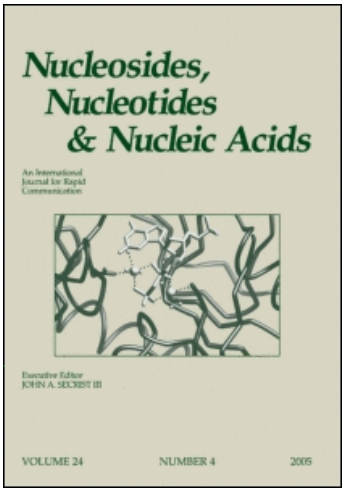

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Stefan Jankowski[a]; Agnieszka B. Olejniczak[b]; Zbigniew J. Lesnikowski[b]
[a] Department of Chemistry, Technical University of Lodz, Lodz, Poland [b] Laboratory of Molecular Virology and Biological Chemistry, Center of Microbiology and Virology, Polish Academy of Sciences, Lodz, Poland

Online publication date: 25 March 2002

To cite this Article Jankowski, Stefan , Olejniczak, Agnieszka B. and Lesnikowski, Zbigniew J.(2002) '*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', Nucleosides, Nucleotides and Nucleic Acids, 21: 2, 177 — 190
To link to this Article: DOI: 10.1081/NCN-120003181
URL: http://dx.doi.org/10.1081/NCN-120003181

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 21(2), 177–190 (2002)

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

Stefan Jankowski,[1] Agnieszka B. Olejniczak,[2] and Zbigniew J. Lesnikowski[2,*]

[1]Technical University of Lodz, Department of Chemistry, Lodz 90-924, Poland
[2]Laboratory of Molecular Virology and Biological Chemistry, Center of Microbiology and Virology, Polish Academy of Sciences, Lodz 93-232, Poland

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.

*Corresponding author. Center of Microbiology and Virology PAS, 106 Lodowa St., Lodz 93-232, Poland. Fax: (+48-42)-677-1230; E-mail: zlesnik@wirus.cmiwpan.lodz.pl, zlesnik@hotmail.com

INTRODUCTION

Methylphosphonate (PMe) oligonucleotides are among the first analogs of oligonucleotides considered as antisense agents[1–3]. 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 non-bridging P-C bond, in addition to the methylphosphonate modification, includes among others, methylphosphonothioates[4], hydroxymethylphosphonates[5,6], benzylphosphonates[7,8], (4,4′-dimethoxytrityl)phosphonate[9], carboranylmethylphosphonates[10], octylphosphonates[11], phenylphosphonates[12], and phenylphosphonothioates[13].

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[14,15] and further emphasized by Lesnikowski and Stec[16]. It is likely that only some of the diastereomers will have desirable physicochemical characteristics facilitating an efficient cellular uptake[17] 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.[18]

Most of the stereocontrolled methods for the formation of the PMe internucleotide linkages are based upon the transesterification of P-stereodefined diastereomeric nucleotide monomers[19–22]. 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)methanephosphonate **(3)**[19]. 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[23], phosphorothioate oligodeoxynucleotides[24] and phosphorothioate oligoribonucleotides[25].

The assignment of the absolute configuration at phosphorus in PMe-oligonucleotides 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[29–31], mobility shift in RP-HPLC[9,32], and chemical correlation[19–21,32,33] 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[19–21,34,35]. 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[20,23].

The absolute configuration in diastereomeric **3** was assigned so far by means of the chemical correlation method[19]. 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 ^{31}P-NMR[36,37]. 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′-*O*-deoxynucleoside 3′-*O*-(*O*-4-nitrophenyl)methylphosphonates **(3)** (B = Thy, Cyt^{Bz}, Ade^{Bz}, Gua^{iBu}) the method previously developed for the preparation

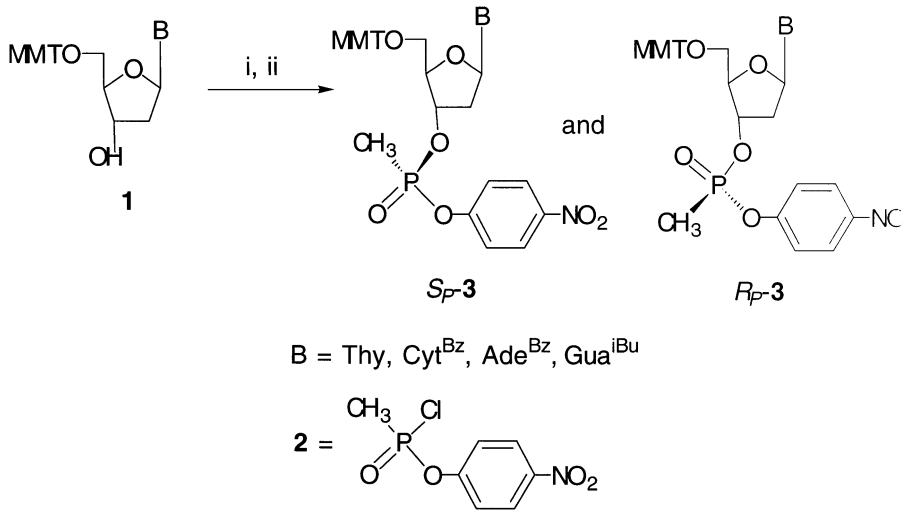

Figure 1. Synthesis of 5′-*O*-monomethoxytrityl-2′-*O*-deoxynucleoside 3′-*O*-(*O*-4-nitrophenyl)-methanephosphonates **(3)**, monomers for the preparation of *P*-stereodefined methanephosphonate oligonucleotide analogues. (i) **2**, 1,2,4-triazole, Et_3N/CH_3CN. (ii) Seperation of S_P-3 and R_P-3 diastereomers of 3.

of base unprotected **3** was employed[19,38]. Thus, suitable 5′-*O*-monomethoxytrityl-N-protected 2′-*O*-deoxynucleosides were treated with prepared in situ [*O*-(4-nitrophenyl)methylphosphonotriazolidate in CH_3CN 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[31], P- and ^{1}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_3 group derived from ROESY experiments[31], taking advantage of the fact that different configurations at phosphorus lead to a different environment of the PCH_3 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_P configuration, though the crosspeak to H4′ was characterized by a much lower intensity. The ROE signals were

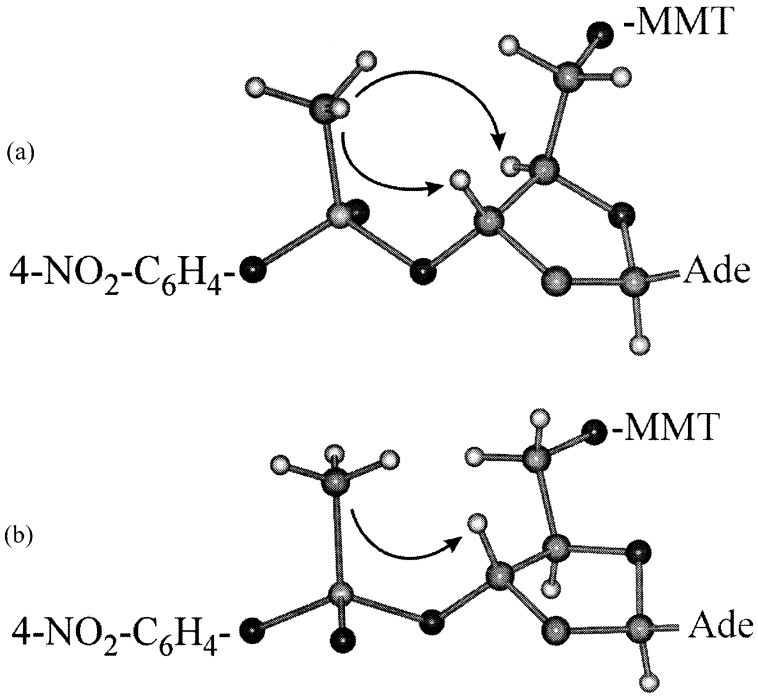

Figure 2. Schematic view of R_P and S_P, 5′-*O*-monomethoxytrityl-2′-*O*-deoxyadenosine 3′-*O*-(*O*-4-nitrophenyl)methanephosphonate **(3)**. (a) R_P5′-*O*-Monomethoxytrityl-2′-deoxyadeno sine3′-*O*-(*O*-4nitrophenyl)methanephosphonate (3, B = Ade^{Bz}). (b) S_P5′-*O*-Monomethoxy-trityl-2′-deoxyadenosine3′-*O*-(*O*-4nitrophenyl)methanephosphonate (3, B = Ade^{Bz}).

integrated and the distances between the PCH_3 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_3/H3′ and PCH_3/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_3 group to H3′ is shorter by more than 50 pm[31].

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_3 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 *Sp* 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*-deoxynucleoside 3′-*O*-(*O*-4-Nitrophenyl)methanephosphonate **(3)** (B = Thy, Cyt^{Bz}, Ade^{Bz}, Gua^{iBu})

Isomer	S_P-dT	R_P-dT	S_p-dC	R_P-dC	S_P-dA	R_P-dA	S_P-dG	R_P-dG
UV λ_{max}[1]	262.4	265.1	261.1	261.8	278.9	279.1	261.1	261.1
UV λ_{min}[1]	245.3	245.2	243.6	245.3	251.1	251.5	241.7	242.4
TLC $[R_f]$[2]	0.66	0.56	0.68	0.55	0.63	0.52	0.44	0.29

[1] In 95% C_2H_5OH. [2] Developing solvent system $CHCl_3$:CH_3OH = 95:5.

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

	R_p-T	S_p-T	R_p-dC	S_p-dC	R_p-dA	S_p-dA	R_p-dG	S_p-dG
^{31}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/iBu			11.27	11.28	11.17	11.20	11.61	11.60
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_3	1.49	1.46						
PCH_3	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′/H5′	3.17/3.	3.25	3.31	3.33	3.20	3.29	3.19	3.23
4-NO_2-Ar	$7.35_{2,6}$	$7.43_{2,6}$	$7.38_{2,6}$	$7.47_{2,6}$	$7.44_{2,6}$	$7.47_{2,6}$	$7.39_{2,6}$	$7.43_{2,6}$
	$8.18_{3,5}$	$8.24_{3,5}$	$8.19_{3,5}$	$8.25_{3,5}$	$8.21_{3,5}$	$8.25_{3,5}$	$8.19_{3,5}$	$8.24_{3,5}$
4-MeO-Ar	$7.18_{2,6}$	$7.21_{2,6}$	$7.20_{2,6}$	$7.22_{2,6}$	$7.14_{2,6}$	$7.16_{2,6}$	$7.13_{2,6}$	$7.15_{2,6}$
	$6.84_{3,5}$	$6.87_{3,5}$	$6.84_{3,5}$	$6.88_{3,5}$	$6.78_{3,5}$	$6.81_{3,5}$	$6.77_{3,5}$	$6.79_{3,5}$
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)			$8.00_{2,6}$	$7.99_{2,6}$	$8.04_{2,6}$	$8.04_{2,6}$		
			$7.50_{3,5}$	$7.50_{3,5}$	$7.54_{3,5}$	$7.54_{3,5}$		
			7.62_4	7.62_4	7.64_4	7.64_4		
i-Pr							1.11 (6H); 2.74 (1H)	1.12 (6H); 2.74 (1H)

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

J [Hz]	R_p-T	S_p-T	R_p-dC	S_p-dC	R_p-dA	S_p-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_3	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

* Overlaps with OH.

means that the PCH_3 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_3 and H3′/H4′ crosspeaks in the ROESY experiments as for dinucleotides.

The crosspeaks of the PCH_3 group were integrated and distances to H3′ and H4′ were estimated (Table 4) using the internal distance *H*2′/*H*2″ (180 pm) for the distance calibration[39]. For the thymidine derivative vicinal 2′/2″ protons are overlapping and distance estimation was impossible.

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

	dGiBu		dABz		dCBz	
	R_p	S_p	R_P	S_P	R_P	S_P
PCH_3/H3′	273	273	246	235	218	278
PCH_3H4′	281	327	302	–	273	368

The estimated distances were used to build (HYPERCHEM 4.5) models illustrating the position of the P-CH_3 group in *Sp* and *Rp* isomers (Fig. 2). As an example, diagnostic parts of the S_P-dA **3** and R_P-dA **3** spectra are shown in Fig. 3 and 4.

The absolute configuration at phosphorus in **3** established by ROESY experiments correlates well with ^{31}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 ^{31}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[40,41]. Each conformer is described by three parameters: the puckering amplitude (Φ_m), the phase angle of pseudorotation (P) and its population. We analyzed ^{31}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

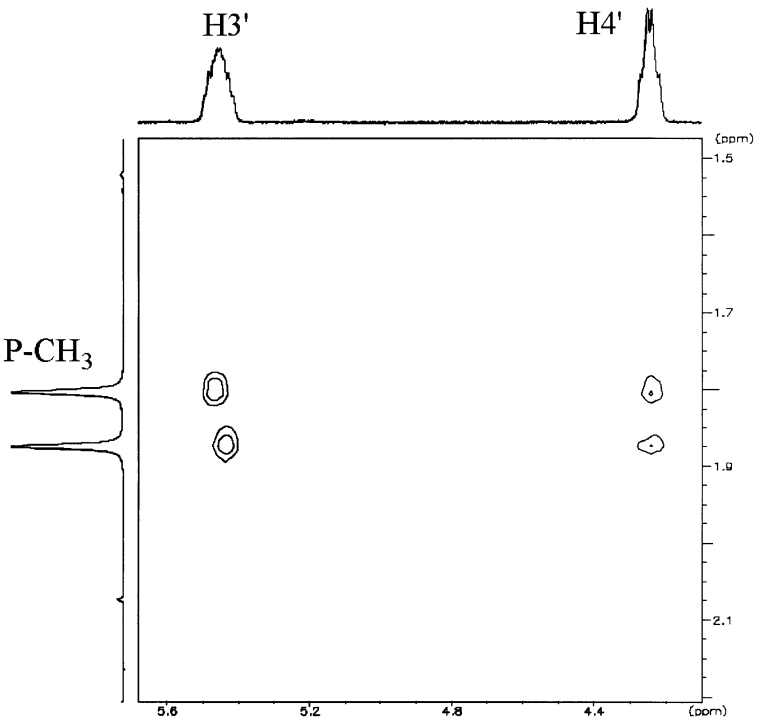

Figure 3. The diagnostic part of R_P-dA **3** ROESY spectrum.

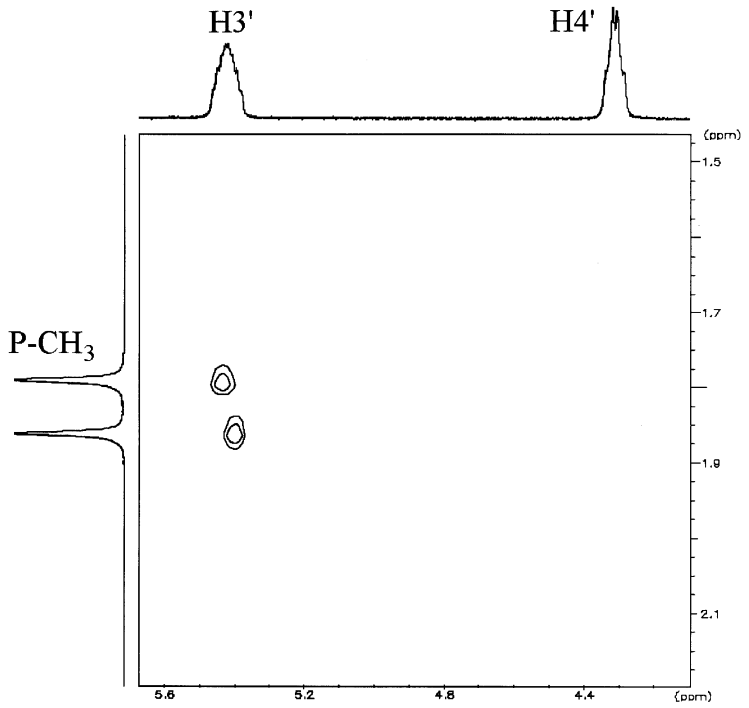

Figure 4. The diagnostic part of S_P-dA **3** ROESY spectrum.

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

Table 5. Pseudorotational Parameters P and Φ (°), Percentage Populations North and South and Rms Errors (Hz)

	Deoxyribonucloside Methanephosphonate							
Parameter	S_p-dG	R_p-dG	S_p-dA	R_p-dA	S_p-dC	R_p-dC	S_p-T	R_p-T
^{S}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
^{N}P	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

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 probe-head. Tetramethylsilane and 85% H_3PO_4 were used as external standards for ^{1}H-NMR and ^{31}P-NMR, respectively.

Methods

5′-O-Monomethoxytrityl-2′-O-deoxynucleoside(B = Thy, Cyt^{Bz}, Ade^{Bz}, Gua^{iBu}) (1) were synthesized as described [42].

R_P and S_P 5′-O-Monomethoxytrityl-2′-O-deoxynucleoside 3′-O-(O-4-nitrophenyl)methyl-phosphonate (3) (B = Thy, Cyt^{Bz}, Ade^{Bz}, Gua^{iBu}). [*O*-(4-nitrophenyl)]methyl-phosphonochloridate (**2**, 3.3 g, 14.0 mmol)[38], 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_3CN (50 mL) under nitrogen. After 15 min at room temperature, to the resultant clear solution of [*O*-(4-nitrophenyl)]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 ($CHCl_3$:CH_3OH = 95:5). After completion of the reaction (ca. 3 h), CH_3CN was evaporated under reduced pressure, then the oily residue was dissolved in $CHCl_3$ (100 mL). The $CHCl_3$ solution was washed with H_2O (3 × 50 mL). The organic fraction was separated, dried over $MgSO_4$, and then $CHCl_3$ 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 $CHCl_3$ as eluting solvent system, or alternatively on normal phase silica using a gradient of CH_3OH in $CHCl_3$. 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, ^{31}P- and ^{1}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_6 (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 λ electro-negativites: λ (C1′) = $\lambda(CH_2OR) = 0.68$, λ (C2′) = 0.72, λ (C3′) = λ(C4′) =0.62, λ = (O4′) =1.40, λ(-O-P(O)(CH_3)O-) = 1.29 as for $OPO(OEt)_2$ group[40,41].

ACKNOWLEDGMENTS

Supported in part by the Polish Foundation for the Promotion of Science (FNP), Project Phare SCI-TECH II, grant number PL9611/98/03-02.9, Polish Committee for Scientific Research (KBN) grant number 6 PO5F 023 20p02 and Technical University of Lodz.

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Received August 14, 2001
Accepted December 7, 2001