Radiation-Induced and Photosensitized Splitting of C5−C5′-Linked Dihydrothymine Dimers: Product and Laser Flash Photolysis Studies on the Oxidative Splitting Mechanism

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Radiation-induced and photosensitized one-electron oxidation of stereoisomeric C5−C5′-linked dihydrothymine dimers (**1a**,**b**[***meso***], meso compound of (5*R*,5′*S*)-bi-5,6-dihydrothymine; **1a**,**b**[***rac***], racemic compound of (5*R*,5′*R*)- and (5*S*,5′*S*)-bi-5,6-dihydrothymines), which are the major products yielded by radiolytic reduction of 1-methylthymine (**2a**) and 1,3-dimethylthymine (**2b**) in aqueous solution, was studied to compare with the photoreactivating repair mechanism of cyclobutane pyrimidine photodimers. Reacting with sulfate radical anion ($SO_4^{\bullet -}$), azide radical ($N_3^{\bullet}$), or photoexcited anthraquinone-2-sulfonate (AQS) as oxidants, the C5−C5′-linked dihydrothymine dimers **1a**,**b** split to regenerate the corresponding thymine monomers **2a**,**b** along with 5,6-dihydrothymines (**3a**,**b**) in a pH dependent manner. The transient absorption spectra of 5,6-dihydrothymin-5-yl radicals (**6a**,**b**) were observed in the nanosecond laser flash photolysis of **1a**,**b** in phosphate buffer under conditions of $SO_4^{\bullet -}$ generation. Both the product study and the laser flash photolysis study indicated an oxidative splitting mechanism by which one-electron oxidation of the C5−C5′-linked dimers **1a**,**b** produces the radical cation intermediates (**4a**,**b**), which undergo facile fragmentation into 5,6-dihydrothymin-5-yl radicals **6a**,**b** and C5-cations (**5a**,**b**), followed by deprotonation at C6 of **5a**,**b** to regenerate the monomers **2a**,**b**.

Introduction

It has been established that DNA is a vital target of genetoxic agents such as a variety of chemical oxidants, ionizing radiation, UV light, and certain antibiotics, that cause chemical modification in DNA.[1] For certain structures of the DNA toxic lesions a number of repair enzymes in the cells have been identified and characterized with respect to their structures and biological functions. Most typically, excision−repair enzymes recognize and excise damaged bases or oligo sequences containing damaged base sites of DNA and then replace them with normal DNA bases by the aid of DNA polymerase.[2] There are also a different class of direct in situ repair enzymes that regenerate normal bases without excising the damaged bases or the DNA backbone. For example, DNA photolyase ($M_r = 55000-65000$) absorbs UV−visible light and thereby catalyzes a redox splitting of the cyclobutane pyrimidine photodimers, which are well-known to be highly mutagenic and carcinogenic lesions induced by UV exposure.[3,4] This enzyme utilizes a deprotonated reduced chromophore ($FADH^-$) of flavin adenine dinucleotide and an antenna chromophore of methyltetrahydrofolate (MTHF) or 8-hydroxy-5-deazaflavin (8-HDF). Thus, in the binding of DNA photolyase to DNA as a light-independent reaction, the cofactor MTHF or 8-HDF absorbs visible light and transfers energy to form the electronically excited state of $FADH^-$ ($FADH^{-*}$), which in turn transfers an electron to the cyclobutane pyrimidine photodimer. Concerted splitting at the C5−C5′ and C6−C6′ bonds of the resulting photodimer radical anion and successive electron transfer back to the flavin reproduce the monomeric pyrimidines.[4a] As demonstrated by photochemical and radiation chemical studies, the splitting reaction of photodimers could also occur by a one-electron oxidation mechanism involving appropriate oxidants such as sulfate radical anion ($SO_4^{\bullet -}$), OH radical ($^{\bullet}OH$),[5] and photoexcited anthraquinone-2-sulfonate (AQS*).[6] The oxidative splitting mechanism is thought to be distinct from the one-electron reduction mechanism,[4,7,8] by which the photodimer radical cation undergoes facile splitting at the C6−C6′ bond in the initial step and successively at the C5−C5′ bond. Such a mechanistic difference in the splitting of photodimers has been accounted for by recent computational studies based on the semiempirical AM1 and ab initio HF and MP2 methods.[9]

In the course of our study on the DNA base damaged structures induced by radiolytic reduction, we have isolated and identified a novel C5−C5′-linked dihydrothymine dimers, the meso compound of (5*R*,5′*S*)-bi-5,6-dihydrothymine (**1a**,**b**[***meso***]) and the racemic compound of (5*R*,5′*R*)- and (5*S*,5′*S*)-bi-5,6-dihydrothymines (**1a**,**b**[***rac***]).[10,11] Interestingly, the three-dimensional structures of these C5−C5′-linked dimers as characterized by X-ray crystallography are similar to the cyclobutane thymine photodimers possessing both C5−C5′- and C6−C6′-bonds. This implied that the C5−C5′-linked dihydrothymine dimers may cause some distortion within a DNA duplex if they were incorporated and could be substrates for DNA photolyase. Our attention has been focused on the splitting of the C5−C5′-linked dihydrothymine dimers into thymine

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monomers to get structural insight into a redox mechanism, compared with the well-characterized fragmentation reactivity of the C5−C5′ and C6−C6′ bonds of pyrimidine photodimers. This paper reports radiation-induced and photosensitized one-electron oxidation of the stereoisomeric C5−C5′-linked dimers of 1-methylthymine (**1a**[***meso***], **1a**[***rac***]) and 1,3-dimethylthymine (**1b**[***meso***], **1b**[***rac***]) by several oxidizing species such as sulfate radical anion $SO_4^{\bullet-}$, azide radical ($N_3^{\bullet}$), and photoexcited anthraquinone-2-sulfonate AQS*. Along with the product study, a laser flash photolysis study was also performed to observe the transient intermediates involved in the oxidative splitting of C5−C5′-linked dihydrothymine dimers under conditions of photochemical $SO_4^{\bullet-}$ generation.

Experimental Section

Materials. 1-Methylthymine (**2a**) (Sigma Chemical) was used as received. Purified 1,3-dimethylthymine (**2b**) was kindly supplied by Fujii Memorial Research Institute, Otsuka pharmaceutical. Anthraquinone-2-sulfonate (AQS), potassium peroxodisulfate ($K_2S_2O_8$), potassium bromide (KBr), sodium azide (NaN_3), and 2-methyl-2-propanol were purchased from Nacalai Tesque and were used without further purification. Reagents for high-performance liquid chromatography (HPLC) including solvents, sodium dihydrogen phosphate (NaH_2PO_4), and methanol (HPLC grade) were used as received from Wako Pure Chemical Industries. C5−C5′-linked dihydrothymine dimers (**1a**,**b**) of 1-methylthymine and 1,3-dimethylthymine were synthesized and purified by following the methods reported previously.[10,11]

Radiolytic Oxidation. Aqueous solutions of C5−C5′-linked dihydrothymine dimers (**1a**,**b**: 0.58 mM) containing [1] 2-methyl-2-propanol (50 mM) and $K_2S_2O_8$ (5.0 mM), [2] KBr (100 mM), or [3] NaN_3 (50 mM) were prepared with water ion-exchanged by Corning Mega-Pure System MP-190 (>16 MΩ cm). The aqueous solutions were buffered at pH 4, 7, and 10 with phosphate buffer (2 mM) and sodium hydroxide and then purged with [1] Ar or [2, 3] N_2O before γ-irradiation. Steady-state γ-irradiation was performed in a sealed ampule at room temperature with a ^{60}Co γ-ray source (dose rate: 0.79−0.70 Gy min^{-1}). The γ-irradiated sample solutions, which were stable at room temperature in the dark, were subjected to HPLC analysis as described below.

Photosensitized Oxidation. Typically, solutions of **1a**,**b** (1 mM) in phosphate buffer (5.0 mM) containing AQS (0.4 mM) were adjusted to pH 4, 7, and 10 and then purged with Ar before photoirradiation. The solutions in sealed Pyrex glass tubes (λ_{ex} > 280 nm) were photoirradiated under magnetic stirring (1000 rpm) at 24 °C with a high-pressure Hg arc (450 W, Eiko-sha 400).

HPLC Analysis. HPLC analyses were performed with Shimadzu 3A and 10A HPLC systems equipped with Rheodyne 7725 sample injector. Aliquots (10 μL) of irradiated sample solutions were injected onto a 5 μm C18 reversed-phase column (Wakosil 5C$_{18}$, ∅ 4.6 mm × 150 mm, Wako). The phosphate buffer solutions (10 mM, pH 3.0) containing various concentrations of methanol (15−25 vol %) were delivered as the mobile phase. The column eluents were monitored by the UV absorbance at 210 nm.

Nanosecond Laser Flash Photolysis. The laser flash photolysis experiments were carried out with a Unisoku TSP-601 flash spectrometer. A Continuum Surelite-I Nd:YAG (Q-switched) laser with the fourth harmonic at 266 nm (ca. 50 mJ per 6 ns pulse) was employed for the flash photoirradiation. The probe beam from a Hamamatsu 150 W xenon short arc (CA 263) was guided with an optical fiber scope to be arranged in an orientation perpendicular to the exciting laser beam. The probe beam was monitored with a Hamamatsu R2949 photomultiplier tube through a Hamamatsu S3701−512Q MOS linear image sensor (512 photodiodes). Timing of the exciting pulsed laser, the probe beam, and the detection system was achieved through a Tektronix model TDS 320 double channel oscilloscope that was interfaced to an NEC PC-9801 computer. Solutions of **1a**[***meso***] and **1a**[***rac***] (1.0 mM) at pH 4, 7, and 10 in phosphate buffer (5.0 mM) containing $K_2S_2O_8$ (50 mM) were deaerated by Ar bubbling prior to the laser flash photolysis experiments.

Results and Discussion

Splitting of C5−C5′-Linked Dihydrothymine Dimers by Oxidizing Radicals Generated in the Radiolysis. The oxidizing sulfate radical anion ($SO_4^{\bullet-}$), as generated from the reaction of peroxodisulfate ion ($S_2O_8^{2-}$) with hydrated electron (e_{aq}^-) in the aqueous radiolysis system, has been conveniently utilized for investigating the one-electron oxidation reactivity of pyrimidines and purines.[12] This radiation chemical method was employed in the present study to get mechanistic insight into one-electron oxidative splitting of C5−C5′-linked dihydrothymine dimers (**1a**,**b**[***meso***] and **1a**,**b**[***rac***]). Thus, Ar-saturated solutions of **1a**,**b**[***meso***] and **1a**,**b**[***rac***] (0.5 mM) in phosphate buffer containing 2-methyl-2-propanol (50 mM) and $K_2S_2O_8$ (5.0 mM) were γ-irradiated up to 800 Gy. In the radiolysis of aqueous solution, strongly oxidizing hydroxyl radicals ($^{\bullet}$OH) and reducing species of e_{aq}^- are generated along with a smaller amount of reducing hydrogen atoms ($^{\bullet}$H) (reaction 1). The G

$$H_2O \rightarrow {}^{\bullet}OH + {}^{\bullet}H + e_{aq}^- \quad (1)$$

$$^{\bullet}OH + (CH_3)_3COH \rightarrow H_2O + {}^{\bullet}CH_2(CH_3)_2COH \quad (2)$$

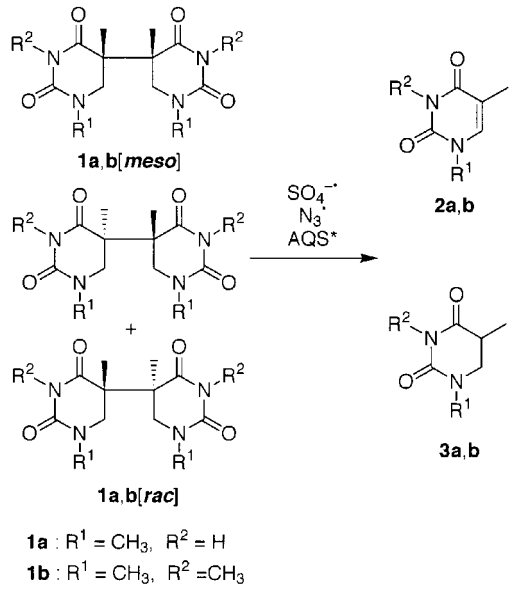
$$e_{aq}^- \ ({}^{\bullet}H) + S_2O_8^{2-} \rightarrow SO_4^{\bullet-} + SO_4^{2-} \ (HSO_4^-) \quad (3)$$

values[13] of such primary water radicals are known as $G(e_{aq}^-) = G(^{\bullet}OH) = 2.8 \times 10^{-7}$ mol J^{-1}, and $G(^{\bullet}H) = 0.6 \times 10^{-7}$ mol J^{-1} in neutral aqueous solution. Under the present conditions, OH radicals are scavenged by 2-methyl-2-propanol into substantially unreactive 2-methyl-2-propanol radicals ($^{\bullet}CH_2(CH_3)_2$-COH) as in reaction 2 ($k(^{\bullet}OH + (CH_3)_3COH) = 6 \times 10^8$ dm^3 mol^{-1} s^{-1}).[1a,14] On the other hand, hydrated electrons e_{aq}^- are scavenged by $S_2O_8^{2-}$ to produce oxidant SO_4 radical anions ($E°(SO_4^{\bullet-}/SO_4^{2-}) = 2.4$ V vs NHE)[15] as in reaction 3 ($k(e_{aq}^- + S_2O_8^{2-}) = 1.2 \times 10^{10}$ dm^3 mol^{-1} s^{-1}, $k(^{\bullet}H + S_2O_8^{2-}) = 2.5 \times 10^7$ dm^3 mol^{-1} s^{-1}).[1a,14,16]

As analyzed by HPLC, **1a**,**b**[***meso***] and **1a**,**b**[***rac***] were oxidized by the radiation chemically generated SO_4 radical anions and their C5−C5′ bonds split to produce the corresponding thymine monomers (**2a**,**b**) and 5,6-dihydrothymine derivatives (**3a**,**b**) (Chart 1), which were accompanied by isomerization from meso dimers **1a**,**b**[***meso***] to racemic dimers **1a**,**b**[***rac***] or vice versa. Figure 1 shows radiation dose responses of the dimer decomposition and the product formation in phosphate buffer at pH 7, the initial slopes of which gave the respective G values as listed in Table 1. The fairly high yields of **2a**,**b** are in accord with a prediction that the C5−C5′-linked dimers **1a**,**b**, as afforded by one-electron reductive dimerization of thymine monomers **2a**,**b**,[11] may undergo the reverse splitting by a one-electron oxidation mechanism.

Table 1 indicates that the G value of dimer decomposition ($G(-\mathbf{1})$) exceeds that of SO_4 radical anion ($G(SO_4^{\bullet-}) < 3.2 \times$

CHART 1



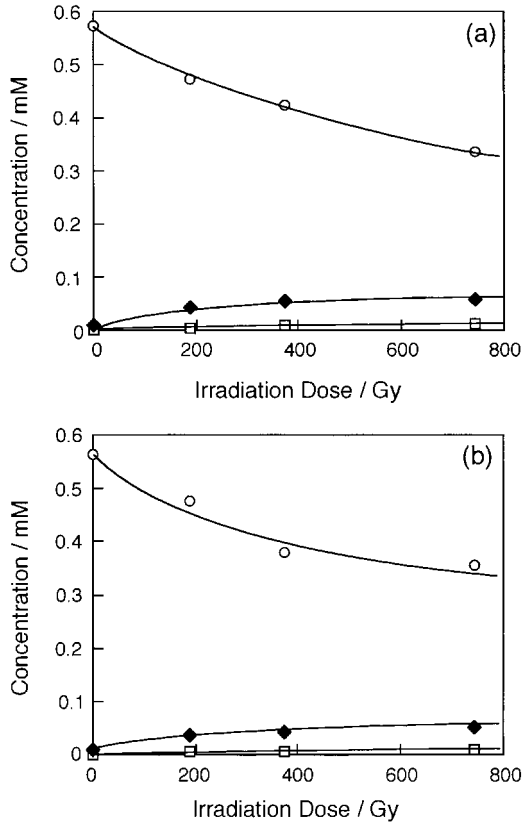


Figure 1. Oxidative splitting of (a) **1a**[***meso***] and (b) **1a**[***rac***] (0.58 mM) in the γ-radiolysis of Ar-purged phosphate buffer solution containing $K_2S_2O_8$ (5.0 mM) and 2-methyl-2-propanol (50 mM): (○) decomposition of **1a**[***meso***] or **1a**[***rac***]; (♦) formation of **2a**; (□) isomerization of **1a**.

10^{-7} mol J^{-1} depending on pH) to some extent. This suggests that some radical intermediates involved in the oxidation of **1a**,**b** by SO_4 radical anions are able to decompose **1a**,**b**. In view of the previous result that the β-oxoalkyl compounds such as 6-hydroxy-5,6-dihydrothymin-5-yl radicals possess oxidizing properties,[17] one-electron oxidation by such oxidizing 5-yl radicals may account for the excess G values of dimer decomposition. Indeed, the oxidative splitting of **1a**,**b** at the C5−C5′ bond may generate 5,6-dihydrothymin-5-yl radicals (**6a**,**b**), as supported by the occurrence of isomerization (see Scheme 1).

TABLE 1: Oxidative Splitting of 1a,b (0.58 mM) in Phosphate Buffer Solutions by Radiation Chemically Generated Oxidizing Radicals $SO_4^{\bullet-}$ or $N_3^{\bullet}$

		$G \times 10^7$/mol J^{-1}			
substrate	pH	**−1**	**2**	**3**	isomerization
		Sulfate Radical Anion ($SO_4^{\bullet-}$)			
1a[***meso***]	4	4.1	2.1 (49%)[a]	2.6 (62%)	0.08 (2%)
	7	3.6	2.3 (43%)	1.6 (42%)	0.31 (9%)
	10	3.7	2.8 (74%)	0.75 (20%)	0.31 (8%)
1a[***rac***]	4	3.2	1.6 (48%)	0.83 (26%)	0.21 (6%)
	7	3.3	2.2 (68%)	0.77 (23%)	0.34 (10%)
	10	2.9	2.3 (77%)	0.99 (34%)	0.23 (8%)
1b[***meso***]	4	5.2	3.0 (58%)	0.52 (10%)	0.33 (6%)
	7	4.3	3.3 (78%)	0.57 (13%)	0.50 (12%)
	10	4.0	3.4 (85%)	0.55 (14%)	0.38 (10%)
1b[***rac***]	4	4.4	2.8 (65%)	0.66 (15%)	0.27 (6%)
	7	3.6	3.1 (86%)	0.65 (18%)	0.31 (9%)
	10	3.4	3.2 (92%)	0.65 (19%)	1.3 (40%)
		Azide Radical ($N_3^{\bullet}$)			
1a[***meso***]	4	0.23	0.18 (77%)	0.10 (45%)	
	7	0.16	0.17(107%)	0.11 (73%)	
	10	0.33	0.37(113%)	0.15 (44%)	

[a] Selectivity based on G values for the decomposition of **1a**,**b**.

The $SO_4^{\bullet-}$-induced splitting of **1a**,**b** to produce **2a**,**b** and **3a**,**b** was dependent on the pH of the aqueous solution. A trend is seen in Table 1 that the restoration of **1a**,**b** to **2a**,**b** becomes more efficient upon increasing the pH value: the yields of **2a**,**b** in basic solution at pH 10 are about 1.5 times greater than those in acidic solution at pH 4. On the other hand, there was substantially no influence of stereoisomerism on the splitting reactivity at each pH. Table 1 also suggests that 1,3-dimethyl-5,6-dihydrothymine dimers **1b**[***meso***] and **1b**[***rac***] are oxidized by SO_4 radical anions somewhat more readily than 1-methyl-5,6-dihydrothymine dimers **1a**[***meso***] and **1a**[***rac***]. It is likely that the inductive electron-donating 3-methyl group may facilitate one-electron oxidation of **1b** relative to **1a**. As a different aspect in this context, a methyl substitution results in a more negative reduction potential of 5,6-dihydropyrimidines.[18]

Less oxidizing Br_2 radical anions ($Br_2^{\bullet-}$; $E°(Br_2^{\bullet-}/2Br^-) = 1.7$ V vs NHE)[15] were also generated by γ-radiolysis of N_2O-saturated phosphate buffer solution containing an excess amount of KBr (100 mM) to examine the oxidative splitting reactivity of the C5−C5′-linked dihydrothymine dimers. Under the present conditions of radiolysis, more than 98% of hydrated electrons are converted into OH radicals by N_2O (reaction 4; $k(e_{aq}^- + N_2O) = 9.1 \times 10^9$ dm^3 mol^{-1} s^{-1}).[14] The OH radicals are in turn scavenged by Br^- ions to produce oxidizing Br_2 radical anions (reactions 5 and 6).[1] In contrast to the reactivity toward

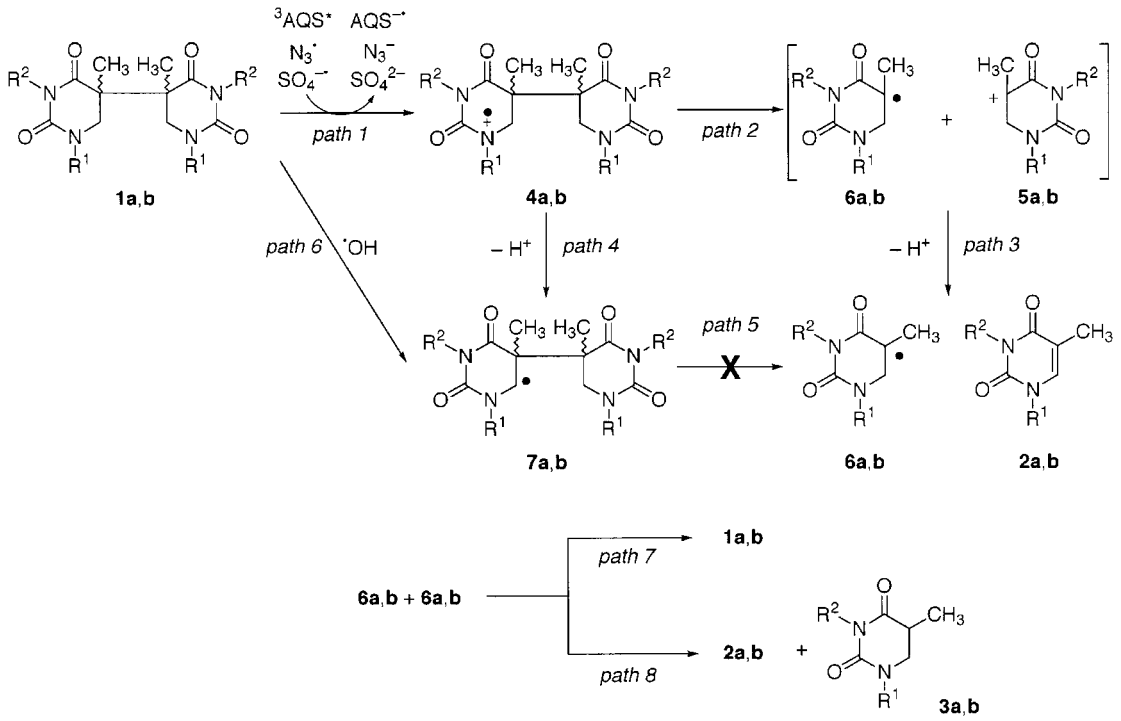
$$e_{aq}^- + N_2O \rightarrow N_2 + {}^{\bullet}OH + OH^- \quad (4)$$

$$Br^- + {}^{\bullet}OH \rightarrow Br^{\bullet} + OH^- \quad (5)$$

$$Br^{\bullet} + Br^- \rightarrow Br_2^{\bullet-} \quad (6)$$

SO_4 radical anions, neither **1a**[***meso***] nor **1a**[***rac***] (0.5 mM) was decomposed by Br_2 radical anions thus generated in phosphate buffer at pH 7. Such an ineffective oxidizing reactivity of Br_2 radical anions has been observed for the reactions with cyclobutane pyrimidine photodimers.[5] Even for electron-rich thymine bearing a C5−C6 double bond, Br_2 radical anions showed practically negligible reactivity ($k(Br_2^{\bullet-} + \text{thymine}) < 10^7$ dm^3 mol^{-1} s^{-1}).[19]

SCHEME 1



Using azide ions (N_3^-; 50 mM NaN_3) instead of Br^- ions in N_2O-saturated phosphate buffer, oxidizing azide radicals ($N_3^•$) were also generated as in reaction 7 ($k = 1.2 \times 10^{10}$ dm^3 mol^{-1} s^{-1}, $E°(N_3^•/N_3^-) = 1.3$ V vs NHE).[14,15] Table 1 shows the results

$$N_3^- + {}^•OH \rightarrow N_3^• + OH^- \quad (7)$$

of $N_3^•$-induced oxidation of **1a**[***meso***] (0.5 mM) in phosphate buffer solutions at pH 4, 7, and 10. The dimer **1a**[***meso***] split into the monomer **2a** in high yield at each pH, despite the reduction potential of the azide radical being more negative by about 0.25 V than that of Br_2 radical anion. While the *G* value of the $N_3^•$-induced splitting of **1a**[***meso***] was reduced to approximately 10% the level of the $SO_4^{•-}$-induced splitting, selectivity of the restoration to **2a** was significantly enhanced (Table 1). A similar difference in oxidizing reactivity between azide radicals and Br_2 radical anions was also observed for the reactions with other nucleic acids. It has been thus suggested that azide radicals react by an outer-sphere electron-transfer mechanism depending on the reduction potential of a given nucleic acid, whereas Br_2 radical anions react by an inner-sphere electron-transfer mechanism.[20] Therefore, the splitting of C5−C5′-linked dihydrothymine dimers **1a**,**b** into **2a**,**b** by SO_4 radical anion or azide radical proceeds via one-electron oxidation (path 1 in Scheme 1) most possibly by an outer-sphere electron-transfer mechanism.

Previously, strongly oxidizing OH radicals with a high reduction potential ($E°(^•OH/OH^-) = 2.7$ V vs NHE)[15] were shown to restore cyclobutane thymine photodimer to thymine with *G* values of 2.0−3.1.[5,21] This reaction might proceed by C6- or C6′-hydrogen abstraction rather than direct electron transfer, on analogy of similar $^•$OH-induced restoration of 5,6-dihydrothymine to thymine involving a hydrogen-abstraction mechanism as in reactions 8−10.[21] For comparison, we further studied the reactivity of **1a**,**b** toward radiation chemically generated OH radicals.

$$TH_2 + {}^•OH \rightarrow TH^• + H_2O \quad (8)$$

$$TH^• + {}^•OH \rightarrow TH^+ + OH^- \quad (9)$$

$$TH^+ + OH^- \rightarrow T + H_2O \quad (10)$$

Upon γ-radiolysis (600 Gy) of N_2O-saturated solution in phosphate buffer **1a**,**b** (0.5 mM) could not be restored to **2a**,**b** in contrast to cyclobutane thymine photodimer. This result clearly rules out a hypothetical one-electron oxidation of the C5−C5′-linked dihydrothymine dimers **1a**,**b** by OH radicals to form the dimer radical cations **4a**,**b**. Although C5−C5′-linked dihydrothymine dimer 6-yl radicals (**7a**,**b**) are most likely intermediates derived from C6-hydrogen abstraction by OH radicals (path 6 in Scheme 1), fragmentation of **7a**,**b** to yield thymines **2a**,**b** and 5,6-dihydrothymin-5-yl radicals **6a**,**b** (path 5) should not occur in the OH radical reaction. Concerning a mechanism of the oxidative splitting of **1a**,**b**, on the other hand, such a reactivity of **7a**,**b** implies that the dimer radical cations **4a**,**b** as the probable intermediates could not yield thymines **2a**,**b**, if deprotonation at the C6 of **4a**,**b** was predominant (path 4). Thus, it is plausible that the dimer radical cations **4a**,**b** are liable to undergo C5−C5′-bond splitting into the corresponding 5-yl radical **6a**,**b** and C5-cation (**5a**,**b**) (path 2).

Splitting of C5−C5′-Linked Dihydrothymine Dimers by Photoexcited Oxidizing Sensitizer. As demonstrated previously by means of CIDNP,[6] oxidative splitting of cyclobutane pyrimidine photodimers (Pyr<>Pyr) into pyrimidine monomers (Pyr) was effectively induced upon photoexcitation of oxidizing sensitizers such as anthraquinone-2-sulfonate (AQS), which possesses a reduction potential of −0.39 V vs NHE.[15] The observation of substrate and product photo-CIDNP signals led to a one-electron oxidation mechanism of the photodimer splitting by which electron transfer from photodimer Pyr<>Pyr to the excited triplet state of AQS (^{3}AQS*) occurs to produce the photodimer radical cation (Pyr<>Pyr$^{•+}$) and ASQ radical

anion ($AQS^{\bullet-}$). The radical cation $Pyr<>Pyr^{\bullet+}$ thus photogenerated may undergo splitting into pyrimidine and its radical cation, the latter of which is one-electron reduced to pyrimidine by the radical anion $AQS^{\bullet-}$.

$$AQS \xrightarrow{h\nu} {}^1AQS^* \xrightarrow{isc} {}^3AQS^* \xrightarrow{Pyr<>Pyr} AQS^{\bullet-} + Pyr<>Pyr^{\bullet+}$$

In this light, we also examined similar AQS-sensitized photooxidation reactivity of the C5−C5′-linked dihydrothymine dimers **1a**,**b**. Upon photoexcitation of AQS (0.4 mM) in deoxygenated phosphate buffer (5.0 mM, pH 4, 7 and 10) containing **1a**,**b** (1.0 mM), efficient splitting of **1a**,**b** into **2a**,**b** and **3a**,**b** was observed in a similar manner as in the one-electron oxidative splitting by SO_4 radical anions or azide radicals. This photooxidative splitting of **1a**,**b** is likely to proceed via electron transfer from **1a**,**b** to the $^3AQS^*$, as in the case of cyclobutane pyrimidine photodimers, since it was suppressed by the addition of diacetyl (4.0 mM), a typical triplet quencher ($E_T = 236$ kJ mol^{-1}).[22] In contrast to the splitting by oxidizing radicals in the radiolysis systems, the photosensitized splitting by AQS involved no isomerization of the dimers **1a**,**b**. This suggests that one-electron reduction of the 5-yl radical intermediates **6a**,**b** by the radical anion $AQS^{\bullet-}$ may occur more efficiently than radical coupling and disproportionation (paths 7 and 8 in Scheme 1), followed by protonation to yield **3a**,**b**. Therefore, in the AQS-sensitized photoreaction system, the electron transfer from $^3AQS^*$ and the back electron transfer from $AQS^{\bullet-}$ would lead to a net splitting of the C5−C5′-linked dihydrothymine dimers **1a**,**b** into the corresponding thymines **2a**,**b** and 5,6-dihydrothymines **3a**,**b** in a 1:1 ratio.

Figure 2 shows the time course of the AQS-sensitized photooxidative splitting of **1a**,**b** in phosphate buffer at pH 7. It is evident that **2a**,**b** and **3a**,**b** were produced in almost equivalent yields in the initial stage of photoirradiation, while the yield of **2a**,**b** became considerably higher than that of **3a**,**b** during the prolonged photoirradiation. This behavior suggests concurrent oxidation of **3a**,**b** to produce **2a**,**b** as a secondary reaction in the AQS-sensitized photooxidation system. In a separate experiment, we confirmed that photoexcitation of AQS (0.2 mM) induced oxidation of **3a** (1.0 mM) to yield **2a** almost quantitatively in Ar-purged phosphate buffer at pH 7 (Figure 3). Therefore, direct one-electron oxidation of dihydrothymines **3a**,**b** by $^3AQS^*$ accounts for the secondary conversion to the corresponding monomers **2a**,**b**. According to a semiquinone−semiquinolate equilibrium with $pK_a = 8.2$ (reaction 11) as

$$AQS^{\bullet-} + H^+ \rightarrow AQSH^{\bullet} \qquad (11)$$

characterized by the previous flash photolysis study,[23] on the other hand, the semiquinone radicals ($AQSH^{\bullet}$) are derived from protonation of the semiquinolate ions $AQS^{\bullet-}$ at an almost diffusion-controlled rate under neutral conditions. An alternative reaction pathway is also possible where the primary photoproduct **3a**,**b** may undergo hydrogen abstraction by the semiquinone radicals $AQSH^{\bullet}$ to yield **2a**,**b**. Similar hydrogen abstraction is also involved in the $^{\bullet}OH$-induced restoration of **3a**,**b** to **2a**,**b** (reactions 8−10).[21]

Table 2 shows the influence of pH on the photosensitized splitting of **1a**,**b** into **2a**,**b** and **3a**,**b** as observed in the initial stage of photoirradiation for 8 min. Although the pH dependence was not clear, the decomposition of **1a**,**b** seemed to become more facilitated with increasing pH of aqueous solution, as expected from the semiquinone−semiquinolate equilibrium, as in reaction 11. As observed in the $SO_4^{\bullet-}$- and $N_3^{\bullet}$-induced

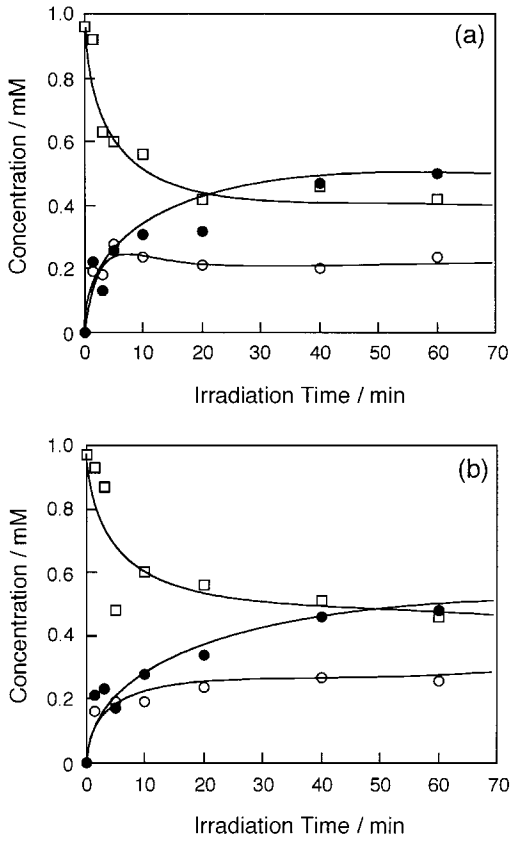


Figure 2. Oxidative splitting of (a) **1a**[***meso***] and (b) **1a**[***rac***] (1.0 mM) in Ar-purged phosphate buffer upon photoexcitation ($\lambda_{ex} > 280$ nm) of AQS (0.4 mM): (□) decomposition of **1a**[***meso***]; formation of (●) **2a** and (○) **3a**.

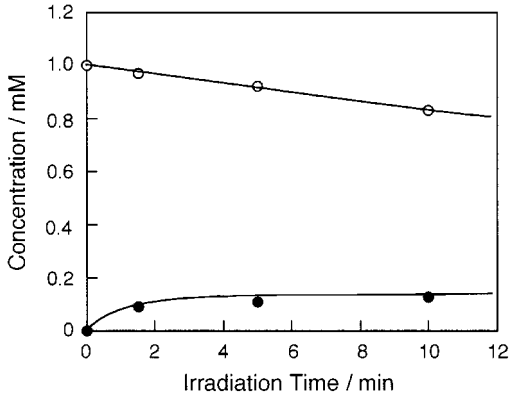


Figure 3. Oxidation of **3a** (1.0 mM) in Ar-purged phosphate buffer upon photoexcitation ($\lambda_{ex} > 280$ nm) of AQS (0.2 mM): (○) decomposition of **3a**; (●) formation of **2a**.

splittings, a trend was also seen in the AQS-sensitized photoreaction that **1b**[***meso***] and **1b**[***rac***] favor a one-electron oxidative splitting more than **1a**[***meso***] and **1a**[***rac***] (Tables 1 and 2).

Laser Flash Photolysis. Laser flash photolyses at 355 nm of aqueous solutions of C5−C5′-linked dihydrothymine dimers **1a**,**b** (1.0 mM) and AQS (50 μM) as an oxidizing sensitizer were first attempted to detect transient absorptions originating from oxidative splitting of **1a**,**b** but were unsuccessful because of overlapping with intense absorption due to $AQS^{\bullet-}$ in the wavelength region of 300−530 nm ($\epsilon(AQS^{\bullet-})_{500} = 6.8 \times 10^3$ $dm^3\ mol^{-1}\ cm^{-1}$; $\epsilon(AQS^{\bullet-})_{385} = 5.37 \times 10^3\ dm^3\ mol^{-1}\ cm^{-1}$).[24] In light of the $SO_4^{\bullet-}$-induced oxidation in the radiolysis experiment as above, oxidizing SO_4 radical anions were

TABLE 2: Oxidative Splitting of 1a,b (1.0 mM) in Ar-Purged Phosphate Buffer upon Photoexcitation (λ_{ex} > 280 nm) of AQS (0.4 mM) for 8 min with a High Pressure Hg Lamp

substrate	pH	conversion of **1a**,**b**/%	yield/% **2a**,**b**	yield/% **3a**,**b**
1a[***meso***]	4	17	65	42
	7	16	63	44
	10	27	48	52
1a[***rac***]	4	17	47	35
	7	17	53	41
	10	24	58	71
1b[***meso***]	4	20	75	65
	7	18	106	106
	10	15	73	100
1b[***rac***]	4	29	55	34
	7	30	60	67
	10	30	43	60

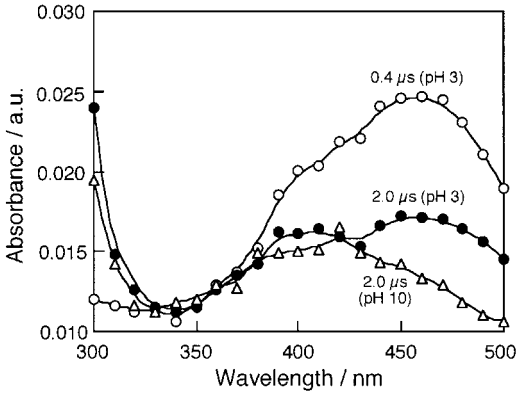


Figure 4. Transient absorption spectra of the intermediates as observed (○, pH 3.3) 0.4 μs and (●, pH 3.3; △, pH 10.2) 2.0 μs after the 266 nm laser photolysis of $S_2O_8^{2-}$ (50 mM) in phosphate buffer solution containing **1a**[***meso***] (1.0 mM).

generated to react with **1a**,**b** by the laser flash photolysis of $S_2O_8^{2-}$ in aqueous solution, as in reaction 12.[25]

$$S_2O_8^{2-} + h\nu \rightarrow 2SO_4^{\bullet -} \qquad (12)$$

In a control experiment, upon laser flash excitation at 266 nm of peroxodisulfate ion $S_2O_8^{2-}$ (50 mM) in deoxygenated aqueous solution, a broad transient absorption band assigned to the SO_4 radical anion at wavelengths of 300−550 nm (λ_{max} = 460 nm) was observed as reported previously.[25] Similar 266 nm laser flash photolyses of Ar-purged acidic solution (pH 3.3) of **1a**[***meso***] (1.0 mM) in the presence of $K_2S_2O_8$ (50 mM) resulted in the transient absorption spectra shown in Figure 4. Substantially the same behavior was observed for a solution of **1a**[***rac***] (1.0 mM) under similar conditions. The spectrum recorded within 1 μs after the laser flash contained an absorption maximum at λ_{max} = 460 nm ($\epsilon(SO_4^{\bullet -})_{455} = 4.6 \times 10^2$ dm^3 mol^{-1} cm^{-1}) characteristic of SO_4 radical anions.[25] The SO_4 radical anions decayed rapidly and thereafter a common absorption maximum at λ_{max} = 400 nm emerged from both aqueous solutions of **1a**[***meso***] and **1a**[***rac***], as was evidently observed 2 μs after the laser flash. This transient may be assigned to 1-methyl-5,6-dihydrothymin-5-yl radical **6a** by reference to the previous pulse radiolysis study, in which 5,6-dihydrothymin-5-yl radicals generated by one-electron reductions of 5-bromo-5,6-dihydrothymine and 5-bromo-5,6-dihydrouracil derivatives by hydrated electrons e_{aq}^- in aqueous solution showed broad absorptions at around 400 nm (λ_{max} = 380 nm, ϵ_{380} = 1150 dm^3 mol^{-1} cm^{-1}).[26] When the laser flash photolysis was performed in a basic solution of **1a**[***meso***] (1.0 mM) and $K_2S_2O_8$ (50 mM) at pH 10.2, the SO_4 radical anions decayed much more rapidly due to a reaction with OH^- to generate OH radicals ($k = 6.5 \times 10^7$ dm^3 mol^{-1} s^{-1}, reaction 13),[27] which occurs in competition with the one-electron oxidation of **1a**[***meso***].

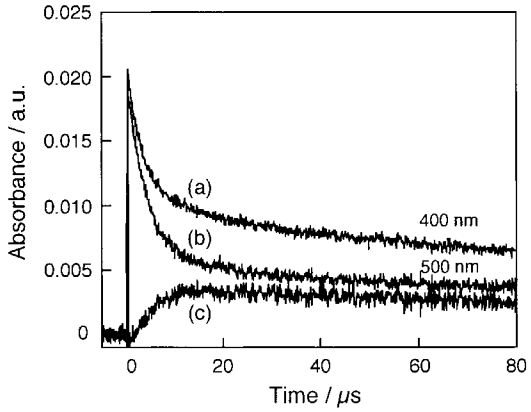


Figure 5. Time courses of the absorbencies at (a) 400 nm and (b) 500 nm in the 266 nm laser photolysis of $S_2O_8^{2-}$ (50 mM) in phosphate buffer solution containing **1a**[***meso***] (1.0 mM). (c) Buildup of the absorbance at 400 nm derived from the decay profile at 400 nm by subtracting a decay component following the kinetics as in the absorbance at 500 nm.

$$SO_4^{\bullet -} + OH^- \rightarrow SO_4^{2-} + {}^{\bullet}OH \qquad (13)$$

In the present experiments, we could not identify the dimer radical cations **4a** that would possess a characteristic absorption at wavelengths shorter than 350 nm. It follows that the dimer radical cations **4a** would be liable to split into C5-cations **5a** and radicals **6a** on the nanosecond time scale.

Parts a and b of Figure 5 compare the decays of absorbancies due to 1-methyl-5,6-dihydrothymin-5-yl radical **6a** and $SO_4^{\bullet -}$ at 400 and 500 nm, respectively. In the initial stage up to 20 μs after the laser flash, the transient absorbance at 400 nm is likely to involve the overlapped absorption tail of $SO_4^{\bullet -}$. The contribution of SO_4 radical anions to the absorbance at 400 nm was therefore estimated from the corresponding decay behavior at 500 nm, taking into account the relative molar extinction coefficients ($\epsilon_{400}(SO_4^{\bullet -})/\epsilon_{500}(SO_4^{\bullet -}) = 1.1$). Thus, the buildup of 1-methyl-5,6-dihydrothymin-5-yl radicals **6a** (λ_{max} = 400 nm) could be obtained by subtracting the contribution of SO_4 radical anions from the apparent absorbance at 400 nm, as shown in Figure 5c. The decay of **6a** was of the second-order kinetics (see Figure 6), indicating the occurrence of disproportionation (formation of **2a** and **3a**) and/or recombination (isomerization), as in reaction 14. The second-order rate constants were

$$TH^{\bullet} + TH^{\bullet} \rightarrow TH_2 + T \text{ or } TH{-}TH \qquad (14)$$

approximately evaluated as $2k = 2.7 \times 10^9$ dm^3 mol^{-1} s^{-1} in the splitting of **1a**[***meso***] and 2.2×10^9 dm^3 mol^{-1} s^{-1} in the splitting of **1a**[***rac***] from the slopes of the reciprocal of absorbance at 400 nm vs time plots (Figure 6), assuming that the molar extinction coefficient of **6a** is equal to the reported value of the 5,6-dihydrothymin-5-yl radical at 380 nm (ϵ_{380} = 1150 dm^3 mol^{-1} cm^{-1}).[26] The rate constants thus evaluated are in good agreement with the literature value ($2k = 2.35 \times 10^9$ dm^3 mol^{-1} s^{-1} at pH 6.8) for the 5,6-dihydrothymin-5-yl radical as derived from the pulse radiolysis study on the reaction of 5,6-dihydrothymine with OH radicals.[28]

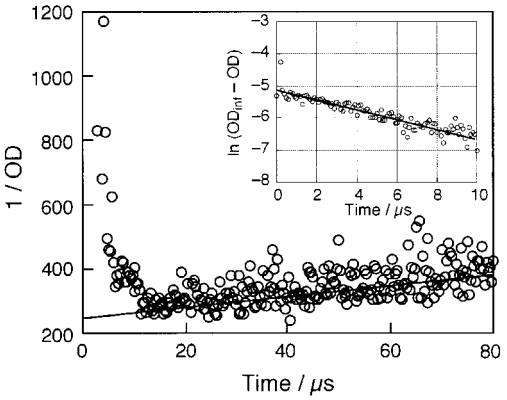


Figure 6. Plot of 1/OD vs time for the buildup component at 400 nm in the 266 nm laser photolysis of $S_2O_8^{2-}$ (50 mM) in phosphate buffer solution containing **1a**[***meso***] (1.0 mM). Inset: pseudo-first-order kinetics for the buildup component with absorbance at 400 nm. OD_{inf} corresponds to a plateau value of the absorbance at 400 nm due to the buildup component.

The SO_4 radical anions decayed following the pseudo-first-order kinetics in the presence of **1a**[***meso***] or **1a**[***rac***], while being of the second-order kinetics in the control photolysis of peroxodisulfate ion $S_2O_8^{2-}$ alone. The pseudo-first-order decays gave the corresponding rate constants for the oxidation of dimers by the SO_4 radical anion as $k = 2.4 \times 10^8$ dm^3 mol^{-1} s^{-1} (**1a**[***meso***] = 1.0 mM) and $k = 1.5 \times 10^8$ dm^3 mol^{-1} s^{-1} (**1a**[***rac***] = 1.0 mM). In accord with the decay behavior of SO_4 radical anions, the buildup of 5-yl radicals **6a** to attain its plateau level was represented in terms of a pseudo-first-order kinetics (the inset in Figure 6), leading to similar rate constants of $k = 1.8 \times 10^8$ dm^3 mol^{-1} s^{-1} (**1a**[***meso***]) and $k = 1.0 \times 10^8$ dm^3 mol^{-1} s^{-1} (**1a**[***rac***]). On the other hand, the $SO_4^{\bullet-}$-induced oxidation of 5,6-dihydrothymine (DHT) at varying concentrations of 0.5−5.0 mM was determined to show a rate constant of $k(\mathrm{DHT} + \mathrm{SO_4^{\bullet-}}) = 8.5 \times 10^7$ dm^3 mol^{-1} s^{-1}. These values were at least 1 order of magnitude smaller than those of 5,6-unsaturated pyrimidines such as 1-methylthymine (k(**2a** + $SO_4^{\bullet-}$) = 5.0×10^9 dm^3 mol^{-1} s^{-1}) and 1,3-dimethylthymine (k(**2b** + $SO_4^{\bullet-}$) = 4.6×10^9 dm^3 mol^{-1} s^{-1}).[29] This is probably attributable to the lower nucleophilicity of 5,6-dihydrothymine compounds including **1a**[***meso***] and **1a**[***rac***] toward SO_4 radical anions.

Oxidative Splitting Mechanism of the C5−C5′-linked dihydrothymine Dimers. Scheme 1 illustrates a proposal for a mechanism by which the C5−C5′-linked dihydrothymine dimers **1a**,**b** undergo splitting into thymines **2a**,**b** and 5,6-dihydrothymines **3a**,**b** by oxidizing radicals such as $SO_4^{\bullet-}$ and $N_3^{\bullet}$, but not $Br_2^{\bullet-}$, and by photoexcited oxidizing sensitizers such as ^{3}AQS*. The failure of the oxidant $Br_2^{\bullet-}$ in reacting toward **1a**,**b** is not surprising in view of the fact that $Br_2^{\bullet-}$ is also unreactive toward the cyclobutane thymine dimers.[5] The laser flash photolysis study has provided spectroscopic evidence in support of 5,6-dihydrothymin-5-yl radical intermediates **6a**,**b** involved in the $SO_4^{\bullet-}$-induced oxidative splitting of **1a**,**b**. The C5−C5′-linked dihydrothymine dimer radical cations **4a**,**b** would be the most likely precursor leading to the observed 5-yl radicals **6a**,**b**, although spectroscopic detection of the transients **4a**,**b** was unsuccessful in the laser flash photolysis experiments. A similar mechanistic aspect has been proposed for the oxidative splitting of cyclobutane pyrimidine photodimers in that the radical cations are generated by electron transfer from the photodimers to a photoexcited oxidizing sensitizer.[4a]

Concerning the fate of dihydrothymine dimer radical cations **4a**,**b**, two possible routes to thymines **2a**,**b** and 5,6-dihydrothymin-5-yl radicals **6a**,**b**, involving C5−C5′-bond splitting followed by deprotonation (paths 2 and 3) or vice versa (paths 4 and 5), can be assumed, as shown in Scheme 1. The latter route is, however, ruled out from the experimental evidence that OH radicals could not produce **2a**,**b**. Thus, the C5−C5′-linked dihydrothymine dimer 6-yl radicals **7a**,**b**, that are potentially derived from C6-deprotonation of **4a**,**b** (path 4) or C6-hydrogen abstraction from **1a**,**b** by OH radicals (path 6) are unlikely to undergo the C5−C5′-bond splitting into **2a**,**b** and **6a**,**b** (path 5). Alternatively, **4a**,**b** may favor the C5−C5′-bond splitting into the 5-yl radicals **6a**,**b** and C5′-cations **5a**,**b** (path 2). In view of the oxidizing property of 5,6-dihydropyrimidin-5-yl radicals,[17] the C5-cations **5a**,**b** must be so unstable as to be deprotonated at C6 into thymines **2a**,**b**. In relation to the C5−C5′-bond splitting reactivity of **4a**,**b**, it is interesting to note that the cyclobutane photodimer radical cation undergoes a stepwise fragmentation involving sequential cleavage of the C6−C6′ and the C5−C5′ bonds, as supported by the successful trapping of a single-bond-cleaved intermediate in a model reaction system.[30] Such a preferential C6−C6′-bond splitting of the photodimer radical cations resulting in intramolecular formation of a pair of a dimer C6 radical and a C6′ cation seems to be rationalized by the reducing property of 5,6-dihydrothymin-6-yl radicals.[1a] Accordingly, a radical and carbocation pair formed by the C6−C6′-bond splitting of the photodimer radical cation would be energetically more stable than that by the C5−C5′-bond splitting of **4a**,**b**.

The C5−C5′-linked dimers **1a**[***meso***] and **1a**[***rac***] are of the N3-protonated forms in the pH range 4−10, since their pK_a values in aqueous solution are 11.7, as measured by the UV-absorption spectral changes (data not shown). The pH dependence of the $SO_4^{\bullet-}$- and $N_3^{\bullet}$-induced splitting of **1a**,**b** that the restoration to **2a**,**b** becomes more efficient with increasing the value (Table 1) can be explained by the more facilitated C6-deprotonation of **5a**,**b** (path 3). On the other hand, base-enhanced C6-deprotonation of **4a**,**b** to form **7a**,**b** is unlikely, because it will reduce the restoration efficiency if occurred.

In accord with the decay of the second-order kinetics shown in Figure 5, two types of bimolecular reactions of 5,6-dihydrothymin-5-yl radicals **6a**,**b** may proceed competitively in the reaction system. Recombination and disproportionation of **6a**,**b** accounts for the apparent isomerization of the dimers **1a**,**b** (path 7) and formation of **2a**,**b** and **3a**,**b** (path 8), respectively. As a concomitant minor reaction, oxidizing radical **6a**,**b** could one-electron oxidize the C5−C5′-linked dimers **1a**,**b**, thereby resulting in the enhanced G value for the decomposition of **1a**,**b** (see Table 1; G(−**1a**,**b**) > $G(SO_4^{\bullet-}) = 3.3 \times 10^{-7}$ mol J^{-1}).[17]

Conclusion

We have demonstrated oxidative splitting of the C5−C5′-linked dihydrothymine dimers **1a**,**b** into thymine monomers **2a**,**b** along with byproducts of 5,6-dihydrothymines **3a**,**b** by oxidizing radicals ($SO_4^{\bullet-}$ and $N_3^{\bullet}$) generated in the radiolysis and by photoexcited oxidizing sensitizer (^{3}AQS*). As characterized by the laser flash photolysis, 5,6-dihydrothymin-5-yl radicals **6a**,**b** are intermediates involved in the oxidative splitting. A mechanism involving generation of the C5−C5′-linked dihydrothymine dimer radical cations **4a**,**b** by electron transfer from **1a**,**b** to an oxidant has been proposed, by reference to a similar oxidative splitting mechanism of cyclobutane pyrimidine photodimers. While the pyrimidine photodimer radical cation prefers the C6−C6′-bond splitting to the counterpart C5−C5′-bond splitting, the C5−C5′-linked dihydrothymine dimer radical

cations **4a**,**b** may undergo the C5−C5′-bond splitting into the 5,6-dihydrothymin-5-yl radicals **6a**,**b** and the C5-cations **5a**,**b**. Because of the oxidizing property, the intermediate radicals **6a**,**b** can also be one-electron oxidants toward **1a**,**b**.

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