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Configurational Determination and Conformational Properties of Diastereomeric Nucleoside-Phosphorothioates

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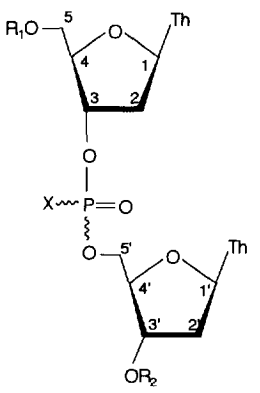
Abstract: The determination of configuration at phosphorous in enantiomerically pure nucleoside-phosphorothioates is reported. T-ROESY experiments and the trend observed in vicinal carbon-phosphorus couplings were used for stereochemical assignment.

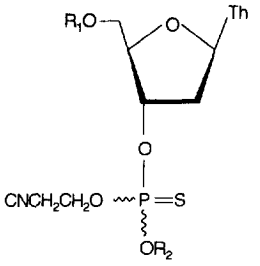
In earlier work the configurational assignment of diastereomeric dinucleotide derivatives was established by the uniformity existing between physico-chemical properties (such as chromatographical mobility, UV and CD data, ^{31}P chemical shifts) and the corresponding configuration of closely related molecules with known stereochemistry.[1-3] However, in most cases solvent dependence of the above parameters and changes in the substitution pattern may prevent direct correlation with literature data. More recently, application of ROESY experiments for deducing the configuration of diastereomeric dinucleoside-methylphosphonates was described.[4-5] For molecules having crowded ^{1}H spectra or broad signals, detection of the relevant connections in the ROESY spectra are often hampered by spectral overlaps or weak crosspeaks.

In a previous paper we reported for some phosphoramidates[6] that the vicinal ^{13}C-^{31}P coupling data can be applied to determine the configuration of the phosphorus atom in the diastereomers. The difference of ^{3}J(C4,P) and ^{3}J(C2,P) values was always larger for the R_p than for the S_p isomer (see e.g. the ΔJ values of the dinucleosidyl phosphoramidates **1a** and **1b** in Table 1.). Our retention pattern for **1a** and **1b** was the same as reported earlier for diastereomeric phosphoranilidate relatives **2a** and **2b** of known configuration at the P atom.[3] Moreover, in agreement with the ^{31}P chemical shifts of Ref. 3, the signal corresponding to the **1b** (S_p) isomer occurred at lower field than that of the **1a** (R_p) isomer.

Though characteristic differences were measured also for the vicinal ^{13}C-^{31}P values of some diastereomeric phosphate methyl esters,[2] scarcity of other examples in the literature prevented generalization of the use of ΔJ values for configurational determination. Here we present the extension of our observations concerning the applicability of the ΔJ values in the stereochemical elucidation of diastereomeric nucleoside-phosphorothioates.

Enantiomerically pure dinucleoside-phosphorothioates **3a** and **3b** were isolated by column chromatography and assigned by T-ROESY[7] experiments and by vicinal ^{13}C-^{31}P couplings (Table 1). In order to avoid total overlap of the pertinent protons the ROESY spectra were recorded in deuteriobenzene solutions. In the spectrum of isomer **3a** distinct ROEs of the OCH_2 protons in the β-cyanoethoxy group were

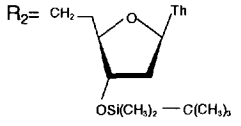




1a, 1b R_1=dimethoxytrityl
R_2=$Si(CH_3)_2$- $C(CH_3)_3$
X=NHBu

2a, 2b R_1, R_2=monomethoxytrityl
X=NHPh

3a, 3b R_1=dimethoxytrityl

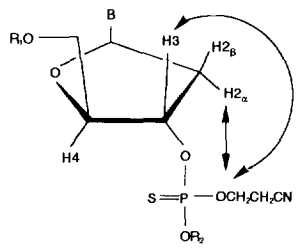


4a, 4b R_1=dimethoxytrityl
R_2=CH_2Ph

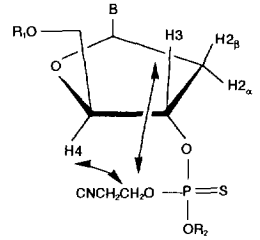
Figure 1. Structure and numbering of compounds **1**-**4**.

measurable to H3 and H4 protons, whereas the crosspeak resulting from the $H2_\alpha$ proton was absent. The ROESY spectrum of **3b** showed peaks correlating the mentioned OCH_2, $H2_\alpha$ and H3 protons. These results are in agreement with those of the diastereomeric methylphosphonates,[4] and allow the assignment of the absolute configuration at phosphorus (**3a** : R_p ; **3b** : Sp).

A common feature of both **3a** and **3b**, that the ^3J(C2,P) couplings are definitely larger (by 1-2 Hz), consequently the ΔJ values are less than in the phosphoramidates and phosphate esters.[5,6]



Sp



Rp

Figure 2. Stereochemistry and ROE connections in compounds **3b** and **3a**

Altered bond length of phosphorus-sulfur compared to phosphorus-oxygen bond and difference in the electron density at the phosphorus atom may be both responsible for the above changes. Since no theoretical estimates of the magnitude of these effects are known to us, we shall interpret our data in a qualitative manner and by analogy of the extensively studied dinucleotides.

Lankhorst and coworkers[8,9] have discussed the variation of the $^3J(C2,P)$ and $^3J(C4,P)$ couplings in terms of ε^t and ε^- populations. Accordingly, for deoxyribonucleotides the ε^t conformer dominates in aqueous solution at room temperature, as evidenced by the much larger vicinal coupling from ^{31}P to C4 than to C2. At higher temperature the differences became less and at 92°C ($^3J(C4,P)$=6.6 Hz and $^3J(C2,P)$=3.6 Hz) the calculations revealed approximately equal population of the two conformers.

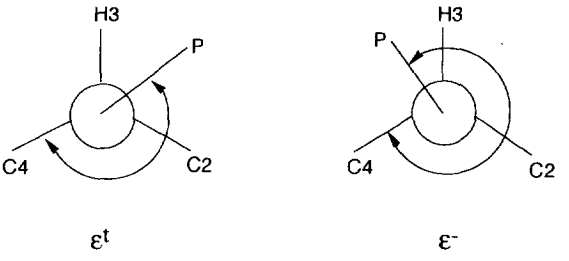


Figure 3. The ε_{trans} and $\varepsilon_{gauche(-)}$ conformers

It is well documented by ^1H NMR studies[1] that while there is a general preference for the ε^t conformer, the overall conformational properties somewhat differ in diastereomeric dinucleotides.The few vicinal ^{13}C-^{31}P coupling data published so far for diastereomers[2,6] supported the dominance of the ε^t conformer for the R_p isomers, while in the S_p isomers the conformational equilibrium shifted somewhat away from the ε^t domain.

In view of the above observations the smaller ΔJ values found for both **3a** and **3b** suggest a substantial increase in the amount of the ε^- relative to the ε^t conformer. These results illustrate that in phosphorothioates altered geometry and not the electronegativity effect is primarily responsible for the changes in vicinal couplings in comparison with the phosphate analogs.

This conclusion is further supported by observing the vicinal ^{13}C-^{31}P couplings of nucleoside-phosphorothioates **4a** and **4b**. In nucleosides, having no bulky substituent at the phosphorus, the overall flexibility is increased. As a result, attempts to apply ^{13}C-^{31}P coupling constants for configurational determination is more difficult than for dinucleotides.

The differences in ΔJ values were less for nucleoside-phosphoramidates[6] (4.5-5.4 Hz for the R_p and 3.1-3.7 Hz for the S_p isomers) than in the dinucleoside-phosphoramidates **1a** and **1b** (Table 1.). The ΔJ value is much less for **4a** (0.1 Hz) and even negative (- 0.3 Hz) for **4b** which reflects the dominance of the ε^- conformer and a higher flexibility for both nucleoside-phosphorothioates. The substantial decrease in the ^{31}P chemical shift difference of diastereomeric phosphorothioates may also be the consequence of the increased rotational freedom. For sake of conformity with the measuring conditions of the T-ROESY experiments the measurement of ^{13}C-^{31}P couplings was repeated in deuteriobenzene solutions. The larger $^3J(C4,P)$ and smaller $^3J(C2,P)$ values (Table 1.) reflect a slight shift towards the ε^t in both **3a** and **3b**. Similar effect was observed for **4a** and **4b** upon changing the solvent.

Table 1. Spectral Properties[a] and Chromatographical Mobility of Diastereomers **1** - **4**[b]

Compd.	Mobility	$\delta(^{31}P)$[c]	$^{3}J(C4,P)$[d]	$^{3}J(C2,P)$[d]	$\Delta J(C4,P\text{-}C2,P)$[d]	Config.
1a	slow	9.43	7.3	2.7	4.6	R_p
1b	fast	9.69	5.1	4.5	0.6	S_p
2a	slow	1.8	e	e	e	R_p
2b	fast	2.3	e	e	e	S_p
3a	fast	67.89	6.8(7.2)[f]	4.8(3.9)[f]	2.0(3.3)[f]	R_p
3b	slow	67.93	6.0(6.8)[f]	5.5(4.3)[f]	0.5(2.5)[f]	S_p
4a	slow	67.94	5.6(6.3)[f]	5.5(4.9)[f]	0.1(1.4)[f]	"R_p"
4b	fast	68.06	5.2(5.9)[f]	5.5(5.1)[f]	- 0.3(0.8)[f]	"S_p"

[a] In $CDCl_3$ solutions. [b] The CIP priority sequence is the same for all the molecules reported in the Table.

[c] Relative to phosphoric acid as external standard. [d] In Hz. [e] Not measured. [f] In C_6D_6 solutions.

In summary, we have demonstrated that vicinal ^{13}C-^{31}P couplings can be applied for the configurational determination of dinucleotide derivatives. The ΔJ values were characteristically different for those diastereomers also where alteration of the internucleotide linkage modified the conformational equilibrium. A limitation for the application of this method is that the assignment of the stereochemistry of any given dimer is only possible with a comparison with the other stereoisomer. We believe that our observations can be extended to other dinucleotides e.g. to the extensively studied dinucleoside-methylphosphonates.

Experimental

General. ^{1}H, ^{13}C and ^{31}P spectra were recorded in $CDCl_3$ and C_6D_6 solutions at ambient temperature using a Varian VXR 400 spectrometer. The chemical shifts were referenced to internal TMS or to external H_3PO_4. The measurement of the ^{13}C spectra was repeated with a spectral width of 5000 Hz (covered by 32K data points) in order to obtain sufficiently accurate ^{13}C-^{31}P couplings. Zero-filling was applied for resolution enhancement giving a digital resolution of 0.16 Hz. The T-ROESY experiments were run on a Varian Unity*.plus* (500 MHz) spectrometer using 250 msec mixing time.

Materials.

5-O-(4,4'-Dimethoxytrityl)-thymidylyl-(3 →5')-3'-O-(t-butyldimethylsilyl)-thymidylyl-O-(2-cyanoethyl)thio-phosphate **3a**,**3b**:

5-Dimethoxytrityl-thymidine (1g) + diisopropylammonium tetrazolide (250 mg) were dissolved in dry CH_2Cl_2 (20 ml) and treated with 2-cyanoethyl-N,N,N,N-tetraisopropyl-diamidite (1 ml). After 1 hr stirring the

reaction mixture was poured into 15 ml aqueous saturated $NaHCO_3$ solution, separated and dried over $MgSO_4$. The residue obtained by evaporation of the solvent was dissolved in 10 ml dry acetonitrile and treated with 200 mg freshly sublimed tetrazole. After 20 min. stirring 250 mg powdered sulfur was added followed by 10 ml dry CH_2Cl_2 to render the mixture homogeneous. Stirring was continued for additional 1 hr and the solution was then concentrated by rotational evaporation of the solvent. The residue was dissolved in 40 ml CH_2Cl_2, washed with 10 ml H_2O and 2x10 ml 1 M aqueous TEAB-solution, separated and dried over $MgSO_4$. Evaporation of the solvent afforded 1.8 g white foam which was purified by chromatography on silica gel (63-200 μ) eluting with chloroform + triethylamine (1%) + methanol (2→10%). Fractions between 360-500 ml of eluent contained 1.42 g 5-O-(4,4'-dimethoxytrityl)-thymidylyl-3-O-thiophosphate-O-(2-cyanoethyl) ester as a white foam which was dissolved in 15 ml dry CH_2Cl_2, 0.8 ml N-methylimidazole was added followed by 1.27 g 2,4,6-triisopropylbenzenesulfonyl chloride.[10] After 90 min strirring the reaction mixture was diluted with 30 ml CH_2Cl_2, washed with 2x15 ml H_2O and dried over $MgSO_4$. The residue obtained after evaporation of solvent was separated by chromatography on 70 g silica gel eluting with chloroform-acetone (3:1) mixture affording 271 mg less polar, 270 mg mixed and 347 mg more polar title product, respectively.

3a: $[\alpha]_D$ = - 113.25 (c = 1.24, C_6H_6), ^1H NMR($CDCl_3$). 2.25+2.28(2H, J_{gem}=13.4, $J_{1',2'}$=6.5 and 6.5, $J_{2',3'}$=6.3 and 4.4 Hz, respectively; 2'-H_2), 2.39(1H, J_{gem}=14.0, $J_{1,2}$=8.8, $J_{2,3}$=5.9, $J_{2,P}$=1.2 Hz; 2-Hβ), 2.62(1H, J_{gem}=14.0, $J_{1,2}$=5.3, $J_{2,3}$=1.6 Hz; 2-H_a), 2.63 + 2.66(2H, J_{gem}=17.0, J_{vic}=6.1 Hz; CH_2CN), 3.42+3.48(2H, J_{gem}=10.6, $J_{4,5}$=2.6 and 2.8 Hz, respectively; 5-H_2), 4.03(1H, $J_{3',4'}$=4.2, $J_{4',5'}$=3.5+3.8, $J_{4',P}$=1.9 Hz; 4'-H), 4.11+4.19(2H, J_{gem}=10.1, J_{vic}=6.3+5.9 and 6.7+5.6, $J_{CH_2,P}$=9.7 and 9.9 Hz, respectively; OCH_2), 4.25(1H, $J_{3,4}$=2.0, $J_{4,5}$=2.6+2.8 Hz; 4-H), 4.25+4.28(2H, J_{gem}=10.5, J_{vic}=3.8 and 3.5, $J_{5',P}$=3.8 and 3.5 Hz, respectively; 5'-H_2), 4.42(1H, $J_{2',3'}$=6.3+4.4, $J_{3',4'}$=4.2 Hz; 3'-H), 5.35(1H, $J_{2,3}$=5.9+1.6, $J_{3,P}$=9.4 Hz; 3-H), 6.17(1H,t, $J_{1',2'}$=6.5 Hz; 1'-H), 6.39(1H, $J_{1,2}$=8.8+5.3 Hz; 1-H). ^{13}C NMR($CDCl_3$): 19.34($J_{C,P}$=8.3 Hz; $\underline{C}H_2CN$), 38.96($J_{C,P}$=4.8 Hz; C2), 40.25(C2'), 62.52($J_{C,P}$=4.3 Hz; OCH_2), 63.23(C5), 67.37($J_{C,P}$=5.8 Hz; C5'), 71.50(C3'), 79.88($J_{C,P}$=4.3 Hz; C3), 84.40(C1), 84.44($J_{C,P}$=6.8 Hz; C4), 84.73($J_{C,P}$=8.8 Hz; C4'), 86.02(C1').

3b: $[\alpha]_D$ = - 199.49 (c = 1.15, C_6H_6), ^1H NMR($CDCl_3$): 2.16+2.25(2H, J_{gem}=13.6, $J_{1',2'}$=6.7 and 6.7, $J_{2',3'}$=6.8 and 4.2 Hz, respectively; 2'-H_2), 2.44(1H, J_{gem}=14.2, $J_{1,2}$=8.9, $J_{2,3}$=5.9, $J_{2,P}$=1.5 Hz; 2-Hβ), 2.62(1H, J_{gem}=14.2, $J_{1,2}$=5.4, $J_{2,3}$=1.5 Hz; 2-Hα), 2.77(2H,t, J_{vic}=6.1 Hz; CH_2CN), 3.42+3.45(2H, J_{gem}=10.5, $J_{4,5}$=2.6 and 2.5 Hz, respectively;5-H_2), 3.97(1H, $J_{3',4'}$=4.2, $J_{4',5'}$=3.2+4.3, $J_{4',P}$=2.0 Hz; 4'-H), 4.16+4.21(2H,J_{gem}=11.3, $J_{4',5'}$=3.2 and 4.3, $J_{5',P}$=7.0 and 7.6 Hz, respectively; 5'-H_2), 4.25(1H, $J_{3,4}$=1.7, $J_{4,5}$=2.5+2.6 Hz; 4-H), 4.21-4.33(2H, unresolved multiplet; OCH_2), 4.37(1H, $J_{2',3'}$=6.8+4.2, $J_{3',4'}$=4.2 Hz; 3'-H), 5.36(1H, $J_{2,3}$=5.9+1.5, $J_{3,4}$=1.7, $J_{3,P}$=9.4 Hz; 3-H), 6.20(1H,t, $J_{1',2'}$=6.7 Hz; 1'-H), 6.41(1H, $J_{1,2}$=8.9+5.4 Hz; 1-H). ^{13}C NMR($CDCl_3$): 19.54($J_{C,P}$=8.3 Hz; $\underline{C}H_2CN$), 39.09($J_{C,P}$=5.5 Hz; C2), 40.39(C2'), 62.74 ($J_{C,P}$=4.0 Hz; OCH_2), 63.34(C5), 67.41($J_{C,P}$=6.0 Hz; C5'), 71.40(C3'), 80.12($J_{C,P}$=4.5 Hz; C3), 84.44(C1), 84.68($J_{C,P}$=6.0 Hz; C4), 84.74($J_{C,P}$=8.8 Hz; C4'), 85.45(C1').

5-O-(4,4'-Dimethoxytrityl)-thymidylyl-3-O-thiophosphate-O-(2-cyanoethyl)-O-benzyl ester **4a, 4b**:
1.30 g 5-O-(4,4'-dimethoxytrityl)-thymidylyl-O-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite was

dissolved in 20 ml dry CH_2Cl_2, 0.05 ml triethylamine and 0.25 ml benzyl alcohol were added followed by 430 mg diethylaniline-hydrochloride. After 40 min 200 mg powdered sulfur was added and stirred for additional 1 hr then diluted with 10 ml CH_2Cl_2, extracted with 2x10 ml H_2O, dried over $MgSO_4$. The residue obtained after evaporation of the solvent was purified and separated by chromatography on 140 g silica gel (63-200 μ) eluting with hexane-ethyl acetate (50→66%). 270 mg less polar and 260 mg more polar products were obtained beside 150 mg mixed product.

4a: ^{1}H NMR($CDCl_3$): 2.38(1H, J_{gem}=14.1, $J_{1,2}$=8.9, $J_{2,3}$=6.0, $J_{2,P}$=1.6 Hz; 2-Hβ), 2.58(1H, J_{gem}=14.1, $J_{1,2}$=5.4, $J_{2,3}$=1.6 Hz; 2-Hα), 2.66(2H,t, J_{vic}=6.3 Hz; CH_2CN), 3.34+3.37(2H, J_{gem}=10.7, $J_{4,5}$=2.6 and 2.7 Hz, respectively; 5-H_2), 4.10(1H, $J_{3,4}$=1.8, $J_{4,5}$=2.6+2.7 Hz; 4-H), 4.13+4.19(2H, J_{gem}=10.3, J_{vic}=6.3, $J_{CH_2,P}$=9.2 and 9.7 Hz, respectively; OCH_2), 5.03+5.08(2H, J_{gem}=11.8, $J_{CH_2,P}$=11.5 and 11.8 Hz, respectively; O<u>C</u>H_2Ph), 5.32(1H, $J_{2,3}$=6.0+1.6, $J_{3,4}$=1.8, $J_{3,P}$=9.6 Hz; 3-H), 6.39(1H, $J_{1,2}$=8.9+5.4 Hz; 1-H). ^{13}C NMR($CDCl_3$):19.34($J_{C,P}$=8.5 Hz; <u>C</u>H_2CN), 39.09($J_{C,P}$=5.5 Hz; C2), 62.30($J_{C,P}$=4.5 Hz; OCH_2), 63.29(C5), 70.56($J_{C,P}$=5.4 Hz;O<u>C</u>H_2Ph), 79.52($J_{C,P}$=4.6 Hz; C3), 84.39(C1), 84.51 ($J_{C,P}$=5.6 Hz; C4).

4b: ^{1}H NMR($CDCl_3$): 2.30(1H, J_{gem}=14.1, $J_{1,2}$=8.7, $J_{2,3}$=6.0, $J_{2,P}$=1.6 Hz; 2-Hβ), 2.44(1H, J_{gem}=14.1, $J_{1,2}$=5.6, $J_{2,3}$=1.7 Hz; 2-Hα), 2.56(2H,t, J_{vic}=6.3 Hz; CH_2CN), 3.40+3.42(2H, J_{gem}=10.6, $J_{4,5}$=2.5 Hz; 5-H_2), 4.08(2H, J_{vic}=6.3, $J_{CH_2,P}$=9.6 Hz; OCH_2), 4.23(1H, $J_{3,4}$=2.0, $J_{4,5}$=2.5 Hz; 4-H), 5.13(2H,d, $J_{CH_2,P}$=12.1 Hz; O<u>C</u>H_2Ph), 5.26(1H, $J_{2,3}$=6.0+1.7, $J_{3,4}$=2.0 Hz; 3-H), 6.38(1H, $J_{1,2}$=8.7+5.6 Hz; 1-H). ^{13}C NMR($CDCl_3$): 19.24($J_{C,P}$=8.4 Hz; <u>C</u>H_2CN), 38.98($J_{C,P}$=5.5 Hz; C2), 62.20($J_{C,P}$=4.4; OCH_2), 63.32(C5), 70.69($J_{C,P}$=5.3 Hz; O<u>C</u>H_2Ph), 79.32($J_{C,P}$=4.7 Hz; C3), 84.39(C1), 84.59($J_{C,P}$=5.2 Hz; C4).

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