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Chemical synthesis and biological characterization of phosphorothioate analogs of 2′, 5′-3′-deoxyadenylate trimer

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ABSTRACT

In continued studies to elucidate the requirements for binding to and activation of the 2′,5′-oligoadenylate (2-5A) dependent endoribonuclease (RNase L), four 2-5A trimer analogs were examined to evaluate the effect of chirality of phosphorothioate substitution on biological activity. The chemical syntheses and purification of the four isomers of P-thio-3′-deoxyadenylyl-(2′-5′)-P-thio-3′-deoxyadenylyl-(2′-5′)-3′-deoxyadenosine, by the phosphoramidite approach, is described. The isolated intermediates were characterized by elemental and spectral analyses. The fully deblocked compounds were characterized by ^{1}H and ^{31}P NMR and HPLC analyses. The 2′,5′-$(3'dA)_3$ cores with either Rp or Sp chirality in the 2′,5′-internucleotide linkages will bind to but will not activate RNase L. This is in contrast to 2′,5′-A_3 core analogs with either RpRp or SpRp phosphorothioate substitution in the 2′,5′-internucleotide linkages which can bind to and activate RNase L. There are also marked differences in the ability of the 2′,5′-A_3 analogs to activate RNase L following introduction of the 5′-monophosphate. For example, the 5′monophosphates of 2′,5′-$(3'dA)_3$-RpRp and 2′,5′-$(3'dA)_3$-SpRp can bind to and activate RNase L, whereas the 5′-monophosphates of 2′,5′-$(3'dA)_3$-RpSp and 2′,5′-$(3'dA)_3$-SpSp can bind to but can not activate RNase L.

INTRODUCTION

The 2′,5′-oligoadenylate (2-5A) synthetase/RNase L system plays a critical role in the antiviral mechanism of mammalian cells following external stimulation by interferon and/or dsRNA and is important in cell growth regulation and differentiation, in regulation of pre-mRNA processing and in the stability of c-myc and n-myc mRNA [1–7]. 2-5A synthetase is an allosterically regulated enzyme, activated by dsRNA, which polymerizes ATP to yield 2-5A, for example: 5′-triphosphoryl-adenylyl-(2′-5′)-adenylyl-(2′-5′)-adenosine [for reviews, see 1,2,8,9]. Primarily, 2-5A exerts its biological effect by binding to and activating its target enzyme, the 2′,5′-oligoadenylate dependent RNase L (EC 3.1.27) [9], although it has also been demonstrated that 2-5A and 2-5A analogs (3′-deoxyadenylate or phosphorothioate analogs) inhibit HIV-1 reverse transcriptase [10–12; Sobol et al., manuscript in preparation]. RNase L is a 2-5A dependent endoribonuclease specific for single-stranded RNA and cleaves on the 3′-side of UN sequences to yield UpNp terminated products [13–16]. Since the enzymatic degradation of 2-5A is rapid in cell-free extracts [17,18], this limits its biological activity in the intact cell [18]. As a result, 2-5A analogs with modifications in the base [19–24], sugar [25–31], or internucleotide linkages [17,32–35] have been designed to explore the biological role of the 2-5A synthetase/RNase L system.

We have examined the requirements for binding to and activation of RNase L via modification of the 2-5A molecule in the phosphodiester backbone, employing phosphorothioate substitution in the 2′,5′-internucleotide linkages [17, 18,34,36–38], as well as modifications in the sugar moiety, employing 3′-deoxyadenylate (cordycepin) substitution [31,39,40]. Changes in the stereochemistry of the phosphodiester backbone of 2-5A does not affect the affinity for RNase L. However, RNase L is a functionally stereoselective enzyme [17,18,35,38], yielding specific agonists and antagonists of RNase L *in vitro* and *in vivo* [17,18,35].

The biological activity of 3′-deoxyadenylate analogs of 2-5A has been reported [31,39,40]. These 3′-deoxyadenosine substituted analogs of 2-5A have extended metabolic stabilities in the absence of cellular toxicity. The 3′-deoxyadenosine substituted 2-5A molecules inhibit protein synthesis in lysed rabbit reticulocytes, prevent transformation of Epstein-Barr virus-infected lymphocytes, and inhibit the synthesis of EBV-induced nuclear antigen, tobacco mosaic virus replication, chondrosarcoma growth in animals and HIV-1 replication and reverse transcriptase [10–12,31,41–44].

Results with phosphorothioate-substituted 2-5A molecules and the metabolic stability and decreased cellular toxicity of 3′-deoxyadenosine analogs of 2-5A prompted us to synthesize the corresponding phosphorothioate analogs of 2′,

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5'-3'-deoxyadenosine trimer core and 5'-monophosphate. The synthesis and characterization of four phosphorothioate analogs of 2',5'-3'-deoxyadenosine, designated 2',5'-p(3'dA)$_3$-RpRp[1], 2',5'-p(3'dA)$_3$-RpSp, 2',5'-p(3'dA)$_3$-SpRp and 2',5'-p(3'dA)$_3$-SpSp, are described herein. The functional contribution of the 3'-hydroxyl groups and the 2',5'-phosphorothioate bonds in the binding and activation process of the 2-5A-dependent RNase L is described.

RESULTS AND DISCUSSION

Chemistry

Synthesis. 3'-Deoxy-5'-O-(monomethoxytrityl)-N^6-(2-(p-nitrophenyl)ethoxycarbonyl)adenosine (1) [40], treated with bis-N,N-diisopropylamino 2-cyanoethoxy phosphane (2) [45] in CH_2Cl_2 in the presence of tetrazole, yielded the corresponding phosphoramidite 3 as a mixture of two diastereomers, which were separated by medium pressure chromatography [46] using EtOAc/toluene (1/1). The higher R_f diastereomer and the lower R_f diastereomer were obtained in 31% and 38% yields, respectively. Each of these isomers were condensed separately with 3'-deoxy-bis-N6-2'-O-(2-(p-nitrophenyl)ethoxycarbonyl) adenosine (4) [40], in the presence of either tetrazole or 3-nitro-1,2,4-triazole in acetonitrile, and after 2 h, was oxidized with sulfur in pyridine. The higher R_f diastereomer phosphoramidite resulted in a yield of 59% of the Rp dimer (5a) and 39% of the Sp dimer (5b), whereas the lower R_f diastereomer phosphoramidite yielded 52% of the Rp dimer and 39% of the Sp dimer, indicating that there is no marked difference in the isomer distribution by using the different diastereomers for the condensation. The isomers could be separated using preparative silica gel plates developed in $CH2Cl_2$/EtOAc (1/1). The Rp dimer (5a) and the Sp dimer (5b) were separately detritylated using 2% p-TsOH in CH_2Cl_2/MeOH (8/2), giving 6a and 6b respectively, in a yield of 85–90%.

For the synthesis of the trimer isomers (RpRp, 7a; SpRp, 7b; RpSp, 8a and SpSp, 8b), the phosphoramidite 3, as the diastereomeric mixture, was condensed with Rp dimer 6a in the presence of 3-nitro-1,2,4 triazole. After oxidation with sulfur, the trimer phosphorothioate isomers RpRp (7a) and SpRp (7b) were obtained as a diastereomeric mixture which could be separated on preparative silica gel plates to give 31% RpRp (7a) and 20% SpRp (7b). Analogously, compound 3 was condensed with the 5'-hydroxy Sp dimer isomer (6b) and following sulfur oxidation and purification yielded 35% of the RpSp isomer (8a) and 24% of the SpSp isomer (8b), although separation of these isomers was difficult due to the small difference in R_f values.

The final deblocking of all the protecting groups from these four isomers was carried out by: (i) DBU treatment to eliminate the phosphate protecting cyanoethyl groups, as well as the base and sugar protecting p-(nitrophenyl)ethoxycarbonyl groups and (ii) acid treatment to remove the 5'-monomethoxytrityl groups. The crude products were purified by DEAE Sephadex A-25 column chromatography with triethylammonium bicarbonate buffer (pH 7.5) as eluant. Final purification was performed by paper chromatography using an isopropanol/ammonia/H_2O system and lyophilization to yield a colorless powder. The yields ranged from 76–86%.

Physical data. The protected nucleosides and nucleotides were characterized by CHN analyses and UV and ^{1}H NMR spectra. The λ_{max} values, molecular extinction coefficients and proton NMR signals are listed in Table I.

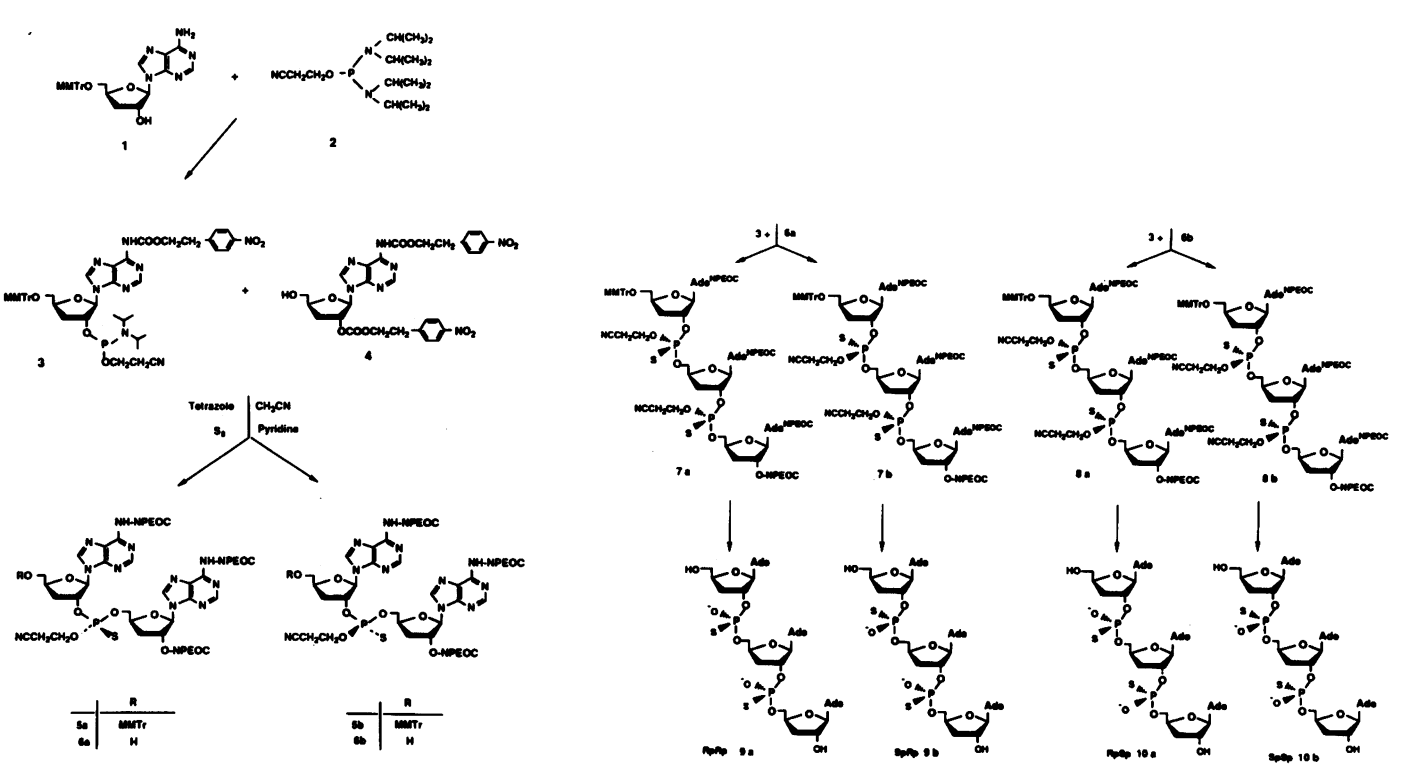

Figure 1. Synthetic scheme for the phosphorothioate analogs of 2',5'-3'-deoxyadenylate trimer.

Biological studies

5'-Monophosphorylation of 2',5'-(3'dA)$_3$ phosphorothioate cores. The 5'-monophosphates of 2',5'-A_3, 2',5'-(3'dA)$_3$ and 2',5'-(3'dA)$_3$ phosphorothioate analogs were synthesized from the corresponding cores and ATP utilizing T4 polynucleotide kinase. 5'-Monophosphorylation was monitored by reverse-phase HPLC analysis and confirmed by the subsequent hydrolysis of each 2',5'-(3'dA)$_3$ phosphorothioate analog by 5'-nucleotidase (data not shown). Yields of phosphorylation ranged from 26% to >99% (Table II). The presence of the Sp isomer in the first internucleotide bond reduced the yield of phosphorylation to as low as 40% (Table II).

Binding affinity of the 2',5'-(3'dA)$_3$ phosphorothioate cores and 5'-monophosphates for RNase L. The affinity of the 2',5'-(3'dA)$_3$ phosphorothioate analogs for binding to RNase L was determined in the 2-5A pCp radiobinding assay, based on the ability of 2-5A or 2-5A analogs to compete with a [^{32}P]2-5A pCp probe for specific binding to RNase L in an unfractionated cell-free extract prepared from L929 cells [47]. Results are expressed as IC_{50}, e.g., the concentration of 2-5A or analog required to inhibit 50% of the binding of the [^{32}P]2-5A pCp probe. 2',5'-(3'dA)$_3$ core and 2',5'-(3'dA)$_3$ phosphorothioate core analogs have IC_{50} values in the range of $1-2\times10^{-5}$ M, compared with an IC_{50} of 10^{-6} M for 2',5'-A_3 and 10^{-9} M for 2',5'-p_3A_3 (Figure 2).

As previously described for the 2',5'-A_3 phosphorothioate analogs [17], 5'-monophosphorylation of the 2',5'-(3'dA)$_3$ phosphorothioates increases affinity for RNase L by approximately 1000-fold. In the 2-5A pCp radiobinding assay, 2',5'-p(3'dA)$_3$ and 2',5'-p(3'dA)$_3$ phosphorothioate analogs have IC_{50} values ranging from 5×10^{-8} M to 1×10^{-7} M (Figure 3), compared with an IC_{50} value of 10^{-9} M for 2',5'-pA_3 or 2',5'-p_3A_3 (Figure 3). These results confirm earlier studies that RNase L cannot distinguish between Rp and Sp chirality with respect to binding [17].

Activation of RNase L by the 2',5'-(3'dA)$_3$ phosphorothioate cores and 5'-monophosphates. Whereas 2',5'-A_3-RpRp and 2',5'-A_3-RpSp phosphorothioate cores can bind to and activate RNase L [17], none of the 2',5'-(3'dA)$_3$ phosphorothioate cores activate RNase L as determined in rRNA cleavage assays using L929 cell extracts as the source of RNase L (Figure 4). 2',5'-p_3A_3 activated RNase L to cleave 28S and 18S rRNA to specific cleavage products (SCP) at 10^{-8} M (lane 2), whereas 2',5'-pA_3 requires a concentration of 5×10^{-6} M to activate RNase L (lane 3). Consistent with results reported from three laboratories, the cordycepin 2-5A analog, 2',5'-p_3(3'dA)$_3$, also activated RNase L to hydrolyze rRNA at 10^{-7} M (lane 9) [36,50,51,52]. The corresponding 5'-monophosphate, 2',5'-p(3'dA)$_3$, was inactive at a concentration of 10^{-7} M (lane 4) or higher (data not shown). However, the phosphorothioate-substituted 2',5'-p(3'dA)$_3$ molecules provide a striking contrast. 2',5'-p(3'dA)$_3$ with either RpRp or SpRp chirality will activate RNase L to hydrolyze 28S and 18S rRNA to SCP (lanes 5 and 6) at 10^{-7} M, whereas 2',5'-p(3'dA)$_3$ with either RpSp or SpSp chirality are without activity (lanes 7 and 8).

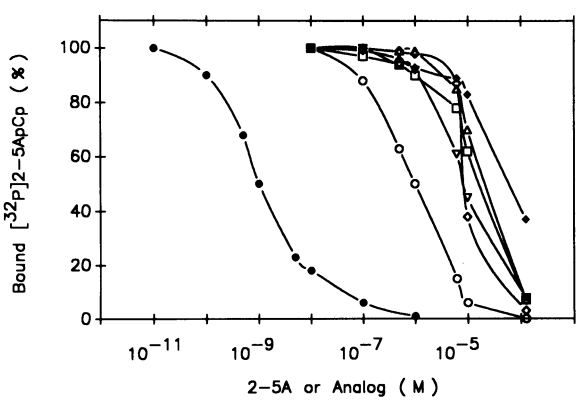

Figure 2. Binding of 2',5'-(3'dA)$_3$ core and its phosphorothioate analogs to RNase L. 2',5'-p_3A_3 (●), 2',5'-A_3 (○), 2',5'-(3'dA)$_3$-RpRp (▽), 2',5'-(3'dA)$_3$-SpRp (□), 2',5'-(3'dA)$_3$-RpSp (◇), 2',5'-(3'dA)$_3$-SpSp (△) and 2',5'-(3'dA)$_3$ (◆) were analyzed for their ability to inhibit binding of [^{32}P]2-5A pCp probe to RNase L in radiobinding assays.

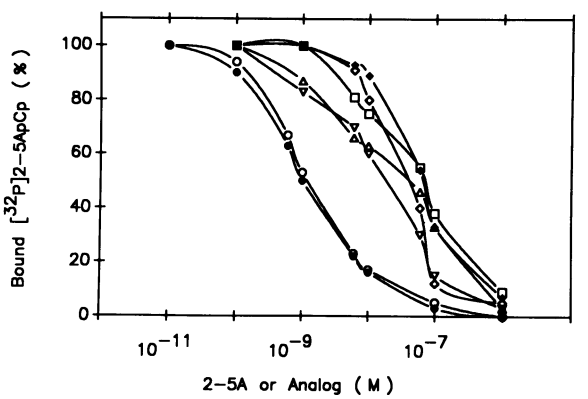

Figure 3. Binding of 2',5'-(3'dA)$_3$ 5'-monophosphate and its phosphorothioate analogs to RNase L. 2',5'-p_3A_3 (●), 2',5'-pA_3 (○), 2',5'-p(3'dA)$_3$-RpRp (▽), 2',5'-p(3'dA)$_3$-SpRp (□), 2',5'-p(3'dA)$_3$-RpSp (◇), 2',5'-p(3'dA)$_3$-SpSp (△) and 2',5'-p(3'dA)$_3$ (◆) were analyzed for their ability to inhibit binding of [^{32}P]2-5A pCp probe to RNase L in radiobinding assays.

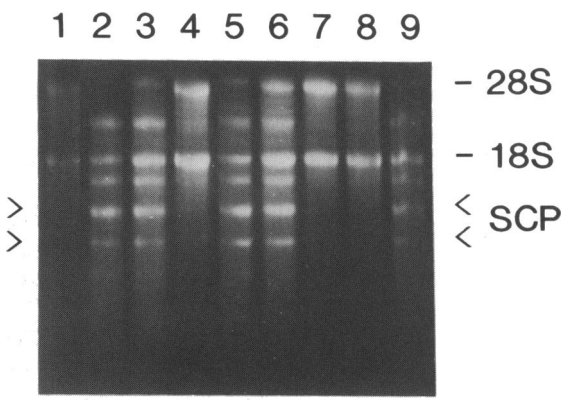

Figure 4: Ribosomal RNA cleavage assay with 2',5'-(3'dA)$_3$ phosphorothioate 5'-monophosphates. L929 extracts were incubated in the absence (lane 1) or presence of 2',5'-p_3A_3 at 10^{-8} M (lane 2), 2',5'-pA_3 at 5×10^{-6} M (lane 3), 2',5'-p(3'dA)$_3$ at 10^{-7} M (lane 4), 2',5'-p(3'dA)$_3$-RpRp at 10^{-7} M (lane 5), 2',5'-p(3'dA)$_3$-SpRp at 10^{-7} M (lane 6), 2',5'-p(3'dA)$_3$-RpSp at 10^{-7} M (lane 7), 2',5'-p(3'dA)$_3$-SpSp at 10^{-7} M (lane 8) and 2',5'-p_3(3'dA)$_3$ at 10^{-7} M (lane 9). The positions of 28S and 18S rRNA and specific cleavage products (SCP) are indicated.

Table I. Physical data for the phosphorothioate analogs of 2′,5′-3′-deoxyadenylate trimer

Cmpd.	UV Spectra (MeOH)		^{1}H-NMR Spectra ($CDCl_3$; δ=ppm)			^{31}P-NMR ($CDCl_3$)	TLC
	λ_{max} (nm)	lg ε	H-C(1′)	H-C-(8)	H-C-(2)		R_f
3a	233 267 275	4.33 4.49 4.42	6.18(s)	8.69(s)	8.22(s)	150.088	0.52[a]
3b	231 267 275	4.33 4.48 4.40	6.24(s)	8.70(s)	8.23(s)	150.858	0.43[a]
5a-Rp	268 275	4.79 4.74	6.21(t) 6.08(t)	8.67(s) 8.58(s)	8.17(s) 8.16(s)	67.855	0.39[b]
5b-Sp	268 276	4.78 4.70	6.22(d) 5.94(d)	8.70(s) 8.66(s)	8.18(s) 8.17(s)	67.300	0.25[b]
6a-Rp	267 274	4.79 4.72	6.13(d) 5.94(d)	8.71(s) 8.68(s)	8.18(s) 8.16(s)		0.22[c]
6b-Sp	267 275	4.79 4.70	6.12(d) 5.97(d)	8.70(s) 8.69(s)	8.19(s) 8.16(s)		0.38[c]
7a-RpRp	234 267 275	4.64 4.96 4.89				67.98 67.64	0.35[d]
7b-SpRp	234 267 275	4.63 4.96 4.91				67.85 67.30	0.25[d]
8a-RpSp	234 267 275	4.63 4.95 4.89				67.96 67.58	0.27[d]
8b-SpSp	236 267 277	4.63 4.95 4.85				67.58 67.48	0.19[d]
9a-RpRp	258[e]		5.95(s) 5.86(s) 5.65(s)	8.16(s) 8.12(s) 8.10(s)	7.98(s)[f] 7.83(s) 7.77(s)		0.20[g]
9b-SpRp	258[e]		5.99(s) 5.92(s) 5.70(s)	8.15(s) 8.11(s) 8.07(s)	8.03(s)[f] 7.93(s) 7.79(s)		0.21[g]
10a-RpSp	258[e]		5.99(s) 5.80(s) 5.66(s)	8.15(s) 8.11(s) 8.06(s)	7.97(s)[f] 7.82(s) 7.80(s)		0.21[g]
10b-SpSp	258[e]		6.01(s) 5.85(s) 5.70(s)	8.13(s) 8.07(s) 8.00(d,2H)	7.88(s)[f] 7.82(s)		0.20[g]

[a] SiO_2, toluene/EtOAc 1:1; [b] CH_2Cl_2/EtOAc 1:1; [c] $CHCl_3$/MeOH 95:5; NH_3/H_2O 65:10:25; s=singlet; d=double; [d] EtOAc (run 3 times); [e] H_2O; [f] D_2O; [g] Cellulose: i-PrOH/conc.

In summary, 2′,5′-(3′dA)$_3$ phosphorothioate cores and their corresponding 5′-monophosphates have been utilized to evaluate the effects of chirality of ophosphorothioate substitution on activation of RNase L. As with the 2′,5′-A$_3$, the 2′,5′-(3′dA)$_3$ phosphorothioate cores exhibited equal affinity for RNase L, similar to authentic 2′,5′-(3′dA)$_3$. The 2′,5′-A$_3$ phosphorothioate cores with the RpRp or SpRp configuration are able to bind to and activate RNase L [36], but there is no activation with the 2′,5′-A$_3$-RpSp or 2′,5′-A$_3$-SpSp phosphorothioate cores. Replacement of the ribosyl moiety of 2′,5′-A$_3$ with the 3′-deoxyribosyl moiety and the introduction of chirality into the 2′,5′-internucleotide linkages (to form the 2′,5′-(3′dA)$_3$ phosphorothioate cores) result in molecules unable to activate RNase L. However, 5′-phosphorylation of 2′,5′-(3′dA)$_3$ and introduction of the correct stereochemistry in the internucleotide linkage (i.e., Rp chirality in the second internucleotide linkage from the 5′-end) yield biologically active isomers. 2′,5′-p(3′dA)$_3$-RpRp and 2′,5′-p(3′dA)$_3$-SpRp can activate RNase L, whereas 2′,5′-p(3′dA)$_3$-RpSp and 2′,5′-p(3′dA)$_3$-SpSp analogs and 2′,5′-p(3′dA)$_3$ are without activity.

Phosphorothioate-substituted analogs of 2-5A (5′-monophosphates and 5′-triphosphates) are effective inhibitors of HIV-1 reverse transcriptase and HIV-1 replication [12]. This inhibition is in part mediated by inhibition of template/primer binding [10,13]. 2′,5′-p(3′dA)$_3$ is also an effective inhibitor of HIV-1 replication when encapsulated in liposomes targeted by antibodies for the T-cell receptor molecule CD3 [11]. Studies are currently underway to determine if the combined effect of phosphorothioate and 3′-deoxyadenylate substitution in the 2-5A molecule as described herein will find application in anti-HIV drug therapy.

Table II. Summary of biological activities of the 2',5'-(3'-deoxyadenylate) phosphorothioate trimer cores and 5'-monophosphates

2',5'-Oligonucleotide	% Transfer by Polynucleotide Kinase[a]	Radiobinding (IC_{50})[b]	rRNA Hydrolysis[c]
2',5'-A_3	26	1 x 10^{-6} M	NA (10^{-5} M)
2',5'-(3'dA)$_3$	75	1 x 10^{-4} M	NA (10^{-5} M)
2',5'-(3'dA)$_3$-RpRp	>99	1-2 x 10^{-5} M	NA (10^{-5} M)
2',5'-(3'dA)$_3$-SpRp	40	1-2 x 10^{-5} M	NA (10^{-5} M)
2',5'-(3'dA)$_3$-RpSp	>99	4-5 x 10^{-5} M	NA (10^{-5} M)
2',5'-(3'dA)$_3$-SpSp	42	4-5 x 10^{-5} M	NA (10^{-5} M)
2',5'-pA_3	---	1 x 10^{-9} M	5 x 10^{-6} M
2',5'-p(3'dA)$_3$	---	8 x 10^{-8} M	NA (10^{-5} M)
2',5'-p(3'dA)$_3$-RpRp	---	2 x 10^{-8} M	1 x 10^{-7} M
2',5'-p(3'dA)$_3$-SpRp	---	5 x 10^{-8} M	1 x 10^{-7} M
2',5'-p(3'dA)$_3$-RpSp	---	8 x 10^{-8} M	NA (10^{-5} M)
2',5'-p(3'dA)$_3$-SpSp	---	5 x 10^{-8} M	NA (10^{-5} M)
2',5'-p$_3A_3$	---	1 x 10^{-9} M	1 x 10^{-9} M
2',5'-p$_3$(3'dA)$_3$	---	1 x 10^{-8} M	1 x 10^{-7} M

[a] Transfer of γ-phosphate from ATP by T4 polynucleotide kinase to the 5'-hydroxyl of 2',5'-oligonucleotides. Percent conversion based on the areas under the HPLC curve for each oligonucleotide core and corresponding 5'-monophosphate. Percent yields are based on an average of three separate determinations.

[b] The concentration of 2-5A or 2-5A analog required to inhibit 50% of the binding of the 2',5'-p$_3A_4$[^{32}P]pCp probe in the radiobinding assay.

[c] The minimum concentration of 2-5A or 2-5A analog required to activate RNase L as determined in the ribosomal RNA cleavage assay. NA (X) = not active at a concentration of X or higher.

EXPERIMENTAL

General. Precoated silica gel thin layer TLC sheets F 1500 LS 254, cellulose thin-layer TLC sheets F 1440 and paper chromatography PC sheets (58×60 cm) were obtained from Schleicher & Schull. Preparative silica gel 60 PF 254 TLC plates and silica gel (Merck-60, 0.063−0.2 mesh) for preparative column chromatography were obtained from Merck. Kiesel-gel LiChroprep Si 60 (15−25 μ) was used for the medium-pressure chromatography (250×24 mm column). The preparation, stabilization and characterization by the determination of the number of theoretical plates are described elsewhere [46]. DEAE Sephadex A-25 was from Pharmacia. ^{1}H-NMR was conducted using a Bruker WM-250 and ^{31}P-NMR was conducted using a Jeol 400 MHz with phosphoric acid as the standard. T4 polynucleotide kinase and T4 RNA ligase were obtained from Bethesda Research Laboratories. [^{32}P]pCp (specific activity 3000 Ci/mmole) was purchased from Amersham. 2',5'-p$_3A_3$ was obtained from Pharmacia-LKB and 2',5'-A_3 core was from Sigma. 2',5'-(3'dA)$_3$ core was synthesized as described [39,40].

3'-Deoxy-5'-O-(monomethoxytrityl)-N^6-[2-(p-nitrophenyl)ethoxycarbonyl]adenosine 2'-(2-cyanoethyl-N,N-diisopropylamino phosphoramidite) (3). Compound 1 (40) (0.716 g, 1 mmole) was dissolved in CH_2Cl_2 (7.5 ml), then tetrazole (0.035 g, 1 mmole) and bis-N,N-diisopropylamino-2-cyanoethoxy phosphane (2) (0.512 g, 1.7 mmole) were added under a nitrogen atmosphere. After stirring at r.t. for 20 h, another 0.5 g (1.63 mmole) of compound 2 was added. After stirring for 4 h, the reaction mixture was diluted with CH_2Cl_2 (100 ml), washed with a saturated $NaHCO_3$/NaCl solution and dried over Na_2SO_4. After evaporation, the product was applied to a silica gel column (15×2 cm) equilibrated in EtOAc/NEt_3 (199/1) and eluted with 300 ml of the same solvent. After evaporation, the residue was coevaporated with CH_2Cl_2 (4×10 ml). For the diastereomeric separation, the product was dissolved in EtOAC/toluene (1/1) and chromatographed with the same solvent system using medium pressure chromatography [46] (500 drops/fraction). The pure higher R_f diastereomer (3a) eluted in fractions 28−38 (yield: 0.284 g, 31%). A mixture of the higher and lower R_f isomers was obtained in fractions 39−41 (yield: 0.03 g, 2.9%) and the lower R_f diastereomer (3b) eluted in fractions 42−58 (yield: 0.348 g, 38%), resulting in a total yield of 72%. Anal. calc. for the higher R_f isomer (3a) $C_{48}H_{53}N_8O_9P$ (916.973): C 62.87, H 5.82, N 12.22. Found: C 62.17, H 5.78, N 12.01. R_f in silica gel toluene/EtOAc (1/1, v/v) = 0.52. ^{31}P NMR ($CDCl_3$): 150.088. Anal. calc. for the lower R_f isomer (3b) $C_{48}H_{53}N_8O_9P$ (916.973): C 62.87, H 5.82, N 12.22. Found: C 62.24, H 5.91, N 12.00. R_f in silica gel toluene/EtOAc (1/1, v/v) = 0.43. ^{31}P NMR ($CDCl_3$): 150.858.

3'-Deoxy-5'-O-(monomethoxytrityl)-N^6-[2-(p-nitrophenyl)-ethoxycarbonyl]P-thioadenylyl-[2'-[O^P-(2-cyanoethyl)]-5']-3'-deoxy-N^6,2'-bis[2-(p-nitrophenyl) ethoxycarbonyl]adenosine: Rp isomer (5a) and Sp isomer (5b). Experiment 1: The higher R_f phosphoramidite diastereomer 3a (0.128 g, 0.14 mmole) and 3'-deoxy-N^6-2'-bis-[2-(p-nitrophenyl) ethoxycarbonyl]-adenosine (4) (0.04 g, 0.076 mmole) were dried overnight at r.t. under high vacuum. The residue was then dissolved in CH_3CN (0.7 ml) and 3-nitro-1,2,4-triazole (0.043 g, 0.377 mmole) was added. After stirring for 2 h at r.t. under a nitrogen atmosphere, S_8 (0.06 g,

0.233 mmole) and pyridine (1.2 ml) were added and stirred at r.t. for 20 h. The reaction mixture was diluted with $CHCl_3$ (200 ml), washed with a saturated NaCl solution (2×100 ml), dried and evaporated to dryness, final coevaporation was done with toluene (2×10 ml). The crude product was purified by a silica gel column (10×2 cm), and both isomers were eluted with $CHCl_3$/MeOH (100/1, 500 ml). After evaporation, the isomers were separated on preparative silica gel plates (20×20×0.2 cm) using EtOAc/CH_2Cl_2 (1/1), developed twice to get clear separation. The higher R_f isomer (Rp, 5a) was obtained in 59% yield (0.066 g) and the lower R_f isomer (Sp, 5b) was obtained in 39% yield (0.044 g). Experiment 2: The lower R_f phosphoramidite diastereomer 3b was used for the condensation and the reaction was carried out analogously. The Rp (5a) and Sp (5b) isomers were obtained in 52% and 39% yields, respectively. Anal. calc. for the higher R_f isomer (5a) $C_{70}H_{65}N_{14}O_{20}PS.H_2O$ (1520.74): C 55.08, H 4.57, N 12.89. Found: C 55.33, H 4.56, N 12.89. R_f in silica gel EtOAc/CH_2Cl_2 (1/1, v/v) = 0.39. ^{31}P NMR ($CDCl_3$): 67.855. Anal. calc. for the lower R_f isomer (5b) $C_{70}H_{65}N_{14}O_{20}PS.H_2O$ (1520.74): C 55.28, H 4.57, N 12.89. Found: C 55.33, H 4.62, N 11.89. R_f in silica gel EtOAc/CH_2Cl_2 (1/1, v/v) = 0.25. ^{31}P NMR ($CDCl_3$): 67.30.

3'-Deoxy-N⁶-[2-(p-nitrophenyl)-ethoxycarbonyl]-P-thioadenylyl-[2'-[O^P-(2-cyanoethyl)]-5']-3'-deoxy-N⁶,2'-O-bis-[2-(p-nitrophenyl)-ethoxycarbonyl] adenosine: Rp isomer (6a) and Sp isomer (6b). The corresponding 5'-monomethoxytrityl dimers 5a and 5b were separately detritylated as follows: 0.13 g (0.0875 mmole) of the fully protected dimer was stirred with 2% p-TsOH in CH_2Cl_2/MeOH (4/1, 1.6 ml) at r.t. for 45 min. The reaction mixture was diluted with $CHCl_3$ (100 ml) and washed with H_2O (2×20 ml), dried and evaporated to dryness. The residue was purified by silica gel column chromatography (10×2.5 cm) and the product was eluted with $CHCl_3$/MeOH (50/1). After evaporation, the pure dimer was dried *in vacuo* at 40°C and yields of 6a and 6b of 87% and 90%, respectively, were obtained. Anal. calc. for the higher R_f isomer (6a) $C_{50}H_{45}N_{14}O_{19}PS$ (1209.02): C 49.67, H 3.75, N 16.21. Found: C 49.08, H 3.55, N 16.19. R_f in silica gel $CHCl_3$/MeOH (95/5, v/v) = 0.22. Anal. calc. for the lower R_f isomer [*6b*] $C_{50}H_{45}N_{14}O_{19}PS$ (1209.02): C 49.67, H 3.75, N 16.21. Found: C 49.62, H 4.47, N 16.37. R_f in silica gel $CHCl_3$/MeOH (95/5, v/v) = 0.38.

3'-Deoxy-5'-O-(monomethoxytrityl)-N⁶-[2-(p-nitrophenyl)ethoxycarbonyl]-P-thioadenylyl-[2'-[O^P-(2-cyanoethyl)]-5']-3'-deoxy-N⁶-[2-(p-nitrophenyl)ethoxycarbonyl]-P-thioadenylyl-[2'-[O^P-(2-cyanoethyl)]-5']-3'-deoxy-N⁶,2'-O-bis-[2-(p-nitrophenyl) ethoxycarbonyl]adenosine: RpRp isomer (7a), SpRp isomer (7b), RpSp isomer (8a) and SpSp isomer (8b). Compound 3 (0.13 g, 0.114 mmole) and 5'-hydroxy dimer (Rp isomer 6a) were dried in high vacuum at 40°C overnight, then dissolved in CH_3CN (0.75 ml) and 3-nitro-1,2,4- triazole (0.042 g, 0.368 mmole) was then added. After 2 h stirring at r.t. under a nitrogen atmosphere, sulfur (0.023 g, 0.089 mmole) and dry pyridine (0.23 ml) were added and further stirred at r.t. for 20 h. The reaction mixture was diluted with CH_2Cl_2 (150 ml), washed with saturated NaCl (2×80 ml), dried and evaporated to dryness, final coevaporation was done with toluene (2×15 ml). The crude product was purified by silica gel column chromatography (12 x 2.5 cm) and eluted with $CHCl_3$ and $CHCl_3$/MeOH (50/2). The pure product fractions were collected and evaporated to yield 0.123 g (81%) of a mixture of RpRp and SpSp isomers. These were then separated on three preparative silica gel plates (20×20 0.2 cm), developed once in CH_2Cl_2/MeOH (1/1) and then three times in EtOAc. The higher R_f isomer (RpRp, 7a) was obtained in 31% yield (0.047 g) and the lower R_f isomer (SpRp, 7b) was obtained in 20% yield (0.029 g). A mixture of the RpRp and SpSp isomers was isolated in 13% yield (0.02 g). Compounds 8a (RpSp isomer; 35% yield, 0.054 g) and 8b (SpSp isomer; 24% yield, 0.0370 g) were obtained in an analogous manner from the 5'-hydroxy dimer (Sp isomer 6b). Anal. calc. for the RpRp isomer (7a) $C_{92}H_{83}N_{21}O_{27}P_2S_2$ (2039.952): C 54.16, H 4.10, N 14.41. Found: C 54.46, H 4.54, N 14.47. R_f on silica gel in EtOAc (run three times) = 0.35. Anal. calc. for the SpRp isomer (7b) $C_{92}H_{83}N_{21}O_{27}P_2S_2$ (2039.952): C 54.16, H 4.10, N 14.41. Found: C 54.93, H 4.77, N 13.14. R_f on silica gel in EtOAc (run three times) = 0.27. Anal. calc. for the RpSp isomer (8a) $C_{92}H_{83}N_{21}O_{27}P_2S_2$ (2039.952): C 54.16, H 4.10, N 14.41. Found: C 54.01, H 4.64, N 13.84. R_f on silica gel in EtOAc (run three times) = 0.27. Anal. calc. for the SpRp isomer (8b) $C_{92}H_{83}N_{21}O_{27}P_2S_2$ (2039.952): C 54.16, H 4.10, N 14.41. Found: C 54.58, H 4.59, N 13.97. R_f on silica gel in EtOAc (run three times) = 0.18.

3'-Deoxy-P-thio-adenylyl-(2'-5')-3'-deoxy-P-thio-adenylyl-(2'-5')-3'-deoxyadenosine: RpRp (9a), SpRp (9b), RpSp (10a), SpSp (10b). 0.03 g (0.0147 mmole) of the appropriate fully protected trimer was first stirred with 0.5 M DBU in dry pyridine (10 ml) and after 24 h stirring, the reaction mixture was neutralized with 1 M acetic acid in dry pyridine (5 ml) and evaporated *in vacuo*. The residue was then treated with 80% acetic acid (10 ml), stirred at r.t. for 16 h and evaporated to dryness, with several coevaporations with H_2O to remove any residual acetic acid. The residue was dissolved in H_2O (10 ml) and applied onto a DEAE Sephadex A-25 column (60× 1 cm) and the pure products were eluted with 0.3 M Et_3NHCO_3 buffer (pH 7.5). After evaporation and coevaporation of the product fractions, the trimers were purified by paper chromatography using 2-propanol/ammonia/H_2O (65/10/35). After eluting the product bands from the paper with water, the solvent was evaporated and lyophilized to give a colorless powder in 75–90% yields. The retention times (R_t) for each purified product by HPLC using the Merck RP-18 column (as described below) are: RpRp (9a), 901 sec; SpRp (9b), 1062 sec; RpSp (10a), 1174 sec; SpSp (10b), 1376 sec.

HPLC. HPLC was conducted with a UV/VIS Uvikon 820, Kontron, and Perkin-Elmer Lambda 5; using a Merck-Hitachi D 2000 reverse-phase column (RP-18; 125×4 mm, 5 μm) at a flow rate of 1 ml/min; using a mobile phase of 50 mM $NH_4H_2PO_4$ (pH 7.0)/MeOH:H_2O (1:1) 70:30. For the purification of the 5'-monophosphates, HPLC was carried out with two Waters Associates Model 6000A pumps controlled by a Model 660 solvent programmer with a Waters C_{18} μBondapak analytical column (3.9 mm×30 cm) at a flow rate 1 ml/min. The column was eluted with solvent A = 50 mM ammonium phosphate, pH 7.0, and solvent B = methanol/H_2O (1:1 v/v) as follows: linear gradient (t = 1 min, 10% B; t = 31 min, 70% B) followed by maintenance at 70% B for 20 min.

Cell culture. L929 cells were maintained in monolayer culture in Dulbecco's modified Eagle medium supplemented with 5% calf serum (GIBCO). Extracts of cells were prepared from confluent monolayer cultures and were used as the source of RNase L for all *in vitro* biological assays [37].

5'-Monophosphorylation of 2',5'-(3'dA)₃ phosphorothioate cores with T4 polynucleotide kinase. The 5'-monophosphates of 2',5'-A_3 and the 2',5'-(3'dA)$_3$ phosphorothioate cores were synthesized from ATP with T4 polynucleotide kinase. The

progress of the reaction was monitored by HPLC as described for the 2',5'-A_4 phosphorothioate cores [18].

Radiobinding assays. Radiobinding assays were performed as described [47], using L929-cell extracts as the source of RNase L. The labelled 2',5'-p_3A_4[^{32}P]pCp probe was synthesized by ligation of [^{32}P]pCp (specific activity 3000 Ci/mmole) onto 2',5'-p_3A_4 with T4 RNA ligase [49].

Ribosomal RNA cleavage assays. rRNA cleavage assays were performed as described [16]. Extracts of L929 cells (prepared as described above) were incubated for 1 h at 30°C in the absence or presence of 2-5A or 2-5A analogs. The total RNA was extracted, denatured, and analyzed by electrophoresis on 1.8% agarose gels. The gels were stained with ethidium bromide and the RNA bands visualized under ultraviolet light.

Radioactive measurements were determined on a Beckman LS-100C liquid scintillation spectrometer with counting efficiency of 99% for ^{32}P.

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ABBREVIATIONS

A_3, pA_3 and p_3A_3, trimers of adenylic acid with 2',5'-phosphodiester linkages and 5'-hydroxyl, 5'-monophosphate or 5'-triphosphate, respectively. $(3'dA)_3$, $p(3'dA)_3$ and $p_3(3'dA)_3$, trimers of 3'-deoxyadenylic acid with 2',5'-phosphodiester linkages and 5'-hydroxyl, 5'-monophosphate, or 5'-triphosphate, respectively. $(3'dA)_3$ -RpRp, -SpRp, -RpSp and -SpSp cores and 5'-monophosphates, phosphorothioate analogs of 2',5'-$(3'dA)_3$ or 2',5'-$p(3'dA)_3$, respectively, with Rp and Sp stereoconfigurations in the two chiral centers; assignment of configuration is from the 5' to the 2'-terminus as described in reference 37.

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