Synthesis and Properties of Oligodeoxyribonucleotides Containing an Ethylated Internucleotide Phosphate†

Michael Weinfeld*,‡ and D. Campbell Livingston§
Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom
Received February 26, 1986; Revised Manuscript Received April 22, 1986

ABSTRACT: Internucleotide phosphotriesters comprise an important class of DNA lesions produced by carcinogenic alkylating agents. To avoid confusion resulting from the presence of other DNA lesions, synthetically prepared oligonucleotides containing ethylated internucleotide phosphates as the sole form of damage were employed to investigate several chemical and biochemical properties of DNA alkyl phosphotriesters. A total of four oligonucleotides were synthesised for this study, the dimers Tp(Et)T and pTp(Et)T and the decamer d-TpTpTp(Et)TpCpTpApTpTpT together with its unmodified analogue. The dimers were characterized by UV and phosphorus NMR spectroscopy and the decamers by two-dimensional homochromatography, alkali hydrolysis, and variable-temperature circular dichroism (CD). Alkali hydrolysis of the ethylated decamer produced strand breaks in approximately 75% of the molecules. This is in close agreement with data previously obtained for dinucleoside ethyl phosphotriesters and triesters in alkylated cellular DNA. Results from the CD study suggest that the ethyl substituent does not disrupt base stacking within the oligomer. The interactions of two enzymes with the alkylated oligonucleotides were examined. First, it was found that ethylation of the internucleotide phosphate renders TpT inactive as a substrate for T4 polynucleotide kinase, implying that a negative charge is required on the 3′-phosphate group of the nucleotide to be phosphorylated. Hence, postlabeling assays of DNA damage that depend upon enzymatic phosphorylation of modified 3′-nucleotides cannot be applied to dinucleoside alkyl phosphotriesters. Second, both decamers, when annealed to a single-stranded plasmid template, were able to prime DNA synthesis, catalyzed by *Escherichia coli* DNA polymerase I, with equal effectiveness. The use of this reaction as a means of site-specifically incorporating phosphotriesters into viral vectors is recognized.

A correlation has been drawn between the carcinogenicity of alkylating agents and their reactivity toward the oxygen atoms of DNA (Pegg, 1977). Numerically, the most significant sites of such attack are the internucleotide phosphates (Singer, 1979). Several properties of the resultant DNA alkyl phosphotriesters suggest that they may be biologically significant lesions. For example, alkylated internucleotide phosphates on the template strand have been shown to severely reduce the rate and extent of template-directed DNA and RNA synthesis in vitro (Miller et al., 1982; Marushige & Marushige, 1983). In addition, esterification of key phosphate groups in promoter sequences inhibits binding of *Escherichia coli* RNA polymerase (Siebenlist & Gilbert, 1980), and similarly the presence of phosphotriesters in the operator regions of DNA markedly reduces the affinity of these regions for their repressor proteins (Siebenlist et al., 1980). Triesterified phosphates are also known to be refractory to the action of exo- and endonucleases in vitro (Miller et al., 1971; Jensen & Reed, 1978) and at least one restriction endonuclease, namely, *Eco*RI (Stec et al., 1985). Recently, a DNA methyltransferase has been isolated from a strain of *E. coli* constitutive for the adaptive response that removes methyl groups from phosphotriesters (McCarthy et al., 1983; Margison et al., 1985), but only those in one of the two stereochemical configurations in which these lesions exist (McCarthy & Lindahl, 1985; Hamblin & Potter, 1985; Weinfeld et al., 1985). In eukaryotic cells, however, triesters appear to persist undiminished for days after treatment with alkylating agents (Frei et al., 1978; Singer et al., 1981). This implies that such modification to the genome of higher organisms is either innocuous or that these cells possess an efficient mechanism for circumventing the damage.

An important chemical property of alkyl phosphotriesters is their lability in strong alkali (Kosolopoff, 1950). Since any one of the three ester bonds can be hydrolyzed, alkali treatment of alkylated DNA produces single-strand breaks at a proportion of the modified internucleotide phosphates and releases the alcohol of the alkyl group at the remainder. This property has been exploited by Shooter and co-workers in assays for DNA phosphotriesters involving either the measurement of strand breaks by sedimentation in alkali sucrose gradients (Shooter, 1976) or collection of the liberated alcohol (Crathorn & Shooter, 1982) following alkali digestion of the DNA.

To date, most investigations concerning phosphotriesters have involved treatment of DNA with alkylating agents. However, because this produces a variety of adducts, it can be difficult to obtain unequivocal information about the biological significance of a single adduct. Therefore, we, like Miller et al. (1982), have turned to the use of synthetic oligonucleotides containing phosphotriesters as the sole lesion. For our study four oligonucleotides were prepared, the dimers Tp(Et)T[1] and pTp(Et)T and the decamers I and II. The

† This work was supported by the Imperial Cancer Research Fund.
‡ Present address: Molecular Genetics and Carcinogenesis Laboratory, Department of Medicine, Cross Cancer Institute, Edmonton, Alberta, Canada T6G 1Z2.
§ Deceased November 27, 1983; to whom this paper is dedicated.

[1] Abbreviations: Np(PhCl)N or NpN, a deoxyribonucleotide 2-chlorophenyl phosphotriester; Np(Et)N, a deoxyribonucleotide ethyl phosphotriester; MSNT, 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole; MeOTr, methoxytrityl; ac, acetyl; bz, benzoyl; EtCN, cyanoethyl; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; PEI, poly(ethylenimine); ODS, octadecasilyl; NMR, nuclear magnetic resonance; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-5083$01.50/0

Table I: Chromatographic and Spectral Properties[a] of Tp(Et)T and pTp(Et)T and Their Precursors

compd	λ_{max} (nm)	λ_{min} (nm)	$\lambda_{260}/\lambda_{280}$	R_f[c] 10% E/C	R_f[c] 15% E/C	R_f[c] system d
MeOTrTp(PhCl)T				0.26	0.43	
MeOTrTp(Et)T				0.22	0.43	
Tp(Et)T	266	234.5	1.48	0.10		
MeOTrTp(Et)Tac				0.44	0.58	
Tp(Et)Tac				0.16	0.35	
$(MeOPhS)_2$pTp(Et)Tac	257[b]	226[b]	2.43	0.36	0.60	
MeOPhSpTp(Et)T						0.50
pTp(Et)T	266	235	1.51			0.23

[a] In 10 mM sodium phosphate buffer (pH 7.4). [b] In 95% ethanol. [c] 10% E/C = EtOH/$CHCl_3$ (1:9 v/v); 15% E/C = EtOH/$CHCl_3$ (3:17 v/v); system d = acetonitrile/acetic acid/water (80:5:15).

sequence of the latter was taken from the polyoma virus genome (bases 5081–5090 inclusive; Tooze, 1980).

d-TpTpTpTpCpTpApTpTpT
|
R

I, R = H
II, R = CH_2CH_3

This paper describes the synthesis and characterisation of these oligonucleotides and some of their chemical and biochemical properties, especially those related to assays for phosphate alkylation. In addition, we report on preliminary investigations into the use of a modified oligonucleotide as a means of introducing an alkyl phosphotriester at a specific site in a viral vector.

Materials and Methods

Nucleosides and 5′-*O*-(mono-*p*-methoxytrityl)thymidine were purchased from Sigma. N^6-Benzoyldeoxyadenosine, N^4-benzoyldeoxycytidine, and the 5′-*O*-(mono-*p*-methoxytrityl) derivative of the latter were prepared by the method of Schaller et al., (1963). 2-Chlorophenyl phosphorodichloridate, 1,2,4-triazole, *p*-toluenesulfonic acid, 2-mesitylenesulfonyl chloride, 2-cyanoethanol, 4-methoxybenzenethiol, and cesium fluoride (Aldrich) and 3-amino-1,2,4-triazole (Koch Light) were used without further purification. 3-Nitro-1,2,4-triazole (Browne, 1969), 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT; Jones et al., 1980), *syn*-4-nitrobenzaldoxime (Reese et al., 1978), and *S*,*S*-bis(4-methoxyphenyl) phosphorodithioate (Hata et al., 1978) were prepared according to published procedures.

Acetonitrile, pyridine, and absolute alcohol were dried by heating under reflux with calcium hydride for 16 h. The solvents were then distilled at atmospheric pressure and stored over molecular sieves (Linde type 4A).

Merck silica gel 60H (TLC grade) was used for short-column chromatography (Hunt & Rigby, 1967), with a ratio of silica gel to unfractionated material of 40:1 (w/w). The columns were eluted with 4–10% absolute alcohol in chloroform. Unless otherwise stated, thin-layer chromatography (TLC) was performed on Merck silica gel 60F 254 precoated aluminum-backed TLC sheets (layer thickness, 0.2 mm), with chloroform:ethanol (9:1 or 17:3) as the mobile phase. High-pressure liquid chromatography (HPLC) was carried out on a Waters Associates Model ALC/GPC 244 instrument using a Whatman Partisil 10SAX M9 (9 × 500 mm) column for ion-exchange chromatography and a Partisil PXS 10/25 ODS (45 × 250 mm) column for reverse-phase chromatography. *Elution conditions* are given in the legends to Figures 2 and 3.

Ultraviolet spectra were recorded on a Cary 17 spectrophotometer. For CD measurements a JASCO J40 CS spectropolarimeter was used together with a Comark electronic thermometer with its thermocouple probe inserted directly into the solution. The CD spectra are reported in terms of a *g* factor (Gioeli et al., 1982), defined as

$$g_\lambda = \Delta A_\lambda / A_{max}$$

where ΔA_λ and A_{max} are the differential and isotropic optical densities of the same solution in the same path length cell. (The *g* factor is a dimensionless quantity that permits one to quote results without having to determine the concentration of the solution. As it is the general shape of the CD vs. temperature curve that is of interest, quoting spectra in terms of this *g* factor is sufficient.)

Phosphorus NMR spectra were recorded at 36.4 MHz on a Bruker HFX 90 spectrometer operating at 28 °C. Chemical shifts are quoted relative to an external standard of H_3PO_4.

The M13mp7/polyoma plasmid was kindly donated by Dr. R. Kamen of the Imperial Cancer Research Fund, London.

Synthesis of Tp(Et)T. The synthetic route followed for the preparation of this compound is outlined in Scheme I. The 2-chlorophenyl ester of 5′-*O*-(methoxytrityl)-TpT [MeOTrTp(PhCl)T] was synthesized by using the phosphorylation and condensation protocols of Chattopadhyaya and Reese (1980). To replace the chlorophenyl moiety with an ethyl group, cesium fluoride (152 mg, 1 mmol) was added to a solution of the 5′-protected Tp(PhCl)T (0.1 mmol) dissolved in a mixture of anhydrous ethanol (1 mL) and dichloromethane (1 mL), and the reaction mixture was stirred at room temperature under dry nitrogen for 20 min. The solvent was evaporated under reduced pressure and the residue taken up in dichloromethane (2 mL) so that undissolved cesium fluoride could be removed by filtration through a Kieselguhr pad. After evaporation of the dichloromethane, the residue was redissolved in chloroform (1 mL), and the product was purified by short-column chromatography. The methoxytrityl group was removed by hydrolysis in 80% acetic acid at 37 °C for 2 h. UV data and R_f values for Tp(Et)T and the R_f values of some of its precursors are given in Table I.

Synthesis of $(MeOPhS)_2$pTp(Et)Tac. (See Scheme I.) MeOTrTp(Et)T (1 mmol) was treated with acetic anhydride (5 mL) in anhydrous pyridine (5 mL) for 2 h at room temperature. Excess acetic anhydride was destroyed by addition of methanol (5 mL), and after a further 1 h, the solvent was evaporated to yield the 3′-O-acetylated product, MeOTrTp-(Et)Tac, as a gum.

The methoxytrityl group was then removed by treatment with 80% aqueous acetic acid (5 mL) at 37 °C for 2 h. After evaporation of the acetic acid under reduced pressure, the residue was dissolved in chloroform (2 mL) and precipitated by dropwise addition to a stirred volume (250 mL) of ether. The precipitate of Tp(Et)Tac (0.87 mmol) was collected by centrifugation.

Table II: R_f Values for Fully Protected Decamers and Their Intermediates

compd	10% E/C[a]	15% E/C[a]
MeOTrTṗT	0.26	0.43
MeOTrTṗTṗEtCN	0.50	0.63
TṗTṗEtCN		0.45
MeOTrTṗTṗTṗTṗEtCN		0.56
MeOTrTp(Et)T	0.22	0.43
MeOTrTp(Et)TṗEtCN	0.51	0.65
Tp(Et)TṗEtCN	0.28	0.45
MeOTrTṗTṗTp(Et)TṗEtCN		0.56
MeOTrC^{bz}ṗT	0.29	0.61
MeOTrC^{bz}ṗTṗA^{bz}	0.23	0.45
MeOTrC^{bz}ṗTṗA^{bz}ṗT	0.19	0.44
MeOTrC^{bz}ṗTṗA^{bz}ṗTṗT	0.18	0.44
MeOTrC^{bz}ṗTṗA^{bz}ṗTṗTṗT	0.15	0.41
MeOTrC^{bz}ṗTṗA^{bz}ṗTṗTṗTac	0.22	0.53
C^{bz}ṗTṗA^{bz}ṗTṗTṗTac	0.12	0.41
fully protected 10-mer	0.18	0.48
fully protected ethylated 10-mer	0.18	0.48

[a] 10% E/C = EtOH/$CHCl_3$ (1:9 v/v); 15% E/C = EtOH/$CHCl_3$ (3:17 v/v).

This was dissolved in anhydrous pyridine (10 mL) together with *S,S*-bis(4-methoxyphenyl) phosphorodithioate (1.3 mmol) and MSNT (4 mmol). After 30 min at room temperature, the reaction was quenched by addition of saturated aqueous sodium hydrogen carbonate (1 mL), and 10 min later, the solution was poured into another 75 mL of aqueous sodium hydrogen carbonate. The resulting mixture was extracted with chloroform (5 × 20 mL), and the combined extracts were dried over magnesium sulfate before the solvent was evaporated. The product, $(MeOC_6H_4S)_2$pTp(Et)Tac, was obtained in 79% yield after purification by short-column chromatography. Spectral and chromatographic properties are presented in Table I.

Synthesis of pTp(Et)T. One of the arylthio groups and the acetyl group were removed by treating $(MeOC_6H_4S)_2$pTp(Et)Tac (0.5 mmol) with ammonia (*d* 0.88)/1,4-dioxane (7:3 v/v) at 37 °C for 24 h. The solvent was evaporated under reduced pressure, and the remaining arylthio group was oxidatively cleaved by treatment with iodine (2.5 g, 10 mmol) in pyridine/water (2:1 v/v) for 5 h. After removal of the solvent, the residue was dissolved in water (50 mL) and washed with ether (5 × 50 mL). The product was purified by chromatography on a DEAE-Sephadex (A-25–120) column (10 g), using a gradient of 0–0.3 M triethylammonium hydrogen carbonate (pH 7.5) to elute the material. The correct fractions were lyophilized to give 7000 A_{260} units of pTp(Et)T. UV spectral characteristics and R_f values are shown in Table I.

Synthesis of the Decanucleotides. (See Scheme II.) The fully protected decamers were prepared by the solution-phase phosphotriester approach (Chattopadhyaya & Reese, 1980), using the bis(triazolide) of 2-chlorophenyl phosphate as the phosphorylating reagent and MSNT as the condensing reagent. Each synthetic step was monitored by silica gel TLC [chloroform/ethanol 17:3 (v/v) as eluant]; terminally phosphorylated products, except those with cyanoethyl protecting groups, remained at the base line while the R_f values of intermediates lacking terminal phosphates were between 0.4 and 0.7 (Table II). Fully protected intermediates were purified by short-column chromatography on TLC-grade silica gel. The decamers were stored at −20 °C, in their fully protected form.

For deprotection, aliquots of the protected oligomers (1–10 μmol) were treated with 0.3 M N^1,N^1,N^3,N^3-tetramethylguanidinium 4-nitrobenzaldoximate in aqueous dioxan (1:1 v/v, 1.5 mL) at room temperature for 15 h. Concentrated aqueous ammonia (*d* 0.88, 1.5 mL) was then added, and the solution was incubated either at room temperature for 24 h or at 37 °C for 5 h. After removal of the solvent under reduced pressure, the residue was dissolved in 80% aqueous acetic acid and left at room temperature until the methoxytrityl group had been completely hydrolyzed from the 5′-terminus of the oligomer, as indicated by TLC. The acetic acid was evaporated in vacuo and the products were partitioned between water (5 mL) and chloroform (5 mL). The aqueous solution was concentrated to 1 mL for further purification of the oligonucleotides by HPLC. Conditions for purification by ion-exchange HPLC are given in the legend to Figure 2. The oligonucleotide solution obtained following HPLC was desalted by passage through a Bio-Gel P-2 column (10 × 750 mm, Bio-Rad), lyophilized, and stored at −20 °C.

The UV spectra of the two oligomers in 0.01 M sodium phosphate buffer (pH 7.4) are essentially identical, with a maximum at 265 nm and a minimum at 234 nm and 260 nm/280 nm ratios of 1.63 for the parent decamer and 1.62 for the ethylated decamer.

Enzymatic Phosphorylation of TpT and Tp(Et)T. A solution (16 μL) containing 0.6 nmol of dinucleoside monophosphate, 6.25 mM Tris-HCl (pH 7.6), 12.5 mM $MgCl_2$, 6.25 mM dithiothreitol, 0.125 mM spermidine, and 0.125 mM EDTA was incubated at 37 °C with 2 μL of [γ-^{32}P]ATP (4 pmol, 5000 Ci/mmol) and 2 μL of T4 polynucleotide kinase (10 units, P-L Biochemicals) for 1 h. Aliquots (2 μL) of the reaction mixture were analyzed by HPLC, using the elution conditions outlined in the legend to Figure 2, and by TLC on PEI–cellulose (Polygram CEL 300 PEI, Machery-Nagel) run in 2 M LiCl/2 M formic acid (1:1 v/v). In both cases the radioactive samples were coeluted with UV-detectable quantities of pTpT or pTp(Et)T. The plates were examined by autoradiography and by scraping and counting the UV-detectable spots.

Total Enzymatic Digestion of the Decamers. Each decamer (1.0 A_{265} unit) was incubated at 37 °C in a 25-μL solution containing 100 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 0.01 unit of snake venom phosphodiesterase (Sigma), and 1.0 unit of bacterial alkaline phosphatase (Sigma) for 5 h. The reaction mixture was applied directly to the ODS HPLC column. Conditions for the reverse-phase chromatography are given in the legend to Figure 3.

Partial Digestion and Sequencing of the Decamers. Standard protocols for 5′-end labeling with ^{32}P, polyacrylamide gel electrophoresis, and subsequent extraction of the decamers were followed (Maxam & Gilbert, 1980). The 5′-labeled oligonucleotide (50–100 pmol) was digested at 37 °C in 6 × 20 μL solutions, each containing 15 μg of partially hydrolyzed yeast RNA, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 10^{-4} unit of venom phosphodiesterase. The reactions were stopped sequentially at 0, 2, 5, 10, 30, and 60 min by addition of 10 μL of 10 mM EDTA and heating for 1 min at 100 °C. After the samples were combined, the digest was lyophilized and redissolved in 2–5 μL of water and the sequence of the oligonucleotide obtained by two-dimensional homochromatography (Jay et al., 1974).

Alkali Hydrolysis of the Decamers. Thirty microliters of an aqueous solution containing approximately 4 μg (0.1 A_{265} unit) of oligonucleotide was added to an equal volume of 1 M sodium hydroxide and incubated in a tightly sealed 500-μL Eppendorf tube at 37 °C for 6 h. The solution was neutralized with 1 M acetic acid (30 μL), and the hydrolysis products were examined by strong anion-exchange HPLC employing the same gradient as that used for the decamer purification.

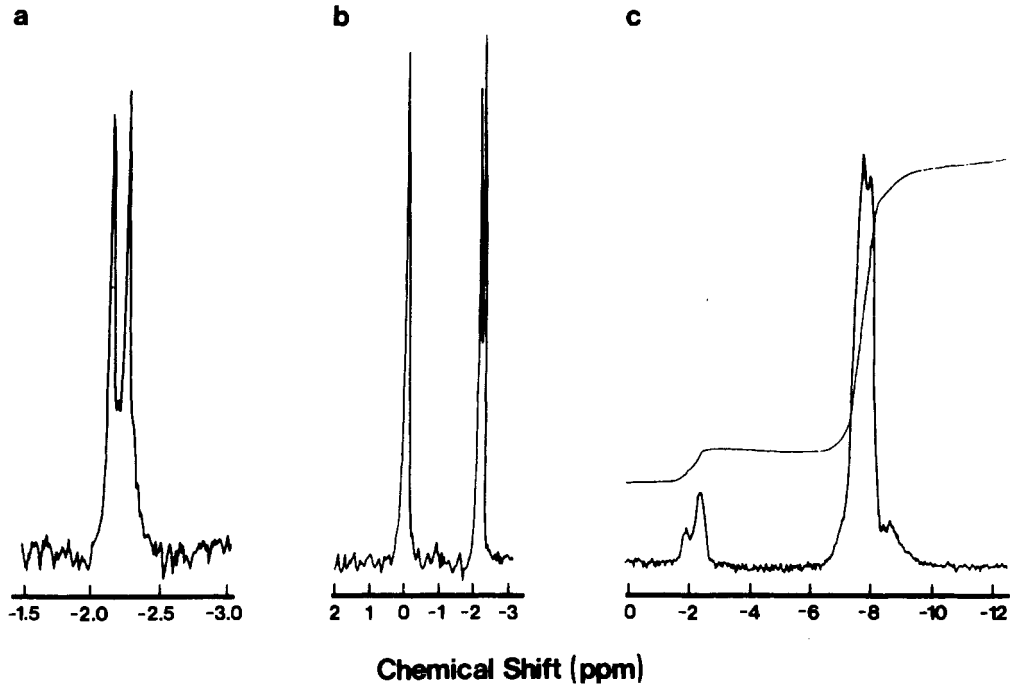


FIGURE 1: Phosphorus NMR spectra of (a) Tp(Et)T in D_2O, (b) pTp(Et)T in D_2O, and (c) the fully protected ethylated decamer in $CDCl_3$, recorded at 36.4 MHz. Chemical shifts (ppm) are relative to an external standard of H_3PO_4.

A 2-μL sample of the neutralized reaction mixture was phosphorylated in the presence of a 100-fold molar excess of [γ-^{32}P]ATP and 15 units of T4 polynucleotide kinase under the temperature and buffer conditions of Maxam and Gilbert (1980). The labeled products were then separated by gel electrophoresis (20% polyacrylamide/7 M urea), and the radioactive bands were cut out of the gel and counted.

Primer Annealing and Extension. The conditions given describe the variation of salt concentration, reaction temperature, and times that yielded the best results in our hands.

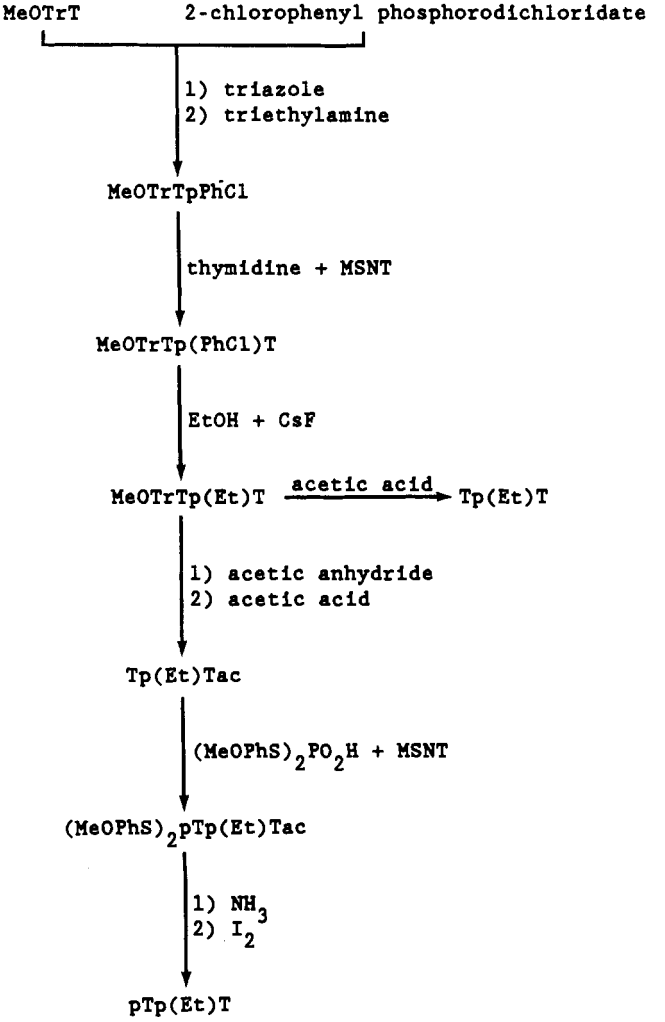
In a sealed capillary tube a mixture (10 μL) containing approximately 100 pmol of labeled primer, 2 pmol of single-stranded M13mp7/polyoma DNA, 40 mM Tris-HCl (pH 7.6), 15 mM $MgCl_2$, 150 mM NaCl, and 2 mM dithiothreitol was heated at 100 °C for 2 min, allowed to cool at room temperature for 10 min, and then kept at 0 °C for 30 min. The capillary was broken, and the contents were added to a solution (10 μL) containing 40 mM Tris-HCl (pH 7.6), 15 mM $MgCl_2$, 100 mM NaCl, 2 mM dithiothreitol, 2 mM of each dNTP, 1 mM ATP, and 4.5 units of DNA polymerase I (Klenow fragment, Boehringer Mannheim). This reaction mixture was held at 4 °C for 5 h, followed by a 15-h incubation at room temperature.

Aliquots (6 μL) of the plasmid DNA were individually digested with 1 unit of restriction enzyme *Hae*III, *Alu*I, or *Acc*I (purchased from New England Biolabs) under conditions recommended by the supplier. After phenolic extraction of the protein from each sample, the DNA was precipitated with ethanol, denatured in an 80% formamide solution, and fractionated by electrophoresis through a strand-denaturing 10% polyacrylamide gel, and the lengths of the restricted extended primer were deduced following autoradiography of the gel (Maxam & Gilbert, 1980).

RESULTS

Synthesis of Tp(Et)T and pTp(Et)T. The preparations of these two compounds are outlined in Scheme I. Conversion of the chlorophenyl ester of MeOTrTpT to its ethyl analogue was readily achieved by fluoride-catalyzed exchange of the phenyl group with ethanol (Ogilvie & Beaucage, 1979). The phosphorus NMR spectrum of Tp(Et)T (Figure 1a) shows two

Scheme I: Synthesis of Tp(Et)T and pTp(Et)T



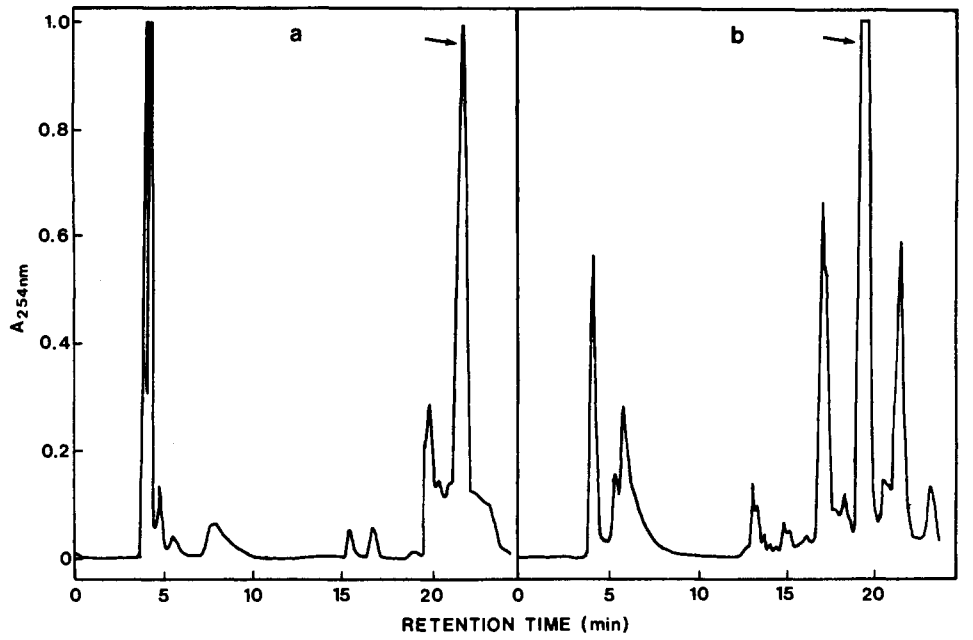


FIGURE 2: HPLC elution profiles of the crude deprotected decanucleotides, (a) the normal decamer and (b) the ethylated compound, from a Whatman Partisil 10 SAX M9 (9 × 500 mm) column, eluting with 99% buffer A (1 mM potassium phosphate in 10% aqueous ethanol, pH 7.0) and 1% buffer B (0.4 M potassium phosphate in 10% aqueous ethanol, pH 7.0) for 5 min, followed by a linear gradient from 1–100% buffer B over 30 min at a flow rate of 1 mL min^{-1}. The arrows indicate the desired product.

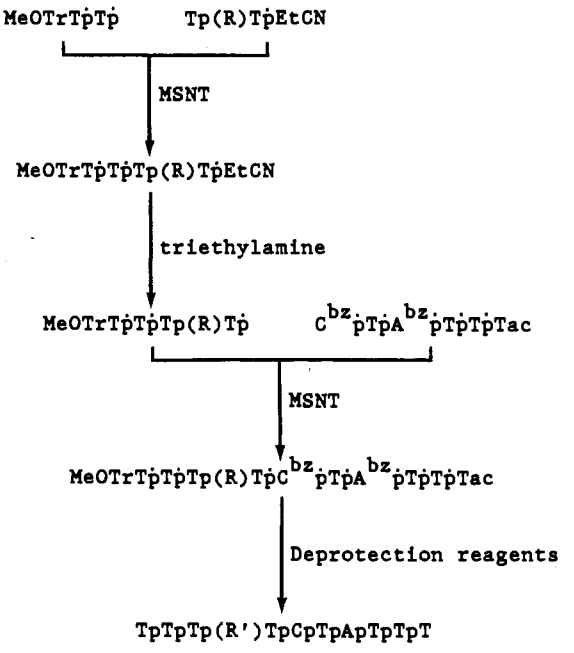
signals resulting from nearly equal quantities of the two diastereoisomers which arise because the phosphorus atom is a chiral center for the molecule. The isomers can be designated R_P and S_P after the manner of Cahn, Ingold and Prelog (Cahn et al., 1966; Weinfeld et al., 1985). By comparison to NMR data obtained for Tp(Me)T (Potter et al., 1983), the S_P isomer probably gave rise to the lower field signal and the R_P to the higher one. The two isomers were not purified but used as an equimolar mixture for all subsequent experiments and syntheses.

S,S-Bis(4-methoxyphenyl) phosphorodithioate (Hata et al., 1978) was chosen as the terminal phosphorylating agent in the synthesis of pTp(Et)T because of the ease and selectivity with which the arylthio groups can be removed, leaving the ethyl phosphotriester intact. After condensation of 3′-acetyl-Tp(Et)T with this reagent, the arylthio groups were hydrolyzed by sequential treatment with ammonia (which also removed the acetyl group) and then iodine. pTp(Et)T was characterized by NMR (Figure 1b) and by treatment with alkaline phosphatase to yield two products with identical retention times on reverse-phase HPLC as the isomers of Tp(Et)T. In addition, pTp(Et)T was found to be completely resistant to snake venom phosphodiesterase as predicted by Bannon and Verly (1972). UV and TLC data for this compound and its precursors are presented in Table I.

Enzymatic Phosphorylation of TpT and Tp(Et)T. Attempts to phosphorylate Tp(Et)T with T4 polynucleotide kinase and [γ-^{32}P]ATP were monitored by ion-exchange HPLC or TLC on PEI–cellulose plates. With standard reaction conditions, under which the radiolabel could be almost totally transferred to TpT, no radiolabeled pTp(Et)T could be detected (data not shown).

Synthesis of the Decanucleotides. The ethylated decamer and its natural analogue were prepared by using the solution-phase phosphotriester approach (Reese, 1978). Each was constructed from two subunits (see Scheme II); a tetramer, composed of the four thymidine residues at 5′-end, and a hexamer, common to both decamers, made from the remaining nucleosides. The tetramers were synthesized by addition of

Scheme II: Synthesis of the Decanucleotides



R = 2-chlorophenyl or ethyl; R' = H or ethyl.

two dimers. In the case of the ethylated tetramer the second dimer contained an ethyl phosphotriester. TLC R_f values for all the intermediates produced following each base addition are given in Table II. Integration of the phosphorus NMR spectrum for the fully protected ethylated decamer showed a ratio of eight aryl internucleotide phosphates to one alkyl phosphate (Figure 1c).

Deprotection of the phosphate groups was achieved by treatment with nitrobenzaldoxime (Reese et al., 1978). The ethyl phosphotriester, unlike the chlorophenyl triesters, was resistant to this reagent, although others have reported that similar treatment with pyridinealdoximate resulted in cleavage

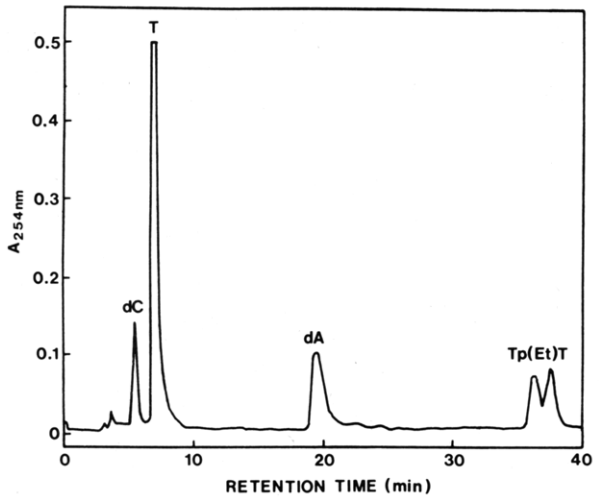


FIGURE 3: HPLC elution profile for the total enzymatic digestion of the ethylated decamer from a Partisil PXS 10/25 ODS (4.5 × 250 mm) column run at 1 mL min^{-1} with a linear gradient of 10–50% methanol in water over 1 h.

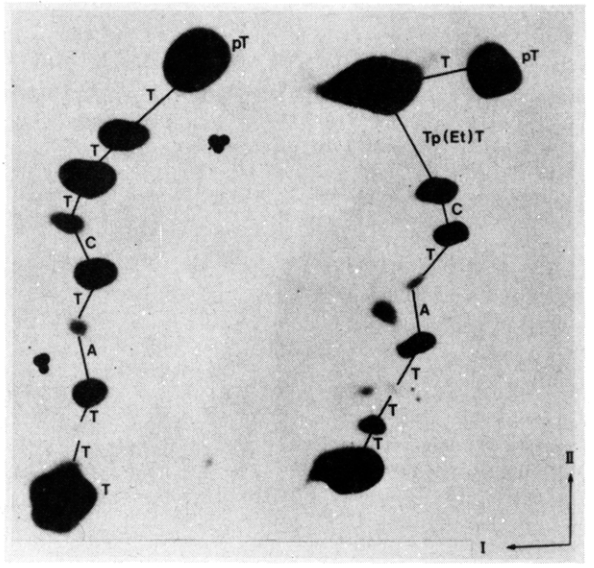


FIGURE 4: Two-dimensional sequences, following partial digestion, of the parent oligomer (left) and ethylated decamer (right). (I) Electrophoresis on cellulose acetate; (II) homochromatography on DEAE-cellulose.

of an internucleotide ethyl phosphotriester (Miller et al., 1982). Following removal of the remaining protecting groups, the decamers were purified by ion-exchange HPLC (Figure 2), desalted, and lyophilized.

Table III: Nucleoside Content of the Decamers

	dC	dA	T	Tp(Et)T
decamer	1.00	0.93	7.65	
ethylated decamer	1.00	0.90	6.20	0.95

Analysis and Sequencing of the Decamers. The nucleoside content of both decamers was established by enzymatic hydrolysis with snake venom phosphodiesterase and alkaline phosphatase, and the resulting nucleosides were separated by reverse-phase HPLC, collected, and quantitated by UV absorption. Figure 3 shows that the ethylated decamer contained almost equal proportions of the diastereoisomers of Tp(Et)T. Ratios for the nucleosides of the hydrolyzed oligomers, taking the relative value of deoxycytidine to be 1, are given in Table III. It is worth noting that occasionally the phosphodiesterase was contaminated with adenosine deaminase. This could be seen by the disappearance of the deoxyadenosine peak and the concomitant appearance of a shoulder on the thymidine peak due to the formation of deoxyinosine (data not shown).

The sequences of the oligonucleotides (Figure 4) were determined, after partial digestion, by two-dimensional mobility shift analysis (Jay et al., 1974). In the trace of the ethylated decamer, the relative positions of the second and third spots in the first dimension are qualitatively consistent with the expected mobilities of the compounds they represent, namely, pTpT and pTpTpTp(Et)T, respectively. The dimer, having a substantially larger charge to mass ratio than the tetramer, would be expected to move faster under the electrophoretic conditions used. The ^{32}P-labeled 5′-terminal mononucleotides of both decamers were shown to be thymidine monophosphate by cochromatography with unlabeled marker on ion-exchange HPLC (data not shown).

Variable-Temperature Circular Dichroism. The variations with temperature of the CD spectra of the two decamers were compared in order to establish whether or not ethylation of the phosphate backbone would lead to destabilization of the base stacking of the modified oligomer. CD spectra of both compounds and their respective CD vs. temperature curves are presented in Figure 5. Clearly, there is no significant difference between the slopes of the two g_{max} vs. temperature curves (Figure 5c).

Alkali Hydrolysis of the Decamers. Triesters of phosphoric acid are readily hydrolyzed in base to the corresponding diesters. Subsequent stages of hydrolysis are relatively slow (Kosolopoff, 1950). In order to gain some measure of the

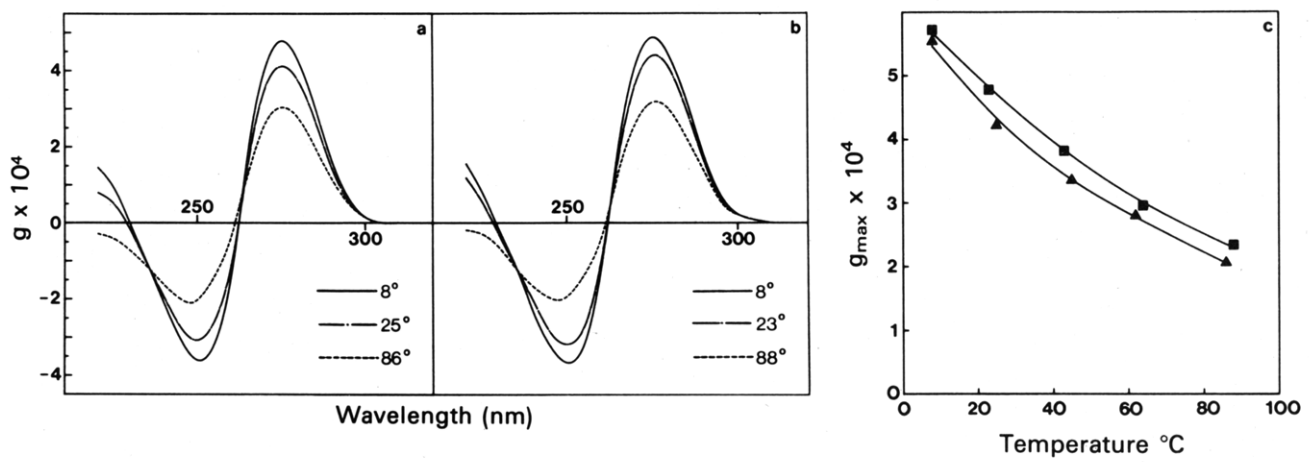


FIGURE 5: Variable-temperature CD spectra obtained for (a) the parent decamer and (b) the ethylated molecule, in 0.01 sodium phosphate (pH 7.4). (c) CD of lowest energy positive maximum (g_{max}) vs. temperature curves for the parent compound (■) and the ethylated molecule (▲).

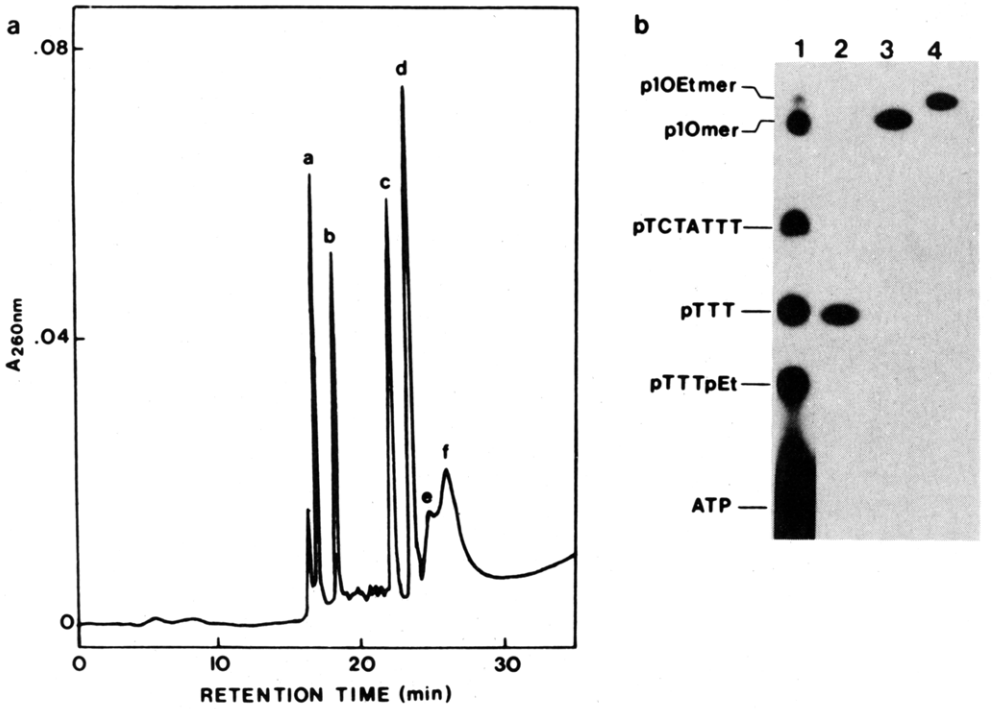


FIGURE 6: (a) HPLC elution profile of the alkali hydrolysis products of the ethylated decamer with the column and conditions described in Figure 2. Peaks: a, TpTpT; b, TpTpTpEt; c, TpCpTpApTpTpT; d, EtpTpCpTpApTpTpT; e, remaining ethylated decamer; f, parent decamer. (b) Gel electrophoresis of the ^{32}P-labeled hydrolysis products, where track 1 is the phosphorylated reaction mixture and tracks 2–4 are labeled markers.

relative lability of the three ester bonds about the triesterified phosphate, and the rate of hydrolysis, the alkylated decamer was digested in 0.5 M sodium hydroxide at 37 °C for 6 h, and the products were analyzed by ion-exchange HPLC and by radiolabeling. The chromatogram (Figure 6a) shows the production of all five possible products as well as a small amount of the starting material. Peak a was identified as TpTpT by coelution with a purchased marker, and peaks b–d were ascribed on the basis of charge. Under the same conditions, no discernible hydrolysis of the parent decanucleotide was observed.

The hydrolysis products of the ethylated decamer were quantified by radiolabeling with ^{32}P. (This avoids the necessity to determine the extinction coefficient of each product when they are assayed by UV.) A sample of the reaction mixture was incubated with an excess of T4 polynucleotide kinase and [γ-^{32}P]ATP and then analyzed by gel electrophoresis. The autoradiogram (Figure 6b) reveals four of the five products; the other one, d-EtpTCTATTT, is not a substrate for the enzyme because it lacks a 5′-hydroxyl group. The ratio of the labeled products indicated that after 6 h hydrolysis only 8% of the ethylated decamer remained, implying a $t_{1/2}$ = 1.65 h and that 27% of the hydrolysis resulted in loss of the ethyl group, 30% in cleavage at the 5′-phosphate bond, and 43% in cleavage at the 3′-phosphate bond.

Enzymatic Extension of the Decamers. It is known that the presence of an ethyl triester, located near the center of a decanucleotide, has no effect on the rate of nucleotide addition catalyzed by terminal nucleotidyl transferase (Miller et al., 1982). We were interested to see whether a similarly placed ethyl group would inhibit oligonucleotide extension on a suitable template catalyzed by *E. coli DNA* polymerase.

The template chosen for this experiment was the single-stranded form of a plasmid constructed by inserting a *Pst*I fragment (bases 4225–484) of polyoma DNA into the *Pst*I site of phage M13mp7 (Figure 7). Both decamers, labeled at their 5′-termini, were annealed to this template and then

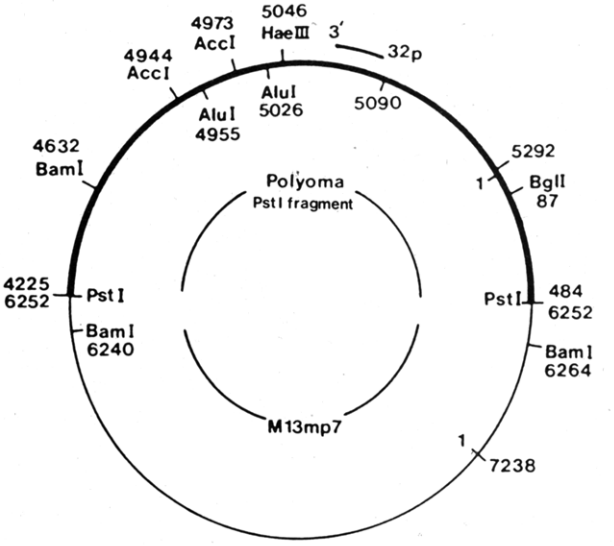


FIGURE 7: Schematic representation of the template DNA constructed from M13mp7 and a *Pst*I fragment of polyoma viral DNA.

Table IV: Predicted Lengths of Enzyme-Digested Extended Primers

restriction enzyme	nucleotide length of extended decamer
*Hae*III	44
*Alu*I	64, 135[a]
*Acc*I	117, 156[a]

[a] Resulting from incomplete digestion.

used as primers for enzymatic synthesis of the complementary strand following well-defined procedures (Gillam et al., 1979; Wasylyk et al., 1980). In order to check for primer extension and to ensure that the decamers, especially the ethylated one, annealed uniquely to the correct site of the template, aliquots of the reaction mixtures were separately treated with one of three restriction enzymes, *Hae*III, *Alu*I, and *Acc*I, and the digested products examined on a strand-denaturing gel. Table

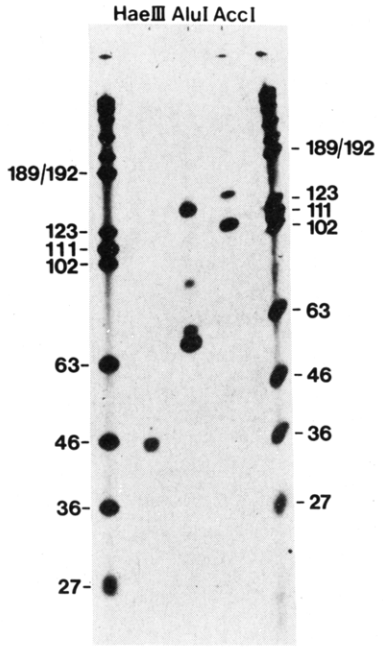


FIGURE 8: Restriction enzyme analysis of the extended ethylated primer. The two outer tracks contain size markers (polyoma DNA digested with *Dde*I).

IV gives the predicted lengths, based on the sequence of polyoma virus (Tooze, 1980), of the extended primers after restriction if the oligomers annealed at the correct site.

The autoradiograph of the gel (Figure 8) demonstrates that the alkylated decamer had indeed functioned as a primer almost exclusively from the desired position. A nearly identical result was obtained with the unmodified decamer (data not shown). The activity of the radioactive bands cut out of the gels indicated that the decamers had acted with equal priming efficiency.

DISCUSSION

As a result of the recent finding that a mechanism exists for the removal of methyl groups from DNA phosphotriesters (McCarthy et al., 1983), more attention is now focusing on this class of lesion. In this report we have described the synthesis of two ethylated oligonucleotides and their use to explore some of the consequences of internucleotide phosphate alkylation.

The chemical and biochemical properties that distinguish these molecules from their natural analogues arise from the loss of charge of the modified phosphate and the steric influence of the alkyl substituent. The former is probably responsible for the inability of Tp(Et)T to act as a substrate for polynucleotide kinase. The enzyme catalyzes the phosphorylation of 5′-hydroxyl residues of DNA, RNA, oligonucleotides, and 3′-mononucleotides but not mononucleosides (Richardson, 1965; Novogrodsky et al., 1966). It requires a 3′-phosphate group on the nucleoside which is to be phosphorylated. Our results imply that a negative charge at the 3′-position, and not just a phosphate group, is necessary for enzyme activity. Within oligonucleotides such interference by alkyl groups is most likely restricted to the phosphate group immediately 3′ to the nucleoside that is to be phosphorylated. The ethylated decamer was just as effectively phosphorylated as the parent molecule. The inability to enzymatically phosphorylate dinucleoside alkyl phosphotriesters means that the postlabeling assay for DNA adducts developed by Randerath et al. (1981), in which 3′-mononucleotides of DNA adducts are converted to radiolabeled 5′-mononucleotides with polynucleotide kinase, cannot be applied to measure triester concentration.

Crathorn and Shooter (1982) noted that because the accurate quantitation of phosphotriesters by their alkali hydrolysis assays is dependent on the relative lability of the three phosphate ester bonds, knowledge of the hydrolysis patterns for the particular alkylating agent is required. Since other alkylation damage can also give rise to strand breaks, the clearest way to obtain such information is from model compounds containing only alkylated internucleotide phosphates. The hydrolysis patterns of methyl and ethyl esters of several dinucleoside monophosphates have been analyzed (Swenson & Lawley, 1978; Jensen & Reed, 1978; Jensen, 1978), but such data have not been produced, until now, for longer oligonucleotides, which would confirm the applicability of the dimer results to full-length DNA. The relative lability of the three phosphate ester bonds of the triester in the decamer is comparable with that of Tp(Et)T. The ratio for bond scission, 3′-oxygen:5′-oxygen:ethyl oxygen of 43:30:27, respectively, found for the decamer is in close accordance with the 47:30:22 ratio obtained for the hydrolysis of the ethylated dimer (Swenson & Lawley, 1978). It also agrees with the finding that approximately three-fourths of the phosphotriesters produced in ethylnitrosourea-treated DNA (clearly a less well-defined system than the specifically esterified oligonucleotide) give rise to strand breaks following treatment with alkali (Crathorn & Shooter, 1982). Collectively, these results suggest that, in terms of relative bond lability, dinucleoside alkyl phosphotriesters serve as good models for phosphotriesters in DNA. However, one factor that has still to be more rigorously investigated is the rate of hydrolysis of DNA triesters. Our data indicate that a minumum 6-h incubation at 37 °C in 0.5 M NaOH is required for >90% hydrolysis of DNA phosphotriesters, assuming that there is no sequence dependence for hydrolysis rate. Crathorn and Shooter, on the other hand, reported that digestion of ethylnitrosourea-treated DNA for just 1 h at 37 °C in 0.5 M NaOH is sufficient for >90% hydrolysis of the alkylated phosphates.

The possiblity that phosphate alkylation may disrupt base stacking (an important steric effect) was investigated by circular dichroism. The slope of the CD vs. temperature curve is related to the cooperativity in the base stacking process (Bush & Scheraga, 1969). Such curves obtained for TpT, Tp(Me)T (Weinfeld et al., 1985), and Tp(Et)T (unpublished data) show that the alkyl group causes considerable destacking to the dimer. It is, therefore, somewhat surprising that the slope of the g_{max} vs. temperature curve for the ethylated decamer (Figure 5c) is very similar to that of the unmodified oligonucleotide. This implies either that in the alkylated decamer, as opposed to the dimers, there is little or no disruption to base stacking or that even if there is destacking at the site of ethylation, CD is not a sensitive enough technique to distinguish distortion about one internal phosphate out of nine.

Miller et al. (1982) employed terminal deoxynucleotidyltransferase to add additional nucleotides to an oligonucleotide containing an ethyl phosphotriester. An alternative, which permits more directed base addition, is to anneal the modified oligomer to a template and extend it by using the Klenow fragment of *E. coli* DNA polymerase. Our results show that ethylation of an internucleotide phosphate, seven nucleotides removed from the 3′-terminus, does not interfere with the annealing or extension of the primer. Consequently, this reaction will enable the investigator to generate viral probes containing alkylated internucleotide phosphates as the sole lesion at defined loci. It is anticipated that such specific probes

should prove to be invaluable in obtaining unequivocal information concerning the fate of phosphotriesters inside mammalian cells.

ACKNOWLEDGMENTS

We thank Drs. J. Jiricny, B. Griffin, R. Kamen, A. Drake, M. C. Paterson, and M. M. Coombs for useful discussions and A. Cowie for her technical assistance.

REFERENCES

Bannon, P., & Verly, W. (1972) *Eur. J. Biochem. 31*, 103–111.

Browne, E. J. (1969) *Aust. J. Chem. 22*, 2251–2254.

Bush, C. A., & Scheraga, H. A. (1969) *Biopolymers 7*, 395–409.

Cahn, R. S., Ingold, C., & Prelog, V. (1966) *Angew. Chem., Int. Ed. Engl. 5*, 385–415.

Chattopadhyaya, J. B., & Reese, C. B. (1980) *Nucleic Acids Res. 8*, 2039–2053.

Crathorn, A. R., & Shooter, K. V. (1982) *Biochim. Biophys. Acta 697*, 259–261.

Frei, J. V., Swenson, D. H., Warren, W., & Lawley, P. D. (1978) *Biochem. J. 174*, 1031–1044.

Gillam, S., Jahnke, P., Astell, C., Phillips, S., Hutchison, C. A., III, & Smith, M. (1979) *Nucleic Acids Res. 6*, 2973–2985.

Gioeli, C., Chattopadhyaya, J. B., Drake, A. F., & Öberg, B. (1982) *Chem. Scr. 19*, 13–17.

Hamblin, M. R., & Potter, B. V. L. (1985) *FEBS Lett. 189*, 315–317.

Hata, T., Yamaguchi, K., Honda, S., & Nakagawa, I. (1978) *Chem. Lett.*, 507–508.

Hunt, B. J., & Rigby, W. (1967) *Chem. Ind. (London)*, 1868–1869.

Jay, E., Bambara, R., Padmanabhan, R., & Wu, R. (1974) *Nucleic Acids Res. 1*, 331–353.

Jensen, D. E. (1978) *Biochemistry 17*, 5108–5113.

Jensen, D. E., & Reed, D. J. (1978) *Biochemistry 17*, 5098–5107.

Jones, S. S., Rayner, B., Reese, C. B., Ubasawa, A., & Ubasawa, M. (1980) *Tetrahedron 36*, 3075–3085.

Kosolopoff, G. (1950) in *Organophosphorus Compounds*, p 235, Wiley, New York.

Margison, G. P., Cooper, D. P., & Brennand, J. (1985) *Nucleic Acids Res. 13*, 1939–1952.

Marushige, K., & Marushige, Y. (1983) *Chem.-Biol. Interact. 46*, 179–188.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.

McCarthy, J. G., Edington, B. V., & Schendel, P. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7380–7384.

McCarthy, T. V., & Lindahl, T. (1985) *Nucleic Acids Res. 13*, 2683–2698.

Miller, P. S., Fang, K. N., Kondo, N. S., & Ts'o, P. O. P. (1971) *J. Am. Chem. Soc. 93*, 6657–6665.

Miller, P. S., Chandrasegaran, S., Dow, D. L., Pulford, S. M., & Kan, L. S. (1982) *Biochemistry 21*, 5468–5474.

Novogrodsky, A., Tal, M., Traub, A., & Hurwitz, J. (1966) *J. Biol. Chem. 241*, 2933–2943.

Ogilvie, K. K., & Beaucage, S. L. (1979) *Nucleic Acids Res. 7*, 805–823.

Pegg, A. E. (1977) *Adv. Cancer Res. 25*, 195–269.

Potter, B. V. L., Connolly, B. A., & Eckstein, F. (1983) *Biochemistry 22*, 1369–1377.

Randerath, K., Reddy, M. V., & Gupta, R. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6126–6129.

Reese, C. B. (1978) *Tetrahedron 34*, 3143–3179.

Reese, C. B., Titmas, R. C., & Yau, L. (1978) *Tetrahedron Lett. 30*, 2727–2730.

Richardson, C. C. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 158–165.

Schaller, H., Weimann, G., Lerch, B., & Khorana, H. G. (1963) *J. Am. Chem. Soc. 85*, 3821–3827.

Shooter, K. V. (1976) *Chem.-Biol. Interact. 13*, 151–163.

Siebenlist, U., & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 122–126.

Siebenlist, U., Simpson, R. B., & Gilbert, W. (1980) *Cell (Cambridge, Mass.) 20*, 269–281.

Singer, B. (1979) *JNCI, J. Natl. Cancer Inst. 62*, 1329–1339.

Singer, B., Spengler, S., & Bodell, W. J. (1981) *Carcinogenesis (London) 2*, 1069–1073.

Stec, W. J., Zon, G., Gallo, K. A., Byrd, R. A., Uznanski, B., & Guga, P. (1985) *Tetrahedron Lett. 26*, 2191–2194.

Swenson, D. H., & Lawley, P. D. (1978) *Biochem. J. 171*, 575–587.

Tooze, J., Ed. (1980) *Molecular Biology of Tumor Viruses, Part 2: DNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Wasylyk, B., Derbyshire, R., Guy, A., Molko, D., Roget, A., Teoule, R., & Chambon, P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4114–4120.

Weinfeld, M., Drake, A. F., Saunders, J. K., & Paterson, M. C. (1985) *Nucleic Acids Res. 13*, 7067–7077.