Radiation-Induced and Photosensitized Splitting of C5−C5′-Linked Dihydrothymine Dimers. 2. Conformational Effects on the Reductive Splitting Mechanism

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Radiation-induced and photosensitized reductive splitting of stereoisomeric C5−C5′-linked dihydrothymine dimers (**1a**,**b**[*meso*], meso compound of (5*R*,5′*S*)- and (5*S*,5′*R*)-bi-5,6-dihydrothymines; **1a**,**b**[*rac*], racemic compound of (5*R*,5′*R*)- and (5*S*,5′*S*)-bi-5,6-dihydrothymines) in aqueous solution were studied to compare with the one-electron oxidative splitting mechanism and the photorepair reaction of cyclobutane pyrimidine photodimers. Reacting with radiation-chemically and photochemically generated hydrated electrons or with photoexcited reduced form of flavin adenine dinucleotide (*FADH$^-$), the C5−C5′-linked dihydrothymine dimers **1a**,**b** produced the corresponding 5,6-dihydrothymine derivatives (**3a**,**b**) along with the thymine monomers (**2a**,**b**) in minor yields. Both the product and laser flash photolysis studies indicated that one-electron adducts of the C5−C5′-linked dimers **1a**,**b** undergo C5−C5′-bond cleavage to generate the 5,6-dihydrothymin-5-yl radicals (**5a**,**b**) and the 5,6-dihydrothymine C5-anions (**6a**,**b**) resulting in the formation of **3a**,**b** by facile protonation at C5. In the reduction by *FADH$^-$, splitting of the 5,6-dihydro-1-methylthymine dimer **1a**[*meso*] into the monomer **2a** was more efficient than that of the racemic isomer **1a**[*rac*]. Conformational analysis by NMR of **1a**[*meso*] and **1a**[*rac*] in solution suggested that **1a**[*meso*] may favor a "closed-shell" conformation and undergo one-electron reduction to form **5a** and **6a**, whereas **1a**[*rac*] may be in a "opened-shell" conformation and undergo successive two-electron reduction by *FADH$^-$ to produce 2 equiv of **6a** as a precursor of **3a**.

Introduction

Ionizing radiation and ultraviolet (UV) light are the etiological agents of gene carcinogenesis and mutagenesis that give rise to a variety of DNA-base modifications.[1,2] Pyrimidine cyclobutane photodimers are among the most representative DNA-base lesions, being produced as a consequence of [2 + 2] photocycloaddition at a tandem pyrimidine moiety in the DNA duplex upon exposure to UV light.[2] The base residues thus modified are subjected to an intracellular repair process of photoreactivation by a photoenzyme, DNA photolyase, consisting of a deprotonated reduced flavin adenine dinucleotide (FADH$^-$) and an antenna chromophore of methenyltetrahydrofolate (MTHF) or 8-hydroxy-5-deazaflavin (8-HDF). While both radical cation and radical anion of the photodimer undergo facile fragmentation into monomers as confirmed by the model photochemical electron-transfer reactions, the enzymatic photoreactivation may favor a reductive fragmentation.[3] Thus, a likely mechanism of the photoreactivation has been drawn from extensive biochemical and photochemical studies:[3] the antenna chromophore of the photolyase complexed to the photodimer in DNA absorbs light in the 300−500 nm range and transfers the excitation energy to the reduced flavin FADH$^-$ moiety, thereby splitting of the cyclobutane photodimer being induced via one-electron transfer from the excited state of FADH$^-$. Concerted C5−C5′ and C6−C6′ bond splitting of the resulting cyclobutane photodimer radical anions, followed by electron transfer back to the flavin, reproduces the monomeric pyrimidines. Previous laser flash photolysis and photoinduced CIDNP spectroscopic studies have detected the radical anion intermediates in the reductive splitting reaction, indicating that the splitting of the photodimer radical anion involves a concerted but nonsynchoronous fragmentation of C5−C5′ and C6−C6′ bonds with a rate constant of the order 1×10^6 s^{-1}.[4,5]

An alternative [2 + 2] photocycloaddition between adjacent pyrimidines is simultaneously induced by UV exposure to result in a similar mutagenic photolesion of pyrimidine(6−4)pyrimidone [(6−4)photoadduct]. A photoenzyme (6−4)photolyase that shows a specific repairing ability toward the (6−4)photoadduct has also been identified.[6] As characterized more recently by a sequence analysis of the genes, the (6−4)photolyase has a high degree of structural identity to the DNA photolyase.[6c] These results strongly suggest a similarity of the repair mechanism between the (6−4)photolyase and DNA photolyase, involving a common electron-transfer process from the excited state of the enzyme to the UV-damaged DNA bases.[6c−e]

Recently, we have identified formation of stereoisomeric C5−C5′-linked dihydrothymine dimers, as fractionated into the meso compounds of (5*R*,5′*S*)- and (5*S*,5′*R*)-bi-5,6-dihydrothymines (**1a**,**b**[*meso*]) and racemic mixtures of (5*R*, 5′*R*)- and (5*S*,5′*S*)-bi-5,6-dihydrothymines (**1a**,**b**[*rac*]), in the radiolytic one-electron reduction of thymine derivatives [1-methylthymine (**2a**) and 1,3-dimethylthymine (**2b**)] in anoxic aqueous solution.[7] The X-ray crystallography demonstrated that the C5−C5′-linked dihydrothymine dimers are structurally similar to the cyclobutane thymine photodimers. Especially, as in the *cis-syn* cy-

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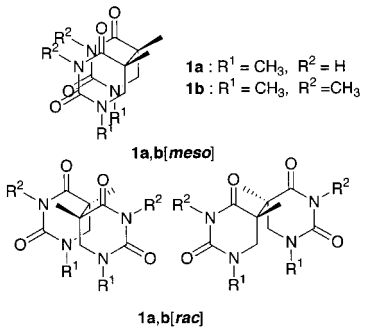
CHART 1



clobutane thymine photodimers, the meso-dimer structure like **1a**[*meso*] seemed to cause insufficient stacking of the pyrimidine rings that will weaken the hydrogen bonds between the dimer and the complementary adenine when formed by chance in a DNA duplex. A remarkable observation with respect to the reactivity was that the C5−C5′-linked dihydrothymine dimers **1a**,**b** undergo oxidative splitting to regenerate the corresponding thymine monomers **2a**,**b** via one-electron transfer from the dimer to radiation chemically or photochemically generated oxidants.[8] Formally, this oxidative splitting of **1a**,**b** is a reverse reaction of the reductive dihydrodimerization of **2a**,**b**.

In view of the fact that the splitting of cyclobutane photodimers into monomers occurs in principle by both oxidative and reductive pathways, further attempt has been made to characterize reductive splitting reactivity of the C5−C5′-linked dihydrothymine dimers **1a**,**b** under conditions of generating hydrated electrons (e_{aq}^-) or photoexcited state of dihydroflavin $FADH^-$. Laser flash photolysis study using *N*,*N*-dimethylaniline as a reducing photosensitizer has also been conducted to identify the mechanism and evaluate the kinetic parameters involved in the reductive splitting of **1a**,**b**.

Experimental Section

Materials. 1-Methylthymine (**2a**) and flavin adenine dinucleotide (FAD) disodium salt were used as received from Sigma Chemical. Purified 1,3-dimethylthymine (**2b**) was kindly supplied by Fujii Memorial Research Institute, Ohtsuka Pharmaceutical. Ethylenediaminetetraacetic acid (EDTA), *N*,*N*-dimethylaniline (DMA), and 2-methyl-2-propanol were purchased from Nacalai Tesque and used without further purification. *N*,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine (TMPD), 2′-deoxythymidine, and 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. For fluorescence quenching experiments, tris(2,2′-bipyridyl)ruthenium(II) chloride hexahydrate ($Ru(bpy)_3Cl_2 \cdot 6H_2O$; Aldrich Chemical), dimethylbenzoquinone (Aldrich Chemical), terephthalaldehyde (Kanto Chemical), 1-methylnicotinamide chloride (Tokyo Chemical Industries), 5-nitrouracil (Tokyo Chemical Industries), methyl viologen (Nacalai Tesque), anthraquinone-2-sulfonate (Nacalai Tesque), and *p*-benzoquinone (Nacalai Tesque) were used as obtained commercially. C5−C5′-linked dihydrothymine dimers **1a**,**b** of 1-methylthymine **2a** and 1,3-dimethylthymine **2b** were synthesized and purified following the methods reported previously.[7] Aqueous solutions for all experiments were prepared using water purified with Corning Mega-Pure System MP-190 (>16 MΩ cm).

HPLC Analysis. Analytical HPLC was performed with Shimadzu 6A HPLC system equipped with Rheodyne 7725 sample injector. Sample solutions were injected onto a 5 μm C18 reversed-phase column (Wakosil $5C_{18}$, o.d. 4.6 mm × 150 mm, Wako). The phosphate buffer solutions (10 mM, pH 3.0) containing various concentrations of methanol (10−25 vol %) were delivered as the mobile phase. The column eluents were monitored by the UV absorbance at 210 nm.

Radiolytic Reduction. Aqueous solutions of C5−C5′-linked dihydrothymine dimers **1a**,**b** (0.5 mM) containing 2-methyl-2-propanol (50 mM) were buffered at pH 7.0 with phosphate buffer, and then purged with Ar before γ-irradiation. Steady-state γ-irradiation was performed in sealed ampules at room temperature with a ^{60}Co γ-ray source (dose rate: 0.75 Gy min^{-1}).

Photosensitized Reduction. Typically, solutions of **1a**,**b** (0.5 mM) in phosphate buffer containing FAD (0.2 mM) were purged with Ar before photoirradiation. For effective generation of the reduced form of FAD ($FADH^-$) in situ, EDTA (20 mM) was added to the solutions. The solutions in sealed Pyrex glass tubes were photoirradiated (λ_{ex} > 280 nm) under magnetic stirring (1000 rpm) at 24 °C with a high-pressure Hg arc (450 W, Eikosha 400).

Nanosecond Laser Flash Photolysis. The laser flash photolysis experiments were carried out with a Unisoku TSP-601 flash spectrometer, as described previously.[8] Aqueous solutions of **1a**,**b**[*meso*] and **1a**,**b**[*rac*] (1.0 mM) at pH 6.7 containing DMA (0.16−0.50 mM) were deaerated by Ar bubbling prior to the laser flash photolysis experiments.

Fluorescence Quenching. Various kinds of quenchers (0.1−4.0 mM) at varying concentrations were dissolved in phosphate buffer (10 mM, pH 7.0) solutions of $Ru(bpy)_3^{2+}$ (10 μM). Each sample solution (2 mL) was purged with Ar for 10 min, sealed in a quartz cell (10 × 10 × 35 mm) with a Teflon cap, and then subjected to the measurement of the fluorescence spectrum which was recorded on a Hitachi F-2000 fluorescence spectrophotometer (slit width: 1.0 nm, λ_{ex} = 450 nm, λ_{flu} = 600 nm).

Results

Splitting of C5−C5′-Linked Dihydrothymine Dimers by Hydrated Electrons Generated in the Radiolysis. γ-Radiolyses of deoxygenated solutions of 1-methyl-5,6-dihydrothymine dimers (**1a**[*meso*] and **1a**[*rac*]; 0.5 mM) and 1,3-dimethyl-5,6-dihydrothymine dimers (**1b**[*meso*] and **1b**[*rac*]; 0.5 mM) in phosphate buffer containing 2-methyl-2-propanol (50 mM) were carried out to characterize the reductive splitting reactivity of the C5−C5′-linked dihydrothymine dimers. In the radiolysis of a diluted aqueous solution, water radicals such as oxidizing hydroxyl radicals ($^\bullet OH$), reducing hydrated electrons (e_{aq}^-), and reducing hydrogen atoms ($^\bullet H$) are primarily generated with the *G* values[9] of $G(^\bullet OH) = 2.8 \times 10^{-7}$ mol J^{-1}, $G(e_{aq}^-) = 2.8 \times 10^{-7}$ mol J^{-1}, and $G(^\bullet H) = 0.6 \times 10^{-7}$ mol J^{-1} (reaction 1). Under the present conditions, the OH radicals are scavenged by excess amount of 2-methyl-2-propanol into substantially unreactive 2-hydroxy-2-methylpropyl radicals ($^\bullet CH_2(CH_3)_2COH$) as in reaction 2. Consequently, the hydrated electrons e_{aq}^-, ($E(nH_2O/e_{aq}^-) = -2.9$ V vs NHE[10] at pH 7.0) along with smaller amount of the hydrogen atoms ($E(H^+/^\bullet H) = -2.4$ V vs NHE[10] at pH 7.0) are involved as the reductants in the reactions of **1a**,**b**.

$$H_2O \rightarrow {}^\bullet OH, {}^\bullet H, e_{aq}^-, H^+, H_2, H_2O_2 \quad (1)$$

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

Analytical HPLC of the γ-irradiated solutions by reference to authentic samples demonstrated that the C5−C5′ linkages

SCHEME 1

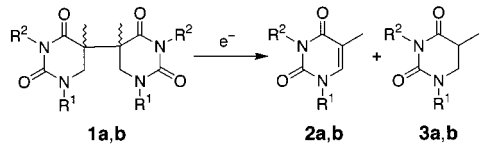


TABLE 1: Reductive Splitting of 1a,b (0.5 mM) in Deoxygenated Aqueous Solution by Radiation-Chemically Generated Hydrated Electrons

	G values × 10^7/mol J^{-1}			
substrate	−1	2	3	isomerizn
1a[*meso*]	2.2	0.59 (13%)[a]	2.1 (48%)	0.25 (11%)
1a[*rac*]	2.3	0.36 (8%)	2.0 (43%)	0.32 (14%)
1b[*meso*]	2.7	0.80 (15%)	2.8 (52%)	0.17 (6%)
1b[*rac*]	2.9	0.96 (17%)	2.9 (50%)	0.31 (11%)

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

of stereoisomeric dimers **1a**,**b**[*meso*] and **1a**,**b**[*rac*], split via one-electron reduction by hydrated electrons to produce the corresponding 5,6-dihydrothymine derivatives (**3a**,**b**) as the major products along with relatively smaller amounts of thymine monomers **2a**,**b** (Scheme 1). The one-electron reductive splitting of **1a**,**b** to **2a**,**b** and **3a**,**b** was accompanied to a lesser extent by isomerization from meso dimers **1a**,**b**[*meso*] to racemic dimers **1a**,**b**[*rac*] or vice versa. Apart from the distribution of product yields (see Table 1), the apparent reaction characteristics of one-electron reductive splitting are essentially the same as in the one-electron oxidative splitting reported recently.[8] Figure 1 shows linear dose−responses of the dimer decomposition and the product formation in the radiolytic reduction, the slopes of which gave the respective *G* values as listed in Table 1. Comparing with the *G* value of hydrated electrons ($G(e_{aq}^-) = 2.8 \times 10^{-7}$ mol J^{-1}) as the substantial species for one-electron reduction, it is apparent that the stereoisomeric 1,3-dimethyl-5,6-dihydrothymine dimers **1b** underwent reductive decomposition almost quantitatively ($G(-\mathbf{1b}) = (2.7-2.8) \times 10^{-7}$ mol J^{-1}), while the stereoisomeric 1-methyl-5,6-dihydrothymine dimers **1a** showed somewhat smaller reactivity toward hydrated electrons ($G(-\mathbf{1a}) = (2.2-2.3) \times 10^{-7}$ mol J^{-1}). In these radiolytic reductions, the major products of 5,6-dihydrothymines **3a**,**b** and thymines **2a**,**b** accounted for about 50% and 15% of the decomposed dimers, respectively. The minor isomerization occurred in 6−14% yields, which is comparable to the isomerization in the previously reported one-electron oxidative splitting.[8]

The above results are rationalized by a mechanism involving the primary intermediates of electron-dimer adducts (**4a**,**b**) that undergo splitting at the C5−C5′ linkages into 5,6-dihydrothymin-5-yl radicals (**5a**,**b**) and C5-anions (**6a**,**b**), as outlined in Scheme 4. The formation of the 5-yl radicals **5a**,**b** is common to both the one-electron reductive and oxidative splittings, while the counterparts in the latter splitting are C5-cations (**7a**,**b**).[8] It has been established that β-oxoalkyl radicals possess oxidizing property for several aromatic amines; e.g., 6-hydroxy-5,6-dihydrothymin-5-yl radical oxidizes TMPD [$E°(\text{TMPD}^{\bullet+}/\text{TMPD}) = 0.16$ V vs NHE][11] readily to form 6-hydroxy-5,6-dihydrothymine C5-anion and TMPD radical cation ($\text{TMPD}^{\bullet+}$).[12] In accord with such an oxidizing property, the 5,6-dihydrothymin-5-yl radicals **5a**,**b** could one-electron oxidize 5,6-dihydrothymine dimers **1a**,**b** to regenerate **5a**,**b** along with the formation of C5-cations **7a**,**b** that produce thymines **2a**,**b** via protonation, as reported recently.[8] Separate experiments with deoxygenated solution of **1a**[*meso*] containing TMPD (0.6 mM)

SCHEME 2

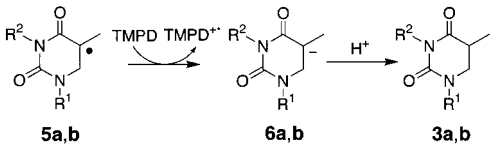


TABLE 2: Reductive Splitting of 1a,b (0.5 mM) by Photoexcited Reduced Form of FAD (*$FADH^-$) in Phosphate Buffer

		conversion/%	yield/%		ratio/%
substrate	additive	−1	2	3	2/3
1a[*meso*]	FAD	4	17	34	50
	FAD/EDTA	30	1	79	1
1a[*rac*]	FAD	5	5	72	7
	FAD/EDTA	26	1	75	1
1b[*meso*]	FAD	11	7	97	7
	FAD/EDTA	42	2	86	2
1b[*rac*]	FAD	11	8	93	9
	FAD/EDTA	28	2	91	2

gave only **3a** as a major product, suggesting that the intermediate 5-yl radical is one-electron reduced almost quantitatively by TMPD into C5-anion which undergoes protonation at C5 to form **3a** (Scheme 2). Consequently, the restoration of **2a** and the isomerization may be suppressed in the presence of TMPD.

Splitting of C5−C5′-Linked Dihydrothymine Dimers by Photoexcited Reducing Sensitizer. It has been suggested that in the DNA photorepair process the cyclobutane photodimer flips out of the DNA helix to fit into the "hole" domain of photolyase,[3k−o] and the fully reduced form of FAD cofactor ($FADH^-$) can catalyze the splitting reaction. In view of the photorepair reaction, $FADH^-$-sensitized photoreductive splitting reaction of the C5−C5′-linked dihydrothymine dimers **1a**,**b** was performed as a related model reaction. Upon photoexcitation of FAD (0.2 mM) in deoxygenated phosphate buffer containing **1a**,**b** (0.5 mM) in the presence of EDTA (20 mM), splitting of **1a**,**b** occurred to afford 5,6-dihydrothymine derivatives **3a**,**b** in considerably high yield (> 75%) along with the minor yield (<10%) of monomeric thymines **2a**,**b**, in which the formation of **3a**,**b** were more efficient than that in the radiation chemical reductions (Table 2). In the presence of a secondary reductant EDTA that converts FAD into a fully reduced form $FADH^-$ in situ, hydrogen transfer from EDTA to intermediate 5,6-dihydrothymin-5-yl radicals **5a**,**b** is possibly involved in the enhanced formation of **3a**,**b**. To remove the influence of the secondary reaction by EDTA, photoreactivity of the dimers in phosphate buffer was also investigated in the absence of EDTA. In a separate pulse radiolysis study, we confirmed that phosphate buffer is a weak reductant toward several radicals.[13] It is therefore likely that FAD may undergo photoreduction by phosphate buffer even in the absence of EDTA to produce $FADH^-$.[14] As shown in Table 2, while the efficiencies for decomposition of **1a**,**b** were decreased to significant extent, the yields of **2a**,**b** relative to **3a**,**b** became higher upon excluding hydrogen-donating EDTA. It is also remarkable that the restoration efficiency of **2a** from **1a**[*meso*] in the absence of EDTA is much greater than those from **1a**[*rac*] **1b**[*meso*], and **1b**[*rac*], and is comparable to those in the reductive γ-radiolysis (see also Table 1).

For comparing the reduction potentials of the four C5−C5′-linked isomeric dimers investigated, a fluorescence quenching kinetic study was carried out using tris(2,2′-bipyridyl)ruthenium(II) complex as a fluorescent electron donor. Recently, the reduction potentials of pyrimidine bases were successfully

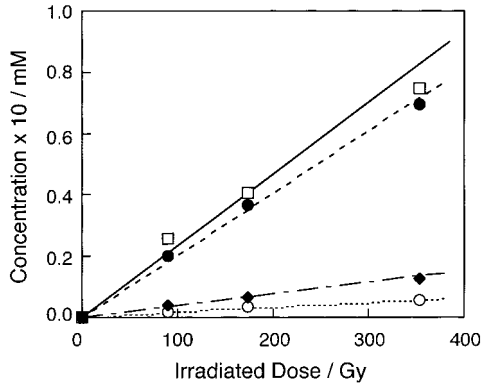


Figure 1. Reductive splitting of **1a**[*rac*] (0.5 mM) in the γ-radiolysis of deoxygenated phosphate buffer containing 2-methyl-2-propanol (50 mM): (□) decomposition of **1a**[*rac*]; formation of (◆) 1-methylthymine **2a**, (●) 5,6-dihydro-1-methylthymine **3a**, and (○) isomeric dimers **1a**[*meso*].

TABLE 3: $Ru(bpy)_3^{2+}$ Fluorescence Quenching Rate Constants and Reduction Potential (vs SCE) of Various Quenchers in Aqueous Solution

quencher	E_{red}/V	$k_q/10^9$ dm^3 mol^{-1} s^{-1}
p-benzoquinone	−0.16[a]	6.32
dimethylbenzoquinone	−0.32[a]	4.56
nitrofurantoin	−0.50[a]	11.4
3,5-dinitrobenzoic acid	−0.58[a]	7.16
anthraquinone-2-sulfonate	−0.63[a]	6.73
methyl viologen	−0.69[a]	5.13
5-nitrouracil	−0.77[a]	4.58
1-methylnicotinamide	−1.01[b]	0.323
terephthaldialdehyde	−1.04[b]	1.40
1,4-dimethylpyridinium	−1.39[b]	0.0379

[a] Reference 10. [b] Reference 19. The reduction potentials vs NHE are converted to the values vs SCE.

estimated from the rate constants of electron-transfer fluorescence quenching of several electron donors with varying oxidation potentials.[15] In general, electron transfer from an excited-state fluorescent molecule (electron donor D*) to a quencher molecule (electron acceptor A) proceeds as follows: D and A diffuse to encounter at a distance where electron-transfer becomes favorable, and thereafter an ion-pair state ($D^{\bullet+}$ $A^{\bullet-}$) forms to undergo dissociation into free radical ions $D^{\bullet+}$ and $A^{\bullet-}$.

$$D + A \xrightarrow{h\nu} D^* + A \underset{k_{-diff}}{\overset{k_{diff}}{\rightleftharpoons}} (D\cdots A)^* \underset{k_{-et}}{\overset{k_{et}}{\rightleftharpoons}} (D^{\bullet+}\ A^{\bullet-}) \xrightarrow{k_{dis}} D^{\bullet+} + A^{\bullet-}$$

Upon 450 nm excitation of $Ru(bpy)_3^{2+}$ (10 μM; $E^*_{ox} = -1.29$ V[11,16]) in aqueous solution, the characteristic fluorescence was observed with a maximum wavelength of 600 nm. The fluorescence quenching rate constants (k_q) of $[Ru(bpy)_3^{2+}]^*$ by a series of electron-accepting quenchers (see Table 3) were determined by a Stern−Volmer analysis.[17] Figure 2 shows a plot of k_q against the reduction potentials of quenchers, in which the Rehm−Weller relationship (eqs 3−6)[18] was fitted to the experimental data.

$$k_q = \frac{k_{diff}}{1 + \frac{k_{-diff}}{k_a K_{eq}}\left\{\exp\left(\frac{\Delta G^{\ddagger}_{et}}{RT}\right)\right\}} \quad (3)$$

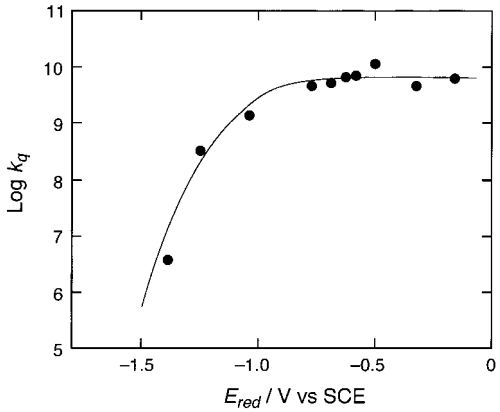


Figure 2. Rehm−Weller plot of the fluorescence quenching rate constant (k_q) in aqueous solution as a function of the reduction potential (E_{red}) of quencher. The solid curve is obtained from a best fit of experimental data to the Rehm−Weller empirical expression (see eqs 3−7).

$$\Delta G^{\ddagger}_{et} = \left[\left(\frac{\Delta G_{et}}{2}\right)^2 + \left(\frac{\lambda}{4}\right)^2\right]^{1/2} + \frac{\Delta G_{et}}{2} \quad (4)$$

$$\Delta G_{et} = 96.5\left(E^*_{ox} = E_{red} - \frac{q^2}{r\epsilon}\right) \quad (5)$$

$$\frac{k_{-diff}}{k_{diff}} = \frac{1}{K_{eq}} \quad (6)$$

where λ refers to solvent reorganization energy, $-q^2/r\epsilon$ is the Coulomb energy of the incipient ion pair formed after electron transfer, and k_a ($=\kappa_{el}\nu_n$) is the preexponential term of the expression for the rate constant of electron transfer.

It is evident in Figure 2 that k_q approaches the diffusion control limit ($k_{diff} = 6.5 \times 10^9$ dm^3 mol^{-1} s^{-1} in aqueous solution) as the reduction potential becomes increasingly positive (Figure 2). The best fit of the Rehm−Weller relationship in Figure 2 was obtained with kinetics parameters of $k_aK_{eq} = 5.0 \times 10^{12}$ dm^3 mol^{-1} s^{-1} and $\lambda = 117$ kJ mol^{-1}. The rate constants of quenching by **1a**,**b**[*meso*] and **1a**,**b**[*rac*] were also determined by a Stern−Volmer analysis (Figure 3), from which the corresponding redox potentials were estimated based on the best fitted Rehm−Weller relationship. Thus, almost the same k_q values for isomers of **1a** (k_q(**1a**[*meso*]) $= 5.48 \times 10^8$ dm^3 mol^{-1} s^{-1}, k_q(**1a**[*rac*]) $= 6.66 \times 10^8$ dm^3 mol^{-1} s^{-1}) and **1b** (k_q(**1b**[*meso*]) $= 2.50 \times 10^8$ dm^3 mol^{-1} s^{-1}, k_q(**1a**[*rac*]) $= 2.58 \times 10^8$ dm^3 mol^{-1} s^{-1}) were obtained, respectively, leading to the reduction potentials of E_{red}(**1a**) ≈ -1.15 and E_{red}(**1b**) ≈ -1.25 V vs SCE independent of isomeric structures. These E_{red} values are slightly positive compared to those of thymine ($E_{red} = -1.34$ V vs SCE) and thymidine ($E_{red} = -1.33$ V) in aqueous solution.[19] It follows that the difference in restoration efficiency between the isomeric dimers cannot be explained in terms of the reduction potentials.

Laser Flash Photolysis Study. Laser flash photolyses with the fourth harmonic at 266 nm of aqueous solutions of the stereoisomeric dimers **1a**,**b** (1.0 mM) containing *N*,*N*-dimethylaniline (DMA; 0.5 mM) as a reducing sensitizer were attempted to detect transient absorptions originating from the splitting reaction. Previous laser flash photolysis study has shown that DMA possessing an excited-state oxidation potential of −3.3 V is a good electron donor for splitting of the cyclobutane photodimers by a single-electron-transfer mechanism.[4] In this study, upon laser photolysis of DMA in deoxygenated aqueous solution, hydrated electrons and radical cations of DMA

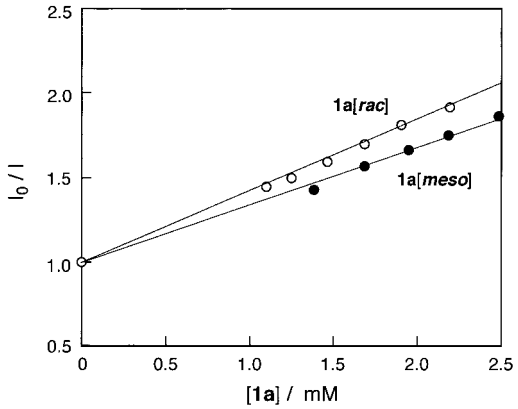


Figure 3. Stern−Volmer plots of the fluorescence quenching of $*Ru(bpy)_3^{2+}$ by (●) **1a**[*meso*] and (○) **1a**[*rac*]. Relative intensity (I_0/I) of the emission at 600 nm was measured in the presence of 10 μM $Ru(bpy)_3^{2+}$ in deoxygenated phosphate buffer solution (10 mM, pH 7).

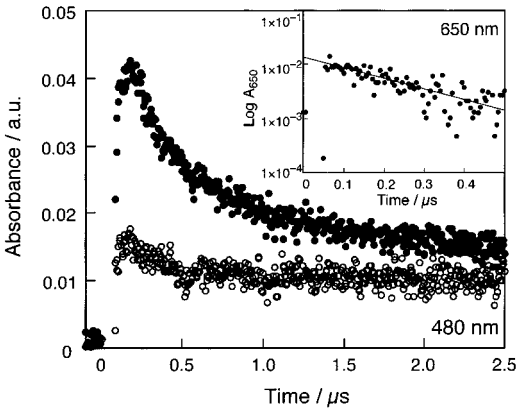


Figure 4. Time courses of the absorbancies at 460 nm in the 266 nm laser photolysis of DMA (0.5 mM) in phosphate buffer containing **1b**[*rac*] (1.0 mM): in the (○) presence and (●) absence of **1b**[*rac*]. Inset: Kinetic plot of the absorption at 650 nm due to e_{aq}^- in the presence of DMA.

($DMA^{\bullet+}$) were generated with the characteristic transient absorptions at $\lambda_{max} = 460$ and 650 nm, respectively.[20,21] Therefore, the hydrated electron generated by the direct photoionization of DMA (reactions 7 and 8) may be responsible for reductive splitting of the C5−C5′-linked dimers, rather than the photoexcited DMA (DMA*).

$$DMA + h\nu \rightarrow DMA^{\bullet+} + e_{aq}^- \quad (7)$$

$$T{-}T + e_{aq}^- \rightarrow T{-}T^{\bullet-} \quad (8)$$

Transient absorption spectra of $DMA^{\bullet+}$ ($\lambda_{max} = 460$ nm) and hydrated electron ($\lambda_{max} = 650$ nm) were detected immediately after the laser flash photolysis of deoxygenated aqueous solutions of **1a**,**b** and DMA. As shown in Figure 4, the absorption at 650 nm due to hydrated electrons decayed following pseudo-first-order kinetics ($k = 2.6-4.4 \times 10^9$ dm^3 mol^{-1} s^{-1}, [**1a**,**b**] = 1.0 mM), possibly as a consequence of the diffusion-controlled reaction with **1a**,**b** (Table 4). Previous pulse radiolysis and laser flash photolysis studies have demonstrated that *N*-substituted 5,6-dihydrothymin-5-yl radicals **5a**,**b** show broad absorption spectra at around 400 nm (**5a**: $\lambda_{max} = 400$ nm;[8] **5b**: $\lambda_{max} = 430$ nm[22]), but such transient absorption spectra of **5a**,**b** were not observed in this study. Alternatively, a slow buildup component of the transient absorption of $DMA^{\bullet+}$ was observed about 5 μs after the laser flash excitation, concomitant with a first-order decay profile for $DMA^{\bullet+}$ generated by photoionization (Figure 4). It is therefore most likely that the oxidizing 5-yl radicals **5a**,**b**, which should be generated by the C5−C5′ bond splitting of the primary intermediate radical anions of **1a**,**b** (reaction 9), are spontaneously reduced by DMA to form the corresponding C5-anions and $DMA^{\bullet+}$ (reaction 10), as in the reaction of **5a**,**b** with TMPD (Scheme 3).[12]

$$T{-}T^{\bullet-} \rightarrow T^- + T^{\bullet} \quad (9)$$

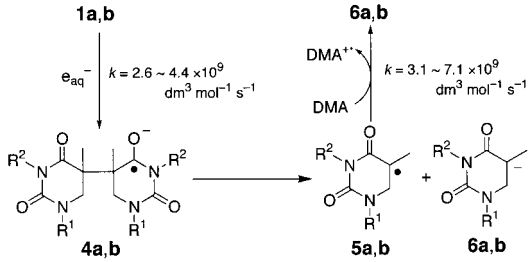
$$T^{\bullet} + DMA\ (TMPD) \rightarrow T^- + DMA^{\bullet+}\ (TMPD^{\bullet+}) \quad (10)$$

In accord with the secondary one-electron oxidation of DMA in the ground state, the slow buildup component of $DMA^{\bullet+}$ could be obtained by subtracting the contribution from the decay of primarily photogenerated $DMA^{\bullet+}$, as occurred within 5 μs after the laser flash, from the apparent overall absorption at 460 nm in the presence of the dimers. Thus, the slow buildup of $DMA^{\bullet+}$ was of the pseudo-first-order kinetics, and the corresponding formal rate constant was in proportion to the concentration of DMA (Figure 5). The slope of the linear plot in Figure 5 gives the intrinsic rate constants for reaction 10, as listed in Table 4. The rate constants thus evaluated are almost in a diffusion-controlled rate limit and are in good accordance with the literature value for the one-electron oxidation of TMPD by 5,6-dihydro-6-hydroxythymin-5-yl radical ($k = 1.3 \times 10^9$ dm^3 mol^{-1} s^{-1}) as derived from pulse radiolysis.[12]

TABLE 4: Rate Constants for One-Electron Reductions of 1a,b and 5a,b with Hydrated Electrons (e_{aq}^-) and DMA, Respectively

	k/dm^3 mol^{-1} s^{-1}	
substrate	$e_{aq}^- + \mathbf{1}$	DMA + **5**
1a[*meso*]	4.4×10^9	3.1×10^9
1a[*rac*]	3.7×10^9	4.8×10^9
1b[*meso*]	2.6×10^9	7.1×10^9
1b[*rac*]	3.0×10^9	6.7×10^9

SCHEME 3



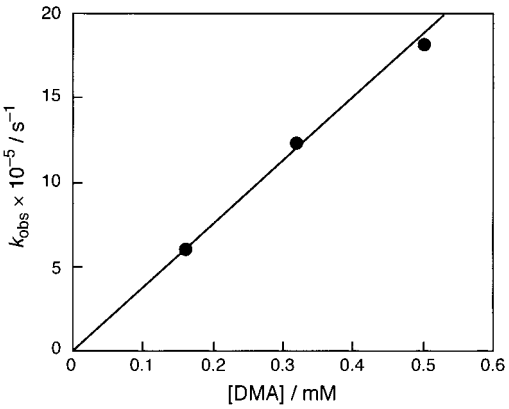
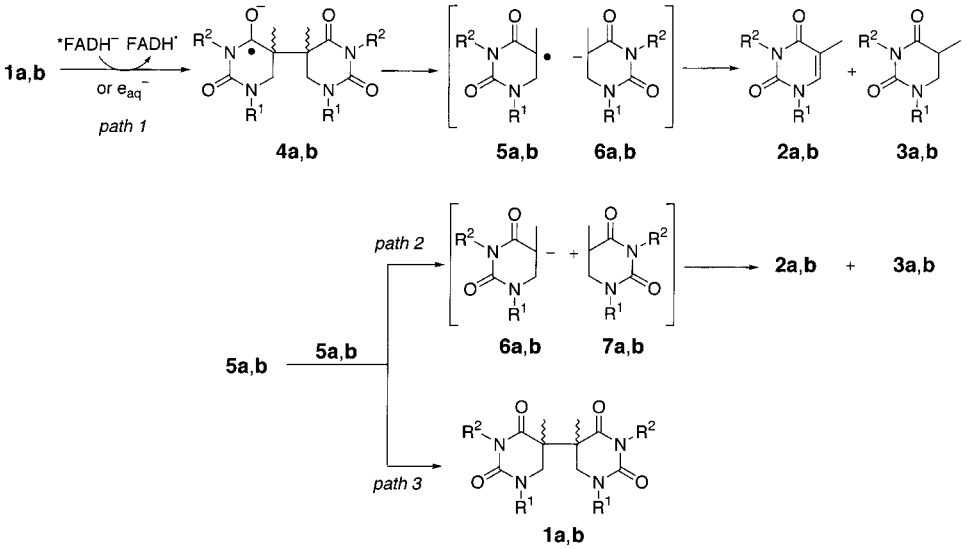
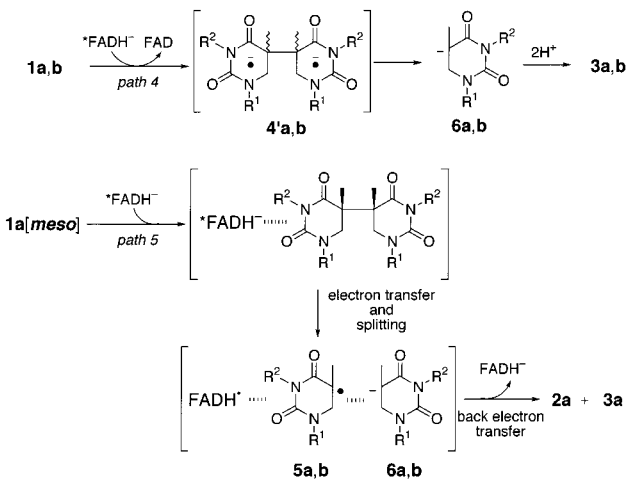


Figure 5. Dependence of k_{obs} of differential absorption growth at 460 nm on the concentration of DMA.

SCHEME 4



SCHEME 5



Discussion

Reductive Splitting Mechanism of C5−C5′-Linked Dihydrothymine Dimers. In light of the proposed mechanisms for the photorepair of cyclobutane pyrimidine dimers[3] and the oxidative splitting of the C5−C5′-linked dihydrothymine dimers,[8] possible pathways for the reductive splitting of C5−C5′-linked dihydrothymine dimers are proposed as illustrated in Schemes 4 and 5. Under the present experimental conditions of photochemical and radiation-chemical generation of hydrated electrons, the splitting is likely to be initiated by one-electron reduction of the C5−C5′-linked dimers into the dimer radical anions **4a**,**b**, although direct observation of the corresponding transient absorption spectra was unsuccessful in the present nanosecond laser flash photolysis. In view of the earlier results that the formation of radical anion intermediate of cyclobutane pyrimidine photodimer (Pyr<>Pyr$^{\bullet-}$) could not be confirmed clearly by nano- and picosecond laser flash photolysis, such radical anion intermediates **4a**,**b** may have much shorter lifetimes.[4,23] Subsequent C5−C5′-bond cleavage of **4a**,**b** produces the C5-anions (**6a**,**b**) and the 5,6-dihydrothymin-5-yl radicals **5a**,**b**, as confirmed by the redox reaction with reducing aromatic amines (DMA and TMPD). The isomerization of C5−C5′-linked dimers supports the formation of **5a**,**b** (Scheme 4, path 3). Concomitant formation of **2a**,**b** in the absence of aromatic amines suggests that disproportionation of **5a**,**b** leading to a pair of C5-anion **6a**,**b** and C5-cation (**7a**,**b**) is involved in the reductive splitting pathway (Scheme 4, path 2), which is a common reaction with the oxidative splitting mechanism.[8]

Flavin-Photosensitized Splitting of C5−C5′-Linked Dihydrothymine Dimers. Because of the existence of multiple protonation−reduction states of the flavin chromophore, the photoreactivation mechanism of photodimers has not been fully understood. The most likely mechanism involves initiation by exoergic one-electron transfer from the excited state of FADH$^-$ in DNA photolyase to the photodimers ($\Delta G_{et} = -125 \pm 30$ kJ mol^{-1})[24] with rate constants of $k_{et} = (5.5-6.5) \times 10^9$ s^{-1}.[25] The Rehm−Weller analysis on the flavin-photosensitized splitting of C5−C5′-linked dimers gave reasonable reduction potentials $E_{red} = -1.15-1.25$ V, which are slightly more positive than those of the monomers **2a**,**b**. Taking into account the reduction (E(FADH$^\bullet$/FADH$^-$) = −0.364 V at pH 7.0),[26] and the excitation energy (E_{00}(*FADH$^-$/FADH$^-$) = 2.58 V)[27] of FADH$^-$, the free energy for the electron transfer from *FADH$^-$ to the dimers **1a**,**b** is calculated from eq 5 as $\Delta G_{et} = -79.4$ kJ mol^{-1}, which shows exothermicity of the reaction.

The remarkably high efficiency of thymine restoration in the photoreduction of **1a**[*meso*] by *FADH$^-$, compared with those of the other dimers (**1a**[*rac*], **1b**[*meso*], and **1b**[*rac*]), could not be explained by the difference in the initial electron transfer efficiency. As a possibility, this feature may be related to the X-ray crystal structures of **1a**[*meso*] that are distinct from those of **1a**[*rac*] **1b**[*meso*], and **1b**[*rac*].[7b] For better understanding, the frame structures of **1a**[*meso*] and **1a**[*rac*] are illustrated in Figure 6.[7b] In a crystal, **1a**[*meso*] has a stacked conformation by which the two pyrimidine rings face each other, while the pyrimidine rings of **1a**[*rac*] are separated to reduce the mutual overlapping. In view of these structures, **1a**[*rac*] may allow successive two-electron reduction by FADH$^-$ (Scheme 5, path 4), whereas **1a**[*meso*] would favor one-electron reduction, rather than two-electron reduction, because of the generation of an electrostatic repulsion between the one-electron-reduced dihydropyrimidine rings. The intermediates (**4′a**,**b**) derived from successive two-electron reduction are expected to afford 2 equiv of C5-anions **6a**,**b**, thus resulting in exclusive formation of **3a**,**b** by protonation at C5. To characterize the conformation of **1a**,**b** in solution, NMR spectra were measured in dimethyl sulfoxide-d_6 at room temperature (Figure 7). In accord with the results of X-ray crystallography, the NOE difference spectra between H6

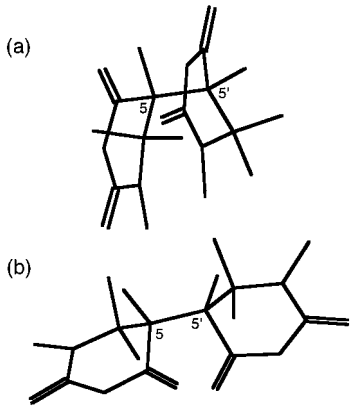


Figure 6. Frame structures of (a) **1a**[*meso*] and (b) **1a**[*rac*] obtained by X-ray crystallography (see ref 7b).

protons and N1-methyl protons of the two pyrimidine rings increased about 16% for **1a**[*meso*] and 6% for **1a**[*rac*], respectively. This result suggests that **1a**[*meso*] exhibits a "closed-shell" conformation in solution in contrast to the "opened-shell" conformation of **1a**[*rac*]. Furthermore, the NOE difference spectra for **1b**[*meso*] and **1b**[*rac*] in solution increased to a much lesser extent and there was no substantial difference between these stereoisomers (data not shown), although the dihydropyrimidine rings of **1b**[*meso*] and **1b**[*rac*] are stacked in a similar manner as those of **1a**[*meso*] in the crystal.[7b] Hence, due to the conformational preference in solution that may relieve the intramolecular electrostatic repulsion between the pyrimidine−electron adducts when formed, **1b**[*meso*] and **1b**[*rac*] could undergo successive two-electron reduction similar to **1a**[*rac*]. In this context, previous EPR spectroscopic studies have demonstrated that the radical anion of *trans-syn*-1,3-dimethyluracil dimer, pyrimidine rings of which are likely to be separated to each other,[2a] is relatively long-lived in contrast to the radical anions of the stacked conformations of *cis-syn*-thymine and uracil dimers that are unstable to split into monomer anions spontaneously.[28]

Another possibility is that the "closed-shell" conformation of **1a**[*meso*] may produce a relatively stable complex state with *FADH$^-$, thereby the 5-yl radical intermediate **5a** formed undergoing readily back electron transfer (BET) (path 5 in Scheme 5). However, such a back electron transfer reaction is less likely in the present case because of the oxidizing properties of pyrimidine 5-yl radicals. This is clearly in contrast to the photoinduced splitting mechanism of photodimers by which thymine monomers are regenerated by BET from the monomer radical anion to the FAD chromophore.

Finally, the reductive splittings of **1b**[*meso*] and **1b**[*rac*] seem to be slightly more effective than those of **1a**[*meso*] and **1a**[*rac*] (see Table 1), although the reduction potentials of **1a** are more positive than those of **1b**. It is likely that *N*-methyl substitution may increase a strain in the dimers to enhance the exothermicity of the splitting reaction. In fact, the enthalpy for splitting of uracil and thymine cyclobutane photodimers tended to become more negative by the methyl-substitution.[29] Accordingly, the instability would make the radical anions of **1b** undergo the C5−C5′-bond cleavage somewhat readily.

Conclusion

We have demonstrated that reductive splitting of C5−C5′-linked dihydrothymine dimers **1a**,**b** by hydrated electrons and the photoexcited reduced form of flavin (*FADH$^-$) affords the 5,6-dihydrothymine derivatives **3a**,**b** along with the corresponding thymine monomers **2a**,**b**. The efficiency for the reductive restoration into **2a**,**b** was significantly lower than those in the previously reported oxidative splitting reaction. According to the proposed reductive splitting mechanism, electron adducts of the dimers undergo the C5−C5′-bond cleavage to produce the 5,6-dihydrothymin-5-yl radicals **5a**,**b** and the C5-anions **6a**,**b**. Disproportionation of **5a**,**b** is the most likely reaction pathway for regeneration of the monomers **2a**,**b**. The relatively high efficiency of restoration into **2a** in the photoreduction of **1a**[*meso*] by *FADH$^-$ suggested that the stacked pyrimidine rings of **1a**[*meso*] does not favor a successive two-electron reduction as in the other dimers (**1a**[*rac*], **1b**[*meso*], and **1b**[*rac*]) with a common conformation of separated pyrimidine rings. This is in accord with the conformational studies by NOESY and X-ray crystallography.

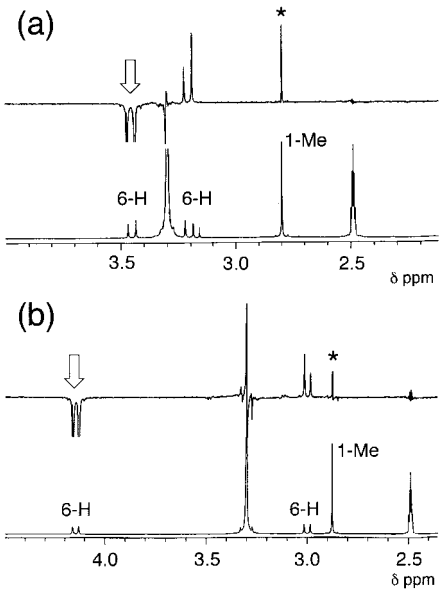


Figure 7. 400 MHz ^{1}H NMR spectra (lower trace) and NOE difference spectra (upper trace) of (a) **1a**[*meso*] and (b) **1a**[*rac*] in dimethyl sulfoxide-d_6 at 303 K. Arrows show irradiated H6 peaks.

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(14) In the absence of EDTA, **3a**,**b** are major products (see Table 2) whereas oxidative splitting of **1a**,**b** by anthraquinone-2-sulfonate (AQS) sensitization affords almost equivalent amounts of **2a**,**b** and **3a**,**b** (ref 8). This indicates the reduction process is predominant in the present case. In addition, photoreduction of FADH$^{\bullet}$ by pyrimidine photodimer has not been observed (Heelis, P. F.; Payne, G.; Sancar, A. *Biochemistry* **1987**, *26*, 6, 4634−4640).

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(17) The dynamic quenching rate constants k_q were determined by the Stern−Volmer equation: $I_0/I = 1 + k_q\tau_0$[Q], where I_0 and I are the intensity of the fluorescence at 600 nm when excited at 450 nm in the absence and presence of a quencher, respectively. The lifetime τ_0 of *Ru(bpy)$_3^{2+}$ in the absence of a quencher is known to be 0.62 μs (Kalyanasundaram, K. *Coord. Chem. Rev.* **1982**, *46*, 159−244).

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