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Nucleic Acids Research, 1996, Vol. 24, No. 22 **4584–4591**

Synthesis and thermodynamics of oligonucleotides containing chirally pure R_P methylphosphonate linkages

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Received May 15, 1996; Revised and Accepted September 24, 1996

ABSTRACT

Methylphosphonate (MP) oligodeoxynucleotides (MPOs) are metabolically stable analogs of conventional DNA containing a methyl group in place of one of the non-bonding phosphoryl oxygens. All 16 possible chiral R_P MP dinucleotides were synthesized and derivatized for automated oligonucleotide synthesis. These dimer synthons can be used to prepare (i) all-MP linked oligonucleotides having defined R_P chirality at every other position (R_P chirally enriched MPOs) or (ii) alternating R_P MP/phosphodiester backbone oligonucleotides, depending on the composition of the 3′-coupling group. Chirally pure dimer synthons were also prepared with 2′-*O*-methyl sugar modifications. Oligonucleotides prepared with these R_P chiral methylphosphonate linkage synthons bind RNA with significantly higher affinity than racemic MPOs.

INTRODUCTION

Antisense oligonucleotides inhibit protein synthesis by binding to mRNA by Watson–Crick pairing (1–4). These compounds prevent processing or translation of mRNA, either by blocking protein recognition or directing nucleases to degrade the message. Methylphosphonate oligonucleotides (MPOs) are among the first oligonucleotide analogs reported to inhibit protein synthesis through a blocking antisense mechanism in cell-free and cell culture assays (5–9). MPOs are highly resistant to metabolic breakdown in biological systems (10,11). Unlike natural phosphodiester oligonucleotides, MPOs contain chiral linkages. The conventional methods for synthesizing MPOs are not capable of forming methylphosphonate bonds without P epimerization. A typical racemic 18mer MPO contains 131 072 (2^{17}) different diastereomers.

Several investigators have produced short strands of chirally enriched (or chirally pure) MPOs through solution phase approaches (12–14). The published procedures take advantage of the differences in chromatographic mobility for 2′-deoxy methylphosphonate (MP) dimers having the R_P versus S_P configuration (the absolute configurations for the 16 MP dimers are assigned and their physical characteristics described in the literature; 15,16). Dimers are separated into R_P and S_P chiral forms and then coupled to a monomer in solution to yield two trimer diastereomers (R_P/S_P and R_P/R_P). These, in turn, are separated and further coupled to produce tetramers. So far, the ability to resolve different diastereomers is limited to septamers and still requires sequential coupling reactions in order to limit the number of diastereomers in each separation step (12). In a separate study, two chiral oligothymidine tetramers were coupled together to generate chirally enriched octamers having a single racemic linkage in the middle of the sequence (13,14). In both cases, R_P chiral MPO–DNA duplexes were significantly more stable to thermal denaturation than S_P chiral MPOs. These results are encouraging, but published solution phase procedures are prohibitive for preparing longer oligonucleotides. Solid phase synthesis of chirally pure MPO dimers was recently described (17) and synthesis of longer sequences is under development (18–22).

In an effort to provide a method for solid phase assembly of R_P chirally enriched MPOs, R_P chiral methylphosphonate dimers were coupled sequentially, producing racemic MP linkages at every other position (Fig. 1a). Each of the 16 possible R_P chiral dimers (‘dimer synthons’) were prepared and derivatized for use in an automated solid phase DNA synthesizer as both the methylphosphonamidites and β-cyanoethyl phosphoramidites to prepare both the MP/MP (Fig. 1a) and MP/phosphodiester (DE) oligomers (Fig. 1b). Additionally, an oligonucleotide was prepared from a dimer synthon with 2′-O-methyl sugar modifications (Fig. 1c). Oligonucleotides prepared with these different dimer synthons were shown to hybridize to their RNA targets with binding constants ranging from 10^5 to 10^{13}.

Finally, oligodeoxynucleotides containing alternating MP/DE backbones are highly nuclease resistant *in vitro*. Oligonucleotides containing MP/DE backbones in combination with 2′-*O*-methyl sugar modifications are almost completely resistant to nuclease degradation *in vivo*.

MATERIALS AND METHODS

Synthesis of chirally pure methylphosphonate dimer synthons

^{1}H NMR spectra were obtained on a 300 MHz Bruker ARX 300 Spectrophotometer. All ^{1}H NMR results were obtained in $CDCl_3$

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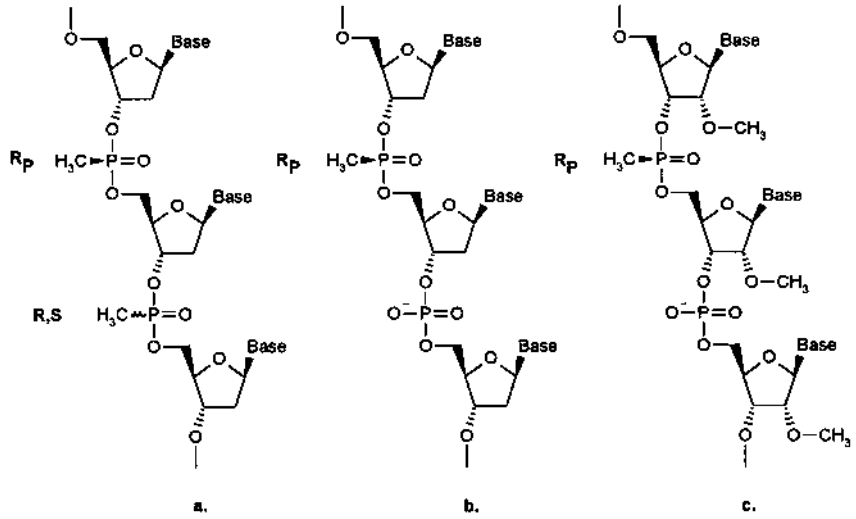

Figure 1. Structures of backbone-modified oligonucleotides containing R_P chiral MP linkages. (**a**) Alternating R_P-MP/MP backbone. (**b**) Alternating R_P-MP/DE backbone. (**c**) 2′-*O*-Methyl (alternating R_P-MP/DE) backbone.

unless otherwise indicated. Mass spectra were recorded on a Trio 2000 Spectrometer.

5′-*O*-Dimethoxytrityl-*N*4-isobutyryl-2′-*O*-methylcytidine was prepared using standard procedures (23). 5′-*O*-Dimethoxytrityl-(base protected)-(2′-deoxy or 2′-*O*-methyl) nucleosides were phosphitylated with chloromethyl-*N*,*N*-diisopropylaminophosphine as published (24). The 3′-*O*-t-butyldimethylsilyl-(base protected)-(2′-deoxy or 2′-*O*-methyl) nucleosides were prepared by 3′-*O*-silylation of the corresponding 5′-*O*-dimethoxytrityl-(base protected)-(2′-deoxy or 2′-*O*-methyl) nucleosides, followed by 5′-*O*-detritylation with benzenesulfonic acid (25). Base protecting groups used in these syntheses were N^6-benzoyl for adenine, N^2-isobutyryl for guanine and N^4-isobutyryl for cytidine.

The general method for preparing 2′-deoxymethylphosphonate dimer synthons is shown in Figure 2. As examples, the syntheses of 2′-deoxy-(CT) and 2′-*O*-methyl-(CU) dimer synthons are described below.

Bis-[2′-Deoxy]-5′-O-dimethoxytrityl-(base protected) R_P methylphosphonate (CT) dinucleotide. The 2′-deoxy R_P methylphosphonate (CT) dinucleotide was prepared essentially as described previously (15). Briefly, 3′-*O*-t-butyldimethylsilyl thymidine was reacted with 5′-*O*-dimethoxytrityl-N^4-isobutyryl-2′-deoxycytidine-3′-*O*-(methyl-*N*,*N*-diisopropylaminophosphonamidite) in the presence of tetrazole and then treated with oxidizer solution (275 ml, iodine/water/lutidine/tetrahydrofuran, 25 g/2.5 ml/100 ml/900 ml). The crude product was separated into fast (R_P) and slow (S_P) isomers by flash column chromatography on silica gel (230–400 mesh). The column was step-eluted with ethyl acetate/dichloromethane (75:25) containing increasing amounts of methanol (1, 2 or 3%). The 3′-*O*-silyl protecting groups were removed by treatment with tetrabutylammonium fluoride (1 M in tetrahydrofuran). The resulting compound was purified by flash column chromatography on silica gel as just described. ^{1}H NMR δ 10.21 and 9.97 (2 br s, 2H, NH), 8.11 (d, J = 7.5 Hz, 1H, cytidine H6), 7.50–7.20 (m, 11H, trityl H and cytidine H5 and thymidine H6), 6.85 (d, J = 8.4 Hz, 4H, trityl H), 6.25 (t, J = 6.2 Hz, 1H, H1′), 6.19 (t, J = 6.2 Hz, 1H, H1′), 5.26 (m, 1H), 4.58 (m, 1H), 4.44–4.23 (m, 3H), 4.11 (m, 1H), 3.80 (s, 6H, DMT OCH_3), 3.43

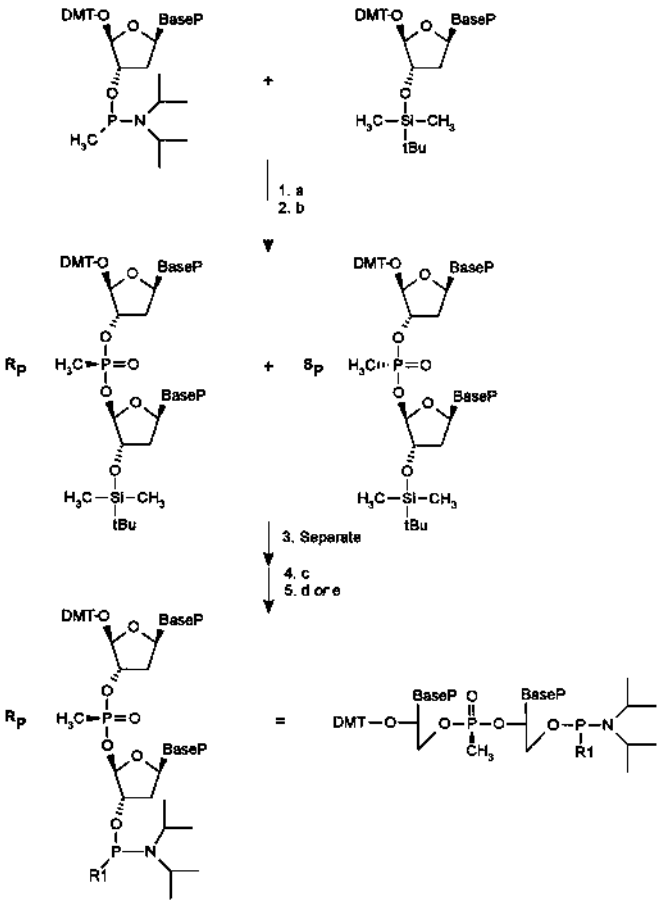

Figure 2. Synthesis of 2′-deoxy R_P MP chiral dimer synthons. (**a**) Tetrazole. (**b**) Iodine/water/2,6-lutidine/THF. (**c**) TBAF. (**d**) *N*,*N*′-Diisopropyl methylphosphonylchloridite. (**e**) *N*,*N*′-Diisopropyl (β-cyanoethoxy)phosphinylchloridite. R1 is either methyl (using reagent d) or β-cyanoethyl (using reagent e). BaseP is a protected base, as described in the text.

(m, 2H, H5′), 2.96 (m, 1H), 2.68 (heptet, J = 6.9 Hz, 1H, isobutyryl CH), 2.45 (m, 2H), 2.23 (m, 1H), 1.87 (s, 3H, thymidine CH_3), 1.53 (d, J = 17.6 Hz, 3H, P-CH_3), 1.17 and 1.07 (2 d, J = 6.9 Hz each, 6H, isobutyryl CH_3); ^{31}P NMR δ 32.31.

Bis-[2′-O-methyl]-5′-O-dimethoxytrityl-(base protected) methylphosphonate (CU) dinucleotide (fast isomer). The 2′-*O*-methyl R_P methylphosphonate (CU) dinucleotide was prepared according to a similar procedure. Briefly, 3′-*O*-t-butyldimethylsilyl 2′-*O*-methyluridine (6 mmol) and 5′-*O*-dimethoxytrityl-*N*^{4}-isobutyryl-2′-*O*-methylcytidine-3′-*O*-(methyl-*N*,*N*-diisopropylaminophosphonamidite) (7 mmol) were dissolved in dry acetonitrile and treated with molecular sieves (3 Å) for 24 h. The two solutions were then mixed under argon and reacted with tetrazole (94 ml, 42 mmol) at room temperature for 4 min. The reaction mixture was then treated with a single addition of cumene hydroperoxide (8.1 mmol). This mixture was rapidly stirred for 10 min and quenched by addition of aqueous sodium bisulfite solution (1 g/ 10 ml). The crude material was fractionated into ‘fast’ and ‘slow’ isomers by flash column chromatography and then desilylated and repurified as described above. ^{1}H NMR δ 10.34 (br s, 2H, NH), 8.62 (d, J = 7.4 Hz, 1H, cytidine H6), 7.71 (d, J = 8.2 Hz, 1H, uridine H6), 7.43 (d, J = 7.3 Hz, 1H, cytidine H5), 7.40–7.20 (m, 9H, trityl H), 6.88 (d, J = 8.7 Hz, 4H, trityl H), 6.01 (s, 1H, H1′), 5.93 (s, 1H, H1′), 5.71 (d, J = 8.1 Hz, 1H, uridine H5), 5.27 (m, 1H), 4.65–4.37 (m, 3H), 4.29 (m, 2H), 4.15 (m, 1H), 3.86 (s, 6H, DMT OCH_3), 3.78 (m, 1H), 3.72 (s, 3H, 2′-OCH_3), 3.63 (m, 1H), 3.59 (s, 3H, 2′-OCH_3), 3.44 (d, J = 10.7 Hz, 1H), 2.56 (heptet, J = 6.8 Hz, 1H, isobutyryl CH), 1.43 (d, J = 17.6 Hz, 3H, P-CH_3), 1.10 and 0.71 (2 d, J = 6.6 Hz each, 6H, isobutyryl CH_3); ^{31}P NMR δ 32.15.

Phosphitylation of dimer dinucleotides. The chirally pure 5′-(*O*-dimethoxytrityl)-(base protected) methylphosphonate dinucleosides were converted to the corresponding methylphosphonamidites or β-cyanoethyl phosphoramidites using standard procedures (23,26,27). Purities were confirmed by ^{1}H and ^{31}P NMR.

Oligonucleotide synthesis

Oligonucleotides were synthesized on a Biosearch Expedite™ Model 8909 DNA synthesizer according to the manufacturer’s recommendations except where indicated. Oligonucleotides containing MP linkages were deprotected as described previously (28) and purified by HPLC with a normal phase column matrix (β-Cyclobond 2000; Astec Inc., Whippany, NJ) using a reverse gradient of 60–30% acetonitrile in 0.1 M ammonium acetate, pH 6. The purity of each oligonucleotide was confirmed by polyacrylamide gel electrophoresis and/or ESI-MS analysis (29).

MP/DE oligodeoxynucleotides. The racemic, alternating MP/DE oligonucleotides were synthesized using a combination of nucleoside β-cyanoethylphosphoramidites (Glen Research Corp., Sterling, VA) and nucleoside methylphosphonamidites (JBL Scientific Inc., San Luis Obispo, CA). Isobutyryl-protected cytidine monomers were used (instead of benzoyl) to prevent transamination when deprotecting with ethylenediamine (28). A specially prepared oxidizing solution containing 0.25% water was used with methylphosphonamidite monomers, since methylphosphonite diester intermediates are highly sensitive to water (30). A commercially available oxidizing solution containing 2% water was used with β-cyanoethylphosphoramidite monomers (Glen Research Corp.). The synthesizer was reprogrammed to include these oxidations in each subroutine. The remainder of the synthetic cycle was identical to that used for a standard MPO synthesis (30).

R_P chirally enriched all-MP oligodeoxynucleotides. Bis-[2′-deoxy]-5′-*O*-dimethoxytrityl-(base protected) R_P methylphosphonate dinucleotide 3′-methylphosphonamidite dimer synthons were dissolved in dry acetonitrile (0.1 M) and dried over 3 Å molecular sieves for at least 24 h before loading onto the synthesizer. The coupling cycle was increased to 1 min/dimer addition. Other coupling reagents were as previously described for conventional racemic MPOs (30).

Alternating all-2′-deoxy-[(Rp MP)/DE] and all-2′-O-methyl-[(fast isomer MP)/DE] oligonucleotides. The appropriate chirally pure dimer synthons were dissolved in dry acetonitrile (0.1 M) and dried over 3 Å molecular sieves as described above before loading onto the synthesizer. Standard reagents and conditions were used as recommended by the manufacturer, except that the coupling times were increased to 2 min/addition for the all-2′-deoxy dimers and 3 min/dimer addition for the all-2′-*O*-methyl dimers.

RNA and DNA oligonucleotides. RNA oligonucleotides were synthesized as previously described (31,32). The phosphodiester oligomer was purchased from Oligos Etc. (Danbury, CT).

Spectroscopic and native gel determination of binding constants for oligonucleotides annealed to complementary synthetic RNA targets

Standard nearest neighbor extinction coefficients (33) were found to be independent of backbone types in this report within experimental error (unpublished data). Annealing reaction mixtures contained equimolar amounts of antisense oligonucleotide analog and RNA target oligonucleotide (~2.4 μM total strand concentration), 20 mM potassium phosphate, pH 7.2, 100 mM sodium chloride, 0.1 mM EDTA and 0.03% potassium sarkosylate. The antisense oligonucleotide/RNA mixtures were heated to 80°C, cooled slowly to room temperature and chilled to 4°C over ≥2 h. The annealed samples were transferred to 1 cm quartz cuvettes (chilled to 4°C) and placed in a Varian Cary Model 3E Spectrophotometer containing a temperature controlled 6 × 6 sample holder (pre-cooled to 5°C). Denaturation was monitored at 260 nM as a function of temperature, increasing from 5 to 80°C at a ramp rate of 1°C/min. These data were fitted to a two-state model of duplex dissociation, assuming sloping linear baselines and temperature-independent ΔH° and ΔS° (34). Each measurement was repeated at least three times at the same concentration, using independent samples. Thermodynamics from $1/T_m$ versus log $C_t/4$ plots were not determined, since measurements were made within a limited concentration range of less than one order of magnitude; typically these plots require data over a concentration range of at least two orders of magnitude.

The duplex binding constant was determined independently by gel mobility shift experiments, similar to published procedures (35). Gel mobility was measured in 0.1 M NaCl, 0.01 M dibasic sodium phosphate and 1 mM EDTA, 0.04 M Tris base, pH 8.0, at 0°C in 16% polyacrylamide gels (19:1 acrylamide:bisacrylamide). RNA was 5′-^{32}P-labeled using Stratagene T4 polynucleotide kinase and NEN [γ-^{32}P]ATP and purified using a NEN Nensorb column. Labeled RNA concentrations were ~10^{-11} M, while oligonucleotides were added over a concentration range of ~10^{-11} to 10^{-5} M. Samples were annealed by slow cooling from 90 to 37°C and incubated at 37°C for 48 h before snap freezing on dry ice. A non-

denaturing loading buffer (15% w/w Ficoll, 0.25% Bromophenol blue, 0.25% Xylene Cyanole) (35) was added and samples loaded on to a running 4°C native gel [0.1 M NaCl, 0.01 M dibasic sodium phosphate, 1 mM EDTA, 0.04 M Tris base, pH 8.0, at 0°C in 16% polyacrylamide gels (19:1 acrylamide:bisacrylamide)]. Native gel band intensities, quantitated with a BioRad GS-363 Molecular Imager, were used to determine the association constant (K_a) from the equation

$$K_a = f_m/[(1 - f_m) \times (B_0 - R_0 \times f_m)]$$

where f_m is the fraction of radiation in the slower, duplex band, B_0 is the total concentration of the unlabeled oligomer and R_0 is the total concentration of the labeled RNA.

In vitro nuclease stability studies of various backbone-modified oligonucleotides

Stability in the presence of fetal calf serum. Fetal bovine serum (Gemini Bioproducts Inc.) was diluted in Dulbecco's phosphate-buffered saline (Mediatech Inc.) to a final concentration of 10% (v/v). Aliquots were mixed with oligonucleotide on ice (0.075 OD_{260} U/sample), incubated at 37°C for the specified time intervals and then quickly frozen in a dry ice/isopropanol bath. Then, samples were thawed individually and analyzed by reversed phase HPLC using a Vydac C4 Protein column (4.6 × 250 mm). Elution was performed with solvents buffer A (50 mM TEAA, pH 7.0, 1% acetonitrile) and buffer B (50 mM TEAA, pH 7.0, 50% acetonitrile). The flow rate was 1.0 ml/min and the gradient conditions for each backbone were as follows: all-DE backbone, 5–25% buffer B (2.5–9.0 min), 25–45% buffer B (9.0–22.5 min), 45–100% buffer B (22.5–28.0 min); 2′-deoxy (R_P MP)/DE backbone, 5–35% buffer B (2.5–12.5 min), 35–50% buffer B (12.5–22.5 min), 50–100% buffer B (22.5–27.5 min); 2′-*O*-methyl (fast isomer MP)/DE backbone, 5–50% buffer B (2.5–17.5 min), 50–65% buffer B (17.5–27.5 min), 65–100% buffer B (27.5–31.0 min). The half-life ($t_{1/2}$) was estimated from the HPLC data as the time required to degrade 50% of the full-length oligonucleotide under a specified *in vitro* incubation.

Stability in the presence of cell lysate from African green monkey kidney (COS-7) cells. COS-7 cells were grown to 90% confluency and harvested in the presence of 0.25% trypsin. The cell pellets were washed with phosphate-buffered saline and then frozen at –20°C. The pellets were resuspended in an equal volume of lysis buffer (2.5 mM HEPES, pH 7.2, 2 mM $MgCl_2$, 0.1% NP-40) and centrifuged at 10 000 *g* for 5 min. Approximately 60% of the supernatant was separated and the remainder was left with the cell pellet, which was resuspended in a dounce homogenizer (type A pestle). This suspension was centrifuged as above and the supernatant was combined with the original aliquots removed from the previous step to give a final lysis suspension. Samples were buffered with either Tris–acetate (25 mM, final pH 7.4) or 2-(*N*-morpholino)ethanesulfonate (MES, 25 mM, final pH 6.0) prior to addition of oligomer. This mixture was incubated at 37°C and analyzed as described.

Stability in the presence of Escherichia coli cell lysate. Approximately 2×10^{11} cells of *E.coli* ES142 were pelleted by centrifugation, resuspended in Tris–HCl buffer (10 ml, 50 mM, pH 7.5) and incubated at room temperature for 5 min. Dithiothreitol and lysozyme were added to give final concentrations of 2 mM and 1 mg/ml respectively and the suspension was incubated at 37°C for 30 min. The mixture was treated to several rounds of sonication and freeze/thawing and centrifuged at 6000 *g* for 5 min. The volume of the final supernatant was estimated to be ~57 times the volume of the original cell pellet. Aliquots of this lysate were mixed with oligonucleotides, incubated at 37°C and analyzed as described.

Stability in the presence of Staphylococcal aureus cell lysate. Cell lysate of *S.aureus* MI273 was prepared and analyzed as described for the *E.coli* preparation with the following modifications: (i) the lysis was conducted with a cell pellet containing ~4×10^{10} cells; (ii) lysostaphin was used instead of lysozyme (500 U; Sigma Chemical Co.).

In vivo stability study in male Sprague–Dawley rats. A tritium labeled, 2′-*O*-methyl alternating MP/DE backbone oligonucleotide was synthesized with the sequence 5′-T-([^{3}H]MP)-2′-*O*-methyl-(C*m*U*p*U*m*C*p*C*m*A*p*U*m*G*p*C*m*A*p*U*m*G*p*C*m*A*p*U*m*G*p*U*m*C*p*C)*m*-T-3′, where *m* represents a racemic methylphosphonate linkage and *p* is a phosphate linkage. The final coupling step was done manually with a thymidine methylphosphonamidite monomer containing a [^{3}H-methyl]phosphonamidite coupling moiety (36). The oligonucleotide was suspended in Dulbecco's phosphate-buffered saline (Mediatech Inc.) and shipped to an outside vendor (Biodevelopment Laboratories Inc., Cambridge, MA) for injection (via tail vein) into cannulated male Sprague–Dawley rats (~250 g) at a dose of 2 mg/kg. Urine and blood samples were collected from animals at specified intervals. Blood samples were separated into plasma and cell pellets by centrifugation. Samples were frozen in liquid nitrogen and then shipped back to Genta Inc. for HPLC analysis. Upon receipt, the urine and plasma samples were thawed and diluted with an equal volume of acetonitrile to precipitate proteins. NP-40 was also added to a final concentration of 0.1%. The samples were then spun for 15 min in a microcentrifuge. Negligible radioactivity was detected in the protein pellets. The supernatants were analyzed by reversed phase HPLC as described above. The gradient used for these samples was as follows: 5–45% buffer B (2.5–12.5 min), 45–90% buffer B (12.5–22.5 min).

RESULTS

Chirally pure dimer synthons with methylphosphonate internucleosidyl linkages

The method for the separation and derivatization of 2′-deoxy R_P MP dimer synthons is illustrated in Figure 2. Each of the 16 possible dimers were prepared as a mixture of R_P and S_P diastereomers. The dimethoxytritylated, base-protected diastereomers were separated by silica gel flash chromatography. In all cases, the R_P diastereomer eluted on silica gel chromatography with faster mobility (15). The desired diastereomer was isolated and derivatized (for coupling on an automated DNA synthesizer). Derivatization at the 3′-end enabled the dimers to be coupled together via either racemic MP linkages, generating all-MP backbones with R_P linkages at every other position, or prochiral DE linkages, yielding alternating R_P MP/DE backbones.

The preparation of 2′-*O*-methyl chiral MP dimer synthons was by analogous procedures, but with several key modifications. First, the reaction time for coupling the two 2′-*O*-methyl monomers together was increased from 3 (as in the case of 2′-deoxy monomers) to 5 min, to drive the reaction to completion. Second, we found cumene hydroperoxide oxidation gave a more favorable ratio of 'fast' versus 'slow' diastereomers compared with iodine

oxidizer reagents (unpublished results). Third, baseline separation of the 'fast' and 'slow' diastereomers was not always achieved using flash column chromatography, even when the amount of silica gel was increased 10-fold. Where necessary, the two diastereomers were further purified by reversed phase HPLC following removal of the 3′-*O*-silyl protecting group.

We chose the 'fast' eluting diastereomers of the 2′-*O*-methyl MP dimers for further derivatization and incorporation into oligonucleotides, analogous to 'fast' eluting 2′-deoxy R_P MP dimers (12,15). This seemed to be a reasonable first choice, since the 2′-*O*-methyl groups were not expected to affect the relative chromatographic mobilities of the two diastereomers. In practice, all of the oligonucleotides prepared with 2′-*O*-methyl (fast isomer MP) dimers have higher binding affinities for their complementary RNA targets compared with the corresponding racemic oligonucleotides (data not shown).

Alternating MP/DE oligonucleotides

Optimized procedures for preparing methylphosphonate oligonucleotides have been described previously (28,30). We found that methylphosphonite (P-III) diester intermediates are highly sensitive to hydrolysis during automated synthesis. Because of this, a specially formulated iodine oxidizer reagent (0.25% water) was used when coupling methylphosphonamidite monomer or dimer synthons. On the other hand, β-cyanoethyl phosphite triester intermediates were not sufficiently oxidized with this reagent (data not shown). Therefore, it was necessary to use both a low-water oxidant (0.25% v/v water) for the MP coupling (29) and a high-water oxidant (2% v/v water) for the DE couplings. An auxiliary well on the DNA synthesizer was used for each oxidant and the oxidation cycles were reprogrammed as part of the respective coupling subroutines.

Isobutyryl-protected cytidine bases were used throughout this work, since benzoyl-protected cytidine is known to transaminate with the ethylenediamine deprotection reagent (28). Most of these bases are commercially available; isobutyryl-protected 2′-*O*-methyl cytidine was synthesized as previously described (24).

Chirally enhanced MP oligonucleotides

The structures of the various backbone analogs prepared with R_P MP dimer synthons are shown in Figure 1. Dimer synthons were coupled sequentially using automated solid phase chemistry (Fig. 3). For alternating (R_P MP)/(racemic MP) oligonucleotides, the synthesis cycles were as reported for racemic MPOs (28,30). Each coupling reaction was run for only 1 min and a low-water oxidizer reagent was used (0.25% water) to minimize hydrolysis of the P-III methylphosphonite intermediate (30). The coupling reactions for all-2′-deoxy and all-2′-*O*-methyl R_P MP/DE oligonucleotides were increased to 2 and 3 min respectively. Normal-water oxidizer reagent (2% water) was used for both types of R_P MP/DE oligonucleotides.

In our initial experiments, extremely poor coupling efficiencies (<10%) were obtained with these dimer synthons, despite their high purity as determined by ^{31}P NMR and HPLC. Based on ^{1}H NMR experiments, we determined that water content greatly attenuates the coupling efficiencies of these dimer synthons, particularly the

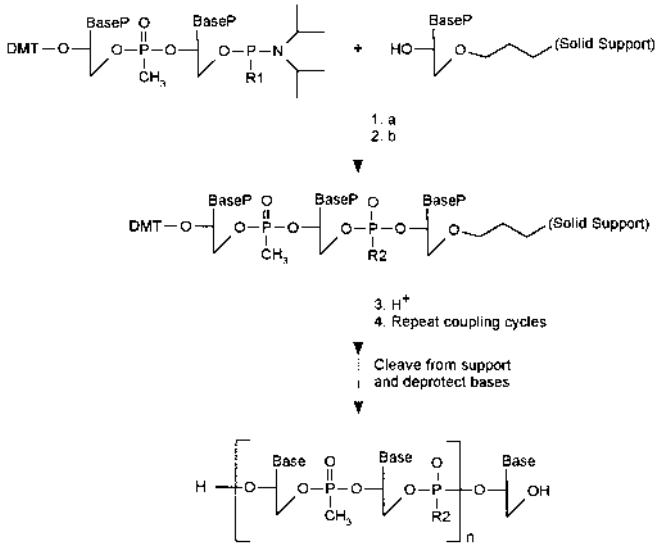

Figure 3. Solid phase coupling of 2′-deoxy R_P MP chiral dimer synthons. (**a**) Tetrazole. (**b**) Iodine/water/2,6-lutidine/THF. R1 is either methyl or β-cyanoethyl (see Fig. 2); R2 is either methyl (if R1 was methyl) or O^- (if R1 was β-cyanoethyl). BaseP is a protected base, as described in the text.

methylphosphonamidite derivatives. Dissolving the synthons in dry acetonitrile and drying over 3 Å molecular sieves for a minimum of 24 h typically increased coupling efficiencies to >95%.

Following coupling of the last dimer, oligonucleotides were cleaved from the support and the base protecting groups were removed using a one-pot procedure initially developed in our laboratory for use with racemic MPOs (28).

Biophysical studies

The effects of R_P versus S_P dimer substitutions on all-MP backbone oligomer–RNA duplex stability are shown in Table 1. These data show that the enhancement in binding affinity that occurs with R_P chiral enrichment is almost equal in magnitude to the reduction in affinity that occurs with S_P chiral enrichment. No attempt was made to correct the two-state model values for the mixture of species found in the racemic MP backbone.

The thermodynamic stability of a racemic methylphosphonate–RNA duplex can increase on replacing the racemic methylphosphonate by the R_P chiral methylphosphonate, a racemic phosphorothioate or a phosphate (Table 2). An additional gain in stability of RNA hybrids is possible by replacing the 2′-deoxyribose by a 2′-*O*-methyl ribose; this modification should shift the sugar conformation to the RNA A-form.

Native gels of selected antisense oligonucleotide/RNA complexes gave binding constants within an order of magnitude of the values from melting curves for phosphodiester, alternating R_P MP/DE and 2′-*O*-methyl alternating (fast isomer MP)/DE backbones (data not shown). No duplex was found for the racemic methylphosphonate–RNA duplex within the experimental concentration range. These gels showed no bands corresponding to triplex or higher order associations, even at extreme ratios of oligomer to RNA.

Table 1. Binding stability data for all-MP oligodeoxynucleotides with their complementary RNA; comparison of effects with R_P versus S_P dimer substitutions[a]

Oligomer	MP sequence[b]	Methylphosphonate configuration (*)	T_m (°C)	ΔT_m (°C)
2286-1	5′-(C*T)$_7$A-3′	R_P	45.5	+10.4
2288-1	5′-(C*T)$_7$A-3′	Racemic	35.1	
2287-1	5′-(C*T)$_7$A-3′	S_P	25.4	–9.7
2323-1	5′-(A*G)$_7$T-3′	R_P	55.2	+7.2
2253-1	5′-(A*G)$_7$T-3′	Racemic	48.0	
2252-1	5′-(A*G)$_7$T-3′	S_P	40.0	–8.0

[a]Melting conditions: 20 mM potassium phosphate, pH 7.2, 100 mM sodium chloride, 0.1 mM EDTA and 0.03% potassium sarkosylate. Approximately 2.4 μM total strand concentration.
[b]MP configuration between the CT or AG dimer is listed under Methylphosphonate configuration. Racemic MPs link the CT or AG dimers together.

Table 2. Thermodynamic data for oligonucleotide analogs determined from a two-state model fit of UV melting curves[a]

Oligomer	Backbone type[b]	T_m (°C)	K_a (per M, 37°C)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)
2288-1	All-MP, racemic	34.3	8.7×10^5	44.9	118
2781-1	2′-*O*-Me-(all-MP, racemic)	37.1	2.0×10^6	45.4	118
2782-2	Alternating MP/DE, racemic	40.6	6.1×10^6	76.1	214
2286-1	All-MP, alternating R_P/racemic	44.0	2.1×10^7	67.0	182
2768-1	2′-*O*-Me-[all-MP, alternating (fast isomer MP)/racemic]	47.4	3.6×10^7	59.0	156
3384-1	Alternating racemic MP/2′-*O*-Me-DE	50.1	3.7×10^8	83.8	231
2760-1	Alternating R_P-MP/DE (chirally pure)	55.1	2.7×10^{10}	107.8	300
2784-1	2′-*O*-Me-[alternating racemic MP/DE]	59.0	3.1×10^9	71.1	186
2795-1	DE	60.8	7.1×10^{11}	111.8	306
2765-1	2′-*O*-Me-[alternating (fast isomer MP)/DE]	70.3	3.6×10^{13}	107.0	283

[a]Sequences are either (CT)$_7$A (for backbones with 2′-deoxyribose sugars) or (CU)$_7$A (for backbones with 2′-*O*-methyl-ribose sugars), where the chiral center, if any, is between the C and T or U. Complementary RNA target U(AG)$_7$. Melting conditions: 20 mM potassium phosphate, pH 7.2, 100 mM sodium chloride, 0.1 mM EDTA and 0.03% potassium sarkosylate. Approximately 2.4 μM total strand concentration.
[b]MP, methylphosphonate; DE, phosphodiester; 2′-*O*-Me, 2′-*O*-methyl. In the methylphosphonate-containing sequences, the CU or CT dimers have methylphosphonate linkages and these linkages may be chirally pure.

Table 3. *In vitro* nuclease stability for different backbone modified oligonucleotides having the sequence (CT)$_7$A (2′-deoxy sugars) or (CU)$_7$A (2′-*O*-methyl sugars)

	Estimated half-life ($t_{1/2}$)			
	Phosphodiester (min)	2′-*O*-Methyl RNA (min)	2′-Deoxy alternating MP/DE (h)	2′-*O*-Methyl alternating MP/DE (h)
10% Fetal bovine serum	12	40	5	>300
COS-7 cell lysate, pH 6.0	<10	300	25	Stable[a]
COS-7 cell lysate, pH 7.4	<5	300	20	Stable[a]
E.coli cell lysate	13	72	65	Stable[a]
S.aureus cell lysate	15	1200	75	Stable[a]

[a]No detectable degradation of full-length oligonucleotide after 24 h incubation.

Nuclease stability studies

Several of the analogs shown in Table 2 were tested for their ability to resist nucleolytic degradation in various biological extracts *in vitro* (Table 3). Oligonucleotides were incubated in the presence of serum or cell lysates at 37°C. Aliquots were removed at specific time intervals and analyzed by reversed phase HPLC to determine the remaining full-length oligonucleotide. The 2′-deoxy alternating R_P MP/DE backbone analog is 25- to 300-fold more resistant to nuclease degradation, as compared with the unmodified DE oligonucleotide. The 2′-*O*-methyl alternating MP/DE backbone analog is almost completely resistant to degradation in these studies.

The 2′-*O*-methyl alternating (fast isomer MP)/DE backbone oligonucleotide was also tested for resistance to metabolic breakdown *in vivo*. This oligonucleotide was labeled with tritium at one of the methylphosphonate groups (36) and injected into male Sprague–Dawley rats via a single bolus tail vein injection (average dose 2 mg/kg). Blood aliquots were removed at specified time intervals via a cannula and centrifuged to separate the plasma for HPLC analysis (see Materials and Methods). Negligible radioactivity was associated with the cell pellets, so only the plasma samples were analyzed. Urine was collected from separate animals and analyzed by HPLC in a similar manner. More than 80% of the full-length oligomer was measured in blood samples taken 45 min post-dosing (the signal-to-noise ratio was too poor at later time points to obtain a reasonable integration of the main peak). Furthermore, >80% of the radioactive sample was excreted into the urine as intact oligomer during the first 4 h.

DISCUSSION

Racemic MPOs are reported to selectively inhibit mRNA translation in cell-free and cell culture assays in the micromolar range (6–9,37). A number of investigators have suggested the possibility of enhancing the potency of MPOs through stereoselective synthesis and/or chiral selection (12–14,17–22,38–40). According to our data, MPOs enriched in R_P linkages bind stronger than MPOs enriched in S_P linkages to complementary RNA. These results suggest that MPO diastereomers prepared by a conventional (racemizing) method have a broad range of binding affinities for RNA.

This study clearly demonstrates the enhanced binding of chirally pure mixed sequence MPOs to complementary RNA. Our binding studies were performed with synthetic RNA targets rather than DNA, since the intended intracellular antisense targets are mRNAs. Furthermore, we recognized that binding data obtained with DNA targets are not necessarily good predictors of binding to RNA due to the structural differences between these two targets. For example, duplexes formed with RNA targets are reported to have an A-form geometry, whereas duplexes formed with DNA can form a variety of different geometries depending on the solvent conditions and crystal packing forces (41).

Computer modeling of the 2′-deoxy MPO/RNA heteroduplex in an A-form helix suggests that S_P chiral methyl groups destabilize the duplex by pointing inward toward the major groove, causing unfavorable steric interactions with the sugar protons and bases of the MPO strand (T.A.Larsen, personal communication). Such observations are supported by molecular mechanics studies of dinucleoside methylphosphonates, wherein the S_P MP linkage is found to produce a greater level of perturbation to conformations around the P–O3′ and glycosyl bonds (42). Similarly, free energy decomposition analysis of palindromic sequences containing a single R_P or S_P methylphosphonate linkage predict steric effects between the C2′ and C3′ substituents on the S_P diastereomer (5′-side) and C5′ substituents on the R_P diastereomer (3′-side); the balance considerably favors the R_P configuration (43). A recent review describes calculated models of B-form helices (44), which show similar trends.

The absolute configuration of the 2′-*O*-methyl fast isomer MP dimer is not known, but it is reasonable to suggest it is the R_P isomer because of its retention on a silica gel in analogy with the 2′-deoxy series (15,16). On the other hand, it is possible that the 2′-*O*-methyl group plays a more significant role in hybridization affinity than we expect. For this reason, we cannot assume the fast isomer is R_P. Dimer configuration will be determined by both X-ray crystallographic and two-dimensional NMR experiments.

While the thermodynamic effects on the R_P MP and S_P MP chiral centers is nearly equal and opposite, the effects of other modifications are not necessarily additive, based on our limited dataset. Converting a single R_P MP linkage to diester gives an average increase of 5-fold in K_a (moving the equilibrium toward the duplex) and +0.4°C in T_m; the average change in the thermodynamic cycle converting a R_P center to racemic methylphosphonate and then from racemic methylphosphonate to a diester shows no change in K_a and only a +0.1°C increase in T_m (these differences were calculated from the average values of converting racemic MP to R_P MP centers, R_P MP to DE centers and racemic MP to DE centers for the oligomers presented here; data from 2′-*O*-methyl-modified oligomers were not included in this calculation). It is clear that describing the thermodynamic effects of R_P MP and S_P MP chiral centers will require a much larger data set than the few oligonucleotides of limited sequence described here. Nevertheless, these data support the relative stabilities of R_P and S_P methylphosphonate centers based on structural models.

These studies do not indicate why chirally pure methylphosphonate: RNA duplexes are less stable than phosphate: RNA duplexes with identical sugar chemistry. Methylphosphonate and other neutral backbones were thought to bind tighter to natural nucleic acids due to lack of charge repulsion. There are many potential reasons for the weaker interaction seen here. The methyl group of the methylphosphonate may be sterically unfavorable, even in the preferred R_P configuration. The neutral backbone in the duplex may perturb the network of ordered water molecules along the major groove typically seen in A-DNA systems (45). Finally, the free methylphosphonate strand may compress, since there is no charge–charge repulsion between phosphates; the collapsed methylphosphonate strand would require additional energy to expand the oligomer for duplex formation and so the single-stranded form would be favored over the duplex. Further study will be required to determine the reason for this difference in hybridization stability, including more in-depth thermodynamic studies, structural studies and comparisons with other uncharged backbones, such as PNAs (46), phosphonates (47) and phosphoramidates (48).

The alternating racemic MP/DE backbone oligonucleotide containing 2′-*O*-methyl sugar modifications is highly resistant to nuclease degradation. This is particularly evident from our *in vivo* rat data, where this analog type is found to be highly stable in the blood and excreted mostly intact into the urine.

CONCLUSION

These chirally pure, partially charged oligomers hybridize better to RNA than racemic MP oligomers, as shown by melting curves and gels. The 2′-*O*-methyl alternating MP/DE compounds tested here degrade more slowly than DE oligomers *in vivo* and *in vitro*. This research shows that these systems have great promise as pharmacological agents. *In vitro* studies will be discussed in other papers (B.D.Brown, manuscripts in preparation).

ACKNOWLEDGEMENTS

We would like to thank Lina I.Borozdina, Eric S.McCampbell and Kevin Young for synthesis and purification of oligomers,

Mike Atkins, Michelle Chapman-Scurria, Chris Garcia, Eric Hasselberth, Jody Hasselfield, Stephanie Hopkins and Mike Scurria for dimer synthesis and purification, Michael D.Metzler and Doreen Bubonovich for running melting curves, Shirley K.Hernandez for HPLC analysis of *in vivo* oligomer degradation and Bob D.Brown and Alexandre Lebedev for enlightening discussions. Peter A.Kollman generously provided the methylphosphonate linkage force fields.

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