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5,6-Dihydropyrimidine Peroxyl Radical Reactivity in DNA

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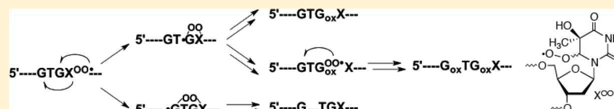
Supporting Information

ABSTRACT: Nucleobase radicals are a major family of reactive species produced in DNA as a result of oxidative stress. Two such radicals, 5-hydroxy-5,6-dihydrothymidin-6-yl radical (**1**) and 5,6-dihydrouridin-6-yl radical (**5**), were independently generated within chemically synthesized oligonucleotides from photochemical precursors. Neither nucleobase radical produces direct strand breaks or alkali-labile lesions in single or double stranded DNA. The respective peroxyl radicals, resulting from O_2 trapping, add to 5′-adjacent nucleobases, with a preference for dG. Distal dG's are also oxidatively damaged by the peroxyl radicals. Experiments using a variety of sequences indicate that distal damage occurs via covalent modification of the 5′-adjacent dG, but there is no evidence for electron transfer by the nucleobase peroxyl radicals.

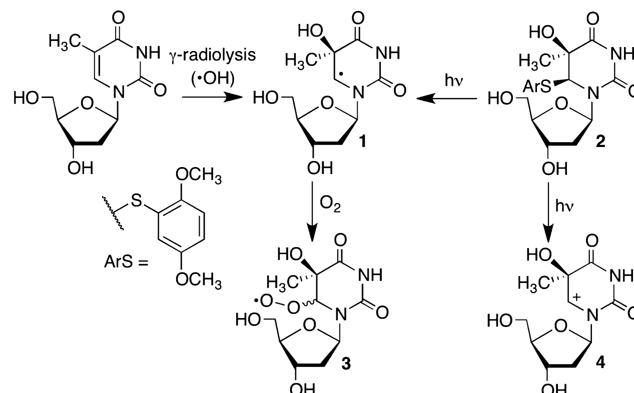


■ INTRODUCTION

Hydroxyl radical (•OH) is a major reactive intermediate produced when water is exposed to ionizing radiation. Its reactions with DNA constitute the "indirect effect" of ionizing radiation and account for as much as 85% of the damage imparted upon the molecular carrier of genetic information in cells.[1,2] Fe·EDTA (and similar metal complexes), an agent that is widely used to probe the structure and binding interactions of DNA (and RNA), relies upon its ability to cleave nucleic acids by producing •OH.[3] Strand scission by •OH is attributed to hydrogen atom abstraction from the C4′- and C5′-nucleotide positions.[4] However, a large number of studies in which ionizing radiation is used to generate •OH indicate that hydrogen atom abstraction from the carbohydrate components of nucleic acids accounts for as little as 7% of the overall reactions.[2,5,6] The major pathway is believed to involve •OH addition to the π-bonds of the nucleobases. The subsequent reactivity of the nucleobase radicals and their respective O_2 trapping products has been a topic of considerable interest to understand the ultimate chemical effects of ionizing radiation on nucleic acids. Pyrimidine •OH radical adduct reactivity has received greater attention than the corresponding purine reactive intermediates. Radiation scientists have employed a variety of sophisticated and clever methods to extract information from experiments in which •OH is generated in the bulk medium (solution, thin films, and glasses) resulting in the formation of multiple reactive intermediates. We and others are studying reactive intermediates in nucleic acids by independently generating individual species from photochemical precursors incorporated at defined sites in chemically synthesized oligonucleotides.[7−9]

Strand scission requires that the spin be transferred from the nucleobase to the carbohydrate backbone of the nucleic acids. Dihydropyrimidine radicals and/or their respective peroxyl radicals have been proposed to induce strand scission by abstracting hydrogen atoms from the carbohydrate components of RNA and to a lesser extent DNA.[6,10−14] Hydrogen atom abstraction may occur from the (2′-deoxy)ribose of another nucleotide (internucleotidyl) or intramolecularly (intranucleotidyl). When internucleotidyl hydrogen atom abstraction occurs at a nucleotide within several base pairs of the original site at which •OH reacted the aggregate damage constitutes a clustered lesion. Clustered (complex) lesions also result from reaction of a nucleotide (peroxyl) radical with another nucleotide's nucleobase and are believed to play an important role in the cytotoxicity of ionizing radiation due to their inefficient repair compared to isolated lesions.[15−19] Tandem lesions are a subset of clustered damage and describe modification on contiguous nucleotides.[20,21] Pyrimidine nucleobase peroxyl radicals have been proposed to produce tandem lesions involving adjacent 2′-deoxyguanosines (dG) by directly oxidizing the purine as well as adding to the purine.[21−23] More recently, pyrimidine peroxyl radicals were proposed to initiate electron transfer (hole migration) within DNA by oxidizing dG.[24] Herein we describe how we have

Scheme 1



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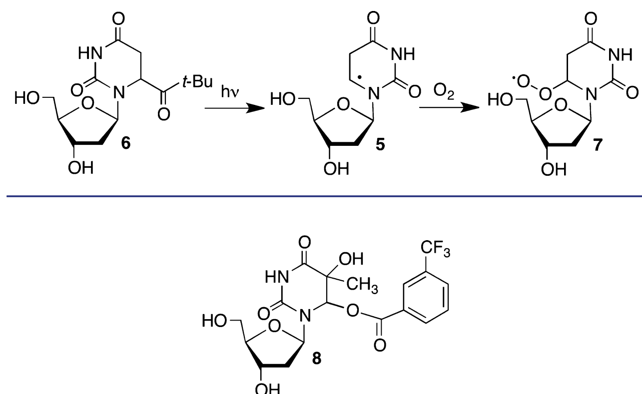
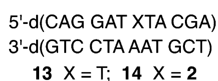
examined these issues by independently generating the major •OH radical adduct of thymidine (**1**, Scheme 1) and the structurally related species, **5** (Scheme 2). (Please note that the same descriptor is used for a compound as a monomer or when it is within a biopolymer.)

Scheme 2



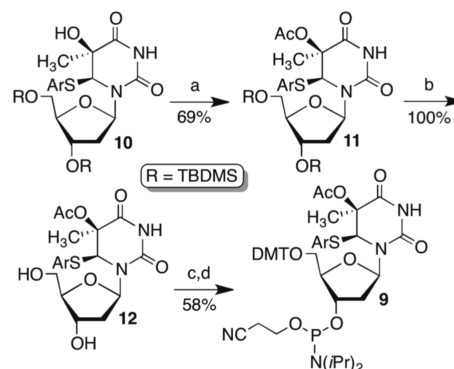
RESULTS AND DISCUSSION

Independent Generation of 5,6-Dihydropyrimidin-6-yl Radicals within Oligonucleotides. Hydroxyl radical is electrophilic and preferentially adds to the more electron-rich C5-position of pyrimidines.[2] In addition to being a synthetically expedient analogue of **1**, radical **5** is the formal product of hydrogen atom addition to 2′-deoxyuridine, which is also generated from ionization of water. 5,6-Dihydro-2′-deoxyuridin-6-yl (**5**) was previously generated in oligonucleotides via Norrish Type I photocleavage of **6** (Scheme 2).[18,19,25] The C5-hydroxyl radical adduct of thymidine (**1**) was independently generated via photoinduced electron transfer to **8**, but this chemistry is incompatible with formation of **1** in chemically synthesized oligonucleotides.[26,27] Formation of **1** by irradiating a phenyl sulfide at 254 nm is also not optimal for working with DNA.[9,28] The dimethoxy substituted aryl sulfide (**2**, Scheme 1) provides **1** along with the corresponding carbocation (**4**) upon 350 nm photolysis.[29] The electron-rich aryl sulfide (**2**) was incorporated into oligonucleotides by solid-phase synthesis using phosphoramidite **9**, which was prepared in a straightforward manner from **10** but required acetylation of the C5-hydroxyl (**11**) to prevent branching during solid phase synthesis (Scheme 3).[29] Following desilylation, the nucleoside (**12**) was carried on to **9** by standard methods. The C5-acetate group was resistant to the typical mild oligonucleotide deprotection conditions (K_2CO_3/CH_3OH) but was removed under more vigorous conditions (concentrated NH_4OH/40% methyl amine; 1:1 by volume; 25 °C, 8 h).[30] Control experiments using **12** showed that the dihydropyrimidine ring did not fragment under these conditions (data not shown). Oligonucleotides containing **2** were characterized by ESI-MS. Duplex DNA containing **2** was destabilized relative to that containing native thymidine. Dodecamer **14** (T_m = 37.6 ± 0.2 °C) melts almost 10 °C lower than **13** (T_m = 47.2 ± 0.3 °C).[31]



5-Hydroxy-5,6-dihydrothymidin-6-yl Radical (1) Does Not Lead to Direct Strand Breaks or Alkali-Labile

Scheme 3^a



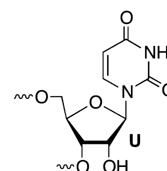
^{a}Key: (a) Ac_2O, pyridine; (b) $Et_3N{\cdot}3HF$; (c)DMTCl, pyridine; (d) phosphitylation.

Lesions Resulting from Hydrogen Atom Abstraction from the Sugar Backbone. Hydrogen atom abstraction from the 2′-deoxyribose ring produces direct strand breaks and/or NaOH labile oxidized abasic sites, depending upon the position from which the hydrogen is removed.[32,33] Previous studies established that monomeric **1** generated from **8** does not abstract hydrogen atoms from its own 2′-deoxyribose ring.[26,27] There also is no evidence for intranucleotidyl or internucleotidyl hydrogen atom abstraction by 5,6-dihydro-2′-deoxyuridin-6-yl radical (**5**) when it is generated in DNA.[15] However, the respective peroxyl radical (**7**) formed by O_2 trapping of **5** abstracts the C1′-hydrogen atom from the 5′-adjacent nucleotide.[15,18,19] Using **2** as a precursor, we observed that the reactivity of 5-hydroxy-5,6-dihydrothymidin-6-yl radical (**1**) paralleled that of **5** in the absence of O_2. Specifically, anaerobic photolyses of duplex DNA containing **2** (5′-^{32}P-**15** or 5′-^{32}P-**16**) failed to produce any direct strand scission or alkali-labile sugar lesions at the nucleotides bonded to the 5′-phosphate of the radical precursor.[31]

The reactivities of 5-hydroxy-5,6-dihydrothymidin-6-yl radical (**1**) and **5** were not as similar to one another when O_2 was present. Like **5**, direct strand scission did not occur when **1** was produced from irradiation of 5′-^{32}P-**15** or 5′-^{32}P-**16** under aerobic conditions. However, as opposed to experiments involving **5**, mild alkaline treatment (0.1 M NaOH, 37 °C, 30 min) of the photolysates in which 5-hydroxy-5,6-dihydrothymidin-6-yl radical (**1**) was formed did not produce any strand scission at the respective 5′-adjacent nucleotides, suggesting that **3** did not effect internucleotidyl hydrogen atom abstraction.[31]

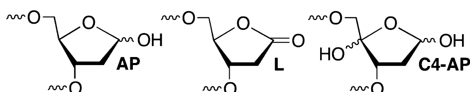
5'-d(GAG CTA GCT CAG GA**X**$_{15}$ **2**TA CGA TCT GCA GCT)
3'-d(CTC GAT CGA GTC CT **Y** AAT GCT AGA CGT CGA)

	X:Y		X:Y
15	T:A	**18**	C:G
16	G:C	**19**	U:A
17	A:T		

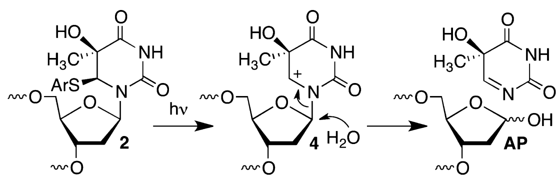


In contrast to the reactivity of 5-hydroxy-5,6-dihydrothymidin-6-yl radical (**1**) at the 5′-adjacent nucleotide, hydroxide treatment following aerobic or anaerobic photolysis of 5′-^{32}P-**15** and 5′-^{32}P-**16** produced strand scission at the site where **2**

was incorporated (19.3−31.3%).[31] This indication of abasic site formation was confirmed via incision by apurinic endonuclease 1 at the nucleotide where **2** was incorporated. The specific structural identity of the abasic site was determined via chemical reactivity. Subjecting photolyzed 5′-^{32}P-**15** and 5′-^{32}P-**16** separately to a series of "fingerprint" reactions ruled out 2-deoxyribonolactone (L) formation and other reactions eliminated formation of the C4′-oxidized abasic site (C4-AP).[31,32,34,35] Rather, the reactivity at the original site of **2** was consistent with AP formation, including the formation of 5′-cleavage products containing sugar fragments resulting from *β*-elimination in addition to phosphate groups at their 3′-termini.[36,37] We ascribe AP formation to the carbocation (**4**), which is also produced upon photolysis of the aryl sulfide (Scheme 4).[29] Attribution of the only NaOH labile damage to **4** also indicates that aerobic photolysis of **2** does not produce diffusible reactive oxygen species (ROS). ROS would have resulted in direct strand scission and/or NaOH labile lesions at the position of **2** and at neighboring nucleotides in both strands. No strand damage is detected in the complementary strand under any conditions.



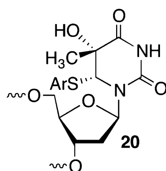
Scheme 4



Previous studies on **7** revealed that distance constraints within helical DNA limit hydrogen atom abstraction from adjacent 2′-deoxyribonucleotides to the 5′-direction and that reaction of diastereomeric C6-peroxyl radicals is coupled to rotation (*syn*/*anti*) about the glycosidic bond.[15,19] Consequently, we considered the possibility that the configuration (5*R*) at C5 in **1** affects the stereoselectivity of O_2 trapping and subsequent reactivity of **3**. To probe this, the diastereomer of **2** (**20**) was incorporated in duplexes (**21**, **22**) of identical sequence as **15** and **16**. The requisite phosphoramidite was prepared in a similar manner as was **9**.[31] The 5*R*-stereochemistry in the precursor (**20**) was controlled via asymmetric dihydroxylation of suitably protected thymidine.[38] However, formation of 5*S*-**1** from **20** upon aerobic photolysis of 5′-^{32}P-**21** or 5′-^{32}P-**22** still did not yield any direct strand breaks or NaOH labile lesions at the 5′-adjacent nucleotides.[31]

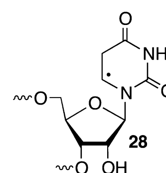
5′-d(GAG CTA GCT CAG GA**X 20** TA CGA TCT GCA GCT)
3′-d(CTC GAT CGA GTC CTY A AT GCT AGA CGT CGA)

	X:Y
21	T:A
22	G:C

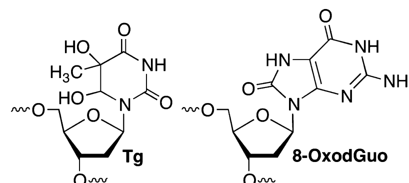


These data suggested that **3** (and its C5-epimer) is less reactive than **7**. The possibility that 5-hydroxy-5,6-dihydrothymidin-6-yl (**1**) and its respective peroxyl radical (**3**) are less reactive than unsubstituted **5** and **7** was probed further using 5′-^{32}P-**19**, in which uridine is the nucleotide bonded to the 5′-phosphate of **2**. 5,6-Dihydro-2′-deoxyuridin-6-yl radical (**5**) yields direct strand breaks by abstracting the C2′-hydrogen atom from a 5′-adjacent uridine.[13,14] The C2′-hydrogen atom of uridine is considerably weaker (∼86.5 kcal/mol) than any carbon−hydrogen bond in a 2′-deoxynucleotide and is accessible in the major groove to the dihydropyrimidine radicals.[39] Despite the more favorable driving force, no evidence for internucleotidyl hydrogen atom abstraction was observed upon photolysis of 5′-^{32}P-**19** under aerobic or anaerobic conditions.[31] Furthermore, irradiation of the analogous single stranded oligonucleotides containing **2** (5′-^{32}P-**23**-**27**) also failed to produce any direct strand scission or NaOH labile cleavage at the 5′-adjacent nucleotides in 5′-^{32}P-**23** and 5′-^{32}P-**24**.[31]

5′-d(GAG CTA GCT CAG GA$\mathbf{X}_{15}$ **2**TA CGA TCT GCA GCT)
23 X = T; **24** X = G; **25** X = A; **26** X = C; **27** X = uridine



Slower hydrogen atom abstraction by **1** and **3** than **5** and **7** correlates with the relative reactivity of monomeric **1** and **28** with thiol.[29,40] Radical **1** reacted ∼5-times more slowly with *β*-mercaptoethanol (BME) than did **28**. The differences in rate constants for reaction with BME between **1** and **28** may be due to greater steric hindrance in the former. Hydrogen atom abstraction from a carbon−hydrogen bond is less favorable thermodynamically than a sulfur−hydrogen bond and likely proceeds through a later transition state. Consequently, any correlation between the reactions of alkyl radicals **1** and **28** (which is very similar to **5**) and peroxyl radicals **3** and **7** should result in similar if not greater differences in reactivity with respect to hydrogen atom abstraction from carbon−hydrogen bonds. Conformations of the radicals may also contribute to the differences in reactivity between the actual hydroxyl radical adduct (**1**, and peroxyl radical **3**) and the respective model radicals (**5** and **7**). C5-disubstitution in **3** should favor a dihydropyrimidine ring conformation in which the methyl group is pseudo-equatorial and will control the orientation of the C6-peroxyl radical.[41−44] Furthermore, the pseudo-axial hydroxyl group will perturb base stacking. Depending on the relative heights of the barriers in the individual steps, the conformational equilibria may play a role in the reactivity of **3**. Radical **7**, which has 2 hydrogen atoms at the C5-position, should encounter smaller conformational isomerization barriers. A recent computational study suggests that conformational effects contribute significantly to the barriers for peroxyl radical reactions in DNA.[45]



Tandem Lesion Formation by Peroxyl Radical Addition to 5′-Adjacent Nucleotides. The peroxyl radical of 5,6-dihydro-2′-deoxyuridin-6-yl radical (**7**) yields tandem lesions by adding to the π-bond of a 5′-adjacent thymidine.[15,18] Oxygen radical addition to pyrimidine nucleobases produces labile lesions, such as thymidine glycol (Tg), which is cleaved by piperidine. Purine addition yields lesions such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OxodGuo), which is cleaved upon incubation with formamido pyrimidine DNA glycosylase (Fpg) or sequential treatment with Na_2IrCl_6 and piperidine but not piperidine by itself.[46,47]

5′-d(CAG GA**X** **Y**TA CGA)

	X	Y		X	Y
29a	G	**2**	**29d**	**32a**	
29b	G	Tg	**29e**	G	AP
29c	G	**31**	**30**	T	**2**

The reactivity of peroxyl radical **3** was examined in four sequences of duplex (**15−18**) and single stranded (**23−26**) DNA in which the identity of the 5′-adjacent nucleotide was varied (Figure 1). As discussed above neither direct strand breaks or abasic lesions are produced at this position, but varying amounts of nucleobase damage were detected. In addition, damage is undetectable in the complementary strand. Labile damage at a 5′-adjacent dG (dG_{15}) was more than 5-times greater than at the other three native nucleotides. Furthermore, cleavage at dG_{15} in photolyzed 5′-^{32}P-**16** was more than 3-fold greater following treatment with Fpg or Na_2IrCl_6 followed by piperidine than with piperidine alone, indicating that 8-OxodGuo is not the only lesion formed. Incision also increased ~3-fold upon Na_2IrCl_6/piperidine treatment in the single stranded substrate (**24**). The formation of all labile lesions was dependent on O_2, consistent with the involvement of **3**.[31]

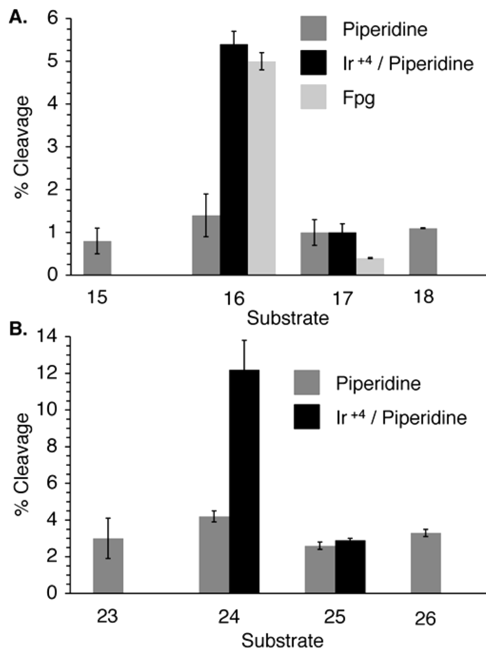
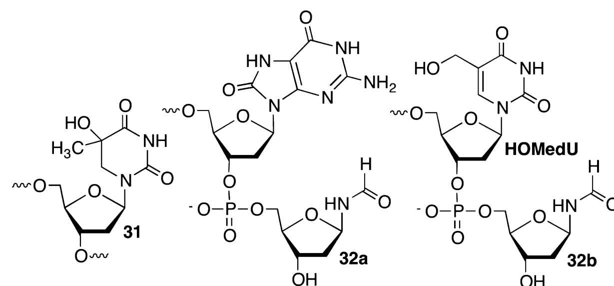


Figure 1. DNA strand lability at nucleotides 5′-adjacent to **2** following aerobic photolysis. (A) Double-stranded substrates (5′-^{32}P-**15-18**). (B) Single-stranded substrates (5′-^{32}P-**23-26**).



More definitive product identification was achieved via MS analysis of photolyzed **29a** (Figure 2) and **30**.[31] Two relatively abundant products from **29a** that were detected under aerobic and anaerobic conditions corresponded to conversion of **2** to Tg (**29b**) or an AP (**29e**) site. As discussed above, AP is attributed to carbocation (**4**) formation. Thymidine glycol was previously shown to result from formation of the radical (**1**) and carbocation (**4**).[29] Another product's m/z (**29c**) is consistent with hydrogen atom transfer to **1** (**31**), which is also consistent with its increased intensity following photolysis under anaerobic conditions without (Figure 2) or with BME (1 mM).[31] Aryl thiol produced upon photolysis of **2** is believed to be the hydrogen atom source in the absence of BME.[29] The peak at m/z = 3630 (**29d**) that is only observed under aerobic conditions is proposed to be the tandem lesion (**32a**) resulting from addition of **3** to the 5′-adjacent dG. The dG is ultimately transformed into 8-OxodGuo, and the peroxyl radical fragments to produce the formamide lesion. Tandem lesion **32a** was previously observed when DNA is exposed to •OH under aerobic conditions.[21−23] Although gel electrophoresis experiments (Figure 1) indicate that tandem lesions other than those containing 8-OxodGuo are formed, none were detected by MALDI-TOF MS (Figure 2). We also identified a tandem lesion from **30** by LC/ESI-MS.[31] An ion that corresponds to

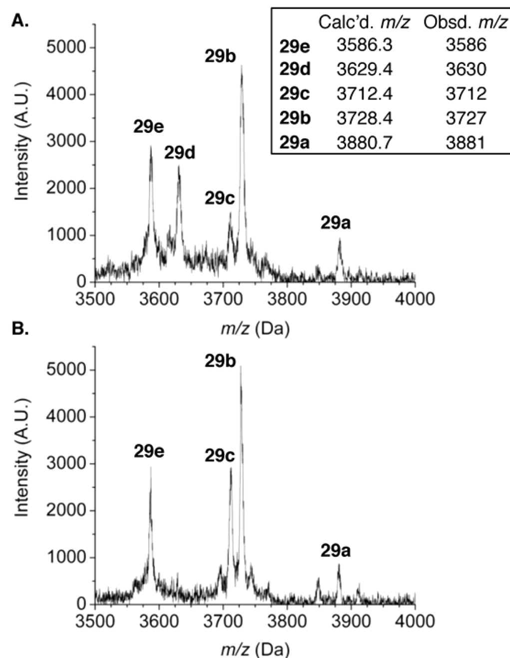
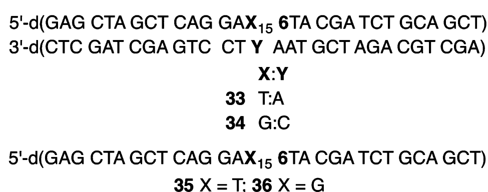


Figure 2. MALDI-TOF MS analysis of photolyzed **29a**. (A) Aerobic and (B) Anaerobic conditions.

the formation of tandem lesion containing formamide and HOMedU (**32b**) was detected.



Previously, the reactivity of **7** had only been examined in sequences containing a 5′-thymidine.[15,18] 5,6-Dihydro-2′-deoxyuridin-6-yl (**5**) was generated in the comparable sequences containing 5′-adjacent dG or T (**33**−**36**) to probe the generality of the preference for reactivity with dG. Overall, the level of alkali-labile damage in DNA was greater when **7** was generated than was **3**. However, the same strong preference for damage at dG_{15} compared to T_{15} was observed (Figure 3).

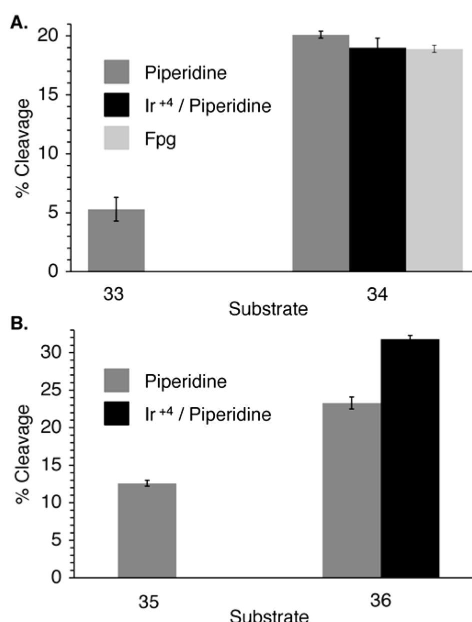


Figure 3. DNA strand lability at nucleotides 5′-adjacent to **6** following aerobic photolysis. (A) Double-stranded substrates (5′-^{32}P-**33**, **34**). (B) Single-stranded substrates (5′-^{32}P-**35**, **36**).

Damage at dG was preferred by almost 3-fold over T in single stranded oligonucleotides (**35**, **36**) and almost 4-fold in duplex substrates **33** and **34** in which peroxyl radical **7** was produced.

To probe whether the higher tandem lesion yields from **6** are due to faster reaction of **7** compared to **3**, we carried out competition experiments using BME (Figure 4, eq 1).

$$\frac{[\text{trapped}]}{[\text{cleaved}]} = \frac{k_{\text{trap}}[\mathbf{3}\text{ or }\mathbf{7}][\text{BME}]}{k_{\text{cleave}}[\mathbf{3}\text{ or }\mathbf{7}]} \quad (1)$$

Assuming that the reactivity−selectivity principle is applicable, the difference between reaction rate constants of the peroxyl radicals with BME will be small compared to 5′-adjacent purine addition. Hence, comparing the slopes of lines obtained from plotting the ratio of thiol trapping to alkali-labile product at the 5′-adjacent nucleotide versus [BME] provides an estimate of the lower limit of relative rate constants for the reaction of peroxyl radicals **3** and **7** with dG_{15} in **16** and **34**, respectively. The amount of alkali-labile product was obtained directly from

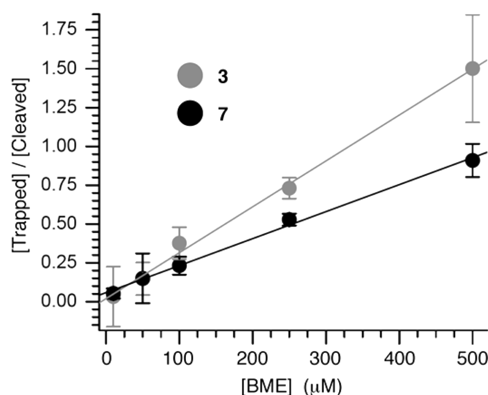


Figure 4. Effect of BME on tandem lesion formation in duplex DNA. 5′-^{32}P-**16** and 5′-^{32}P-**34** were photolyzed.

the amount of cleavage product following treatment of the photolysate with excess Fpg or Na_2IrCl_6/piperidine. (The ratios of rate constants were independent of the postphotolysis treatment.) Subtracting the amount of strand scission at dG_{15} in the presence of thiol at a given concentration from that in the absence of thiol provided the amount of thiol trapping. These experiments were carried out at sufficiently low thiol levels so that BME trapping of **1** and **5** did not compete with O_2. By assuming that k_{trap} of the peroxyl radicals in DNA by BME is 2×10^2 M^{-1} s^{-1},[48] we estimate that **3** ($k_{cleave} = 7.3 \pm 0.9 \times 10^{-2}$ s^{-1}) reacts with the 5′-adjacent dG_{15} approximately half as fast as does **7** ($k_{cleave} = 12.2 \pm 1.5 \times 10^{-2}$ s^{-1}).

The thiol trapping data are consistent with the observations noted above regarding the relative abilities of **3** and **7** to abstract hydrogen atoms from the 2′-deoxyribose portions of DNA and react with adjacent nucleobases (Figures 1 and 3). The difference likely represents a maximum that will be reduced by any differences in reactivity between BME and the peroxyl radicals. Comparing k_{cleave} for **7** with that reported previously for this peroxyl radical's reaction with a 5′-T also reinforces the qualitative comparisons of peroxyl radical reactivity showing that dG is more readily damaged than T (Figures 1 and 3).[18] The competitive kinetic experiments indicate that **7** reacts with a 5′-dG ~28-times faster than with a 5′-T.

Distal Oxidation via 5,6-Dihydropyrimidine Peroxyl Radical Formation. DNA oxidation occurs over long distances via electron transfer (often referred to as hole transfer/migration).[49−53] The damage ultimately settles preferentially at 2′-deoxyguanosine because it is the most readily oxidized nucleotide.[54] ^{18}O-Labeling studies indicate that pyrimidine peroxyl radicals add into a guanine ring, but it is not known if the subsequently formed radical is capable of initiating hole migration.[22] More recently, electron transfer between dG and pyrimidine peroxyl radicals has been proposed to account for approximately one-half of the 8-OxodGuo produced by •OH, despite electron transfer from dG to a peroxyl radical being thermodynamically uphill by ~0.23 V.[24,55]

We combined our ability to independently generate **3** and **7** with the wealth of information available regarding hole transfer in DNA to examine the proposal that a pyrimidine peroxyl radical can initiate electron transfer by oxidizing dG. 5′-dGGG is the most readily oxidized trinucleotide sequence, and it is frequently used as a trap for holes in DNA.[7,56−59] Depending upon the flanking sequence, either the 5′-terminal dG or the central dG within 5′-dGGG is most readily oxidized as a result

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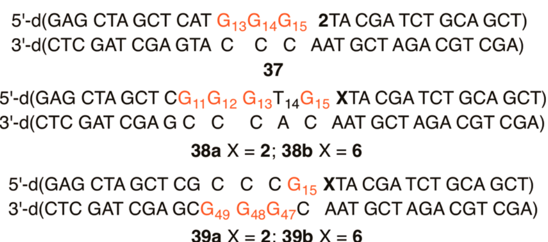


of hole transfer.[60] A series of duplexes containing a 5′-dGGG sequence and either **2** or **6** were prepared to probe for electron transfer (**37**–**39**). Curiously, strand damage in **37** was less than in the comparable duplex (**16**) containing a single dG adjacent to **2** (Figure 1A). Moreover, strand damage at dG_{15} (1.5 ± 0.4%) of **37**, which is the 3′-terminal nucleotide in the 5′-dGGG sequence was greater than at dG_{14} (0.5 ± 0.1%) and dG_{13} (none detected).[31] This is the opposite selectivity for damage expected if electron transfer is involved.

Although experiments with **37** suggested that **3** was unable to oxidize a 5′-dG by outer sphere electron transfer, we considered the possibility that addition of peroxyl radical **3** to an adjacent dG produces an intermediate(s) that initiates electron transfer. Consequently, duplexes (**38a** and **38b**) containing a 5′-dGGG sequence separated from the 5′-adjacent dG by one base pair were prepared. Overall alkali-labile damage was 4–5 times greater in **38b** (Figure 5B) than in **38a**. This is consistent with

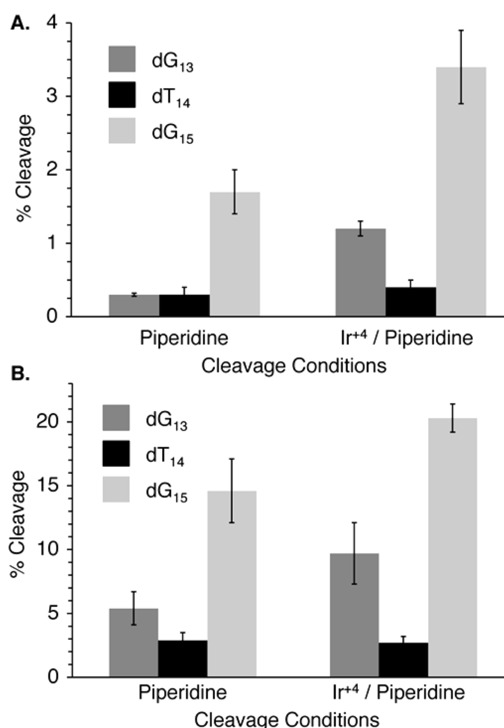
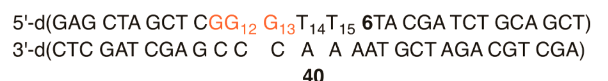


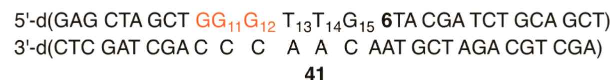
Figure 5. DNA strand lability following aerobic photolysis. (A) 5′-^{32}P-**38a**. (B) 5′-^{32}P-**38b**.

the greater nucleobase radical yield from **6** than from **2** and the higher reactivity described above for **7** compared to **3**.[29] Per above, no damage was detected under anaerobic conditions. Overall strand damage was greater in **38a** (Figure 5A) than in **37**, and the 3′-terminal dG of the 5′-dGGG sequence was most susceptible to either piperidine or Na_2IrCl_6/piperidine cleavage. Preferential damage at dG_{13}, the 3′-terminal dG in 5′-dGGG, over dG_{12} and dG_{11} (not detected), is inconsistent with the damage pattern expected for an electron transfer process.[31] Moreover, the same trend was observed from alkaline cleavage in the 5′-dGGG sequence of **38b**. The damage detected at dG_{13} was 2–3 times greater than at dG_{12} and dG_{11}, again inconsistent with electron transfer. As an aside, the cleavage yields at the dG's in each substrate were different when treated with piperidine or Na_2IrCl_6/piperidine, suggesting that 8-OxodGuo was not the only lesion formed at these nucleotides.[46,47]

Final tests for electron transfer were carried out using **39a** and **39b**. Holes migrate from one strand to another in duplex DNA, whereas an addition mechanism will be more limited by conformational constraints imposed by the biopolymer.[7] While alkaline damage was detected at dG_{15} in **39a** and **39b**, no strand damage was detected at dG_{47-49} in either substrate. In addition to providing additional evidence against dG oxidation by electron transfer following pyrimidine peroxyl radical (**3**, **7**) formation, the absence of strand damage at dG_{47-49} in **39a** and **39b** provides additional evidence against the involvement of a diffusible reactive oxygen species.[31]

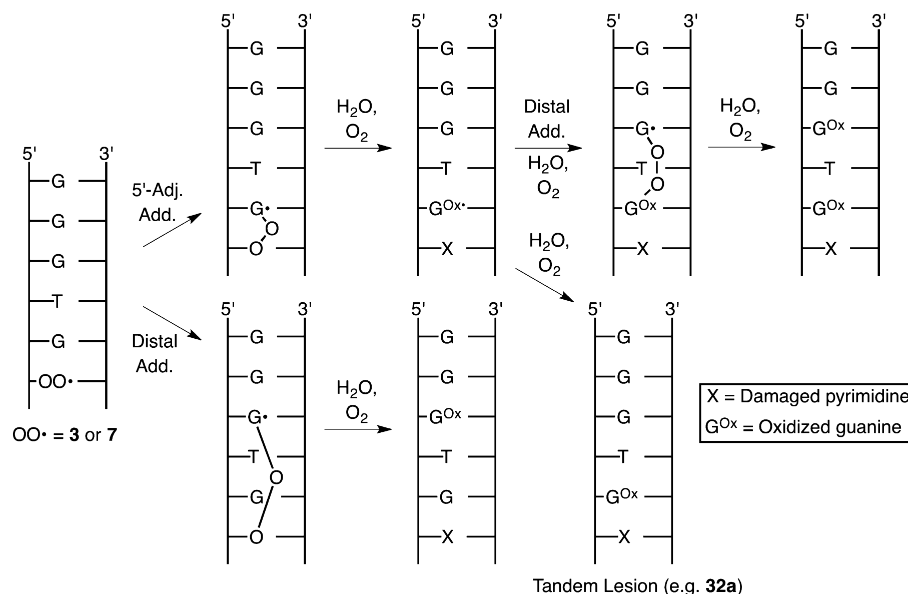
Having ruled out electron transfer and diffusible reactive species, an alternative explanation for distal oxidation (dG_{13}) in **38a,b** was sought. One possibility involves addition of the initially generated peroxyl radical (**3**, **7**) to the distal purine. Reaction at dG_{13} requires the duplex to adopt a conformation that enables the peroxyl radical to approach the purine 3 nucleotides removed (Scheme 5). UV melting experiments reveal that the dihydropyrimidine photochemical precursors destabilize the duplexes, and computations on related molecules suggest that the peroxyl radicals are likely to as well.[41,42] However, we are unaware of a reaction between two nucleotides this far away from one another in duplex DNA. Alternatively, the peroxyl radical initially formed could react with 5′-adjacent dG_{15} and a reactive intermediate on the purine that results from this process could act as a shuttle and transfer damage to dG_{13} (Scheme 5). Reaction between guanyl radicals in single stranded and duplex DNA with a nucleotide two positions away has been observed.[61,62] Recently, such lesions were even detected in irradiated HeLa cells.[63]





These possibilities were explored by comparing the damage induced in **38b** with that in **40** and **41**. Replacing dG_{15} in **38b** (Figure 5B, alkali-labile cleavage at dG_{13}: 9.7 ± 2.4%) with thymidine resulted in a large reduction in damage at dG_{13} in **40** (Figure 6, alkali-labile cleavage at dG_{13}: 3.8 ± 1.0%). This is consistent with generation of a reactive species at dG_{15} of **38b** that acts as a shuttle to transfer damage two nucleotides further in the 5′-direction to dG_{13}. The effect of distance between dG_{15} and the 3′-terminal dG in the 5′-dGGG sequence was examined by adding a thymidine (**41**). Greater damage is observed at T_{13} (4.1 ± 0.2%, Figure 6) than at a T in any other duplex. Furthermore, alkali-labile damage at dG_{12} is the same within experimental error in **38b** (3.7 ± 1.5%), **40** (2.7 ± 0.8%), and **41** (2.2 ± 0.5%, Figure 6). These observations are also consistent with formation of a reactive intermediate at dG_{15} capable of reacting two nucleotides away.

Scheme 5



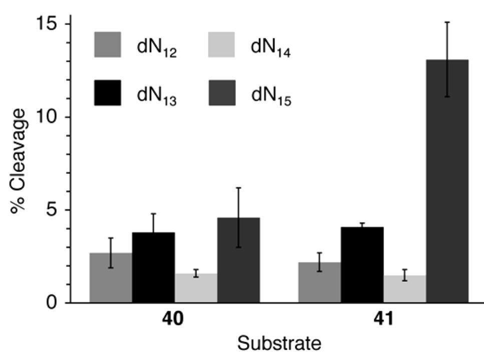


Figure 6. DNA strand lability following aerobic photolysis of 5′-^{32}P-**40** and 5′-^{32}P-**41**. Cleavage was induced with piperidine following treatment with Na_2IrCl_6.

In contrast, the presence of dG_{15} between **7** and dG_{13} has no apparent effect in the more conformationally mobile single stranded oligonucleotides (Figure 7). The alkali-labile damage at dG_{13} in **42** and **44** is within experimental error of one another. Furthermore, the alkali-labile damage yield at T_{13} in **43** is not any greater than at any thymidine in any of the other substrates examined, while the yield of damage at dG_{12} in **43** is double that in dG_{12} in **41** (Figure 6). These observations further illustrate how greater conformational mobility in single stranded substrates facilitates reactivity toward the most readily oxidized nucleotides, which are the dGs.

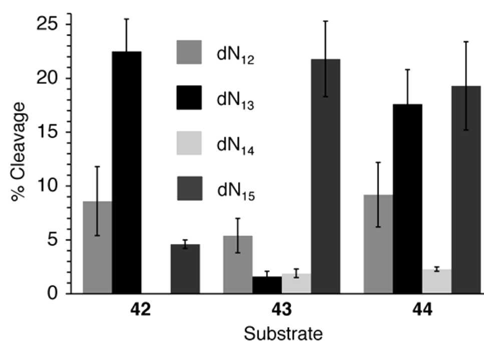
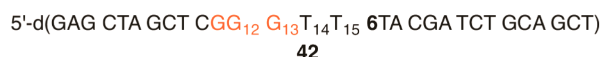
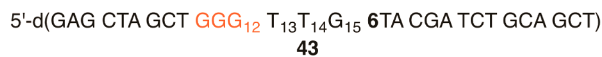
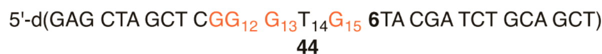


Figure 7. DNA strand lability following aerobic photolysis of 5′-^{32}P-**42**–**44**. Cleavage was induced with piperidine following treatment with Na_2IrCl_6.







Overall, these experiments indicate that a 5′-adjacent dG can react with a pyrimidine peroxyl radical and help transfer damage to a more distal nucleotide in duplex DNA. However, these experiments do not rule out a small contribution from direct reaction between a pyrimidine peroxyl radical and a nucleobase up to three nucleotides away, as observed for single stranded **42** (Figure 7, Scheme 5). These reactions will produce complex, multiply damaged lesions, which are increasingly common in ionizing radiation and believed to be biologically significant.[16,64] Attempted characterization of the complex lesions by LC/MS was unsuccessful. This may be due to the formation of multiple combinations of damaged nucleotides at up to three positions in one oligonucleotide.

■ CONCLUSIONS

These experiments reveal that the major hydroxyl radical adduct of thymidine (**1**) does not produce detectable levels of direct strand breaks or alkali-labile clustered lesions. The respective peroxyl radical (**3**) of 5-hydroxy-5,6-dihydrothymidin-6-yl radical (**1**) is less reactive than unsubstituted analogue **7** and does not yield measurable levels of hydrogen atom abstraction products. The source of the lower reactivity of **3** compared to **7** is uncertain but sterics could play a role. Disubstitution at C5 of the dihydropyrimidine destabilizes base stacking and may increase the energy of conformations necessary to achieve the internucleotide reactions discussed.[41,42,65] Recent computational studies on dinucleotide reactions involving peroxyl radicals affirm the importance of the approach trajectory.[45]

Despite steric differences, **3** and **7** react with adjacent and nearby nucleobases, producing potentially mutagenic clustered lesions. The clustered lesions result from covalent reactions of the peroxyl radicals with neighboring nucleotides, preferably dG. The initial species produced from reaction at a 5′-adjacent dG may act as a shuttle by transferring damage to more distal nucleotides, creating clustered lesions that consist of three oxidatively modified nucleotides. There is no evidence for peroxyl radicals **3** or **7** initiating electron transfer in duplex DNA by oxidizing dG. Biochemically deleterious lesions consisting of multiply damaged nucleotides are produced by ionizing radiation, and these experiments suggest that they may result from a single event between hydroxyl radical and DNA.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Procedures for all experiments, MS of photolyzed **29a** and **30**, UV melting curves, representative autoradiograms, NMR spectra of new compounds, and mass spectra of oligonucleotides containing nonnative nucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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