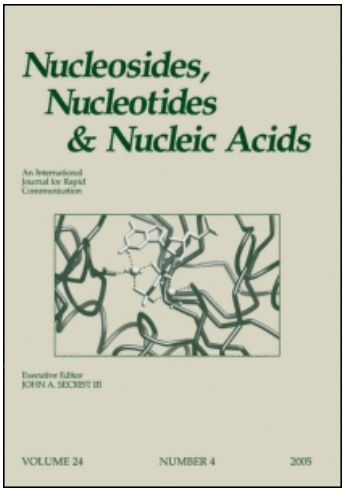

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PHOSPHONOACETATE DERIVATIVES OF OLIGODEOXYRIBONUCLEOTIDES

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PHOSPHONOACETATE DERIVATIVES OF OLIGODEOXYRIBONUCLEOTIDES

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ABSTRACT. A deoxyribodinucleotide phosphonoacetate derivative has been prepared, separated into individual diastereomers, and incorporated into oligodeoxyribonucleotides possessing alternating phosphodiester and phosphonoacetate backbone linkages. The hybridization properties and enzymatic stabilities of these oligonucleotides have been studied.

A variety of modifications to the phosphodiester backbone of oligonucleotides have recently been made in attempts to avoid the inherent limitations of natural oligonucleotides. Modifications at phosphorus have included neutral, as well as positively and negatively charged species. In terms of neutral modifications to the backbone, oligonucleotide methyl phosphonates have been most extensively investigated[1-4]. Other neutral backbone modifications include phosphoramidates, in which one of the non-bridging atoms has been replaced by a nitrogen-containing moiety[5,6] and hydroxymethyl phosphonates[7]. Backbone modifications have also been prepared in which one of the non-bridging oxygen atoms attached to phosphorus has been replaced by a positively charged alkylammonium group, and some oligodeoxynucleotides with an alternating alkylammonium phosphonate/phosphodiester backbone have been shown to hybridize to complementary phosphodiester sequences more strongly than their natural counterparts.[8,9] Oligonucleotides with negatively charged backbone

modifications, such as phosphorothioates, have also been thoroughly investigated[10,11] and some compounds in this series are being clinically evaluated. A few other negatively charged backbone modifications, such as phosphorodithioates[12,13] have been reported, although no examples of negatively charged phosphonate derivatives have been described in the literature. Phosphonoacetate derivatives of mononucleotides have been prepared and shown to exhibit antiviral activity,[14-16] but to our knowledge, this modification has not been incorporated into an oligonucleotide backbone. Phosphonoacetate derivatives of oligonucleotides might be of biological interest, since the phosphonoacetate groups could provide the negative charges that appear to be essential for induction of RNase activity whilst being resistant to degradation by nucleases. In addition, the negative charges might play an important role in maintaining water solubility. Initial compounds in this series are described in this report.

EXPERIMENTAL PROCEDURES

Materials and Methods. Thermal denaturation experiments were performed on a Gilford Response temperature controlled spectrophotometer with 400 μL quartz cuvettes. ^{1}H NMR spectra were recorded at 500 MHz on a Varian Unity Plus spectrometer in DMSO-d_6 using adventitious DMSO-H_6 as an internal reference. ^{31}P spectra were recorded at 202 MHz on a Varian Unity Plus 500 MHz spectrometer or at 162 MHz on a Varian VXR-400 spectrometer in DMSO-d_6 using phosphoric acid as an external standard. Preparative normal phase HPLC (NP-HPLC) was performed using a Rainin Dynamax silica column. Silica gel high performance thin layer chromatography (HPTLC) plates were obtained from E. Merck, Gibbstown, NJ.

Methyl phosphonoacetate, triethylammonium salt (1). Trimethyl phosphonoacetate (14.9 g, 82 mmol) in dry acetonitrile (80 mL) was cooled in an ice/salt bath under dry nitrogen and bromotrimethylsilane (25 g, 163 mmol) was slowly added. The ice bath was removed and the mixture was allowed to stir at room temperature for 1 h. The solvents were evaporated and the residue treated with dry triethylamine (TEA, 23 mL, 163 mmol) in dry methanol (80 mL). The solvents were evaporated to give an oil which was purified by column chromatography on silica gel (200 g), using a gradient of methanol (0-5%) in dichloromethane, followed by neat methanol. Fractions containing pure product were evaporated to give 18 g of **1** as a pale yellow oil (86% yield). TLC (9:1

CH_2Cl_2 / MeOH), R_f = 0.15. ^1H NMR (DMSO-d_6) δ (ppm) 6.3 (br s, 2H, NH+), 3.67 (s, 3H, OCH_3), 3.10 (q, 6H, J = 7.3 Hz, C_2H_5), 2.69 (d, 2H, J_{PH} = 20.5 Hz, PCH_2), 1.31 (t, 9H, J = 7.3 Hz, C_2H_5). ^{31}P NMR (DMSO-d_6) δ 9.8 (t, J_{PH} = 20.5 Hz).

5'-*O*-(Dimethoxytrityl)thymidine-3'-(methoxycarbonylmethyl)-phosphonate, triethylammonium salt (2). Compound **1** (16.2 g, 63 mmol) and 5'-*O*-(dimethoxytrityl)thymidine (8.6 g, 16 mmol) were coevaporated with dry pyridine (2 x 50 mL), dissolved in dry pyridine (50 mL) and the volume reduced to 30 mL by evaporation. Dicyclohexylcarbodiimide (37.6 g, 182 mmol) was coevaporated with dry pyridine (80 mL), dissolved in dry pyridine (80 mL) and the volume reduced to 50 mL by evaporation. The dicyclohexylcarbodiimide (DCC) solution was transferred to the reaction flask via canula, the flask rinsed with dry pyridine (40 mL), and this solution was added to the reaction flask. The reaction mixture was stirred under nitrogen at 37°C for 16 h and cooled to room temperature. Water (10 mL) was added and the mixture was stirred for 3 h. The resulting solid was filtered through a sintered glass Büchner funnel containing a layer (10 cm) of sand and the solid washed with methanol. The filtrate was concentrated and the crude product purified by column chromatography on silica gel (300 g), eluting with a gradient of methanol (0-25%) in dichloromethane. Fractions containing pure material were evaporated to obtain 4.9 g of **2** as a white foam (40% yield). TLC (8:2 CH_2Cl_2/MeOH) R_f = 0.15. ^1H NMR (DMSO-d_6) δ (ppm) 11.32 (s, 1H, H_3), 9.67 (br s, 1H, NH+), 7.50 (s, 1H, H_6), 7.38-6.87 (m, 13H, aromatic), 6.19 (m, 1H, $H_{1'}$), 4.76 (m, 1H, $H_{3'}$), 4.07 (m, 1H, $H_{4'}$), 3.72 (s, 6H, 2 x OCH_3), 3.42 (s, 3H, OCH_3), 3.17 (m, 2H, $J_{5',5''}$ = 10.3 Hz, $H_{5',5''}$), 3.04 (m, 4H, C_2H_5), 2.42 (d, 2H, J_{PH} = 19.8 Hz, PCH_2), 2.28 (m, 2H, $H_{2',2''}$), 1.34 (s, 3H, CH_3), 1.15 (m, 6H, C_2H_5). ^{31}P NMR (DMSO-d_6) δ 7.99 (t, J_{PH} = 20.5 Hz).

***O*-[5'-*O*-(Dimethoxytrityl)thymid-3'-yl]-*O*-(thymidin-5'-yl)-[(methoxycarbonyl)methyl]phosphonate (3a & 3b).** Compound **2** (4.9 g, 6.3 mmol) and thymidine (2.0 g, 8.2 mmol) were coevaporated with dry pyridine (25 mL) and dissolved in dry pyridine (25 mL). A solution of 1-(2,4,6-trimethylbenzenesulfonyl)-3-nitrotriazolide (MSNT, 4.9 g, 16.4 mmol) in dry pyridine (25 mL) was prepared and added to the reaction flask via canula. The reaction mixture was stirred under nitrogen for 3 h at room temperature. This mixture was poured into 5% aqueous sodium bicarbonate (200 mL) and

extracted with dichloromethane (3 x 200 mL). The combined organic layers were washed with saturated brine (250 mL), the brine layer backwashed with dichloromethane (2 x 75 mL), and the combined organic layers dried over anhydrous sodium sulfate. The solvents were evaporated and the residue coevaporated with toluene, methanol, hexane and dichloromethane (2 x 50 mL). The resulting pale yellow foam was purified by NP-HPLC using 99:1:0.1 dichloromethane/methanol/TEA as solvent to obtain 5.0 g of a mixture of **3a** and **3b** (88% yield). HPTLC (9:1 CH_2Cl_2/EtOH), R_f = 0.41, 0.37. The mixture of isomers (4.45 g) was loaded in 250 mg portions dissolved in 3 mL of dichloromethane onto the NP-HPLC column and eluted with 95:5 dichloromethane/methanol. The eluant was monitored by HPTLC and fractions containing the pure faster eluting isomer **3a** were pooled and the remaining fractions were concentrated, loaded onto the NP-HPLC column, and eluted with a gradient of methanol in dichloromethane (4-5%) over 60 min. Fractions containing the pure slower eluting isomer **3b** were pooled and evaporated. By this procedure 610 mg (11% yield) of **3a** and 840 mg (15% yield) of **3b** were isolated, together with 1.8 g (32% yield) of the mixture of isomers.

3a: ^{1}H NMR (DMSO-d_6) δ (ppm) 11.32 (s, 1H, H_3), 9.67 (br s, 1H, NH+), 7.50 (s, 1H, H_6), 7.38-6.87 (m, 13H, aromatic), 6.19 (m, 1H, $H_{1'}$), 4.76 (m, 1H, $H_{3'}$), 4.07 (m, 1H, $H_{4'}$), 3.72 (s, 6H, 2 x OCH_3), 3.42 (s, 3H, OCH_3), 3.17 (m, 2H, $J_{5',5''}$ = 10.3 Hz, $H_{5',5''}$), 3.04 (m, 4H, C_2H_5), 2.42 (d, 2H, J_{PH} = 19.8 Hz, PCH_2), 2.28 (m, 2H, $H_{2',2''}$), 1.34 (s, 3H, CH_3), 1.15 (m, 6H, C_2H_5). ^{31}P NMR (DMSO-d_6) δ (ppm) 22.5 (m).

3b: ^{1}H NMR (DMSO-d_6) δ (ppm) 11.32 (s, 1H, H_3), 9.67 (br s, 1H, NH+), 7.50 (s, 1H, H_6), 7.38-6.87 (m, 13H, aromatic), 6.19 (m, 1H, $H_{1'}$), 4.76 (m, 1H, $H_{3'}$), 4.07 (m, 1H, $H_{4'}$), 3.72 (s, 6H, 2 x OCH_3), 3.42 (s, 3H, OCH_3), 3.17 (m, 2H, $J_{5',5''}$ = 10.3 Hz, $H_{5',5''}$), 3.04 (m, 4H, C_2H_5), 2.42 (d, 2H, J_{PH}= 19.8 Hz, PCH_2), 2.28 (m, 2H, $H_{2',2''}$), 1.34 (s, 3H, CH_3), 1.15 (m, 6H, C_2H_5). ^{31}P NMR (DMSO-d_6) δ (ppm) 21.8 (m).

***O*-[5'-*O*-(Dimethoxytrityl)thymid-3'-yl]-*O*-(thymidin-5'-yl)-(carboxymethyl)phosphonate (4a & b).** Compound **3a** (60 mg, 0.07 mmol) was dissolved in a mixture of methanol (7.5 mL), water (7.5 mL), and piperidine (150 μL; 1.5 mmol) and stirred at room temperature for 6 h. The solvents were evaporated and the residue coevaporated with methanol. The crude product was purified by reverse phase C_4 HPLC, eluting with a gradient of acetonitrile (30-50%) in 0.1 M triethylammonium acetate (TEAA) over 50 min. The fractions

containing pure product were extracted with dichloromethane (6 x 20 mL) and the organic layers were dried over anhydrous sodium carbonate. After filtration, the solvents were evaporated, and the residue was coevaporated with methanol to give 24 mg of **4a** as a white solid (41% yield). ^{1}H NMR (DMSO-*d*$_6$) δ (ppm) 11.29 (br s, 2H, 2 x H3), 7.52 (s, 1H, H6), 7.47 (s, 1H, H6), 7.36-6.86 (m, 13H, aromatic), 6.19 (dd, 1H, J = 6.1 Hz, 8.1 Hz, H1'), 6.14 (t, 1H, J = 6.7 Hz, H1'), 5.76 (br s, 1H, 3'-OH), 5.18 (m, 1H, H3'), 4.29 (m, 1H, H3'), 4.18 (m, 1H, H5'), 4.13 (m, 1H, H4'), 3.92 (m, 1H, H5''), 3.80 (m, 1H, H4'), 3.72 (s, 6H, 2 x OCH3 trityl), 3.27 (m, 1H, H5'), 3.16 (m, 1H, H5''), 2.67 (d, 2H, JPH = 19.6 Hz, PCH2), 2.52-2.50 (m, 2H, H2'), 2.43-2.37 (m, 1H, H2''), 2.19-2.14 (m, 1H, H2'), 2.04-1.99 (m, 1H, H2''), 1.74 (s, 3H, CH3), 1.40 (s, 3H, CH3). ^{31}P NMR (DMSO-*d*$_6$) δ (ppm) 21.2 (m).

Using a similar procedure 87 mg of **3b** was treated for 7.5 h to give 30 mg of **4b** as a white solid (35% yield) after identical workup and purification. ^{1}H NMR (DMSO-*d*$_6$) δ (ppm) 11.35 (s, 1H, H3), 11.27 (s, 1H, H3), 7.53 (s, 1H, H6), 7.45 (s, 1H, H6), 7.37-6.87 (m, 13H, aromatic), 6.21 (t, 1H, J = 7 Hz, H1'), 6.15 (t, 1H, J = 7.0 Hz, H1'), 5.21 (m, 1H, H3'), 4.23 (m, 1H, H3'), 4.19 (m, 1H, H4'), 4.15-4.14 (m, 2H, H5',5''), 3.91 (m, 1H, H4'), 3.72 (s, 6H, 2 x OCH3 trityl), 3.25-3.16 (m, 2H, J5',5'' = 8.2 Hz, H5',5''), 3.03 (d, 2H, JPH = 19.6 Hz, PCH2), 2.44 (m, 2H, H2',2''), 2.14-2.07 (m, 2H, H2',2''), 1.74 (s, 3H, CH3), 1.36 (s, 3H, CH3),. ^{31}P NMR (DMSO-*d*$_6$) δ (ppm) 19.7 (m).

O-(Thymid-3'-yl)-O-(thymidin-5'-yl)-(carboxymethyl)phosphonate (5a). Compound **3a** (40 mg, 0.044 mmol) was dissolved in methanol (1 mL), water (1 mL), and piperidine (19.8 μL, 0.2 mmol) and shaken at room temperature for 17 h. The solvents were evaporated and the residue treated with 3% dichloroacetic acid in dichloromethane (2 mL). After agitating the mixture for 10 min, ether (10 mL) and water (5 mL) were added. The ether layer was decanted and the aqueous layer extracted with ether (10 mL). The aqueous layer was concentrated and the crude product was purified by reverse phase C4 HPLC, eluting with a gradient of acetonitrile (5-60%) in water over 45 min. The solvents were evaporated to give **5a** as a white solid. ^{1}H NMR (DMSO-*d*$_6$) d (ppm) 11.31 (br s, 1H, H3), 11.28 (br s, 1H, H3),7.68 (s, 1H, H6), 7.51 (s, 1H, H6), 6.18 (m, 2H, 2 x H1'), 5.07 (m, 1H, H3'), 4.28 (m, 1H, H3'), 4.21 (m, 1H, H5'), 4.11 (m, 1H, H5''), 4.02 (m, 1H, H4'), 3.90 (m, 1H, H4'), 3.59 (m, 2H, H5',5''), 2.95 (d, 2H, JPH = 20.5 Hz, PCH2), 2.35 (m, 1H, H2'), 2.26 (m, 1H, H2''), 2.16 (m, 1H, H2'), 2.06 (m, 1H, H2''), 1.79 (s, 3H, CH3), 1.40 (s, 3H, CH3). ^{31}P NMR (DMSO-*d*$_6$) δ (ppm) 23.8 (m).

Compound **5b** was prepared using essentially the same procedure. ^{1}H NMR (DMSO-d_6) δ (ppm) 11.30 (br s, 1H, H_3), 11.26 (br s, 1H, H_3), 7.69 (s, 1H, H_6), 7.57 (s, 1H, H_6), 6.17 (m, 2H, 2 x $H_{1'}$), 5.07 (m, 1H, $H_{3'}$), 4.26 (m, 1H, $H_{3'}$), 4.16 (m, 1H, $H_{5',5''}$), 4.05 (m, 1H, $H_{4'}$), 3.90 (m, 1H, $H_{4'}$), 3.60 (m, 2H, $H_{5',5''}$), 2.94 (d, 2H, J_{PH} = 20.5 Hz, PCH_2), 2.30 (m, 2H, $H_{2',2''}$), 2.14 (m, 1H, $H_{2'}$), 2.06 (m, 1H, $H_{2''}$), 1.78 (s, 3H, CH_3), 1.76 (s, 3H, CH_3). ^{31}P NMR (DMSO-d_6) δ (ppm) 24.3 (m).

O-[5'-O-(Dimethoxytrityl)thymidin-3'-yl]-O-[thymidine-3'-O-(cyanoethoxy-N,N-diisopropylaminophosphine)-5'-yl]-[(methoxycarbonyl)methyl]phosphonate (6a). Compound **3a** (610 mg, 0.67 mmol) was dried over phosphorus pentoxide for 16 h, dissolved in dry dichloromethane (10 mL) containing dry diisopropylethylamine (DIPEA, 350 μl, 2.0 mmol). Chloro-β-cyanoethyl N,N-diisopropyl-phosphoramidite (250 μl, 1.0 mmol) was added dropwise over 5 min and the reaction was stirred under dry nitrogen for 2 h at room temperature. The solvents were evaporated, the residue dissolved in ethyl acetate/0.1% TEA (75 mL), and the mixture was washed with 5% aqueous sodium bicarbonate (50 mL). The aqueous layer was extracted with ethyl acetate/0.1% TEA (75 mL) and the combined organic layers were washed with saturated brine. The brine layer was back extracted with ethyl acetate/0.1% TEA (2 x 50 mL) and the combined organic layers were dried over anhydrous sodium sulfate. The solvents were evaporated to give a pale yellow foam which was purified by NP-HPLC using a gradient of methanol in CH_2Cl_2 (0.5-2%) containing TEA (0.1%) to obtain 530 mg (71% yield) of pure dimer phosphoramidite **6a**. HPTLC (9:1 CH_2Cl_2/EtOH) R_f = 0.47. ^{31}P NMR (DMSO-d_6) δ (ppm) 148.7 (m), 22.5 (m).

Compound **6b** was obtained by the same procedure. HPTLC (9:1 CH_2Cl_2/EtOH) R_f = 0.54. ^{31}P NMR (DMSO-d_6) δ (ppm) 148.7 (m), 21.9 (m).

Oligonucleotide Synthesis. Oligodeoxynucleotides were synthesized on a 1 μmol scale using thymidine derivatized controlled-pore glass columns on a DNA synthesizer. The alternating 13-mers **7a** and **7b** were synthesized from dimer phosphoramidites **6a** and **6b**, respectively, dissolved in acetonitrile (0.2 M) using a standard 1 μmol synthesis cycle modified with 5 min coupling times. The average stepwise coupling yield was >98%. Cleavage of the oligomers from the support and partial deprotection of the phosphonoacetate groups was accomplished by treatment of the resin with 0.1 M piperidine in 1:1

methanol/water (1 mL) for 16 h at room temperature. The supernatant was decanted, evaporated to dryness and analysis of this material by HPLC showed the presence of mostly methyl ester protected oligomer. This material was treated with 0.1 M piperidine in 1:1 methanol/water (1mL) for 72 h at room temperature, and the solvents evaporated to dryness. Analysis of the trityl-on oligomers showed complete removal of the methyl ester protecting groups. The crude, protected, trityl-on oligomers were purified on a preparative C_4 reverse phase column with a gradient of acetonitrile in TEAA (5-15%) over 10 min, and acetonitrile in TEAA (15-45%) over 15 min at a flow rate of 7 mL/min. Fractions containing the pure oligomers were evaporated to dryness. Detritylation of the oligomers was carried out by treatment with 0.2 M aqueous acetic acid for 45 min at room temperature followed by extraction of the dimethoxytrityl alcohol with ethyl acetate (2 x 1 mL) and ether (1 mL). The aqueous layer was lyophilized and the crude trityl-off oligomers were purified and desalted on a semi-preparative C_4 reverse phase column with a gradient of acetonitrile (5-15%) in TEAA over 10 min, followed by 15-45% over 15 min at a flow rate of 7 mL/min. Fractions containing pure trityl-off oligomer were lyophilized to give pure **7a** (21.9 OD_{260}) and **7b** (34.3 OD_{260}). These materials were converted to the sodium form by passing an aqueous solution of the oligomers through a sodium ion exchange resin, followed by lyophilization.

Thermal Denaturation Experiments. Hybridization studies were carried out in low salt (150 mM NaCl, 10 mM Na_2HPO_4) and high salt (1 M NaCl, 10 mM Na_2HPO_4) buffers at pH 7 with equimolar amounts of **7a** or **7b** and d-A(pA)$_{12}$ as the complementary strand. Changes in absorbance were monitored at 260 nm whilst heating or cooling at a rate of 1°C/min between 5 and 75°C. Two complete heating and cooling cycles were performed with no change in the shapes of the curves or the transition temperatures. The transition temperatures were obtained from the first derivative of the absorbance versus temperature plot. Extinction coefficients for the oligomers were calculated by a published method.[17]

Enzymatic Degradation. Solutions of **7a**, **7b**, or d-T(pT)$_{12}$ (0.3 OD_{260}) in 2 μL of water, 55 μL 0.1 M TEAA buffer (pH 8.8) and 3 μL (0.01 unit) snake venom phosphodiesterase in water were incubated at 37°C. Aliquots (10 μL) were periodically removed and analyzed on a PRP-1 column using a gradient of acetonitrile (5-15%) in TEAA over 3 min, followed by 15-35% over 30 min

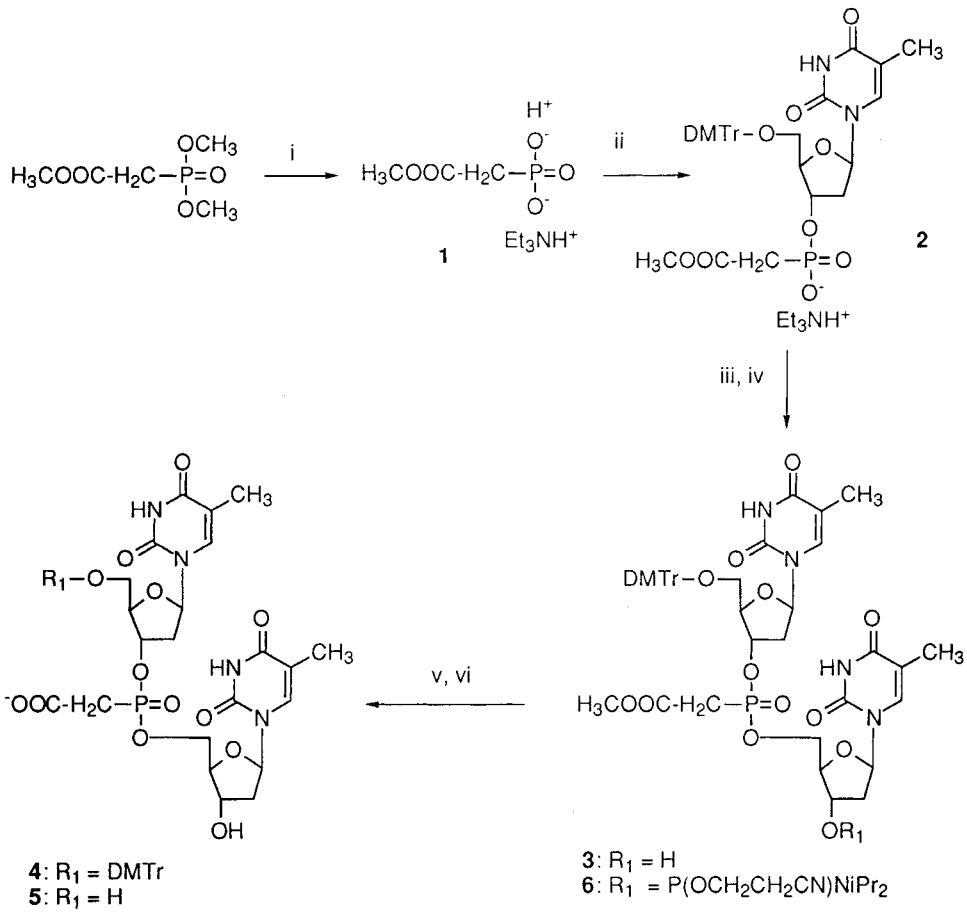
with a flow rate of 1 mL/min. Degradation was determined by measurement of the decrease in the area of the peak corresponding to starting material.

Thermal Stability of the Deprotected Dimers 5a and 5b. Solutions of **5a** or **5b** (5 mg) in water (1 mL) were incubated at 37°C for 96 h and analyzed on an analytical reverse phase C_{18} column using a gradient of acetonitrile (5-40%) in TEAA over 5 min, followed by 40-60% over 20 min with a flow rate of 1 mL/min. Dimers **5a** and **5b** were unchanged by this treatment.

RESULTS AND DISCUSSION

Synthetic Strategy. The synthesis of a phosphonoacetate derivative of a dinucleotide can in principle be carried out using one of two general nucleotide-linking chemistries: a) a phosphotriester type of coupling involving a pentavalent phosphorus nucleotide monomer, or b) a phosphoramidite type of coupling involving a trivalent phosphorus intermediate. Since mononucleotide phosphonoacetates have been previously prepared using a readily available pentavalent phosphonoacetate derivative[14-16], this factor made the phosphotriester route a more reasonable approach for the preparation of dinucleotide phosphonoacetates. These dimers can then be coupled using phosphoramidite chemistry to give oligonucleotides with alternating phosphonoacetate/phosphodiester backbones. This approach was followed in the present work.

Dimer Synthesis. Trimethyl phosphonoacetate was used as the starting material and treatment with bromotrimethylsilane[18] followed by hydrolysis of the intermediate methyl phosphonoacetate bis-trimethylsilyl anhydride with TEA in anhydrous methanol gave the monotriethylammonium salt of methyl phosphonoacetate (**1**, Figure 1) as an oil in 85% yield. Compound **1** was condensed with 5'-(dimethoxytrityl)thymidine using DCC in pyridine at 37°C for 16 h to give **2** as a white foam in 40% yield after purification. Variation of the conditions did not produce any further improvement. No reaction was observed using 2,4,6-triisopropylbenzenesulfonyl chloride as a condensing agent, which is surprising in view of the fact that this reagent is known to be a powerful condensing agent for the synthesis of nucleotides. Reaction with DCC in pyridine at room temperature gave no reaction after 7 days and reaction at 50°C gave lower yields of monomer. Condensation of the mononucleotide phosphonoacetate **2** with thymidine was carried out in pyridine with MSNT as



(i) (a) $(CH_3)_3SiI$, (b) TEA/MeOH; (ii) DCC; (iii) MSNT/thymidine; (iv) tetrazole/CH_3CN/chloro-β-cyanoethyl-diisopropylamino-phosphite; (v) 0.1 M piperidine in 1:1 MeOH/H_2O, (vi) 3% Dichloroacetic acid.

FIGURE 1

the condensing agent for 3 h at room temperature, and HPTLC of the reaction mixture showed a major spot, together with much smaller amounts of faster running material, which was assumed to be the 3',3'-dimer. The formation of 3',5'-dimers in preference to 3',3'-dimers is to be expected due to the greater reactivity of the primary hydroxyl group. After purification of the reaction mixture by normal phase HPLC, a mixture of the Rp and Sp dimers **3a** and **3b** was obtained in 88% yield.

The individual R and S isomers were separated by repeated purification using normal phase HPLC and 610 mg (11% yield from **2**) of the faster running Sp isomer **3a** and 810 mg (15% yield from **2**) of the slower running Rp isomer **3b** were obtained by this procedure.

A variety of conditions for cleavage of the methyl ester functionality of **3** were explored using the mixture of Sp and Rp isomers as the starting material. Treatment with 29% ammonium hydroxide at room temperature for 15 min cleaved the dinucleotide bond to give thymidine. Conditions previously used for deprotection and cleavage of base labile oligonucleotide methyl phosphonates were therefore explored.[19] Treatment of the dimer with hydrazine for 24 h, followed by ethylenediamine in anhydrous ethanol for 6 h, again resulted in cleavage of the internucleotide bond. Organic amines in aqueous organic solvent mixtures were also considered for deprotection of the methyl ester, because of the poor solubility of the protected dimer in aqueous solution. The dimer was stable to a solution of 0.1 M TEA or piperidine in acetonitrile or methanol, but was partially cleaved to thymidine by treatment with 0.1 M solutions of piperidine or TEA in water at room temperature for 24 h. Hydrolysis of the methyl ester proceeded slowly in 0.1 M solutions of TEA or piperidine containing 0.1 M water. Deprotection of the methyl phosphonoacetate dinucleotides **3a** and **3b** to give the phosphonoacetate salts **4a** and **4b** was accomplished by stirring with a solution of 0.1 M piperidine in 1:1 methanol/water. Thus, **3a** was stirred for 6 h at room temperature to give complete conversion to **4a**, with no cleavage to thymidine as observed by HPLC. An amount of 24 mg of the deprotected dimer **4a** was isolated in 24% yield after purification by HPLC on a semi-preparative C_4 reverse phase column. Similarly, isomer **3b** was stirred for 7.5 h at room temperature to give 30 mg of **4b** in 35% yield. Detritylation of the Sp and Rp phosphonoacetate dinucleotides **4a** and **4b** was carried out by treatment with 3% dichloroacetic acid in dichloromethane for 10 min, followed by removal of the dimethoxytrityl alcohol by extraction with ether and purification of the crude residue by reverse phase HPLC.

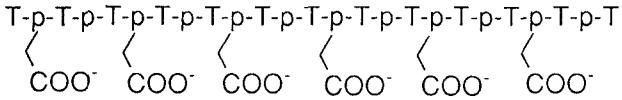
Oligonucleotide Synthesis. The protected dimers **3a** and **3b** were phosphitylated according to a standard protocol[20] and the corresponding phosphoramidates **6a** and **6b** were obtained in good yield. The dimer phosphoramidites were dissolved in anhydrous acetonitrile to give 0.2 M solutions and coupled to a commercial CPG-T support for 5 min using a

standard phosphoramidite coupling cycle on a DNA synthesizer. The alternating phosphodiester/phosphonoacetate backbone modified 13-mer **7a** (Figure 2), prepared from the Sp dimer phosphoramidite, was synthesized with stepwise coupling yields of amost 100%, as measured by spectrophotometric assay of the cleaved dimethoxytrityl groups at 498 nm. The alternating 13-mer **7b** was prepared in the same way, with average stepwise coupling yields of >98%.

Cleavage of the oligonucleotides from the CPG support was achieved by treatment with 0.1 M piperidine in 1:1 methanol/water for 16 h at room temperature; these conditions were shown to completely cleave the control sequence d-T(pT)$_6$ from the support. Analysis of the crude oligomers by HPLC revealed the presence of trityl-on, partially methyl esterified species.

Retreatment of these mixtures with additional 0.1 M piperidine in 1:1 methanol/water for a total of 72 h, followed by analysis of the crude mixture by HPLC showed primarily single peaks, indicating complete hydrolysis of the methyl ester groups, without any increase in the amounts of failure sequences which would be produced by internucleotide cleavage. The crude oligomers were purified from failure sequences by HPLC on a semi-preparative C_4 column, and detritylated by treatment with 0.2 M aqueous acetic acid. The trityl-off oligomers were then purified and desalted by HPLC on a semi-preparative C_4 column to yield 21.9 OD_{260} of **7a** and 34.3 OD_{260} of **7b**. The purified, deprotected, trityl off oligomers were converted to the sodium form by passing through a column of ion exchange resin. Analysis of the oligomers **7a** and **7b** by ion exchange HPLC showed that these compounds elute at roughly the same retention time as d-T(pT)$_{12}$, an oligonucleotide with the same number of negative charges.

Assignment of Stereochemistry at Phosphorus. A preliminary assignment of the stereochemistry at phosphorus of the partially deprotected dimers **4a** and **4b** was made by 2-D ROESY NMR, using a modification of the technique of Löschner and Engels.[21] These investigators previously investigated the NMR of a dinucleotide methyl phosphonate and reported a difference in the observed intensities of interaction between the 3'- and 4'-hydrogens of the 3'-phosphorylated sugar moiety and the hydrogens of the methyl phosphonate group. The faster eluting dimer **4a** showed a weaker interaction between the phosphonate methylene and the 4'-hydrogens of the 5'-terminal sugar as compared with **4b**, suggesting that **4a** is of Sp



7a: Sp ISOMER
7b: Rp ISOMER

FIGURE 2

ALTERNATING PHOSPHONOACETATE/PHOSPHODIESTER OLIGONUCLEOTIDES

stereochemistry. Analysis of the ROESY NMR spectra of the fully deprotected isomers **5a** and **5b** showed a similar result. A weaker interaction between the phosphonate methylene and the 4'-hydrogens was observed for **5a** as compared with **5b**, indicating that **5a** is probably of Sp stereochemistry. This sterochemical assignment is consistent with the hybridization properties of the oligonucleotides derived from these dimers as described below.

Hybridization Studies. The alternating phosphodiester/phosphonoacetate oligomers **7a** and **7b** were hybridized to the complementary deoxynucleotide d-A(pA)$_{12}$ and the results are shown in Table 1. The duplex of oligonucleotide **7a**, prepared from the Sp dimer, showed a melting temperature 17°C lower than the corresponding natural duplex at both low and high salt concentrations. This represents almost 3°C of duplex destabilization for each Sp phosphonoacetate group relative to the corresponding natural duplex.

The oligomer **7b**, prepared from the Rp dimer, displayed a melting temperature 4°C higher than a native control at low salt and 5°C higher under high salt conditions. A recent investigation of the hybridization of octa(thymidine-Rp-methanephosphonate) to a natural complementary sequence reported a melting temperature 25°C higher than a native control, which again represents a 3°C duplex stabilization for each Rp methyl phosphonate backbone modification.[22] In contrast, the corresponding duplex of the octa(thymidine Sp methanephosphonate) melted at <2°C. These investigators postulated that the Rp methyl isomer with an "outward" or "pseudoequatorial" orientation can form stable duplexes, but the Sp methyl isomer perturbs the base stacking and destabilizes duplex formation. The Sp

TABLE 1

HYBRIDIZATION OF MODIFIED OLIGONUCLEOTIDES TO d-A(pA)12

Oligonucleotide	Backbone	Isomer	Melting Temp (°C) Low Salt	High Salt
d-T(pT)12	P-O⁻	n/a	39	50
7a	P-O⁻/P-CH2COO⁻	Sp	22	33
7b	P-O⁻/P-CH2COO⁻	Rp	43	55

phosphonoacetate would be expected to perturb the base stacking due to steric interactions in the same way as the methyl phosphonate. The Rp phosphonoacetate/phosphodiester alternating 13-mer forms a duplex with similar stability to a native duplex. This is to be expected since the negatively charged phosphonoacetate group does not give rise to any additional duplex stabilization as compared with the neutral Rp methyl phosphonate group, where the charge-charge repulsion in the duplex is removed.[22] Standard sigmoidal, biphasic transition curves were observed in all cases, suggesting transitions between single-stranded and duplex forms; no evidence for triplex formation was obtained.

Enzymatic Stability. The stability of phosphodiester/phosphonoacetate backbone-modified oligomers towards exonuclease activity was measured by incubating **7a** and **7b** with snake venom phosphodiesterase at 37°C. The Sp oligomer **7a** had a half-life of approximately 2 h, whereas the Rp oligomer **7b** was less stable, with a half-life of approximately 1 h. The natural sequence d-T(pT)12 was completely degraded within 1 h under these conditions. Thus the presence of phosphonoacetate groups in the backbone of an oligonucleotide produces a marked increase in the stability towards nucleases. In a separate experiment, the dimers **5a** and **5b** were unchanged after incubation for 4 days at 37°C in water, indicating that the phosphonoacetate linkage is not susceptible to hydrolysis in aqueous solution at neutral pH.

CONCLUSIONS

Dinucleotide phosphonoacetate derivatives have been prepared and the individual isomers have been separated. These dimers have been coupled via phosphoramidite chemistry to produce oligomers with alternating phosphodiester/phosphonoacetate backbones. Phosphonoacetates were found to be more labile to alkaline conditions than phosphodiesters, so that modified deprotection conditions were therefore required to accommodate the ammonia sensitivity of the phosphonoacetate group. A duplex of oligomer **7b** (prepared from the Rp dimer) with d-A(pA)$_{12}$ displayed a thermal stability profile similar to that of the corresponding natural duplex, whereas the corresponding duplex with the Sp isomer **7a** was much less stable. Further experiments will be needed to determine the full potential of oligonucleotides of this type as antisense agents.

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