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Efficient Synthesis of γ–Methyl-Capped Guanosine 5'-Triphosphate as a 5'-Terminal Unique Structure of U6 RNA *via* a New Triphosphate Bond Formation Involving Activation of Methyl Phosphorimidazolidate Using $ZnCl_2$ as a Catalyst in DMF under Anhydrous Conditions

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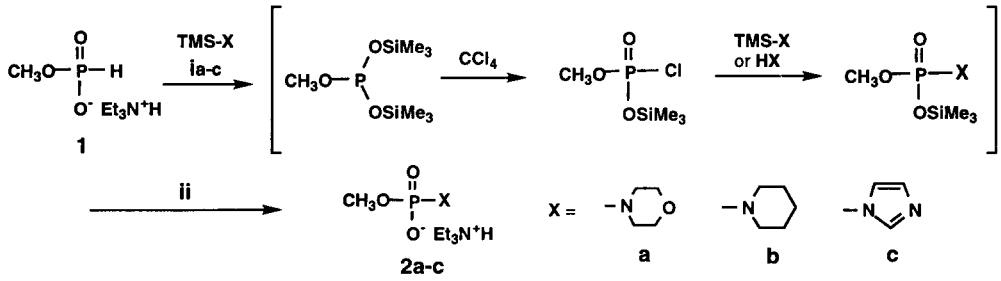
Abstract: P^1-Methyl P^3-(5'-guanosyl) triphosphate (CH_3pppG), which exists as a unique structure at the 5'-terminal region of U6 RNA, was synthesized in an excellent yield by methyl phosphorimidazolidate (**2c**) with the bis(tetrabutylammonium) salt of GDP in the presence of $ZnCl_2$ as an effective catalyst in dry DMF. © 1997 Elsevier Science Ltd.

U6 RNA is a small nuclear RNA and plays an important role in splicing of pre-mRNAs.[1] The 5'-terminus of U6 RNA has a unique methyl-capped structure, CH_3pppGUGC···, in which the γ-position of guanosine 5'-triphosphate is methylated. To clarify the mechanism of mRNA splicing, a chemical synthesis of U6 RNA and its partial fragments is highly desired. To date, however, the only published research has been that of Eckstein *et al.*[2] who synthesized CH_3pppG in a low yield using a sluggish diphenyl phosphorochloridate-mediated coulpling of GTP with methanol. Here, we report an effective method for the synthesis of CH_3pppG from GDP by use of a metal salt catalyst of $ZnCl_2$ for activation of methyl phosphorimidazolidate (**2c**) in anhydrous DMF under homongenous conditions.

Nucleoside 5'-phosphoromorpholidate or 5'-phosphorimidazolidate derivatives have frequently been utilized as the useful intermediates for the synthesis of nucleoside 5'-triphosphates and oligonucleotides containing a 5'-terminal triphosphate group such as pppA(2'-5')A(2'-5')A.[3] These active phosphoramidates have been successfully utilized because they can be used with a large excess amount (10-20 equiv) of inorganic pyrophosphate. In general, however, this method has not yielded satisfactory results when applied to the synthesis of dialkyl triphosphates. Particularly, in the synthesis of triphosphate derivatives containing guanosine derivatives such as CH_3pppG and $m^7G^{5'}$pppG, the desired products have been obtained in low yields due to the inherent poor solubility of the starting acceptor material, GMP or GDP, in organic solvents. To improve the solubility of these guanylic acid derivatives, lipophilic protecting groups of the exo-amino group or organic salts as counter cations of the phosphate group have been used.[4] However, the phosphoramidate derivatives have shown a lack of reactivity when used as the phosphoryl donors in the stoichiometric reaction with alkyl mono- or di-phosphate derivatives dissolved or suspended in organic solvents.[3,5] In fact, Wang *et al.* reported the use of 1*H*-tertrazole as a catalyst of guanosine 5'-phosphoromorpholidate in the synthesis of GDP-Fucose and related compounds.[6] On the other hand, Sawai *et al.* have recently reported an effective method for triphosphate bond formation from nucleoside 5'-phosphorimidazolidates and nucleoside 5'-diphosphates in aqueous solution by use of Mn^{2+} or Cd^{2+} ion as the catalyst.[7] This method has proved to be practically useful since water-soluble unprotected nucleotidic substances can be used. The only drawbacks in this strategy are the rather slow reaction and the serious competitive hydrolysis of phosphorimidazolidate derivatives in aqueous solution, such that the yield of products did not exceed 40% for 4 days when 1 equiv of phosphorimidazolidates was used.[7] In consideration of these precedents, we have searched for a new method for the synthesis of CH_3pppG *in homogeneous solution using anhydrous organic solvents.*

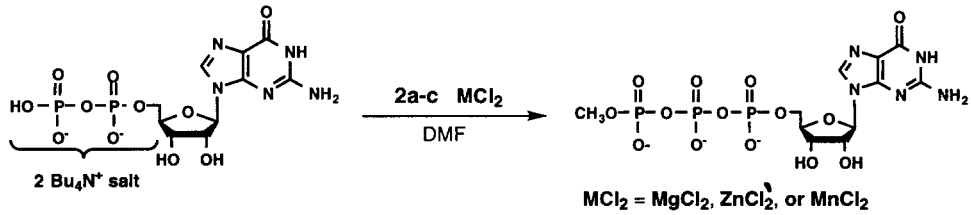
Recently, we have developed a method for the oxidative phosphoramidate formation using trimethylsilylamines and CCl_4 from nucleoside 5'-*H*-phosphonate derivatives.[8] We applied this method to the preparation of methyl phosphorimidazolide **2c** required for the synthesis of CH_3pppG. Hydrolysis of dimethyl phosphonate with THF-Et_3N-H_2O (8:1:1, v/v/v) under reflux for 30 min gave quantitatively methyl phosphonate **1** [^{31}P NMR (DMSO-d_6–DMF, 1:8, v/v): δ 5.85 ppm], which was converted *in situ* to methyl phosphorimidazolidate **2c** (^{31}P NMR : δ -7.5 ppm) by treatment with 1-(trimethylsilyl)imidazole in the presence of CCl_4 followed by addition of methanol (Scheme 1). All the volatile materials were removed by evaporation under reduced pressure to give **2c** (purity: > 99%), which *in situ* was used as a methoxyphosphoryl donor for the synthesis of CH_3pppG. In a similar manner, methyl phosphoromorpholidate **2a** (^{31}P NMR: δ 6.99 ppm) and methyl phosphoropiperididate **2b** (^{31}P NMR; δ 9.68 ppm) were synthesized *in situ* from **1** by treatment with 1-(trimethylsilyl)morpholine and 1-(trimethylsilyl)piperidine, respectively.



Scheme 1

(i) a. 1-(Trimethylsilyl)morpholine (2 equiv), b. 1-(Trimethylsilyl)piperidine (2 equiv), c. 1-(Trimethylsilyl)imidazole (1.2 equiv), triethylamine (4 equiv), CH_3CN-CCl_4 (1:1, v/v), r.t., 1.5 h; (ii) MeOH, r.t., 1 min.

The triphosphate bond formation was carried out as follows. Equimolar amounts of methoxyphosphoryl donor **2a-c** (0.05 M) and guanosine-5'-diphosphate bis(tetrabutylammonium) salt (0.05 M, purity: 93% contaminated with 7% of GMP)[9] were mixed in dry DMF (0.4 ml). First, these reactions were carried out in suspension in the absence of a metal salt and monitored by anion exchange HPLC (Scheme 2) .

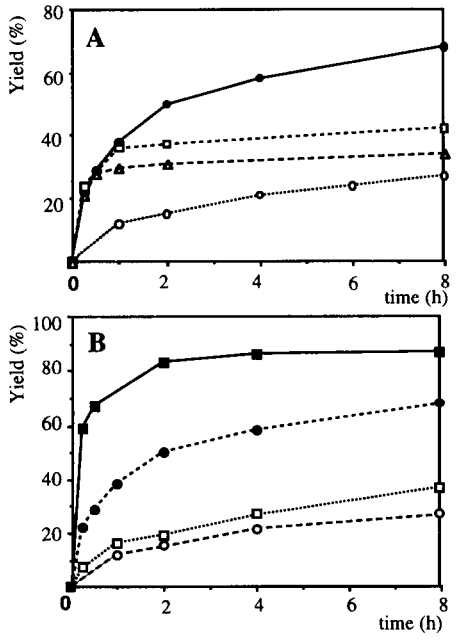


Scheme 2

As shown in Table 1, the reactions using **2a** and **2b** were rather slow and gave low yields of CH_3pppG even after 2 weeks, when half amounts of **2a** and **2b** remained unchanged as evidenced by ^{31}P NMR. Among the three reagents **2a-c**, **2c** was found to be the most reactive donor in this triphosphate bond formation, but the yield of CH_3pppG did not exceed 40%. This is because a half amount of **2c** decomposed during the reaction after 24 h along with formation of CH_3ppCH_3. Next, to an equimolar suspension of **2c** (0.05 M) and GDP bis(tetrabutylammonium) salt (0.05 M) in dry DMF was added 4 equiv of an appropriate divalent metal chloride ($MgCl_2$, $ZnCl_2$, or $MnCl_2$) (Scheme 2). The time course of the coupling reaction was analyzed by anion exchange HPLC (Figure 1A).

It is noteworthy that addition of these metal chlorides resulted in *clear homogeneous solutions*. In addition to this effect, the coupling reaction was considerably accelarated. In particular, addition of $ZnCl_2$ markedly improved the conversion of GDP to CH_3pppG. It is also interesting that among the three metal salts $ZnCl_2$ was the best catalyst in our anhydrous medium, since Sawai reported that $MnCl_2$ was the most effective in aqueous

solution while $ZnCl_2$ was less effective. It seems that in aqueous solution the Zn^{2+} ion might be solvated by water to lose its activity as a Lewis acid. Since the Mg^{2+}, Mn^{2+} and Zn^{2+} ions have high affinity for the phosphate oxygen,[10] it is likely that these ions play a role as a template as proposed by Sawai *et al*[11] so that both the coupling time and the yield were improved in such an organic solvent as DMF. Among the three metal ions tested, the Zn^{2+} ion has high affinity not only for the phosphate oxygen but also for the nitrogen atom of the methyl phosphorimidazolidate **2c**. Accordingly, this metal cation can also serve as a Lewis acid which can activate the P-N bond. Therefore, the $ZnCl_2$ mediated double activation of the phosphate and imidazole residues gave CH_3pppG in a better yield of ca. 70% after 8 h, as shown in Figure 1A.



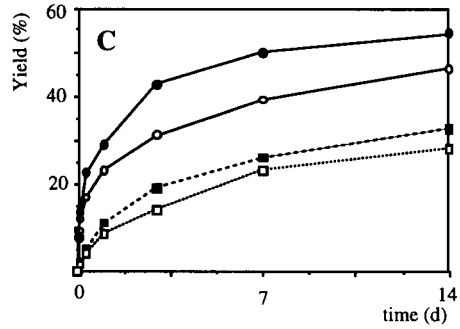


Table 1. Effects of Phosphoramidates 2a-c on the Synthesis of CH_3pppG

capping regent	time	yield of CH_3pppG
2a	14 d	35%
2b	14 d	28%
2c	1 d	39%

Figure 1. **A**: Effects of various metal chlorides (4 equiv) on the coupling reaction between **2c** and GDP giving rise to CH_3pppG. (●): $ZnCl_2$; (□): $MgCl_2$; (△): $MnCl_2$; (○): No additive. **B**: Effects of the addition of $ZnCl_2$. on the coupling yield of CH_3pppG. (○): No additive; (□): 1.2 equiv of $ZnCl_2$; (●): 4 equiv of $ZnCl_2$; (■): 10 equiv of $ZnCl_2$. **C**: Effects of addition of 4 equiv of $ZnCl_2$ on the coupling reaction of GDP with **2a** or **2b**. (●): **2a** + $ZnCl_2$; (○): **2b** + $ZnCl_2$; (■): **2a** alone; (□): **2b** alone.

Next, this reaction was carried out by using 1.2, 4 or 10 equiv of $ZnCl_2$. When 1.2 equiv of $ZnCl_2$ was used, the reaction mixture became suspension and the coupling yield was considerably decreased. In contrast, when 10 equiv of $ZnCl_2$ was added, the reaction was almost completed after 4 h and the yield of CH_3pppG was 87% (Figure 1B). Since the zinc ion can coordinate with many atoms, such as the phosphate oxygens and the imidazole residue of the guanine base in GDP and **2c**, more than 4 equiv of $ZnCl_2$ must be needed. A similar rate enhancement was observed by addition of $ZnCl_2$ when the coupling reaction was carried out by use of **2a** or **2b** (Figure 1C). However, the reactions under these conditions were slower than that of GDP with **2c** and took two weeks for completion.

When an equimolar mixture of **2c** and GDP (purity: 98% contaminated with 2% of GMP) was used, the yield of CH_3pppG did not exceed 90% because of the competitive decomposition of **2c**. To ensure these results, further experiments were performed under different conditions, as shown in Table 2. Thus, we found that, when this reaction was carried out using 2 equiv of **2c** in the presence of 8 equiv of $ZnCl_2$ in dry DMF, the yield of CH_3pppG was optimized to an almost quantitative yield of 98% after 4 h. When the reaction mixture obtained in Entry 5 was separated using DEAE Sephadex column chromatography, CH_3pppG could be isolated

in 90% yield with a purity of 98%. The structure of CH_3pppG was characterized by ^{31}P NMR, ^1H NMR and FAB Mass spectroscopy.[12]

Table 2. The Synthesis of CH_3pppG under Various Conditions[a]

Entry	**2c** equiv	$ZnCl_2$ equiv	time h	yield of CH_3pppG %
1	1	0	4 (24)	21 (39)
2	1	4	4 (24)	57 (72)
3	1	10	4	86
4	2	4	4 (8)	88 (92)
5	2	8	4	98

[a]The reactions were carried out at r.t using 20 mM GDP, 20 or 40 mM **2c** and 0, 80, or 200 mM $ZnCl_2$ in dry DMF (0.4 ml).

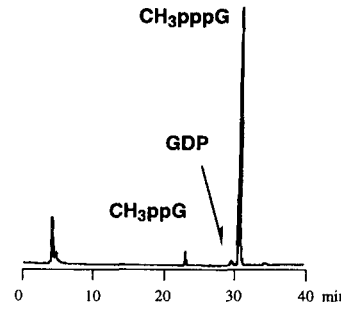


Figure 2. Anion exchange HPLC profile of the reaction mixture obtained after 4 h by using the conditions of Entry 5

In conclusion, a sufficient amount of $ZnCl_2$ in DMF was found to serve as an effective catalyst for triphosphate bond formation between GDP bis(tetrabutylammonium) salt and **2c.** This clear homogeneous reaction system could be applied to the chemical synthesis of other triphosphate bond-bridged naturally occurring products which exist at the 5'-terminal cap structure of mRNA or U1 RNA, as well as to the synthesis of γ-methyl-capped U6 RNA fragments. These studies are now under investigation.

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12. CH_3pppG: ^1H NMR (270 MHz, D_2O) δ 3.43 (3H, d, J_{PH}= 11.2 Hz, $POCH_3$), 4.02 (2H, m, 5'-H), 4.16, 4.32, 4.51 (1H, br, 2',3',4'-H), 5.74 (1H, d, $J_{1',2'}$= 4.9 Hz, 1'-H), 7.89 (1H, s, 8-H); ^{31}P NMR (109.25 MHz, D_2O) δ -8.54, -10.30 (d, $J_{\alpha,\beta} = J_{\gamma,\beta}$ = 18.3 Hz, α,γ–phosphate of triphosphate bridge), -21.47 (t, $J_{\alpha,\beta} = J_{\gamma,\beta}$ = 18.3 Hz, β–phosphate of triphosphate bridge): FAB MS Calcd for m/z $C_{11}H_{15}O_{14}N_5P_3Na_4$ $(M+Na)^+$ 625.9420. Observed for m/z 625.9409.

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