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 OsO₄ produced two isomers of thymine glycol. Their configurations were determined by NMR spectroscopy after protection of the hydroxyl functions. The ratio of (5R,6S) and (5S,6R) 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 5R- or 5S-thymidine glycol were treated with Escherichia coli endo III, no stabilized covalent intermediate was observed regardless of the stereochemistry at C5. The 5S 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-hydroxuracil (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 1,2,3,6-tetrahydroxanthine (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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the intermediate with sodium borohydride (NaBH₄), 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 NaBH₄ 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). 1H-NMR spectra were measured on a JEOL AL-400 or Varian INOVA 600 spectrometer, and 31P-NMR spectra were measured on a JEOL GX270 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 NaHCO₃ and then with saturated aqueous NaCl. The organic layer was dried with Na₂SO₄ and after evaporation and coevaporation with toluene, the residue was chromatographed on silica gel (30 g). The product was eluted with chloroform and was obtained as foam after evaporation.

Yield: 5.13 g (7.69 mmol, 91%). TLC (CHCl₃/MeOH, 10/1, v/v); Rᵱ: 0.81. ¹H NMR (400 MHz, CDC₁₃, 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; H'), 4.48–4.44 (m, 1H; H⁴'), 3.75 (s, 3H; -OCH₃), 3.74 (s, 3H; -OCH₂), 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; -CH₃). HRMS (ESI): m/z: 689.2273 ([M+Na⁺], C₃₈H₃₅O₈N₂FNa: 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’,5’-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 Na₂SO₄. 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.

5’-O-(4,4’-Dimethoxytrityl)-3’-O-benzoyl-5,6-dihydro-5,6-dihydroxy-2'-fluorothymidine (1)

5,6-dihydroxy-2’-fluorothymidine (2a) and 2b)

5,6-dihydroxy-2’-fluorothymidine (2a) (443 mg, 633 μmol) in N,N-dimethylformamide (7 ml), imidazole (431 mg, 6.33 mmol) and tert-butylidimethylchlorosilane (500 mg, 3.31 mmol) were added, and the mixture was stirred at 76°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 Na₂SO₄, 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); Rᵱ: 0.59. ¹H NMR (400 MHz, CDCl₃, 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; H'), 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, 3H; -OCH₃), 3.43 (d, J = 4.8 Hz, 2H; H⁵'), 1.47 (s, 3H; -CH₃), 0.87 (s, 9H; TBDMDS), 0.84 (s, 9H; TBDMDS), 0.27 (s, 3H; TBDMDS), 0.22 (s, 3H; TBDMDS), 0.16 (s, 3H; TBDMDS). HRMS (ESI): m/z: 951.4040 ([M+Na⁺], C₅₀H₆₅O₁₀N₂FSi₂Na: 951.4053).

5’-O-(4,4’-Dimethoxytrityl)-3’-O-benzoyl-(5S,6R)-5,6-dihydro-5,6-dihydroxy-2’-fluorothymidine (3b)

To a solution of 5’-O-(4,4’-dimethoxytrityl)-3’-O-benzoyl-(5S,6R)-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-butylidimethylchlorosilane (363 mg, 2.41 mmol) were added, and the mixture was stirred at 76°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 Na₂SO₄, 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); Rᵱ: 0.62. ¹H NMR (400 MHz, CDCl₃, 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; H'), 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; -OCH₃), 3.39 (d, J = 4.4 Hz, 2H; H⁵'), 1.53 (s, 3H; -CH₃), 0.88 (s, 9H; TBDMDS), 0.85 (s, 9H; TBDMDS), 0.26 (s, 3H; TBDMDS), 0.21 (s, 3H; TBDMDS), 0.12 (s, 3H; TBDMDS), 0.10 (s, 3H; TBDMDS). HRMS (ESI): m/z: 951.4039 ([M+Na⁺], C₅₀H₆₅O₁₀N₂FSi₂Na: 951.4053).
5'-O-(4,4'-Dimethoxytrityl)-(5S,6R)-5, 6-dihydro-5,6-di[(tert-butyl)dimethylsilyloxy]-2'-'fluorothymidine (4a)

5'-O-(4,4'-Dimethoxytrityl)-3'-O-benzoyl-(5R,6S)-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 Na2SO4, and after evaporation, the residue was chromatographed on silica gel (30 g) with a step gradient of ethyl acetate in hexane containing 0.1% pyridine. 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): Rf 0.43. 1H NMR (400 MHz, CDCl3, 30°C, TMS): δ 7.44 (d, J = 7.7 Hz, 2H; DMT), 7.32 (d, 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; OCH3), 3.41–3.31 (m, 2H; H5'), 2.02 (dd, J = 7.9, 3.8 Hz, 1H; 3'-OH), 1.46 (s, 3H; -CH3), 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]+, C43H61O9N2FSi2Na: 847.3791).

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 μBondaplace 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 μBondaplace C18 15 μm 300 Å column (7.8 × 300 mm) (Waters) was used, and the acetonitrile gradient was 8–13% for 30 min.

32P-labeling of a CPD-containing oligonucleotide

A 34mer, d(GGCTTGTACATCGCGTCGTA)[T]TGC(GTACTACCATGA), where T4[T]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

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[γ-32P]ATP and T4 polynucleotide kinase (10 U) (Takara Bio) in a buffer (50 μl) containing 50 mM Tris–HCl (pH 9.5), 10 mM MgCl2, 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-32P]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 32P-labeled 13 bp duplexes (10 nM) containing the 5’- or 3’-32P-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 32P-labeled 13 bp duplexes (10 nM) containing the 5R or 5S isomer of thymidine glycol or 2’-fluorothymidine glycol, 32P-d(ACCGGTAGTGACAAGCC), 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 NaBH4. The mixtures were incubated at 30°C for 30 min and were subjected to 15% SDS–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 complex was detected by SDS–PAGE and was compared with that formed between the enzyme and a normal substrate under the NaBH4-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 NaBH4 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-32P]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’-32P-labeled substrate analog to confirm that the entire chain was intact in the complex, prior to the Endo III studies.

A 34mer duplex, d(GGCTTGTGACTATCGCGT_F)[TGCGCTACGTTAAGTG]-d(CACTTACTGTACAACGCCAGGGATAGTGAACAG), where T_F 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 (T_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 (T_F, 1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)-5,
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 1H-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) group.

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 32P-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.

Scheme 2. Synthesis of phosphoramidite building blocks of the two isomers of TgF. (i) OsO4, pyridine, room temperature, 2 h; (ii) TBDMS-Cl (five equivalents), imidazole, DMF, 37°C, 24 h; (iii) K2CO3, MeOH, room temperature, 2 h; (iv) [(CH3)2CH]2NP(Cl)OCH2CH2CN, [(CH3)2CH]2NC2H5, THF, room temperature, 1 h.
The H6 resonance was saturated in the NOE experiments. 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(ACGCCGATgACGCCCA), the Endo III reaction with the fluorine-containing substrate analogs was analyzed. After 32P-labeling, the 13mers containing the 5R and 5S isomers of TgF were separately hybridized to the complementary strand, d(TGCCGTATCGCGT). For comparison, 32P-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 5'-fluoro-5S-thymidine glycol (5S-TgF). Binding was not detected for 5R-TgF. 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 5S-TgF is an inhibitor of the E.coli...
Endo III reaction, although the effect of the fluorine atom is not the same as for T4 Endo V.

To characterize the duplexes containing Tgf as inhibitors of the Endo III reaction further, 32P-labeled, thymine glycol-containing duplexes without fluorine were treated with Endo III in the presence of the 13 bp duplex containing Tgf. Consistent with the result of the EMSAs shown in Figure 6, the activity of Endo III for either duplex containing 5R- or 5S-thymine glycol was inhibited by the 5S-Tgf-containing duplex in a concentration-dependent manner, but the 5R-Tgf-containing one did not work (Figure 8). This result supported the distinct binding capacities of the 5R-Tgf and 5S-Tgf duplexes for Endo III. For quantitative analysis of the inhibition by the 5S-Tgf-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 NaBH₄, and the reaction mixtures were analyzed by 10% SDS-PAGE.

**Figure 6.** Binding of endonuclease III to the duplexes containing T₅F. 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.
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 TgF, the OsO₄ oxidation of the protected 2′-fluorothyridine resulted in formation of the (5S,6S)- and (5S,6R)-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 (5S,6R) 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 5R- and 5S-thymine glycols in DNA (27–29), the higher yield of the (5S,6R) isomer is favorable for studies comparing the stereoisomers of this damaged base. Using the phosphoramidite building blocks (5a and 5b), oligonucleotides containing TgF 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 TgF 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 5R- and 5S-thymine glycol. The band was detected at a lower enzyme concentration in the case of the 5S-thymine glycol-containing substrate. This difference can be attributed to the higher affinity of E.coli Endo III for the 5S 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 synthesized in the present study. We were mainly interested in whether the duplex containing TgF could form a stable covalent intermediate with Endo III that was similar to that obtained in our previous study on 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.

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for the 5S-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 5S-TGF, incision of this substrate analog was not detected. These results indicate that the inhibition of the Endo III reaction by 5S-TGF 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 5S-TGF after the Endo III treatment and the untreated one migrated identically on the gel, and no shorter fragment was detected (Figure 7, the 5S-TGF 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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