Synthesis, Characterization, and Biological Properties of 8-Azido- and 8-Amino-Substituted 2′,5′-Oligoadenylates

Hiroaki Sawai,*[,†] Atushi Hirano,[†,§] Hiroyuki Mori,[†,⊥] Kazuo Shinozuka,[†] Beihua Dong,[‡] and Robert H. Silverman[‡]

Department of Applied Chemistry, Faculty of Engineering, Gunma University, Kiryu, Gunma 376-8515 Japan, and Department of Cancer Biology, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

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A series of 8-azido- and 8-amino-substituted 2′,5′-oligoadenylatyes was prepared by a uranyl-ion catalyzed polymerization of the corresponding 8-substituted adenosine phosphorimidazolide. Subsequent 5′-dephosphorylation of the resulting 5′-phosphoryl 2′,5′-linked oligomers with alkaline phosphatase gave the corresponding core oligomers. The CD spectra indicated that the 8-aminoadenosine analogue of the 2′,5′-linked trimer has an anti-orientation as in naturally occurring 2′,5′-oligoadenylates, while 8-azido-substituted 2′,5′-oligoadenylates have a syn-orientation. The 8-substituted oligomers showed enhanced resistance against digestion by snake venom phosphodiesterase. The 2′,5′-linked 8-azidoadenylate trimer and tetramer displayed strong RNase L binding and activating ability, although the corresponding dimer is devoid of such activities. In contrast, very low or no RNase L binding and activating ability were observed in the 8-aminoadenosine analogue of 2′,5′-oligoadenylates. Results indicate that the bulkiness and ionic character of the 8-substituting group have significant effects on the ability of these analogues to bind and activate RNase L. Furthermore, the orientation of the glycosidic base in the 2−5A analogues may change from syn to anti during binding to RNase L. The 8-azidoadenosine analogues of 2−5A will be useful tools in the photoaffinity labeling of RNase L, due to their strong RNase L binding ability. In addition, these 8-azidoadenosine compounds may be considered as candidates for experimental therapeutic agents because they have enhanced stability to enzyme degradation while retaining the ability to activate RNase L.

Introduction

2′,5′-Oligoadenylates (2−5A) are formed from ATP when viral double-stranded RNA binds to 2−5A synthetase in interferon-treated cells. The 2−5A binds and activates 2−5A dependent RNase (RNase L) which degrades viral mRNA, resulting in an inhibition of the protein synthesis.[1−6] The 2−5A plays an important role in the mechanism of interferon's antiviral activity, but is also involved in other cellular processes. During the past two decades, various 2−5A analogues have been synthesized to study the biological function of 2−5A and its structure−activity relationship and to explore the potential for deriving an antiviral agent based on 2−5A. Several 8-substitued analogues of 2−5A were prepared and their biological activities were evaluated. 2−5A analogues with 8-bromo,[7,8] 8-hydroxy,[9] 8-azido,[10,11] and 8-methyl[12] groups in the 2′-terminal residue have been reported to have strong RNase L binding and activating ability, equal to or stronger than that of naturally occurring 2−5A, while the corresponding 2−5A analogues bearing fully 8-substituted adenosine showed 10- to 100-fold reduction of the biological activities. On the other hand, 2−5A analogues containing an 8-mercapto[13] or 8-hydroxypropyl[9] group have been reported to possess very low or no RNase L binding and activating abilities. The difference in their biological activities was partly explained by the difference in the syn-anti conformation around the base-glycosydic bonds of 2−5A.[8−14] However, the detailed mechanism of 2−5A and its congener to bind and activate RNAse L is still unknown. Among the 8-substituted adenosine 2−5A analogues, 8-azidoadenosine analogue is very interesting, because it has strong biological activities despite its bulky azido group at the 8-position, and it can be useful as a photoaffinity labeling agent for RNase L to study the binding mechanism.[15] Enzymatically, an 8-azido analogue of 2−5A timer is produced from 8-azido ATP by 2−5A synthetase, although the yield and the scale of the enzymatic synthesis are very low.[10] 2−5A trimer bearing 8-azidoadenosine at the 2′-terminal was prepared chemically by a conventional phosphotriester method, in which tedious blocking, coupling, and deprotection are required in each reaction step.[11] Thus, we undertook the preparation of a series of 5′-monophosphorylated 2′,5′-oligo-8-azidoadenylates from the dimer to the tetramer by uranyl-ion catalyzed polymerization of 8-azidoadenosine 5′-phosphorimidazolide in aqueous solution, as the procedure is very easy to perform with high 2′,5′ regioselectivity without using any protecting group. We have also prepared a series of 8-aminoadenosine analogues of 2−5A by the same methodology to examine the effect of an amino group at the 8-position on the biological activity. We have evaluated biological activities of these 2−5A analogues, along with the corresponding core analogues prepared from the correspond-

* To whom correspondence should be addressed. Tel: 81−277−30−1220. Fax: 81−277−30−1224. E-mail: sawai@chem.gunma-u.ac.jp.
† Gunma University.
‡ The Cleveland Clinic Foundation.
§ Present address: Banyu Tsukuba Research Institute, Okubo-3, Tsukuba, Ibaraki, 300-2611 Japan.
⊥ Present address: Fine Chemical Division, J. F. E. Chemical Co., 9-2 Koukan-chou, Kasaoka, Okayama, 714-0063 Japan.

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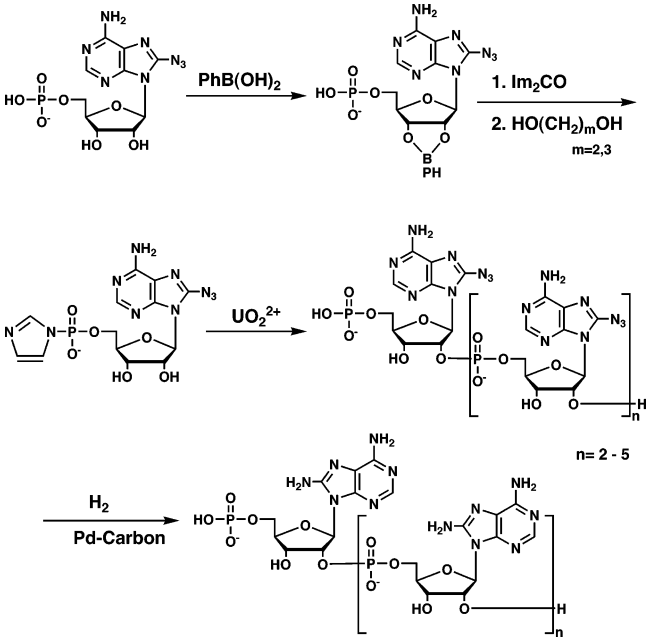
Scheme 1. Synthetic Scheme of 2′,5′-Linked Oligoadenylates with 8-Azido and 8-Amino Groups



ing 5′-phosphorylated analogues with alkaline phosphatase.

Results and Discussion

Synthesis and Characterization of 2′,5′-Oligoadenylate Analogues Bearing an 8-Azido or 8-Amino Group. 8-Aminoadenosine phosphorimidazolide was prepared by the published method from 8-aminoadenosine 5′-phosphate and imidazole using triphenyl phosphine and dipyridyl disulfide as a condensing agent.[16] However, this method could not be applied for the synthesis of 8-azidoadenosine 5′-phosphorimidazolide, because an azido group reacts easily with triphenylphosphine forming triphenylphosphine-nitride. Thus, we employed a carbonyldiimidazole method[17] for the formation of 8-azidoadenosine phosphorimidazolide after transient protection of the 2′,3′-dihydroxy groups of 8-azidoadenosine with phenylboronic acid as shown in Scheme 1. The phenyl boronate ester of 2′,3′-diol was deblocked by treatment with ethylene glycol and 1,3-propandiol in methanol without degrading the phosphorimidazolide bond. We conducted polymerization of 8-azidoadenosine 5′-phosphorimidazolide by an uranyl-ion catalyst in neutral aqueous solution for the synthesis of 2′,5′-oligomers of 8-azidoadenosine, as the reaction proceeds with high 2′,5′ regioselectivity without using any protecting group.[18] Table 1 shows the HPLC yield data of oligo 8-azidoadenylates under several conditions. Preferential formation of 2′,5′-linked oligomers from the dimer to the octamer was observed under the optimum reaction conditions. 2′,5′-Linkage in the resulting oligomers was confirmed by the resistance against nuclease P1 digestion. Polymerization of 8-aminoadenosine 5′-phosphorimidazolide took place in the same way, although yields and chain length of the resulting oligomers were very low as shown in Table 1. The amino group is likely located close to the phosphoryl group and catalyzes the hydrolysis of the phosphorimidazolide bond intramolecularly suppressing the polymerization

Table 1. Synthesis of 8-Azido- and 8-Amino-Substituted Oligoadenylates by Uranyl Ion-Catalyzed Polymerization of ImpA-X[a]

[ImpA-X]/ [uranyl ion]	yield (%)*					
	pA-X	(pA-X)2	(pA-X)3	(pA-X)4	(pA-x)5	(pA-X)6−8
X-N_3						
5	5.6	18.8	23.5	4.8	2.9	1.5
10	4.6	18.6	24.8	7.6	2.7	2.6
20	2.9	23.6	25.9	9.0	2.7	2.1
50	0.6	30.9	22.0	3.9	0.7	0.3
100	4.7	25.8	18.7	2.4	0.2	
X-NH_2						
5	50.6	12.8	6.6	2.1		
10	67.7	6.9	6.0			
50	78.2	2.6	2.9			

[a] Polymerization of ImpA-X (50 mM) was carried out at 20 °C for 24 h in 0. 2 M *N*-ethylmorpholine buffer (pH 7.0). [b] No correction for the hypochromicity of each oligomer was done on the yield.

reaction. 5′-Phosphoryl 2′,5′-oligoadenylates bearing 8-azido group or 8-amino group from the dimer to the tetramer were prepared in a large scale under the optimum reaction condition and purified by DEAE-Sephadex anion exchange column chromatography followed by preparative HPLC or paper chromatography. The corresponding core analogues were obtained by dephosphorylation of the 5′-phosphoryl 2′,5′-oligoadenylates analogues with alkaline phosphatase. The structures of these 2−5A analogues were assigned by ESI-mass and the analyses of enzyme digestion products, as well as NMR when sufficient quantity is available. The results are listed in Tables 2 and 3, with hypochromicity data of the 5′-phosphoryl oligonucleotides.

Figure 1 shows the CD spectra of the 2′,5′-linked trimers of 8-azidoadenosine and 8-aminoadenosine at several temperatures. The CD spectrum of the trimer of 8-aminoadenosine showed positive ellipticity at longer wavelength and negative ellipticity at lower wavelength around 270 nm, indicative of a right-handed helix with an anti-orientation about the base-glycoside bonds as a naturally occurring 2−5A.[14,19] The 8-amino group of adenine interacts with 5′-phosphate in the 2−5A analogue forming anti-conformation. On the contrary, the CD spectrum of the trimers of 8-azidoadenosine showed negative ellipticity at longer wavelength and positive ellipticity at lower wavelength around 280 nm, which indicates that two adjacent 8-azide adenosine moieties are in a strongly right-handed stacking arrangement with a syn-orientation about the base-glycoside bonds.[19] The presence of a bulky 8-azido group in the 2−5A analogues could make syn-conformation as the 8-bromoadenosine analogue of 2−5A.

The stability of 8-azido- and 8-aminoadenosine analogues of 2−5A against snake venom phosphodiesterase (SVPD) and bacterial alkaline phosphatase was assessed using the corresponding 2,′5′-linked dimers. 8-Azido and 8-amino substitution enhanced the stability against SVPD. The half-lives of the intact 2′,5′-linked dimers of 8-azido-, 8-amino-, and the unsubstituted adenosine against SVPD were 330, 37, and 7.5 min, respectively, under the same condition. Thus 8-azido group in the 2−5A markedly enhance the stability against SVPD. The enhancement of stability against phosphodiesterase has been reported in other 8-substituted 2-5A analogues.[8−12] Both 2−5A analogues were dephosphorylated at the same rate as the corresponding

Table 2. ESI-Mass, Characteristic NMR, and Hypochromicity of 8-Azido- and 8-Amino-Substituted 2′,5′-Oligoadenylates

2−5A analogue	ESI-mass in negative mode (calcd) [M − H]*, [M − 2H]/2**, [M − 3H]/3***	NMR H-2	NMR H-1′	hypochromicity (%)
ApA-N_3	676.9 (677.1)*		ND[a]	
pApA-N_3	757.1 (757.1)*, 378.0 (378.1)**	8.05 (s, 1H)	6.09 (d, 0.6 Hz, 1H)	
		8.18 (s, 1H)	5.62 (d, 6.8 Hz, 1H)	13
ApApA-N_3	1047.0 (1047.2)*, 522.9 (523.1)**		ND	
pApApA-N_3	1127.1 (1127.2)*, 563.0 (563.1)**	8.03 (s, 2H)	6.00 (d, 2.2 Hz, 1H)	
		8.16 (s, 1H)	5.74 (d, 3.1 Hz, 1H)	17
			5.59 (d, 6.9 Hz, 1H)	
ApApApA-N_3	708.2 (708.2)**		ND	
pApApApA-N_3	748.1 (748.1)**, 498.6 (498.4)***	8.03 (s, 3H)	6.01 (d, 2.7 Hz, 1H)	21
		8.13 (s, 1H)	5.77 (d, 2.9 Hz, 1H)	
			5.74 (d, 2.6 Hz, 1H)	
			5.62 (d, 6.9 Hz, 1H)	
ApA-NH_2	625.3 (625.2)*		ND	
pApA-NH_2	705.1 (705.1)*, 352.3 (352.1)**	8.05 (s, 1H)	6.20 (d, 6.5 Hz, 1H)	12
		8.16 (s, 1H)	5.85 (d, 6.1 Hz, 1H)	
ApApA-NH_2	969.2 (969.2)*, 484.2 (484.1)**		ND	
pApApA-NH_2	1049.1 (1049.2)*		ND	21
ApApApA-NH_2	1313.5 (1313.3)*, 656.2 (656.2)**		ND	
pApApApA-NH_2	696.2 (696.2)**, 463.8 (463.8)***		ND	24

[a] ND: Not meassured, as the sample quantity is too small.

Table 3. Enzyme Digestion of 8-Azido- and 8-Aminoadenosine Analogues of 2−5A

2−5A analogue (retention time)	BAP digest product (retention time)	SVP digest product (retention time)	NP1 digestion
ApA-N_3		A-N_3 + pA-N_3	no digestion
pApA-N_3 (7.8)	ApA-N_3 (2.8)	pA-N_3 (2.9)	no digestion
ApApA-N_3		A−N_3 + 2pA-N_3	no digestion
pApApA-N_3 (10.7)	ApApA-N_3 (10.0)	pA-N_3 (2.9)	no digestion
ApApApA-N_3		A−N_3 + 3pA-N_3	no digestion
pApApApA-N_3 (13.7)	ApApApA-N_3 (15.8)	pA-N_3 (2.9)	no digestion
ApA-NH_2		A-NH_2 + pA-NH_2	no digestion
pApA-NH_2 (10.8)	ApA-NH_2 (2.8)	pA-NH_2 (4.4)	no digestion
ApApA-NH_2		A-NH_2 + 2 pA-NH_2	no digestion
pApApA-NH_2 (14.8)	ApApA-NH_2 (9.2)	pA-NH_2 (4.4)	no digestion
ApApApA-NH_2		A-NH_2 + 3pA-NH_2	no digestion
pApApApA-NH_2 (20.7)	ApApApA-NH_2 (16.1)	pA-NH_2 (4.4)	no digestion

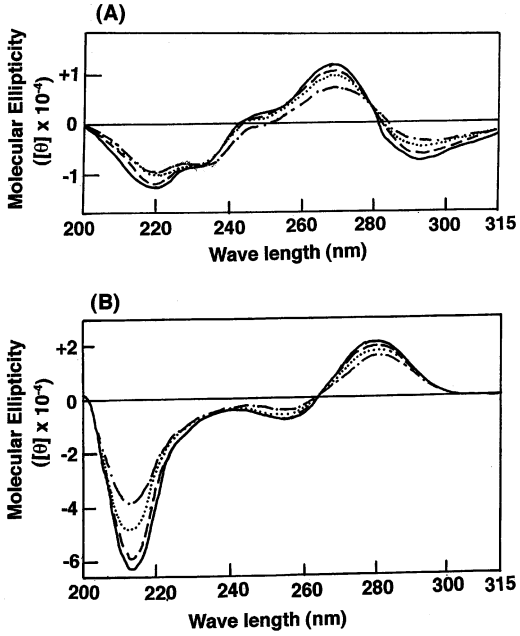


Figure 1. CD spectra of 2′,5′-linked triadenylates with 8-azido and 8-amino groups at 4 —, 22 − − −, 52 ···, and 82 °C −·−. (A) pApApA-N_3 and (B) pApApA-NH_2.

unsubstituted 2−5A by bacterial alkaline phosphatase. The half-lives of the intact 5′-phosphoryl dimers were 5−6 min when 0.04 μmol of the substrate and 0.01 unit of the enzyme were used.

RNase L Binding and Activating Activity of 8-Azidoadenosine and 8- Aminoadenosine Analogues of 2−5A. Activity of the 8-azido- or 8-aminoadenosine analogues of 2−5A to bind with RNAse L was evaluated by the radiobinding assay described previously using ^{125}I-labeled 2−5A analogue as a probe.[20] Mouse liver extract was used as a source of RNase L. The binding ability is expressed as a IC_{50}, a concentration of the 2−5A analogue to inhibit binding of the radiolabeled 2−5A probe by 50%. The results are summarized in Table 4, along with the relative binding ability to the corresponding unmodified 2−5A, pA2′p5′A2′p5′A. The trimer and tetramer of 5′-phosphoryl 8-azidoadenosine analogues of 2−5A were bound to the RNAse L slightly more effectively than the unmodified 2−5A trimer, pA2′p5′A2′p5′A, and the corresponding core analogues had a slightly weaker binding ability. The dimer of 2′,5′-linked 8-azidoadenosine showed very low or no binding ability. On the other hand, 2−5A analogues of 8-aminoadenosine showed very poor binding ability. The tetramers, pApApApA-NH_2 and ApApApA-NH_2, showed over 100-fold decrease in the binding with RNase L. The trimer and dimer of 2′,5′-linked 8-aminoadenosine have no binding ability at 10 μM concentration.

Assay for activation of RNase L with the 2−5A analogues was carried out by a previously described method that uses human recombinant RNase L.[21,22] ^{32}P-Labeled oligoribonucleotide, C_7UUC_{12}[^{32}P]pCp, was used as an

Table 4. Binding Ability of 8-Azido- and 8-Amino-Substituted Analogues of 2−5A to RNase L in Mouse Liver Extract

oligomer	IC_{50} (M)*	relative activity to pApApA
ApA-N_3	$>10^{-5}$	<0.0004
ApApA-N_3	7×10^{-8}	0.6
ApApApA-N_3	$5 \times 10-8$	0.8
pApA-N_3	5×10^{-6}	0.0008
pApApA-N_3	2×10^{-9}	2.0
pApApApA-N_3	2×10^{-9}	2.0
ApA-NH_2	$>10^{-5}$	<0.0004
ApApA-NH_2	$>10^{-5}$	<0.0004
ApApApA-NH_2	1×10^{-6}	0.004
pApA-NH_2	$>10^{-5}$	<0.0004
pApApA-NH_2	$>10^{-5}$	<0.0004
pApApApA-NH_2	5×10^{-7}	0.008
pApApA	4×10^{-9}	1.0
pppApApA	3×10^{-9}	1.3

[a] Binding ability was determined by radiobinding assay. *IC_{50} = concentration required to inhibit binding of the ^{125}I-labeled 2−5A by 50%.

RNA substrate.[23] The resulting RNA product was analyzed after electrophoresis on 20% polyacrylamide/7 M urea gels, and the extent of the degradation was measured with a phosphorimager. 5′-Monophosphoryl 2−5A tetramer, pA2′p5′A2′p5′A2′p5′A, was used as a positive control, as human RNase L can be activated with 2−5A with 5′-monophosphate, contrary to mouse RNase L which requires 2−5A with 5′-di- or triphosphate for its activation.[21] The results of the RNA degradation with the activated RNase L are shown in Figure 2. The cleaved RNA product was C_{12}[^{32}P]pCp, because RNase L cleaves RNA between the UN bond preferentially.[24,25] The 5′-phosphoryl trimer and tetramer 2−5A analogues of 8-azidoadenosine, pApApA-N_3 and pApApApA-N_3, activates RNase L significantly although <10-fold less effectively compared with the unmodified 2−5A tetramer (Figure 2A). The corresponding trimer and tetramer core, pApApA-N_3 and ApApApA-N_3, showed 100- to 1000-fold decrease in activating RNase L. Therefore, the core analogues had low level of RNase L activating ability in contrast to the parent 2−5A core which is devoid of RNase L activating ability. The dimers, pApA-N3 and ApA-N3, at concentrations as high as 1 μM concentration did not activate RNase L. The 8-aminoadenosine 2−5A analogues exhibited no RNase L activating ability, although the 5′-phosphoryl tetramer, pApApApA-NH_2, was an activator of RNase L at 1 μM concentration, a 1000-fold reduction of 2−5A (Figure 2B). The loss of RNase L activating ability was related to a loss of capability to bind to RNase L.

The CD studies suggest that the 8-azidoadensoine ananlogue of 2−5A adopts syn-conformation around the base-glycosidyl bond in the free state in contrast to the unsubstituted 2−5A which prefers anti-conformation.[14] 8-Bromo- or 8-methyladenosine analogues of 2−5A which possess syn-conformation are also effective in the binding and activating of RNase L.[7,8,12] These bulky 8-substitutent groups in the adenosine residue make the syn-conformation; however, they are not sufficient to block the rotation around the base-glycosyl bond of the 2−5A analogues.[26] It is reported that the 8-bromadenosine analogue of NAD changes its conformation from syn in the free-state to anti when it is bound to horse liver alcohol dehydrogenase.[27] The 8-substitued adenosine analogues of 2−5A may alter the conformation similarly when they are bound to RNase L. The conformation of the active form of 2−5A or 2−5A analogues is not clear, and NMR, CD, or X-ray crystallographic studies of the bound state with RNase L are needed for its detailed analysis. 8-Aminoadenosine analogues of 2−5A that occur in the anti-conformation in the free state could not activate RNase L. In contrast, the 8-hydroxyadenosine analogue of 2−5A has been shown to possess strong biological activities.[9] The 8-amino group of the 2−5A analogue likely forms guanidinium type resonance cation in the 8-aminoimidazole ring under the physiological condition and interacts with the negatively charged 5′-phosphoryl group. The large diversities in the RNase L binding and activating of the 8-substituted 2−5A analogues found in this and other previous studies demonstrate that the bulkiness and ionic character of the 8-substituent groups is an important factor for their biological activity.

In conclusion, a series of 8-azido- and 8-aminoadenosine analogues of 2−5A were prepared easily and at reasonable scales by polymerization of the phosphorimidazolide of 8-azidoadenosine in aqueous solution without employing protecting groups. The 8-azidoadenosine analogue of 2−5A will be useful as a photoaffinity labeling agent for the study of 2−5A-RNase L binding and activation after labeling with ^{32}P at the 5′-phosphate with kinase. The 5′-phopshoryl trimer and tetramer analogues of 8-azidoadenosine have significant RNase L binding and activating ability, in contrast to the corresponding 8-aminoadenosine analogues which are devoid of biological activity. Because the 8-azidoadenosine analogues of 2−5A are relatively stable while retaining the ability to activate RNase L, they may also be considered candidates for experimental therapeutic agents.

Experimental Section

Materials and Spectrometric Method. Adenosine-5′-phosphate and nuclease P1 were purchased from Seikagaku Kogyo. Venom phosphodiesterase and alkaline phosphatase were from Worthington Biochemicals, RNase T2 from Sigma, and T4 RNA ligase from Invitrogen. [5′-32P]-pCp (3000 Ci/mmol) was from Perkin-Elmer. DEAE-TOYOPEARL was from Toso Co., and DEAE-Sephadex A-25 from Pharmacia. Other reagents were obtained commercially. *N*-Ethylmorpholine was distilled before use. 8-Bromo-, 8-azido-, and 8-aminoadenosine 5′-monophosphate were prepared by the method described previously.[28]

UV measurements were carried out on a Hitachi 3200 instrument or a Shimazu UV-1200 spectrophotometer. Concentrations of 2′,5′-oligonucleotides were determined spectrophotometrically using the molar coefficients of adenosine-, 8-bromoadenosine-, 8-azidoadenosine-, and 8-aminoadenosine-5′-monophosphate, $\epsilon_{260} = 15.3 \times 10^3$, $\epsilon_{265} = 16.7 \times 10^3$, $\epsilon_{281} = 14.9 \times 10^3$, and $\epsilon_{273} = 16.4 \times 10^3$, respectively, after correcting for the hypochromicity of each oligonucleotide. Hypochromicity of the oligonucleotides was determined by the ratio of absorbance before and after alkaline hydrolysis. CD spectra were recorded with a JASCO J-720 spectrometer in 10 mM phosphate buffer (pH 6.7). The CD results are expressed in terms of molecular ellipticity per mole of nucleotide residue, [θ]. ^{1}H NMR spectra were taken on a Varian XL200 spectrometer with sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]-propionate as an internal standard. ESI-mass spectra were taken with a Perkin-Elmer Sciex API-100 instrument in a negative mode.

HPLC. HPLC was taken with a Hitachi 638 or a JASCO PU880/UV870 apparatus on an RPC-5 or an ODS-silica gel (Wakosil 5C18) column (40 × 250 mm). HPLC on an ODS-

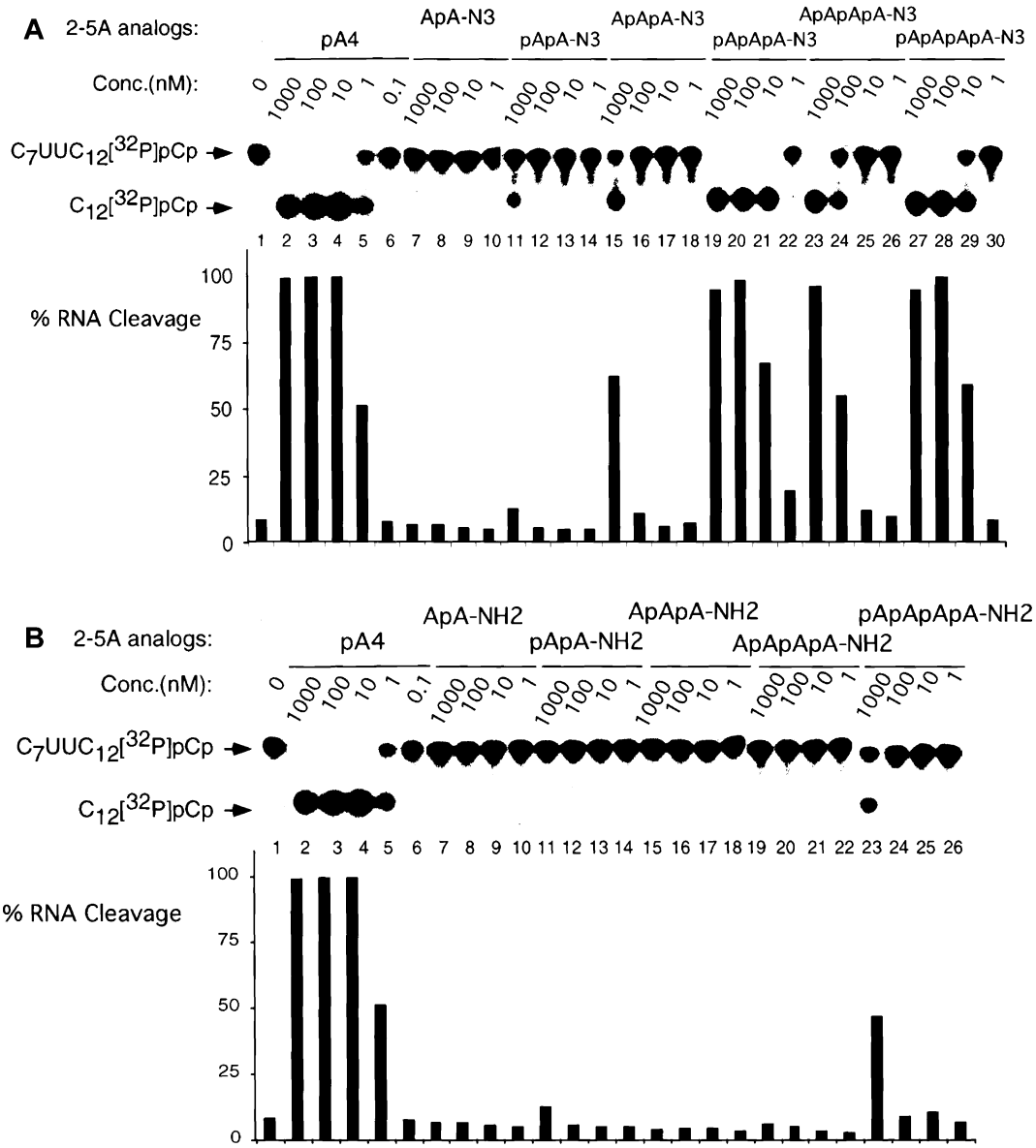


Figure 2. RNase L activating ability of 8-azidoadenosine and 8-aminoadenosine analogues of 2−5A. (A) 8-Azidoadenosine analogues of 2−5A. (B) 8-aminoadenosine analogues of 2−5A. 2−5A and analogues at different concentrations (as indicated) were incubated with human recombinant RNase L on ice for 30 min followed by incubation with $C_7U_2C_{12}$-[^{32}P]pCp at 30 °C for 30 min. An autoradiogram of the dried gel is shown. Arrows indicate the intact and cleaved RNA. The graph represents percent RNA cleavage as determined by phosphorimager analysis.

silica gel was carried out with a linear gradient elution of methanol (2.4−51.2%) buffered with 10 mM triethylammonium acetate (pH 7.3) in 60 min at flow rate of 1.0 mL/min. HPLC on an RPC-5 was conducted with a linear gradient of $NaClO_4$ solution (0−50 mM) buffered with 2 mM Tris-acetate (pH 7.5) and 0.1 mM EDTA in 60 min at a flow rate of 1.0 mL/min. Yields were calculated from the peak integrals of the oligonucleotides on the HPLC chromatogram, and no correction for the hypochromicity of each oligonucleotide was made on the yield. Preparative HPLC was carried out on an ODS-silica gel (10 × 250 mm) with a linear gradient elution of methanol (12−20%) buffered with 10 mM triethylammonium acetate (pH 7.3) in 60 min at flow rate of 2.0 mL/min. Paper chromatography was performed on Whatman 3MM paper by a descending technique using 1-propanol-concentrated ammonia−water (55:10:35).

Preparation of 8-Aminoadenosine-5′-phosphorimidazolide (ImpA-NH_2). 8-Aminoadenosine 5′-phoshorimidazolide was prepared in the same way as adenosine 5′-phosphorimidazolide.[16] 8-Aminoadenosine-5′-phosphate (1.64×10^4 ODU_{273}, 1.0 mmol) was coevaporated with water and then pyridine several times to remove a trace of triethylammonium hydrogen carbonate and water and then dissolved in dry DMF. Imidazole (0.68 g, 10 mmol), triethylamine (1.0 mL), trioctylamine (0.5 mL), triphenylphosphine (0.79 g, 3.0 mmol), and 2,2′-dipyridyl disulfide (0.66 g, 3.0 mmol) were added to the solution. The reaction mixture was stirred for 2 h at room temperature. The completion of the reaction was checked by cellulose-TLC (solvent: 2-propanol−concentrated ammonia−water = 7:2:1). The reaction mixture was poured into a solution of acetone (200 mL)−ether (100 mL)−triethylamine (1 mL)−acetone saturated with $NaClO_4$ (1 mL). The resulting white precipitate was collected in a glass filter with a slow stream of dry nitrogen, washed with dry acetone then dry ether several times, and dried in a desiccator. 8-Aminoadenosine-5′-phosphorimidazolide was obtained in 58% yield (0.95×104 ODU_{273}, 0.58 mmol) as a sodium salt. Purity was checked by TLC and HPLC.

Preparation of 8-Azidoadenosine 5′-phosphorimidazolide (ImpA-N_3). We cannot apply the conventional method of the synthesis of adenosine 5′-phosphorimidazolide as described above, because triphenylphosphine reacts easily with

an azido group of 8-azidoadenosine-5′-phosphate with conversion into the 8-aminoadenosine-5′-phosphate. Thus we employed another method for the 5′-phosphorimidazolide synthesis.[17] After coevaporation of 8-azidoadenosine-5′-phosphate (1.49×10^4 ODU_{281}, 1.0 mmol) with water and then pyridine several times to remove a trace of triethylammonium hydrogen carbonate and water, dry DMF (10 mL), triethylamine (1.0 mL), trioctylamine (0.5 mL), and phenyl boric acid (200 mg, 1.6 mmol) were added and the solution was kept for 30 min at 40 °C for blocking of the 2′,3′-dihydroxyl group of the ribose ring by a boronate ester formation. Then, *N*,*N*-carbonyldiimidazole (648 mg, 4.0 mmol) and imidazole (289 mg, 4.0 mmol) were added to the reaction mixture, and the solution was kept for 2 h with stirring at room temperature. The completion of the reaction was checked by cellulose-TLC (solvent: 2-propanol−concentrated ammonia−water = 7:2:1). After the reaction, a methanol solution (16.6 mL) containing ethylene glycol (0. 5 mL) and 1,3-propandiol (1.0 mL) was added to the solution and stirred for 5 min at room temperature, then overnight at 4 °C to remove the phenyl boronate from the 2′,3′-diol. Methanol was evaporated under reduced pressure, and the mixture was poured into a solution of acetone (200 mL)−ether (100 mL)−triethylamin (1 mL)−acetone saturated with $NaClO_4$ (1 mL). The resulting white precipitate was collected in a glass filter with a slow stream of dry nitrogen, washed with dry acetone and then dry ether several times, and dried in a desiccator. 8-Azidoadenosine-5′-phosphorimidazolide was obtained in 61% yield (0.91×10^4 ODU_{281}, 0.61 mmol) as a sodium salt.

Synthesis of 2′,5′-Linked Oligonucleotides of 8-Azido- and 8-Aminoadenosine by Uranyl Ion-Catalyzed Oligomerization of ImpA-N_3 and ImpA-NH_2. Reactions were carried out in an Eppendorf tube. The reaction mixture was prepared on an ice bath by addition of compounds in the following order: buffer solution, ImpA-N_3 solution in water, and uranyl nitrate solution. Samples of the mixture were agitated vigorously and kept at 25 °C for various periods of times. A typical reaction mixture (50 μL) contained ImpA-N_3 (50 mM) and a catalytic amount of uranyl nitrate (1−10 mM) in *N*-ethylmorpholine−HNO_3 buffer (0.2 M, pH 7.0). The reactions were stopped by adding 10 mL of 0.25 M EDTA solution, and the solutions were analyzed by HPLC on an RPC-5 column. Polymerization of ImpA-NH_2 was carried out in the same way as described above for ImpA-N_3.

Large-scale synthesis of oligomers of 8-azidoadenylate by polymerization of ImpA-N_3 was carried out in a reaction mixture (11.8 mL) containing 50 mM ImpA-N_3 (8790 ODU_{281}), 2.5 mM uranyl nitrate in 0.2 M *N*-ethylmorpholine−HNO_3 buffer (pH 7.0) at 25 °C for 1 d with stirring. The reaction mixture was passed through a Chelex 100 column (Na^+ form, 16 × 150 mm) to remove the uranyl ion and was concentrated under vacuum to 4.2 mL to which acetate buffer (0.2 M, pH 5.5, 4.3 mL) and nuclease P1 solution (0.3 mg in 0.3 mL) were added, and the reaction mixture was kept overnight at 37 °C to degrade 3′,5′ linkage. After confirming formation of a series of 2′,5′-linked 8-azidoadenylate oligomers by HPLC, the solution was subjected to DEAE-TOYOPEARL 650 M anion column chromatography (HCO_3^- form, 30 × 600 mm). The elution was carried out with a linear gradient [0.15 M (3 l) to 1.0 M (3 l)] of triethylammonium hydrogen carbonate buffer (pH7.5) containing 80% methanol at a flow rate of 1.35 mL/min. The fractions containing UV absorption were collected. The first fractions (3150 ODU_{281}) contained 2′,5′-linked oligomers of 8-azidoadenylate from dimer to pentamer. The second fractions (2400 ODU_{281}) contained the oligomers from trimer to heptamer. HPLC analysis showed that the separation of the oligonucleotides was not satisfactory. Thus one-fifth portion of the first fraction and a quarter portion of the second fraction were further separated by preparative HPLC on an ODS-silica gel (10 × 250 mm), each several times. The 2′,5′-linked dimer, trimer, tetramer, and pentamer were isolated in 95, 161, 150, and 64 ODU_{281}, respectively, in a purified form. The purified oligomers were lyophilized several times with water to remove triethylammonium acetate.

Large-scale synthesis of oligomers of 8-aminoadenylates by polymerization of ImpA-NH_2 was carried out in a reaction mixture (6.7 mL) containing 50 mM ImpA-NH_2 (5490 ODU_{273}), 25 mM uranyl nitrate in 0.2 M *N*-ethylmorpholine−HNO_3 buffer (pH 7.0) at 25 °C for 1 d with stirring. The reaction mixture was passed through a Chelex 100 column (Na^+ form, 16 × 150 mm) to remove the uranyl ion and was concentrated under vacuum to 2.0 mL to which acetate buffer (0.2 M, pH 5.5, 4.0 mL) and nuclease P1 solution (0.3 mg in 0.3 mL) were added, and the reaction mixture was kept overnight at 37 °C to degrade 3′,5′ linkage. The solution was subjected to QAE-Sephadez A-25 anion column chromatography (HCO_3^- form, 30 × 800 mm). The elution was carried out with a linear gradient [0.23 M (3 l) to 0.8 M (3 l)] of triethylammonium hydrogen carbonate buffer (pH7.5) at a flow rate of 1.35 mL/min. The fractions containing the dimer and trimer were collected and concentrated. A portion of the dimer and trimer fractions were further purified by paper chromatography on Whatman 3MM paper with *n*-propanol:concentrated ammonia: water (55:10:35) as an eluent using a descending technique. The 2′,5′-linked dimer and trimer were obtained in 150 and 24 ODU_{273}, respectively, in a purified form. The 2′,5′-linked tetramer of 8-aminoadenylate was prepared by hydrogenation of the tetramer of 8-azidoadenylate. The tetramer of 8-azidoadenylate (10 ODU_{281}) was reduced on Pd-black (5 mg) in 0.15 mL of aqueous solution under an atmospheric pressure of hydrogen for 2 d at room temperature. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The product was purified by preparative HPLC on ODS-silica gel and lyophilised several times with water. The tetramer was obtained in 58% yield (6.3 ODU_{273}).

Cores of 2′,5′-linked oligomers of 8-azido- and 8-aminoadenylates with a 5′-hydroxyl group were prepared by dephosphorylation of the 5′-phosphates of the corresponding oligomers (2−10 ODU_{260}) with bacterial alkaline phosphatase in nearly quantitative yield and purified by HPLC on ODS-silica gel.

Characterization of 2′,5′-Linked Oligomers of 8-Azido- and 8-Aminoadenylates. Identification of the products was determined from the ^{1}ESI-mass spectrum and NMR spectrum and by enzyme digestion with snake venom phosphodiesterase, nuclease P1, and alkaline phosphatase.

Enzyme Digestion. Snake venom phosphodiesterase degrades both 2′,5′- and 3′-5′-linked oligonucleotides. Digestion with venom phosphodiesterase was carried out at 37 °C for 2.5 h in a mixture (50 μL) containing the substrate (1−2 ODU_{260}), 0.01−0.5 unit of the enzyme, 0.01 M $MgCl_2$ in 0.02 M Tris-acetate (pH 8.8). An aliquot of the solution was analyzed by HPLC.

Nuclease P1 degrades 3′-5′-linked oligonucleotides but is inactive against the 2′,5′-linked oligonucleotides. The reaction with nuclease P1 digestion was carried out at 37 °C for 1 d in a mixture (50 μL) containing the substrate (1−2 ODU_{260}), 1 mg of the enzyme, 2 mM EDTA, and 1/35 M Veronal-acetate (pH 4.5). The sample was analyzed by HPLC.

Bacterial alkaline phosphatase (BAP) cleaves 5′-phosphate of the oligonucleotides. The reaction with BAP was carried out at 37 °C for 2.5 h in a mixture (50 μL) containing the substrate (1−2 ODU_{260}), 0.01 unit of the enzyme, 0.001 M $MgCl_2$, and 0.01 M Tris-HCl (pH 8.05). The product was analyzed by HPLC on ODS−silica gel.

RNase L Binding and Activating Activities of 2′,5′-Linked Oligomers of 8-Azido- and 8-Aminoadenylates. Binding of the 2′,5′-linked oligomers of 8-azido- and 8-aminoadenylates with RNase L from mouse liver extract was evaluated by radiobinding assay described previously using ^{125}I-labeled 2−5A as a probe.[20] Binding activity of the oliogmer was expressed by IC_{50}, the oligomer concentration which requires 50% inhibition of binding of ^{125}I-labeled 2−5A.

Assay for activation of RNase L was done using ^{32}P-labeled C_7UUC_{12} as an RNA substrate.[23] The synthetic RNA, C_7UUC_{12}, which was prepared on an ABI model 380 DNA synthesizer, was labeled at its 3′-terminus with [5′-^{32}P]pCp (3000 Ci/mmol) with T4 RNA ligase. Different 2−5A analogues at 1 to 1000 nM, compared with 0.1 to 1000 nM of pA4 [pA(2′p5′A)$_3$] as a

positive control, were incubated on ice for 30 min with 0.2 μg/reaction of RNase L expressed from a baculovirus vector in insect cells.[21,22] Reaction mixtures were further incubated with the RNA substrate, 80 nM of $C_7UUC_{12}[^{32}P]pCp$, for 30 min at 30 °C. RNA was analyzed in 20% acrylamide/7 M urea gels, and the extent of degradation was measured with a phosphorimager (Amersham Biosciences).

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