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# Nonionic Nucleic Acid Analogues. Synthesis and Characterization of Dideoxyribonucleoside Methylphosphonates†

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ABSTRACT: A series of dideoxyribonucleoside methylphosphonate analogues, dN*p*N and dN*p*N*p*, which contain a nonionic 3′–5′ methylphosphonyl internucleoside linkage were prepared. The two diastereoisomers, designated isomers 1 and 2, of each dimer differ in configuration of the methylphosphonate group and were separated by column chromatography. The diastereoisomers of each dimer have different conformations in solution as shown by ultraviolet hypochromicity data and their circular dichroism spectra. For example, dA*p*A isomer 1 is more highly stacked than isomer 2, although both isomers are less stacked than the dinucleoside monophosphate, dApA. The circular dichroism spectrum of isomer 1 is very similar to that of dApA, while the CD spectrum of isomer 2 shows a loss of molecular ellipticity, [θ], at 270 nm and a greatly diminished [θ] at 250 nm. These results suggest that the stacked bases of dA*p*A isomer 1 tend to orient in an oblique manner, while those in isomer 2 tend to orient in a parallel manner. This interpretation is verified by the $^1$H NMR study of these dimers (L. S. Kan, D. M. Cheng, P. S. Miller, J. Yano, and P. O. P. Ts'o, unpublished experiments). Both diastereoisomers of dA*p*A form 2U:1A and 2T:1A complexes with poly(U) and poly(dT), respectively. The higher $T_m$ ($T_m$ of poly(U)–isomer 1, 15.4 °C; $T_m$ of poly(U)–isomer 2, 19.8 °C; $T_m$ of poly(dT)–isomer 1, 18.7 °C; $T_m$ of poly(dT)–isomer 2, 18.4 °C) values of these complexes vs. those of the corresponding dApA–polynucleotide complexes ($T_m$ of poly(U)–dApA, 7.0 °C; $T_m$ of poly(dT)–dApA, 9.2 °C) result from decreased charge repulsion between the nonionic dimer backbone and the negatively charged polymer backbone. The difference in conformations between dA*p*A isomer 1 and dA*p*A isomer 2 is reflected in the $T_m$ of the isomer 1-poly(U) complex which is 4.4 °C lower than that of the isomer 2-poly(U) complex. Since these nonionic oligonucleotide analogues are taken up by cells in culture, they show promise as molecular probes for the function and structure of nucleic acids inside living cells.

Studies on nucleic acid analogues and derivatives possessing modified internucleoside linkages have made important contributions to understanding nucleic acid conformation in solution and have provided materials for various biochemical and biological studies (Jones et al., 1970, 1973; Pitha et al., 1971; Letsinger et al., 1976; Blob et al., 1977; Mungall & Kaiser, 1977; Vosberg & Eckstein, 1977). In a series of seven papers, our laboratory has reported studies on the physical, biochemical, and biological properties of one class of nonionic nucleic acid derivative, the oligonucleotide alkyl phosphotriesters. The physical properties of dinucleotide methyl and ethyl phosphotriesters have been studied by ultraviolet, circular dichroism, infrared, and proton nuclear magnetic resonance spectroscopy (Miller et al., 1971; Deboer et al., 1973). The interaction of deoxyribooligonucleotide ethyl phosphotriesters with sequences complementary to the amino acid accepting stem and anticodon region of transfer RNA has been characterized (Miller et al., 1974), and their inhibitory effects on in vitro aminoacylation have been studied (Barrett et al., 1974). More recently, the inhibitory effect of a 2′-*O*-methyl ribooligonucleotide triester, $G_p^m(Et)G_p^m(Et)U$,[1] on cellular protein synthesis and growth of mammalian cells in culture has been reported (Miller et al., 1977). In addition, selective binding of an octathymidylate ethyl phosphotriester, $[Tp(Et)]_7T$, to poly(deoxyadenylic acid) has been extensively investigated (Pless & Ts'o, 1977).

In this and subsequent papers in this series (Kan et al., unpublished experiments; Cheng et al., unpublished experiments), we describe the synthesis, the interaction with com-

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[1] Abbreviations used: Np(Et)N, an oligonucleotide ethyl phosphotriester; dN*p*N, a deoxyribonucleotide dimer analogue containing a 3′–5′ internucleoside methylphosphonate linkage (in this abbreviation the italic p represents the methylphosphonate linkage); MST, mesitylenesulfonyl tetrazolide; TPSCl, 2,4,6-triisopropylbenzenesulfonyl chloride; DCC, dicyclohexylcarbodiimide. The symbols used to represent protected nucleosides and dideoxyribonucleoside methylphosphonates follow the IUPAC–IUB Commission on Biochemical Nomenclature recommendations (1970).

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plementary polynucleotides, and the general conformational features of a novel type of nonionic dideoxyribonucleotide analogue, the dideoxyribonucleoside methylphosphonates. In these analogues, the natural phosphodiester linkage is replaced by an isosteric 3′–5′-linked methylphosphonate group. In terms of size, substitution of a methyl residue for the nonesterified oxygen of the phosphate group represents the smallest possible structural change which can be made in the nucleic acid backbone and which results in the removal of the electrostatic charge while still retaining the geometrical features of the phosphodiester linkage. Thus, this modification was expected to have only a relatively small steric perturbation on the conformation of the dimer in solution and on the interaction of the dimer with complementary polynucleotides.

Our synthetic procedure resulted in the separation of the two diastereoisomers of each dimer analogue. This synthetic scheme also allowed the preparation of analogues containing a $^{13}C$-enriched phosphonate methyl group. The influence of backbone configuration on overall dimer conformation was studied by ultraviolet, circular dichroism, and $^{1}H$, $^{13}C$, and $^{31}P$ nuclear magnetic resonance techniques, and the results were compared to the conformations of their parent dideoxyribonucleoside monophosphates. Furthermore, the effects of backbone configuration and the removal of the negative charge on the interaction of deoxyadenosine-containing dimers with poly(uridylic acid) and poly(thymidylic acid) were assessed.

## Materials and Methods

Thymidine and 2′-deoxyadenosine were purchased from P-L Biochemicals and were checked for purity by paper chromatography before use. 5′-(*p*-Methoxytrityl)thymidine, 5′-(di-*p*-methoxytrityl)-*N*-benzoyldeoxyadenosine (Schaller et al., 1963), 3′-*O*-acetylthymidine, and 3′-*O*-acetyl-*N*-benzoyldeoxyadenosine (Zorbach & Tipson, 1968) were prepared according to published procedures. Diethyl [$^{13}C$]methylphosphonate was prepared by reaction of [$^{13}C$]methyl iodide (96% enriched) with triethyl phosphate followed by vacuum distillation of the product (bp 64–66 °C/2 mmHg) according to the procedure of Ford-Moore & Williams (1947). The pyridinium salt of methylphosphonic acid was prepared by hydrolysis of dimethyl methylphosphonate (K & K Laboratories) or diethyl [$^{13}C$]methylphosphonate in 4 N hydrochloric acid, followed by isolation of the product as the barium salt (Hõly, 1967). The barium salt was converted to the pyridinium salt by passage through a Dowex 50X pyridinium ion-exchange column. Mesitylenesulfonyl chloride (Aldrich Chemical Co.) was treated with activated charcoal and recrystallized from pentane immediately before use. 1*H*-Tetrazole was prepared by the method of Henry & Finnegan (1954). All solvents and reagents were purified as previously described (Miller et al., 1974).

Silica gel column chromatography was performed by using Baker 3405 silica gel (60–200 mesh). Thin-layer silica gel chromatography and thin-layer cellulose chromatography were done on plates manufactured by E. Merck Co. Paper chromatography was carried out on Whatmann 3MM paper using the following solvent systems: solvent A, 2-propanol–concentrated ammonium hydroxide–water (7:1:2 v/v); solvent C, 1 M ammonium acetate–95% ethanol (3:7 v/v); solvent F, 1-propanol–concentrated ammonium hydroxide–water (50:10:35 v/v); solvent I, 2-propanol–water (7:3 v/v). High-pressure liquid chromatography (LC) was performed on a Laboratory Data Control instrument using columns (2.1 mm × 1 m) packed with Du Pont Permaphase ODS reverse-phase material. Linear gradients (40 mL) from 0 to 75% methanol in water were used at a flow rate of 1 mL/min. The LC mobility refers to the percentage of methanol in water required to elute the compound from the column.

For reactions carried out in pyridine, the reactants were dried by repeated evaporation with anhydrous pyridine and were then dissolved in anhydrous pyridine. Unless otherwise noted, all reactions and operations were performed at room temperature.

*Preparation of Mesitylenesulfonyl Tetrazolide.* Although the preparation of MST has been published (Stawinski et al., 1977), we followed a modified procedure originally suggested to us by Letsinger and co-workers (R. L. Letsinger, personal communication). A solution of 1*H*-tetrazole (3.36 g, 48 mmol) in 40 mL of dry methylene chloride containing 5.6 mL (40 mmol) of triethylamine was added dropwise to 40 mL of anhydrous ether containing mesitylenesulfonyl chloride (8.76 g, 40 mmol) at room temperature. After 2 h, the precipitated triethylammonium chloride was removed by filtration and washed with 50 mL of methylene chloride–ethyl ether (1:1 v/v). The filtrate was cooled to 0 °C and pentane was added until the solution became cloudy. This procedure was repeated until a total volume of 25 mL had been added over a 4-h period. After storage overnight at 4 °C, the resulting white solid was removed by filtration on a sintered glass filter. The solid was dissolved in 500 mL of anhydrous ethyl ether. The solution was then filtered to remove a small amount of insoluble material. The filtrate was evaporated to dryness, and the resulting mesitylenesulfonyl tetrazolide (4.46 g) was obtained in 44% yield. The MST was pure as indicated by silica gel TLC, $R_f$ ($C_6H_6$) 0.11 (mp 109–110 °C), and was stored in a desiccator at −80 °C. Under these storage conditions, the MST maintained its condensing activity for at least 1 month.

*Preparation of (MeOTr)TpCE.* (MeOTr)T (10.3 g, 20 mmol), the pyridinium salt of methylphosphonic acid (40 mmol), and Dowex pyridinium resin (0.5 g) were treated with dicyclohexylcarbodiimide (41.2 g, 200 mmol) in 100 mL of pyridine at 37 °C for 3 days. The resulting (MeOTr)T*p*, $R_f$ (silica gel TLC) 0.00 (EtOAc–THF, 1:1), appeared to be formed in approximately 86% yield as determined by LC. The material is eluted from the LC column with 37% methanol–water. Hydracrylonitrile (100 mL) was added to the reaction mixture, which was kept at 37 °C for 2 days. Aqueous pyridine (200 mL) was then added and the resulting dicyclohexylurea was removed by filtration. The filtrate was evaporated and dissolved in 250 mL of ethyl acetate, and the solution was extracted with three (250-mL) portions of water. The ethyl acetate solution was dried over anhydrous sodium sulfate. After filtration and evaporation, the mixture was chromatographed on a silica gel column (5.4 × 37 cm) which was eluted with ether (1 L), ethyl acetate (1.2 L), and tetrahydrofuran (1.6 L). Pure (MeOTr)T*p*CE (7.5 g) was isolated in 55% yield after precipitation from tetrahydrofuran by addition of hexane. The monomer has $R_f$ values of 0.32 (EtOAc–THF, 1:1) and 0.66 (20% MeOH–$CHCl_3$) on silica gel TLC and is eluted from the LC column with 54% methanol–water. The UV spectrum gave $\lambda_{max}$ 267 and 230 nm (sh) and $\lambda_{min}$ 250 nm ($\epsilon_{260}/\epsilon_{280}$ 1.44) in 95% ethanol.

*Preparation of (MeOTr)Tp Pyridinium Salt.* (MeOTr)-T*p*CE (2.17 g, 3.36 mmol) was treated with 16.8 mL of 1 N sodium hydroxide in a solution containing 126 mL of dioxane and 25 mL of water for 15 min. The solution was neutralized by addition of Dowex 50X pyridinium resin. After filtration, the solution was evaporated and the residue was rendered anhydrous by evaporation with pyridine. (MeOTr)T*p* (1.90 g, 2.83 mmol) was obtained in 84% yield after precipitation from pyridine by addition to anhydrous ether. The material

Table I: Preparation of Protected Dideoxyribonucleoside Methylphosphonates

| monomers (mmol) | condensing agent[a] (mmol) | reaction time | dimer (mmol) | yield (%) |
|---|---|---|---|---|
| d(MeOTr)T*p* (0.20) + dTOAc (0.22) | DCC (0.73) | 3 days, 37 °C | d(MeOTr)TpTOAc (0.031) | 16 |
| d(MeOTr)T*p* (2.40) + dT*p*CE (3.60) | MST (9.60) | 3 h, rt[b] | d(MeOTr)T*p*T*p*CE (1.32) | 55 |
| d[$(MeO)_2$Tr]bA*p* (1.26) + d(bz)AOAc (1.50) | TPSCl (2.0) | 4 days, 37 °C | d[$(MeO)_2$Tr]bA*p*bzAOAc (0.50) | 39 |
| d[$(MeO)_2$Tr]bzA (0.70) [$^{13}$C]*p* + d(bz)AOAc (1.05) | MST (2.8) | 4 h, rt | d[$(MeO)_2$Tr]bzA[$^{13}$C]*p*bzAOAc (0.29) | 41 |
| d[$(MeO)_2$Tr]bA*p* (0.85) + d(bz)A*p*CE (1.28) | MST (4.0) | 6 h, rt | d[$(MeO)_2$Tr]bzA*p*bzA*p*CE (0.39) | 46 |
| d(MeOTr)T*p* (1.0) + d(bz)AOAc (1.0) | TPSCl (3.0) | 16 h, 37 °C | d(MeOTr)T*p*bzAOAc (0.35) | 35 |
| d[$(MeO)_2$Tr]bA*p* (1.0) + dTOAc (1.3) | TPSCl (1.5) | 46 h, 37 °C | d[$(MeO)_2$Tr]bA*p*TOAc (0.38) | 38 |

[a] DCC, dicyclohexylcarbodiimide; TPSCl, triisopropylbenzenesulfonyl chloride; MST, mesitylenesulfonyl tetrazolide. [b] rt, room temperature.

has $R_f$ values of 0.00 (EtOAc–THF, 1:1) and 0.04 (20% MeOH–$CHCl_3$) on silica gel TLC. The UV spectrum gave $\lambda_{max}$ 267 and 230 nm (sh) and $\lambda_{min}$ 250 nm ($\epsilon_{230}/\epsilon_{267}$ 1.68; $\epsilon_{260}/\epsilon_{280}$ 1.44) in 95% ethanol.

The monomethoxytrityl group was removed from 70 mg (0.1 mmol) of d(MeOTr)T*p* by treatment with 80% aqueous acetic acid. The resulting T*p* (874 $A_{267}$ units, 0.095 mmol) was isolated in 95% yield by chromatography on a DEAE-Sephadex A-25 column (3 × 8.5 cm) using a linear gradient of ammonium bicarbonate (0.01–0.20 M, 500 mL). The monomer has the following $R_f$ values on cellulose TLC: 0.41 (solvent A), 0.77 (solvent C), and 0.69 (solvent F). The UV spectrum gave $\lambda_{max}$ 267 nm and $\lambda_{min}$ 235 nm in water, pH 7.0. The $^1$H NMR spectrum was consistent with the structure of the monomer (Kan et al., unpublished results).

*Preparation of TpCE.* (MeOTr)T*p*CE (3.26 g, 5.04 mmol) dissolved in 20 mL of methanol was treated with 80 mL of 80% acetic acid solution for 5 h at 37 °C. The solvents were removed by evaporation, and the residue was evaporated repeatedly with 50% toluene–tetrahydrofuran to remove the acetic acid. T*p*CE (1.80 g, 4.8 mmol) was obtained in 96% yield after precipitation from tetrahydrofuran (10 mL) by addition of hexane (200 mL). The material has $R_f$ values of 0.08 (EtOAc–THF, 1:1) and 0.16 (15% MeOH–$CHCl_3$) on silica gel TLC. The UV spectrum gave $\lambda_{max}$ 265 nm and $\lambda_{min}$ 233 nm ($\epsilon_{260}/\epsilon_{280}$ 1.61) in absolute ethanol.

*Preparation of d[(MeO)$_2$Tr]bzApCE.* A solution containing d[$(MeO)_2$Tr]bzA (10.5 g, 16 mmol), methylphosphonic acid (32 mmol), and Dowex 50X pyridinium resin (0.5 g) in 80 mL of anhydrous pyridine was treated with dicyclohexylcarbodiimide (25 g, 121 mmol) for 3 days at 37 °C. Examination of the reaction mixture by LC showed essentially quantitative conversion of d[$(MeO)_2$Tr]bzA to d-[$(MeO)_2$Tr]bzA*p*, which has a LC retention time of 22.8 min. The reaction mixture was treated with 80 mL of hydracrylonitrile for 2 days at 37 °C. After filtration and evaporation of the solvents, the residue was dissolved in 200 mL of ethyl acetate, and the solution was extracted with three (200-mL) portions of water. The ethyl acetate solution was dried over anhydrous sodium sulfate, concentrated to 50 mL, and chromatographed on a silica gel column (5.4 × 37 cm). The column was eluted with ether (1.5 L), ethyl acetate (1.5 L), and tetrahydrofuran (1.5 L). The resulting d[$(MeO)_2$Tr]-bzA*p*CE weighed 5.4 g (6.84 mmol, 43%) after precipitation from tetrahydrofuran (100 mL) with hexane (500 mL). The material elutes from the LC column with 68% methanol–water and has silica gel TLC $R_f$ values of 0.13 (EtOAc–THF, 1:1 v/v) and 0.27 (THF). The UV spectrum shows $\lambda_{max}$ 279 and 234 nm and $\lambda_{min}$ 258 and 223 nm ($\epsilon_{234}/\epsilon_{279}$ 1.44; $\epsilon_{260}/\epsilon_{280}$ 0.67) in 95% ethanol.

*Preparation of d[(MeO)$_2$Tr]bzAp.* A solution containing d[$(MeO)_2$Tr]bzA*p*CE (3.79 g, 4.8 mmol) in 180 mL of dioxane and 36 mL of water was treated with 24 mL of 1 N sodium hydroxide for 7 min. The solution was neutralized with Dowex 50X pyridinium resin and then was passed through a Dowex 50X pyridinium ion-exchange column (3 × 30 cm). The eluate was evaporated and the residue was rendered anhydrous by evaporation with pyridine. The resulting d-[$(MeO)_2$Tr]bzA*p* (2.9 g, 3.56 mmol) was obtained in 74% yield after precipitation from anhydrous ether. The material has $R_f$ values of 0.00 (THF) and 0.36 (50% MeOH–$CHCl_3$) on silica gel TLC. The UV spectrum showed $\lambda_{max}$ 280 and 233 nm and $\lambda_{min}$ 255 and 225 nm ($\epsilon_{233}/\epsilon_{280}$ 1.37; $\epsilon_{260}/\epsilon_{280}$ 0.67) in 95% ethanol.

The protecting groups were removed from a small sample of d[$(MeO)_2$Tr]bzA*p* (80 mg, 0.1 mmol) by sequential treatment with concentrated ammonium hydroxide in pyridine and 80% acetic acid. The monomer dA*p* (1400 $A_{260}$ units, 0.09 mmol) was isolated by chromatography on a DEAE-Sephadex A-25 column (3 × 8.5 cm) using a linear gradient of ammonium bicarbonate (0.01–0.2 M, 600 mL). The monomer has the following $R_f$ values on cellulose TLC: 0.45 (solvent A), 0.56 (solvent C), 0.67 (solvent F), and 0.44 (solvent I). The UV spectrum showed $\lambda_{max}$ 259 nm and $\lambda_{min}$ 227 nm ($\epsilon_{260}/\epsilon_{280}$ 6.13) in water, pH 7.0. The $^1$H NMR spectrum was consistent with the structure of the monomer (Kan et al., unpublished experiments).

*Preparation of d(bz)ApCE.* A solution of d[$(MeO)_2$Tr]-bzA*p*CE (1.58 g, 2 mmol) in 6.3 mL of methanol was treated with 25 mL of 80% acetic acid for 1.5 h. The solvents were evaporated, and the residue was repeatedly evaporated with toluene and tetrahydrofuran to remove acetic acid. The residue was precipitated from 20 mL of tetrahydrofuran by dropwise addition to 250 mL of hexane to give 0.95 g (1.95 mmol) of d(bz)A*p*CE in 98% yield. The material has $R_f$ values of 0.09 (THF) and 0.25 (20% MeOH–$CHCl_3$) on silica gel TLC and is eluted from the LC column with 12% methanol–water. The UV spectrum shows $\lambda_{max}$ 280 and 233 nm (sh) and $\lambda_{min}$ 247 nm ($\epsilon_{233}/\epsilon_{280}$ 0.65; $\epsilon_{260}/\epsilon_{280}$ 0.60) in 95% ethanol.

*Preparation of Dinucleoside Methylphosphonates.* The general procedure for the preparation of protected dinucleoside methylphosphonates is given in this section. Table I shows the specific reaction conditions and yields for each dimer. The protected nucleoside 3′-methylphosphonate and protected nucleoside or nucleoside 3′-methylphosphonate cyanoethyl ester were dried by evaporation with anhydrous pyridine. The condensing agent was added, and the reactants were taken up in anhydrous pyridine to give a 0.2 M solution. After completion of the reaction as indicated by TLC and/or LC, an equal volume of water was added and the solution was kept at room temperature for 30 min. The solvents were then evaporated and the residue was dissolved in ethyl acetate or chloroform. The organic solution was extracted with water and then dried over anhydrous sodium sulfate. After concentration, the organic solution was applied to a silica gel column (3 × 28 cm for a 1-mmol scale reaction). The column was

Table II: Chromatographic Mobilities and Ultraviolet Spectral Properties of Protected Dideoxyribonucleoside Methylphosphonates

| dimer | mobility ($R_f$) on silica gel TLC[a] THF | 10% MeOH–$CHCl_3$ | 15% MeOH–$CHCl_3$ | LC mobility[c] (% methanol–water) | UV spectral properties[b] $\lambda_{max}$ (nm) | $\lambda_{min}$ (nm) | |
|---|---|---|---|---|---|---|---|
| d(MeOTr)T*p*TOAc | 0.44 | 0.63, 0.53 | | | 267, sh 235 | 246 | $\epsilon_{235}/\epsilon_{267} = 0.91$ |
| d(MeOTr)T*p*T*p*CE | | | 0.28, 0.22 | | 265, sh 235 | 245 | $\epsilon_{235}/\epsilon_{265} = 0.93$ |
| d[$(MeO)_2$Tr]bzA*p*bzAOAc | 0.34, 0.29 | | 0.51, 0.43 | 68 | 281, sh 233 | 256 | $\epsilon_{233}/\epsilon_{281} = 1.10$ |
| d[$(MeO)_2$Tr]bzA*p*bzA*p*CE | 0.09 | 0.32, 0.28 | 0.39, 0.35 | | 281, sh 230 | 256 | $\epsilon_{230}/\epsilon_{281} = 1.24$ |
| d(MeOTr)T*p*bzAOAc | 0.47 | 0.24, 0.19 | 0.59 | 62 | 276, 230, sh 260 | 247, 227 | $\epsilon_{230}/\epsilon_{276} = 1.07$ |
| d[$(MeO)_2$Tr]bzA*p*TOAc | 0.49, 0.41 | | 0.52, 0.48 | 66 | 277, 235, sh 263 | 255, 227 | $\epsilon_{235}/\epsilon_{277} = 1.18$ |

[a] Two $R_f$ values refer to the mobilities of the individual diastereoisomers. [b] Ultraviolet spectra were measured in 95% ethanol at room temperature. [c] Percentage of methanol in water required to elute the compound from the LC column (Du Pont Phermaphase ODS).

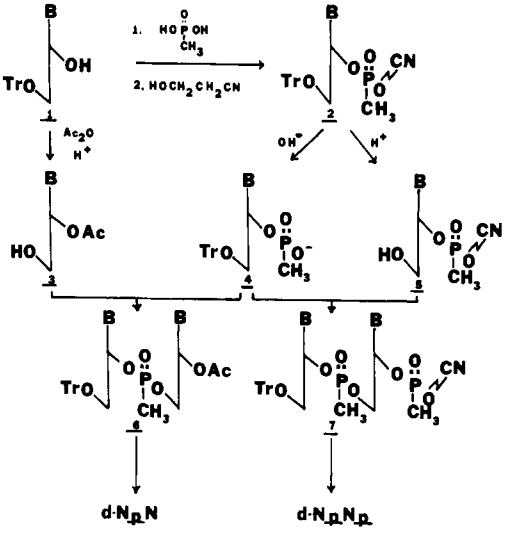
Table III: Chromatographic Mobilities and Ultraviolet Spectral Properties of Dideoxyribonucleoside Methylphosphonates

| dimer | mobility on paper chromatography $R_f$ (A) | $R_f$ (C) | $R_f$ (I) | UV spectral properties $\lambda_{max}$ (nm) | $\lambda_{min}$ (nm) | $\epsilon_{260}/\epsilon_{280}$ |
|---|---|---|---|---|---|---|
| dT*p*T | | 0.67 | 0.73 | 267 | 234 | 1.91 |
| dA*p*A | | 0.48 | 0.62 | 258 | 223 | 6.65 |
| dA*p*A*p* | 0.30 | | 0.38 | 258 | 227 | 4.52 |
| dT*p*A | 0.42 | 0.57 | 0.63 | 262 | 230 | 2.80 |
| dA*p*T | 0.33 | 0.55 | 0.61 | 261 | 233 | 3.22 |

eluted with ethyl acetate, ethyl acetate–tetrahydrofuran (1:1 v/v), and tetrahydrofuran. The progress of the elution was monitored by silica gel TLC. Dimers terminating with a 3′-acetyl group separated into their individual diastereoisomers on the column and were eluted as pure isomer 1, a mixture of isomer 1 and 2, and pure isomer 2. The dimers were isolated as white solids, by precipitation from tetrahydrofuran solution upon addition of hexane. The $R_f$ values on silica gel TLC, the mobilities on the LC column, and the ultraviolet spectral characteristics of the protected dimers are given in Table II.

The base-labile protecting groups were removed from the dimers by treatment with 50% concentrated ammonium hydroxide–pyridine solution for 3 days at 4 °C. Alternatively, the *N*-benzoyl protecting groups of dimers containing deoxyadenosine could be removed by treatment with 85% hydrazine hydrate in 20% acetic acid–pyridine buffer overnight at room temperature (Letsinger et al., 1968). This treatment also partially removed the 3′-*O*-acetyl group. The acetyl group was completely removed by further treatment with 50% concentrated ammonium hydroxide–pyridine solution for 2 h at 4 °C. After complete removal of solvents, the trityl protecting groups were removed by treatment with 80% acetic acid–methanol (8:2 v/v) solution at room temperature. The solvents were then removed and the dimers were chromatographed on Whatmann 3MM paper by using solvent A. The dimers were eluted from the paper with 50% aqueous ethanol. For dimers terminating with 3′-OH groups, the ethanol solutions were passed through small (0.5 × 1 cm) DEAE-cellulose columns to remove trace impurities eluted from the paper chromatogram. Dimers terminating with 3′-methylphosphonate groups were adsorbed to small DEAE-cellulose columns and then eluted with 0.5 M ammonium bicarbonate solution. The dimers were stored as standard solutions in 50% ethanol at 0 °C and were found to be completely stable under these conditions for at least 9 months. For physical and NMR studies, aliquots containing the required amount of dimer were evaporated to remove the ethanol and then lyophilized from water or $D_2O$ before use. The $R_f$ values and UV spectral characteristics of the dimers are given in Table III. The $^1H$ NMR

Scheme I




spectra and tentative chemical shift assignments of the two diastereoisomers of dA*p*A and dT*p*T are shown in Figure 1. Similar $^1H$ NMR spectra were obtained for dA*p*T and dT*p*A (data not shown). The spectra are consistent with the structures of the dimers. The complete characterization of all these dimers by $^1H$ NMR spectroscopy will be described in a subsequent paper (Kan et al., unpublished experiments).

*Physical Studies and Interaction with Polynucleotides.* Ultraviolet and circular dichroism spectra were recorded respectively on a Cary 15 spectrophotometer and a Cary 60 spectropolarimeter with CD attachment. The continuous variation experiments, melting experiments, and circular dichroism experiments were carried out as previously described (Miller et al., 1971). The molar extinction coefficient of poly(U) is $9.2 \times 10^3$ (265 nm) and of poly(dT) is $8.52 \times 10^3$ (264 nm). The molar extinction coefficients of the dideoxyadenosine methylphosphonates were determined by comparing the absorption of a solution of the dimer at pH 7.4 with the absorption of the same solution at pH 1.0. The dimer extinction coefficient was then calculated from the observed hyperchromicity of the dimer at pH 1.0 by using an extinction coefficient for deoxyadenosine at pH 1.0 of $14.1 \times 10^3$.

## Results

*Preparation of Dinucleoside Methylphosphonates.* The synthetic route used to prepare the dinucleoside methylphosphonates is shown in Scheme I. 5′-(*p*-Methoxytrityl)thymidine and 5′-(di-*p*-methoxytrityl)-*N*-benzoyldeoxyadenosine were converted to the corresponding 3′-methylphosphonate β-cyanoethyl esters **2** by sequential reac-

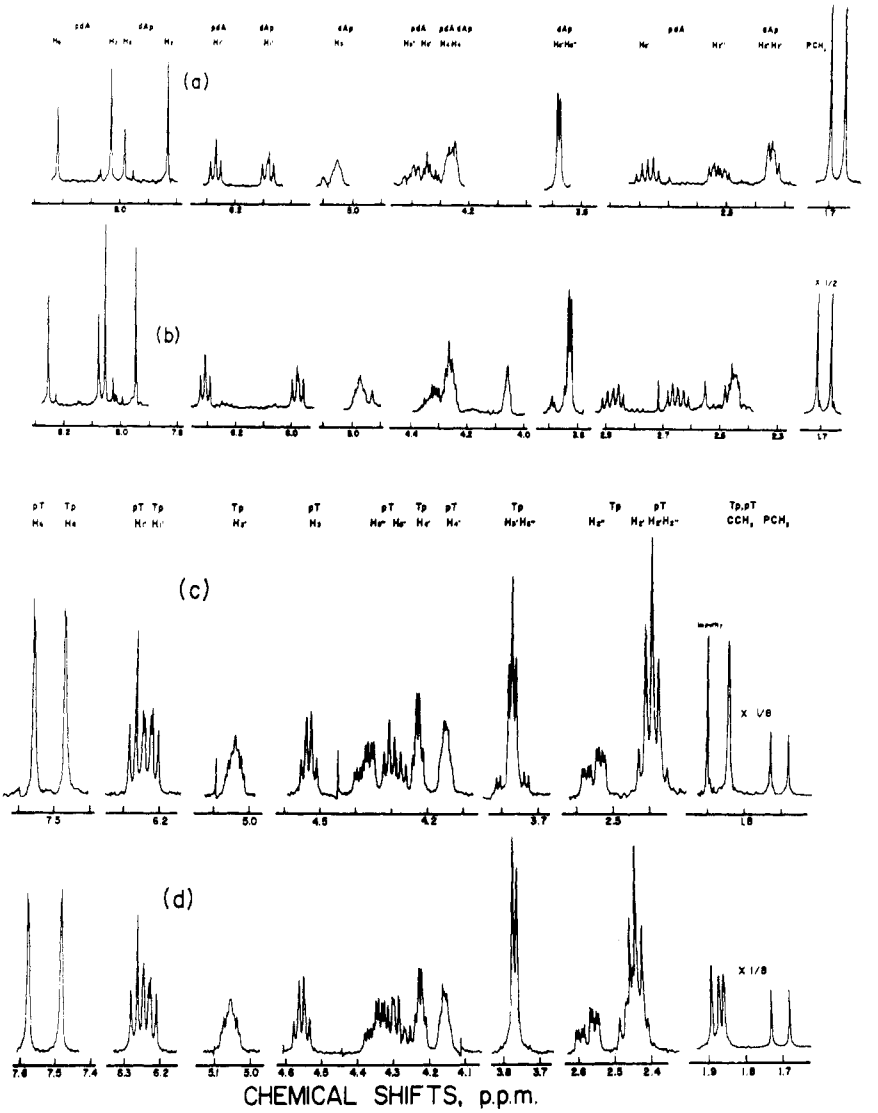



FIGURE 1: The 360-MHz $^1H$ NMR spectra of (a) dA*p*A[1], (b) dA*p*A[2], (c) dT*p*T[1], and (d) dT*p*T[2] at 25 °C in $D_2O$ containing 1 mM ethylenediaminetetracetate–10 mM sodium phosphate, pH 7.0. The tentative chemical shift assignments appear above each dimer.

tion of **1** with methylphosphonic acid and β-cyanoethanol in the presence of dicyclohexylcarbodiimide. The preparation of nucleoside 5′-methylphosphonates by reaction of a suitably protected nucleoside with methylphosphonic acid has been previously reported (Gulyaev et al., 1971; Nichol et al., 1967; Hôly, 1969; Hôly & Hong, 1971; Wigler & Lozzio, 1972). Direct conversion of the protected nucleoside 3′-methylphosphonate to its β-cyanoethyl ester allows purification of this intermediate on a large scale by silica gel column chromatography, thus avoiding the use of ion-exchange chromatography. The trityl or β-cyanoethyl protecting groups can be selectively removed from **2** by treatment with either 80% acetic acid or 0.1 N sodium hydroxide solution, respectively, at room temperature.

Protected nucleoside 3′-methylphosphonate (**4**) was condensed with either 3′-*O*-acetylthymidine or 3′-*O*-acetyl-*N*-benzoyldeoxyadenosine to give fully protected dinucleoside methylphosphonate **6**. Alternatively, **4** was condensed with the β-cyanoethyl ester of thymidine 3′-methylphosphonate or *N*-benzoyldeoxyadenosine 3′-methylphosphonate to give **7**. The condensing agents used in these reactions were dicyclohexylcarbodiimide, triisopropylbenzenesulfonyl chloride, or mesitylenesulfonyl tetrazolide (Stawinski et al., 1977). The reaction conditions and yields are given in Table I.

The fully protected dimers were readily purified by silica gel column chromatography. For dimers terminating with 3′-*O*-acetyl groups, the two diastereoisomers were sufficiently separated on the silica gel column that fractions containing each pure diastereoisomer were obtained. These isomers were designated isomer 1 and isomer 2 in reference to their order of elution from the column. The diastereoisomers were generally formed in a 4:6 ratio of isomer 1 to isomer 2. Alternatively, the diastereoisomers could be obtained in pure form by thick-layer chromatography on silica gel plates. The dimers terminating in a 3′-(*O*-β-cyanoethyl methylphosphonate) group (**7**) consist of four diastereoisomers, although only two separate bands were observed on silica gel thin-layer chromatography (see Table II). For the deoxyadenosine-containing dimer, these two bands turned out to be the two isomers with opposite configurations (*R* and *S*, see Figure 5) about the methylphosphonyl internucleoside linkage.

Removal of the protecting groups from **6** and **7** was accomplished by sequential treatment with concentrated ammonium hydroxide in pyridine for 3 days at 4 °C, followed by treatment with 80% acetic acid. In the case of the dideoxyadenosine methylphosphonates, some hydrolysis of the phosphonate linkage was noted when the ammonium hydroxide treatment was carried out at room temperature. However, the hydrolysis

Table IV: Hypochromicity of Dideoxyadenosine Methylphosphonate Analogues

| compd | $\epsilon$ (M)[a] | % hypochromicity |
|---|---|---|
| dpA | $15.3 \times 10^3$ | |
| dApA | $12.7 \times 10^3$ | 17 |
| dA*p*A$^1$ | $13.7 \times 10^3$ | 11.0 |
| dA*p*A$^2$ | $14.3 \times 10^3$ | 7.1 |
| dA*p*Ap$^1$ | $13.0 \times 10^3$ | 13.3 |
| dA*p*Ap$^2$ | $13.3 \times 10^3$ | 11.3 |

[a] The extinction coefficient per base residue at 258 nm was measured in 1 mM Tris-HCl, pH 7.4, at 27 °C.

was suppressed at low temperature. Alternatively, the *N*-benzoyl protecting groups of these dimers could be removed by treatment with hydrazine hydrate (Letsinger et al., 1968). The dimers were then purified by paper chromatography. The individual diastereoisomers of each deprotected dimer had the same chromatographic mobilities on paper chromatography in all solvent systems tested (see Table III).

*Ultraviolet and Hypochromicity Measurements.* The ultraviolet spectral properties of the dinucleoside methylphosphonates are recorded in Table III. Qualitatively, the spectra are similar to those of 3′–5′-linked dinucleoside monophosphates. The spectra of the individual diastereoisomers are qualitatively similar to each other.

Hypochromicity measurements for the dideoxyadenosine methylphosphonates were carried out in water at pH 7.4 and are shown in Table IV. The percent hypochromicity of the methylphosphonate dimers is from 4 to 10% lower than the percent hypochromicity of dApA. Each diastereoisomer has an unique molar extinction coefficient. The hypochromicity of isomer 1, the isomer eluted first from the silica gel column, is greater than that of isomer 2, reflecting differences in the extent of base–base overlap in these dimers.

*Circular Dichroism Spectra.* Differences in the extent and mode of base-stacking interactions are observed for individual diastereoisomers within a given dimer sequence as reflected by the CD spectra of the dimers. The profile of the CD spectrum of dA*p*A$^1$ (Figure 2a) is qualitatively similar to that of the parent dinucleoside monophosphate, dApA (Miller et al., 1971). However, the magnitudes of the molecular ellipticity, [θ], at 267 and 270 nm of dA*p*A$^1$ are approximately half of those found for dApA. A very dramatic difference in the CD spectrum of dA*p*A$^2$ is observed. Only negative [θ] is found at 250 nm and the amplitude of the molecular ellipticity is approximately threefold less than that of dA*p*A$^1$. Similar results were observed for dA*p*Ap$^1$ and dA*p*Ap$^2$ (data not shown).

In the case of dA*p*T (Figure 2b), the profiles of the CD spectra of both isomers 1 and 2 are qualitatively similar to that of dApT (Cantor et al., 1970). However, the magnitudes of the ellipticity of the peak (272 nm) and trough (253 nm) of the dinucleoside methylphosphonate are less than those in the dinucleoside monophosphate. For dA*p*T$^1$, the peak is reduced 1.8-fold and the trough is reduced 1.3-fold compared to those of dApT while for dA*p*T$^2$ the reductions are 8.1- and 5.0-fold.

The CD spectra of dT*p*A$^1$ and dT*p*A$^2$ (Figure 2c) show differences in both the magnitude of the molecular ellipticity and the position of the positive and negative bands. Isomer 2 has a CD spectrum which is virtually identical with that observed for dTpAp (Cantor et al., 1970). Isomer 1, on the other hand, has a lower magnitude of the [θ] value, while the positions of the peak and trough are shifted to shorter wavelengths.

The CD results for dA*p*T$^1$, dA*p*T$^2$, dT*p*A$^1$, and dT*p*A$^2$ are qualitatively similar to those obtained by Jensen & Reed

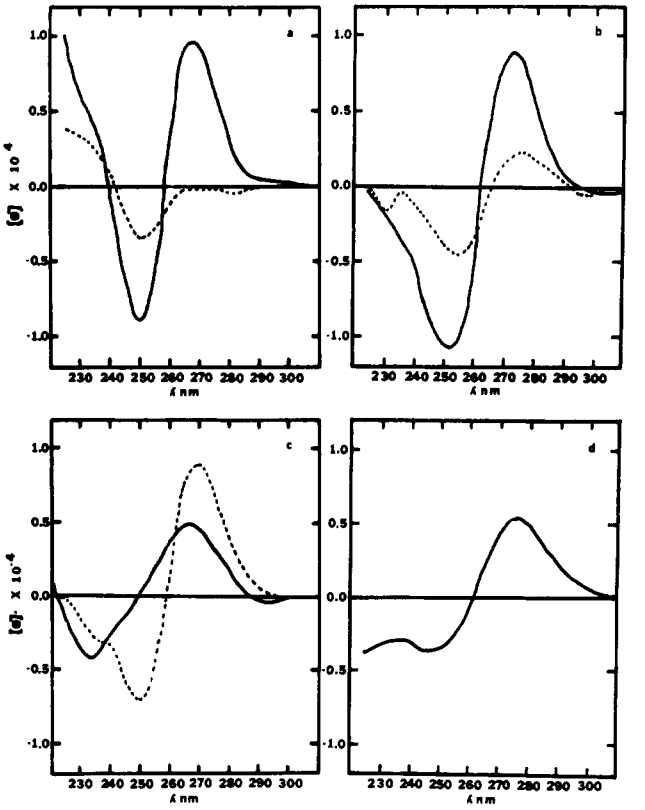



FIGURE 2: Circular dichroism spectra of (a) dA*p*A$^1$ (—) and dA*p*A$^2$ (---), (b) dA*p*T$^1$ (—) and dA*p*T$^2$ (---), (c) dT*p*A$^1$ (---) and dT*p*A$^2$ (—), and (d) dT*p*T in 10 mM Tris-HCl and 10 mM $MgCl_2$, pH 7.5, at 27 °C. The molecular ellipticity, [θ], is given per base residue.

(1978) on the CD spectra of the separated diastereoisomers of the dinucleoside ethyl phosphotriesters, dAp(Et)T and dTp(Et)A. In the case of these triesters, one isomer has a spectrum which is almost identical with that of the corresponding dinucleoside monophosphate. The other isomer shows significant reductions in the magnitudes of both the positive and negative CD absorption bands. It is not possible at this time to make detailed comparisons between our results and those on the triesters, since the absolute configurations of the modified phosphate groups in the triesters are not known.

Figure 2d shows the CD spectrum of a 1:1 mixture of the diastereoisomers of dT*p*T. The spectrum of this mixture is clearly different from the spectrum of dTpT (Cantor et al., 1970). For dTpT, positive [θ] occurs at 280 nm with a magnitude approximately 1.8-fold greater than that of the 275-nm band of dT*p*T. Similarly, dTpT shows negative [θ] at 250 nm which is approximately 1.8-fold greater than that of the negative band at 245 nm in dT*p*T.

*Interaction of Dideoxyadenosine Methylphosphonates with Poly(U) and Poly(dT).* Both diastereoisomers of dA*p*A form complexes with poly(U) at 0 °C. The mixing curves for dA*p*A$^1$ and dA*p*A$^2$ with poly(U) (Figure 3) show that complex formation occurs with a base stoichiometry of 2U:1A. Similar results were obtained for the interaction of dA*p*Ap$^1$ and dA*p*Ap$^2$ with poly(U) and for the interaction of dA*p*A with poly(dT).

As shown in Figure 4, the methylphosphonate–polynucleotide complexes exhibit a cooperative thermal transition with a well-defined melting temperature. The melting temperature of the dA*p*A$^1$–poly(U) complex is 4.4 °C higher than that of the dA*p*A$^2$–poly(U) complex. A similar difference in melting temperatures for the dA*p*Ap–poly(U) complexes was

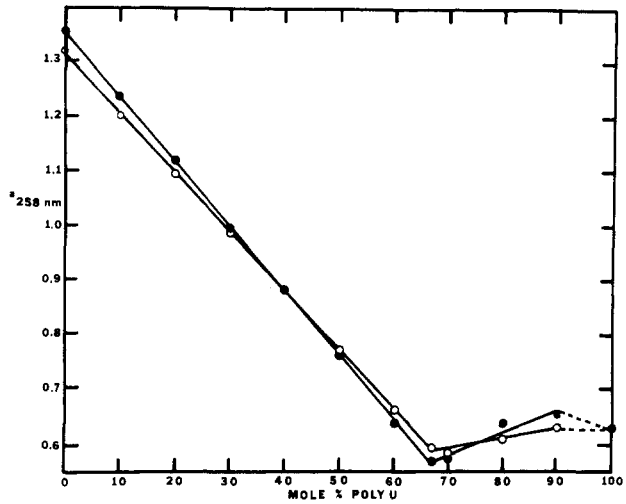



FIGURE 3: Mixing experiment between poly(uridylic acid) and dA*p*A[1] (○) or dA*p*A[2] (●) in 10 mM Tris and 10 mM $MgCl_2$, pH 7.5, at 0 °C. The total nucleotide concentration is $1 \times 10^{-4}$ M.

Table V: Melting Temperatures of Complexes Formed between Dideoxyadenosine Methylphosphonate Analogues and Poly(uridylic acid) or Poly(thymidylic acid)

| complex[a] | $T_m$ (°C) [poly(U)][b] | $T_m$ (°C) [poly(dT)] |
|---|---|---|
| dApA | 7.0 | 9.2 |
| dA*p*A[1] | 15.4 | 18.7 |
| dA*p*A[2] | 19.8 | 18.4 |
| dA*p*Ap[1] | 13.5 | |
| dA*p*Ap[2] | 17.4 | |

[a] Complex stoichiometry: 2U:1A or 2T:1A. Total nucleotide concentration: $5 \times 10^{-5}$ M. [b] 10 mM Tris-HCl and 10 mM $MgCl_2$, pH 7.5.

also observed (Table V). Essentially no difference is observed between the $T_m$ values of the two dA*p*A–poly(dT) complexes, however.

Significant increases are observed in the thermal stabilities of the dinucleoside methylphosphonate–polynucleotide complexes as compared to similar complexes formed between dApA and poly(U) or poly(dT). The nonionic dA*p*A forms complexes with $T_m$ values 8.4 and 12.4 °C higher than that of dApA–poly(U), while the singly charged dA*p*Ap forms complexes with $T_m$ values 6.5 and 10.4 °C higher than that of dApA–poly(U). Similarly, the complexes formed between dA*p*A and poly(dT) each melt approximately 10 °C higher than the dApA–poly(dT) complex.

## Discussion

Dinucleoside methylphosphonates are novel nucleic acid analogues in which the phosphodiester internucleoside linkage is replaced by a 3′–5′-linked internucleoside methylphosphonyl group. Unlike the dinucleoside methylenephosphonates prepared by Jones et al. (1970), the methylphosphonate analogues do not contain a negatively charged backbone and are nonionic molecules at pH 7. The methylphosphonate group is isosteric with respect to the phosphate group of dinucleoside monophosphates. Thus, these analogues should present minimal steric restrictions to interaction with complementary polynucleotides or single-stranded regions of nucleic acid molecules. Since the methylphosphonyl group is not found in naturally occurring nucleic acid molecules, this internucleoside linkage may be resistant to hydrolysis by various nuclease and esterase activities, and this has in fact been observed (Miller, unpublished data). These properties make analogues of this type potentially useful as vehicles for exploring the interactions of

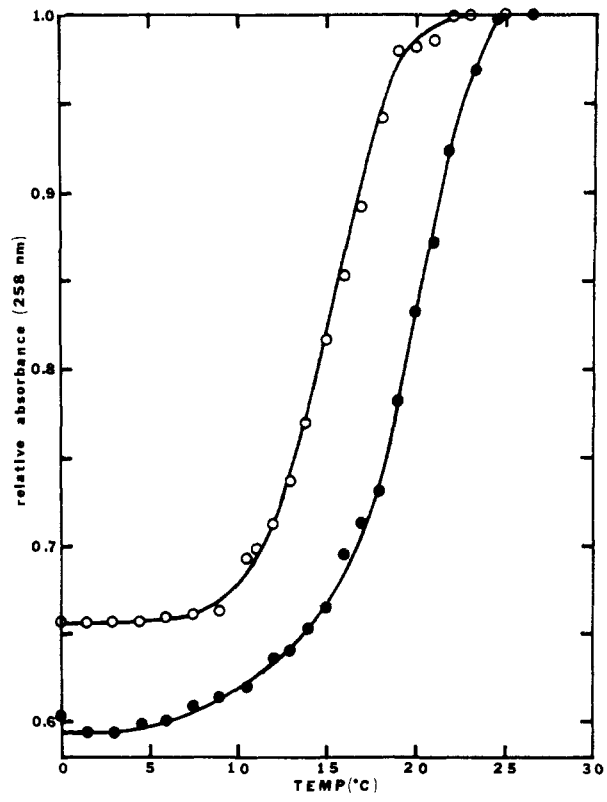



FIGURE 4: Melting curves of poly(U) plus dA*p*A[1] (○) and poly(U) plus dA*p*A[2] (●) in 10 mM Tris and 10 mM $MgCl_2$, pH 7.5. The stoichiometry of each complex is 2U:1A and the total nucleotide concentration is $5 \times 10^{-5}$ M.

selected oligonucleotide sequences with nucleic acids and nucleic acid related enzymes within the living cell (Miller et al., 1977).

The preparation of the oligonucleoside methylphosphonates follows the basic strategy used for the preparation of protected oligonucleotide phosphotriesters (Letsinger & Ogilvie, 1969). The synthetic scheme which has been adopted first involves preparation of a protected nucleoside 3′-methylphosphonate β-cyanoethyl ester (Scheme I). This two-step preparation can be carried out in a one-flask reaction and proceeds in high overall yield. Since the product is readily purified by silica gel column chromatography, multigram quantities of this key intermediate can be prepared. By selective removal of the 5′-trityl group or the β-cyanoethyl group, chain extension can proceed in either direction. Thus, compound **1** in Scheme I serves as a basic building block for the preparation of longer oligomers. This type of synthetic scheme was originally developed by Catlin & Cramer (1973) for the preparation of oligonucleotide β,β,β-trichloroethyl phosphotriesters and has more recently been used by Narang and co-workers (Itakura et al., 1975; Stawinski et al., 1977) for the preparation of oligonucleotide *p*-chlorophenyl phosphotriesters. This procedure also allows the preparation of specifically $^{13}C$-enriched dimers by use of [$^{13}C$]methylphosphonic acid in the synthesis of **1**. Dimers and oligomers containing [$^{13}C$]methylphosphonate groups could be very useful for probing the physical and biological properties of oligonucleoside methylphosphonates by nuclear magnetic resonance spectroscopic techniques (Cheng et al., unpublished experiments).

In the present study (Scheme I), the β-cyanoethyl group was removed from **1** and chain extension was continued in the 3′ direction. Two types of condensation reactions were carried out: (1) condensation with a 3′-*O*-acetylated nucleoside to give dimers with the general structure **6** and (2) condensation with

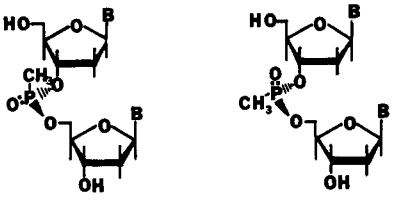



FIGURE 5: Diastereoisomers of dideoxyribonucleoside methylphosphonates.

a nucleoside 3′-methylphosphonate β-cyanoethyl ester to give dimers with general structure 7. The latter type of dimer can be further extended by removal of the β-cyanoethyl group, followed by condensation with other oligonucleoside methylphosphonate blocks. In this way, oligonucleoside methylphosphonates containing up to four deoxyadenosine residues and up to nine thymidine residues have been prepared (P. S. Miller and J. Yano, unpublished results).

Different condensing agents were used in these reactions, including dicyclohexylcarbodiimide (DCC), triisopropylbenzenesulfonyl chloride (TPSCl), and mesitylenesulfonyl tetrazolide (MST). The order of condensing efficiency was found to be MST > TPSCl > DCC. Although DCC did bring about condensation, several days at elevated temperatures were required and the yields were quite low. Considerable improvement in reaction yield was obtained when TPSCl was used. However, again prolonged reaction periods were required and noticeable buildup of side products was observed. The reagent of choice for these reactions is MST. The reaction occurs within a period of several hours, with little or no side products. The efficiency of a particular condensing agent depends not only upon its structure but also upon the nature of the phosphorous-containing substituent which is activated. Thus, when MST was used as a condensing agent, we observed that reactions involving nucleoside 3′-methylphosphonates or nucleoside 3′-ethyl phosphates usually proceed in lower yield than those involving nucleoside 3′-*p*-chlorophenyl phosphates.

The ability to separate the individual diastereoisomers of each dimer sequence allowed examination of the effect of the configuration of the phosphonyl methyl group on the overall dimer conformation. As shown in Figure 5, the isomers differ in configuration at the internucleoside linkage with the methyl group, assuming either a pseudoaxial (*S*) or pseudoequatorial (*R*) position when the dimers are drawn in a stacked conformation. The unique conformational properties of each diastereoisomer of dA*p*A and dA*p*A*p* are most readily seen by examining the percent hypochromicity of each diastereoisomer (Table IV). Isomer 1 of both dA*p*A and dA*p*A*p* exhibits a greater percent hypochromicity than does isomer 2 of this series. Since the percent hypochromicity is related to the extent of base–base overlap in dimers of this type (Ts'o, 1974), the result suggests that dA*p*A$^1$ and dA*p*A*p*$^1$ are more highly stacked in solution than are dA*p*A$^2$ and dA*p*A*p*$^2$. Comparison of the percent hypochromicities of the methylphosphonate dimers with that of dApA shows that these dimers are less stacked than the parent dinucleoside monophosphate. A similar result was observed for the methyl and ethyl phosphotriesters of dApA (Miller et al., 1971). Thus, nonionic methyl or ethyl phosphotriester or methylphosphonate internucleoside linkages appear to perturb the stacking interactions between the bases in these dimers.

The circular dichroism spectra of dinucleoside monophosphates are indicators of both the extent and mode of base stacking, as well as the population of right-handed vs. left-handed stacks (Kondo et al., 1972; Ts'o, 1974). The CD spectra of each diastereoisomer for the methylphosphonate dimer sequences dA*p*A, dA*p*A*p*, dA*p*T, and dT*p*A suggest that each diastereoisomer has an unique stacking mode in solution. The profiles of the CD spectra of dA*p*A$^1$ and dA*p*A*p*$^1$ are very similar to those of dApA and rApA and differ only in the magnitude of the molecular ellipticity. This result and the results of the hypochromicity measurements suggest that the stacking modes of the bases in these dimers are similar to those of dApA and rApA. On the other hand, the profiles of the CD spectra of dA*p*A$^2$ and dA*p*A*p*$^2$ are quite different. The magnitudes of the molecular ellipticities of dA*p*A$^2$ and dA*p*A*p*$^2$ are greatly diminished, with complete loss of [θ] at 270 nm. Since the hypochromicity measurements suggest that the bases in these dimers have substantial overlap, the mode of stacking in these dimers must be quite different from that found for isomer 1 or for dApA. The magnitude of the molecular ellipticity in dimers of this type is sensitive to the angle, θ, between the transition dipoles of the bases (Ts'o, 1974). The value of the molecular ellipticity is greatest when θ is 45° and diminishes to 0 when θ is 0, 90, or 180°. Thus, the most reasonable interpretation of the CD results is that, in dA*p*A$^1$ and dA*p*A*p*$^1$, the bases tend to orient in an oblique manner, while, in dA*p*A$^2$ and dA*p*A*p*$^2$, the bases tend to orient in a parallel or perpendicular manner. This interpretation is supported by the base–base stacking patterns as determined by $^1$H NMR spectroscopy (Kan et al., unpublished experiments). The substantial change in the CD profile of dA*p*A$^2$ rather than a simple diminution of the amplitude of the [θ] values suggests that variation of the population of right-handed vs. left-handed stacks would not provide an adequate explanation of the CD results.

The CD spectra of dA*p*T isomers 1 and 2 have the same shape as the CD spectrum of dApT but with diminished molecular ellipticity. For dT*p*A, the spectrum of isomer 2 is identical with that of dTpAp, while the spectrum of isomer 1 shows diminished [θ] values of the peak and trough regions. Thus, the stacking modes in these methylphosphonate dimers are expected to be basically similar to the stacking modes of the parent dinucleoside monophosphates but with perhaps different degrees of base–base overlap or different populations of right- and left-handed stacks.

The dimer dA*p*A forms stable complexes with both polyribo- and polydeoxyribonucleotides. These poly(U) and poly(dT) complexes have greater stability than similar complexes formed by the parent dinucleoside monophosphate, dApA. Similar observations have previously been made for triple helix formation between the alkyl phosphotriesters dAp(Me)A or dAp(Et)A and poly(U) (Miller et al., 1971), for duplex formation between oligonucleotide triesters and tRNA (Miller et al., 1974), and for helical duplex formation between the octathymidylate ethyl phosphotriester d[Tp(Et)]$_7$T and poly(dA) (Pless & Ts'o, 1977). It should be noted, however, that d-[Tp(Et)]$_7$T, in contrast to dA*p*A, exhibits selective binding to polydeoxyribonucleotides vs. polyribonucleotides in duplex formation.

Our previous analyses indicate that the increased stability of the complexes formed between nonionic oligomers and complementary polynucleotides results from the reduction in charge repulsion between the nonionic backbone of the oligomer and the negatively charged sugar–phosphate backbone of the polynucleotide (Miller et al., 1971; Pless & Ts'o, 1977). Although both dA*p*A*p* and dApA possess a formal negative charge, the dA*p*A*p*–poly(U) complexes are more stable than the dApA–poly(U) complex. The 3′-terminal methylphosphonate group of dA*p*A*p* is free to rotate away from the

negatively charged phosphate backbone of poly(U) without disrupting the base-pairing and base-stacking interactions in the complex. In contrast, repulsion between the negative charge of the phosphodiester linkage in dApA and the polymer backbone directly opposes base pairing and stacking. Thus, the presence of a negative charge at the *internucleotide* linkage contributes much more effectively to the charge repulsion effect between the dimers and polynucleotides.

Under the conditions of the present experiments, the $T_m$ values of dAp(Me)A–poly(U) and dAp(Et)A–poly(U) are 13 and 12 °C, respectively. These $T_m$ values are lower than those of dA*p*A and dA*p*A*p* complexes with poly(U). These results suggest that the increasing size of the methyl and ethyl side chains in the phosphotriester dimers may provide a greater steric hindrance to complex formation. The methyl group of the phosphonate dimers should be only slightly larger in size than the oxygen of the phosphate group and thus would be expected to have the least steric effect. A similar phenomenon has been observed when the stabilities of poly(U) complexes with the ethyl phosphotriester and methylphosphonate analogues of dApApApA are compared (Jayaraman et al., 1979).

The differences in the conformations of the individual diastereoisomers of dA*p*A and dA*p*A*p* are reflected in their interactions with poly(U). For each dimer, the diastereoisomer with greater base–base overlap (isomer 1) forms a complex of lower stability with poly(U). In a previous analysis of the influence of C-2′ substituents of adenine polynucleotides on the $T_m$ values of the helices (Alderfer et al., 1974), we reasoned that the conformation free-energy difference ($F_D - F_S$) at the melting temperature is directly related to the $T_m$ value, where $F_D$ represents the free energy of the double-stranded duplex and $F_S$ represents the free energy of the base-stacked single strand. The values of $F_D - F_S$ reflect the conformation of the duplex state and the single-stranded state. The data indicate that $F_D - F_S$ for isomer 1 of dA*p*A or dA*p*A*p* is slightly less than $F_D - F_S$ for isomer 2 of dA*p*A or dA*p*A*p*. This reduction may reflect a higher $F_S$ value of isomer 1 since this isomer indeed has a greater degree of stacking, assuming that $F_D$ for isomer 1 and isomer 2 remains the same. In contrast to the behavior with poly(U), both diastereoisomers of dA*p*A form complexes with poly(dT) which have similar $T_m$ values. Since the geometry of the triple helix of dA*p*A–**2**–poly(U) is likely to be different than the geometry of the dA*p*A–**2**–poly(dT) triple helix, the difference in $F_S$ of isomer 1 vs. $F_S$ of isomer 2 may be compensated by a difference in $F_D$ of isomer 1 vs. $F_D$ of isomer 2.

The studies reported in this paper have shown that dideoxyribonucleotide analogues containing nonionic 3′–5′ internucleoside methylphosphonate linkages can be readily synthesized. The configuration of the methyl group in the backbone of these dimers influences their conformation in solution and their ability to form complexes with complementary polyribonucleotides. More detailed descriptions of the conformations of these molecules based on NMR spectroscopic results will be presented in subsequent papers in this series (Kan et al., unpublished experiments; Cheng et al., unpublished experiments). In addition, preliminary studies have shown that oligodeoxyribonucleoside methylphosphonates are resistant to nuclease hydrolysis, are taken up in intact form by mammalian cells in culture, and can exert specific inhibitory effects on cellular DNA and protein synthesis. Unlike 2′-*O*-methyl oligonucleotide ethyl phosphotriesters, the methylphosphonates appear to have relatively long half-lives within the cells. Thus, oligonucleoside methylphosphonates of specific sequence could complement oligonucleotide phosphotriesters as probes and regulators of nucleic acid function within living cells.

## Added in Proof

Recently Agarwal & Riftina (1979) reported the syntheses of some dideoxyribonucleoside methylphosphonates using synthetic procedures different from those reported here.

## Acknowledgments

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# Inhibition of Intractable Nucleases with Ribonucleoside–Vanadyl Complexes: Isolation of Messenger Ribonucleic Acid from Resting Lymphocytes†

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ABSTRACT: Human lymphocyte lysates prepared by detergent treatment of intact, normal resting cells contain ribonucleases that are insensitive to many inhibitors commonly used with eucaryotic cells. Phenol-extracted ribonucleic acid (RNA) obtained directly from unfractionated cytoplasm is sometimes degraded, but after fractionation of the cytoplasmic material by sucrose density gradient centrifugation, the polyadenylated RNA, in particular, is inevitably destroyed. An extensive survey of ribonuclease inhibitors, undertaken as a consequence, indicated that the complexes formed between the oxovanadium ion and the four ribonucleosides were unique in their ability to suppress lymphocyte nuclease activity. It proved possible to isolate intact ribosomal RNA and polyadenylated messenger RNA from lymphocyte cytoplasm fractionated on sucrose gradients when 2.5 mM each of the four ribonucleoside–vanadyl complexes was used throughout the procedure. The data showed that the size distribution of poly(A)-bearing RNA remained unchanged, with a peak at ~16 S under denaturing conditions, regardless of whether the mRNA was originally associated with polysomes or was nonpolysome bound. The cytoplasmic RNAs were completely free of contamination by either intact nuclear RNA or nuclear fragments. Furthermore, exogenous globin mRNA mixed with lymphocytes and reisolated together with endogenous cytoplasmic polyadenylated RNA was fully translatable only when ribonucleoside–vanadyl complexes were employed during the preparation. The use of this inhibitor should therefore be considered for all tissues in which ribonucleases impede isolation of intact RNA.

The isolation of intact RNA from most animal cells relies on the use of exogenous ribonuclease inhibitors. Many substances including diethyl pyrocarbonate, polyvinyl sulfate, heparin, bentonite, macaloid, an assortment of ribonucleotides, sodium dodecyl sulfate, and proteinase K have been employed routinely for this purpose (Poulson, 1977). Recent investigations, however, have revealed that pancreatic ribonuclease (Jones, 1976) as well as other nucleases in crude cell homogenates (Gray, 1974; Egberts et al., 1977) retains enzymatic activity in the presence of these agents. Although the studies were carried out under test conditions removed from those actually confronted in preparative situations, in all cases the warning implicit in these findings is unmistakable. The ability to obtain intact RNA from HeLa cells (Milcarek et al., 1974), hen oviducts (Schimke et al., 1974), or fibroblasts (Johnson et al., 1974), for example, is probably a fortuitous event stemming from low endogenous levels of nucleases that are sensitive to one or more of the substances listed above. It is also possible that subcellular compartmentalization maintains the separation between RNA and RNases more effectively during lysis of some cells than during disruption of others. Clearly, the ability to carry out quantitative studies of RNA, particularly under conditions in which protein denaturation and deproteinization are incompatible with the aim of the experiment, depends upon a restricted choice of biological material. Those systems in which the nucleases are known to be intractable (Spradling et al., 1975; Cooper & Kay, 1969) receive little attention.

In order to work with resting lymphocytes, it was necessary to overcome this problem. Toward this end, we have tested a number of ribonuclease inhibitors for their ability to protect RNA in crude lysates of these cells. The criteria for choosing suitable inhibitors were the following: (1) the substance must be compatible with cell lysis techniques in which subcellular organelles are to be purified; (2) RNA must not leak out of the nuclei; (3) cytoplasmic components such as ribonucleoprotein particles and polysomes should be unaffected; and (4) RNA must remain undegraded in disrupted cells during sucrose gradient centrifugation of cytoplasm, a technique requiring several hours in the presence of proteins. Of the inhibitors tested, only one related set, the complexes formed between $VOSO_4$ and each of the four ribonucleosides, proved satisfactory. The use of the mixed vanadyl complexes not only facilitated the isolation of structurally intact, translatable RNA but also increased the yield of polyadenylated mRNA from resting lymphocytes fourfold.

## Materials and Methods

*Materials.* Iodoacetic acid, EGTA,[1] diethyl pyrocarbonate, and the ribonucleoside 2′,3′-monophosphates (cyclic) of adenine, guanine, cytosine, and uracil were purchased from Sigma. Other compounds and their suppliers were as follows: polyuridylic acid, Schwarz/Mann; vanadyl sulfate, Fisher; proteinase K, E. Merck Darmstadt; ribonucleoside 3′,5′-bis-

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[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.