Octa(thymidine methanephosphonates) of partially defined stereochemistry: synthesis and effect of chirality at phosphorus on binding to pentadecadeoxyriboadenylic acid

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ABSTRACT
Block condensation of MePOCl₂ or MeP(NEt₂)₂ with appropriately protected tetra(thymidine methanephosphonates) of predetermined sense of chirality at asymmetric phosphonate centres gave two pairs of diastereomeric mixtures, namely (SpSpSpSpSpSp + SpSpSpRpSpSpSp) 5a and (RpRpRpRpRpRp + RpRpRpRpRpRp) 5b. A comparison of the CD spectra of 5a and 5b with those of octathyminidylic acid (7) and a random mixture of diastereomers of octa(thymidine methanephosphonate) (6), and also a comparison of the Tₘ of complexes formed between 5a, 5b, 6 or 7, and pentadecadeoxyriboadenylic acid (8), Indicates that octamer 5b and its complex with its complementary oligonucleotide has a well-ordered structure due to the ‘outward’ or ‘pseudoequatorial’ orientation of the methyl group of each internucleotide methanephosphonate function of Rp configuration. Results presented in this report clearly indicate that the stability of hybrids formed between octa(thymidine methanephosphonate) and pentadecadeoxyriboadenylic acid depends on the stereochemistry of each internucleotide methanephosphonate function and strongly suggests that stereoselective synthesis of P-chiral oligonucleotide analogues is an important goal.

INTRODUCTION
Oligonucleotide analogues possessing asymmetric centres at phosphorus atoms involved in modified internucleotide linkages have found wide application as models for the study of nucleic acids structure and dynamics⁹ and as probes for the elucidation of specific interactions of DNA/RNA with proteins and enzymes³⁻⁶. Their ability to modulate gene expression has also been extensively explored⁷,⁸. When non-bridging oxygens at internucleotide phosphorus atoms are replaced by sulphur, alkyl or alkoxy groups, in many cases the configuration of asymmetric phosphorus centres drastically influences the interaction between modified oligonucleotides and their complementary sequences as well as the conformation of single- and double-stranded oligonucleotides. Unfortunately the nature of these phenomena is not fully understood. Studies on the relationship between the absolute configuration at phosphorus and oligonucleotide conformation or binding have so far concerned dinucleotides⁹,¹⁰, oligonucleotides with one P-chiral centre¹¹⁻¹⁴, oligonucleotides possessing alternating modified/unmodified internucleotide linkages¹⁵,¹⁶, or oligonucleotides possessing n P-chiral centres and consisting of a mixture of all 2ⁿ possible diastereomers¹⁷,¹⁸.

Recently we have reported on stereospecific synthesis of phosphorothioate¹⁸,¹⁹ and methanephosphonate²⁰⁻²² oligonucleotide analogues with defined sense of chirality at phosphorus. In this paper we wish to present the results of our studies on the synthesis and binding properties of two octa(thymidine methanephosphonates) 5a and 5b, each composed of two diastereomers of known absolute configuration, namely (SpSpSpSpSpSp + SpSpSpRpSpSpSp) and (RpRpRpRpRp + RpRpRpRpRpRp).

EXPERIMENTAL
Materials and methods
Column chromatography and TLC were performed on silica gel 230–400 mesh and on silica gel F 254 plates, respectively, (both from E.Merck). Reversed phase high performance liquid chromatography (RP-HPLC) was performed with a LDC/Milton Roy system, using ODS Hypersil 5µ, 4.6×300 mm column. The elution conditions are given below. UV spectra were recorded and Tₘ measurements were performed with a Uvikon 860 spectrometer (Kontron Instruments AG). ³¹P-NMR spectra were recorded with a Bruker MSL 300 spectrometer operating at 121.47 MHz, with 85% H₃PO₄ as an external standard. Positive chemical shift values are assigned for compounds absorbing at lower field than standards. CD spectra were recorded on Dichrograf Mark III (Jobin-Yvon).

(SpSpSp)- and (RpRpRp)-Isomers of 5’-O-monomethoxytrityl-3’-O-acetyl tetra (thymidine methanephosphonate) (1a and 1b)
The title compounds were prepared as described previously²².
The removal of 5'-monomethoxytrityl group was performed according to the literature method\(^{23}\). Thus, individual isomers of 1 (58.5 mg, 0.04 mmol) were treated with 80% aqueous acetic acid (1.0 mL) at room temperature. The reaction progress was monitored by means of TLC. After 1–3 h AcOH was removed by coevaporation with n-BuOH. The oily residue was dissolved in pyridine and dropped into hexane. The precipitate was washed with n-pentane and dried under reduced pressure.

2a: \( R_f 0.04 \) vs. 1a: \( R_f 0.17 \); 2b: \( R_f 0.07 \) vs. 1b: \( R_f 0.19 \), [CHCl\(_3\)-CH\(_3\)OH (9:1) as developing solvent system].

To the solution of individual isomers of 1 (50 mg, 0.034 mmol) in 0.75 mL of methanol, 0.5 mL 25% aq ammonia solution was added. The reaction progress was monitored by means of TLC. After 2–3 h the post-reaction mixture was evaporated to dryness. The residue was redissolved in 2 mL of chloroform and extracted with 2 mL of water. The organic layer was separated, dried over MgSO\(_4\), and evaporated to dryness. The crude product was precipitated from hexane.

3a: \( R_f 0.05 \) vs. 1a: \( R_f 0.17 \); 3b: \( R_f 0.08 \) vs. 1b: \( R_f 0.19 \), [CHCl\(_3\)-CH\(_3\)OH (9:1) as developing solvent system].

<table>
<thead>
<tr>
<th>octa(thymidine methanephosphonate)</th>
<th>Yield [%]</th>
<th>TLC(^a)</th>
<th>( {^31P-NMR}^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a-(SpSpSpSpSpSpSpSp + SpSpSpRpSpSpSp)</td>
<td>5.0</td>
<td>0.65</td>
<td>33.88</td>
</tr>
</tbody>
</table>

\(^{a}\) Developing solvent system: CH\(_3\)Cl-CH\(_3\)OH (6:4)  
\(^{b}\) in \( C_6D_6N (4a) \) or CD\(_3\)OD (4b).
resin was washed with CH$_3$CN/H$_2$O (1:1, 0.5 mL) and the combined washings were evaporated to dryness. The purification of crude 6 was accomplished by means of HPLC using the following elution conditions: a linear gradient of acetonitrile in 0.1 M TEAB (pH 7.0) at a flow-rate of 1.5 mL/min, starting from 1.2% CH$_3$CN, gradient 1.4%/min., R, 15.66 min.

Octa(thymidine phosphate) (7) and pentadeca(deoxyriboadenosine phosphate) (8)

7 And 8 were prepared on a Biosearch Cyclone DNA Synthesizer that employed phosphoramidite chemistry according to standard protocol$^{26}$. The resultant 7 and 8 were purified by means of two steps of RP-HPLC using the following eluting conditions: 5'-DMT-protected oligomer: gradient from 5% to 30% CH$_3$CN in 0.1 M TEAB, 5'-deprotected oligomer: gradient from 5% to 15% CH$_3$CN, 1.5 mL/min., 7, R, 9.83 min. 8 in addition to RP-HPLC was purified by means of polyacrylamide gel electrophoresis.

Circular dichroism measurements

The CD spectra of 5a, 5b, 6 and 7 were measured in double distilled water solution at concentrations of 0.43 g/L, 1.06 g/L, 0.93 g/L and 0.82 g/L, respectively. The following molar extinction coefficients were used: 5a, $e_{265}$=8500 [as for d(T$_{265}$=8300 [as for d(T$_{265}$=8700 [as for d(T$_{265}$=8450 (average value for 5a and 5b).

T$_m$ measurements

Melting experiments were performed on a Uvikon 860 spectrometer (Kontron Instruments AG). The temperature control was through a programmable Tischkryostat KT6 (VEB MLW. Prüfgeräte-Werk Meadingen). Temperature was monitored by a Uvikon 860 thermistor unit with the temperature transducer connected to a thermostated sample cell holder. Cuvettes had Teflon stoppers and were of 1 cm pathlength. Nitrogen gas was passed continuously through the sample and reference compartment of the Uvicon 860 during low temperature measurements. Digitalized absorbance values were stored by the computer and next plotted as a function of temperature on the Plotter 800 (Kontron Instrument AG). The computer collected 10 absorbance readings and averaged them for each point on the Tm curve to improve signal-to-noise ratio. Tm values were established on the basis of first order derivative of Tm curves. The measurements were initiated at 0°C or slightly below 0°C and the temperature ramp was 0.5°C/min. These experiments were carried out in 0.1 M sodium cacodylate, pH 6.8, at a total nucleotide concentration of 1X 10$^{-4}$M and proportions of ldTldA and 2dTldA of both oligonucleotide components$^{1}$. All samples were pre-melted at 60°C and then cooled slowly (0.5°C/min) to 0°C.

RESULTS

P-Homochiral 5'-O-monomethoxytrityl-3'-O-acetyl tetra (thymidine methanephosphonates) (l) were prepared, as described previously$^{22}$, by stepwise, stereospecific coupling of a 5'-hydroxyl activated growing oligonucleotide chain with (Sp)-
FIGURE 3. $^{31}$P-NMR spectrum (121.47 MHz) of (RpRpRpRpRpRpRp+RpRpRpSpRpRpRp) octa(thymidine methanephosphonate) $(4b)$. Chemical shifts are given in ppm $(\delta)$ relative to $85\%$ H$_3$PO$_4$.


The mixtures of 5'- and 3'-protected diastereomers $(\text{SpSpSpSpSpSpSp+SpSpSpRpSpSpSp})$ $(4a)$ and $(\text{RpRpRpRpRpRpRp+RpRpRpSpRpRpRp})$ $(4b)$ of octa(thymidine methanephosphonate) $(4)$ were prepared via block condensation of suitably deprotected tetramers 2 and 3. Deprotection of the 5'-hydroxyl function of 1a and 1b was achieved by the treatment of fully protected tetrancleotides 1 with 80% acetic acid. After standard work up, chromatographically homogeneous byproducts 2 suitable for the coupling reaction were obtained and used for synthesis of 4 without further purification.

Removal of the 3'-O-acetyl group in 1a and 1b was performed under alkaline conditions by treatment of 1 with a mixture of concentrated aqueous ammonia and methanol (1:1.5 v/v) at room temperature. No degradation products were observed by means of TLC and additional purification of deprotected byproducts 3 was not necessary for further condensation.

Octamer 5a was prepared by condensation of appropriately protected tetramer blocks 2a and 3a according to the methodology described previously by Miller for the synthesis of di- and tri(nucleotide methanephosphonates)$^{(27)}$.

Thus, the 3'-hydroxyl group of 3a was phosphorylated with methanephosphonic dichloride and then 2a was added. The preparative yield of fully protected octamer 4a was low (see Table I) (ca. 5%) which may be explained by the fact that relatively long oligonucleotides were coupled together.

Whereas octamer 5b could not be synthesised according to the method described, an alternative procedure applying phosphonamidite chemistry was successful. Phosphonation of the 3'-OH function of 3b was performed by means of (N,N,N',N'-tetraethyl)methanephosphonamidite. Intermediate 5'-O-MMT tetra(thymidine methanephosphonate)-3'-O-(N,N-diethyl methanephosphonamidite) was reacted, without isolation, with 2b and the central methanephosphonic group of the resultant octamer was oxidized with I$_2$ in H$_2$O/2,6-lutidine/THF. The preparative yield of 5b was 10%.

The resultant octanucleotides 4a and 4b were characterized by means of $^{31}$P-NMR. After deprotection, resulting 5a and 5b were analysed by means of UV and HPLC. Since in the course of the first step of the coupling reaction formation of a symmetrical product is possible, the presence of 3'-O-acetyl and 5'-O-monomethoxytrityl protecting groups in 4 was proved by means of selective deprotection and analysis by TLC (for MMT removal) or HPLC (acetyl removal from 5'-deprotected 4).

Because the coupling of the two tetramers 2 and 3, leading to octamers 4, is not a stereospecific reaction, compounds 4a and 4b consist of a mixture of two diastereomers epimeric at the phosphorus atom of central methanephosphonate internucleotide linkage. However, it should be pointed out that the analogous octamers (i.e. 6) obtained by non-stereospecific synthesis consists of a mixture of 128 diastereomers.

Attempts at separation of diastereomers constituting pairs $(\text{SpSpSpSpSpSpSp+SpSpSpRpSpSpSp})$-5a and $(\text{RpRpRpRpRpRpRp+RpRpRpSpRpRpRp})$-5b by means of RP-HPLC were unsuccessful. This is in contrast to the described fractionation of decathymidylic acid bearing only one, centrally located methanephosphonate internucleotide linkage$^{(28)}$. The reasons for this difference in chromatographic behavior are not known at the present. Perhaps, the higher lipophilicity of the molecule, 5a and 5b, compared to the monomethanephosphonate analogue of decathymidylic acid, may be responsible.
Tm measurements of the duplexes between octanucleotides 5a or 5b and pentadecadeoxyriboadenyllic acid matrix were performed and compared with those formed between unmodified octathymidylic acids (7) or octa(thymidine methanephosphonate) consisting of the random mixture of 128 diastereoisomers (6). Tm studies and CD measurements on 5a, 5b, 6 and 7 are discussed below.

**DISCUSSION**

The key step in the synthesis of octanucleotides 5a and 5b was the condensation of two suitably protected tetranucleotide blocks: 2a–3a and 2b–3b, respectively. This was achieved according to phosphotriester (Method A) or phosphoramidite (Method B) chemistries. Unfortunately, in both cases the efficiencies of the coupling steps were low. Similarly, low yields (7–50%) were reported by Miller for coupling of d[DMTT₆₋₈] and d[T₉₋₆] (n = 1–6).

The ³¹P-NMR spectra of both 4a and 4b reveal five signals (Figures 2 and 3). Their relative integrations are consistent with the fact that both 5a and 5b consist of a mixture of two diastereomeric octa(thymidine methanephosphonates), epimeric at the phosphorus of the central methanephosphonate internucleotide linkage and each containing seven phosphorus centres. The different compositions of overlapping signals observed for 5a and 5b reflect the perturbation introduced by the central methanephosphonate function of 'doubled' sense of chirality in both pairs of diastereomers.

CD measurements have shown a strong influence of configuration at internucleotide methanephosphonate functions on the CD spectra of 5a, 5b, 6 and 7 which exhibit the same shape but drastically different molecular ellipticity values.

Octa(thymidine methanephosphonates): 5b, 6 and 7, all exhibit a positive CD signal between 295 and 265 nm, while the CD spectrum of octa(thymidine methanephosphonate) 5a is entirely negative in the wavelength range 220–320 nm. The molecular ellipticity of 5a, 5b, 6 and 7 decrease in the order 7 > 5b > 6 > 5a and is reduced by 26%, 60% and 108% (negative sign of CD signal) for 5a, 6 and 5b, respectively, as compared with 7. It should be mentioned that these observations are in agreement with similar measurements reported previously for homochiral tetra(thymidine methanephosphonates), but it should be pointed out however, that this dependence is more strongly pronounced in octanucleotides than in tetranucleotides, which may suggest that the effect of absolute configuration of methanephosphonate groups upon oligonucleotide conformation is additive.

Taking into account that molecular ellipticity is sensitive to the extent and mode of base stacking and that the nonionic methanephosphonate group of internucleotide linkage perturbs the stacking interactions between the bases of modified oligonucleotides, the conclusion can be drawn that the stacking perturbation is much stronger for the (Sp)-configuration of internucleotide methanephosphonates than that observed for the (Rp)-configuration. This correlates with NMR studies of duplexes of complementary oligonucleotides bearing internucleotide methanephosphonate function with defined sense of chirality as well as with results of Tm measurements.

Dependence of Tm upon the stereochimistry of the modified phosphate group is a well known phenomenon. However, it has not been studied so far for longer oligomers possessing adjacent P-chiral centres with defined sense of chirality. Our results of Tm measurements for duplexes of octa(thymidine methanephosphonates)/pentadecadeoxyriboadenyllic acid illustrate very dramatically the relationship between 'tacticity' of oligo(thymidine methanephosphonates) and Tm values. The Tm values decrease in the order: 5b > 7 > 6 > 5a (Table 2 and Figure 5).

Since according to Miller oligo(thymidine methanephosphonates) can form triplex structure with complementary poly(oligo)deoxy(ribo)adenyllic acid, Tm measurements of 1dT·1dA and 2dT·1dA mixtures were also performed for all oligonucleotide studied 5a, 5b, 6 and 7. No substantial differences of Tm were observed.

**TABLE 2. Tm values of complexes formed between octathymidylic acid 7, and its analogues 5 and 6, and pentadecadeoxyriboadenyllic acid (8)**

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Ratio dT:dA</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T₃₈₄)₅, 5a</td>
<td>1:1</td>
<td>&lt; 2 (n.d.)</td>
</tr>
<tr>
<td>(T₃₈₄)₅, 5b</td>
<td>2:1</td>
<td>&lt; 2 (n.d.)</td>
</tr>
<tr>
<td>(T₃₈₄)₅ random, 6</td>
<td>1:1</td>
<td>38.2</td>
</tr>
<tr>
<td>(T₃₈₄)₅, 7</td>
<td>1:1</td>
<td>39.3</td>
</tr>
<tr>
<td>(T₃₈₄)₅</td>
<td>1:1</td>
<td>13.4</td>
</tr>
<tr>
<td>(T₃₈₄)₅</td>
<td>2:1</td>
<td>13.3</td>
</tr>
</tbody>
</table>

- a) The experiments were carried out in 0.1 M sodium cacodylate, pH 6.8, at a total nucleotide concentration of 1×10⁻⁴ M.
- b) The Tm values are the transition midpoints of 1T:1dA or 2T:1dA mixtures of suitable oligonucleotides.

![Figure 5](image-url)
Tm values for 7(dA)15 and 6(dA)15 are quite similar: 13.4°C and ca. 12.9°C (for 1dT:1dA mixture, respectively. It should be pointed out, however, that the melting curve observed for 6(dA)15 is markedly broader and is characterized by lower hypochromicity: 17% vs. 22.4%. The broad melting curve for 6(dA)15 might result from the superimposition of a multitude of octa(thymidine methanephosphonates)/pentadecadeoxyriboadenylic acid transitions with different stabilities\(^{36}\). This supposition is strongly supported by the comparison of melting curves obtained for complexes of 5a/(dA)15 and 5b/(dA)15 which are completely different in their character. The curve for 5a/(dA)15 is flat, with hypochromicity of 3.8% and no shoulder point. By contrast, the melting curve for complex 5b/(dA)15 is sigmoidal with a distinct shoulder at 38.2°C and hypochromicity of 35.2%.

The shape of the curve is consistent with the assumption that 5b forms, in the range of temperatures studied, a well ordered, stable complex and that 5a does not form a complex with (dA)15 at all, and that the binding properties of 5a and 5b depend upon the absolute configuration of internucleotide methanephosphonate functions.

The presence of a methyl group in the pro-S position decreases the duplex stability. The possible factors influencing the binding properties, related to the configuration of intermediate methanephosphonate groups, are discussed below.

If the complexes of octa(thymidine methanephosphonate)/pentadecadeoxyriboadenylic acid have B-type geometry, the P-CH\(_3\) bond of the methyl group of internucleotide methanephosphonate function of (Sp)-isomer is oriented 'inward' towards the DNA double-helix, near the hydrophobic base-stacking region of the complex, and that of the (Rp)-isomer is oriented 'outward' away from the DNA double helix (into the solvent)\(^{11}\). The geometry of oligothymidine/oligodeoxyribadenosine may, however, deviate from classical B-DNA since such deviations were observed for poly(dA):poly(dT) complexes\(^{35,36}\).

At present it is not possible to discuss the exact features of perturbations due to the introduction of methanephosphonate functions into oligonucleotide chains without further NMR studies. However, both CD and Tm data seem to indicate that the interaction between oligo(thymidine methanophosphonates) and pentadecadeoxyriboadenylic acid is an entropy driven process, and binding of oligothymidine methanophosphonate of exclusive Sp configuration suffers from constraints, as indicated by molecular mechanics calculations\(^{37}\), due to interactions between P-methyl groups and the 5-CH\(_3\) groups of thymines.

It has been proposed that the effect of the methylation of phosphates on the stability of duplexes results from three main factors\(^{22}\): 1) elimination of the phosphate charge, 2) electronic and other substituent effects, 3) the steric effect of the substituent group. At least one more factor can be added, namely, 4) the differences in hydration of the analogue/phosphodiester backbone of the oligomer in the duplex compared to that in the single-stranded forms\(^{1,29}\).

From our and other observations, the hypothesis emerges that the influence of factors mentioned above is potetiated by the desired 'tacticity' of oligomers, which results from the predetermined sense of chirality at each internucleotide methanephosphonate function.

Thus, comparison of the Tm of octa(thymidine methanephosphonate) 5a and 5b suggests that the destabilizing effect of factors 2 and 3 is successfully neutralized if internucleotide methanephosphonate groups exist in the (Rp)-configuration (P-CH\(_3\) bond pointing towards the solvent); on the other hand it seems that (Sp)-configuration is especially unfavourable. A similar relationship was also observed for triester analogues\(^{11}\). It is true for methanephosphonates as well as for triester oligonucleotide analogues\(^{33}\) that the isomer with a 'pseudoequatorial configuration' of methanephosphonates (Rp-configuration) forms more stable complexes than their counterparts with 'pseudoxial' orientation of the P-methyl group.

Our studies suggest that although the duplex stabilization or destabilization results from the balance between many factors, the balance itself can be shifted into a desirable direction by a carefully designed sequence of absolute configurations of modified P-chiral internucleotide functions.

**ACKNOWLEDGMENTS**

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This paper is dedicated to Professor J. Michalski on the occasion of his 70th birthday.

**REFERENCES**

37. Hausheer, F.—private information.