Oligonucleotides with novel, cationic backbone substituents: aminoethylphosphonates

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Received July 20, 1994; Revised and Accepted October 14, 1994

ABSTRACT

Oligonucleotide (2-aminoethyl)phosphonates in which the backbone consisted of isomerically pure, alternating (2-aminoethyl)-phosphonate and phosphodiester linkages have been prepared and characterized. One of these single isomer oligonucleotides (Rp) formed a more stable duplex with DNA or RNA than its corresponding natural counterpart. Hybrid stability was more pH-dependent, but less salt-dependent than a natural duplex. The specificity of hybridization was examined by hybridization of an oligonucleotide containing one (2-aminoethyl)phosphonate to oligonucleotides possessing mismatches in the region opposite to the aminoethyl group. In contrast to oligonucleotides containing (aminomethyl)-phosphonate linkages, oligonucleotide (2-aminoethyl)phosphonates were completely stable to hydrolysis in aqueous solution. These oligonucleotides were resistant to nuclease activity but did not induce RNase H mediated cleavage of a complementary RNA strand. Incubation in a serum-containing medium resulted in minimal degradation over 24 hours. Studies of cell uptake by flow cytometry and confocal microscopy demonstrated temperature dependent uptake and intracellular localization. (2-Aminoethyl)phosphonates represent a novel approach to the introduction of positive charges into the backbone of oligonucleotides.

INTRODUCTION

For oligonucleotides to be effective as antisense agents, they need to penetrate cell membranes and be resistant to degradation by nucleases. These requirements have stimulated efforts to prepare backbone-modified derivatives which might be capable of penetrating membranes more readily whilst retaining their resistance to degradation. One widely used approach to improve cell penetration involves removal of the negative charges to produce neutral backbones such as, for example,

methyl phosphonates [1], phosphoramidates [2] or peptide nucleic acids [3]. Methylphosphonates have been shown to be transported into cells by endocytosis [1] and were more resistant to degradation by nucleases [4].

An alternate approach to backbone modification is to replace the anionic phosphodiester groups with cationic groups. The introduction of a single cationic group could be advantageous in enhancement of binding to a specific site of a target protein or receptor, whereas the introduction of cationic groups at various positions throughout the oligonucleotide provides a method for producing molecules with widely differing charge densities. Cationic molecules have previously been used to enhance the delivery of a variety of conjugates into cells. The iron-transport protein transferrin, for example, has been conjugated to cationic molecules such as protamine or poly-lysine in order to transport nucleic acids [5], and cationic lipids have been used in a liposome-mediated transfection protocol for the introduction of DNA into the nuclei of animal cells. The delivery of proteins and peptides such as albumin [6] and enkephalin [7] across the blood-brain barrier has been enhanced by conjugation to cationic molecules such as hexamethylenediamine, and a variety of drugs, including methotrexate and daunomycin, have been conjugated to poly-lysine in attempts to improve their uptake and efficacy [8].

A few cationic molecules have been attached to oligonucleotides in attempts to improve their cellular uptake and/or activity *in vivo*. The cationic protein avidin enhanced the cellular uptake of a biotin-oligonucleotide conjugate, whereas the anionic protein streptavidin did not facilitate uptake of the same oligonucleotide [9]. Conjugation of poly-lysine to oligonucleotides directed against VSV [10] and HIV [11] sequences has been shown to produce enhanced effects as compared with their unmodified counterparts. Other methods which have been used for the introduction of positive charges into oligonucleotides include the synthesis of 2'-amino [12] or 2'-alkylamino substituents [13] and the attachment of amino-containing groups to the purine or pyrimidine bases [14]. Cationic substituents have been attached to the phosphorus atom via phosphoramidate linkages [15], although the biological properties of these compounds have not been reported.

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Cationic groups can be introduced into oligonucleotides via phosphonate derivatives in which the anionic oxygen is replaced by a cationic group via a phosphorus-carbon bond. A previous paper in this series [16] described the synthesis of oligonucleotide (aminomethyl)phosphonates, the simplest example in this series. During the course of this prior work, it became evident that although (aminomethyl)phosphonates possessed interesting properties, they were shown to be hydrolytically labile in aqueous solution. The present paper describes the synthesis and properties of a more stable representative of this class of compounds, (2-aminoethyl)-phosphonates.

EXPERIMENTAL PROCEDURES

Materials and methods

Synthesis and purification of oligonucleotides was carried out as previously described [16]. Extinction coefficients of oligonucleotides were obtained using a published procedure [17]. NMR spectra were recorded using a Varian Unity Plus 500 MHz spectrometer. Unless otherwise stated, NMR spectra were run in DMSO- d_6 . ^{31}P NMR were broad band decoupled, referenced to H_3PO_4 as an external standard, and ^1H NMR spectra were referenced to external tetramethylsilane as standard.

Pyridinium [2-(3,4,5,6-tetrabromophthalimido)ethyl]phosphonate

(2-Aminoethyl)phosphonic acid (2g, 16 mmol) was dissolved in water (20 mL), neutralized with 1N NaOH to pH 7.0 and passed through a column of Dowex 50X2-400 (40 mL, pyridinium form) ion exchange resin. The column was washed with water (3×50 mL) and the combined eluates were concentrated *in vacuo* to give 2.33 g (52%) of the pyridinium salt of (2-aminoethyl)phosphonic acid as a white solid. A portion of this material (1g, 3.53 mmol) was suspended in DMF (100 mL) and treated with 3,4,5,6-tetrabromo-phthalic anhydride (9.8 g, 21.2 mmol) under reflux overnight. The reaction mixture was cooled to room temperature to precipitate the product. The precipitate was filtered, washed with DMF (3×50 mL) followed by hexane (2×50 mL) and dried *in vacuo* over P_2O_5 to give the title compound (2.57 g, 70%) as an off-white solid. ^1H NMR: δ (ppm) 8.5, 7.7, 7.3 (m, 12H, pyridinium), 3.7 (m, 2H, CH_2), 1.8 (m, 2H, CH_2). ^{31}P NMR: δ (ppm) 21.6 (s).

The tetrachloro derivative was prepared using a similar procedure in which pyridinium (2-aminoethyl)phosphonate (1g, 3.53 mmol) was suspended in DMF (100 mL) and treated with 3,4,5,6-tetrachlorophthalic anhydride (6.05 g, 21.2 mmol). After isolation 1.94 g (89%) of pyridinium [2-(3,4,5,6-tetrachloro-phthalimido)-ethyl] phosphonate was obtained. ^1H NMR: δ (ppm) 7.8, 7.7, 7.3 (m, 12H, pyridinium), 3.7 (m, 2H, CH_2), 1.8 (m, 2H, CH_2). ^{31}P NMR: δ (ppm) 21.4 (s).

Synthesis of 5'-O-(Dimethoxytrityl)thymidine-3'-[2-(3,4,5,6-tetrabromophthalimido)ethyl]phosphonate (1)

5'-O-(Dimethoxytrityl)thymidine (0.822 g, 1.5 mmol) was dried by coevaporation with pyridine (2×5 mL), dissolved in dry pyridine (40 mL), and treated with triisopropylbenzenesulfonyl chloride (TPS-Cl, 1.45g, 4.8 mmol) followed by pyridinium [2-(3,4,5,6-tetrabromo-phthalimido)ethyl]phosphonate (1.0 g, 1.814 mmol). The resulting suspension was stirred overnight under a dry nitrogen atmosphere, diluted with water (150 mL),

Figure 1. Synthesis of dinucleotide (2-aminoethyl)phosphonates.

and extracted with ethyl acetate (2×75 mL). The combined organic layers were washed with water (100 mL), dried over anhydrous sodium sulfate, filtered, and the filtrate was evaporated to dryness to give crude 1. This material was dissolved in $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (99:1, 10 mL) and filtered through a one micron glass filter. The filtrate was purified on a Dynamax normal phase silica column (8 μ , 21.4 mm $\times$ 25 cm) with a gradient of 1–20% CH_3OH in CH_2Cl_2 containing 0.1% triethylamine (TEA). The fractions eluting at 26–34 minutes were collected and concentrated to yield 0.32 g (19%) of pure 1 as an off-white foam. ^1H NMR: δ (ppm) 11.33 (s, 1H, NH), 7.49 (s, 1H, H_6), 6.8–7.38 (m, 13H, trityl), 6.19 (t, 1H, H_1), 4.77 (m, 1H, H_3), 4.09 (s, 1H, H_4), 3.69 (s, 6H, OMe), 3.62 (m, 2H, CH_2), 3.2–3.6 (m, 2H, $\text{H}_{5',5'}$), 2.32 (m, 2H, $\text{H}_{2',2'}$), 1.68 (m, 2H, CH_2), 1.4 (s, 3H, 5- CH_3). ^{31}P NMR: δ (ppm) 17.35 (s).

The corresponding tetrachloro derivative 3 was prepared as an off-white foam by a similar procedure (63% yield) ^1H NMR: δ (ppm) 11.32 (s, 1H, NH), 7.47 (s, 1H, H_6), 6.84–7.35 (m, 13H, trityl), 6.12 (t, 1H, H_1), 4.75 (m, 1H, H_3), 4.04 (m, 1H, H_4), 3.68 (s, 6H, OMe), 3.64 (m, 2H, H_2), 3.16–3.23 (m, 2H, $\text{H}_{5',5'}$), 2.31 (m, 2H, $\text{H}_{2',2'}$), 1.70 (m, 2H, H_2), 1.38 (s, 3H, CH_3). ^{31}P NMR: δ (ppm) 16.4 (s).

O-[5'-O-(Dimethoxytrityl)thymidin-3'-yl]-O-(thymidin-5'-yl)-[2-(3,4,5,6-tetrabromophthalimido)ethyl]phosphonate (2)

Crude 1 (9 g, 7.6 mmol) and thymidine (1.5 g, 6.2 mmol) were dried by coevaporation with pyridine (2×10 mL), redissolved in dry pyridine (100 mL), and treated with TPS-Cl (8.06 g, 26.6

mmol) followed by tetrazole (0.53 g, 7.6 mmol). The reaction mixture was stirred under dry nitrogen for 4 h and partitioned between 2.5% aqueous NaHCO_3 (300 mL) and methylene chloride (2×300 mL). The organic layer was washed with water (100 mL), dried over sodium sulfate and evaporated to a foam. This material (10 g) was dissolved in CH_2Cl_2 (30 mL), filtered through a glass funnel to remove insoluble materials, and injected in three separate aliquots onto a normal phase silica column which was eluted at 40 mL/min with a linear gradient from 0–10% isopropanol in CH_2Cl_2 containing 0.1% pyridine. **2a** and **2b** were eluted at 32.2 and 31.0 minutes respectively. The appropriate fractions were pooled and evaporated to give white solids. This procedure yielded 470 mg of **2a**, 500 mg of **2b**, and 690 mg of a mixture of isomers.

2a: ^1H NMR: δ (ppm) 11.35 (s, 1H, NH), 11.27 (s, 1H, NH), 7.44 (s, 2H, H_6), 6.8–7.43 (m, 13H, trityl), 6.15 (t, 1H, H_1), 6.09 (t, 1H, H_1), 5.41 (d, 1H, 3'-OH), 5.12 (m, 1H, H_3), 4.05–4.21 (m, 3H, $\text{H}_3, \text{H}_{3',5'}$), 3.88 (m, 1H, H_4), 3.68 (m, 1H, H_4), 3.6 (s, 6H, OCH_3), 3.22 (m, 2H, CH_2), 3.15 (m, 2H, $\text{H}_{5',5'}$), 2.42 (m, 2H, $\text{H}_{2',2'}$), 2.2 (m, 4H, $\text{H}_{2',2'}, \text{CH}_2$), 1.67 (s, 3H, CH_3), 1.35 (s, 3H, CH_3). ^{31}P NMR: δ (ppm) 30.45 (s).

2b: ^1H NMR: δ (ppm) 11.36 (s, 1H, NH), 11.26 (s, 1H, NH), 7.45 (s, 1H, H_6), 7.39 (s, 1H, H_6), 6.8–7.34 (m, 13H, trityl), 6.16 (t, 1H, H_1), 6.08 (t, 1H, H_1), 5.4 (d, 1H, 3'-OH), 5.15 (m, 1H, H_3), 4.1 (m, 1H, H_3), 3.66–4.0 (m, 3H, $\text{H}_4, \text{H}_{4',5'}$), 3.6 (s, 6H, OCH_3), 3.4 (m, 2H, CH_2), 3.18 (m, 2H, $\text{H}_{5',5'}$), 2.45 (m, 2H, $\text{H}_{2',2'}$), 2.18–2.32 (m, 4H, $\text{H}_{2',2'}, \text{CH}_2$), 1.68 (s, 3H, CH_3), 1.4 (s, 3H, CH_3). ^{31}P NMR: δ (ppm) 31.03 (s).

The chloro derivatives **4a** and **4b** were prepared by a similar method.

4a: ^1H NMR: δ (ppm) 11.34 (s, 1H, NH), 11.26 (s, 1H, NH), 7.45 (s, 2H, H_6), 6.8–7.3 (m, 13H, trityl), 6.16 (t, 1H, H_1), 6.10 (t, 1H, H_1), 5.1 (m, 1H, H_3), 4.1–4.26 (m, 3H, $\text{H}_3, \text{H}_{3',5'}$), 4.14 (m, 1H, H_4), 3.88 (m, 1H, H_4), 3.7 (s, 6H, OCH_3), 3.35 (m, 2H, CH_2), 3.21–3.31 (m, 2H, $\text{H}_{5',5'}$), 2.44 (m, 2H, $\text{H}_{2',2'}$), 2.25 (m, 2H, CH_2), 2.07 (m, 2H, $\text{H}_{2',2'}$), 1.69 (s, 3H, CH_3), 1.4 (s, 3H, CH_3). ^{31}P NMR: δ (ppm) 29.52 (s).

4b: ^1H NMR: δ (ppm) 7.46 (s, 1H, H_6), 7.40 (s, 1H, H_6), 6.8–7.3 (m, 13H, trityl), 6.18 (t, 1H, H_1), 6.09 (t, 1H, H_1), 5.13 (m, 1H, H_3), 4.0–4.12 (m, 3H, $\text{H}_3, \text{H}_{3',5'}$), 3.9 (m, 1H, H_4), 3.77 (m, 3H, H_4, CH_2), 3.70 (s, 6H, OCH_3), 3.01–3.3 (m, 2H, $\text{H}_{5',5'}$), 2.46 (m, 2H, $\text{H}_{2',2'}$), 2.24 (m, 2H, CH_2), 2.0 (m, 2H, $\text{H}_{2',2'}$), 1.70 (s, 3H, CH_3), 1.43 (s, 3H, CH_3). ^{31}P NMR: δ (ppm) 29.26 (s).

O-[5'-O-(Dimethoxytrityl)thymidin-3'-yl]-O-[thymidin-3'-(cyanoethoxy-N,N-diisopropylaminophosphine)-5'-yl]-[2-(3,4,5,6-tetrabromophthalimido)ethyl]phosphonate (6)

A sample of **2** (500 mg, 0.38 mmol) was dried by coevaporation with pyridine, dissolved in dry acetonitrile (5 mL) and methylene chloride (7 mL) under nitrogen, and treated with stirring with 2-cyanoethoxy-N,N,N',N'-tetraisopropylphosphorodiamidite (1.2 mL, 3.8 mmol), diisopropylamine (0.3 mL, 2.1 mmol), and tetrazole (106 mg, 1.52 mmol). After 2 h at room temp the mixture was partitioned between 5% aqueous sodium bicarbonate and methylene chloride (50 mL of each). The aqueous layer was extracted with methylene chloride (2×70 mL) and the combined

organic layers were washed with water (2×50 mL) and dried over magnesium sulfate. The solids were removed by filtration and the filtrate was concentrated to a gum which was purified by column chromatography on silica gel (55 g). The column was eluted with a linear gradient of 0–15% ethanol in methylene chloride containing Et_3N (0.1%) and the appropriate fractions were combined and evaporated to yield the purified material as a foam. This material was dissolved in methylene chloride (10 mL) and added dropwise with stirring to pentane (450 mL). The precipitate was collected and dried to yield 448 mg (78%) of pure **6**. ^{31}P nmr δ (ppm) 31.239 (s), 150.858 (s).

The chloro derivative **7** was prepared by a similar method. Yield 173 mg (45%), ^{31}P nmr δ (ppm) 29.09 (s), 148.65 (s).

O-[5'-O-(Dimethoxytrityl)thymidin-3'-yl]-O-(thymidin-5'-yl)-(2-aminoethyl)phosphonate (5)

A sample of **2b** (36 mg, 0.03 mmol, Sp isomer) was dissolved in anhydrous CH_3CN (800 μL) and treated with ethylenediamine (400 μL). After one hour at room temperature the reaction mixture was dried under reduced pressure and coevaporated with ethanol (5×500 mL) and toluene (2×500 mL). The residue was purified on a C_4 semipreparative HPLC column (Waters RCM, 25mm $\times$ 10 cm) with a gradient of 35–80% acetonitrile in 0.1 M TEAA. The deprotected compound, eluting at 14.9 min, was collected and evaporated to dryness to give **5b**, Sp isomer, as a white solid, 9.3 mg (39%).

^1H NMR: δ (ppm) 7.47 (s, 1H, H_6), 7.46 (s, 1H, H_6), 6.86–7.43 (m, 13H, trityl), 6.20 (t, 1H, H_1), 6.15 (t, 1H, H_1), 5.09 (m, 1H, H_3), 4.16 (m, 1H, H_3), 4.11 (m, 1H, H_4), 3.94–4.07 (m, 2H, $\text{H}_{5',5'}$), 3.84 (m, 1H, H_4), 3.71 (s, 6H, OCH_3), 3.21–3.28 (m, 2H, $\text{H}_{5',5'}$), 2.74 (m, 2H, CH_2), 2.43 (m, 2H, $\text{H}_{2',2'}$), 2.06 (m, 2H, $\text{H}_{2',2'}$), 1.97 (m, 2H, $\text{CH}_2\text{-P}$, $J = 18.0$ Hz), 1.74 (s, 3H, CH_3), 1.43 (s, 3H, CH_3). ^{31}P NMR: δ (ppm) 33.014 (s).

An identical procedure was used to prepare the Rp isomer **5a**, using (70 mg, 0.05 mmol) of **2a**. The deprotected material was eluted at 16.19 min and these fractions were collected and evaporated to dryness to give **5a** as a white solid, 46.5 mg (82%).

^1H NMR: δ (ppm) 7.49 (s, 1H, H_6), 7.46 (s, 1H, H_6), 6.87–7.37 (m, 13H, trityl), 6.20 (t, 1H, H_1), 6.15 (t, 1H, H_1), 5.11 (m, 1H, H_3), 4.21 (m, 1H, H_3), 4.03–4.19 (m, 3H, $\text{H}_3, \text{H}_{5',5'}$), 3.90 (m, 1H, H_4), 3.72 (s, 6H, OCH_3), 3.20–3.27 (m, 2H, $\text{H}_{5',5'}$), 2.71 (m, 2H, CH_2), 2.41 (m, 2H, $\text{H}_{2',2'}$), 2.09 (m, 2H, $\text{H}_{2',2'}$), 1.92 (m, 2H, $J = 17.5$ Hz, $\text{CH}_2\text{-P}$), 1.73 (s, 3H, CH_3), 1.41 (s, 3H, CH_3). ^{31}P NMR: δ (ppm) 32.754 (s).

O-(Thymidin-3'-yl)-O-(thymidin-5'-yl)-(2-aminoethyl)-phosphonate (10)

A sample of **5b** (9.3 mg, 0.01 mmol, Sp isomer) was dissolved in 1% dichloroacetic acid in CH_2Cl_2 (1 mL). After one hour at room temperature the reaction mixture was dried under reduced pressure, redissolved in 1mL H_2O and extracted with 3×1 mL ethyl acetate. The aqueous layer was purified on a C_4 semipreparative HPLC column (Waters RCM, 25 mm $\times$ 10 cm) with a gradient of 5–70% acetonitrile in 0.1 M TEAA. The detritylated compound, eluting at 11.8 min, was collected and evaporated to dryness to give **10b**, Sp isomer, as a white solid. ^1H NMR: δ (ppm) 7.67 (s, 1H, H_6), 7.48 (s, 1H, H_6), 6.18 (m, 2H, H_1), 5.0 (m, 1H, H_3), 4.2 (m, 1H, H_3), 4.12 (m, 2H,

Figure 2. Oligonucleotide (2-aminoethyl)phosphonates.

H_{5',5'}), 4.01 (m, 1H, H_{4'}), 3.9 (m, 1H, H_{4'}), 3.58 (m, 2H, H_{5',5'}), 2.77 (q, 2H, CH₂), 2.11–2.31 (m, 4H, H_{2',2'}), 1.98 (m, 2H, CH₂), 1.79 (s, 3H, CH₃), 1.76 (s, 3H, CH₃). ³¹P NMR: 29.71 (s).

An identical procedure was used to prepare the Rp isomer **10a**, using (46.5 mg, 0.053 mmol) of the compound **5a**. The detritylated material was eluted at 8.48 min. Fractions were collected and evaporated to dryness to give **10a** as a white solid. ¹H NMR: δ (ppm) 7.67 (s, 1H, H₆), 7.50 (s, 1H, H₆), 6.17 (t, 2H, H_{1'}), 5.0 (m, 1H, H_{3'}), 4.23 (m, 1H, H_{3'}), 4.09–4.19 (m, 2H, H_{5',5'}), 4.0 (m, 1H, H_{4'}), 3.9 (m, 1H, H_{4'}), 3.58 (m, 2H, H_{5',5'}), 2.79 (m, 2H, CH₂), 2.07–2.33 (m, 4H, H_{2',2'}), 2.0 (m, 2H, CH₂), 1.78 (s, 3H, 5-CH₃), 1.76 (s, 3H, CH₃). ³¹P NMR: 29.71 (s).

Synthesis of oligonucleotides

Oligonucleotides were synthesized using a standard 1 μmol scale phosphoramidite cycle except that a coupling time of 1 min/cycle was used, with the dimer phosphoramidites (**6** or **7**, **a** or **b**) being delivered at a concentration of 0.15 M in acetonitrile. Coupling yields of >96% were routinely obtained. For **9a**, **9b**, **11a** and **11b**, additions of a conventional thymidine phosphoramidite were employed to introduce phosphodiester linkages at the appropriate positions. At the conclusion of the synthesis, the solid support containing the tritylated oligonucleotides was dried *in vacuo* overnight, transferred to a screw cap glass vial and treated with ethylenediamine (500 μL) for 30 min at 55°. The supernatant was collected, evaporated to dryness, and the support was washed with ethanol (2×200 μL) followed by TEAA (0.1 M, 2×200 μL) and ethanol (2×200 μL). The washes were com-

bined, evaporated to dryness, and the residues from the supernatants and washes were dissolved in water (250 μL each), combined, filtered through a 0.45 μm membrane and loaded onto a preparative reverse phase C₄ HPLC column. The column was eluted with 0.1 M TEAA/CH₃CN according to the following conditions: 0–5 min 5% CH₃CN; 5–20 min 5–30% CH₃CN; 20–25 min 30–50% B, flow rate of 10 mL/min. The fractions containing product, which were generally eluted between 22–26 min, were combined, lyophilized, and detritylation was performed using 80% acetic acid (1 mL) for 30 min at room temp. The solution was lyophilized, and the residues were dissolved in TEAA (1 mL) and injected onto a C₄ column. The fractions containing pure material, which were generally eluted between 14–18 min, were combined, evaporated to dryness and the residue was converted into the sodium form and lyophilized to give the required oligonucleotide. Yields varied between 7 and 64 OD₂₆₀ units.

The fluoresceinated oligonucleotide **12b** was synthesized as described above except that a fluorescein phosphoramidite (100 μM, Glen Research, Stirling, VA) was used for addition of fluorescein to the 5'-terminus. The fluorescein amidite was dissolved in dry acetonitrile (0.75 mL) and a double coupling procedure (15 min each) was employed; under these conditions coupling yield of >95% was obtained, as determined by trityl assay. A trityl-off ending procedure was employed, followed by deprotection and HPLC purification as described above. The peak eluting at 16.75–17.4 min was collected, lyophilized and converted into the sodium form to give pure **12b** (25 OD₂₆₀ units).

Thermal denaturation experiments

These experiments were carried out using a Gilford Response II temperature controlled spectrophotometer by monitoring the changes in absorbance at 260 nm versus temperature, with a heating rate of 1°/min from 0–70°. Melting curves were obtained both in low salt (150 mM NaCl, 10 mM Na₂HPO₄) and high salt (1 M NaCl, 10mM Na₂HPO₄) conditions at pH 7.0. Transition temperatures were obtained from the first order derivative plot of absorbance versus temperature.

Hydrolytic stability of **10a** and **10b**

The dinucleotides **10a** and **10b** were incubated at pH 7.1 in TEAA buffer at 37° over 50 h and aliquots were removed at intervals and injected onto a C₄ reverse phase HPLC column. The rate of degradation was determined by measurement of the area remaining under the peak corresponding to starting material. The half lives as determined by this method were 51 days for **10a** and 45 days for **10b**.

Stability to S1 nuclease

Solutions of the natural oligomer d-T(pT)₁₂ and the aminoethyl modified oligomer **8a** or **8b** (3 OD₂₆₀) in 150 μL of buffer (50 mM sodium acetate, 250 mM sodium chloride, 1 mM zinc chloride, pH 4.6, containing 50 μg/mL of bovine serum albumin) were equilibrated at 25°C for 30 min, treated with 10 units of S1 nuclease (US Biochemicals) and the solutions were stored at 25°C. Aliquots (10 μL) of the reaction mixture were injected onto an analytical reverse phase HPLC column, eluted with a gradient of 5–30% acetonitrile in 0.1 M TEAA buffer (pH 7.1) and the area under the peak corresponding to starting material was plotted versus time to determine t_{1/2} values. The procedure for incubation of dimers **10a** and **10b**

Table 1. Hybridization of oligonucleotide aminoethylphosphonates to complementary DNA and RNA sequences

Oligo-nucleotide	Isomer	Target	Melting Temp (°C)	
			Low Salt ^a	High Salt ^b
d-T(pT) ₁₂	n/a	d-A(pA) ₁₂	34	47
8a	Rp	d-A(pA) ₁₂	51	51
8b	Sp	d-A(pA) ₁₂	12	17
d-T(pT) ₁₂	n/a	r-A(pA) ₁₂	30	39
8a	Rp	r-A(pA) ₁₂	35	37
8b	Sp	r-A(pA) ₁₂	—	—

^a150mM NaCl, 10mM Na₂HPO₄, pH 7.^b1M NaCl, 10 mM Na₂HPO₄, pH 7.

was as previously described for the aminomethyl derivatives [16].

Induction of RNase H activity

A single-stranded DNA–RNA hybrid target molecule was synthesized consisting of 8-base deoxynucleotide flanks and a central r-pA₁₃ core. This molecule was end labeled with T4 polynucleotide kinase in the presence of 400 μCi of [³²P]-ATP (ICN, 7000 Ci/mmol). The labeled compounds were heated (95°, 5 min) in 50% formamide and purified by separation on a 12% PAGE with 7M urea using Tris-borate-EDTA buffer (TBE). The isolated bands were frozen, crushed and extracted with 2mM EDTA, filtered through cellulose acetate (0.45 μ), extracted with phenol/chloroform and precipitated with ethanol. The target molecule (~7 fmol, 40,000 cpm) was annealed to 10-, 100- or 1000-fold excess of either **11a**, **11b** or d-T(pT)₁₂ as a control by heating to 65° for 10 min. in 10 μL GB (20 mM Hepes pH 7.4, 140 mM NaCl, 5 mM KOAc, 5 mM MgCl₂) followed by slow cooling to room temp. One unit of RNase H (Promega) was added and the solutions were incubated at 37° for 20 min. Formamide was added to 50% concentration and the solutions were heated (95°, 5 min), and electrophoresed on a 16% PAGE with 7M urea in TBE. The gels were fixed and dried prior to autoradiography.

Serum stability

Ten pmol of d-T(pT)₁₂ or the alternating aminoethyl oligomers **11a** or **11b** were 5'-end labelled and isolated as described above. For each time point examined, each oligomer (50,000 cpm) was incubated at 37° in a 5% humidified CO₂ atmosphere with 50 μL of Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% heat-inactivated (56°, 45 min) fetal bovine serum (FBS). Control samples were incubated in plain DMEM. At the end of 0, 1, 4 or 24 hours, the samples were mixed with an equal volume of formamide, and analyzed by 12% urea–PAGE. The gels were fixed, washed and dried before autoradiography (Kodak XAR-5).

Flow cytometry

JY cells (a human B lymphoblastoid cell line) in log phase were washed and cultured in RPMI containing 10% FBS (heat inactivated to 65°C for 30 min.), 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamate at 1.5 × 10⁶ cells/ml for the indicated times and temperatures with or without oligonucleotide as indicated. Cells were then washed twice in HBSS with 1% BSA. Data on viable cells were

collected on a FACScan (Becton-Dickinson, Mountain View, CA) containing a 15-mW argon-ion laser with Lysis II software.

Confocal microscopy

This was performed following JY cell culture as described above using a Biorad MRC600, with a krypton/argon laser, Nikon optiphot optics with a 60X 1.4 NA objective, and images were printed with a SONY UP5000 video printer. Cells were washed but not fixed, and were analyzed in BSS within 15 min.

RESULTS AND DISCUSSION

Our initial strategy for the synthesis of nucleotide (2-aminoethyl)-phosphonates employed the phthalimido group for protection of the primary amine of the (2-aminoethyl)phosphonate moiety, since this group was previously used for the preparation of (aminomethyl)-phosphonate derivatives. Accordingly, the thymidine dinucleotide-(2-phthalimidoethyl)phosphonate was prepared, but difficulties were experienced in the removal of the phthalyl group. A variety of deprotection conditions were evaluated, including treatment with ethylenediamine in acetonitrile for up to 6.5 h at room temp, but no cleavage of the phthalyl group was observed. This result is in marked contrast to the results with the corresponding dinucleotide-(phthalimido-methyl)phosphonate as previously reported [16] which could be completely deprotected and isolated in excellent yield upon treatment with ethylenediamine under the same conditions. Hydrazine has routinely been used for the removal of phthalyl protecting groups [18] and deprotection of the base protecting groups of oligonucleotide methylphosphonates [19] but this reagent was not satisfactory for removal of the phthalyl groups since decomposition was observed.

Because of the difficulties experienced with removal of the phthalyl group, other groups were evaluated for protection of the amino functionality. Since halogenated phthalimido groups have been employed for other purposes [20,21], these were evaluated for protection of the amino group. Reaction of tetrabromophthalic anhydride with (2-aminoethyl)phosphonic acid produced pyridinium [2-(3,4,5,6-tetrabromophthalimido)ethyl]phosphonate which could be isolated as a solid. Reaction of this compound with 5'-dimethoxytritylthymidine using TPS-Cl as the coupling agent produced the mononucleotide **1**, although this reaction was inefficient due to the poor solubility of the starting material in the reaction medium. Coupling of **1** with one equivalent of thymidine gave the dimer as a mixture

of isomers **2a** and **2b** which could be separated by HPLC, with no evidence for 3',3'-coupling. After removal of the acid labile dimethoxytrityl group, the tetrabromophthalyl group could readily be removed by treatment with ethylenediamine in acetonitrile. Thus the introduction of the halogen substituents into the phthalimido group produced a protecting group which is expected to be compatible with other nucleic acid components.

Although the tetrabromophthalimido group was suitable for protection of the amino function in the synthesis of oligonucleotides, the low yields obtained in the synthesis of the mononucleotide prompted an evaluation of the corresponding tetrachloro derivatives. Reaction of pyridinium [2-(3,4,5,6-tetrachlorophthalimido)ethyl] phosphonate with thymidine was much more efficient than for the tetrabromo derivative and the mononucleotide **3** could be isolated in 63% yield. The dimer **4** was prepared as described for **2**, and deprotection of the tetrachlorophthalyl group could be easily accomplished using ethylenediamine for 10 min at 55°. Thus the tetrachlorophthalimido group appears to be the protecting group of choice for the aminoethyl series.

Stereochemistry at phosphorus

A preliminary determination of the stereochemistry at phosphorus was obtained by an adaptation of the 2D-NMR method of Loschner and Engels [22]. These authors determined the stereochemistry about phosphorus of dinucleoside methylphosphonates based upon the level of interactions between the methylphosphonate and the C_{3'} and C_{4'} sugar protons. In this work the 2D-ROESY NMR spectra of the partially protected dimers **5a** and **5b** indicated that the relative interactions of the P-CH₂ and C_{4'} protons as compared with the C_{3'} protons were enhanced for one isomer, which was tentatively assigned as the Rp isomer **5a**; details of the NMR data will be described elsewhere. This assignment is consistent with data obtained from the hybridization experiments which showed that

the oligonucleotide with Rp isomer exhibited a much higher T_m than the corresponding Sp isomer (see below).

Hybridization experiments

The alternating sequences **8a** and **8b** were hybridized to the complementary sequence d-A(pA)₁₂ and the results are shown in Table 1. As with the aminomethyl series, one isomer (Rp) hybridized more strongly than its natural counterpart, whereas the other did not form a stable hybrid. It is of interest that under low salt conditions the Rp isomer of the aminoethyl oligomer formed a more stable hybrid than the corresponding aminomethyl analog (T_m = 51° versus 45° for the latter). The effects of salt concentration on the dissociation temperatures of hybrids of the Rp isomer were also investigated. For the hybrid with DNA, no salt dependence was observed, whereas for the hybrid with RNA, the salt dependence was relatively low as compared with a natural duplex. This type of reduced salt dependence has previously been observed for other oligonucleotides with cationic groups [15]. The relatively high T_m of the Rp isomer versus the Sp isomer is consistent with data previously obtained for stereoisomers of oligonucleotide methylphosphonates [23].

Since the presence of positive charges might potentially increase the likelihood of non-specific hybridization, the stability of hybrids of oligonucleotides **9a** and **9b**, each possessing one aminoethylphosphonate moiety, with a fully complementary sequence and with a series of partially mismatched complements was examined. The mismatched bases were introduced opposite to the aminoethyl group and the results are shown in Table 2. In terms of hybridization to the fully complementary targets, a substantial difference was observed for the two isomers, one producing an elevation of 2° versus natural whereas the other caused a marked depression of 6°. In all cases the introduction of mismatches produced a substantial depression of melting temperature, i.e. 12–13° for the Sp isomer **9b**, 13–14° for the Rp isomer **9a**, versus 13–14° for the natural sequence. Since the magnitude of depression for each mismatched pair was approximately the same in all cases, this suggests that the introduction of an aminoethyl group does not result in a loss of specificity. When similar mismatch experiments were carried out with the alternating sequence **8a**, a drop in T_m of 9–11° was observed for the mismatched duplexes as compared with the fully complementary sequences. Thus the presence or absence of paired bases appears to be the primary factor for the stability of these duplexes.

Since the ionization of the cationic groups depends on the pH of the medium, a series of hybridization experiments was carried out with **8a** in which the pH of the solution was varied and these results are summarized in Table 3. For the natural sequences d-T(pT)₁₂ and d-A(pA)₁₂ the dissociation temperature

Table 2. Hybridization of **9a** and **9b** to partially complementary sequences possessing a mismatch opposite to the aminoethyl group

Oligonucleotide	Melting temperature (°C) of duplex with matched or mismatched sequence ^a		
	9a	9b	d-T(pT) ₁₂
d-A(pA) ₁₂	38	30	36
A(pA) ₅ pC(pA) ₆	24	17	22
A(pA) ₅ pG(pA) ₆	25	18	23
A(pA) ₅ pT(pA) ₆	24	18	23
A(pA) ₆ pC(pA) ₅	25	17	22
A(pA) ₆ pG(pA) ₅	25	17	23
A(pA) ₆ pT(pA) ₅	25	17	23

^a150mM NaCl, 10mM Na₂HPO₄, pH 7.

Table 3. Effect of pH on the melting temperature of duplexes of d-T(pT)₁₂ or **8a** with d-A(pA)₁₂

Duplex	Dissociation temperature ^a					
	5	6	7	pH 8	9	10
d-A(pA) ₁₂ + d-T(pT) ₁₂	34	34	34	36	35	30
8a + d-A(pA) ₁₂	43	51	51	51	47	43

^a150mM NaCl, 10mM Na₂HPO₄

was relatively constant over the pH range of 4–9, with a decrease being observed at pH 10. This latter decrease might be due to the destabilization caused by the ionization of the imino protons of the thymine residues. The hybridization of **8a** versus d-A(pA)₁₂ showed a much more dramatic variation with changes of pH, with the T_m 's being in all cases substantially higher than the natural duplex. A T_m of 51° was observed over the pH range of 5–7 with a substantial decrease at pH 8–10. These data suggest that at pH 5–7 the ionized aminoethyl groups play an important role in enhancing stability either by interstrand or intrastrand ionic attractions, whereas at higher pH values these attractive forces are absent since the amino groups are unionized, or only partially ionized. These data are consistent with the pK values reported for (2-aminoethyl)phosphonic acid [24].

The hybridization of the alternating-backbone oligonucleotides **8a** and **8b** to RNA was also studied, using r-A(pA)₁₂ as the target. Under low salt conditions the Rp isomer **8a** exhibited a T_m 5° higher than the natural counterpart, whereas under high salt conditions the T_m of **8a** was actually lower than the natural sequence. As with the hybridization to DNA, the modified oligonucleotides show less salt dependence, as would be expected for a hybrid in which the charge-charge repulsions of the backbone have been reduced. The Sp isomer **8b** did not exhibit a detectable transition under any salt conditions.

Figure 3. Serum stability of d-T(pT)₁₂ (panel A), and **11a** (panel B). Lanes 1,3,5,7 were incubated in the presence of FBS, and lanes 2,4,6,8 without FBS. Incubation times: lanes 1 and 2, 0 h; lanes 3,4, 1 h; lanes 5,6, 4 h; lanes 7,8, 24 h.

Resistance to S1 nuclease

The unprotected dinucleotides **10a** and **10b** were incubated with S1 nuclease, which is capable of degrading single-stranded DNA, and the digestion products were examined by HPLC. The aminoethyl-phosphonate derivatives were found to be completely resistant after a 30 minute incubation period whereas the natural dimer was completely degraded after this time. The action of S1 endonuclease on the alternating sequences **8a** and **8b** was also examined. The natural sequence d-T(pT)₁₂, which was used as a control, was rapidly degraded with a $t_{1/2}$ of only four minutes, whereas the Sp isomer **8b** was completely undegraded after 24 hours and the Rp isomer **8a** showed a small amount (approx. 6%) of degradation. At the end of these experiments the activity of the enzyme was confirmed by addition of d-T(pT)₁₂ to the digestion mixture followed by a short incubation period and HPLC analysis, which confirmed that the enzyme was still capable of degrading the natural oligonucleotide.

Figure 4. A: Time course of oligonucleotide uptake. Fluorescein conjugated alternating aminoethyl oligonucleotide **12b** or phosphodiester (PO) oligonucleotide (2 μ M concentration) was cultured with JY cells at the indicated temperature and times before washing and flow cytometry to determine the mean fluorescence intensity (MFI). B: Concentration dependence of oligonucleotide uptake. Cells were cultured for 3 h with the oligos from panel A at the concentrations shown prior to washing and analysis.

Figure 5. Intracellular oligonucleotide localization. Panels A and B, FITC-phosphodiester; panels C and D, FITC-alternating aminoethyl oligonucleotide 12b (2 μ M concentration). Panels A and C, phase contrast; panels C and D, confocal microscopy.

Thus the enzyme was apparently incapable of degrading phosphodiester linkages which are flanked by aminoethylphosphonates. This protective effect might be steric in nature, or alternatively the aminoethyl groups might exert a protective effect by the formation of internal ion pairs. Similar results on the enzymatic degradation of oligonucleotides with stereoregular, alternating methylphosphonate/phosphodiester backbones have been reported by Miller *et al* [25].

Induction of RNase H activity

It was of interest to determine if the alternating oligomers which form stable hybrids would induce RNase H activity when hybridized to a target RNA molecule. For this experiment an oligonucleotide with an rA₁₃ core and deoxynucleotide flanking regions was synthesized and ³²P-end labelled for use as the target. Cleavage of the RNA region of the target would thus be detected by the appearance of a shorter ³²P-labelled fragment. After incubation of samples of **8a**, **8b** and d-T(pT)₁₃ with labelled target, the products were examined by native PAGE. Analysis of the autoradiograms confirmed the lack of hybridization for the Sp isomer **8b**, whereas the expected retardations due to hybridization for the control oligomer and the Rp isomer **8a** were observed (data not shown). Under the conditions employed, the d-T(pT)₁₃ control was effective in inducing RNase H activity with as little as a 10-fold molar excess

over target, whereas the Rp isomer **8b** showed no sign of such activity even with a 1000-fold excess of oligonucleotide.

Serum stability

³²P labelled oligonucleotides were needed in order to assess the serum stability of the alternating aminoethylphosphonate/phosphodiester backbones. Preliminary experiments showed that **8a** and **8b** could not be labelled effectively, presumably due to the presence of the aminoethyl moiety adjacent to the 5'-terminus. Oligonucleotides **11a**, **11b**, possessing phosphodiester linkages adjacent to the 5'-terminus, were therefore prepared and ³²P labelled at the 5'-position. The labelled oligonucleotides were incubated in conditioned medium containing 10% FBS and the products were examined by autoradiography. As shown in Figure 3, the nuclease stability of the alternating aminoethyl linkages was superior to the d-T(pT)₁₂ oligonucleotide control. The natural oligomer showed significant degradation after only one hour, and full-length oligomer was virtually undetectable after 24 hours. In contrast, the degradation of **11a** and **11b** was barely visible after 24 hours. The slight retardation observed on PAGE after longer time periods up to 24 hours might be an indication of cleavage of the phosphodiester at the 3'-position to produce a 12mer with one less negative charge. Although a 12mer would normally be expected to migrate faster for a phosphodiester, the removal of a negative charge in the alternating oligomer could have a greater retarding effect than the loss of one base.

Cell uptake

Flow cytometry was used to determine whether an oligonucleotide with alternating positive and negative charges on the backbone might be taken up by cells. This technique has been demonstrated to be useful for studying and comparing the uptake and intracellular localization of different oligonucleotides conjugated to fluorescent molecules such as fluorescein [26]. Oligonucleotide uptake in cell lines has previously been shown to be saturable, sequence independent, and temperature and energy dependent [27]. JY cells were cultured with the 5'-fluoresceinated oligonucleotide **12b** (Sp isomer), or with a 5'-fluoresceinated, twenty base, mixed sequence oligonucleotide as control, at 2 mM concentration at 4°C for varying times and compared to those cultured at 37°C for the same time before washing and flow cytometry to determine the mean fluorescence intensity (MFI). As shown in Figure 4, (panel A), uptake of both oligonucleotides was highly temperature-dependent, compatible with uptake through an endocytic pathway. Cell surface binding (defined as uptake in cells cultured at 4°C) of both oligonucleotides was very similar. To evaluate the concentration dependence, oligonucleotides were cultured for 3 hours at the indicated temperature and concentration before washing and flow cytometry as above. In contrast, the level of cellular uptake of the oligonucleotides at the three hour time point studied was highly dependent on the oligonucleotide concentration (Figure 4, panel B). Both experiments were performed three times with similar results. It is of interest that at lower concentrations (less than 1 μ M) the level of phosphodiester uptake generally exceeded that of the alternating aminoethyl, while at higher concentrations, the converse was true. Thus the oligonucleotide with alternating positive and negative charges on the backbone appears to be taken up as well as, or under certain conditions better than, the control (phosphodiester) se-

quence. Further studies are needed to determine if uptake is dependent upon the stereochemistry of the aminoethyl group.

The temperature dependence of cellular oligonucleotide association seen in the experiments above suggested that culture at 37°C resulted in oligonucleotide internalization. To test this hypothesis, confocal microscopy was used to confirm that both the phosphodiester and alternating aminoethyl oligonucleotides were present intracellularly (Figure 5). The top panels show the phase and confocal images for same field of view of two representative cells that had been incubated with the fluoresceinated phosphodiester oligonucleotide for 3 hours and the bottom panels show the phase and confocal images for a cell incubated with **12b** for the same time. The pattern of this fluorescence was typically speckled and cytoplasmic with very little nuclear fluorescence. This result suggests that the oligonucleotides were sequestered in endosomal compartments.

CONCLUSIONS

(2-Aminoethyl)phosphonates represent an interesting new type of oligonucleotide backbone modification which introduces positive charges into the molecule. The stereoisomers of thymine dinucleotides are readily resolved by procedures that can be applied to the synthesis of isomers of dimers of other nucleotides. The studies described above using oligonucleotides with alternating (2-aminoethyl)phosphonate/phosphodiester backbones indicate that the Rp isomers are capable of forming more stable duplexes than their natural counterparts. The Sp isomers do not form stable hybrids, presumably due to unfavorable steric interactions between the aminoethyl groups and other portions of the duplex. The increase in duplex stability is more marked with DNA than with RNA as the complementary strand, and less dependent upon salt concentration. More stable hybrids are formed at pH's which favor protonation of the amino groups. Surprisingly, oligonucleotide (2-aminoethyl)-phosphonates form more stable hybrids than the corresponding (aminomethyl)phosphonate derivatives even though the former possess more steric bulk. Backbones of this type are not degraded by S1 nuclease to any significant extent. The most striking difference between (aminomethyl)- and (2-aminoethyl)phosphonates is their difference in stability in aqueous solution. The (aminomethyl)-phosphonate linkage is rapidly hydrolyzed at pH 7 to aminomethyl mononucleotides, whereas the corresponding (2-aminoethyl)-phosphonate is completely stable under the same conditions. This difference in stability may be due to the reduction in the electropositivity of the phosphorus atom due to the additional intervening methylene group. A preliminary study on cell uptake indicated that under certain conditions a net neutral oligonucleotide was taken up as well as, or somewhat better than a phosphodiester control sequence. Since these studies demonstrate that oligonucleotides with aminoethyl substituents hybridize well to nucleic acid targets and are taken up by cells, their potential value as antisense therapeutic agents deserves further investigation.

ACKNOWLEDGEMENTS

A.M.K. is a Pfizer Scholar and was supported by grants from the Veterans Administration, Carver Foundation and NIH R29 AR 42556-01.

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