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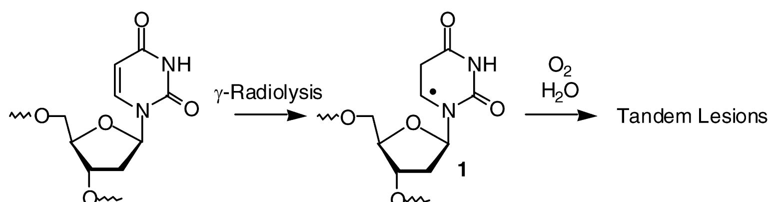

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Tandem Lesions Are the Major Products Resulting from a Pyrimidine Nucleobase Radical

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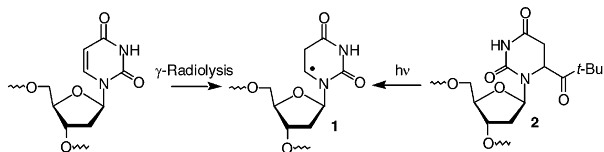
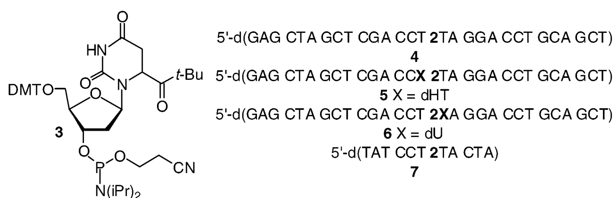
DNA damage is believed to play a significant role in the cytotoxic effects of γ-radiolysis, which is a common treatment for cancer. The chemistry of DNA damage resulting from ionizing radiation is very complex and manifests itself by the formation of direct strand breaks and a multitude of base and sugar modifications, many of which are alkali-labile.[1] Clustered damage and the subset of tandem DNA lesions have attracted attention recently due to the possible biological effects of these species.[2,3] Tandem lesions attributable to initially formed nucleobase radicals have been detected following enzymatic digestion of irradiated DNA and in model systems. However, it is not known what fraction of induced damage these lesions represent.[4-6] We have studied the chemistry of a nucleobase radical (**1**) independently generated in DNA and report the surprising observation that alkali-labile tandem lesions account for the majority of damage emanating from this reactive intermediate.



When monomeric pyrimidine nucleosides are exposed to γ-radiolysis, the formal addition of HO• or H• to the C5-position accounts for as much as 80% of the reactive intermediates produced. The large preference for forming nucleobase radicals is evident in DNA as well and is a unique aspect of DNA damage resulting from γ-radiolysis.[1] Although nucleobase radicals have been implicated in tandem lesion formation, most studies have focused on their transformation into direct strand breaks and modified nucleotides.[1] We recently described the generation and reactivity of a member of this family of nucleobase radicals, 5,6-dihydro-2′-deoxyuridin-6-yl (**1**), from **2**.[7] In the present study, the radical precursor (**2**) was incorporated into oligonucleotides **4**−**7** via phosphoramidite **3**. The oligonucleotides were characterized by HPLC analysis of the nucleosides liberated upon enzymatic digestion and by ESI-MS.[8]



A very small amount of direct strand scission is observed when aerobic solutions of duplex DNA (5′-^{32}P-**8**) are photolyzed at 350 nm (Figure 1A), and adventitious cleavage of alkali-labile lesions cannot be ruled out as its source. Similar alkaline lability is observed under degassed conditions, but control experiments with thiol indicate that residual O_2 is responsible for strand damage.[9]

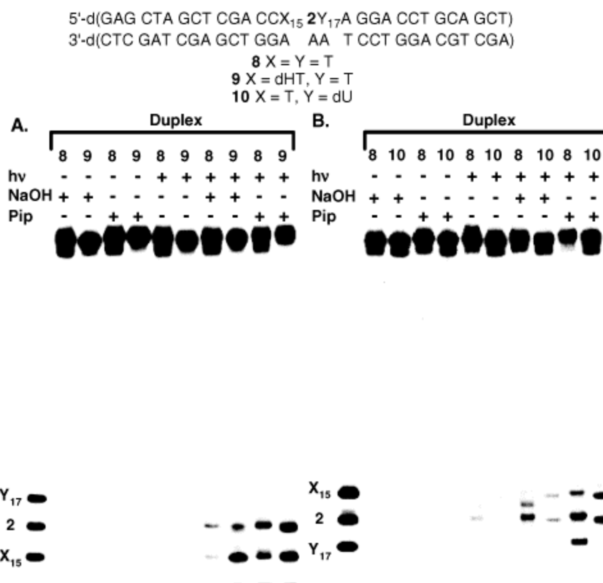


Figure 1. Gel electrophoresis analysis of aerobic photolyses (10 mM pH 7.5 phosphate, 100 mM NaCl), followed by indicated treatment of 5′-^{32}P (A) or 3′-^{32}P (B) labeled DNA containing **2**. Independently synthesized oligonucleotides are used as markers.

Table 1. Quantitative Analysis of Observed Strand Scission in **8**−**10**

duplex	site[a]	% of total cleavage[b] NaOH	piperidine	relative cleavage piperidine/NaOH
5′-^{32}P-**8**	T_{15}	37 ± 5	40 ± 5	3.3 ± 1.4
	1 (**2**)	63 ± 2	52 ± 2	2.4 ± 0.5
	T_{17}		8.0 ± 1.4	
3′-^{32}P-**8**	T_{15}	33 ± 7	8.4 ± 3.9	0.83 ± 0.10
	1 (**2**)	67 ± 7	51 ± 4	2.9 ± 0.5
	T_{17}		41 ± 7	
5′-^{32}P-**9**	dHT_{15}	74 ± 2	59 ± 1	1.4 ± 0.1
	1 (**2**)	26 ± 3	38 ± 4	2.8 ± 0.4
	T_{17}		3.0 ± 0.1	
3′-^{32}P-**10**	T_{15}	49 ± 14	38 ± 7	2.9 ± 0.6
	1 (**2**)	51 ± 14	54 ± 4	3.9 ± 1.2
	dU_{17}		8.0 ± 4	

[a] **1**(**2**) describes the position in duplex DNA where radical **1** is generated from **2**. [b] Values are calculated on the basis of total alkali-labile lesions. Each value is determined from a minimum of two experiments carried out in triplicate.

Treatment of photolyzates with NaOH (0.1 M, 37 °C, 20 min) or piperidine (1 M, 90 °C, 20 min) shows cleavage at T_{15} and the original site of **1** (Table 1). Labeling the complementary strand of **8** reveals only trace amounts of alkali-labile lesions, indicating that an oxygen radical resulting from O_2 trapping of **1**, and not a diffusible species, is responsible for the majority of alkali-labile products. Although the ratio of strand scission at these sites is dependent upon the method of base treatment (Table 1), enzymatic end-group analysis reveals that all products contain 3′-phosphate termini.[8]

The majority of lesions revealed upon NaOH treatment are presumably abasic sites, which are known to cleave under mild

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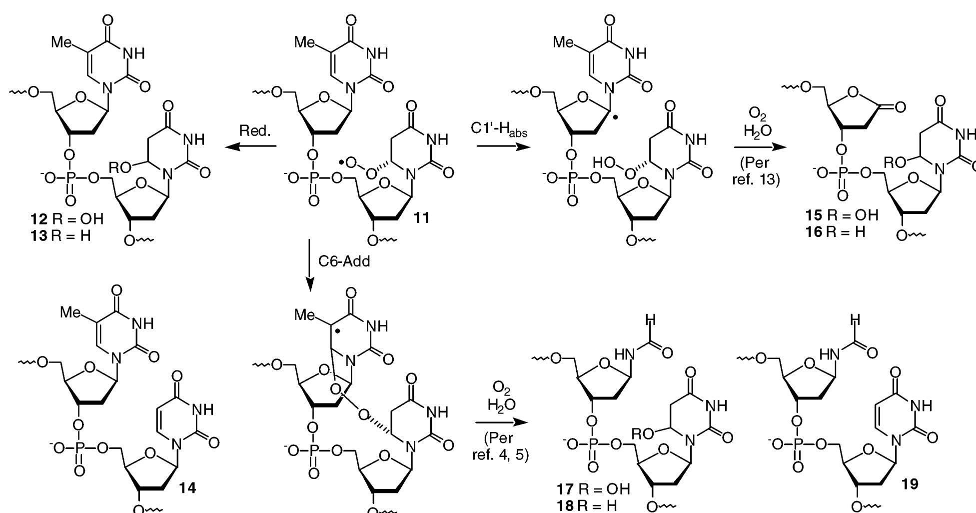
Scheme 1



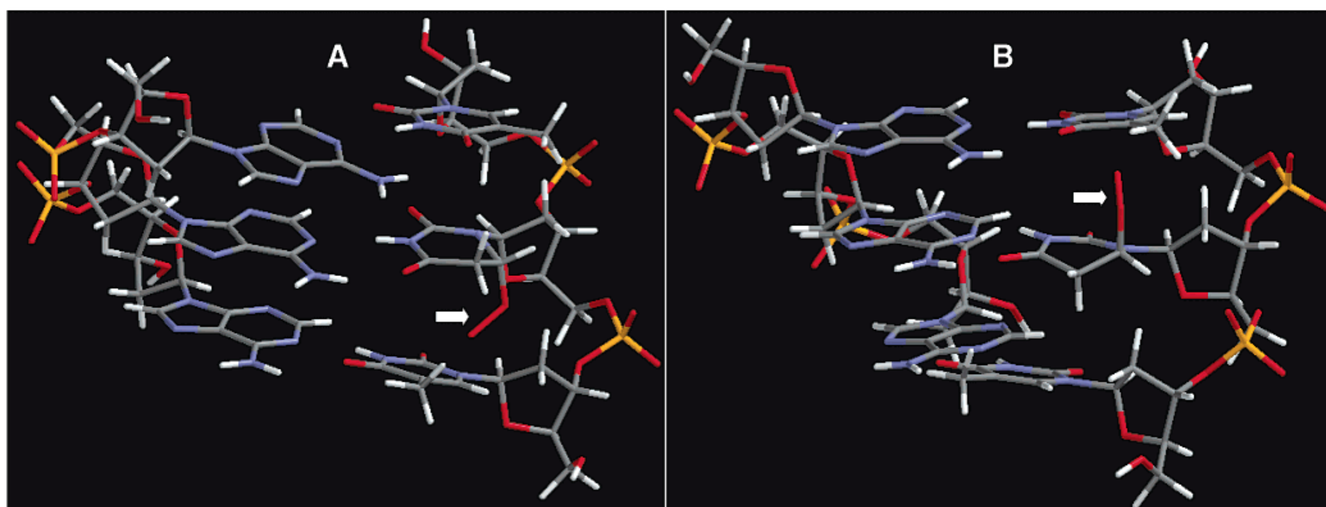
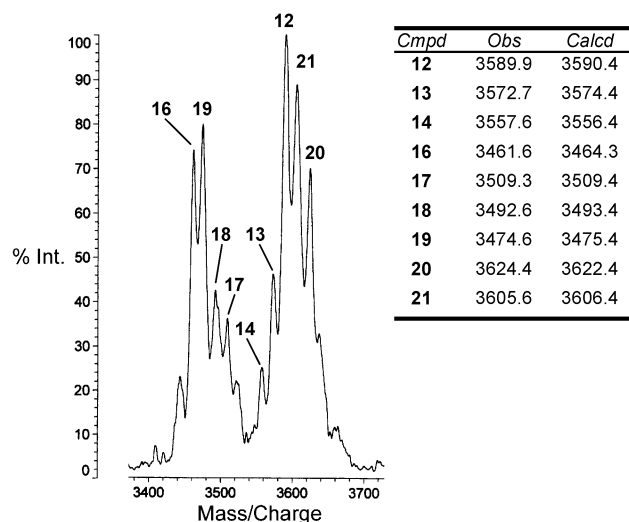


Figure 2. Molecular models of *anti*-6*R*-**11** (A) and *anti*-6*S*-**11** (B) in **8**. Arrows indicate the position of peroxyl radicals.

conditions.[10−12] Treatment of photolyzed 5′-^{32}P-**8** with hydrazine did not give rise to any of the pyridazine cleavage product that is characteristic of the C4′-oxidized abasic site.[11] In contrast, experimental evidence is consistent with formation of the 2- deoxyribonolactone oxidized abasic site. This labile product has been observed as part of a tandem lesion generated by a nucleobase oxygen radical, and deuterium isotope effect experiments showed that the lesion is formed via C1′-hydrogen atom abstraction.[6] C1′-radicals yield 2-deoxyribonolactone under aerobic conditions following O_2 trapping and subsequent superoxide elimination.[13] The occurrence of a similar process involving **11** was examined using a chemically synthesized oligonucleotide in which one thymidine adjacent to a radical precursor (**2**) was deuterated at C1′.[14] A KIE (4.4 ± 0.1), consistent with C1′-hydrogen atom abstraction from the 5′-adjacent thymidine by the oxygen radical, was observed in NaOH treated samples (Scheme 1).[8]

Piperidine treatment enhances cleavage at T_{15} and at the radical site in 5′-^{32}P-**8** greater than 2-fold (Table 1), indicating that abasic sites are not the exclusive variety of DNA damage products. The possibility that piperidine-labile lesions were derived from addition to the double bond of T_{15} was explored by substituting 5,6-dihydrothymidine (dHT) at this site (5′-^{32}P-**9**). Indeed, the yield of NaOH-labile lesions increased at the expense of lesions cleaved only by piperidine treatment (Figure 1A, Table 1). The higher yield of the NaOH-labile product enabled us to confirm its identity as 2-deoxyribonolactone using a set of reactions that convert this lesion to a characteristic set of cleavage products that are discernible by gel electrophoresis.[8,12b] Molecular modeling demonstrates that the above observations may be rationalized on the basis of DNA secondary structure.[15] The C1′-hydrogen atom and C6-position of T_{15} are less than 2.5 Å from the terminal 6*R*-peroxyl oxygen atom when the nucleotide is in the anti-conformation (Figure 2A).

Experiments using 3′-^{32}P-**8** reveal strand damage at T_{17} and the site of **1** (Figure 1B, Table 1). Although all products contain 5′-phosphate termini, insignificant amounts of NaOH-labile lesions are formed at T_{17}, and a deuterium isotope effect is not observed upon deuteration of the C1′-position. The absence of reaction at the C1′-position in T_{17} is consistent with molecular modeling, which illustrates the effects of the helical twist in DNA. A model duplex reveals that the terminal oxygen in 6*S*-**11** and the T_{17} C1′-hydrogen atom are ∼5.5 Å apart (Figure 2B), but the hydrogen of the thymine methyl group is less than 2 Å from the radical center. It is notable that the respective methyl group in T_{15} is ∼5.3 Å from the terminal peroxyl oxygen atom in 6*R*-**11**. The involvement of the methyl group in T_{17} on alkali-labile lesion formation was probed by photolyzing the respective duplex in which 2′-deoxyuridine was substituted at this position (3′-^{32}P-**10**, Figure 1B). Cleavage at dU_{17} in 3′-^{32}P-**10** was reduced by ∼80% relative to that observed at T_{17} in 3′-^{32}P-**8** (Table 1). This suggests that the majority of tandem



Cmpd	Obs	Calcd
12	3589.9	3590.4
13	3572.7	3574.4
14	3557.6	3556.4
16	3461.6	3464.3
17	3509.3	3509.4
18	3492.6	3493.4
19	3474.6	3475.4
20	3624.4	3622.4
21	3605.6	3606.4

Figure 3. MALDI-TOF MS of photolyzed **7**. Product structures are shown in Schemes 1 and 2.

lesions involving the 3′-adjacent nucleotide are derived from hydrogen atom abstraction from the thymine methyl group.

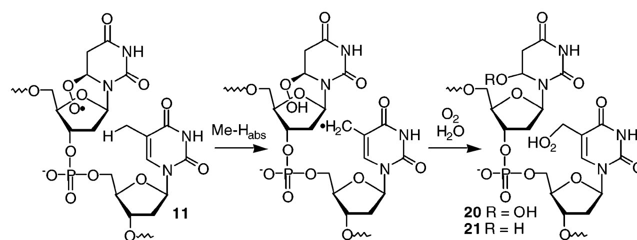
The products were qualitatively characterized further by MALDI-TOF MS analysis of photolyzed **7** (Figure 3). Isolated lesions observed include hydroperoxide (**12**), C6-hydrate (**13**), and a small amount of 2′-deoxyuridine (**14**, Scheme 1). The latter product could result from radical−radical reactions during photolysis and elimination from **12** or **13** during postphotolysis handling. Several ions attributable to tandem lesions were observed. One of these contains 2-deoxyribonolactone and C6-hydrate (**16**). The C6-hydrate component is undoubtedly derived from **1**, but the mechanism of its formation is uncertain. We cannot distinguish between hydrogen atom abstraction by the alkoxyl radical produced from reduction of **11** or reaction of the C6-hydrogen peroxide (**15**) after radical reactions.[16]

Molecular ions corresponding to products containing **12** and **13** in tandem with formamide lesions are also observed (**17**, **18**, Figure 3). The formamide is attributed to initial oxygen radical addition to the thymidine double bond (Scheme 1).[4,5,16] The deoxyuridine-containing lesion (**19**) may be attributed to decomposition of the hydroperoxide (**17**) or hydrate (**18**).[1,16] This is consistent with the observation that the relative amounts of **17**−**19** were variable.[8] On the basis of experiments using **8**−**10** (Figure 1), literature precedent, and molecular modeling, we suggest that formamide-containing lesions result from oxygen radical addition to C6 and are the major piperidine-labile products formed at T_{15} (Scheme 1).[4,5]

Formamide formation at T_{17} cannot be excluded. However, the secondary structure of DNA and gel electrophoresis experiments with 3′-^{32}P-**8** and -**10** suggest a different family of tandem lesions that is also observed by MS dominates reaction with the 3′-adjacent nucleotide. Products corresponding to the addition of four and three oxygen atoms to **1** were observed. Bis-hydroperoxide **20** and its reduction product **21** (Scheme 2) are consistent with the MS observations and gel electrophoresis experiments.

MALDI-TOF MS affirms tandem lesion formation, but is not informative with respect to the efficiency of these processes. The amount of tandem lesions relative to isolated lesions derived from **1** was estimated from quantitative analysis of electrophoresis experiments using **8** (Table 1).[8] Tandem and isolated lesions involving T_{15} give rise to strand breaks at this position in 5′-^{32}P-**8**. Similarly, tandem and isolated lesions involving T_{17} are observed as strand breaks in 3′-^{32}P-**8**. Isolated lesions are observed as strand breaks at T_{15} in 3′-^{32}P-**8** and at T_{17} in 5′-^{32}P-**8**. By subtracting the

Scheme 2



amount of isolated lesions, we could estimate the percentage of DNA damage due to tandem lesions. Tandem lesions involving T_{15} and T_{17} represent 32% and 33%, respectively, of the total amount of alkali-labile damage.

A great deal of research has been carried out regarding the formation of direct strand breaks and isolated lesions from pyrimidine nucleobase radicals.[1] Our results indicate that these lesions account for a minor amount of the reactions of **1**. Tandem lesions, whose biological role are uncertain, are the major lesions derived from **1**.[2,17] Furthermore, independent generation of 5,6-dihydro-2′-deoxyuridin-6-yl (**1**) reveals that the distribution of tandem lesions is dependent upon the local secondary structure of DNA. The generality and biological relevance of these findings remain to be addressed, but could be significant with respect to how the effects of γ-radiolysis on DNA are interpreted.

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Supporting Information Available: Synthetic procedures for the preparation of **3**. ESI-MS of **4**−**7**. HPLC analysis of the enzyme digest of **4**. Additional MALDI-TOF MS of photolyzed **7**. Phosphor images of KIE experiments, enzymatic end-group analysis, and fingerprint reactions for detecting deoxyribonolactone. Description of analysis for estimating the quantity of tandem lesions (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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