VOLUME 120, NUMBER 20
MAY 27, 1998

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Release of Superoxide from Nucleoside Peroxyl Radicals, a Double-Edged Sword?

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Received September 11, 1997

Abstract: 5,6-Dihydrothymidin-5-yl (**1**) and 2′-deoxyuridin-1′-yl (**3**) were independently generated in solution under aerobic conditions. The release of superoxide ($O_2^{\bullet-}$) from the respective peroxyl radicals derived from **1** and **3** was determined spectrophotometrically. Competition studies enable one to estimate that the rate constant for elimination of $O_2^{\bullet-}$ from the peroxyl radical (**4**) derived from **3** is $\sim$1 s^{-1}. This process is competitive with the anticipated rate of trapping of **4** in DNA by glutathione. Relative rate studies indicate that $O_2^{\bullet-}$ generation resulting from the formation of **1** under aerobic conditions competes effectively with trapping of the peroxyl radical by Bu_3SnH. Superoxide elimination from the peroxyl radical of **1** (**2**) restores the damaged nucleoside to its unaltered form, implying that this reactive intermediate has a naturally occurring detoxification pathway available to it. However, the freely diffusible superoxide can react further to generate other reactive species capable of damaging nucleic acids, suggesting that the elimination of $O_2^{\bullet-}$ from **2** is a potential double-edged sword.

Alkylperoxyl radicals (ROO•) are involved in a variety of chemical processes that result in the degradation of biomolecules. Although the reactivity of alkylperoxyl radicals as chain carriers in lipid autoxidation has been rigorously characterized, the role of these reactive intermediates in nucleic acid damage is less understood.[1,2] The importance of ROO• in nucleic acid damage is reflected in the observation that the presence of O_2 during γ-radiolysis of DNA results in an approximately 3-fold enhancement in damage.[2a] Trapping of the myriad of alkyl radicals produced in this mechanism by O_2 has been referred to as a damage-fixing event. In some instances, the resulting peroxyl radicals are believed to contribute to further DNA damage by abstracting hydrogen atom(s) from the carbohydrate backbone of the biopolymer.[3] Evidence for this brand of DNA damage amplification has been reported for peroxyl radical **2**, which was produced at defined sites within single-stranded oligonucleotides from a modified nucleotide (eq 1).[4] In contrast, the trapping of alkyl radicals by thiols is generally considered to be a protective process which can compete with the fixing of DNA damage by ROO• formation. Using generic rate constants for the trapping of alkyl radicals by O_2 and by thiols (in water), these two competing chemical processes are expected to account for a significant fraction of the reactivity of DNA radicals.[5] In fact, independent studies on 2′-deoxyuridin-1′-yl (**3**) produced at defined sites in oligonucleotides revealed that physiologically relevant levels of thiol (5 mM) compete with

(1) (a) Porter, N. A. In *Organic Peroxides*; Ando, W., Ed.; John Wiley & Son, Ltd.: Chichester, England, 1992. (b) Porter, N. A.; Mills, K. A.; Carter, R. L. *J. Am. Chem. Soc.* **1994**, *116*, 6690. (c) Porter, N. A.; Mills, K. A.; Caldwell, S. E.; Dubay, G. R. *J. Am. Chem. Soc.* **1994**, *116*, 6697.

(2) (a) von Sonntag, C. *The Chemical Basis of Radiation Biology*; Taylor and Francis: London, 1987. (b) Breen, A. P.; Murphy, J. A. *Free Radical Biol. Med.* **1995**, *18*, 1033.

(3) (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.

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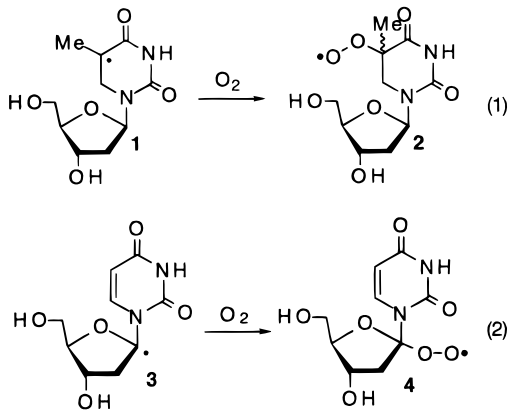
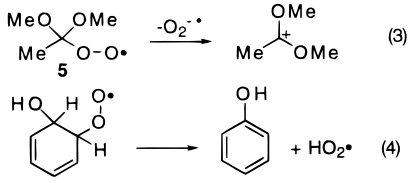
S0002-7863(97)03200-9 CCC: $15.00
Published on Web 04/29/1998



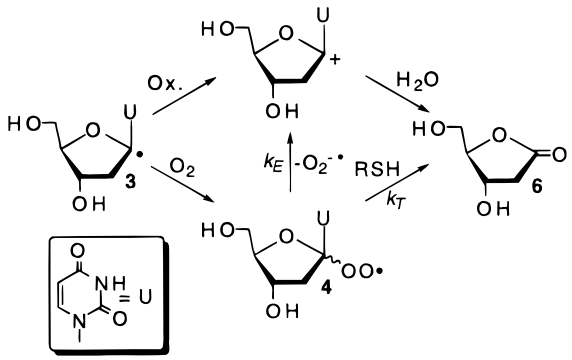
O_2 (0.2 mM) for **3**.[6] As with most generalizations, there are exceptions to these principles. The formation of premutagenic α-nucleotides via trapping of the respective nucleotide radical by thiols is contrary to the role that these hydrogen atom donors play as protecting agents.[7] Similarly, one could ask whether the formation of an alkylperoxyl radical is always a damage-fixing event. Herein, we describe experiments on two nucleoside radicals (**1**, **3**) that address this question.

Alkylperoxyl radicals can undergo heterolytic cleavage to form a carbocation and a molecule of superoxide ($O_2^{\bullet-}$).[8] The rate constant of this dissociative pathway is highly dependent upon the stability of the incipient carbocation. Simple alkyl-substituted peroxyl radicals (ROO•) eliminate superoxide with rate constants that are too small to compete with their trapping by thiols, or possibly even less activated intramolecular sources of hydrogen atoms, or their bimolecular self-reactions. Heteroatom substitution of the carbon atom bearing the peroxyl moiety significantly increases the rate of superoxide elimination. The ROO• derived from the dimethyl acetal of acetaldehyde (**5**) eliminates $O_2^{\bullet-}$ at pH 5 with a rate constant ($6.5 \times 10^4\ s^{-1}$) that should enable this unimolecular process to compete effectively with trapping by physiologically relevant levels of thiol (5−10 mM) (eq 3).[2a,9] An alternative unimolecular decomposition pathway is available to ROO• containing a hydrogen atom in the β-position of the molecule. Given a suitable thermodynamic driving force, such as rearomatization (eq 4), the elimination of hydroperoxy radical (HOO•), which

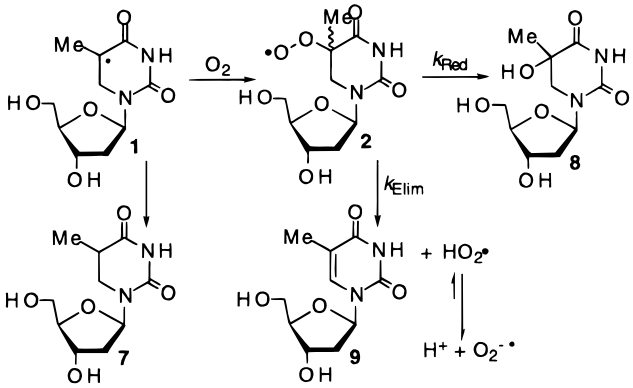


essentially completely dissociates under neutral conditions, can occur with a rate constant greater than $10^5\ s^{-1}$.[8] The viability of such processes in nucleobase radical chemistry has been investigated. Support for an analogous reaction in studies on

Scheme 1



Scheme 2



dihydrouracil was obtained, but not for the peroxyl radical derived from 5,6-dihydrothymin-5-yl.[10]

Nucleoside peroxyl radicals **2** and **4** are candidates for the above homolytic and heterolytic processes, respectively. Elimination of superoxide from **4**, followed by trapping by H_2O, yields the same deoxyribonolactone product (**6**, Scheme 1) that one would obtain from its reduction by thiol. When formed within a biopolymer, the deoxyribonolactone (**6**) represents an alkaline labile lesion.[11] Hence, elimination of $O_2^{\bullet-}$ from **4** would not result in a deviation from the principle of O_2 trapping as a lesion-fixing event. However, elimination of HOO• from **2** reconstitutes thymidine (Scheme 2). Considering that **2** is derived from a DNA lesion (**1**), elimination of $O_2^{\bullet-}$ could be defined as a naturally occurring detoxification pathway, and would represent an exception to the principle of O_2 fixation of lesions.

In the above processes, only the fate of the peroxyl radicals has been considered in the context of the nucleoside components. The other product of these reactions is $O_2^{\bullet-}$, which in itself is not deleterious to nucleic acids. However, in the presence of redox active metal ions, $O_2^{\bullet-}$ is transformed into the highly damaging hydroxyl radical.[2,12] In addition, in the presence of nitric oxide (NO), $O_2^{\bullet-}$ forms peroxy nitrite (ONO_2^-) which selectively damages deoxyguanosine in DNA.[13] Hence, depending upon the source of the $O_2^{\bullet-}$ (e.g., **4**) and the reaction

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(12) (a) Walling, C. *Acc. Chem. Res.* **1975**, *8*, 125. (b) Haber, F.; Weiss, J. *Proc. R. Chem. Soc. London, Sect. A* **1934**, *147*, 332. (c) Fenton, H. J. H. *J. Chem. Soc.* **1894**, *65*, 899.

(13) Tamir, S.; Burney, S.; Tannenbaum, S. R. *Chem. Res. Toxicol.* **1996**, *9*, 821.

Table 1. Generation and Reactivity of **1** under Anaerobic Conditions[a]

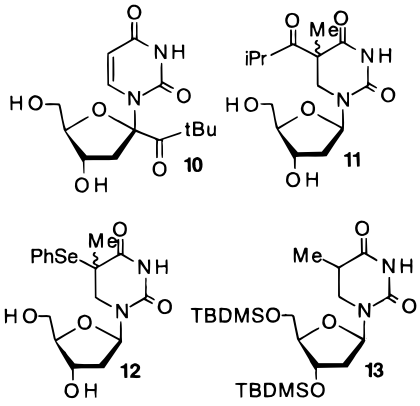
method of gen of **1**	**7**	% yield **8**	**9**	% conv	% mass balance
hν	51 ± 4	2 ± 1	17 ± 3	96 ± 2	74 ± 6
hν[b]	78 ± 4	3 ± 0	1 ± 0.1	100	81 ± 4
Δ	86 ± 5	1 ± 1	0.5 ± 0.1	100	88 ± 6
Δ[b]	93 ± 0	1 ± 1	0.7 ± 0.1	100	94 ± 1

[a] [**12**] = 0.1 mM; [Bu_3SnH] = 1.7 mM. [b] [$(Bu_3Sn)_2$] = 0.2 mM.

conditions, the elimination of $O_2^{\bullet-}$ could result in further DNA damage. The participation of $O_2^{\bullet-}$ in DNA damage processes makes its elimination from nucleoside peroxyl radicals (in particular **2**) a potential double-edged sword.

Results and Discussion

Methods for Nucleoside Radical Generation. The efficient generation of **3** via Norrish type I photocleavage of **10** was described previously.[14] No evidence for singlet oxygen ($^1O_2$) formation during UV irradiation of an oligonucleotide containing **10** was detected. This is fully consistent with the short lifetime for the excited state of **10** detected by Stern−Volmer analysis. Norrish type I photocleavage of **11** was considerably less efficient, and irradiation of **11** resulted in the production of $^1O_2$.[4] Consequently, phenyl selenide **12** was designed as a potential photochemical and/or thermal precursor to **1**. The synthesis of **12** was completed in a straightforward manner starting from bis-silyl-protected dihydrothymidine (**13**), as previously described for the synthesis of **11**.[4] Although the diastereomers of **12** were separable, this proved to be unnecessary for the experiments described below.

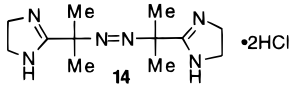


Phenyl selenide **12** proved to be a versatile precursor to **1**. Photolysis of **12** under anaerobic conditions resulted in a moderate yield of 5,6-dihydrothymidine (**7**) as a 1:1 mixture of diastereomers (Table 1). The reactions were carried out in a mixture of THF and H_2O (D_2O) (1:1 by volume) in order to strike a balance between the solubility requirements of the tin hydride and the desire to carry out the experiments in an aqueous environment.[15] Employing an aqueous solvent system makes extrapolation of the experimental results to biological systems more reasonable. In addition, utilizing D_2O in conjunction with a nonexchangeable hydrogen atom donor (Bu_3SnH) provides an additional mechanistic handle regarding the origin of **7**. Deuterated **7** is indicative of involvement of a nonradical pathway and/or alternative hydrogen atom donor (e.g., benzeneselenol). Only trace amounts of 5,6-dihydro-5-hydroxythymidine (**8**) are observed under degassed conditions.[16] We believe that **8** is produced as a result of trace amounts of O_2 that are not removed during the freeze−pump−thaw degas cycles. Anaerobic photolysis of **12** in the absence of $(Bu_3Sn)_2$ yields significant amounts of thymidine (**9**), which are reduced to trace amounts when photolyses are carried out in the presence of the ditin reagent. The majority of **9** produced under these conditions is believed to result from a side reaction involving the disproportionation of two radicals, presumably the radical pair produced upon direct irradiation of **12**. Minor amounts of **9** may also result from an O_2 dependent pathway which will be discussed in detail below. The significant reduction in the amount of **9** produced upon photolysis of **12** in the presence of $(Bu_3Sn)_2$ is attributed to the more efficient photoinitiation of radical chain reactions involving $Bu_3Sn^{\bullet}$ as the chain transfer agent. Thermolysis (37 °C) of **12** in THF−D_2O (1:1 by volume) under anaerobic conditions using a water soluble azo initiator (**14**) in the presence of Bu_3SnH produces high yields of 5,6-

Table 2. Generation and Reactivity of **1** under Aerobic Conditions[a]

method of gen of **1**	% yield **8**	% yield **9**	**9:8**	% conv	% mass balance
hν	8 ± 1	48 ± 8	6 ± 2	95 ± 7	62 ± 5
hν[b]	12 ± 1	51 ± 1	4 ± 1	100	64 ± 3
Δ	4 ± 1	28 ± 3	7 ± 2	28 ± 7	103 ± 6
Δ[b]	4 ± 1	34 ± 7	9 ± 4	30 ± 16	108 ± 7

[a] [**12**] = 0.1 mM; [Bu_3SnH] = 1.7 mM. [b] [$(Bu_3Sn)_2$] = 0.2 mM.



dihydrothymidine (**7**) as a 1:1 mixture of diastereomers (Table 1). The thermolysis conditions produce only trace amounts of **8** and **9** regardless of whether $(Bu_3Sn)_2$ is present, consistent with the expected efficient chain reactions involving $Bu_3Sn^{\bullet}$. Mass spectral analysis of bis-silylated dihydrothymine, produced via formic acid hydrolysis and subsequent persilylation of crude reaction mixtures, confirms that the majority of **7** formed under anaerobic conditions is due to trapping of **1** by Bu_3SnH. Regardless of the reaction conditions (see Table 1), the deuterium content of dihydrothymine was never greater than 8%. The deuterated dihydrothymine detected could be attributed to small amounts of benzeneselenol produced during the reactions.

Dihydrothymidine (**7**) is absent from product mixtures resulting from decomposition of **12** under aerobic conditions. This is consistent with the strongly favored trapping of **1** by O_2 in competition with Bu_3SnH, resulting in the formation of a 1:1 diastereomeric mixture of 5,6-dihydro-5-hydroxythymidine (**8**, Table 2).[16] When **12** is decomposed under aerobic conditions in $H_2{}^{18}O$, none of the heavier isotope is incorporated in **8**, indicating that O_2 is the sole source of the hydroxyl group in this product. Although thermolyses were carried out using stoichiometric initiator (**14**), the extent of conversion of **12** was significantly reduced from that achieved under anaerobic conditions (100%), or upon photolysis.[17] This is presumably due to O_2 quenching of radical chain initiation and propagation. It is important to note that the ratio of **9** to **8** is significantly greater than one in the presence of low, but pseudo-first-order,

(14) Goodman, B. K.; Greenberg, M. M. *J. Org. Chem.* **1996**, *61*, 2.
(15) Experiments carried out using a water soluble tin hydride reagent (tris[3-(2-methoxyethoxy)propyl]stannane) complicated the product analysis, and will be reported on at a later date. For the synthesis of this water soluble tin hydride reagent see: Light, J.; Breslow, R. In *Organic Syntheses*; Coffen, D. L., Ed.; Wiley: New York, 1994; Vol. 72, p 199.
(16) Barvian, M. R.; Greenberg, M. M. *J. Org. Chem.* **1993**, *58*, 6151.
(17) Mayer, S.; Prandi, J. *Tetrahedron Lett.* **1996**, *37*, 3117.

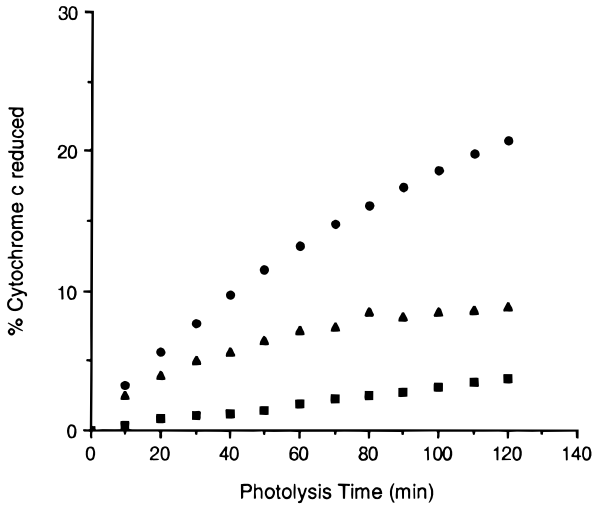


Figure 1. Cytochrome *c* reduction upon photolysis of (●) **12** (50 μM) in the presence of cyt *c* (50 μM), EDTA (0.1 mM), and catalase (12 μg), (▲) **12** (50 μM), cyt *c* (50 μM), EDTA (0.1 mM), catalase (12 μg), and superoxide dismutase (5 units), and (■) cyt *c* (50 μM), EDTA (0.1 mM), and catalase (12 μg).

concentrations of Bu_3SnH. This indicates that the formation of thymidine (**9**) is not due to a depletion of the hydrogen atom donor.

Hydroperoxyl Radical (Superoxide) Elimination from 2. The large amount of thymidine (**9**) formed during decomposition of **12** under aerobic conditions is consistent with the elimination of HOO$^\bullet$ from **2** (Scheme 2). The formation of thymidine (**9**) from **2** is drawn as a single-step homolytic process, rather than a two-step process involving loss of $O_2^{\bullet-}$ followed by deprotonation. This proposal is based upon the expected instability of the intermediate carbocation α to the carbonyl moiety. Furthermore, the absence of any ^{18}O incorporation in **8** from water (~27.7 M) does not support the two-step heterolytic process. Admittedly, a two-step heterolytic process cannot definitively be ruled out, as the same labeling result would be obtained if deprotonation overwhelmed hydration. The possible involvement of a carbocation does not alter the discussion below. The low pK_a (4.8) of HOO$^\bullet$ ensures that $O_2^{\bullet-}$ will be the ultimate product whether the concerted process (as shown in Scheme 2) or two-step process is operative.

Evidence for the release of $O_2^{\bullet-}$ from **2** was obtained using a spectrophotometric assay involving the reduction of cytochrome *c*.[18] The reduced form of cytochrome *c* possesses a strong chromophore at 550 nm. Irradiation of **12** (0.05 mM) in the presence of 1 equiv of cytochrome *c* results in steady growth of the reduced product (Figure 1). Photolysis of **12** in the presence of superoxide dismutase (5 units) quenches 74.3% of the amount of cytochrome *c* reduction, supporting the contention that $O_2^{\bullet-}$ is responsible for the observed reaction. Additional superoxide dismutase (100 units, data not shown) results in no further reduction in the amount of reduced cytochrome *c*. We suggest that the remainder of the reduced product is due to a superoxide independent process involving the phenylselenyl group released upon photolysis. The amount of cytochrome *c* reduced that is attributable to $O_2^{\bullet-}$ corresponds to 36% of the observed thymidine (as determined by reversed-phase HPLC). Considering that more than one-third of the thymidine formed under aerobic photolysis of **12** is attributable to radical cage processes (see Tables 1 and 2), more than half

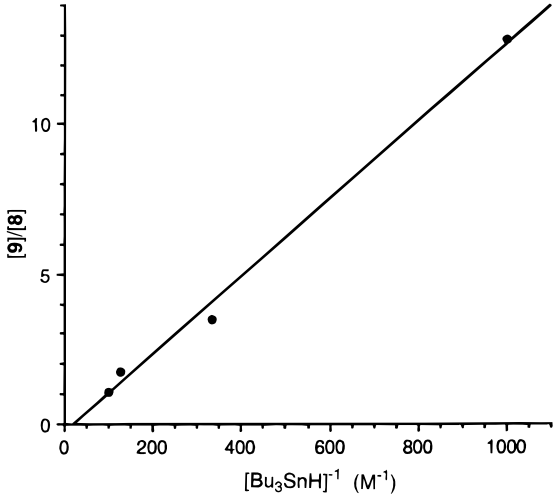


Figure 2. Ratio of thymidine (**9**) versus 5,6-dihydro-5-hydroxythymidine (**8**) formed upon photolysis of **12** (50 μM) as a function of $[Bu_3SnH]^{-1}$. The slope of the line = $k_{Elim}/k_{Red} = 1.3 \times 10^{-2}$ M.

of the expected amount of $O_2^{\bullet-}$ formed is accounted for by this spectrophotometric assay. We believe that the remainder of the $O_2^{\bullet-}$ released upon formation of thymidine is consumed by disproportionation. These observations were qualitatively confirmed using the oxidation of epinephrine to adrenochrome (λ_{max} = 480 nm) by $O_2^{\bullet-}$.[19,20]

To gauge the facility of the formal elimination of $O_2^{\bullet-}$ from **2**, the ratio of **9** to **8** was determined as a function of Bu_3SnH concentration (Figure 2). The tin hydride was present in pseudo-first-order concentration in all experiments. In contrast to the data reported above (Table 2), these experiments were carried out at 55 °C in a mixture of THF−H_2O (19:1 by volume) in order to ensure reduction of the hydroperoxide to **8** and complete solubility of Bu_3SnH over its entire concentration range. The ratio of **9** to **8** (12.8 ± 1.9) formed in the presence of the Bu_3SnH (1 mM) under these less polar solvent conditions is slightly larger than the respective ratio of products formed under comparable reaction conditions at 37 °C in 1:1 THF−H_2O in the presence of 1.7 mM tin hydride (Table 2). Due to the alteration of more than one experimental variable (trap concentration, solvent, temperature), the significance of solvent and/or temperature effects on these reactions from this single comparison will not be overinterpreted. However, the change in the ratio of **9** to **8** is consistent with the expected reduction in k_{Red} (Scheme 2) with decreasing solvent polarity, and cannot be accounted for solely by the change in tin hydride concentration.[21]

The dependency of the **9**:**8** ratio as a function of Bu_3SnH concentration is consistent with the postulated competing reactive pathways available to **2** (Scheme 2). The data clearly demonstrate that elimination of HOO$^\bullet$ effectively competes with physiologically relevant concentrations of an efficient hydrogen atom donor. The slope of Figure 2 (1.3×10^{-2} M) represents the ratio k_{Elim}/k_{Red}. The rate constant for the elimination process ($k_{Elim} \approx 65\ s^{-1}$) is estimated assuming that the bimolecular rate constant for the reaction between Bu_3SnH and **2** is approximately the same as for *tert*-butyl thiol reacting with ROO$^\bullet$

(18) (a) Armitage, B.; Yu, C.; Devadoss, C.; Schuster, G. B. *J. Am. Chem. Soc.* **1994**, *116*, 9847. (b) McCord, J. M.; Fridovich, I. *J. Biol. Chem.* **1969**, *244*, 6049.

(19) Misra, H. P.; Fridovich, I. *J. Biol. Chem.* **1972**, *247*, 3170.

(20) See the Supporting Information.

(21) Tronche, C.; Martinez, F. N.; Horner, J. H.; Newcomb, M.; Senn, M.; Giese, B. *Tetrahedron Lett.* **1996**, *37*, 5845.

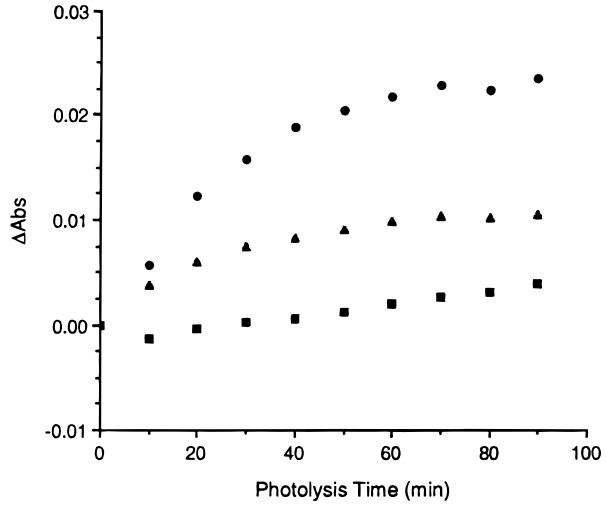


Figure 3. Adrenochrome formation ($\Delta Abs = Abs_t - Abs_0$) upon photolysis of (●) **10** (0.1 mM) in the presence of (*R*)-(−)-epinephrine (0.1 mM) and desferal (1 mM), (▲) **10** (0.1 mM), (*R*)-(−)-epinephrine (0.1 mM), desferal (1 mM), and superoxide dismutase (100 units), and (■) (*R*)-(−)-epinephrine (0.1 mM) and desferal (1 mM).

(5×10^3 M^{-1} s^{-1}).[22] The estimated rate constant for elimination of HOO• from **2** is more than 1000 times slower than that measured for rearomatization of the peroxyl radical derived from the hydrogen atom adduct of benzene (eq 4), and is reflective of the smaller thermodynamic driving force for this process.[8] This estimate is indeed a rough one, and is based upon the fact that alkanethiols and Bu_3SnH react with alkyl radicals at similar rates.[23] One should note that these respective families of radical traps show opposite reactivity trends for electrophilic perfluoroalkyl radicals.[24] Therefore, the above bimolecular rate constant for the reaction between Bu_3SnH (k_{Red}) and electrophilic **2** (and in effect k_{Elim}) may be underestimated.

Superoxide ($O_2^{\bullet-}$) Elimination from 4. Quantitative determination of superoxide release using cytochrome *c* during irradiation of **10** was not possible. Control experiments demonstrated that one or more of the alkyl radicals were capable of inducing reduction of the cytochrome.[25] However, as was the case for **2**, qualitative determination that **4** releases $O_2^{\bullet-}$ was addressed using the oxidation of epinephrine as a probe (Figure 3). Irradiation of **10** in the presence of superoxide dismutase results in significant diminution of adrenochrome formation. However, increasing the amount of superoxide dismutase (200 units) does not completely quench adrenochrome formation (data not shown). This indicates that a second oxidant is present which is capable of oxidizing epinephrine. We suggest that the *tert*-butylperoxyl and/or pivaloylperoxyl radicals (also derived from Norrish type I photocleavage of **10**) is this other oxidant. However, we do not believe that these other ROO• radicals are responsible for an appreciable amount of the indirectly detected $O_2^{\bullet-}$. In general, geminal heteroatoms are required in order for $O_2^{\bullet-}$ elimination to compete with dimerization of the corresponding peroxyl radicals.[8,9]

The viability for competition of $O_2^{\bullet-}$ elimination in the presence of biologically relevant reductants (e.g., thiols) was

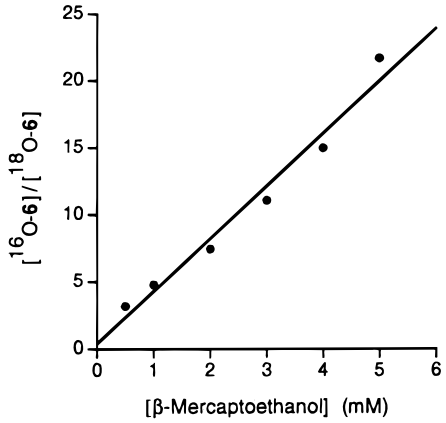
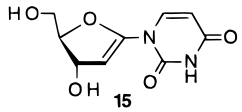


Figure 4. Ratio of [^{16}O]**6** to [^{18}O]**6** formed upon photolysis of **10** (0.05 mM) in $H_2{}^{18}O$ as a function of β-mercaptoethanol concentration. The slope of the line $= k_T/k_E = 4.3 \times 10^3$ M^{-1}.

investigated by measuring the extent of ^{18}O incorporation in **6** (resulting from aerobic photolysis of **10** in $H_2{}^{18}O$) as a function of β-mercaptoethanol concentration (Scheme 1). It was anticipated that the deoxyribonolactone (**6**) produced during photolysis of **10** in $H_2{}^{18}O$ via superoxide elimination would contain ^{18}O, whereas reduction of the peroxyl radical by thiol would yield [^{16}O]**6** (Scheme 1, Figure 4).[26] Alternative pathways for the formation of **6** were considered. For example, disproportionation of **3** and the *tert*-butyl radical to form the unstable 1′,2′-dehydronucleoside (**15**) would yield **6** upon hydrolysis.[27] This



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pathway was discounted as a major source of **6** on the basis of the anaerobic photolysis of **10** in D_2O, which produced **6** containing only 6% deuterium.[26] In general, O_2 independent pathways for the formation of **6** were eliminated by carrying out all photolyses in the presence of a minimum level of β-mercaptoethanol (=0.5 mM). No deoxyribonolactone (**6**) is produced upon photolysis of **10** (50 μM) under these conditions in the absence of O_2. Therefore, any **6** formed in the presence of O_2 and thiol must result from decomposition of the alkylperoxyl radical (**4**).[28] Formation of [^{18}O]**6** from radical−radical reactions involving **4** (such as dimerization, followed by decomposition of the tetroxide) are inconsistent with the observed dependence of the [^{16}O]**6**:[^{18}O]**6** ratio on thiol concentration. In addition, processes involving tertiary alkylperoxyl radicals are relatively slow ($\sim 10^4$ M^{-1} s^{-1}), and would not compete with thiol trapping of **4** under the photolysis conditions.[29] Nonetheless, photolysis experiments were carried out under the least intense conditions possible, to minimize the steady-state concentration of **3** (and in effect **4**). Furthermore,

(22) (a) Schulte-Frohlinde, D.; Behrens, G.; Önal, A. *Int. J. Radiat. Biol.* **1986**, *50*, 103. (b) Chenier, J. H. B.; Furimsky, E.; Howard, J. A. *Can. J. Chem.* **1974**, *52*, 3682.

(23) Newcomb, M. *Tetrahedron* **1993**, *49*, 1151.

(24) Rong, X. X.; Pan, H.-Q.; Dolbier, W. R. Jr.; Smart, B. E. *J. Am. Chem. Soc.* **1994**, *116*, 4521.

(25) EPR spin trapping experiments using 5,5-dimethylpyrroline *N*-oxide were also unfruitful. Multiple trapping products were observed. Furthermore, the superoxide adduct is unstable under the photolysis conditions.

(26) It was independently demonstrated that the deoxyribonolactone (**1**) does not undergo deuterium or oxygen exchange under the reaction conditions.

(27) Robins, M. J.; Trip, E. M. *Tetrahedron Lett.* **1974**, 3369.

(28) The yield of **6** was >90% of the amount of **10** consumed during photolysis. Consumption of **10** was established by reversed-phase HPLC. The yield of **6** was determined by GC/MS analysis following persilylation. Both compounds were quantitated using internal standards.

(29) (a) Bennett, J. E. *J. Chem. Soc., Faraday Trans.* **1990**, *86*, 3247. (b) During the photolysis (2 h), ~25% of a solution of **10** (50 μM) is converted in the RPR-100 photoreactor (equipped with one lamp). On the basis of this level of conversion, we estimate that the steady-state concentration of **4** during photolysis is at the nanomolar level. Consequently, the rate of reaction of two molecules of **4** (using the rate constant in ref 29a) is significantly slower than trapping of **4** by thiol.

Table 3. Effect of NaCl on the Reactivity of **4**

[NaCl] (mM)	k_T/k_E ($\times 10^{-3}$, M^{-1})	k_E (s^{-1})
0	4.3	1.2
25	3.8	1.3
50	3.5	1.4

it is more likely that dimerization of **4**, or reaction of **4** with an alkyl radical (e.g., *tert*-butyl or **3**), would yield [^{16}O]**6**.[8] Hence, we propose that the [^{18}O]**6** formed in $H_2{}^{18}O$ during photolysis of **10** results from $O_2^{\bullet-}$ elimination from **4**.

The ratio of the rate constants for peroxyl radical trapping by β-mercaptoethanol relative to $O_2^{\bullet-}$ elimination (3.5×10^3 M^{-1}) is extracted from the data in Figure 4. However, due to the limited body of absolute rate constants available concerning the trapping of alkylperoxyl radicals by thiols as discussed above ($k_T \approx 5.0 \times 10^3$ M^{-1} s^{-1}), the absolute rate constant for superoxide elimination (k_E) from **4** can only be estimated to be $\sim$1.2 s^{-1}.[22] The possibility that the heterolytic fragmentation of **4** might be significantly accelerated by increasing the ionic strength was investigated (Table 3). The isotopic partitioning in **6** as a function of thiol concentration varied only slightly upon adding NaCl to the photolysis mixtures. The lack of a strong salt effect on the isotope content of **6** could be a consequence of the fact that both competing processes (thiol trapping and $O_2^{\bullet-}$ elimination, Scheme 1) are accelerated upon increasing the ionic strength of the solvent.[5,21] Overall, these results suggest that the additional stabilization by the pyrimidine nitrogen of the incipient carbocation formed upon $O_2^{\bullet-}$ elimination from **4** is less than that of a second ethereal group of an acetal (as in **5**).[8]

Conclusions

The experiments described above indicate that nucleoside peroxyl radicals participate in unimolecular reactions that produce $O_2^{\bullet-}$. At first glance, the estimated rate constant for $O_2^{\bullet-}$ elimination from **4** ($\sim$1 s^{-1}, Table 3) may appear to be too slow to contribute significantly to DNA damage. However, it has recently been shown that glutathione (which is present in vivo) reacts with DNA peroxyl radicals at $<2 \times 10^2$ M^{-1} s^{-1}.[30] Since the concentration of glutathione in cells is estimated to be $\sim$5 mM, $O_2^{\bullet-}$ elimination from **4** within biopolymers could compete with its reduction by thiol (k_T[RSH]/k_E $\sim$1).[2a]

In absolute terms, elimination of $O_2^{\bullet-}$ from **2** is considerably faster than from **4**. Superoxide elimination from **2** is also more facile than from the analogous radical derived from the free base, thymine.[10a] This process competes favorably with bimolecular trapping by Bu_3SnH in an aqueous solvent system. The competition between hydrogen atom transfer to **2** and its unimolecular reconstitution of thymidine is directly relevant to the efficiency of the recently reported DNA damage amplification mechanism involving this alkylperoxyl radical.[4] While Bu_3SnH is a superior hydrogen atom donor compared to molecules such as THF (a model for a deoxyribose ring) in bimolecular reactions, it is unclear at this time what the effective molarity of an adjacent nucleotide's carbohydrate is in a biopolymer containing **2**.[22,31] Hence, the viability of the process described in Scheme 2 requires further investigation within biopolymers, and would constitute a naturally occurring detoxification pathway.

Considering the relatively high effective concentration of superoxide generated by the decomposition of peroxyl radicals in nucleic acids (compared to its production in bulk solution), such processes could result in DNA damage amplification when effected in the presence of NO or redox active metal ions.[12,13] When a radical such as **2** is the progenitor to superoxide, elimination of this reactive oxygen species could very well be a double-edged sword.

Experimental Section

General Methods. All reactions were carried out in oven-dried glassware, under a nitrogen atmosphere, unless specified otherwise. DMSO, pyridine, and CH_2Cl_2 were distilled from CaH_2. Bu_3SnH was distilled from itself immediately prior to use. THF was distilled from Na/benzophenone ketyl. D_2O and $H_2{}^{18}O$ (>97%) were from Cambridge Isotope Labs. BSTFA, β-mercaptoethanol, and KO_2 were from Sigma-Aldrich. Doubly distilled H_2O (dd H_2O) was obtained from a Barnstead Nanopure still. Azo initiator **14** was from Wako. Ketone **10** was prepared as previously described.[32] 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 300 or 350 nm.

5-(Phenylseleno)-3′,5′-*O,O*-bis(*tert*-butyldimethylsilyl)-5,6-dihydrothymidine. *sec*-BuLi (17.5 mL (1.3 M), 22.8 mmol) was added to a solution of bis-TBDMS-5,6-dihydrothymidine (**13**, 3.67 g, 7.77 mmol) in THF (26 mL) at −78 °C.[4] After 2 h, DMPU (4.98 g, 38.9 mmol) was added to the reaction mixture, followed by a solution of PhSeBr (2.05 g, 8.70 mmol) in THF (12 mL). The reaction mixture was slowly warmed to room temperature. After 6 h, the reaction mixture was quenched with NH_4Cl (4 mL), poured into H_2O (100 mL), and extracted with ether (3 × 100 mL). The organics were washed with brine (100 mL) and dried over $MgSO_4$. Flash chromatography (19:1 CH_2Cl_2−EtOAc to 9:1 CH_2Cl_2−EtOAc) yielded the bis-silyl-protected phenyl selenide as a white foam (3.03 g, 62%) as a ($\sim$2:1) mixture of diastereomers. Running a slower gradient enables one to separate the diastereomers. Diastereomer A (minor): ^{1}H NMR ($CDCl_3$) δ 7.75 (br s, 1H) 7.59−7.23 (m, 5H), 6.23 (t, 1H, J = 7.2 Hz), 4.34 (m, 1H), 3.91−3.70 (m, 4H), 1.93 (m, 1H), 1.44 (s, 3H), 0.90 (s, 9H), 0.87 (s, 9H), 0.10 (s, 3H), 0.09 (s, 3H), 0.06 (s, 3H), 0.05 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 170.6, 151.4, 138.0, 129.6, 128.8, 125.5, 86.7, 83.9, 72.5, 63.2, 47.4, 43.5, 37.4, 25.9, 25.7, 20.8, 18.3, 17.9, −4.7, −4.8, −5.3, −5.4; IR (KBr) 3202, 3079, 2954, 2928, 2895, 1712, 1472, 1438, 1377, 1362, 1253, 1222, 1116, 1092, 1029, 834 cm^{-1}. Diastereomer B (major): ^{1}H NMR ($CDCl_3$) δ 7.73 (br s, 1H), 7.55−7.23 (m, 5H), 6.35 (dd, 1H, J = 6.2, 8.1 Hz), 4.38 (m, 1H), 3.71−3.47 (m, 5H), 2.24−2.15 (m, 1H), 2.06−1.99 (m, 1H), 1.40 (s, 3H), 0.91 (s, 9H), 0.89 (s, 9H), 0.08 (s, 3H), 0.07 (s, 3H), 0.06 (s, 3H), 0.05 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 170.7, 152.3, 137.8, 129.7, 129.0, 125.1, 86.4, 83.4, 71.9, 62.9, 48.5, 43.8, 37.9, 25.9, 25.7, 20.7, 18.3, 17.9, −4.6, −4.8, −5.3, −5.4; IR (KBr) 3212, 3076, 2954, 2928, 2897, 2857, 1715, 1697, 1472, 1437, 1377, 1361, 1255, 1216, 1115, 1092, 1028, 835 cm^{-1}.

5-(Phenylseleno)-5,6-dihydrothymidine (12). NH_4F (1.81 g, 48.8 mmol) was added to a solution of the above bis-silyl compound (3.05 g, 4.85 mmol) in anhydrous MeOH (65 mL) and stirred at 60 °C for 27 h. The reaction mixture was quenched with $NaHCO_3$ (1 mL) and the solvent removed in vacuo. The crude product was dissolved in MeOH, filtered, and purified by flash chromatography (19:1 CH_2Cl_2−MeOH to 4:1 CH_2Cl_2−MeOH). The product was isolated as a white foam (1.64 g, 85%). Reaction of the separated diastereomers in the same manner enabled their spectral characterization. Diastereomer A: ^{1}H NMR (MeOH-d_4) δ 7.62−7.59 (m, 2H), 7.44−7.39 (m, 1H), 7.34−7.29 (m, 2H), 6.19 (dd, 1H, J = 8.3, 6.0 Hz), 4.31 (m, 1H), 3.90−3.83 (m, 2H), 3.72 (d, 2H, J = 4.1 Hz), 3.7 (d, 1H, J = 13.6 Hz), 2.19−2.10 (m, 1H), 2.0−1.93 (m, 1H), 1.43 (s, 3H); ^{13}C NMR (MeOH-d_4) δ 173.3, 154.1, 150.2, 139.4, 138.5, 130.8, 130.1, 127.2, 87.8, 85.6, 72.9, 63.7, 45.4, 37.5, 21.2; IR (KBr) 3400 (br), 3057, 2922, 1684, 1473, 1436, 1379, 1291, 1220, 1086, 1049, 744 cm^{-1}. Diastereomer

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B: mp 171−173.5 °C; ^{1}H NMR (MeOH-d_4) δ 7.58−7.55 (m, 2H), 7.45−7.39 (m, 1H), 7.34−7.29 (m, 2H), 6.30 (dd, 1H, J = 6.6, 7.8 Hz), 4.31 (m, 1H), 3.71−3.53 (m, 5H), 2.39−2.30 (m, 1H), 2.10−2.00 (m, 1H), 1.42 (s, 3H); ^{13}C NMR (MeOH-d_4) δ 173.2, 154.9, 139.2, 130.9, 130.2, 126.9, 87.5, 84.9, 72.4, 63.4, 45.7, 38.1, 21.1; IR (KBr) 3400 (br), 2923, 2861,1703, 1477, 1438, 1380, 1364, 1286, 1218, 1156, 1087, 1048, 1000, 980 cm^{-1}.

General Procedure for Thermolysis of 12. Stock solutions of **12**, Bu_3SnH, and $(Bu_3Sn)_2$ were prepared in THF. Stock solutions of water soluble azo initiator, **14**, were prepared in H_2O. Thermolysis samples were prepared with the appropriate reagents having a final volume of 100−400 μL (1:1 or 19:1 THF−H_2O). Isotopic studies were carried out in D_2O or $H_2{}^{18}O$. Aerobic experiments were conducted so that O_2 was not the limiting reagent. The reaction tubes were left open to the atmosphere and heated at 37 °C (water bath) or 55 °C (heat block) for 24 h. Anaerobic thermolyses were carried out in sealed tubes, which were degassed with four freeze−pump−thaw cycles (3 min each). Anaerobic thermolyses were run at 37 °C for 1.5 h, unless otherwise specified.

General Procedure for Photolyses. Samples were prepared with **10** (or **12**) and the appropriate trap in (1:1 or 19:1) THF−H_2O (for **12**) or H_2O (for **10**). Samples for experiments under anaerobic conditions were degassed with at least four freeze−pump−thaw cycles (3 min each). Aerobic samples were left open to the atmosphere. Photolyses of samples containing **12** were carried out in Pyrex reaction tubes in a Rayonet photoreactor at 300 nm (four lamps) for 45 min, with an internal temperature of 25 °C. Photolyses of samples containing **10** were carried out in a similar manner using one lamp (350 nm).

General Workup of Thermolysis and Photolysis Samples. Samples containing Bu_3SnH were transferred to 1 dram vials fitted with Teflon septa. The tubes were rinsed with dd H_2O (200 μL) and THF (2 × 200 μL), and lyophilized. The residue was subsequently lyophilized from hexanes (500 μL) to facilitate the removal of tin. The residue was taken up in dd H_2O (200 μL), vortexed, and filtered through a 0.45 μm syringe filter. The vials were rinsed with dd H_2O (3 × 200 μL), filtered, and pooled. The internal standard was added, and the samples were lyophilized on a Savant Speed Vac concentrator. The residue was taken up in dd H_2O (100 μL) for HPLC analysis.

All other samples were transferred directly to eppendorf tubes (1.5 mL). The reaction tubes were washed with dd H_2O (200 μL) and THF (2 × 200 μL). The internal standard was added and the sample lyophilized. For HPLC analysis the residue was taken up in dd H_2O (100 μL).

HPLC Analysis of Thermolysis and Photolysis Samples. The products were detected at 205 and/or 254 nm using a gradient solvent program of H_2O and MeOH, unless otherwise specified. For analysis of experiments involving **12**, the gradient was 5% MeOH at t_0 for 10 min, linearly to 70% MeOH at 30 min, and linearly to 5% MeOH at 40 min at a flow rate of 1.0 mL/min. For analysis of experiments involving **10**, the gradient was 0% MeOH at t_0, linearly to 50% MeOH at 22 min, and held at this solvent mixture for 13 min, at a flow rate of 1.0 mL/min.

Mass Spectral Analysis of Persilylated Derivatives. An aliquot of the HPLC sample (50 μL) was transferred to a 1 mL Kontes conical reaction vial fitted with a Teflon septa. The sample was treated with 96% formic acid (450 μL) and heated at 100 °C for 1 h. The formic acid was removed in vacuo, and the samples were lyophilized from absolute ethanol (3 × 50 μL). The residue was dissolved in BSTFA (40 μL) and anhydrous pyridine (10 μL). The solution was heated at 100 °C for 2 h. GC/MS analysis was carried out immediately. Samples were reheated for 1 h if analysis was not completed within 3 h of silylation. Note: The formic acid treatment steps were omitted for sample preparation from experiments involving **10**. Pyridine was omitted from the persilylation procedures involving **10**.

GC/MS analysis was carried out directly on the BSTFA solution containing the persilylated nucleosides using a 30 m 5% phenyl methyl silicone capillary column. Data were collected in the SIM mode for isotopic studies with a dwell time of 100 ms. Analysis of cleaved nucleosides from experiments involving **12** was carried out using the following temperature program: T_1, 150 °C (2 min); T_f, 190 °C at 10 °C/min. Ions monitored were 255.1 (thymine), 257.1 (5,6-dihydrothymine), 258.1, 345.1 (5,6-dihydro-5-hydroxythymine), and 347.1. Ions 258.1 and 347.1 were detected to determine ^{2}H and ^{18}O incorporation, respectively. Analysis of experiments involving **10** was carried out using the following temperature program: T_1, 120 °C (2 min); T_f, 300 °C at 10 °C/min. Ions monitored in bis-silylated **6** were 261.1 (^{16}O) and 263.1 (^{18}O).

Superoxide Detection with Epinephrine. A solution of **10** or **12** (0.1 mM) and (*R*)-(−)-epinephrine (0.1 mM) was prepared in phosphate buffer (50 mM, pH 7.5) treated with desferal (1 mM). The epinephrine was prepared in HCl (10 mM, pH 2). SOD was prepared in phosphate buffer. The samples were prepared in eppendorf tubes (1 mL total volume), vortexed, and transferred to a UV cell. The initial absorbance was measured and the sample photolyzed at 350 nm (one lamp). The UV absorbance was measured at 480 nm at the specified time intervals.

When experiments were conducted with KO_2, a sample of epinephrine (0.1 mM) and/or Bu_3SnH (0.17 or 1.7 mM) was prepared in 1:1 THF−H_2O (1 mM desferal) and transferred to a UV cell. The initial absorbance was measured at 480 nm. A solution of KO_2 (0.1 mM) in anhydrous DMSO was added dropwise at 0.06 mL/h over 2 h, and the final absorbance at 480 nm was measured.

Superoxide Detection with Cytochrome *c*. A solution of **12** (50 μM), cytochrome *c* (50 μM), and catalase (12 μg) was prepared in phosphate buffer (50 mM, pH 8) treated with EDTA (0.1 mM). SOD was prepared in phosphate buffer. The samples were prepared in eppendorf tubes (1 mL total volume), vortexed, and transferred to a UV cell. The initial absorbance was measured at 550 nm. The samples were photolyzed at 350 nm (four lamps) for 2 h, measuring the absorbance in 10 min intervals. The percentage of reduced cytochrome *c* was calculated as previously described.[18a] Aliquots of the sample before and after photolysis were analyzed by reversed-phase HPLC as described above, to determine the extent of conversion of **12** and the yield of thymidine.

Acknowledgment. Financial support of this work from the National Institutes of Health (Grant GM-54996) and the Petroleum Research Fund, administered by the American Chemical Society (Grant AC-30027), is greatly appreciated. M.M.G. is an Alfred P. Sloan Research Fellow. We thank Brian K. Goodman for carrying out initial experiments on **3**.

Supporting Information Available: ^{1}H and ^{13}C NMR spectra of the separated diastereomers of **12** and the respective bis-silyl phenyl selenides and oxidation of epinephrine upon aerobic photolysis of **12** (9 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA973200S