Synthesis and NMR Structural Analysis of 2′-OMe-Uridin-3′-yl (3′,5′)-5′-*O*-(*N*-Isobutyryl-2′-OMe-Cytidine) Methylthiophosphonates

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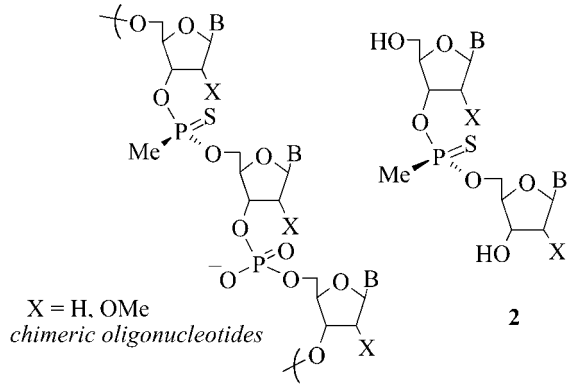
Detailed analysis of the molecular structures of both the **Fast**-eluted and the **Slow**-eluted (the notation **Slow** and **Fast** corresponds to the relative mobility properties of compounds in silica gel column chromatography under normal phase conditions, see Exp. Sect. for details) diastereomers of 5′-OH-2′-OMe-uridin-3′-yl (3′,5′)-5′-*O*-(*N*-isobutyryl-2′-OMe-cytidine) methylthiophosphonate (**1**) was performed with the aid of ^{1}H, ^{13}C, 1D and 2D homo- and heteronuclear PFG (Pulse Field Gradient) NMR studies. The absolute configurations [(R_P) and (S_P)] of the diastereomers of **1** were unambiguously assigned by ^{1}H ROESY experiments. The pseudorotation parameters of the ribose ring were calculated with the aid of the PSEUDOROT program, and compared with data obtained from X-ray structure analysis.

Introduction

Synthetically modified non-ionic oligonucleotides provide a convenient tool for study of the consequences of inhibited expression of target genes, including both target validation[1] and therapeutic applications,[2] or determination of protein function and interactions.[3,4] Recently, the properties of *chimeric constructs*[5,6] containing a mixed backbone structure, with P-modified dinucleoside (3′,5′) methylphosphonates d(N_{PMe}N) incorporated into different positions of the oligonucleotide chain, have been reported.[7−9] It has been established that oligonucleotides containing (R_P)-methylphosphonate bonds form heteroduplexes with complementary DNA or RNA of higher thermodynamic stability than those formed between the isosequential oligonucleotides with incorporated methylphosphonate linkages with (S_P) configurations at the internucleotide phosphorus centres.[5,10] Recent NMR studies revealed that PMe-modified oligonucleotide strands exhibit greater mobility relative to the DNA strand.[7]

The search for efficient methods for the synthesis of the tailored chimeric oligonucleotides has focused our attention on the potential of dinucleoside (3′,5′) methylthiophosphonates **2**, which could be regarded as dimeric building blocks for the preparation of chimeric oligonucleotides. As they are isosteric with dinucleoside methylphosphonates, these analogues might be expected to possess P-stereodependent binding properties towards complementary DNA or RNA targets when incorporated into chimeric oligomers. Further improvement in the binding properties was expected after introduction of 2′-OMe derivatives in place of the 2′-deoxynucleosides.[11] Moreover, the above modifications could be particularly interesting in the light of the anticipated changes in the conformational properties of 2′-OMe oligonucleotides existing in RNA-like conformations.[12] Although a few reports on the synthesis of di(2′-deoxynucleoside methylthiophosphonates) had been published previously,[13,14] their properties had never been studied in detail.



In this paper we report on a new and efficient synthesis and structural analysis of (R_P)- and (S_P)-5′-OH-2′-OMe-uridin-3′-yl (3′,5′)-5′-*O*-(*N*-isobutyryl-2′-OMe-cytidine) methylthiophosphonates (**1**) and demonstrate the unam-

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biguous assignment of the absolute configuration at phosphorus by NMR techniques.

Results and Discussion

The potential use of chimeric oligonucleotides with *P*-chiral centres[3,4,6] creates a demand not only for efficient syntheses of their dimeric building blocks but also for the evaluation of straightforward spectral methods of analysis, applicable for fast and unequivocal assignment of absolute configuration at the phosphorus centres in partially protected, diastereomerically pure, dimeric building blocks. The intention of the reported studies was to compare results of structural analysis for the diastereomeric dinucleoside (3′,5′) methylthiophosphonates **1**, both in solution and in solid state, and to assign the absolute configurations for these isomers. Previously, comprehensive studies by Engels et al. had allowed the determination of the absolute configuration of d(N_{PMe}N) by NMR, UV and CD, and application of multivariate statistics.[15,16]

We reported recently that diastereomeric dinucleotides **2** could be obtained through *one-pot* condensation reactions between 5′-*O*-DMT-2′-OMe (*N*-protected) ribonucleosides **3** and 3′-*O*-*tert*-butyldimethylsilyl-*N*-protected-2′-OMe ribonucleosides **4** with the aid of methylphosphono-bis(1,2,4-triazolidite) (**5**) generated in situ, followed by treatment with elemental sulfur (Scheme 1).[17]

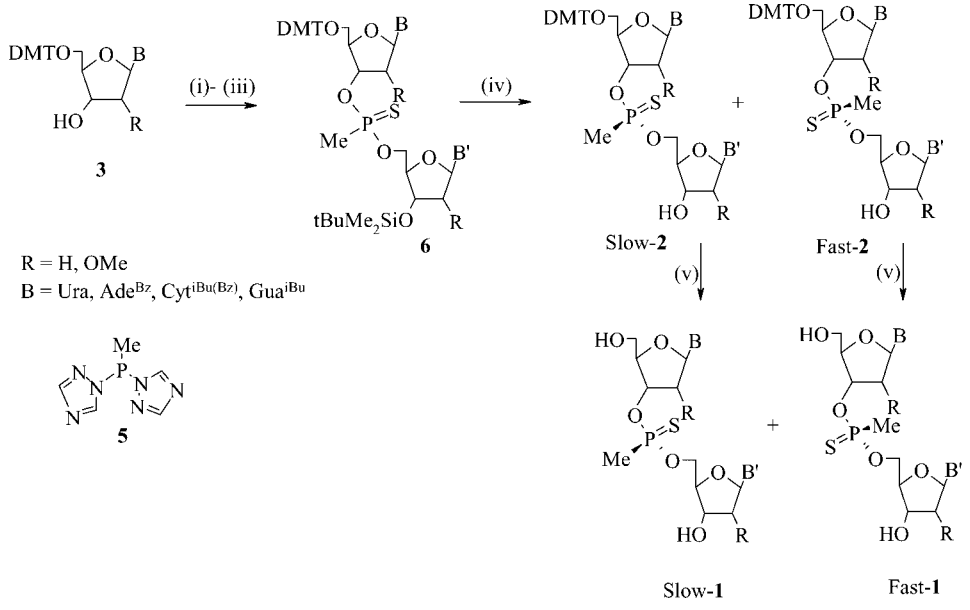
After the condensation, without isolation of the fully protected dinucleotides **6**, obtained as mixtures of diastereomers, the *tert*-butyldimethylsilyl protecting group was removed by treatment with $Et_3N{\cdot}3HF$ in 1:1 ratio. As calculated from the ^{31}P NMR spectrum of a crude reaction mixture, the diastereomeric ratios of dinucleotides **6** were almost 1:1 for 2′-deoxy derivatives and about 1:2 (Fast/Slow) for 2′-OMe derivatives. Total yields of both diastereomers **2** varied (^{31}P NMR) from 86% for TC to about 65% for AT. It was found that the partially protected dinucleoside (3′,5′) methylphosphonothiates **2** could be chromatographically separated (silica gel column) into their isomers more efficiently than the fully protected compounds **6**.

The assignment of the absolute configurations of partially protected dinucleotides **2** by fast and unambiguous NMR analysis is of significant value if they are to be used as *dimeric building blocks* and incorporated into *chimeric oligonucleotides*. We had previously demonstrated the diagnostic value of ROESY spectra for conformational analysis of diastereomeric 5′-*O*-DMT-nucleoside 3′-*O*-(*S*-methyl methylphosphonothiolates) and the corresponding *Se*-methyl methylselenophosphonates,[18] monomers for stereospecific synthesis of dinucleoside (3′,5′) methylphosphonates.[19,20]

Preliminary studies revealed that the removal of the 5′-protected group could simplify interpretation of NMR spectra. Therefore, each isomer (**Fast-2** and **Slow-2** eluting from silica gel plates) was separately treated with 2% dichloroacetic acid, affording partially protected dinucleotide **1**, which was purified by flash column chromatography and subjected to NMR analysis.

^1H and ^{13}C NMR Studies in Solution

In this project, we were attracted by the potential to correlate the conformations of the furanose rings with the absolute configurations of the partially protected dinucleoside (3′,5′) methylthiophosphonates (S_P)-**1** and (R_P)-**1** by NMR spectroscopy. In addition to standard one-dimensional ^1H and ^{13}C NMR spectra, we also carried out several



Scheme 1. Reagents and reaction conditions: (i) dropwise addition of **3** to **5**, ice bath, 45 min; (ii) 3′-protected nucleoside **4** in THF, room temp, 1.5 h; (iii) elemental sulfur, room temp., overnight, followed by aqueous workup, precipitation; (iv) $Et_3N{\cdot}3HF/Et_3N$ in THF, followed by silica gel column chromatography; (v) 2% dichloroacetic acid, CH_2Cl_2, 10 min, room temp.

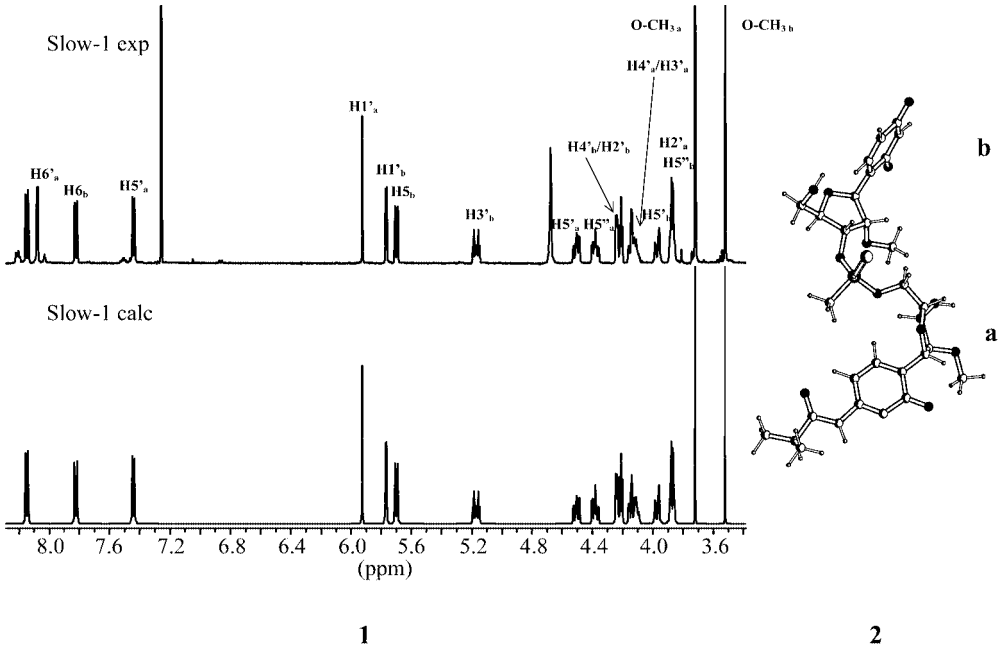


Figure 1. (1) Spectrum of **Slow-1** recorded in $CDCl_3$ and the theoretical spectrum modelled with the WINDAISY program;[21] (2) the 3D structure of diastereomer **Slow-1** obtained by the PM3 semiempirical method[22]

two-dimensional experiments in order to achieve complete assignment of proton and carbon chemical shifts and to elucidate unequivocally the structures of **Fast-1** and **Slow-1**. In some experiments, we took advantage of the Pulse Field Gradient (PFG) system to reduce the time of measurement and to improve the quality of spectra (e.g., reduction of T_1 noise). The accuracy of δ_{iso} and J assignments was confirmed by comparison of experimentally measured and theoretically predicted spectra obtained by use of the WINDAISY program (Figure 1, a).[21]

The ^1H and ^{13}C chemical shifts and the proton-proton J coupling constants established for **Fast-1** and **Slow-1** by ^1H-^1H PFG COSY, ^1H-^{13}C PFG HMQC and ^1H-^{13}C PFG HMBC experiments are collected in Table 1−4.

Table 1. ^1H NMR chemical shifts (ppm) for diastereomers of (S_P)-**1** and (R_P)-**1** at 25 °C

	Fast-1		**Slow-1**	
	b[a]	**a**	**b**	**a**
H1′	5.69	5.92	5.77	5.92
H2′	4.34	3.88	4.21	3.87
H3′	5.27	4.11	5.17	4.11
H4′	4.24	4.14	4.24	4.14
H5′	3.94	4.40	3.97	4.50
H5′′	3.81	4.37	3.87	4.38
H5	5.74	7.47	5.70	7.44
H6	7.74	8.21	7.82	8.15
O−CH_3	3.54	3.75	3.52	3.72
P−CH_3	1.98		1.97	

[a] The term **b** corresponds to a 2'-OMe-uridine moiety and **a** corresponds to a 2′-OMe-cytidine moiety.

Table 2. $J_{H,H}$ and $J_{P,H}$ coupling constants (Hz) for **Fast-1** and **Slow-1** at 25 °C

	Fast-1		**Slow-1**	
	b	**a**	**b**	**a**
H1′-*H2′*	5.2	0.8	4.2	0.8
H2′-*H3′*	5.0	5.0	4.9	4.8
H3′-*H4′*	4.2	9.2	4.8	8.1
H4′-*H5′*	2.0	2.1	2.0	1.8
H4′-*H5′′*	1.3	3.0	2.0	3.3
H5′-*H5′′*	12.4	11.0	12.4	11.7
H5−H6	7.8	7.8	8.1	7.5
P−CH_3	15.9		15.5	
P−$H_{3'}$	11.9		13.5	
P−H5′,H5′′		7.1		8.0

Table 3. $J_{C,P}$ coupling constants (Hz) and torsion angles from PM3 calculations for **Fast-1** and **Slow-1** at 25 °C

	Fast-1	**Slow-1**	P−O−C−Cx′$_b$ (S_P)	P−O−C−Cx′$_b$ (R_P)
P−C2′b	3.6	2.4	119.0	119.7
P−C3′b	6.1	7.3		
P−C4′b	6.1	4.8	−124.3	123.4
P−C5′a	6.1	7.3		
P−C4a	8.5	9.7	155.0	124.9
P−CH_3	113.8	116.3		

The conformations of different pentose rings in nucleosides and nucleotides have been extensively discussed by Leeuw and Altona.[23] The relationship between vicinal coupling constants and pseudorotation parameters (phase angle P and puckering amplitude Φ_m) is also known.[24]

Table 4. ^{13}C NMR chemical shifts (ppm) for diastereomers of **Fast-1** and **Slow-1** at 25 °C

	Fast-1		**Slow-1**	
	b	**a**	**b**	**a**
C1′	90.5	88.9	89.3	89.4
C2′	80.5	82.6	81.5	83.0
C3′	73.5	67.4	72.6	67.9
C4′	83.7	81.6	83.5	81.9
C5′	60.6	63.0	60.5	64.4
C5	102.3	95.7	102.7	96.3
C6	141.8	143.8	141.1	144.0
$O-CH_3$	58.7	58.6	58.7	59
$P-CH_3$	21.4		21.7	

From the previously reported[17] X-ray data for SLOW (R_P)-**1**, reported previously,[17] it could be concluded that both ribose rings were in the *N*-type conformation in the solid state. The *P* parameters for the **b** (uracyl) and **a** (cytidine) rings had been determined to be 8.0° and 13.4°, respectively. In solution, accurate pseudorotational parameters fitting best with the experimentally obtained data can be calculated by use of the PSEUROT 6.2 computer program;[23] in the current studies the vicinal coupling constants taken from the WINDAISY simulation were used as input data. A procedure based on analysis of *J* couplings at four temperatures (25°, 35°, 45° and 55 °C) was adopted for these calculations. The puckering amplitudes for *N*- and *S*-type conformers were held constant, and then raised in 1° steps between 30° and 42°, while the phase angles and the percentage populations were allowed to vary freely, until the optimized value had been found. Five parameters – P_N, Φ_N, P_S, Φ_S and f_S (the sum of f_S and f_N is equal to 1) – fully characterize the conformations of pentose rings. The calculated pseudorotation parameters are collected in Table 5.

Table 5. Comparison of the calculated pseudorotation parameters for **Fast-1** and **Slow-1** and data obtained from X-ray analysis of **Slow-1**

	b							**a**						
	%N	RMS	N		S			%N	RMS	N		S		
			P	Φ	P	Φ				P	Φ	P	Φ	
Fast-1	45	0.06	14	31	160	36	100	0.27	23	36	169	25		
Slow-1	53	0.05	9	34	163	34	99	0.01	14	32	149	34		
Slow-1 (X-ray)	N		8.02	–	–	–	N			13.50	–	–	–	

From the above data it is apparent that for **Slow-1** there is a very good consistency between X-ray and NMR results. Comparison of NMR spectroscopic data for **Slow**-(R_P)-**1** and **Fast**-(S_P)-**1** diastereomers discloses small differences in phase parameters, however, the existence of *N* conformers has been confirmed in both cases.

The important structural information can be also acquired by analysis of NOE effects, which allows direct proton-proton connectivities resulting from dipolar interactions to be established within distances up to 5 Å. For medium-sized molecules with molecular weights of ca 1 kD, however, NOE measurements are often hampered by the vanishing cross-relaxation rates. Since the measurement of transverse cross-relaxation (ROE) does not suffer from this problem, the chemical shifts of the protons and their spatial connectivity can be assigned unambiguously by employing the ROESY technique. Moreover, as reported by Löschner and Engels,[15] in the case of d($N_{PMe}N$), the absolute configuration at the phosphonyl residue could be determined on the basis of NOE distance constraints.

The ROESY experiments were performed both for the diastereomer **Fast-1** and for **Slow-1**, with the methyl group directly bonded to phosphorus regarded as indicative of their chirality. Although the potential of this method has been reported previously,[25] the assignment of stereochemistry in dinucleoside (3′,5′) methylthiophosphonates **1** has been equivocal because the cross-peaks from the methyl group are very weak (at the same level as noise). However, we found that the quality of the spectra could be greatly improved if the phosphorus decoupling was turned on during acquisition. Figure 2 presents ^{31}P-decoupled ROESY spectra of **1** (spectra without phosphorus decoupling are attached in the Supporting Information; for Supporting Information see also the footnote on the first page of this article).

The presence of cross-peaks between the methyl group and H5′′a, H3′a, and OCH_3 in **Fast-1** (Figure 2, b), and their absence in **Slow-1** (Figure 2, a) provide an unambiguous distinction between isomers. The appropriate distances between protons in the structure obtained by molecular modelling are displayed (right) and unambiguously confirm the (S_P) configuration at the phosphorus centre in **Fast-1**. In the light of this result, the lack of cross-peaks from the methyl group in the diastereomer with (R_P) configuration can readily be interpreted (Figure 3, b).

It is interesting to note that in the case of thio derivatives, due to strong magnetic shielding of the thiophosphonyl residue, the chemical shift of CH_2 protons at 5′ position is a sensitive indicator of stereochemistry on phosphorus.

Figure 3 illustrates the expanded region of the H5′/5′′ protons for both diastereomers. The distinction between protons for (R_P)-**1** is ca 0.15 ppm, while for (S_P)-**1** these signals are overlapped. The local magnetic shielding effect of the thiophosphonyl group on H5′/H5′′ protons is illustrated in the top inset.

^{31}P CP/MAS NMR Studies in the Solid State

Solid-state (SS) NMR spectroscopy is a technique that provides a link between NMR spectroscopic data in the solution or liquid phases and results obtained from single-crystal X-ray or neutron diffraction studies. Comparison of the isotropic chemical shifts and further structural parameters that characterize the geometries of molecules in crystal lattices allows conclusions regarding changes in conformation, as well as the nature of the intermolecular contacts in both phases. Since a powder specimen (i.e., not only one selected single crystal) is used for SS NMR, this method

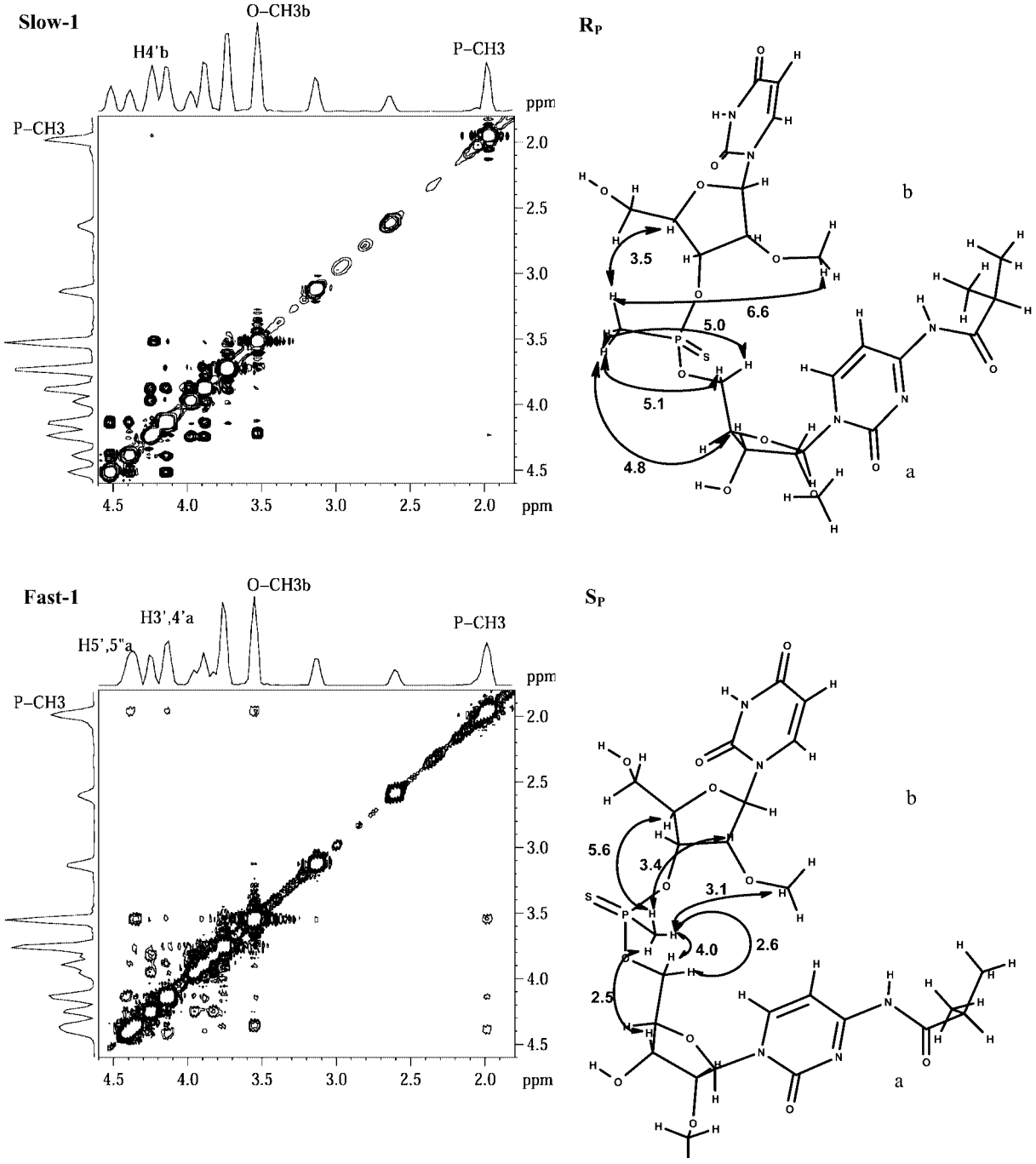


Figure 2. ^{31}P NMR decoupled ROESY spectra of **Slow**-(R_P)-**1** (a) and **Fast**-(S_P)-**1** (b) registered at 25 °C

can provide immediate identification of polymorphs, solvates, inclusion complexes etc. that may exist in the crystalline state.

The room-temperature ^{31}P CP/MAS spectrum of the diastereomer (R_P)-**1**, crystallized from ethanol, is displayed in Figure 4. As can be seen, a single resonance line in the isotropic part of the spectrum represents the compound under investigation. This observation is consistent with X-ray analysis assigning one molecule of (R_P)-**1** in the asymmetric unit.[17]

The principal components of the ^{31}P chemical shift, tensors δ_{ii}, were established from the spinning sideband intensities by use of the WINMAS program, on the basis of the Berger–Herzfeld algorithm.[26,27] The calculated values of principal tensors elements δ_{ii} and the shielding parameters are given in Table 6, with the accuracy of calculations confirmed by comparison of experimental and theoretical spectra (Figure 4).

In contrast, attempts to crystallize the diastereomer (S_P)-**1** were not successful, and provided only amorphous pow-

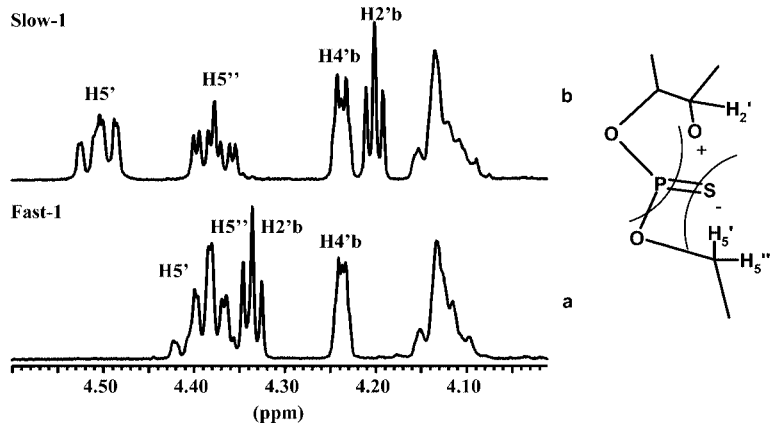


Figure 3. Expanded region of the H5′ protons for (R_P)-**1** (**Slow**) and (S_P)-1 (**Fast**)

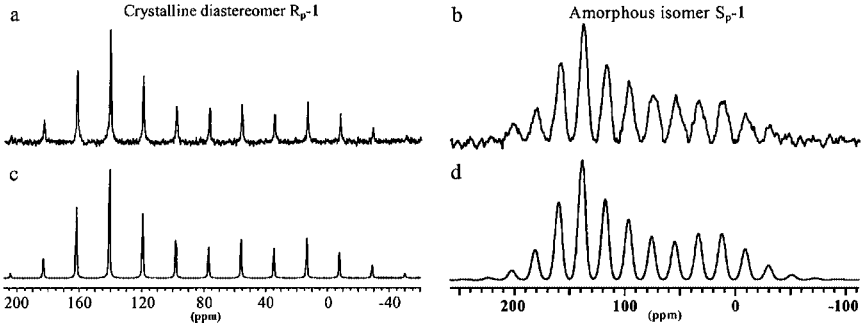


Figure 4. Room-temperature ^{31}P CP/MAS spectra of (R_P)-**1**, crystallized from ethanol (a), and amorphous (S_P)-**1** (b), together with the corresponding WINMAS program simulations (c and d)

Table 6. ^{31}P chemical shift NMR parameters for diastereomeric **1** and (for comparison) (deoxyxylo)thymidyl-3′-*O*-acetylthymidyl (3′,5′)-*O*-(2-cyanoethyl) thiophosphate (**7**)

Compound	δ_{11} [a]	δ_{22}	δ_{33}	Ω	κ	δ_{iso}
1 (crystalline)	185	150	−40	220	–	98.3
1 (amorphous)	189	139	−38	–	–	96.7
7 (crystalline)	152	131	−81	233	0.81	67.8
(S_P)-**7** (amorphous)	151	128	−91	242	0.81	62.6
(R_P)-**7** (amorphous)	154	130	−92	246	0.85	63.6

[a] Estimated errors in δ_{11}, δ_{22}, δ_{33} and $\Delta\delta$ are −/+5 ppm; errors in δiso are −/+0.2 ppm; the principal components of the chemical shift tensor are defined as follows: $\delta_{11} > \delta_{22} > \delta_{33}$; the isotropic chemical shift is given by $\delta iso = (\delta_{11} + \delta_{22} + \delta_{33})/3$.

der. The δ_{ii} elements were therefore established only for the amorphous form of (S_P)-**1**. The experimentally obtained and theoretically predicted spectra are given in Figure 4, and the calculated parameters are collected in Table 6. It is interesting to note the difference between isotropic values of the crystalline and amorphous phases (≈ 1.6 ppm). Interestingly, small changes in the δ_{ii} parameters are observed for both modifications.

We had previously also noted a distinction between the ^{31}P δ_{ii} parameters for the (S_P) and (R_P) diastereomers of (deoxyxylo)thymidyl-3′-*O*-acetylthymidyl (3′,5′)-*O*-(2-cyanoethyl) thiophosphate (**7**) in the crystalline and the amorphous phases.[28] In the case of the dinucleotides **7**, however, we observed the most significant changes in the δ_{33} parameter. From this we concluded that this effect was related to the formation of weak P=S···H−C hydrogen bonds,[29] and the decrease in electronic shielding of the phosphorus as the thiophosphoryl group became more polarized during the bond formation. Unexpectedly, in the cases of samples (R_P)-**1** and (S_P)-**1** the biggest differences were seen for the δ_{22} element.

In order to answer the questions regarding the influence of inter- or intramolecular contacts on shielding parameters, knowledge about the orientations of principal elements of chemical shift tensors with respect to the molecular skeletons of **1** and **7** was required. Information about orientation of the principal axes can be gained either experimentally from NMR goniometric measurements of single crystals or from quantum chemical calculations. Here we employed the latter approach, using the DFT GIAO method included in the Gaussian 98 program package.[30] Rather than performing the calculations for the dinucleoside methylthiophosphonate **1**, we ran them for the simpler model, since we had recently demonstrated that such model calculations gave results only slightly poorer than calcu-

lations for the larger molecule of actual interest.[31] According to these calculations, the principal axis *3* (most shielded) would be expected to be very close to the P=S double bond, while principal axis *2* should bisect the plane O−P=S. The least shielded axis *1* should therefore be close to the P−CH_3 bond. The orientation of principal elements of chemical shift tensors with respect to the molecular skeletons for samples **1** and **7** is shown in pictorial form in Figure 5.

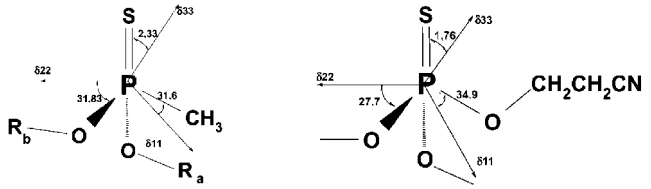


Figure 5. Principle elements of chemical shift tensors for compounds **1** and **7**

From these results and from theoretical calculations we can conclude that for methylthiophosphonates **1** the P=S···H contacts play rather small roles in crystallization process, and other effects therefore dominate molecular packing.

Conclusions

This paper reports the complete assignment of the structures of the diastereomers of 2′-OMe-uridin-3′-yl (3′,5′)-5′-*O*-(*N*-isobutyryl-2′-OMe-cytidine) methylthiophosphonate by NMR spectroscopy. Conformational features of 2′-OMe ribonucleotides are to a large extent determined by the presence of 2′-bulky substituents, and so molecules made up of 2′-OMe ribonucleotides would be expected to possess RNA-like conformations. These studies have confirmed that assumption, both in solution and in the solid state. We have demonstrated agreement between the conformation parameters of dinucleoside methylthiophosphonate **1** obtained from studies carried out in solution and in the solid phase. We have also demonstrated the feasibility of unequivocal assignment of the absolute configuration at phosphorus by means of ROESY experiments. Preliminary thermodynamic studies on chimeric oligonucleotides with **1** incorporated are described elsewhere, and exhibit profound differences in the thermodynamic properties depending on the absolute configurations at the methylthiophosphonate centres.[32]

Experimental Section

Reactions were carried out under positive pressures of dry argon. Solvents and reagents were purified by standard laboratory techniques and distilled directly into reaction vessels. Column chromatography and TLC analyses were performed on silica gel (Kieselgel 60, 240−400 mesh, E.Merck Inc.) and silica gel HP TLC precoated F_{254} plates (purchased from E.Merck Inc.), respectively. NMR spectra were recorded on a Bruker Advance DRX 500 spectrometer, operating at 500.13 MHz (^{1}H), and 202.46 MHz (^{31}P). Chemical shifts (δ) are reported relative to TMS (^{1}H) and 80% H_3PO_4 (^{31}P) as external standards. Positive chemical shift values were assigned for compounds resonating at lower fields than the standards. Mass spectra were recorded on a Finnigan Mat 95 (NBA, Cs^+ gun operating at 13 keV).

1. Synthesis of (R_P)- and (S_P)-5′-*O*-DMT-2′-OMe-uridin-3′-yl (3′,5′)-3′-*O*-(*N*-Isobutyryl-2′-OMe-cytidine) 3′-Methylthiophosphonates (2): A solution of 1,2,4-triazole (2.5 equiv.) and triethylamine (3 equiv.) in dry THF was cooled in an ice bath, and methyldichlorophosphane (1.1 equiv.) was added with vigorous stirring. After 20−25 min, a solution of 5′-*O*-DMT-2′-OMe-uridine (**3**, 1 equiv.) in THF was added dropwise at 0 °C. Stirring was continued for 45 min, followed by addition of 3′-*O*-*tert*-butyldimethylsilyl-*N*^{4}-isobutyryl-2′-OMe-cytidine (**4**) in THF. The reaction mixture was warmed to room temperature and stirred for an additional 1.5 h, and sulfur (twofold molar excess) was added in one portion. Stirring was continued overnight. The solvents were evaporated under reduced pressure (ca. 1/3 vol.), and an oily residue was dissolved in chloroform and washed twice with $NaHCO_3$ (0.1 M) and water. The organic layer containing **6** was dried with $MgSO_4$, concentrated to dryness and coevaporated twice with toluene. This crude reaction mixture was dissolved in a small volume of dry THF and treated with $Et_3N{\cdot}3HF/Et_3N$ (3:1 v/v). After deprotection was complete (3−4 h), the reaction mixture was diluted with chloroform, washed twice with $NaHCO_3$ (0.1 M), concentrated and subjected to silica gel column chromatography (gradient 0−6% EtOH in chloroform, with addition of 0.05% of Et_3N). The collected fractions of pure diastereomers **2** were concentrated, precipitated with hexane and stored as white powders. Total yield 61%.

Fast-(S_p)-2: (R_f = 0.5, $CHCl_3$/EtOH, 9:1), ^{31}P NMR (202.46 MHz, $CDCl_3$): δ = 100.1 ppm. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.22 [d, $^3J_{H,H}$ = 7.59, 6 H, CH(CH_3)$_2$], 1.94 (d, $^2J_{PH}$ = 15.61, 3 H, P−CH_3), 2.1 [dq, 1 H, CH(CH$_3$)$_2$], 2.56 (m, 1 H), 3.44 (dd, 1 H), 3.6 [s, 3 H, (C)CH_3O−C2′], 3.74 [s, 3 H, (Ura)CH_3O−C2′], 3.8 [s, 6 H, (Ura)CH_3O−DMT], 3.92 (m, 1 H), 4.18 (dd, 1 H), 4.26 [d, $^3J_{H,H}$ = 6.56, 1 H, (Ura)*H*2′], 5.36 [m, 1 H, (C)*H*2′], 5.87 [s, 1 H, (C)*H*1′], 6.05 [d, $^3J_{H,H}$ = 3.41, 1 H, (Ura) *H1′*] ppm. FAB$^-$MS [M − H]: 962.6 ($C_{46}H_{55}N_5O_{14}PS$: calcd. 963.99).

Slow-(R_p)-2: (R_f = 0.45, $CHCl_3$/EtOH, 9:1): ^{31}P NMR ($CDCl_3$): δ = 98.26 ppm. ^{1}H NMR ($CDCl_3$): δ = 1.22 [d, $^3J_{H,H}$ = 7.59, 6 H, CH(CH_3)$_2$], 1.76 (d, $^2J_{PH}$ = 15.32, 3 H, P−CH_3), 2.1 [dq, 1 H, CH(CH$_3$)$_2$], 2.56 (m, 1 H), 3.44 (dd, 1 H), 3.55 [s, 3 H, (C)CH_3O−C2′], 3.74 [s, 3 H, (Ura)CH_3O−C2′], 3.81 [s, 6 H, (Ura)CH_3O−DMT], 3.92 (m, 1 H), 4.18 (dd, 1 H), 4.26 [d, $^3J_{H,H}$ = 6.56, 1 H, (Ura)2′*H*], 5.36 [m, 1 H, (C)*H2′*], 5.95 [s, 1 H, (C)*H1′*], 6.02 [d, $^3J_{H,H}$ = 3.43, 1 H, (Ura)*H1′*], 7.42 [d, $^3J_{H,H}$ = 7.4, 1 H, (C)5 H], 7.92 [d, $^3J_{H,H}$ = 8.2, 1 H, (Ura)6 H], 8.16 [d, $^3J_{H,H}$ = 7.5, 1 H, (C)6 *H*], FAB-MS [M − H]: 962.4 (calcd. 963.99).

1.2 Removal of the 5′-*O*-DMT Protecting Group: Diastereomerically pure isomers **2** (20 mg) were separately dissolved in 2% dichloroacetic acid in CH_2Cl_2 (2 mL) and stirred for 10 min at room temperature. After extraction (twice with sat. $NaHCO_3$), each was purified by flash silica gel column chromatography (0−10% MeOH in $CHCl_3$). The obtained white foams were dissolved in chloroform and precipitated from hexane to give **1** as white powders.

Slow-1: (R_f = 0.3, $CHCl_3$/EtOH, 9:1), ^{31}P NMR ($CDCl_3$): δ = 98.44 ppm. $C_{25}H_{36}N_5O_{12}PS$, *FW* = 661.62, FAB^- [M − H]: 660.3, FAB^+ [M + H]: 662.2.

Fast-1: (R_f = 0.32, $CHCl_3$/EtOH, 9:1). ^{31}P NMR ($CDCl_3$): δ = 98.54 ppm. FAB^- [M − H]: 660.3.

NMR Measurements in Solution: Samples of diastereomerically pure **1** (5 mg) were dissolved in $CDCl_3$ (0.5 mL). All spectra were recorded with a Bruker Advance DRX 500 spectrometer, operating at 500.1300 MHz for ^1H, 125.2578 MHz for ^{13}C, and 202.46 MHz for ^{31}P. Original Bruker pulse programs were used for all experiments. The chemical shift of the DMSO signal was used as a reference (δ = 2.49 for ^1H and δ = 39.5 for ^{13}C). Phosphoric acid (85%) was used as an external standard for ^{31}P spectra. The spectrometer was fitted with a pulse field gradient unit (50 G/cm). The *inverse* broadband probe head was used. The COSY90 spectra were obtained from 1024 experiments with 4 scans of each. The relaxation delay was 1.5 s, the spectral width was 10 ppm (5000 Hz) in both dimensions, and the data size in F2 was 4 K. Digital quadrature detection (DQD) was applied. Two 10-μs *z*-gradient pulses, strength about 5 G/cm each, were applied with 1 ms delay for gradient recovery. The FID was apodized with a sine-bell function in both dimensions. Final data were zero-filled twice in both dimensions and symmetrized about the diagonal. The ROESY spectra were recorded in 2 K × 1 K (F2 × F1) data matrix. Digital quadrature detection was applied, and 32 scans were accumulated in each experiment. The experiment was run in phase-sensitive mode with 3650 ms cw pulse for ROESY spin lock. The spectral width was 4500 Hz (δ = 9 ppm) in both dimensions. Data were processed with sine-bell shape apodization function in both directions and TPPI in F1. No zero filling was applied.

The PFG-HMQC spectra were acquired in a 1 K × 4 K [F1 (^{13}C) × F2 (^1H)] data matrix. Three 1-ms *z*-gradient pulses, strengths about 25 G/cm, 15 G/cm and 20 G/cm, were applied in sequence with 1 ms delay for gradient recovery. The spectral width was 4000 Hz (δ = 8 ppm) in F2 (^1H) and 25 kHz (δ = 200 ppm) in F1 (^{13}C). The Garp decoupling sequence was employed. Final data were processed with sine function in F1 and qsine in F2 dimension.

The PFG-HMBC experiment was acquired in a 0.5 K × 4 K (F1 × F2) data matrix and with 8 scans for each experiment. Three 1-ms *z*-gradient pulses, strengths about 25 G/cm, 15 G/cm and 20 G/cm, were applied in sequence with 50 μs delay for gradient recovery. Final data were processed with sine-bell and qsine-bell functions in F1 and F2 dimensions, respectively.

NMR Measurements in the Solid State: Cross-polarization magic angle spinning solid-state ^{31}P NMR spectra were recorded with a Bruker DSX 300 spectrometer with high-power proton decoupling at 121.46 MHz for ^{31}P. The ^{31}P CP/MAS NMR spectra were recorded in the presence of high-power proton decoupling. Powder samples of (S_P)-**1** and (R_P)-**1** were placed in a cylindrical rotor and spun at 2.0−4.5 kHz. The field strength for ^1H decoupling was 1.05 mT, a contact time of 5 ms, a repetition of 6 s and spectral width of 50 kHz were used, and 8 K data points represented the FID. Spectra were accumulated 500 times, giving a reasonable signal-to-noise ratio. ^{31}P chemical shifts were calibrated indirectly with reference to bis[(dineopentoxy)thiophosphonoyl] disulfide set at δ = 84.0 ppm. The principal elements of the ^{31}P chemical shift tensor and shielding parameters were calculated by employing the WINMAS program. The details describing the method and accuracy of calculations have been exhaustively discussed.[22,33]

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