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Synthesis of specific diastereomers of a DNA methylphosphonate heptamer, d(C*p*C*p*A*p*A*p*A*p*C*p*A), and stability of base pairing with the normal DNA octamer d(TpGpTpTpTpGpGpC)

Ekaterina V.Vyazovkina, Elena V.Savchenko, Sergei G.Lokhov, Joachim W.Engels[1], Eric Wickstrom[2,*] and Alexander V.Lebedev
Institute of Bioorganic Chemistry, 8 Lavrentyev Prospect, Novosibirsk 630090, Russia, [1]Institute for Organic Chemistry, Johann Wolfgang Goethe University, Marie-Curie-Strasse 11, Frankfurt 60439, Germany and [2]Department of Pharmacology, Thomas Jefferson University, 233 S. Tenth St., Philadelphia, PA 19107, USA

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ABSTRACT

DNA methylphosphonates are candidate derivatives for use in antisense DNA therapy. Their efficacy is limited by weak hybridization. One hypothesis to explain this phenomenon holds that one configuration of the chiral methylphosphonate linkage, Rp, permits stronger base pairing than the other configuration, Sp. To test this hypothesis, four specific pairs of Rp and Sp diastereomers of the DNA methylphosphonate heptamer d(C*p*C*p*A*p*A*p*A*p*C*p*A) were prepared by block coupling of different combinations of individual diastereomers of d(C*p*C*p*A*p*A) and d(A*p*C*p*A). Each pair of the diastereomers of the heptamer was separated into individual diastereomers using affinity chromatography on a Lichrosorb-NH_2 silica column with a covalently attached complementary normal DNA octamer, d(pTpGpTpTpTpGpGpC). The stabilities of complementary complexes of phosphodiester d(TpGpTpTpTpGpGpC) with 8 individual diastereomers of methylphosphonate d(C*p*C*p*A*p*A*p*A*p*C*p*A) were studied by measuring their melting temperatures (Tm). A direct correlation of Tm values with the number of Rp methylphosphonate centers in the heptamer was found: the more Rp centers, the higher the stability of the complex. Tm values for the diastereomers with 6 all-Rp or all-Sp methylphosphonate centers were found to be 30.5° and 12.5°C, respectively, in 100 mM NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, pH 7.0 with 15 μM of each oligomer. On the average, each substitution of one Rp-center to an Sp-center in the heptamer decreased the Tm by 3°C. Under the same conditions, the Tm of the normal DNA heptamer with its complement was 21°C. These results are consistent with the model that all-Rp methylphosphonate DNAs hybridize much more tightly to complementary normal DNA than do racemic methylphosphonate DNAs, and may therefore exhibit greater potency as antisense inhibitors.

INTRODUCTION

The simplest way to target a specific gene sequence in a naturally occurring nucleic acid lies in the synthesis of a complementary oligonucleotide (1). Cellular expression of a target gene may in theory be ablated by hybridization of a single-stranded DNA complementary to an accessible sequence in the mRNA transcript from that gene (2), or to some sequence in the transcript which is required for post-transcriptional modification (3), or to a purine-rich sequence in the gene itself, capable of forming a triple helix (4). Genetic regulation by these methods is called antisense or antigene inhibition, and may prove valuable for functional probing, diagnosis, and therapy of aberrant genes in viral, bacterial, plant, and animal systems (5,6). A variety of synthetic DNA derivatives have been applied to control many different genes in cell culture, and a few in whole organisms, such as mice (7–10).

Antisense DNA synthesized with normal phosphodiester linkages is useful as a research tool for probing biological function. However, the relatively high concentrations of DNA necessary for activity in cell culture, and the rapid degradation of normal DNA in serum (11), make its use in therapy unrealistic. Attempts to improve the therapeutic efficiency have involved many different approaches to derivatization (12–14) to improve nuclease resistance, specificity of binding to target nucleic acids, and cellular uptake. Substitutions on the backbone include those which reduce nuclease sensitivity, while maintaining a negative charge, such as phosphorothioates (15) or borane phosphonates

*To whom correspondence should be addressed

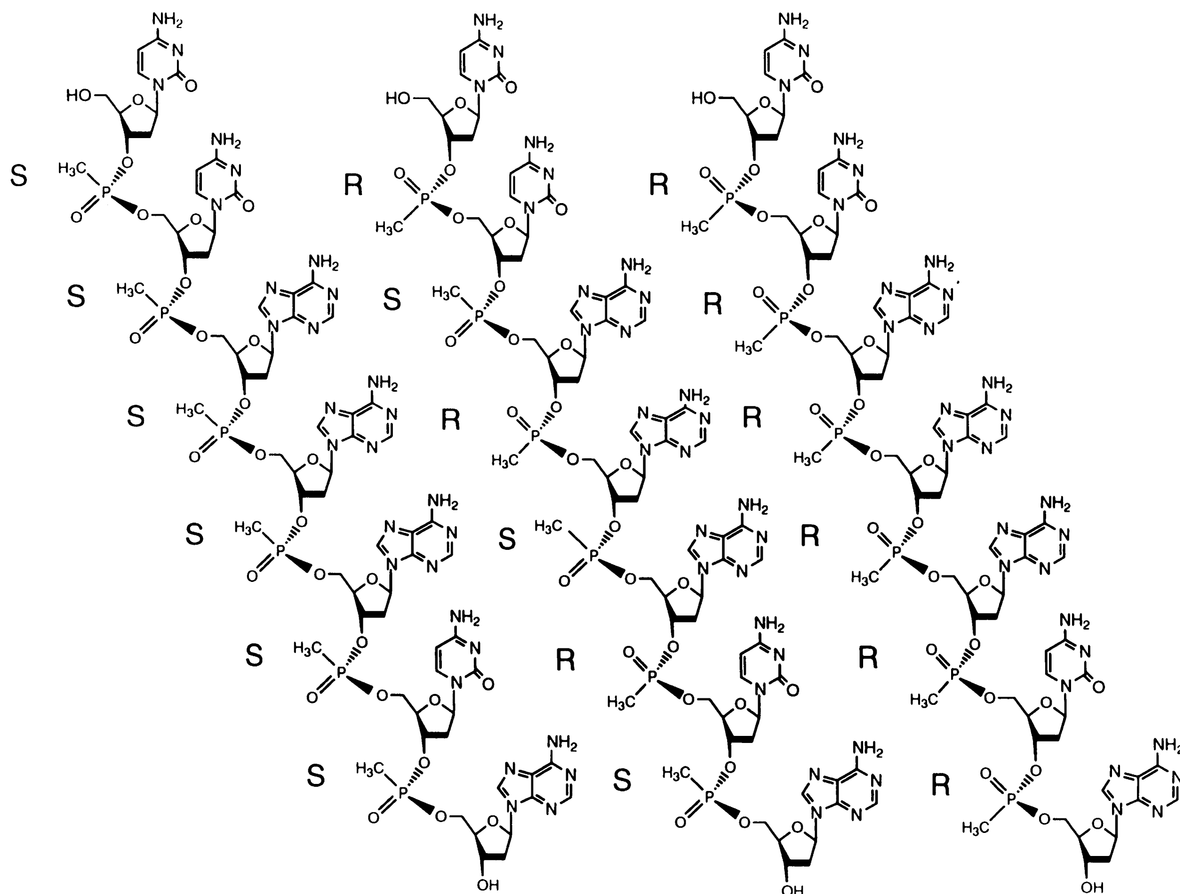

Figure 1. Individual diastereomers of d(C*p*C*p*A*p*A*p*A*p*C*p*A) methylphosphonate. Left, SpSpSpSpSpSp; middle, RpSpRpSpRpSp; right, RpRpRpRpRpRp.

(16), and those which replace ionic moieties, in order to allow the DNA to diffuse through cellular, organellar, or nuclear membranes, such as methylphosphonates (17) or phosphoramidates (18).

In particular, uncharged methylphosphonate DNAs (Fig. 1) are unrecognized by nucleases, enter animal cells rapidly without utilizing the DNA receptor, and specifically inhibit expression of targeted genes, distinguishing even a single mismatch (19). In c-*myc*-transgenic mice, intravenous administration of an antisense DNA methylphosphonate lowered the expression of oncogene c-*myc* (8). However, significant inhibition only achieved at high concentrations, as others have also observed for DNA methylphosphonates. This requirement for a high DNA methylphosphonate concentration is probably due to lack of cleavage of DNA methylphosphonate/RNA hybrids by RNase H, and to the existence of asymmetric Rp and Sp diastereomers at each methylphosphonate linkage (17). The steric limitation applies equally to all asymmetric phosphodiester derivatives, e.g., phosphorothioates (20). To bypass the stereochemical problems brought on by modifying the phosphate, a number of non-phosphate linkages have been synthesized (21); these modifications do not necessarily provide cellular uptake and strong hybridization.

One may predict from molecular mechanics calculations that all-Rp DNA methylphosphonates would display the greater hybridization strength expected originally (22,23). To test this idea experimentally, octathymidylates were synthesized, by Grignard reagent activation of the 5′-OH, with all-Rp or all-Sp methylphosphonate linkages, except for the central one, which was racemic (24). In a physiological buffer, the melting temperature of the Rp-enriched form with $(dA)_{15}$ was 36° higher than that of the Sp-enriched form, and 25° higher than that of normal $(dT)_8$. Evidently, an Sp residue does not stack or hydrogen bond as strongly as an Rp residue, in a thymidylate octamer. This was a powerful experimental demonstration of the importance of stereochemistry in the hybridization of methylphosphonates.

Limited progress has been achieved so far in developing stereospecific solution phase coupling methods for methylphosphonate (25,26). In order to produce a few testable oligomers with heterogeneous sequences, racemic coupling followed by chromatographic separation has been utilized to prepare and assign individual diastereomers of dimers (27), trimers, and tetramers (28). In the work below, we report coupling of defined trimers and tetramers, followed by chromatographic separation, to yield individual diastereomers of a methylphosphonate heptamer, d(C*p*C*p*A*p*A*p*A*p*C*p*A) (Fig. 1). The latter sequence was chosen for comparison with the phosphodiester heptamer, d(CpCpApApApCpA), used as a model oligomer in a number of earlier physical chemical studies (29). Measurements of melting temperatures of the duplexes of the methylphosphonate heptamers with a complementary normal DNA in a physiological buffer revealed stabilization by Rp linkages, and destabilization by Sp linkages.

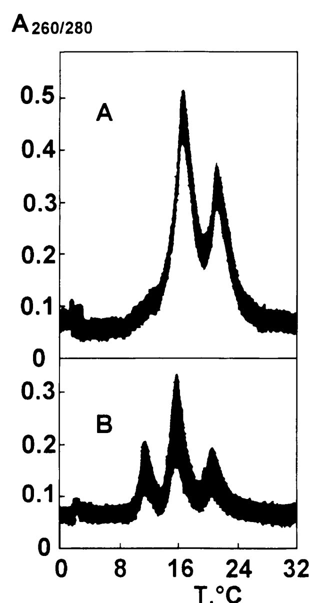

Figure 2. Separation of diastereomers of d(C*p*C*p*A*p*A*p*A*p*C*p*A) methylphosphonate by complementary DNA chromatography with a sorbent containing covalently attached phosphodiester d(pTpGpTpTpTpGpGpC). Eluent: 20% acetonitrile in water; flow rate: 50 μl/min; linear increase of the column temperature: 1°C/min; ultraviolet absorbance detection: 260/280 nm, alternating every 15 sec. Upper edge of trace is A_{260}; lower edge is A_{280}. **A** — separation of the mixture of RpRpRpRpRpRp and RpRpRpSpRpRp diastereomers; **B** — separation of the mixture of RpRpRpRpRpRp, RpRpRpSpRpRp, RpSpRpRpRpSpRp and RpSpRpSpRpSpRp diastereomers.

MATERIALS AND METHODS

Synthesis of individual diastereomers of base-protected DMT-d(C*p*C*p*A*p*A)-Ac methylphosphonate and d(A*p*C*p*A)-Ac methylphosphonate

The preparation and assignment of the individual methylphosphonate diastereomers of d(C*p*C*p*A*p*A) and d(A*p*C*p*A) has been described (28). Briefly, the trimers and tetramers were prepared by block coupling of diastereomerically pure dimers (27) with either monomers or other diastereomerically pure dimers. These oligomers were separated chromatographically into individual diastereomers, and the configurations of the methylphosphonate linkages were assigned. Three types of methods were used to assign configuration of a new methylphosphonate linkage: preparation of the same diastereomer through multiple synthetic pathways, base hydrolysis, and acid hydrolysis. Hydrolysis of the diastereomerically pure oligomers into component dimers and monomers was followed by chromatographic comparison with control dimers of known configuration. In all cases studied, oligomers with Rp configurations displayed faster elution from silica gel than did oligomers with the respective Sp configuration. Two-dimensional NMR spectra of individual diastereomers of d(A*p*C*p*A) were characterized and assigned.

Deacetylation and methylphosphonylation of individual diastereomers of base-protected DMT-d(C*p*C*p*A*p*A)-Ac methylphosphonate

The following configurations of individual diastereomers were used: RpRpRp, RpSpRp, SpRpSp, and SpSpSp. Each individual tetramer diastereomer (ca. 8 mg) was dissolved in 400 μl of pyridine–ethanol mixture (1:3), then cooled to 0°C, after which 200 μl of 2 N KOH at 0°C were added to the solution. After 4.5–5 min., the reaction was stopped by the addition of Dowex H^+ 50W×4 resin (50–100 mesh, Serva); the suspension was filtered, and the resin was washed with a mixture of pyridine–ethanol–water (1:1:1). The combined filtrate and wash solutions, containing the product, were extracted into chloroform from the aqueous phase, and the chloroform phase was evaporated and purified by chromatography on silica gel eluted with a gradient of methanol in chloroform (0–10%). The yields of deacetylated tetramer were 30–50%. To incorporate a methylphosphonate function on the 3′-end of a tetramer, each individual deacetylated diastereomer of the tetramer (ca. 3 mg) was dried under vacuum and dissolved in 20 μl of pyridine. Next, a 3-fold molar excess of 1 M methyphosphono-bis-imidazolide in acetonitrile was added to the solution (30). The reaction was monitored by TLC on silica gel plates developed in methanol–chloroform (1:9); the resulting products with a 3′-methylphosphonate had low mobility and stayed at the origin. After completion of the reaction (3 hours at 20°C), 25 μl of aqueous 1 M $NaHCO_3$ were added, followed 5 min. later by 50 μl of water, and the mixture was extracted several times with chloroform. The chloroform layer was washed with water, evaporated to dryness, redissolved in chloroform, then precipitated by the addition of hexane. The product was used without further purification. The yield of each 3′-methylphosphonylated tetramer was about 2–3 mg (60–80%).

Coupling of individual diastereomers of base-protected DMT-d(C*p*C*p*A*p*A)-methylphosphonate and d(A*p*C*p*A)-Ac methylphosphonate

The following pairs of diastereomers of tetramers and trimers were used: RpRpRp+RpRp; RpSpRp+RpSp; SpRpSp+SpRp; SpSpSp+SpSp. Individual diastereomers of DMT-d(A*p*C*p*A)-Ac were detritylated as described (28). The ratio of tetramer to trimer was about 1:2. An excess of trimer was used because of the presence of a side reaction of sulfonylation of the 5′-OH group with an excess of TPS. Trimer, (2–3 μmol), tetramer (1–1.5 μmol), and a 3-fold molar excess of TPS over tetramer were dried overnight under vacuum, dissolved in 20–30 μl of pyridine to 50 mM tetramer and 100 mM trimer, and a 2-fold molar excess of N-methylimidazole (300 mM) over TPS (150 mM) was added. The reaction was monitored by TLC on silica gel plates developed in methanol–chloroform (1:9); the resulting d(C*p*C*p*A*p*A*p*A*p*-C*p*A) heptamers with a DMT group and higher mobility than the initial tetramer appeared. After completion of the reaction (5 hr. at 20°C), 10 μl of water were added, the mixture was evaporated, and dissolved in chloroform. The heptamer was isolated by chromatography on silica gel eluted with a gradient of methanol (0–20%) in chloroform. The yields of each heptamer were 1–2 mg (30–50%). None of the pairs of diastereomers of fully protected heptamer were separable by silica gel HPLC, nor fully deprotected heptamers by reversed phase HPLC. Deprotection of heptamers was performed as described previously (28), yielding about 0.1 mg of each fully deprotected heptamer with a single racemic methylphosphonate linkage.

Separation of diastereomers of d(*CpCpApApApCpA*) methylphosphonate by microcolumn complementary chromatography

Thirty μl of each pair of heptamer diastereomers (ca. 2 μg) in 20% acetonitrile in water was applied at low temperature

Table 1. Separation of diastereomers of d(C*p*C*p*A*p*A*p*A*p*C*p*A) methylphosphonate by complementary DNA chromatography on a Lichrosorb-NH_2 column with covalently attached d(pTpGpTpTpTpGpGpC).

Diastereomer Pair	Temperature of elution (°C) First Eluted	Second Eluted
RpRpRpSpRpRp + RpRpRpRpRpRp	22	25
RpSpRpSpRpSp + RpSpRpRpRpSp	18	21
SpRpSpSpSpRp + SpRpSpRpSpRp	13	15
SpSpSpSpSpSp + SpSpSpRpSpSp	11	12

Diastereomers were eluted under the conditions of Figure 2.

(0–1°C) to a water-jacketed thermostatted column (2×64 mm) with a sorbent (Lichrosorb-NH_2) containing a covalently attached normal octamer, d(pTpGpTpTpTpGpGpC) (31). Elution was performed with 20% acetonitrile in water at a flow rate of 50 μl/min. During elution, the temperature of the column was increased linearly from 1° to 30°C at 1°C/min. Eluent absorbance was measured alternately at 260 nm or 280 nm, changing every 15 sec., allowing continuous monitoring of the 260/280 ratio of each peak. Fractions containing the first and second eluted diastereomers were collected separately and evaporated to dryness.

Measurements of d(*CpCpApApApCpA*) methylphosphonate-d(TpGpTpTpTpGpGpC) phosphodiester melting temperatures

Each purified individual diastereomer of d(C*p*C*p*A*p*A*p*A*p*C*p*A) was dissolved in 100 mM NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, pH 7.00, to a final oligomer concentration of 100 μM. Next, an equal volume of 100 μM d(TpGpTpTpTpGpGpC) in the same buffer was added, and the final concentration of the double-stranded complex was adjusted to 15 μM. For melting curves in 1.0 M NaCl, insufficient samples of each separated diastereomer remained, so samples of each unseparated pair were measured instead. Melting experiments were carried out using a custom-made microscale instrument based on the Milichrom-2 spectrophotometric detector (PO Nauchpribor, Orel, Russia) as described (32). The microcell used was 2 mm in pathlength, 1.1 mm in diameter, and 2 μl in volume. Melting temperatures (Tm) were determined as the maximum point on the peaks of the first derivatives of the melting curves.

RESULTS

Four pairs of diastereomers of the DNA methylphosphonate heptamer d(C*p*C*p*A*p*A*p*A*p*C*p*A) (Table 2) were synthesized, then separated into individual diastereomers using affinity chromatography on a silica column (Lichrosorb-NH_2) with covalently attached complementary phoshodiester DNA, d(pTpGpTpTpTpGpGpC). Separation of the two diastereomers of the remaining racemic linkage was performed using a linear increase in the column temperature, or separation at constant melting temperature were used. Figure 2 shows examples of the temperature increase method of separation of the mixture of RpRpRpSpRpRp and RpRpRpRpRpRp diastereomers (Fig. 2A). It was assumed that the first eluting peak was the Sp diastereomer, and the second peak, which hybridizes more tightly, was the Rp form. In the case when two pairs of diastereomers RpSpRpSp-RpSp/RpSpRpRpRpSp, and RpRpRpSpRpRp/RpRpRpRpRpRp

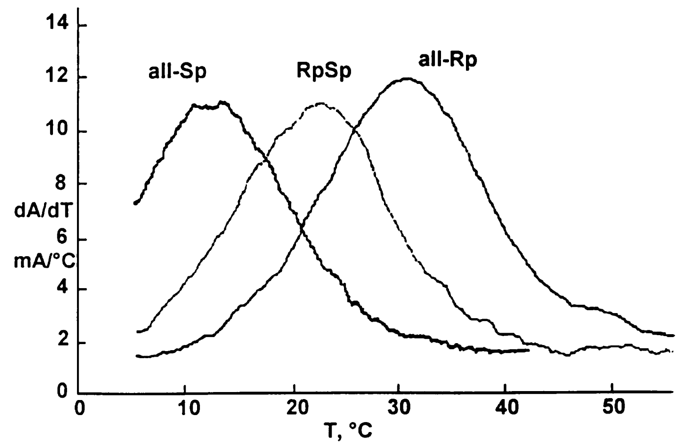

Figure 3. First derivatives of the melting curves for duplexes of normal DNA octamer d(TpGpTpTpTpGpGpC) with individual diastereomers of the methylphosphonate DNA heptamer d(C*p*C*p*A*p*A*p*A*p*C*p*A) in 100 mM NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, pH 7.00. All-Sp: SpSpSpSpSpSp; RpSp: RpSpRpSpRpSp; all-Rp: RpRpRpRpRpRp.

Table 2. Melting temperatures of the duplexes of individual diastereomers of methylphosphonate heptamer d(C*p*C*p*A*p*A*p*A*p*C*p*A) with d(pTpGpTpTpTp-GpGpC).

Diastereomer	Tm, °C 0.1 M NaCl	1.0 M NaCl
RpRpRpRpRpRp	30.5	28*
RpRpRpSpRpRp	27	
RpSpRpRpRpSp	25	24*
RpSpRpSpRpSp	23	
SpRpSpRpSpRp	14.5	15*
SpRpSpSpSpRp	13	
SpSpSpRpSpSp	13.5	14*
SpSpSpSpSpSp	12.5	
d(CpCpApApApCpA)	21	35

Concentration of each double-stranded duplex was 15 μM. The melting temperatures of complexes (Tm) were determined at the maximum of the first derivative plot.
*for a mixture of a pair of diastereomers.

were mixed before separation, resulting in three peaks (Fig. 2B), the first peak was assumed to contain the most Sp linkages, the broad middle peak a mixture of one and two Sp linkages, and the latest eluting peak was assumed to be all-Rp. While under identical separation conditions the elution temperature was reproducible and the order of elution of diastereomers was not dependent on the rate of increase in column temperature, the absolute value of the elution temperature for diastereomers does depend on the rate of column temperature change. Table 1 displays the elution temperatures of each of the individual diastereomers of all four pairs, under the conditions described in Fig. 2.

Figure 3 shows the first derivatives of the melting curves of the complexes of the DNA octamer d(TpGpTpTpTpGpGpC) with three diastereomers of the methylphosphonate heptamer d(C*p*C*p*A*p*A*p*A*p*C*p*A). The least stable diastereomer, SpSpSpSpSpSp, peaked at 12.5°C, while the most stable, RpRpRpRpRpRp, peaked at 30.5°C. The alternating form, RpSpRpSpRpSp, peaked at 23°C. In Table 2, the melting

temperatures of all 8 diastereomers in 100 mM NaCl are presented, along with the Tm of normal d(CpCpApApApCpA) in that buffer, 21°C. For comparison, Table 2 also shows the melting temperatures of the unseparated pairs of diastereomers in 1.0 M NaCl. It is assumed that the latter results represent the average of the melting temperatures of each pairs of diastereomers.

DISCUSSION

Upon reviewing of the body of work dealing with the predicted and observed effects of stereochemistry of modified phosphodiester linkages on duplex stability (30), it was reasonable to hypothesize that one configuration of the chiral methylphosphonate linkage, Rp, would allow more stable hybridization than the opposite configuration, Sp. To test this hypothesis, four specific pairs of Rp and Sp diastereomers of the DNA methylphosphonate heptamer d(CpCpApApApCpA) were prepared by block coupling of different combinations of individual diastereomers of d(C*p*C*p*A*p*A) and d(A*p*C*p*A). Each pair of the diastereomers of the heptamer was separated into individual diastereomers using affinity chromatography on a Lichrosorb-NH_2 silica column with a covalently attached complementary normal DNA octamer, d(pTpGpTpTpTpGp-GpC). The stabilities of complementary complexes of phosphodiester d(TpGpTpTpTpGpGpC) with 8 individual diastereomers of methylphosphonate d(C*p*C*p*A*p*A*p*A*p*C*p*A) were then studied by measuring their melting temperatures (Tm). A direct correlation of Tm values with the number of Rp methylphosphonate centers in the heptamer was found: the more Rp centers, the higher the stability of the complex (Table 2).

As the number of Rp centers increased from zero to three, the Tm in 100 mM NaCl increased from 12.5° to 14.5°. This modest increase, however, was followed by a sharp jump in Tm from 14.5° to 23° for the structural transition from SpRpSpRpSpRp to RpSpRpSpRpSp. From this point on, Tm increased at 2° per Rp center from three to five centers; for the final step to the all-Rp heptamer, the Tm increased by 3.5°. Thus, the average increase in Tm was 3° per Rp center, with the normal phosphodiester d(CpCpApApApCpA) displaying a Tm equivalent to alternating Rp and Sp centers.

The pattern, however, showed only moderate increases in duplex stability as the all-Sp form was perturbed by the addition of Rp centers. The jump in Tm in the middle of Table 2 for the heptamers with a preponderance of Rp centers implies a cooperative transition to a noticeably more stable duplex conformation. In 100 mM NaCl, the all-Rp methylphosphonate heptamer was significantly more stable in a duplex with complementary DNA than was normal DNA. This result allows the possibility that all-Rp methylphosphonate diastereomers might be able to invade a duplex DNA structure, as was found for polyamide DNA (34). As would be expected for a neutral oligomer, the methylphosphonate Tm's showed little dependence on salt concentration, while the Tm of the normal heptamer climbed above that of the all-Rp methylphosphonate in 1 M NaCl.

The preparation of individual diastereomers of DNA methylphosphonates with heterogeneous sequences and assignment of absolute configurations at asymmetric centers still remain very difficult tasks, and obviously are not convenient for routine procedures. On the other hand, the preparation of the individual diastereomers of at least a few different DNA methylphosphonate sequences is necessary in order to answer the question as to whether or not real differences exist in the stability of their complexes with complementary nucleic acid sequences. Our data showed a dramatic difference in stability of the complementary complexes of phosphodiester DNA with the all-Rp or all-Sp individual diastereomers of a DNA methylphosphonate heptamer, d(C*p*C*p*A*p*A*p*A*p*C*p*A). It will be of great interest to determine the stabilities of the corresponding complexes with phosphodiester RNA, the stabilities of other sequences of defined stereochemistry, and the efficacy of stereoregular DNA methylphosphonates as antisense inhibitors.

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