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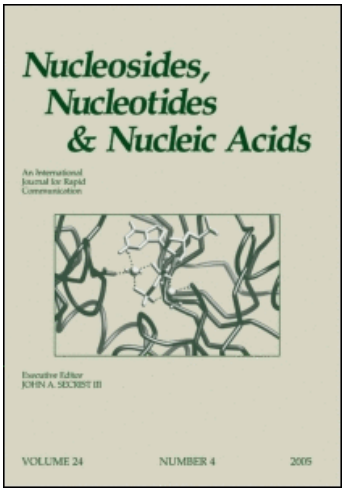

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ENZYMATIC SYNTHESIS OF STEREOREGULAR (all Rp) OLIGONUCLEOTIDE PHOSPHOROTHIOATE AND ITS PROPERTIES

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Abstract: A protocol has been established for the synthesis of stereoregular (all Rp) oligonucleotide phosphorothioates. A 25-mer oligodeoxynucleotide phosphorothioate has been synthesized and studied for biophysical and biochemical properties.

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Oligodeoxynucleotide phosphorothioates (PS-oligonucleotides) are being widely studied for their properties as antisense oligonucleotides . The PS-oligoncleotides used for all the studies have been obtained after chemical synthesis using either the phosphoramidite or H-phosphonate approach. Since the phosphorothioate linkage is chiral, it gives Rp and Sp diastereoisomers. In chemically synthesized PS-oligonucleotides, there is no control on the stereospecificity, it generally gives a mixture of 40% Rp and 60% Sp population. These diastereosomers may have different biochemical and biophysical properties.

The present study has been carried out to establish procedure to synthesize stereoregular PS-oligonucleotides and compare the biochemical and biophysical properties of the stereoregular PS-oligonucleotide (PS-Rp) and synthetic PS-oligonucleotide (PS).

To carry out the study, we have selected a 25-mer PS-oligonucleotide (5'-CTCTCGCACCCATCTCTCTCCTTCT) which has shown significant anti-HIV activity in various assays and is presently in human clinical trials[1].

The procedure established for synthesizing stereoregular PS-olignucleotides (all Rp) is outlined in Figure 1. In earlier studies, carried out by Eckstein[2], it has been shown that DNA polymerase uses only Sp isomer of 2'-deoxynucleotide-5'-(∝-thio) triphosphate during extension to produce Rp linkage. However, the studies are limited to incorporation of a single phosphorothioate linkage. To obtain a defined oligonucleotide sequence, we have designed an appropriate template and primer.

Synthesis of the template was carried out using β-cyanoethyl phosphoramidite chemistry. During the synthesis of the primer, we incorporated a ribonucleotide, at position shown by using ribonucleotide phosphoramidite. After the synthesis,

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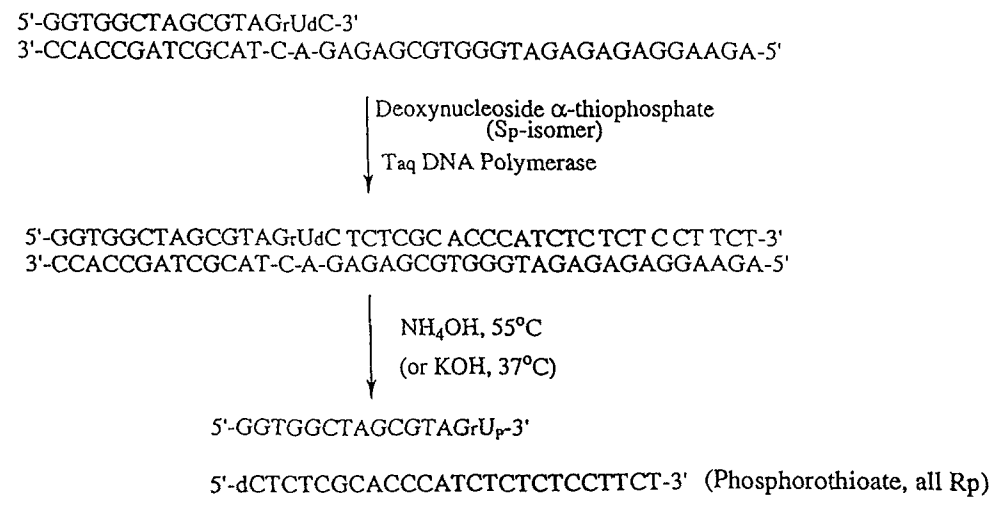

Fig. 1. Scheme showing synthesis of stereoregular (all Rp) PS-oligonucleotide. In a 30 µl volume of 50 mM tris pH 9.0, 10 mM $MgCl_2$, 8 nmoles primer, d-GGTGGCTAGCGTAGrUC and 2.5 nmoles template, d-AGAAGGAGA-GAGATGGGTGCGAGAGACTACGCTAGCCACC were preannealed by heating at 90°C for 3 min and allowed to cool at room temperature for 1 hour. The reaction volume was increased to 1.5 ml containing 50 mM tris pH 9.0, 10 mM $MgCl_2$, 5 mM DTT, 100 mM deoxyguanosine 5'-(∝-thio) triphosphate, 100 mM deoxyadenosine 5'-(∝-thio) triphosphate, 200 mM deoxycytidine 5'-(∝-thio) triphosphate and 200 mM thymidine 5'-(∝-thio) triphosphate. The reaction was initiated by the addition of 2500 units of sequence grade Taq DNA polymerase. The mixture was incubated for 4 hours at 37°C. The reaction mixture was desalted and treated with of ammonium hydroxide solution (28-30%) at 55°C for 24 hours. The product was dried and purified on a 20% denaturing polyacrylamide gel electrophoresis. The product was excised from the gel under UV shadowing and eluted with 500 mM ammonium acetate and desalted by dialysis.

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deprotection was carried out using an RNA protocol. This ribonucleotide was introduced to cleave off the synthesized stereoregular oligonucleotide by treatment with base.

Extension of the primer was carried out with Taq DNA polymerase using all four 2'-deoxynucleotide 5'-(∝-thio) triphosphates. After the extension, the synthesized stereoregular (all Rp) PS-oligonucleotide was obtained by treating the duplex with 0.3 M KOH or conc. NH_4OH. The base treatment causes cleavage at the ribonucleotide linkage. After the treatment, the product was purified on 20% polyacrylamide gel electrophoresis. The band under UV shadowing was excised and desalted. Generally, synthesis carried out using 8 nmoles of primer and 2.5 nmoles of template gave 1 nmole of oligonucleotide product.

We have studied the comparative nuclease resistance of PS-oligonucleotides against T4 DNA polymerase, DNA polymerase (Pol I), bovine and human serum (Figure 2).

The results showed that Rp PS-oligonucleotide is less stable than chemically synthesized PS-oligonucleotide in bovine and human serum. Similar results were obtained with T4 DNA polymerase and DNA polymerase (Pol I) (data not shown).

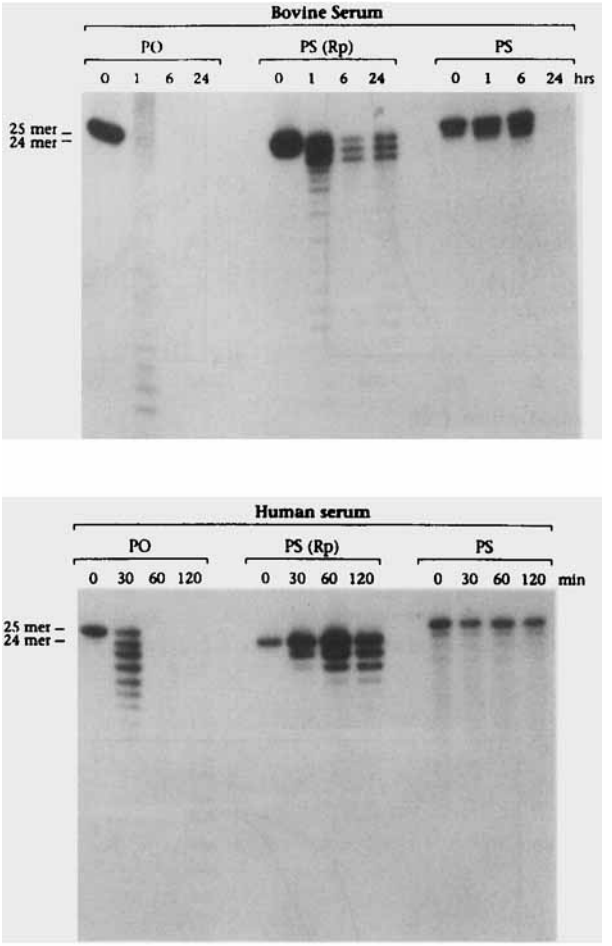

Fig. 2. Stability of phosphodiester (PO), stereoregular (PS-Rp) and synthetic PS-oligonucleotide (PS) against bovine and human serum. Experiment was carried out as described earlier[3].

Both Rp PS-oligonucleotide and synthetic PS-oligonucleotide were also compared for their affinity to the complementary DNA and RNA targets, as measured by Tm. Both PS-oligonucleotides showed the same Tm with DNA target (51.8° and 51.9°), however, Rp-PS-oligonucleotide had higher Tm (68.9°) compared to synthetic PS-oligonucleotide (64°) with RNA target. The difference in CD spectra for RNA duplexes is more significant than that for DNA duplexes, indicating that RNA target is more sensitive to the conformational change of Rp-PS vs. PS oligonucleotides than DNA target (Figure 3).

RP PS-oligonucleotide was found to be a stronger inhibitor of RT (Figure 4) and a better substrate for RNaseH (Figure 5) compared to synthetic PS-oligonucleotide. Rp PS-

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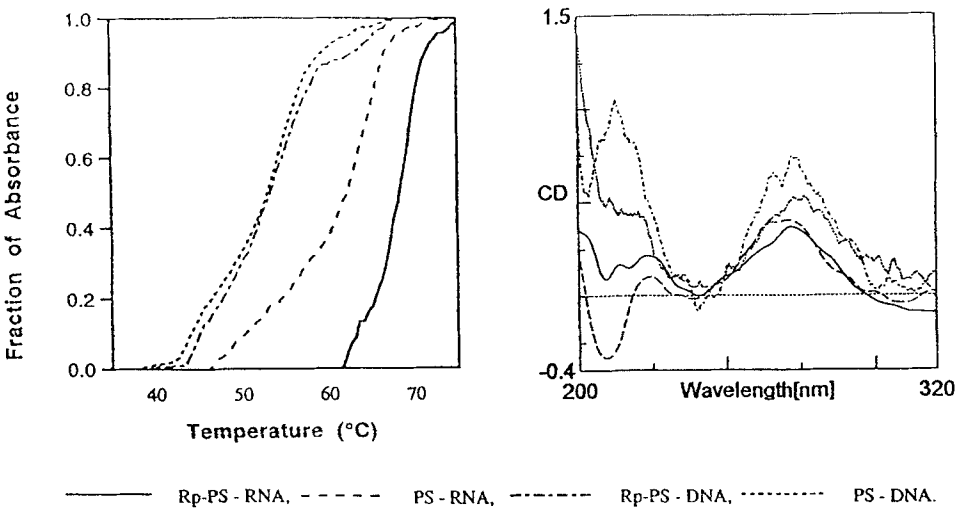

Fig. 3. Melting temperature (Tm) and circular dichroism of stereoregular (PS-Rp) and synthetic (PS) PS-oligodeoxynucleotide. Melting temperature studies were carried in 10 mM phosphate, 100 mM NaCl (pH 7) at 1.06 X 10^{-6} M oligonucleotide concentration using GBC 920 Spectrophotometer. CD spectra were obtained under the same sample condition as Tm using Jasco J-710 Spectropolarimeter.

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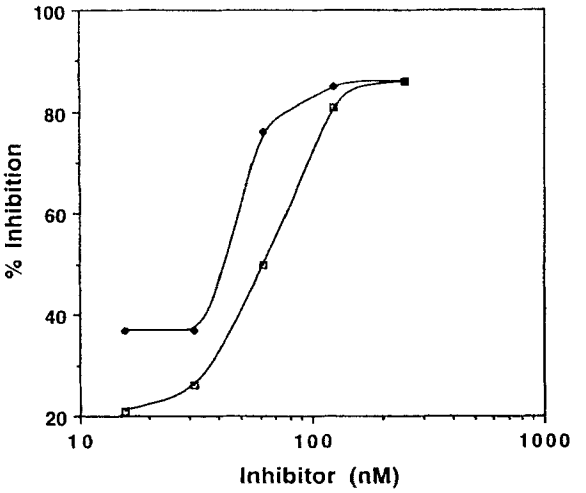

Fig. 4. *Inhibition of rHIV Reverse Transcriptase of Rp-PS* (♦) *and PS-Oligonucleotide* (□). A 10 µl reaction mixture contained 1 mM of the preannealed ^{32}P 5'-end labeled primer, d-GATTCAGCTAGTCCA and the template, d-CCAACTGTGATACGATGGACTAG-CTGAATC, 50 mM tris pH 8.3, 10mM $MgCl_2$, 50 mM KCl, 5mM DTT, 0.25 mM deoxynucleoside 5'-triphosphates (dNTPs) and various concentrations of oligonucleotide. After the addition of 0.8 units rHIV reverse transcriptase, the mixture was incubated at 37°C for 1 hour. The reaction was arrested with 10 µl formamide and then analyzed on a 20% denaturing polyacrylamide gel electrophoresis. CPMs were measured for the extension product, remaining primer bands and percentage of inhibition was calculated.

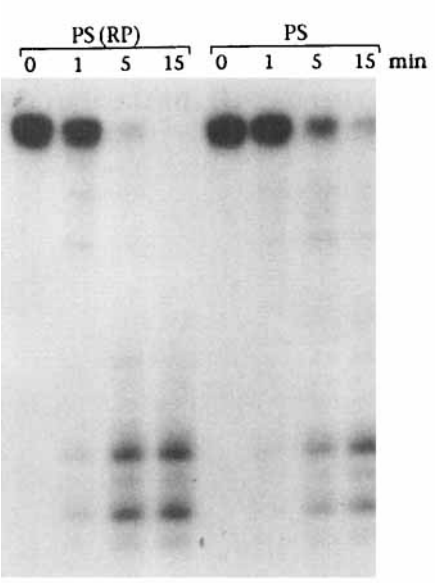

Fig. 5 *RNaseH Activity Assay;* 1 pmole of ^{32}P 3'-end labeled 39-mer HIV-I gag RNA (AGAAGGA-GAGAGAUGGGUGCGAGAGCGUCAGUAUUAA GC) was preannealed with 10 pmole of oligonucleotide in 10 µl buffer (20 mM tris pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM DTT, 5% glycerol). The reaction was brought up to a volume of 30 µl with adding the same buffer and 40 units of RNaseH. An aliquot of 7 µl was removed as time zero. 0.5 units of RNaseH was added to the remainder of the mixture and incubated at 37° C for 15 min. Aliquots of 7 µl were taken and quenched with 10 µl of formamide at time points of 1, 5 and 15 min. The mixture was analyzed on a 20% denaturing polyacrylamide gel electrophoresis and autoradiographed.

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oligonucleotides are being studied presently for their cellular uptake and gene regulation activity.

While this manuscript was in preparation, a similar procedure for Rp PS-oligonucleotide synthesis has been reported[4].

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