Nucleic Acids Research, Vol. 19, No. 15 4103–4108

8-Methyladenosine-substituted analogues of 2-5A: synthesis and their biological activities

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Received May 24, 1991; Revised and Accepted July 9, 1991

ABSTRACT

8-Methyladenosine-substituted analogues of 2-5A, p5'A2'p5'A2'p5'(me^{8}A), p5'A2'p5'(me^{8}A)2'p5'(me^{8}A), p5'(me^{8}A)2'p5'(me^{8}A)2'p5'(me^{8}A), and p5'(me^{8}A) 2'p5'A2'p5'A, were prepared *via* a modification of a lead ion-catalyzed ligation reaction. These 2-5A monophosphates were converted into the corresponding 5'-triphosphates. Substitution of an 8-methyladenosine residue at the third position (2'-terminus) of the oligonucleotides increased the stability to snake venom phosphodiesterase digestion. Both binding and activation of mouse liver 2-5A dependent ribonuclease (RNase L) by the various 8-methyladenosine-substituted 2-5A analogues were examined. Among the 8-methyladenosine-substituted trimer analogues, the analogues with 8-methyladenosine residing in the 2'-terminal position showed the strongest binding affinity and were several times more effective than 2-5A itself as an inhibitor of translation.

INTRODUCTION

A variety of base-substituted analogues of 2-5A (1–4) have been proven valuable in determining the structural and conformational factors that govern the interaction of the 2-5A-dependent endonuclease, RNase L, with its activator 2-5A (ppp5'A2'p5'A2'p5'A). For instance, 2',5'-oligonucleotides in which all adenosines were replaced by either uridine, cytidine or inosine showed the vital role of one or more of the adenosine nucleoside components of 2-5A (5). Sequence-specific 2-5A analogues, in which each adenosine was specifically replaced in turn by inosine, provided evidence that the N6-amino/N1 functionality of the adenosine at the 5'-terminus of 2-5A was critical for endonuclease binding, while the middle adenosine of trimeric 2-5A played a minor role in binding or activation, and the N6-amino/N1 grouping of the third adenosine was not critical for effective binding but was a requirement for activation of RNase L (6). In another example, 8-bromoadenosine analogues of 2-5A (7,8) not only suggested a relationship between oligonucleotide conformation and nuclease binding and activation (9), but also provided a powerful photoaffinity labelling reagent for RNase L (10). Herein we describe the synthesis and properties of another modification to the 2-5A structure, 8-methyladenosine (me^{8}A). This nucleoside introduction to the 2-5A molecule is of interest since it can, due to the 8-methyl substituent, force the nucleoside to adopt a *syn* orientation about the base-sugar glycoside bond. It is, however, a less electronically perturbing substituent introduction than the more electronegative bromine atom.

EXPERIMENTAL

Snake venom phosphodiesterase was a product of Cooper Biomedical, alkaline phosphatase from Pharmacia, nuclease P1 from Yamasa Shoyu Co., Ltd. The 270 MHz proton NMR spectra were recorded on a JEOL JNX-270 spectrometer using HDO as an internal standard. Ms were taken on a JEOL JMS-D300 machine operating at 70 eV. All UV measurements were carried out on a Shimadzu UV-260 spectrophotometer. High-performance liquid chromatography was executed with a Shimadzu LC-6A 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. Column chromatographic separation was accomplished by using silica gel (Wakogel C-200).

Normal-pressure ion-exchange column chromatography on DEAE-Sephadex A-25 was carried on at 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.

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Biochemical assays to evaluate the synthetic oligonucleotides have been described in detail elsewhere (15, 17, 19, 20).

8-Methyladenosine. A mixture of 1.038 (3 mmol) of 8-bromoadenosine and a catalytic amount of ammonium sulfate in 30 mL of hexamethyldisilazane was heated at 100°C for 8 h. A remaining insoluble material was filtrated off and washed with chloroform. The combined mixture was evaporated *in vacuo* and the residue was dissolved in 40 mL of dry tetrahydrofuran (THF) in the presence of 200 mg of tetrakistriphenylphosphine palladium (0) under argon atmosphere. The mixture was refluxed for 5 min and then 6 mL of trimethylaluminum (1 M solution in n-hexane) was added dropwise. The reaction mixture was refluxed overnight and poured into 50 mL of water. The solution was extracted with chloroform and the resulting emulsion was filtrated thoroughly a layer of Celite-sand, and the filter aid was washed with chloroform. The organic layer was separated and evaporated under diminished pressure. The oily residue was dissolved in a mixture of methanol and ammonium chloride solution (0.802 g dissolved in 5 mL of water) and refluxed for 3 h. The solvent was removed under reduced pressure and the residue was chromatographed on a silica gel column with chloroform−methanol (10: 1). The appropriate fractions were collected and the solvent was removed under reduced pressure to give 8-methyladenosine. Recrystallization from water gave analytically pure 8-methyladenosine (11) (0.639 g, 62%), mp 207−209°C. MS m/z: 281 (M^+). ^{1}H nmr δ [$(CD_3)_2SO$] 8.08 (1H, s, H-2), 7.26 (2H, br, NH_2), 5.91 (1H, m, OH), 5.80 (1H, d, J = 7.3 Hz, H-1′), 5.40 (1H, d, OH), 5.25 (1H, d, OH), 4.86 (1H, dd, J = 12.4 and 7.3 Hz, H-2′), 4.18 (1H, br, H-3′), 4.02 (1H, br, H-4′), 3.73−3.53 (2H, m, H-5′), 2.57 (3H, s, CH_3).

8-Methyladenosine 5′-Monophosphate. A mixture of 8-methyladenosine (84.4 mg, 0.3 mmol) in trimethyl phosphate (900 μL) and water (38 μL) was allowed to stand for 10 min. Phosphorus oxychloride (458 μL) was added dropwise to the mixture and stored at a refrigerator overnight. The mixture was poured into ice-cold water (30 mL) and extracted with ether (30 mL×3). The water-layer was neutralized with ammonium hydroxide. The solution was diluted with water to 300 mL and applied to a DEAE-Sephadex A-25 column (16×20 cm) preequilibrated with water. Elution was with a linear gradient of 0.0 M (1000 mL) to 0.3 M (1000 mL) triethylammonium bicarbonate (pH 7.6). Appropriate fractions were collected and concentrated *in vacuo*, and water was added to and evaporated from the residue to remove residual triethylammonium bicarbonate. The desired product was obtained as the triethylammonium salt (4200 OD_{260}, 90%). ^{1}H nmr (D_2O) δ 8.04 (1H, s, H-2), 5.87 (1H, br, H-1′), 4.88 (1H, br, H-2′), 4.36 (1H, br, H-3′), 4.11 (1H, br, H-4′), 3.98 (2H, m, H-5′), 2.52 (3H, s, CH_3).

Preparation of 5′-Phosphoromorpholidates and 5′-Phosphoroimidazolidates. Typical Method — 8-Methyladenosine 5′-phosphoromorpholidate. Triphenylphosphine (260 mg, 1.0 mmol) and morpholine (0.45 ml, 5 mmol) were added to a mixture of 8-methyladenosine 5′-monophosphate (0.2 mmol, 3080 OD_{260}, triethylammonium salt) in DMF (2 mL) and DMSO (0.5 mL). The mixture was stirred for 5 min and 2,2′-dipyridyldisulfide (220 mg, 1 mmol) was added to the mixture. The mixture was stirred for 2 h. The whole mixture was poured into 0.1 M sodium iodide solution in acetone (10 mL) with stirring. The resulting precipitate was collected by centrifugation and washed several times with fresh acetone until the yellow color was gone. After drying at room temperature for 1 h under vacuum, the sodium salt, Mop5′(me^{8}A), was used directly in the next step of the synthesis. Other nucleotide 5′-phosphoromorpholidates and 5′-phosphoroimidazolidates were prepared in a similar manner. The yields were as follows: Mop5′(me^{8}A), 80%; Im5′(me^{8}A), 92%; Imp5′(me^{8}A)2′p5′(me^{8}A) 80%.

Preparation of p5′(me^{8}A)2′p5′(me^{8}A) and p5′(me^{8}A)2′p5′(me^{8}A)2′p5′(me^{8}A). $Pb(NO_3)_2$ (0.25 M, 150 mL) was added to a mixture of Imp5′(me^{8}A) (132.3 mg, 0.3 mmol as sodium salt) in 1-methylimidazole buffer (0.2 M, 2 mL, pH 7.5) at 4°C. After 10 h of stirring at 4°C, Chelex 100 (NH_4^+ form, 3 mL) was added to the turbid reaction mixture which gradually became clear. The Chelex was removed by filtration, and the filtrate was acidified with acetic acid. The mixture was incubated at 37°C for 24 h and the solvent was removed under reduced pressure. The residue was dissolved in water (35 mL) and the pH of the solution was adjusted to 5.75 with acetic acid. Nuclease P_1 (100 unit) was added to the mixture and incubated at 37°C for 24 h. The solution was treated at boiling water temperature for 5 min and diluted with water to 300 ml and applied to a DEAE-Sephadex A-25 column (1.6×20 cm), preequilibrated with water. Elution was with a linear gradient of 0.0 M (1000 mL) to 0.5 M (1000 mL) triethylammonium bicarbonate (pH 7.6). Appropriate fractions were pooled and concentrated *in vacuo*, and water was added to and evaporated from the resulting residue to remove residual triethylammonium bicarbonate. The desired product, p5′(me^{8}A)2′p5′(me^{8}A), was isolated as the triethylammonium salt (630 OD_{260}). The trimer, p5′(me^{8}A)2′p5′(me^{8}A)2′p5′(me^{8}A), was obtained as the triethylammonium salt (176 OD_{260}, 4%).

Additional preparations. The dimer and trimer were obtained in 30% and 9%, respectively, by the reaction of Mop(me^{8}A) and Imp(me^{8}A) under the analogous conditions. The trimer was also isolated in 13% from Mop(me^{8}A)p(me^{8}A) and Imp(me^{8}A).

Preparation of p5′A2′p5′A2′p5′(me^{8}A). $Pb(NO_3)_2$ (0.25 M, 150 mL) was added to a mixture of MopApA (2400 OD_{260}, 0.09 mmol) and Imp(me^{8}A) (2400 OD_{260}, 0.16 mmol) in 1-methylimidazole buffer (0.2 M, 2 mL, pH 7.5) at 4°C. After 10 h of stirring at 4°C, Chelex 100 (NH_4^+ form, 3 mL) was added to the turbid reaction mixture which gradually became clear. The Chelex was removed by filtration, and the filtrate was acidified with acetic acid. The mixture was incubated at 37°C for 24 h and the solvent was removed under reduced pressure. The residue was dissolved in water (35 mL) and the pH of the solution was adjusted to 5.75 with acetic acid. Nuclease P_1 (100 unit) was added to the mixture and incubated at 37°C for 24 h. The solution was treated with boiling water for 5 min and diluted with water to 300 mL and applied to a DEAE-Sephadex A-25 column (1.6×20 cm), preequilibrated with water. Elution was with a linear gradient of 0.0 M (600 mL) to 0.5 M (600 mL) triethylammonium bicarbonate (pH 7.6). Appropriate fractions were pooled and concentrated *in vacuo*, and water was added to and evaporated from the resulting residue to remove residual triethylammonium bicarbonate. The desired product, pApAp(me^{8}A), was isolated as the triethylammonium salt (220 OD_{260}, 9%).

Preparation of p5'(me^{8}A)2'p5'A2'p5'A. $Pb(NO_3)_2$ (0.25 M, 150 mL) was added to a mixture of Mop5'(me^{8}A) (1600 OD_{260}, 0.1 mmol) and ImpApA (1700 OD_{260}, 0.065 mmol) in 1-methylimidazole buffer (0.2 M, 2 mL, pH 7.5) at 4°C. After 10 h of stirring at 4°C, Chelex 100 (NH_4^+ form, 3 mL) was added to the turbid reaction mixture which gradually became clear. The Chelex was removed by filtration, and the filtrate was acidified with acetic acid. The mixture was incubated at 37°C for 24 h and the solvent was removed under reduced pressure. The residue was dissolved in water (35 mL) and the pH of the solution was adjusted to 5.75 with acetic acid. Nuclease P_1 (100 unit) was added to the mixture and incubated at 37°C for 24 h. The solution was treated with boiling water for 5 min, and diluted with water to 300 ml, applied to a DEAE-Sephadex A-25 column (1.6×20 cm), preequilibrated with water. Elution was with a linear gradient of 0.0 M (600 mL) to 0.5 M (600 mL) triethylammonium bicarbonate (pH 7.6). Appropriate fractions were pooled and concentrated *in vacuo*, and water was added to and evaporated from the resulting residue to remove residual triethylammonium bicarbonate. The desired product, p(me^{8}A)pApA, was isolated as the triethylammonium salt (350 OD_{260}, 20%).

Preparation of p5'A2'p5'(me^{8}A)2'p5'(me^{8}A). $Pb(NO_3)_2$ (0.25 M, 150 mL) was added to a mixture of MopA (500 OD_{260}, 0.03 mmol) and Imp5'(me^{8}A)2'p5'(me^{8}A) (700 OD_{260}, 0.027 mmol) in 1-methylimidazole buffer (0.2 M, 2 mL, pH 7.5) at 4°C. After 10 h of stirring at 4°C, Chelex 100 (NH_4^+ form, 3 mL) was added to the turbid reaction mixture which gradually became clear. The Chelex was removed by filtration, and the filtrate was acidified with acetic acid. The mixture was incubated at 37°C for 24 h and the solvent was removed under reduced pressure. The residue was dissolved in water (35 mL) and the pH of the solution was adjusted to 5.75 with acetic acid. Nuclease P_1 (100 unit) was added to the mixture and incubated at 37°C for 24 h. The solution was treated with boiling water for 5 min and diluted with water to 300 ml and applied to a DEAE-Sephadex A-25 column (1.6×20 cm), preequilibrated with water. Elution was with a linear gradient of 0.0 M (600 mL) to 0.5 M (600 mL) triethylammonium bicarbonate (pH 7.6). Appropriate fractions were pooled and concentrated *in vacuo*, and water was added to and evaporated from the resulting residue to remove residual triethylammonium bicarbonate. The desired product, p5'A2'p5'(me^{8}A)2'p5'(me^{8}A), was isolated as the triethylammonium salt (84 OD_{260}, 17%).

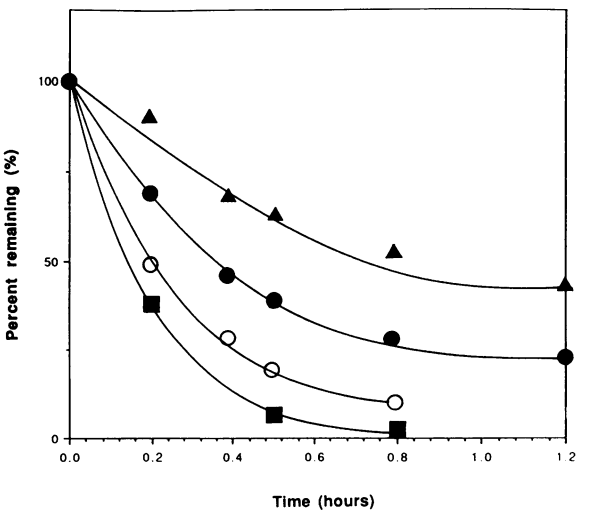
Preparation of 8-methyladenosine-substituted 2-5A 5'-triphosphates. All 2-5A 5'-monophosphates were converted to the corresponding 5'-triphosphates using following method described earlier. For example, p5'A2'p5'A2'p5'(me^{8}A) (60 OD_{260}, 1.71 mmol, triethylammonium salt) was converted to the imidazole derivatives, Imp5'A2'p5'A2'p5'(me^{8}A), by reaction with N,N'-carbonyldiimidazole (1.88 g, 8.55 mmol). The mixture was treated with methanol (10 mL) for 30 min, and tri-n-butylammonium pyrophosphate in DMF (1 mL, 0.5 M) was added to the mixture, which reaction mixture was kept at room temperature for 20 h. After dilution with H_2O (100 mL) the solution was applied to DEAE-Sephadex A-25 (1×20 cm) and eluted with a linear gradient of 0.0 M (300 mL) to 0.7 M (300 mL) triethylammonium bicarbonate (pH 7.6). The final 5'-triphosphate derivatives were isolated after addition and evaporation of water. The yields were as follows: pppA2'p5'A2'p5'(me^{8}A), 53%; pppA2'p5'(me^{8}A)2'p5'(me^{8}A), 62%; ppp(me^{8}A)2'p5'(me^{8}A)2'p5'(me^{8}A), 30%; ppp(me^{8}A) 2'p5'A2'p5'A, 23%.

Enzymatic and Chemical Stabilities. Kinetics of snake venom phosphodiesterase showed that introduction of 8-methyladenosine residue at the third position (2'-terminal) of the oligonucleotides such as p5'A2'p5'A2'p5'(me^{8}A) and p5'(me^{8}A)2'p5'(me^{8}A) 2'p5'(me^{8}A) increased their stability to SVP digestion. The 5'-monophosphate p5'(me^{8}A)2'p5'A2'p5'A, however, was more quickly degraded than 2-5A 5'-monophosphate itself. Under SVP digestion assay conditions, and at an initial concentration of 0.86×10^{-6} M, the half-life times of the analogues were as follows: pApApA, 0.2 h; pApAp(me^{8}A), 0.33 h; p(me^{8}A) p(me^{8}A)p(me^{8}A), 0.94 h; p(me^{8}A)pApA, 0.11 h (Figure 1). However, when the alkaline hydrolysis of the analogues were examined, no difference of the half-times was observed (data not shown).

Enzymatic and Chemical Degradation. 5'-Dephosphorylation was effected *via* alkaline phosphatase exposure using 0.4 OD_{260} of substrate and 0.06 unit of enzyme in Tris-acetate (0.2 M, pH 8.8), $MgCl_2$ (0.001 M), and a total volume of 100 μL. Incubation was 37°C for 30 min. Snake venom phosphodiesterase degradation was performed with 0.4 OD_{260} of substrate and 0.02 unit of enzyme in Tris-acetate (0.01 M, pH 8.8), $MgCl_2$ (0.001 M), in a total volume of 100 μl, with incubation at 37°C for 1 h. The digested products were identified by comparison with authentic materials. Ratios of nucleotide and/or nucleoside products were determined *via* integration of the chromatogram and correction for difference in ε values. Table 2 provides the results of such experiments as well as HPLC systems employed.

Chemical characterization was performed with 0.3 OD_{260} of substrate in 0.1N NaOH solution (100 μL), with incubation at 37°C for 12 h. Table 3 shows the results of such experiments.

Figure 1. Stability of Methylated 2',5'-Oligoadenylates against SVP



p5'A2'p5'A2'p5'A, ○: p5'(me^{8}A)2'p5'(me^{8}A)2'p5'(me^{8}A), ▲: p5'A2'p5'A2'p5'(me^{8}A), ●: p5'(me^{8}A)2'p5'A2'p5'A, ■.

RESULTS

Chemistry

8-Methyladenosine (11) was obtained with ease by reaction of 8-bromoadenosine with trimethylaluminum in the presence of palladium catalyst according to a newly developed methodology (12). Using previously published procedures (13), four 2',5'-linked oligonucleotides could be generated; namely p5'(me^{8}A)2'p5'A2'p5'A, p5'A2'p5'A2'p5'(me^{8}A), p5'A2'p5'(me^{8}A)2'p5'(me^{8}A), and p5'(me^{8}A)2'p5'(me^{8}A)2'p5'(me^{8}A) (Scheme 1). The assigned structures were confirmed by degradation methods as well as NMR (Table 1, 2 and 3). These trimer 5'-monophosphates were further phosphorylated to the corresponding 5'-triphosphates by reaction with pyrophosphate using N,N'-carbonyldiimidazole as a condensing agent (14). Due to paucitTy of materials, the structures of these 2-5A analogs were assigned only by the analyses of enzyme digestion products (Table 2).

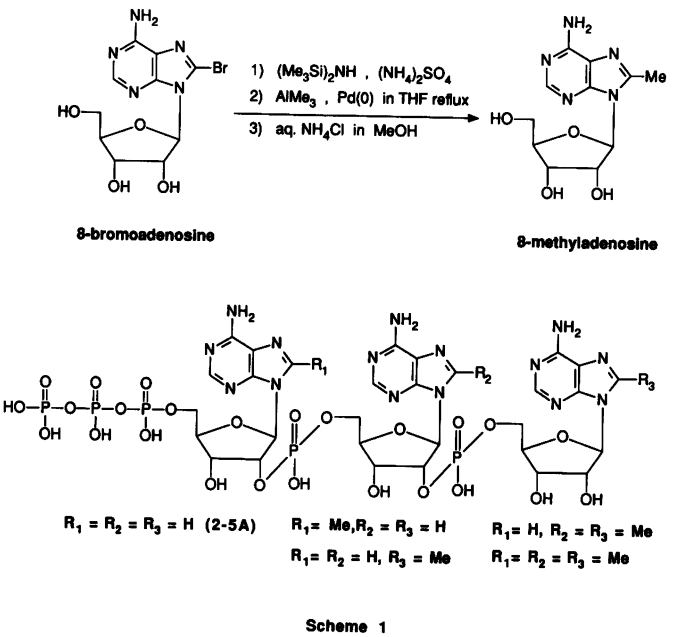
Table 1. Characteristic Proton NMR Signals of Methylated 2',5'-Oligoadenylates

Oligomer	Adenine C_2-H, C_8-H	Adenine C_8-CH_3	Anomeric Protons
p5'(me^{8}A)2'p5'(me^{8}A)	8.00(s)	2.43(s)	5.96(d, 4.4Hz)
	7.82(s)	2.28(s)	5.01(d, 4.4Hz)
p5'(me^{8}A)2'p5'A2'p5'A	8.06(s)	2.35(s)	5.89(d, 2.4Hz)
	7.85(s)		5.74(d, 3.2Hz)
	7.82(s)		5.72(d, 4.9Hz)
	7.80(s)		
	7.74(s)		
p5'A2'p5'A2'p5'(me^{8}A)	8.10(s)	2.30(s)	5.93(d, 2.9Hz)
	7.98(s)		5.84(d, 1.3Hz)
	7.94(s)		5.53(d, 5.6Hz)
	7.84(s)		
	7.78(s)		
p5'A2'p5'(me^{8}A)2'p5'(me^{8}A)	8.26(s)	2.30(s)	6.01(d, 1.3Hz)
	8.04(s)	2.35(s)	5.81(d, 2.9Hz)
	7.90(s)		5.65(d, 5.6Hz)
	7.85(s)		
p5'(me^{8}A)2'p5'(me^{8}A)2'p5'(me^{8}A)	8.06(s)	2.57(s)	6.01(d, 1.3Hz)
	7.91(s)	2.39(s)	5.82(d, 4.1Hz)
	7.86(s)	2.22(s)	5.68(d, 8.8Hz)

Biological studies

The methylated oligonucleotides were evaluated for their ability to activate the RNase L of mouse liver extracts using a modification of an assay originated by Silverman (15, 16). The results of these determinations are provided in Figure 2. RNase L activating ability could be summarized as follows: ppp5'A2'p5'A2'p5'(me^{8}A) > ppp5'A2'p5'A2'p5'A > ppp5'A2'p5'(me^{8}A)2'p5'(me^{8}A) > ppp5'(me^{8}A)2'p5'(me^{8}A) 2'p5'(me^{8}A) ppp5'(me^{8}A)2'p5'A2'p5'A.

The ability of these methyladenosine analogues of 2-5A to bind to mouse liver RNase L was assayed using a modification of the radiobinding assay described by Knight et al (17), and the results are given in Table 4.



Scheme 1

Table 2. Enzymatic Characterization of Methylated 2',5'-Oligoadenylates

Oligomer (Retention Time)	AP Digest Product (Retention Time)	SVP Digest Moles Product	AP/SVP Digest Moles Product
ppp(me^{8}A)p(me^{8}A)p(me^{8}A) (13.5)	(me^{8}A)p(me^{8}A)p(me^{8}A) (33.0)	p(me^{8}A)	ND
p(me^{8}A)pApA (14.9)	(me^{8}A)pApA (33.7)	2 pA + 1 p(me^{8}A)	1 (me^{8}A) + 2 pA
pApAp(me^{8}A) (17.4)	ApAp(me^{8}A) (29.3)	2 pA + 1 p(me^{8}A)	1 A + 1 pA + 1 p(me^{8}A)
pppApAp(me^{8}A) (15.3)	ApAp(me^{8}A) (29.3)	2 pA + 1 p(me^{8}A)	ND
pAp(me^{8}A)p(me^{8}A) (17.3)	Ap(me^{8}A)p(me^{8}A) (28.0)	1 pA + 2 p(me^{8}A)	1 A + 2 p(me^{8}A)
pppAp(me^{8}A)p(me^{8}A) (16.9)	Ap(me^{8}A)p(me^{8}A) (28.0)	1 pA + 2 p(me^{8}A)	ND
p(me^{8}A)p(me^{8}A)p(me^{8}A) (16.2)	(me^{8}A)p(me^{8}A)p(me^{8}A) (33.0)	p(me^{8}A)	1 (me^{8}A) + 2 p(me^{8}A)
ppp(me^{8}A)pApA (13.5)	(me^{8}A)pApA (33.7)	2 pA + 1 p(me^{8}A)	ND

Alkaline phosphatase (AP) or snake venom phosphodiesterase (SVP) digestion of monophosphatases and triphosphates to dephosphorylated linkage isomer products was performed as described in the material and methods. HPLC was performed on two Shimadzu system model LC-6A pumps and a SCL-6A system controller. The detector was a Shimadzu SPD-6A UV spectrophotometric detector. The intergration of the chromatograph was determined with a C-R3A chromatopac. The column employed was Wakosil 5C18 (Wako). Buffer A (50 mM ammonium phosphate, pH 7.0) in 0 to 50% Buffer B (methanol: water, 1:1) over 30 minutes at 1 mL/min.

DISCUSSION

Several conclusions are apparent from inspection of the above results.

1. Introduction of an 8-methyladenosine in place of adenosine at the 2'-terminus of 2-5A trimer yielded an oligonucleotide which was significantly more effective as an activator of RNase L than was 2-5A itself.

2. When a similar substitution of 8-methyladenosine for adenosine was carried out at the 5'-terminus of 2-5A trimer, a dramatic loss (about 100-fold) of RNase L activating ability was observed. This loss of activity was related to a loss (28-fold compared to p5'A2'p5'A2'p5'A) of capacity to bind to the 2-5A-dependent endonuclease.

Table 3. Chemical Characterization of Methylated 2',5'-Oligoadenylates

Oligomer	1/10 NaOH Digestion (Molar Ratio)
p(me^{8}A)pApA	p(me^{8}A)2'(3')p + A2'(3')p + A (1: 1: 1)
pApAp(me^{8}A)	pA2'(3')p + A2'(3')p + (me^{8}A) (1: 1:1)
pAp(me^{8}A)p(me^{8}A)	pA2'(3')p + (me^{8}A)2'(3')p + (me^{8}A) (1: 1: 1)
p(me^{8}A)p(me^{8}A)p(me^{8}A)	p(me^{8}A)2'(3')p + (me^{8}A)2'(3')p + (me^{8}A) (1: 1: 1)

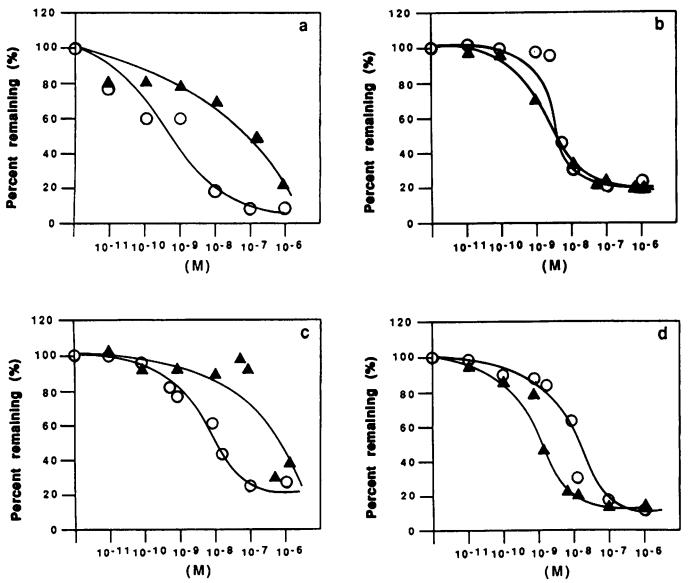


Figure 2. ability of 8-methyladenosine substituted 2-5A analogues to stimulate the degradation of RNA. Oligonucleotides were added in the indicated concentrations to reaction mixture containing mouse L cell extract RNase L absorbed to 2-5A hexamer core agarose and containing the nuclease substrate poly(U)[^{32}P]pCp. After 60 min incubation, aliquots were analyzed for ammonium acetate-ethanol-inosoluble radioactivity.
a: ppp5'(me^{8}A)2'p5'(me^{8}A)2'p5'(me^{8}A), ▲, vs 2-5A trimer, ○:
b: ppp5'A2'p5'(me^{8}A)2'p5'(me^{8}A), ▲, vs ppp5'A2'p5'A2'p5'A, ○:
c: ppp(me^{8}A)2'p5'A2'p5'A, ▲, vs 2-5A trimer, ○:
d: ppp5'A2'p5'A2'p5'(me^{8}A), ▲, vs ppp5'A2'p5'A2'p5'A, ○:

3. This dependence of nuclease activating ability on the nature of the nucleoside at the 5'-terminus was also clear from the behavior of the di- and trimethylated 2-5A analogues. The triphosphate ppp5'A2'p5'(me^{8}A)2'p5'(me^{8}A) was approximately as effective as 2-5A as an RNase L activator. However, when 8-methyladenosine was substituted for the adenosine at the 5'-terminus, a 100-fold drop in activation occurred once more. In this case, there was only a 5-fold decrease in RNase L binding at the triphosphate level.

Other 8-substituted purine analogues of 2-5A also have shown a dramatic variation in interaction with RNase L according to the extent and pattern of 8-substitution in the oligonucleotide. Thus, when all three of the adenosines of 2-5A trimer triphosphate were converted to 8-bromoadenosine (7), a reduction in extent of binding to and activation of RNase L was observed. In addition, when only the 5'-adenosine or else the second adenosine of ppp5'A2'p5'A2'p5'A was transformed to 8-bromoadenosine (8), a significant decrease in binding and activation occurred. On the other hand, replacement of just the 2'-terminus of ppp5'A2'p5'A2'p5'A by 8-bromoadenosine brought about no significant change in RNase L binding, but did result in an analogue that was about ten times more potent as an activator of RNase L than was 2-5A (8). Conformational studies on such brominated 2',5'-adenylates, together with the previously established role of the adenosine N6-amino/N1 position in influencing endonuclease binding, led to the hypothesis that , so far as the 5'-terminal adenosine is concerned, RNase L binding requires an *anti* orientation of the base about the glycosidic bond (9). Furthermore, based on similar considerations, it has been suggested that a *syn* base-sugar orientation or an S-type sugar conformation may have a positive influence on binding to the 2-5A-dependent endonuclease (9). The extremely poor RNase L binding of the 2-5A analogue in which all adenosines were converted to 8-hydroxypropyladenosine (18) is likely at least partly related to the *syn* glycosidic conformation that would obtain at all three residues.

Table 4. Activity of Methylated 2',5'-Oligoadenylates in the Radiobinding Assay with the RNase L of Mouse Liver Extracts

Oligomer	IC_{50}[a] Molar	Standard Deviation	C_{rel}[b]
triphosphates:			
ppp5'(me^{8}A)2'p5'A2'p5'A	6.5×10^{-9}	1.3×10^{-9}	30
ppp5'A2'p5'A2'p5'(me^{8}A)	1×10^{-10}	0.6×10^{-10}	0.4
ppp5'A2'p5'(me^{8}A)2'p5'(me^{8}A)	4.5×10^{-10}	2×10^{-10}	2
ppp5'(me^{8}A)2'p5'(me^{8}A)2'p5'(me^{8}A)	2.5×10^{-9}	1×10^{-9}	10
monophosphates:			
p5'A2'p5'A2'p5'A	2.3×10^{-10}	1.5×10^{-10}	1
p5'(me^{8}A)2'p5'A2'p5'A	7×10^{-8}	0.6×10^{-8}	300
p5'A2'p5'A2'p5'(me^{8}A)	9×10^{-11}	1.7×10^{-11}	0.4
p5'A2'p5'(me^{8}A)2'p5'(me^{8}A)	1×10^{-9}	0.7×10^{-9}	4
p5'(me^{8}A)2'p5'(me^{8}A)2'p5'(me^{8}A)	7×10^{-10}	0.9×10^{-10}	3

a: IC_{50} = concentration (M) necessary to inhibit binding of the radiolabelled 2-5A probe by 50%.
b: C_{rel} was defined as the relative concentration of analogue to displace 50% of the probe. The greater the value, the less effective the analogue was at competing with probe: 2-5A 5'-monophosphate was arbitrarily set equal to 1; analogue values were calculated from the quotient:
(IC_{50} analogue)/(IC_{50} pApApA).

ACKNOWLEDGEMENTS

This work was supported, in part, by the Grant-in-Aid for Scientific Research from the Ministry of Education of Japan, which is gratefully acknowledged. The authors thank Dr. Akira Matsuda, Hokkaido University, for the useful discussion on the conformation of methylated 2′,5′-oligoadenylates.

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