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Synthesis and biological activities of a phosphorodithioate analog of 2′,5′-oligoadenylate

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ABSTRACT

To enhance the resistance of 2-5A (pppA2′p5′A2′p5′A) to degradation by exo- and endonucleases, a phosphorodithioate analog was synthesized using a solid-phase phosphite triester approach with N^6-benzoyl-5′-*O*-dimethoxytrityl-3′-*O*-*t*-butyldimethylsilyladenosine 2′-[*S*-(β-thiobenzoylethyl)-pyrrolidinophosphorothioamidite]. 5′-Monophosphorylation was accomplished with 2-[2-(4,4′-dimethoxytrityloxy)-ethylsulfonyl]ethyl-(2-cyanoethyl)-(*N,N*-diisopropyl)-phosphoramidite. The resulting product, p5′A2′(s2p)- 5′A2′(s2p)5′A, was ~10-fold less effective as an activator of purified human recombinant 2-5A-dependent RNase than was 2-5A itself. This loss of activation ability was related directly to the loss of binding ability of the phosphorodithioate analog. As predicted, p5′A2′(s2p)5′A2′(s2p)5′A was stable to snake venom phosphodiesterase and the nucleolytic activities of both human lymphoblastoid CEM cell extracts and human serum, under conditions that led to facile degradation of parent 2-5A. This nuclease stability permitted the observation of the CEM cell extracts and human serum phosphatase activity which led to 5′-dephosphorylation of p5′A2′(s2p)5′A2′(s2p)5′A.

INTRODUCTION

The antiviral proteins, interferons [reviewed in (1)], can induce mammalian, reptilian and avian cells to synthesize a family of new enzymes called 2-5A synthetases [reviewed in (2)]. While particular properties of these isoenzymes vary, upon stimulation with double-stranded RNA, they all share the ability to elaborate oligoadenylates with 2′–5′ internucleotide linkages. These unique oligoadenylates, known as 2-5A (3), can in turn activate a latent 2-5A-dependent ribonuclease (4,5). Considerable evidence exists that the foregoing 2-5A system is responsible for the antiviral effect of interferon against picornaviruses such as mengovirus and encephalomyocarditis virus [reviewed in (6,7)]. Additionally, suppositions have been advanced that 2-5A may be involved in cell growth regulation and differentiation [reviewed in (6,8)].

Efforts toward capitalizing on the 2-5A system for development of a potential antiviral or antitumor therapeutic agent, either directly or using the recently introduced 2-5A-antisense strategy for the selective degradation of RNA (10–13), have to contend with stabilization of the molecule against premature degradation (7). A host of stabilizing modifications have been applied to the 2-5A molecule, perhaps the most recent being alteration of the 2′–5′ internucleotide linkages to phosphorothioates, thereby giving rise to diastereomers [(9) and references contained therein].

To alleviate the diastereoisomer problem, we prepared **p5′A2′(s2p)5′A2′(s2P)5′A**, replacing the internucleotide phosphates with phosphorodithioates. This modification is known to endow 3′,5′-oligonucleotides with nuclease resistance and is achiral, unlike oligonucleotide phosphorothioates (14).

MATERIALS AND METHODS

General materials and methods

N^6-Benzoyl-2′-*O*-*t*-butyldimethylsilyl-5′-*O*-dimethoxytrityladenosine and N^6-benzoyl-3′-*O*-*t*-butyldimethylsilyl-5′-*O*-dimethoxytrityladenosine were obtained from ChemGenes Corporation (Waltham, MA). The other reagents for the synthesis of phosphorothioamidite **2** and reagents for the solid-phase sulfurization were obtained from Aldrich (Milwaukee, WI). Reagents for oligonucleotide synthesis were from Applied Biosystems, Inc. (Foster City, CA) and the 5′-phosphorylation reagent 2-[2-(4,4′-dimethoxytrityloxy)ethylsulfonyl]ethyl-(2-cyanoethyl)-(*N,N*-diisopropyl)-phosphoramidite was obtained from Glen Research (Sterling, VA). Aminomethyl polystyrene (PS, Applied Biosystems) derivatized (15) with N^6-benzoyl-2′-*O*-*t*-butyldimethylsilyl-5′-*O*-dimethoxytrityladenosine was used as a solid support. RP-HPLC purification and analysis was performed on an

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ABI HPLC instrument utilizing a Hamilton PRP-1 (250×4.1 mm) column. Nuclear magnetic resonance spectra were recorded on a Varian Gemini 400 spectrometer operating at 161.947 MHz for ^{31}P and 400.075 MHz for ^{1}H with 85% phosphoric acid and 1% tetramethylsilane as external standard respectively.

Synthesis of N^6-benzoyl-5′-*O*-dimethoxytrityl-3′-*O*-*t*-butyldimethylsilyl adenosine 2′-[*S*-(β-thiobenzoylethyl)pyrrolidinophosphorothioamidite] (2)

N^6-Benzoyl-5′-*O*-dimethoxytrityl-3′-*O*-*t*-butyldimethylsilyladenosine (1) (1 g, 1.27 mmol) was dissolved in anhydrous dichloromethane (CH_2Cl_2) (20 ml) containing 0.5 g of 3 Å molecular sieves. Tris(pyrrolidino)phosphine (292 ml, 1.27 mmol) and 0.5 M tetrazole in anhydrous acetonitrile (CH_3CN) (added in seven 0.2 ml aliquots at 2 min intervals) were first added to the reaction mixture. Trimethylsilylimidazole (20 ml, 0.13 mmol) was then added and after 10 min 0.5 M tetrazole (6.8 ml) in anhydrous CH_3CN followed immediately by ethanedithiol monobenzoate (280 ml, 1.6 mmol). The reaction was allowed to proceed for 4 min and then quenched by pouring it into CH_2Cl_2 (60 ml) containing triethylamine (TEA, 4 ml). The reaction mixture was washed successively with saturated sodium bicarbonate, sodium carbonate and brine. The organic layer was dried over Na_2SO_4 for 15 min, filtered TEA (4 ml) was added to the filtrate and the solution was concentrated *in vacuo*. The resulting syrup was dissolved in minimal toluene and precipitated into vigorously stirred hexanes. The resulting white precipitate was filtered off and dried *in vacuo* to give the desired product (0.91 g, 84%). ^{31}P NMR ($CDCl_3$) δ 173.9, 168.3.

Chemical synthesis of p5′A2′(s2p)5′A2′(s2p)5′A

The oligonucleotide was synthesized using the solid-phase phosphite-triester method on a polystyrene support derivatized with N^6-benzoyl-5′-*O*-dimethoxytrityl-2′-*O*-*t*-butyldimethylsilyl-adenosine on an Applied Biosystems 394 DNA/RNA synthesizer according to Matulic-Adamic *et al.* (unpublished observations). The standard ABI 10 μmol RNA cycle was modified as follows. Thioamidite **2** and tetrazole solutions were delivered in two pulses instead of one. Immediately after coupling, the column was removed from the synthesizer and treated, by syringe addition, with elemental sulfur (1.5 g) in carbon disulfide/2,6-lutidine (1:1, 10 ml) for 1 h and then washed with carbon disulfide/2,6-lutidine (1:1, 20 ml) followed with CH_3CN (20 ml). The column was reattached to the synthesizer and the assembly continued with capping and detritylation. Attachment of the terminal 5′-phosphate group using 2-[2-(4,4′-dimethoxytrityloxy)-ethylsulfonyl]ethyl-(2-cyanoethyl)- (*N,N*-diisopropyl)-phosphoramidite (Glen Research) was accomplished using the standard ABI 10 μmol RNA cycle. The average stepwise coupling yield, determined by colorimetric quantitation of trityl fractions, was 99.3%.

Cleavage and deprotection of p5′A2′(s2p)5′A2′(s2p)5′A

Cleavage from the support and cleavage of the cyanoethyl and benzoyl protecting groups was accomplished by treating the dried, PS-bound oligonucleotide with ammonium hydroxide/ethanol (3:1, v/v, 40 ml) in a stainless steel sealed container for 1 h at room temperature, followed by 20 h at 55°C. Silyl protecting groups were removed by treatment with tetrabutylammonium fluoride (Aldrich, 1 M in THF, 0.2 ml in 1 ml THF) for 24 h at room temperature. The solution was concentrated *in vacuo*, dissolved in water (10 ml) and applied to a DEAE Sephadex A-25 (HCO_3^-) column (15 × 250 mm). The column was eluted with a linear gradient of triethylammonium bicarbonate buffer (0–0.5 M, 1/1 l). Fractions containing the product were pooled, evaporated *in vacuo* and coevaporated several times with methanol to remove the buffer. The residual syrup was further purified by RP-HPLC using a linear gradient of 0–40% CH_3CN in 50 mM TEAA in 30 min. The product eluted at 26 min (42 A_{260} U, 12%). ^{31}P NMR (D_2O) showed the expected 2:1 ratio of phosphorodithioate (δ 115.5) to phosphomonoester (δ 1.6) signal by integration. ^{1}H NMR (D_2O) δ 8.57, 8.19, 7.95, 7.92 (4 s, 6H, adenine 8 and 2H's, ratio 1:3:1:1), 6.13 (d, $J_{1'2'}$ = 2.16, 1H, H1′a), 6.01 (d, J = 5.76, 1H, H1′ b), 5.83 (d, 1H, $J_{1'2'}$ = 2.13, H-1′c).

2-5A-dependent RNase activities

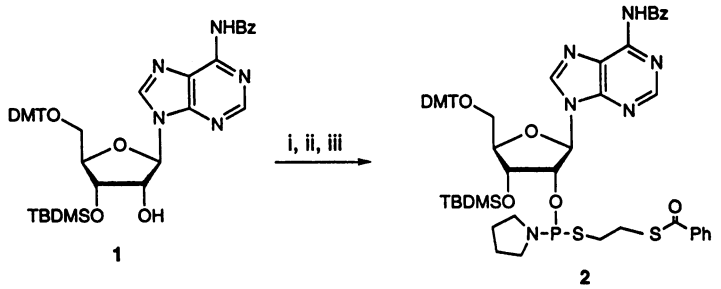
Binding of the synthetic analogs to the 2-5A-dependent RNase was examined using a modification of the radiobinding assay originally reported by Knight *et al.* (16) and further refined by Silverman and Krause (17). The procedure employed the oligonucleotide **ppp5′A2′p5′A2′p5′A2′p5′A3′[^{32}P]p5′Cp** as a radioactive probe which would be displaced in a competitive manner by 2-5A or an analog. Nitrocellulose filters were employed to trap the radioprobe–endonuclease complex. Analyses of the ability of the dithioate analogs to activate RNase L were performed using modifications of the procedure originally developed by Silverman (18). In this methodology, the degradation of poly(U)[^{32}P]pCp was followed by monitoring the disappearance of trichloroacetic acid-insoluble radioactivity. The precipitated poly(U)pCp was isolated, together with carrier yeast RNA, by filtration on glass filter discs. The 2-5A-dependent RNase was expressed in SF21 insect cells from a human cDNA subcloned in a baculovirus vector (19). It was purified with the aid of three fast protein liquid chromatography (FPLC) columns (Pharmacia).

Assays for degradation by 2′–5′-phosphodiesterase activities

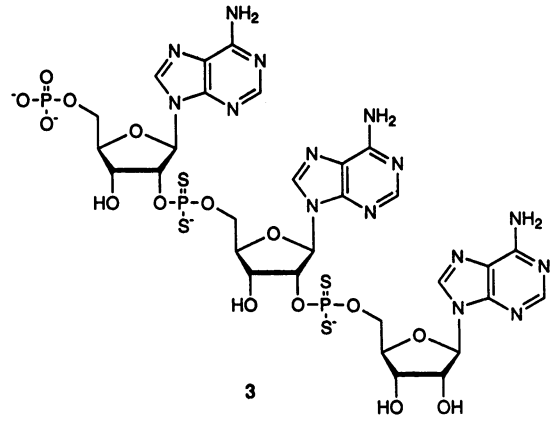
Snake venom phosphodiesterase degradation. The reaction mixture contained in a total of 200 μl of 50 mM Tris, pH 7.8, 0.5 mM $MgCl_2$: 0.37 A_{260} U of **p5′A2′p5′A2′p5′A** or 0.39 A_{260} U of **p5′A2′(s2p)5′A2′(s2p)5′A** and 0.015 U or 0.15 U of snake venom phosphodiesterase (20 U/mg). Incubation was at 37°C.

CEM cell extract degradation. The reaction mixture contained in a total volume of 200 μl of 50 mM Tris, pH 7.8, 0.5 mM $MgCl_2$: 0.37 A_{260} U **p5′A2′p5′A2′p5′A** or 0.39 A_{260} U of **p5′A2′(s2p)5′A2′(s2p)5′A** and 30 μl of CEM cell extract centrifuged free of any visible precipitate. Incubation was at 37°C.

Human serum degradation. The reaction mixture contained in a total of 200 μl of 50 mM Tris, pH 7.8, 0.5 mM $MgCl_2$: 0.37 A_{260} U of **p5′A2′p5′A2′p5′A** or 0.39 A_{260} U of **p5′A2′(s2p)5′A2′(s2p)5′A** with 50 μl human serum. Incubation was at 37°C. Samples (20 μl) for analysis were collected at 0, 60, 180 and 1440 min and mixed with methanol (20 μl). The protein precepitate was spun down with the aid of a microcentrifuge and



[a] Reagents: (i) tris(pyrrolidino)phosphine, tetrazole; (ii) 1-(trimethylsilyl)imidazole; (iii) ethanedithiol monobenzoate, tetrazole.



Scheme 1. Synthesis of the phosphorodithioate analog, **3**, of 2-5A trimer 54-monophosphate.

the supernatant (20 µl) was mixed with 30 µl of HPLC buffer and injected into the HPLC.

HPLC conditions. The HPLC used for analysis of degradation was controlled by Beckman System Gold software and employed an IBM PS/2 computer with two Beckman 110B solvent delivery modules and a Beckman 167 UV/VIS variable wavelength detector (set to operate at 260 and 280 nm). An analytical Ultrasphere ODS column (reversed phase C_{18}, 4.9 × 250 mm) was used to determine products of the various degradations above. The program used for elution of the products involved a 20 min wash with 100% solvent A, then a linear gradient to 57% solvent B, followed by a 5 min ramp to 100% solvent B. The column was washed for 10 min at 100% solvent B before return to initial conditions. Solvent A was 100 mM ammonium phosphate (pH 5.5) and solvent B was methanol/water (1:1).

RESULTS

We undertook synthesis of the dithioate analog of 2-5A core 5′-monophosphate **3** (Scheme 1) using a solid-phase phosphite-triester method because of the speed and simplicity (20,21) of this approach. Currently only the solid-phase synthesis of dithioate DNA is known (21), the only reported RNA-related phosphorodithioate chemistry is the phosphorothioamidite solution-phase synthesis of dimer ribonucleotides containing internucleotidic phosphorodithioate linkages (22). We used a solid-phase synthetic method for the synthesis of **3** based on the 3′-phosphorothioamidite **2** (Matulic-Adamic, J., Haeberli, P., Karpeisky, A., Beigelman, L., Usman, N. unpublished observations). The compound was synthesized from N^6-benzoyl-3′-*O*-*t*-butyldimethylsilyl-5′-*O*-dimethoxytrityl adenosine **1** in 84% yield by phosphitylation with tris(pyrrolidino)phosphine in the presence of tetrazole with subsequent introduction of a *S*-β-thiobenzoylethyl group according to method of Wiesler *et al.* (21) (Scheme 1). Using synthon **2**, trimer **3** was prepared on a polystyrene support derivatized with N^6-benzoyl-5′-*O*-dimethoxytrityl-2′-*O*-*t*-butyldimethylsilyl adenosine utilizing the standard ABI 10 µmol RNA cycle with a 99.3% average coupling yield. Off-machine sulfurization was performed manually by syringe addition of elemental sulfur in CS_2-2,6-lutidine for 2 h (not optimized) (Matulic-Adamic, J., Haeberli, P., Karpeisky, A., Beigelman, L., Usman, N. unpublished observations and 23). The terminal 5′-phosphate was introduced by coupling 2-[2-(4,4′-dimethoxytrityloxy)-ethylsulfonyl]-ethyl-(2-cyanoethyl)- (*N,N*-diisopropyl)-phosphoramidite (24, Glen Research). Standard deprotection (25) and HPLC purification afforded analog **3** in 12% yield.

The dithioate 2-5A analog, **p5′A2′(s2p)5′A2′(s2p)5′A**, was evaluated for its ability to bind to purified recombinant human 2-5A-dependent RNase by measuring its capacity to displace a labelled 2-5A probe from the endonuclease. The results are shown in Figure 1. The dithioate analog was bound ~10-fold less effectively to the 2-5A-dependent RNase than was the tetramer, **p5′A2′p5′A2′p5′A2′p5′A**. Earlier experiments have shown that there exists little, if any, difference in ribonuclease binding between trimeric and tetrameric 2′,5′-oligoadenylates (reviewed in 6,7).

The ability of **p5′A2′(s2p)5′A2′(s2p)5′A** to activate the 2-5A-dependent nuclease was determined by monitoring the disappearance of trichloroacetic acid precipitable poly(U) [^{32}P]pCp in the presence of purified recombinant human enzyme

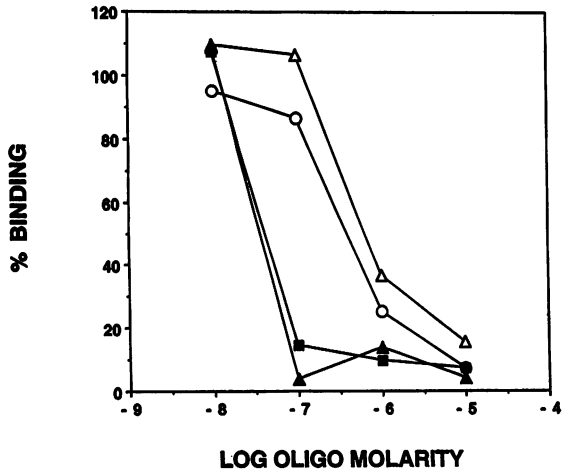


Figure 1. Binding of **p5′A2′(s2p)5′A2′(s2p)5′A** (Δ, ○) to purified recombinant human 2-5A-dependent RNase as compared to tetramer p5′(A2′p)$_3$A (▲ ●) as measured by displacement of the probe p5′(A2′p)$_3$A. The assay was performed with 1 µg of the 2-5A-dependent RNase in a final assay volume of 20 µl corresponding to a RNase concentration of 0.6 µM. The results of two separate experiments are shown.

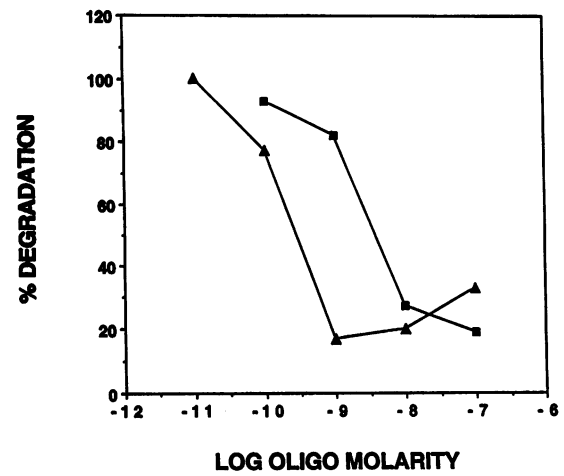


Figure 2. Activation of the pure recombinant human 2-5A-dependent RNase as measured by the degradation of poly(U)[^{32}P]pCp: **p5′A2′(s2p)5′A2′(s2p)5′A** (■), tetramer p5′(A2′p)$_3$A (▲). Reaction mixtures contained 0.1 µg of 2-5A-dependent RNase in final volumes of 25 µl containing 12 nM poly(U). Incubations were at 30°C for 30 min.

with and without varying concentrations of either the known activator **p5′A2′p5′A2′p5′A2′p5′A**, equipotent with trimer (12) or the dithioate analog **p5′A2′(s2p)5′A2′(s2p)5′A**. The concentration for 50% RNA degradation was ~2 × 10^{-10} M for **p5′A2′p5′A2′p5′A2′p5′A**, but it was at least 10-fold greater for the dithioate, **p5′A2′(s2p)5′A2′(s2p)5′A** (Fig. 2).

The oligonucleotide **p5′A2′(s2p)5′A2′(s2p)5′A** was compared to **p5′A2′p5′A2′p5′A2′p5′A** for its ability to act as a substrate for a phosphodiesterase, specifically that from snake venom. Under conditions where **p5′A2′p5′A2′p5′A2′p5′A** had a half-life of <30 min, no detectable degradation of the dithioate, **p5′A2′(s2p)5′A2′(s2p)5′A**, was observed (data not shown). When the degradation of **p5′A2′(s2p)5′A2′(s2p)5′A** was examined in either cytoplasmic extracts of human CEM cells or in human serum, a significantly different result was obtained (Fig. 3a and b). The dithioate rapidly disappeared from the incubation mixture at about the same rate as the parent tetramer **p5′A2′p5′A2′p5′A2′p5′A**. The critical difference in the course of the degradations for the two oligonucleotides was the nature of the degradation products. For **p5′A2′p5′A2′p5′A2′p5′A**, the products were due to scission of the internucleotide bonds as well as dephosphorylation, in accord with many earlier studies (6). However for the dithioate, **p5′A2′(s2p)5′A2′(s2p)5′A**, the only product formed (Fig. 3c) was that identified tentatively as the core dithioate, **5′A2′(s2p)5′A2′(s2p)5′A**, a product of 5′-dephosphorylation. No other product, nor any evidence for internucleotide cleavage, could be obtained even after 24 h incubation in human serum or after 3 h incubation in CEM cell extract.

DISCUSSION

A variety of base and sugar-substituted analogs of 2-5A (6,7) have provided valuable information for understanding the structural and conformational factors that govern the interaction of 2-5A-dependent endonuclease with its activator 2-5A (ppp5′A2′p5′A2′p5′A). At the same time only few modifications of the 2′,5′-phosphodiester bond have been explored (6,7,9). Since 2-5A is subject to facile degradation by phosphodiesterases (27) including one with 2′,5′-phosphodiester bond specificity (28), additional stabilization of internucleotide linkages could be beneficial for the biological activity of 2-5A. Recently the synthesis of phosphorodithioate oligodeoxyribonucleotides by a solid phase methodology has been described (29–32). Phosphorodithioate internucleotide linkages are isosteric and isoelectronic with natural phosphodiester linkages and are also nuclease resistant (14).

The building block for the synthesis of **3**, that is 3′-phosphorothioamidite **2** was synthesized from the suitably 3′-protected adenosine derivative **1** by the method of Wiesler *et al.* (21). These authors have recently introduced a new *S*-protecting group for the protection of internucleotidic dithioate linkages, the β-thiobenzoylethyl group (21). The use of this protecting group helped reduce phosphorothioate contamination in the final products when compared to the use of the β-cyanoethyl (22) or 2,4-dichlorobenzyl protection (23) protecting groups. The ^{31}P NMR spectrum of the phosphorothioamidite **2** showed the expected two singlets at δ 173.9 and 168.3 respectively, for two diastereoisomers. The NMR data confirmed that no migration of the 3′-*O*-*t*-butyldimethylsilyl group to the 2′-*O*-position occurred during the phosphitylation reaction (25). After completion of the synthesis and standard deprotection (25), trimer **3** was obtained in 45% crude yield. Two step purification using ion-exchange column chromatography and reverse-phase HPLC gave compound **3** in 12% yield and 95% purity. The NMR spectrum of trimer **3** corroborated the structure. ^{31}P Chemical shifts were observed at δ 115.5 and 1.6 p.p.m., corresponding to the presence of dithioate linkages and a 5′-monophosphate (9,39) in the correct ratio of 2:1. No phosphorus NMR spectrum resonances were observed δ 56–58 (44) which could correspond to the presence of monothioate linkages which are a possible contaminant of dithioate preparations [e.g. see (21)]. The proton NMR spectrum of trimer **3** revealed the three sets of base protons (H-2 and 2-8) in the δ 8.57–7.91 region as well as the anomeric H-1′ protons in the

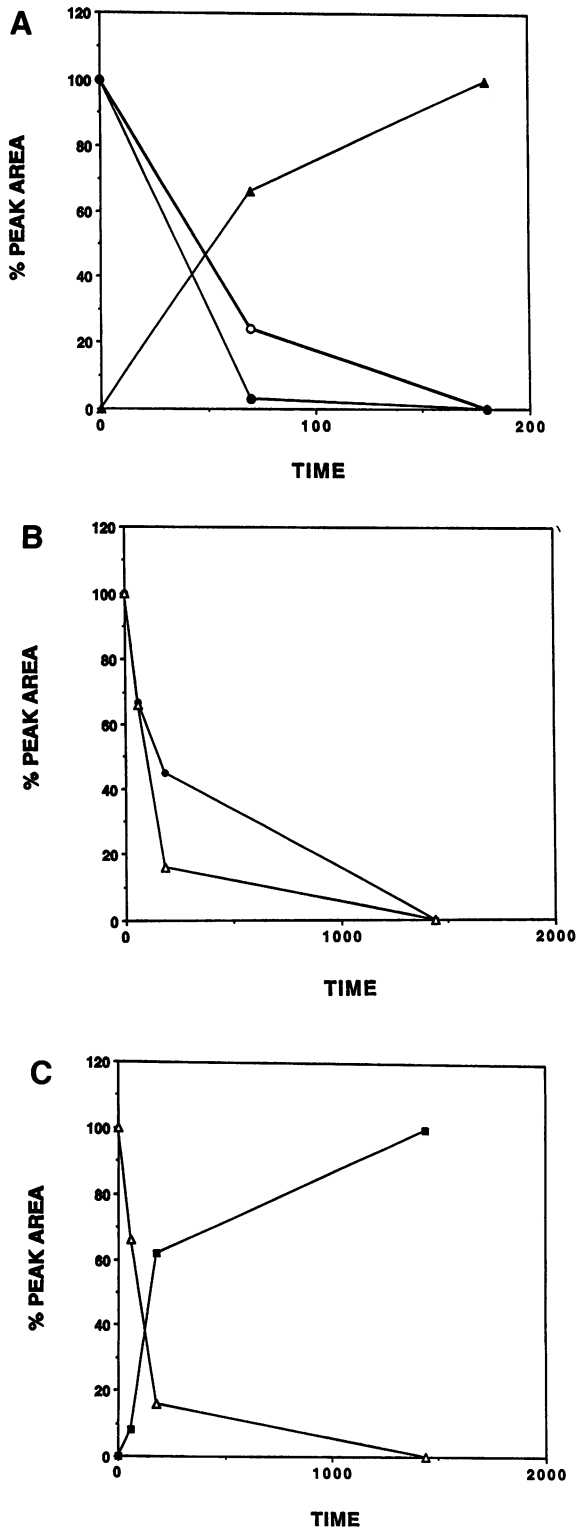


Figure 3. (A) Degradation of 2′,5′-oligoadenylates in the presence of CEM cell extract upon incubation at 37°C: **p5′A2′(s2p)5′A2′(s2p)5′A** (○), **p5′A2′p5′A2′p5′A** (●). The appearance of putative 'core' 5′-dephosphorylated phosphorodithioate, **A2′(s2p)5′A2′(s2p)5′A** (▲), paralleled the disappearance of starting oligomer, **p5′A2′(s2p)5′A2′(s2p)5′A** (●). (B) Degradation of **p5′A2′(s2p)5′A2′(s2p)5′A** (▲) and p5′A2′p5′A2′p5′A (●) in 25% human serum at 37°C. (C) Degradation of **p5′A2′(s2p)5′A2′(s2p)5′A** (Δ) in 25% human serum at 37°C and the appearance of putative 'core' **A2′(s2p)5′A2′(s2p)5′A** (■).

region of δ 5.8–6.15, in accord with literature reports for related compounds (9,39).

Inspection of Figure 1 and Figure 2 leads to the conclusion that the loss of ability of **p5′A2′(s2p)5′A2′(s2p)5′A** to activate the 2-5A-dependent RNase was related directly to its loss of binding affinity, thereby separating the dithioate from a number of other 2-5A analogs which bind well to the nuclease, but fail to activate it to degrade substrate RNA (6,7). This decreased 2-5A-dependent RNase binding is difficult to rationalize given the observation that internucleotide phosphorothioate diastereomers of **p5′A2′p5′A2′p5′A2′p5′A** all have been reported to bind equally well to the 2-5A-dependent RNase and with the same affinity as **p5′A2′p5′A2′p5′A2′p5′A** itself. However, the relative inactivity of **p5′A2′(s2p)5′A2′(s2p)5′A** might well be related to the reported differences among various phosphorothioate substituted diasterioisomers as activators of the RNase. For example, the *SpSp* diastereomer of the analog, **p5′A2′(sp)5′A2′(sp)5′A**, bound the 2-5A-dependent RNase as well as **p5′A2′p5′A2′p5′A2′p5′A**, but was unable to activate the enzyme. It is possible that the activated form of the enzyme is conformationally more restrained. It would follow that 2′,5′-oligonucleotides might be bound to the nuclease, but not effect sufficient conformational change to switch on nuclease activity as has been hypothesized for several base and sugar analogs of 2-5A (6,26,33–42). By the same token, sufficient conformational flexibility could exist in such a relaxed enzyme form that binding of a variety of stereoisomers could be accommodated. Only when the enzyme undergoes a needed conformational change for activation would discrimination at the stereoisomer level become critical. The dithioate 2-5A analog, **p5′A2′(s2p)5′A2′(s2p)5′A**, would challenge the conformational flexibility of the more relaxed enzyme form since it would present no charged phosphate oxygens to the enzyme.

Therefore, the relatively greater hydrophobicity and/or size of the dithioate linkages (14), compared to either phosphodiesters or phosphorothioates, could also be a liability in this interaction with the 2-5A-dependent RNase. In addition, the rigid rod-like structure of dithioate oligonucleotides (14) may contribute to the reduced binding of **p5′A2′(s2p)5′A2′(s2p)5′A**.

The considerably enhanced resistance of the dithioate 2-5A analog to degradation by phosphodiesterases of snake venom, CEM cells and human serum, was expected based on previous studies of dithioate oligonucleotides. What was most dramatic, however, was the rapid dephosphorylation of **p5′A2′(s2p)5′A2′(s2p)5′A** by phosphatase activities in human serum and CEM cells. This 5′-dephosphorylation of 2-5A itself is not usually seen when the degradation of 2-5A or similar molecules is studied since the phosphodiesterase degradation mode is predominant. What the results of this current study emphasize, however, is that such phosphatase activity would likely be limiting in attempts to use unmodified 2-5A and its congeners as potential therapeutic agents (43).

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