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The influence of oligodeoxyribonucleotide phosphorothioate pyrimidine strands on triplex formation

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Analogues of the homopyrimidine oligonucleotide dT_{15} that contained phosphorothioate bonds of a mixture of diastereoisomers or one of the two stereoisomers (either Rp or Sp) were synthesized. The analogues were mixed under conditions conducive to the formation of triple-stranded assemblies. The mixtures were characterized by their thermal stabilities (T_m values), CD spectra, and gel electrophoresis pattern. The 34-mer duplexes containing 15 central purines on one strand and 15 complementary pyrimidines on the other strand gave no detectable triple helix upon combination with $dT_{15}S_{14}$. On the other hand, 34-bp duplexes with $dT_{15}S_1$, having Rp or Sp, formed triple helixes. This suggests that a steric factor plays an important role in triple helix formation.

Triplex formation; Phosphate modification; T_m value; CD spectra; Conformation; Native polyacrylamide gel electrophoresis

1. INTRODUCTION

Triple-helical nucleic acid structures can be formed from synthetic polymers, as the result of internal disproportionation of polypurine arrays in duplexes. These structures may involve binding of the third strand to the major groove of the DNA duplex. Binding results in the formation of hydrogen-bonded T·AT and C^+·GC triplets in which pyrimidines in the thirdstrand are Hoogesteen-bound to the purines of the DNA duplex [1–3]. Several researchers have used DNA triplexes including modified bases and phosphate groups on the third strand in order to develop highly selective drugs [4–12]. We report the effect of phosphorothioate substitution on the conformation of a triplex of DNA.

For synthetic nucleic acids to be effective in chemotherapy, their molecular structure must provide nuclease resistance and membrane permeability. Nuclease-resistant phosphorothioate oligomers were more effective antisense inhibitors of HIV, and would be more effective for duplex formation, and retention of the water solubility of charged phosphates (cellular uptake via receptor) [13–16]. To determine the effect of the phosphorothioate substitution on the conformation of triplex DNA, we chose oligomers having phosphorothioate internucleotidic bonds of a mixture of diastereoisomers or one of the two stereoisomers (either Rp or Sp).

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2. MATERIALS AND METHODS

Oligomers were prepared with a synthesizer using our new phosphite approach and were purified by reverse-phase chromatography using published procedures [16]. The presence of P–S bonds in phosphorothioates was confirmed using ^{31}P-NMR spectroscopy. The phosphorothioate oligomer was oxidized with a 0.1 M I_2 solution to phosphodiester. The product was used for determination of base composition by enzymatic degradation to nucleosides followed by HPLC.

2.1. *Thermal denaturation profiles*

Thermal transitions were recorded at 260 nm using a Shimadzu UV-260. The insulated cell compartment was warmed from 5°C to 90°C at increments of 1°C with equilibration for 1 min after attaining each temperature using a temperature controller SPR-8 (Shimadzu). Samples were heated in masked 1 cm path length quartz cuvettes fitted with Teflon stoppers. Each thermal denaturation was performed in 10 mM sodium phosphate buffer (pH 7.2), 1 mM EDTA, and NaCl at various concentrations and 1 μM of each strand. In the case of $dT_{15}S_{14}$ and $dT_{15}S_1$ (R,S), the oligomers were used for the triplex formation without separation of the diastereoisomers. The mixture of duplex and single strands was kept at 90°C for 5 min and cooled to 5°C. At a temperature below 20°C, N_2 gas was continuously passed through the sample compartment to prevent formation of condensate.

2.2. *CD spectroscopy*

The CD spectra were recorded with a Jasco J-600 spectropolarimeter, equipped with a thermostatically controlled cuvette holder that allowed measurements to be made at a controlled temperature. Buffer and oligomer conditions were the same as those used for melting studies.

2.3. *Gel electrophoresis*

The duplex DNA was made by combining equimolar amounts of oligonucleotides in a 40 mM Tris-acetate (pH 7.0) buffer/100 mM NaCl/10 mM $MgCl_2$, keeping the mixture at 90°C for 10 min, and slowly cooling it to room temperature. The triplex DNA was made by the addition of an equimolar amount of the third strand to the duplex followed by overnight incubation at 4°C. The concentration of each strand was 1.23×10^{-3} μM in a total vol. of 10 μl. Electrophoresis

experiments were conducted using gels containing 15% polyacrylamide (acrylamide/bisacrylamide, 19:1) prepared in a Bio-Rad Protean II gel apparatus with 20 × 22 × 0.75 cm glass slabs. 90 mM TBM buffer (pH 8.3)/5 mM $MgCl_2$ was used in the electrophoresis reservoirs. Experiments were conducted at a constant temperature (4°C) and 200 V for 16 h. The gel was stained using Methylene blue.

3. RESULTS AND DISCUSSION

For the thermal melting experiments of triple strand oligomers, we chose a non-self complementary 34-mer duplex consisting of sequences with nine and ten bases of non-identical termini surrounding a core of 15 purines/15 pyrimidines (purine 34-mer = 5'-TGAGTG-$AGTA_{15}$TGAGTGCCAA and pyrimidine 34-mer = 5'-TTGGCACTCAT_{15}ACTCACTCA), and a parallel/anti-parallel third strand comprised of 15 pyrimidines (Table I). Table II shows the results of the thermal melting of the modified oligo-T bound to the 34-mer duplex. At pH 7.2 and a 100 mM salt concentration, none of the single strands alone nor the pyrimidine 34-mer/T_{15} mixture showed any melting transition (data not shown). The 34-mer duplex underwent a normal duplex transition at 63°C (Table II; entry 1; Fig. 1A). However, when the 34-mer duplex was mixed with dT_{15}, two transitions were observed: one, at 63°C, which coincided with the 34-mer duplex melting, and one, at 48°C, which was typical of a triple helix (Table II; entry 2; see Fig. 1A).

The influence of the salt concentration on the T_m was determined at 100, 500, and 1000 mM NaCl for the 34-mer duplex/T_{15} mixture. In the case of 1000 mM NaCl, the two transitions were not observed. Triplex formation of the third (modified oligo-T) strand was investigated in a 100 mM NaCl solution. The addition of $dT_{15}S_{14}$ and $dT_{15}S_1$ with a mixture of diastereoisomers (Table II; entry 3, 6; Fig. 1A,B) gave no detectable triple helix. This might have been caused by the mixture of the diastereoisomers of the phosphorothioate bonds. A steric factor is partially responsible for the altered stability of the hybrid triplexes.

On the other hand, with $dT_{15}S_1$ (R) (Table II; entry 4; Fig. 1B) and $dT_{15}S_1$ (S) (Table II; entry 5; Fig. 1B), the triplex transition also occurred at 35°C and 25°C, respectively, but not with dT_{15}. In the case of oligomer analogues containing one phosphorothioate bond, the

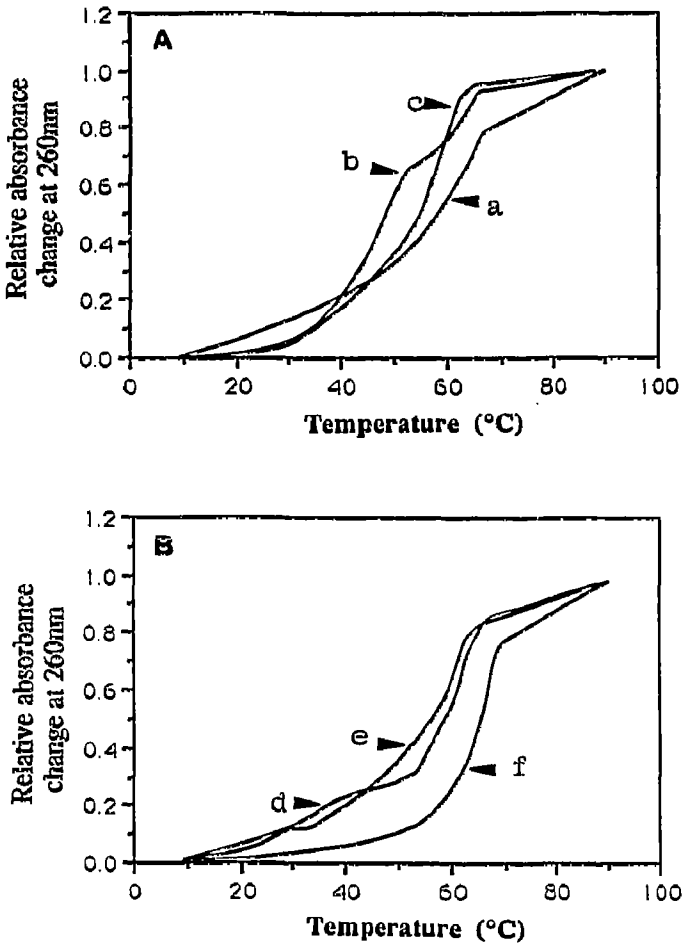

Fig. 1. Representative melting profiles of 1 μM nucleotides in 10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl: A: duplex (a); duplex-dT_{15} (b); duplex-$dT_{15}S_{14}$ (c) and B: duplex-$dT_{15}S_1$ (R) (d); duplex-$dT_{15}S_1$ (S) (e); duplex $dT_{15}S_1$ (R,S) (f). $[A_{260}(T°C)-A_{260}(5°C)]/[A_{260}(90°C)-A_{260}(5°C)]$.

Table I

Sequences of the third strands with duplex 34-mer

duplex 34-mer	5'TGAGTGAGTAAAAAAAAAAAAAAATGAGTGCCAA3'
	3'ACTCACTCATTTTTTTTTTTTTTTTACTCACGGTT5'
triplex N_{15}	5'NNNNNNNNNNNNNNN3'
	5'TGAGTGAGTAAAAAAAAAAAAAAATGAGTGCCAA3'
	3'ACTCACTCATTTTTTTTTTTTTTTTACTCACGGTT5'
triplex N_{15}	
dT_{15}	5'TTTTTTTTTTTTTTT3'
$dT_{15}S_{14}$	5'TsTsTsTsTsTsTsTsTsTsTsTsTsTsT3'
$dT_{15}S_1$(R,S)	5'TTTTTTTsTTTTTTTT3'
$dT_{15}S_1$(R)	5'TTTTTTTsTTTTTTTT3'
$dT_{15}S_1$(S)	5'TTTTTTTsTTTTTTTT3'

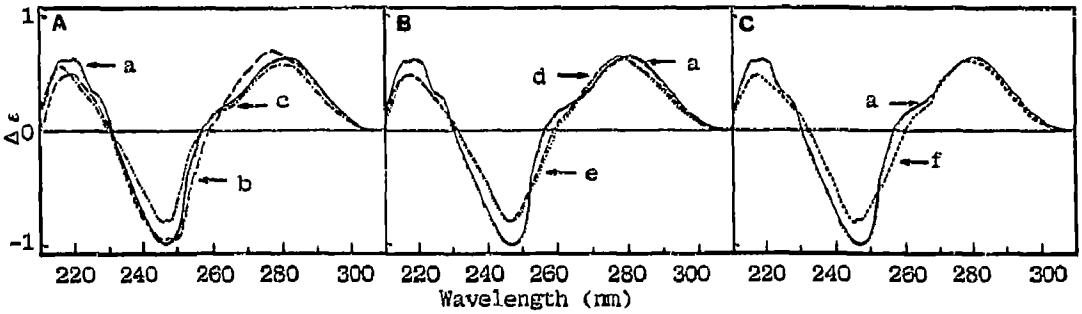

Fig. 2. CD spectra for A: duplex (curve a); duplex-dT_{15} (curve b); duplex-$dT_{15}S_{14}$ (curve c), B: duplex $dT_{15}S_1$ (R) (curve d); duplex $dT_{15}S_1$ (S) (curve e), and C: duplex $dT_{15}S_1$ (R,S) (curve f). The spectra were recorded at 4°C in 10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl.

small conformation changes must be due to the large size of sulfur and its electronic 'softness' compared to oxygen [6]. Of particular interest is $dT_{15}S_1$ (R) which possesses a slightly higher stability for the triplex structure than $dT_{15}S_1$ (S). The formation and stability of the triplexes containing phosphorothioate oligonucleotides were also confirmed by the CD spectra. As can be seen in Fig. 2A–C, the Cotton band of the 34-mer duplex-dT_{15} (b) or 34-mer duplex-$dT_{15}S$ (R = d or S = e) shifted from 280 nm to 275 nm, and a negative band at 246 nm, as well as a positive band at 215 nm, decreased in amplitude. The change of these CD curves suggested the triplex form as described above. By contrast, the 34-mer duplex-$dT_{15}S_{14}$ (c) and $dT_{15}S_1$ (RS mixture, f) showed CD profiles similar to that of the 34-mer duplex. These CD findings on the effects of phosphorothioate substitution on the conformation of the oligonucleotide triplex suggested that the presence of a mixture of diastereoisomers in the internucleotide bonds of the oligonucleotide hinders the formation of a triplex helix, because of steric interactions between the duplex and single strands.

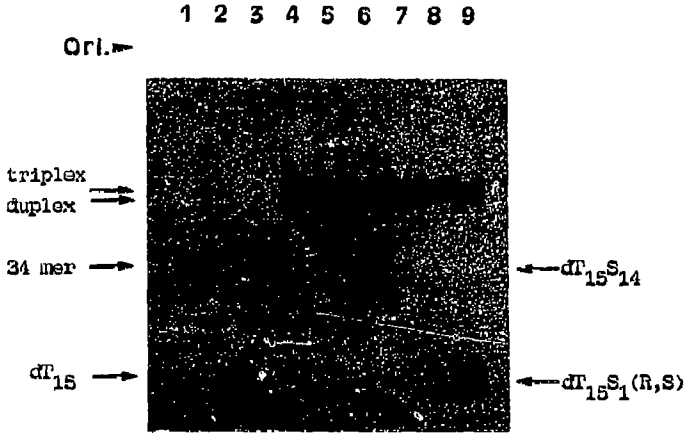

Fig. 3. 15% Non-denaturing polyacrylamide gel electrophoresis (pH 8.3). Lane 1, pyrimidine 34-mer; 2, dT_{15}; 3, $dT_{15}S_{14}$; 4, duplex 34-mer; 5, duplex-dT_{15}; 6, duplex $dT_{15}S_{14}$; 7, duplex $dT_{15}S_1$ (R); 8, duplex $dT_{15}S_1$ (S); 9, duplex $dT_{15}S_1$ (R,S).

Finally, the formation and stability of the triplexes containing phosphorothioate oligonucleotides were confirmed by the gel retardation assay [17]. As Fig. 3 shows, the single-, double-, and triple-stranded species can be separated on a 15% polyacrylamide gel at a low temperature. A band corresponding to the duplex (lane 4) and three bands corresponding to the triplexes were formed by the addition of dT_{15} (lane 5), $dT_{15}S_1$ (R) (lane 7) and $dT_{15}S_1$ (S) (lane 8), respectively, to the duplex. However, a band with mobility similar to that in lane 4 (duplex) was obtained for $dT_{15}S_{14}$ and $dT_{15}S_1$ (RS) (lane 5 and 9). The instability of the triplex $dT_{15}S_{14}$ and $dT_{15}S_1$ (RS), found in the above T_m (Table II) and CD (Fig. 2) measurements, was supported by the band corresponding to the duplex in lane 4.

In conclusion, the oligonucleotides having phosphorothioate internucleotidic bonds of a mixture of diastereoisomers can not form triple helical structures, whereas one of the two stereoisomers (Rp or Sp) can form triple helical structures with double strands. In particular, the whole phosphorothioate–diester oligomer having Rp or Sp had a marked affect on triplex formation [6]. Thus thio–DNAs (Rp or Sp) are more resistant to nuclease than the parent polymers [15,16]. Therefore, they may be useful as 'antisense' compounds for inhibiting viral replication or transcription from specific genes.

Table II

Melting temperature [T_m (°C)] of the 34-mer duplex and triplexes

Entry	Strands	100 mM NaCl		500 mM NaCl		1000 mM NaCl	
1	Duplex		63		73		76.5
2	dT_{15}	48	63	53.5	73	NT	76.5
3	$dT_{15}S_{14}$	NT	63	NT	73	NT	76.5
4	$dT_{15}S_1$(R)	35	63				
5	$dT_{15}S_1$(S)	25	63				
6	$dT_{15}S_1$(R,S)	NT	63	NT	73		

NT indicates that no transition larger than 0.01 absorbance units was observed.

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