Reactivity of 5,6-Dihydro-5-hydroxythymid-6-yl Generated via Photoinduced Single Electron Transfer and the Role of Cyclohexa-1,4-diene in the Photodeoxygenation Process

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Abstract: The major reactive species formed via reaction of hydroxyl radical and the pyrimidine nucleoside thymidine, 5,6-dihydro-5-hydroxythymid-6-yl (**1**), is generated photochemically under anaerobic conditions from **6** via photoinduced single electron transfer. Under the conditions in which it is generated, **1** is trapped by hydrogen atom donors to form thymidine C5-hydrate (**7**), and undergoes oxidation, resulting in the formation of thymidine glycol (**8**). Isotopic (^{2}H, ^{18}O) labeling experiments indicate that dehydration of **1** is not competitive with intermolecular hydrogen atom donation by 3,3,6,6-tetradeuteriocyclohexa-1,4-diene. Extrapolation of the known rate constants for hydrogen atom donation by cyclohexa-1,4-diene to alkyl radicals suggests that intramolecular hydrogen atom abstraction and dehydration are $<2\ s^{-1}$, and are not kinetically competent to be involved in nucleic acid strand scission that arises from **1**. Relative quantum yields for disappearance of **6** in the presence and absence of cyclohexa-1,4-diene suggest that the diene reduces the *N*-methylcarbazole cation radical, preventing back electron transfer. Labeling studies using 3,3,6,6-tetradeuteriocyclohexa-1,4-diene and 1,2,3,4,5,6-hexadeuteriocyclohexa-1,4-diene suggest that the resulting olefin cation radical, or other reactive species derived from the trap, competes with cyclohexa-1,4-diene for **1**.

Examples abound of natural products and synthetic analogues possessing antitumor activity that oxidatively cleave nucleic acids.[1] Oxidative reagents are also immensely useful for analyzing nucleic acid binding interactions and structure.[2] There is evidence to suggest that common reactive intermediates are produced in different methods of oxidative nucleic acid damage. From this perspective, ionizing radiation, which degrades nucleic acids via several pathways, can be regarded as the most general method for oxidizing nucleic acids. γ-Radiolysis is also possibly the oldest method employed for oxidizing nucleic acids, having been used to treat tumors since 1897.[3] Despite significant advances in the understanding of the chemical mechanisms involved in the γ-radiolysis of nucleic acids, many questions remain unanswered. The rich chemistry associated with the myriad of reactive intermediates produced via the interaction between ionizing radiation and nucleic acids contributes to the overall complexity of this process. Recently, we and others have sought to elucidate the mechanistic pathways of oxidative nucleic acid damage by studying the reactivity of putative reactive intermediates which are independently generated from photolabile precursors.[4] Herein, we describe the chemistry of the major adduct of hydroxyl radical and thymidine (**1**) with respect to its role in nucleic acid strand scission under anaerobic conditions.

Reactive oxygen species are often responsible for inducing oxidative nucleic acid damage, including several pathways initiated by ionizing radiation. The most commonly invoked and well studied (*vis-à-vis* nucleic acid damage) reactive oxygen species produced during γ-radiolysis of water is hydroxyl radical (OH•). In addition, hydroxyl radical is believed to be generated by Fenton-like systems, photolysis of nitrite and nitrate, as well as via the photochemical rearrangement of a hydroperoxyphthalimide.[5-7] Studies on the reactivity of OH• with nucleic acids are carried out under anaerobic conditions, in order to reduce the number of chemical processes, as well as to model hypoxic cellular conditions. Under anaerobic conditions, approximately 41% of the reactions between OH• and DNA are believed to lead to strand scission.[8] Independent electron paramagnetic resonance (EPR) experiments indicate that reaction of OH• with thymidine and polypyrimidines generates the C5

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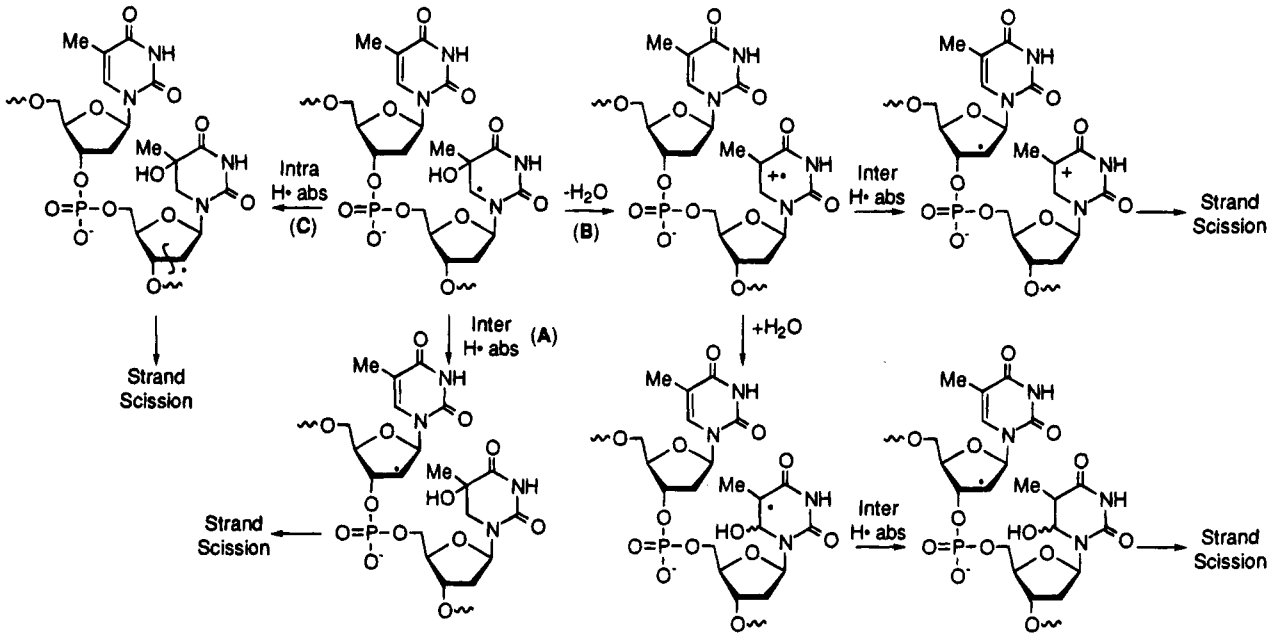
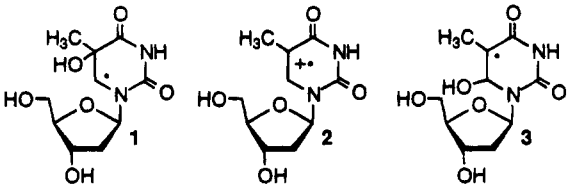
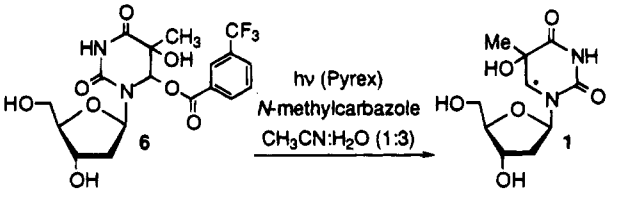
Scheme 1



nucleobase adduct **1** in greater than 60% yield.[3,9] Taken together, the results of these experiments suggest that a fraction of intermediates such as **1** ultimately produce strand breaks. Although the relative importance of the indirect and direct effects of ionizing radiation *in vivo* are debatable, it is generally accepted that **1** plays a significant role in strand scission produced by OH• *in vitro*.[3,10]



The mechanism by which C5-pyrimidine nucleoside radical adducts such as **1** lead to oxidation of the carbohydrate moiety under anaerobic conditions is uncertain. Whatever the rate-determining step in strand scission involving **1** may be, time-resolved light scattering, electron spin resonance (ESR), and conductivity experiments show that it is a slow process ($k_{\text{strand scission}} \leq 38\ s^{-1}$).[11] Most time-resolved studies estimate that the rate constant for strand scission in homopolymers (oligoribonucleotides and oligodeoxyribonucleotides) and single-stranded DNA are $<10\ s^{-1}$.[12] The rate-limiting step in strand scission from **1** has been suggested to involve hydrogen atom abstraction from an adjacent nucleotide in a biopolymer (Scheme 1, pathway A).[13] Alternatively, the rate-determining step of strand scission resulting from generation of a nucleobase-centered radical was suggested to involve protonation, followed by dehydration to the radical cation (e.g., **2**). The radical cation has been suggested to transfer spin from the nucleobase to the carbohydrate moiety either via abstracting a hydrogen atom from the adjacent nucleotide's carbohydrate moiety or via initial hydration at C6, followed by hydrogen atom abstraction by the resulting dihydrothymid-5-yl (Scheme 1, pathway B).[12d,14,15] While **1** and **3** are examples of stabilized radicals, it is possible that the conformational constraints of a biopolymer would facilitate hydrogen atom abstraction by **3** relative to **1**. Common among these mechanistic proposals is the contention that direct intramolecular hydrogen atom abstraction within 2′-deoxypyrimidine nucleosides and nucleotides does not play a role in the chemistry of the reactive intermediates involved in these important processes (Scheme 1, pathway C). Proposals that eliminate intramolecular hydrogen atom abstraction by **1** and **2** are based on steady state EPR experiments, in which these reactive intermediates are found to be long lived.[14a,b] Utilizing photolabile substrate **6** to independently generate **1**, we assessed its potential to effect intramolecular hydrogen atom abstraction, and to undergo dehydration to **2**.



Results and Discussion

Generation of hydroxyl radical adduct **1** from *m*-(trifluoromethyl)benzoate **6**, via a photoinduced single electron transfer reaction involving *N*-methylcarbazole as the electron donor, was

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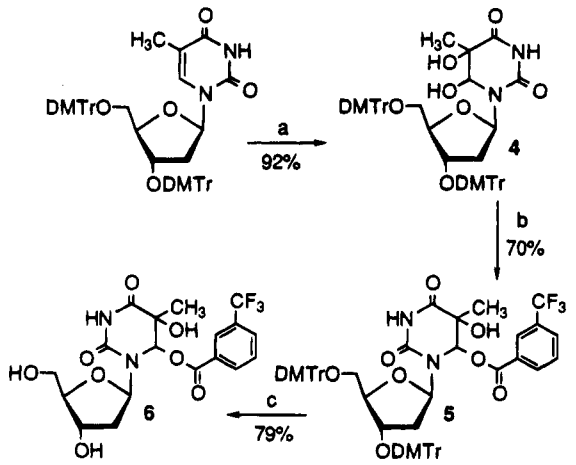
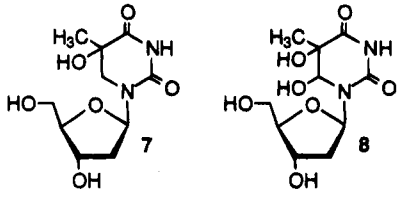
Scheme 2



[a] Key: (a) OsO_4, *N*-methylmorpholine *N*-oxide, tBuOH, H_2O, THF, 40 °C; (b) *m*-(trifluoromethyl)benzoyl chloride, DMAP, THF, −10 °C; (c) AcOH−H_2O (1:4), 25 °C.

reported previously.[4b,16] The synthesis of **6** was improved by protecting the nucleoside's hydroxyl groups with the more acid labile 4,4′-dimethoxytrityl group (Scheme 2). Diastereoselectivity of the osmylation reaction, which favors (5*R*,6*S*)-**4** relative to (5*S*,6*R*)-**4** was not affected.[17] This minor change enabled us to effect the final deprotection in 79% yield. Consequently, all photolysis experiments described below employed the (5*R*,6*S*) diastereomer of **6**.

Generation and Trapping of 1. Photolysis of **6** and stoichiometric *N*-methylcarbazole proceeded smoothly in a degassed mixture of acetonitrile and H_2O (73:27, v/v), using either the Pyrex-filtered output of a high-pressure Hg/Xe lamp or a Rayonet photoreactor equipped with lamps exhibiting maximum emission at 350 nm. In the presence of O_2, or absence of *N*-methylcarbazole, extended photolysis induced only slight decomposition of **6**, and glycol **8** was the only product detected. Anaerobic photolysis in the presence of a hydrogen atom donor led to formation of (5*R*)-thymidine C5-hydrate (**7**), as well as a mixture of *cis*-(5*R*,6*S*)- and *trans*-(5*R*,6*R*)-thymidine glycols (**8**) as the major products (Table 1).[17,18] Both products were identified by ^1H NMR, reversed-phase HPLC, and GC/MS (following persilylation with BSTFA)[19] using comparisons to authentic materials.



The dependence of the ratio of **7** to **8** on the particular hydrogen atom donor and its concentration is consistent with the proposal that both products are derived from **1**. Thymidine C5-hydrate (**7**) isolated from photolysis of **6** in CH_3CN/D_2O and 1,4-cyclohexadiene contains no deuterium, supporting the assumption that **7** is formed via a radical pathway. Thymidine C5-hydrate (**7**), isolated by trapping **1** with thiols in D_2O, contains equal amounts of the two possible stereoisomers that are deuterated at C6, suggesting that both faces of the radical are accessible. Thymidine glycol (**8**) can arise from Bouveault−Blanc type cleavage of the radical anion, or oxidation of **1**.[16b] Stabilization in **1** due to the nitrogen atom adjacent to the alkyl radical center caused us to consider the former pathway to be the less likely of the two to account for **8**. Furthermore, oxidation of **1** by *N*-methylcarbazole cation radical is consistent with its known behavior. Its ability to act as a reducing radical has been exploited during its characterization by EPR spectroscopy.[20] These two pathways are distinguishable using $H_2{}^{18}O$ as cosolvent with CH_3CN (Scheme 3). Quantitative examination of persilylated **8** by GC/MS using selected ion monitoring indicates that 6% of the glycol **8** formed contains ^{16}O (Table 2). Equilibration of **[^{16}O]8** in $H_2{}^{18}O$ for 6 h (a period 3 times longer than the time over which the photolysate is exposed to water) results in less than 3% exchange. After accounting for residual $H_2{}^{16}O$ (4%) in the $H_2{}^{18}O$, we conclude that no more than 3% of the glycol **8** formed is attributable to Bouveault−Blanc cleavage.

Table 1. Product Ratios Obtained from Photolysis of **6**[a]

[**6**] (mM)	H• donor (mM)	[**7**]:[**8**]	mass balance (%)
5	1,4-CHD[b] (300)	9.4	100
25	dithiothreitol (75)	4.6	100
25	2-methylpropane-2-thiol (75)	4.2	67
5	2-methylpropane-2-thiol (15)	4.2	88
5	1,4-CHD[b] (150)	4.0	99
25	1,4-CHD[b] (75)	0.7	97
5	1,4-CHD[b] (15)	0.4	99

[a] Determined by HPLC. [b] Cyclohexa-1,4-diene.

Intramolecular Hydrogen Atom Abstraction by 1. The kinetic competency of this process with respect to strand scission from **1** was probed by constructing a competition with an external deuterium atom donor (Scheme 4). Using ^2H NMR to analyze the deuterium content of 5,6-dihydro-5-hydroxythymidine (**7**) isolated from the photoreaction (64% yield), we reported previously that intramolecular hydrogen atom abstraction within **1** does not compete with intermolecular trapping by deuterated 2-methylpropane-2-thiol (15 mM).[4b] A similar experiment was carried out using the deuterium atom donor 3,3,6,6-tetradeuteriocyclohexa-1,4-diene (cyclohexadiene-d_4) as trap (Figure 1).[21] Cyclohexa-1,4-diene typically reacts with structurally similar alkyl radicals between 40 and 190 times more slowly than 2-methylpropane-2-thiol.[22] The ^2H NMR spectrum recorded after repeated lyophilization from H_2O of the crude photolysate obtained from **6** (5 mM) and cyclohexadiene-d_4 (50 mM) revealed the presence of deuteron(s) that resonated between δ 2.8 and δ 2.9, in addition to those assigned to C6 of **7** (δ 3.3). The additional resonances do not correlate with any resonance in thymidine C5-hydrate (**7**), and were absent after HPLC purification of **7**.

While *before and after* analysis by ^2H NMR eliminates the involvement of intramolecular hydrogen atom transfer as a

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(21) Batches of cyclohexadiene-d_4 were assayed by the manufacturers Merck, Sharpe and Dohme Laboratories and Cambridge Isotopes, and found to be 99.1% and 99.3%, respectively.

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Scheme 3

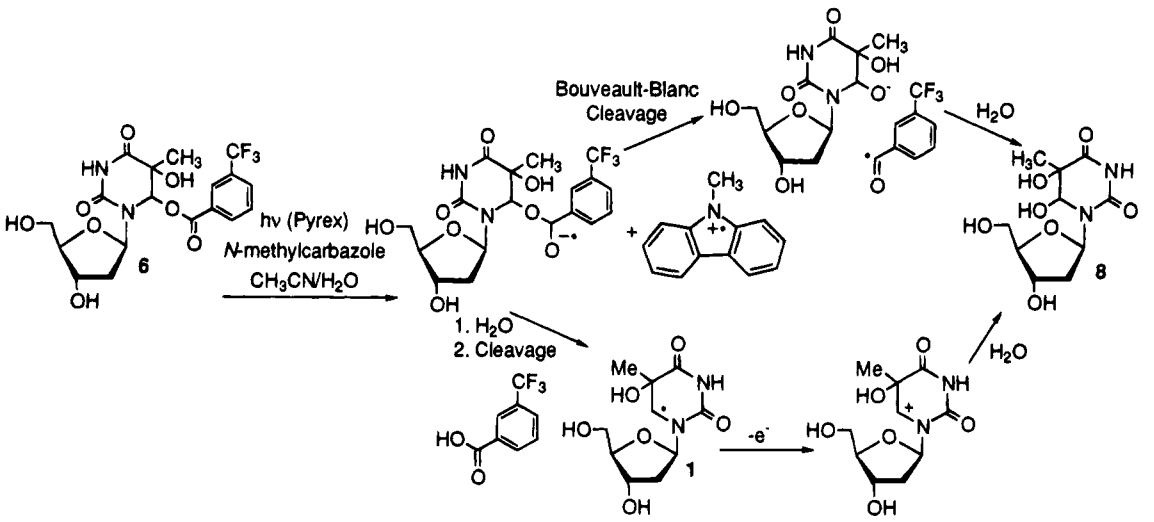
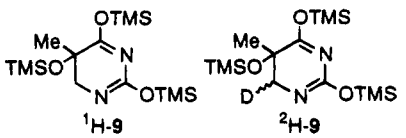


Table 2. Isotopic Content of **8** Produced in a Photolysis of **6** with 50 mM Cyclohexadiene-d_4 Using $H_2{}^{18}O$ as Cosolvent[a]

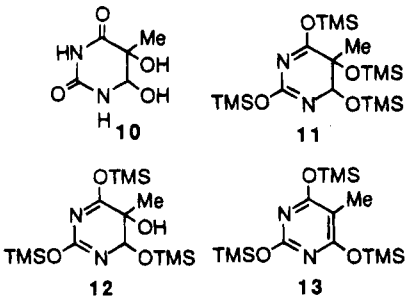
cosolvent	[^{16}O]**8** (%)	[^{18}O]**8** (%)
H_2O	100	0
$H_2{}^{18}O$[a]	6.0	100
control[b]	100	2.6

[a] 96% enriched $H_2{}^{18}O$. [b] [^{16}O]**8** after 6 h of equilibration in 27% $H_2{}^{18}O/CH_3CN$.

pathway for formation of **7**, it leaves open the general issue concerning intramolecular hydrogen atom abstraction within **1**. The results obtained utilizing cyclohexadiene-d_4 point out a limitation of the ^{2}H NMR experiment. By analyzing the deuterium content of **7**, the ^{2}H NMR enables one to determine the relative rate constant (compared to an external deuterium atom donor) with which a specific hydrogen atom is abstracted by the nucleobase-centered reactive intermediate, provided that the sugar radicals formed via intramolecular hydrogen atom abstraction are efficiently trapped to give **7** containing a deuterio-enriched deoxyribose moiety. Similarity in the magnitudes of k_{intra} and k_{inter}[D•] (Scheme 4) is a necessary, but insufficient, criterion for the success of the ^{2}H NMR experiment. In order for this experiment to unequivocally detect intramolecular hydrogen atom abstraction in **1**, the intermolecular trapping of any deoxyribose-centered radicals (k_2[D•]) must be significantly greater than any subsequent reactions of these radicals (k_{dec}). Such a coincidental cooperation of rate constants would ensure that **7** would be formed from any intramolecular process. In view of this analysis, a plausible alternative source of the additional signal in Figure 1a is that it is attributable to a product(s) containing protiothymine C5-hydrate that is derived from a rearranged (or oxidized) deoxyribose-centered radical generated via intramolecular hydrogen atom abstraction in **1**, and undergoes further reaction prior to being trapped by cyclohexadiene-d_4. Alternatively, the resonance at 2.8 ppm can be ascribed to a process that is unrelated to **1**.

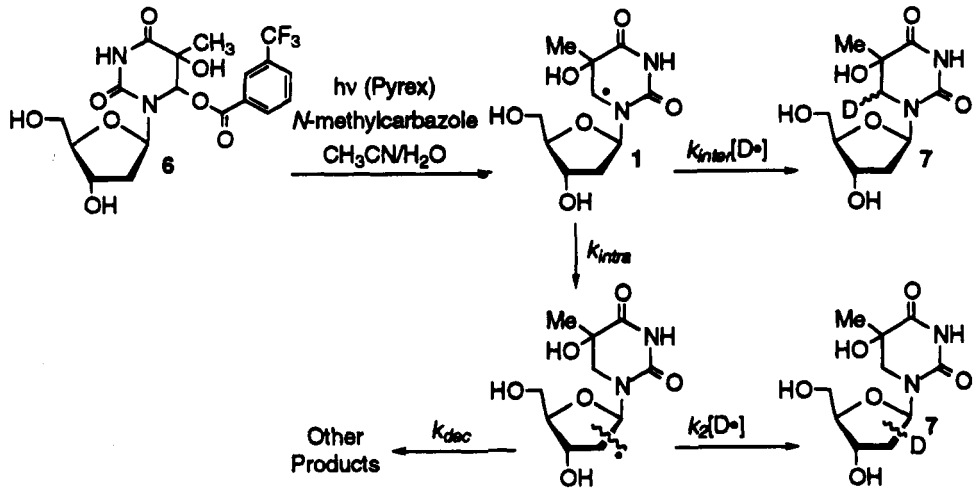


Analysis of the isotopic content of the nucleobases released upon formic acid treatment of the crude photolysate enables us to determine k_{inter}/k_{intra} (Scheme 4), regardless of the relative magnitudes of k_{dec} and k_2[D•]. If the only processes responsible for formation of [^{1}H]**9** and [^{2}H]**9** are intramolecular and intermolecular hydrogen atom abstraction processes, then the ratio of these products will vary linearly with the concentration of the hydrogen atom donor. The slope of the line obtained will equal k_{inter}/k_{intra}. The relative amounts of protio and deuterio incorporation within thymine C5-hydrate were determined via ion selective GC/MS analysis of persilylated material (**9**), which enabled us to separate **9** from products **11** and **12** derived from **10** that contain common ions.



Photolyses of **6** (5 mM) carried out in the presence of cyclohexadiene-d_4 using CH_3CN as cosolvent showed that the ratio of m/z = 346 to m/z = 345 was proportional to the concentration of the hydrogen atom donor. This qualitative trend strongly suggested that one or more intramolecular processes were competing with intermolecular trapping of **1** by cyclohexadiene-d_4. The contribution of naturally occurring m/z = 346 to the above ratio was determined experimentally using independently synthesized **7**. In order to determine the amount of [^{1}H]**9** attributable to residual protio material in cyclohexadiene-d_4, we needed to determine the kinetic isotope effect associated with hydrogen atom transfer from this trap to **1**. Cyclohexadiene-d_4 was 99.1−99.3 atom % enriched, making it statistically improbable that there will be a significant concentration of cyclohexadiene-d_4 that contains two residual hydrogen atoms on a single methylene carbon.[21] The competition of interest is between abstraction of a deuteron from a center containing two such atoms and abstraction of a hydrogen atom from a center that also contains a deuteron. The contribution due to abstraction of a deuteron from a methylene carbon containing a hydrogen atom was ignored. Taking into account the effect of the change in hybridization with respect to the remaining deuteron at each center, one realizes that the transfer of a hydrogen atom from cyclohexadiene-d_3 relative to the

Scheme 4



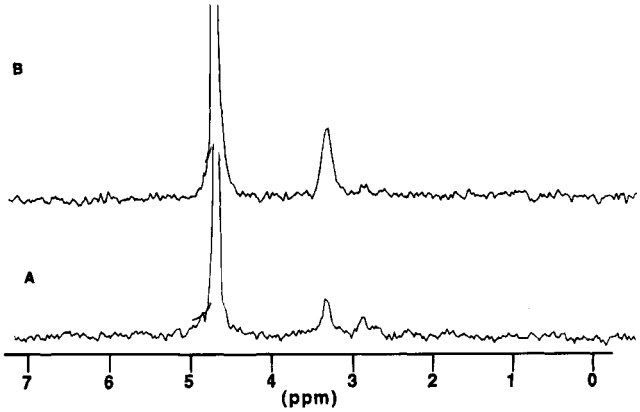
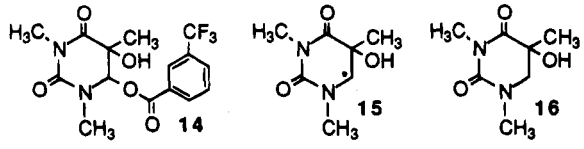


Figure 1. ^{2}H NMR spectra of **6** photolyzed in the presence of cyclohexadiene-d_4 (50mM): (a) crude photolysate, (b) following HPLC purification of **7**.

transfer of a deuterium atom from cyclohexadiene-d_4 is favored by the magnitude of the primary kinetic isotope effect.



m-(Trifluoromethyl)benzoate **14** was designed in order to measure the kinetic isotope effect of the reaction between cyclohexadiene-d_4 and **1**. Synthesis of **14** was based upon that carried out for **6**. The primary kinetic isotope effect in the reaction between **15** and cyclohexadiene-d_4 was extracted from two independent competition studies (Figure 2). Measurements carried out in protio- and deuterioacetonitrile demonstrated that there was an insignificant contribution from the solvent in the formation of **16** under these trapping conditions. The product of the primary and α-secondary kinetic isotope effects was obtained by measuring the ratio of **[^{1}H]16** to **[^{2}H]16** in the presence of 1,4-cyclohexadiene and 3,3,6,6-tetradeuterio-1,4-cyclohexadiene (cyclohexadiene-d_4). Residual hydrogen in cyclohexadiene-d_4 was accounted for during the calculation of the product of the isotope effects. Measuring the same product ratio in the presence of **17** (instead of **18**) yielded the value of the primary kinetic isotope effect divided by the secondary kinetic isotope effect (Figure 2). Our assumption in preparing **17** is that the β-secondary kinetic isotope effect introduced by incorporating deuterium at the vinylic positions is small. From

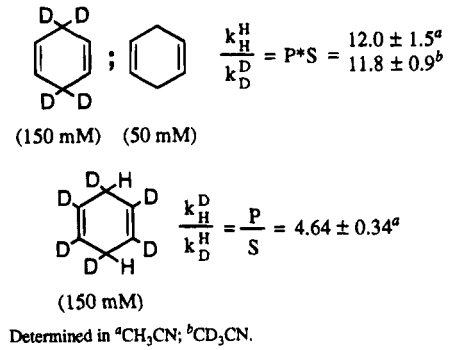
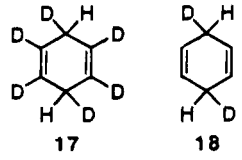


Figure 2. Determination of kinetic isotope effects for cyclohexadiene-d_4. Adopted notation is that reported by Hanzlik et al. (ref 23a).

these two independent measurements, we determined that the primary kinetic isotope effect with cyclohexadiene-d_4 for the reactions of **15** and therefore **1** is 7.5.[23]



After correcting for the contribution from residual hydrogen in cyclohexadiene-d_4 and naturally occurring isotopomers of **[^{1}H]9**, the ratio of **[^{2}H]9** to **[^{1}H]9** formed from photolysis of **6** in a mixture of CH_3CN and H_2O varied linearly with [cyclohexadiene-d_4], suggesting that intramolecular hydrogen atom abstraction within **1** is competitive with trapping by cyclohexadiene-d_4 (Figure 3). However, this interpretation is refuted by other observations made using *m*-(trifluoromethyl)benzoate **14**, which was designed to produce a model (**15**) of **1** which cannot undergo intramolecular hydrogen atom abstraction. Photolysis of **14** under identical conditions ([cyclohexadiene-d_4] = 50 mM) employed for **6** produces **[^{2}H]16** and **[^{1}H]16** in a ratio (5.36 ± 0.5) that is within experimental error of that measured for **9**. Furthermore, no thymine C5-hydrate is observed when **6** is irradiated in the absence of cyclohexadiene-d_4. Both observations argue against the formation of **[^{1}H]9** via intramolecular hydrogen atom abstraction within **1**. The latter observation also

(23) For other examples in which primary and secondary kinetic isotope effects in radical reactions were independently determined, see: (a) Hanzlik, R. P.; Hogberg, K.; Moon, J. B.; Judson, C. M. *J. Am. Chem. Soc.* **1985**, *107*, 7164. (b) Jones, J. P.; Trager, W. F. *J. Am. Chem. Soc.* **1987**, *109*, 2171.

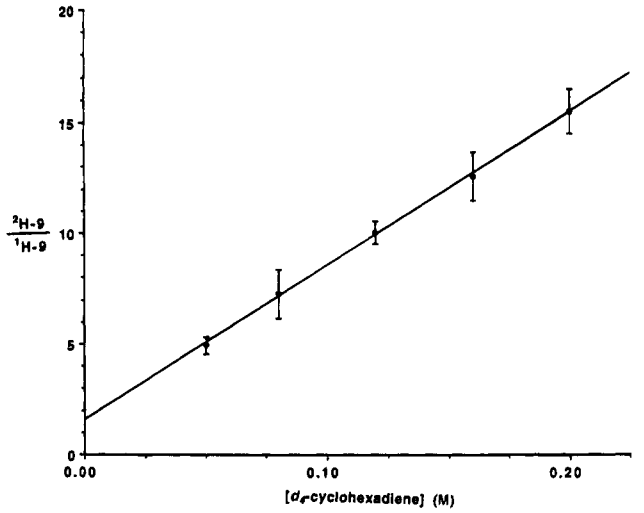


Figure 3. Dependence of the ratio of [^{2}H]**9** to [^{1}H]**9** formed from photolysis of **6** versus [cyclohexadiene-d_4]. Ratios are corrected for the primary kinetic isotope effect. Each data point represents an average of two photolyses with replicate analyses.

Table 3. Upper Limits for k_{intra} in **1** Derived from Absolute Values for k_{inter} from Cyclohexa-1,4-diene by Alkyl Radicals

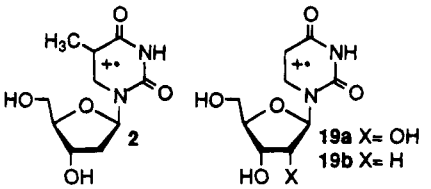
radical	$k_{H^{\bullet}Abs}$ (M^{-1} s^{-1})	$k_{D^{\bullet}Abs}$ (M^{-1} s^{-1})	k_{intra}[a] (s^{-1})
$CH_3CH_2^{\bullet}$ [b]	5.8×10^4	5.0×10^3	<12.5
$(CH_3)C^{\bullet}$ [b]	9.4×10^3	8.0×10^2	<2.0
$MeO(C{=}O)C(Me)_2^{\bullet}$ [c]	$<2.2 \times 10^3$	$<1.9 \times 10^2$	<0.5

[a] Calculated on the basis of [cyclohexadiene-d_4] = 0.05 M. [b] Reference 24a. [c] Reference 24b.

proves that CH_3CN does not donate a hydrogen atom to **1**. These observations raise two questions: (1) If intramolecular hydrogen atom abstraction within **1** does not compete with trapping by cyclohexadiene-d_4, what is the upper limit for k_{intra}? (2) What is the source of [^{1}H]**9**?

The latter question will be addressed at a later point in this discussion. In order to place an upper limit on k_{intra}, we must estimate a value for k_{inter}. Absolute rate constants for hydrogen atom donation from 1,4-cyclohexadiene to three alkyl radicals are listed in Table 3. Using the product of the primary and secondary kinetic isotope effects measured with **14** and the observed reactivity of nucleobase radicals in polymers with thiols enables us to estimate the rate constant for transfer of a deuterium atom from cyclohexadiene-d_4 to **1**. The nucleobase radicals analogous to **1** in polyuridine and single-stranded DNA react with dithiothreitol at rates as slow as 5×10^5 M^{-1} s^{-1}.[24] This corresponds to a reduction in bimolecular trapping by more than an order of magnitude compared to the reactions of small organic radicals with 2-methylpropane-2-thiol.[22c,d] While part of this diminution in reactivity between thiols and radicals in polymers is typically associated with the slower diffusional rates of the biopolymers, this effect is not believed to account for the entire magnitude of the observed rate reduction.[3] The slower rate constants for hydrogen atom abstraction from cyclohexadiene relative to thiols by alkyl radicals suggests that this retardation should be even more pronounced using cyclohexadiene.[22] Consequently, as a secondary alkyl radical which is stabilized by the adjacent nitrogen, **1** should be less reactive than ethyl radical, but more reactive than the methyl isobutyrate radical studied by Engel.[22a,b] On the basis of the observed reactivity of thiols with nucleobase radicals in biopolymers, we believe that the rate constant measured for the reaction between cyclohexadiene and *tert*-butyl radical represents a conservative upper limit for the reaction between **1** and cyclohexadiene (50 mM). Accounting for the kinetic isotope effect encountered when utilizing cyclohexadiene-d_4 (as measured with **14**) and placing the limit of detection of [^{1}H]**9** at 5% results in an estimate for k_{intra} of 2 s^{-1} (Table 3).[25] The upper limit for k_{intra} is less than or equal to the rate constant measured for the rate-limiting step in nucleic acid strand scission measured by several techniques, and suggests that intramolecular hydrogen atom abstraction within **1** is not a kinetically viable pathway for producing nucleic acid strand scission.[11,12]

Dehydration of 1 in Competition with Hydrogen Atom Trapping. Time resolved experiments show that the rate of strand scission in poly(U) induced by hydroxyl radical increases as pH is decreased.[12b,d,14b] In addition, the relative intensity of carbohydrate-centered radicals versus nucleobase radicals detected by EPR also increases as pH is lowered. These observations have been rationalized by invoking rate-limiting dehydration of the uridine hydroxyl radical adduct analogous to **1**. The resulting cation radicals are proposed to undergo either direct hydrogen atom abstraction or rehydration to the regioisomeric C6-hydroxyl radical adduct **3** which then abstracts a hydrogen atom from an adjacent nucleotide (Scheme 1).[12d] While independent generation of monomeric nucleoside cation radicals indicates that intramolecular hydrogen atom abstraction within ribonucleosides **19a** is far more rapid than in the analogous 2′-deoxyribonucleosides **2** and **19b**, the rate constants for dehydration of the respective hydroxyl radical adducts should not depend upon the substitution pattern of the carbohydrate moiety.[14a] Hence, estimation of the rate constant for cation radical formation from **1** is of general interest with respect to this issue.



Independent experiments on model compounds support the proposal that α,β-heteroatom-substituted alkyl radicals undergo elimination in aqueous media.[26] Rate constants for radical cation formation are strongly dependent on the nature of the leaving group at the β-position, and can be as fast as 10^7 s^{-1}. The presence of a hydroxyl group in the β-position of a radical (such as in **1**) significantly retards dehydration. Photolysis of **6** in $H_2{}^{18}O$ enabled us to determine the relative magnitudes of the rate constants for dehydration versus hydrogen atom abstraction from cyclohexadiene-d_4. For this experiment, there is a very distinct advantage to generating **1** from **6**. When **1** is generated from thymidine in H_2O via γ-radiolysis, isotopic labeling cannot be used to determine the extent to which **2** is responsible for the formation of products such as **7**, because the nucleobase hydroxyl group is derived from the solvent, regardless of whether **1** or **2** is its precursor. Using **6**, dehydration is potentially detectable using ^{18}O-enriched H_2O and mass spectral product analysis.

The intermediacy of **2** can be detected in a number of ways. Thymine C5-hydrate production is attributable to direct hydrogen atom abstraction by **2** at C6 (Scheme 5), followed by

(24) (a) Rao, P. J. P.; Bothe, E.; Schulte-Frohlinde, D. *Int. J. Radiat. Biol.* **1992**, *61*, 577. (b) Lemaire, D. G. E.; Bothe, E.; Schulte-Frohlinde, D. *Int. J. Radiat. Biol.* **1987**, *51*, 319.

(25) The precision of the mass spectral anlysis suggests that 5% is a very conservative estimate of the limit of detection for [^{1}H]**9**.

(26) (a) Behrens, G.; Koltzenburg, G.; Ritter, A.; Schulte-Frohlinde, D. *Int. J. Radiat. Biol.* **1978**, *33*, 163. (b) Behrens, G.; Koltzenburg, G.; Ritter, A.; Schulte-Frohlinde, D. *Z. Naturforsch.* **1982**, *37C*, 1205.

Scheme 5

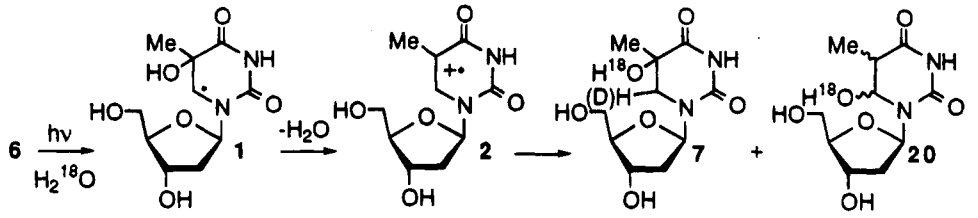


Table 4. Mass Spectral Analysis of **9** Produced Using Aqueous Acetonitrile and Cyclohexadiene-d_4 (50mM)

cosolvent	m/z^a 346	m/z^a 347	m/z^a 348
H_2O	100	32.50 ± 0.3	12.65 ± 0.5
$H_2{}^{18}O^b$	100	33.20 ± 0.7	13.50 ± 0.6

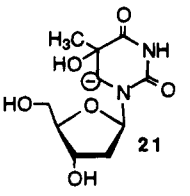
[a] m/z= 346, 347, and 348 are the $M^+ - 15$ ions of **[^{2}H]9**, **[^{1}H,^{18}O]9**, and **[^{2}H,^{18}O]9**, respectively. [b] 96% enriched $H_2{}^{18}O$.

nucleophilic attack by H_2O. As was the case for **1** (Scheme 1), **2** can give rise to thymine C5-hydrate through pathways that involve intramolecular or intermolecular hydrogen atom abstraction. The intermediacy of **2** was examined by determining the isotopic composition of **9** formed following the photolysis of **6** in the presence of cyclohexadiene-d_4 and $H_2{}^{18}O$. Control experiments were carried out using unenriched water. Photolyses contained a relatively low concentration of cyclohexadiene-d_4 (50 mM), in order to maximize the competition of dehydration and hydrogen atom abstraction by **1**. Dehydration of **1**, followed by intramolecular hydrogen atom abstraction by **2** and quenching of the resulting cation by $H_2{}^{18}O$, ultimately produces (after formic acid treatment as described previously) **[^{18}O]9**, regardless of the fate of the deoxyribose radical(s). Similarly, **[6-^{2}H-5-^{18}OH]9** is formed from **2** via intermolecular hydrogen atom abstraction from cyclohexadiene-d_4. Since intramolecular hydrogen atom abstraction involving **1** has already been ruled out, only the $M^+ - 15$ ions of **[^{2}H]9**, **[^{18}O,^{1}H]9**, and **[^{18}O,^{2}H]9** were quantitated by mass spectrometry (m/z = 346, 347, 348). The ratios of these ions to each other were independent of the isotopic composition of the water cosolvent (Table 4), indicating that if dehydration of **1** to give **2** occurs under these conditions, it does not produce thymine C5-hydrate.

Alternative reaction pathways available to **2** that would not produce thymine C5-hydrate include (1) intermolecular hydrogen atom abstraction at C5, followed by hydration, (2) initial hydration at C6, followed by radical quenching, (3) hydration at C6, followed by reduction of the C5 radical, and protonation by water, (4) deprotonation of the methyl group, followed by quenching of the allylic radical. Pathways 1−3 would produce thymidine C6-hydrate (**20**; Scheme 5). Alternatives 2 and 3 are consistent with the known reactivity of pyrimidine cation radicals.[15,20] Pathway 4 has been ascribed to **2**, but deprotonation occurs more slowly than hydration.[20c] Independently generated thymidine cation radical (**2**) is attacked by water exclusively at C6.[14a,b,15] ^{1}H NMR analysis of crude photolysates of **6** (in the presence of 50 mM d_4 -cyclohexadiene) indicated the presence of small quantities of **20**, as evidenced by the distinctive chemical shift of the C6-proton in **20**. The lability of **20** to acid and heat precluded determination of its isotopic content by mass spectral procedures utilized in other aspects of this study. Furthermore, **20** was formed in quantities too small to permit its isolation and analysis of the underivatized nucleoside by fast atom bombardment (FAB)MS.

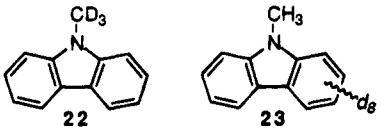
Consequently, the formation of **20** during the photolysis of **6** was corroborated by taking advantage of its facile acid-mediated dehydration, and concomitant glycosidic bond cleavage that results in the formation of thymine. Uracil and thymine formation have been used previously as a means for indirect detection of the respective C6-hydrates.[9,27] Subjection of crude photolysates of **6** to acid hydrolysis, and analysis of the persilylated mixture by GC/MS, clearly shows the presence of thymine. However, detection of thymine does not require that it be derived from **20**. Using authentic samples, we showed that thymine is not produced from **6**, or **8**, but is formed to a small extent from **7** during preparation of the analytical samples. Furthermore, formation of **20** does not necessitate that it be derived from **2**. Artifactual pathways for producing **20**, such as benzoyl migration in **6** (or a reactive species derived from it), which would produce 5,6-dihydro-5-hydroxythymid-5-yl directly, are distinguishable from that involving **1** by determining the dependency of the formation of thymine on the concentration and/or type of hydrogen atom donor. If **20** is formed through the hydroxyl radical adduct, then dehydration must compete with intermolecular quenching of **1**. Using 2′-deoxyuridine as an internal standard, we determined that the amount of thymine produced increased by 50% when 2-methylpropane-2-thiol (100 mM) was used in place of cyclohexadiene-d_4 (50 mM), suggesting that formation of thymidine C6-hydrate (**20**) during the photolysis of **6** does not arise via dehydration of **1**.[28] Using the value for k_{inter} for the reaction between cyclohexadiene-d_4 and **1** estimated above results in a similar conclusion regarding the upper limit (<2 s^{-1}) for the formation of **2**. Hence, this process is not kinetically competent to be involved in nucleic acid strand scission.

Source of [^{1}H]9 Not Accounted for by Residual Hydrogen in cyclohexadiene-d_4. At 50 mM cyclohexadiene-d_4 and 5 mM **6**, 20−25% of **9** produced contained unaccounted for ^{1}H. The possibility that residual **[^{1}H]9** is unrelated to **1**, and is produced from an unrelated product upon formic acid treatment of the crude photolysate, was explored by carrying out the glycosidic cleavage using deuterated formic acid. However, the ratio of **[^{2}H]9** to **[^{1}H]9** was independent of the isotopic content of the acid.[29] The possibility of a hydrogen atom transfer process in a bimolecular reaction between **1** and another nucleoside was eliminated by demonstrating that the extent of protio incorporation in **9** was independent of the concentration of **6**, and was unaffected by the addition of 2′-deoxyuridine prior to photolysis. Finally, ionic mechanisms involving **21** were eliminated on the basis of the observation that **[^{2}H]9** was not produced during photolyses of **6** in CH_3CN, 1,4-cyclohexadiene, and D_2O.



These experiments leave *N*-methylcarbazole and cyclohexadiene-d_4 as the remaining possible sources of the as yet unaccounted for **[^{1}H]9**. Semiempirical calculations suggest that the methyl group of *N*-methylcarbazole could serve as a

hydrogen atom donor to **1**.[30] Alternatively, the *N*-methylcarbazole· cation radical is similar to those of thianthrene and 9-phenylanthracene. Hence, the possibility that this cation radical reacts with nucleophilic solvents to produce cyclohexadienyl radicals was also considered.[31] Consequently, attribution of any **[^{1}H]9** to the *N*-methylcarbazole (or species derived from it) was eliminated by demonstrating that deuteration of the methyl group (**22**) or the aromatic portion (**23**) of *N*-methylcarbazole did not affect the ratio of **[^{1}H]9** to **[^{2}H]9** produced.



22 **23**

Elimination of the carbazole or any intermediate formed from it as the source of residual hydrogen in **9** leaves the 3,3,6,6-tetradeuteriocyclohexa-1,4-diene (cyclohexadiene-d_4) as the only remaining component which could serve in this capacity. Invocation of 1,4-cyclohexadiene in this capacity requires deuterium atom (hydride) shifts in cyclohexadiene-d_4, or one or more of the reactive intermediates derived from it. Deuterium atom migration increases the effective number of hydrogen atom equivalents available for abstraction by alkyl radicals by transforming sp^2 carbons containing hydrogens into sp^3 atoms containing a hydrogen and a deuterium. Neither ^{1}H NMR examination of cyclohexadiene-d_4 remaining after photolysis nor mass spectral analysis of benzene formed during the photoreaction provided evidence for this type of process. These observations suggest that any migration that occurs must be relatively small, and if this is the source of residual **[^{1}H]9**, the species responsible must be one of high energy in order to compete kinetically with a large excess of cyclohexadiene-d_4. A cyclohexadienyl radical which has undergone hydrogen migration is an example of a species that will react with **1** at the diffusion-controlled rate (several orders of magnitude greater than the rate of reaction with cyclohexadiene-d_4). If an open shell species which has undergone migration is responsible for the residual **[^{1}H]9**, then the level of protio incorporation should remain approximately constant if migration does not alter the number of transferable protio equivalents. Indeed, the ratio of **[^{1}H]9** to **[^{2}H]9** formed upon photolysis of **6** increases only slightly as **17** is increased from 50 to 200 mM (Figure 4), suggesting that a portion of **7** results from the trapping of **1** by an open shell cyclohexadiene species. The slightly positive slope of the plot in Figure 4 is fully consistent with the above mechanism. As the concentration of **17** decreases, more of **9** is formed from the open shell species, which is expected to display a smaller kinetic preference for protio transfer, resulting in a smaller **[^{1}H]9**:**[^{2}H]9** ratio.

In light of these observations, it is worthwhile to revisit the kinetic isotope effect (KIE) issue which played an integral part

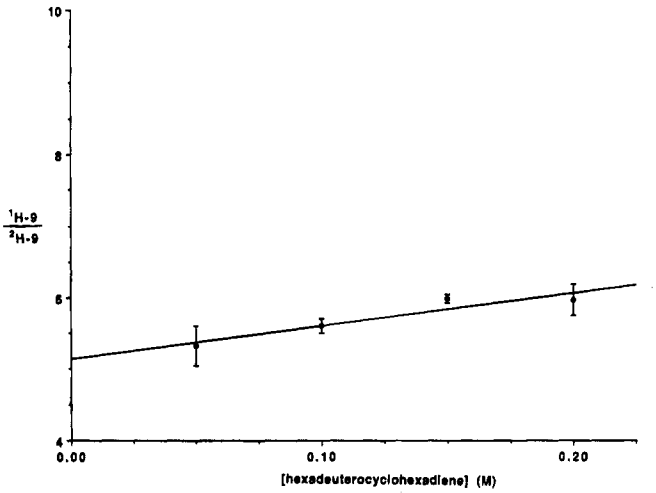
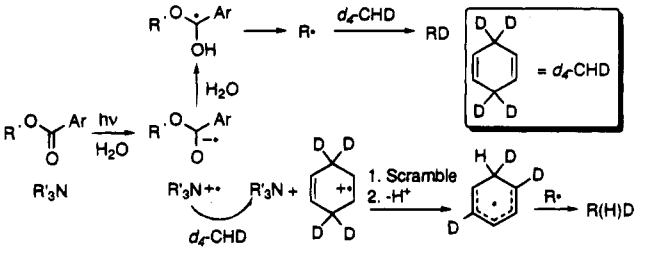


Figure 4. Dependence of the ratio of **[^{1}H]9** to **[^{2}H]9** formed from photolysis of **6** versus [**17**]. Each data point represents an average of two photolyses with replicate analyses.

Scheme 6



in estimating k_{inter} and k_{intra}. Trapping of **1** by an isomerized open shell form of cyclohexadiene-d_4 (or protiocyclohexadiene) results in the measurement of the product of a primary and secondary KIE that is larger than the true value. However, in the presence of 200 mM cyclohexadiene-d_4 (the total diene concentration in the KIE experiment) less than 6% of the total amount formed is attributable to the open shell species. Consequently, the product of the primary and secondary kinetic isotope effect and in effect the magnitudes of k_{inter} and k_{intra} may be underestimated by as much as 12%. This uncertainty does not change the biological significance of the experiments described above.

Although one expects cyclohexadienyl radical to be formed from cyclohexadiene-d_4 reacting directly with **1**, a reasonable question to ask is whether open shell species derived from cyclohexadiene are produced by other pathways. Observation by ^{1}H NMR that **6** is consumed more than twice as fast in the presence of cyclohexadiene-d_4 than in the absence of any exogenous hydrogen atom donor suggests that this is possible. One possible interpretation of the affect of cyclohexadiene on the quantum yield for disappearance of **6** utilizes the olefin as a reducing agent for the cation radical of *N*-methylcarbazole. A step by step rationalization of a mechanism (Scheme 6) that is consistent with the data presented above is described as follows.

(1) Cyclohexadiene increases the quantum yield for disappearance of **6** by competing with back electron transfer in the photochemically produced ion pair. This results in the formation of the cation radical of 1,4-cyclohexadiene. The reduction potential of 1,4-cyclohexadiene is only slightly higher than that of cyclohexa-1,3-diene, which reacts with aminium ions extremely rapidly.[32,33]

(27) Teoule, R.; Bonciel, A.; Bert, C.; Fouque, B. *J. Am. Chem. Soc.* **1974**, *100*, 6749.

(28) If the increase in thymine production was due solely to the greater yield of **7** formed during photolysis of **6** in the presence of thiol, then on the basis of the relative yields of products (**7**:**8**, Table 1) under the different conditions, one would have expected an increase of several-fold in thymine production.

(29) It is important to note that this control experiment only eliminates the formation of **[^{1}H]**thymine C5-hydrate via initial protonation of some other undefined product.

(30) Korzekwa, K. R.; Jones, J. P.; Gillette, J. R. *J. Am. Chem. Soc.* **1990**, *112*, 7042.

(31) (a) Parker, V. D.; Tilset, M. *J. Am. Chem. Soc.* **1987**, *109*, 2521. (b) Hammerich, O.; Parker, V. D. In *Advances in Physical Organic Chemistry*; Gold, V., Bethell, D., Eds.; Academic Press: New York, 1984; Vol. 20. (c) Bard, A. J.; Ledwith, A.; Shine, H. J. In *Advances in Physical Organic Chemistry*; Gold, V., Bethell, D., Eds.; Academic Press: New York, 1976; Vol. 13.

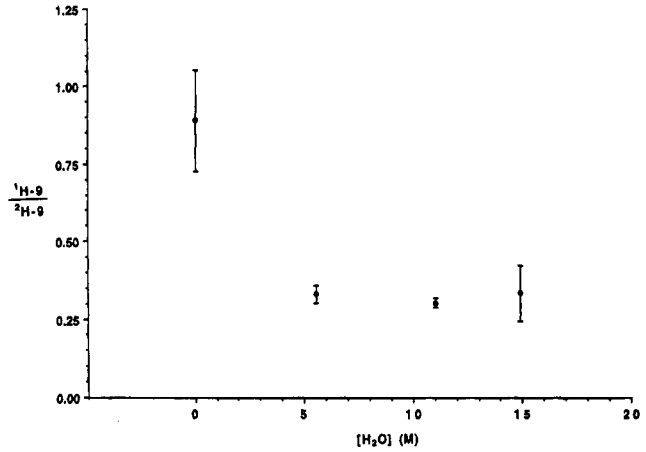


Figure 5. Dependence of the ratio of **[^{1}H]9** to **[^{2}H]9** formed from photolysis of **6** versus [H_2O]. All photolyses were carried out in the presence of cyclohexadiene-d_4 (50 mM). Each data point represents an average of two photolyses with replicate analyses.

(2) The cyclohexadienyl cation radical undergoes rapid 1,3-hydride (deuteride) transfers, which increases the number of hydrogen equivalents in the molecule. This step in the process is consistent with the fact that lifetimes for such processes in the gas phase are on the subnanosecond scale.[34]

(3) It is possible that the cyclohexadiene cation radical donates a hydrogen atom to **1**. Alternatively, the cation radical derived from cyclohexadiene-d_4 may compete with water as a proton source for the *m*-(trifluoromethyl)benzoate radical anion derived from **6**, resulting in formation of the very reactive hydrogen (deuterium) atom donor cyclohexadienyl radical.[35] This proposal is consistent with the trend in the ratio of **[^{1}H]9** to **[^{2}H]9** as a function of [H_2O] (Figure 5).

(4) The scrambled cyclohexadienyl radical (or possibly the cyclohexadienyl cation radical) reacts with **1** at diffusion-controlled rates, in competition with cyclohexadiene-d_4. The amount of carbazole cation radical formed during the course of the photolyses is independent of the amount of cyclohexadiene-d_4 present in the samples. Hence, the ratio of cyclohexadienyl radical versus cyclohexadiene increases as the overall concentration of cyclohexadiene-d_4 decreases, resulting in the observed **[^{2}H]9**:**[^{1}H]9** ratio as a function of cyclohexadiene-d_4 (Figure 3).

Conclusions

The reactivity of **1** in aqueous solvent was investigated with the aid of isotopic labeling. Hydroxyl radical adduct **1**, generated under anaerobic conditions from **6**, is trapped efficiently by mercaptans. Increasing amounts of thymidine glycol (**8**) are formed when **1** is trapped by the less efficient hydrogen atom donor cyclohexa-1,4-diene. Quantitative product analysis of anaerobic photolyses of **6** in $H_2{}^{18}O$ shows that neither dehydration of **1** nor intramolecular hydrogen atom abstraction within this radical competes with trapping by 50 mM cyclohexadiene-d_4. Estimation of k_{inter} using literature values for k_{abs} from cyclohexa-1,4-diene by alkyl radicals, and independent measurement of the kinetic isotope effect associated with cyclohexadiene-d_4, enables us to place a conservative upper limit of 2 s^{-1} on the rate constants for intramolecular hydrogen atom abstraction and dehydration within **1**. The upper limits for these processes lie in the lower range of the observed rates for nucleic acid strand scission, indicating that neither intramolecular hydrogen atom abstraction nor dehydration at neutral pH is kinetically competent to account for nucleic acid strand scission induced via **1**.

Experimental Section

General Methods. ^{1}H NMR spectra were recorded at 300, 270, or 200 MHz. IR spectra were obtained using a Perkin-Elmer 1600 Series FT-IR. GC/MS analyses were carried using a Hewlett-Packard 5988A or Hewlett-Packard 5970 mass spectrometer interfaced to a Hewlett-Packard 5890 gas chromatograph equipped with a DB-1701 or a DB-5 (30 m) fused silica column. Injections were made in the split mode (split ratio approximately 30:1) with an injection port temperature at 275 °C. In cases where isotopic ratioing was performed, data were acquired in the selected ion monitoring (SIM) mode, with a dwell time of 80 ms. This ensured that at least 10 data points were acquired during elution of individual products, which was required in order to obtain reproducible results.

All reactions were carried out in oven-dried glassware, under a nitrogen atmosphere, unless specified otherwise. Cyclohexadiene-d_4 was obtained from Merck, Sharpe and Dohme Laboratories and from Cambridge Isotopes. Deuterated acetonitrile, benzene-d_6, iodomethane-d_3 and carbazole-d_8 were obtained from Cambridge Isotopes. Silylating reagents *N*-(*tert*-butyldimethylsilyl)(trifluoromethyl)acetamide (TBDMSFA) and *N,O*-bis(trimethylsilyl)(trifluoromethyl)acetamide containing 1% (TMS)Cl (BSTFA/TMS) were obtained from Sigma. Pyridine was distilled from CaH_2. THF was distilled from Na/benzophenone ketyl. Acetonitrile was purified according to literature procedures.[36]

(5*R*,6*S*)-3′,5′-*O*,*O*′-Bis(dimethoxytrityl)thymidine Glycol (4). 3′,5′-*O*,*O*′-Bis(dimethoxytrityl)thymidine (2.0 g, 2.36 mmol) was added to *N*-methylmorpholine *N*-oxide (554 mg, 4.73 mmol) and osmium tetroxide (25 mg, 0.1 mmol) in a mixture of THF (10 mL), *tert*-butyl alcohol (5 mL), and water (2 mL). The mixture was stirred and heated in an oil bath at 40 °C. After 14 h, the solution was cooled in an ice bath, and sodium sulfite (590 mg, 4.73 mmol) was added. After 30 min, the mixture was poured into water (50 mL) and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried over sodium sulfate and concentrated in vacuo. Glycol (1.91 g, 92%) was isolated by flash chromatography using a solvent gradient (30:65:5 to 50:45:5, EtOAc−hexanes−MeOH): mp 136−139 °C; ^{1}H NMR ($CDCl_3$) δ 7.56 (br ds, 1H), 7.42−7.14 (m, 18 H), 6.76 (m, 8H), 6.26 (dd, 1H, J = 6, 8 Hz), 5.07 (s, 1H), 4.43 (d, 1H, J = 6 Hz), 3.89 (s, 1H), 3.76 (s, 6H), 3.75 (s, 6H), 3.47 (m, 2H), 3.00 (s, 1H), 2.70 (s, 1H), 2.02 (m, 1H), 1.75 (dd, 1H, J = 6, 8 Hz), 1.27 (s, 3H); IR (KBr) 3400 (br d), 2940, 2928, 1690, 1666, 1637, 1510, 1245 cm^{-1}. Anal. Calcd for $C_{52}H_{52}N_2O_{11}$: C, 70.89; H, 5.95; N 3.18. Found: C, 70.64; H, 6.17; N, 2.98.

(5*R*,6*S*) 3′,5′-*O*,*O*′-Bis(*O*-dimethoxytrityl) *m*-(Trifluoromethyl)-benzoate 5. *m*-(Trifluoromethyl)benzoyl chloride (544 mg, 2.61 mmol) was added via syringe to a solution of **4** (1.91 g, 2.17 mmol) and DMAP (531 mg, 4.35 mmol) in THF (15 mL) at −10 °C. After 2 h, the reaction mixture was poured into water (15 mL) and extracted with Et_2O (3 × 75 mL). The combined organic layers were washed with brine (50 mL), dried over $MgSO_4$, and concentrated. Protected ester **5** (1.59 g, 70%) was isolated via flash chromatography using a solvent gradient (20:75:5 to 50:45:5 EtOAc−hexanes−MeOH): mp 140−142 °C; ^{1}H NMR ($CDCl_3$) δ 8.2 (s, 1H), 8.03 (d, 1H, J = 8 Hz), 7.86 (d, 1H, J = 8 Hz), 7.75 (s, 1H), 7.61 (dd, 1H, J = 8 Hz), 7.42−7.08 (m, 18H), 6.88 (d, 4H, J = 7 Hz), 6.69 (d, 4H, J = 7 Hz), 6.30 (s, 1H), 6.18 (dd, 1H, J = 6,7 Hz), 4.30 (m, 1H), 4.13 (m, 1H), 3.80 (s, 6H), 3.68 (s, 6H), 3.27 (m, 2H), 1.53 (s, 3H), 1.07 (dd, 1H, J = 6,7 Hz), 0.89 (m, 1H); IR (KBr) 3550 (br d), 3200 (br d), 2960, 2930, 1719,

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1607, 1508, 1470, 1243, 1204 cm^{-1}. Anal. Calcd for $C_{60}H_{55}N_2O_{12}F_3$: C, 68.43; H, 5.26; N, 2.66. Found: C, 68.39; H, 5.49; N, 2.58.

(5*R*,6*S*)-*m*-(Trifluoromethyl)benzoate Ester 6. Bistrityl ester **5** (420 mg, 0.4 mmol) was stirred in a mixture (4:1) of HOAc−H_2O (10 mL) for 12 h. Following removal of solvents in vacuo, **6** (142 mg, 79%) was isolated via flash chromatography (MeOH−CH_2Cl_2, 1:19) as a foam: mp 108−109 °C; ^{1}H NMR (MeOH-d_4) δ 8.20 (m, 2H), 7.93 (d, 1H, J = 8 Hz), 7.70 (t, 1H, J = 8 Hz), 6.57 (s, 1H), 6.23 (dd, 1H J = 6, 7 Hz), 4.28 (m, 1H), 3.82 (m, 1H), 3.72 (m, 2H), 2.00 (m, 2H), 1.54 (s, 3H); IR (KBr) 3437 (br d), 2954 (w), 2926 (w), 2869 (w), 1709 (s), 1453 (m), 1335 (m), 1236 (s), 1131 (m), 1065 (m), 980 (m) cm^{-1}. Anal. Calcd for $C_{18}H_{19}N_2O_8F_3$: C, 48.22; H, 4.27; N, 6.25. Found: C, 48.41; H, 4.30; N, 6.11.

***N,N'*-Dimethyl-5,6-dihydro-5,6-dihydroxythymine.** *N,N'*-Dimethylthymine (280 mg, 1.82 mmol) was subjected to osmylation conditions described for **4**. *N,N'*-Dimethyl-5,6-dihydroxythymine (225 mg, 66%) was isolated via flash chromatography (MeOH−$CHCl_3$, 1: 19) as a colorless oil. ^{1}H NMR (MeOH-d_4) δ 4.82 (s, 1H), 3.12 (s, 3H), 3.08 (s, 3H), 1.36 (s, 3H); ^{13}C NMR (MeOH-d_4) δ 175.6, 154.5, 86.8, 73.3, 35.1, 28.1, 23.5; IR (film) 3400 (br d), 2941 (m), 1672 (s), 1482 (m), 1292 (m), 1235 (w), 1182 (w), 1056 (m) cm^{-1}. FAB HRMS for $C_7H_{12}N_2O_4$ (M + H), calcd 189.0875, found 189.0883.

***N,N'*-Dimethyl-5,6-dihydro-5-hydroxy-6-[*m*-(trifluoromethyl)benzoyl]thymine (14).** *N,N'*-Dimethyl-5,6-dihydroxythymine (200 mg, 1.06 mmol) was esterified as described for **6**. The respective *m*-(trifluoromethyl)benzoate **14** (299 mg, 83%) was isolated via flash chromatography (EtOAc−CH_2Cl_2, 1:4) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 8.18 (s, 1H), 8.08 (d, 1H, J = 8 Hz), 7.81 (d, 1H, J = 8 Hz), 7.56 (t, 1H, J = 8 Hz), 6.17 (s, 1H), 3.65 (br d s, 1H), 3.30 (s, 3H), 3.21 (s, 3H), 1.57 (s, 3H); IR (KBr) 3450 (br d), 2990 (w), 2935 (w), 1725 (s), 1687 (s), 1471 (m), 1331 (s), 1241 (s), 1171 (s), 1129 (s), 1071 (s), 980 (m). Anal. Calcd for $C_{15}H_{15}N_2O_5F_3$: C, 50.01; H, 4.20; N, 7.78. Found: C, 50.11; H, 4.30; N, 7.79.

***N,N'*-Dimethyl-5-hydroxy-5,6-dihydrothymine (16).** Ester **14** (80 mg, 0.22 mmol), *N*-methylcarbazole (40 mg, 0.22 mmol), and 2-methylpropane-2-thiol (180 mg, 2 mmol) were dissolved in 20 mL of CH_3CN−H_2O (2:1, v/v) in a 50 mL Schlenk flask. The solution was degassed (three freeze−pump−thaw cycles) and photolyzed in a Rayonet photoreactor for 3 h (λ_{max} = 350 nm). After removal of the solvents in vacuo, flash chromatography (EtOAc−CH_2Cl_2, 1:4) afforded the desired product (6 mg, 16%). The low yield of pure **16** was a consequence of difficulties encountered with separation from *N,N'*-dimethyl-5,6-dihydro-5,6-dihydroxythymine: mp 75 °C; ^{1}H NMR ($CDCl_3$) δ 3.52 (br d, 1H), 3.47 (d, 1H, J = 12.3 Hz), 3.18 (s, 3H), 3.15 (d, 1H, J = 12.3 Hz), 3.07 (s, 3H), 1.41 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 174.0, 153.0, 68.0, 54.5, 36.3, 28.2, 24.4; IR (KBr) 3404 (br d), 1714, 1667, 1491, 1421, 1287, 1179, 1052 cm^{-1}. FAB HRMS (M + H) calcd 173.0926, found 173.0922.

***N*-Methylcarbazole-d_3 (22).** Sodium hydride (40 mg, 50% dispersion in oil) was washed with hexanes (1 mL) and then suspended in THF (1.5 mL). Carbazole (100 mg, 0.60 mmol) was dissolved in THF (1 mL) and then added to the hydride suspension via cannula. After stirring for 30 min, methyl-d_3 iodide (303 mg, 2.09 mmol) was added via syringe. After 60 min, the reaction was quenched by adding EtOH (100 μL) and the reaction was poured into water (10 mL) and extracted with Et_2O (3 × 15 mL). The organic layers were combined and washed with brine solution (10 mL), dried over anhydrous $MgSO_4$, and concentrated. The product was isolated by flash chromatography (hexanes−EtOAc, 6:1) and then recrystallized once from EtOH to afford 91 mg (83%): mp 91 °C (commercial material 90−92 °C).

***N*-Methylcarbazole-d_8 (23).** Sodium hydride (10 mg, 50% dispersion in oil) was washed with hexanes (2 × 0.5 mL) and suspended in THF (1 mL). Carbazole-d_8 (25 mg, 0.14 mmol) was dissolved in THF (0.5 mL) and added to the hydride suspension via cannula. After stirring for 30 min, methyl iodide (28 mg, 0.2 mmol) was added via syringe. After 60 min, the reaction was quenched by adding EtOH (100 μL) and the reaction was poured into water (10 mL) and extracted with Et_2O (3 × 15 mL). The organic layers were combined and washed with brine (10 mL), dried over anhydrous $MgSO_4$, and concentrated. The product was purified by flash chromatography (hexanes−EtOAc, 6:1) and then recrystallized once from EtOH to afford **23** (21 mg, 77%): mp 91 °C (commercial material 90−92 °C).

1,2,3,4,5,6-Hexadeuteriocyclohexa-1,4-diene (16). To a 100 mL three-necked flask, cooled to −78 °C, was added freshly distilled (from sodium) liquid ammonia (40 mL). Anhydrous ethanol (3.7 g, 83.3 mmol) and benzene-d_6 (3.0 g, 25.2 mmol) were added sequentially, via a dropping funnel. Sodium metal (1.73 g, 75.7 mmol) was added in small pieces over 3.5 h at a rate sufficient to preserve the blue color. After 2.5 h the cold bath was removed and the ammonia was allowed to evaporate. Saturated NH_4Cl (15 mL) was added to the reaction mixture dropwise over 15 min. The resulting slurry was poured into a separatory funnel and washed three times with pentanes (5 mL). The organic layers were combined and dried over Na_2SO_4. The pentanes were removed by careful distillation using an oil bath heated to 50 °C. The residue contained 0.95 g (42%) of the product.

General Procedure for the Photolysis of 6. Stock solutions of **6**, *N*-methylcarbazole, and hydrogen atom donor were prepared in CH_3CN and stored at −20 °C. Aliquots of these solutions were loaded into Pyrex tubes and diluted to a final volume (typically 200 μL). The tubes were then connected to vacuum adapters fitted with Cajon Ultra-Torr vacuum seals and Teflon stopcocks. The solutions were degassed with three freeze−pump−thaw cycles and flame sealed. Photoreactions were carried out in a Rayonet photoreactor (model RPR-100) equipped with λ_{max} = 350 bulbs for 2 h.

General Procedure for the Mass Spectral Analysis of 9. After photolysis, the solvents were removed using a Savant Speed Vac concentrator. *N*-Methylcarbazole and *m*-(trifluoromethyl)benzoic acid were removed from the crude reaction mixture by adding distilled water (500 μL) and filtering the resulting suspension through a 0.45 μm nylon filter disk into a 1 mL Kontes reaction vial fitted with a Teflon septum. The excess water was removed on a Savant Speed Vac concentrator. Glycosidic bond cleavage was effected by treating the residue with formic acid (88%, 500 μL) at 100 °C. After 60 min, the formic acid was removed on a Savant Speed Vac concentrator. Residual formic acid was removed by repeated lyophilization from absolute ethanol (50 μL) and dried under vacuum for at least 8 h. The samples were prepared for GC/MS analysis by heating with *N,O*-bis(trimethylsilyl)-(trifluoromethyl)acetamide (containing 1% (TMS)Cl) (BSTFA; 50 μL) at 100 °C for 1 h. Aliquots (1−5 μL) of the silylated mixture were analyzed directly using a DB-1701 column. The column temperature was maintained at 190 °C for 2 min and then increased linearly at 15 °C/min. Spectra were acquired continuously after the volatile silylating reagent emerged. Compound **9** elutes 4 min after injection.

Persilylated nucleobases, including **9** often give weak parent ions under electron ionization (EI) conditions, due to facile loss of a methyl group from the trimethylsilyl moiety.[19] Consequently, the ion currents for the respective $M^+ - 15$ ions (m/z = 345, 346) of **[^{1}H]9** and **[^{2}H]9** were integrated. Care must be taken when analyzing the isotopic distribution in **9**. The competitive kinetics experiments were carried out under conditions where intramolecular hydrogen atom transfer has the greatest chance to compete with intermolecular trapping of **1**. Thymidine glycol (**8**) is the major isolable product obtained from the photolysis of **6** under these trapping conditions. Persilylation of the free base of thymine glycol (**10**) yields **11**. This product is readily separable from **9** on nonpolar capillary GC columns. However, incomplete silylation and/or adventitious hydrolysis of one silyl group from **11** produces **12** (and possibly other regioisomers). Trisilylated thymine glycol (**12**) coelutes with **11** on commonly used methyl silicone capillary columns, and fragments to yield an ion with m/z = 345, which is the ion used to measure the amount of **[^{1}H]9**. Hence, coelution of **9** and **12** results in overestimating the amount of **[^{1}H]9** formed in the photolysis mixtures. Further complications in the analysis of **9** stem from the presence of varying amounts of **13**, which also reacts under the electron ionization conditions to yield a fragment containing m/z = 345. Compound **13** arises via elimination from **11**, as demonstrated by its formation via formic acid hydrolysis and silylation of independently synthesized thymidine glycol (**8**).[17] Baseline separation was achieved using a cyanopropyl fused silica capillary GC column. Homogeneity of the peak assigned to **9** was confirmed via high-resolution mass spectral analysis.

General Procedure for the HPLC Quantitation of 7 and 8. Samples were prepared, degassed, and photolyzed as described above. After photolysis, a 5 μL aliquot of 1 mM 5-bromo-2′-deoxyuridine (internal standard) was added to 50 μL of the crude photolysate. The

solvents were removed on a Savant Speed Vac. The *N*-methylcarbazole and *m*-(triflouromethyl)benzoic acid were removed as described above. After lyophilization, the samples were redissolved in 50 μL of distilled water.

Samples were analyzed by reversed-phase HPLC on a μ-Bondapak C-18 8 × 10 RCM cartridge (Waters) using a 3% CH_3CN−water mobile phase. Both **7** and **8** were detected at 205 nm, and were quantitated using linear regions of calibration curves established using independently prepared materials.

General Procedure for the Photolysis of Dimethyl Ester 14 and Mass Spectral Analysis of Product Mixtures. The samples were prepared, degassed, and photolyzed as previously described. After photolysis, the samples were transferred to 1 mL microvials, lyophilized, and dried at high vacuum for 8 h. The samples were prepared for GC/MS analysis by heating with 100 μL of *N*-(*tert*-butyldimethylsilyl)-(trifluoromethyl)acetamide (TBDMSFA) at 100 °C for 1 h. The samples were analyzed using a 30 m DB-5 fused silica column. The underivatized product, **16**, elutes 2.9 min after injection. In cases where ^2H:^1H ratios were measured, data were acquired in the SIM mode where the M^+ ions of **[^{2}H]16** and **[^{1}H]16** (m/z = 173 and 172) were monitored. The area observed for $M^+ - 1$ (m/z = 171) of **[^{1}H]16** was less than 1% that of 172. **[^{1}H]16** was equilibrated in D_2O for 2 h, dried, and analyzed by GC/MS to ensure that ratios obtained were not influenced by the solvent used.

Quantitation of the Fraction of 6 That Undergoes Bouveault−Blanc Type Cleavage. A solution of **6** (5 mM), *N*-methylcarbazole (5 mM), and cyclohexadiene-d_4 (50 mM) in 27% $H_2{}^{18}O$ was photolyzed as previously described. The crude photolysate was lyophilized to dryness, and prepared for mass spectral analysis as described above. The ions corresponding to the M^+ of **7** and **[^{18}O]7** (m/z = 636 (raw) and 638 (raw)) were monitored. The amount of Bouvealt-Blanc type cleavage was found by correcting 638 (raw) for heavy isotopomers of 636. An experimentally determined correction factor of 0.322 was found. The area for 636 (raw) was then corrected for the **[^{16}O]7** formed from residual $H_2{}^{16}O$ in the $H_2{}^{18}O$ (4%).

The contribution of isotopic enrichment at C6 due to "washing in" under the reaction conditions was determined by allowing a sample of **[^{16}O]7** allowed to stand in 27% $H_2{}^{18}O/CH_3CN$ for 6 h. The sample was lyophilized and analyzed by GC/MS in the manner described.

Determination of the Amount of ^{18}O Incorporation in 9. A solution of **6** (5 mM), *N*-methylcarbazole (5 mM), and cyclohexadiene-d_4 (50 mM) in 27% $H_2{}^{18}O$ was photolyzed and subsequently analyzed as described above. Ions corresponding to the $M^+ - 15$ for **[^{2}H]9**, **[^{1}H,^{18}O]9**, and **[^{2}H,^{18}O]9** were monitored (m/z = 346, 347, and 348, respectively).

Quantitation of Thymine Produced via Acidolysis of Photolysates Obtained via Photolysis of 6 with Cyclohexadiene-d_4 and 2-Methylpropane-2-thiol. To samples that were photolyzed as previously described, an aliquot of 2′-deoxyuridine (10 μL, 5 mM) was added as an internal standard. The *m*-(trifluoromethyl)benzoic acid and *N*-methylcarbazole were removed as previously described. The samples were dissolved in 0.1 N HCl (500 μL) and heated to 80 °C. After 12 h, the solvents were removed in vacuo and the residue was lyophilized from ethanol (2 × 100 μL). Samples were silylated and analyzed using GC/MS as previously described. Ions corresponding to $M^+ - 15$ were monitored for thymine and uracil (m/z = 255 and 241, respectively).

Determination of the Relative Quantum Yield for Disappearence of 6 by ^{1}H NMR. Samples containing **6** (25 mM) and *N*-methylcarbazole (25 mM) with and without 1,4-cyclohexadiene (200 mM) were loaded into NMR tubes fitted with Pyrex extensions and degassed with three freeze−pump−thaw cycles and flame sealed. ^{1}H NMR spectra were recorded before and after a 20 min irradiation (Oriel lamp). The consumption of **6** was determined by comparing the ratio of the integral of the proton on C6 (δ 6.75) in **6** versus the total intensity of anomeric protons (C1′, δ 6.28−6.15) in the deoxyribose moiety.

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Supplementary Material Available: Text giving an explanation and equations for the determination of kinetic isotope effects and protio:deuterio ratios in products (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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