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# Chirality at phosphorus: hybrid duplexes of chimeric oligonucleotides containing methylphosphonothioate linkages with complementary DNA and RNA

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**Abstract**

Chimeric oligonucleotides with incorporated diastereomerically pure dinucleoside(3′,5′)-methylphosphonothioates and their oxo- and seleno- congeners of known absolute configuration are reported. The relation between stability of the hybrid duplexes with complementary DNA and RNA and their structure is analyzed in context of absolute configuration of the P-chiral internucleotide bonds.


*Keywords:* Chimeric stereoregular oligonucleotides; Methylphosphonates; Methylphosphonoselenoates; Methylphosphonothioates; P-chirality; Stereochemistry

## 1. Introduction

Efficient solid-phase methods of synthesis of oligonucleotides and their analogs, the development of versatile phosphoramidite reagents, and efficient scale-up have expanded the application of modified oligonucleotides to diverse areas of fundamental and applied biological research, including therapeutic applications [1,2]. The availability of different backbone modifications have added to the utility of such oligonucleotides [3].

Methylphosphonate analogues of nucleic acids belong to the oldest candidates for *antisense therapeutics* [5]. Recently, they have been considered as convenient structural elements for *chimeric oligonucleotides*, i.e., oligonucleotides with modified internucleotide centres in particular positions of the oligomer. Such modifications allow for precise tuning of the properties of these constructs (higher stability in presence of exonucleases, reduction of total negative charge, improved transport into cells etc.) [6]. They have also been successfully applied as structural and mechanistic probes [7] for mapping of nucleic acid–protein interactions [8,9] as well as spectroscopic analysis of biochemical reactions and nucleic acid structures structural studies [10].

In this paper, we review the range, scope, and practical utility of a *dimeric building block approach* for synthesis of such chimeric oligonucleotides. We report here efficient methods of synthesis and properties of oligonucleotides modified with incorporated diastereomerically pure methylphosphonothioates (Oligo-MePS-**2**) or methylphosphonates (oligo MePO-**4**), and the influence of the sense of P-chirality of the internucleotide methylphosphonothioate [11–13] linkage on the stability of heteroduplexes formed between chimeric oligonucleotides **3** and complementary DNA and RNA.

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## 2. Results and discussion

### 2.1. Synthesis and properties of dinucleoside(3′,5′)-methylphosphonothioates

Our recent approach to a large-scale laboratory synthesis of dinucleoside (3′,5′)-methylphosphonothioates (**1a**) and their selenium congeners (**1b**), is based on *in situ* generated methylphosphono bis-(1,2,4-triazolidite) (**5**), as a selective although formally bifunctional phosphitylating agent [14]. We found that such transient activation of methyldichlorophosphine with 1,2,4-triazole in the presence of triethylamine prior to phosphitylation of the 5′-*O*-protected nucleoside leads to substantial improvement of the reaction, and almost complete elimination of the unwanted symmetrical by-products, usually present in the case of the use of $MePCl_2$ as phosphitylating agent [15] (Fig. 1).

The corresponding dinucleoside (3′,5′)-methylphosphonothioates (**1a**) and dinucleoside (3′,5′)-methylphosphonoselenoates (**1b**) were synthesized in good yields [83% for d($T_{MePS}C^{Bz}$) to 65% for d($G^{ibu}{}_{MePS}C^{Bz}$)] in one-pot-reaction, using protected nucleosides and methylphosphono bis-(1,2,4-triazolidite) [$^{31}$P NMR: $\delta$ 73.47 ppm; $^{1}$H NMR: 2.25 (d, 3H, $^{2}J_{PH}$ = 15.6 Hz), 8.15, 9.05] (**5**), as depicted in Scheme 1. After addition of elemental sulfur or selenium, and without purification, fully protected dimers **8** were treated with triethylamine tris-hydrofluoride to remove the 3′-*O*-*tert*-butyldimethylsilyl protecting group. Partially deprotected dinucleoside (3′,5′)-methylphosphonothio(seleno)ates **1** were separated chromatographically into pure diastereomers. It is important to note that separation of diastereomers **8** is less efficient than separation of partially deprotected dimers **1a** or **1b**, therefore silica gel column chromatography is not required after synthesis of fully protected dinucleotides **8** but is recommended after 3′-*O*-deprotection.

The diastereomerically pure $R_P$- or $S_P$-isomers **1a** or **1b** [16] were 3′-*O*-phosphitylated with ($CNCH_2CH_2O$)($iPr_2N$)$_2$P/tetrazole and further used without purification, for condensation *via* the phosphoramidite method on a solid support. Alternatively, 3′-*O*-methylphosphonamidites **9** were purified by a flash column chromatography [17].

### 2.2. Assignment of the absolute configuration and structure of dinucleotides *1a*

The isomer SLOW-**1a** (B = U, B′ = $C^{Bz}$, R = OMe, R′ = H) was crystallized from ethanol (Fig. 2). The absolute configuration of the isomer slowly eluted during chromatographic separation under normal phase conditions was assigned as $R_P$. It was found that in crystalline form both nucleosides existed in the *3′-endo* conformation, characteristic for RNA structure [18]. We also demonstrated that $^{1}$H NMR spectroscopy (2D NMR, COSY, ROESY) can be used for assignment of an absolute configuration of partially protected dinucleotides **1** [19].

Following structural studies, fully deprotected $S_P$-**1a** (B = U, B′ = C, R = OMe, R′ = H, X = S) was analyzed by $^{1}$H NMR (2D ROESY, $^{31}$P NMR–$^{1}$H NMR correlations, and NOESY) in order to assign the conformation of both 2′-*O*-methylribose rings in water (10 mM Tris–HCl, 100 mM NaCl, and 10 mM $MgCl_2$ pH 7.4). The conformation of the ribose rings was studied applying a two-state model, and described in terms of equilibrium between two conformations in solution, or a ratio of the populations of the corresponding *N*- and *S*-conformers [19]. The *3′-endo* pucker of the ribose ring is typical for an A-RNA, and introduction of an electronegative 2′-*O*-methyl modification leads to further *3′-endo* pucker stabilization [20]. However, we found that unlike in crystalline $R_P$-**1a** [18], $R_P$-**1a** in organic solution has a considerable fraction of the *2′-endo* population for the uridine moiety in equilibrium, with even more populated

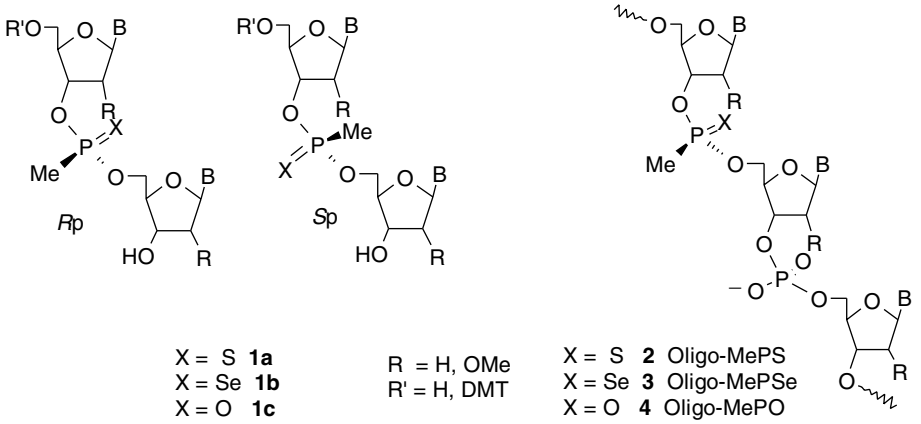
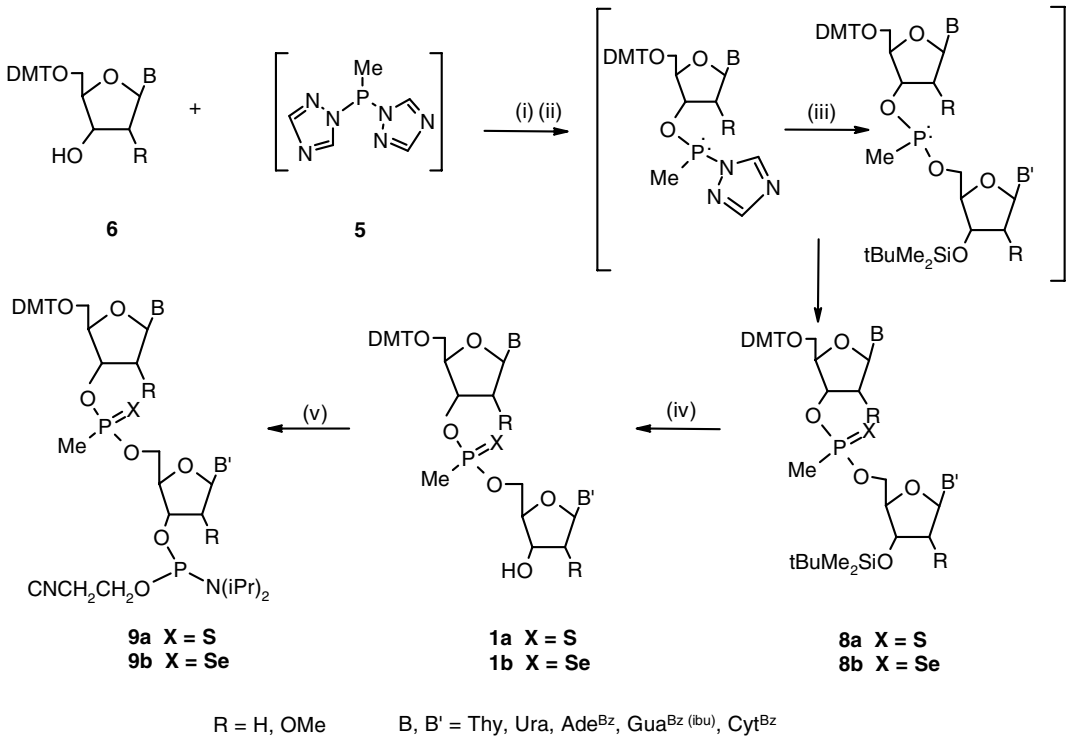



Fig. 1. Chimeric oligonucleotides and diastereomeric dinucleoside-(3′,5′)-methylphosphonothioates (**1a**) and their seleno-(**1b**) and oxo-(**1c**) congeners. The $R_P$-methyl group points out from the major groove causing minimal interactions with the sugar, whereas the $S_P$-methyl group points inward the major groove with possible steric interactions with H3′ sugar protons [4].




Scheme 1. Reagents and reaction conditions: (i) **5** and **6** in THF, ice bath, 45 min at RT; (ii) **7** in THF, RT, 1.5 h; (iii) sulfur or selenium, RT, overnight (iii) aqueous work-up; (iv) $Et_3N \cdot 3HF$ in THF, silica gel column chromatography; (v) $(CNCH_2CH_2O)(iPr_2N)_2P$/tetrazole, column chromatography.

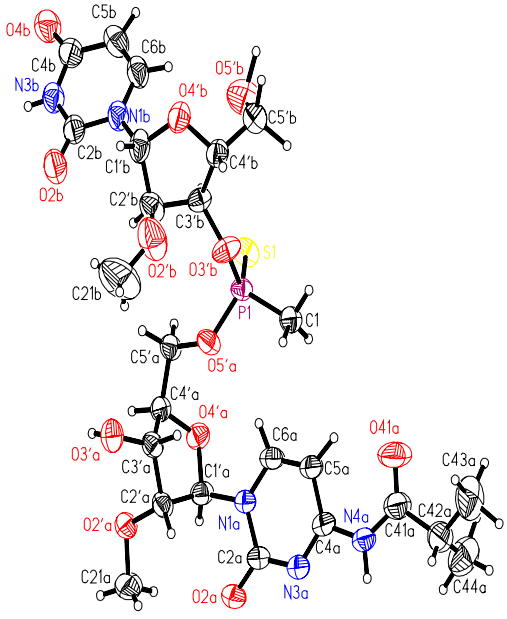



Fig. 2. The ORTEP drawing of SLOW-**1a** in the crystal (displacement ellipsoids drawn with 50% probability level).

*2′-endo*-fraction observed for $S_P$-**1a** (B = U, B′ = $C^{Bz}$, R = OMe, R′ = H) (*N* – 45% at 25 °C in $CDCl_3$) [19]. The H1′ signals for $S_P$-**1a** appeared as doublets with significantly different coupling constants $J_{1',2'}$ for uridine ($J_{1',2'} = 5.2$ Hz) and cytidine ($J_{1',2'} = 0.8$ Hz) moieties. The small value for cytidine indicated the preferred *N*-conformation of this sugar moiety, whereas for uridine moiety was indicative of higher conformational flexibility of this moiety in solution, and a significant content of the *2′-endo* puckering conformer in dynamic equilibrium between *2′-endo* and *3′-endo* conformations [21]. In water these coupling constants were $J_{1',2'} = 5.85$ Hz for uridine and $J_{1',2'} = 2.9$ Hz for cytidine in $S_P$-**1a**, respectively, relating to 62% of *2′-endo* conformation for the uridine moiety and 26% for the cytidine moiety. These results indicate that the $S_P$-methylphosphonothioate modification of the internucleotide bond in 2′-*O*-methylribonucleotides has increased significantly the population of the *S*-conformers in comparison to the non-modified internucleotide phosphodiester bonds in 2′-*O*-methylribonucleotides [22].

### 2.3. Synthesis of chimeric oligonucleotides

We have expected further enhancement of the influence of P-chirality in chimeric Oligo-MePS (**3**) strands in comparison to Oligo-MePO (**4**) [4,6,23] on their avidity towards complementary DNA and RNA. Since strong affinity towards complementary RNA is of special interest, mostly due to potential therapeutic applications of **2** or **4**, we first studied chimeric oligonucleotides consisting of 2′-*O*-methylribonucleosides, and these data are given here.

Table 1
MALDI TOF MS analysis of chimeric 2′-*O*-methyloligonucleotides containing methylphosphonothioate (MePS), methylphosphonoselenoate (MePSe) or methylphosphonate (PMePO) linkages

| Oligonucleotides | | *M*/*z*[a] |
|---|---|---|
| 5′-$U_{MePS}C$ UC UC UC $U_{MePS}C$ U-3′ | Thio-$R_P R_P$-**2a** | 3479 (3478) |
| 5′-$U_{MePS}C$ UC UC UC $U_{MePS}C$ U-3′ | Thio-$S_P S_P$-**2b** | 3481 (3478) |
| 5′-UC UC $U_{MePS}C$ UC UC U-3′ | Thio-$R_P$-**2c** | 3467 (3463) |
| 5′-UC UC $U_{MePS}C$ UC UC U-3′ | Thio-$S_P$-**2d** | 3466 (3463) |
| 5′-$U_{MePS}C$ $U_{MePS}C$ $U_{MePS}C$ $U_{MePS}C$ $U_{MePS}C$ U-3′ | Thio-$R_P R_P R_P R_P$R$_P$-**2e** | 3521 (3523) |
| 5′-$U_{MePS}C$ $U_{MePS}C$ $U_{MePS}C$ $U_{MePS}C$ $U_{MePS}C$ U-3′ | Thio-$S_P S_P S_P S_P S_P$-**2f** | 3523 (3523) |
| 5′-UC UC $U_{MePO}C$ UC UC U-3′ | Oxo-$R_P$-**4a** | 3452 (3447) |
| 5′-UC UC $U_{MePO}C$ UC UC U-3′ | Oxo-$S_P$-**4b** | 3452 (3447) |
| 5′-TCTC$T_{MePSe}$CTC TCT-3′ | Seleno-$R_P$-**3a** | 3270 (3270) |
| 5′-TCTC$T_{MePSe}$CTCTCT-3 | Seleno-$S_P$-**3b** | 3270 (3270) |
| 5′-TCTC$U_{MePS}$CTC TCT-3′ | Thio-$S_P$-**2g** | 3221 (3222) |
| 5′-TGACG$T_{MePSe}$CATTTTTGACGTCA-3′ | Seleno-$R_P$-**16c** | 6159 (6158) |
| 5′-TGACG$T_{MePS}$CATTTTTGACGTCA-3′ | Thio-$R_P$-**16a** | 6111 (6111) |

[a] Negative ions were registered. Calculated molecular weights are given in brackets.

Roelen et al. [24] reported incorporation of dinucleoside methylphosphonothioates into chimeric hexamers. However, neither an absolute configuration nor stability studies on heteroduplexes formed with complementary DNA or RNA strands were reported. In our earlier studies aimed at the stereocontrolled synthesis of Oligo-MePO (**4**) [25–27], we prepared diastereomerically pure dinucleoside (3′,5′)-methylphosphonothioates (**1a**) in a stereoselective way [28,29], and elaborated an efficient method for the stereospecific P(X) → P(O) (X = S, Se) conversion [30].

Chimeric oligonucleotides were prepared via "*dimeric block* " approach [31] on 1 μmol scale using an ABI 392 Synthesizer. Incorporation of the *dimeric building blocks* **9a** or **9b** was performed using the standard protocol for phosphoramidite chemistry, with coupling time for the *dimeric building block* incorporation prolonged to 5–10 min.

The methodology was verified for several homopyrimidine oligo-(2′-OMe nucleotide)s **2** and **3** but also for dodecamers **16** of mixed sequence (Thio-$R_P$-**16a** and Seleno-$R_P$-**16c**) which are now being used for structural studies.

The average yields of coupling (trityl assay) are usually above 90%. Oligonucleotides were deprotected and released from the support and after concentration were purified by HPLC [32].

The oligomers Oxo-$R_P$-**4a** and Oxo-$S_P$-**4b** were prepared *via* stereoretentive oxidation of the internucleotide methylphosphonothioate bond in $R_P$-**2c** and $S_P$-**2d** by means of Oxone® in water–MeCN solution, respectively [33] (see Table 1).

As references for stability studies reported here, isosequential phosphodiesters d(TC)$_5$T (**10**) and (2′-OMe)-(UC)$_5$U (**11**) were used, while phosphodiesters d(AG)$_5$A (**12**) and r(AG)$_5$A (**13**) were prepared as the complementary templates.

### 2.4. Thermal stability of hybrid duplexes – influence of the absolute configuration

Melting temperature measurements of hybrid duplexes obtained from hybridization of unmodified oligonucleotides **10** and **11** and stereoregular chimeric oligonucleotides **3** with complementary templates were performed at $\lambda = 260$ nm [34].

Data collected in Table 2 confirm the correlation between the absolute configuration at the phosphorus atom of the modified internucleotide bond and the stability of hybrid duplexes with DNA and RNA. Generally, oligomers containing incorporated $R_P$ methylphosphonothioates (**2a**, **2c**, **2e**) elicit much stronger (and increasing with the extent of modifications) avidity towards complementary DNA and RNA than those with $S_P$ modifications. In the case of a single P-chiral bond located in the middle of Oligo-MePS, the difference of $T_m$ caused by the opposite absolute configuration (**2c**/RNA versus **2d**/RNA) is

Table 2
Thermal stability of hybrid duplexes formed with complementary DNA and RNA

| Chimeric oligonucleotides | $T_m$ (°C) of duplexes[a] | |
|---|---|---|
| | DNA-**12** | RNA-**13** |
| Thio-$R_P R_P$-**2a** | 40.4 | 75.5 |
| Thio-$S_P S_P$-**2b** | 28.1 | 65.9 |
| Thio-$R_P$-**2c** | 41.4 | 76.3 |
| Thio-$S_P$-**2d** | 29.0 | 71.3 |
| Thio-$R_P R_P R_P R_P R_P$-**2e** | 41.0 | 71.2 |
| Thio-$S_P S_P S_P S_P S_P$-**2 f** | n.d. | 37.0 |
| Oxo-$R_P$-**4a** | 43.8 | 74.4 |
| Oxo-$S_P$-**4b** | 36.0 | 70.0 |
| Reference Oligo-**10** | 44.0 | 57.7 |
| Reference Oligo-**11** | 41.0 | 74.9 |

[a] $T_m$ calculated from the first derivative [35]; with the error of temp not exceeding 0.3 °C.

5 °C, while $\Delta T_m = 12.4$ °C is found for hybrid duplexes $R_P$-**2c**/DNA and $S_P$-**2d**/DNA.

Oligonucleotide Oxo-$R_P$-**4a**, modified with a single methylphosphonate linkage appeared to form hybrid duplexes with complementary DNA and RNA of stability close to those determined for Thio-$R_P$-**2c**, however, the stability of hybrid duplex formed by $S_P$-**2d** was significantly lower than this for Oxo-$S_P$-**4b**. The difference in stabilities caused by incorporation of a single modification into a chimeric Oxo-$R_P$-**4a** or Oxo-$S_P$-**4b** ($\Delta T_m = 4.4$ °C for RNA and $\Delta T_m = 7.8$ °C for DNA) is in this case relatively lower than the corresponding difference between **2c** and **2d**, indicating major disruption caused by exchange of oxygen by sulfur at the $S_P$ position. Hybrid duplexes formed between the target RNA **13** and the $R_P$-modified chimeric oligomers **2a** and **2c** are about 18 °C more stable than those formed between **13** and the reference phosphodiester d(TC)$_5$T **10**, but of similar stability as **13** with **11**, leading to conclusions about close structural and conformational similarities of the above duplexes. Chimeric oligonucleotide **2e** furnishes complex with **13** of $T_m$ 71.2 °C, and 2′-OMe-modified oligonucleotide **11** with **13** provides the complex of similar thermal stability, as well as hybrid duplexes **2a/13** and **2c/13**. It can be further concluded that $R_P$-methyl group pointing out from the major groove causes minimal interactions with the sugar and has negligible influence on the stabilization of duplexes in comparison to non-modified oligonucleotides. In such context, destabilizing effect of $S_P$-methylphosphonothioate function with methyl group pointing inward the major groove and creating possible steric interactions with H3′ protons and increasing position of sulfur should be noticed. The effect of stabilization of duplexes formed by **2a** and **2b** is similar to that formed by **2c** and **2d** and stays in agreement with the studies of chimeric oligonucleotides with incorporated $N_{MePO}N'$ reported by Reynolds et al. [6] (see Fig. 2).

The presented data offer an efficient method to confirm that the P-chirality of methylphosphonothioate modification in chimeric oligonucleotides can be a very useful and sensitive probe to study local changes of structure of complexes between these oligonucleotides and other biomolecules (nucleic acids, proteins etc.).

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[17] $R_P$-**9a** (B = Ura, B′ = $C^{Bz}$, X = S, R = OMe)$^{31}$P NMR (MeCN-$d_3$) 98.15, 150.58, 150.25; $S_P$-**9a**: 99.32, 150.59, 150.44.
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