HPLC system, with the UV detector set at 230 nm. Two major fractions, TM1 ($k' = 2.3$) and TM2 ($k' = 6.2$), were collected and freeze-dried. TM2 was also detected by fluorescence. The attribution of the ^{1}H NMR signals was carried out by comparison of the chemical shifts with those of the corresponding TpT photoproducts (Table 1). Two additional small peaks were observed in the HPLC elution profile. However, the corresponding products were isolated in a amount too low to be characterized.

TM1, TpT *cis–syn* cyclobutane dimer: FAB-MS negative mode, $m/z = 545$ ([M – H]; relative intensity 35%). ^{1}H NMR (D_2O, 400.13 MHz, TSP) δ (ppm): Tp, 5.73 (1 H, H1′), 2.67 (1 H, H2′), 2.40 (1 H, H2″), 4.70 (1 H, H3′), 4.22 (1 H, H4′), 1.54 (3 H, methyl), 4.39 (1 H, H6); pT, 6.03 (1 H, H1′), 2.39 (1 H, H2′), 2.17 (1 H, H2″), 4.39 (1 H, H3′), 4.04 (1 H, H4′), 1.59 (3 H, methyl), 4.31 (1 H, H6).

Table 1: ^{1}H NMR 400.13 MHz Chemical Shifts (in ppm with Respect to TSP as Internal Reference) of Photoproducts of TpT, dCpT, TpdC, and m^{5}dCpT in D_2O

		H1′	H2′	H2″	H3′	H4′	H5/Me	H6
cis–syn Cyclobutane Dimers								
(dU)pT	CT1	5.69	2.75	2.40	4.73	4.27	3.56	4.50
dUp(T)		6.09	2.40	2.28	4.27	3.98	1.71	4.47
(T)pT	TT1	5.71	2.68	2.40	4.72	4.23	1.54	4.40
Tp(T)	MT3 TM1	6.04	2.40	2.17	4.38	4.04	1.59	4.30
trans–syn Cyclobutane Dimers								
(m^{5}C)pT	MT4	5.47	3.34	2.46	4.84	4.24	1.63	4.09
m^{5}Cp(T)		5.76	2.23	2.05	4.60	3.95	1.57	4.24
(T)pT	TT3	5.36	3.39	2.68	4.84	4.21	1.58	4.22
Tp(T)		5.80	2.27	2.07	4.63	3.98	1.52	4.33
(6–4) Photoproducts								
(dC)pT	CT3	6.25	1.40	2.20	4.01	3.78	5.30	5.45
dCp(T)		6.55	3.15	2.70	4.88	4.23	2.40	8.10
(T)pdC	TC2	6.26	1.77	2.35	4.03	3.82	1.58	5.06
Tp(dC)	MC2	6.58	3.06	2.71	4.87	4.15	6.88	8.37
(T)pT	TT2	6.27	1.55	2.25	4.08	3.76	1.83	5.17
Tp(T)	MT5 TM2	6.60	3.15	2.67	4.82	4.23	2.40	8.09
(m^{5}C)pT	MT2	6.33	1.45	2.16	4.07	3.75	1.85	5.08
m^{5}Cp(T)		6.58	3.12	2.66		4.22	2.38	8.06
Dewar Valence Isomers								
(dC)pT	CT1	5.65	2.25	2.25	4.45	3.90	4.90	4.90
dCp(T)		6.35	2.50	2.25	4.60	3.85	2.10	5.70
(T)pdC	TC1	5.81	2.39	2.39	4.54	4.02	1.54	4.78
Tp(dC)	MC1	6.41	2.57	2.55	4.80	3.93	6.91	5.56

TM2, TpT (6–4) photoproduct: FAB-MS negative mode, $m/z = 545$ ([M – H], relative intensity 9%); $m/z = 527$ ([M – H – H_2O], relative intensity 6%); UV (λ_{max}, H_2O): 325 nm. ^{1}H NMR (D_2O, 400.13 MHz, TSP) δ (ppm): Tp, 6.27 (1 H, H1′), 1.55 (1 H, H2′), 2.24 (1 H, H2″), 4.09 (1 H, H3′), 3.77 (1 H, H4′), 1.81 (3 H, methyl), 5.17 (1 H, H6); pT, 6.61 (1 H, H1′), 3.14 (1 H, H2′), 2.67 (1 H, H2″), 4.82 (1 H, H3′), 4.24 (1 H, H4′), 2.42 (3 H, methyl), 8.08 (1 H, H6).

Isolation of Photoproducts of m^{5}dCpT. m^{5}dCpT (**5**) (50 mg) was irradiated for 1 h in aqueous solution with the germicidal lamp. Five fractions, detected by UV monitoring at 230 nm, were isolated by reverse-phase HPLC, including MT1 ($k' = 1.0$), MT2 ($k' = 1.8$), MT3 ($k' = 2.4$), MT4 ($k' = 5.2$), and MT5 ($k' = 6.3$). MT2 and MT5 exhibited fluorescence properties and absorbed at 325 nm. All compounds were analyzed by FAB mass spectrometry and 400.13 MHz ^{1}H NMR. The attribution of the ^{1}H NMR signals was carried out by comparison with the ^{1}H NMR features of the photoproducts of TpT.

MT1, mixture of two photoproducts: FAB-MS negative mode, $m/z = 544$ (relative intensity 10%, [M – H]).

MT2, m^{5}dCpT (6–4) photoproduct: FAB-MS negative mode, m/z = 544 ([M – H], relative intensity 100%). UV (λ_{max}, H_2O): 325 nm. ^{1}H NMR (D_2O, 400.13 MHz, TSP) δ (ppm): m^{5}dCp, 6.33 (1 H, H1′), 1.45 (1 H, H2′), 2.16 (1 H, H2″), 4.07 (1 H, H3′), 3.75 (1 H, H4′), 1.85 (3 H, methyl), 5.08 (1 H, H6); pT, 6.58 (1 H, H1′), 3.12 (1 H, H2′), 2.66 (1 H, H2″), 4.22 (1 H, H4′), 2.38 (3 H, methyl), 8.06 (1 H, H6).

MT3, TpT *cis–syn* cyclobutane dimer: FAB-MS negative mode, m/z = 545 ([M – H], relative intensity 87%). ^{1}H NMR (D_2O, 400.13 MHz, TSP) δ (ppm): Tp, 5.72 (1 H, H1′), 2.69 (1 H, H2′), 2.38 (1 H, H2″), 4.72 (1 H, H3′), 4.23 (1 H, H4′), 1.54 (3 H, methyl), 4.39 (1 H, H6); pT, 6.05 (1 H, H1′), 2.39 (1 H, H2′), 2.17 (1 H, H2″), 4.39 (1 H, H3′), 4.03 (1 H, H4′), 1.59 (3 H, methyl), 4.31 (1 H, H6).

MT4, m^{5}dCpT *trans–syn* cyclobutane dimer: FAB-MS negative mode, m/z = 544 ([M – H], relative intensity 33%). ^{1}H NMR (D_2O, 400.13 MHz, TSP) δ (ppm): m^{5}dCp, 5.47 (1 H, H1′), 3.34 (1 H, H2′), 2.46 (1 H, H2″), 4.84 (1 H, H3′), 4.24 (1 H, H4′), 1.63 (3 H, methyl), 4.09 (1 H, H6); pT, 5.76 (1 H, H1′), 2.23 (1 H, H2′), 2.05 (1 H, H2″), 4.60 (1 H, H3′), 3.95 (1 H, H4′), 1.57 (3 H, methyl), 4.24 (1 H, H6).

MT5, TpT (6–4) photoproduct: FAB-MS negative mode, m/z = 545 ([M – H], relative intensity 37%). UV (λ_{max}, H_2O): 325 nm. ^{1}H NMR (D_2O, 400.13 MHz, TSP) δ (ppm): Tp, 6.27 (1 H, H1′), 1.55 (1 H, H2′), 2.27 (1 H, H2″), 4.07 (1 H, H3′), 3.75 (1 H, H4′), 1.83 (3 H, methyl), 5.18 (1 H, H6); pT, 6.61 (1 H, H1′), 3.14 (1 H, H2′), 2.66 (1 H, H2″), 4.83 (1 H, H3′), 4.23 (1 H, H4′), 2.41 (3 H, methyl), 8.10 (1 H, H6).

Isolation of Photoproducts of m^{5}dCpdC. m^{5}dCpdC (**7**) (50 mg) was irradiated for 1 h in aqueous solution. Reverse-phase HPLC separation of the concentrated solution provided two main fractions, MC1 (k' = 2.5) and MC2 (k' = 6.4). Then, the corresponding fractions were freeze-dried. The assignment of the ^{1}H NMR signals of the corresponding compounds was inferred from a comparison with the spectroscopic features of the TpdC photoproducts (Table 1). MC2 was found to be fluorescent.

MC1, TpdC Dewar valence isomer: FAB-MS negative mode, m/z = 530 ([M – H], relative intensity 12%). ^{1}H NMR (D_2O, 400.13 MHz, TSP) δ (ppm): Tp, 5.82 (1 H, H1′), 2.40 (1 H, H2′), 2.40 (1 H, H2″), 4.55 (1 H, H3′), 4.02 (1 H, H4′), 1.55 (3 H, methyl), 4.79 (1 H, H6); pdC, 6.40 (1 H, H1′), 2.58 (1 H, H2′), 2.53 (1 H, H2″), 4.81 (1 H, H3′), 3.93 (1 H, H4′), 6.91 (1 H, H5), 5.57 (1 H, H6).

MC2, TpdC (6–4) photoproduct: FAB-MS negative mode, m/z = 530 ([M – H], relative intensity 100%). UV (λ_{max}, H_2O): 315 nm. ^{1}H NMR (D_2O, 400.13 MHz, TSP) δ (ppm): Tp, 6.26 (1 H, H1′), 1.77 (1 H, H2′), 2.34 (1 H, H2″), 4.02 (1 H, H3′), 3.80 (1 H, H4′), 1.59 (3 H, methyl), 5.06 (1 H, H6); pdC, 6.59 (1 H, H1′), 3.05 (1 H, H2′), 2.71 (1 H, H2″), 4.88 (1 H, H3′), 4.15 (1 H, H4′), 6.86 (1 H, H5), 8.38 (1 H, H6).

Photosensitization of m^{5}dCpT. m^{5}dCpT (**5**) (50 mg) was irradiated in 30 mL of a 0.01 M aqueous solution of acetophenone. The UV-B light was provided by a Rayonet photoreactor (The Southern New England Ultraviolet Co., Handem, MA) equipped with 16 15-W tubes emitting mainly at 300 nm. The overall UV dose was 1 kJ m^{-2}. The photolyzed solution was freeze-dried, and two main photoproducts were isolated by reverse-phase HPLC. The second eluting compound (k' = 2.3) was identified as TT1, by comparing its ^{1}H NMR spectrum and chromatographic features with those of the *cis–syn* cyclobutane dimer of TpT. The first eluting product (k' = 1.1) slowly converted into TT1 when left in aqueous solution at room temperature.

Table 2: Capacity Factors (k')[a] of Photoproducts of TpT, dCpT, TpdC, M^{5}dCpT, m^{5}dCpdC, and Tpm^{5}dC on ODS Column[b]

	1	2	3	4	5
TpT	2.3	6.2	8.8		
dCpT	0.6	1.1	1.6		
TpdC	2.5	6.3			
m^{5}dCpT	1.0	1.8	2.4	5.2	6.3
m^{5}dCpdC	2.5	6.4			
Tpm^{5}dC[c]	2.3	6.2			

[a] $k' = (V - V_0)/V_0$, where V and V_0 are the elution volume of the compound and the void volume of the chromatographic system, respectively. [b] The system was equipped with a semipreparative (250 × 7 mm i.d., particle size, 10 μm) Nucleosil 100-10 C_{18} octadecylsilyl silica gel column (Macherey-Nagel, Düren, Germany). The isocratic eluent was a 25 mM ammonium formate solution, and the flow rate was 3 mL min^{-1}. [c] Two other photoproducts were observed, but generated in too low yield to be characterized.

RESULTS

Characterization of Photoproducts of TpT, TpdC, dCpT, and dCpdC. Comparison of the spectroscopic features of the photoproducts with available data allowed the identification of the compounds isolated after photolysis of TpT (Kan et al., 1988), TpdC (Franklin et al., 1985; Taylor & Lu, 1990), and dCpT (Douki & Cadet, 1992; Douki et al., 1991). Only one major photoproduct (CC1) was generated upon far-UV photolysis of dCpdC. CC1 was produced in, at least, a 10-fold lower yield than the photoproducts of the other dinucleoside monophosphates, as inferred from the comparison of the area of the peaks of the HPLC profile. CC1 was likely to be only modified on one of the two cytosine moieties, as shown by the observation of signals corresponding to the H5 and H6 protons of a cytosine moiety in the ^{1}H NMR spectrum. Moreover, the presence of a maximum around 260 nm in the UV absorption spectrum was indicative of the presence of an unsaturated pyrimidine ring. Even though no further characterization of CC1 was performed due to the small amount available, the modified base has a likely photohydrate structure (Johns et al., 1965; Liu & Yang, 1978b).

Characterization of Photoproducts of Tpm^{5}dC. The two main photoproducts isolated from the far-UV irradiated aqueous solution of Tpm^{5}dC (**3**) were unambiguously characterized as photoproducts of TpT. TM1 and TM2 were identified as the *cis–syn* cyclobutane dimer and the (6–4) adduct of TpT, respectively. This was inferred from their mass and ^{1}H NMR spectra (Table 1) and chromatographic features (Table 2), which are identical to those of the photoproducts prepared by far-UV photolysis of TpT.

The characterization of TM2 as the TpT (6–4) photoproduct was further confirmed by the observation in the negative FAB mass spectrum of a peak at m/z = 527. This was rationalized in terms of the loss of a water molecule from the C5–C6 bond of the 5′ end pyrimidine ring (Figure 4). A loss of a NH_3 group would have been observed if TM2 was the Tpm^{5}dC (6–4) adduct. However, the formation, in a very low yield, of 5-methylcytosine-containing photoproducts cannot be ruled out since a few unidentified photoproducts corresponding to peaks of very low intensity were detected by HPLC analysis of the photolyzed solution of Tpm^{5}dC.

Characterization of Photoproducts of m^{5}dCpT. Five fractions were isolated after 254-nm photolysis of m^{5}dCpT (**5**). The ^{1}H NMR (Table 1) and chromatographic features

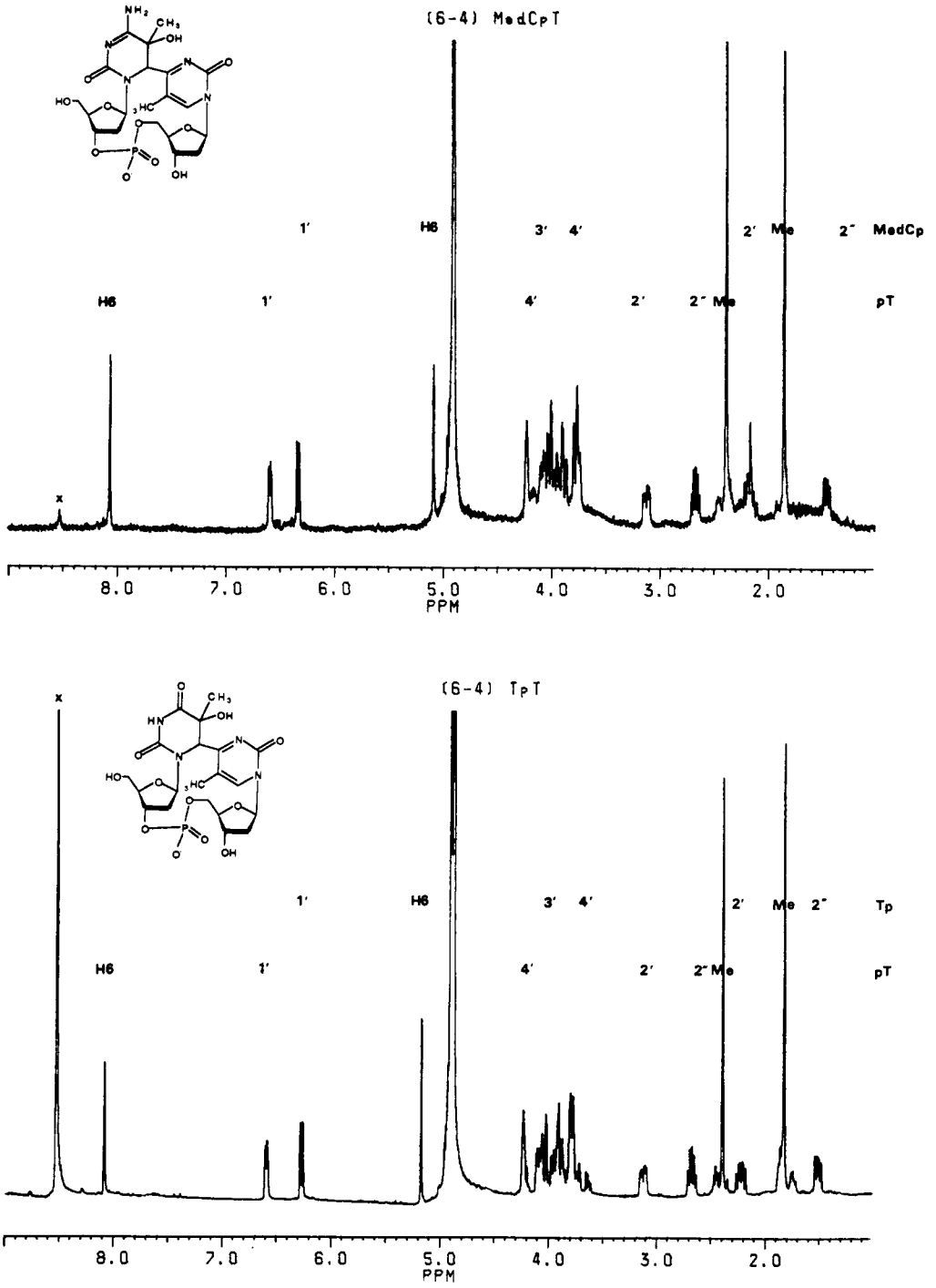


FIGURE 3: ^{1}H NMR 400.13 MHz spectra of the (6–4) photoproducts of m^{5}dCpT (MT3) and TpT (TT2 and MT5) in D_2O. Chemical shifts are expressed with respect to TSP used as the internal reference. (x, ammonium formate from the HPLC buffer).

(Table 2) of MT5 were identical to those of the authentic TpT (6–4) adduct (TT2). The spectroscopic characteristics of MT3 were found to be identical to those of the *cis–syn* cyclobutane dimer of TpT (TT1). In addition, both photoproducts exhibited a pseudo-molecular ion [M – H] at m/z = 545 in the negative FAB mass spectrum. Altogether, these data clearly showed that MT3 and MT5 are the *cis–syn* cyclobutane dimer and the (6–4) photoproduct of TpT, respectively.

MT2 and MT4 exhibited ^{1}H NMR features similar to those of the (6–4) adduct (Figure 3) and the *trans–syn* cyclobutane dimer of TpT, respectively. However, their FAB-MS spectra in the negative mode exhibited a pseudo-molecular ion at m/z = 544 [M – H]. In addition, MT2 and MT4 were found to be eluted faster than the related TpT photoproducts on the ODS HPLC column. A similar behavior has been previously reported for the dCpT photoproducts with respect to their dUpT deamination products (Douki et al., 1991). This indicates that MT2 and MT4 are the (6–4) photoproduct and the *trans–syn* cyclobutane dimer of m^{5}dCpT, respectively. Similar ^{1}H NMR nuclear Overhauser effects were observed for the (6–4) adducts MT2 and TT2 (data not shown) upon selective irradiation of the methyl and the H6 protons. This indicates that the configuration of the 5′ base in MT2 is likely to be 5*R*,6*S* as in TT2 (Taylor et al., 1988).

Fraction MT1 was shown to contain two compounds (MT1a and MT1b) in a 1/3 ratio as inferred from the integration of the respective ^{1}H NMR signals. The ^{1}H NMR spectra of the mixture exhibited resonance signals similar to those found in

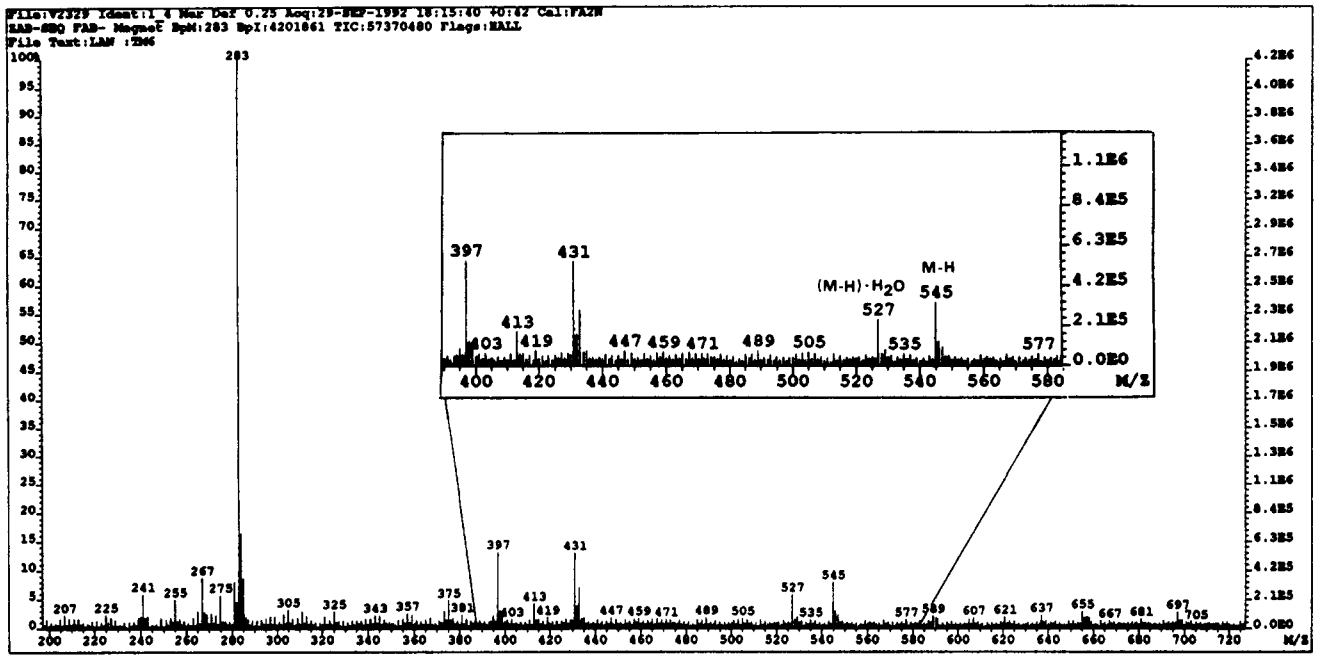


FIGURE 4: FAB mass spectrum in the negative mode of TM2, the TpT (6–4) photoproduct generated by far-UV photolysis of Tpm^{5}dC.

the spectra of the *cis–syn* cyclobutane dimer and the Dewar valence isomer of TpT. However, the chromatographic behavior of MT1a and MT1b was different from those of the two TpT photoproducts. Moreover, their mass spectra exhibited a pseudo-molecular ion at m/z = 544, indicative of 5-methylcytosine–thymine derivatives. Consequently, the two photoproducts coeluting in fraction MT1 were identified as the *cis–syn* cyclobutane dimer and the Dewar valence isomer of m^{5}dCpT. Further support for the presence of the m^{5}dCpT Dewar valence isomer was provided by the observation that UV-B (300 nm) irradiation of the (6–4) adduct of m^{5}dCpT (MT2) quantitatively generated a photolysis product coeluting with MT1 on the ODS column. Further evidence that the second compound in fraction MT1 was a cyclobutane dimer was provided by photosensitization of m^{5}dCpT with an aromatic ketone. This reaction is known to specifically generate cyclobutadipyrimidines (Lamola, 1970). UV-B irradiation of m^{5}dCpT in a 0.01 M acetophonone solution provided both the TpT *cis–syn* dimer and a faster eluting compound coeluting with MT1. The latter compound was gradually converted into the TpT *cis–syn* cyclobutane dimer when left in aqueous solution (*vide infra*). This observation, indicative of a deamination reaction, allowed the characterization of the cyclobutane dimer coeluting with MT1 as the *cis–syn* cyclobutane dimer of m^{5}dCpT. Additional support for this characterization was provided by the observation of the splitting of MT1 upon far-UV irradiation, due to the characteristic photosplitting of cyclobutane dimers. It should be added that compounds with retention times identical to those of the *trans–syn* cyclobutane dimers of m^{5}dCpT and TpT were observed in the HPLC profile of the photosensitized solution of m^{5}dCpT. However, the rate of the latter photoproducts was lower than those of the *cis–syn* diastereoisomers.

Deamination of m^{5}dCpT Photoproducts. The deamination, in a 0.01 M phosphate buffer (pH 7, 25 °C), of the *cis–syn* cyclobutane dimer of m^{5}dCpT (MT1) obtained by photosensitization was followed by reverse-phase HPLC analysis. The deamination rate constant was shown to be 10^{-5} min^{-1}. A similar study was carried out with the (6–4) photoproduct MT2 and the *trans–syn* cyclobutane dimer MT4. No deamination products (TT2 and TT3, respectively) were observed in the aqueous solution of MT2 and MT4, even after 4 weeks at room temperature.

Table 3: Photoproducts[a] Generated upon Far-UV Irradiation of TpT, dCpT, TpdC, dCpdC, m^{5}dCpT, m^{5}dCpdC, and Tpm^{5}dC in H_2O[b]

	TpT	TpdC	dCpT	dCpdC	m^{5}dCpT	m^{5}dCpdC	Tpm^{5}dC
				Undeaminated Photoproducts			
c,s	+	–	–	–	+	–	–
t,s	+	–	–	–	+	–	–
(6–4)	+	+	+	–	+	–	–
Dewar	–	+	+	–	+	–	–
				Deaminated Photoproducts			
c,s	/	–	+	–	+	–	+
t,s	/	–	–	–	–	–	–
(6–4)	/	–	–	–	+	+	+
Dewar	/	–	–	–	–	+	–

[a] *c,s* = *cis–syn* cyclobutane dimer; *t,s* = *trans–syn* cyclobutane dimer; (6–4) = pyrimidine (6–4) pyrimidone photoproduct; Dewar = Dewar valence isomer. [b] (+) isolated; (–) not detected.

Characterization of Photoproducts of m^{5}dCpdC. MC1 and MC2 (m/z = 531) were characterized as the (6–4) photoproduct and the Dewar valence isomer of TpdC since their chromatographic behavior and their FAB mass spectrum in the negative mode and ^{1}H NMR features were identical with those of the authentic samples.

DISCUSSION

Photochemistry of 5-Methylcytosine in Dinucleoside Monophosphates. Three 5-methylcytosine-containing bipyrimidine dinucleoside monophosphates were irradiated with 254-nm light. The main photoproducts were isolated and characterized (Table 3) as cyclobutane type dimers, (6–4) adducts, and related Dewar valence isomers. Four thymine- and cytosine-containing dinucleoside monophosphates were also exposed to far-UV light. The concentration of the irradiated aqueous solution and the overall UV dose were identical for all the samples, as shown by the equal 254-nm UV absorption of 1 OD. Therefore, the monitoring of HPLC analysis of the photolyzed solutions at 230 nm allowed a semiquantitative estimation of the relative yield of formation of the photoproducts, even though the molecular extinction coefficient of

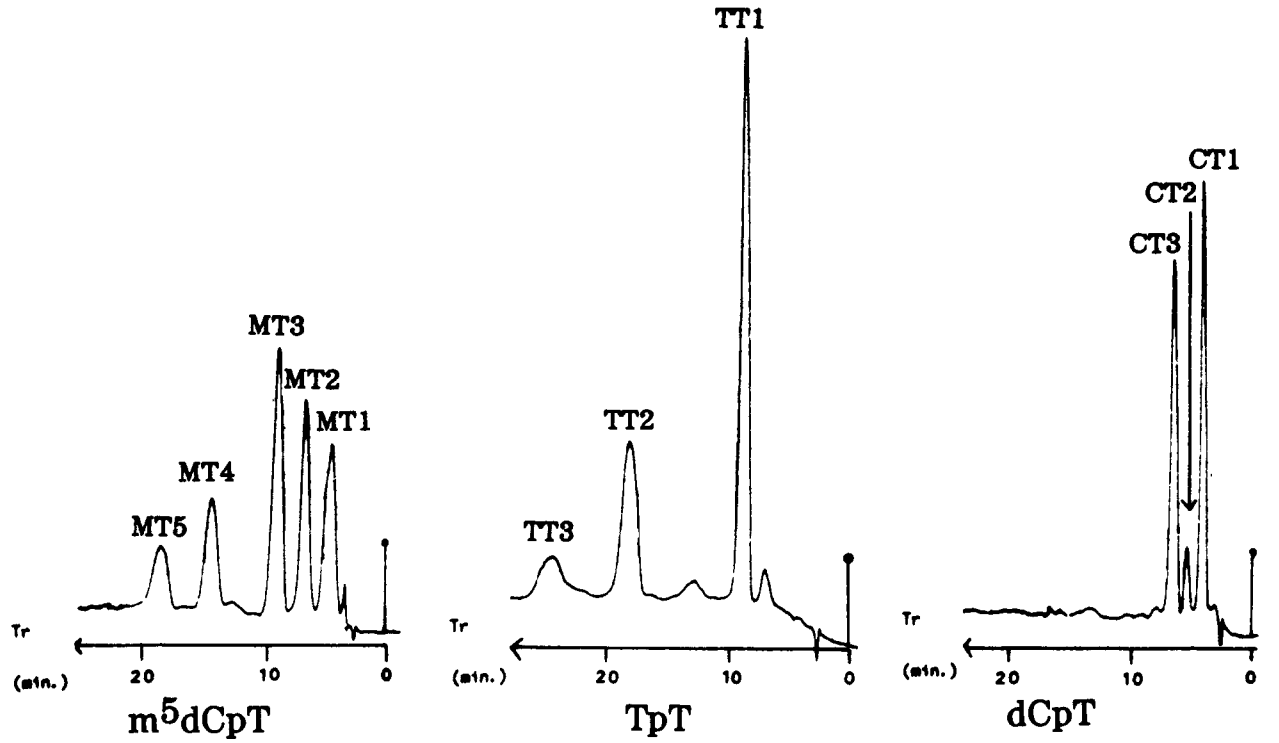


FIGURE 5: HPLC elution profile of the aqueous solutions of m^{5}dCpT, TpT, and dCpT exposed to 254-nm light in the same condition of concentration and UV dose. Injection volume for all samples, 200 μL; octadecylsilyl silica gel column (250 × 7 mm; particle size, 10 μm); flow rate, 3 mL min^{-1}; eluent, 25 mM ammonium formate. UV detection set at 230 nm. m5dCpT: MT1, Dewar valence isomer and *cis–syn* cyclobutane dimer; MT2, (6–4) adduct; MT3, deaminated *cis–syn* cyclobutane dimer; MT4, *trans–syn* cyclobutane dimer; MT5, deaminated (6–4) adduct, TpT: TT1, *cis–syn* cyclobutane dimer; TT2, (6–4) adduct; TT3, *trans–syn* cyclobutane dimer. dCpT: CT1, Dewar valence isomer; CT2, deaminated *cis–syn* cyclobutane dimer; CT3, (6–4) adduct.

the compounds was not determined. The comparison of the elution profile of the irradiated samples, as shown in Figure 5 for dCpT, m^{5}dCpT, and TpT, indicates that the degradation rate is in the same range for all the model compounds, with the exception of dCpdC, which was found to be far less photoreactive.

These results provided evidence that the presence of a 5-methylcytosine moiety does not prevent the formation of either cyclobutadipyrimidines or (6–4) photoadducts. In addition, no ring-opening photoproducts, reported to be the main products of the 254-nm photolysis of 5-methyl-2′-deoxycytidine (Shaw & Shetlar, 1989, 1990; Celewicz & Shetlar, 1992) were detected. These results confirm that intramolecular dimerization is favored in bipyrimidine dinucleoside monophosphates by comparison with monomeric compounds. However, this does not exclude the possibility for ring-opening products to be generated in DNA when 5-methylcytosine is located between two purine bases.

Comparison of the photolysis of dCpdC with that of one of its methylated analogs, m^{5}dCpdC, provides further evidence that 5-methylcytosine does not prevent but enhances the dimerization reaction with the adjacent 3′ end cytosine residue. In dCpdC, only a monomeric photoproduct was generated in a low yield upon exposure to UV light, whereas the photolysis of m^{5}dCpdC gave rise to the (6–4) photoproduct and its Dewar valence isomer. This result, which in some way is opposite to the conclusions raised by Glickman et al. (1986), also indicates that the formation of dimeric photoproducts involving 5-methylcytosine and cytosine is likely to occur via exciplex intermediates (Cadet & Vigny, 1990) with the 5-methylcytosine preferentially excited. However, it remains to be shown that 5-methylcytosine residues exhibit the same photochemical behavior within double-stranded DNA.

It is also worth mentioning that the methylation of cytosine did not change the relative amount of the different types of photoproducts (Table 3). The decreasing order in the yield of formation of the compounds generated by photolysis of m^{5}dCpT was found to be the following: (6–4) adduct MT2 > Dewar valence isomer MT1a > *cis–syn* cyclobutane dimer MT1b > *trans–syn* cyclobutane dimer MT4, as observed for dCpT: (6–4) photoproduct CT3 > Dewar valence isomer CT1 > *cis–syn* cyclobutane dimer CT2.

Deamination. Deamination is an hydrolytic process giving rise to uracil from cytosine (Shapiro & Klein, 1966). 5-Methylcytosine also deaminates, giving rise to thymine (Ehrlich et al., 1986b; Kusmierek et al., 1989). This reaction occurs with C5–C6 saturated derivatives of cytosine in a much higher rate. For instance, this was observed for 5,6-dihydrocytosine (Green & Cohen, 1957) and a series of far-UV photoproducts of dinucleoside monophosphates (Koning et al., 1991; Douki & Cadet, 1992; Lemaire & Ruzsicska, 1993b). Deamination of thymine–cytosine cyclobutane dimers was also shown to occur in cellular DNA (Ruiz-Rubio & Bockrath, 1989) and was recently proposed to be involved in delayed far-UV mutagenesis (Jiang & Taylor, 1993; Tessman et al., 1994).

The isolation of 5-methylcytosine containing m^{5}dCpT photoproducts (MT1, MT2, and MT4) allowed the study of the influence of the C5 methylation of the cytosine moieties on the deamination. The reaction rate constant for the *cis–syn* cyclobutane dimer (MT1) ($k' = 10^{-5}$ min^{-1}) was found to be 100 times lower than that of the corresponding dCpT dimer ($k' = 10^{-3}$ min^{-1}) (Douki & Cadet, 1992; Lemaire & Ruzsicska, 1993b). In addition, the deamination products were not observed even after several weeks when the (6–4) adduct (MT2) and the *trans–syn* cyclobutane dimer (MT4) of m^{5}dCpT were left in phosphate buffer pH 7 at 25 °C,

whereas the corresponding photoproducts of dCpT underwent deamination with a rate constant around 10^{-5} min^{-1} (Douki et al., 1991; Douki & Cadet, 1992; Lemaire & Ruzsicska, 1993b). This indicates that methylation of the C5 atom of a cytosine residue dramatically decreases the deamination rate of 5,6-saturated derivatives of m^{5}dCpT. This result can be extended to other 5-methylcytosine-containing bipyrimidine photoproducts since comparison of the deamination of the dCpT and TpdC cyclobutane dimers showed that the location of the cytosine residue at the 3′ or the 5′ end does not modify the reaction rate constant (Douki & Cadet, 1992; Lemaire & Ruzsicska, 1993b).

Photodeamination. Due to the high stability of the 5,6-saturated 5-methylcytosine-containing photoproducts towards deamination, the formation of thymine derivatives as major products of the photolysis of Tpm^{5}dC, m^{5}dCpT, and m^{5}dCpdC cannot be rationalized in terms of secondary deamination. In addition, the (6–4) adduct of TpT and its Dewar valence isomer were the only photoproducts detected after irradiation of Tpm^{5}dC. However, these compounds cannot be obtained by deamination of the corresponding Tpm^{5}dC derivatives since their 5-amino-5,6-dihydrothymine moiety is expected to be stable (as shown by the stability of the (6–4) photoproduct of TpdC) and would not be hydrolyzed to produce the 5-hydroxy-5,6-dihydrothymine residue.

Another mechanism is thus needed to explain these observations. Deamination of the 5-methylcytosine residues in the unmodified dinucleoside monophosphates is very unlikely to be involved. The deamination rate constant for cytosine and 5-methylcytosine is low, around 5×10^{-7} min^{-1} at pH 7.4 and 95 °C (Wang et al., 1982; Ehrlich et al., 1986b). In addition, the lack of any detectable amount of TpT in the solution of m^{5}dCpT after irradiation was established by reverse-phase HPLC analysis. Moreover, no detectable deamination of the (6–4) adduct and the *cis–syn* cyclobutane dimer of m^{5}dCpT was observed upon exposure to far-UV light, as inferred from HPLC analysis. The peaks corresponding to the related TpT photoproducts were not observed in any of the two samples, over a wide range of total UV dose (0–1.5 kJ). Further support for the lack of photoinduced deamination of the *cis–syn* cyclobutane dimer of m^{5}dCpT is provided by the observation of its deamination product upon UV-B photosensitization since it does not absorb at this wavelength.

Since deamination does not occur, at least significantly, neither in the starting dinucleoside monophosphate nor in the photoproducts, one possible mechanism for this reaction would involve the excited state of the starting molecule. This hypothesis received further support from the observation that in all cases the relative amount of deaminated photoproducts was in the same range as those obtained by direct photolysis of the deaminated dinucleoside monophosphate. For example, the *cis–syn* cyclobutane dimer and the (6–4) photoproduct of TpT were obtained by photolysis of m^{5}dCpT in the same relative yield as that observed after far-UV photolysis of TpT. A similar result was obtained for the (6–4) adduct of TpdC and its Dewar valence isomer, which were the only photoproducts isolated after irradiation of m^{5}dCpdC. It should also be added that both singlet and triplet excited states are involved in this reaction. This was inferred from the observation of deaminated derivatives of the (6–4) adducts of m^{5}dCpT, m^{5}dCpdC, and Tpm^{5}dC that were generated by 254 nm irradiation *via* a likely singlet state. On the other hand, the formation of the deaminated *cis–syn* cyclobutane dimer of m^{5}dCpT by ketone photosensitization specifically involves a triplet state.

The comparison between the products generated by photolysis of m^{5}dCpT and Tpm^{5}dC provides evidence for the influence of the sequence on the "photodeamination" process. The UV-C photolysis of m^{5}dCpT gives rise to both the 5-methylcytosine–thymine and the thymine–thymine photoproducts in similar yields, as inferred from the area of the peaks in the HPLC elution profile. On the other hand, photolysis of Tpm^{5}dC generated only the deaminated adducts, without a detectable amount of 5-methylcytosine derivatives.

CONCLUSION

The work provides evidence that 5-methylcytosine does undergo dimerization upon exposure to far-UV light, as inferred from the isolation and the characterization of the resulting photoproducts. These results, which provide new insights in the photochemistry of 5-methylcytosine, should be taken into account for the study of far-UV-induced DNA lesions. An extension of this work is aimed at studying the photolysis of 5-methylcytosine after incorporation in longer oligonucleotides to better mimic the sequence and structure effects of DNA. In addition, a new photochemical process was observed, suggesting that deamination can take place in the excited state of 5-methylcytosine, giving rise to the related thymine photoproducts. However, further work is required to establish the mechanism of this fast reaction, which is likely to involve short-lived excited species.

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