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Synthesis and enzymatic properties of deoxyribooligonucleotides containing methyl and phenylphosphonate linkages

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ABSTRACT

Chemical methods for the synthesis of short deoxyribooligonucleotides containing methyl and phenylphosphonodiester linkages have been developed. The interaction of two such nonionic dinucleotide analogs, $T(pCH_3)T$ and $T(pC_6H_5)T$, with several enzymes has been investigated. Because of the phosphonate linkage each dinucleotide exists as a diastereomeric pair as shown by thin layer chromatography and enzymatic studies. Both isomers of each dinucleotide can be phosphorylated by T_4-polynucleotide kinase in the presence of [γ-^{32}P]ATP. Only one of the diastereoisomers of each dinucleotide is slowly hydrolyzed by snake venom phosphodiesterase and acts as an inhibitor of the enzyme-catalyzed hydrolysis of 5'-labeled oligothymidylic acid. Both isomers of each dinucleotide analog are completely resistant to hydrolysis by spleen phosphodiesterase.

INTRODUCTION

A great number of nucleotide analogs in which either the heterocyclic base or the sugar is modified have been synthesized and studied while only a small number of analogs are known in which the phosphate group is altered. These include nucleoside phosphites[1], nucleoside phosphonates[2], and nucleoside phosphorothioates[3]. Oligonucleotides containing altered phosphate groups can be classified into three major groups: (a) polyionic oligonucleotides, (b) nonionic oligonucleotides, and (c) mixed ionic-nonionic oligonucleotides. Oligonucleotides with a phosphorothioate group in place of a phosphate group were synthesized by Eckstein and have proved to be useful substrates in studying enzyme mechanisms[4]. Nonionic analogs such as oligonucleotide triesters have been synthesized by Ts'o and coworkers[5] who used them as model compounds for studies on the physical chemistry of nucleic acids. More recently these authors have obtained deoxyribodinucleoside methylphosphonates and studied their physical properties by CD and pmr spectroscopy[6]. However, chemical synthesis of these nonionic analogs has not been reported by these authors.

Independently, we have developed chemical procedures for the synthesis of oligonucleotides containing nonionic as well as mixed diester linkages with the hope of acquiring potential pseudosubstrates for the nucleic acid enzymes. In this paper we wish to report the synthesis of oligonucleotides carrying methyl and phenylphosphonate linkages, their phosphorylation by T_4-polynucleotide kinase, and their interaction with snake venom and spleen phosphodiesterases.

RESULTS AND DISCUSSION

Although several procedures for the synthesis of dinucleoside methyl and phenylphosphonates were attempted, only three procedures were found satisfactory and are described below. Methods A and B produced dinucleoside methylphosphonates, but in each case the reaction was slow and the yield of final product was low. In Method A methylphosphonodichloridate was used directly for phosphorylation of the 3'-hydroxyl group of a 5'- and amino-protected nucleoside. The 3'-phosphorylated nucleoside thus obtained was further treated with the 5'-hydroxyl group of incoming nucleoside. While the first step of phosphorylation was quantitative in 2 hr, the second step of phosphorylation was slow, requiring 12 hr for the formation of diester in 40% yield. Although dinucleoside methylphosphonates, $d(Me_2O)TrT(pCH_3)T$ and $d(Me_2O)Tr\ anC\ (pCH_3)anC$[7], were isolated in reasonable yields (36% and 28%, respectively) using Method A, the dinucleotide, $d(Me_2O)Tr\ ibG(pCH_3)ibG$ was isolated in only 12% yield. Several unidentified side products formed during the synthesis made isolation of the desired product difficult. Some of the side products may have resulted from the prolonged exposure of the reaction products to pyridine HCl formed during the reaction. However, formation of these side products was avoided by using methylphosphonoditriazolide (II, Figure 1) as the phosphorylating reagent. This reagent II is similar to the reagent used by Narang and coworkers[8] in the synthesis of deoxyoligonucleotide triesters.

Methods B and C use reagent (II) in the phosphorylation of the 3'-hydroxyl group of a 5'-protected nucleoside. The reagent was prepared *in situ* by stirring equimolar quantities of methylphosphonodichloridate, triethylamine, and triazole in anhydrous tetrahydrofuran at room temperature. The reaction of triazole with methylphosphonodicholoridate was slow and required 4 hr for completion. Formation of phenylphosphonoditriazolide was even slower and required 8 hr with stirring for completion. Both methyl and

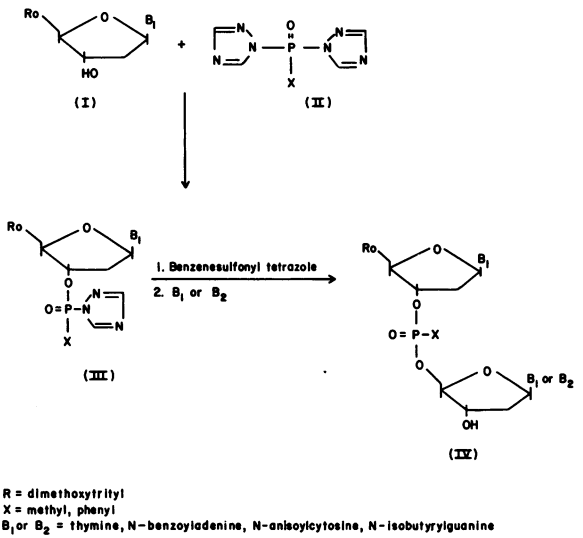

FIGURE 1. Synthesis of dinucleoside methyl and phenylphosphonate by one step procedure.

phenylphosphonoditriazolide were used directly, without isolation for the phosphorylation of the 3'-hydroxyl group of a 5'-dimethoxytritylated nucleoside. Phosphorylation of the 3'-hydroxyl group was rapid and reached completion within 30 minutes. Recently, we have shown[8] that in the synthesis of triesters that an intermediate similar to intermediate (III) did not phosphorylate the second nucleoside, and that further activation of this intermediate was necessary for an efficient second step phosphorylation. Further activation of intermediate (III), as described in Method B, was accomplished with triethylammonium benzenesulfonate. The activated intermediate thus obtained reacted slowly with the second nucleoside requiring 12 hr for 60% reaction. The yields of the isolated phosphonate diesters were in the range of 40-50%.

Although satisfactory yields of the dinucleoside phosphonates were obtained by Method B, synthesis by this method on a microscale (10-20 μmol) was unsatisfactory, and in each case the yield of the isolated product was very low (15-20%). In most cases, the second step of phosphorylation reached only 20-30% of completion. This may be due to partial hydrolysis of the activated intermediate (III). In order to improve the efficiency of the

second step of phosphorylation in microscale reactions, we investigated the activation of intermediate (III) by benzenesulfonyl tetrazole[9]. This reagent was prepared in situ and was added to intermediate (III) in the presence of the second nucleoside. A highly reactive phosphorylating intermediate was formed which reacted with the second nucleoside completely within one hr. By following this modification (Method C), oligonucleoside methylphosphonates were isolated in 60-70% yield (Table 1). This procedure was equally efficient for the synthesis of oligonucleoside phosphonates on a large scale (1-5 mmol). From the experiments described above it is clear that Method C is the method of choice for the synthesis of oligonucleotides carrying methyl or phenylphosphonate linkages.

Reaction products were readily separated by silica gel column chromatography using $CHCl_3$ containing 1% pyridine as the solvent. The columns were developed at a moderate pressure of 50 psi. The dinucleoside phosphonates eluted in 2-4% methanol while the tetranucleoside phosphonates eluted in 6-8% methanol. The reaction products from microscale synthesis were separated by silica gel preparative thin layer chromatography in solvent D and the desired product was isolated by elution with $CHCl_3$-MeOH-pyridine (7.8:2.0:0.2; v/v).

Both stepwise and blockwise elongation procedures were employed successfully for the synthesis of oligonucleoside methyl or phenylphosphonates. For

TABLE 1. Reaction conditions and isolated yields of the various dinucleoside methyl and phenylphosphonates.

[a]DMTr Comp.	5'-OH Comp.	[b]$MePOCl_2$	[c]$PhPOCl_2$	Triazole	Et_3N	[d]BST	Nucleoside Phosphonate	[e]Yield
(equiv)	(equiv)	(equiv)	(equiv)	(equiv)	(equiv)	(equiv)		
1. DMTr T (1.0)	dT (1.0)	1.0	-	2.5	2.5	1.0	DMTr T(pCH_3)T	65%
2. DMTr bzA (1.0)	dbzA (1.05)	1.0	-	2.5	2.5	1.1	DMTr bzA(pCH_3)bzA	62%
3. DMTr anC (1.0)	danC (1.05)	1.0	-	2.5	2.6	1.2	DMTr anC(pCH_3)anC	69%
4. DMTr ibG (1.0)	dibG (1.1)	1.05	-	2.8	2.8	1.3	DMTr ibG(pCH_3)ibG	58%
5. DMTr T (1.0)	dT (1.0)	-	1.0	2.5	2.5	1.1	DMTr T(pC_6H_5)T	60%
6. DMTr T(pCH_3)T (1.0)	dT(pCH_3)T (1.1)	1.15	-	3.0	3.0	1.8	Tetranucleoside phosphonate	53%
7. DMTr T(ClC_6H_4)T (1.0)	dT (1.0)	1.1	-	3.0	3.0	1.5	Trinucleoside phosphonate	50%
8. DMTr T(ClC_6H_4)T (1.0)	dT(ClC_6H_4)T (1.2)	1.15	-	3.0	3.0	1.1	Tetranucleoside phosphonate	62%

[a]Abbreviation for dimethoxytrityl group; [b]abbreviation for methylphosphonodichloridate; [c]abbreviation for phenylphosphonodichloridate; [d]abbreviation for benzenesulfonyl tetrazole; [e]isolated yields.

blockwise elongation, oligonucleoside phosphonate blocks of the appropriate size were synthesized in a stepwise manner. The dimethoxytrityl group was removed and the 5'-unprotected oligonucleotide was then condensed with 5'-protected oligonucleotide using Method C. For example, in the synthesis of the tetranucleoside methylphosphonate $d(Me_2O)TrT(pCH_3)T(pCH_3)T(pCH_3)T$, two dinucleotides, $d(Me_2O)TrT(pCH_3)T$ and $T(pCH_3)T$, were synthesized in a stepwise manner and coupled to give a tetranucleotide.

For the synthesis of oligonucleotides possessing the usual diester as well as phosphonate diester linkages, we have used a triester method[8] in combination with Method C. Synthesis of the tetranucleotide $d(Me_2O)TrT(ClC_6H_4)T(pCH_3)T(ClC_6H_4)T$ was accomplished by coupling two dinucleotides $d(Me_2O)TrT(ClC_6H_4)T$ and $T(ClC_6H_4)T$ in the presence of reagent (III) and benzenesulfonyl tetrazole as described in Method C. Removal of the protecting groups under appropriate conditions (described in Methods and discussed below) provided a tetranucleotide containing a single methylphosphonate linkage.

Oligonucleoside methylphosphonates were unstable in 1 M NaOH at room temperature. Under these conditions as much as 40% of the methylphosphonate diester linkage was hydrolyzed in one hr. Analysis of the cleaved products by silica gel tlc in the presence of the appropriate markers clearly showed that the hydrolysis of the methylphosphonate diester was random. Dithymidine phenylphosphonate, on the other hand, was more stable to hydrolysis under these conditions. Only a trace amount of hydrolysis (2-4%) was observed in one hour. However both phosphonate analogs of these oligonucleotides were resistant to hydrolysis by concentrated ammonium hydroxide at 36° for up to 16 hr; thereafter slow hydrolysis was observed. Treatment with concentrated ammonium hydroxide at elevated temperatures (>50°) resulted in rapid hydrolysis of both methyl and phenylphosphonate analogs. Since treatment with concentrated ammonium hydroxide at 36° for 16 hr does not produce any side reactions, these conditions were employed for the removal of p-chlorophenyl and amino protecting groups.

Both methyl and phenylphosphonate analogs were stable to mild acid. Treatment of these analogs with benzenesulfonic acid under the conditions used for removal of the dimethyoxytrityl group[9] produced no side products as judged by tlc. Removal of the dimethyoxytrityl group on a microscale (0.1 to 0.5 μmol) was achieved by treatment with pyridine-acetic acid-H_2O (3:14:1, v/v, pH 3.5) for 4 hr at room temperature. Even under these conditions no undesired products were formed.

To show that under the present set of conditions the major product of

synthesis carries a 5'→3' internucleotide linkage, we synthesized the dinucleotide d(Me_2O)TrT(pCH_3)T-OAC. The 3'-acetyl group was hydrolyzed by mild NaOH treatment at 4°. The dimethoxytrityl group was removed from the dinucleotide d(Me_2O)TrT(pCH_3)T by benzenesulfonic acid treatment as described in Methods. The dinucleotide was identical to the dinucleotide synthesized by using thymidine as shown by tlc and its interactions with snake venom phosphodiesterase and T4 polynucleotide kinase. Further evidence in support of this will come from studies of the separated isomers.

In dideoxynucleoside methyl or phenylphosphonates, the phosphorous atom is asymmetric so that two stereoisomers exist. This was shown by thin-layer chromatography and enzymatic studies. Thin-layer chromatography of the dinucleosidephosphonates gave two UV-absorbing spots moving close to each other indicating the presence of two stereoisomers. Separation, isolation, and identification of these two stereoisomers is presently under investigation.

No hydrolysis of 5'→3'-dithymidine methylphosphonate by spleen phosphodiesterase was detected after incubation for 24 hr at 37° using 50 times the amount of enzyme necessary to hydrolyze T-T in one hr. However, slow hydrolysis by snake venom phosphodiesterase was detected in the presence of excess enzyme. Only 50% of the methylphosphonate was hydrolyzed in 24 hr using 42 times the amount of enzyme needed to hydrolyze the same amount of T-T in 10 min. After 24 hr, no further hydrolysis was observed even after the addition of the same amount of enzyme initially used. Under identical conditions of enzyme excess, the phenylphosphonate analog of T-T was also hydrolyzed to 50% but at a much slower rate. These observations suggest that the snake venom phosphodiesterase may bind to and cleave only one stereoisomer. It is possible, however, that both stereoisomers bind to the enzyme equally well and that only one isomer interacts properly with the functional groups of the active site and can be cleaved. These alternatives are currently under study. Assuming that both T-T and T(pCH_3)T are hydrolyzed by the same active site, the slow hydrolysis of the preferred nonionic isomer may be due to the slow dissociation of the enzyme-product complex compared to T-T. Presumably the rapid hydrolysis of T-T is due to the fast dissociation of the enzyme-product complex. This phenomenon may be due to the electrostatic repulsion between the newly formed phosphomonoester and the negatively charged functional groups of the active site of the enzyme. In the case of nonionic analogs, this repulsive force is considerably lowered and thus reduces the rate of hydrolysis. If this is the case,

then the rate of hydrolysis of T-T and the oligonucleotides should be affected by the presence of these nonionic analogs. In fact the rate of hydrolysis of T-T was greatly reduced in the presence of equal amounts of $T(pCH_3)T$ (data not shown). In an analogous experiment, the rate of hydrolysis of 5'-(^{32}P)-labeled oligo$(dT)_{15}$ (50 pmole) was markedly reduced in the presence of 5 nmol of $T(pCH_3)T$, while the addition of 5 nmol of T-T had very little effect upon the hydrolysis of the 5'-labeled oligo$(dT)_{15}$ as illustrated in Figure 2. These observations suggest that nonionic analogs may compete with the ionic substrates in a snake venom diesterase-catalyzed reaction. These results are preliminary and require further studies to determine the nature and mechanism of inhibition by these nonionic analogs.

Mixed ionic-nonionic analogs were also substrates for snake venom and spleen phosphodiesterases. For example, the tetranucleotide $T\text{-}T(pCH_3)T\text{-}T$ was rapidly hydrolyzed to completion by snake venom phosphodiesterase to trinucleotide $T\text{-}T(pCH_3)T$ (the trinucleotide had the same mobility on tlc as the chemically synthesized marker) as judged by tlc. However, further hydrolysis of the trinucleotide to thymidine was very slow and required 24 hr for 50% completion. Similarly the tetranucleotide $T\text{-}T(pCH_3)T\text{-}T$ was rapidly hydrolyzed to $T(pCH_3)T\text{-}T$ by spleen phosphodiesterase as judged by tlc. These observations suggest that the presence of a methylphosphonate

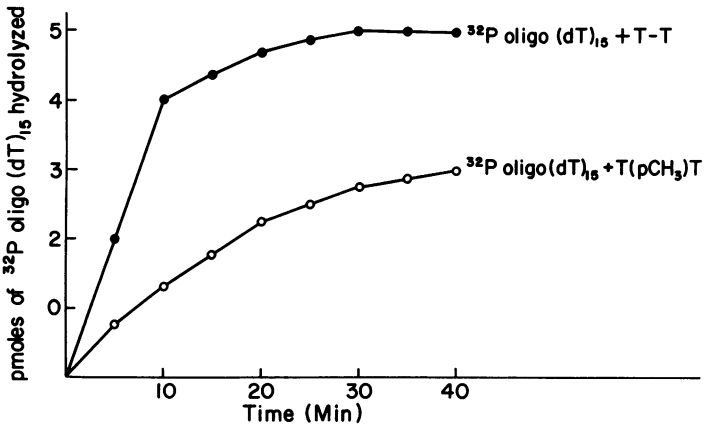

FIGURE 2. Rate of hydrolysis of 5'-(^{32}P)-labeled oligo$(dT)_{15}$ by snake venom phosphodiesterase in the presence of T-T or $T(pCH_3)T$. Hydrolysis of ^{32}P-oligo$(dT)_{15}$ (100 nM) in the presence of T-T (100 μM) is shown by (●—●) and the hydrolysis of ^{32}P-oligo$(dT)_{15}$ (100 nM) in the presence of $T(pCH_3)T$ (100 μM) is shown by (o—o). Experimental details are described in Methods.

next to the phosphodiester does not affect the exonuclease activity.

In order to show that the nonionic analogs may also be substrates for other nucleic acid enzymes, we investigated the phosphorylation of $T(pCH_3)T$ and $T(pC_6H_5)T$ by T4-polynucleotide kinase in the presence of [γ-^{32}P]ATP. Our results showed that both analogs were phosphorylated and that the phosphorylation was several times slower than that of T-T. In each case the phosphorylated products were analyzed by PEI-cellulose thin layer chromatography which showed the presence of two radioactive spots as illustrated in Figure 3. Presumably the two radiolabeled spots correspond to the two stereoisomers. As may be seen in Figure 3, the slower migrating isomer of $^{32}pT(pCH_3)T$ contains more radioactivity than the faster moving isomer,

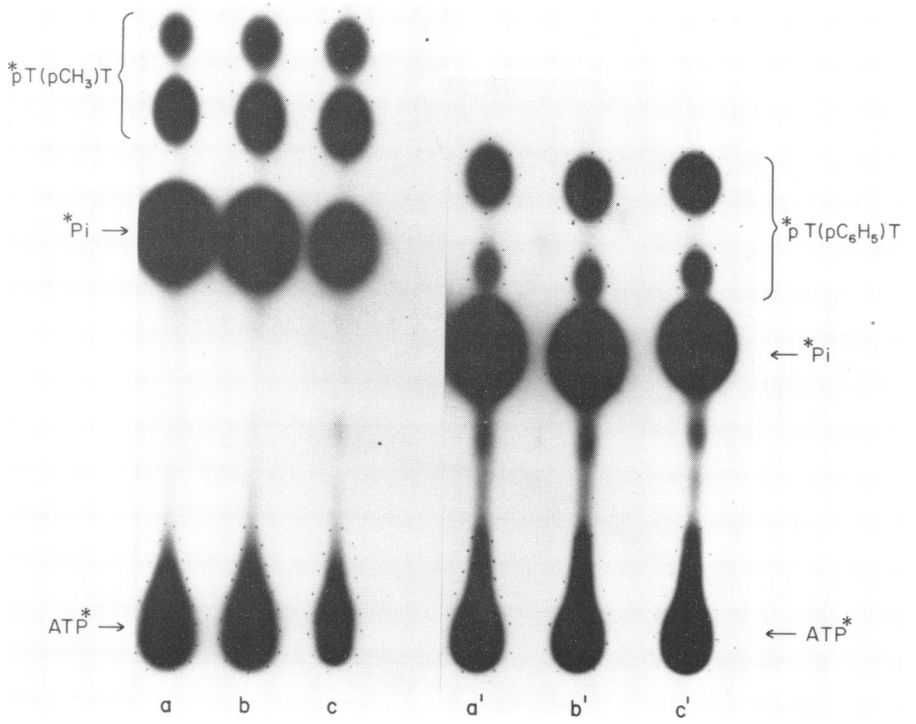

FIGURE 3. Autoradiogram of the phosphorylation of $T(pCH_3)T$ and $T(pC_6H_5)T$ by T4-polynucleotide kinase and [γ-^{32}P]ATP. Conditions of phosphorylations are described in Methods. Phosphorylation of $T(pCH_3)T$ is shown on the left. a, b, and c are 2, 4, and 8 hr time intervals and a', b', and c' are 4, 8, and 12 hr time intervals.

suggesting that one isomer is preferentially phosphorylated by the T4-polynucleotide kinase. In the case of $T(pC_6H_5)T$, the faster moving isomer contains more radioactivity than the slower migrating isomer. Phosphorylation of the methylphosphonate analog was several times faster than that of the phenylphosphonate analog. The difference in the rate of phosphorylation of the two analogs may be due to the bulky phenyl group in the phenylphosphonate analog.

Enzymatic studies on the nonionic analogs described above clearly show that they are ideal substrates for studying the mechanism of nucleic acid enzymes. Separation and characterization of the stereoisomers of these nonionic analogs will aid in precise understanding of their interaction with the various enzymes. Further studies along these lines are in progress and will be reported elsewhere.

CONCLUDING REMARKS

Availability of nonionic oligonucleotides as analogs of nucleic acids provides an opportunity to study the mechanism of action of nucleic acid enzymes. For the first time it is possible to synthesize pseudosubstrates for endonucleases which can be used to study DNA-protein complexes by physical and biochemical methods.

EXPERIMENTAL

Pyridine, tetrahydrofuran, and triethylamine were distilled and stored as previously described[8]. Thionyl chloride was refluxed over linseed oil for 10 hr and then distilled at atmospheric pressure in the presence of dry nitrogen stream. Methyl iodide was purified by filtration through a small plug of silica gel. Phenylphosphonodichloridate was purchased from Aldrich and was vacuum distilled before use. All condensation reactions were carried out by repeated evaporation *in vacuo* of a solution of component or components with added dry pyridine (at least four times) and the reaction flask was opened into a dry box which had a positive pressure of dry air[10].

All four nucleosides were obtained from Calbiochem. 1,2,4-Triazole, 1-(H)-tetrazole, and dimethyoxytrityl chloride were obtained from Aldrich. DEAE-cellulose plates were from Anal Tech. PEI-cellulose plates were from Brinkmann and were washed with 1.2 M pyridinium formate (pH 3.5) followed by water before use. $Oligo(dT)_{15}$ was purchased from P.L. Biochemicals.

Snake venom and spleen phosphodiesterases were purchased from Worthington Biochemicals. T4-polynucleotide kinase was isolated by a modified procedure of Panet et al.[11] and was completely free of contaminating exonucleases. [γ-^{32}P]ATP of high specific activity (1400 ci/mmol) was prepared by the procedure of Maxam and Gilbert[12] and was used without further purification. The dimethoxytritylated-amino protected nucleosides were prepared according to the published procedure[13]. Merck silica gel 60 F_{254} were used for thin layer chromatography in the following solvent systems: A, $CHCl_3$-EtOH (9.5:0.5; v/v); B, $CHCl_3$-EtOH (9:1; v/v); C, $CHCl_3$-EtOH (8.2; v/v); D, CH_3CN-H_2O (9:1; v/v); E, CH_3CN-H_2O (8.5:1.5; v/v); F, isopropanol-aqueous NH_3(d0.88)-H_2O (7:1:2; v/v).

Preparation of dimethyl methylphosphonate. This compound was prepared by the procedure described by Kosolopoff[14] except that the product was twice vacuum distilled (b.p. 80° at 25 mm) before use.

Preparation of methylphosphonodichloridate. To a stirred solution of thionyl chloride (150 ml) and pyridine (1.5 ml), dimethyl methylphosphonate (25 ml) was added slowly over a period of 2 hr. After the addition was complete, the solution was slowly brought to reflux and the refluxing was continued for a total of 24 hr. Excess thionyl chloride was removed by distillation at atmospheric pressure and the product was distilled twice under reduced pressure (b.p. 65° at 25 mm). Colorless low melting (m.p. 36°) white solid was isolated in 80% yield. Anal. Calcd. for CH_3POCl_2: Cl, 53.38. Found: Cl, 53.13.

Synthesis of the dinucleotide, d(Me_2O)TrT(pCH_3)T.

Method A

An anhydrous pyridine solution (10 ml) of 5'-O-dimethoxytritylthymidine (545 mg, 1.0 mmol) was added to a stirred pyridine solution (10 ml) of methylphosphonodichloridate (134 mg, 1.0 mmol). After 4 hr stirring at room temperature, the mixture was analyzed by silica gel tlc in solvents A (Rf 0.0) and B (Rf 0.18) which showed quantitative reaction. The second step of phosphorylation involved addition of an anhydrous pyridine solution (10 ml) of thymidine (243 mg, 1.0 mmol). The total reaction mixture was concentrated to 5.0 ml and stirred for 12 hr in the absence of moisture. Analysis of the reaction mixture by silica gel tlc in solvents B (Rf 0.66) and D (Rf 0.80) showed about 40% reaction. The reaction was terminated by cooling in dry ice-ethanol and adding $NaHCO_3$ (1 M, 20 ml) followed by $CHCl_3$ (100 ml). The oligonucleoside methylphosphonate was extracted in $CHCl_3$ (2 x 50 ml), concentrated under vacuum, dissolved in $CHCl_3$ (5 ml) contain-

ining 1% pyridine, and then chromatographed on silica gel column (2 x 30 cm) pre-equilibrated with $CHCl_3$-1% pyridine. The column was developed under a pressure of 50 psi using a gradient of methanol (0-5%) in the same solvent and was monitored by tlc. The fractions containing the required product were pooled and concentrated in the presence of pyridine. The dinucleoside methylphosphonate (261 mg, 30%) was isolated from the solvent by precipitation with hexane.

Method B

To a solution of 1,2,4-triazole (105 mg, 1.5 mmol) and triethylamine (0.21 ml, 1.5 mmol) in anhydrous tetrahydrofuran (10 ml) was added methylphosphonodichloridate (80 mg, 0.6 mmol). The reaction mixture was stirred for 6 hr at room temperature and then filtered. To the filtrate was added a previously dried pyridine solution of 5'-O-dimethoxytritylthymidine (272 mg, 0.5 mmol) and the reaction solution was concentrated in vacuo to 5 ml. After 30 min at room temperature the reaction mixture was analyzed by silica gel tlc in solvent B (Rf 0.18) which showed quantitative phosphorylation. The second step of phosphorylation was carried out by adding to the above reaction mixture an anhydrous pyridine (10 ml) solution of benzenesulfonic acid (264 mg, 1.65 mmol) and triethylamine (0.23 ml, 1.65 mmol) followed by an anhydrous pyridine solution of thymidine (145 mg, 0.6 mmol). After 12 hr at room temperature, the reaction mixture was analyzed by silica gel tlc in solvents B (Rf 0.64) and D (Rf 0.83). The reaction mixture was terminated and worked up as described above. The dinucleoside methylphosphonate diester (430 mg, 50% yield) was isolated in homogeneous form, m.p. 157-159° C. λ_{max} (EtOH), 267 (ε 19,987). Anal. Calcd. for $C_{42}H_{47}N_4O_{13}P$: C, 59.50; H, 5.54; N, 5.61. Found: C, 59.38; H, 5.46; N, 6.71.

Method C

The first step of phosphorylation of 5'-O-dimethyoxytritylthymidine was on the same scale as described in Method B. After 30 min at room temperature, the phosphorylation was complete as judged by silica gel tlc. The second step of phosphorylation was carried out by addition of benzenesulfonyl tetrazole (0.6 mmol) followed by a pyridine solution (10 ml) of thymidine (145 mg, 0.6 mmol). The total reaction mixture was concentrated to 5 ml and kept at room temperature for 1 hr. Silica gel tlc of the reaction mixture in solvents B (Rf 0.66) and D (Rf 0.79) showed about 80% reaction. The reaction was terminated and worked up as described in Method A. The dithymidine methylphosphonate was isolated in homogeneous form in 65% yield. m.p. 157-159° C. Anal. Calcd. for $C_{42}H_{47}N_4O_{13}P$: C, 59.50;

H, 5.54; N, 6.61. Found: C, 59.41; H, 5.43; N. 6.71.

Synthesis of dinucleotide, d(Me_2O)TrT(pC_6H_5)T. To a solution of 1,2,4-triazole (210 mg, 3.0 mmol) and triethylamine (0.43 ml, 3 mmol) in anhydrous tetrahydrofuran (25 ml) was added phenylphosphonodichloridate (0.14 ml, 1 mmol). The reaction mixture was stirred for 8 hr at room temperature and then filtered. To the filtrate a solution of 5'-O-dimethoxytritylthymidine (544 mg, 1 mmol) in anhydrous pyridine (10 ml) was added and the reaction mixture was concentrated to 10 ml. After 2 hr at room temperature, the reaction mixture was analyzed by silica gel tlc in solvent A (Rf 0.01) which showed over 90% phosphorylation. The second step of phosphorylation involved the addition of benzenesulfonyl tetrazole (1.5 mmol) followed by a pyridine solution (10 ml) of thymidine (284 mg, 1 mmol). The reaction mixture was concentrated to 10 ml and kept at room temperature for 6 hr. Silica gel tlc of the reaction mixture in solvents B (Rf 0.63) and C (Rf 0.86) showed about 70% reaction. Termination and work up of the reaction mixture was as described in Method A. The reaction products were separated on a silica gel column (2 x 30 cm) using a gradient of methanol (0-5%). Fractions containing the required product were pooled and concentrated in the presence of pyridine. The dithymidine phenylphosphonate, d(Me_2O)TrT(pC_6H_5)T, was isolated (545 mg, 60% yield) from the solvent by precipitation with hexane. λ_{max} (EtOH), 269 (ε 21,100); m.p. 171-174° C. Anal. Calcd. for $C_{47}H_{49}N_4O_{13}P$: C, 62.04; H, 5.39; N, 6.16. Found: C, 61.97; H, 5.37; N, 6.03.

Synthesis of d(Me_2O)TrT(ClC_6H_4)T(pCH_3)T. To an anhydrous solution of 1,2,4-triazole (8.4 mg, 124 µmole) and triethylamine (17 µl, 120 µmol) in tetrahydrofuran (200 µl) was added methylphosphonodichloridate (6.4 mg, 48 µmol). After six hr stirring at room temperature the reaction mixture was filtered and the filtrate was treated with an anhydrous solution of d(Me_2O)TrT(ClC_6H_4)T (39.1 mg, 40 µmol) in pyridine (500 µl). The light yellow solution was concentrated to 100 µl and kept at room temperature for 30 minutes. Silica gel tlc of the reaction mixture showed complete phosphorylation. The second step involved an addition of benzenesulfonyl tetrazole (60 µmol) followed by a pyridine solution (200 µl) of thymidine (11.6 mg, 48 µmol). The reaction mixture was concentrated to 100 µl and kept at room temperature for 1 hr. Analysis of the reaction mixture by silica gel tlc showed that the trinucleotide, d(Me_2O)TrT(ClC_6H_4)T(pCH_3)T was formed. The reaction mixture was separated by preparative tlc developed in solvent D. The trinucleotide was eluted from the thin layer plate with solvent D containing 2% pyridine and was isolated in 50% yield (24 mg).

Synthesis of $d(Me_2O)TrT(ClC_6H_4)T(pCH_3)T(ClC_6H_4)T$. A mixture of 1,2,4-triazole (8.4 mg, 120 μmol) and triethylamine (17 μl, 120 μmol) in anhydrous tetrahydrofuran (400 μl) was treated with methylphosphonodichloridate (6.2 mg, 46 μmol). After 6 hr at room temperature, the reaction mixture was filtered and the filtrate was treated with an anhydrous solution of $d(Me_2O)TrT(ClC_6H_4)T$ (33 mg, 40 μmol) in pyridine (200 μl). The combined solution was concentrated to 150 μl and was kept at room temperature. After 30 minutes it was analyzed by silica gel tlc. The second step of phosphorylation involved the addition of benzenesulfonyl tetrazole (45 μmol) followed by a pyridine solution (200 μl) of $T(ClC_6H_4)T$ (32.4 mg, 48 μmol). The total reaction mixture was concentrated to 100 μl and kept at room temperature for 1 hr. Silica gel tlc of the reaction mixture in solvents B (Rf 0.22) and C (Rf 0.46) showed 85% reaction. Termination and work up of the reaction mixture was as described in Method A. The reaction products were separated by preparative tlc developed in solvent D. The tetranucleotide was eluted from the thin layer plate with solvent D containing 2% pyridine and was isolated in 62% yield (41 mg).

Synthesis of $T(pCH_3)T$. $d(Me_2O)TrT(pCH_3)T$ was dissolved in ice-cold $CHCl_3$-n-butanol (9:1, v/v; 2 ml) and was treated with an equal volume of a 2% solution of benzenesulfonic acid in the same solvent. After 2 minutes at 4° C, the reaction was terminated by adding ice-cold $NaHCO_3$ (0.6 ml, 1 M) and the organic phase was recovered and evaporated under vacuo. The product (31 mg) was isolated from pyridine solution by precipitation into petroleum ether. λ_{max} (EtOH) 267 (ε 19,931). Anal. Calcd. for $C_{21}H_{29}N_4O_{11}P$: C, 46.23; H, 5.32; N, 10.27. Found: C, 46.20; H, 5.36; N, 10.33.

Synthesis of $T\text{-}T(pCH_3)T$. The trinucleotide, $d(Me_2O)TrT(ClC_6H_4)T(pCH_3)T$ (10 mg), was treated with concentrated ammonia (d 0.88) in pyridine (2 ml, 2:1; v/v) at 36° for 12 hr. Silica gel tlc in solvents D (Rf 0.31) and E (Rf 0.46) showed only one trityl positive spot. Ammonia and pyridine were removed by concentration in vacuo and the residue was treated with pyridine-acetic acid-H_2O (2 ml, 3:14:1; v/v, pH 3.5) for 90 minutes at room temperature. The solution was concentrated to dryness in vacuo and the residue was dissolved in H_2O (1 ml) and extracted with ether (2 x 3 ml). The aqueous layer was evaporated to dryness and dissolved in 1.0 ml of 5 mM Tris HCl (pH 7.5) and stored frozen.

Synthesis of $T\text{-}T(pCH_3)T\text{-}T$. The tetranucleotide, $d(Me_2O)TrT(ClC_6H_4)T\text{-}(pCH_3)T(ClC_6H_4)T$, was treated with concentrated ammonia followed by pyridinium acetate buffer pH 3.5 as described above for the trinucleotide.

Synthesis of $T(pC_6H_5)T$. For removal of dimethoxytrityl group from $d(Me_2O)TrT(pC_6H_5)T$, the procedure described for the synthesis of $T(pCH_3)T$ was used. After usual work up, the isolated product was homogeneous as judged by tlc in solvents D (Rf 0.82) and C (Rf 0.63). The product was isolated directly from the solvent by precipitation into hexane. λ_{max}(EtOH) 269 (ε 21,131). Anal. Calcd. for $C_{26}H_{31}N_4O_{11}P$: C, 51.40; H, 5.10; N, 9.22. Found: C, 51.32; H, 4.96; N, 9.63.

Enzyme Reactions

Snake venom phosphodiesterase-catalyzed hydrolysis of $T(pCH_3)T$ and $T(pC_6H_5)T$. Dithymidine methylphosphonate (5.76 OD_{267}, 300 nmol) was dissolved in 1 ml buffer (10 mM Tris HCl pH 8.0, 5 mM sodium phosphate, and 2 mM $MgCl_2$). Snake venom phosphodiesterase (1.5 U; this amount of enzyme is sufficient to completely hydrolyze 12 μmol of T-T at 37° in 2 hr) was added and the reaction was incubated at 37°. Aliquots of 100 μl were withdrawn at an interval of 2 hr and spotted on Whatman paper, and the paper was developed in solvent F. UV-absorbing spots were eluted from the paper with 1% aqueous NH_3 in 50% aqueous ethanol and their UV absorbance was measured.

For the hydrolysis of dithymidine phenylphosphonate, the procedure and the quantities of various components were the same as described above.

Hydrolysis of 5'-[^{32}P]labeled oligo$(dT)_{15}$ + T-T by snake venom phosphodiesterase in the presence and absence of $T(pCH_3)T$. 5'-[^{32}P]-labeled oligo$(dT)_{15}$ (50 pmol, 1.1 x 10 cpm/pmol) and $T(pCH_3)T$ (5 nmol, 0.01 OD_{267}) was dissolved in 40 μl of buffer (10 mM Tris HCl pH 8.0, 5 mM sodium phosphate, and 2 mM $MgCl_2$) and treated with snake venom phosphodiesterase (10 μl of 1.0 mg/ml stock solution). The reaction mixture was incubated at room temperature and 5 μl aliquots were withdrawn at 5 minute intervals and applied to a DEAE-cellulose thin layer plate. The plate was developed in homomix-IV of Jay et al.[15] The radioactive spots were located by autoradiography. The radioactive band corresponding to oligo$(dT)_{15}$ was scraped and counted. Decrease in radioactivity with time provided a measure of oligo$(dT)_{15}$ hydrolyzed. Similarly in a parallel experiment $T(pCH_3)T$ was replaced by the same amount of T-T.

Phosphorylation of $T(pCH_3)T$ and $T(pC_6H_5)T$ by T4-polynucleotide kinase and [γ-^{32}P]ATP. The dinucleotides, $T(pCH_3)T$ or $T(pC_6H_5)T$ (200 pmol), and [γ-^{32}P]ATP (710 pmol, 2000 cpm/pmol of the ATP) were suspended in 10 μl of buffer (50 mM Tris HCl pH 9.0, 5 mM DTT, 2 μM spermidine, and 10 mM $MgCl_2$). The reaction mixture was heated at 95° for 2 minutes and then cooled to 37°.

The T4-polynucleotide kinase (1 μl, 1 unit/μl) was added and the reaction was incubated at 37°. Aliquots of 2 μl were withdrawn at intervals of 2, 4, and 12 hr in the case of $T(pCH_3)T$ and 4, 8, and 24 hr in the case of $T(pC_6H_5)T$. The aliquots were directly applied to prewashed PEI-cellulose thin layer plates and the plates were developed in 1.5 M pyridinium formate pH 3.5. Figure 3 shows the autoradiogram of the phosphorylated products.

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7 Abbreviations for synthetic oligonucleotides are as suggested by IUPAC-IUB, Biochemistry (1976) 9, 4022. The phosphodiester bond is represented by a hyphen; a phosphonodiester bond is represented as nucleoside (pCH_3) nucleoside and a phosphotriester bond is represented as suggested by Pless, R. and Ts'o, P.O.P. (1977) Biochemistry 16, 1239. TEAB, triethylammonium bicarbonate.

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