Synthesis and Kinetic Study of the Deamination of the *Cis* Diastereomers of 5,6-Dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine†

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The main objectives of the present work were the synthesis of the two *cis* diastereomers of 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine and the kinetic study of their hydrolytic deamination. The preparation of the two glycols, two main •OH-mediated oxidation products of 5-methyl-2′-deoxycytidine, was achieved in two steps. The first one involved the synthesis of the two *trans*-(5*R*,6*S*)- and (5*S*,6*R*)-5-bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine. In a subsequent step, the bromohydrins were specifically converted into the *cis*-(5*S*,6*S*) and (5*R*,6*R*) diastereomers of 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine, respectively, under slightly alkaline conditions. The resulting glycols were purified by reverse phase high performance liquid chromatography and characterized by extensive spectroscopy measurements including ^{13}C- and ^{1}H-NMR analyses. Exact mass determination was inferred from high resolution fast atom bombardment mass spectrometry measurements. Circular dichroism spectroscopy confirmed the diastereomeric relationship existing between the pair of glycols. Kinetic study of the deamination of the above glycols was carried out in phosphate buffer solutions (pH 7) at two different temperatures (37 °C and 25 °C) in order to determine the thermodynamic and kinetic parameters of the reaction.

Introduction

5-Methylcytosine is a minor pyrimidine nucleobase of the DNA of mammalians and higher eukaryotes (1−7% of the DNA bases in mammalian cells). The distribution of methylated cytosines in vertebrates differs from species and tissues[1,2] and occurs largely at the CpG dinucleotide sequence.[3−5] The methylation process is catalyzed by either DNA methylase or transferase with the methyl donor being *S*-adenosylmethionine.[6,7] Methylation of cytosine residues at C5 in eukaryotic DNA has been suggested to be involved in the control of transcription, cell differentiation,[8−10] and maintenance of chromosome structure such as the *Z* conformation.[11,12] It is well documented that 5-methylcytosine derivatives are converted into related thymine compounds by a hydrolytic deamination reaction. However, deamination is more efficient for 5,6-saturated 5-methylcytosine derivatives.[13,14] Recently, it has been suggested that the mechanism of action of DNA methyltransferases involves the formation of a 5,6-dihydrocytosine intermediate which is highly susceptible to hydrolytic deamination.[15] Only a few attempts have been made to prepare 5,6-dihydro-5-methylcytosine derivatives. As a few major exceptions, we may mention the recent works on the isolation of far-UV photoproducts of 5-methylcytosine DNA model compounds.[16,17] In particular, it was shown that far-UV irradiation of m^{5}dCpT and Tpm^{5}dC leads to the formation of three major types of photoproducts including cyclobutane dimers, pyrimidine (6-4) pyrimidone adducts and their Dewar valence isomers.[16] On the basis of radical oxidation studies of thymidine,[18] we expect 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine to be a major oxidation product of m^{5}dCyd. We report here the synthesis and the characterization of the two *cis* diastereomers of 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3** and **4**). The kinetics of deamination of the above glycols was also investigated. Attempts were made to search for the formation of the *cis* diastereomers of 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3** and **4**) in a γ-irradiated aerated aqueous solution of m^{5}dCyd by using the above standards. Under the latter conditions, we observed that both glycols were formed in significant amounts.

Experimental Section

Chemicals. Thymidine was obtained from Sigma Chemical Co. (St. Louis, MO). Bromine, sodium acetate, and ammonium hydrogen carbonate were purchased from Prolabo (Paris, France). HPLC grade methanol was from Carlo Erba (Farmitalia Carlo Erba, Milan, Italy). 5-Methyl-2′-deoxycytidine was synthesized from thymidine according to an adaptation of a general procedure described by Fox *et al.*[19]

High-performance Liquid Chromatography Separations. The *trans* diastereomers of 5-bromo-6-hydroxy-5,6-

† Abbreviations: CD, circular dichroism; D_2O, deuterium oxide; dCMP, 2′-deoxycytidine 5′-monophosphate; HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; m^{5}dCyd, 5-methyl-2′-deoxycytidine; m^{5}Cyt, 5-methylcytosine; TLC, thin-layer chromatography.

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Table 1. 400.13 MHz ^{1}H NMR Chemical Shifts[a] (ppm) and Proton−Proton Coupling Constants[b] (Hz) of (5*S*,6*R*)- and (5*R*,6*S*)-5-Bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (1 and 2) and (5*R*,6*R*)- and (5*S*,6*S*)-5,6-Dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (3 and 4) Obtained in D_2O as Inferred from Computer Iterative Analysis (LAOCOON III program)

	δ H1′	δ H2′	δ H2″	δ H3′	δ H4′	δ H5′	δ H5″	δ CH_3	δ H6
(**1**) *trans*-(5*S*,6*R*)	6.29	2.55	2.34	4.50	4.03	3.87	3.82	2.12	5.41
(**2**) *trans*-(5*R*,6*S*)	6.32	2.48	2.30	4.51	4.04	3.85	3.83	2.11	5.46
(**3**) *cis*-(5*R*,6*R*)	6.23	2.36	2.30	4.49	4.01	3.88	3.82	1.55	5.03
(**4**) *cis*-(5*S*,6*S*)	6.33	2.43	2.24	4.49	4.00	3.83	3.80	1.54	5.05
	$J_{1'2'}$	$J_{1'2''}$	$J_{2'2''}$	$J_{2'3'}$	$J_{2''3'}$	$J_{3'4'}$	$J_{4'5'}$	$J_{4'5''}$	$J_{5'5''}$
(**1**) *trans*-(5*S*,6*R*)	7.7	6.5	−14.1	6.6	3.8	3.9	3.7	5.1	−12.3
(**2**) *trans*-(5*R*,6*S*)	8.5	6.1	−14.0	6.2	3.0	3.0	3.9	4.4	−12.3
(**3**) *cis*-(5*R*,6*R*)	7.6	6.6	−13.9	6.6	3.9	3.9	3.7	4.9	−12.2
(**4**) *cis*-(5*S*,6*S*)	9.0	5.9	−14.0	6.2	2.7	2.9	4.1	5.0	−12.2

[a] The accuracy of the measurement is provided to ±0.01 ppm. [b] Accuracy of the measurement is provided to ±0.1 Hz.

Table 2. 100.7 MHz ^{13}C NMR Chemical Shifts (ppm) of *trans*-(5*S*,6*R*)-5-Bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (1), *trans*-(5*R*,6*S*)-5-Bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (2), *cis*-(5*R*,6*R*)-5,6-Dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (3), and *cis*-(5*S*,6*S*)-5,6-Dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (4) obtained in D_2O

	C1′	C2′	C3′	C4′	C5′	C2	C4	C5	C6	CH_3
(**1**) *trans*-(5*S*,6*R*)	85.3	37.3	71.9	86.1	61.7	151.8	161.8	54.0	80.6	23.6
(**2**) *trans*-(5*R*,6*S*)	84.5	36.9	71.4	86.2	62.0	151.7	161.7	53.6	80.2	23.5
(**3**) *cis*-(5*R*,6*R*)	85.2	37.3	70.6	85.5	61.3	nd[a]	175.7	69.9	79.1	22.7
(**4**) *cis*-(5*S*,6*S*)	84.3	36.2	71.2	85.1	61.9	nd[a]	nd[a]	70.0	77.9	22.9

[a] Not determined.

dihydro-5-methyl-2′-deoxycytidine (**1** and **2**) and the *cis* diastereomers of 5,6-dihydoxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3** and **4**) were purified by using a HPLC system consisting of a M6000A dual pump Waters Associate (Mildford, MA) equipped with a Model 7125 Rheodyne injection loop (Berkeley, CA) and a Waters R401 differential refractometer. Samples were injected onto a homemade semipreparative Nucleosil octadecylsilyl silica gel column (300 × 7.5 mm i.d., mean particle size 10 μm) from Macherey-Nagel (Düren, Germany).

Two different chromatographic systems were used: System A for the purification of the two *trans* diastereomers of m^{5}dCyd bromohydrins **1** and **2**: eluent, H_2O/MeOH (70/30); flow-rate, 2 mL/min.

System B for the purification of the two *cis* diastereomers of m^{5}dCyd glycols **3** and **4**: eluent, H_2O; flow-rate, 2 mL/min.

The deamination kinetics were followed by using a HPLC system consisting of a L-6000 pump model (Merck, Darmstadt, Germany) associated with a L-4000 UV detector (Merck, Darmstadt, Germany) set at 230 nm. The mobile phase consisted of water (pH 6.0) with a flow rate of 1 mL/min. Samples were injected onto an analytical Hypersil C18 octadecylsilyl silica gel Interchrom column (250 × 4.6 mm i.d., mean particle size 5 μm) (Interchim, Montluçon, France). Under these conditions, the following values of capacity factors (k' = retention volume/[retention volume − void column volume]) are the following: $k' = 2.12$ and 1.52 for *cis*-(5*S*,6*S*) and *cis*-(5*R*,6*R*)-5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**4** and **3**), respectively, and $k' = 1.38$ and 1.36 for *cis*-(5*S*,6*S*)- and *cis*-(5*R*,6*R*)-5,6-dihydroxy-5,6-dihydrothymidine, respectively.

Thin-Layer Chromatography Analysis. Two-dimensional thin-layer chromatography (TLC) separations were performed on Merck 60 F_{254} silica gel plates (Merck, Darmstadt, Germany) (0.2 mm thickness) using the lower phase of chloroform−methanol−water (4:2:1) (v:v:v) to which 5% of methanol was added (solvent A) and ethyl acetate−2-propanol−water (75:16:9) (solvent B) as the developing solvents.[20]

Spectrometric Measurements. Fast atom bombardment (FAB) mass spectrometry analyses were carried out on a Model ZAB 2-SEQ spectrometer (Fisons-V. G. Manchester, United Kingdom) in the positive mode. The nucleosides were dissolved in a mixture of thioglycerol/glycerol (50:50) (v:v) matrix. Circular dichroism spectra were recorded in water on a DICHRO III instrument (Jobin-Yvon, Lonjumeau, France). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a AM400 Brüker spectrometer in the Fourier transform mode (Brüker, Wissemburg, France). The chemical shifts are expressed in ppm with reference to 3-(trimethylsilyl)propionate-2,2,3,3-d_4 used as the internal standard in D_2O (99.96% D). ^{1}H-NMR spectra were simulated for best fit by using the LAOCOON III software. Nuclear Overhauser effect (NOE) experiments were carried out with the decoupling field gated off during the acquisition of the data. The recycle delay was 1 s.

Synthesis of the *trans*-(5*S*,6*R*) Diastereomer of 5-Bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (1). 5-Methyl-2′-deoxycytidine (20 mg) was dissolved in 4 mL of 0.1 M sodium acetate solution (pH 7). Bromine solution was added dropwise until the persistence of a yellow color. Then, the mixture was stirred at 25 °C for 30 min. Under these conditions, the conversion of 5-methyl-2′-deoxycytidine was complete as inferred from the two-dimensional TLC analysis of the mixture. The excess of bromine was removed from the solution with a stream of air. Then, the mixture was purified by HPLC (*vide supra*). Evaporation to dryness of the main HPLC fraction ($k' = 1.32$) yielded 17.9 mg of the *trans*-(5*S*,6*R*)-5-bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**1**) (yield = 64%). ^{1}H-NMR (D_2O) and *J* (H−H) see Table 1. ^{13}C-NMR (D_2O) see Table 2. FAB-MS positive mode (relative intensity): *m*/*z* 126 (100; [base − BrOH + 2H]$^+$); *m*/*z* 242 (90; [M − BrOH + H]$^+$; *m*/*z* 340 (10; [M + H]$^+$, ^{81}Br); *m*/*z* 338 (9; [M + H]$^+$, ^{79}Br); *m*/*z* 109 (6; [sugar moiety − H_2O]$^+$).

Synthesis of the *trans*-(5*R*,6*S*) Diastereomer of 5-Bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (2). The second HPLC fraction ($k' = 1.48$) obtained by purification of the above mixture was evaporated under reduced pressure. The minor product (yield = 32%) was the *trans*-(5*R*,6*S*)-5-bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**2**) as inferred from extensive spectroscopic analyses. ^{1}H-NMR (D_2O) and *J* (H−H) see Table 1. ^{13}C-NMR (D_2O) see Table 2. FAB-MS positive mode (relative intensity): *m*/*z* 126 (100; [base − BrOH + 2H]$^+$); *m*/*z* 242 (63; [M − BrOH + H]$^+$; *m*/*z* 483.(45; [2(M − BrOH) + H]$^+$); *m*/*z* 340 (42; [M + H]$^+$, ^{81}Br); *m*/*z* 338 (39; [M + H]$^+$, ^{79}Br); *m*/*z* 264 (28; [M − BrOH + Na]$^+$); *m*/*z* 117 (10; [sugar moiety]$^+$).

Synthesis of *cis*-(5*R*,6*R*)-5,6-Dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (3). The *trans*-(5*S*,6*R*) diastereomer of 5-bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycy-

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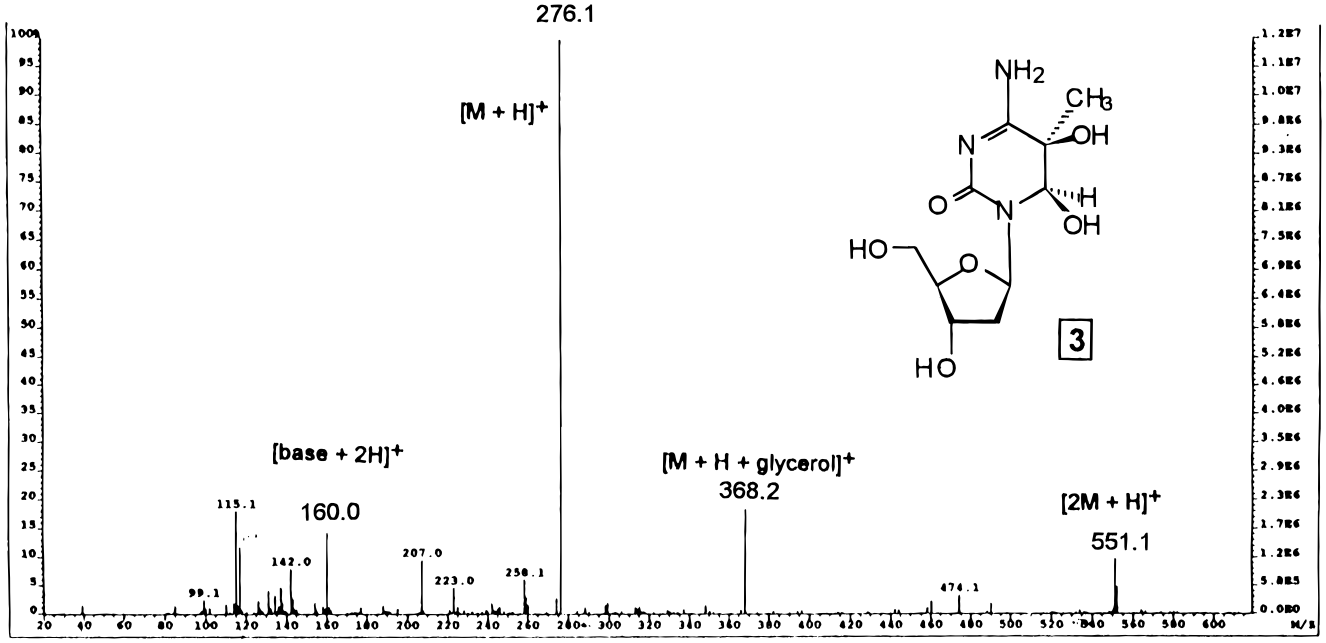

Figure 1. FAB mass spectrum in the positive mode of the *cis*-(5*R*,6*R*)-5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3**).

tidine (**1**) (10 mg) was dissolved in 3 mL of 0.1 M ammonium hydrogen carbonate (pH 8.3). The mixture was stirred at 25 °C for 45 min. Then, the main product of the reaction was purified by HPLC. Evaporation to dryness of the pooled fractions ($k' = 1.33$) gave 4 mg of the *cis*-(5*R*,6*R*)-5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3**) (yield = 49%). ^{1}H-NMR (D_2O) and *J* (H−H) see Table 1. ^{13}C-NMR (D_2O) see Table 2. FAB-MS positive mode (Figure 1) (relative intensity): *m*/*z* 276 (100; $[M + H]^+$); *m*/*z* 368 (20; [M + glycerol + H]$^+$); *m*/*z* 160 (15; [baseH + H]$^+$); *m*/*z* 117 (13; [sugar moiety]$^+$); *m*/*z* 551 (10; $[2(M + H)]^+$); *m*/*z* 258 (7; [base − H_2O + H]$^+$). In addition, the exact mass measurement of the pseudomolecular ion of **3** (*m*/*z* 276.1195) was obtained from high resolution FAB-MS analysis. This is indicative of an empirical formula of $C_{10}H_{18}O_6N_3$ $[M + H]^+$. UV (λ_{max}, H_2O): 210, 242 nm.

Synthesis of *cis*-(5*S*,6*S*)-5,6-Dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (4). The *trans*-(5*R*,6*S*) diastereomer of 5-bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**2**) (9 mg) was dissolved in 3 mL of (0.1 M) ammonium hydrogen carbonate (pH 8.3). The resulting solution was stirred at 25 °C for 45 min, and the mixture was purified by HPLC. The main HPLC fractions ($k' = 2.0$) were pooled and then evaporated to dryness under reduced pressure. This provided 3 mg (yield = 41%) of an oily compound which was assigned as the *cis*-(5*S*,6*S*)-5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**4**). ^{1}H-NMR (D_2O) and *J* (H−H) see Table 1. ^{13}C-NMR (D_2O) see Table 2. FAB-MS positive mode (relative intensity): *m*/*z* 276 (100; $[M + H]^+$); *m*/*z* 117 (25; [sugar moiety]$^+$); *m*/*z* 160 (20; [baseH + H]$^+$); *m*/*z* 258 (7; [base − H_2O + H]$^+$). In addition, the exact mass measurement of the pseudomolecular ion of **4** (*m*/*z* 276.1174) was inferred from high resolution FAB-MS measurement. This is indicative of an empirical formula of $C_{10}H_{18}O_6N_3$ $[M + H]^+$. UV (λ_{max}, H_2O): 210, 242 nm.

Deamination of the Two *Cis* Diastereomers of 5,6-Dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (3 and 4). A 6.4 mg amount of each of the *cis* diastereomer of 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3** and **4**) was dissolved in 10 mL of phosphate buffer (pH 7) (SDS, Peypin, France). The resulting solution was then divided into two equal volume fractions, which were incubated at 25 °C and 37 °C, respectively. The kinetic of deamination was followed by HPLC analyses (*vide supra*). Samples of 50 μL were taken at increasing periods of time and directly injected on the above HPLC system.

γ Radiolysis of an Aerated Aqueous Solution of 5-Methyl-2′-deoxycytidine. An aqueous aerated solution (90 mL) of 2 mM m^{5}dCyd was exposed to the γ rays (overall dose = 10.8 kGy) emitted from a ^{60}Co source. The dose-rate was 50 Gy/min. The water was removed by rotary evaporation under reduced pressure (<30 °C) and the resulting solid residue was resuspended in 200 μL of water prior to HPLC analysis. The two *cis* diastereomers of 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3** and **4**) were isolated by using HLPC system B (*vide supra*).

Results and Discussion

The preparation of the two *cis* diastereomers of 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3** and **4**) involves the initial synthesis of the *trans* diastereomers of 5-bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**1** and **2**). Then, each of these intermediates **1** and **2** was specifically converted into the corresponding glycol **3** and **4**. This follows a general procedure which was previously applied to the synthesis of the *cis* diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine.[21]

Structural Determination of the Two *Trans* Diastereomers of 5-Bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (1 and 2). ^{1}H-NMR Analysis. The 400 MHz ^{1}H-NMR spectrum of each of the two *trans* diastereomers of 5-bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**1** and **2**) in D_2O exhibits nine signals which were totally assigned (Table 1). The ^{1}H-NMR features of the sugar moiety constitute the fingerprint of the C5−C6 saturated pyrimidine derivatives exhibiting a hydroxyl group at C6 such as the diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine, 5-bromo-6-hydroxy-5,6-dihydrothymidine, and 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine.[22,23] Moreover, the values of ^{1}H chemical shifts as well as those of scalar proton−proton coupling constants allow insight into the conformation and configuration of the latter compounds. It was clearly established that $J_{1'2'}$ coupling constant is smaller for 6*R* diastereomers than for 6*S* compounds. Therefore, ^{1}H-NMR data obtained for both m^{5}dCyd bromohydrins

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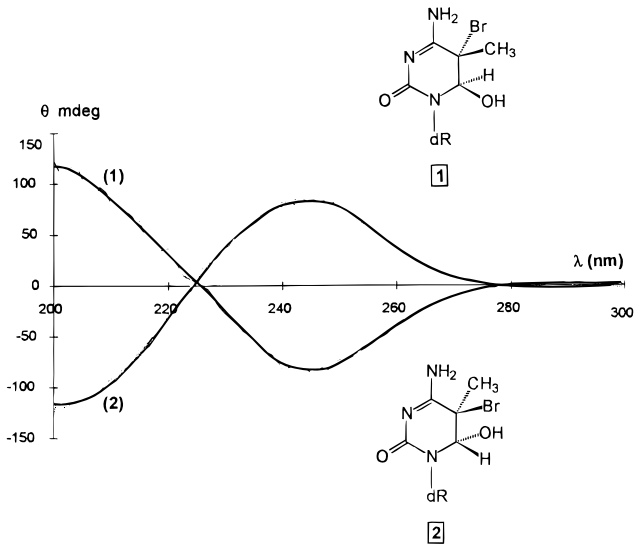

Figure 2. CD spectra of the *trans*-(5*S*,6*R*)-5-bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**1**) and *trans*-(5*R*,6*S*)-5-bromo-6-hydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**2**) in water.

1, **2** and glycols **3**, **4** allowed assignment of the absolute configuration of these modified nucleosides. The two trans diastereomers of bromohydrins **1**, **2** exhibited similar ^{1}H-NMR features with only slight differences. As it was previously reported for the bromohydrins of thymidine,[22] the 6*R* diastereomer **1** exhibited a coupling constant $J_{1'2'} = 7.7$ Hz which was approximately 0.8 Hz smaller than that of the 6*S* diastereomer **2** ($J_{1'2'} = 8.5$ Hz).

^{13}C-NMR Analysis. The ^{13}C-NMR spectrum of each of the two *trans* bromohydrins **1**, **2** exhibits ten carbon resonances (see Table 2). The unambiguous assignment of the 2-deoxyribose carbons, the methyl group, and C6 carbons was inferred from two-dimensional ^{1}H−^{13}C heteronuclear-correlated NMR experiments (XHCORRC) (data not shown). The quaternary carbons C2, C4, and C5 were assigned by comparison with the ^{13}C NMR features of the thymidine bromohydrins for which the structure and stereochemistry have been established.[24]

Circular Dichroism. The two diastereomeric m^{5}dCyd bromohydrins **1**, **2** exhibit opposite ($\pi-\pi^*$) transition whose maximum is centered about 230 nm (Figure 2). The observed opposite Cotton effects provide further support for the diastereomeric relationship existing between the pair of bromohydrins.

Structural Analyses of the Two Cis diastereomers of 5,6-Dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine. ^{1}H-NMR Analysis. The 400 MHz ^{1}H-NMR spectra of the two *cis* diastereomers of 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3** and **4**) in D_2O exhibit nine signals which were unambiguously assigned. The related chemical shifts are reported in Table 1. The stereochemistry of the two *cis* diastereomers of 5-methyl-2′-deoxycytidine glycols **3** and **4** was assigned on the basis of the comparison of their ^{1}H-NMR features with those of the two *cis* diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine.[22] The respective chemical shifts are similar for the two *cis* diastereomers **3** and **4**. However, slight but significant differences were noted concerning the scalar proton−proton coupling constants of the sugar moiety. The coupling constant $J_{1'2'}$ is about 1 Hz smaller for the diastereomer **3** than for the diastereomer **4**. In addition, the two coupling constants $J_{2''3'}$ and $J_{3'4'}$ are about 1 Hz smaller for **4** than for **3**. Similar differences were observed for the two *cis* glycols of thymidine, whose absolute configuration was attributed by using a chemical method[24] and further confirmed by X-ray crystallography.[25] In conclusion, these observations are indicative of a 5*R*,6*R* stereochemistry for **3** and a 5*S*,6*S* configuration for the diastereomer **4**. This received further confirmation from deamination studies. It was shown that the hydrolytic deamination of glycols **3** and **4** gives rise specifically to the *cis*-(5*S*,6*R*) and *cis*-(5*R*,6*S*) diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine, respectively.

^{13}C-NMR Analysis. The ^{13}C chemical shifts of the two *cis* glycols **3**, **4**, with the exception of a few quaternary carbons, are reported in Table 2. The above assignment of the absolute configuration of the two *cis* diastereomers of the 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3** and **4**) receives further support from the comparison of their ^{13}C chemical shifts with those of the thymidine glycols. The most significant differences in the ^{13}C chemical shifts observed between the two *cis* diastereomers of m^{5}dCyd **3**, **4** concern the C1′ and C2′ of the sugar moiety and the C6 of the base. It appears that these differences are correlated with the C6 absolute configuration of **3** and **4**. Thus, the 6*R* configuration of **3** is associated with a 1 ppm downfield shift of the C1′, C2′, and C6 resonances with respect to the 6*S* stereochemistry of **4**. Similar trends in the ^{13}C chemical shifts were also observed for the *cis* diastereomers of thymidine glycols.

Mass Spectrometry. The positive fast atom bombardment mass spectrum of both *cis* diastereomers of 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3** and **4**) exhibits a pseudomolecular ion at *m*/*z* 276.1 ($[M + H]^+$). In addition, two relevant fragments are observed at *m*/*z* 160.0 ($[BH + H]^+$) and *m*/*z* 117.0 ($[S]^+$). These correspond to the aglycon and the sugar moiety, respectively. Exact mass measurement of the pseudomolecular ions (*m*/*z* 276.1195 and *m*/*z* 276.1174 for **3** and **4**, respectively) is indicative of a molecular formula of $C_{10}H_{18}O_6N_3$ for both modified nucleosides. The experiments allow to confirm that the hydrolytic deamination process does not occur during the synthesis.

Circular Dichroism. Inspection of the CD features provides further confirmation of the diastereomeric relationship existing between the pair of m^{5}dCyd glycols **3**, **4**. Both glycols exhibited similar but opposite absorption curves centered around 230 nm and 255 nm, respectively (Figure 3). The *cis*-(5*R*,6*R*) diastereomer of m^{5}dCyd glycol **3** exhibits a negative Cotton effect at about 250 nm which was also observed for the related diastereomer of 5,6-dihydroxy-5,6-dihydrothymidine.[21] On the other hand, a similar but positive Cotton effect was observed for **4** and the *cis*-(5*S*,6*S*) diastereomer of thymidine glycol at about 250 nm. The CD observations further support the assignment of the absolute configuration of the two *cis* diastereomers **3** and **4**.

Conformational Analysis. The furanose ring conformation of nucleosides is usually described in terms of a dynamic equilibrium between two C2′ *endo* and C3′ *endo* puckered forms.[26,27] The C2′ *endo* characterizes the conformation in which C2′ is displaced toward the C5′

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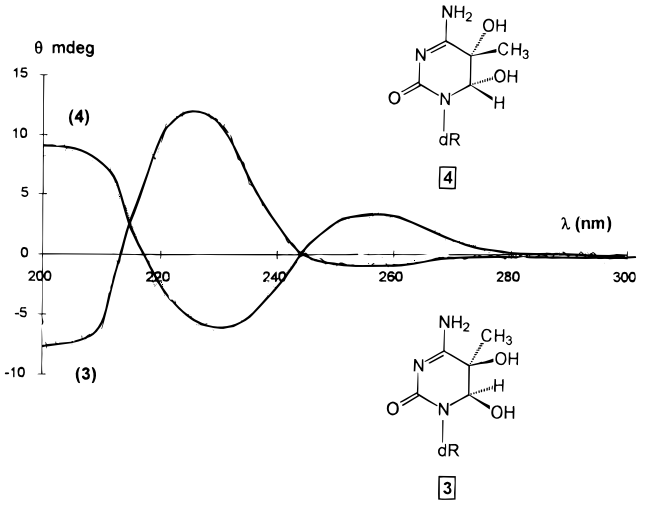

Figure 3. CD spectra of the *cis*-(5*R*,6*R*)-5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3**) and *cis*-(5*S*,6*S*)-5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**4**) in water.

Table 3. Conformational Features of Nucleosides 1−4 in D_2O as Inferred from ^{1}H-NMR Measurements

	% $C_{2'}$ *endo*[a]	% gg[b]	% gt + tg[c]
(**1**) *trans*-(5*S*,6*R*)	66	51	49
(**2**) *trans*-(5*R*,6*S*)	74	55	45
(**3**) *cis*-(5*R*,6*R*)	66	53	47
(**4**) *cis*-(5*S*,6*S*)	76	47	53

[a] Percentage of C2′ *endo* = $J_{1'2'}/[J_{1'2'} + J_{3'4'}]$.[28] [b] Percentage of rotamer gg = $[13.7 - (J_{4'5'} + J_{4'5''}]100/9.7$. [28] [c] Percentage of rotamer (gt + tg) = 100 − %gg.

from the mean plane of the other atoms of the sugar ring. Our analysis gave clues that the furanose ring of 5-methyl-2′-deoxycytidine bromohydrins **1**, **2** and glycols **3**, **4** exists predominantly in a C2′ *endo* conformation (Table 3). In addition, the C2′ *endo* population was enhanced for both the bromohydrin **2** and the glycol **4** which both exhibit a 6*S* configuration. This is inferred from the increase in the $J_{1'2'}$ coupling constant and the decrease in the $J_{2''3'}$ and $J_{3'4'}$ coupling constants for the 6*S* diastereomers **2**, **4**. Similar differences are also observed for various 5,6-dihydrothymidine nucleosides having a hydroxyl group at C6.[22] The conformation of the C5′-exocyclic hydroxymethyl group is usually described in terms of equilibrium between three main low energy rotamers, namely *gauche-gauche* (gg), *trans-gauche* (tg), and *gauche-trans* (gt). The distribution of the staggered conformers about the C4′−C5′ bond was estimated by using the equation %gg = $[13.7 - \sum]100/9.7$ where $\sum$ is the experimental sum $J_{4'5'} + J_{4'5''}$.[28] The population of gg rotamer is predominant and almost similar for all four compounds **1**-**4**. A pronounced downfield shift of H2′ is usually indicative of a *syn* conformation for pyrimidine nucleosides where the carbonyl group of the base moiety is constrained to lie over the sugar ring.[28] The lack of such an effect for both *cis* m^{5}dCyd glycols **3**, **4** is in favor of a predominant *anti* conformation. Confirmation of a preferential *anti* conformation was also provided by NOE experiments. Irradiation of the H6 proton of **3** and **4** gave rise to significant NOE effects on the H1′ and H2′ signals (data not shown) as expected for a nucleoside in a preferential *anti* conformation.

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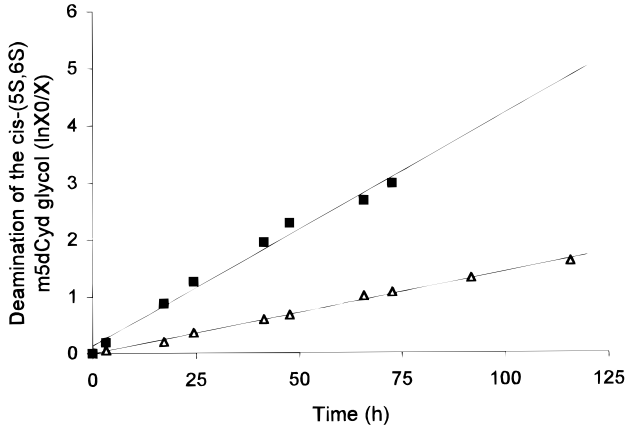

Figure 4. Deamination of the *cis*-(5*S*,6*S*)-5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**4**) in phosphate buffer (pH 7) at 25 °C (△) and 37 °C (■). X0: Initial % of the *cis*-(5*S*,6*S*) m^{5}dC glycol (100%). X: % of the *cis*-(5*S*,6*S*) m^{5}dC glycol.

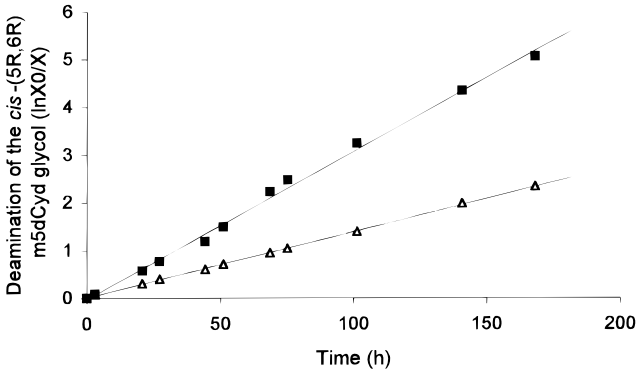

Figure 5. Deamination of the *cis*-(5*R*,6*R*)-5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (**3**) in phosphate buffer (pH 7) at 25 °C (△) and 37 °C (■). X0: Initial % of the *cis*-(5*R*,6*R*) m^{5}dC glycol (100%). X: % of the *cis*-(5*S*,6*S*) m^{5}dC glycol.

Kinetic of Deamination of the Two *Cis* Diastereomers of 5,6-Dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (3) and (4). The deamination study of the *cis* glycols of m^{5}dCyd **3**, **4** was carried out at neutral pH in phosphate buffer at two different temperatures (25 °C and 37 °C). The kinetics of deamination of the latter modified nucleosides was monitored by reverse-phase HPLC analysis by following the decrease in the peak area of the eluting fraction of m^{5}dCyd glycols **3**, **4**. It should be noted that for each of the m^{5}dCyd glycols **3**, **4** no degradation products other than the corresponding *cis* diastereomer of 5,6-dihydroxy-5,6-dihydrothymidine, which is due to deamination, were observed. The linear regression analysis of the data shown in Figures 4 and 5 indicates that the deamination reaction obeys a first order kinetic ($r^2 = 0.993$ at 25 °C and 0.983 at 37 °C for **4** and $r^2 = 0.999$ at 25 °C and 0.996 at 37 °C for **3**). The calculated Arrhenius parameters are $k = (147 \pm 2) \times 10^{-4}$ h^{-1} at 25 °C and $(406 \pm 2) \times 10^{-4}$ h^{-1} at 37 °C for the *cis*-(5*S*,6*S*) glycol diastereomer **4** and $(138 \pm 2) \times 10^{-4}$ h^{-1} at 25 °C and $(309 \pm 4) \times 10^{-4}$ h^{-1} at 37 °C for the *cis*-(5*R*,6*R*) diastereomer **3**. The activation energy E_a is 51.7 ± 1.0 kJ mol^{-1} for **3** and 66.3 ± 1.0 kJ mol^{-1} for **4**. The thermodynamic parameters including the enthalpy of activation ($\Delta H^{\ddagger}$ and the entropy of activation ($\Delta S^{\ddagger}$) were also determined.[23] The results are summarized in Table 4.

The deamination of cytosine derivatives can be described as the hydrolysis of the amino functional group. An increasing interest in the deamination of cytosine is

Table 4. Thermodynamic Parameters of the Deamination Reaction of the Two *Cis*-Diastereomers of (5*R*,6*R*)- and (5*S*,6*S*)-5,6-Dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine (3 and 4)

	(**3**) *cis*-(5*R*,6*R*)		(**4**) *cis*-(5*S*,6*S*)	
	25 °C	37 °C	25 °C	37 °C
E_a (kJ mol^{-1})	51.7 ± 1.0		66.3 ± 1.0	
k (h^{-1})	(138 ± 2) × 10^{-4}	(309 ± 4) × 10^{-4}	(147 ± 2) × 10^{-4}	(406 ± 2) × 10^{-4}
$\Delta H^{\ddagger}$ (kJ mol^{-1})[a]	49.2 ± 1.0	49.1 ± 1.0	63.8 ± 1.0	63.7 ± 1.0
$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	−176 ± 2	−177 ± 2	−127 ± 3	−127 ± 3

[a] $\Delta H^{\ddagger} = E_a - RT$ and $A = (k_B T/h)e^{(1 + \Delta S^{\ddagger})/R}$ where A is the frequency factor derived from Arrhenius plots, whereas K_B and h are the thermochemical Boltzmann and Planck constants, respectively.

correlated with the fact that CpG sites constitute hot spots for mutation in DNA.[14] The deamination process of cytosine and 5-methylcytosine gives rise to uracil and thymine, respectively. Hydrolytic deamination of 5-methylcytosine, which is 3.5 times faster than that of cytosine, results in T/G mismatches.[29] Moreover, Lindahl *et al.*[30] obtained evidence that the rate of deamination of 5-methyldCMP at neutral pH was approximately four times faster than that of dCMP itself. It has been demonstrated that deamination reaction occurs with C5−C6 saturated derivatives of m^{5}Cyt at much higher rates. For instance, 5,6-dihydrocytosine derivatives lose ammonia spontaneously[31,32] in aqueous solution at neutral pH. The latter hydrolytic deamination is likely to explain the presence of the *cis* and *trans* isomers of 5,6-dihydroxy-5,6-dihydrouracil in the γ-irradiated aqueous solution of cytosine.[33] The role of both methylation at C5 position and saturation of the C5−C6 bond was recently investigated with m^{5}dCpT photoproducts. The presence of a methyl group at C5 appears to dramatically decrease the deamination rate of the C5−C6 saturated cytosine photoproducts.[16] It should be noted that both *cis* m^{5}dCyd glycol diastereomers undergo faster deamination than the *cis-syn* cyclobutane m^{5}CpT dimer under similar conditions. This could be explained by both the steric hindrance of the cyclobutane ring and the electron-withdrawing inductive effect of the hydroxyl group at C5 of m^{5}dCyd glycols **3**, **4**. The latter effect is likely to facilitate a nucleophilic attack by water. The *cis*-(5*R*,6*R*) glycol **3** has an activation energy and an activation enthalpy smaller than those of the *cis*-(5*S*,6*S*) diastereomer **4**. On the other hand, the deamination rate is slower for **3**. The strongly negative activation entropy value of **3** is in agreement with a lower activation energy. This involves an ordinate transition state as the result of immobilization of water molecules, a process which is correlated with a decrease in the reaction rate.[34]

The structure of the double-stranded DNA has been shown to protect cytosine residues from deamination by decreasing the accessibility of hydroxyl ions to cytosine residues. Cytosine is deaminated around 140-fold slower when present in the double helix.[35] It is likely that 5-methylcytosine glycols which are more resistant to deamination than cytosine glycols should be stable enough within double stranded DNA.

Conclusion

The synthesis of m^{5}dCyd bromohydrins **1**, **2** and glycols **3**, **4** was optimized. The deamination of the latter nucleosides was avoided by using a relatively mild method of synthesis. Thermodynamic and kinetic parameters of the deamination reaction, including rate constant, enthalpy, and entropy were determined. Using the products isolated previously as standards for HPLC assay, both diastereomeric *cis* glycols of m^{5}dCyd **3**, **4** were detected in the γ-irradiated aerated aqueous solution of m^{5}dCyd. Work is in progress to search for the formation of the *cis* m^{5}dCyd glycols **3**, **4** within DNA. In addition, attempts are currently made to determine if 5,6-dihydroxy-5,6-dihydro-5-methylcytosine is a substrate for the glycosylase action of the endonuclease III repair enzyme.[36−39]

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Supporting Information Available: Copies of ^{1}H and ^{13}C NMR spectra of **1**−**4** (13 pages). This material is contained in libraries on microfiche, immediately follows this article in microfilm version of the journal, and can be ordered from ACS; see any current masthead page for ordering information.

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