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A comparative conformational study of thymidylyl(3′ → 5′)-thymidine, thymidylyl(3′ → 5′)-5′-thio-5′-deoxythymidine and thymidinylacetamido-[3′(O) → 5′(C)]-5′-deoxythymidine

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Summary

A comparative 270 MHz NMR spectroscopic study on the solution structure of the dimer d(TpT) **1**, and its two analogues, namely, d(TpST) **2**, and NH_2d(TcmT) **4** has been reported. Analysis of chemical shifts and coupling constants indicate that: (*i*) The sugar moieties of the constituent nucleotides are not affected by modification of the internucleotide linkages and adopt preferentially an S-type conformation. (*ii*) The C4′-C5′ bond in the pT part of the modified dimers **2** and **4** shows a large conformational freedom ($\gamma^+ = 32\%$ and 35%, respectively) compared to **1** ($\gamma^+ = 75\%$). (*iii*) The population of the *trans* conformer about C5′-O5′ is less important in d(TpST) **2** compared to d(TpT) **1**. (*iv*) The C3′-O3′ bond in **2** adopts a trans conformation as in **1**. (*v*) The glycosidic bonds in the modified dimers **2** and **4** showed preferential *syn* conformation. UV and CD data show that the modified dimers **2** and **4** have poor tendency to stack intramolecularly, they also base pair less efficiently with d(ApA) as compared to d(TpT) **1**.

Key words: Thymidylyl(3′ → 5′)thymidine; Thymidylyl(3′ → 5′)-5′-thio-5′-deoxythymidine; Thymidinylacetamido-[3′(O) → 5′(C)]-5′-deoxythymidine

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Introduction

Analogues of oligodeoxynucleotides with modified internucleotide linkages are of considerable interest because of their potential use as therapeutic or diagnostic reagents [1–4], and also as models for the study of nucleic acid structure and function [4–6]. DNA analogues with chiral phosphorothioate linkage have so far turned out to be most biochemically useful [6]. Some of these DNA analogues have also shown promising antiviral activity in vitro [7,8], while DNA analogues with internucleotide phosphorothioate, phosphoramidate and phosphonate linkages have received considerable attention [1–11]; however, very little work has been reported on the category of isosteric and isopolar DNA analogues in which the bridging oxygen at 3′- or 5′- has been replaced by sulfur such as [nucleoside-3′-*S*-PO_2-*O*-5′-nucleoside] or [nucleoside-3′-*O*-PO_2-*S*-5′-nucleoside]. Their syntheses have been reported, and some of them have turned out to be relatively stable toward cellular nuclease [12–20]; however, their biochemical use as antisense repressor has not been investigated. Similarly, oligodeoxynucleotide analogues in which the carbohydrate, purine and pyrimidine moieties are the same as those of natural polynucleotides but in which the internucleotide phosphate linkage has been replaced with flexible aliphatic, non-ionic, achiral spacers are of potential interest. One such category of DNA analogue containing all four naturally-occurring nucleobases with [3′(O) → 5′(C)]oxyacetamide as the internucleoside linkage, as in NH_2d(TcmT) **4**, has been recently synthesized by us [21]. Some of the requirements for a DNA analogue to be useful as an antisense repressor in antiviral chemotherapy are that (*i*) they are easily available; (*ii*) they should be soluble under physiological conditions; (*iii*) they are resistant to enzymatic degradation; and (*iv*) they should be able to form a stable Watson-Crick base pair. The potential utility of such antisense analogues in biological systems is related to the degree to which the geometry of the analogue resembles the geometry to that of the natural oligonucleotide. Therefore, in this study, we wanted to investigate the conformational implication of the replacement of the internucleotidyl (3′ → 5′)-phosphodiester moiety in thymidylyl(3′ → 5′)thymidine **1** with a (3′ → 5′)-thiophosphate linkage, as in thymidylyl(3′ → 5′)-5′-thio-5′-deoxythymidine **2**, or with a nonpolar, achiral spacer of approximately the same distance between sugar residues as those of natural polynucleotides (7.1 Å) [22] such as in thymidinylacetamido[3′(O) → 5′(C)]-5′-deoxythymidine [NH_2TcmT] **4**.

We herein report a comparative study on the solution structure of the dimer d(TpT) **1** and its two analogues, namely, d(TpST) **2** [19], and NH_2d(TcmT) **4** [21] by 270 MHz NMR, CD and UV spectroscopy. It is of interest to understand, for the reasons stated above, the conformational implication of introducing a bridging sulfur atom, as in dimer **2**, compared to d(TpT) **1**, due to the differences in the electronegativity of the constituent sulfur and oxygen, respectively, and also due to the larger size of sulfur which results in a slight elongation of the sugar phosphate backbone in **2** compared to **1**. It was also of interest to show how a completely 'unnatural' [3′(O) → 5′(C)]oxyacetamido internucleoside linkage, such as in NH_2d(TcmT) **4**, affects its conformational properties in comparison with that of d(TpT) **1** in order to understand the structural criteria of an appropriate antisense repressor which will conformationally mimic the natural oligo-DNA.

Results and Discussion

Assignment and ^1H chemical shifts

The chemical shifts (δ) and coupling constants (J) for compounds **1**, **2** and **4** at 25 and 70 °C are listed in Tables 1 and 2. The NMR data for NH_2d(TcmA) **3** are also presented, because of its usefulness in the analysis of the ^{1}H-NMR spectrum of NH_2d(TcmT) **4**. The resonances of the sugar protons were assigned by means of homonuclear decoupling experiments and by 2-D COSY experiments (Figs. 1a–c). 2-D NOE experiments which provide information about the spatial proximities between the sugar and its base (*vide supra*) were used to assign the resonances of the base protons. In each case, the lower-field proton at C5′ was assigned as the 5′ proton, and the higher-field proton was assigned as the 5″ proton in accordance with the proposal put forward by Remin and Shugar [23]. When δ(H2′) was different from δ(H2″), the discrimination between the 2′ and 2″ protons was made using the rules devised by Altona et al [24]. These rules state that Σ1′ values of 13.3 Hz or more indicate a predominance of S-type conformer, and in this case the $J_{1'2'}$ coupling constant is greater than the $J_{1'2''}$. A predominant S-type sugar conformation means that $\Sigma 2' > \Sigma 2''$ ($\Sigma 1' = J_{1'2'} + J_{1'2''}$; $\Sigma 2' = J_{1'2'} + J_{2'3'} + J_{2'2''}$; $\Sigma 2'' = J_{1'2''} + J_{2''3'} + J_{2'2''}$ obtained from the separation of the outer peaks of the H1′, H2′ and H2″ regions in Hz, respectively).

The chemical shifts, coupling constants and conformational parameters observed for d(TpT) **1**, are in good agreement with previous reports [25]. The small differences can be due to different sample concentrations and experimental temperature.

A comparison of the chemical shifts of d(TpT) **1** and d(TpST) **2** shows that the main difference is the large upfield shift (1 ppm) of the 5′ and 5″ protons in the latter which is attributed to the difference in electronegativity between a sulfur and an oxygen atom. The other sugar protons and the base protons are not affected by the modification of the internucleotide linkage. The characterization of compounds **2**, **3** and **4** have been described elsewhere [19,21]. Also for the dimer NH_2d(TcmT) **4**, the 5′ and 5″ protons next to the internucleoside linkage experience an upfield shift (0.5 ppm) as compared to d(TpT) **1**. The upfield shift of H5′/H5″ in **4** is smaller than in the case of d(TpST) **2** which is due to the higher electronegativity of the nitrogen atom, and also to the delocalisation of the nitrogen lone pair in the amide group which leads to a downfield shift of the 5′ and 5″ protons. The 5′ and 5″ protons of the 5′-NH_2 terminal nucleoside move upfield by 0.6 ppm as compared to a 5′-OH terminal nucleoside. The 3′ proton of the Tcm part is shielded by 0.7 ppm due to the substitution of the phosphorus atom by a CH_2 group.

A concentration-dependence study was also performed (results not shown). Variation of the chemical shifts and coupling constants were monitored at 1, 5 and 10 mM solution of compounds **1**–**4**. No change was observed, suggesting that the intermolecular association in water is not important at these concentrations.

Effect of temperature on the chemical shifts

In order to investigate possible base stacking in these dimers, the variation of the H6 proton chemical shift was followed upon increasing the temperature. The H6

TABLE 1

*^{1}H-NMR chemical shifts (δ, CH_3CN set at 2.0 ppm) of d(TpT) **1**, d(TpST) **2**, NH_2d(TcmA) **3** and NH_2d(TcmT) **4***

	d(TpT) **1**				d(TpST) **2**				NH_2d(TcmA) **3**				NH_2d(TcmT) **4**			
	Tp		pT		Tp		pT		Tcm		cmA		Tcm		cmT	
	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C
H1′	6.147	6.143	6.248	6.220	6.214	6.183	6.188	6.159	5.875	5.894	6.356	6.356	6.106	6.109	6.132	6.075
H2′	2.278	2.273	2.317	2.315	2.365	2.351	2.236	2.341	2.270	2.330	2.826	2.820	2.380	2.419	2.296	2.347
H2″	2.490	2.475	2.304	2.290	2.556	2.540	2.285	2.277	2.190	2.160	2.563	2.547	2.380	2.419	2.296	2.347
H3′	4.717	4.705	4.531	4.481	4.806	4.803	4.393	4.383	4.095	4.081	4.525	4.509	4.195	4.174	4.332	4.313
H4′	4.111	4.092	4.068	4.045	4.162	4.142	4.075	4.070	3.985	4.007	4.146	4.127	4.128	4.105	4.012	4.004
H5′	3.766	3.758	4.096	4.022	3.762	4.769	3.096	3.084	3.168	3.154	3.618	3.574	3.132	3.103	3.558	3.515
H5″	3.697	3.685	4.002	3.981	3.720	3.714	2.989	2.985	3.027	3.021	3.445	3.468	3.016	2.994	3.471	3.494
H6	7.571	7.511	7.605	7.562	7.572	7.531	7.504	7.478	7.278	7.264			7.377	7.346	7.403	7.369
CH_3	1.809	1.805	1.822	1.821	1.823	1.818	1.827	1.825	1.807	1.806			1.822	1.819	1.822	1.819
H8											8.222	8.191				
H2											8.167	8.187				
CH_2									3.955	3.963			4.075	4.06		

TABLE 2

*$J_{H,H}$ and $J_{H,P}$ coupling constants (Hz) of d(TpT) **1**, d(TpST) **2**, NH_2d(TcmA) **3** and NH_2d(TcmT) **4***

	d(TpT) **1**				d(TpST) **2**				NH_2d(TcmA) **3**				NH_2d(TcmT) **4**			
	Tp		pT		Tp		pT		Tcm		cmA		Tcm		cmT	
	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C	25 °C	70 °C
1′-2′	7.40	7.12	6.85	6.80	7.51	7.41	6.66	6.70	7.47	7.28	6.22	6.32	7.20 [a]	7.14 [a]	6.83 [a]	6.83 [a]
1′-2″	6.18	6.32	6.85	6.80	6.30	6.23	6.66	6.70	6.60	6.80	6.71	6.59	7.20 [a]	7.14 [a]	6.83 [a]	6.83 [a]
2′-2″	14.04	14.11	14.18	14.20	14.28	14.22	14.20	14.47	14.28	14.56	14.16	14.15	/	/	/	/
2′-3′	6.46	6.71	7.02	6.50	6.51	6.70	6.88	6.72	6.22	6.32	6.30	6.32	5.63	5.86	6.23	6.59
2″-3′	3.42	3.62	4.00	4.30	3.16	3.01	4.15	4.28	3.19	3.57	5.13	4.81	5.00	5.13	4.76	4.63
3′-4′	3.50	3.50	3.80	4.15	3.11	3.24	4.01	4.07	3.11	3.13	4.80	4.53	3.30	3.78	4.13	4.27
4′-5′	3.56	3.65	2.69	2.98	3.24	3.43	4.67	4.93	3.91	3.98	6.18	6.25	4.39	4.03	6.83	6.10
4′-5″	4.49	4.69	3.35	4.51	4.99	4.99	6.85	6.44	8.99	8.51	4.12	4.53	8.48	8.18	4.64	4.27
5′-5″	12.54	12.41	11.70	11.98	12.41	12.46	13.51	13.53	13.39	13.46	14.42	14.42	13.55	13.30	14.28	/
H6, CH_3	1.27	1.27	1.27	1.26	1.22	1.20	1.22	1.27	1.22	1.24			1.25	1.22	1.25	1.22
3′-P	6.51	6.68			9.61	9.56										
5′-P			4.28	4.40			11.26	11.68								
5″-P			4.25	5.06			11.54	11.70								

[a] Only the sum of the coupling constants $J_{1'2'} + J_{1'2''}$ could be determined since $\delta 2' \sim \delta 2''$.

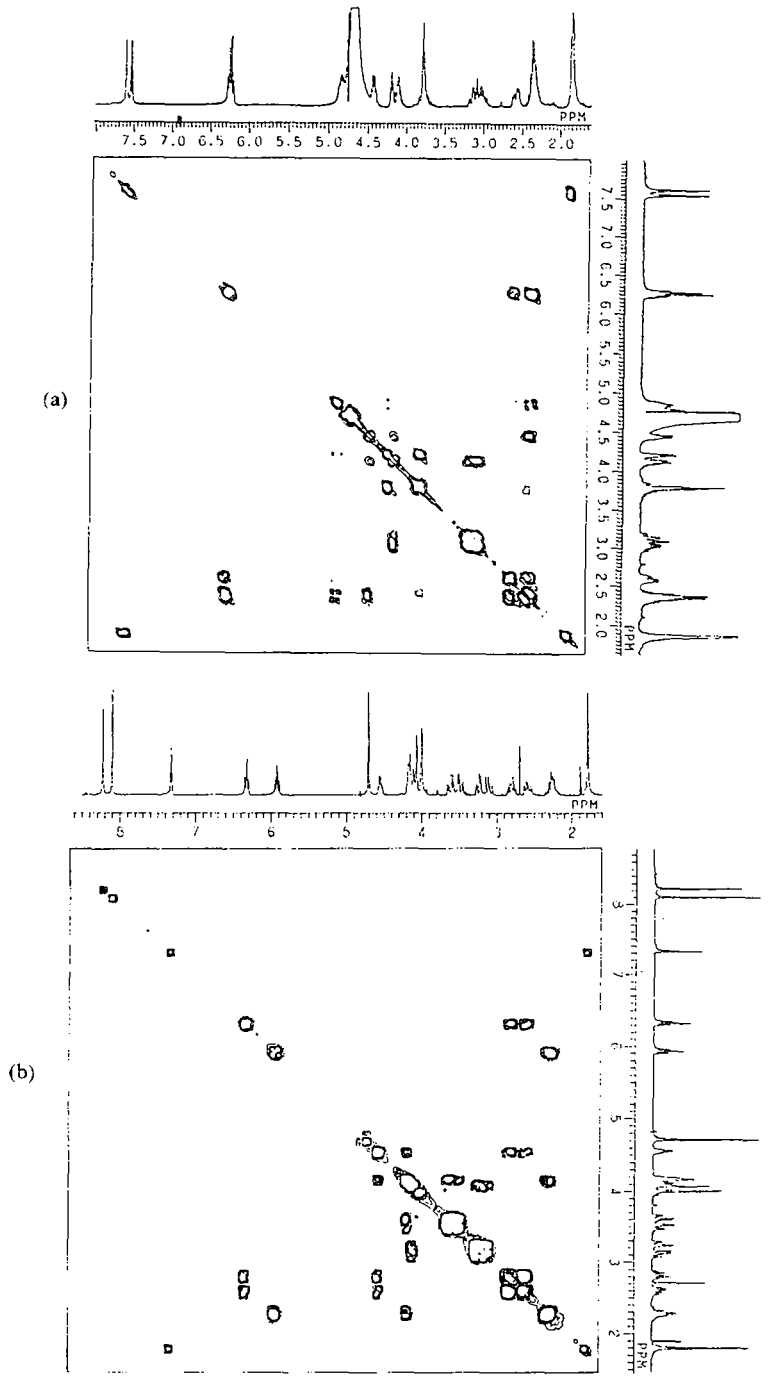


Fig. 1. (a) 2-D DQFCOSY spectrum of d(TpST) **2** at 298 K. (b) 2-D DQFCOSY spectrum of NH_2d(TcmA) **3** at 298 K. (c) 2-D DQFCOSY spectrum of NH_2d(TcmT) **4** at 298 K.

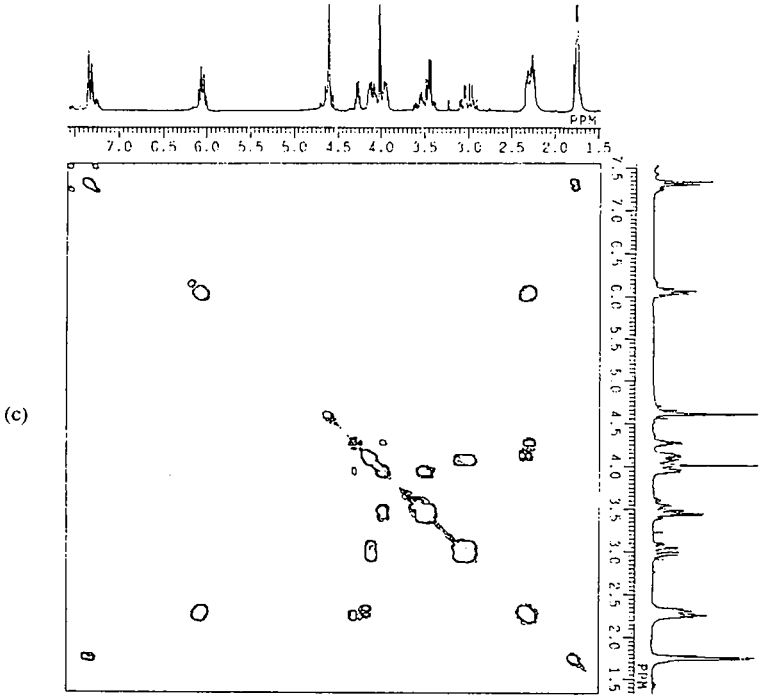


Fig. 1 (continued).

proton is known to be sensitive to base stacking. Fig. 2 shows the δ(H6) versus temperature profiles measured for **1**, **2** and **4**. As the temperature is increased from 5 to 80 °C, all H6 protons experience an upfield shift. Theoretical calculations have shown that the H6 proton is in a deshielded region when stacked with another pyrimidine [26]. The upfield shift is more important in d(TpT) **1**, which suggests that modification of its internucleotide linkage has some influence on the intramolecular stacking.

Sugar conformation

The furanose ring in deoxyribonucleotides is known to exist in an equilibrium consisting of two rapidly interconverting conformers which are denoted as N (C3′ *endo*) and S (C2′ *endo*). From the five coupling constants, $J_{1'2'}$, $J_{1'2''}$, $J_{2'3'}$, $J_{2''3'}$ and $J_{3'4'}$, the geometries of the N and S type conformer expressed as their phase angle of pseudorotation (P_N and P_S) and puckering amplitude (ϕ_N and ϕ_S), together with their molar fractions can be deduced. The deoxyribose conformation was deduced from a pseudorotational analysis of the sugar ring using, the coupling constants (Table 2) obtained after simulation using the program PSEUROT [27]. The results are reported in Table 3 and show: (*i*) All sugar moieties adopt preferentially an S-type conformation. Constraint of the pseudorotational parameters of the minor conformer did not change the population of S-type conformer,

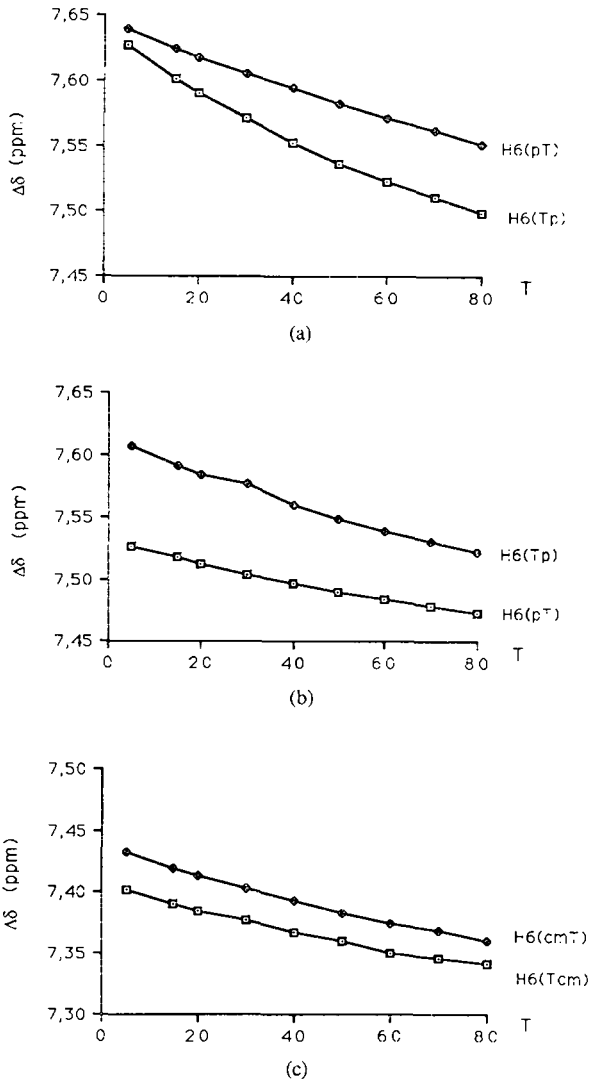


Fig. 2. ^{1}H-NMR chemical shifts vs. temperature profiles of the H6 protons of (a) d(TpT) **1**, (b) d(TpST) **2** and (c) NH_2d(TcmT) **4**.

neither did the constraint of both ϕ_N and ϕ_S and therefore the calculation of χ_S (population of S-type conformer) is quite reliable. (*ii*) The similarity between the pseudorotational parameters of d(TpT) **1** and d(TpST) **2** suggests that the replacement of an oxygen atom by a sulfur atom in the internucleotide linkage has no influence on the sugar conformation of the constituent sugar units. (*iii*) Due to the isochronicity of the H2′ and H2″ resonances in NH_2d(TcmT) **4**, the individual coupling constants involving 2′ and 2″ protons could not be obtained by computer

TABLE 3

Pseudorotational parameters P, ϕ and population of S type conformer for the sugar rings of d(TpT) 1, d(TpST) 2, NH_2d(TcmA) 3 and NH_2d(TcmT) 4

	d(TpT) **1**		d(TpST) **2**		NH_2d(TcmA) **3**		NH_2d(TcmT) **4**	
	Tp	pT	Tp	pT	Tcm	cmA	Tcm	cmT
P_S	153	155	153	152	164	155	– [b]	– [b]
ϕ_S	34	30	34	34	34	38	– [b]	– [b]
P_N	−3	3	−7	15	17	21	– [b]	– [b]
ϕ_N	35	35	33	34	34	38	– [b]	– [b]
%S 25 °C	74 (68) [a]	66 (66) [a]	76 (68) [a]	64 (65) [a]	75 (74) [a]	55 (49) [a]	(78) [a]	(65) [a]
Error (Hz) [c]	0.30	0.35	0.20	0.31	0.24	0.21	– [b]	– [b]
%S 70 °C	71 (65) [a]	58 (67) [a]	76 (64) [a]	63 (58) [a]	72 (74) [a]	58 (53) [a]	(76) [a]	(65) [a]
error (Hz) [c]	0.21	0.95	0.41	0.29	0.35	0.22	– [b]	– [b]

[a] Calculated by the 'sum rule' using Eqn 1.

[b] Could not be calculated due to resonance overlap.

[c] Sum of the differences (absolute values) between the five experimental coupling constants and their calculated values determined from P_S, P_N, ϕ_S, ϕ_N and %S.

simulation. However, the percentage of S-type conformer could be estimated from the separation of absorption in Hz between the outer peaks of the H1′ resonances by the "sum rule" using the equation [24]:

$$P_S = (\Sigma H1' - 9.8)/5.9 \quad (1)$$

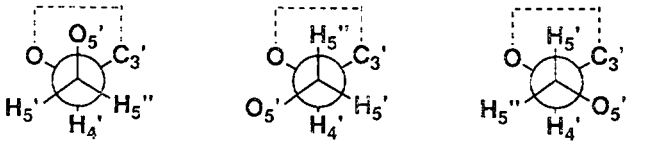
From Table 3, it can be seen that both calculations based on the PSEUROT program and calculations using the 'sum rule' reveal the same trend. Replacement of a phosphate linkage by a non-ionic oxyacetamido linkage has, as could be expected, no major influence on the conformation of the sugar ring: the C-3′ substituent is preferentially in an axial position. A comparison of the percentage of S-type conformer calculated by using the ΣH1′ shows that NH_2dT part in **3** and **4** is more in a S type (74% and 78%, respectively) compared to d(TpT) **1** (68%). (*iv*) Increasing the temperature to 70 °C mainly does not affect the N ⇌ S equilibrium. The fact that the N ⇌ S equilibrium is not very sensitive to temperature changes suggests that the intramolecular base stacking is not very important at ambient temperature.

Conformation around C4′-C5′ (γ)

The torsion angle γ(C3′-C4′-C5′-O5′) is known to occur in three staggered conformations, namely *gauche*$^+$ (γ^+), *trans* (γ^t) and *gauche*$^-$ (γ^-) (Fig. 3). In natural oligomers, a preference for γ^+ is generally observed. The conformational behavior about γ is monitored by means of the coupling $J_{4'5'}$ and $J_{4'5''}$. The population around γ can be estimated from the coupling constants in terms of distribution of population of three rotamers:

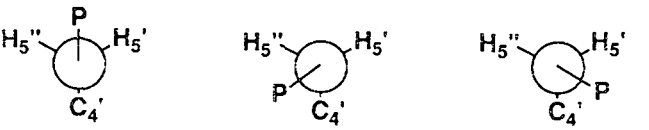
$$J_{4'5'} = (\chi_{\gamma^+} J'_{\gamma^+}) + (\chi_{\gamma^-} J'_{\gamma^-}) + (\chi_{\gamma^t} J'_{\gamma^t}) \quad (2)$$

γ (C4′- C5′) bond conformations



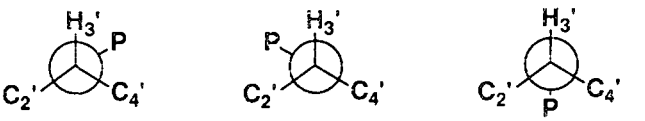
gauche $^+$ $\gamma = 60°$ | trans $\gamma = 180°$ | gauche $^-$ $\gamma = 300°$

β (O5′- C5′) bond conformations



Trans $\beta = 180°$ | gauche $^-$ $\beta = 300°$ | gauche $^+$ $\beta = 60°$

ε (C3′- O3′) bond conformations



gauche $^-$ $\varepsilon = 300°$ | trans $\varepsilon = 180°$ | gauche $^+$ $\varepsilon = 60°$

Fig. 3.

$$J_{4'5''} = (\chi_\gamma \text{-} J''_{\gamma^-}) + (\chi_\gamma \text{-} J''_{\gamma^-}) + (\chi_{\gamma'} J''_{\gamma^+}) \tag{3}$$

The J couplings of the individual rotamers were calculated by using the generalized Karplus equation [28]:

$$^3J_{\mathrm{HH}} = 13.22\cos^2\phi - 0.99\cos\phi + \Sigma\left(0.87 - 2.46\cos^2(\zeta_i\phi + 19.9\,|\Delta\chi_i|)\right)|\Delta\chi_i| \tag{4}$$

where ϕ represents the proton-proton torsion angle, ζ_i is $+1$ or -1 depending on the orientation of the substituents with respect to its geminal coupled proton. $\Delta\chi$ is the difference in electronegativity between the substituents of the HCCH fragments and hydrogen ($\Delta\chi = \chi_{\mathrm{subst}} - \chi_{\mathrm{H}}$).

Table 2 shows that the coupling constants $J_{4'5'}$ and $J_{4'5''}$ are larger in the 5′-S- part in d(TpST) **2** and 5′-N- part in NH_2d(TcmA) **3** or NH_2d(TcmT) **4** compared to the natural 5′-O- part in d(TpT) **1**. Calculations of the limiting coupling constants in the individual rotamers using equation 4 were performed with $\Delta\chi = 0.40$ for the sulfur substituent and $\Delta\chi = 0.85$ for nitrogen using Higgins' electronegativity constants [39]. The population around γ was then calculated using Eqs. 2 and 3 and the results are listed in Table 4. The rotamer populations in the Tp part of compound **2** is similar to that of the unmodified dimer d(TpT) **1**. At 25 °C, the Tp part of d(TpST) **2** shows a slight preference for the γ^+ conformation. Replacement of the oxygen atom by a sulfur atom in the internucleotide linkage decreases the γ^+ population of the pT fragments from 75 to 32% at 25 °C, increases the γ^t population from 22 to 47% and the γ^- population from 3 to 21% which indicates a large conformational freedom across the C4′-C5′ in d(TpST) **2**. In the case of compounds **3** and **4**, where the phosphate group is replaced by a nonionic linkage, the population of the γ^- conformer is decreased to 47 and 35%, respectively, while the population of the γ^- conformer is increased to 38 and 47%. The C4′-C5′ bond of the NH_2 thymidine shows a large preference for a *trans* conformation (74 and 67% in NH_2d(TcmT) **3** and NH_2d(TcmT) **4**, respectively at 25 °C). In the event the H5′ and H5″ are assigned incorrectly, the effect is to interchange the γ^- with the γ^t

TABLE 4

Rotamer distribution around C4′ – C5′ for d(TpT) 1, d(TpST) 2, NH_2d(TcmA) 3 and NH_2d(TcmT) 4

	°C	γ^+	γ^t	γ^-
d(TpT) **1**				
Tp	25	56	31	13
	70	53	34	14
pT	25	75	22	3
	70	60	34	6
d(TpST) **2**				
Tp	25	53	38	9
	70	51	37	12
pT	25	32	47	21
	70	35	42	23
NH_2d(TcmA) **3**				
Tcm	25	11	74	15
	70	16	69	15
cmA	25	47	15	38
	70	42	19	39
NH_2d(TcmT) **4**				
Tcm	25	13	67	20
	70	19	66	15
cmT	25	35	18	47
	70	46	17	37

Calculated from Eqns. 2, 3 and 4.

conformer. Increasing the temperature to 70 °C reduces in all cases the population of the major conformer.

Conformation around O5′-C5′ (β)

The conformational preference about β(C4′-C5′-O5′-P) is monitored by means of the coupling constants $J_{H5'P}$, $J_{H5''P}$ and $J_{C4'P}$ (Fig. 3). An estimation of the molar fraction of the preferred *trans* conformer β^t can be obtained independently from ^1H and ^{13}C data using the equations [29]:

$$\beta^t = (25.5 - J_{H5'P} - J_{H5''P})/20.5 \quad (5)$$

$$\beta^t = (J_{C4'P} - 0.73)/10.27 \quad (6)$$

The influence of electronegativity on the coupling constants must be assessed in the case of d(TpST) **2**. The values of the coupling constants $^3J_{PH}$ in **5** and **6** (Fig. 4)

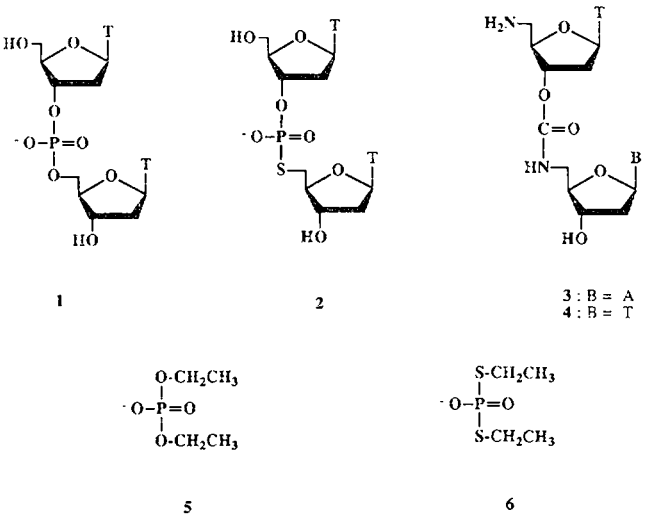


Fig. 4. Schemes of compounds **1**, **2**, **3**, **4**, **5** and **6**.

TABLE 5

$^{13}C - ^{31}P$ coupling constants (Hz) of d(TpT) 1 and d(TpST) 2

	d(TpT) **1**		d(TpST) **2**	
	Tp	pT	Tp	pT
C2′-P	3.36		2.29	
C3′-P	5.87		6.04	1.50
C4′-P	6.85	8.85	6.10	7.63
C5′-P		5.87		3.05

TABLE 6

Population of trans rotamer around O5′ – C5′ (β)

	25 °C	70 °C
d(TpT) **1**	83% [a] (79%) [b]	78% [a]
d(TpST) **2**	57% [a] (52%) [b]	54% [a]

[a] Calculated from Eqn. 5.
[b] Calculated from Eqn. 6.

are 8.01 and 12.62 Hz, respectively. A correction of 4.5 Hz was therefore adopted on the observed coupling constants $J_{H5'P}$ and $J_{H5''P}$ in d(TpST) **2**. The values of the coupling constants $^3J_{CP}$ are 5.86 and 7.32 Hz in compounds **5** and **6** and a correction of 1.5 Hz was made on $J_{C4'P}$ of the pT part of d(TpST) **2** (Table 5). The results in Table 6 show that the data obtained from J_{PH} and J_{CP} are in good agreement. The population of the β^t conformer decreases from 83 to 58% when comparing d(TpT) **1** and d(TpST) **2**. Due to the approximation made on the coupling constants in d(TpST) **2**, these results have to be considered qualitatively, but it has been shown previously that a reduction of the population of the γ^+ conformer is accompanied by a reduction of the population of the β^t conformer.

Conformation around C3′-O3′ (ε)

The conformation about ε(C4′-C3′-O3′-P) bond is monitored by the coupling constants $J_{C2'P}$, $J_{C4'P}$ and $J_{H3'P}$ (Fig. 3). Table 2 shows that $J_{C4'P}$ is larger than $J_{C2'P}$(~ 6–7 and 2–3 Hz, respectively) which suggests a preference for the *trans* conformer over the *gauche*$^-$. The conformations of exocyclic bonds can be interpreted in terms of rigid angles: Lankhorst et al. [29] have derived Karplus relationships between the torsion angles φ and $^3J_{HP}$ and $^3J_{CP}$ values:

$$^3J_{CCOP} = 6.9 \cos^2\phi - 3.4 \cos\phi + 0.7 \quad (7)$$

$$^3J_{HCOP} = 15.3 \cos^2\phi - 6.1 \cos\phi + 1.6 \quad (8)$$

The assumption of trigonal projection symmetry leads to:

Torsion angle: C2′-C3′-O3′-P = ε − 120

Torsion angle: C4′-C3′-O3′-P = ε

Torsion angle: H3′-C3′-O3′-P = 240 − ε (9)

The Karplus equation was used to determine the Karplus torsion angles from which the dihedral angles were calculated. The results are listed in Table 7. For d(TpST) **2**, the $J_{C2'P}$, $J_{C4'P}$ and $J_{H3'P}$ translate into $\phi_{C2'P} = \pm 38°(\pm 107°)$, $\phi_{C4'P} = \pm 132°$ and $\phi_{H3'P} = \pm 21°(\pm 122°)$. The combination of these torsion angles gives dihedral angles which fall into two groups; 117 – 158° or 219 – 227°. This last ε

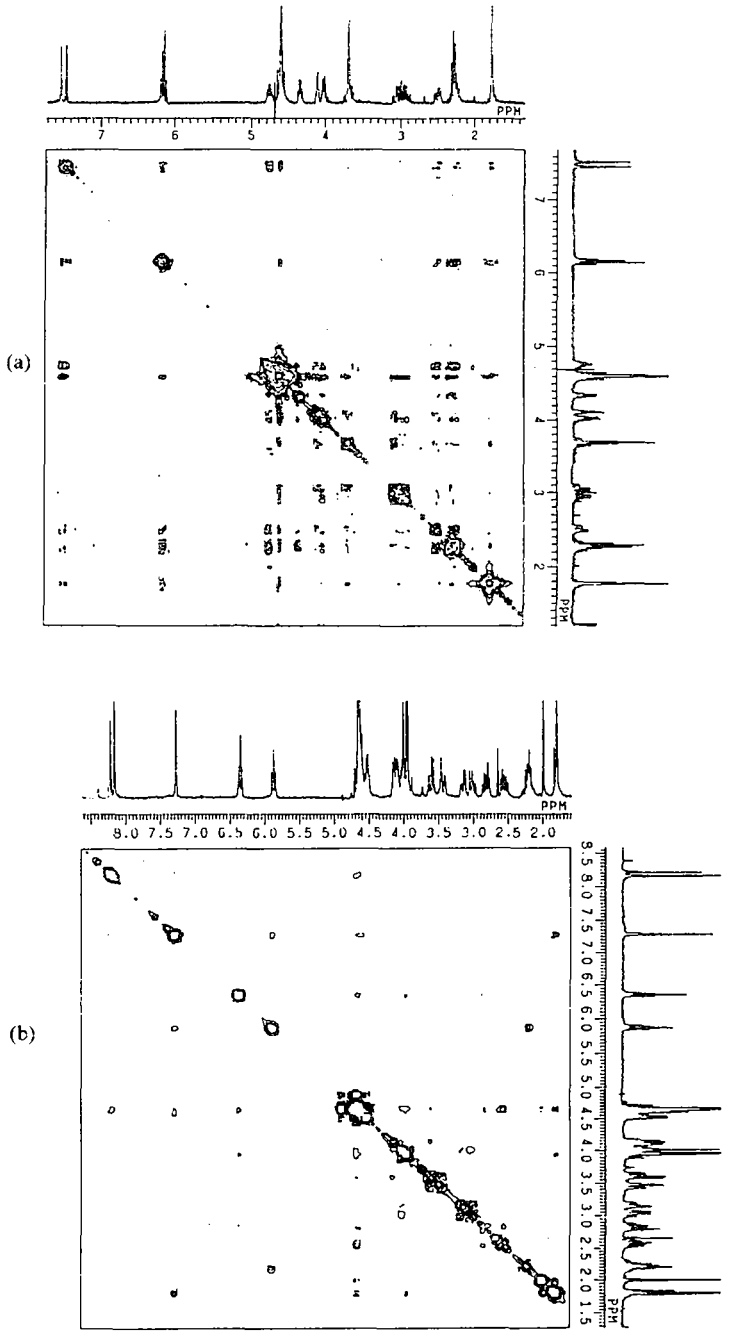


Fig. 5. (a) 2-D NOE spectrum of d(TpST) **2** at 298 K, mixing time of 500 ms. (b) 2-D NOE spectrum of NH_2d(TcmA) at 298 K, mixing time of 900 ms. (c) 2-D NOE spectrum of NH_2d(TcmT) **4** at 298 K, mixing time of 500 ms.

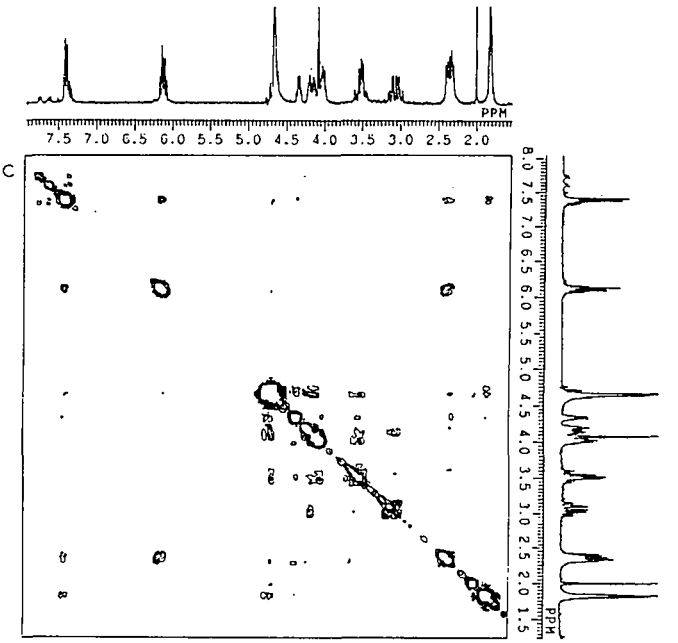


Fig. 5 (continued).

range is a conformation in the *trans* class (average: 220°). No information about the backbone torsion angles α and ζ can be obtained from $^{1}H-{}^{1}H$ and $^{1}H-{}^{31}P$ coupling constants data.

Conformation around the glycosidic bond

2-D NOE experiments are useful to obtain information about the spatial proximities between nucleotide residues and about the orientation of a base (*syn* or *anti*) relative to its sugar ring. An *anti* conformation is preferred when a strong NOE between the H6 of thymidine (H8 of adenosine) and H2′ or H3′ of the sugar is

TABLE 7

Calculated Karplus angles and dihedral angles about C3′ – O3′ (ε) from Eqns. 7, 8 and 9

	d(TpT) **1**			d(TpST) **2**	
	Karplus angle	Dihedral angle		Karplus angle	Dihedral angle
ϕ C2′-P	±24°	144°, 96°	ϕ C2′ – P	±38°	158°, 82°
	±115°	235°, 5°		±107°	227°, 13°
ϕ C4′-P	±137°	223°, 137°	ϕ C4′ – P	±132°	228°, 132°
ϕ H3′-P	±37°	277°, 203°	ϕ H3′ – P	±18°	258°, 222°
	±114°	354°, 126°		±123°	363°, 117°

TABLE 8

^{31}P-NMR chemical shifts temperature dependence of d(TpT) 1 and d(TpST) 2

	5°C	15°C	20°C	30°C	40°C	50°C	60°C	70°C	80°C
d(TpT) **1**	−1.134	−1.058	−1.011	−0.944	−0.875	−0.815	−0.754	−0.692	−0.650
d(TpST) **2**	19.096	18.896	18.939	18.841	18.736	18.643	18.539	18.440	18.342

observed while a strong NOE between H6/H8 and H1′ reveals a preferred *syn* conformation. In the case of comparable NOE strengths between H6/H1′ and H6/H2′ or H3′, a prefered *syn* conformation is attributed because of the fact that the minimum possible distance to H1′ to H6/H8 is greater than for H2′ or H3′ to H6/H8.

In d(TpT) **1**, H6 of Tp part shows a NOE with H3′ and H6 of pT part shows a NOE with H2′, suggesting a predominance of *anti* conformation for the constituent units around the glycosidic bond. In d(TpST) **2** (Fig. 5a), H6 of both Tp and pT parts show a NOE with their H1′ and H2′ which suggests that the glycosidic bonds adopt more of a *syn* conformation as compared to d(TpT). In NH_2d(TcmA) **3** (Fig. 5b) and NH_2d(TcmT) **4** (Fig. 5c), a strong NOE is observed between H1′ of NH_2T and its H6 which indicates a *syn* orientation around the glycosidic bond. The H6 of cmT in NH_2d(TcmT) **4** shows a NOE which its H1′, H2′ and H3′, suggesting a slight preference for the *syn* conformation. For the adenosine part of NH_2d(TcmA) **3**, no NOE was observed between H8 of the base and the sugar protons. However, these arguments to determine the conformation of the glycosidic bond must be considered carefully because significant NOE may occur due to conformational states which do not correspond to minimum energy conformations [30]. Small amounts of *syn* conformer can make an appreciable contribution to NOE.

It has also been shown that a *syn* conformer is associated with a larger population of S conformer if stabilization occurs by hydrogen bonding between the 5′ substituent and the base [30]. We have already noticed that the population of S conformer is slightly higher for the NH_2T part in NH_2d(TcmT) **4** and NH_2d(TcmA) **3** as compared to d(TpT) **1**. Also in a *syn* conformation, the repulsion between the $C^2=O$ carbonyl group and the 5′ substituent leads to a destabilization of the *gauche*$^+$ conformer while the *trans* becomes the predominant conformer about the C4′-C5′ bond.

Dependence of phosphorus chemical shifts on temperature

It has been proposed [31] that the main factor which determines the phosphorus chemical shift change in oligonucleotides is the torsional angles around the P-O ester bond which is correlated with the O-P-O bond angle (O3′-P-O5′). In addition, other components in the phosphorus screening have been used to interpret ^{31}P chemical shifts in nucleic acids: ring-current effect of the bases and conformation about the C-O bonds [C3′-O3′: ε, and C5′-O5′: β]. When the temperature is increased, two opposing effects contribute to the variation of chemical shifts: A down-field shift due to the change of conformation of the phosphate backbone and

an upfield shift due to a partial dehydration of the phosphate group [32]. Upon increasing the temperature from 5 to 80 °C, ^{31}P chemical shifts of d(TpT) **1** experience a down-field shift (0.48 ppm) due to a decrease of the constraint about the phosphate backbone (Table 8). d(TpST) **2** shows an anomalous behavior upon

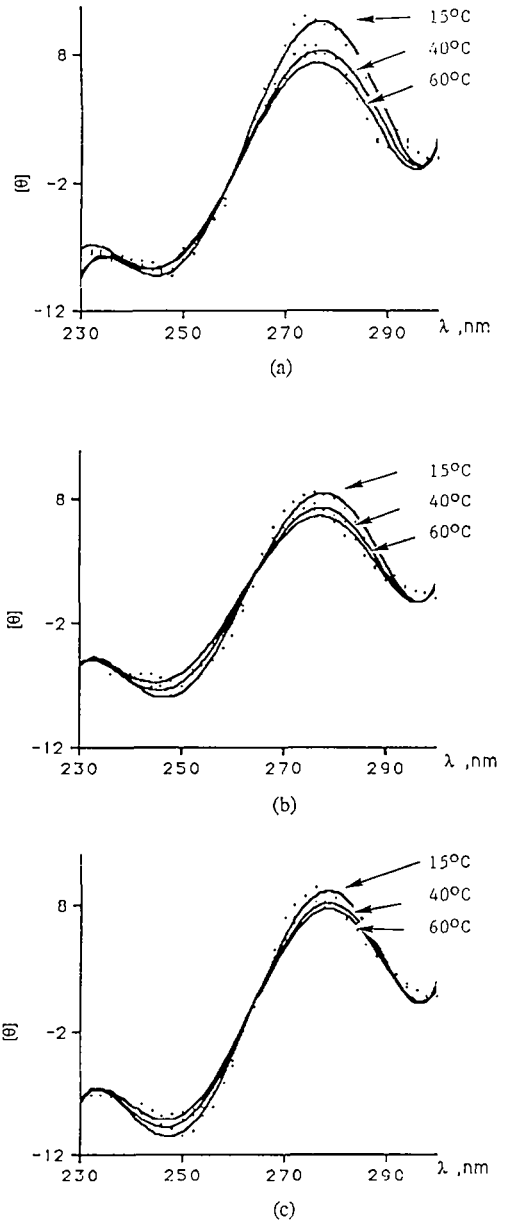


Fig. 6. Temperature-dependent circular dichroism (CD) spectra of (a) d(TpT) **1**, (b) d(TpST) **2** and (c) NH_2d(TcmT) **4**.

increasing the temperature: the ^{31}P chemical shift moves upfield by 0.75 ppm (from 19.096 ppm at 25 °C to 18.342 ppm at 80 °C) (Table 8). The reason for this is not clear for us.

Temperature dependent CD and UV of 2 and 4 and their comparison with 1

Fig. 6 represents the variation of the molar ellipticities for **1**, **2** and **4** at three

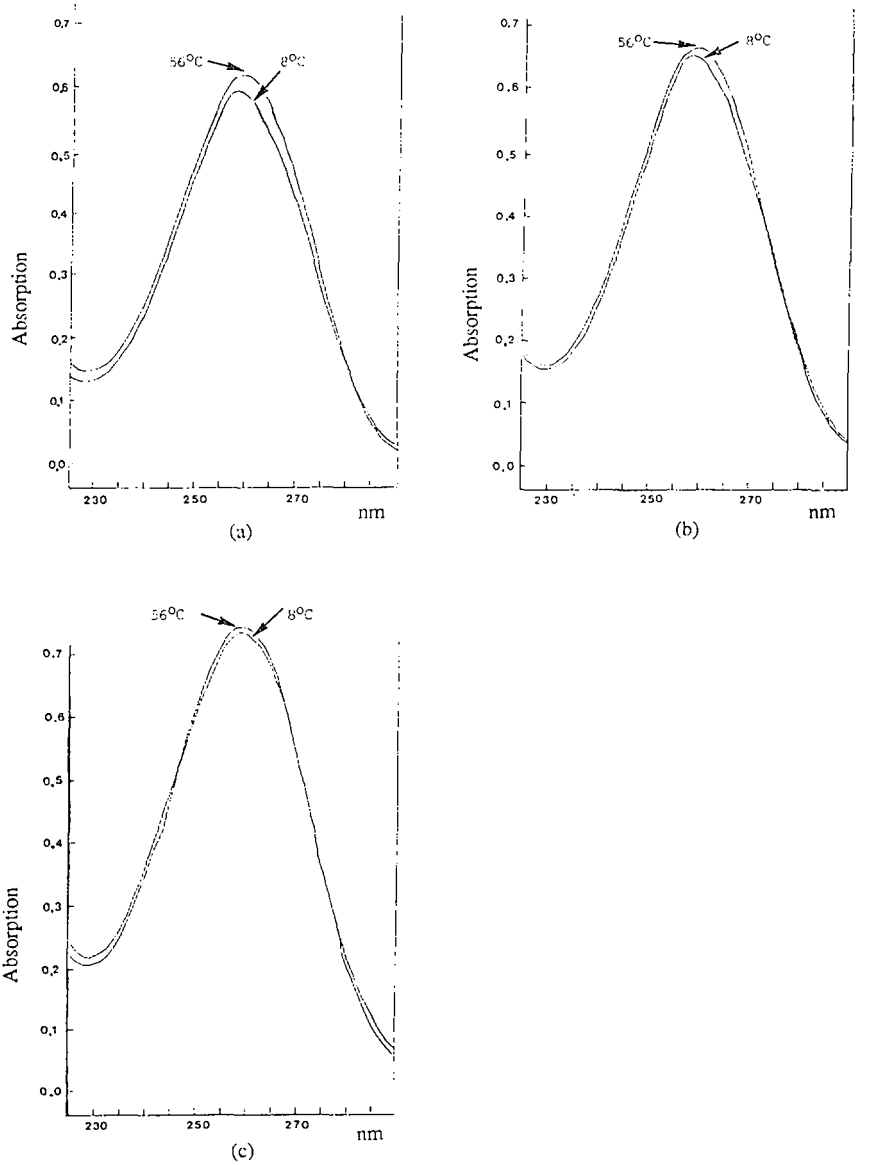


Fig. 7. Temperature dependent UV absorption spectra of an equimolar solution of d(ApA) with; (a) d(TpT) **1**, (b) d(TpST) **2** and (c) NH_2d(TcmT) **4**.

different temperatures (15, 40 and 60 °C). Increasing the temperature leads to a reduction in the amplitude of the peaks. It can be seen that d(TpT) **1** shows the maximum change upon increase of temperature compared to d(TpST) **2** and NH_2d(TcmT) **4**, which confirms the conclusion from our ^{1}H-NMR study (*vide supra*) that d(TpST) **2** and NH_2d(TcmT) **4** are poorer intramolecular stackers than d(TpT) **1**.

UV hyperchromicity experiments were also performed to study the extent of base pairing of d(TpST) **2** and NH_2d(TcmT) **4** with d(ApA) as compared to d(TpT) **1**. The UV absorption of equimolecular solutions of d(ApA) with compounds **1**, **2** and **4** were measured in the temperature range 8–56 °C (Fig. 7). Increasing the temperature results in the destabilization of the duplex which leads to a higher UV absorption. Only the equimolar mixture d(TpT) **1** and d(ApA) showed noticeable change in the UV absorption upon increasing the temperature and this could suggest that the base pairing ability of d(TpST) **2** and NH_2d(TcmT) **4** is reduced due to the modification of the internucleotide linkage.

Conclusion

From this study, it appears that modification of the internucleotide linkage as in compounds **2**, **3** and **4** has little effect on the conformation of the sugar ring which adopts preferentially an S-type conformation. The backbone conformation is, however, affected. The population of the γ^+ conformer about the C4′-C5′ bond decreased when the oxygen atom at the 5′ position is replaced by a sulfur or by a nitrogen atom. The preferred conformation in **2** about C5′-O5′ (β) is *trans*. The dihedral angle of the C3′-O3′ bond (ε) is very similar in d(TpT) **1** and d(TpST) **2** and corresponds to a *trans* conformation. 2-D NOE experiments seem to indicate that the glycosidic bond of Tp and pT part in **2** and **4** adopts more of a *syn* conformation as compared to the natural d(TpT) **1** where the conformation is *anti*.

Letsinger et al. [33] studied the conformational changes induced by the replacement of the oxygen atom at the C5′ position by a nitrogen atom and the same conformational changes were found with the exception that the conformation about the glycosidic bond was *anti* in the natural d(TpT) **1**.

The overall conformations of the modified dimers **2**, and **4** are not quite similar to the conformation of d(TpT), since **2** and **4** do not base pair with d(ApA) as effectively as d(TpT). Clearly, conformational studies with larger oligomeric analogues of **2** and **4** are required to understand the details of their intra- and intermolecular stacking abilities, which may have important implications for base pairing with a complementary strand in order to be able to act as the antisense repressor of DNA or RNA.

Experimental

NMR sample preparation

^{1}H-NMR samples were lyophilized twice from 99.8% D_2O and coevaporated in

99.8% D_2O. The samples were then dissolved in 0.5 ml of 99.96% D_2O and transferred into 5-mm tubes. The pH was adjusted to 7 for NH_2d(TcmA) and NH_2d(TcmT) by addition of HCl. A trace of dry acetonitrile was added to the sample as an internal reference (set at 2 ppm). Three different sample concentrations were prepared: 1, 5 and 10 mM. ^{13}C-NMR samples (in 5-mm tubes) were prepared in a similar way, the internal reference being CD_3OD. The sample concentration were approximately 10 mM. ^{31}P-NMR samples (in 10-mm tubes) were dissolved in 2 ml of double-distilled water. 10% H_3PO_4 was used as an external reference [34]. The sample concentrations were about 5 mM.

NMR acquisitions. ^{1}H, ^{13}C- and ^{31}P-NMR spectra were recorded on a Jeol GX 270 MHz spectrometer. ^{1}H-NMR spectra were recorded on 16K data points. The FIDs were zero filled to 32K data points before Fourier transformation. The resolution was 0.11–0.15 Hz. The water peak was suppressed by using a WEFT pulse sequence. ^{13}C-NMR spectra were recorded at 67.80 MHz using broad band proton decoupling. ^{31}P-NMR, proton decoupled, spectra were recorded at 109.40 MHz.

Two-dimensional acquisitions. 2-D COSY: 2-D double-quantum filtered COSY were recorded using the pulse sequence described in Ref 35. 96 scans were acquired for each t_1 value. A sinus square window was applied on a zero filled (512 × 1K) matrix and the spectrum was symmetrized.

2-D NOESY. The 2-D NOE spectra were recorded by the basic pulse sequence NOESY [36] where the mixing time t_m was systematically changed as t_1 increments in order to minimize the peaks arising from J contributions [37]. 128 FIDs of 512 data points were recorded. Each FID constituted of 208 scans and the pulse delay was 3 s. A sine bell apodization was applied on a zero filled (512 × 1024) matrix.

Spectral simulation. The chemical shifts and coupling constants listed in Tables 1 and 2 were obtained by using the iterative spin-spin simulation program LAOCOON [38]. Simulations were performed on proton NMR data obtained at 270 MHz. The calculated rms errors for the parameters were between 0.005 and 0.39 Hz.

CD spectra. Circular dichroism spectra were measured with a Jasco J-41 A spectropolarimeter using cells with 0.1 cm path-lengths and equipped for temperature control with a water bath. Each dimer was dissolved in a phosphate buffer solution (pH 6.9) at a concentration of 10^{-3} M.

UV spectra. Ultraviolet hyperchromicity experiments were performed on a Varian Cary 2200 spectrophotometer. The concentration of each dimer was $5 \cdot 10^{-4}$ M in water (pH 7). The UV absorption of each dimer was monitored when the temperature was increased from 8 to 56 °C, and no change in the UV absorption was found at this temperature range. An equimolar solution of d(ApA) was then added in the solution containing **1**, **2** or **4**, and the change of UV absorption spectra was then monitored with increasing temperature from 8 to 56 °C. It turned out that only the equimolar solution of d(TpT) and d(ApA) showed an appreciable change in absorption with the increase of temperature. This was interpreted as a pertubation of the base pairing between the two dimers.

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