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Preparation of trimers and tetramers of mixed sequence oligodeoxynucleoside methylphosphonates and assignment of configurations at the chiral phosphorus

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ABSTRACT

Synthesis of stereoregular DNA methylphosphonates has been accomplished for homo-oligomers, but remains a formidable problem for oligomers of a defined antisense target sequence. In this work, four trimer and tetramer deoxynucleoside methylphosphonates of mixed sequence (dACA, dCCAA, dAGGG, and dGCAT) were prepared by block coupling of diastereomerically pure dimers with either monomers or other diastereomerically pure dimers. These oligomers were separated chromatographically into individual diastereomers, and the configurations of the chiral 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 R configurations displayed faster elution from silica gel than did oligomers with the respective S configuration. NMR spectra of individual diastereomers of dACA were studied, revealing characteristic differences in chemical shifts which may prove useful in configurational assignments of longer oligomers. Thus, these data provide a methodological basis for synthesis and configurational assignment of longer methylphosphonate oligomers to use as antisense probes.

INTRODUCTION

Oligodeoxyribonucleoside methylphosphonates have been shown to be attractive analogs for use as antisense DNAs because of their nuclease resistance (1–4), ease of penetration into cells (1,5–7), and ability to hybridize with complementary oligonucleotides and polynucleotides (1,5,6). However, coupling of methylphosphonates results in asymmetric phosphorus centers, either in the Rp or Sp configuration, with differing physical properties (1,2,8–13). Most studies carried out so far indicate that the Rp diastereomer is more desirable because it allows for more stable complex formation with complementary oligo- and polynucleotides (2,9,14,15). However in a few cases the Sp diastereomer showed a higher stability of complexes, especially in the case of triplex formation (5,14,16).

Previously, the only stereoregular oligodeoxynucleoside methylphosphonates prepared, longer than dimers, were the all Rp and all Sp tetrathymidylates (17). They were synthesized from 5′-activated monomers that were coupled stereoregularly through a Grignard reaction. Two tetrathymidylates were later coupled to yield an octathymidylate, which was racemic only at the central methylphosphonate linkage (9). This method has recently been extended to diadenylate, dicytidylate, and diguanylate (18).

To achieve the goal of obtaining a heterogeneous stereoregular oligomethylphosponate, we relied on racemic coupling (19) and separation at each step. Thus, after the separation of the individual diastereomers, it was necessary to assign the configuration of the newly formed chiral center. Previously, all 16 pairs of methylphosphonate dimers were synthesized, separated into individual diastereomers, and their absolute configurations were assigned (11–13). Here we report the preparation and configurational assignments of several diastereomerically pure trimers and tetramers.

EXPERIMENTAL

NMR spectroscopy

One-dimensional NMR spectra and 2D ROESY NMR spectra were recorded on a Bruker AM-400 spectrometer at 27°C. Samples were prepared as 450 μl of ca. 5–10 mM solutions in C^2HCl_3 (for protected oligomer) or 2–3 mM solutions in ^2H_2O (for deprotected oligomers). The spectra were calibrated using internal signals of CH_3CN (1.93 ppm) for ^2H_2O solutions or $CHCl_3$ (7.24 ppm) for C^2HCl_3 solutions. The details of ROESY experimental parameters were similar to those used by Löschner and Engels (10). 2D ROESY NMR pulse programs were kindly provided by Prof. C. Griesinger. Polynomial baseline corrections

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were performed by a program kindly provided by Dr. J. Zimmermann.

Liquid chromatography

Preparative HPLC (isolation and separation of diastereomers) was performed with a Waters 600 HPLC on a Nucleosil silica gel (5 μm) column (1×50 cm) or a Nucleosil reversed phase (10 μm) RP-18 column (0.4×25 cm). Analytical microcolunm HPLC was performed with a Milichrom HPLC (Orel, Nauchpribor, Russia) on a Zorbax reversed phase (5 μm) RP-18 column (2×64 mm).

Synthesis of DMT-*dNp(Me)*dN-Ac

All dimers necessary for the synthesis of the trimers and tetramers were previously described (11). The diastereomers were separated by silica gel HPLC as described except for DMT-*dGp(Me)*dG-Ac which was found to have a much better separation without the DMT blocking group in a MeOH/$CHCl_3$ gradient.

Synthesis of HO-*dNp(Me)*dN-Ac

The general method for removing the 5′ DMT blocking group was to dissolve DMT-*dNp(Me)*dN-Ac in 80% CH_3COOH for 30−60 minutes at 20°C. Following detritylation the reaction mixture was extracted several times with $CHCl_3$ and 1 M $NaHCO_3$. Water was removed from the organic layer by drying with Na_2SO_4 or by co-evaporation with EtOH. The organic layer was concentrated to an oil and purified by silica gel HPLC with a gradient of MeOH (0−10%) in $CHCl_3$.

Synthesis of DMT-*dNp(Me)*dN-OH

The general method for removing the 3′ Ac blocking group was to dissolve DMT-*dNp(Me)*dN-Ac in a pyridine/EtOH mixture (1:3) cooled to 0°C, and then to add half of the resulting volume of 3N KOH, cooled to 0°C. The reaction mixture was kept at 0°C for about 6 min. and then transferred to Dowex 50W X4, 50−100 mesh (Serva) in the H^+ form. The resin was washed with a mixture of EtOH/pyridine/H_2O (1:1:1). The washes were extracted twice with $CHCl_3$, dried with Na_2SO_4, filtered, evaporated to an oil, redissolved in $CHCl_3$, and purified by silica gel HPLC with a gradient of MeOH (0−10%) in $CHCl_3$.

Total deprotection of oligodeoxyribonucleoside methylphosphonates

Fully protected trimer or tetramer (1−2 mg) was dissolved in 1 ml of 80% CH_3COOH and kept for 60 min at 20°C. Then 0.5 ml of pyridine was added, the mixture was evaporated to an oil, and dissolved in 1 ml of $CHCl_3$. Detritylated oligodeoxynucleoside methylphosphonate was isolated by silica gel HPLC with a gradient of MeOH (0−10%) in $CHCl_3$. The fractions containing product were combined, evaporated to dryness and dissolved in 0.5 ml of ethylenediamine/EtOH (1:1). After 10 h the reaction mixture was evaporated to an oil, twice dissolved and evaporated with absolute EtOH, dissolved in 1 ml of water, and the product was isolated by reversed phase HPLC with a gradient of CH_3CN (0−40%) in water. The overal yields were 40−60%.

Acid hydrolysis of deprotected oligodeoxyribonucleoside methylphosphonate

Deprotected oligodeoxynucleoside methylphosphonate (2−4 $A_{260}U$) was dissolved in 0.05 ml of 0.01 M HCl at 37°C. After sufficient time for about 50% reaction (2−3 h) the reaction was stopped by neutralization with 0.1 M NH_3 (aq) up to pH 7. The solution was lyophilized, dissolved in 0.05ml of water, and analysed by microcolumn reversed phase HPLC.

Base hydrolysis of deprotected oligodeoxyribonucleoside methylphosphonate

Deprotected oligodeoxynucleoside methylphosphonate (2−4 $A_{260}U$) was dissolved in 0.05 ml 0f 0.1 M KOH at 20°C. After sufficient time for about 50% reaction (5−10 min) the reaction was stopped by neutralization with 0.1 M HCl down to pH 7. The solution was passed through Dowex 1×4, 50−100 mesh (Serva) in the Cl^- form. The resin was washed with water, and the fractions containing uncharged reaction products were combined. The resulting solution was lyophilized, dissolved in 0.05 ml of water and analysed by by microcolumn reversed phase HPLC.

Microcolumn HPLC analysis of hydrolyzed products

Five μl of hydrolyzed oligodeoxynucleoside methylphosphonate (see above) were applied to an analytical Zorbax C18 column (2×64 mm), eluted at 100 μl/min. A step gradient of CH_3CN in 0.05 M $NaClO_4$ was used: 0%, 5.0%, 7.5%, 10.0% and 25.0% CH_3CN (0.4 ml or 4 min for each step) for diastereomers of the tetramer dAp(Me)dGp(Me)dGp(Me)dG; 0% (0.1 ml, 1 min), 2.5%, 5.0%, 7.5%, 10.0%, and 25.0% CH_3CN (0.4 ml or 4 min for each step) for diastereomers of the trimer dAp(Me)dCp(Me)dA and the tetramers dCp(Me)dCp-(Me)dAp(Me)dA and dGp(Me)dCp(Me)dAp(Me)dT.

Synthesis of diastereomers of DMT-*dAp(Me)*dCp(Me)*dA-Ac by multiple pathways (Scheme 1)

Routes 1 and 4: DMT-*dA (66 mg, 0.1 mmol) was dissolved in 0.05 ml of pyridine and added to 0.1 ml of 1.19 M $P(CH_3)O(Im)_2$ in CH_3CN. Preparation of this phosphonylating reagent was described previously (11,18). After 2 h the reaction mixture was divided into two equal parts and coupled to either the Rp or Sp diastereomer of HO-*dC(Me)*dA-Ac. In Route 1, 22 mg of the Rp isomer in 0.03 ml pyridine and 7.5 mg of tetrazole in 0.015 ml of pyridine were added to one part; in Route 4, 16 mg of the Sp isomer in 0.02 ml of pyridine and 6.0 mg of tetrazole in 0.012 ml of pyridine were added to the second part. The following steps were performed separetely for each diastereomer. The mixture was kept at room temperature for 3 h, then 0.05 ml of 1 M $NaHCO_3$ were added, and the nucleotide material was extracted with 0.01 ml of $CHCl_3$. Organic layers were combined, washed 3 times with 0.1 ml of water, dried with

Table 1. The retention times (Rt) of individual diastereomers of trinucleoside methylphosphonate (DMT)-*dAp(Me)*dCp(Me)*dA-(Ac), synthesized through multiple pathways.

Configuration	Rt (min)[a]	Route[b] 1	2	3	4
RR	5.25	+	−	+	−
SR	6.75	+	−	−	+
RS	6.13	−	+	+	−
SS	8.75	−	+	−	+

[a] Coincidence of Rt for identical diastereomers in different analyses was within 0.25 min; the conditions of analytical silica gel (Nucleosil, 5 μm, 4×250 mm) chromatography were 5% MeOH in $CHCl_3$ eluted at 5 ml/min.
[b] See route designation in Scheme 2; (+) and (−) means that diastereomer was or was not prepared by the appropriate route, respectively.

Na_2SO_4, and evaporated to an oil. The oil was dissolved in $CHCl_3$ and the diastereomers were purified and separated by silica gel HPLC with a 0–15% MeOH gradient in $CHCl_3$/EtOAc (1:1). The yields were 11 mg (30%) for the RpRp and 11 mg (30%) for the SpRp diastereomers of Route 1, and 9 mg (25%) for the RpSp and 9 mg (25%) for the SpSp diastereomers of Route 4.

Route 2: DMT-*dAp(Me,R)*dC-OH (20 mg) was dissolved in 0.02 ml of pyridine and added to 0.022 ml of 1.19 M $P(CH_3)O(Im)_2$ in CH_3CN. The mixture was kept at room temperature for 1.5 h and a mixture of 15 mg of *dA-Ac and 4 mg of tetrazole in 0.01 ml of pyridine was added. After 2 h the mixture was treated identically as described for routes 1 and 4. The oil was dissolved in $CHCl_3$ and the diastereomers were purified and separated by silica gel HPLC with a 0–10% MeOH gradient in $CHCl_3$. The yields were 6.5 mg (25%) for RpRp and 6.5 mg (25%) for RpSp diastereomers.

Route 3: DMT-*dAp(Me,S)*dC-OH (11 mg) was dissolved in 0.02 ml of pyridine and added to 0.015 ml of 1.19 M $P(CH_3)O(Im)_2$ in CH_3CN. The mixture was kept at room temperature for 1.5 h, and then a mixture of 10 mg of *dA-Ac and 3 mg of tetrazole in 0.01 ml of pyridine was added. After 2 h the mixture was treated identically as described for Method 1. The oil was dissolved in $CHCl_3$ and the diastereomers were purified and separated by silica gel HPLC with a 0–10% MeOH gradient in $CHCl_3$. The yields were 2.5 mg (18%) for SpRp and 2.5 mg (18%) for SpSp diastereomers.

RESULTS AND DISCUSSION

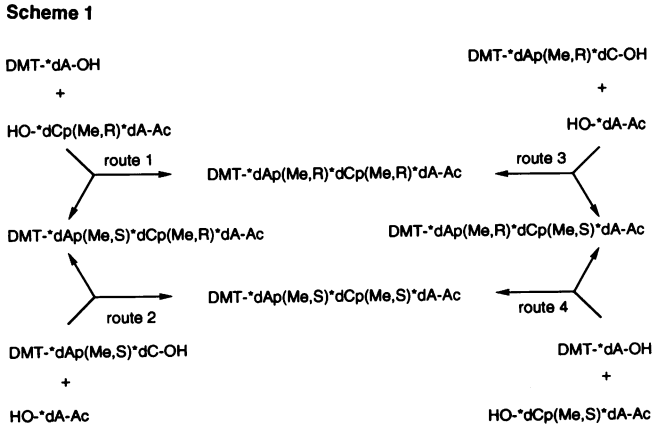
Synthesis and assignment of configuration

Two different approaches were developed to assign the configuration of the new asymmetric phosphorus. The first method (scheme 1) depended on synthesizing the same compound from different directions. For example, DMT-*dA-OH can be coupled to HO-*dCp(Me,R)*dA-Ac (route 1) yielding DMT-*dAp(Me,R)*dCp(Me,R)*dA-Ac and DMT-*dAp(Me,S)*dCp-(Me,R)*dA-Ac after separation. Conversely, DMT-*dAp(Me,R)*dC-OH can be coupled to HO-*dA-Ac yielding DMT-*dAp(Me,R)*dCp(Me,R)*dA-Ac and DMT-*dAp(Me,R)-*dCp(Me,S)*dA-Ac after separation (route 2). By comparing the retention times of the separated diastereomers of both pathways the common diastereomer, DMT-*dAp(Me,R)*dCp(Me,R)*dA-Ac, can be identified on the coincident elution point (Table 1). The assignment of the two remaining diastereomers (RpSp and SpRp) was clear from similar syntheses and can be further confirmed by preparation of each diastereomer by using another route (3 or 4, respectively). A similar synthetic procedure was used to assign the configurations of the SpSp diastereomer. It may be seen from Table 1 that for each diastereomeric center that the Rp diastereomer elutes before the Sp diastereomer. In principle this method of assignment could be used for longer oligomers, but the large number of syntheses makes this methodology unwieldy. In general the fully protected oligomers were separated by silica gel HPLC with a gradient of MeOH in $CHCl_3$. However, in the case of the mixture of DMT-*dGp(Me,S)*dCp(Me,R)*dAp(Me,S)dT-Ac and DMT-*dGp(Me,S)*dCp(Me,S)*dAp(Me,S)dT-Ac, and in the case of the mixture of DMT-*dCp(Me,S)*dCp(Me,R)*dAp(Me,S)*-dA-Ac and DMT-*dCp(Me,S)*dCp(Me,S)*dAp(Me,S)*dA-Ac, the diastereomers were only separable once the 5′-DMT protecting group was removed.

The second method of configurational assignment involves the partial hydrolysis of the diastereomerically pure oligomer and comparison of the retention times of the dimer products with authentic samples of the component dimers in question. Two types of hydrolysis were used. The first type uses the previous observation that acidic conditions produce chain cleavage through an apurinic intermediate yielding nucleosides with 5′ and 3′ terminal hydroxyl groups (20). The reaction conditions used yielded significant amounts of unhydrolyzed dimers. Figure 1 is an example which shows the reversed phase chromatography of dAp(Me,R)dCp(Me,R)dA acid hydrolyzed products (1B), base hydrolyzed products (1B), and the mixture of all four possible dimer standards (1A). The comparison of chromatography positions of respective peaks clearly indicates the coincidence of two peaks with the dCp(Me,R)dA and dAp(Me,R)dC standards. This method requires that the subject dimer be flanked by purine nucleosides at both the 3′ and 5′ ends, or terminate the oligomer. This same method was used for several tetramers. Figure 2 shows the chromatogram of the acid treated tetramer dAp(Me,R)dGp(Me,R)dGp(Me,R)dG (2A) and the chromatograms of this reaction mixture plus the addition of control dGp(Me,S)dG dimer (2B) or control dGp(Me,R)dG dimer (2C). The growth of existing peaks or the appearance of new peaks clearly demonstrate the configuration of the putative dimer internal fragment dGp(Me,R)dG being Rp. The later eluting peaks in the chromatogram are the two possible trimers and unreacted tetramer.

Basic hydrolysis was necessary when the above conditions were not met; these conditions can be used for all cases. In the presence

Scheme 1



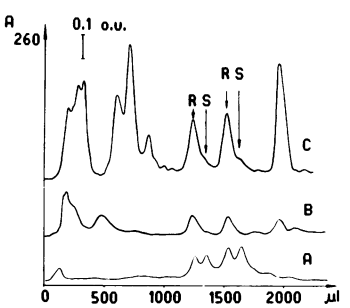


Figure 1. Hydrolysis of the methylphosphonate trimer dAp(Me,R)dCp(Me,R)dA. (**A**) Control micro-column HPLC analysis of dAp(Me,R)dC, dAp(Me,S)dC, dCp(Me,R)dA, and dCp(Me,S)dA, Rp and Sp diastereomers of each dimer. (**B**) Acid hydrolysis of the trimer. (**C**) Base hydrolysis of the trimer.

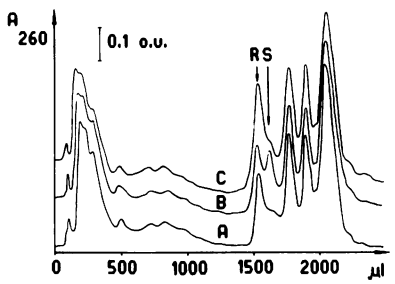


Figure 2. Acid hydrolysis of the methylphosphonate tetramer dAp(Me,R)dGp(Me,R)dGp(Me,R)dG. (A) Reaction mixture after hydrolysis. (B) Reaction mixture plus dGp(Me,S)dG dimer as a control. (C) Reaction mixture plus dGp(Me,R)dG dimer as a control.

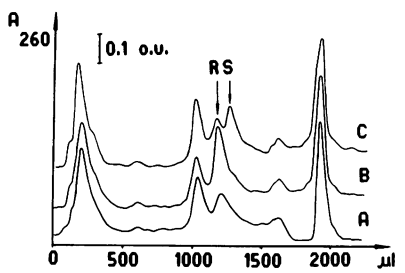


Figure 3. Base hydrolysis of the methylphosphonate tetramer dCp(Me,R)dCp(Me,R)dAp(Me,R)dA. (A) Reaction mixture after hydrolysis. (B) Reaction mixture plus dCp(Me,R)A dimer as a control. (C) Reaction mixture plus dCp(Me,S)dA dimer as a control.

of base, methylphosphonate linkages are hydrolyzed with a hydroxyl group remaining on one fragment and the other fragment maintaining the charged methylphosphonate group (21). Chain scission occurs to either the 5′ or 3′ direction of the methylphosphonate. In some small fraction of cases two chain scissions will result in the putative dimer having hydroxyl groups at both the 5′ and 3′ positions. Figure 1C shows reversed phase chromatograms of dAp(Me,R)dCp(Me,R)dA basic hydrolysis. The peak positions show that both asymmetric centers are in the Rp configuration. This method is useful for oligomers with both pyrimidines and purines, but results in a larger number of products than acidic hydrolysis. When this was compared to the acidic hydrolysis of the same trimer (Figure 1B), a number of additional peaks were seen in the earlier portion of the chromatogram.

The majority of these products were charged methylphosphonates containing oligomers and monomers that can be removed when the reaction mixture is applied to an anion exchange column. The tetramer dCp(Me,R)dCp(Me,R)dAp(Me,R)dA was hydrolyzed in basic conditions and passed over an anion exchange column with Dowex 50×4 in Cl^- form. The void volume peak was then concentrated and analyzed by reversed phase HPLC (Figure 3A). When compared to the chromatogram of the trimer dAp(Me,R)dCp(Me,R)dA shown in Figure 1C, this method showed the utility of removing the earlier eluting peaks. Figure 2B shows the chromatogram of the base hydrolyzed tetramer plus the addition of control dCp(Me,R)A dimer, or control dCp(Me,S)dA dimer in Figure 2C. The growth of existing peaks or the appearance of new peaks clearly demonstrate the configuration of the putative dimer internal fragment dCp(Me,R)dA being Rp.

From the methods described above, configurations were assigned for all of the trimers and tetramers synthesized. The trimers dAp(Me,R)dCp(Me,R)dA, dAp(Me,R)dCp(Me,S)dA, dAp(Me,S)dCp(Me,R)dA, and dAp(Me,S)dCp(Me,S)dA were redundantly characterized by acid hydrolysis, base hydrolysis and dual synthetic routes. The tetramers dCp(Me,R)dCp(Me,R)dAp-(Me,R)dA, dCp(Me,R)dCp(Me,S)dAp(Me,R)dA, dCp(Me,S)-dCp(Me,R)dAp(Me,S)dA, and dCp(Me,S)dCp(Me,S)dAp-(Me,S)dA were characterized by the base hydrolysis method, as were the tetramers dGp(Me,R)dCp(Me,R)dAp(Me,R)dT, dGp(Me,R)dCp(Me,S)dAp(Me,R)dT, dGp(Me,S)dCp(Me,R)-dAp(Me,S)dT, and dGp(Me,S)dCp(Me,S)dAp(Me,S)dT. In contrast, the tetramers dAp(Me,R)dGp(Me,R)dGp(Me,R)dG, dAp(Me,R)dGp(Me,S)dGp(Me,R)dG, dAp(Me,S)dGp(Me,R)-dGp(Me,S)dG, and dAp(Me,S)dGp(Me,S)dGp(Me,S)dG were more easily characterized by the acid hydrolysis method.

Structural analysis by NMR spectroscopy

To study further the effect of configuration on molecular structure, the separated diastereomers were examined by NMR spectroscopy in ^2H_2O without protecting groups to evaluate the variations of chemical shifts. As an example, the ^1H NMR spectra of the RpSp diastereomers of the trimer dAp(Me)dCp(Me)dA are shown in Figure 4A (fully deprotected) and 4B (fully protected). Assignment of the proton signals of all diastereomers was accomplished by ROESY 2D NMR spectroscopy.

In the chromatographic analysis of hydrolysis of RpRp diastereomers of trimers and tetramers (Figs. 1−3), the peaks which coincide with Sp diastereomers should not necessarily be assigned exclusively to Sp diastereomeric impurities. They may also represent some unidentified hydrolysis products which have similar retention times. Therefore quantitation of characteristic Rp and Sp crosspeaks allowed estimation of chiral purity of each diastereomer. For dAp(Me)dCp(Me)dA, dCp(Me)dCp(Me)-dAp(Me)dA, and dGp(Me)dCp(Me)dAp(Me)dT, NMR data for fully deprotected oligomers were used. For dAp(Me)dGp(Me)-dGp(Me)dG, aggregation of the deprotected tetramers in aqueous solution precluded NMR analysis, so NMR data for fully protected tetramers were used to assess chiral purity. For dAp(Me)dCp(Me)dA the following purities were observed: RpRp, 96%; RpSp, 97%; SpRp, 94%; SpSp, 92%. For dCp(Me)dCp(Me)dAp(Me)dA: RpRpRp, 95%; RpSpRp, 97%; SpRpSp, 98%; SpSpSp, 95%. For dAp(Me)dGp(Me)dGp-(Me)dG: RpRpRp, 97%; RpSpRp, 98%; SpRpSp, 94%; SpSpSp, 92%. For dGp(Me)dCp(Me)dAp(Me)dT: RpRpRp, 96%; RpSpRp, 97%; SpRpSp, 98%; SpSpSp, 88%.

For deprotected diastereomers of the dimer dCp(Me)dA (Table 2) and the trimer dAp(Me)dCp(Me)dA (Table 3), nearly complete assignment of the proton signals was carried out. For protected diastereomers only selected signals of the base and sugar protons were unambiguously assigned due to complex overlapping signals in aromatic region and in sugar H5′ H5″ and H4′ region (data not shown). Analysis of the data led to several conclusions, whose general pattern may be seen in a detailed examination of the trimer data. The sensitivity of the aromatic adenine base protons to the change of configuration of the nearest neighbor methylphosphonate residue in most cases was not significant. The H8(A1) signal was shifted by 0.04 ppm to lower field upon transition from RpRp to SpRp diastereomer, and by 0.035 ppm

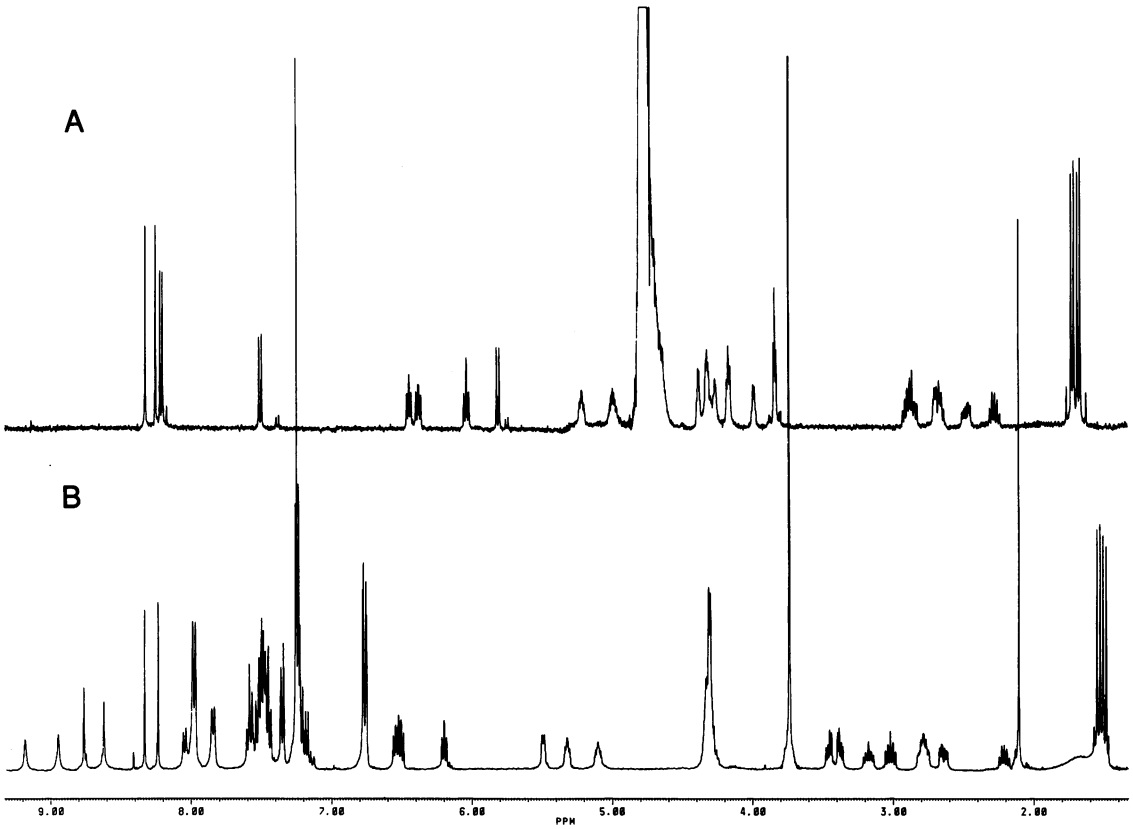


Figure 4. One dimensional ^1H NMR spectra of deprotected (**A**) and fully protected (**B**) dAp(Me,R)dCp(Me,S)dA trimer. The deprotected trimer was measured in ^2H_2O, and the protected trimer in C^2HCl_3, at 27°C, on a Bruker AM-400 spectrometer.

Table 2. Chemical shifts of protons of Rp and Sp diastereomers of dimer dCp(Me)dA without protecting groups (in ^2H_2O).

dC	R	S	R-S	dA	R	S	R-S
6	7.595	7.730	−0.135	2	8.260	8.330	−0.070
5	6.035	6.100	−0.065	8	8.390	8.420	−0.030
1′	6.120	6.130	−0.010	1′	6.510	6.560	−0.050
3′	4.870	4.900	−0.030	3′	4.820	4.920	−0.100
4′	4.160	3.950	−0.210	4′	4.360	4.360	0.000
5′5″	3.750	3.700	+0.050	5′5″	4.380	4.380	0.000
	3.680	3.700	−0.020		4.320	4.320	0.000
2″	2.330	2.480	−0.150	2′	3.000	3.050	−0.050
2′	1.860	2.150	−0.290	2″	2.720	2.780	−0.060
CH_3-P	1.640	1.690	−0.050				

to lower field upon transition from RpSp to SpSp diastereomer. No significant variations (> 0.01 ppm) were found for the chemical shifts of the signals of other protons: H8(A3), H2(A1), H2(A3). RpRp → SpRp and SpRp → SpSp transitions led to 0.025 ppm and 0.01 ppm low field shifts for H2(A1) signals and 0.035 ppm and 0.03 ppm low field shifts for H2(A3) signals, while no variations were found for the chemical shifts of H8 signals.

Significant low field shift changes were observed for H6 and H5 signals of the C2 residue. RpRp → SpRp and SpRp → SpSp transitions led to 0.08 ppm and 0.05 ppm decreases for the H6 signals, and 0.16 ppm and 0.10 ppm decreases for the H5 signals, respectively. RpRp → RpSp and SpRp → SpSp transitions led to 0.155 ppm and 0.12 ppm decreases for the H6 signals, and 0.075 ppm and 0.015 ppm decreases for the H5 signals, respectively. The closer the chiral center was to H6 or H5 protons of the dC residue, the more pronounced was the effect observed due to the change of configuration at the methylphosphonate linkage.

The difference of the chemical shifts of H1′ signals in the various diastereomers was noticeable only for the A1 residue. Thus the RpRp → SpRp transition led to a 0.025 ppm low field shift, and the SpRp → SpSp transition led to a 0.04 ppm low field shift. There were essentially no changes for H1′ signals of the two other residues. RpRp → RpSp and SpRp → SpSp transitions led to small variations of chemical shifts of all H1′ signals. Similarly, the H3′ signal of the dC residue reacted to all Rp → Sp transitions by shifting to lower field, from 0.025 to 0.045 ppm. A low field shift of the H3′(A1) signal upon Rp → Sp transition was also noticeable (from 0.01 to 0.025 ppm).

Table 3. Chemical shifts of protons of diastereomers of trimer dAp(Me)dCp(Me)dA without protecting groups (in ^2H_2O).

proton	RR	SR	RS	SS	RR-SR	RR-RS	RS-SS	SR-SS
dA1								
2	8.178	8.186	8.204	8.197	−0.008	−0.026	−0.007	−0.011
8	8.231	8.270	8.236	8.270	−0.039	−0.005	−0.035	−0.000
1′	6.376	6.399	6.360	6.404	−0.023	−0.016	−0.044	−0.005
3′	5.212	5.220	5.194	5.196	−0.008	0.018	−0.002	0.024
4′	4.371	4.360	4.365	4.370	0.011	0.006	−0.005	−0.010
5′,5″	3.828	3.820	3.828	3.835	0.008	0.000	−0.007	−0.015
	3.814	3.810	3.810	3.812	0.004	0.004	−0.002	−0.002
2′	2.877	2.920	2.881	2.903	−0.043	−0.005	−0.022	−0.017
2″	2.709	2.740	2.666	2.725	−0.031	−0.043	−0.059	−0.015
dC2								
6	7.354	7.437	7.509	7.557	−0.083	−0.155	−0.048	−0.120
5	5.719	5.879	5.793	5.893	−0.160	−0.084	−0.100	0.014
1′	6.018	6.017	6.019	6.013	0.001	−0.001	0.006	0.004
3′	4.953	4.982	4.974	5.005	−0.029	−0.021	−0.034	−0.023
4′	4.279	4.304	3.971	4.035	−0.025	0.208	−0.064	0.269
5′,5″	4.279	4.300	4.154	4.179	−0.021	0.125	−0.025	0.121
2′	1.923	1.999	2.258	2.272	0.024	−0.335	−0.014	−0.273
2″	2.326	2.392	2.462	2.511	−0.066	−0.136	−0.049	−0.119
dA3								
2	8.153	8.163	8.187	8.193	−0.010	−0.034	−0.006	−0.040
8	8.307	8.309	8.310	8.313	0.002	−0.003	−0.003	−0.004
1′	6.418	6.418	6.428	6.442	0.000	−0.010	−0.014	−0.024
3′	n.a.	n.a.	n.a.	n.a.				
4′	4.280	4.300	4.249	4.268	0.020	0.031	−0.019	0.032
5′,5″	4.300	4.300	4.305	4.310	0.000	−0.005	−0.005	−0.010
2′	2.867	2.870	2.854	2.886	−0.003	0.013	−0.032	−0.016
2″	2.628	2.640	2.660	2.667	−0.012	−0.032	−0.007	−0.027
CH_3-P								
A1C2	1.729	1.750	1.700	1.724	−0.021	0.029	−0.024	0.026
C2A3	1.634	1.640	1.675	1.674	−0.006	−0.041	0.001	−0.034

n.a.- not assigned, overlaps with $^1HO^2H$ signal at ca. 4.8 ppm.

Variations in the position of the H3′(A3) signal were not detectable due to overlap with the $^1HO^2H$ signal.

The areas of resonances of H4′ and H5′,5″ protons were well resolved for the RpSp diastereomer as well as for the SpSp diastereomer, but these signals were partially overlapped in the cases of SpRp and RpRp diastereomers. Therefore for later diastereomers it was difficult to estimate the exact values of the chemical shifts for most of the proton signals. The H4′ and H5′,5″ protons of the dC residue were found to be the signals most sensitive to RpRp → RpSp and SpRp → SpSp transitions: low field shifts of 0.21 ppm and 0.27 ppm, respectively, for H4′; 0.125 ppm and 0.12 ppm, respectively, for H5′,5″. RpRp → SpRp and RpSp → SpSp transitions led to smaller effects: 0.025 ppm and 0.064 ppm low field shifts, respectively, for H4′; 0.021 ppm and 0.025 ppm, respectively for H5′,5″. For the H4′ and H5′,5″ protons of the two other nucleoside residues, no significant variations of the chemical shifts were found.

Among the three pairs of H2′,2″ protons of the three deoxynucleoside residues, again the protons of the dC residue were most sensitive to the absolute configuration of the nearest chiral center: RpRp → RpSp and SpRp → SpSp transitions led to 0.335 ppm and 0.275 ppm low field shifts, respectively for H2′; 0.135 ppm and 0.12 ppm, respectively, for H2″. Similar behavior was found for the H2′,2″ protons of the dA1 residue. RpRp → SpRp and RpSp → SpSp transitions led to 0.045 ppm and 0.015 ppm low field shifts, respectively for H2′; low field shifts of 0.03 ppm and 0.06 ppm, respectively, were found for H2″. As may be expected for protons much more distant from the chiral center, variations upon RpRp → RpSp and SpRp → SpSp transitions were smaller: low field shifts of 0.005 ppm and 0.015 ppm, respectively, for H2′, and 0.04 ppm and 0.015 ppm, respectively for H2″. No significant variations were found for 2′,2″ signals of the dA3 residue.

Phosphonate methyl resonances also showed dependence of the chemical shifts on the configuration at the chiral center. RpRp → SpRp and SpRp → SpSp transitions led to 0.02 ppm and 0.025 ppm downfield shifts, respectively, for methyl signals of the dAp(Me)dC portion of the trimer, and 0.06 ppm and 0.005 ppm shifts to lower field, respectively, for methyl signals of the dCp(Me)dA linkage. RpRp → RpSp and SpRp → SpSp transitions led to 0.3 ppm and 0.26 ppm low field shifts, respectively, for methyl signals of the 5′ dAp(Me)dC domain, and 0.04 ppm and 0.035 ppm downfield shifts, respectively, for methyl signals of the 3′ dCp(Me)dA domain.

Summarizing the data for dAp(Me)dCp(Me)dA (Table 3), one can conclude that the protons most sensitive to Rp→Sp transitions are those of the 2′-deoxycytidine residue. The H6 and H5 aromatic protons and the H2″ proton displayed a significant low field shift for both 5′ or 3′ Rp→Sp configuration transitions, while H4′ and H5′,5″ protons showed high field shifts only for 3′ Rp→Sp configuration transitions. Comparison of the data for the dimer dCp(Me)dA (Table 2) and the trimer dAp(Me)dCp-(Me)dA (Table 3) illustrates that the presence of the 2′-dA residue on the 5′ side of 2′-dC does not influence significantly the values of changes in chemical shifts for the above protons upon Rp→Sp transition on the 3′ side of 2′-dC. In contrast to the behavior of the chemical shifts of the 2′-dC protons, only minor changes of the proton chemical shifts for both 2′-dA residues of the trimer

are observed for Rp→Sp configuration transitions. These observations probably reflect the fact that the 2′-dC residue, being between the two 2′-dA residues, is more flexible and adjustable to conformational changes of the molecule upon inversion of configuration at the asymmetric methylphosphonate center. In general, it seems that the overall relative conformation of 2′-dA residues in trimers (especially dA3) does not change significantly with position. The same general patterns were observed for the tetramer dCp(Me)dCp(Me)dAp(Me)dA without protecting groups, and for the tetramer dGp(Me)dCp(Me)dAp(Me)dT without protecting groups.

CONCLUSIONS

The preparation of individual diastereomers of heterogeneous oligodeoxynucleoside methylphosphonates and assignment of absolute configurations at asymmetric centers remain very difficult tasks, and obviously are not convenient for routine synthetic procedures. On the other hand, the preparation of individual diastereomers of oligodeoxynucleoside methylphosphonates of mixed sequences is very important for determination of meaningful differences in stabilities of methylphosphonate complexes with complementary sequences of normal nucleic acids.

For subsequent synthesis of dCp(Me)dCp(Me)dAp(Me)dAp-(Me)dAp(Me)dCp(Me)dA heptamers from dCp(Me)dCp-(Me)dAp(Me)dA tetramer and dAp(Me)dCp(Me)dA trimer described above (22), new syntheses of trimer and tetramer were performed, where the separations of diastereomers were repeated several times. Thus all the synthons used for subsequent work were at least 95% chirally pure. Our preliminary data concerning dCp(Me)dCp(Me)dAp(Me)dAp(Me)dAp(Me)dCp(Me)dA revealed a dramatic difference in stability of the complementary complexes of oligodeoxyribonucleotides with all-Rp or all-Sp individual diastereomers of oligodeoxynucleoside methylphosphonate heptamers.

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ABBREVIATIONS

1D, one-dimensional; 2D, two-dimensional; Ac, 3′-acetyl; DMT, 5′-dimethoxytrityl; HPLC, high performance liquid chromatography; Py, pyridine; *dN, protected deoxyribonucleoside: N-benzoyl deoxyribocytidine, N-benzoyl deoxyriboadenosine or N-isobutyryl deoxyriboguanosine; NMR, nuclear magnetic resonance; ROESY, rotational spin correlation spectroscopy; TPS, 2,4,6-triisopropylbenzene sulfonyl chloride.

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