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Synthesis and characterization of oligonucleotides containing 2′-fluorinated thymidine glycol as inhibitors of the endonuclease III reaction

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ABSTRACT

Endonuclease III (Endo III) is a base excision repair enzyme that recognizes oxidized pyrimidine bases including thymine glycol. This enzyme is a glycosylase/lyase and forms a Schiff base-type intermediate with the substrate after the damaged base is removed. To investigate the mechanism of its substrate recognition by X-ray crystallography, we have synthesized oligonucleotides containing 2′-fluorothymidine glycol, expecting that the electron-withdrawing fluorine atom at the 2′ position would stabilize the covalent intermediate, as observed for T4 endonuclease V (Endo V) in our previous study. Oxidation of 5′- and 3′-protected 2′-fluorothymidine with OsO_4 produced two isomers of thymine glycol. Their configurations were determined by NMR spectroscopy after protection of the hydroxyl functions. The ratio of (5*R*,6*S*) and (5*S*,6*R*) isomers was 3:1, whereas this ratio was 6:1 in the case of the unmodified sugar. Both of the thymidine glycol isomers were converted to the corresponding phosphoramidite building blocks and were incorporated into oligonucleotides. When the duplexes containing 2′-fluorinated 5*R*- or 5*S*-thymidine glycol were treated with *Escherichia coli* endo III, no stabilized covalent intermediate was observed regardless of the stereochemistry at C5. The 5*S* isomer was found to form an enzyme–DNA complex, but the incision was inhibited probably by the fluorine-induced stabilization of the glycosidic bond.

INTRODUCTION

Endonuclease III (Endo III) is an enzyme that initiates base excision repair of oxidatively damaged pyrimidine bases in DNA (1). Its activity prevents the lethal effects of ionizing radiation. This important enzyme is highly conserved in evolution from bacteria to human cells (2–4), and *Escherichia coli* Endo III has been studied intensively. The major substrates for Endo III are 5,6-saturated pyrimidines, such as 5,6-dihydrothymine, 5,6-dihydro-5-hydroxythymine and thymine glycol (5,6-dihydro-5,6-dihydroxythymine), and 5-hydroxy-5-methylhydantoin, which is derived by spontaneous cyclization of fragmented thymine glycol, formed by γ-irradiation in DNA (5). This enzyme also recognizes 5-hydroxycytosine and 5-hydroxyuracil (6), which have an unsaturated C5–C6 bond, deoxyribosylurea and an apurinic/apyrimidinic (AP) site (7,8). Recent studies revealed that hydantoins derived by further oxidation of 8-oxoguanine (9), 8-oxoguanine mispaired with guanine (10), and a pyrimidine ring-opened derivative of 1,N^6-ethenoadenine (11) were removed by Endo III, although this enzyme was less active for these substrates.

Endo III has two catalytic activities, i.e. DNA glycosylase and AP lyase. This enzyme removes the damaged base by the scission of its glycosidic bond first and subsequently cleaves the phosphodiester linkage on the 3′ side of the resultant AP site by a β-elimination reaction. After the first step, a Schiff base-type, covalently-bonded intermediate is formed between the enzyme and the substrate, as shown in Scheme 1. This mechanism was originally found in the T4 endonuclease V (Endo V) reaction (12,13) and was demonstrated for other glycosylase/AP lyases including Endo III (14). A stable, covalent enzyme–DNA complex can be obtained by reduction of

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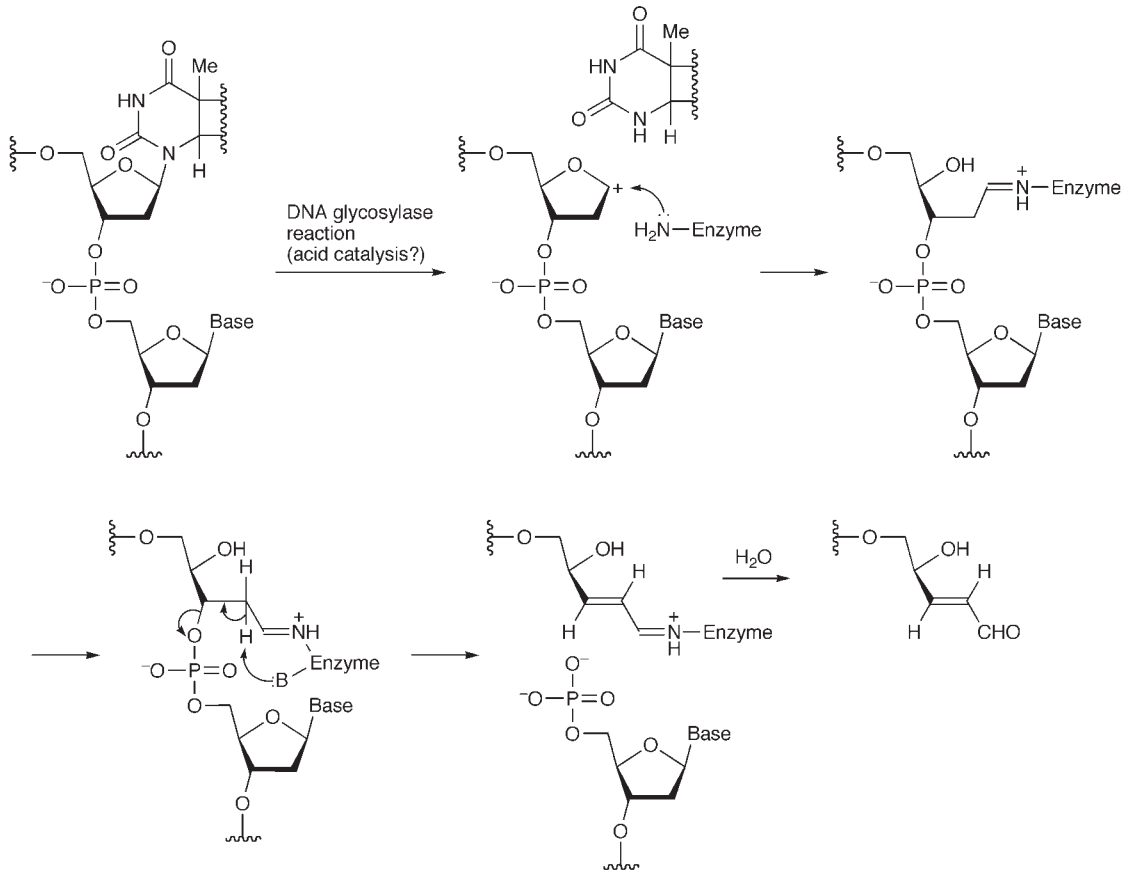


Scheme 1. The proposed mechanism for the T4 Endo V and Endo III reactions.

the intermediate with sodium borohydride ($NaBH_4$), which was used to show the presence of such a covalent intermediate (13,14). This method was applied to crystallization of an Endo III–DNA complex, and its tertiary structure has been determined (15). In this structure study, the C–N bond of the reduced Schiff base was shown clearly, and a candidate for the amino acid side chain responsible for the β-elimination reaction was given from the active site structure. However, the substrate recognition mechanism of this enzyme was not elucidated because the oxidized base was lost in the reduced enzyme–DNA complex. Therefore, an alternative crystallization method is required to understand the wide substrate specificity of Endo III.

Previously, we reported that an oligonucleotide duplex containing a 2′-fluorinated sugar moiety at the cyclobutane pyrimidine dimer (CPD) site inhibited the T4 Endo V reaction by stabilizing the covalent enzyme–DNA complex without the $NaBH_4$ reduction (16). We reasoned that the electron-withdrawing fluorine atom at the C2′ position stabilized the cyclic hemiacetal form of the sugar moiety, which is inactive in the β-elimination reaction, after the N-terminal α-amino group of the enzyme formed a covalent bond with the 1′ carbon of the substrate. Since a crystal structure of the T4 Endo V–DNA complex was successfully determined using a mutant enzyme (17), this system was not applied to crystallization. Here we describe the synthesis of oligonucleotides containing 2′-fluorinated thymidine glycol, which are possible inhibitors of the Endo III reaction and may be used for crystallization of the enzyme–DNA complex. The enzyme reaction with the modified oligonucleotide duplexes is also reported.

MATERIALS AND METHODS

For the chemical synthesis, the general methods are basically the same as those reported previously (18,19). The starting material, 2′-fluorothymidine [1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)thymine], was purchased from R.I. Chemical (Orange, CA). ^{1}H-NMR spectra were measured on a JEOL AL-400 or Varian INOVA 600 spectrometer, and ^{31}P-NMR spectra were measured on a JEOL GSX270 spectrometer using trimethyl phosphate as an internal standard. Mass spectra were obtained on a Micromass LCT spectrometer. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra of oligonucleotides were measured in the negative ion mode on an Applied Biosystems Voyager DE PRO spectrometer, using 3-hydroxypicolinic acid as a matrix.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-2′-fluorothymidine (1)

To a solution of 5′-*O*-(4,4′-dimethoxytrityl)-2′-fluorothymidine (4.76 g, 8.46 mmol) in pyridine (32 ml), benzoyl chloride (1.11 ml, 9.60 mmol) was added, and the mixture was stirred in an ice bath for 3 h. Chloroform (80 ml) was added, and the mixture was washed with saturated

aqueous $NaHCO_3$ and then with saturated aqueous NaCl. The organic layer was dried with Na_2SO_4, and after evaporation and coevaporation with toluene, the residue was chromatographed on silica gel (80 g). The product was eluted with chloroform and was obtained as foam after evaporation. Yield: 5.13 g (7.69 mmol, 91%). TLC ($CHCl_3$/MeOH, 10/1, v/v): R_f: 0.81. ^{1}H NMR (400 MHz, $CDCl_3$, 30°C, TMS): δ 8.10 (s, 1H; -NH-), 8.03 (dd, J = 8.4, 1.3 Hz, 2H; bz), 7.62 (tt, J = 7.4, 1.8 Hz, 1H; bz), 7.53 (d, J = 1.3 Hz, 1H; H6), 7.47 (t, J = 8.1 Hz, 2H; bz), 7.39 (dd, J = 6.9, 1.6 Hz, 2H; DMT), 7.30–7.15 (m, 7H; DMT), 6.79 (t, J = 8.1 Hz, 4H; DMT), 6.20 (dd, J = 16.2, 3.1 Hz, 1H; H1′), 5.67–5.60 (m, 1H; H3′), 5.50 (dd, J = 4.8, 3.1 Hz, 0.5H; H2′), 5.37 (dd, J = 4.7, 3.1 Hz, 0.5H; H2′), 4.48–4.44 (m, 1H; H4′), 3.75 (s, 3H; -OCH_3), 3.74 (s, 3H; -OCH_3), 3.63 (dd, J = 11.2, 2.4 Hz, 1H; H5′), 3.49 (dd, J = 11.2, 2.8 Hz, 1H; H5′), 1.51 (s, 3H; -CH_3). HRMS (ESI): m/z: 689.2273 ([M+Na]$^+$, $C_{38}H_{35}O_8N_2FNa$: 689.2270).

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-5,6-dihydro-5,6-dihydroxy-2′-fluorothymidine (2a and 2b)

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-2′-fluorothymidine **(1)** (2.62 g, 3.93 mmol) was mixed with osmium tetroxide (1.0 g, 3.93 mmol) in pyridine (10 ml), and this mixture was stirred at room temperature for 2 h. Sodium hydrogen sulfite (1.8 g) dissolved in water (30 ml) and pyridine (20 ml) was added, and the mixture was stirred further for 30 min. The product was extracted with chloroform (100 ml), and the organic layer was dried with Na_2SO_4. After evaporation, the pyridine was removed by coevaporation with toluene, and the residue was chromatographed on silica gel (70 g). Two isomers were separated by elution with diethyl ether, and their configurations were determined after the protection of the hydroxyl functions, as described in the text.

*5R,6S-Isomer **(2a)***. Yield: 1.24 g (1.77 mmol, 45%). TLC (diethyl ether): R_f: 0.31. ^{1}H-NMR (400 MHz, $CDCl_3$, 30°C, TMS): δ 8.01 (dd, J = 8.5, 1.4 Hz, 2H; bz), 7.60 (tt, J = 7.6, 1.2 Hz, 1H; bz), 7.52 (s, 1H; -NH-), 7.45 (t, J = 8.0 Hz, 2H; bz), 7.40 (dd, J = 8.6, 1.5 Hz, 2H; DMT), 7.31–7.15 (m, 7H; DMT), 6.79–6.74 (m, 4H; DMT), 6.15 (dd, J = 18.8, 2.4 Hz, 1H; H1′), 5.67–5.59 (m, 1H; H3′), 5.60 (dd, J = 4.9, 2.2 Hz, 0.5H; H2′), 5.46 (dd, J = 4.9, 2.7 Hz, 0.5H; H2′), 5.31 (s, 1H; H6), 4.36–4.32 (m, 1H; H4′), 3.72 (s, 3H; -OCH_3), 3.71 (s, 3H; -OCH_3), 3.59 (dd, J = 11.2, 2.4 Hz, 1H; H5′), 3.56 (s, 1H; 6-OH), 3.43 (dd, J = 11.0, 3.2 Hz, 1H; H5′), 3.26 (s, 1H; 5-OH), 1.43 (s, 3H; -CH_3). HRMS (ESI): m/z: 723.2349 ([M+Na]$^+$, $C_{38}H_{37}O_{10}N_2FNa$: 723.2324).

*5S,6R-Isomer **(2b)***. Yield: 438 mg (625 μmol, 16%). TLC (diethyl ether): R_f: 0.15. ^{1}H-NMR (400 MHz, $CDCl_3$, 30°C, TMS): δ 8.04 (dd, J = 8.3, 1.2 Hz, 2H; bz), 7.62 (tt, J = 7.6, 1.4 Hz, 1H; bz), 7.47 (t, J = 8 Hz, 2H; bz), 7.44 (s, 1H; -NH-), 7.37 (dd, J = 7.1, 1.5 Hz, 2H; DMT), 7.27 (dd, J = 9.0, 1.7 Hz, 4H; DMT), 7.24–7.16 (m, 3H; DMT), 6.81–6.75 (m, 4H; DMT), 6.23 (dd, J = 19.5, 3.4 Hz, 1H; H1′), 5.57–5.49 (m, 1H; H3′), 5.37 (dd, J = 5.4, 3.4 Hz, 0.5H; H2′), 5.24 (dd, J = 5.2, 3.4 Hz, 0.5H; H2′), 4.94 (d, J = 1.9 Hz, 1H; H6), 4.37–4.32 (m, 1H; H4′), 3.72 (s, 6H; -OCH_3), 3.67 (d, J = 2.0 Hz, 1H; 6-OH), 3.53 (d, J = 2.7 Hz, 2H; H5′), 3.21 (s, 1H; 5-OH), 1.52 (s, 3H; -CH_3). HRMS (ESI): m/z: 723.2351 ([M+Na]$^+$, $C_{38}H_{37}O_{10}N_2$ FNa: 723.2324).

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-(5*R*,6*S*)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]-2′-fluorothymidine (3a)

To a solution of 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-benzoyl-(5*R*,6*S*)-5,6-dihydro-5,6-dihydroxy-2′-fluorothymidine (**2a**) (443 mg, 633 μmol) in *N*,*N*-dimethylformamide (7 ml), imidazole (431 mg, 6.33 mmol) and *tert*-butyldimethylchlorosilane (500 mg, 3.31 mmol) were added, and the mixture was stirred at 37°C for 24 h. This mixture was diluted with chloroform (50 ml) and was washed with 0.5 M sodium phosphate (pH 5.0). The organic layer was dried with Na_2SO_4, and after evaporation and coevaporation with toluene, the residue was chromatographed on silica gel (30 g) with a step gradient of ethyl acetate in hexane. The product was eluted with 15% ethyl acetate in hexane and was obtained as foam after evaporation. Yield: 528 mg (568 μmol, 90%). TLC (hexane/ethyl acetate, 3/2, v/v): R_f: 0.59. ^{1}H NMR (400 MHz, $CDCl_3$, 30°C, TMS): δ 8.00 (dd, J = 8.0, 1.0 Hz, 2H; bz), 7.58 (tt, J = 7.4, 1.2 Hz, 1H; bz), 7.45 (d, J = 8.0 Hz, 2H; bz), 7.45–7.41 (m, 2H; DMT), 7.31 (dt, J = 8.8, 2.1 Hz, 4H; DMT), 7.22–7.13 (m, 3H; DMT), 7.10 (s, 1H; -NH-), 6.74 (d, J = 8.3 Hz, 4H; DMT), 5.61 (dd, J = 5.6, 2.6 Hz, 0.5H; H2′), 5.48 (dd, J = 5.7, 2.6 Hz, 0.5H; H2′), 5.45–5.35 (m, 1H; H3′), 5.18 (dd, J = 24.0, 2.6 Hz, 1H; H1′), 4.67 (s, 1H; H6), 4.31–4.25 (m, 1H; H4′), 3.73 (s, 6H; -OCH_3), 3.43 (d, J = 4.8 Hz, 2H; H5′), 1.47 (s, 3H; -CH_3), 0.87 (s, 9H; TBDMS), 0.84 (s, 9H; TBDMS), 0.27 (s, 3H; TBDMS), 0.23 (s, 3H; TBDMS), 0.22 (s, 3H; TBDMS), 0.16 (s, 3H; TBDMS). HRMS (ESI): m/z: 951.4040 ([M+Na]$^+$, $C_{50}H_{65}O_{10}N_2FSi_2Na$: 951.4053).

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-(5*S*,6*R*)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]-2′-fluorothymidine (3b)

To a solution of 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-benzoyl-(5*S*,6*R*)-5,6-dihydro-5,6-dihydroxy-2′-fluorothymidine (**2b**) (338 mg, 482 μmol) in *N*,*N*-dimethylformamide (6 ml), imidazole (330 mg, 4.82 mmol) and *tert*-butyldimethylchlorosilane (363 mg, 2.41 mmol) were added, and the mixture was stirred at 37°C for 30 h. This mixture was diluted with chloroform (50 ml) and was washed with 0.5 M sodium phosphate (pH 5.0). The organic layer was dried with Na_2SO_4, and after evaporation and coevaporation with toluene, the residue was chromatographed on silica gel (30 g) with a step gradient of ethyl acetate in hexane. The product was eluted with 15% ethyl acetate in hexane and was obtained as foam after evaporation. Yield: 408 mg (439 μmol, 91%). TLC (hexane/ethyl acetate, 3/2, v/v): R_f: 0.62. ^{1}H NMR (400 MHz, $CDCl_3$, 30°C, TMS): δ 8.00 (dd, J = 7.2, 1.2 Hz, 2H; bz), 7.58 (tt, J = 7.4, 1.2 Hz, 1H; bz), 7.44 (d, J = 7.9 Hz, 2H; bz), 7.42–7.38 (m, 2H; DMT), 7.27 (d, J = 8.9 Hz, 4H; DMT), 7.21–7.14 (m, 3H; DMT), 7.07 (s, 1H; -NH-), 6.72 (dd, J = 9.0, 2.6 Hz, 4H; DMT), 5.83 (d, J = 4.8 Hz, 0.5H; H2′), 5.69 (d, J = 4.9 Hz, 0.5H; H2′), 5.62–5.55 (m, 1H; H3′), 5.20 (d, J = 24.4 Hz, 1H; H1′), 4.72 (s, 1H; H6), 4.36–4.30 (m, 1H; H4′), 3.72 (s, 6H; -OCH_3), 3.39 (d, J = 4.4 Hz, 2H; H5′), 1.53 (s, 3H; -CH_3), 0.88 (s, 9H; TBDMS), 0.85 (s, 9H; TBDMS), 0.26 (s, 3H; TBDMS), 0.21 (s, 3H; TBDMS), 0.12 (s, 3H; TBDMS), 0.10 (s, 3H; TBDMS). HRMS (ESI): m/z: 951.4039 ([M+Na]$^+$, $C_{50}H_{65}O_{10}N_2FSi_2Na$: 951.4053).

5′-*O*-(4,4′-Dimethoxytrityl)-(5*R*,6*S*)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]-2′-fluorothymidine (4a)

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-(5*R*,6*S*)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]-2′-fluorothymidine (**3a**) (465 mg, 500 μmol) was dissolved in a 50 mM solution of potassium carbonate in anhydrous methanol (14 ml). This mixture was stirred at room temperature for 3 h. After the solution was cooled in an ice bath, 0.5 M sodium phosphate (pH 5.0, 14 ml) was added, and the product was extracted with chloroform (50 ml). The organic layer was dried with Na_2SO_4, and after evaporation, the residue was chromatographed on silica gel (30 g) with a step gradient of ethyl acetate in hexane. The product was eluted with 20% ethyl acetate in hexane and was obtained as foam after evaporation. Yield: 394 mg (478 μmol, 96%). TLC (hexane/ethyl acetate, 3/2, v/v): R_f: 0.43. ^{1}H NMR (400 MHz, $CDCl_3$, 30°C, TMS): δ 7.44 (d, J = 7.7 Hz, 2H; DMT), 7.32 (dt, J = 8.9, 2.1 Hz, 4H; DMT), 7.27–7.16 (m, 3H; DMT), 7.12 (s, 1H; -NH-), 6.80 (d, J = 8.9 Hz, 4H; DMT), 5.28 (dd, J = 5.5, 2.7 Hz, 0.5H; H2′), 5.14 (dd, J = 5.5, 2.6 Hz, 0.5H; H2′), 5.11 (dd, J = 24.3, 2.8 Hz, 1H; H1′), 4.64 (s, 1H; H6), 4.40–4.30 (m, 1H; H3′), 3.89–3.83 (m, 1H; H4′), 3.78 (s, 6H; -OCH_3), 3.41–3.31 (m, 2H; H5′), 2.02 (dd, J = 7.9, 3.8 Hz, 1H; 3′-OH), 1.46 (s, 3H; -CH_3), 0.87 (s, 9H; TBDMS), 0.83 (s, 9H; TBDMS), 0.26 (s, 3H; TBDMS), 0.22 (s, 3H; TBDMS), 0.19 (s, 3H; TBDMS), 0.15 (s, 3H; TBDMS). HRMS (ESI): *m*/*z*: 847.3785 ([M+Na]$^+$, $C_{43}H_{61}O_9N_2FSi_2Na$: 847.3791).

5′-*O*-(4,4′-Dimethoxytrityl)-(5*S*,6*R*)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]-2′-fluorothymidine (4b)

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-(5*S*,6*R*)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]-2′-fluorothymidine (**3b**) (372 mg, 400 μmol) was dissolved in a 50 mM solution of potassium carbonate in anhydrous methanol (10 ml). This mixture was stirred at room temperature for 2 h. After the solution was cooled in an ice bath, 0.5 M sodium phosphate (pH 5.0, 10 ml) was added, and the product was extracted with chloroform (50 ml). The organic layer was dried with Na_2SO_4, and after evaporation, the residue was chromatographed on silica gel (25 g) with a step gradient of ethyl acetate in hexane. The product was eluted with 20% ethyl acetate in hexane and was obtained as foam after evaporation. Yield: 307 mg (372 μmol, 85%). TLC (hexane/ethyl acetate, 3/2, v/v): R_f: 0.43. ^{1}H NMR (400 MHz, $CDCl_3$, 30°C, TMS): δ 7.42 (dd, J = 7.1, 1.6 Hz, 2H; DMT), 7.31 (d, J = 8.9 Hz, 4H; DMT), 7.25–7.16 (m, 3H; DMT), 7.10 (d, J = 10 Hz, 1H; -NH-), 6.79 (d, J = 8.0 Hz, 4H; DMT), 5.51 (d, J = 4.8 Hz, 0.5H; H2′), 5.38 (d, J = 4.9 Hz, 0.5H; H2′), 5.09 (d, J = 25.9 Hz, 1H; H1′), 4.70 (s, 1H; H6), 4.57–4.46 (m, 1H; H3′), 3.89–3.84 (m, 1H; H4′), 3.78 (s, 6H; -OCH_3), 3.38–3.30 (m, 2H; H5′), 2.04–1.98 (m, 1H; 3′-OH), 1.53 (s, 3H; -CH_3), 0.88 (s, 9H; TBDMS), 0.86 (s, 9H; TBDMS), 0.25 (s, 3H; TBDMS), 0.20 (s, 3H; TBDMS), 0.14 (s, 3H; TBDMS), 0.12 (s, 3H; TBDMS). HRMS: (ESI) *m*/*z*: 847.3778 ([M+Na]$^+$, $C_{43}H_{61}O_9N_2FSi_2Na$: 847.3791).

5′-*O*-(4,4′-Dimethoxytrityl)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]-2′-fluorothymidine 3′-[(2-cyanoethyl)-*N*,*N*-diisopropyl]phosphoramidite (5a and 5b)

To a solution of 5′-*O*-(4,4′-dimethoxytrityl)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]-2′-fluorothymidine (**4a** or **4b**) (166 mg, 201 μmol) in tetrahydrofuran (4 ml), *N*,*N*-diisopropylethylamine (208 μl, 1.20 mmol) and (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite (134 μl, 600 μmol) were added. This mixture was stirred for 1 h, diluted with ethyl acetate, and washed with 2% $NaHCO_3$ and water. The organic layer was dried with Na_2SO_4, and after evaporation, the residue was chromatographed on silica gel (5 g) with a step gradient of ethyl acetate in hexane containing 0.1% pyridine. The product was eluted with 20% ethyl acetate, and after evaporation, the pyridine was removed by coevaporation with acetonitrile. (**5a**) Yield: 145 mg (141 μmol, 70%). ^{31}P-NMR (109.25 MHz, $CDCl_3$, 30°C, trimethyl phosphate): δ 148.3, 147.8 p.p.m. HRMS (ESI): *m*/*z*: 1025.5058 ([M+H]$^+$, $C_{52}H_{79}FN_4O_{10}PSi_2$: 1025.5051). (**5b**) Yield: 169 mg (165 μmol, 82%). ^{31}P-NMR (109.25 MHz, $CDCl_3$, 30°C, trimethyl phosphate): δ 148.7, 147.7 p.p.m. HRMS (ESI): *m*/*z*: 1025.5045 ([M+H]$^+$, $C_{52}H_{79}FN_4O_{10}PSi_2$: 1025.5051).

Oligonucleotide synthesis

The phosphoramidite building blocks of 2′-fluorothymidine glycols (**5a** and **5b**) were dissolved in anhydrous acetonitrile at a concentration of 0.1 M and were installed on an Applied Biosystems Model 394 or 3400 DNA synthesizer. Nucleoside phosphoramidites for ultramild DNA synthesis (Glen Research), as well as the base-unprotected thymidine phosphoramidite, were also dissolved in acetonitrile to make 0.1 M solutions and were installed on the synthesizer. Oligonucleotides were synthesized on a 0.2 or 1.0 μmol scale, and the reaction time for the coupling of **5a** and **5b** was prolonged to 5 min. After chain assembly and removal of the 4,4′-dimethoxytrityl (DMT) group at the 5′ end on the synthesizer, the solid supports containing the oligonucleotides were treated with 28% aqueous ammonia (2 ml) at room temperature for 2 h. The resulting ammoniac solutions were concentrated to dryness on a rotary evaporator equipped with a vacuum pump. The residues were dissolved in triethylamine trihydrofluoride (500 μl) (Aldrich), and the mixtures were kept at 40°C overnight. After desalting on a NAP-10 column (Amersham Biosciences), the oligonucleotides were analyzed and purified by high-performance liquid chromatography (HPLC). For analysis, a μBondasphere C18 5μm 300 Å column (3.9 × 150 mm) (Waters) was used with a linear gradient of acetonitrile [6–11% (13mers) or 7–13% (40-mers) for 20 min] in 0.1 M triethylammonium acetate (pH 7.0). For purification, a μBondasphere C18 15 μm 300 Å column (7.8 × 300 mm) (Waters) was used, and the acetonitrile gradient was 8–13% for 30 min.

^{32}P-labeling of a CPD-containing oligonucleotide

A 34mer, d(GGCTTGTCACTATCGCGT$_F$[]TGCGCTACAGTAAGTG), where T$_F$[]T represents the *cis–syn* CPD with a fluorine atom attached at the 2′ upper position of the 5′ component, was prepared as described previously (16). For 5′-labeling, this oligonucleotide (60 pmol) was incubated with

[γ-^{32}P]ATP and T4 polynucleotide kinase (10 U) (Takara Bio) in a buffer (50 µl) containing 50 mM Tris–HCl (pH 9.5), 10 mM $MgCl_2$, 5 mM DTT and 5% glycerol at 37°C, and after 30 min, the mixture was heated to 75°C for 15 min. For 3′-labeling, the 34mer (60 pmol) was incubated with [α-^{32}P]ATP and terminal deoxynucleotidyl transferase (40 U) (Amersham Biosciences) in a buffer (50 µl) containing 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate and 50 mM potassium acetate at 37°C, and after 15 min, 0.5 M EDTA (5 µl) was added. In both cases, the labeled oligonucleotide was purified using a ProbeQuant G-50 Micro Column (Amersham Biosciences).

Formation of the T4 Endo V–DNA complex

The ^{32}P-labeled 34mer was hybridized with a 1.25-fold excess of the complementary strand by heating the solution to 80°C for 5 min and cooling it to 4°C. The binding reaction (20 µl) contained the 5′- or 3′-^{32}P-labeled duplex (1 pmol) and T4 Endo V (10 pmol) in 32 mM Tris–HCl (pH 7.5), 9.6 mM EDTA and 100 mM NaCl. The mixtures were incubated at 30°C for 30 min and were subjected to 15% SDS–PAGE, followed by detection of the bands by autoradiography.

Trapping of the covalent intermediate in the Endo III reaction

The ^{32}P-labeled 13 bp duplexes (10 nM) containing the 5*R* or 5*S* isomer of thymidine glycol or 2′-fluorothymidine glycol, ^{32}P-d(ACGCGATgACGCCA)·d(TGGCGTATCGCGT), in which Tg represents thymine glycol, were incubated with *E.coli* Endo III (10 or 100 nM) at 25°C for 30 or 60 min in a buffer (10 µl) containing 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.1 mg/ml BSA and 0 or 50 mM $NaBH_4$. The mixtures were analyzed by 10% SDS–PAGE.

Binding of Endo III

The ^{32}P-labeled 13 bp duplexes (10 nM) containing the 5*R* or 5*S* isomer of 2′-fluorothymidine glycol were incubated with *E.coli* Endo III (0–100 nM) on ice for 30 min in a buffer (10 µl) containing 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA and 0.1 mg/ml BSA. The mixtures were analyzed by 6% non-denaturing PAGE.

Analysis of the Endo III reaction

The ^{32}P-labeled 13 bp duplexes (10 nM) containing the 5*R* or 5*S* isomer of thymidine glycol or 2′-fluorothymidine glycol were incubated with *E.coli* Endo III (10 nM) at 25°C for 10 or 30 min in a buffer (10 µl) containing 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA and 0.1 mg/ml BSA. The enzyme reactions were performed at 25°C because of the low melting temperature (ca. 35°C) of the thymine glycol-containing duplexes. The mixtures were analyzed by 16% denaturing PAGE. To analyze the inhibitory effect of 2′-fluorothymidine glycol on the Endo III reaction, the ^{32}P-labeled 13 bp substrate duplexes without fluorine (10 nM) were incubated with *Escherichia coli* Endo III (10 and 2 nM in the cases of 5*R*- and 5*S*-thymine glycols, respectively) at 25°C for 20 min, in the presence of the 13 bp duplex containing the 5*R* or 5*S* isomer of 2′-flurothymidine glycol (0–160 nM) as a competitor. The products were analyzed by denaturing PAGE, as described above. For the kinetic analysis, similar reactions were performed using the substrate duplexes (2.5–150 nM) and the competitor (80 nM). The parameters were obtained by the Lineweaver–Burk plots.

RESULTS

Complex formation of T4 Endo V

In our previous study (16), an oligonucleotide duplex containing a fluorine atom at the 2′ position of the 5′ component of the CPD was prepared, and its reaction with T4 Endo V, which is a DNA glycosylase/AP lyase specific for the CPD, was analyzed. Because the enzyme reaction with this substrate analog was inhibited, two sets of experiments were carried out to verify the formation of an enzyme–DNA complex with a stabilized covalent bond. In one experiment, the covalent complex was detected by SDS–PAGE and was compared with that formed between the enzyme and a normal substrate under the $NaBH_4$-reducing conditions. In the other experiment, the reaction with the normal substrate was inhibited by pre-treatment of the enzyme with an equimolar amount of the fluorinated analog. Although these experiments clearly showed that the 2′-fluorine atom stabilized the covalently-bonded intermediate without the $NaBH_4$ treatment, we recently noticed an important point to be confirmed. For the SDS–PAGE analysis, the 5′ end of the CPD-containing strand was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. This means that only the 5′ side of the CPD site was proven to be intact. If there is an equilibrium between the cyclic hemiacetal and acyclic imino forms of the sugar moiety, the fluorine atom might have inhibited only the hydrolysis of the C–N bond at the product release step, and the chain might have been cleaved because the hydrogen at the 2′ 'upper' position that should be abstracted in the β-elimination reaction (20) was present in this substrate analog. Therefore, we analyzed the T4 Endo V reaction using the 3′-^{32}P-labeled substrate analog to confirm that the entire chain was intact in the complex, prior to the Endo III studies.

A 34mer duplex, d(GGCTTGTCACTATCGCGT$_F$[]TGCGCTACAGTAAGTG)·d(CACTTACTGTAGCGCAACGCGATAGTGACAAGCC), where T$_F$[]T represents the *cis–syn* CPD with a fluorine atom attached at the 2′ 'lower' position, was used. The 5′ and 3′ ends of the CPD-containing strand were labeled using T4 polynucleotide kinase and terminal deoxynucleotidyl transferase, respectively, and these oligonucleotides were hybridized to the complementary strand. Formation of the stabilized intermediate in the T4 Endo V reaction was analyzed by SDS–PAGE. As shown in Figure 1, the result of the experiment using the duplex labeled at the 3′ end (lane 4) was identical to that using the 5′-labeled one (lane 2).

Synthesis of the building blocks for the incorporation of 2′-fluorothymidine glycol (Tg$_F$)

The lack of chain cleavage for the T4 Endo V reaction with the 2′-fluorinated substrate analog indicated the practicality of these types of mechanism-based inhibitors for crystallographic studies. For application to Endo III, we planned to synthesize oligonucleotides containing 2′-fluorothymidine glycol (Tg$_F$, 1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)-5,

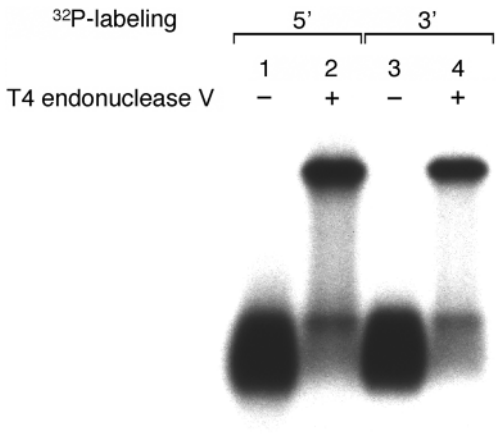
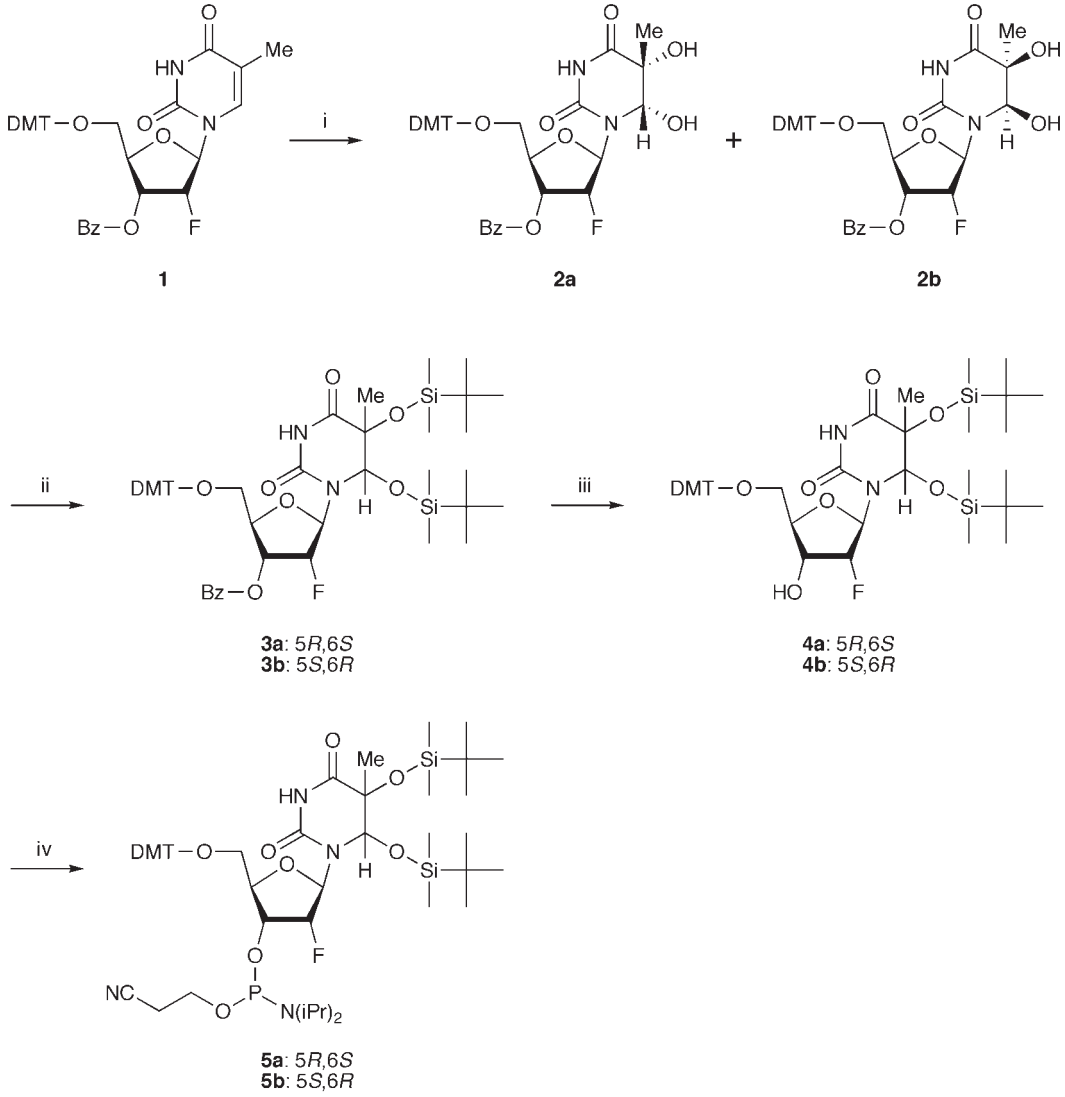


Figure 1. Formation of the covalent complex between T4 Endo V and a CPD-containing oligonucleotide duplex fluorinated at the 2′ position. The 5′ and 3′ ends of the CPD-containing strand were ^{32}P-labeled. After hybridization with the complementary strand, the duplexes were incubated with T4 Endo V at 30°C for 30 min, and the mixtures were subjected to 15% SDS–PAGE.

6-dihydro-5,6-dihydroxythymine). For this purpose, we prepared the phosphoramidite building blocks, as shown in Scheme 2, separately using the two stereoisomers of thymine glycol.

Our procedure was basically the same as that developed for the building block of thymidine glycol without fluorine (18,19). Oxidation of 2′-fluorothymidine (1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)thymine) gave two products, which were supposed to be the two isomers of *cis*-thymine glycol. The ratio of the isolation yields of these products was 3:1. In our previous study, the configuration of each isomer was determined from the NOESY spectra in ^{1}H-NMR spectroscopy, on the basis of the observation that the base moiety was in the *anti* conformation about the glycosidic bond (18). However, NOESY crosspeaks supporting this conformation were not obtained for our current products (**2a** and **2b** in Scheme 2), probably because the sugar pucker was changed from C2′-*endo* to C3′-*endo* by the fluorine substitution (21–24). Therefore, the two hydroxyl functions of the base moiety were protected with the *tert*-butyldimethylsilyl (TBDMS)



Scheme 2. Synthesis of phosphoramidite building blocks of the two isomers of Tg_F. (i) OsO_4, pyridine, room temperature, 2 h; (ii) TBDMS-Cl (five equivalents), imidazole, DMF, 37°C, 24 h; (iii) K_2CO_3, MeOH, room temperature, 2 h; (iv) $[(CH_3)_2CH]_2NP(Cl)OCH_2CH_2CN$, $[(CH_3)_2CH]_2NC_2H_5$, THF, room temperature, 1 h.

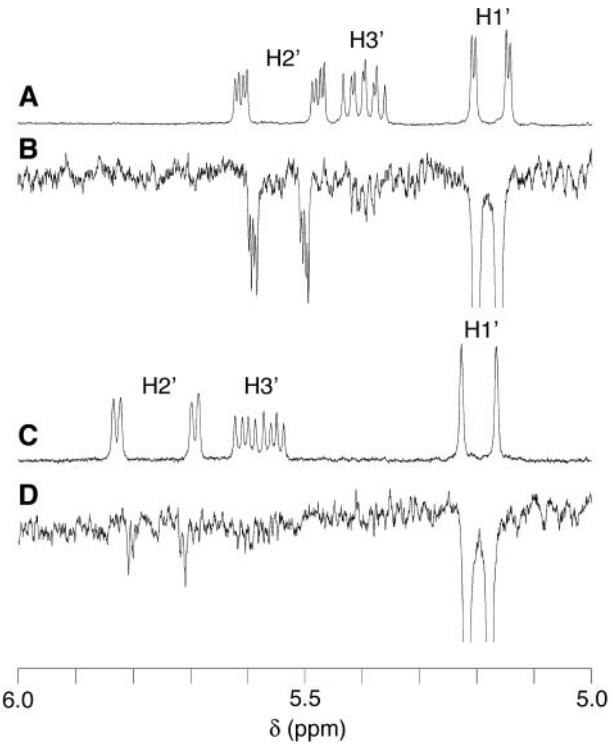


Figure 2. ^{1}H-NMR spectra (**A** and **C**) and NOE difference spectra (**B** and **D**) of compounds **3a** and **3b**. (**A** and **B**) the major isomer; (**C** and **D**) the minor isomer. The H6 resonance was saturated in the NOE experiments.

group, and the two isomers bearing the bulky TBDMS groups (**3a** and **3b**), which would fix the glycosyl torsion angle in the *syn* conformation, were used to determine the stereochemistry. As shown in Figure 2, a strong nuclear Overhauser effect (NOE) was detected between H6 and H1′ for both isomers, which showed that the base moiety was in the *syn* conformation. For the major isomer obtained by the OsO_4 oxidation, a relatively strong NOE was observed between H6 and H2′, whereas the minor one showed only a slight effect. From this result, the thymine glycols in the major and minor products were assigned as (5*R*,6*S*) (**2a**) and (5*S*,6*R*) (**2b**), respectively.

After the benzoyl (Bz) group at the 3′ position was removed with potassium carbonate, the 3′ hydroxyl function of each isomer was phosphitylated to obtain the phosphoramidite building blocks (**5a** and **5b**) for oligonucleotide synthesis (19). Because the phosphitylation reaction was slow, an excess amount (three equivalents) of the chlorophosphoramidite reagent was used, and the reaction time was prolonged to 1 h.

Synthesis of oligonucleotides containing Tg_F

Assembly of oligonucleotides on a DNA synthesizer, using compounds **5a** and **5b**, was carried out in the same way as reported previously for thymidine glycol without fluorine (18,19). The coupling yield in each cycle was monitored by a conductivity sensor on the synthesizer. Although the values obtained by this method contained relatively large errors, the coupling yields of **5a** and **5b** were between 94 and 108%, which were similar to those of the 2′-deoxynucleoside 3′-phosphoramidites with the normal bases. Since thymine glycol is labile under alkaline conditions used for oligonucleotide deprotection, commercially available nucleoside 3′-phosphoramidites bearing base-labile protecting groups, i.e. phenoxyacetyl, (4-isopropylphenoxy)acetyl and acetyl groups for adenine, guanine and cytosine, respectively, were used. A 13mer, d(ACGCGATg_FACGCCA) and a 40mer, d(GCATGCCTGCACGGTg_FCATGGCCAGATCCCCGGGTACCGAG), were synthesized, separately incorporating the thymine glycol isomers.

After the removal of the last DMT group on the synthesizer, the oligonucleotides were cleaved from the solid support with ammonium hydroxide at room temperature for 2 h, and the amino groups on the base moieties and the internucleotide phosphates were deprotected simultaneously by this treatment. The procedure for the subsequent removal of the TBDMS group was changed from the original method. We previously used tetrabutylammonium fluoride (TBAF) at this step and found the formation of 5-hydroxy-5-methylhydantoin as a byproduct (19). Glen Research reported that removal of the TBDMS group with triethylamine trihydrofluoride, which was originally used for oligoribonucleotide synthesis (25), did not cause this side reaction [*The Glen Report*, Vol. 16, No. 1, page 4 (2003)]. We adopted this method, and the results are shown in Figure 3. Because the C6 position of deprotected thymine glycol is epimerized in solution (26), we describe the stereochemistry only at the C5 position for the obtained oligonucleotides. HPLC analysis on a reversed-phase column gave a single main peak in all cases. The retention times of the oligonucleotides containing 5*R*-thymine glycol were longer than that of each 5*S* counterpart. This result is identical with that obtained for the sugar-unmodified oligonucleotides in our previous study (19). The products were purified by reversed-phase HPLC, and the molecular weights of the 13mers were confirmed by MALDI-TOF mass spectrometry, as shown in Figure 4.

Analysis of the Endo III reaction

Using the 13mer, d(ACGCGATg_FACGCCA), the Endo III reaction with the fluorine-containing substrate analogs was analyzed. After ^{32}P-labeling, the 13mers containing the 5*R* and 5*S* isomers of Tg_F were separately hybridized to the complementary strand, d(TGGCGTATCGCGT). For comparison, ^{32}P-labeled, thymine glycol-containing duplexes without fluorine were prepared in the same way. We first tried to detect the stable covalent intermediate in the reaction between *E.coli* Endo III and the fluorine-containing duplexes. Contrary to our expectation, however, such an intermediate was not observed in the analysis by SDS–PAGE, as shown in Figure 5, whereas the Schiff base-type intermediate was trapped with sodium borohydride in the reaction with the non-fluorinated substrates. Then, we analyzed the binding and the incision reaction of Endo III. As shown in Figure 6, it was found by electrophoretic mobility shift assays (EMSAs) that *E.coli* Endo III formed a complex with the 13 bp duplex containing 2′-fluoro-5*S*-thymidine glycol (5*S*-Tg_F). Binding was not detected for 5*R*-Tg_F. Cleavage of the thymine glycol-containing strand was not detected for either of the fluorinated substrate analogs (Figure 7). From these results, it is concluded that the duplex containing 5*S*-Tg_F is an inhibitor of the *E.coli*

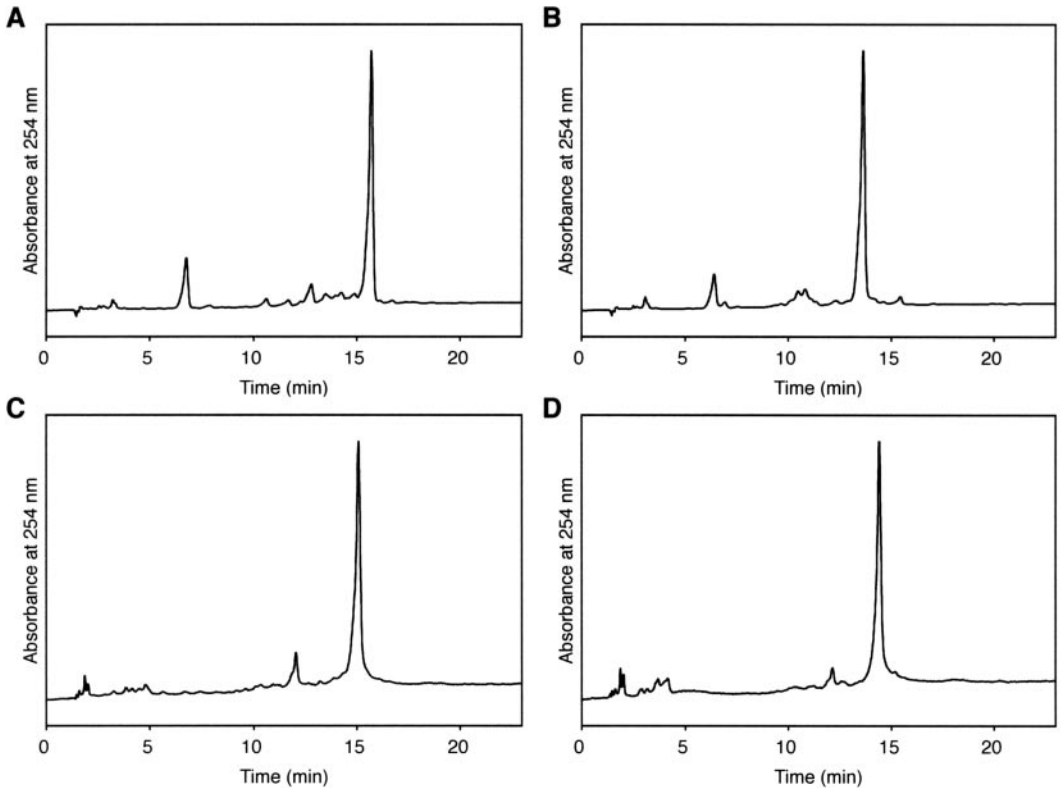


Figure 3. HPLC analyses of crude oligonucleotides on a reversed phase column. (**A**) 5*R*-Tg_F 13mer, (**B**) 5*S*-Tg_F 13mer, (**C**) 5*R*-Tg_F 40mer and (**D**) 5*S*-Tg_F 40mer. The acetonitrile gradients were 6–11% and 7–13% for the 13 and 40mers, respectively.

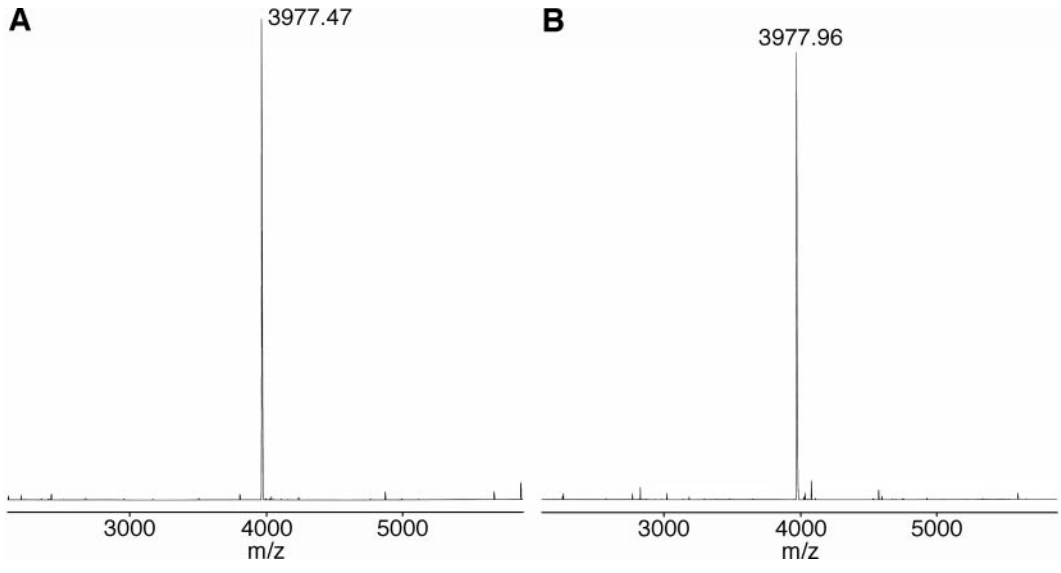


Figure 4. Analysis of the 5*R*-Tg_F (**A**) and 5*S*-Tg_F (**B**) 13mers by MALDI-TOF mass spectrometry. The calculated molecular weight is 3977.69.

Endo III reaction, although the effect of the fluorine atom is not the same as for T4 Endo V.

To characterize the duplexes containing Tg_F as inhibitors of the Endo III reaction further, ^{32}P-labeled, thymine glycol-containing duplexes without fluorine were treated with Endo III in the presence of the 13 bp duplex containing Tg_F. Consistent with the result of the EMSAs shown in Figure 6, the activity of Endo III for either duplex containing 5*R*- or 5*S*-thymine glycol was inhibited by the 5*S*-Tg_F-containing duplex in a concentration-dependent manner, but the 5*R*-Tg_F-containing one did not work (Figure 8). This result supported the distinct binding capacities of the 5*R*-Tg_F and 5*S*-Tg_F duplexes for Endo III. For quantitative analysis of the inhibition by the 5*S*-Tg_F-containing duplex, enzymatic parameters (k_{cat} and K_m) and the inhibition constant (K_i) were determined. Comparison of the enzymatic parameters

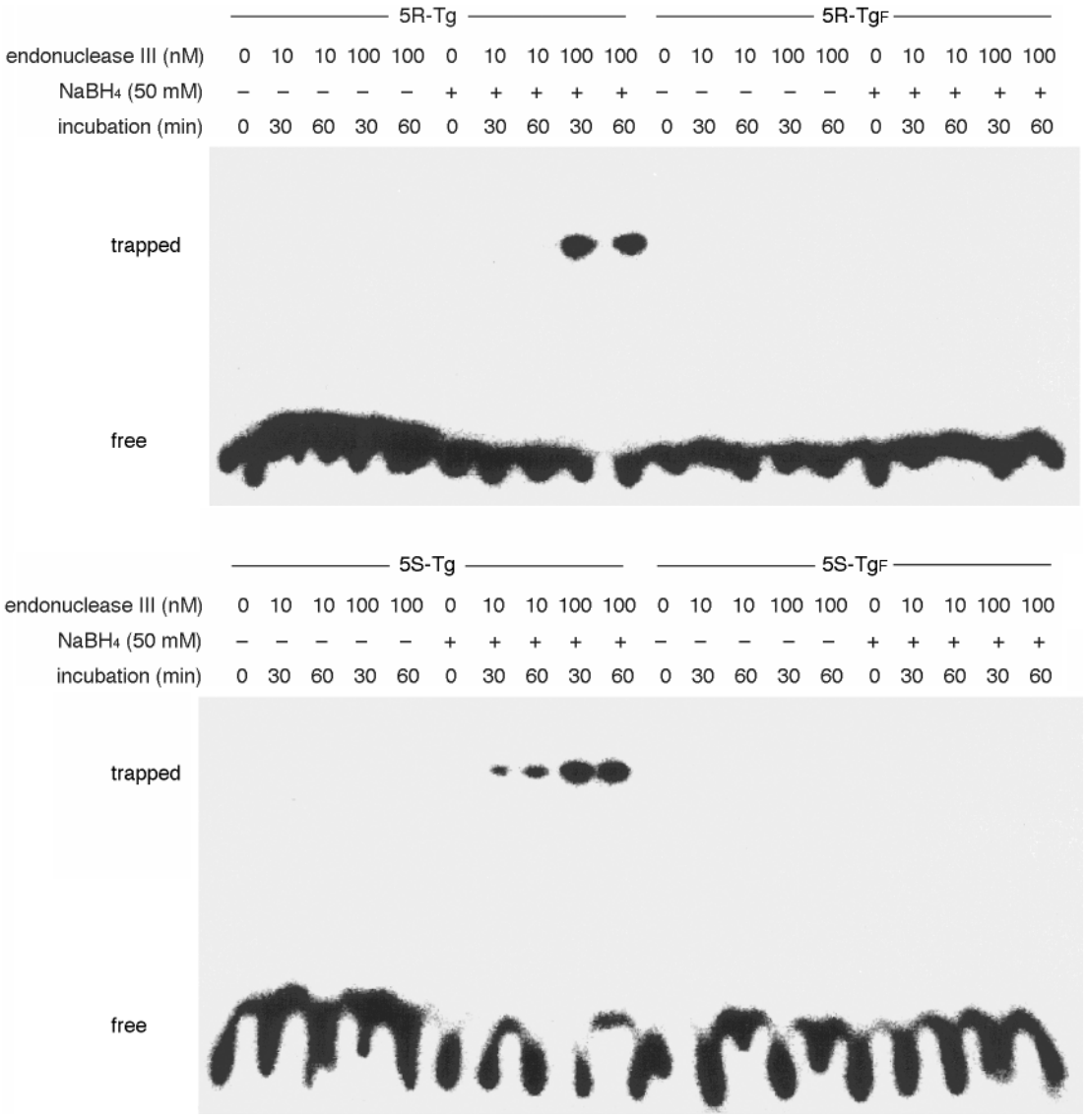


Figure 5. Formation of the covalent intermediates in the Endo III reaction. The 13 bp duplexes (10 nM) were incubated with *E.coli* Endo III in the presence or absence of $NaBH_4$, and the reaction mixtures were analyzed by 10% SDS–PAGE.

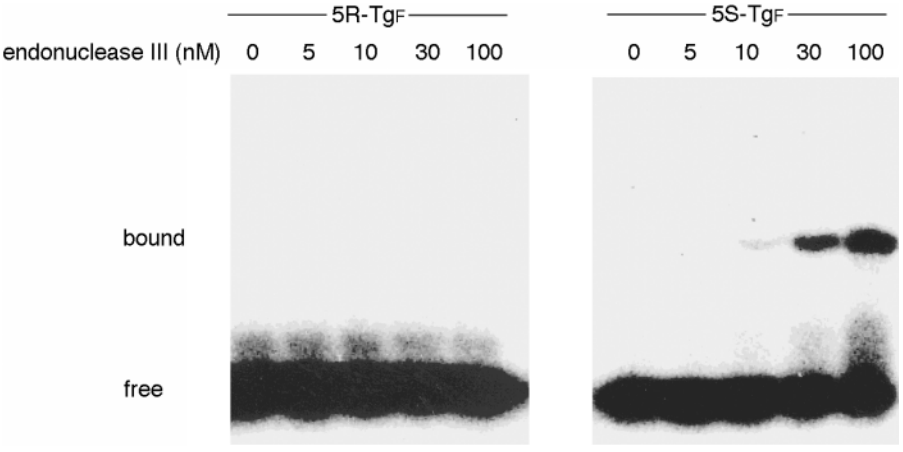


Figure 6. Binding of endonuclease III to the duplexes containing Tg_F. The 13 bp duplexes (10 nM) were incubated with *E.coli* Endo III at 0°C for 30 min, and the mixtures were analyzed by 6% non-denaturing PAGE.

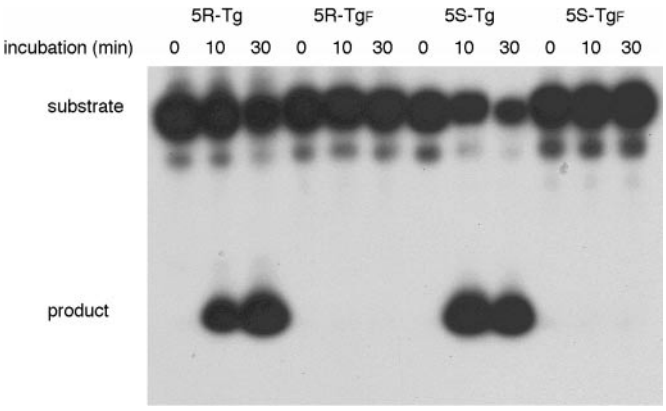


Figure 7. Analysis of the Endo III reaction. The 13 bp duplexes (10 nM) were incubated with *E.coli* Endo III (10 nM) at 25°C, and the reaction mixtures were analyzed by 16% denaturing PAGE.

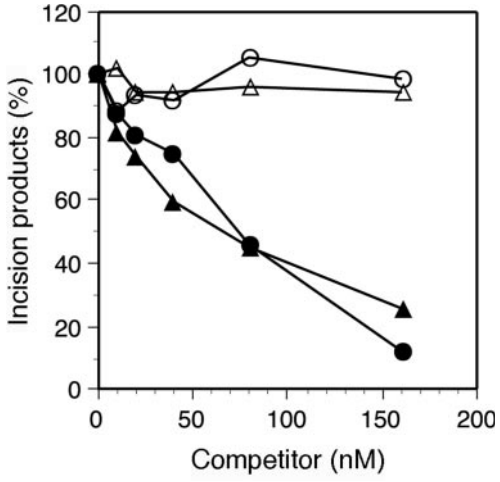


Figure 8. Competition of the 13 bp substrate duplexes and the Tg_F-containing duplexes in the Endo III reaction. The ^{32}P-labeled substrates without fluorine were incubated with Endo III in the presence of the competitors containing Tg_F. The amounts of the nicked products (standardized to those without the competitors) were plotted against the concentrations of the competitor. Open circles, ^{32}P-5*R*-Tg + 5*R*-Tg_F; filled circles, ^{32}P-5*R*-Tg + 5*S*-Tg_F; open triangles, ^{32}P-5*S*-Tg + 5*R*-Tg_F; filled triangles, ^{32}P-5*S*-Tg 5*S*-Tg_F.

Table 1. Enzymatic parameters obtained for the 13 bp duplexes containing 5*R*- and 5*S*-thymine glycols and the inhibition constant of the duplex containing 5*S*-Tg_F

Substrate	Competitor	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m (min^{-1} nM^{-1})	K_i (nM)
5*R*-Tg	None	0.36	132	0.0027	
5*S*-Tg	None	0.45	31	0.015	
5*S*-Tg	5*S*-Tg_F	0.48	40		276

(k_{cat}/K_m) showed that the 5*S*-thymine glycol-containing duplex had a higher affinity for Endo III than the 5*R* counterpart (Table 1). The Lineweaver–Burk plot obtained in the presence of the 5*S*-Tg_F competitor exhibited a feature characteristic of the competitive reaction. The k_{cat} values without and with the 5*S*-Tg_F-containing duplex as a competitor were comparable, whereas the K_m value with the competitor was notably higher than that without it. The inhibition constant (K_i = 276 nM in Table 1) was significantly higher than the K_m values obtained for the duplexes containing 5*S*- and 5*R*-thymine glycols (31 and 132 nM, respectively), and this result shows that the 5*S*-Tg_F-containing duplex is a relatively weak inhibitor of Endo III.

DISCUSSION

In this study, we intended to use a fluorinated sugar moiety as a mechanism-based inhibitor of Endo III. This enzyme forms a covalent Schiff base-type intermediate with the substrate DNA, in the same way as T4 Endo V (Scheme 1). We expected that a fluorine atom attached at the 2′ position would inhibit the Endo III reaction by stabilizing the cyclic hemiacetal form of the enzyme-linked sugar moiety, and such a substrate analog would be useful for elucidation of the recognition mechanism of this enzyme by X-ray crystallography.

In the synthesis of the building block of Tg_F, the OsO_4 oxidation of the protected 2′-fluorothymidine resulted in formation of the (5*R*,6*S*)- and (5*S*,6*R*)-thymine glycols (**2a** and **2b**, respectively) in the ratio of 3:1. This ratio was 6:1 when thymidine with the same protecting groups was oxidized under the same conditions (18), and a large-scale preparation was required to obtain the (5*S*,6*R*) isomer in a yield sufficient for its incorporation into oligonucleotides (19). This change in stereoselectivity can be attributed to the C3′-*endo* sugar pucker induced by the fluorine substitution (21–24), and the building blocks of both isomers were obtained at the same time in the present study. Since several groups have recently reported that *E.coli* Endo III and its mammalian counterpart distinguished the 5*R*- and 5*S*-thymine glycols in DNA (27–29), the higher yield of the (5*S*,6*R*) isomer is favorable for studies comparing the stereoisomers of this damaged base. Using the phosphoramidite building blocks (**5a** and **5b**), oligonucleotides containing Tg_F were synthesized, incorporating the thymine glycol isomers separately, in the same way as those without the sugar modification (19). We confirmed that triethylamine trihydrofluoride prevented the side reaction in the deprotection of thymine glycol-containing oligonucleotides.

The Endo III reaction was analyzed using the 13mers synthesized in the present study. We were mainly interested in whether the duplex containing Tg_F could form a stable covalent intermediate with Endo III that was similar to that obtained in our previous study on T4 Endo V (Figure 1). As shown in Figure 5, such an intermediate was not formed with *E.coli* Endo III. In this experiment, the covalent complex trapped with sodium borohydride was obtained when the thymine glycol-containing duplexes without fluorine were used, and there was a difference between 5*R*- and 5*S*-thymine glycol. The band was detected at a lower enzyme concentration in the case of the 5*S*-thymine glycol-containing substrate. This difference can be attributed to the higher affinity of *E.coli* Endo III for the 5*S* isomer, which was revealed in our previous study (29). To determine why the covalent intermediate was not formed with the fluorine-containing substrate analogs, enzyme binding was analyzed by EMSAs. A complex was detected for the duplex containing 5*S*-Tg_F, but not for the 5*R* isomer (Figure 6). The enzyme binding to the 5*S*-Tg_F-containing duplex was confirmed by the competition experiment (Figure 8). Although *E.coli* Endo III has a higher affinity

for the 5*S*-thymine glycol, as described above, these all-or-none results shown in Figures 6 and 8 suggest that the sugar conformation affects the substrate recognition because the fluorine atom at the 2′ position alters the sugar pucker (21–24). Finally, the DNA glycosylase/AP lyase reaction of Endo III was analyzed, as shown in Figure 7. Although the enzyme bound to the duplex containing 5*S*-Tg_F, incision of this substrate analog was not detected. These results indicate that the inhibition of the Endo III reaction by 5*S*-Tg_F was caused by the effect of the fluorine atom that is different from the mechanism discussed in our previous study on T4 Endo V (16). The electron-withdrawing fluorine atom at the 2′ position affects the reaction of the base excision repair enzyme in two ways. It stabilizes the glycosidic bond of the damaged base and changes the equilibrium between the cyclic hemiacetal and linear aldehyde forms of the deoxyribose at the AP site towards the former that prevents the β-elimination reaction. If the glycosidic bond is cleaved, backbone breakage occurs during electrophoresis (30), and the oligonucleotide containing an AP site migrates slightly faster than the intact one in the analysis by denaturing PAGE (31). In our results, both the substrate analog containing 5*S*-Tg_F after the Endo III treatment and the untreated one migrated identically on the gel, and no shorter fragment was detected (Figure 7, the 5*S*-Tg_F lanes). These results indicate that the fluorine atom at the 2′ position of thymidine glycol inhibits the DNA glycosylase activity, not the AP lyase step, in the Endo III reaction, by stabilizing the glycosidic bond.

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Conflict of interest statement. None declared.

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