Covalent Modification and Single-Strand Scission of DNA by a New Antitumor Antibiotic Kapurimycin A3

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ABSTRACT: Kapurimycin A3 is a new antitumor antibiotic isolated from a *Streptomyces*. It contains the anthrapyrone skeleton and a β,γ-unsaturated δ-keto carboxylic acid moiety in the structure. In vitro, kapurimycin causes single-strand cleavage of supercoiled pBR322 DNA. The diminished cytotoxicity and DNA cleaving activity for 13-decarboxykapurimycin A3 indicates that the β,γ-unsaturated δ-keto carboxylic acid moiety is important for the activity of kapurimycin. Kapurimycin A3 binds to calf thymus DNA at 4 °C, and the thermal treatment of this adduct results in release of a guanine covalently attached to C-16 of kapurimycin via one of its nitrogen atoms. Thus, the epoxide is the alkylating functional group of kapurimycin, and this is consistent with the lack of DNA cleaving and cytotoxic activities for 14,16-deoxy-14,16-dihydroxykapurimycin. These findings have revealed that DNA strand scission by kapurimycin is due to the alkylation of guanine by ring opening of the epoxide group of kapurimycin, depurination of modified guanine, and presumably subsequent hydrolysis of the phosphate ester backbone at the resultant apurinic sites.

DNA recognition and strand scission by small molecules are currently topics of intense investigation. Of particular interest are antitumor compounds that interact with DNA (Graves et al., 1984; Tomasz et al., 1984, 1987; Kappen & Golgberg, 1989; Hara et al., 1990a). The discovery of new antitumor agents that interact with DNA has provided insight into the mechanistic principles that can be used to mediate DNA recognition and cleavage.

From our search for new antitumor antibiotics of microbial origins, we have isolated a new antitumor antibiotic, kapurimycin A3 (Figure 1, **1**), produced by a *Streptomyces* sp. (Hara et al., 1990b; Yoshida et al., 1990). It constitutes a new class of polycyclic microbial metabolites possessing the tetrahydroanthrapyrone skeleton. It also contains the β,γ-unsaturated δ-keto carboxylic acid structure, which corresponds to the C_5 unit composed of 4, 4a, 5, and 13 atoms and its neighboring carboxyl carbon atom in the molecule. **1** exhibited cytotoxic activity against cultured mammalian cells and prolonged the life of mice bearing P388 and adriamycin-resistant P388 ascitic tumors. It also showed inhibition of growth of Gram-positive bacteria. In the present study, we undertook to examine the interaction of **1** with DNA in vitro. The results are discussed in relation to the structural characteristics of kapurimycin A3.

Experimental Procedures

Materials. **1** was isolated from a cultured broth of *Streptomyces* sp. as reported previously (Hara et al., 1990b). **2** was prepared by thermal decarboxylation of **1** dissolved in DMSO solution. 14,16-Deoxy-14,16-dihydroxykapurimycin A3 (**3**) was isolated from the reaction mixture containing **1** and calf thymus DNA by HPLC. Stock solutions of **1** and **3** were prepared in MeOH. Since **2** was only slightly soluble in MeOH, a stock solution was prepared in DMSO. Use of DMSO instead of MeOH did not affect the reaction of drug with DNA. All other reagents used were of commercial reagent grade.

Antimicrobial Activity and Cytotoxic Activity. The in vitro antimicrobial activity against *Bacillus subtilis* was determined in nutrient agar by a 2-fold serial dilution method. The lowest concentration that inhibited growth of a bacterial strain after an 18-h incubation at 37 °C was recorded as the minimum inhibitory concentration (MIC).

H-*ras*-transfected mouse BALB 3T3 (BALB 3T3/H-*ras*)[1] cells were culturd in Dulbecco's modified Eagle's medium containing 10% calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). For determination of drug cytotoxicities, BALB 3T3/H-*ras* cells were preincubated for 24 h at 37 °C in 96-well plastic plates and then treated with different dilutions of drugs for 1 h. After washing with PBS, the cells were cultured in fresh medium for 3 days. The concentration of drug required for 50% inhibition of cell growth (IC_{50}) was determined by the Giemsa staining method, as described by Mirabelli et al. (1985).

Reaction of Drugs with pBR322 DNA. The DNA cleavage activity was determined by using purified pBR322 DNA. A typical reaction mixture included 20 μL of 20 mM Tris-HCl buffer, pH 7.5, 0.3 μg of pBR322 DNA, and various concentrations of drugs. In some studies, different oxygen radical scavengers were added to the buffer solution before adding pBR322 DNA and drugs. The reaction mixtures were incubated at 37 °C for 60 min. After the addition of 3.5 μL of 0.02% bromophenol blue and 50% sucrose, 20 μL of the mixture was loaded onto a 1.2% agarose slab gel. Equal amounts of DNA were applied to each lane of the agarose gel. The agarose was supplemented with EtBr (2 μg/mL), if necessary.

Agarose Gel Electrophoresis of pBR322 DNA. Electrophoresis was carried out in a pH 8.3 89 mM borate–2 mM EDTA buffer containing 0.01% SDS at 50 mV for 12 h. EtBr (2 μg/mL) was added to the above buffer solution, if necessary. Following electrophoresis, gels were stained with an aqueous solution of ethidium bromide (1 μg/mL). DNA bands were visualized by transillumination with ultraviolet light (300 nm) and photographed with Kodak Nos. 24A and 12 filters with Polaroid type 665 positive/negative film. The amount of DNA

[1] Abbreviations: AP, apurinic,; BALB 3T3/H-*ras*, BALB 3T3 cells transformed by the H-*ras* oncogene; PBS, phosphate-buffered saline; TMS, tetramethylsilane; COSY, correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy.

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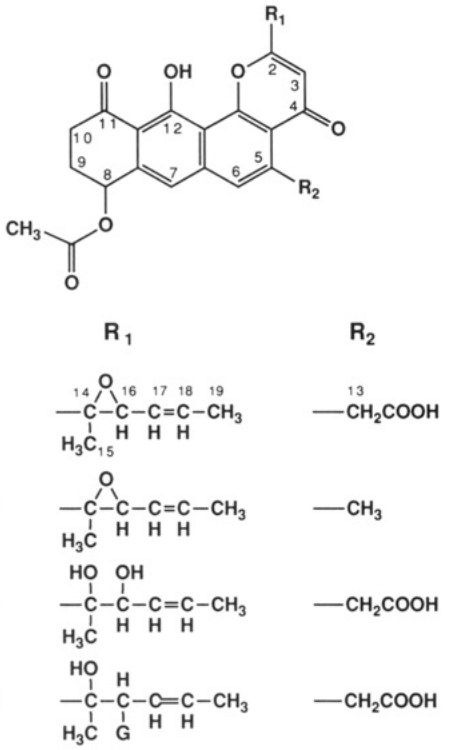

FIGURE 1: Structure of kapurimycin A3 and its related compounds. G denotes guanine.

was quantitated by scanning negatives with a Shimadzu scanning densitometer.

Reaction of Drugs with Calf Thymus DNA. Various amounts of drugs were added to 200 μL of 20 mM Tris-HCl buffer solution containing 0.096 μmol (DNA nucleotide) of calf thymus DNA (Sigma, type I). The reaction mixtures were incubated for 5 days at 4 °C. The reaction was stopped by adding 1/10 volume of 3 M NaOAc and 2.5 volumes of cold ethanol. The calf thymus DNA recovered by ethanol precipitation was subjected to HPLC analysis (YMC AM312 ODS column). The column was run with MeOH–50 mM phosphate buffer, pH 7 (7:3), at a flow rate of 1 mL/min. Drug and DNA eluted from the column were detected with a multichannel photo-diode-array detector (Union Giken, Model MCPD-350). The amount of DNA-bound drug was estimated from its absorbance at 380 nm with an extinction of 10 500 M^{-1} cm^{-1} for guanine-bound kapurimycin A3, and the nucleic acid was estimated from its absorption at 259 nm with an extinction of 6500 M^{-1} cm^{-1} (Swenson et al., 1982).

Thermal Treatment of Kapurimycin-Modified DNA. Kapurimycin-modified DNA was collected by ethanol precipitation and dissolved in 50 mM phosphate buffer, pH 6.7. The solutions were incubated at 37 or 55 °C. Samples (30 μL) were taken at indicated times and subjected to the HPLC analysis. The amount of DNA-bound drug was estimated as described above. The amount of modified kapurimycin (**4**) released from the DNA–drug complex was estimated by calculating the HPLC peak areas.

Large-Scale Preparation and Isolation of **4**. **1** (36 mg) was dissolved in 100 mL of 20 mM Tris-HCl buffer, pH 7.5, containing 100 mg of calf thymus DNA. The reaction mixture was incubated at 4 °C for 5 days with stirring. Modified DNA was recovered by ethanol precipitation and dissolved in 80 mL of 50 mM phosphate buffer, pH 6.7. After incubation at 55 °C for 3 h, the sample was subjected to HPLC (YMC R335-20, ϕ 50 × 500 mm) with MeOH–50 mM phosphate buffer, pH 7 (7:3), solvent at a flow rate of 50 mL/min. The

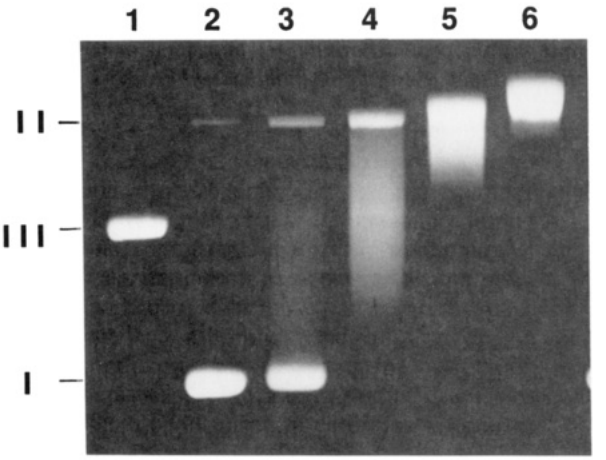

FIGURE 2: Agarose gel electrophoresis of pBR322 DNA treated with kapurimycin A3 (**1**). Lane 1, pBR 322 linear DNA; lane 2, pBR322 DNA alone; lanes 3–6, pBR322 DNA plus **1** at 1.6, 6.3, 25, and 100 μM, respectively. The positions of form I, II, and III DNA were included.

fraction that eluted at retention time 27 min was collected and concentrated. The samples were then desalted on a Diaion HP20 column, concentrated, and dried in vacuo to yield 12.3 mg of **4**.

NMR Experiments. All experiments were performed on a Bruker AM500 spectrometer operating at 500 MHz for proton and 125 MHz for carbon observations. Samples were dissolved in methanol-d_4, and chemical shifts were referenced internally to tetramethylsilane (TMS). All proton and carbon assignments were based on 2D homonuclear (DQF-COSY, NOESY) and 2D-HETCOR experiments.

Results

Reaction of Kapurimycin A3 (**1**) *with pBR322 Plasmid DNA.* The effect of **1** on plasmid pBR322 DNA was analyzed by agarose gel electrophoresis. Figure 2 shows that conversion of supercoiled DNA (form I) to open circled (nicked or closed) DNA is apparent with increasing concentrations of **1**. A smear from the supercoiled to the open circled position was also observed. Form III (linear duplex) DNA was not observed, even at drug concentrations as high as 100 μM, indicating that **1** did not cause double-strand breaks in supercoiled plasmid DNA. The presence of EDTA did not suppress DNA cleavage. Furthermore, no changes were observed in the presence of divalent metal cations. The DNA cleaving activity was not enhanced by the presence of reducing agents such as DTT or $NaBH_4$, nor was it suppressed by the presence of oxygen radical scavengers (data not shown). Figure 2 also shows that **1** produced subtle but significant changes in the electrophoretic mobility of supercoiled as well as open circled DNA. This was also observed when the sample was ethanol precipitated to remove unreacted drug. Furthermore, DNA bands of form I and II DNA in gels after electrophoresis, but before ethidium staining, showed a faint blue fluorescence characteristic of **1** upon UV irradiation (data not shown). These results suggest that **1** forms a covalent linkage to DNA.

To characterize further the effect of **1** on pBR322 plasmid DNA, gel electrophoresis was performed by using an agarose gel containing 2 μg/mL EtBr (Figure 3A). A smear from the supercoiled to the open circled position became positively twisted during electrophoresis, as judged from the increase in the supercoiled DNA when run in the presence of EtBr, an intercalating agent that induces positive superhelical turns (Figures 3A and 4). Thus, drug binding unwinds DNA, and the resulting variously relaxed not nicked DNA migrated

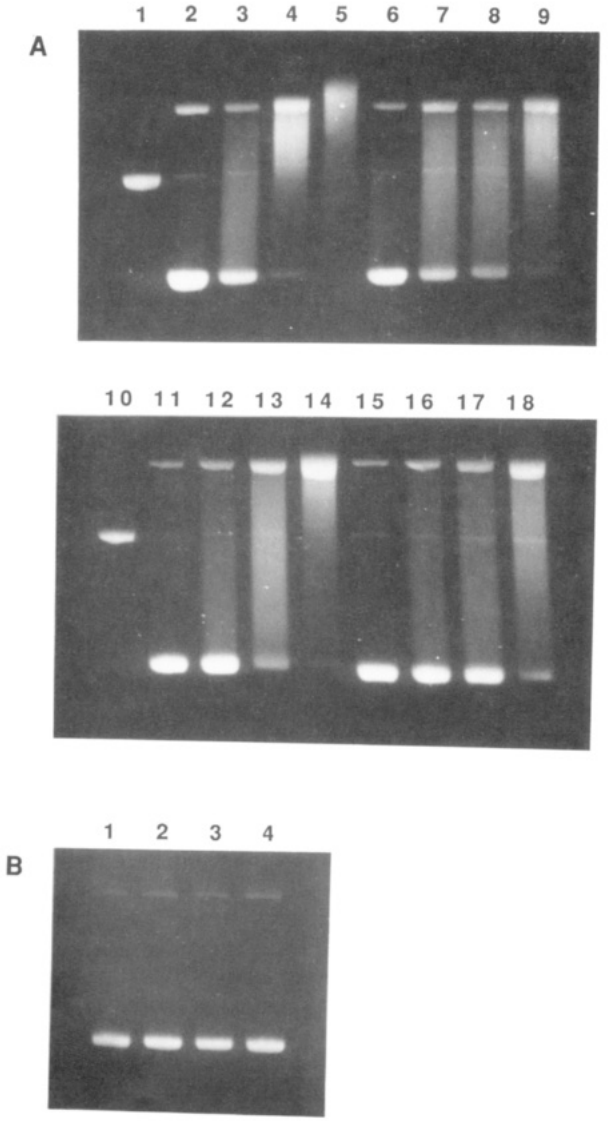

FIGURE 3: Agarose gel electrophoresis of pBR322 DNA treated with **1** and its related compounds. (A) The samples were electrophoresed on 1.2% agarose (lanes 1–9) or 1.2% agarose containing 2 μg/mL EtBr (lanes 10–18). Lanes 1 and 10, pBR322 linear DNA; lanes 2, 6, 11, and 15, pBR322 DNA alone; lanes 3–5 and 12–14, pBR322 DNA plus **1** at 1, 10, and 100 μM; lanes 7–9 and 16–18, pBR322 DNA plus **2** at 1, 10, and 100 μM. (B) Lane 1, pBR322 DNA alone; lanes 2–4, pBR322 DNA plus **3** at 25, 100, and 200 μM, respectively.

anywhere from the positions of supercoiled to open circled DNA. It is also to be noted that the open circled DNA ran at a form II position in the presence of EtBr, indicating that open circled DNA induced by **1** is nicked form II DNA. From these results, it is concluded that **1** binds and causes single-strand breaks of pBR322 plasmid DNA.

Reaction of 13-Decarboxy- and 14,16-Deoxy-14,16-dihydroxykapurimycin A3 (**2** *and* **3**) *with pBR322 Plasmid DNA.* Toward understanding the nature of the covalent binding and cleaving activity of **1**, two derivatives of kapurimycin A3 were examined for their effect on plasmid DNA. 13-Decarboxykapurimycin A3 (**2**), which is formed by thermal decarboxylation of the β,γ-unsaturated δ-keto carboxylic acid group,[2] is less cytotoxic than **1**. 14,16-Deoxy-14,16-dihydroxykapurimycin A3 (**3**) is inactive in cytotoxicity tests on cultured cells; viz., the IC_{50} against BALB 3T3 cells transformed by the H-*ras* gene was more than 25 μg/mL, while those for **1** and **2** were 0.1 μg/mL and 4.5 μg/mL, respectively. Consistent with its weak cytotoxic activity, **3** exhibited no DNA cleaving activity, nor did it induce the change in electrophoretic mobility of pBR322 plasmid DNA (Figure 3B). As shown in Figure 3A, **2** caused single-strand scission of pBR322 plasmid DNA, but the extent of strand breakage was less than that of **1**. As noted in the former section, the covalently closed open circled DNA because positively twisted so as to run at a supercoiled position in an agarose gel containing EtBr, and thereby one can readily distinguish nicked form II DNA from relaxed. Therefore, quantitation of the amount of DNA was performed for the data obtained by an agarose gel supplemented with EtBr. As shown in Figure 4, the drug concentrations required for disappearance of 50% of form I DNA are 2.5 and 30 μM for **1** and **2**, respectively. The drug concentrations necessary for 40% accumulation of form II DNA are 10 and 100 μM for **1** and **2**, respectively. Thus, the DNA cleaving activity of **2** is less than approximately 10% of **1**. All of the above data indicate that the presence of the epoxide group is necessary for the interaction of **1** with DNA, and the β,γ-unsaturated δ-keto carboxylic acid moiety also contributes to the DNA cleaving ability.

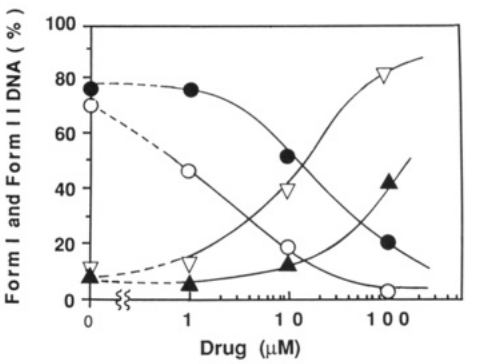

FIGURE 4: Quantification of the degree of strand scission in the conversion of form I to form II DNA. The amount of form I and form II DNA in an agarose gel containing EtBr (data shown in lanes 11–18 in Figure 3A) was quantitated as described under Experimental Procedures. Symbols are as follows: form I DNA in the presence of **1** (○) and **2** (●); form II DNA in the presence of **1** (▽) and **2** (▲).

Interaction of **1** *and* **2** *with Calf Thymus DNA.* As described in the former section, the changes in the electrophoretic mobility of form I as well as form II plasmid DNA and a smear from form I to form II position induced by **1** are thought to be due to covalent binding of the drug to DNA. To explore this observation further, we examined the interaction of **1** with calf thymus DNA. Various amounts of **1** were incubated with calf thymus DNA at 4 °C and subjected to HPLC analysis, as described under Experimental Procedures. A typical HPLC elution profile is shown in Figure 5C. After 5 days of incubation, almost all of **1** had disappeared and a new peak with the same UV-absorbing component as **1** appeared with a retention time of 11 min. Spectroscopic analysis of this compound identified it as 14,16-deoxy-14,16-dihydroxykapurimycin A3 (**3**). Antibacterial and cytotoxic activities for **3** have been described in the former section. As for **2**, however, the corresponding 14,16-deoxy-14,16-dihydroxy derivative was not obtained under the same conditions as was **3**. Calf thymus DNA treated with **1** was recovered by ethanol precipitation and analyzed by HPLC (Figure 5B). As shown in Figure 6B, it gave rise to an adduct with a UV absorption

[2] The mechanism of thermal decarboxylation of the β,γ-unsaturated δ-keto carboxylic acid moiety is not fully understand at the present stage but is supposed to be analogous to that for decarboxylation of the β,γ-unsaturated acid (malonic acid), that is, a cyclic process of elimination in which hydrogen bonding plays an important role.

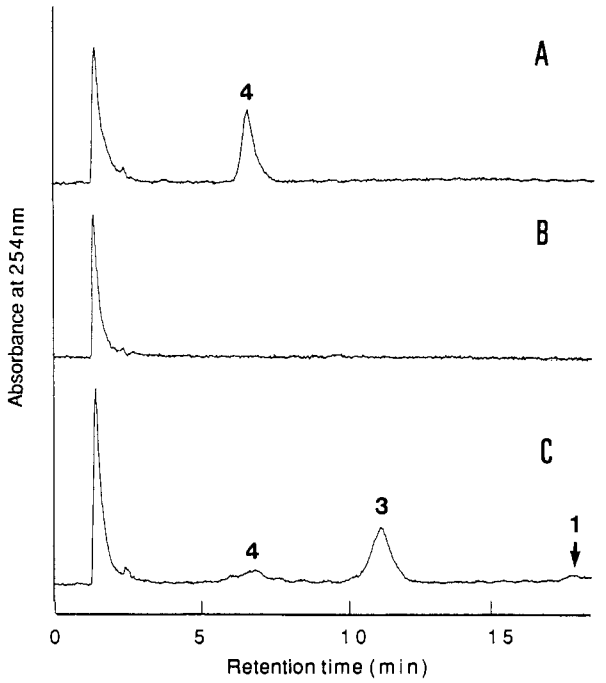

FIGURE 5: HPLC pattern for **1** incubated with calf thymus DNA. (A) **1**–DNA complex incubated at 55 °C for 3 h. (B) **1**–DNA complex recovered by ethanol precipitation. (C) **1** incubated with calf thymus DNA at 4 °C for 5 days. HPLC was carried out on YMC AM-312, 5 mm (i.d.) × 15 mm, and monitored at 254 nm. The column was developed with MeOH–50 mM phosphate buffer, pH 7 (7:3), at a flow rate of 1 mL/min.

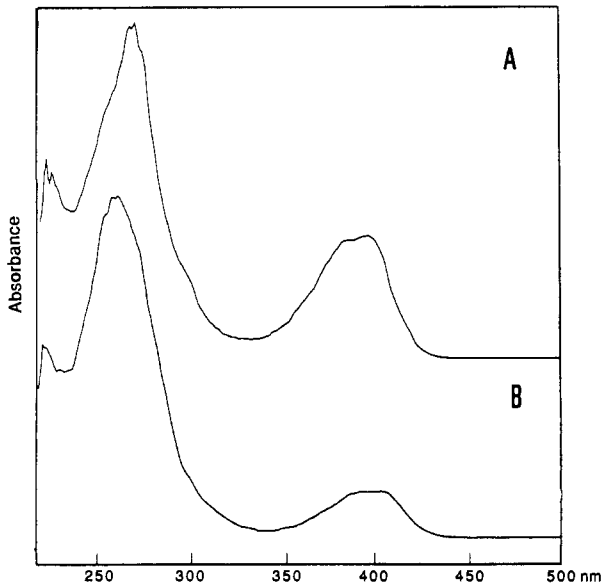

FIGURE 6: UV absorption spectra for **4** (A) and **1** complexed with calf thymus DNA (B). UV spectra were recorded by the multichannel photo-diode-array detector, as described under Experimental Procedures.

maximum at 380 nm in addition to the expected DNA absorption at 259 nm, suggesting the covalent binding to **1** to DNA.

Figure 7 illustrates the binding of **1** and **2** to calf thymus DNA at various drug concentrations. **1** bound to DNA in a dose-dependent manner and reached a maximum of about 1 drug bound per 9 base residues with an input ratio of 1 drug per 1 base residue in the reaction. On the other hand, **2** bound to calf thymus DNA to a lesser extent and reached saturation at about 1 drug bound per 22 base residues with an input ratio of 1 drug per 1 base residue. It therefore seems possible that the reduced binding of **2** to DNA could be responsible for its

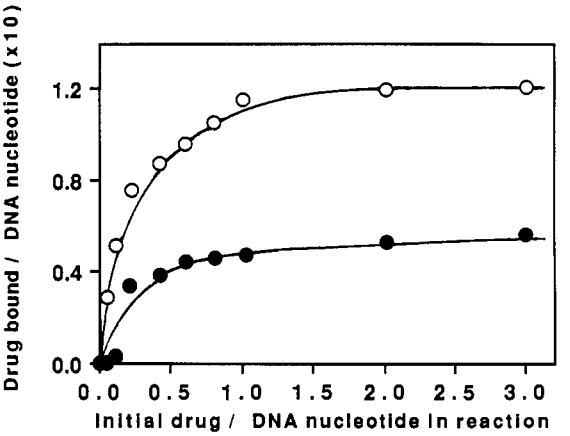

FIGURE 7: Binding of drugs to calf thymus DNA at various concentrations of **1** (○) and **2** (●). Various amounts of drugs were incubated with 0.096 μmol (DNA nucleotide) of calf thymus DNA at 4 °C for 5 days. DNA recovered by ethanol precipitation was analyzed by HPLC to record UV absorption spectra of the drug–DNA complex. The amount of drug bound to DNA was estimated as described under Experimental Procedures.

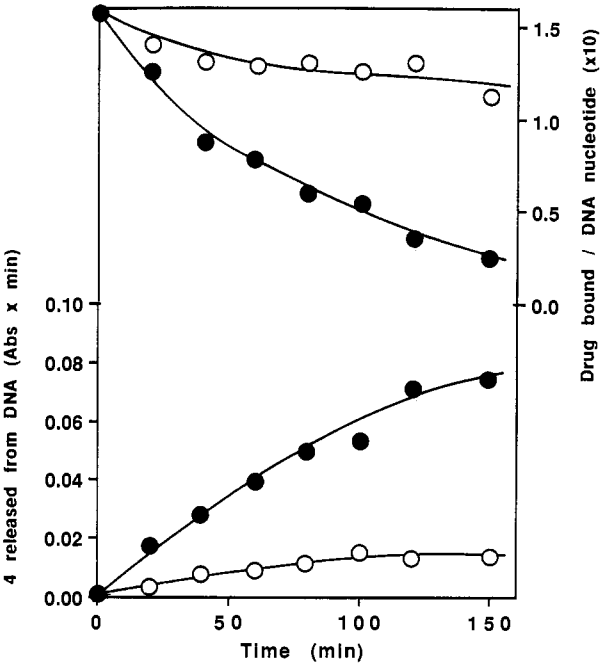

FIGURE 8: Time-dependent release of **4** from the **1**–DNA adduct at 37 (○) or 55 °C (●). The **1**–DNA adduct in 50 mM phosphate buffer, pH 6.7, was incubated at 37 or 55 °C. Sample (30 μL) were taken at the indicated times and subjected to HPLC analysis. The amount of drug remaining bound to DNA and **4** released from the **1**–DNA complex were estimated as described under Experimental Procedures.

diminished biological and DNA cleaving activity.

Thermal Treatment of DNA Modified by **1**. When the drug–DNA adduct was incubated at 37 or 55 °C, peak absorption at 380 nm decreased and one compound (**4**) with UV absorption characteristic of kapurimycins newly appeared (Figures 5A and 6A). Compared to UV absorption maxima of DNA-bound **1**, a slight red shift of peak absorption around 280 nm was observed for **4**. **4** was clearly different from **1**, **2**, or **3** by its retention time on HPLC analysis. It is to be noted that a small but significant amount of **4** was found by the incubation of **1** with calf thymus DNA at 4 °C for 5 days (see Figure 5C). When 0.3 μg of pBR322 DNA was incubated with 50 μM of **1** (an input ratio of 2 drug per 1 base residue) at 4 °C for 5 days, single-strand scission of plasmid DNA occurred (data not shown). The release of **4** from the drug–

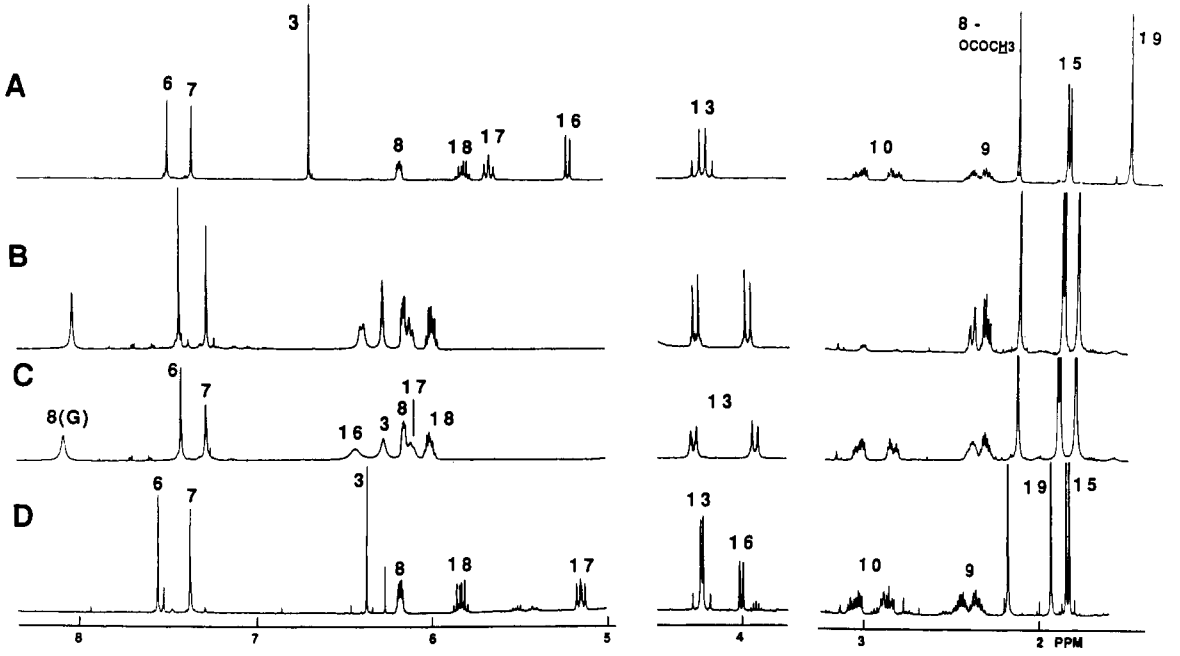

FIGURE 9: 500-MHz ^{1}H NMR spectra for kapurimycin A3 and its related compounds. (A) **3** at 22 °C; (B) **4** at 45 °C; (C) **4** at 22 °C; (D) **1** at 22 °C. Spectra were recorded in methanol-d_4. The assignments of the resonances are included. In spectrum B, the H_{10} resonance was not observed due to the hydrogen–deuterium exchange. H–D exchange of this resonance was also observed for **1**, **2**, and **3** when spectra were recorded 2 days or more after dissolving the sample in methanol-d_4.

DNA complex occurred in a time- and temperature-dependent manner, as shown in Figure 8. The amount of **4**, which was estimated by an area of peak **4** in the HPLC analysis, gradually increased concomitantly with a decrease in DNA-bound **1**. The release of **4** occurred more readily at higher temperatures. These observations indicate that the drug–DNA complex is thermolabile, and so thermal treatment of the complex results in the dissociation of **1** from the complex so as to afford **4**.

Isolation and Characterization of **4**. In order to gain further insight into the mechanisms of DNA binding and breakage by kapurimycin A3, we conducted a large-scale preparation and isolation of **4**, which was used to obtain both ^{1}H and ^{13}C NMR spectra (Figure 9 and Table I). Since the decarboxylation of the β,γ-unsaturate δ-keto carboxylic acid moiety occurs by dissolving **1** in DMSO, NMR spectra were recorded in methanol-d_4. COSY and HETCOR experiments of **4** together with a comparison of the spectra of **1**, **2**, and **3** provided definite assignments of all of ^{1}H and ^{13}C resonances arising from the kapurimycin-derived portion of **4**. In the ^{1}H-NMR spectrum of **4**, all of the resonances attributable to kapurimycin A3 and the additional one resonance at 8.08 ppm were found. The only base residue that gives one nonexchangeable resonance is guanine. Furthermore, the chemical shift value of this resonance showed a close correlation with the guanine H_8 resonance. These data suggest that **4** is an kapurimycin–guanine adduct. This was supported by the secondary ion mass spectrum of **4**, which gave the corresponding molecular ion peak $[M + H]^+$ at m/z 643. As for the ^{13}C resonances attributable to the guanine moiety in **4**, one tentatively assigned C_5 resonance at 109.3 ppm and two unassigned resonances were observed, but the remaining two ^{13}C peaks were not detected, probably due to sizeable broadening of the resonances (Table I). The ^{1}H NMR spectrum of **4** was compared to that of **1**. There is a downfield shift of 2.2 ppm for the H_{16} resonances, 0.86 ppm for the H_{17} resonance, and 0.25 ppm for the H_{18} resonance. While the 2.2 ppm downfield shift of the H_{16} resonance was larger than that expected, the anisotropic effect of the guanine base residue may explain the observed changes in chemical shift in this resonance upon adduct formation. In 2D NOESY experiments, four NOE cross peaks were observed connecting the guanine H_8 proton with the protons (H_{16}, H_{17}, and H_{18}) in the pyrone side chain and one proton (H_3) in the pyrone ring. These results indicate that guanine locates in close proximity to the pyrone side chain. The change in chemical shifts of the drug resonances upon formation of an adduct were

Table I: ^{13}C NMR Assignments for Kapurimycin A3 (**1**) and the Kapurimycin–Guanine Adduct (**4**)[a]

carbon atom	1	3	4
2	166.6 (s)	173.7 (s)	171.0 (s)
3	111.5 (d)	111.4 (d)	111.2 (d) br
4	181.0 (s)	181.6 (s)	181.0 (s)
4a	120.9 (s)	121.1 (s)	120.8 (s)
5	138.2 (s)	140.9 (s)[b]	141.2 (s)[b]
6	129.7 (d)	128.8 (d)	128.6 (d)
6a	141.9 (s)	141.2 (s)[b]	141.3 (s)[b]
7	118.1 (d)	118.3 (d)	118.0 (d) br
7a	141.9 (s)	142.0 (s)	142.0 (s)
8	70.7 (d)	70.6 (d)	70.7 (d)
9	28.7 (t)	28.8 (t)	28.7 (t)
10	35.2 (t)	35.2 (t)	35.2 (t)
11	205.7 (s)	205.8 (s)	205.5 (s)
11a	113.0 (s)	112.6 (s)	112.7 (s)
12	165.5 (d)	165.7 (s)	166.0 (d)
12a	114.7 (s)	114.6 (s)	114.7 (s)
12b	158.9 (s)	158.6 (s)	158.6 (s)
13	42.7 (t)	42.9 (t)	45.6 (t)
13-*C*OOH	175.5 (s)	178.0 (s)	178.9 (s)
14	62.2 (s)	77.5 (s)	76.3 (s) br
15	19.8 (q)	23.3 (q)	24.1 (q) br
16	62.9 (d)	70.9 (d)	60.5 (d) br
17	124.1 (d)	129.4 (d)[b]	125.1 (d) br
18	134.7 (d)	129.5 (d)[b]	132.9 (d)
19	13.7 (q)	13.7 (q)	13.8 (q)
8-O*C*OCH$_3$	172.2 (s)	172.0 (s)	172.0 (s)
8-OCO*C*H$_3$	21.1 (q)	21.1 (q)	21.1 (q)
guanine C_5			109.3 (s)
			154.6 (s)
			144.7 (s)

[a] Spectra (125 MHz) were obtained in methanol-d_4. br: sizable broadening of the resonances was observed. Abbreviations: s, singlet, d; doublet. t; triplet, q; quartet. [b] Assignments may be reversed.

in accord with a structure resulting from opening of the epoxide ring. The alternative mode of epoxide ring opening, which would result in attachment of C_{14} of **1** to guanine, was eliminated by consideration of the ^{13}C chemical shift value of C_{14} in **4**. Comparison of ^{13}C NMR spectra of **4** with **3** revealed that the C_{16} resonance of **4** exhibited a 10 ppm upfield shift, while only small changes were observed for the other resonances. This finding is consistent with the lack of DNA binding and cleaving actiity for **3** and thereby confirmed the essential role of the epoxide for the activity of **1**.

Discussion

The results reported in this paper demonstrate a covalent modification and single-strand scission of DNA by **1** in vitro. The diminished ability to react with DNA for **2** as well as lack of this activity for **3** are consistent with their cytotoxic activity, suggesting that antitumor activity of **1** is most likely a direct result of binding and single-strand scission of DNA. These results are consistent with our previous observation for strong activity of kapurimycin A3 against the recombination-deficient bacterial strains (Hara et al., 1990b). The examination of the molecular nature of the covalent interaction between **1** and DNA revealed that **1** binds to guanine at the C_{16} position of the anthrapyrone side chain. Thus, the epoxide is the alkylation group on **1**, and this is the reason why the 14,16-dihydroxy derivative (**3**) is inactive.

Thermal treatment of the **1**–DNA adduct leads to release of a guanine covalently attached to kapurimycin A3 moiety (**4**). A small but significant amount of **4** was released even at 37 °C and thus is likely to be associated with single-strand scission observed in agarose gel electrophoresis of pBR322 treated with **1**. One would then expect the pathway of degradation of alkylated DNA to be via apurinic (AP) site; i.e., thermal treatment of the **1**–DNA adduct results in depurination and subsequent hydrolysis of the AP site, leading to single-strand scission. Although this interpretation remains the best working hypothesis, it can be proved conclusive by sequencing experiments, which distinguish breaks at AP sites produced by depurination from those produced by the other mechanisms. These are open to further investigation.

Several DNA-damaging agents produce AP sites (Lown & MacLaughlin, 1979a,b; Bose et al., 1980; Povirk & Goldberg, 1985). Among these compounds, CC-1065 is well-known to form a covalent adduct with DNA through N_3 of adenine (Hurley et al., 1984; Reynolds et al., 1985; Swenson et al., 1982). The AP sites produced as a result of thermal treatment of the CC-1065–(N_3-adenine)–DNA adduct are accompanied by strand breakage in close proximity to the covalent binding site. Temperature dependence of CC-1065-induced DNA strand breaks revealed that significant strand breakage occurs only at temperatures above 70 °C. Compared with the above temperature-dependent features of CC-1065-induced DNA strand breaks, covalent adducts of **1** to DNA are much more thermolabile so that extensive DNA strand breaks occur even at physiologically relevant temperature of 37 °C. These labile features of the **1**–DNA adduct, which is liable to produce DNA breaks, are characteristic of kapurimycin A3.

The chemical shift of ^1H and ^{13}C resonances at position 16 of **4** suggests that the binding site of the base moiety is at one of the nitrogen atoms of guanine. Two nitrogen atoms in guanine are reported to be the sites of alkylation by antitumor agents. Alkylation of DNA by the antitumor antibiotics anthramycin and mitomycin C takes place at the 2-NH_2 position (Tomasz et al., 1984, 1987; Petrusek et al., 1981; Graves et al., 1984, 1985). However, depurination and extensive DNA breakage by these antibiotics have not been reported. 7-Alkylguanine is identified for alkylating agents, such as nitrosourea (Gombar et al., 1980; Tong & Ludlum, 1981; Hsiung et al., 1976). The alkylation as well as subsequent depurination and strand cleavage at the AP site is known to occur. Along these lines, a possible alkylation site of guanine by **1** might by N_7 of guanine. N_3 in guanine, which also has a lone pair of electrons in a σ orbital in the plane of the ring, could be the site of potential alkylation by **1**. As can be seen in Figure 9 and Table I, several ^1H and ^{13}C resonances of **1** experience progressive broadening upon formation of an adduct with guanine.[3] Long-range ^{13}C–^1H coupling data were not obtained because of this sizable braodening of resonances and therefore hampered the unequivocal assignment of the nitrogen atom of guanine covalently attached to kapurimycin A3. Other efficient physicochemical approaches are necessary for determination of the alkylation site.

The results of the DNA cleaving and cytotoxic activity for **2** compared to **1** establish the importance of the β,γ-unsaturated δ-keto carboxylic acid moiety. The diminished DNA cleaving activity as well as cytotoxic activity for **2** appear to be the result of its reduced DNA binding activity. Since the decarboxylated species **2** binds DNA and induces single-strand breaks, the carboxyl group is not required for covalent binding and cleavage of DNA but appears to facilitate the interaction of drug with DNA. As was expected from the previous results for decarboxylation of **1** in DMSO solution (Yoshida et al., 1990), the decarboxylation from the β,γ-unsaturated δ-keto carboxylic acid moiety of **4** immediately occurred. The resulting decarboxylated derivative of **4** is labile and degraded, resulting in a complex NMR spectrum within 1 h (data not shown). Since the ^{13}C chemical shift value of the resonance of the carboxyl carbon atom is in accord with that for carboxylate, we would have the following implication that the drugs might be held to the DNA by ionic bonding between carboxylate anion and a certain part of the guanine. This appears one of the reasons why the 13-decarboxylated derivative of **4** is unstable and degrades rapidly. However, these arguments are indirect, and it is safe to say that covalent bonding together with certain noncovalent interaction prior to covalent bond formation between kapurimycin and DNA is necessary for the formation of the **1**–DNA adduct. The present study suggests that synthesis of small molecules which contain both the β,γ-unsaturated δ-keto carboxylic acid and the epoxide groups might provide a novel approach for the design of new DNA-damaging cancer chemotherapeutic agents.

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[3] Broadening of resonances was most prominent for those arising carbon and proton atoms which are thought to be located in close proximity to guanine, such as H_3, H_{15}, H_{16}, H_{17}, C_3, C_{14}, C_{15}, C_{16}, and C_{19}. The line widths of these resonances decreased when the temperature was increased from 28 to 45 °C (Figure 9B). It is therefore likely that the structural portion of kapurimycin A3 adjacent to guanine moiety adopts several conformations which differ in their chemical shift on NMR, and the chemical exchange among these conformers is intermediate so that broad resonances were observed.

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Formation and Stability of Repairable Pyrimidine Photohydrates in DNA†

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ABSTRACT: Ultraviolet irradiation of poly(dG-dC) and poly(dA-dU) in solution produces pyrimidine hydrates that are repaired by bacterial and mammalian DNA glycosylases [Boorstein et al. (1989) *Biochemistry 28*, 6164–6170]. *Escherichia coli* endonuclease III was used to quantitate the formation and stability of these hydrates in the double-stranded alternating copolymers poly(dG-dC) and poly(dA-dU). When poly(dG-dC) was irradiated with 100 kJ/m^2 of 254-nm light at pH 8.0, 2.2% of the cytosine residues were converted to cytosine hydrate (6-hydroxy-5,6-dihydrocytosine) while 0.09% were converted to uracil hydrate (6-hydroxy-5,6-dihydrouracil). To measure the stability of these products, poly(dG-dC) was incubated in solution for up to 24 h after UV irradiation. Cytosine hydrate was stable at 4 °C and decayed at 25, 37, and 55 °C with half-lives of 75, 25, and 6 h. Uracil hydrate produced in irradiated poly(dA-dU) was stable at 4 °C and at 25 °C and decayed with a half-life of 6 h at 37 °C and less than 0.5 h at 55 °C. Uracil hydrate and uracil were also formed in irradiated poly(dG-dC). These experiments demonstrate that UV-induced cytosine hydrate may persist in DNA for prolonged time periods and also undergo deamination to uracil hydrate, which in turn undergoes dehydration to yield uracil. The formation and stability of these photoproducts in DNA may have promoted the evolutionary development of the repair enzyme endonuclease III and analogous DNA glycosylase/endonuclease activities of higher organisms, as well as the development of uracil–DNA glycosylase.

The mutagenicity and carcinogenicity of ultraviolet (UV) radiation have been attributed to the effects of base modifications formed in DNA as a consequence of such radiation (Witkin, 1976; Harm, 1980; Hall & Mount, 1981; Hutchinson, 1987). The most readily formed UV-induced base modifications are the cyclobutane pyrimidine dimers and the pyrimidine 6–4 photoproducts (Setlow & Carrier, 1963; Fisher & Johns, 1976; Patrick & Rahn, 1976; Franklin & Haseltine, 1986; Mitchell & Mairn, 1989). However, all the biologic consequences of UV irradiation cannot result from the effects of these lesions. In particular, mutations formed at cytosine residues (Brash & Haseltine, 1982; Lebkowski et al., 1985; Glazer et al., 1986; Schaaper et al., 1987; Brash et al., 1987) that are not adjacent to other pyrimidines cannot be ascribed to either of the dimeric photoproducts. Instead, these mutations might result from the UV-induced modification of single cytosine residues.

The most readily formed UV-induced modification of single cytosine residues is the photohydrate resulting from addition of water to the 5,6 double bond (Kochetkov & Budovskii, 1972; Fisher & Johns, 1976). UV-induced formation of this mod-

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