Biological Radicals

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Oxidation and Reduction of the 5-(2′-Deoxyuridinyl)methyl Radical**

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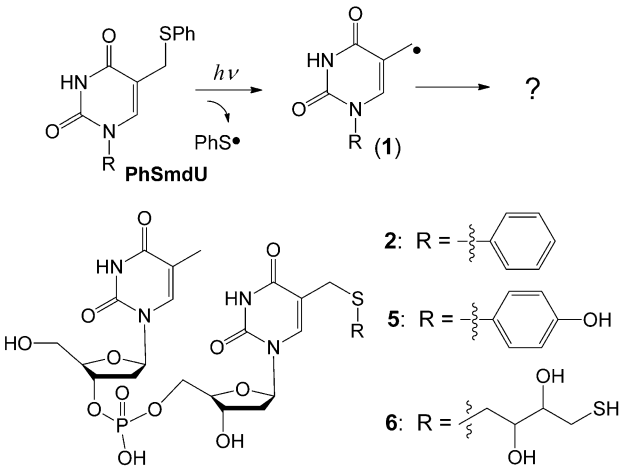
Thymine is susceptible toward ionizing radiation, a typical source of reactive oxygen species (ROS). The 5-(2′-deoxyuridinyl)methyl radical (**1**) is a key intermediate of thymine radical reactions involving either a hydroxyl radical-mediated H atom abstraction at the methyl moiety[1] or a one-electron oxidation followed by deprotonation process.[2] Species **1** was implied to possess neither reducing nor oxidizing properties.[3] It can add to atom C8 of a vicinal guanine or adenine under anaerobic conditions.[4] A similar 5-(2′-deoxycytidinyl)methyl radical gave rise to an intrastrand cross-link lesion in dinucleotides d(mCpG) and d(GpmC).[5] Further studies of **1** by Greenberg et al. found that it also reacts with the opposing 2′-deoxyadenosine in duplex DNA to yield a cross-linked TpA dimer with the thymine methyl group added to the adenine N6.[6] Such a lesion is readily detected in γ-irradiated DNA and accounts for at least 25% of the DNA interstrand cross-links.[6b]

These cross-linked base dimers are suggested to block replication and transcription and are quite toxic to cells. It is thus of great significance to understand the reactivity of **1**. O_2 reacts with **1**, forming a peroxyl radical intermediate, which then decomposes to yield 5-(hydroxymethyl)-2′-deoxyuridine (HmdU), 5-(hydroperoxy methyl)-2′-deoxyuridine (HpmdU), and 5-formyl-2′-deoxyuridine (fdU).[1e,7] Specifically, the formation of HpmdU requires one electron to reduce the peroxyl radical precursor, which may be provided by a superoxide radical ($O_2^{\bullet-}$)[8] or a thiol compound.[7a] The reaction between **1** and O_2 is reversible;[9] however, it is still two orders of magnitude faster than its reaction with thiol (H abstraction),[7a] suggesting that O_2 may prevent the nucleobase cross-linking reaction. However, a recent study revealed that after complete consumption of the radical precursor, only 25% of the generated **1** were involved in the crosslinking reaction with adjacent nucleobases. The yield of the cross-linking product remains the same under degassed or aerobic conditions.[6a] The fact that the presence of O_2 did not further lower the reaction yield implies that other routes may also be involved in the quenching process of **1**.

We thus re-examined the reactivity of **1** generated by photolytic cleavage of the C–S bond in 5-(phenylthiomethyl)-2′-deoxyuridine (PhSmdU, Scheme 1). The PhSmdU was linked to another thymidine by phosphoramidite chemistry to yield the dinucleotide TpPhSmdU (**2**).[10] The photoreaction of **2** was allowed to proceed for 5 min under 254 nm UV light in the presence or absence of O_2 (Figure 1), which usually consumed about 15% of **2**. The reaction was then analyzed by HPLC; the yields for the major products in the anaerobic reactions are shown in Table 1. Our results suggest that **1** can undergo both oxidative and reductive reactions in the absence of O_2; the resulting anion and cation are quenched by the general acid and base in solution respectively. Under an oxidizing environment, the anion formation is inhibited. Under a reducing environment, the cation formation is suppressed.



Scheme 1. Generation of **1** by photolytic cleavage of the C–S bond in 5-(phenylthiomethyl)-2′-deoxyuridine (PhSmdU) as well as thiol addition products in the dinucleotide context.

The aerobic reaction generated TpHmdU, TpHpmdU, and TpfdU (Figure 1 A), in agreement with the previous reports that **1** is prone to O_2 oxidation.[1e,7a] Surprisingly, an unknown product X was also generated. In contrast, the anaerobic reaction yields TpT and X as the major products and TpHmdU as the minor product. The TpT formation is puzzling as it was suggested to be formed by an H-abstraction mechanism, with thiols such as glutathione or thiophenol serving as the H-donor.[1e,7a] However, no thiol was added to our reaction. One possibility is that the PhS· generated may

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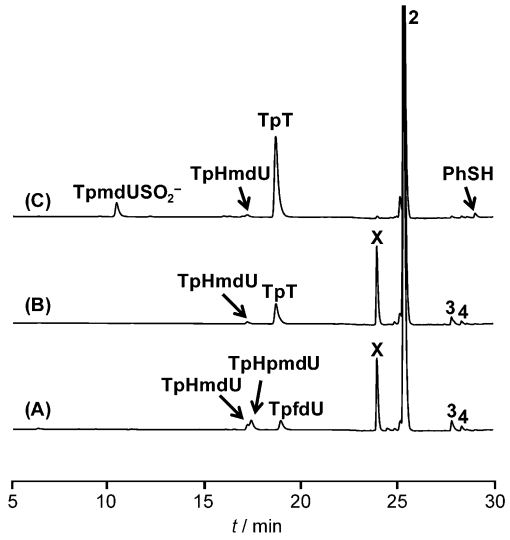


Figure 1. HPLC chromatograph of TpPhSmdU (**2**, 200 μM) photoreaction under 254 nm UV light. A) In H_2O in air; B) in degassed H_2O in a Coy anaerobic chamber; C) in degassed H_2O in a Coy anaerobic chamber and supplemented by 1 mM sodium dithionite.

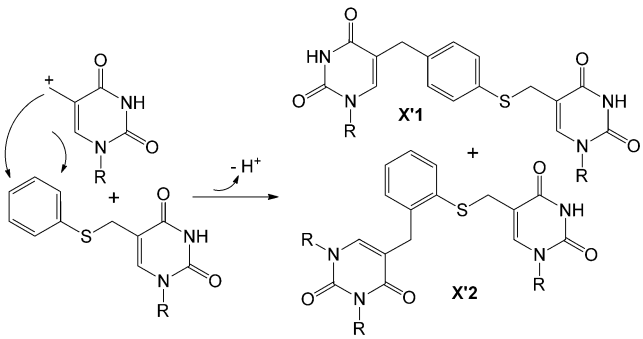
Table 1: Product yields in photoreactions of **2** at the absence of O_2.

Reactions	Yield [%][a]			Mass balance
	Anion	Cation		
	TpT	Adduct X	Other thiol adduct	
none	44.5 ± 2.3	43.2 ± 4.1	–	89.2 ± 5.9[b]
$Na_2S_2O_4$	77.5 ± 3.4	–	–	91.3 ± 4.3[c]
PhSH	85.4 ± 5.7	–	–	87.3 ± 7.0
4-OHC_6H_4SH	39.0 ± 2.5	–	46.6 ± 1.9	86.2 ± 3.0
DTT (1 mM)	48.5 ± 2.1	37.4 ± 3.9	5.1 ± 0.7	88.7 ± 5.4
DTT (10 mM)	44.0 ± 1.9	26.1 ± 1.5	20.7 ± 1.4	89.1 ± 4.2
DTT (40 mM)	36.3 ± 4.0	13.8 ± 1.9	33.8 ± 3.3	86.5 ± 7.5

[a] The yields were based upon the amount of **2** reacted and were calculated by HPLC peak integrations (See the Supporting Information). [b] The yield of TpHmdU was about 2.5%. [c] The yield of $TpmdUSO_2H$ was 12.1 ± 2.8%.

be reduced to PhSH and subsequently donates an H atom to **1** to yield TpT. To exclude this possibility, 1 mM PhSH was added to the solution before **2** was irradiated. Such a large excess of PhSH only improved the TpT yield by about 20% (Figure 3B), proving that PhSH can serve as the H donor; however, such an H donation process is not the major reaction pathway to quench **1**.

Besides TpT, species X is another major product, whose nature needs to be revealed before the reactivity of **1** can be understood. X exhibits a mass of 1199.26 in the positive-ion mode, corresponding to a formula of [TpT + **2**-2H]. Such a compound can be obtained either by TpT radical addition to **2** followed by elimination of an H atom or by TpT cation addition followed by loss of a proton. ^{1}H NMR analysis suggests X to be a mixture of products with the thymine methyl group added to the phenyl ring of **2** (Figure 2A); however structural details cannot be obtained for the compounds that comprise the spectrum owing to signal overlap. This addition pattern was further supported by the MS/MS spectrum of X, in which three major fragments were found (Figure 2B), with the corresponding chemical structures shown in Figure 2A. To reveal the structure of X, we examined the PhSmdU photoreaction as a model system.[10] As expected, the reaction produced thymidine and HmdU. More importantly, two species X′1 and X′2 were formed and separated by HPLC, both of which possess a mass of [T + PhSmdU-2H]. ^{1}H NMR analyses confirm that the thymine methyl moiety is added to the *para* position of the phenyl ring in X′1 and to the *ortho* position in X′2 (Scheme 2). The yield of isolated X′2 is about 50% higher than that of X′1. As the ring possesses two positions *ortho* and one position *para* to the SR substituent, our data



Scheme 2. Addition of the thymine methyl cation to the *para* position of the phenyl ring in X′1 and to the *ortho* position in X′2.

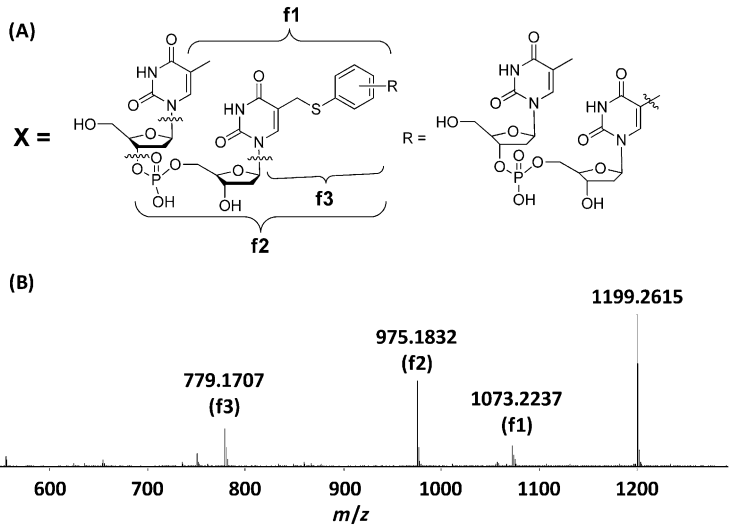


Figure 2. A) Proposed structure of product X, suggesting that a TpT moiety is added to the phenyl ring of -SPh. B) LC-MS/MS analysis of ion $[X+H]^+$ at 1199.26. The structures of the three fragmentation ions (f1, f2, and f3) are indicated in (a).

suggest that the *ortho* positions are slightly less active toward the addition reaction probably due to the steric hindrance of the SR moiety.

Such a reaction pattern suggests that X is formed by a cation addition mechanism. If a radical addition is involved, three products, with the thymine methyl group added randomly to any of the five positions at the phenyl ring, are expected. The thymine cation is still formed in the presence of O_2, as indicated by the generation of X in Figure 1 A. Its formation may be explained by competitive elimination of the superoxide radical (O_2^{-}) from the peroxyl radical precursor, similar to what was observed in the addition of O_2 to 2′-deoxyuridin-1′-yl radical.[11] Alternatively, the peroxyl radical may undergo a reverse process,[9] resulting in **1** and O_2 with **1** being subsequently oxidized to the cation. Although HmdU is typically formed by O_2 oxidation to **1**,[1e,7a,12] the TpHmdU formed in the absence of O_2 likely results from the H_2O addition to the cation followed by loss of a proton. After diluting **2** by tenfold, the TpHmdU yield is improved at the expense of X,[10] further supporting this mechanism. Moreover, if the reaction is conducted in methanol, the corresponding methoxy adduct TpMeOmdU is produced.[10]

The presence of thiol compounds barely enhances the TpT formation, contrasting with the fourfold enhancement induced by 1 mM sodium dithionite in Figure 1 C. Photoreaction of **2** in the presence of 1 mM PhSH results in the disappearance of X (Figure 3 B). As PhSH is an excellent nucleophile, it likely quenches the thymine cation, reproducing **2**, which escapes from the HPLC detection. To test this hypothesis, we repeated the photoreaction in the presence of 1 mM 4-hydroxythiophenol (4-OH-C_6H_4-SH). As expected, formation of X was suppressed and a new species **5** was generated (Figure 3 C). NMR and MS analyses confirm that **5** is the addition product Tp(4-OH)C_6H_4SmdU formed between the TpT cation and 4-OH-C_6H_4-S^- (Scheme 1).[10] Equal amounts of **5** and X were produced as shown in Figure 3 A and C, indicating that **5** is formed at the expense of X (Table 1). The cation can also be trapped by other thiols, such as DTT. Addition of 1–40 mM DTT did not markedly increase the yield of TpT (Figure 3 D–F), again suggesting that H-abstraction is unlikely to play a major role. Under 1 mM DTT, formation of X decreased by only about 10%, contrasting to the nearly complete quenching by 1 mM PhSH. With 40 mM DTT, formation of X decreased by 50%. These results are in line with the rationale that DTT is a better reductant, but a much worse nucleophile than PhSH. The reactions also yield the expected TpmdU-DTT adduct **6** (Scheme 1).[10] **6** exist as a pair of DL isomers, as indicated by the doublet peak in HPLC chromatograph, which result from the DL mixture of DTT used.

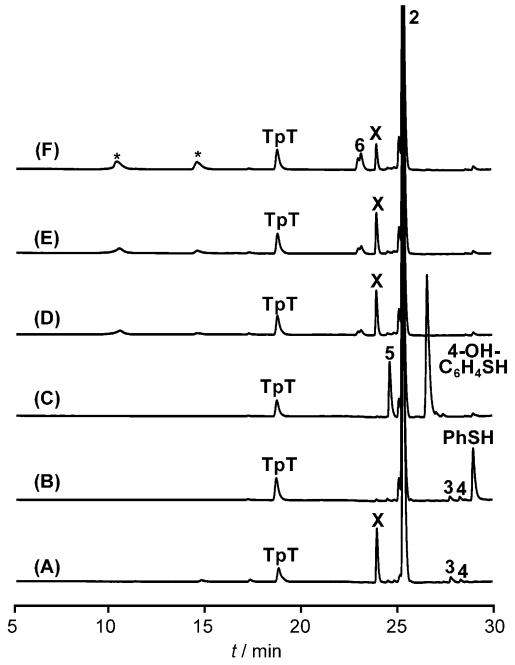


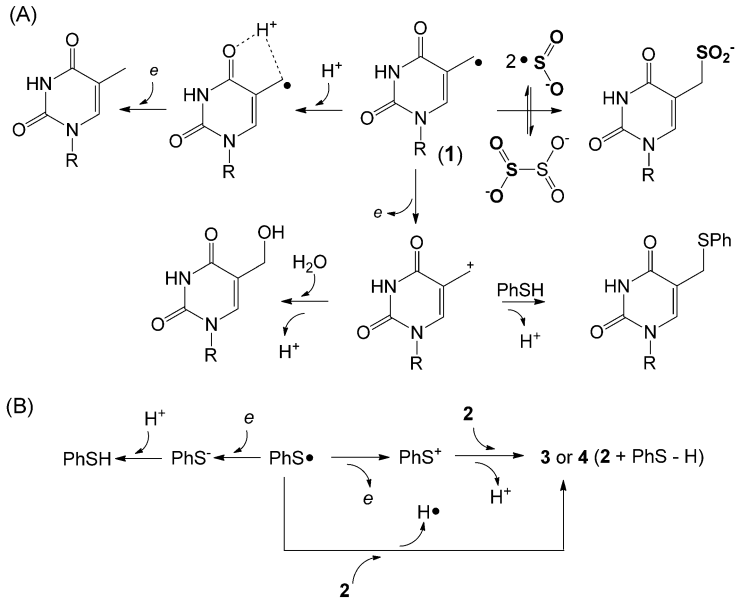
Figure 3. HPLC chromatograph of anaerobic TpPhSmdU (**2**, 200 μM) photoreaction under 254 nm UV light. A) In H_2O; B) with 1 mM PhSH; C) with 1 mM 4-OH-C_6H_4SH; D) with 1 mM DTT; E) with 10 mM DTT; and F) with 40 mM DTT. As the DTT utilized was a DL mixture, the resulting adducts **6** were a mixture as well, as indicated by the doublet peak in the HPLC chromatograph. The two peaks marked by * correspond to DTT and DTT disulfide, respectively, which already exist in the DTT used.[13]

Formation of thymine cation from **1** is a one-electron oxidation process. As no obvious electron acceptor can be identified in the anaerobic reaction, it is intriguing to suggest that another molecule of **1** accepts the electron to yield a methyl anion, which then obtains a proton from water, yielding TpT. This hypothesis is supported by the reaction in D_2O. The bond dissociation energy (BDE) for the O–H bond in water is 119 kcal mol^{-1},[14] which is about 30 kcal mol^{-1} higher than the C–H bond in the thymine CH_3 group.[15] It is highly unlikely that **1** would abstract a deuterium atom from D_2O. However, more than 95% of the TpTs formed contain one deuterium, which is located on the methyl group, as shown by ^{1}H NMR spectroscopy.[10] When **2** was irradiated in [D_1]methanol (CH_3OD), more than 95% of the TpTs are [D_1]TpTs as well,[10] showing that the stronger O–D bond in CH_3OD is involved in TpT formation. As 2-deoxyribose is prone for H-abstraction reactions,[16] these labeling studies also allow us to exclude the possible involvement of the 2-deoxyribose. Collectively, our data discriminate against the H-abstraction mechanism, but support the anion reaction. The formed anion takes a deuteron from solvent to yield [D_1]TpT.

Should **1** be reduced by another thymine radical, it would be reduced by a stronger reductant. We thus examined the reactivity of **1** in the presence of 1 mM sodium dithionite. As expected, the reducing environment totally abolished the cation-related products; the radical reduction product TpT was increased about fourfold (Figure 1 C), confirming that the presence of a strong reductant facilitates the thymine anion formation. The reaction also produced TpmdUSO_2^- as a minor product (Figure 1 C),[17] with its yield roughly 1/6 of that for TpT. TpmdUSO_2^- is likely formed by a radical recombination mechanism. The dithionite dianion has a dissociation constant $K_d \approx 10^{-6}$ mM in water,[18] which results in the formation of $^{\bullet}SO_2^-$. UV irradiation may promote the S–S bond cleavage in dithionite dianion, making the concentration of $^{\bullet}SO_2^-$ even higher. It should be fairly favorable for **1** to

recombine with $^{\bullet}SO_2^-$, yielding $TpmdUSO_2^-$. Although the $TpmdUSO_2^-$ formation rate cannot be obtained owing to the difficulty to determine the exact concentrations of **1** and $^{\bullet}SO_2^-$, the lower yield of $TpmdUSO_2^-$ indicates that the radical reduction reaction to produce TpT likely occurs with a rate that is at least comparable to that of the radical recombination reaction.

These experiments clearly indicate that **1** can undergo both oxidation and reduction reactions (Scheme 3A); it disproportionates in the absence of stronger redox reagents.



Scheme 3. Oxidation and reduction reactions of **1** and PhS•.

Pyrimidine radicals were known to disproportionate under anaerobic conditions.[3,19] However, the reaction of **1** is different as the disproportionations of previous pyrimidine radicals and traditional alkyl radicals require an H atom β to the radical center and the reactions proceed by a radical mediated H-abstraction mechanism.[20] We tentatively ascribe the difference to the polar solvents used here, which stabilize the charged reaction intermediates by electrostatic interactions. Moreover, although **1** can be reduced, a direct one-electron reduction followed by protonation to yield thymine is unfavorable owing to the poor stability of the thymine methyl anion intermediate. Compound **1** is likely to be protonated at the C4=O bond to a radical cation before the reduction occurs (Scheme 3A).

The mechanistic discussions above only involve the carbon-based radical **1**. Considering the similar electronegativity between C (2.544) and S (2.589),[21] the PhS• generated after the C–S bond cleavage in **2** is likely involved in the redox reactions as well. We thus carefully quantified all of the PhS• related redox products under varying pH. The photoreaction produces two minor products **3** and **4** (Figure 1A,B and Supporting Information), both of which exhibit a mass of 761.14 at the negative-ion mode,[10] corresponding to a formula of [PhS• + **2**-H]. Limited by the low yields, we were unable to isolate enough compound for NMR spectroscopy characterization. However, **3** and **4** are likely to be formed either by PhS^+ addition to a double bond in **2** followed by loss of a H^+, similar to the generation of X, or by PhS• addition to **2** followed by loss of a H• ($e + H^+$). The released H• can combine with PhS• or **1**, resulting in the reducing products PhSH and TpT, respectively. Both routes indicate that **3** or **4** can be treated as a cation product of PhS• (Scheme 3B). Furthermore, the formed PhSH, the anion (reducing) product of PhS•, was extracted with hexane and quantified by GC-MS spectroscopy (Table 2).

Excellent mass balance was obtained for the redox process in all of the reactions in Table 2. When pH ≤ 7, the amounts of cationic and anionic products of **1** are roughly equal. Assuming PhS• and **1** do not interact with each other, disproportionation of **1** becomes a simple interpretation for our data. In contrast, under basic pH where $[H^+]$ is low, the thymine anion formation is unfavored. Species **1** is still oxidized to a cation; however, the preferred electron acceptor becomes PhS•, yielding PhS^-. Formation of PhS^- was implied by a 5,6-dihydro-5-hydroxythymidin-6-yl radical study.[22] Furthermore, we observed the dimer of **1** in our reaction, the formation of which was suggested in previous studies.[3,16b,19b,c] The dimer yield is very low under neutral or acidic pH, which is consistent with the low yield obtained in $TpmdUSO_2^-$ formation. These results re-confirm that the radical recombination pathway is unfavorable; dimer formation increases only when the disproportionation reaction slows down at pH 11. As PhS• is the electron acceptor in this case, this result also indicates the electron transfer between **1** and PhS• to be slightly slower than that between two molecules of **1**, which likely rationalizes why disproportionation of **1** is favored in most of our reactions.

It is worth pointing out that the electron transfer process between two **1** molecules (radical disproportionation reaction) is unlikely to happen in vivo as it is implausible to have two thymine radicals close to each other in duplex DNA. Our

Table 2: Products isolated [nmol] in photoreactions of **2** [40 nmol].

	Anion products		Cation products			Dimer of **1**
pH	TpT	PhSH	TpTOH	X	**3** + **4**	
3–7	2.73 ± 0.22	0.78 ± 0.05	0.25 ± 0.03	2.50 ± 0.20	0.55 ± 0.03	< 0.02
9	2.88 ± 0.10	0.94 ± 0.06	0.29 ± 0.02	2.83 ± 0.17	0.66 ± 0.07	0.05 ± 0.01
11	0.38 ± 0.05	2.59 ± 0.12	0.85 ± 0.07	2.18 ± 0.14	0.20 ± 0.05	0.39 ± 0.04

data should be interpreted as the indication that **1** possesses both oxidizing and reducing properties; thus the current paradigm in DNA biochemistry may not be correct.[3] Formation of cation or anion from **1** may dominate in vivo given a different local redox environment. Also, without O_2, **1** was proposed to be quenched by an H-abstraction reaction with the H atom provided by thiol compounds.[1e,7a] Our report suggests that this conclusion may need to be re-examined.

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