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Thymidine Hydroperoxides: Structural Assignment, Conformational Features, and Thermal Decomposition in Water

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Abstract: The primary products of DNA oxidation by free radicals are thymidine hydroperoxides, which include eight diastereomers of 5(6)-hydroxy-6(5)-hydroperoxy-5,6-dihydrothymidine and 5-(hydroperoxymethyl)-2′-deoxyuridine. The hydroperoxides were prepared by trifluoroperacetic acid oxidation of thymidine, which gave the four *trans* and *cis* diastereomers of 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine, and sensitized photooxidation of thymidine with 2-methyl-1,4-naphthoquinone and near-UV light, which gave the four *trans* and *cis* diasteromers of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine as well as 5-(hydroperoxymethyl)-2′-deoxyuridine. ^{1}H and ^{13}C NMR analyses suggested that the pyrimidine ring of thymidine 5,6-hydroxyhydroperoxides adopts four puckered conformations in which the orientations of the C_6 hydroxy or hydroperoxy substituents are predominantly axial. The kinetics of decomposition of 5(6)-hydroxy-6(5)-hydroperoxy-5,6-dihydrothymidine were studied at 22, 37, and 55 °C in ultrapure water. The *cis* diastereomers of each group were generally found to be more stable than the corresponding *trans* diastereomers. The enthalpy (ΔH^*) and entropy (ΔS^*) of decomposition were in the range of 22.9–25.2 kcal mol^{-1} (ΔH^*) and −7.4–+3.7 cal mol^{-1} deg^{-1} (ΔS^*) for 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine and in the range 28.5–35.2 kcal mol^{-1} (ΔH^*) and +9.7–+30 cal mol^{-1} deg^{-1} (ΔS^*) for 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine. The mechanism of decomposition was studied by analysis of stable and intermediate products: the major decomposition products of the *trans* and *cis* diastereomers of 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine were *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylbarbituric acid and N^1-(2-deoxy-β-D-*erythro*-pentofuranosyl-N^3-tartronoylurea in neutral aqueous solutions; in contrast, the *trans* and *cis* diastereomers of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine were observed to undergo isomerization and ultimately decomposed into the 5*R** and 5*S** diastereomers of *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin. On the basis of the above results, the mechanism of decomposition was proposed to involve either dehydration for 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine or ring–chain tautomerism followed by α-cleavage of an intermediate hydroperoxy aldehyde and subsequent ring closure for 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine.

Introduction

Free radical induced oxidation of DNA plays a central role in the deleterious effects of ionizing radiation and similarly appears to contribute to spontaneous and oxidant induced mutagenesis in aerobic organisms.[1] Hydroxy (OH) radicals are likely the main species responsible for oxidative DNA damage in biological

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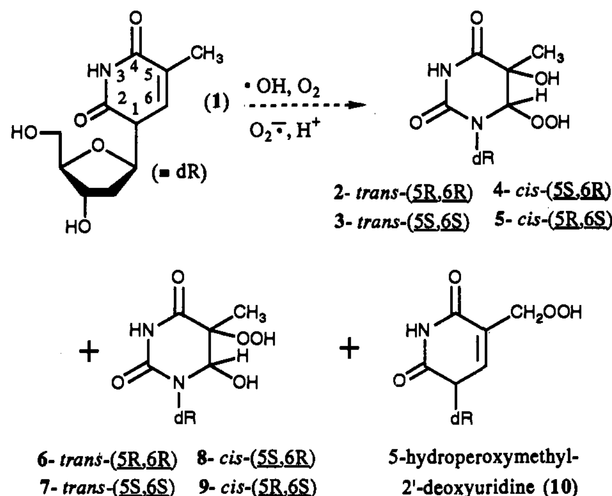
Scheme 1. Formation of Thymidine Hydroperoxides **2–10** by OH Radical Induced Oxidation of Thymidine (**1**) in Aerated Aqueous Solutions



systems. They are generated upon exposure to ionizing radiation by the radiolysis of water. Alternatively, OH radicals may be formed from the reduction of biological peroxides, i.e. H_2O_2, by reactive metal ions, i.e. Fe^{2+} or Cu^+ (the Fenton reaction). The main reaction of OH radicals with DNA involves addition to the C_5 and C_6 positions of cytosine and thymine and to the C_4, C_5, and C_8 positions of guanine and adenine.[2] Also, for the latter substrates, electron abstraction takes place, resulting in the corresponding base radical cations.[2] H-atom abstraction by OH radicals occurs to a minor extent and involves either the 2-deoxy-*β*-*erythro*-pentofuranose moiety (10–15%) or the C_5 methyl group for thymine derivatives (5%). A multitude of stable products has been identified from the radiolysis of aqueous aerated solutions containing uracil, cytosine, 2′-deoxycytidine, thymine, thymidine, and purine derivatives.[3] However, the exact mechanism of OH radical induced decomposition of DNA still remains unclear because of the large number of intermediates and the possible influence of DNA structure and environment on their chemistry.

The addition of OH radicals across the C_5–C_6 pyrimidine double bond of thymidine followed by incorporation of O_2 and subsequent reduction of *β*-hydroxyperoxy radicals leads to the formation of eight diastereomers of thymidine 5,6-hydroxyhydroperoxides (Scheme 1). The transformation of *β*-hydroxyperoxy radicals into the corresponding hydroperoxides is the predominant pathway for the oxidation of pyrimidines within DNA. For example, the yield of intact hydroperoxides immediately following *γ*-irradiation of DNA in aqueous solutions represents as much as 50–70% of the initial yield of OH radicals.[4] In contrast, the yield of hydroperoxides is much lower when DNA monomers are irradiated in solution since intermediate *β*-hydroxyperoxy radicals undergo bimolecular modes of decay. Stable products are then formed by the breakdown of transient tetraoxides. Although thymidine hydroperoxides are the primary products of radical induced DNA oxidation, the chemical properties of these relatively unstable compounds have not been well characterized because of problems related to their synthesis and isolation. We have overcome these problems and herein report the structural features of the eight diastereomers of thymidine 5,6-hydroxyhydroperoxides as well as 5-(hydroperoxymethyl)-2′-deoxyuridine. In addition, the mechanism of decomposition of these compounds has been studied in water by analysis of their thermodynamic parameters and by chemical analysis of the intermediate and stable products of decomposition.

Experimental Section

Materials and Methods. Thymidine (**1**) and reagents were obtained from Sigma (St. Louis, MO) and Aldrich (Milwaukee, WI). High-performance liquid chromatography (HPLC) was performed with dual piston pumps (Model M6000, Millipore, Mildford, MA) equipped with an UV–visible light detector (Model 441, Millipore) or a refractive index detector (Model 401, Millipore). ^{1}H and ^{13}C NMR spectra were recorded in Fourier transform mode using a Bruker AM400 spectrometer equipped with an Aspect 3000 computer (Wissembourg, France). Chemical shifts were calibrated in D_2O with reference to 3-(trimethylsilyl)propionate-2,2,3,3-d_4. ^{1}H NMR spectra were simulated for best fit with a precision of 0.05 Hz by LAOCOON III software. Proton assignments were determined by homonuclear decoupling experiments. Mass spectrometry analyses were carried out on an Hewlett Packard 5988A spectrometer using electron impact (EI), fast atom bombardment (FAB), and thermal spray liquid chromatography (LC) ionization techniques.

Stable Oxidation Products of Thymidine (1). Oxidation products were prepared by reported procedures: *trans*-(5*R*,6*R*)-5-bromo-6-hydroxy-5,6-dihydrothymidine (**13**) and *trans*-(5*S*,6*S*)-5-bromo-6-hydroxy-5,6-dihydrothymidine (**14**);[5a] *trans*-(5*R*,6*R*)-5,6-dihydroxy-5,6-dihydrothymidine (**18**), *trans*-(5*S*,6*S*)-5,6-dihydroxy-5,6-dihydrothymidine (**19**), *cis*-(5*S*,6*R*)-5,6-dihydroxy-5,6-dihydrothymidine (**20**), and *cis*-(5*R*,6*S*)-5,6-dihydroxy-5,6-dihydrothymidine (**21**);[5b] *N*-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylbarbituric acid (**22**);[5c] N^1-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-N^3-tartronoylurea (**23**);[5d] (5*R**)-*N*-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (**24**) and (5*S**)-*N*-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (**25**);[5e] pyruvamide (**26**);[5f] *N*-(2-deoxy-*β*-D-*erythro*-pentofuranosylformamide (**27**;[5g] 5-(hydroxymethyl)-2′-deoxyuridine (**28**) and 5-formyl-2′-deoxyuridine (**29**).[5h]

5-Hydroxy-6-hydroperoxy-5,6-dihydrothymidine (2–5). Thymidine (**1**, 250 mg, 1 mmol) was mixed with 600 μL of 30% (v/v) H_2O_2 and 400 μL of CF_3COOH for 2 h at 22 °C. The solution was lyophilized, and excess H_2O_2 was removed by successive additions of water and pump–dry cycles. The sticky white residue was then dissolved in 500 μL of water and purified by HPLC using an Ultrasphere octadecylsilyl silica gel (ODS) column (7.6-mm i.d. × 25-cm length) and water as the mobile phase. Four hydroperoxide fractions were obtained (k' (capacity factor) = retention volume/(retention volume − column void volume): *trans*-(5*R*,6*R*)-5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**2**) $k' = 4.8$; yield = 5 mg, 1.7%), *trans*-(5*S*,6*S*)-5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**3**) ($k' = 5.2$; yield = 5 mg, 1.7%); *cis*-(5*S*,6*R*)-5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**4**) ($k' = 5.6$; yield = 50 mg, 17%), and *cis* (5*R*,6*S*)-5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**5**) ($k' = 6.8$; yield = 50 mg, 17%). In addition, a novel non-peroxide product was characterized from the reaction mixture: O^6,5′-cyclo-5-hydroxy-5,6-dihydrothymidine (**12**; $k' = 8.5$; yield = 10 mg, 3.2%). ^{1}H NMR (250 MHz, D_2O): δ 6.20 (1H, d, $J = 6.8$ Hz, H1′), 4.79 (1H, s, H6), 4.31 (1H, d, $J = 7.2$ Hz, H3′), 4.18 (1H, s, H4′), 4.00 (1H, d, $J = -12.5$ Hz, H5′), 3.59 (1H, d, $J = -12.5$ Hz, H5″), 2.31 (1H, dd, $J = 7.2, -14.0$ Hz, H2′), 2.10 (1H, dd, $J = 6.8, -14.0$ Hz, H2″), 1.23 (3H, s, 5-CH_3). LC/MS (m/z, relative intensity) 276 (MNH_4^+, 20), 259 (MH^+, 10).

5-Hydroperoxy-6-hydroxy-5,6-dihydrothymidine (6–9) and 5-(hydroxyperoxymethyl)-2′-deoxyuridine (10). Thymidine (**1**, 3.7 g, 14.5 mmol) and 2-methyl-1,4-naphthoquinone (10 mg, 1.2 mmol) were dissolved in 750 mL of water and exposed to near-UV 365-nm light using

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two medium pressure 200-W Hg lamps for 4 h at 4 °C. The solution was well oxygenated and stirred during photolysis by a stream of oxygen. Under these conditions, we obtained about 40% decomposition of thymidine (1). The mixture of oxidation products was separated by HPLC using an Ultraphere ODS column (7.6-mm i.d. × 25-cm length) with 3% aqueous methanol as the mobile phase. Five hydroperoxide fractions were collected: *trans*-(5*R*,6*R*)-5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**6**) (k' = 3.8), *trans*-(5*S*,6*S*)-5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**7**) (k' = 4.5), *cis*-(5*S*,6*R*)-5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**8**) (k' = 7.7), *cis*-(5*R*,6*S*)-5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**9**) (k' = 8.0), and 5-(hydroperoxymethyl)-2′-deoxyuridine (**10**) (k' = 16.7). The yields after purification were about 20 mg of **6** and **7** (1%), 10 mg of **8** and **9** (0.5%), and 50 mg of **10** (2.5%). 5-(Hydroperoxymethyl)-2′-deoxyuridine (**10**): ^{1}H NMR (400 MHz, D_2O) δ 8.04 (1H, s, H6), 6.29 (1H, dd, J = 6.7, 6.5 Hz, H1′), 4.73 (2H, s, 5-CH_2OOH), 4.47 (1H, dd, J = 4.1, 6.7 Hz, H3′), 4.05 (1H, ddd, J = 3.4, 4.1, 4.9 Hz, H4′), 3.85 (1H, dd, J = 4.9, −12.5 Hz, H5′), 3.77 (1H, dd, J = 3.4, −12.5 Hz, H5″), 2.44 (1H, ddd, J = 4.3, 6.7, −14.3, Hz, H2″), 2.40 (1H, ddd, J = 6.5, 6.7, −14.3 Hz, H2′); ^{13}C NMR (100.62 MHz, D_2O) δ 151.6 (C2), 142.9 (C6), 109.2 (C5), 86.8 (C4′), 85.7 (C1′), 71.1 (C3′), 70.4 (CH_3), 61.1 (C5′), 40.0 (C2′).

Decomposition Rates and Thermodynamic Parameters. Decomposition studies were carried out using quintriply distilled (5×) water, with the final two distillation cycles in quartz. The hydroperoxides were carefully purified in 5× water prior to experiments. They were placed in capped glass vials (2.0 mL) which contained either a single hydroperoxide (1.5 mL, 100 μM) or a mixture of four hydroperoxides (1.5 mL, 25 μM each), including mixtures 1 (**4**, **5**, **8**, and **9**), 2 (**2**, **5**, **6**, and **7**), and 3 (**3**, **5**, **7**, and **10**). The samples were kept at room temperature (22 °C) or incubated in a thermostated water bath (±0.1 °C) at 37 and 55 °C for periods of several minutes to 1 week, depending on the temperature. The concentration of hydroperoxide(s) was determined by HPLC-PC analysis at 8–12 time points of decomposition. The hydroperoxides were detected by the formation of a Fe^{3+}–xylenol orange complex in a postcolumn reaction which was monitored on line by its absorption at 540 nm.[6] Using this system, the eight diastereomers of thymidine 5,6-hydroxyhydroperoxides (**2–9**) are well resolved and may be quantitated in the range 100 nmol to 10 pmol. The rates of decay (k_d) were calculated by best fit analysis of the data to a single exponential decay, $A_t = A_0 \exp(-k_d t + c)$, where A_0 and A_t are the concentrations of hydroperoxide at initial time and periods of time thereafter (t), respectively, and where c is a constant. In order to minimize sample to sample variation, the rates of decay for hydroperoxides **2–4** and **6–9** were determined from mixtures of purified hydroperoxides (mixtures 1–3, see above), and the values were normalized to the rate of decay of the reference hydroperoxide (**5**) which was common in each of the mixtures. The rate of decay of **5** was determined precisely in samples containing only this hydroperoxide (n = 10). The activation energy ($\Delta E^{\ddagger}$) and the thermochemical frequency factor (A) were obtained by Arrhenius analysis of the kinetic data ($\ln(k_d) = \ln(A) - (\Delta E^{\ddagger}/RT)$); the enthalpy of activation ($\Delta H^{\ddagger}$) was estimated from $\Delta H^{\ddagger} = \Delta E^{\ddagger} - RT$, where R is the gas constant and T is the temperature in Kelvin, and the entropy of activation ($\Delta S^{\ddagger}$) was taken from the expression $A = kT/h \exp((1 + \Delta S^{\ddagger})/R)$, with T equal to 295 K, where k and h were Boltzman and Planck constants.

Stable Decomposition Products of Thymidine Hydroperoxides. The analysis of the stable products was carried out by two-dimensional thin layer chromatography (2D-TLC) after nearly complete decomposition (four half-lives) of purified [2-^{14}C]-labeled thymidine hydroperoxides in quintriply distilled water at 37 °C. 2D-TLC analysis was performed on silica gel plates (Merck, Darmslodt, Germany) by migration with CH_3Cl:CH_3OH:H_2O (4:2:1, organic phase with 5% CH_3OH) followed at right angles by a second migration with $CH_3CH_2COOCH_3$:$CH_3CH_2OHCH_3$:H_2O (75:16:9). The quantitation of radiolabeled thymidine oxidation products involved autoradiography of the TLC plates followed by recuperation of the radioactive spots and liquid scintillation counting.

Results and Discussion

Synthesis of Thymidine Hydroperoxides. Two complementary routes were necessary for the preparation of thymidine hydroperoxides (**2–10**). The first route involved the oxidation of thymidine (**1**) with trifluoroacetic acid in the presence of H_2O_2

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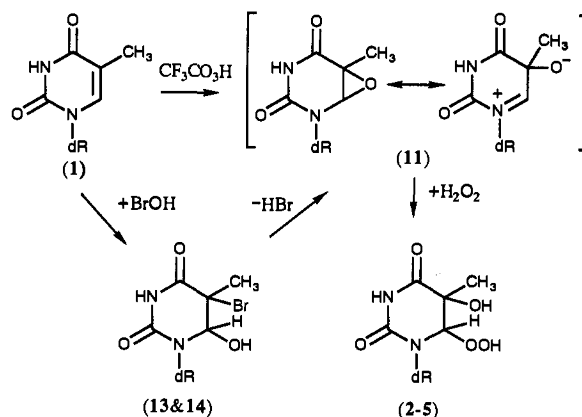
Scheme 2. Preparation of the *Trans* and *Cis* Diastereomers of 5-Hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**2–5**) by Trifluoroperacetic Acid Oxidation of Thymidine (**1**) and by Substitution of Thymidine Bromohydrins **13** and **14** with H_2O_2 in the Presence of Ag_2O



and gave the four *trans* and *cis* diastereomers of 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**2–5**). The formation of these products (**2–5**) as well as O^6,5′-cyclo-5-hydroxy-5,6-dihydrothymidine (**12**) may be explained by intermolecular (H_2O_2) and intramolecular (C_5′-OH) addition of the nucleophile, respectively, to the C_6 position of an intermediate epoxide or zwitterion (**11**) (Scheme 2). The most likely precursor of hydroperoxides **2–5** was the zwitterion (**11**) since the addition of H_2O_2 led to the *cis* products (**4** and **5**) in large excess over the *trans* products (**2** and **3**) with a 10-fold greater yield of the former. Furthermore, the proposed mechanism (Scheme 2) is consistent with the transformation of thymidine bromohydrins into hydroperoxides in the presence of H_2O_2 and Ag_2O (**13** to **4** and **14** to **5**) such that the hydroperoxide group was incorporated exclusively at C_6 of the bromohydrin and the cabon center at C_5 was inverted during the reaction. Previously, on the basis of ^{18}O-labeling experiments, the hydrolysis of thymine bromohydrins into 5,6-glycols was shown to involve transfer of the hydroxy group from the C_6 to the C_5 position of the bromohydrin in aqueous solution.[7a] Thymidine bromohydrins also appear to undergo a similar reaction.[7b] Therefore, since the transformation of thymidine bromohydrins into hydroperoxides gives mainly *cis* products with inversion of C_5, we expect that the predominant reaction pathway involves the addition of H_2O_2 to an intermediate zwitterion (**11**), which is analogous to the epoxidation of thymidine by trifluoroperacetic acid as discussed above.

The photooxidation of thymidine (**1**) by near-UV light and 2-methyl-1,4-naphthoquinone afforded the four *trans* and *cis* diastereomers of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**6–9**) and 5-(hydroperoxymethyl)-2′-deoxyuridine (**10**). The mechanism of photooxidation of thymidine (**1**) has been well studied (Scheme 3).[8] The initial step involves charge transfer from **1** to the excited triplet state of 2-methyl-1,4-naphthoquinone, giving thymidine radical cations (**15**) together with sensitizer radical anions. In aerated aqueous solutions, thymidine radical cations (**15**) undergo two main types of reactions, which include either hydration to 6-hydroxy-5-yl radicals (**16**) or deprotonation to neutral methylene radicals (not shown). The formation of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**6–9**) occurs by the rapid addition of oxygen to intermediate **16** followed by

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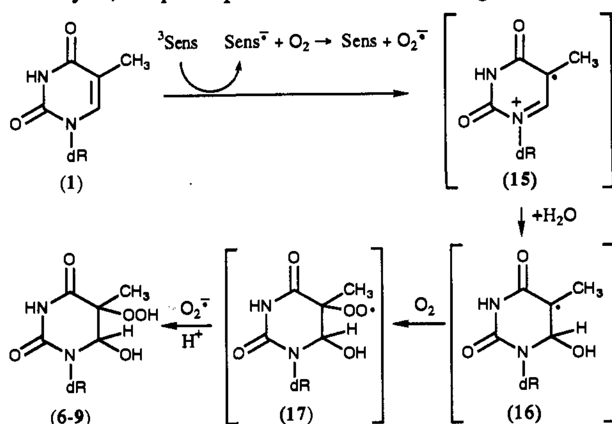
Scheme 3. Preparation of the *Trans* and *Cis* Diastereomers of 5-Hydroperoxy-6-hydroxy-5,6-dihydrothymidine (6–9) by Photooxidation of Thymidine (1) with 2-Methyl-1,4-naphthoquinone and Near-UV Light



reduction of the resulting β-hydroxyperoxy radicals (17). Previously, we have shown that only four of the eight possible diastereomers of thymidine 5,6-hydroxyhydroperoxides including 6–9 are produced by photooxidation.[6a] These studies indicated that water specifically adds to the C_6 position of initial thymidine radical cations (15). It should also be noted that photooxidation does not lead to detectable amounts of thymine or products that arise from modification of the osidic moiety.[8b] These two features greatly simplify the mixture of thymidine oxidation products making it possible to single out thymidine hydroperoxides by chromatography. Alternatively, the radiolysis of aerated aqueous solutions containing thymidine (1) leads to a poorly resolved mixture of oxidation products including hydroperoxides 2–10 as well as similar hydroperoxides of the nucleobase.[3e]

Structural Assignment of Thymidine Hydroperoxides. The structure and stereochemistry of the eight diastereomers of thymidine 5,6-hydroxyhydroperoxides (2–9) were determined by ^{1}H and ^{13}C NMR analyses. ^{1}H NMR spectra of 2–9 in D_2O depicted 11 signals assignable to H_6, CH_3, $H_{1'}$, $H_{2'}$, $H_{2''}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, and $H_{5''}$ of the 2-deoxy-β-D-*erythro*-pentofuranose moiety (Table 1). $H_{2'}$ and $H_{2''}$ protons were assigned from coupling constant arguments.[9a] The lower field component of the $H_{5'}$ and $H_{5''}$ pattern may be attributed to $H_{5'}$, in line with previous studies of nucleosides and nucleotides.[9b,c] ^{13}C NMR analysis of thymidine 5,6-hydroxyhydroperoxides 2–9 depicted 10 carbon signals which were assigned by heteronuclear decoupling experiments (Table 2). The position of the hydroperoxide group was deduced by comparing the amplitudes of the chemical shifts for the α-substituted carbons of 2–9 with those for thymidine 5,6-glycols 18–21. Hence, the presence of an hydroperoxide group at either C_5 or C_6 resulted in about a 10-ppm downfield shift of the attached carbon in comparison to those of the alcohol substituted derivatives. Furthermore, the substition of an hydroperoxide group gave an upfield shift of the associated β-carbons for C_5 of 2–5, C_6 of 6–9, and CH_3 of 6–9 (Table 2). The stereochemistries of thymidine 5,6-hydroxyhydroperoxides 2–9 were based on their ^{1}H NMR spectra with reference to the NMR spectra and crystal structures of thymidine 5,6-glycols 18–21.[10b] The configurations of diastereomers (6*R* or 6*S*) were determined by the coupling constants of $H_{1'}$ and $H_{2'}$, which were about 1 Hz smaller for 6*R* diastereomers (2, 4, 6, and 8) than for 6*S* diastereomers (3, 5, 7, and 9) (Table 1). In addition, the ^{13}C NMR spectra revealed marked differences in the chemical shifts of the CH_3 groups between *trans* and *cis* diastereomers (Table 2). Lastly, the absolute configurations of thymidine 5,6-hydroxyhydroperoxides 2–9 were further supported by their reduction with Zn powder in the presence of acetic acid, which resulted in the corresponding thymidine 5,6-glycols 18–21 with retention of the configurations about C_5 and C_6.

Conformational Analysis of Thymidine Hydroperoxides. The conformations of the furanose ring and exocylic hydroxymethyl group of the 2-deoxy-β-D-*erythro*-pentofuranose moiety were estimated by analysis of the proton coupling constants (Tables 1 and 3). For these analyses, the furanose ring was assumed to reside in a pseudorotational cycle composed of interconverting 2′-*exo*-3′-*endo* and 2′-*endo*-3′-*exo* domains.[9] The analyses indicate that the furanose ring of thymidine 5,6-hydroxyhydroperoxides (2–9) exists predominantly in the 2′-*endo*-3′-*exo* puckered form and that a change in the configuration from 6*S* to 6*R* brings about a shift in the population of 2′-*exo*-3′-*endo* from 26–29% to 35–36%, respectively (Table 3). The exocyclic hydroxymethyl group may be described by three low-energy staggered rotamers in which the orientation of $C_{5'}$-OH is *gauche–gauche* ($g^+(\psi)$), *gauche–trans* ($t(\psi)$), and *trans–gauche* ($g^-(\psi)$) with respect to the O_4'–C_3' axis.[9] The population of $g^+(\psi)$ was similar for six out of the eight diastereomers (3, 5, and 6–9, 40–46%). However, there was a notable difference for the other two diastereomers (2, 33%; 4, 35%) in which the configuration of the hydroperoxide group at C_6 was 6*R*. These findings indicate that the hydroperoxide group at C_6 interacts to some extent with the exocyclic hydroxymethyl moiety by stabilizing the population of $t(\psi)$ at the expense of $g^+(\psi)$. In contrast, the presence of an hydroxy group at the C_6 position did not shift the population of $g^+(\psi)$ rotamers for either 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (6–9, $g^+(\psi)$ = 42–46%) or thymidine 5,6-glycol (18–21, $g^+(\psi)$ = 39–43%).[10a]

Thymidine 5,6-hydroxyhydroperoxides 2–9 are proposed to adopt four preferential conformations in aqueous solution (Figure 1). The CH_3 signal was shifted 5.5 ppm toward higher field for the *trans* diastereomers (2 and 3) in comparison to the corresponding *cis* diastereomers (4 and 5) (Table 2). A similar effect with a difference of about 4 ppm was observed for 6–9. The difference in the chemical shift of the CH_3 group may be attributed to the orientation of the γ-substituted electronegative groups (the γ-effect).[11] Thereby, the C_5-CH_3 substituent for the *trans* diastereomers 2, 3, 6, and 7 likely adopts a *gauche* orientation with respect to the C_6 hydroxy or hydroperoxy groups since maximum deshielding was observed (4–5.5 ppm). Conversely, the CH_3 group for the *cis* diastereomers 4, 5, 8, and 9 is likely *anti* with respect to the γ-substituted hydroxy or hydroperoxy groups. The preferential conformations of hydroperoxides 2–9 are also supported by the chemical shift of the C_4=O group, which was 2–3 ppm farther downfield for *cis* compared to *trans* diastereomers (Table 2). The shift of the C_4=O group to lower ppm may be attributed to H-bonding with the C_5 hydroxy and hydroperoxy groups. Thus, the *cis* diastereomers likely adopt a conformation in which the C_5 electronegative group is equatorial with the pyrimidine ring, i.e. coplanar with the C_4=O group. Alternatively, the C_5 hydroxy or hydroperoxy group must be axial and out of the C_4=O plane for the *trans* diastereomers. The γ-effect and H-bonding suggest that the eight diastereomers of thymidine 5,6-hydroxyhydroperoxides (2–9) adopt four preferential puckered conformations in aqueous solution such that the C_6 hydroxy or hydroperoxy substituents are always axial with respect to the pyrimidine ring while the corresponding C_5 substituents may be either axial or equatorial (Figure 1).

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Table 1. Proton Chemical Shifts and Coupling Constants of Thymidine 5,6-Hydroxyhydroperoxides **2–9**[a]

	proton chemical shifts								
	CH_3	H_6	$H_{1'}$	$H_{2'}$	$H_{2''}$	$H_{3'}$	$H_{4'}$	$H_{5'}$	$H_{5''}$
trans-(5*R*,6*R*)-5-OH-6-OOH (**2**)	1.58	5.32	6.11	2.42	2.28	4.38	3.95	3.76	3.68
trans-(5*S*,6*S*)-5-OH-6-OOH (**3**)	1.59	5.39	6.26	2.43	2.25	4.37	3.93	3.77	3.70
cis-(5*S*,6*R*)-5-OH-6-OOH (**4**)	1.54	5.36	6.04	2.46	2.31	4.39	3.96	3.77	3.69
cis-(5*R*,6*S*)-5-OH-6-OOH (**5**)	1.52	5.43	6.27	2.46	2.26	4.40	3.95	3.77	3.72
trans-(5*R*,6*R*)-5-OOH-6-OH (**6**)	1.56	5.20	6.19	2.31	2.23	4.40	3.93	3.79	3.72
trans-(5*S*,6*S*)-5-OOH-6-OH (**7**)	1.56	5.20	6.21	2.37	2.20	4.40	3.92	3.76	3.71
cis-(5*S*,6*R*)-5-OOH-6-OH (**8**)	1.60	5.40	6.19	2.33	2.26	4.43	3.95	3.80	3.74
cis-(5*R*,6*S*)-5-OOH-6-OH (**9**)	1.59	5.46	6.23	2.44	2.24	4.44	3.95	3.77	3.74
	proton coupling constants								
	$J_{1'2'}$	$J_{1'2''}$	$J_{2'2''}$	$J_{2'3'}$	$J_{2''3'}$	$J_{3'4'}$	$J_{4'5'}$	$J_{4'5''}$	$J_{5'5''}$
trans-(5*R*,6*R*)-5-OH-6-OOH (**2**)	7.1	6.6	−14.0	6.5	4.2	4.0	3.9	5.8	−12.2
trans-(5*S*,6*S*)-5-OH-6-OOH (**3**)	8.4	6.2	−14.0	6.6	3.4	3.5	3.7	5.3	−12.4
cis-(5*S*,6*R*)-5-OH-6-OOH (**4**)	7.0	6.6	−14.0	6.7	4.2	3.9	3.9	5.6	−12.2
cis-(5*R*,6*S*)-5-OH-6-OOH (**5**)	8.1	6.3	−14.1	6.4	3.4	3.2	3.9	5.0	−12.3
trans-(5*R*,6*R*)-5-OOH-6-OH (**6**)	7.4	6.6	−14.0	6.4	4.0	4.0	3.7	5.0	−12.3
trans-(5*S*,6*S*)-5-OOH-6-OH (**7**)	8.6	6.1	−14.0	6.4	3.0	3.0	3.9	4.9	−12.4
cis-(5*S*,6*R*)-5-OOH-6-OH (**8**)	7.5	6.6	−14.1	6.7	4.0	4.1	3.6	4.8	−12.3
cis-(5*R*,*S*)-5-OOH-6-OH (**9**)	8.4	6.2	−14.0	6.3	3.1	3.1	3.9	4.9	−12.3

[a] Obtained by simulation of 400.13-MHz spectra in D_2O. Chemical shifts referenced against 3-(trimethylsilyl)propionate-2,2,3,3-d_4.

Table 2. Carbon Chemical Shifts of Thymidine 5,6-Hydroxyhydroperoxides **2–9** and Thymidine 5,6-Glycols **18–21**[a]

	carbon chemical shifts									
	CH_3	C_2	C_4	C_5	C_6	$C_{1'}$	$C_{2'}$	$C_{3'}$	$C_{4'}$	$C_{5'}$
trans-(5*R*,6*R*)-5-OH-6-OOH (**2**)	17.9	153.1	172.2	69.3	90.5	86.4	37.0	70.8	85.9	61.8
trans-(5*S*,6*S*)-5-OH-6-OOH (**3**)	17.9	153.0	172.0	69.0	88.9	84.5	37.3	70.0	85.6	61.6
cis-(5*S*,6*R*)-5-OH-6-OOH (**4**)	23.4	153.0	175.4	69.9	90.6	86.7[b]	37.3	70.7	86.0	61.7[b]
cis-(5*R*,6*S*)-5-OH-6-OOH (**5**)	23.6	152.8	175.5	69.6	88.7	84.6[b]	37.1	70.9[b]	85.6	61.6
trans-(5*R*,6*R*)-5-OOH-6-OH (**6**)	14.2	152.4	169.2	81.7	76.8	84.9[b]	37.2	70.5	85.5	61.3
trans-(5*S*,6*S*)-5-OOH-6-OH (**7**)	14.1	152.3	169.4	81.8	76.0	84.1[b]	36.7	71.0	85.5	61.7
cis-(5*S*,6*R*)-5-OOH-6-OH (**8**)	17.9	152.4	171.3	81.8	77.1	84.9[b]	37.4	70.5	85.6	61.3
cis-(5*R*,6*S*)-5-OOH-6-OH (**9**)	18.0	151.8	171.4	81.9	76.2	84.3[b]	36.5	71.1	85.6	61.7
trans-(5*R*,6*R*)-5-OH-6-OH (**18**)	18.8	153.4	172.9	71.5	80.4	85.4	37.8	71.2	86.1	62.1
trans-(5*S*,6*S*)-5-OH-6-OH (**19**)	18.9	153.1	172.9	71.6	79.7	84.8	37.6	71.6	86.1	62.5
cis-(5*S*,6*R*)-5-OH-6-OH (**20**)	22.4	153.5	175.9	73.2	80.4	85.6	38.1	71.2	86.2	62.0
cis-(5*R*,6*S*)-5-OH-6-OH (**21**)	22.6	152.9	175.8	73.2	79.5	84.9	37.2	71.9	86.3	62.6

[a] Obtained at 100.62 MHz in D_2O. Chemical shifts referenced against 3-(trimethylsilyl)propionate-2,2,3,3-d_4. [b] Carbon position confirmed by homonuclear proton decoupling.

Likewise, the conformations of the *trans* and *cis* diastereomers of thymidine 5,6-glycols may be deduced from their ^{13}C NMR data (Table 2, **18–21**), which is consistent with the crystal structure of *cis*-(5*R*,6*S*)-thymidine 5,6-glycol.[10b]

Decomposition of Thymidine Hydroperoxides: Intermediate and Stable Products. Peroxide intermediates that occur during the decomposition of thymidine 5,6-hydroxyhydroperoxides **2–9** were monitored by HPLC coupled to the specific detection of peroxides by postcolumn reaction (see the Experimental Section). These studies indicated that thymidine hydroperoxides do not undergo hydrolytic cleavage to give H_2O_2 and thymidine 5,6-glycols in contrast to an earlier report.[4a] More information was obtained by HPLC-PC decomposition analysis of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**6–9**). These hydroperoxides were observed to interconvert in aqueous solution with an efficiency of between 5 and 20% according to ring–chain tautomerism, i.e. *trans*-(5*R*,6*R*) (**6**) interconverted with *cis*-(5*R*,6*S*) (**9**) and *trans*-(5*S*,6*S*) (**7**) interconverted with *cis*-(5*S*,6*R*) (**8**). In addition, a novel peroxide was observed in the decomposition analysis of **8** which did not coelute on HPLC-PC with any of the other thymidine hydroperoxides (**2–9**). The rate of decay of this peroxide was nearly identical with that of **8**. Its retention on reversed-phase HPLC indicated that it was considerably more polar than **8** ((k' = 1.2 compared to k' = 7.8; ODS column, H_2O). These results suggest that the novel peroxide may have either been the open ring tautomer of **8**, an hydroperoxy aldehyde (see **30**, Scheme 5), or the corresponding hemiacetal which arises by the addition of water to the aldehyde. The hemiacetal is the most likely candidate. For example, silyl-protected hydroperoxy aldehydes, which are relatively stable in solution, have been shown to transform into the corresponding hemiacetal upon deprotection in methanol, whereas intermediate hydroperoxy aldehydes were not detected.[12] Similarly, hydroperoxy ketones have been shown to form hemiketals upon decomposition in base or nucleophilic solvents.[13]

The decomposition of each group of four thymidine 5,6-hydroxyhydroperoxides (**2–5** and **6–9**) led to specific stable product distributions. The decomposition of 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**2–5**), labeled with [2-^{14}C], resulted in a major radioactive spot which remained at the origin on 2D-TLC analyses. The percentage of the total radioactivity was 50–53% from the *trans* diastereomers **2** and **3** and 85–90% from the *cis* diastereomers **4** and **5**. Subsequently, the decomposition mixtures of hydroperoxides **4** and **5** were separated into two peaks by HPLC analysis. The fast eluting peak (k' = 0.2; HPLC, ODS column, H_2O) and the slow eluting one (k' = 8.0) were identified as N^1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-N^3-tartronoylurea (**23**) and *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-

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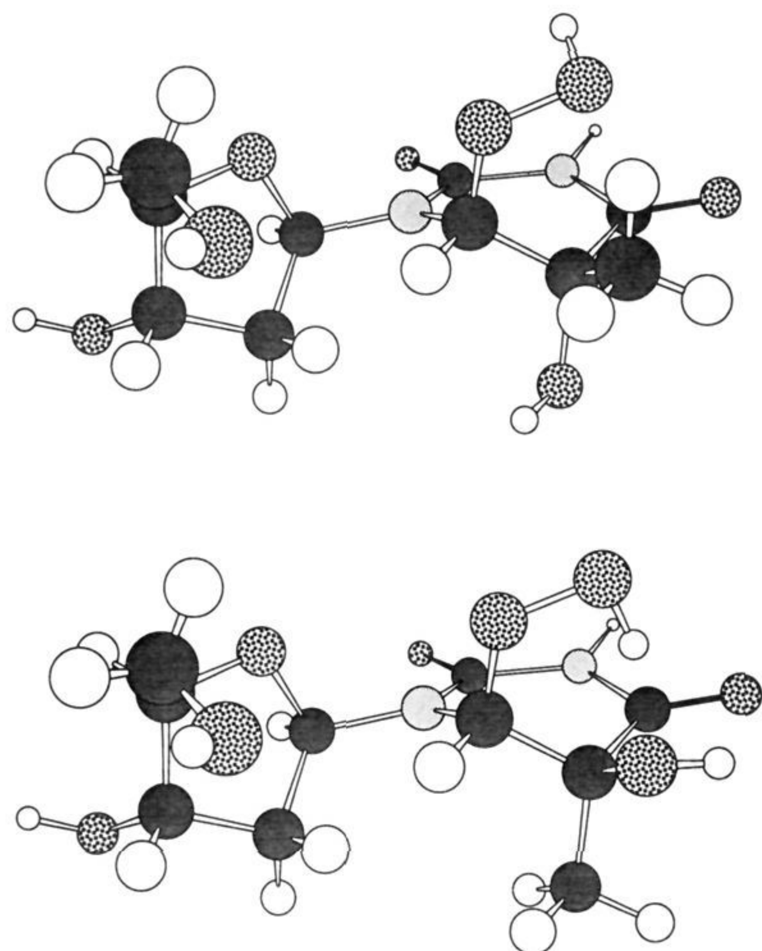


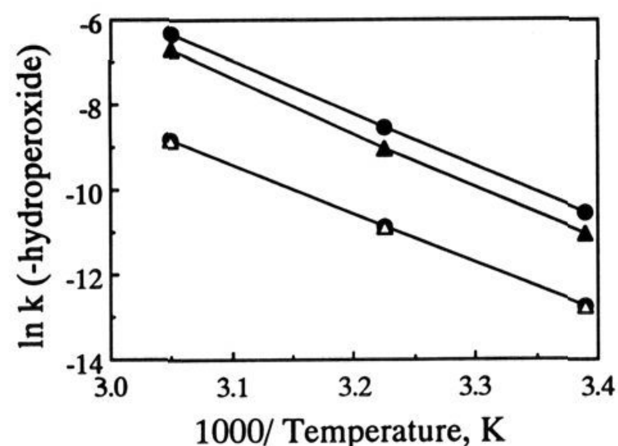
Figure 1. Molecular models of thymidine 5,6-hydroxyhydroperoxides (**2**–**9**). Models are shown for *trans*-(5*R*,6*R*)- (**2**) (top) and *cis*-(5*S*,6*R*)- (**4**) (bottom) 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine. They were constructed from the crystal structure of *cis*-(5*S*,6*R*)-thymidine 5,6-glycol.[10b] The structures of **3** and **5** may be obtained by reflecting the C_5 and C_6 substituents on the pyrimidine plane. The structures of the four diastereomers of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**6**–**9**) are similar except that the hydroxy and hydroperoxy substituents are interchanged.

Table 3. Populations of Furanose Ring and Exocyclic Hydroxymethyl Conformations of the 2-Deoxy-β-D-*erythro*-pentofuranosyl Moiety of Thymidine 5,6-Hydroxyhydroperoxides **2**–**9**[a]

population	% 2′-exo-3′-endo	% g⁺ (ψ)	% *t* (ψ)	% g⁻ (ψ)
trans-(5*R*,6*R*)-5-OH-6-OOH (**2**)	36	33	43	24
trans-(5*S*,6*S*)-5-OH-6-OOH (**3**)	29	40	38	22
cis-(5*S*,6*R*)-5-OH-6-OOH (**4**)	36	35	41	24
cis-(5*R*,6*S*)-5-OH-6-OOH (**5**)	28	41	35	24
trans-(5*R*,6*R*)-5-OOH-6-OH (**6**)	35	43	35	22
trans-(5*S*,6*S*)-5-OOH-6-OH (**7**)	26	42	34	24
cis-(5*S*,6*R*)-5-OOH-6-OH (**8**)	35	46	33	21
cis-(5*R*,6*S*)-5-OOH-6-OH (**9**)	27	42	34	24

[a] Percentages: % 2′-*exo*-3′-endo = 100 × $J_{3'4'}/J_{1'2'} + J_{3'4'}$. % $g^+(\psi)$ = 13.75 − ($J_{4'5'} + J_{4'5''}$)/10.65. % $t(\psi) = J_{4'5''} - 1.5/10$. % $g^-(\psi)$ = % $g^+(\psi)$ − % $t(\psi)$.[10]

5-hydroxy-5-methylbarbituric acid (**22**), respectively, by comparison of their ^{1}H NMR and MS spectra with those of authentic compounds. Furthermore, the initial product of decomposition of hydroperoxides **2**–**5** appeared to be **22** since it was observed to convert quantitatively into **23** when left at room temperature for several hours in neutral aqueous solution. The conversion of **22** into **23** is consistent with previous studies.[5d] Several other decomposition products of hydroperoxides **2**–**5** were also detected by 2D-TLC and HPLC analyses. Thymidine 5,6-glycols **18**–**21** represented 34–40% of the total radioactivity from the decomposition of **2** and **3**, whereas the yield was only 5–7% from the decomposition of **4** and **5**. Trace amounts of pyruvamide (**26**) were also detected (<2%). In contrast, the decomposition of the *trans* and *cis* diastereomers of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**6**–**9**), labeled with [2-^{14}C], resulted in the appearance of two major well-separated spots by 2D-TLC analysis with equal intensity and a combined radioactivity of 75%. These



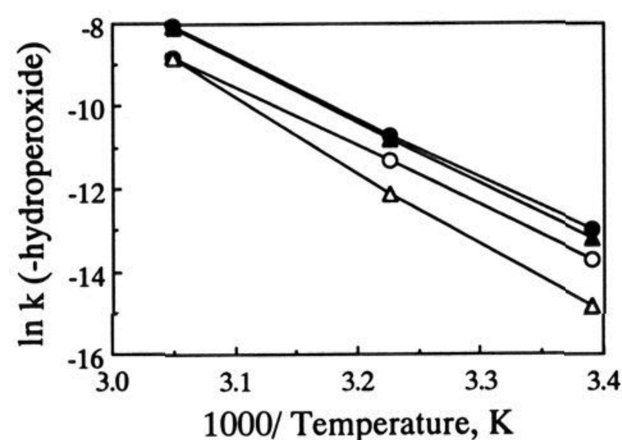


Figure 2. Arrhenius plots of the thermal decomposition of thymidine 5,6-hydroxyhydroperoxides (**2**–**9**). Top frame shows the effect of temperature on the decomposition of 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**2**–**5**): solid circles, **2**; solid triangles, **3**; open circles, **4**; open triangles, **5**. Lower frame shows the decomposition of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**6**–**9**): solid circles, **6**; solid triangles, **7**; open circles, **8**; open triangles, **9**.

products were identified by ^{1}H NMR analysis as the 5*R** and 5*S** diastereomers of *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (**24** and **25**). The remainder of the radioactivity may be divided up among other well-characterized oxidation products of thymidine (**1**) including the thymidine 5,6-glycols (**18**–**21**, 4-11%), pyruvamide (**26**, 7–11%), and *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)formamide (**27**, detected by cysteine spray reagent). Therefore, the thermal decomposition of thymidine 5,6-hydroxyhydroperoxides **2**–**9** involves two major pathways: hydroperoxides **2**–**5** decompose into product **22** which then transforms into **23**, whereas hydroperoxides **6**–**9** decompose into products **24** and **25**. These results sharply contrast previous studies with three isomers of thymine 5,6-hydroxyhydroperoxides in which the yields of thymine 5,6-glycols were much higher, 50–70%.[3d] However, the extensive formation of 5,6-glycols in these studies may likely be attributed to metal-catalyzed reactions since no precautions were taken to eliminate metal ions from the decomposition solutions.

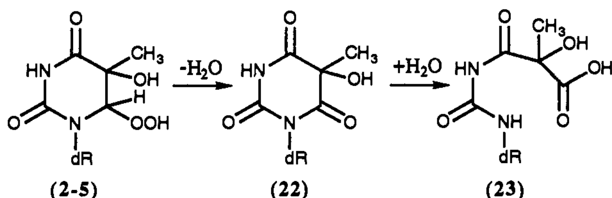
Decomposition of Thymidine Hydroperoxides: Kinetics and Thermodynamics. The rates of decomposition for thymidine 5,6-hydroxyhydroperoxides **2**–**9** were examined in ultrapure water at 22, 37, and 55 °C, from which the half-lives, enthalpies ($\Delta H^{\ddagger}$) and ($\Delta S^{\ddagger}$) were calculated (Figure 2, Table 4). The four diastereomers of 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**2**–**5**) displayed a notable difference in their stability with the *cis* diastereomers (**4** and **5**) being more stable by 5–12-fold than the *trans* diastereomers (**2** and **3**). The order of stability for hydroperoxides **2**–**5** (*cis* > *trans*) appeared to be related to the entropy of activation since it was more negative by about 10 cal mol^{-1} deg^{-1} for *cis* diastereomers than for *trans* diastereomers, whereas the enthalpy of activation was significantly lower by about 2 kcal mol^{-1} for the *cis* diastereomers (Table 4). The differences in stability between the *trans* and *cis* diastereomers may be attributed to differences in their structure. Within *cis* diastereomers **4** and **5**, the C_5 hydroxy groups are equatorial and

Table 4. Thermodynamic Parameters Describing the Thermal Decomposition of Thymidine 5,6-Hydroxyhydroperoxides **2–9**

thermodynamic parameters	half-life, h[a] 22 °C	37 °C	55 °C	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, cal/mol deg
trans-(5*R*,6*R*)-5-OH-6-OOH (**2**)	7.3	1.0[b]	0.11	24.5 ± 0.3	+2.3 ± 1.2
trans-(5*S*,6*S*)-5-OH-6-OOH (**3**)	12	1.6	0.16	25.2 ± 0.4	+3.7 ± 1.2
cis-(5*S*,6*R*)-5-OH-6-OOH (**4**)	66	10.4	1.30	22.9 ± 0.6	−7.4 ± 1.9
cis-(5*R*,6*S*)-5-OH-6-OOH (**5**)	66	10.4	1.30	22.9 ± 0.3	−7.4 ± 0.9
trans-(5*R*,6*R*)-5-OOH-6-OH (**6**)	85	8.5	0.60	28.9 ± 0.9	+12.4 ± 2.8
trans-(5*S*,6*S*)-5-OOH-6-OH (**7**)	104[b]	9.6	0.62	29.9 ± 0.8	+15.3 ± 2.3
cis-(5*S*,6*R*)-5-OOH-6-OH (**8**)	172[b]	16.4	1.30	28.5 ± 1.5	+9.7 ± 4.9
cis-(5*R*,6*S*)-5-OOH-6-OH (**9**)	542[b]	34.6	1.30[b]	35.2 ± 1.5	+30.0 ± 4.7

[a] Errors in the range of 3–8%. [b] Error is 12–15%.

Scheme 4. Principle Mechanism of Decomposition of the *Trans* and *Cis* Diastereomers of 5-Hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**2–5**)

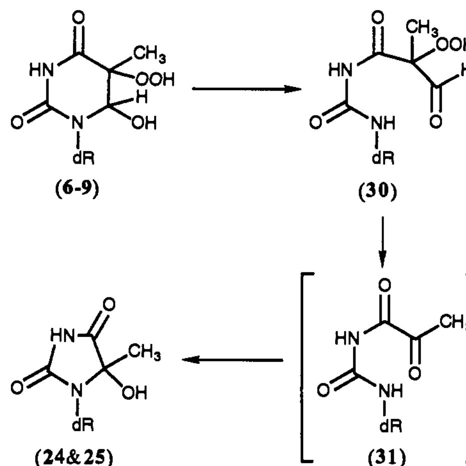


the C_6 hydroperoxy groups are axial which would allow for the formation of a strong intramolecular H-bond (closest contact was 2.1 Å, as in Figure 1). Alternatively, the C_5 hydroxy and C_6 hydroperoxy groups may not form an intramolecular bond within *trans* diastereomers **2** and **3** because the groups are diaxial. Therefore, we may explain the greater stability of *cis* diastereomers in thermodynamic terms by an increase in the solvation of the molecule in the ground state, which would lead to a greater demand for entropy in the transition state.

In comparison to hydroperoxides **2–5**, the decomposition of **6–9** was characterized by a higher enthalpy of activation (>4 kcal mol^{-1}) and a more positive entropy of activation (>10 cal mol^{-1} deg^{-1}) (Table 4). The contrasting thermodynamic parameters support different pathways of decomposition for the two groups of hydroperoxides. In particular, the relatively large positive entropies of activation for **6–9** imply that their decomposition involves cleavage of the 5,6-saturated pyrimidine ring. Indeed, the decomposition of hydroperoxides **6–9** may be compared to the isomerization of thymidine 5,6-glycols, which involves ring–chain tautomerism. For example, the *cis* diastereomers of thymidine 5,6-glycols are thermodynamically the most stable forms in aqueous solution at room temperature, i.e. *trans* and *cis* diastereomers form an equilibrium with a ratio of about 1 to 4, respectively.[10] Also, the equilibrium favors the 6*S* diastereomers over the 6*R* diasteromers, suggesting that the former are more stable. Thus, the stabilities of thymidine 5,6-glycols at room temperature are similar to those of thymidine 5,6-hydroxyhydroperoxides **6–9**, *cis*-(5*R*,6*S*) (**9**) > *cis*-(5*S*,6*R*) (**8**) > *trans*-(5*S*,6*S*) (**7**) > *trans*-(5*R*,6*R*) (**6**), estimated from their half-lives at 22 °C (Table 4). The difference in stability of the *cis* diastereomers, however, was remarkable for thymidine hydroperoxides **8** and **9**. The *cis*-(5*R*,6*S*) diastereomer (**9**) was more stable than the *cis*-(5*S*,6*R*) diastereomer (**8**) by about 3-fold at 22 °C and 2-fold at 37 °C. Furthermore, the yields of the *trans* diastereomers during decomposition of the *cis* diastereomers were about twice as high for the unstable diastereomer (**8**) than for the more stable diastereomer (**9**). These results suggest that the difference in stability between hydroperoxides **8** and **9** is closely related to the ability of each diastereomer to undergo ring–chain tautomerism.

Mechanism of Decomposition of Thymidine Hydroperoxides. We propose two main pathways for the decomposition of thymidine 5,6-hydroxyhydroperoxides **2–9** in water (Schemes 4 and 5). The major products of the *trans* and *cis* diastereomers of 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**2–5**) were

Scheme 5. Principle Mechanism of Decomposition of the *Trans* and *Cis* Diastereomers of 5-Hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**6–9**)



N-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylbarbituric acid (**22**) and N^1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-N^3-tartronoylurea (**23**). The formation of these products is consistent with initial dehydration of the C_6 hydroperoxide group giving rise to **22**, which undergoes subsequent hydrolysis into **23** (Scheme 4). In contrast, the major products of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**6–9**) were the 5*R** and 6*S** diastereomers of *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (**24** and **25**). The formation of these products likely involves a sequence of steps which includes ring–chain tautomerism of the 5,6-saturated pyrimidine ring, followed by α-cleavage of the open chain hydroperoxide and subsequent ring closure (Scheme 5). The existence of the intermediate hydroperoxy aldehyde (**30**) was supported by HPLC-PC which indicated that the *trans* and *cis* diastereomers interconvert according to ring–chain tautomerism. We expect that the decomposition of the hydroperoxy aldehyde (**30**), or the corresponding hemiacetal, takes place by α-cleavage in agreement with studies of alkyl hydroperoxy ketones.[13] This would lead to N^1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-N^3-pyruvoylurea (**31**). Ring closure of **31** then gives the final products **24** and **25**. Previously, the precursor of hydantoin oxidation products from pyrimidines was suggested to involve 5,6-cleavage of intermediate 5,6-hydroxyhydroperoxides, leading to a dicarbonyl intermediate which cyclizes by intramolecular nucleophilic addition followed by loss of formic acid.[14] However, the formation of N_1-substituted hydantoin derivatives **24** and **25** by a similar pathway is not likely because the additional substitution at N_1 would inhibit ring closure of the 5,6-cleavage dicarbonyl intermediate; rather further fragmentation overwhelms this mode of decomposition.[5f]

Analysis and Decomposition of 5-(Hydroperoxymethyl)-2′-deoxyuridine. The structure of 5-(hydroperoxymethyl)-2′-deoxy-

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uridine (**10**) was determined by ^1H and ^{13}C NMR analyses. The associated downfield proton and carbon shifts of the pyrimidine methylene group in comparison with those of 5-(hydroxymethyl)-2′-deoxyuridine (**28**) are consistent with the presence of an hydroperoxide group at this position. The half-life of decomposition of **10** in aqueous solution was about 40-fold greater than that of the most stable thymidine 5,6-hydroxyhydroperoxides (**9**). The main stable product of decomposition was 5-(hydroxymethyl)-2′-deoxyuridine (**28**, >90%); in addition, traces of 5-formyl-2′-deoxyuridine (**29**) were detected.

Fate of Thymidine Hydroperoxides in DNA. The yield of DNA hydroperoxides has been shown to be 50–70% of the total base damage immediately following γ-irradiation of DNA in aqueous aerated solutions.[4] Intact DNA hydroperoxides may be located on either thymine or cytosine residues, although they are unlikely to involve purines which, during radical oxidation, consume relatively low amounts of O_2 and do not form stable hydroperoxides.[15] Therefore, hydroperoxides are the principal intermediates of the free radical induced oxidation of pyrimidines in DNA. The decomposition of intact DNA hydroperoxides has been studied for the complete mixture of hydroperoxides derived from both cytosine and thymine residues. The decay exhibited exponential kinetics with fast (half-life of 1 h, 22 °C) and slow (half-life of 100 h, 22 °C) components which constituted 35 and 65% of the total hydroperoxides, respectively.[4a] The half-life of the slow component agrees well with the half-life of a mixture of thymidine 5,6-hydroxyhydroperoxides **2–9** estimated to be 91 h at 22 °C using our data (Table 4) and the relative yields of each diastereomer from the radiolysis of aerated aqueous solutions of thymidine.[3e] Thus, the slow component likely represents the decay of thymidine hydroperoxides in DNA. More importantly, the data imply that the mechanism of decomposition is the same for thymidine hydroperoxides which are attached to DNA or which are free in solution. Therefore, we may propose that *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylbarbituric acid (**22**), *N*[1]-(2-deoxy-β-D-*erythro*-pentofuranosyl)-*N*[3]-tartronoylurea (**23**), and *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-methyl-5-hydroxyhydantoin (**24** and **25**) are the major stable products of DNA when it is oxidized by OH radicals under aerated aqueous conditions. The relative yields of these products, however, have been difficult to determine because of limitations in detecting them from DNA samples. In a series of studies, 5-hydroxy-5-methylhydantoin, though, appears to be a major oxidation product from DNA exposed to a mixture of H_2O_2 and reactive metal ions.[16]

The reaction of metal ions with thymidine hydroperoxides may also be important when considering the fate of these intermediates, for DNA in solution or in cellular DNA. For example, the addition of μM Fe^{2+} to γ-irradiated DNA immediately reduces the level of intact hydroperoxides by about 50%, probably leading to the formation of 5,6-glycols.[16,17] In contrast, the half-life of 5-(hydroperoxymethyl)-2′-deoxyuridine (**10**) was reduced to hours at room temperature in the presence of various metal ions, Fe^{2+}, Cu^+, and their complexes, although the concentration required was greater than or equal to 250 μM.[18] The distribution of stable products **28** and **29** was dependent on the type, valence, and chelation of the metal ions. Thus, redox active metal ions may dramatically affect the fate of thymidine hydroperoxides in DNA. However, the relatively long half-life of DNA hydroperoxides suggests that metal ions which are bound to DNA have a reduced reactivity with respect to those which are "added" or free in solution. Clearly, more studies will be required to determine the exact role of metal ions in the fate of intact hydroperoxides within naked and cellular DNA.

Conclusions

Thymidine hydroperoxides are primary free radical products of thymidine in aerated solutions. They consist of eight diastereomers of 5(6)-hydroxy-6(5)-hydroperoxy-5,6-dihydrothymidine (**2–9**) as well as 5-(hydroperoxymethyl)-2′-deoxyuridine (**10**). Previously, the complete characterization of these relatively unstable products was not possible because of difficulties in their preparation and purification. In the present work, we report the preparation of thymidine hydroperoxides using mild methods for their synthesize in combination with HPLC separation to obtain homogeneous samples. These methods have set the stage for the detailed chemical analyses of thymidine hydroperoxides: the stereochemistries and conformations of **2–9** were determined by ^1H and ^{13}C NMR analyses; the decompositions of **2–9** were investigated by the analysis of intermediate products, stable products, and thermodynamic parameters. On the basis of these results, we have proposed two major pathways of thermal decomposition in water. The four diastereomers of 5-hydroxy-6-hydroperoxy-5,6-dihydrothymidine (**2–5**) decompose by dehydration of the secondary hydroperoxide to give *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)barbituric acid (**22**) which then undergoes hydrolysis into *N*[1]-(2-deoxy-β-D-*erythro*-pentofuranosyl)-*N*[3]-tartronoylurea (**23**), whereas the four diastereomers of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (**6–9**) decompose by ring–chain tautomerism, α-cleavage of an intermediate hydroperoxy aldehyde, and ring closure to give *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (**24** and **25**). These studies have shed light on the chemical properties of unique intermediates in free radical reactions, β-hydroxy hydroperoxides, and thereby have provided new insights into the complex mechanism of oxidative DNA damage.

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