Independent Generation of the 5-Hydroxy-5,6-dihydrothymidin-6-yl Radical and Its Reactivity in Dinucleoside Monophosphates

Qibin Zhang and Yinsheng Wang*

Contribution from the Department of Chemistry-027, University of California at Riverside, Riverside, California 92521-0403

Received March 15, 2004; E-mail: yinsheng.wang@ucr.edu

Abstract: Hydroxyl radical is a major reactive oxygen species produced by γ-radiolysis of water or Fenton reaction. It attacks pyrimidine bases and gives the 5-hydroxy-5,6-dihydropyrimidin-6-yl radical as the major product. Here we report the synthesis of all four stereoisomers of 5-hydroxy-6-phenylthio-5,6-dihydrothymidine (T*), which, upon 254 nm UV irradiation, give rise to the 5-hydroxy-5,6-dihydrothymidin-6-yl radical (**I**). We also incorporated the photolabile radical precursors into dinucleoside monophosphates d(GT*) and d(TT*) and characterized major products resulting from the 254-nm irradiation of these dinucleoside monophosphates. Our results showed that, under anaerobic conditions, the most abundant product emanating from the 254-nm irradiation of d(GT*) and d(TT*) is an abasic site lesion. Products with the thymine portion being modified to thymine glycol and 5-hydroxy-5,6-dihydrothymine were also observed. In addition, we demonstrated that radical **I** can attack the C8 carbon atom of its 5′ neighboring guanine and give rise to a novel cross-link lesion. Moreover, LC−MS/MS results showed that γ-radiation of d(GT) under anaerobic condition yielded the same type of cross-link lesions.

Introduction

Oxidative DNA damage plays an important role in a number of pathological conditions, including cancer,[1] neurodegeneration,[2] and aging.[3] Hydroxyl radical is one of the major reactive oxygen species (ROS) formed from Fenton reaction[4−6] or the γ-radiolysis of water.[7] It is well-documented that hydroxyl radical reacts with thymine either by adding to the C5=C6 double bond or by abstracting a hydrogen atom from the methyl group (Scheme 1a). The addition to the C5 carbon atom, however, is the major pathway (60%).[7] The secondary radicals formed from the hydroxyl radical attack may further decompose or react with neighboring nucleoside to form stable diamagnetic products.
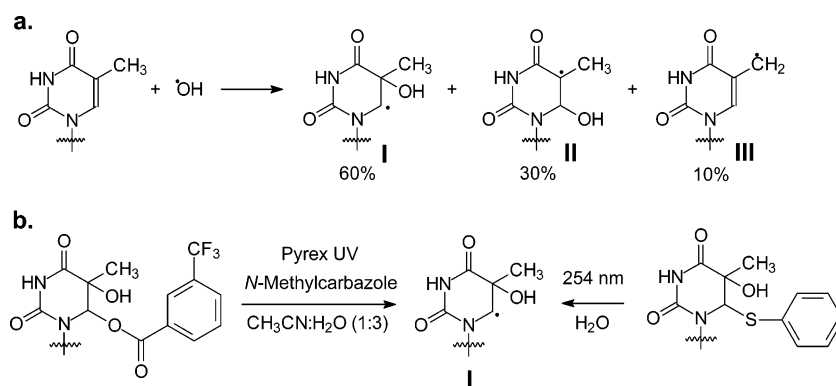
The independent generation of reactive intermediates of nucleosides, especially those from their photolabile precursors, facilitates the examination of the roles of these reactive intermediates in DNA damage.[8] Precursors for a number of radical intermediates, which are generated either on the pyrimidine base, such as pyrimidin-6-yl radical,[9,10] 5-hydroxy-5,6-dihydropyrimidin-6-yl radical,[11] and pyrimidin-5-methyl radical,[12−15] or from the deoxyribose ring, such as radicals on the C1′,[16−21] C3′,[22] and C4′[23−29] carbon atoms, have been synthesized, and their reactivities have been examined. Recently, we reported the independent generation of the 5-methyl radical of 5-methyl-2′-deoxycytidine and the facile formation of an

(1) Dizdaroglu, M. *NATO ASI Ser., Ser. A* **1999**, *302*, 67−87.
(2) Rolig, R. L.; McKinnon, P. J. *Trends Neurosci.* **2000**, *23*, 417−424.
(3) Finkel, T.; Holbrook, N. J. *Nature* **2000**, *408*, 239−247.
(4) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 2nd ed.; Oxford University Press: New York, 1989.
(5) Halliwell, B.; Gutteridge, J. M. C. *FEBS Lett.* **1992**, *307*, 108−112.
(6) Henle, E. S.; Linn, S. *J. Biol. Chem.* **1997**, *272*, 19095−19098.
(7) von Sonntag, C. *The Chemical Basis of Radiation Biology*; Taylor & Francis: London, 1987.
(8) Greenberg, M. M. *Chem. Res. Toxicol.* **1998**, *11*, 1235−1248.
(9) Carter, K. N.; Greenberg, M. M. *J. Org. Chem.* **2003**, *68*, 4275−4280.
(10) Carter, K. N.; Greenberg, M. M. *J. Am. Chem. Soc.* **2003**, *125*, 13376−13378.
(11) Barvian, M. R.; Barkley, R. M.; Greenberg, M. M. *J. Am. Chem. Soc.* **1995**, *117*, 4894−4904.
(12) Anderson, A. S.; Hwang, J. T.; Greenberg, M. M. *J. Org. Chem.* **2000**, *65*, 4648−4654.
(13) Bellon, S.; Ravanat, J. L.; Gasparutto, D.; Cadet, J. *Chem. Res. Toxicol.* **2002**, *15*, 598−606.
(14) Romieu, A.; Bellon, S.; Gasparutto, D.; Cadet, J. *Org. Lett.* **2000**, *2*, 1085−1088.
(15) Zhang, Q.; Wang, Y. *J. Am. Chem. Soc.* **2003**, *125*, 12795−12802.
(16) Gimisis, T.; Chatgilialoglu, C. *J. Org. Chem.* **1996**, *61*, 1908−1909.
(17) Goodman, B. K.; Greenberg, M. M. *J. Org. Chem.* **1996**, *61*, 2−3.
(18) Chatgilialoglu, C.; Gimisis, T.; Guerra, M.; Ferreri, C.; Emanuel, C. J.; Horner, J. H.; Newcomb, M.; Lucarini, M.; Pedulli, G. F. *Tetrahedron Lett.* **1998**, *39*, 3947−3950.
(19) Gimisis, T.; Ialongo, G.; Chatgilialoglu, C. *Tetrahedron* **1998**, *54*, 573−592.
(20) Tronche, C.; Goodman, B. K.; Greenberg, M. M. *Chem. Biol.* **1998**, *5*, 263−271.
(21) Hwang, J.-T.; Greenberg, M. M. *J. Am. Chem. Soc.* **1999**, *121*, 4311−4315.
(22) Koerner, S.; Bryant-Friedrich, A.; Giese, B. *J. Org. Chem.* **1999**, *64*, 1559−1564.
(23) Giese, B.; Dussy, A.; Elie, C.; Erdmann, P.; Schwitter, U. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1941−1944.
(24) Giese, B.; Beyrich-Graf, X.; Erdmann, P.; Petretta, M.; Schwitter, U. *Chem. Biol.* **1995**, *2*, 367−375.
(25) Gugger, A.; Batra, R.; Rzadek, P.; Rist, G.; Giese, B. *J. Am. Chem. Soc.* **1997**, *119*, 8740−8741.
(26) Giese, B.; Dussy, A.; Meggers, E.; Petretta, M.; Schwitter, U. *J. Am. Chem. Soc.* **1997**, *119*, 11130−11131.
(27) Dussy, A.; Meggers, E.; Giese, B. *J. Am. Chem. Soc.* **1998**, *120*, 7399−7403.
(28) Dussy, A.; Giese, B. *Nucleosides Nucleotides* **1999**, *18*, 1343−1344.
(29) Strittmatter, H.; Dussy, A.; Schwitter, U.; Giese, B. *Angew. Chem., Int. Ed.* **1999**, *38*, 135−137.

Scheme 1

Scheme 2
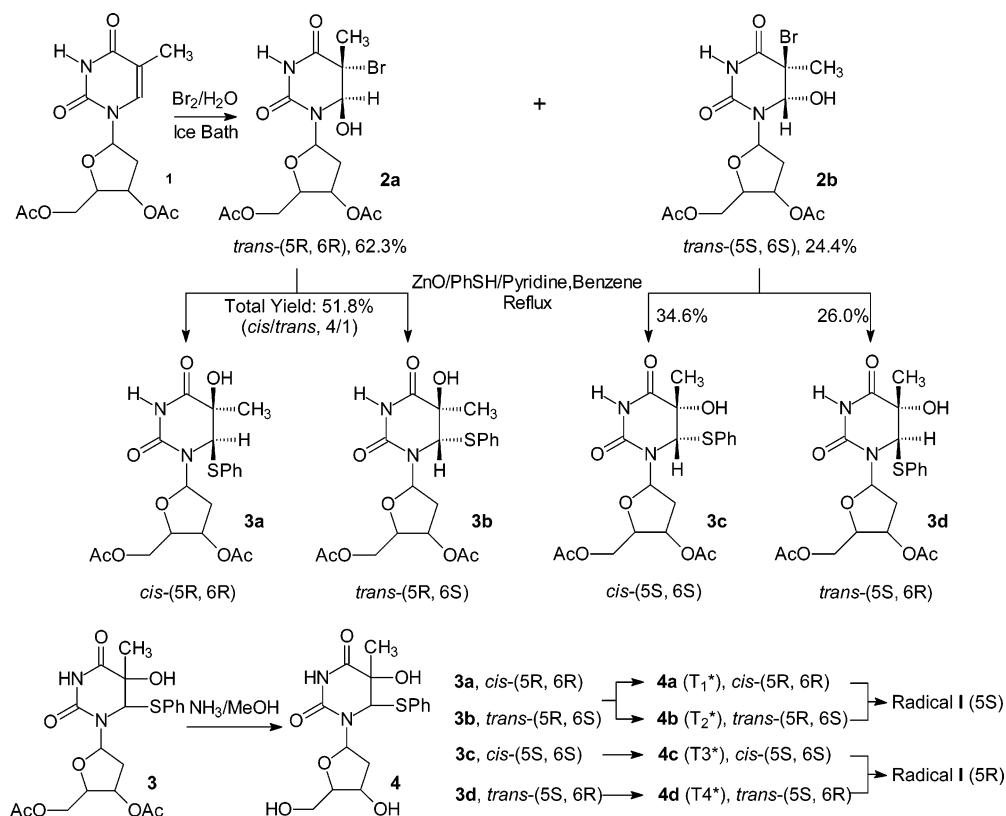
intrastrand cross-link lesion, in which the methyl carbon atom of 5-methylcytosine and the C8 carbon atom of its adjacent guanine are covalently bonded, from this reactive intermediate.[15]

The independent generation of the 5-hydroxy-5,6-dihydro-thymidin-6-yl radical has been previously realized by Barvian et al.[11] through photoinduced electron transfer from *N*-methyl-carbazole (Scheme 1b). The photoirradiation, however, was carried out in a solvent mixture of acetonitrile and H_2O (27:73, v/v), and the efficiency for the generation of the radical was very low in the presence of O_2 or the absence of the electron donor.[11]

We sought to investigate the fate of reactive intermediate **I** (Scheme 1) under both aerobic and anaerobic conditions. Moreover, it is desirable to be able to generate **I** in pure aqueous solution for the future investigation of its reactivity in duplex DNA. With these goals in mind, here we report the synthesis of all four diastereomers of 5-hydroxy-6-phenylthio-5,6-dihydro-thymidine (Scheme 2), their incorporation into dinucleoside

monophosphates, and the characterizations of major products from the 254-nm irradiation of these dinucleoside monophos-phates in aqueous solution under anaerobic conditions.

Results and Discussion

1. Synthesis of the Photolabile Precursor of the 5-Hy-droxy-5,6-dihydrothymidin-6-yl Radical (I). Phenylthio-substituted nucleosides have been shown to have high efficiency in generating carbon-centered radicals through homolytic cleav-age of the C−SPh bond upon 254-nm UV irradiation.[13−15] In addition, the release of the radicals can be achieved in aqueous solution without adding organic cosolvent. Therefore, we decided to adopt similar chemistry for the independent genera-tion of **I**.

Our synthetic strategy for the preparation of 5-hydroxy-6-phenylthio-5,6-dihydrothymidine (**4a−4d**, Scheme 2) was in-spired by previous work of Harayama et al.,[30,31] which showed that thymidine bromohydrin can react with amines and L-amino

Scheme 3 [a]
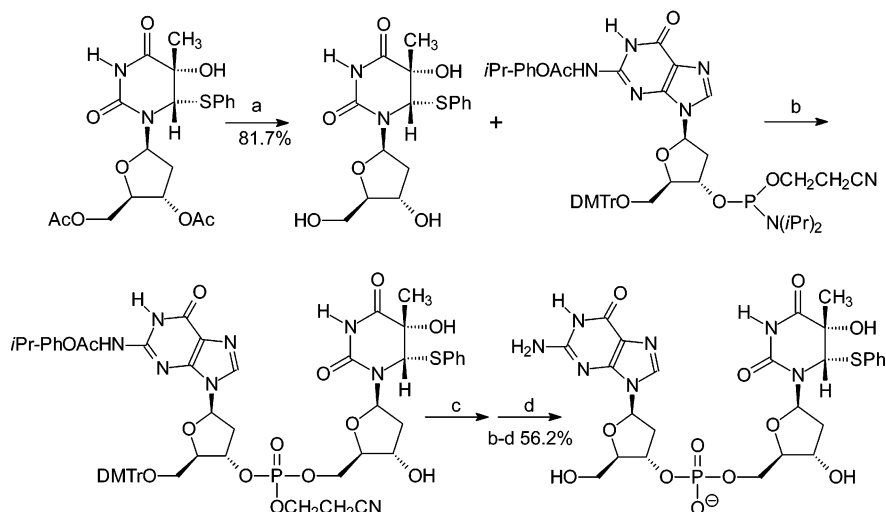
[a] Reagents. a: NH_3/MeOH. b: (i) Dicyanoimidazole/DMF, (ii) t-BuOOH in nonane. c: 80% CH_3COOH. d: NH_3/MeOH.

acid ethyl esters. Moreover, the reaction was proposed to proceed through an epoxide intermediate, which gives rise to thymidine derivatives with a hydroxyl group and the amino group of amines or L-amino acid ethyl esters being substituted to the C5 and C6 carbon atoms, respectively.[30,31] The incorporation of the phenylthio group was achieved through in situ generation of phenylthiolate (PhS⁻) as described previously.[32] The trans (5R,6R) isomer of thymidine bromohydrin (**2a**) gave rise to two isomers of 5-hydroxy-6-phenylthio-5,6-dihydrothymidine, i.e., cis (5R,6R) (**3a**) and trans (5R,6S) (**3b**), with a ratio of 4:1 as estimated from ^{1}H NMR spectrum (Scheme 2). It is worth noting that we attempted but failed to resolve **3a** and **3b** by silica gel column chromatography with several different solvent systems. The chemistry also occurs for bromohydrin **2b**, but we were able to separate **3c** and **3d** from each other. Treatment of compounds **3a**–**3d** with methanolic ammonia gave the desired photolabile precursors **4a**–**4d** (Scheme 2).

It has been demonstrated previously that the tertiary hydroxyl group does not have to be protected during solid-phase DNA synthesis.[33,34] These precursors, therefore, were employed directly for the synthesis of dinucleoside monophosphates d(GT*) and d(TT*), where T* represents the photolabile radical precursor, by using solution-phase phosphoramidite chemistry (Scheme 3 gives an example for the preparation of d(GT₃*)). To prevent the decomposition of saturated thymine portion, deprotection needs to be carried out under mild conditions. Therefore, commercially available ultramild dG phosphoramidite was used for the synthesis of d(GT*) (Scheme 3). We were able to separate the two isomeric dinucleoside monophosphates d(GT₁*) and d(GT₂*) that were initiated from **3a** and **3b** by reverse-phase HPLC. On the basis of the peak areas in the HPLC traces, we estimated that the ratio between these two isomers was again 4:1 with the cis isomer being more abundant.

The structure of each stereoisomer was determined by 2-D NMR experiments (spectra shown in the Supporting Information). In this regard, 2-D COSY results showed strong correlation between the OH proton and the H6 proton for the starting thymidine bromohydrin in DMSO-d_6; no correlation, however, was observed between the H6 proton and the OH proton of the thymine moiety of all the four isomers of T* (Scheme 2), demonstrating that the hydroxyl group is substituted to the C5 carbon atom of the thymine portion (data shown in the Supporting Information). Moreover, 2-D nuclear Overhauser effect (NOE) spectra of the four isomeric d(GT*) showed strong correlation between the protons on the phenyl group and the H₁′ proton of the 3′ nucleoside, which furnishes evidence supporting that the phenylthio group is incorporated to the C6 position of the thymine moiety.

To establish the absolute stereochemistry of the C5 and C6 carbon atoms of the modified thymidine by NMR, we need to consider first whether the glycosidic bond in the modified thymidine adopts a syn or anti conformation. In this respect, substituted pyrimidine nucleosides with a glycosidic bond in the syn conformation are known to lead to a downfield shift of the H₂′ proton (by ∼0.6 ppm) of the deoxyribose because of the placement of C2 carbonyl moiety on the top of the deoxyribose ring.[35,36] ^{1}H NMR results showed that the H₂′ protons of the thymidine portion in d(GT₂*) and d(GT₃*) appeared at approximately 2.9 ppm, whereas the chemical shifts of the respective protons in d(GT₁*) and d(GT₄*) were slightly less than 2.4 ppm and very similar to that of the H₂′ proton of the unmodified thymidine. The significant downfield displacement of the H₂′ protons of the 3′ nucleoside in d(GT₂*) and d(GT₃*) supports that the glycosidic bonds of the thymidine moiety adopt a syn conformation. The lack of alterations in chemical shifts of the corresponding protons in d(GT₁*) and d(GT₄*), however, shows that the glycosidic bonds of the modified nucleoside in d(GT₁*) and d(GT₄*) are in their anti conformation.

(30) Harayama, T.; Yanada, R.; Taga, T.; Machida, K.; Cadet, J.; Yoneda, F. *J. Chem. Soc., Chem. Commun.* **1986**, 1469–1471.
(31) Harayama, T.; Yanada, R.; Tanaka, M.; Taga, T.; Machida, K.; Yoneda, F.; Cadet, J. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2555–2562.
(32) Rajanikanth, B.; Ravindranath, B. *Indian J. Chem., Sect. B* **1984**, *23*, 1043–1045.
(33) Iwai, S. *Angew. Chem., Int. Ed.* **2000**, *39*, 3874–3876.
(34) Iwai, S. *Chem.—Eur. J.* **2001**, *7*, 4343–4351.

(35) George, A. L.; Hruska, F. E.; Ogilvie, K. K.; Holy, A. *Can. J. Chem.* **1978**, *56*, 1170–1176.
(36) Cadet, J.; Ducolomb, R.; Hruska, F. E. *Biochim. Biophys. Acta* **1979**, *563*, 206–215.

We then employed semiempirical PM3 method [37] and optimized the geometry of the four isomeric photolabile precursors (T_1*-T_4*) (structures shown in Scheme 2) with the glycosidic bond in both anti and syn conformations. It turned out that, in equilibrium geometry, the distances between the C2′ proton and the phenyl proton that are in closest proximity are predicted to be 5.8 and 5.0 Å for T_1* and T_4* in anti glycosidic conformation, respectively (Figure S27), whereas the corresponding distance is approximately 2.6 Å for both T_2* and T_3* in syn glycosidic conformation (Figure S28). From this theoretical prediction, we anticipate to observe, in 2-D NOE spectra, strong correlation between the phenyl protons and the protons on the C2′ carbon atom of the 3′ nucleoside for d(GT_2*) and d(GT_3*), whereas no such correlation should be present for d(GT_1*) and d(GT_4*). Indeed, that is exactly what we observed (spectra are shown in the Supporting Information). These results, therefore, demonstrate that the C6 carbon atom of the thymine portion in d(GT_2*) and d(GT_3*) is in *S* configuration, whereas that of d(GT_1*) and d(GT_4*) is in *R* configuration.

After establishing the absolute stereochemistry of the C6 carbon atom, the stereochemistry of the C5 carbon atom can be determined since there is no NOE between the phenyl protons and the methyl protons observed for the cis isomers, d(GT_1*) and d(GT_3*). However, strong NOE was found between these two groups of protons for the two trans isomers, d(GT_2*) and d(GT_4*). In addition, our assignment of the absolute stereochemistry of the C5 carbon atom of the photolabile precursors is in accord with previous observation of the products initiated from the reaction of thymidine bromohydrin with amines and L-amino acid esters.[30,31]

2. Abasic Site Lesion Was the Major Product Initiated from the 254-nm Irradiation of d(GT*) and d(TT*). To avoid the complicating effects of molecular oxygen and to mimic the hypoxic cellular environments, we chose to first examine the reactivities of **I** under anaerobic conditions. To this end, the above dinucleoside monophosphates were purified by HPLC and irradiated for 15−20 min with 254-nm UV light under anaerobic conditions. Negative-ion ESI-MS for the irradiation mixture showed that the starting material is almost undetectable (data not shown), showing that the release of intermediate **I** is efficient. The resulting products were separated again by HPLC and characterized by mass spectrometry and NMR. Here we will focus our discussion on the products isolated from the 254-nm irradiation of d(GT_1*).

It turned out that the major product emanating from the photoirradiation is the dinucleoside monophosphate with the thymidine portion being degraded to an abasic site (a typical chromatogram for the separation of the irradiation mixture of d(GT_1*) is shown in Figure 1). Negative-ion ESI-MS for the 17.0-min fraction gave an ion of *m/z* 462.1, which is the [M − H]⁻ ion of the lesion (Figure 2a). Exact mass measurement of the product gave *m/z* 462.1062, which is consistent with the calculated *m/z* 462.1062 for the [M − H]⁻ ion. Collisional activation of this ion yields major fragment ions forming from the eliminations of a H_2O molecule (*m/z* 444.0) or the 3′ remnant nucleoside (*m/z* 346.0, spectrum shown in Figure 2b).

As reported previously, the abasic site is unstable, and it decomposes completely upon evaporation to dryness.[38] Indeed
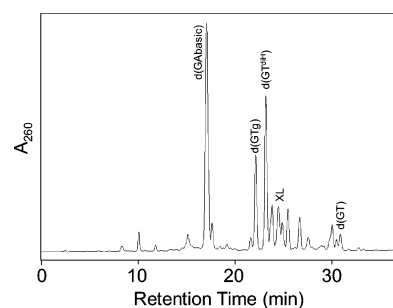
(37) Stewart, J. J. P. *J. Comput. Chem.* **1989**, *10*, 209−220.

Figure 1. HPLC trace for the separation of the 254-nm irradiation mixture of d(GT_1*) under anaerobic condition, in which d(GAbasic), d(GTg), and d(GT^{diH}) represent the dinucleoside monophosphate with the thymidine portion being replaced with an abasic site lesion, thymidine glycol, and 5-hydroxy-5,6-dihydrothymidine, respectively. "XL" designates the cross-link lesion.
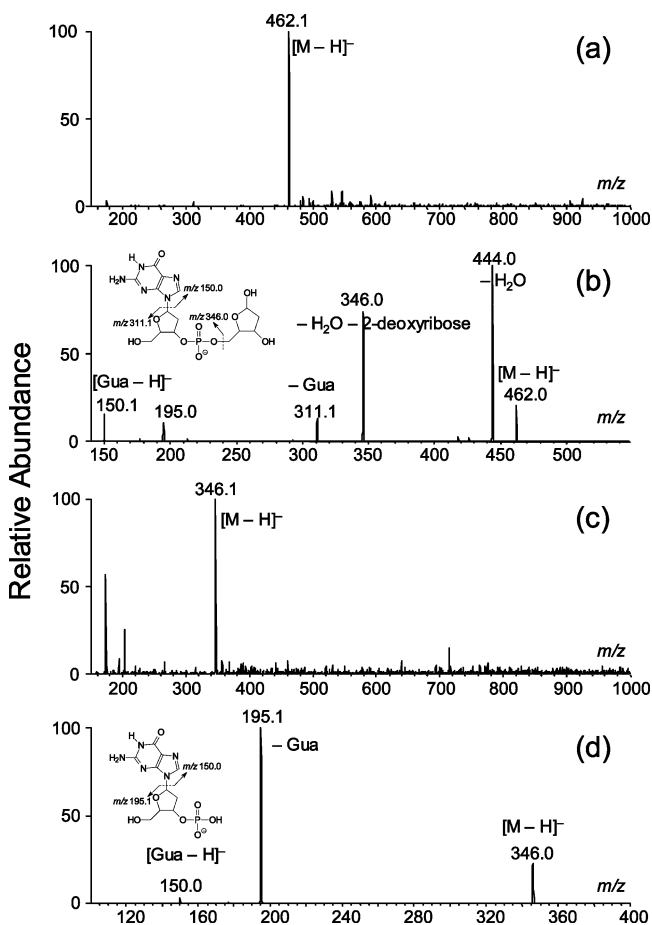

Figure 2. Negative ESI-MS (a) and MS/MS (b) of d(GAbasic), and negative ESI-MS (c) and MS/MS (d) of 2′-deoxyguanosine-3′-monophosphate. The latter compound forms from the decomposition of the d(GAbasic) upon the drying by Speed-vac (see text).

after being dried in a Speed-vac, the major decomposition product gave an ion of *m/z* of 346.1 in negative-ion ESI-MS (Figure 2c). The decomposition product was purified by HPLC; ESI-MS/MS and ¹H NMR results showed its identity as 2′-deoxyguanosine-3′-monophosphate (designated as d(Gp)). The product-ion spectrum of the ion of *m/z* 346.1 showed a dominant fragment ion of *m/z* 195.1 and an ion of *m/z* 150.0 of low abundance. These two fragment ions are induced from the cleavage of the *N*-glycosidic bond (Figure 2d). ¹H NMR

(38) Shishkina, I. G.; Johnson, F. *Chem. Res. Toxicol.* **2000**, *13*, 907−912.

Scheme 4
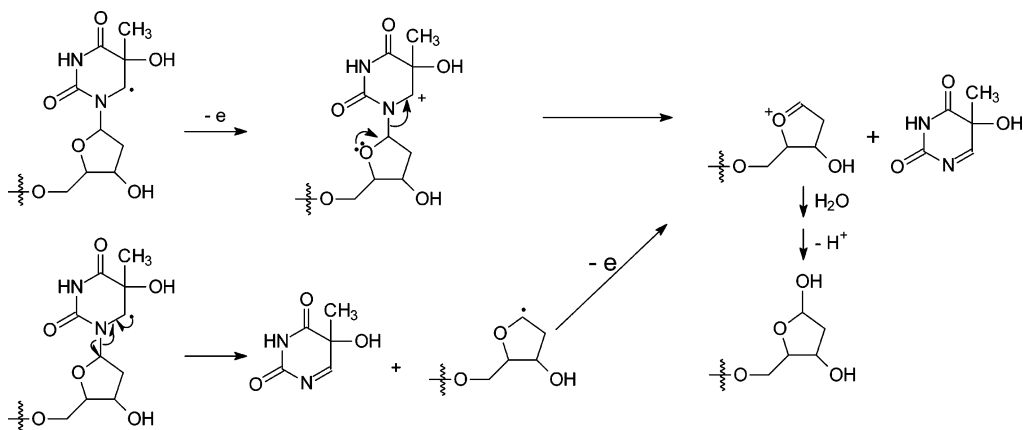
spectrum showed the presence of only one deoxyribose, demonstrating that the deoxyribose of the 3′ nucleoside has been degraded (spectrum shown in the Supporting Information).

For d(TT$_1$*) and all four isomers of d(GT*) that we examined, the abasic site lesion is the most abundant product that we observed (Table S1 gives the estimated yields of the formation of various products initiated from the 254-nm irradiation of these dinucleoside monophosphates). It is well-documented that the abasic site lesion can be generated during ionizing radiation.[7] Given that reactive intermediate **I** is the major secondary radical induced from •OH attack on thymidine and the above finding that this radical can lead to the facile formation of the abasic site lesion, our results indicate that radical **I** may constitute an important reactive intermediate resulting in the formation of abasic site lesion.

Several possible mechanistic pathways may give rise to the abasic site lesion. Reactive intermediate **I** is recognized as a reducing radical because of the adjacent N1 nitrogen atom,[39] and the reducing property of this radical has been demonstrated in that it reduces tetranitromethane with a rate constant of ∼10^9 M^{-1} s^{-1}.[40] With that in mind, we reason that reactive intermediate **I** can be oxidized to give a C6 carbon cation (Scheme 4), which can lead to the facile elimination of the thymine portion with the charge being transferred to the C1′ carbon atom of the deoxyribose moiety. The latter intermediate can undergo consecutive hydration and deprotonation to afford the abasic site lesion. Some precaution, however, should be taken for the interpretation of the formation of the abasic site lesion from the 254-nm irradiation of d(GT*). In this regard, previous work by Kropp et al.[41] showed that 254-nm irradiation of phenyl thioethers leads to the formation of both the carbon-centered radical and carbon cation. The latter was proposed to form from electron transfer within the initially formed radical pair cage.[41] In the γ-radiation-induced DNA damage, the electron transfer from radical **I** may not be as facile as what occurs in the photolytically generated radical pair cage. In this regard, parallel LC−MS/MS analyses of the γ-radiation mixture of d(GT) and the 254-nm irradiation of d(GT$_1$*) showed that this is indeed the case (vide infra).

Alternatively, the spin at the C6 carbon atom can be transferred to the C1′ carbon atom of the deoxyribose portion via the elimination of the nucleobase moiety. The resulting C1′ radical is expected to possess reducing property because of the adjacent oxygen atom on the deoxyribose ring. Similar to radical **I**, the C1′ radical may lose an electron to give a carbon cation at the C1′ position, which can again undergo consecutive hydration and deprotonation to yield the abasic site lesion (Scheme 4).

3. Other Lesions Formed from I. In addition to the abasic site lesion described above, intrastrand cross-link and lesions with the thymine portion of the dinucleoside monophosphates being modified to thymine glycol, 5-hydroxy-5,6-dihydrothymine, or thymine, have been isolated and characterized (Scheme 5).

a. Thymine Glycol and 5-Hydroxy-5,6-dihydrothymine. The LC fraction eluting at 22.1 min is a product with the thymine portion being replaced with a thymine glycol (labeled as d(GTg) in Figure 1). The product-ion spectrum of the ESI-produced [M − H]$^-$ ion (*m/z* 604) of the thymine glycol-bearing dinucleoside monophosphate shows a unique elimination of a 143-Da fragment (Figure 3a). Similar fragmentation has been previously observed in the product-ion spectra of the [M − H]$^-$ ions of thymine glycol-bearing mononucleosides and dinucleotides.[42,43] This facile loss was due to the cleavages of the N1−C2 and N1−C6 bonds of the modified thymine portion.[42,43] Our structure assignment is further supported by 1-D and 2-D NMR data (spectra shown in the Supporting Information).

We also observed that the thymine portion can be transformed to 5-hydroxy-5,6-dihydrothymine. Exact mass measurement of the 23.1-min fraction (labeled as d(GT^{diH}) in Figure 1) gives *m/z* 588.1436 for the [M − H]$^-$ ion, which is consistent with the calculated *m/z* of 588.1455 for the deprotonated ion of d(GT^{diH}). The product-ion spectrum of the [M − H]$^-$ ion shows the facile elimination of a water molecule. In addition, we observed the cleavages of the *N*-glycosidic and the 3′ C−O bonds of the 5′ nucleoside to give ions of *m/z* 437.1 and 346.1, respectively (Figure 3b). We also acquired 2-D COSY and NOESY data for the lesion, which allow us to assign all the protons in ^{1}H NMR without ambiguity. In the NOE spectrum,

(39) O'Neill, P.; Fielden, E. M. In *Advances in Radiation Biology*; Lett, J. T., Sinclair, W. K., Eds.; Academic Press: New York, 1993; Vol. 17, pp 53−121.
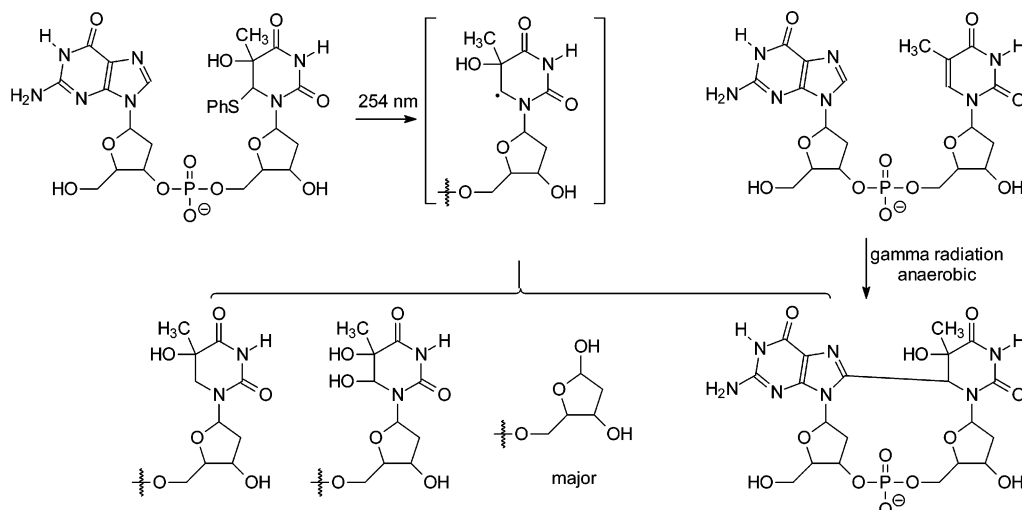(40) Fujita, S.; Steenken, S. *J. Am. Chem. Soc.* **1981**, *103*, 2540−2545.
(41) Kropp, P. J.; Fryxell, G. E.; Tubergen, M. W.; Hager, M. W.; Harris, G. D., Jr.; McDermott, T. P., Jr.; Tornero-Velez, R. *J. Am. Chem. Soc.* **1991**, *113*, 7300−7310.

(42) Wang, Y.; Vivekananda, S.; Zhang, K. *Anal. Chem.* **2002**, *74*, 4505−4512.
(43) Wang, Y. *Chem. Res. Toxicol.* **2002**, *15*, 671−676.

Scheme 5

two protons at 3.5 ppm, which are the H-6 protons of the thymine portion, show correlation with the protons on the methyl group and the C2′ and C3′ carbons of the 3′ nucleoside. Furthermore, the methyl protons show correlation with protons on the C2′ carbon atom of the 3′ nucleoside, indicating that the C5 carbon remains in its *S* configuration. The above MS and NMR results support our structural assignment of this lesion. The formation of thymidine glycol and 5-hydroxy-5,6-dihydro-thymidine has also been previously observed by Barvian et al.[11] from the independently generated radical **I** in mononucleoside.

b. Intrastrand Cross-Link Lesion. As observed with the 5-methyl radical of thymine[13,14] and 5-methylcytosine,[15] reactive intermediate **I** in d(GT) can also couple with the C8 carbon atom of its 5′ neighboring guanine to give a cross-link lesion (24.4-min fraction, labeled as XL in Figure 1).

The structure of the cross-link lesion was established by extensive characterizations with MS and NMR. Negative-ion ESI-MS of the cross-link lesion gives an ion of *m/z* 586, which is the [M − H]⁻ ion of the cross-link lesion. Exact mass measurement of this ion gives *m/z* 586.1275, which is consistent with the calculated *m/z* of 586.1299. The product-ion spectrum of the ion of *m/z* 586 does not show the elimination of an unmodified guanine (Figure 3c), whereas this elimination is the most facile cleavage resulting from the collisional activation of the [M − H]⁻ ion of the unmodified d(GT) (data not shown). Instead, the most abundant fragment ion (*m/z* 488.0) is due to the elimination of a 2-deoxyribose (−98 Da). This ion can further lose [HPO₃ + H₂O] to yield an ion of *m/z* 390.1. The assignments of these two fragment ions are supported by high-resolution MS/MS acquired on a Fourier transform mass spectrometer, which gives *m/z* 488.0908 and 390.1160. The results are in line with the calculated *m/z* values of 488.0931 and 390.1162 for these two product ions.

Given the above MS and MS/MS results and that the cross-link lesion is initiated from a structurally defined radical, we believe that the cross-link lesion has a covalent bond formed between the C8 carbon atom of guanine and the C6 carbon atom of the thymine moiety. Indeed, the ¹H NMR spectrum clearly shows the disappearance of H-8 proton of guanine (Figure 4). All the proton resonances in ¹H NMR were again assigned from the 2-D COSY and NOESY results (data shown in the Supporting Information).

It is interesting to note that the yield for the production of the cross-link lesion depends on the conformation of C5 carbon atom (LC traces for the separation of the irradiation mixtures of d(GT₂*), d(GT₃*), and d(GT₄*) are shown in the Supporting Information). In this respect, d(GT₁*) and d(GT₂*), which give rise to the 5*S* isomer of radical **I** upon 254-nm irradiation (Scheme 2), have a yield of approximately 4% (Table S1), whereas d(GT₃*) and d(GT₄*), which yield the 5*R* isomer of radical **I** (Scheme 2), show a lower yield for the formation of the cross-link lesion, that is, approximately 1 to 2% (Table S1).
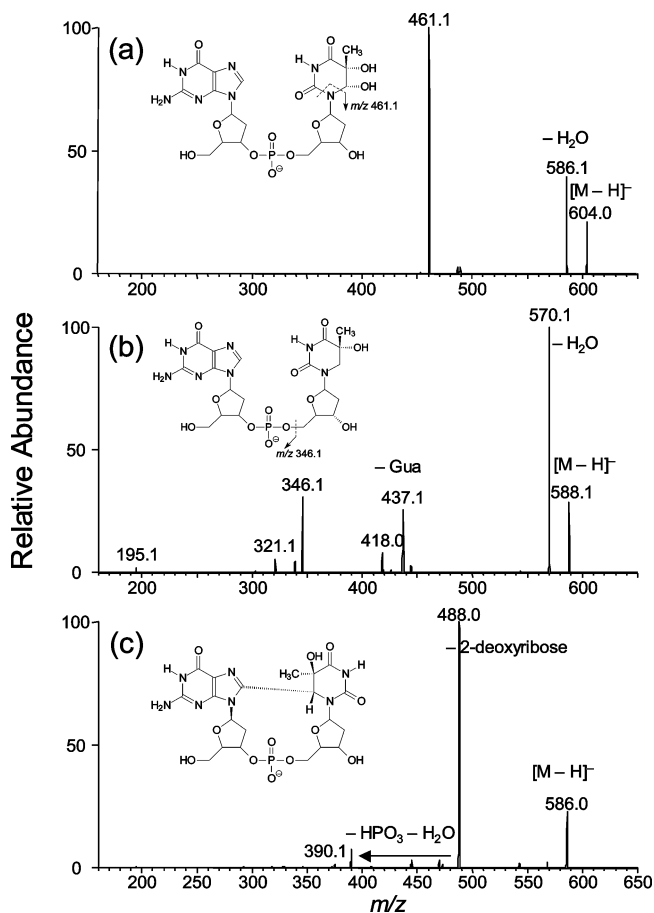

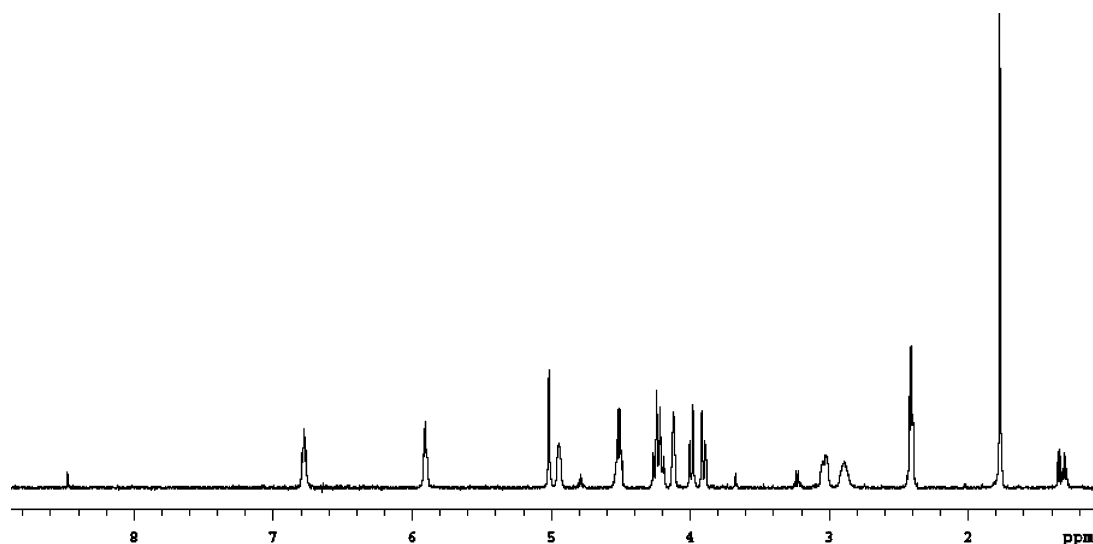**Figure 3.** Product-ion spectra of the ESI-produced [M − H]⁻ ions of d(GTg) (a), d(GT^diH) (b), and the XL (c).

Figure 4. ¹H NMR spectrum of the cross-link lesion (XL) initiated from the 254-nm irradiation of d(GT₁*) (500 MHz, D₂O. The peak at 8.5 ppm is due to impurity.)

The difference in yield for the formation of the lesion is likely due to different steric hindrance imposed by the C5 carbon atom of the two isomeric radicals.

We also prepared d(TT₁*), irradiated it with 254-nm UV light under anaerobic condition, and characterized the products by mass spectrometry. It turned out that d(TT₁*) gave very similar products as d(GT*), that is, d(TAbasic), d(TT^diH), d(TTg), and d(TT), except that we failed to isolate any cross-link lesion from the irradiation mixture of d(TT₁*) (Figure S29).

4. Identification of the Cross-Link Lesions from the γ-Irradiation of d(GT) under Anaerobic Conditions. Previously, Box et al.[44] reported a cross-link lesion that was isolated from the X-ray irradiation of d(GT) under anaerobic conditions, and they proposed that the cross-link lesion was initiated from the coupling of the 6-hydroxy-5,6-dihydrothymidin-5-yl radical (**II**, Scheme 1a) with the C8 carbon atom of guanine. We, however, found that the ¹H NMR spectrum reported by Box et al.[44] is nearly identical to that for the 5S isomer of the cross-link lesion that we isolated. This motivated us to propose that the lesion isolated by Box et al.[44] is the 5S isomer of the cross-link lesion initiated from radical **I**.

To support such a proposal, we carried out the γ-irradiation of d(GT) under anaerobic conditions and identified the cross-link lesions induced under this condition by LC−MS/MS. It turns out that the 5S isomer is indeed the major cross-link lesion of this kind formed from γ-irradiation of d(GT). Total-ion chromatogram for the fragmentation of the ion of m/z 588, which corresponds to the [M + H]⁺ ion of the cross-link lesion, in the positive-ion mode gave four peaks (Figure 5a). The product-ion spectra of the two earlier eluting fractions gave characteristic elimination of a 98-Da moiety, which is identical to the fragmentation of the authentic cross-link lesions that we isolated from the 254-nm irradiation of d(GT*). The two later eluting factions at 33.1 and 35.5 min are not cross-link lesions because the product-ion spectra of the ion of m/z 588 show fragment ions of m/z 323, which are assigned as the [M + H]⁺ ion of thymidine-5′-phosphate. Combining the results from both positive- and negative-ion MS/MS, we determined that the 35.5-

min fraction is a guanine oxidation product with 8-oxoguanine being most likely (spectra shown in Figure S36). The nature of the product eluting at 33.1 min remains unknown.

Selected-ion chromatograms for the m/z 588 → 490 transition, which corresponds to the elimination of the 2-deoxyribose from the precursor ion, of the γ-irradiation sample shows that there are two major peaks at 27.5 and 30.0 min (Figure 5b). Furthermore, we carried out LC−MS/MS experiments with the co-injection of the γ-irradiation mixture and the authentic cross-link lesion that was isolated from the 254-nm irradiation of each of the four isomeric d(GT*), that is, d(GT₁*)−d(GT₄*). It turned out that the 27.5-min eluting fraction comigrates with the cross-link lesion initiated from the photoirradiation of d(GT₁*) or d(GT₂*) (Figure 5c). The fraction eluting at 30.0 min, however, comigrates with the authentic cross-link lesion that was initiated from the irradiation of d(GT₃*) or d(GT₄*) (Figure 5d). These results demonstrate that, as expected, d(GT₁*) and d(GT₂*) give rise to the same cross-link lesion, and the same is true with d(GT₃*) and d(GT₄*). Moreover, the yield for the formation of the 5S isomer of the cross-link lesion from the γ-irradiation mixture of d(GT) is higher than that for the formation of the 5R isomer, which is consistent with the formation of these two isomeric cross-link lesions from the two independently generated isomeric radicals.

The above results demonstrate that γ-irradiation of d(GT) under anaerobic condition can induce the formation of both isomers of the cross-link lesions that we have isolated from the 254-nm irradiation of d(GT*). It should be noted that the analogous 6-hydroxy-5,6-dihydrothymidin-5-yl radical may also give rise to a cross-link lesion via coupling to the C8 carbon atom of its adjacent guanine. In this respect, we recently discovered that γ-irradiation of duplex synthetic oligodeoxynucleotide (ODN) can give rise to a cross-link lesion, with the C8 carbon atom of guanine and the C5 carbon atom of its 3′ neighboring cytosine covalently bonded.[45] This type of lesion was proposed to form from the dehydration of the coupling product of the 6-hydroxy-5,6-dihydrocytosin-5-yl radical and its adjacent guanine.[46] The cross-link lesions initiated from the

(44) Box, H. C.; Budzinski, E. E.; Dawidzik, J. B.; Gobey, J. S.; Freund, H. G. *Free Radical Biol. Med.* **1997**, *23*, 1021−1030.

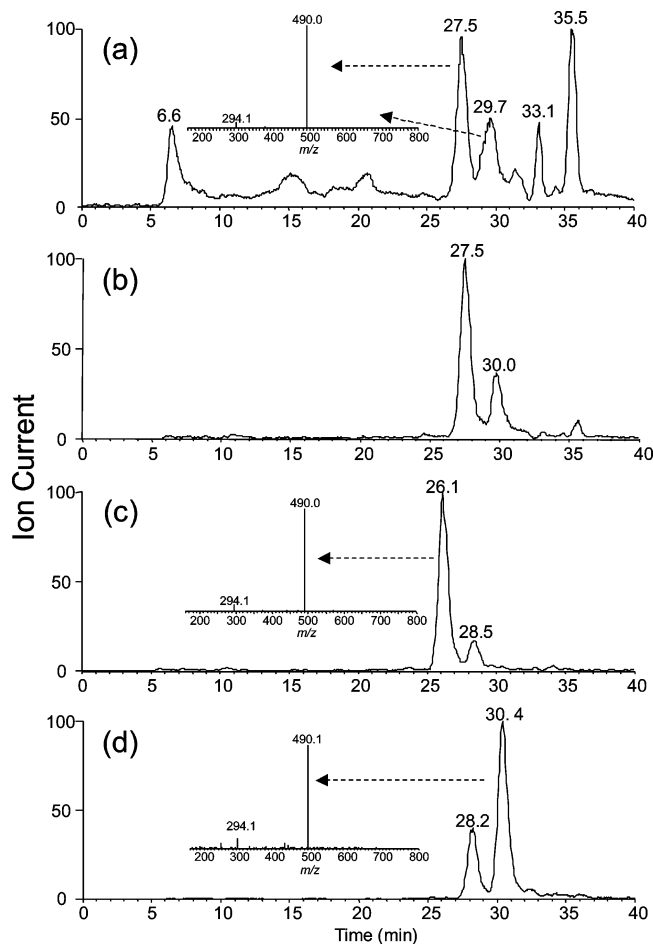(45) Gu, C.; Wang, Y. *Biochemistry* **2004**, *43*, 6745−6750.

Figure 5. LC−MS/MS of the γ-irradiation mixture of d(GT) under anaerobic condition. The mass spectrometer was set at monitoring the fragmentation of the ion of m/z 588 in the positive-ion mode: (a) Total ion chromatogram for the fragmentation of the m/z 588 ion with the injection of 4-nmol γ-irradiation mixture of d(GT). (b) Selected-ion chromatogram for the m/z 588 → m/z 490 transition for the same LC−MS/MS analysis as in (a). (c) Selected-ion chromatogram for the m/z 588 → m/z 490 transition with the co-injection of the 1.3-nmol γ-irradiation mixture of d(GT) and 5 pmol of the XL lesion initiated from d(GT$_1$*). (d) Selected-ion chromatogram for the m/z 588 → m/z 490 transition with the co-injection of the 0.8-nmol γ-irradiation mixture of d(GT) and 3 pmol of the XL lesion initiated from d(GT$_3$*). Shown in the insets are the product-ion spectra for the ion of m/z 588 for the two fractions containing the cross-link lesion.

6-hydroxy-5,6-dihydrothymidin-5-yl radical (**II**, Scheme 1a) and radical **I** have the same molecular weight, and the two lesions are expected to have similar ionization efficiency. Thus, the above LC−MS/MS studies show that the cross-link lesion initiated from radical **I** is more abundant than that formed from radical **II**, if the cross-link lesion can be initiated from the latter radical.

To examine to what extent the abasic site lesion was formed from electron transfer within the initial radical pair cage as alluded to in the foregoing discussion, we employed LC−MS/MS and determined the relative yields of formation of the abasic site lesion over cross-link lesion from the 254-nm irradiation of d(GT$_1$*) and the γ-irradiation of d(GT) under anaerobic conditions. In the course of this study, we noticed that the abasic site lesion d(GAbasic) and its decomposition product d(Gp) do not ionize well in positive-ion ESI-MS. Thus, the LC−ESI-

(46) Box, H. C.; Budzinski, E. E.; Dawidzik, J. B.; Wallace, J. C.; Iijima, H. *Radiat. Res.* **1998**, *149*, 433−439.

MS/MS experiments were carried out in the negative-ion mode, and the mass spectrometer was set to monitor alternatively the fragmentations of the [M − H]$^-$ ions of the cross-link lesion (m/z 586), d(GAbasic) (m/z 462), and d(Gp) (m/z 346).

By taking into consideration the different ionization and fragmentation efficiencies of the three compounds, we estimated that the yield for the formation of the abasic site lesion, that is, d(GAbasic), from the 254-nm irradiation of d(GT$_1$*) is approximately eight times as high as that of the cross-link lesion, which is in reasonable agreement with what we obtained from the LC trace as monitored by UV absorbance at 260 nm (Table S1). The decomposition product of the abasic site lesion, d(Gp), was undetectable under these UV irradiation conditions (LC−MS/MS traces and the relevant calculations are shown in Figures S33−35 and page S40 in the Supporting Information).

The distribution of the three products from the γ-irradiation of d(GT), however, is very different from that of the UV irradiation of the d(GT$_1$*). In this regard, the formation of d(GAbasic) from the γ-irradiation is approximately 11 times less efficient than the formation of the 5S isomer of the cross-link lesion, though the decomposition product of d(GAbasic), that is, d(Gp), is produced at a similar efficiency as the 5S isomer of the cross-link lesion. Therefore, the relative yield for the formation of the abasic site lesion over that of the cross-link lesion is much higher from the 254-nm UV irradiation of d(GT$_1$*) than from the γ-irradiation of d(GT). Moreover, the d(GAbasic) and d(Gp) may also be initiated from deoxyribose radicals and other types of nucleobase radicals under γ-irradiation.[7] Therefore, the C6 carbon cation, which forms from the oxidation of the reactive intermediate **I** and is produced more readily from the 254-nm irradiation of d(GT$_1$*) than from the γ-radiation of d(GT), makes a significant contribution to the formation of the abasic site lesion.

Experimental Section

Materials. Common reagents for solid-phase DNA synthesis were obtained from Glen Research Co. (Sterling, VA). Silica gel and TLC plates were purchased from EM Science (Gibbstown, NJ). All chemicals unless otherwise specified were from Sigma-Aldrich (St. Louis, MO).

Mass Spectrometry and NMR. ESI-MS and MS/MS experiments were carried out on an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). An equal-volume solvent mixture of acetonitrile and water was used as solvent for electrospray, and a 2-μL aliquot of a ∼5 μM sample solution was injected in each run. The spray voltages were 4.0 and 3.4 kV for experiments in the positive- and negative-ion modes, respectively. The capillary temperature was maintained at 150 °C. An IonSpec HiResESI external ion source FTICR mass spectrometer (IonSpec Co., Lake Forest, CA) equipped with a 4.7 T unshielded superconducting magnet was used for high-resolution MS and MS/MS measurements as described previously.[15] Dinucleoside monophosphate ApA and an oligodeoxynucleotide d(AATTGA) were used as internal standards for mass calibration.

All NMR spectra were recorded on a Varian Unity Inova 500 MHz instrument (Palo Alto, CA). The residual proton signal of the solvent served as internal reference.

UVC Irradiation. An unbuffered aqueous solution of dinucleoside monophosphate d(GT*) or d(TT*) with AU$_{260}$ of 0.4 in a quartz tube was degassed by three cycles of freeze−pump−thaw. The solution was irradiated for 15−20 min with 254-nm light generated by a Rayonet photochemical reactor that was equipped with 16 light tubes (The Southern New England Ultraviolet Company, CT). The resulting solution was lyophilized to dryness.

HPLC. The HPLC separation was performed on a system composed of a Hitachi L-6200A pump (Hitachi Ltd., Tokyo, Japan), a HP-1050 UV detector (Agilent Technologies, Palo Alto, CA), and a Peak Simple Chromatography Data System (SRI Instruments Inc., Las Vegas, NV). A 4.6 × 250 mm Apollo C18 column (5 μm in particle size and 300 Å in pore size, Alltech Associates Inc., Deerfield, IL) was used for the separation.

A solution of 50 mM ammonium formate (solution A) and a mixture of 50 mM ammonium formate and methanol (60/40, v/v) (solution B) were used as the mobile phases. For the purification of d(GT*) and d(TT*), a gradient of 20-min 0−100% B followed by 10-min 100% B was used. Appropriate fractions were pooled, evaporated to dryness, and redissolved in doubly distilled water for photoirradiation. For the separation of the 254-nm irradiation products of d(GT*) and d(TT*), a gradient of 5-min 0−15% B, 30-min 15−55% B, and 5-min 55−100% B was used. The flow rate was 1.0 mL/min, and the effluents were monitored by UV detection at 260 nm. The HPLC condition for LC−MS experiments was different and described below.

γ-Irradiation of d(GT) and LC−MS/MS. A 1.5-mL solution of d(GT) (80 nmol) in a 35-mL Pyrex tube was degassed by three cycles of freeze−pump−thaw. The tube was filled with high-purity argon and exposed to a Mark I ^{137}Cs Irradiator (JL Shepherd and Associates, San Fernando, CA) at a dose rate of 2.0 Gy/min for 4 h (total dose: 480 Gy). The resulting solution was dried by the Speed-vac, redissolved in 40 μL water, and subjected directly to LC−MS/MS analysis.

A 0.32 × 250 mm C18 column with a particle size of 5 μm and a pore size of 300 Å (Micro-Tech Scientific, Vista, CA) was employed for the separation. A homemade precolumn splitter was incorporated between the HPLC pump and the injector, the flow rate was 4−6 μL/min after splitting, and a 100-min gradient of 0−35% acetonitrile in 10 mM ammonium acetate was used. The flow from HPLC column was coupled directly to the LCQ Deca XP ion-trap mass spectrometer. The mass spectrometer was set up to monitor the fragmentation of the [M + H]$^+$ ion (*m/z* 588) of the cross-link lesions, and 4 nmol of the above γ-irradiated d(GT) was injected in each run. In comigration experiments, standard 5S or 5R isomer of the cross-link lesion was mixed with the above γ-irradiation sample and injected to the column with identical experimental setup as for the γ-irradiation mixture.

Slightly different conditions were employed for the determination of the relative yields of the formation of different products from 254-nm irradiation of d(GT*) and the γ-irradiation of d(GT). In this regard, the mass spectrometer was run in the negative-ion mode with the monitoring of the fragmentation of the ions of *m/z* 346, 462, and 586, which correspond to the deprotonated ions of 2′-deoxyguanosine-5′-monophosphate, d(GAbasic), and the cross-link lesion, respectively, in an alternative manner. In addition, the flow splitter was placed after the injector. Therefore, the retention times are different from those LC−MS/MS experiments performed in the positive-ion mode.

Synthesis and Characterization of Compounds. Preparation of Bromohydrins 2a and 2b (Scheme 2). Compound **1** (1.63 g, 5 mmol) and 200 mL of water were added to a 500-mL flask, and the mixture was vigorously stirred for 30 min until compound **1** was completely dissolved. The flask was then immersed in an ice bath for 15 min, and bromine (282 μL, 1.1 equiv) was slowly added to the vigorously stirred solution over 15 min. After being stirred for another 15 min, the volume of the solution was reduced to 30 mL by rotavap. The same procedures were repeated for another three batches of reaction. The concentrated reaction mixtures were combined and extracted with chloroform twice (120 mL each). The organic layer was washed with brine and dried with anhydrous Na$_2$SO$_4$, and the solvent was evaporated off to give a white foam.

The white foam was loaded onto a silica gel column and eluted with CH$_2$Cl$_2$/EtOAC (5/6, v/v). Appropriate fractions were combined, and the solvent was evaporated off under reduced pressure to afford compounds **2a** (5.27 g, 62.3%) and **2b** (2.07 g, 24.4%) as colorless crystal and amorphous white powder, respectively.

Compound **2a**: R_f: 0.48 (CH$_2$Cl$_2$/EtOAC, 5/6, v/v). ESI-MS: *m/z* 445.1, 447.1 [M + Na]$^+$. ^{1}H NMR (DMSO-d_6): 10.86 (s, 1H, NH), 6.82 (br, s, 1H, 6-OH), 6.04 (dd, 1H, H-1′, J = 8.7, 5.9 Hz), 5.19 (s, 1H, H-6), 5.14 (m, 1H, H-3′), 4.18 (d, 2H, J = 4.8 Hz, H-5′ and H-5″), 4.11−4.07 (m, 1H, H-4′), 2.48−2.41 (m, 1H, H-2′), 2.15−2.09 (m, 1H, H-2″), 2.06 (s, 6H, COCH$_3$), 1.82 (s, 3H, CH$_3$).

Compound **2b**: R_f: 0.33 (CH$_2$Cl$_2$/EtOAC, 5/6, v/v). ESI-MS: *m/z* 445.1, 447.1 [M + Na]$^+$. ^{1}H NMR (DMSO-d_6): 10.88 (s, 1H, NH), 7.14 (br, s, 1H, 6-OH), 6.06 (dd, 1H, J = 9.1, 5.5 Hz, H-1′), 5.13 (m, 1H, H-3′), 5.08 (s, 1H, 6-H), 4.24−4.15 (m, 2H, H-5′ and H-5″), 4.07 (m, 1H, H-4′), 2.48−2.40 (m, 1H, H-2′), 2.14−2.08 (m, 1H, H-2″), 2.07 (s, 6H, COCH$_3$), 1.81 (s, 3H, CH$_3$).

Preparation of Compounds 3a and 3b (Scheme 2). The incorporation of the phenylthio group was achieved by in situ generation of phenylthiolate (PhS$^-$) as described previously.[32] To a 50-mL flask, which was equipped with a dean-stark tube and a condenser, was added benzene (20 mL), ZnO (211.7 mg, 0.55 equiv), and PhSH (0.534 mL, 1.1 equiv). The solution was refluxed for 4 h and cooled, and to the cooled solution was added compound **2a** (2.0 g, 4.73 mmol) and anhydrous pyridine (765 μL, 2 equiv). The mixture was refluxed for overnight and cooled to room temperature. Some white precipitates formed during the reaction, and they were removed by filtration. The resulting filtrate was washed with HCl (0.05 M), 1% NaHCO$_3$, and brine. The benzene layer was further dried with anhydrous Na$_2$SO$_4$ and evaporated to dryness under reduced pressure to give a light orange oil. The oily product was loaded onto a silica gel column and eluted with 0−4% MeOH in CH$_2$Cl$_2$. The appropriate fractions were pooled, and the solvent was evaporated to give slight yellowish oil. This yellowish oil was loaded to a silica gel column and eluted with hexane/EtOAc (1/2, v/v). The appropriate fractions were combined, and the solvent was evaporated off to afford a mixture of **3a** and **3b** as white foam (1.11 g, total yield of **3a** and **3b**: 51.8%). The ratio of **3a** to **3b** was 4:1 as established by ^{1}H NMR. Compounds **3a** and **3b**: ESI-MS: *m/z* 450.9 [M − H]$^-$, MS/MS 341.1 [M − H − PhSH]$^-$; 452.9 (weak) [M + H]$^+$, 475.1 (strong) [M + Na]$^+$.

^{1}H NMR of compound **3a** (DMSO-d_6): 10.62 (s, 1H, NH), 7.54−7.49 (m, 2H, H-PhS), 7.33−7.29 (m, 3H, H-PhS), 6.19 (s, 1H, 5-OH), 5.39 (dd, 1H, J = 7.1, 6.7 Hz, H-1′), 5.10 (s, 1H, H-6), 4.99 (m, 1H, H-3′), 3.96−3.86 (m, 3H, H-4′, H-5′ and H-5″), 2.30−2.23 (m, 1H, H-2′), 2.00 (s, 3H, COCH$_3$), 1.94 (s, 3H, COCH$_3$), 1.65−1.60 (m, 1H, H-2″), 1.40 (s, 3H, CH$_3$).

^{1}H NMR of compound **3b** (DMSO-d_6): 10.28 (s, 1H, NH), 7.47−7.44 (m, 2H, H-PhS), 7.38−7.33 (m, 3H, H-PhS), 6.24 (s, 1H, 5-OH), 5.92 (dd, J = 6.3, 5.9 Hz, 1H, H-1′), 5.13−5.11 (m, 1H, H-3′), 4.92 (s, 1H, H-6), 4.20−4.15 (m, 3H, H-4′, H-5′ and H-5″), 2.67−2.60 (m, 1H, H-2′), 2.05−1.97 (m, overlap with COCH$_3$ signal, 1H, H-2″), 2.05 (s, 3H, COCH$_3$), 2.03 (s, 3H, COCH$_3$), 1.94 (s, 3H, CH$_3$).

Synthesis of Compounds 3c and 3d (Scheme 2). Compounds **3c** and **3d** were prepared according to the same procedures as described for the syntheses of compounds **3a** and **3b**. These two compounds, however, could be separated from each other by silica gel column chromatography, and they were obtained as colorless glass.

Compound **3c** (yield: 34.6%): R_f = 0.44 (hexane/EtOAc, 1/2, v/v). ESI-MS: *m/z* 450.9 [M − H]$^-$, MS/MS 341.1 [M − H − PhSH]$^-$; 452.8 (weak) [M + H]$^+$, 475.1 (strong) [M + Na]$^+$. ^{1}H NMR (DMSO-d_6): 10.67 (s, 1H, NH), 7.52−7.49 (m, 2H, H-PhS), 7.37−7.33 (m, 3H, H-PhS), 6.28 (s, 1H, 5-OH), 6.01−5.98 (m, 1H, H-1′), 4.97 (m, 1H, H-3′), 4.83 (s, 1H, H-6), 4.08 (m, 2H, H-5′ and H-5″), 3.99 (m, 1H, H-4′), 2.24−2.18 (m, 1H, H-2′), 2.02 (s, 3H, COCH$_3$), 1.93 (s, 3H, COCH$_3$), 1.56−1.52 (m, 1H, H-2″), 1.32 (s, 3H, CH$_3$).

Compound **3d** (yield: 26.0%): R_f = 0.28 (hexane/EtOAc, 1/2, v/v). ESI-MS: *m/z* 450.9 [M − H]$^-$, MS/MS 341.2 [M − H − PhSH]$^-$; 452.7 (weak) [M + H]$^+$, 475.1 (strong) [M + Na]$^+$. ^{1}H NMR (DMSO-d_6): 10.48 (s, 1H, NH), 7.50−7.47 (m, 2H, H-PhS), 7.35−7.30 (m, 3H, H-PhS), 6.42 (s, 1H, 5-OH), 5.81 (t, J = 5.9 Hz, 1H, H-1′), 5.04−5.01 (m, 1H, H-3′), 4.97 (s, 1H, H-6), 4.06−4.04 (m, 1H, H-4′), 3.99−

3.97 (m, 2H, H-5′ and H-5″), 2.37−2.31 (m, 1H, H-2′), 2.04 (s, 3H, COCH$_3$), 1.96 (s, 3H, COCH$_3$), 1.95−1.93 (m, 1H, H-2″), 1.42 (s, 3H, CH$_3$).

Synthesis of (5R,6R)-*cis*-5-Hydroxy-6-phenylthio-5,6-dihydro-thymidine (4a) and (5R,6S)-*trans*-5-Hydroxy-6-phenylthio-5,6-di-hydrothymidine) (4b) (Scheme 2). The above **3a/3b** mixture (0.500 g, 1.11 mmol) was dissolved in 100 mL of saturated methanolic ammonia and stirred at room temperature for 9 h. The solvent was removed under reduced pressure. The dried residue was redissolved in 1 mL of MeOH/EtOAc (1/80, v/v), loaded onto a silica gel column, and eluted with 4% MeOH in EtOAc. Appropriate fractions were pooled, and the solvent was removed to afford colorless glass (349 mg, 86.1%). ESI-MS: 367.0 [M − H]$^-$, MS/MS 257.1 [M − H − PhSH]$^-$; 368.9 [M + H]$^+$. Compounds **4a** and **4b** again could not be resolved from each other by silica gel column chromatography, and the proton resonances in ^{1}H NMR were assigned based on 2-D COSY and NOESY experiments.

^{1}H NMR of compound **4a** (DMSO-d_6): 10.48 (s, br, 1H, NH), 7.53−7.49 (m, 2H, H-PhS), 7.31−7.28 (m, 3H, H-PhS), 6.00 (s, 1H, 5-OH), 5.38 (t, J = 6.3 Hz, 1H, H-1′), 5.25 (s, 1H, H-6), 4.99 (d, J = 4.3 Hz, 1H, 3′-OH), 4.68 (t, J = 5.1 Hz, 1H, 5′-OH), 4.05−4.01 (m, 1H, H-3′), 3.61−3.57 (m, 1H, H-4′), 3.26 (m, 2H, H-5′ and H-5″), 2.06−1.99 (m, 1H, H-2′), 1.74−1.69 (m, 1H, H-2″), 1.38 (s, 3H, CH$_3$).

^{1}H NMR of compound **4b** (DMSO-d_6): 10.38 (s, br, 1H, NH), 7.48−7.44 (m, 2H, H-PhS), 7.37−7.32 (m, 3H, H-PhS), 6.07 (s, 1H, 5-OH), 6.01 (t, overlapped with 5-OH of compound **4a**, 1H, H-1′), 5.20 (s, 1H, H-6), 5.10 (d, J = 4.0 Hz, 1H, 3′-OH), 4.89 (dd, J = 5.5, 5.1 Hz, 1H, 5′-OH), 4.15−4.11 (m, 1H, H-3′), 3.65 (m, 1H, H-4′), 3.48 (m, 2H, H-5′ and H-5″), 2.38−2.32 (m, 1H, H-2′), 1.78−1.74 (m, 1H, H-2″), 1.29 (s, 3H, CH$_3$).

Synthesis of (5S,6S)-*cis*-5-Hydroxy-6-phenylthio-5,6-dihydro-thymidine (4c) (Scheme 2). Similar to the synthesis of compounds **4a** and **4b**, compound **3c** (364 mg) was deacetylated with methanolic ammonia to afford compound **4c** as glass (242 mg, 81.7%). R_f = 0.48 (MeOH/EtOAc, 1/25, v/v). ESI-MS: 367.0 [M − H]$^-$, MS/MS 257.1 [M − H − PhSH]$^-$; 368.9 [M + H]$^+$. ^{1}H NMR (DMSO-d_6): 10.58 (s, br, 1H, NH), 7.46−7.42 (m, 2H, H-PhS), 7.33−7.29 (m, 3H, H-PhS), 6.07 (s, 1H, 5-OH), 6.04 (t, J = 5.5 Hz, 1H, H-1′), 5.15 (s, 1H, H-6), 5.04 (d, J = 4.0 Hz, 1H, 3′-OH), 4.83 (t, 1H, 5′-OH), 4.01 (m, 1H, H-3′), 3.61 (m, 1H, H-4′), 3.40−3.35 (m, 1H, H-5′), 3.29 (m, 1H, H-5″), 2.06−2.00 (m, 1H, H-2′), 1.42−1.39 (m, 1H, H-2″), 1.38 (s, 3H, CH$_3$).

Synthesis of (5S,6R)-*trans*-5-Hydroxy-6-phenylthio-5,6-dihydro-thymidine (4d) (Scheme 2). Compound **3d** (278 mg) was deacetylated with methanolic ammonia and separated on silica gel column with MeOH/EtOAc (1/10, v/v) to afford compound **4d** as glass (168 mg, 74.2%). R_f = 0.42 (MeOH/EtOAc, 1/10, v/v). ESI-MS: 367.1 [M − H]$^-$, MS/MS 257.1 [M − H − PhSH]$^-$; 368.9 [M + H]$^+$. ^{1}H NMR (DMSO-d_6): 10.34 (s, 1H, NH), 7.53−7.51 (m, 2H, H-PhS), 7.34−7.32 (m, 3H, H-PhS), 6.27 (s, 1H, 5-OH), 5.77 (dd, J = 7.1, 5.9 Hz, 1H, H-1′), 5.12 (s, 1H, H-6), 5.04 (d, J = 4.3 Hz, 1H, 3′-OH), 4.74 (t, J = 5.1 Hz, 1H, 5′-OH), 4.10−4.07 (m, 1H, H-3′), 3.68−3.66 (m, 1H, H-4′), 3.38−3.35 (m, 2H, H-5′ and H-5″), 2.15−2.10 (m, 1H, H-2′), 1.86−1.81 (m, 1H, H-2″), 1.35 (s, 3H, CH$_3$).

Synthesis of d(GT$_1$*) and d(GT$_2$*) (T$_1$* and T$_2$* Represent 4a and 4b, Respectively). To a 25-mL flask, which contained a mixture of compounds **4a** and **4b** (117 mg), was added 4,5-dicyanoimmidazole (100 mg) and DMF (0.8 mL). The flask was kept under argon atmosphere, and to the stirred solution was added dropwise *i*Pr-PAC-dG-CE phosphoramidite (0.25 g in 0.8 mL of DMF) over 15 min. The solution was stirred for another 15 min, to which solution *t*-BuOOH (100 μL, 5 M solution in nonane) was added. The solution was stirred for still another 25 min, and the reaction mixture was extracted with EtOAc. The organic layer was washed with 5% NaHCO$_3$ solution, followed by saturated NaCl solution, and dried with anhydrous Na$_2$SO$_4$. The solvent was removed by evaporation under reduced pressure. The dried residue was separated on a silica gel column with a gradient

of 4−15% MeOH in EtOAc. Appropriate fractions were pooled, and the solvent was evaporated to afford white foam (210 mg, 53.8%). ESI-MS: *m/z* 1227.2 [M − H]$^-$.

The above foam was deprotected with 7 N methanolic ammonia (100 mL) for 12 h at room temperature, and the solvent was removed to give colorless solid. ESI-MS: *m/z* 998.3 [M − H]$^-$, MS/MS 888.1 [M − H − PhSH]$^-$. The resulting solid was detritylated with 80% acetic acid at room temperature for 6 h. The solvent was removed under reduced pressure. The dried residue was dissolved in water and extracted with ether. The aqueous layer was dried by Speed-vac to yield light yellowish powder. ESI-MS: *m/z* 696.1 [M − H]$^-$, MS/MS 586.1 [M − H − PhSH]$^-$.

The above mixture was separated by HPLC as described in an earlier section. The ratio between d(GT$_1$*) and d(GT$_2$*) was estimated to be approximately 4:1 from the peak areas in the HPLC trace, which is consistent with the ratios of compounds **3a/3b** and **4a/4b**.

Compound d(GT$_1$*): ESI-MS: *m/z* 696.2 [M − H]$^-$. HRMS (ESI-FTICR) calcd 696.1489 [M − H]$^-$, found 696.1501. ^{1}H NMR (D$_2$O): 7.96 (s, 1H, H-8 of dG), 7.45−7.37 (m, 3H, H-PhS), 7.28−7.23 (m, 2H, H-PhS), 6.24 (t, J = 6.4 Hz, 1H, H-1′ of dG), 6.01 (t, J = 6.9 Hz, 1H, H-1′ of dT*), 5.05 (s, 1H, H-6 of dT*), 4.97 (m, 1H, H-3′ of dG), 4.49 (m, 1H, H-3′ of dT*), 4.26 (m, 1H, H-4′ of dG), 4.21−4.15 (m, 1H, H-4′ of dT*), 4.14−4.10 (m, 2H, H-5′ and H-5″ of dT*), 3.89−3.80 (m, 2H, H-5′ and H-5″ of dG), 2.89−2.83 (m, 1H, H-2′ of dG), 2.79−2.74 (m, 1H, H-2″ of dG), 2.41−2.35 (m, 1H, H-2′ of dT*), 2.21−2.16 (m, 1H, H-2″ of dT*), 1.52 (s, 3H, CH$_3$).

Compound d(GT$_2$*): ESI-MS: *m/z* 696.3 [M − H]$^-$. HRMS (ESI-FTICR) calcd 696.1489 [M − H]$^-$, found 696.1482. ^{1}H NMR (D$_2$O): 8.00 (s, 1H, H-8 of dG), 7.49−7.46 (t, J = 7.3 Hz, 1H, H-PhS), 7.37−7.31 (m, 4H, H-PhS), 6.33 (dd, J = 7.7, 6.4 Hz, 1H, H-1′ of dG), 6.14 (dd, J = 7.7, 6.9 Hz, 1H, H-1′ of dT*), 5.03 (s, 1H, H-6 of dT*), 5.02 (m, 1H, H-3′ of dG), 4.62 (m, 1H, H-3′ of dT*), 4.33 (m, 1H, H-4′ of dG), 4.18−4.13 (m, 3H, H-4′ and H-5′, H-5″ of dT*), 3.90−3.83 (m, 2H, H-5′ and H-5″ of dG), 2.94−2.86 (m, 2H, H-2′ of dG and H-2′ of dT*), 2.82−2.77 (m, 1H, H-2″ of dG), 2.29−2.24 (m, 1H, H-2″ of dT*), 1.72 (s, 3H, CH$_3$).

Synthesis of d(GT$_3$*) (Scheme 3). Compound d(GT$_3$*) was prepared by using the same procedures as described for the synthesis of d(GT$_1$*)/d(GT$_2$*), and it was obtained as white powder (158 mg, 56.2%). ESI-MS: *m/z* 696.3 [M − H]$^-$. HRMS (ESI-FTICR) calcd 696.1489 [M − H]$^-$, found 696.1489. ^{1}H NMR (D$_2$O): 8.02 (s, 1H, H-8 of dG), 7.49−7.42 (m, 3H, H-PhS), 7.35−7.32 (dd, J_1 = 7.3 Hz, J_2 = 7.7 Hz, 2H, H-PhS), 6.35 (m, 1H, H-1′ of dG), 6.18 (m, 1H, H-1′ of dT*), 5.04 (s, 1H, H-6 of dT*), 4.99 (m, 1H, H-3′ of dG), 4.55 (m, 1H, H-3′ of dT*), 4.33 (m, 1H, H-4′ of dG), 4.12 (m, 3H, H-4′ and H-5′, H-5″ of dT*), 3.89−3.82 (m, 2H, H-5′ and H-5″ of dG), 2.90−2.75 (m, 3H, H-2′, H-2″ of dG and H-2′ of dT*), 2.19−2.14 (m, 1H, H-2″ of dT*), 1.54 (s, 3H, CH$_3$).

Synthesis of d(GT$_4$*). The title compound was prepared by using the same procedures as described for the synthesis of d(GT$_3$*), and it was obtained as white powder (180 mg, 57.5%). ESI-MS: *m/z* 696.3 [M − H]$^-$. HRMS (ESI-FTICR) calcd 696.1489 [M − H]$^-$, found 696.1494. ^{1}H NMR (D$_2$O): 7.95 (s, 1H, H-8 of dG), 7.40 (t, 1H, H-PhS), 7.32 (d, 2H, H-PhS), 7.25 (t, 2H, H-PhS), 6.22 (dd, J = 6.4, 6.0 Hz, 1H, H-1′ of dG), 6.15 (dd, J = 6.9, 6.4 Hz, 1H, H-1′ of dT*), 5.02 (s, 1H, H-6 of dT*), 5.01−4.97 (m, 1H, H-3′ of dG), 4.52 (m, 1H, H-3′ of dT*), 4.25−4.14 (m, 4H, H-4′ of dG, H-4′, H-5′, and H-5″ of dT*), 3.92−3.83 (m, 2H, H-5′ and H-5″ of dG), 2.93−2.87 (m, 1H, H-2′ of dG), 2.81−2.76 (m, 1H, H-2″ of dG), 2.42−2.36 (m, 1H, H-2′ of dT*), 2.21−2.17 (m, 1H, H-2″ of dT*), 1.66 (s, 3H, CH$_3$).

Synthesis of d(TT$_1$*). This compound was prepared according to the same procedure as for the preparation of d(GT$_1$*) except that dT-CE phosphoramidite was used in place of the *i*Pr-PAC-dG-CE phosphoramidite. Yield for the coupling step: 83.0%. The resulting mixture of d(TT$_1$*) and d(TT$_2$*) was again separated by HPLC. d(TT$_1$*): ESI-MS: *m/z* 671.1 [M − H]$^-$, MS/MS: 561.1 [M − H −

PhSH]⁻, 435.1 [M − H − PhSH − T]⁻ (T is thymine). ¹H NMR (D₂O): 7.57 (s, 2H, H-PhS), 7.56 (s, 1H, H-6 of dT), 7.48 (t, *J* = 7.3 Hz, 1H, H-PhS), 7.37 (m, 2H, H-PhS), 6.25 (dd, *J* = 6.9, 6.4 Hz, 1H, H-1′ of dT*), 5.96 (dd, *J* = 6.8, 6.4 Hz, 1H, H-1′ of dT), 5.15 (s, 1H, H-6 of dT*), 4.83 (m, 1H, H-3′ of dT), 4.47 (m, 1H, H-3′ of dT*), 4.16 (m, 1H, H-4′ of dT*), 4.11−4.04 (m, 3H, H-4′ of dT, H-5′ and H-5″ of dT*), 3.89−3.79 (m, 2H, H-5′ and H-5″ of dT), 2.65−2.60 (m, 1H, H-2′ of dT), 2.52−2.47 (m, 1H, H-2″ of dT), 2.42−2.36 (m, 1H, H-2′ of dT*), 2.22−2.17 (m, 1H, H-2″ of dT*), 1.84 (s, 3H, CH₃ of dT), 1.58 (s, 3H, CH₃ of dT*).

Conclusions

We synthesized photolabile precursors for the generation of both the 5*R* and 5*S* isomers of 5-hydroxy-5,6-dihydrothymidin-6-yl radical (**I**) and examined their reactivities in dinucleoside monophosphates through rigorous product analyses.

We observed that, under anaerobic conditions, radical **I** can attack the C8 carbon atom of its 5′ neighboring guanine and give rise to a cross-link lesion. The yield for the formation of this lesion is affected by the stereochemistry of the C5 carbon. The 5*S* radical forms the cross-link lesion more efficiently than the 5*R* radical. Moreover, we showed by LC−MS/MS that γ-irradiation of d(GT) under anaerobic condition can also lead to the formation of the two isomeric cross-link lesions again with the formation of the 5*S* isomer being more facile than that of the 5*R* isomer. Our study also demonstrated that the cross-link lesion previously isolated by Box et al.[44] from the X-ray irradiation mixture of d(GT) under anoxic condition is the lesion that is initiated from the 5*S* isomer of radical **I**, not from the 6-hydroxy-5,6-dihydrothymidin-5-yl radical as was originally proposed.[44]

We also found that the major lesion initiated from the 254-nm irradiation of the dinucleoside monophosphates d(GT*) and d(TT*) is the dinucleoside monophosphate with the thymidine portion being degraded to an abasic site. However, the relative yield for the formation of the abasic site lesion over the cross-link lesion is much higher from the 254-nm irradiation of d(GT₁*) than that from the γ-radiation of d(GT). This result demonstrates that the C6 carbon cation, which forms from the oxidation of the reactive intermediate **I** and is generated more readily from the 254-nm irradiation of d(GT₁*) than from the γ-radiation of d(GT), contributes to a significant extent to the formation of the abasic site lesion.

The realization of the independent generation of this radical also sets the stage for the examination of the reactivity of this radical in double-stranded DNA. In this respect, we are in the process of synthesizing the phosphoramidite building block for the precursor.

Supporting Information Available: NMR spectra of synthetic compounds, LC traces, LC−MS/MS data, and spectroscopic characterizations of products isolated from the irradiation mixtures. This material is available free of charge via the Internet at http://pubs.acs.org.

JA048492T