DNA damage induced via independent generation of the radical resulting from formal hydrogen atom abstraction from the C1′-position of a nucleotide

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Background: Deoxyribonucleotide radicals resulting from formal C1′-hydrogen atom abstraction are important reactive intermediates in a variety of DNA-damage processes. The reactivity of these radicals can be affected by the agents that generate them and the environment in which they are produced. As an initial step in determining the factors that control the reactivity of these important radical species, we developed a mild method for their generation at a defined site within a biopolymer.

Results: Irradiation of oligonucleotides containing a photolabile nucleotide produced C1′-DNA radicals. In the absence of potential reactants other than O_2, approximately 90% of the damage events involve formation of alkaline-labile lesions, with the remainder resulting in direct strand breaks. The ratio of alkaline-labile lesions to direct strand breaks (~ 9:1) is independent of whether the radical is generated in single-stranded DNA or double-stranded DNA. Strand damage is almost completely quenched under anaerobic conditions in the presence of low thiol concentrations. Competition studies with O_2 indicate that the trapping rate of C1′-DNA radicals by β-mercaptoethanol is ~ $1.1 \times 10^7\ M^{-1}s^{-1}$.

Conclusions: The mild generation of the C1′-DNA radical in the absence of exogenous oxidants makes it possible to examine their intrinsic reactivity. In the absence of other reactants, the formation of direct strand breaks from C1′-radicals is, at most, a minor pathway. Competition studies between β-mercaptoethanol and O_2 indicate that significantly higher thiol concentrations than those *in vivo* or some means of increasing the effective thiol concentration near DNA are needed for these reagents to prevent the formation of DNA lesions arising from the C1′-radical under aerobic conditions.

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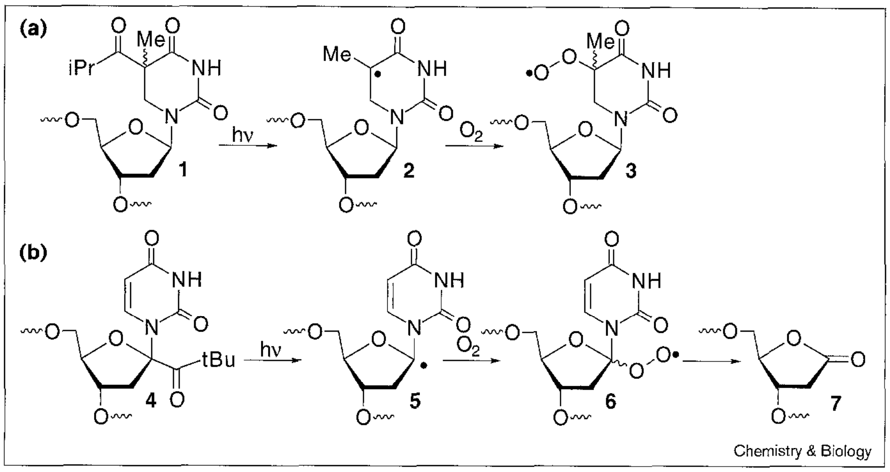
Introduction

The C1′-position of nucleotides is involved in a variety of nucleic acid damage processes. Reaction at this center can give rise to premutagenic α-deoxyribonucleotides, alkaline-labile lesions or direct strand breaks [1–7]. Direct two-electron oxidation of the anomeric position has been proposed to explain DNA damage mediated by some ruthenium complexes [8,9]. In many instances, however, DNA damage processes involving the anomeric position of nucleotides are believed to proceed through radical intermediates. For instance, the neocarzinostatin chromophore, esperamicin A and bis(1,10-phenanthroline)copper are believed to abstract the C1′-hydrogen directly from nucleotides in duplex DNA [4,10–12]. Yet, only bis(1,10-phenanthroline)copper is believed to produce direct strand breaks via a C1′-deoxyribonucleotide radical. More recently, the peroxyl radical (**3**, Figure 1a) derived from 5,6-dihydrothymidin-5-yl (**2**) was proposed to participate in DNA damage amplification by selectively abstracting the C1′-hydrogen atom from the 5′-adjacent thymidine in a single-stranded oligonucleotide [13]. In addition, the formal product resulting from C1′-hydrogen atom abstraction is produced during γ-radiolysis via a two-step process involving nucleobase ionization, followed by deprotonation from the anomeric position [14]. Often anomeric nucleotide radicals (e.g. 2′-deoxyuridin-1′-yl, **5**; Figure 1b) are believed to be involved in DNA-damage processes, but there is very little information on the fundamental chemistry of these reactive intermediates. In attempts to remedy this situation, we, and others, have reported on the reactivity of anomeric nucleoside radicals independently generated from unnatural precursors (e.g., *t*-butyl ketone, **4**; Figure 1b) [15,16]. The ability to probe the reactivity of independently generated intermediates in DNA and the reactive moieties of naturally occurring damaging agents has proved to be very useful for examining nucleic acid damage processes [17,18]. Here, we describe initial studies on the reactivity of 2′-deoxyuridin-1′-yl (**5**), a radical that has been independently generated for the first time at a defined site in single- and double-stranded oligonucleotides. The results provide a basis for the reactivity of C1′-DNA radicals that will be useful for studying

Figure 1



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Independent generation of reactive intermediates involved in DNA damage. **(a)** Photolysis of **1** results in the formation of 5,6-dihydrothymidin-5-yl (**2**), and **2** is trapped by O_2 to form the respective peroxyl radical (**3**). **(b)** Photolysis of **4** results in the formation of the reactive intermediate formed via C1′-hydrogen atom abstraction (**5**) that forms the deoxyribonolactone lesion (**7**) via the respective peroxyl radical (**6**).

the effects of exogenous agents on the fate this important reactive intermediate in biopolymers.

Results

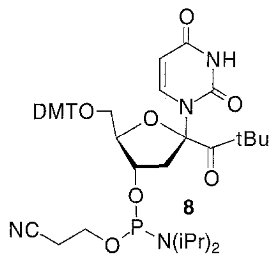
Oligonucleotide synthesis and characterization

The ability to use **4** in standard automated oligonucleotide synthesis conditions was determined by testing the stability of the free nucleoside under the oxidation and acidic deprotection conditions. The *t*-butyl ketone (**4**) was unaffected by stirring for 4 h in solutions of either I_2 (0.1 M in tetrahydrofuran (THF):pyridine:H_2O) or 3% trichloroacetic acid in CH_3CN. Moreover, **4** was unaltered after heating to 55°C for 4 h in concentrated aqueous ammonia, indicating that oligonucleotides containing the ketone could be synthesized and deprotected using commercially available reagents and standard deprotection conditions. The free nucleoside was elaborated into the requisite phosphoramidite (**8**, Figure 2), and, in conjunction with commercially available fast deprotecting phosphoramidites, **8** was used in the synthesis of oligonucleotides **9** and **10** (Figure 2) [19,20]. Following concentrated aqueous ammonia deprotection and gel purification, the stability of **4** under these conditions was demonstrated by characterizing **9** using electrospray mass spectrometry. The observed mass for **9** was within 1 amu of the calculated mass.

Formation of DNA strand damage under aerobic and anaerobic conditions

Photolysis of 5′-^{32}P-**10** or 3′-^{32}P-**10** produces direct strand breaks and alkaline-labile lesions at the site where **5** is produced. (For simplicity, **4** and **5** are referred to as such, regardless of whether one is discussing the monomeric species, or the respective molecule in an oligonucleotide.) Although it was possible to completely convert **10** to damaged products under aerobic conditions, photolyses were typically carried out to < 30% conversion. Phosphorimaging analysis reveals that the damage (in the form of direct strand breaks or alkaline-labile lesions) at the adjacent deoxyadenosines are less than one-fifth as intense as the damage at the nucleotide where **5** is generated. In the absence of thiol, the extent of direct strand scission and the total amount of strand damage (defined as the summation of direct strand breaks and alkaline-labile lesions) are independent of O_2 (Table 1), and direct strand breaks account for a very minor (~ 10%) amount of the total strand damage. Anaerobic photolysis of **10** in the presence of thiol (10 mM) reduces total strand damage by more than 90%. Increasing the thiol concentration to 50 mM does not reduce the extent of total strand damage any further. In contrast, β-mercaptoethanol (10 mM, Table 1)

Figure 2



8

5'-d(TGC A**4**T GT)

9

5'-d(GTC ACG TGC TGC A**4**A CGA CGT GCT GAG CCT)

10

5'-d(GTC ACG TGC TGC A**4**A CGA CGT GCT GAG CCT)
d(CAG TGC ACG ACG TAT GCT GCA CGA CGC GGA)

11

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The synthesis of **9**, **10** and **11**. Oligonucleotides **9** and **10** containing **4** were synthesized via standard methods using phosphoramidite **8**. Hybridization of **10** to its complement yielded the double-stranded DNA substrate (**11**).

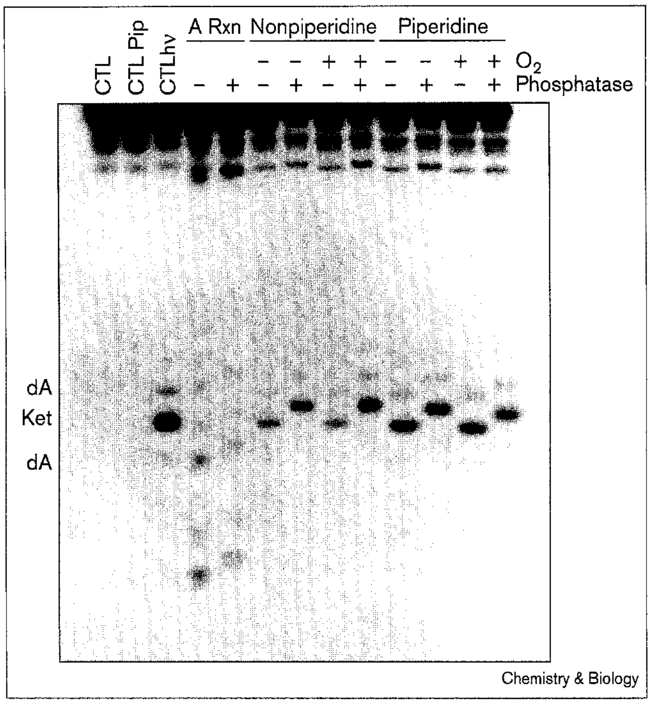
Table 1

Effect of O_2 on strand cleavage in photolyzed single-stranded DNA (10).

β-Mercapto-ethanol (mM)	$-O_2$ – Piperidine	$-O_2$ + Piperidine	$+O_2$ – Piperidine	+O + Piperidine
0	2.8 ± 0.5	29.1 ± 3.7	2.8 ± 0.8	29.8 ± 2.5
10	1.2 ± 0.3	2.0 ± 1.4	3.2 ± 0.6	27.6 ± 1.8

Cleavage is expressed in terms of percent of total DNA cleaved. Values reported represent average of at least four experiments ± standard deviation from this average. Samples were exposed to light for 20 min.

Figure 3



An autoradiogram of photolyzed 3′-^{32}P-**10**. Lanes: CTL, unphotolyzed; CTL Pip, piperidine treated unphotolyzed; CTLhv, photolyzed material incubated with calf intestine alkaline phosphatase buffer; A rxn, deoxyadenosine sequencing reaction; nonpiperidine, photolyzed substrate; piperidine, piperidine-treated photolyzed substrate. Lanes are not loaded with equal amounts of radiation. dA, deoxyadenosine; Ket, **4**. –/+ correspond to whether the reagent indicated at the end of the row was used.

has no statistical effect on the extent or distribution of strand damage in **10** under aerobic conditions.

A similar dependency of strand damage on O_2 and thiol is observed when **1** is generated in double-stranded DNA (dsDNA, **11**). Direct strand breaks represent approximately 10% of the total strand damage, and 10 mM β-mercaptoethanol effectively quenches total strand damage under anaerobic conditions, but not under aerobic conditions (Table 2). Photolytically induced strand damage in dsDNA (**11**) is less efficient than in single-stranded DNA (ssDNA, **10**), however. Although **11** was irradiated three times longer (1 h) than **10**, less than one third the amount of strand damage was observed (Tables 1 and 2).

End group analysis

Treatment of photolyzed 3′-^{32}P-**10** with calf intestine alkaline phosphatase, which produces slower migrating 5′-hydroxy DNA fragments via dephosphorylation of 5′-phosphate groups, reveals that cleavage arising from **5** produces 5′-phosphate groups exclusively (Figure 3). Photolytic generation of **5** in dsDNA (**11**) yields the same result (data not shown). Denaturing polyacrylamide gel electrophoretic analysis of direct strand breaks formed from photolysis of 5′-^{32}P-**10** reveals the presence of a major strand scission product and a minor product that migrates slightly more slowly (Figure 4). Only the faster moving product is observed upon piperidine treatment. Treatment of photolyzed 5′-^{32}P-**10** with T4 polynucleotide kinase, which dephosphorylates 3′-phosphate groups, causes a reduction in the migration of the major cleavage product, indicating that the major product contains a 3′-phosphate terminus (Figure 4). The resulting 3′-hydroxyl terminus comigrates with the minor product of direct strand scission observed prior to kinase treatment. An additional product, whose migration is significantly retarded relative to those observed in samples not treated with piperidine, is also formed upon kinase treatment. This product is not observed in samples subjected to kinase following piperidine treatment. The product is formed upon exposing non-piperidine-treated samples with the cocktail (containing Tris (pH 8.8) and dithiothreitol; DTT) used in the kinase reaction (Figure 4, lane CTLhv), however, suggesting the slower moving minor product of direct strand scission, and possibly a precursor to it, gives rise to the product that has significantly retarded gel mobility.

Table 2

Effect of O_2 on strand cleavage in photolyzed double-stranded DNA (11).

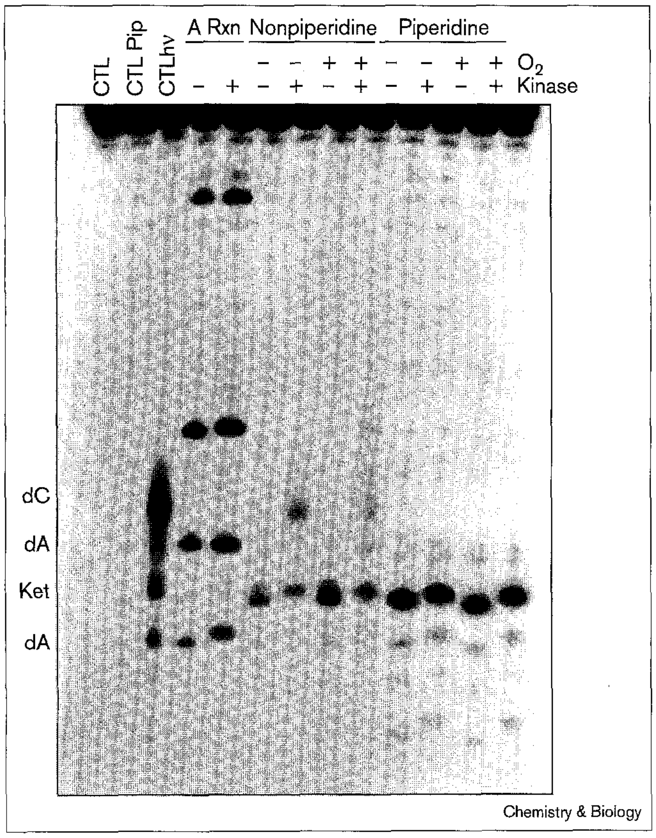
β-Mercapto-ethanol (mM)	$-O_2$ – Piperidine	$-O_2$ + Piperidine	$+O_2$ – Piperidine	$+O_2$ + Piperidine
0	0.4 ± 0.2	2.6 ± 0.6	0.9 ± 0.3	8.6 ± 1.1
10	0.8 ± 0.4	1.3 ± 0.6	0.8 ± 0.4	6.1 ± 1.8

Cleavage is expressed in terms of percent of total DNA cleaved. Values reported represent average of at least four experiments ± standard deviation from this average. Samples were exposed to light for 1 h.

Chemical probes for diffusible reactive oxygen species

Previous studies on **2** suggested that the system involving **1** produced $^1O_2$ that was responsible for some of the strand damage produced upon irradiation of oligonucleotides containing **1** [13]. In contrast, strand damage was unaffected upon photolysis of **10** in the presence of NaN_3, indicating that $^1O_2$ is not involved (Table 3). Furthermore, photolysis of **10** in the presence of mannitol

Figure 4



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An autoradiogram of photolyzed 5′-^{32}P-**10**. Lanes: CTL, unphotolyzed; CTL Pip, piperidine treated unphotolyzed; CTLhv, photolyzed material incubated with T4 polynucleotide kinase buffer + dithiothreitol; A rxn, deoxyadenosine sequencing reaction; nonpiperidine, photolyzed substrate; piperidine, piperidine treated photolyzed substrate. Note: Lanes are not loaded with equal amounts of radiation. dA, deoxyadenosine; dC, deoxycytidine; Ket, **4**. –/+ correspond to whether the reagent indicated at the end of the row was used.

Table 3

Effect of diffusible species scavengers on strand cleavage in photolyzed single-stranded DNA (10).

Additive (mM)	$-O_2$ – Piperidine	$-O_2$ + Piperidine	$+O_2$ – Piperidine	$+O_2$ + Piperidine
NaN_3 (10)	0.7 ± 0.3	0.8 ± 0.2	0.9 ± 0.3	0.7 ± 0.2
Mannitol (10)	1.1 ± 0.4	0.8 ± 0.2	1.6 ± 0.6	0.8 ± 0.3

Cleavage is expressed in terms of percent of total DNA cleaved relative to total DNA cleaved in the absence of any additives. Values reported represent average of at least four experiments ± standard deviation from this average. Samples were exposed to light for 20 min.

rules out the involvement of hydroxyl radical in strand damage (Table 3) [1].

Competition between β-mercaptoethanol and O_2 for 5 in DNA

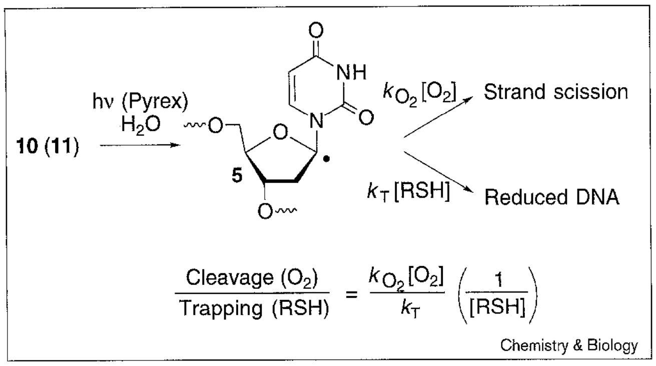
As the concentration of O_2 in H_2O is known, and its rate constant for trapping **5** can be assumed to be $2 \times 10^9\ M^{-1}\ s^{-1}$, the rate constant with which **5** is trapped by the reducing agent can be extracted from measurements of the extent of strand scission as a function of reducing agent concentration (Figure 5). The amount of reduced DNA produced by thiol trapping of **5** is measured indirectly, and is presumed to be equal to the difference in the amount of total strand damage formed in the absence of thiol and that observed in the presence of a given concentration of thiol. There are three main assumptions inherent in applying this treatment. First, the amount of total strand damage observed is attributable to O_2 trapping of **5**. Second, under aerobic conditions, the fraction of O_2-trapped radicals (**6**) that gives rise to strand breaks upon piperidine treatment is independent of thiol concentration. Reduction of **6** by thiol yields **7** but in the absence of thiol the peroxyl radical also yields **7** via superoxide elimination [21]. Third, the thiol does not affect the photoconversion of **4** within the biopolymer. The first assumption is substantiated by studies on monomeric **4** (50 μM) in which **5** was efficiently trapped under anaerobic conditions in the presence of as little as 0.5 mM β-mercaptoethanol. No deoxyribonolactone (**7**) was detected under these conditions. In contrast, **7** was formed in > 90% yield under aerobic conditions in the presence of < 5 mM β-mercaptoethanol [21], an observation that supports the second assumption. Finally, the thiol does not absorb light in the irradiation region, and **4** does not undergo photoreduction in competition with Norrish Type I photocleavage to produce **5**. Hence, the photoconversion of **10** (or **11**) should be unaffected by the presence of varying concentrations of thiol.

Plotting the ratio of observed cleavage to reduced DNA versus the reciprocal of the β-mercaptoethanol concentration, yielded a straight line and the rate constant for trapping of **5** by thiol in **10** was extracted from the slope (Figure 6). As all the measurements are dependent upon the extent of cleavage under aerobic conditions in the absence of thiol, this measurement was made 15 times. Using 0.2 mM as the concentration of O_2 in aerated H_2O and $2 \times 10^9\ M^{-1}s^{-1}$ as the rate constant for trapping of **5** by O_2, the rate constant for trapping by β-mercaptoethanol (k_T) is $1.1 \times 10^7\ M^{-1}s^{-1}$ in ssDNA (**10**) [22,23]. If one assumes that the residual strand damage measured under anaerobic conditions in the presence of thiol (Table 1) is attributable to radical-cage processes, or a species other than **5**, k_T increases slightly to $1.2 \times 10^7\ M^{-1}s^{-1}$. Similar examination of the thiol-dependent cleavage in **11** gives rise to a rate constant for trapping of **5** by β-mercaptoethanol of ~ $1.0 \times 10^7\ M^{-1}s^{-1}$ (data not shown).

Discussion

The purpose of designing **4** as a photochemical source of a C1′-deoxyribonucleotide radical was to develop a probe that permits the dissection of the secondary roles of

Figure 5

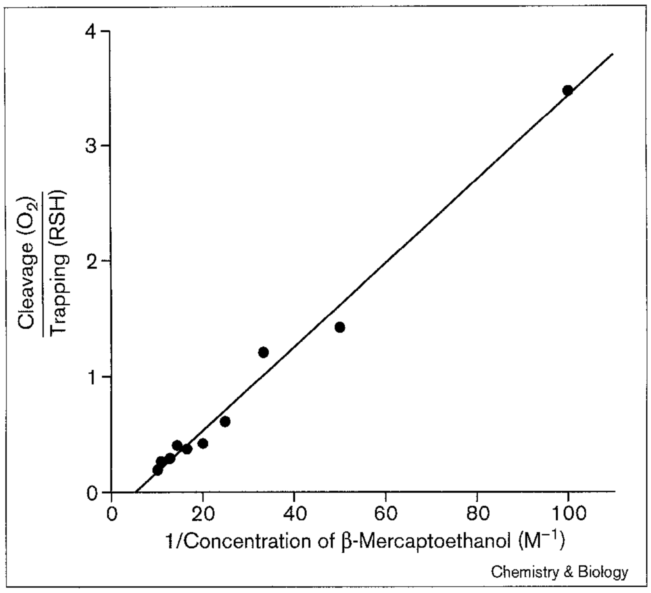


The partitioning of **5** between trapping by thiol (to yield nonlabile product(s)) and O_2 (to yield labile product(s)).

exogenous agents in the chemical reactions of the radical intermediate that they produce from the means by which the agents generate the transient radical species within the biopolymer. One criterion for a useful probe of reactivity of the C1′-radical in DNA is that random damage within the biopolymer should be minimized. Irradiation of oligonucleotides containing **4** results in the majority of strand damage at the nucleotide where **5** is produced, and a minor amount of damage at the deoxyadenosines adjacent to the site at which **5** is generated. Experiments using NaN_3 and mannitol indicate that cleavage at the 3′- and 5′-deoxyadenosines are not caused by $^1O_2$ and OH•, respectively. Damage at the deoxyribonucleotides adjacent to **5** are attributed to diffusible radicals (e.g., *t*-butyl radical) produced via Norrish Type I photocleavage of the ketone (**4**).

Strand damage at the site where **5** is generated consists mostly (~ 90%) of alkaline-labile lesions. This suggests that **4** is a satisfactory precursor of **5**, because, when **5** is formed in the presence of agents that are not expected to interact further with the C1′-radical (e.g., hydroxyl radical), alkaline-labile lesions are the expected product. In addition, the similarity in the distribution between direct strand breaks and alkaline-labile lesions in ssDNA (**10**, Table 1) and dsDNA (**11**, Table 2) is consistent with the generation of a common intermediate. Attributing the difference in the efficiency with which strand damage is induced in **10** and **11** to the involvement of one or more diffusible species in ssDNA damage can be dismissed, because there is no reason to assume that dsDNA should be less susceptible than ssDNA to diffusible species. In support of this argument is the finding that strand damage is not observed when the complementary strand in **11** is radiolabeled (data not shown). A diffusible species generated in the minor groove via **4** would be expected to damage both strands of **11** [24]. We suggest that the decreased efficiency of photoinduced cleavage in **11** relative to **10** is a result of less efficient Norrish Type I photocleavage of **4** in **11** than in **10**. A portion of this reduced efficiency is attributable to a competition for light by the complementary oligonucleotide in **11** and the remainder of the effect is attributed to the photophysics of **4**. It is possible that the less polar environment of the minor groove of **11** relative to **10** could enhance the quantum yield for Norrish Type I cleavage of **4** [25]. Energy quenching of the excited state of **4** by the adjacent nucleotides should be more efficient in **11** than in ssDNA (**10**), however, resulting in the observed decrease in photochemical efficiency [26].

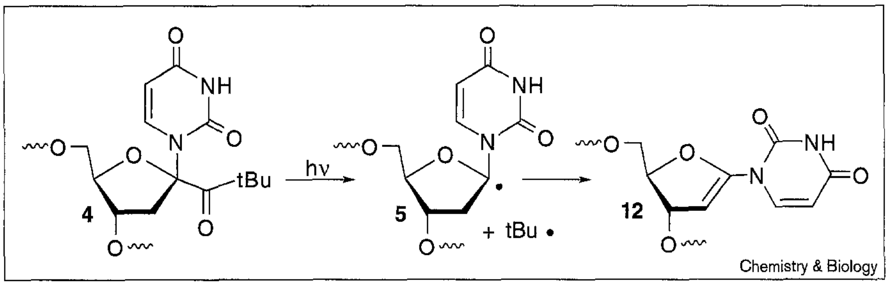
Figure 6



The dependence of strand damage in 5′-^{32}P-**10** on β-mercaptoethanol concentration photolyzed under aerobic conditions. The slope of the line is 3.6×10^{-2} M.

The formation of alkaline-labile lesions under anaerobic conditions is ascribed to radical–radical reactions and is consistent with observations involving monomeric **5** [15]. Quenching of the formation of alkaline-labile lesions by low concentrations of thiol is consistent with this proposal. The small fraction (~ 6%) of those lesions that are not quenched by thiol are attributed to reactions that occur within the solvent cage. The 1′,2′-dehydronucleotide (**12**, Figure 7), which has recently been shown not to give rise to direct strand breaks, is a candidate for the alkaline-labile lesion that arises via a radical–radical reaction [27,28]. Under aerobic conditions, the majority of the alkaline-labile lesions are attributed to O_2 trapping of **5**, and are consistent with the lack of an effect of low concentrations of thiol on their formation. Oxygen trapping of **5** will compete very effectively with non-cage radical–radical chemical reactions and the intermediate peroxyl radical (**6**) will give rise to the alkaline-labile deoxyribonolactone (**7**) regardless of the presence of thiol (Figure 8) [21].

Figure 7



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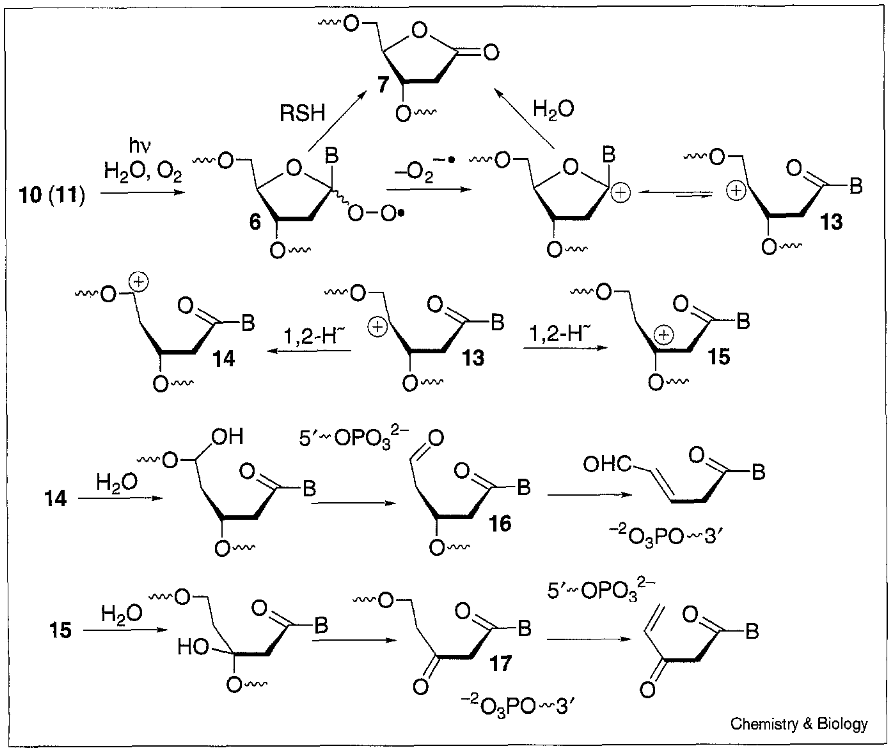
Alkaline-labile lesions such as **12** can be formed from **5** under anaerobic conditions via radical–radical reactions.

Direct strand breaks are a minor product (~ 10%) from **4** (or **5**) in biopolymers under these conditions. Determining the sugar degradation products and mechanism for their formation is complicated by the fact that only a single molecule of **5** is produced from each molecule of **10** (or **11**), and **10** and **11** are themselves synthesizable on the submicromole scale. There are a number of possible explanations at this time for the formation of the small proportion of direct strand breaks from **5**, resulting in 3′-phosphate termini. Under aerobic conditions, in which direct strand breaks are not diminished in the presence of low concentrations of β-mercaptoethanol, direct strand breaks may arise from the carbocation formed following loss of superoxide ($O_2^{-\bullet}$) from **7** (Figure 8). Such a process is supported by a recent study on monomeric **5** in which $O_2^{-\bullet}$ elimination has been detected, and by pulse radiolysis experiments which revealed that reactions of thiols with DNA are dependent upon the charge of the thiol and that glutathione trapping of DNA peroxyl radicals are slower than believed previously [21,29,30].

Under anaerobic conditions, **5** may undergo β scission to form the less stable C4′-radical (**18**, Figure 9). The analogous reaction involving C4′-nucleoside radicals of this highly speculative process has been detected in model compounds [31]. The driving force for this initially uphill process would be the formation of direct strand breaks via phosphate elimination from the nonstabilized secondary radical [32–35]. The anticipated slow rate constant for this process can also explain why alkaline-labile lesions are formed predominantly. Considering the speculative nature of this process and the low concentrations (< 10 nM) of

Figure 8



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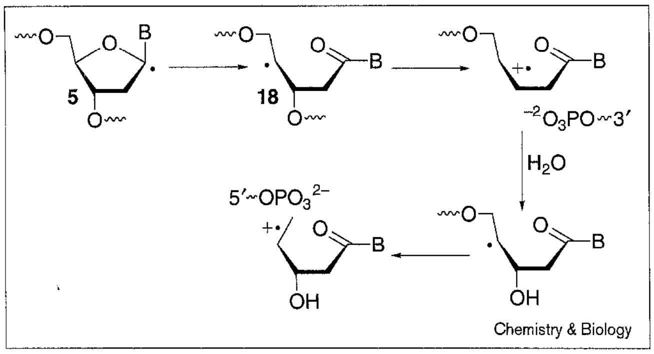
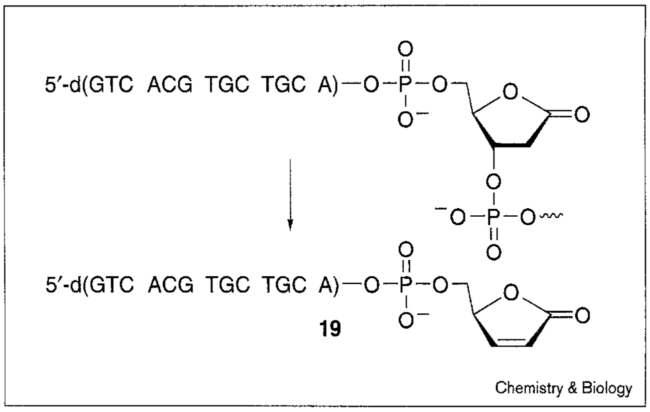
The postulated mechanism for the formation of damage products from **5** in the presence of O_2.

Figure 9



The postulated mechanism for the formation of direct strand breaks from **5** in the absence of O_2.

Figure 10



Alkaline- and thiol-labile lesion **19** can be formed from **7**.

radiolabeled **10** used in these experiments, the trapping of **5** by traces of O_2 cannot be ruled out.

The structure of the slower moving, minor product of direct strand scission is also speculative at this time. It is certainly not the 3′-hydroxyl product, because it is not present in the piperidine-treated samples (Figure 4). One suggestion for the structure of the minor product from the minor pathway is the butenolide (**19**, Figure 10), which has previously been proposed as a product of DNA damage mediated by bis(1,10-phenanthroline)copper and ruthenium complexes [4,8]. The alkaline lability of this lesion is consistent with this proposal. Furthermore, the lability of the observed product to DTT in the T4 polynucleotide kinase reaction buffer (pH 8.8), to yield the product that has a significantly retarded electrophoretic migration (Figure 4) is consistent with β elimination of labile lesions under mild alkaline conditions in the presence of other thiols [36,37]. In addition, prolonged exposure of photolyzed **10** to the above alkaline buffer conditions results in even greater amounts of the suggested DTT–furanone adduct. We attribute this to at least partial conversion of **7** to **19**, which is trapped by thiols.

Although exceptions exist, O_2 trapping of nucleotide radicals are generally considered to be damage-fixing events, whereas thiols generally (but not always) act as protecting agents [1,6,7,38]. For instance, it was recently shown that thiols can modulate the bistranded damage of DNA by esperamicins A and C, and that anomeric nucleotide radicals are involved in DNA damage mediated by esperamicin A [12,39]. Thiol trapping of C1′-nucleotide radicals also result in the formation of premutagenic α-deoxyribonucleotides [6,7]. Hence, determination of the efficiency of thiol trapping of **5** relative to that by O_2 is useful for calibrating competing repair and fixation pathways. The experiments involving **10** and **11** demonstrate that the relative rate constants for O_2 and β-mercaptoethanol trapping of **5** are comparable in ssDNA and dsDNA. In addition, the estimated absolute rate constants (~ 1.1×10^7 $M^{-1}s^{-1}$) are very close to those reported for thiols reacting with alkyl radicals in H_2O at neutral pH [22,23]. Considering that glutathione should react even more slowly with nucleic acids than β-mercaptoethanol, the above rate constant suggests that the amount of premutagenic α-nucleotide lesions formed would be small under aerobic conditions in the presence of physiologically relevant levels of thiol (5–10 mM) [29,30]. In addition, the observed competition between O_2 and β-mercaptoethanol suggests that, if thiols are to quench the formation of bistranded lesions by esperamicin A, then the effective concentration of the thiols with respect to the DNA duplex must be significantly higher than the concentration of such thiols in bulk solution [39].

Significance

A nucleotide radical resulting from formal C1′-hydrogen atom abstraction (5) has been independently generated at a defined site in single-stranded (ss) DNA and double-stranded (ds) DNA for the first time. As expected on the basis of DNA damage induced by the enediynes and ionizing radiation, the anomeric radical gives rise predominantly to alkaline-labile lesions. Direct strand breaks are produced at least an order of magnitude less efficiently than alkaline-labile lesions in ssDNA and dsDNA. Although possible explanations have been proposed, further studies will be necessary to elucidate the deoxyribosyl degradation products, and the mechanisms responsible for their formation.

The proportions of different types of strand damage observed from independently generated 5 are identical, within experimental error, to those previously reported involving peroxyl radical 3, and provide further support for the DNA-damage-amplification mechanism proposed involving 5,6-dihydrothymidin-5-yl (2) in single-stranded

oligonucleotides [13]. These studies also demonstrate that if bis(1,10-phenanthroline)copper-mediated strand scission involves the formation of a C1′-radical, then there must be a bifurcation in the reaction mechanism at the stage of the radical (or later) which results in direct strand break formation.

The rate constant for trapping of **5** by an uncharged thiol, β-mercaptoethanol, was estimated ($k_T \approx 1.1 \times 10^7\ M^{-1}s^{-1}$) by examining the dependency of DNA cleavage on thiol concentration under aerobic conditions. To our knowledge, this is the first estimate of an absolute rate constant for the thiol trapping of a nucleotide radical generated a defined site in DNA, and should provide the impetus to investigate previously reported processes in greater detail.

Materials and methods

General methods

Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Phosphorimager equipped with Imagequant® Version 3.3 software. Qualitative analysis of radiolabeled oligonucleotides was carried out using X-OMAT AR film obtained from Kodak. Synthesis of oligonucleotides was carried out on an Applied Biosystems Incorporated 380B DNA synthesizer using standard protocols. β-Mercaptoethanol was from Sigma. 2-Cyanoethyl-*N,N*-diisopropylchlorophosphoramidite was from Aldrich. Phenoxyacetyl-protected deoxyadenosine, isobutyryl-protected deoxycytidine, and isopropylphenoxyacetyl-protected deoxyguanosine β-cyanoethyl phosphoramidites were obtained from Pharmacia Biotech. Thymidine β-cyanoethyl phosphoramidite and all other oligonucleotide synthesis reagents were obtained from Glen Research.

DNA manipulations, including enzymatic end group analysis were carried out using standard procedures [40]. Oligonucleotides were sequenced using a reaction specific for adenine [41]. Alkaline-labile lesions were revealed via treatment with piperidine (1 M) at 90°C for 20 min. Preparative and analytical oligonucleotide separations were carried out on 20% polyacrylamide denaturing gels (5% cross-link, 45% (urea by weight)). T4 polynucleotide kinase and calf intestine alkaline phosphatase were obtained from New England Biolabs. Terminal deoxynucleotidyl transferase was from US Biochemical. [α-^{32}P]-ddATP and [γ-^{32}P]-ATP were from Amersham. Electrospray mass spectrometry samples were prepared by precipitating from NH_4OAc prior to photolysis at 302 nm.

Reactions were carried out in oven dried glassware, under a nitrogen atmosphere, unless specified otherwise. Diisopropylethylamine (Hünig's base), pyridine, and CH_2Cl_2 were distilled from CaH_2. All photolyses of oligonucleotides were carried out in Pyrex tubes (0.25" i.d.) using a Rayonet Photoreactor (RPR-100) equipped with lamps having a maximum output at 350 nm. Oligonucleotide photolyses were carried out in 10 mM phosphate buffer (pH 7.0) and 100 mM NaCl. Anaerobic photolyses were carried out in tubes which were flame sealed under vacuum, following freeze–pump–thaw degassing (three cycles).

*t-Butyl ketone phosphoramidite (**8**)*

Ketone (**4**) (78.8 mg, 0.25 mmol) was dried azeotropically with pyridine (2 × 5 ml), taken up in pyridine (4 ml), and 4,4′-dimethoxytritylchloride (96.8 mg, 0.29 mmol) was added at 0°C.[19] The reaction vessel was purged with N_2 for 20 min, and stirred for 12 h at 4°C. The reaction was quenched with MeOH (5 ml), and then H_2O (30 ml) and EtOAc (50 ml) were added. The aqueous layer was washed with EtOAc (2 × 40 ml). The combined organic layers were washed with brine (30 ml), dried over $MgSO_4$, and removed *in vacuo*. The product was purified by silica gel flash chromatography (1:1; EtOAc:Hexanes, followed by 1:4:5; MeOH:EtOAc:hexanes to elute unreacted **4**) to yield 60 mg of dimethoxytritylated ketone (71% based on unrecovered starting material) as a yellow powder. Unreacted **4** was recovered (35.8 mg, 45%). ^{1}H NMR ($CDCl_3$) δ 8.54 (bd s, 1H), 7.81 (d, 1H, J=8.3 Hz), 7.35-7.15 (m, 9H), 6.81 (d, 4H, J=8.3 Hz), 5.50 (dd, 1H, J=2.3, 8.4 Hz), 4.41-4.35 (m, 1H), 4.17-4.09 (m, 1H), 3.78 (s, 6H), 3.45-3.32 (m, 3H), 3.17 (d, 1H, J = 9 Hz), 2.27-2.20 (m, 1H), 1.16 (s, 9H). IR (film) 3215, 3081, 2973, 1704, 1610, 1503 cm^{-1}. HRMS FAB (M+Na$^+$) calc'd 637.2526, found 637.2536.

To the dimethoxytritylated ketone (55 mg, 0.09 mmol) and Hünig's base (51.9 mg, 0.40 mmol) was added CH_2Cl_2 (3 ml). The mixture was cooled to 0°C. 2-Cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (32 mg, 0.134 mmol) was added and the reaction allowed to warm to room temperature over 20 min. The mixture was poured into EtOAc (50 ml) and quickly washed with $NaHCO_3$ (20 ml), H_2O (20 ml), and brine (20 ml). The EtOAc was dried over Na_2SO_4 and removed *in vacuo*. Flash chromatography (10 g dry silica 1:4; EtOAC:CH_2Cl_2, freshly distilled) gave 55.4 mg (76%) of a diastereomeric mixture of **8** as an oil. The oil was powdered from Et_2O:hexanes. ^{1}H NMR ($CDCl_3$) δ 8.16 (d, 0.5H, J = 8.2 Hz), 8.08 (d, 0.5H, J = 8.3 Hz), 7.5-7.1 (m, 9H), 6.9-6.7 (m, 4H), 5.34-5.30 (m, 1H), 4.68-4.62 (m, 0.5H), 4.62-4.50 (m, 0.5H), 4.00-3.30 (m, 16H), 2.63-2.60 (m, 2H), 2.42-2.30 (m, 2H), 1.40-0.70 (m, 29H). ^{31}P NMR ($CDCl_3$, H_3PO_4 reference) δ 150.5, 149.7.

Supplementary material

Supplementary material available on the internet includes an electrospray mass spectrum of **9** and a plot of the dependence of aerobic strand damage in **11** on β-mercaptoethanol concentration.

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