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# P-CHIRAL OLIGONUCLEOTIDES. 2D ROESY NMR ASSIGNMENT OF ABSOLUTE CONFIGURATION AT PHOSPHORUS AND CONFORMATIONAL ANALYSIS OF 5'-O-MONOMETHOXYTRITYL-(2'-O-DEOXYRIBONUCLEOSIDE) 3'-O-[O-(4-NITROPHENYL)]METHANEPHOSPHONATES

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# P-CHIRAL OLIGONUCLEOTIDES. 2D ROESY NMR ASSIGNMENT OF ABSOLUTE CONFIGURATION AT PHOSPHORUS AND CONFORMATIONAL ANALYSIS OF 5'-O-MONOMETHOXYTRITYL-(2'-O-DEOXYRIBONUCLEOSIDE) 3'-O-[O-(4-NITROPHENYL)]METHANEPHOSPHONATES

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# ABSTRACT

Fast and simple methodology for the assignment of the absolute configuration at the phosphorus atom in diastereomerically pure  $R_P$  and  $S_P$  5'-O-monomethoxytrityl-2'-O-deoxynucleoside 3'-O-(O-4-nitrophenyl) methanephosphonate (3) was established. The method utilizes 2D ROESY NMR and can be used for the stereochemical analysis of other P-chiral mononucleotides. Configurational analysis shows that the major conformation of the sugar residue in 3 is of the S (South) type. This study will facilitate synthesis of stereoregular methylphosphonate oligonucleotide analogues via the transesterification method.

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## **INTRODUCTION**

Methylphosphonate (PMe) oligonucleotides are among the first analogs of oligonucleotides considered as antisense agents<sup>1–3</sup>. The PMe-internucleotide group, in contrast to the natural phosphodiester internucleotide linkage, is uncharged and contains a chirality center at phosphorus.

The family of modified non-ionic oligonucleotide containing a nonbridging P-C bond, in addition to the methylphosphonate modification, includes among others, methylphosphonothioates<sup>4</sup>, hydroxymethylphosphonates<sup>5,6</sup>, benzylphosphonates<sup>7,8</sup>, (4,4'-dimethoxytrityl)phosphonate<sup>9</sup>, carboranylmethylphosphonates<sup>10</sup>, octylphosphonates<sup>11</sup>, phenylphosphonates<sup>12</sup>, and phenylphosphonothioates<sup>13</sup>.

PMe-oligonucleotides are characterized by high lipophilicity, resistance to nucleases, weak interactions with plasma proteins, wide distribution in various tissues and promotion of easy nonreceptor-mediated cellular uptake. The major drawback of these analogs is that they do not activate RNase-H. This however is compensated to some extent by the fact that PMe-oligonucleotides are relatively low in toxicity which allows administration of higher doses of PMe-oligomers. Another strategy to avoid the problems related to lack of RNase H induction by PMe-oligonucleotides while capitalizing on their unique aspects is to use short PMe-oligomers as a part of chimerical or hybrid oligonucleotide constructs.

The P-diastereomerism of PMe-oligonucleotides and the influence of P-chirality on the physicochemical properties of PMe-oligomers have been shown in the pioneering work of Miller<sup>14,15</sup> and further emphasized by Lesnikowski and Stec<sup>16</sup>. It is likely that only some of the diastereomers will have desirable physicochemical characteristics facilitating an efficient cellular uptake<sup>17</sup> and the most favorable biological effects. This causes a serious concern regarding the optimization of potential oligonucleotide pharmaceuticals based on PMe chemistry and creates a need for effective chirotechnology for P-chiral P-stereodefined oligonucleotide synthesis.<sup>18</sup>

Most of the stereocontrolled methods for the formation of the PMe internucleotide linkages are based upon the transesterification of P-stereodefined diastereomeric nucleotide monomers<sup>19–22</sup>. The first successful method for the stereocontrolled synthesis of PMe-oligonucleotides was based on the process of stereospecific nucleophilic substitution at a pentavalent phosphorus atom in P-chiral, diastereomerically pure monomer type of 5'-O-monomethoxytrityl-2'-O-deoxynucleoside 3'-O-(O-4-nitrophenyl)methane-phosphonate (3)<sup>19</sup>. The substitution of 4-nitrophenyl group by the 5-OH group of the nucleoside component, activated with Grignard reagent (tBuMgCl), proceeds with an inversion of absolute configuration at phosphorus. This method was successfully used for the step by step, in solution synthesis of short, stereoregular PMe oligonucleotides ranging from dimers to pentanucleotides<sup>23</sup>, phosphorothioate oligodeoxynucleotides<sup>24</sup> and phosphorothioate oligoribonucleotides<sup>25</sup>.

The assignment of the absolute configuration at phosphorus in PMeoligonucleotides is crucial for the discussion of the relationship between the stereochemistry of the internucleotide linkage and the physicochemical and biological properties of PMe-oligomers. For the assignment of absolute configuration in PMe-dinucleotides X-ray crystallography  $[S_P-d(A_{PMe}T)^{26}, R_P-d(C_{PMe}G)^{27}, d(T_{P(S)Me}T)^{28},], {}^{1}$ H-NMR analysis<sup>29–31</sup>, mobility shift in RP-HPLC<sup>9,32</sup>, and chemical correlation<sup>19–21,32,33</sup> were employed. It should be emphasized however, that the direct assignment of absolute configuration of the internucleotide PMe group becomes very difficult or impractical for longer than dimer oligomers.

In the transesterification methods used for the stereoselective synthesis of PMe-oligonucleotides, the formation of the internucleotide linkage occurs with defined stereochemistry<sup>19–21,34,35</sup>. Thus, in the case of monomer type of **3** and Grignard reagent assisted coupling, the reaction takes place with the inversion of absolute configuration at phosphorus of **3**. Consequently, the absolute configuration at phosphorus of the PMe-internucleotide linkage can be deduced for each subsequent linkage of the growing oligonucleotide chain if the absolute configuration at phosphorus of monomer is known<sup>20,23</sup>.

The absolute configuration in diastereomeric **3** was assigned so far by means of the chemical correlation method<sup>19</sup>. Briefly, after 5'-deprotection of **3**, and conversion of the resulting 2'-O-deoxynucleoside 3'-[O-(4-nitrophenyl)]methanephosphonate to 2'-O-deoxynucleoside (3',5') cyclic methylphosphonate, the absolute configuration of the cyclic product was determined by means of <sup>31</sup>P-NMR<sup>36,37</sup>. Since the cyclization reaction is stereospecific, and occurs with inversion of configuration at phosphorus, the absolute configuration in the acyclic precursor, and consequently in **3** could be assigned.

Herein we present a convenient and fast method based on 2D NMR for assignment of absolute configuration at phosphorus and preliminary results of configurational analysis of base-protected monomers type **3** (Fig. 1 and 2).

#### **RESULTS AND DISCUSSION**

# Synthesis of Base Protected 5'-O-Monomethoxytrityl-2'-O-deoxynucleoside 3'-O-(O-4-nitrophenyl)methylphosphonates (3)

For the synthesis of base protected 5'-O-monomethoxytrityl-2'-Odeoxynucleoside 3'-O-(O-4-nitrophenyl)methylphosphonates (3) (B = Thy, Cyt<sup>Bz</sup>, Ade<sup>Bz</sup>, Gua<sup>iBu</sup>) the method previously developed for the preparation



*Figure 1.* Synthesis of 5'-O-monomethoxytrityl-2'-O-deoxynucleoside 3'-O-(O-4-nitrophenyl)methanephosphonates (3), monomers for the preparation of *P*-stereodefined methanephosphonate oligonucleotide analogues. (i) 2, 1,2,4-triazole, Et<sub>3</sub>N/CH<sub>3</sub>CN. (ii) Seperation of  $S_{P}$ -3 and  $R_{P}$ -3 diastereomers of 3.

of base unprotected **3** was employed<sup>19,38</sup>. Thus, suitable 5'-O-monomethoxytrityl-N-protected 2'-O-deoxynucleosides were treated with prepared in situ [O-(4-nitrophenyl)methylphosphonotriazolidate in CH<sub>3</sub>CN solution. The resulting mixture of diastereomeric **3**, after standard work up, was separated into individual diastereomers by means of silica gel chromatography. Diastereomerically pure **3** were then analyzed by means of TLC, UV spectroscopy<sup>31</sup>, P- and <sup>1</sup>H-NMR (Fig. 1, Table 1–3).

#### **NMR** Measurements

The absolute configuration at phosphorus in dinucleoside methylphosphonates was assigned using NOE's of the PCH<sub>3</sub> group derived from ROESY experiments<sup>31</sup>, taking advantage of the fact that different configurations at phosphorus lead to a different environment of the PCH<sub>3</sub> group. The *Rp* configuration in dinucleotides was unequivocally derived from the ROESY spectra on the basis of two diagnostic crosspeaks of similar intensity for most dinucleotides as expected. In the case of  $d(T_{PMe}T)$  and  $d(T_{PMe}A)$ dimers, however, only one ROE signal to H3' was detected within the sensitivity limits of the experiment. Two diagnostic crosspicks were observed for dimers with tentatively assigned *S*<sub>*P*</sub> configuration, though the crosspeak to H4' was characterized by a much lower intensity. The ROE signals were



*Figure 2.* Schematic view of  $R_P$  and  $S_P$ , 5'-O-monomethoxytrityl-2'-O-deoxyadenosine 3'-O-(O-4-nitrophenyl)methanephosphonate (3). (a)  $R_P5'$ -O-Monomethoxytrityl-2'-deoxyadeno sine3'-O-(O-4nitrophenyl)methanephosphonate (3, B = Ade<sup>Bz</sup>). (b)  $S_P5'$ -O-Monomethoxytrityl-2'-deoxyadenosine3'-O-(O-4nitrophenyl)methanephosphonate (3, B = Ade<sup>Bz</sup>).

integrated and the distances between the PCH<sub>3</sub> group and H3' or H4' were estimated for  $d(A_{PMe}T)$ ,  $d(A_{PMe}A)$  and  $d(T_{PMe}A)$  dimers. The average sum of the distances PCH<sub>3</sub>/H3' and PCH<sub>3</sub>/H4' is equal to 626 pm. For the  $S_P$  isomers both distances were equal within the experimental error, and for the  $R_P$  isomers the distance of the PCH<sub>3</sub> group to H3' is shorter by more than 50 pm<sup>31</sup>.

We applied the same method to the deoxyribonucloside methylphosphonates **3**, keeping in mind that the flexibility of mononucleosides is supposed to be higher than the flexibility of the dimers due to lower steric hindrance and lack of stacking stabilization. In all cases ROEs were visible. The diastereomers **3**-SLOW (the SLOW descriptor is used for the diastereomer characterized by the lower mobility on a TLC plate) were assigned as the  $R_P$  isomers on the basis of two diagnostic crosspeaks between PCH<sub>3</sub> and H3' and H4'. For the FAST diastereomer, including 2'-O-deoxyadenosine and thymine derivatives, only ROE to H3' was detected, as expected for the opposite  $S_P$  isomer (Table 4). 2'-O-Deoxycytidine and 2'-O-deoxyguanosine methylphosphonates **3**-FAST isomers are giving ROE signals to H4' weaker than to H3', but recognizable. This observation

*Table 1.* UV and Chromatographic Characteristics of 5'-O-Monomethoxytrityl-2'-O-deoxy-nucleoside 3'-O-(O-4-Nitrophenyl)methanephosphonate (3) ( $B = Thy, Cyt^{Bz}, Ade^{Bz}, Gua^{iBu}$ )

Isomer	S <sub>P</sub> -dT	$R_P$ -dT	$S_p$ -dC	$R_P$ -dC	S <sub>P</sub> -dA	<i>R</i> <sub><i>P</i></sub> -dA	S <sub>P</sub> -dG	<i>R</i> <sub>P</sub> -dG
UV $\lambda_{max}^{1}$	262.4	265.1	261.1	261.8	278.9	279.1	261.1	261.1
UV $\lambda_{\min}^{man_1}$	245.3	245.2	243.6	245.3	251.1	251.5	241.7	242.4
TLC $[\mathbf{R}_f]^2$	0.66	0.56	0.68	0.55	0.63	0.52	0.44	0.29

<sup>1</sup> In 95% C<sub>2</sub>H<sub>5</sub>OH. <sup>2</sup> Developing solvent system CHCl<sub>3</sub>:CH<sub>3</sub>OH = 95:5.

	$R_p$ -T	$S_p$ -T	$R_p$ -dC	$S_p$ -dC	$R_p$ -dA	$S_p$ -dA	$R_p$ -dG	$S_p$ -dG
<sup>31</sup> P	30.36	29.96	30.25	29.99	30.19	29.82	30.25	29.85
NH	11.39	11.38					12.06	12.05
NHBz/i			11.27	11.28	11.17	11.20	11.61	11.60
Bu								
H8					8.59	8.59	8.16	8.14
H6	7.47	7.47	8.15	8.15				
H5			7.20	7.15				
H2					8.59	8.59		
CH <sub>3</sub>	1.49	1.46						
PCH <sub>3</sub>	1.79	1.76	1.79	1.76	1.84	1.83	1.82	1.80
H1′	6.21	6.20	6.17	6.14	6.53	6.49	6.28	6.25
H2′	2.50	2.43	2.45	2.39	2.73	2.65	2.68	2.60
H2″	2.50	2.43	2.70	2.61	3.21	3.24	3.04	2.99
H3′	5.21	5.19	5.17	5.16	5.45	5.42	5.23	5.24
H4′	4.09	4.16	4.21	4.27	4.24	4.32	4.21	4.21
H5′/	3.17/3.	3.25	3.31	3.33	3.20	3.29	3.19	3.23
H5′								
4-NO <sub>2</sub> -	7.35 <sub>2,6</sub>	7.43 <sub>2,6</sub>	7.38 <sub>2,6</sub>	7.47 <sub>2,6</sub>	7.44 <sub>2,6</sub>	7.47 <sub>2,6</sub>	7.39 <sub>2,6</sub>	7.43 <sub>2,6</sub>
Ar	8.18 <sub>3,5</sub>	8.24 <sub>3,5</sub>	8.19 <sub>3,5</sub>	8.25 <sub>3,5</sub>	8.21 <sub>3,5</sub>	8.25 <sub>3,5</sub>	8.19 <sub>3,5</sub>	8.24 <sub>3,5</sub>
4-MeO-	7.18 <sub>2,6</sub>	7.21 <sub>2,6</sub>	7.20 <sub>2,6</sub>	$7.22_{2,6}$	7.14 <sub>2,6</sub>	7.16 <sub>2,6</sub>	7.13 <sub>2,6</sub>	7.15 <sub>2,6</sub>
Ar	6.84 <sub>3,5</sub>	6.87 <sub>3,5</sub>	6.84 <sub>3,5</sub>	6.883,5	6.78 <sub>3,5</sub>	6.81 <sub>3,5</sub>	6.77 <sub>3,5</sub>	6.79 <sub>3,5</sub>
MeO	3.71	3.72	3.70	3.72	3.70	3.70	3.70	3.71
Ph	7.21-7.36	7.24-7.40	7.22-7.35	7.25-7.40	7.16-7.34	7.16-7.35	7.16-7.30	7.18-7.34
Ph(Bz)			8.002,6	7.99 <sub>2,6</sub>	8.04 <sub>2,6</sub>	8.04 <sub>2,6</sub>		
			$7.50_{3,5}$	$7.50_{3,5}$	7.54 <sub>3,5</sub>	7.54 <sub>3,5</sub>		
			7.624	7.624	7.644	7.644		
i-Pr							1.11	1.12
							(6H);	(6H);
							2.74	2.74
							(1H)	(1H)

Table 2. Chemical Shifts (ppm) at 25°C

J [Hz]	$R_p$ -T	$S_p$ -T	$R_p$ -dC	$S_p$ -dC	$R_p$ -dA	S <sub>p</sub> -dA	$R_p$ -dG	$S_p$ -dG
1'2'	6.9	7.0	6.2	6.2	6.9	6.7	6.0	6.1
1'2''	6.9	7.0	6.4	6.4	6.9	6.7	7.7	7.3
2'3'	6.3	6.4	6.1	6.7	6.1	6.0	6.3	6.0
2"3'	3.3	3.3	3.7	3.7	3.2	3.2	2.2	3.1
2'2''	-14.0	- 14.6	- 14.4	- 14.3	-14.0	- 14.3	-14.4	- 14.2
3'4'	2.9	3.7	4.0	3.8	3.4	3.3	2.5	2.8
4'5'	4.0	3.7	4.0	4.0	5.2	6.1	4.9	4.9
4'5''	4.0	3.7	4.0	4.0	5.2	6.1	4.9	4.9
5'5''	-12.2	*	-12.3	*	-10.4	-15.1	-14.5	- 14.5
PCH <sub>3</sub>	17.7	17.8	17.7	17.7	17.8	17.7	17.8	17.7
3'P	8.2	7.0	7.5	7.0	7.8	8.1	7.9	7.0

Table 3. J(H,H) Coupling Constants (Hz) at 25°C

\* Overlaps with OH.

means that the PCH<sub>3</sub> group is shifted towards H3', but still close enough to H4' to generate ROE, as previously was detected for dinucleotides  $d(T_{PMe}T)$  and  $d(T_{PMe}A)^{31}$ .

The chirality at phosphorus is designed as  $R_P$  and  $S_P$ , according to the Cahn-Ingold-Prelog rules. It should be pointed out that, because of the replacement of the nucleosidyl residue in the dinucleotide discussed above for the 4-nitrophenyl group in **3**, and the substituent priority rules (4-nitrophenyl > nucleosidyl), the same spatial orientation of the ligands at phosphorus atom corresponds to different configurational descriptors according to the R/S nomenclature. However, it seems that the rotational freedom around the P-O and O-C bonds in **3** leads to the same relation between R/S configuration at P-atom and the relative intensities of P-CH<sub>3</sub> and H3'/H4' crosspeaks in the ROESY experiments as for dinucleotides.

The crosspeaks of the PCH<sub>3</sub> group were integrated and distances to H3' and H4' were estimated (Table 4) using the internal distance H2'/H2'' (180 pm) for the distance calibration<sup>39</sup>. For the thymidine derivative vicinal 2'/2'' protons are overlapping and distance estimation was impossible.

dGiBu dABz dCBz  $\mathbf{R}_p$  $\mathbf{S}_p$  $R_P$  $S_P$  $R_P$  $\mathbf{S}_P$ PCH<sub>3</sub>/H3' 273 273 246 235 218 278 PCH<sub>3</sub>H4' 273 281 327 302 368 \_

**Table 4.** Estimated Distances in Pm Between  $PCH_3$  and H3' or H4' for (3)

The estimated distances were used to build (HYPERCHEM 4.5) models illustrating the position of the P-CH<sub>3</sub> group in *Sp* and *Rp* isomers (Fig. 2). As an example, diagnostic parts of the *S*<sub>*P*</sub>-dA **3** and *R*<sub>*P*</sub>-dA **3** spectra are shown in Fig. 3 and 4.

The absolute configuration at phosphorus in **3** established by ROESY experiments correlates well with <sup>31</sup>P NMR chemical shifts and chromatographic mobility on TLC plates. In all cases diastereomers with assigned  $S_P$  configuration and higher mobility on TLC (designed as FAST) were shifted up-field by about 0.4 ppm in the <sup>31</sup>P NMR spectra.

The sugar rings in nucleic acids exist in an equilibrium between two conformers – called north (N) and south (S). We were interested if the conformation on the phosphorus atom may affect this equilibrium in the case of monomers **3**. The conformational analysis of a five-membered ring is facilitated by the Altona-Sundaralingam concept<sup>40,41</sup>. Each conformer is described by three parameters: the puckering amplitude ( $\Phi_m$ ), the phase angle of pseudorotation (P) and its population. We analyzed <sup>31</sup>P NMR decoupled spectra by the program PSEUROT, which calculates the best fit of the five pseudorotational parameters and defines the two-state equilibrium. The puckering amplitudes were held constant and equal to one another, and in



*Figure 3.* The diagnostic part of  $R_P$ -dA 3 ROESY spectrum.



Figure 4. The diagnostic part of  $S_P$ -dA 3 ROESY spectrum.

every fitting raised in 1° steps between  $30^{\circ}$  and  $42^{\circ}$ . The herein described preliminary analysis was limited to the spectra recorded at  $25^{\circ}$ C. It was found that in all cases, independently of the P atom configuration, the south conformation dominates.

	Deoxyribonucloside Methanephosphonate								
Parameter	S <sub>p</sub> -dG	$R_p$ -dG	$S_p$ -dA	$R_p$ -dA	$S_p$ -dC	$R_p$ -dC	$S_p$ -T	$R_p$ -T	
<sup>s</sup> P	200.0	211.1	174.9	176.5	142.4	154.0	175.3	179.4	
${}^{S}\Phi$	33.0	32.0	31.0	31.0	30.0	32.0	31.0	30.0	
NР	13.8	39.2	13.4	27.0	-16.3	0.50	40.3	19.3	
$^{N}\Phi$	33.0	31.0	31.0	32.0	30.0	32.0	31.0	30.0	
%S	0.71	0.77	0.67	0.65	0.60	0.59	0.61	0.70	
Rms error	0.068	0.117	0.186	0.134	0.148	0.290	0.092	0.079	

*Table 5.* Pseudorotational Parameters P and  $\Phi$  (°), Percentage Populations North and South and Rms Errors (Hz)

#### EXPERIMENTAL

#### Materials

Deoxynucleosides (B = Thy, Cyt, Ade, Gua) were obtained from Pharma Waldhof GmbH (Düsseldorf, Germany). Column chromatography was performed on silica gel 230-400 mesh obtained from Sigma-Aldrich. (Steinheim, Germany) or reverse phase silica gel C8 from Fluka (Steinheim, Germany). TLC was performed on silica gel F254 plates purchased from Sigma-Aldrich (Steinheim, Germany). Solvents were purchased in the highest available quality. UV measurements were performed on GBC Cintra10e UV-VIS spectrometer (Dandenong, Australia). NMR spectra were recorded with a Bruker Avance DPX 250 spectrometer equipped with BB inverse probehead. Tetramethylsilane and 85% H<sub>3</sub>PO<sub>4</sub> were used as external standards for <sup>1</sup>H-NMR and <sup>31</sup>P-NMR, respectively.

#### Methods

5'-O-Monomethoxytrityl-2'-O-deoxynucleoside(B = Thy, Cyt<sup>Bz</sup>, Ade<sup>Bz</sup>, Gua<sup>iBu</sup>) (1) were synthesized as described <sup>42</sup>.

R<sub>P</sub> and S<sub>P</sub> 5'-O-Monomethoxytrityl-2'-O-deoxynucleoside 3'-O-(O-4nitrophenyl)methyl-phosphonate (3) ( $B = Thy, Cyt^{Bz}, Ade^{Bz}, Gua^{iBu}$ ). [O-(4-nitrophenyl)]methyl-phosphonochloridate (2, 3.3 g, 14.0 mmol)<sup>38</sup> 1,2,4-triazole (3.6 g, 52.0 mmol) and triethylamine (2.7 g, 3.7 mL, 26.5 mmol) were mixed together in anhydrous CH<sub>3</sub>CN (50 mL) under nitrogen. After 15 min at room temperature, to the resultant clear solution of [O-(4nitrophenyl)]methylphosphonotriazolidate a solution of the proper  $O_{N}$ protected nucleoside 1 (5.6 mmol) was added under nitrogen. The reaction progress was monitored by TLC (CHCl<sub>3</sub>:CH<sub>3</sub>OH = 95:5). After completion of the reaction (ca. 3h), CH<sub>3</sub>CN was evaporated under reduced pressure, then the oily residue was dissolved in CHCl<sub>3</sub> (100 mL). The CHCl<sub>3</sub> solution was washed with  $H_2O$  (3 × 50 mL). The organic fraction was separated, dried over MgSO<sub>4</sub>, and then CHCl<sub>3</sub> was evaporated under reduced pressure yielding crude 3 as a mixture of  $R_P$  and  $S_P$  diastereomers. Monomer **3** was then purified and separated into individual diastereomers on reverse phase C8 silica gel column using CHCl<sub>3</sub> as eluting solvent system, or alternatively on normal phase silica using a gradient of CH<sub>3</sub>OH in CHCl<sub>3</sub>. The yield of **3** as  $S_P$ ,  $R_P$  and  $S_P + R_P$  fraction was 35–50%. The UV and chromatographic characteristics are presented in Table 1, <sup>31</sup>P- and <sup>1</sup>H-NMR data are shown in Tables 2 and 3.

**NMR measurements.** NMR spectra were recorded at 25°C. Samples were prepared by dissolving a quantity of **3** (10 mg) in DMSO-d<sub>6</sub> (0.7 mL, 99.5% D, final concentration of **3** 1.7-2.0 mM) and solutions were purged

with dry Ar for 30 min. Spectra were referenced to the residual DMSO signal at 2.49 ppm. ROESY experiments were carried out with an average field of 2.2 kHz and 350 ms duration. The transmitter was set to 6 ppm. The coupling constants were derived from 1D homodecoupling experiments and verified through the simulation procedure by the DAISY (Bruker) procedure. Integration of 2D spectra was performed using X-WIN NMR 2.1 (Bruker) program subroutine. For the PSEUROT analyses [PSEUROT 6.2, J. van Wijk and C. Altona, Leiden Institute of Chemistry, Leiden University, Netherlands], we have used the following  $\lambda$  electro-negativites:  $\lambda$  (C1') =  $\lambda$ (CH<sub>2</sub>OR) = 0.68,  $\lambda$  (C2') = 0.72,  $\lambda$  (C3') =  $\lambda$ (C4') = 0.62,  $\lambda$  = (O4') = 1.40,  $\lambda$ (-O-P(O)(CH<sub>3</sub>)O-) = 1.29 as for OPO(OEt)<sub>2</sub> group<sup>40,41</sup>.

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#### REFERENCES

- Miller, P.S.; McParland, K.B.; Jayaraman, K.; Ts'o, P.O.P. Biochemical and Biological Effects of Nonionic Nucleic Acid Methylphosphonates. Biochemistry 1981, 20, 1874–1880.
- a) Miller, P.S.; Ts'o, P.O.P. Oligodeoxyribonucleoside Alkyl and Arylphosphonates, U.S. Patent 4.507.433; **1985**, b) Ts'o, P.O.P.; Aurelian, L.; Chang, E.; Miller, P.S. Nonionic Oligonucleotide Analogs (Matagen) as Anticode Agents in Duplex and Triplex Formation. Ann. NY Acad. Sci. **1992**, *660*, 159–177.
- Smith, C.C.; Aurelian, L.; Reddy, M.P.; Miller, P.S.; Ts'o, P.O.P. Proc. Natl. Acad. Sci. USA 1986, 83, 2787–2791.
- Habus, I.; Devlin, T.; Iyer, R.P.; Agrawal, S. Improved Synthesis of Oligonucleoside Methylphosphonate Analogs. Bioorg. Med. Chem. Lett. 1996, 6, 1393–1398.
- Lesnikowski, Z.J. Hydroxymethylphosphonate: Novel Oligonucleotide Analogue. Bioorg. Chem. 1994, 22, 128–139.
- Wada, T.; Sekine, M. Chemical Synthesis of Oligothymidylate having Hydroxymethylphosphonate Internucleotide Linkages. Tetrahedron Lett. 1995, 36, 8845–8848.
- Mauritz, R.P.; Meier, C.; Uhlman, E. Synthesis of 3',5'-Dithymidylyl-α-Hydroxyphosphonate Dimer Building Blocks for Oligonucleotide Synthesis. Nucl. Nucl. 1997, 16, 1209–1212.
- Samstag, W.; Eisenhardt, S.; Offensperger, W.B.; Engels, J.W. Synthesis and Properties of New Antisense Oligodeoxynucleotides Containing Benzylphosphonate Linkage. Antisense Nucl. Acid Drug Develop. 1996, 6, 153–156.

- Stec, W.J.; Zon, G.; Egan, W.; Byrd, R.A.; Phillips, L.R.; Gallo, K.A. Solid-Phase Synthesis, Separation, and Stereochemical Aspects of P-Chiral Methane- 4,4'-Dimethoxytriphenylmethanephosphonate Analogues of Oligodeoxyribonucleotides. J. Org. Chem. 1985, 50, 3908–3913.
- Lesnikowski, Z.J.; Lloyd, R.M. Jr.; Schinazi, R.F. Comparison of Physicochemical and Biological Properties of (*o*-carboran-1-yl)methylphosphonate and Methylphosphonate Oligonucleotides. Nucleosides and Nucleotides 1997, 16, 1503–1505.
- Mag, M.; Jahn, K.; Kretzschmar, G.; Peyman, A.; Uhlman, E. Synthesis and Duplex Stability of Oligodeoxynucleotides Containing Stereoregular or Stereorandom Octylphosphonate Linkages. Tetrahedron 1996, 52, 10,011–10,024.
- Agarwal, K.L.; Riftina, F. Synthesis and Enzymatic Properties of Deoxyribooligonucleotides Containing Methyl and Phenylphosphonate Linkages. Nucl. Acids Res. 1979, 6, 3009–3023.
- Mag, M.; Muth, J.; Jahn, K.; Peyman, A.; Kretzschmar, G.; Engels, J.W.; Uhlman, E. Synthesis and Binding Properties of Oligonucleotides Containing Phenylphosphonothioate Linkages. Bioorg. Med. Chem. 1997, 5, 2213–2220.
- Miller, P.S.; Annan, N.D.; Mcparland, K.B.; Pulford, S.M. Oligothymidylate Analogues having Stereoregular, Alternating Methylphosphonate/Phosphodiester Backbones as Primers for DNA Polymerase. Biochemistry 1982, 21, 2507–2512.
- Miller, P.S. Development of Antisense and Antigene Oligonucleotide Analogs. Nucl. Acids. Res. 1996, 52, 261–291.
- Lesnikowski, Z.J.; Jaworska, M.; Stec, W.J. Octa(Thymidine Methanephosphonates) of Partially Defined Stereochemistry: Synthesis and Effect of Chirality at Phosphorus on Binding to Pentadecadeoxyriboadenylic Acid. Nucl. Acid Res. 1990, 18, 2109–2115.
- Lesnikowski, Z.J.; Przepiorkiewicz, M.; Tamura, Y.; Kaji, H.; Wickstrom, E. P-Chiral Oligonucleotides. Effect of Configuration at Phosphorus on Transport of Tetra(Thymidine Methylphosphonate)s Across Organic Liquid Membrane. Collect. Czech. Chem. Commun. 2001-accepted for publication.
- Lesnikowski, Z.J. Stereocontrolled Synthesis of P-chiral Analogues of Oligonucleotides. Bioorg. Chem. 1993, 21, 127–155.
- Lesnikowski, Z.J.; Wolkanin, P.J.; Stec, W.J. Stereospecific Synthesis of (R<sub>P</sub>) and (S<sub>P</sub>)-Thymidylyl(3',5')Thymidylyl Methanephosphonate. Tetrahedron Lett. **1987**, 28, 5535–5538.
- Wozniak, L.; Pyzowski, J.; Wieczorek, M.; Stec, W.J. New Stereospecific Method of Synthesis of [R<sub>P</sub>]- and [S<sub>P</sub>]-Dinucleoside (3',5')-Methanephosphonates. J. Org. Chem. **1994**, *59*, 5843–5846.
- Stec, W.J.; Wozniak, L.A.; Pyzowski, J.; Niewiarowski, W. Novel Cost-Effective Methanephosphonoanilidothioate Approach to the Stereoselective Synthesis of Dinucleoside (3',5')-Methanephosphonates. Antisense Nucl. Acid Drug. Develop. 1997, 7, 381–395.
- Lesnikowski, Z.J.; Zabawska, D.; Jaworska-Maslanka, M.M.; Schinazi, R.F.; Stec, W.J. Base Catalyzed Transeterification Method for Internucleotide Linkage Formation. New J. Chem. **1994**, *18*, 1197–1204.
- Jaworska-Maslanka, M.M.; Kacperczyk, W.; Korczynski, D.; Lesnikowski, Z.J. Studies on Stereospecific Formation of P-chiral Internucleotide Linkage.

Synthesis of All- $R_P$  and All- $S_P$  Methylphosphonate Oligonucleotide d(MMTrA<sub>PMe</sub>T<sub>PMe</sub>T<sub>PMe</sub>C<sub>PMe</sub>T<sub>Ac</sub>) *via* Grignard Activated Coupling. Antisense Nucl. Acid Drug Develop. **1997**, *7*, 23–30.

- Lesnikowski, Z.J.; Jaworska, M.M. Studies on Stereospecific Formation of P-Chiral Internucleotide Linkage. Synthesis of (*R<sub>P</sub>R<sub>P</sub>*)- and (*S<sub>P</sub>S<sub>P</sub>*)-Thymidylyl (3',5')Thymidylyl(3',5') Thynidine Di(*O*, *O*-Phosphorothioate) using 2-Nitrobenzyl Group as S-Protection. Tetrahedron Lett. **1989**, *30*, 3821–3824.
- 25. Lesnikowski, Z.J. The First Stereocontrolled Synthesis of Thiooligoribonucleotide:  $(R_P R_P)$ - and  $(S_P S_P)$ -U<sub>PS</sub>U<sub>PS</sub>U, Nuleosides and Nucleotides **1992**, *11*, 1621–1638.
- Chacko, K.K.; Lindner, K.L.; Saenger, W.; Miller, P.S. Molecular Structure of Deoxyadenylyl-3'-Methylphosphonate-5'-Thymidyne Dihydrate, (d-Apt × 2H<sub>2</sub>O), a Dinucleoside Monophosphate with Neutral Phosphodiester Backbone. An X-ray Crystal Study. Nucl. Acid Res. **1983**, *11*, 2801–2814.
- Han, F.; Watt, W.; Callahan, L.; Kezdy, F.J.; Agarwal, K. Molecular Structure Of Deoxycytidyl-3'-Methylphosphonate (R<sub>p</sub>) 5'-Deoxyguanidine, d[C<sub>p</sub>(CH<sub>3</sub>)G]. A Neutral Dinucleotide with Watson- Crick Base Pairing and a Right Handed Helical Twist. Nucl. Acid Res. **1990**, *18*, 2759–2767.
- Szabo, T.; Noreus, D.; Norrestam, R.; Stawinski, J., Molecular and Crystal Structure of SP-Thymidin-3'-yl 4-Thiothymidin-5'-yl Methylphosphonate. Nucl. Acid Res. 1993, 21, 3921–3926.
- Kan, L.S.; Cheng, D.M.; Miller, P.S.; Yano, J.; Ts'o, P.O.P. Proton Nuclear Magnetic Resonance Studies on Dideoxyribonucleoside Methylphosphonates. Biochemistry, **1980**, *19*, 2122–2132.
- Lebedev, A.V.; Frauendorf, A.; Vyazovkina, E.V.; Engels, J.W. Determination and Prediction of the Absolute Configuration of Dinucleoside Alkylphosphonates using Conformational Analysis and Multivariant Statistics. Tetrahedron, 1993, 49, 1043–1052.
- Löshner, T.; Engels, J.W. Diastereomeric Dinucleoside-Methylphosphonates: Determination of Configuration with the 2-D NMR ROESY Technique, Nucl. Acid Res. 1990, 18, 5083–5088.
- Lesnikowski, Z.J.; Jaworska-Maslanka, M.M.; Stec, W.J. Stereospecific Synthesis of P-chiral di(2-O-deoxyribonucleoside)methanephosphonates. Nucleosides and Nucleotides 1991, 10, 733–736.
- Wozniak, L.A.; Wieczorek, M.; Pyzowski, J.; Majzner, W.; Stec., W.J. Stereochemistry of the DBU/LiCl-Assisted Nucleophilic Substitution at Phosphorus in Nucleoside-3'-O-(Se-Methylmethanephosphonoselenoate)s. J. Org. Chem. 1998, 63, 5395–5402.
- 34. Lesnikowski, Z.J.; Wolkanin, P.J.; Stec, W.J. Determination of Absolute Configuration at Phosphorus in Diastereomers of Thymidylyl(3',5')Thymidylyl Methanephosphonate. In Biophosphate and their Analogues-Synthesis, Structure, Metabolism and Activity, Proceedings of the 2nd International Symposium on Phosphorus Chemistry Directed Towards Biology, K.S., Bruzik, W.J., Stec Eds.; Lodz: Poland, September 8–12, 1986.
- 35. Cormier, J.F.; Pannunzio, T. An Improvement in the Stereospecific Synthesis of Dinucleoside Methylphosphonates. Tetrahedron Lett. **1991**, *32*, 7161–7164.

- Bajwa, G.S.; Bentrude, W.G. Preparation of Diastereomeric Thymidine 3',5'-Cyclic Methylphosphonates. Assignment of Rp and Sp Configuration by <sup>13</sup>C NMR. Tetrahedron Lett. 1980, 21, 4683–4686.
- Stec, W.J.; Zielinski, W.S. <sup>31</sup>P-<sup>15</sup>N Spin-Spin Coupling as a Probe for the Assignment of Absolute Configuration in Rp- and Sp-Thymidine Cyclic 3',5'-Phosphordimethylamidates, Tetrahedron Lett. **1980**, *21*, 1361–1364.
- Stec, W.J.; Lesnikowski, Z.J. Stereospecific Synthesis of P-Chiral Analogs of Oligonucleotides, In Methods in Molecular Biology Vol. 20: Protocols for Oligonucleotide and Analogs, S. Agrawal Ed.; Humana Press Inc.; Totowa: NJ, 1993.
- Wutrich, K., NMR of Proteins and Nucleic Acids, Wiley-Interscience Publication; John Wiley & Sons: New York, 1986.
- Altona, C.; Ippel, J. H.; Westra Hoekzema, A.J.A.; Erkelens, C.; Groesbeek, M.; Donders, L.A. The Relationship Between Proton-Proton NMR Coupling Constants and Substituent Electronegativities. V. Empirical Substituent Constants Derived from Ethanes and Propanes. Magn. Res. Chem. 1989, 27, 564–576.
- Altona, C.; Francke, R.; de Haan, R.; Ippel, J.H.; Daalmans, G.J.; Westra Hoekzema, A.J.A.; van Wijk, J. Empirical Group Electronegativities for Vicinal NMR Proton-Proton Coupling Along a C-C Bond: Solvent Effects and Reparameterization of the Haasnoot Equation. Magn. Res. Chem. 1989, 32, 670–678.
- Ti, G.S.; Gaffney, B.L.; Jones, R.A. Protected Deoxynucleosides. J. Am. Chem. Soc. 1982, 104, 1316–1319.

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