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SYNTHESIS OF A 5'-THIOPHOSPHATE ANALOGUE OF 2-5A, A PHOSPHATASE RESISTANT ACTIVATOR OF THE 2-5A-DEPENDENT RIBONUCLEASE

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Abstract: The 5'-thiophosphate analogue of the small oligonucleotide mediator of the anti-picornavirus action of interferon was synthesized and shown to be highly resistant to enzymic 5'-dephosphorylation and to activate the 2-5A-dependent ribonuclease as effectively as parent p5'A2'p5'A2'p5'A itself.

We have described [1-3] a new approach to the selective regulation of mRNA expression. This technique involves the synthesis of a chimeric molecule which contains an antisense domain to target a given RNA and a 2',5'-oligoadenylate moiety which attracts and activates a latent cellular nuclease, the 2-5A-dependent RNase, which then cleaves the object RNA. This targeted genetic code strategy for the degradation of mRNA promises to be a powerful research tool to establish gene function and holds obvious potential for the therapy of disease. Research on 2-5A/antisense chimeras has established that the 5'-monophosphate moiety at the 5'-terminus of the 2-5A domain is required for activation of degradation of the targeted mRNA and the activation of the purified recombinant human 2-5A-dependent RNase.[1-3] Earlier work had established[4] that 5'-phosphorylation of 2-5A itself was needed for effective 2-5A-dependent RNase activation; *i.e.*, A2'p5'A2'p5'A, the 2-5A core with a free 5'-hydroxyl group, was virtually without 2-5A-dependent RNase binding and activation abilities.

Since phosphatases and related nucleotidases are of widespread occurrence in natural systems and are responsible for the dephosphorylation of nucleotide 5'-monophosphates to nucleosides,[6] it would be expected that one possible mechanism for the physiologic inactivation of 2-5A/antisense chimeras would be a similar enzymatic dephosphorylation. We have demonstrated that although the primary mechanism for the biological degradation of 2-5A oligonucleotides is through 2',5'-phosphodiesterase action, when phosphodiesterase resistance is built into a 2-5A analogue by chemical modification, 5'-dephosphorylation becomes the predominant mode of degradation. Extensive studies[7-9] have documented that nucleoside thiophosphates

(nucleoside phosphorothioates), such as adenosine 5'-O-thiophosphate (AMPS), are extremely resistant to the action of such dephosphorylating enzymes. For instance, AMPS was degraded about 2000 times slower than AMP by alkaline phosphatase from calf intestine or *E. coli*, [7] and was dethiophosphorylated much slower than AMP was dephosphorylated by nucleotidase from *Crotalus*.

To provide such a 2-5A/antisense chimera with enhanced resistance to metabolic deactivation through dephosphorylation, we first have explored the effect of the 5'-thiophosphate modification on the non-conjugated 2-5A trimer, **p5'A2'p5'A2'p5'A**; specifically, **sp5'A2'p5'A2'p5'A**. Extensive studies[10] have been conducted with the internucleotide phosphorothioate congeners to enhance resistance to phosphodiesterase degradation, but the 5'-thiophosphate has not been reported.

The objective oligonucleotide, **sp5'A2'p5'A2'p5'A**, was synthesized by first building the 2',5'-oligonucleotide skeleton from the 2'-terminus on a solid support, followed by 5'-phosphorylation, sulfurization, and cleavage from the support and deprotection. Specifically, trimer 2',5'-ApApA was prepared on a Applied Biosystems 391 DNA synthesizer. A 1 μmol A CPG 500 column (N^6-benzoyl-5'-dimethoxytrityl-2'(3')-O-acetyladenosine linked to long chain alkylamine CPG via a 2'(3')-succinate) (Glen Research, Sterling,VA)[11] was used. For chain extension, 5'-O-(4,4'-dimethoxytrityl)-3'-O-(tert-butyldimethylsilyl)-N^6-benzoyladenosine-2-cyanoethyl-N,N-diisopropyl-phosphoramidite (Chem Genes Corp., Waltham, MA) was employed. The synthesis cycle was modified by simply increasing the coupling time of the standard 1 μmol scale synthesis cycle from 15 seconds to 600 seconds. Under these conditions, the average coupling efficiency was above 95-97% as determined by quantitating spectrophotometrically the release of the dimethoxytrityl cation. The oligonucleotides were synthesized in the trityl-off mode and 5'-end modification was performed manually according the procedure presented in the Table 1. The 5'-terminus phosphorylation reagent was 2-[2-(4,4'-dimethoxytrityloxy)ethylsulfonyl]ethyl-2-cyanoethyl-N,N-diisopropylphosphoramidite (Glen Research, Sterling, VA),[12] and the sulfurizing reagent was tetraethylthiuram disulfide (TETD).[13]

The synthesized oligonucleotides were cleaved from the CPG solid support by treatment with a mixture of concentrated ammonium hydroxide and ethanol (3:1) for 2 h at room temperature. N^6-benzoyl and 3'-O-acetyl groups were removed by incubating the resulting alcoholic solutions at 55° C for 8 h. Finally, the 3'-*O-tert* - butyldimethylsilyl protecting groups were removed by treatment with 1 M tetrabutylammonium fluoride in THF overnight at room temperature. Crude deprotected oligonucleotides first were desalted on C-18 Sep-Pak cartridges (Waters, Milford, MA),and then HPLC purified on a Beckman Ultrasphere ODS (10 x 250 mm) column under the following conditions: linear gradient of 0-50% B in A during 30 min and then 50% B for 10 min (flow rate of 2 mL/min). Solvent A was 50 mM ammonium acetate (pH 7.0) and solvent B was methanol/water (1:1 v/v). Finally, the purified oligonucleotides were desalted on C-18 Sep-Pak cartridges and quantitated as A_{260} OD units.

Synthetic 2',5'-oligoadenylates were characterized by enzymatic digestion, capillary electrophoresis,[14] ^1H NMR and ^{31}P NMR.[15] Capillary electrophoretic analyses of the oligonucleotides were performed

Table 1. Synthesis Procedure for 5'-end modification of core trimer (2',5') ApApA

Step	Solvent/reagent	Time	Volume
1. coupling	0.2 M phosphorylation reagent in tetrazole/CH_3CN	3 min.	0.15 mL
2. washing	acetonitrile		3 mL
3. drying	argon	3 min.	
(4.) oxidation*	0.1 M I_2 in lutidine:THF:water, 20:80:1	0.75 min.	1 mL
(4.) sulfurization*	TETD in acetonitrile	15 min.	2.5 mL
5. washing	acetonitrile		3 mL
6. drying	argon	3 min.	
7. detritylation	3% TCA in CH_2Cl_2	1.5 min.	1 mL
8. washing	2% pyridine in CH_3CN		1 mL
9. washing	acetonitrile		3 mL

* The oxidation procedure was carried out for preparation of pApApA and the sulfurization process was performed when the desired product was the thiophosphate spApApA.

on an Applied Biosystems 270A-HT capillary electrophoresis instrument using MICRO-GEL$_{100}$ (Applied Biosystems Inc.) gel-filled capillaries (50 mM i.d.; effective length 27 cm; running buffer, 75 mM Tris phosphate, pH 7.6, 10% methanol). Detection was at 260 nm. Capillary gel electrophoresis was carried out with a sample concentration of approximately 0.1 OD/mL using electrokinetic injection for 2 s at -5kV. Run voltage was -10 kV (13 μA), and the operation temperature was 30 °C. An oligomer standard [d(AGTC)3] was coinjected with the analyzed sample (Figure 1).[14] When the oligonucleotide **p5'A2'p5'A2'p5'A** was

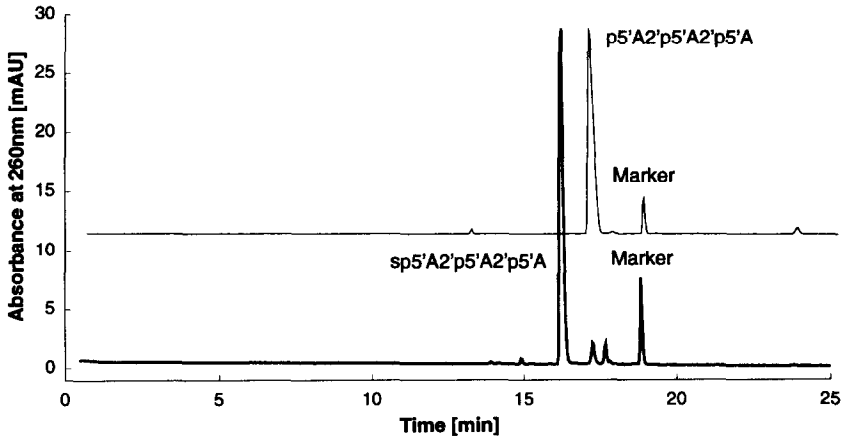

Figure 1. Capillary gel electropherogram of **p5'A2'p5'A2'p5'A** (top) and **sp5'A2'p5'A2'p5'A** (bottom) under conditions described in text. Each was coinjected together with a marker oligo, d(AGTC)$_3$.

digested with *C. adamanteus* venom phosphodiesterase, only AMP was formed as a hydrolysis product while **sp5'A2'p5'A2'p5'A** yielded AMP and AMPS in a molar ratio of 2:1. **^{31}P NMR** (D_2O, 85% H_3PO_4 as external reference, 0 ppm) of **sp5'A2'p5'A2'p5'A** δ (ppm): 45.99 (s, 1P, **P**=S), -1.07 (s, 1P, internucleotide **P**=O), -1.32 (s, 1P, internucleotide **P**=O). **^{1}H NMR** (D_2O, solvent peak 4.78 ppm as internal reference) of **sp5'A2'p5'A2'p5'A** δ (ppm): 8.21, 8.16, 7.96, 7.92, 7.91, 7.87 (s^{6x}, 1H, adenine H-2 and H-6 protons), 6.05 (d, 1H-1', J = 2.9 Hz), 5.91 (d, 1H-1', J = 3.6 Hz), 5.81 (d, 1H-1', J = 4.3 Hz), 3.95-5.05 (m, 15H, 3H-2', 3H-3', 3H-4', 6H-5').

To examine the relative stabilities of these **p5'A2'p5'A2'p5'A** and **sp5'A2'p5'A2'p5'A** toward enzymatic dephosphorylation,[16] their hydrolyses by calf intestinal alkaline phosphatase (CIP) and bacterial alkaline phosphatase (BAP) were monitored by HPLC during digestion under the following conditions: for calf intestinal alkaline phosphatase (CIP), the reaction was carried out in 90 μL of 10 mM Tris/HCl buffer (pH 8.0) containing 0.4 A_{260} OD units of oligonucleotide, 5 μL of 10 x CIAP buffer and 0.005 unit of CIP. For bacterial alkaline phosphatase (BAP), the reaction was carried out in 95 μL of 10 mM Tris/HCl buffer (pH 8.0) containing 0.4 A_{260} OD units of oligonucleotide and 0.005 units of BAP (48 units/mg protein). The reaction mixtures were incubated at 37 °C. Periodically, 10 μL of the reaction solution was withdrawn and immediately put in dry-ice to stop the reaction. The samples were diluted to 100 μL with 50 mM ammonium acetate and analyzed by HPLC to measure the peak area corresponding to the starting material and hydrolyzed product. The oligonucleotides **p5'A2'p5'A2'p5'A**, **sp5'A2'p5'A2'p5'A**, and core trimer **A2'p5'A2'p5'A** were used as the standard markers. Under the above conditions, **p5'A2'p5'A2'p5'A**, and **sp5'A2'p5'A2'p5'A** both had retention times of 11.5 min. and core trimer **A2'p5'A2'p5'A** had a retention time of 21.5 min. The digestion mixtures were monitored at different times and the % digestions were determined by monitoring the peaks of the starting oligonucleotide and the core digestion product. The stabilities of **p5'A2'p5'A2'p5'A** and **sp5'A2'p5'A2'p5'A** against CIP and BAP are presented in Figure 2. The halflives of **p5'A2'p5'A2'p5'A** in the presence of calf intestinal alkaline and bacterial alkaline phosphatases were 75 min and 45 min, respectively. The oligomer, **sp5'A2'p5'A2'p5'A**, was resistant toward calf intestinal phosphatase and bacterial alkaline phosphatase under the conditions where unmodified **p5'A2'p5'A2'p5'A** was hydrolyzed rapidly. Under the same conditions, the **sp5'A2'p5'A2'p5'A** was unaffected after 1 h of incubation. The observed hydrolysis (approx. 6.8%) with both enzymes could be attributed to the 4.4% **p5'A2'p5'A2'p5'A** present as the impurity in the **sp5'A2'p5'A2'p5'A** preparation.

The interaction of **sp5'A2'p5'A2'p5'A** with pure, recombinant human 2-5A-dependent RNase[5] was examined using a modification of the nuclease assay originated by Silverman.[17] This assays measures the ability of a 2-5A analogue to activate the 2-5A-dependent RNase to degrade [^{32}P]-poly(U). The parent trimer, **p5'A2'p5'A2'p5'A**, was used as a reference, and results were obtained as IC_{50}'s; that is, the concentration of oligonucleotide required to cause 50% degradation of the radioactive poly(U) substrate. Under previously described conditions, [3,17] both **sp5'A2'p5'A2'p5'A** and **p5'A2'p5'A2'p5'A** showed identical IC_{50}'s of

5 x 10^{-10} M, in excellent agreement with earlier observations.[4,5,17] Thus, the thiophosphoryated analogue was equipotent with its oxygenated parent as an activator of the 2-5A-dependent RNase.

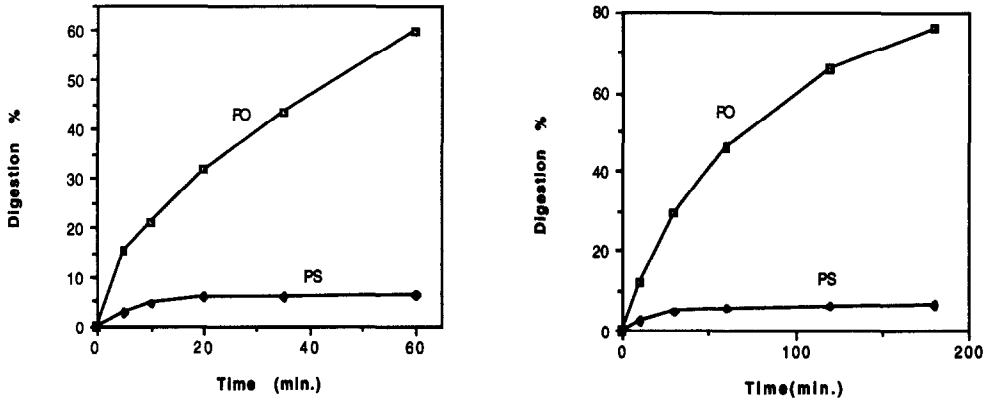

Figure 2. Stabilities of **sp5'A2'p5'A2'p5'A** (♦) and **p5'A2'p5'A2'p5'A** (□) toward bacteria alkaline phosphatase (right panel) and calf intestinal phosphatase (left panel). Assays were at 37°. Digestion % were determined from the HPLC peak area of the oligonucleotide and the digested product as measured at 260 nm. Whether dephosphorylation was determined by disappearance of starting oligo or by appearance of core dephosphorylated product, the same result was obtained.

Several conclusions are apparent based on the above results: (1) the 5'-thiophosphate of 2-5A trimer core can be prepared readily through reaction of tetraethylthiuram disulfide (TETD) with the intermediate phosphoramidite produced by reaction of 2-[2-(4,4'-dimethoxytrityloxy)ethyl sulfonyl]ethyl-2-cyanoethyl-N,N-diisopropylphosphoramidite with the CPG support-bound 5'-detritylated oligonucleotide trimer; (2) capillary gel electrophoresis provides a powerful analytical method to distinguish between 5'-phosphorylated and 5'-thiophosphorylated 2',5'-oligoadenylate trimers; (3) replacement of the 5'-phosphate moiety of **p5'A2'p5'A2'p5'A** with a 5'-thiophosphate group provides an analogue, **sp5'A2'p5'A2'p5'A**, with dramatically enhanced resistance to degradation by two representative phosphatases, calf intestinal alkaline phosphatase and bacterial alkaline phosphatase; (4) at the same time, the 5'-thiophosphorylated 2-5A analogue, **sp5'A2'p5'A2'p5'A**, was equipotent with **p5'A2'p5'A2'p5'A** as an activator of the 2-5A-dependent RNase. Thus 5'-thiophosphorylation provides an effective approach to stabilizing 2-5A against enzymic 5-dephosphorylation and therefore extending the biologic lifetime of such analogues.

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14. The synthetic **p5'A2'p5'A2'p5'A** and **sp5'A2'p5'A2'p5'A** were not resolved by reverse-phase HPLC under several different conditions. To determine the purities of these oligonucleotides, we found gel capillary electrophoresis technique (GCE) to be most valuable. The oligo trimers **sp5'A2'p5'A2'p5'A** and **p5'A2'p5'A2'p5'A** were resolved well by GCE. The thiophosphate, **sp5'A2'p5'A2'p5'A**, had approximately 91% purity and **p5'A2'p5'A2'p5'A** was identified as one of the contaminants (4.4%). There was also a second unidentified impurity present (approx. 3.6%). The synthesized **p5'A2'p5'A2'p5'A** showed about 99% purity as determined by GCE. Thus, in contrast to HPLC, GCE can be used to determine the degree of the 5'-end sulfurization by TEDT.
15. ^{1}H NMR and ^{31}P NMR were determined in D_2O on either Varian XL-300 or Gemini 300 MHz instruments. Chemical shifts are reported in ppm (δ units) relative to solvent peak (4.78 ppm, proton NMR) or relative to H_3PO_4 (0 ppm, external reference, ^{31}P NMR). Enzymatic digestion: substrate (0.2 A_{260} ODunits) was incubated with 0.15 unit of SVPD in 50 mM Tris/HCl (pH 8.0), 0.5 mM $MgCl_2$ at 37 °C for at least 3 h. The resulting hydrolyzed products were analyzed on a Beckman Ultrasphere ODS (0.46 x 250 mm) column. The solvent system used was 5% solvent B in A at a flow rate of 0.5 mL/min. where solvent A was 100 mM ammonium phosphate, pH 7.0 and solvent B was methanol/water (1:1). Adenosine 5'-thiophosphate (AMPS) and 5'-AMP (Aldrich, Milwaukee, WI) were used as the standard markers for products analyses.
16. Snake venom phosphodiesterase (SVPD) was from Pharmacia (Piscatway, NJ), bacterial alkaline phosphatase (BAP) was from Sigma (St. Louis, MO), and calf intestinal alkaline phosphatase (CIP) was from Promega (Madison, WI). For the dephosphorylation studies , a Beckman Ultrasphere ODS (4.6 x 250 mm) column was used under the following conditions: linear gradient of 0 - 60% B in A during 30 min. at a flow rate of 1 mL/min. and then 60% B for 10 min. where solvent A was 50 mM ammonium acetate, pH 7.0 and solvent B was MeOH/water (1:1).
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