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Evidence for Glycosidic Bond Rotation in a Nucleobase Peroxyl Radical and Its Effect on Tandem Lesion Formation

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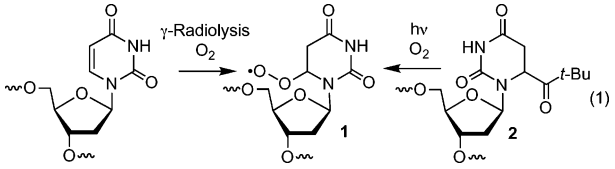
Nucleobase peroxyl radicals are the major reactive intermediates formed in DNA when the biopolymer is exposed to γ-radiolysis under aerobic conditions. The major reaction pathways for the peroxyl radical (**1**) derived from 5,6-dihydro-2′-deoxyuridin-6-yl involve π-bond addition to or hydrogen atom abstraction from the adjacent nucleotides to produce tandem lesions. The ability to independently generate **1** at a defined site in DNA enabled us to probe its reactivity by varying the local DNA structure. The effect of DNA structure variation reveals that **1** reacts from its *syn*- and *anti*-conformations in competition with trapping by thiol. These experiments also reveal that tandem lesions will be produced as a mixture of diastereomers, which could impact their biological effects.

Exposing DNA to γ-radiation, a common treatment for cancer, produces an array of lesions via radical intermediates. The identification and study of DNA lesions that exert mutagenic and/or cytotoxic effects are of particular interest and importance.[1−3] Because of their potential biological effects, there is increasing interest in tandem lesions.[4−10] Tandem lesions are a subset of clustered lesions that consist of two contiguously damaged nucleotides in DNA.[11,12] In a formal sense, tandem lesions are also produced from the UV-irradiation of DNA containing 5-bromodeoxyuridine, in which lesions such as 2-deoxyribonolactone are formed adjacent to 2′-deoxyuridine.[13−17] Relatively little is known about tandem lesions. In contrast to other clustered lesions, tandem lesions may result from amplification of a single DNA-damaging event and in order to do so require radical propagation from one nucleotide to another. Several processes resulting in tandem lesions discovered thus far involve alkyl radical (often σ-radicals) addition to an adjacent nucleobase. Hydrogen atom abstraction by σ-radicals or peroxyl radicals is also precedented. For instance, we recently demonstrated that tandem lesions are the major products produced from nucleobase peroxyl radical **1**, which was independently generated at defined sites in DNA from a photochemical precursor (**2**) (eq 1, Scheme 1).[18] Nucleobase radicals are the major family of DNA radicals generated by γ-radiolysis, suggesting that tandem lesions



(1)

form in significant amount.[19] The dearth of examples of mechanistic studies on tandem lesion formation and their potential biological importance led us to further investigate the reactivity of **1** in DNA. As in previous studies, we have taken advantage of independent generation of **1** to use local DNA structure as a tool in probing the peroxyl radical's reactivity.[18]

Previously, **1** was found to react with neighboring nucleotides by multiple pathways. The partitioning of the radical (**1**) among these pathways was found to be dependent upon local DNA structure.[18] Substituting 5,6-dihydrothymidine (dHT) for thymidine at the 5′-adjacent

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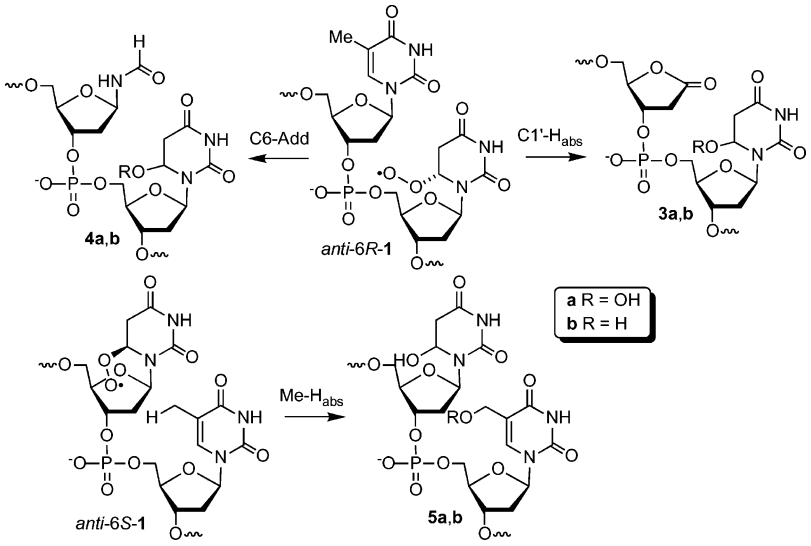
SCHEME 1



nucleotide increased the yield of NaOH-labile 2-deoxyribonolactone (**3**) at the expense of piperidine-labile (e.g., **4**) lesions. These experiments supported the proposal that **1** abstracted the C1′-hydrogen atom and added to the thymine ring. In contrast, replacing the 3′-thymidine, where there was no evidence for hydrogen atom abstraction from the deoxyribose, with 2′-deoxyuridine (dU) significantly reduced the amount of piperidine-labile tandem lesions (**5**) at this site. Molecular modeling provided an explanation for the effects of local DNA structure on radical reactivity by suggesting that the environments of the diastereomeric peroxyl radicals (6*S*- and 6*R*-**1**) in their respective *anti*-conformations were very different as a result of the helical twist of DNA. We have now utilized local DNA structure variation in conjunction with independent generation of **1** to demonstrate that the diastereomeric peroxyl radicals produce tandem lesions when present in their *anti*- and *syn*-conformations. Conformational isomerization is rapid compared to thiol trapping and has consequences on the stereochemistry of the observed lesions.

Results and Discussion

Substitution at the C6-position of a pyrimidine destabilizes the *anti*-conformation, although deplanarization of a dihydropyrimidine ring mitigates the steric interactions responsible for enhancing the relative stability of the *syn*-isomer.[20,21] Molecular modeling of 6*R*- and 6*S*-**1** revealed that the peroxyl group was pseudoaxial in the minimum energy structure, which is consistent with other studies on C6-oxygenated pyrimidines.[22,23] Incorporation of the diastereomeric peroxyl radicals into the 5′-d(T**1**T) duplex suggests that *anti*- and *syn*-6*S*-**1** are positioned to react with the 3′- and 5′-adjacent nucleotides, respectively, when the molecules are intrahelical

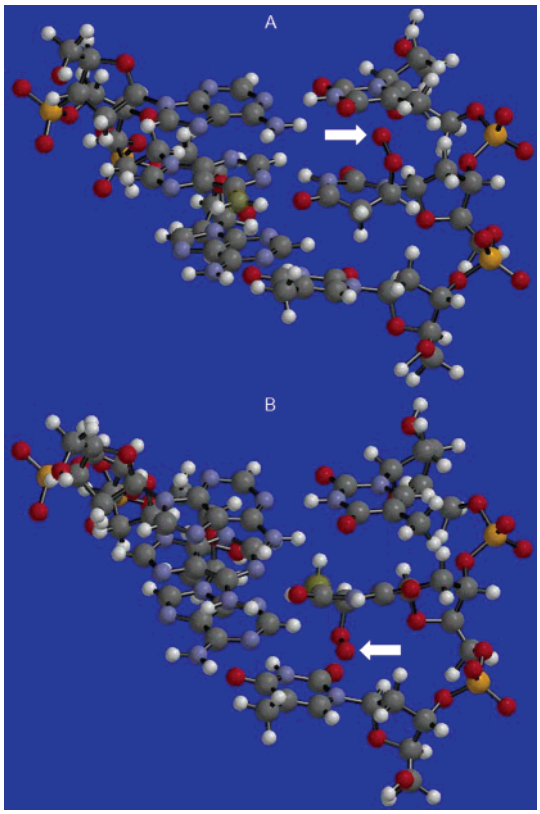
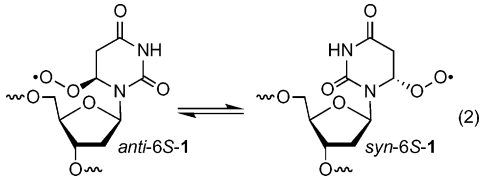


FIGURE 1. Molecular models of *anti*-6**S**-**1** (A) and *syn*-6*S*-**1** (B) in 5′-d(T**1**T)/d(AAA). Arrows indicate the position of the peroxyl radicals.

(Figure 1, eq 2). In contrast, *anti*- and *syn*-6*R*-**1** are oriented to react with the 5′- and 3′-adjacent nucleotides, respectively. The proximity of the peroxyl radical oxygen



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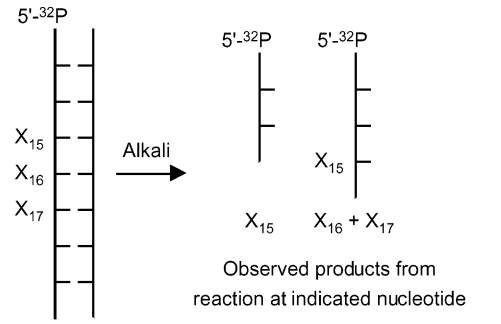
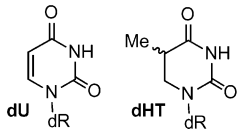


5'-d(GAG CTA GCT CGA CCX$_{15}$ **2**Y$_{17}$A GGA CCT GCA GCT)
3'-d(CTC GAT CGA GCT GGA AA T CCT GGA CGT CGA)

6a X = Y = T
6bX = dU, Y = T
6c X = T, Y = dU
6d X = Y = dU
6e X = T, Y = dHT



of *syn*-6*S*-**1** to the C1′-hydrogen atom of the 5′-thymidine (1.5 Å) is a notable difference from *anti*-6*S*-**1**, which is distant from the respective hydrogen atom of the 3′-adjacent thymidine (5.5 Å). Previously, we showed that **1** produces 2-deoxyribonolactone via C1′-hydrogen atom abstraction from the 5′-adjacent thymidine, whereas reaction at the sugar of the 3′-adjacent nucleotide was not observed (Scheme 1).[18] Molecular models suggest *anti*-6*R*-**1** and *syn*-6*S*-**1** (Figure 1B) are well positioned to react with the deoxyribose of the 5′-adjacent nucleotide.[18]

Although one could be confident that *anti*-6*R*-**1** contributes to strand damage at the 5′-adjacent nucleotide, it is not possible to determine whether *syn*-6*S*-**1** is also involved in a duplex containing the sequence 5′-d(T**1**T). We reasoned that if rotation about the glycosidic bond in *anti*-6S-**1** was rapid enough to compete with its chemical reactivity, decreasing its ability to react with the 3′-adjacent thymidine should result in an increase in reaction at the 5′-thymidine by *syn*-6*S*-**1** (Figure 1B). In contrast, decreasing the reactivity of the 3′-adjacent nucleotide would have no effect on alkali-labile lesion formation at the 5′-adjacent nucleotide if *anti*- and *syn*-**1** interconvert much more slowly than the peroxyl radical reacts. Given the fact that the lifetimes of peroxyl radicals in DNA can be as high as seconds, glycosidic bond rotation does not have to be very fast in order to play a role in the reactivity of **1**.[24−26] This effect on reactivity should be discernible using gel electrophoresis. When the DNA strand is labeled at its 5′-terminus, alkali-labile products resulting from reaction at the 5′-adjacent nucleotide (X_{15}) are separable by denaturing polyacrylamide gel electrophoresis from isolated lesions formed at **1** (X_{16}) and tandem lesions involving the 3′-adjacent nucleotide (X_{17}, Scheme 2). Repartitioning of the reactivity of 6*S*-**1** would be evident by an increase in the relative amount of 2-deoxyribonolactone (which can be selectively detected via NaOH treatment) formed at the 5′-adjacent nucleotide versus NaOH-labile lesions observed at the site at which **1** is generated in 5′-^{32}P-labeled DNA.[27] Piperidine-labile lesions at the 5′-adjacent nucleotide should also increase as a result of the proximity of *syn*-6*S*-**1** to the pyrimidine's double bond.

TABLE 1. Effect of Local DNA Sequence on Alkali-Labile Lesion Formation at the Original Radical Position and 5′-Adjacent Nucleotide

	% NaOH cleavage		% piperidine cleavage	
duplex (local seq)[a]	X_{15}	X_{16}[a]	X_{15}	X_{16}[a]
6a (T**1**T)	22 ± 1	78 ± 1	24 ± 1	54 ± 1
6b (U**1**T)	25 ± 2	75 ± 2	17 ± 1	55 ± 1
6c (T**1**U)	53 ± 3	47 ± 3	42 ± 1	44 ± 1
6d (U**1**U)	52 ± 2	48 ± 2	30 ± 1	49 ± 1
6e (T**1**dHT)	60 ± 6	40 ± 9	43 ± 2	40 ± 4

[a] X_{16} refers to site at which radical (**1**) is generated from **2**.

Consequently, the relative amount of lesions derived from sugar damage formed in 5′-^{32}P-labeled duplexes containing deoxyuridine or 5,6-dihydrothymidine (dHT) on the 3′-side of **1** were compared to one containing thymidine at this position (Table 1). Previous experiments suggested that removing the allylic methyl group or saturating the double bond of the 3′-proximal thymidine would result in decreased reactivity at this nucleotide.[18] Sugar damage products such as 2-deoxyribonolactone (**3**) were selectively revealed by mild base treatment (0.1 M NaOH, 37 °C, 20 min) and analyzed by denaturing gel electrophoresis, as previously described.[18] The relative amount of NaOH-labile lesions produced from **1** at the 5′-adjacent nucleotide (X_{15}) compared to NaOH-labile products at the site of the original radical (X_{16}, Scheme 2) increased significantly when dU was incorporated at the 3′-adjacent nucleotide (**6c**, **6d**, Table 1, Figure 2). Substitution of dU for thymidine at the 5′-position had no effect on the relative amounts of NaOH-induced strand scission (**6b**, Table 1, Figure 2). The relative amount of NaOH-labile products at X_{15} also increased when the double bond in the 3′-adjacent nucleobase was removed by substituting dHT for thymidine (**6e**, Table 1, Supporting Information). Piperidine cleavage, which reveals sugar and nucleobase lesions, was affected similarly by substituting dU or dHT for thymidine at X_{17} (Table 1, Figure 2).

The increase in relative NaOH and piperidine lability at X_{15} compared to that of X_{16} upon substitution of T_{17} by dU or dHT in **6a**−**e** is consistent with an increased yield of tandem lesions such as those containing 2-deoxyribonolactone (**3**). More definitive evidence regarding the formation of **3** was gleaned using a previously reported set of fingerprint reactions that characterize this lesion.[27,28] Treatment of photolyzed **6d** containing dU at the 5′- and 3′-adjacent nucleotides with the appropriate reagents produces a fingerprint (Figure 3) that is identical to that observed from independently generated 2-deoxyribonolactone.[27] In contrast, we previously reported that the yield of **3** produced from photolysis of **6a** was too low to detect using this procedure.[18]

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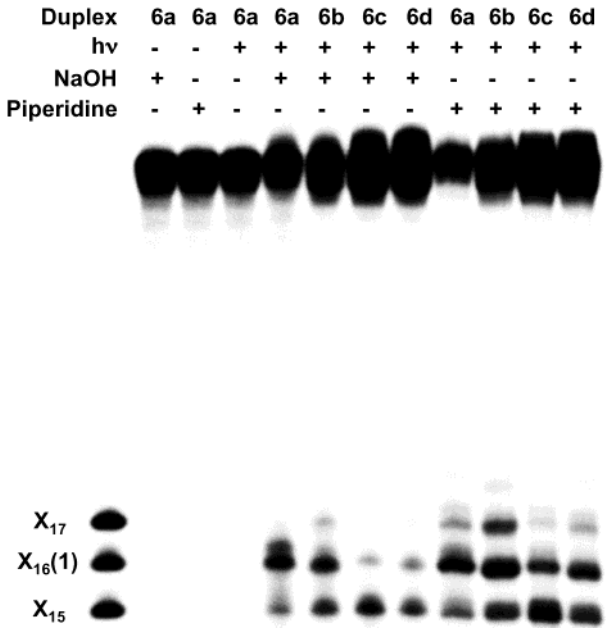


FIGURE 2. Phosphorimage of 5′-^{32}P-**6**(**a**−**d**) showing the effect of adjacent nucleotides on ratio of alkali induced cleavage at original site of radical (**1**, X_{16}) versus 5′-adjacent nucleotide (X_{15}).

The increased lactone yield (and that of piperidine labile lesions) at the 5′-adjacent nucleotides is ascribed to a redistribution in the reactions involving **1** caused by variation in the reactivity of the 3′-adjacent nucleotides. Substituting dU for thymidine at X_{17} (**6c**, **6d**) reduces alkali-labile lesion formation at this nucleotide.[18] The absence of an effect upon substituting the 5′-thymidine (X_{15}) by dU (**6b**) indicates that the altered reactivity patterns in **6c** and **6d** are not due to changes in duplex structure. If the distribution of alkali-labile lesions were altered only when using the oligonucleotide containing dHT (**6e**), one might ascribe this to structural deformation of the duplex, despite calculations indicating that the lesion does not perturb DNA.[23] However, given the altered reactivity observed when more conservative nucleotide substitutions are made, we propose that the effect of dHT substitution (**6e**) be attributed at least in part to altered reactivity. If conformational isomerization were slow compared to the reactivity of **1**, the peroxyl radicals (*anti*-6*S*-**1** and/or *syn*-6*R*-**1**) oriented toward the 3′-adjacent nucleotide would not form tandem lesions but would still produce alkali-labile lesions detectable in 5′-labeling experiments at the original site of radical generation (X_{16}). In this situation the nucleotide changes would not affect the ratio of alkali-labile lesions at X_{15}:X_{16}.

The effect of substitution at the 3′-adjacent nucleotide on the reactivity of **1** with the 5′-proximal nucleotide leads us to propose that an individual diastereomer of **1** reacts from both its *syn*- and *anti*-conformation. The hypothesis that **1** forms tandem lesions from either conformation about the glycosidic bond was explored further using glutathione (GSH) trapping of the peroxyl radical, which is a biologically relevant competing reaction to approximate how rapid conformational isomerization is. If interconversion of *anti*- and *syn*-**1** is rapid compared to GSH trapping, the ratio of NaOH-induced cleavage at X_{15}:X_{16}(**1**) is independent of thiol concentration (Scheme 3), but not if isomerization is competitive

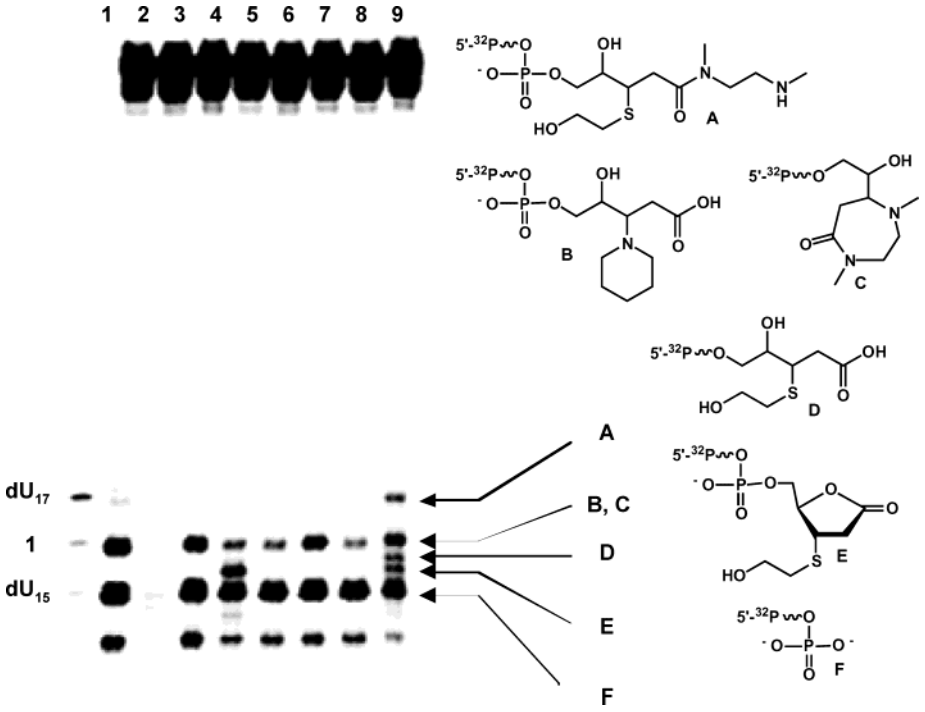


FIGURE 3. Fingerprint of adducts formed from 2-deoxyribonolactone produced by photolysis of 5′-^{32}P-**6d**: (lane 1) oligonucleotide size markers; (lane 2) photolyzate treated with 1.0 M piperidine (90 °C, 20 min); (lane 3) photolyzate, no further treatment. Lanes 4−9, photolyzate treated with the appropriate reagent(s) at 37 °C for 20 min: (lane 4) piperidine (100 mM); (lane 5) piperidine (100 mM), BME (50 mM); (lane 6) DMEDA (100 mM); (lane 7) DMEDA (5 mM); (lane 8) DMEDA (5 mM), BME (5 mM); (lane 9) DMEDA (100 mM), BME (50 mM). DMEDA = *N*,*N*′-dimethylethylenediamine; BME = β-mercaptoethanol.

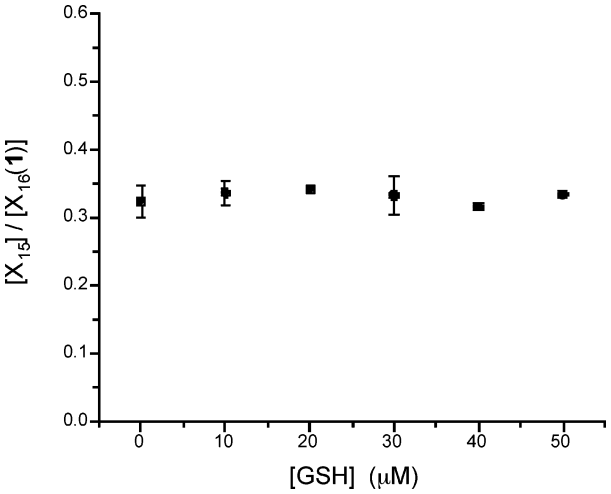
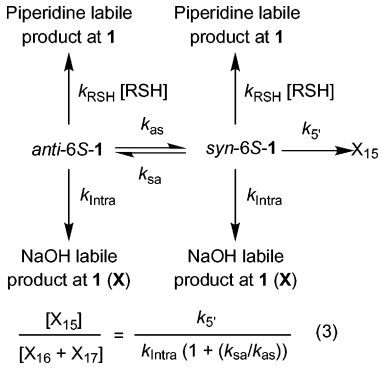


FIGURE 4. Ratio of NaOH-labile lesions (**3**) formed from at the 5′-thymidine (T_{15}) versus the original radical site (**1**) in **6a** as a function of glutathione (GSH) concentration.

SCHEME 3



with thiol tapping (see Supporting Information). The ratio of alkali-labile lesions at these sites will also be independent of [GSH] if conformational isomerization is much slower than thiol trapping, but this scenario is excluded by the data above (Table 1). The kinetic scheme is drawn for 6*S*-**1**, but a complementary one can be drawn for the diastereomeric peroxyl radical (6*R*-**1**), which is in position to react with the 5′-adjacent nucleotide when present in the *anti*-conformation.[18] Experiments were carried out using a range of glutathione concentration that reduces the amount of NaOH-labile product by 30%. The maximum [GSH] (50 μM) used is low enough that it will not compete ($k_{RSH} \approx 1 \times 10^7$ M^{-1} s^{-1}) with O_2 trapping ($k_{O_2} = 2 \times 10^9$ M^{-1} s^{-1}, $[O_2] \approx 0.2$ mM) of the original nucleobase radical. The lack of an effect on the cleavage ratio demonstrates that *syn*-*anti* isomerization is rapid compared to thiol trapping (Figure 4). The lower limit for thiol reaction with peroxyl radicals is $\sim 10^2$ M^{-1} s^{-1}, and the upper limit is $\sim 5 \times 10^3$ M^{-1} s^{-1}.[29,30] This would indicate that the rate constant for isomerization is significantly greater than 5×10^{-3} or 0.25 s^{-1}, respectively. The lifetime of DNA peroxyl radicals range from a few tenths of a second to seconds.[24−26] Hence, these experiments suggest that tandem lesions will arise from multiple configurational and conformational isomers of a DNA peroxyl radical (e.g., **1**).

The results above are based upon the premise that the diastereomers of **1** can adopt intrahelical orientations (e.g., Figure 1). It is possible that intrahelical models do not represent the lowest energy state of the duplexes containing **1**. NMR and UV-melting experiments indicate that a stable C6-substituted dihydropyrimidine, thymidine glycol, does not form stable Watson−Crick base pairs in DNA.[31,32] Bolton estimates that ∼40% of the thymine glycol surface area is exposed to solvent. This is significantly more than a typical base pair (∼15%) but considerably less than other extrahelical nucleotides, where surface accessibility can reach ∼80%. Moreover, such studies do not account for dynamics. Since the lifetime of DNA peroxyl radicals (0.1 to >1 s) are considerably longer than that required for base pair breathing, which is often on the order of milliseconds, it is possible that isomerization occurs from an extrahelical state, which is in equilibrium with a less stable but more reactive intrahelical structure.[24−26,33]

Conclusions

These experiments demonstrate that tandem lesions arise from the reaction of a mixture of nucleobase peroxyl radical (**1**) conformational and stereoisomers. Previously, we showed that DNA helicity controls the chemoselectivity of tandem lesions derived from 6*R*- and 6*S*-**1**. We have now shown that conformational isomerization also contributes to the product distribution, resulting in stereoisomeric tandem lesions. For instance, tandem lesions composed of a 5′-deoxyribonolactone will contain both diastereomers of 3′-pyrimidine C6-hydrate (and/or hydroperoxide). Diastereomeric DNA lesions can exhibit different physicochemical and/or mutagenic effects and can interact differently with repair enzymes.[31,34−36] Hence, conformational isomerization adds stereochemistry to the list of parameters that must be considered when determining the biological effects of tandem lesions.

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Supporting Information Available: Experimental procedures for oligonucleotide synthesis, characterization, and photolysis reactions. Sample phosphor image of experiments involving **6e**. Derivations of kinetic schemes describing product dependence upon thiol concentration for slow and fast conformational isomerization. ESI-MS of oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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