A "Capping" Agent: P1-S-Phenyl P2-7-Methylguanosine-5'-Pyrophosphorothioate

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Messenger ribonucleic acids (mRNAs) from eukaryotic cells and viruses have been found to contain a 5'-terminal "cap" structure. In 1975 Miura and Furutachi first reported the characteristic structure from cytoplasmic polyadenylated virus as presented by mGppppApGpUP... Their structures of the 5'-terminus of mRNAs can be described generally as mGppppX(m)pYpZ... (X, Y, and Z are nucleoside residues) and mGppp in a part of the confronting structure is called the "cap" of the mRNAs. Chemical synthesis of the confronting structure is necessary to study the relation between structure and functions of the 5'-terminus of mRNAs.

While the syntheses of α,β-, and γ-dinucleoside diphosphates have been well established, very little is known of the synthesis of 5' dinucleoside triphosphates. More recently, we have found a method for the synthesis of mGpppXpYpZ... another synthetic pattern, in which the pyrophosphorylation reaction is achieved by activating the 7-methylguanosine diphosphate component, is required for the synthesis of the 5'-terminus of mRNAs. A deprotected oligonucleotide bearing a 5'-phosphate group (pXpYpZ...) had to be employed to synthesize the 5'-terminal structure containing the cap because of the instability of 7-methylguanosine residue under alkaline conditions.

In this report, we describe a new method of pyrophosphorylation which is applicable to the synthesis of the 5'-terminus of mRNAs. P1-S-Phenyl P2-7-Methylguanosine-5'-pyrophosphorothioate (1a) was proposed as a pyrophorylating agent. The S-phenylthio group was chosen as an activatable group since the 5'-phenylphosphorothioate derivatives of guanine nucleotides have already been reported in our laboratory. From the results of Cook and Nussmann on the S-ethylthio group, 1a should be activated with iodine through the sulfoxide iodide intermediate which, in turn, reacts with the nucleotide (pX) to give the mGpX. The reagent 1a was prepared by the one-flask reaction of methyl phosphoroiodochloridate with benzethiol and 7-methylguanosine 5'-phosphate in dry pyridine by a modification of the procedure of Smr" and Rubinstein.

The α,γ-dinucleoside triphosphate, mGγpppA was synthesized by use of 1a as follows: A mixture of 1a (1 equiv) and adenosine 5'-phosphate (1 equiv) was treated with tri-n-butylamine (4 equiv) in formamide. The reaction mixture was concentrated in vacuo and the residue was dissolved in pyridine. Iodine (10 equiv) was added. After workup in the usual manner mGγpppG was obtained in 51% yield. Similarly, mGγpppG was obtained in 48% yield.

Instead of iodine, silver acetate and silver nitrate could be also used for the activation of phenylthio group, however, mercury(II) chloride or copper(II) chloride was not effective for this purpose.

The structures of the reported compounds were confirmed by phosphorus analysis by and enzymic degradation to pmG and the corresponding nucleoside 5'-phosphate with snake venom phosphodiesterase which cleaved not only internucleotide bonds but also pyrophosphate bonds. Penicillium phosphatase cleaved selectively pyrophosphate linkages.

P1-S-Phenyl P2-7-Methylguanosine-5'-Pyrophosphorothioate (1a): To a cold solution of methyl phosphoroiodochloridate (0.3 mmol) in dry pyridine (5 ml), benzethiol (0.3 ml, 3 mmol) is added dropwise within 20 min under vigorous stirring followed by gradual, portion-wise addition of well ground 7-methylguanosine 5'-phosphate (377 mg, 1 mmol). The mixture is stirred continuously for 5 h under cooling with an ice bath and left to stand at room temperature overnight. The reaction is quenched by addition of water (5 ml) and the solution is concentrated in vacuo to remove pyridine. The residue is dissolved in water (15 ml). The aqueous solution is washed with ether (3 x 10 ml) to remove benzethiol and concentrated to a small volume. It is applied to a column chromatography on Dowex 1 x 2 (Cl- form); 1a is eluted using the mixed solvent system: 0.01 normal hydrochloric acid/0.025 molar lithium chloride solution. The eluate is concentrated to a small volume while adjusting the pH to 7 by addition of lithium hydroxide solution. The concentrate is further applied to a column (3 x 20 cm) of charcoal and is washed with water to remove lithium salts. Washing has to be repeated until no lithium chloride is detected by a solution of silver nitrate. A mixture of 2 molar triethylammonium hydroxide carbonate solution (50 ml) and 50% ethanol (1000 ml) is used for the elution of 1a. The eluate is neutralized with Dowex 50W-X2 (pyridinium form) and concentrated to a small volume. It is then applied to paper (Toyo Roshi No. 51 A). Paper electrophoresis is performed in 0.2 molar phosphate buffer at pH 8; yields: 373 mg (monosodium salt, 64%).

Phosphorus analysis: mGγp/phaseate = 1.00/2.06.

U.V. (H2O, pH 7): λ_{max} = 247 (ε = 10,500); 280 nm (sh; λ_{max} = 235 nm (ε = 9300).

U.V. (H2O, pH 1): λ_{max} = 252, 280 nm (sh; λ_{max} = 231 nm.

Relative mobilities (Rm; = 0.75 (to pG) and 0.69 (to pmG).

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P-5-Pheny1 P-7-Adenosine 5'-Pyrophosphothioate (1b):
In a similar fashion, compound 1b is obtained; yield: 59%.
Phosphorus analysis: Ado/phosphate = 1.00/2.04.
U.V. (H2O): λmax = 258 nm (ε = 18000); λmin = 229 (ε = 8700).
Relative mobilities (Rm): 2.4 (to pA) at pH 3.5 and 1.0 (to pA) at pH 8.

P-Adenosine-5'-P-7-Methylguanosine-5' Triphosphate (2a):
A mixture of 1a (27 mg, 0.05 mmol) and adenosine 5'-phosphate (17 mg, 0.05 mmol) is treated with tri-n-butylamine (0.05 ml, 0.21 mmol) in formamide (0.2 ml). After removal of excess tri-n-butylamine in vacuo, the residue is dissolved in dry pyridine (0.5 ml). Iodine (127 mg, 0.5 mmol) is added and the mixture is stirred at room temperature overnight. After being concentrated in vacuo, the mixture is dissolved in water (10 ml) and washed with ether (3 × 5 ml). The aqueous layer is concentrated to a small volume and applied to paper (Toyo Roshi No. 51 A). Electrophoresis is performed in acetate buffer at pH 3.5; yield: 20 mg (diammonium salt, 51%).
Phosphorus analysis: Ado/phosphate = 1.00/2.93; pa/pm^3G = 1.00/0.91.
U.V. (H2O, pH 7): λmax = 257 nm (ε = 22500); λmin = 232 nm.
Relative mobilities (Rm): 0.95 (to pA) and 0.93 (to pG) at pH 3.5.

P-Guanosine-5'-P-7-Methylguanosine-5' Triphosphate (2b):
Similarly, m^5G^7pppG is obtained from the reaction of 1a (27 mg, 0.05 mmol) with tri-n-butylammonium salt of guanosine 5'-phosphate (22 mg, 0.05 mmol) in the presence of iodine (127 mg, 0.5 mmol) in dry pyridine (0.25 ml); yield: 19 mg (diammonium salt, 48%).
Phosphorus analysis: Guo/phosphate = 1.00/2.85; pG/pm^3G = 1.00/0.93.
U.V. (H2O, pH 7): λmax = 254 nm (ε = 18200); λmin = 230 nm.

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15 The S-ethylthio group, known to be a useful protecting group for phosphate in oligonucleotide synthesis, is removed by using iodine as well as activation by the same method to form pyrophosphate linkages: A. F. Cook, M. J. Holman, A. L. Nussbaum, J. Am. Chem. Soc. 91, 1522 (1969).