# Independent Generation of 5,6-Dihydrothymid-5-yl in Single-Stranded Polythymidylate. $O_2$ Is Necessary for Strand Scission†

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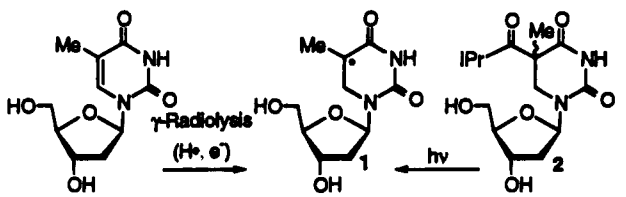
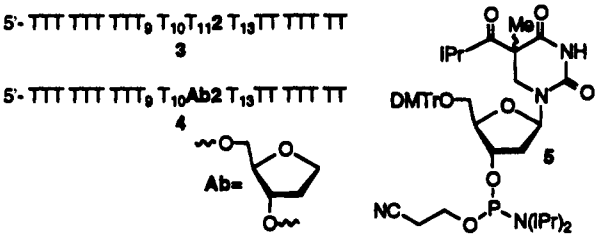
Independent generation of putative reactive intermediates is a powerful tool for the study of reaction mechanisms in organic chemistry.[1] Unambiguous generation of radical species from judiciously designed precursors has been successfully employed for testing theory, as well as examining the viability of such entities on reaction surfaces. While significant advances have been made in the elucidation of the mechanism of action of a number of nucleic acid damaging agents, only recently have these processes been examined by independent generation of the putative reactive intermediates.[2−4] Our efforts have focused on mechanistic questions resulting from studies of the effects of ionizing radiation on nucleic acids. Ionizing radiation produces a myriad of reactive intermediates within nucleic acids.[5] Consequently, elucidation of damage mechanisms at the molecular level would be facilitated by the ability to generate a single putative intermediate site specifically in biopolymers. We now report the independent generation of 5,6-dihydrothymid-5-yl (**1**) (Scheme 1) in single-stranded oligonucleotides and

**Scheme 1**




observations regarding its participation in nucleic acid strand scission.

5,6-Dihydrothymid-5-yl (**1**) is the major reactive species produced upon the reaction of hydrogen atoms or solvated electrons with thymidine. Radical **1** is also formed via the direct effect of ionizing radiation on nucleic acids, and its reactivity should be similar to that of the analogous hydroxyl radical adduct.[5,6] While the generation of **1** in nucleic acids is well accepted, there is considerable discord concerning its role in strand scission.[4a,6a,7] Radical adduct **1** is believed to amplify DNA damage by inducing strand breaks in single-stranded nucleic acids via abstracting a hydrogen atom from either the C1′ or C2′ position of the 5′-linked nucleoside.[6a,7] Producing **1** through Norrish type I photocleavage of **2** enabled us to show that 5,6-dihydrothymid-5-yl does not abstract hydrogen atoms from isopropyl alcohol or glycolaldehyde dimethyl acetal.[4a] These observations suggest that **1** is incompetent at inducing nucleic acid strand scission via direct hydrogen atom abstraction from a sugar moiety of an adjacent nucleotide.




In order to unequivocally address this issue, **2** was site specifically incorporated into chemically synthesized oligonucleotides using previously reported methodology.[8] A diastereomeric mixture of **2** was introduced into **3** and **4** as its β-cyanoethyl phosphoramidite (**5**). Evidence that **2** remained intact was gleaned from GC/MS analysis of formic acid cleaved material.[9] Irradiation of 5′-$^{32}$P-labeled **3** or **4** under anaerobic conditions for 6 h did not induce direct strand breaks, or alkaline labile lesions (Figure 1).[10,11] In marked contrast, irradiation of **3** under aerobic conditions results in direct strand scission and alkaline labile lesions (Figure 1). Enzymatic analysis indicates that cleavage produces oligonucleotides containing 3′ terminal phosphates.[10] Strand breaks are induced at the position where **1** is generated, and they continue in the 5′ direction through $T_{10}$.[12] Identical cleavage patterns are observed upon separate incorporation of (*5R*)-**2** and (*5S*)-**2** into **3**, indicating that strand scission does not involve the excited state of **2**.[9] The proximity of the deoxyribose moiety of $T_{11}$ to the peroxyl radical derived from **1**, as revealed by inspection of a model of the oligonucleotide, is consistent with the observed cleavage pattern.

In order to probe the source of cleavage at $T_{10}$ in **3**, $T_{11}$ was replaced by a tetrahydrofuran moiety (**Ab** in **4**) as a model of an abasic site.[13] The tetrahydrofuran moiety changes two parameters in **4**. In addition to eliminating the thymine moiety at $T_{11}$ in **3**, **Ab** changes the inherent reactivity of the C1′ hydrogen atom at $T_{11}$. The damage at $T_{10}$ relative to the total damage induced during photolysis is significantly diminished

† Dedicated to Professor Jerome A. Berson.

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(10) Oligonucleotides were radioactively labeled at the their 5′ termini using [$\gamma$-$^{32}$P]-ATP and polynucleotide $T_4$ kinase. Photolyses were analyzed via 20% denaturing polyacrylamide gel electrophoresis (PAGE). 3′-$^{32}$P labeling of single-stranded oligonucleotides was carried out using terminal deoxynucleotidyl transferase and [$\alpha$-$^{32}$P]-ddATP. Standard procedures for enzymatic labeling and analysis by PAGE can be found in the following: Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: New York, 1989.

(11) All samples (10 mM phosphate buffer, pH 7.0; 100 mM NaCl) were irradiated in Pyrex tubes for 6 h inside the chamber of a Rayonet RPR-100 photoreactor equipped with $\lambda_{max}$ = 350 nm lamps. Anaerobic samples were subjected to 3 "freeze−pump−thaw" degas cycles and sealed.

(12) Densitometric analysis: photolysis of **3**: nonpiperidine, $T_{10}$:$T_{11}$:**2** = 1.0:2.1:1.0; piperidine, $T_{10}$:$T_{11}$:**2** = 1.0:1.7:1.0. Photolysis of **4**: nonpiperidine, $T_{10}$:**Ab**($T_{11}$):**2** = 1.0:5.0:1.2: piperidine, $T_{10}$:**Ab**($T_{11}$):**2** = 1.0: 3.1:1.2. The extent of cleavage in **3** and **4** observed following piperidine treatment is 2−3 times greater than that resulting from direct strand breaks. The ratios are averages of 6 experiments. Densitometry was carried out using software from Technology Resources Inc., Nashville, TN 37212. Images were recorded using a CCD camera. All band intensities are within the linear range of the film. Photographs of autoradiograms and tables of densitometric data are included in the supporting information.

(13) The requisite β-cyanoethyl phosphoramidite was purchased from Glen Research Inc., Sterling, VA.

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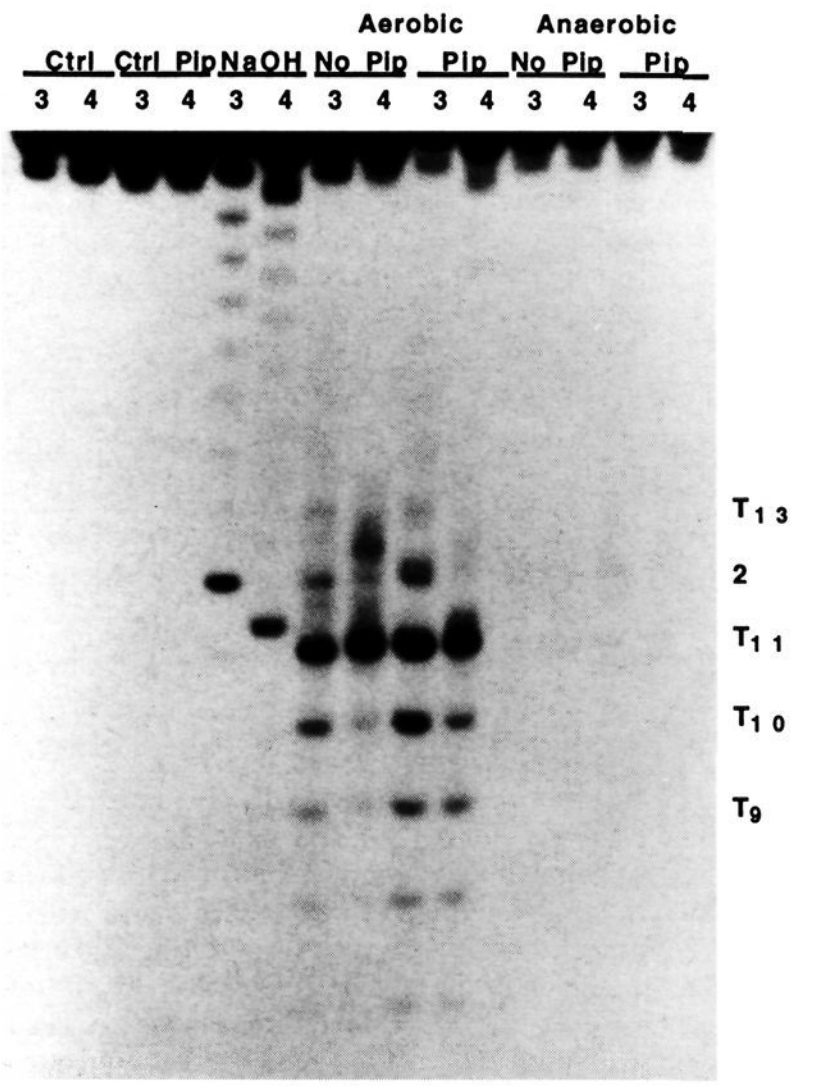


**Figure 1.** Denaturing 20% polyacrylamide gel electrophoresis of [5′-$^{32}$P]-**3** and [5′-$^{32}$P]-**4**. NaOH induces strand scission at **2**. Cleavage sites marked on the right side of the gel correspond to cleavage at the indicated nucleotides in **3**. Note that the presence of **Ab** in **4** increases the migration of fragments of this oligonucleotide containing **Ab** (e.g., **2**, $T_{13}$).

in **4** compared to that in **3**.[12] In addition, a small amount of direct strand scission, which disappears upon piperidine treatment, is observed at $T_{13}$ in **4**. Models suggest that replacement of $T_{11}$ by **Ab** does not significantly alter access of the peroxyl radical derived from **1** to $T_{10}$. Hence, diminution of strand damage at $T_{10}$ in **4** is not attributable to direct reaction between the $O_2$ adduct of **1** and the deoxyribose moiety of this nucleotide. The effect of the abasic moiety on the cleavage pattern, as well as the absence of cleavage at $T_{13}$ in **3,** makes the invocation of a diffusible species to explain cleavage at this distal site ($T_{10}$) unlikely.[14] We also believe that the effect of **Ab** on the cleavage pattern implies that the pyrimidine moiety is involved in the propagation of oligonucleotide damage. These interpretations are supported by results obtained using 3′-$^{32}$P-labeled **3**.[9]

(14) The involvement of a diffusible species in the observed cleavage pattern can not be eliminated completely. This is particularly true for the less intense cleavage products observed in Figure 1.

Additional information on the reactivity of the $O_2$ adduct of **1** is gleaned from **4**. Since **Ab** reduces the reactivity of C1′ at $T_{11}$, the appearance of strand cleavage at $T_{13}$ in **4** implies that the peroxyl radical derived from **1** abstracts the C1′ hydrogen from $T_{11}$ in **3**. Substitution of **Ab** for $T_{11}$ results in an increase in the relative reactivity of $T_{13}$ in **4**. Furthermore, the disappearance of the cleavage product at $T_{13}$ in **4** upon piperidine treatment suggests that damage at $T_{13}$ and damage at the original site of **1** are interdependent rather than the result of a randomly reacting diffusible species.

The results described above obtained under anaerobic conditions confirm that **1** is incompetent at inducing nucleic acid damage via abstracting hydrogen atoms from adjacent nucleotides within single-stranded DNA.[4a] The induction of strand breaks and alkaline labile lesions upon photolysis under aerobic conditions is consistent with previous proposals involving the formation of peroxyl radicals from nucleobase radicals, which subsequently participate in internucleotidyl hydrogen atom abstraction reactions.[15]

These observations raise new questions regarding the oxidative damage of nucleic acids. For instance, the mechanism by which strand scission propagates in the 5′ direction of a polythymidylate merits further investigation, as well do the products at the cleavage sites. Nonetheless, the independent generation of 5,6-dihydrothymid-5-yl (**1**) site specifically in oligonucleotides unambiguously shows that $O_2$ is required for the production of strand breaks arising through this reactive intermediate. Furthermore, these experiments support proposals regarding the amplification of DNA damage that is initiated through the formation of nucleobase-centered radicals.[16] The ability to independently generate reactive intermediates site specifically within nucleic acids, employed in conjunction with other mechanistic probes, will facilitate the elucidation of these and other chemical processes involving biopolymers.

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**Supporting Information Available:** Experimental procedures for the preparation of **5**, GC/MS chromatogram of the nucleobase constituents of **3**, autoradiograms of [5′-$^{32}$P]-**3** containing (*5R*)-**2** or (*5S*)-**2** separately and [3′-$^{32}$P]-**3**, and densitometric results from **3** and **4** (21 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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