Synthesis and Template Properties of an Ethyl Phosphotriester Modified Decadeoxyribonucleotide†

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ABSTRACT: The phosphate groups of nucleic acids are often the targets of mutagenic and carcinogenic alkylating agents. In order to study the effects of alkyl phosphotriester modification on the physical and biochemical properties of DNA, two diastereomeric ethyl phosphotriester modified decadeoxyribonucleotides, d-CpCpApApGp(Et)ApTpTpGpG isomer I and isomer II, were prepared. A phosphotriester synthetic procedure was used to specifically place ethyl triester groups with either an *R* or *S* configuration in the central dimer region of the decamer. Terminal deoxynucleotidyl transferase was used to add oligodeoxyadenylate tails to the 3′ end of the decamers. The resulting oligomers were tested as templates for *Escherichia coli* DNA polymerase I with d-$(pT)_8$pCpC as a primer. The rates and extents of polymerization directed by the modified templates were 25% (isomer I) and 50% (isomer II) less than those of an unmodified control template. Thus the presence of an ethyl triester group inhibits polymerization, the effectiveness of which is determined by the orientation of the ethyl group relative to the rest of the template backbone. These results suggest ethyl phosphotriester lesions could inhibit replication rates of cellular DNA.

Many mutagenic and carcinogenic alkylating agents react with the phosphate groups of nucleic acids to produce alkyl phosphotriesters (Singer & Fraenkel-Conrat, 1975; Sun & Singer, 1975; Singer et al., 1978). In the case of some alkylating agents such as ethylnitrosourea, phosphotriester formation represents the major product of the reaction. Alkylation of RNA phosphates gives unstable linkages that are readily hydrolyzed, thus leading to strand scission. In contrast, phosphate alkylation in DNA yields phosphotriester groups that are stable under physiological conditions (Bannon & Verly, 1972). Indeed, alkyl phosphotriesters in cellular DNA are not readily repaired and persist in DNA long after exposure of cells to alkylating agents (Bodell et al., 1979; Singer et al., 1981).

The alkyl phosphotriester group is asymmetric and can have either an *R* or *S* configuration. Phosphotriester groups of both configurations were found when poly(dA-dT) was exposed to ethylnitrosurea (Jensen & Reed, 1978; Jensen, 1978). The bases of a phosphotriester-modified oligonucleotide can participate in normal H-bonding interactions. However, studies on deoxyribodinucleotide methyl and ethyl phosphotriesters have shown that the phosphotriester modification alters the conformation of these dimers (Miller et al., 1971; Kan et al., 1973). Phosphotriester modification can also alter the ability of oligonucleotides to bind to complementary polyribonucleotides and polydeoxyribonucleotides (Miller et al., 1974, 1977; Pless & Ts'o, 1977). Thus a single phosphotriester group in the backbone of a nucleic acid could potentially lead to conformational changes and/or to changes in the stability of the nucleic acid helix in the region of the modification.

The alkyl phosphotriester modification introduces a hydrophobic group into the backbone of the nucleic acid and results in the loss of the negative charge of the phosphate. In addition to causing changes in the structure of the nucleic acid, the phosphotriester substitution could alter interactions between proteins and the modified region of the nucleic acid.

To examine the effects of phosphotriester modification on the structural and enzymological properties of DNA, we have prepared two decadeoxyribonucleotides with the base sequence d-CpCpApApGp(Et)ApTpTpGpG.[1] The decanucleotides contain an ethyl phosphotriester group with either an *R* or *S* configuration specifically located in the central dimer region of the molecule. When combined with the complementary decanucleotide d-CpCpApApTpCpTpTpGpG, the modified decanucleotides form short DNA helixes that serve as models for phosphate-alkylated DNA. Extensive NMR investigations on the conformation of these helices are in progress. In this paper we describe the chemical syntheses of the modified decanucleotides. Addition of oligodeoxyadenylate tails to the 3′ ends of the decamers provided molecules that were tested as templates for DNA polymerase. In this way, the potential effect of ethyl phosphotriester groups on DNA replication has been investigated.

Materials and Methods

Nucleosides, 5′-deoxyadenosine triphosphate, d-Tp-$(Tp)_7$CpC, deoxynucleotidyl terminal transferase, and DNA polymerase were purchased from P-L Biochemicals Inc. Tritium-labeled dATP and α-^{32}P-labeled dGTP were purchased from New England Nuclear Corp. All reagents and solvents were purified as previously described (Miller et al., 1980). *p*-Toluenesulfonyl-4-nitroimidazole was prepared according to the procedure of Gough et al. (1979). The protected oligonucleotides d-[$(MeO)_2$Tr]bzCṗbzCṗbzAṗbzAṗCNEt and d-TṗTṗibuGṗibuGOBz and the monomers d-[$(MeO)_2$Tr]-ibuGṗCNEt and d-bzA were prepared as previously described (Miller et al., 1980). The decanucleotide d-CpCpApAp-GpApTpTpGpG was prepared by the phosphotriester method. A complete description of its synthesis will be presented elsewhere. The general synthetic procedures and techniques used to prepare fully protected deoxyribooligonucleotides have been previously reported (Miller et al., 1980). Thin-layer chromatography (TLC) was carried out on Merck silica gel

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[1] Abbreviations: d-NṗN, a deoxyribooligonucleotide *p*-chlorophenyl phosphotriester; d-Np(Et)N, a deoxyribooligonucleotide ethyl phosphotriester; TSNI, *p*-toluenesulfonyl-4-nitroimidazole; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl. The symbols used to represent oligonucleotides and their protected derivatives follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (1970).

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Table I: Preparation of Protected Deoxyribooligonucleotide *p*-Chlorophenyl Phosphotriesters

3′-p component (mmol)	5′-OH component (mmol)	TSNI (mmol)	oligomer (mmol)	yield (%)
d-[$(MeO)_2$Tr]ibuGṗ (7.2)	d-bzA (6.0)	18	d-[$(MeO)_2$Tr]ibuGṗbzA (4.03)	67
d-[$(MeO)_2$Tr]bzCṗbzCṗbzAṗbzAṗ (0.075)	d-ibuGp(Et)acAṗCNEt isomer I (0.053)	0.03	d-[$(MeO)_2$Tr]bzCṗbzCṗbzAṗbzAṗibuGp(Et)acAṗCNEt isomer I (0.034)	64
	isomer II (0.06)	0.03	isomer II (0.031)	52
d-[$(MeO)_2$Tr]-bzCṗbzCṗbzAṗbzAṗibuGp(Et)acAṗ	d-TṗTṗibuGṗibuGOBz (0.055)		d-[$(MeO)_2$Tr]-bzCṗbzCṗbzAṗbzAṗibuGp(Et)acAṗTṗTṗibuGṗibuGOBz	
isomer I (0.034)		0.175		
isomer II (0.031)		0.175		

Table II: Ultraviolet Spectral Properties and Chromatographic Mobilities of Protected Deoxyribooligonucleotide *p*-Chlorophenyl Phosphotriesters

	ultraviolet spectral properties						R_f values on silica gel TLC		
	calcd			obsd					
oligomer	E_{260}/E_{240}	E_{280}/E_{240}	E_{306}/E_{240}	E_{260}/E_{240}	E_{280}/E_{240}	E_{306}/E_{240}	5% MeOH/$CHCl_3$	10% MeOH/$CHCl_3$	15% MeOH/$CHCl_3$
d-[$(MeO)_2$Tr]ibuGṗbzA	0.88	1.00	0.20	0.81	0.93	0.24	0.00	0.27, 0.22	
d-[$(MeO)_2$Tr]bzCṗbzCṗbzAṗbzAṗibuGp(Et)acAṗCNEt									
isomer I	1.18	1.11	0.43	1.21	1.06	0.38		0.25	0.46
isomer II	1.18	1.11	0.43	1.20	1.08	0.39		0.24	0.45
d-[$(MeO)_2$Tr]bzCṗbzCṗbzAṗbzAṗibuGp(Et)acAṗTṗTṗibuGṗibuGOBz									
isomer I								0.10	0.45
isomer II								0.10	0.45

60F plastic-backed silica gel sheets. Poly(ethylenimine) (PEI)–cellulose chromatography was carried out on Brinkmann Polygram CEL 300 precoated plastic sheets. High-pressure liquid chromatography (HPLC) was carried out on a Varian LC 5000 instrument. Analytical reversed-phase HPLC was run on a Whatman Partisil ODS column with water/acetonitrile gradients buffered with 0.2 M ammonium acetate, pH 5.8. Analytical silica gel HPLC was performed on a Whatman Partisil 10 silica gel column with linear chloroform/methanol gradients. Both analytical columns were run for 20 min at a flow rate of 2.5 mL/min. Preparative silica gel HPLC was carried out on a Whatman M-9 Partisil 10 column with linear methanol/chloroform gradients. Preparative ion-exchange HPLC was performed on a Whatman M-9 Partisil SAX column with linear gradients of ammonium sulfate in 25% aqueous ethanol. Both preparative columns were operated at a flow rate of 4.5 mL/min.

Preparation of d-[$(MeO)_2$Tr]ibuGṗbzA. The reaction conditions and yield are reported in Table I. The dimer was purified by silica gel column chromatography (5 × 20 cm) with 0.75 L of chloroform and 0.75 L of 1%, 0.75 L of 2%, 0.75 L of 4%, and 1.8 L of 5% methanol in chloroform as solvents. Fractions containing the product were pooled, and the solvents were evaporated. The dimer was isolated by precipitation from hexane. The ultraviolet spectral characteristics and the mobilities are given in Table II. A sample of the protected dimer (58 mg, 0.05 mmol) was treated with 2 mL of 50% ammonium hydroxide/pyridine solution for 3 h at 50 °C. After evaporation of solvents, the residue was treated with 0.5 mL of 80% aqueous acetic acid for 30 min at 22 °C. The residue, after evaporation of solvents, was dissolved in 1 mL of water, and the solution was extracted with three, 1-mL, portions of ethyl acetate. The aqueous solution was shown by ODS-2 HPLC to contain only d-GpA (retention time 11.7 min, 2–10% acetonitrile/water gradient). The dimer (1.1 A_{254} units) was incubated with 5 μg of snake phosphodiesterase in 50 μL of buffer containing 10 mM Tris-HCl (pH 8.2) and 2 mM magnesium chloride for 1 h at 37 °C. The digest was examined by ODS-2 HPLC. Only two products, d-pA (retention time 5.8 min) and dG (retention time 6.2 min), were observed. No d-GpA remained.

Preparation of d-[$(MeO)_2$Tr]ibuGp(Et)bzA. d-[$(MeO)_2$Tr]ibuGṗbzA (4.4 g, 3.78 mmol) was treated with tetra-*n*-butylammonium fluoride (23.9 g, 7.56 mmol) in 10 mL of anhydrous ethanol for 48 h at 37 °C. After evaporation of the solvents, the residue was dissolved in 200 mL of chloroform, and the solution was extracted with two, 100-mL, portions of 5% aqueous sodium bicarbonate. The chloroform extract was dried over anhydrous sodium sulfate. The product was purified by silica gel column chromatography (3 × 25 cm) with 1.5 L of chloroform, 0.8 L of 5% methanol in chloroform, and 1 L of 10% methanol in chloroform as solvents. The dimer (2.0 g, 1.85 mmol) was isolated in 49% yield by precipitation from hexane. The dimer migrated as a single peak on silica gel HPLC (retention time 11.2 min, 0–20% methanol in chloroform). The UV spectrum was similar to that of the *p*-chlorophenyl phosphotriester derivative.

Preparation of d-[$(MeO)_2$Tr]ibuGp(Et)A Isomers I and II. d-[(MeO)Tr]ibuGp(Et)bzA (1.91 g, 1.76 mmol) was stirred with 3.52 mL of 80% hydrazine hydrate in 35.2 mL of 20% acetic acid/pyridine solution overnight at 22 °C. The solution was diluted with 100 mL of 5% aqueous sodium bicarbonate and extracted with four, 100-mL, portions of chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate. The diastereoisomers of the protected dimer appeared as two spots on silica gel TLC (R_f 0.27 and 0.19, 30% methanol in chloroform). They were separated on an M-9 Partisil 20 silica gel column with a 0–30% linear gradient of methanol in chloroform at a flow rate of 4.0 mL/min. Mixed fractions were rechromatographed. Pure isomer I (0.25 mmol) and isomer II (0.23 mmol) were obtained in 14% and 13% yields, respectively. The retention times on analytical silica gel HPLC were 14.8 min for isomer I and 15.8 min for isomer II with a 0–30% gradient of methanol in chloroform. Both isomers had the following UV spectral characteristics in 95% ethanol: E_{260}/E_{240} 0.94; E_{280}/E_{240} 0.47; E_{306}/E_{240} 0.02.

Portions of the protected dimers (0.01 mmol of isomer I and 0.007 mmol of isomer II) were each treated with 0.5 mL of

50% ammonium hydroxide–pyridine solution for 2 days at 0 °C. The solvents were evaporated, and the residue was treated with 0.5 mL of 80% aqueous acetic acid for 1.5 h at 22 °C. After evaporation of the solvent, the residue was dissolved in water, and the solution was extracted with three, 1-mL, portions of ethyl acetate. The dimers were purified by preparative HPLC on a M-9 Partisil ODS-2 column with a linear gradient of 10–15% acetonitrile in water (240 mL) at a flow rate of 4.0 mL/min. Isomer I (190 A_{254} units, 0.007 mmol) was obtained in 71% yield, while isomer II (120 A_{254} units, 0.0045 mmol) was obtained in 64% yield. The retention times on analytical ODS-2 HPLC were 10.7 min for isomer I and 10.6 min for isomer II. The UV spectra in water of each isomer showed λ_{max} 255 nm, λ_{min} 225 mn, and E_{255}/E_{280} 2.61. The ^1H and ^{31}P spectra of each isomer were consistent with the structure.

Preparation of d-[(MeO)$_2$Tr]ibuGp(Et)acAOTBS Isomers I and II. The diastereoisomers of d-[(MeO)$_2$Tr]ibuGp(Et)A (isomer I, 0.227 mmol; isomer II, 0.187 mmol) were each treated with *tert*-butyldimethylsilyl chloride (isomer I, 0.68 mmol; isomer II, 0.56 mmol) and imidazole (isomer I, 1.14 mmol; isomer II, 0.94 mmol) in anhydrous pyridine (isomer, I, 0.46 mL; isomer II, 0.38 mL) for 20 h at 37 °C. The reaction mixtures were each treated with 0.5 mL of 50% pyridine/water solution of 1 h. The solvents were evaporated, each residue was dissolved in 2 mL of chloroform, and the solutions were extracted with three, 1-mL, portions of 5% aqueous sodium bicarbonate. After the samples were dried over anhydrous sodium sulfate, the chloroform was evaporated, and the residues were each treated with 2 mL of acetic anhydride in 2 mL of anhydrous pyridine for 2 days at 22 °C. The solutions were diluted with 2 mL of methanol and kept at 0 °C for 2 days. The solvents were evaporated, and the residues were each incubated with 2 mL of 50% pyridine/water at 40 °C for 7 h. The resulting d-[(MeO)$_2$Tr]ibuGp(Et)-acAOTBS diastereoisomers were recovered in quantitative yield by evaporation of the solvent. Each isomer had the following ultraviolet spectral characteristics in 95% ethanol: E_{260}/E_{240} 1.00; E_{280}/E_{240} 0.79; E_{306}/E_{240} 0.05. The isomers had identical mobilities on silica gel TLC: R_f 0.17 in 10% methanol/chloroform solution.

Preparation of d-[(MeO)$_2$Tr]ibuGp(Et)acA Isomers I and II. Each diastereoisomer of d-[(MeO)$_2$Tr]ibuGp(Et)-acAOTBS (isomer I, 0.22 mmol; isomer II, 0.18 mmol) was treated with 0.75 mL of 1 M tetra-*n*-butylammonium fluoride in tetrahydrofuran for 1 h at 22 °C. The solutions were diluted with 5 mL of 5% aqueous sodium bicarbonate and extracted with three, 1-mL, portions of chloroform. The chloroform extracts were dried over anhydrous sodium sulfate, and the dimers were recovered by evaporation of the chloroform solutions. Each diastereosimer of d-[(MeO)$_2$Tr]ibuGp(Et)acA had the following UV spectral characteristics in 95% ethanol: E_{260}/E_{240} 0.91; E_{280}/E_{240} 0.78; E_{306}/E_{240} 0.05. Each isomer had the same mobility on silica gel TLC: R_f 0.00 in 10% methanol in chloroform; R_f 0.13 in 20% methanol in chloroform. Removal of the protecting groups from samples of each isomer by sequential treatment with 50% ammonium hydroxide/pyridine solution and by 80% aqueous acetic acid as previously described gave d-Gp(Et)A as the sole nucleotidic reaction product as shown by reversed-phase HPLC.

Preparation of d-[(MeO)$_2$Tr]ibuGp(Et)acAṗCNEt Isomers I and II. Each diastereoisomer of d-[(MeO)$_2$Tr]ibuGp(Et)acA (isomer I, 0.22 mmol; isomer II, 0.18 mmol) was phosphorylated with 0.375 mmol of the bis[triazole] derivative of *p*-chlorophenyl phosphate by procedures identical with those described for the preparation of d-[(MeO)$_2$Tr]NṗCNEt (Miller et al., 1980). After workup by extraction, the dimer reaction product, which consisted of a mixture of d-[(MeO)$_2$Tr]ibuGp(Et)acAṗ and d-[(MeO)$_2$Tr]ibuGp(Et)-acAṗCNEt, was evaporated with three, 1-mL, portions of anhydrous pyridine. The residue was dissolved in a solution containing 1 mL of anhydrous pyridine and 0.17 mL (2.5 mmol) of anhydrous hydracrylonitrile. The solution was treated with 0.40 g (1.5 mmol) of *p*-toluenesulfonyl-4-nitroimidazole for 16 h at room temperature. The reaction mixture was diluted with 1 mL of 50% pyridine/water and 2.5 mL of 5% aqueous sodium bicarbonate. The solution was extracted with three, 1-mL, portions of chloroform, and the combined extracts were dried over anhydrous sodium sulfate. The dimers were purified by silica gel column chromatography (1.2 × 20 cm) with 100 mL of chloroform and 200 mL of 5%, 50 mL of 7.5%, 100 mL of 10%, and 100 mL of 25% methanol in chloroform as solvents. d-[(MeO)$_2$Tr]ibuGp(Et)acAṗCNEt isomer I (0.053 mmol, 24%) and isomer II (0.06 mmol, 33%) were isolated by evaporation of solvents. Each isomer had the following UV spectral properties in 95% ethanol: E_{260}/E_{240} 0.96; E_{280}/E_{240} 0.85; E_{306}/E_{240} 0.10. The two isomers had the following TLC mobilities in 20% methanol in chloroform: isomer I, R_f 0.47 and 0.45; isomer II, R_f 0.37 and 0.34.

Preparation of d-[(MeO)$_2$Tr]bzCṗbzCṗbzAṗbzAṗibuGp-(Et)acAṗCNEt Isomers I and II. The dimethoxytrityl group was removed from d-[(MeO)$_2$Tr]ibuGp(Et)acAṗCNEt (isomer I, 0.053 mmol; isomer II, 0.06 mmol) by treatment of a solution of each dimer in 0.44 mL of 75% methanol/chloroform with 1.76 mL of 2% benzenesulfonic acid in chloroform for 4 min at 0 °C. The reaction mixture was extracted with 2 mL of 5% aqueous sodium bicarbonate, and the chloroform layer was dried over anhydrous sodium sulfate. The dimers, d-ibuGp(Et)acAṗCNEt, were isolated by evaporation of the chloroform solution. Each isomer had the same mobility on silica gel TLC: R_f 0.005 in 15% methanol/chloform.

The condensation reactions were carried out as described in Table I. Each protected hexanucleotide was isolated by preparative HPLC on an M-9 Partisil 10 silica gel column with a linear gradient of 0–20% methanol in chloroform (isomer I, 180 mL; isomer II, 260 mL) at a flow rate of 4.0 mL/min. Appropriate fractions (10 mL) were pooled as indicated by silica gel TLC, and the hexamers were recovered by evaporation of solvents. The UV spectral characteristics and TLC mobilities are given in Table II.

Preparation of d-CpCpApApGp(Et)ApTpTpGpG Isomers I and II. The condensation reactions were carried out under the conditions described in Table I. The chromatographic mobilities of the fully protected decamers are given in Table II. The crude protected decamers were isolated from the reaction mixtures by extraction into chloroform. Each decamer was treated as follows. The residue obtained after evaporation of the chloroform was dissolved in 1 mL of pyridine, and the solution was treated with 10 mL of 0.017 M tetra-*n*-butylammonium fluoride in tetrahydrofuran/pyridine/water (8:1:1 v/v) for 2 days at 22 °C. The solution was diluted with 20 mL of pyridine and 40 mL of concentrated ammonium hydroxide and was further incubated at 22 °C for 3 days. The solvents were evaporated, and the residue was dissolved in 100 mL of 60% ethanol/water. The solution was passed through a DEAE-cellulose column (2 × 20 cm) that was washed with 100 mL of 0.1 M triethylammonium bicarbonate and then eluted with 1 M triethylammonium bicarbonate in 60% ethanol/water. The 1 M bicarbonate eluate was evaporated, and the residue was treated with 4 mL of 80%

Table III: Base Ratio Analysis of d-CpCpApApGp(Et)ApTpTpGpG

products of snake venom phosphodiesterase digest	theoretical	ratios	
		isomer I	isomer II
dC	1.00	1.11	1.00
d-pC	1.00	1.00	0.88
d-pA	2.00	2.03	2.06
d-pGp(Et)A	1.00	1.19	1.15
d-pT	2.00	1.85	1.93
d-pG	2.00	1.88	2.05

acetic acid for 30 min at 22 °C. The solvents were evaporated, the residue was dissolved in 5 mL of water, and the solution was extracted with three, 5-mL, portions of ethyl acetate. The pH was adjusted to 7, and 1.8 mL of 95% ethanol was added. The deprotected decamers were purified on a M-9 Partisil SAX preparative HPLC column with a 270-mL linear gradient of 0.001–0.20 M ammonium sulfate in 25% ethanol/water. Fractions were monitored at 280 nm. Fractions containing the desired decamer were pooled and desalted on a DEAE-cellulose column (Miller et al., 1980). A total of 329 A_{257} units (0.00365 mmol, 11% overall yield) of d-CpCpApApGp(Et)ApTpTpGpG isomer I and 658 A_{257} units (0.0073 mmol, 24% overall yield) of isomer II was obtained. Each isomer had the following UV spectral properties in 25% ethanol/water solution: λ_{max} 257 nm; λ_{min} 228 nm; E_{260}/E_{280} 1.91. Each isomer eluted as a single peak at 0.19 M ammonium sulfate on a Partisil SAX column with a linear gradient of 0.001–0.30 M ammonium sulfate in 25% ethanol/water.

The extinction coefficients and base ratios of the decamers were determined as follows. (1) Extinction coefficients. The UV spectrum of each decamer (0.5 A_{254} unit) was recorded in 1 mL of solution containing 0.25 mM Tris-HCL (pH 8.2) and 0.05 mM magnesium chloride. The solution was treated with 3 μg of snake venom phosphodiesterase for 2 h at 37 °C. The UV spectrum was again recorded, and the extinction coefficients were calculated by using the following monomer extinction coefficients at 254 nm: d-pT, 7250; d-pC, 6260; d-pA, 13 200; d-pG, 13 600; d-pGp(Et)A isomer I 23 070; d-pGp(Et)A isomer II, 24 670. The average results of three separate determinations were as follows: E_{254} for isomer I 82 560; E_{254} for isomer II 84 090. (2) Base ratio analysis. The decamer (0.55 A_{254} unit) was hydrolyzed with 3 μg of snake venom phosphodiesterase in 1 mL of buffer containing 0.25 mM Tris-HCl (pH 8.2) and 0.05 mM magnesium chloride for 2 h at 37 °C. The solution was heated for 1 min at 75 °C and then cooled. Aliquots were analyzed by ODS-2 reversed-pulse HPLC with the following gradient at a flow rate of 2.5 mL/min: 1% acetonitrile in 0.1 M ammonium acetate (pH 5.8) (0–5 min) and 1–15% acetonitrile in 0.1 M ammonium acetate (5–15 min). The column was monitored at 254 nm, and the areas under product peaks were calculated by using the E_{254} values given above. Under these conditions the phosphodiester linkages of the decamers were completely digested while the ethyl phosphotriester linkage was not hydrolyzed. The digest products had the following retention times on the HPLC column: d-pC, 1.5 min; d-pT, 3.0 min; d-pG, 3.8 min; d-pA, 8.0 min; dC, 4.6 min; d-pGp(Et)A isomer I, 11.4 min; d-pGp(Et)A isomer II, 11.2 min. The base ratios are given in Table III.

Addition of Oligodeoxyadenylate Tails to the Decamers. Oligodeoxyadenylate tails were added to d-CpCpApApGpApTpTpGpG and to d-CpCpApApGp(Et)ApTpTpGpG isomer I and isomer II with terminal deoxynucleotidyl transferase. Each oligomer (50 μM) was incubated with 20–40 units of enzyme in 400 μL of buffer containing 150 mM potassium cacodylate (pH 7.8), 8 mM magnesium chloride, 1 mM β-mercaptoethanol, 200 μM zinc acetate, and 100 μM d-[^{3}H]ATP (5 mCi/mmol). The reaction process was monitored by spotting 5 μL of reaction mixture on a PEI strip (1 cm × 6 cm), eluting the strip with 1 N hydrochloric acid, cutting the strip in two pieces, 1 cm above the origin, and counting each piece in 6 mL of Betafluor scintillation mixture. In this assay the oligonucleotide remains at the origin while the nucleoside triphosphate migrates with the solvent front. The percentage of d-p[^{3}H]A remaining at the origin was determined, and the reactions were terminated by being heated in a boiling water bath when approximately 10 dAMP residues were incorporated. The tailed oligonucleotides were purified on a Bio-Gel P-6 column with water as the eluant.

DNA Polymerase I Reactions. Polymerization reactions were initiated by adding 0.2 unit of *Escherichia coli* DNA polymerase I to a buffer containing the following: 20 mM Tris-HCl (pH 7.6), 5 mM magnesium chloride, 100 mM potassium chloride, 20 μg/mL bovine serum albumin, 2.5 μM dCCAAGATTGG(pA)$_n$ template, 2.5 μM d-(pT)$_8$pCpC, and 33 μM each of dATP, dCTP, dTTP, and d-[α-^{32}P]GTP. The concentration of each template was determined from the known specific activity and chain length of the ^{3}H-labeled oligo(dA) tail. The reactions were run at 37 °C, and the incorporation of d-[^{32}P]GMP into oligomeric material was assayed on PEI–cellulose as described in the preceding section. To demonstrate that the length of the oligo(dA) tail did not affect the rate of the polymerization reactions, we prepared two control templates, d-CpCpApApGpApTpTpGpG(pA)$_{9or14}$. The rates of polymerization with these two nonmodified templates were identical.

Results

Scheme I outlines the preparation of the two diastereoisomers of the fully protected ethyl phosphotriester modified dimer d-[(MeO)$_2$Tr]ibuGp(Et)acApCNEt. The ethyl group was introduced by fluoride-catalyzed exchange of the *p*-chlorophenyl group of d-[(MeO)$_2$Tr]ibuGpbzA with ethanol (Ogilvie & Beaucage, 1979). In order to separate the two diastereoisomers, it was first necessary to selectively remove the *N*-benzoyl protecting group by hydrazine treatment (Letsinger et al., 1968). The resulting isomers of d-[(MeO)$_2$Tr]ibuGp(Et)A could then be isolated by preparative silica gel HPLC.

Each dimer was then reprotected. The 3′-hydroxyl group was temporarily blocked with a *tert*-butyldimethylsilyl group (Ogilvie & Iwacha, 1973). The N-6 amino group of deoxyadenosine was acetylated with acetic anhydride. Finally the 3′-silyl protecting group was selectively removed by reaction with tetra-*n*-butylammonium fluoride in tetrahydrofuran, and the resulting 3′-hydroxyl group was esterified with β-cyanoethyl *p*-chlorophenyl phosphate. The dimers along the synthetic pathway were purified by extraction procedures, while the final product, d-[(MeO)$_2$Tr]ibuGp(Et)acApCNEt, was purified by silica gel column chromatography.

The two modified decadeoxyribonucleotides, d-CpCpApApGp(Et)ApTpTpGpG isomer I and isomer II, were prepared by the procedure shown in Scheme II. A similar approach was used to synthesize d-CpCpApApGpApTpTpGpG. The yields obtained and the physical properties of the protected oligonucleotide intermediates are given in Tables I and II.

The protected decanucleotides were not purified after the final condensation reaction. Instead, the protecting groups were removed from material in the crude reaction mixture.

Scheme I: Preparation of d-[$(MeO)_2$Tr]ibuGp(Et)acAp̈CNEt

The *p*-chlorophenyl phosphate protecting groups were partially removed by treatment with aqueous tetra-*n*-butylammonium fluoride. Complete removal of the phosphate and base protecting groups was achieved by reaction with concentrated ammonium hydroxide/pyridine solution. After removal of the dimethoxytrityl group, the decamers were purified by ion-exchange HPLC. The phosphodiester groups of the decamers were completely hydrolyzed by snake venom phosphodiesterase. The resistance of the ethyl phosphotriester group to cleavage by this enzyme (Barrett et al., 1974) resulted in the formation of d-pGp(Et)A. The products of the enzymatic digests were separated by analytical reversed-phase HPLC and were obtained in the expected ratios (see Table II).

Terminal deoxynucleotidyl transferase was used to add oligodeoxyadenylate tails to the 3′ ends of d-CpCpApApGp-(Et)ApTpTpGpG and d-CpCpApApGpApTpTpGpG. The length of the tails was controlled by monitoring the incorporation of d-p[^{3}H]A into the oligonucleotide with PEI–cellulose chromatography. The reactions were stopped when the average chain length was between 9 and 12 adenylate residues. The oligomers were then purified by Bio-Gel P-6 gel filtration.

The tailed decamers were tested as templates for *E. coli* DNA polymerase I. In these reactions, d-$(pT)_8$pCpC, which can bind to the -GpG$(pA)_n$ region of the template, was used as a primer. Figure 1 shows the incorporation d-[^{32}P]GMP vs. time. All three oligomers serve as templates for the DNA polymerase I. The extent of the reaction directed by the unmodified template levels off after approximately one dGMP residue has been incorporated. Reactions using the ethyl

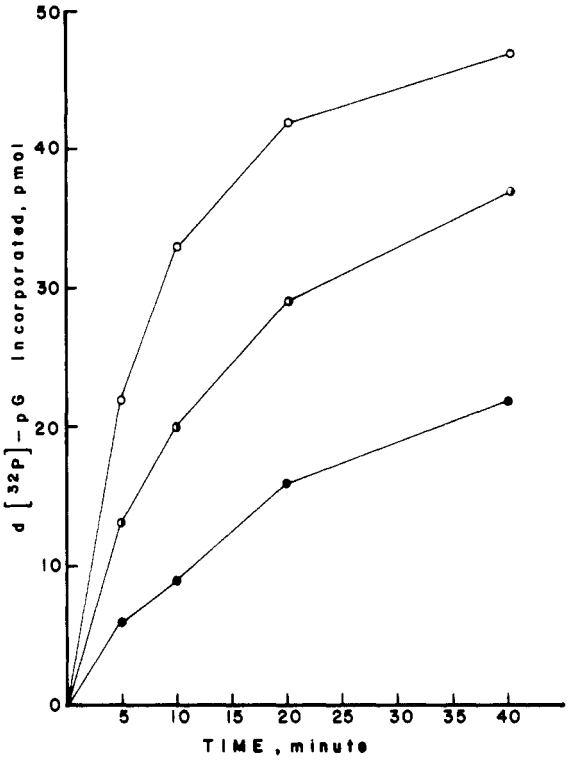


FIGURE 1: DNA polymerase I catalyzed polymerization directed by (○) d-CpCpApApGpApTpTpGpG(pA)$_9$, (◑) d-CpCpApApGp-(Et)ApTpTpGpG(pA)$_{12}$ isomer I, and (●) d-CpCpApApGp(Et)-ApTpTpGpG(pA)$_{11}$ isomer II. The reactions were run at 37 °C under the conditions described under Materials and Methods.

phosphotriester modified templates show both a lower rate and extent of incorporation. No polymerization was observed in the absence of DNA polymerase I or in the absence of dTTP. The difference in reaction rates is not due to different lengths of the oligo(dA) tail. Thus d-CpCpApApGpApTpTpGpG-(pA)$_{9or14}$ gave identical rates and extents of polymerization.

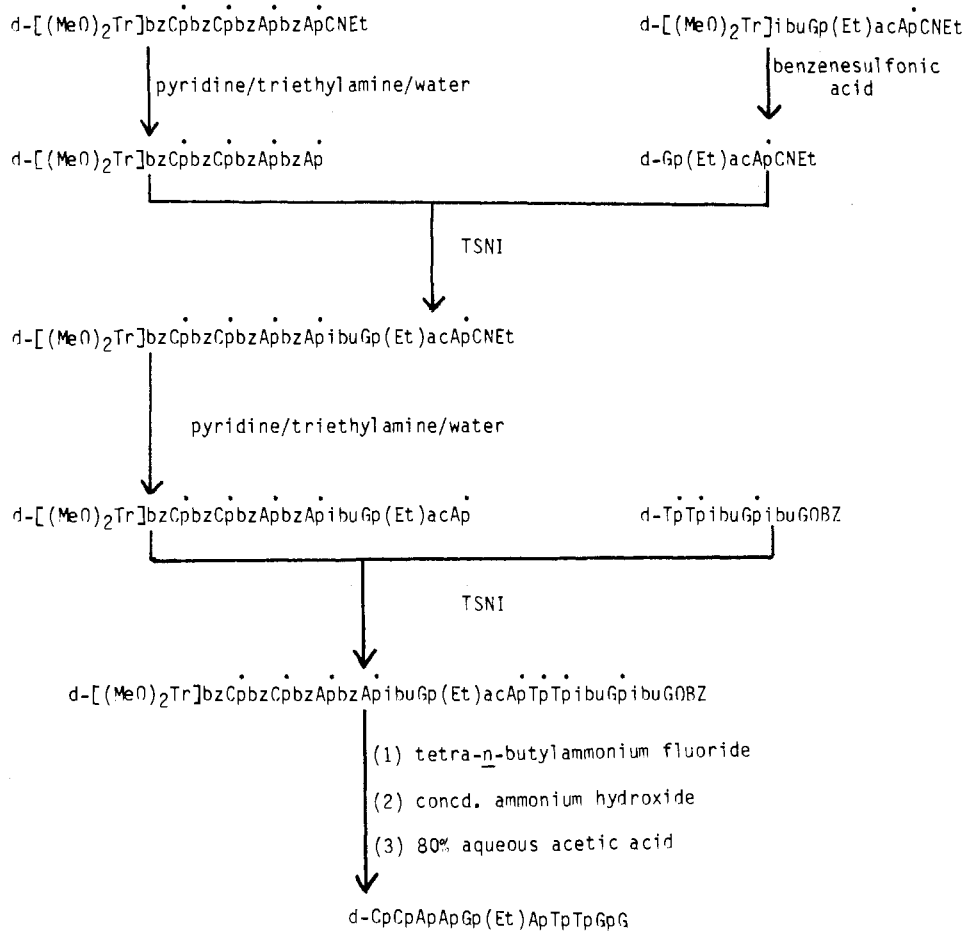
Discussion

Two decadeoxyribonucleotides, d-CpCpApApGp(Et)-ApTpTpGpG isomer I and isomer II, containing ethyl phosphotriester groups of opposite configuration were synthesized. These modified decamers were prepared by a phosphotriester synthetic procedure previously used in our laboratory for the preparation of deoxyribooligonucleotides on a large scale (Miller et al., 1980). The procedure, which is outlined in Scheme II, allowed us to place the ethyl phosphotriester modification at a specific position within the decanucleotide chain and also allowed us to control the configuration of ethyl phosphotriester linkage.

The key element in the synthesis involved the preparation and separation of the two protected ethyl phosphotriester dimers, d-[$(MeO)_2$Tr]ibuGp(Et)A isomers I and II. This was accomplished as outlined in Scheme I. Certain features of this synthesis deserve further comment. First, the dimer d-[$(MeO)_2$Tr]ibuGp̈bzA was prepared by condensation of d-[$(MeO)_2$Tr]ibuGp̈ with d-bzA. Although the 3′-hydroxyl group of the nucleoside was not protected, the condensation reaction proceeded in a selective manner to give only the 3′–5′-linked dimer. This was confirmed by removing the protecting groups and showing that the resulting dimer, d-GpA, was completely hydrolyzed by snake venom phosphodiesterase.

The two diastereoisomers of d-[$(MeO)_2$Tr]ibuGp̈bzA had significantly different mobilities on silica gel TLC and HPLC. Conversion to the ethyl phosphotriester derivative, d-

Scheme II: Preparation of d-CpCpApApGp(Et)ApTpTpGpG



$[(MeO)_2Tr]$ibuGp(Et)bzA, by fluoride ion catalyzed transesterification in absolute ethanol (Ogilvie & Beaucage, 1979) gave a single broadened spot on TLC and a broad peak on HPLC. No solvent system could be found that would cleanly separate the protected ethyl phosphotriester dimers. Removal of the protecting groups gave d-Gp(Et)A, the diastereoisomers of which appeared as two well-resolved peaks on reversed-phase HPLC.

The following experiment was performed to determine if the transesterification reaction proceeded stereospecifically. A portion of d-$[(MeO)_2Tr]$ibuGpbzA was separated into its constituent diastereoisomers, and each isomer was then converted to the ethyl phosphotriester derivative. After removal of the protecting groups, the resulting d-Gp(Et)A from each transesterification reaction was found to consist of a mixture of both diastereoisomers. Thus the transesterification reaction proceeds with racemization.

The ethyl phosphotriester diastereoisomers could be obtained in pure form after selective removal of the *N*-benzoyl group from d-$[(MeO)_2Tr]$ibuGp(Et)bzA. This partial deprotection was accomplished by reaction of the dimer with hydrazine hydrate (Letsinger et al., 1968). The two isomers of the resulting d-$[(MeO)_2Tr]$ibuGp(Et)A were separated by preparative silica gel HPLC. Each pure isomer was then carried through the remaining synthetic pathways.

A procedure was required to reprotect the amino group of the deoxyadenosine portion of the dimer without irreversibly blocking the 3′-hydroxyl group toward further reaction. Benzoylation or acetylation resulted in esterification of the 3′-hydroxyl groups as well as acylation of the amino group. Therefore, the 3′-hydroxyl group was first protected with a *tert*-butyldimethylsilyl group by treating d-$[(MeO)_2Tr]$ibuGp(Et)A with *tert*-butyldimethylsilyl chloride in pyridine. This reaction was found to be selective for the 3′-hydroxyl group of the dimer. The N-6 amino group was then acetylated with acetic anhdyride, and the silyl protecting group was selectively removed by treatment of the dimer with fluoride ion. The resulting d-$[(MeO)_2Tr]$ibuGp(Et)acA was then phosphorylated with the bis[triazole] derivative of *p*-chlorophenyl phosphate.

Construction of the decamer chains followed standard phosphotriester methods. The protected decamers were not purified after the final condensation reaction. Instead, the protecting groups were first removed, and the resulting deprotected oligomers were separated by preparative ion-exchange HPLC. Two methods were tested to remove the *p*-chlorophenyl phosphate protecting groups. Treatment of the protected decamer with pyridine aldoximate (Reese et al., 1978) followed by further deblocking with concentrated ammonium hydroxide/pyridine solution and 80% aqueous acetic acid gave as major components two pentanucleotides in addition to the decanucleotide and the starting tetra- and hexanucleotides. In contrast, overnight pretreatment of the protected decamer with fluoride ion (Itakura et al., 1975; Olgilvie & Beaucage, 1979) followed by the standard ammonium hydroxide/pyridine–acetic acid deblocking procedure gave only the decanucleotide and the starting tetra- and hexanucleotides as the major components. This result suggests that pyridine aldoximate cleaves the internucleotide ethyl phosphotriester linkage as well as the *p*-chlorophenyl groups.

The phosphodiester linkages of the decanucleotides were readily hydrolyzed by snake venom phosphodiesterase, while

the ethyl phosphotriester linkage remained intact. Thus the limit digest contained in addition to deoxycytidine and deoxynucleoside 5′-phosphates, the 5′-phosphorylated dimer, d-pGp(Et)A. Removal of the phosphate group from this dimer by alkaline phosphatase gave a dimer identical with authentic d-Gp(Et)A. Both d-Gp(Et)A and the decanucleotides have been completely characterized by high-resolution nuclear magnetic resonance spectroscopy. The results of these studies will be published in future papers.

The ethyl phosphotriester modified decanucleotides were tested as templates for polymerization reactions catalyzed by *E. coli* DNA polymerase I. To carry out these experiments, we used calf thymus terminal deoxynucleotidyl transferase to add an oligodeoxyadenylate tail to the 3′ end of each decamer. The length of the tail was controlled by following the incorporation of deoxyadenylate residues by PEI–cellulose TLC. The presence of the ethyl phosphotriester group, which is located five nucleotide residues from the 3′ terminus, had no effect on the rate of the addition reaction. Previous studies have shown that the enzyme requires a primer with a minimum of three contiguous phosphodiester linkages (Kato et al., 1967).

The polymerization reactions were carried out at 37 °C with d-$(pT)_8$pCpC as a primer. This oligonucleotide is expected to bind to the -GpG$(pA)_n$ portion of the template. Thus polymerization will be initiated three nucleotides away from the ethyl phosphotriester group. The progress of the polymerization reaction was followed by observing the incorporation of dGMP into higher molecular weight material. For polymerization to occur, the polymerase must copy through the ethyl phosphotriester modified region of the template. The unmodified oligomer d-CpCpApApGpApTpTpGpG$(pA)_9$ was prepared and used as a control template.

As shown in Figure 1, the rates and extents of the polymerization reactions directed by the ethyl phosphotriester templates were considerably less than that directed by the unmodified template. The rates of polymerization using the modified templates depended upon the configuration of the phosphotriester group. The extent of polymerization using the unmodified template levels off after approximately one dGMP residue was incorporated. This result suggests that the 3′–5′ exonucleolytic activity of the polymerase prevents complete copying of the 5′ end of the template. Similar plateaus are observed for both modified templates. This result is expected if only the rate of the polymerization reaction but not the nuclease reaction is affected by the presence of the triester group.

The observation that polymerization occurs at a slower rate with the isomer II template suggests the orientation of the ethyl group relative to the rest of the template backbone is an important factor in determining the effectiveness of inhibition. The ethyl triester group could slow the rate of polymerization in a number of ways. The triester group could induce a change in the conformation of the template at the site of modification. Previous studies on ethyl phosphotriester modified dinucleotides and the closely related dinucleoside methyl phosphonates have shown that conformational changes occur and that these changes are sensitive to the configuration of the phosphotriester or methyl phosphonate group (Miller et al., 1979; Kan et al., 1980). If similar alterations in template conformation occur, this could affect binding of the polymerase to the template during initiation or subsequent translocation steps. The presence of the ethyl group could result in unfavorable steric interactions with the polymerase, which could physically block the polymerase during initiation or translocation events. Alternatively the absence of a negatively charged phosphate group could prevent correct binding of the polymerase with the template.

The observation that a single ethyl phosphotriester group can significantly affect DNA polymerization rates in vitro suggests such groups could similarly alter the rates of cellular DNA replication. In addition to its effect on DNA polymerase, the triester group could also modify interactions with other DNA replication and transcription proteins such as single-stranded binding proteins and RNA polymerase. Recent experiments have suggested that alkyl triester groups accumulate in cellular DNA during prolonged exposure to alkylating agents (Shooter, 1978). The persistance of such lesions in cellular DNA could ultimately lead to alterations in the expression of DNA.

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