

Identification and Quantitation of the Lesion Accompanying Base Release in Bleomycin-Mediated DNA Degradation

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Abstract: Interaction of bleomycin, Fe²⁺, and limiting O₂ or bleomycin, Fe³⁺, and H₂O₂ with DNA results in the production of free nucleic acid base and an oxidatively damaged sugar lesion that undergoes strand scission subsequent to treatment with alkali. The hexamer d(CGCGCG) has been utilized to develop a protocol to establish the identity of this lesion as a 2'-deoxy-4'-pentulose moiety and to establish its stoichiometric production with respect to free nucleic acid base. The protocol developed has been extended to a variety of heterogeneous DNAs with similar results. The predominant pathway for base release in bleomycin-mediated DNA degradation is the result of chemistry at the C-4' carbon of the sugar moiety.

Bleomycin (BLM) is an antitumor antibiotic isolated from *Streptomyces verticillilis* by Umezawa and co-workers in 1966.¹ Its cytotoxicity is thought to be related to its ability to bind to double-stranded DNA, and in the presence of cofactors Fe²⁺ and O₂ or Fe³⁺ and H₂O₂, it causes single- and double-stranded breaks.^{2–4}

The two major monomeric products formed when Fe–BLM interacts with DNA are base propenal and nucleic acid base. 3'-Phosphoglycolate- and 5'-phosphate-modified DNA fragments associated with formation of base propenal have been identified.^{5–7} The nature of the modified DNA that accompanies base release has proven to be a less easily resolvable issue. It has been known since the 1970s that the liberation of DNA base results in the destabilization of the DNA sugar–phosphate backbone such that additional single-strand cleavage of the DNA occurs upon alkaline treatment.⁸ Thus this lesion has been referred to as "alkaline labile".

Studies using specifically tritium-labeled poly(dA–dU) led to a proposal for the chemical identity of these alkaline-labile sites^{9–12} (Figure 1). Isotope effect studies with poly(dA–[4'-³H]dU) indicated that both nucleic acid base and base propenal resulted from partitioning of a common intermediate hypothesized to be a 4'-carbon-centered radical.^{9,10} Additional studies utilizing poly(dA–dU) isotopically labeled at the 2'-*proS*-, 3'-, and 5'-positions of dU revealed a heat- and pH-dependent labilization of tritium that could be suppressed by treatment of the alkaline-labile lesions with NaBH₄.^{9,11} These results in toto led to the prediction that a 4'-ketone moiety is generated (1, Figure 1) concomitant with base release.

In the past few years, efforts have been focused on identification of this oxidized sugar.^{11,13,14} Sugiyama et al.^{13,14} have attempted to define the stable products generated when the oligomer d(CGCTTTAAAGCG) was treated with Fe²⁺, BLM, and O₂, followed by strand cleavage either with NaOH (0.2 M) at 90 °C for 10 min or with hydrazine. From their reaction mixture treated with NaOH, they observed two products that comigrated on a reverse-phase HPLC column with a 3'-(3-hydroxy-5-oxo-1-cyclopentenyl) modified dimer prepared by chemical synthesis (Figure 2a). A hydrazine-trapped 3'-(3-pyridazinylmethyl)-modified dimer also coeluted with an authentic chemical standard (Figure 2b). However, no quantitative comparisons of these products to base release were reported.

We have taken an alternative approach to identifying this oxidized sugar: stabilization of the lesion by NaBH₄ reduction, degradation into sugar moieties utilizing enzymatic methods, and

GC/MS identification of the derivatized alditol (Figure 3). In preliminary studies we showed that the hexamer d(CGCGCG) served as an excellent DNA model for BLM-mediated DNA degradation.¹⁵ In the present studies we have been able to identify the structure and quantitate the amount of intact modified strand formed when cytosine is released. The same results are obtained when BLM is activated by Fe³⁺ and H₂O₂ anaerobically or by Fe²⁺ and O₂. The ultimate precursor of these products, 2'-deoxy-4'-pentulose, has also been demonstrated to exist at the sites of alkaline lability of poly(dA–dU), poly(dG–dC), and calf thymus DNA.

Experimental Section

Materials. The hexanucleotide d(CGCGCG) was generously provided by John Gerlt at the University of Maryland. Blenoxane, a clinical mixture containing 60% BLM A₂ and 30% BLM B₂, was a gift of Bristol-Myers. DNA polymerase large fragment (Klenow fragment, 3.5 units μL⁻¹) was isolated from a cloned overproducer provided by Dr. Nigel Grindly at Yale University. P₁ nuclease [specific activity 800 units mg⁻¹, where 1 unit hydrolyzes 1 μmol of 3'-AMP min⁻¹ at 37 °C (pH 7.2)], snake venom phosphodiesterase (PDE I) [46 units mg⁻¹, 1 unit hydrolyzes 1 μmol of *p*-nitrophenyl phosphate min⁻¹ at 25 °C (pH 8.9)], spleen 3'-exonuclease (phosphodiesterase II) [1 unit produces an absorbance change of 0.2 at 260 nm in 30 min at 37 °C when incubated with an excess of RNA in 125 mM succinate hydrochloride (pH 6.5)], poly(dG–

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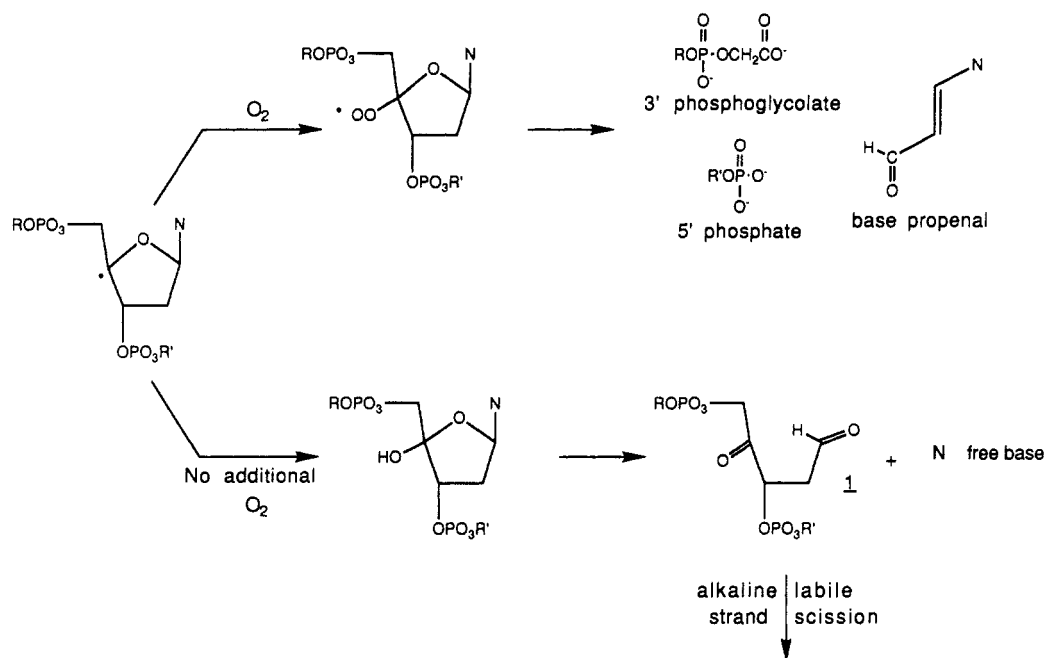


Figure 1. Products produced upon interaction of activated BLM with DNA.

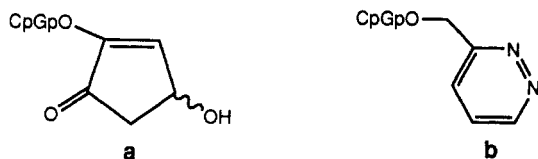
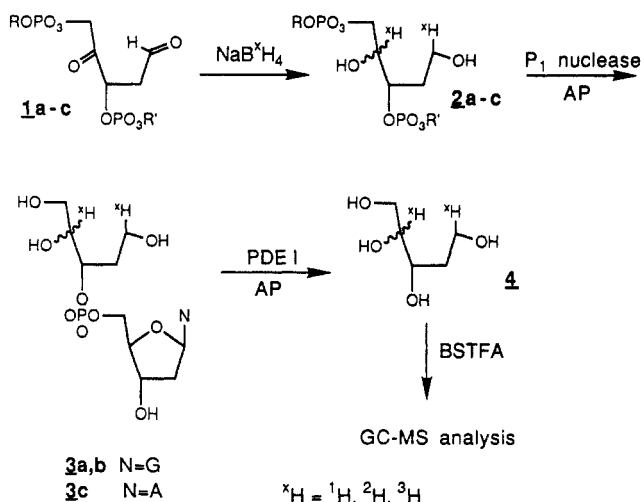


Figure 2. Interaction of Fe^{2+} -BLM- O_2 with d(CGCTTAAAGCG), followed by treatment with (a) 0.2 M NaOH to produce 3-hydroxy-5-oxo-1-cyclopenten-1-yl 2'-deoxycytidylyl-(3'→5')-2'-deoxy-3'-guanylate or (b) NH_2NH_2 to produce 3-pyridazinylmethyl 2'-deoxycytidylyl-(3'→5')-2'-deoxy-3'-guanylate.



Substrate	R	R'
a d(CGCGCG)	d(CGCG)	dG
b poly(dGdC)	d(CG) _n	d(GC) _n
c poly(dAdU)	d(UA) _n	d(AU) _n

Figure 3. General method to isolate and quantitate the oxidatively damaged sugar accompanying base release when activated BLM interacts with DNA.

dC), poly(dA-dU), deoxynucleoside triphosphates, and d(CG) were purchased from Pharmacia. T4 polynucleotide kinase (10^4 units mL^{-1} ,

1 unit catalyzes the production of 1 nmol of acid-insoluble ^{32}P in 30 min at 37°C and pH 7.6) was obtained from New England Biolabs. *Escherichia coli* alkaline phosphatase (specific activity 45 units mg^{-1} , where 1 unit hydrolyzes 1 μmol of *p*-nitrophenyl phosphate min^{-1} at 37°C and pH 10.4), calf intestinal alkaline phosphatase, tRNA, calf thymus DNA, NaBH_4 , NaB^3H_4 (98 atom %), thiobarbituric acid, and methyl β -D-xylopyranoside were obtained from Sigma. Nuclease Bal-31 (1 unit produces 1 μg of acid-soluble nucleotide from denatured calf thymus DNA in 1 min at 30°C , pH 8.1) was a product of Bethesda Research Laboratories. NaB^3H_4 (25 mCi, 15 Ci mmol^{-1}) was supplied by Amersham. 2-Deoxy-D-ribose, 2-methoxypropene, 1,1'-thiocarbonyldiimidazole, tri-*n*-butyltin hydride, and silica gel (230–400 mesh) were purchased from Aldrich. Cellulose F254 TLC plates (0.1 mm thick, 20×20 cm sheets), silica gel 60 F254 TLC plates (0.2 mm thick, 20×20 cm sheets), and PEI-cellulose F-TLC plates (0.1 mm thick, 20×20 cm sheets) were products of EM Reagents. Kodak X-Omat AR 50 X-ray film was used for autoradiography. *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) and dry acetonitrile used as silylation cosolvents were purchased from Pierce Chemical Corp. C_{18} reverse-phase HPLC columns were obtained from Alltech (10 μm) of Beckman (5 μm). Enhance spray, ScintA, and [γ - ^{32}P]ATP (3000 Ci mmol^{-1}) were obtained from New England Nuclear. All other reagents were of the highest available laboratory grade.

HPLC analyses were performed by using a Beckman system with Model 110A pumps. Detection was by UV absorption at 254 or 260 nm. FPLC was performed on a Pharmacia system using an analytical Mono-Q column with UV detection at 254 nm. ^1H NMR spectra were obtained by using a Bruker 270-MHz spectrometer, and ^{13}C NMR spectra were taken on a Bruker 200-MHz instrument (at 50.3 MHz). After trimethylsilyl (TMS) derivatization, samples were analyzed by GC/MS on a Kratos MS 25 system with a 10-m OV-17 capillary column (Alltech) or a 30-m homemade equivalent and on a Hewlett-Packard 5890 GC/5970A MS with a 30-m DB-5 capillary column (J&W Scientific) with detection as total ion current. UV/visible spectra were taken on a Cary 210 or Beckman DU-50 instrument. A Bioscan gas radioactivity scanner and Packard Tri-Carb 300 liquid scintillation counter were used to measure radioactivity.

The following extinction coefficients were utilized to quantitate starting materials: bleomycin (λ_{292} , $\epsilon = 14.5 \text{ mM}^{-1} \text{ cm}^{-1}$), calf thymus DNA (λ_{260} , $\epsilon = 6.6 \text{ mM}^{-1} \text{ cm}^{-1}$), poly(dA-dU) (λ_{260} , $\epsilon = 6.7 \text{ mM}^{-1} \text{ cm}^{-1}$), and poly(dG-dC) (λ_{266} , $\epsilon = 8.4 \text{ mM}^{-1} \text{ cm}^{-1}$). The following extinction coefficients were utilized to quantitate products isolated by HPLC: cytosine (λ_{267} , $\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$), dC (λ_{271} , $\epsilon = 8.93 \text{ mM}^{-1} \text{ cm}^{-1}$ at pH 5.5), cytosine propenal (λ_{312} , $\epsilon = 29.1 \text{ mM}^{-1} \text{ cm}^{-1}$), 5'-dGMP (λ_{252} , $\epsilon = 13.7 \text{ mM}^{-1} \text{ cm}^{-1}$), and dG (λ_{252} , $\epsilon = 13.7 \text{ mM}^{-1} \text{ cm}^{-1}$). Poly(dG-dC) and poly(dA-dU) were synthesized by using the Klenow fragment of DNA polymerase as previously described.¹⁶ Phosphate was determined by the

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procedure of Ames and Dubin¹⁷ and H₂O₂ by the colorimetric procedure of Hildebrandt et al.¹⁸ Quantitation of base prepenals was determined by reaction with thiobarbituric acid (TBA) to produce a chromophore characteristic of malondialdehyde (MDA) (λ_{432} , $\epsilon = 160 \text{ mM}^{-1} \text{ cm}^{-1}$).^{19,20}

Reaction of BLM-Fe³⁺-H₂O₂ with d(CGCGCG). All solutions were rendered anaerobic by a 15–20 min purge with argon scrubbed with Fieser's solution²¹ or copper catalyst at 80 °C (BASF catalyst R3-11 obtained from Chemical Dynamics Corp.). A typical reaction mixture in a final volume of 0.5 mL contained 0.21 mM BLM, 0.21 mM FeN-H₄(SO₄)₂·12H₂O, and 0.84 mM d(CGCGCG) (in nucleotides: $\epsilon_{260} = 8.3 \text{ mM}^{-1} \text{ cm}^{-1}$) in 10 mM HEPES (pH 7.5) under an argon atmosphere. Reactions were initiated with argon-purged H₂O₂ to give a final concentration of 0.6 mM. H₂O₂ was added either as a single aliquot at time zero or as several aliquots over 30 min. The reactions were allowed to proceed for 30 min to 2.5 h at room temperature. At the end of this period the products were analyzed directly by using a reverse-phase HPLC column (10 μm : 4.6 mm \times 25 cm) (flow rate 1 mL min⁻¹). Buffer A contained 5 mM NH₄OAc or potassium phosphate at pH 5.5, and buffer B was CH₃OH. Elution was effected by using a 0–20% CH₃OH linear gradient over 10 min, followed by isocratic elution for an additional 15 min. Products, amounts, and retention times were as follows: cytosine, 23.0 nmol, 7.8 min; **1a**, 16 nmol, 10.7 min; d(CGCGCG), 27.2 nmol, 12.4 min.

Reaction of BLM-Fe²⁺-O₂ with d(CGCGCG). Experiments involving atmospheric O₂ contained 0.21 mM BLM, 0.84 mM d(CGCGCG) (in nucleotides), and 10 mM HEPES (pH 7.5). A solution of Fe(NH₄)₂(SO₄)₂·6H₂O (final concentration 0.21 mM) in deionized H₂O was added to initiate the reaction. The temperature was maintained at 4 °C and the reaction was allowed to proceed for 20 min. Products of a typical experiment (Figure 6) were analyzed directly by HPLC as described above. Products, amounts, and retention times were as follows: cytosine, 11.8 nmol, 6 min; dGMP, 10.8 nmol, 10.7 min; d(CGCG) glycolate, 13.1 nmol, 11.5 min; **1a**, 10.2 nmol, 12.8 min; d(CGCGCG) 8.6 nmol, 14.8 min; cytosine prepenal, 15.2 nmol, 15.7 min.

Conversion of **1a to **3a**: NaB³H₄ ($x = 1, 2, 3$) Reduction and Enzymatic Digestion.** Compound **1a** was isolated as described above by using a semipreparative reverse-phase HPLC column, flow rate 2 mL min⁻¹, and the solvent volume was reduced in vacuo without heat. A typical reduction was carried out in 40 mM CHES (pH 9.0) and 60 mM NaBH₄ for 30 min at 4 °C or in 500 mM HEPES (pH 6.8) and 200 mM NaBH₄ at 4 °C for 30 min. In certain cases NaB³H₄ (25 mCi, 1.67 μmol) (5.2 mM) or NaB³H₄ replaced NaBH₄. For the NaB³H₄ reaction, the incubation was for 3.5 h at room temperature, followed by addition of unlabeled NaBH₄ to a final concentration of 200 mM and incubation for an additional 45 min. At the end of the incubation, acetic acid was added to destroy the excess NaBH₄ and the solution neutralized. The product was isolated by reverse-phase HPLC chromatography as described above. Compound **2a** eluted at 11 min.

In a typical reaction, 7.2 nmol of **1a** resulted in the isolation of 6.6 nmol of **2a** by reverse-phase HPLC (92% recovery) when the reduction was conducted at pH 9.0. Compound **2a** was shown to elute at 9 min as a single peak (76% recovery) from a Mono-Q-column. Buffer A was 12.5 mM Tris-HCl (pH 7.5), and buffer B was identical with buffer A but contained 1 M NaCl. Elution was effected by using 5% buffer B for 5 min, followed by a 5–100% buffer B linear gradient over 15 min.

Compound **2a** (91 nmol) was added to 20 mM NaOAc (pH 5.5) containing 1.4 mM ZnCl₂. Five to ten units of P₁ nuclease was added, and the reaction was allowed to proceed for 1.5 h at 37 °C. The pH was raised to 7.5 by the addition of Tris-HCl (final concentration 0.056 M), 3 units of alkaline phosphatase was added, and the incubation was continued at 37 °C for an additional 30 min. The reaction was stopped by heating the solution in a boiling H₂O bath for 2.5 min. The solutions were cooled and the protein was removed by centrifugation. The products of the reaction were analyzed by three HPLC systems. (a) Compound **3a** eluted from a C₁₈ reverse-phase column by an isocratic elution with 5 mM NH₄OAc (pH 5.5) for 5 min, followed by a linear gradient from 0 to 20% CH₃OH over 20 min. Products, amounts, and retention times were as follows: dC, 191 nmol, 18 min; **3a**, 91.3 nmol, 19.2 min; dG, 191.5 nmol, 27.9 min. (b) Compound **3a** was also shown to elute as a single peak (retention time 18 min) by ion-pairing reverse-phase chromatography and isocratic elution with 12% CH₃OH and 88% 50 mM

potassium phosphate (pH 4.8) containing 5 mM tetrabutylammonium bromide, flow rate 1 mL min⁻¹. (c) Compound **3a** was eluted at 3 min from the reverse-phase column with H₂O as eluent, flow rate 1 mL min⁻¹. Compound **3a** comigrated in all three systems with the authentic D-erythro-**3a** prepared as described subsequently.

Conversion of **3a to **4**.** Compound **3a** (11.5 nmol) was incubated in 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl₂ with 3 units of PDE I at 37 °C for 1.5 h. The reaction was stopped by heating the solution in a boiling H₂O bath for 2.5 min. The protein was removed by centrifugation and the products were analyzed by ion-pairing reverse-phase HPLC (HPLC system b listed above). The sole UV-absorbing species observed was 5'-dGMP (10.0 nmol, 87%). When isolation of the deoxypentitol products **4** was desired, reverse-phase HPLC with H₂O elution was used (HPLC system c). Compound **4** eluted near the void volume of the column, between 3.5 and 5.0 min.

TLC of [³H]-4** and Deoxypentitol Standards.** [³H]-**4** isolated by HPLC was compared to chemically prepared 2-deoxy-L-threo-pentitol (**11**) and 2-deoxy-D-erythro-pentitol (**5**) (see below) by thin-layer chromatography. Three TLC systems were used. (a) Cellulose plates were impregnated with tungstate by dipping the plates in a 5% tungstate solution, pH 7.4 or 8.0 (adjusted with sulfuric acid). Plates were developed with a 5:3:2 acetone:1-butanol:H₂O mixture. (b) Silica gel plates were impregnated with tungstate by dipping the plates in a 5% tungstate solution, pH 8 or 7.4, and developed with a 2:2:1 ethyl acetate:2-propanol:H₂O mixture. (c) Silica gel plates were eluted with a 9:1 1-butanol:H₂O mixture. Detection of the chemical standards for system a used alkaline silver nitrate. Air-dried plates were dipped into a saturated solution of AgNO₃ in acetone and allowed to dry. The plates were developed by spraying with 0.5 M NaOH in 95% ethanol. For systems b and c, the plates were developed by spraying with 5% sulfuric acid in ethanol and subsequent charring. Radioactivity was determined by (a) scanning with a gas radioactivity counter, (b) scintillation counting, and/or (c) autoradiography. Autoradiography was performed by spraying the developed TLC plate with Enhance spray 3–4 times and exposing it at –78 °C for 1 week. Alternatively, [³H]-**4** was chromatographed adjacent to lanes containing standards, and the TLC plates were then cut into rectangular sectors that were counted by liquid scintillation in ScintA after a 30-min elution of the radioactivity from the thin-layer plate with 500 μL of H₂O. The radioactivity was observed at the expected R_f in comparison with the standards. Identical results were obtained from **4** derived from peak **1a** produced from either the Fe³⁺-H₂O₂- or Fe²⁺-O₂-activated bleomycin reactions.

GC/MS Analysis of Deoxypentitols. Compound **4** was eluted with H₂O from a C₁₈ Sep-Pak cartridge (Waters Associates) prewashed with methanol. The aqueous solution was deionized by successive passage over 0.6-mL ethanol-washed Dowex-1-X2 (HCOO⁻ or OH⁻ form) and Dowex-50W-X4 (H⁺ form) columns, and the aqueous washes were combined and lyophilized. The compound was transferred to the silylation vessel by rinsing the lyophilization flask 3–4 times with methanol. After several ethanol evaporations, the silylation vessel and contents were dried in vacuo over P₂O₅ for at least 12 h. The compounds were silylated with a 1:1 acetonitrile:BSTFA (with 1% TMCS) mixture at 100 °C for 30 min (10- μL total volume), and aliquots (5 μL or less) were analyzed by GC/MS. The (TMS)₄ derivatives typically eluted at 200 °C.

Reaction of Polymeric DNAs with Fe²⁺-BLM and Limiting O₂. Poly(dG-dC) (1.2 or 0.84 mM in nucleotides), poly(dA-dU) (1 mM in nucleotides), or DNA from calf thymus (1 mM in nucleotides) in a final volume of 0.5 mL was reacted with 0.21 mM Fe²⁺-BLM and 0.42 mM O₂ in 40 mM CHES, pH 9, in the presence of 60 mM NaB³H₄ for 90 min at 4 °C (limiting O₂ conditions³⁷). The reaction was quenched with acetic acid, and the products and unreacted oligonucleotides were digested in situ with P₁ nuclease (5 units) and alkaline phosphatase (3 units) by using standard buffers under anoxic conditions. The products were analyzed by reverse-phase HPLC using a 5-min isocratic elution with 5 mM NH₄OAc, pH 5.5, followed by 0–20% methanol over 20 min for poly(dG-dC), or a 5-min elution with 0% methanol, followed by 0–30% methanol over 30 min for poly(dA-dU). The product of these reactions, **3b** from poly(dG-dC) or **3c** from poly(dA-dU), was further digested with PDE I, and the deoxypentitols **4** were prepared for GC/MS as described above. The entire reaction mixture was digested with PDE I when calf thymus DNA was the substrate.

Two-Dimensional TLC Sequencing of Oligonucleotides **2a.** A method for sequencing small oligonucleotides using two-dimensional thin-layer chromatography (TLC) has recently been developed.²⁵ The technique was used to analyze d(CGCGCG) and **2a** from Fe³⁺-BLM-H₂O₂ and Fe²⁺-BLM-O₂ reactions. The substrate, at a concentration of 10 nmol/*n* where *n* = the number of bases, was reacted with 60 milliunits of spleen 3'-phosphodiesterase II (PDE II) in 50 μL of 10 mM sodium 2-(*N*-morpholino)ethanesulfonate (MES) buffer, pH 6.5, at 37 °C. Aliquots of 5 μL each were removed at 1, 3, 6, 10, 15, 20, 30, 40, 50, and 60 min,

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frozen on dry ice, and then heated at 100 °C for 5 min to inactivate the enzyme. Each of these aliquots was then combined with 5 μ L of 250 mM Tris-HCl, pH 7.6, 10 μ L of water, and 58 milliunits of calf intestinal alkaline phosphatase and incubated for 15 min at 37 °C prior to heat inactivation of the enzyme. The entire solution was added to 33 pmol of dried [γ - 32 P]ATP (75 Ci mmol $^{-1}$), and 5 μ L of 50 mM MgCl $_2$ /25 mM dithiothreitol and 2 units of T $_4$ polynucleotide kinase were added. After a 45-min incubation at 37 °C, 1–2 μ L of the reaction mixture was applied to a PEI-cellulose plate (prewashed with 10% NaCl, then washed twice with doubly distilled H $_2$ O). Chromatography in the first dimension was effected with a 1:1 formamide:Tris-HCl (2.7 N) (pH 8.3) solution. After development, the plate was air-dried and the eluting solution removed by three washes with methanol. The chromatographed oligomers were then digested with 100 μ L of a solution containing 20 mM Tris-HCl, pH 8, 12 mM CaCl $_2$, 12 mM MgCl $_2$, 1 mM EDTA, 1 OD $_{260}$ unit of tRNA, and 3 units of Bal-31 nuclease along the oligomer line; the plate was wrapped with Saran wrap, pressed between two sheets of Lucite plastic, and incubated at 37 °C for 1 h. Chromatography in the second dimension was effected with a sodium acetate buffer, pH 4.35 (0.2 M Na $^+$). TLC plates were subjected to autoradiography using Kodak X-Omat AR 50 X-ray film.

Chemical Standards. (A) **Synthesis of 3-O-[5'-(2'-Deoxyguanylyl)]-2-deoxy-D-erythro-pentitol (D-erythro-3a).** The cytosine-specific cleavage reaction of the Maxam-Gilbert sequencing technique was utilized to prepare one diastereomer of 3a.²² 5'-d(CG)-3' (100 OD $_{260}$ units, 5.3 μ mol, Na $^+$ salt) was dried for several hours over P $_2$ O $_5$. To this was added 20 μ L (630 μ mol) of freshly distilled anhydrous hydrazine, and the solution was heated at 60 °C in a sealed tube for 3 h. The unreacted hydrazine was removed in vacuo and the residue redissolved in 100 μ L of H $_2$ O, and 20 μ L of 10% ethanolic benzaldehyde solution was added and the mixture stirred for 12 h at room temperature to effect an aldehyde exchange. This solution was extracted 3 times with diethyl ether after addition of 1 mL of H $_2$ O. The aqueous layer was retained and the volume diminished. A 200-fold excess of NaBH $_4$ was added at 0 °C and the reaction stirred for 1 h. Acetic acid was added at 0 °C and the solvents were removed in vacuo after 30 min, followed by several evaporations with methanolic HCl. The desired compound (D-erythro-3a) was then isolated by C $_{18}$ reverse-phase HPLC (flow rate 1 mL min $^{-1}$; H $_2$ O or aqueous buffer; isocratic elution for 5 min, followed by a 0–20% linear gradient with methanol over 20 min) at a retention time of 18 min. This peak contained 10% of the starting OD units.

This material (D-erythro-3a) could be degraded with PDE I under standard conditions (3 units of PDE I, 10 mM MgCl $_2$, 50 mM Tris-HCl, pH 8.0, at 37 °C for 1.5 h) to produce 5'-dGMP as the sole UV-absorbing species. Digestion with alkaline phosphatase (3 units) for an additional 30 min at 37 °C produced dG. The liberated sugar could be isolated from a C $_{18}$ reverse-phase HPLC column with H $_2$ O as the eluent (3.5–4.5-min retention time on an analytical column at a flow rate of 1 mL min $^{-1}$). The species (R_f = 0.44) comigrated with 2-deoxy-D-erythro-pentitol (5, R_f = 0.45) but not 2-deoxy-L-threo-pentitol (11, R_f = 0.26) on the cellulose TLC system (a) described above. The TMS derivative of the material isolated by HPLC exhibited the characteristic fragmentation pattern for 2-deoxypentitols when subjected to GC/MS analysis after standard workup and silylation (see above).

(B) **Chemical Synthesis of 2-Deoxypentitols.** (1) **Synthesis of 2-Deoxy-D-erythro-pentitol (5) from 2-Deoxy-D-ribose.** 2-Deoxy-D-ribose (2 g, 14.9 mmol) was dissolved in ethanol. NaBH $_4$ (800 mg, 21.2 mmol, 5.6 equiv) was added and the reaction allowed to proceed at room temperature for 1 h. The apparent pH was adjusted to 4 with glacial acetic acid to destroy the excess borohydride, and the solution was decarbonized by passage through a Dowex-50W-X8 column (8.7 \times 2 cm); the solvent was removed in vacuo, and the borate esters were removed by repeated evaporation after redissolution of the residue in methanol. The synthetic procedure closely followed the methods of Tymiak and Rinehart.²³ 1 H NMR (270 MHz, D $_2$ O, HDO = 4.65 ppm) of 5: δ 3.45 (4 H, m), 3.2 (2 H, m), 1.57 (1 H, dtd, J = 14.5, 7.35, 1.57 Hz, H-2), 1.35 (1 H, tdd, J = 14.6, 5.53, 4.45 Hz, H-2). 13 C NMR (50.3 MHz, D $_2$ O, dioxane external standard at 66.5 ppm) of 5: δ 74.5 (C-4), 68.7 (C-3), 62.2 (C-5), 58.3 (C-1), 34.0 (C-2). (Please note that the carbon numbers relative to 2-deoxyribose have been retained, although IUPAC conventions would reverse the numbering scheme for the corresponding sugar alcohol). GC/MS of the (TMS) $_4$ derivative of 5 (Kratos MS 25): m/z 321, 307, 231, 219, 205, 103 (base peak). This compound served as a MS standard for the deoxypentitols.

(2) **Synthesis of 2-Deoxy-L-threo-pentitol (11) from Methyl β -D-Xylopyranoside (6) (Figure 4).** Methyl 2,3-O-isopropylidene- β -D-xylo-

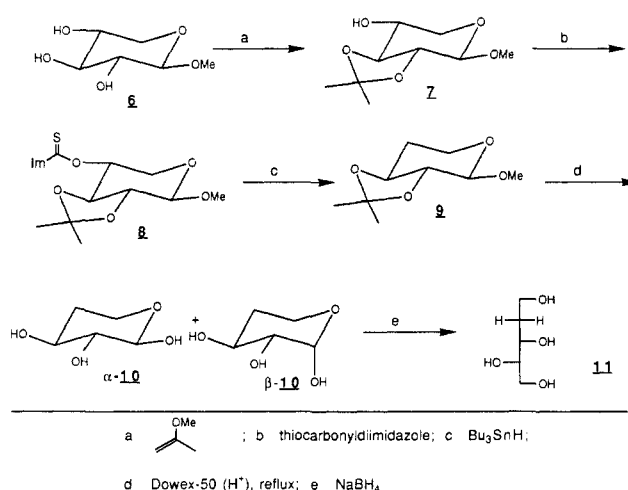


Figure 4. Synthesis of 2-deoxy-L-threo-pentitol (11).

pyranoside (7) was prepared from methyl β -D-xylopyranoside (6) in 72% yield by the procedure previously described.²⁴

(a) **Synthesis of Methyl 2,3-O-Isopropylidene-4-O-(imidazolylthiocarbonyl)- β -D-xylopyranoside (8) from 7.** The protected pyranose compound 7 (850 mg, 4.17 mmol) and 1,1'-thiocarbonyldiimidazole (1.49 g, 8.33 mmol) were dissolved in 20.8 mL of dry 1,2-dichloroethane under N $_2$ and reacted at 40 °C for 3 h. The product was isolated as a clear oil following flash chromatography (silica gel, 1:1 EtOAc:CHCl $_3$ eluent) and rotary evaporation of the appropriate fractions identified by TLC (980 mg, 75%). 1 H NMR (270 MHz, CDCl $_3$, TMS = 0.0 ppm) of 8: δ 8.36 (1 H, s, imidazole ring proton), 7.65 (1 H, s, imidazole ring proton), 7.05 (1 H, s, imidazole ring proton), 5.74 (1 H, dt, J = 8.4, 4.3 Hz, H-4), 4.79 (1 H, d, J = 6.7 Hz, H-1), 4.36 (1 H, dd, J = 13.2, 5.1 Hz, H-5e), 4.09 (1 H, dd, J = 9.9, 8.6 Hz, H-3), 3.71 (1 H, dd, J = 13.3, 3.8 Hz, H-5a), 3.62 (1 H, dd, J = 10.0, 6.8 Hz, H-2), 3.52 (3 H, s, OMe), 1.51 (3 H, s, CH $_3$), 1.48 (3 H, s, CH $_3$). The thiocarbonylimidazole group causes a large downfield shift of the C-4 proton, as evidenced by the coupling of this proton to the C-5 protons. This serves to establish the regiochemistry of the initial isopropylidination.

(b) **Synthesis of Methyl 4-Deoxy-2,3-O-isopropylidene- α -L-threopyranoside (9) from 8.** Tri-*n*-butyltin hydride (525 mL, 1.95 mmol) was dissolved in 84.9 mL of freshly distilled toluene (dried over sodium metal) under an inert atmosphere and heated to reflux. The sugar derivative 8 (400 mg, 1.27 mmol) was dissolved in an additional 21 mL of dry toluene and added to the refluxing mixture over 30 min, and the reaction was allowed to proceed for 2.25 h total. The toluene was removed in vacuo, and the residue was dissolved first in three 50-mL portions of acetonitrile and then the remaining material dissolved in 50 mL of hexanes. The combined acetonitrile washes were extracted 4 times with 50 mL of hexanes, and then the acetonitrile solvent was removed in vacuo. The crude product was purified by flash chromatography (silica gel, 1:1 EtOAc:CHCl $_3$ eluent), and the appropriate fractions were pooled and the solvents removed by rotary evaporation to give 200 mg of a clear liquid (84% yield) that crystallized upon standing, mp 93–97 °C. 1 H NMR (270 MHz, CDCl $_3$, TMS = 0.0 ppm) of 11: δ 4.44 (1 H, d, J = 7.6 Hz, H-1), 4.12 (1 H, ddd, J = 12.3, 4.9, 1.2 Hz, H-5e), 3.56 (1 H, ddd, J = 12.0, 9.4, 4.3 Hz, H-3), 3.55 (3 H, s, OMe), 3.47 (1 H, tdd, J = 12.0, 2.6 Hz, H-5a), 3.23 (1 H, dd, J = 8.8, 7.9 Hz, H-2), 2.07 (1 H, dtd, J = 12.1, 3.8, 2.0 Hz, H-4e), 1.86 (1 H, qd, J = 11.8, 4.8 Hz, H-4a), 1.46 (6 H, s, CH $_3$).

(c) **Synthesis of 4-Deoxy-L-threo-pentose (10) from 9.** The deprotection reaction was carried out in water (120 mg of 9 in 25 mL of H $_2$ O) in the presence of an acid catalyst [0.6 g of Dowex-50W-X4 (H $^+$ form)]. The reaction was followed by TLC (12:8:1 1-butanol:acetic acid:H $_2$ O eluent). After 1.5 h at room temperature, the starting compound 9 had been converted to a new product that was not isolated (presumably methyl 4-deoxy- β -L-threo-pentopyranoside). The solution was then heated to 100 °C over 45 min and refluxed for an additional 4.5 h, during which time the first product was converted to 10. The Dowex was removed by vacuum filtration and the solvent evaporated in vacuo to yield 70 mg (82%) of a clear oil.

The resulting 1 H NMR spectrum of 10 was complex, consisting of a mixture of α and β anomers. The C-2, C-3, and C-5 protons were not clearly separated and the C-4 axial and equatorial protons were not

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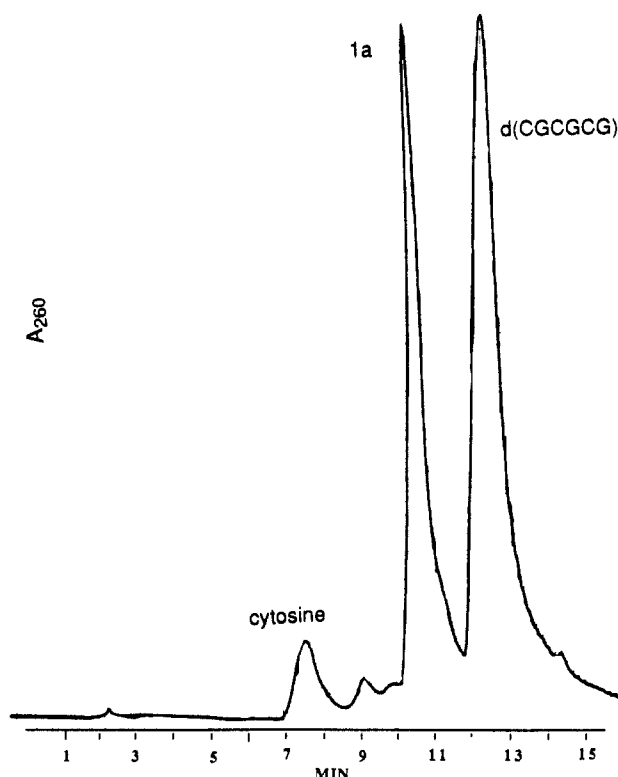


Figure 5. HPLC trace of the anaerobic products produced when d(CGCGCG) is incubated with Fe^{3+} , H_2O_2 , and BLM.

clearly resolved. The α anomer contains all equatorial hydroxyls (H-1, δ 4.29, 70%) while the β anomer possesses an axial hydroxyl group at the anomeric carbon (H-1, δ 5.01, 30%). GC/MS analysis of the $(\text{TMS})_3$ derivatives of **10** is consistent with the proposed structures (data available on request). ^1H NMR (270 MHz, D_2O , HDO = 4.65 ppm) of **10**: δ 5.01 (d, J = 3.4 Hz, H-1), 4.29 (d, J = 7.9 Hz, H-1), 2.8–3.8 (4 H, m), 1.6–1.84 (1 H, m), 1.36–1.53 (1 H, m). The general features of the spectrum of this 4-deoxy sugar can be compared to that of 2-deoxyribose. 2-Deoxyribose forms an α and β anomeric mixture of both the pyranose and furanose rings, all of which are clearly visible in the NMR. The pyranose form is preferred. In contrast, **10** is a 4-deoxy sugar and can only form the 6-membered ring. Hence the absence of any 5-membered rings (anomeric protons would be shifted downfield) is further proof of Bu_3SnH reduction at C-4.

(d) Synthesis of 2-Deoxy-L-threo-pentitol (11) from 10. The synthesis of **11** is completely analogous to that described for 2-deoxy-D-erythro-pentitol (**5**).²³ The 4-deoxy sugar **10** (60 mg, 0.45 mmol) was dissolved in 2 mL of ethanol, to which was added 14 mg of NaBH_4 (0.37 mmol). After 1 h at room temperature, the solution was acidified with acetic acid and decationized by passage over a Dowex-50W-X4 (H^+ form) column (1.2 \times 6.5 cm) in ethanol. The ethanol was removed in vacuo and borate esters were removed by repeated (5 times) dissolution of the product in 1.5 mL of methanol, followed by rotary evaporations. Recovery of **11** was approximately 84%. ^1H NMR (270 MHz, D_2O , HDO = 4.65 ppm) of **11**: δ 3.52 (4 H, m), 3.39 (2 H, m), 1.55 (2 H, m). ^{13}C NMR [50.3 MHz, D_2O , dioxane (external standard) = 66.5 ppm]: δ 74.0 (C-4), 68.1 (C-3), 62.5 (C-5), 58.2 (C-1), 34.5 (C-2). GC/MS of the $(\text{TMS})_4$ derivative of **11** (Kratos MS 25): m/z 321, 307, 231, 219, 205, 103 (base peak).

Results and Discussion

Reaction of "Activated" BLM with d(CGCGCG): Products and Quantitation. The oligonucleotide d(CGCGCG) was chosen initially as a model DNA system for investigations of the interaction of nucleic acids with Fe-BLM.¹⁵ The distribution of the products arising from the interaction of Fe-BLM with the hexanucleotide was dependent on the method of activation (Fe^{3+} - H_2O_2 or Fe^{2+} - O_2) and the O_2 concentration. Evidence will be presented that the major lesion occurs at the third cytidine from the 5' end (the fifth position in the hexamer, designated dC-5) under all conditions. These observations have allowed us to isolate and identify the structure of the sugar accompanying base release and to determine its stoichiometry. Methods developed with the

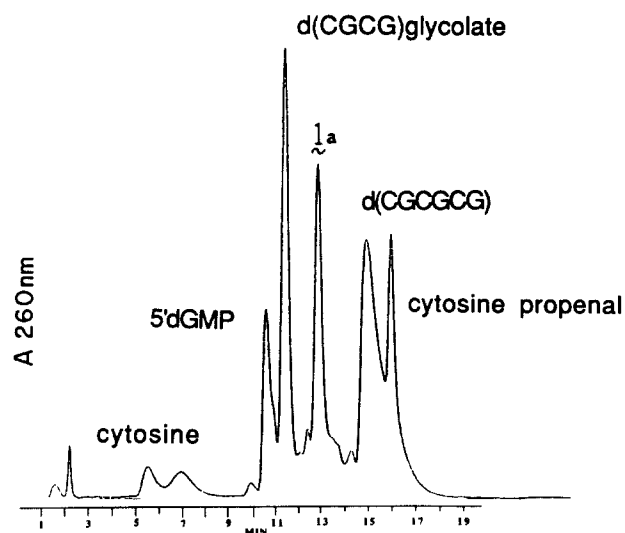


Figure 6. HPLC trace of the products produced when d(CGCGCG) is incubated with Fe^{2+} -BLM and O_2 .

hexamer have been extended to DNA polymers.

When Fe^{3+} and H_2O_2 were used to activate BLM in the absence of O_2 and d(CGCGCG) (56 nmol) was added, the HPLC traces revealed essentially no aerobic products (cytosine propenal or oligonucleotides containing glycolic acid) (Figure 5). The observed products were cytosine (23 nmol), oligonucleotide **1a** (16 nmol), and unreacted starting material d(CGCGCG) (27.2 nmol). In a large number of experiments, **1a** typically accounted for 70.5% of the amount of cytosine produced, suggesting that it represented the major lesion accompanying base release. Upon prolonged anaerobic incubation of Fe^{3+} -BLM- H_2O_2 with hexamer, significant quantities of a second oligomeric product occurred; it represents a single-stranded oligonucleotide (λ_{max} 256 nm, ϵ = 40.1 $\text{mM}^{-1}\text{cm}^{-1}$) with cytosine at the dC-3 and dC-5 positions released (data not shown).

When BLM was activated with atmospheric O_2 in the presence of d(CGCGCG), similar amounts of aerobic and anaerobic products were formed (Figure 6). Cytosine (14.3 nmol) and **1a** (12.2 nmol) are produced in a ratio (0.85) very similar to that in the anaerobic activation described above. Furthermore, cytosinepropenal (18.1 nmol), 5'-dGMP (18.6 nmol), and d(CGCG)glycolate (15.5 nmol) were produced in a ratio of 1:1:0.85. Cleavage appears to be occurring specifically at dC-5. When the oligonucleotide was reacted with Fe^{2+} -BLM in one atmosphere of 100% O_2 , the ratio of aerobic to anaerobic products changed from 1:1 (atmospheric O_2) to 2–3:1. The same oligomeric and monomeric products were formed. These results indicate that d(CGCGCG) is an excellent model for structural identification and quantification of the lesion accompanying base release.

Stabilization and Characterization of the Alkaline-Labile Oligonucleotide 1a. Compound **1a** is chemically unstable. Preliminary studies from our laboratory¹⁵ had established that NaBH_4 (x = 1, 2, 3) could be utilized to stabilize **1a**, allowing structural characterization and quantitation. Compound **2a** produced by reduction of **1a** migrated as a single species in both reverse-phase and anion-exchange chromatography. In contrast to compound **1a**, **2a** can be stored at -20°C for 1 month without any apparent decomposition. Compound **2a** was produced by reduction of **1a** isolated from either the Fe^{3+} - H_2O_2 (anaerobic) or Fe^{2+} - O_2 -activated BLM reactions. Available evidence from two-dimensional TLC and enzymatic digestion suggests that **2a** isolated by HPLC is single-stranded and contains a lesion at dC-5.

Evidence for the site of lesion being dC-5 was provided by using a two-dimensional TLC sequencing method developed by Black and Gilham.²⁵ As predicted from the method, sequencing of the hexamer gives 5'-CGCGC-3'. The 3'-terminal residue is not detected by this method. Sequencing of **2a** revealed 5'-CGCG-3'

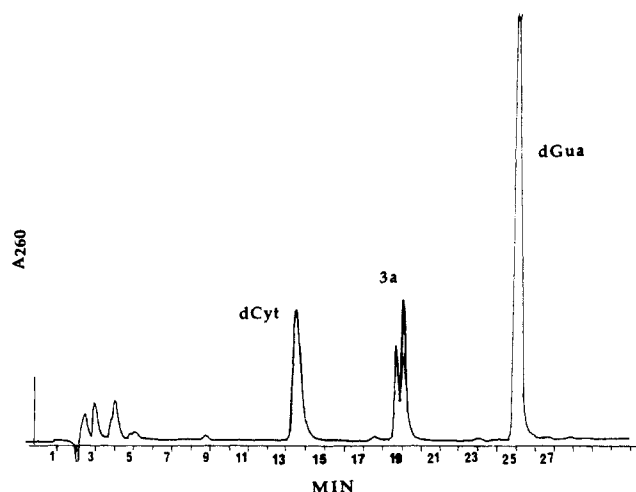


Figure 7. HPLC trace of the products produced subsequent to digestion of **2a** (Figure 3) with P_1 nuclease and alkaline phosphatase.

Table I. Enzymatic Digestion of NaBH_4 -Reduced Oligonucleotides **2a**

product 2a ^a	deoxycytidine (dC), nmol	deoxyguanosine (dG), nmol	product 3a , nmol
P_1/AP , $n = 6$			
amount, nmol	62.9	64.6	30.5
ratio ^b	1.99	2.04	0.97
$\text{PDE II}/\text{AP}$, $n = 1$			
amount, nmol	11.8	10.5	5.4
ratio ^b	2.13	1.90	0.97

^a Product **2a** derived from reductions at pH 7.8 and 9.0; n = number of determinations. ^b $R = (\text{dC} + \text{dG} + \text{3a})/5$ used to give mean recovery; dC, dG, or **3a** divided by R to determine ratio.

and is therefore consistent with the lesion being at dC-5. Comparison of the results of cosequencing hexamer and **2a** with the data for **2a** alone reveals that **2a** as isolated by HPLC is single-stranded (data not shown). The isolation of d(CGCG) glycolate from aerobic reactions provides additional support for dC-5 cleavage.

Moreover, digestion of **2a** with P_1 nuclease and bacterial alkaline phosphatase produced four products identified as dC, dG, and an epimeric mixture of **3a** (Figure 7). Separation of the epimers of **3a** requires a 5- μm reverse-phase column. The products were quantified by using experimentally determined extinction coefficients. The extinction coefficient for **3a** ($13.7 \text{ mM}^{-1} \text{ cm}^{-1}$) was assumed to be identical with that determined for dG, since the UV spectra of **3a** and dG were superimposable. Compound **3a** gave a ratio of phosphate to dG of 1:1. The ratios of dC:dG:**3a** from the P_1 nuclease/alkaline phosphatase cleavage of **2a** were 2:2:1 and were found to be independent of the method used to activate BLM (Table I). NaBH_4 reduction of **1a**, produced from either method of activation, resulted in ^3H incorporation into **3a** but not dC or dG. Considering the known specificity of P_1 nuclease,^{26,27,41} these results are consistent with the major lesion occurring at dC-5 of the hexamer.⁴⁰

The postulated structure for **2a** predicts that complete enzymatic digestion with PDE II should yield the same product **3a** as produced from P_1 nuclease digestion. Since PDE II, in contrast to P_1 nuclease, possesses only exonuclease activity, an abasic sequence in the middle of the molecule would prevent complete degradation.^{28,29} Digestion of 5.5 nmol of peak **2a** with PDE II followed by the standard alkaline phosphatase digestion gave the results

Table II. Characterization of ^3H -**4** from Fe^{3+} - H_2O_2 -BLM Reactions and from Fe^{2+} - O_2 -BLM Reactions by TLC

(A) From Fe ³⁺ -H ₂ O ₂ -BLM Reactions					
	% radioactivity, cellulose W ₈ ^a		R _f values		
	[³ H]-4 ^b	[³ H]-4 and standards ^b	[³ H]-4 ^c	standards ^d	
erythro	42	43	0.5	0.5 (5)	
threo	58	57	0.28	0.28 (11)	
% recovery	87	96			
% radioactivity, silica W ₈ ^a					
	[³ H]-4 ^b	[³ H]-4 ^e	R _f values		
			[³ H]-4 ^c	standards ^d	
erythro	33	38	0.44	0.425 (5)	
threo	67	62	0.39	0.38 (11)	
% recovery	92				
% radioactivity, silica (9:1 1-BuOH:H ₂ O elution), [³ H]-4 ^b			R _f values		
			[³ H]-4 ^b	standards ^d	
erythro	single spot		0.33	0.34 (5)	
threo				0.33 (11)	
% recovery	88			0.335 av	
(B) From Fe ²⁺ -O ₂ -BLM Reactions					
	% radioactivity, cellulose W ₈ ^a			R _f values	
	[³ H]-4 ^{b,f}	[³ H]-4 ^{b,h}	overall	[³ H]-4 ^{c,g}	[³ H]-4 ^{c,h} standards ^d
erythro	48.3	29.7	42.5	0.425	0.431
threo	51.7	70.3	57.5	0.193	0.207
% recovery	84.9	75			0.22 (11)
% radioactivity, silica W ₈ ^a			R _f values		
	[³ H]-4 ^{b,f}	[³ H]-4 ^{b,h}	overall	[³ H]-4 ^{c,g}	[³ H]-4 ^{c,h} standards ^d
erythro	43.9	27.9	39	0.396	0.394
threo	56.1	72.1	61	0.338	0.336
% recovery	93.7	75			0.328 (11)

^a Refers to pH of tungstate solution. ^b Scintillation counting of sectors. ^c Autoradiography. ^d Alkaline AgNO_3 detection. ^e Gas radioactivity detection. ^f Sulfuric acid charring. ^g Fraction eluting at 3.5–4 min (C_{18} HPLC, H_2O elution). ^h Fraction eluting at 4–4.5 min (C_{18} HPLC, H_2O elution). ⁱ Sulfuric acid detection.

indicated in Table I (dC:dG:3 is 2:2:1). Thus the ratio and recovery of products are again consistent with dC-5 being the location of the lesion.

To further establish its identity, D-erythro-**3a** was prepared chemically from d(CG) by hydrazine-induced depyrimidination followed by NaBH_4 reduction^{22,30,31} and compared to **3a** isolated from an Fe^{3+} -BLM- H_2O_2 reaction. HPLC analysis of both samples of **3a** revealed that they coeluted in three solvent systems.

Isolation and Characterization of dGMP and 4 from 3a. Treatment of **3a** with PDE I released 5'-dGMP in 86% yield. This result is consistent with the postulated structure of **3a** and indicates that PDE I can be used as an analytical reagent for the conversion of **3a** to the sugar alcohols **4** for further analysis. Thus, treatment of ^3H -**3a** isolated from both the aerobically and anaerobically activated BLM reactions with PDE I resulted in production of 5'-dGMP and ^3H -**4**, which eluted from a reverse-phase HPLC column between 3.5 and 4.5 min with 92% recovery of radioactivity. This material was utilized for further characterization by TLC and GC/MS analysis.

On the basis of our hypothesis (Figure 3), **4** is predicted to be a mixture of the epimers 2-deoxy-D-erythro-pentitol (**5**) and 2-deoxy-L-threo-pentitol (**11**). The deoxypentitol **5** is readily prepared by reduction of 2-deoxy-D-ribose with NaBH_4 .²³ However, no synthesis of the other epimer, **11**, exists in the chemical literature. The strategy used for synthesis of **11** depended upon synthesis of the protected sugar derivative **7** (Figure 4²⁴). The

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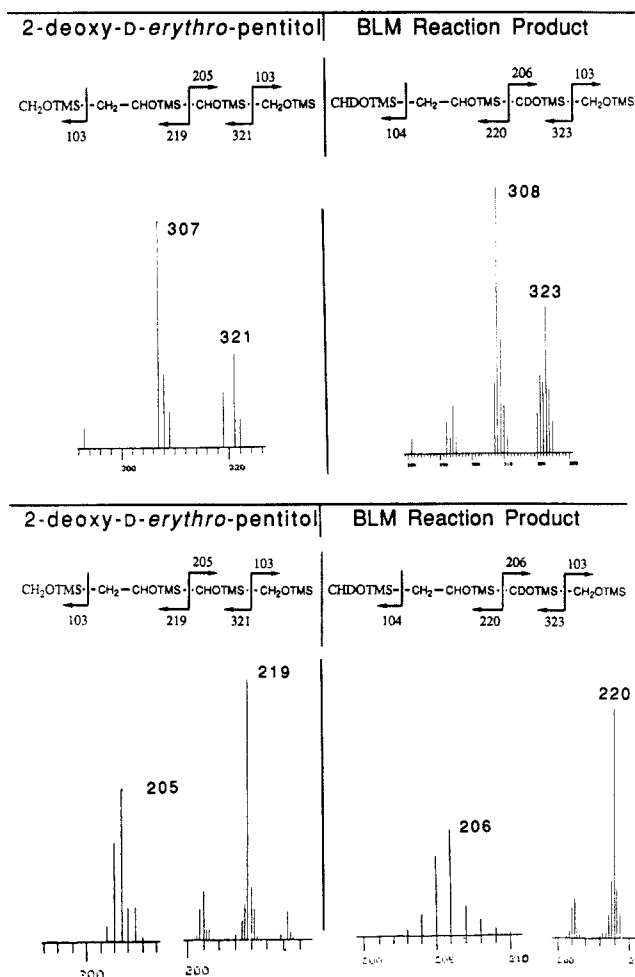


Figure 8. Comparison of the mass spectrum of 2-deoxy-D-erythro-pentitol (**5**) with that of the deoxypentitol products, [^2H]-**4**, isolated by methods described in Figure 3.

remainder of the synthesis is original but closely followed published procedures.^{32,33} The [^3H]-**4** isolated from the reaction of Fe^{3+} -BLM- H_2O_2 with d(CGCGCG) was collected in two 0.5-min fractions from the HPLC column. The earlier migrating fraction (3.5–4 min) contained 41% of the ^3H but also contained salt that interfered with the TLC analysis. The 4–4.5 min fraction, which contained less salt, was concentrated in vacuo, redissolved in a small volume, and chromatographed by using three TLC systems. Tungstate-impregnated cellulose completely resolved **4** into two components that comigrated with the chemically synthesized standards,^{34,35} and scintillation counting of sectors from the TLC plate revealed a 60:40 threo:erythro ratio (see Table IIA). While the R_f values of the epimers of **4** were similar on tungstate-impregnated silica, TLC gas radiography and autoradiography clearly showed the presence of two peaks (Table II). On silica TLC plates (9:1 1-BuOH:H₂O eluent), the diastereomers of **4** were not resolved and migrated as a single peak [88% recovery of radioactivity (Table IIA)]. Similar results were obtained with **1a** from an Fe^{2+} - O_2 -activated BLM reaction (Table IIB).

This analysis provides strong evidence that two diastereomeric 2-deoxypentitols are the modified carbohydrates produced in the BLM reaction with subsequent workup. Furthermore, the loss of stereochemical integrity at the C-4 position as indicated by comigration of [^3H]-**4** with the C-4 epimers **5** and **11** provides

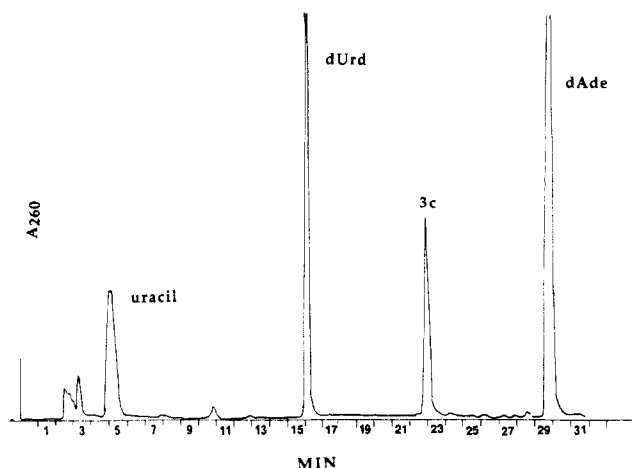


Figure 9. HPLC trace of products isolated subsequent to treatment of poly(dA-dU) with Fe^{2+} - O_2 -BLM, followed by NaB^2H_4 reduction and P_1 nuclease/alkaline phosphatase digestion.

Table III. Direct Quantitative Comparison of Base to 3-*O*-[5'-(2'-Deoxynucleotidyl)]-2-deoxy-D-erythro-pentitol

substrate ^a	cytosine, nmol	product 3a,b , nmol	product 3a,b / cytosine, %	dG, nmol	dC, nmol
d(CGCGCG)	23.1	22.9	99	141.3	143.0
poly(dG-dC) ^b	22.0	10.4	48	75	88
substrate	uracil, nmol	product 3c , nmol	product 3c / uracil, %	dA, nmol	dU, nmol
poly(dA-dU)	54.7	44.2	81	355.2	353.6

^a Average of duplicate determinations. ^b Guanine is also produced, 1.2 nmol.

compelling evidence for the intermediacy of a 4'-ketone compound.

Mass Spectral Identification of Deoxypentitols. Compound [^2H]-**4** prepared by reduction of **1a** with NaB^2H_4 was derivatized for GC/MS analysis. It was possible to partially resolve the two (TMS)₄ derivatives of [^2H]-**4** with a 30-m DB-5 column. The mass spectrum of the epimeric mixture afforded a single fragmentation pattern [m/z 323, 308, 233, 220, 206, 104 (base peak)] that is entirely consistent with the putative structures of 1,4-di-deuterio-2-deoxypentitols. Comparison of derivatized [^2H]-**4** with authentic TMS-derivatized **5** allowed for facile recognition of the shifted mass-to-charge (m/z) ratios (Figure 8).

The highest mass fragment is observed at m/z 323 for derivatized [^2H]-**4**. This is two mass units higher than the unlabeled standard. Comparison of the m/z 205 fragment in the standard with the m/z 206 fragment in the derivatized [^2H]-**4** establishes deuteriation at C-4. The m/z 104, 220, and 308 peaks (this last peak is a product of hydrogen atom abstraction and rearrangement^{36,37}) are indicative of monodeuteriation at C-1. This proves that a 4'-ketone and a 1'-aldehyde were present in the original structure prior to NaB^2H_4 reduction. This carbohydrate modification has also been observed as a consequence of ionizing radiation damage to DNA.³⁸

Isolation and Identification of Deoxypentitols from Reactions with Polymeric DNAs. Poly(dA-dU) was used as a substrate with limiting amounts of O_2 , Fe^{2+} -BLM, and NaB^2H_4 to trap 2'-deoxy-4'-pentulose-modified DNA. In situ enzymatic digestion with P_1 nuclease/alkaline phosphatase allowed the isolation of sugar alcohols linked to dAMP (**3c**). The HPLC trace for this reaction is shown in Figure 9. Isolation of a peak of material eluting at 23 min and treatment with PDE I resulted in the production of 5'-dAMP and [^2H]-**4**, identified by GC/MS as having one deuterium each at carbons 1 and 4. This result indicates that the

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species eluting at 23 min is 3-*O*-[5'-(2-deoxyadenylyl)]-2-deoxypentitol. Similar limiting O₂ experiments were carried out with sonicated calf thymus DNA and poly(dG-dC). In both cases [1,4-²H₂]-2-deoxypentitols were identified.

Quantitation of 3a-c and Correlation to Base Release. To circumvent any uncertainties associated with differential recovery of monomeric and oligomeric products through multiple chromatographic procedures, a simplified degradation procedure was used for quantitative analysis. Subsequent to treatment of DNA (polymer or oligomer) with activated BLM, the lesion was reduced with NaBH₄ and digested directly with P₁ nuclease/alkaline phosphatase. HPLC analysis then allowed a direct comparison to be made between uracil and 3c (Figure 3) in the case of poly(dA-dU) and cytosine and 3a and 3b in the case of d-(CGCGCG) and poly(dG-dC) (Figure 3). The results are summarized in Table III. For d(CGCGCG), quantitative recovery (99%) compared to cytosine release occurs under these conditions. With poly(dA-dU), 81% of the uracil released could be accounted for by isolation of 3c. The recovery of 3b relative to cytosine release from poly(dC-dG) was only 48%. The poor quantitation with poly(dG-dC) may reflect difficulty associated with the synthesis and hence heterogeneity of this polymer.³⁹ Such factors

could influence the stability of the lesion toward elimination prior to trapping with NaBH₄.

The results in Table III establish that NaBH₄ trapping is efficient compared to elimination across the C-2'-C-3' bond and prove that formation of a 4'-ketone accounts for the majority of the alkaline-labile sites produced by Fe-BLM.

Conclusions

The results reported here firmly establish the identity of the alkaline-labile lesion in the BLM reaction as a 4'-keto-1'-aldehyde. Our findings constitute the first verification of a near stoichiometric production of this lesion with respect to nucleic acid base release. Moreover, the generality of the analysis is demonstrated by our studies on a variety of heterogeneous DNAs.

A different approach to identification of the alkaline-labile lesion has recently been reported by Sugiyama et al.^{13,14} The method involves strongly basic conditions to effect the rearrangement of the lesion and has not been quantitated with respect to base release. Our attempts to utilize this approach with 1a (Figure 3) resulted in a mixture of products intractable to identification and quantitation. The results obtained by their procedures may therefore constitute a specialized case lacking the generality of the procedures reported here.

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(40) P₁ nuclease is an endo- and exonuclease that can act as a monoesterase. Both single-stranded and double-stranded DNAs are substrates. The *p*-nitrophenyl ester of 3'-TMP can be hydrolyzed to thymidine and *p*-nitrophenyl phosphate, while the corresponding 5'-TMP is not hydrolyzed.²⁶ No hydrolysis of deoxyribose 3'-phosphate is observed.²⁷

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Identification of the Source of Oxygen in the Alkaline-Labile Product Accompanying Cytosine Release during Bleomycin-Mediated Oxidative Degradation of d(CGCGCG)

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Abstract: Interaction of bleomycin, Fe²⁺, and limiting O₂ or bleomycin, Fe³⁺, and H₂O₂ with the hexamer d(CGCGCG) results in the production of an oxidatively damaged sugar lesion, 2'-deoxy-4'-pentulose, that is produced stoichiometrically with respect to free nucleic acid base release (Rabow et al., *J. Am. Chem. Soc.*, preceding paper in this issue). Similar studies using either ¹⁸O-labeled O₂ or H₂O have established that the source of oxygen in the 2'-deoxy-4'-pentulose is the solvent. The implications of these results with respect to the mechanism of nucleic acid base release in bleomycin-mediated DNA degradation are presented.

Bleomycin, an antitumor antibiotic,¹ in the presence of the required cofactors Fe²⁺, O₂, and reductant or Fe³⁺ and hydroperoxide oxidatively degrades DNA to produce two types of lesions in a ratio of approximately 1:1.^{2–4} This ratio can be modulated by the O₂ concentration.⁵ Under anaerobic conditions, the predominant lesion results in the release of free nucleic acid base, which is accompanied by strand scission upon subsequent treatment with hydroxide. At elevated O₂ concentrations, the predominant lesion results in the production of base propenal, which is accompanied by strand scission under neutral conditions to produce 3'-phosphoglycolate and 5'-phosphate ends.^{2–5}

Previous studies from our laboratories^{6,7} using 4'-³H-labeled DNA models established that both types of lesions were the result of labilization of the 4'-carbon-hydrogen bond of a pyrimidine

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