was added to 600 mL of cold H_2O. The mixture was stirred vigorously as it was allowed to warm to room temperature. After 25 min, to the mixture was added 2.60 g (0.0310 mol) of $NaHCO_3$ (pH was still strongly acidic). After 1 h and 10 min, another 2.60 g (0.0310 mol) of $NaHCO_3$ was added (pH still was strongly acidic). After 1 h and 25 min, another 1.86 g (0.0221 mol) of $NaHCO_3$ was added, and the pH of the mixture was 4–5. After 1 h and 40 min, the ether was removed under vacuum at 30 °C. The remaining mixture was adjusted to pH 5–6 with 0.41 g (0.00488 mol) of $NaHCO_3$ and was extracted with 2 × 50 mL of CH_2Cl_2. The combined CH_2Cl_2 extract was washed with 250 mL of water, dried over Na_2SO_4, and filtered. The filtrate was concentrated to a pale yellow oil: yield 11.0 g. The oil was chromatographed on silica gel in a Waters Prep 500 A apparatus with 3:2 hexanes/EtOAc: yield 7.58 g (69%) of a colorless oil **51**, which solidified; mp 44–47 °C; IR (Nujol) 1745 (CO), 3428 and 3540 (NH) cm^{-1}; NMR ($CDCl_3$) δ 0.98 (2 t's, 6 H, propyl CH_3), 1.2–1.9 (m, 4 H, propyl C-2 CH_2), 2.05 (s) and 2.12 (s) (9 H, acetyl CH_3), 2.63 (2 t's, 4 H, propyl C-1 CH_2), 3.8–4.4 (m, 3 H, C-1 H and C-5 CH_2), 4.84 (br s, 2 H, NH_2), 4.9–5.2 (m, 1 H, C-4 H), 5.27 (dd, J = 7.5 and 2.5 Hz, 1 H, C-2 H), 5.73 (dd, J = 7.5 and 2.5 Hz, 1 H, C-3 H); $[\alpha]^{20}_D$ +24.6° (*c* 1.05, $CHCl_3$). Anal. ($C_{18}H_{31}NO_8S_2$) C, H, N, S.

2,3-*O*-Isopropylidene-D-ribono-1,4-lactone 5-(Di-*tert*-butyl phosphate) (49). The lactone **26** was treated with Ph_3P in CBr_4 to obtain the 5-bromo derivative. The bromo compound (1.2 g, 0.0047 mol) in 5 mL of dry DME was added dropwise to a refluxing solution of tetramethylammonium di-*tert*-butyl phosphate (1.3 g, 0.0047 mol) in 19 mL of dry DME under N_2.[39] This mixture was refluxed for 1 h, cooled, and filtered. The filtrate was evaporated to an oil, which was dissolved in EtOAc and washed with H_2O. The EtOAc solution was dried (Na_2SO_4) and evaporated to a pale yellow oil **49**: yield 1.7 g (94%); NMR (Me_2SO-d_6) δ 1.3–1.5 (several s, 24 H), 4.1 (dd, J_{PH} = 6 Hz, J_{45} = 3 Hz, 2 H, H-5), 4.75 (s and m, 3 H, H-2, H-3, H-4).

Isomerase Assay.[24] A mixture containing 170 μL of 0.1 M histidine buffer at pH 7.5, 30 μL of inhibitor solution, and 50 μL of enzyme preparation was preincubated for 2 min at 37 °C. The reaction was initiated by the addition of 50 μL of 10 mM D-arabinose 5-phosphate or D-ribulose 5-phosphate and was terminated at 0, 3, 6, 9, and 12 min by the addition of 50 μL of 1.5% cysteine hydrochloride solution, followed immediately by 1.5 mL of 25 N H_2SO_4. After being mixed, the samples were treated with 50 μL of 0.12% carbazole in 95% EtOH and incubated at 37 °C for 30 min. The absorbance was read at 540 nm. The conversion of 1 μmol of D-A5P to 1 μmol of D-ribulose 5-phosphate gave a ΔOD of 8.2 ± 0.34.

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Supplementary Material Available: NMR data for compounds **44–46**, **50**, and **72–78** (2 pages). Ordering information is given on any current masthead page.

Oligonucleotide Structural Parameters That Influence Binding of 5′-*O*-Triphosphoadenylyl-(2′→5′)-adenylyl-(2′→5′)-adenosine to the 5′-*O*-Triphosphoadenylyl-(2′→5′)-adenylyl-(2′→5′)-adenosine Dependent Endoribonuclease: Chain Length, Phosphorylation State, and Heterocyclic Base

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A number of 2′,5′-linked oligoadenylates and their analogues were prepared and evaluated for their ability to interact with the 5′-*O*-triphosphoadenylyl-(2′→5′)-adenylyl-(2′→5′)-adenosine (2-5A) dependent endoribonuclease of mouse L cells. The oligonucleotides were assayed for their ability to antagonize the action of 2-5A, to displace a radiolabeled probe from the 2-5A-dependent nuclease, or to inhibit translation in a cell-free system. These experiments demonstrated the following: (1) Three AMP residues in a 5′-phosphorylated oligonucleotide were needed for maximum interaction with the endonuclease, and higher oligomers (≥4 AMP residues) did not show significantly higher binding. (2) The third (2′-terminal) adenosine residue was required for optimal binding activity. (3) 5′-Phosphorylation of the oligonucleotide was necessary for maximum binding to the endonuclease, but the first (from the 5′ terminus) internucleotide phosphate of higher unphosphorylated or core oligomers, such as A2′p5′A2′p5′A2′p5′A, may partly replace the requirement for a 5′-monophosphate moiety; in agreement with this, the 5′-methyl ester of 5′pA2′p5′A2′p5′A, i.e., Me-p5′A2′p5′A2′p5′A, was bound to the endonuclease as well as or better than the higher core oligomers but approximately 100 times more effectively than the trimer core, A2′p5′A2′p5′A. (4) Base-modified analogues, such as p5′C2′p5′C2′p5′C, p5′U2′p5′U2′p5′U, or p5′I2′p5′I2′p5′I, were at least 2000 times less effectively bound to the endonuclease than p5′A2′p5′A2′p5′A. (5) The triphosphate ppp5′I2′p5′I2′p5′I was 10 000 times less active than 2-5A as an inhibitor of translation. These latter two points implied the critical role of the adenine N^1-nitrogen and/or exocyclic amino group in the binding of 2-5A to the endonuclease.

The 2′,5′-phosphodiester bond linked oligoriboadenylate 5′-*O*-triphosphoadenylyl-(2′→5′)-adenylyl-(2′→5′)-adenosine (2-5A) is believed to be a mediator of interferon action (for reviews, see ref 1–4). Although it is probably not involved in all the biological effects of interferon, there is strong evidence that 2-5A may be responsible for the inhibition of encephalomyocarditis virus and reovirus replication by interferon.[5,6] Other data have suggested a possible role for the 2-5A system in the normal regulation

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of cellular differentiation or development.[7-16]

Considerations such as the above suggest the use of 2-5A or its derivatives as potential antiviral or antitumor agents; moreover, 2-5A antagonists[17] may find a niche in the treatment of interferon-induced disease.[18-20] However, the following two difficulties stand in the way of such applications:[21,22] (1) 2-5A is not readily taken up by the intact eukaryotic cell, and (2) 2-5A is rapidly degraded by a phosphodiesterase(s) present in cell extracts. While chemical modification of the 2-5A structure might provide solutions to these difficulties, it is first necessary to define what positions of the 2-5A molecule are critical in its interaction with its target enzyme, RNase L or 2-5A-dependent endonuclease, which after activation degrades mRNA, resulting in inhibition of translation.[23-27] In this report, we examine the effects of oligonucleotide chain length, the extent of oligonucleotide phosphorylation, and the nature of the heterocyclic base on the ability of 2-5A analogues to interact with the 2-5A-dependent endonuclease.[28a,b]

Results and Discussion

Chemistry. The 2′,5′-linked base-modified oligonucleotides p5′I2′p5′I2′p5′I, p5′U2′p5′U2′p5′U,[29a,b] and the previously unreported p5′C2′p5′C2′p5′C were prepared by lead ion catalyzed polymerization of the 5′-phosphoroimidazolidate of inosine, uridine, and cytidine, respectively, in a similar manner to that used for the synthesis of 2′,5′-linked oligoadenylates.[30] The oligomers formed in each reaction were separated by QAE-Sephadex A25 column chromatography and then further purified by paper chromatography. Purity was established by both HPLC and TLC. The yields of the trinucleotides p5′I2′p5′I2′p5′I, p5′U2′p5′U2′p5′U, and p5′C2′p5′C2′p5′C were 4.5–6% based on starting phosphoroimidazolidate. The oligoinosinate p5′I2′p5′I2′p5′I was converted to ppp5′I2′p5′I2′p5′I by the phosphoroimidazolidate method in the same way as already described for 2-5A.[31] Thus, the trimer 5′-monophosphate was reacted with N,N'-carbonyldiimidazole to form the corresponding 5′-phosphoroimidazolidate, which was reacted with pyrophosphate to give ppp5′I2′p5′I2′p5′I and pp5′I2′p5′I2′p5′I in 26 and 41% yield, respectively.

Characteristic proton NMR signals (Table VII) and mobilities of the base-modified oligonucleotides on TLC (Table VI) were in agreement with the assigned structures. The assigned structures of the trinucleotides were further confirmed by enzyme digestion. Treatment of p5′I2′p5′I2′p5′I, p5′U2′p5′U2′p5′U, and p5′C2′p5′C2′p5′C with venom phosphodiesterase gave p5′I, p5′U, and p5′C, respectively, as the sole products. The above trinucleotides were, however, insensitive to RNase T2 or nuclease P1, which are specific for 3′,5′ internucleotide bonds. Alkaline phosphatase digestion of p5′I2′p5′I2′p5′I, pp5′I2′p5′I2′p5′I, or ppp5′I2′p5′I2′p5′I gave the core, I2′p5′I2′p5′I, as the only product. When I2′p5′I2′p5′I was degraded by venom phosphodiesterase, it gave I and p5′I in a 1:2 ratio. Similarly, digestion of p5′C2′p5′C2′p5′C or p5′U2′p5′U2′p5′U with alkaline phosphatase gave the corresponding core oligomers, C2′p5′C2′p5′C or U2′p5′U2′p5′U.

The route chosen for the preparation of the 5′-methylphosphoryl derivative A2′p5′A2′p5′A, i.e., Me-p5′A2′p5′A2′p5′A, was patterned after the earlier work of Schaller et al.,[32] and Cramer and Neunhoffer,[33] who employed acid-catalyzed methanolysis to obtain the methyl ester of AMP. Since formation of 2′,3′-cyclic carbonate is sometimes typical of carbonyldiimidazole generation of an imidazolidate,[34] oxidation–reduction condensation[35] was used to prepare the 5′-phosphoroimidazolidate of A2′p5′A2′p5′A. Thus, the imidazolidate obtained as a

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Table I. Interaction of Oligonucleotides with 2-5A-Dependent Endonuclease as Determined by Antagonism of 2-5 Action and Radiobinding Assays

no.	oligonucleotide	antagonism of 2-5A action[a]	radiobinding assay[b]
1	p5'A2'p5'A	$>2 \times 10^{-4}$ (>200)[c]	2×10^{-5} (40000)
2	p5'A2'p5'A2'p5'A	1×10^{-6} (1)	8×10^{-10} (1.6)
3	p5'A2'p5'A2'p5'A2'p5'A	1×10^{-6} (1)	7×10^{-10} (1.4)
4	p5'A2'p5'A2'p5'A2'p5'A2'p5'A	5×10^{-7} (0.5)	9×10^{-10} (1.8)
5	p5'A2'p5'A2'p5'A2'p5'A2'p5'A2'p5'A		1×10^{-9} (2.0)
6	A2'p5'A	$\geqslant 1 \times 10^{-3}$ (>1000)[c]	
7	A2'p5'A2'p5'A	$\geqslant 1 \times 10^{-4}$ (⩾100)	8×10^{-7} (1600)
8	A2'p5'A2'p5'A2'p5'A	1×10^{-5} (10)	2.2×10^{-8} (40)
9	A2'p5'A2'p5'A2'p5'A2'p5'A	7×10^{-6} (7)	1.3×10^{-8} (26)
10	A2'p5'A2'p5'A2'p5'A2'p5'A2'p5'A		1.3×10^{-8} (26)
11	Me-p5'A2'p5'A2'p5'A		2×10^{-9} (4)
12	p5'A2'p5'A3'p		4×10^{-6} (8000)
13	p5'A2'p5'A2'p		4×10^{-7} (800)
14	p5'A2'p5'A2'p5'A2'(3')p	3×10^{-7} (0.33)	5×10^{-10} (1)
15	p5'C2'p5'C2'p5'C	$\geqslant 1 \times 10^{-4}$ (>100)[c,d]	3.3×10^{-6} (6600)
16	p5'U2'p5'U2'p5'U	$\geqslant 1 \times 10^{-4}$ (>100)[c,d]	1.1×10^{-6} (2200)
17	p5'I2'p5'I2'p5'I	$>1 \times 10^{-4}$ (>100)[c,d]	3.3×10^{-6} (6600)
18	ppp5'I2'p5'I2'p5'I	$>1.3 \times 10^{-4}$ (>130)	2.6×10^{-7} (520)
19	ppp5'A2'p5'A2'p5'A		5×10^{-10} (1)

[a] Molar concentration of oligonucleotide needed to give 50% prevention of 2-5A action. The value in parentheses refers to the relative activity of the compound. Relative activity is defined as the relative amount ot achieve the half maximal effect. The higher the value, the less active was the oligomer. [b] Molar concentration of oligonucleotide required to displace 50% of the radiolabeled probe from the endonuclease-nitrocellulose complex. Relative activity is given in parentheses (see footnote *a*). [c] Compound had no effect at this, the highest concentration tested. [d] At very high concentrations, these analogues caused some inhibition of translation. p5'C2'p5'C2'p5'C gave 50% inhibition at 3×10^{-5} M and p5'U2'p5'U2'p5'U gave 50% inhibition at 1×10^{-4} M, while p5'I2'p5'I2'p5'I required more than 1×10^{-4} M to cause a 50% inhibition. This inhibition was not due to activation of the 2-5A-dependent endonuclease, since the inhibitions were not prevented by p5'A2'p5'A2'p5'A (1.8×10^{-4} M).

product of the reaction of p5'A2'p5'A2'p5'A with triphenylphosphine, imidazole, and dipyridyl disulfide[35] gave, upon reaction with methanol under conditions of acid catalysis, Me-p5'A2'p5'A2'p5'A in good yield. In the same fashion, the 5'-methylphosphoryl derivative of the dimer, A2'p5'A, also could be prepared.

Two separate approaches were employed for the synthesis of 2' (3') terminally phosphorylated oligonucleotides. For the synthesis of p5'A2'p5'A2'p5'A2'p, p5'A2'p5'A2'p5'A2'p5'A was subjected to periodate oxidation and base-catalyzed elimination of adenine according to previously described methodology.[22,36,37] Structure confirmation was obtained by alkaline phosphatase digestion, which gave A2'p5'A2'p5'A as the sole product. Different methodology was used in the preparation of the dimer diphosphate p5'A2'p5'A3'p. In this case, the linkage isomer[30] p5'A2'p5'A3'p5'A2'p5'A was digested with RNase T_2. The other isomer, p5'A2'p5'A2'p, was prepared by periodate oxidation and base elimination of p5'A2'p5'A2'p5'A.

Both p5'A2'p5'A2'p and p5'A2'p5'A3'p were completely digested to A2'p5'A by bacterial alkaline phosphatase digestion for 3.5 h at 37 °C. The 2' (or 3') terminal phosphate group was removed first, since TLC during the early part of the digestion revealed the presence of p5'A2'p5'A. On the other hand, digestion of the above oligonucleotides with nuclease P_1 gave differing results. Incubation of p5'A2'p5'A3'p with nuclease P_1 rapidly gave the expected product p5'A2'p5'A within 1 h of incubation. To the contrary, the other isomer, p5'A2'p5'A2'p, was resistant to the action of nuclease P_1, showing no change after a 1-h incubation. Prolonged incubation (3.5 h) gave a small yield (~10%) of p5'A2'p5'A. Since it has been previously established that hydrolysis of 2'-AMP is 3000 times slower than the hydrolysis of 3'-AMP by nuclease P_1,[38] these results served to further confirm the assigned structures.

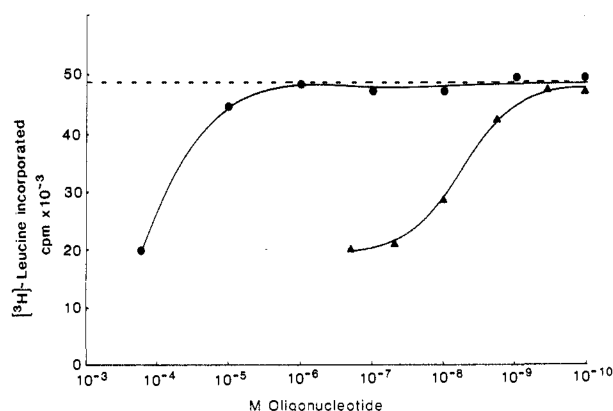

Figure 1. Comparison of ppp5'A2'p5'A2'p5'A (△) and ppp5'I2'p5'I2'p5'I (○) as inhibitors of translation in extracts of mouse L cells programmed with encephalomyocarditis virus RNA. The dashed line represents the level of translation in the absence of any additions.

Biological Activities of the Oligonucleotides. Interaction of the various oligonucleotides with the 2-5A-dependent endonuclease has been followed with three different assays. The first involved determination of the ability of the oligonucleotide to prevent the protein synthesis inhibitory effects of 2-5A in a mouse L-cell-free protein synthesis system programmed with encephalomyocarditis virus RNA.[17] Since this antagonism of 2-5A action is a result of competition of the oligonucleotide for the 2-5A binding site of the endonuclease,[39,40] it provided

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a measure of the ability of the analogue to bind to the endonuclease. In this case, the concentration of analogue necessary to effect a 50% reversal of the inhibition of translation caused by 20 nM 2-5A was determined. The second assay revealed the ability of the oligonucleotide 5′-triphosphate to inhibit protein synthesis in the above cell-free system. In this instance, the concentration of oligomer necessary to cause half-maximal inhibition of translation was determined. The third method was the radiobinding assay developed by Knight et al.[39,40] Here the concentration of oligomer needed to prevent 50% of the added ppp5′A2′p5′A2′p5′A2′p5′A3′[^{32}P]p5′C3′p from binding to the endonuclease was established. The results of such studies are presented in Table I and Figure 1, and the following conclusions may be drawn from them. In Table I are given the concentrations of oligonucleotides necessary to achieve a 50% effect. Relative activity is defined as the relative amount required to give the 50% effect. The higher the value, the less active the oligomer.

(1) When 5′-monophosphorylated and 5′-unphosphorphorylated oligonucleotides were considered, it was clear that for optimal antagonistic activity and endonuclease binding, a 5′-terminal monophosphate moiety was required. No unphosphorylated oligoadenylate (**6–9**) was as active as the 5′-monophosphorylated oligoadenylates (**1–5**) in antagonistic or radiobinding activity; i.e., removal of the 5′-terminal monophosphate moiety led to an approximate 10- to 100-fold increase in the concentration of oligomer required to prevent 2-5A action or to displace radiolabeled probe from the endonuclease. However, even though the trimer core (**7**) was approximately 100-fold less active as an antagonistic than its corresponding 5′-monophosphate (**2**) and also 1000 times less efficient than **2** in displacing the probe from endonuclease, the tetramer, pentamer, and hexamer cores (**8–10**) were only about 7–10 times less active than the tetramer or pentamer 5′-monophosphate (**3** and **4**) as 2-5A antagonists and only 15–30 times less efficient in displacing the probe. Thus, while 5′-unphosphorylated oligomers did not bind to the endonuclease as well as 5-phosphorylated oligomers, the differential was not so great in the tetramer and higher oligonucleotides as at the trimer level. Since elongation of the chain of the 5′-monophosphates (**2–5**, vide infra) beyond three nucleotides did not lead to substantial increases in antagonistic or radiobinding activity, it may be possible that the increased activity of the tetramer, pentamer, or hexamer core (**8–10**) as compared to the trimer core (**7**) may be rationalized by assuming that the first internucleotide residue (from the 5′ end) may "slip" onto the binding site normally occupied by the 5′-monophosphate group. Knight et al.[39,40] also have reported that the trimer core had substantially reduced binding activity when compared to the 5′-mono- or 5′-triphosphate.

The behavior of the 5′-methyl ester of p5′A2′p5′A2′p5′A, i.e., **11**, was consistent with this idea. Although, Me-p5′A2′p5′A2′p5′A was bound to the endonuclease about 3 times less effectively than p5′A2′p5′A2′p5′A itself, it was about 400 times more active in binding than the trimer core, A2′p5′A2′p5′A. Nonetheless, for optimal endonuclease binding, the presence of a free 5′-terminal monophosphate seems to be a minimal requirement.

(2) At least three adenosine residues were necessary for optimal antagonistic or endonuclease binding activity. Thus, p5′A2′p5′A (**1**) was more than 200 times less effective than higher oligomers (**2–5**) as antagonist of 2-5A action and even less effective at displacing the probe from the endonuclease. The activity of the core oligoadenylates (**6–10**) also lend support to this conclusion of the "slippage" mechanism alluded to above. While the dimer and trimer "cores" (**6** and **7**) showed little or no evidence of endonuclease binding, the tetramer, pentamer, and hexamer cores (**8–10**) showed at least 10-fold higher activities, since the internucleotide linkage adjacent to the 5′-terminal adenosine may "slip" into the 5′-monophosphate binding site.

The relative importance of the third (2′-terminal) residue of 2′,5′-$(pA)_3$ (2-5A trimer triphosphate) can be further discerned by partly dissecting the terminal residue. When adenosine was removed from the tetramer **4**, the resulting diphosphorylated oligonucleotide (**14**) became a more effective antagonist of 2-5A action and possessed the same apparent affinity for the endonuclease as did the tetramer (**4**) itself. On the contrary, a similar shortening of the trimer (**3**) to give the diphosphorylated dimer (**13**) produced a compound with dramatically reduced binding activity (Table I). The terminal isomer of **13**, namely, **12**, was bound even less effectively to the endonuclease, in accord with the observation[41] that substitution of 3′,5′-phosphodiester bonds for 2′,5′-phosphodiester bonds in 2-5A results in a decrease of affinity for the endonuclease. Previous observations[42] have established that 2′ (3′) terminal phosphorylation increases the resistance of the 2-5A-derived molecule to degradation. The increased antagonistic activity of **14** was in agreement with this. The results obtained with the 2′ (3′) terminally phosphorylated dimers (**12** and **13**) suggest that the third adenosine moiety may contribute substantially to endonuclease binding. That the ribose moiety of the terminal adenosine residue may not be critical in endonuclease binding was suggested by the data of Imai et al.,[22] who found that conversion of the 2′-terminal ribose moiety of the trimer p5′A2′p5′A2′p5′A to an *N*-hexylmorpholine derivative increased antagonistic capacity relative to the parent compound, thereby implying the lack of importance of the ribose ring of the 2′-terminal adenosine residue in binding to the endonuclease. However, Drocourt et al.[43] found that while compounds of the general formula ppp5′A2′p5′A2′p5′N (where N was any of the common nucleic acid bases) could not activate the endonuclease, they could antagonize 2-5A action as effectively as pA2′p5′A2′p5′A. This result implied that replacement of the 2′-terminal residue with other nucleosides did not decrease endonuclease binding. These data were obtained in a calcium phosphate coprecipitation system in which the concentrations of oligonucleotide actually entering the cell are impossible to ascertain.

The significantly lower protein synthesis inhibitory activity of ppp5′A2′p5′A as compared to ppp5′A2′p5′A2′p5′A[44] is a reflection of the fact that three adenosine residues are required for maximum binding to the 2-5A-activated endonuclease.

(3) As the oligonucleotide chain length was increased beyond three nucleotide residues no substantial increase in antagonistic activity or endonuclease binding occurred. The addition of single nucleotide units to the trimer (**2**) to give the tetramer (**3**) or pentamer (**4**) had only a marginal effect on activity. A similar situation obtains in

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comparison of the core tetramer, pentamer, and hexamer (**8–10**). These data agree with the earlier observations[44] that the various oligomers of 2-5A, i.e., ppp5′A2′p5′A2′p5′A, ppp5′A2′p5′A2′p5′A2′p5′A, and ppp5′A2′p5′A2′p5′A2′p5′A2′p5′A, were equally effective as protein synthesis inhibitors.

(4) All base-modified analogues of p5′A2′p5′A2′p5′A, including p5′C2′p5′C2′p5′C (**15**), p5′U2′p5′U2′p5′U (**16**), and p5′I2′p5′I2′p5′I (**17**), showed dramatically reduced antagonistic and endonuclease binding activity. Apparently, just the simple elimination of the adenosine amino group was sufficient to lead to a >2000-fold loss of endonuclease binding affinity. Moreover, since the inosinate analogue (**17**) showed comparable activities to the cytidylate (**15**) and uridylate (**16**) analogues, it would appear that the adenine 6-amino group and possibly its N-1 nitrogen may be the greatest contributors to the binding of the adenine rings.

Three reports have appeared concerning oligoinosinate analogues of 2-A.[45–47] In these studies, the inosinate analogue was prepared by nitrous acid deamination[45] or by enzymatic synthesis by using the 2-5A synthetase[46] from mouse L cells, although the former group of investigators found that ITP was not a substrate for the reticulocyte enzyme. In addition, other studies[48–50] have indicated that nucleoside triphosphates other than ATP did not lead to homooligonucleotides but would incorporate into a 2′,5′-oligonucleotide chain only in the presence of ATP or an adenosine terminated trimer. In these latter cases, the nonadenosine NTP acts as a terminator of chain elongation. Finally, it may be noted that no structure confirmation of the putative oligoinosinates of the quoted studies[45–47] have been reported.

Since the synthetic p5′I2′p5′I2′p5′I of this study was inefficiently bound to the 2-5A-dependent endonuclease, as judged by both antagonism and radiobinding assays, it was unlikely that the corresponding 5′-triphosphate would be an effective endonuclease activator. In order to check the remote possibility that the 5-triphosphate moiety might be more critical in endonuclease binding in the case of the inosinate analogue than it is in the case of 2-5A, the triphosphate ppp5′I2′p5′I2′p5′I was prepared by standard procedures. As is evident from Table I, the triphosphate of 2′,5′-oligoinosinate (**18**) was ineffective as an antagonist of 2-5A action and was more than 500 times less effective than 2-5A in displacing radiolabeled probe from the endonuclease. The experiments of Figure 1 document that, in accord with the findings of Table I, ppp5′I2′p5′I2′p5′I was a very poor inhibitor of protein synthesis, at least 10 000 times less potent than 2-5A.

Conclusions

From this and related studies, it is possible to present the following tentative picture of the interaction of oligonucleotides with the 2-5A-activated endonuclease. Optimal binding of 5′-phosphorylated 2′,5′-oligoadenylates occurs when three or more AMP residues are present in the chain: longer oligomers do not show greater apparent affinities for the endonuclease.[21,44,51] The 2′-terminal adenosine residue of 2-5A may be required for maximum endonuclease binding, as well as the presence of 5′-terminal mono- or triphosphate groups, since nonphosphorylated or core oligomers show greatly reduced apparent affinities.[21,39] This difference is, however, most dramatic at the trimer level. Tetramer and higher core oligomers show enhanced binding compared to the trimer core, albeit significantly less than the 5′-phosphorylated oligonucleotides (Table I). This raises the possibility that one of the internucleotide phosphates may replace, albeit less effectively, the 5′-terminal phosphate group in binding to the endonuclease. Interaction of 2-5A with the endonuclease also occurs through the adenine rings; moreover, the adenine N^1 nitrogen and/or adenine 6-amino group probably plays a critical role in the binding interaction (Table I and Figure 1). This conclusion is also supported by the observation[52] that the 1,N^6-ethenoadenosine analogue of 2-5A binds very poorly to the 2-5A-dependent endonuclease. Endonuclease binding also involves the nature of the internucleotide bond, since replacement of one or the other 2′,5′-linkages of 2-5A by 3′,5′ linkages gives isomers that bind to the endonuclease 20–50 times less effectively than 2-5A itself; moreover, 3-5A, i.e., ppp5′A3′p5′A3′p5′A, is 10 000 times less effective than 2-5A in endonuclease binding or as an inhibitor of translation.[41] Finally, interaction with the 3′-hydroxyl groups of 2-5A is not important for oligonucleotide binding to the endonuclease but is vital for endonuclease activation, since analogues in which all of the 3′-hydroxyl groups have been replaced by methoxy or hydrogen bind well to the 2-5A-dependent endonuclease (albeit less effectively than 2-5A) but do not activate it to degrade RNA or to inhibit protein synthesis.[53–55]

Table II. Preparation and Purification of 2′,5′-Oligoadenylate 5′-Monophosphates

compd	HPLC t_R, min	yield,[b] %	TEAB gradient for DEAE-Sephadex col[c]
$(pA)_2$	19.7	29.5	0.5–0.30 M (0.27 M)[d]
$(pA)_3$	25.8	7.7	0.20–0.35 M (0.305 M)[d]
$(pA)_4$	30.6	2.2	0.25–0.40 M (0.34 M)[d]
$(pA)_5$	33.3	0.85	0.30–0.45 M (0.37 M)[d]
$(pA)_6$	35.1	0.55	0.35–0.50 M (0.41 M)[d]

[a] Column: Zorbax ODS (21.2 mm × cm). Buffer A: 50 mM ammonium phosphate; buffer B: $MeOH/H_2O$, 1:1. Flow rate: 9.0 mL/min. Gradient 0–20% B (in 40 min). Detector wavelength: 280 nm. [b] Yields are calculated based on the are integration of each peak in the HPLC chromatogram. [c] Column size: 1.6 × 8 cm; the column was eluted with a linear gradient (each 250 mL) of TEAB buffer. [d] Concentration of TEAB buffer at which each oligoadenylate was eluted from the column.

Experimental Section

High-performance liquid chromatography was carried out with either a Hitachi 638 apparatus using an RPC-5 column (4 mm × 25 cm) and elution with a linear gradient of $NaClO_4$

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Table III. Characteristic Proton NMR Signals of 2′,5′-Oligoadenylate 5′-Monophosphates

compd	chemical shift,[a] δ: Ar ring protons (C-2 or C-8)[b]	chemical shift,[a] δ: anomeric protons (C-1′)[c]
$(pA)_2$	8.08 (1 H), 7.93 (1 H), 7.83 (1 H), 7.75 (1 H)	5.97 (1 H, d, J = 3.0 Hz), 5.64 (1 H, br s)
$(pA)_3$	7.98 (1 H), 7.97 (1 H), 7.80 (1 H), 7.76 (2 H), 7.73 (1 H)	5.89 (1 H, d, J = 3.0 Hz), 5.75 (1 H, d, J = 3.7 Hz), 5.64 (1 H, d, J = 4.0 Hz)
$(pA)_4$	7.96 (1 H), 7.93 (1 H), 7.70 (3 H), 7.65 (1 H), 7.62 (1 H), 7.61 (1 H)	5.85 (1 H, d, J = 2.7 Hz), 5.68 (1 H, d, J = 3.6 Hz), 5.60 (2 H, m)
$(pA)_5$	7.94 (1 H), 7.91 (1 H), 7.70 (1 H), 7.68 (1 H), 7.67 (1 H), 7.64 (1 H), 7.63 (1 H), 7.61 (1 H), 7.59 (1 H), 7.53 (1 H)	5.84 (1 H, d, J = 3.0 Hz), 5.67 (1 H, d, J = 4.1 Hz), 5.56 (3 H, m)
$(pA)_6$	7.94 (1 H), 7.93 (1 H), 7.72 (1 H), 7.71 (2 H), 7.69 (1 H), 7.68 (2 H), 7.67 (1 H), 7.65 (1 H), 7.56 (1 H), 7.54 (1 H)	5.90 (1 H, d, J = 4.0 Hz), 5.69 (1 H, d, J = 3.8 Hz), 5.60 (4 H, m)

[a] Chemical shifts are determined in D_2O, with acetone (δ 2.05) as an internal standard. [b] All aromatic protons appeared as singlets. [c] Doublets and multiplets are indicated as d and m.

(0.0–0.05 M) buffered with Tris-acetate (pH 7.5) or with a Beckman instrument with Model 110A pumps with columns and eluents as indicated in the text.

Paper chromatography was performed with Whatman 3MM paper by the descending technique and with concentrated 1-propanol/concentrated NH_4OH/H_2O (55:10:35) as developing solvent.

Thin-layer chromatography (TLC) was performed on PEI-cellulose F in 0.25 M NH_4HCO_3 (solvent 1), on cellulose F in 1-propanol/concentrated NH_4OH/H_2O (55:10:35) (solvent 2), or on cellulose F in saturated ammonium sulfate/0.1 M sodium acetate/2-propanol (79:19:2) (solvent 3).

Materials. Inosine 5′-monophosphate (Na salt), uridine 5′-monophosphate, and cytidine 5′-monophosphate were from Yamasa Co. All 2′–5′ linked core oligoadenylates, except A2′p5′A and A2′p5′A2′p5′A, were from Calbiochem. (La Jolla, CA). Sigma Chemical Co. (St. Louis, MO) was the source for A2′p5′A, A3′p5′A, and A3′p5′A3′p5′A. TLC plates were from Merck. DEAE-Sephadex and QAE-Sephadex were from Pharmacia. Enzymes such as bacterial alkaline phosphatase or venom phosphodiesterase were from Worthington Biochemical Corp. Nuclease P_1 was obtained either from Sigma or Yamasa. All other materials obtained commercially were either reagent or HPLC grade. 2-5A and 2-5A trimer core were prepared as described earlier.[56,57] Imidazole was purchased from J. T. Baker Chemical Co. Triphenylphosphine, dipyridyl disulfide, and AMP were from Aldrich.

NMR Spectra. The 400-MHz proton NMR spectra were recorded with a JEOL GX-400 instrument at 27 °C. The 220-MHz proton NMR spectra were obtained on a Varian HR 220 spectrometer. In some instances, samples first were purified by paper chromatography on Whatman 3MM paper and then passed through a short column of Dowex 50W X 8 (Na^+ form). The solution was evaporated in vacuo, and then D_2O was added and evaporated (3 × 0.2 mL). Samples were generally made 10–20 mM in D_2O and in some cases were buffered with sodium phosphate (pH 6.6). Chemical shifts were recorded relative to an external TSP standard.

The preparation of mouse L cell extracts, encephalomyocarditis virus RNA, and the techniques and conditions for cell-free protein synthesis and antagonism of 2-5A action assays have been described elsewhere.[17,58] Radiobinding assays were performed according to Knight et al.,[39,40] with ppp5′A2′p5′A2′p5′A2′p5′A3′[^{32}P]p5′C3′p of specific activity 3000 Ci/mmol (Amersham, Chicago, IL). This assay measures the capacity of an oligonucleotide to displace the radiolabeled probe from the endonuclease as determined by a nitrocellulose filter binding assay.

Preparation of 2′,5′-Oligoadenylate 5′-Monophosphates [2′,5′-$(pA)_n$, n = 2–6]. 2′,5′-Oligoadenylate monophosphates $(pA)_n$ were prepared by lead ion catalyzed polymerization of adenosine 5′-phosphoimidazolidate (ImpA) basically according to the method described by Sawai et al.[30] For the purification of each oligoadenylate from the crude reaction mixture, however, RNase P_1 digestion and HPLC purification was employed instead of direct application of the mixture to the QAE-Sephadex column chromatography.[30]

Table IV. Characteristics of Synthetic Methylphosphoryl Oligoadenylates on HPLC

compd	HPLC t_R,[a] min: Accupak (anal.)	HPLC t_R,[a] min: Zorbax (prep)
$(pA)_2$	7.25, 7.26[b]	10.22, 10.26[b]
$Me(pA)_2$	9.01	11.49
$(pA)_3$	7.28	10.58
$Me(pA)_3$	9.95	11.83

[a] HPLC experiments were carried out on a Beckman instrument with Model 110 A pumps with detector at 280 nm, with an Accupak column (flow rate 1 mL/min), and with a Zorbax column (flow rate 2.5 mL/min). Both columns were eluted with a linear gradient of 50 mM $NH_4H_2PO_4$ (pH 7.0) (A) mixed up to 70% of methanol/water (1:1) (B) in 10 min. Elution was then continued to 100% (B) in 5 min. [b] Elution to 100% in 10 min. [c] After 10 min, the flow rate was reduced to 0.75 mL/min.

The polymerized reaction mixture (75 000 OD_{260}) was first treated with Chelex 100 (100–200 mesh, NH_4^+ form, 30 mL) at room temperature for 1 h to remove Pb^{2+} ion. After filtration of Chelex, the filtrate was evaporated under vacuum, and ethanol (40 mL) was added to the dried residue. The insoluble precipitate was collected by filtration and redissolved in H_2O (40 mL) for the absorbance measurement (68 000 OD_{260}). To this solution were added 100 mM morpholine ethane sulfonic acid buffer (pH 6.0, 5.0 mL), 1 mM $ZnCl_2$ (5.5 mL), and RNase P_1 (1.0 mg, 800 units). The reaction mixture was incubated at 40 °C for 2.5 h, followed by heating at 100 °C for 10 min and deproteinization with a mixture of $CHCl_3$/isoamyl alcohol (5:2, 10.0 mL). The aqueous layer was separated and concentrated under vacuum to approximately 10 mL. The crude mixture of 2′,5′-$(pA)_n$ (n = 2–6) was then separated by reverse-phase HPLC (Zorbax ODS column, 2.12 × 25 cm). Approximately 4000 OD_{260} units of the crude mixture was injected, and the column was eluted with a linear gradient of 0–20% buffer B in 40 min (buffer A: 50 mM ammonium phosphate, pH 7.0; buffer B: 1:1 methanol/H_2O, flow rate 9.0 mL/min). Corresponding peaks for 2′,5′-$(pA)_n$ (n = 2–6) were collected separately. In these separation conditions, all desirable 2′,5′-linked oligoadenylates were eluted from the column before 25 min so that the all other byproducts that stayed in the column longer could be separated from them. The column was then washed with 100% buffer B to remove other products. The whole HPLC operation was repeated several times until the substantial amount of higher oligoadenylates were pooled. The collected 2′,5′-$(pA)_n$ (n = 2–6) eluates were applied individually to a short DEAE-Sephadex column (1.6 cm × 8 cm) to remove phosphate buffer. The retention time, the triethylammonium bicarbonate (TEAB) buffer gradient condition on the DEAE-Sephadex column, and the yield for each oligomer are listed in Table II. The proper fractions from the DEAE-Sephadex column were combined and concentrated under vacuum. The resulting residue was coevaporated with H_2O several times until the TEAB buffer was

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Table V. Characteristic Proton NMR Signals of 5′-*O*-(Methylphosphoryl) 2′,5′-Oligoadenylates

compd	chemical shifts,[a] δ: Ar ring protons (C-2 or C-8)[b]	anomeric protons (C-1′)[c]	$CH_3O-P(=O)-O$
Me(pA)[d]	8.19 (1 H), 7.93 (1 H)	5.87 (1 H, d, J = 5.0 Hz)	3.38 (3 H, d, J = 9.5 Hz)
$Me(pA)_2$	8.02 (1 H), 7.92 (1 H), 7.88 (1 H), 7.76 (1 H)	6.13 (1 H, d, J = 2.5 Hz), 6.06 (1 H, d, J = 3.0 Hz)	3.03 (3 H, d, J = 9.5 Hz)
$Me(pA)_3$	7.98 (1 H), 7.90 (1 H), 7.85 (1 H), 7.76 (1 H), 7.73 (1 H), 7.65 (1 H)	5.88 (1 H, br s), 5.76 (1 H, d, J = 5.0 Hz), 5.60 (1 H, d, J = 4.0 Hz)	2.99 (3 H, d, J = 9.5 Hz)

[a] Chemical shifts were determined in D_2O with acetone (δ 2.05 ppm) as an internal standard. [b] All aromatic protons appeared as singlets. [c] Doublets are indicated as d. [d] Calcium salt.

completely removed. A small amount of each 2′,5′-oligoadenylate thus prepared was reinjected into the HPLC column to assure the purity. All compounds [2′,5′-$(pA)_n$, n = 2–6] prepared above showed resistance to RNase P_1 and RNase T_2 but were degraded completely to 5′-AMP with snake venom phosphodiesterase. ^1H NMR data for these compounds are presented in Table III.

Preparation of 5′-*O*-(Methylphospho)adenylyl-(2′→5′)-adenylyl-(2′→5′)-adenosine (Me-p5′A2′p5′A2′p5′) and 5′-*O*-(Methylphosphoryl)adenylyl-(2′→5′)-adenosine (Me-p5′A2′p5′A). According to the preparation of adenosine 5′-phosphoroimidazolidate described by Mukaiyama and Hashimoto,[35] p5′A2′p5′A2′p5′A (1080 A_{258} units, 29.21 μmol, triethylammonium salt) was dissolved in dry Me_2SO (150 μL). Imidazole (150 μmol), triphenylphosphine (60 μmol, 15.75 mg), freshly redistilled triethylamine (5 μL), and dipyridyl disulfide (60 μmol, 13.2 mg) were added to the initial solution. The yellow mixture was stirred at room temperature for 40 min and then transferred dropwise and under stirring to a 10-mL solution of sodium iodide in dry acetone (60 μmol/mL). The resulting precipitate was centrifuged and washed three times with dry acetone (each 5 mL), and the white residue was dried over P_2O_5 in vacuo for 3 h. The imidazolidate (916 A_{258} units, 24.7 μmol) was added to 500 μL of anhydrous methanol, and the mixture was treated at 4 °C with 47.8 μmol (5 mg) of imidazole hydrochloride. The reaction mixture was kept stirring for 3 days at 4 °C. The excess methanol was then removed under vacuum. The residue was dissolved in Me_2SO, and the crude sodium salt was collected as described above. The precipitated salt was dissolved in water (1 mL) and purified by HPLC (reverse phase, Zorbax ODS column, 0.94 × 25 cm). The column was eluted with two stepwise linear gradients of 50 mM $NH_4H_2PO_4$ (pH 7.0) (A) mixed up to 70% of methanol/water (1:1) (B) in 10 min and then to 100% (B) in 10 min. The methyl ester was collected at a retention time of 11.85 min. It's concentration in the reaction mixture was 82% according to the integration of the area of each peak in the HPLC chromatogram. The collected fractions containing the 5′-methyl ester were pooled, evaporated under vacuum, and applied to a DEAE-Sephadex A 25 (HCO_3^-) column (20 × 1.0 cm), in order to remove ammonium phosphate buffer. The column was eluted with a linear gradient (250/250 mL) of 0.1 to 0.3 M TEAB buffer (pH 7.6). Fractions from 21 to 35 contained the 5′-*O*-methyl ester of the trimer monophosphate. They were pooled and evaporated repeatedly with water to remove TEAB buffer. The dry residue was dissolved in anhydrous methanol, and the sodium salt was isolated in the same manner as described above. The yield was 68.5% (740 A_{258} units based on monophosphate).

A similar procedure was employed to prepare the 5′-*O*-(methylphospho)adenylyl-(2′→5′)-adenosine. Thus, starting from 850 A_{258} units (32.44 μmol) of the dimer monophosphate, esterification and HPLC purification were carried out as previously described. The elution gradient was from 0.05 to 0.15 M TEAB; fractions from 52 to 70 were collected and contained 450 A_{258} units (17.18 μmol). The yield was 55% based on the monophosphate.

HPLC characteristics and pertinent NMR data on these compounds are presented in Tables IV and V.

Synthesis of the Base-Modified 2′,5′-Linked Trinucleotides p5′I2′p5′I2′p5′I, p5′C2′p5′C2′p5′C, and p5′U2′p5′U2′p5′U. 2′,5′-Linked triinosinate (p5′I2′p5′I2′p5′I) and triuridylate (p5′U2′p5′U2′p5″U) were prepared by the published procedures.[28,29] The synthesis of the tricytidylate (p5′C2′p5′C2′p5′C) was by a method similar to that used for the preparation of 2′,5′-linked oligoadenylates (Sawai et al.). Cytidine 5′-monophosphate (1 mmol) was converted to cytidine 5′-phosphoroimidazolidate (ImpC) by reaction with imidazole, triphenylphosphine, and 2,2′-dipyridyl disulfide[35] and was obtained as the sodium salt in a yield of >90%. The polymerization of ImpC was accomplished by using lead nitrate as a catalyst. The reaction mixture (8 mL) contained 0.4 mmol of ImpC and 0.1 mmol of lead nitrate in 0.2 M imidazole buffer (pH 7.0). The mixture was maintained at room temperature for 5 days with constant stirring. Treatment of the reaction mixture with EDTA (0.5 mL of 0.25 M) gave a homogeneous mixture, indicating formation of the lead–EDTA complex. The resultant solution was applied to a column (30 mm × 45 cm) of QAE-Sephadex A25, and the products were eluted with a stepwise linear gradient of triethylammonium bicarbonate buffer. The fractions containing p5′C2′p5′C2′p5′C were collected and evaporated in vacuo at <30 °C. Lyophilization of the residue afforded p5′C2′p5′C2′p5′C as the triethylammonium salt (yield 5.3% based on the UV spectrum after correction for hypochromicity). The hypochromicity was 14.5% as calculated from the UV absorption before and after alkali hydrolysis of p5′C2′p5′C2′p5′C. Chromatographic and NMR spectral characteristics of the base-modified oligomers can be found in Tables VI and VII.

Table VI. TLC Mobilities of the Base-Modified Trinucleotides

compd	R_f^1 [a]	R_f^2 [b]	R_f^3 [c]
pC	0.70	0.42	0.79
pC2′p5′C2′p5′C	0.48	0.21	0.70
C2′p5′C2′p5′C	0.74	0.47	0.67
pU	0.60	0.40	0.80
pU2′p5′U2′p5′U	0.11	0.28	0.69
U2′p5′U2′p5′U	0.14	0.45	0.61
pI	0.52	0.41	0.73
pI2′p5′I2′p5′I	0.10	0.31	0.69
I2′p5′I2′p5′I	0.14	0.48	0.56
ppI2′p5′I2′p5′I	0.08	0.24	0.76
pppI2′p5′I2′p5′I	0.04	0.22	0.82

[a] Solvent system 1. [b] Solvent system 2. [c] Solvent system 3.

Synthesis of pppI2′p5′I2′p5′I and pp5′I2′p5′I2′p5′I. The oligomer p5′I_2′p5′I_2′p5′I (150 A_{260} units, 13 μmol) was dissolved in water and passed through a column of Dowex 50W X-8 (pyridinium form) to give the pyridinium salt. The eluate was evaporated in vacuo, and then dry pyridine (1 mL) was added and removed by evaporation three consecutive times. This dried residue was dissolved in dry DMF (1 mL), and then triethylamine (20 μL) and tri-*n*-octylamine (20 μL) were added. *N*,*N*-Carbonyldiimidazole (35 mg, 200 μmol) was then added, and the mixture was kept at room temperature for 2 h. After phosphoroimidazolidate formation was complete, methanol (30 μL) was added to destroy excess *N*,*N*′-carbonyldiimidazole.

After evaporation of the solvent, tri-*n*-butylammonium pyrophosphate (200 μmol) in dry DMF (400 μl) was added, and the mixture was kept at room temperature for 1 day. The entire reaction mixture was then poured into a solution containing acetone (5 mL), ether (5 mL), and an acetone solution (0.5 mL) saturated with $NaClO_4$. A white precipitate formed. The precipitate was collected by centrifugation, washed with ether, dried in vacuo, and then dissolved in water. The solution was applied to a column (15 mn × 30 cm) of QAE-Sephadex A25 (HCO_3^-) and

Table VII. Characteristic Proton NMR Spectra of the Base-Modified Trinucleotides

compd	chemical shift,[a] δ: anomeric protons: H(1′)	H(6) or H(8)	H(5) or H(2)
pI2′p5′I2′p5″I	6.11 (1 H, d, J = 3.4 Hz)	8.26 (1 H, s)	8.04 (2 H, s)
	6.01 (1 H, d, J = 3.4 Hz)	8.16 (1 H, s)	8.00 (1 H, s)
	5.90 (1 H, d, J = 4.3 Hz)	8.08 (1 H, s)	
pU2′p5′U2′p5′U	6.13 (1 H, d, J = 3.4 Hz)	7.95 (1 H, d, J = 6.1 Hz)	5.93 (1 H, d, J = 6.1 Hz)
	6.07 (1 H, d, J = 3.2 Hz)	7.86 (1 H, d, J = 6.1 Hz)	5.89 (1 H, d, J = 8.7 Hz)
	5.92 (1 H, d, J = 2.0 Hz)	7.75 (1 H, d, J = 8.2 Hz)	5.86 (1 H, d, J = 6.5 Hz)
pC2′p5′C2′p5′C	5.96 (1 H, d, J = 3.0 Hz)	8.12 (1 H, d, J = 7.3 Hz)	6.03 (1 H, d, J = 7.6 Hz)
	5.80 (1 H, d, J = 1.5 Hz)	7.93 (1 H, d, J = 7.6 Hz)	6.00 (2 H, d, J = 7.3 Hz)
	5.79 (1 H, d, J = 2.1 Hz)	7.91 (1 H, d, J = 7.7 Hz)	

[a] Chemical shifts were determined in D_2O with TSP as an external standard.

eluted with a linear gradient of triethylammonium bicarbonate buffer, 0.25 M (1 L) to 0.75 M (1 L). Evaporation and lyophilization of the UV-absorbing fractions gave ppp5′I2′p5′I2′p5′I (26%), pp5′I2′p5′I2′p5′I (41%), and p5′I2′p5′I2′p5′I (22%) as the triethylammonium salts.

Procedures for Enzymic Digestion of the Base-Modified Trinucleotides. Digestion with bacterial alkaline phosphatase was carried out by incubation of the trinucleotide (1–5 A_{260} units) in a solution (50 mL) of 0.1 M Tris-HCl (pH 8.05), 0.01 M $MgCl_2$, and 0.1 unit of enzyme for 2 h at 37 °C. The digested products were isolated by paper chromatography on Whatman 3MM paper in nearly quantitative yield. Digestion with venom phosphodiesterase was carried out at 37 °C for 2.5 h in a solution (50 mL) containing oligonucleotide (1–3 A_{260} units), 0.01 M Tris–acetate (pH 8.8), 0.001 M $MgCl_2$, and the enzyme (0.01 unit). Nuclease P_1 digestion was performed at 37 °C for 2.5 h in a mixture (50 μL) containing the trinucleotide (1–5 A_{260} units), 0.006 M EDTA/acetate buffer (pH 5.75), and the enzyme (5 mg in 5 mL).

Preparation of p5′A2′p5′A2′p5′A2′p. Sodium metaperiodate (0.1 M, 60 μL) was added to an ice-cold solution of p5′A2′p5′A2′p5′A2′p5′A (208 A_{260} units, 5 μmol) in water (250 μL). After the reaction mixture had been stirred at 4 °C for 20 min, hexylamine (20 μL) was added, and the pH of the solution was adjusted to 9.5 with 10% acetic acid. The reaction mixture was incubated at 45 °C for 90 min and then applied to a DEAE-Sephadex A25 (HCO_3^-) column (1.0 × 20 cm), which was eluted with a linear gradient (250 mL/250 mL) of 0.35 to 0.45 M triethylammonium bicarbonate. After the appropriate fractions were pooled and evaporated, p5′A2′p5′A2′p5′A2′p was isolated as the sodium salt by precipitation from methanol (200 μL) with sodium iodide/acetone (100 mM, 3 mL), followed by washing with dry acetone and drying in vacuo.

Preparation of pA2′p5′A3′p. RNase T2 Digestion of pA2′p5′A3′p5′A2′p5′A. The oligomer p5′A2′p5′A3′p5′A2′p5′A (416 OD_{260} units, 10 μmol) was incubated in 50 mM NH_4OAc (500 μL, pH 4.5) at 37 °C for 2 h in the presence of RNase T2 (40 units). The reaction mixture was heated at 100 °C for 5 min to destroy the enzyme and then centrifuged. The supernatant was applied to a DEAE-Sephadex A25 column (HCO_3^- form, 1.0 × 20 cm), which was eluted with a linear gradient (250 mL/250 Ml) of 0.10 to 0.35 M triethylammonium bicarbonate. Fractions of 3.7 mL (total 140 fractions) were collected and monitored by UV absorbance at 260 nm. The first peak, which appeared between fractions 9 and 16, was identified as A2′p5′A on a PEI-cellulose plate developed in solvent 1 by direct comparison with authentic A2′p5′A (189 OD_{260} units, 7.5 μmol). The second peak, which appeared between fractions 120 and 140, was concentrated under vacuum, and evaporated with water several times until the triethylammonium bicarbonate was completely removed. The compound thus obtained (193 OD_{260} units, 7.7 μmol) was identified as p5′A2′p5′A3′p by digestion with nuclease P_1 (p5′A2′p5′A was obtained as the sole product) and bacterial alkaline phosphatase (A2′p5′A as the only product).

Preparation of p5′A2′p5′A2′p. To an ice-cold solution of 2′,5′-$(pA)_3$ (426 OD_{260} units, 12 μmol) was added a 0.1 M aqueous solution of sodium metaperiodate (164 μL). After the solution was stirred at 0 °C for 45 min, n-hexylamine (20 μL) was added to the mixture, and the pH of the solution was adjusted to 11.0 with 10% acetic acid (approximately 22 μL). The reaction mixture was then incubated at 37 °C for 2.5 h. Completion of the reaction was monitored by the PEI-cellulose, 0.1 M NH_4HCO_3. The whole reaction mixture was applied to a DEAE-Sephadex A25 column (HCO_3^-, 1.0 × 20 cm, equilibrated with 0.3 M TEAB), which was eluted with a linear gradient of 0.30–0.40 M TEAB (pH 7.6, total volume 500 mL, 136 fractions). After the proper fractions were pooled and evaporated (fractions 40–65), p5′A2′p5′A2′p was isolated as a sodium salt (152 OD_{260} units, 6.1 μmol; yield 51%).

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