Synthesis and characterization of a substrate for T4 endonuclease V containing a phosphorodithioate linkage at the thymine dimer site

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ABSTRACT

A dodecadeoxyribonucleotide containing a cis-syn thymine dimer with a phosphorodithioate linkage was synthesized on a solid support using a dinucleotide coupling unit prepared by UV-irradiation of dithymidine monophosphorodithioate followed by *S*- and 5′-*O*-protection and 3′-phosphitylation. A photodimer-containing dodecamer without phosphate modification was also synthesized. The dodecamers were hybridized to the complementary sequence, and the duplexes used as substrates for T4 endonuclease V. This enzyme cleaved the phosphate-modified substrate more slowly than the unmodified duplex with the same dissociation constant.

INTRODUCTION

Many kinds of oligodeoxyribonucleotides containing modified internucleotide linkages have been synthesized for use as antisense agents. For instance, synthetic methods and properties of oligonucleotides containing the methylphosphonate linkage and the phosphorothioate linkage have been studied intensively, and gene expression and virus replication have been inhibited using these analogs (1−6). In recent years, the phosphorodithioate linkage, which is achiral and conserves the ionic character, has been developed (7−12). It is of interest to utilize these analogs for investigation of interactions between proteins and the phosphate moiety of DNA and for elucidation of the mechanisms for recognition of the substrates by enzymes.

In this paper, we describe the synthesis of a dodecadeoxyribonucleotide containing a cis-syn thymine dimer, into which a phosphorodithioate linkage is incorporated, and its use as a substrate for endonuclease V from bacteriophage T4 (13), which cleaves a thymine dimer and the 3′-phosphodiester bond of the consequent apyrimidinic site via a β-elimination mechanism (14−17). The phosphorodithioate linkage was prepared via the phosphonodithioate as described (11), and after deprotection the dithymidine phosphorodithioate was irradiated using acetone as a photosensitizer to form the cyclobutane ring between adjacent thymines (18). The cis-syn dimer was separated from the trans-syn isomer by high-performance liquid chromatography (HPLC) and phosphitylated after protection of the internucleoside linkage and the 5′-hydroxyl function. Using this coupling unit, a dodecamer (dGCACGT[PS2]TGCACG, T[PS2]T is the thymine dimer containing the phosphorodithioate linkage) was synthesized on controlled pore glass (CPG). The phosphate-unmodified dodecamer (dGCACGT[ ]TGCACG, T[ ]T is the thymine dimer containing a phosphodiester linkage) was also synthesized in the same way. These dodecamers were hybridized to the complementary sequence (dCGTGCAACGTGC), and the catalytic properties of T4 endonuclease V for each substrate were assayed.

EXPERIMENTAL

General methods

Thin layer chromatography (TLC) was performed on Kieselgel $60F_{254}$ plates (Merck) with chloroform-methanol (10:1, v/v). For reversed-phase TLC, Kieselgel $60F_{254}$ silanisiert plates (Merck) were used with a solvent system of acetone-20 mM triethylammonium acetate (TEAA, pH 7.0). For column chromatography, Wakogel C-300 (Wako Pure Chemical Industries) and PREPARATIVE C18 (Waters Associates) were used.

^{1}H-NMR spectra of the protected nucleosides and nucleotides were measured at 100 MHz with a JEOL JNM-FX100 spectrometer. For dithymidine monophosphorodithioate containing the cis-syn thymine dimer **11**, JEOL JNM-GX270 and Brucker AM500 spectrometers were used at 270 and 500 MHz respectively. ^{31}P-NMR spectra were measured at 36.25 MHz with a JEOL JNM-FX90Q spectrometer using trimethyl phosphate as an internal standard or 85% phosphoric acid as an external standard.

Separation of the cis-syn thymine dimer derivative containing the phosphorodithioate linkage from the trans-syn isomer was performed on a Shimadzu LC-6A system using an Inertsil PREP ODS column (20 mm I.D.×250 mm L., Gasukuro Kogyo) with a linear gradient of acetonitrile in 0.1 M TEAA at a flow rate of 9.0 ml/min. For HPLC analysis of oligonucleotides, a YMC AM-303 column (4.6 mm I.D.×250 mm L., Yamamura Chemical Laboratories) or a CHEMCOSORB 5-ODS-H column (4.6 mm I.D.×250 mm L., Chemco Scientific) was used at a flow rate of 1.0 ml/min on a Waters ALC/GPC 608 system.

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Purification of oligonucleotides was performed on a GILSON system using a YMC AM-324 column (10 mm I.D.×300 mm L., Yamamura Chemical Laboratories) at a flow rate of 2.0 ml/min. For anion-exchange HPLC, a TSK gel DEAE-2SW column (4.6 mm I.D.×250 mm L., Tosoh Corporation) was used with a linear gradient of ammonium formate in 20% aqueous acetonitrile at a flow rate of 1.0 ml/min on a Shimadzu LC-3A system.

T4 endonuclease V was obtained by expression of a synthetic gene as described previously (20).

Preparation of 3'-*O*-levulinylthymidine (2)

5'-*O*-(4, 4'-Dimethoxytrityl)thymidine (5.45 g, 10 mmol) was dissolved in 1, 4-dioxane (100 ml), and 4-dimethylaminopyridine (0.10 g, 0.82 mmol) and 1, 3-dicyclohexylcarbodiimide (5.16 g, 25 mmol) were added. To this solution, levulinic acid (2.05 ml, 20 mmol) was added, and the mixture was stirred for 1 hr. The solution was washed with saturated $NaHCO_3$ and dried with Na_2SO_4. After evaporation, the residue was purified by chromatography on silica gel eluted with chloroform-methanol (10:1, v/v) and then by reversed-phase chromatography eluted with acetone-0.1% aqueous pyridine (45:55, v/v). The product was precipitated with hexane (500 ml) from a chloroform solution (30 ml). yield: 5.12 g (7.97 mmol, 80%). ^{1}H-NMR ($CDCl_3$/TMS) δ: 8.02 (br, 1H, -NH-), 7.56 (s, 1H, H-6), 7.4−6.7 (m, 13H, DMTr), 6.42 (t, 1H, H-1'), 5.5−5.3 (m, 1H, H-3'), 4.2−4.0 (m, 1H, H-4'), 3.79 (s, 6H, DMTr), 3.6−3.4 (m, 2H, H-5'), 2.9−2.3 (m, 6H, H-2' and Lev), 2.19 (s, 3H, Lev), 1.57(s, 3H, CH_3-).

5'-*O*-Dimethoxytrityl-3'-*O*-levulinylthymidine (3.86 g, 6 mmol) was dissolved in 80% aqueous acetic acid (120 ml). After 30 min, the mixture was concentrated and coevaporated with pyridine and toluene in turn. The residue was purified by chromatography on silica gel eluted with 3% methanol in chloroform. 3'-*O*-Levulinylthymidine **2** was obtained as a foam by evaporation. yield: 2.01 g (5.90 mmol, 98%).

Preparation of protected dithymidine monophosphate (3)

5'-*O*-Dimethoxytritylthymidine 3'-(2-cyanoethyl)-*N*, *N*-diisopropylphosphoramidite **1** (2.23 g, 3.0 mmol) and 3'-*O*-levulinylthymidine **2** (0.85 g, 2.5 mmol) were dissolved in anhydrous acetonitrile (40 ml), and 1H-tetrazole (0.70 g, 10 mmol) was added. After 10 min, the solution was washed with water, dried with Na_2SO_4, and concentrated. To this residue, 0.11 M iodine in tetrahydrofuran-pyridine-water (80:18:2, v/v/v) (50 ml) was added, and the mixture was stirred for 40 min. Then 1 M aqueous $Na_2S_2O_3$ (10 ml) and chloroform (100 ml) were added. After separation and concentration of the organic layer, the product was purified by chromatography on silica gel eluted with 2% methanol in chloroform and precipitated with hexane (250 ml) from a chloroform solution (15 ml). yield: 1.84 g (1.84 mmol, 74%). ^{31}P-NMR ($CDCl_3$/trimethyl phosphate) δ: −5.12.

The fully-protected dimer (1.84 g, 1.84 mmol) was dissolved in 80% aqueous acetic acid (30 ml). After 3 hr, the mixture was concentrated and coevaporated with pyridine and toluene in turn. The residue was purified by chromatography on silica gel eluted with 4% methanol in chloroform. The product **3** was precipitated with hexane-ethyl ether (1:1, v/v) (100 ml) from a chloroform solution (5 ml). yield: 0.93 g (1.34 mmol, 73%). ^{1}H-NMR ($CDCl_3$/TMS) δ: 8.8−8.6 (m, 2H, -NH-), 7.40 (s, 1H, H-6), 7.30 (s, 1H, H-6), 6.4−6.0 (m, 2H, H-1'), 5.4−5.1 (m, 2H, H-3'), 4.4−4.1 (m, 6H, H-4' and 5'), 4.0−3.7 (m, 2H, CE), 2.9−2.3 (m, 10H, H-2', CE, and Lev), 2.19 (s, 3H, Lev), 1.96 (s, 3H, CH_3-), 1.90 (s, 3H, CH_3-).

Photodimerization of protected dithymidine monophosphate (3)

Protected dithymidine monophosphate **3** (0.91 g, 1.3 mmol) was dissolved in 30% aqueous acetonitrile containing 5% acetone (400 ml). After nitrogen purge, the solution was irradiated at 0°C for 13 hr in a 500 ml capacity Pyrex immersion well apparatus fitted with a 400 W high pressure mercury lamp (Rikoh Scientific Industries UVL-400P). After evaporation, the products (four isomers due to the thymine dimer and chirality on phosphorus) were isolated by chromatography on silica gel eluted with chloroform-methanol (9:1, v/v). An aliquot of each product was deprotected with aqueous ammonia and analyzed by reversed-phase HPLC. Two isomers were co-eluted with the authentic cis-syn dimer prepared by irradiation of completely deprotected dithymidine monophosphate. The cis-syn and trans-syn derivatives (**4** and **5** respectively) were collected separately. yield: cys-syn 0.38 g (0.54 mmol, 42%), trans-syn 0.18 g (0.26 mmol, 20%).

Protection and phosphitylation of the dimer (4)

The cis-syn thymine dimer derivative **4** (0.38 g, 0.54 mmol) was dissolved in pyridine (5 ml), and 4, 4'-dimethoxytrityl chloride (0.22 g, 0.65 mmol) was added. After 3 hr, methanol (1 ml) was added, and the solvent was evaporated. The residue was dissolved in chloroform and washed with water. After concentration and coevaporation with toluene, the 5'-dimethoxytrityl derivative was purified by chromatography on silica gel eluted with 4% methanol in chloroform and precipitated with hexane (50 ml) from a chloroform solution (3 ml). yield: 0.41 g (0.41 mmol, 76%).

The 5'-tritylated dimer (0.41 g, 0.41 mmol) was dissolved in pyridine (5 ml), and 0.82 M hydrazine monohydrate in pyridine-acetic acid (3:2, v/v) (5 ml, 4.1 mmol) was added. After 5 min, acetone (0.6 ml) was added, and the mixture was diluted with chloroform and washed with water. The organic layer was further washed with 10% aqueous $NaHCO_3$ and saturated NaCl in turn and dried with Na_2SO_4. After evaporation, the 3'-deprotected dimer was purified by chromatography on silica gel eluted with 5% methanol in chloroform and precipitated with hexane (30 ml) from a chloroform solution (2 ml). yield: 0.29 g (0.32 mmol, 78%).

The 3'-deprotected dimer (0.20 g, 0.22 mmol) was dried by coevaporation with pyridine and dissolved in tetrahydrofuran (2.2 ml) containing *N*, *N*-diisopropylethylamine (0.15 ml, 0.88 mmol). To this solution, 2-cyanoethyl *N*, *N*-diisopropylchlorophosphoramidite (0.10 ml, 0.44 mmol) was added. After stirring for 80 min, the mixture was diluted with ethyl acetate, washed with saturated aqueous $NaHCO_3$, and dried with Na_2SO_4. The 3'-phosphoramidite derivative **6** was purified by chromatography on silica gel eluted with 1% methanol in chloroform containing 0.1% pyridine and precipitated with pentane (20 ml) from a chloroform solution (1 ml). yield: 0.13 g (0.12 mmol, 54%). ^{31}P-NMR ($CDCl_3$/trimethyl phosphate) δ: 147.27, 145.86, −5.79, −7.54.

Synthesis of a 12mer containing the thymine dimer

The building block **6** (0.13 g, 0.12 mmol) was dissolved in anhydrous acetonitrile (0.86 ml) and attached to a DNA synthesizer (Applied Biosystems 381A). The dodecamer containing the cis-syn thymine dimer (dGCACGT[ ]TGCACG)

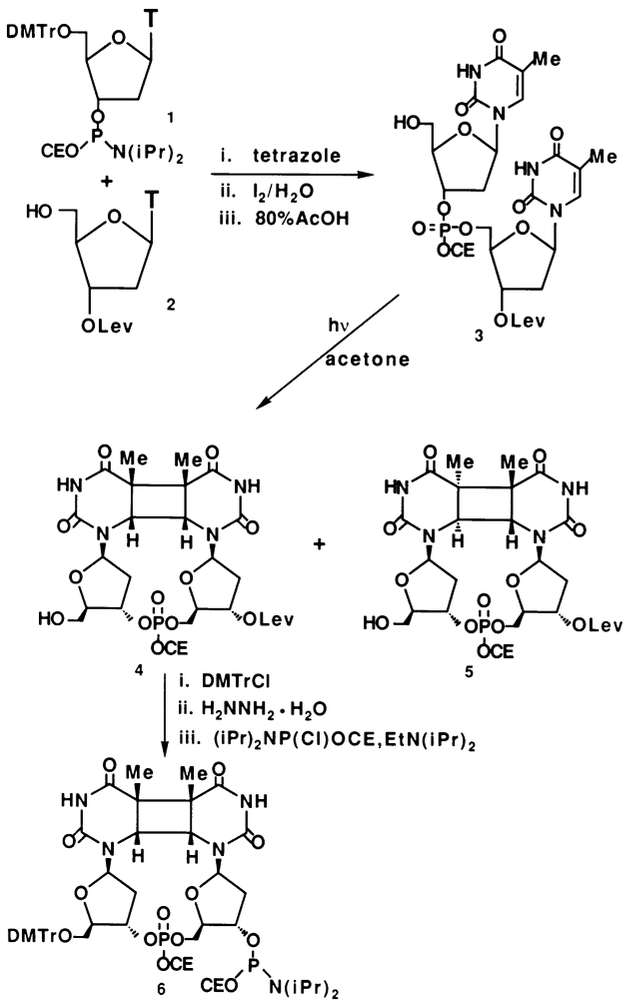

Figure 1. Synthesis of the building block containing the cis-syn thymine dimer.

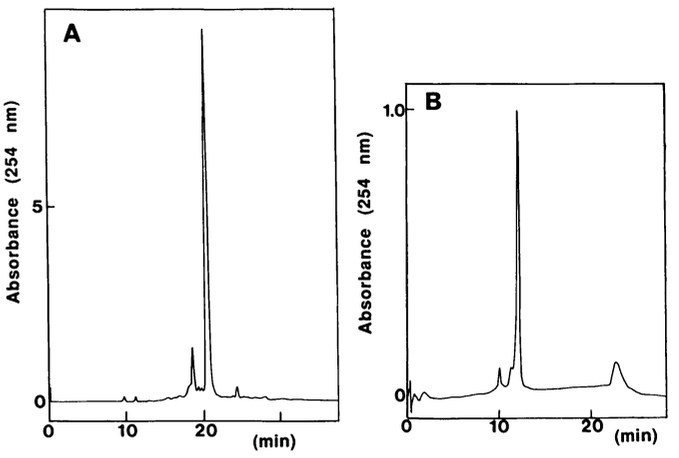

Figure 2. Purification of dGCACGT[ ]TGCACG. The deprotected 12mer was purified first by reversed-phase HPLC using a YMC A-324 column with a linear gradient of acetonitrile (from 9 to 13% during 20 min) in 0.1 M TEAA (**A**) and further by anion-exchange HPLC using a TSK gel DEAE-2SW column with a linear gradient of ammonium formate (from 0.3 to 0.7 M during 20 min) in 20% aqueous acetonitrile (**B**).

was synthesized on a 10 μmol scale. The reactions for chain elongation were as follows; 1) detritylation with trichloroacetic acid (4.8%, w/v) in dichloromethane for 3 min, 2) coupling of the phosphoramidite derivative (49–56 mM) using 1H-tetrazole (0.26 M) as an activator in anhydrous acetonitrile for 3 min (The reaction time was prolonged to 30 min in the case of **6.**), 3) capping of the remaining 5′-hydroxyl function with acetic anhydride (0.44 M) and 1-methylimidazole (0.88 M) in tetrahydrofuran containing 2, 6-lutidine (0.42 M) for 3 min, and 4) oxidation of the phosphite linkage with iodine (0.11 M) in tetrahydrofuran-pyridine-water (80:18:2, v/v/v) for 3 min. After the reaction cycle was repeated ten times, the CPG support was treated with aqueous ammonia (2.5 ml×4) for 1 hr, and the resulting solution was heated in a sealed vial at 55°C for 5 hr. Then ammonia and water were evaporated in vacuo, and the product was purified by reversed-phase chromatography. Elution was performed with a linear gradient of acetonitrile (0–50%) in 50 mM TEAA, and the fractions eluted later were collected. After concentration and coevaporation with water, the oligonucleotide with the dimethoxytrityl group at the 5′-end was treated with 80% aqueous acetic acid for 30 min. Acetic acid was removed by evaporation and coevaporation with water, and the residue was dissolved in water and washed with chloroform. The deprotected dodecamer was purified by both reversed-phase and anion-exchange HPLC as shown in Figure 2. An aliquot of the dodecamer dissolved in water was irradiated for 2 hr in a quartz cell under a 10 W germicidal lamp to confirm the presence of the thymine dimer in the isolated product. The result is shown in Figure 3. yield: 100 A_{260} units.

Preparation of 5′-*O*-dimethoxytritylthymidine 3′-phosphonodithioate (8)

A mixture of phosphorus trichloride (3.49 ml, 40 mmol), triethylamine (36.2 ml, 260 mmol), and 1H-tetrazole (8.41 g, 120 mmol) in tetrahydrofuran (160 ml) was stirred at room temperature for 30 min under a nitrogen atmosphere, and a solution of 5′-*O*-dimethoxytritylthymidine (5.45 g, 10 mmol) in tetrahydrofuran (50 ml) was added dropwise at −30°C. After 10 min, the reaction mixture was bubbled with hydrogen sulfide at room temperature for 20 min and stirred for 1 hr under a nitrogen atmosphere, followed by bubbling of nitrogen for 20 min. The resulting mixture was diluted with ethyl acetate, washed with 1 M triethylammonium bicarbonate (TEAB), and dried with Na_2SO_4. The product **8** was purified by chromatography on silica gel eluted with ethyl acetate-chloroform-methanol-triethylamine (60:30:5:5, v/v/v/v) and then by reversed-phase chromatography eluted with acetone-0.1% aqueous pyridine (35:65, v/v) and obtained as a foam by evaporation. yield: 4.67 g (6.29 mmol, 63%). ^{1}H-NMR ($CDCl_3$/TMS) δ: 8.70 (d, 1H, P-H, J=547 Hz), 8.36 (br, 1H, -NH-), 7.6–6.7 (m, 14H, H-6 and DMTr), 6.6–6.3 (m, 1H, H-1′), 5.5–5.2 (m, 1H, H-3′), 4.6–4.3 (m, 1H, H-4′), 3.78 (s, 6H, DMTr), 3.6–3.3 (m, 2H, H-5′), 3.22 (q, 2H, Et_3N, J=7.3 Hz), 3.0–2.2 (m, 2H, H-2′), 1.6–1.2 (m, 6H, CH_3- and Et_3N). ^{31}P-NMR ($CDCl_3$/85% H_3PO_4) δ: 84.5, 84.1.

Preparation of dithymidine monophosphorodithioate (10)

5′-*O*-Dimethoxytritylthymidine 3′-phosphonodithioate **8** (1.56 g, 2.1 mmol) and 3′-*O*-levulinylthymidine **2** (0.36 g, 1.1 mmol) were dissolved in pyridine-acetonitrile (5:1, v/v) (24 ml), and pivaloyl chloride (0.26 ml, 2.1 mmol) was added dropwise under a nitrogen atmosphere. The reaction mixture was stirred for 20 min, diluted with ethyl acetate, and washed with 1 M TEAB. The organic layer was further washed with saturated aqueous

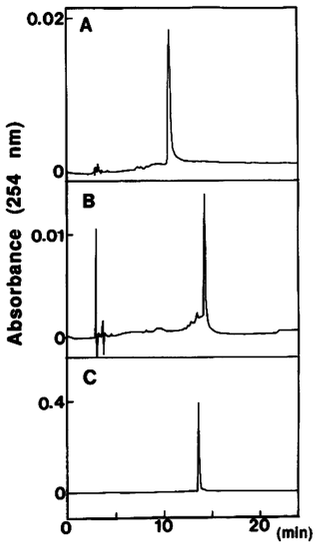

Figure 3. Elution profiles of dGCACGT[ ]TGCACG before and after photolysis (**A** and **B**, respectively) and dGCACGTTGCACG (**C**). A YMC A-303 column was used with a linear gradient of acetonitrile (from 9 to 17% during 20 min) in 0.1 M TEAA.

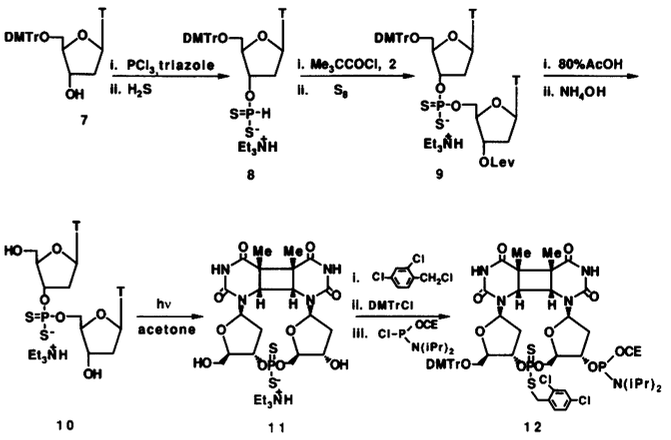

Figure 4. Synthesis of the building block containing the thymine dimer and the phosphorodithioate linkage.

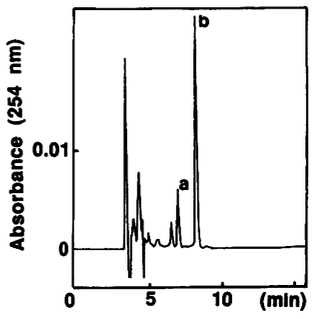

Figure 5. Separation of the photoreaction products by HPLC. An Inertsil PREP ODS column was used with a linear gradient of acetonitrile (from 11 to 13% during 20 min) in 0.1 M TEAA. Peak **a** and peak **b** were identified with the trans-syn and cis-syn thymine dimers respectively.

NaCl, dried with Na_2SO_4, and concentrated. The residue was dissolved in chloroform (6 ml), precipitated with pentane (100 ml), and added to a solution of sulfur (1.34 g, 10.5 mmol) in pyridine (40 ml). This mixture was stirred for 30 min, diluted with ethyl acetate, and washed with 1 M aqueous $Na_2S_2O_3$. The organic layer was filtered and dried with Na_2SO_4. The product **9** was purified by chromatography on silica gel eluted with 5% methanol in chloroform containing 0.5% triethylamine and then by reversed-phase chromatography eluted with acetone-0.1% aqueous pyridine (35:65, v/v) and obtained as a foam by evaporation. yield: 0.71 g (0.66 mmol, 63%). ^{1}H-NMR ($CDCl_3$/TMS) δ: 9.4–8.8 (m, 2H, -NH-), 8.8–7.7 (m, 15H, H-6 and DMTr), 6.6–6.3 (m, 2H, H-1′), 5.7–5.3 (m, 2H, H-3′), 4.6–4.0 (m, 4H, H-4′ and 5′), 3.78 (s, 6H, DMTr), 3.6–3.3 (m, 2H, H-5′), 3.28 (q, 2H, Et_3N, J=7.3 Hz), 2.9–2.2 (m, 8H, H-2′ and Lev), 2.18 (s, 3H, Lev), 2.02 (s, 3H, CH_3-), 1.5–1.1 (m, 6H, CH_3- and Et_3N). ^{31}P-NMR ($CDCl_3$/85% H_3PO_4) δ: 112.9.

Protected dithymidine monophosphorodithioate **9** (0.87 g, 0.81 mmol) was dissolved in 80% aqueous acetic acid (30 ml), and the solution was stirred for 30 min. Acetic acid was removed by evaporation and coevaporation with water, and the residue was dissolved in water and washed with chloroform. The aqueous layer was concentrated, and 28% aqueous ammonia (50 ml) was added. After 30 min, ammonia and water were evaporated, and deprotected dithymidine monophosphorodithioate **10** was purified by reversed-phase chromatography. Elution was performed with a linear gradient of acetonitrile (5–25%) in 50 mM TEAA. yield: 0.40 g (0.59 mmol, 73%). ^{31}P-NMR (D_2O/85% H_3PO_4) δ: 113.3.

Photodimerization of dithymidine monophosphorodithioate (10)

Dithymidine monophosphorodithioate **10** (0.50 g, 0.74 mmol) was dissolved in 5% aqueous acetone (400 ml). After nitrogen purge for 10 min, the solution was irradiated at 0°C for 6 hr in the apparatus described above. After evaporation, the products were separated from the starting material by reversed-phase chromatography eluted with a linear gradient of acetonitrile (0–20%) in 50 mM TEAA. The cis-syn isomer **11** was partitioned by HPLC as shown in Figure 5. An aliquot of **11** was passed through columns (5.5 mm I.D.×3 cm L.) of DOWEX 50W-X2 (pyridinium form), CHELEX 100, and DOWEX 50W-X2 (sodium form) in turn. ^{1}H-NMR spectra of the sodium salt of **11** were measured at 500 MHz, and all signals were assigned using the DQF-COSY technique. A NOESY spectrum was also measured to confirm the structure (Figure 6). yield: 0.17 g (0.25 mmol, 34%). ^{1}H-NMR (D_2O/TMS) δ: 5.81 (dd, 1H, H-1′ of Tp, J=2.93, 5.87 Hz), 5.49 (dd, 1H, H-1′ of pT, J=3.30, 5.31 Hz), 4.8–4.7 (m, 1H, H-3′ of pT), 4.20 (d, 1H, H-6 of Tp, J=6.04 Hz), 4.2–4.1 (m, 1H, H-3′ of Tp), 4.10 (d, 1H, H-6 of pT, J=6.04 Hz), 4.1–4.0 (m, 1H, H-4′ of pT), 4.0–3.9 (m, 2H, H-5′ of Tp), 3.9–3.8 (m, 1H, H-4′ of Tp), 3.61 (d, 2H, H-5′ of pT, J=3.67 Hz), 2.6–2.5 (m, 1H, H-2″ of pT), 2.3–2.2 (m, 1H, H-2′ of pT), 2.2–2.1 (m, 1H, H-2′ of Tp), 2.0–1.9 (m, 1H, H-2″ of Tp), 1.37 (s 3H, Me

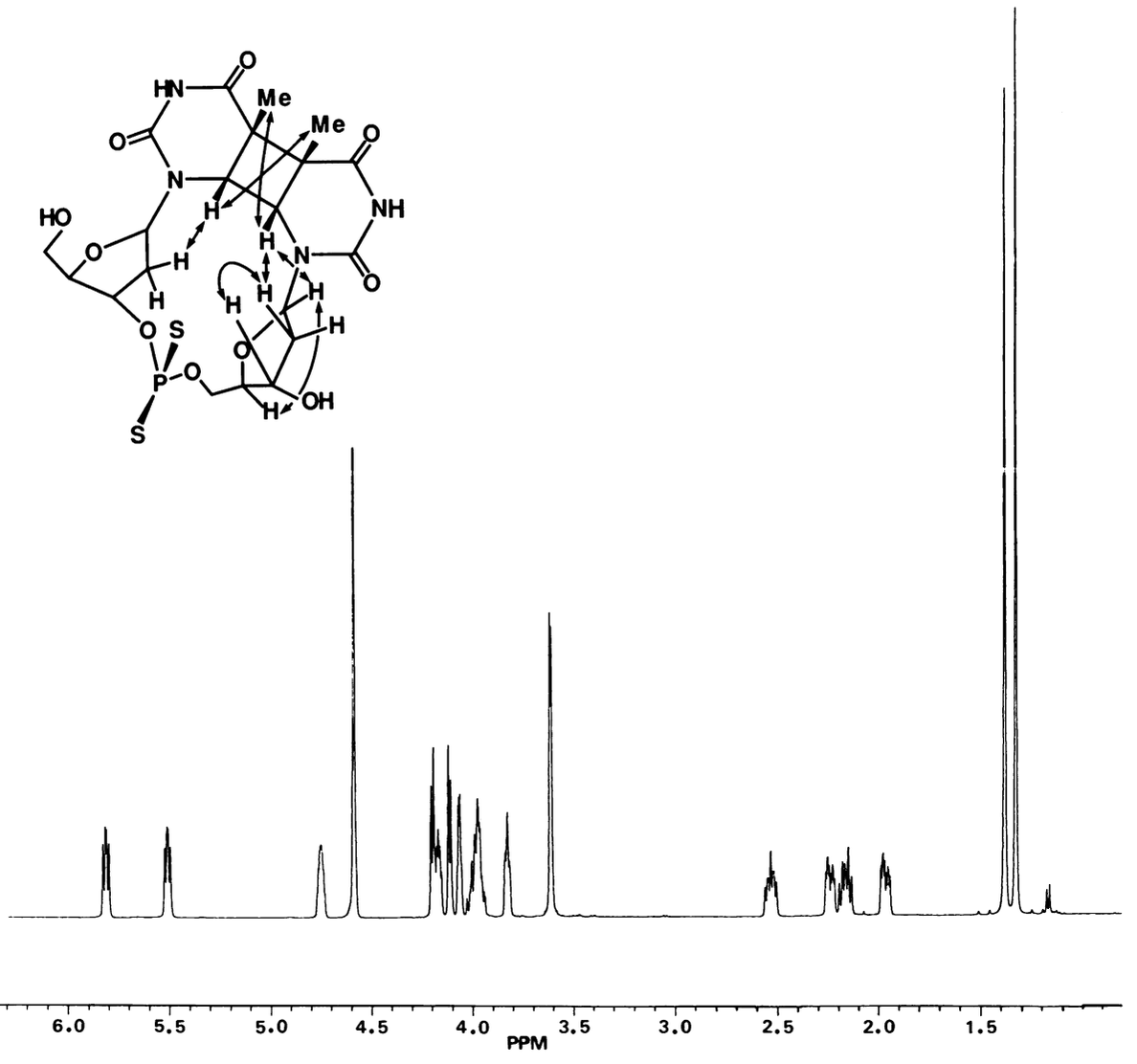

Figure 6. A 500 MHz ^{1}H-NMR spectrum of the cis-syn thymine dimer derivative containing the phosphorodithioate linkage **11**. The NOE crosspeaks observed in a NOESY spectrum are represented schematically in the upper part.

of Tp), 1.32 (s, 3H, Me of pT). ^{31}P-NMR (D_2O/85% H_3PO_4) δ: 110.5.

Protection and phosphitylation of the phosphorodithioate dimer (11)

The cis-syn thymine dimer derivative containing the phosphorodithioate linkage **11** (0.41 g, 0.6 mmol) and 2, 4-dichlorobenzyl chloride (1.7 ml, 12 mmol) were dissolved in pyridine-acetonitrile (1:1, v/v) (30 ml), and the mixture was stirred at 60°C for 2 hr. After concentration, chloroform and water were added, and the organic layer and precipitate were homogenized by addition of methanol. The solvents were evaporated, and the residue was purified by chromatography on silica gel. The phosphorodithioate-protected dimer was eluted with 5% methanol in chloroform. yield: 0.22 g (0.30 mmol, 50%). ^{31}P-NMR (DMSO-d_6/85% H_3PO_4) d: 90.8, 88.0. FAB$^+$-MS: 737 (M^+).

This intermediate (0.22 g, 0.30 mmol) was dried by coevaporation with pyridine and dissolved in pyridine (8 ml). 4, 4'-Dimethoxytrityl chloride (0.20 g, 0.60 mmol) was added, and the mixture was stirred for 3 hr. After addition of methanol (1 ml), the resulting mixture was concentrated, dissolved in chloroform, and washed with water. The organic layer was concentrated, and the residue was coevaporated with toluene. The 5'-dimethoxytrityl derivative was purified by chromatography on silica gel eluted with 3% methanol in chloroform. yield: 0.20 g (0.19 mmol, 65%). ^{31}P-NMR ($CDCl_3$/85% H_3PO_4) δ: 94.9, 91.9. FAB$^+$-MS: 1040 (M^+).

The 5'- and phosphorodithioate-protected dimer (0.19 g, 0.18 mmol) was dissolved in tetrahydrofuran (2 ml) containing *N*, *N*-diisopropylethylamine (0.13 ml, 0.72 mmol). To this solution, 2-cyanoethyl *N*, *N*-diisopropylchlorophosphoramidite (0.12 ml, 0.54 mmol) was added dropwise. After stirring for 90 min, the mixture was diluted with ethyl acetate, washed with saturated aqueous $NaHCO_3$, and dried with Na_2SO_4. The 3'-phosphoramidite derivative **12** was purified by chromatography on silica gel eluted with 3% methanol in chloroform containing 0.1% pyridine and by re-chromatography eluted with ethyl acetate and obtained as a foam by evaporation. yield: 128 mg (103 μmol, 56%). ^{31}P-NMR ($CDCl_3$/85% H_3PO_4) δ: 149.7, 148.6, 148.2, 93.7, 90.3.

Synthesis of a 12mer containing the thymine dimer and the phosphorodithioate linkage

The building block **12** was dissolved in anhydrous acetonitrile (0.15 M) and attached to a DNA synthesizer (Applied Biosystems 381A). The phosphorodithioate-containing dodecamer (dGCACGT[$_{PS2}$]TGCACG) was synthesized on a 1 μmol scale. The reagents are described in the previous section, and the reaction times are as follows; 1) detritylation for 2 min, 2) coupling for 1 min (for 20 min in the case of **12**), 3) capping for 30 sec, and 4) oxidation for 1 min. After the reaction cycle was repeated ten times, the CPG support was treated with thiophenol-triethylamine-dioxane (1:1:2, v/v/v) (1 ml×4) for 2 hr and then with aqueous ammonia (0.5 ml×4) for 1 hr, and the resulting solution was heated in a sealed vial at 60°C for 5 hr. Ammonia and water were evaporated in vacuo, and the product was purified by reversed-phase chromatography. Elution was performed with a linear gradient of acetonitrile (10−40%) in 50 mM TEAA, and the fractions eluted later were collected. After concentration and coevaporation with water, the oligonucleotide with the dimethoxytrityl group at the 5′-end was treated with 80% aqueous acetic acid for 30 min. Acetic acid was removed by evaporation and coevaporation with water, and the residue was dissolved in water and washed with chloroform. The deprotected dodecamer containing the phosphorodithioate linkage was purified by reversed-phase HPLC. yield: 6.9 A_{260} units.

5′-Labeling of the 12mers containing the thymine dimer

The dodecamer containing the thymine dimer (20 pmol) was dissolved in water (4 μl) and a buffer (2 μl) containing 250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, and 50 mM 2-mercaptoethanol. To this solution, [γ-^{32}P]ATP (3 μl, 1.1 MBq) and T4 polynucleotide kinase (1 μl, 10 units) were added. The mixture was incubated at 37°C for 1 hr, diluted with water (40 μl) and a pH 7.7 solution (350 μl) of 0.1 M Tris-HCl, 10 mM triethylamine, and 1 mM EDTA, and applied to a NENSORB 20 column (DuPont). This column was rinsed with the above solution and then with water, and the 5′-labeled oligonucleotide was eluted with 50% aqueous methanol.

Cleavage of the 12mer duplex by T4 endonuclease V

The 5′-labeled dodecamer (2 pmol) and the complementary dodecamer (2 pmol) were dissolved in reaction buffer (10 μl) containing 64 mM Tris-HCl (pH 7.5), 19.2 mM EDTA, and 200 mM NaCl and heated at 75°C for 10 min. After cooling to room temperature, a solution of T4 endonuclease V (0−2 μg/ml) (10 μl) in a buffer containing 32 mM Tris-HCl (pH 7.5), 9.6 mM EDTA, 100 mM NaCl, and 0.2% (w/v) bovine serum albumin was added. The mixture was incubated at 30°C for 30 min and concentrated after addition of 95% aqueous formamide containing 0.1% xylene cyanol and 0.1% bromophenol blue (10 μl). The products were separated by electrophoresis on a 20% polyacrylamide gel containing 7 M urea, and the amount of radioactivity in each band was determined in a liquid scintillation counter (Figure 9).

Filter binding assay

To a solution (50 μl) of the substrate (2 pmol) annealed in the reaction buffer as described above, the enzyme solution (0−300 nM) (50 μl) was added. After incubation in an ice water bath for 2 min, the mixture was diluted with a solution (1 ml) of 3.0 M NaCl and 0.03 M sodium citrate and passed through a cellulose nitrate membrane filter (ADVANTEC TOYO A045A025A) equilibrated with the above solution. The filter was rinsed with the same solution (1 ml×3) and dried. The amount of radioactivity retained on the filter was determined in a liquid scintillation counter.

RESULTS AND DISCUSSION

Synthesis of an oligonucleotide containing a thymine dimer with normal internucleotide linkages

The cis-syn thymine dimer is the major lesion of DNA caused by UV irradiation, which is recognized and cleaved by T4 endonuclease V. Two procedures have been reported for incorporation of this type of lesion into synthetic DNA fragments; one is the irradiation of oligodeoxyribonucleotides containing two adjacent thymidines (19, 20), and the other is the synthesis of oligonucleotides using a dinucleotide building block containing

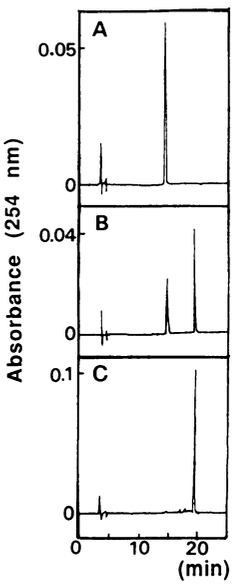

Figure 7. Elution profiles of dGCACGT[$_{PS2}$]TGCACG before and after photolysis (**A** and **B**, respectively) and dGCACGT$_{PS2}$TGCACG (**C**). A CHEMCOSORB 5-ODS-H column was used with a linear gradient of acetonitrile (from 9 to 15% during 20 min) in 0.1 M TEAA.

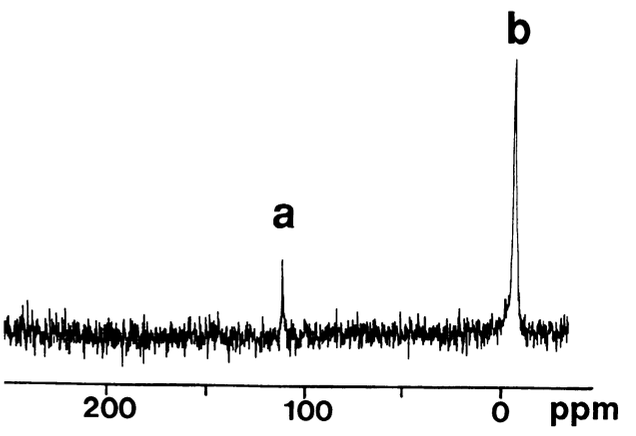

Figure 8. A ^{31}P-NMR spectrum of dGCACGT$_{PS2}$TGCACG. The phosphorodithioate and phosphodiester signals are assigned to **a** and **b** respectively.

a thymine dimer (18). We have chosen the latter method because of the high yield of product and no restriction in sequence.

The building block was synthesized as shown in Figure 1. Dithymidine monophosphate **3** was prepared using the 2-cyanoethyl and levulinyl groups, which can be removed very easily, for protection of the internucleoside linkage and the 3′-hydroxyl function respectively, and photodimerization was performed using acetone as a sensitizer. The product containing the cis-syn thymine dimer **4**, which was co-eluted with the authentic cis-syn dimer prepared by irradiation of dithymidine monophosphate after deprotection with aqueous ammonia, was separated from the trans-syn isomer **5** by chromatography on silica gel and phosphitylated after protection of the 5′-hydroxyl function and removal of the levulinyl group.

A dodecadeoxyribonucleotide containing the cis-syn thymine dimer (dGCACGT[ ]TGCACG) was synthesized on a DNA synthesizer using the dimer coupling unit **6**. The coupling yield of **6** was relatively low, although the reaction time was extended to 30 min. After cleavage and deprotection with aqueous ammonia, the product was purified by reversed-phase chromatography. Final purification was performed by HPLC as shown in Figure 2 after removal of the 4, 4′-dimethoxytrityl group from the 5′-end. The presence of the thymine dimer in the resulting oligonucleotide was confirmed by HPLC analysis after UV irradiation (Figure 3).

Synthesis of an oligonucleotide containing a thymine dimer with the phosphorodithioate linkage

Methods for the formation of the phosphorodithioate internucleotide linkage have been developed using both the phosphoramidite (7−10) and H-phosphonate (11) approaches. In our work, the phosphorodithioate linkage between two thymidines was formed via the phosphonodithioate intermediate **8** (Figure 4), because the 4-chlorobenzyl or 2, 4-dichlorobenzyl group used for protection of the internucleotide linkage in the phosphoramidite procedure may interfere with the photoreaction. The 4, 4′-dimethoxytrityl group protecting the 5′-hydroxyl function was also removed before the photoreaction.

The photodimerization of the phosphorodithioate derivative **10** was performed using acetone as a photosensitizer in the same way as described in the above section. We have compared the photoproducts starting from 3′-deprotected dithymidine monophosphorodithioate with those from the 3′-levulinyl derivative by HPLC. In the latter case, the ratio of the trans-syn isomer was relatively high, and two extra peaks were detected besides the cis-syn and trans-syn thymine dimers. These by-products were converted into the starting material by UV irradiation after separation by HPLC with little increase in extinction coefficients and were regarded as other photolesions such as the (6−4) photoproduct and Dewar pyrimidinone (21). Therefore, the completely deprotected dimer **10** was used for the photoreaction. The cis-syn and trans-syn thymine dimer derivatives were purified by HPLC (Figure 5) and identified by ^{1}H-NMR spectroscopy (Figure 6).

The phosphorodithioate linkage and the 5′-hydroxyl function of the cis-syn thymine dimer derivative **11** were protected by the 2, 4-dichlorobenzyl and 4, 4′-dimethoxytrityl groups respectively, and the 3′-hydroxyl function was phosphitylated with 2-cyanoethyl *N*, *N*-diisopropylchlorophosphoramidite. This coupling unit **12** was used for the synthesis of the dodecadeoxyribonucleotide containing the cis-syn thymine dimer with the phosphorodithioate linkage (dGCACGT[PS2]TGCACG). After chain elongation, the CPG support containing the oligonucleotide was treated with thiophenol-triethylamine-dioxane and then with aqueous ammonia. The 5′-tritylated oligonucleotide was purified by reversed-phase chromatography, and after treatment with acetic acid the deprotected oligonucleotide was further purified by HPLC. The presence of the thymine dimer and the phosphorodithioate linkage in the isolated product was confirmed by photolysis (Figure 7) and ^{31}P-NMR (Figure 8) respectively.

Cleavage of the phosphate-modified substrate by T4 endonuclease V

Very little has been reported about the properties of the phosphorodithioate linkage or oligonucleotides containing this modification. The only report is on the resistance of the phosphorodithioate linkage to hydrolysis with spleen and snake venom phosphodiesterases (10). In this study, we have assayed the affinity and sensitivity of the phosphorodithioate-containing substrate to T4 endonuclease V.

The dodecamers containing a cis-syn thymine dimer with and

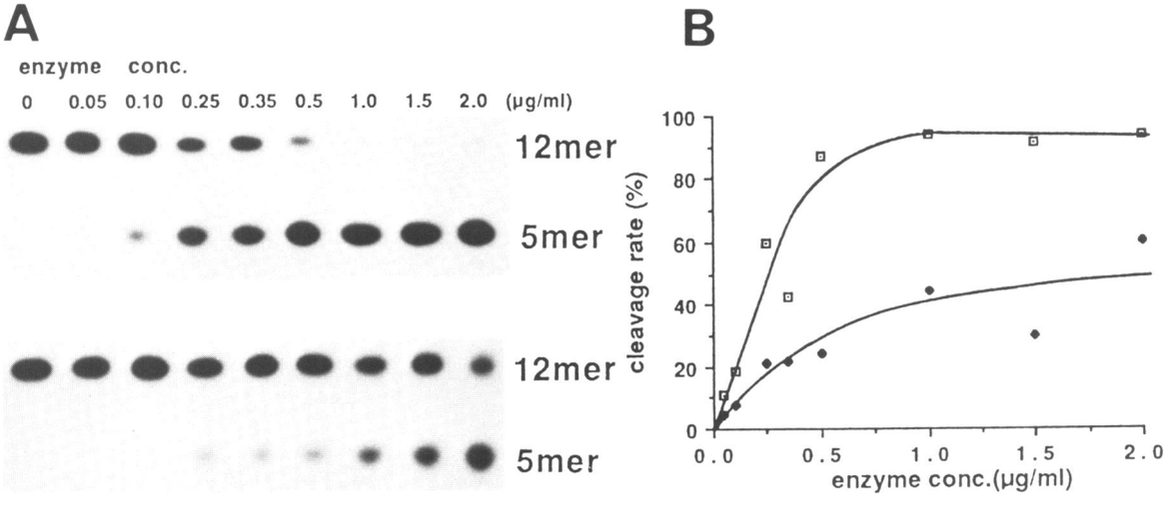

Figure 9. (**A**) Cleavage of dGCACGT[ ]TGCACG (upper) and dGCACGT[$_{PS2}$]TGCACG (lower) by T4 endonuclease V. (**B**) Cleavage rates of dGCACGT[ ]TGCACG (o) and dGCACGT[$_{PS2}$]TGCACG (●).

without the phosphorodithioate linkage were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and hybridized to the complementary dodecamer. Each duplex was used as a substrate for T4 endonuclease V under the conditions described previously (20), and cleavage rates at various concentrations of the enzyme were obtained (Figure 9). The phosphorodithioate linkage was cleaved by T4 endonuclease V, although the rate was low compared with the normal phosphodiester linkage. The dissociation constants were also obtained after a filter binding assay (22) and found to be almost the same (2.3×10^{-8} M for the phosphorodithioate-containing substrate and 1.6×10^{-8} M for the phosphate-unmodified substrate).

From these results, the phosphorodithioate internucleotide linkage at the photolesion has no effect on the binding property of T4 endonuclease V but reduces the rate of overall chain cleavage (shown in Figure 9) as well as the rate of the glycosyl bond cleavage (data not shown). This reduction may be due to local distortions at the catalytic sites in the enzyme-substrate complex caused by the change from phosphate to phosphorodithioate. Side reactions of the 5′-phosphorodithioate hexamer produced after cleavage by the enzyme may have some influence, as many products were detected by HPLC in addition to the expected peaks after the enzyme reaction with the phosphorodithioate-containing substrate (data not shown). Using the reactivity of the phosphorodithioate residue for alkylation, for example, this modified linkage has the potential to be applied to construction of functional oligonucleotides.

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