40 μL of this solution was used for each 1-mL kinetic run. The solvent for all experiments is thus 4% ethanol/96% water, by volume. Exact conditions for experiments are listed in the tables and figure legends.

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Registry No. GSH, 70-18-8; DL-DTT, 27565-41-9; CDNB, 97-00-7; GSH transferase, 50812-37-8.

Bleomycin-Mediated Degradation of Aristeromycin-Containing DNA. Novel Dehydrogenation Activity of Iron^{II}–Bleomycin

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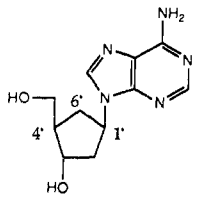
Abstract: The antitumor antibiotic bleomycin (BLM) induces C–H bond scission at the C4′ of deoxyribose moiety of DNA in the presence of Fe(II) and O_2. To investigate the primary oxidation step, 2′-deoxyaristeromycin (Ar) possessing a cyclopentane ring instead of a ribofuranose ring was incorporated into the BLM-cleaving site of synthetic oligonucleotide d(GGArAGG). It was found that an unprecedented dehydrogenation occurs effectively at the C4′ and C6′ positions of the Ar moiety to give 2′-deoxyneplanocin A containing oligonucleotide together with a minor but stereospecific C4′ hydroxylation in the Fe^{II}–BLM-mediated degradation of duplex d(GGArAGG)–d(CCTTCC). An intermediate C4′ carbocation derived from one-electron oxidation of initially formed C4′ radical has been proposed for the dehydrogenation reaction.

While various types of site specifically modified oligonucleotides have been used as a probe for DNA–ligand interactions, e.g., DNA–protein and DNA–drug interactions,[1] the use of such modified oligonucleotides as a probe for the mechanism of DNA degradation by antitumor antibiotics has attracted only little attention. Antitumor antibiotic bleomycins (BLMs) are known to induce C–H bond scission at the C4′ of the deoxyribose moiety of DNA in the presence of Fe(II) and O_2.[2] Under aerobic conditions, O_2-dependent products such as base propenals and 5′-phosphate and 3′-phosphoglycolate termini are produced as the major products,[3] whereas at limiting O_2 concentrations C4′ hydroxylation occurs preponderantly to give alkali-labile products with concomitant release of free bases.[3,4] While the structures of these ultimate degradation products have been well established,[3b,5] the exact nature of the initial step of the C4′ oxidation is still a matter of debate.[2,6] One major difficulty in getting closer insight into the mechanism of the primary oxidation step is due to the inherent instability of the initial product of hemiketal structure derived from deoxyribose oxidation (Scheme I).

We reasoned that if 2′-deoxyaristeromycin (Ar, **1**), a carbocyclic analogue of 2′-deoxyadenosine possessing a cyclopentane ring instead of a ribofuranose ring, is incorporated into the BLM-



2'-deoxyaristeromycin (1)

cleaving site of a synthetic oligonucleotide, such modified oligonucleotide would then serve as a useful probe for investigating the primary oxidation step, e.g., the stereochemical outcome of C4′ hydroxylation of the deoxyribose moiety. Such an Ar-containing oligonucleotide would also serve as a useful probe for an O_2-labeling experiment due to the lack of the exchange of functional groups of sugar oxidation products with solvent water, which is inevitable in the oxidation of normal DNA.[3c,7]

We describe here our results on the degradation of an Ar-containing deoxyhexanucleotide induced by Fe^{II}–peplomycin

[†] Kyoto University.
[‡] Takeda Chemical Industries, Ltd.

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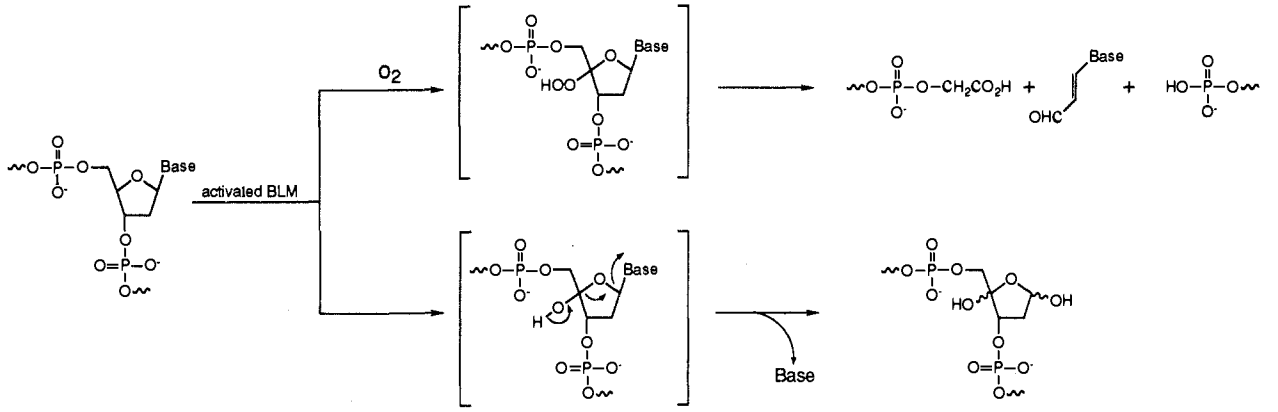
Scheme I



(PEM), a derivative of naturally occurring BLMs (Figure 1). Careful product analysis of the reaction of heteroduplex d(GGArAGG)–d(CCTTCC) with Fe^{II}–PEM revealed that an unprecedented dehydrogenation occurs effectively at the C4′ and C6′ positions of the Ar moiety together with a minor but stereospecific C4′ hydroxylation at the same site.

Results and Discussion

Since iron–BLM is known to cleave double-strand DNA preferentially at 5′-GC, 5′-GT, and 5′-GA sites,[8] we selected heteroduplex d($G_1G_2Ar_3A_4G_5G_6$)–d(CCTTCC) as a BLM substrate in which Ar was incorporated into the 3′-side of 2′-deoxyguanosine$_2$ (G_2). In a preliminary control experiment using parent duplex d(GGAAGG)–d(CCTTCC), we confirmed that the cleavage at the 5′-GA site amounts to 40% of the total event occurring in the Fe^{II}–BLM-mediated modification. Thus, we synthesized the Ar-containing hexamer d(GGArAGG) by a conventional phosphoramidite method.[9] Since the Ar moiety contained in DNA has been reported to behave like 2′-deoxyadenosine in DNA replication of the host cell,[10] conformational difference between Ar and 2′-deoxyadenosine in duplex DNA would not be so significant. Melting temperature experiments also indicated that d(GGArAGG)–d(CCTTCC) (T_m 23.8 °C at 0.034 mM base concentration) forms a duplex with a stability comparable with that of d(GGAAGG)–d(CCTTCC) (T_m 27 °C at 0.036 mM base concentration). It is therefore very likely that the adenine base of the Ar moiety forms a base pairing with a complementary thymine base.

Heteroduplex d(GGArAGG)–d(CCTTCC) thus prepared was oxidized with PEM in the presence of Fe(II) and O_2. Direct HPLC analysis of the reaction mixture indicated that only the d(GGArAGG) strand was selectively oxidized with formation of a major product eluting at 15.2 min as illustrated in Figure 2. The product was separated by HPLC and subjected to enzymatic digestion with snake venom phosphodiesterase (sv PDE) and alkaline phosphatase (AP). HPLC analysis of the hydrolysate indicated the presence of two major peaks eluted at 13.4 and 19.2 min (Figure 3). The peak at 13.4 min was 2′-deoxyguanosine, whereas the latter peak consisted of 2′-deoxyadenosine and an unknown product. Attempted separation of these two components by preparative HPLC under several conditions was unsuccessful. Accordingly, the latter peak was subjected to acid treatment (0.1 N HCl, 90 °C, 5 min). By this procedure, 2′-deoxyadenosine was converted to adenine and roughly half of the peak remained unchanged (Figure 4b), suggesting that the acid-insensitive unknown product is derived from the oxidation of the Ar moiety of d(GGArAGG).

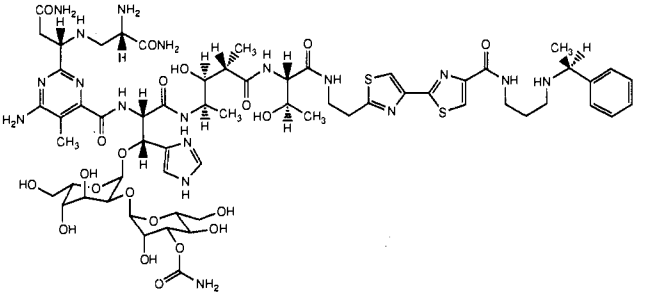


Figure 1. Structure of peplomycin (PEM).

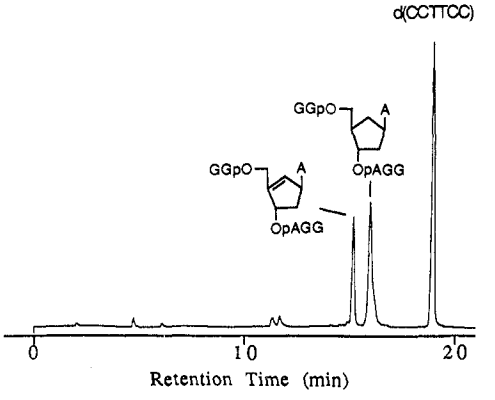


Figure 2. HPLC analysis of Fe^{II}–PEM-treated d(GGArAGG)–d(CCTTCC). The reaction mixture was incubated at 0 °C for 15 min under aerobic conditions and analyzed immediately by HPLC on a Cosmosil 5C_{18} ODS column. Elution was with 0.05 M ammonium formate containing 0–10% acetonitrile; linear gradient, 20 min, at a flow rate of 1.5 mL/min.

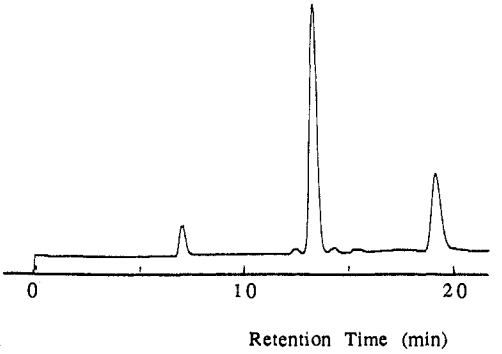


Figure 3. HPLC analysis of the enzymatic digestion of the peak at 15.2 min. The peak eluting at 13.4 min was 2′-deoxyguanosine. The peak at 19.2 min was a mixture of 2′-deoxyadenosine and **5**. HPLC conditions: Cosmosil 5C_{18} ODS column; 0.05 M ammonium formate containing 0–10% acetonitrile; linear gradient, 30 min; flow rate of 1.5 mL/min.

(8) (a) Takeshita, M.; Grollman, A. ref 2, p 207. (b) Takeshita, M.; Grollman, A. P.; Ohtsubo, E.; Ohtsubo, H. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5983. (c) D'Andrea, A. D.; Haseltine, W. A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3608.

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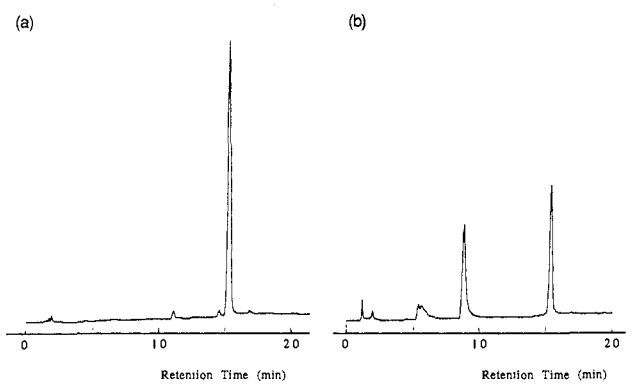


Figure 4. HPLC analysis of the peak at 19.2 min (a) before and (b) after acid treatment (0.1 N HCl, 90 °C, 5 min). HPLC conditions: Cosmosil $5C_{18}$ ODS column; 0.05 M ammonium formate containing 0–10% acetonitrile; linear gradient, 20 min; flow rate of 1.5 mL/min.

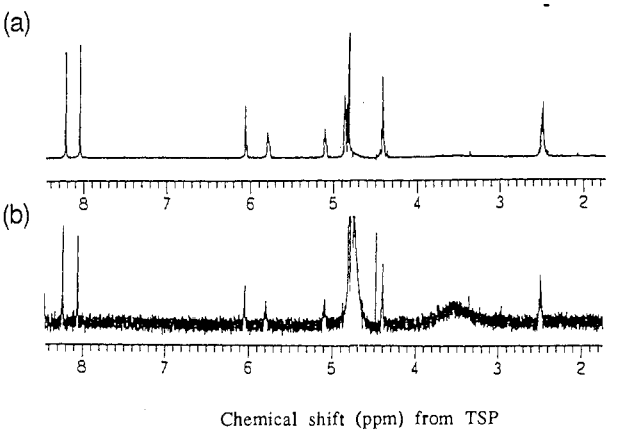
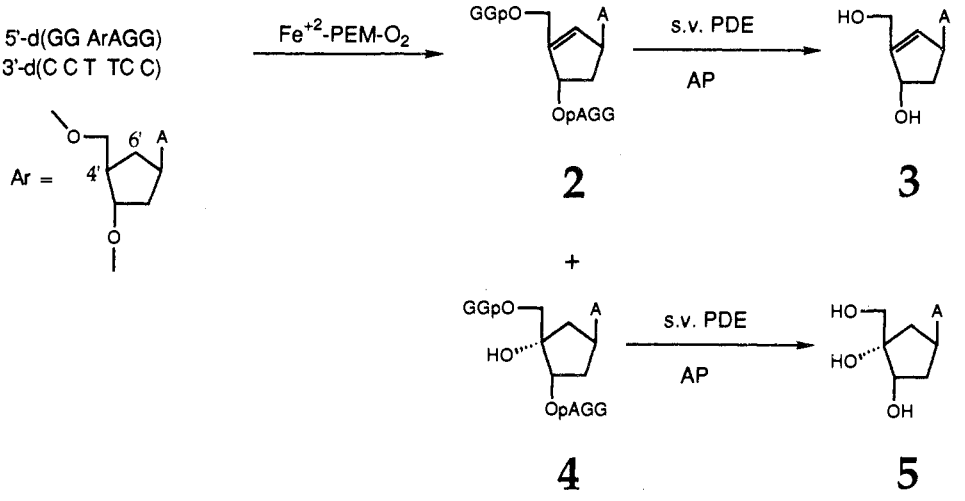
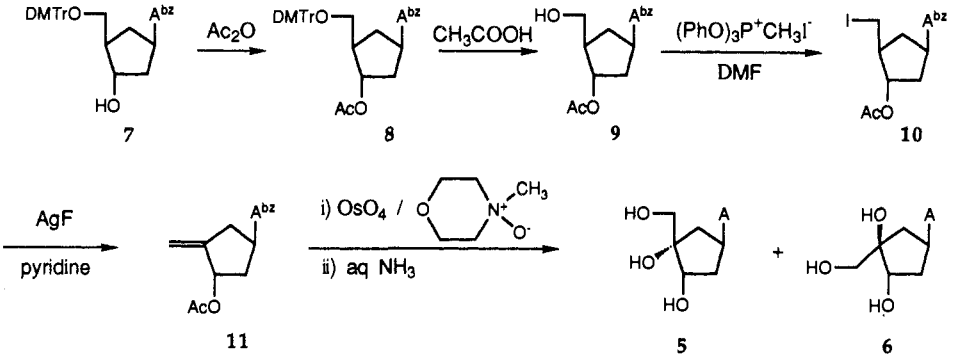


Figure 5. ^{1}H NMR (400 MHz) of 2′-deoxyneplanocin A (**2**) obtained from the Fe^{II}–PEM reaction (b) and the authentic sample (a).

Scheme II



Scheme III



In order to elucidate the structure of the modified residue, a larger scale reaction of d(GGArAGG)–d(CCTTCC) with Fe^{II}–PEM was carried out. The major peak was separated by HPLC and then subjected to enzymatic digestion and subsequent acid treatment as described above. After repeated HPLC purification, the unknown product was obtained in a pure form. A ^{1}H NMR spectrum of the isolated product showed a characteristic olefinic proton at δ 6.05 as a singlet. The product was finally confirmed to be 2′-deoxyneplanocin A (**3**)[11] by comparison of its ^{1}H NMR spectrum (Figure 5) and HPLC profile with those of authentic sample.

These results indicate that dehydrogenation occurs specifically at the C4′ and C6′ positions of the Ar moiety of d(GGArAGG) by action of Fe^{II}–PEM (Scheme II). Interestingly, this dehydrogenation proceeded only in the presence of complementary strand d(CCTTCC). Without the complementary strand, d(GGArAGG) was not oxidized at all by Fe^{II}–PEM, indicating the requirement of a double-strand structure for this reaction. A similar requirement of a double-strand structure has been observed in the cleavage of d(GGAAGG)–d(CCTTCC) and ^{32}P-end-labeled DNA fragments by an Fe^{II}–BLM–O_2 system.[12]

Also expected was the C4′ hydroxylation of the Ar moiety of d(GGArAGG). We therefore synthesized two diastereoisomers of 2′-deoxy-4′-hydroxyaristeromycin, **5** and **6**, and examined their possible formation in the Fe^{II}–PEM-mediated reaction after enzymatic digestion. Authentic samples of **5** and **6** were prepared from *N*-benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxyaristeromycin (**7**)

(11) Fukukawa, K.; Ueda, T.; Hirano, T. *Chem. Pharm. Bull.* **1981**, *29*, 597.

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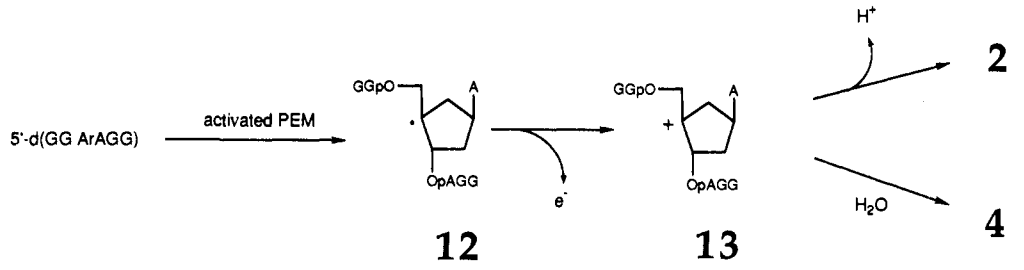
Scheme IV



by the route shown in Scheme III. OsO_4 oxidation of **11** gave a mixture of diastereoisomers **5** and **6** in a 3:7 ratio, which were completely separated by means of HPLC. The 4′*S* configuration of **5** was confirmed by NOE relationship between $H_{3'}$ and $H_{5'}$. Likewise, 4′*R* configuration of **6** was determined by a NOE relationship between $H_{6'\alpha}$ and $H_{5'}$.

After the large-scale reaction of d(GGArAGG)–d(CCTTCC) with Fe^{II}–PEM, the mixture was directly subjected to enzymatic digestion with sv PDE and AP followed by acid treatment. HPLC analysis of the mixture indicated the presence of **3** and unreacted Ar together with adenine, guanine, dC, and dT. In addition, a minor peak corresponding to **5** was observed (Figure 6). The formation of **5** was confirmed by comigration of this peak with synthetic authentic sample on HPLC in several different solvent systems. However, the peak corresponding to **6** was not detected at all. Quantitative analysis of the reaction mixture indicated that 38% (0.64 nmol) of the Ar moiety (1.66 nmol) initially used was consumed to afford **3** (0.60 nmol) and a minor amount of **5** (0.04 nmol). This implies that activated PEM selectively dehydrogenates $H_{4'}$ and $H_{6'}$ of the Ar moiety of d(GGArAGG) to afford 2′-deoxyneplanocin A containing hexamer **2** in 94% yield based on consumed hexamer, whereas C4′ hydroxylation giving **4** is only a minor path.

The possibility that **3** may arise from dehydration of initially formed **5** during reaction or the workup process was unambiguously excluded by careful control experiments. For instance, treatment of **5** with 0.1 N HCl (90 °C, 1 h) never produced **3** with quantitative recovery of **5**, whereas **5** was quite stable upon treatment with 0.1 N NaOH (90 °C, 5 min). In addition, the formation of dehydrogenated oligomer **2** was clearly obsevable by HPLC even at the initial stage of the Fe^{II}–PEM-mediated reaction. Thus, the dehydrogenated oligomer **2** should arise from a direct attack of activated PEM on the Ar moiety of d(GGArAGG). To our knowledge, this is the first example of the dehydrogenation activity observed for BLM–metal complexes.[2] Such a dehydrogenation reaction of saturated hydrocarbons has not been reported for P-450 model systems as well.[13] Of special interest is that the product ratio of **3** to **5** (96:4) was not changed by changing the O_2 concentration from atmospheric to 20 atm pressure. Formation of O_2-incorporated products has not been observed even at 20 atm O_2 pressure. These results strongly suggest that both **3** and **5** are derived from a common precursor via an O_2-independent process.

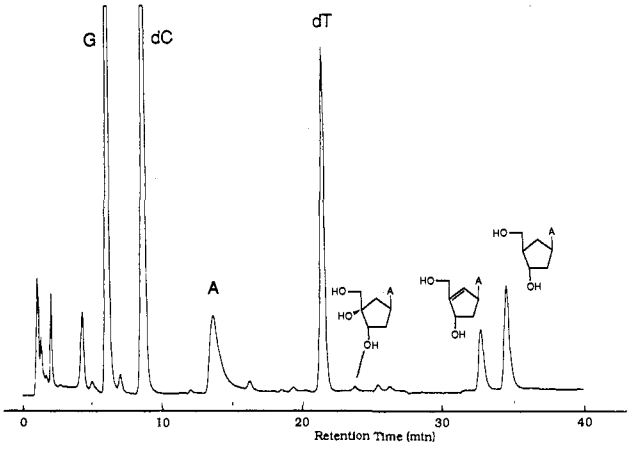


Figure 6. HPLC analysis of the enzymatic digestion of Fe^{II}–PEM-treated d(GGArAGG)–d(CCTTCC). The same reaction mixture (total volume 20 μL) described in Figure 2 was subjected to enzymatic digestion with sv PDE and AP. The resulting hydrolysate was analyzed by HPLC on a Cosmosil 5C_{18} ODS column. Elution was with 0.05 M ammonium formate containg 0–6% acetonitrile; linear gradient, 40 min, at a flow rate of 1.5 mL/min.

Formation of **2** from d(GGArAGG) apparently involves two-electron oxidation. A reasonable mechanism to accommodate the formation of both **2** and **4** is provided in Scheme IV, wherein C4′ carbocation intermediate **13** derived from one-electron oxidation of initially formed C4′ radical **12** has been proposed. Since an analogous regioselective proton loss has precedent in carbocation chemistry,[14] exclusive formation of **2** from carbocation **13** via selective proton loss from the C6′ position is easily understandable. The formation of **4** as a minor product (4% based on consumed hexamer) is assumed to arise from the trapping of cation **13** with water, although attempts to prove this possibility by an $H_2{}^{18}O$-labeling experiment have been fruitless because of the extremely low isolated yield of pure **5**. Steric constraint imposed by duplex formation would probably be relevant to such an exclusive formation of 4′*S* isomer **5**.

While the exact nature of the activated BLM is still a matter of debate,[6] recent studies[2b,6a-g] based on the analogy with various heme model systems and with cytochrome P-450 have strongly suggested that a high-valent iron–oxo species such as heterolytically activated perferryl (BLM·Fe^{V}=O) is responsible for the oxidative DNA cleavage reaction. Thus, it seems likely that the species responsible for one-electron oxidation of C4′ radical **12** would be Fe^{4+}—OH resulting from hydrogen abstraction by activated BLM (BLM·Fe^{V}=O) or a second molecule of activated BLM. In this context, it has recently been reported that solvent $H_2{}^{18}O$ is incorporated into the 4′-keto moiety of the alkali-labile product derived from BLM-treated d(CGCGCG), suggesting the carbocation intermediate for the C4′ hydroxylation of deoxyribose moiety.[7a] Insensitivity of the product ratio (3:5) to oxygen pressure observed here is also consistent with the carbocation precursor. If **5** is derived from the oxygenation of C4′ radical **12**, then the ratio should depend on oxygen pressure. This was not the case. Our results do not, however, preclude other possibilities, e.g., a concerted mechanism in which C4′ hydrogen abstraction and $H_{6'}$ elimination occur simultaneously by the action of activated BLM. The results obtained in this study indicate that the oxidation of the cyclopentane ring of the Ar moiety by activated iron–BLM is mechanistically discrete from the oxidation by cytochrome P-450 model systems, where the radical recombination (rebound) mechanism has been generally accepted.[15]

In summary, we have demonstrated for the first time a novel dehydrogenation activity of activated iron–BLM. Furthermore, the present work has demonstrated that modified oligonucleotides

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(14) (a) Olah, G. A. *Carbocations and Electrophilic Reactions*; Wiley: New York, 1974. (b) Rudolph, A.; Weedon, A. C. *J. Am. Chem. Soc.* **1989**, *111*, 8756.

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containing the 2′-deoxyaristeromycin residue at cleaving sites can serve as a powerful probe for the mechanism of oxidative DNA degradation by other DNA-damaging agents.

Experimental Section

Materials and Methods. Peplomycin sulfate was obtained from Nippon Kayaku CO., Ltd. through the courtesy of Dr. T. Takita. Calf intestine alkaline phosphatase (AP) (1000 units/mL) and snake venom phosphodiesterase (sv PDE) (3 units/mL) were purchased from Boehringer Mannheim. Sep-Pak C18 cartridges were purchased from Waters. An authentic sample of 2′-deoxyneplanocin A was obtained from Toyo Jozo Corp. Aristeromycin was obtained from Takeda Chemical Industries, Ltd. *N*-Benzoyl-2′-deoxyaristeromycin and *N*-benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxyariteromycin (**7**) were prepared according to the published procedure.[10,16] *N*-Benzoyl-2′-deoxyaristeromycin: ^{1}H NMR (CD_3OD) δ 1.88–2.05 (m, 1 H), 2.11–2.33 (m, 2 H), 2.40–2.64 (m, 2 H), 3.62–3.80 (m, 2 H), 4.28–4.37 (m, 1 H), 5.15–5.33 (m, 1 H), 7.39–7.68 (m, 3 H), 7.97–8.12 (m, 2 H), 8.50 (s, 1 H), 8.69 (s, 1 H). **7**: ^{1}H NMR ($CDCl_3$) δ 1.82–2.01 (m, 1 H), 2.25–2.58 (m, 4 H), 3.19–3.31 (m, 1 H), 3.39–3.48 (m, 1 H), 3.82 (s, 6 H), 4.43–4.54 (m, 1 H), 5.09–5.28 (m, 1 H), 6.82–6.91 (m, 4 H), 7.21–7.68 (m, 12 H), 7.99 (s, 1 H), 8.01–8.10 (m, 2 H), 8.76 (s, 1 H), 9.02 (br s, 1 H).

2-Cyanoethyl phosphoramidite of **7** was prepared by the procedure of van Boom[9] and was directly applied to an automated DNA synthesizer without further purification. Deoxyhexanucleotides d(GGArAGG), d(GGAAGG), and d(CCTTCC) were synthesized by an ABI 381A DNA synthesizer, and their concentrations were determined by complete digestion with sv PDE and AP to corresponding 2′-deoxymononucleosides. ^{1}H NMR spectra were recorded on a JEOL JNM-GX400 spectrometer.

Synthesis of *N*-Benzoyl-3′-*O*-acetyl-2′,5′-dideoxy-5′-iodoaristeromycin (10). *N*-Benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxyaristeromycin (**7**; 228 mg, 0.35 mmol) was dissolved in 20 mL of pyridine. To this solution were added acetic anhydride (71 mg, 0.7 mmol) and 4-(dimethylamino)pyridine (4.2 mg, 0.34 mmol), and the resultant solution was stirred for 1 h at room temperature. After evaporation of the solvent, the residue was subjected to silica gel column chromatography. Elution with dichloromethane–methanol (96:4) afforded *N*-benzoyl-3′-*O*-acetyl-5′-*O*-(dimethoxytrityl)-2′-deoxyaristeromycin (**8**) as a yellow syrup: yield 150 mg (59%); ^{1}H NMR ($CDCl_3$, TMS) δ 2.06 (s, 3 H), 2.07 (m, 1 H), 2.28–2.64 (m, 4 H), 3.22 (m, 2 H), 3.84 (s, 6 H), 5.12 (m, 1 H), 5.25 (m, 1 H), 6.61 (m, 4 H), 7.20–7.60 (m, 12 H), 8.02 (s, 1 H), 8.05 (m, 2 H), 8.71 (s, 1 H).

8 (150 mg, 0.21 mmol) was treated with 80% aqueous acetic acid (50 mL) at room temperature for 1 h, and the solvent was evaporated under reduced pressure. The residue was subjected to silica gel column chromatography (dichlomethane–methanol (96:4)) to afford *N*-benzoyl-3′-*O*-acetyl-2′-deoxyaristeromycin (**9**) as a yellow syrup: yield 65 mg (76%); ^{1}H NMR ($CDCl_3$, TMS), δ 2.08 (s, 3 H), 2.15 (m, 1 H), 2.31–2.47 (m, 2 H), 2.53–2.70 (m, 2 H), 3.70–3.93 (m, 2 H), 5.14 (m, 1 H), 5.25 (m, 1 H), 7.45–7.64 (m, 3 H), 8.01 (m, 2 H), 8.08 (s, 1 H), 8.76 (s, 1 H), 9.03 (br s, 1 H). To a solution of **9** (65 mg, 0.16 mmol) in 1 mL of DMF was added methyltriphenoxyphosphonium iodide (154 mg, 0.34 mmol) in DMF (0.6 mL). The solution was stirred at room temperature for 3 h and the solvent was evaporated under reduced pressure. The residue was subjected to silica gel column chromatography (dichloromethane–methanol (96:4)) to afford **10** as a yellow syrup: yield 59 mg (69%); ^{1}H NMR ($CDCl_3$, TMS) δ 2.01 (s, 1 H), 2.08 (s, 3 H), 2.33–2.49 (m, 2 H), 2.60–2.77 (m, 2 H), 3.48 (m, 2 H), 5.06–5.22 (m, 2 H), 7.46–7.63 (m, 3 H), 8.01 (m, 2 H), 8.08 (s, 1 H), 8.79 (m, 1 H), 8.94 (br s, 1 H). **10** (57 mg, 0.11 mmol) and AgF (34.4 mg, 0.27 mmol) were dissolved in 3 mL of pyridine, and the resultant solution was stirred for 12 h at room temperature. After evaporation of the solvent, the residue was subjected to silica gel column chromatography (dichloromethane–methanol (96:4)) to give **11** as a yellow syrup: yield 29 mg (69%); ^{1}H NMR ($CDCl_3$, TMS) δ 2.12 (s, 3 H), 2.40–2.54 (m, 1 H), 2.61–2.77 (m, 1 H), 2.86–3.01 (m, 1 H), 3.14–3.30 (m, 1 H), 5.21–5.36 (m, 2 H), 5.46 (m, 1 H), 5.77 (m, 1 H), 7.49–7.66 (m, 3 H), 8.04 (m, 2 H), 8.06 (s, 1 H), 8.81 (s, 1 H), 9.01 (br s, 1 H).

Synthesis of (4′*S*)-2′-Deoxy-4′-hydroxyaristeromycin (5) and (4′*R*)-2′-Deoxy-4′-hydroxyaristeromycin (6). **11** (29.4 mg, 0.078 mmol), 4-methylmorpholine *N*-oxide (10.3 mg, 0.088 mmol), and 75 μL of 10% OsO_4 in *tert*-butanol were dissolved in aqueous acetone (acetone 310 μL, H_2O 780 μL). The solution was stirred for 12 h at room temperature under nitrogen. After addition of 0.1 mg of $Na_2S_2O_4$, the mixture was treated with concentrated ammonia (18 mL, 55 °C, 12 h). After evaporation of excess ammonia, the solution was neutralized with 1 N acetic acid and concentrated. The residue was purified by HPLC (μ-Bondasphere 5C$_{18}$ 300 Å (19 × 150 mm); 0.05 M ammonium formate containing 0–10% acetonitrile; linear gradient, 20 min; flow rate of 10 mL/min). Two major fractions with retention times of 14.4 and 16.9 min were collected. The former fraction was further purified by HPLC under isocratic conditions (μ-Bondasphere 5C$_{18}$ 300 Å (19 × 150 mm); 0.05 M ammonium formate containing 2% acetonitrile; flow rate of 10 mL/min) to afford **5** as a white powder: yield 3.1 mg (15%); ^{1}H NMR (D_2O, TSP) δ 2.09–2.26 (m, 2 H, $H_{2'\alpha}$, $H_{6'\beta}$), 2.32–2.47 (m, 2 H, $H_{2'\beta}$, $H_{6'\alpha}$), 3.66 (s, 2 H, $H_{5'}$), 4.37–4.44 (m, 1 H, $H_{3'}$), 5.15–5.25 (m, 1 H, $H_{1'}$), 8.24 (s, 1 H, H_2), 8.46 (s, 1 H, H_8); SIMS (positive ion) m/z 266 $(M + 1)^+$. The 4′*S* configuration was assigned by NOE relationship between $H_{3'}$ and $H_{5'}$.

The combined latter fraction was further purified in a similar manner as described above to provide **6**: yield 7.3 mg (35%); ^{1}H NMR (D_2O, TSP) δ 2.03 (dd, 1 H, J = 15.5, 3.4 Hz, $H_{6'\beta}$), 2.44 (dd, 1 H, J = 13.7, 8.6 Hz, $H_{2'\alpha}$), 2.61 (dd, 1 H, J = 15.5, 9.5 Hz, $H_{6'\alpha}$), 2.66 (dd, 1 H, J = 8.6, 4.6 Hz, $H_{2'\beta}$), 3.74 (d, 1 H, J = 11.9 Hz, $H_{5'}$), 3.83 (d, 1 H, J = 11.9 Hz, $H_{5'}$), 4.27 (d, 1 H, J = 4.6 Hz, $H_{3'}$), 5.29 (ddd, 1 H, J = 13.7, 9.5, 3.4 Hz, $H_{1'}$), 8.25 (s, 1 H, H_2), 8.28 (s, 1 H, H_8); SIMS (positive ion) m/z 266 $(M + 1)^+$. The 4′*R* configuration was assigned by NOE relationship between $H_{6'\alpha}$ and $H_{5'}$.

Digestion of d(GGArAGG)–d(CCTTCC) with Fe^{II}–PEM. The reaction mixture (total volume 20 μL) contained d(GGArAGG)–d(CCTTCC) (1 mM, final base concentration) and PEM (1 mM) in 50 mM sodium cacodylate at pH 7.0. The reaction was initiated by addition of freshly prepared aqueous $Fe^{2+}(NH_4)_2(SO_4)_2$ solution (1 mM), and the mixture was incubated at 0 °C for 15 min under aerobic conditions. A 10-μL aliquot was subjected to direct HPLC analysis. HPLC conditions: Cosmosil 5C$_{18}$ ODS column; 0.05 M ammonium formate containing 0–10% acetonitrile; linear gradient, 20 min; flow rate of 1.5 mL/min. The HPLC profile of the mixture is shown in Figure 2. The peak at 15.2 min was collected. After the same experiment was repeated for several times, the combined fraction was evaporated to dryness. The residue (ca. 1 OD) was dissolved in water and then subjected to enzymatic digestion with sv PDE (0.3 unit/mL) and calf intestine AP (100 units/mL). The HPLC peak at 19.2 min was collected and the fraction was concentrated. The residue was dissolved in water and then subjected to acid treatment (0.1 N HCl, 90 °C, 5 min). HPLC profiles of the solution before and after acid treatment are shown in Figure 4. Another 10-μL aliquot was directly subjected to enzymatic digestion with sv PDE (0.3 unit/mL) and calf intestine AP (100 units/mL followed by acid treatment), and then the mixture was subjected to HPLC analysis. HPLC conditions: Cosmosil 5C$_{18}$ ODS column; 0.05 M ammonium formate containing 0–6% acetonitrile; linear gradient, 20 min; flow rate of 1.5 mL/min. The HPLC profile of the mixture is shown in Figure 6.

Digestion of d(GGArAGG) with Fe^{II}–PEM. The reaction mixture (total volume 20 μL) containing d(GGArAGG) (0.5 mM, final base concentration) and PEM (1 mM) in 50 mM sodium cacodylate at pH 7.0. The reaction was initiated by addition of freshly prepared aqueous $Fe^{2+}(NH_4)_2(SO_4)_2$ solution (1 mM), and the mixture was incubated at 0 °C for 15 min under aerobic conditions. A 10-μL aliquot was subjected to direct HPLC analysis. HPLC conditions: Cosmosil 5C$_{18}$ ODS column; 0.05 M ammonium formate containing 0–10% acetonitrile; linear gradient, 20 min; flow rate of 1.5 mL/min. Neither formation of **2** nor the degradation of d(GGArAGG) was observed under the conditions.

Digestion of d(GGArAGG)–d(CCTTCC) with Fe^{II}–PEM under High Oxygen Pressure (20 atm). An ice-cooled solution (total volume 20 μL) containing d(GGArAGG)–d(CCTTCC) (1 mM, final base concentration) and PEM (1 mM) in 50 mM sodium cacodylate at pH 7.0 in a plastic vial was placed in an autoclave. A 2-μL aliquot of aqueous $Fe^{2+}(NH_4)_2(SO_4)_2$ (10 mM) solution fixed on a magnetic stirrer bar was placed over the reaction mixture. Oxygen was added to the autoclave to 20 atm and then the magnetic stirrer bar was dropped into the reaction mixture by use of a magnet. The reaction mixture was stirred for 30 min at 0 °C. The reaction mixture was subjected to enzymatic digestion and acid treatment as described above. The products **3** and **5** were produced in a 96:4 ratio as determined by HPLC.

Isolation of 2′-Deoxyneplanocin A (3) from Fe^{II}–PEM-Treated d(GGArAGG)–d(CCTTCC). The reaction mixutre (total volume 9.5 mL) contained d(GGArAGG)–d(CCTTCC) (1 mM, final base concentration) and PEM (1 mM) in 50 mM sodium cacodylate at pH 7.0. The reaction was initiated by addition of freshly prepared aqueous $Fe^{2+}(NH_4)_2(SO_4)_2$ solution (1 mM), and the mixture was incubated at 0 °C for 30 min under aerobic conditions. The reaction mixture was loaded on four Sep-Pak cartridges and washed with 2 mL of distilled water. Oligonucleotides were eluted with 0.05 M triethylammonium (pH 7.0) containing 10% acetnitrile. After lyophilization, the peak corresponding to **2** was collected as described previously, and the combined fraction was

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evaporated to dryness. The residue was dissolved in water and subjected to enzymatic digestion with sv PDE (0.3 unit/mL) and calf intestine AP (100 units/mL) and then subjected to acid treatment (0.1 N HCl, 90 °C, 5 min). The HPLC peak at 15.4 min was collected and the fraction was concentrated to afford ca. 20 μg of pure material. HPLC conditions: Cosmosil $5C_{18}$ ODS column; 0.05 M ammonium formate containing 0–10% acetonitrile; linear gradient, 20 min; flow rate of 1.5 mL/min. The ^1H NMR spectrum and HPLC behavior of the product **3** were identical with those of the authentic sample: HPLC retention time, 19.2 min; ^1H NMR (D_2O, TSP) δ 2.42–2.55 (m, 2 H, $H_{2'}$), 4.40 (br s, 1 H, $H_{5'}$), 5.06–5.12 (m, 1 H, $H_{3'}$), 5.75–5.82 (m, 1 H, $H_{1'}$), 6.05 (br s, 1 H, $H_{6'}$), 8.04 (s, 1 H, H_2), 8.21 (s, 1 H, H_8); SIMS (positive ion) m/z 248 $(M + 1)^+$.

Stability of 5 under Acidic and Basic Conditions. A 20-μL sample of **5** (0.1 mM) was treated with 6 N HCl (90 °C, 1 h), and the solution was neutralized with 6 N NaOH and subjected to HPLC analysis. Neither formation of **4** nor the decomposition of **5** was observed. Another 20 μL of **5** (0.1 mM) was treated with 0.1 NaOH (90 °C, 5 min), and the solution was neutralized with 1 N HCl and then subjected to HPLC analysis. Neither formation of **4** nor the degradation of **5** was observed.

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Theoretical Approach to Drug Design. 2. Relative Thermodynamics of Inhibitor Binding by Chicken Dihydrofolate Reductase to Ethyl Derivatives of Trimethoprim Substituted at 3'-, 4'-, and 5'-Positions

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Abstract: The relative binding thermodynamics of trimethoprim [2,4-diamino-5-(3',4',5'-trimethoxybenzyl)pyrimidine] congeners to chicken dihydrofolate reductase are determined by free energy simulation methods. The congeners considered in this study represent methoxy to ethyl substitutions at the 3'-, 4'-, and 5'-positions of trimethoprim found at the benzyl portion of the inhibitor. Molecular dynamics simulations of the protein–inhibitor complexes, and the evaluation of potential energy interactions between inhibitor and protein, were evaluated for their ability to predict trends similar to those found for the thermodynamic simulations. The relative free energy trends for the 3',5'-diethyl and 3',4',5'-triethyl derivatives of trimethoprim follow those observed experimentally for identical inhibitor compounds. An observation of a reversal of enzyme preference for species with 3'- or 5'-ethyl functional groups, depending on the absence or presence of a 4'-ethyl group, demonstrates complex influences by active site atoms on the relative thermodynamics of inhibitor binding. Potential energy data derived from molecular dynamics of inhibitor–protein complexes show no demonstrable correlation to experimental results or calculated thermodynamic data.

Introduction

The process of inhibitor binding by proteins or enzymes is a complex thermodynamic one, which is driven by both energetic and entropic changes. Changes to the free energy of inhibitor binding, resulting from the addition or deletion of functional groups, arise from differences in the solvation/desolvation thermodynamics of the inhibitor and protein, energetic differences associated with different inhibitor–protein contacts, and conformational entropy changes to the protein and inhibitor.[1] Information regarding relative free energy differences between different inhibitors bound to a common receptor is accessible via experimental methods[2,3] or more recently with theoretical methods using the thermodynamic cycle perturbation (TCP) approach.[4-7] In addition, quantitative structure–activity relationship (QSAR) studies can be used to augment thermodynamic data.[16] In this article, free energy simulation methods are used to investigate the principles that determine the structural and thermodynamic aspects of inhibitor binding and specificity. In particle, with this work we extend earlier thermodynamic simulations for the binding of trimethoprim [2,4-diamino-5-(3',4',5'-trimethoxybenzyl)pyrimidine] and congeners to dihydrofolate reductase (DHFR) derived from chicken liver.[5,8,9] This system is a strong candidate for testing the utility of these methodologies given the number of crystallographic structures for DHFR derived from several species,[10-14] and the experimental characterization and QSAR analyses of inhibitor–DHFR complexes,[15,16] as well as the importance of DHFR as a target for inhibition in the treatment of bacterial

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