

# DNA Damage by Ptaquiloside, a Potent Bracken Carcinogen: Detection of Selective Strand Breaks and Identification of DNA Cleavage Products

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**Abstract:** Ptaquiloside (**1**) is a potent carcinogen isolated from bracken fern. Under weakly alkaline conditions, the carcinogen is converted into dienone **2** which is thought to be the ultimate agent responsible for bracken fern carcinogenicity. This study details the selective alkylation and strand scission of DNA with dienone **2**. Dienone **2** forms covalent adducts through N-3 of adenine or N-7 of guanine with opening of the cyclopropyl ring. Under physiological conditions, spontaneous cleavage of the N-glycosidic linkage occurs primarily at the modified adenines to produce abasic sites. The abasic sites are so unstable that subsequent backbone breakage occurs via a  $\beta$ -elimination reaction. Product analyses on sequencing gels and HPLC reveal evolution of the structures of the 5'- and 3'-termini that result from the abasic sites. In addition, the sequence selectivity for the DNA cleavage is demonstrated. The cleavage rates at the target adenine residues are affected by both 5'- and 3'-flanking nucleotides. The rank orders are 5'-AT > 5'-AG > 5'-AC > 5'-AA for 3'-flanking nucleotides and 5'-AA > 5'-TA > 5'-GA > 5'-CA for 5'-flanking nucleotides (where A is a site of cleavage). The most favorable sequence is estimated to be 5'-AAAT. The present results on dienone **2** have also been compared with those of CC-1065 containing a similar reactive cyclopropyl ring.

## Introduction

Bracken fern, *Pteridium aquilinum*, has been known to cause intestinal and bladder carcinomas in animals<sup>1-3</sup> and also to enhance esophageal cancer risk in human being.<sup>4</sup> After the intensive search for the carcinogenic principle(s), a new type of potent carcinogenic compound termed ptaquiloside (**1**) was isolated from bracken fern.<sup>5,6</sup> Under weakly alkaline conditions, ptaquiloside (**1**) is converted, with liberation of D-(+)-glucose, into an unstable compound dienone **2** which subsequently leads to the aromatic compounds such as pterisin B (**3**).<sup>5a,b</sup> Dienone **2** is assumed to be the ultimate agent responsible for bracken fern carcinogenicity. Indeed, the cyclopropyl group in dienone **2** is strongly electrophilic and dienone **2** can readily react with amino acids, nucleosides, and nucleotides, under mild conditions.<sup>6</sup> Since dienone **2** forms a covalent adduct with DNA and causes DNA strand breaks,<sup>7</sup> DNA is the principal biological target of ptaquiloside (**1**).

Recently, DNA cleaving activities of both enantiomers of dienone **2** have been evaluated.<sup>8</sup>

In general, reaction of an ultimate carcinogen with DNA is regarded as a crucial step in the initiation of chemical carcinogenesis.<sup>9,10</sup> Such reactions usually produce a number of DNA adducts and abasic sites in different portions and in different locations. The adducts and abasic sites have been proposed as intermediates in mutagenesis by a variety of genotoxic agents.<sup>11</sup> In the present study, we have disclosed the mechanism, sequence preference, and product distribution of the DNA strand scission induced by dienone **2**. So far, the 3'-terminal structures at the DNA cleavage sites produced by alkylating agents such as CC-1065 have remained unknown.<sup>12</sup> This problem has also been solved in the present study.

## Experimental Section

**Materials.** Ptaquiloside (**1**), dienone **2**, and pterisin B (**3**) were prepared as previously described.<sup>6</sup> Stock drug concentrations were made by dissolving weighed amounts of the drugs in acetonitrile. When stored at -20 °C, the drug solutions were stable for several weeks as judged by <sup>1</sup>H NMR. Plasmid pBR322 DNA was isolated from *Escherichia coli* C600. *Escherichia coli* DNA polymerase I large fragment, bacterial alkaline phosphatase, and polynucleotide kinase were purchased from New England Biolabs. Restriction enzymes and plasmid pUC19 DNA were from Takara (Kyoto, Japan). Radiochemicals were obtained from Amersham or DuPont. Water was distilled and purified through a Sybron Nanopure II System. All other chemicals used were of commercial reagent grade.

**Preparation and Labeling of DNA Restriction Fragments.** Restriction *Bam*HI-*Sall* fragment from pBR322 was labeled at *Bam*HI cut. The

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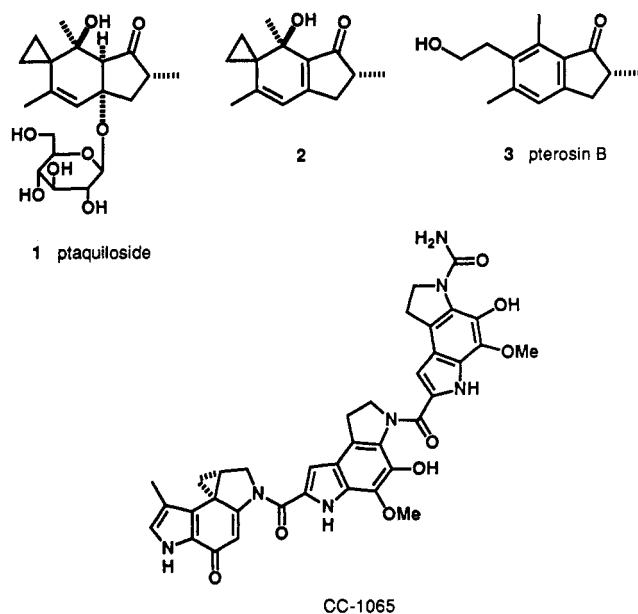
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Chart 1



5'-end was labeled with polynucleotide kinase and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  after treatment with bacterial alkaline phosphatase. The 3'-end was labeled with  $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$  by using the filling-in function of *Escherichia coli* DNA polymerase I large fragment. After digestion with the second enzyme, *SalI*, the singly labeled fragment was purified on a nondenaturing 5% (w/v) polyacrylamide gel. The other fragments used in this study were labeled in a similar manner.

**Assay for Damage to Supercoiled DNA.** Ptaquiloside (1) or dienone 2 was incubated with 0.25  $\mu\text{g}$  of pure covalently closed circular (form I) pBR322 DNA in 25% (v/v) acetonitrile/TBE buffer (89 mM Tris-borate, 2.5 mM  $\text{Na}_2\text{EDTA}$ , pH 8.0) at 37 °C for 30 min. The samples were successively precipitated in ethanol to remove all unreacted drug. The DNA pellets were dried and dissolved in 20  $\mu\text{L}$  of TBE buffer (pH 8.0) and incubated again in sealed tubes at 37 °C for 25 h. After ethanol precipitation, the samples were dissolved in 10  $\mu\text{L}$  of loading buffer containing 0.05% (w/v) bromophenol blue and 10% (v/v) glycerol for electrophoresis. The different forms of DNA were separated at 15 °C on a 1% (w/v) agarose gel stained with ethidium bromide (0.5  $\mu\text{g}/\text{mL}$ ). The gels were placed on a UV transilluminator and photographed with Polaroid 665 film.

**Cleavage Specificity with End-Labeled Restriction Fragments.** Dienone 2 (250  $\mu\text{M}$ ) was incubated with 0.25  $\mu\text{g}$  of calf thymus DNA and a trace ( $<0.01$   $\mu\text{g}$ ) of end-labeled DNA ( $\approx 10\,000$  cpm) in a total volume of 20  $\mu\text{L}$ . The reactions were carried out at 37 °C for various lengths of time in 25% acetonitrile/TBE buffer (pH 8.0). When required, the samples were subsequently treated with 1 M piperidine at 90 °C for 30 min. Each lyophilized sample was dissolved in 3  $\mu\text{L}$  of 90% (v/v) deionized formamide containing 1 mM EDTA and 0.01% (w/v) bromophenol blue and loaded into a 10% (w/v) polyacrylamide sequencing gel. For product analysis, a 15% (w/v) polyacrylamide sequencing gel was used. Electrophoresis was performed at 2000 V in TBE buffer (pH 8.0). DNA sequencing was carried out by the Maxam–Gilbert method.<sup>13</sup>

**Alkylation of d(GTAC) with Dienone 2.** A solution of dienone 2 (105 mg, 0.482 mmol) in acetonitrile (4.0 mL) was added to a solution of deoxytetranucleotide d(GTAC) (119 mg, 0.097 mmol) in  $\text{H}_2\text{O}$  (12 mL, pH 7.5), and the mixture was stirred at 0 °C for 15 h and extracted with ethyl acetate (6  $\times$  18 mL) at 0 °C. The combined extracts were concentrated to give crystalline pterisin B (3) (99 mg). The aqueous layer was freeze-dried to give a colorless amorphous powder. The powder was purified by HPLC (Develosil ODS-10, 20  $\times$  250 mm, 0.02 M  $\text{NH}_4\text{OAc}$ –acetonitrile, 100:0  $\rightarrow$  40:60, 256 min linear gradient elution, 7 mL/min) to give guanine adduct 4 (8.2 mg, 1.2% based on dienone 2), adenine adduct 5 (3.5 mg, 0.5% based on dienone 2), and unreacted d(GTAC) (86 mg, 73%) as a colorless amorphous powder, respectively.

**Guanine adduct 4:** UV ( $\text{H}_2\text{O}$ )  $\lambda_{\text{max}}$  197 ( $\epsilon$  77 500), 260 nm (31 600);  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  1.22 (br d,  $J$  = 7.0 Hz, 3 H), 1.53 (br s, 3 H), 1.82–2.09 (m, 1 H), 2.10–2.44 (m, 2 H), 2.24 (br s, 3 H), 2.28 (br

s, 3 H), 2.45–2.95 (m, 8 H), 3.08–3.20 (m, 1 H), 3.33–3.48 (m, 1 H), 3.86–4.46 (m, 15 H), 4.54–5.06 (m, 3 H), 5.78–5.96 (m, 1 H), 6.00–6.22 (m, 4 H), 6.95 (br s, 1 H), 7.41–7.46 (m, 1 H), 7.77 (br s, 1 H), 8.28 (br s, 1 H), 9.17 (br s, 1 H), a proton of a nucleic acid base in 4 was not observed; FABMS (glycerol)  $m/z$  1396 ( $\text{M} + \text{Na}$ )<sup>+</sup>, 1374 ( $\text{M} + \text{H}$ )<sup>+</sup>.

**Adenine adduct 5:** UV ( $\text{H}_2\text{O}$ )  $\lambda_{\text{max}}$  192 ( $\epsilon$  61 800), 263 nm (35 100);  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  1.18 (d,  $J$  = 7.3 Hz, 3 H), 1.78 (s, 3 H), 1.97–2.06 (m, 1 H), 2.20–2.30 (m, 2 H), 2.21 (s, 3 H), 2.24 (s, 3 H), 2.33–2.41 (m, 1 H), 2.50–2.62 (m, 3 H), 2.64 (dt,  $J$  = 3.7, 7.3 Hz, 1 H), 2.92 (br dd,  $J$  = 13.4, 4.6 Hz, 1 H), 3.00–3.08 (m, 1 H), 3.20 (dd,  $J$  = 17.4, 7.9 Hz, 1 H), 3.28 (dt,  $J$  = 15.3, 7.0 Hz, 1 H), 3.36 (dt,  $J$  = 15.3, 7.3 Hz, 1 H), 3.73 (dd,  $J$  = 12.8, 3.9 Hz, 1 H), 3.78 (dd,  $J$  = 12.8, 3.4 Hz, 1 H), 3.94–3.99 (m, 1 H), 4.02–4.23 (m, 9 H), 4.52–4.57 (m, 2 H), 5.02–5.06 (m, 1 H), 5.90 (br d,  $J$  = 7.0 Hz, 1 H), 6.03 (dd,  $J$  = 8.9, 3.1 Hz, 1 H), 6.06 (dd,  $J$  = 13.7, 7.6 Hz, 1 H), 6.20 (dd,  $J$  = 6.4, 6.4 Hz, 1 H), 6.50 (dd,  $J$  = 8.6, 3.7 Hz, 1 H), 7.04 (s, 1 H), 7.46 (s, 1 H), 7.83 (d,  $J$  = 7.0 Hz, 1 H), 7.83–7.89 (br s, 1 H), 8.02 (s, 1 H), 8.59 (s, 1 H), three protons were overlapped with the solvent signals; FABMS (glycerol)  $m/z$  1374 ( $\text{M} + \text{H}$ )<sup>+</sup>.

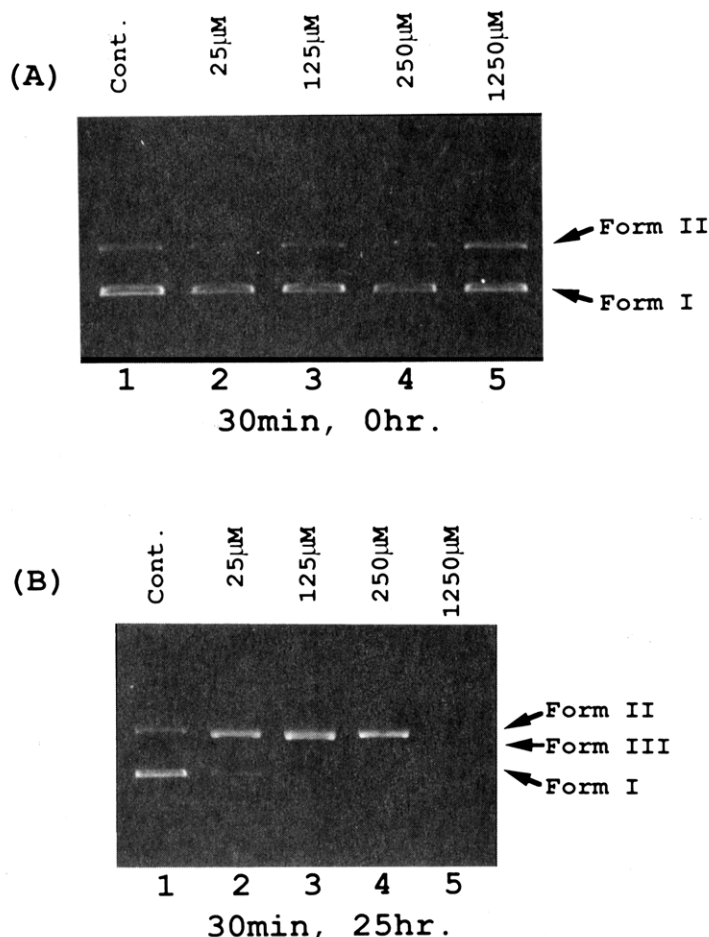
**Thermal Hydrolysis of Alkylated Deoxytetranucleotides 4 and 5.** (i) **Thermal Hydrolysis of Guanine Adduct 4.** A solution of guanine adduct 4 (1.7 mg, 1.2  $\mu\text{mol}$ ) in  $\text{H}_2\text{O}$  (5 mL) was heated at 90 °C for 5 min and extracted with 1-butanol (4  $\times$  7 mL). The combined extracts were concentrated, and the residual solid was washed with methanol (2 mL) to give N-7 alkylguanine 8<sup>7</sup> (0.4 mg, 95%). The aqueous layer was freeze-dried and purified by HPLC (Develosil ODS-10, 20  $\times$  250 mm, 0.02 M  $\text{NH}_4\text{OAc}$ –acetonitrile, 100:0  $\rightarrow$  40:60, 256 min linear gradient elution, 7 mL/min) to give d(deoxyribose-TAC) 6 (0.8 mg, 63%) as a colorless amorphous powder: UV ( $\text{H}_2\text{O}$ )  $\lambda_{\text{max}}$  198 ( $\epsilon$  44 000), 261 nm (28 400);  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  1.75–1.92 (m, 2 H), 1.83 (s, 3 H), 2.01–2.14 (m, 1 H), 2.21–2.30 (m, 2 H), 2.36 (ddd,  $J$  = 11.3, 6.4, 4.6 Hz, 1 H), 2.75 (ddd,  $J$  = 14.0, 5.8, 2.5 Hz, 1 H), 2.87 (ddd,  $J$  = 14.0, 7.0, 7.0 Hz, 1 H), 3.52–3.69 (m, 1 H), 3.82–4.02 (m, 4 H), 4.05–4.28 (m, 6 H), 4.40 (br s, 1 H), 4.52–4.57 (m, 1 H), 5.00 (br s, 1 H), 5.22 (m, 0.7 H), 5.54 (d,  $J$  = 4.0 Hz, 0.2 H), 5.59 (t,  $J$  = 4.9 Hz, 0.1 H), 5.92 (br d,  $J$  = 7.6 Hz, 1 H), 6.00–6.05 (m, 1 H), 6.19 (dd,  $J$  = 6.4, 6.4 Hz, 1 H), 6.35 (dd,  $J$  = 8.2, 5.8 Hz, 1 H), 7.40 (s, 0.5 H), 7.42 (s, 0.5 H), 7.79 (d,  $J$  = 7.6 Hz, 1 H), 8.15 (s, 1 H), 8.41 (s, 1 H), two protons were overlapped with the solvent signals; FABMS (glycerol)  $m/z$  1063 ( $\text{M} + \text{Na}$ )<sup>+</sup>, 1041 ( $\text{M} + \text{H}$ )<sup>+</sup>.

(ii) **Thermal Hydrolysis of Adenine Adduct 5.** According to the procedure described above, adenine adduct 5 (3.2 mg, 2.2  $\mu\text{mol}$ ) gave d(GT-deoxyribose-C) 7 (2.5 mg, 100%) and N-3 alkyladenine 9<sup>7</sup> (0.7 mg, 93%) as a colorless amorphous powder, respectively: 7 UV ( $\text{H}_2\text{O}$ )  $\lambda_{\text{max}}$  265 nm ( $\epsilon$  15 400);  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  1.71 (s, 3 H), 2.02–2.28 (m, 2 H), 2.29–2.50 (m, 4 H), 2.67–2.79 (m, 2 H), 3.78 (dd,  $J$  = 12.5, 3.9 Hz, 1 H), 3.82 (dd,  $J$  = 12.5, 3.4 Hz, 1 H), 3.89–4.21 (m, 7 H), 4.25–4.30 (m, 1 H), 4.30–4.35 (m, 1 H), 4.51–4.57 (m, 1 H), 5.62 (m, 1 H), 6.07 (d,  $J$  = 7.6 Hz, 0.33 H), 6.09 (d,  $J$  = 7.6 Hz, 0.67 H), 6.19–6.28 (m, 3 H), 7.57 (s, 0.33 H), 7.58 (s, 0.67 H), 7.87 (d,  $J$  = 7.6 Hz, 0.33 H), 7.88 (d,  $J$  = 7.6 Hz, 0.67 H), 7.93 (br s, 1 H), four protons were overlapped with the solvent signals; FABMS (glycerol)  $m/z$  1079 ( $\text{M} + \text{Na}$ )<sup>+</sup>, 1057 ( $\text{M} + \text{H}$ )<sup>+</sup>.

**Thermal Stabilities of Alkylated Deoxytetranucleotides 4 and 5.** The disappearance of guanine adduct 4 or adenine adduct 5 in 0.02 M Tris-borate buffer (pH 7.5) at 32 °C was monitored by HPLC analysis (Develosil ODS-5, 4.6  $\times$  250 mm, 0.02 M  $\text{NH}_4\text{OAc}$ –acetonitrile, 100:0  $\rightarrow$  40:60, 60 min linear gradient elution, 1 mL/min). The amount of the compound in the injected sample was determined by the peak area at 260 nm.

**Cleavage of d(GT-Deoxyribose-C) 7.** A solution of d(GT-deoxyribose-C) 7 (1.25 mg, 1.13  $\mu\text{mol}$ ) in Tris-borate buffer (pH 7.5) was heated at 90 °C for 7 h. The reaction mixture was freeze-dried and separated by HPLC (Develosil ODS-5, 10  $\times$  250 mm, 0.02 M  $\text{NH}_4\text{OAc}$ –acetonitrile, 100:0  $\rightarrow$  40:60, 128 min linear gradient elution, 4 mL/min) to give fractions A ( $t_R$  = 4 min), B ( $t_R$  = 14 min), and C ( $t_R$  = 16 min). Fraction A was further purified by HPLC (Develosil ODS-5, 10  $\times$  250 mm, 0.02 M  $\text{NH}_4\text{OAc}$ –acetonitrile, 100:0  $\rightarrow$  40:60, 128 min linear gradient elution, 4 mL/min) to give 2'-deoxycytidine 5'-monophosphate (0.2 mg, 51%):  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  2.31 (ddd,  $J$  = 13.7, 6.7, 6.7 Hz, 1 H), 2.39 (ddd,  $J$  = 13.7, 6.7, 3.7 Hz, 1 H), 3.91–4.01 (m, 2 H), 4.12–4.19 (m, 1 H), 4.53–4.59 (m, 1 H), 6.12 (d,  $J$  = 7.6 Hz, 1 H), 6.33 (t,  $J$  = 6.7 Hz, 1 H), 8.06 (d,  $J$  = 7.6 Hz, 1 H); FABMS (glycerol)  $m/z$  374 ( $\text{M} + 3\text{Na}$ )<sup>+</sup>, 352 ( $\text{M} + 2\text{Na} + \text{H}$ )<sup>+</sup>.

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**Figure 1.** DNA damage by dienone **2**. (A) Supercoiled pBR322 DNA (12.5  $\mu\text{g/mL}$ ) was incubated at 37  $^{\circ}\text{C}$  for 30 min in the presence of various concentration of dienone **2**. The final concentrations of **2** were 0 mM (lane 1), 0.025 mM (lane 2), 0.125 mM (lane 3), 0.25 mM (lane 4), and 1.25 mM (lane 5). The ratios  $[\mathbf{2}]/[\text{DNA nucleotide}]$  were 0, 0.65, 3.3, 6.5, and 33, respectively. The DNA samples were precipitated in ethanol to remove unreacted drug and separated on a 1% (w/v) agarose gel stained with ethidium bromide. (B) The same DNA samples as in panel A were dissolved in TBE buffer (pH 8.0) and incubated again at 37  $^{\circ}\text{C}$  for 25 h. The positions of the supercoiled (form I), relaxed nicked circular (form II), and linear (form III) plasmid molecules are indicated.

Fraction B was concentrated to give alcohol **10**<sup>14</sup> (0.5 mg, 58%) as an amorphous powder: UV ( $\text{H}_2\text{O}$ )  $\lambda_{\text{max}}$  255 nm ( $\epsilon$  14 300);  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  1.78 (s, 3 H), 1.89–2.04 (m, 0.67 H), 2.14–2.24 (m, 1 H), 2.31–2.38 (m, 0.33 H), 2.44–2.65 (m, 2 H), 2.81–2.90 (m, 2 H), 3.83–3.91 (m, 2 H), 3.92–4.07 (m, 3 H), 4.08–4.44 (m, 4.33 H), 4.48 (m, 0.33 H), 4.58–4.61 (m, 0.33 H), 5.64 (m, 0.67 H), 5.76 (m, 0.33 H), 6.28 (dd,  $J$  = 6.4, 6.4 Hz, 1 H), 6.36 (m, 1 H), 7.62 (s, 1 H), 8.00 (br s, 1 H), two protons were overlapped with the solvent signals; FABMS (glycerol)  $m/z$  790 ( $\text{M} + \text{Na}$ )<sup>+</sup>, 768 ( $\text{M} + \text{H}$ )<sup>+</sup>.

Without concentration fraction C was subjected to reduction by  $\text{NaBH}_4$  (1.0 mg, 0  $^{\circ}\text{C}$ , 1 h). The reaction mixture was purified by HPLC (Develosil ODS-5, 10  $\times$  250 mm, 0.02 M  $\text{NH}_4\text{OAc}$ -acetonitrile, 100:0  $\rightarrow$  40:60, 128 min linear gradient elution, 4 mL/min) to give a 2:1 mixture of allylic alcohols **12a** and **12b** (0.3 mg, 35%) as an amorphous powder:  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  1.77 (s, 3 H), 2.41–2.45 (m, 1 H), 2.51–2.75 (m, 1 H), 2.77–2.82 (m, 2 H), 3.83–3.87 (m, 4 H), 4.13–4.38 (m, 6 H), 5.60 (dd,  $J$  = 11.0, 10.0 Hz, 0.67 H), 5.79 (dd,  $J$  = 15.0, 5.0 Hz, 0.33 H), 5.84 (dt,  $J$  = 11.0, 6.0 Hz, 0.67 H), 5.99 (dt,  $J$  = 15.0, 6.0 Hz, 0.33 H), 6.26 (dd,  $J$  = 6.4, 6.4 Hz, 1 H), 6.34 (dd,  $J$  = 6.4, 6.4 Hz, 1 H), 7.62 (s, 1 H), 7.97 (s, 1 H), three protons were overlapped with the solvent signals; FABMS (glycerol)  $m/z$  774 ( $\text{M} + \text{Na}$ )<sup>+</sup>.

**Reduction of Alcohol 10.** To the solution of alcohol **10** (0.5 mg, 0.65  $\mu\text{mol}$ ) in  $\text{H}_2\text{O}$  (0.2 mL) was added  $\text{NaBH}_4$  (1.0 mg, 0.03 mmol) at 0  $^{\circ}\text{C}$ . The mixture was stirred at 0  $^{\circ}\text{C}$  for 1 h, diluted with acetone (0.1 mL), and concentrated. The residue was purified by HPLC (Develosil ODS-5, 10  $\times$  250 mm, 0.02 M  $\text{NH}_4\text{OAc}$ -acetonitrile, 100:0  $\rightarrow$  40:60, 256 min linear gradient elution, 3 mL/min) to give alcohol **13a** (0.4 mg, 80%) and alcohol **13b** (0.1 mg, 20%) as a colorless amorphous powder, respectively.

(14) Alcohol **10** was shown to consist of two diastereomers at C-3 of the deoxyribose moiety in the ratio of 4:1.

(15) There was no background cleavage due to any of protocols that use heat, piperidine, or long incubation times.

**Alcohol 13a:** UV ( $\text{H}_2\text{O}$ )  $\lambda_{\text{max}}$  193 ( $\epsilon$  26 100), 256 nm (16 400);  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  1.64–1.74 (m, 1 H), 1.75 (s, 3 H), 1.89–1.99 (m, 1 H), 2.43 (ddd,  $J$  = 14.1, 7.1, 7.1 Hz, 1 H), 2.53 (ddd,  $J$  = 14.1, 6.1, 3.1 Hz, 1 H), 2.75–2.85 (m, 2 H), 3.72–3.90 (m, 6 H), 3.96 (dt,  $J$  = 11.5, 6.2 Hz, 1 H), 4.07 (ddd,  $J$  = 10.4, 5.8, 3.3 Hz, 1 H), 4.18 (br d,  $J$  = 12.4 Hz, 1 H), 4.25 (br d,  $J$  = 12.4 Hz, 1 H), 4.28–4.39 (m, 2 H), 4.88–4.98 (m, 2 H), 6.25 (t,  $J$  = 6.7 Hz, 1 H), 6.33 (t,  $J$  = 7.0 Hz, 1 H), 7.60 (s, 1 H), 7.98 (s, 1 H); FABMS (glycerol)  $m/z$  792 ( $\text{M} + \text{Na}$ )<sup>+</sup>, 770 ( $\text{M} + \text{H}$ )<sup>+</sup>.

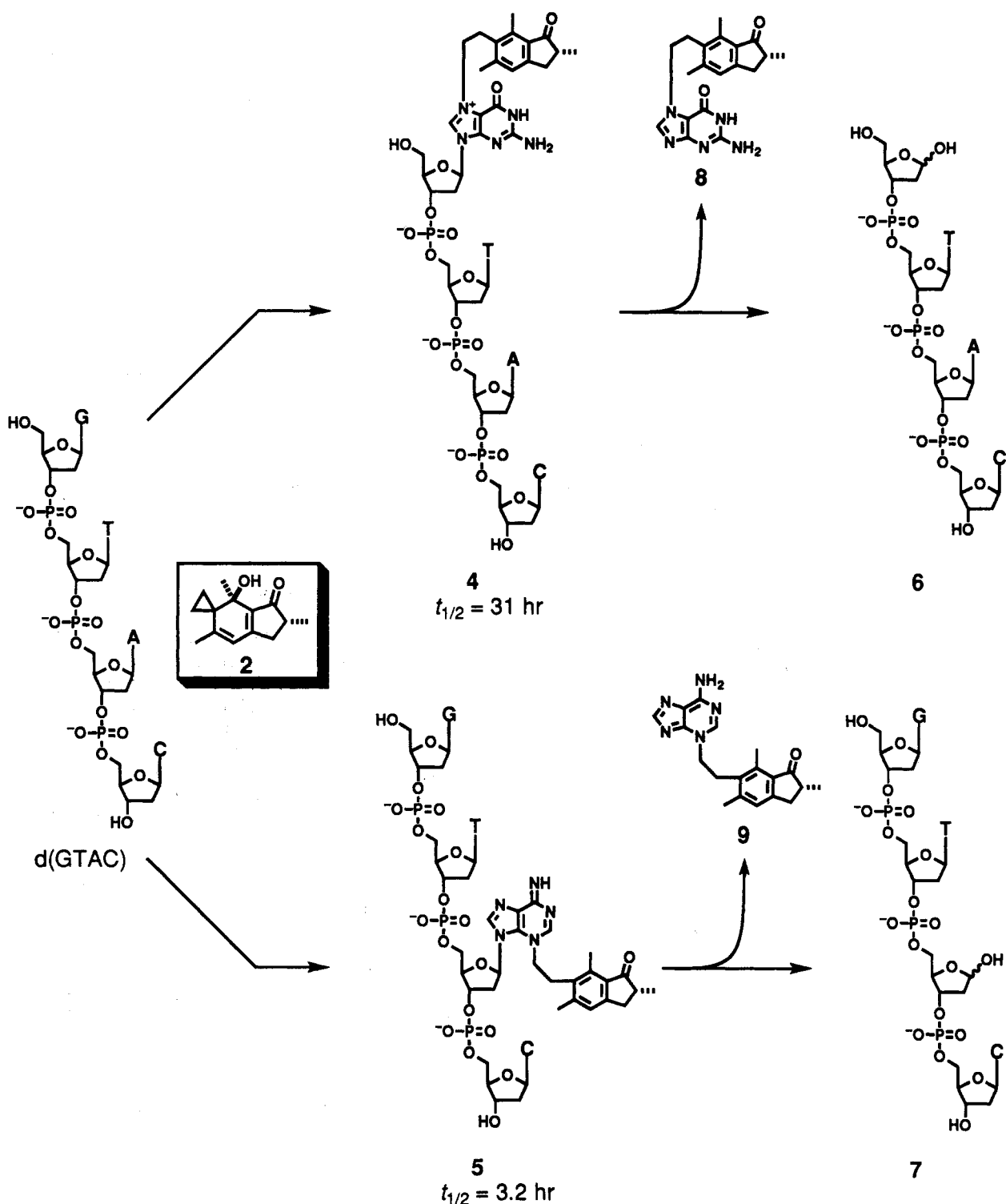
**Alcohol 13b:** UV ( $\text{H}_2\text{O}$ )  $\lambda_{\text{max}}$  193 ( $\epsilon$  32 100), 256 nm (17 600);  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  1.75 (s, 3 H), 1.75–1.90 (m, 2 H), 2.43 (ddd,  $J$  = 14.0, 7.0, 7.0 Hz, 1 H), 2.53 (ddd,  $J$  = 14.0, 6.0, 3.0 Hz, 1 H), 2.79–2.83 (m, 2 H), 3.74–3.81 (m, 3 H), 3.83–3.91 (m, 3 H), 3.95 (ddd,  $J$  = 11.0, 6.0, 6.0 Hz, 1 H), 4.02 (ddd,  $J$  = 11.0, 5.5, 5.5 Hz, 1 H), 4.18 (br d,  $J$  = 12.2 Hz, 1 H), 4.26 (br d,  $J$  = 12.2 Hz, 1 H), 4.31–4.37 (m, 2 H), 4.91–4.97 (m, 2 H), 6.25 (dd,  $J$  = 6.7, 6.7 Hz, 1 H), 6.34 (dd,  $J$  = 7.0, 7.0 Hz, 1 H), 7.60 (s, 1 H), 7.98 (br s, 1 H); FABMS (glycerol)  $m/z$  792 ( $\text{M} + \text{Na}$ )<sup>+</sup>, 770 ( $\text{M} + \text{H}$ )<sup>+</sup>.

## Results

**DNA Cleavage by Dienone 2.** The DNA-cleaving activity of dienone **2** was demonstrated by monitoring the conversion of covalently closed circular (form I) pBR322 DNA to nicked circular (form II) and linear duplex (form III) DNAs. A single-strand break changes form I DNA to form II DNA, while a double-strand break, produced either in a direct manner or as a result of two close-spaced single-strand breaks in complementary strands, changes either form I or form II DNA into form III DNA. As shown in Figure 1A, at the reaction with dienone **2** for 30 min at 37  $^{\circ}\text{C}$ , no strand scissions were observed. Further



Scheme 1



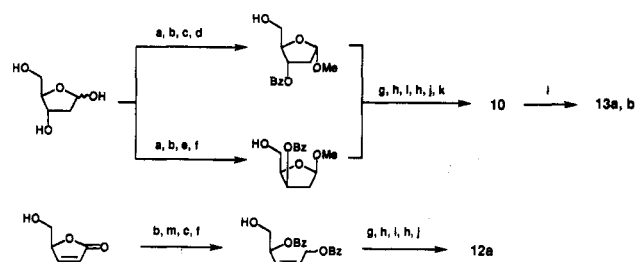
deoxytetranucleotide adducts **4** (1.2% based on dienone **2**), **5** (0.5%)<sup>17</sup> and unreacted starting material d(GTAC).

Thermal treatment of **4** (90 °C, 5 min) afforded d(deoxyribose-TAC) **6** with liberation of N-7 alkylguanine **8**<sup>7</sup> (Scheme 1), while that of **5** provided d(GT-deoxyribose-C) **7** and N-3 alkyladenine **9**.<sup>7</sup> The structures of **6** and **7** were determined by spectral data and confirmed by the HPLC analysis of the nucleosides formed by the enzymatic digestions of **6** and **7** ((1) snake venom

phosphodiesterase and alkaline phosphatase and (2) spleen phosphodiesterase and alkaline phosphatase). On the basis of these findings, the structures of the two major adducts **4** and **5** have been established. The stabilities of the adducts **4** and **5** in Tris-borate buffer (pH 7.5) at 32 °C were measured by using HPLC analysis. Their half-lives ( $t_{1/2}$ ) are as follows: **4**,  $t_{1/2} = 31 \text{ h}$ ; **5**,  $t_{1/2} = 3.2 \text{ h}$ . Thus, the depurination reaction of the adduct **5** was found to proceed much faster than that of the adduct **4**.

**Characterization of the Cleavage Products.** On heating in Tris-borate buffer (pH 7.5, 90 °C, 7 h), backbone cleavage of d(GT-deoxyribose-C) **7** took place. This cleavage reaction also

(17) While the reaction of dienone **2** and d(GTAC) affords **4** (7-G modified oligomer) and **5** (3-A modified oligomer) in a 2.4:1 ratio, the A cleavage bands on the sequencing gels are more intense than the G cleavage bands upon heating the DNA. This conflicting result may be due to the difference between the single-<sup>16</sup> and double-stranded substrates.

Scheme 2<sup>a</sup>

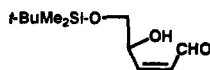
<sup>a</sup> (a) 1% HCl in MeOH, 26 °C; (b) *t*-BuMe<sub>2</sub>SiCl, imidazole, DMF, 23 °C; (c) BzCl, pyridine, 23 °C; (d) Bu<sub>4</sub>NF, THF, 23 °C; (e) (EtOOCN=)<sub>2</sub>, PPh<sub>3</sub>, PhCOOH, THF, 23 °C; (f) HF·Py, THF, 23 °C; (g) thymidine phosphoramidite, 1*H*-tetrazole, THF, acetonitrile, 23 °C; then *t*-BuOOH, 23 °C; (h) CHCl<sub>2</sub>COOH, CH<sub>2</sub>Cl<sub>2</sub>, 23 °C; (i) guanine phosphoramidite, 1*H*-tetrazole, THF, acetonitrile, 23 °C; then *t*-BuOOH, 23 °C; (j) 28% NH<sub>3</sub>, 50 °C; (k) 0.05 M HCl, 0 or 23 °C; (l) NaBH<sub>4</sub>, H<sub>2</sub>O, 0 °C; (m) *i*-Bu<sub>2</sub>AlH, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C.

proceeded very slowly under a physiological condition (pH 7.5, 32 °C, 29 days). HPLC analysis of the cleavage mixture (Figure 6) showed three major peaks **a**, **b**, and **c**. The compound obtained from the peak **a** was identified as deoxycytidine-5'-phosphate by spectral comparison with the authentic specimen. The spectral analysis suggested that the material obtained from peak **b** was alcohol **10**. In order to establish the structures of this alcohol **10**, it was reduced with NaBH<sub>4</sub> and separated by HPLC to afford the major alcohol **13a** and the minor one **13b**. The structures of the two alcohols **13a** and **13b** were established by the spectral data and were unambiguously confirmed by the synthesis (Scheme 2). The material in peak **c** was so unstable that it was decomposed during concentration of the solution. Therefore, the solution of peak **c** obtained by HPLC separation was immediately treated with NaBH<sub>4</sub> to afford an inseparable 2:1 mixture of the stable allyl alcohols **12a** and **12b**. The spectral analysis of the mixture of **12a** and **12b** suggested the structure of the major alcohol to be **12a**, which was confirmed by the synthesis (Scheme 2). These findings indicated that the peak **c** consisted of hemiacetal **11a** and aldehyde **11b** in the ratio of 2:1.<sup>18</sup> Further, the mixture of **11a** and **11b** in Tris-borate buffer (pH 7.5) was shown to be converted into alcohol **10** at 32 °C for seven days (or 90 °C, 5 h). Thus, alcohol **10** was formed by the Michael addition of water to **11a** and **11b**. Further, all of the cleavage products, **10**, **11a**, and **11b**, were shown to lead to the dinucleotide d(GpTp) on heating with piperidine (90 °C, 30 min).

## Discussion

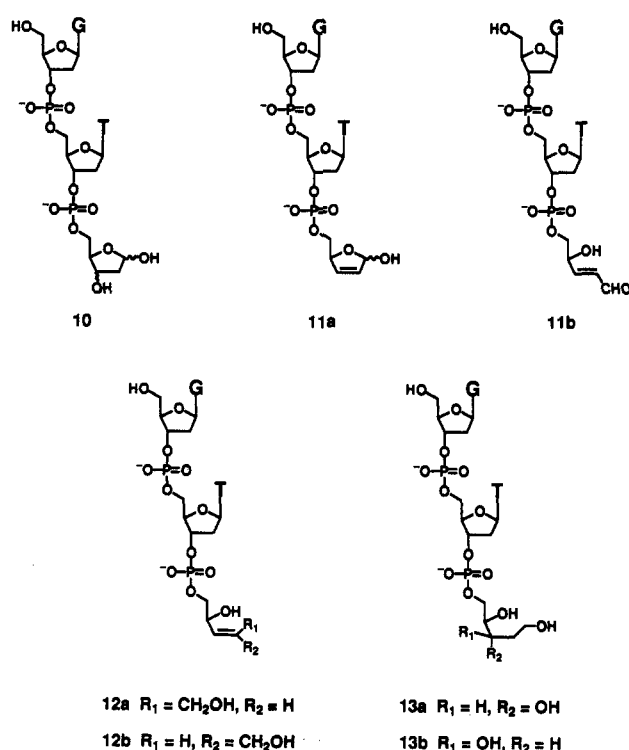
**Mechanism of DNA Cleavage.** Agarose gel electrophoretic results of pBR322 plasmid DNA treated with dienone **2** suggest formation of the single-strand breaks in DNA. Of special interest is the fact that this DNA cleavage requires no thermal or base treatments and causes under physiological conditions. In order to obtain information on a possible mechanism for the DNA strand scission induced by dienone **2**, the positions of the strand breakage were investigated by examination of the Maxam-Gilbert sequencing gels. By the incubation for 25 h, the cleavage sites were detected only at adenine residues (Figure 2, lane 4). Further incubation for 130 h additionally induced the cleavage at guanine residues (Figure 2, lane 5). This observation indicates that the spontaneous DNA cleavage occurs primarily at adenine residues.

(18) Under the conditions employed for the NaBH<sub>4</sub> reduction of hemiacetal **11a** and aldehyde **11b** to allyl alcohols **12a** and **12b**, the model compound **i** afforded the *cis*-allyl alcohol exclusively. Therefore, during the NaBH<sub>4</sub> reduction, isomerization of **11a** to **11b** would not take place.



**i**

Chart 2



Dienone **2** was previously reported to alkylate purine residues at N-7 of guanine and N-3 of adenine.<sup>7</sup> The depurination rates of adducts **4** and **5** (*t*<sub>1/2</sub>: 3 h and 3.2 h)<sup>19</sup> strongly suggest that the spontaneous DNA strand scission in the present case is due in large part to facile depurination of the N-alkylated adenine residues (Scheme 1). The N-modification of adenine bases with dienone **2** weakens the N-glycosidic linkage between the alkyladenine adducts and the deoxyribose backbone, and accordingly the alkyladenine adducts are gradually eliminated from the deoxyribose moiety. Whereas, the alkylguanine adducts are more stable under physiological conditions. The resulting abasic sites are so unstable that they are decomposed by a β-elimination reaction under physiological conditions, necessarily causing DNA strand breaks (Scheme 3).

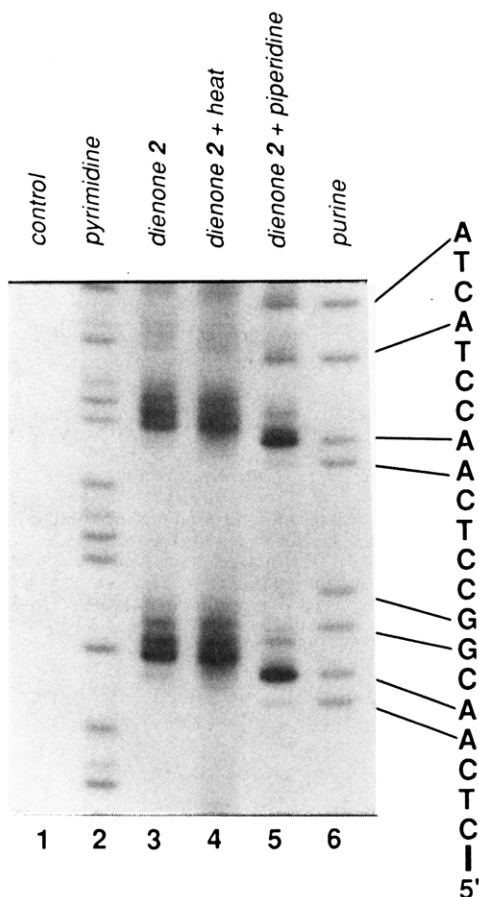
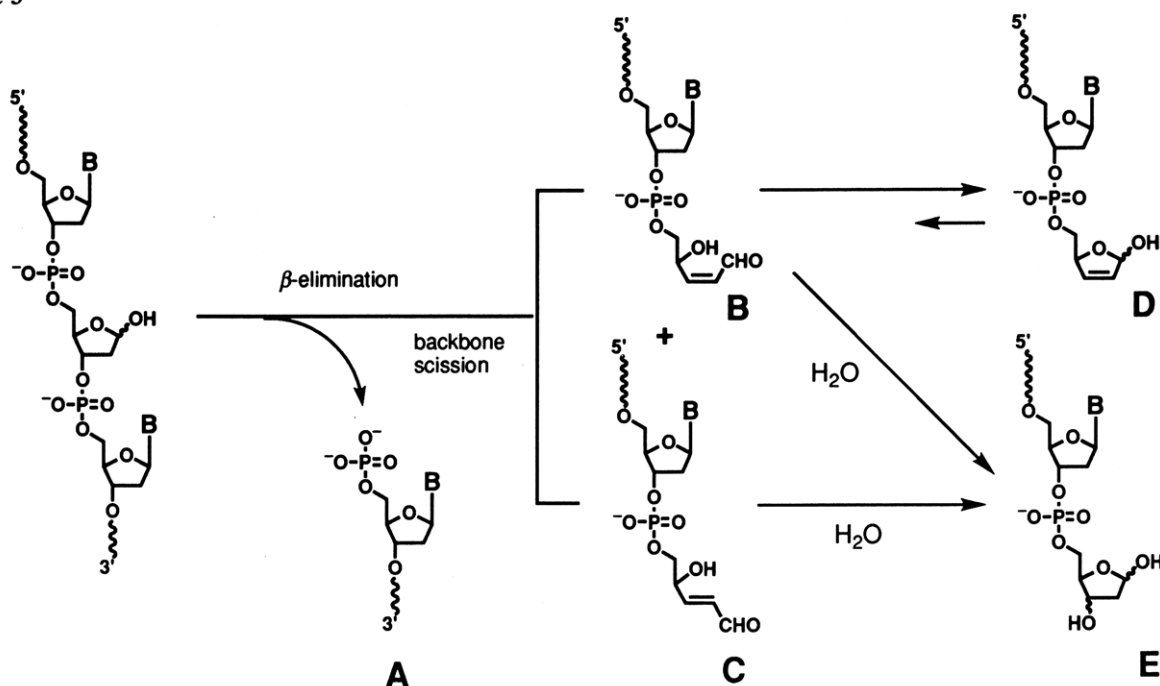
What terminal structures do the DNA fragments bear after the β-elimination reaction? While the CC-1065-directed thermal cleavage was shown to result in production of a 5'-phosphate, the identity of the 3'-end terminus is unclear.<sup>12a</sup> In this study, we have fully determined both the 3'- and 5'-terminal structures derived from abasic sites after the β-elimination reaction. The data (Figure 3, lane 3) clearly show electrophoretic comigration of the 3'-end-labeled fragments with a Maxam-Gilbert marker that is known to contain a 5'-phosphate. Thus, the 5'-terminal structure is a phosphate. On the other hand, the 5'-labeling experiment in Figure 4 demonstrates formation of three kinds of 3'-end termini. The 3'-terminal structures were unambiguously determined to be **10**, **11a**, and **11b** by using a DNA model system, d(GTAC).

The above findings allow us to propose a molecular mechanism of the DNA cleavage by dienone **2**. In the first step, dienone **2** forms covalent adducts through N-3 of adenine or N-7 of guanine with opening of the cyclopropyl ring. Under physiological conditions, spontaneous cleavage of the N-glycosidic linkage

(19) The loss of adducts from **4** and **5** comes from single-stranded DNA.<sup>16</sup> In general, the rates of depurination of alkyladenine and alkylguanine adducts from single-stranded DNA are thought to be faster than those observed for double-stranded DNA. Indeed, the *t*<sub>1/2</sub>'s of depurination of dienone-guanine and dienone-adenine adducts from double-stranded calf thymus DNA were determined by HPLC to be 40 and 18 h, respectively. In either case, however, the dienone-adenine adducts show a more rapid rate of depurination than the dienone-guanine adducts.



Scheme 3



**Figure 4.** Analysis of 3'-termini on a 15% sequencing gel. The 5'-labeled *SphI*-*Bam*HI fragment from pBR322 was treated with dienone 2 at 37 °C for 25 h (lane 3). The ratio [2]/[DNA nucleotide] was 6.5. After the treatment, DNA samples were subjected to thermal treatment (lane 4) or piperidine treatment (lane 5).<sup>15</sup> Lane 1 shows no drug treatment. Lanes 2 and 6 are the Maxam–Gilbert pyrimidine and purine sequencing ladders.

occurs mainly at the modified adenine residues to produce abasic sites.<sup>20</sup> The abasic sites show a series of evolution as indicated in Scheme 3.<sup>21</sup> An abasic site is so unstable that subsequent

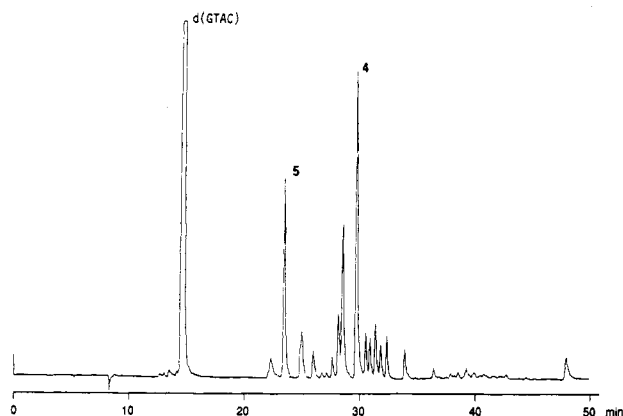
backbone breakage occurs via a  $\beta$ -elimination reaction to leave a 5'-phosphate on the 3'-side of the break (A) and a *cis* (or *trans*)  $\alpha,\beta$ -unsaturated aldehyde on the 5'-side (B or C). The *cis*  $\alpha,\beta$ -unsaturated aldehyde B would predominantly exist in the cyclic hemiacetal form (D), while the *trans* isomer C would remain to be in the ring-opened form. A portion of B and C are further converted into diol E by the Michael addition of water. All of the products can also be transformed by piperidine treatment into a single product, a 3'-phosphate, through a mechanism of piperidine-catalyzed  $\beta$ -elimination followed by a vinylogous  $\beta$ -elimination. Therefore, the triplet in Figure 4 is presumed to consist of C, D, and E (see Scheme 3).

**Sequence Preference.** To explore sequence preference of the DNA cleavage induced by dienone 2, over 1000 base pairs were examined by using many fragments of pBR322 DNA such as *Bam*HI-*Sal*I, *Sal*I-*Bam*HI, *Sph*I-*Sal*I, and *Bam*HI-*Sph*I restriction fragments. The results reveal that both 5'- and 3'-flanking nucleotides affect the cleavage rates at target adenines. The rank orders are 5'-AT > 5'-AG > 5'-AC > 5'-AA for 3'-flanking nucleotides and 5'-AA > 5'-TA > 5'-GA > 5'-CA for 5'-flanking nucleotides (where A is a site of cleavage). Interestingly, 5'-adenine residues in 5'-AA steps are not cleaved (as shown in lane 5 of Figure 4), while 3'-adenine residues in 5'-AA steps are most preferentially attacked. The most preferable sequence of dienone 2 was estimated to be 5'-AAAT. This preference is considerably similar to that of CC-1065; a consensus sequence analysis of CC-1065 binding site on DNA has revealed two distinct classes of sequences, 5'-PuNTTA and 5'-AAAAA.<sup>12a,22</sup>

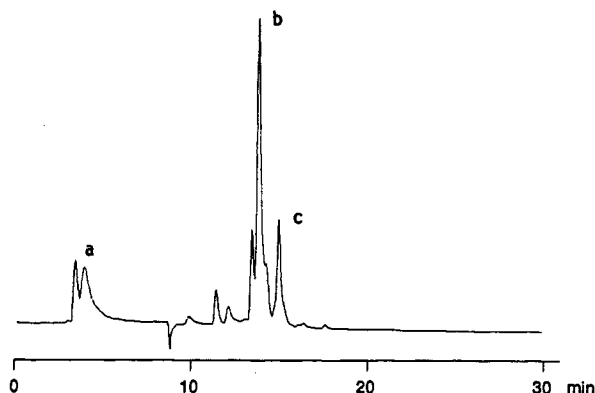
(20) NMR studies have shown that the predominant form of the abasic site is a 40:60 mixture of  $\alpha$ - and  $\beta$ -hemiacetals and that the ring-opened aldehyde tautomer represents less than 1% of the total abasic sites. (a) Manoharan, M.; Ransom, S. C.; Mazumder, A.; Gerlt, J. A.; Wilde, J. A.; Withka, J. A.; Bolton, P. H. *J. Am. Chem. Soc.* **1988**, *110*, 1620–1622. (b) Wilde, J. A.; Bolton, P. H.; Mazumder, A.; Manoharan, M.; Gerlt, J. A. *J. Am. Chem. Soc.* **1989**, *111*, 1894–1896.

(21) As to the cleavage mechanism of the deoxyoligonucleotide possessing an abasic site, Sugiyama et al. has reported similar results: Sugiyama, H.; Tashiro, T.; Hosoda, M.; Saito, I. *Abstracts of Papers II*, 61st National Meeting of the Chemical Society of Japan, Yokohama; Chemical Society of Japan: Tokyo, 1991; 1B133.

(22) (a) Hurley, L. H.; Warpehoski, M. A.; Lee, C.-S.; McGovern, J. P.; Scallion, T. A.; Kelly, R. C.; Mitchell, M. A.; Wicnienski, N. A.; Gebhard, I.; Johnson, P. D.; Bradford, V. S. *J. Am. Chem. Soc.* **1990**, *112*, 4633–4649. (b) Reynolds, V. L.; McGovern, J. P.; Hurley, L. H. *J. Antibiot.* **1986**, *39*, 319–334. (c) Hurley, L. H.; Reynolds, V. L.; Swenson, D. H.; Petzold, G. L.; Scallion, T. A. *Science* **1984**, *226*, 843–844.



**Figure 5.** HPLC analysis of carcinogen-d(GTAC) adducts. Analysis was performed on a Develosil ODS-5 column (4.6 × 250 mm), elution with 0.02 M  $\text{NH}_4\text{OAc}$ -acetonitrile (100:0 → 50:50, 50 min linear gradient) at a flow rate of 1 mL/min.



**Figure 6.** HPLC analysis of cleavage reaction of depurinated deoxytetranucleotide 7. Conditions are described in Figure 5.

This analogy of selectivity is surprising because CC-1065 is believed to present an affinity site inside of the minor groove.<sup>23</sup> In dienone **2**, it appears to be only a chemical reactivity rather than a preliminary binding of the drug within the DNA grooves that governs the adduct formation. There is a possibility that 3'-adenine residues in a sequential AT run are especially reactive with a cyclopropyl ring.

**Comparison with CC-1065.** It is proposed that the DNA cleavage by dienone **2** and CC-1065 occurs through a similar

(23) (a) Boger, D. L.; Munk, S. A.; Zarrinmayeh, H. *J. Am. Chem. Soc.* **1991**, *113*, 3980–3983. (b) Lin, C. H.; Beale, J. M.; Hurley, L. H. *Biochemistry* **1991**, *30*, 3597–3602. (c) Scallan, T. A.; Jansen, R. M.; Swenson, J. D.; Hatzenbuehler, N. T.; Petzold, G.; Wierenga, W.; Brahme, N. D. *Biochemistry* **1990**, *29*, 2852–2860.

mechanism, namely depurination of the alkyladenine adducts. However, there are significant differences observed in the characteristics for the DNA damage between dienone **2** and CC-1065: (1) dienone **2** forms adducts at guanine residues in addition to adenine residues and (2) dienone **2** induces the spontaneous cleavage at adenine base sites under physiological conditions, whereas CC-1065 causes the cleavage at higher temperature (>70 °C).<sup>12a</sup> The covalent adenine-adduct of CC-1065 has been reported to undergo a retrohomologous Michael reaction to regenerate the initial cyclopropylpyrroloindole structure and, presumably, intact DNA.<sup>24</sup> This reaction occurs to a significant extent at 37 °C in neutral aqueous solution, and at higher temperature competes with depurination of the N-3 alkyladenine. In contrast, the N-3 adenine adduct of dienone **2** appears to depurinate spontaneously at 37 °C within 25 h.

Ptaquiloside (**1**) is a typical carcinogen, whereas CC-1065 is an antitumor agent. The biological action of both the compounds is believed to be attributed to their ability to bind covalently to DNA. The different effects between ptaquiloside (**1**) and CC-1065 may be due to significant distinction in the stability of the alkylpurine adducts and the reactivity toward adenine and guanine residues. It is well documented that N-3 alkyladenine adducts are particularly toxic unless repaired<sup>25</sup> and that spontaneous apurinic sites are rapidly repaired. Therefore, the N-7 guanine adduct of dienone **2**, as it is with aflatoxin,<sup>26</sup> may be responsible for the mutagenicity and carcinogenicity of ptaquiloside (**1**).

**Acknowledgment.** This work was supported by Grants-in-Aid for Scientific Research on Priority Area (No. 04220106 & 04226205) and for Scientific Research (No. 04403009) from the Ministry of Education, Science, and Culture, Japan and by the grant from the Asahi Glass Foundation (to H.K.). A fellowship of the Japan Society for the Promotion of Science to M.U. is also acknowledged.

**Supplementary Material Available:** Experimental procedures for the synthesis of **10**, **11a**, **11b**, and **12a** (11 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(24) Warpehoski, M. A.; Harper, D. E.; Mitchell, M. A.; Monroe, T. J. *Biochemistry* **1992**, *31*, 2502–2508.

(25) (a) Sun, D.; Hurley, L. H. *Biochemistry* **1992**, *31*, 2822–2829. (b) Weiland, K. L.; Dooley, T. P. *Biochemistry* **1991**, *30*, 7559–7565.

(26) Aflatoxin is among the most powerful mutagens in bacterial assays [McCann, J.; Spingarn, J. E.; Kobori, J.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 979–983]. This mutagen reacts almost exclusively with the guanine N-7 position, and this reaction is believed to be responsible for its toxic, mutagenic, and carcinogenic properties [Busby, W. F., Jr.; Wogan, G. N. In *Chemical Carcinogenesis*; Searle, C. E., Ed.; American Chemical Society: Washington, DC, 1984; pp 945–1136].