phosphate. Thus, metal ion catalysis is much more effective than specific acid catalysis via protonation of a quinoline nitrogen. This suggests that the metal must interact directly with the departing oxygen of the leaving group (Scheme IX), while the proton is much less effective in this interaction (Scheme VII). Proton transfer from N $\rightarrow$ O in the transition state is not important as shown by the absence of a deuterium solvent kinetic isotope effect in the hydrolysis of protonated (deuterated) bis(8-hydroxyquinoline) phosphate.

The following putative roles for metal ion as catalyst have not been observed in this study. The metal ion does not act to deliver HO^- to the phosphorus center with specific acid catalysis of departure of the leaving group (Scheme X) or with metal ion catalysis of departure of the leaving group (Scheme XI) because the loose preassociation of metal ion does not allow these mechanisms to compete with those in which the quinoline nitrogen acts as the attacking nucleophile at the phosphorus center. Studies of models are under investigation which may exhibit the mechanisms of Schemes X and/or XI.

Acknowledgment. This work was supported by grants from the Office of Naval Research and the National Science Foundation.

New Insight into the Mechanism of Base Propenal Formation during Bleomycin-Mediated DNA Degradation

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Abstract: The mechanism of interaction of the antitumor antibiotic bleomycin (BLM) and cofactors with DNA to produce base propenal and 3′-phosphoglycolate and 5′-phosphate ends has been investigated. Analytical methods have been developed that allow the isolation of glycolic acid from the modified DNA without washout of isotopic labels. Results using $^{18}O_2$, $H_2{}^{18}O$, and $H_2{}^{18}O_2$ under a variety of conditions show that 1 mol of ^{18}O is found per mole of glycolate in the carboxylate group and is derived from the $^{18}O_2$ not used in the formation of activated BLM. The second oxygen in the carboxylate of glycolate is derived from the 4-oxygen in the deoxyribose moiety from which it is generated. The oxygen in the aldehyde of the base propenal appears to be derived exclusively from H_2O. Studies using poly[dA(2′-*pro-R*-^{3}H)dU] and poly[dA(2′-*pro-S*-^{3}H)dU] demonstrate, as originally proposed by Burger et al. [Burger, R. M.; Projan, S. J.; Horwitz, S. B.; Peisach, J. *J. Biol. Chem.* **1986**, *261*, 15855], that 2′-*pro-R*-hydrogen cleavage and DNA strand scission occur more rapidly than the rate of base propenal formation. An alternative mechanism to the one currently favored involving intermediate **6** (Scheme I) is proposed to accommodate all of the available data.

The bleomycins are a group of antitumor antibiotics first isolated by Umezawa in 1966.[1] These compounds are used clinically in the treatment for head and neck cancer, testicular cancer, and squamous cell carcinomas;[2] their cytotoxicity is thought to be related to their ability to bind to and degrade double-stranded DNA.[3] The DNA cleavage reaction requires two cofactors: a metal, either Fe^{2+} or Cu^+, and O_2.[4,5] Extensive efforts by numerous investigators in the past decade have provided much information about the products produced during BLM-mediated degradation of DNA and the chemistry of their formation (Scheme I).[6,7]

Two monomeric products, trans base propenal **7** and nucleic acid base, have been shown to ultimately result from a putative common intermediate **1**[8-10] (Scheme I). This intermediate is produced by cleavage of the 4′ carbon-hydrogen bond of a nucleotide residue by activated BLM.[10] This reaction exhibits sequence-specificity for a pyrimidine moiety to the 3′-side of a deoxyguanosine residue.[11,12] Activated iron-BLM results from the interaction of Fe^{2+}, BLM, and O_2 and requires the presence of an additional electron.[13] The actual structure of the activated BLM species remains to be elucidated. While the pathway that leads to free nucleic acid base release has been established using a variety of methods (Scheme I, pathway B),[14-18] the pathway

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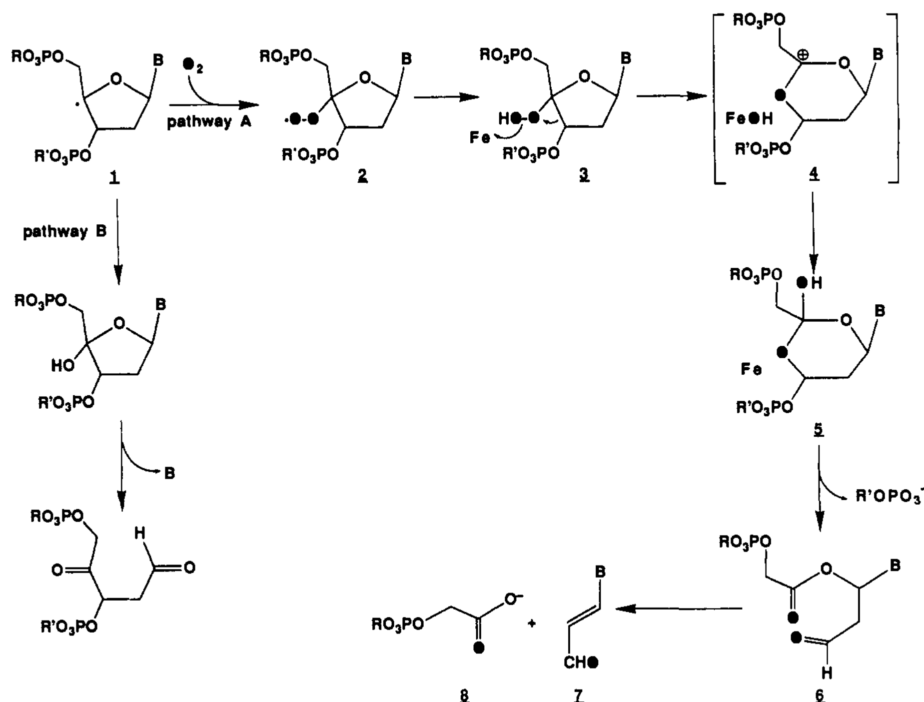
Scheme I



leading to base propenal formation still remains relatively obscure (Scheme I, pathway A).[8,19-25]

Studies of Peisach and co-workers revealed that production of base propenal (7) required an additional O_2 over that required to form activated BLM.[26] Production of 7 was shown to be accompanied by "immediate" strand scission and formation of modified 3′-phosphoglycolate and 5′-phosphate ends. Kinetic studies revealed that the O_2 required for the production of activated BLM (the pulse) could be distinguished from the additional O_2 (the chase) required to form base propenal.[13,27-29] These observations facilitated the preliminary $^{18}O_2$-labeling studies of McGall et al. that demonstrated that one oxygen in the carboxylate of the 3′-phosphoglycolate moiety was derived from O_2 in the chase and suggested that the other carboxylate oxygen was derived from the oxygen within the deoxyribose moiety.[22]

Studies of Ajmera et al. using a DNA model system, poly-[dA(2′-*pro-R*-^{3}H)dU], revealed that the 2′-*pro-R*-hydrogen of the deoxyuridine moiety was specifically removed, resulting in the production of only trans base propenal.[21] Subsequent studies of Burger et al. using [1′,2′,5-*methyl*-^{3}H]dT incorporated into DNA reported that DNA strand scission occurred concomitant with ^3H_2O release ($t_{1/2}$ = 1.8 min at 5 °C) and was 1 order of magnitude faster than base propenal production ($t_{1/2}$ = 45 min). The rapid ^3H_2O washout from the putative 2′-position was difficult to reconcile with the mechanism postulated in pathway A, Scheme I, and prompted Burger et al. to propose an alternative mechanism.[25]

The present report is focused on investigating in more detail the mechanism by which BLM mediates base propenal formation. Specifically, ^{18}O-labeling experiments using $^{18}O_2$, $H_2{}^{18}O_2$, and $H_2{}^{18}O$ are presented in detail, focusing on the source of oxygen in both the 3′-phosphoglycolate moiety and the accompanying base propenal. In addition, the kinetics of ^3H_2O production from poly[dA(2′-*pro-R*-^{3}H)dU] and poly[dA(2′-*pro-S*-^{3}H)dU] was investigated in a systematic fashion. These studies have led to the formulation of an alternative mechanism for base propenal formation, consistent with all of the available experimental facts.

Materials

Blenoxane, a clinical mixture of BLM A_2(60%) and BLM B_2 (30%), was generously provided by Bristol-Myers. Solutions of the drug were standardized optically.[30] Calf thymus DNA was obtained from Sigma and purified as described by Povirk.[31] The alternating copolymer poly[d(AT)] was prepared according to a published procedure,[10] and the hexanucleotide d(CGCGCG) was kindly provided by John Gerlt at the University of Maryland. Bacterial alkaline phosphatase (AP) and bovine liver catalase were purchased from Sigma and P_1 nuclease from Pharmacia. Glycolic acid, thiobarbituric acid, 2,7-dihydroxynaphthalene, $NaBH_4$, *N,O*-bis(trimethylsilyl)trifluoroacetamide, and trimethylchlorosilane were all obtained from Aldrich. $H_2{}^{18}O$ (98 atom %) was supplied by MSD Isotopes, and $^{18}O_2$ (96 atom %) was a product of Amersham. The syntheses of *trans*-1-(3-oxopropenyl)thymine, *trans*-1-(3-[^{18}O]oxopropenyl)thymine, and $H_2{}^{18}O_2$[19,32] are reported elsewhere using materials obtained from Aldrich. The ^{18}O content of the $H_2{}^{18}O_2$ was determined by the method of Ortiz de Montellano,[33] and the concentration was determined iodometrically.[34]

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HPLC separations were carried out on a Beckman system with 110A pumps. UV–visible spectrophotometry was performed on a Cary 210 (Varian) or a Beckman DU-50 instrument. Mass spectra were obtained on either a Kratos MS-25 or a Hewlett-Packard 5890GC/5970MS system.

[1,1-$^{18}O_2$]Glycolic Acid. Aqueous glycolic acid (1 mL, 1 μmol) was lyophilized to dryness, dissolved in 100 μL of $H_2{}^{18}O$ (98 atom %), and acidified with 1 μL of 12 M HCl. After 48 h, the solution was made alkaline by the addition of 5 μL of concentrated NH_4OH and stored in a sealed vial. To determine the ^{18}O content in the product, 1 μL of the stock solution was dried by adding and evaporating 200 μL of anhydrous ethanol, and the glycolate was converted to a TMS derivative for analysis by GC–MS (see below).

Calibration of the Glycolate Assay Using 2,7-Dihydroxynaphthalene. An aqueous solution of glycolic acid (GA) was prepared and titrated with standard NaOH (Dilut-It; J. T. Baker). This was used to prepare more dilute stock solutions of 1.00 mM concentration. Aliquots in the range 2–50 μL of 1.00 mM GA were combined with a 0.01% solution of 2,7-dihydroxynaphthalene in concentrated H_2SO_4 to a final volume of 1.00 mL. The total volume of aqueous solution used could not exceed 50 μL/mL.[35] The mixture was heated in a sealed vial at 100 °C for 20 min and cooled, and the absorbance at 540 nm was measured. From the calibration curve, which was linear in the range 2–50 nmol, an effective extinction coefficient of $(2.3 \pm 0.1) \times 10^4$ M^{-1} cm^{-1} was obtained.[35]

General Procedure for the Enzymatic Release of Glycolate from BLM-Modified DNA. After incubation with Fe–BLM, reaction mixtures, which typically contained 500 nmol of DNA (total nucleotides) in a 500-μL volume, were adjusted to pH 5.5 with 100 μL of 0.1 M sodium acetate buffer. Zinc chloride (0.2 mM final concentration) and 5 units of P_1 nuclease were then added, and the mixture was maintained at 37 °C for 90 min. The pH of the solution was readjusted to 8.0–8.5 by adding a few microliters of concentrated NH_4OH, and the mixture was incubated with 3 units of bacterial alkaline phosphatase at 37 °C for another 90 min. The enzymes were then removed by heating the solution briefly at 100 °C, cooling on ice, and filtering through a Centricon-10 ultrafiltration device from Amicon. Glycolic acid was isolated from the filtrate on DEAE Sephadex either directly or after separation from the other products by HPLC.

General Procedure for the Isolation and GC–MS Analysis of Glycolic Acid. Solutions containing glycolic acid were applied to a 0.7 × 4 cm column of DEAE Sephadex-A25, which had been washed with 1 M ammonium formate and then water prior to use. The column was washed with 10 mL of water and then 8 mL of 1% formic acid solution to elute the GA. The acid wash was immediately neutralized with a few drops of concentrated NH_4OH and reduced to a final volume of ~1 mL using a Speed Vac concentrator. The concentrated solution of glycolate was then lyophilized for 4–8 h to ensure removal of most of the residual ammonium formate. Recoveries of GA by this procedure were typically greater than 85%.

The remaining residue of ammonium glycolate was rinsed from the lyophilized flask with methanol and transferred into a 300-μL derivatization vial (Pierce) where it was dried by the repeated addition and evaporation of 50-μL volumes of absolute ethanol. The sample was dried over P_2O_5 and then heated in the sealed vial with 10–20 μL each of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane and anhydrous acetonitrile at 75 °C for 1 h.

Aliquots (1–5 μL) of the silylation mixture were analyzed directly by GC–MS using a Kratos MS-25 mass spectrometer interfaced to a Carlo-Erba gas chromatograph. The GC columns used were 30 m × 0.53 mm (i.d.) fused silica capillaries with a bonded nonpolar stationary phase (RSL-150; 1-μm film thickness) obtained from Alltech Associates. Injections were made in the splitless mode with the injector port at 250–270 °C. The column temperature was maintained isothermally at 45 °C for 2–5 min and then increased at a rate of 3–6 °C/min. An ion gauge was used to monitor the emergence of volatile solvent and reagents before activating the source (70 eV). Spectra were then acquired continuously, scanning from 600–30 m/z at 0.3 s/decade. The bis-TMS derviative of GA eluted as a sharp peak 14–16 min after injection. In cases where accurate isotope ratios were desired, the spectrometer was scanned from m/z 220 to 160, and the partial spectra so obtained were integrated. Mass range calibration was obtained using perfluorokerosene as a standard.

Calculation of ^{18}O Enrichment in Glycolic Acid by MS. The ^{18}O content in glycolate samples was determined from the relative intensities of the labeled and unlabeled parent M − 15 ions which resulted from loss of methyl radical from the molecular ions.[36] These ions have m/z 205, 207, and 209 for the unlabeled, singly labeled, and doubly labeled compounds, respectively. Each parent peak (mass = n) is accompanied by "satellite" peaks ($n + 1$, $n + 2$) due to the natural abundance of heavy isotopes (^{13}C, ^{18}O, and ^{29}Si) in the molecule. Therefore, in an ^{18}O-enriched sample, the observed intensity of the ^{18}O-labeled ion must be corrected for the contribution to the "$n + 2$" peaks arising from the presence of other heavy isotopes in the molecule.[37,38] A correction factor of 0.08 was obtained from the mass spectrum of the natural abundance glycolate–TMS derivative (I_{207}/I_{205}).

The overall % ^{18}O was calculated as follows:

$$\%\ ^{18}O = \frac{I_{209}(\text{corr}) + 0.5I_{207}(\text{corr})}{I_{209}(\text{corr}) + I_{207}(\text{corr}) + I_{205}(\text{obsd})}$$

where $I_{207}(\text{corr}) = I_{207}(\text{obsd}) - 0.08I_{205}(\text{obsd})$ and $I_{209}(\text{corr}) = I_{209}(\text{obsd}) - 0.08I_{207}(\text{corr})$. For most purposes, it was desirable to express enrichment as a percentage of the product, singly or doubly labeled:

$$\%\text{ singly labeled} = \frac{100I_{207}(\text{corr})}{I_{209}(\text{corr}) + I_{207}(\text{corr}) + I_{205}(\text{obsd})}$$

$$\%\text{ doubly labeled} = \frac{100I_{209}(\text{corr})}{I_{209}(\text{corr}) + I_{207}(\text{corr}) + I_{205}(\text{obsd})}$$

Quantitative Analysis of Glycolic Acid from BLM-Modified DNA. (i) Aerobic Degradation of DNA with Fe^{2+}–Bleomycin. A freshly prepared solution of BLM (250 nmol) in sodium phosphate buffer (25 mM, pH 7.2) was purged with humidified O_2 for several seconds at 2 °C. Ferrous ammonium sulfate (250 nmol) was added to activate the drug, and after 10 s, calf thymus DNA (1.2 μmol in nucleotides) was introduced. Final concentrations were as follows: sodium phosphate, 25 mM; BLM, 250 μM; $Fe(NH_4)_2(SO_4)_2$, 250 μM; DNA, 1.2 mM in nucleotides. The reaction was maintained at 2 °C under a stream of O_2 for 25 min, at which time three 25-μL aliquots were withdrawn and assayed for total base propenals using thiobarbituric acid.[26,39] The remainder of the reaction mixture was subjected to the general P_1/AP digest (see above) to release glycolic acid from the modified DNA, and then it was divided into equal portions for analysis of GA by independent methods as described below.

Three controls were also prepared lacking BLM, but otherwise having the same final concentrations of Fe^{2+}, DNA, and buffer as the reaction described above. Each control was digested with P_1/AP according to the general method. To two of the controls was added 58 nmol of [1,1-$^{18}O_2$]GA (97.5 atom %), and the third was left unchanged.

(ii) Determination of GA with 2,7-Dihydroxynaphthalene. After the phosphatase treatment, one of the two portions from the BLM–DNA reaction was injected onto an analytical C_{18} reverse-phase HPLC column and eluted at 1.0 mL/min with 5 mM sodium acetate buffer (pH 5.5). The GA, eluting with the void volume, was recovered in a single fraction collected between 1 and 6 min. This fraction was reduced in volume to 500 μL on a Speed Vac concentrator and then injected onto an analytical SAX HPLC column (Whatman). The SAX column was eluted at 1.5 mL/min with 5 mM sodium acetate buffer, and 3-mL fractions were collected, frozen at −80 °C, and lyophilized. The recovered GA in each fraction was assyed by adding a 1.0-mL volume of 2,7-dihydroxynaphthalene reagent (0.01% in concentrated H_2SO_4) to the lyophilization flask, mixing thoroughly to dissolve the residue, heating at 100 °C for 20 min, and measuring the absorbance at 540 nm.

Two of the controls described above, one containing [1,1-$^{18}O_2$]GA (58 nmol) and the other none at all, were analyzed by the same method. The A_{540} readings in fractions 6–9 of the glycolate-free control were taken as background and subtracted from the A_{540} values measured for the sample and control which did contain GA in these fractions. The quantity of GA was calculated (eq 1) from these corrected values using the extinction coefficient of 2.30×10^4 M^{-1} cm^{-1} determined previously. A final correction was applied in order to account for subquantitative recoveries (63 ± 11%) from the isolation procedure (eq 1). The percent recovery by

$$\text{total GA} = \left(\frac{A_{540}(\text{exp}) - A_{540}(\text{control})}{(0.63) \times 23.0\ \text{mM}^{-1}}\right)(\text{volume}) \quad (1)$$

this method was determined beforehand using known amounts of authentic GA. For the positive control which had been spiked with 58 nmol of GA, a value of 59 ± 14 nmol was obtained by the preceding analysis, thus confirming the overall accuracy of the method.

(iii) Determination of GA by GC–MS Using an Internal Standard. To the second portion of the phosphatase-digested reaction mixture was

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added 65 nmol of [1,1-$^{18}O_2$]GA as an internal standard. The mixture of glycolic acids was partially purified by reverse-phase HPLC as described in part ii, isolated on DEAE Sephadex, and converted to bis-TMS derivatives according to the general methods described earlier in this section. The ^{18}O-labeled GA in the remaining spiked control sample was isolated and derivatized in the same manner. Both sample and control GA derivatives were then analyzed by GC–MS using narrow mass range scanning (m/x 220–160) as previously described.

The absolute mole fraction (F) of bis-^{18}O-labeled GA in the "standard only" control was determined from the $[M - CH_3]^+$ ion intensities after correcting for natural abundance $n + 2$ ions according to eq 2.

$$F = \frac{I_{209}(\mathrm{corr})}{I_{205} + I_{207}(\mathrm{corr}) + I_{209}(\mathrm{corr})} \quad (2)$$

The amount of unlabeled GA present in the standard only control, expressed as a fraction of the doubly labeled component, was also calculated (eq 3):

$$C = \frac{I_{205}}{I_{209}(\mathrm{corr})} \quad (3)$$

For a mixture of the labeled standard with unlabeled GA, a ratio can be determined from the corrected ion intensities according to eq 4:

$$\frac{\mathrm{GA(sample)}}{\mathrm{GA(standard)}} = \frac{F[I_{205} - CI_{209}(\mathrm{corr})]}{I_{209}(\mathrm{corr})} \quad (4)$$

In this way, the amount of GA released from the BLM reaction mixture was calculated from the quantity of labeled standard that had been added. In test mixtures containing a known amount of unlabeled GA (110 nmol) as the analyte and 50 nmol of labeled GA as an internal standard, a value of 113 ± 7 nmol was obtained, which confirmed the accuracy of the method.

(iv) Analysis of GA from the Anaerobic Degradation of DNA by BLM. A solution of Fe^{3+}–BLM was prepared in unbuffered solution by combining equal quantities of the drug and $Fe(NH_4)(SO_4)_2$ in water at 2 °C. After 2 min, a solution of aqueous Tris·HCl (pH 7.5) was added. The resulting mixture was degassed by alternately freezing and thawing three times under vacuum and finally repressurizing the flask with argon. Calf thymus DNA and H_2O_2 were added as argon-saturated solutions via a gastight syringe, and the reaction was allowed to proceed for 90 min at 2 °C. Final concentrations were as follows: Tris·HCl (pH 7.5), 10 mM; DNA, 1 mM (in nucleotides); BLM, 250 μM; $Fe(NH_4)(SO_4)_2$, 250 μM; H_2O_2, 600 μM.

A 25-μL aliquot of the reaction mixture was removed for analysis of total base propenals using thiobarbituric acid.[26,39] To the remainder was added 1 mg of bovine liver catalase to remove H_2O_2, and then digestion with P_1/AP was carried out by the general method. Free glycolic acid was then determined by GC–MS using [1,1-$^{18}O_2$]GA (59 nmol) as an internal standard according to the protocol described in part iii.

^{18}O Incorporation in Glycolic Acid from BLM-Modified DNA. (i) Excess $^{18}O_2$. A buffered solution containing BLM and DNA (d-(CGCGCG), poly[d(A)d(T)], or DNA from calf thymus) was placed in a small flask equipped with a septum-stoppered side arm on a vacuum line. The solution was degassed by three consecutive freeze–thaw cycles under vacuum and then repressurized with $^{18}O_2$ from a reservoir. A sufficient quantity of aqueous $Fe(NH_4)_2(SO_4)_2$ (previously degassed and stored under argon) was then added via a gastight syringe to activate the drug, and the mixture was incubated for 20–30 min at 2 °C. Final concentrations were as follows: Tris·HCl (pH 7.5), 10 mM; DNA, 1 mM (total nucleotides); BLM, 1 mM; $Fe(NH_4)_2(SO_4)_2$, 1 mM in a total volume of 500 μL. A small aliquot was usually withdrawn at this point for total base propenal determination using thiobarbituric acid.[26,39] Yields of base propenal in the 45–50-nmol range were noted.

In an initial series of experiments, reaction mixtures were digested with P_1/AP after exposure to the atmosphere. However, subsequent experiments involved carrying out the enzyme digest in situ so that atmospheric oxygen would not contaminate the reaction during this stage of the workup. All of the necessary solutions were purged with argon prior to addition. Glycolic acid was isolated and analyzed by GC–MS according to the general procedures.

(ii) $^{16}O_2$ Pulse/$^{18}O_2$ Chase. Each of the following three solutions was degassed by three consecutive freeze–thaw cycles under vacuum and then repressurized with argon and equilibrated at 2 °C: (1) BLM (588 nmol) in 50 μL of Tris·HCl (50 mM, pH 7.5) buffer; (2) calf thymus DNA (525 nmol total nucleotides) in 1.75 μL of Tris·HCl buffer (15 mM, pH 7.5); (3) aqueous $Fe(NH_4)_2(SO_4)_2$ (10 mM). Also, a saturated solution of $^{16}O_2$ was prepared by degassing 1 mL of water and equilibrating under $^{16}O_2$ at ambient pressure and temperature for 1 h before use. The O_2 concentration was determined from published data[40] to be 1.3 mM.

To the solution of BLM was added 50 μL of anaerobic Fe^{2+} (500 nmol) via a gastight syringe to form the pink Fe^{2+}–BLM complex. The complex was then activated by the addition of 210 μL of saturated $^{16}O_2$ (275 nmol), giving the yellow color of the activated complex.[29,41] After 60 s, the flask was rapidly evacuated and repressurized with $^{18}O_2$ gas, and then the DNA was immediately added via a gastight syringe. After 30 min at 2 °C (22 nmol total base propenals), the general P_1/AP digest was carried out in situ using argon-purged enzyme and buffer solutions. Free GA was isolated and analyzed by GC–MS.

(iii) $^{18}O_2$ Pulse/$^{16}O_2$ Chase. Essentially the same protocol used in the previous experiment was employed. A limiting pulse of $^{18}O_2$-saturated water was used to activate the drug, but instead of evacuating the flask at this point, $^{16}O_2$ was introduced by simply purging the solution vigorously with $^{16}O_2$ through a hypodermic needle for several seconds before adding the DNA. Final concentrations were as follows: Tris·HCl, 10 mM (pH 7.5); DNA, 1.3 mM (total nucleotides); BLM, 0.13 mM; $Fe(NH_4)_2(SO_4)_2$, 0.12 mM; $^{18}O_2$, 0.12 mM (pulse) in a total volume of 2.0 mL.

After 25 min at 2 °C (30 nmol total base propenal formed), the reaction mixture was submitted to the standard P_1/AP digest, and GA was isolated and analyzed by GC–MS.

(iv) $^{18}O_2$/$Fe^{3+}$$H_2{}^{16}O_2$. A solution of Fe^{3+}–BLM was prepared in unbuffered solution by combining equal quantities of the drug and $Fe(NH_4)(SO_4)_2$ in water at 2 °C. After 2 min, aqueous Tris·HCl (pH 7.5) was added and the resulting solution was degassed by three consecutive freeze–thaw cycles under vacuum. The reaction flask was repressurized with $^{18}O_2$, and an argon-purged solution of calf thymus DNA was added via a gastight syringe. An argon-purged solution of H_2O_2 was then added to activate the drug, and the reaction was maintained at 2 °C for 90 min. Final concentrations were as follows: Tris·HCl, 10 mM; DNA, 1 mM (total nucleotides); BLM, 250 μM; $Fe(NH_4)(SO_4)_2$, 250 μM; H_2O_2, 600 μM in a total volume of 1.0 mL.

When the incubation was complete, the entire reaction mixture was passed through a 0.5 × 1 cm column of Chelex resin (BioRad) to remove the iron, using 3 mL of water to elute the other components of the mixture. Bovine liver catalase (1 mg) was added to the eluent to destroy the remaining H_2O_2, and then it was worked up in the usual way for GA analysis.

(v) $^{16}O_2$/$H_2{}^{18}O_2$$Fe^{3+}$. The same procedure used in the previous experiment was employed to activate the drug with Fe^{3+} and $H_2{}^{18}O_2$ under an atmosphere of $^{16}O_2$.

(vi) [^{18}O]Water. A 250-μL solution containing 10 mM Tris·HCl (pH 7.5), 5 mM DNA from calf thymus, and 1 mM BLM was frozen and lyophilized. The residue was reconstituted with 200 μL of $H_2{}^{18}O$ (98 atom %) at 2 °C. A 50-μL volume of $Fe(NH_4)_2(SO_4)_2$ (10 mM, 50 μL) was added to activate the drug, and the solution was maintained at 2 °C under a stream of dry O_2 for 25 min. Free GA was analyzed by GC–MS after the usual workup.

^{18}O Incorporation into Base Propenals. (i) From $^{18}O_2$. In two experiments, a 900-μL solution containing 11 mM buffer (HEPES, pH 7.5, or phosphate, pH 7.0), 1.1 mM BLM, and 1.1 mM poly[d(AT)] (total nucleotides) was degassed by three consecutive freeze–thaw cycles under vacuum and repressurized with $^{18}O_2$. An argon-purged $Fe(NH_4)_2(SO_4)_2$ solution (10 mM, 55 μL) was then added via a gastight syringe to activate the drug, and the mixture was maintained at 2 °C for 30 min. Argon-purged sodium borohydride (25 mg/mL in 50 mM CHES, pH 9; 50 μL) was then added, and the mixture was left standing at 2 °C for another 15 min.

Acetic acid (~20 μL) was added cautiously, and when the bubbling had ceased, the solution was eluted on a C_{18} Sep Pak (Waters Associates) column washed with 2 mL of water and then 2 mL of methanol. The methanol wash, containing the reduced thymine propenal, was evaporated in a derivatization vial where the residue was dried under vacuum over P_2O_5. The sample was then heated for 1 h at 80 °C in the sealed vial with 20 μL of 50% BSTFA/1% TMCS in dry acetonitrile. Aliquots of 1–3 μL were analyzed directly on a Hewlett-Packard 5890GC/5970MS system. The column used was a 30 m × 0.32 mm (i.d.) fused silica capillary column with a bonded nonpolar stationary phase (DB-5; 1-μm film thickness) obtained from Suppleco. Injections were made in the splitless mode with the injector port maintained at 260 °C, and the column temperature was increased from 60 to 280 °C at a rate of 10–20 °C/min after sample injection.

The percent ^{18}O in the thymine propenal was determined from the relative intensities of the $CH{=}CHCH_2OSi(Me)_3^+$ fragment ions at m/z

(40) Dean, J. A., Ed. *Lange'ss Handbook of Chemistry*, 11th Ed.; McGraw-Hill: New York, 1973; pp 10–6, 10–7.

(41) Horwitz, S. B.; Sauseville, E. A.; Peisach, J. In *Bleomycin Chemical, Biochemical and Biological Aspects*; Hecht, S. M., Ed.; Springer-Verlag: New York, 1979; pp 170–183.

129 and 131 according to eq 5, which includes a correction[38] for the occurrence of natural abundance ions at m/z 131.

$$\%\ ^{18}O = \frac{I_{131} - 0.05I_{129}}{0.95I_{129} + I_{131}} \quad (5)$$

(ii) From $H_2^{18}O$. To 300 μL of $H_2^{18}O$ (81.5 atom %) were added 100 μL of 4.6 mM poly[d(AT)], 40 μL of 6.5 mM BLM, and 10 μL of 0.5 M sodium phosphate (pH 7.0) at 2 °C. The reaction was initiated by adding 50 μL of 10 mM $Fe(NH_4)_2(SO_4)_2$, bringing the % ^{18}O in the solvent to a final value of 48.9 ± 1%. The reaction was maintained at 2 °C under a stream of O_2 for 30 min, at which time 1 mg of $NaBH_4$ was added. After another 15 min, 10 μL of acetic acid was added to destroy the borohydride, and the reduced thymine propenal was isolated and analyzed as described in the previous experiment.

Synthesis of Poly[dA(2'-^{3}H)dU] Polymers. Synthesis of [2'-*S*-^{3}H]-UTP. [2'-*S*-^{3}H]UTP was synthesized using an eight-step procedure starting from uridine.

(1) 3',5'-*O*-TIPDS-uridine. A mixture of uridine (1.22 g, 5.0 mmol) and 1,3-dichloro-1,1,3,3-tetraisopropyl-1,3-disiloxane (1.8 mL, 5.5 mmol) in 10 mL of pyridine (distilled from CaH_2) was stirred for 24 h. The pyridine was evaporated and the residue partitioned between Et_2O and H_2O. The organic phase was washed successively with H_2O, ice-cold 1 N HCl, saturated aqueous $NaHCO_3$, and saturated NaCl, dried over Na_2SO_4, filtered, and evaporated to give the product (2.1 g, 80% yield) as a colorless foam: ^{1}H NMR ($CDCl_3$) δ 8.72 (br s, 1 H, NH), 7.64 (d, 1 H, J = 8.0, H-6), 5.67 (s, 1 H, H1'), 5.63 (dd, 1 H, J = 1.3, 8.0, H-5), 4.29 (dd, 1 H, J = 4.9, 8.7, H-3'), 4.13 (complex pattern with H2' and one H5'), 4.05 (m, 1 H, H4' with $J_{3',4'}$ = 8.7), 3.93 (dd, J = 2.6, 13.2, H5'), 1.0 (m, 14 H, ^{i}Pr).

(2) 3',5'-*O*-TIPDS-2'-ketouridine. CrO_3 (300 mg, 3 mmol) was suspended in 7 mL of dry CH_2Cl_2 (from 3A molecular sieves) and cooled on ice. Freshly distilled acetic anhydride (0.3 mL, 3 mmol) and pyridine (0.5 mL, 6 mmol, from CaH_2) were added in sequence. The mixture was warmed to and maintained at room temperature for 30 min then recooled on ice. A solution of 3',5'-*O*-TIPDS-uridine (487 mg, 1 mmol) in 0.5 mL of CH_2Cl_2 was added, and the mixture was stirred for 1 h at 0 °C. The mixture was poured into 25 mL of EtOAc with vigorous stirring, and the precipitate was removed by vacuum filtration through a 1 cm thick layer of SiO_2. The SiO_2 was washed thoroughly with EtOAc, and the combined filtrates were evaporated to dryness. The brownish residue was chromatographed by flash chromatography (1:1 EtOAc/hexanes), yielding 427 mg (88% yield) of an orange foam. Crystallization from a concentrated solution in $CHCl_3$ by careful addition of hexane gave 315 mg of white needles: ^{1}H NMR ($CDCl_3$) δ 8.16 (br s, 1 H, NH), 7.14 (d, 1 H, J = 8.0, H-6), 5.75 (dd, 1 H, J = 2.2, 8.0, H-5), 5.06 (d, 1 H, J = 9.1, H-3'), 4.96 (s, 1 H, H1'), 4.12 (m, 2 H, H-5'), 3.91 (ddd, J = 3.7, 3.9, 9.1, H4'), 1.1 (m, 14 H).

(3) [2'-^{3}H]-3',5'-*O*-TIPDS-arabinouridine. A solution of 3',5'-*O*-TIPDS-2'-ketouridine (250 mg, 0.52 mmol) in 5 mL of freshly distilled THF cooled to −15 °C was added to a stirred solution of [^{3}H]$NaBH_4$ (100 mCi, 6 μmol) in 1 mL of 1:1 ^{i}PrOH/H_2O cooled to −15 °C. After 30 min, the mixture was warmed to and maintained at room temperature for 60 min. After being cooled back to −15 °C, a solution of $NaBH_4$ (50 mg, 1.4 mmol) in 1 mL of 1:1 ^{i}PrOH/H_2O was added. After 15 min, the reaction was warmed to and maintained at room temperature for 30 min. Saturated NaCl was added until the phases separated. The mixture was extracted three times with Et_2O, and the combined organic phases were washed with saturated NaCl and evaporated. The residue was evaporated three times from $CHCl_3$ to remove H_2O and then dissolved in $CHCl_3$ and applied to a 1.5 × 5 cm column of flash SiO_2. The column was washed with $CHCl_3$ and then with EtOAc to elute the product (0.44 mmol as a 95:5 mixture of arabino- to ribouridine by UV, 9 × 10^7 cpm/μmol): ^{1}H NMR ($CDCl_3$) δ 9.80 (br s, 1 H, NH (δ is variable)), 7.82 (d, 1 H, J = 8, H-6), 6.02 (d, 1 H, J = 6, H1'), 5.64 (br d, 1 H, J = 8, H-5), 4.52 (ddd, 1 H, J = 5, 6, 9, H-2'), 4.22 (d, 1 H, J = 5, OH (δ is variable)), 4.08 (dd, 1 H, J = 9, 10, H-3'), 4.06 (d, 1 H, J = 12, H-5'), 3.94 (dd, J = 3, 12, H-5'), 3.70 (br d, 1 H, J = 10, H-4'), 1.0 (m, 14 H).

(4) [2'-^{3}H]-3',5'-*O*-TIPDS-2'-chloro-2'-deoxyuridine. A mixture of [2'-^{3}H]-3',5'-*O*-TIPDS-arabinouridine (250 mg, 0.5 mmol) and triphenylphosphine (250 mg, 0.95 mmol) in 10 mL of CCl_4 was heated at reflux for 36 h (the reaction was followed by TLC [SiO_2, 1:1 EtOAc/hexane] until the starting material at R_f 0.27 disappeared; product R_f = 0.50). The solvent was evaporated, the residue was taken up in 75:25 hexane/EtOAc, and the precipitated Ph_3PO filtered out. The filtrate was applied to a 1.5 × 10 cm column of flash SiO_2 equilibrated in hexane. The column was washed successively with hexane (elutes Ph_3P), 75:25 hexane/EtOAc, and finally 1:1 hexane/EtOAc which eluted the product. Evaporation of solvent yielded the product (230 mg, 89%) as a colorless oil, which crystallized upon standing: ^{1}H NMR ($CDCl_3$) δ 8.90 (br s, 1 H, NH), 7.89 (d, 1 H, J = 8, H-6), 5.94 (s, 1 H, H1'), 5.69 (d, 1 H, J = 8, H-5), 4.42 (dd, 1 H, J = 5, 9, H-3'), 4.31 (d, 1 H, J = 5, H-2'), 4.26 (d, 1 H, J = 14, H-5'), 4.19 (br d, 1 H, J = 9, H-4'), 3.98 (dd, J = 2, 14, H-5'), 1.0 (m, 14 H).

(5) [2'-^{3}H]-2,2'-Anhydrouridine. A mixture of [2'-^{3}H]-3',5'-*O*-TIPDS-2'-chloro-2'-deoxyuridine (100 mg, 198 μmol) and 0.4 mL of 1 M tetrabutylammonium fluoride in THF in 2 mL of freshly distilled THF was stirred for 12 h at room temperature, during which time the product usually crystallized. This material was collected by centrifugation. The supernatant was evaporated, and the residue was redissolved in 2 mL of MeOH. Upon cooling, more crystals formed. The combined crystalline product (30 mg, 67% yield) was dried under vacuum: ^{1}H NMR (D_2O) δ 7.78 (d, 1 H, J = 8, H-5), 6.41 (d, 1 H, J = 7, H-1'), 6.06 (d, 1 H, J = 8, H-5), 5.34 (d, 1 H, J = 7, H-2'), 4.55 (s, 1 H, H-3'), 4.26 (m, 1 H, H-4'), 3.45 (m, 2 H, H-5'); UV (H_2O) λ_{max} 224, 252, 270 (sh). If crystallization could not be induced, initial purification was obtained through chromatography on a Sephadex LH-20 column (2.5 × 50 cm), eluting with H_2O. Material purified in this manner still contained some tetrabutylammonium salts.

(6) [2'-^{3}H]Uridine. A mixture of vacuum-dried [2'-^{3}H]-2,2'-anhydrouridine (173 μmol), benzoic acid (20 mg, 164 μmol), and sodium benzoate (100 mg, 695 μmol) in 1 mL of freshly distilled HMPA was heated at 150 °C for 10 h. The mixture was cooled to room temperature, diluted with $CHCl_3$, and washed three times with H_2O (both the $CHCl_3$ and H_2O phases were saved). The $CHCl_3$ phase was chromatographed on SiO_2 (2.7 × 6 cm), eluting first with $CHCl_3$ and then with 10% MeOH/$CHCl_3$. Fractions were analyzed by TLC (SiO_2, 10% MeOH/$CHCl_3$), and the fractions containing the uridine benzoates were pooled and evaporated. [Note: There are two products formed, the 2'-benzoate and the 3'-benzoate, which equilibrate (R_f = 0.19, 0.25).] The residue was dissolved in 5 mL of MeOH and treated with 50 mg of NaOMe. After 15 min, Dowex-50W (H^+) was added for neutralization, and the mixture was filtered and evaporated. The residue was partitioned between $CHCl_3$ and H_2O, and the aqueous phase was lyophilized. Final purification was achieved by HPLC (2% MeOH/H_2O). On occasion (depending on the dryness of the reagents and the solvents), some loss of the benzoate esters occurred during the inversion reaction, leading to uridine formation. This uridine was found in the aqueous phase of the initial extraction along with a lot of HMPA. It could be recovered by initial chromatography on Sephadex LH-20 (H_2O) followed by the final purification by HPLC. Total recovery was 63.9 μmol (37%).

(7) [2'-^{3}H]UMP. [2'-^{3}H]UMP was prepared by the procedure of Yoshikawa et al.[42] Freshly distilled $POCl_3$ (14 mL, 150 μL) was added to a solution of [2'-^{3}H]uridine (64 μmol) in 0.5 mL of triethyl phosphate at 0 °C. After 4 h, the reaction was warmed to room temperature and kept for an additional 2 h. The mixture was poured into 50 mL of cold H_2O, and the solution was neutralized by the addition of KOH. The product was chromatographed using a linear gradient (0–0.4 Et_3N–H_2CO_3) on DEAE–Sephadex A-25. UMP and P_i cochromatographed, and the excess P_i was removed by barium precipitation: UMP was dissolved in H_2O, and the pH was adjusted to 8.0 with NH_4OH. $BaBr_2$ (2× over total phosphate) was added to precipitate the P_i. The pellet was removed by centrifugation and the supernatant decanted. The pellet was washed with H_2O, and the supernatant was combined and rechromatographed on DEAE–Sephadex as above. The yield was 40 μmol (62%) of product.

(8) [2'-^{3}H]UTP. [2'-^{3}H]UTP was prepared by the procedure of Hoard and Ott.[43] [2'-^{3}H]UMP (40 μmol) was dissolved in 2 mL of dry DMF and treated with 10 μL of tributylamine. The solution was evaporated three times from dry DMF. The residue was dissolved in 1 mL of dry DMF and treated with 32 mg (198 μmol) of carbonyldiimidazole for 12 h. At that time, 3 μL of H_2O was added to the reaction mixture and was stirred for 30 min. Tributylammonium pyrophosphate (1 mL of 0.2 M) was added in DMF, and the mixture was stirred for 5 h. H_2O (5 mL) was then added and the solution evaporated to dryness. The residue was dissolved in 40 mL of H_2O and chromatographed on DEAE–Sephadex A-25 (0–0.8 M Et_3N–H_2CO_3).

Conversion of UTP to dUTP with Ribonucleoside Triphosphate Reductase (RTPR). A typical reaction mixture of 300 μL contained 30 mM dithiothreitol, 1 mM EDTA, 250 mM Tris Cl (pH 8.2), 1 M sodium acetate, 150 μM adenosyl cobalamin (AdoCbl), 12 mM UTP, and 1.14 mg of RTPR from *Lactobacillus leichmannii* (1.02 units) in ^3H_2O (0.67 Ci/g) for the synthesis of [2'-*R*-^{3}H]dUTP. AdoCbl was added under reduced light to initiate the reaction, which was incubated in the dark for 3 h at 37 °C. To monitor conversion of UTP to dUTP, an aliquot (1 μL) was removed and treated with alkaline phosphatase (3 units) in

(42) Yoshikawa, M.; Kato, T.; Takenishi, T. *Tetrahedron Lett.* **1967**, *50*, 5066.

(43) Hoard, D. E.; Ott, P. G. *J. Am. Chem. Soc.* **1965**, *87*, 1785.

50 mM Tris Cl, pH 8, with 10 mM $ZnCl_2$ for 0.5 h at 37 °C. The mixture was analyzed for uridine and deoxyuridine on an Alltech 10-μm C_{18} reversed-phase HPLC with 2% methanol in H_2O at a flow rate of 1.7 mL/min (compound, retention time: uridine, 6.0 min; deoxyuridine, 9.5 min). Upon complete conversion to product, the ^3H_2O from the reaction mixture was removed by repeated bulb-to-bulb distillation. The [^{3}H]dUTP was then combined with an equal amount of unlabeled dUTP, and the mixture was applied to a (3 × 10 cm) DEAE-Sephadex A-25 column and eluted with a 1-L linear gradient of 0.1–0.8 M triethylammonium bicarbonate. Appropriate dUTP fractions were pooled and desalted. Analogous reactions with [2'-*S*-^{3}H]UTP (10^7 cpm/μmol) were conducted in 75 μL total volume, and the converted dUTP was diluted 12-fold prior to ion exchange chromatography.

Synthesis of Poly(dA-dU) Copolymers. The *R* and *S* poly[dA(2'-^{3}H)dU] copolymers were synthesized as previously described.[10] Final yields of 6.6 μmol of poly[dA(2'-*R*-^{3}H)dU] (specific activity 8.5 × 10^5 cpm/μmol) and 12 μmol of poly[dA(2'-*S*-^{3}H)dU] (specific activity 3.8 × 10^5 cpm/μmol) were obtained.

Base Propenal and ^{3}H Release from *R* and *S* Poly[dA(2'-^{3}H)dU]. In a typical reaction volume of 1 mL, poly[dA(2'-*R*-^{3}H)dU] (1.0 mM, 8.5 × 10^5 cpm/μmol) or poly[dA(2'-*S*-^{3}H)dU] (1.0 mM, 3.8 × 10^5 cpm/μmol), 0.1 mM BLM, 20 mM sodium phosphate (pH 7.0), and 0.12 mM ferrous ammonium sulfate were incubated at 0 °C. Ferrous ammonium sulfate solution (5 mM, prepared immediately before use) was combined with BLM solutions (7–15 mM) and vortexed, and aliquots were added to DNA reactions after allowing 15 s for bleomycin activation.[13] After a 5 min to 7 h incubation, 200-μL aliquots were removed and added to a 27-μL solution of 8.6 M LiCl/2.1 mM calf thymus DNA in an Eppendorf tube and briefly vortexed, three volumes of cold ethanol were added, and the solution was incubated in a dry ice/acetone bath for 20 min. Removal of the precipitate was accomplished by centrifugation at 15 500 rpm for 20 min in a JA-21 rotor at –20 °C using a Beckman J2-21 centrifuge. This procedure reproducibly precipitated 62–70% of the base propenal precursors at 5 min. Base propenal in the supernatant was assayed by reacting one-quarter of the total volume (225 μL) with 600 μL of 2-thiobarbituric acid solution (42 mM 2-thiobarbituric acid, 1 mM EDTA), adjusting the final volume to 1 mL with 75% ethanol, and heating at 92 °C for 20 min.[26,39] Cooled assay mixtures were quantitated at 532 nm (ϵ = 160 mM^{-1} cm^{-1}). The remaining supernatant portion (three-quarters of the total volume, 675 μL) was shell-frozen in a 5-mL flask, and the volatile ^{3}H was removed by bulb-to-bulb distillation. A portion of the distillate was combined with ScintA for liquid scintillation counting. Pellets were resuspended in 20 mM sodium phosphate, pH 7, to the original volume (200 μL) and incubated at 37 °C for 2 h to release base propenal precursors. An 8.3 M solution of LiCl (27 μL) and 3 volumes of ice-cold ethanol were added, and DNA was precipitated in dry ice/actone and centrifuged as described above. This procedure allowed for the pellet to be fractionated into a second pellet and supernatant. The supernatant derived from the pellet was analyzed for base propenal by the thiobarbituric acid assay and volatile ^{3}H by bulb-to-bulb distillation as described above for the initial supernatant. Supernatant fractions recovered from the walls of the distillation flasks were analyzed for monomeric products by C_{18} reversed-phase HPLC: 100% buffer A (10 mM potassium phosphate, pH 6.8) for 5 min, followed by a linear gradient of 0–30% buffer B (methanol) for 30 min at a flow rate of 1 mL/min (compound, retention time: uracil, 4.5 min; uracil propenal, 31 min).

^{3}H Release from *R* and *S* Poly[dA(2'-^{3}H)dU] under Limiting O_2. *R* and *S* poly[dA(2'-^{3}H)dU] (1 mM in nucleotides) in a final volume of 300 μL were reacted with 0.21 mM Fe^{2+}–BLM and 0.42 mM O_2 in 20 mM sodium phosphate, pH 7.0, for 45 min at 0 °C (limiting O_2 conditions). At the conclusion of the reaction, the solution was shell-frozen and subjected to bulb-to-bulb distillation as described above. The DNA solution was resuspended in 40 mM triethanolamine, pH 7.8 (300 μL), and an aliquot (50 μL) was removed for base propenal determination by the thiobarbituric acid assay. The remaining solution was sealed with a rubber septum and Ar-purged for 20 min, at which time $NaBH_4$ (final concentration 100 mM) was added. After 90 min, the reaction was quenched with acetic acid and digested in situ with P_1 nuclease (5 units) and alkaline phosphatase (3 units). Products were analyzed using the HPLC gradient described above [compound, retention time, ϵ: uracil, 5 min, 8.2 mM^{-1} cm^{-1} at 260 nm; deoxyuridine, 16 min, 10.2 mM^{-1} cm^{-1}; 2''-deoxypentitol 3'',5'-dAMP (**9**), 23 min, 15 mM^{-1} cm^{-1}; deoxyadenosine, 30 min, 15 mM^{-1} cm^{-1}]. Concentrations were measured optically, and the radioactivity was quantitated by scintillation counting.

Results

Recovery of GA from BLM–DNA. Analytical methods were developed to determine the source of oxygen in the 3'-phosphoglycolate ends and the base propenal when BLM mediates DNA degradation via pathway A (Scheme I). The release of free glycolic acid (GA) from the 3'-termini of BLM-mediated oligonucleotide fragments was accomplished by consecutive treatments with P_1 nuclease and alkaline phosphatase. The glycolate obtained in this way was quantitated by two analytical methods. In one method, GA was assayed spectrophotometrically using 2,7-dihydroxynaphthalene, which formed a colorled product (ϵ_{540} = 2.3 × 10^4 M^{-1} cm^{-1}) after heating in concentrated H_2SO_4.[35] A purification scheme involving reversed-phase HPLC followed by anion exchange HPLC and lyophilization was necessary to remove substances which could interfere with this assay. A comparison of the elution profiles of GA recovered in this manner from BLM-modified DNA with the control samples containing unmodified DNA with and without added GA confirmed the authenticity of this analysis. Since the net recovery of GA was less than quantitative, a correction factor, determined with known quantities of GA, was required to account for material losses. The procedure yielded accurate, if somewhat imprecise (22% relative uncertainty), determinations when applied to test samples supplemented with authentic GA.

Table I. Determination of Glycolate Released Enzymatically from BLM-Modified DNA

	total base propenals (nmol)[a,b]	total glycolate (nmol)[a]
	Control	
DNA/BLM/glycolate (128 nmol)		126 (±28)[c]
DNA/BLM/glycolate (110 nmol)		113 (±7)[d]
	Reaction	
DNA/Fe^{2+}–BLM (aerobic)	144 (±8)	129 (±8)[d] 130 (±29)[c]
DNA/Fe^{3+}–BLM/H_2O_2 (anaerobic)	<3	7 (±3)[d]

[a] Data are for reactions containing 1 mM calf thymus DNA and 0.25 mM BLM in a total volume of 1.0 mL. [b] Determined by thiobarbituric acid method.[9] [c] Determined by 2,7-dihydroxynaphthalene method.[35] [d] Determined by GC-MS method.

A second, more precise method employed in the quantitation of GA involved the addition of a known quantity of [1,1-$^{18}O_2$]-labeled glycolate as an internal standard. Since the standard and sample GA co-purify, losses can be ignored, and a ratio can be calculated directly by mass spectrometric analysis of the material recovered. Only a minor correction was needed to account for isotope dilution from the labeled standard caused by solvent exchange during the recovery (2–3%). The amount of GA from BLM-modified DNA determined by this procedure is in excellent agreement with the value obtained by the colorimetric assay.[35] These values are, within the limits of experimental uncertainty, equivalent to the total yield of base propenals, and this is in accordance with the known 1:1 stoichiometry of strand scission and propenal production.[8,26,39] When DNA was degraded anaerobically with Fe^{3+}–BLM and H_2O_2, oxygen-dependent strand scission was suppressed. This accounts for the low levels of both GA and base propenals observed under these conditions (Table I).

While a combination of reversed-phase and strong anion exchange HPLC was the method of choice when purifying the GA for colorimetric analysis, a more direct isolation using DEAE–Sephadex was preferred when preparing samples for GC–MS analysis. This routine procedure involved binding the glycolate at neutral pH, washing, eluting with dilute formic acid, and then neutralizing the eluate with NH_4OH. Lyophilization removes the water and formate, leaving the glycolate in a form suitable for silylation using standard reagents. Control experiments with ^{18}O-labeled GA showed that the combined enzyme treatment and isolation procedures resulted in a consistent but minor degree of isotope dilution (4 ± 2%). Previously reported methods for the quantitative isolation of GA from BLM-cleaved DNA required conditions which would have washed out the label completely.[8]

^{18}O Incorporation into Glycolic Acid. In an initial series of

Table II. Percent Incorporation of ^{18}O into Glycolic Acid from the Degradation of DNA with Fe^{2+}–BLM under $^{18}O_2$

substrate	conditions during P_1/AP workup	% [^{18}O]Glycolate[a]
calf thymus DNA	aerobic	97 102
calf thymus DNA	anaerobic	102
poly[d(AT)]	aerobic	80
poly[d(AT)]	anaerobic	96
d(CGCGCG)	aerobic	99

[a] Data are corrected for % ^{18}O in the source (98%) and for isotope dilution during isolation (4%). Error ±3%.

Table III. Percent Incorporation of ^{18}O into Glycolic Acid from the Degradation of DNA with Fe–BLM and $^{18}O_2$ in Pulse–Chase Experiments

activation method	reaction chase	% [^{18}O]glycolate[a]
$Fe^{2+}/^{18}O_2$	$^{16}O_2$	4
$Fe^{2+}/^{16}O_2$	$^{18}O_2$	93
$Fe^{3+}/H_2{}^{18}O_2$	$^{16}O_2$	3
$Fe^{3+}/H_2{}^{16}O_2$	$^{18}O_2$	99
$Fe^{3+}/H_2{}^{16}O_2$/d(CGCGCG)[b]	$^{18}O_2$	79.5
$Fe^{2+}/^{16}O_2$/d(CGCGCG)/$H_2{}^{18}O$[c]		−0.4

[a] Data are corrected for % ^{18}O in the source (98%) and for isotope dilution during isolation (4%). Error ±3%. [b] Excess H_2O_2 not destroyed with catalase prior to workup results in washout of ^{18}O from GA. [c] 95 atom %.

experiments, DNA substrates were degraded with Fe^{2+}–BLM in solution saturated with $^{18}O_2$ gas at 2 °C. The Fe^{2+} was added last in these experiments so that the labeled oxygen was present during both drug activation and the subsequent oxygen-dependent strand scission. In all cases, the GA released and isolated from the cleaved DNA contained high levels of a single atom of ^{18}O in the carboxyl group (Table II). No label was incorporated at C-2, as indicated by the lack of enrichment at m/z 161,[36,37] nor was any di-^{18}O-labeled product observed. The observed incorporation levels were somewhat dependent on the conditions of the workup: consistently lower values were observed if reactions were exposed to the atmosphere during the P_1/AP digestion steps. This we attribute to residual drug activity during the extended periods at 37 °C necessary for the enzyme digestions. Interestingly, while the effect was rather small with heterogeneous DNA, it was particularly evident when the alternating copolymer poly[d(AT)] was employed as the substrate. A significantly slower time course for the cleavage of poly[d(AT)] would account for this, if it were such that the reaction was only partially complete when the enzyme treatments were initiated. The effect was alleviated by simply avoiding contamination with atmospheric oxygen during the workup. Under anaerobic work-up conditions, ^{18}O incorporation levels of 96–102% were observed for all three substrates. The lack of ^{18}O (<1%) in GA isolated from reactions performed in $H_2{}^{18}O$ demonstrates that the other oxygen atom of the carboxyl group is retained from the deoxyribose ring.

In order to distinguish between the O_2 involved in drug activation and the second O_2 required for the formation of 3′-phosphoglycolate termini, a "pulse–chase" method was employed. In a typical experiment, an anaerobic solution of Fe^{2+}–BLM was activated with a "pulse" of $^{16}O_2$ or $^{18}O_2$. The stoichiometry of O_2 consumption in the absence of DNA has been shown to be 0.5 mol of O_2 per mole of Fe^{2+}–BLM, resulting in a 1:1 mixture of Fe^{3+}–BLM and activated BLM.[26,29] In these experiments, the oxygen pulse was added as a saturated solution in H_2O. After activation, residual oxygen was removed by evacuation prior to admitting a "chase" of $^{18}O_2$ or $^{16}O_2$ and DNA from calf thymus. In a final series of experiments, DNA was combined with Fe^{3+}–BLM and $H_2{}^{16}O_2$ (or $H_2{}^{18}O_2$) under an atmosphere of $^{18}O_2$ (or $^{16}O_2$). Under these conditions, the molecular oxygen does not participate in drug activation.[13,27] The results of these experiments, summarized in Table III, clearly demonstrate that the oxygen incorporated in the glycolate product derives primarily, if not exclusively (93–99%) from the second O_2 requirement and not the bound oxygen(s) of the activated drug.

Table IV. Incorporation of Oxygen from Solvent or O_2 into Thymine Propenal Produced by the Degradation of Poly[d(AT)] with Fe^{2+}–BLM

solvent	O_2	% [^{18}O]thymine propenal[a]
$H_2{}^{16}O$	$^{18}O_2$	0
$H_2{}^{18}O$	$^{16}O_2$	99

[a] Data are corrected for % ^{18}O in the sources. Error ±4%.

It is worth noting that additional precautions are necessary in the experiments involving hydrogen peroxide activation to remove residual peroxide before isolating the GA. Control experiments reveal that oxygen from H_2O_2 becomes incorporated into GA to the extent of about 30% under typical experimental conditions. The nature of this exchange reaction was not investigated, but it could be eliminated by destroying the excess hydrogen peroxide with catalase prior to initiating the enzymatic release of glycolate.

^{18}O Incorporation into Base Propenal. In order to determine the source of the aldehydic oxygen in the base propenals produced by O_2-dependent strand scission, poly[d(AT)] was degraded by Fe^{2+}–BLM in the presence of $^{18}O_2$ or $H_2{}^{18}O$. The alternating copolymer was chosen as a substrate in these experiments for two reasons. First, the production of a single predominant product, thymine propenal, afforded a simpler and more sensitive analysis. Second, difficulties which had been encountered in preparing derivatives of the other base propenals for GC–MS could be avoided. After incubating the DNA with the drug, thymine propenal was reduced with $NaBH_4$ in situ, and the resulting propenol was isolated by reversed-phase chromatography. Control experiments with authentic ^{18}O-labeled thymine propenal established that isotope dilution due to solvent exchange occurred to an extent of less than 10% during a typical reaction (30 min, pH 7.0, 2 °C). The product obtained from the degradation of poly[d(AT)] contained oxygen derived *exclusively* from the solvent and not from the O_2 involved in strand scission (Table IV).

Kinetics of ^3H Release from *R* and *S* Poly[dA(2′-^3H)dU]. Studies of Burger et al.[25] using [1′,2′,5-*methyl*-^3H]dT in DNA were interpreted to indicate that DNA strand scission and [2′-*pro-R*-^3H] C–H bond cleavage occurred 1 order of magnitude more rapidly than the formation of base propenal. Alternative interpretations of these results are possible. First, it was not clear that the ^3H_2O observed by Burger et al. was the result of 2′-*pro-R* C–H bond cleavage since multiple sites in the deoxynucleoside were labeled and the distribution of the label at C-2′ of dT was unknown. Second, ^3H_2O could be the result of a secondary exchange reaction(s) from the nucleic acid base pathway B (Scheme I) or the base propenal pathway A (Scheme I). In the former case, the 2′-hydrogens are adjacent to the C-1′ aldehyde, and in the latter case, the 2′-hydrogens are determined adjacent to the putative C-3′ aldehyde (intermediate **6**, Scheme IA). Third, the products of the reaction and their specific activities were not isolated and determined.

Given the importance of the timing of 2′-C–H bond cleavage to DNA strand scission and mechanistic proposals, the Burger et al. experiments previously described[25] were repeated using specifically [^3H]-labeled DNA models: poly[dA(2′-*pro-R*-^3H)dU] and poly[dA(2′-*pro-S*-^3H)dU]. Poly[dA(2′-*R*-^3H)dU] was incubated with Fe–BLM, and aliquots were removed; the DNA was ethanol precipitated, and the supernatants were analyzed for base propenal release and volatile tritium. The precipitated DNA was resuspended in buffer; base propenals released by heat treatment and the DNA in the solution reprecipitated to perform the same measurements with the supernatant and pellet fractions. The data in Table V demonstrate that 85% of the volatile ^3H is released into the supernatant in 5 min, at which time 39% of the base propenal precursors have been released. The uracil propenal isolated from both the pellet and supernatant fractions contained no ^3H above background (detection limit: 3.5% of the original specific radioactivity). Thus, rapid release of ^3H from precursors to base propenals does occur from the 2′-*pro-R* position, in agreement with the proposal of Burger et al. (1986)[25] based on their studies using [1′,2′(*pro-R*, *pro-S*),5-*methyl*-^3H]T-labeled DNA.

Table V. Kinetics of Base Propenal Formation and Release of ^{3}H from Poly[dA(2'-*R*-^{3}H)dU]

	supernatant fractions (nmol)		pellet fractions (nmol)	
time	base propenals[a]	volatile ^{3}H[b]	base propenals[a]	volatile ^{3}H[b]
5 min	1.61	3.43	2.55	0.57
45 min	2.65	3.71	1.88	0.44
3 h	3.30	4.06	1.21	0.40
7 h	2.85	4.04	0.67	0.33

[a] Determined by thiobarbituric acid assay as described in Methods. [b] Recovered by bulb-to-bulb distillation of ethanol/water solution; specific activity 850 cpm/nmol.

Table VI. Control Experiments Demonstrating That Anaerobic Product **9** Is Not Responsible for Release of Tritium from *R* or *S* Poly[dA(2'-^{3}H)dU]

	poly[dA(2'-*R*-^{3}H)dU][b]	poly[dA(2'-*S*-^{3}H)dU][c]
base propenal (nmol)[a]	0.55	0.74
^3H_2O release (nmol)	0.45	0.34
uracil (nmol)	21.3	8.75
2''-deoxypentitol 3'',5'-dAMP (**9**, nmol)	11.9	3.4
specific activity of **9** (cpm/nmol)	804.8	340.1
specific activity of reisolated dU (cpm/nmol)	821.4	353.3

[a] Determined by thiobarbituric acid assay. [b] Specific activity 850 cpm/nmol. [c] Specific activity 380 cpm/nmol.

The low level of tritium volatilization from the pellet fraction during the liberation of propenals from BLM-treated poly[dA-(2'-*R*-^{3}H)dU] suggests that significant release of tritium from the oxygen-independent uracil formation pathway does not occur. This issue was addressed specifically by conducting BLM reactions under limiting oxygen conditions. In one such experiment in which uracil formation predominated (12.8 nmol, 88% of monomeric products), the release of tritium was equivalent to base propenal production (1.8 nmol and 1.7 nmol, respectively). Identical results are obtained in the presence or absence of 1 M LiCl, suggesting that high salt does not catalyze [2'-^{3}H] exchange. The ability to trap the alkaline-labile product that accompanied uracil release as 2''-deoxypentitol 3'',5'-dAMP (**9**) allowed for a direct determination of the radioactivity remaining in the 2'-position.[14,15] When such a limiting oxygen experiment was conducted using poly[dA(2'-*R*-^{3}H)dU], the specific radioactivity of **9** isolated by HPLC (805 cpm/nmol) was 98% of that of the unreacted deoxyuridine (821 cpm/nmol, Table VI), demonstrating that the tritium was specifically retained in the alkaline-labile product.

We have previously observed that the *pro-R* hydrogen is lost stereospecifically from the C2'-position in the formation of uracil propenal.[21] However, the high salt and ethanol required to precipitate DNA in the present experiments might accelerate exchange from the *pro-S* 2'-position. For example, enolization of the postulated 3'-aldehyde precursor could cause exchange of both the 2'-*pro-R* and 2'-*pro-S* hydrogens prior to base propenal formation. Therefore, the copolymer poly[dA(2'-*S*-^{3}H)dU] was used to investigate directly the consequences of the DNA precipitation protocol on [2'-^{3}H] labilization. The release of uracil propenal precursors is 40% complete at 5 min and 77% complete after 7 h (Table VII), comparable to the results with poly[dA(2'-*R*-^{3}H)dU]. However, only small amounts of ^{3}H are released to the supernatant (3–6% of base propenal formed at any given time). The uracil propenal isolated from the supernatant and subsequently released from the pellet has a specific activity of 330–360 cpm/nmol, which indicates very little loss of the *pro-S* hydrogen (deoxyuridine of the copolymer has a specific activity of 380 cpm/nmol). The low levels of ^{3}H release observed on analysis of both the supernatant and pellet fractions suggest further that the alkaline-labile lesion, which accounts for approximately half

Table VII. Kinetics of Base Propenal Formation and Release of ^{3}H from Poly[dA(2'-*S*-^{3}H)dU]

	supernatant fractions (nmol)		pellet fractions (nmol)	
time	base propenals[a]	volatile ^{3}H[b]	base propenals[a]	volatile ^{3}H[b]
5 min	1.70	0.06	2.55	0.57
45 min	3.41	0.15	3.10	0.43
3 h	4.44	0.25	1.75	0.34
7 h	5.29	0.31	1.60	0.26

[a] Determined by thiobarbituric acid assay as described in Methods. [b] Recovered by bulb-to-bulb distillation of ethanol/water solution; specific activity 380 cpm/nmol.

of the BLM-induced DNA damage, is also not subject to rapid 2'-hydrogen exchange. This is confirmed by analysis of the trapped product **9** from poly[dA(2'-*S*-^{3}H)dU] as described above for the *pro-R* isomer; the specific activity of this product (340 cpm/nmol) is the same as that of deoxyuridine (353 cpm/nmol, Table VI). The results of *R* and *S* poly[dA(2'-^{3}H)dU] are complementary and support the notion that release of ^{3}H from the *pro-R* C2'-position is rapid compared with the uracil propenal formation that is the ultimate consequence of its release. These results essentially confirm the findings of Burger et al. (1986)[25] and their assumption that release of ^{3}H into the supernatant from [1',2',5-*methyl*-^{3}H]thymidine-containing DNA occurs primarily from the *pro-R* 2'-position.

Discussion

The results summarized in the introduction and presented in the Results section must be incorporated into a coherent mechanistic picture for base propenal formation. The "favored" mechanism (Scheme I, pathway A) in the literature postulates that a putative 4'-hydroperoxide **3** undergoes heterolytic O–O bond cleavage via a Criegee-type rearrangement to produce an aldehyde intermediate **6**, presumed to be the "stable" base propenal precursor. As recently demonstrated using the 4'-α,β epimers of 3'-*O*-benzoyl-5'-deoxy-4'-hydroperoxythymidine, heterolytic O–O bond cleavage requires activation either via acylation of the terminal oxygen of the hydroperoxide or via catalysis with H^+ or a Lewis acid.[19] In BLM-mediated DNA degradation, activation of the putative hydroperoxide intermediate toward heterolysis might be envisioned via migration of either the 3'- or 5'-phosphate moiety to the terminal oxygen of the hydroperoxide. In the case of the 3'-phosphate, this migration would require 3'-P–O bond scission, which is contrary to the previously demonstrated 3'-C–O bond cleavage.[21] Migration of the 5'-phosphate would not result in production of the observed 3'-phosphoglycolate termini. Thus neither of these possibilities is mechanistically viable. If this rearrangement is to be considered a reasonable mechanism, an Fe^{3+}–BLM-assisted O–O bond cleavage, i.e., metal–BLM acts as a Lewis acid, must therefore be invoked. The possibility of the involvement of BLM in this process is supported by the observation that Fe–BLM coprecipitates in ethanol/high salt with the DNA.[44] Therefore, the observed decomposition of the precursor to base propenal always occurs in the presence of Fe–BLM. Furthermore, DNA hydroperoxides can be formed by treatment with ionizing radiation although the distribution of sugar and base hydroperoxides is not precisely known. Interestingly, ~80% of DNA hydroperoxides produced in this manner can be decomposed by Fe^{3+}–BLM, while the base-modified C-5 and C-6 hydroperoxides of thymine are unaffected by the drug.[45] Thus, site-specific O–O bond cleavage catalyzed by Fe–BLM is a realistic possibility.

Two experimental facts however are difficult to reconcile with the postulated mechanism for the Criegee rearrangement as defined in pathway A, Scheme I. The first is the observation that a single atom of ^{18}O derived from $^{18}O_2$ in the chase is observed in the 3'-phosphoglycolate-modified DNA. This result requires

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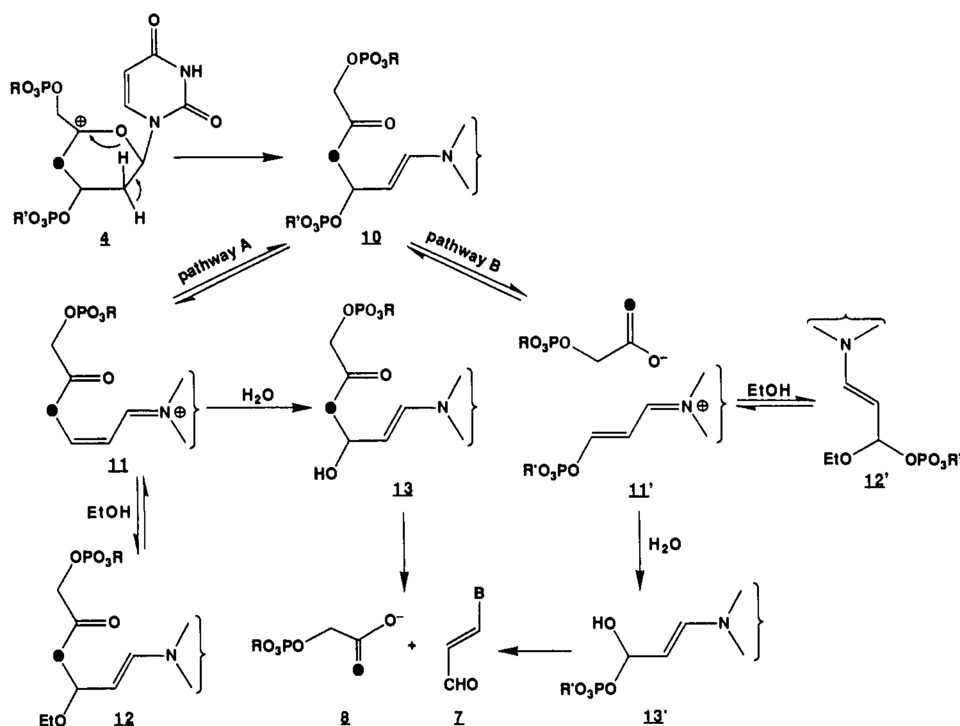
Scheme II



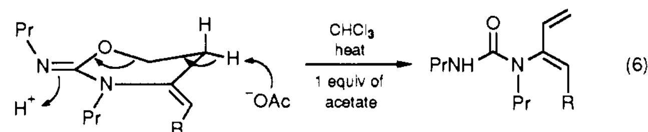
that, subsequent to O–O heterolytic bond cleavage, no significant exchange of the putative iron-bound hydroxide occurs prior to trapping of the carbocation intermediate (**4**). The second is that the rapid release of ^{3}H from the *pro-R* 2'-position is inconsistent with aldehyde **6** as the long-lived base propenal precursor, given that the ^3H_2O does not appear to be a salt-mediated exchange process. In this mechanism, base propenal would be produced at a rate greater than or equal to the rate of ^3H_2O production.

It is also possible that Fe^{3+}–BLM mediates homolytic cleavage of the 4'-hydroperoxy O–O bond.[46,47] The immediate consequence of homolytic cleavage would be the production of a 4'-alkoxy radical. Cleavage of the C3'–C4' bond would then occur by a β-scission reaction to generate a 3'-carbon radical. Recombination with a hydroxy radical equivalent (rebound mechanism) or one-electron oxidation at C-3 followed by H_2O addition would generate a phosphate hemiacetal that would decompose to generate the putative 3'-aldehyde **6** (pathway A, Scheme I). This proposal would give labeling patterns different from those predicted from the heterolytic mechanism. Furthermore, this mechanism still cannot reasonably accommodate the rapid rate of [2'-*pro-R*-^{3}H] washout. Finally, recent studies of Hecht and co-workers[47] suggest that, in contrast to the proposal of Padbury et al.,[46] homolytic O–O cleavage is an unlikely possibility.

One additional experiment has been undertaken in an effort to identify the "long-lived" base propenal precursor as **6** (Scheme I). We postulated that if aldehyde **6** (Scheme I) was the intermediate, it might be reductively trapped with $NaBH_4$ to generate 1,3-propanediol and free nucleic acid base. A protocol for trapping this putative 1,3-propanediol by forming a stable 6-membered ring with phenylboronic acid was developed.[48] Control experiments demonstrated facile recovery of authentic 1,3-propanediol (1–100 nmol) as the propane-1,3-diol phenylboronate. This product could then be directly identified by GC–MS analysis. However, in authentic BLM reactions in which 9–13 nmol of 1,3-propanediol would have been expected to be produced, no evidence for this product was obtained. This result, while preliminary, suggests that the 3'-aldehyde **6** is not the long-lived base propenal precursor as predicted by Scheme I, pathway A. Thus, at this stage, evidence suggests that an alternative mechanism to the one described in Scheme IA must be considered.

The following alternative mechanism for base propenal formation can be put forth to accommodate the available information (Scheme II). The initial step would be identical to that postulated in pathway A, Scheme I: iron-catalyzed rearrangement to form a 4'-carbonium ion **4**. Removal of the 2'-*pro-R* proton and concomitant anti elimination of O4' would generate intermediate **10**. Intermediate **10** could be converted to either intermediate **11** or **11'** by elimination of the 3'-phosphate moiety or 3'-phosphoglycolate moiety, respectively (pathways A or B, Scheme II). Either of these intermediates could function as the ethanol-precipitable base propenal precursor (**12** or **12'**, Scheme II). Addition of H_2O to **11** or **11'** would result in the formation of highly reactive hemiacetals **13** or **13'**, which would be expected to rapidly decompose to form base propenal **7** and the 3'-phosphoglycolate moiety **8**.[50]

Chemical precedent for an anti elimination similar to that proposed in the conversion of **4** to **10** (Scheme II) has been provided by recent studies of Clegg et al.[49] (eq 6). This proposal



accounts for the required removal of the 2'-*pro-R* hydrogen from the DNA at a rate greater than or equal to that of DNA strand scission. A model system for the breakdown of intermediate **10** has not been found. Scheme II offers the proposal that its breakdown might proceed by either of two pathways, both of which involve DNA strand scission. To our knowledge, however, no precedent exists for such heterocyclic base-assisted elimination reactions. Model systems need to be designed to further explore this proposition.

Studies of Burger et al.[25] also require the existence of a "long-lived" intermediate subsequent to DNA strand scission and prior to base propenal formation. Intermediate **11** or **11'** would

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of necessity be this putative intermediate(s). The lifetime of **11** or **11′** might be expected to be enhanced if it is in equilibrium with intermediate **10**. This proposal is not unreasonable given the geometric constraints imposed on the system by the three-dimensional structure of DNA. Alternatively, **10** might be the long-lived intermediate that could undergo strand scission as an artifactual consequence of the method using ethidium bromide fluorescence changes to monitor strand scission.[25] Finally, Michael addition of H_2O to either **11** or **11′** would result in the production of **13** or **13′**, respectively, which on the basis of recent studies of Sorensen and Jencks,[44] would rapidly be converted to the observed products **7** and **8** (Scheme II).

Summary. The following pieces of information must be accounted for in any mechanism proposed for base propenal formation by activated BLM: (1) the stereospecific removal of the 2′-*pro-R* proton; (2) the incorporation of one atom of ^{18}O into the carbonyl group of glycolic acid derived from the second O_2 equivalent; (3) the incorporation of ^{18}O from solvent into the aldehyde group of base propenal; (4) the 3′-C–O bond cleavage to generate a 5′-phosphate terminus; and (5) the kinetically more rapid release of the 2′-*pro-R* proton and DNA strand scission relative to the release of base propenal from DNA. While the traditionally accepted mechanism for base propenal formation (Scheme I, pathway A) meets the first four requirements, it cannot reconcile the kinetic uncoupling of 2′-*pro-R* proton release and strand scission from this process. The present studies, which have verified and expanded the observations of Burger et al.,[25] suggest that the putative "long-lived" precursor of base propenal is consistent with intermediate **10** in equilibrium with either **11** or **11′** (Scheme II).

DNA Binding and Photocleavage by Uranyl(VI) (UO_2^{2+}) Salts

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Received November 16, 1990. Revised Manuscript Received February 1, 1992

Abstract: The interaction of the uranyl(VI) ion (UO_2^{2+}) with DNA and its light-induced cleavage of DNA has been studied using flow-linear dichroism and ^{32}P-endlabeled oligonucleotides. It was found that binding of uranyl ion to DNA is a prerequisite for photocleavage; from run-off experiments the binding constant was estimated to be of the order of 10^{10} M^{-1} at pH 4. The angular orientation of the $[O{=}U{=}O]^{2+}$ chromophore is consistent with binding by bridging phosphate groups on opposite strands of the minor groove of DNA; at higher DNA concentration aggregation indicates intermolecular bridging as well. The uranyl-mediated photocleavage of DNA is not influenced by the presence of O_2, is more efficient at low pH (<7), and is virtually absent at pH 8.5. Subsequent treatment of uranyl photocleaved DNA with hot piperidine does not significantly increase the cleavage. The free nucleobases (adenine, cytosine, guanine, and thymine) were the major ethanol-soluble products to be observed after uranyl photocleavage of calf thymus DNA. From experiments using ^{32}P-endlabeled and methylphosphonate-containing oligonucleotides, it was concluded that upon irradiation attack by the uranyl ion occurs next to the phosphate to which it is bound, with equal preference for the 3′- and 5′-deoxyribose.

Introduction

Analysis of the structure and conformation of DNA complexes relies heavily on the employment of small probes that react with DNA either by forming covalent adducts or by inducing DNA strand scissions. These probes include alkylating agents, such as dimethyl sulfate (which primarily methylates N7 of guanine) or ethylnitrosourea (which ethylates oxygen on the DNA phosphates) or cleaving reagents such as EDTA/Fe(II) and various phenanthroline and bipyridyl complexes of transition metal ions (e.g., Cu(I), Ru(II), and Co(II)). The strand scission is typically caused by oxidation of the deoxyribose moiety of the DNA backbone.[1-5]

We have recently found that the uranyl(VI) ion (UO_2^{2+}) induces single strand nicks in DNA upon irradiation with long wavelength ultraviolet light[6] which can be exploited for photofootprinting of phosphate backbone contacts in protein–DNA complexes such as λ-repressor/O_R1,[6] *Escherichia coli* RNA polymerase/promoter,[7] and transcription factor IIIA-ICR interaction.[8] The uranyl ion, furthermore, serves as a sensitive probe of DNA conformation as exemplified in the modulation of the photocleavage of bent DNA and various other DNA sequences.[9,10]

Because of the potential of the uranyl ion mediated DNA photocleavage in molecular biology for studying DNA ligand complexes and DNA conformations it is important to understand the interaction of the uranyl ion, UO_2^{2+}, with DNA.

The dynamic behavior of the uranyl/DNA system in the dark, its pH, and ionic strength dependencies as well as the photoinduced reactions resulting in DNA cleavage are complex. Previous studies[11] exploiting uranyl staining of DNA for electron microscopy have concluded that at low pH (<3.5) one uranyl ion is bound for every two phosphates of the DNA with an association constant

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