Chemistry of Thermal Degradation of Abasic Sites in DNA. Mechanistic Investigation on Thermal DNA Strand Cleavage of Alkylated DNA

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Received May 12, 1994®

The chemistry of thermal degradation of aldehydic abasic sites in DNA was investigated. Sequencing gel analysis of duocarmycin A-treated 5′-^{32}P-end-labeled DNA fragment indicated that upon heating at neutral pH alkylated DNA was cleaved to provide fragments possessing a modified sugar moiety which is readily decomposed to 3′-phosphate terminus by piperidine treatment. To identify the structure of modified sugar product and to investigate the mechanism of thermal cleavage, thermal degradation of various oligonucleotides containing abasic sites was investigated in detail. It was found that heating the DNA containing an abasic site induces β-elimination to provide 3′-termini possessing a *trans*-α,β-unsaturated aldose residue together with 5′-phosphate termini. Upon prolonged heating at pH 7.0, the *trans*-α,β-unsaturated aldose terminus is isomerized to a *cis* isomer or is further degraded to its hydrated products and a 3′-phosphate terminus via δ-elimination. This type of thermal degradation also occurs in the abasic site-containing calf thymus DNA. Investigation of the stereochemical course of the thermal β-elimination reaction using a 2-*pro-R*-D-containing abasic site has demonstrated that the reaction proceeds via a *syn*-elimination process as observed for the enzymatic reaction of UV endonuclease V and endonuclease III.

Introduction

Hydrolytic cleavage of the *N*-glycoside bond of a purine base in DNA generates a baseless site, called an "abasic" site. Abasic sites in DNA are intermediates in the base excision repair pathway initiated by the action of DNA *N*-glycosylases and are also formed spontaneously under physiological conditions (*1–3*). Abasic sites have been shown to be mutagenic in transcription and to be repaired by the action of a series of enzymes (*4*). The structures in solution and the enzymatic repair mechanism of these DNA lesions have been extensively investigated (*5–9*). The predominant form of the abasic site is a 40:60 mixture of α- and β-hemiacetals (*5*), and the proportion of ring-opened aldehydic tautomer is less than 1% (*7*). Recently, Gerlt and co-workers have demonstrated that DNA containing an abasic site is cleaved by repair enzymes such as UV endonuclease V (*6, 8*) or endonuclease III (*9*) to give *trans* α,β-unsaturated aldose products by *syn*-β-elimination mechanisms. In contrast, they demonstrated that the degradation under alkaline conditions or in the presence of tripeptide Lys-Trp-Lys proceeds by *anti*-β-elimination mechanisms (*9*).

Abasic sites are chemically generated by alkylating agents (*10*) and by ionizing radiation (*11*), as well as simply treatment by acid or base (*1, 12*). Recently, DNA alkylation by antitumor antibiotics and related synthetic drugs has been intensively studied at the molecular level (*13–24*). Minor groove alkylating antitumor antibiotics such as CC-1065 (*25–27*) and duocarmycin A (*28–33*) have been shown to alkylate duplex DNA specifically at the N3 nitrogen of adenine at the 5′ side of three or four consecutive A·T base pairs. Sequence specificity for the DNA alkylation by these drugs can be determined by high-resolution polyacrylamide gel electrophoresis after thermal elimination of drug–base adducts followed by piperidine treatment, which is equivalent to the Maxam–Gilbert chemical sequencing reaction (*29, 35*). While strand cleavage at the alkylated sites is accomplished simply by heating at neutral pH (100 °C for 30 min) (*7, 26, 28–30*), the mechanism of strand scission and the structures of the modified sugar moieties have not been elucidated.

In recent years we have been investigating the chemistry associated with DNA cleavage induced by antitumor antibiotics by utilizing oligonucleotides of defined sequences as DNA substrates (*36–40*). We have demonstrated that the cyclopropane subunit of duocarmycin A alkylates N3 of adenine$_6$ (A_6) of d(CGTATACG)$_2$ to provide the metastable covalent adduct **1** ($t_{1/2}$ 7.5 h at 37 °C) (*15*). Upon brief heating (90 °C, 5 min) at pH 7.0, **1** was decomposed to an abasic site-containing oligomer **3** with concomitant release of duocarmycin A–adenine adduct **2** (Scheme 1) (*15*). We have also found that under prolonged heating conditions (100 °C, 30 min) **3** is decomposed to d(pCG) and d(CGTATp), the latter bearing a modified sugar fragment at the 3′ end (*15*). In the present study, we have clarified the chemical structures of the products obtained from the thermal degradation of oligodeoxynucleotides containing abasic sites. Furthermore, we have demonstrated for the first time (i) that, under heating conditions, abasic sites in DNA selectively decompose to 3′-termini possessing a *trans*-α,β-unsaturated aldose residue and 5′-phosphate termini via *syn*-β-elimination and (ii) that the resulting *trans*-α,β-unsaturated aldose terminus is converted to a *cis*

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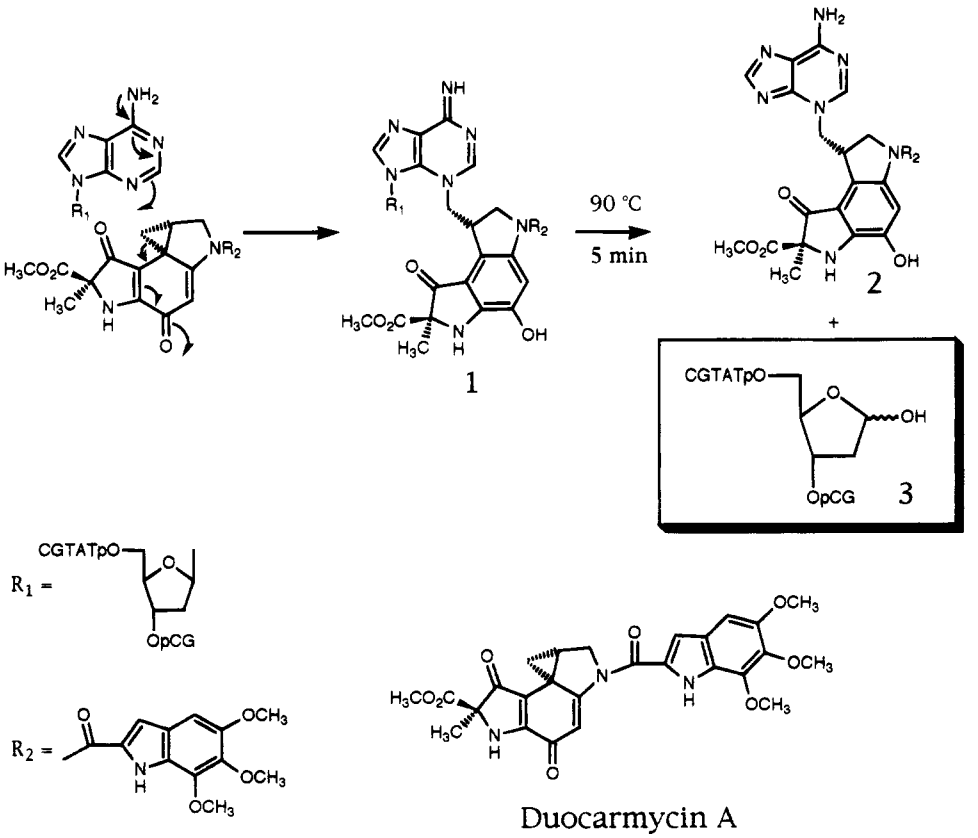
® Abstract published in *Advance ACS Abstracts,* August 15, 1994.

0893-228x/94/2707-0673$04.50/0

Scheme 1



isomer and their hydrates upon prolonged heating at neutral pH.

Experimental Section

Materials and Methods. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Du Pont–New England Nuclear (Boston, MA). Restriction enzymes (*Ava*I, *Xba*I) and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). Calf intestine alkaline phosphatase (AP, 1000 unit/mL) and snake venom phosphodiesterase (s.v. PDE;[1] 3 units/mL) were purchased from Boehringer Mannheim (Mannheim, Germany). The 2′-*pro*-*R*-*D*(86%)-2′-deoxyadenosine was prepared by the reported method (*41*). *N*-Benzoylation and dimethoxytritylation of 2′-*pro*-*R*-D-2′-deoxyadenosine were carried out according to the reported procedure (*42*). Calf thymus DNA was purchased from Pharmacia P-L Biochemicals (Milwaukee, WI) and sonicated to a 150–200-base-pair range by the reported procedure (*43*), whose concentration was determined by complete digestion with s.v. PDE and AP to 2′-deoxymononucleosides. Duocarmycin A was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). The concentration of duocarmycin A was determined spectrophotometrically (ϵ_{358} = 28 000 mM^{-1} cm^{-1} in methanol) (*44*). The Cosmosil 5C$_{18}$ column was purchased from Nacalai Tesque Inc. (Kyoto, Japan). The μBondasphere 5C$_{18}$ column was purchased form Millipore Waters (Milford, MA). The Ultron 5C$_{18}$ column was obtained form Shinwa Chemical Industries (Kyoto, Japan). The CHEMCOBOND 5-ODS-H column was purchased from Chemco (Osaka, Japan). The YMC-AQ 302 column was purchased from YMC (Kyoto, Japan). Preparative TLC was carried out on Merck silica gel 60 PF$_{254}$ plates. 2,5-Dimethoxy-2,5-dihydrofurfuryl 2′-deoxy-3′-cytidylate was prepared by the reported procedure (*45*). 3′-*O*-[(5*R*)-2-Hydroxy-2,5-dihydrofurfuryl] 2′-deoxycytidylate (*12*) was prepared by photoisomerization of **11** according to the reported procedure (*9*). ^{1}H NMR spectra were recorded on a JEOL-JNM-GX400 spectrometer.

[1] Abbreviations: s.v. PDE, snake venom phosphodiesterase; AP, alkaline phosphatase; FABMS, fast atom bombardment mass spectroscopy.

Preparation of DNA Fragments. The 5′-^{32}P-end-labeled DNA fragment was prepared from plasmid pbcN1, which carries a 6.6-kilobase *Bam*HI chromosomal DNA segment containing human c-Ha-*ras*-1 protooncogene (*46*), according to the method described previously (*47*). A singly labeled 261-base-pair fragment (*Ava*I* 1645–*Xba*I 1965) was obtained. The asterisk indicates that ^{32}P-labeling and nucleotide numbering starts with the *Bam*HI site (*46*).

DNA Cleavage by Duocarmycin A. A standard reaction mixture in a microtube (Eppendorf) contained [^{32}P]DNA fragment, sonicated calf thymus DNA (50 μM nucleotide), and duocarmycin A (5 μM) in 50 mM sodium cacodylate buffer (pH 7.0). After incubation of the mixture at room temperature for 1 h, duocarmycin A-treated DNA was isolated by ethanol precipitation and the workup procedure indicated in Figure 1. The sample solutions were electrophoresed on an 18 × 50 cm gel using a DNA sequencing system (LKB 2010 Macrophor) as previously described (*48*). Autoradiograms were obtained by exposure of X-ray film to the gel.

Synthesis of 1-*O*-Methyl-5-*O*-(dimethoxytrityl)-2-deoxy-D-ribose (25). 2-Deoxyribose (1.0 g, 7.46 mmol) was dissolved in 100 mL of methanol. To this solution were added 50 μL of concentrated HCl and 1 g of molecular sieves, and the solution was stirred overnight. The reaction mixture was filtered, and the solution was concentrated under reduced pressure. The residue was dried by coevaporation with dry pyridine three times. The residue was dissolved in 100 mL of dry pyridine, and then 3.8 g (11.2 mmol) of 4,4′-dimethoxytrityl chloride was added to the reaction mixture. The solution was stirred overnight at room temperature. The reaction mixture was concentrated, and the residue was purified by silica gel chromatography. The diastereomeric mixture of **25** was obtained as a yellow syrup: yield 2.1 g (58%). ^{1}H NMR [$CDCl_3$, $(CH_3)_4$-Si] δ 1.97–2.30 (m, 2 H), 3.12–3.22 (m, 2 H), 3.28–3.39 (m, 3 H), 3.79 (s, 6 H), 3.90–4.22 (m, 2 H), 5.02–5.16 (m, 1 H), 6.82 (d, 4 H, J = 9.0 Hz), 7.31–7.34 (m, 4 H), 7.20–7.50 (m, 5 H); exact mass calcd for $C_{27}H_{30}O_6$ 450.20424, found 450.20358.

Synthesis of Deoxyhexanucleotides. Oligonucleotides were prepared by the β-cyanoethyl phosphoramidite method (*49*) on controlled-pore glass supports (1 μmol) by using an ABI 381

A DNA synthesizer. 2-Cyanoethyl phosphoramidites of 1-*O*-methyl-5-*O*-(dimethoxytrityl)-2-deoxy-D-ribose (**25**) and 2′-*pro*-*R*-D-2′-deoxyadenosine were prepared by the procedure of van Boom (*50*) and directly used in an automated DNA synthesizer without further purification. The precursor of the protected trimer **24** was prepared by using 3′-*O*-[methoxy(*N*,*N*-diisopropylaminyl]phosphino-5′-*O*-(4,4′-dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine. After automated synthesis, the oligomer was detached from the support and deprotected without thiophenol treatment as described previously (*40*). After lyophilization, approximately 20 OD of pure oligomer was isolated. The purity and concentration of all oligodeoxynucleotides were determined by complete digestion with s.v. PDE and AP to 2′-deoxymononucleosides.

Thermal Degradation of Abasic Site-Containing Oligomer from Duocarmycin A Reaction. A reaction mixture (total volume 98 μL) containing d(CGTATACG) (1.02 mM base concentration) in 51 mM sodium cacodylate buffer (pH 7.0) was prepared. To this reaction mixture was added 2 μL of duocarmycin A stock solution (5 mM in dimethylformamide) to initiate the reaction. The reaction mixture was incubated at 0 °C for 12 h and then heated at 90 °C for 5 min. A 10-μL aliquot was then subjected to HPLC analysis (Wakosil $5C_{18}$ DNA column; 0.05 M ammonium formate containing 0−7% acetonitrile/20-min linear gradient; 7% (isocratic)/20−30 min; flow rate 1.0 mL/min) for the detection of the abasic site-containing oligomer **3** (Figure 2a). The rest of the reaction mixture (40 μL) was heated at 90 °C for 30 min, and an aliquot (10 μL) was subjected to HPLC analysis. The result is shown in Figure 2b. The formation of pd(CG) and d(CGTAT)p was confirmed by the comparison with the authentic materials. The sharp peak eluted at 16.6 min was found to be d(CG) which is assumed to be formed from p(CG) by dephosphorylation. The reaction mixture (30 μL) then was treated with 0.1 M $NaBH_4$ at room temperature for 15 min. After quenching the reaction with 3 μL of 1 N acetic acid, the solution was subjected to HPLC analysis. The HPLC peak at 24.7 min corresponding to **5** was collected, and the fractions were concentrated. The residue was dissolved in water and then subjected to enzymatic digestion with s.v. PDE and calf intestine AP. After incubation at 37 °C for 2 h, the mixture was subjected to HPLC analysis. The formation of **6** together with dC, dG, dT, and dA was observed.

Formation of 3′-*O*-[(2″*R*)-2″,5″-Dihydroxy-*trans*-3″-pentenyl] Thymidylate (6) from d(TAT). A solution (500 μL) containing d(TAT) (10 mM base concentration) in 0.1 N HCl was heated at 90 °C for 5 min. HPLC analysis of a 10-μL aliquot indicated the formation of abasic site-containing trimer **7**. A 49-μL aliquot of 1 M NaOH was added to neutralize the reaction mixture. To this solution was added 49 μL of 500 mM sodium cacodylate buffer (pH 7.0), and the resulting solution was heated at 90 °C for 40 min. A solution (66 μL) of 1 M $NaBH_4$ was added. The solution was kept standing at room temperature for 15 min, and then the reaction was quenched with 1 M acetic acid. The resulting solution was concentrated and the residue was purified by HPLC (Cosmosil $5C_{18}$ column; 0.05 M ammonium formate containing 2−6% acetonitrile/20-min linear gradient; flow rate 1.5 mL/min; retention time 13.7 min). After lyophilization, white powder was obtained: **6**; yield 0.5 mg (70%). ^{1}H NMR (D_2O) δ 1.74 (d, 3 H, J = 1.0 Hz, $5CH_3$), 2.26 (ddd,1 H, J = 13.3, 6.7, 3.2 Hz, 2′), 2.39 (ddd, 1 H, J = 13.3, 6.5, 3.2 Hz, 2′), 3.61−3.78 (m, 5 H, 5′, 1″, 2″), 3.96 (dd, 2 H, J = 5.4, 1.5 Hz, 5″), 4.04 (dd, 1 H, J = 7.8, 3.3 Hz, 4′), 4.21−4.26 (m, 1 H, 3′), 4.80 (ddt, 1 H, J = 15.1, 6.2, 1.5 Hz, 3″), 5.80 (dtd, 1 H, J = 15.1, 5.4, 1.1 Hz, 4″), 6.16 (dd, 1 H, J = 6.7, 6.5 Hz, 1′), 7.50 (d, 1 H, J = 1.0 Hz, 6); FABMS (positive ion) m/z 423 [M + 1].

Thermal Degradation of 10 at pH 7.0. A solution (200 μL) containing **9** (333 μM) in 0.1 N HCl was heated at 90 °C for 5 min. The solution was then neutralized by the addition of 20 μL of 0.9 N NaOH and 20 μL of 500 mM sodium cacodylate (pH 7.0). The HPLC profile of the aliquot (12 μL) indicates the formation of **10** (Figure 3a). The solution is heated at 90 °C for 45 min. HPLC analysis of the aliquot (12 μL) is shown in Figure 3b. The reaction mixture was treated with 0.1 M $NaBH_4$ at room temperature for 15 min and then quenched by addition of 1 N CH_3COOH. The HPLC profile of the aliquot (14 μL) is shown in Figure 3c.

Isolation of 2′-Deoxycytidylyl-(3′−5″)-(4″*R*)-4″-hydroxy-*trans*-2″-pentenal (11) and 2′-Deoxycytidylyl-(3′−5″)-(4″*R*)-4″-hydroxy-*cis*-2″-pentenal (12). d(CGT) (2 mg) was dissolved in 3 mL of 0.1 N HCl, and the reaction mixture was heated at 90 °C for 5 min. To neutralize this mixture, 270 μL of 1 N NaOH and 650 μL of 500 mM of sodium cacodylate (pH 7.0) were added. The solution was heated at 90 °C for 30 min. HPLC analysis of aliquots (10 μL) indicated the formation of **11**. The fraction with a retention time of 7.8 min was collected (Ultron $5C_{18}$ column; 0.05 M triethylammonium acetate, pH 7.0, containing 5% acetonitrile; flow rate 1.2 mL/min). To remove acetonitrile, the combined fractions were evaporated for 30 min at room temperature and subjected to HPLC desalting (μBondasphere $5C_{18}$ column; 100% H_2O for 30 s, then 20% acetonitrile; flow rate 10 mL/min). After lyophilization, white powder (0.5 mg) of a mixture of **11** and **12** was obtained. Two isomers were separated by HPLC (CHEMCOBOND 5-ODS-H column; 0.05 M ammonium formate; flow rate 1.0 mL/min; retention times: **11**, 62 min; **12**, 76 min). After HPLC desalting (CHEMCOBOND 5-ODS-H column; 100% H_2O for 3 min, then 20% acetonitrile; flow rate 1 mL/min) an aqueous solution of **11** and **12** was obtained and used for thermal degradation study. After lyophilization of the rest of fractions containing **11**, white powder of **11** (0.2 mg) was obtained. ^{1}H NMR (D_2O) δ 2.33 (m, 2′), 2.54 (m, 2′), 3.77 (m, 5′), 3.97 (m, 1″, 2″), 4.28 (m, 4′), 4.70 (m, 3′), 6.08 (d, J = 6.8 Hz, 6), 6.24 (m, 1′), 6.87 (ddd, J = 15.7, 8.1, 1.8 Hz, 4″), 7.10 (ddd, J = 15.7, 4.1, 1.6 Hz, 3″), 7.83 (d, J = 6.8 Hz, 5), 9.45 (dd, J = 8.1, 1.6 Hz, 5″).

Thermal Degradation of Isolated 11 and 12. A solution containing 100 μM **11** or **12** in sodium cacodylate (pH 7.0) was heated at 90 °C for 1 h. A 10-μL aliquot was treated with 0.1 M $NaBH_4$ at room temperature for 15 min and then subjected to HPLC analysis after quenching the reaction with 1 μL of 1 N acetic acid (CHEMCOBOND 5-ODS-H column; 0.05 M ammonium formate; flow rate 1.0 mL/min). The results are summarized in Tables 1 and 2.

Thermal Degradation of 15b. A solution containing 100 μM of **15b** in sodium cacodylate (pH 7.0) was heated at 90 °C for 26 h. A 10-μL aliquot was treated with 0.1 M $NaBH_4$ at room temperature for 15 min and then subjected to HPLC analysis after quenching the reaction with 1 μL of 1 N acetic acid (CHEMCOBOND 5-ODS-H column; 0.05 M ammonium formate; flow rate 1.0 mL/min).

Preparation of Authentic 15b. A solution containing d(CA) (1.0 mM) was dissolved in 1 mL of 0.1 N HCl, and the reaction mixture was heated at 90 °C for 5 min. After neutrarization, the aliquot (10 μL) was subjected to HPLC analysis. The fraction with a retention time of 5.0 min was collected (Cosmosil $5C_{18}$ column; flow rate 1.0 mL/min). After lyophilization, white powder was obtained: **15b**; yield 0.1 mg. The purity of **15b** was more than 95% as determined by HPLC. ^{1}H NMR (D_2O) δ 1.87 (m, 2″β), 2.15 (m, 2″α), 2.34 (m, 2′), 2.41 (m, 2″β), 2.57 (m, 2′), 3.77 (dd, J = 7.2, 2.5 Hz, 5′), 3.83 (dd, 7.2, 1.9 Hz, 5′), 3.92 (m, 5″), 4.01 (m, 4″β), 4.22 (m, 4′, 4″α), 4.32 (m, 3″β), 4.43 (m, 3″α), 4.74 (m, 3′), 5.55 (m, 1″β), 5.59 (m, 1″α), 6.03 (d, J = 7.7 Hz, 6), 6.27 (m, 1′), 7.80 (d, J = 7.7 Hz, 5).

Isolation of 3′-*O*-[(2″*R*)-2″,5″-Dihydroxy-*trans*-3″-pentenyl] 2′-Deoxycytidylate (13). To a heated solution (660 μL) containing **11** from the experiment described above was added 66 μL of 1 M $NaBH_4$. The solution was allowed to stand at room temperature for 15 min, and the reaction was quenched with 66 μL of 1 M acetic acid. The resulting solution was concentrated, and the residue was purified by HPLC (Cosmosil $5C_{18}$ column; 0.05 M ammonium formate containing 0−10% acetonitrile/15-min linear gradient; flow rate 1.5 mL/min; retention time 7.9 min). After lyophilization, a white powder was obtained: yield 0.2 mg. **13**: ^{1}H NMR (D_2O, TSP) δ 2.19 (ddd, 1H, J = 12.9, 7.2, 6.9 Hz, 2′), 2.44 (ddd, 1 H, J = 12.9, 6.4, 3.5 Hz, 2′), 3.59−3.78 (m, 5 H, 5′, 1″, 2″), 3.96 (d, 2 H, J = 5.3 Hz, 5″), 4.07 (dd, 1 H, J = 7.6, 4.0 Hz, 4′), 4.22−4.27 (m, 1 H, 3′),

5.60 (dd, 1 H, J = 15.6, 6.3 Hz, 3″), 5.79 (ddd, 1 H, J = 15.6, 5.3, 5.3 Hz, 4″), 5.90 (d, 1 H, J = 7.6 Hz, 5), 6.15 (dd, 1 H, J = 6.9, 6.4 Hz, 1′), 7.68 (d, 1 H, J = 7.6 Hz, 6); FABMS (negative ion) m/z 406 $(M - 1)^-$.

Synthesis of 5″-*O*-[3′-(2′-Deoxycytidylyl)]-2″-deoxy-D-*erythro*-pentitol (18a) and 5″-*O*-[3′-(2′-Deoxycytidylyl)]-2″-deoxy-L-*threo*-pentitol (18b). 50 mg of N^4-benzoyl-5′-*O*-(dimethoxytrityl)-3′-*O*-(2-chlorophenyl)-2′-deoxycytidylyl-(3′−5′)-N^2-isobutyryl-2′-deoxyguanosine-3′-*O*-(2-chlorophenyl phosphate) (**19**) was treated with concentrated aqueous ammonia−pyridine (2:1, 30 mL) at 65 °C for 4 h and then concentrated. The residue 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 purified by HPLC (Cosmosil $5C_{18}$ column; 0.05 M ammonium formate containing 0−20% acetonitrile/20-min linear gradient; flow rate 1.5 mL/min; retention time 20 min). After lyophilization, 2′-deoxycytidylyl-(3′−5′)-2′-deoxyguanosine-3′-*O*-(2-chlorophenyl phosphate) (**20**) was obtained as a white powder: yield 5 mg (19%). **20**: ^{1}H NMR (D_2O, TSP) δ 1.38−1.42 (m, 1 H), 2.36 (dd, 1 H, J = 13.8, 5.2 Hz), 2.68 (dd, 1 H, J = 13.0, 4.8 Hz), 2.89−2.95 (m, 1 H), 3.60−3.70 (m, 2 H), 3.98−4.14 (m, 3 H), 4.58−4.62 (m, 2 H), 5.14−5.19 (m, 1 H), 5.89 (d, 1 H, J = 7.6 Hz), 6.06 (dd, 1 H, J = 9.8, 4.8 Hz), 6.11 (dd, 1 H, J = 9.0, 5.2 Hz), 6.78 (t, 1 H, J = 7.8 Hz), 7.10 (t, 1 H, J = 7.8 Hz), 7.18 (d, 1 H, J = 7.8 Hz), 7.31 (d, 1 H, J = 7.8 Hz), 7.51 (d, 1 H, J = 7.6 Hz), 8.13 (s, 1 H). Compound **20** (5 mg) was dissolved in 1 mL of 0.1 N HCl, and the solution was heated at 90 °C for 5 min to produce **21**. To this solution were added 100 μL of 1 N NaOH and 100 μL of 500 mM sodium cacodylate (pH 7.0), and the mixture was heated at 90 °C for 30 min. To this solution was added 100 μL of 1 M $NaBH_4$ solution. After 15 min the reaction was quenched by the addition of 100 μL of 1 M CH_3COOH. The resulting solution was concentrated, and the residue was purified by HPLC (YMC-AQ 302 column; 0.05 M triethylammonium acetate (pH 7.0); flow rate 1 mL/min; retention time 26.7 and 29.5 min). After lyophilization, a white powder containing **18a** (0.6 mg, 24%) and **18b** (0.4 mg, 16%) was obtained. **18a**: ^{1}H NMR (D_2O, TSP) δ 1.45−1.65 (m, 2 H, 2″), 2.19 (ddd, 1 H, J = 14.0, 7.2, 6.8 Hz, 2′), 2.44 (ddd, 1 H, J = 14.0, 6.5, 3.2 Hz, 2′), 3.53−3.85 (m, 8 H, 5′, 1″, 3″, 4″, 5″), 4.06 −4.09 (m, 1 H, 4′), 4.56−4.74 (m, 1 H, 3′), 5.90 (d, 1 H, J = 7.6 Hz, 5), 6.13 (dd, 1 H, J = 6.8, 6.5 Hz, 1′), 7.69 (d, 1 H, J = 7.6 Hz, 6); FABMS (negative ion) m/z 410 $(M - 1)^-$. **18b**: ^{1}H NMR (D_2O, TSP) δ 1.51(ddd, 1 H, J = 19.9, 10.0, 4.5 Hz, 2″), 1.72 −1.80 (m, 1 H, 2″), 2.16−2.24 (m, 1 H, 2′), 2.44 (ddd, 1 H, J = 14.1, 6.5, 3.4 Hz, 2′), 3.54−3.73 m, 6 H, 5′, 1″, 3″, 4″), 3.74−3.81 (m, 1 H, 5″), 3.88 (ddd, 1 H, J = 11.1, 6.0, 3.2 Hz, 5″), 4.06−4.10 (m, 1 H, 4′), 4.59−4.67 (m, 1H, 3′), 5.89 (d, 1 H, J = 7.6 Hz, 5), 6.14 (dd, 1 H, J = 6.9, 6.5 Hz, 1′), 7.67 (d, 1 H, J = 7.6 Hz, 6); FABMS (negative ion) m/z 410 (M - 1)$^-$. The authentic sample of **18b** was independently prepared from d(CG) by acid depurination and subsequent $NaBH_4$ reduction.

Synthesis of 3′-*O*-[(2″*R*)-2″,5″-Dihydroxy-*cis*-3″-pentenyl] 2′-Deoxycytidylate (14). 2,5-Dimethoxy-2,5-dihydrofurfuryl 2′-deoxy-3′-cytidylate (2 mg) was dissolved in 1 mL of 0.1 N HCl, and the reaction mixture was incubated at room temperature for 1 h. A 50-μL aliquot was taken out and neutralized with 5 μL of 1 N NaOH. To the reaction mixture (950 μL) was added 200 μL of 1 M $NaBH_4$, and the reaction mixture was incubated at room temperature. The reaction was quenched by 200 μL of 1 M CH_3COOH, the solvent was evaporated to dryness, and the residue was subjected to HPLC purification (Cosmosil $5C_{18}$ column; 0.05 M ammonium formate containing 0−10% acetonitrile/20-min linear gradient; flow rate 1.5 mL/min; retention time: **14**, 10.4 min). Pure **14** (0.5 mg) was obtained together with its diastereomer (retention time 10.8 min). **14**: ^{1}H NMR (D_2O, TSP) δ 2.18 (m, 1 H, 2′), 2.42 (ddd, 1 H, J = 14.1, 6.6, 2.9 Hz, 2′), 3.59−3.71 (m, 4 H, 5′, 5″), 4.01−4.13 (m, 3 H, 4′, 1″), 4.50−4.62 (m, 2 H, 3′, 2″), 5.41 (dd, 1 H, J = 11.2, 9.3 Hz, 3″), 5.64 (dt, 1 H, J = 11.2, 6.5 Hz, 4″), 5.89 (d, 1 H, J = 7.8 Hz, 5), 6.13 (dd, 1 H, J = 7.0, 6.6 Hz, 1′), 7.67 (d, 1 H, J = 7.8 Hz, 6). **26**: ^{1}H NMR (D_2O, TSP) δ 2.18 (m, 1 H, 2′), 2.43 (ddd, 1 H, J = 14.1, 6.7, 2.9 Hz, 2′), 3.59−3.71 (m, 4 H, 5′, 5″), 4.01−4.13 (m, 3 H, 4′, 1″), 4.50−4.62 (m, 2 H, 3′, 2″), 5.41 (dd, 1 H, J = 11.2, 9.3 Hz, 3″), 5.65 (dt, 1 H, J = 6.7, 6.6 Hz, 4″), 5.89 (d, 1 H, J = 7.6 Hz, 5), 6.14 (dd, 1 H, J = 6.7, 6.6 Hz, 1′), 7.67 (d, 1 H, J = 7.6 Hz, 6).

HPLC Analysis of Thermal Degradation Products of 10. The reaction mixture (100 μL) containing 0.33 mM of **9** and 0.1 N HCl was heated at 90 °C for 5 min. To neutralize the mixture 10 μL of 0.9 N NaOH and 10 μL of 500 mM sodium cacodylate (pH 7.0) were added. A 10-μL aliquot was subjected to HPLC analysis (Cosmosil $5C_{18}$ column; 0.05 M ammonium formate containing 0−8% acetonitrile/20-min linear gradient; flow rate 1.0 mL/min) to confirm the production of the abasic site-containing oligomer **10** (Figure 3a). The rest of the reaction mixture (48 μL) was heated at 90 °C for 45 min, and an aliquot (12 μL) was subjected to HPLC analysis. The results are shown in Figure 3b. The reaction mixture (36 μL) was treated with 0.08 M $NaBH_4$ at room temperature for 15 min. After quenching the reaction with 3 μL of 1 N acetic acid, 14 μL of the aliquot was subjected to HPLC analysis. The results are shown in Figure 3c.

Quantitative Analysis of the Thermal Degradation Products of 10. The reaction mixture (100 μL) containing 0.33 mM of d(CAT) was heated in 0.1 N HCl at 90 °C for 5 min and was adjusted to pH 7.0. A 10-μL aliquot was subjected to HPLC analysis for the abasic site-containing trimer **10**. Then, the reaction mixture was heated at 90 °C for the indicated period (10, 20, 30, 45, 60, 90 min), and an aliquot (10 μL) was subjected to HPLC analysis (Cosmosil $5C_{18}$ column; 0.05 M ammonium formate containing 0−15% acetonitrile/20-min linear gradient; flow rate 1.5 mL/min). The results are summarized as shown in Figure 4.

Detection of β-Elimination Products from Depurinated Calf Thymus DNA. A solution (100 μL) containing calf thymus DNA (1 mM base concentration) in 0.1 N HCl was heated at 90 °C for 2 min. To this solution was added 10 μL of 0.9 N NaOH and 10 μL of 500 mM sodium cacodylate buffer (pH 7.0), and the solution was heated at 90 °C for 40 min. The reaction mixture was treated with s.v. PDE and AP at 37 °C for 2 h and then with 0.1 M $NaBH_4$. The reaction was quenched by 1 M CH_3COOH, and the solution was subjected to HPLC analysis (Cosmosil $5C_{18}$ column; 0.05 M ammonium formate containing 0−5.6% acetonitrile/40-min linear gradient; flow rate 1.0 mL/min). HPLC analysis of the mixture is shown in Figure 5.

Stereochemical Course of the Thermal β-Elimination Reaction. Compound **22** (3.0 mg) was dissolved in 491 μL of D_2O in an NMR tube (5-mm diameter). To this solution was added 55 μL of 1 N DCl, and the solution was heated at 90 °C for 5 min. The solution mixture was neutralized by the addition of 55 μL of 1 N NaOD solution. To this solution was added 60 μL of 500 mM sodium cacodylate buffer of D_2O solution (pD 6), and the solution was heated at 90 °C for 30 min. The reaction mixture was subjected to ^{1}H NMR spectroscopy. The peaks corresponding to **11** were observed. The result are shown in Figure 6.

Quantitative Analysis of Thermal Degradation Products of Protected Nucleotide 24. A reaction mixture (100 μL) containing 1 mM of **24** was heated in 0.1 N HCl at 90 °C for 5 min, and the mixture was adjusted to pH 7.0. Then, the reaction mixture was heated at 90 °C for 20 min. HPLC analysis of the reaction mixture indicated the decomposition of **24** together with the formation of **11** and uridine 5′-monophosphate. Quantitative analysis of the disappearance of **24** at pH 7.0 (90 °C) indicated that the decomposition reaction has first-order kinetics with a rate constant of 5.1×10^{-4}.

Results and Discussion

Thermal Strand Cleavage of Duocarmycin A-Treated DNA Fragments. In order to elucidate the mechanism of thermal strand cleavage of DNA containing abasic sites, we have examined the strand cleavage of duocarmycin A-treated DNA by polyacrylamide gel elec-

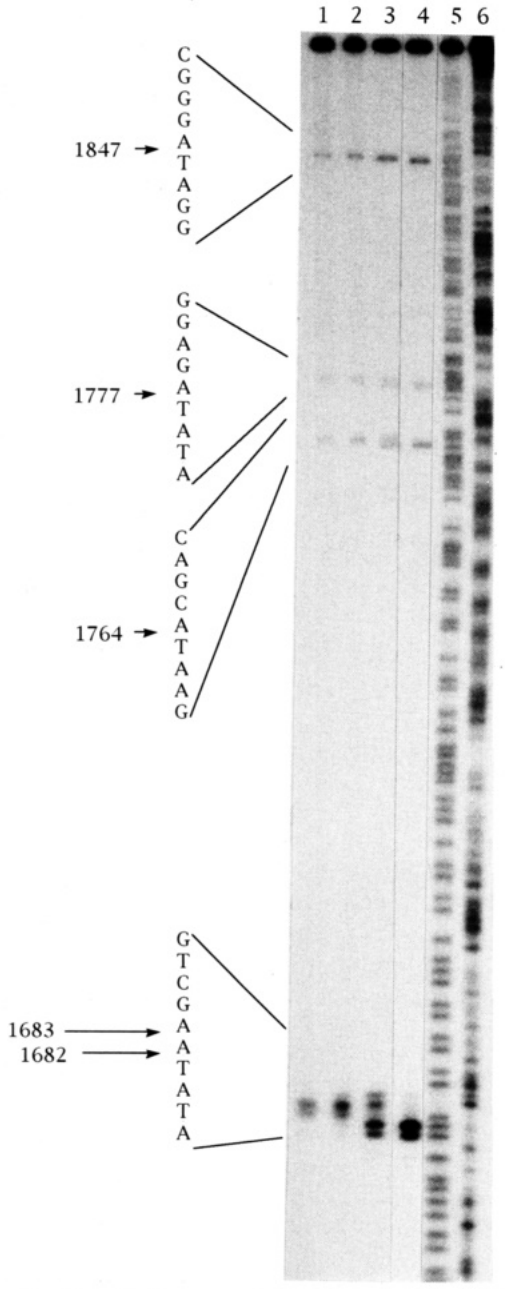


Figure 1. Strand cleavage of duocarmycin A-treated DNA fragment containing human c-Ha-*ras*-1 protooncogene. The duocarmycin A-treated DNA fragment was isolated by ethanol precipitation, and cleavage fragments were analyzed by polyacrylamide gel electrophoresis following workup treatments. Lane 1: no workup. Lane 2: heated at 90 °C for 5 min. Lane 3: heated at 90 °C for 5 min and subsequent heating in 1 M piperidine (90 °C, 5 min). Lane 4: heated at 90 °C for 5 min and subsequent heating in 1 M piperidine (90 °C, 20 min). Lanes 5 and 6 are Maxam–Gilbert chemical sequencing G+A and C+T lanes.

trophoresis. Figure 1 shows the autoradiograph of a 5′-^{32}P-end-labeled DNA fragment which has been treated with duocarmycin A. This 261-base-pair fragment has four alkylation sites, 5′-ATAT***AA***GCTG-3′ (1682 and 1683), 5′-GAAT***A***CGAC-3′ (1764), 5′-ATAT***A***GAGG-3′ (1777), and 5′-GGAT***A***GGGC-3′ (1847), the major alkylation sites being the adenine residues at 1682 and 1683 (*33*). The duocarmycin A-treated DNA fragment without any workup treatment showed only very faint bands which migrated more slowly than the 3′-phosphate cleavage band at 5′-ATAT***AA***GCTG-3′ sites (1682 and 1683 in the 261-bp fragment; lane 1). Upon heating at neutral pH, the cleavage at these sites was considerably enhanced (lane 2). Subsequent piperidine treatment (90 °C, 5 min) induced an additional cleavage at the same sites, and some of the slower migrating bands were shifted to faster migrating bands (lane 3) which comigrated with the fragment possessing a 3′-phosphate terminus produced by the Maxam–Gilbert sequencing reaction (*35*). It was also found that heating in 1 M piperidine for 20 min is a minimum requirement for the complete conversion of the modified sugar moiety to a 3′-phosphate terminus (lane 4). These results indicate that strand cleavage of the alkylated DNA occurs only simply by heating at neutral pH and that subsequent piperidine treatment converts the modified sugar termini to 3′-phosphate termini. A similar heat-dependent formation of modified sugar termini from alkylated DNA was observed previously on gel electrophoresis in the reactions with CC-1065 (*26*), duocarmycin A (*28, 31*), ptaquiloside dienone (*24*), and bromoacetyl distamycin (*17*). Although the structure of one of the modified sugar termini was proposed to be that of a *cis*-α,β-unsaturated aldose (*17, 24, 31*), the exact structures and the relevant chemistry still remain to be clarified.

Characterization of Modified Sugar Termini in Thermal Degradation of Abasic Sites from Duocarmycin A-Treated d(CGTATACG). Since we have already demonstrated that the abasic site in oligodeoxynucleotide **3** is produced from primary adduct **1** by brief heating, we employed this reaction to investigate the chemistry of thermal degradation of DNA containing abasic sites (*15*). The HPLC profile of duocarmycin A-treated d(CGTATACG)$_2$ after brief heating (90 °C, 5 min) reveals the formation of the abasic site-containing octamer **3** as a major peak at 25.9 min together with the formation of unknown product at 27.4 min (Figure 2a). Upon further heating at 90 °C for 30 min, the unknown peak was increased with release of pd(CG) (10.6 min) and d(CGTAT)p (21.8 min) (Figure 2b). Treatment of the unknown product with hot alkali (0.1 N NaOH, 90 °C, 5 min) provided d(CGTATp), indicating that the material is d(CGTAT)p possessing a modified sugar at the 3′-phosphate terminus. The structure of this unknown product was assigned as **4** on the basis of the chemical transformations shown in Scheme 2 (*9*). $NaBH_4$ reduction of **4** gave the reduced product **5**, and subsequent digestion with snake venom phosphodiesterase (s.v. PDE) and alkaline phosphatase (AP) provided **6** together with equivalent amounts of dC, dG, dT, and dA. A quantity of **6** large enough to allow structure determination (^{1}H NMR, FABMS) was independently obtained from the thermal degradation of abasic site-containing deoxynucleotide **7** and following $NaBH_4$ reduction. The peak eluted at 11.6 min was found to be an adenine base which is assumed to be derived from a thermal reversal reaction of **2** (*51*). It is apparent from these data that thermal degradation of **3** at neutral pH mainly proceeds by a β-elimination mechanism via a ring-opened aldehyde tautomer of the abasic site, to produce **4** at neutral pH (Scheme 2).

Detailed Analysis of Thermal Degradation of Abasic Site. In order to obtain a detailed picture of the thermal degradation of abasic sites, a careful product analysis of the heat-treated abasic site-containing oligomer was undertaken. Figure 3a shows the HPLC profile of an abasic site-containing dinucleotide **10** which was generated by acid treatment of **9**. The reaction mixture was then adjusted to pH 7.0 and heated at 90 °C for 45 min. HPLC analysis of the reaction mixture indicates

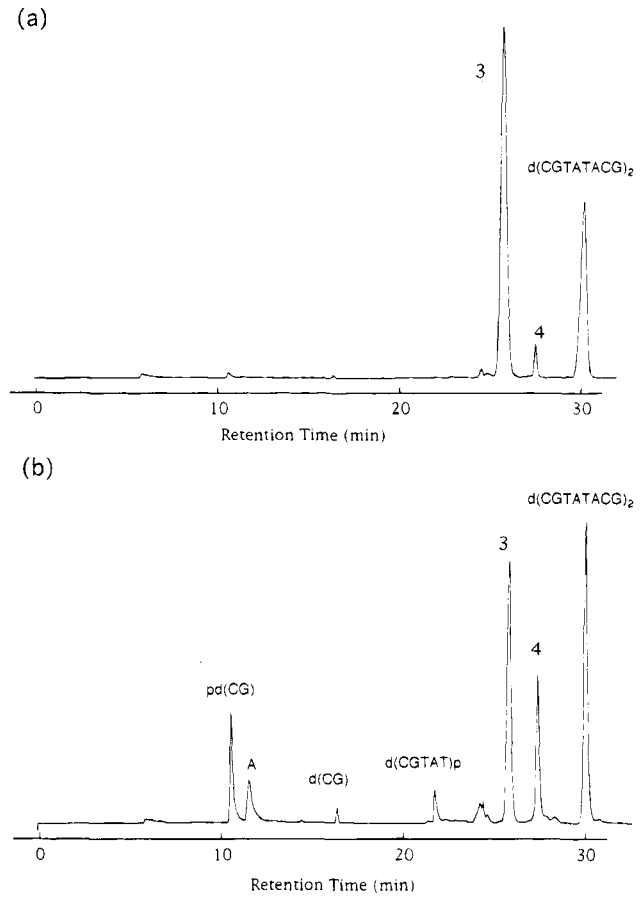
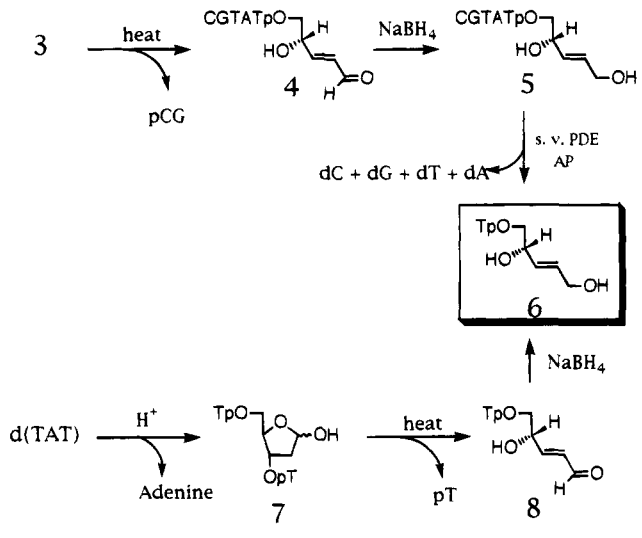


Figure 2. HPLC profiles of duocarmycin A-treated d(CGTATACG)$_2$ after brief heating (90 °C, 5 min). The reaction mixture was analyzed (a) directly and (b) after further heating at 90 °C for 20 min. Analysis was carried out as described in the Experimental Section.

Scheme 2



the formation of pT, and unknown products which were eluted at 8.8 and 11.7 min, respectively (Figure 3b). The peak eluted at 11.7 min was resolved into two peaks in a ratio of 7:3 under different HPLC separation conditions. The structures of major and minor components were assigned as **11** and **12**, respectively, by the following experiments described in Scheme 3. ^{1}H NMR analysis of the major component showed the presence of characteristic signals at δ 9.32, 7.17, and 6.42 ascribable to the *trans*-α,β-unsaturated aldose **11** as reported for the enzymatic reactions (*8*). $NaBH_4$ reduction of the product gave rise to the formation of alcohol **13**. The minor component was obtained by photoisomerization of **11** (*9*). $NaBH_4$ reduction of this component gave alcohol **14**. The structure of the unknown material eluted at 8.8 min, which is also resolved into two peaks under different HPLC separation conditions, was assigned as a diastereomeric mixture of **15a** and **15b** on the basis of the following experiments. The structure of **15b** was determined by comparison with the authentic sample which was prepared by acid depurination of d(CG). $NaBH_4$ reduction of the mixture of **15a** and **15b** gave a diastereomeric mixture of **18a** and **18b**. Larger quantities of **18a** and **18b** amenable for structure determination were obtained by thermal degradation of the protected dinucleotide **21** as outlined in Scheme 4. The structures of **18a** and **18b** were determined by ^{1}H NMR and FABMS together with comparison to the authentic samples.

Table 1. Thermal Degradation Products of Isolated 11 upon Heating at pH 7.0

condition	**11** (μM)	**12** (μM)	Cp (μM)	**15a** (μM)	**15b** (μM)
	100	0	0	0	0
90 °C, 1 h	70	8.6	13	4.5	4.1

Table 2. Thermal Degradation Products of Isolated 12 upon Heating at pH 7.0

condition	**11** (μM)	**12** (μM)	Cp (μM)	**15a** (μM)	**15b** (μM)
	1.8	98	0	0	0
90 °C, 1 h	8.9	72	14	0.7	0.7

The time-dependent quantitative assay of the degradation products from **10** under pH 7.0 is shown in Figure 4. The decomposition of **10** at pH 7.0 showed first-order kinetics with a rate constant of 4.5×10^{-4} s^{-1}. It is important to note that, at the early stage of the degradation (10 or 20 min), *trans* isomer **11** was predominantly formed: the ratio of **11** and **12** at 10 min was 10:1. Upon further heating, the ratio gradually converged to almost 1:1. The results indicate that *trans* isomer **11** is produced as a primary product of β-elimination which gradually isomerizes to the *cis* isomer **12** upon heating through its hydrate **16**. In fact, the isolated **11** and **12** were converted to each other upon heating at 90 °C as shown in Tables 1 and 2. The formation of **15a** and **15b** results from the cyclization of **16**. Upon prolonged heating (90 °C, 26 h), **15b** was converted to Cp, **11**, and **12** in 56%, 3%, and 1.9% yield, respectively. Interestingly, **15a** and **15b** were formed more efficiently from **11** than from **12**. Cp is produced from **11** and/or **12** through its ring-opened form **17** by δ-elimination. These results clearly indicate that the thermal degradation of the abasic site proceeds via a ring-opened form of aldehydic tautomer by β-elimination mechanism to produce a *trans*-α,β-unsaturated aldose terminus in a regioselective fashion. Possible mechanisms for the isomerization of **11** to **12** and the formation **15a** and **15b** are summarized in Scheme 3.

Thermal β-Elimination of Abasic Site in DNA. In order to find out whether abasic sites in longer DNA substrates are also decomposed in a similar manner, we examined the thermal degradation of an abasic site-containing calf thymus DNA. The abasic site-containing DNA was prepared by acid depurination of calf thymus DNA. After heating at 90 °C for 20 min at neutral pH, the reaction mixture was subjected to enzymatic digestion with s.v. PDE and AP. HPLC analysis of the

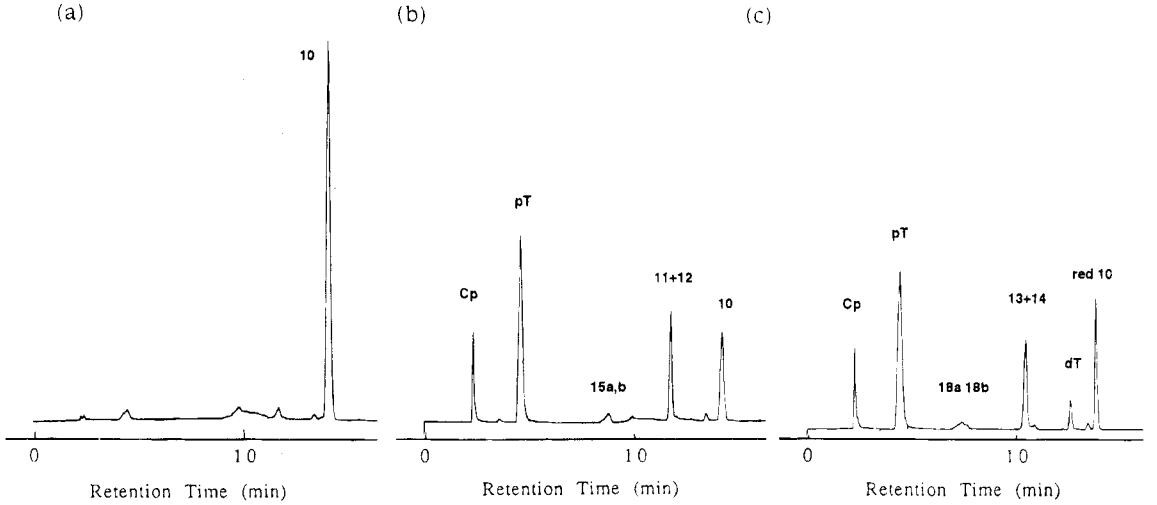
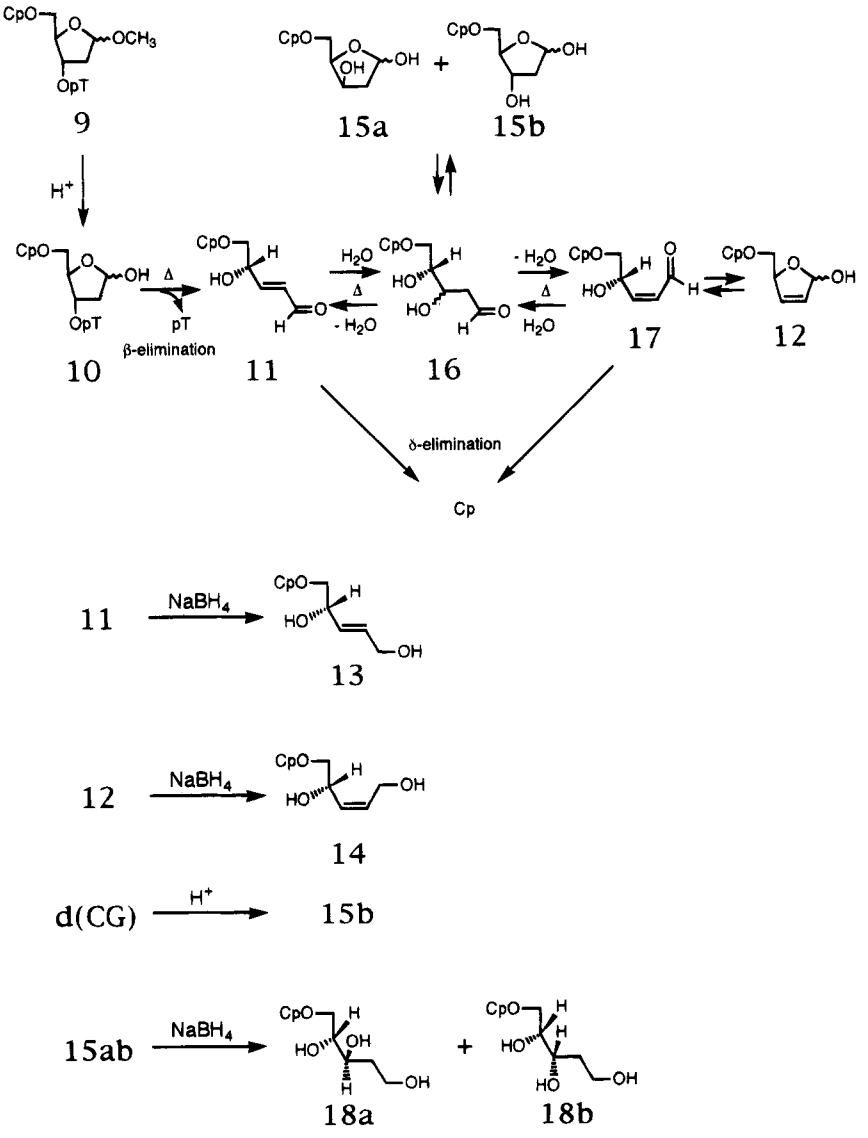


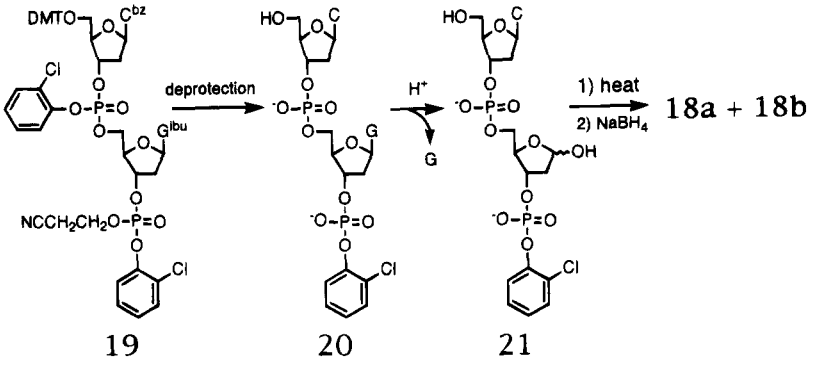
Figure 3. HPLC profiles of the thermal degradation of **10**. (a) Before heat treatment; (b) heated at 90 °C for 45 min; (c) the same sample as (b) but after $NaBH_4$ reduction.

Scheme 3



reaction mixture revealed the formation of only pyrimidine base-containing *trans*-α,β-unsaturated aldoses **8** and **11** (Figure 5a). After $NaBH_4$ reduction of the reaction mixture, the formation of the corresponding alcohols **6** and **13** was observed (Figure 5b). Under different HPLC conditions, the formation of **18a** and **18b** was also observed. The formation of **8** and **11** was also observed in the case of partially depurinated calf thymus DNA. These results clearly indicate that abasic sites in longer DNA fragments also undergo β-elimination to provide

Scheme 4



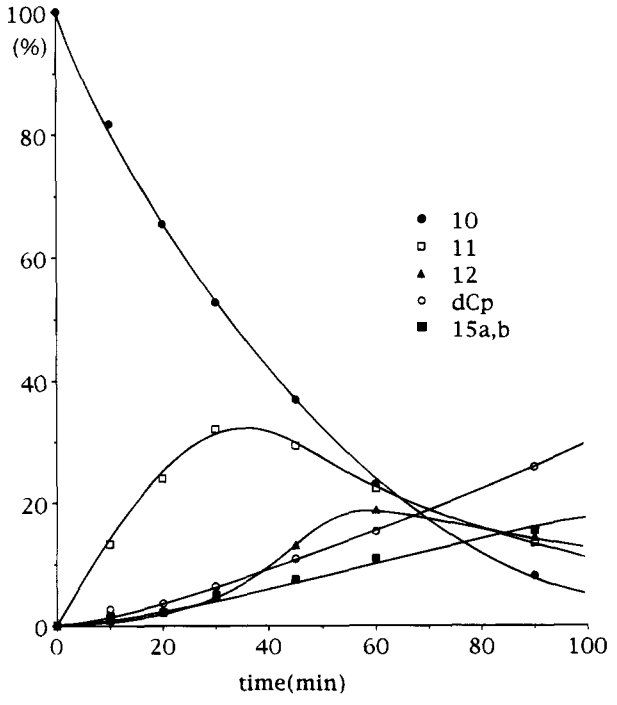


Figure 4. Time-dependent product distribution in the thermal degradation of **10**.

trans-α,β-unsaturated aldose termini as shown in Scheme 5.

Stereochemical Course for β-Elimination of Heat-Treated Abasic Sites. In order to ascertain the stereochemical course of the β-elimination reaction, thermal degradation of oligomer **23** containing a 2-*pro-R* deuteriated deoxyribose was investigated. In this system *syn*-β-elimination should lead to the retention of deuterium, whereas *anti*-β-elimination would result in a loss of deuterium as shown in Scheme 6. The compound **23** was prepared by depurination of **22** which was synthesized by an automated DNA synthesizer using 2′-*pro-R*-D-2′-deoxyadenosine (D content 86%) (*41*) as a phosphoramidite monomer. The D_2O solution containing **23** was heated at 90 °C for 20 min under neutral conditions and directly subjected to ^1H NMR analysis. Figure 6a shows the ^1H NMR spectrum of the reaction mixture, indicating that the deuterium is mostly retained in **11**. After $NaBH_4$ reduction of the reaction mixture, product **13-*d*** was isolated and subjected to ^1H NMR analysis. ^1H NMR spectrum of **13-*d*** indicated that most of the deuterium (96%, D content 82%) was retained in the double bond as shown in Figure 6b. These results indicate that under heating conditions the β-elimination reaction proceeds via syn orientation. The fact that the methyl phosphate **24**

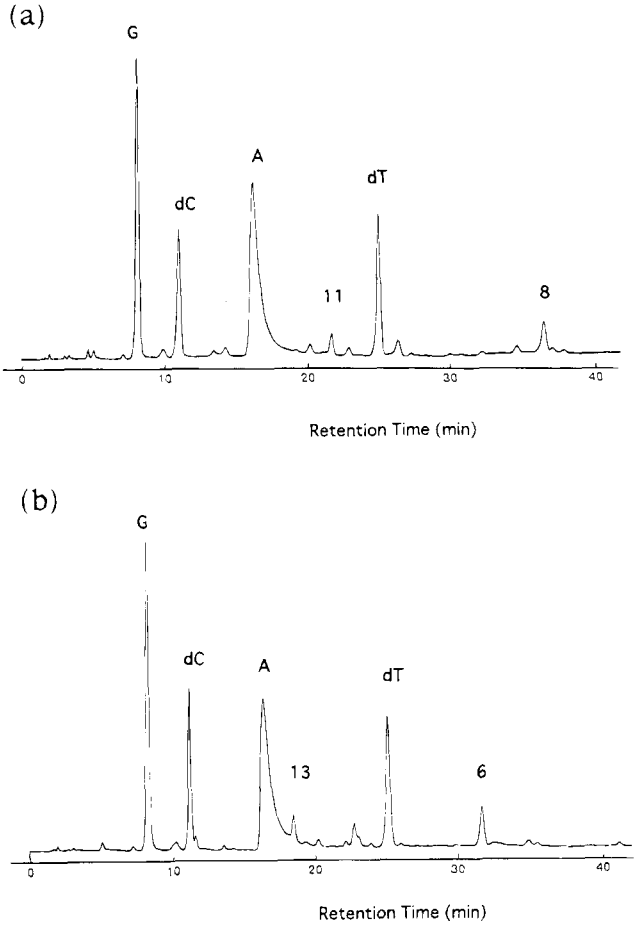
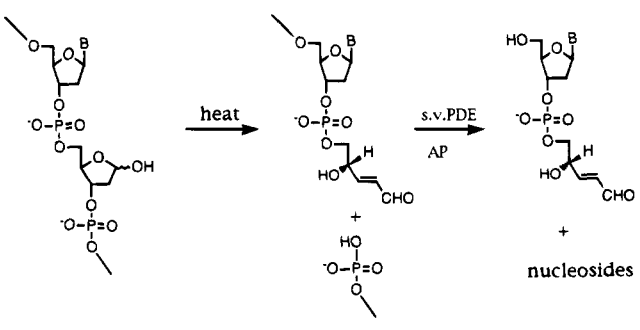


Figure 5. HPLC profiles of the heat-treated abasic sites of calf thymus DNA: (a) after heating at 90 °C for 20 min; (b) the same sample as in (a) but after $NaBH_4$ reduction.

Scheme 5



also undergoes β-elimination to **11** in a similar manner suggests that the β-elimination reaction does not involve general-base-catalyzed abstraction of the 2′-*pro-S* hydrogen by the anion of the 3′-phosphate leaving group, but

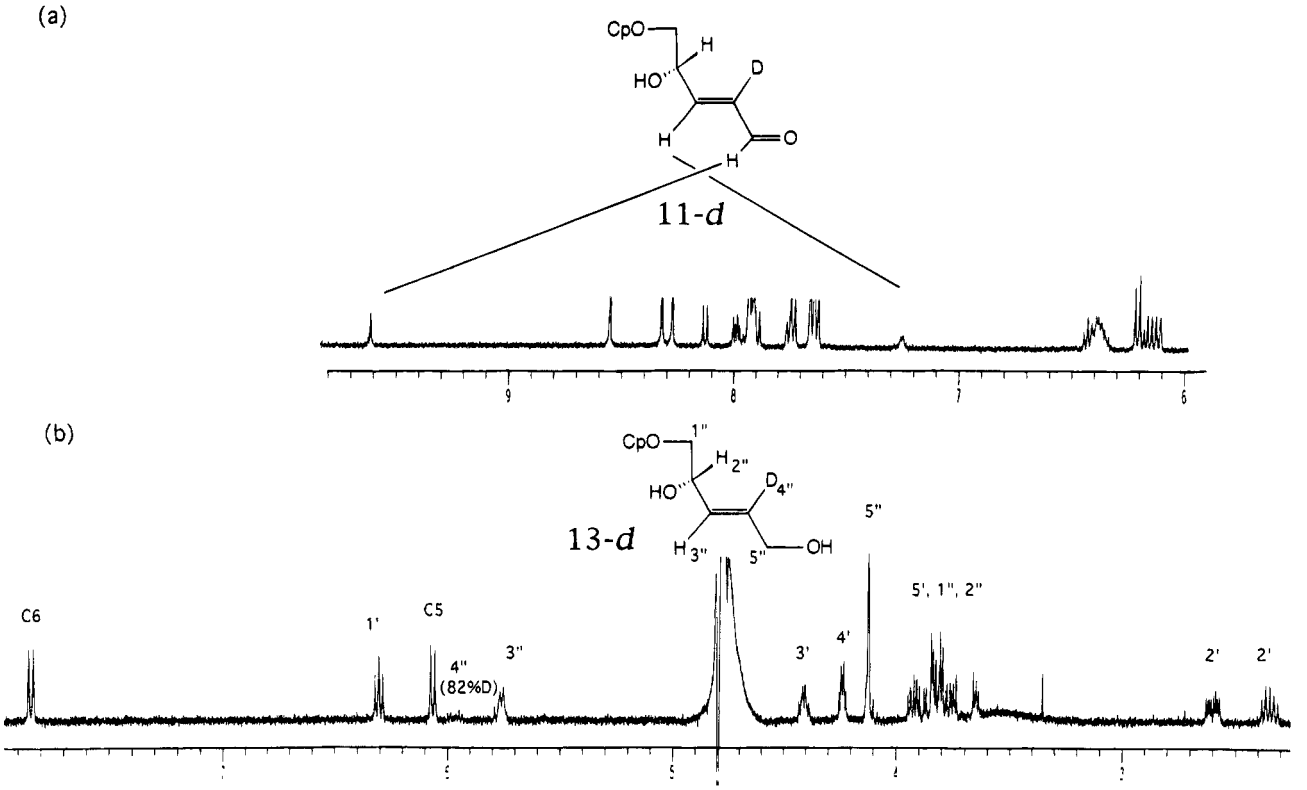
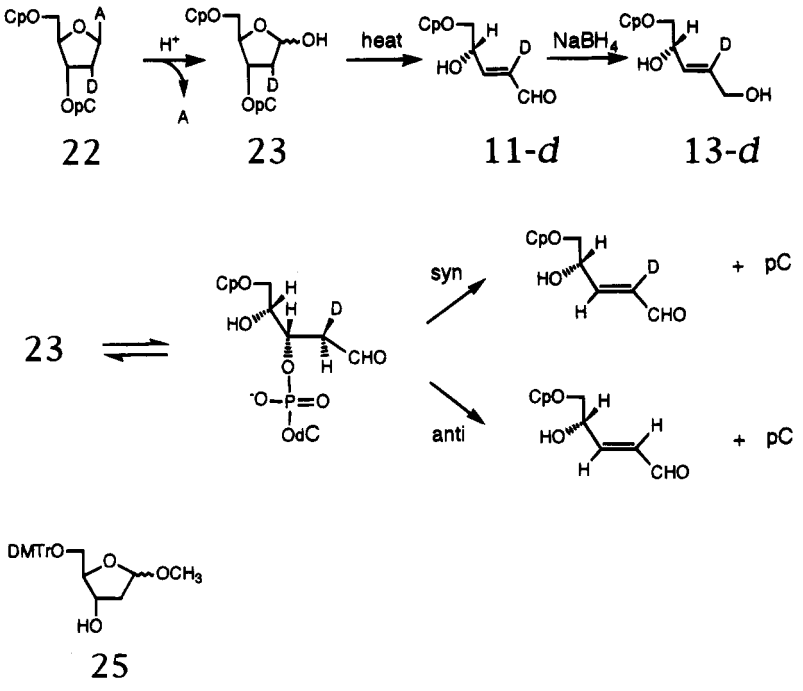
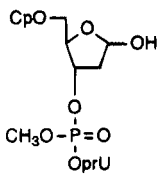


Figure 6. ^{1}H NMR of **11** after heating at neutral pH (90 °C, 20 min): (a) after heating at 90 °C for 20 min; (b) the isolated **13-*d*** after $NaBH_4$ reduction.

Scheme 6



proceeds via an Ei mechanism generally observed in pyrolytic elimination as depicted in Scheme 7 (*52*).



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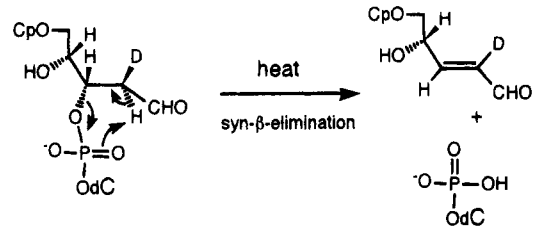
In conclusion, abasic sites in DNA are shown to be cleaved to the fragments containing *trans*-α,β-unsaturated aldose and 5′-phosphate termini under neutral heating conditions. Upon prolonged heating, the *trans*-α,β-unsaturated aldose terminus is converted to its hydrates, *cis* isomer, and 3′-phosphate terminus via δ-elimination. The stereochemical course of the β-elimination is *syn*, which is identical to that observed for the enzymatic reactions of UV endonuclease V and endonuclease III. The fact that *syn* elimination can readily occur in the nonenzymatic reaction will be very important for

Scheme 7



understanding the mechanism of the enzymatic β-elimination reaction.

Acknowledgment. This work was supported by a Grant-in-Aid for Priority Research from the Ministry of Education, Japan, Kawakami Memorial Foundation, and the CIBA-GEIGY Foundation.

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