Synthesis and Reactions of Nucleoside 5′-Diphosphate Imidazolide. A Nonenzymatic Capping Agent for 5′-Monophosphorylated Oligoribonucleotides in Aqueous Solution

Hiroaki Sawai,* Hiromichi Wakai, and Akiko Nakamura-Ozaki

Department of Chemistry, Faculty of Engineering, Gunma University, Kiryu, Gunma 376-8515, Japan

Received February 17, 1999

We have synthesized adenosine and 7-methylguanosine 5′-diphosphate imidazolides from imidazole and the corresponding nucleoside 5′-diphosphates. The phosphorimidazolide bond of the compounds was susceptible to hydrolysis and hydrolyzed gradually in neutral aqueous solution, but it was more stable than that of the corresponding imidazolides of nucleoside 5′-monophosphate. The 7-methylguanosine 5′-diphosphate imidazolide reacted with guanosine 5′-monophosphate or 5′-monophosphorylated oligoribonucleotides in neutral aqueous solution in the presence of an Mn^{2+} ion catalyst converting to the cap portion of mRNA or the capped m7Gppp-oligoribonucleotides in substantial yields. The condensation reaction of adenosine 5′-diphosphate imidazolide with adenosine 5′-monophosphate took place similarly in neutral aqueous solution by a divalent metal ion-catalyst such as Mg^{2+} or Mn^{2+}, yielding diadenosine 5′,5′-triphosphate.

Introduction

The eucaryotic and viral messenger RNAs have a unique cap structure, m7GpppNu- - - -, at their 5′ terminus.[1] The cap portion plays important roles in the processing and maturation of the transcript RNA in the nucleus,[2] in the transport of the mRNA from the nucleus to the cytoplasm,[3] in the mRNA stability,[4] in the binding of mRNA to ribosome[5] and in the initiation of protein synthesis.[6] The preparation of capped oligoribonucleotides has been done by either the chemical or enzymatic method. Hata and Sekine have developed several methods for the chemical synthesis of capped mRNAs.[7] Their methods use an anhydrous organic solvent as media, thereby requiring a complicated procedure to solubilize the capping agent and the oligoribonucleotides. Tertiary alkylammonium ions as countercations and protecting groups for the sugar and base parts of oligoribonucleotides and the capping agent are required for the solubilization and to promote the target triphosphorylation reaction. In the enzymatic method, 5′-triphosphorylated RNA is synthesized in vitro by transcription of a suitable plasmid DNA, followed by capping reaction by guanylyl transferase in the presence of GTP and *S*-adenosylmethionine.[8] Further, Brownlee et al. reported the chemical solid-phase synthesis of 5′-diphosphorylated oligoribonulceotides and their enzymatic conversion to the capped oligoribonucleotides by guanylyl transferase with GTP and *S*-adenosylmethionine.[9] However, no enzymatic capping reaction takes place for the 5′-monophosphorylated oligoribonucleotides, which can be obtained easily either by solid-phase synthesis or by kination of the 5′-OH group of oligoribonucleotides with polynucleotide kinase using ATP. Thus, we have explored a facile nonenzymatic capping reaction of 5′-phosphorylated oligoribonucleotides, which can proceed in aqueous solution. Previously, we have shown the Mn^{2+} ion-catalyzed triphosphorylation between 7-methylguanosine 5′-monophosphate imidazolide and nucleoside 5′-diphosphates in aqueous solution giving the cap portion of mRNAs, 7mGpppN.[10] If 7-methylguanosine 5′-diphosphate imidazolide could be prepared and reacted with 5′-monophosphorylated oligoribonucleotides in aqueous solution to form the triphosphate bond, the capped oligoribonucleotides could be synthesized easily. So far, no report has appeared on the synthesis and properties of the nucleoside 5′-diphosphate imidazolide, although the corresponding imidazolides and related azolides of nucleoside 5′-monophosphates have been prepared widely.[11] They are used as a starting material for the nonenzymatic oligoribonucleotide synthesis,[12] especially from the point of the prebiotic synthesis,[13] and for the synthesis of nucleotides containing a pyrophosphate bond.[14]

(1) (a) Furuuchi, Y.; Miura, K. *Nature* **1975**, *253*, 374. (b) Shatkin, A. J. *Cell* **1976**, *9*, 645. (c) Miura, K. *Adv. Biophys.* **1981**, *14*, 205. (d) Sonenberg, N. *Prog. Nucleic Acids Res. Mol. Biol.* **1988**, *35*, 173.

(2) (a) Edery, I.; Sonenberg, N. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7590. (b) Konarska, M. M.; Padgett, R. A.; Sharp, R. A. *Cell* **1984**, *38*, 731.

(3) Hamm, J.; Mattaj, I. W. *Cell* **1990**, *63*, 109.

(4) (a) Furuuchi, Y.; Lafindra, A. J.; Shatkin, A. J. *Nature* **1977**, *266*, 235. (b) Simotohno, K.; Kodama, Y.; Hashimoto, J.; Miura, K. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 2734. (c) Murthy, K. G. K.; Park, P.; Manley, J. L. *Nucleic Acids Res.* **1991**, *19*, 2685.

(5) (a) Yamaguchi, K.; Hidaka, S.; Miura, K.. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 1012. (b) Kozak, M. *Cell* **1986**, *44*, 283.

(6) Kozak, M. *J. Biol. Chem.* **1991**, *266*, 19867.

(7) (a) Kamimura, T,; Osaki, Y.; Sekine, M.; Hata, T. *Tetrahedron Lett.* **1984**, *25*, 2683. (b) Sekine, M.; Nishiyama, S.; Kamimura, Y.; Osaki, Y.; Hata, T. *Bull. Chem. Soc. Jpn* **1985**, *58*, 850. (c) Iwase, R.; Sekine, M.; Hata, T.; Miura, K. *Tetrahedron Lett.* **1988**, *29*, 2969. (d) Sekine, M.; Iwase, R.; Hata, T.: Miura, K. *J. Chem. Soc., Perkin Trans. 1* **1989**, 969. (e) Iwase, R.; Maeda, M. Fujisawa, T.; Sekine, M.; Hata, T. Miura, K. *Nucleic Acids Res.* **1992**, *20*, 1643. (f) Fukuoka, K.; Suda, F.; Suzuki, R.; Takaku, H.; Ishikawa, M.; Hata, *Tetrahedron Lett.* **1994**, *35*, 1063.

(8) Chung, T. D. Y.; Cianci, C.; Hagen, M.; Terry, B.; Matthews, J. T.; Krystal, M.; Colonno, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2372.

(9) Brownlee, G. G.; Fodor, E.; Pritlove, D. C.; Gould K. G.; Dalluge, J. J. *Nucleic Acids Res.* **1995**, *23*, 2641.

(10) Sawai, H.; Wakai, H.; Shimazu, M. *Tetrahedron Lett.* **1991**, *32*, 6905.

(11) (a) Cramer, F.; Schaller, H.; Staab, H. A. *Chem. Ber.* **1961**, *94*, 1612. (b) Schaller, H.; Staab, H. A.; Cramer, F. *Chem. Ber.* **1961**, *94*, 1621. (c) Lohrmann, R.; Orgel, L. E. *Tetrahedron* **1978**, *34*, 853. (d) Inoue, T.; Orgel, L. E. *J. Am. Chem. Soc.* **1981**, *103*, 7666. (e) Sawai, H.; Shibusawa, T.; Kuroda, K. *Bull. Chem. Soc. Jpn* **1990**, *63*, 1776.

10.1021/jo990286u CCC: $18.00
Published on Web 07/16/1999

In this paper we report a synthesis and the hydrolytic stability of nucleoside 5′-diphosphate imidazolide and an application of 7-methyylguanosine 5′-diphosphate imidazolide to the nonenzymatic synthesis of the capped oligoribonucleotides from 5′-phosphorylated oligoribonucleotides in aqueous solution.

Materials and Methods

Materials. Adenosine and guanosine 5′-monophosphates (pA and pG) were purchased from Seikagaku Kogyo. Adenosine 5′-diphosphate was from Boeringer. The 5′-monophosphate of hexariboadenylate (pAAAAAA) was prepared by partial digestion of poly(A) with nuclease SW as described previously.[15] The chemically synthesized 5′-monophosphate of RNA 11mer (pACACUUGCUUU) was purchased from Genset. Nuclease P1 was from Seikagaku Kogyo, and Venom phosphodiesterase and alkaline phosphatase were from Worthington Biochemicals. 7-Methylguanosine 5′-monophosphate (p7mG) was prepared from pG and methyl iodide by slight modification of the published procedure.[16] Adenosine 5′-phosphorimidazolide (ImpA)[11c] and 7-methylguanosine 5′-phosphorimidazolide (Imp7mG)[10] were prepared from pA and p7mG, respectively, as described previously. All other chemicals were of reagent grade.

HPLC. HPLC on an ODS-silica gel column (4 mm × 250 mm) was done with a linear gradient elution from 2.4 to 12 (system A), 24 (system B), or 40% (system C) methanol in 0. 1 M triethylammonium acetate at pH 7.0 in 30 min at a flow rate of 1.0 mL/min. HPLC on an RPC-5 column (4 mm × 250 mm) was carried out with a linear gradient elution from 0 to 0.08 M $NaClO_4$ in 10 mM Tris-acetate (pH 7.5) containing 1.0 mM EDTA in 30 min at a flow rate of 1.0 mL/min. The eluate was monitored by UV absorption at 260 nm and by fluorescence with excitation at 279 nm and emission at 393 nm.

Synthesis of Adenosine 5′-Diphosphate Imidazolide (ImppA). Adenosine 5′-diphosphate (ppA) was at first purified by anion-exchange column chromatography on Sephadex A-25 using a linear gradient (0−0.5 M) of triethylammonium hydrogen carbonate buffer (pH 7.5), as commercially available ppA contains some amounts of adenosine 5′-monophosphate (pA). The triethylammonium salt of ppA (1440 ODU at 260 nm, 0.09 mmol) was lyophilized several times by adding small amounts of water to remove excess triethylammonium bicarbonate and coevaporated three times with pyridine and then twice with dry DMF. Imidazole (63 mg, 0.9 mmol), di-2-pyridyl disulfide (203 mg, 0.9 mmol), triphenylphosphine (244 mg, 0.9 mmol), and dry DMF (10 mL) containing triethylamine (0.5 mL) and tri-*n*-octylamine (0.2 mL) were added to the residue, and the solution was stirred overnight at 24 °C. The completion of the reaction was checked by HPLC. The reaction mixture was poured into a solution containing dry acetone (100 mL), dry ether (50 mL), acetone saturated with sodium perchlorate (0. 5 mL), and triethylamine (2 mL) with stirring. The sodium salt of ImppA was obtained in 82% yield (48 mg, 1180 ODU at 260 nm) as white precipitates which were collected by a glass filter, washed with dry acetone and then with dry ether, and dried in a desiccator. The ImppA is stable under dry conditions at low temperature and can be stored in a refrigerated desiccator. Analytical HPLC: t_R = 24.2 min (system B). λ_{max} = 260 nm. ^{1}H NMR δ (D_2O): 8.39 (s, 1H), 8.26 (s, 1H), 7.94 (s, 1H), 7.30 (s, 1H), 6.98 (s, 1H), 6.10 (d, 1H, $J_{1',2'}$ = 5.9 Hz), 4.71 (dd,1H), 4.40 (dd,1H), 4.33 (m, 1H), 4.09 (m, 2H).

Synthesis of 7-Methylguanosine 5′-Diphosphate (pp7mG). The triethylammonium salt of 7-methylguanosine 5′-monophosphate[16] (15120 ODU at 260 nm, 1.5 mmol) was dried by coevaporation with DMF twice. *N*,*N*′-Carbonyldiimidazole (2.4 g, 14.7 mmol) and dry DMF (10 mL) were added to the residue, and the solution was stirred for 1 h at 40 °C. After an excess amount of *N*,*N*′-carbonyldiimidazole was degraded by addition of a small amount of dry methanol to the reaction mixture, tris(tri-*n*-butylammonium)phosphate (10 mmol) in dry DMF (20 mL) was added and the solution was stirred for 3 d at room temperature. The completion of the reaction was checked by HPLC. The reaction mixture was poured into a solution containing dry acetone (300 mL), ether (150 mL), $NaClO_4$-saturated acetone (6 mL), and triethylamine (2 mL) to precipitate 7-methylguanosine 5′-diphosphate as a sodium salt. The precipitates were collected with a glass filter, washed with acetone and ether, and dried. The resulting precipitates were dissolved in water, passed through a column on activated carbon, washed with water to remove the phosphate, and eluted with a solution of pyridine:ethanol:water (1:20:20). The eluate was evaporated to dryness and applied to an anion-exchange column (3 × 50 cm) on QAE-Sephadex (bicarbonate form) with a linear gradient elution of triethylammonium bicarbonate buffer (0−0.5 M). Appropriate fractions were collected and lyophilized several times to remove triethylammonium bicarbonate. 7-Methylguanosine 5′-diphosphate triethylammonium was obtained in 57% yield, 8450 ODU at 260 nm. Analytical HPLC: t_R = 11.9 min (system B). λ_{max} = 258 and 280 nm. ^{1}H NMR δ (D_2O): 9.18 (s, 1H), 6.05 (d, 1H, $J_{1',2'}$ = 3.5 Hz)), 4.67 (t, 1H), 4.51 (m, 1H), 4.40 (m, 1H), 4.33, 4.21 (m, 2H), 4.11 (s, 3H).

Synthesis of 7-Methylguanosine 5′-Diphosphate Imidazolide (Impp7mG). The Impp7mG was prepared from pp7mG and imidazole by a similar procedure for the synthesis of ImppA. In brief, the triethylammonium salt of pp7mG (1183 ODU at 260 nm, 0.1 mmol) was condensed with imidazole (78 mg, 12.2 mmol) in dry DMF (15 mL) using triphenylphosphine (301 mg, 1.2 mmol) and di-2-pyridyl disulfide (253 mg, 1.2 mmol) as a condensing agent at room temperature overnight. The completion of the reaction was checked by HPLC. Impp7mG was isolated as a sodium salt by pouring the above reaction mixture into a solution of dry acetone and ether containing sodium perchlorate with stirring. The resulting white precipitates were collected by centrifuge, washed with acetone and ether, and dried in a desiccator. The isolated yield was 71% (840 ODU at 260 nm, 53 mg). Impp7mG was kept in a desiccator stored in a freezer. Analytical HPLC: t_R = 16.8 min (system B). λ_{max} = 258 and 280 nm. ^{1}H NMR δ (D_2O): 8.68 (s, 1H), 7.94 (s, 1H), 7.32 (s, 1H), 7.03 (s, 1H), 6.04 (d, 1H, $J_{1',2'}$ = 3.2 Hz), 4.65 (m, 1H), 4.51 (m, 1H), 4.38 (m, 1H), 4.20−4.30 (m, 2H), 4.12 (s, 3H**)**.

Hydrolytic Stability of Impp7mG and ImppA. The Impp7mG (25 mM) was dissolved in 0.25M Tris-acetate buffer (pH 7.5) and kept at 25 °C. An aliquot of the sample solution was withdrawn at appropriate intervals and analyzed by HPLC. Hydrolysis of Impp7mG to pp7mG was observed. The half-life of Impp7mG against hydrolysis was estimated from the time course of the reaction. Hydrolysis of Imp7mG was also carried out under the same conditions as that for Impp7mG to compare the hydrolytic stability of the phosphorimidazolide bond of nucleoside 5′-diphosphate and 5′-monophosphate. Hydrolytic stabilities of ImppA and ImpA were also examined by the same procedure as described above.

Diadenosine 5′,5′-Triphosphate (ApppA) Formation from ImppA and pA by Divalent Metal Catalyst in

(12) (a) Sawai, H.; Shibata, T.; Ohno, M. *Tetrahedron* **1981**, *37*, 481. (b) Sawai, H.; M.; Ohno, M. *Bull. Chem. Soc. Jpn* **1981**, *54*, 2759. (c) Sawai, H.; Kuroda, K.; Hojo, T. *Bull. Chem. Soc. Jpn.* **1989**, *62*, 2018. (d) Sawai, H.; Higa, K.; Kuroda, K. *J. Chem. Soc., Perkin Trans. 1* **1992**, 505.

(13) (a) Orgel, L. E.; Lohrmann, R. *Acc. Chem. Res.* **1973**, *7*, 368. (b) Sawai, H. *J. Am. Chem. Soc.* **1976**, *98*, 7037. (c) Lohrmann, R.; Bridson, P. K.; Orgel, L. E. *Science* **1983**, *219*, 8590. (d) Inoue, T.; Orgel, L. E. *Science* **1983**, *219*, 859. (e) Ferris J. P.; Ertem, G. Science **1992**, *257*, 1387. (f) Kawamura, K.; Ferris, J. P. *J. Am. Chem. Soc.* **1994**, *116*, 7564. (g) Ferris J. P.; Hill. A. R.; Liu, R.; Orgel. L. E. *Nature* **1996**, *381*, 59. (h) Ertem, G.; Ferris, J. P. *J. Am. Chem. Soc.* **1997**, *119*, 7179. (i) Kozlov, I. A.; Pitsch, S.; Orgel, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13448.

(14) (a) Staab, H. A. *Angew. Chem.* **1962**, *74*, 407. (b) Hoard, D. E.; Otto, D. G. *J. Am. Chem. Soc.* **1965**, *87*, 1785. (c) Kozarich, J. W.; Chinault, A. C.; Hecht, S. M. *Biochemistry* **1973**, *12*, 4458. (d) Shimazu, M.; Shinozuka, K.; Sawai, H. *Tetrahedron Lett.* **1990**, *31*, 235. (e) Sawai, H.; Shimazu, M.; Wakai, H. Wakabayashi, H.; Shinozuka, K. *Nucleosides Nucleotides* **1992**, *11*, 973.

(15) Sawai, H.; Kuroda, K. Seki, J.; Ozaki, H. *Biopolymers* **1996**, *39*, 173.

(16) Jones, R. W.; Robins, R. K. *J. Am. Chem. Soc.* **1963**, *34*, 853.

Neutral Aqueous Solution. A reaction mixture (50 μL) containing ImppA (20 mM), pA (20mM), and divalent metal chloride or nitrate (20 mM) in 0.2 M *N*-ethylmorpholine·HCl buffer (pH 7.0) was kept at 30 °C for 4 d. The reaction mixture was treated with versenol solution (0.25 M × 5 μL) to remove the metal ion as a versenol−metal chelate and stored in a freezer until analysis by HPLC. Identification of the products was carried out by coinjection with the authentic samples. Yields were calculated from the peak integral of the HPLC chromatogram after correction for the hypochromicity of ApppA.

Synthesis of a 5′-Cap Portion of Messenger RNA (7mGpppG) from Impp7mG and pG by Divalent Metal Ion Catalyst in Neutral Aqueous Solution. TheImpp7mG (20 or 100 mM) was reacted with pG (20mM) in the presence of divalent metal chloride (20 or 100 mM) in 50 μL of 0.2 M *N*-ethylmorpholine·HCl buffer (pH 7.0) at 30 °C for 4 d. The reaction mixture was treated with a versenol solution (0.25 M × 5 μL) to remove the metal ion as a versenol−metal chelate and analyzed by HPLC. Identification of the products was carried out by coinjection with the authentic sample.[10] Yields were calculated from the peak integral of the HPLC chromatogram after correction for the hypochromicity of 7mGpppG.

Capping Reaction of 5′-Monophosphorylated Oligoribonucleotides with Impp7mG by Mg^{2+} or Mn^{2+} Catalyst in Neutral Aqueous Solution. The capping reaction of the 5′-monophosphate of oligoribonucleotide with Impp7mG was tried at first using hexariboadenylate (pAAAAAA) as a model substrate. A reaction mixture (100 μL) containing pAAAAAA (1.0 mM), Impp7mG (10 mM), and $MgCl_2$ or $MnCl_2$ (10 mM) in 0.2 M *N*-ethylmorpholine·HCl buffer (pH 7.0) was kept at 30 °C for 4 d. The reaction mixture was treated with versenol solution (0.25 M × 5 μL) to remove the metal ion as a versenol-metal chelate and analyzed by HPLC. The capped product, 7mGpppAAAAAA, was isolated by HPLC followed by evaporation with a rotavac several times to remove the triethylammonium acetate buffer. The 7mGpppAAAAAA was then digested with nuclease P1 in a 30 μL reaction mixture containing 0.15 ODU of the substrate and 3 μl of 1 mg/ml of nuclease P1 in 50 mM acetate buffer (pH 5.4) for 12 h at 37 °C, followed by analysis by HPLC on the ODS-silica gel column. NuceaseP1 cleaves the 3′−5′ internucleotide bond but not the triphosphate bond.

Second, the capping reaction of pACACUUGCUUU was conducted to prepare the 5′-end of rabbit β-globin mRNA. A reaction mixture (50 μL) containing pACACUUGCUUU (10 ODU at 260 nm, 9.7 nmol, 0.2 mM), Impp7mG (10 ODU at 260 nm, 97 nmol, 2 mM), and $MgCl_2$ or $MnCl_2$ (10 mM) in 0.2 M *N*-ethylmorpholine·HCl buffer (pH 7.0) was kept at 30 °C for 6 d. The reaction without a divalent metal ion catalyst was also carried out as a control reaction. The reaction mixture was treated with versenol solution (0.25 M × 5 μL) to remove the metal ion as a versenol−metal chelate and analyzed by HPLC. The capped product, 7mGpppACACUUGCUUU, was isolated by HPLC, digested with nuclease P1 and then with alkaline phosphatase, and analyzed by HPLC to confirm the introduction of the cap portion at the 5′-terminus. The reaction mixture containing 27 μL of 0.15 ODU of the substrate in 0.05 M acetate buffer (pH 5.4) and 3 μL of 1 mg/mL of nuclease P1 in 50 mM acetate buffer (pH 5.4) was kept for 12 h at 37 °C. To 15 μL of the above reaction mixture, 10 μL of 10 mM $MgCl_2$, 15 μL of 50 mM Tris·HCl (pH 9.0), and 1 μL of alkaline phosphatase (1 unit/μL) were added, and the solution was kept at 37 °C for 12 h to remove the 5′-terminal phosphate, followed by analysis by HPLC.

Results and Discussion

Synthesis and Hydrolytic Stability of Nucleoside 5′-Diphosphate Imidazolide. First, we studied the synthesis and stability of adenosine 5′-diphosphate imidazolide, since no report has been published on the synthesis and properties of nucleoside 5′-diphosphate imidazolide, although imidazolides or related azolides of nucleoside 5′-monophosphates have been prepared widely[11] and used as a monomer for the oligoribonucleotides synthesis[12,13] or as an intermediate for the synthesis of nucleotides bearing a pyrophosphate bond.[14] Condensation of adenosine 5′-diphosphate with imidazole using di-2-pyrydyl disulfide and triphenylphosphine as a condensing agent gave the adenosine 5′-diphosphate imidazolide. In a similar way, 7-methylguanosine 5′-diphosphate imoidazolide was prepared from 7-methylguanosine 5′-diphosphate, which was obtained from guanosine 5′-monophosphate by 7-methylation followed by diphosphorylation. Adenosine and 7-methylguanosine 5′-diphosphate imidazolides were moderately stable and could be kept in a desiccator stored in a freezer. However, the phosphorimidazolide bond of the compounds is susceptible to moisture and hydrolyzes in aqueous solution, forming the corresponding nucleoside 5′-diphosphate. We monitored the hydrolysis reaction of the phosphorimidazolide bond of adenosine and 7-methylguanosine 5′-diphosphate imidazolides in neutral aqueous solution by HPLC. Figure 1 shows the time course of the hydrolysis reaction of adenosine and 7-methylguanosine 5′-diphosphate imidazolides along with that of the corresponding adenosine and 7-methylguanosine 5′-monophosphate imidazolides. The hydrolysis of the phsophorimidazolide bond obeys pseudo-first-order kinetics. The phosphorimidazolide bond of nucleoside 5′-diphosphate imidazolides was more stable than that of the corresponding 5′-monophosphate imidazolide against hydrolysis in neutral aqueous solution. The half-lives of the phosphorimidazolide bond were 142, 40, 92, and 50 h for the imidazolides of 7-methyguanosine 5′-diphosphate, 7-methylguanosine 5′-monophosphate, adenosine 5′-diphosphate, and adenosine 5′-monophosphate, respectively, in aqueous solution at 25 °C and pH 7.5.

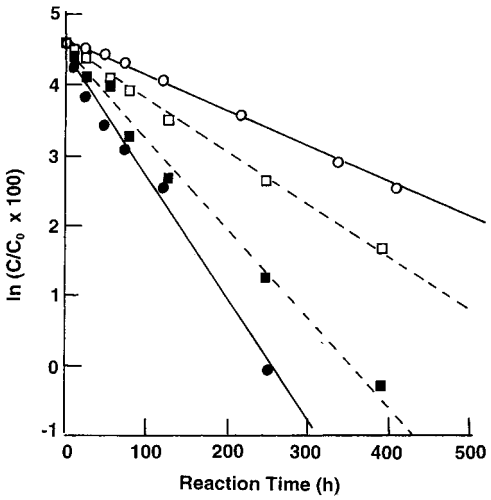


Figure 1. Time course of hydrolysis of imidazolides of nuceloside 5′-diphosphates and 5′-monophosphates: *C*, concentration of the imidazolide; C_0, initial concentration of the imidazolide; ○, Impp7mG; ●, Im7mpG; □, ImppA; ■, ImpA.

Diadenosine 5′,5′-Triphosphate (ApppA) Formation by the Reaction of ImppA and pA in Aqueous Solution by a Divalent Metal Ion Catalyst. We first examined the diadenosine 5′,5′-triphosphate (ApppA) formation from ImppA and pA using various metal ion catalysts to explore an efficient catalyst for the triphosphate formation in aqueous solution. The reaction was carried out in a reaction mixture containing equimolar

Scheme 1. Imidazolides of Nucleoside 5′-Monophosphate (ImpN) and 5′-Diphosphate (ImppN)

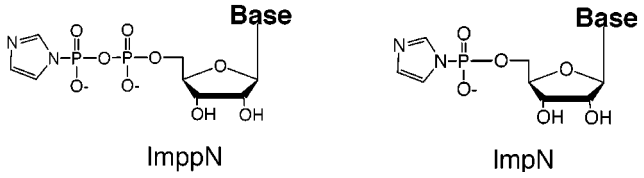


Table 1. ApppA Formation from ImppA and PA Catalyzed by Metal Ions

catalyst	yield (%)[a]	catalyst	yield (%)[a]
Mg^{2+}	34.4	Ba^{2+}	5.5
Mn^{2+}	47.8	Pb^{2+}	0.9
Cd^{2+}	36.7	Co^{2+}	9.5
Ca^{2+}	26.0	Fe^{2+}	9.2
Sn^{2+}	2.2	Ni^{2+}	5.6
Cu^{2+}	0.7	Hg^{2+}	2.3
Zn^{2+}	3.7	None	1.3

[a] Yields were based on the starting pA. Reactions were run at 30 °C for 4 d using 20 mM ImppA, 20 mM pA, and 20 mM Metal chloride in 0.2 M *N*-ethylmorpholine buffer (pH 7.0).

Table 2. Yield of the Cap Portion of mRNA, 7mGpppG, from Impp7mG and by Mn^{2+}, Mg^{2+}, or Cd^{2+} Ion Catalyst

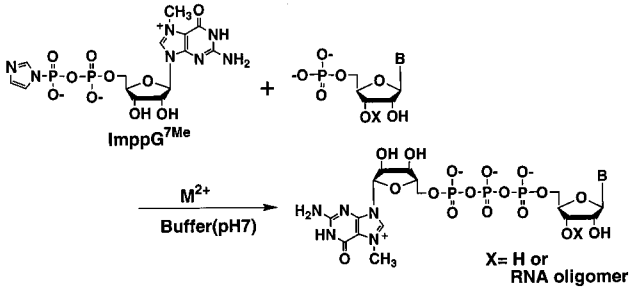
	yield (%)[a]	
catalyst	method A[b]	method B[c]
Mn^{2+}	41.7	76.8
Mg^{2+}	23.9	74.3
Cd^{2+}	19.2	66.5
none	7.9	21.5

[a] Yields were based on the starting pG. [b] 20 mM Impp7mG, 20 mM pG, and 20 mM metal chloride were used in the reaction. [c] 100 mM Impp7mG, 20 mM pG, and 100 mM metal chloride were used in the reaction. Reactions were run at 30 °C for 4 d in 0.2 M *N*-ethylmorpholine buffer (pH 7.0).

amounts of ImppA, pA, and metal chloride (each in 20 mM) in 0.2 M *N*-ethylmorpholine buffer (pH 7.0) at 30 °C for 4 d. The products were analyzed by HPLC. The formation of ApppA was observed along with ppA, pA, and a very small amount of AppA and AppppA, which could be formed from ImpA with pA and ImppA with ppA, respectively (Scheme 1). Table 1 shows the yield data of ApppA formation. The Mn^{2+} was the most efficient catalyst for the triphosphate formation, and ApppA was obtained in 48% yield. The Mg^{2+} and Cd^{2+} ions were the second most efficient catalyst. A very small amount of ApppA was formed in the control reaction where no metal ion was used as a catalyst. The catalytic effect of the various metal ions on the ApppA formation is nearly in accord with that in the AppA formation from ImpA and pA.[14d]

Preparation of Cap Portion of mRNA, 7mGpppG, by Mn^{2+}, Mg^{2+}, or Cd^{2+} Ion-Catalyzed Triphosphorylation from Impp7mG and pG. The reaction of the capping agent, Impp7mG, with pG was carried out using Mn^{2+}, Mg^{2+}, or Cd^{2+} ion as a catalyst to check the triphosphate formation in aqueous solution. The reaction was done at 30 °C for 4 d at the 1:1:1 molar ratio of Impp7mG, pG, and metal ion or at the 5:1:5 molar ratio. The yield data of 7mGpppG formation from Impp7mG and pG are listed in Table 2. The Mn^{2+} is the most effective for the 7mGpppG formation. The 7mGpppG was obtained in 77% yield based on pG when 5 times molar equivalent of Impp7mG and Mn^{2+} ion to pG were used. Without a metal ion catalyst, Impp7mG hydrolyzed

Scheme 2. Capping Reaction of 5′-Monophosphorylated Nucleosides or Oligoribonucleotides with Im7MppG



predominantly and only a small amount of 7mGpppG was formed. The metal-ion catalyzed formation of 7mGpppG from Impp7mG and pG was comparable to that from Imp7G and ppG, in which Mn^{2+} and Mg^{2+} ions were also effective for the pyrophosphate bond formation.[10] An excess amount of Impp7mG and the metal ion catalyst resulted in increase in the yield of 7mGpppG based on the acceptor pG.

Preparation of the Capped Oligoribonucleotides by the Capping Reaction of 5′-Monophosphorylated Oligoribonucleotides with Impp7mG in Aqueous Solution by Mn^{2+} or Mg^{2+} Catalyst. The capping reaction of 5′-monophoshorylated oligoribonucleotides was carried out with Impp7mG in the presence of Mn^{2+} or Mg^{2+} ion as a catalyst, since the 5′-monophosphorylated oligoribonucleotides can be obtained easily by conventional solid-phase synthesis or by kination of natural or synthetic 5′-hydroxyl oligoribonucleotides with polynucleotide kinase. A large excess molar ratio of the Impp7mG and the metal ion to the 5′-monophosphorylated oligoribonucleotide were used for the reaction to increase the yield of the capped oligoribonucleotide based on the starting oligoribonucleotide. Initially, we examined the capping reaction of hexaadenylate, pAAAAAA as a model compound in *N*-ethylmorpholine buffer (pH 7.0) for 10 d at 30 °C (Scheme 2). The product was analyzed by HPLC after treatment of the reaction mixture with versenol to remove the metal ion as a versenol−metal chelate. The capped product, 7mGpppAAAAAA, which has fluorescence due to 7-methylguanosine, had an HPLC retention time just shorter than that of the starting pAAAAAA. The yield of 7mGpppAAAAAA was 49% based on the starting pAAAAAA, when the Mn^{2+} ion was used as the catalyst. The yield decreased to 16% in the presence of the Mg^{2+} ion catalyst under the same conditions. Only 1% of the capped oligoribonucleotide was formed in the control reaction where no metal ion catalyst was used. The 7mGpppAAAAAA was isolated by HPLC and subjected to nuclease P1 digestion to confirm the structure. The HPLC analysis of the digestion mixture showed the presence of 7mGpppA and pA in nearly a 1:5 ratio.

Second, we studied the capping reaction of the oligoribonucleotide, pACACUUGCUUU, for the preparation of the 7mGpppACACUUGCUU, the 5′-end of rabbit *β*-globin mRNA but lacking the 2′-*O*-methyl group on the first A residue. The capping reaction of pACACUUGCUUU (0.2 mM) was done with Impp7mG (2 mM) in the presence of the Mn^{2+} or Mg^{2+} ion catalyst (10mM) in *N*-ethylmorpholine buffer (pH 7.0) for 6 d at 25 °C. The HPLC profiles of the reaction mixtures are shown in Figure 2. The capped product showed fluorescence due to 7-meth-

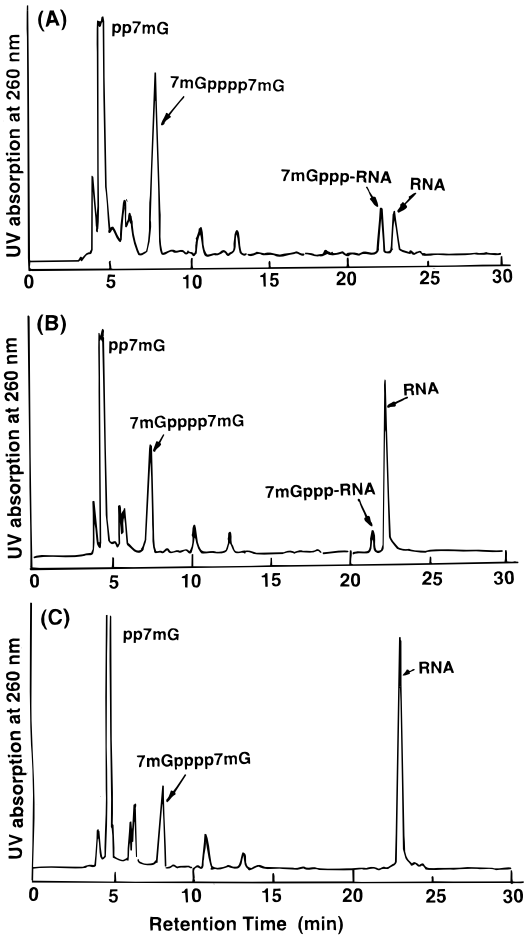


Figure 2. HPLC profiles of the reaction mixtures obtained from pACACUUGCUUU with Impp7mG. The capping reaction of pACACUUGCUUU (0.2 mM) with Im7mppG (2mM) was run at 30 °C for 6 days in 0.2 M *N*-ethylmorpholine buffer (pH 7.0) using (A) $MnCl_2$ (10 mM), (B) $MgCl_2$ (10 mM), or (C) without metal ion catalyst.

ylguanosine. The Mn^{2+} or Mg^{2+} ion catalyst gave the capped product in 35% or 6% yield, respectively, based on the starting oligoribonucleotide. A very small capped product was obtained in the control reaction where no metal ion catalyst was used. The capped product was isolated by HPLC and characterized by enzyme digestion with nuclease p1 followed by bacterial alkaline phosphatase. The successive treatment of 7mGpppACACUUGCUUU with the two enzymes gave a mixture of 7mGpppA, A, G, C, and U in a ratio of nearly 1:1:5:3 as shown in Figure 3.

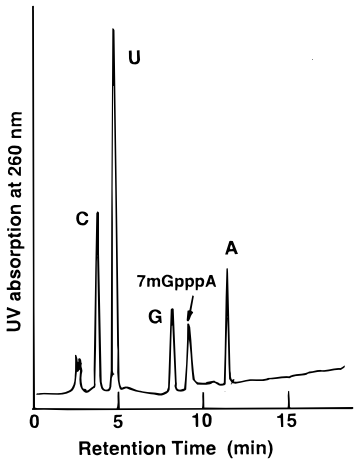


Figure 3. HPLC profile of the mixture of 7mGpppA, A, G, C, and U which was obtained after treatment of the capped 7mGpppACACUUGCUUU with nuclease p1 and alkaline phosphatase.

As a preliminary model reaction of the capping of longer RNA, we examined the capping reaction of $tRNA^{Phe}$ from yeast using a large excess of the capping agent and the Mn^{2+} ion catalyst, as this tRNA is commercially available and has 72 chain length with 5′-terminal monophosphate. The introduction of the cap portion was observed by HPLC on an RPC-5 column in roughly 40% yield, but the separation of the capped RNA from the uncapped one was unsuccessful. The result suggests that the capping reaction of the long messenger RNA could also occur in a modest yield, but the separation of the capped long messenger RNA is problematical.

In conclusion, nucleoside 5′-diphosphate imidazolides, ImppN, could be prepared easily from the nucleoside 5′-diphosphates and used for the triphosphorylation by the Mn^{2+} or Mg^{2+} catalyst. Thus, the Impp7mG reacted with 5′-monophosphorylated nucleosides or oligoribonucleotides in aqueous solution using the Mn^{2+} ion catalyst giving the cap portion of mRNA or the capped oligoribonucleotide in substantial yield. This procedure provides a very simple method for the synthesis of the capped oligoribonucleotides from the 5′-monophosphorylated oligoribonucleotides which can be obtained easily, since the reaction proceeds in a neutral aqueous solution without using any protecting group or enzyme. The capped oligoribonucleotide could ligate with an appropriate RNA by ligase giving the full length messenger RNA which can be applied to molecular biology.

JO990286U