Chemical synthesis and biological activities of analogues of 2',5'-oligoadenylates containing 8-substituted adenosine derivatives

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ABSTRACT
The synthesis of sequence-specific 2'-5' oligonucleotides and analogues of 2'-5' linked oligoadenylates containing 8-substituted adenosine derivatives [8-hydroxypropyladenosine (AHPr) and 8-hydroxyadenosine (A0H)] is reported. The reaction of 5'-phosphorimidazolidate of 8-substituted adenosines under conditions of lead ion catalyst did not give the corresponding 2'-5' oligoadenylates containing pA HPr and pA0H. When these reactions were carried out in the presence of uranyl ion (UO2²⁺) in place of lead ion as a catalyst, the desired 2'-5' oligoadenylates were obtained. The p5'A HPr 2'p5'A HPr 2'p5'A HPr and p5'A OH 2'p5'A OH 2'p5'A OH, p5'A2'p5'A2'pA OH were slightly resistant to snake venom phosphodiesterase. The both circular dichroism and ¹H-NMR spectra studies were used to characterize the modified 2'-5' oligoadenylates. Further, the biological activity evaluations of 8-substituted analogues of 2-5A are also described.

INTRODUCTION
2'-5' Oligoadenylate 5'-triphosphates (2-5A) synthesized from ATP in the presence of dsRNA by 2-5A synthetase and activators of the 2-5A dependent endoribonuclease L (RNase L) are mediators in the antiviral mechanism of interferon [1,2]. The RNase L cleaves viral and cellular mRNA or rRNA, thereby inhibiting protein synthesis [3-6]. This degradation may play a role in the antiviral action of interferon. However, 2'-5' linked oligoadenylates as therapeutic agents introduces some problems, such as the degradation of 2'-5' phosphodiester linkages of 2-5A by an exonucleolytic 2'-5' phosphodiesterase [7-9] and the incorporation into cells by a high negatively charged molecule. With the objective of decreasing, or suppressing completely, the hydrolytic action of an exonucleolytic 2'-5'-phosphodiesterase, many analogues of 2-5A have been already obtained with modified base, ribose or phosphate moieties [10-29]. While these have provided some important information about the enzyme-activator interactions, the cell permeability problem still remains unsolved.

Recently Torrence et al. have reported that the oligo-8-bromoadenylate 5'-di- or 5'-triphosphates possessed about 5% of the protein synthesis inhibitory potency of 2-5A itself, but of great interest was the observation that the corresponding oligo-8-bromoadenylate 5'-monophosphate had 0.2% of the translational inhibitory activity of 2-5A [26,27]. Importantly, under these conditions the 5'-monophosphate in the 2-5A series, i.e. pA'pA'pA had less than 0.001% of the activity of 2-5A. Their findings implied a significant role for conformational factors, particularly glycosidic torsion angle, in determining oligonucleotide-RNase L interaction.

In this paper, we wish to report a rapid chemical synthesis and biological activity evaluations of analogues of 2'-5' linked oligoadenylates containing 8-substituted adenosine derivatives [8-hydroxypropyladenosine (AHPr) (1a) and 8-hydroxyadenosine (A0H) (1b)].

RESULTS AND DISCUSSION
Chemistry: Preparation and characterization of 2'-5' linked oligoadenylates containing 8-substituted adenosine derivatives (4a,c,d)
First, we examined the synthesis of p5'A HPr 2'p5'A HPr 2'p5'A HPr (4a) by polymerization of 8-hydroxypropyladenosine 5'-phosphorimidazolide (2a), using a modification of the lead ion catalysis procedure introduced by Sawai et al [30]. The reaction was carried out by use of imidazole buffer at pH 6.5 in 2 days at room temperature. After treatment with nuclease P1, which hydrolyzes only 3'-5'-internucleotidic bonds, the reaction mixture was separated by means of DEAE-Sephadex

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A-25 column chromatography, and certain components were purified further on HPLC reverse phase ODS column. The yield of 4a was 1.4%. The trinucleotide (4a) was completely resistant to degradation by nuclease P1 or RNase T2, but was completely digested with snake venom phosphodiesterase to give pA\(^{HP}Pr\). Further, in the proton NMR spectrum of this sample (Figure 1b), three sets of the pA\(^{HP}Pr\) proton resonances were observed with the same intensities, indicating that this nucleotide consists of three pA\(^{HP}Pr\) units. As compared with the H2' proton resonance of pA\(^{HP}Pr\) monomer (Figure 1a), one of the three H2' proton resonances of this nucleotide is down-field shifted by 0.3 ppm, while the other two are only slightly shifted and therefore overlapped with each other (Figure 1). Further, the down-field shifted H2' proton resonance shows a \(^1H\)-J\(^3P\) coupling (9.3 Hz), whereas the others do not. This indicates that only one pA\(^{HP}Pr\) unit has a phosphate group at the 2' position which is involved in the 2'-5' linkage. On the other hand, none of the three H3' protons are coupled phosphorus. This suggests that the other two phosphate groups form a 5'-5' pyrophosphate linkage, or one of them is bonded to the hydroxyl group of the 8-hydroxypropyl adenine moiety. Clearly, the unsequential introduction of phosphates on the 2'-hydroxyl functions at 8-hydroxypropyladenosine is unable to be synthesized 2'-5A containing modified adenosine. In order to overcome this problem, we tested various metal ions for the synthesis of 2'-5' linked oligonucleotides containing modified adenosine derivatives and have found that the uranyl ion catalysis procedure introduced by Sawai et al.\[31\] is much more effective as a metal ion for the polymerization of 8-hydroxypropyladenosine than other metal ions.

In order to study the catalytic effect of uranyl ion, polymerization of adenosine 5'-phosphoroimidazolidate (ImpA) (1.0 mmol) (2b) was carried out first in the presence of uranyl ion (0.01 - 0.05 mmol) in N-ethylmorpholine-CH\(_3\)COOH buffer (0.2 M, pH 6.8 and 7.0) for 24 h. Table I shows the effect for the oligoadenylate formation under various of catalyst concentration or pH of the medium. As shown in Table I, high catalyst concentration increased oligomers of the same chain length containing the side product 3'-5' linkages, whereas the N'-ethylmorpholine buffer at pH 6.8 and 7.0 did not change the 2'-5' regioselectivity. Further, we found that UO\(_2\)(OMe)\(_2\) is effective as a catalyst for the polymerization of 8-substituted adenine 5'-phosphorimidazolides than UO\(_2\)(NO\(_3\))\(_2\).

From above results, we chose the conditions of 0.05 mmol of UO\(_2^{2+}\) at pH 7.0 for the synthesis of 2'-5' oligoadenylate trimers containing modified adenosines (A\(^{HP}Pr\), A\(^{OH}Pr\)). ImpA\(^{HP}Pr\) (2a) (0.77 mmol) was treated with UO\(_2^{2+}\) (0.05 mmol) in 0.2M
N-ethylmorpholine-CH₃COOH (pH 7.0) at room temperature for 24 h. After treatment with nuclease P₁, which hydrolyzes only 3'-5' internucleotidic linkages, the reaction mixture was separated by means of DEAE-Sephadex A-25 column chromatography. However, 2-5A trimer 4a containing A₃p₃HPr is unable to be isolated from the reaction mixture. Crude trimer thus obtained was further purified by anion exchange HPLC column. The yield obtained after both purification steps was 18% for 4a.

In similar manner, the UO₂²⁺ ion catalyzes the polymerization of 8-hydroxyadenosine 5'-phosphoroimidazolidate (ImpA(OH)) (2c), giving the trimer, p5'A₃OH2'p5'A₃OH2'p5'A₃OH (4c) (20%).

In support of the assigned structures, compounds 4a-c were all resistant to the action of nuclease P₁ and RNase T₂ but were degraded by snake venom phosphodiesterase. It was also found that the compounds 4a-c were cleaved approximately 7 times more slowly than unmodified 2-5A trimer 4b and this enhanced potency could be related to a greatly increased stability against degradation by enzyme activity.

Additional confirmation of the assigned structures was based on the proton NMR spectra analyses. The proton NMR spectrum

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<th>TABLE I. Oligoadenylate formation catalyzed by uranyl ion under various conditions</th>
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<td><strong>UO₂OAc₂ (mmol)</strong></td>
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<td>UO₂(NO₃)₂⁴⁺</td>
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The reactions were carried out in the presence of ImpA (1.0 mmol) at 20°C in 0.2 M N-ethylmorpholine-acetate buffer.  
a) Reaction was carried out at 20°C in 0.2 M N-ethylmorpholine-HNO₃ buffer.

Figure 2. 65-ms mixing time pure absorption 2D HOHAHA spectrum of p5'A₃HPr2'p5'A₃HPr2'p5'pA₃HPr in ²H₂O at 500 MHz, 25°C. The connection of J network from each sugar moiety is shown from each H1'.
shown in Figure 1c, confirms that there are three pAHP units in this nucleotide. This time, two of the three H2' proton resonances are coupled with 31P and markedly down-field shifted as compared with that of pAHP monomer, whereas the other one is slightly upfield shifted without any 1H-31P coupling. This clearly shows that the three units are connected through two 2'-5' linkages. For each of the three pAHP units, spin-couplings among the ribose protons H1', H2', H3' and H4' were found by two-dimensional homonuclear Hartmann-Hahn spectroscopy (HOHAHA) as shown in Figure 2. Vicinal spin-coupling constants between H1' and H2' protons are 6.1, 5.9 and 6.1 Hz, respectively, and those between H3' and H4' protons are 4.1, 4.5 and 4.1 Hz, respectively, for these units. Analysis of these values as described [32], it was found that the ribose ring of each unit is in a conformation mixture between the C2'-endo form (ca. 40%) and C3'-endo form (ca. 60%).

Nuclear Overhauser effect (NOE) was measured by the two-dimensional NOE spectroscopy (NOESY) as shown in Figure 3. As for the methyl proton resonances of two of the 2-hydroxypropyl groups, strong NOEs were observed with the H1' proton but not with the 2' and 3' protons. Therefore, in these two units, the 2-hydroxypropyl group at position 8 is much closer to the H1' proton than to the 2'/3' protons, indicating that the nucleotide units take the syn form rather than the anti form. Probably, the anti form is extremely unstable because of the steric repulsion between the bulky substituent at position 8 of adenine ring and the phosphate or methylene group at position 5' of the ribose moiety. As compared with these two units, NOE cross peaks are much weaker for the other unit, possibly because of a longer correlation time of this unit [33]. The preference for the syn form due to the bulkiness of the substituent may also be the case for this unit.

Similarly, the trimer, 4c was established by proton NMR spectra analysis (Figure 4a). The two of the three H2' proton resonances are coupled with 31P and markedly down-field shifted, whereas the other one is slightly shifted without any 1H-31P coupling.

Finally, we examined the synthesis of 2-5A trimer bearing 2'-terminal 8-hydroxyadenosine using modification of the procedure reported by Imai et al. [34]. The required 2-5A trimer, p5'A2'p5'A2'p5'AOH (4d) was readily prepared by allowing the dinucleotide-5'-phosphoromorpholidate, Mop5'A2'p5'A (5) prepared from morpholine and p5'A2'p5'OH to react with ImpA (2c) in the presence of U2+ at pH 7.0 for 24 h. After treatment with nuclease P1, which hydrolyzes only 3'-5' internucleotidic linkages, the reaction mixture was separated by means of DEAE-Sephadex A-25 column chromatography. The yield obtained after purification step was 35% for 4d. When this coupling reaction was carried out under a lower pH (6.0), demorpholine products, p5'A2'p5'A or 4d were formed and lead to a further decrease in the yield of desired 2-5A trimer.

The trimer 4d obtained was completely resistant to degradation by either nuclease P1 or RNase T2, but was completely digested with snake venom phosphodiesterase to give pA and pA0H in a 2:1 ratio. However, this compound 4d was also cleaved approximately 5 times more slowly than unmodified 2-5A trimer 4b. The structure assigned to 4d was also supported by proton NMR spectra analysis (Figure 4b).

Circular Dichroism (CD) Spectra of 2-5A trimers (4a-d)

The CD spectra of 2-5A trimers (4a-4d) at several temperatures are presented in Figure 5. We note a number of interesting features in these CD spectra. Uesugi et al. reported 8-substituted purine nucleosides to have a negative band at around 280 nm and two positive bands around 260 and 225 nm in CD [35]. However, the trimer 4a and 4c gave Cotton curves of the different profile. Most importantly significant differences were observed between the CD spectra of 4a,c,d. The Cotton effect amplitudes of 4a,c,d are smaller than the natural 2-5A trimer (4b), which
suggests a decreased stacking. Further, in case of 4a trinucleoside triphosphate, in which three bases are maintained in the syn form and stacked along the left-hand helical axis. On the other hand, in the CD spectrum of 2'-5A trimer (4c), in which base and sugar moieties are fixed in the anti position. Further, the CD absorption spectra of 4b and 4d gave Cotton curves of the same profile. They have a negative band at around 250 nm and two positive bands around 270 and 220 nm. Also, 2'-5A trimers (4a-d) exhibit decreased Cotton effect amplitudes with increasing temperature. The difference of the CD spectra suggests that the 2'-5A analogues have different conformations from 2'-5A. We believe that the conformation of 2'-5A is an important factor for determining the biological activity of the 2'-5A.

Biological studies: Binding of the adenosine-8-substituted analogues to RNase L

As one measure of the ability of the 8-substituted 2'-5A analogues to interact with RNase L, the radiobinding assay developed by Knight et al. [36] was employed to determine the binding assay of the synthetic analogues. This assay for the capacity of a given oligonucleotide to displace the radiolabeled probe p3A[32P]pCp, from a nitrocellulose filter-endonuclease complex. This assay are carried out with the endonuclease of extracts of mouse L929 cells and the results of the assay are presented Figure 6. The results can be summarized in terms of concentrations necessary to inhibit binding of 50% of the applied 32P-probe; that is, the IC50. Under these conditions, the analogues with 8-substituted adenosines, p5'A'OH2'p5'A'OH2'p5'A'OH (4c) and p5'A2'p5'A2'p5'A'OH (4d) were bound to the endonuclease only slightly less effectively than 2'-5A with IC50 values of 3 × 10^-9 and 3 × 10^-9 M, respectively. The analogue which showed the poorest binding was p5'A'HPr2'p5'A'HPr2'p5'A'HPr (4a) with an IC50 of 1 × 10^-4 M.

Capacity of the adenosine-8-substituted analogues to activate RNase L as determined by Ribosomal RNA cleavage

Although more difficult to quantify, the ribosomal RNA cleavage assay of Wrechnner et al. [37] is a useful adjunct to the translational inhibition assay since it gives a measure of the direct action of RNase L. Cell extracts were incubated with or without 2'-5A for 1 h before isolation of the rRNA and subsequent electrophoresis. The results obtained in extracts of present mouse L929 cells are presented in Figure 7. The p5'A'O'O'p5'A'O'O'p5'A'O (4c) and p5'A2'p5'A2'p5'A'O (4d) activated RNase L to cleavage 28S and 18S rRNA to specific
Figure 6. Radiobinding assay of adenosine-8-substituted analogues of 2–5A compared to 2–5A itself. The ability of each oligomer to inhibit the binding of p5A2p5'A2'p5'A (4a) (1 × 10⁻⁶M) did not activate RNase L (Figure 10, lane 2). This lack of hydrolysis is consistent with the inability of the p5'A2'p5'A (4a) (1 × 10⁻⁶M) to activate RNase L in intact cells, after microinjection. From these results, it clear that the p5'A2'p5'A2'p5'A (4a) was clearly antagonistic to effective RNase L binding. It seems likely that the negative effect on RNase L binding of 4a may be related to its conformation. Although the nucleotide conformations such 8-hydroxypropylated 2–5A analogues have been established from 1H-NMR and CD spectra, it is reasonable to expect that such a preference for syn conformation would be maintained in such oligomer.

Finally, note that in the series of 8-hydroxylated analogues, the 5'-monophosphate itself can directly activate RNase L or that the 5'-monophosphate can be phosphorylated in the cell extract to the requisite 5'-di- or 5'-triphosphate. In distinct contrast to adenylate oligomers such as p5'A2'p5'A2'p5'A or p5'A2'p5'A2'p5'A, which show no significant inhibitory activity in this system and which are, in fact, excellent antagonists of 2–5A action [17], the oligo(8-hydroxyadenylate) 5'-monophosphates, p5'A2'p5'A2'p5'A (4c) and p5'A2'p5'A2'p5'A (4d), both possessed substantial activity as inhibitors of protein synthesis. Further, the 2',5'-oligo(8-hydroxyadenylates) were significantly more resistant to degradation than the corresponding 2',5'-oligoadenylates, and that this substitution also may be due to prior phosphorylation to the corresponding 5'-di- or 5'-triphosphate.

It is reasonable to conclude from the work described in this paper that the uranyl ion is likely to be most suitable catalyst for the 2',5'-intermolecular bond formation of 8-substituted adenosine-5'-phosphorimidazolidates. These compounds have the different profiles of CD spectra and the properties such as resistance to degradation by the enzymes. Further, 2',5'-oligo(8-hydroxyadenylates) might, as a 5'-monophosphate, be taken up by a cell either to activate the nucleic directly, or indirectly, after conversion to the corresponding di or triphosphate.

EXPERIMENTAL

Materials

Adenosine 5'-phosphate (free acid) was purchased from Yamasa. 8-hydroxypropyladenosine 5'-phosphate (free acid) and 8-hydroxyadenosine 5'-phosphate (free acid) were prepared according to the published procedures [39,40]. Imidazole, triphenylphosphine and 2,2'-dipyridyl disulfide from Tokyo Kasei were recrystallized before use. N-ethylmorpholine and triethylamine were distilled before use. Uranyl acetate, lead nitrate, and uranyl nitrate were purchased from Kanto Chemical Co. Dowex 50W-X2 and DEAE-Sephadex were Pharmacia Fine Chemicals. Nuclease P1 was from Yamasa, bacterial alkaline phosphatase and snake venom phosphodiesterase were from Boehringer Mannheim Co., and RNase T2 was from Sigma.

TLC analysis

Analytical TLC was done on precoated PEI-Cellulose F plates (Merck) with solvent systems A (0.1 M NH₄HCO₃) or B (0.25 M NH₄HCO₃).

Spectral measurements

The nucleosides were dissolved in H₂O (99.95% H₂; commissariat a l’Energie Atomique). The 400-MHz proton NMR spectra were recorded on a Bruker AM-400 spectrometer at a probe temperature of 25 °C. Chemical shifts were determined relative to the methyl proton resinates of internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Two-dimensional homonuclear Hartmann-Hahn spectroscopy (HOHAHA) [41] was
performed with a trim pulse of 1.5 ms followed by a mixing time of 65 ms. Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) [42] was performed at a mixing time of 100 ms. In two-dimensional NMR measurements, 512 free induction decays (32 or 48 scans in total for each free induction decay) of 2K data points were accumulated in the phase sensitive mode using time-proportional phase increment [42], and the spectra of 2K×1K data points were obtained with zero-filling prior to Fourier transformation. UV spectra were recorded with a Shimadzu UV-160 spectrophotometer. CD spectra were measured by a Jasco J-600 spectrometer using a 2-mm cell in 0.1 M phosphate buffer (pH 7.0).

High-performance liquid chromatography

HPLC was carried out with Shimazu LC-6A pumps controlled by a Model SCL-6A solvent programmer. Reverse phase chromatography was performed with a Inertil ODS column (46×250 mm) (flow rate 1 ml/min). The column was eluted with a linear gradient of MeOH-H2O (1:1, v/v) (0—50%) in 50 mM ammonium phosphate (pH 7.0) in 25 min. Anion exchange chromatography was performed with a TSKgel DEAE-25W column (46×250 mm) (flow rate 1 ml/min). The column was eluted with a linear gradient of 0.1—1.5 M ammonium formate (pH 6.8) in 20% CH3CN in 40 min.

Enzymatic digestion of the various trimers was carried out with the following enzymes under the indicated conditions

Digestion with snake venom phosphodiesterase was carried out by incubation of trimer (1.0 OD unit) in a solution (100 μl) of 0.01 M Tris-acetate (pH 8.8), 0.01 M MgCl2, and 0.2 unit of enzyme for 0.25—2h at 37°C. The ratio of the resulting mononucleotides was determined by HPLC, which permits one to estimate the chain length. The HPLC of the mononucleotides was performed on an anion exchange column (TSKgel DEAE-25W) with a linear gradient of 0.1—0.5 M ammonium formate (pH 6.8) in 20% CH3CN in 30 min or a reverse phase column (TSKoligo-DNA RP) with 0.1 M triethylammonium acetate (pH 7.0) in 10% CH3CN in 30 min.

Digestion with nuclease P1 was carried out at 37°C for 2.5 h in a mixture (50 μl) containing the trimer (1.0 OD unit), 0.02 M ammonium acetate buffer (pH 5.75) and an enzyme solution (2.5 μg in 150 μl).

Preparation of nucleoside 5'-phosphorimidazolodinitriles (2a–c)
Nucleoside 5'-phosphate (1a-c, free acid, 2.0 mmol) and Et3N (1.8 ml) were dissolved in a mixture of dry DMSO (3.0 ml) and dry DMF (0.5 ml). Imidazole-triphenylphosphine (1.572 g, 6 mmol) and 2,2'-dipyridyl disulfide (1.320 g, 6 mmol) were added successively to this solution, and the reaction mixture was stirred for 4 h at room temperature. After that time, TLC (solvent system A) indicated that reaction was completed. The mixture was poured into 0.1 M sodium iodide solution in acetone (150 ml) with stirring. The white precipitate was collected by centrifugation and washed several times with fresh acetone until the yellow color was gone. After drying over P2O5, the sodium salt of nucleoside 5'-phosphorimidazolodinitriles (2a-c) were obtained in 85—90% yields.

Polymerization of nucleoside 5'-phosphorimidazolodinitriles (2a–c)

The nucleoside 5'-phosphorimidazolodinitriles (2a–c, 1.0 mmol) were added to a mixture of catalytic amount of lead nitrate (0.05 mmol) or uranyl acetate (0.01—0.05 mmol) dissolved in 0.2 M N-ethylmorpholine-CH3COOH buffer (pH 6.8 and 7.0) or imidazole-HNO3 buffer (pH 6.5) on an ice bath. The mixture were stirred and kept at 20°C for various periods of time. After the reactions were quenched with 0.5 M EDTA, samples were analyzed by high performance liquid chromatography (HPLC) (Table I).

Metal ions were removed by treatment with Dowex 50W-X2, Na+ form, 5 ml of wet resin. The resin was filtered off and washed with water (2×30 ml). The solution was concentrated to about 20 ml and ethanol (200 ml) was added in order to precipitate dimer and higher oligomers. The ethanol-insoluble material was dissolved in 0.02 M ammonium acetate (pH 5.75, 15 ml) and incubated for 12 h at 37°C with nuclease P1 (2.5 mg/ml, 150 μl) in order to digest any 3'-5' linked isomers. The enzyme was denatured by shaking with a mixture of CHCl3 and isoamyl alcohol (7:3, v/v, 20 ml). The water layer was washed with ether (3×20 ml) and the 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 (1.5× 35 cm, HCO3− form). Elution was with a linear gradient of 0—0.8 M triethylammonium bicarbonate (TEAB) buffer, pH 7.5. The fractions containing trimers (4a,c) were further purified by means of HPLC. The yields were as follows: p5'AHPr2'p5'AHPr2'pA (4a) (6440 A260, 18%); p5'AHPr2'p5'AOH2'pA (4c) (5600 A280, 20%). Each oligomer was homogenous by TLC (system A and B) and by HPLC.

Uranyl ion catalyzed coupling of dinucleotide 5'-phosphoromorpholodinitrile (5) and nucleoside 5'-phosphorimidazolodinitrile (2c)

Dinucleotide 5'-phosphate, pApA (free acid, 431 mg, 0.6 mmol) was dissolved in a mixture of dry DMSO (0.8 ml) and dry DMF (2.0 ml). 2,2'-Dipyridyl disulfide (229 mg, 1.0 mmol), morpholine (0.2 ml, 2.0 mmol), and triphenylphosphine (262 mg, 1.0 mmol) were added to this solution, and the reaction mixture was stirred at room temperature for 3 h. The mixture was poured into 0.1 M sodium iodide solution in acetone (20ml) with stirring. The white precipitate was collected by centrifugation and washed several times with fresh acetone until the yellow color was gone. After drying over P2O5, the sodium salt of nucleoside 5'-phosphoromorpholodinitrile, MopApA (5) was obtained in 83% (395 mg, 0.5 mmol) yields.

MopApA (318 mg, 0.4 mmol as sodium salt) and ImpA(44 mg, 0.1 mmol as sodium salt) were added to a mixture of 0.2 M N-ethylmorpholine-CH3COOH buffer (pH 7.0, 4 ml) containing uranyl acetate (0.001 mmol) on an ice bath. The mixture was stirred and kept at 20°C for 6 h. Metal ions were removed by treatment with Dowex 50W-X2 (Na+ form, 1 ml of wet resin). The resin was filtered off and washed with water (2×30 ml). The solution was concentrated to about 20 ml and ethanol (200 ml) was added in order to precipitate dimer and higher oligomers. The ethanol-insoluble material was dissolved in 0.02 M ammonium acetate (pH 5.75, 8 ml) and incubated for 12 h at 37°C with nuclease P1 (2.5 mg/ml, 80 μl) in order to digest any 3'-5' linked isomers. The enzyme was denatured by shaking with a mixture of CHCl3 and isoamyl alcohol (7:3, v/v, 20 ml). The water layer was washed with ether (3×20 ml) and the concentrated to a oil. The residue was dissolved in 10% AcOH. The pH of the aqueous solution was adjusted to 4.0 and the mixture was incubated at 37°C for 5 h. After the completion of the hydrolysis was assured by TLC (solvent system A),
solution was neutralized with Et₃N and evaporated in vacuo. The residue was dissolved in H₂O (1 ml) and applied to a DEAE Sephadex A-25 column (1.85 x 34 cm, HCO₃⁻ form). Elution was with a linear gradient of 0–0.35 M triethylammonium bicarbonate (TEAB) buffer, pH 7.5. The appropriate fractions were collected and concentrated in vacuo. The desired product, p5′A2′p5′A2′p5′A (4d) was isolated in 35% yield as triethylammonium salt (983 A₂₆₀ unit, 35 μmol, 35%).

**CD spectra measurements**

The CD spectra of oligomers were measured on a JASCO J-600 spectropolarimeter in 0.1 M phosphate buffer (pH 7.0) (Figure 8).

**Radioiodination assays**

Radioiodination assays were performed according to Knight et al. [36] with p3A₄[3²P]pCp of specific activity 3000 Ci/mmol (Amersham) and L929 cell extracts as the source of Rnase L.

**Ribosomal RNA cleavage assays**

The total RNA was extracted, denatured, and analyzed by ribosomal RNA cleavage assays performed according to procedure described (Kariko and Ludwing) [43] and incubated for 1 h at 30°C in the presence of 2′-5′A or 2′-5′A analogues. The rRNA cleavage assays were performed according to procedure described by Wreschner et al. [37]. Extracts of L929 cells were prepared as described (Kariko and Ludwing) [43] and incubated for 1 h at 30°C in the presence of 2′-5′A or 2′-5′A analogues. The total RNA was extracted, denatured, and analyzed by electrophoresis on 1.8% agarose gels. The gels were stained with ethidium bromide and the RNA bands visualized under ultraviolet light.

Radioactive measurement was determined on a Beckman LS-100C liquid scintillation spectrometer with counting efficiencies of 99%, 90%, and 50% for ³²P.

**REFERENCES**