Solid-Phase Syntheses of Oligodeoxyribonucleoside Methylphosphonates†

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ABSTRACT: Oligodeoxyribonucleoside methylphosphonates of defined sequence of the type d-Np(N*p*)$_n$N, where n is 6–13, are readily prepared on insoluble polystyrene supports by use of protected 5′-(dimethoxytrityl)deoxyribonucleoside 3′-(methylphosphonic imidazolides) as synthetic intermediates. The imidazolides are prepared in situ by reaction of protected 5′-(dimethoxytrityl)deoxyribonucleoside with methylphosphonic bis(imidazolide) and can be stored in the reaction solution for up to 2 weeks at 4 °C with no loss in activity. The condensation reaction is accelerated by the presence of tetrazole, which appears to act as an acid catalyst. The half-life for dimer formation on the polystyrene support is 5 min, and the reaction is 95% complete after 60 min. Although similar kinetics are observed when controlled pore glass is used as the support, the extent of the reaction does not go beyond 78%, even after prolonged incubation. In order to simplify purification and sequence analysis of the oligomer, the 5′-terminal nucleoside unit is linked via a phosphodiester bond. This linkage may be introduced by either an *o*-chlorophenyl phosphotriester method or a cyanoethyl phosphoramidite method. The latter procedure simplifies the deprotection step, since the cyanoethyl group is readily cleaved by ethylenediamine, which also removes the base protecting groups and cleaves the oligomer from the support. The singly charged oligomers are easily purified by affinity chromatography on DEAE-cellulose. The chain lengths of the oligomers were confirmed after 5′-end labeling with polynucleotide kinase by partial hydrolysis of the methylphosphonate linkages with 1 M aqueous piperidine followed by polyacrylamide gel electrophoresis of the hydrolysate. The positions of purine and pyrimidine bases were confirmed by treatment of 5′-end labeled oligomers with acid and hydrazine, respectively. These experiments show that oligodeoxyribonucleoside methylphosphonates can be prepared and characterized by procedures analogous to those used to prepare oligodeoxyribonucleotides.

Recent reports from our laboratory have demonstrated that nonionic oligodeoxyribonucleoside methylphosphonates are capable of inhibiting mRNA translation in mammalian cells and certain bacterial cells (Miller et al., 1981, 1983a; Jayaraman et al., 1981; Blake et al., 1985). In addition, oligodeoxyribonucleoside methylphosphonates complementary to the splice junctions of SV40 and herpes simplex virus precursor mRNA have been shown to selectively inhibit virus protein synthesis in virus-infected cells (Miller et al., 1983a; Smith et al., 1986). These nonionic nucleic acid analogues contain a neutral methylphosphonate linkage that replaces the negatively charged phosphodiester internucleotide bond normally found in nucleic acids. The methylphosphonate linkage, which is resistant to hydrolysis by nucleases, enables the oligomer to be taken up intact by mammalian cells in culture and by a cell wall mutant of *Escherichia coli*.

In the course of our biochemical and cell culture experiments, we required a procedure that would allow efficient synthesis of oligomers of defined sequence up to 15 nucleoside units in length. In previous papers, we have described the syntheses of oligomers in solution and on a polystyrene support (Miller et al., 1983b,c). In this paper we describe a new solid-phase approach that makes use of protected nucleoside 3′-(methylphosphonic imidazolides) as synthetic intermediates. Using this method, it is possible to prepare 15-mers of defined sequence in sufficient quantities for biochemical experiments.

Materials and Methods

Protected deoxyribonucleosides, protected deoxyribonucleoside 3′-(cyanoethyl diisopropylphosphoramidites), and derivatized controlled pore glass supports were purchased from American Bionuclear Inc. 5′-Protected deoxyribonucleoside 3′-(*o*-chlorophenylphosphates) and 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT)[1] were purchased from Vega Biochemicals Inc. Methylphosphonic dichloride was purchased from Alfa Chemical Co. and distilled under vacuum prior to use. The derivatized polystyrene support was a product of ChemGenes Inc. All solvents were reagent- or HPLC-grade. Tetrahydrofuran and acetonitrile, which were used for washing the support, were dried over a 4-Å molecular sieves 24 h prior to use. Anhydrous acetonitrile was prepared by refluxing reagent-grade acetonitrile with anhydrous calcium chloride for 6 h. After distillation, the acetonitrile was further refluxed with calcium hydride and then distilled onto calcium hydride chips in a 10-mL V-vial and sealed with a Teflon-faced silicone septa. Anhydrous tetrahydrofuran was prepared by refluxing dry tetrahydrofuran with calcium hydride for 6 h followed by distillation into sealed 10-mL V-vials containing several chips of calcium hydride. Anhydrous pyridine was prepared as previously described (Miller et al., 1980). High-performance liquid chromatography was carried out on a Whatman ODS-3 column (0.9 × 25 cm) with a gradient of 0.5–30% or 0.5–35% acetonitrile in 0.1 M ammonium acetate buffer at pH 5.8. The flow rate was 2.5 mL/min, and the total volume of solvent in the gradient was 50 mL.

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[1] Abbreviations: d-Np(N*p*)$_n$N or d-Np*NNNN*, an oligodeoxyribonucleoside methylphosphonate that terminates with a phosphodiester linkage at its 5′-end; *p* or italicized bases, 3′–5′-linked methylphosphonate internucleoside bonds; ṗ, an (*o*-chlorophenyl)phosphoryl group; MSNT, 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; TEAA, triethylammonium acetate; CPG, controlled pore glass; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Preparation of Protected 5'-(Dimethoxytrityl)nucleoside 3'-(Methylphosphonic imidazolides), d-[(MeO)$_2$Tr]NpIm. The following operations were carried out in septum-sealed V-vials at room temperature. Solution transfers were made with gas-tight syringes. Base-protected 5'-(dimethoxytrityl)deoxyribonucleoside (0.396 mmol) and imidazole (1.84 mmol) were dried under vacuum overnight in the presence of phosphorus pentoxide. The imidazole was dissolved in 400 μL of anhydrous acetonitrile in a 3-mL V-vial containing a stirring bar. Liquified methylphosphonic dichloride (0.360 mmol; 33 μL) was added to this solution, and the solution was stirred for 60 min during which time a white precipitate of imidazolinium hydrochloride formed. The dried nucleoside was dissolved in 400 μL of anhydrous tetrahydrofuran, and this solution was added with stirring to the solution of methylphosphonic bis(imidazolide). Stirring was continued for 6 h at which time formation of d-[(MeO)$_2$Tr]N*p*Im is complete. A 5-μL aliquot was removed and added to 100 μL of 10% methanol in chloroform. This solution was subjected to silica gel TLC. Complete reaction was indicated by disappearance of the starting nucleoside and formation of d-[(MeO)$_2$Tr]N*p*Me, whose mobilities are given in Table I. The reaction solution was stored in a desiccator at 4 °C.

Kinetics of Dimer Formation on Insoluble Polymer Supports. The reactions were carried out on either 5'-(dimethoxytrityl)thymidylyl 1% cross-linked polystyrene or 5'-(dimethoxytrityl)thymidylyl-controlled pore glass support. The dimethoxytrityl group was removed by treatment with 1 M zinc bromide in methylene chloride/2-propanol (85:15 v/v) solution as described in Table II, and the support was then dried under vacuum for 30 min. Ten-milligram portions of the dried support were weighed out into 1-mL V-vials and dried overnight under vacuum in the presence of phosphorus pentoxide. A solution containing 20 mg of tetrazole dissolved in 200 μL of d-[(MeO)$_2$Tr]N*p*Im was prepared. Forty-microliters of this solution was added to each support. The supports were incubated for various lengths of time and were then washed with methylene chloride/2-propanol solution. The support was dried under vacuum and treated with 20 μL of 50% ethylenediamine/ethanol for 4 h at 37 °C. The solution was removed and evaporated to dryness, and the residue was coevaporated twice with 95% ethanol. The residue was treated with 50 μL of 80% acetic acid for 30 min, and the solvents were evaporated. The residue was dissolved in 50% ethanol solution and examined by reversed-phase HPLC. The conversion of thymidine to dinucleoside methylphosphonate, d-N*p*T, was determined from the chromatograms.

Preparation of Oligodeoxyribonucleoside Methylphosphonates. The reactions were carried out on 100 or 200 mg of 1% cross-linked polystyrene support derivatized with 5'-(dimethoxytrityl)deoxyribonucleoside in a 4-mL Bio-Rad Econo column fitted with a three-way Teflon valve and a No. 33 Suba seal rubber stopper (Aldrich Chemical Co.). Transfer of anhydrous solutions was made with a gas-tight syringe. The steps for each reaction cycle are given in Table II. For a 100 mg scale reaction, the coupling mixture contained 80 mg of dry tetrazole, dissolved in 400 μL of the d-[(MeO)$_2$Tr]N*p*Im solution.

The final coupling reaction was carried out with a solution containing 0.1 mmol of d-[(MeO)$_2$Tr]Np·Et$_3$NH and 0.25 mmol of MSNT dissolved in 410 μL of anhydrous pyridine. The support was washed with eight 1-mL portions of methylene chloride/isopropyl alcohol (85:15 v/v) and three 1-mL portions of acetonitrile and then dried under vacuum.

Alternatively, the final coupling step was carried out with a solution containing 0.128 mmol of 5'-(dimethoxytrityl)-deoxynucleoside 3'-(cyanoethyl diisopropylphosphoramidite) and 0.286 mmol of tetrazole in 600 μL of anhydrous acetonitrile. In this case, after the coupling reaction the support was treated with a solution containing 0.1 M iodine in 1.2 mL of tetrahydrofuran, 0.4 mL of lutidine, and 0.4 mL of water for 5 min. The support was washed with six 1-mL portions of tetrahydrofuran/lutidine/water (3:1:1 v/v) and six 1-mL portions of tetrahydrofuran and then dried under vacuum.

Oligomers that terminated with an *o*-chlorophenyl phosphotriester internucleotide bond were first treated with 1 mL of 0.017 M tetra-*n*-butylammonium fluoride solution in tetrahydrofuran/water/pyridine (8:1:1 v/v) for 40 h to remove the *o*-chlorophenyl protecting group. The support was washed with ten 2-mL portions of tetrahydrofuran and then dried under vacuum. The remaining deprotection procedure was then the same for oligomers that had been prepared by the phosphotriester or the phosphite method to add the 5'-terminal nucleotide unit. The support was incubated with 200 μL of ethylenediamine/ethanol (1:1 v/v) solution for 7 h and then washed with two 1-mL portions of pyridine/ethanol (1:1 v/v). The solvent was evaporated, and the residue was coevaporated with 0.5 mL of 95% ethanol 5 times. The support was further washed with three 1-mL portions of pyridine/water (1:1 v/v) and three 1-mL portions of pyridine, and these washings were added to the residue obtained above. After evaporation of solvents, the residue was treated with 1 mL of 80% acetic acid for 60 min. The solvents were removed by evaporation, and the residue was dissolved in 20 mL of 25% ethanol/water. This solution was passed through a DEAE-cellulose column (2.5 × 8 cm) in the bicarbonate form. The column was washed with water to remove all noncharged UV-absorbing material. The d-Np(N*p*)$_n$N was then eluted from the column with 0.15 M ammonium bicarbonate. The buffer was removed by repeated evaporation with water, and the oligomer was recovered by lyophilization from water.

Characterization of Oligodeoxyribonucleoside Methylphosphonates. The 5'-OH of d-Np(N*p*)$_n$N was phosphorylated with [γ-^{32}P]ATP, and the resulting d-[^{32}P]pNp(N*p*)$_n$N was purified by polyacrylamide gel electrophoresis as previously described (Murakami et al., 1985). The chain length of d-[^{32}P]pNp(N*p*)$_n$N was confirmed by partially hydrolyzing the oligomer with 1 M aqueous piperidine at 37 °C for 20 min for the 8-mers and 10 and 20 min for the 15-mers. The digest was then subjected to electrophoresis for 120 min at 800 V on a 32-cm 10% polyacrylamide gel that contained 7 M urea. The gel running buffer contained 89 mM Tris, 89 mM boric acid, and 10 mM EDTA, pH 8.3 (Maniatis et al., 1982).

The positions of purine and pyrimidine bases were determined by treating 0.2 pmol of 5'-end labeled oligomer with 6 μL of 2 M hydrochloric acid for 5, 10, or 20 min or 6 μL of 90% hydrazine hydrate for 20 min at 37 °C (Murakami et al., 1985). The reactions were placed on ice, and the hydrochloric acid was neutralized by addition of 2 M ammonium hydroxide. The resulting oligomer fragments were desalted on a SEP-PAK column (Lo et al., 1984). After lyophilization, each reaction mixture was dissolved in 5 μL of loading buffer containing 90% formamide and 0.04% bromophenol blue in gel running buffer and electrophoresed on a 32 × 0.75 cm 15% polyacrylamide/7 M urea gel at 800 V for 1.75 h. The gel was then dried and autoradiographed at −80 °C.

Results and Discussion

A number of methods have been developed to synthesize oligodeoxyribonucleoside methylphosphonates either in solution

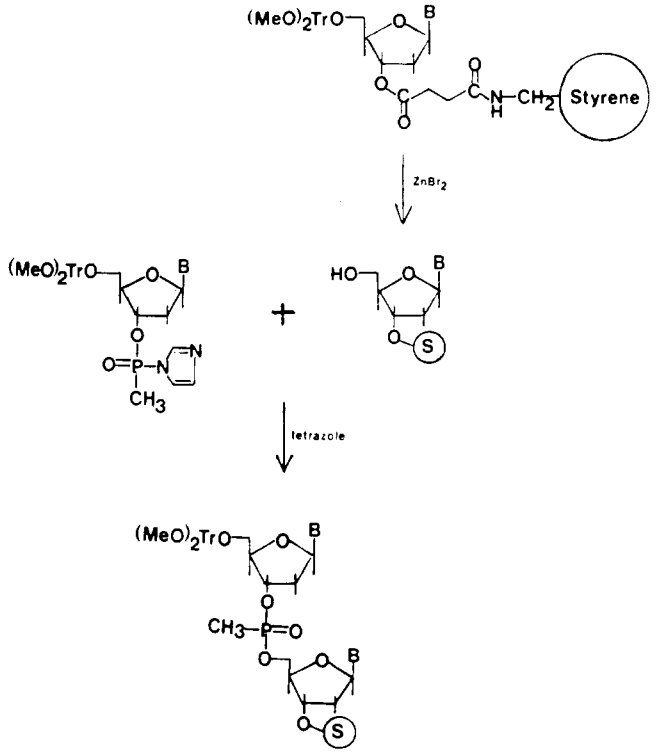

FIGURE 1: Synthesis of oligodeoxyribonucleoside methylphosphonates on a polystyrene support.

or on insoluble polymer supports. Agarwal and Riftina (1979) and our laboratory have reported the use of methylphosphonic dichloride (Miller et al., 1983b) as a combined phosphonylating/condensing reagent for preparation of oligomers of up to four nucleoside units in length. Longer oligomers have been prepared with 5′-protected nucleoside 3′-(methylphosphonic acid) as the reaction intermediate in conjunction with (mesitylenesulfonyl)-3-nitrotriazole on a 1% cross-linked polystyrene support (Miller et al., 1983c). This procedure is analogous to that used to prepare oligonucleotide phosphotriesters (Ito et al., 1982), although the rates of condensation are slower than those for the triester reactions. Although oligomers up to 12 nucleoside units were obtained by this method, low yields were usually encountered with oligomers having two or more deoxyguanosine residues in the sequence. Recent reports from Koester's laboratory show that oligonucleoside methylphosphonates can be synthesized on controlled pore glass supports with 5′-protected nucleoside 3′-(methylchlorophosphines) (Sinha et al., 1983). In analogy to the phosphite methodology used to synthesize oligonucleotides (Letsinger & Lunsford, 1975; Ogilvie & Nemer, 1980; Matteucci & Caruthers, 1981), the coupling reactions are quite fast and proceed in high yields. However, the reagents are difficult to handle because they are air- and moisture-sensitive, and there is some indication that isomerization of the internucleoside linkage can occur during the coupling step (Jager & Engels, 1984).

Our studies with 5′-protected nucleoside 3′-(methylphosphonic chlorides), d-[$(MeO)_2$Tr]N*p*Cl, suggested that similar reagents having a leaving group that could be activated just prior to the coupling step might be very suitable intermediates for solid-support syntheses. Experiments with 5′-protected nucleoside 3′-(methylphosphonic imidazolides), d-[$(MeO)_2$Tr]N*p*Im, whose structure is shown in Figure 1, suggest that these intermediates meet this requirement. Methylphosphonic bis(imidazolide) is prepared by reacting methylphosphonic dichloride with excess imidazole in anhydrous acetonitrile. Further reaction with 1.1 equiv of base-protected 5′-(dimethoxytrityl)deoxynucleoside yields the desired 5′-(dimethoxytrityl)deoxynucleoside 3′-(methylphosphonic imidazolide), d-[$(MeO)_2$Tr]N*p*Im, and the 3′–3′ dimer, d-[$(MeO)_2$Tr]$N^{3'}p^{3'}$N[$(MeO)_2$Tr]. The latter is formed by reaction of excess d-[$(MeO)_2$Tr]N with d-[$(MeO)_2$Tr]-N*p*Im. Since the 3′–3′ dimer is inert, no attempts were made to remove it from the reaction mixture.

Table I: Chromatographic Mobilities and ^{31}P Chemical Shifts of 5′-Protected Deoxyribonucleoside 3′-(Methylphosphonic imidazolides)

protected deoxyribonucleoside 3′-(methylphosphonic imidazolide)	R_f[a]	δ (ppm)[b]
d-[$(MeO)_2$Tr]T*p*Im	0.53; 0.57	−1.3899; −0.6269
d-[$(MeO)_2$Tr]bzC*p*Im	0.54; 0.56	−1.0774; −0.6744
d-[$(MeO)_2$Tr]bzA*p*Im	0.51; 0.54	−1.6880; −1.1654
d-[$(MeO)_2$Tr]ibuG*p*Im	0.43; 0.46	−1.3061; −0.6631

[a] Mobility of the methyl ester d-[$(MeO)_2$Tr]N*p*Me on silica gel TLC with 10% methanol in chloroform as the solvent. [b] Chemical shifts were measured in anhydrous tetrahydrofuran/acetonitrile (1:1 v/v) and are relative to the phosphorus resonance of dimethyl methylphosphonate observed in the same solvent system.

Formation of d-[$(MeO)_2$Tr]N*p*Im takes approximately 6 h at room temperature. The completeness of the reaction and the activity of the product are readily determined by TLC. When an aliquot of the reaction mixture is added to aqueous pyridine, 90% of the material remains at the origin of the chromatogram and corresponds to the methylphosphonic acid derivative, d-[$(MeO)_2$Tr]N*p*. The remaining trityl-containing material has an R_f value between 0.5 and 0.6 in 10% methanol–chloroform solution and corresponds to the 3′–3′ dimer. If the d-[$(MeO)_2$Tr]N*p*Im is added to 10% methanol–chloroform, the methyl ester d-[$(MeO)_2$Tr]N*p*Me is formed, which has the TLC mobilities shown in Table I. Each methyl ester appears as two close-moving spots corresponding to the two diastereoisomers. Little or no trityl-containing material is observed at the origin of the chromatogram. The formation of d-[$(MeO)_2$Tr]N*p*Im can also be followed by ^{31}P NMR. The proton-decoupled phosphorus resonance appears as two peaks corresponding to the two diastereoisomers with chemical shifts characteristic of the nucleoside base as shown in Table I.

Although attempts to isolate d-[$(MeO)_2$Tr]N*p*Im by precipitation from the reaction mixture were not successful, this proved to be unnecessary since the reagent can be stored in the reaction solution at 4 °C for at least 2 weeks with no apparent loss of activity. This offers a distinct advantage over d-[$(MeO)_2$Tr]N*p*Cl, which cannot be stored and must be prepared just prior to use (Miller et al., 1983b). Unlike previously used 5′-protected nucleoside 3′-methylphosphonates, d-[$(MeO)_2$Tr]N*p*, whose synthesis and purification involve several steps (Miller et al., 1983c), the nucleoside 3′-(methylphosphonic imidazolides) are prepared by a simple one-pot, two-step procedure. Thus, it is possible to prepare all four reagents on the same day and then use them as required for oligomer synthesis on the support.

The rates of internucleotide bond formation on a 1% cross-linked polystyrene support and on a controlled pore glass support are shown in Figure 2. When d-[$(MeO)_2$Tr]bzA*p*Im was reacted with the thymidylylpolystyrene support, the reaction required 3 h to reach 90% completion. The rate of the coupling reaction is accelerated approximately 6-fold by addition of tetrazole. Similar kinetics were observed for the other three nucleoside imidazolides. Addition of triethylamine to the reaction mixture causes the reaction rate to return to that

Table II: Reaction Cycle for Preparation of Oligodeoxyribonucleoside Methylphosphonates[a]

step	reagent	amount	time (min)
detritylation	1 M $ZnBr_2$ in methylene chloride/2-propanol[b]	4 × 1 mL 2 × 2 mL	2, each (C,T) 2, each (G,A)
wash	methylene chloride/2-propanol[b]	3 × 1 mL	5, last wash
wash	0.5 M TEAA in dimethylformamide	3 × 1 mL	
wash	acetonitrile	6 × 1 mL	
dry	vacuum pump		30
condensation	coupling mixture	400 μL[c]	60
wash	tetrahydrofuran	5 × 1 mL	
acetylation	capping solution[d]	2 × 1 mL	5, last wash
wash	methylene chloride/2-propanol[b]	6 × 1 mL	

[a] Reactions were carried out on 100 or 200 mg of polystyrene support at room temperature. [b] At 85:15 v/v. [c] Use 800 μL for 200 mg of support. [d] Contained 1 mL of acetic anhydride, 1 mL of anhydrous pyridine, and 10 mg of (dimethylamino)pyridine.

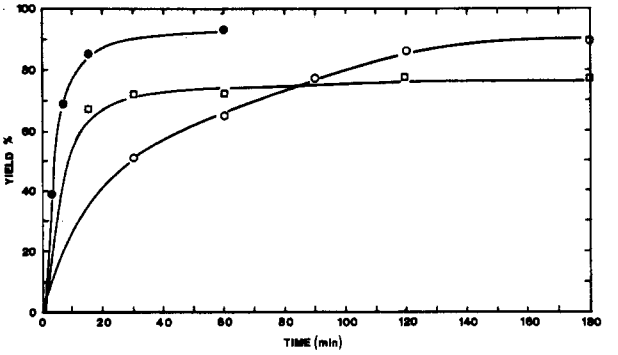

FIGURE 2: Kinetics of the coupling reaction between d-[$(MeO)_2$Tr]bzA*p*Im and thymidine-derivatized polystyrene in the absence (○) or presence (●) of tetrazole and thymidine-derivatized controlled pore glass in the presence (□) of tetrazole at 22 °C.

observed in the absence of tetrazole (data not shown). This observation suggests that tetrazole serves as an acid catalyst by protonating the imidazole group of d-[$(MeO)_2$Tr]N*p*Im. The protonated imidazole would be expected to be a much better leaving group than imidazole itself, and thus the rate of the coupling reaction is increased. Tetrazole could also act as a nucleophile to form the tetrazolide intermediate, d-[$(MeO)_2$Tr]N*p*tet. No change in the ^{31}P NMR spectrum of d-[$(MeO)_2$Tr]T*p*Im was observed, however, when tetrazole was added. This result indicates that either the tetrazolide is not formed as a distinct intermediate or, more unlikely, that d-[$(MeO)_2$Tr]T*p*Im and d-[$(MeO)_2$Tr]T*p*tet have identical chemical shifts.

Reaction of d-[$(MeO)_2$Tr]bzA*p*Im with thymidine bound to a controlled pore glass support occurred at approximately the same initial rate as with thymidine bound to the polystyrene support. However, the extent of the reaction was much lower. After 60 min, the yield of d-A*p*T was 74%, and this increased only slightly to 78% after 3 h. The reason for the failure of the reaction to go to completion is not understood. It does not appear to be due to side reactions, since dT and d-A*p*T were the only two nucleoside compounds observed on the support. The d-[$(MeO)_2$Tr]bzA*p*Im–tetrazole solution recovered from the support was still active after 3 h as evidenced by its conversion to d-[$(MeO)_2$Tr]bzA*p*Me when it was added to 10% methanol in chloroform. Addition of fresh d-[$(MeO)_2$Tr]bzA*p*Im–tetrazole solution to the support does not increase the yield.

A possible explanation would be steric hindrance by the support. However, the CPG support has a long, 20-Å linker arm that attaches the nucleoside to the support. This linkage should reduce the effects of steric hindrance to reaction between the nucleoside methylphosphonic imidazolide and the support-bound nucleoside. Low yields were also encountered on subsequent coupling steps between d-[$(MeO)_2$Tr]bzA*p*Im and the growing oligomer chain on the CPG support. Thus, for example, the average yield per coupling step for the hexamer d-A*p*A*p*A*p*A*p*A*p*T was 75%. This observation also suggests that steric hindrance is not the cause of the low yields, since the steric effects should be reduced as the 5′ end of the growing oligomer chain moves further away from the surface of the support.

Polystyrene cross-linked with divinylbenzene appears to be a suitable matrix for the synthesis of oligonucleoside methylphosphonates with d-[$(MeO)_2$Tr]N*p*Im. Table II shows the steps involved in one complete reaction cycle. The reactions were carried out in a polypropylene Econo column fitted with a three-way valve, which allowed the column to be attached directly to a vacuum pump with a cold trap. The top of the column was fitted with a tight fitting rubber septum cap. Detritylation was carried out with 1 M zinc bromide. We also tried 2% dichloroacetic acid in methylene chloride. However, this procedure resulted in lower yields in subsequent coupling steps even when the support was washed extensively after detritylation. The support was dried under vacuum prior to the coupling step. Drying could also be accomplished by washing the support with dry acetonitrile. In our hands, the former procedure proved to be simpler and more convenient for manual synthesis.

We have used this procedure to synthesize oligomers of defined sequence containing up to 15 nucleoside units. As shown in Table III, the average yield per condensation step as determined by trityl analysis is 88–92%, even for oligomers containing several deoxyguanosines. This represents a sig-

Table III: Syntheses of Oligodeoxyribonucleoside Methylphosphonates on a Polystyrene Support[a]

oligomer	average yield for phosphonate condensation step (%)[b]	isolated yield (%)	HPLC retention time (min)[c]
d-Tp*CCTCCTG*	89	15	13.8
d-Gp*AATCCTG*	90	21	14.1
	92[d]	24	14.1
d-Tp*GTTGGTC*	91	25	14.2
		18[e]	14.2
d-Ap*ACAGACAT*	88	12	15.8
d-Tp*AAATAAAAAAAATT*	91	4[e]	16.5
d-Cp*ATTTTTGGTTTCCA*	91	4	15.6

[a] The reactions were carried out on 100 mg of support, and the last nucleotide was added by the phosphotriester method unless otherwise noted. [b] Determined by analysis of the dimethoxytrityl group after each coupling step. [c] ODS-3 reversed-phase HPLC with a 50-mL gradient of 0.5–30% acetonitrile (8- and 9-mers) or 0.5–35% acetonitrile (15-mers) in 0.1 M ammonium acetate (pH 5.8) at a flow rate of 2.5 mL/min. [d] Reaction run on 200 mg of polystyrene support. [e] 5′-Terminal nucleotide added by phosphoramidite method.

nificant improvement over our previously described methods in which low yields were generally encountered for synthesis of oligomers containing one or more deoxyguanosine units (Miller et al., 1983b,c). The use of d-[$(MeO)_2$Tr]N*p*Im in these reactions avoids the necessity of using the condensing agent MSNT, which can lead to side reactions during the course of the synthesis. Because each reaction cycle takes approximately 2 h to complete, it is possible to synthesize a 15-mer on the support in 3.5 days.

Each oligomer terminates at the 5′ end with an internucleotide phosphodiester bond. This linkage was introduced by two different methods. In the first method, a suitably protected nucleoside 3′-(*o*-chlorophenyl phosphate), d-[$(MeO)_2$Tr]Np·Et_3NH, was coupled with the 5′-OH of the oligomer chain by use of MSNT as the condensing agent. This reaction results in the formation of an *o*-chlorophenyl phosphotriester internucleotide bond at the 5′ end of the oligomer. When treated with aqueous tetra-*n*-butylammonium fluoride, the phosphotriester group is converted to a phosphodiester linkage while the methylphosphonate linkages are not affected. The oligomer is then cleaved from the support, and the base-protecting groups are hydrolyzed in one step by treatment with 50% ethylenediamine in ethanol (Miller et al., 1983c).

The second method uses a protected nucleoside 3′-(cyanoethyl diisopropylphosphoramidite) to form a cyanoethyl phosphite internucleotide linkage. Upon oxidation with iodine/water, this linkage is converted to a β-cyanoethyl phosphotriester internucleotide bond. The β-cyanoethyl group was readily removed by the ethylenediamine treatment used to cleave the oligomer from the support. This approach avoids potential side reactions resulting from the use of MSNT and is faster than the other method because prolonged deblocking with fluoride ion is unnecessary. As shown in Table III, both methods gave comparable isolated yields of the oligomer d-Tp*GTTGGTC*. In this example, the support carrying the protected methylphosphonate oligomer was divided into two equal amounts. One portion was terminated by the phosphotriester procedure, and the other portion was terminated by the phosphoramidite method. The oligomers synthesized by these two different methods had identical mobilities on reversed-phase HPLC and gave identical piperidine hydrolysis ladders upon polyacrylamide gel electrophoresis.

We have observed that ethylenediamine reacts with support-bound *N*-benzoyldeoxycytidine to give two products: deoxycytidine and 4-(aminoethyl)deoxycytidine in 95% and 5% yields, respectively. The latter product presumably forms via direct nucleophilic attack by ethylenediamine at C-4 of *N*-benzoylcytosine. The extent of this side reaction can be followed indirectly by observing the formation of benzamide, which has a characteristic retention time of 8.5 min on the reversed-phase HPLC column when a 0–30% acetonitrile in a 0.1 M ammonium acetate gradient is used. When longer oligomers containing one or more protected C residues are deblocked with ethylenediamine, the extent of formation of 4-(aminoethyl)-C appears to be between 0% and 5% as monitored by the appearance of benzamide.

Oligomers having one or more 4-(aminoethyl)-C residues are readily separated from the desired oligomer by DEAE-cellulose chromatography. The desired product contains a single negative charge and is absorbed by the column, whereas the 4-aminoethyl-substituted oligomers have a net charge of 0 or a net positive charge since the 4-aminoethyl group is positively charged at pH 7. This procedure also removes shorter, noncharged methylphosphonate oliogmers from the desired oligomer.

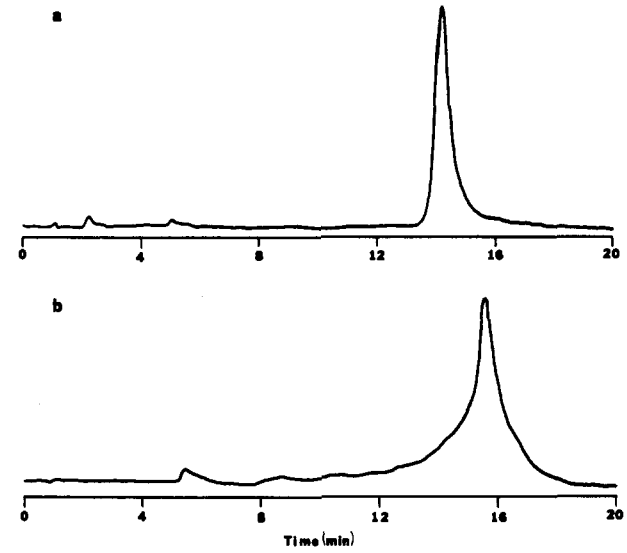

FIGURE 3: Reversed-phase HPLC chromatograms of (a) d-Tp*GTTGGTC* eluted with 0.5–30% acetonitrile in 0.1 M ammonium acetate, pH 5.8, at a flow rate of 2.5 mL/min and (b) d-Cp*ATTTTTGGTTTCCA* eluted with 0.5–35% acetonitrile in 0.1 M ammonium acetate, pH 5.8, at a flow rate of 2.5 mL/min.

During the course of the synthesis, each condensation gives a methylphosphonate linkage with an *R* and *S* configuration. Thus, an oligomer with *n* methylphosphonate linkages consists of 2^n diastereoisomers. The mobilities of these diastereoisomers are very similar since each purified oligomer gave a single peak on reversed-phase HPLC as shown in Figure 3 for the 8-mer d-Tp*GTTGGTC* and the 15-mer d-Cp*ATTTTTGGTTTCCA*. The retention times of the oligomers are given in Table III. As expected, the longer oligomers require higher concentrations of acetonitrile for elution from the column.

The oligomers were end-labeled with [^{32}P]phosphate by polynucleotide kinase (Murakami et al., 1985). The phosphorylated oligomers were partially hydrolyzed with 1 M aqueous piperidine, and their chain lengths were confirmed by polyacrylamide gel electrophoresis. The positions of purine and pyrimidine base residues in the oligomer were confirmed by treatment of the oligomer with either acid or hdyrazine. Figure 4 shows the characterization of the 15-mer d-[^{32}P]-pCp*ATTTTTGGTTTCCA* by these procedures. Treatment with piperidine gives two sets of end-labeled oligomers (lane 2). One set terminates with a 3′-hydroxyl group whereas the other set with a 3′-(methylphosphonate) group (Miller et al., 1983b; Murakami et al., 1985). The chain length of the oligomer is determined by counting either set of bands starting with the dimer, since the phosphodiester linkage of the oligomer is not cleaved under these conditions. Treatment of the oligomer with acid cleaves the 15-mer at G and A residues and gives a series of 5′-end labeled oligomers that terminate with a 3′-hydroxyl group (Miller et al., 1983b). This serves to locate the purine residues (lane 3). The C and T residues are located by treatment of the 15-mer with hydrazine (lane 4). Again, a series of 5′-end labeled oligomers are produced that terminate with a 3′-hydroxyl group. With the exception of the monomer produced by cleavage of the first A residue of d-[^{32}P]pCp*ATTTTTGGTTTCAA*, the oligomer bands in lanes 3 and 4 correspond to the 3′-hydroxyl-terminated set of the oligomer bands produced by piperidine hydrolysis of the 15-mer (lane 2).

The results presented in this paper show that oligodeoxyribonucleoside methylphosphonates of defined sequence may be prepared in an efficient manner on polystyrene supports. Combined with our previous paper on the characterization of

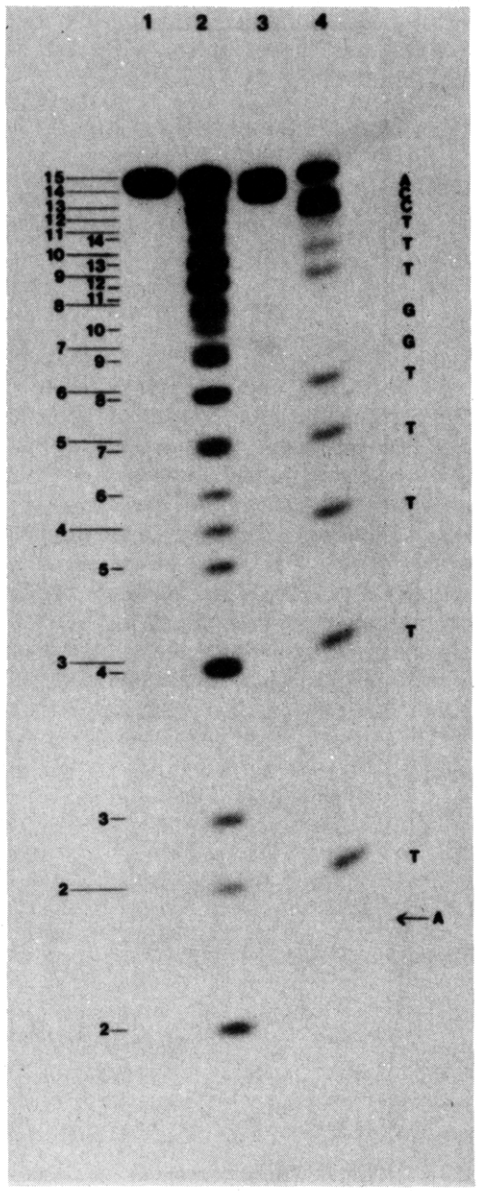

FIGURE 4: Characterization of d-[^{32}P]pCp*ATTTTTGGTTTCCA*. The autoradiogram shows (lane 1) 15-mer, (lane 2) 15-mer treated with 1 M aqueous piperidine at 37 °C, (lane 3) 15-mer treated with 2 M hydrochloric acid at 37 °C, and (lane 4) 15-mer treated with 90% hydrazine at 37 °C. The numbers at the far left show the chain length of oligomers terminating with a 3′-hydroxyl group while the next set of numbers shows the chain length of oligomers terminating with a 3′-(methylphosphonate) group. The positions of purine and pyrimidine residues are shown at the right. The arrow indicates the position of the band that is observed in longer exposures of the gel. This band corresponds to the monomer formed by cleavage of the first A residue in the oligomer.

oligodeoxyribonucleoside methylphosphonates, this paper demonstrates that these nucleic acid analogues may be prepared and handled by methods analogous to those used for oligodeoxyribonucleotides. Thus, these analogues should be readily accessible to laboratories interested in using these compounds in biochemical or cell biology experiments.

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Registry No. d-[$(MeO)_2$Tr]T*p*Im, 103562-78-3; d-[$(MeO)_2$Tr]-bzC*p*Im, 103562-79-4; d-[$(MeO)_2$Tr]bzA*p*Im, 103562-80-7; d-[$(MeO)_2$Tr]ibuG*p*Im, 103562-81-8; d-Tp*CCTCCTG*, 98014-37-0; d-Gp*AATCCTG*, 103590-61-0; d-Tp*GTTGGTC*, 103590-62-1; d-Ap*ACAGACAT*, 103590-63-2; d-Tp*AAATAAAAAAAATT*, 103691-68-5; d-Cp*ATTTTTGGTTTCCA*, 103691-67-4; d-[$(MeO)_2$Tr]T, 40615-39-2; d-[$(MeO)_2$Tr]bzC, 67219-55-0; d-[$(MeO)_2$Tr]bzA, 64325-78-6; d-[$(MeO)_2$Tr]ibuG, 68892-41-1; methylphosphonic bis(imidazolide), 99450-90-5.

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