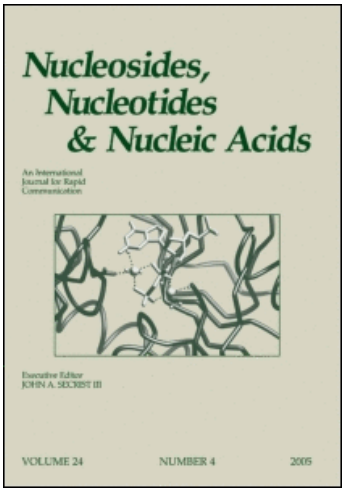

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OLIGONUCLEOTIDE (CYANOMETHYL)PHOSPHONATES

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Abstract. A dinucleoside (cyanomethyl)phosphonate has been prepared, and its properties have been studied. This compound was converted into an oligonucleotide possessing alternating (cyanomethyl)phosphonate and phosphodiester backbone groups and its hybridization to complementary DNA and RNA sequences was studied versus methylphosphonate and phosphodiester controls.

Introduction.

One approach to the design of backbone-modified oligonucleotides is to replace the anionic oxygen of a phosphodiester moiety with a neutral group, such as an alkyl- or arylphosphonate. Such substitutions might enhance cellular uptake by removal of the negative charges whilst retaining the ability of the oligonucleotide to hybridize to its complementary sequence in an antisense mode. Methylphosphonates represent the simplest example of neutral backbones, and several reports of the properties of oligonucleotide methylphosphonates have been described.[1-4] Relatively few syntheses of other types of oligonucleotide alkyl or aryl phosphonates have been reported. Short oligonucleotides containing phenylphosphonate groups have been described,[5] and relatively bulky dimethoxytritylmethylphosphonates of dinucleotides have been synthesized in low yield,[6] but neither of these phosphonates would be expected to produce stable hybrids due to their steric bulk. A dinucleotide difluoromethylphosphonate has been described and the individual isomers separated[7] but oligonucleotides possessing this type of modified backbone have not been evaluated. A dinucleotide hydroxymethylphosphonate has been prepared and the enzymatic and chemical properties of the mixture of isomers have been examined;

this approach was proposed as a methylphosphonate analog with improved solubility in water.[8] As part of a program on functionalized alkylphosphonate derivatives of oligonucleotides, we have prepared some di- and oligonucleotide (cyanomethyl)-phosphonates. These compounds might be of interest as antisense agents, since the the cyanomethyl group is relatively compact and may permit the formation of duplexes with RNA. The stability of hybrids of this type might be enhanced since the introduction of neutral backbone groups would reduce the ionic repulsions between strands produced by the phosphodiester groups. In addition, they might serve as useful synthetic precursors to (2-aminoethyl)phosphonates[9] if the nitrile could be reduced to an amino group without affecting the remainder of the molecule. The synthesis and properties of some oligonucleotides possessing (cyanomethyl)phosphonate groups are therefore described below.

RESULTS AND DISCUSSION

Synthesis.

Diethyl (cyanomethyl)phosphonate **1** (Figure 1) served as a useful starting material for the synthesis of dinucleotide (cyanomethyl)phosphonates. De-esterification of **1** with bromotrimethylsilane, followed by treatment of the de-ethylated intermediate with triethylamine produced the bis(triethylammonium) salt of **2**. Reaction of **2** with 5'-dimethoxytritylthymidine (**3**) using triisopropylbenzenesulfonyl chloride (TPS-Cl) gave the 3'-mononucleotide **4**, which was obtained in pure form by column chromatography. Condensation of this mononucleotide with thymidine using TPS-Cl as the condensing agent produced the partially protected dinucleotide **5** as a mixture of isomers which could be separated by column chromatography. As expected, the primary 5'-hydroxyl group of thymidine reacts preferentially to give the 3',5'-dimer **5**, as confirmed by the presence of an exchangeable doublet in the ^{1}H NMR spectrum, due to the secondary hydroxyl group. Small amounts of impurities were also produced, presumably including 3',3'-isomers, although these were not isolated or characterized. Presumably, the difference in reactivity between the 5'- and 3'-hydroxyl groups is sufficient to ensure the regiospecificity of the reaction to form the 3',5'-dimer. An infrared spectrum of one of the isomers of **5** showed the

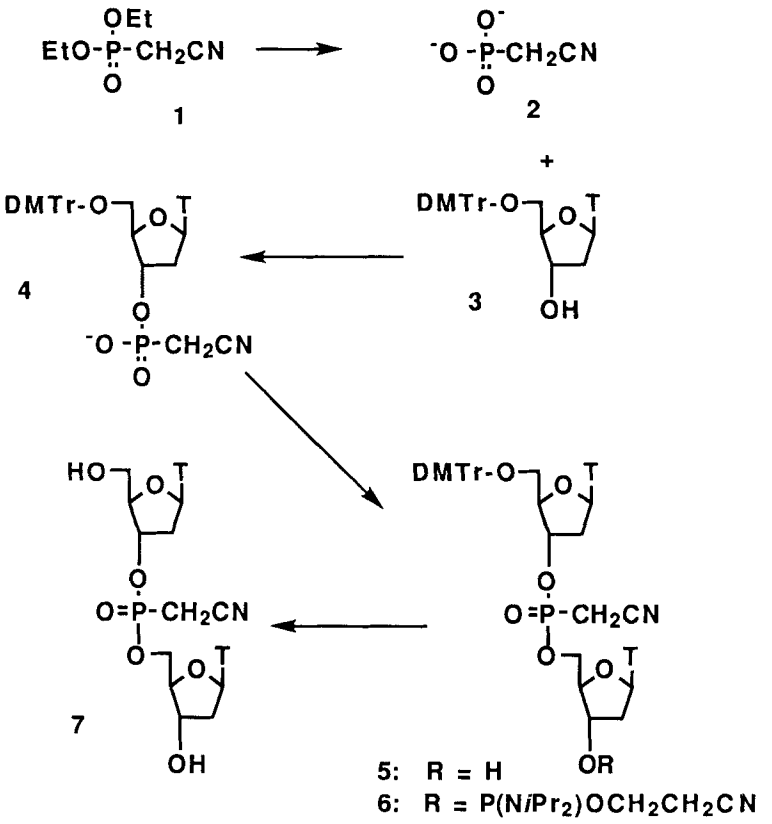


FIGURE 1

presence of a clearly distinguishable band at 2254 cm^{-1} which was attributed to the nitrile group, and the mass spectrum gave a molecular ion of 894, corresponding to the sodium adduct. Detritylation of **5** gave the unprotected dimer **7** which was used for stability and reactivity studies.

The stereochemical assignment of the individual isomers of **5** relied primarily on the relative retention times on HPLC together with ^{31}P NMR chemical shift data as compared with other phosphonate derivatives. These assignments should be considered as tentative and more rigorous analysis would be required for a definitive assignment. An attempt to apply a 2D ROESY NMR technique which has previously been used to determine the configuration at phosphorus of a series of dinucleoside

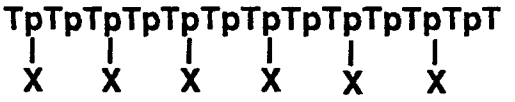
methylphosphonates,[3] was inconclusive for isomers of **5** because of the overlap between the methylene protons of the cyanomethyl groups and the methoxy groups of the trityl functionalities.

Since the nitrile group is a versatile functionality which could potentially be converted into a variety of other substituents, a few reactions directed towards transformation of the nitrile group of **7** (Rp isomer) were evaluated. Treatment with an excess of lithium aluminum hydride in THF led to decomposition as evidenced by the fact that several peaks were obtained from an HPLC analysis of the reaction mixture. Efforts to convert the cyanomethyl group to an amide by treatment with ammonium formate under reflux was also unsuccessful due to rapid cleavage of the P-O bond. Thus it appears likely that the dinucleotide is too labile to permit derivatization of the nitrile group.

The dinucleotide **5** (mixed isomers) was converted to the corresponding phosphoramidite **6** and coupled to produce the oligonucleotide **8** (Figure 2) in which the backbone consisted of alternating (cyanomethyl)phosphonate and phosphodiester linkages. The same series of reactions was also carried out on the Rp isomer, and the hybridization of these oligomers was examined as described below. The Sp isomer was not converted into an oligonucleotide since Sp isomers of other phosphonates have been shown not to produce stable duplexes.[9,10,11]

Hybridization.

The alternating (cyanomethyl)phosphonate/phosphodiester **8** (mixed isomers and Rp isomer) was hybridized to complementary DNA and RNA sequences to determine the stability of duplexes of this type. Other oligonucleotide phosphonates, including alternating methyl phosphonate/phosphodiester and (2-aminoethyl)-phosphonate/phosphodiester oligonucleotides were used as controls, and the results are displayed in Table 1. Duplexes of the (cyanomethyl)phosphonate/phosphodiester **8** with DNA or RNA were less stable than with the alternating (2-aminoethyl)-phosphonate/phosphodiester **10**, which is surprising in view of the fact that **10** possesses similar steric bulk, although the rigidity imposed by the cyano group may impart unfavorable steric interactions. Duplexes of **8** with DNA or RNA were also shown to be less stable than the methylphosphonate analog **9**. Hybrids with the Rp isomer of **8** were somewhat more stable than with the mixed



8: X = CH_2CN

9: X = CH_3

10: X = $CH_2CH_2NH_3^+$

FIGURE 2

TABLE 1

HYBRIDIZATION OF ALTERNATING BACKBONE dT_{13} OLIGONUCLEOTIDE PHOSPHONATES TO DNA AND RNA

OLIGOMER	BACKBONE TYPE	ISOMER	MELTING TEMP[a] DNA[b]	RNA
-	P-O^-	-	35	30
8	P-CH_2CN/P-O^-	Mixed	26	17
8	P-CH_2CN/P-O^-	Rp	32	21
9	P-CH_3/P-O^-	Mixed	32	19
10	P-$CH_2CH_2NH_3^+$/P-O^-	Mixed	38	23
10	P-$CH_2CH_2NH_3^+$/P-O^-	Rp	51	35

[a] In 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.

[b] DNA target = d-pA_{13}, RNA target = r-pA_{13}.

isomers, which lends support to the tentative stereochemical assignment, since previous results with methylphosphonates and (aminoalkyl)phosphonates have all shown that the Rp isomers were more stable than the mixed isomers.[9,10,11] Thus the (cyanomethyl)phosphonates are of less interest as antisense compounds due to the relatively low stability of the duplexes as compared with other phosphonate derivatives.

Hydrolytic stability.

The dinucleotide (cyanomethyl)phosphonate **7** was stored at 37°, pH 7 and aliquots were removed at intervals and analyzed by HPLC to determine the extent of degradation. Under these conditions about 25% hydrolysis was observed after several days, whereas the corresponding dinucleotide methylphosphonate was found to be completely stable. The corresponding dinucleotide (aminomethyl)phosphonate was previously reported to be quite labile, with a half life of 42 hours under the same conditions.[10] This relatively slow degradation of **7** versus the aminomethyl compound is interesting since the strongly electron-withdrawing nitrile group would be expected to make the phosphorus atom more electropositive and thus more susceptible to nucleophilic attack.

The hydrolysis of diethyl (cyanomethyl)phosphonate (**1**) was also studied to determine the effect of the different ester groups on the rate of hydrolysis. At 37° and pH 7 the diethyl ester **1** was more stable than the dinucleotide **7**, demonstrating that hydrolysis depends in part upon the nature of the ester groups. Hydrolysis of **1** was achieved with concentrated ammonium hydroxide and was almost complete after 24 h, as determined by ^{31}P NMR.

EXPERIMENTAL

General methods.

Diethyl (cyanomethyl)phosphonate was purchased from Lancaster Synthesis Inc. (Windham, NH). Ultraviolet spectra were obtained with a Shimadzu UV 160 spectrophotometer using 1 mL quartz cuvettes. Oligonucleotides were synthesized using an Applied Biosystems Model 394 DNA synthesizer. HPLC purifications were performed using a Waters 600E system controller equipped with multisolvent delivery system and a Model 991 photodiode array detector. Preparative reversed phase HPLC was performed using a Waters RCM (8 mm x 10 cm) C_4 column with a gradient of 0.1 M triethylammonium acetate buffer pH 7.0 (TEAA)/acetonitrile. NMR spectra were recorded using a Varian 500 MHz UNITY-Plus spectrometer. Unless otherwise stated, ^{31}P NMR were run in DMSO-d6, broad band decoupled, referenced to H_3PO_4 as an internal standard, and ^{1}H NMR spectra were referenced to external tetramethylsilane as standard.

Bis(Triethylammonium) (cyanomethyl)phosphonate (2).
Diethyl (cyanomethyl)phosphonate (**1**, 5.0 g, mmol) was dissolved in chloroform (40 mL), and bromotrimethylsilane was added dropwise to the solution at 0°C. After 3.5 h the reaction mixture was concentrated under reduced pressure, and the residue was redissolved in chloroform (40 mL). Triethylamine (60 mL) was added dropwise with cooling in an ice bath and after stirring at room temperature for 2 h, the mixture was concentrated to dryness. The residual oil was directly used for the next step without any further purification.

5'-Dimethoxytritylthymidine-3'-(cyanomethyl)phosphonate (4). The triethylammonium salt of **2** (5 g, 15.47 mmol) was dried by coevaporation with pyridine (3 x 10 mL), dissolved in dry pyridine (80 mL) and treated with TPS-Cl (14 g, 45 mmol) followed by a solution of 5'-dimethoxytrityl-thymidine (**3**, 8.42 g, 15.47 mmol) in dry pyridine (20 mL) which had also been previously dried by coevaporation with pyridine. The resulting mixture was stirred at room temperature for 4 h. The reaction mixture was quenched by addition of 5% sodium bicarbonate, extracted with ethyl acetate (3 x 150 mL), dried over anhydrous sodium sulfate and concentrated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using a gradient of methanol (0-7%) in CH_2Cl_2 containing 0.5% Et_3N. The appropriate fractions were collected and combined to yield 6.3 g (54% yield) of pure mononucleotide **4**.
^1H NMR (DMSO-d6): δ (ppm) 11.32 (bs, 1H, NH), 7.42 (s, 1H, H_6), 6.8-7.4 (m, 13 H, trityl), 6.0-6.19 (t, 1H, $H_{1'}$), 4.84 (m, 1H, $H_{3'}$), 4.0 (m, 1H, $H_{4'}$), 3.7 (s, 6H, OCH_3), 3.12-3.27 (m, 4H, CH_2, $H_{5',5''}$), 2.65 (m, 2H, $H_{2',2''}$), 1.3 (s, 3H, CH_3). ^{31}P NMR: δ (ppm) 6.42 (s). IR (KBr): 2245 cm^{-1}.

5'-Dimethoxytritylthymidyl-3'-(cyanomethyl)phosphonyl-5'-thymidine (5).
Compound **4** (6.3 g, 8.41 mmol) was dried by coevaporation with pyridine (3 x 15 mL), redissolved in dry pyridine (50 mL) and treated with TPS-Cl (10.0 g, 33.01 mmol) and tetrazole (0.5 g, 7.1 mmol). Thymidine (2.0 g, 8.41 mmol) was added to this mixture and after 3 h the solution was diluted with aqeous sodium bicarbonate (5%, 450

mL) and extracted with ethyl acetate (3 x 100 mL). The organic layers were combined, dried over anhydrous sodium sulfate and concentrated under reduced pressure to yield 18 g of crude material. A portion (8 g) was purified on a normal phase silica column (Dynamax 60 Å, 41.4 mm x 25 cm, Rainin, Emeryville, CA) with a linear gradient from 0-10% isopropanol in CH_2Cl_2 containing 0.1% pyridine. The desired compound was eluted at 68 minutes to yield 3.1 g of pure **5**.

Separation of Isomers of 5 by HPLC.

The mixture of isomers of **5** (1.7 g, 1.95 mmol) was dissolved in 0.1 M TEAA/acetonitrile (1:1, 15 mL) and injected in 3 separate aliquots onto a reversed-phase C_4 Vydac column (5 x 25 cm). The column was eluted with a linear gradient from 35-80% acetonitrile in 0.1 M TEAA over 55 minutes and the Sp and Rp isomers were eluted at 26-28.2 and 32-34 minutes, respectively. The appropriate fractions were pooled, an equal amount of water was added, and the solution was extracted with ethyl acetate (2 x 150 mL). The organic layer was dried over anhydrous sodium sulfate overnight and the solids were removed by filtration. The solution was evaporated and dried over P_2O_5 *in vacuo* to yield 600 mg of the faster (Sp) isomer, 440 mg of the slower (Rp) isomer and 370 mg of mixed isomers, total yield 78%.

^1H NMR (DMSO-d6): Sp isomer, δ (ppm) 11.38 (s, 1H, NH), 11.30 (s, 1H, NH), 7.46 (s, 1H, H_6), 7.39 (s, 1H, H_6), 6.8-7.3 (m, 13H, trityl), 6.22 (t, 1H, $H_{1'}$), 6.15 (t, 1H, $H_{1'}$), 5.46 (ex d, 1H, 3'-OH), 5.24 (m, 1H, $H_{3'}$), 4.14-4.21 (m, 4H, $H_{3'}$, $H_{4'}$, $H_{5',5''}$) 3.87 (m, 1H, $H_{4'}$), 3.77 (d, 2H, CH_2), 3.72 (s, 6H, OCH_3), 3.2 (m, 2H, $H_{5',5''}$), 2.46-2.52 (m, 2H, $H_{2',2''}$), 2.06-2.11 (m, 2H, $H_{2',2''}$), 1.74 (s, 3H, CH_3),1.40 (s, 3H, CH_3). IR (KBr) 2254 cm^{-1}, Mass spectrum: $(M + Na)^+$ Found 894, theory 894.

^1H NMR (DMSO-d6): Rp isomer, δ (ppm) 11.32 (s, 1H, NH), 11.28 (s, 1H, NH), 7.48 (s, 1H, H_6), 7.44 (s, 1H, H_6), 6.86-7.34 (m, 13H, trityl), 6.23 (t, 1H, $H_{1'}$), 6.14 (t, 1H, $H_{1'}$), 5.47 (ex d, 1H, 3'-OH), 5.23 (m, 1H, $H_{3'}$), 4.18-4.31 (m, 4H, $H_{3'}$, $H_{4'}$, $H_{5',5''}$) 3.93 (m, 1H, $H_{4'}$), 3.76 (d, 2H, CH_2), 3.73 (s, 6H, OCH_3), 3.17-3.20 (m, 2H, $H_{5',5''}$), 2.46-2.49 (m, 2H, $H_{2',2''}$), 2.07-2.13 (m, 2H, $H_{2',2''}$), 1.73 (s, 3H, CH_3),1.36 (s, 3H, CH_3).

^{31}P NMR (DMSO-d6): δ (ppm) Sp isomer, 21.2 (s); Rp isomer, 20.344 (s).

Dimer phosphoramidite (6).

A sample of **5** (mixture of isomers, 1.58 g, 1.8 mmol) was dried by coevaporation with pyridine, dissolved in dry acetonitrile (20 mL) under nitrogen, and treated with stirring with 2-cyanoethoxy-bis-(N,N-diisopropylamino)phosphine (1.43 mL, 4.5 mmol), diisopropylamine (0.35 mL, 2.52 mmol) and tetrazole (0.127 g, 1.8 mmol). After 2 h the reaction mixture was partitioned between water (150 mL) and CH_2Cl_2 (3 x 70 mL). The organic layers were separated, combined and evaporated to dryness. The residue was purified by silica gel column chromatography (100 g, 230-400 mesh) using a gradient of ethanol (0-1.5%) in CH_2Cl_2 containing 0.5% Et_3N. Fractions containing the desired material were combined to yield 46% of pure **6** as a white precipitate. ^{31}P NMR (DMSO-d6) δ (ppm) 150.76, 20.4, 21.2.

Isolation and hydrolytic stability of dimer 7.

The isomers of **6** were detritylated as previously described[10] and purified by reverse phase HPLC. The Rp and Sp isomers of **7** were eluted at 14.66 and 15.18 min respectively. The Sp isomer of **7** was incubated at pH 7.1 in TEAA buffer (0.1 M) at 37° and aliquots were removed at intervals and injected onto a C_4 reversed phase HPLC column. The column was eluted with a linear gradient of 30-80% acetonitrile in 0.1 M TEAA and the degradation of the dinucleotide was determined by measurement of the area remaining under the peak corresponding to starting material versus time.

Synthesis of oligonucleotides.

Oligonucleotides were synthesized on a 1 μmol scale using standard coupling cycles and reagents. For the synthesis of **8**, the dimer amidite **6** was used at a concentration of 0.1 M in acetonitrile and coupling yields of 96% were obtained as determined by trityl assay. Upon completion of the synthesis, the oligonucleotide was cleaved from the support using ethylenediamine (0.4 mL) for 30 minutes at room temperature. The supernatant was evaporated to dryness, and the support was washed with absolute ethanol (2 x 0.2 mL), TEAA (2 x 0.2 mL, 0.1 M, pH 7.0), and ethanol (2 x 0.2 mL). The washings were combined with the residue from the supernatant and the solution was evaporated to dryness. The residue was redissolved in distilled water (0.5 mL), filtered through a 0.45 μ nylon filter and the filtrate

was loaded into a semipreparative reversed phase C_4 HPLC column. The column was eluted with a gradient of 0-70% acetonitrile in 0.1 M TEAA, and the fractions eluting between 23.5-27.8 minutes were collected, lyophilized and detritylated using 0.1 M acetic acid at 45°C for 45 minutes. After lyophilization, the residue was redissolved in TEAA (1.0 mL, pH 7.0) and injected onto a C_4 column. The fractions eluting at 15-18 minutes were collected, concentrated to dryness and converted to the sodium form to yield 19.7 OD of pure **8**.

Thermal stability of duplexes.

Studies of the thermal dissociation of duplexes were carried out using a Gilford Response II temperature-controlled spectrophotometer by monitoring absorbance at 260 nm versus temperature. Oligonucleotides were mixed in an equimolar ratio, using the method of Rychlik and Rhoads[12] to determine extinction coefficients. The samples were heated at a rate of 0.5°C/min from 0-65°C in 150 mM NaCl, 10 mM Na_2HPO_4, pH 7. Transition temperatures were obtained from the first order derivative plot of absorbance versus temperature.

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