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Enhanced P-stereodependent stability of complexes formed by phosphorothioate oligonucleotides due to involvement of sulfur as strong hydrogen bond acceptor*

Piotr Guga‡, Małgorzata Boczkowska, Magdalena Janicka, Anna Maciaszek, Barbara Nawrot, Sławomir Antoszczyk, and Wojciech J. Stec

Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Department of Bioorganic Chemistry, Sienkiewicza 112, 90-363 Łódź, Poland

Abstract: The antisense or antigene properties are exerted not only by natural oligonucleotides, but also by their different analogs. Among them, phosphorothioate oligonucleotides (PS-DNA), in which the sugar-phosphate backbone is modified due to substitution of the sulfur atom for one of the nonbridging oxygens, are much more resistant toward nucleases and, simultaneously, maintain good hybridization properties. However, the substitution induces the P-chirality of dinucleoside phosphorothioate moiety and even short PS-DNA synthesized using standard chemical methods exist as a mixture of hundreds or thousands of diastereoisomers. Diastereomerically pure oligomers of [PS]-d$(CG)_4$ and [PS]-d$(GC)_4$ series, obtained using the oxathiaphospholane method, were investigated with respect to their ability to adopt the left-handed conformation at high sodium chloride concentration. Obtained data allow us to postulate the formation of a strong intramolecular hydrogen bond with anionic sulfur atom as an acceptor. Homopurine [All-R_P-PS]-oligos, but not [All-S_P-PS]-oligos, form with the RNA templates extremely stable triplex structures, so far not described in the literature. Most likely, the triplexes are stabilized by hydrogen bonds or water bridges with the participation of sulfur atoms of internucleotide linkage. Notably, target RNA molecules are "arrested" by properly designed [All-R_P-PS]-DNA probes at submicromolar concentration, and as the result, they *are not* recognized by reverse transcriptase.

Keywords: oligo(nucleoside phosphorothioate)s; P-chiral phosphorothioates; antisense strategy; B-Z transition; triplexes.

INTRODUCTION

Oligonucleotides and their analogs have recently found broad application in biochemistry and molecular biology as new tools for studying interactions of nucleic acids with other biomolecules, and as potential candidates for therapeutics. Research on application of synthetic oligodeoxyribonucleotides for the inhibition of gene expression, as well as on their implementation in anticancer and antiviral therapy, is based upon the observations that oligonucleotide probes complementary to selected fragments of

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‡Corresponding author

DNA (so-called *antigene strategy*) or mRNA molecules (*antisense strategy*) may hybridize with those templates, affecting in that way their biological functions. Thus, DNA molecules acting as potential therapeutic agents, if stable enough to survive in the body fluids and if efficiently delivered into the cells to reach sufficient concentration, may "arrest" unwanted mRNA to form DNA/RNA duplexes, further cleaved by RNase H, and in this way inhibit expression of encoded onco-proteins or prevent viral RNA from reverse transcription into DNA (Fig. 1). Any of these processes would be beneficial to the organism, provided that the interactions are highly sequence-specific, so the probe used does not affect other important metabolic pathways.

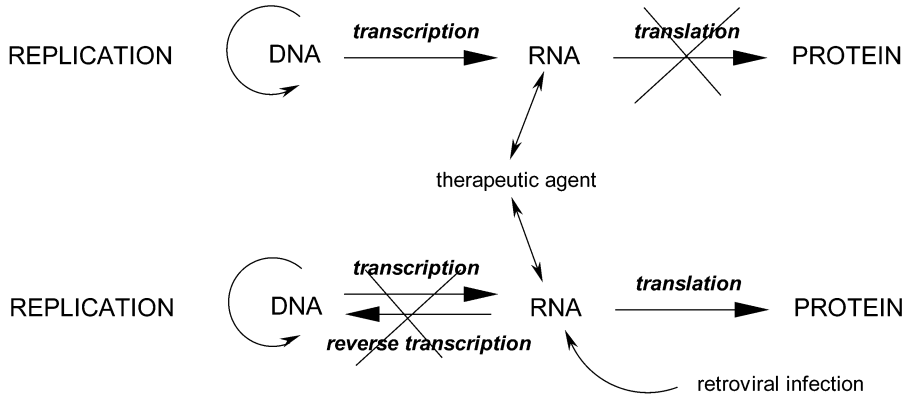


Fig. 1 Possible places of interactions with RNA to prevent translation or reverse transcription.

Properties of antisense probes and discussion of the mechanism of their action can be found in several excellent reviews [1]. Other explored ways of action of oligonucleotide therapeutics include use of: triplex-forming oligos, ribozymes, DNAzymes, siRNA, and others.

The antisense properties are exerted not only by oligonucleotides, but also by their analogs, sometimes of structures very much different as compared to the original DNA molecule (Fig. 2), like PNA (*peptide nucleic acids*) [2] or LNA (*locked nucleic acids*) [3].

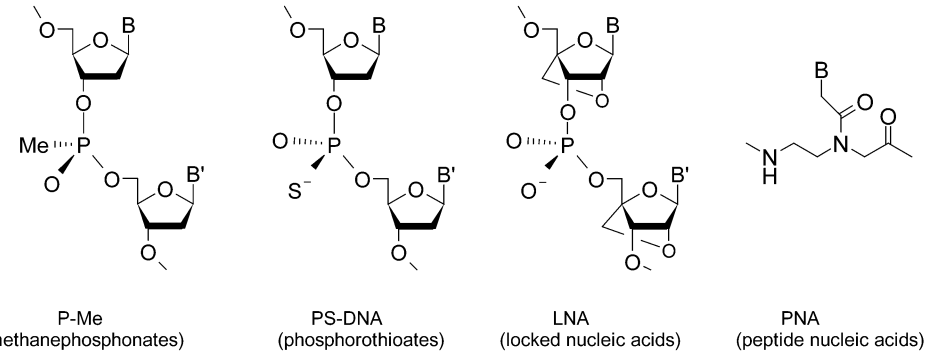
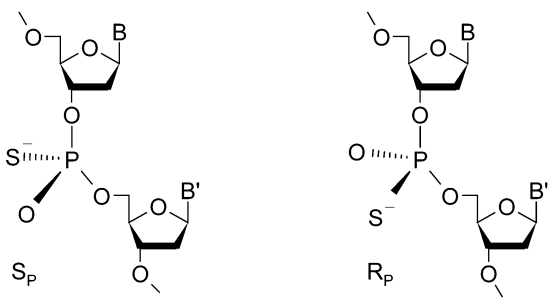


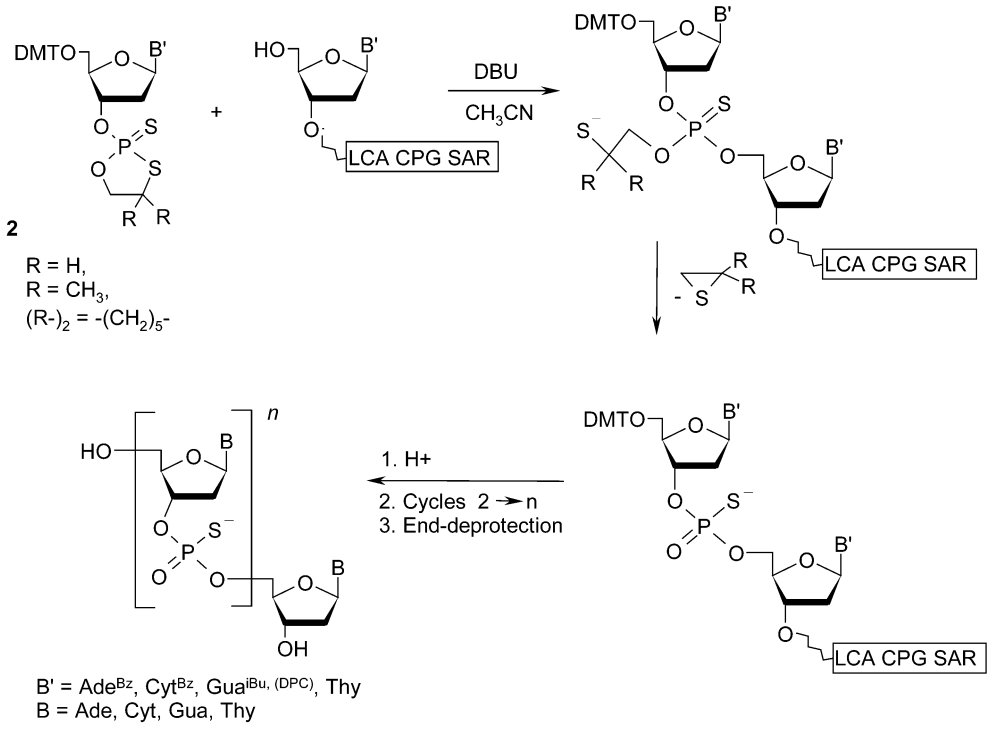
Fig. 2 Examples of DNA analogs possessing antisense properties. B = nucleobase.

PHOSPHOROTHIOATE ANALOGS OF DNA

The ability of short single-stranded DNA probes to act in vivo according to any of the above-mentioned therapeutic approaches requires their sufficient half-life in the inter- and intracellular media. Stability

against nuclease-assisted degradation can be achieved by appropriate sugar-phosphate backbone modification. One of the early introduced modifications was a change within the phosphate linkage, namely, substitution of sulfur atom for one of the nonbridging oxygen atoms attached to the phosphorus [4]. In this way, phosphorothioate DNA analogs (PS-DNA) were obtained and introduced into research as specific tools in numerous areas of molecular and cell biology. However, the substitution creates a new center of asymmetry at the phosphorus atom. The shortest possible PS-DNA fragment (i.e., 3',5'-dinucleoside phosphorothioate), if prepared in the nonstereospecific manner, consists of the mixture of two P-diastereomers of either S_P or R_P absolute configuration (**1a** or **1b**, respectively; B = adenine, cytosine, guanine, or thymine) which differ in spatial location of sulfur atoms in internucleotide phosphorothioate linkages. For longer oligonucleotides containing *n* modified internucleotide bonds, the number of existing P-diastereomers *N* is given by the equation $N = 2^n$. Commonly used methods for chemical synthesis of PS-DNA, such as, for example, the phosphoramidite method with sulfurization of the P^{III}-intermediate triester [4c], provide the final product as a mixture of all possible P-diastereomers. Such polydiastereoisomerism means that even for relatively short PS-oligos (10–15 mers) thousands of diastereomers [5] would be involved in interactions with other chiral biomolecules, like DNA, RNA, or proteins, and, in principle, each diastereomer may interact in a slightly different mode. Although PS-DNA are much more resistant toward nucleases, compared to natural DNA, the enzymes present in blood plasma, as well as those present inside the cells, are able to hydrolyze slowly internucleotide phosphorothioate linkages [6]. Therefore, the typical half-time of their presence under in vivo conditions does not exceed 24 h [7]. Most often, the nucleases are P-stereoselective and hydrolyze phosphorothioate bonds preferably of one absolute configuration—either R_P (snake venom phosphodiesterase or *Serratia marcescens* endonuclease) [8] or S_P (nuclease S1 and nuclease P1) [9]. We found that PS-DNA are hydrolyzed in blood plasma as well as inside the cells by action of R_P-specific 3'-exonucleases [6]. Also, it was found that the phosphorothioate modification affects thermodynamics of interactions with target DNA and RNA strands, as independent of absolute configuration of phosphorus centers the PS-DNA/DNA and PS-DNA/RNA duplexes have lower thermal stability compared to unmodified species [7b,10]. The data also showed that relative stability of the duplexes ([All-R_P]-PS-DNA/DNA vs. [All-S_P]-PS-DNA/DNA) depends on their sequential composition, rather than on the absolute configuration of PS-oligos [11]. Although, formally, PS-DNA are isoelectronic with natural DNA, the modification alters the distribution of negative charge within ambident anionic phosphorothioate moiety. The above-mentioned polydiastereoisomerism hampered an application of PS-DNA in mechanistic studies of its interactions with other biomolecules. Albeit several stereodefined PS-DNA oligomers were synthesized using dimer-block approach or enzymatic synthesis, a method for chemical synthesis of such constructs with any combination of absolute configuration of P-atoms was highly demanded. Such a method, based on condensation of diastereomerically pure oxathiaphospholane monomers **2**, was developed in our laboratory [12], and its principles are shown in Scheme 1.





Scheme 1 Synthesis of stereodefined oligonucleotides using the oxathiaphospholane method. LCA CPG SAR = long-chain alkylamino controlled pore glass with DBU-resistant sarcosinyl linker.

The stereodefined PS-DNA were successfully used in numerous studies, like structural aspects of DNA (formation of duplexes [11], P-chirality-dependent stability of the i-motif [13]), analysis of factors affecting ability of $(CG)_4$ and $(GC)_4$ oligomers to perform the B-Z transition [14], elucidation of the mode of action of nucleolytic enzymes [6,15] and deoxyribonucleotidyl transferase [16], as well as investigation on immunostimulating effect of ...C_PG... motif. [17] and aptameric effect [18]. Some of these results, together with new observations reported below, prompted us to reconsider commonly accepted opinion on poor hydrogen bond acceptor properties of phosphorothioate function [19].

P-STEREODEPENDENCE OF B-Z TRANSITION OF PS-DNA

In 1985, using natural and chimeric, partially modified, stereodefined PS-DNA octamers, Cosstick and Eckstein showed that: (1) $d(CG)_4$ undergoes the B-Z transition with a midpoint of transition at 3 M NaCl; (2) $d(GC)_4$ does not convert because of difficult nucleation without dG residue at the 3'-end; (3) [S_P]-d-[$C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G$ converts at 4 M NaCl; and (4) [R_P]-d[$C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G$] does not undergo transition into the Z-form [20]. We extended that work by synthesis of fully modified stereodefined [All-R_P-PS]-$d(CG)_4$ and [All-R_P-PS]-$d(GC)_4$, as well as their [All-S_P-PS] counterparts. Their B-Z conversion at increasing salt concentrations was followed by circular dichroism (CD) spectroscopy, where characteristic changes, i.e., disappearance of the negative band around 255 nm and increasing abundance of positive band at 275 nm were observed. Our CD measurements gave results that could be explained by the assumption that the sulfur atom of phosphorothioate moiety can interact strongly as hydrogen bond or water bridge acceptor [14]. This hypothesis was verified by synthesis of [All-S_PR_P-PS]-$d(CG)_4$ and [All-R_PS_P-PS]-$d(GC)_4$ possessing R_P and S_P configuration of P atoms at juxtaposed positions. Their CD spectra (shown in Fig. 3) indicate that [All-S_PR_P-PS]-$d(CG)_4$ converts

into the Z form at 2.5 M NaCl (i.e., easier than unmodified $d(CG)_4$, panel A) and [All-R_PS_P-PS]-$d(GC)_4$ is ca. 50 % converted at 5 M NaCl, while unmodified $d(GC)_4$ does not undergo the B-Z transition at all.

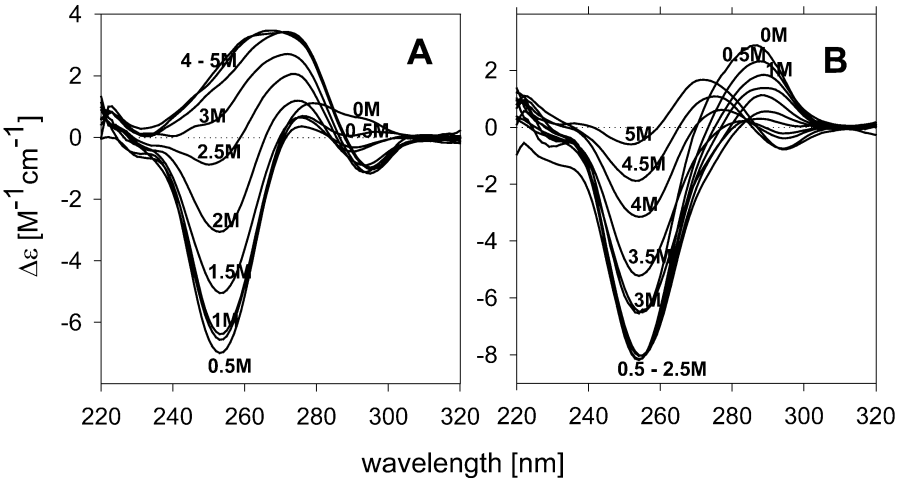


Fig. 3 CD spectra for [All-S_PR_P-PS]-$d(CG)_4$ (panel A) and [All-R_PS_P-PS]-$d(GC)_4$ (panel B) at increasing NaCl concentration, ranging from 0.1 to 5 M and marked at corresponding curves.

These results support our assumption and can be explained in terms of already known interactions taking place during the B-Z transition (Fig. 4). The dotted line in panel A shows interaction stabilizing the intermediate formed during nucleation, when the guanine base makes rotation into *syn*-conformation. The dotted lines in panel B show a water bridge stabilizing final Z-form. In [All-S_PR_P-PS]-$d(CG)_4$ and [All-R_PS_P-PS]-$d(GC)_4$ we have Y = S and X = O at the 5'-side and X = S at the 3'-side. Thus, if sulfur is involved in strong hydrogen bonding (which, in fact, is a charge-assisted hydrogen bonding), strong stabilization of the final Z-form occurs with slightly weaker stabilization of the intermediate shown in panel A. Even lack of the dG residue at the 3'-end of [All-R_PS_P-PS]-$d(GC)_4$, which slows down the nucleation, does not hamper the conversion. If in the intermediate shown on panel A Y = O and X = S, like in Eckstein's [R_P]-d[$C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G_{PO}C_{PS}G$] and in our [All-$R_P$-PS]-$d(CG)_4$, strong interactions N^2–H•••$^-$S–P anchor the guanine ring and do not allow the completion of the rotation around the glycosidic bond. As a result, the B-Z conversion does not take place. A few reports in the literature indicate that despite the comparatively diffused character of the acceptor electron pair, the ionized sulfur atom is able to form strong hydrogen bonds. X-ray analysis of thiolate salt revealed strong SH•••$^-$S hydrogen bond (3.454 vs. 4.35 Å calculated for pure van der Waals contacts) [21] and H-bonded dimers were identified in crystalline dithiophosphinic acids [22]. In the area of phosphorothioate compounds, intermolecular hydrogen bonds N^3–H•••$^-$S–P (3.596 Å) between anionic sulfur and endocyclic nitrogen atoms were detected in crystals of tetramethylammonium salt of 2-oxo-2-thio-1,3,2-oxazaphosphorinane, where an alternative interaction N^3–H•••$^-$O–P could be expected due to identified equilibrium between two mesomeric forms S=P–O$^-$ and O=P–S$^-$ [23]. Regarding biophosphates, Uhlenbeck and coworkers showed that a single-site-modified 15-nucleotide RNA molecule binds 20-fold tighter to MS2 coat protein than the unmodified RNA when the pro-R_P oxygen atom of internucleotide linkage, involved in hydrogen bonding with asparagine moiety, was replaced with a sulfur atom [24]. When a sulfur atom was substituted for the pro-S_P oxygen atom, which does not interact with asparagine side chain, the binding was only 1.6 times strengthened.

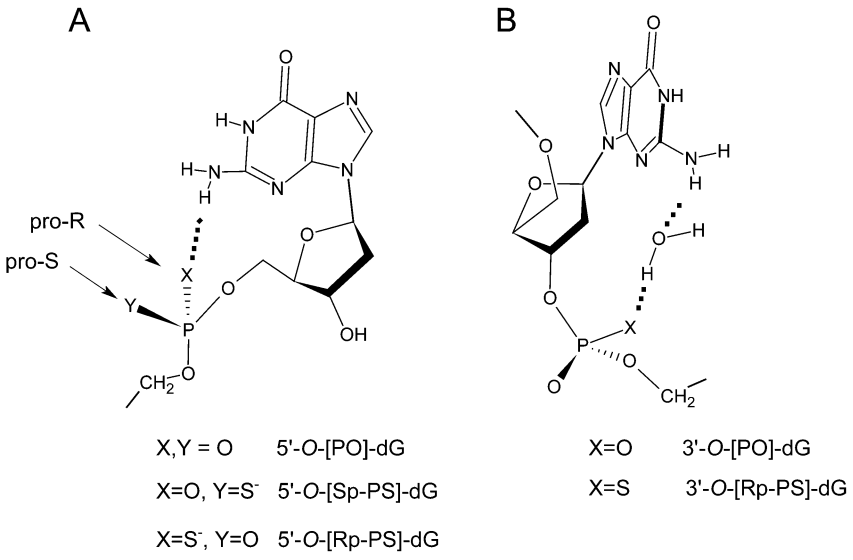


Fig. 4 Schematic diagram showing the hydrogen bonding (dotted lines) between the amino group at position 2 of the guanine and the 5'-phosphate group (X, Y = O), 5' S_P-phosphorothioate group (X = O, Y = S) or 5' R_P-phosphorothioate group (X = S, Y = O) during nucleation (panel A) and the water bridge formed with participation of the amino group at position 2 of the guanine and the 3' phosphate group (X = O) or the 3' R_P-phosphorothioate group (X = S) (panel B).

HIGH THERMAL STABILITY OF PS-DNA/RNA COMPLEXES

As mentioned earlier, usually PS-DNA/DNA and PS-DNA/RNA complexes have lower thermal stability compared to unmodified congeners [10]. However, we found that this generally accepted opinion was not true for certain homopurine [All-R_P-PS]-DNA oligomers interacting with complementary (in the Watson–Crick sense) RNA templates. Detailed analysis, including titration experiments, indicates that if the homopurine sequences of at least six nucleotides are palindromic, parallel triplexes RNA/PS-DNA/RNA are formed, while only duplexes RNA/DNA are observed for [PO]-, [Mix-PS]- and [All-S_P-PS]-DNA. Two examples, where whole strands are perfectly palindromic, are shown in Table 1, entries 1 and 2. The parallel orientation of the third strand means that the triplex is stabilized by normal Hoogsteen hydrogen bonds [25] (see Fig. 5). If the palindromic tracks are too short (Table 1, entry 3, fragments 5'-d(…GAAAG… or 5'-d(…GAGAG)-3', the thermally stable triplex is formed only when the RNA strand complementary in the Hoogsteen sense (5'-CUCCUUUCUCUC-3') is added to the mixture of homopurine [All-R_P-PS]-DNA and Watson–Crick complementary RNA, giving rise to the formation of structure **3** (T_m = 60 °C). In the absence of the Hoogsteen RNA strand, only imperfect triplexes of low thermal stability can be formed. Two of them are exemplified by structures **4** (the mismatches are underlined) and **5**. Interestingly, the triplexes formed by phosphorothioate DNA dodecamers containing 4–6 dG residues are thermally stable at pH 7.4 (T_m 55–80 °C), but their stability is significantly enhanced at pH 5.3. To the best of our knowledge, such triplex structures have not so far been described in the literature.

5’- CUCCUUUCUCUC-3’	5’- CUCUCUUUCCUC-3’	5’- CUCUCUUUCCUC-3’
5’-d(GAGGAAAGAGAG)-3’	5’-d(GAGGAAAGAGAG)-3’	5’-d(GAGGAAAGAGAG)-3’
3’- CUCCUUUCUCUC-5’	3’- CUCCUUUCUCUC-5’	3’- CUCCUUUCUCUC-5’
3	**4**	**5**

Table 1 Melting temperatures (T_m) for complexes of [PO]-DNA and [PS]-DNA with complementary RNA mixed at molar ratio 1:1. Buffer 10 mM tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4. Temperature gradient 0.2 °C/min.

Entry	Sequence of DNA strand	Form of DNA	T_m (°C)
1	5'-d(AAAAAAAAAAAA)-3'	[PO]	23
		[All-R_P-PS]	37
		[All-S_P-PS]	18
2	5'-d(GAGAAAAAAGAG)-3'	[PO]	26
		[All-R_P-PS]	54
		[All-S_P-PS]	16
3	5'-d(GAGGAAAGAGAG)-3'	[PO]	40 (40)[a]
		[All-R_P-PS]	35 (60)[a]
		[Mix-PS]	33 (36)[a]

[a]Melting temperature for an equimolar mixture of 5'-d(GAGGAAAGAGAG)-3' and two RNA strands complementary in Watson–Crick and Hoogsteen sense.

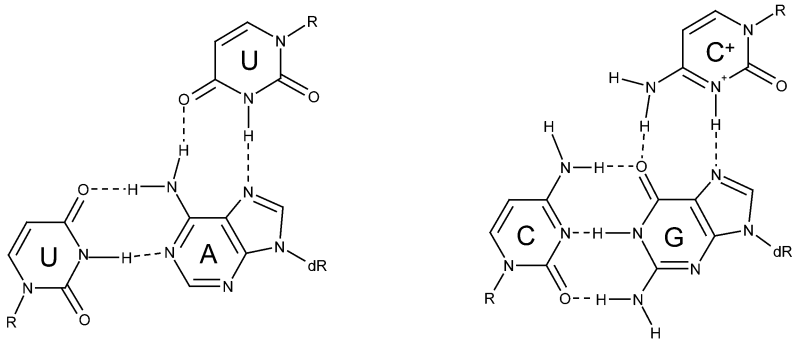


Fig. 5 Normal Hoogsteen (vertical dashed lines) and Watson–Crick (horizontal dashed lines) hydrogen bonds present in triplets U-A-U and C-G-C^+ in a parallel triplex. R = ribose, dR = deoxyribose.

The following two observations are corollary for explanation of such phenomena at molecular level: (a) only R_P-PS-DNA (and not S_P counterpart) can form the triplex, (b) only homopurine DNA, and, hence, homopyrimidine RNA strands interact so strongly. Thus, the spatial orientation of the sulfur atoms is crucial for the overall stability at the DNA edge, while the structural motif involved in the stabilization at the Hoogsteen RNA edge must be common for uridine and cytidine (e.g., oxygen atom in position 2 of pyrimidine ring). Since aforementioned results of studies on B-Z transition could be explained by the mechanism involving formation of strong hydrogen bonding with phosphorothioate moiety as the acceptor, we believe that similar interactions may stabilize the triplex. One of the possible structures, where a water bridge spans DNA and Hoogsteen RNA strands, is depicted on Fig. 6, although we realize that unambiguous information may be obtained from X-ray crystallography experiments or NMR studies, which are currently in progress.

It is worth mentioning that our reverse transcription polymerase chain reaction (RT-PCR) experiments showed that RNA molecules can be selectively "arrested" by properly designed [All-R_P-PS]-DNA probes at submicromolar concentration, and as a result, the amount of RNA available as a template for reverse transcriptase is substantially reduced (data not shown). This observation kindles hopes for novel gene-silencing strategy.

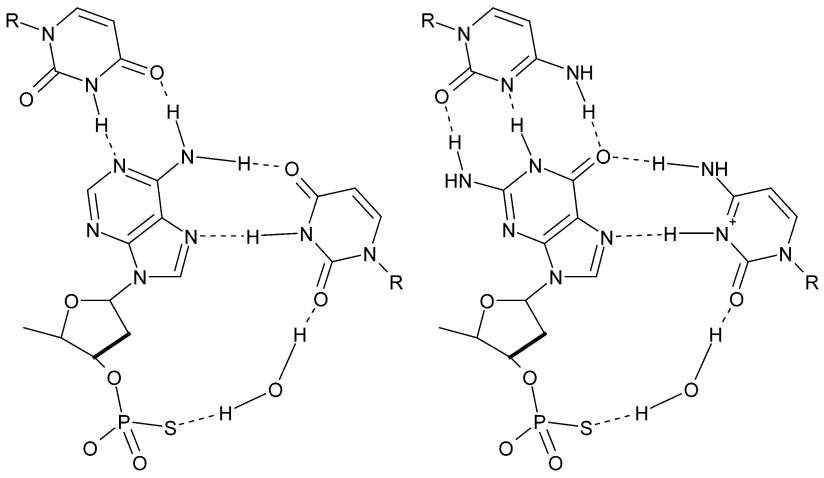


Fig. 6 Proposed scheme of hydrogen bonding and water bridge in the RNA/[All-R_P-PS]-DNA/RNA triplex. R = ribose.

SUMMARY

The oxathiaphospholane method allowed for the first time the preparation of PS-DNA of predetermined sense of chirality at each phosphorus atom of internucleotide phosphorothioate linkage, although in the meantime, other methods claiming the accessibility of stereodefined PS-oligos have been published [26]. Unusual properties observed for stereodefined constructs, like high avidity toward RNA strands presented for the first time in this communication, reflect astonishing functionality of phosphorothioate moieties, where sulfur atoms present in proper spatial orientation confer the phosphorothioate oligomers unique hybridization and thermodynamic properties, as compared to unmodified oligonucleotides. In our interpretation, these properties may be due to strong hydrogen bonds resulting from uneven distribution of negative charge between sulfur and nonbridging oxygen atom within phosphorothioate moiety. Also, we anticipate that greater atomic radii of sulfur atom, as compared with oxygen, may allow for the formation of new hydrogen bonds, which are not present (or are relatively weak) in unmodified complexes because of too long distance between donor and acceptor atoms. Thus, on the interface of DNA and RNA world, stereodefined PS-DNA, which are relatively stable in body fluids, due to strong association with target RNA molecules in the sequence-dependent manner, may be applied for modulation of gene expression and, on that way, used to combat diseases caused by retroviruses.

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