Synthetic dinucleotide mRNA cap analogs with tetraphosphate 5′,5′ bridge containing methylenebis(phosphonate) modification†‡

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Received 10th June 2009, Accepted 4th August 2009
First published as an Advance Article on the web 7th September 2009
DOI: 10.1039/b911347a

An effective and facile synthesis of six novel tetraphosphate cap analogs modified with a methylenebis(phosphonate) moiety (**1–6**) is presented. Analogs have been rationally designed to bind tightly to the eukaryotic initiation factor 4E (eIF4E) responsible for cap binding during the initiation of translation, and have increased stability owing to resistance to enzymatic degradation. Final compounds turned out to have significantly higher association constant values (K_{AS}) for binding to eIF4E (5–9 fold higher than standard). Four of the analogs were resistant towards enzymatic degradation by human Decapping Scavenger enzyme (DcpS). Binding studies of non-hydrolyzable analogs with DcpS revealed a broad range of K_{AS} values for different analogs. All of the analogs were potent inhibitors of translation in a rabbit reticulocyte lysate system (RRL) and those resistant to DcpS turned out to be stable under an elongated time of preincubation while the inhibitory potency of standard was diminished in these conditions. For Anti Reverse Cap Analog (ARCA) dinucleotides (**4–6**), we have shown that they are effectively incorporated into mRNA and transcripts capped with these analogs undergo translation *in vitro*.

Introduction

A distinctive feature of all eukaryotic cellular mRNAs is the presence of a cap on their 5′ end. A typical cap structure consists of 7-methyl guanosine joined *via* a 5′,5′ triphosphate linkage to the first nucleotide of the mRNA chain. This modification plays a crucial role in the functioning of mRNA in a variety of cellular processes,[1] including translation, splicing, transport from nucleus to cytoplasm and regulation of gene expression by micro RNAs.[2] It also takes a vital part in mRNA turnover.[3]

Synthetic cap analogs have become promising tools in structural biology[4] and biotechnology,[5] and have attracted attention as potent therapeutic agents.[6]

From the medical point of view, it seems that the most important role of the cap structure is its participation in the initiation of translation. In this process, the cap is specifically recognized by eukaryotic initiation factor 4E,[7] which is claimed to be the rate-limiting step for the initiation of translation under normal conditions[8] (in the absence of viral infection, cellular stress, *etc.*) and plays a central role in the regulation of translation. It is known that eIF4E is overexpressed in many types of tumor cells and that an elevated eIF4E level selectively increases the translation of mRNAs, which is important in malignant transformation and metastasis.[9] Previous studies demonstrated that targeting eIF4E may inhibit the growth of tumor cells and induce apoptosis.[10] From this fact, the idea of employing cap analogs in a therapy, as specific inhibitors that might counteract elevated eIF4E levels in tumor cells, has originated. Several synthetic cap analogs have been synthesized and characterized in order to check their inhibitory properties towards eIF4E protein. It has been observed that cap derivatives resistant to enzymatic degradation are more stable under cellular conditions and accordingly have better inhibitory properties.[11]

The fragments of capped mRNAs (up to 20 nt), as well as synthetic dinucleotide cap analogs, are hydrolyzed by a Decapping Scavenger enzyme known as DcpS.[12] It cleaves naturally occurring cap structures between the β and γ phosphates and releases 7-methylGMP. DcpS belongs to the HIT hydrolases family and is a nucleocytoplasmic shuttling protein. It is postulated that DcpS not only plays a role in well-characterized mRNA decay, but is also involved in a broader range of RNA processing,[13] including pre-mRNA splicing.[14] Therefore it represents a novel therapeutic target for modulating gene expression.[15]

On the other hand, synthetic dinucleotide cap analogs serve as potent tools for obtaining 5′ capped mRNAs *via* transcription *in vitro*. Capped mRNAs have found a broad range of application in *in vitro* protein synthesis as well as in obtaining expression in living cells.[16] During an *in vitro* transcription process, cap is incorporated into the end of an mRNA chain. Unfortunately, a polymerase is incapable of distinguishing between guanosine and 7-methylguanosine structures, which leads to a mixture of transcripts having both caps in the reverse and correct orientations,

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† Symbol G refers to guanosine. All compounds described in this paper abbreviated as Gp (and their derivatives) refer to 5′ phosphates. The α, β, γ *etc*. phosphate designations used in this work arise from the nomenclature commonly used in biochemistry, in which the α phosphate in a dinucleotide cap analog is always the one that is the closest to the mRNA chain after its correctly-oriented incorporation into the mRNA transcript.

‡ Electronic supplementary information (ESI) available: ^{1}H NMR, ^{31}P NMR, ESI MS and HPLC profiles of newly described compounds. See DOI: 10.1039/b911347a

i.e. Gpppm^{7}GpNpN... instead of m^{7}GpppGpNpN.[17] The exclusively correct orientation can be ensured by introducing a modification at the C2′ or C3′ position of 7-methylguanosine, such as replacing the hydroxyl group with a methoxy moiety or hydrogen. These kind of modified cap analogs are referred to as Anti Reverse Cap Analogs (ARCA).[18-20] ARCA-capped mRNAs were shown to have higher translational efficiency *in vitro*[18,20] as well as in cultured cells.[21] Moreover, it is possible to increase mRNA translational efficiency by introducing further modifications, *e.g.*, extending the length of the 5′,5′-bridge.[20]

The superior properties of modified cap analogs offer the possibility of using them to improve gene delivery systems based on mRNAs in order to treat genetic disorders, obtain anti-tumor vaccines or to use them in cancer immunotherapy.[6]

One of the first trials to obtain enzymatically stable dinucleotide cap analogs was to substitute one of the bridging oxygen atoms in 5′,5′-triphosphate bridge with a methylene group.[22] The methylene group precludes any enzymatic cleavage due to the extreme stability of P–C bonds.[23] The analogs obtained, m^{7}GpCH$_2$ppG, m$_2^{7,3'\text{-O}}$GppCH$_2$pG, and m$_2^{7,3'\text{-O}}$GpCH$_2$ppG, were resistant towards enzymatic hydrolysis by DcpS due to the presence of a methylene group in certain position in the 5′,5′-triphosphate bridge.[22] Introduction of a methoxy group at the 3′ position of the ribose ring led to ARCA-type analogs being correctly incorporated into the mRNA chain. However, it was also shown that the affinity for eIF4E protein of ARCA analogs with a methylene modification in the triphosphate bridge was significantly lower in comparison to unmodified ARCAs, which manifested itself in a lower translation efficiency of mRNA capped with such analogs.[21]

Previous studies have shown that elongation of the 5′,5′-triphosphate bridge increases the affinity of cap analogs for eIF4E by one order of magnitude per one additional phosphate group.[20] Accordingly, novel cap analogs were designed—a compromise between stability towards enzymatic degradation and maintaining high affinity for the eIF4E protein (Fig. 1). Six dinucleotide tetraphosphate cap analogs (**1–6**) have been examined from the perspective of hydrolytic resistance and eIF4E binding. Three of them (**4–6**) are of the ARCA type, where a C2′ methoxy modification was chosen. There is no significant difference between binding affinities to eIF4E as well translational efficiency of ARCA analogs modified with a methoxy group at C2′ and C3′.[20] However, the C2′ modified guanosine is cheaper and commercially available as an intermediate for oligonucleotide synthesis.

Results and discussion

Synthesis

Six tetraphosphate cap analogs in which one of the bridging oxygens was replaced by a methylene group were prepared. Three of these compounds (**1–3**) are tetraphosphate derivatives of a standard cap structure m^{7}GpppG, while compounds **4–6** are ARCA-type analogs that bear a 2′-O methyl group in addition to the modified tetraphosphate bridge. Each step of synthesis is easy to carry out and allows isolation of product with reasonable yield.

The crucial step of the syntheses of the tetraphosphate dinucleotides (**1–6**) was the coupling of two nucleotide subunits to form dinucleotide 5′,5′-tetraphosphates. In all examples, one subunit was activated as a P-imidazolide[24] and the second acted as a nucleophilic agent in the pyrophosphate bond formation process, while zinc chloride served as a Lewis acid catalyst.[18,20,25] The structures of the substrates and products of each coupling and HPLC yields are shown in the Table 1.

There are several factors which have to be taken into account when choosing appropriate subunits to be coupled. Our previous studies have shown that some reagents couple more effectively when changed into an imidazolide. On the contrary, for some units the yield increases when they are used as nucleophilic agents (unpublished results). Also, as mononucleotide compounds containing 7-methylguanosine are less stable than their non-methylated compounds, it is worth reducing the number of reaction steps in which they are involved. Some of the P-imidazolides, such as m^{7}Gpp-Im (**12**), GpCH$_2$p-Im (**31**) or triphosphate P-imidazolides, are more difficult to synthesize in comparison to imidazolide derivatives of unmodified nucleotides *i.e.* Gp (**17**) or Gpp (**19**). It is also worth noting that it is easier to separate the product from any remaining substrate by ion-exchange chromatography when two diphosphate subunits are coupled to form a tetraphosphate dinucleotide than when triphosphate and monophosphate are coupled.

For analogs **1** and **4**, bearing methylene modification at the γ-δ position, two diphosphate units have been chosen as intermediates for the coupling reaction. Guanosine diphosphate (**19**) was selected to be activated as the electrophilic P-imidazolide derivative. m^{7}GpCH$_2$p (**7**) and m$_2^{7,2'\text{-O}}$GpCH$_2$p (**14**) were prepared in a two-step procedure involving methylation of guanosine (**20**) or 2′-*O*-methyl guanosine (**21**) with iodomethane and subsequent conversion into bisphosphonate derivative. As these intermediates contained both a methylene modification in the phosphate chain

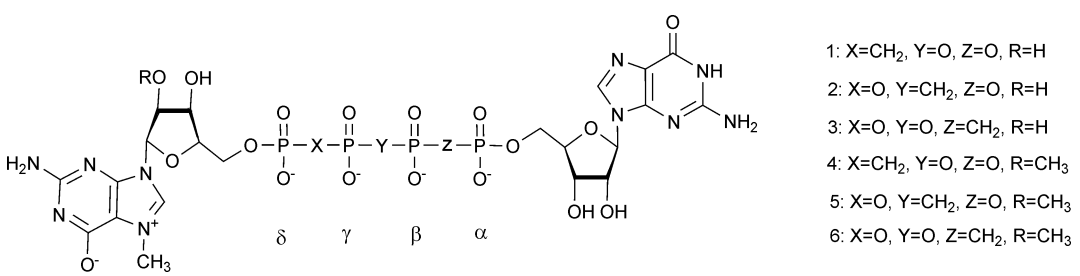
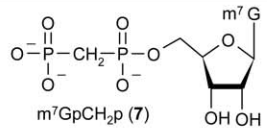
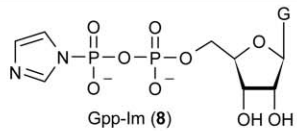
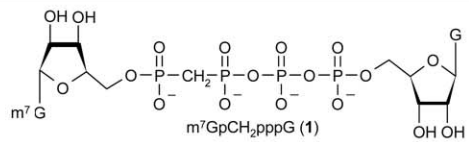
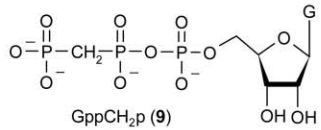
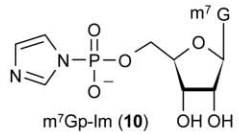
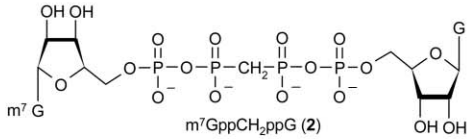
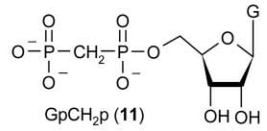
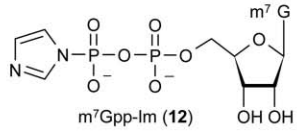
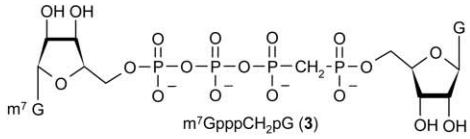
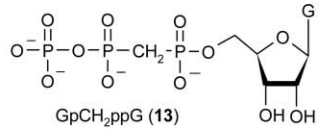
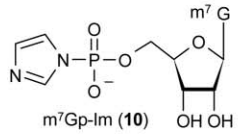
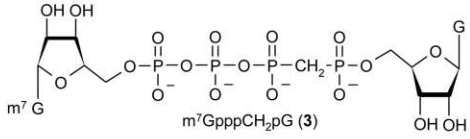
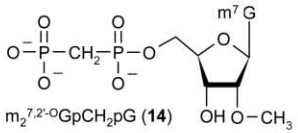
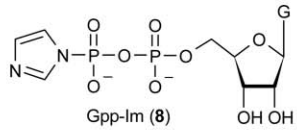
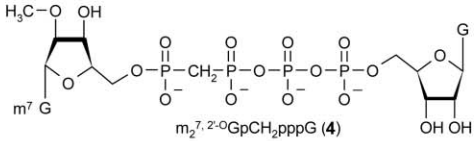
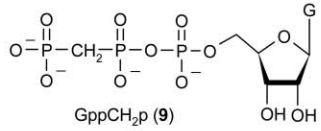
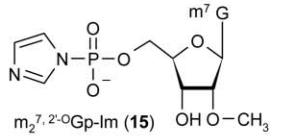
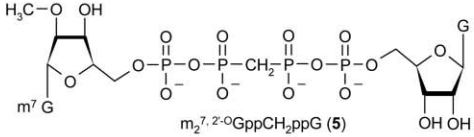
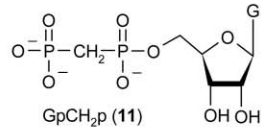
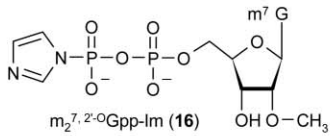
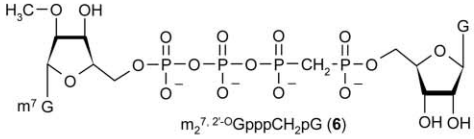
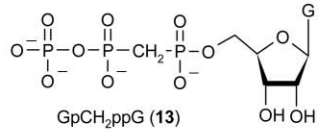
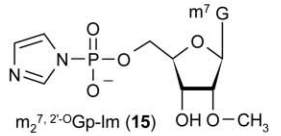
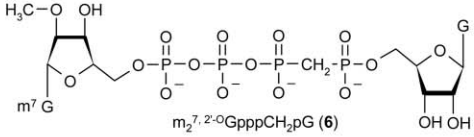


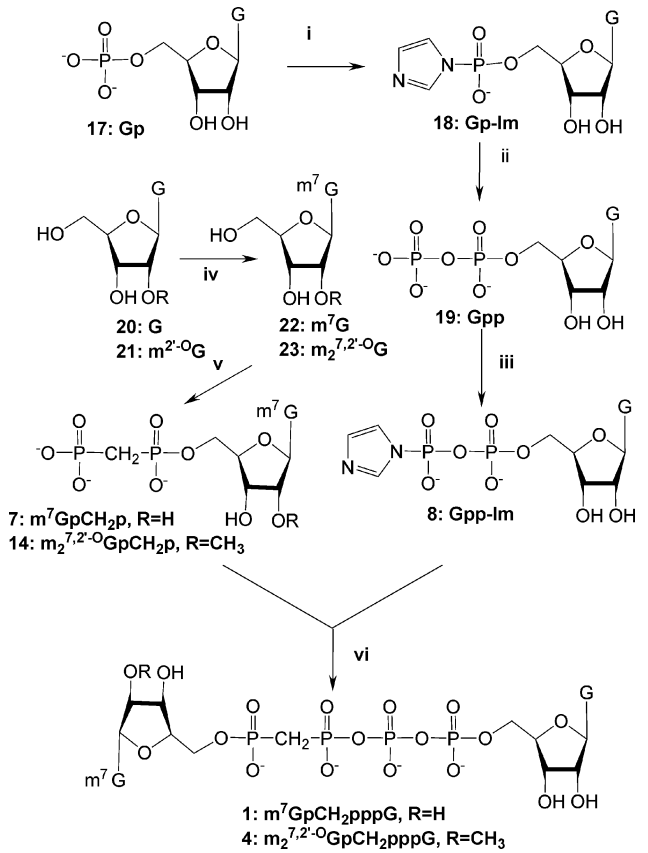
Fig. 1 Structure of methylenebis(phosphonate) tetraphosphate cap analogs **1–6**.

Table 1 Synthesis of methylenebis(phosphonate) tetraphosphate cap analogs[a]

Nucleophile	Activated nucleotide	Product	HPLC yield %[a]
 m^{7}GpCH$_2$p (**7**)	 Gpp-Im (**8**)	 m^{7}GpCH$_2$pppG (**1**)	77
 GppCH$_2$p (**9**)	 m^{7}Gp-Im (**10**)	 m^{7}GppCH$_2$ppG (**2**)	91
 GpCH$_2$p (**11**)	 m^{7}Gpp-Im (**12**)	 m^{7}GpppCH$_2$pG (**3**)	75
 GpCH$_2$ppG (**13**)	 m^{7}Gp-Im (**10**)	 m^{7}GpppCH$_2$pG (**3**)	68
 m$_2^{7,2'\text{-O}}$GpCH$_2$pG (**14**)	 Gpp-Im (**8**)	 m$_2^{7,2'\text{-O}}$GpCH$_2$pppG (**4**)	88
 GppCH$_2$p (**9**)	 m$_2^{7,2'\text{-O}}$Gp-Im (**15**)	 m$_2^{7,2'\text{-O}}$GppCH$_2$ppG (**5**)	89
 GpCH$_2$p (**11**)	 m$_2^{7,2'\text{-O}}$Gpp-Im (**16**)	 m$_2^{7,2'\text{-O}}$GpppCH$_2$pG (**6**)	84
 GpCH$_2$ppG (**13**)	 m$_2^{7,2'\text{-O}}$Gp-Im (**15**)	 m$_2^{7,2'\text{-O}}$GpppCH$_2$pG (**6**)	80

[a] HPLC yields were calculated as percentage conversion of nucleophilic agent into corresponding product, differences in extinction coefficients were taken into account.

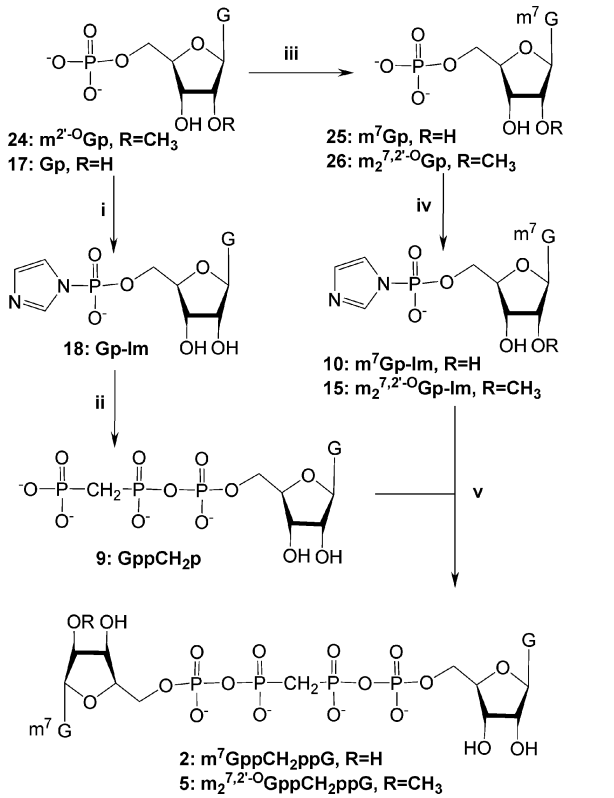
and were methylated at the N7 position, it was recommended not to convert them further but use them in the final step of synthesis. Syntheses of **1** and **4** are outlined in Scheme 1.



Scheme 1 Synthesis of analogs **1** and **4**; reagents: i. imidazole, 2,2′-dithiodipyridine, Ph_3P, DMF; ii. phosphate triethylammonium salt, $ZnCl_2$, DMF; iii. imidazole, 2,2′-dithiodipyridine, Ph_3P, DMF, TEA; iv. CH_3I, DMSO; v. 1. methylenebis(phosphonic dichloride), trimethyl phosphate, 0 °C, 2. triethylammonium bicarbonate; vi. $ZnCl_2$, DMF.

Analogs **2** and **5** bearing a methylene group between the β and γ phosphorus atoms could only be coupled using tri- and monophosphate subunits, as the pyrophosphate bond formed in zinc chloride-mediated coupling reactions is only present between α-β and γ-δ phosphorus atoms. In order to reduce the number of steps involving N7 derivatives, guanosine monophosphate P–imidazolide (**18**) was converted into $GppCH_2p$ (**9**) by coupling with methylenebis(phosphonate) triethylammonium salt. Finally, triphosphates were coupled with m^7Gp-Im (**10**) and $m_2^{7,2'\text{-O}}$Gp-Im (**15**), respectively. Syntheses and reaction conditions for analogs **2** and **5** are presented in Scheme 2.

Two synthetic routes were applied for analogs **3** and **6**, having a methylene modification between the α and β position. The first of them employed the coupling of two diphosphate units (Scheme 3). Both P-imidazolides, of m^7Gpp (**29**) and $GpCH_2p$ (**11**), are not easily formed, but it is easier to produce m^7Gpp-Im (**12**), so the route where m^7Gpp (**29**) is activated and $GpCH_2p$ (**11**) acts as the nucleophile was chosen. Guanosine diphosphate (**19**) was obtained by addition of an excess of triethylammonium phosphate to the imidazolide derivative of guanosine (**18**) in the presence of zinc dichloride. Then guanosine diphosphate (**19**) was methylated

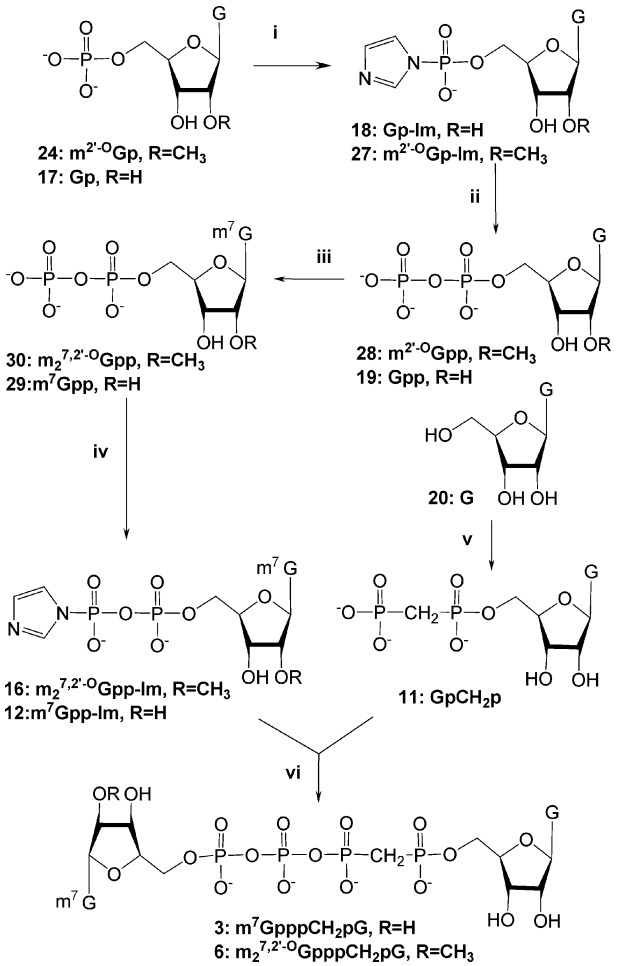


Scheme 2 Synthesis of analogs **2** and **5**; reagents: i. imidazole, 2,2′-dithiodipyridine, Ph_3P, DMF; ii. methylenebis(phosphonate) triethylammonium salt, $ZnCl_2$, DMF; iii. CH_3I, DMSO; iv. imidazole, 2,2′-dithiodipyridine, Ph_3P, DMF (for **26**) or DMSO (for **25**); v. $ZnCl_2$, DMF.

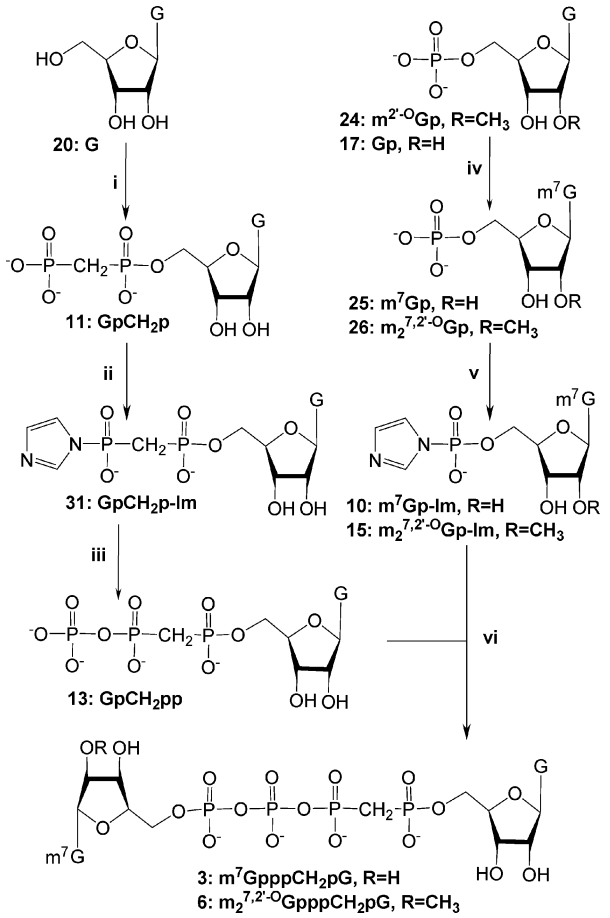
at the N7 position and converted into a P-imidazolide. $GpCH_2p$ (**11**) was synthesized from methylenebis(phosphonic dichloride) and guanosine (**20**). For the corresponding ARCA analog, a similar synthetic pathway was used. It is worth mentioning that derivatives containing 2′-methoxy and 7-methyl groups are more stable than their counterparts methylated only at the N7 position. Employing this reaction pathway, we were able to obtain analogs **3** and **6** with 75 and 84% HPLC yields, respectively.

In the second approach, tri- and monophosphate units were used (Scheme 4). Triphosphate $GpCH_2pp$ (**13**) was made from the earlier synthesized $GpCH_2p$ (**11**) after its activation as P-imidazolide. Coupling of $GpCH_2pp$ (**13**) with m^7Gp-Im (**10**) or $m_2^{7,2'\text{-O}}$Gp-Im (**15**) gave analogs **3** and **6**, with 68 and 80% yield by HPLC conversion, respectively. The yields obtained were not significantly different from those for the first synthetic route. In conclusion, both approaches can be successfully applied.

Intermediate compounds were prepared as described earlier.[20,22,26] The syntheses of diphosphate analogs bearing methylene modifications were carried out by phosphonylation of the appropriate nucleoside with methylenebis(phosphonic dichloride) *via* Yoshikawa's phosphorylation conditions.[27] Imidazolides were obtained by employing imidazole, 2,2′-dithiodipyridine and triphenylphosphine activation system in DMF,[24] except for m^7Gp-Im (**10**) where DMSO was used due to the poor solubility of 7-methylguanosine (**22**) in DMF. The change of solvent increased the reaction yield from 53 to 95%. Methylation reactions were performed using iodomethane in DMSO.



Scheme 3 Synthesis of analogs **3** and **6**—approach 1; reagents: i. imidazole, 2,2′-dithiodipyridine, Ph_3P, DMF; ii. phosphate triethylammonium salt, $ZnCl_2$, DMF; iii. CH_3I, DMSO; iv. imidazole, 2,2′-dithiodipyridine, Ph_3P, DMF; v. 1. methylenebis(phosphonate dichloride), trimethyl phosphate, 0 °C, 2. triethylammonium bicarbonate; vi. $ZnCl_2$, DMF.



Scheme 4 Synthesis of analogs **3** and **6**—approach 2; reagents: i. 1. methylenebis(phosphonic dichloride), trimethyl phosphate, 0 °C, 2. triethylammonium bicarbonate; ii. imidazole, 2,2′-dithiodipyridine, Ph_3P, DMF; iii. phosphate triethylammonium salt, $ZnCl_2$, DMF; iv. CH_3I, DMSO; v. imidazole, 2,2′-dithiodipyridine, Ph_3P, DMF (for **26**), DMSO (for **25**); vi. $ZnCl_2$, DMF.

Binding to eIF4E factor

The binding affinities of cap analogs **1–6** to mouse eIF4E were determined by a fluorescence quenching method.[28] The K_{AS} values are presented in Table 2, along with the same data for their unmodified parent compounds and triphosphate methylene analogs.

We found that in the tetraphosphate series, similarly to the triphosphate series,[29] methylene modification resulted in a decrease in the binding affinity to eIF4E.

However, all analogs **1–6** were characterized by significantly higher association constants than their triphosphate counterparts and unmodified cap structure m^7GpppG. Compared to m^7GpppG, binding affinities for analogs **1**,**2**,**4** and **5** are approximately 5 fold higher and for analogs **3** and **6** association constants were only slightly lower than for m^7GppppG and approximately 9 fold higher than for the standard.

Susceptibility to hydrolysis by DcpS

The analogs **1–6** were subjected to hydrolysis by human DcpS enzyme. In this experiment, m^7GpppG was used as a positive control. Besides m^7GpppG, also m^7GppppG, $m_2{}^{7,2'\text{-O}}$GpppG and $m_2{}^{7,2'\text{-O}}$GppppG were included in the assay as reference compounds. The samples collected from reaction mixtures at various time intervals were analyzed by RP HPLC. The amount of DcpS enzyme was optimized to provide complete degradation of the control substrate within 15 min. The examined compound was assigned as non-hydrolyzable when after two hours of incubation with hDcpS there was no trace of cleavage products.

The cap structure cleavage catalysed by hDcpS proceeds through nucleophilic attack of His277 on the γ phosphate group within the triphosphate bridge of substrates.[12] Unmodified cap structure, m^7GpppG, is degradated to m^7Gp and Gpp. We found that the tetraphosphate cap analog m^7GppppG undergoes cleavage between the δ and γ phosphates, as we noticed that m^7Gp and Gppp were released.

As it was previously shown, replacing the bridging oxygen atom in the polyphosphate chain with a methylene group resulted in enhanced stability of the P–C bond towards enzymatic degradation.[22,23] It was expected that introducing methylene modification between the γ and δ phosphorus atoms of a tetraphosphate cap analog would protect such an analog towards hydrolysis by DcpS. The experiment confirmed this thesis, as analogs **1** and **4**

Table 2 Properties of cap analogs

Cap analog	Hydrolysis by hDcpS	K_{AS} hDcpS [μM^{-1}]	K_{AS} eIF4E [μM^{-1}]	R (K_{AS} hDcpS/ K_{AS} eIF4E)	IC_{50} exp. A RRL system	IC_{50} exp. B RRL system
m^7GpppG	yes		9.4 ± 0.4	-	8.3 ± 0.2	N.D. (>20)
$m_2^{7,2'\text{-O}}$GpppG	yes	-	10.8 ± 0.3[b]	-	-	-
$m_2^{7,3'\text{-O}}$GpppG	yes[a]	-	10.2 ± 0.3[b]	-	-	-
m^7GpCH_2ppG	no[a]	234 ± 14[a]	6.3 ± 0.3[c]	37.1	-	-
m^7GppCH_2pG	yes[a]	-	8.6 ± 0.4[c]	-	-	-
$m_2^{7,3'\text{-O}}GpCH_2ppG$	no[a]	43.0 ± 4.0[a]	4.65 ± 0.03[c]	9.2	-	-
$m_2^{7,3'\text{-O}}GppCH_2pG$	no[a]	37.0 ± 3.0[a]	4.4 ± 0.2[c]	8.4	-	-
m^7GppppG	yes	-	110.9 ± 6.0	-	5.0 ± 0.5	N.D. (>20)
$m_2^{7,2'\text{-O}}$GppppG	yes	-	99.8 ± 6.0[b]	-	2.8 ± 0.2	4.2 ± 0.1
m^7GpCH_2pppG (**1**)	no	160 ± 17	51.8 ± 0.8	3.1	3.5 ± 0.3	3.8 ± 0.6
m^7GppCH_2ppG (**2**)	no	38.4 ± 3.9	48.6 ± 2.1	0.79	4.6 ± 0.6	3.8 ± 0.2
$m^7GpppCH_2pG$ (**3**)	yes	-	86.5 ± 1.4	-	3.6 ± 0.5	20.0 ± 2.7
$m_2^{7,2'\text{-O}}GpCH_2pppG$ (**4**)	no	13.6 ± 0.9	44.8 ± 2.0	0.30	3.0 ± 0.3	3.3 ± 0.1
$m_2^{7,2'\text{-O}}GppCH_2ppG$ (**5**)	no	37.7 ± 2.2	44.0 ± 3.2	0.86	2.8 ± 0.2	2.9 ± 0.3
$m_2^{7,2'\text{-O}}GpppCH_2pG$ (**6**)	yes	-	84.9 ± 3.6	-	3.3 ± 0.1	3.4 ± 0.4

[a] Data from reference 23. [b] Data from reference 20. [c] Data from reference 31.

turned out to be resistant towards DcpS. We also observed that extension of the triphosphate bridge resulted in a slight decrease in the hydrolysis rate, as tetraphosphate cap was cleaved slower than its triphosphate counterpart. This effect probably has a significant contribution to the stability of analogs **2** and **5**, which unexpectedly were also resistant towards enzymatic cleavage by DcpS. In the case of an ARCA-type analog (**5**), this observation can be also supported by extra stabilization due to the presence of a 2′-O methyl group. Similarly in the triphosphate series, analog $m_2^{7,3'\text{-O}}GppCH_2pG$ was non-hydrolyzable while m^7GppCH_2pG was susceptible to hydrolysis.

Analogs **3** and **6** undergo hydrolysis but with a much slower rate than m^7GpppG or m^7GppppG. After 15 min, when m^7GpppG was digested completely, there remained still 40% and 83% of **3** and **6** respectively.

The enzymatic resistance of triphosphate cap analogs modified in the β-γ position or tetraphosphate analogs **1** and **4** modified in the γ-δ position is easily explainable, since the phosphate closest to the m^7Guo moiety is the position at which nucleophilic attack of His277 in the DcpS active site occurs,[12b] hence the P–C binding is the one that should be cleaved during enzymatic reaction. However, the enzymatic resistance of triphosphate ARCAs modified in the α-β position or tetraphosphate analogs **2** and **5** modified in the β-γ position was rather unexpected, since the modification is in a position that is not directly involved in the catalytic mechanism. Thus, we presume that the resistance of analogs **2** and **5** might result from steric hindrances or conformational changes in the cap, introduced by the additional phosphate group and methylene modification (and for ARCA analogs additionally by the 2′-OMe group).

Fig. 2 depicts HPLC profiles for digestion of cap analogs with DcpS enzyme. Along with example cap analogs (**2**, **3**, **4**), a positive control (m^7GpppG) and analogs unmodified in the polyphosphate bridge (m^7GppppG, $m_2^{7,2'\text{-O}}$GppppG) are also shown.

Binding to hDcpS

The binding affinity of hDcpS to non-hydrolyzable cap analogs (**1**, **2**, **4**, **5**) was determined by monitoring of intrinsic Trp fluorescence quenching employing the Time Synchronized Titration method.[28]

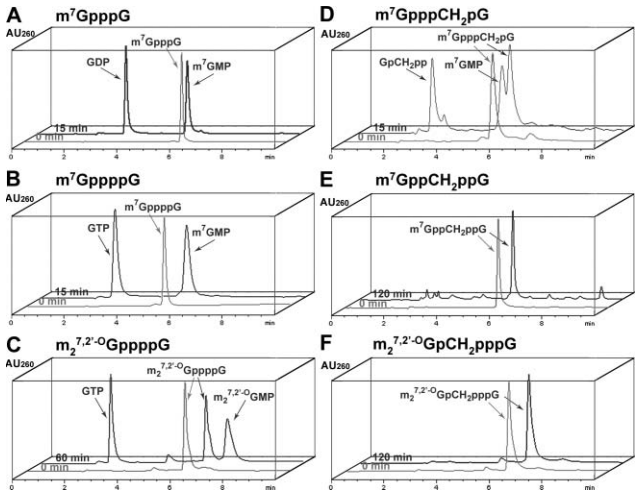


Fig. 2 Example HPLC profiles from the hDcpS susceptibility assay. Reaction conditions were optimized to provide complete degradation of m^7GpppG (positive control) within less than 15 min (A). m^7GppppG and $m_2^{7,2'\text{-O}}$GppppG were also substrates for DcpS, and were hydrolyzed regiospecifically to produce GTP and either m^7GMP or $m_2^{7,2'\text{-O}}$GMP, respectively (B and C). Among methylenebis(phosphonate) analogs only those modified at the α-β-position were hydrolyzed by DcpS (D). Analogs which remained undigested after 120 min of incubation with DcpS in these conditions (*e.g.* E and F) were assigned as *DcpS-resistant* (Table 2).

Equilibrium association constants of complexes of hDcpS with cap analogs are summarized in Table 2.

Analog **1** is characterized with the highest association constant (160.0 μM^{-1}), while its ARCA counterpart (**4**) has the lowest value of K_{AS} (13.6 μM^{-1}). Analogs **2** and **5** bind to DcpS with similar affinity (K_{AS} = 38.4 μM^{-1} and K_{AS} = 37.7 μM^{-1}, respectively). Comparison between corresponding analogs in tri- and tetraphosphate series leads to the conclusion that an additional phosphate group in the polyphosphate chain decreases binding affinity to DcpS. According to previous results,[22,30] introducing a methoxy group to the ribose moiety also resulted in a decrease of binding affinity, since ARCAs have lower K_{AS} values. That trend can be also noticed in the experiment with the tetraphosphate series. Apparently, the presence of a methylene group in the γ-δ

position does not interfere significantly with binding to DcpS, as analog **1** has the highest K_{AS} value. Similarly in the triphosphate series, m^7GpCH_2ppG was described with the highest K_{AS}.

Among characterized non-hydrolyzable analogs, two interesting types of compound can be discerned. We have introduced a new parameter, defined as a ratio of $K_{AS(DcpS)}$ and $K_{AS(eIF4E)}$ ($R = K_{DcpS}/K_{eIF4E}$), which enables a better description of those analogs. According to the definition, compounds with low R values have high affinity to eIF4E protein yet low for binding to DcpS. On the contrary, if the R value is high, K_{AS} for DcpS binding is much higher than for eIF4E. Compounds with a low R parameter are particularly valuable as translation inhibitors, as they bind strongly to eIF4E protein and additionally their effective concentration in cytoplasm would not be significantly decreased by formation of cap analog–DcpS complex. On the other hand, a high R value could ensure good properties of the cap analog as a DcpS inhibitor without significant interference with eIF4E. On the basis of its R parameter, the utility of an analog in designing affinity resins can be determined, as it might be easier to choose between those directed towards eIF4E or DcpS purification.

At this early stage of research, we are able to use R as a qualitative value, since no exact values of R below which eIF4E would be inhibited selectively or over which the compound would be bound mainly to DcpS were assigned.

Among the presented set of cap analogs, we have observed a difference of two orders of magnitude (*ca.* 120 fold) between the analog with the lowest R value (**4**) and the previously synthesized m^7GpCH_2ppG. The values obtained prove that modified cap analogs could be considered as good tools for selective inhibition of cap-dependent cellular processes.

The differences in the binding affinity of different cap analogs for eIF4E and DcpS can be explained by the geometry of interactions within the cap-binding pockets of these proteins. The cap–eIF4E complex, besides the stacking of 7-methylguanine between Trp-56 and Trp-102, is stabilized by numerous hydrogen bonds and salt bridges between the negatively charged triphosphate bridge and positively charged amino acid residues in the cap binding pocket. The eIF4E cap binding pocket is relatively loose and therefore the protein is not "sensitive" to steric hindrances and, as shown in numerous studies,[20] accepts different modifications within the cap structure. Hence, it is rather unlikely that the decrease in affinity of the methylene analogs to eIF4E is a result of structural or conformational changes, but could be explained by the inability of the CH_2 group to accept an H-bond interaction, which is important for stabilizing the cap–eIF4E complex. This is consistent with the fact that such interactions can be observed in the eIF4E–cap crystal structures *e.g.* between one of the phosphate bridging oxygen atoms and Lys 135.[28] On the contrary, the crystal structure of DcpS[12b] reveals that the active site is very tight. In agreement with that, in this and in our previous works,[22,30] we found that DcpS is very sensitive to even minor structural modifications within the cap structure (both in the 7-methylguanosine moiety as well as the triphosphate chain).

Inhibition of translation in RRL system

The ability of the new methylene analogs to inhibit cap-dependent translation was assayed in a rabbit reticulocyte lysate system. In our study, an *in vitro* transcribed, ARCA-capped, β-globin 5′ UTR containing mRNA encoding firefly luciferase was used to allow luminometric determination of protein synthesis.

Two sets of experiments were performed. In experiment A, the cap analog along with $m_2^{7,3'-O}$GpppG-5′UTRβglob-LUC mRNA were added to the translation reaction mixture. The assay was preincubated at 30 °C for 60 minutes prior to addition of mRNA and a cap analog. Reaction was stopped 60 min after addition of mRNA and the luciferase activity was measured (Fig. 3A). In experiment B, the cap analog was added to the translation reaction mixture at the beginning of the experiment, *i.e.* 60 min before the addition of mRNA (Fig. 3B). IC_{50} values for analogs **1–6** calculated from experimental data are summarized in Table 2. In the experiment A, all analogs **1–6** turned out to have better inhibitory properties than unmodified cap m^7GpppG. Extension of the triphosphate chain resulted in lower IC_{50} values, which is in agreement with the higher binding affinity to eIF4E in the tetraphosphate series. For methylene analogs **1–3**, although their binding to eIF4E is weaker than binding of $m^7GppppG$, methylene modification in the α-β (**3**) and γ-δ (**1**) positions of the tetraphosphate chain resulted in even lower IC_{50} values as compared to parent tetraphosphate cap analog.

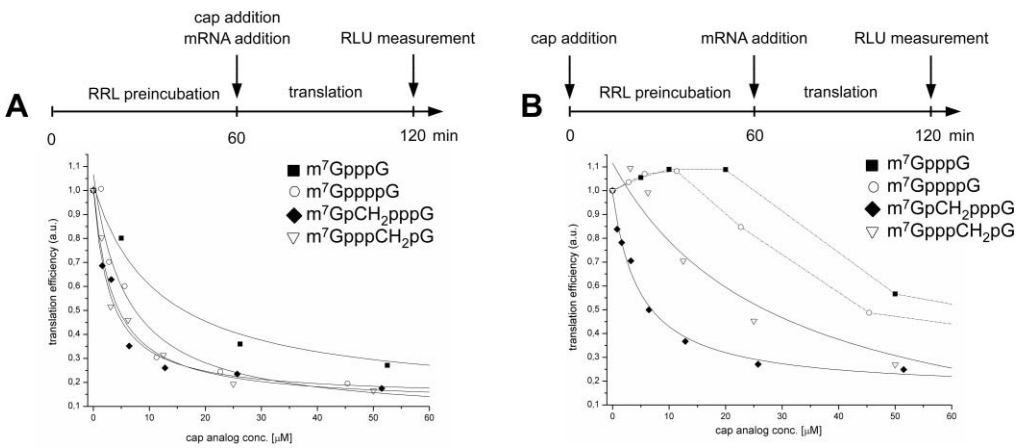


Fig. 3 Inhibition of translation in RRL system. A—experiment without preincubation of cap analogs in the RRL system, B—experiment with preincubation of cap analogs in the RRL system prior to addition of mRNA.

Experiment B revealed that the inhibitory potency of unmodified cap analogs is diminished if they are preincubated in RRL before addition of mRNA to the system.[11] We observed that preincubation diminishes the inhibitory properties of both m^7GpppG and m^7GppppG. Analogs, which were not susceptible to enzymatic hydrolysis (**1**, **2**, **4**, **5**) by DcpS enzyme, retained their inhibitory properties. Tetraphosphate ARCA-type analogs, which had increased resistance towards DcpS even without introducing a methylene moiety into the polyphosphate bridge, were comparably potent inhibitors in both experiments. On the basis of these results, it can be seen that decomposition of cap analogs vulnerable to enzymatic hydrolysis occurs during preincubation (exp. B). The rapidity of cap analog hydrolysis by DcpS correlates with the differences in the IC_{50} values obtained in experiments A and B. Analogs fully resistant towards degradation (**1**, **2**, **4**, **5**) did not change their inhibitory properties during preincubation. For analog **6**, which was hydrolyzed very slowly, the IC_{50} value changed only slightly, whereas for tetraphosphate ARCA $m^{7,2'\text{-}O}$GppppG, hydrolysis was faster and the change was more significant. For even more rapidly hydrolysed analog **3** the IC_{50} value increased strikingly.

The stability pattern of cap analogs **1–6** in the RRL system is in agreement with the properties of analogs **1–6** towards hydrolysis with DcpS. This indicates that the RRL system is a good model for predicting the stability of synthesized compounds in cellular systems.

ARCAs (**4–6**) are particularly designed as analogs aimed at obtaining capped mRNAs since 2′ methoxy modification provides only the correct orientation of building into the mRNA chain. In order to determine whether modified ARCA-type cap analogs are effectively incorporated into mRNA chain, we performed *in vitro* transcription reactions where short RNA transcripts are synthesized (uncapped 5-mer, pppGCCCC or capped 6-mer). Analysis of the reaction products in a denaturating polyacrylamide/7M urea gel showed that all the methylene-modified tetraphosphate cap analogs are incorporated into RNA transcript with an efficiency indistinguishable from standard cap m^7GpppG or GTP (Fig. 4A).

Long transcripts encoding luciferase mRNA capped with analogs **4–6** were also obtained (Fig. 4B) and used in *in vitro* translation experiments. In Fig. 4C, a plot of luciferase activity as a function of mRNA concentration is shown. These results indicate that synthesized ARCA cap analogs (**4–6**) can be considered as useful tools for obtaining capped mRNAs and *in vitro* protein expression. Further studies on this subject are in progress and will be described elsewhere.

Conclusions

Efficient syntheses of six novel tetraphosphate cap analogs bearing a methylene group in the polyphosphate moiety were performed. The methodology developed is general and thus can be applied to the synthesis of other important nucleotide analogs, *e.g.* Ap_4N modified in the tetraphosphate bridge with a methylene group.

We have found that all of the synthesized tetraphosphate cap analogs have significantly higher affinity to eIF4E protein (5–9 fold higher than control).

Analogs modified with the methylene group in the γ-δ and β-γ positions of the polyphosphate bridge were resistant against enzymatic hydrolysis by hDcpS.

Binding studies with DcpS enzyme revealed that some of the non-hydrolyzable cap analogs bind tightly to DcpS, and yet for some of them the K_{AS} value is low.

The diversity of cap analogs with respect to interactions with various proteins offers a set of analogs that could be useful in selective inhibition of processes in which cap is involved.

We have shown that methylene tetraphosphate cap analogs are efficient inhibitors of translation in an RRL system and are much more stable than their unmodified counterparts. These observations are very promising in the context of their cellular applications.

We have proved that tetraphosphate ARCAs are efficiently incorporated into the mRNA chain during *in vitro* transcription and that the obtained transcripts undergo cap-dependent translation. Further studies on *in vitro* and *in vivo* translational properties,

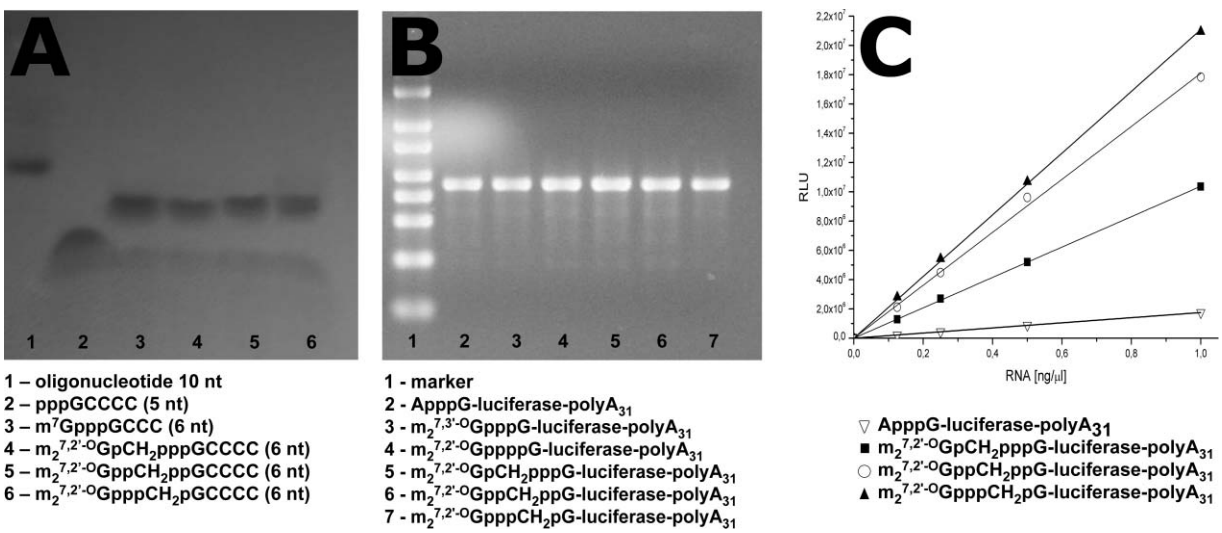


Fig. 4 A, Electrophoretic analysis of short transcripts capped with **4–6**. B, Electrophoretic analysis of long transcripts capped with **4–6**. C, Luciferase activity as a function of concentration of mRNA capped with ARCA analog.

stability in cellular conditions and utility in mRNA based therapy of transcripts capped with novel ARCA analogs are in progress.

Experimental

General

Intermediate nucleotides were separated by ion-exchange chromatography on DEAE-Sephadex A-25 (HCO_3^- form) column using a linear gradient of triethylammonium bicarbonate (TEAB) in deionized water and after evaporation under reduced pressure with addition of ethanol, isolated as triethylammonium (TEA) salts.

Final products were either converted into sodium salts by passing through Dowex 50 W × 8 (200-400 mesh) resin (Na^+ form) or, in case of any remaining impurities, further purification was performed employing semi-preparative RP HPLC and after repeated freeze-drying products were isolated as ammonium salts.

Yields were calculated either based on sample weight or optical units of the product. Optical units are defined as absorption of compound solution in phosphate buffer 0.1 M (pH = 7) at 260 nm multiplied by volume of the solution in ml.

Analytical HPLC was performed on Agilent Tech. Series 1200 using Supelcosil LC-18-T RP column (4.6 × 250 mm, flow rate 1.3 mL/min) with a linear gradient 0–25% of methanol in 0.05 M ammonium acetate buffer (pH 5.9) in 15 min, UV-detection at 260 nm and fluorescence detection (excitation at 280 nm and detection at 337 nm). Semi-preparative HPLC was performed on Waters 600E Multisolvent Delivery System apparatus using Discovery RP Amide C-16 HPLC column (25 cm × 21.2 mm, 5 μm, flow rate 5.0 mL/min) with linear gradient of methanol in 0.05 M ammonium acetate buffer (pH 5.9) and UV-detection at 260 nm.

The structure and homogeneity of each final product was confirmed by re-chromatography on RP HPLC, mass spectrometry using negative electrospray ionization (MS ESI-) and ^{1}H NMR and ^{31}P NMR spectroscopy.^{1}H NMR and ^{31}P NMR spectra were recorded at 25 °C on a Varian UNITY-plus spectrometer at 399.94 MHz and 161.90 MHz respectively. ^{1}H NMR chemical shifts were reported to sodium 3-trimethylsilyl-[2,2,3,3-D4]-propionate (TSP) in D_2O as an internal standard. ^{31}P NMR chemical shifts were reported to 20% phosphorus acid in D_2O as an external standard. Mass spectra were recorded on Micromass QToF 1 MS spectrometer.

Preparation of 2′-*O*-methylguanosine (21)

2′-*O*-Methylguanosine (**21**) was prepared as previously described[33] by methylation of 6-O-ethyl derivative of guanosine with diazomethane. The resulting mixture of 2′-O-methyl-6-O-ethylguanosine and 3′-methyl-6-O-ethylguanosine was resolved by column chromatography using Dowex 1 × 2 Ion-Exchange Resin (200–400 mesh) and 25% methanol in water as eluent in an isocratic system.

Commercially available compounds

Guanosine (**20**) was purchased from US Biological. Methylenebis-(phosphonic dichloride) tetrachloride was purchased from Sigma-Aldrich.

Guanosine monophosphate disodium salt was purchased from Sigma-Aldrich and converted into triethylammonium salt (**17**) using Dowex 50 W × 8 (200–400 mesh) ion-exchange resin. Methylenebis(phosphonate) tetrasodium salt was purchased from Sigma-Aldrich and changed into TEA form employing the same procedure.

General procedure for obtaining nucleotide imidazolide derivatives

Imidazolides (**8**, **12**, **15**, **16**, **18**, **27**) were prepared according to previously described procedure.[20] An appropriate nucleotide (1 eq., TEA salt), imidazole (8 eq.), 2,2′-dithiodipyridine (3 eq.) were mixed in DMF (~2.5 ml/100 mg of nucleotide). Triethylamine (2 eq.) and triphenylphosphine (3 eq.) were added and the mixture was stirred for 6–8 h. The product was precipitated from reaction mixture with a solution of anhydrous $NaClO_4$ (1 eq. per one negative charge) in dry acetone (~8 ml/1 ml of DMF). After cooling at 4 °C, the precipitate was filtered, washed repeatedly with cold, dry acetone and dried *in vacuum* over P_4O_{10}. Yields: 80–100%.

Synthesis of **10** and **31** were performed in different conditions according to the poor yields obtained with the previous methodology.

Synthesis of m^{7}Gp-Im (10), 7-methylguanosin-5′-yl monophosphate P-imidazolide

m^{7}Gp/TEA (**25**) (1.916 g, 4 mmol), imidazole (4.060 g, 60 mmol), 2,2′-dithiodipyridine (5.250 g, 24 mmol) were mixed in anhydrous DMSO (20 ml). Then triethylamine (1.12 ml, 8 mmol) and triphenylphosphine (6.270 g, 24 mmol) were added. Mixture was stirred at room temperature overnight and poured into a beaker containing 490 mg sodium perchlorate (4 mmol) dissolved in cool anhydrous acetone. The precipitate was filtered, washed with acetone and dried in vacuum desiccator over P_4O_{10}. Product was obtained as a white powder (1.712 g, 3.8 mmol, 95% yield).

^{1}H NMR (D_2O, 400 MHz): δ 7.88 (1H, s, $H2_{im}$), 7.22 (1H, s, $H4_{im}$), 7.04 (1H, s, $H3_{im}$), 5.99 (1H, bs, H1′), 4.69 (1H, m, H2′), 4.38 (1H, m, H3′), 4.34 (1H, m, H4′), 4.23 (1H, m, H5′), 4.13 (1H, m, H5″), 4.09 (3H, s, CH_3). ^{31}P NMR (D_2O, 163 MHz): δ −7.73 (1P, s, α).

Synthesis of GpCH$_2$p-Im (31), P1-guanosin-5′-yl 1,2-methylene-diphosphate P2- imidazolide

GpCH$_2$p/TEA (**11**) (4500 opt. u.; 0.33 mmol, 210 mg), imidazole (449 mg, 6.6 mmol), 2,2′-dithiodipyridine (726 mg, 3.3 mmol) were mixed in anhydrous DMF (10 ml). Then triethylamine (460 μl, 3.3 mmol) and triphenylphosphine (865 mg, 3.3 mmol) were added. The mixture was stirred at room temperature overnight and poured into a beaker containing 81 mg (0.66 mmol) sodium perchlorate dissolved in 50 ml of cool anhydrous acetone. The precipitate was filtered, washed and dried in vacuum desiccator over P_4O_{10}. The product was obtained as a white powder (118 mg, 0.22 mmol, 67% yield).

^{1}H NMR (D_2O, 400 MHz): δ 8.17 (1H, s, $H2_{im}$), 8.09 (1H, s, H8), 7.38 (1H, s, $H4_{im}$), 7.17 (1H, s, $H3_{im}$), 5.92 (1H, d, $J_{1'-2'}$=5.2 Hz, H1′), 4.45 (1H, m, H2′), 4.27 (1H, m, H3′), 4.20 (1H, m, H4′), 4.05 (2H, m, H5′,5″), 2.44 (2H, t, J=19.2 Hz, P–CH_2–P). ^{31}P NMR (D_2O, 163 MHz): δ 14.82 (1P, m, α), 10.20 (1P, m, β).

Preparation of 7-methyl derivatives—selective methylation

7-Methylguanosine (**22**) was prepared by treatment of guanosine with CH_3I in DMSO according to previously reported procedure.[32] 7,2′-*O*-dimethylguanosine (**23**) was obtained similarly from 2′-*O*-methylguanosine. 7,2′-*O*-dimethylguanosine diphosphate (**30**) was synthesized as described before[20] employing CH_3I in DMSO. In similar pattern, 7-methylguanosine monophosphate (**25**), 7,2′-*O*-dimethylguanosine monophosphate (**26**) and 7-methylguanosine diphosphate (**29**) were prepared.

Polyphosphate chain elongation

The elongation of polyphosphate chain by one phosphate unit was performed by coupling corresponding imidazolide with phosphate TEA salt. 2′-*O*-Methyl guanosine diphosphate (**28**) was prepared as was described before[20] from 2′-*O*-methyl guanosine monophosphate P-imidazolide (**27**). Similarly, guanosine diphosphate (**19**) was synthesized from guanosine monophosphate P-imidazolide (**18**).

Synthesis of nucleotides containing methylenebis(phosphonate) unit was prepared using the same methodology. In case of compound **9**, methylenebis(phosphonate) was used instead of phosphate.

Synthesis of $GpCH_2pp$ (13), P1-guanosin-5′-yl 1,2-methylene-triphosphate

$GpCH_2p$-Im (**31**) (168 mg, 0.31 mmol) and PO_4^{3-}/TEA (348 mg, 1.4 mmol) were mixed in anhydrous DMF (5 ml). $ZnCl_2$ (381 mg, 2.8 mmol) was added. Reaction was stirred at room temperature for 4 h and progress was monitored by HPLC. To stop the reaction, a solution of EDTA (1.042 g, 2.8 mmol) in water was added and pH was brought to 7 by addition of solid $NaHCO_3$. The product was purified employing DEAE–Sephadex in linear 0-1.2 M gradient of TEAB. GpCH2pp (**13**) was obtained as triethylammonium salt (1100 opt. u., 76 mg, 0.092 mmol) with 30% yield.

^{1}H NMR (D_2O, 400 MHz): δ 8.14 (1H, s, H8), 5.92 (1H, d, $J_{1'-2'}$=6.4 Hz, H1′), 4.83 (1H, dd, $J_{1'-2'}$=6.4 Hz, $J_{2'-3'}$=4.0, H2′), 4.56 (1H, dd, $J_{2'-3'}$=4.0 Hz, $J_{3'-4'}$=4.4 Hz, H3′), 4.33 (1H, m, H4′), 4.17 (2H, m, H5′, H5″), 2.36 (2H, t, J=20.4 Hz, P-CH_2-P). ^{31}P NMR (D_2O, 163 MHz): δ 17.83 (1P, m, Pα), 6.87 (1P, m, Pβ), –10.31 (1P, d, J=25.3 Hz, Pγ). ESI MS: calcd for $C_{11}H_{17}N_5O_{13}P_3$ 520.0036. Found 519.9490 $[M - H]^-$.

Synthesis of $GppCH_2p$ (9), P1-guanosin-5′-yl 2,3-methylene-triphosphate

9 was synthesized by mixing methylenebis(phosphonate)/TEA (500 mg, 1.32 mmol) with guanosine 5′-monophosphate imidazolide (150 mg, 0.34 mmol) in DMF (4 ml) as a solvent. Zinc chloride (400 mg, 2.9 mmol) was added and reaction was stirred at room temperature and was stopped after 2 h by addition of EDTA (1.1 g, 2.9 mmol) solution in water. pH was adjusted to 7 with solid $NaHCO_3$. The product was purified by ion-exchange chromatography on DEAE–Sephadex. Yield after chromatographic purification: 68% (3550 opt. u., 190 mg, 0.23 mmol).

^{1}H NMR (D_2O, 400 MHz): δ 8.09 (1H, s, H8), 5.85 (1H, d, $J_{1'-2'}$=6.0 Hz, H1′), 4.72 (1H, dd, $J_{1'-2'}$=5.6 Hz, $J_{2'-3'}$=5.2 Hz, H2′), 4.47 (1H, dd, $J_{2'-3'}$=5.2 Hz, $J_{3'-4'}$=3.3 Hz, H3′), 4.27 (1H, m, H4′), 4.14 (2H, m, H5′, H5″), 2.24 (2H, t, J=20.4Hz, P-CH_2-P). ^{31}P NMR (D_2O, 163 MHz): δ 14.91 (1P, dt, J1=8.7 Hz, J2=19.9 Hz, Pγ), 8.80 (1P, m, Pβ), –10.77 (1P, d, J=26.2 Hz, Pα). ESI MS: calcd for $C_{11}H_{17}N_5O_{13}P_3$ 520.0036. Found 519.9407 $[M - H]^-$.

General procedure for phosphonylation and phosphorylation

Phosphorylation of 2′-*O*-methylguanosine (**21**) resulting in 2′-*O*-methylguanosine monophosphate (**24**) was performed as described previously[20] employing Yoshikawa reaction (Yoshikawa) with phosphorus oxychloride in trimethyl phosphate as a solvent.[27]

Phosphonylation of guanosine (**20**) with methylenebis-(phosphonic dichloride), resulting in **11**, was achieved by modified Yoshikawa procedure as described in the literature.[26]

Compounds **7** and **14** were synthesized following the same procedure.

Synthesis of m^7GpCH_2p (7), P1-(7-methylguanosin-5′-yl) 1,2-methylenediphosphate

m^7G (**22**) (42 mg, 0.14 mmol) was dissolved in 2 ml of trimethyl phosphate and cooled to 0 °C on an ice bath. Methylenebis(phosphonic dichloride) (140 mg, 0.56 mmol) was added to the stirred mixture. Reaction was performed at 0 °C and after 1 h, when HPLC showed disappearance of substrate, it was stopped by addition of 0.7 M TEAB to pH = 7. The product was purified by ion–exchange chromatography on DEAE-Sephadex as TEA salt (85 mg, 1230 opt.u., 0.11 mmol, 77%).

^{1}H NMR (400 MHz, D_2O): δ 6.07 (1H, d, $J_{1'-2'}$=3.3 Hz, H1′), 4.68 (1H, dd, J_1=3.3 Hz, J_2=4.7 Hz, H2′), 4.53 (1H, dd, J_1=4.7 Hz, J_2=5.4 Hz, H3′), 4.40 (1H, dt, J_1=5.4 Hz, J_2=2.0 Hz, H4′), 4.31 (1H, ddd, J_1=2.0 Hz, J_2=11.7 Hz, J_3 =4.3 Hz, H5′), 4.20 (1H, ddd, J_1=2.0 Hz, J_2=11.7 Hz, J_3=5.7 Hz, H5″), 4.13 (3H, s, CH_3), 2.21 (2H, t, P-CH_2-P, J=19.6 Hz). ^{31}P NMR (163 MHz, D_2O): δ 19.18 (1P, m, Pα), 15.11 (1P, dt, J_1=10.5 Hz, J_2=19.6 Hz, Pβ).

Synthesis of $m_2^{7,2'-O}GpCH_2p$ (14), P1-(7,2′-*O*-dimethylguanosin-5′-yl) 1,2-methylenediphosphate

Compound **14** was obtained (133 mg, 0.20 mmol, 63%) starting from $m^{7,2'-O}G$ (100 mg, 0.32 mmol) and following the same procedure as for **7**.

^{1}H NMR (D_2O, 400 MHz): δ 9.36 (1H, s, H8), 6.17 (1H, d, $J_{1'-2'}$=6.2 Hz, H1′), 4.63 (1H, dd, $J_{2'-3'}$=6.2 Hz, $J_{3'-4'}$=4.6 Hz, H3′), 4.28–4.42 (4H, m, H2′, H4′, H5′, H5″), 4.14 (3H, s, CH_3), 3.60 (3H, s, OCH_3), 2.23 (2H, t, J=19.8 Hz, P-CH_2-P). ^{31}P NMR (163 MHz, D_2O): δ 18.67 (1P, m, Pα), 15.15 (1P, dt, J_1=10.6 Hz, J_2=19.7 Hz, Pβ).

m^7GpCH_2pppG (1), P1-(7-methylguanosin-5′-yl) P4-guanosin-5′-yl 1,2-methylenetetraphosphate

m^7GpCH_2p (**7**) (55 mg, 0.084 mmol) and Gpp-Im (**8**) (82 mg, 0.15 mmol) were mixed in anhydrous DMF (3 ml). $ZnCl_2$ (170 mg, 1.25 mmol) was added. Reaction was stirred at room temperature for 4 h and progress was monitored by HPLC. To stop the reaction, solution of EDTA (465 mg, 1.25 mmol) in water was added and pH was brought to 7 by addition of solid $NaHCO_3$. The product

was purified employing DEAE–Sephadex. **1** was obtained as triethylammonium salt (906 opt. u., 57 mg, 0.043 mmol). Further purification was performed using preparative HPLC (buffers: A- 0.05M ammonium acetate, pH = 5.9, B- 50%A, 50% methanol; linear gradient from 0 to 50% B in 60 min, rt = 20 min). The product was collected, methanol was evaporated and solution was lyophilized to give **1** as white powder (ammonium salt, 680 opt. u., 32 mg, 0.032 mmol); yield after further HPLC purification 40%. Percentage HPLC conversion of m^7GpCH_2p into **1**—77%, yield of purification by ion exchange chromatography—66%, yield of purification by preparative HPLC—75%, final yield—38%.

^{1}H NMR (400 MHz, D_2O): δ 9.36 (1H, s, $H8_{m7G}$), 8.08 (1 H, s, $H8_G$), 5.99 (1H, d, $J_{1'-2'}$=3.4 Hz, $H1'_{m7G}$), 5.84 (1H, d, $J_{1'-2'}$=6.2 Hz, $H1'_G$), 4.64 (1H, dd, $J_{1'-2'}$=3.4 Hz, $J_{2'-3'}$=6.4 Hz, $H2'_{m7G}$), 4.73 (1H, t, $J_{1'-2'}$=6.2 Hz, $J_{2'-3'}$=5.8 Hz, $H2'_G$), 4.53 (2H, m, $H3'_{m7G}$, $H3'_G$), 4.40–4.17 (6H, m; $H4'_{m7G}$, $H4'_G$, $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.07 (3H, s, CH_3), 2.45 (2H, t, J=20.4 Hz, P-CH_2-P); ^{31}P NMR (162 MHz, D_2O) δ −11.09 (1P, α), −22.57 (1P, β), 7.54 (1P, γ), 17.69 (1P, δ). ESI MS: calcd for $C_{22}H_{31}N_{10}O_{20}P_4$ 879.0666. Found 879.0549 $[M - H]^-$.

Synthesis of m^7GppCH_2ppG (2), P1-(7-methylguanosin-5′-yl) P4-guanosin-5′-yl 2,3-methylenetetraphosphate

m^7Gp-Im (**10**) (81 mg, 0.18 mmol) and $GppCH_2p$ (**9**) (100 mg, 0.12 mmol) were mixed in anhydrous DMF (2.5 ml). $ZnCl_2$ (135 mg, 1.0 mmol) was added. Reaction was stirred at room temperature for 4 h and progress was monitored by HPLC. To stop the reaction, solution of EDTA (370 mg, 1.0 mmol) in water was added and pH was brought to 7 by addition of solid $NaHCO_3$. The product was purified employing DEAE–Sephadex. **2** was obtained as TEA salt (1666 opt. u., 102 mg, 0.079 mmol). Further purification was performed using preparative HPLC (buffers: A - 0.05M ammonium acetate, pH = 5.9, B - 50%A, 50% methanol; linear gradient from 0 to 50% B in 60 min, rt = 22 min). The product was collected, methanol was evaporated and solution was lyophilized to give **2** as white powder (ammonium salt, 729 opt. u., 58 mg, 0.035 mmol). Percentage HPLC conversion of $GppCH_2p$ into **2**—91%, yield of purification by ion exchange chromatography—73%, yield of purification by preparative HPLC—44%, final yield—29%.

^{1}H NMR (400 MHz, D_2O): δ 8.09 (1 H, s, $H8_G$), 6.00 (1H, d, $J_{1'-2'}$=3.6 Hz, $H1'_{m7G}$), 5.87 (1H, d, $J_{1'-2'}$=6.4 Hz, $H1'_G$), 4.60 (1H, t, $J_{1'-2'}$=3.6 Hz, $J_{2'-3'}$=5.6 Hz, $H2'_{m7G}$), 4.76 (1H, dd, $J_{1'-2'}$=6.4 Hz, $J_{2'-3'}$=5.8 Hz, $H2'_G$), 4.50 (1H, dd, $J_{2'-3'}$=5.6 Hz, $J_{3'-4'}$=4.8 Hz $H3'_{m7G}$), 4.55 (1H, m, $H3'_G$), 4.40–4.20 (6H, m; $H4'_{m7G}$, $H4'_G$, $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.09 (3H, s, CH_3), 2.52 (2H, t, J=21 Hz, P-CH_2-P); ^{31}P NMR (162 MHz, D_2O) δ −10.76 (2P; α,δ), 7.75 (2P; β,γ). ESI MS: calcd for $C_{22}H_{31}N_{10}O_{20}P_4$ 879.0666. Found 879.0566 $[M - H]^-$.

Synthesis of $m^7GpppCH_2pG$ (3), P1-(7-methylguanosin-5′-yl) P4-guanosin-5′-yl 1,2-methylenetetraphosphate

Method 1. m^7Gpp-Im (**12**) (30 mg, 0.055 mmol) and $GpCH_2p$ (**11**) (31 mg, 0.0475 mmol) were mixed in anhydrous DMF (2 ml). $ZnCl_2$ (61 mg, 0.45 mmol) was added. Reaction was stirred at room temperature for 4 h and progress was monitored by HPLC. To end the reaction, solution of EDTA (167 mg, 0.45 mmol) in water was added and pH was brought to 7 by addition of solid $NaHCO_3$. The product was purified employing preparative HPLC (buffers: A- 0.05M ammonium acetate, pH = 5.9, B- 50%A, 50% methanol; linear gradient from 0 to 50% B in 60 min, rt = 19 min). The product was collected, methanol was evaporated and solution was lyophilized to give **3** as white powder (ammonium salt, 8.6 mg, 0.009 mmol). Percentage HPLC conversion of $GpCH_2p$ into **3**—75%, yield of purification by preparative HPLC—25%, final yield—19%.

^{1}H NMR (400 MHz, D_2O): δ 8.10 (1 H, s, $H8_G$), 5.98 (1H, d, $J_{1'-2'}$=3.5 Hz, $H1'_{m7G}$), 5.85 (1H, d, $J_{1'-2'}$=6.0 Hz, $H1'_G$), 4.63 (1H, dd, $J_{1'-2'}$=3.5 Hz, $J_{2'-3'}$=5.3 Hz, $H2'_{m7G}$), 4.72 (1H, dd, $J_{1'-2'}$=6.0 Hz, $J_{2'-3'}$=4.8 Hz, $H2'_G$), 4.51 (2H, m, $H3'_{m7G}$, $H3'_G$), 4.41–4.17 (6H, m; $H4'_{m7G}$, $H4'_G$, $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.08 (3H, s, CH_3), 2.43 (2H, t, J=20.4 Hz, P-CH_2-P); ^{31}P NMR (162 MHz, D_2O) δ 17.36 (1P, α), 8.33 (1P, β), −22.07 (1P, γ), −11.04 (1P, δ). ESI MS: calcd for $C_{22}H_{31}N_{10}O_{20}P_4$ 879.0666. Found 879.0514 $[M - H]^-$.

Method 2. $GpCH_2pp$ (**13**) (10 mg, 0.012 mmol) and m^7Gp-Im (**10**) (8 mg, 0.018 mmol) were mixed in anhydrous DMF (0.5 ml). $ZnCl_2$ (20 mg, 0.15 mmol) was added. Reaction was stirred at room temperature for 4 h and progress was monitored by HPLC. To end the reaction, solution of EDTA (55 mg, 0.15 mmol) in water was added and pH was brought to 7 by addition of solid $NaHCO_3$. HPLC conversion of $GpCH_2pp$ into **3**—68%. The product was purified employing preparative HPLC (buffers: A- 0.05M ammonium acetate, pH = 5.9, B- 50%A, 50% methanol; linear gradient from 0 to 50% B in 60 min, rt = 19 min). The product was collected, methanol was evaporated and solution was lyophilized to give **3** as white powder (ammonium salt, 4 mg, 0.0042 mmol).

Percentage HPLC conversion of $GpCH_2p$ into **3**—68%, yield of purification by preparative HPLC—52%, final yield—35%.

Synthesis of $m_2^{7,2'-O}GpCH_2pppG$ (4), P1-(7, 2′-*O*-dimethylguanosin-5′-yl) P4-guanosin-5′-yl 1,2-methylenetetraphosphate

$m_2^{7,2'-O}GpCH_2p$ (**14**) (54 mg, 0.081 mmol) and Gpp-Im (**8**) (80 mg, 0.15 mmol) were mixed in anhydrous DMF (2 ml). $ZnCl_2$ (57 mg, 0.42 mmol) was added. Reaction was stirred at room temperature for 2 h and progress was monitored by HPLC. To stop the reaction, solution of EDTA (156 mg, 0.042 mmol) in water was added and pH was brought to 7 by addition of solid $NaHCO_3$. The product was purified employing DEAE–Sephadex. **4** was obtained as triethylammonium salt (960 opt. u., 59 mg, 0.046 mmol). Further purification was performed using preparative HPLC (buffers: A- 0.05M ammonium acetate, pH = 5.9, B- 50%A, 50% methanol; linear gradient from 0 to 50% B in 50 min, rt = 21 min). The product was collected, methanol was evaporated and solution was lyophilized to give **4** as white solid (ammonium salt, 750 opt. u., 35 mg, 0.036 mmol). Percentage HPLC conversion of $GpCH_2pp$ into **4**—88%, yield of purification by ion exchange chromatography—65%, yield of purification by preparative HPLC—77%, final yield—44%.

^{1}H NMR (400 MHz, D_2O): δ 9.36 (1H, s, $H8_{m7G}$), 8.09 (1 H, s, $H8_G$), 6.07 (1H, d, $J_{1'-2'}$=2.4 Hz, $H1'_{m7G}$), 5.86 (1H, d, $J_{1'-2'}$=6.4 Hz, $H1'_G$), 4.75 (1H, dd, $J_{1'-2'}$=6.4 Hz, $J_{2'-3'}$=6.0 Hz, $H2'_G$), 4.62 (1H, m, $H3'_{m7G}$), 4.55 (1H, m, $H3'_G$), 4.40–4.31 (3H, m; $H2'_{m7G}$, $H4'_{m7G}$, $H4'_G$), 4.31–4.18 (4H, m; $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.10 (3H, s,

CH_3), 3.61 (3H, s, OCH_3), 2.47 (2H, t, J=20.4 Hz, P-CH_2-P); ^{31}P NMR (162 MHz, D_2O) δ -11.02 (1P, α), –22.63 (1P, β), 7.58 (1P, γ), 17.69 (1P, δ). ESI MS: calcd for $C_{23}H_{33}N_{10}O_{20}P_4$ 893.0823. Found 893.0533 $[M - H]^-$.

Synthesis of $m_2^{7,2'\text{-}O}$GppCH$_2$ppG (5), P1-(7, 2′-*O*-dimethylguanosin-5′-yl) P4-guanosin-5′-yl 2,3-methylenetetraphosphate

$m_2^{7,2'\text{-}O}$Gp-Im (**15**) (31 mg, 0.066 mmol) and GppCH$_2$p (**9**) (750 opt. u., 52 mg, 0.063 mmol) were mixed in anhydrous DMF (2 ml). $ZnCl_2$ (60 mg, 0.44 mmol) was added. Reaction was stirred at room temperature for 4.5 h and progress was monitored by HPLC. In order to stop the reaction, solution of EDTA (164 mg, 0.44 mmol) in water was added and pH was brought to 7 by addition of solid $NaHCO_3$. The product was purified employing DEAE–Sephadex. **5** was obtained as triethylammonium salt (705 opt. u., 44 mg, 0.034 mmol). Further purification was performed using preparative HPLC (buffers: A- 0.05M ammonium acetate, pH = 5.9, B- 50%A, 50% methanol; linear gradient from 0 to 50% B in 50 min, rt = 28 min). The product was collected, methanol was evaporated and solution was lyophilized to give **5** as white solid (ammonium salt, 578 opt. u., 27 mg, 0.028 mmol). Percentage HPLC conversion of GppCH$_2$p into **5**—89%, yield of purification by ion exchange chromatography—61%, yield of purification by preparative HPLC—81%, final yield—44%.

^{1}H NMR (400 MHz, D_2O): δ 8.07 (1 H, s, $H8_G$), 6.07 (1H, d, $J_{1'\text{-}2'}$=2.8 Hz, $H1'_{m7G}$), 5.85 (1H, d, $J_{1'\text{-}2'}$=6.2 Hz, $H1'_G$), 4.74 (1H, m, $H2'_G$), 4.57 (1H, m, $H3'_{m7G}$), 4.53 (1H, m, $H3'_G$), 4.37–4.31 (3H, m; $H2'_{m7G}$, $H4'_{m7G}$, $H4'_G$), 4.31–4.20 (4H, m; $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.09 (3H, s, CH_3), 3.60 (3H, s OCH_3), 2.50 (2H, t, J=21 Hz, P-CH_2-P); ^{31}P NMR (162 MHz, D_2O) δ –10.81 (2P; α,δ), 7.71 (2P; β,γ). ESI MS: calcd for $C_{23}H_{33}N_{10}O_{20}P_4$ 893.0823. Found 893.0516 $[M - H]^-$.

Synthesis of $m_2^{7,2'\text{-}O}$GpppCH$_2$pG (6), P1-(7, 2′-*O*-dimethylguanosin-5′-yl) P4-guanosin-5′-yl 3,4-methylenetetraphosphate

Method 1. $m_2^{7,2'\text{-}O}$Gpp-Im (**16**) (13 mg, 0.023 mmol) and GpCH$_2$p (**11**) (10 mg, 0.015 mmol) were mixed in anhydrous DMF (0.5 ml). $ZnCl_2$ (17 mg, 0.13 mmol) was added. Reaction was stirred at room temperature for 4 h and progress was monitored by HPLC. To stop the reaction, solution of EDTA (46 mg, 0.13 mmol) in water was added and pH was brought to 7 by addition of solid $NaHCO_3$. The product was purified employing preparative HPLC (buffers: A- 0.05M ammonium acetate, pH = 5.9, B- 50%A, 50% methanol; linear gradient from 0 to 50% B in 60 min, rt = 19 min). Product was collected, methanol was evaporated and solution was lyophilized to give **6** as white powder (ammonium salt, 6.9 mg, 0.0072 mmol). Percentage HPLC conversion of GpCH$_2$p into **6**—84%, yield of purification by preparative HPLC—57%, final yield—48%.

^{1}H NMR (400 MHz, D_2O): δ 9.19 (1H, s, $H8_{m7G}$), 8.09 (1 H, s, $H8_G$), 6.10 (1H, d, $J_{1'\text{-}2'}$=2.6 Hz, $H1'_{m7G}$), 5.99 (1H, d, $J_{1'\text{-}2'}$=4.3 Hz, $H1'_G$), 4.71 (1H, t, $J_{1'\text{-}2'}$=4.3 Hz, $J_{2'\text{-}3'}$=5.1 Hz, $H2'_G$), 4.62 (1H, m, $H3'_{m7G}$), 4.56 (1H, m, $H3'_G$), 4.45–4.20 (7H, m; $H2'_{m7G}$, $H4'_{m7G}$, $H4'_G$, $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.11 (3H, s, CH_3), 3.61 (3H, s OCH_3), 2.50 (2H, t, J=20.7 Hz, P-CH_2-P); ^{31}P NMR (162 MHz, D_2O) δ 18.41 (1P, α), 8.34 (1P, β), –22.12 (1P, γ), –10.67 (1P, δ). ESI MS: calcd for $C_{23}H_{33}N_{10}O_{20}P_4$ 893.0823. Found 893.0775 $[M - H]^-$.

Method 2. $m_2^{7,2'\text{-}O}$Gp-Im (**15**) (40 mg, 0.086 mmol) and GpCH$_2$pp (**13**) (760 opt. u., 52 mg, 0.063 mmol) were mixed in anhydrous DMF (2 ml). $ZnCl_2$ (61 mg, 0.45 mmol) was added. Reaction was stirred at room temperature overnight and progress was monitored by HPLC. To stop the reaction, solution of EDTA (167 mg, 0.45 mmol) in water was added and pH was brought to 7 by addition of solid $NaHCO_3$. The product was purified employing DEAE–Sephadex. **6** was obtained as triethylammonium salt (560 opt. u., 25.4 mg, 0.027 mmol). Then the compound was changed into sodium salt on Dowex resin. The product was collected and lyophilized to give **6** as white solid.

Percentage HPLC conversion of m^7GpCH$_2$p into **6**—80%, yield of purification by ion exchange chromatography—54%, yield of exchange into sodium salt—95%, final yield—41%.

Biological assays

eIF4E binding. Fluorescence titration measurements were carried out on LS-55 spectrofluorometer (Perkin Elmer Co.), in 50 mM HEPES/KOH (pH 7.2), 100 mM KCl, 0.5 mM EDTA, 1 mM DTT at 20.0 ± 0.3 °C. Aliquots of 1 μl of solutions with increasing concentration of cap analog were added to 1.4 ml of 0.1 μM protein solutions. Fluorescence intensities (excitation at 280 nm with 2.5 nm bandwidth and detection at 337 nm with 4 nm bandwidth and 290 nm cut-off filter) were corrected for sample dilution and the *inner filter* effect. Equilibrium association constants (K_{AS}) were determined by fitting the theoretical dependence of the fluorescence intensity on the total concentration of cap analog to the experimental data points according to equation described previously.[28] The concentration of protein was fitted as a free parameter of equilibrium equation showing amount of "active" protein. The final K_{AS} was calculated as a weighted average of three to five independent titrations, with the weights taken as the reciprocals of the numerical standard deviations squared. Numerical nonlinear least-squares regression analysis was performed using ORIGIN 6.0 (Microcal Software Inc., USA).

Hydrolysis by hDcpS. Human DcpS was expressed in *E. coli* as described previously,[33] and stored at ~15 μM concentration in 20 mM Tris buffer, pH 7.5, containing 50 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 20% glycerol at –80 °C. Enzymatic reactions were carried out similarly as described earlier.[34] An appropriate cap analog at 40 μM concentration was treated with 4.0 μl of hDcpS in 500 μl of 50 mM TRIS buffer, pH = 7.9, containing 20 mM of $MgCl_2$ and 60 mM of $(NH_4)_2SO_4$ at 30 °C. After 15, 30, 60 and 90 min a 100 μl aliquot was collected from each reaction mixture and deactivated by incubation in 90 °C for 3 min. Collected samples were analyzed without further treatment by analytical HPLC using a linear gradient from 0–50% of methanol in 0.1 M KH_2PO_4 pH = 6.0 within 30 min and UV-detection at 260 nm.

hDcpS binding. Association constants were calculated from the fluorescence titration curves (time-synchronized titration method) as described in the literature.[28]

Fluorescence experiments were performed on LS-55 spectrofluorometer (Perkin Elmer Co.) in a quartz cuvette (Hellma) with

optical path length of 4 mm for absorption and 10 mm for emission. All measurements were performed at 20 °C, in 50 mM Tris buffer, pH 7.6, containing 200 mM KCl, 1 mM DTT and 0.5 mM EDTA. Sample excitation wavelength was 280 nm and emission was observed at 340 nm. Obtained data points were corrected for sample dilution and for the *inner filter* effect.

Inhibition of translation in RRL system. Inhibition of cap-dependent translation in RRL (Flexi Rabbit Reticulocyte Lysate, Promega) by cap analogs, analysis of their stability in RRL and calculation of IC_{50} values were performed as described previously.[11]

Briefly, the *in vitro* translation reaction was performed in 12.5 µl volume for 60 minutes at 30 °C, in conditions determined for cap-dependent translation. The reaction mixture was pre-incubated for 60 minutes at 30 °C prior to addition of dinucleotide cap analog (inhibitor) and $m_2^{7,3'\text{-O}}$GpppG-capped luciferase mRNA to start the translation reaction.

To analyse stability of studied cap analogs in rabbit reticulocyte lysate translation system, each cap analog was incubated in a translation mixture for 60 minutes at 30 °C and then luciferase mRNA was added to start translation. Reactions were stopped by chilling on ice and the luciferase activity was measured in a luminometer (Glomax, Promega).

Translation efficiency in RRL of luciferase mRNA capped *in vitro*. Capped, polyadenylated luciferase mRNAs were synthesized *in vitro* on a dsDNA template (amplified by PCR reaction) that contained: SP6 promoter sequence of DNA-dependent RNA polymerase, 5′UTR sequence of rabbit β-globin, the entire firefly luciferase ORF and a string of 31 adenosines. A typical *in vitro* transcription reaction mixture (40 µl) contained: SP6 transcription buffer (Fermentas), 0.7 µg of DNA template, 1 U/µl RiboLock Ribonuclease Inhibitor (Fermentas), 0.5 mM ATP/CTP/UTP and 0.1 mM GTP and 0.5 mM dinucleotide cap analog (molar ratio cap analog:GTP 5:1). The reaction mixture was preincubated at 37 °C for 5 minutes before addition of SP6 RNA polymerase (Fermentas) to final concentration 1 U/µl and reaction was continued for 45 minutes at 37 °C. After incubation, reaction mixtures were treated with DNase RQ1 (Promega), in transcription buffer, for 20 min at 37 °C at concentration 1U per 1 µg of template DNA.

RNA transcripts were purified using NucAway Spin Columns (Ambion), integrity of transcripts was checked on a non-denaturating 1% agarose gel and concentrations were determined spectrophotometrically.

A translation reaction in RRL was performed in 10 µl volume for 60 minutes at 30 °C, in conditions determined for cap-dependent translation. A typical reaction mixture contained: 40% RRL lysate, mixture of amino acids (0.01 mM), $MgCl_2$ (1.2 mM), potassium acetate (170 mM) and 5′-capped mRNA. Four different concentrations of each analysed transcript were tested in *in vitro* translation reaction.

Activity of synthesized luciferase was measured in a luminometer.

Synthesis of short capped RNA transcripts. Short RNA transcripts were synthesized on a synthetic dsDNA template (28 bp) where SP6 promoter sequence is directly followed by GCCCC sequence, which undergoes transcription to RNA.

An *in vitro* transcription reaction mixture (25 µl) contained: SP6 transcription buffer (Fermentas), 3.4 µM DNA template, 1.2 U/µl of SP6 RNA polymerase, 2 U/µl RiboLock Ribonuclease Inhibitor (Fermentas), 2 mM CTP and either 1 mM GTP or 1 mM dinucleotide cap analog. The reaction was incubated at 37 °C for 60 minutes.

After incubation, short RNA transcripts (5 and 6 nucleotides in length) were separated on 20% polyacrylamide gel (19:1) with 7M urea in 1X TBE buffer (30 minutes at 15 mA). Directly after electrophoresis RNA transcripts were visualised by UV shadowing.

Acknowledgements

We are indebted to Mike Kiledjian (Rutgers University) for providing the hDcpS encoding plasmid, to the Laboratory of Biological NMR (Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, IBB PAS) for access to the NMR apparatus and to the Laboratory of Mass Spectrometry (IBB PAS) for recording MS spectra. Financial support from the Howard Hughes Medical Institute to ED (No. 55005604) and National Science Support Project 2008-2010 No. PBZ-MNiSW-07/I/2007 is gratefully acknowledged.

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