Measurement of Pyrimidine (6-4) Photoproducts in DNA by a Mild Acidic Hydrolysis-HPLC Fluorescence Detection Assay

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Received September 9, 1994[⊗]

Pyrimidine (6-4) pyrimidone photoproducts constitute one of the major classes of DNA lesions induced by far-UV irradiation. However, their biological role remains difficult to assess partly because of the lack of a specific and sensitive assay for monitoring their formation in DNA. Here is presented a measurement method based on the release of the (6-4) base adducts from DNA followed by an HPLC separation associated with a sensitive and specific fluorescence detection. The quantitative and mechanistic aspects of the chemical hydrolysis, based on the use of hydrogen fluoride stabilized in pyridine, were investigated, using dinucleoside monophosphate (6-4) photoproducts as model compounds. The final hydrolysis products were isolated and characterized by UV, fluorescence, mass, and ^{1}H NMR spectroscopies. Application of the assay to far-UV irradiated calf thymus DNA provided information on the sequence effect on the rate of formation of three of the four possible bipyrimidine (6-4) photoproducts.

Introduction

Pyrimidine (6-4) pyrimidone photoproducts, a major class of DNA photolesions (*1*), are involved in the mutagenic and lethal effects of far-UV[1] light (*2*), although their exact biological significance still remains open to debate (*3, 4*). Characterization of several (6-4) photoproducts has been achieved by using model compounds including bases and nucleosides (*5-7*) and, more recently, dinucleoside monophosphates (*8-10*). Their formation involves an unstable intermediate, generated *via* a singlet excited state. The transient cycloadduct has either an oxetane (Figure 1) or an azetidine structure depending on whether the base located on the 3′ end is a thymine or a cytosine, respectively. The presence of a pyrimidone moiety in (6-4) photoadducts induces a red shift in the UV absorption spectrum maximum centred around 320 nm. This is associated with a high fluorescence emission (*11, 12*). Another peculiar behavior of the (6-4) photoproducts is their efficient conversion into the corresponding Dewar valence isomer upon exposure to UVB radiation (*13*).

Measurement of (6-4) photoproducts in DNA has been achieved by using various methods. Emphasis has been placed on the development of immunological techniques, including radioimmunoassays (RIA) (*14*), enzyme linked immunosorbent assays (ELISA) (*15*), immunofluorescence laser cytometry (*16*), and immunoprecipitation measurements (*17*). However, the sequence specificity of the available immonulogical assays is too low to permit the individual measurement of the different (6-4) adducts (*18, 19*). In addition, immunological techniques for the detection of (6-4) photoproducts lack calibration and provide only relative information on the amount of lesion. Other indirect assays for (6-4) photoproducts have been developed, based on either a combination of repair enzymes (photolyase and UvrABC) allowing specific cleavage of far-UV irradiated DNA (*20*) or a ligation mediated polymerase chain reaction for the measurement at the gene level (*21*). Another approach involving gel sequencing analysis subsequent to alkaline treatment of DNA has been proposed to provide individual information on the different (6-4) photoproducts (*22, 23*). However, it has been recently shown that the Dewar valence isomers, rather than their (6-4) adduct precursors, are alkali labile (*24-26*). Therefore, the previous results using this technique were mostly related to the formation of Dewar valence isomers which can be produced not only by near-UV conversion of the (6-4) photoproducts but also by 254 nm photolysis of pyrimidine bases, as observed with dCpdT and dTpdT (*10,27*). Further support for this result was provided by the observation that exposure of far-UV irradiated DNA to UVB light dramatically increased the number of alkali labile sites (*28*). This is likely to explain why the rate of (6-4) photoproducts has been underestimated in the early applications of the gel sequencing assay.

Most of these assays consisting in indirect measurements do not provide information on individual photoadducts. Acidic hydrolysis, followed by chromatography, was proposed as a method for monitoring the formation of (6-4) photoproducts in far-UV irradiated DNA. This led to unsuccessful attempts when formic acid was used. However, isolation of the thymine-cytosine (6-4) adduct, in its dehydrated form, was achieved by employing trifluoroacetic acid under milder hydrolytic conditions (*29, 30*). It should be noted that the quantitative aspect of the method has never been investigated.

We wish to report a mild acidic hydrolysis assay which allows a quantitative release of three of the four possible

[⊗] Abstract published in *Advance ACS Abstracts,* January 15, 1995.

[1] Abbreviations: 1,3-DMU, 1,3-dimethyluracil; CpC, cytidylyl-(3′-5′)-cytidine; dCpdC, 2′-deoxycytidylyl-(3′-5′)-2′-deoxycytidine; dCpdT, 2′-deoxycytidylyl-(3′-5′)-thymidine; dUpdC, 2′-deoxyuridylyl-(3′-5′)-2′-deoxycytidine; dUpdT, 2′-deoxyuridylyl-(3′-5′)-thymidine; ELISA, enzyme linked immunosorbent assay; HF/pyridine, 70% (w/w) solution of hydrogen fluoride in pyridine; LC-thermospray MS, liquid chromatography-thermospray mass spectrometry; ODS, octadecylsilyl silica gel; RP-HPLC, reverse phase high performance liquid chromatography; RIA, radioimmuno assay; dTpdC, thymidylyl-(3′-5′)-2′-deoxycytidine; TSP, [2,2,3,3-^{2}H]-3-(trimethylsilyl)propionate; dTpdT, thymidylyl-(3′-5′)-thymidine; UV, ultraviolet.

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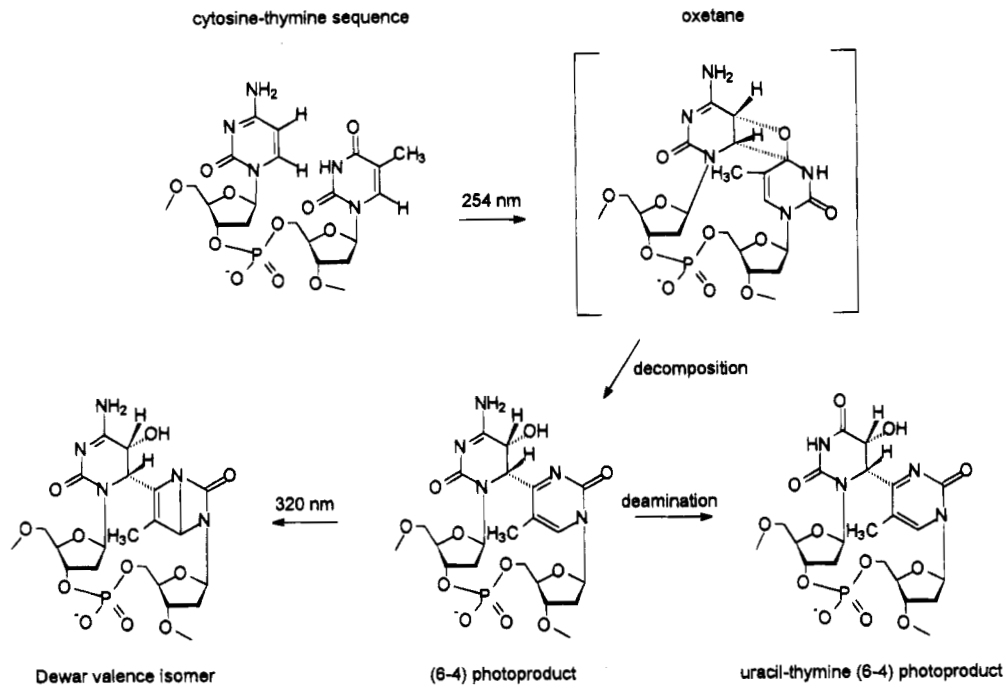


Figure 1. Formation, isomerization, and deamination of the (6–4) photoproduct of dCpdT.

(6–4) photoproducts. This is based on the use of hydrogen fluoride stabilized in pyridine which was recently applied to the acidic hydrolysis of various oxidized nucleosides (*31*). This mild acidic treatment does not induce any detectable dehydration of the (6–4) adducts, allowing the development of a sensitive method taking advantage of their fluorescence properties for their detection (*11, 12*). The released base photoproducts were unambiguously characterized, and the quantitative aspect of the hydrolysis was investigated by using dinucleoside monophosphate (6–4) adducts as model compounds. The mechanism of HF/pyridine hydrolysis is also discussed, on the basis of kinetic studies and the identification of some intermediate compounds of the reaction. Then, the rate of formation of the (6–4) adducts in far-UV irradiated DNA was determined.

Materials and Methods

Chemicals. ***Caution:*** *HF/Pyridine is a very corrosive chemical and must be used with care. It must be handled in polypropylene vials and not in glass since hydrogen fluoride can dissolve it.* Thymidine and 2′-deoxycytidine were obtained from Pharma-Waldhof (Geneva, Switzerland). 2′-Deoxyuridine was purchased from Sigma (St Louis, MO). [*methyl*-^{3}H]Thymidine (70–85 Ci/mmol) and [5′-^{3}H]Thymidine (5–20 Ci/mmol) were provided by Amersham (Bucks, U.K.). [*methyl*-^{14}C]Thymine was obtained from Commissariat à l'Energie Atomique (Saclay, France). Benzoyl chloride, triazole, 4-chlorophenyl dichlorophosphate, 2,4,6-triisopropylbenzenesulfonyl chloride, *N*-methylimidazole, and 70% hydrogen fluoride in pyridine were purchased from Aldrich-Chemie (Steinheim, Germany). Acetic acid, methanol, and concentrated ammonia were provided by Prolabo (Paris, France). Deuterium oxide (99.96%) used for ^{1}H NMR spectroscopy was purchased from Merck (Darmstadt, Germany).

High Performance Liquid Chromatography. The chromatographic system consisted of a L 6200 Hitachi (Tokyo, Japan) intelligent pump equipped with either a semipreparative 250 × 7.4 mm i.d. (particle size 10 μm) ODS silica gel column (Nucleosil 100-10 C_{18}, Macherey Nagel, Düren, Germany) or a 250 × 4.6 mm i.d. (particle size 5 μm) LiChrospher 100 NH_2 (Merck, Darmstadt, Germany) amino silica gel column. The detection was provided by either a LKB-Bromma 2151 UV spectrometer (Pharmacia-LKB, Uppsala, Sweden), a R 401 Waters differential refractometer, or a F 1050 Hitachi (Tokyo, Japan) fluorimeter. Quantitative measurements were carried out by using either a LKB 2220 recording integrator (Pharmacia-LKB, Uppsala, Sweden) or a Hitachi (Tokyo, Japan) D 2500 integrator after appropriate calibration. The on-line radioactivity detection was provided by a Flo One/β radioactivity flow detector (Radiomatic Instruments, Tampa, FL) used with Luna Flo II (Lumac LSC, Olen, Belgium) liquid scintillator.

Spectrometric Measurements. ^{1}H NMR analyses were carried out in D_2O solutions either on a AC 200 Bruker apparatus or on a AM 400 Bruker spectrometer (Wissenbourg, France) operating at 200.13 and 400.13 MHz, respectively. Chemical shifts were inferred from first order spectrum analysis and expressed with respect to [2,2,3,3-^{2}H]potassium 3-(trimethylsilyl)propionate. Circular dichroism spectra were obtained in water by using a Dichrograph III apparatus (Jobin-Yvon, Longjumeau, France). UV measurements were carried out in water with a DU-88 Beckmann (Irvine, CA) spectrophotometer. Mass spectra were obtained by using a Trio II (Fisons Instrument, Manchester, U.K.) mass spectrometer coupled with an HPLC system through a thermospray source. The chromatographic system consisted of an ODS analytical (25 × 4.6 mm i.d., particle size 5 μm) Hypersyl column (Interchrom, Montluçon, France) used with a mixture of 50 mM ammonium acetate solution and methanol (98:2) as the isocratic eluent.

Synthesis of the Dinucleoside Monophosphates. The synthesis of dCpdT, dTpdT, dTpdC, dUpC, and dUpdT was carried out by using a triester method (*32*). The phosphorylation of the protected nucleoside was achieved by using bis(triazole)–4-chlorophenyl phosphate according to Chattopadahyaya and Reese (*33*). The condensation of the second nucleoside was made by using triisopropylbenzenesulfonyl chloride as the coupling agent and *N*-methylimidazole as the activating reagent, following the procedure reported by Miyoshi and Itakura (*34*). The purification of the deprotected product was carried out by reverse phase high performance liquid chromatography (RP-HPLC) in the ion suppression mode with a mixture of 0.025 M ammonium formate and acetonitrile (95:5) as the isocratic eluent. The detection of the compound was made by differential refractometry. The overall yield of isolated and purified dinucleoside monophosphate was about 40%.

Actinometry. Actinometry measurement was adapted from the method reported by Shaw and Shetlar (*35*). This was based on the formation of 6-hydroxy-5,6-dihydro-1,3-dimethyluracil by UVC photolysis of 1,3-dimethyluracil (1,3-DMU). Typically, 4 mL of a 3.8 mM aqueous solution of 1,3-DMU was exposed to

the UV light emitted by a 15 W VL-15 G (Bioblock, Illkirch, France) germicidal lamp. Aliquots of 100 μL were analyzed by RP-HPLC (C_{18}, 250 × 7 mm i.d., particle size 10 μm) after increasing periods of exposure. The isocratic eluent consisted of a 85:15 water/methanol mixture, and the UV detector was set at 220 nm. The capacity factors (k') were 3.46 and 1.42 for 1,3-DMU and its photohydrate, respectively. The area of the peaks was corrected by taking into account the molecular absorption coefficient at 220 nm of 1,3-DMU and its hydrate (5.4×10^3 and 6.2×10^3 L·mol^{-1}·cm^{-1}, respectively). The rate constant for photohydration was found to be 3.9×10^{-3} min^{-1}. Since the quantum yield is 0.0106 and the molecular absorption coefficient of 1,3-DMU is 6.14×10^3 L·mol^{-1}·cm^{-1} at 254 nm, it was inferred that the irradiation intensity was 0.13 J·s^{-1}·m^{-2}. A similar experiment was carried out for a 30 W VL-215 G germicidal lamp (Bioblock, Illkirch, France) that was found to provide a fluence of 0.43 J·s^{-1}·m^{-2}.

Preparation of the Dinucleoside Monophosphate (6–4) Photoproducts. A millimolar solution (50 mL) of each of the dinucleoside monophosphates was irradiated in a Petri dish ($\phi = 16$ cm) with the 30 W germicidal lamp for 4 h. The overall UV dose was 6 kJ·m^{-2}. Then, the irradiated solution was concentrated to 2 mL, and the (6–4) photoproducts were isolated by RP-HPLC in the ion suppression mode. The isocratic eluent consisted of a 25 mM ammonium formate solution. The increasing order for the value of the capacity factor (k') of the dinucleoside monophosphate (6–4) photoproducts was the following: 5.8 for dUpC (**5**); 10.0 for dCpdT (**3**); 11.7 for dUpdT (**4**); 22.5 for ddTpdC (**2**); and 23.3 for dTpdT (**1**). The photoproducts were characterized by comparison of their UV absorption, ^{1}H NMR, and FAB$^-$ mass spectroscopy features with those of authentic samples.

Hydrolysis of the Dinucleoside Monophosphate (6–4) Photoproducts. The hydrolysis solutions were neutralized to prevent the formation of gaseous HF during the freeze-drying of the samples and facilitate the removal of pyridine. Calcium carbonate was chosen as a base because it is poorly soluble in water as the resulting calcium fluoride. This prevents a too high concentration of salts in the final hydrolysis solutions which would interfere with the HPLC separations.

(5-Hydroxy-5,6-dihydrothyminyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (1a). A solution of **1** (2 mg) was freeze-dried and hydrolyzed in 1 mL of HF/pyridine for 3 h at 37 °C in a water bath. Then, the solution was diluted to 5 mL with water and neutralized by addition of 3 g of calcium carbonate under stirring. The resulting suspension was filtered and the liquid phase freeze-dried. The residue obtained was dissolved in 500 μL of water prior to reverse phase HPLC analysis. This was achieved by using a 0.025 M ammonium formate solution as the isocratic eluent. The fraction corresponding to the major peak absorbing at 310 nm ($k' = 2.2$) was collected and freeze-dried, providing a homogeneous compound in a 90% yield. The UV, ^{1}H NMR, and chromatographic features were identical to those of (5-hydroxy-5,6-dihydrothyminyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (Figure 3) obtained by irradiation of thymine in aqueous solution (*5*). UV (H_2O, λ_{max}): 314 nm; ϵ_{314}: 8300 L·mol^{-1}·cm^{-1}; LC-thermospray MS, m/z (rel intensity) 253 ([M + H]$^+$, 90), 235 ([M + H − H_2O]$^+$, 100), 270 ([M + NH_4]$^+$, 85); ^{1}H NMR (D_2O, TSP, 400.13 MHz) δ (ppm) 7.92 (1 H, s, Pyo H-6), 4.93 (1 H, s Pyr H-6), 2.27 (3 H, s, Pyo CH_3), 1.84 (3 H, s, Pyr CH_3).

5-Amino-5,6-dihydrothyminyl)-(6–4′)-(1′,2′-dihydropyrimidin-2′-one) (2a). One milligram of **2** was freeze-dried, and the resulting sample was hydrolyzed in 1 mL of HF/pyridine for 4 h at 37 °C in a water bath. Then, the solution was diluted to 5 mL with water, neutralized by adding 3 g of calcium carbonate, and filtered. The solution was freeze-dried, and the resulting dry sample was dissolved in 500 μL of water. The hydrolysis products were separated by RP-HPLC by using a 25 mM ammonium formate solution as the isocratic eluent. The main 310 nm absorbing HPLC fraction ($k' = 1.53$) was collected and freeze-dried. The resulting homogeneous compound was obtained in a 85% yield. UV (H_2O, λ_{max}): 307 nm; ϵ_{307}: 3800 L·mol^{-1}·cm^{-1}; ^{1}H NMR (D_2O, TSP, 200.13 MHz) δ (ppm) 8.16 (1 H, d, Pyo H-6), 6.78 (1 H, d, Pyo H-5), 1.66 (3 H, s, Pyr CH_3).

(5-Hydroxy-5,6-dihydrocytosinyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (3a). A freeze-dried sample of **3** (1.5 mg) was hydrolyzed for 4 h in 500 μL of HF/pyridine, and the solution was neutralized by adding 1 g of calcium carbonate and 2 mL of water. The resulting suspension was filtered and freeze-dried. Then, the residue was dissolved in water prior to injection on the ODS silica gel column. The separation was achieved with a 0.025 M ammonium formate solution at a flow rate of 1.5 mL·min^{-1}. The fastest eluting peak ($k' = 1.25$) was collected (yield inferred form HPLC peak area: 85%), and the corresponding adduct was obtained by freeze-drying. The sample was analyzed by LC-thermospray mass spectrometry, and the ion at m/z 220 ([M + H − H_2O]$^+$) was observed. UV (H_2O, λ_{max}): 315 nm; ϵ_{315}: 6700 mol·l^{-1}·cm^{-1}; ^{1}H NMR (D_2O, TSP, 400.13 MHz) δ (ppm) 7.94 (1 H, s, Pyo H-6), 5.25 (1 H, d, Pyr H-6), 5.09 (1 H, d, Pyr H-5), 2.27 (3 H, s, Pyo CH_3).

Stability of the Radiolabeled 5-Hydroxy-5,6-dihydrothyminyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) under Various Acidic Conditions. [*Me*-^{14}C]-5-Hydroxy-5,6-dihydrothyminyl)-(6–4′)-5′-methyl-1′,2′-dihydroprimidin-2′-one was prepared by far-UV irradiation in aqueous solution of [*Me*-^{14}C]thymine (*5*). The (6–4) photoproduct was purified by RP-HPLC separation ($k' = 2.2$), using a 25 mM ammonium formate solution as the isocratic eluent. The sample was split into four aliquot fractions which were submitted to four different acidic treatments, including pure formic acid (170 °C, sealed tube, 60 min), trifluoroacetic acid (170 °C, sealed tube, 60 min), 0.5 N hydrochloric acid (90 °C, sealed tube, 60 min), and HF/pyridine (37 °C, 60 min). The samples were dried by evaporation *in vacuo* and dissolved in 100 μL of water. The solutions were analyzed by two-dimensional TLC on 10 × 10 cm F_{254} silica gel plates (Merck, Darmstadt). The first development was achieved with a mixture of ethyl acetate, 2-propanol, and water (75/16/9 v/v). The lower layer of a mixture of chloroform, methanol, and water (4/2/1 v/v) was used as eluent for the second direction. The location of the spots corresponding to the products generated by the various treatments was determined by autoradiography.

Partial Hydrolysis of the dTpdT (6–4) Photoproduct. A sample of the (6–4) photoproduct of dTpdT **1** (2 mg) was freeze-dried in a 1.5 mL polypropylene vial and subsequently hydrolyzed in 100 μL of HF/pyridine for 30 min at 0 °C. Then, the solution was neutralized under stirring in a suspension of 150 mg of calcium carbonate in 2 mL of water (final pH: 7). The resulting suspension was centrifuged, and the supernatant was freeze-dried. The hydrolysis products were isolated by RP-HPLC on the ODS silica gel column. The isocratic eluent was a 25 mM ammonium formate solution with a flow rate of 2 mL·min^{-1}. The UV detector was set at 310 nm. The fastest eluting compound was identified as (5-hydroxy-5,6-dihydrothyminyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one (**1a**) by comparison of its chromatographic and ^{1}H NMR and UV features with those of the authentic sample.

[1-(2-Deoxy-α-D-*erythro*-pentopyranosyl)-5-hydroxy-5,6-dihydrothyminyl]-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (1b). The second fastest eluting compound ($k' = 10.7$) was collected and freeze-dried. It represented 47% of the overall amount of 310 nm absorbing products. UV (H_2O, λ_{max}): 315 nm; LC-thermospray MS, m/z (rel intensity) 369 ([M + H]$^+$, 100), 351 ([M + H − H_2O]$^+$, 5), 386 ([M + NH_4]$^+$, 55), 256 ([M − deoxyribose + 2H]$^+$, 15), 235 ([M − deoxyribose + 2H −H_2O]$^+$, 50); ^{1}H NMR (D_2O, TSP, 400.13 MHz) δ (ppm) 7.88 (1 H, s, Pyo H-6), 5.64 (1 H, m, H-1′), 5.07 (1 H, s, Pyr H-6), 4.11 (1 H, m, H-3′), 3.92 (1 H, s, H-4′), 3.85 (1 H, d, H-5′), 3.65 (1 H, d, H-5″), 2.37 (3 H, s, Pyo CH_3), 2.32 (1 H, m, H-2′), 2.01 (1 H, m, H-2″), 1.84 (3 H, s, Pyr CH_3).

[1-(2-Deoxy-α-D-*erythro*-pentofuranosyl)-5-hydroxy-5,6-dihydrothyminyl]-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (1c). The slowest eluting HPLC peak ($k' = 14.3$), which contained 24% of the 310 nm absorbing compounds, was collected and freeze-dried. The resulting homogeneous compound was characterized as 5-hydroxy-6–4′-[1-(2-deoxy-α-D-*erythro*-pentofuranosyl)-5-methylpyrimidine-2′-one] 5,6-dihydrothymine. UV (H_2O, λ_{max}): 315 nm; LC-thermospray MS, m/z (rel intensity) 369 ([M + H]$^+$, 25), 386 ([M + NH_4]$^+$, 10), 256 [M

– deoxyribose + 2H]$^+$, 7), 235 ([M – deoxyribose + 2H – H_2O]$^+$, 13); ^{1}H NMR (D_2O, TSP, 400.13 MHz) δ (ppm) 7.88 (1 H, s, Pyo H-6), 6.19 (1 H, m, H-1′), 5.20 (1 H, s, Pyr H-6), 4.51 (1 H, dxd, H-3′), 4.26 (1 H, d, H-4′), 3.60 (2 H, m, H-5′,5″), 2.33 (3 H, s, Pyo CH_3), 2.34 (1 H, d, H-2′), 2.22 (1 H, m, H-2″), 1.83 (3 H, s, Pyr CH_3).

Partial Hydrolysis of the dTpdC (6–4) Photoproduct. A solution of the (6–4) photoproduct of dTpdC **2** (2 mg) was hydrolyzed in 100 μL of HF/pyridine for 30 min at 0 °C. Then, the solution was neutralized by addition of 150 mg of calcium carbonate in 2 mL of water. After freeze-drying and filtration of the sample, three main products were isolated by RP-HPLC using a 0.025 M ammonium formate isocratic eluent with a elution flow rate of 2 mL·min^{-1}. Evaporation of the fraction containing the fastest eluting compound (k′ = 1.53) provided (5-amino-5,6-dihydrothyminyl)-(6–4′)-(1′,2′-dihydropyrimidin-2′-one) (**2a**) (yield 20%), identified on the basis of its HPLC behavior and its spectroscopic features (*vide supra*).

[5-Amino-1-(2-deoxy-α-D-*erythro*-pentopyranosyl)-5,6-dihydrothyminyl]-(6–4′)-(1′,2′-dihydropyrimidin-2′-one) (2b). The HPLC fraction containing the second faster eluting product (*k*′ = 8.2) was collected and freeze-dried. This provided a homogeneous compound in a 38% yield. UV (H_2O, λ_{max}): 310 nm; LC-thermospray MS, *m*/*z* (rel intensity) 354 ([M + H]$^+$, 20), 371 ([M + NH_4]$^+$, 8), 238 ([M – deoxyribose + 2H]$^+$, 22), 221 ([M – deoxyribose + 2H – NH_3]$^+$, 35); ^{1}H NMR (D_2O, TSP, 200.13 MHz) δ (ppm) 8.09 (1 H, d, Pyo H-6), 6.86 (1 H, d, Pyo H-5), 5.65 (1 H, dxd, H-1′), 4.12 (1 H, dxd, H-3′), 3.91 (1 H, s, H-4′), 3.88 (1 H, d, H-5′), 3.68 (1 H, d, H-5″), 2.31 (1 H, dxd, H-2′), 2.01 (1 H, m, H-2″), 1.67 (3 H, s, Pyr CH_3).

[5-Amino-1-(2-deoxy-α-D-*erythro*-pentopyranosyl)-5,6-dihydrothyminyl]-(6–4′)-(1′,2′-dihydropyrimidin-2′-one) (2c). The fraction containing the slowest eluting compound (*k*′ = 13.8) absorbing at 310 nm was collected and freeze-dried. The yield of the resulting product was 36%. UV (H_2O, λ_{max}): 309 nm; ^{1}H NMR (D_2O, TSP, 400.13 MHz) δ (ppm) 8.17 (1 H, d, Pyo H-6), 6.68 (1 H, d, Pyo H-5), 6.30 (1 H, m, H-1′), 4.47 (1 H, m, H-3′), 4.12 (1 H, d, H-4′), 3.58 (2 H, m, H-5′,5″), 2.77 (1 H, m, H-2′), 2.18 (1 H, m, H-2″), 1.64 (3 H, s, Pyr CH_3).

Sodium Periodate Oxidation of 1b and 1c. Samples of **1b** or **1c** (50 μg) were left for 18 h in 100 μL of a 0.3 M aqueous solution of $NaIO_4$. After completion of the reaction, the solution was diluted by addition of 1 mL of acetronitrile, prior to injection on the amino silica gel column. The HPLC separation was achieved by using a mixture of acetonitrile and 0.025 M ammonium formate aqueous solution (80:20 v/v) as the isocratic eluent. The detection was provided by a spectrofluorimeter with excitation and emission wavelengths set up at 310 and 380 nm, respectively.

Thyminyl-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one). (5-Hydroxy-5,6-dihydrothyminyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (**1a**) (1 mg) was dissolved in 5 mL of 4 N HCl and treated for 90 min at 80 °C. The solution was neutralized by addition of 4 mL of 5 N sodium hydroxide and concentrated to 500 μL by evaporation under vacuum. In a subsequent step, insoluble sodium chloride was removed by centrifugation. Thyminyl-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (*k*′ = 2.4) was isolated by RP-HPLC by using a mixture of 0.025 M ammonium formate solution and methanol (9:1) as the eluent with a flow rate of 3 mL·min^{-1}. It should be noted that when the eluent is a 0.025 M ammonium formate aqueous solution, as used for the elution of the base (6–4) adducts, the value of the capacity factor was 15.6. UV (H_2O, λ_{max}): 320 nm; ^{1}H NMR (D_2O, TSP, 400.13 MHz) δ (ppm) 8.50 (1 H, s, Pyo H-6), 2.16 (3 H, s, Pyo CH_3), 1.83 (3 H, s, Pyr CH_3).

Determination of the Molecular Absorption Coefficients. The radiolabeled (6–4) photoproducts of dTpdT*, T*pdC, and dCpdT* (where T* is [*Me*-^{3}H]thymidine), were prepared following the procedure described for the unlabeled dinucleoside monophosphates (*vide supra*). The specific activity was 893, 873, and 2460 Bq/mg, respectively. Aliquot fractions of each of the dinucleoside monophosphate (6–4) adducts were hydrolyzed in 500 μL of HF/pyridine. In a subsequent step, the solution was diluted by addition of water and neutralized by addition of 1.5 g of calcium carbonate. After filtration, the samples were purified by reverse phase HPLC. Under these conditions, only the (6–4) photoproducts **1a**, **2a**, and **3a** were found to be produced, as inferred from the comparison of their retention times with those of authentic samples. The amount of (6–4) adduct was determined by measuring the activity of the samples. The molecular absorption coefficients were determined at 320 nm for the dinucleoside monophosphate (6–4) photoproducts and at 310 nm for the corresponding base adducts.

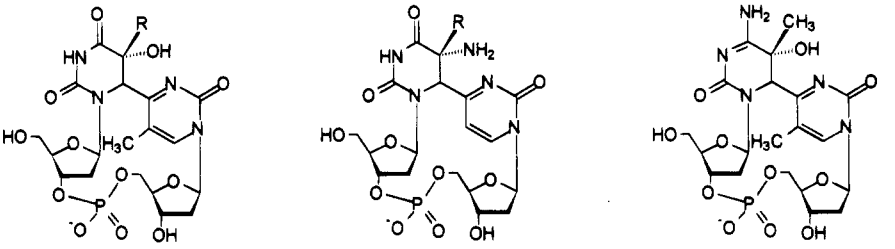
Isolation of 1a and 1b from Irradiated DNA. DNA (1 g) was dissolved in 10 mL of water and split into 6 equal fractions. Each of these solutions were placed in a Petri dish (ϕ = 140 mm) and irradiated for 5 h with the 15 W germicidal lamp. The fractions were combined, and the resulting solution was concentrated by evaporation and split into 50 aliquot fractions which were transferred into 1.5 mL polypropylene vials. Then, the aliquot samples were freeze-dried and hydrolyzed by 200 μL of HF/pyridine (*vide supra*). The hydrolysis products were eluted on the ODS column, and two major fluorescent fractions were collected. The corresponding products were identified as **1a** and **1b** on the basis of their chromatographic and ^{1}H NMR features.

Analysis of 254 nm Irradiated DNA. Calf thymus DNA (Sigma, St. Louis, MO) samples (150 μg) were irradiated in 3 mL of water with increasing UV doses by using the 15 W germicidal lamp. The solutions were split into three equal fractions which were subsequently freeze-dried in polypropylene vials. Each of the resulting residues were hydrolyzed in 50 μL of HF/pyridine for 2 h at 37 °C. The acidic solution was diluted with 2 mL of water and neutralized by addition of 150 mg of calcium carbonate. The suspension was centrifuged and the supernatant freeze-dried. The resulting dry residue was solubilized in 500 μL of a 90:10 acetonitrile/water mixture and analyzed by HPLC on an amino silica gel column after appropriate calibration. A mixture of acetonitrile and 25 mM ammonium formate solution (9:1) was used as the isocratic eluent, and the flow rate was 1 mL·min^{-1}. The excitation and emission wavelengths of the fluorescence detector were set at 310 and 380 nm, respectively.

Results and Discussion

Formation of the (6–4) Photoproducts in Dinucleoside Monophosphates. As previously reported, the (6–4) photoproducts of dTpdT (*9*), dTpdC (*8*), and dCpdT (*10*) (Figure 2) were generated upon exposure of the corresponding dinucleoside monophosphates to 254 nm radiation in aqueous solution. Attempts to generate the (6–4) adduct of dCpdC failed, not only upon irradiation of the dinucleoside monophosphate in aqueous solution but also as either a thin ice layer or frozen droplets. This was further confirmed by using a sensitive fluorescence HPLC assay that showed the lack of the formation of any fluorescent compound over a wide range of far-UV doses. These results are in agreement with the identification of cyclobutane dimers and photohydrates as the main far-UV photoproducts of CpC (*36*). It should be noted that the deaminated (6–4) photoproduct **5**, which was produced by photolysis of dUpdC, was also not observed upon far-UV irradiation of dCpdC.

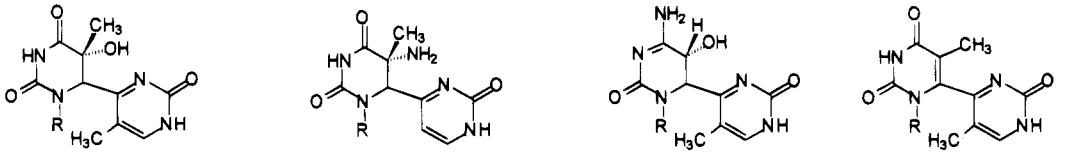
Characterization of the Hydrolyzed Photoproducts. Base (6–4) adducts **1a**, **2a**, and **3a** (Figure 3) were isolated as the hydrolysis products of the corresponding dinucleoside monophosphate (6–4) photoproducts. Their ^{1}H NMR spectra (Figure 4) did not exhibit any signal corresponding to osidic protons, indicating that the hydrolysis has been completed. Moreover, the chemical shifts of the H-6, H-5, and/or methyl protons are consistent with a (6–4) adduct structure. A downfield shift is observed for the H-6 proton of the 3′ end moiety, in agreement with the presence of a vinylic proton in the pyrimidone ring. This also applied to the H-5 proton of compound **2a**. The upfield shift of the ^{1}H NMR signal of



1: R = CH_3 2: R = CH_3 3

4: R = H 5: R = H

Figure 2. Structure of dinucleoside monophosphate (6–4) photoproducts.



1 a,b,c 2 a,b,c 3a 6

a: R = H

b: R = 2-deoxy-α-D-erythro-pentopyranosyl

c: R = 2-deoxy-α-D-erythro-pentofuranosyl.

Figure 3. Hydrolysis products of the (6–4) photoproducts of dTpdC, dTpdT, and dCpdT and dehydrated T-T (6–4) adduct.

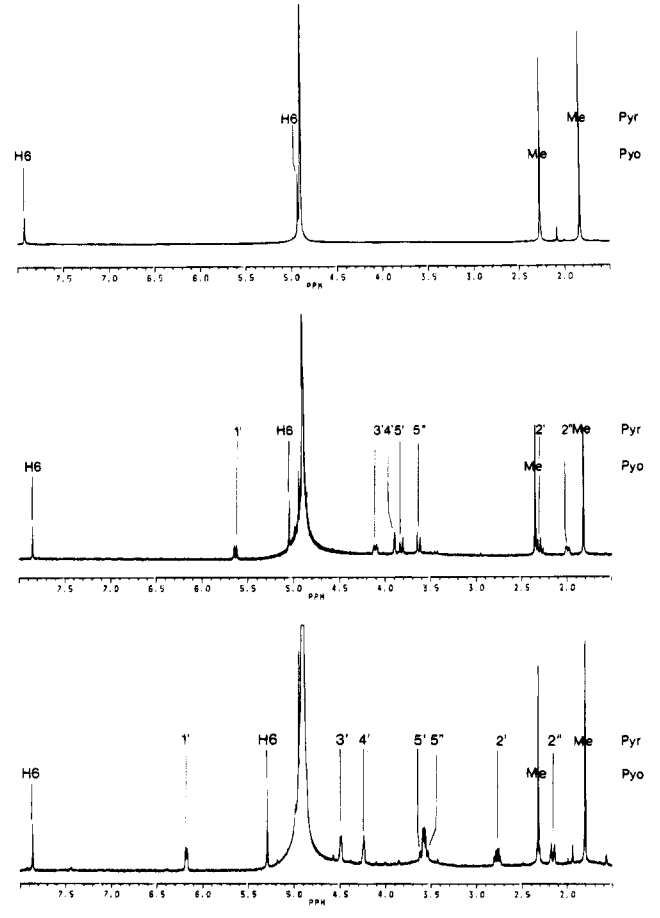


Figure 4. 400.13 MHz ^{1}H NMR spectra of hydrolyzed dTpdT (6–4) adducts: (upper) **1a**, (middle) **1b** (lower) **1c**.

the H-6 (and H-5 in **3a**) protons of the 5′ base is consistent with the saturation of the C-5/C-6 bond. It is worth mentioning that these features are similar to those of the pyrimidine (6–4) pyrimidone structure in the corresponding dinucleoside monophosphate (6–4) adducts. In addition, all the compounds exhibited an absorption maximum in the UVB region and fluorescence properties (Table 1). It should be added that the fragmentation observed in the thermospray–mass spectrum of **1a** (Figure 5) is in complete agreement with the proposed structure.

Table 1. Spectroscopic Features[a] of Dinucleoside Monophosphate and Base (6–4) Photoproducts

compd	UV λ_{max} (nm)	ϵ^b (mol·L^{-1}·cm^{-1})	Φ_{fluo}
1	325	4600	0.028
2	315	1400	0.007
3	325	3500	0.015
1a	314	8000	0.20
2a	307	3400	0.04
3a	315	6700	0.05
6	315	ND[c]	0.006

[a] Previously published in ref 12. [b] Wavelength: 320 nm for dinucleoside monophosphates (**1**, **2**, **3**) and 310 nm for base derivatives (**1a**, **2a**, **3a**). [c] Not determined.

Quantitative Aspects of the Hydrolysis. A comparative study of the stability of ([*methyl*-^{14}C]-5-hydroxy-5,6-dihydrothyminyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) was carried out under different acidic conditions (Figure 6). Formic acid hydrolysis led to a complete destruction of the (6–4) photoproduct. Trifluoroacetic acid was found to be only slightly less destructive, while hydrochloric acid generated the 5,6-dehydrated (6–4) adduct **6**. On the other hand, **1a** was found to be completely stable in HF/pyridine under the conditions used for DNA analysis (*vide infra*).

Hydrolysis of the (6–4) photoproducts of dTpdT*, T*pdC, and dCpdT* (where T* was [*methyl*-^{3}H]thymidine) by HF/pyridine followed by HPLC analysis monitored by on-line radioactivity detection showed that (i) the total amount of initial radioactivity in the sample of (6–4) adduct of dinucleoside monophosphate was recovered after the hydrolysis; (ii) no initial product remained; and (iii) no dehydrated (6–4) photoproduct was generated. The absence of dehydration is a major advantage of this hydrolysis method with respect to previous acidic assays.

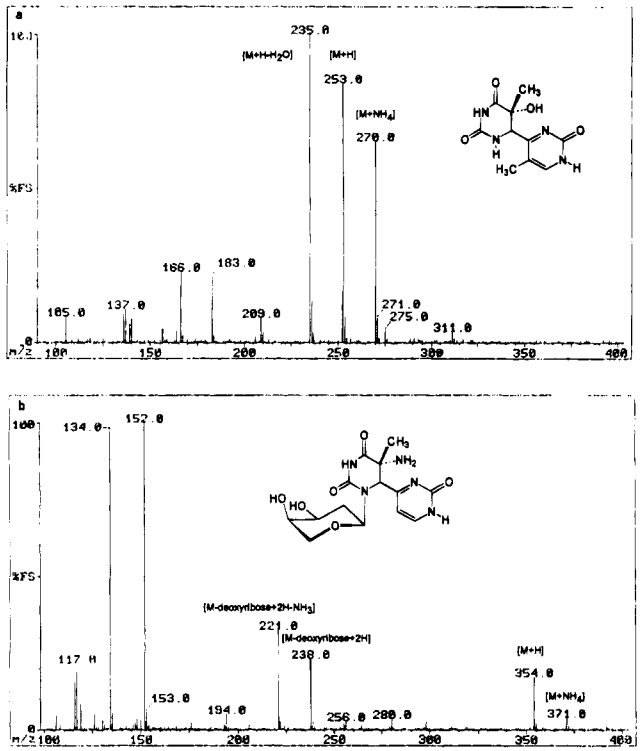


Figure 5. LC-thermospray mass spectra of (a) (5-hydroxy-5,6-dihydrothyminyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (**1a**) and (b) [5-amino-1-(2-deoxy-α-D-*erythro*-pentopyranosyl)-5,6-dihydrothyminyl]-(6–4′)-(1′,2′-dihydropyrimidin-2′-one (2b).

This indicates that the hydrolytic process occurring in the HF/pyridine mixture, likely to involve a $(HF)_x$/pyridine complex, is much milder than those of acidic reagents used previously. The dehydrated product **6**, exhibiting an unsaturated 5′ end pyrimidine ring, has a 33-fold lower quantum yield of fluorescence than its precursor **1a** (Table 1). The conversion of the (6–4) adducts into 5,6-unsaturated pyrimidine derivatives would thus limit the use of the fluorescence detection for monitoring their formation.

The three dinucleoside monophosphate (6–4) photoproducts were quantitatively converted into their corresponding base derivatives. The use of HF/pyridine allowed the isolation of (5-amino-5,6-dihydrothyminyl)-(6–4′)-(1′,2′-dihydropyrimidin-2′-one) (**2a**) and (5-hydroxy-5,6-dihydrocytosinyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (**3a**), which had never been described before. This result clearly shows that hydrogen fluoride stabilized in pyridine is able to cleave both N-glycosidic and phosphodiester bonds of the dinucleoside monophosphate photoadducts. These observations are in agreement with previous results involving the use of either anhydrous or aqueous HF mediated hydrolysis of various biopolymers, including polysaccharides (*37*), glycoproteins (*38*), teichoic acid (a phosphoric polyester of glycerol) (*39*), and nucleotides (*40–42*).

Deamination of the (6–4) Photoproducts of dCpdT in HF/Pyridine. (5-Hydroxy-5,6-dihydrocytosinyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (**3a**) was shown to be quantitatively released without undergoing any detectable deamination that would generate the uracil–thymine (6–4) adduct. The HF/pyridine mediated hydrolysis of **3** produced only one (6–4) adduct which was slowly converted ($t_{1/2}$ = 3.5 days at 20°C) into a slower eluting compound on the ODS silica gel column when left in aqueous solution. This secondary product exhibited a retention time identical to that of the hydrolyzed dUpdT (6–4) adduct and was therefore assigned to be the deamination product of **3a** (data not shown). The deamination of **3a** in aqueous solution was found to be a slow process, in agreement with a similar observation made on the dCpdT (6–4) photoproduct (*10*). Altogether, these observations provided evidence that the hydrolyzed compound is still the cytosine adduct and that no deamination occurred during the hydrolysis step. Final support for the lack of deamination of **3a** during

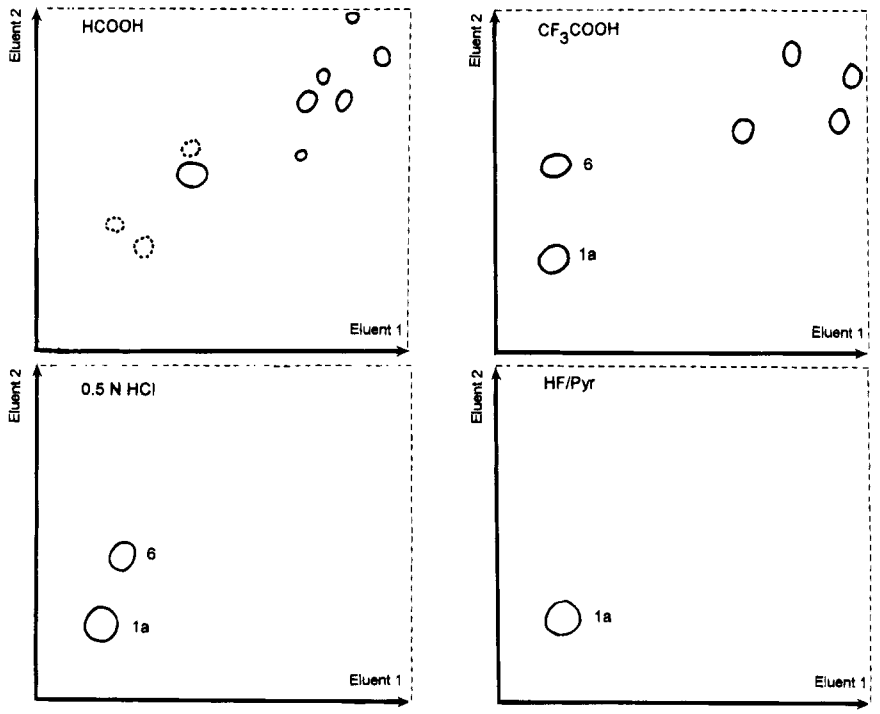


Figure 6. Comparison of the stability of radiolabeled thymine–thymine (6–4) adduct in formic acid, trifluoroacetic acid, hydrochloric acid, and hydrogen fluoride stabilized in pyridine. **1a**: (5-hydroxy-5,6-dihydrothyminyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one). **6**: (thyminyl)-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one). TLC eluent 1: mixture of ethyl acetate, 2-propanol, and water (75/16/9 v/v); eluent 2: lower layer of a mixture of chloroform, methanol, and water (4/2/1 v/v).

Table 2. 400.13 MHz ^{1}H NMR Chemical Shifts[a] of the Hydrolysis Products of dTpdT, dTpdC and dCpdT (6–4) Photoadducts in D_2O

adduct	1a	1b	1c	2a	2b	2c	3a
			5′-Pyr				
H5/Me	2.27	2.37	2.33	1.66	1.67	1.94	5.09
H6	4.95	5.07	5.20	4.90	4.90	4.90	5.25
H1′		5.64	6.19		5.65	6.30	
H2′		2.32	2.34		2.31	2.77	
H2″		2.01	2.22		2.01	2.18	
H3′		4.11	4.51		4.12	4.47	
H4′		3.92	4.26		3.91	4.12	
H5′		3.85	3.6		3.88	3.62	
H5″		3.65	3.6		3.68	3.62	
			3′-Pyo				
H5/Me	1.84	1.84	1.83	6.78	6.86	6.68	2.27
H6	7.92	7.67	7.88	8.16	8.09	8.17	7.94

[a] In ppm with respect to 3-(trimethylsilyl)propionate as internal reference.

HF/pyridine treatment was the observation in the chromatogram of its LC-thermospray mass spectra of **3a** of a pseudo-molecular at m/z 219. This corresponds to the dehydration product, induced by the temperature of the source (280 °C), whereas an ion at m/z 220 would have been observed with the deaminated (6–4) adduct.

Partially Hydrolyzed (6–4) Photoproducts. The use of milder conditions for the hydrolysis of the (6–4) adducts of dTpdT and dTpdC, including a shorter period of reaction (10 min) and a lower temperature (0 °C), allowed the isolation of two intermediates, together with a small amount of the (6–4) photoproducts **1a** and **2a**. The major product was identified as containing a mononucleoside residue with a 2-deoxy-α-D-*erythro*-pentofuranosyl structure (*vide infra*). The presence of a (6–4) adduct with an osidic moiety exhibiting a α-pyranosyl isomer was also observed, but its relative yield was found to be dependent on the concentration of (6–4) photoproduct. The α-pyranosyl isomers **1b** and **2b** were not observed when 100 μg of dinucleoside monophosphates (6–4) adduct was hydrolyzed in 500 μL of HF/pyridine. In contrast, they were the main hydrolysis products when 2 or 3 mg of **1a** or **2a** was hydrolyzed in 25 μL of HF/pyridine, even after 7 h of incubation at 37 °C.

The products obtained by partial hydrolysis of the (6–4) photoproducts of dTpdT and dTpdC (**1b** and **1c**, and **2b** and **2c**, for dTpdT and dTpdC, respectively) were eluted more slowly than the corresponding base (6–4) adducts (**1a** and **2a**) on the ODS column. However, they exhibited UV absorption and fluorescence properties identical to those of the (6–4) adducts **1a** and **2a** *(12)*. In addition, characteristic signals corresponding to the protons of the pyrimidine and pyrimidone moieties of the (6–4) adducts were observed in the ^{1}H NMR spectra (Figure 4). These results indicate that the (6–4) structure was not lost in **1b**, **1c**, **2b**, and **2c**. The ^{1}H NMR spectra also exhibited the presence of signals corresponding to those of a 2-deoxy-*erythro*-pentosyl residue (Table 2), therefore showing that one of the two N-glycosidic bonds was not cleaved. It was clearly established, by using dTpdT with the 3′ end thymidylyl unit specifically labeled at the 5′ position, that the remaining osidic moiety was linked to the 5′ base of the (6–4) photoproduct, since the two partially hydrolyzed (6–4) adducts were not radioactive anymore. Both the (6–4) adduct structure and the presence of a deoxyribose ring were confirmed by mass spectrometric analysis. The thermospray mass spectra exhibited the expected pseudomolecular ions and fragments corresponding to the loss of either a H_2O or NH_3 molecule for the derivatives of dTpdT and dTpdC, respectively, as shown in Figure 5 for **2b**.

Table 3. Coupling Constants (in Hz) of the Sugar Moiety of Partially Hydrolyzed (6–4) Adducts 1b, 1c, 2b and 2c

adduct	1′–2′	1′–2″	2′–2″	2′–3′	2″–3′	3′–4′	4′–5′	4′–5″	5′–5″
1b	3.1	11.3	−12.9	3.0	11.4	<1	<1	<1	−13.1
1c	7.3	3.8	−14.8	7.6	4.0	3.9	3.9	4.8	−12.7
2b	3.0	11.2	−12.7	3.1	11.6	<1	<1	<1	−13.0
2c	7.4	3.9	−14.6	7.4	3.8	3.8	3.9	4.9	−12.6

The configuration of the sugar moiety of **1b** and **2b** was inferred from detailed analysis of the sugar ^{1}H NMR coupling constants (Table 3), which were in agreement with available data *(43)*. This unambiguously established that the 2-deoxyribose moiety of **1b** and **2b** has an α-pyranosyl configuration. First, the magnitude of $J_{4'-5'}$ and $J_{4'-5''}$ is very low (<1 Hz). This observation strongly suggests that the osidic moiety does not have a furanosyl structure since the smallest value for the sum of the coupling constants $J_{4'-5'} + J_{4'-5''}$ cannot be lower than 2.5 Hz when the rotameric population of the sugar adopts a preferential *gauche-gauche* conformation *(44)*. In addition, the *trans* coupling constants between H-1′ and H-2″ on one hand, and H-2″ and H-3′ on the other hand, are very high (11 Hz), which is indicative of a preferential axial orientation of these protons. The latter features could be rationalized in terms of either a α-pyranosyl isomer in a $^1C_4$ conformation or a α-furanosyl isomer in a C3′*endo* conformation. However, for the latter structure, the coupling constant between H-3′ and H-4′ should be high since both H-3′ and H-4′ protons are axial. Since the value of $J_{3'-4'}$ is around 1 Hz, compounds **1b** and **2b** can only be assigned as 2-deoxy-α-D-*erythro*-pentopyranosyl nucleoside adopting a preferential $^1C_4$ conformation. Further support for this assignment was provided by the observation of low coupling constants between the H-2′ equatorial proton, and either the anomeric (3 Hz) or the H-3′ (5 Hz) protons. Moreover, H-4′ exhibits a very low coupling constant (J <1 Hz) with either H-3′ or H-5′ or H-5″ protons. This indicates that H-4′ is also in a preferential equatorial orientation, as expected for a pyranosyl 2′-deoxynucleoside in a $^1C_4$ conformation.

Coupling constant arguments were also used for the assignment of the osidic moiety of **1c** and **2c** (Table 3). The magnitude of $J_{4'-5'}$ is expected to be very low for a 2-deoxy-β-D-*erythro*-pentopyranosyl isomer in a $^1C_4$ conformation. An opposite result is expected for a $^4C_1$ conformer. Since H-4′ exhibits coupling constants with H-5′ and H-5″ of only 3.9 and 4.9 Hz, it may be concluded that **1c** and **2c** cannot be either $^1C_4$ or $^4C_1$ β-pyranosyl derivatives. Moreover, for a mixture of these two conformers in dynamic equilibrium, the value of the coupling constant for two protons in a *gauche* relationship is given by $J_{3'-4'}$ (3.8 Hz). Since H-1′ and H-3′ would be on the opposite sides of the β-pyranose ring, each of H-2′ and H-2″ would exhibit a *cis* and a *trans* scalar coupling. However, one of these two protons is highly coupled with both H-1′ and H-3′ (7.4 Hz) whereas one of the two coupling constants should be around 3.8 Hz as inferred from the $J_{3',4'}$ value. Consequently, **1c** and **2c** are not a mixture of $^1C_4$ and $^4C_1$ β-pyranosyl derivatives and can only be compounds with a furanosyl 2′-deoxyribose ring. Since $J_{2''-3'}$ and $J_{3'-4'}$ are low (3.8 Hz), the sugar ring was assigned to be in a preferential C2′*endo* conformation, as it is generally observed for most of the nucleosides *(45)*. The percentage of C2′*endo* conformation was determined

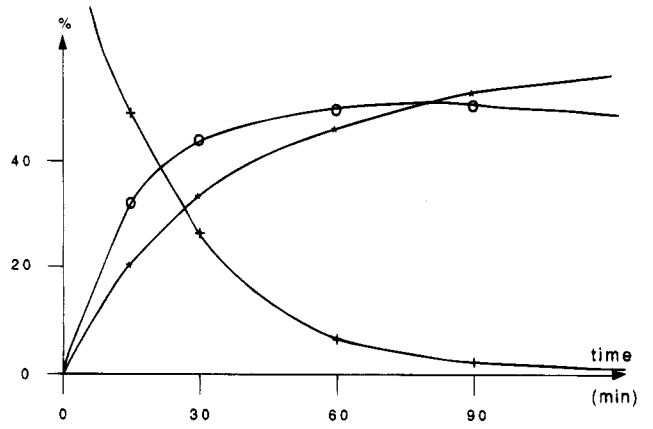


Figure 7. Kinetic of the hydrolysis of the dTpdC (6–4) photoproduct under suboptimal conditions. (+) [5-Amino-1-(2″-deoxy-α-D-*erythro*-pentofuranosyl)-5,6-dihydrothyminyl]-(6–4′)-(1′,2′-dihydropyrimidin-2′-one). (○) [5-Amino-1-(2″-deoxy-α-D-*erythro*-pentopyranosyl)-5,6-dihydrothyminyl]-(6–4′)-(1′,2′-dihydropyrimidin-2′-one). (*) (5-Amino-5,6-dihydrothyminyl)-(6–4′)-(1′,2′-dihydropyrimidin-2′-one).

to be 65%, using the equation $\%_{C2'endo} = 100(J_{1'-2'}/(J_{1'-2'} + J_{3'+4'}))$ *(45)*. For a β-furanosidic isomer, $J_{1'-2'}$ should be high since both H-1′ and H-2′ protons would be in a preferential axial orientation. Therefore, the observed value (7.4 Hz) is more consistent with an equatorial H-1′ and an axial H-2′. This shows that the deoxyribose has an α-pyranosyl structure. In addition, $J_{1'-2''}$ and $J_{2''-3'}$ are low (3.9 and 3.8 Hz, respectively). This provides further support for a preferential equatorial orientation of the H-1′, H-3′ and H-2″ protons as expected in a 2-deoxy-α-*erythro*-pentofuranosyl exhibiting a preferential C2′*endo* conformation *(45)*.

It is worth mentioning that chemical shifts of compounds **1b**, **1c**, **2b**, and **2c** are very similar to those of modified nucleosides exhibiting either 2-deoxy-α-D-*erythro*-pentopyranosyl or 2-deoxy-α-D-*erythro*-pentofuranosyl residues *(46, 47)*. The downfield shift of H-5′ and the upfield shift of H-3′ in **1b** and **2b** are characteristic of an α-pyranosyl configuration. Similarly, the value of the chemical shifts of H-4′ and H-2′ for compounds **1c** and **2c** are in agreement with a α-furanosyl sugar ring structure. Further support for the proposed structures were provided by experiments of oxidation by sodium periodate, a specific oxidative agent of *cis*-diols *(48)*. Upon $NaIO_4$ treatment, the chromatographic behavior of **1b** was deeply modified whereas that of **1c** remained unchanged, as expected for the proposed structures.

Mechanistic Hypothesis. Kinetic study of the partial hydrolysis of the dTpdT and dTpdC (6–4) photoproducts in highly concentrated solutions provided information about the mechanism of hydrolysis by HF/pyridine (Figure 7). The 2-deoxy-α-D-*erythro*-pentofuranosyl derivatives (**1c**, **2c**) were found to be generated very rapidly. Then, their amount decreased with increasing reaction time while those of the base (6–4) photoproducts and the α-pyranosyl derivatives increased. These observations provided evidence for a sequential mechanism, as illustrated for the dTpdT (6–4) adduct **1** in Figure 8.

The first step is likely to be the cleavage of the bonds involving the osidic moieties and the phosphate group as the result of the nucleophilic action of fluoride ion. This is in agreement with the fact that hydrogen fluoride preferentially hydrolyzes the phosphodiester bond of nucleotides *(42)*. In a subsequent step, the N-glycosidic bond of the pyrimidone moiety is preferentially hydrolyzed, giving rise to the monodeoxyribose adduct **1c**. This specific reaction could be explained by the difference in stability of the N-glycosidic bond of the two base moieties. It is likely that a fast epimerization of the anomeric carbon also occurs at this stage since the 2-deoxy-β-D-*erythro*-pentofuranosyl derivative was not isolated.

In diluted samples, the concentration of hydrogen fluoride relative to that of (6–4) adduct is high enough to induce a fast hydrolysis of the remaining N-glycosidic bond of the 5′ end pyrimidine moiety, giving rise to **1a**. On the other hand, in highly concentrated solutions, an additional reaction was found to occur. This involves the isomerization of the [1-(2-deoxy-α-D-*erythro*-pentofuranosyl)-5-hydroxy-5,6-dihydrothyminyl]-(6–4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) form into the more stable α-pyranosyl isomer **1b**. The latter compound is then hydrolyzed with a much lower rate. This would account for the fact that **1b** and **2b** are the major adducts isolated upon hydrolysis of large amounts of **1** and **2**, respectively.

Measurement of (6–4) Photoproducts in Irradiated DNA. The separation of (5-hydroxy-5,6-dihydrothyminyl)-(6–4′)(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (**1a**), (5-amino-5,6-dihydrothyminyl)-(6–4′)-(1′,2′-dihydropyrimidin-2′-one) (**2a**), and (5-hydroxy-5,6-dihydro-

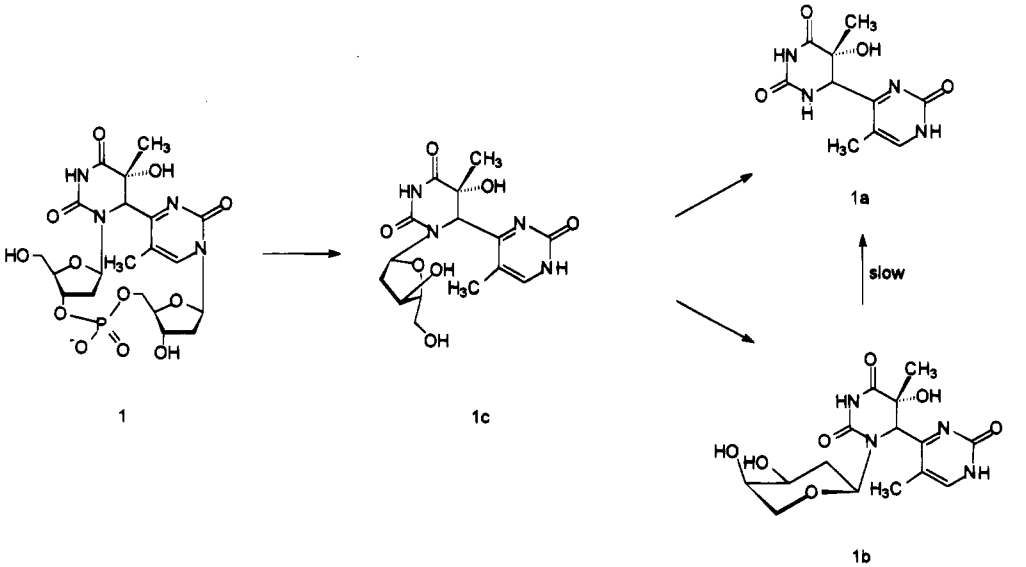


Figure 8. Mechanism of hydrolysis of the dTpdT (6–4) photoproduct.

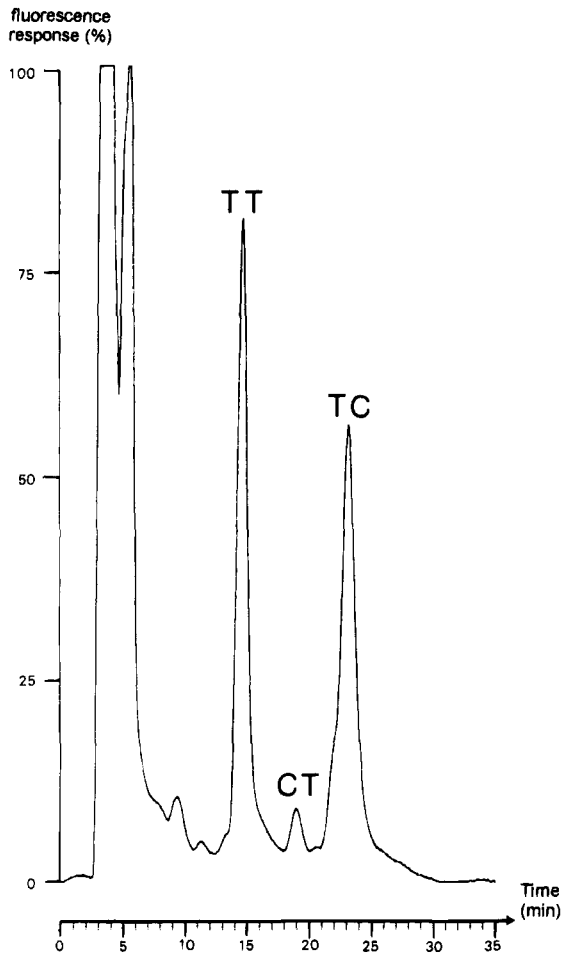


Figure 9. HPLC elution profile of the hydrolysate of 50 μg of calf thymus DNA exposed to 254 nm light (overall UV dose: 70 J·m^{-2}) on an amino column (see conditions in Materials and Methods). The detection was provided by a fluorimeter with excitation and emission wavelengths set at 315 and 380 nm, respectively.

cytosinyl)-(6−4′)-(5′-methyl-1′,2′-dihydropyrimidin-2′-one) (**3a**) was achieved on an analytical amino silica gel column. It was thus shown that HF/pyridine mediated hydrolysis of UVC irradiated DNA provided **1a**, **2a**, and **3a**, on the basis of both fluorescence and chromatographic properties (Figure 9). To prevent the formation of partially hydrolyzed products, 1 μL of HF/pyridine was used per microgram of DNA. Hydrolysis of a large amount of DNA with a lower volume of hydrolyzing reagent also made possible the isolation of **1a** and **1b** in amounts allowing their unambiguous characterization by ^{1}H NMR. It should be mentioned that normal bases are also quantitatively released from DNA, as inferred from the observation of the corresponding peaks on the RP-HPLC elution profile of the hydrolyzed samples (data not shown).

The detection limit of isolated thymine−thymine (6−4) adduct is around 1 pmol. However, this value is 10-fold higher in far-UV irradiated DNA, because of the presence of fluorescent impurities in the hydrolysis mixture. This corresponds to the amount of (6−4) photoproducts produced in 30 μg of DNA irradiated with 5 J·m^{-2}. This value is low enough to make the method applicable at the biological level.

The yield of the (6−4) photoproducts in DNA was estimated by analysis of DNA irradiated with increasing doses of 254 nm light in aqueous solution, by using preliminary calibration of the HPLC system with authentic samples. A first observation is the linear formation of the (6−4) adducts with the applied dose. In addition, the rate of formation of (6−4) photoproducts at different sequences was found to be in the following order: T-C ≫ T-T > C-T (Figure 10). It should be noted that the peaks corresponding to **1a** and **2a** are almost of equal intensity on the HPLC profile because of the difference of UV and fluorescence features. These results represent the first data available on the relative rate of formation of the (6−4) photoproducts within DNA. The formation of the thymine−cytosine (6−4) adduct as the main (6−4) photoproducts generated in far-UV irradiated DNA is in agreement with the trend shown by the gel sequencing technique *(22)*. Works are under progress aiming at searching for the cytosine−cytosine (6−4) adduct in far-UV irradiated DNA.

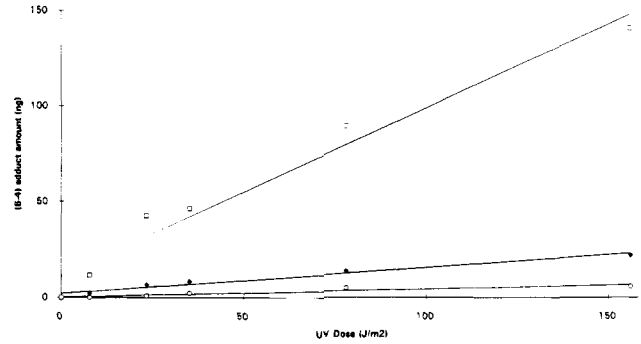


Figure 10. Dose course formation of the (6−4) photoproducts in calf thymus DNA (50 μg) upon exposure to 254 nm radiation. (□) Thymine−cytosine; (♦) thymine−thymine; (○) cytosine−thymine.

Acknowledgment. This work was partly supported by a grant from European Commissions (HCM Program−Contract CHRX CT 93.0275).

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