Phosphorothioate Analogues of 2′,5′-Oligoadenylate. Enzymatic Synthesis, Properties, and Biological Activities of 2′,5′-Phosphorothioates from Adenosine 5′-*O*-(2-Thiotriphosphate) and Adenosine 5′-*O*-(3-Thiotriphosphate)†

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ABSTRACT: The chiral and achiral phosphorothioate analogues of 2′,5′-oligoadenylates (2-5A) have been enzymatically synthesized from the S_P and R_P isomers of adenosine 5′-*O*-(2-thiotriphosphate) [(S_P)-ATPβS and (R_P)-ATPβS, respectively] and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) by 2-5A synthetase from L929 cells and lysed rabbit reticulocytes. These 2′,5′-phosphorothioate analogues were separated, purified, and structurally characterized. While ATPγS and (S_P)-ATPβS were as efficient substrates for the 2-5A synthetase as was ATP, (R_P)-ATPβS was more than 50-fold less efficient a substrate. The β- and γ-phosphorothioates were more resistant to enzymatic hydrolysis than was authentic 2-5A. Compared to 2-5A, there were marked differences in the biological activities of the 2′,5′-phosphorothioates as determined by (i) binding to 2-5A-dependent endoribonuclease (RNase L), (ii) activation of RNase L to hydrolyze RNA, and (iii) inhibition of protein synthesis in intact L929 cells. These studies extend previous reports on the elucidation of the stereochemical requirements of 2-5A synthetase and RNase L [Karikó, K., Sobol, R. W., Jr., Suhadolnik, L., Li, S. W., Reichenbach, N. L., Suhadolnik, R. J., Charubala, R., & Pfleiderer, W. (1987) *Biochemistry* (first of three papers in this issue); Karikó, K., Li, S. W., Sobol, R. W., Jr., Suhadolnik, R. J., Charubala, R., & Pfleiderer, W. (1987) *Biochemistry* (second of three papers in this issue)] with the phosphorothioate analogues of 2-5A. The γ-phosphorothioates displaced the p_3A_4[^{32}P]pCp probe from RNase L with equivalent affinity to p_3A_3 (IC_{50} 5×10^{-9} M), while the (S_P)-β-phosphorothioates had a slightly increased binding affinity (IC_{50} 1×10^{-9} M). However, the (R_P)-β-phosphorothioates had a 1000-fold lower binding affinity (IC_{50} 1×10^{-6} M). This same dramatic difference was observed in the activation process of RNase L as determined in the core–cellulose and rRNA cleavage assays; i.e., the (S_P)-β-, γ-, and (R_P)-β-phosphorothioates activated RNase L to hydrolyze poly(U)[^{32}P]pCp 50% at 5×10^{-10}, 1×10^{-9}, and 3×10^{-7} M, respectively. The proposed structures of the 2′,5′-phosphorothioate trimer analogues synthesized from (S_P)-ATPβS, (R_P)-ATPβS, and ATPγS are 5′-*O*-[(S_P)-2-*P*-thiotriphosphoryl]adenylyl-(2′–5′)adenylyl(2′–5′)adenosine, 5′-*O*-[(R_P)-2-*P*-thiotriphosphoryl]adenylyl(2′–5′)adenylyl(2′–5′)adenosine, and 5′-*O*-[3-thiotriphosphoryl]adenylyl(2′–5′)adenylyl(2′–5′)adenosine, respectively.

As described in the preceding two papers in this issue, we have focused our studies on the effect of backbone modification of the 2-5A[1] molecule on the binding and activation of RNase L by the introduction of R_P and S_P chirality (Karikó et al., 1987a,b). To progress in the elucidation of substrate specificity of the 2-5A synthetase and requirements for binding to and activation of RNase L, we have expanded our stereochemical approach by utilizing β- and γ-phosphorothioate analogues of ATP. In this paper, we report the enzymatic synthesis, structural characterization, and biological properties of the trimer and tetramer phosphorothioate analogues of 2-5A synthesized from (S_P)-ATPβS, (R_P)-ATPβS, and ATPγS (Figure 1). These studies provide a comparative description of phosphorothioate-containing 2-5A analogues bearing sulfur substitution either in the 2′,5′-internucleotide linkages and/or in the α-, β-, or γ-phosphorus of the 5′-terminus with respect to substrate specificity and stereoselectivity for 2-5A synthetase and RNase L. Several reports of the synthesis and biological activities of other 5′-terminus-modified 2-5A analogues have appeared (Baglioni et al., 1981; Bisbal et al., 1985; Imai & Torrence, 1984; Karikó & Ludwig, 1985; Krause et al., 1986; Schryver et al., 1985; Watling et al., 1985); these analogues demonstrated widely varying biological activities (refer to Discussion for further description).

We have observed marked differences in the substrate specificity of the 2-5A synthetase and in the binding and activation of RNase L following introduction of sulfur at the β-phosphorus and at the γ-phosphorus of the 5′-triphosphate terminus of ATP and 2-5A. The significance of access to 2-5A analogues with β or γ substitution and resistance to phosphatase activity as a biological modifier to explore the 2-5A synthetase/RNase L/interferon system in virus-infected cells are discussed.

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[1] Abbreviations: ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); (R_P)- and (S_P)-ATPβS, R_P and S_P isomers of adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); (R_P)-β-2′,5′-phosphorothioate trimer [(R_P)-p_3A_3βS], 5′-*O*-[(R_P)-2-*P*-thiotriphosphoryl]adenylyl(2′–5′)adenylyl(2′–5′)adenosine; (S_P)-β-2′,5′-phosphorothioate trimer [(S_P)-p_3A_3βS], 5′-*O*-[(S_P)-2-*P*-thiotriphosphoryl]adenylyl(2′–5′)adenylyl(2′–5′)adenosine; γ-2′,5′-phosphorothioate trimer (p_3A_3γS), 5′-*O*-[3-thiotriphosphoryl]adenylyl(2′–5′)-adenylyl(2′–5′)adenosine; NP-40, Nonidet P-40; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloroacetic acid. All other abbreviations used were as described in the preceding two papers in this issue (Karikó et al., 1987a,b).

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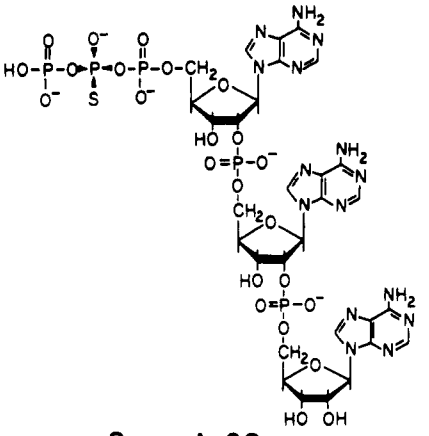
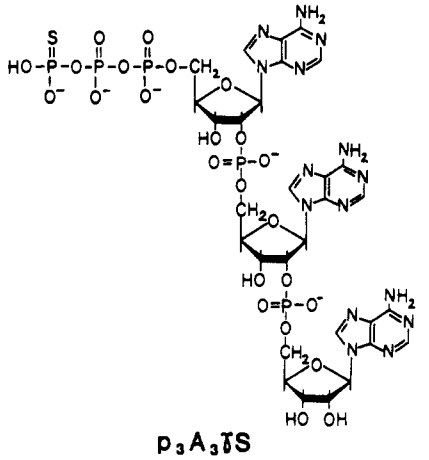


Sp-p$_3$A$_3$βS



p$_3$A$_3$γS

FIGURE 1: Structures of (S_P)-β-2′,5′-phosphorothioate trimer 5′-triphosphate [(S_P)-p$_3$A$_3$βS] and γ-2′,5′-phosphorothioate trimer 5′-triphosphate (p$_3$A$_3$γS).

Experimental Procedures

Many experimental procedures were as described in the preceding two papers in this issue (Karikō et al., 1987a,b). Additional procedures are described below.

Materials. (S_P)-[β-^{35}S]ATPβS (1300 Ci/mmol), [γ-^{35}S]-ATPγS (650 Ci/mmol), [α-^{32}P]ATP (410 Ci/mmol), p$_3$A$_4$-[^{32}P]pCp (3000 Ci/mmol), and [4,5-^{3}H]-L-leucine (120 Ci/mmol) were purchased from Amersham. (S_P)-ATPβS, (R_P)-ATPβS, and ATPγS were kindly provided by Dr. P. A. Frey. AMPS and ADPβS were from Boehringer Mannheim.

Synthesis of 2-5A and β- or γ-2′,5′-Phosphorothioates. Reaction conditions for the synthesis of 2-5A and the (S_P)-β-2′,5′-phosphorothioates were as described for the α-phosphorothioates (Lee & Suhadolnik, 1985). For the synthesis of the γ-2′,5′-phosphorothioates, the reaction mixture contained 2.5 mM [γ-^{35}S]ATPγS (0.2 Ci/mmol), but the energy-regenerating system (creatine phosphate/creatine phosphokinase) was omitted. Syntheses were at 30 °C for 20 h, after which the poly(rI)·poly(rC)–agarose was pelleted and the supernatant containing synthetic products was collected and analyzed by DEAE-cellulose column chromatography and HPLC. In addition, partially purified 2-5A synthetase from rabbit reticulocyte lysates was used to synthesize 2′,5′-phosphorothioates from (S_P)-ATPβS, (R_P)-ATPβS, and ATPγS. The 2-5A synthetase was purified approximately 100 000-fold from rabbit reticulocytes as described by Wu and Eslami (1983). The reaction mixture for oligonucleotide synthesis (total volume = 40 μL) contained 20 mM Tris-HCl, pH 8.5, 25 mM KCl, 20 mM Mg(OAc)$_2$, 1 mM DTT, 0.1 mg/mL bovine serum albumin, 7.5 mM creatine phosphate, 22.5 units/mL creatine phosphokinase, 5 μg/mL poly(rI)·poly(rC), 5 mM ATP [or (S_P)-ATPβS, (R_P)-ATPβS, or ATPγS], 10 μCi [α-^{32}P]ATP [or (S_P)-[β-^{35}S]ATPβS or [γ-^{35}S]ATPγS], and 15 μL of 2-5A synthetase (0.2 M KCl fraction). Incubations were for 18 h at 30 °C. Synthesis of 2-5A or 2′,5′-phosphorothioates was followed by radioactive measurement [from ATP, (S_P)-ATPβS, and ATPγS] or ultraviolet measurement [from (R_P)-ATPβS].

Isolation and Structural Determination of β- and γ-Phosphorothioates. The 2′,5′-phosphorothioates isolated from the 350 mM KCl eluates from DEAE-cellulose columns and subsequently purified by HPLC were further characterized by digestions with BAP, SVPD, and T$_2$ RNase under the same conditions used for the α-phosphorothioates (Lee & Suhadolnik, 1985; Karikō et al., 1987a).

Metabolic Stability Studies in Cell-Free Extracts. The stability of 2-5A and the 2′,5′-phosphorothioates was measured in NP-40 extracts prepared from HeLa cells and L929 cells according to the method of Weber et al. (1975). Conditions for the hydrolysis were as described (Lee & Suhadolnik, 1985); 5 μM [^{32}P]p$_3$A$_n$ or [^{35}S]-2′,5′-phosphorothioate analogue was incubated in a reaction mixture (140 μL) containing 2.5 mM Mg(OAc)$_2$, 120 mM KCl, 20 mM Hepes, pH 7.4, 1 mM DTT, and 70 μL of cell extract (300–400 μg of protein). At the times indicated, an aliquot of sample was withdrawn and the amount of unhydrolyzed 2′,5′-oligonucleotide was determined by DEAE-cellulose chromatography (Doetsch et al., 1981). The recovered 2′,5′-oligonucleotides were then further analyzed and identified by HPLC according to Bayard et al. (1984).

Assay of Cellular Protein Synthesis. HPLC-purified 2′,5′-phosphorothioates were tested for their ability to inhibit protein synthesis in intact L929 cells seeded into 24-well plates at 2 × 10^5 cells/well 1 day prior to use. Compounds were introduced into the cells by the calcium phosphate coprecipitation technique as described by Lee and Suhadolnik (1983) with the exception that [4,5-^{3}H]-L-leucine was used instead of [^{35}S]-L-methionine.

Results

Enzymatic Synthesis and Purification of the β- and γ-Phosphorothioate Analogues of 2-5A. The enzymatic synthesis and characterization of the α-phosphorothioate dimer, trimer, and tetramer analogues of 2-5A from (S_P)-ATPαS by 2-5A synthetase from L929 cell extracts have been previously reported from this laboratory (Lee & Suhadolnik, 1985; Karikō et al., 1987a). The syntheses of the phosphorothioate analogues of 2-5A from (S_P)-ATPβS and achiral ATPγS were accomplished with 2-5A synthetase from interferon-treated L929 cell extracts bound to poly(rI)·poly(rC)–agarose. The yields of the 2′,5′-phosphorothioate oligomers as determined by radioactivity or ultraviolet absorbance at 256 nm synthesized from (S_P)-[β-^{35}S]ATPβS and [γ-^{35}S]ATPγS were 11% and 16%, respectively. The conversion of [α-^{32}P]ATP to 2-5A was 18% under the same conditions. Similar results were obtained with Daudi cell extracts and lysates of rabbit reticulocytes (unpublished results). The synthesis of 2′,5′-phosphorothioates from (S_P)-ATPβS and (R_P)-ATPβS was also accomplished with the 2-5A synthetase purified from lysates of rabbit reticulocytes; yields were 14% and 0.7%, respectively. The 2′,5′-phosphorothioates enzymatically synthesized from (S_P)-ATPβS and ATPγS were each resolved into two major radioactive peaks by reverse-phase HPLC (Figure 2). On the basis of the elution profiles with authentic

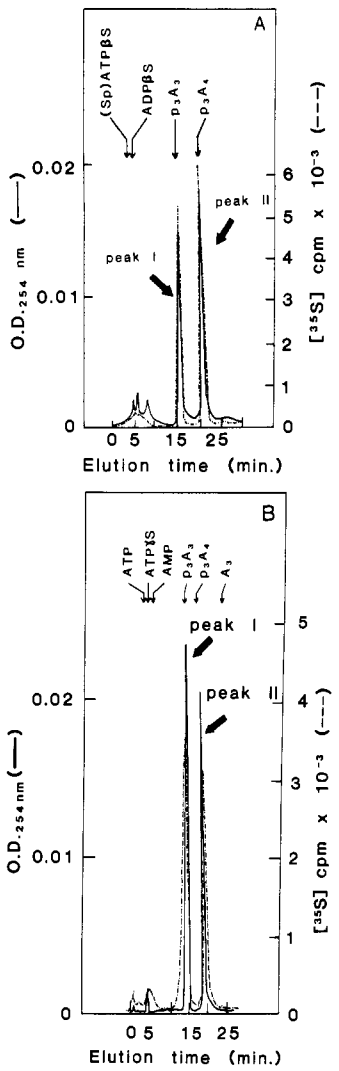


FIGURE 2: HPLC analysis of the β-phosphorothioate analogues synthesized from (S_P)-[β-^{35}S]ATPβS (panel A) and the γ-phosphorothioate analogues (panel B) synthesized from [γ-^{35}S]ATPγS. The phosphorothioate analogues displaced from DEAE-cellulose columns with 350 mM KCl buffer were analyzed by HPLC injection of 200 μL as described by Lee and Suhadolnik (1985). Ten-microliter samples of each 0.5-mL fraction were removed, and the radioactivity was determined. The elution times of UV standards [AMP, ADPβS, (S_P)-ATPβS, ATPγS, p_3A_3, p_3A_4, and A_3] are indicated by arrows. Panel A: Peak I, putative β-phosphorothioate trimer analogue from (S_P)-ATPβS; peak II, putative β-phosphorothioate tetramer analogue. Panel B: Peak I, putative γ-phosphorothioate trimer analogue; peak II, putative γ-phosphorothioate tetramer analogue. The β-phosphorothioate trimer and tetramer analogues synthesized from (R_P)-ATPβS had the same elution times as peaks I and II (panel A).

2-5A trimer and tetramer 5′-triphosphate in parallel syntheses, peak I (Figure 2A) was assigned as the putative (S_P)-β-2′,5′-phosphorothioate trimer analogue [i.e., (S_P)-p_3A_3βS] and peak II as the putative tetramer analogue [i.e., (S_P)-p_3A_4βS]. Similarly, peak I (Figure 2B) was identified as putative γ-phosphorothioate trimer analogue (i.e., p_3A_3γS) and peak II as putative γ-phosphorothioate tetramer analogue (i.e., p_3A_4γS). The amount of unreacted (S_P)-[β-^{35}S]ATPβS and [γ-^{35}S]ATPγS detected by HPLC was negligible. The 2′,5′-phosphorothioates enzymatically synthesized from (R_P)-ATPβS and purified by HPLC resolved into two peaks (with the same elution times as peak I and peak II in Figure 2, panel B) and were identified as putative (R_P)-p_3A_3βS and (R_P)-p_3A_4βS, respectively (data not shown).

Structural Characterization of the 2′,5′-Phosphorothioates by Enzymatic Hydrolysis. To elucidate the structure of the (R_P)- and (S_P)-β- and γ-phosphorothioates, the HPLC-purified 2′,5′-phosphorothioates were further characterized by enzymatic hydrolysis and subsequent analysis by HPLC and biological assay. The 2′,5′ linkage in the phosphorothioate analogues was determined by treatment with T_2 RNase (a 3′,5′-phosphodiesterase). Hydrolysis of the (S_P)-β- and γ-phosphorothioates with SVPD resulted in cleavage at the 2′,5′-internucleotide linkages and hydrolysis of the 5′-triphosphate terminus to inorganic [^{35}S]thiophosphate. Proof of the 2′,5′ linkage in the (S_P)-β- and γ-phosphorothioates was revealed by the lack of hydrolysis with T_2 RNase. The hydrolysis of 2-5A by BAP produced dephosphorylated 2-5A ("core") and inorganic phosphate (Kerr & Brown, 1978). It has been demonstrated that the α-phosphorothioate trimer analogue synthesized from (S_P)-[α-^{35}S]ATPαS is hydrolyzed by BAP (90-min incubation) to yield [^{35}S]-5′-monophosphorothioate 2′,5′-α-phosphorothioate analogue, whereas 24-h incubation resulted in complete dephosphorylation to the core molecule (Lee & Suhadolnik, 1985; Karikō et al., 1987a). Treatment of the (S_P)-β-phosphorothioate tetramer analogue with BAP for 90 min yielded ^{35}S-labeled products eluting between the position of authentic 2-5A tetramer 5′-triphosphate (p_3A_4) and 2-5A tetramer core (A_4). The observation that no inorganic [^{35}S]thiomonophosphate was generated is in agreement with the inability of BAP to cleave the phosphorothiodiester bond in 90-min incubations. This is further evidenced by the observation that the γ-phosphorothioate trimer and tetramer analogues are completely resistant to hydrolysis by BAP (90 min); the γ-phosphorothioate trimer and tetramer triphosphate analogues were recovered intact with no formation of inorganic [^{35}S]thiomonophosphate. Therefore, substitution of sulfur for the nonbridging oxygen at the α-, β-, and γ-phosphorus at the 5′-terminus of the 2-5A molecule renders the molecule resistant to complete dephosphorylation by BAP. An additional proof of structure of the (R_P)- and (S_P)-β-phosphorothioates was accomplished by BAP digestion followed by radiobinding and rRNA cleavage assays. Incubation of the (R_P)- and (S_P)-β-phosphorothioates with BAP for 90 min resulted in the formation of p_2A_3βS; this p_2A_3βS was without activity in binding and rRNA cleavage assays at concentrations as high as 10^{-7} M. These data are in agreement with the earlier report that, while ATP is hydrolyzed by BAP to adenosine, BAP treatment of ATPαS, ATPβS, and ATPγS produces AMPS, ADPβS, and ATPγS, respectively (Eckstein & Goody, 1976). In addition, SVPD hydrolyzes ATP, ATPαS, ATPβS, and ATPγS to AMP, AMPS, AMP, and AMP, respectively (Lee & Suhadolnik, 1985).

Metabolic Stability of 2′,5′-Phosphorothioate Analogues to 2′-Phosphodiesterase. The stability of the β- and γ-phosphorothioate analogues was determined in L929 cell extracts and compared with stability of authentic 2-5A (Figure 3). Authentic 2-5A was degraded 50% in 2.5 min, whereas the α-, β-, and γ-phosphorothioate analogues were hydrolyzed 45%, 75%, and 65%, respectively, after 30 min (Figure 3).

Binding Affinity of β- and γ-Phosphorothioates to RNase L. The ability of the phosphorothioate analogues of 2-5A to bind to RNase L was examined in radiobinding assays. A dose-dependent displacement of the radioactive probe, p_3A_4-[^{32}P]pCp, from RNase L by the (R_P)-β-, (S_P)-β-, and γ-phosphorothioates was observed (Figure 4A). The achiral γ-phosphorothioate trimer (●) showed a binding affinity equal to that of authentic p_3A_3 (□) (IC_{50} 5×10^{-9} M). The β-phosphorothioate with the S_P stereoconfiguration (▲) showed higher affinity (IC_{50} 1×10^{-9} M) for RNase L compared to authentic p_3A_3 (□). However, the (R_P)-β-phosphorothioate

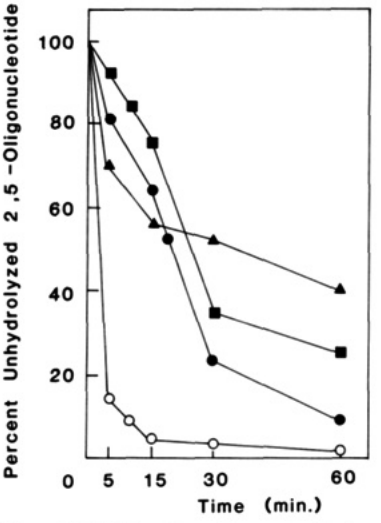


FIGURE 3: Stability of 2′,5′-phosphorothioates to 2′-phosphodiesterase in L929 cell extracts. Hydrolysis of the [^{35}S]-α-, β-, and γ-2′,5′-phosphorothioate tetramer analogues and authentic [^{32}P]p_3A_4 was performed following isolation by 350 mM KCl buffer from DEAE-cellulose columns (see Experimental Procedures). Hydrolysis of the 2′,5′-oligonucleotides was determined by monitoring the radioactive nucleotides displaced from DEAE-cellulose columns first with 90 mM KCl buffer and then with 350 mM KCl buffer. $p_3A_4\alpha S$ (▲); (S_P)-$p_3A_4\beta S$ (●); $p_3A_4\gamma S$ (■); p_3A_4 (○).

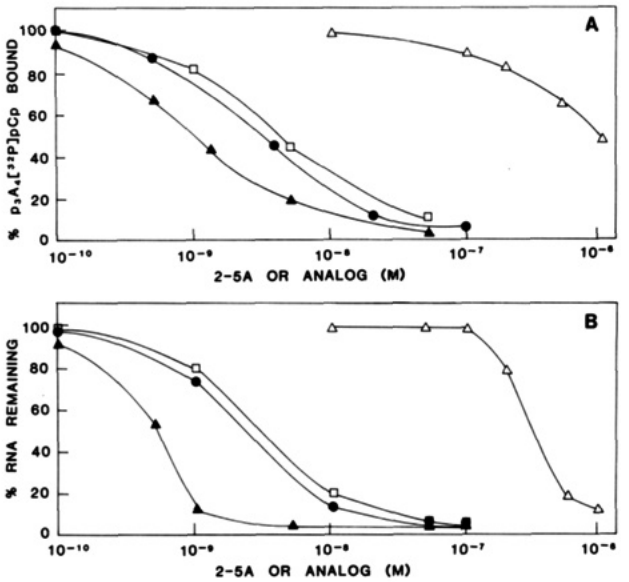


FIGURE 4: (A) Ability of β- and γ-2′,5′-phosphorothioates to compete with p_3A_4[^{32}P]pCp for binding to the RNase L in mouse L929 cell extracts in radiobinding assays (Knight et al., 1981). Approximately 50% of the p_3A_4[^{32}P]pCp was bound in the absence of added oligonucleotide (total dpm = 9500). (B) Ability of β- and γ-2′,5′-phosphorothioates to activate partially purified RNase L from L929 cell extracts as determined by the hydrolysis of poly(U)-3′-[^{32}P]pCp in the core–cellulose assay (Silverman, 1985). Activation of RNase L was determined by conversion of poly(U)-3′-[^{32}P]pCp to acid-soluble fragments after incubation. One hundred percent represents 10000 dpm of labeled poly(U)-3′-[^{32}P]pCp bound to glass fiber filters. (S_P)-$p_3A_3\beta S$ (▲); (R_P)-$p_3A_3\beta S$ (△); $p_3A_3\gamma S$ (●); p_3A_3 (□).

(△) exhibited approximately a 1000-fold lower affinity for RNase L (IC_{50} 1 × 10^{-6} M) compared to its S_P isomer (▲).

Activation of Partially Purified RNase L by (R_P)-β-, (S_P)-β-, *and* γ-*Phosphorothioates As Determined by Hydrolysis of Poly(U)-3′-*[^{32}*P]pCp.* The ability of the (R_P)-β-, (S_P)-β-, and γ-phosphorothioates to activate partially purified RNase L was determined in the core–cellulose assay (Silverman, 1985) by monitoring the degradation of poly(U).

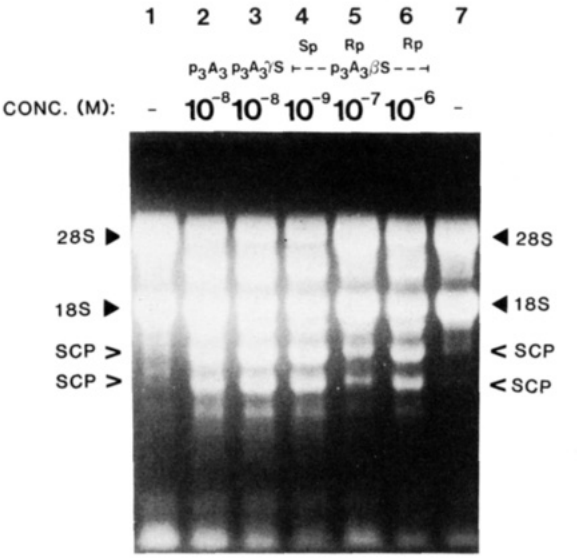


FIGURE 5: Ribosomal RNA cleavage assay with β- and γ-2′,5′-phosphorothioates. L929 cell extracts were incubated in the absence (lane 1) or presence of p_3A_3 at 10^{-8} M (lane 2), $p_3A_3\gamma S$ at 10^{-8} M (lane 3), (S_P)-$p_3A_3\beta S$ at 10^{-9} M (lane 4), and (R_P)-$p_3A_3\beta S$ at 10^{-7} and 10^{-6} M (lanes 5 and 6, respectively); cell extracts without incubation are shown in lane 7. The positions of 28S and 18S rRNA as well as specific cleavage products (SCP) are indicated by arrows.

Degradation of poly(U) increased proportionately with an increase in the concentration of 2′,5′-phosphorothioate (Figure 4B). The (S_P)-β-phosphorothioate (▲) was more active than either the authentic p_3A_3 (□) or the γ-phosphorothioate (●). The (R_P)-β-phosphorothioate (△) was 1000-fold less active than its S_P diastereomer. The extent of activation of RNase L by the (R_P)-β-, (S_P)-β-, or γ-phosphorothioates was identical with the binding affinity (Figure 4A), thereby demonstrating that sulfur substitution on the β- or γ-phosphorus of the 5′-triphosphate terminus exclusively affected the binding process and *not* the activation process.

Activation of RNase L by (R_P)-β-, (S_P)-β-, *and* γ-*Phosphorothioates To Cleave Ribosomal RNA.* Activation of RNase L by (R_P)-β-, (S_P)-β-, and γ-phosphorothioates was also measured in L929 cell extracts by using the rRNA cleavage assay (Wreschner et al., 1981). The achiral γ-phosphorothioate could activate RNase L from L929 cell extracts to the same extent as authentic 2-5A. At 10^{-8} M final concentration, the γ-phosphorothioate (Figure 5, lane 3) and authentic p_3A_3 (Figure 5, lane 2) resulted in equal amounts of specific cleavage products (SCP). The β-phosphorothioate with S_P chirality at the β-phosphorus resulted in the same amount of SCP at 10^{-9} M (lane 4) as were seen with authentic p_3A_3 at 10^{-8} M (lane 2). The β-phosphorothioate with R_P chirality required higher concentrations for detectable cleavage products (10^{-7} M, lane 5; 10^{-6} M, lane 6).

Inhibition of Cellular Protein Synthesis by (R_P)-β-, (S_P)-β-, *and* γ-*Phosphorothioates.* On the basis of in vitro assays (i.e., the core–cellulose and the rRNA cleavage assays) with the (R_P)-β-, (S_P)-β-, and γ-phosphorothioates, studies were done with intact cells to determine whether the in vitro data could be extrapolated to the inhibition of protein synthesis in the intact cell. In addition, the inhibitory effect of the 2′,5′-phosphorothioates on in vitro translation had been studied in the rabbit reticulocyte lysate system. The 2′,5′-phosphorothioate analogues were more potent inhibitors of in vitro protein synthesis than authentic 2-5A, with 45–50% inhibition being achieved by 40 nM α-, β-, or γ-phosphorothioates in 60–80-min assays (data not shown). The ability of the 2′,5′-phosphorothioates to inhibit cellular protein synthesis was

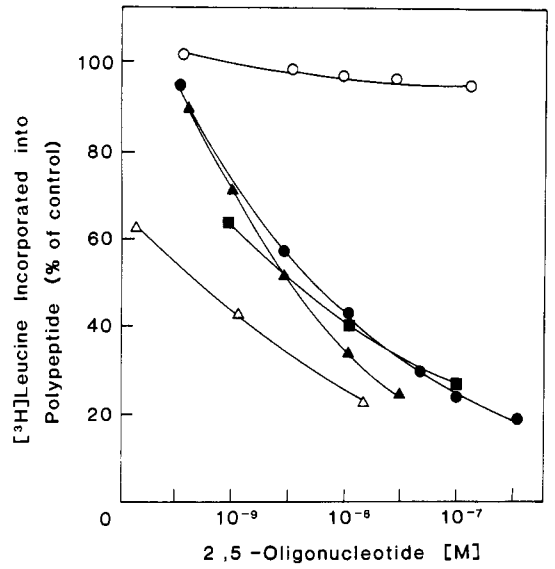


FIGURE 6: Inhibition of cellular protein synthesis by the 2′,5′-phosphorothioate analogues of 2-5A in intact L929 cells. The procedure for the calcium phosphate coprecipitation technique was as described (Lee & Suhadolnik, 1983). Control cultures were treated with calcium phosphate but no oligonucleotides. The incorporation of [³H]leucine in control cultures was taken as 100% (5200 dpm). There was no inhibition of protein synthesis by (S_P)-ATPαS, (S_P)-ATPβS, (R_P)-ATPβS, or ATPγS. (R_P)-p_3A_3βS (○); p_3A_4αS (△); (S_P)-p_3A_4βS (▲); p_3A_4γS (■); p_3A_4 (●).

examined by using the calcium phosphate coprecipitation technique to introduce the 2′,5′-phosphorothioates into intact L929 cells. The α-, (S_P)-β-, and γ-phosphorothioate analogues (at 10^{-6}–10^{-10} M), but *not* the (R_P)-β-phosphorothioate analogue, inhibited cellular protein synthesis in a dose-dependent manner, as measured by the incorporation of [³H]leucine into TCA-precipitable polypeptides (Figure 6). Results from radiobinding assays and rRNA cleavage assays indicate that the observed inhibition of protein synthesis in intact cells by the 2′,5′-phosphorothioates proceeds via binding to and activation of RNase L (Figures 4 and 5). In order to assess the possibility that the observed inhibition might be due to the action of degradation products of the 2′,5′-phosphorothioates, the effect of (S_P)-ATPαS, (S_P)-ATPβS, (R_P)-ATPβS, and ATPγS was also examined. There was no protein synthesis inhibition by any of these nucleotides (not shown).

Discussion

By utilization of the properties of R_P and S_P chirality in phosphorothioate analogues of ATP and 2-5A, several new characteristics of 2-5A synthetase and RNase L have been revealed. Replacement of the P–O group with a P–S group provides new insight into the stereoconfiguration of the naturally occurring P–O groups in 2-5A when RNase L is activated by 2-5A. The findings described in this and the preceding two papers in this issue show that the formation of the 2-5A–RNase L–RNA productive complex favors the R_P stereoconfiguration of the 2′,5′-phosphodiester backbone and the S_P stereoconfiguration at the α- and β-pyrophosphoryl groups at the 5′-triphosphate terminus of 2-5A.

With respect to the 2-5A synthetase (either isolated from mammalian cell extracts or highly purified from lysed rabbit reticulocytes), we have observed a stereoselectivity for the ATPβS. The (S_P)-ATPβS is approximately 20 times more effective a substrate for the synthesis of the 2′,5′-β-phosphorothioates than is (R_P)-ATPβS. Although (S_P)-ATPβS, but not (R_P)-ATPβS, is a substrate analogue of ATP for nucleotidyltransferases, this is the first report that the β-phosphorus of ATP shows a requirement for the S_P stereoconfiguration for this type of nucleotidyltransferase. These data support the stereoselectivity of the 2-5A synthetase in distorting the α–β pyrophosphoryl bond to form the adenylyl group and subsequent formation of the 2′,5′-phosphodiester bond. This is accomplished by recognition of the specific S_P configuration at the β-phosphorus and productive ATP–synthetase complex formation. Experiments are under way to determine whether this stereospecificity at the β-phosphorus of the 2′,5′-phosphorothioates is reversed when Mg^{2+} is replaced with Cd^{2+} [as was recently reported for dynein action on ATPβS (Shimizu & Furusawa, 1986)].

With respect to the binding to RNase L, several heretofore unknown observations have been made. p_3A_3αS has binding affinity equivalent to authentic p_3A_3 [Karikó et al. (1987a), see Figure 5A]. Altered stereochemical configuration in the internucleotide linkages of the 2′,5′-phosphorothioate trimer cores and 5′-monophosphates also does not affect binding affinity [Karikó et al. (1987b), Figure 2A]. Further, the 5′-monophosphates of 2-5A bind as well as the 5′-di- or triphosphates (Haugh et al., 1983), suggesting that the β- and γ-phosphorothioates are not involved in the binding process. However, replacement of the nonbridging oxygen of the β-phosphorus of 2-5A with sulfur and subsequent introduction of chirality have a marked influence on binding affinity to RNase L. Introduction of sulfur at the β-phosphorus of p_3A_3 in the S_P stereoconfiguration increased the binding affinity to RNase L compared to p_3A_3, whereas the R_P-β stereoconfiguration revealed a 1000-fold decreased binding affinity (Figure 4A, compare △ and ▲). In view of the dramatic differences observed in binding affinities of the *chiral* (S_P)- and (R_P)-β-2′,5′-phosphorothioates (Figure 4A) [in contrast to sulfur substitution at the γ-phosphorus of p_3A_3 to form the *achiral* γ-phosphorothioate which does not influence binding affinity (Figure 4A)], it was essential to characterize the effect of sulfur substitution in an *achiral* β-phosphorothioate molecule (i.e., p_2A_3βS). This was accomplished by treatment of the (R_P)- and (S_P)-β-phosphorothioates with BAP to yield the achiral p_2A_3βS. We could not detect any binding to RNase L by the achiral p_2A_3βS at concentrations as high as 10^{-7} M (not shown). It is of utmost interest that the *achiral* monophosphorothioate formed from BAP digestion of p_3A_3αS (i.e., pA_3αS) had the same binding affinity as authentic p_3A_3 [see Karikó et al. (1987a), Figure 5A].

Activation of RNase L by 2-5A results in the hydrolysis of RNA and subsequent inhibition of protein synthesis. Therefore, the ability of the β- and γ-phosphorothioate analogues of 2-5A to activate RNase L was measured by three independent techniques: core–cellulose assay, rRNA cleavage assay, and inhibition of protein synthesis in intact cells (Figures 4–6). The activation of RNase L by the 2′,5′-phosphorothioates in these three assays is consistent with the affinities measured in the binding assays (Figure 4A). We therefore conclude that the introduction of chirality at the β-phosphorus of the 5′-terminus of 2-5A alters the binding process but not the activation process. There are striking biological differences when R_P or S_P chirality is introduced into the 2′,5′-phosphodiester bond compared to the introduction of R_P or S_P chirality into the β-phosphorus of the 5′-terminus. Whereas all four 2′,5′-phosphorothioate trimer cores and their 5′-monophosphates bind equally well to RNase L but *activate* differently [see Karikó et al. (1987b)], the introduction of chirality at the β-phosphorus of p_3A_3 altered the binding affinity but *not* the activation ability of p_3A_3 to RNase L (Figure 4).

The effect of alteration of the α-, β-, and γ-phosphorus of the 5′-terminal phosphate groups of 2-5A on binding and

activation of RNase L has been examined in several laboratories in order to explain the nearly equal binding affinities of authentic p_3A_3, p_2A_3, and pA_3 as opposed to the 1000 times decreased activation of RNase L by pA_3 compared to p_2A_3 and p_3A_3. Chemical modification at the α-phosphate of pA_3 to form the methylthio analogue ($CH_3SpA2'pA2'pA2'pp3'OCH_3$) did not alter the binding affinity, but activation ability was completely lost (Watling et al., 1985). We have shown that, with sulfur substitution on the 5'-monophosphate, instead of a methylthio group, the binding affinity of the resultant $pA_3\alpha S$ was the same as that of authentic pA_3, but activation ability was greater than that of pA_3 (Karikó et al., 1987a). Modifiction of the β-phosphorus of p_2A_3 or p_3A_3 has also revealed marked differences in binding and activation of RNase L. A "capped" derivative (i.e., Ap_2A_3) showed the same binding activity (Imai & Torrence, 1984) as p_3A_3 but could not activate RNase L (Krause et al., 1986). When a methylene group replaced the oxygen between the 5'-terminal β- and γ-phosphates, the resultant $pCH_2p_2A_3$ cannot activate RNase L as the consequence of totally abolished binding ability (Baglioni et al., 1981). New aspects of the stereochemical specificity of RNase L binding and activation have been revealed in our current studies by using stereochemically modified $p_3A_3\beta S$ and $p_2A_3\beta S$. As was observed with $pCH_2p_2A_3$ (Baglioni et al., 1981), activation of RNase L by $p_2A_3\beta S$ was abolished. With (S_P)-$p_3A_3\beta S$, the binding and activation processes of RNase L were slightly enhanced compared to p_3A_3 (Figures 4–6); however, (R_P)-$p_3A_3\beta S$ shows a 1000-fold decreased binding affinity and thus a decreased activation ability. As was observed with Ap_3A_3, modification of the γ-phosphorus of p_3A_3 with a 5'-adenosine cap did not effect the binding to RNase L (Imai & Torrence, 1984) but did result in a 1000-fold decrease in activation of RNase L (Krause et al., 1986). In stark contrast, substitution on the γ-phosphorus with either *n*-decyl-NH_2 or sulfur resulted in 2-5A analogues with the same biological activities as authentic p_3A_3 (Bisbal et al., 1985; Karikó & Ludwig, 1985; Schryver et al., 1985). Here we have shown that the biological activity of the γ-phosphorothioate analogues of 2-5A is due to a direct effect on RNase L binding (Figure 4A).

The 1000-fold difference in binding of (R_P)-$p_3A_3\beta S$ [compared to (S_P)-$p_3A_3\beta S$] was not totally unexpected in view of the knowledge that most ATP-dependent enzymes prefer a β,γ-bidentate/metal chelate/ATP complex (Eckstein et al., 1982; Eckstein, 1985). It is well established that Mg^{2+} coordination to phosphorus occurs though oxygen, whereas Cd^{2+} has a strong tendency to form a chelate complex through sulfur (Pecoraro et al., 1984). Although it is known that RNase L requires Mg^{2+} for activity (Wreschner et al., 1982), the type of complex formed is not established. Therefore, if a β,γ-bidentate complex forms with Mg^{2+} and (S_P)-$p_3A_3\beta S$, such a complex might fit into the binding domain of RNase L, form a productive complex, and result in the hydrolysis of RNA. Such a mechanism would be similar to the β,γ-bidentate complex which forms with (S_P)- and (R_P)-ATPβS in binding to hexokinase (Jaffe & Cohn, 1979). Therefore, the formation of a β,γ-bidentate complex of (S_P)-$p_3A_3\beta S$ with Mg^{2+} might place the 2',5'-phosphorothioate in a stereochemical configuration favorable for binding in a mechanism similar to that which occurs with (S_P)-ATPβS and other enzymes (Jaffe & Cohn, 1979; Eckstein, 1985; Shimizu & Furusawa, 1986). Precedents for the β,γ-bidentate complex have been provided by Cleland and co-workers (Merritt et al., 1978).

On the basis of the observations reported here, we can also speculate that the amino acids in the 2-5A binding domain of RNase L interact with the P–S group of the (S_P)-β-phosphorothioates. Such interaction can facilitate the binding of the β-phosphorothioate analogue when the P–S group is in the S_P stereoconfiguration and in juxtaposition to positively charged amino acid residue(s) or can decrease the binding of the β-phosphorothioate when the P–S group is in the R_P stereoconfiguration and in juxtaposition to negatively charged amino acid residues of RNase L (Plaut et al., 1979). This hypothesis has further basis in the reports of Frey and Sammons (1985) and Eckstein (1985) in which they have reported electronic differences and differences in bond length of P–S vs P–O groups. Further, lowering of the pK_a upon replacement of the P–S with a P–O in the β-pyrophosphoryl group at the 5'-terminus could result in increased ionic interaction between the P–S group and amino acid residues (i.e., histidinyl, arginyl, lysinyl) (Jaffe & Cohn, 1978; Plaut et al., 1979). These physicochemical properties of the P–S bonds in either the R_P or S_P stereoconfiguration could result in an altered ionic interaction between the amino acid residues of RNase L with the P–S group of the 2',5'-phosphorothioate analogues that is not observed with the P–O group. By having access to chiral ATP and 2-5A analogues (i.e., with chirality at the α- or β-phosphorus introduced with ^{18}O substitution in lieu of sulfur substitution), it would be possible (i) to confirm the type of asymmetry found with authentic 2-5A once it binds to RNase L and (ii) to determine if electronic interactions (i.e., P–S vs P–O) are responsible for the differences observed in enzymatic activity of 2-5A synthetase and binding to RNase L observed with the 2',5'-phosphorothioates.

The fact that the α-, β-, and γ-phosphorothioate analogues of 2-5A show enhanced metabolic stability in vitro together with the inhibition of protein synthesis in intact cells adds to the concept that this new class of biological modifiers (particularly the S_PS_P trimer 5'-monophosphate) will be excellent probes in the analysis of the 2-5A pathway. A logical extension of the studies presented in these three papers is the use of the specific stereochemically modified 2-5A molecules to test the mechanism by which some virus-infected cells overcome the 2-5A synthetase/RNase L system. Three mechanisms by which viruses may alter the 2-5A molecule have been suggested: (i) conversion of 2-5A to a nonfunctional molecule that binds to and inactivates RNase L by acting as a competitive inhibitor, (ii) hydrolysis of 2-5A by 2'-phosphodiesterase, and (iii) hydrolysis of the 5'-triphosphate by a virus-mediated phosphatase that converts functional 2-5A to nonfunctional 2-5A (Cayley et al., 1982, 1984; Rice et al., 1984; Williams et al., 1986; Paez & Esteban, 1984; Esteban et al., 1984). In view of the observed biological properties of the α-, β-, and γ-phosphorothioate analogues of 2-5A reported here, this class of biological modifiers is ideally suited to assess the 2-5A/RNase L system in virus infection, antineoplastic chemotherapy, cell growth, and differentiation.

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SUPPLEMENTARY MATERIAL AVAILABLE

HPLC analysis of products following enzymatic digestion (BAP, SVPD, T_2 RNase) of the β- and γ-2',5'-phosphorothioate analogues (3 pages). Ordering information is given on any current masthead page.

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