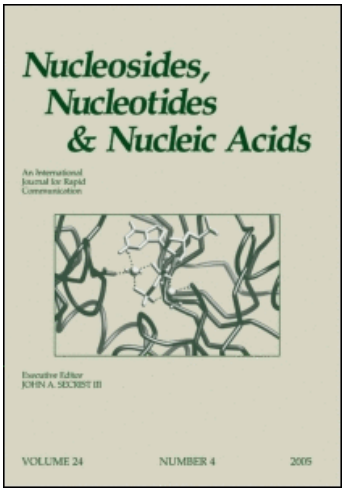

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PHOSPHOROTHIOATE ANALOGS OF 2-5A: ELUCIDATION OF THE STEREOCHEMICAL COURSE OF THE ENZYMES OF THE 2-5A SYNTHETASE / RNASE L SYSTEM ¶

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Abstract

Enzymatically and chemically synthesized 2-5A phosphorothioates stereochemically altered in the 2',5'-internucleotide linkages gave a series of metabolically stable chiral 2-5A derivatives that have been used to study the stereochemical requirements for binding to and activation of RNase L. The RpRp and SpRp, but not the RpSp or SpSp, trimer core isomers could bind to and activate RNase L.

Many biochemical events in cells are triggered by interferon, including induction of 2-5A synthetase which produces 2',5'-linked oligoadenylates [2-5A][†] upon activation by dsRNA[1,2]. The only known biochemical effect of 2-5A is activation of a 2-5A dependent endoribonuclease (RNase L); this enzyme hydrolyzes mRNA and rRNA, thereby resulting in an inhibition of protein synthesis. The 2-5A synthetase / RNase L system has been implicated in the inhibition of virus replication, regulation of cell growth and differentiation[1,2].

Numerous reports have appeared on the use of various dsRNAs and modified ATP analogs in an attempt to further characterize the 2-5A

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synthetase. Similarly, 2-5A† analogs have been used to describe the relationship between 2-5A structure and RNase L binding/activation in order to modulate the 2-5A system in an interferon-independent mode. With respect to RNase L, the requirements for binding and activation have been shown to be: (i) at least one 5'-phosphate group[3], (ii) at least three adenylate or adenylate-like residues with (iii) 2',5'-internucleotide linkages[4].

Numerous 2-5A analogs have been synthesized to examine RNase L activation. We have reported that the enzymatically and chemically synthesized cordycepin analogs of 2-5A inhibit protein synthesis in intact L cells and activate RNase L to cleave rRNA[5,6]. Eppstein and Torrence and coworkers have also reported that the cordycepin analogs activate L cell RNase L[7]. Our strategy is based on the use of phosphorothioate analogs of ATP to investigate the substrate specificity of 2-5A synthetase. Phosphorothioate analogs of ATP have provided an experimental method of stereochemical analysis of nucleophilic substitution reactions at phosphorus catalyzed by nucleotidyl transferases, phosphotransferases, phosphohydrolases, and certain ATP dependent synthetases.

Enzymatically and chemically synthesized phosphorothioate analogs of 2-5A have been prepared and have served as a most interesting species for studies of the relationship of 2-5A structure/chirality to RNase L binding and activation. The results presented here provide dramatic new information on the stereochemical requirements for recognition by RNase L.

†Abbreviations: 2-5A, 2,5-p_3A_n, 5'-O-triphosphoryladenylyl-(2'-5')-adenosine (n=2), 5'-O-triphosphoryladenylyl-(2'-5')-adenylyl-(2'-5')-adenosine (n=3); 2,5-A_3 core, adenylyl-(2'-5')-adenylyl-(2'-5')-adenosine; 2,5-$p_3A_n\alpha S$, 5'-O-(Sp)-1-P-thiotriphosphoryl-(Rp)-P-thioadenylyl-(2'-5')-adenosine (n=2), 5'-O-(Sp)-1-P-thiotriphosphoryl-(Rp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine (n=3); Rp dimer core, (Rp)-P-thioadenylyl-(2'-5')-adenosine; Sp dimer core, (Sp)-P-thioadenylyl-(2'-5')-adenosine; RpRp trimer core, (Rp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine; SpRp trimer core, (Sp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine; RpSp trimer core, (Rp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine; SpSp trimer core, (Sp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine.

MATERIALS AND METHODS

The α-phosphorothioate analogs of 2-5A were enzymatically synthesized from Sp-ATPαS and isolated in yields of 7-10% with the 2-5A synthetase from lysed rabbit reticulocytes as previously described from this laboratory[8]. The two isomeric dimer cores (Rp, Sp) and the four diastereomeric trimer cores (RpRp, SpRp, RpSp, SpSp) were chemically synthesized (manuscript in preparation). Mouse L929 cells were grown in monolayer culture and cell extracts were prepared as described[9]. Stability of the phosphorothioate analogs of 2-5A was measured in interferon-treated L929 cell extracts[8]. Radiobinding assays were performed according to Knight et al.[10] with 2,5-$p_3A_4[^{32}P]pCp$ of specific activity 3000 Ci/mmole. Ribosomal RNA cleavage assays were performed using the basic procedure of Wreschner et al.[11] The core-cellulose assay was performed according to Silverman[12].

RESULTS AND DISCUSSION

Structural proof and stabilities of chemically synthesized phosphorothioate dimer and trimer cores. The chemically synthesized Rp- and Sp-phosphorothioate dimer cores and four phosphorothioate trimer cores (RpRp, SpRp, RpSp, SpSp) were shown to be chromatographically pure by HPLC, TLC and ^{31}P-NMR. Proof of structure was based on a combination of ^{31}P-NMR results and enzymatic hydrolyses. A. Snake venom phosphodiesterase (SVPD). The Rp dimer core was completely hydrolyzed by SVPD to yield AMPS and adenosine as determined by HPLC and TLC[8]; the Sp dimer core was not a substrate for the enzyme. In contrast to previous reports that SVPD hydrolyzes both the Rp- and Sp-3,5-linkages (with a preference for the hydrolysis of the Rp linkage)[13-15], we have shown that SVPD hydrolyzes the Rp-2,5-phosphorothioate linkage, but not the Sp-2,5-phosphorothioate linkage. Based on these results, hydrolyses with SVPD[8] were performed with the four 2,5-phosphorothioate trimer cores (TABLE 1). Isomer 1 was a substrate for SVPD, revealing an accumulation of Rp dimer core and AMPS in a molar ratio of 1:1 following 30 min incubation (RpRp configuration assigned). Isomer 2 required 24 hr for complete hydrolysis to Sp dimer core (SpRp configuration assigned). Neither isomer 3 or 4 was a substrate for SVPD. Because SVPD was not able to distinguish between isomers 3 and

TABLE 1. Stabilities of the Chemically Synthesized Phosphorothioate Trimer Core Analogs of 2-5A

Trimer core isomer	Hydrolysis by:				Stereo-configuration assigned[c]
	SVPD		L cell extract		
	Dimer core isolated	Half-life	Dimer core isolated	Half-life	
1	Rp	1 hr	Rp	18 hr	RpRp
2	Sp	a	Sp	15 hr	SpRp
3	--	b	Rp	20 days	RpSp
4	--	b	--	b	SpSp
2,5-A_3 core	--	5 min	--	10 min	--

[a]Complete degradation in 24 hr.
[b]No degradation detected.
[c]Assignment confirmed by ^{31}P-NMR.

4, hydrolyses by 2'-phosphodiesterase from L cell extracts were used to identify these two diastereoisomers.

B. L cell extracts. Hydrolysis of authentic 2,5-A_2 and 2,5-A_3 cores by 2'-phosphodiesterase (2'-PDE) from L cell extracts was compared with that of the two dimer and four trimer phosphorothioate cores. 2,5-A_2 core is rapidly hydrolyzed by 2'-PDE[16]. However, the Rp and Sp dimer phosphorothioate cores were not substrates for 2'-PDE from L cell extracts (24 hr incubations). The resistance to hydrolysis of the Rp and Sp dimer cores, therefore, permitted the analysis of stereo-configuration of the four phosphorothioate trimer cores. Incubation of isomer 1 with L cell extracts resulted in 60% hydrolysis to Rp dimer core (RpRp configuration assigned). Hydrolysis of isomer 2 resulted in 68% hydrolysis and the isolation of Sp dimer core (SpRp configuration assigned). Isomer 3 showed 5% hydrolysis with the accumulation of the Rp dimer core (RpSp configuration assigned). There was no hydrolysis of isomer 4 (SpSp configuration assigned). Similar stereospecificity for hydrolysis by phosphodiesterase has also been reported using racemic mixtures of phosphorothioate trimer cores[17,18]. Eppstein et

al.[17] reported that these mixtures had anticellular and antiviral activity. On the basis of enzymatic hydrolyses and confirmation by ^{31}P-NMR it is now possible to assign the stereoconfigurations of the Rp and Sp dimer and the RpRp, SpRp, RpSp, and SpSp phosphorothioate trimer cores (please refer to FIG. 2 for the HPLC separation of the phosphorothioate trimer cores).

Structural proof and stability of the enzymatically synthesized α-phosphorothioate analogs of 2-5A. Earlier studies from this laboratory reported the enzymatic synthesis and characterization of the α-phosphorothioate trimer and tetramer analogs of 2-5A (i.e., 2,5-$p_3A_3\alpha S$ and 2,5-$p_3A_4\alpha S$) from Sp-[α-^{35}S]ATPαS[8] (FIG. 1). Both 2,5-$p_3A_3\alpha S$ and 2,5-$p_3A_4\alpha S$ were shown to inhibit protein synthesis in intact L cells[8]. With the four chemically synthesized trimer core phosphorothioates, it is now possible to firmly establish the stereoconfiguration of the 2,5-phosphodiester linkages in the enzymatically synthesized 2,5-$p_3A_3\alpha S$. The hydrolysis of the 2,5-$p_3A_3\alpha S$ with bacterial alkaline phosphatase (BAP) followed by HPLC analysis revealed that all of the ^{35}S resided under the peak equivalent to RpRp trimer core (FIG. 2). These results unequivocally show that the enzymatic synthesis of 2,5-$p_3A_3\alpha S$ from Sp-ATPαS proceeds via inversion to the Rp configuration. SVPD hydrolysis of 2,5-$p_3A_3\alpha S$ revealed that the 5'-O-1-thiotriphosphoryl group had the Sp configuration.[8]

Biological activity of chemically synthesized phosphorothioate trimer cores. Binding to and activation of RNase L have been clearly established as discrete phenomena[2]. Therefore, three assays were employed to examine the effect of stereochemical modification on the structural parameters of 2-5A recognized by RNase L. A. Radiobinding assay. The chemically synthesized phosphorothioate trimer cores (RpRp, SpRp, RpSp, SpSp) were able to displace the 2,5-$p_3A_4[^{32}P]pCp$ probe from the RNase L in mouse L929 cell extracts. All of the four phosphorothioate trimer cores were equally as potent as 2,5-A_3 core (FIG. 3). The concentrations of the trimer cores tested which were required for 50% displacement of probe were 2×10^{-6} to 5×10^{-6} M (FIG. 3), in good agreement with values previously reported for the 2,5-A_3 core[19]. The chemically synthesized Rp and Sp dimer cores did not show any binding activity up to 10^{-3} M.

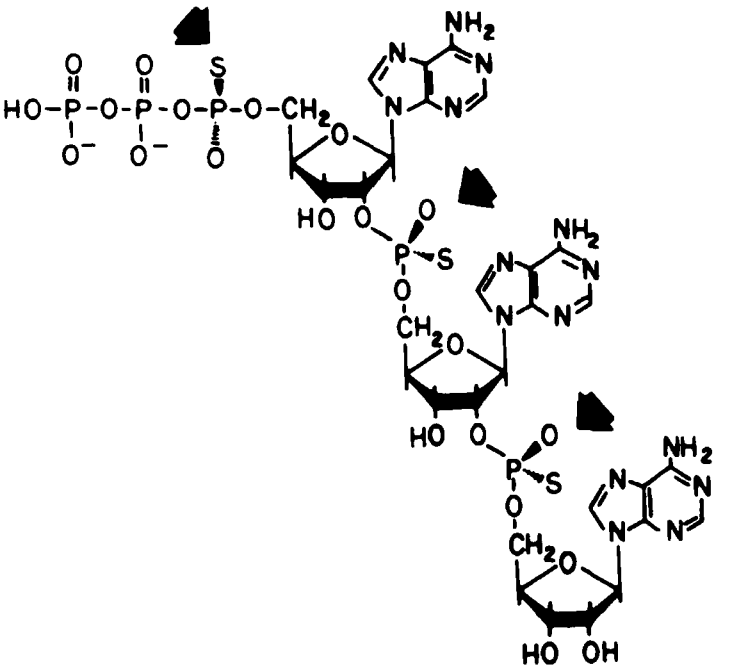

FIGURE 1. Structure of the α-phosphorothioate trimer analog of 2-5A synthesized from Sp-ATPαS, i.e., 5'-O-(Sp)-1-P-thiotriphosphoryl-(Rp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine.

B. Ribosomal rRNA cleavage. The effect of the chemically synthesized phosphorothioate trimer cores on the activation of RNase L to degrade 18S and 28S rRNA from intact ribosomes was compared with that of authentic 2,5-A_3 core. The most specific functional assay, the rRNA cleavage assay, was used to examine this effect[11]. The degradation of 18S and 28S rRNA by RNase L with the phosphorothioate trimer cores revealed dramatic new information about the activation of RNase L. The RpRp and SpRp diastereomers of the phosphorothioate trimer cores are effective in the rRNA cleavage assay at concentrations as low as 5×10^{-6} M (FIG. 4, lanes 3 and 7). In contrast, the RpSp and SpSp diastereomers did not cause any noticeable rRNA degradation, even at concentrations as high as 10^{-4} M (FIG. 4, lanes 4 and 8). Authentic 2,5-A_3 core was also without RNase L activating ability (FIG. 4, lane 1) as had been previously reported[12].

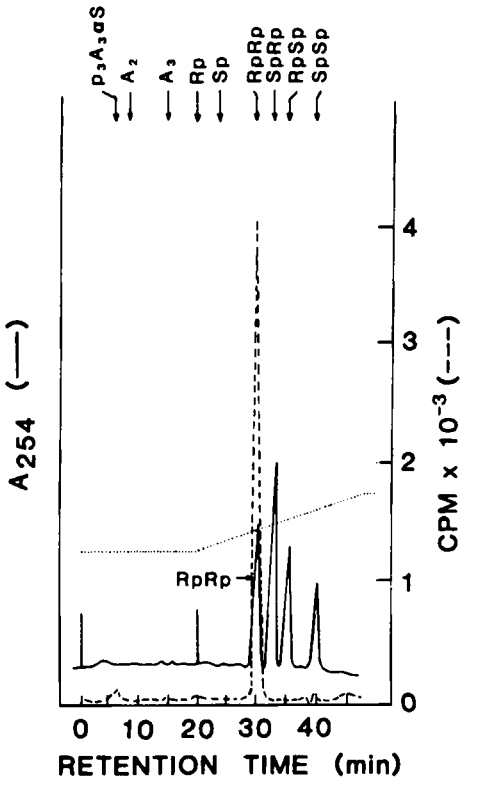

FIGURE 2. HPLC analysis of enzymatically synthesized 2,5-$p_3A_3\alpha S$ following BAP hydrolysis for 18 hr. Elution times of standard $p_3A_3\alpha S$, A_2, A_3, Rp dimer core, Sp dimer core, and RpRp, SpRp, RpSp and SpSp trimer cores are indicated by arrows. The dashed line indicates the radioactivity from the BAP hydrolysis product of 2,5-$[^{35}S]p_3A_3\alpha S$. The dotted line indicates the gradient used. HPLC conditions: reverse phase chromatography, Waters C_{18} μBondapak analytical column using an isocratic gradient: solvent A: 50 mM ammonium phosphate, pH 7.0; solvent B: methanol:water (1:1)(t=0-20 min, 30%B) followed by a linear gradient (t=20 min, 30%B; t=50 min, 40%B), flow rate = 1 ml/min.

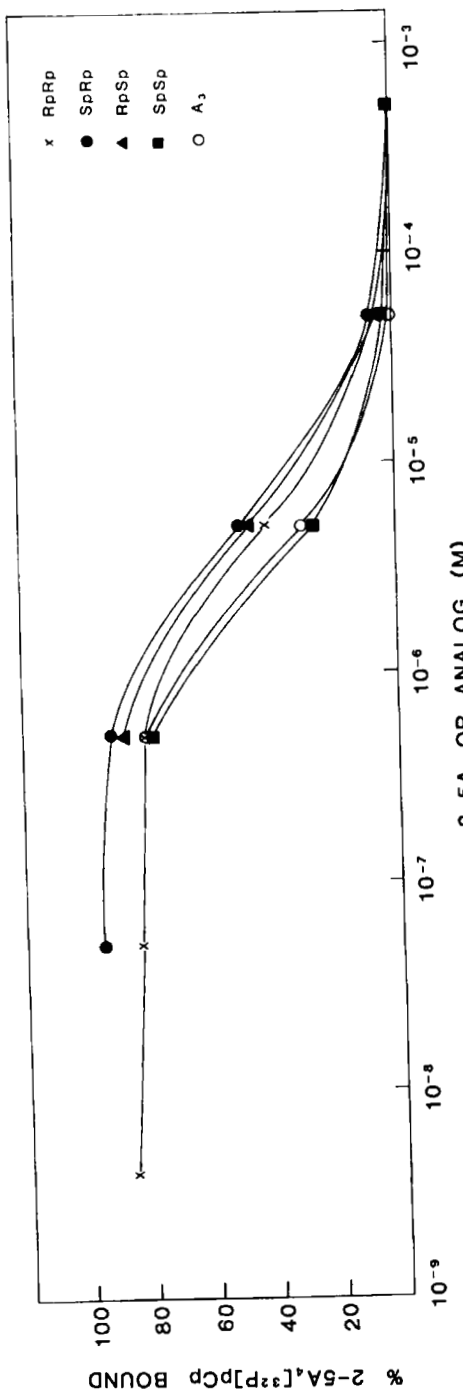

FIGURE 3. Radiobinding assay of the phosphorothioate trimer core analogs of 2-5A. The ability of the analogs to displace 2,5-$p_3A_4[^{32}p]pCp$ bound to RNase L in mouse L929 cell extracts was determined as described[10]. Approximately 60% of the 2,5-$p_3A_4[^{32}p]pCp$ was bound in the absence of added oligonucleotide (total cpm ≅ 23,000).

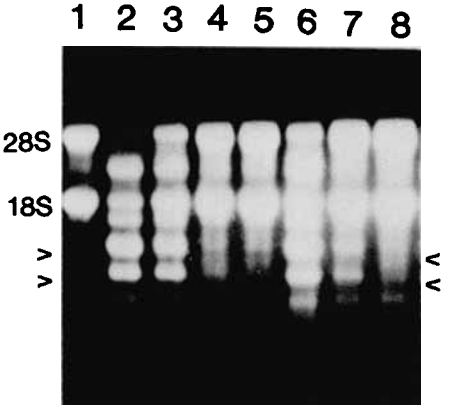

FIGURE 4. rRNA cleavage assay of phosphorothioate trimer core analogs of 2-5A. L929 cell extracts were incubated in the presence of authentic 2,5-A_3 core at 10^{-4} M (1) or phosphorothioate trimer cores at the following concentrations: RpRp, 10^{-5} M (2) and 5 x 10^{-6} M (3); RpSp, 10^{-4} M (4) and 1 x 10^{-5} M (5); SpRp, 10^{-5} M (6) and 5 x 10^{-6} M (7); SpSp, 10^{-4} M (8). The positions of 28S and 18S rRNA are shown; the arrows indicate the positions of the well-characterized specific cleavage products (SCP) of RNase L.

C. Degradation of polyU[^{32}P]pCp. Enzymatically synthesized α-phosphorothioate analogs of 2,5-p_3A_3 and 2,5-p_3A_4 were compared with authentic 2,5-p_3A_4 in their abilities to activate RNase L using a recently developed method based on the immobilization and partial purification of the RNase L on core cellulose[12]. In this technique, the hydrolysis of polyU[^{32}P]pCp to acid-soluble fragments after incubation with various 2-5A analogs can be quantitatively measured. The enzymatically synthesized α-phosphorothioate analogs, 2,5-$p_3A_3\alpha S$ and 2,5-$p_3A_4\alpha S$, are able to activate the purified RNase L as well as does authentic 2,5-p_3A_4 (FIG. 5).

Our strategy to create stereochemically modified 2-5A analogs has proven successful. The chemically synthesized diastereomers of the phosphorothioate trimer cores, with the same chemical structure

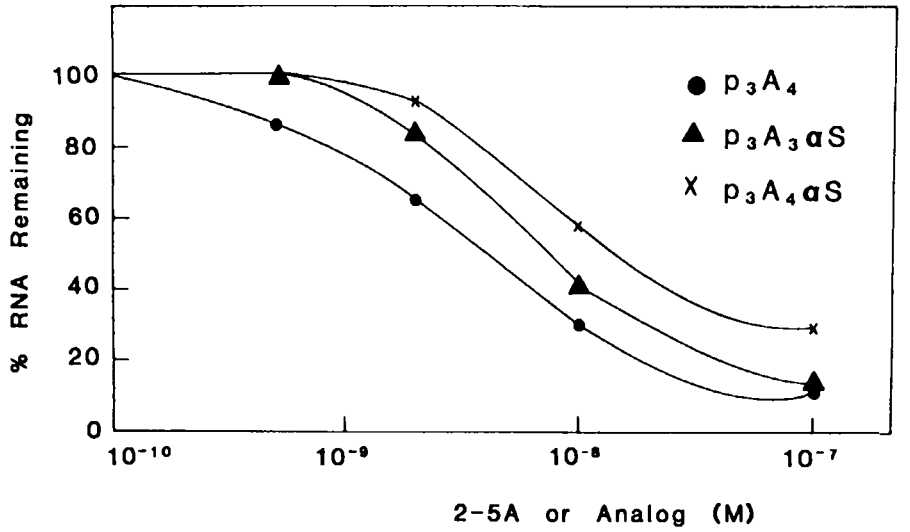

FIGURE 5. Core cellulose assay of the enzymatically synthesized α-phosphorothioate analogs of 2-5A. The core-cellulose assay was performed using the RNase L from L929 cell extracts as described[12]. Activation of RNase L was determined by conversion of polyU[^{32}P]pCp to acid-soluble fragments after incubation. 100% represents 15,000 cpm of labeled polyU[^{32}P]pCp bound to glass fiber filters in the absence of added 2-5A.

but different stereoconfigurations, show markedly increased stability, which is essential in the design of 2-5A analogs. Stereochemical modification of the 2,5-phosphodiester linkage changed the stability of the isomers towards SVPD and 2'-PDE (TABLE 1), but did not affect their binding affinity for RNase L (FIG. 3). However, the introduction of chirality into the internucleotide linkages conferred new properties on the 2,5-A_3 core molecule. The RpRp and SpRp stereomers of the phosphorothioate trimer cores are able to activate the RNase L to cleave 28S and 18S rRNA in rRNA cleavage assays (FIG. 4, lanes 3,7) at 5 x 10^{-6} M (the same concentration needed for 50% displacement of 2,5-p_3A_4[^{32}P]pCp in the radiobinding assay (FIG. 3)). The RpSp and SpSp trimer cores failed to activate RNase L even at concentrations as high as 10^{-4} M (the concentration which was sufficient to replace 100% of the 2,5-p_3A_4[^{32}P]pCp in the binding assay (FIG. 3)). Our results also suggest that binding of the 2-5A molecule

to the allosteric site of RNase L is followed by a conformational change in the enzyme.[3] Because we now have synthesized phosphorothioate trimer cores with different stereoconfigurations in the 2,5-phosphodiester linkages, we propose that in the case of the RpRp and SpRp stereomers the stereochemical modification facilitated the conformational change in RNase L that is required for its activation, whereas in the case of the RpSp and SpSp stereomers, the conformational change was hindered.

We have demonstrated that the enzymatically synthesized phosphorothioate analogs, 2,5-$p_3A_2\alpha S$ and 2,5-$p_3A_4\alpha S$, bind to (not shown) and activate (FIG. 5) RNase L as well as the authentic 2,5-p_3A_4. The enzymatically synthesized 2,5-phosphorothioates with the reported chirality in the phosphodiester backbone and in the pyrophosphoryl group (FIG. 1) gave optimal recognition and activation of RNase L.

Because 2,5-phosphorothioates containing the Sp configuration can not be synthesized enzymatically, we have chemically synthesized and fully characterized the 2,5-phosphorothioate dimer and trimer cores and their 5'-monophosphates (manuscript in preparation). Preliminary studies with these 2,5-phosphorothioate trimer cores and their 5'-monophosphates reveal marked differences in their binding to and activation of RNase L (manuscript in preparation). The 2,5-phosphorothioates have been used to further elucidate the effects of introducing chirality, possible conformational changes in the 2-5A molecule and/or RNase L as well as differences in charge distribution and charge density resulting from the P-O and P-S groups which could result in changes in the hydrophobic character of the 2-5A oligomer.

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