VOLUME 121, NUMBER 18
MAY 12, 1999

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Kinetics and Stereoselectivity of Thiol Trapping of Deoxyuridin-1′-yl in Biopolymers and Their Relationship to the Formation of Premutagenic α-Deoxynucleotides

Jae-Taeg Hwang and Marc M. Greenberg*

Contribution from the Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523

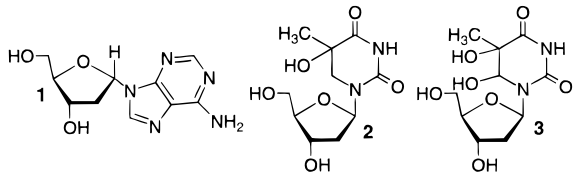
Received January 15, 1999

Abstract: α-Deoxynucleotides are potentially deleterious lesions when produced in DNA. They are presumably formed in part via misrepair of the respective C1′-nucleotide radicals by thiols. However, the selectivity and extent to which these lesions are formed via this pathway has not been ascertained. Using the ability to independently generate deoxyuridin-1′-yl (**4**) at a defined site in a biopolymer, we have determined that thiol trapping in duplex DNA occurs with high stereoselectivity from the α-face, resulting in restoration of the naturally occurring β-deoxynucleotide. The observed stereoselectivity of thiol trapping in duplex DNA suggests that **4** is intrahelical. The rate constant for hydrogen atom donation to **4** is reduced 2−3-fold in double-stranded DNA compared to single-stranded DNA. This decrease is attributed to the relative inaccessibility of the C1′-position in duplex DNA. The combination of these two properties of **4** indicates that, at O_2 concentrations present in aerated water, α-deoxynucleotide formation should constitute a minor component of the reactivity of C1′-radicals. Accordingly, the chemical biology of other lesions derived from formal damage at C1′-position could be significant.

α-Deoxynucleotides, such as α-deoxyadenosine (**1**), are produced during γ-radiolysis of DNA under anaerobic conditions.[1] α-Deoxyadenosine (**1**) has been shown to be premutagenic in vitro, inducing DNA polymerase to incorporate deoxyadenosine and deoxycytidine opposite it.[2] A variety of pathways have been proposed to account for α-deoxynucleotide formation, some of which require high fluxes of reactive intermediates.[3] One pathway leading to anomerization that does not require reaction of two reactive intermediates with a single nucleotide involves reversible ring opening of an initially formed C4′-radical, which is subsequently reduced. Chemical evidence for such a ring opening in a model system has been reported recently.[4] α-Deoxynucleotides may also be formed under oxidative stress conditions from hydrogen atom donation by thiol to the radical resulting from formal C1′-hydrogen atom abstraction.[5−7] The C1′-radical which undergoes misrepair may be produced either by hydrogen atom abstraction from the native nucleotide by species such as hydroxyl radical, via internucleotidyl hydrogen atom abstraction, or via deprotonation from C1′ of the nucleobase cation radical.[7−9] Unlike other lesions, such as thymidine C5-hydrate (**2**) and the thymidine glycols (**3**),

(1) Lesiak, K.; Wheeler, K. T. *Radiat. Res.* **1990**, *121*, 328.
(2) Ide, H.; Yamaoka, T.; Kimura, Y. *Biochemistry* **1994**, *33*, 7127.
(3) Raliegh, J. A.; Fuciarelli, A. F.; Kulatunga, C. R. In *Anticarcinogenesis and Radiation Protection*; Cerutti, P. A., Nygaard, O. F., Simic, M. G., Eds.; Plenum Press: New York, 1987.
(4) (a) Cadet, J.; Berger, M.; Shaw, A. In *Mechanisms of DNA Damage and Repair*; Simic, M. G., Grossman, L., Upton, A. C., Eds.; Plenum Press: New York, 1986. (b) Crich, D.; Yao, Q. *Tetrahedron* **1998**, *54*, 305.
(5) (a) Goodman, B. K.; Greenberg, M. M. *J. Org. Chem.* **1996**, *61*, 2. (b) Tronche, C.; Goodman, B. K.; Greenberg, M. M. *Chem. Biol.* **1998**, *5*, 263.
(6) Daniels, J. S.; Gates, K. S.; Tronche, C.; Greenberg, M. M. *Chem. Res. Toxicol.* **1998**, *11*, 1254.
(7) Greenberg, M. M. *Chem. Res. Toxicol.* **1998**, *11*, 1235.

10.1021/ja990152y CCC: $18.00
Published on Web 04/27/1999



resulting from irreversible hydroxyl radical addition to the native nucleoside, the mechanism of formation of an α-deoxynucleotide must compete with a pathway which reconstitutes the undamaged nucleotide.[10] Consequently, it is difficult to gauge the efficiency by which the initially formed C1′-radical is transformed into the α-anomer of the respective nucleotide. Furthermore, the efficiency of α-deoxynucleotide formation must also be addressed in the context of other competing reactions, most notably quenching of the radical precursor by O_2, which would ultimately give rise to the deoxyribonolactone lesion.[5,11,12]

We have addressed these issues by utilizing the ability to independently generate the radical (**4**) resulting from C1′-hydrogen atom abstraction from deoxyuridine (Scheme 1).[5,13] Utilizing deoxyuridine enables one to probe the reactivity of the C1′-radical by taking advantage of the uniqueness of the products derived from it, while at the same time constructing biopolymers containing all four naturally occurring nucleotides. For instance, independent generation of deoxyuridin-1′-yl (**4**) guarantees that any α,*β*-deoxyuridine (**6**) present in the biopolymer must be derived from radical **4**. Consequently, the stereoselectivity of the reduction of the C1′-radical can be determined in a direct manner. Furthermore, independent generation of deoxyuridin-1′-yl (**4**) enables one to determine the competition between O_2 and thiol for the radical by measuring the dependence of direct strand breaks and alkaline labile lesions on thiol concentration (Scheme 1).[5]

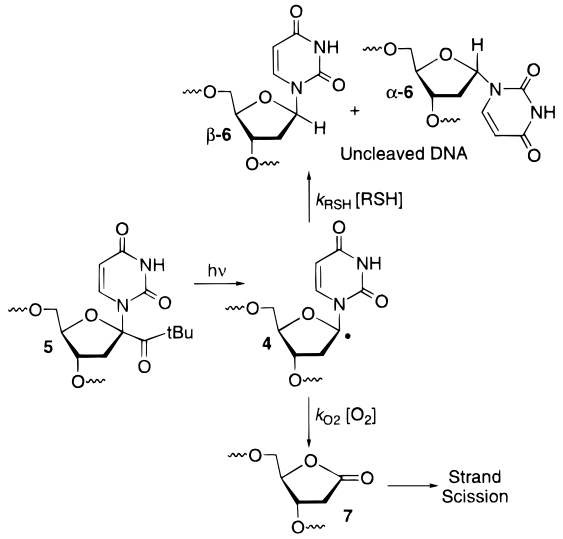
Results and Discussion

Independent generation of deoxyuridin-1′-yl (**4**) at a defined site in an oligonucleotide was achieved via UV irradiation of **5**. The experiments described herein were facilitated by an improved synthesis of the photolabile ketone (Scheme 2), which made biopolymers containing **5** more readily available.[14,15] The integrity of the oligonucleotides (**10**, **11**) containing **5** were verified by electrospray mass spectrometry.[15]

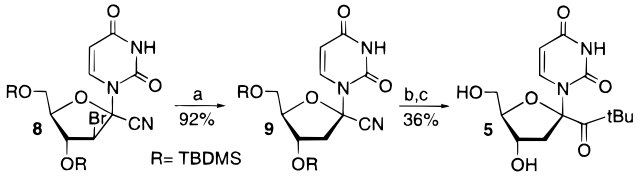
5′-d(ATA GCG A**5**A CGT)
10

5′-d(GTC ACG TGC TGC A**5**A CGA CGT GCT GAG CCT)
11

Scheme 1



Scheme 2[a]



[a] Key: (a) Bu_3SnH (1.2 equiv), AIBN (0.03 equiv), benzene, Δ. (b) *t*-BuLi (7 equiv), CuI (cat.), THF, −78 °C; then H_2O/AcOH, 16 h. (c) NH_4F (20 equiv), MeOH, 16 h, Δ.

Effects of 5 on Duplex Structure and Stability. One concern which must be addressed when utilizing **5** as a source for deoxyuridin-1′-yl (**4**) is whether the observed reactivity pattern of the radical is reflective of its structure in duplex DNA when produced as a result of oxidative stress. While no structural information is available for radical intermediates in DNA, it is reasonable to assume that a C1′-radical such as **4** would be accommodated within a duplex. If **5** is extrahelical, then it is likely that **4** will be born in such a state as well. This in itself does not mean that subsequent studies on the reactivity of **4** are not biologically relevant. If **4** is intrahelical when produced in DNA as a result of oxidative stress, generation of **4** extrahelically from **5** would be biologically relevant, provided the lifetime of the radical is long compared to the time required for conformational isomerization of the DNA duplex. Assuming that bimolecular reactions with O_2 ($k_{O_2} = 2 \times 10^9$ $M^{-1}s^{-1}$, [O_2] = 0.2 mM) and thiol(s) ($k_{RSH} \approx 1-10 \times 10^6$ M^{-1} s^{-1} (vide infra), [RSH] = 5 mM) are the predominant reaction pathways available to **4**, its half-life should be on the microsecond time scale. This period of time should be large compared to that of DNA breathing.[16] If **5** is intrahelical, then it is even more likely that the time needed for structural adjustment will be small compared to the time frame for radical reactivity. To gain some insight into the orientation of **5** in duplex DNA, we examined its effect on the stability of duplex DNA by measuring the thermodynamics of melting via UV spectroscopy and its effect on duplex structure via CD spectroscopy.[17,18]

The presence of the photolabile ketone **5** destabilizes a dodecameric duplex (**12a**) by approximately 2.0 kcal/mol at 298

(8) (a) Deeble, D. J.; Schulz, D.; Von Sonntag, C. *Int. J. Radiat. Biol.* **1986**, *49*, 915. (b) von Sonntag, C. *The Chemical Basis of Radiation Biology*; Taylor & Francis Inc.: Philadelphia, PA, 1987. (c) Greenberg, M. M.; Barvian, M. R.; Cook, G. P.; Goodman, B. K.; Matray, T. J.; Tronche, C.; Venkatesan, H. *J. Am. Chem. Soc.* **1997**, *119*, 1828.

(9) (a) Decarroz, C.; Wagner, J. R.; Cadet, J. *Free Rad. Res. Commun.* **1987**, *2*, 295. (b) Weiland, B.; Huttermann, J.; Malone, M. E.; Cullis, P. M. *Int. J. Radiat. Biol.* **1996**, *70*, 327.

(10) (a) Matray, T. J.; Greenberg, M. M. *Biochemistry* **1997**, *36*, 14071. (b) Clark, J. M.; Beardsley, P. *Biochemistry* **1987**, *26*, 5398.

(11) Tallman, K. A.; Tronche, C.; Greenberg, M. M. *J. Am. Chem. Soc.* **1998**, *120*, 4903.

(12) Goldberg, I. H. *Acc. Chem. Res.* **1991**, *24*, 191.

(13) For simplicity, molecules are referred to by the same number whether they are present as a monomeric nucleoside or as part of a biopolymer.

(14) (a) Itoh, Y.; Haraguchi, K.; Tanaka, H.; Gen, E.; Miyasaka, T. *J. Org. Chem.* **1995**, *60*, 656. (b) Chatgilialoglu, C.; Gimisis, T. *Chem. Commun.* **1998**, 1249.

(15) See the Supporting Information.

(16) Guest, C. R.; Hochstrasser, R. A.; Sowers, L. C.; Millar, D. P. *Biochemistry* **1991**, *30*, 3271.

(17) Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry, Part II: Techniques for the Study of Biological Structure and Function*; W. H. Freeman: New York, 1980.

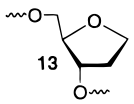
Table 1. Effect of 5 on the T_m and Thermodynamic Stability of **12**

duplex	T_m (° C)[a]	ΔH[b]	ΔS[c]	ΔG° (25 °C)[b]
12a	47.5	57.6	160	9.9
12b	54.6	66.2	182	11.9

[a] [duplex] = 13.5 mM. [b] kcal/mol. [c] cal/(mol·deg).

K relative to an otherwise identical one which contains deoxyuridine (**12b**) (Table 1). The destabilization is dominated by the enthalpy of melting, suggesting that the duplex containing **5** is not significantly less ordered. In addition, the decrease in duplex stability is less than when a molecule (**13**) which is

5′-d(A T A GGG A **X** A CGT)
(T A T CGC T A T GCA)d-5′
12a X = **5**
12b X = dU



incapable of base pairing, and has been determined by NMR to be extrahelical, is incorporated in a duplex.[19] The thermodynamic destabilization and melting temperature depression induced by the presence of **5** in a DNA duplex are also less than when (*5R*)-**2** and **3** are incorporated in duplexes of equal length.[20] Moreover, duplexes containing (*5R*)-**2** experience a considerably less favorable entropic change upon melting than an analogous duplex containing thymidine, consistent with a duplex containing less order. Thymidine glycol (**3**) has been shown by NMR to exist in an extrahelical conformation.[20a,b] Thymidine C5-hydrate (*5R*-**2**) and **3** are predicted to affect DNA structure in a similar manner, and their effects on DNA polymerase activity support this scenario.[10,21] Taken together, these UV-melting experiments support a duplex structure containing **5** in an intrahelical manner. Furthermore, analysis of the CD spectra of **12a**,**b** clearly show that the duplex structure is retained when **5** is incorporated in the center of this duplex, again indicating that the ketone could be intrahelical.[15]

Stereoselectivity of Thiol Trapping of Deoxyuridin-1′-yl (4). When monomeric **5** is irradiated in the presence of thiol under anaerobic conditions, high yields of β- and α-deoxyuridine (**6**) (3:2 ratio) are obtained.[5a] MALDI-TOF mass spectral analysis of the polymeric products produced from **12a** upon irradiation under anaerobic conditions in the presence of β-mercaptoethanol (5 mM) reveals the presence of the unadulterated complement (**14**) and the expected deoxyuridine containing oligonucleotide(s) (**15**) as the only products, suggesting that deoxyuridin-1′-yl (**4**) is formed cleanly and trapped efficiently when **5** is irradiated within this biopolymer.[15]

5′-d(ACG TAT CGC TAT)
14

5′-d(ATA GCG A**X**A CGT)
15 X = α,β-dU

Table 2. Stereoselectivity of Thiol Trapping of Deoxyuridin-1′-yl (**4**) in Oligonucleotides

thiol	concn (mM)	β:α-deoxyuridine **10**	**12a**
β-mercaptoethanol	5	4.1 ± 0.1	6.2 ± 0.1
β-mercaptoethanol	50	4.3 ± 0.3	6.7 ± 0.2
dithiothreitol	5	4.1 ± 0.1	8.3 ± 0.1
dithiothreitol	50	4.5 ± 0.3	7.8 ± 0.5

The stereoselectivity of thiol mediated reduction of **4** in biopolymers was determined by HPLC analysis of the mixture of α,β-deoxyuridine (**6**) released upon enzymatic digestion. Preparation of an epimeric mixture of deoxyuridine enabled us to establish separation conditions for the mixture and to determine that their relative concentrations were equal to the ratio of their respective peak areas.[22] It is known that α-deoxynucleotides are processed more slowly by phosphodiesterases than their naturally occurring counterparts.[23,24] Hence, enzymatic digestion conditions which resulted in complete digestion of a chemically synthesized dodecamer containing a mixture of α,β-**6** at a defined site were developed. Digestion of **15**, which was prepared from a mixture of β- and α-deoxyuridine β-cyanoethyl phosphoramidites (β:α, 2.4:1), with snake venom phosphodiesterase, nuclease P1, and calf intestine alkaline phosphatase, followed by reverse phase HPLC analysis of the liberated nucleosides yielded a β:α ratio of 3.5. Extending the digestion period and supplementation with additional aliquots of enzymes had no effect on this ratio, suggesting that digestion was complete. The larger β-**6**:α-**6** ratio found in the polymer compared to that in the phosphoramidite used to prepare **15** is attributed to slower coupling of the α-anomer compared to the respective phosphoramidite of β-deoxyuridine and is consistent with the significantly longer coupling time employed to incorporate α-deoxynucleotides during automated oligonucleotide synthesis.[23]

The stereoselectivity of trapping of deoxyuridin-1′-yl (**4**) in single-stranded DNA (**10**) by β-mercaptoethanol and dithiothreitol is independent of the nature of the thiol and of thiol concentration at 5 and 50 mM (Table 2). Selectivity for formation of the β-anomer is increased for both thiols, albeit to different degrees, in duplex DNA (**12a**) (Table 2). Importantly, the stereoselectivity is still independent of the concentration of either thiol over the range studied and suggests that the same population of conformational isomers is trapped under both conditions.

The observed lack of dependence on thiol concentration, coupled with the effect of oligonucleotide hybridization on the selectivity of thiol trapping of **4**, is consistent with the radical existing in an intrahelical conformation. The greater stereoselectivity exhibited by dithiothreitol, which reacts faster with **4** than β-mercaptoethanol (vide infra), also argues against trapping the radical in an extrahelical position. Moreover, the observations imply that **4** generated photolytically from ketone **5** is trapped in a conformation that is comparable to that present when the radical is formed as a result of oxidative stress. These observations are also consistent with the suggestion that the photolabile precursor (**5**) does not perturb the structure of the

(18) Breslauer, K. J. In *Methods in Molecular Biology, Volume 26: Protocols for Oligonucleotide Conjugates*; Agrawal, S., Ed.; Humana Press: New Jersey, 1994.

(19) (a) Gelfand, C. A.; Plum, G. E.; Grollman, A. P.; Johnson, F.; Breslauer, K. J. *Biochemistry* **1998**, *37*, 7321. (b) Zhen, L.; Hung, K.-N.; Grollman, A. P.; De Los Santos, C. *Nucleic Acids Res.* **1998**, *26*, 2385.

(20) (a) Kao, J. Y.; Goljer, I.; Phan, T. A.; Bolton, P. H. *J. Biol. Chem.* **1993**, *268*, 17787. (b) Kung, H. C.; Bolton, P. H. *J. Biol. Chem.* **1997**, *272*, 9227. (c) Sambandam, A.; Greenberg, M. M. In preparation.

(21) (a) Miaskiewicz, K.; Miller, J.; Ornstein, R.; Osman, R. *Biopolymers* **1995**, *35*, 113. (b) Miaskiewicz, K.; Miller, J.; Osman, R. *Biochim. Biophys. Acta* **1994**, *1218*, 283.

(22) Ward, D. I.; Jeffs, S. M.; Coe, P. L.; Walker, R. T. *Tetrahedron Lett.* **1993**, *34*, 6779.

(23) Morvan, F.; Rayner, B.; Leonetti, J.-P.; Imbach, J.-L. *Nucleic Acids Res.* **1988**, *16*, 833.

(24) (a) Séquin, U. *Helv. Chim. Acta* **1974**, *57*, 68. (b) Thuong, N. T.; Asseline, U.; Roig, V.; Takasugi, M.; Hélene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5129. (c) Bacon. T. A.; Morvan, F.; Rayner, B.; Imbach, J.-L.; Wickstrom, E. *J. Biochem. Biophys. Methods* **1988**, *16*, 311.

Table 3. Kinetics of Thiol Trapping of Deoxyuridin-1′-yl (**4**) in Oligonucleotides

	k_{O_2}/k_{RSH}[a]		$k_{RSH} \times 10^{-6}\ M^{-1}\ s^{-1}$	
thiol	**11**	**16**	**11**	**16**
β-mercaptoethanol	450	1100	4.4 ± 0.4	1.8 ± 0.6
dithiothreitol	150	270	13.0 ± 3.0	7.5 ± 2.4

[a] Taken from the average value of plots of the effect of thiol on cleavage.

DNA duplex to a significant extent, or if it does, then annealing of the duplex occurs rapidly compared to bimolecular trapping reactions.

The greater stereoselectivity for reduction of **4** in duplex DNA by dithiothreitol than by β-mercaptoethanol can be attributed to the larger size of the dithiol. However, one cannot rule out other physicochemical reasons for this selectivity, such as preferred thiol binding in the minor groove. The latter explanation gains credence given the fact that the facial selectivity for **4** by dithiothreitol in duplex DNA is within experimental error of that observed for the trapping of a C4′-radical in duplex DNA, whereas the C4′-radical is trapped with little selectivity in a single-stranded host.[25] While the source of the selectivity is clearly speculative, the indisputable fact is that α-deoxyuridine (**6**) formation from **4** accounts for a minor fraction of this radical's trapping reactions with thiols in duplex DNA.

Efficiency of Thiol Trapping of Deoxyuridin-1′-yl (4) by Thiols in the Presence of O_2. Under most circumstances α-deoxynucleotide formation via thiol trapping of anomeric nucleotide radicals must compete with O_2. The concentration of O_2 in cells is highly variable, but the solubility (1 mM) of air (~20% O_2, 0.2 mM) often serves as an upper limit.[26] Thiol trapping rate constants of deoxyuridin-1′-yl in single-stranded (**11**) and double-stranded (**16**) DNA were estimated by establishing a competition between the thiol and O_2 and measuring the extent of labile lesions produced as a function of thiol concentration (eq 1, Table 3). The rate constants measured for

5′-d(G T C A C G T G C T G C A **5** A C G A C G T G C T G A G C C T)
(C A G T G C A C G A C G T A T G C T G C A C G A C T C G G A)d-5′
16

$$\frac{\text{Cleavage } (O_2)}{\text{Trapping (RSH)}} = \frac{k_{O2}[O_2]}{k_{RSH}}\left(\frac{1}{[RSH]}\right) \qquad (1)$$

trapping **4** were verified by multiple experiments. The measured value was ≈3-fold less than that previously reported in a polymer but within experimental error of that determined for trapping of the monomer.[5] Dithiothreitol is more effective than β-mercaptoethanol at trapping the deoxyuridin-1′-yl (**4**) radical.[27] Moreover, the rate constant for trapping **4** by each thiol was smaller when the radical was produced in a double-stranded substrate than in a single-stranded oligonucleotide. The ratio of rate constants for thiol and O_2 trapping are independent of any assumptions regarding the absolute rate constant for reaction of deoxyuridin-1′-yl (**4**) with O_2 (Table 3). However, the absolute magnitude of the rate constants determined in double-stranded DNA assume that bimolecular trapping by O_2 is independent of the secondary structure of the substrate. Considering that the assumed rate constant for O_2 trapping is near the diffusion-controlled upper limit, the estimated absolute rate constant for trapping **4** in duplex DNA must also represent an upper limit. The dependence of the thiol trapping rate constants on the hybridization of the substrate provide further support for the proposal that the radical is intrahelical in the double-stranded substrate and, more importantly, is present in a conformation that is at least similar to that which would be populated if **4** were produced in DNA as a result of oxidative stress.

To our knowledge, this is the first demonstration that the rate constant for thiol trapping of a nucleotide radical (**4**) is dependent on hybridization of the oligonucleotide in which the radical is produced. In contrast, thiol trapping of the radical resulting from C4′-hydrogen atom abstraction was not affected by hybridization of the oligonucleotide.[25] The decrease in trapping of the radical when it is buried in the minor groove in duplex DNA is consistent with the relative inaccessibility of the C1′-radical upon the basis of results from recent computational experiments and application of the principle of microscopic reversibility.[28]

Conclusions. These studies on the reactivity of deoxyuridin-1′-yl (**4**) indirectly suggest that the presence of anomeric radicals in duplex DNA does not significantly perturb the structure of the biopolymer. The above experiments also suggest that α-deoxynucleotide lesions are unlikely to be a significant family of DNA lesions in vivo, provided that C1′-nucleotide radicals constitute their source. Stereoselectivity studies reveal that α-deoxyuridine (**6**) constitutes less than 15% of the mixture of nucleotides formed when **4** is trapped by thiols in duplex DNA (Table 2). Selection against the α-anomer is compounded further by the measured ratio of rate constants (k_{O_2}/k_{RSH}, Table 3). These indicate that α-deoxynucleotide formation at the concentration of O_2 present in aerated water (0.2 mM) and physiologically relevant thiol concentrations (~5 mM) will constitute as little as 0.4% of the products resulting from C1′-nucleotide radical production and trapping by β-mercaptoethanol in duplex DNA. Furthermore, considering that glutathione is the likely hydrogen atom donor in vivo and that this species might react even more slowly with DNA radicals due to charge repulsion between the biopolymer and the thiol, it is possible that thiol trapping of C1′-nucleotide radicals might make up an even smaller fraction of products arising from this species in vivo.[29] In the final analysis, the extent to which α-deoxynucleotides are formed will depend strongly upon the O_2 concentration in the nucleus and possibly upon the local structure of the DNA.

Experimental Section

General Methods. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics phosphorimager equipped with Imagequant version 3.3 software. Oligonucleotide synthesis was carried out on an Applied Biosystems Incorporated 380B DNA synthesizer using standard protocols. 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 labeling, were carried out using standard procedures.[30] Oligonucleotides were sequenced using a reaction specific for adenine.[31] Preparative and analytical oligonucle-

(25) Giese, B.; Dussy, A.; Meggers, E.; Petretta, M.; Schwitter, U. *J. Am. Chem. Soc.* **1997**, *119*, 11130.
(26) Zander, R. *Z. Naturforsch.* **1976**, *31c*, 339.
(27) Neta, P.; Grodkowski, J.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1996**, *25*, 709.
(28) Miaskiewicz, K.; Osman, R. *J. Am. Chem. Soc.* **1994**, *116*, 232.
(29) Hildenbrand, K.; Schulte-Frohlinde, D. *Int. J. Radiat. Biol.* **1997**, *71*, 377.
(30) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: New York, 1989.
(31) Iverson, B. L.; Dervan, P. B. *Nucleic Acids Res.* **1987**, *15*, 7823.

otide 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. Nuclease P1 and snake venom phosphodiesterase were from Boehringer Mannheim. [γ-^{32}P]-ATP was from Amersham. Electrospray and MALDI-TOF mass spectrometry samples were prepared by precipitating from NH_4OAc.

All photolyses of oligonucleotides were carried out in Pyrex tubes (0.25 in. i.d.) using a Rayonet Photoreactor (RPR-100) equipped with lamps having a maximum output at 350 nm. Anaerobic photolyses were carried out in tubes which were flame sealed under vacuum, following freeze−pump−thaw degassing (3 cycles).

CD Experiments. Samples (112 μM) were prepared in phosphate buffer (10 mM, pH 7.2) and NaCl (100 mM). Duplexes **12a,b** were prepared by heating the sample to 90 °C for 5 min, then allowing the sample to cool to room temperature overnight. Solutions containing duplex DNA (22.4 μM) were analyzed in a cell (0.5 mm path length) from 300 to 200 nm.

DNA Melting Temperatures. Samples (400 μL) for thermal denaturing studies contained a 1:1 molar ratio of complementary oligonucleotides. Solutions of **12a,b** were prepared by the addition of appropriate volumes of stock solutions of the requisite complementary oligonucleotides to 200 μL of PIPES (1,4-piperazine bis(ethanesulfonate) sesquisodium salt) buffer (20 mM PIPES, pH 7.0, 20 mM $MgCl_2$, 200 mM NaCl), followed by dilution with water to a volume of 400 μL. The complementary oligonucleotides were hybridized by heating the samples at 90 °C for 5 min, followed by slow cooling overnight to room temperature. The melting studies were carried out in 1 cm path length quartz cells using a Beckman DU 640 spectrophotometer equipped with a thermoprogrammer. Absorbance was monitored at 260 nm while the temperature was increased at a rate of 0.5 °C/min over a range of 45 °C (25 to 70 °C).

MALDI-TOF Analysis of Photolysis of 5 under Anaerobic Conditions in the Presence of β-Mercaptoethanol. Duplex **12a** was hybridized in the phosphate−sodium chloride buffer system described above and precipitated twice from ammonium acetate. A sample of **12a** (0.1 OD_{260}) and β-mercaptoethanol (5 mM) in H_2O was photolyzed for 2 h, after being degassed (three freeze−pump−thaw degassing cycles) and sealed. The photolyzed solution was transferred to an eppendorf tube, and the photolysis tube was washed with H_2O (50 μL). The combined photolyzate was concentrated in vacuo and examined by MALDI-TOF mass spectrometry using a matrix composed of 3-hydroxypicolinic acid and ammonium citrate in H_2O and CH_3CN.

Analysis of α- and β-Deoxyuridine (6) in Oligonucleotides via Enzymatic Digestion and HPLC. A mixture of α- and β-deoxyuridine (**6**) was prepared as reported in the literature.[22] The ratio of anomers (β:α = 2.4:1) was determined by ^{1}H NMR, and it was determined that this ratio was equal to the area ratio of separated species on reverse-phase HPLC. Reversed-phase HPLC analysis was carried out on a 0.4 × 25 cm Rainin C-18 Microsorb-MV column. Gradient conditions: A, 0.01 M KH_2PO_4, pH 6.8, 1% methanol; B, 0.01 M KH_2PO_4, pH 6.8, 25% methanol; T_i: 100% A maintained for 20 min, 0−5% B linearly over 5 min, 5−100% B linearly over 5 min, maintained at 100% B for 20 min. Flow rate: 1 mL/min. Retention times: α-**6**, 18.3 min; β-**6**, 19.2 min.

The mixture of anomers of **6** was transformed into the respective mixture of β-cyanoethyl phosphoramidites using the standard procedures and then used to synthesize **15**.[5b,32] Desalted samples of **15** that had been purified by denaturing PAGE were taken up in 86 μL of buffer (Tris-HCl, pH 7.9, 50 mM; NaCl, 100 mM; DTT, 1 mM) and $MgCl_2$ (100 mM). Nuclease P1 (10 μL, 0.3 units/μL), snake venom phosphodiesterase (2 μL, 0.003 units/μL), and calf intestine alkaline phosphatase (2 μL, 10 units/μL) were added to the oligonucleotide solution. The tube was vortexed, spun briefly, and incubated at 37 °C for 12 or 48 h. Each sample was passed through a 0.45 μm filter disk, which was then washed with H_2O (5 × 100 μL). The filtrate was concentrated in vacuo, resuspended in H_2O (80 μL), and analyzed by reverse-phase HPLC. In other experiments, additional aliquots of enzyme (equal to the original amount) were added at 12 and 24 h. However, the ratio of anomers of **6** was invariant.

Enzymatic Digestion of Photolyzed Oligonucleotides Containing 5. Samples (1.0 $OD_{260} \approx$ 10 nmol) of **10** or **12a** and 5 or 50 mM of β-mercaptoethanol or dithiothreitol were degassed and sealed. Samples of **10** were photolyzed in H_2O for 4 h, whereas samples of **12a** in phosphate buffer (10 mM, pH 7.2) and NaCl (100 mM) were irradiated for 6 h. The photolyzed solutions were transferred to an eppendorf tube, and the photolysis tube was washed with H_2O (3 × 100 μL). Samples of **10** were concentrated in vacuo and subjected to enzymatic digestion as described above for 48 h. The photolyzate obtained from **12a** was desalted using a Sep-Pak C-18 cartridge prior to enzymatic digestion.

Competition between Thiols and O_2 for 4 in Single-Stranded (11) and Double-Stranded (16) DNA. Photolysis tubes were loaded with oligonucleotide in phosphate buffer (10 mM, pH 7.2) and NaCl (100 mM). Thiol was present between 0 and 100 mM. Single-stranded DNA samples were photolyzed for 20 min and duplex DNA samples for 2 h under aerobic conditions. After photolysis, the solution was transferred to an eppendorf tube, the photolysis tube was washed with H_2O (70 μL), and the combined material was precipitated. The DNA pellets were resuspended in 1 M piperidine (100 μL) and heated at 90−95 °C for 20 min. The samples were then lyophilized, resuspended in H_2O, and lyophilized (2 × 100 μL). Prior to loading on a gel (20% denaturing PAGE), the DNA pellets were resuspended in formamide loading buffer by vortexing for 2 min, heating at 55 °C for 5 min, and vortexing again for 1 min. Duplex DNA samples were heated at 95 °C for 5 min and quickly placed in an ice−water bath, prior to loading on a gel. Loading of samples in adjacent lanes were staggered in time, so as to facilitate quantitating intact DNA. In a typical experiment, five samples containing no thiol were irradiated and the effect of thiol on cleavage at each thiol concentration was carried out in duplicate. The amount of cleavage (fraction of total DNA in a sample) was determined by measuring the volume of the cleavage band corresponding to where **5** is incorporated and dividing by the sum of this band and that corresponding to intact DNA. The amount of trapping by thiol was determined by subtracting the amount of cleavage observed in a sample containing a given thiol concentration from the average amount of cleavage observed in the samples in which no thiol is present. Since the samples containing no thiol affect every measurement, five such samples were employed in each experiment.

Acknowledgment. This manuscript is dedicated to Dr. Keith U. Ingold on the occasion of his 70th birthday. We are grateful for support of this research by the National Institutes of Health (GM-54996). Mass spectra were obtained on instruments supported by the National Institutes of Health shared instrumentation grant (GM-49631). M.M.G. thanks the Alfred P. Sloan Foundation for a fellowship.

Supporting Information Available: Text describing the experimental procedure for the synthesis of **5** from **8**, CD spectra of **12a**,**b**, MALDI-TOF spectrum of the photolysis of **12a**, and electrospray mass spectra of **10** and **11** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA990152Y

(32) Beaucage, S. L.; Caruthers, M. H. In *Bioorganic Chemistry Nucleic Acids*; Hecht, S. M., Ed.; Oxford University Press: Oxford, U.K., 1996.