Use of methylphosphonic dichloride for the synthesis of oligonucleoside methylphosphonates

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ABSTRACT

Methylphosphonic dichloride was used to prepare protected deoxyribonucleoside 3'-methylphosphonate β -cyanoethyl esters, $d-[(\text{MeO})_2\text{Tr}]\text{NpCNEt}$, and protected oligonucleoside methylphosphonates in solution. Reaction of $d-[(\text{MeO})_2\text{Tr}]\text{N}$ with methylphosphonic dichloride gives $d-[(\text{MeO})_2\text{Tr}]\text{NpCl}$. The phosphorylation and subsequent esterification or condensation reactions are each complete within 60 min. The products are readily purified by "flash chromatography" on silica gel columns. $d-[(\text{MeO})_2\text{Tr}]\text{NpCl}$, or its tetrazole derivative, $d-[(\text{MeO})_2\text{Tr}]\text{Nptet}$, were tested as intermediates for the synthesis of oligothymidine methylphosphonates on a silica gel polymer support. The average yield per coupling step was 76% and did not increase with addition of more $d-[(\text{MeO})_2\text{Tr}]\text{TpCl}$. The formation of (5'-5') linked thymidine dimers indicated that the thymidine monomers are clustered closely together on the support. When N is *ibuG*, the yield for the coupling step on the support is very low. This may be due to steric hindrance of the 3'-phosphonate group by the N-2 isobutryl protecting group.

INTRODUCTION

Oligonucleoside methylphosphonates, nonionic nucleic acid analogs which contain a 3'-5' methylphosphonate internucleoside linkage, have unique physical and biochemical properties (1,2). These properties include their resistance to hydrolysis by nucleases and their ability to be taken up by mammalian cells and certain bacterial cells in culture. We have previously described the synthesis and biochemical properties of methylphosphonate oligomers whose sequences are complementary to transfer RNA (3) and to the Shine-Dalgarno sequence of 16S rRNA (4). These oligomers were shown to be capable of specifically inhibiting tRNA aminoacylation and protein synthesis both in cell-free systems and in living cells by virtue of their ability to bind to complementary sequences of cellular nucleic acid. Thus, these analogs show considerable promise as sequence specific inhibitors of nucleic acid function within living cells.

A number of methods have been developed to prepare oligonucleotides containing methylphosphonate linkages. These include the use of

1) dicyclohexylcarbodiimide and arenesulfonyl chlorides or tetrazolides as condensing agents (1,3,4,5); 2) methylphosphonic dichloride in combination with arenesulfonyl tetrazolide as a phosphorylating and condensing agent (6); and 3) methylchlorophosphine as a bifunctional phosphinylating/condensing reagent (7,8). In this paper we describe the use of methylphosphonic dichloride as a bifunctional phosphorylating/condensing agent for the rapid preparation of fully protected di- and trinucleoside methylphosphonates in solution. The utility of this reagent for the synthesis of oligonucleoside methylphosphonates on an insoluble polymer support was also examined.

EXPERIMENTAL

Materials

Protected nucleosides, d-[(MeO)₂Tr]T, d-[(MeO)₂Tr]bzA, d-[(MeO)₂Tr]bzC, and d-[(MeO)₂Tr]ibuG, were purchased from P.L. Biochemicals and were used without further purification. Methylphosphonic dichloride was prepared by reaction of dimethylmethylphosphonate (K+K Laboratories) with thionyl chloride according to the method of Agarwal and Riftina (6) and was checked for purity by ³¹P NMR. Anhydrous pyridine was prepared as previously described (9). It was further refluxed with calcium hydride and distilled onto calcium hydride chips in 5 ml V-vials (Wheaton Scientific). The vials were sealed with Teflon-lined silicone septa and were stored in desiccators containing anhydrous calcium sulfate. Hydracrylonitrile was distilled under vacuum before use. Solvents for chromatography were reagent grade. Chloroform was passed through a column of alumina prior to use. Flash chromatography was carried out according to the procedure of Still *et al.* (10). Aminopropyl succinyl derivatized silica gel was prepared according to the procedure of Matteucci and Caruthers (11) and was esterified with d-[(MeO)₂Tr]T or d-[(MeO)₂Tr]ibuG *via* reaction with dicyclohexylcarbodiimide.

High performance liquid chromatography (HPLC) was carried out on C-18 reversed phase columns (Whatman ODS-2 or ODS-3). The columns were eluted with a 50 ml linear gradient of water to 25% acetonitrile at a flow rate of 2.5 ml/min. Silica gel thin layer chromatography (TLC) was carried out on EM precoated plastic backed plates.

Preparation of Fully Protected Deoxyribonucleoside 3'-methylphosphonate β -Cyanoethyl Esters, d-[(MeO)₂Tr]NpCNEt

A solution containing 6 mmol of methylphosphonic dichloride in 6.0 ml of anhydrous pyridine was added dropwise *via* a gas-tight syringe to a stirred solution of 2 mmol of d-[(MeO)₂Tr]N in 5.0 ml of anhydrous pyridine over a

period of 50 min at room temperature. When N is ibuG, the reaction was carried out at 40°C. The reaction mixture was stirred an additional 30 min and then 18 mmol of anhydrous hydracrylonitrile was added via a syringe. The reaction was stopped by addition of 2 ml of 50% aqueous pyridine and was further diluted with 50 ml of 5% sodium bicarbonate. The mixture was extracted with two 100 ml portions of chloroform and the chloroform extract was dried over anhydrous sodium sulfate. The product was purified by flash chromatography on a silica gel column (4.5 x 15 cm). The column was prepared with chloroform. The concentrate from the chloroform extract was applied and the column was eluted with 750 ml of 1% methanol in chloroform to ensure removal of bis(cyanoethyl)methylphosphonate. The column was then eluted with the appropriate percentage of methanol/chloroform (see Table I). Fractions containing the product were pooled and d-[(MeO)₂Tr]NpCNEt was isolated by evaporation of solvents. The TLC R_f values and isolated yields are given in Table I. The UV spectra of the methylphosphonate β-cyanoethyl esters were identical to those of corresponding protected nucleosides d-[(MeO)₂Tr]N. Preparation of Fully Protected Di- and Trinucleoside Methylphosphonates

The reaction conditions and yields are described in Table II. A general procedure is given below.

Method A. Both trityl-containing nucleoside and 5'-OH component were dried under vacuum in the presence of phosphorus pentoxide overnight. The reactions were carried out in 1 or 5 ml V-vials (Wheaton Scientific) fitted with Teflon-faced silicone septum caps. Solutions were transferred via gas-tight syringes (Hamilton). A 0.67 M solution of d-[(MeO)₂Tr]N (1 equivalent) in anhydrous pyridine was added dropwise to a 0.53 M solution of methylphosphonic dichloride (0.8 equivalents) in anhydrous pyridine over a period of 20 min. The solution was stirred an additional 30 min at room temperature. The reaction was checked for completeness by removing a 5 μl aliquot and quenching in tetrahydrofuran/triethylamine/water (90:5:5 v/v). This was chromatographed on a silica gel TLC slide using 10% methanol in chloroform as solvent. The starting material was absent and trityl-containing spots appeared at the origin, corresponding to d-[(MeO)₂Tr]Np, and at approximately 1.3 times the mobility of d-[(MeO)₂Tr]N, which corresponded to (3'-3') linked dimer. In some cases a precipitate of pyridinium hydrochloride formed and the reaction mixture was centrifuged. The supernatant was removed via a syringe and was then added to a 1.6 M solution of the 5'-OH component (0.5 equivalents) in anhydrous pyridine. The reaction mixture was stirred at room temperature for 1 hour. The reaction was stopped by adding 50% aqueous

pyridine or by diluting 10-fold with 5% sodium bicarbonate. The mixture was extracted twice with chloroform. The chloroform extract was dried over anhydrous sodium sulfate, concentrated and chromatographed on a silica gel column using either gravity flow or the "flash" column technique. Column fractions were monitored by silica gel TLC. The product was recovered by evaporation of the solvents. Yields were determined either spectrophotometrically or by weighing the product after precipitation from hexane. Silica gel TLC Rf values and UV spectral characteristics are given in Table III.

Method B. A 1 M solution of tetrazole/triethylamine was prepared by dissolving 0.94 mmol of tetrazole and 0.93 mmol of triethylamine in 0.81 ml of anhydrous pyridine. One equivalent of this solution was added to 1 equivalent of a 1 M solution of methylphosphonic dichloride in anhydrous pyridine with stirring. After 10 min the solution was centrifuged and the supernatant (0.04 equivalents of phosphorylating agent) was transferred to a 1 ml V-vial containing a stirring bar. d-[(MeO)₂Tr]N (0.33 M solution, 0.033 equivalents) in anhydrous pyridine was added dropwise with stirring. The reaction was complete after 30 min at room temperature as shown by TLC. For N = ibuG the reaction was run at 40°C. The solution was then added directly via a syringe to dried 5'-OH component and the solution was kept at room temperature (40°C for N = ibuG) for 60 min. The reaction mixture was worked up and the product was isolated and characterized as described under Method A above.

Synthesis of Oligonucleotides on a Silica Gel Polymer Support

The following general procedure was used. The yields from the various condensation reactions are given in Tables IV and V. The silica gel support (50 to 150 mg) esterified with the appropriate nucleoside (40 to 100 μmol nucleoside/g support) was treated with 3 ml of fresh 3% trichloroacetic acid in chloroform or 2% benzenesulfonic acid in acetonitrile in a 2 ml sintered glass funnel for 3 min at room temperature. The support was washed with four 1 ml portions of chloroform, four 1 ml portions of pyridine, and four 1 ml portions of diethyl ether. The support was transferred to a 1 ml V-vial fitted with a Teflon-faced silicone septum and a 22 gauge syringe needle attached to a Drierite-filled syringe was inserted into the septum. The support was dried by evaporation with two 200 μl portions of anhydrous pyridine by placing the vial in a vacuum bottle attached to an oil pump. After each evaporation, dry argon was admitted into the bottle. A 0.63 M solution of d-[(MeO)₂Tr]NpCl or d-[(MeO)₂Tr]NpCl/tet (5 to 15 fold excess over 5'-OH group on support) in anhydrous pyridine prepared as described in the previous section under Method A or B was added via a syringe and the reaction

mixture was kept for 1 hour at room temperature. When d-[(MeO)₂Tr]NpCl/tet was used additional tetrazole (1.3 equivalents) was added. The support was then transferred via a Pasteur pipet to a 2 ml sintered glass funnel and was washed with four 1 ml portions of pyridine, four 1 ml portions of acetonitrile, four 1 ml portions of chloroform and four 1 ml portions of ether. The support was dried under vacuum. A small portion of the support (0.2 to 0.5 mg) was weighed accurately on a microbalance and treated with 3% trichloroacetic acid in chloroform to release the support bound trityl group. The amount of dimethoxytrityl cation and hence the condensation yield was determined spectrophotometrically at 510 nm using a molar extinction coefficient of 7.1×10^4 (11). For extended synthesis the cycle was repeated using the next incoming nucleoside methylphosphonate.

The oligomer was removed from the support by treatment with 1 ml of ethanol/concentrated ammonium hydroxide (1:1, V/V) for 4 to 6 days at 4°C. The support was removed via filtration and the filtrate was evaporated. The residue was chromatographed on a C-18 reversed phase column (ODS-2 or ODS-3, Whatman, Inc.). Non-tritylated oligomers were eluted with 0 to 25% linear gradient of acetonitrile in water while the tritylated product was eluted with 50% acetonitrile in water. The trityl group was then removed by treatment with 80% acetic acid for 30 min at 37°C or 1 hour at room temperature. The deprotected oligomer was then purified by C-18 reversed phase HPLC using a 0 to 25% linear gradient of acetonitrile in water.

Characterization

The protected oligomers were characterized by their UV spectral properties which were compared with calculated values (see Table III and Ref. 12). The HPLC mobilities of deprotected oligomers on C-18 reversed phase columns were compared to those of authentic samples prepared in solution as previously described (1,3). For some of the deprotected dimers and trimers the ratio of nucleoside(tide) units was determined by base hydrolysis followed by analysis by reversed phase HPLC. A complete description of this procedure will be presented in a future publication. In all cases the HPLC mobilities were identical to those of authentic samples and the observed nucleoside(tide) ratios agreed with calculated values.

RESULTS

Fully protected nucleoside 3'-methylphosphonate β-cyanoethyl esters, d-[(MeO)₂Tr]NpCNEt, were prepared by a two-step procedure. Reaction of a 5'-O-dimethoxytrityl nucleoside with methylphosphonic dichloride in anhydrous

TABLE I. Preparation of Protected Deoxyribonucleoside 3'-Methylphosphonate

Nucleoside 3'-Methylphosphonate	Isolated Yield	% Methanol Required for Elution (a)	Rf Silica Gel TLC (10% MeOH in CHCl ₃)
d-[(MeO) ₂ Tr]TpCNET	70%	9%	0.41
d-[(MeO) ₂ Tr]bzApCNET	64%	7%	0.43
d-[(MeO) ₂ Tr]bzCpCNET	74%	7%	0.42
d-[(MeO) ₂ Tr]ibuGpCNET	52%	10%	0.46

(a) Percentage of methanol in chloroform used to elute nucleoside 3'-methylphosphonate from a silica gel column.

pyridine gave the protected nucleoside 3'-methylphosphonic chloride, d-[(MeO)₂Tr]NpCl. This reaction was complete within 60 min at room temperature or at 40° when the nucleoside was d-[(MeO)₂Tr]ibuG. Although the reaction was

Figure 1. Preparation of Oligonucleoside Methylphosphonates in Solution.

TABLE II. Preparation of Protected Oligonucleoside Methylphosphonates in Solution

3'-Methylphosphonate Component (mmol)	Method of Preparation	5'-OH Component (mmol)	Product (mmol)	Yield (%)
d-[(MeO) ₂ Tr]bzApC1 (0.32)	A	d-ibuG0Bz (0.16)	d-[(MeO) ₂ Tr]bzApibuG0Bz (0.068)	43%
d-[(MeO) ₂ Tr]bzApC1 (1.05)	A	d-ibuGpCNEt (0.41)	d-[(MeO) ₂ Tr]bzApibuGpCNEt (0.16)	38%
d-[(MeO) ₂ Tr]bzApC1 (0.136)	A	d-bzApibuG0Bz (0.068)	d-[(MeO) ₂ Tr]bzApbzApibuG0Bz (0.0195)	29%
d-[(MeO) ₂ Tr]ibuGpC1/tet (0.033)	B	d-T0TBS (0.0825)	d-[(MeO) ₂ Tr]ibuGpT0TBS (0.0114)	35%
d-[(MeO) ₂ Tr]ibuGpC1 (2.2)	A	d-TpCNEt (1.1)	d-[(MeO) ₂ Tr]ibuGpTpCNEt (0.418)	38%
d-[(MeO) ₂ Tr]ibuGpC1/tet (0.052)	B	d-ibuGpT0TBS (0.052)	d-[(MeO) ₂ Tr]ibuGpibuGpT0TBS (0.019)	37%
d-[(MeO) ₂ Tr]ibuGpC1 (0.40)	A	d-ibuGpTpCNEt (0.20)	d-[(MeO) ₂ Tr]ibuGpibuGpTpCNEt (0.04)	20%
d-[(MeO) ₂ Tr]TpC1 (1.75)	A	d-ibuG0Bz (1.00)	d-[(MeO) ₂ Tr]TpibuG0Bz (0.49)	49%
d-[(MeO) ₂ Tr]bzCpbzCpC1/tet (0.115)	B	d-bzApCNEt (0.23)	d-[(MeO) ₂ Tr]bzCpbzCpbzApCNEt (0.082)	29%

TABLE III. Chromatographic Mobilities and Ultraviolet Spectral Properties of Protected Oligonucleoside Methylphosphonates

Oligomer	Silica Gel TLC Rf			UV Spectra			
	in MeOH-CHCl ₃			λ_{max} (nm)	λ_{min} (nm)	E_{260}/E_{240}	
	5%	10%	15%			calcd.	obsd.
d-[(MeO) ₂ Tr]bzApIbuGObz	0.10	0.46	0.59	235	226	0.65	0.65
	0.08	0.44	0.56	262	258		
				283	267		
d-[(MeO) ₂ Tr]bzApIbuGpCNEt	-	-	0.36	237	224	0.86	0.85
				260	257		
				281	267		
d-[(MeO) ₂ Tr]bzApbzApIbuGObz	0.03	0.10	0.44	234	230	0.73	0.70
			0.40	263	257		
				281	267		
d-[(MeO) ₂ Tr]IbuGpTOTBS	-	0.51	-	235	230	1.02	0.99
		0.41		260	245		
				275 sh			
d-[(MeO) ₂ Tr]IbuGpTpCNEt	-	0.30	0.36	237	225	1.02	1.05
				257	245		
				280 sh			
						1.37	1.37
						1.45	1.48

Table III (cont.)

d-[(MeO) ₂ Tr]ibuGpibuGpTOTBS	-	0.21	0.43	258	226	1.26	1.22	1.38	1.37
		0.18	0.41	238 sh					
				275 sh					
d-[(MeO) ₂ Tr]ibuGpibuGpTpCNEt	-	-	0.21	235	227	1.26	1.30	1.38	1.33
				260	243				
				280 sh					
d-[(MeO) ₂ Tr]TpibuG0Bz	-	0.41	0.55	233	223	0.71	0.73	1.34	1.37
				260					
				275 sh					
d-[(MeO) ₂ Tr]bzCpbzCpbzApCNEt	-	0.14	0.28	233	247	1.09	1.02	1.42	1.25
				263					
				280 sh					
				310 sh					

carried out using an excess of methylphosphonic dichloride, some (3'-3') d-[(MeO)₂Tr]NpN[Tr(MeO)₂] was formed. This dimer formation was greatly reduced by slowly adding the nucleoside to a rapidly stirred solution of the dichloride.

Esterification with an excess of anhydrous hydracrylonitrile yields the β-cyanoethyl ester, d-[(MeO)₂Tr]NpCNEt. This reaction is usually complete within 60 min. The product is then purified by the "flash chromatography" procedure of Still *et al.* (10) on a silica gel column. The isolated yields from these reactions are shown in Table I.

Methylphosphonic dichloride was also used to prepare fully protected di- and trinucleoside methylphosphonates. As shown in Figure 1, two methods of preparation were used. In Method A, d-[(MeO)₂Tr]NpCl (1) was prepared as described above and was subsequently condensed with a protected nucleoside or dinucleotide bearing a free 5'-OH group. In Method B, 5'-protected nucleoside was reacted with tetrazole methylphosphonic chloride (2) to give d-[(MeO)₂Tr]Nptet (3) or d-[(MeO)₂Tr]NpCl, which was then used in the condensation reaction without further isolation. The reaction conditions are described in Table II. The products from these reactions were purified by silica gel column chromatography. The yields obtained and the spectral and

TABLE IV. Synthesis of Oligothymidine Methylphosphonates on a Silica Gel Support

Protected Oligomer on Support	Yield of Condensation Step	(a)	Retention time of Deprotected Oligomer (min)(b)
d-[(MeO) ₂ Tr]TpT-(S)	63%		13.3, 13.6
d-[(MeO) ₂ Tr]TpTpT-(S)	63%		15.8, 16.2
d-[(MeO) ₂ Tr]Tp(Tp) ₂ T-(S)	72%		17.4, 17.7
d-[(MeO) ₂ Tr]Tp(Tp) ₃ T-(S)	80%		18.6
d-[(MeO) ₂ Tr]Tp(Tp) ₄ T-S	93%		19.8
d-[(MeO) ₂ Tr]Tp(Tp) ₅ T-(S)	85%		20.5

(a) Yields are based upon analysis of the dimethoxytrityl group bound to the support after each condensation step.

(b) Retention time on an ODS-3 column (0.46 cm x 25 cm) using a 50 ml linear gradient of 0 to 25% acetonitrile in water at a flow rate of 2.5 ml/min. The retention time of thymidine is 6.5 min.

chromatographic properties of the oligomers are given in Tables II and III, respectively.

5'-Protected nucleoside 3'-methylphosphonic chloride was used to synthesize oligonucleotides on a silica gel support. The silica gel was derivatized with a nucleoside linked to the support via an aminopropyl succinyl linker arm as described by Matteucci and Caruthers (11). The reaction cycle consisted of the following steps: 1) remove the 5'-O-dimethoxytrityl group by treatment with 3% trichloroacetic acid in chloroform or 2% benzenesulfonic acid in acetonitrile; 2) dry the support by evaporation with anhydrous pyridine; 3) couple with a solution of 1 or d-[(MeO)₂Tr]NpCl/tet; and 4) acetylate unreacted 5'-OH groups with acetic anhydride. This procedure was applied to the synthesis of oligothymidine methylphosphonates as shown in Table IV and to a number of heterooligomers as shown in Table V.

The oligothymidine methylphosphonates were cleaved from the support by treatment with ethanol/concentrated ammonium hydroxide (1:1, V/V). The dimethoxytrityl group was removed with 80% acetic acid. The HPLC retention times of the oligomers are given in Table IV. The two diastereoisomers of the

TABLE V. Syntheses of Oligonucleoside Methylphosphonates on Silica Gel Polymer Supports

Protected Oligomer on Support	Yield of Condensation Step (a)
d-[(MeO) ₂ Tr]TpT-(S)	66%
d-[(MeO) ₂ Tr]bzApT-(S)	45%
d-[(MeO) ₂ Tr]bzCpT-(S)	51%
d-[(MeO) ₂ Tr]ibuGpT-(S)	13%
d-[(MeO) ₂ Tr]IpT-(S)	30%
d-[(MeO) ₂ Tr]bzApi buG-(S)	43%
d-[(MeO) ₂ Tr]bzApbzApi buG-(S)	31%
d-[(MeO) ₂ Tr]bzCpbzCpT-(S)	36%
d-[(MeO) ₂ Tr]bzCpbzApT-(S)	29%
d-[(MeO) ₂ Tr]bzCpbzCpbzApT-(S)	28%

(a) Yields are based upon analysis of the dimethoxytrityl group bound to the support after addition of the 5'-terminal nucleotide.

deprotected dimer appear as two well-resolved peaks. The trimer and tetramer appear as two peaks, which indicates only partial resolution of the diastereoisomers has occurred. The pentamer appears as a broad peak while both the hexamer and heptamer appear as reasonably sharp single peaks. This suggests the various diastereoisomers of the longer oligomers have very similar physical properties with respect to their mobilities on the reversed phase column.

DISCUSSION

Methylphosphonic dichloride can be used as a bifunctional phosphorylating/condensing agent to prepare protected nucleoside 3'-methylphosphonates and protected di- and trinucleoside methylphosphonates in solution. Previously Agarwal and Riftina (6) used a combination of methylphosphonic dichloride, triazole and benzenesulfonyl tetrazole to prepare a series of di- through tetrathymidine methylphosphonates. They found this combination of reagents to be superior to the use of methylphosphonic dichloride alone, which gave low yields and required long reaction times.

We have found methylphosphonic dichloride allows rapid preparation of fully protected nucleoside 3'-methylphosphonate β -cyanoethyl esters when the reactions are carried out under strictly anhydrous conditions. The procedure involves first preparing protected nucleoside 3'-methylphosphonic chloride, $d-[(\text{MeO})_2\text{Tr}]\text{NpCl}$, followed by esterification with hydrazylacrylonitrile. Both reactions are carried out in the same reaction vessel and require two hours to complete. Purification of $d-[(\text{MeO})_2\text{Tr}]\text{NpCNEt}$ is accomplished by silica gel "flash" column chromatography which can be accomplished within one or two hours. Thus, the entire synthesis may be easily completed in one day, and as shown in Table I, proceeds in good overall yield. This procedure represents a considerable improvement in terms of time and effort over our previously described method which involved esterification of protected nucleosides with methylphosphonic acid in the presence of dicyclohexylcarbodiimide (1).

The cyanoethyl group can be removed quantitatively by overnight treatment with an aqueous triethylamine/pyridine solution. The triethylammonium salts of the nucleoside 3'-methylphosphonates may be easily isolated by precipitation from hexane and the stable solids may be stored indefinitely at 0°. Fully protected nucleoside 3'-methylphosphonate triethylammonium salts serve as convenient intermediates for the preparation of oligonucleoside methylphosphonates in solution (1,4,5), and on polystyrene polymer supports (13).

Two methods were used to prepare fully protected di- and trinucleoside methylphosphonates (see Figure 1). The first (Method A) involves condensation of protected nucleoside 3'-methylphosphonic chloride, $d-[(\text{MeO})_2\text{Tr}]\text{NpCl}$, with the 5'-OH group of a protected nucleoside or dinucleoside methylphosphonate. The nucleoside 3'-methylphosphonic chloride was prepared by reaction of a slight excess of 5'-protected nucleoside (1 equivalent) with methylphosphonic dichloride (0.8 equivalent). This resulted in the formation of approximately 10% (3'-3') dimer which does not participate in subsequent condensation reactions. The initial phosphorylation reaction and subsequent condensation reaction are most conveniently carried out in septum sealed reaction vials under dry argon. Anhydrous pyridine solutions of the reagents are transferred via gas-tight syringes. When these precautions are taken the condensation reaction proceeds in about 1 hour at room temperature. For reactions involving condensation of $d-[(\text{MeO})_2\text{Tr}]\text{ibuGpCl}$, the reaction is carried out at 40°C.

We also examined the use of tetrazole to further "activate" the methylphosphonic dichloride via formation of the tetrazolide intermediate. Addition of 2 equivalents of tetrazole to an anhydrous pyridine solution of methylphosphonic dichloride in the presence of anhydrous triethylamine resulted in immediate formation of a precipitate, triethylammonium hydrochloride, and presumably the formation of the bis-tetrazole methylphosphonate. Reaction of this intermediate with 5'-protected nucleoside gave low yields of the nucleoside 3'-methylphosphonate. When one equivalent of tetrazole was added to methylphosphonic dichloride, again immediate precipitation of triethylammonium hydrochloride occurred. The supernatant from this reaction, which presumably contains tetrazole methylphosphonic chloride (2), reacted efficiently with 5'-protected nucleoside to give either the nucleoside 3'-methylphosphonic chloride, $d-[(\text{MeO})_2\text{Tr}]\text{NpCl}$ (1) or the nucleoside 3'-methylphosphonic tetrazolide, $d-[(\text{MeO})_2\text{Tr}]\text{Nptet}$ (3). The nature of this intermediate is currently being investigated by ^{31}P NMR. Under this condition (Method B), we found less formation of (3'-3') dimer than occurred when Method A was used. The nucleoside methylphosphonate intermediate designated as $d-[(\text{MeO})_2\text{Tr}]\text{NpCl}/\text{tet}$, readily reacted with the 5'-OH groups of nucleosides or dinucleoside methylphosphonates. As shown in Table II, the yields obtained by this procedure were similar to those obtained by Method A.

The protected dimers and trimers were purified by silica gel column chromatography. In some cases, the mobility of the (3'-3') dimer was similar to that of the product and a second column was required in order to obtain

pure oligomer. Generally the dimers were observed as two closely moving spots on silica gel TLC; these spots correspond to the two diastereoisomers. The amount of separation depends on the base composition of the dimer. The products were characterized by comparison of their UV spectra with calculated spectra (12) as shown in Table III.

Protected di- and trinucleoside methylphosphonates may be prepared and purified in approximately one day by the methods described above. The reactions may be carried out on multimillimole scales. Since oligomers which terminate in 3'-methylphosphonate groups can be prepared, they may be further utilized as intermediates to prepare longer oligomers by block condensation methods (3 and unpublished results).

We have also explored the possibility of using 1 and 3 as intermediates for the syntheses of oligonucleoside methylphosphonates on an insoluble polymer support. In these reactions, silica gel derivatized according to the procedure of Matteucci and Caruthers (11) was used as the support matrix. The preparation of d-[(MeO)₂Tr]TpT-5 was used as a test reaction. A number of silica gels were examined including HPLC-grade Partisil 20 and Vydac, silica gel-60 and macroporous silicas such as Fractosil 200. The best yields were consistently obtained with Fractosil 200 at a nucleoside loading level of 50 to 150 μ mol of nucleoside/gram of support.

The yield of dimer obtained on this support was 63% when a five-fold excess of d-[(MeO)₂Tr]TpCl/tet was used. Attempts to improve the yield by using larger excesses of nucleotide in the coupling reaction or retreating the support with more nucleotide were not successful. This result suggests that support-bound thymidine residues are unavailable for further reaction with the nucleotide. When the products of the dimerization reaction were cleaved from the support with ethanol/concentrated ammonium hydroxide (1:1, V/V), two major products, thymidine and d-[(MeO)₂Tr]TpT, were observed by HPLC. Small amounts of two other nucleotide products were also observed. These were shown to correspond to d-pT and the (5'-5') linked dimer d-TpT. Thymidine 5'-methylphosphonate results from reaction of trace amounts of unreacted phosphorylating agent present in the coupling mixture with support bound thymidine. Attempts to remove this unreacted reagent by evaporation or by selective precipitation of d-[(MeO)₂Tr]NpCl/tet were not successful. The (5'-5') dimer is formed by reaction of phosphorylated thymidine with adjacent support bound thymidine residues. The formation of these side products will of course lower the overall yield of the condensation reaction.

The formation of (5'-5') dimer implies that the thymidine units are

clustered very near each other on the surface of the support. Indeed, (5'-5') dimer formation is observed even on supports which have low nucleoside loading levels. The clustering effect is consistent with recent studies on the surface properties of aminopropyl derivatized silicas (14). This suggests that a thymidine residue could be sterically hindered from reacting once dimer formation has occurred at an adjacent thymidine. Thus, steric hindrance could account for the inability of the support-bound thymidines to further react with 1 or d-[(MeO)₂Tr]TpCl/tet. As shown in Table IV, the yields of the coupling reactions dramatically increase after the trimer stage. This may result from less steric restriction to reaction as the 5'-OH end of the support bound oligomer moves further away from the surface of the support.

Coupling reactions employing other nucleosides proceeded in lower yield than those using thymidine (Table V). This may be due to the greater steric size of the base-protected purine and pyrimidine nucleosides. The lowest yield was encountered in the preparation of d-[(MeO)₂Tr]ibu GpT 5. All attempts to increase the yield above 13% failed despite the presence of unreacted thymidine residues on the support. Examination of space-filling molecular models show that the N-2 isobutryl group can interact with the 3'-methylphosphonate group when the base is rotated about the N-glycosyl bond. This interaction could severely limit the reactivity of this nucleotide if steric factors are particularly critical for efficient reactions on the support. To test this possibility we synthesized d-[(MeO)₂Tr]IpCl, a purine nucleoside which lacks an N-2 isobutryl group. This nucleotide reacted with support bound thymidine to the extent of 30% or approximately 2.3 fold better than d-[(MeO)₂Tr]ibuGpCl. This observation suggests low yields which are generally encountered when protected deoxyguanosine-containing nucleotides are used in coupling reactions on either polymer supports or in solution may be due in part to steric interference by the N-2 protecting groups.

The results of our studies show methylphosphonic dichloride may be used as a convenient bifunctional phosphorylating/coupling reagent for the rapid preparation of mono- and oligonucleoside methylphosphonates in solution. These compounds may be readily synthesized on large scales by this method. The protected intermediates can be further used for synthesis of longer oligomers. It would appear this procedure is less useful when applied to polymer support synthesis. However, we have found oligonucleoside methylphosphonates can be efficiently synthesized on polystyrene supports via the use of nucleoside 3'-methylphosphonic acid intermediates. This procedure will be described in a future communication.

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