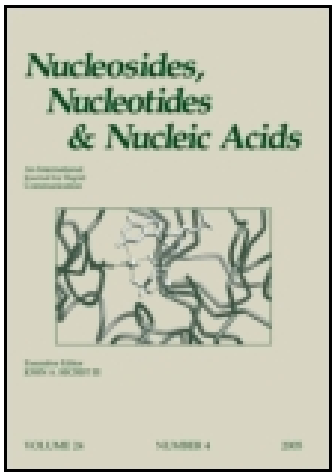

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Stereochemistry of Internucleotide Bond Formation by the H-Phosphonate Method. 1. Synthesis and ^{31}P Nmr Analysis of 16 Diribonulceoside (3′-5′)-H-Phosphonates and the Corresponding Phosphorothioates

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STEREOCHEMISTRY OF INTERNUCLEOTIDE BOND FORMATION BY THE *H*-PHOSPHONATE METHOD. 1. Synthesis and ^{31}P NMR Analysis of 16 Diribonulceoside (3′-5′)-*H*-Phosphonates and the Corresponding Phosphorothioates

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□ *Sixteen diribonucleoside (3′-5′)-H-phosphonates were synthesized via condensation of the protected ribonucleoside 3′-H-phosphonates with nucleosides, and the influence of a nucleoside sequence on the observed stereoselectivity was analyzed. ^{31}P NMR spectroscopy was used to evaluate a relationship between chemical shift and absolute configuration at the phosphorous center of the H-phosphonate diesters as well as of the corresponding phosphorothioate diesters. Although for the most cases such correlation was found, there was however several exceptions to the rule where the relative positions of resonances arising from R_P and S_P diastereomers were reversed.*

Keywords *H*-Phosphonates; Phosphorothioates; Nucleotides; Stereoselective coupling; ^{31}P NMR spectroscopy

INTRODUCTION

One of the fundamental questions of nucleoside and nucleotide chemistry is to find out how a nucleobase may influence a chemical or stereochemical outcome of the reactions. In this context, understanding factors governing stereoselectivity during condensations of suitably protected ribo-

In honor and celebration of the life and career of John A. Montgomery.

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nucleoside 3′-*H*-phosphonates with various hydroxylic components,[2–8] may have a pivotal role in controlling a ratio of the diastereomers of the *H*-phosphonate diesters formed.

In reactions of ribonucleoside *H*-phosphonates with nucleosides, only the structure of a nucleotidic component (an *H*-phosphonate monoester) was considered to be responsible for the observed stereoselectivity.[5,7,8] Although in the deoxy series the stereoselectivity is usually rather low (if any),[3,6] some reports indicated that in certain instances it was a nucleosidic component that directed the stereochemical course of the reaction.[3] All these data were, however, fragmentary and incomplete, making it difficult to draw more general conclusions concerning sources of stereoselectivity in *H*-phosphonate diester synthesis.

To fill up this gap, we carried out syntheses of 16 possible diribonucleoside (3′-5′)-*H*-phosphonates to assess the influence of both the nucleotidic and nucleosidic component on the stereoselectivity of the internucleoside *H*-phosphonate bond formation.

Having at hand a set of diastereomeric diribonucleoside (3′-5′)-*H*-phosphonates and a possibility to convert them stereospecifically into the corresponding *P*-chiral dinucleoside phosphorothioates,[5,9,10] another objective of this work was to evaluate a correlation between configuration at the phosphorous center and the ^{31}P NMR chemical shifts of the investigated compounds.

Absolute configuration of *P*-chiral dinucleoside phosphorothioates can be unambiguously determined by enzymatic methods, by taking advantage of the fact that snake venom phosphodiesterase (SVPD) digests preferentially the R_P diastereomers,[11,12] while nuclease P1, the S_P isomers.[13] This enzymatic method is reliable but rather time consuming. To facilitate the configurational assignment, Eckstein postulated that absolute configuration at the phosphorous center of di(deoxyribonucleoside) phosphorothioates could be correlated with ^{31}P NMR chemical shifts of these compounds.[14] According to this correlation rule (referred further to as the Eckstein's rule), the R_P diastereomers of deoxy-[14] and ribonucleoside[5] phosphorothioates resonate at lower field in the ^{31}P NMR spectroscopy, relatively to the corresponding S_P diastereomers. For other classes of nucleotide analogues, e.g., phosphoranilidate monoesters,[15] aryl phosphoranilidate diesters,[15] aryl phosphorothioate triesters,[15] tervalent phosphoramidites,[16] and oxathiaphospholanes,[17] an analogous correlation between configuration at the phosphorous center and the ^{31}P NMR chemical shifts, was observed.

There are, however, notable exceptions from the Eckstein's rule, for which the positions of ^{31}P NMR resonances of S_P and R_P diastereomers are reversed. For example, S_P diastereomers of protected ribo- and deoxyribonucleoside *H*-phosphonate diesters,[5,18] *H*-phosphonothioates,[18,19] and dinucleoside phosphorothioates[5] do not resonate upfield but downfield,

relatively to the corresponding R_P diastereomers. Nevertheless, in all instances, within a given class of nucleotide analogues, no variations in the order of chemical shifts between R_P and S_P diastereomers were observed. Thus, until recently, for closely related compounds, a correlation between ^{31}P NMR shifts and the sense of chirality seemed to be a reliable means for the assignment of absolute configuration at the phosphorous center for *P*-chiral nucleotide analogues. The present studies, however, have shown that one should exercise some caution, since even for structurally closely related compounds, this correlation rule may not hold.

RESULTS AND DISCUSSION

This paper addresses two important issues of nucleotide chemistry: (a) an influence of nucleotidic and nucleosidic components on stereoselectivity during formation of *P*-chiral diribonucleoside *H*-phosphonates and (b) a generality of a relationship between ^{31}P NMR chemical shifts and absolute configurations of the phosphorous center of ribonucleoside *H*-phosphonate and phosphorothioate diesters.

To get some insight into these problems, sixteen diribonucleoside (3′-5′)-*H*-phosphonates of type **4** were synthesized via condensation of 5′-*O*-dimethoxytrityl-2′-*O*-*t*-butyldimethylsilyl ribonucleoside *H*-phosphonates (**1**) with four 2′,3′-*O*-dibenzoyl ribonucleosides (**2**) in the presence of pivaloyl chloride as a condensing agent (Figure 1). For comparison, also ethanol was used as a hydroxylic component. The reactions were carried out either in neat pyridine or in methylene chloride (DCM) containing limited amount of pyridine (3 equiv., relatively to *H*-phosphonate) and progress of the condensations was monitored by ^{31}P NMR spectroscopy.

Degree of stereoselectivity in the investigated reactions was assessed by integration of the resonance signals of the corresponding R_P and S_P diastereomers formed. The obtained *H*-phosphonate diesters **4aa-de**[20] were then stereospecifically sulfurized with elemental sulfur[5,9,10] to the corresponding phosphorothioates **5aa-de** and the reaction mixtures were again analyzed by ^{31}P NMR. The stereochemical results of these experiments are presented in Table 1.

As it is apparent from the above data, stereoselectivity in the reactions investigated was controlled primarily by a nucleotidic component **1** as the stereochemical outcomes of the condensations with nucleosides and ethanol (or other aliphatic alcohols, e.g., isopropanol, *t*-butanol; results not shown), in most cases were similar. Some fluctuations in stereoselectivity related to the nature of a hydroxylic component occurred, however, these did not exceed ca. 10% (in absolute values). Usually, in couplings with pyrimidine nucleosides (e.g., **2b**, **2d**), the stereoselectivity was higher than for purine ones (e.g., **2a**, **2c**). The highest stereoselectivity was observed for

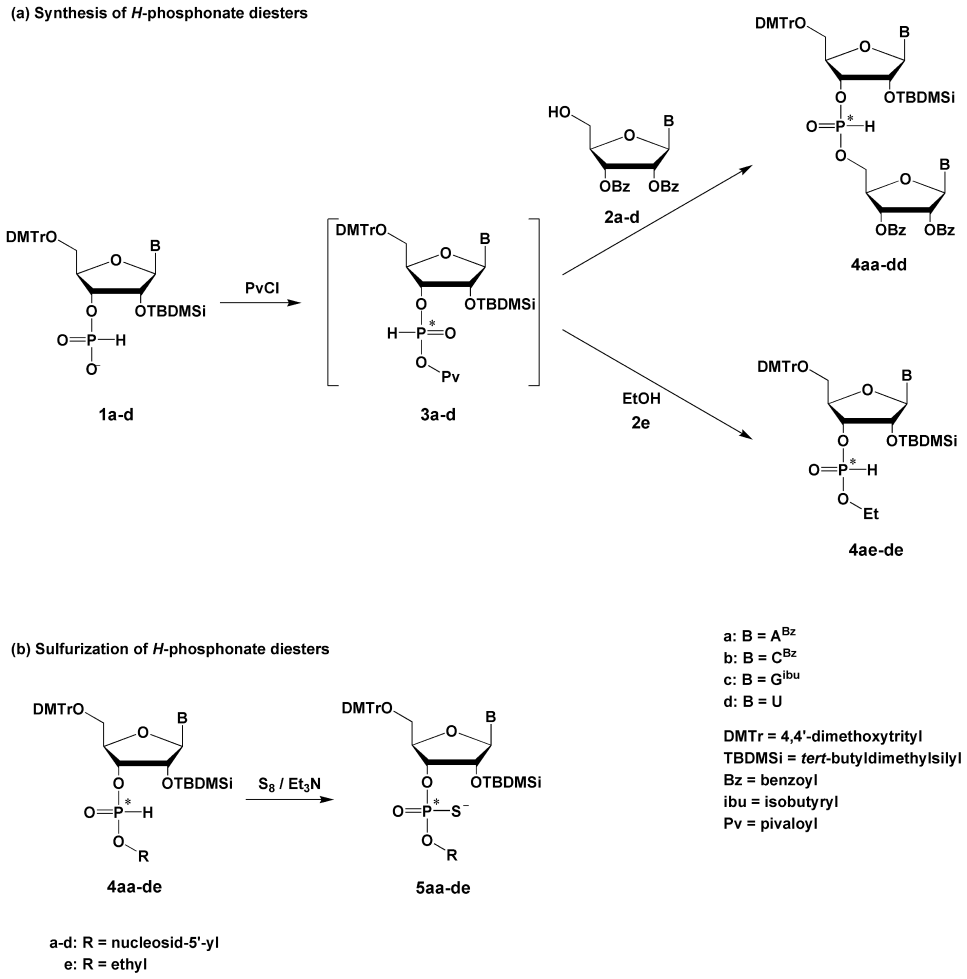

FIGURE 1 Substrates, intermediates, and products during synthesis of *H*-phosphonate diesters and the corresponding phosphorothioates.

the formation of $G_{PH}C$ diester (**4cb**), while the lowest one for dinucleoside *H*-phosphonate $C_{PH}G$ (**4bc**). The reason for these remains obscure and is a subject of further studies in our laboratories.

As to the role of the solvent composition, amounts of pyridine had rather moderate effect on the stereoselectivity of the condensations of nucleoside *H*-phosphonates **1a**, **1c**, and **1d**, and only for the reactions of cytidine *H*-phosphonate (C_{PH}, **1b**), a distinct increase in stereoselectivity with decreasing concentration of pyridine was observed.[8] In contrast to the previous report,[8] we did not observe an exceptionally high stereoselectivity for condensations involving guanosine *H*-phosphonate (G_{PH}, **1c**).

TABLE 1 (a) Stereoselectivity (% of a Low Field Signal in ^{31}P SMR Spectra) in Condensations of Ribonucleoside *H*-Phosphonates **1a-d** (c = 0.1 M) with Nucleosides **2a-d** or Ethanol (1.2 Equiv.) Using Pivaloyl Chloride (3 Equiv.) as a Condensing Agent in Neat Pyridine (Py) or in Methylene Chloride Containing 3 Equiv. of Pyridine (2.4% v/v) (DCM-Py). (b) The Same Reaction Mixtures after Sulfurization with S_8 in the Presence of TEA (% of a High Field Signal)

(a)

	Reactions in Py (%)					Reactions in DCM-Py (%)					
	2a (A)	**2b** (C)	**2c** (G)	**2d** (U)	EtOH		**2a** (A)	**2b** (C)	**2c** (G)	**2d** (U)	EtOH
1a (A_{PH})	78	82	70	81	79	**1a** (A_{PH})	79	86	73	85	82
1b (C_{PH})	59	*45*	52	59	*46*	**1b** (C_{PH})	71	*30*	61	75	71
1c (G_{PH})	77	82	80	*19*	*24*	**1c** (G_{PH})	76	90	79	*14*	*21*
1d (U_{PH})	76	78	71	81	71	**1d** (U_{PH})	80	86	74	86	80

(b)

	Reactions in Py (%)					Reactions in DCM-Py (%)					
	2a (A)	**2b** (C)	**2c** (G)	**2d** (U)	EtOH		**2a** (A)	**2b** (C)	**2c** (G)	**2d** (U)	EtOH
1a (A_{PH})	81	78	69	79	78	**1a** (A_{PH})	76	85	71	86	81
1b (C_{PH})	57	56	*48*	60	51	**1b** (C_{PH})	69	73	*37*	75	n.d.[a]
1c (G_{PH})	77	82	80	80	76	**1c** (G_{PH})	*24*	*10*	79	*15*	*22*
1d (U_{PH})	76	75	74	80	70	**1d** (U_{PH})	80	85	74	86	81

[a]Signals not resolved due to overlapping.

For most of the reactions investigated, the main diastereomer of *H*-phosphonate diesters **4** resonated at lower field, while that of the corresponding phosphorothioates at higher field. This was in agreement with the previously reported data.[5,8,18,19] However, in several cases (the values underlined in Table 1) the intensity of the low field signals of *H*-phosphonate diesters **4** were significantly smaller than those at high field, which might have indicated that the diastereomers with opposite configuration were formed as major products.[21] This phenomenon was observed in condensations involving both nucleosides and ethanol, and was also reflected in some reaction mixtures after sulfurization.

A change in stereoselectivity in these particular instances would be an interesting exception to the commonly observed preference for the formation S_P diribonucleoside (3′-5′)-*H*-phosphonate diesters in the condensation reactions. However, since the anomalies observed for *H*-phosphonate diesters **4** did not coincide with those for the corresponding phosphorothioates **5**, we assumed that the phenomenon was probably due to changes in relative positions of the ^{31}P NMR signals of the phosphorous diastereomers of compounds **4** and **5**. This seemed plausible, although it clashed with the aforementioned Eckstein's rule.

To substantiate this assumption, a more detailed ^{31}P NMR analysis was carried out for the four representative cases, for which anomalous patterns of ^{31}P NMR signals were observed, namely the condensations U_{PH} + U, C_{PH} + G, G_{PH} + U, and G_{PH} + EtOH. These reactions were carried out in acetonitrile (ACN) containing 3 equiv. of pyridine and after completion, ACN was evaporated and replaced, consecutively, with DCM, pyridine, and toluene. For the reaction mixtures in various solvents, the ^{31}P NMR spectra were recorded. The same protocol was used after sulfurization of the corresponding *H*-phosphonate diesters **4** (Table 2).

For some of the investigated diastereomers of **4** and **5**, the order of the ^{31}P NMR resonances was independent of the solvent used, showing either typical ($U_{PH}U$, $U_{PS}U$, $C_{PH}G$) or an anomalous ($G_{PH}U$, $G_{PH}Et$) pattern of signals. Thus, on this basis it was not possible to conclude what was responsible for the observed pattern of signals in the ^{31}P NMR spectra: a change in stereoselectivity or a change in chemical shifts. Fortunately, for three phosphorothioates investigated, namely $C_{PS}G$, $G_{PS}U$, $G_{PS}Et$, the order of the ^{31}P NMR signals varied with solvents and indicated that a relative position of the resonances of diastereomeric compounds in ^{31}P NMR spectra depended not only on the stereochemistry at the phosphorous center but also on the solvent used. Further studies showed that even for deprotected dinucleoside phosphorothioates the order of the ^{31}P signals of the corresponding diastereomers followed the Eckstein's rule only in water, while in ACN, the positions of the signals were reversed (Figure 2).

TABLE 2 Positions and Values of ^{31}P NMR Resonances of Protected *H*-Phosphonate Diesters of Type **4** and the Corresponding Phosphorothioates of Type **5** in Various Solvents. The Anomalies Are Marked with Grey Background

Solvent	Pattern of signals[a] and chemical shifts in the ^{31}P NMR spectra (ppm)							
	$U_{PH}U$	$U_{PS}U$	$C_{PH}G$	$C_{PS}G$	$G_{PH}U$	$G_{PS}U$	$G_{PH}Et$	$G_{PS}Et$
ACN	9.25 9.04	58.38 57.70	11.36 9.10	57.72 57.53	9.80 9.31	58.96 58.26	8.67 8.28	57.85 57.74
DCM	8.53 7.35	58.32 57.29	10.23 7.39	57.80 57.44	8.51 8.14	58.88 58.25	7.71 7.04	58.01 57.39
Pyridine	9.21 8.75	61.30 60.24	10.11 8.74	59.30 58.80	9.38 9.04	58.97 58.58	7.74 7.55	58.84 58.32
Toluene	8.04 8.93[b]	60.02 58.20	13.27 7.90[b]	58.58 58.38[b]	9.12 8.24	59.48 59.30[b]	8.07 7.33	59.11 58.86

[a]The inserts are symbolic representations of the signals pattern, not the actual spectra.
[b]Due to poor solubility of the diester in neat toluene, a mixture containing 20% of DCM and 80% (v/v) of toluene was used.

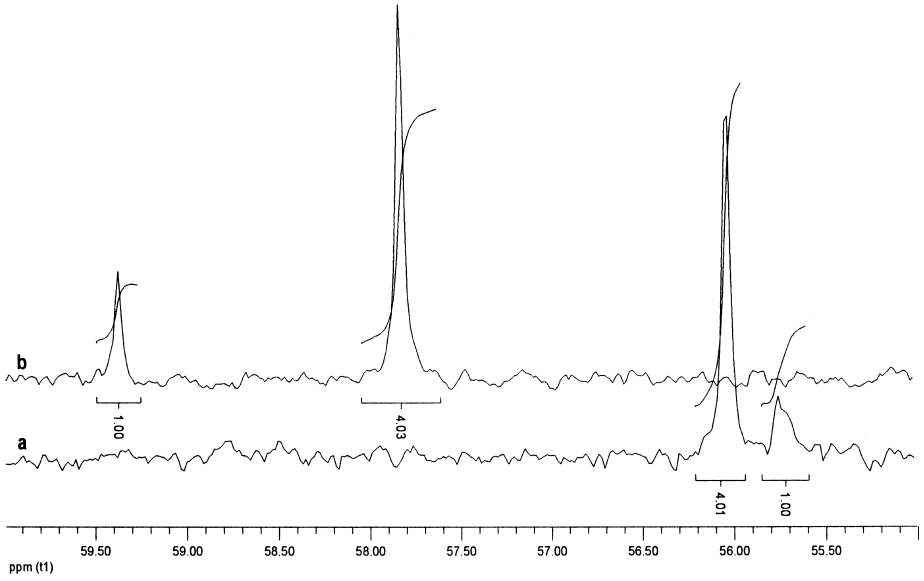

FIGURE 2 The ^{31}P NMR spectra of unprotected diester $G_{PS}U$ in (**a**) H_20 and (**b**) ACN.

Finally, for one of the investigated dinucleoside phosphorothioate that showed a solvent-dependent relative position of the ^{31}P NMR signals ($G_{PS}U$, **5cd**), the absolute configurations of the separate diastereomers were determined by enzymatic methods (see the Experimental). As expected, only the major diastereomer of **5cd** was the substrate for SVPD (Figure 3, chromatograms c and d). This confirmed its R_P configuration and excluded the possibility of a change in stereochemical preference during condensation in this, and most likely also in other analogous instances.

A solvent-dependence of ^{31}P NMR chemical shifts was further investigated by recording the spectra of the fully protected phosphorothioate diester $G_{PS}U$ (**5cd**) in ACN and in DCM containing various amounts of pyridine (0, 25, 50, 75, and 100%; Figure 4). Since for both ACN and DCM, the presence of more than 50% pyridine caused change in the order of the ^{31}P NMR signals of the diastereomers, one can suppose that a relative position of the signals is mainly determined by the major component of the solvent system. For the DCM-Py mixtures the diagram consists of roughly two intersecting lines, while for the ACN-Py mixtures, two concave curves with minima at different pyridine concentrations indicate probably more complex interactions with the solutes in this case.

Also, variable-temperature ^{31}P NMR spectra of the selected derivatives were recorded to find out the influence of temperature on a relative position of the resonances of *P*-diastereomers of the investigated compounds. To this end the samples in toluene were warmed up between 20 and 70°C, and ^{31}P NMR spectra were recorded every 10°C. For the *H*-phosphonate diester $U_{PH}Et$ (**4de**) no significant changes could be detected, neither in the signals positions nor in the ratio of the diastereomers. For the phosphorothioate diester $U_{PS}Et$ (**5de**), a moderate downfield shift (0.35 ppm) for both diastereomers in the investigated range of temperatures was observed. In contrast to this, the chemical shifts of the diastereomers of the mixed anhydride $U_{PH}Pv$ (**3d**) were much more sensitive to the temperature (Figure 5). Although signals from both diastereomers showed upfield shifts with temperature, at $t > 50$°C the order of the resonances changed, indicating higher sensitivity to temperature of the diastereomer **3d** resonating at lower field.

EXPERIMENTAL

^{31}P NMR spectra were recorded at 121 MHz on a Varian Unity BB VT spectrometer. ^{31}P NMR experiments were carried out in 5 mm tubes using 0.1 M concentrations of phosphorous-containing compounds in appropriate solvents (0.5 mL) and the spectra were referenced to 2% H_3PO_4 in D_2O (external standard). Pyridine, dichloromethane (POCh, Poland), and

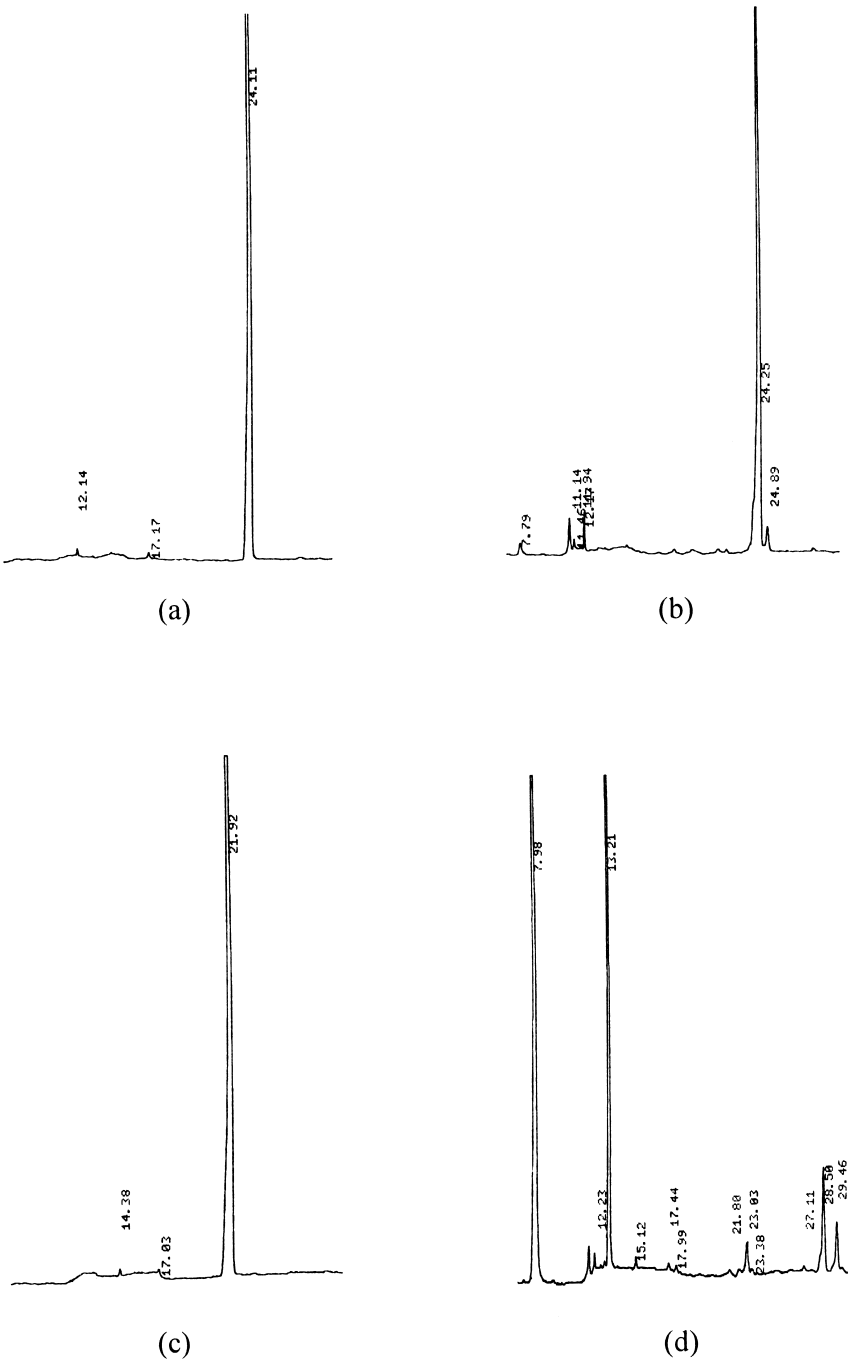

FIGURE 3 R*P*-HPLC profiles of digestion of diastereomers of the $G_{PS}U$ diester with SVPD (incubation time 48 h). Minor diastereomer: (a) before and (b) after digestion; major diastereomer: (c) before and (d) after digestion.

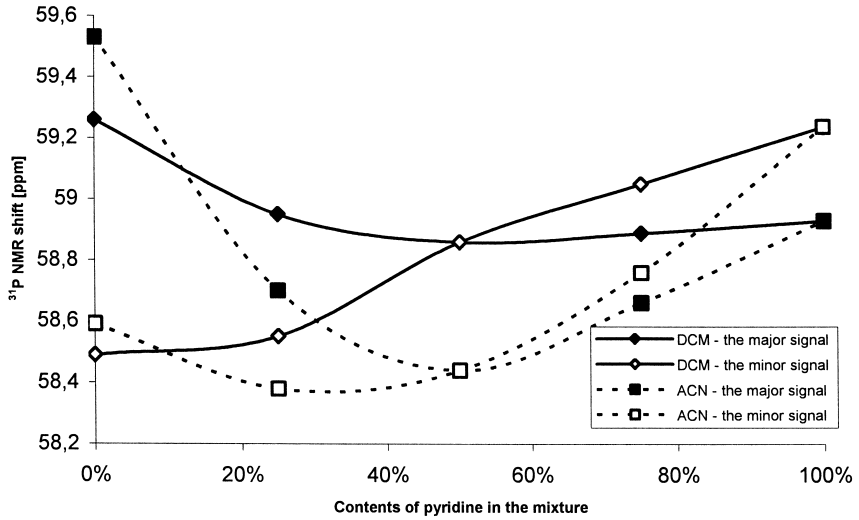

FIGURE 4 Positions of ^{31}P NMR signals of diastereomers of fully protected phosphorothioate diester $G_{PS}U$ (**5cd**) in ACN or DCM mixed with various amounts of pyridine.

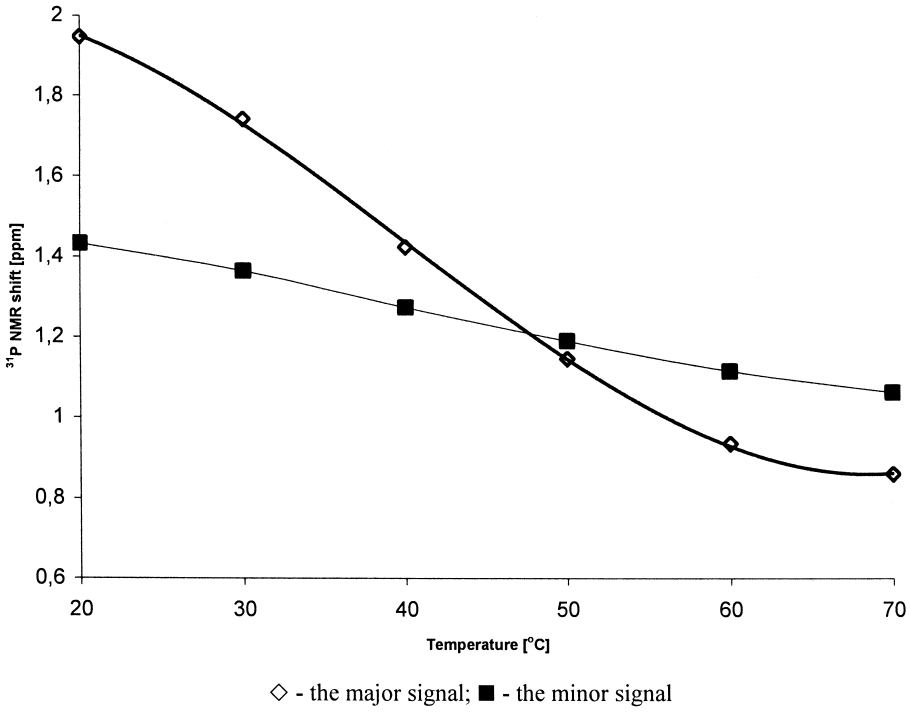

FIGURE 5 Positions of ^{31}P NMR signals of the in situ produced mixed anhydride $U_{PH}Pv$ (**3d**) diastereomers in toluene as a function of temperature.

acetonitrile (Merck) were refluxed with P_2O_5, distilled, and stored over molecular sieves 4 Å until the amount of water was below 20 ppm (Karl Fischer coulometric titration, Metrohm 684 KF coulometer.). Pivaloyl chloride (Fluka) was distilled and used within one month. Nucleoside *H*-phosphonates of type **1** were obtained according to the published method.[22] Snake venom phosphodiesterase (SVPD, from *Crotalus adamanteus*) was purchased from Sigma. All HPLC analyses and purifications were performed on ODS-Hypersil column (30 cm × 4.6 mm) detecting the eluate at 256 nm. ^{31}P NMR data of all intermediates and products are given in Table 3.

General Procedure for Condensations of *H*-Phosphonates of Type 1 with Nucleosides of Type 2

Nucleoside *H*-phosphonate **1** (0.05 mmol) and nucleoside **2** (0.06 mmol) were mixed together and rendered anhydrous by evaporation of added pyridine (3 mL) under reduced pressure. After a period of drying under vacuum (15 min, 0.5 Torr), the flask was filled with air dried by passing through Sicapent (Merck). The residue was dissolved in 0.5 mL of appropriate solvent (DCM or ACN containing 3 equiv. of pyridine, or neat pyridine) and pivaloyl chloride (1.2 equiv) was added. After ca. 10 min a ^{31}P NMR spectrum was recorded. The same mixture was sulfurized subsequently by adding elemental sulfur (3 equiv.) and Et_3N (50 μL) and after ca. 10 min a consecutive ^{31}P NMR spectrum was recorded.

General Procedure for Condensations of *H*-Phosphonates of Type 1 with Aliphatic Alcohols

The same procedure as above was used, with the exception that *H*-phosphonate was dried separately and dry alcohol was added to its solution (prior to pivaloyl chloride).

General Procedure for in situ Preparation of the Mixed Anhydrides of Type 3

Nucleoside *H*-phosphonate **1** (0.05 mmol) was rendered anhydrous by evaporation of added pyridine (3 mL) under reduced pressure. After drying under vacuum (15 min, 0.5 Torr), the flask was filled with air, dried by passing it through Sicapent (Merck). The residue was dissolved in 0.5 mL of the appropriate solvent (DCM or ACN containing 3 equiv. of pyridine), and pivaloyl chloride (1.2 equiv) was added. The ^{31}P NMR spectra showed that in such mixtures the mixed anhydrides of type **3** were stable for at least a few hours.

TABLE 3 ^{31}P NMR Data of Intermediates and Products (Chemical Shifts δ_P in ppm, Coupling Constants J_{HP} in Hz, and Ratios of Diastereomers). All Measurements Were Done Using Crude Reaction Mixtures

	Pyridine							DCM-pyridine (2.4% v/v)						
	R_P			S_P				R_P			S_P			
Compound	δ_P	$^1J_{HP}$	$^3J_{HP}$	δ_P	$^1J_{HP}$	$^3J_{HP}$	R_P/S_P	δ_P	$^1J_{HP}$	$^3J_{HP}$	δ_P	$^1J_{HP}$	$^3J_{HP}$	R_P/S_P
3d ($U_{PH}Pv$)	—	—	—	—	—	—	—	**1.46**	755.5	9.2	**1.34**	739.0	10.1	70:30
4aa ($A_{PH}A$)	**8.60**	721.6	9.2	**9.20**	729.9	7.8	22:78	**7.51**	722.6	8.2	**8.41**	732.6	7.3	21:79
4ab ($A_{PH}C$)	**8.68**	720.8	8.9	**9.37**	730.7	6.8	18:82	**7.48**	722.6	9.2	**8.49**	734.5	7.8	14:86
4ac ($A_{PH}G$)	**8.46**	722.7	[a]	**10.11**	729.8	7.5	30:70	**7.39**	728.7	[a]	**10.18**	731.7	7.0	27:73
4ad ($A_{PH}U$)	**8.56**	720.7	[a]	**9.27**	730.8	[a]	19:81	**7.13**	723.5	[a]	**8.55**	731.7	7.3	15:85
4ae ($A_{PH}Et$)	**7.39**	702.1	9.6	**7.69**	714.0	8.6	21:79	**6.54**	705.7	8.2	**7.12**	717.2	8.7	18:82
4ba ($C_{PH}A$)	**8.97**	720.0	8.9	**9.08**	726.8	8.3	41:59	**7.72**	726.0	[a]	**8.38**	730.5	7.9	29:71
4bb ($C_{PH}C$)	**9.22**	720.4	8.7	**9.14**	720.3	[a]	45:55	**8.60**	698.7	9.0	**8.45**	720.7	7.1	30:70
4bc ($C_{PH}G$)	**9.99**	712.7	8.7	**8.67**	728.6	8.5	48:52	**7.39**	727.5	7.2	**10.23**	733.1	7.0	39:61
4bd ($C_{PH}U$)	**8.89**	720.7	9.2	**9.18**	728.0	8.2	41:59	**7.56**	719.8	8.6	**8.43**	730.8	7.3	25:75
4be ($C_{PH}Et$)	**7.63**	699.8	9.5	**7.48**	710.5	9.1	46:54	**6.73**	698.9	[a]	**6.79**	712.7	8.9	29:71
4ca ($G_{PH}A$)	**9.19**	735.2	[a]	**9.10**	725.8	8.3	23:77	**8.30**	724.5	9.1	**8.79**	738.2	[a]	24:76
4cb ($G_{PH}C$)	**9.34**	720.2	[a]	**9.00**	730.0	6.7	18:82	**7.75**	726.2	[a]	**8.54**	735.4	7.3	10:90
4cc ($G_{PH}G$)	**10.02**	726.9	9.3	**9.74**	729.2	8.2	20:80	**9.44**	719.6	[a]	**9.76**	732.1	7.9	21:78
4cd ($G_{PH}U$)	**9.38**	718.1	8.9	**9.04**	732.3	[a]	19:81	**9.07**	716.2	8.4	**8.07**	738.6	[a]	14:86
4ce ($G_{PH}Et$)	**7.87**	700.4	9.7	**7.61**	714.5	8.8	24:76	**7.52**	706.1	9.6	**7.09**	717.5	8.8	21:79
4da ($U_{PH}A$)	**8.79**	721.6	8.2	**9.22**	729.9	9.2	24:76	**7.67**	723.8	9.1	**8.42**	734.8	8.1	20:80
4db ($U_{PH}C$)	**9.00**	718.0	9.2	**9.28**	729.9	8.2	22:78	**7.90**	716.2	9.2	**8.51**	733.6	7.3	14:86
4dc ($U_{PH}G$)	**8.80**	720.4	8.7	**9.86**	731.8	7.7	29:71	**7.17**	732.6	8.6	**9.90**	735.1	8.3	26:74
4dd ($U_{PH}U$)	**8.75**	720.7	9.2	**9.21**	731.7	8.2	19:81	**7.35**	722.6	9.2	**8.53**	736.3	7.3	14:86
4de ($U_{PH}Et$)	**7.38**	701.4	9.4	**7.66**	713.9	9.0	29:71	**6.56**	703.7	9.1	**7.07**	717.7	8.8	20:80

(*continued*)

TABLE 3 (*Continued*)

	Pyridine							DCM-pyridine (2.4% v/v)						
	R_P			S_P				R_P			S_P			
Compound	δ_P	$^1J_{HP}$	$^3J_{HP}$	δ_P	$^1J_{HP}$	$^3J_{HP}$	R_P/S_P	δ_P	$^1J_{HP}$	$^3J_{HP}$	δ_P	$^1J_{HP}$	$^3J_{HP}$	R_P/S_P
5aa ($A_{PS}A$)	**59.97**	—	[a]	**60.33**	—	[a]	81:19	**57.07**	—	9.2	**57.25**	—	[a]	76:24
5ab ($A_{PS}C$)	**60.24**	—	[a]	**61.15**	—	[a]	78:22	**57.26**	—	7.3	**57.67**	—	[a]	85:15
5ac ($A_{PS}G$)	**59.95**	—	[a]	**60.79**	—	[a]	69:31	**57.18**	—	7.3	**57.34**	—	[a]	71:29
5ad ($A_{PS}U$)	**60.15**	—	[a]	**60.99**	—	[a]	79:21	**57.53**	—	7.3	**58.09**	—	[a]	86:14
5ae ($A_{PS}Et$)	**60.31**	—	[a]	**61.33**	—	[a]	78:22	**57.46**	—	8.2	**58.23**	—	9.2	81:19
5ba ($C_{PS}A$)	**60.28**	—	[a]	**60.50**	—	[a]	57:43	**57.82**	—	8.5	**58.02**	—	[a]	69:31
5bb ($C_{PS}C$)	**60.49**	—	[a]	**61.13**	—	[a]	56:44	**57.99**	—	[a]	**58.31**	—	[a]	73:27
5bc ($C_{PS}G$)	**59.84**	—	[a]	**60.60**	—	[a]	52:48	**57.64**	—	7.8	**57.46**	—	[a]	63:37
5bd ($C_{PS}U$)	**60.13**	—	[a]	**61.00**	—	[a]	60:40	**57.65**	—	8.5	**58.45**	—	[a]	75:25
5be ($C_{PS}Et$)	**60.79**	—	11.0	**61.00**	—	11.0	51:49	**57.87**	—	10.1	**57.92**	—	[a]	[a]
5ca ($G_{PS}A$)	**60.45**	—	[a]	**59.92**	—	[a]	77:23	**58.29**	—	8.3	**57.87**	—	8.7	76:24
5cb ($G_{PS}C$)	**60.35**	—	[a]	**61.72**	—	[a]	82:18	**57.99**	—	9.2	**57.78**	—	[a]	90:10
5cc ($G_{PS}G$)	**60.04**	—	[a]	**61.01**	—	[a]	80:20	**57.58**	—	8.2	**58.11**	—	[a]	79:21
5cd ($G_{PS}U$)	**58.58**	—	7.3	**58.97**	—	8.3	80:20	**59.56**	—	7.3	**58.19**	—	[a]	85:15
5ce ($G_{PS}Et$)	**58.32**	—	[a]	**58.84**	—	[a]	76:24	**57.74**	—	10.0	**57.60**	—	[a]	78:22
5da ($U_{PS}A$)	**60.34**	—	[a]	**60.94**	—	[a]	76:24	**57.34**	—	9.1	**58.21**	—	9.0	80:20
5db ($U_{PS}C$)	**60.15**	—	8.2	**61.05**	—	9.2	75:25	**57.47**	—	8.3	**58.28**	—	[a]	85:15
5dc ($U_{PS}G$)	**60.06**	—	[a]	**61.08**	—	[a]	74:26	**57.59**	—	8.3	**57.89**	—	8.0	74:26
5dd ($U_{PS}U$)	**60.24**	—	[a]	**61.30**	—	[a]	80:20	**57.29**	—	8.2	**58.32**	—	6.4	86:14
5de ($U_{PS}Et$)	**60.75**	—	[a]	**61.55**	—	[a]	70:30	**57.46**	—	9.2	**58.34**	—	11.0	81:19

[a]Signals not resolved.

Preparative Synthesis and Enzymatic Digestion of Guanosin-3′-yl Uridin-5′-yl Phosphorothioate (Unprotected 5cd)

(a) Synthesis. The fully protected phosphorothioate diester **4cd** was prepared according to the above procedure at the 0.25 mmol scale. The diastereomers of the product were isolated on silica-gel column using 0–4% gradient of MeOH in toluene containing 1% of Et_3N.

The faster eluting diastereomer: 51 mg (15% yield); δ_P 57.98 ppm (ACN); R_f 0.54 (toluene-ACN-Et_3N 45:45:10).
The slower eluting diastereomer: 235 mg (67% yield); δ_P 58.67 ppm, R_f 0.41 (toluene-ACN-Et_3N 45:45:10).

Each diastereomer was subjected separately to the deprotection with conc. ammonia (48h, rt) and the tritylated product was purified on a silica-gel column using 0–2% gradient of 25% NH_3 *aq.* in *i*PrOH containing 2% of water. The dimethoxytrityl group was removed with 80% aq. AcOH. Triethylammonium hydrofluoride (TEA·3HF)[23] was used to cleave 2′-*O*-silyl ether. Purification according to the published method,[5] yielded the corresponding unprotected dinucleoside phosphorothioate diesters: 18 mg (68%) of the minor isomer, and 87 mg (74%) of the major isomer. The ^1H and ^{31}P NMR spectra of both diastereomers were in agreement with the literature data.[5] For enzymatic experiments, the samples were purified additionally on RP HPLC yielding ca. 100 OD of each isomer (combined fractions from three runs).

(b) Nuclease Digestion. The nuclease digestion was performed according to Cummins et al.[24] Separate diastereomers of phosphorothioate **5cd** (0.25 OD) and SVPD (2 mg) were incubated in 50 μL of the buffer solution (pH 8.5)[24] for 48 h at room temperature, and then analyzed directly by RP HPLC (see Figure 2).

CONCLUSIONS

In conclusion, our data lend support to the earlier findings by Almer et al. that the structure of an *H*-phosphonate monoester was crucial for the degree of stereoselectivity observed in the condensation reactions.[8] Although hydroxylic component had rather minor influence on the stereochemistry of the condensations, purine nucleosides usually gave slightly lower stereoselectivity than pyrimidine ones and simple alcohols. Among the compounds investigated, *H*-phosphonate C_{PH} (**1b**) consistently gave the poorest results, and for the reaction in neat pyridine hardly any stereoselectivity could be observed for this derivative. In all instances, decreasing of the pyridine content in the reaction mixture had beneficial effect on the

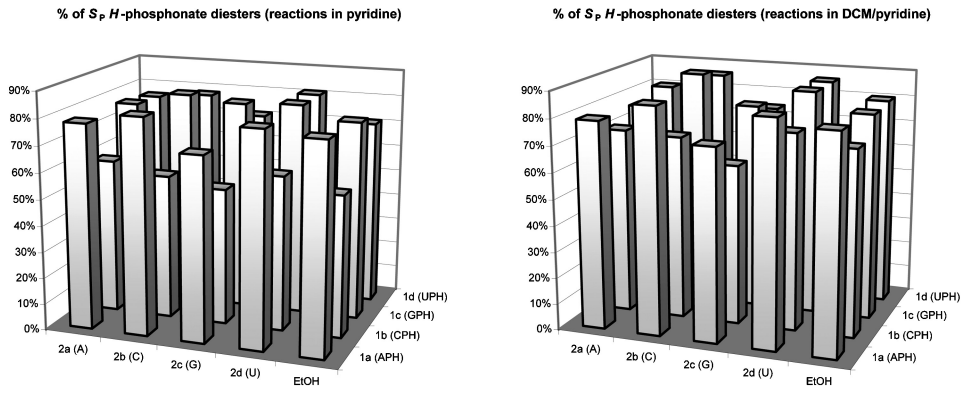

FIGURE 6 Stereoselectivity (% of S_P diastereomer of diesters **4aa-de**) during condensation of ribonucleoside *H*-phosphonates **1a-d** with nucleosides **2a-d** and ethanol.

stereoselectivity. For G_{PH} (**1c**), although high stereoselectivity was observed in most cases, we could not attain the value of >99% reported by Almer et al. In all instances, the major product of the condensations had R_P configuration (Figure 6).

Concerning a correlation between ^{31}P NMR chemical shifts and a sense of chirality at the phosphorous center, it was found that the mutual positions of the resonances of *P*-diastereomers were governed not only by the absolute configuration at the phosphorous center, but also by a nucleotide sequence, solvent composition, and temperature. Anomalous positions of the ^{31}P NMR signals occurred mainly for nucleotide analogues bearing protecting groups, although even for unprotected phosphorothioates variation in the order of *P*-diastereomers resonances was observed. Thus, one should exercise considerable caution while using these correlation rules for assigning absolute configurations at the phosphorous center.

REFERENCES

1. Sobkowski, M.; Jankowska, J.; Stawinski, J.; Kraszewski, A. A cautionary note on the use of the ^{31}P NMR spectroscopy in stereochemical correlation analysis. Nucleosides, Nucleotides & Nucleic Acids, **2005**, 24(5–7), 1033–1036.
2. Battistini, C.; Brasca, M.G.; Fustinoni, S. High stereoselectivity in the formation of the interribonucleotidic phosphorothioate bond. Nucleosides Nucleotides **1991**, 10(1–3), 723–725.
3. Seela, F.; Kretschmer, U. Diastereomerically pure R_P and S_P dinucleoside *H*-phosphonates—The stereochemical course of their conversion into *P*-methylphosphonates, phosphorothioates, and [^{18}O] chiral phosphates. J. Org. Chem. **1991**, 56(12), 3861–3869.
4. Battistini, C.; Brasca, M.G.; Fustinoni, S.; Lazzari, E. An efficient and stereoselective synthesis of 2′,5′-oligo-(S_P)-thioadenylates. Tetrahedron **1992**, 48(15), 3209–3226.
5. Almer, H.; Stawinski, J.; Strömberg, R.; Thelin, M. Synthesis of diribonucleoside phosphorothioates via stereospecific sulfurization of *H*-phosphonate diesters. J. Org. Chem. **1992**, 57(23), 6163–6169.

6. Battistini, C.; Fustinoni, S.; Brasca, M.G.; Borghi, D. Stereoselective synthesis of cyclic dinucleotide phosphorothioates. Tetrahedron **1993**, 49(5), 1115–1132.
7. Almer, H.; Stawinski, J.; Stromberg, R. Synthesis of Stereochemically homogeneous oligoribonucleoside all-R_P-phosphorothioates by combining *H*-phosphonate chemistry and enzymatic digestion. J. Chem. Soc., Chem. Commun. **1994**, (12), 1459–1460.
8. Almer, H.; Stawinski, J.; Strömberg, R. Solid support synthesis of all-R_P-oligo(ribonucleoside phosphorothioate)s. Nucleic Acids Res. **1996**, 24(19), 3811–3820.
9. Almer, H.; Stromberg, R. Intramolecular transesterification in thiophosphate-analogues of an RNA-dimer. Tetrahedron Lett. **1991**, 32(30), 3723–3726.
10. Seela, F.; Kretschmer, U. Stereochemistry of oxidation of diastereoisomeric d(TpA) phosphonates with sulphur and iodine-[^{18}O]water. Chem. Commun. **1990**, (17), 1154–1159.
11. Burgers, P.M.J.; Eckstein, F. Diastereoisomers of 5′-*O*-adenosyl 3′-*O*-uridyl phosphorothioate: Chemical synthesis and enzymatic properties. Biochemistry **1979**, 18(4), 592–596.
12. Bryant, F.R.; Benkovic, S.J. Stereochemical course of the reaction catalyzed by 5′-nucleotide phosphodiesterase from snake venom. Biochemistry **1979**, 18(13), 2825–2828.
13. Potter, B.V.; Connolly, B.A.; Eckstein, F. Synthesis and configurational analysis of a dinucleoside phosphate isotopically chiral at phosphorus. Stereochemical course of *Penicillium citrum* nuclease P1 reaction. Biochemistry **1983**, 22(6), 1369–1377.
14. Eckstein, F. Phosphorothioate analogues of nucleotides: Tools for the investigation of biochemical processes. Angew. Chem. Int. Ed. **1983**, 22(6), 423–439.
15. Lesnikowski, Z.J.; Niewiarowski, W.; Zielinski, W.S.; Stec, W.J. 2′-Deoxyribonucleoside 3′-aryl phosphoranilidates. Key intermediates in the stereospecific synthesis of 2′-deoxyribonucleoside cyclic 3′,5′-phosphorothioates and dinucleoside(3′->5′)-phosphorothioates. Tetrahedron **1984**, 40(1), 15–32.
16. Stec, W.J.; Zon, G. Stereochemical studies of the formation of chiral internucleotide linkages by phosphoramidite coupling in the synthesis of oligodeoxyribonucleotides. Tetrahedron Lett. **1984**, 25(46), 5279–5282.
17. Stec, W.J.; Grajkowski, A.; Koziolkiewicz, M.; Uznanski, B. Novel route to oligo(deoxyribonucleoside phosphorothioates). Stereocontrolled synthesis of *P*-chiral oligo(deoxyribonucleoside phosphorothioates). Nucleic Acids Res. **1991**, 19(21), 5883–5888.
18. Stawinski, J.; Stromberg, R.; Zain, R. Stereospecific oxidation and oxidative coupling of *H*-phosphonate and *H*-phosphonothioate diesters. Tetrahedron Lett. **1992**, 33(22), 3185–3188.
19. Stawinski, J.; Thelin, M. 3-*H*-2,1-benzoxathiol-3-one 1-oxide—A new reagent for stereospecific oxidation of nucleoside *H*-phosphonothioate diesters. Tetrahedron Lett. **1992**, 33(22), 3189–3192.
20. The extended numbering system adopted in this paper was intended to reflect a composition of the synthesized dinucleoside *H*-phosphonates **4** and dinucleoside phosphorothioates **5**. For example, "**4bc**" means that the compound was obtained from *H*-phosphonate **1b** and nucleoside **2c**, while the compound **5ad**, from **1a** and **2d**, with intermediacy of **4ad**.
21. An unexpected signals positions were found also at the level of phosphono-acyl mixed anhydrides (obtained by pre-activation of *H*-phosphonate monoesters). In DCM the signal of the main diastereomer of this intermediate was located at higher field, while in toluene it was at a lower field.
22. Jankowska, J.; Sobkowski, M.; Stawinski, J.; Kraszewski, A. Studies on aryl *H*-phosphonates. 1. An efficient method for the preparation of deoxyribo- and ribonucleoside 3′-*H*-phosphonate monoesters by transesterification of diphenyl *H*-phosphonate. Tetrahedron Lett. **1994**, 35(20), 3355–3358.
23. Westman, E.; Stromberg, R. Removal of *t*-butyldimethylsilyl protection in RNA-synthesis. Triethylamine trihydrofluoride (TEA, 3HF) is a more reliable alternative to tetrabutylammonium fluoride (TBAF). Nucleic Acids Res. **1994**, 22(12), 2430–2431.
24. Cummins, L.L.; Owens, S.R.; Risen, L.M.; Lesnik, E.A.; Freier, S.M.; McGee, D.; Guinosso, C.J.; Cook, P.D. Characterization of fully 2′-modified oligoribonucleotide hetero- and homoduplex hybridization and nuclease sensitivity. Nucleic Acids Res. **1995**, 23(11), 2019–2024.