in chloroform (50 mL) at 0 °C. The hydrochloride salt (0.77 g, 75%) is collected by filtration and dried at room temperature under high vacuum: mp 118–121 °C; IR (KBr) 3320, 3200, 3100 (OH, NH_2), 2800 (N^+H_2), 1650 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$, free base) δ 1.1 (d, 3 H, J = 6.5), 1.7 (m, 2 H), 3.8 (s, 3 H), 4.67 (m, 1 H). Anal. ($C_{22}H_{27}N_3O_2$·HCl) H, N; C: calcd, 65.74; found, 63.92.

Acknowledgment. We thank M. Stanciulescu, J. Corriveau, and G. Gavin for technical assistance in the synthesis and Dr. A. Treasurywala for useful discussions. We also acknowledge the assistance of D. Loughney in obtaining and analyzing the results from the search of the Cambridge Crystallographic data base.

Registry No. **4**, 36894-69-6; **5**, 101565-18-8; **5**·HCl, 101544-35-8; **6**, 101544-37-0; **6**-HCl, 101544-36-9; **7**, 16078-30-1; **8**, 76139-03-2; **9**, 101544-38-1; **10**, 101544-39-2; **11**, 101544-40-5; **12**, 101629-42-9; **13**, 101544-41-6; **13** (diazonium fluoroborate salt), 101544-48-3; **14**, 101544-42-7; **15**, 101544-43-8; **16**, 101544-44-9; **17**, 101544-45-0; **17** (amine), 101544-49-4; **18**, 101544-46-1; $ClCH_2COCl$, 79-04-9; $(Bz)_2NH$, 103-49-1; $C_6H_5(CH_2)_2COCH_3$, 2550-26-7.

Synthesis and Biological Activities of Oligo(8-bromoadenylates) as Analogues of 5′-*O*-Triphosphoadenylyl(2′→5′)adenylyl(2′→5′)adenosine

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The 8-bromoadenosine analogue of 5′-O-triphosphoadenylyl(2′→5′)adenylyl(2′→5′)adenosine (2-5A) and its derivatives were synthesized, and their biological activity was evaluated in mouse L cell extracts. All compounds, except 5′-dephosphorylated "cores" bound to the 2-5A-dependent endonuclease with a relative activity, depending on the derivative, of 1 to 0.035 of that of 2-5A trimer. 8-Bromoadenylate trimer 5′-mono-, -di-, and -triphosphates inhibited protein synthesis with a relative activity of 0.0023, 0.050, and 0.015 compared to 2-5A. Tetramer 5′-monophosphate also inhibited protein synthesis (relative activity 0.0033). The corresponding pentamer 5′-monophosphate did not; however, the pentamer 5′-diphosphate was able to inhibit translation (relative activity 0.0092). All compounds that possessed inhibitory activity in the protein synthesis inhibition assay gave ribosomal RNA cleavage patterns characteristic of the action of 2-5A-dependent endonuclease. Thus, 8-bromination of the all of the adenine rings of 2-5A leads to 20- to 70-fold reduction in the biological activity of the corresponding 5′-di- and -triphosphate, respectively; however, this same alteration of the three adenine moieties gives rise to a 5′-monophosphate with much enhanced translational inhibitory activity compared to the parent 2-5A trimer 5′-monophosphate.

One of the events that takes place after interferon interaction with cell surface receptors is induction of an enzyme named 2-5A synthetase, which in the presence of dsRNA synthesizes from ATP a mixture of 2′,5′-linked oligoadenylates, ppp5′A(2′p5′A)$_n$ ($n > 2$ to about 10), often referred to as 2-5A.[1-4]

2-5A is a very potent inhibitor of translation in cell-free systems[5] or in intact cells after introduction by cell permeabilization methods[6-8] or microinjection.[9] This protein synthesis inhibitory action is due to the action of ribonuclease L, which is activated by 2-5A to degrade viral and/or cellular RNAs with a preference for cleavage after UNp sequences.[10,11] 2-5A at a concentration sufficient to inhibit protein synthesis has been found in EMC virus-infected, interferon-treated mouse L cells or HeLa cells.[12-14] In addition, ribosomal RNA cleavage patterns characteristic of 2-5A-dependent ribonuclease L activity has been observed in interferon-treated mouse L cells infected with EMC virus[15] and reovirus-infected HeLa cells.[16]

The likely role of 2-5A in some of the antiviral actions of interferon and its potential role in cell regulation processes[17-19] has led to considerable activity in an attempt to develop analogues of 2-5A that might be active in the intact cell or animals.[20-24] Only a limited amount of in-

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Table I. Chromatographic Mobilities of 2′,5′-(8-Bromoadenylates)

no.	oligonucleotide	TLC,[a] R_f	HPLC t_R,[b] min	HPLC t_R,[c] min
1	p5′A (5′-AMP)	1.00	15.7	
2	p5′(br⁸A) (5′-8BrAMP)	0.88	21.1	
3	p5′(br⁸A)2′p5′(br⁸A)	0.72	21.9	
4	p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	0.59	23.0	16.8
5	p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	0.49	23.9	
6	p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	0.37	24.2	
7	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	0.82	40.1	
8	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	0.73	39.1	
9	pp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	0.31		15.2
10	ppp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	0.15		14.6
11	pp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	0.17		17.6

[a] PEJ-Cellulose F (Merck), 0.25 M ammonium bicarbonate. [b] Zorbax ODS (DuPont), 9.4 mm × 25 cm, 0–50% B in buffer A, 30 min, and then 50–100% B, 10 min. [c] Bondapak C_{18} (Waters) 3.9 mm × 30 cm, 0–50% B in buffer A, 25 min. A: 0.05 M ammonium phosphate, pH 7.0. B: methanol–water, 1:1.

Table II. ^1H NMR Data of 2′,5′-(8-Bromoadenylates)

no.[a]	chemical shift,[b] δ: anomeric protons (H-1′)	chemical shift,[b] δ: aromatic protons (H-2)
4	5.64 (d, 7.1 Hz), 5.78 (d, 3.2 Hz), 6.04 (d, 1.8 Hz)	7.93 (s, 2 protons), 8.01 (s)
5	5.76 (d, 7.0 Hz), 5.88 (d, 3.6 Hz), 5.90 (d, 3.6 Hz), 6.15 (d, 1.8 Hz)	7.92 (s), 7.96 (s), 7.99 (s), 8.03 (s)
6	5.78 (d, 6.8 Hz), 5.86 (d, 3.6 Hz, 2 protons), 5.91 (d, 3.5 Hz), 6.17 (d, 2.5 Hz)	7.99 (s, 3 protons), 8.02 (s), 8.13 (s)
9	5.74 (d, 7.0 Hz), 5.90 (d, 4.0 Hz), 6.16 (d, 4.0 Hz)	7.97 (s, 2 protons), 8.04 (s)
10	5.74 (d, 8.0 Hz), 5.88 (d, 4.0 Hz), 6.14 (nd, 4.0 Hz)	7.96 (s, 2 protons), 8.08 (s)

[a] See Table I. [b] Chemical shifts were determined in D_2O with acetone (δ 2.05) or dioxane (δ 2.75) as internal standards. Multiplicity of signal (s, singlet; d, doublet; m, multiplet) and coupling constants are given in parentheses.

formation is available, however, on the biological activity of base-modified analogues of 2-5A.[25–28]

In this work we present the synthesis and biological activity evaluations of 8-bromo-substituted analogues of 2-5A. The introduction of the bulky bromine atom at the 8-position of the adenine ring results in a switch of sugar–base conformation from anti in adenine nucleosides and nucleotides[29,30] to syn in 8-bromoadenosine,[31] its 5′-monophosphate,[34] and 3′,5′-linked polymers.[33] On the other hand, since there is no evidence available that 2-5A has a dramatically different conformation than 3′,5′-oligoadenylates,[32] it was reasonable to assume that 8-bromo-substituted analogues of 2-5A should prefer a syn conformation. We asked the question: how might this base substitution and resulting major change in oligonucleotide conformation influence its biological activity?

Results

Chemistry: Synthesis and Characterization of 2′,5′-Oligo(8-bromoadenylates). The mixture of 2′,5′-oligo(8-bromoadenylates) was obtained by polymerization of 8-bromoadenosine 5′-phosphoroimidazolidate, using a modification of the lead ion catalysis procedure introduced by Sawai et al.[35] Use of 1-methylimidazolium buffer at pH 7.5[36] resulted in completion of polymerization in 2 days at 4 °C. After treatment with nuclease P1, which hydrolyzes only 3′,5′-internucleotide bonds, the reaction mixture was separated by means of DEAE-Sephadex A-25 column chromatography, and certain components were purified further on HPLC reverse-phase ODS column (Zorbax) (Table I). The yields obtained after both purification steps were 9.7%, 3.1%, and 1.4% for p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A), p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′5′-(br⁸A), and p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)-2′p5′(br⁸A), respectively.

Table III. ^{31}P NMR Data of 2′,5′-(8-Bromoadenylates)

no.[a]	chemical shift,[b] δ: internucleotide phosphates	chemical shift,[b] δ: α, β, γ
4	−0.17 (s, 1 P), −0.25 (s, 1 P)	0.58 (s, 1 P)
5	−0.70 (s, 2 P), −0.84 (s, 1 P)	3.25 (br, 1 P)
6	−0.70 (s, 2 P), −0.78 (s, 2 P)	3.54 (br, 1 P)
9	−0.49 (s, 1 P), −0.67 (s, 1 P)	−10.4 (d, 1 P), −5.9 (d, 1 P)
10	−0.53 (s, 1 P), −0.65 (s, 1 P)	−10.8 (d, 1 P), −20.9 (t, 1 P), −5.8 (d, 1 P)

[a] See Table I. [b] Spectra were recorded in D_2O with 0.85% phosphoric acid as and external standard; s, singlet; d, doublet; t, triplet; $^3J_{P-O-,P}$ = 19–21 Hz.

The structure assignments of the products were based on ^1H and ^{31}P NMR spectra analyses. All compounds revealed the signals for required numbers of anomeric, H-1′, and aromatic, H-2 protons (Table II). ^{31}P NMR showed the presence of the requisite number of phosphorus atoms (Table III). Product purity and identity were further ascertained by means of HPLC before and after digestion with snake venom phosphodiesterase, bacterial alkaline phosphatase, and nuclease T2 (Tables I and IV).

5′-Dephosphorylated "cores" of trimer and tetramer [(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A) and (br⁸A)2′p5′(br⁸A)2′p5′-

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Table IV. Analysis of the Enzymic Digestion Products

no.[a]	compound	nuclease[b] P1	nuclease[c] T_2	snake venom[d] phosphodiesterase (SVPD)	bacterial alkaline[e] phosphatase (BAP)
4	p(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	nr	nr	p5′(br⁸A)	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)
5	p(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′-(br⁸A)	nr	nr	p5′(br⁸A)	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′-(br⁸A)
6	p(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′-(br⁸A)2′p5′(br⁸A)	nr	nr	p5′(br⁸A)	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′-(br⁸A)2′p5′(br⁸A)
7	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)			br⁸A + p5′(br⁸A)	
8	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)			br⁸A + p5′(br⁸A)	
9	pp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)			p5′(br⁸A)	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)
10	ppp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)			p5′(br⁸A)	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)
11	pp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′-(br⁸A)2′p5′(br⁸A)			p5′(br⁸A)	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′-(br⁸A)2′p5′(br⁸A)

[a] See Table I. [b] 1.5 OD units of substrate in 0.05 M sodium acetate, pH 5.75, 5 μL (2.5 mg/mL) of enzyme, 2 h, 37 °C. [c] 1.5 OD units of substrate in 0.05 M sodium acetate, pH 4.5, 4 units of enzyme, 2 h, 37 °C. [d] 1.5 OD units of substrate in 0.01 M Tris–acetate, pH 8.8, 0.01 M $MgCl_2$, 2 units of enzyme, 2 h, 37 °C. [e] 1.5 OD units of substrate, in 0.1 M Tris-HCl, pH 8.0, 0.001 M $MgCl_2$, 5 μL (125 units) of enzyme, 2 h, 37 °C.

Table V. Biological Activity of 8-Bromoadenylate Analogues of 2-5A

no.	compound	concn of 50% inhibn of protein synthesis, M	relative activity	ability to 50% replacement of radioactive probe, M	relative ability
	ppp5′A2′p5′A2′p5′A	2.3×10^{-9}	1	7.0×10^{-10}	1
	p5′A2′p5′A2′p5′A₈	2.0×10^{-4}	<0.00001	7.0×10^{-10}	1
4	p5(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	1.0×10^{-6} [a]	0.0023	2.0×10^{-8}	0.035
5	p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	7.0×10^{-7} [a]	0.0033	4.0×10^{-9}	0.18
6	p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	$\gg 4.0 \times 10^{-5}$	<0.00006	3.0×10^{-9}	0.23
7	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	$\gg 5.0 \times 10^{-5}$	<0.00005	6.0×10^{-7}	0.0012
8	(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	$>2.0 \times 10^{-4}$	<0.00001	3.0×10^{-8}	0.023
9	pp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	4.6×10^{-8}	0.050	3.0×10^{-9}	0.23
10	ppp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	1.5×10^{-7}	0.015	8.0×10^{-9}	0.088
11	pp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)	2.5×10^{-7} [a]	0.0092	7.0×10^{-10}	1

[a] Concentration required to obtain the same effect on protein synthesis as IC_{50} for 2-5A. The maximum inhibition effects caused by these analogues were 80%, 66%, and 60% of that of 2-5A, for compounds **4**, **5**, and **11**, respectively.

(br⁸A)2′p5′(br⁸A)] were prepared by bacterial alkaline phosphatase treatment of the corresponding 5′-monophosphates. Snake venom phosphodiesterase hydrolysis of both compounds gave 8-bromoadenosine and p5′(br⁸A) in the expected ratio (Table IV).

For the preparation of 8-bromoadenylate trimer 5′-triphosphate and both trimer and pentamer 5′-diphosphates, a modification of earlier procedures was applied.[37,38] The 5′-monophosphates were converted into 5′-phosphoroimidazolidates by modification of the procedure of Mukaiyama and Hashimoto[39] and, after separation as sodium salts, were reacted with tributylammonium pyrophosphate or phosphate, respectively. The structure of both trimer derivatives were established by ^{31}P NMR spectra analyses (Table III), which showed a characteristic pattern for 5′-triphosphate group (doublets for P_α and P_γ, and double doublet for P_β) and 5′-diphosphate group (doublets for both P_α and P_β phosphorus atoms). Coupling constants, ^{31}P–O–P were in the range 19–21 Hz, which is characteristic for this group of compounds.[40] Due to paucity of material, the structure of 8-bromoadenylate pentamer 5′-diphosphate was assigned only by the analysis of enzyme digestion products (Table IV). Snake venom phosphodiesterase hydrolysis gave p5′(br⁸A) as the only product and bacterial alkaline phosphatase treatment gave product identical with that obtained from starting pentamer 5′-monophosphate.

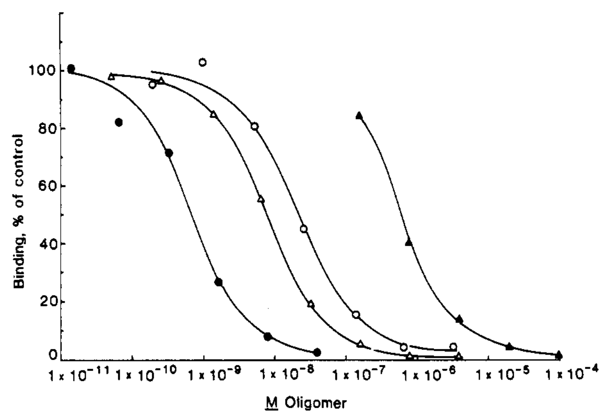

Figure 1. Ability of 8-bromoadenylates to inhibit the binding of radioactive probe, ppp5′A2′p5′A2′p5′A2′p5′A3′[^{32}P]pCp to the 2-5A-dependent RNase L in mouse L cell extracts. Approximately 30–40% of added radioactive probe was bound to the nitrocellulose filters. 100% corresponds to total probe binding in the absence of any other oligonucleotide. (●) ppp5′A2′p5′A2′p5′A, (△) ppp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A), (○) p5′(br⁸A)2′p5′(br⁸A)-2′p5′(br⁸A), (▲) (br⁸A)2′p5′(br⁸A)2′p5′(br⁸A).

Biological Studies. The biological activities of the synthetic 2′,5′-linked 8-bromoadenylate oligomers were compared with the activity of 2-5A itself by the following assays: (1) competition assay for binding to the enzyme RNase L,[41] (2) activation of RNase L established by measuring protein synthesis inhibition in vitro in mouse

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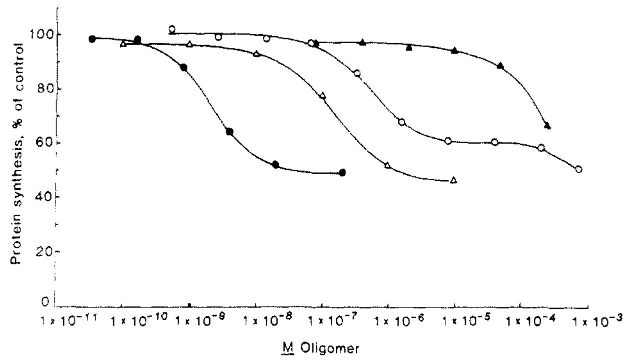

Figure 2. Inhibition of translation caused by 8-bromoadenylates in mouse L cell extracts programmed with EMCV RNA. 100% corresponds to protein synthesis level measured by the amount of [^{3}H]leucine incorporated into hot trichloroacetic acid insoluble material in the absence of any oligonucleotide added. (●) ppp5′A2′p5′A2′p5′A, (△) ppp5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A), (○) p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A), (▲) (br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A).

L cell extracts programmed with encephalomyocarditis virus RNA,[42,43] (3) nuclease activation analyzed by rRNA cleavage pattern characteristic of RNase L action.[15]

Ability of 2′,5′-Oligo(8-bromoadenylates) To Bind to 2-5A-Dependent RNase L. All prepared analogues were tested and compared to 2-5A trimer in the binding competition assay.[41] Crude mouse L cell extracts were used as a source of RNase L and ppp5′A2′p5′A2′p5′A2′p5′A3′[^{32}P]p5′Cp as a radioactive probe. The ability of a given analogue to bind to RNase L was expressed as the concentration required to bring about a 50% replacement of radioactive probe from endonuclease–nitrocellulose complex. The binding of all analogues was less efficient than the binding of ppp5′A2′p5′A2′p5′A (Figure 1, Table V). Only one analogue, 2′,5′-(8-bromoadenylate) pentamer 5′-diphosphate bound with the same efficiency as 2-5A itself. The relative binding abilities of other 5′-mono-, 5′-di-, and 5′-triphosphate analogues were similar (in the range 0.23–0.035 as compared 2-5A (Table V). For 5′-monophosphates the order of decreasing activities was as follows: p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A) > p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A) > p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A). For 5′-di- and 5′-triphosphates: pp5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A) > pp5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A) > ppp5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A). The binding ability of 8-bromoadenylate trimer 5′-triphosphate was ca. 2.5 times lower than that of the corresponding 5′-diphosphate. 5′-Dephosphorylated "cores" were bound with very low efficiency (0.0023–0.0012 of that of 2-5A).

RNase L Activation by 2′,5′-Oligo(8-bromoadenylates). All synthesized 8-bromoadenylates except the 5′-dephosphorylated "cores" and 8-bromoadenylate pentamer 5′-monophosphate were inhibitors, albeit less effective than 2-5A, of protein synthesis in mouse L cell extracts programmed with EMCV RNA (Figure 2, Table V). The presence of a 5′-di- or 5′-triphosphate group was not a necessary requirement for this activity. As presented in Table V, the relative inhibitory activities (compared to 2-5A) of 8-bromoadenylate trimer and tetramer 5′-monophosphates were 0.0023 and 0.0033, respectively. In contrast, the 5′-monophosphate of 2′,5′-adenylate trimer did not have any inhibitory activity in this system even at a concentration as high as 10^{-4} M.[43] Introduction of an additional phosphate or pyrophosphate group at the 5′-end of the bromoadenylate trimer monophosphate increased the activity 22 and 6 times, respectively. Although pentamer 5′-monophosphate was not an inhibitor of protein synthesis in the mouse L cell system, the corresponding 5′-diphosphate had activity.

The extent of protein synthesis inhibition caused by some analogues, i.e., 8-bromoadenylate trimer and tetramer 5′-monophosphates and pentamer 5′-diphosphate, was smaller than that caused by 2-5A and other analogues. This phenomenon was also reflected by the kinetics of protein synthesis (Figure 3). The inhibition profiles were generally similar for all tested compounds except that at the time of maximal inhibition the level of protein syn-

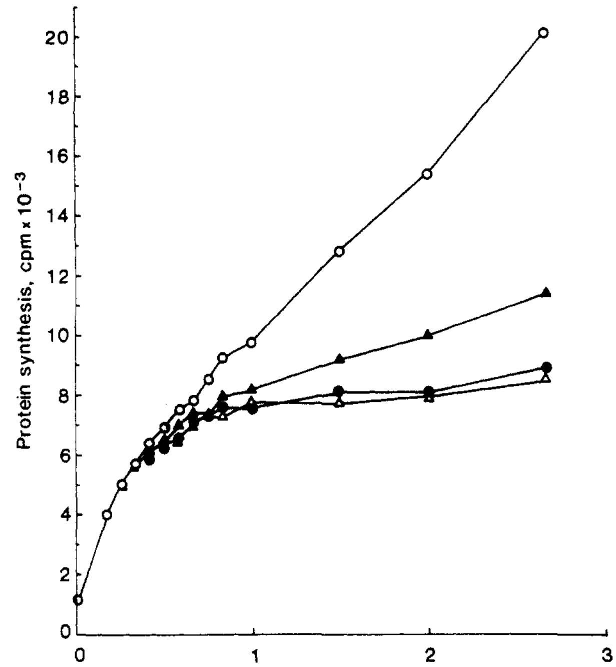

Figure 3. Kinetics of protein synthesis inhibition by 8-bromoadenylates in mouse L cell extracts as measured by the radioactivity of [^{3}H]leucine incorporated into hot trichloroacetic acid insoluble material. Five-microliter aliquots were taken at given times and were applied on filter paper disks, presoaked with 15% solution of trichloroacetic acid. (○) No additions; (●) ppp5′A2′p5′A2′p5′A, concentration 2 × 10^{-8} M; (△) ppp5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A), concentration 2 × 10^{-6} M; (▲) p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A), concentration 2 × 10^{-6} M.

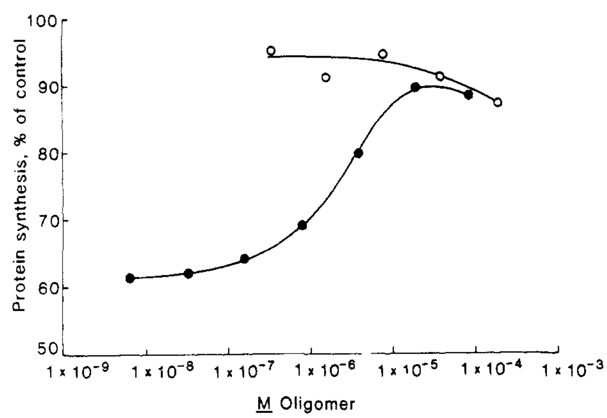

Figure 4. Prevention of the protein synthesis inhibitory activity of p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A) (concentration 2 × 10^{-5} M) by various concentrations of p5′A2′p5′A2′p5′A. 100% corresponds to the protein synthesis level without any oligonucleotide added. (○) p5′A2′p5′A2′p5′A, (●) p5′A2′p5′A2′p5′A in the presence of p5′(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A), concentration 2 × 10^{-5} M.

(42) Torrence, P. F.; Friedman, R. M. *J. Biol. Chem.* **1979**, *254*, 1259.
(43) Torrence, P. F.; Imai, J.; Johnston, M. I. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 5993.

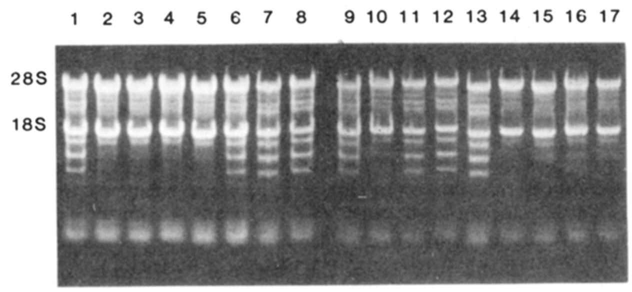

Figure 5. Ability of 8-bromoadenylates to activate RNase L as determined by characteristic degradation patter of ribosomal RNA of mouse L cells. Lanes: 1 and 9, pppA2′p5′A2′p5′A, concentration 2×10^{-7} M; 2, no additions; 3 and 4, pA2′p5′A2′p5′A, concentration 2×10^{-5} and 2×10^{-4} M; 5–8, ppp(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A), concentration 2×10^{-8}, 2×10^{-7}, 2×10^{-6}, and 2×10^{-5} M; 10–13, pp(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A), concentration 2×10^{-8}, 2×10^{-7}, 2×10^{-6}, and 2×10^{-5} M; 14–17, p(br^{8}A) 2′p5′(br^{8}A), concentration 2×10^{-7}, 2×10^{-6}, 2×10^{-5}, and 2×10^{-4} M.

thesis was higher for 8-bromoadenylate trimer 5′-monophosphate as compared with corresponding 5′-triphosphate.

Proof that observed inhibition of protein synthesis by 8-bromoadenylate trimer 5′-monophosphate was caused by activation of RNase L was provided by experiments (Figure 4) in which the translational inhibition could be prevented by p5′A2′p5′A2′p5′A, an established antagonist of 2-5A action.[43] In addition, in a separate assay, but under conditions of protein synthesis, all of the above 8-bromoadenylate oligomers that showed inhibition of protein synthesis also gave rise to the well-established ribosomal RNA cleavage pattern typical of 2-5A itself (Figure 5).

Possible 5′-Terminal Phosphorylation of 2′,5′-(8-Bromoadenylate) Trimer 5′-Monophosphate to 5′-Di- or 5′-Triphosphates. In the first approach to this problem, γ[^{32}P]ATP was used as a radioactive marker of phosphorylation. Two reaction mixtures, (i) control with no added oligonucleotide and (ii) 8-bromoadenylate trimer 5′-monophosphate (1×10^{-4} M), were incubated for 1 h at 30 °C with γ[^{32}P]ATP (total activity 67 μCi) in the same conditions as for protein synthesis but without EMCV RNA and [^{3}H]leucine. After workup and purification, comparison of the UV absorption with radioactivity profiles (Figure 6) provided no indications of any products of phosphorylation of 5′-monophosphate (in mouse L cell extracts), at concentrations greater than the limit of detection, ca. 2×10^{-8} M (this concentration was determined by radioactivity background level). On the other hand, the phosphorylation of 5′-diphosphate to 5′-triphosphate clearly occurred during incubation with mouse L cell extract, as described previously.[44] As calculated from the activity and purity of starting γ[^{32}P]ATP and total radioactivity of triphosphate peak, the concentration of obtained ^{32}P-labeled 5′-triphosphate was about 4.5×10^{-7} M, representing a 0.45% conversion.[44]

In a separate series of experiments, 2-aminopurine, an established inhibitor of protein kinases[45,46] and previously employed putatively to block phosphorylation of a 2-5A analogue,[24] was added to the protein synthesis assay at a final concentration of 0.1 M. 2-Aminopurine had no effect on the protein synthesis inhibitory properties of 2-5A or 8-bromoadenylate trimer 5′-monophosphate (data not illustrated). In fact, 2-aminopurine elevated the control levels of protein synthesis, in agreement with earlier observations.[45]

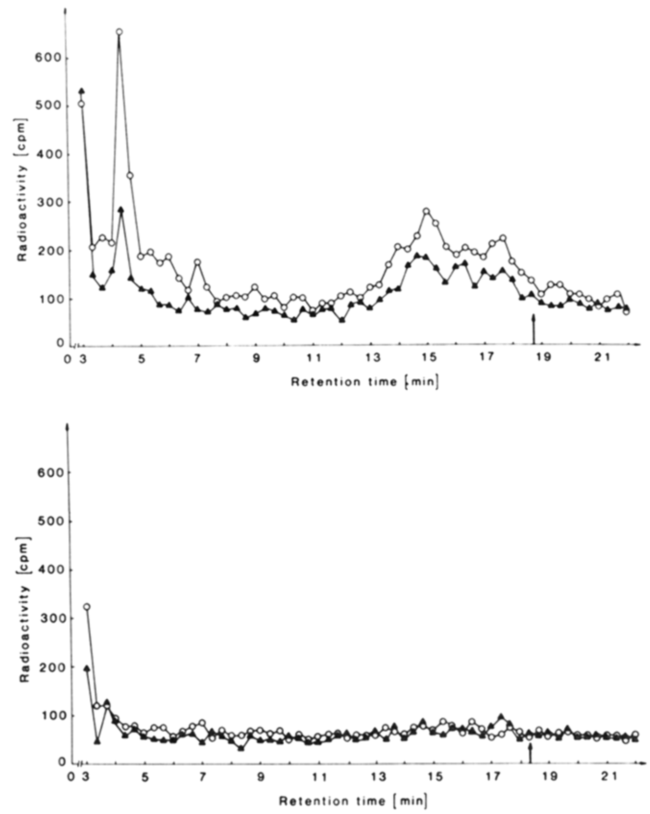

Figure 6. The possibility of 5′-terminal phosphorylation of p(br^{8}A)2′p5′(br^8)2′p5′(br^{8}A) determined after 1-h incubation at 30 °C with γ[^{32}P]ATP (total activity 67 μCi) in 20% mouse L cell extract under the protein synthesis assay conditions but without creatine kinase, amino acids, and EMCV RNA added. The two reaction mixtures (300 μL), (A) without any addition and (B) with p(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A), concentration 1×10^{-4} M, were first separated by means of HPLC (Bondapak C_{18}, 3.9 mm × 25 cm column, 0–50% B in buffer A) in order to remove γ[^{32}P]ATP and products of its degradation. Broad fractions, with retention times between 11 and 19 min, were collected. About 10 OD units of 8-bromoadenylate trimer 5′-mono-, -di-, and -triphosphate were added to each A and B to make possible UV detection and the mixtures were separated on DEAE-Sephadex A-25 column (1 × 15 cm, eluted with 0.05–0.6 M triethylammonium buffer, pH 7.5, 300 mL). Fractions containing p(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A), pp(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A), and ppp(br^{8}A)2′p5′(br^{8}A)2′p5′-(br^{8}A) were again, after concentration, applied on the same HPLC column. Twenty-second fractions were collected, and their radioactivity was measured. (Top) Distribution of radioactivity for pp(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A). (Bottom) Distribution of radioactivity for ppp(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A) obtained from experiments A (O) and B (▲), respectively. Arrows indicate the retention times of pp(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A) (top) and ppp(br^{8}A)2′p5′(br^{8}A)2′p5′(br^{8}A) (bottom) determined from UV detection.

In a third series of experiments, no ATP or other nucleoside triphosphate was added to the reaction mixture, and in addition, the mouse L cell lysates were depleted of endogenous ATP (as established by HPLC and TLC) by a prior 10-min incubation with glucose hexokinase.[47] The observed cleavage patterns were the same as those seen in the presence of exogenously added ATP.

Stability of 2′,5′-(8-Bromoadenylate) Oligomers in Mouse L Cell Extracts. All experiments were done un-

(44) Lesiak, K.; Torrence, P. F. *Biochem. Biophys. Res. Commun.* **1985**, *126*, 917.
(45) West, D. K.; Lenz, J. R.; Baglioni, C. *Biochemistry* **1979**, *18*, 624.
(46) Farell, P.; Balkcow, K.; Hunt, T.; Jackson, R.; Trachsel, H. *Cell* **1977**, *11*, 187.
(47) Dougherty, J. P.; Samanta, H.; Farrell, P. J.; Lengyel, P. *J. Biol. Chem.* **1980**, *255*, 3813.

Table VI. Stability of p(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A), pp (br⁸A)2′p5′(br⁸A)2′p5′(br⁸A), and ppp(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A) in 20% Mouse L Cell Extracts[a]

time	p(br⁸A)2′p5′(br⁸A)-2′p5′(br⁸A), %		pp(br⁸A)2′p5′(br⁸A)-2′p5′(br⁸A), %		ppp(br⁸A)2′p5′(br⁸A)-2′p5′(br⁸A), %	
	+ATP	−ATP	+ATP[b]	−ATP[c]	+ATP[d]	−ATP[d]
0	100	100	100	100	71	48
30 min				80		8.0
40 min	99	88	87		54	
75 min	89	82	71		46	
90 min				70		3
120 min			67		36	
150 min	84	77		55		
220 min			70		16	
240 min		76		0		
12 h	53	22	47	0	0	

[a] Conditions are as for the protein synthesis assay but amino acids and EMCV RNA were omitted. In one set of experiments also ATP, GTP, and CTP were not added. [b] The main product of degradation was the corresponding 5′-monophosphate (16.5% after 220 min of incubation). Phosphorylation to 5′-triphosphate was also observed (14% after 120 min of incubation). [c] 31% of 5′-monophosphate after 240 min of incubation. [d] The main product of degradation was the corresponding 5′-diphosphate (65% after 220 min of incubation in the presence of ATP and 84% after 30 min of incubation in the absence of ATP).

der the conditions used for the protein synthesis assay, but creatine kinase and amino acids were omitted. In one set of experiments also ATP, GTP, CTP, and creatine phosphate were not added.

In comparison with 2′,5′-adenylates, all 2′,5′-(bromoadenylates) were more resistant to degradation. The half-life of 2-5A trimer 5′-monophosphate was about 30 min under these conditions,[20,48] but its 8-bromoadenylate analogue was degraded only 20% after $2^1/_2$ h of incubation (Table VI). In contrast to 2-5A, the main degradation pathway was not chain cleavage but rather dephosphorylation (7% of trimer "core" after 4-h incubation). The stabilities of 2′,5′-(8-bromoadenylate) trimer 5′-di- and 5′-triphosphates were dependent on the presence of ATP. At an ATP concentration of 1×10^{-3} M, the half-life of 8-bromoadenylate trimer 5′-triphosphate was about 40 min, and the main product of degradation was the corresponding 5′-diphosphate. In the absence of ATP and without incubation at 30 °C, 52% of the triphosphate was already degraded to 5′-diphosphate (Table VI).[49]

In the presence of 1×10^{-3} M ATP, the amount of 5′-diphosphate decreased to 71% after 75-min incubation time, but further degradation was very slow (47% of 5′-diphosphate remained after 12-h incubation). The main product of degradation was 5′-monophosphate; however, the 5′-diphosphate was also phosphorylated to 5′-triphosphate (14% after 2 h of incubation). This phosphorylation, which had been also observed with γ [^{32}P]ATP[44] (vide supra), did not occur in the absence of ATP. In addition, the stability of the 5′-diphosphate was lower than in the previous experiment (Table VI).

Discussion

In regard to RNase L binding (Table V), the 2′,5′-oligo(8-bromoadenylate) 5′-mono-, -di-, or -triphosphates displayed a behavior that was dependent on the exact nature of the congener. Affinities for the 2-5A-dependent endonuclease of L cells ranged from an activity of 0.035 of that of 2-5A, in the case of p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A), to an activity equivalent to 2-5A in the case of pp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A). This amounts to a range of about 30-fold in activity, which is much greater than that obtained in the case of the parent 2′,5′-oligoadenylates. For instance, in a recent study, there was only a twofold difference in activity of oligoadenylate trimer, tetramer, pentamer, and hexamer 5′-monophosphates as compared to that of 2-5A trimer triphosphate.[26] Also unusual in this oligo(bromoadenylate) series was the significantly reduced affinity of the 5′-triphosphate, ppp5′(br⁸A)2′p5′(br⁸A) 2′p5′(br⁸A), as compared to that of the trimer 5′-diphosphate, pp5′(br⁸A)-2′p5′(br⁸A)2′p5′(br⁸A), and the pentamer 5′-diphosphate, pp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A). In other cases that have been examined, the binding activity of the 5′-triphosphate has been at least equivalent to the binding activity of the corresponding 5′-mono- or 5′-diphosphate.[12,22,26]

The "core" oligobromoadenylates, (br⁸A)2′p5′(br⁸A)-2′p5′(br⁸A) and (br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A), showed behavior typical of other cases;[26] that is a significant reduction in affinity for RNase L with the tetramer "core" having somewhat more affinity than the trimer "core".

The ability of the oligo(bromoadenylates) to activate RNase L (Table V), as judged by their translational inhibitory capacity, also was anomalous in comparison to parent oligoadenylates and other analogues that have been examined so far. First of all, as would be expected, the "core" trimeric and tetrameric unphosphorylated oligo(bromoadenylates) were without discernible inhibitory activity. The 5′-di- and 5′-triphosphates, pp5′(br⁸A)-2′p5′(br⁸A)2′p5′(br⁸A), pp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)-2′p5′(br⁸A)2′p5′(br⁸A), and ppp5′(br⁸A)2′p5′(br⁸A)2′p5′-(br⁸A), had 1.5–6.0% of the inhibitory activity of 2-5A trimer triphosphate, with the trimer 5′-diphosphate being 3–4 times more active than the corresponding bromoadenylate 5′-triphosphate. This behavior departs from that of the parent oligoadenylates in that that ppp5′A2′p5′A2′p5′A and pp5′A2′p5′A2′p5′A have equivalent activities in the mouse system.[22,23] The most remarkable facet of the oligo(bromoadenylates) is the ability of the 5′-monophosphorylated species to inhibit protein synthesis in the mouse L cell system. In distinct contrast to adenylate oligomers such as p5′A2′p5′A2′p5′A or p5′A2′p5′A2′p5′A2′p5′A, which have no significant inhibitory activity in this system and which are, in fact, excellent antagonists of 2-5A action[22,43,50], the oligobromo-

(48) Schmidt, A.; Zilberstein, A.; Shulman, L.; Federman, P.; Berissi, H.; Revel, M. *FEBS Lett.* **1978**, *95*, 257.

(49) Recent results that we obtained from examination by HPLC of the degradation of 2-5A trimer 5′-di- and 5′-triphosphates indicated the existence of two reactions: ppA2′p5′A2′p5′A ⇌ ppp5′A2′p5′A. The phosphorylation reaction depends on the presence of ATP in the mixture.

(50) Miyamoto, N. G.; Jacobs, B. L.; Samuel, C. E. *J. Biol. Chem.* **1983**, *258*, 15232–15237.

adenylate 5'-monophosphates, p5'(br^{8}A)2'p5'(br^{8}A)2'p5'-(br^{8}A) and p5'(br^{8}A)2'p5'(br^{8}A)2'p5'(br^{8}A)2'p5'(br^{8}A), both possessed substantial activity as inhibitors of protein synthesis. In contrast, the pentamer 5'-monophosphate was devoid of significant activity. As shown by antagonism studies (in the case of 8-bromoadenylate trimer) as well as rRNA cleavage studies, both these oligomers most probably owe their inhibitory properties to activation of RNase L.

The protein synthesis inhibitory properties of the 8-bromoadenylate trimer and tetramer 5'-monophosphates could be due to either (i) an effective phosphorylation to the corresponding 5'-diphosphate or (ii) a direct activation of RNase L by the 5'-monophosphate itself. In the latter case, it could be that the unique, possible syn nucleotide conformation generated by the introduction of the 8-bromo substituent might effect the requisite endonuclease conformational change normally induced by the presence of the di- or triphosphate moiety. Alternatively, in the former instance, it could be argued that 8-bromination provides a much superior substrate for any kinase enzyme that could act on a 2',5'-linked oligonucleotide 5'-monophosphate or that the increased resistance of oligobromoadenylates to degradation (Table VI) might effectively reinforce the resultant action of any kinase that way be operative. However, resistance to degradation cannot be a sufficient criterion for such activity; a 2'-terminally modified tetramer 5'-monophosphate did not inhibit protein synthesis.[26]

We have not been able to provide any evidence of the first alternative, that is, phosphorylation of the oligobromoadenylate 5'-monophosphate to the 5'-diphosphate. In the presence of p5'(br^{8}A)2'p5'(br^{8}A)2'p5'(br^{8}A), the rRNA cleavage pattern characteristic for activated RNase L persisted even when the cell extracts were depleted of ATP by glucose hexokinase treatment; moreover, we could not detect, using γ[^{32}P]ATP, any formation of pp5'-(br^{8}A)2'p5'(br^{8}A)2'p5'(br^{8}A) ($>2 \times 10^{-8}$ M) under conditions where phosphorylation of pp5'(br^{8}A)2'p5'(br^{8}A)-2'p5'(br^{8}A) to ppp5'(br^{8}A)2'p5'(br^{8}A)2'p5'(br^{8}A) was readily witnessed.[44] These results, therefore, tend to suggest the second alternative raised above, i.e., direct activation of RNase L by the oligo(8-bromoadenylate) 5'-monophosphate. However, further experimentation along this line must await preparations of highly purified or homogeneous RNase L.

The enhanced resistance to degradation of oligo(bromoadenylates) also is of interest since it is the first reported example of increasing the metabolic stability of a 2',5'-oligonucleotide by altering the nature of the heterocyclic base. This had been previously accomplished only by modifying the ribose elements of the oligonucleotide.[20,22,24,50,52]

Thus modification of the 2',5'-oligoadenylate structure by substitution of all of the purine 8-hydrogens by bromine produces 2-5A analogues with the following properties: (a) Depending on the oligonucleotide chain length, 2',5'-oligo(8-bromoadenylate) 5'-di- and 5-triphosphates are produced with 20–100% of the RNase L binding ability and 1.5–6.0% of the RNase L activation ability of the parent 2',5'-oligoadenylates. (b) In distinct contrast to 2',5'-oligoadenylate 5'-monophosphates, the 2',5'-oligo(8-bromoadenylate) 5'-monophosphates possessed substantial RNase L activating abilities; however, this was markedly dependent on oligonucleotide chain length. At this time, we have been unable to determine unequivocally whether this nuclease activation ability may be due to direct activation of RNase L or to prior phosphorylation to the corresponding 5'-di- or 5'-triphosphate. (c) The 2',5'-oligo(8-bromoadenylates) were significantly more resistant to degradation than the corresponding 2',5'-oligoadenylates.

The latter two findings that RNase L activation by a 5'-monophosphate can be dramatically enhanced by 8-bromo substitution and that this substitution also increases the metabolic stability of such 2',5'-oligonucleotides may represent useful observations in the development of a 2-5A analogue that would show activity in the intact cell.

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(52) Sawai, H.; Imai, J.; Lesiak, K.; Johnston, M. I.; Torrence, P. F. *J. Biol. Chem.* **1983**, *258*, 1671.

Experimental Section

8-Bromoadenosine 5'-monophosphate, phosphoglucose hexokinase, and ribonuclease T2 were from Sigma (St. Louis, MO). Snake venom phosphodiesterase was a product of Worthington (Freehold, NJ), bacterial alkaline phosphatase from Bethesda Research Laboratories (Bethesda, MD), nuclease P1 from Calbiochem. (LaJolla, CA). γ[^{32}P]ATP, sp act. 10–40 Ci/mmol, was from New England Nuclear (Boston, MA) and pppA2'p5'A2'p5'Ap5'A3'[^{32}P]pCp, sp act. 3000 Ci/mmol, from Amersham (Chicago, IL).

The 220-Hz protein NMR spectra were recorded on a Varian HR220 NMR spectrometer. ^{31}P NMR spectra were obtained on a JEOL JNM-FX100 instrument operating at 40 MHz and chemical shifts were reported relative to external 0.85% H_3PO_4 as a standard. All UV measurements were carried out on a Hewlett-Packard 8450A UV/vis spectrophotometer.

Thin-layer chromatography was on E. Merck precoated PEI-cellulose F plates. High-performance liquid chromatography was executed with a Beckman instrument with a Model 110A pump with columns and solvent systems indicated in text. Solvent A refers to 0.05 M ammonium phosphate, pH 7.0, solvent B to methanol–water, 1:1.

Normal-pressure ion-exchange column chromatography on DEAE-Sephadex A-25 was carried on a 4 °C, with various concentrations of triethylammonium bicarbonate, pH 7.5, as an elution buffer. Buffer was removed by repeated coevaporation with water. Triethylammonium salts of oligonucleotides were usually exchanged into sodium salts by precipitation from 1% acetone solution of sodium iodide. Purity of all obtained compounds was determined by means of HPLC chromatography.

The preparation of mouse L cell extracts, encephalomyocarditis virus RNA, as well as the techniques and conditions for in vitro protein synthesis and endonuclease L binding assay have been described previously.[41,42]

Ribosomal RNA cleavage assay was carried on at 30 °C under conditions essentially as for protein synthesis inhibition assay[15] but without creatine kinase, amino acid mixture, and EMCV RNA. The reaction mixtures were diluted 10 times with a denaturing buffer (50 mM sodium acetate, 10 mM EDTA, 0.5% sodium dodecyl sulfate, pH 5.0) and extracted two times with an equal volume of water saturated phenol–chloroform (1:1) solution. Before the second extraction the aqueous phase was made 100 mM in sodium acetate (pH 5.5). RNA was precipitated with 2.5 volumes of ethanol at −20 °C, denatured with glyoxal, and analyzed by electrophoresis on 1.8% agarose gel, in the presence of 1.6 M urea in a buffer containing 40 mM Tris–acetate, 10 mM sodium acetate, and 1 mM EDTA, at pH 7.2. The gel was stained with ethidium bromide and photographed under UV light (302 nm).

Polymerization of 8-Bromoadenosine Phosphoroimidazolidate. 8-Bromoadenosine-5-monophosphoric acid (852 mg, 2 mmol) and Et_3N (1.8 mL) were dissolved (with gentle heating) in DMF (40 mL). Imidazole (680 mg, 10 mmol), triphenylphosphine (1572 mg, 6 mmol), 2,2'-dipyridyl disulfide (1320 mg, 6 mmol) were added, and the reaction mixture was stirred for 30 min at room temperature. After that time, TLC (*n*-BuOH–EtOH–H_2O–30% NH_3, 60:20:10:1; silica gel) indicated that reaction was completed. Imidazolidate was separated as a sodium salt by precipitation from acetone solution of sodium iodide (500 mL, 1% NaI). Yield 990 mg (26400 OD_{264} units, 79%).

8-Bromoadenosine 5′-phosphoroimidazolidate (700 mg, 1.12 mmol, 18700 OD_{264} units) was dissolved in 22 mL of 0.2 M *N*-methylimidazolium buffer (pH 7.5) and 1.1 mL of 0.25 M $Pb(NO_3)_2$ was added with vigorous stirring. After 48 h at 4 °C all imidazolidate reacted (TLC, PEI-cellulose F, 0.1 M ammonium bicarbonate). Pb^{2+} ions were removed by treatment with Chelex ion-exchange resin (100–200 mesh, Na^+ form, 10 mL of wet resin). The resin was filtered off and washed with water (3 × 20 mL). The filtrate contained 16300 OD_{263} units of oligonucleotide mixture (87.2%).

The solution was concentrated to about 20 mL and ethanol (200 mL) was added in order to precipitate dimer and higher oligomers. This gave 12640 OD_{263} units of ethanol-insoluble and 4190 OD_{263} units of ethanol-soluble fractions.

The ethanol-insoluble material was dissolved in 25 mL of 0.02 M ammonium acetate (pH 5.75) and incubated overnight at 37 °C with nuclease P1 (250 μL, 2.5 mg/mL) in order to digest any 3′,5′-linked isomers. The enzyme was denatured by shaking with an equal volume of chloroform–isoamyl alcohol (7:3). The water layer was extracted with ethyl ether (3 × 10 mL) and then concentrated to a volume of about 5 mL. The pH of the aqueous solution was adjusted to 7.5, and the mixture was applied to a DEAE-Sephadex A-25 column (1.6 × 30 cm, HCO_3^- form). Elution was with 2 L of 0.05–0.75 M triethylammonium bicarbonate (TEAB) buffer, pH 7.5. The following fractions were collected: 8BrAMP, 3830 OD_{264} units; p(br⁸A)2′p5′(br⁸A), 2840 OD_{263} units; p(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A) 1500 OD_{263} units; p(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A), 1100 OD_{263} units; p(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A), 560 OD_{263} units.

All fractions were characterized by TLC and HPLC (Table I). Tetramer and pentamer fractions were further repurified by means of HPLC. Trimer fraction was also repurified by column chromatography.

Purification of p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A). The trimer fraction from the first separation of the polymerization mixture was dissolved in 5 mL of 0.02 M ammonium acetate, pH 5.75, and again incubated overnight at 37 °C with nuclease P1 (50 μL, 2.5 mg/mL). Enzyme was denatured as described previously and oligonucleotide purified again on a DEAE-Sephadex A-25 column (1.6 × 30 cm), eluted with a linear gradient of 0.05–0.6 M (1 L) TEAB buffer, pH 7.5. Yield 1480 OD_{263} units (9.7%). Hypochromicity effect was 18% as determined by phosphorus analysis or 16% as obtained by snake venom phosphodiesterase digestion.

Purification of p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A) and p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A). The products from the first separation were again treated with nuclease P1 as for the trimer and then purified by means of HPLC (Zorbax, 2.12 × 25 cm, flow rate 5 mL/min, linear gradient 0–40% solvent B in solvent A in 60 min). Compounds were desalted on a DEAE-Sephadex A25 column eluted with triethylammonium buffer. Yields: tetramer, 516 OD_{263} units (3.1%), hypochromicity 21% (SVPD method); pentamer, 216 OD_{263} units (1.4%), hypochromicity 21% (SVPD method).

Synthesis of (br⁸A)2′p5′(br⁸A)2′p5′(br⁸A). Trimer core 5′-monophosphate (50 OD_{263} units) was incubated for 5 h at 37 °C with 5 μL (125 units) of bacterial alkaline phosphatase in 0.1 M Tris-HCl, pH 8.0, and 0.001 M $MgCl_2$. The enzyme was denatured by shaking with an equal volume of chloroform–isoamyl alcohol (7:3), and the product was purified on a DEAE-Sephadex A-25 column, 1 × 15 cm, eluted with 0.05–0.5 M triethylammonium buffer, 500 mL, pH 7.5. Yield 44 OD units (88%).

Synthesis of (br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A). This compound was obtained in the same way as its trimer analogue, starting from 125 OD_{263} units of 2′,5′-(8-bromoadenylate) tetramer 5′-monophosphate and 10 μL (250 units) of bacterial alkaline phosphatase. Yield 100 OD_{263} units (80%).

Synthesis of ppp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A). An aqueous solution of 8-bromoadenylate trimer 5′-monophosphate, triethylammonium salt (400 OD_{263} units, 10 μmol), was evaporated to dryness and the oligonucleotide was rendered anhydrous by coevaporations with DMF (2 × 10 mL, gentle heating was required for dissolving). The residue was suspended in fresh DMF (5 mL) and triethylamine (15 μL), imidazole (14 mg, 200 μmol), triphenylphosphine (26 mg, 100 μmol), and 2,2′-dipyridyl disulfide (21 mg, 100 μmol) were added. The mixture was stirred for 1 h at room temperature. The product, 5′-phosphoroimidazolidate of trimer, was precipitated as a sodium salt by pouring the reaction mixture into a 1% solution of sodium iodide in acetone (50 mL). The precipitate was spun down and dried by coevaporation with DMF (5 mL). Tributylammonium pyrophosphate (400 μL, 0.5 M in DMF) was added to the residue, and the mixture was kept overnight at room temperature.

HPLC analysis of the crude mixture revealed the presence of 79% of 5′-triphosphate and 9% of 5′-monosphosphate. Triethylammonium buffer (0.05 M, 1 mL) was added, and the solution was directly applied to a DEAE-Sephadex A-25 column (1 × 15 cm) and eluted with 0.3–0.6 M TEAB, pH 7.5, 500 mL). Yield 293 OD_{263} units (73%).

Synthesis of pp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A). The 8-bromoadenylate trimer 5′-diphosphate was prepared in the same way as 5′-triphosphate, but instead of pyrophosphate, tributylammonium phosphate (400 μL, 0.5 M in DMF) was added. The HPLC taken after 24 h at room temperature revealed the presence of 73.3% of the desired product, 5′-diphosphate and 12.5% of 5′-monophosphate. After addition of TEAB buffer (1 mL, 0.05 M), the mixture was separated on DEAE-Sephadex A-25 column (1 × 15 cm, eluted with 0.3–0.6 M TEAB, pH 7.5, 500 mL). Yield 255 OD_{263} units (64%).

Synthesis of pp5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A)2′p5′(br⁸A). The 8-bromoadenylate pentamer 5′-diphosphate was prepared in the same way as corresponding trimer 5′-diphosphate, starting from 200 OD_{263} units of pentamer 5′-monophosphate, except that Me_2SO instead of DMF was used as solvent. After 24 h the mixture contained 69% of 5′-diphosphate and 23% of 5′-monophosphate (HPLC analysis). The reaction mixture was diluted with 1 mL of 0.05 M TEAB, applied to a DEAE-Sephadex A-25 column (1 × 15 cm, 0.2–0.75 M TEAB, pH 7.5, 400 mL). Yield 110 OD_{263} units (55%).

Registry No. 1, 61-19-8; 2, 23567-96-6; 3, 84877-17-8; 4, 84311-64-8; 5, 84877-20-3; 6, 100899-86-3; 7, 100899-87-4; 8, 100899-88-5; 9, 84824-01-1; 10, 84824-00-0; 11, 100899-89-6; ppp5′A2′p5′A2′p5′A, 65954-93-0; p5′A2-p5′A2′p5′A, 61172-40-5; RNase L, 76774-39-5; 8-bromoadenosine 5′-phosphoroimidazolidate, 100899-90-9; 8-bromoadenylate trimer 5′-phosphoroimidazolidate, 100899-91-0; 8-bromoadenylate pentanamer 5′-phosphoroimidazolidate, 100909-12-4.