Analysis of Products Formed during Bleomycin-Mediated DNA Degradation†

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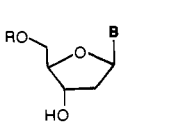
ABSTRACT: By the use of DNA, copolymers of defined nucleotide composition, and a synthetic dodecanucleotide having putative bleomycin cleavage sites in proximity to the 5′- and 3′-termini, the products formed concomitant with DNA strand scission have been isolated and subjected to structural identification and quantitation via direct comparison with authentic synthetic samples. The products of DNA strand scission by Fe(II)–bleomycin include oligonucleotides having each of the four possible nucleoside 3′-(phosphoro-2″-*O*-glycolates) at their 3′-termini, as well as the four possible base propenals. At least for 3-(adenin-9′-yl)propenal and 3-(thymin-1′-yl)propenal, the products formed were exclusively of the trans configuration.

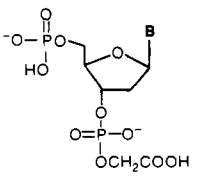
The bleomycins are a family of clinically useful antitumor antibiotics that are believed to mediate their therapeutic effects at the level of DNA strand scission. Bleomycin-mediated DNA degradation can be effected in vitro with bleomycin + Fe(II) + O_2 and has been shown to result in concomitant formation of some unusual products including base propenals (Burger et al., 1980; Giloni et al., 1981) and oligonucleotide 3′-(phosphoro-2″-*O*-glycolic acid) derivatives (Giloni et al., 1981; Uesugi et al., 1984). Recently, it has also been shown that Cu (Oppenheimer et al., 1981; Ehrenfeld et al., 1985; Kilkuskie et al., 1984), Co (Chang & Meares, 1982, 1984), and Mn (Ehrenfeld et al., 1984) derivatives of BLM[1] can be activated for DNA strand scission. Moreover, both Fe(III)·BLM and Cu(II)·BLM can also be activated anaerobically in the presence of oxygen surrogates such as iodosobenzene (Murugesan et al., 1982; Murugesan & Hecht, 1984) and enzymatically via the agency of NADPH-cytochrome P-450 reductase, NADPH, and O_2 (Kilkuskie et al., 1984). Much less is known about the chemistry of DNA strand scission mediated by metallobleomycins other than Fe·BLM, or following anaerobic or enzymatic activation.

Presently, we employ synthetic nucleoside 3′-(phosphoro-2″-*O*-glycolic acid) derivatives to establish the formation of all four such species from *Escherichia coli* DNA concomitant with DNA strand scission by Fe(II)·BLM in the presence of O_2. The formation of the glycolate moiety at the 3′-terminus of the cleaved oligonucleotide was further established by the finding that treatment of d(CGCTTTAAAGCG) with Fe(II)·BLM + O_2 provided a dinucleotide identical with synthetic 2′-deoxycytidylyl(3′→5′)[2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolic acid)]. Also investigated was the extent and stereochemistry of base propenal formation. All four base propenals were observed following degradation of calf thymus DNA by bleomycin A_2. By comparison with synthetic samples of *cis*- and *trans*-3-(thymin-1′-yl)propenal and *cis*- and *trans*-3-(adenin-9′-yl)propenal, it was shown that Fe–bleomycin-mediated DNA strand scission resulted exclusively in the formation of the trans base propenals. The mechanistic consequences of this observation are discussed.

Experimental Procedures

The nucleoside precursors employed for the synthesis of **5–8** and **18** were purchased from Sigma Chemical Co., as was QAE-Sephadex 25. DEAE-cellulose (DE-23) was obtained





1 B = T, R = DMTr
2 B = C^{Bz}, R = MTr
3 B = A^{Bz}, R = MTr
4 B = G^{iBu}, R = MTr

5 B = T
6 B = C
7 B = A
8 B = G

from Whatman; flash chromatography was carried out on Macherey-Nagel silica gel 60 (230–400 mesh). TLC was carried out on silica gel 60 plates manufactured by E. Merck (aluminum support, 0.2 mm; glass support, 0.25 and 0.5 mm). T4 polynucleotide kinase was purchased from Bethesda Research Laboratories; 1 unit was defined as the amount of enzyme needed to catalyze the incorporation of 1 nmol of [γ-^{32}P]ATP into acid-insoluble material in 30 min under the assay conditions. Lambda exonuclease was obtained from New England Biolabs; 1 unit was defined as the amount of enzyme needed to catalyze the release of 10 nmol of acid-soluble nucleotide from duplex DNA in 30 min at 37 °C. *Escherichia coli* DNA was purchased from P-L Biochemicals; calf thymus DNA was from Sigma. The concentrations of all DNA oligonucleotide samples are expressed as total nucleotide concentrations. Bleomycin was obtained from Bristol Laboratories through the courtesy of Dr. William Bradner; it was fractionated as described previously (Chien et al., 1977; Oppenheimer et al., 1979).

Methyl Glycolate 2-O-Phosphorodichloridite. A 10.6-mL sample (16.7 g, 122 mmol) of phosphorus trichloride was stirred and cooled to −5 °C and then treated with 5.0 mL (5.49 g, 61 mmol) of methyl glycolate over a period of 30 min. The

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[1] Abbreviations: BLM, bleomycin; DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-pressure liquid chromatography.

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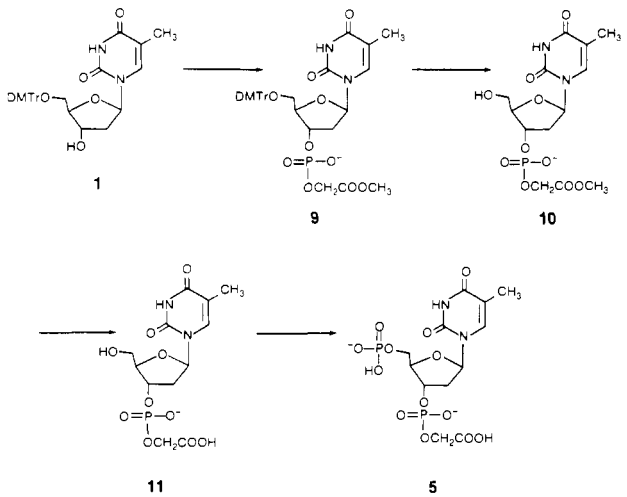
reaction mixture was stirred at −5 °C for an additional 30 min and then at 25 °C for 2 h. Following removal of excess PCl_3 by distillation at atmospheric pressure, the desired phosphorodichloridite was isolated by vacuum distillation: yield 4.66 g (40%); bp 85–90 °C (18–20 mmHg); ^1H NMR [CCl_4, $(CH_3)_4Si$] δ 3.76 (s, 3) and 4.66 (d, 2, J = 9 Hz).

Methyl $O^{5'}$-(Dimethoxytrityl)thymidine 3'-(Phosphoro-2''-O-glycolate) (**9**). A solution of 1.6 mL of dry tetrahydrofuran and 0.8 mL (5.0 mmol) of collidine was stirred under N_2 in a septum-stopped flask and cooled to −78 °C.



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Methyl glycolate 2-*O*-phosphorodichloridite (220 μL, 1.50 mmol) was added, followed by dropwise addition of 261 mg (0.48 mmol) of $O^{5'}$-(dimethoxytrityl)thymidine (**1**) in 2 mL of dry tetrahydrofuran over a period of 15 min. The reaction mixture was stirred at −78 °C for 1 h and then at 25 °C for 30 min. A solution containing 480 mg (1.88 mmol) of iodine in 6 mL of 1:1:1 pyridine–water–tetrahydrofuran was added, and the combined solution was stirred at room temperature for 30 min. The dark red solution was concentrated, and the resulting semisolid product mixture was partitioned between $CHCl_3$ and 3% aqueous $NaHSO_3$. The aqueous layer was back-extracted with $CHCl_3$, and the combined organic extract was dried (Na_2SO_4) and concentrated to afford a solid. Preparative silica gel TLC (development with 3:1 $CHCl_3$–CH_3OH) afforded methyl $O^{5'}$-(dimethoxytrityl)thymidine 3'-(phosphoro-2''-*O*-glycolate) (**9**) as colorless plates: yield 293 mg (90%); (partial) ^1H NMR [[$^2H_6$]Me_2SO, $(CH_3)_4Si$] δ 1.46 (s, 3), 2.23–2.60 (m, 2), 3.63 (s, 3), 3.80 (s, 6), 4.22 (d, 2, J = 7 Hz), 4.80 (m, 1), 6.23 (t, 1, J = 6 Hz), 6.9–7.5 (m, 13), and 7.16 (s, 1); silica gel TLC (7:3 $CHCl_3$–CH_3OH) R_f 0.40.

Methyl Thymidine 3'-(Phosphoro-2''-O-glycolate) (**10**). A solution of 80 mg (117 μmol) of methyl $O^{5'}$-(dimethoxytrityl)thymidine 3'-(phosphoro-2''-*O*-glycolate) (**9**) in 0.5 mL of 80% aqueous acetic acid was stirred at 25 °C for 12 h. The solution was concentrated under diminished pressure, and the residue was treated with portions of water and again concentrated. The resulting solid residue was dissolved in 3 mL of water and washed with ether (3 × 5 mL). The aqueous phase was then lyophilized, affording methyl thymidine 3'-(phosphoro-2''-*O*-glycolate) (**10**) as a white solid: yield 37 mg (94%); λ_{max} (pH 7) 265 nm; (partial) ^1H NMR (D_2O) δ 1.79 (s, 3), 2.16–2.26 (m, 2), 3.69 (m, 5), 4.11 (m, 1), 4.39 (d, 2, J = 7 Hz), 6.10 (t, 1, J = 6 Hz), and 7.50 (s, 1); poly(ethylenimine) TLC (1:1 1.5 M LiCl–DMF) R_f 0.76.

Thymidine 3'-(Phosphoro-2''-O-glycolate) (**11**). A solution of 25 mg (36.7 μmol) of methyl thymidine 3'-(phosphoro-2''-*O*-glycolate) (**10**) in 0.5 mL of aqueous 0.1 N NaOH was stirred at 25 °C for 30 min. The reaction mixture was neutralized with 80% aqueous acetic acid, diluted to 25 mL with H_2O, and applied to a DEAE-cellulose column (HCO_3^- form, 1.2 × 40 cm). Elution with an NH_4^+ HCO_3^- gradient (0–0.35 M, 1-L total volume, 6-mL fractions) afforded thymidine 3'-(phosphoro-2''-*O*-glycolate) in fractions 55–65. These fractions were pooled and desalted, affording compound **11** as a white solid: yield 19 mg (80%); λ_{max} (pH 7) 267 nm; ^1H NMR (D_2O) δ 1.74 (s, 3), 2.25–2.41 (m, 2), 3.65–3.71 (m, 2), 4.03 (m, 1), 4.06 (d, 2, J = 6.7 Hz), 4.73 (m, 1), 6.16 (t, 1, J = 6.9 Hz), and 7.51 (s, 1); FAB mass spectrum, m/z (positive ion) 381 $(M + H)^+$, (negative ion) 379 $(M - H)^-$; poly(ethylenimine) TLC (1:1 1.5 M LiCl–DMF) R_f 0.44; silica gel TLC (7:3:2 2-propanol–NH_4OH–H_2O) R_f 0.58.

Phosphorylation of Thymidine 3'-(Phosphoro-2''-O-glycolic acid). Method A. A reaction mixture (100-μL total volume) containing 0.1 mg (0.26 μmol) of thymidine 3'-(phosphoro-2''-*O*-glycolate), 10 mM ATP, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, and ~10 units of T4 polynucleotide kinase in 50 mM Tris-HCl buffer, pH 7.6, was incubated at 37 °C for 5 h. The solution was then applied to a column of QAE-Sephadex 25 (HCO_3^- form, 1 × 15 cm). Elution with an $NH_4^+HCO_3^-$ gradient (0–0.45 M, 400-mL total volume, 1.5-mL fractions) at a flow rate of 30 mL/h afforded thymidine 3'-(phosphoro-2''-*O*-glycolic acid) 5'-phosphate (**5**) in fractions 155–170. These fractions were pooled and desalted, affording compound **5** as a white solid: yield 1.8 A_{260} units (92%); λ_{max} (pH 7) 262 nm; FAB mass spectrum, m/z (positive ion) 461 $(M + H)^+$, (negative ion) 459 $(M - H)^-$; silica gel TLC (7:3:2 2-propanol–NH_4OH–H_2O) R_f 0.24.

Method B. A stock solution was prepared by combining (at 0 °C) freshly distilled phosphorus oxychloride (670 mg, 400 μL, 4.4 mmol), water (50 μL, 2.8 mmol), pyridine (380 mg, 4.8 mmol), and acetonitrile (770 mg, 18.9 mmol). A total of 10 μL of this cold stock solution was added to a vial containing 0.1 mg (0.26 μmol) of thymidine 3'-(phosphoro-2''-*O*-glycolate). The reaction mixture was stirred in an ice bath for 4 h. Ice–water (100 μL) was added, and the solution was stirred and cooled for another hour. The solution was then applied to a column of QAE-Sephadex (HCO_3^- form, 1 × 15 cm) and purified as indicated under Method A. The yield was 0.86 A_{260} unit (44%) of compound **5**: λ_{max} (pH 7) 262 nm; silica gel TLC (7:3:2 2-propanol–NH_4OH–H_2O) R_f 0.24.

2'-Deoxycytidine 3'-(Phosphoro-2''-O-glycolic acid) 5'-Phosphate (**6**). The preparation of nucleoside phosphoroglycolic acid derivative **6** was accomplished by starting from N^4-benzoyl-$O^{5'}$-(monomethoxytrityl)-2'-deoxycytidine (**2**) in analogy with the preparation of thymidine derivative **5** from deoxynucleoside **1**. Following condensation with methyl glycolate 2-*O*-phosphorodichloridite and I_2 oxidation, methyl N^4-benzoyl-$O^{5'}$-(monomethoxytrityl)-2'-deoxycytidine 3'-(phosphoro-2''-*O*-glycolic acid (84% isolated yield) was deblocked by successive treatments with 80% aqueous acetic acid (25 °C, 4 h), 0.1 N NaOH, and 28% aqueous NH_4OH (50 °C, 12 h). The desired product, 2'-deoxycytidine 3'-(phosphoro-2''-*O*-glycolic acid), was obtained as a white solid in 53% yield (overall for three steps): λ_{max} (pH 7) 268 nm; ^1H NMR (D_2O) δ 2.22–2.51 (m), 3.70 (m), 4.11 (d, J = 6.7 Hz), 4.75 (m), 5.98 (d, J = 7.7 Hz), 6.18 (t, J = 6.7 Hz), and 7.78 (d, J = 7.7 Hz); poly(ethylenimine) TLC (1:1 1.5 M LiCl–DMF) R_f 0.35; silica gel TLC (7:3:2 2-propanol–NH_4OH–H_2O) R_f 0.54. Phosphorylation was accomplished both enzymatically (92% yield) and chemically (40% yield) in analogy with the procedures outlined above for the thymidine derivative. 2'-

Deoxycytidine 3′-(phosphoro-2″-*O*-glycolic acid) 5′-phosphate (**6**) was obtained as a white solid: λ_{max} (pH 7) 267 nm; silica gel TLC (7:3:2 2-propanol–NH_4OH–H_2O) R_f 0.22.

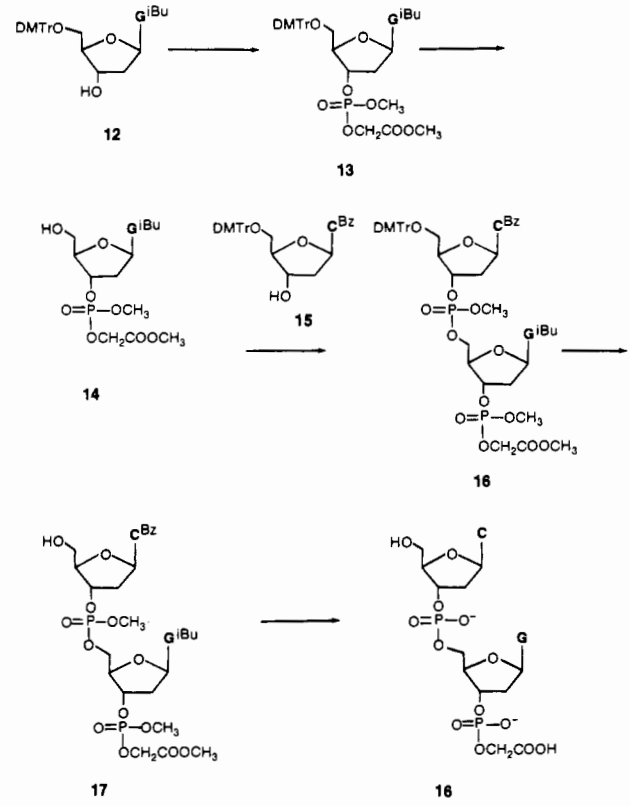
2′-Deoxyadenosine 3′-(Phosphoro-2″-O-glycolic acid) 5′-Phosphate (**7**). Condensation of N^6-benzoyl-$O^{5'}$-(monomethoxytrityl)-2′-deoxyadenosine (158 mg, 0.24 mmol) and methyl glycolate 2-*O*-phosphorodichloridite (110 μL, 0.75 mmol) was carried out as described above for the corresponding thymidine derivative; following iodine oxidation, methyl N^6-benzoyl-$O^{5'}$-(monomethoxytrityl)-2′-deoxyadenosine 3′-(phosphoro-2″-*O*-glycolate) was isolated as a white solid, yield 165 mg (85%). Deblocking was accomplished in 49% overall yield by successive treatments with 80% aqueous acetic acid (25 °C, 20 min), 0.1 N NaOH (25 °C, 1.5 h), and 28% aqueous NH_4OH (50 °C, 15 h) to provide 2′-deoxyadenosine 3′-(phosphoro-2″-*O*-glycolic acid) as a white solid: λ_{max} (pH 7) 259 nm; ^1H NMR (D_2O) δ 2.49–2.75 (m), 3.65 (m), 4.05 (d, J = 6.9 Hz), 4.16 (m), 4.88 (m), 6.31 (q, J = 6.1 Hz), 8.05 (s), and 8.14 (s); FAB mass spectrum, m/z (positive ion) 390 $(M + H)^+$, (negative ion) 388 $(M - H)^-$; poly(ethylenimine) TLC (1:1 1.5 M LiCl–DMF) R_f 0.44; silica gel TLC (7:3:2 2-propanol–NH_4OH–H_2O) R_f 0.67. Phosphorylation was accomplished enzymatically in 98% yield with T4 polynucleotide kinase as described above for the corresponding thymidine derivative. 2′-Deoxyadenosine 3′-(phosphoro-2″-*O*-glycolic acid) 5′-phosphate (**7**) was obtained as a white solid: λ_{max} (pH 7) 258 nm; silica gel TLC (7:3:2 2-propanol–NH_4OH–H_2O) R_f 0.29.

2′-Deoxyguanosine 3′-(Phosphoro-2″-O-glycolic acid) 5′-Phosphate (**8**). In analogy with the procedures employed above, methyl N^2-isobutyryl-$O^{5'}$-(monomethoxytrityl)-2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolic acid) was prepared as a white solid in 88% isolated yield by condensation of N^2-isobutyryl-$O^{5'}$-(monomethoxytrityl)-2′-deoxyguanosine and methyl glycolate 2-*O*-phosphorodichloridite. These procedures were similar to those described previously (Henner et al., 1983). Deblocking was carried out by successive treatments with 80% aqueous acetic acid (25 °C, 12 h), 0.1 NaOH (25 °C, 1.5 h), and 28% aqueous NH_4OH (50 °C, 15 h); 2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolic acid) was obtained in 71% overall yield for the three deblocking steps. The desired product was obtained as a white solid: λ_{max} (pH 7) 253 and 271 (sh) nm; ^1H NMR (D_2O) δ 2.53–2.71 (m), 3.66 (m), 4.14 (d, J = 7.1 Hz), 4.82 (m), 6.15 (t, J = 6.9 Hz), and 7.86 (s); ^{13}C NMR (D_2O) δ 47.21 (d, J = 3.2 Hz), 71.00 (s), 73.25 (dt, J = 5.7 and 146 Hz), 85.17 (dt, J = 5.12 and 158 Hz), 93.54 (d, J = 205 Hz), 95.85 (dd, J = 6.0 and 191 Hz), 125.72 (s), 147.25 (s), 160.47 (s), 163.07 (s), 168.04 (s), and 185.04 (s); IR (KBr) 1648 and 1640 cm^{-1}; FAB mass spectrum, m/z (positive ion) 409 $(M + H)^+$, (negative ion) 404 $(M - H)^-$; poly(ethylenimine) TLC (1:1 1.5 M LiCl–DMF) R_f 0.33; silica gel TLC (7:3:2 2-propanol–NH_4OH–H_2O) R_f 0.53. Enzymatic phosphorylation with T4 polynucleotide kinase as described above provided 2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolic acid) 5′-phosphate as a white solid in 90% yield: λ_{max} (pH 7) 251 and 275 (sh) nm; silica gel TLC (7:3:2 2-propanol–NH_4OH–H_2O) R_f 0.20.

Dimethyl O⁵′-(Dimethoxytrityl)-N²-isobutyryl-2′-deoxyguanosine 3′-(Phosphoro-2″-O-glycolate) (**13**). A solution containing 140 μL (0.95 mmol) of methyl phosphorodichloridite in 3.2 mL of dry tetrahydrofuran and 0.25 mL of dry collidine under N_2 was cooled to −78 °C and treated with 610 mg (0.95 mmol) of $O^{5'}$-(dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine in 6 mL of tetrahydrofuran over a period of 20 min. The combined solution was stirred at −78 °C for 15 min and then treated with 96 mg (1.0 mmol) of methyl glycolate in 2.5 mL of tetrahydrofuran over a period of 15 min. The reaction mixture was stirred at −78 °C for 30 min and then at 25 °C for 1 h. The reaction mixture was then treated with a solution consisting of 300 mg (1.2 mmol) of iodine in 1 mL of H_2O, 1 mL of pyridine, and 2 mL of tetrahydrofuran. After an additional 10 min at 25 °C, the reaction mixture was concentrated under diminished pressure, and the brownish syrupy residue was dissolved in $CHCl_3$ and washed with 1% aqueous $NaHSO_3$ and H_2O. The organic phase was dried (Na_2SO_4) and concentrated to afford a colorless syrup. Flash chromatography (Still et al., 1978) on a 25-g silica gel column, elution with 5% CH_3OH in $CHCl_3$, afforded dimethyl $O^{5'}$-(dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolate) (**13**) as a foam: yield 585 mg (76%); $\lambda_{max}^{CH_3OH}$ 235 and 258 nm; (partial) ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 1.10 (m, 6), 3.66 (s, 9), 6.20 (m, 1), 6.80–7.30 (m, 13), and 7.73 (s, 1); silica gel TLC (10% CH_3OH in $CHCl_3$) R_f 0.41.

Dimethyl N²-Isobutyryl-2′-deoxyguanosine 3′-(Phosphoro-2″-O-glycolate) (**14**). A solution of 550 mg (0.68 mmol) of dimethyl $O^{5'}$-(dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolate) (**13**) in 15 mL of 80% aqueous acetic acid was stirred at 25 °C for 30 min. The solution was concentrated under diminished pressure, and the residue was treated with portions of water and again concentrated. The gummy white solid thus obtained was purified by flash chromatography on a 25-g silica gel column, elution with 7% CH_3OH in $CHCl_3$. Dimethyl N^2-isobutyryl-2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolate) was obtained as a colorless foam: yield 301 mg (88%); $\lambda_{max}^{CH_3OH}$ 256 nm; (partial) ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 1.30 (m, 6), 3.77 (s, 3), 4.64 (d, 2, J = 9 Hz), 6.20 (m, 1), and 7.97 (s, 1); silica gel TLC (10% CH_3OH in $CHCl_3$) R_f 0.28.

Trimethyl N⁴-Benzoyl-O⁵′-(dimethoxytrityl)-2′-deoxycytidylyl(3′→5′)[N²-isobutyryl-2′-deoxyguanosine 3′-(phosphoro-2″-O-glycolate)] (**16**). A solution containing 70 μL (0.48 mmol) of methyl phosphorodichloridite in 2 mL of dry



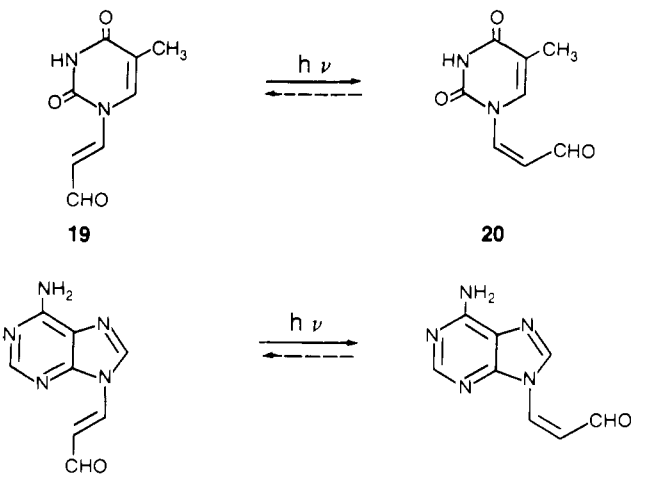
tetrahydrofuran and 0.5 mL of dry collidine under N_2 was cooled to −78 °C and treated with 317 mg (0.50 mmol) of N^4-benzoyl-$O^{5'}$-(dimethoxytrityl)-2′-deoxycytidine (**15**) in 2.5 mL of dry tetrahydrofuran over a period of 30 min. The combined solution was stirred at −78 °C for 10 min and then treated with 250 mg (0.50 mmol) of dimethyl N^2-isobutyryl-2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolate) (**14**) in 2.5 mL of dry tetrahydrofuran over a period of 25 min. The reaction mixture was stirred at −78 °C for 30 min and then at 25 °C for 30 min. The reaction mixture was then treated with a solution consisting of 150 mg (0.59 mmol) of iodine in 1 mL of H_2O, 1 mL of pyridine, and 1 mL of tetrahydrofuran. After an additional 10 min at 25 °C, the reaction mixture was concentrated, and the residue was dissolved in $CHCl_3$ and washed with 1% aqueous $NaHSO_3$ and H_2O. The organic phase was dried (Na_2SO_4) and concentrated to afford a colorless syrup. Flash chromatography on a 10-g silica gel column, elution with 5% CH_3OH in $CHCl_3$, afforded dinucleotide **16** as a colorless syrup: yield 308 mg (15%); $\lambda_{max}^{CH_3OH}$ 235, 261, and 273 (sh) nm; (partial) ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 1.15 (m, 6), 3.66 (s, 3), 3.70 (s, 6), 4.61 (d, 2, J = 9 Hz), 6.20 (m, 2), 6.80–7.60 (m, 20), and 7.90 (m, 1); silica gel TLC (10% CH_3OH in $CHCl_3$) R_f 0.35.

Trimethyl N^4-Benzoyl-2′-deoxycytidylyl(3′→5′)[N^2-isobutyryl-2′-deoxyguanosine 3′-(phosphoro-2″-O-glycolate)] (**17**). A solution of 60 mg (49 μmol) of dinucleotide **16** in 0.5 mL of 80% aqueous acetic acid was stirred at 25 °C for 20 min. The solution was concentrated under diminished pressure, and the residue was treated with portions of water and again concentrated. The residue was purified by flash chromatography on a 7-g silica gel column, elution with 10% CH_3OH in $CHCl_3$. Trimethyl N^4-benzoyl-2′-deoxycytidylyl[3′→5′)[N^2-isobutyryl-2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolate)] (**17**) was obtained as a colorless syrup: yield 32 mg (71%); $\lambda_{max}^{CH_3OH}$ 258 and 271 (sh) nm; silica gel TLC (10% CH_3OH in $CHCl_3$) R_f 0.14.

2′-Deoxycytidylyl(3′→5′)[2′-deoxyguanosine 3′-(phosphoro-2″-O-glycolate)] (**18**). A solution of 32 mg (35 μmol) of trimethyl N^4-benzoyl-2′-deoxycytidylyl(3′→5′)[N^2-isobutyryl-2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolate)] (**17**) in 1 mL of 0.1 N NaOH was stirred at 25 °C for 1 h. The reaction mixture was neutralized with 80% aqueous acetic acid and concentrated. The residue was dissolved in 0.5 mL of a 1:1:1 thiophenol–triethylamine–dioxane solution and stirred under N_2 at 25 °C for 1.5 h. Concentration of this solution under diminished pressure afforded a syrup that was partitioned between water and benzene. The aqueous phase was concentrated to dryness, and the residue was dissolved in 2 mL of 28% NH_4OH and maintained at 50 °C for 12 h in a sealed tube. Concentration of the solution afforded a white residue that was dissolved in 2 mL of water and applied to a DEAE-cellulose column (HCO_3^- form, 1 × 30 cm). Elution with a gradient of ammonium bicarbonate (0.005–0.5 M, 1-L total volume, 6-mL fractions) at a flow rate of 25 mL/h provided the desired compound in fractions 94–110. These fractions were pooled and desalted, affording 2′-deoxycytidylyl(3′→5′)[2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolic acid)] (**18**) as a white solid: yield 7.3 mg (30%); λ_{max} (pH 7) 252 and 271 (sh) nm; (partial) ^1H NMR (D_2O) δ 4.13 (d, 2, J = 6.9 Hz), 5.86 (d, 1, J = 7.5 Hz), 5.97 (q, 1, J = 6.3 Hz), 6.13 (t, 1, J = 6.6 Hz), 7.48 (d, 1, J = 7.5 Hz), and 7.95 (s, 1) [cf. Uesugi et al. (1984)]; FAB mass spectrum, m/z (positive ion) 695 $(M + H)^+$, (negative ion) 693 $(M - H)^-$; poly(ethylenimine) TLC (1:1 1.5 M LiCl–DMF) R_f 0.13; silica gel TLC (7:3:2 2-propanol–NH_4OH–H_2O) R_f 0.45.

Phosphorylation of 2′-Deoxycytidylyl(3′→5′)[2′-deoxyguanosine 3′-(phosphoro-2″-O-glycolic acid)]. A reaction mixture (100-μL total volume) containing 0.1 mg (0.14 μmol) of 2′-deoxycytidylyl(3′→5′)[2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolic acid)] (**18**), 10 mM ATP, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, and ~10 units of T4 polynucleotide kinase in 50 mM Tris-HCl, pH 7.6, was incubated at 37 °C for 5 h. The solution was applied to a (20 cm × 20 cm) silica gel TLC plate; development was with 7:5:3 2-propanol–NH_4OH–H_2O. The appropriate band was eluted from the plate with CH_3OH; lyophilization of the crude product afforded 2′-deoxycytidylyl(3′→5′)[2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolic acid)] 5′-phosphate as a white powder: yield 1.1 A_{260} units (90%); λ_{max} (pH 7) 251 and 270 (sh) nm; silica gel TLC (7:3:2 2-propanol–NH_4OH–H_2O) R_f 0.18.

cis-3-(Thymin-1′-yl)propenal (**20**). A solution of 5 mg (27 μmol) of *trans*-3-(thyminyl-1′-yl)propenal (**19**) (Giloni et al., 1981) in 30 mL of methanol was placed in a Pyrex flask and irradiated (Rayonet photoreactor, 250 W) with stirring at ambient temperature for 24 h. The reaction mixture was



19 **20**

21 **22**

concentrated, and the residue was applied to a silica gel column (1.5 × 60 cm). Elution with 99:1 ethyl acetate–ethanol provided *cis*-3-(thymin-1′-yl)propenal (**20**) as a white powder: yield 4.5 mg (90%); $\lambda_{max}^{CH_3OH}$ 298 nm; ^1H NMR [[$^2H_6$]Me_2SO, $(CH_3)_4Si$] δ 1.82 (s), 5.82 (q, J = 9.0 and 7.2 Hz), 7.42 (d, J = 9.0 Hz), 7.88 (s), and 9.81 (d, J = 7.2 Hz); silica gel TLC (99:1 ethyl acetate–ethanol) R_f 0.67.

cis-3-(Adenin-9′-yl)propenal (**22**). A methanolic solution of 43 mg (220 μmol) of *trans*-3-(adenin-9′-yl)propenal (**21**) (Giloni et al., 1981) was irradiated and worked up as indicated above for thymine derivative **19**. Silica gel chromatography (1.5 × 60 cm column), elution with 90:10 ethyl acetate–ethanol, provided *cis*-3-(adenin-9′-yl)propenal (**22**) as a yellowish powder: yield 25 mg (58%); $\lambda_{max}^{CH_3OH}$ 257 nm; ^1H [[$^2H_6$]-Me_2SO, $(CH_3)_4Si$] δ 6.00 (q, J = 9.0 and 7.9 Hz), 7.54 (s), 7.84 (d, J = 9.0 Hz), 8.23 (s), 8.65 (s), and 10.03 (d, J = 7.9 Hz); silica gel TLC (90:10 ethyl acetate–ethanol) R_f 0.34.

Synthesis of d(CGCTTTAAAGCG). The dodecanucleotide of interest was prepared by solid-phase synthesis with protected nucleoside phosphite intermediates. The procedure employed was essentially that of Matteucci & Caruthers (1981), but with the deoxynucleoside phosphoramidite intermediates developed by Beaucage & Caruthers (1981). Fully blocked dodecanucleotide d(5′-DMTr-$C^{Bz}_{OCH_3}G^{iBu}_{OCH_3}C^{Bz}_{OCH_3}T_{OCH_3}T_{OCH_3}T_{OCH_3}A^{Bz}_{OCH_3}A^{Bz}_{OCH_3}A^{Bz}_{OCH_3}G^{iBu}_{OCH_3}C^{Bz}_{OCH_3}G^{iBu}$)–silica gel was prepared in ~20% overall yield on the basis of analysis of the efficiency

of individual coupling reactions (via trityl group analysis of small aliquots of the derivatized silica gel following each coupling). The desired oligodeoxynucleotide was isolated following treatment with 1:1:2 thiophenol–triethylamine–dioxane, followed by concentrated ammonium hydroxide. The presence of the tritylated oligomer d(5′-DMTr-CGCTTTAAAGCG) was verified by HPLC analysis on a C_{18} reverse-phase column; elution was with 0.1 M triethylammonium acetate containing 26% CH_3CN, as described by Matteucci & Caruthers (1981). Purification was carried out by cellulose TLC, developed with 55:10:35 1-propanol–NH_4OH–H_2O; the band with R_f 0.68 was shown to be the desired product, as judged by its behavior on the C_{18} reverse-phase column. The sample was deblocked with 80% acetic acid (25 °C, 15 min) and again purified by cellulose TLC (55:10:35 1-propanol–NH_4OH–H_2O). The identity of the dodecamer was verified by nucleotide sequence analysis.

Preparation of [5′-^{32}P]d(CGCTTTAAAGCG). A solution of 20 μL of 0.4 M Tris-HCl buffer, pH 7.6, containing 75 mM $MgCl_2$, 750 μM spermidine, and 70 pmol of d($_{HO}$CGCTTTAAAGCG) was heated at 75 °C for 90 s and then immersed in a ice bath for 5 min. The solution containing the oligomer was then treated with 15 μL of a 50 mM dithiothreitol solution, 0.5 mCi of [γ-^{32}P]ATP (sp act. >3000 Ci/mmol), and 50 units of T4 polynucleotide kinase, and the combined solution (150-μL total volume) was incubated at 37 °C for 45 min. The reaction was quenched by the addition of 100 μL of 300 mM NaOAc, pH 4.5, and 750 μL of cold ethanol. The mixture was maintained at −20 °C overnight, and the precipitated DNA was collected by centrifugation and washed twice with 1-mL portions of ethanol.

Initial purification of the 5′-^{32}P-labeled dodecanucleotide was carried out on a 12% polyacrylamide gel; following electrophoresis at 15 V/cm for 3.5 h, the gel was visualized by autoradiography, and the band of interest was excised from the gel and placed in 600 μL of a 500 mM NH_4OAc solution containing 100 mM MgOAc, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 10 μg/mL carrier tRNA. Following incubation at 37 °C for 16 h, the solution was filtered through siliconized glass wool and treated with 3 volumes of ethanol to effect oligonucleotide precipitation. The precipitated material was reprecipitated from 50 mM NaOAc, pH 4.5, containing 5 mM $MgCl_2$. The precipitate was washed with ethanol and dried; it was found to contain >70 μCi of ^{32}P-labeled oligonucleotide.

A portion of the labeled dodecamer (7×10^5 cpm) was purified further on a Rainin Microsorb C_{18} column (0.46 × 10 cm); elution was with 10% CH_3CN in 0.01 M triethylammonium acetate, pH 7.0, at a flow rate of 1.5 mL/min. Fractions (0.75 mL) were collected, and those containing [5′-^{32}P]d(CGCTTTAAAGCG) (6×10^4 cpm) were combined and isolated by lyophilization. The dodecamer was precipitated from 200 μL of 0.3 M NaOAc, pH 5.2, with 600 μL of cold ethanol. Centrifugation of the precipitated material afforded 48 000 cpm of [5′-^{32}P]d(CGCTTTAAAGCG). This material was diluted with unlabeled dodecamer as required.

Bleomycin-Mediated Formation of Nucleoside 3′-(Phosphoro-2″-O-glycolic acids) from DNA. To a solution (1-mL total volume) of 50 mM sodium cacodylate, pH 7.0, containing 1 mM *E. coli* DNA and 300 μM $CuCl_2$ or $Fe(NH_4)_2(SO_4)_2$ was added bleomycin B_2 to a final concentration of 300 μM. Each reaction mixture was maintained at 25 °C for 30 min. A portion (50 μL) of this material was analyzed for malondialdehyde (precursor) as described below. The remainder of the reaction mixture was adjusted to 0.3 M NaOAc and treated with 3 volumes of cold ethanol. Precipitation was carried out for 10 min in a dry ice–acetone bath; the precipitate was isolated by centrifugation, washed once with ethanol, and dried. The metallobleomycin-digested samples of DNA were then dissolved in 50 mM glycine buffer, pH 9.4 (total volume 200 μL), containing 3 mM $MgCl_2$, 3 mM dithiothreitol, and 50 units of lambda exonuclease. The reaction was run at 37 °C for 2 h and then utilized for HPLC analysis.

Bleomycin-Mediated Production of Malondialdehyde (Precursors). A 50-μL aliquot of the BLM-treated DNA described above was combined with 950 μL of 50 mM thiobarbituric acid containing 1 mM EDTA. The combined solution was heated at 85 °C for 15 min prior to measurement of A_{532} (Waravdekar & Saslaw, 1959; Burger et al., 1980). The results are shown in Table I.

Degradation of d(CGCTTTAAAGCG) by Bleomycin. Degradation of the dodecanucleotide (1 mM) was carried out in a solution (50-μL total volume) of 50 mM sodium cacodylate buffer, pH 7.0, and 1 mM BLM A_2. The reaction was initiated by the addition of $Fe(NH_4)_2(SO_4)_2$ to a final concentration of 1 mM; after incubation at 25 °C for 30 min, the reaction mixture was analyzed immediately by HPLC (Figures 3 and 4).

Cleavage of [5′-^{32}P]d(CGCTTTAAAGCG) (~5 μM, 48 000 cpm; diluted to 1 mM with unlabeled dodecamer) was carried out in 25 μL (total volume) of 50 mM sodium cacodylate, pH 7.0, containing 1 mM BLM A_2 and 1 mM $Fe(NH_4)_2(SO_4)_2$. The reaction was initiated and incubated as described above. A 20-μL aliquot of the reaction mixture was admixed with authentic (unlabeled) 2′-deoxycytidylyl(3′→5′)[2′-deoxyguanosine 3′-(phosphoro-2″-O-glycolic acid)] 5′-phosphate and analyzed by HPLC. The results are shown in Figure 6.

Bleomycin-Mediated Formation of Base Propenals. To 100 μL (total reaction volume) of 50 mM sodium cacodylate, pH 7.0, containing 1 mM calf thymus DNA and 1 mM bleomycin A_2 was added 1 mM $Fe(NH_4)_2(SO_4)_2$. The combined solution was maintained at 0 °C for 30 min and then utilized immediately for HPLC analysis. Samples were analyzed on a Rainin Microsorb C_{18} column (0.46 × 10 cm); elution was carried out at 25 °C over a period of 20 min with a linear gradient of 0→100% methanol (in water) at a flow rate of 1.0 mL/min. The A_{254} profile was monitored; the profile obtained with Fe(II)·BLM is shown in Figure 7.

RESULTS

Formation of Nucleoside Glycolates from DNA and d(CGCTTTAAAGCG). To permit direct analysis of the products of bleomycin-mediated DNA degradation, the four possible 2′-deoxynucleoside 3′-(phosphoro-2″-O-glycolic acid) 5′-phosphates (**5**–**8**) were prepared synthetically as illustrated for the thymidine derivative (**5**) (i.e., **1** → **9** → **10** → **11** → **5**). Following condensation of $O^{5'}$-(dimethoxytrityl)thymidine with methyl glycolate 2-O-phosphorodichloridite and oxidation of the intermediate dialkyl phosphite with I_2/H_2O, methyl $O^{5'}$-(dimethoxytrityl)thymidine 3′-(phosphoro-2″-O-glycolate) (**9**) was isolated as colorless plates in 90% yield. Deblocking afforded thymidine 3′-(phosphoro-2″-O-glycolate) (**11**) as a white solid in 75% overall yield from nucleotide **9**; structural verification of **11** was accomplished by ^{1}H NMR spectroscopy [δ 1.74 (s, CH_3) and 4.06 (d, $-OCH_2COOH$)] and FAB mass spectrometry (M_r 380). Phosphorylation of nucleotide **11** was accomplished enzymatically in 92% yield by incubation in the presence of ATP and T4 polynucleotide kinase; the same species was also obtained by phosphorylation via the agency of $POCl_3$ and pyridine in acetonitrite, albeit only in 44% yield.

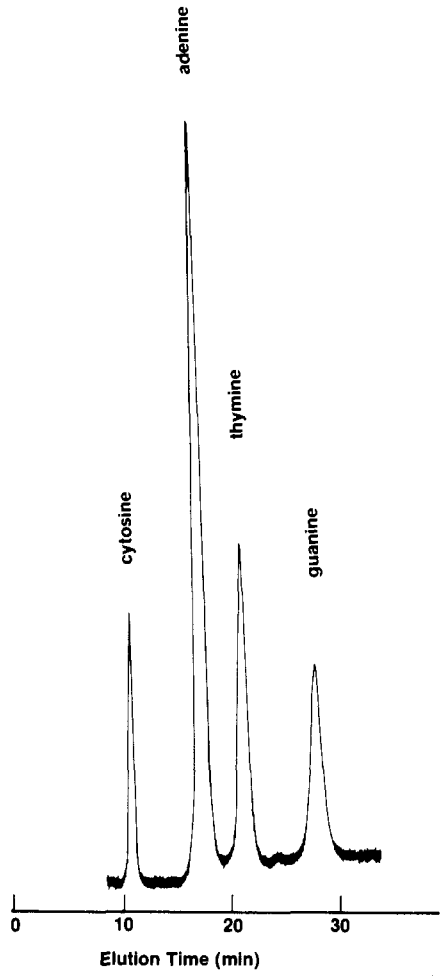


FIGURE 1: Separation of synthetic 2′-deoxynucleoside 3′-(phosphoro-2″-*O*-glycolic acid) 5′-phosphates (**5**–**8**) by anion-exchange HPLC. Compounds **5**–**8** (identified above by their respective purine or pyrimidine bases) were coinjected onto a Du Pont Zorbax SAX column and eluted with 0.25 M ammonium phosphate buffer, pH 3.3, at a flow rate of 1.5 mL/min. The retention times under these conditions were 11.0, 16.9, 20.9, and 27.1 min for the cytosine, adenine, thymine, and guanine nucleoside glycolate derivatives, respectively. The elution profile was monitored at 254 nm.

The structure of thymidine 3′-(phosphoro-2″-*O*-glycolic acid) 5′-phosphate (**5**) was verified by positive and negative ion mass spectrometry. Depicted in Figure 1 are the HPLC profiles of nucleotides **5**–**8** following coinjection on a Zorbax-SAX column. As shown, complete separation of these four nucleotides was obtained routinely.

Following treatment of *E. coli* DNA with Fe(II)·BLM, the hydrolyzed DNA oligonucleotides were isolated by precipitation and centrifugation, then treated further with lambda exonuclease to liberate individual nucleotides. Analysis of a typical reaction mixture is illustrated in Figure 2; the HPLC profile clearly reflected the presence of 2′-deoxycytidine 3′-(phosphoro-2″-*O*-glycolic acid) 5′-phosphate (**6**), 2′-deoxyadenosine 3′-(phosphoro-2″-*O*-glycolic acid) 5′-phosphate (**7**), and 2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolic acid) 5′-phosphate (**8**). Also noted was a peak with the same mobility as thymidine 3′-(phosphoro-2″-*O*-glycolic acid) 5′-phosphate (**5**); while only a minor component of the reaction mixture analyzed in Figure 2, this peak was considerably more prominent in other experiments (data not shown).

The identities of the individual nucleotide glycolate derivatives were supported by the demonstration that each coeluted with the authentic (i.e., synthetic) species following coinjection under the conditions employed in Figures 1 and 2. It was also

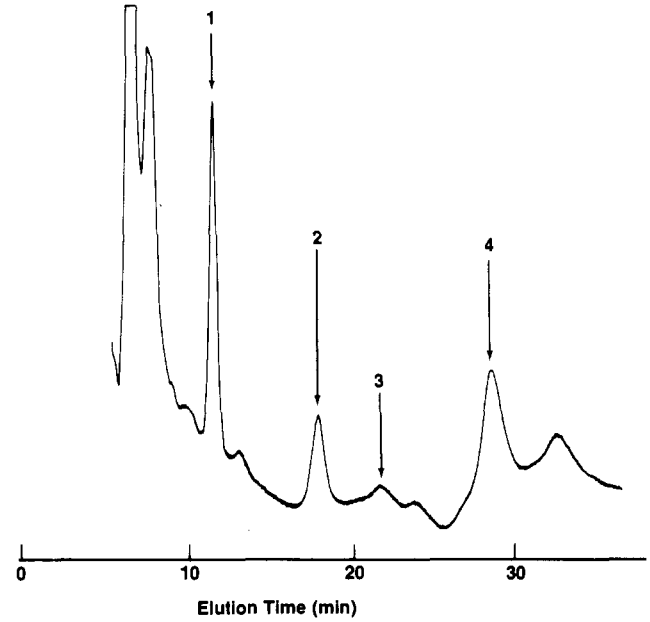
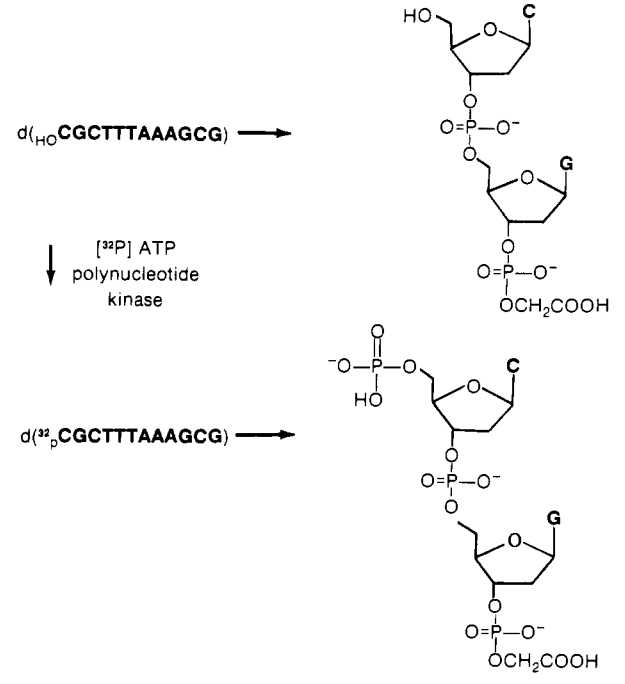


FIGURE 2: Production of nucleotide glycolate derivatives by Fe(II)·BLM digestion of DNA. *E. coli* DNA, treated with Fe(II)·BLM and lambda exonuclease as described under Experimental Procedures, was analyzed by HPLC on a Du Pont Zorbax SAX column with 0.25 M ammonium phosphate buffer, pH 3.3 (flow rate 1.5 mL/min, monitored by A_{254}). Peaks 1–4 (retention times of 11.2, 17.5, 21.6, and 27.7 min, respectively) were identified as nucleotide glycolate derivatives of deoxycytidine, deoxyadenosine, thymidine, and deoxyguanosine, respectively, by coinjection with synthetic samples.

Scheme I: Derivation of 2′-Deoxycytidylyl(3′→5′)-[2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolic acid)] (**18**) and Its 5′-Phosphate from Self-Complementary Dodecanucleotides via the Agency of Fe·BLM



shown that authentic **5**–**8** were unaffected by the treatments used for the generation and isolation of putative **5**–**8** from *E. coli* DNA.

Also prepared for use as a bleomycin substrate was the dodecanucleotide d(CGCTTTAAAGCG). The preparation was carried out by solid-phase synthesis by the methods of Matteucci & Caruthers (1981), as described under Experimental Procedures. A 5′-^{32}P-labeled sample of d-

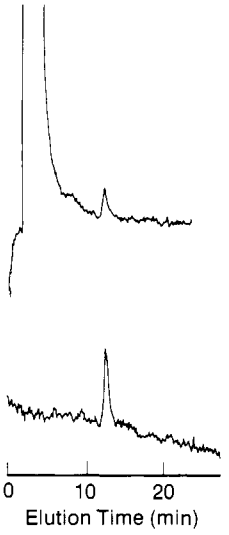


FIGURE 3: Characterization of dCpGpCOOH (**18**) by anion-exchange HPLC. The dodecanucleotide d(CGCTTTAAAGCG) (1 mM) was digested with 1 mM Fe(II)·BLM A_2 for 30 min at 25 °C in 50 mM sodium cacodylate, pH 7.0. The reaction mixture was analyzed on a Du Pont Zorbax SAX column (monitored at 254 nm) with 0.2 M ammonium phosphate, pH 3.3, at a flow rate of 1.5 mL/min (top). Authentic dCpGpCOOH (**18**) was analyzed under identical conditions (bottom).

(CGCTTTAAAGCG) was prepared by the use of T4 polynucleotide kinase and [γ-^{32}P]ATP. The products whose formation was anticipated upon treatment of these self-complementary oligonucleotides with bleomycin (cf. Scheme I), i.e., 2′-deoxycytidylyl(3′→5′)[2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolic acid)] (**18**) and the respective 5′-phosphate, were prepared synthetically to facilitate their identification as products of the bleomycin reactions. As illustrated, the pathway employed for the elaboration of **18** (i.e., **12** → **13** → **14** → **16** → **17** → **18**) was analogous to those used for the synthesis of nucleotides **5**–**8**. One needed modification was the introduction of the phosphoro-2″-*O*-glycolic acid moiety by successive additions of methyl phosphorodichloridite and methyl glycolate; following oxidation, this procedure afforded a phosphotriester that could be employed without further functional group modification for synthesis of the required dinucleotide. The structure of dinucleotide **18** was verified by ^{1}H NMR spectroscopy and FAB mass spectrometry. Phosphorylation was effected by the use of ATP and T4 polynucleotide kinase.

Following treatment of dodecanucleotide d(CGCTTTAAAGCG) with Fe(II)·BLM A_2 at 25 °C for 30 min, the digest was analyzed directly by HPLC in comparison with synthetic dinucleotide **18**. In separate assays, these conditions were shown to completely degrade the dodecanucleotide while cleaving selectively (>85%) at the two GC sites within the molecule (data not shown). Analysis was carried out on an anion-exchange column (Figure 3) and by chromatography on a C_{18} reverse-phase column (Figure 4); in each case, authentic **18** coeluted with a major component of the dodecamer digest. While the yields of compound **18** and the analogous product derived from cleavage of the other strand of the duplex were found to vary somewhat with reaction conditions, under optimal conditions they constituted 70% of the products formed during d(CGCTTTAAAGCG) degradation. Also subjected to digestion with Fe(II)·BLM A_2 was the 5′-^{32}P end-labeled dodecanucleotide (Scheme I). HPLC analysis on an anion-exchange column, elution with 0.4 M ammonium phosphate, pH 3.3, gave a peak of radioactivity having the same elution time as authentic 2′-deoxycytidylyl-(3′→5′)[2′-deoxyguanosine 3′-(phosphoro-2″-*O*-glycolic acid)] 5′-phosphate (Figure 5).

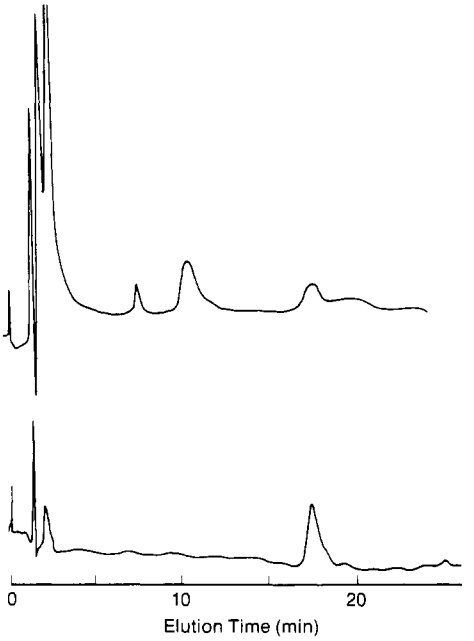


FIGURE 4: Characterization of dCpGpCOOH (**18**) by reverse-phase HPLC. After digestion of the dodecanucleotide d(CGCTTTAAAGCG) (1 mM) with 1 mM Fe(II)·BLM at pH 7.0 and 25 °C for 30 min, the sample was analyzed on a Rainin Microsorb Short-One C_{18} column (monitored by A_{254}) with 0.1 M ammonium formate buffer, pH 6.0, at a flow of 1 mL/min (top). Authentic dCpGpCOOH (**18**) was analyzed under the same conditions (bottom).

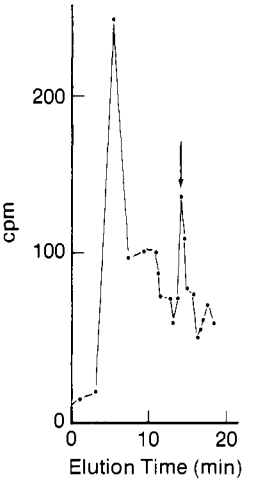


FIGURE 5: BLM-mediated release of ^{32}P-labeled d(pCpGpCOOH). [5′-^{32}P]d(CGCTTTAAAGCG) (1 mM, ~48 000 cpm) was treated with 1 mM Fe(II)·BLM at pH 7.0 as described under Experimental Procedures. The resulting solution was analyzed on a Du Pont Zorbax SAX anion-exchange HPLC column with 0.4 M ammonium phosphate buffer, pH 3.3, at a flow rate of 1.0 mL/min. Fractions were collected for determination of ^{32}P radioactivity by liquid scintillation counting. The arrow shows the position at which authentic d(pCpGpCOOH) was eluted from the column as monitored by A_{254}.

Quantitative Analysis of Base and Base Propenal Release from DNA. Production of base propenals concomitant with DNA strand scission was assayed initially by the use of aliquots of reaction mixtures employed for the analysis of nucleoside 3′-(phosphoro-2″-*O*-glycolic acids). These aliquots were heated in the presence of thiobarbituric acid, which has been shown to form an adduct with base propenal derived malondialdehyde (Burger et al., 1980). The adduct has an absorption of known molar absorptivity at 532 nm, which provides the basis for quantitative assay of this DNA degradation product. As

Table I: Production of Malondialdehyde (Precursors) during Bleomycin-Mediated DNA Degradation[a]

additions	malondialdehyde (nmol)
300 μM BLM B_2, 300 μM Fe(II)	9.0
300 μM Fe(II)	0[b]
none	0[b]

[a] Reaction mixtures containing *E. coli* DNA (1 mM) and the additions shown above were incubated at 25 °C and pH 7.0 for 30 min. An aliquot (50 μL) was analyzed for malondialdehyde (precursors) as described under Experimental Procedures. [b] <0.1 nmol.

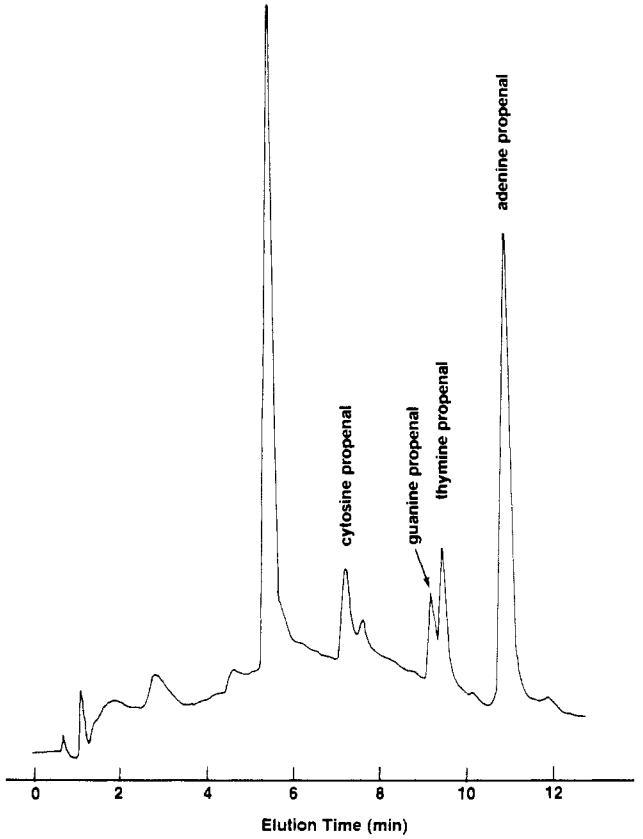


FIGURE 6: Formation of base propenals from DNA by Fe(II)·BLM. Calf thymus DNA (1 mM) was digested aerobically with 1 mM Fe(II)·BLM A_2 at pH 7.0 for 30 min at 0 °C as described under Experimental Procedures. Base propenal release was analyzed on reverse-phase HPLC (Rainin Microsorb Short-One C_{18} column) with a linear gradient of 0–100% methanol (in water) at a flow rate of 1.0 mL/min (monitored at 254 nm). The base propenals identified had retention times of 7.5, 9.5, 9.8, and 11.1 min for cytosine, guanine, thymine, and adenine propenals, respectively.

shown in Table I, digestion of *E. coli* DNA with 300 μM Fe(II)·BLM provided 9.0 nmol of malondialdehyde (precursor).

Analysis of the formation of individual base propenals was carried out by reversed-phase HPLC following treatment of calf thymus DNA with Fe(II)·BLM A_2. A typical profile obtained for Fe(II)·BLM A_2 is shown in Figure 6. Peaks corresponding to all four base propenals were observed and the structures verified by direct comparison with authentic synthetic samples (Giloni et al., 1981; Johnson et al., 1984) and by conversion (0.1 N HCl, 90 °C, 10 min) to the respective bases. The largest peak in Figure 6 corresponded to free thymine (elution time 5.6 min) and was followed by a smaller (partially resolved) peak consisting of 3-(cytosin-1′-yl)propenal (7.4 min) and adenine (8.0 min). As expected, much larger amounts of 3-(cytosin-1′-yl)propenal were formed when poly(dG·dC)·poly(dG·dC) (P-L Biochemicals) or d-

Table II: Extinction Coefficients for Base Propenals[a]

compd	λ_{max} (nm)	$\epsilon_{\lambda_{max}}$
3-(thymin-1′-yl)propenal	303	26 300
3-(adenin-9′-yl)propenal	257	34 300
3-(cytosin-1′-yl)propenal	312	28 700
3-(guanin-9′-yl)propenal	327	6 800
	266	11 300
	240	10 500

[a] Extinction coefficients were measured in 10 mM sodium cacodylate buffer, pH 7.0.

Table III: Release of Bases and Base Propenals from DNA by Fe(II)·BLM Degradation[a]

compd	retention time (min)[b]	amount (nmol)
thymine	5.3	3.8
3-(thymin-1′-yl)propenal	9.8	1.9
cytosine	2.9	1.4
3-(cytosin-1′-yl)propenal	7.4	1.4
adenine	8	0.10
3-(adenin-9′-yl)propenal	11.1	2.3
guanine	4.5	0.92
3-(guanin-9′-yl)propenal	9.5	1.5

[a] Calf thymus DNA (1 mM) was treated with 1 mM Fe(II) + 1 mM BLM A_2 at pH 7 for 30 min in a total volume of 0.1 mL as described under Experimental Procedures. [b] Products were separated by reverse-phase HPLC as described under Experimental Procedures.

(CGCTTTAAAGCG) was treated with Fe(II)·BLM. Although 2-mercaptoethanol has been reported to decompose base propenals (Giloni et al., 1981), under the experimental conditions employed here the inclusion of 1 mM dithiothreitol in DNA degradation reactions containing 1 mM Fe(II)·BLM had only a limited effect on the ratio of products formed. The isolated base propenals were found to react with DTT, but under the reaction conditions employed here base propenal formation could be verified by HPLC. Further, the total amount of base propenals observed was comparable to the amount of malondialdehyde measured when the same reaction mixtures were assayed with 2-thiobarbituric acid (Burger et al., 1980).

Irradiation of synthetic *trans*-3-(thymin-1′-yl)propenal with Pyrex-filtered light produced *cis*-3-(thymin-1′-yl) propenal (**20**) in 90% yield as a white powder. The latter exhibited the expected vinylic coupling in the ^{1}H NMR (J = 9.0 Hz vs. 15.1 Hz for the trans isomer) and was slowly converted back to the trans isomer on standing. *cis*-3-(Adenin-9′-yl)propenal (**21**) was produced analogously. The isomers of each base propenal were easily separable by Microsorb C_{18} HPLC, as illustrated by Figure 7 for *cis*- and *trans*-3-(thymin-1′-yl)propenal.

HPLC analysis of samples of calf thymus DNA that had been treated with Fe(II)·BLM revealed that 3-(thymin-1′-yl)propenal and 3-(adenin-9′-yl)propenal were formed exclusively as the respective trans isomers. Further, authentic samples of *cis*-3-(thymin-1′-yl)propenal and *cis*-3-(adenin-9′-yl)propenal were shown to be stable under the reaction conditions that produced base propenals from DNA, indicating that the observed *trans*-propenals were primary products of bleomycin-mediated DNA degradation.

To facilitate the quantitation of reaction products observed in this study, the molar absorptivities of the synthetic base propenals were measured and are reported in Table II. Using these values, it was determined that treatment of 1 mM calf thymus DNA (29% thymidine, 29% adenosine content) with 1 mM Fe(II)·BLM resulted in the release of ~6% of all thymidines as *trans*-3-(thymin-1′-yl)propenal and ~13% of all thymidines as free thymine. Also observed was concomitant formation of *trans*-3-(adenin-9′-yl)propenal and adenine, as

FIGURE 7: Separation of *cis*- and *trans*-3-(thymin-1′-yl)propenals (**20** and **19**, respectively) by HPLC. Synthetic samples of *cis*- and *trans*-3′-(thymin-1′-yl)propenals were separated on reverse-phase HPLC with a Rainin Microsorb Short-One C_{18} column as described under Experimental Procedures.

well as the other base propenals and free bases; these are quantitated in Table III.

Discussion

Oligonucleotide 3′-(phosphoro-2″-*O*-glycolic acid) derivatives are believed to result from γ-irradiation of DNA (Henner et al., 1983) and by treatment with bleomycin (Giloni et al., 1981; Uesugi et al., 1984), as well as certain synthetic DNA-specific reagents that bring an attached Fe(II)·EDTA moiety in proximity to the double helix (Hertzberg & Dervan, 1982; Schultz et al., 1982). To date, direct analysis of this DNA modification has been carried out only for the case of γ-irradiated DNA, where individual modified nucleotides are released (Henner et al., 1983). For Fe(II)·BLM-treated DNA, analysis for free glycolic acid was carried out after enzymatic or acid hydrolysis of the bleomycin-derived oligonucleotides (Giloni et al., 1981). The point of attachment of glycolic acid to the initially formed DNA oligomers was inferred from the known substrate specificity of the enzymes employed for analysis and by the use of DNA radiolabeled at specific positions; the analytical procedure precluded the identification of the nucleotides to which glycolic acid was attached. Recently, Uesugi et al. (1984) have demonstrated the presence of dinucleotide **18** in a reaction mixture containing Fe(II)·BLM B_2 and d(CGCGCG). In the present case, we have analyzed bleomycin-digested DNA samples following initial treatment with lambda exonuclease; this enzyme was found to convert oligonucleotides to single nucleotides without hydrolysis of phosphoroglycolic acid from the 3′-terminal nucleoside. The derived nucleoside 3′-(phosphoro-2″-*O*-glycolic acid) 5′-phosphates were analyzed by direct comparison with authentic samples of the four possible derivatives (**5**–**8**), all of which were prepared by chemical synthesis. As illustrated in Figure 2, the extent of appearance of these four derivatives (especially **5**) was reasonable in the context of the known (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Mirabelli et al., 1983) sequence specificity of Fe(II)·BLM. However, the use of enzymatic hydrolysis and subsequent workup of the DNA in order to generate these products for analysis made quantitative assessment of their formation difficult. In addition, the efficiency of lambda exonuclease in complete digestion of the DNA to release *all* nucleoside glycolates is not known. It is probably worth noting that the formation of additional oligonucleotide-bound products concomitant with DNA strand scission by other reagents [see, e.g., Uesugi et al. (1982)] suggests that lambda exonuclease may be of more general utility for DNA product analysis.

Also employed as a substrate for Fe(II)·BLM was the self-complementary dodecanucleotide d(CGCTTTAAAGCG) predicted (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Mirabelli et al., 1983) to have a preferred bleomycin cleavage site three nucleotides from the 5′-end (cf. Scheme I). Treatment of this oligonucleotide with Fe(II)·BLM produced a reaction mixture that was analyzed directly by HPLC on anion-exchange and reverse-phase columns in comparison with an authentic sample of the expected dinucleotide (**18**). The observation of dinucleotide **18** as a product of bleomycin-mediated dodecanucleotide degradation demonstrated the formation of an oligonucleotide 3′-(phosphoro-2″-*O*-glycolic acid) derivative in a system that employed no workup subsequent to bleomycin treatment. Also established by this experiment was the retention in a small oligonucleotide of the sequence specificity noted for bleomycin in much larger DNA fragments (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Mirabelli et al., 1983).

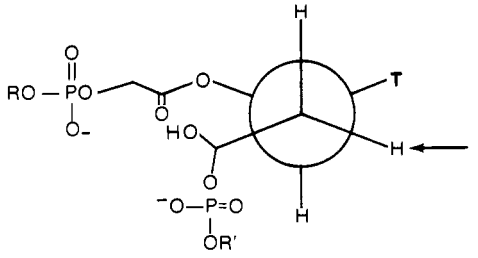
Earlier reports have established that DNA degradation mediated by Fe(II)·BLM results in the formation of all four base propenals and that the aliphatic carbon atoms are derived from C-1′–C-3′ of ribose (Burger et al., 1980, 1982; Giloni et al., 1981; Wu et al., 1983). Assay has been carried out by chromatographic analysis and by reaction of base propenal derived malondialdehyde with thiobarbituric acid. Although the latter assay method has provided a convenient measure of total base propenal production, reports that malondialdehyde and related species can react with DNA (Brooks & Klamerth, 1968; Moschel & Leonard, 1976; Czarnik & Leonard, 1981; Nair et al., 1981) and the absence of any analysis of the relative amounts of individual base propenals produced have prompted us to carry out an analysis of the extent of formation of certain of the primary products of Fe(II)·BLM-mediated DNA degradation, including the four base propenals.

On the basis of DNA labeling experiments and mechanistic analysis of formed reaction products, it seems likely that the oligonucleotide 3′-(phosphoro-2″-*O*-glycolic acid) derivatives formed from DNA by Fe(II)·BLM + O_2 incorporate C-4′ and C-5′ of a cleaved deoxyribose moiety, while C-1′, C-2′, and C-3′ of the same deoxyribose are incorporated into the derived base propenals (Giloni et al., 1981; Saito et al., 1983; Wu et al., 1983). Accordingly, aliquots of reaction mixtures employed for the analysis of individual nucleoside 3′-(phosphoro-2″-*O*-glycolates) (**5**–**8**) were employed to measure thiobarbituric acid reactive material. Additionally, following degradation of d(CGCTTTAAAGCG) with Fe(II)·BLM, the formation of **18** and 3-(cytosin-1′-yl)propenal was monitored simultaneously by HPLC. Roughly comparable amounts of (oligo)nucleotide 3′-(phosphoro-2″-*O*-glycolates) and base propenals were formed.

Direct measurement of base propenal formation required some modification of reaction conditions to provide increased sensitivity. In addition, it was found that individual base propenals differed significantly in λ_{max} and molar absorptivity

(Table II), necessitating a determination of peak areas of authentic synthetic samples. As shown in Table III, a total of ~7 nmol of base propenal was produced from 100 nmol of DNA nucleotide. This compared favorably with the measurements made for malondialdehyde production from a different substrate (*E. coli* DNA, 18 nmol; cf. Table I). These data thus suggest that three different substrates [d-(CGCTTTAAAGCG), *E. coli* DNA, and calf thymus DNA] all release comparable amounts of similar products [i.e., oligonucleotide 3′-(phosphoro-2″-*O*-glycolates) and base propenals].

Also analyzed directly in the present study was the geometry of the 3-(thymin-1′-yl)propenal and 3-(adenin-9′-yl)propenal produced during BLM-mediated DNA degradation. By comparison with authentic cis and trans isomers of each base propenal, it was shown that bleomycin produced exclusively *trans*-3-(thymin-1′-yl)propenal and *trans*-3-(adenin-9′-yl)-propenal and that both were primary reaction products. The formation of essentially equal amounts of oligonucleotide 3′-(phosphoro-2″-*O*-glycolates) and base propenals and the finding that at least two of the latter are formed exclusively with trans geometry suggest that DNA strand scission may occur from a single conformation by specific loss of the α-2′-H of deoxyribose, i.e.



This result is entirely consistent with the data of Wu et al. (1983), which demonstrated specific abstraction of α-2′-H. The reason(s) for this specificity is (are) not entirely clear at present but could relate to constraints on conformation of the damaged deoxyribose moiety imposed by association with the undamaged strand of the DNA duplex. However, bleomycin is also known to cause double-strand breaks; if the mechanism for scission of the "second" strand also involved base propenal formation, it would be more difficult to envision DNA duplex enforced conformational constraints as a source of geometrical selectivity. Alternatively, it seems possible that bleomycin may also participate mechanistically in a second step in DNA strand scission that results in removal of a specific H from the C-2′ of deoxyribose or that the pathway leading to the same isomer is inherently more favorable.

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