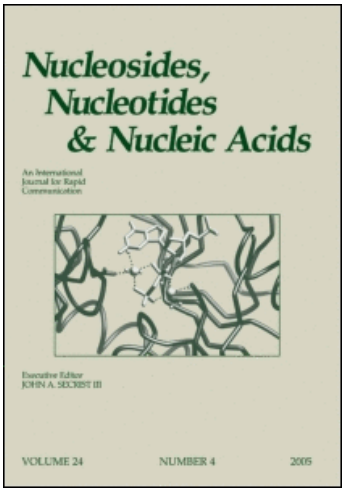

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SYNTHESIS AND SUBSTRATE VALIDATION OF CAP ANALOGS CONTAINING 7-DEAZAGUANOSINE MOIETY BY RNA POLYMERASE

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Life Technologies Corporation, Bioorganic Chemistry Division, Austin, Texas, USA

□ *An efficient synthesis of new cap analogs containing 7-deazaguanosine moiety such as* $m^7G[5']ppp[5']^{7\text{-deaza}}G$ *and* $m_2^{7,3'O}G[5']ppp[5']^{7\text{-deaza}}G$ *is described. The biological substrate validation of these new cap analogs is evaluated with respect to its capping efficiency and in vitro T7 RNA polymerase transcription using standard cap* $m^7G[5']ppp[5']G$ *as a control. The capping efficiency and HPLC data reveal that these new analogs are not the substrate for T7 RNA polymerase or SP6 RNA polymerase. The present study highlights the importance of the presence of nitrogen atom at N7-position of the guanosine moiety for the polymerase recognition.*

Keywords 7-Deazaguanosine; cap analogs; T7 RNA polymerase, in vitro transcription; capping efficiency

INTRODUCTION

The 5′-end of cellular and eukaryotic viral RNAs that are synthesized by various RNA polymerase contains a dinucleotide cap structure $m^7G[5']ppp[5']N$ (where N can be any one of the four nucleosides), in which a 7-methylguanosine residue is linked to the 5′-end of the transcribed RNA via a 5′-5′ triphosphate bridge (Figure 1).[1,2] The cap plays an important role in several stages of gene expression, such as promoting mRNA splicing, facilitating mRNA export to the cytoplasm, and protecting mRNA against nucleolytic degradation.[3,4] The presence of cap structure serves as binding site of enzymes and in particular, cap structure is being specifically recognized by the protein eukaryotic initiation factor eIF4E.[5] The presence of the positive charge of the *N*7-methylguanosine base is crucial in recognition that acts as a docking point for the cap-protein complex. In addition to eIF4E proteins, the cap structure is also recognized by other cap-binding proteins such as vaccinia virus methyltransferase VP39[6] and the CBC80/20 nuclear cap-binding complex.[7]

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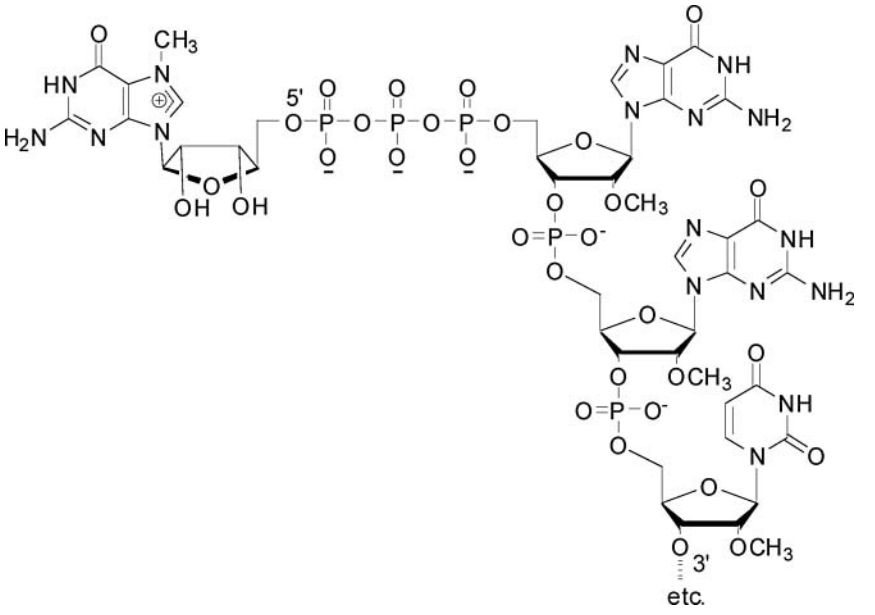


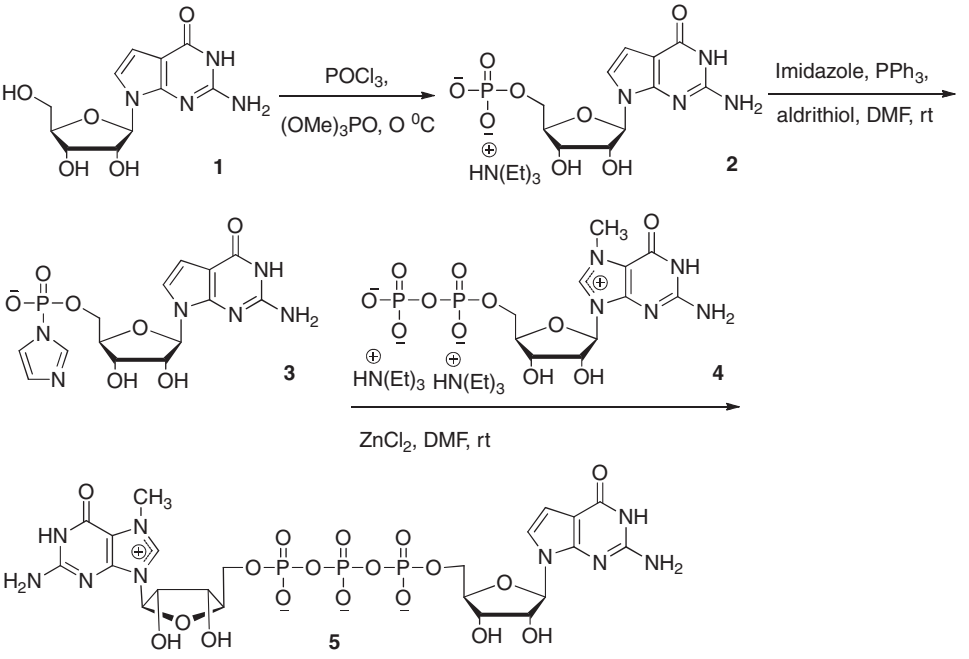
FIGURE 1 The chemical structure of the 5′ terminus of a 'capped' mRNA with standard mCAP.

The use of synthetic dinucleotide cap analog $m^7G[5']ppp[5']G$ has been the most widely used method for the in vitro synthesis of 5′-capped mRNAs as an initiation of transcription.[8] However, this analog results in the synthesis of two isomeric RNAs in approximately equal proportions depending upon the ionic conditions of the transcription reaction due to the presence of a 3′-OH group on both guanosine and m^7guanosine moieties in which both 3′-OH groups serve as the initiating nucleophile.[8] It should be noted that mRNAs possessing correctly incorporated transcripts, that is, $m^7G[5']ppp[5']G[pN]^n$, are properly recognized, while the reverse capped caps, that is, $G[5']ppp[5']m^7G[pN]^n$, are not recognized during translation.[8] This problem has been addressed by the use of 3′-OH chemical modification on m^7G moieties such as $m_2{}^{7,3'O}G$ [5′]ppp[5′]G and $m^7$3′dG[5′]ppp[5′]G. These analogs, called anti-reverse cap analogs, incorporate exclusively in the forward orientation and reverse-orientation is not possible because of the absence of a free 3′-OH group on the m^7G moiety.[9,10] The noteworthy feature is that these analogs display two-fold higher translational efficiency in vitro compared to RNAs capped with the conventional cap analog. In addition to 3′-OH chemical modifications on m^7 moiety, the chemical modification of the 2′-OH group also incorporates exclusively in the forward orientation even though the 2′-OH group is not responsible in the phosphodiester linkage.[11] While several substituted nucleosides such as m^7guanosine,[12,13] 2′-*O*-methylguanosine,[14] nucleosides (A,C,G or U),[15] 2′-deoxyguanosine[16], N^7-$O^{2'}$-dimethylguanosine[14], 2,6-diaminopurine,[17]

and N^6,N^6,$O^{2'}$-trimethyladensoine[18] have been used at the other end of m^7G moiety of the dinucleotide cap analog, to the best of our knowledge, no example using a 7-deazaguanosine moiety has been reported. The recent literature report reveals that 7-deazaguanosine triphosphate (DTP)-eIF4E binding is slightly greater than the guanosine triphosphate (GTP)-eIF4E binding based on free energy perturbation (FEP) simulations using Monte Carlo (MC) as the sampling technique.[19] It has been reported that the single nucleotide, 7-deaza-GTP incorporates at position +2 as efficiently as GTP, but it is much less able to incorporate at position +1.[20] Our continuing a interest in the design of new cap analogs for biological application prompted us to explore the possibility of synthesizing dinucleotides containing a 7-deazaguanosine moiety.[12–14,17,21–23] In this article, we report the first example of the synthesis of cap analogs containing 7-deazaguanosine moiety and its validation using RNA polymerase.

RESULTS AND DISCUSSION

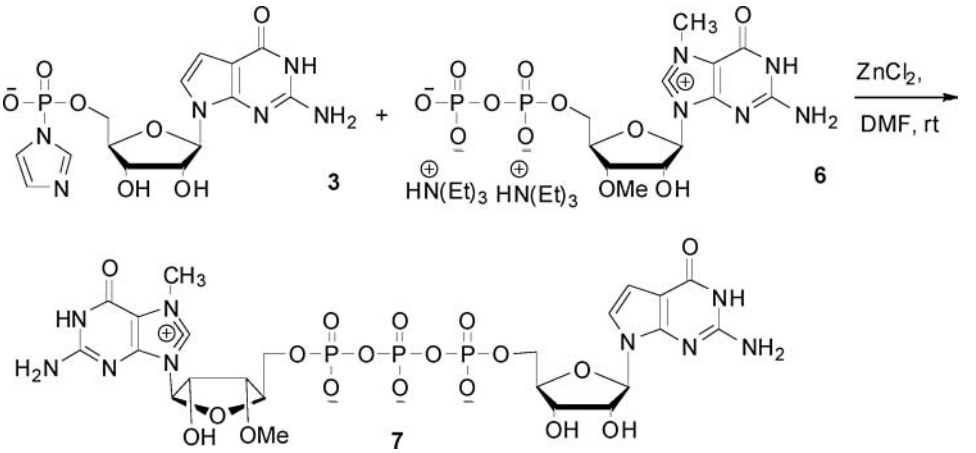
The reaction pathway leading to the formation of the desired $m^7G[5']ppp[5']^{7\text{-}deaza}G$ **5** for biological testing is depicted in Scheme 1. The monophosphorylation reaction of commercially available 7-deazaguanosine



SCHEME 1 Synthesis of $m^7G[5']ppp[5']^{7\text{-}deaza}G$ **5**.

1 was carried out using $POCl_3$ and trialkyl phosphate to furnish the corresponding 7-deaza GMP **2** in 79% yield. The monophosphorylated product **2** was converted into the corresponding imidazolide salt **3** using imidazole, triphenylphosphine, and aldrithiol in 89% yield. The other precursor, m^7GDP (**4**) was prepared by the methylation of guansoine diphospate using dimethyl sulfate as the methylating agent.[24] Finally, treatment of m^7GDP **4** with 7-deaza ImGMP (**3**) in the presence of zinc chloride as the catalyst and DMF as the solvent afforded $m^7G[5']ppp[5']^{7\text{-}deaza}G$ (**5**) in 64% yield.

The chemical modification at 3′ or 2′ on m^7G moiety has had a great impact on the nature of orientation and the translational efficiency. In order to study the effect of chemical modifications on m^7G moiety, we have synthesized $m_2^{7,3'O}G[5']ppp[5']^{7\text{-}deaza}G$(**7**). The synthesis of the desired cap analog **7** is depicted in Scheme 2. The required intermediate, $m_2^{7,3'O}GDP$ (**6**) was prepared in four steps starting from 3′-*O*-methylguanosine.[13] The coupling reaction of **3** with **6** in the presence of zinc chloride as the catalyst and DMF as the solvent afforded the corresponding cap analog **7** in 70% yield. The structure of compounds **5** and **7** was confirmed by ^1H and ^{31}P NMR and mass data.



SCHEME 2 Synthesis of $m_2^{7,3'O}G[5']ppp[5']^{7\text{-}deaza}G$ **7**.

In order to see whether the newly synthesized cap analogs **5** and **7** are substrates for RNA polymerase, the capping efficiency was determined in an in vitro transcription system by using pTri β actin vector (Life Technologies Corporation, Austin, TX, USA) in the presence of T7 RNA polymerase. During transcription, only purine nucleotide triphosphates (ATP and GTP) were used that resulted in a transcript containing only six nucleotides in length. All reactions were performed in the presence of (α-^{32}P) ATP to internally label the transcript. The gel shift assay was used to analyze the resulting 6-mer transcription products and the capping efficiency was determined by

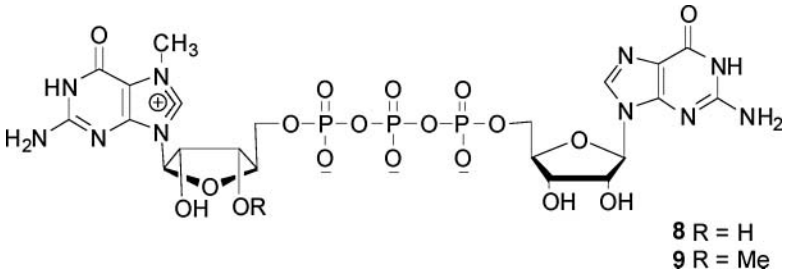


FIGURE 2 The chemical structure of standard mCAP **8** and ARCA **9**.

quantitating the intensities of capped versus uncapped RNA by normalizing with the background intensity. The chemical structure of standard cap $m^7G[5']ppp[5']G$ (**8**) and ARCA $m_2^{7,3'O}G[5']ppp[5']G$ (**9**) is depicted in Figure 2. From the data, it appears that the capping efficiencies of mCAP **8**, ARCA **9** and **5** are 74%, 71%, and 64%, respectively. The capping efficiency is slightly higher because of the "plus" polymerase, which has a single-base active-site mutation. T7 RNA Polymerase-Plus and SP6 RNA Polymerase-Plus by Life Technologies Corporation are recombinant enzymes that are encoded by DNA sequences having single-base active-site mutations in the respective T7 or SP6 RNA polymerase gene. These active-site mutations enable the corresponding T7 or SP6 polymerase to incorporate NTPs into full-length transcripts much more efficiently than the corresponding wild-type enzymes, while retaining the same catalytic activity for incorporation of canonical NTPs and the same high promoter specificity as the respective wild-type T7 or SP6 RNA Polymerase. Unlike **5**, the ARCA version of cap analog **7** does not show any 5′-capped RNA bands for either T7 and SP6 RNA polymerase, indicating the absence of any incorporation products in a correct orientation (Figure 3).

In order to determine the incorporation orientation of new cap analogs **5** and **7**, RNAs were synthesized similar to the capping assay experiment except for the addition of radioactive material and that the crude reaction mixtures were analyzed by HPLC (Figure 4). From the HPLC data, it seems that the standard cap **8** shows two peaks at 6.92 and 6.97 minutes indicating the presence of both reverse and forward orientation products, whereas ARCA **9** shows a single peak at 6.95 minutes due to the presence of exclusive forward orientation product. The HPLC data for the new cap analog **7** does not show any peak around 6.95 minutes indicating the absence of any orientation products, whereas cap analog **5** shows a single peak at 6.99 minutes indicating the presence of reverse orientation product based on the results of ARCA version of cap analog **7**. Based on the HPLC data and capping efficiency data, it is clear that the cap analog **7** is not a substrate for T7 RNA polymerase. The capping efficiency for **5** is 64%, which could be accounted from the reverse orientation product, that is, $^{7\text{-deaza}}G[5']ppp[5']m^7G[pN]^n$.

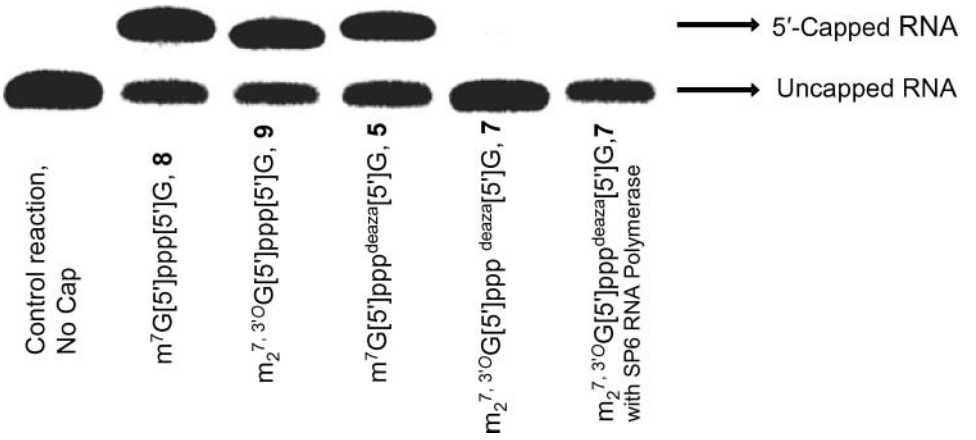


FIGURE 3 Twenty percent of dPAGE gel showing capping efficiency of standard mCAP **8**, ARCA **9**, deazacap **5**, and ARCA Deazacap **7** with T7 RNA polymerase. The last lane shows ARCA Deazacap **7** with SP6 RNA polymerase. The control reaction was normal in vitro transcription reaction, in which no cap analog was added. Radiation in the gel bands of interest was quantified by a phosphorimager (GE Healthcare, USA).

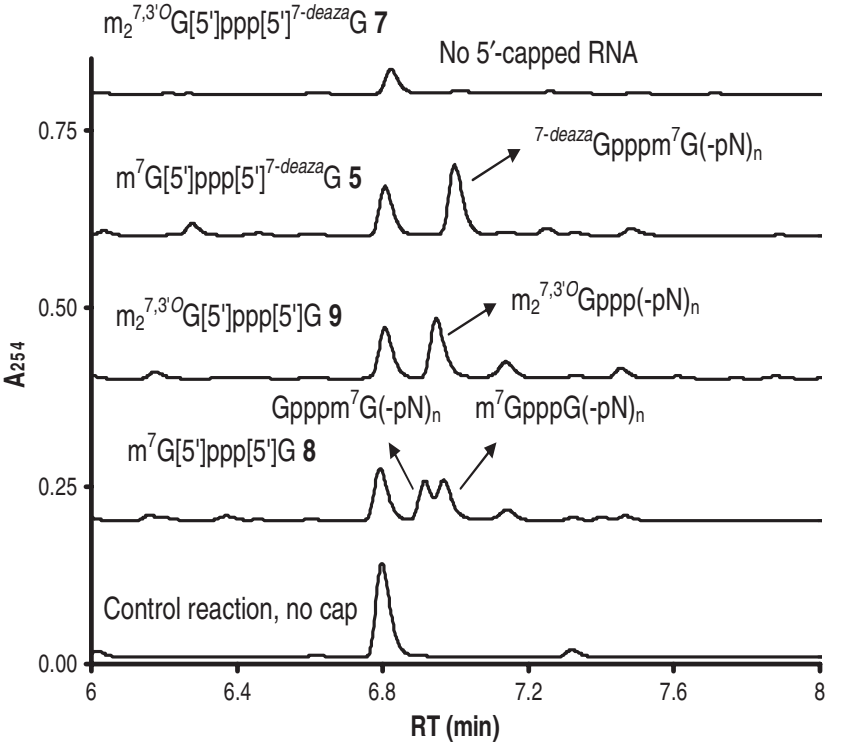


FIGURE 4 Ion exchange HPLC analysis of forward and reverse orientation of 5′-capped RNA by using mCAP **8**, ARCA **9**, deazacap **5**, and ARCADeazacap **7**.

It is to be noted that the reverse orientation product will not be recognized during translation.[8]

It is interesting to compare the results of mCAP **8** and ARCA **9** with cap analogs **5** and **7**. The striking structural feauture of cap anlogs **5** and **7** with mCAP **8** and ARCA **9** is the absence of a nitrogen atom at the N7-position of the guanosine moiety. Although the exact reason for the reactivity differences is not entirely clear, it seems from the present results that the presence of nitrogen atom in the 7-position of the guanosine is necessary for the recognition, in addition to the presence of m^7G moiety.[20]

In conclusion, we have reported the first example of the synthesis of cap analogs bearing 7-deazagunaosine moiety such as $m^7G[5']ppp[5']^{7\text{-}deaza}G$ and $m_2{}^{7,3'O}G[5']ppp[5']^{7\text{-}deaza}G$. The capping efficiency and HPLC data reveals that cap analogs **5** and **7** are not substrates for T7 or SP6 RNA polymerase. The present study establishes the importance of the presence of the nitrogen atom at 7-position of the guanosine moiety for recognition.

EXPERIMENTAL

^1H NMR spectra were recorded in $CDCl_3$ on a Bruker 400. Chemical shifts are reported in ppm, and signals are described as s (singlet), d (doublet), t(triplet), q (quartet), and m (multiplet). Electrospray ionization (ESI) mass was recorded on a Applied Biosystems/Sciex MDX API 150 model (Applied Biosystems, USA). HPLC was run on a Waters 2996 (Waters Corporation, USA) using ion exchange column.

Synthesis of 7-Deaza GMP (2)

To a stirred solution of $POCl_3$ (1.40 g, 12.12 mmol) and $(MeO)_3PO$ (25.0 mL) at 0°C under argon atmosphere, 7-deaza guanosine **1** (1.40 g, 4.05 mmol) was added and the reaction mixture was stirred for 4 hours at 0°C. After 4 hours, 50.0 mL water was added to the reaction mixture. The resulting reaction mixture was washed with dichloromethane (2 × 50 mL) to remove phosphorylating agent. The collected aqueous solution was adjusted to pH 1.5 and allowed to stir at 4°C for 15 hours. After 15 hours, the aqueous solution was adjusted to pH 6.5 and loaded on a DEAE Sepharose column The desired product was eluted using a linear gradient of 0–1M TEAB (triethylammonium bicarbonate, pH 7.5) and the fractions containing the product were pooled, evaporated and dried in a vacuum desiccator over phosphorous pentoxide to give a fine white powder **2** (Yield: 1.81 g, 79%).

Synthesis of 7-Deaza ImGMP (3)

To a stirred solution of 7-deaza GMP (1.31g, 2.99 mmol) in 30 mL dry DMF, imidazole (0.92 g, 14.92 mmol), triphenylphosphine (1.49 g, 5.99 mmol), aldrithiol (1.25 g, 6.00 mmol), and triethylamine (0.29 g, 2.97 mmol)

were added. The reaction mixture was stirred under argon atmosphere at room temperature for 16 hours. To a solution of sodium perchlorate (2.0 g) in 100 mL acetone in a centrifuge tube at 0°C, the above reaction mixture was added slowly for 5 minutes. The resulting mixture was centrifuged and the supernatant liquid was removed. The solid was ground with a new portion of acetone (100 mL), cooled, and centrifuged again. This process was repeated two more times, and the resulting solid was dried in a vacuum desiccator over P_2O_5 to give a white powder **3** (Yield: 1.10 g, 89%).

Synthesis of $m^7G[5']ppp[5']^{7\text{-}deaza}G$ (5)

To a stirred solution of m^7GDP **4** (0.20 g, 0.29 mmol) and 7-deaza ImGMP **3** (0.132 g, 0.28 mmol) in 10 mL dry DMF, zinc chloride (0.20 g, 1.40 mmol) was added under argon atmosphere and the reaction mixture was stirred at room temperature for 60 hours. After 60 hours, the reaction mixture was added to a solution of EDTA disodium (0.52 g, 1.40 mmol) in 100 mL of water at 0°C. The resulting aqueous solution was adjusted to pH 6.5 and loaded on a DEAE Sepharose column. The desired product was eluted using a linear gradient of 0–1M TEAB and the fractions containing the product were pooled, evaporated and concentrated to 10 mL TEA salt of **5**. The resulting 10 mL was passed through a Strata-X-AW column and washed with 10 mL water followed by 10 mL MeOH. Then, the desired compound was eluted with 15 mL of $NH_4OH/MeOH/H_2O$ (2/25/73) and the collected solution was evaporated and dried to give a fine white powder **5**. (Yield: 0.17 g, 64%). Data for **5**. ^{1}H NMR (D_2O, 400 MHz) δ 7.03 (d, $J = 3.6$ Hz, 1H), 6.41 (d, $J = 4.0$ Hz, 1H), 5.91 (d, $J = 6.4$ Hz, 1H), 5.87 (d, $J = 2.4$ Hz, 1H), 4.51–4.20 (m, 10H), 3.98 (s, 3H); ^{31}P NMR (D_2O, 162 MHz) δ −10.34 (d, $J = 18.1$ Hz, 1P), −10.43 (d, $J = 19.4$ Hz, 1P), −21.87 (t, $J = 18.6$ Hz, 1P); MS (m/z): 800 $[M\text{-}H]^-$.

Synthesis of $m_2^{7,3'O}G[5']ppp[5']^{7\text{-}deaza}G$ (7)

To a stirred solution of $m_2^{7,3'O}$GDP **6** (0.16 g, 0.24 mmol) and 7-deaza ImGMP **3** (0.10 g, 0.23 mmol) in 10 mL dry DMF, zinc chloride (0.16 g, 1.18 mmol) was added under argon atmosphere and the reaction mixture was stirred at room temperature for 60 hours. After 60 hours, the reaction mixture was added to a solution of EDTA disodium (0.44 g, 1.18 mmol) in 100 mL of water at 0°C. The resulting aqueous solution was adjusted to pH 6.5 and loaded on a DEAE Sepharose column. The desired product was eluted using a linear gradient of 0–1M TEAB and the fractions containing the product were pooled, evaporated, and concentrated to 10 mL TEA salt of **7**. The resulting 10 mL was passed through a Strata-X-AW column and washed with 10 mL water followed by 10 mL MeOH. Then, the desired compound was eluted with 15 mL of $NH_4OH/MeOH/H_2O$ (2/25/73) and

the collected solution was evaporated and dried to give a fine white powder **7**. (Yield: 0.14 g, 70%). Data for **7**. ^{1}H NMR (D_2O, 400 MHz) δ 7.01 (d, J = 3.6 Hz, 1H), 6.42 (d, J = 4.0 Hz, 1H), 5.90 (d, J = 6.4 Hz, 1H), 5.87 (d, J = 3.6 Hz, 1H), 4.68 (t, J = 4.4 Hz, 1H), 4.51 (t, J = 6.0 Hz, 1H), 4.43–4.20 (m, 7H), 4.10 (t, J = 5.2 Hz, 1H), 4.01 (s, 3H), 3.49 (s, 3H); ^{31}P NMR (D_2O, 162 MHz) δ −10.17 (d, J = 21.0 Hz, 1P), −10.41 (d, J = 20.7 Hz, 1P), −21.90 (t, J = 19.8 Hz, 1P); MS (m/z): 814 $[M\text{-}H]^-$.

Gel Shift (Capping Efficiency) and HPLC Assay

The capping efficiency of new cap analogs **5** and **7** was compared with the standard cap $m^7G[5']ppp[5']G$ (**8**) and ARCA $m_2^{7,3'O}G[5']ppp[5']G$ (**9**). The pTri β actin template (Life Technologies Corporation) has all three promoters embedded in the template for T7, SP6, and T3 RNA polymerase. The pTri β actin template was used in an in vitro transcription reaction omitting pyrimidine nucleotides, resulting in the termination of transcription after the first 7 coded nucleotides, all purines. Syntheses of the capped and uncapped oligoribonucleotides performed by using the MAXIscript kit (Life Technologies Corporation) following manufacturer's protocol. Typically, 20 μl of the transcription reactions contained the following final concentrations of components: linearized pTri β actin vector template, 25 ng/μl (0.5 μg total); ATP, 2 mM; GTP, 0.4 mM; standard mCAP (**8**), ARCA (**9**), $m^7G[5']ppp[5']^{7\text{-}deaza}G$ (**5**), and $m_2^{7,3'O}G[5']ppp[5']^{7\text{-}deaza}G$ (**7**), 1.6 mM each in separate reaction; reaction buffer, 1X; T7 RNA Polymerase-Plus, 20 units/μl; and (α-^{32}P) ATP, 800 (Ci/mmol). In the control reaction, no cap analog was added. For SP6 RNA polymerase reaction, the same experimental conditions as above were used except the GTP was replaced with UTP 0.4 mM and **7**, and the transcription reaction was carried out in presence of SP6 RNA Polymerase-Plus. The transcription reactions were incubated at 37°C for 2 hours, after which the reaction mixtures (10 μl) were then applied to a 20% dPAGE gel. Radiation in the gel bands of interest were quantified by a phosphorimager (GE Healthcare). In order to determine the orientation of the incorporated cap analog the HPLC assay was developed. For this purpose, the above transcription reaction was scaled up 3 times and the use of (α-^{32}P) ATP was eliminated from the reaction mixture. The remaining plasmid pTri β actin was hydrolyze by adding 6 μl of turbo DNase to the reaction mixture, and further incubated at 37°C for 15 minutes. The resulting crude reaction mixture was used as such for HPLC analysis using anion exchange column.

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