Oxygen-Dependent DNA Damage Amplification Involving 5,6-Dihydrothymidin-5-yl in a Structurally Minimal System

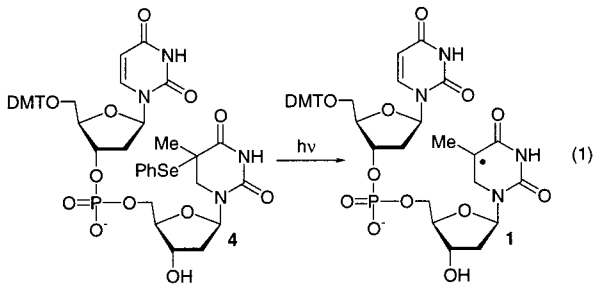
Keri A. Tallman and Marc M. Greenberg*

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

Received January 22, 2001

Abstract: 5,6-Dihydrothymidin-5-yl (**1**) was independently generated in a dinucleotide from a phenyl selenide precursor (**4**). Under free radical chain propagation conditions, the products resulting from hydrogen atom donation and radical-pair reaction are the major observed products in the absence of O_2. The stereoselectivity of the trapping process is dependent on the structure of the hydrogen atom donor. No evidence for internucleotidyl hydrogen atom abstraction by **1** was detected. The tandem lesion (**17**) resulting from hydrogen atom abstraction from the C1′ position of the adjacent 2′-deoxyuridine by the peroxyl radical derived from **1** (**3**) is observed under aerobic conditions. The structure of this product is confirmed by independent synthesis and its transformation into a second independently synthesized product (**24**). Internucleotidyl hydrogen atom abstraction is effected selectively by the 5*S*-diastereomer of the peroxyl radical. The formation of dinucleotide **17** provides further support for the novel O_2-dependent DNA damage amplification mechanism involving **1** reported previously (Greenberg, M. M.; et al. *J. Am. Chem. Soc.* **1997**, *119*, 1828).

Nucleic acids are oxidatively damaged by a variety of natural and unnatural products. Some of these molecules, such as the neocarzinostatin chromophore and members of the enediyne family of antitumor antibiotics, are capable of producing bistranded lesions due to the biradical nature of their reactive form.[1] Additionally, a single molecule of bleomycin is capable of forming multiple lesions via recycling of its redox-active metallocenter.[2] Investigations of DNA damage mediated by γ-radiolysis brought to light the possibility that a single initial nucleic acid damaging event could yield bistranded and tandem lesions.[3,4] Currently, there is significant interest in the possible role of these types of lesions in DNA repair and mutagenesis.[5] Using γ-radiolysis studies as a guide, we carried out chemical studies, from which it was concluded that the nucleobase radical 5,6-dihydrothymidin-5-yl (**1**)[6] produces tandem lesions in oligodeoxynucleotides via an O_2-dependent DNA damage amplification mechanism (Scheme 1).[7,8] We now wish to report product studies employing the minimal chemical unit capable of sustaining such a process, a dinucleotide (**4**), which support this nucleic acid strand damage mechanism. Analysis of photolysis experiments on **4** by HPLC using independently synthesized potential products as standards corroborates the conclusions reached previously and provides additional details about this chemical process (eq 1).



5,6-Dihydrothymidin-5-yl (**1**) is a reactive intermediate that is formed in significant amounts upon exposure of DNA to γ-radiolysis.[3,9,10] This nucleobase radical is produced as a consequence of the indirect effect of ionizing radiation via hydrogen atom addition to the pyrimidine double bond of thymidine, and represents the major reactive intermediate formed as a result of the reaction between these two entities. The analogous hydroxyl radical adduct (**5**) is also produced in significant amounts. In addition, the favorable reduction potential of thymidine, compared to those of the other three native nucleotides in DNA, results in the formation of **1** via sequential electron addition and protonation as perhaps the most abundant

(1) Xi, Z.; Goldberg, I. H. In *Comprehensive Natural Products Chemistry, Volume 7, DNA and Aspects of Molecular Biology*; Kool, E. T., Ed.; Pergamon: Oxford, 1999; pp 553−592.

(2) (a) Steighner, R. J.; Povirk, L. F. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8350. (b) Absalon, M. J.; Kozarich, J. W.; Stubbe, J. *Biochemistry* **1995**, *34*, 2065. (c) Absalon, M. J.; Wu, W.; Kozarich, J. W.; Stubbe, J. *Biochemistry* **1995**, *34*, 2076.

(3) (a) von Sonntag, C. *The Chemical Basis of Radiation Biology*; Taylor and Francis, London, 1987. (b) Greenberg, M. M. In *Comprehensive Natural Products Chemistry, Volume 7, DNA and Aspects of Molecular Biology*, Kool, E. T., Ed.; Pergamon: Oxford, 1999; pp 371−426. (c) Greenberg, M. M. *Chem. Res. Toxicol.* **1998**, *11*, 1235.

(4) Aslam Siddiqi, M.; Bothe, E. *Radiat. Res.* **1987**, *112*, 449.

(5) (a) Sutherland, B. M.; Bennett, P. V.; Sidorkina, O.; Laval, J. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 103. (b) Satoh, M. S.; Jones, C. J.; Wood, R. D.; Lindahl, T. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6335.

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

(7) (a) Barvian, M. R.; Greenberg, M. M. *J. Org. Chem.* **1995**, *60*, 1916. (b) Barvian, M. R.; Greenberg, M. M. *J. Am. Chem. Soc.* **1995**, *117*, 8291. (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.

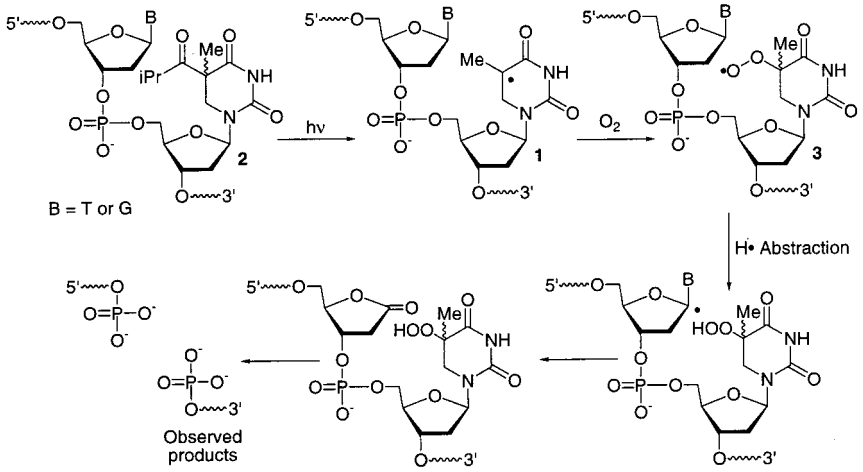
(8) (a) Deeble, D. J.; von Sonntag, C. *Int. J. Radiat. Biol.* **1986**, *49*, 927. (b) Schulte-Frohlinde, D.; Bothe, E. *Z. Naturforsch.* **1984**, *39c*, 315.

(9) (a) Symons, M. C. R. *J. Chem. Soc., Faraday Trans. 1* **1987**, *83*, 1. (b) Boon, P. J.; O'Connell, A.; Podmore, I. D.; Symons, M. C. R. *J. Chem. Soc., Perkin Trans. 2* **1993**, 2061. (c) Cullis, P. M.; Langman, S.; Podmore, I. D.; Symons, M. C. R. *J. Chem. Soc., Faraday Trans.* **1990**, *86*, 3267.

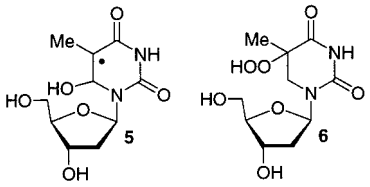
(10) Cullis, P. M.; McClymont, J. D.; Malone, M. E.; Mather, A. N.; Podmore, I. D.; Sweeney, M. C.; Symons, M. C. R. *J. Chem. Soc., Perkin Trans 2* **1992**, 1695.

10.1021/ja010180s CCC: $20.00
Published on Web 05/11/2001

Scheme 1



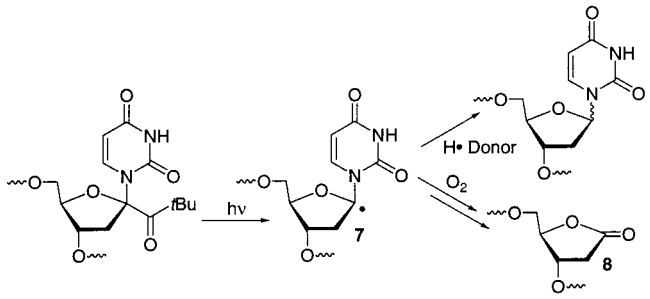
reactive intermediate produced from thymidine as a consequence of this two-step process. 5,6-Dihydrothymidin-5-yl (**1**) has been suggested to play a significant role in nucleic acid strand damage. It was proposed that **1** amplifies the initial strand damage event (its formation) by directly abstracting a hydrogen atom from the nucleotide bonded to its 5′-phosphate, resulting in the formation of tandem lesions.[9]



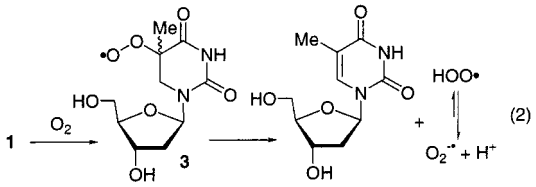
Chemical studies in which **1** was generated from a ketone precursor (**2**) via Norrish type I photocleavage were inconsistent with such a proposal. At the monomeric level, **1** failed to abstract hydrogen atoms from molecules chosen to mimic the carbohydrate components of nucleosides.[6] Furthermore, direct strand breaks or alkali-labile lesions were not observed when **1** was produced from **2** under anaerobic conditions in single-stranded oligodeoxynucleotides.[7b,c] The consequences of generating **1** from **2** under aerobic conditions were considerably different. At the monomeric level, a diastereomeric mixture of the unstable tertiary hydroperoxide (**6**) was isolated.[7a] When produced in single-stranded oligodeoxynucleotides under aerobic conditions, **1** gave rise to alkali-labile lesions, and to a lesser extent direct strand breaks, predominantly at the nucleotide bonded to the 5′-phosphate of **1**. Furthermore, the initial site of **1** was transformed into an alkali-labile lesion, indicating that two lesions are formed from one initial interaction between DNA and ionizing radiation. This mechanism for O_2-dependent DNA damage amplification represented a pathway that was distinct from any other DNA-damaging pathway documented in the chemical literature.

A variety of experiments, including studies of kinetic (product) isotope effects and enzymatic end group analysis, led to a proposed mechanism involving selective C1′ hydrogen atom abstraction by the nucleobase peroxyl radical derived from **1** (Scheme 1).[7] No evidence (as indicated by the lack of an isotope effect upon deuteration of the C4′ position or the formation of 3′-phosphoglycolate termini) for competing hydrogen atom abstraction from the slightly stronger carbon−hydrogen bond at the C4′ position was obtained.[3b,11,12] This selectivity is consistent with that expected for a peroxyl radical (e.g., **3**), as

Scheme 2



opposed to an alkoxyl radical, which may result from reduction of **3**. Alkoxyl radicals are expected to be far more promiscuous in their hydrogen atom abstraction reactions from carbon−hydrogen bonds.[14,15] Later studies on monomers and biopolymers, in which the C1′ radical of 2′-deoxyuridine (**7**) was independently generated, corroborated the O_2-dependent DNA damage amplification mechanism (Scheme 2).[16−18] In addition, further investigation of the reactivity of **1** at the monomeric level under aerobic conditions revealed the existence of a competing pathway for the peroxyl radical **3** that reconstitutes the native nucleoside with concomitant release of superoxide (eq2).[17] Although these reports provide significant information,



a number of issues regarding this DNA damage amplification

(11) Kozarich, J. W.; Worth, L., Jr.; Frank, B. L.; Christner, D. F.; Vanderwall, D. E.; Stubbe, J. *Science* **1989**, *245*, 1396.

(12) (a) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107. (b) Dussy, A.; Meggers, E.; Giese, B. *J. Am. Chem. Soc.* **1998**, *120*, 7399.

(13) (a) Colson, A.-O.; Sevilla, M. D. *J. Phys. Chem.* **1995**, *99*, 3867. (b) Miaskiewicz, K.; Osman, R. *J. Am. Chem. Soc.* **1994**, *116*, 232.

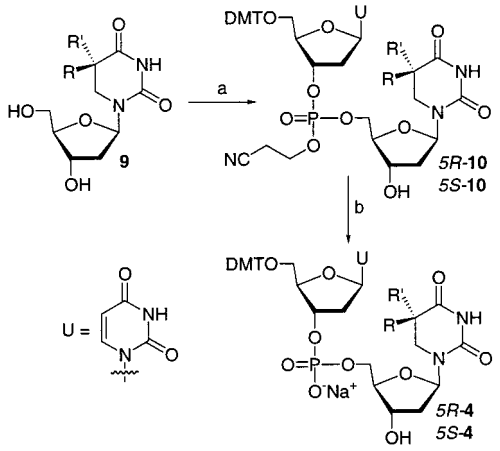
(14) Ho, W. F.; Gilbert, B. C.; Davies, M. J. *J. Chem. Soc., Perkin Trans. 2* **1997**, 2525.

(15) (a) Avilla, D. V.; Brown, C. E.; Ingold, K. U.; Lusztyk, J. *J. Am. Chem. Soc.* **1993**, *115*, 466. (b) Weber, M.; Fischer, H. *J. Am. Chem. Soc.* **1999**, *121*, 7381.

(16) (a) Tronche, C.; Goodman, B. K.; Greenberg, M. M. *Chem. Biol.* **1998**, *5*, 263. (b) Hwang, J.-T.; Greenberg, M. M. *J. Am. Chem. Soc.* **1999**, *121*, 4311.

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

Scheme 3[a]



Monomer	R	R'	Coupling (a)	Deprotection (b)
5R-**9**	PhSe	CH_3	51% (*5R*-**10**)	72% (*5R*-**4**)
5S-**9**	CH_3	PhSe	58% (*5S*-**10**)	59% (*5S*-**4**)

[a] Reagents: (a) 2′-deoxyuridine β-cyanoethyl phosphoramidite, tetrazole/CH_3CN (0.5 M), THF, 0 °C; 1.0 M I_2, THF/2,6-lutidine/H_2O (2:2:1 by volume); (b) 50% Et_3N/THF, reflux.

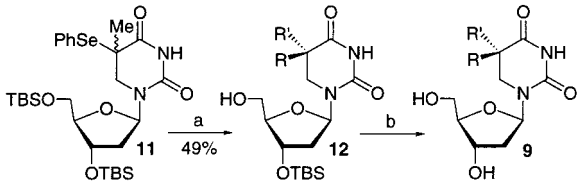
process remain unresolved. This collection of independent experiments did not provide any information on the competition (and hence efficiency of the internucleotidyl hydrogen atom transfer) between superoxide elimination and hydrogen atom abstraction by **3**. Studies using oligodeoxynucleotides did not provide detailed product analysis in support of the proposed mechanism (Scheme 1). Finally, experiments involving biopolymers also did not rule out the possibility that **1** abstracts a hydrogen atom from an adjacent nucleotide, but did show that this event does not result in the formation of an alkali-labile lesion or direct strand break in the absence of O_2. These issues are addressed in the studies described below.

Results and Discussion

Design, Synthesis, and Analysis of the Dinucleotide and Possible Products Derived from 5,6-Dihydrothymidin-5-yl (1). To more fully characterize the reactivity of **1** and its respective peroxyl radical (**3**) with an adjacent nucleotide in an oligonucleotide, we reduced the system to the minimal structural entity that could sustain such a reaction process, a dinucleotide (**4**). The dinucleotide **4** was designed with a dimethoxytrityl group at its 5′-terminus in order to facilitate synthesis and purification, as well as to enhance detection of the starting material and products by UV absorption. 2′-Deoxyuridine was chosen as the 5′-nucleotide relative to the radical center (instead of thymidine) in order to distinguish the possible formation of thymidine via **1** in experiments where the photolysates are subjected to enzymatic digestion. The phenyl selenide **4** was chosen as a precursor for **1** due to its greater efficiency for photochemical generation of the radical than the isopropyl ketone **2**.[7b,17]

Initially, the diastereomeric dinucleotides ((5*S*)-,(5*R*)-**4**) were synthesized individually via standard phosphoramidite coupling methods (Scheme 3), although our previous studies had shown that a diastereomeric mixture of the ketone radical precursor **2** did not adversely affect mechanistic studies of **1** and **3**.[7b,19] However, characterization of the synthetic intermediates and

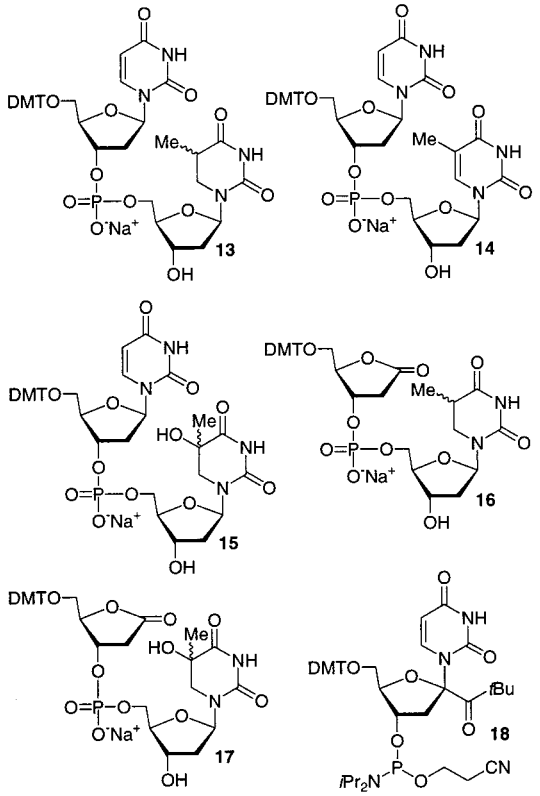
Scheme 4[a]



	R	R'	%Yd. **9**
5R-**12** (**9**)	PhSe	CH_3	93
5S-**12** (**9**)	CH_3	PhSe	88

[a] Reagents: (a) 10% TFA/H_2O, THF, 0 °C; (b) $Et_3N{\cdot}3HF$, THF.

the ultimate radical precursor was facilitated by using single diastereomers of **9**. We also wanted to independently determine that the stereochemistry of the phenyl selenide precursor **4** did not affect the observed products. The diastereomers of **9** were separated as their respective 3′-*O*-*tert*-butyldimethylsilyloxy compounds (**12**), which were prepared from the previously reported bis-silyl protected substrate (**11**) using aqueous TFA in THF (Scheme 4).[20] Following removal of the remaining silyl group, the fully deprotected phenyl selenides **9** were coupled to the β-cyanoethyl phosphoramidite of 2′-deoxyuridine. Products corresponding to coupling at the 3′ hydroxyl group were not observed. Following cleavage of the β-cyanoethyl group from a diastereomeric mixture of phosphate triesters, the desired products were obtained as their sodium salts (Dowex Na^+).



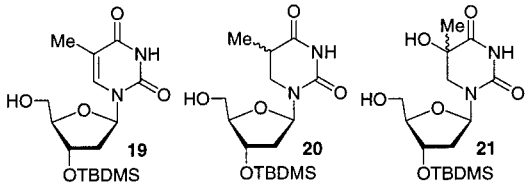
Solubility limitations necessitated that the anticipated products (**13**−**17**) be synthesized by coupling the respective 3′-silyloxy monomers (**19**−**21**) to either **18** or the β-cyanoethyl phosphoramidite of 2′-deoxyuridine. The requisite 3′-*O*-*tert*-butyldimeth-

(18) (a) Chatgilialoglu, C.; Ferreri, C.; Bazzanini, R.; Guerra, M.; Choi, S.-Y.; Emanuel, C. J.; Horner, J. H.; Newcomb, M. *J. Am. Chem. Soc.* **2000**, *122*, 9525. (b) Emanuel, C. J.; Newcomb, M.; Ferreri, C.; Chatgilialoglu, C. *J. Am. Chem. Soc.* **1999**, *121*, 2927.

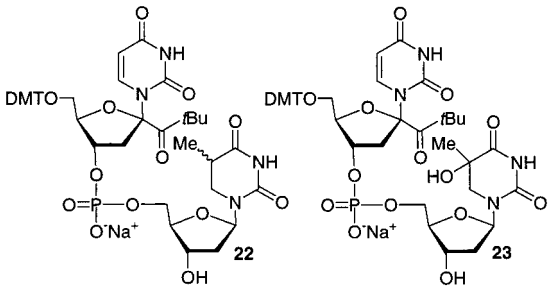
(19) (a) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859. (b) Matteucci, M. D.; Caruthers, M. H. *Tetrahedron Lett.* **1980**, *21*, 719.

(20) Robins, M. J.; Sarker, S.; Samano, V.; Wnuk, S. F. *Tetrahedron* **1997**, *53*, 447.

ylsilyloxy nucleosides **19**−**21** were obtained via selective desilylation of the bis-silyl-protected compounds as described above for **12**. 5,6-Dihydrothymidine (**20**) was coupled to the 2′-deoxyuridine phosphoramidite as a mixture of diastereomers (5*S*:5*R*, 3:2), which were obtained via epimerization of the dihydrothymidine mixture initially isolated upon hydrogenation (5*S*:5*R*, 9:1). Similarly, the analogous dinucleotide containing 5,6-dihydro-5-hydroxythymidine (**15**) was prepared using a diastereomeric mixture (5*R*:5*S*, 4:1) of the 3′-silyloxy derivative of thymidine C5 hydrate (**21**). The desirability of using a mixture of diastereomers enabled us to prepare the protected thymidine C5 hydrate **21** by a more expedient route than that previously reported.[21] The C5 hydroxyl group was incorporated by quenching the dianion used to prepare **11** with O_2. The coupling of the 3′-silyloxy-protected nucleosides **19**−**21** was carried out as described above (Scheme 3). Following deprotection of the mixture of phosphate triesters, the 3′-silyl groups were cleaved from the dinucleotides using $Et_3N{\cdot}3HF$. The workup of this reaction was crucial in order to prevent detritylation of the dinucleotides. Passing the crude reaction mixture through Amberlite anion-exchange resin was determined to be the most suitable method for neutralizing the reaction mixtures. Subsequent chromatography and passage through a cation-exchange column (Dowex Na^+) yielded the desired products as their sodium salts.



The lability of the 2-deoxyribonolactone (**8**) to alkaline conditions required that the independent synthesis of dinucleotide products containing this lesion (**16**, **17**) be carried out using a latent form of the lactone.[22] We and others have shown that 2-deoxyribonolactone can be produced photochemically from nucleoside analogues that are stable to the conditions of oligonucleotide synthesis/deprotection (Scheme 2).[16−18,23,24] The dinucleotides **22** and **23** containing the photochemical precursor to the 2-deoxyribonolactone and the diastereomeric mixtures of 5,6-dihydrothymidine (**20**) or thymidine C5 hydrate (**21**) were prepared as described above for **13**−**15**. The lactone-containing dinucleotides **16** and **17** were generated by aerobic photolysis (350 nm) of **22** and **23**, respectively, and purified by flash chromatography.

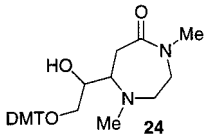


Although it was unnecessary to synthesize the diastereomers of **13** and **15**−**17** separately, quantitation of each isomer produced from the generation of **1** was desirable. No single solvent gradient was suitable for separating every product. Consequently, samples were analyzed by reverse-phase HPLC using two different gradients which had complementary advantages. The samples were first analyzed using CH_3CN as the organic solvent. These conditions allowed separation of (5*R*)-**15**, (5*R*)-**13**, and (5*S*)-**13**, as well as the diastereomers of **16** and **17**. However, (5*S*)-**15** and **14** coeluted under these conditions. To determine the yields of (5*S*)-**15** and **14**, the samples were analyzed with a second gradient program using MeOH as the organic cosolvent. These conditions resulted in coelution of (5*R*)-**15** and (5*S*)-**15**, which were resolved from **14**. Although (5*S*)-**15** was now separable from **14**, both diastereomers of **16** and **17** coeluted with **14** under these conditions. Since the total yield of **15** was determined using the MeOH gradient, and the yield of (5*R*)-**15** was determined from analysis under the CH_3CN conditions, the amount of (5*S*)-**15** formed was derived from the difference between these two measurements. Similarly, once the yield of (5*S*)-**15** was calculated, it could be subtracted from the total yield of (5*S*)-**15** and **14** that was obtained during the analysis of the samples under the CH_3CN conditions, providing the yield of the latter dinucleotide. In addition, conversion of starting material (**4**) and the yield of **24**, which is formed upon elimination from **16** and **17** in the presence of *N*,*N*′-dimethylethylenediamine, were determined using the CH_3CN gradient.

Table 1. Anaerobic Photolysis of Phenyl Selenide Dinucleotide (**4**)[a]

trap	yield (%)[b] **13**	yield (%)[b] **14**	(5*S*)-**13**: (5*R*)-**13**	mass balance (%)[b]
t-BuSH	38 ± 12	44 ± 9.0	1.5 ± 0.4	82 ± 13
β-mercaptoethanol	28 ± 3	42 ± 2	0.6 ± 0.1	70 ± 1

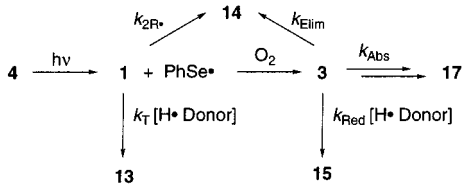
[a] [**4**] = 0.1 mM; [trap] = 1.1 mM; solvent, CH_3CN/H_2O, 1:1 by volume. [b] Reported yields and mass balances represent average yields from individual experiments ± the standard deviation measured from these averages.



Photolysis of 4 under Anaerobic Conditions. Product yields were found to depend strongly on the nature of the trap employed but, as expected, not on the stereochemistry at the C5 position of the phenyl selenide **4**. Consequently, all subsequent photolysis experiments employed a diastereomeric mixture of **4** which was enriched in the 5*R*-diastereomer (typically between 2:1 and 3:1, (5*R*)-:(5*S*)-**4**). When the dinucleotide **4** was photolyzed in the presence of *t*-BuSH or *β*-mercaptoethanol under degassed conditions, a significant amount of **14** was observed in addition to the product resulting from hydrogen atom donation to **1** (**13**, Table 1). The formation of **14** in the presence of thiol is consistent with relatively inefficient radical chain propagation (via reaction between thiyl radicals and **4**) when this trap is used, compared to Bu_3SnH.[17] Consequently, a larger fraction of **4** is decomposed by direct photolysis in the presence of thiol. This results in higher yields of thymidine-containing product **14**, which can be formed by reaction between **1** and the benzene selenyl radical ($k_{2R^\bullet}$, Scheme 5).

(21) Barvian, M. R.; Greenberg, M. M. *J. Org. Chem.* **1993**, *58*, 6151.
(22) (a) Kappen, L. S.; Goldberg, I. H. *Biochemistry* **1989**, *28*, 1027. (b) Urata, H.; Yamamoto, K.; Akagi, M.; Hiroaki, H.; Uesugi, S. *Biochemistry* **1989**, *28*, 9566.
(23) Kotera, M.; Bourdat, A. G.; Defrancq, E.; Lhomme, J. *J. Am. Chem. Soc.* **1998**, *120*, 11810.
(24) Hwang, J.-T.; Tallman, K. A.; Greenberg, M. M. *Nucleic Acids Res.* **1999**, *19*, 3805.

Scheme 5



The stereoselectivity of hydrogen atom donation was also dependent upon the nature of the hydrogen atom donor. Reaction of monomeric **1** with hydrogen atom donors produces 5,6-dihydrothymidine as a 1:1 mixture of diastereomers.[7a] In contrast, when **1** is generated in the dinucleotide, formation of (5*S*)-**13** is favored when *t*-BuSH is employed as a trap, presumably because attack from the *si* face of **1** is hindered by the 5′-*O*-dimethoxytrityl-2′-deoxyuridine nucleotide (Table 1). The opposite stereoselectivity is observed when *β*-mercaptoethanol reacts with **1**. Preferential attack from the more hindered face to form (5*R*)-**13** may be due to delivery of the hydrogen atom donor to the *si* face of **1** via hydrogen bonding of the thiol to the uracil moiety, and is discussed further below.

Probing Internucleotidyl Hydrogen Atom Abstraction by 1. Thermodynamic considerations led us to predict that if **1** abstracts hydrogen atom(s) from an adjacent nucleotide, abstraction from the C1′ and/or C4′ positions is most likely.[13] Addressing this possibility in oligonucleotides was limited by the necessity of such a process to give rise to direct strand scission or alkali-labile lesions in order to be detected by gel electrophoresis.[7] The absence of direct strand breaks under anaerobic conditions was sufficient to eliminate C4′ hydrogen atom abstraction by **1** as a possible pathway, because this process is known to result in elimination of the adjacent phosphate.[12b,25] However, the reliance on gel electrophoresis could allow hydrogen atom abstraction from the C1′ position to go undetected since alkali-labile lesions and direct strand breaks are produced inefficiently from C1′ radicals under anaerobic conditions when exogenous hydrogen atom donors are present.[16] Using the detritylated form of dinucleotide **4** as a mechanistic probe, we anticipated that we could employ the stereoselectivity of the reduction of 2′-deoxyuridin-1′-yl (**7**) as a means for detecting internucleotidyl hydrogen atom abstraction by **1**. Hydrogen atom donation to **7** by hydrogen atom donors (e.g., thiols) occurs preferentially from the *si* face to yield *β*-2′-deoxyuridine as the major product.[16b,26] The preference for this process to yield *β*-2′-deoxyuridine varies from 1.5 to 8.3, depending on the hydrogen atom donor and whether the radical is produced in the monomeric nucleoside, single-stranded DNA, or double-stranded DNA. We reasoned that α-2′-deoxyuridine formation resulting from thiol trapping of **7** would serve as a clear indication for internucleotidyl hydrogen atom abstraction by 5,6-dihydrothymidin-5-yl (**1**) in this system. α-2′-Deoxyuridine, which could have been observed in amounts as small as 2% relative to the *β*-anomer, was not detected when the crude photolysate of a sample of detritylated **4** that was irradiated in the presence of *β*-mercaptoethanol was subjected to enzymatic digestion.[16b] Using the observed stereoselectivity for reduction of monomeric 2′-deoxyuridin-1′-yl (**7**, $\beta{:}\alpha = 1.5$) and $(2.3-8.0) \times 10^6$ M^{-1} s^{-1} as the rate constant for reaction of **1** with *β*-mercaptoethanol, an upper limit for internucleotidyl hydrogen atom abstraction is estimated to be between 115 and 440 s^{-1} (eq 3).[18,26,27]

$$\frac{[\alpha\text{-dU}]}{[\beta\text{-dU}]} = \left(\frac{k_\alpha}{k_\alpha + k_\alpha}\right)\frac{k_{inter}}{k_H[RSH]} \quad (3)$$

Further attempts were made to detect hydrogen atom abstraction from the C1′ position of 2′-deoxyuridine by **1** using tetranitromethane as a mechanistic probe. Tetranitromethane is known to react with alkyl radicals, and in particular with reducing radicals such as 2′-deoxyuridin-1′-yl (**7**).[18a,28] We anticipated that if **7** was formed via hydrogen atom abstraction by **1**, reaction of tetranitromethane with the C1′ radical would yield 2-deoxyribonolactone (**8**). Hence, internucleotidyl hydrogen atom abstraction by **1** might be expected to yield **16** in the presence of tetranitromethane. However, **14** and **15** were the only products observed when **4** was irradiated under anaerobic conditions in the presence of this trapping agent. As discussed above, the former product may have arisen from reaction between the radical pair formed upon photolysis of **4**. The thymidine C5 hydrate-containing product **15** is believed to be formed via trapping of **1** by tetranitromethane, which is presumably much faster than the internucleotidyl process that was being probed for, and is consistent with the reactivity of other nucleobase radicals with nitroaromatic compounds.[29]

O_2-Dependent DNA Damage Amplification by 1. Previous studies suggested that hydroperoxyl radical elimination ($O_2^{-\bullet} + H^+$) and reduction of **3** by exogenous hydrogen atom donors would be competing processes for internucleotidyl hydrogen atom abstraction (Scheme 5).[17,30,31] Moreover, independent investigations of the reactivity of 5,6-dihydrothymidin-5-yl (**1**) and 2′-deoxyuridin-1′-yl (**7**) indicated that **17** should be expected from internucleotidyl C1′ hydrogen atom abstraction by the peroxyl radical **3** in the presence of a reducing agent.[6,7,16−18,24,26] As expected from studies on monomeric **1**, dinucleotides containing thymidine (**14**) and thymidine C5 hydrate (**15**) were observed under aerobic conditions, the former due at least in part to hydroperoxyl radical elimination from **3** (Table 2). Most significant is the observation of **17**, the product expected from the O_2-dependent DNA damage amplification process under reducing conditions (Scheme 1). The selective reduction of the diastereomeric peroxyl radicals to yield (5*S*)- and (5*R*)-**15** exhibited a dependence on hydrogen atom donor similar to that of the formation of **13** under anaerobic conditions (Table 3). Trapping of the diastereomeric peroxyl radicals by thiol competes with elimination to **14**. Formation of the 5*R*-diastereomer of **15** is favored by attack from the less hindered face of **3** by *t*-BuSH. In contrast, we believe that hydrogen bonding of *β*-mercaptoethanol to the uracil component of the dinucleotide results in preferential trapping of the diastereomeric peroxyl radical that yields (5*S*)-**15**. Inherent assumptions in this analysis are that O_2, which reacts with **1** at close to diffusion-controlled rates, produces **3** with little or no facial selectivity, and that the diastereomeric peroxyl radicals eliminate superoxide at comparable rates.

Although the identity of the signature product of DNA damage amplification (**17**) was corroborated via co-injection

(25) Giese, B.; Dussy, A.; Meggers, E.; Petretta, M.; Schwitter, U. *J. Am. Chem. Soc.* **1997**, *119*, 11130.
(26) Goodman, B. K.; Greenberg, M. M. *J. Org. Chem.* **1996**, *61*, 2.
(27) Newcomb, M. *Tetrahedron* **1993**, *49*, 1151.
(28) Eibenberger, J.; Schulte-Frohlinde, D.; Steenken, S. *J. Phys. Chem.* **1980**, *84*, 704.
(29) (a) Jagannadham, V.; Steenken, S. *J. Am. Chem. Soc.* **1988**, *110*, 2188. (b) Steenken, S.; Jagannadham, V. *J. Am. Chem. Soc.* **1985**, *107*, 6818.
(30) (a) Chatgilialoglu, C.; Timokhin, V. I.; Zaborovskiy, A. B.; Lutsyk, D. S.; Prystansky, R. E. *J. Chem. Soc., Chem. Commun.* **1999**, 405. (b) Chatgilialoglu, C.; Timokhin, V. I.; Zaborovskiy, A. B.; Lutsyk, D. S.; Prystansky, R. E. *J. Chem. Soc., Perkin Trans. 2* **2000**, 577.
(31) (a) Hildebrand, K.; Schulte-Frohlinde, D. *Int. J. Radiat. Biol.* **1997**, *71*, 377. (b) Schulte-Frohlinde, D.; Behrens, G.; Önal, A. *Int. J. Radiat. Biol.* **1986**, *50*, 103. (c) Chenier, J. H. B.; Furimsky, E.; Howard, J. A. *Can. J. Chem.* **1974**, *52*, 3682.

Table 2. Aerobic Photolysis of Phenyl Selenide Dinucleotide (**4**)[a]

trap	yield (%)[b] **13**	**14**	**15**	**17**	mass balance (%)[b]
β-mercapto-ethanol	4 ± 2	28 ± 17	33 ± 3	0.15 ± 0.10	65 ± 22
t-Butyl thiol	1.5 ± 1.5	33 ± 0.1	10.5 ± 0.5	0.13 ± 0.05	52 ± 1

[a] [**4**] = 0.1 mM; [trap] = 1.1 mM; solvent, CH_3CN/H_2O, 1:1 by volume. [b] Reported yields and mass balances represent average yields from individual experiments ± the standard deviation measured from these averages.

Table 3. Stereoselectivity for the Formation of **15** and **17** from the Phenyl Selenide Dinucleotide[a]

trap	(5*S*)-**15**:(5*R*)-**15**[b]	(5*S*)-**17**:(5*R*)-**17**[b]
β-mercaptoethanol	2.4 ± 1.0	6.4 ± 0.1
tert-butyl thiol	0.9 ± 0.2	*c*

[a] [**4**] = 0.1 mM; [trap] = 1.1 mM; solvent, CH_3CN/H_2O, 1:1 by volume. [b] Reported selectivities represent averages from individual experiments ± the standard deviation measured from these averages. [c] The amount of (5*R*)-**17** was below the limit of detection.

on reverse-phase HPLC with independently synthesized material, further evidence for its formation was desired due to the small amount of product formed during the photolysis of **4**. This evidence was obtained by taking advantage of a recent study in which oligonucleotides containing 2-deoxyribonolactone were shown to give rise to condensation products with nucleophiles after undergoing β-elimination to the butenolide.[24] In this previous study, reaction of *N*,*N*′-dimethylethylenediamine and 5-*O*-dimethoxytritylated furanone produced **24**. An analogous butenolide trapping product was detected upon reaction of 2-deoxyribonolactone in an oligonucleotide with this same diamine. Treatment of a crude photolysate of **4** with *N*,*N*′-dimethylethylenediamine yielded **24** (confirmed via co-injection with independently synthesized material) in a yield comparable (0.41 ± 0.15%) to that in which **17** was observed. Taken together, these results confirm that the lactone-containing product **17** is produced upon generation of **1** under aerobic conditions in the presence of exogenous reducing agents, and provides strong evidence for the previously proposed O_2-dependent DNA damage amplification mechanism (Scheme 1).[7b,c]

The relative yields of thymidine C5 hydrate (**15**)- and thymidine (**14**)-containing dinucleotides compared to lactone product indicate that the rate constant for internucleotidyl hydrogen atom abstraction by **3** is modest. A more quantitative value for the facility of this process was estimated by making assumptions regarding rate constants for the reactions that compete with internucleotidyl hydrogen atom abstraction (Scheme 5). The approximate rate constant obtained using the competition between hydroperoxyl radical elimination to yield **14** (k_{elim}) and internucleotidyl hydrogen atom abstraction (k_{abs}) to form **17** is estimated to be $k_{abs} \approx 0.1-0.3\ s^{-1}$.[32] Estimating the magnitude of k_{abs} by using the competition with peroxyl radical reduction (k_{red}) is even less exact, due to the range of rate constants available for reaction between alkylperoxyl radicals and thiols ($(0.4-5.0) \times 10^3\ M^{-1}\ s^{-1}$).[31] The measured product ratios ([**15**]:[**17**], Table 3) and literature values for k_{red} suggest as an upper limit $k_{abs} \approx 0.1\ s^{-1}$.[33] These rate constants are approximated using only a single concentration of exogenous hydrogen atom donor (in effect, a single data point) and should be considered to be crude estimates. Given the low yields of internucleotidyl hydrogen atom abstraction product **17** and the desire to carry out experiments under pseudo-first-order reaction conditions in hydrogen atom donor, it was not deemed practical to carry out the competition experiments at a variety of thiol concentrations.

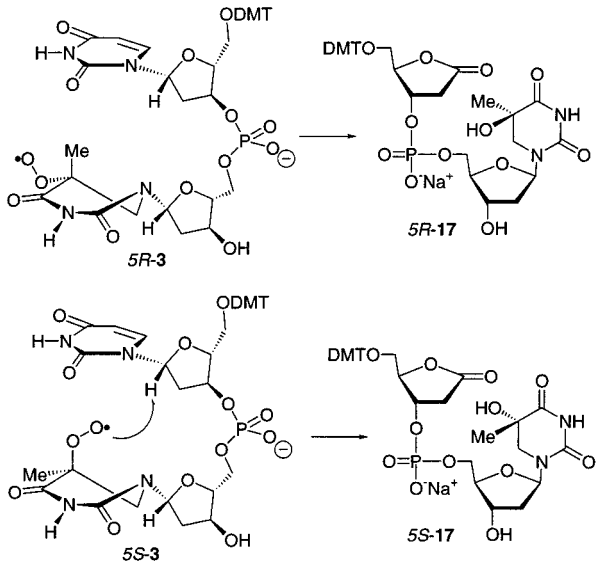


Figure 1. Illustration of the greater proximity of the peroxyl radical in (5*S*)-**3** to the C1′ hydrogen of the adjacent nucleotide compared to the epimeric peroxyl radical ((5*R*)-**3**).

Furthermore, given the number of assumptions involved in arriving at these approximate rate constants, it is quite possible that the difference between the independent estimates is insignificant. The small rate constant and corresponding low yield of product resulting from internucleotidyl hydrogen atom abstraction caused us to consider the possibility that **17** was formed via an alternative pathway, such as one involving a diffusible species. Although precautions were taken to eliminate metal ions in the reaction, we addressed the possibility that superoxide released during the formation of **14** was transformed via Haber−Weiss and Fenton chemistry into hydroxyl radical. The hydroxyl radical could subsequently abstract the C1′ hydrogen from the 2′-deoxyuridine of a molecule of **15** that was produced independently from **4**. This possibility was eliminated by the absence of an effect by mannitol (a known hydroxyl radical scavenger) on the yield of **17**.[3a] Moreover, further evidence in support of the intramolecular mechanism was gleaned from photolyses carried out in the presence of a competing dinucleotide (TpT). The yield of **17** was unchanged when **4** (0.1 mM) and β-mercaptoethanol (1.1. mM) were photolyzed in the presence of 0.1 mM (yield of **17** = 0.15 ± 0.10%) or 0.2 mM (yield of **17** = 0.21 ± 0.10%) TpT.

The observed stereoselectivity for formation of **17** is also consistent with the mechanism involving internucleotidyl C1′ hydrogen atom abstraction by a selective nucleobase peroxyl radical (Table 3). Formation of **17** containing the 5*S*-diastereomer of thymidine C5 hydrate is significantly favored over that of the dinucleotide containing the epimeric modified thymidine. This is consistent with the expected better proximity of the 5*S*-peroxyl radical ((5*S*)-**3**) to the C1′ hydrogen atom of the deoxyuridine bonded to its 5′-phosphate than that of the 5*R*-peroxyl radical (Figure 1), and is clearly visible in a three-dimensional model of detritylated (5*S*)-**3**.[34] The 5*R*-peroxyl

(32) The value estimated for k_{elim} (23.4 s^{-1}) is slightly lower than that reported previously (see ref 17). This change is made in order to accommodate the recently measured rate constant for the reaction of the cumyl peroxyl radical with Bu_3SnH ($(1.6-1.8) \times 10^3\ M^{-1}\ s^{-1}$; see ref 30) which is used as an approximation for k_{red} in Scheme 5.

(33) In these calculations, it is assumed that transformation of the initially trapped peroxyl radical (**3**) by thiol to **15** and transformation of the radical formed upon internucleotidyl hydrogen atom abstraction to **17** occur with equal efficiency.

(34) See Supporting Information.

radical ((5*R*)-**3**) is drawn in a pseudoequatorial conformation (Figure 1), because this arrangement of the dihydropyrimidine ring brings the peroxyl radical marginally closer to the 5′-nucleotide. The (5*R*)-**3** radical must adopt the syn conformation in order to bring the radical center into close proximity of the 5′-adjacent nucleotide's C1′ hydrogen atom. Although the barrier to such a conformational change in this molecule is unknown, UV-melting studies, NMR experiments, and molecular dynamics simulations on related dihydropyrimidine molecules ((5*R*)-thymidine C5 hydrate and 2′-deoxy-5,6-dihydrouridine) in DNA are consistent with these molecules existing in DNA as the respective anti isomers.[35−38]

Conclusions

These experiments provide additional evidence for the formation of tandem lesions from a nucleobase radical (5,6-dihydrothymidin-5-yl, **1**) that is produced in DNA by γ-radiolysis. Independent synthesis of putative products and independent generation of **1** in a dinucleotide provides further substantiation for the requirement of O_2 in the amplification of DNA damage that is initiated by the formation of this radical. Product analyses suggest that the 5*S*-peroxyl radical (**3**) is more efficient at effecting internucleotidyl hydrogen atom abstraction than its epimer. This result is not surprising, based upon the assumptions that the peroxyl radicals exist predominantly in the anti conformation (Figure 1) and that in the current experiment there is no 2′-deoxynucleotide bonded to the 3′ position of **1**. The estimated rate constant for the process is modest, and rate constants are not available for comparable bimolecular reactions between nucleosides and peroxyl radicals in order to estimate the effective molarity of the substrate in this system. However, if one uses the reaction between THF and *t*-BuOO$^{\bullet}$ as a rough comparison ($k_{H^{\bullet}\,abs} = 0.085\ M^{-1}\ s^{-1}$), the effective molarity of the abstracted hydrogen atom in **3** is between 1.2 and 3.4 M, depending upon which estimate of k_{abs} is employed (Scheme 5).[39] The rate constant for internucleotidyl hydrogen atom abstraction (k_{abs}) estimated in this work may be considered a lower limit for the comparable process in duplex DNA due to the absence of significant secondary structure in the dinucleotide substrate. It is possible that internucleotidyl hydrogen atom abstraction will be faster in duplex DNA, where the more rigid secondary structure will increase the effective molarity of the nucleotide bonded to the 5′-phosphate of **1**, resulting in higher yields of lesions analogous to **17**. However, it is interesting to note that the yield of tandem lesion **17** observed in this structurally minimal system (**4**) is consistent with those estimated in plasmid DNA for the formation of similar lesions.[40]

Experimental Section

General Procedure for the Photolysis of 4. Samples containing **4** (0.1 mM) and the appropriate trap (1.1 mM) were prepared in CH_3CN/H_2O (1:1 by volume) having a final volume of 500 μL. Anaerobic samples were degassed using the freeze−pump−thaw method (four 3-min cycles), and aerobic samples were left open to the atmosphere. The samples were photolyzed at 350 nm in a Rayonet photoreactor for the appropriate time.

Upon completion of the reaction, the samples were transferred to Eppendorf tubes (1.5 mL) and lyophilized. The residue was taken up in benzophenone (10 μL) and CH_3CN/NH_4HCO_3 buffer (90 μL, 25 mM, pH 6.8, 1:1 by volume). Samples containing tin were extracted with hexanes (3 × 200 μL). The samples were analyzed by reverse-phase (C8) HPLC using a gradient of NH_4HCO_2 (0.2 M, pH 6.2) and either CH_3CN (method A) or MeOH (method B) at a flow rate of 1.0 mL/min. Complete resolution was not obtained under a single gradient program. Therefore, the samples were analyzed using both methods A (Supporting Information, Table 1) and B (Supporting Information, Table 2). Typically, 50 μL of the sample was injected using an AUFS of 1.0 and monitored at 240 nm.

The products were quantitated using the calculated response factors listed below with benzophenone as the internal standard (Supporting Information, Table 3). The yields of (5*R*)-**15**, (5*R*)-**13**, (5*S*)-**13**, (5*R*)-**16**, (5*S*)-**16**, (5*R*)-**17**, (5*S*)-**17**, and **24** and conversion of **4** were determined using method A. Under these conditions, (5*S*)-**15** and **14** coelute. Therefore, the samples were also analyzed by method B, in which (5*R*)-**15** and (5*S*)-**15** coelute but are separable from **14**. The yield of (5*S*)-**15** was obtained by subtracting the yield of (5*R*)-**15** determined using method A from the total yield found in method B. The yield of **14** was determined by subsequently subtracting (5*S*)-**15** from the total yield of **14** + (5*S*)-**15** determined in method A.

Anaerobic Photolysis of Detritylated 4 and Subsequent Enzymatic Digestion. Samples containing detritylated **4** (0.1 mM) and *t*-BuSH (1.1 mM) in CH_3CN/H_2O (500 μL, 1:1 by volume) were degassed and photolyzed at 350 nm (96 W) for 1 h. The samples were transferred to Eppendorf tubes and lyophilized. One set of samples was taken up in a solution of 2′-deoxycytidine (10 μL) and H_2O (90 μL) and analyzed directly by reverse-phase (C18) HPLC. The second set was subjected to enzymatic digest. The residue was taken up in buffer (86 μL, Tris-HCl, pH 7.9, 50 mM; NaCl, 100 mM; DTT, 1 mM) and $MgCl_2$ (100 mM). Nuclease P1 (10 μL, 0.3 unit/μL), snake venom phosphodiesterase (2 μL, 0.003 unit/μL), and calf intestine alkaline phosphatase (2 μL, 10 units/μL) were added to the sample and incubated at 37 °C for 12 h. 2′-Deoxycytidine (10 μL) was added to the sample and analyzed by reverse-phase (C18) HPLC using method C (Table 4). The sample was analyzed at 205 nm at an AUFS of 1.0 with dC as the internal standard. The response factors listed below were estimated on the basis of the extinction coefficients of dU and dC at 205 nm (Supporting Information, Table 5).

Anaerobic Photolysis of 4 in the Presence of Tetranitromethane. Samples containing **4** (0.15 mM), tetranitromethane (1.04 mM), and βME (1.4 mM) were prepared in CH_3CN/NH_4HCO_2 buffer (25 mM, pH 6.8, 1:1 by volume) with a final volume of 500 μL. The samples were degassed using the freeze−pump−thaw method (four 3-min cycles) and photolyzed at 350 nm (96 W) for 1 h. The samples were transferred to Eppendorf tubes and lyophilized. The residue was taken up in benzophenone (10 μL) and CH_3CN/NH_4HCO_2 buffer (90 μL, 25 mM, pH 6.8, 1:1 by volume) and analyzed by reverse-phase (C8) HPLC using methods A and B (Supporting Information, Tables 1 and 2), as discussed above.

Aerobic Photolysis of 4 in the Presence of Mannitol. Samples containing **4** (0.10 mM) and mannitol (1.1 mM) were prepared in CH_3CN/H_2O (500 μL, 1:1 by volume) and photolyzed at 350 nm (96 W) for 1 h. The samples were worked up as above and analyzed by reverse-phase (C8) HPLC using method A (Supporting Information, Table 1) for formation of **17**.

See Supporting Information for synthetic procedures.

Acknowledgment. Financial support of this work from the National Institutes of Health (GM-54996) is greatly appreciated. We thank Brian C. Bales and K. Nolan Carter for technical assistance, and the reviewers for helpful comments.

Supporting Information Available: Procedures for the synthesis of all dinucleotides used in this study, and three-dimensional structures of detritylated **1**, (5*S*)-**3**, and (5*R*)-**3** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA010180S

(35) Sambandam, A.; Greenberg, M. M. *Nucleic Acids Res.* **1999**, *27*, 3597.

(36) Villanueva, J. M.; Pohl, J.; Doetsch, P. W.; Marzilli, L. G. *J. Am. Chem. Soc.* **1999**, *121*, 10652.

(37) Grand, A.; Cadet, J. *Acta Crystallogr.* **1978**, *B34*, 1524.

(38) Miaskiewicz, K.; Miller, J.; Ornstein, R.; Osman, R. *Biopolymers* **1995**, *35*, 113.

(39) Howard, J. A. In *The Chemistry of Free Radicals Peroxyl Radicals*; Alfassi, Z. B., Ed.; John Wiley: New York, 1997.

(40) Milligan, J. R.; Aguilera, J. A.; Nguyen, T.-T. D.; Ward, J. F.; Kow, Y. W.; He, B.; Cunningham, R. P. *Radiat. Res.* **1999**, *151*, 334.