Recent Developments in 5′-Terminal Cap Analogs: Synthesis and Biological Ramifications

Anilkumar R. Kore,* Irudaya Charles, Muthian Shanmugasundaram, Zejun Xiao and Richard C. Conrad

Ambion, Inc., An Applied Biosystems Business, Bioorganic Chemistry Division, 2130, Woodward Street, Austin, TX 78744-1832, USA

Abstract: The 5′-terminus of eukaryotic messenger RNA (mRNA) molecules contains a unique 'cap' structure: 7-methylguanosine (m^7G) linked by a 5′-5′-triphosphate bridge to the first nucleoside in the standard polymer chain. This 5′-cap is recognized by numerous enzymes involved in the transport and translation of mRNA, as well as its processing, both in terms of generating mature mRNA from the initial transcript and its natural degradation. *In vitro* generation of capped RNAs uses a cap analog in which two nucleosides are joined in a 5′-5′ triphosphate linkage, allowing incorporation of the cap along with the first nucleotide in the polymer chain. The conventional cap analog, $m^7G[5']ppp[5']G$, has a 3′-OH on both nucleoside moieties, resulting in the incorporation of the dinucleotide in either orientation. This results in one-third to one-half of the mRNAs containing a non-methylated cap and a methylated initial nucleotide. These 'reverse cap' structures bind poorly to elF4E and other cap binding proteins, greatly reducing the overall translational efficiency *in vitro* and biological activity *in vivo*. This drawback of the conventional cap analog has been addressed by creating an "anti-reverse cap analog" (ARCA), $m_2^{7,3'\text{-}O}G[5']ppp[5']G$, in which the 3′-OH of the ribose connected to the m^7Guo moiety is replaced with a 3′-*O*-methyl group, allowing incorporation only in the forward orientation due to the presence of the single extendable 3′-OH group. In addition to the chemical modification at the 3′-OH group of the ribose connected to the m^7G moiety, modifications at the 2′-OH group also preclude attachment of a second nucleotide by polymerase, again forcing the forward orientation. This mini-review focuses on recent developments in the synthesis of novel cap analogs, highlighting modifications at several positions including; the heterocyclic bases, the sugars, and the α-, β-, and γ-phosphates in the triphosphate chain, and assessing their impact on biological activity.

Key Words: Cap analogs, capping efficiency, *in vitro* transcription, translation efficiency, luciferase activity, HeLa cells, anti-reverse cap analog (ARCA), elF4E, cap analog inhibition, mammalian cells.

1. INTRODUCTION

Eukaryotic cap-dependent gene regulation at the post-transcriptional level involves recognition of specific molecular structures on the mRNA by protein factors. These structures include a 5′-"cap" and a tract of several hundred adenosine nucleotide residues at the 3′-end, both of which are added after transcription. The 5′-cap structure consists of m^7G (N^7-methyl guanosine) nucleoside connected to the first nucleoside of mRNA by a 5′-5′-triphosphate bridge. The addition of the 5′-cap requires three steps in *in vivo*. Nascent pre-mRNAs have a triphosphate group at their 5′-termini which is first converted to a diphosphate. This pyrophosphate group is the substrate for the addition of a guanosine monophosphate moiety in a phosphodiester-bond formation reaction, creating a triphosphate bridge between the 5′-OH groups of the terminal nucleoside and the added guanosine. Finally, a methylase adds a methyl group from S-adenosylmethionine (SAM or AdoMet) to the N^7 group of the cap's guanine base moiety to form a structure m^7GpppN, where m represents the methyl group and N can be any nucleotide, as shown in Fig. (**1**) [1, 2]. This unique structure is found in various viral [3-12] and cellular mRNAs [11, 13-16]. The mRNA cap plays vital roles during the life cycle of mRNA [1], the primary one arguably being a binding site for eukaryotic translational initiation factor eIF4E, initiating the translation of the mRNA to protein [17-21]. In addition to this, its presence has been shown to be required for the accurate and efficient splicing of pre-mRNA [22-24] in the nucleus, transport of the processed mRNA from the nucleus to cytoplasm [25, 26], and protection against 5′-exonucleases [27-30] in the cytoplasm until it is removed in the process of mRNA turnover [31].

Many current practices in Molecular Biology utilize RNA synthesized *in vitro* of known DNA templates. Bacteriophage RNA polymerases, especially those from the T3, T7 and SP6 phage strains, are especially useful for these procedures, as they initiate transcription of known sequences and they create hundreds to thousands of copies of each template. In some applications, notably *in vitro* translation systems using reticulocyte or wheat germ extracts and cell culture transfections, the presence of the cap is necessary to ensure efficient translation. Although the enzymes responsible for the sequential addition of the cap as described above can be used *in vitro*, the process is very inefficient. A much more efficient method is to introduce the cap structure into an RNA transcript during incorporation of the first nucleotide in *in vitro* transcription. In this method, the reaction mixture contains not only all four ribonucleotide triphosphates, but also a 'cap analog' that contains the initial nucleotide pre-linked to a m^7G by a 5′-5′ triphosphate bond. Since the initial nucleotide is usually G, the synthetic cap dinucleotide most commonly used has the form m^7GpppG.

*Address correspondence to this author at the Ambion, Inc., An Applied Biosystems Business, Bioorganic Chemistry Division, 2130, Woodward Street, Austin, Texas 78744-1832, USA; Tel: +1 512 721 3589; Fax: +1 512 651 0201; E-mail: anil.kore@appliedbiosystems.com

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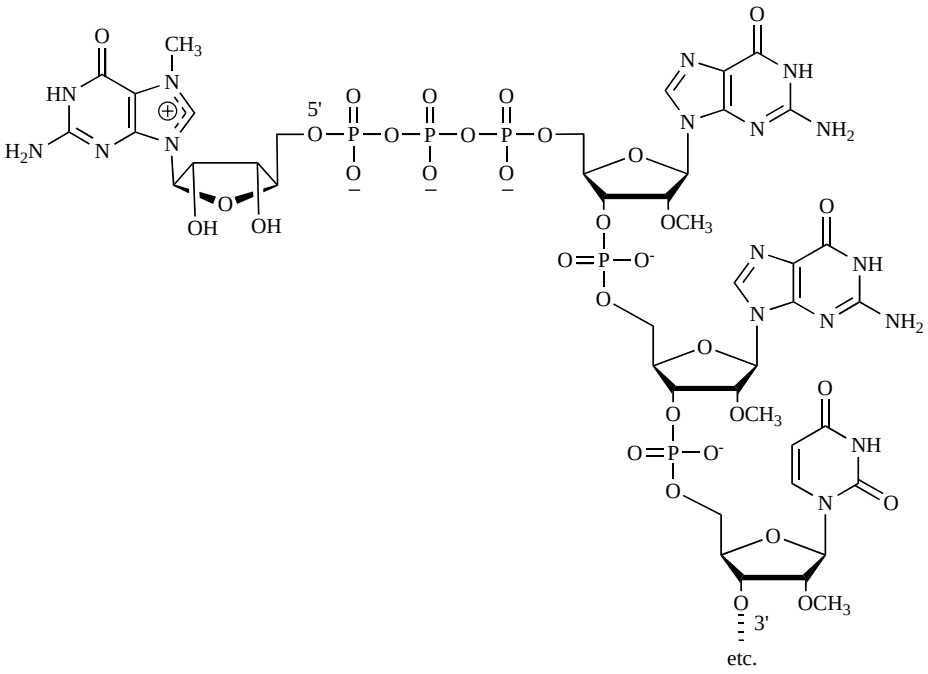


Fig. (1). The chemical structure of the 5' terminus of a 'capped' mRNA.

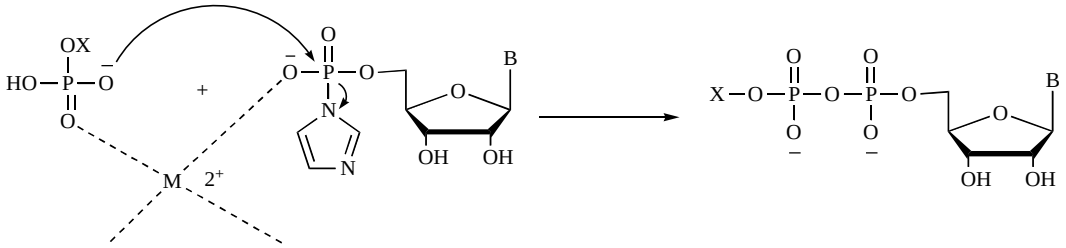
The polymerase can only use this analog to initiate transcription, since incorporation of subsequent nucleotides relies on the presence of a free 5′-triphosphate group to initiate nucleophilic attack on the 3′-OH of the previous riboguanosine. Cleavage of the bond between the α- and β- phosphate groups of this next templated nucleoside triphosphate to release pyrophosphate provides the energy to drive the reaction. However, the phage RNA polymerases can fit either nucleoside into their active site since both ends of the standard synthetic cap dinucleotide contain a free 3′-OH riboguanosine. This will direct either the methylated or the non-methylated G can serve as the intiating nucleotide. In practice, the resulting capped RNA obtained from *in vitro* transcription using the standard cap analog contains 30-50% of the products containing a "reverse cap", [Gpppm^{7}GpNm] instead of exclusively forward-capped RNA [m^{7}GpppGp Nm]. The absence of m^{7}G at the cap site of the RNA transcript has significant disadvantages in cap-dependent translation such as deranged transport of reverse capped mRNA in the cytoplasm and poor translational efficiency. This issue has been addressed, to some extent, by using synthetic anti-reverse cap analogs which do not carry a free hydroxyl group at 3′-end of m^{7}G, thereby blocking transcription elongation from this side of the analog. This strategy gave exclusive forward oriented capped mRNA with higher translational efficiency. This review focuses on various modifications to the 5′-cap to reduce or eliminate reverse capping, increase capping efficiency, and improve translational efficiency, and also details mRNA synthesis.

CHEMICAL SYNTHESIS OF CAP ANALOGUES

The most commonly used method to make synthetic cap analogs involves the coupling between two nucleotides of either same or different with one of them being activated by the presence of a good leaving group. It is believed that activated nucleotide acts as an electrophile, while the non-activated nucleotide acts as a nucleophile. There are three different classes of activation methods available in the literature, involving the use of: phenylthio and methoxyphenylthio groups; imidazolide and morpholidate groups; and 5-chloro-8-quinolyl as leaving groups [32]. The general method to make simple cap analogs has been extensively reviewed in 2005 [32]. This present review details the synthesis of various sugar-modified cap analogs, phosphate modifications, and tetranucleotides using imidazolide chemistry.

In general, the coupling reaction using an imidazolide activating group is promoted by the presence of a divalent metal catalysts such as $MnCl_2$, $MgCl_2$, $CdCl_2$, $CaCl_2$, and $ZnCl_2$ [33-35]. This reaction has been carried out in aqueous or anhydrous conditions. Although there hasn't been protecting group or hydrophobic counter ion requirements and solubility issues, the chemistry using an aqueous solutions is limited, because of the increased reaction time (typically days) and poor yields of final cap analog. The recent discovery of the use of zinc chloride catalyzed coupling reaction under anhydrous condition using DMF as the solvent paves the way for a wide variety of substituted cap analogs [36]. The prerequisite for the non-imidazolide nucleotide is that it should have a hydrophobic counter ion. The typical nucleotide coupling involves reaction of the triethylammnonium salt of GDP with ImGMP, using the $ZnCl_2$/DMF system described above, which affords the corresponding cap analogs with improved yields. Our experience indicates that the coupling reaction under anhydrous conditions is superior over aqueous conditions in terms of both reaction time and yield.

While the exact mechanistic function of a metal ion during the coupling reaction is not entirely clear, it is likely that it brings the phosphorimidazolide of ImpN and the phosphate group of the phosphate donor into close proximity by coordination (Scheme **1**) [35]. In other words, it acts as a



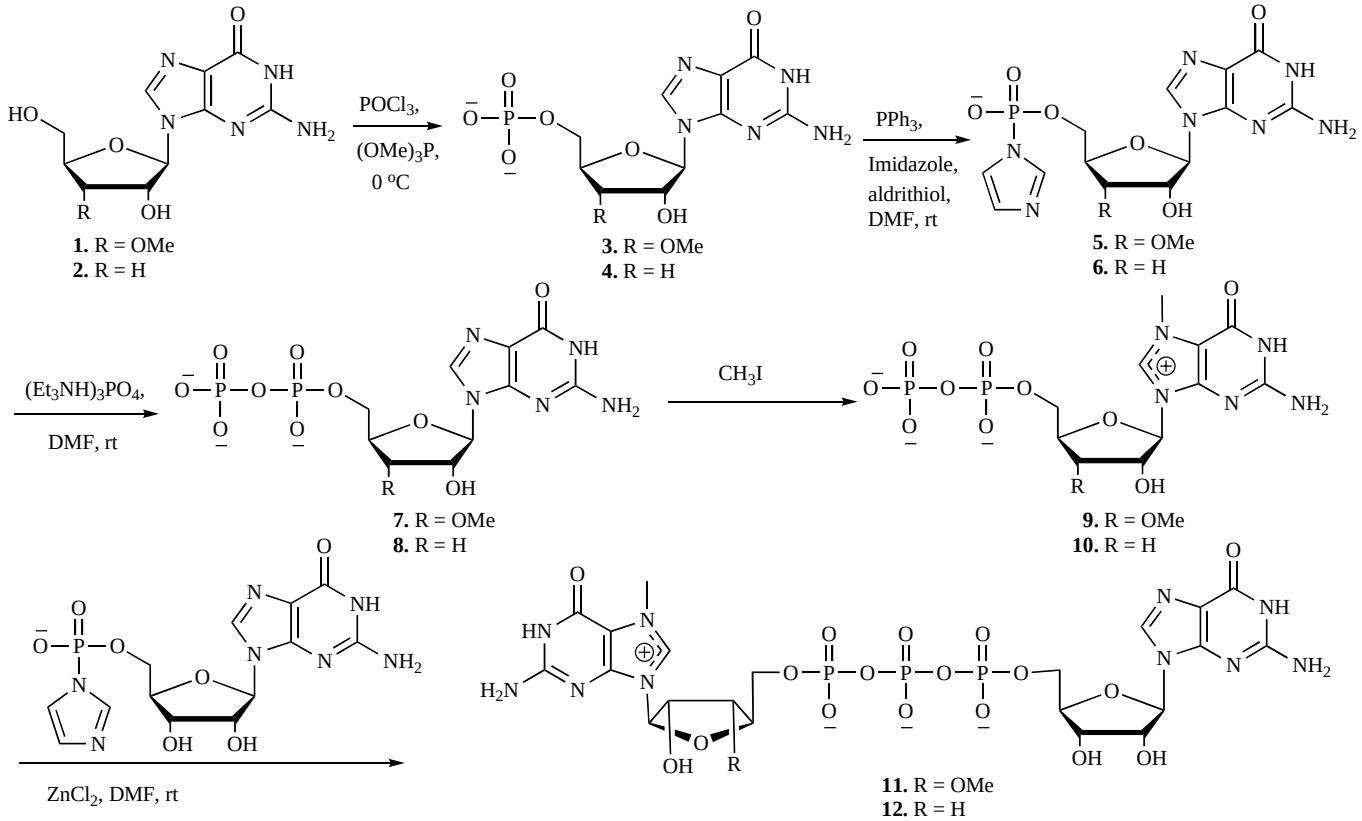
Scheme 1. Mechanistic Approach for the Metal-Catalyzed Pyrophosphate Bond Formation.

template for the reaction. The reaction involves displacement of a leaving group by nucleophilic attack of the phosphate donor on the phosphorus atom connected to the activating (imidizole) group, leading to the formation of pyrophosphate bond.

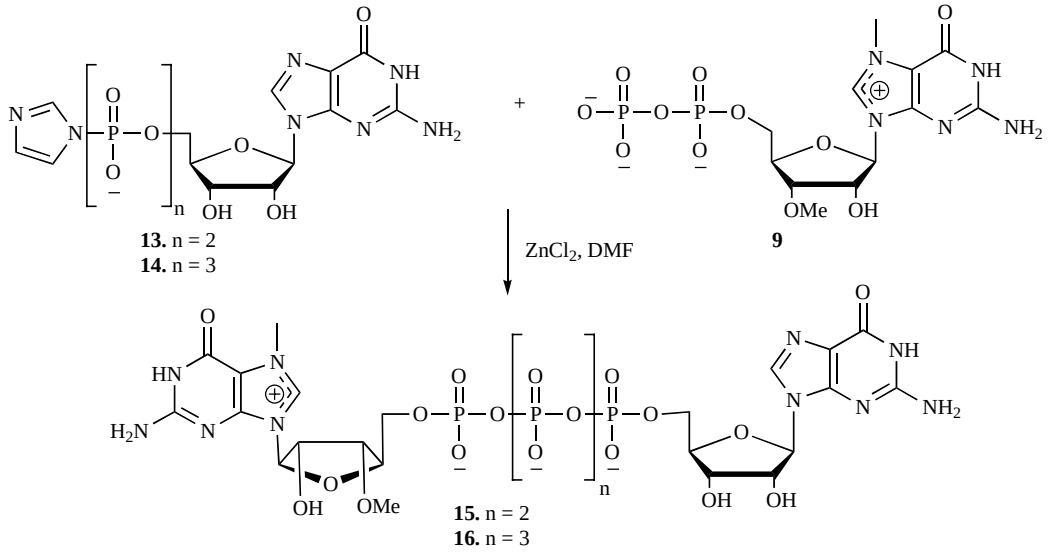
2.1. Chemical Synthesis of 3′-Substituted Cap Analogs

Although the method for synthesis of simple cap analog m^7G[5']ppp[5']G has been known for more than three decades [37], the first syntheses of cap analogs with modified sugars such as $m_2^{7,3'\text{-}O}$G[5']ppp[5']G **11** and $m^7$3'dG[5']ppp[5']G **12** were first performed by Stepinski *et al.*, only in 2001 [38]. Since these 3′-modified molecules **11** and **12** are incorporated exclusively in the forward orientation because of the modifications at the 3′-OH group of N^7-methylguanosin, they are called "anti-reverse" cap analogs (ARCA). The synthetic scheme to prepare these two novel molecules is depicted in Scheme **2**. The synthesis of **11** begins with 3′-*O*-methylguanosine **1**. The monophosphorylation of 3′-*O*-methylguanosine **1** is achieved using $POCl_3$ and trialkyl phosphate according to the method developed by Yoshikawa [39]. The 3'-*O*-Me GMP **3** is then converted into the corresponding imidiazolide salt **5** using imidazole, triphenyl phosphine, and aldrithiol. Next, the resulting imidazolide salt **5** is further phosphorylated in the presence of zinc chloride that affords the corresponding 3'-*O*-Me GDP **7**. The methylation reaction of 3'-*O*-Me GDP **7** using methyl iodide as the methylating agent affords the corresponding $m_2^{7,3'\text{-}O}$GDP **9**. The final coupling reaction of **9** with ImGMP in the presence of aqueous Mn^{2+} results in 42% of **11**. The best coupling result is obtained when the reaction is carried out for 16 h using $ZnCl_2$ as the catalyst and DMF as the solvent to afford 78% of **11**. The other ARCA compound, $m^7$3'dG[5']ppp[5']G **12** is obtained in a similar manner, starting from 3'-deoxyguanosine **2**.

Peng *et al*., reported an independent synthetic strategy to make **11** in 2002 [40]. There are two striking synthetic feature differences that deserve comment in comparison to the



Scheme 2.

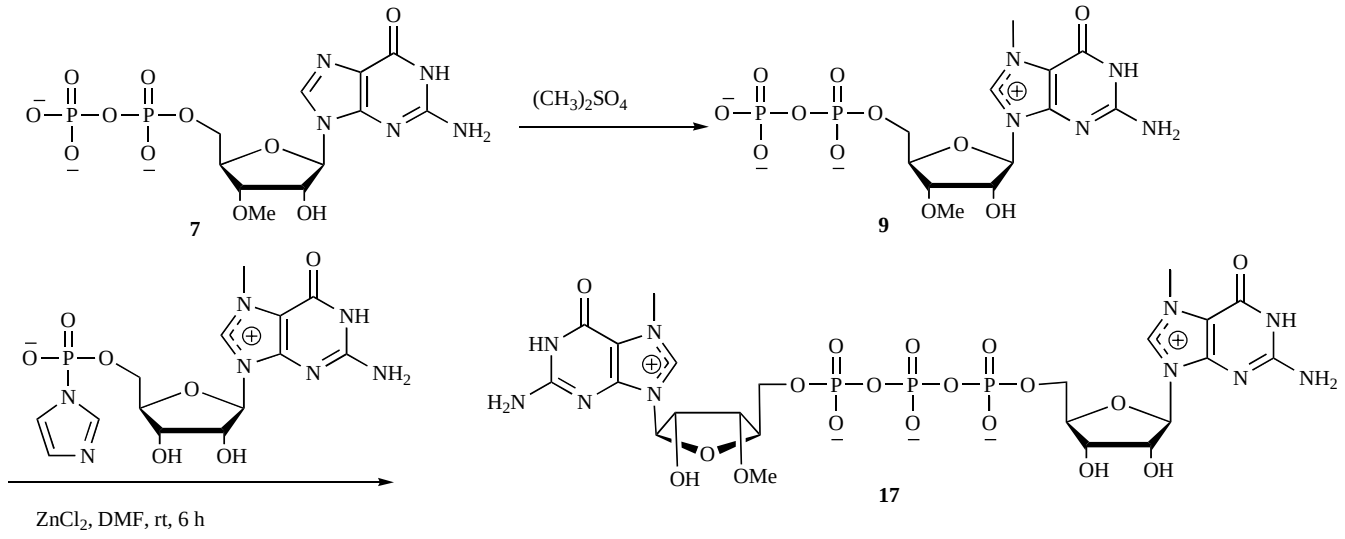


Scheme 3.

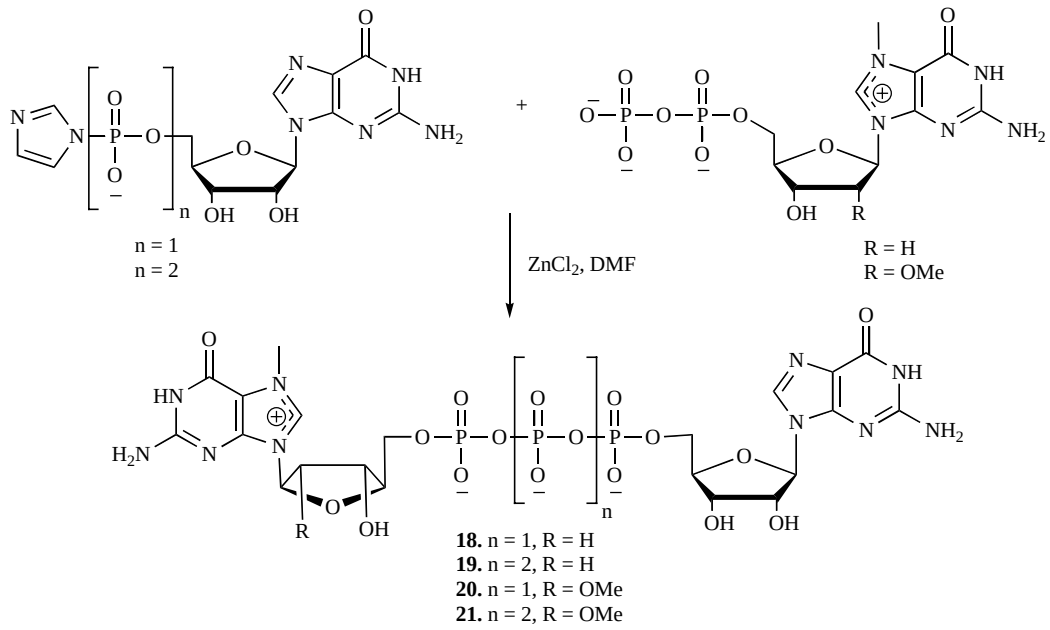
Stepinski method. First, methylation of guanosine preceded formation of the diphosphate group to furnish the required intermediate **9** for the coupling reaction. This was done using the standard methylation reaction of 3'-*O*-Me GMP to form $m_2^{7,3'\text{-}O}$GMP, and subsequently phosphorylating with tributylammonium orthophosphate using the carbonyldiimidazole coupling method. Second, the coupling reaction was carried out with activated GMP-morpholidate. Thus, the reaction of **9** with GMP-mopholidate in the presence of 1H-tetrazole as the catalyst for 2.5 days afforded **11** in 60% yield. Comparing with the original method (78% yield in 16 h), it appears that the coupling reaction with imidazolide as the leaving group is superior to the one using the morpholidate group in terms of both reaction time and yield.

Furthermore, the synthesis of the tetraphosphate, $m_2^{7,3'\text{-}O}$G[5']pppp[5']G **15** and pentaphosphate, $m_2^{7,3'\text{-}O}$G[5']ppppp [5']G **16** versions of ARCA were reported by Jemielity *et al.*, in 2003 [41]. Thus, reactions of $m_2^{7,3'\text{-}O}$GDP **9** with the polyphosphate imidazolide derivatives of GDP **13** or **14** with $ZnCl_2$ as the catalyst afford the corresponding cap analogs **15** and **16** with 51 % and 64% yields, respectively (Scheme **3**). The behavior of these cap analogs is quite interesting in that they bind eIF4E more strongly than their triphosphate counterparts but actually decrease translational production in test systems.

We have recently reported a new trimethylated cap analog, $m_2^{7,3'\text{-}O}$G[5']ppp[5']m^7G **17**. In these compounds we have placed methyl groups on the N^7 of both guanosine moieties, as well as at the 3'-OH of one of the ribose moieties (Scheme **4**) [42]. In this synthetic process, the strategy to make the key intermediate **9** was different from the literature method. The most commonly used methylating agent to perform methylation of the guanosine nucleoside is methyl iodide, using DMF [43] or DMSO [38] as the reaction solvent. In our hands, scaling up of the N^7 methylation of guanosine nucleotides using the traditional methyl iodide method was not successful. GDP is only sparingly soluble in DMF or DMSO and the heterogeneous reaction mixture gave a mixture of products when carried out in large scale. To address this issue, we have recently reported an industrial scale re-



Scheme 4.



Scheme 5.

gioselective production of N^7 methylated guanosine nucleotides using dimethyl sulfate as a methylating agent [44]. This method has general applicability in that the reaction can be carried out at acidic pH, at which only the N^7 nitrogen can be methylated. This method was successfully extended to the preparation of $m_2^{7,3'\text{-}O}$GDP **9**. Thus, the methylation reaction of 3'-*O*-Me GDP **7** using dimethyl sulfate at pH 4.0 afforded the corresponding single m^7 methylated product, $m_2^{7,3'\text{-}O}$GDP **9** at 78% yield. Treatment of $m_2^{7,3'\text{-}O}$GDP with m^7ImGMP in the presence of $ZnCl_2$ as catalyst afforded the desired $m_2^{7,3'\text{-}O}$G[5']ppp[5']m^7G **17** at 61% yield.

2.2. Chemical Synthesis of 2'-Substituted Cap Analogs

In addition to 3'-OH modifications, the synthesis of 2'-OH modifications was reported by Jemielity *et al.*, in 2003 [41]. The synthetic protocol to make $m^7$2'dG[5']ppp[5']G **18**, $m^7$2'dG[5']pppp[5']G **19**, $m_2^{7,2'\text{-}O}$G[5']ppp[5']G **20**, and $m_2^{7,2'\text{-}O}$G[5']pppp[5']G **21** was similar to the strategy developed for the 3'-cap analogs by Stepinski *et al.*, (Scheme **5**). It is noteworthy that chemical modifications at the 2' position of N^7-methylguanosine forced the cap to be incorporated solely in the forward direction, even though the 2'-OH group does not participate in the phosphodiester linkage [41].

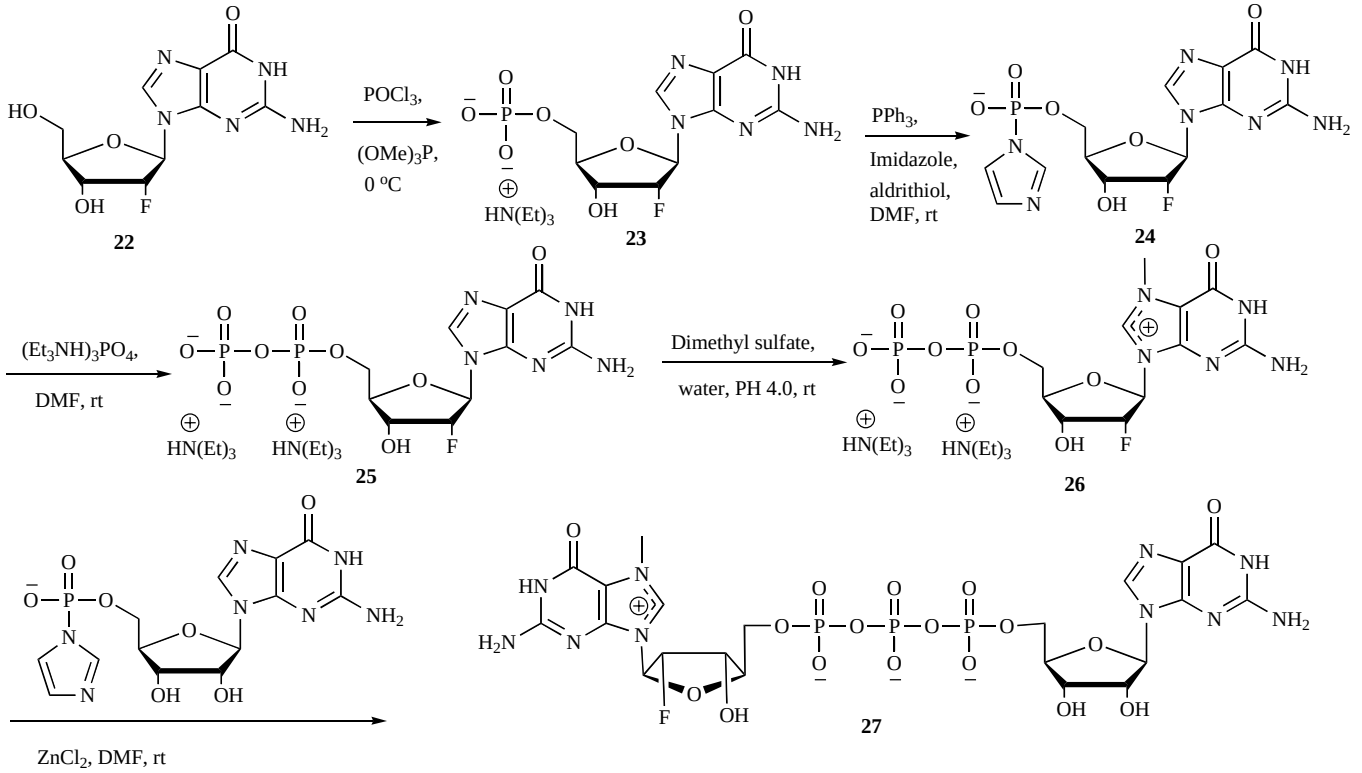
We have recently reported the synthesis of $m^{7,2'\text{-}F}$G[5']ppp[5']G **27** and $m^{7,2'\text{-}F}$G[5']ppp[5']m^7G **28** in order to understand the effect of the 2'-fluoro-substituted ribose moiety with respect to their capping efficiency, *in vitro* T7 RNA polymerase transcription efficiency, and translational activity using transfected HeLa cells [45]. The reaction pathway leading to the desired product formation of $m^{7,2'\text{-}F}$G[5']ppp[5']G **27** is depicted in Scheme **6**. Mono phosphorylation of 2'-fluoro guanosine **22** in the presence of $POCl_3$ and trimethyl phosphate afforded the corresponding 2'-F GMP **23** with 87% yield. 2'-F GMP **23** was further converted to its corresponding imidiazolide salt by the standard procedure to get 81% of 2'-F ImGMP **24**. The resulting imidazolide salt **24** was further phosphorylated in the presence of zinc chloride that furnished the corresponding 2'-F GDP **25** with 72% yield. Regioselective N^7 methylation using dimethyl sulfate afforded $m^{7,2'\text{-}F}$GDP **26** with 83% yield. The final coupling reaction of $m^{7,2'\text{-}F}$GDP **26** with ImGMP in the presence of zinc chloride as catalyst gave $m^{7,2'\text{-}F}$G[5']ppp[5']G **27** with 58% yield.

The formation of $m^{7,2'\text{-}F}$G[5']ppp[5']m^7G **28** is depicted in Scheme **7**. Treatment of $m^{7,2'\text{-}F}$GDP **26** with m^7ImGMP in the presence of $ZnCl_2$ as catalyst afforded $m^{7,2'\text{-}F}$G[5']ppp[5']m^7G **28** with 64% yield.

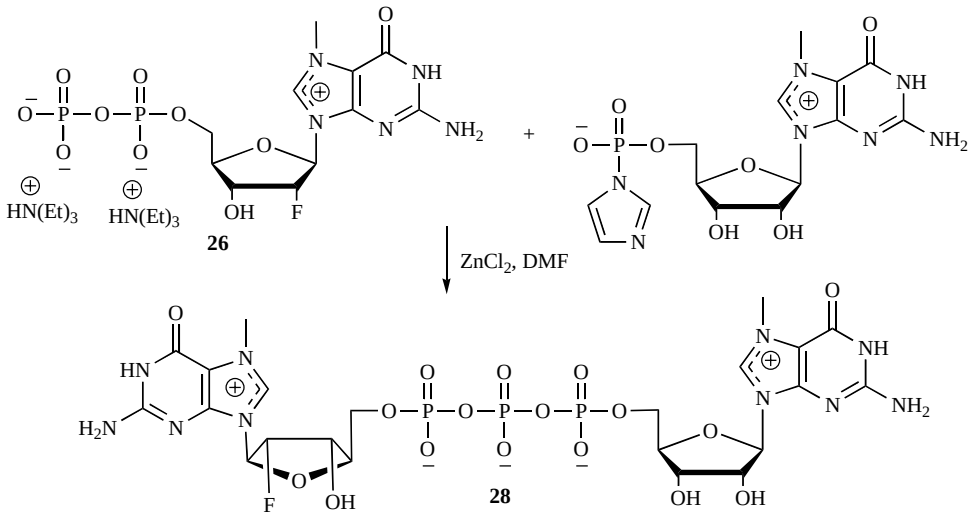
2.3. Chemical Synthesis of Phosphate Chain Modified Cap Analogs

An important class of cap analogs containing a phosphorothioate moiety in the α, β, or γ position of the 5′-5′-triphosphate chain such as m^7G[5']$p_{(s)}$pp[5']G **31**, m^7G[5']$pp_{(s)}$p[5']G **34**, and m^7G[5']$ppp_{(s)}$[5']G **32** were reported by Kowalska *et al.*, in 2005 [46]. The synthetic path to make **31** and **32** is shown in Scheme **8**. 5'-*O*-thiophosphorylation of guanosine with $PSCl_3$ in triethyl phosphate provided $GMP_{(s)}$ **30**. The yield of the reaction was significantly improved with the presence of the organic base, 2,6-dimethylpyridine. Similarly, 5'-*O*-thiophosphorylation of N^7 guanosine afforded $m^7GMP_{(s)}$ **29** under identical conditions. The coupling reaction of m^7ImGDP with $GMP_{(s)}$ **30** in the presence of $ZnCl_2$ as catalyst furnished the corresponding m^7G[5']$ppp_{(s)}$[5']G **32**. In a similar manner, m^7G[5']$p_{(s)}$pp[5']G **31** was obtained from the reaction of ImGDP with $m^7GMP_{(s)}$ **29**. Both phosphorothioated cap analogs were obtained as diastereomeric mixtures.

A different strategy was used to synthesize m^7G[5']$pp_{(s)}$p[5']G **34** starting from ImGMP (Scheme **9**). ImGMP was reacted with a two molar excess of triethylammonium phosphorothioate in the presence of $ZnCl_2$, to afford guanosine 5'-*O*-(1-thiodiphosphate), $GDP_{(\beta\text{-}s)}$ **33**. The final coupling of $GDP_{(\beta\text{-}s)}$ **33** with m^7ImGMP in the presence of $ZnCl_2$ af-



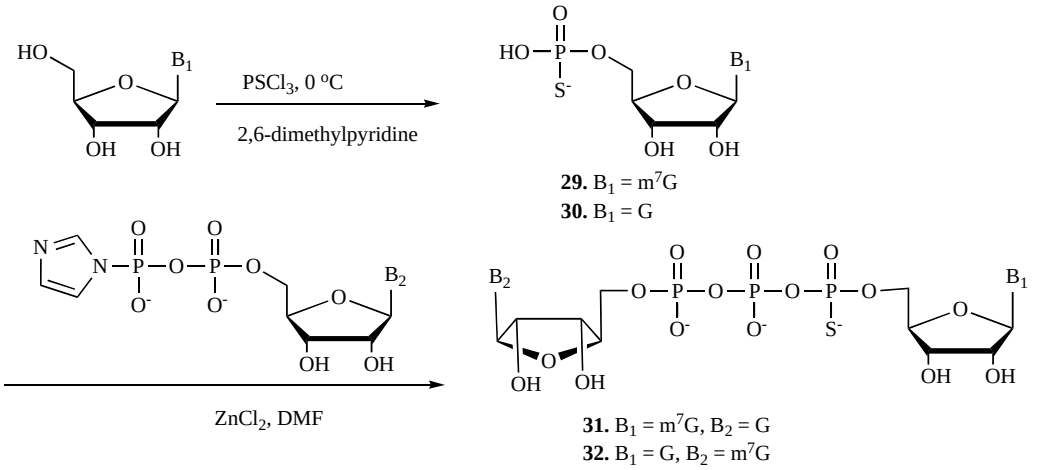
Scheme 6.



Scheme 7.

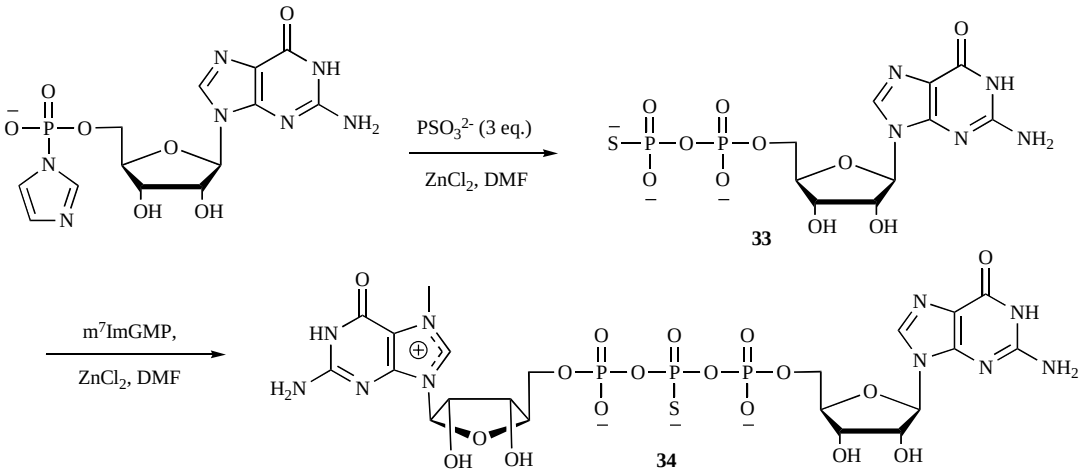
forded 20% $m^7G[5']pp_{(s)}p[5']G$ **34**, again as a diasteromeric mixture. The poor yield was attributed to the presence of m^7GMP as a contaminate of m^7ImGMP that led to formation of $m^7G[5']pp[5']m^7G$ as a side product.

Darzynkiewicz and coworkers have reported a new series of cap analogs with one of the pyrophosphate bridge oxygen atom of the triphosphate linkage replaced with a methylene group. This is resistant to the human scavenger decapping hydrolase, DcpS [47, 48]. The synthetic pathway leading to the formation of two new methylene-modified cap analogs $m^7G[5']pCH_2pp[5']G$ **39** and $m^7G[5']pppCH_2[5']G$ **37** is shown in Scheme **10**. The required key intermediates guanosine 5'-bisphosphonate **35** and N^7-methyl guanosine 5'-bisphosphonate **38** were achieved by replacing $POCl_3$ with methylenebis(phosphonic dichloride) by a modification of Yoshikawa's 5'-phosphorylation conditions. The reaction is highly regioselective to afford the corresponding products **35** and **38** with 83% and 77% yields, respectively. It is to be noted that methylation of **35** using methyl iodide in DMSO led to **38** in only the moderate yield of 37%, probably due to the poor solubility of **35** in the reaction media. Compound **35** was converted into the corresponding imidazolide salt **36** and subsequently coupled with m^7ImGMP in the presence of $ZnCl_2$, affording the corresponding cap analog **37** with 23% yield. In a similar manner, the coupling reaction of **38** with ImGMP afforded the corresponding cap analog **39** with 38% yield.



29. $B_1 = m^7G$
30. $B_1 = G$
31. $B_1 = m^7G$, $B_2 = G$
32. $B_1 = G$, $B_2 = m^7G$

Scheme 8.



33
34

Scheme 9.

Furthermore, the synthesis of the ARCA versions of methylene-modified cap analogs such as $m_2^{7,3'\text{-}O}$G[5']pCH$_2$pp[5']G **40** and $m_2^{7,3'\text{-}O}$G[5']pppCH$_2$[5']G **41** starting from 3'-*O*-methyl guanosine is depicted in Scheme **11**.

2.4. Chemical Synthesis of Tetranucleotide Containing Cap Analogs

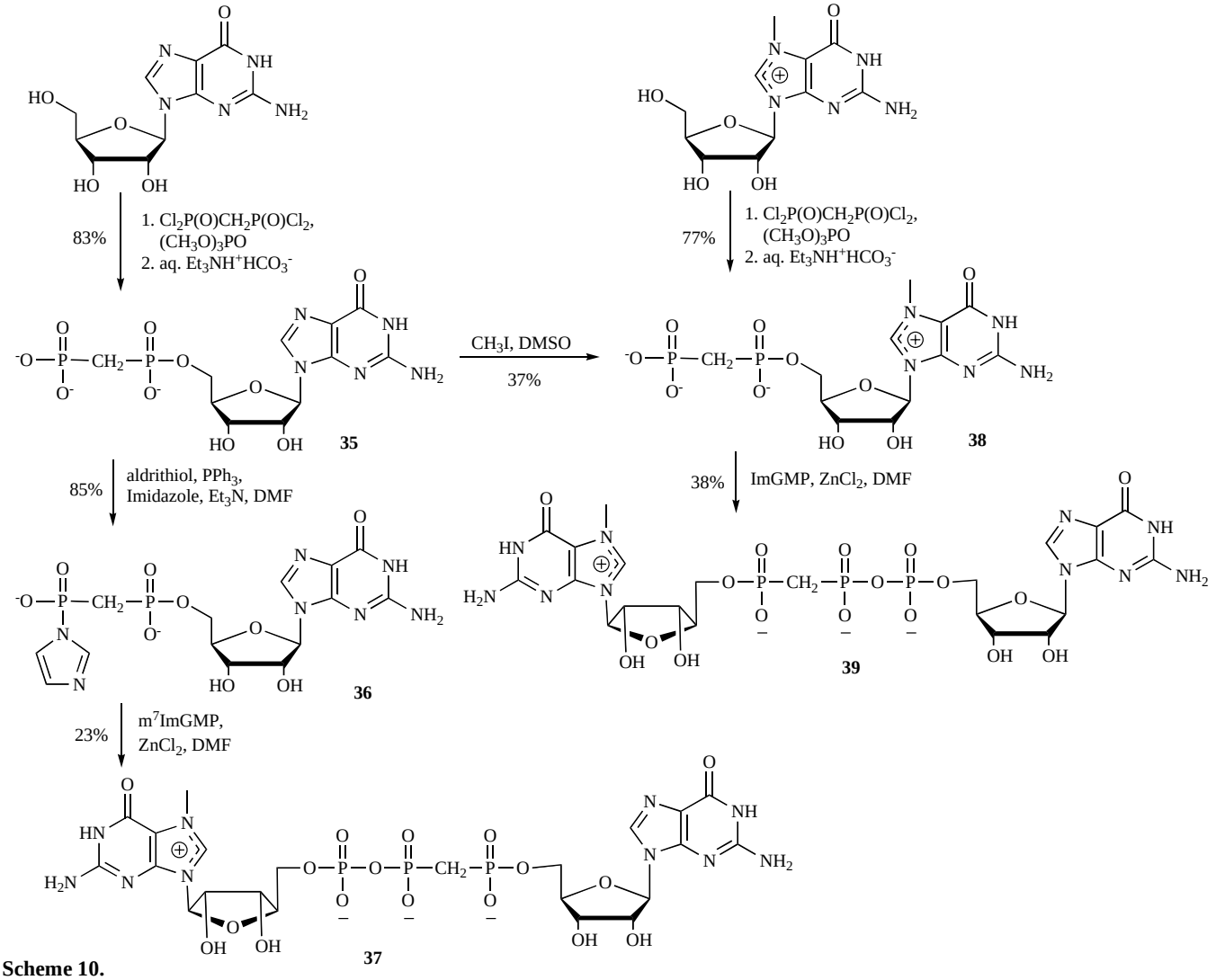
Worch *et al*., reported a novel approach to preparing an mRNA-based tetramer in order to examine the possible synergistic effects of an extended mRNA chain in specific recognition by the human nuclear cap-binding complex (CBC) [49]. The synthesis of the tetranucleotide, m^7GpppA$^{m2'}$pU$^{m2'}$pA$^{m2'}$ **43** was achieved by the coupling of a 5'-phosphorylated trimer **42** with m^7ImGDP in the presence of $ZnCl_2$ using DMF as the solvent (Scheme **12**).

3. CHEMICAL MODIFICATIONS AND THEIR BIOLOGICAL RAMNIFICATIONS

3.1. Nucleobase Modifications

Base modification of the cap nucleotide of capped RNA has significant effects in transcription and translational processes. The literature reveals that N^7 substitution is critical but a specific substituent is not required for the RNA transcript to be recognized by protein initiation factors [50]. During the translation initiation process, the three consecutive phosphate groups in the capped RNA form the basis of a "molecular anchor" which is followed by a sandwiched π-stack interaction of the N^7-substituted guanosine with two tryptophan rings (Trp-102 and Trp-56) of eIF4E [51, 52]. It is expected that replacement of the N^7-methyl group of the 5′-cap guanosine nucleotide by a different substituent will result in steric hinderance as well as stronger hydrophobic interactions with nearby Trp-166. Among these two counteractive forces, the one that dominates for a given substituent will determine whether that substituent is suitable for replacing the N^7-methyl group or not (Table **1**). In other words, any significant change in the N^7-substitution will influence the entire translational process (Scheme **13**).

With the substitutions given in Table **1**, the N^7-benzyl substituted cap analog exhibited 1.87-fold better relative translational efficiency compared to the methyl substitution, whereas the N^7-ethyl substitution showed a negative impact on the translational efficiency (0.68-fold) [53]. Presumably, N^7-benzyl substitution increases π-stack interactions of the cap with the indole ring of Trp-166. This attractive effect

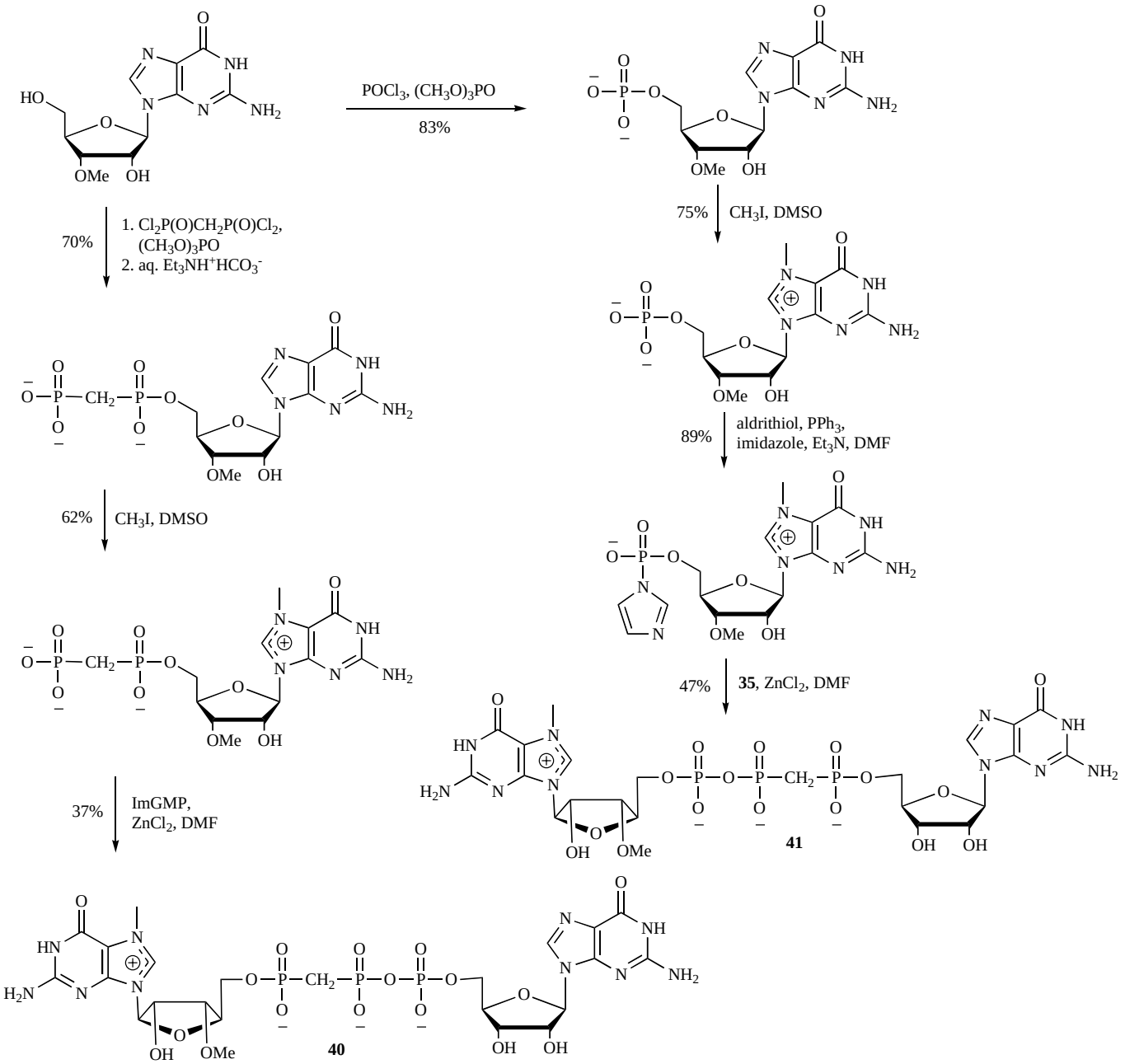


Scheme 10.

might dominate over steric hinderance effects, resulting in a net positive effect. In the case of N^7-ethyl substitution, a steric hinderance effect apparently dominates the hydrophobic interaction of the ethyl group with Trp-166 resulting in poor translational efficiency. Using an *in vitro* wheat germ system with capped reovirus mRNA, protein output was inhibited 70-80% by free N^7-substituted GDP, but not GDP, inferring that the methylated nucleotide provides a much more effective competitive inhibitor for the binding of ribosomes to the cap. Methylation at the N^2 position is seen in nature, notably in the nematodes where this position is dimethylated in addition to the N^7 methyaltion, creating a trimethylguanosine ($m_3^{2,2,7}$Gppp–, TMG) cap. Rather than added *de novo*, this cap is added to the 5′ end of the RNA along with a short sequence, called a splice leader (SL), by trans-splicing. Current estimates are that, at least in the well-studies nematode species *C. elegans*, about 70% of the mRNAs contain a 5′ SL and TMG cap. However, dimethylation of the standard cap analog at N^2 position ($m_3^{2,2,7}$GpppG) and using it in standard assays indicated a ~63% reduction in the translational efficiency in rabbit reticulocyte lysate system with a slight preference for forward orientation. A recent study showed that the translational efficiency of naturally occurring-capped mRNAs is markedly improved by the additional presence of the spliced leader (SL) sequence in the 5′ end. The synergistic action of both spliced leader sequence and TMG cap were shown to be responsible for their equilibrated translation [54]. Little work has been performed with N^2 mono methylation of the 5′-end guanosine nucleotide, but it is known that this moiety increases the capping efficiency over standard cap analog with a nearly 25% increase in the forward orientation.

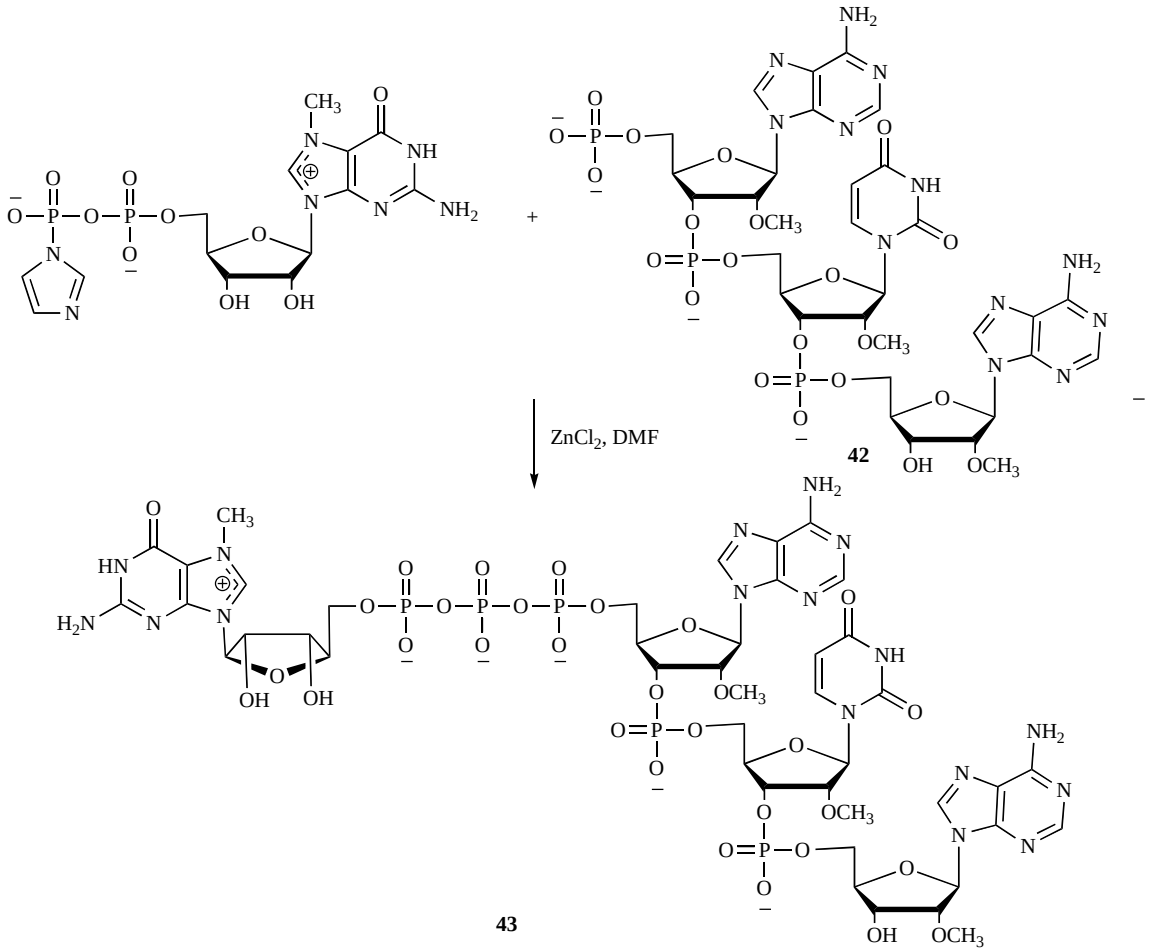
3.2. Sugar Modifications

The inviolate prerequisite for the sugar group of the cap analog is that the initiating nucleotide must have a 3′-OH for addition of the second nucleotide. However, during transcription initiation, the 3′-OH of either of the guanosine moieties of m^7GpppGpNm can serve as an initiating nucleophile [55]. This can lead to the formation of two isomeric RNAs of the forms m^7GpppGpNm and Gpppm^{7}GpNm in approximately equal proportion. The former RNA is referred to as capped RNA with the cap in forward orientation and the latter as in reverse orientation. One of the ways to obtain exclusively forward-oriented capped RNA is to start with a capped dinucleotide having only one 3′-OH available for nucleophilic attack. Stepinski *et al.* exploited this principle

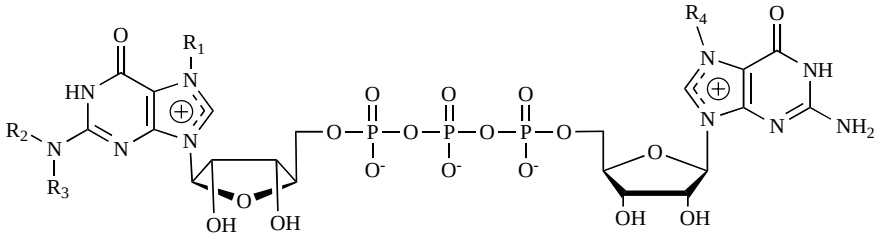


Scheme 11.

by using p[1]-3′-deoxy-7-methylguanosine-5′ P[3]-guanosine-5′-triphosphate ($m^7$3′dGpppG) and p[1]-3′-*O*,7-dimethylguanosine-5′ P[3]-guanosine-5′-triphosphate ($m_2^{7,3'-O}$GpppG) [38, 40] for transcription to obtain, solely, forward oriented capped RNA (Scheme **14**) (Table **2**). Both these dinucleotides caps were incorporated solely in the forward orientation as anticipated. This exclusive forward-oriented incorporation was confirmed by digesting the ARCA capped RNA transcript with RNase T2 followed by tobacco acid pyrophosphatase (TAP) [38]. Also, both of these capped dinucleotides exhibited a competitive inhibition of translation roughly equivalent to m^7GpppG in the rabbit reticulocyte lysate system. This contrasts with GpppG, which was not an inhibitor but rather stimulated the protein synthesis at low concentrations. *In vitro* translation experiments revealed that $m^7$3′dGpppG- and $m_2^{7,3'-O}$GpppG-capped RNAs were translated 2.6- and 2.3-fold more efficiently than m^7GpppG capped RNA. The interesting conclusion presented in the above experiments was there is a negative impact of the reverse capped mRNA. *In vitro* transcription reaction with m^7GpppG generally gives ~33% reverse capped RNA as reported by Pasquinelli *et al.*, [55] and Stepinski *et al.* [38]. So after transcribing under the same experimental conditions with these variant ARCAs, one would expect that this 33% would now be correctly capped, resulting in a 1.5-fold increase in the number of functional RNAs in the capped population and a similar increase in translational efficiency. This relies on several assumptions: the proportion of capped RNA (versus uncapped from initial GTP incorporation) is equivalent for all cap variants, all cap variants are equivalently recognized by ribo-



Scheme 12.



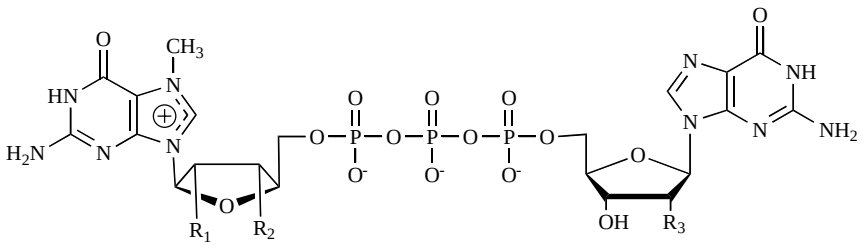
Scheme 13.

Table 1. Nucleobase Modifications of Cap Analogs

Entry	R_1	R_2	R_3	R_4
1	$-CH_3$	H	H	H
2	$-CH_2C_6H_5$	H	H	H
3	C_2H_5-	H	H	H
4	$-CH_3$	$-CH_3$	H	H
5	-OH	$-CH_3$	$-CH_3$	H
6	$-CH_3$	H	H	$-CH_3$

somes, and that the reverse-capped molecules are neutral in the translation process. But the translational data showed a much higher increase than 1.5. Since the first two assumptions have the supporting evidence presented above, the conclusion was that reverse capped RNAs, rather than being neutral, exhibited an inhibitory effect.

Even though not involved directly in the inter-nucleotide linkages, modifications at the 2′-OH of the ribose can affect coupling effeciency. When the 2′-OH of the non-methylated base was protected with a methyl group, only 24% of the forward oriented capped RNA was formed, resulting in a deleterious reduction in translational efficiency (Scheme **14**, entry 6) [53]. Jemielity *et al.* extended their study by including modifications at the C2′ position of the N^7-methyl guanosine residue [41]. Their results indicated that sugar modifications such as substitution of hydrogen or methoxy groups for the C2′-OH of the N^7-methyl guanosine ribose also belong to a family of ARCA, yielding exclusively forward-oriented capped RNAs. The binding affinities of both $m_2^{7,2'-O}$GpppG and $m^7$2′dGpppG for eIF4E are similar to m^7GpppG but the cap-dependent translation inhibition of the



Scheme 14.

former was 1.9-fold more than the latter and 1.2-fold more than m^7GpppG.

The above results reveal that the RNA polymerase used is very flexible in recognizing these di-ribonucleotides with 2′-deoxy, 3′-deoxy, 2′-methoxy and 3′-methoxy groups present on the cap-position nucleotide, and that even the 2′-substitutions force correct orientation. This prompted us to synthesize a 2′-fluoro-modified cap analog to explore not only whether it can be a substrate for transcription and translation, but also whether its incorporation is biased to the forward direction. Additionally, 2′-fluoro modification of nucleotides incorporated into the polynucleotide chain has been shown to offer better stability towards nucleases compared to hydroxyl, methoxy and deoxy nucleotides, so this cap analog may be more resistant to decapping activity as well.

Table 2. Sugar Modifications of Cap Analogs

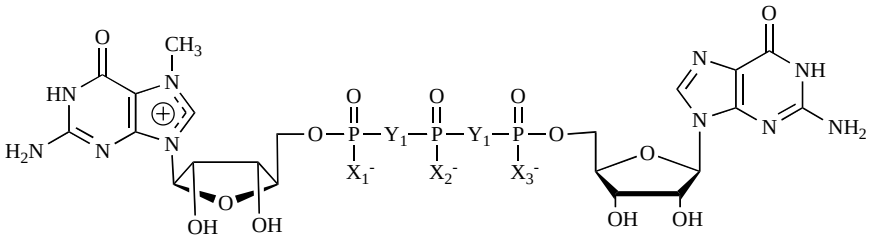
Entry	R_1	R_2	R_3
1	-OH	-OH	-OH
2	-OH	$-OCH_3$	-OH
3	$-OCH_3$	-OH	-OH
4	-OH	H	-OH
5	H	-OH	-OH
6	-OH	-OH	$-OCH_3$
7	F	-OH	-OH

Indeed, we have demonstrated that 2′-F substituted cap analogs of the form $m_2^{7,2'-F}GpppG$ and $m_2^{7,2'-F}Gpppm^7G$ were substrates for T7 RNA polymerase under transcription conditions and their transcription yields were comparable to m^7GpppG-capped RNA. The capping efficiency of $m_2^{7,2'-F}GpppG$ was 9% better than m^7GpppG but N^7-methylation of the second guanosine ($m_2^{7,2'-F}Gpppm^7G$) was capped 9% less efficiently than regular cap. The translational efficiency of $m_2^{7,2'-F}GpppG$ and $m_2^{7,2'-F}Gpppm^7G$ was 2.4- and 2.5-fold better than regular m^7GpppG, respectfully [45]. When a trimethylated cap analog $m_2^{7,3'-O}Gpppm^7G$, bearing modifications both at sugar and base moieties, was used in an *in vitro* transcription reaction, some compromise in the capping efficiency was observed compared to conventional cap. However, its translational efficiency was 2.6 fold higher than regular capped RNA. The advantage of using $m_2^{7,3'-O}Gpppm^7G$ is that it carries only one 3′-OH for transcriptional initiation which will result, exclusively, in forward oriented cap analog. The translational efficiency of $m_2^{7,3'-O}GpppG$ and m^7Gpppm^7G, by comparison, were only 2.2- and 1.6- fold higher than the regular cap [42].

3.3. Phosphate Chain Modifications

The phosphate groups of the cap structure play a significant role during the early stages of eIF4E-cap complex formation [51]. The first step of the two-step mechanism proposed for the binding of cap structures into the binding slot of eIF4E involves electrostatic interaction of the triphosphate chain of the cap and the positively charged amino acid side chains at the entrance of the eIF4E cap binding slot. This "molecular anchoring" enables sandwich stacking of the 7-substituted guanine of the cap between two Trp rings of eIF4E. The complex will be further stabilized by van der Waals interactions and hydrogen bond formation. Chemical modification will not only impact their initial complex formation and, thereby translational efficiency, but also their stability towards cellular nucleases. When Grudzien *et al*. introduced four phosphate groups in the cap structure, up-to a 10-fold increase in the equilibrium association constant (K_{AS}) with eIF4E was observed. However, as a competitive inhibitor $m^7GppppG$ exhibited only a 1.5-fold increase in the inhibition of *in vitro* translation compared to triphosphate, which the authors quantified by calculating K_Is from their data. The authors explained that the additional phosphate charge in the tertraphosphate cap structure might cause non-specific binding of this cap to additional proteins in reticulocyte lysate, lowering its effective concentration and interfering with its binding to eIF4E. Alternatively, since K_{AS} values represent an equilibrium measurement whereas inhibition of translation is a steady-state measurement, it could be that binding of the tertraphosphate cap to eIF4E may not be the rate determining step, so that tighter binding would not be reflected in a proportionally higher K_I. Insertion of one more phosphate group(s) to the base and/or sugar modified triphosphate cap structure always provided an increase in K_{AS}, K_I and translational efficiency compared to their triphosphate counterparts [53]. It is the addition of one more phosphate group to a regular cap m^7GpppG and not the base or sugar modifications which provided the best translational efficiency, K_{AS} and K_I. Interestingly, the pentaphosphate cap structure showed no significant change in the translational efficiency even though K_A and K_I were both a little higher than its triphosphate analog [41]. Effects on capping efficiency were slightly deleterious and the percent of cap analogs in the correct orientation were not determined.

Other researchers have looked at replacing the oxygen atoms in the triphosphate chain (Table **3**). Although this may not directly affect translational efficiency, it could extend the lifetime of the transcripts in *in vivo* systems. Also, since it is known from *in vitro* studies that synthetic cap analogs are effective competitive inhibitors of protein synthesis by bind-



Scheme 15.

Table 3. Phosphate Chain Modifications of Cap Analogs

Entry	X_1	X_2	X_3	Y_1	Y_2
1	S	O	O	O	O
2	O	S	O	O	O
3	O	O	S	O	O
4	O	O	O	$-CH_2-$	O
5	O	O	O	O	$-CH_2-$

ing to eIF4E during translation initiation [41, 56], they could be used in this role *in vivo*. This observation has several potential applications, since many tumors express very high levels of eIF4E and the availability of eIF4E for protein production is critical step for disease progression [57]. The superior binding capacities of modified cap analogs can be exploited for therapeutical applications where protein production needs to be stopped or at least reduced. However, the cytoplasm contains numerous enzymes which would attack the relatively labile triphosphate bridge after introduction into the cell. Thus, for these kind of applications, a more stable form of synthetic cap analogs is warranted. Another reason for exploring more stable capped analogs is to extend the life cycle of capped mRNAs *in vivo*. There are two major exonucleolytic pathways by which polyadenylated mRNAs are degraded in eukaryotic cells, a 3′→5′ pathway and a 5′→3′ pathway [58]. Both pathways are intimately involved with the removal of the 5′ cap. 5′-Decapping is carried out by the mRNA-decapping complex Dcp1–Dcp2, in which Dcp2 is the catalytic subunit [29]. Both Dcp2 and Dcp1–Dcp2 complex degrade mRNA releasing m^7GDP which suggests that the site of cleavage is between the two phosphate groups distal from the cap. The exposed 5′-end is then digested by Xrn1, a 5′-exoribonuclease. In the 3′→5′ decay pathway, the mRNA body continues to be degraded from the 3′-end by the exosome complex following deadenylation [59, 60]. The resulting capped oligonucleotide of ten nucleotides or fewer is a substrate for DcpS, a scavenger decapping enzyme. DcpS releases m^7GMP during decapping, suggesting that cleavage also occurs between the proximal- phosphate groups (Scheme **15**). It was initially thought that the 3′→5′ decay pathway predominates in mammalian system. The observation of m^7GMP but not m^7GDP was the reason for this conclusion. Thus, increasing the stability of α-, β- and γ-phosphate groups of capped mRNA might decrease the turnover of mRNA resulting in longer gene expression.

Grudzien *et al*. synthesized methylene-bridged cap analogs of both regular and a 3′-methoxy ARCA and studied their biophysical properties [61-63]. Methylene bridged cap analogs of the form m_2^7GppCH$_2$pG and m_2^7GpCH$_2$ppG showed 11% and 23% reduction in the relative translational efficiency compared to regular cap analog, respectively [61]. The ARCA version analogs, $m_2^{7,3'\text{-}O}$GppCH$_2$pG and $m_2^{7,3'\text{-}O}$GpCH$_2$ppG, also showed 34% and 41% reduction in the relative translational efficiency compared to the triphosphate ARCA, respectively. Also, all four methylene analogs exhibited slightly lower equilibrium association constants to eIF4E compared to their counterparts. The possible explanations given for these results are that the replacement of oxygen atoms with methylene groups changes the geometry and charge distribution of the polyphosphate chain in the cap analogs [62, 63]. This will reduce the electrostatic interactions of cap analogs with positively charged amino acid residues at the entrance to cap binding slot of eIF4E, causing poor binding efficiency. Although mRNAs containing methylene bridged cap analogs were only moderately translated *in vitro*, their translational efficiencies were slightly better than m^7GpppG. The purpose of methylene bridged cap analog design was to increase its resistance to decapping. Indeed, the stability of $m_2^{7,3'\text{-}O}$Gpp CH$_2$pG capped RNA to recombinant human (hDcp2) Dcp2 activity *in vitro* was found to have increased. When mRNA capped with $m_2^{7,3'\text{-}O}$GppCH$_2$pG was electroporated into MM3MG cells, they were found to be more stable ($t_{1/2}$=330 min) compared to mRNAs capped with either m^7GpppG or $m_2^{7,3'\text{-}O}$GpppG ($t_{1/2}$=156 and 282 min, respectively). On the other hand, $m_2^{7,3'\text{-}O}$Gp CH$_2$ppG was resistant to hydrolysis by DcpS but mRNA capped with $m_2^{7,3'\text{-}O}$GpCH$_2$ppG was found to be less stable ($t_{1/2}$=180 min) than $m_2^{7,3'\text{-}O}$GpppG ($t_{1/2}$=282 min).

When the non-bridging oxygen atoms of triphosphate chain were replaced by sulfur atoms, no compromise in the relative translational efficiency was observed relative to their non-sulfurated analogs, as opposed to those with a methylene-bridged triphosphate chain modification [46, 64]. Three different S-ARCAs containing phosphorothioate moieties either at the α-, β- and γ-positions of the triphosphate chain were synthesized. Each S-ARCA was obtained as two diastereomers as the introduction of sulfur groups create a new

stereogenic P-center. The diastereomers were separated on a reverse phase HPLC. Each diastereomer was capped to 48mer oligonucleotide and their stability towards Dcp2 hydrolysis was examined *in vitro*. The D2 isomer of $m_2^{7,3'-O}Gpp_{(S)}pG$ was completely resistant to hDcp2 hydrolysis, whereas, oligonuclotides capped with isomers $m_2^{7,3'-O}Gppp_sG$ (D1), $m_2^{7,3'-O}Gppp_{(S)}G$ (D2), $m_2^{7,3'-O}Gpp_{(S)}pG$ (D1) were only partially resistant. Both isomers of $m_2^{7,3'-O}Gp_{(S)}ppG$ showed no additional stability compared to their parent ARCA. The intracellular stability of mRNA capped with $m_2^{7,3'-O}Gpp_{(S)}pG$ (D2) ($t_{1/2}$=257 min) was ~ 3-fold better than regular cap and ~1.7-fold better than its parent ARCA in culture cells. Its other isomer, $m_2^{7,3'-O}Gpp_{(S)}pG$ (D1) was only ~2.1 fold better than its parent ARCA. No significant improvement in the stability was observed for mRNA capped with either of the isomers of $m_2^{7,3'-O}Gppp_{(S)}G$, while mRNA capped with either of the isomers of $m_2^{7,3'-O}Gp_{(S)}ppG$ showed a slight increase in stability compared to their parent ARCA. The results of the translational efficiencies of phosphorothioate modified ARCAs are more intriguing. Luciferase mRNA capped with $m_2^{7,3'-O}Gpp_{(S)}pG$ (D2) and $m_2^{7,3'-O}Gpp_{(S)}pG$ (D1) were translated 2.4- and 1.3-fold better than their parent ARCA, reflecting the former isomer's increased resistance to Dcp2 and higher stability, once incorporated into RNA, in cultured cells. No significant difference in the translational efficiencies of both isomers of $m_2^{7,3'-O}Gp_{(S)}ppG$ and $m_2^{7,3'-O}Gppp_{(S)}G$, compared to regular ARCA, was observed.

4. CONCLUSION

The wide applications of synthetic mRNAs in molecular biology and cell biology justify the importance of achieving higher translational yields from these molecules, either *in vitro* or in cell-based assays. Various chemically-modified cap analogs have been used to achieve improved capping and translational efficiency. Changing the 2′- or 3′-hydroxy groups of N^7 methyl guanosine moiety groups such as 3′- & 2′-methoxy, 3′- & 2′-deoxy and 2′-fluoro, exclusively, yielded forward-oriented capping. Since the RNA polymerase only interacts with the nucleotide used to initiate attack on the next NTP, it seems reasonable that the enzyme's preferences for ribonucleotides lacking modifications like these imposes a strong bias to incorporate only those ribonucleotides without modification into the polymer chain. 2′-Fluoro and 3′-*O*, $m^{7,7}$-trimethylated cap analogs also showed higher translation compared to conventional cap analogs. Additional benefits such as nuclease stability can be realized with 2′-fluoro analogs. Among base-modified cap analogs, the N^7-benzylated cap analog exhibited better translational efficiency but the N^7-ethyl cap analog exhibited decreased function, possibly due to steric hindrance during RNA binding to eIF4E. No significant improvement in translational efficiency was achieved with tetra- and penta-phosphate-bridged cap analogs in spite of their higher equilibrium constants for binding eIF4E. However, the translational efficiency was better when ARCA was modified into its tetraphosphate analog. Methylene bridged ARCA analogs were more stable to Dcp2 and DcpS but they did not significantly improved translational efficiency. Among phosphodiester modifications, S-ARCAs containing phosphorothioate were shown to provide greater translational efficiency and stability to transfected mRNAs compared to the original ARCA. Considering the role of cap analogs as tools for studying molecular mechanisms of gene expression and therapeutics, there is a strong impetus not only for identifying cap analogs that can provide greater capping and translational efficiency but also extend the stablility of transfected capped RNAs *in vivo*. Studies are continuing to find these novel analogs and demonstrate their utilities in both *in vitro* and *in vivo* applications.

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