Acyl Migration in the Production of Thymine Propenal from 3'-*O*-Benzoyl-5'-deoxy-4'-hydroperoxythymidine: A Reinterpretation of a Putative Model for Bleomycin-Mediated DNA Degradation

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Received June 22, 1990

Studies of Saito et al. (Saito, I.; Morii, T.; Matsuura, T. *J. Org. Chem.* **1987**, *52*, 1008) analyzing the decomposition of 3'-*O*-benzoyl-5'-deoxy-4'-hydroperoxythymidine (**7**) claimed to model the decomposition of the putative 4'-hydroperoxynucleotide intermediate in the bleomycin (BLM) mediated production of base propenal, 3'-phosphoglycolate, and 5'-phosphate termini from double-stranded DNA. A number of puzzling observations reported in this paper prompted a reinvestigation of this model system in detail. [4'-$H^{18}O_2$]-**7** and its 4' epimer **8** were prepared and their fate in aqueous solution as a function of pH was examined. Compound **7** decomposed in aqueous solution to produce thymine propenal accompanied by stoichiometric formation of benzoate containing 1 atom of ^{18}O. In addition, thymine accompanied by stoichiometric amounts of malondialdehyde and [^{18}O]benzoate was also observed. Acetate containing 1 atom of ^{18}O accompanied production of both thymine and thymine propenal. The ratio of thymine propenal to thymine varied as a function of pH and temperature. Production of [^{18}O]benzoate and a detailed kinetic analysis of the decomposition of **7** unequivocally demonstrated that conversion of **7** to thymine propenal required the intermediacy of a 4'-perbenzoate ester. This perester produced by migration of the 3'-benzoyl blocking group of **7** to the terminal oxygen of its 4'-hydroperoxy moiety would then greatly facilitate heterolytic cleavage of the oxygen–oxygen bond. For stereochemical reasons a similar intramolecular benzoyl migration cannot occur with **8**, explaining its lack of reactivity. These results call into question the relevance of the model proposed by Saito et al. to understanding the base propenal pathway in the BLM-catalyzed degradation of DNA. In addition, preparation of a second model of a putative intermediate in the base propenal pathway, [1-[[2-(acetyloxy)acetyl]oxy]-3-oxopropyl]thymine (**12**), is reported. The detailed kinetics of its decomposition as well as identification of the products accompanying its decomposition are reported. The relevance of these two model systems to the mechanism of degradation of DNA by BLM is discussed.

Introduction

The bleomycins (BLM)[1] are a family of glycopeptide antibiotics isolated from *Streptomyces verticillus* in the late 1960s.[1,2] Their cytotoxicity is thought to be related to their ability to bind to double-stranded DNA and, in the presence of metal and O_2, mediate DNA strand scission.[2-4]

Numerous studies from several laboratories have demonstrated that incubation of Fe^{+2}, O_2, and a reductant with BLM results in the production of "activated BLM," which leads to two monomeric products: nucleic acid base and nucleic acid base propenals.[5-8] Formation of base propenals requires O_2 in addition to that required to form activated BLM and is accompanied by DNA strand scission and production of 3'-phosphoglycolate and 5'-phosphate termini (Figure 1, A).[9,10] Production of nucleic acid base is accompanied by formation of an oxidatively damaged sugar (4'-ketone 1'-aldehyde, Figure 1, B) within an intact DNA strand that undergoes phosphodiester bond cleavage only after "alkaline" treatment.[11,12]

A modification of the mechanism proposed by Giloni et al. to account for the base propenal pathway is outlined in Figure 2.[10] Studies using [4'-^{3}H]DNA models and more recently [4'-^{2}H]DNAs have established that the first step in the reaction involves cleavage of the 4' carbon–hydrogen bond to produce the "putative" C-4' radical **1**.[13,14] This intermediate can be trapped by O_2 to produce 4'-peroxy radical **2**. This radical may then be reduced to yield 4'-hydroperoxide intermediate **3**. Compound **3** has been proposed to undergo a Criegee rearrangement and eventually collapse to produce 3'-aldehyde intermediate **5**, the direct precursor to the observed products.

The conversion of **3** to **4** (Figure 2) is chemically unattractive because degradation of DNA by BLM occurs in neutral aqueous solution. Previous work has indicated that a Criegee-type rearrangement requires strong acid catalysis to effect heterolytic oxygen–oxygen bond scission.[1] Recent studies of Saito et al.,[15] analyzing the decomposition of 3'-*O*-benzoyl-5'-deoxy-4'-hydroperoxythymidine (**7**) (Figure 3), a model for the decomposition of **3** (Figure 2), seemed

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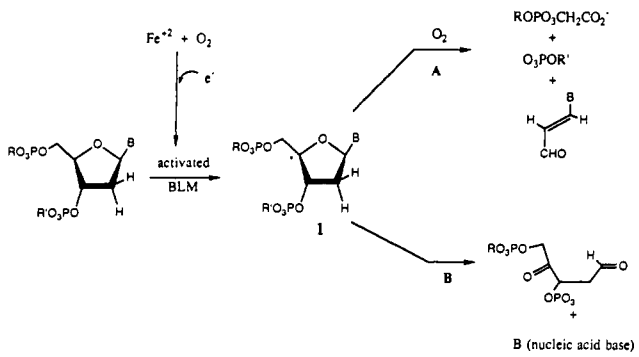

Figure 1. Degradation of DNA via a bleomycin-induced 4′-radical.

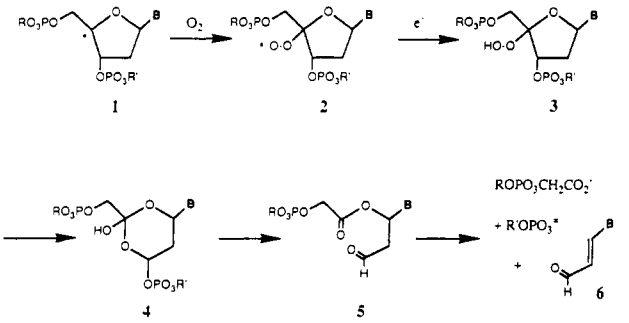

Figure 2. Proposed mechanism (pathway A, Figure 1) for the production of base propenals, 3′-phosphoglycolate, and 5′-phosphate termini.

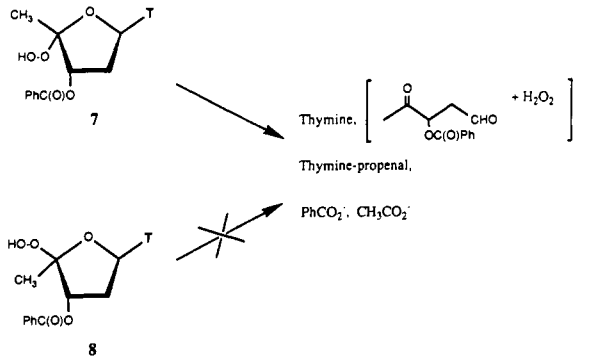

Figure 3. Aqueous decomposition of the isomeric 4′-hydroperoxynucleosides **7** and **8** as described by Saito et al.[15]

to alleviate these concerns when they demonstrated that in aqueous acetonitrile (pH 6 to 8) **7** could decompose to produce thymine propenal and thymine. A puzzling and unexplained observation also reported by Saito and coworkers was that the corresponding 4′*R* isomer **8** (Figure 3) was stable under conditions in which **7** decomposed. Since there should be no stereochemical imperative for the Criegee rearrangement in this five-membered ring system, the observed differences in chemical reactivity between the stereoisomers suggested to us an alternative explanation for their results. We propose in the case of **7** that the 3′-benzoyl group can migrate to the terminal oxygen of the hydroperoxide to produce perester **9** (Figure 4). A similar intramolecular rearrangement could not occur with the 4′*R* isomer **8** due to geometric constraints. In contrast to hydroperoxides, peresters (**9**, Figure 4) are known to undergo facile heterolytic cleavage under mild conditions.[30] If in fact, perester **9** is the precursor to thymine propenal, then the validity of using this reaction to model the BLM-mediated DNA degradation must be questioned.

In the present paper, evidence is presented using [4′-$H^{18}O_2$]-**7** that unequivocally demonstrates that the benzoyl migration postulated in Figure 4 is required for thymine

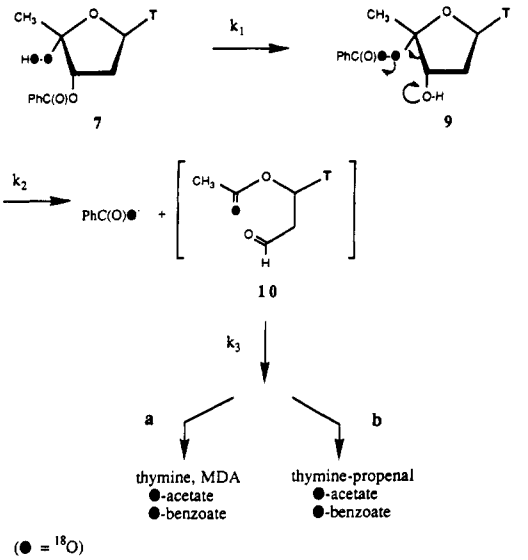

Figure 4. Proposed mechanism of formation of the observed breakdown products of [$H^{18}O_2$]-**7** in aqueous solution.

propenal production. In addition, we have synthesized a model (**12**) for a second "putative" intermediate **5** (Figure 2). The detailed kinetics of decomposition of both **7** and **12** as well as the isolation and identification of products formed are reported. The relevance of these model systems as aids to our understanding of the mechanism of degradation of DNA by BLM is discussed.

Experimental Section

Materials and Methods. Acetoxyacetic acid, α,*p*-dibromoacetophenone, and thiobarbituric acid were obtained from Aldrich. Tetrahexylammonium bromide (Sigma) was converted to the hydrogen sulfate salt by ion exchange chromatography. $H_2{}^{18}O$ (95 atom %) and $^{18}O_2$ (95 atom %) were from MSD isotopes and Amersham, respectively.

Malondialdehyde,[16] 3-acetoxypropenal,[17] and *trans*-1-(3-oxoprop-1-enyl)thymine (thymine propenal)[18a] were prepared as previously described. Ozone was assayed iodometrically.[19] Analytical HPLC separations employed Alltech C_{18} reverse phase columns (10 μm, 1 × 25 cm) and elution with 5 mM ammonium acetate (pH 5.5) and CH_3OH increasing from 0 to 50% over 25 min. The flow rate was 1.5 mL min^{-1}. The following retention times and extinction coefficients were used to identify and quantitate products: malondialdehyde, 3.5 min, ϵ_{267} = 31 800 M^{-1} cm^{-1}; thymine, 11.0 min, ϵ_{267} = 7900 M^{-1} cm^{-1}; thymine propenal, 19.8 min, ϵ_{303} = 26 300 M^{-1} cm^{-1}; benzoic acid, 6 min, ϵ = 11 500 M^{-1} cm^{-1}; and 1-(5-*O*-acetyl-2,3-dideoxy-3,4-didehydro-β-D-*erythro*-pentofuranosyl)thymine (**11**), 26.9 min, A_{267} = 8500 M^{-1} cm^{-1}.

GC-mass spectra were obtained on a 30-m Suppelco fused silica capillary column (0.32-mm i.d.) with a 1-μm thickness DB-5 (nonpolar) bonded stationary phase. Injections were made in splitless mode with the injector port maintained at 260 °C. Specific column temperature gradients used are outlined in the appropriate sections below. The mass spectrometer was scanned continuously from 600 to 30 amu at 0.3 s/decade. Between 3 and 10 complete spectra were acquired as each peak eluted and ion intensities were obtained from these as average values after background subtraction.

1-(3-*O*-Benzoyl-2,5-dideoxy-4-([^{18}O]hydroperoxy)-β-D-*glycero*-pentofuranosyl)thymine (7). Aqueous $H_2{}^{18}O_2$ (~0.5

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M in dilute HCl) was prepared from $^{18}O_2$ as described by Sawaki and Foote[20] (25% yield) and the isotope content was determined by the method of Ortiz de Montellano.[21] One milliliter of the aqueous peroxide was added dropwise to a vigorously stirred suspension of $MgSO_4$ in 20 mL of ether. The ether was decanted and dried again with $MgSO_4$. The $H_2{}^{18}O_2$ concentration was determined iodometrically[19] and then the ether solution was reduced in volume to give a final concentration in $H_2{}^{18}O_2$ of ~2.5 M. The ethereal $H_2{}^{18}O_2$ was used to prepare a mixture of isomeric hydroperoxides 7 and 8 from 1-(3-*O*-benzoyl-2,5-dideoxy-β-D-*glycero*-pent-4-enofuranosyl)thymine as described by Saito and co-workers.[15] The crude product mixture was resolved by preparative normal-phase HPLC on a silica gel column (Alltech, 10 μm, 1 × 25 cm) eluted with 2% methanol in absolute chloroform at a flow rate of 2.5 mL/min. The 4′*R* (8) and 4′*S* (7) hydroperoxides, obtained in a 1:3 ratio, eluted at 9.0 and 10.3 min, respectively, and were stored in dry chloroform at 4 °C. The solutions were assayed spectrophotometrically using the reported extinction coefficients.[15]

1-(3-([^{18}O]Oxo)prop-1-enyl)thymine. A 50-mg sample of [^{16}O]thymine propenal was heated in a sealed vial for 4 h at 80 °C with 200 μL of 1:1 $H_2{}^{18}O$ and acetonitrile containing 1% concentrated HCl. The solvents were removed and the product was dried under vacuum. Isotope content was determined by reducing a small quantity of the propenal with $NaBH_4$ in methanol. After being quenched with acetic acid and evaporating the methanol, the [^{18}O]propenol was dissolved in water and isolated on a Waters reverse phase C_{18} SepPak. The propenol was eluted with methanol, dried, and converted to a TMS derivative for GC-MS analysis as described below.

1-(5-*O*-Acetyl-2,3-dideoxy-3,4-didehydro-β-D-*erythro*-pentofuranosyl)thymine (11). Acetic anhydride (1 mL, 10 mmol) and 1-(2,3-dideoxy-3,4-didehydro-β-D-*erythro*-pentofuranosyl)-thymine (223 mg, 1 mmol)[23] were combined in 10 mL of dry pyridine and left standing at 5 °C overnight. The mixture was partitioned between water and chloroform and the organic phase was separated and washed with saturated $NaHCO_3$ and then water. After drying over $MgSO_4$, the chloroform was evaporated and the product was purified by flash chromatography (silica gel/1% methanol in chloroform). The product was obtained as a colorless oil and stored at −20 °C: ^{1}H NMR (Brücker 250 MHz, $CDCl_3$) δ 9.92 (1 H, s), 7.08 (1 H, d, J = 0.7 Hz), 6.77 (1 H, m), 5.17 (1 H, br s), 4.68 (2 H, s), 3.28 (1 H, m), 2.64 (1 H, m), 2.12 (3 H, s), 1.94 (3 H, s); UV (EtOH) λ_{max} = 265 nm (ϵ = 8500 M^{-1} cm^{-1}).

Ozonolysis of 11. In a series of experiments, 25 to 50 μmol of ozone was passed through a rapidly stirring solution of 11 (25 μmol) in 5 mL of dry methylene chloride at −45 °C. After an additional 5 min of stirring at −45 °C, 2 equiv of dimethyl sulfide (50 to 100 μmol) was added and the mixture was allowed to warm slowly to room temperature. The solvent was evaporated at 10 °C and the residue was redissolved in dry $CDCl_3$.

Analysis of the samples by ^{1}H NMR indicated that treatment of 11 with 1 equiv of ozone resulted in a 1:1 mixture of the starting material and a product having a spectrum consistent with the aldehyde 1-[[[2-(acetyloxy)acetyl]oxy]-3-oxopropyl]thymine (12): ^{1}H NMR (Brüker 250 MH, $CDCl_3$) δ 9.72 (1 H, s), 9.02 (1 H, br s), 7.18 (1 H, s), 6.89 (1 H, t, J = 6.4 Hz), 4.64 (1 H, s), 4.62 (1 H,s), 3.44 (1 H_A, 9, J_{AB} = 18.6 Hz, J_{AX} = 7.7 Hz, J_{BX} = 8.1 Hz), 3.38 (1 H_B, 9, J_{AB} = 18.6 Hz, J_{AX} = 7.7, J_{BX} = 8.1 Hz), 2.17 (3 H, s), 1.95 (3 H, s).

Approximately 2 equiv of ozone was necessary to completely oxidize the starting material, but NMR analysis showed the product to be of inferior purity. Even partially ozonolyzed samples of 11 usually contained ~5% free thymine as a byproduct. Chromatography of 12 led to extensive decomposition and solutions in $CDCl_3$ could be stored at 5 °C without significant degradation for no more than 2–3 days.

Samples of the ozonolyzed product used for aqueous decomposition studies were prepared by partial ozonation (1 equiv of O_3) as described above, reduced in volume to obtain a final concentration of about 50 mM (11 and 12), and used within 24 h.

Reaction of Ozonation Product with Triethylamine. A 25-μmol sample of 11 was partially ozonated with an equivalent amount of ozone as described above. After addition of dimethyl sulfide and warming to room temperature, an excess (7 μL, 50 μmol) of dry triethylamine was added. The solution was stirred for 15 min and evaporated to dryness under vacuum. Analysis of the residue by ^{1}H NMR in DMSO-d_6 revealed it to consist of approximately equivalent amounts of unreacted 11, thymine propenal, and triethylammonium acetoxyacetate. This was established by direct comparison with spectra of the authentic materials. The products were taken up in 250 μL of H_2O and an aliquot was withdrawn for determination of total malondialdehyde (MDA) equivalents using the TBA assay.[9] The remainder was analyzed by reverse phase HPLC as described above. The amount of thymine propenal observed was equivalent to the total amount of TBA-reactive material in the sample.

Decomposition of 7 and 12 in Aqueous Solutions: Product Analysis by HPLC. Aliquots (0.25 μL) of a 25 mM solution of 7 or 12 (in $CHCl_3$) were evaporated at 5–10 °C under a stream of dry argon. The compound was then redissolved with vigorous stirring in 1 mL of aqueous buffer containing 35% (vol) acetonitrile.[15] When the reaction was complete, the acetonitrile was removed by evaporating to a final volume of 250 μL. A small portion of this solution was assayed for total MDA equivalents using TBA and the remainder was analyzed by reverse phase HPLC as described above. In the case of 12, the amount of thymine determined by HPLC analysis was adjusted to account for its presence as a contaminant in the original sample. The percentage of thymine in solutions of 12 was determined by HPLC analysis after triethylamine treatment (see above).

Isotopic Analysis of Products. A 100-μL aliquot of a stock solution of 7 or 12 (~25 mM in $CHCl_3$) was evaporated to dryness at 5–10 °C under a stream of dry argon. The substrate was then dissolved by vigorous stirring for several minutes in 2 mL of an aqueous buffer containing 35% (vol) acetonitrile. In experiments employing $H_2{}^{18}O$, solutions were prepared by first lyophilizing the desired H_2O–CH_3CN buffer and then reconstituting with $H_2{}^{18}O$–CH_3CN. The solution was then incubated at 25 °C for 1 to 10 reaction half-lives before product isolation and analysis. Workup of the samples for derivatization and mass-spectrometric analysis was dependent on the product(s) to be analyzed and is described subsequently.

(a) Benzoate and Thymine Propenol. After the desired incubation period (see Results section), solutions of 7 were quenched by rapidly cooling on ice and adding 2 mg of $NaBH_4$. After stirring for 5 min, the excess borohydride was destroyed by carefully adding acetic acid to give an apparent pH 4. Acetonitrile was then removed by evaporating to a final volume of 0.5 mL and the resulting aqueous solution was eluted onto a Waters reverse phase C_{18} SepPak. The column was washed with 1 mL of water and the products were eluted with 2 mL of 1% methanolic NH_4OH. The products were dried in a microderivatization vial, after removing the methanol, by repeatedly adding and evaporating small portions of anhydrous acetonitrile. The dried thymine propenol and ammonium benzoate were then converted to TMS derivatives by heating for 1 h at 80 °C in a sealed vial with 20 μL of 50% *N,O*-bis(trimethylsilyl)trifluoroacetamide/1% trimethylchlorosilane in acetonitrile. Aliquots of 1–3 μL were analyzed directly by GC-MS. Column temperature was increased from 60 to 280 °C at a rate of 10–20 °C per minute after sample injection.

Percent incorporated ^{18}O in the TMS benzoate was computed from the relative intensities (I) of the M − CH_3 ions at m/e 179 and 181 according to eq 1, which includes a correction[24] for the

$$\%\ ^{18}\text{O excess} = \frac{I_{181} - 0.05I_{179} \times 100}{0.95I_{179} + I_{181}} \quad (1)$$

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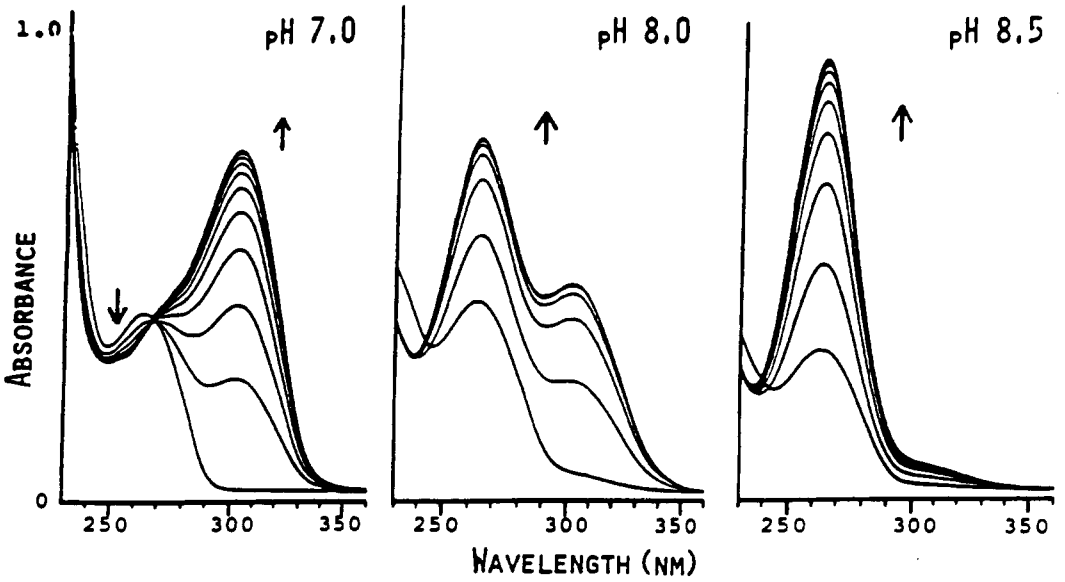

Figure 5. UV spectral changes associated with the decomposition of 7 in aqueous acetonitrile (35%) solutions at varying pH values.

occurrence of natural abundance ions at m/e 181. The percent incorporated ^{18}O in the thymine propanol was derived in an analogous manner from the $CH_2OSi(Me)_3$ fragment (m/e 129 and 131) ions of the 3-*O*-TMS-thymine propenol derivative.[18]

In control experiments, samples of authentic [3-^{18}O]thymine propenal were subjected to the same reaction conditions and workup to determine the extent of ^{18}O washout due to exchange with the solvent.

(b) Acetate. The acetate resulting from the decomposition of 7 was analyzed as the *p*-bromophenacyl ester by using an extractive alkylation technique described by L'Emeillat et al.[25] Samples of 7 were decomposed in 50 mM potassium phosphate buffer (pH 6.0 or 8.0) in 35% aqueous acetonitrile as described above. After complete reaction, the co-solvent was removed by evaporation. The remaining solution was acidified to pH 6 with H_3PO_4 and stirred vigorously for 16 h at 50 °C in a sealed vial with 1 mL of methylene chloride containing 36 mM α,*p*-dibromoacetophenone and 1 mM tetrahexylammonium hydrogen sulfate. The organic layer was separated, dried over Na_2SO_4, and concentrated to ~100 μL. Aliquots (1–2 μL) were analyzed directly by GC-MS. The GC column temperature was maintained at 50–60 °C for 5 min and then increased at a rate of 5 °C per min. The percent incorporated ^{18}O in the acetate derivative was determined from the intensities of the CH_3CO^+ fragment ions appearing at m/e 43 and 45 by using eq 2, which includes corrections for natural abundance as well as the retention of only half of the carboxylate ^{18}O in the CH_3CO^+ fragment.

$$\%\ ^{18}O\ \text{excess} = \frac{2(I_{45} - 0.04I_{43}) \times 100}{I_{45} + 0.96I_{43}} \qquad (2)$$

(c) Kinetics. Aqueous solutions for kinetic studies contained 50 mM buffer at the desired pH and NaCl to adjust the total ionic strength. Approximately 1–2 μL of the substrate (25 mM in $CHCl_3$) was dissolved in 1 mL of buffer in a UV cuvette thermostatted at 25 °C. Reaction progress was monitored by measuring the absorbance at 245, 267, and 303 nm until stable infinity values were reached. First-order rate constants were obtained from the slopes of plots of log ($A_\infty - A_t$) versus time, where A_t and A_∞ are absorbance values at time t and infinity, respectively. In the case of compound 7, where biphasic kinetics were observed, rate constants for the fast phase were calculated from absorbance–time data collected during the first 3–4 half-lifes by the Guggenheim method.[26] Rate constants for the second, slow phase were calculated as described for the general case above after editing off the early time points associated with the fast phase.

After the reaction was complete, the amount of thymine propenal was determined from the absorbance at 303 nm. Total malondialdehyde equivalents (free malondialdehyde + thymine propenal) were obtained by assaying an aliquot with TBA,[18] and the amount of thymine was established by subtracting the amount of thymine propenal from the total MDA. The method was shown to be valid by previous HPLC quantitation of products at several pH values.

Results

Preparation of [4′-H$^{18}O_2$]-7: Identification and Quantitation of Its Decomposition Products in Aqueous Solution. The nucleoside was prepared in which the peroxyl oxygens were labeled with ^{18}O to probe the detailed mechanism of the decomposition of hydroperoxide 7. The two-step chromatographic purification of the isomeric peroxides described by Saito and coworkers[15] was replaced by a more rapid and direct resolution of the epimers by silica gel HPLC. As previously reported, the 4′*S* hydroperoxide 7 decays in aqueous solution to give thymine, thymine propenal, and acetic and benzoic acids. A fifth major product detected was malondialdehyde (MDA), surprisingly overlooked in the previous study, which accounts for the fate of the 1′, 2′, and 3′ carbon atoms of 7 after the release of thymine. This is inconsistent with the hypothesis of Saito et al. that thymine is accompanied by the production of H_2O_2 and 3-(benzoyloxy)-4-oxopentanal. In the present study, neither of these products was observed. The results of a quantitative HPLC analysis of the products (except acetate) at pH 7.0 in 35% (vol) acetonitrile–water are shown in Table I. From these data it is apparent that thymine and MDA are produced in equivalent amounts and that the benzoic acid produced is equal to the total yield of thymine (or MDA) and thymine propenal combined. This is consistent with the occurrence of two modes of breakdown of 7 (Figure 4).

Kinetics of the Breakdown of 7. The kinetics of the production of both thymine and thymine propenal were examined in detail. The relative proportion of thymine produced (Figure 4, pathway a) is enhanced with increasing pH, as is the overall rate of the reaction. The increase in thymine and MDA production is illustrated in Figure 5, which shows the UV spectral changes accompanying the reaction at increasing pH values. At pH 7, thymine propenal (λ_{max} = 303 nm) is the major product, while at

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Table I. Products from the Decomposition of 7 in Aqueous Solution[a]

product	no. of moles
MDA	7.7[b]
	7.6[c]
benzoic acid	35.5[b]
thymine	8.0[b]
thymine propenol	28.0[b]
	27.7[c]

[a] At 25 °C in 40 mM phosphate buffer–35% (vol) CH_3CN. Products isolated by HPLC. [b] Direct spectrophotometric determination. [c] Determined by TBA assay.

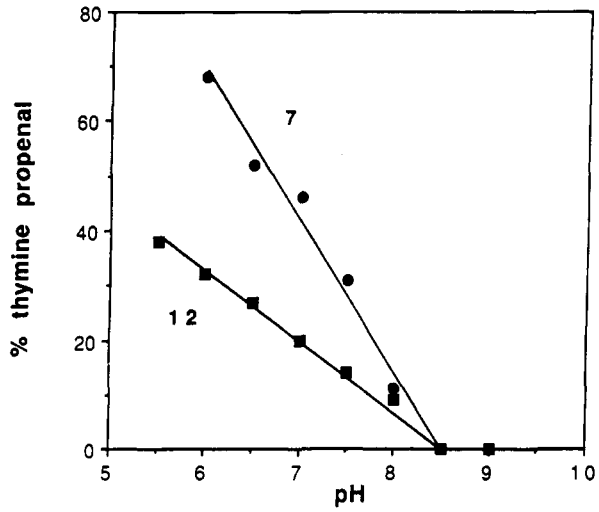


Figure 6. Product–pH profile for the decomposition of 7 and 12 in aqueous solution (μ = 0.5, 25 °C).

pH 8.5 thymine and MDA (λ_{max} = 267 nm) are the predominant products. More complete data, obtained in 100% aqueous solution at 25 °C and ionic strength = 0.5, are shown in Figure 6. The relative amount of thymine propenal (Figure 4, pathway b) decreased in an approximately linear fashion with increasing pH under these conditions.

Kinetic studies of the decomposition of 7 were carried out by following the UV absorbance changes associated with the reaction at several wavelengths (245, 267, and 303 nm). Contrary to the previous report,[15] isosbestic points were not observed. Rather, the absorbance changes at all wavelengths were biphasic. This is illustrated in Figure 7. At the longer wavelengths, an initial "lag" period was observed, and this was followed by a first-order increase in absorbance. At 245 nm, instead of the "lag" phase, there was an initial rapid decrease followed by a subsequent slower absorbance increase. The rapid kinetic phase observed at 245 nm took place over a period that corresponds to the "lag" at longer wavelengths. These observations are consistent with the formation and decay of an intermediate. The two processes were sufficiently different in rate that kinetic constants could be evaluated for both by appropriate editing of the absorbance–time data. Rates for the fast phase were calculated from the data collected during the first 3–4 half-lives at 245 nm using the Guggenheim approximation.[26] Rates for the second (slow) phase were obtained from plots of log A versus time after deleting the early time points associated with the fast phase. The rate constants for the second kinetic phase were independent of the wavelength (245, 267, 310 nm) at which the absorbance change was monitored. This indicates that following completion of the first phase, formation of all products is subject to the same rate-determining step that preceeds the partitioning event.

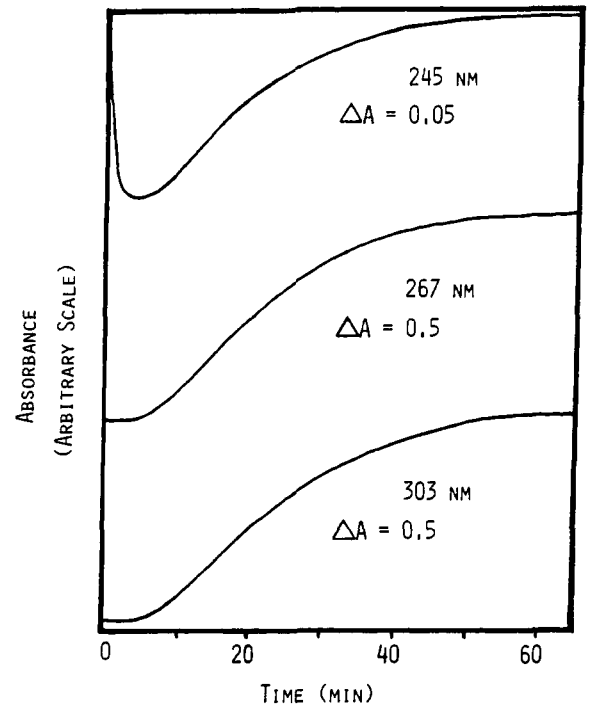


Figure 7. Absorbance changes at 245 nm, 267 nm, and 303 nm accompanying the decomposition of 7 in aqueous solution (μ = 0.5, 25 °C). Note change in scale (ΔA).

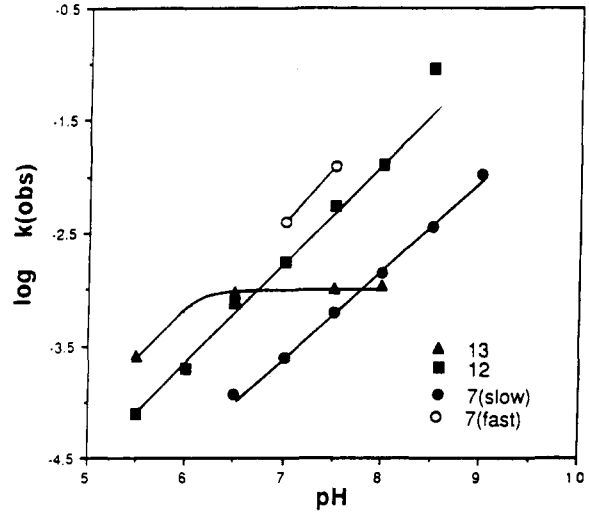


Figure 8. Rate–pH profiles for the decomposition of 7, 12, and 13 in aqueous solution (μ = 0.5, 25 °C).

As is apparent from Figure 5, the kinetics of the decomposition of 7 were dependent on pH, and this was examined more closely in Figure 8. Although the observed rates were found to be somewhat dependent on buffer concentration, this aspect of the reaction was not pursued in detail. At the buffer concentrations employed in this study, rate contributions from buffer catalysis were negligible (data not shown). The data presented in Figure 8 indicate that both kinetic processes exhibit approximately linear dependence on hydroxide ion concentration. The slopes of log k(obs) versus pH for the fast and slow kinetic phases are close to the theoretical value of unity.

^{18}O Distribution in the Products from [4′-$H^{18}O_2$]-7. The products arising from the ^{18}O-labeled hydroperoxide were analyzed by GC-MS to test the hypothesis that the breakdown of 7 involves the formation of an intermediate 4′-peroxybenzoate ester 9 (Figure 4). The results, summarized in Table II, indicate that both benzoic acid and acetic acid contain one of the labeled oxygen atoms ori-

Table II. Incorporation of ^{18}O in the Products of Decomposition of 7 in Aqueous Solution

product	conditions[a]	% ^{18}O[b]
benzoic acid	65 mM Pipes, pH 6.5	85.4
benzoic acid	65 mM Hepes, pH 7.0	92.1
benzoic acid	10 mM Hepes, pH 7.5	96.0
benzoic acid	65 mM NH_4HCO_3, pH 10.0	91.5
acetic acid	50 mM KP, pH 6.0	101
acetic acid	50 mM KP, pH 8.0	98.4
thymine propenol	65 mM Hepes, pH 7.5 ($H_2^{16}O$)	0
thymine propenol	65 mM Hepes, pH 7.5 ($H_2^{18}O$)	73

[a] At 25 °C, 35% (vol) CH_3CN in water. [b] Incorporation of a single atom. Values are corrected for percent enrichment in the source.

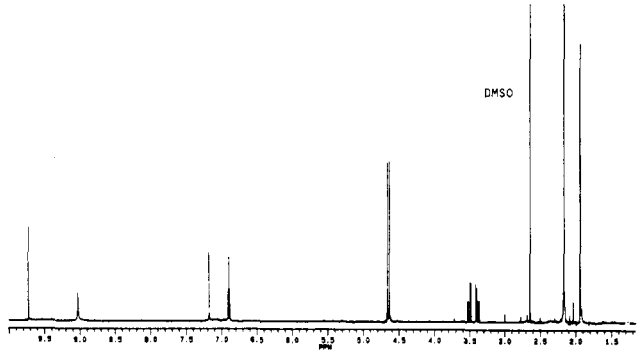
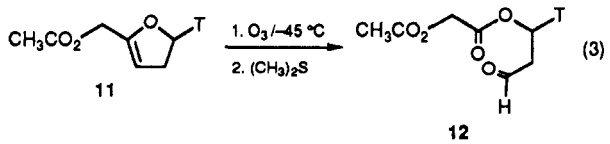


Figure 9. ^1H NMR spectrum (500 MHz, $CDCl_3$) of **12** from a partially ozonized solution of **11**. Spectrum of unreacted **11** has been subtracted.

ginating from the hydroperoxy group. None of the labeled oxygen was incorporated into the thymine propenal. The actual origin of the aldehydic oxygen of the propenal was determined by GC-MS analysis after reactions of **7** were carried out in ^{18}O water (Table II). About $^3/_4$ of the 3′ oxygen of the propenal was derived from the solvent. The remainder therefore originates from the 3′ oxygen of the hydroperoxide. The extent to which simple oxygen exchange between the propenal and solvent could account for the observed labeling was determined in control experiments with authentic thymine propenal in $H_2^{18}O$. These experiments showed that only about 8% of the observed labeling could be attributed to solvent exchange with the propenal itself, indicating that solvent oxygen must be incorporated at some stage prior to formation of the final product.

Synthesis of [1-[[2-(Acetyloxy)acetyl]oxy]-3-oxopropyl]thymine (12): A Model of Intermediate 5 (Figure 2) in the BLM-Mediated DNA Degradation. A new model intermediate (**12**) was prepared to provide further insight into the breakdown of the model hydroperoxide **7**, as well as the putative aldehyde intermediate **5** (Figure 2) implicated in DNA strand scission by Fe-BLM. This compound was obtained by the partial ozonolysis of 1-(5-*O*-acetyl-2,3-dideoxy-3,4-didehydro-β-D-*erythro*-pentofuranosyl)thymine (**11**), eq 3. Under optimal



11 12 (3)

conditions approximately half of **11** could be converted to **12**. Assignment of the structure of **12** is based on both its spectral and chemical properties. The ^1H NMR spectrum of partially ozonolyzed **11** in $CDCl_3$ is shown in Figure 9 and is consistent with the proposed structure. Interestingly, as has been observed in other similar aldehydes, the

Table III. Products Obtained from the Decomposition of the Aldehyde 11 in Aqueous Solution

	nmol	
product	pH 6.5	pH 9.0
MDA	72	117
thymine	72	118
thymine propenol	40	1

[a] At 25 °C in 50 mM phosphate or NH_4OAc buffer–35% (vol) CH_3CN.

aldehydic proton at 9.72 ppm does not exhibit significant coupling to the adjacent 2′ protons.[27] Off-resonance decoupling confirms that the 1′ proton (6.89 ppm) couples with the diastereotopic 2′ protons, which appear at ~3.44 ppm as a symmetrical eight-line multiplet of an ABX pattern. The 5′ hydrogens of **12** (4.64 ppm) are also diastereotopic.

The chemical stability of **12** was also consistent with the proposed structure. Incubation of **12** in $CHCl_3$ with triethylamine resulted in its complete conversion to thymine propenal and the triethylammonium salt of acetoxyacetate, the expected products of β-elimination.

Attempts to improve the yield of **12** with increasing exposure to ozone gives a product of inferior purity as judged by ^1H NMR. The major contaminating side product was identified as free thymine, which could arise from oxidation of the 1′ position of either **11** and **12**.[28] Oxidation of the pyrimidine ring olefin is also possible. The ozonation of **11** was therefore allowed to proceed to no more than 50% completion to avoid excessive amounts of these side products in samples of **12** prepared for this study. Also, the product was not separated from unreacted starting material as all attempts to do so resulted in its decomposition. Control experiments with the anhydronucleoside **11** established that its presence in samples of **12** did not interfere with the study of **12** in aqueous or other media. Under all of the conditions to which **12** and its decomposition products were subjected, **11** was completely inert and easily separated after reactions by HPLC.

Identification and Kinetics of the Products Produced from the Decomposition of 12. In aqueous solution **12** decomposed in a manner that was directly analogous to the hydroperoxide **7**. A quantitative product analysis (Table III) showed that, like **7**, compound **12** gave thymine propenal and thymine/MDA in a ratio that was dependent on pH. As seen in Figure 6, the steady decrease in the amount of propenal with increasing pH paralleled that observed for hydroperoxide **7**. At every pH, however, the percentage of thymine propenal produced was only about half of that observed for **7**.

Product formation from **12** was also very similar to the hydroperoxide from a kinetic standpoint. Changes in the UV spectrum obeyed simple first-order kinetics at all wavelengths. Notable in the case of **12** was the absence of the initial rapid spectral change that was observed in the decomposition of **7**. The rate constants for the decomposition of **12** also showed a linear dependence on hydroxide ion concentration (Figure 8). The breakdown of **12** was about ten times faster than the second kinetic phase of breakdown of the hydroperoxide **7** under the same conditions.

Structure of the Precursor to MDA? It was conceivable that the MDA accompanying thymine production

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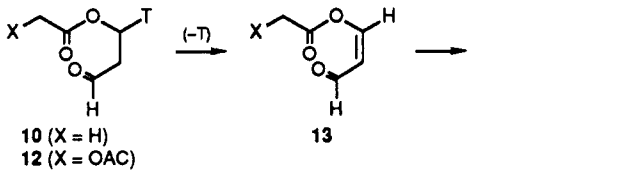
(29) Burger, R. M.; Projan, S. J.; Horwitz, S. B.; Peisach, J. *J. Biol. Chem.* **1986**, *261*, 15955–15959.

from breakdown of 7 and 12 arises from hydrolysis of an (acyloxy)propenal precursor 13 (eq 4). 3-Acetoxypropenal



$XCH_2CO_2^-$ + malondialdehyde (4)

13 (X = H) was prepared to establish whether 13 is a kinetically competent intermediate, and hydrolysis rate constants were obtained under the same conditions used for kinetic studies of 7 and 12. A partial rate–pH profile for 13 (X = H) is shown in Figure 8. The formation of an intermediate (acyloxy)propenal can be ruled out since 13 hydrolyzes at pH 8 with a rate constant slower than the decay of either 7 or 12.

Discussion

The mechanism shown in Figure 2 was originally proposed by Giloni et al.[10] to account for the degradation of DNA to base propenals, 5′-phosphate, and 3′-phosphoglycolate termini mediated by "activated" BLM in the presence of oxygen. While isotopic labeling studies using [4′-^{3}H]- and [4′-^{2}H]-DNA substrates have shown that cleavage of the 4′ C–H bond is the rate-determining step in this reaction,[13,14] the identities of the intermediates leading to base propenal formation remain to be established. The existence of at least one intermediate in this pathway has been demonstrated kinetically by Burger and co-workers who reported that DNA strand scission by "activated" BLM ($t_{1/2}$ = 2.5–5 min at 4 °C, pH 7) is followed by the slow release of base propenal ($t_{1/2}$ ~40 min) from a species still bound to the cleaved polymer. Unfortunately, this intermediate has not been further characterized.

Recently, a compound intended to model the putative DNA hydroperoxide 3 (Figure 2) has been prepared by Saito et al. and its decomposition was studied in some detail.[15] The observed production of thymine propenal from this model hydroperoxide 7 led to the conclusion that it was capable of undergoing a simple Criegee rearrangement in neutral aqueous solution, thereby providing a direct analogy for the spontaneous breakdown of 3 (Figure 2). The concomitant formation of thymine from 7 was explained in terms of hydroxide ion attack on the terminal peroxide oxygen, producing H_2O_2, and a 4′-hydroxythymidine intermediate that then fragments to give thymine and 3-(benzoyloxy)-4-oxopentanal.

Several puzzling observations prompted us to investigate the properties of the epimeric hydroperoxides 7 and 8 in greater detail. First, there is no known precedent for a Criegee rearrangement of alkyl hydroperoxides at neutral pH. In fact, strongly acidic conditions are normally required to induce such rearrangements.[30] Second, the contention that the breakdown of 7 is the result of a proton-assisted Criegee rearrangement is inconsistent with the observation that the reaction rate increased with increasing pH. Finally, the lack of reactivity displayed by 8 under identical conditions[31] suggested to us the presence of an important stereochemical component in the reaction, which was left uninterpreted by Saito et al.[15] These inconsistencies prompted a reevaluation of the proposed mechanism using the labeled hydroperoxide [4′-H$^{18}O_2$]-7.

The most revealing observation, with regard to the mechanism of breakdown of [4′-H$^{18}O_2$]-7, is the isotope distribution among the products. One atom of ^{18}O was found in both the acetate and benzoate obtained from this hydroperoxide over the entire pH range (Table II). These results are consistent with the initial step in the reaction involving intramolecular transfer of the C3′-*O*-benzoyl group to the terminal oxygen of the hydroperoxide. This process, which is precluded in 8 due to geometric constraints, gives a perester (9) capable of a facile Criegee-type breakdown to the aldehyde 10 (Figure 4). This mechanism also accounts for the biphasic kinetics observed in the decay of 7. The initial fast kinetic phase, which is catalyzed by hydroxide ion, we attribute to the acyl group migration (Figure 4). The slow kinetic phase would then correspond to either the formation (k_2) or decay (k_3) of the aldehyde 10. The observed catalysis of the slow step by hydroxide ion would be consistent with either possibility.

While the formation of the intermediate aldehyde 10 could not be established by direct observation, it is strongly supported by two pieces of evidence. The first of these is the observation that 73% of the thymine propenal obtained from 7 at neutral pH contains ^{18}O derived from the solvent at the 3′ (aldehyde) position. Since the propenal product itself exchanges very slowly with the solvent (only 8% incorporation of solvent oxygen under identical reaction conditions), this level of incorporation in the product is best explained by a more rapid exchange in the intermediate 10 prior to its decay. A simple aliphatic aldehyde of this type undergoes very rapid exchange in water,[32] k of 10^{-2} s^{-1} under conditions analogous to those used in present investigations.

Further evidence for intermediate 10 is provided by a comparison with the model compound 12. The rate– and product–pH profiles of 12 are remarkably similar to those associated with the breakdown of 7 (Figure 6 and Figure 8). In addition to the products originally reported (acetate, benzoate, thymine, and thymine propenal) by Saito et al.,[15] we have identified a fifth major product from the decomposition of 7: malondialdehyde (MDA). The amount of MDA is equivalent to the amount of thymine, indicating that cleavage of the C3′–C4′ bond is also involved in the release of thymine. The products anticipated by Saito et al.[15] (H_2O_2 and 3-(benzoyloxy)-4-oxopentanal) were not observed. Like 7, compound 12 gives rise to both thymine/MDA and thymine propenal. Also, the ratio of these products and their rate of formation increases with pH (Figures 6 and 8) in a manner that is analogous to the hydroperoxide. Notably absent in the breakdown of 12 is the rapid kinetic phase, which preceded product formation from the hydroperoxide. This supports the idea that this rapid kinetic phenomenon is associated with the postulated acyl migration step (Figure 4). The close similarity between compounds 7 and 12, with regard to products and kinetics, strongly suggests that intermediate 10 is involved in the breakdown of hydroperoxide 7.

The mechanistic basis for the pH dependence of the product ratios from 10 and 12 is not entirely clear. One possibility is that a competition occurs between hydrolysis of the ester (perhaps intramolecular), which leads to thymine and MDA, and a direct β-elimination reaction, which gives thymine propenal. An elimination mechanism to account for the thymine/MDA formation, involving the (acyloxy)propenal intermediate 13 (eq 4), was ruled out

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(31) The 4′*R* hydroperoxide 8 slowly decomposes but does not yield thymine propenal.[15]
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on the basis of the hydrolysis kinetics of authentic 13 (X = H).

Relevance of 7 and 12 to the Mechanism of DNA Strand Scission by BLM. While the model hydroperoxide 7 mimics the BLM-DNA reaction in its ability to produce base propenal in neutral solution, a closer examination of this compound reveals that it may not provide an appropriate model for the putative DNA hydroperoxide 3. The results of the present study indicate that the observed breakdown of 7 is initiated by a rearrangement to the perester 9, which polarizes the O-O bond sufficiently to enable heterolytic cleavage. Although one might speculate that the postulated DNA hydroperoxide 3 could be similarly activated by an internal migration of the 3'-phosphoryl group, this would require scission of the (3')-P-O bond, inconsistent with our previous oxygen labeling studies, which have established that this bond remains intact.[33]

Thus, it appears that perester formation in the model system is an undesirable artifact due to the unfortunate choice of a 3' blocking group. A more appropriate model of 3 would be a 4'-hydroperoxynucleoside with a 3' substituent unable to migrate. From this point of view, 8 would provide a suitable model since, for stereochemical reasons, it does not rearrange to a perester. In aqueous solution, 8 decays at a very slow rate ($t_{1/2}$ = several hours) and does not give rise to either thymine propenal or MDA (data not shown). This observation strongly suggests that the introduction of a hydroperoxy group at C4' alone may not be sufficient to account for the cleavage of DNA, which is observed in the presence of activated BLM. Since this calls into question an independent role of 3 in the cleavage of the C3'-C4' bond of DNA (Figure 2), one might consider the possibility that this process is mediated by further intervention of the Fe-BLM complex beyond its initial role in radical formation at C-4'. It is not unreasonable to assume that the drug remains in close proximity to the initial lesion for a significant period and provides the necessary catalysis for a Criegee rearrangement in 3 by acting as a Lewis acid.[11]

Burger and co-workers demonstrated that the formation of base propenals from DNA by activated BLM is a relatively slow process, resolvable kinetically from the more rapid strand cleaving event.[29] Although the intermediate propenal precursor could be coprecipitated with the DNA following strand scission, its exact structure has not been determined. Compound 12 provided an opportunity to evaluate the possibility that Burger's intermediate corresponds to the aldehyde 5 (Figure 2). In several respects, 12 does resemble the species described by Burger et al.[29] At 4 °C in pH 7 buffer, 12 decomposes to give thymine propenal at a rate of 0.03 min^{-1}, which is essentially identical with the reported rate of 0.02 min^{-1} in DNA under the same conditions. The breakdown of 12, however, differs from that of the DNA intermediate in that thymine and MDA are also formed in substantial quantities. Extensive investigations undertaken in our laboratory indicate that base release from DNA occurs predominantly through pathway B (Figure 1) and is not accompanied by MDA.[13] We have, however, found the observed thymine/thymine propenal ratio from 12 to be very sensitive to a variety of factors including pH (Figure 6), temperature, solvent composition, and ionic strength (data not shown). Given that these physical changes can dramatically alter the product distribution in 12, we would not rule out the possibility that the generation of its equivalent (5) in the constrained environment of a DNA polymer might favor the formation of base propenals exclusively. Experiments are underway in order to resolve this discrepancy between the natural and model systems.

Acknowledgment. This work was supported by the National Institutes of Health GM 34454.

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Naurol A and B, Novel Triterpene Alcohols from a Pacific Sponge

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Received December 28, 1989 (Revised Manuscript Received July 19, 1990)

Two new stereoisomeric triterpene alcohols with an uncommon symmetrical carbon skeleton centered about a linear conjugated tetraene moiety and having only two carbocyclic rings have been isolated from a sponge from Nauru. The structures were determined primarily from ^1H and ^{13}C NMR data. Both alcohols are mildly cytotoxic to murine leukemia cells (P388).

Although triterpenes of varied skeletal arrangements are abundant among terrestrial plant products,[1] they are far less prominent among secondary metabolites reported to date from marine organisms.[2] The majority of all triterpenes reported have the conventional skeletons arising from cyclization of 2,3-squalene epoxide to fused polycyclic products. More unusual are incompletely cyclized compounds or ones exhibiting cyclization within the chain rather than with cyclizations beginning at one end. In our continuing search for bioactive compounds from marine organisms we have isolated two stereoisomeric triterpene alcohols that have a novel, partially cyclized skeleton which has been noted in only one other natural product.[3] The new compounds display cytotoxicity against murine lymphocytic leukemia.

The new triterpenes were isolated from several sponge specimens collected at Nauru Is. These specimens looked superficially the same and hence were extracted together, but, unfortunately, subsequent taxonomic analysis revealed that the collection was a mixture of a *Rhaphisia* sp. (order

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