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Double-Strand Breaks from a Radical Commonly Produced by DNA-Damaging Agents

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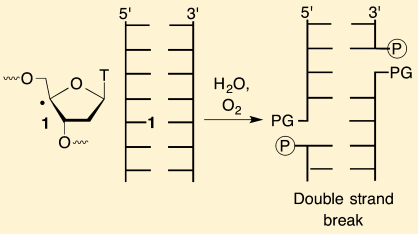
Supporting Information

ABSTRACT: Double-strand breaks are widely accepted to be the most toxic form of DNA damage. Molecules that produce double-strand breaks via a single chemical event are typically very cytotoxic and far less common than those that form single-strand breaks. It was recently reported that a commonly formed C4′-radical produces double-strand breaks under aerobic conditions. Experiments described herein indicate that a peroxyl radical initiates strand damage on the complementary strand via C4′-hydrogen atom abstraction. Inferential evidence suggests that a C3′-peroxyl radical induces complementary strand damage more efficiently than does a C4′-peroxyl radical. Complementary strand hydrogen atom abstraction by the peroxyl radical is efficiently quenched by thiols. This mechanism could contribute to the higher than expected yield of double-strand breaks produced by ionizing radiation.



INTRODUCTION

Double-strand breaks (dsb) are considered to be one of the most toxic forms of DNA damage that threaten the integrity of the genome and cell death.[1,2] Failure to repair even a single dsb can be cytotoxic.[3] Although there are many molecules that cleave nucleic acids, the size of the human genome (~3 billion base pairs) makes the probability that two molecules acting independently on DNA will produce a dsb low. Consequently, molecules and chemical mechanisms that lead to dsbs are of great interest. We recently identified a mechanism for dsb formation that could arise from a single chemical reaction with DNA and involves radical transfer from one strand to another (Schemes 1 and 2).[4] The mechanism of this unusual process is expanded upon in this article.

Calicheamicin and C-1027 are examples of rare molecules that directly produce dsbs by abstracting hydrogen atoms from opposite strands within duplex DNA.[5,6] Most recently, another natural product, lomaiviticin A, has been shown to produce dsbs.[7] A single molecule of bleomycin can also produce dsbs via a mechanism in which the iron-containing antibiotic is reactivated following oxidation of one DNA strand while it remains bound to the duplex.[8−11] Several other structurally related antitumor antibiotics exist that produce bistranded lesions that can be converted to dsbs.[12−14] The bistranded lesions produced are examples of clustered lesions (2 or more lesions within ~1.5 turns of duplex DNA) that are converted to dsbs as a result of DNA repair or by interactions with amines, such as those present in the histone proteins within nucleosomes.[15−21]

Although ionizing radiation can produce dsbs via two hydroxyl radicals reacting with opposite DNA strands, this pathway would be expected to be dependent on the square of the dose (second order in OH•). However, dsb yield increases linearly at low ionizing radiation doses. The mechanism put forth to explain this phenomenon that is widely accepted is that multiple OH• ("spurs") are produced in the vicinity of DNA due to the ability of the radiation track to ionize several water molecules.[22,23] A second mechanism that has been considered involves the formation of a radical on one DNA strand by OH• addition to a nucleobase or abstraction of a hydrogen atom by it.[24,25] A sequence of reactions then ensue resulting in cleavage of the original strand and formation of a radical on the opposing DNA strand that ultimately leads to dsb formation.

Chemical support for the latter mechanism was difficult to attain using ionizing radiation as a source for initiating DNA damage, possibly due to the large number of reactive intermediates produced throughout the biopolymer. However, we recently discovered a process whereby a C4′-nucleotide radical (**1**) yields a dsb (Schemes 1 and 2).[4] A C4′-radical (**1**) was independently generated from a previously reported photochemical precursor (**2**, Scheme 2).[26] Double-strand breaks (and bistranded lesions) are produced in an O_2-dependent manner and are composed of cleavage at the site of the originally formed radical on one strand and at one of three nucleotides on the opposite strand (these are indicated by asterisks in Scheme 1). The three nucleotides cleaved on the complementary strand are opposite those immediately 5′ to the position of the originally formed radical. Reaction with the opposing strand is made possible by cleavage of the original strand to form a cation radical (**3**, Scheme 2).[26−29] Cation radical (**3**) generation is critical for dsb formation due to concomitant strand scission, which provides the necessary conformational freedom. Water addition to **3** produces two regioisomeric radicals (**5** and **6**, Scheme 2),[30,31] which could yield as many as four diastereomeric peroxyl radicals (**7** and **8**)

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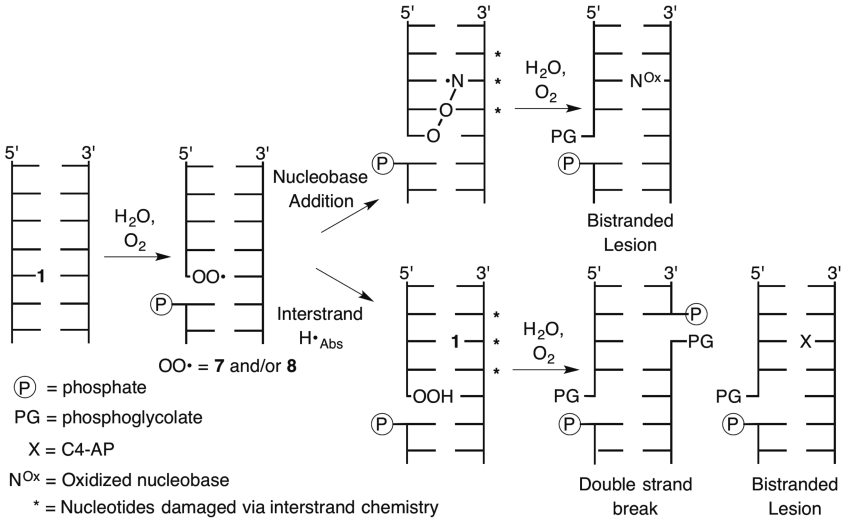
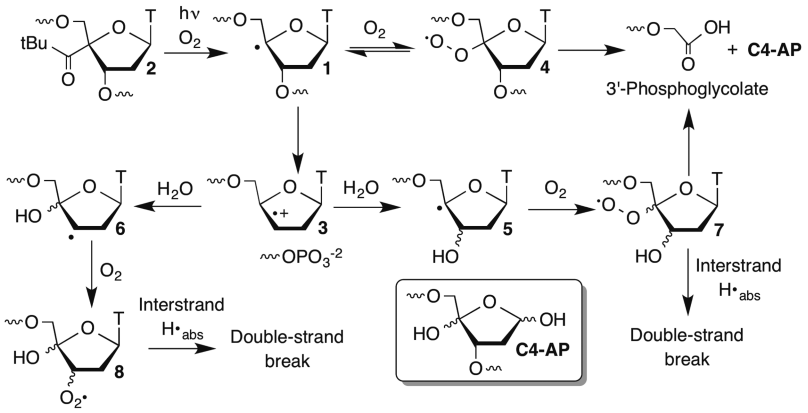
Scheme 1



Scheme 2



that may carry out interstrand hydrogen atom abstraction. Peroxyl radicals **7** and **8**, or **4** (which is formed reversibly from **1**), yield either 3′-phosphoglycolate (PG) or C4-AP.[32,33] Mass spectral analysis of the cleavage products in the complementary strand suggested that the respective C4′-hydrogen atoms were abstracted from the corresponding nucleotides in the opposing strand.[4] Whether this pathway was a major contributor and how fast the process occurred were unknown. In addition, the identity of the reactive species responsible for complementary strand damage was uncertain.

EXPERIMENTAL PROCEDURES

General Methods. Oligonucleotides were synthesized via standard automated DNA synthesis on an Applied Biosystems Inc. model 394 instrument. DNA synthesis reagents were obtained from Glen Research. Oligonucleotides were purified by 20% denaturing gel electrophoresis and desalted using C18-Sep-Pak cartridges. Oligonucleotides were characterized by ESI (Thermoquest LCQ Deca) or MALDI-TOF (Bruker Autoflex III) mass spectrometry.

Radiolabeling was carried out using standard protocols and is briefly described below.[34] T4 polynucleotide kinase (PNK) was purchased from New England Biolabs. γ-^{32}P-ATP was purchased form PerkinElmer. C18-Sep-Pak cartridges were obtained from Waters. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics phosphorimager equipped with ImageQuant TL software. Radiolabeled samples were counted using a Beckman Coulter LS 6500 scintillation counter. Photolyses were carried out in a Rayonet RPR-100 photoreactor (Southern New England Ultraviolet) equipped with 16 lamps with maximum emission at 350 nm. BME and piperidine solutions were freshly prepared.

General Procedure for the Preparation of Modified Oligonucleotides Containing 2. The syntheses of oligonucleotides containing radical precursor **2** were carried out on a 1 μmol scale. The requisite phosphoramidite was prepared as previously described.[35] The standard method for 2-cyanoethylphosphoramidites provided by the instrument manufacturer was used except that the coupling of the modified phosphoramidite was extended to 15 min. Deprotection of the nucleobases and phosphate moieties as well as cleavage of the linker was carried out using 28% aq. NH_3 at 55 °C overnight.

General Procedure for Oligonucleotide Photolysis. The strands of interest were labeled at their 5′-termini with γ-^{32}P-ATP using T4 PNK in T4 PNK buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 45 min, 37 °C). Radiolabeled oligonucleotides were separated from unincorporated ^{32}P-nucleotides by gel filtration using Sephadex G-25. Prior to photolysis, labeled strands were hybridized to the complementary strand(s) (1.5 equiv) in PBS (0.1 M NaCl, 10 mM sodium phosphate, pH 7.2) by heating at 90 °C for 5 min and slow cooling to room temperature. DNA was photolyzed (350 nm) for 4−10 h under aerobic conditions in Pyrex glass tubes (5 mm i.d.).

Postphotolysis Oligonucleotide Treatments. Aliquots were treated with NaOH (0.1 M, 30 min, 37 °C; neutralized with 0.1 M HCl) or piperidine (1 M, 30 min, 90 °C). Piperidine-treated samples

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were frozen (dry ice/acetone) and evaporated to dryness under vacuum. The residue was taken up in H_2O (50 μL), vortexed, spun briefly, frozen, and evaporated to dryness under vacuum. Samples were analyzed by dissolving in formamide loading buffer prior to separating by denaturing PAGE.

RESULTS AND DISCUSSION

Isotopic Labeling Detection of Interstrand C4′-Hydrogen Atom Abstraction. Cleavage products from the complementary strand from which **1** was initially generated containing 3′-phosphoglycolate termini (PG, Scheme 2) were detected by LC/MS.[4] This was strong evidence for C4′-hydrogen atom abstraction en route to dsbs. However, it did not provide quantitative information regarding the contribution of C4′-hydrogen atom abstraction to overall dsb formation. More insight into the overall contribution of C4′-hydrogen atom abstraction from the complementary strand was gleaned from experiments in which deuterium was selectively incorporated at the C4′-position of the nucleotide, which is the major cleavage site on the complementary strand. It was anticipated that deuteration of the C4′-position would give rise to a kinetic isotope effect (KIE), resulting in reduced cleavage at the deuterated nucleoside.[36−40] The reduction in the amount of strand scission at the nucleotide would be proportional to the KIE for the hydrogen atom abstraction reaction and the fraction of overall cleavage at the nucleotide attributable to this process and are referred to here as product KIEs.[38]

The C4′-position of the nucleotide (T_{50}), which is the major site where strand damage is induced in the complementary strand of **9a** (Figure 1), was deuterated (**9b**).[4] Denaturing PAGE analysis following photolysis of 5′-^{32}P-c-**9a** and -**9b** (c indicates that the strand complementary to the one containing **2** is ^{32}P-labeled; k indicates that the strand containing **2** is ^{32}P-labeled) reveals visibly significant reduction in direct strand scission and alkali-labile lesion formation at T_{50} in the latter (Figure 2). The product KIEs were estimated by normalizing the cleavage at T_{50} in 5′-^{32}P-c-**9a** and -**9b** using the strand scission at A_{51}. This method removed the extent photochemical conversion as a variable from the calculation. The values calculated are the averages of two experiments, with each containing three samples. The error in the product KIE for direct strand scission is greatest because the absolute amount of cleavage is closest to background in these samples (particularly at 4′-^{2}H-T_{50} in **9b**) and the percentage variation is greatest. Although the magnitudes of product KIEs are similar when direct strand scission (3.9 ± 1.9) or NaOH (3.4 ± 0.8) induced cleavage is measured, the isotope effect is significantly smaller following piperidine cleavage (1.7 ± 0.3). Direct and NaOH-induced strand scission detect damage resulting from oxidation of the carbohydrate backbone.[41] The cleavage observed upon NaOH treatment encompasses direct strand breaks and oxidized carbohydrates that are labile to the alkali. That the product KIEs under these conditions are within experimental error of one another suggests that hydrogen atom abstraction from other positions that give rise to NaOH-labile lesions (e.g., C1′), from which hydrogen atom abstraction would not be affected by C4′-deuteration, do not contribute significantly to damage on the complementary strand.[42] Furthermore, the small amount of direct and NaOH-labile cleavage observed at 4′-^{2}H-T_{50} in **9b** suggests that abstraction of this hydrogen is a major contributor to damage resulting from carbohydrate oxidation at this nucleotide. In contrast, 4′-^{2}H-T_{50} has a smaller effect on strand scission at this nucleotide following piperidine treatment of photolyzed 5′-^{32}P-c-**9b**. This suggests that there is one, or more, pathway(s) that does not involve C4′-hydrogen atom abstraction, which yields damaged products on the

5′-d(GGT GAC AGC TAT TGA TCT T T A A **2**T TGT GAT GCT AG)
3′-d(CCA CTG TCG ATA ACT AGA AA$_{51}$N$_{50}$ T$_{49}$ AA ACA CTA CGA TC)
9a N$_{50}$ = T
9b N$_{50}$ = 4′-^{2}H-T

5′-d(GGT GAC AGC TAT TGA TCT TA T T **2**T TGT GAT GCT AG)
3′-d(CCA CTG TCG ATA ACT AGA AT$_{51}$A$_{50}$ A$_{49}$ AA ACA CTA CGA TC)
10

5′-d(GGT GAC AGC TAT TGA TCT TA T F **2**T TGT GAT GCT AG)
3′-d(CCA CTG TCG ATA ACT AGA AT$_{51}$A$_{50}$ A$_{49}$ AA ACA CTA CGA TC)
11

5′-d(GGT GAC AGC TAT TGA TCT TA T F **2**^{OH} T TGT GAT GCT AG)
3′-d(CCA CTG TCG ATA ACT AGA AT$_{51}$A$_{50}$ A$_{49}$ A A ACA CTA CGA TC)
12

5′-d(GGT GAC AGC TAA TTG ATA TCC C T **2** TGC GAT GCT AG)
3′-d(CCA CTG TCG ATT AAC TAT AGG$_{51}$G$_{50}$ A$_{49}$ A ACG CTA CGA TC)
13

5′-d(GGT GAC AGC TAA TTG ATA TCC C T **2**^{OH} TGC GAT GCT AG)
3′-d(CCA CTG TCG ATT AAC TAT AGG$_{51}$G$_{50}$ A$_{49}$ A ACG CTA CGA TC)
14

5′-d(GGT GAC AGC TAA TTG ATA TCC C F **2** TGC GAT GCT AG)
3′-d(CCA CTG TCG ATT AAC TAT AGG$_{51}$G$_{50}$ A$_{49}$ A ACG CTA CGA TC)
15

5′-d(GGT GAC AGC TAA TTG ATA TCC C F **2**^{OH} TGC GAT GCT AG)
3′-d(CCA CTG TCG ATT AAC TAT AGG$_{51}$G$_{50}$ A$_{49}$ A ACG CTA CGA TC)
16

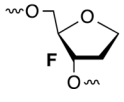

Figure 1. DNA substrates used in this study.

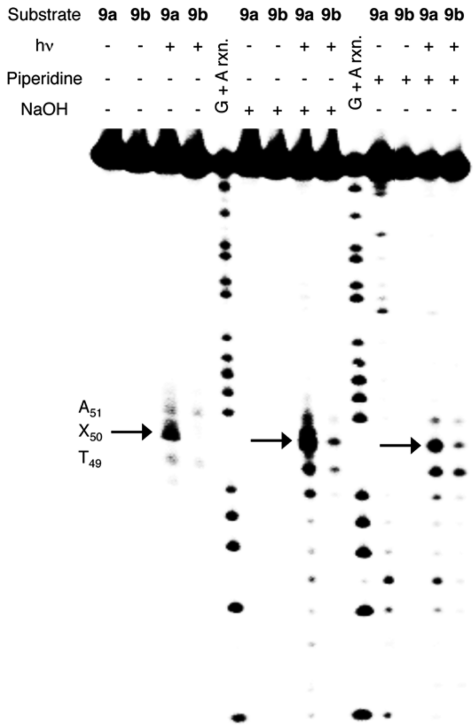

Figure 2. Autoradiogram of cleavage in 5′-^{32}P-**9a** and 5′-^{32}P-**9b** following photolysis with no further treatment (direct strand scission), NaOH treatment, or piperidine treatment.

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complementary strand that are labile to piperidine treatment. Previously, this property was attributed to nucleobase damage, which would not be affected by C4′-deuteration, and the reduced product KIE is consistent with this proposal.[4,41]

Effects of Thiols on Double-Strand Break Formation. Product and isotope labeling experiments indicate that peroxyl radical mediated interstrand C4′-hydrogen atom transfer from the complementary strand is the major pathway for dsb formation. However, neither the peroxyl radical (e.g., **7**, **8**) nor the efficiency/rate of this process are known. Insight into the latter was obtained by examining the effects of thiols (glutathione, GSH; β-mercaptoethanol, BME) on complementary strand damage. The respective form of damage (direct strand break, NaOH labile cleavage, piperidine labile cleavage) was measured as a function of thiol concentration (0−300 μM). The amount of trapping by thiol was estimated to be the difference in the amount of uncleaved DNA (5′-^{32}P-c-**10**) in the presence and absence of the reducing agent. Plotting the ratio of the trapped intermediate versus cleavage as a function of thiol concentration (eq 1) yielded a straight line (GSH (Figure 3); BME (Figure S1)). The slope of these plots represent the ratio of k_{Trap}/k_{Dam}, where k_{Trap} is the rate constant for reaction between the thiol and reactive intermediate(s) (identified below), and k_{Dam} is the rate constant for complementary strand damage by the same reactive intermediate(s).[43] The slopes of the respective plots for the dependence of direct strand scission (Figure 3A) and NaOH-labile strand scission (Figure 3B) on GSH concentration were within error of one another and were approximately 2-fold greater than those when piperidine cleavage (Figure 3C) was measured. Comparable observations were made when BME was used (Figure S1). Assuming that the direct strand breaks and alkali-labile lesions are derived from a common intermediate that reacts with the thiols, the decreased k_{Trap}/k_{Dam} ratio is consistent with the approximate doubling of the total strand scission upon piperidine treatment compared to that with NaOH reported previously.[4] This also suggests that the rate constant for hydrogen atom abstraction from the 2′-deoxyribose ring is approximately equal to that for nucleobase addition.

$$\frac{[\text{Trapped}]}{[\text{Cleaved}]} = \frac{k_{Trap}}{k_{Dam}}[\text{GSH}] \quad (1)$$

The response of strand damage to thiol concentration also provides insight with respect to the species that reacts with the reductant. The thiols can, in principle, quench complementary strand damage by intercepting the originally formed C4′-radical (**1**) or peroxyl radical(s) (**7** and **8**). Trapping of **1** in 5′-^{32}P-k-**10** is not considered to be viable because neither thiol is expected to compete (k = 2.2 × 10^6 M^{-1} s^{-1}) at these concentrations with O_2 (0.2 mM, k = 2 × 10^9 M^{-1} s^{-1}), which reacts reversibly with the nucleotide radical.[29] Peroxyl radical reduction by the thiols is expected to more effectively compete with hydrogen atom abstraction by these reactive intermediates.[29,44] If one assumes that hydrogen atom abstraction from thiol by a DNA peroxyl radical(s) (**7**, **8**) occurs with rate constant k_{Trap} = 2 × 10^2 M^{-1} s^{-1},[45] then the rate constants for complementary strand cleavage (k_{Dam}) range from ∼1−2 × 10^{-2} s^{-1} (Table 1). On the basis of the previously described products in the complementary strand and what is known about direct strand scission from various 2′-deoxyribose radicals, we propose that C4′-hydrogen atom abstraction by **7** and/or **8** is the rate-determining step.[4,46] Although there are not many examples of inter- or intrastrand hydrogen atom abstraction events by nucleic acid peroxyl radicals that have been characterized kinetically, the above estimated rate constant is 2−5-fold faster than C1′-hydrogen atom abstraction by **17**.[47]

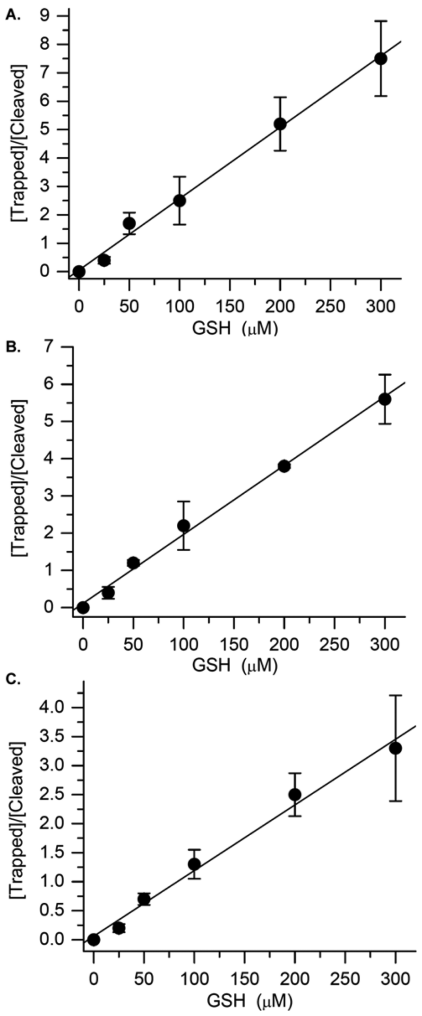

Figure 3. Effect of GSH on cleavage in 5′-^{32}P-**10** following photolysis with (A) no further treatment (direct strand scission), (B) NaOH treatment, or (C) piperidine treatment.

Table 1. Estimated Rate Constants for Complementary Strand Damage in 5′-^{32}P-10

	k_{Dam} (×10^{-2} s^{-1})[a]	
treatment	GSH	BME
none[b]	0.8 ± 0.4	0.9 ± 0.1
NaOH[c]	1.1 ± 0.5	1.3 ± 0.1
piperidine[d]	1.8 ± 0.8	1.9 ± 0.1

[a]Rate constants are the average ± SD of two independent experiments each carried out in triplicate. [b]Direct strand scission. [c]0.1 M NaOH, 37 °C, 30 min. [d]1.0 M piperidine, 90 °C, 30 min.

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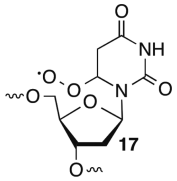


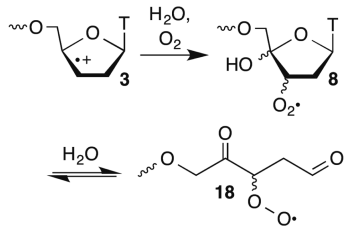
Identifying the Peroxyl Radical(s) Responsible for Interstrand Damage. Complementary strand damage requires cleavage within the strand in which the C4′-radical (**1**) is originally generated. This provides the conformational flexibility to react with nucleotides in the complementary strand. Formation of the nicked strand from **1** proceeds through the cation radical (**3**) and ultimately provides two regioisomeric peroxyl radicals (**7** and **8**). Preferential formation of 4′-peroxyl radical (**7**) is expected.[30,31] Insight into the relative contributions of 3′-peroxyl (**8**) and/or 4′-peroxyl (**7**) radicals to interstrand damage was gleaned by comparing the reactivity of DNA substrates in which the C4′-radical precursor (**2**) is either within an intact strand (e.g. **11**) or at the 3′-terminus of an otherwise identical ternary complex (e.g. **12**). Substrates containing **2** at the 3′-terminus of an oligonucleotide within a ternary complex do not produce cation radical **3** and can form only diastereomeric 4′-peroxyl radicals (**7**) by reacting with O_2 (Scheme 2). We previously reported that complementary strand damage is enhanced in substrates containing a stable abasic site analogue (F), presumably by reducing the barrier for the required conformational reorganization.[4] For instance, complementary strand damage in **11** was ∼2−3-fold greater than that in **10** (Table 2). However, in a side-by-side comparison, the complementary strand damage in **12** was ∼2.5−3.5-fold lower than that in **11**. The same pattern was evident, albeit to a lesser extent, when analogous substrates containing 5′-dGGG sequences (**13**−**16**) were photolyzed. These sequences produce lower levels of sugar damage than those containing AT sequences. However, the amounts of all types of complementary strand damage are reduced in ternary complexes, which cannot form radical cation **3** (**14** and **16**) compared to duplexes that can (**13** and **15**) (Table 2). These data suggest that the anticipated minor regioisomeric peroxyl radicals (**8**) are more effective at inducing complementary strand damage than are C4′-peroxyl radicals (**7**). One possible reason for this is that hydration at C4′-of the cation radical yields a hemiacetal (**8**), which can equilibrate under the aqueous conditions with an acyclic peroxyl radical (**18**) that enjoys greater conformational freedom (Scheme 3).

Table 2. Effect of the Ability To Form Cation Radical 3 on Complementary Strand Damage

		% complementary strand cleavage[a]		
		treatment		
substrate	form **3**?	none[b]	NaOH[c]	piperidine[d]
10	yes	3.7 ± 0.5	5.5 ± 0.8	9.9 ± 1.5
11	yes	7.6 ± 2.3	14.8 ± 1.8	20.1 ± 2.4
12	no	2.3 ± 0.3	5.3 ± 0.4	8.5 ± 0.9
13	yes	0.4 ± 0.1	0.8 ± 0.2	6.6 ± 0.6
14	no		0.5 ± 0.1	4.5 ± 1.0
15	yes	1.6 ± 0.5	2.5 ± 0.8	13.0 ± 2.5
16	no		0.6 ± 0.1	10.6 ± 0.4

[a]Cleavage is the average ± SD of three independent measurements. [b]Direct strand scission. [c]0.1 M NaOH, 37 °C, 30 min. [d]1.0 M, 90 °C, 30 min.

Scheme 3



CONCLUSIONS

Hydrogen atom abstraction from the C4′-position of nucleotides is common among DNA damaging agents that bind in the minor groove.[46] Diffusible species, such as hydroxyl radical, also react at this site because of the relatively low carbon−hydrogen bond dissociation energy and high solvent accessibility.[38] Double-strand break formation from an initially formed C4′-radical under aerobic conditions verifies general mechanistic proposals based upon ionizing radiation studies and unveils a possible pathway for designing molecules that produce this type of DNA damage via a single chemical event.[24,25] The experiments described above provide mechanistic insight into how a dsb is produced from abstraction of a single hydrogen atom from one strand of DNA. Isotopic labeling reveals that the C4′-hydrogen atom is the major site of reaction on the complementary strand. Experiments with ternary complexes containing a C4′-radical precursor at the 3′-terminus of an oligonucleotide further substantiate the importance of conformational freedom for interstrand hydrogen atom abstraction and suggest that a 3′-peroxyl radical (**8**) more efficiently reacts with the complementary strand than does a regioisomeric C4′-peroxyl radical (**7**). Competitive kinetic experiments using thiols reveal that interstrand hydrogen atom transfer by a peroxyl radical(s) is slow and would be quenched by physiologically relevant levels of reductant (millimolar). The rate constants estimated for interstrand hydrogen atom transfer by the peroxyl radical are consistent with their expected reactivity. These experiments suggest that dsb formation emanating from a single initial hydrogen atom abstraction on one DNA strand is possible but would be more practical if one could utilize a radical trap other than O_2, which produces a more reactive intermediate that carries out interstrand hydrogen atom transfer more efficiently.

ASSOCIATED CONTENT

Supporting Information

Mass spectra of oligonucleotides containing nonnative nucleotides. Plots of the effect of BME on complementary strand damage. 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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■ ABBREVIATIONS

double strand break, dsb; hydroxyl radical, OH•

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