Bioorganic & Medicinal Chemistry 20 (2012) 1699–1710

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and properties of mRNA cap analogs containing imidodiphosphate moiety—fairly mimicking natural cap structure, yet resistant to enzymatic hydrolysis

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ARTICLE INFO

Article history:
Received 7 December 2011
Revised 4 January 2012
Accepted 9 January 2012
Available online 20 January 2012

Keywords:
Bioorganic chemistry
Nucleotides
mRNA cap
Enzymatic resistance
Translation inhibition
Imidodiphosphate

ABSTRACT

We describe synthesis and properties of eight dinucleotide mRNA 5′ cap analogs containing imidodiphosphate moiety within 5′,5′-tri- or tetraphosphate bridge (NH-analogs). The compounds were obtained by coupling an appropriate nucleoside 5′-imidodiphosphate with nucleotide *P*-imidazolide mediated by divalent metal chloride in anhydrous DMF. To evaluate the novel compounds as tools for studying cap-dependent processes, we determined their binding affinities for eukaryotic translation initiation factor 4E, susceptibilities to decapping pyrophosphatase DcpS and, for non-hydrolysable analogs, binding affinities to this enzyme. The results indicate that the O to NH substitution in selected positions of oligophosphate bridge ensures resistance to enzymatic decapping and suggest that interactions of NH-analogs with cap binding proteins fairly mimic interactions of unmodified parent compounds. Finally, we identified NH-analogs as potent inhibitors of cap-dependent translation in cell free system, and evaluated their utility as reagents for obtaining 5′ capped mRNAs in vitro to be rather moderate.

1. Introduction

Nucleotide analogs modified within oligophosphate bridge are important tools for structural, biochemical and physiological studies. They serve as building blocks for enzymatic syntheses of modified nucleic acids, reagents for molecular biology, have found several biotechnological applications and may serve as enzymatically resistant counterparts of natural compounds with potential medicinal applications. For the purpose of conferring enzymatic resistance to nucleotides, two main types of phosphate modifications have been exploited: the bridging (e.g. (methylenebis)phosphonate and imidodiphosphate) as well as the non-bridging modifications (e.g., phosphorothioate, phosphoroselenoate and boranophosphate).[1,2] Generally, mononucleoside triphosphates have been most intensively studied and explored for biotechnology, and their synthesis can be achieved through several well-established chemistries. Nonetheless, dinucleoside 5′,5′-oligophosphates are being studied more intensively due to their involvement in regulatory processes and, hence, methods for their synthesis are being constantly developed.[3–6] Among dinucleoside 5′,5′-oligophosphates, of our particular interest is the cap structure present on the 5′ end of eukaryotic mRNAs, which consists of N^7-methylguanosine connected by 5′,5′-triphosphate bridge to the first transcribed nucleotide. This fragment of mRNA molecule plays important roles during several steps of mRNA synthesis, expression and turnover, and suitable chemical modifications of the cap offer a facile way for interfering with this processes to achieve desired biological effects.[7]

The cap, being a nucleotide, consists of three types of chemically interesting parts—nucleobase, sugar moiety and oligophosphate chain. Each of these parts can be considered as a site for modification in order to obtain analogs with interesting properties and hence, a large number of cap structures differently modified at these sites have been synthesized.[7] In this study, we analyzed for the first time the effects of modifying the cap's triphosphate bridge with imidodiphosphate moiety, that is, substituting one of the bridging oxygens with NH group.

Several synthetic methods have been developed for imidodiphosphate-modified nucleoside 5′-di- and triphosphates.[8–15] However, the area of NH-substituted dinucleoside 5′,5′-polyphosphates appears to be far less explored. In the Miller's lab synthesis of AppNHppA, AppNHppG and AppNHppU by a combination of chemical and enzymatic methods[16] and synthesis of AppNHpppU via EDC-mediated coupling of AppNHp with UDP have been carried

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doi:10.1016/j.bmc.2012.01.013

out,[17] but to our knowledge a more general synthetic approach for such compounds has not yet been proposed.

Here, we report syntheses of eight imidodiphosphate-modified, dinucleotide analogs of mRNA cap bearing the NH for O substitution at different positions of the tri-or tetraphosphate bridge via metal(II) chloride-mediated coupling of two nucleotide subunits. The new cap analogs not only contribute to the development of new synthetic methods for modified dinucleoside 5′,5′-oligophosphates, but may also be useful for biophysical and biochemical studies on proteins and enzymes involved in mRNA metabolism by a specific recognition of cap structure. These proteins include eukaryotic translation initiation factor 4E (eIF4E)[18,19] which binds mRNA cap during translation initiation, or mRNA decapping enzymes (DcpS, Dcp2, NUDT16) which are involved in the regulation of mRNA stability.[20–24] In our opinion, the major advantage of replacing certain oxygen atom(s) in polyphosphate chain by another atom or moiety is the possibility of obtaining caps or capped mRNAs resistant towards enzymatic hydrolysis and hence more stable in vivo. Such compounds would benefit all potential medicinal applications, which include the use of small-molecular cap analogs as inhibitors of excessive cap-dependent translation in cancer cells,[25] or in vitro synthesis of capped mRNAs that have high stability and translation efficiency when introduced into cells or organisms.[7,26,27]

In our previous works, we have developed dinucleotide cap analogs modified at various positions of the 5′,5′-triphosphate bridge with either (methylenebis)phosphonate[28–30] or phosphorothioate groups[31–33] (i.e., bridging O to CH_2 and non-bridging O to S substitutions, respectively). The analogs bearing CH_2 group, depending on the position of modification, were resistant to different decapping enzymes (DcpS or Dcp2), and stabilized either caps or capped mRNAs in cell lysates or cultured cells. However, the O to CH_2 substitution caused decrease in affinity to eIF4E by ~40%, which consequently decreased potency of CH_2-analogs as translation inhibitors and also notably diminished the translation efficiency of mRNAs capped with them.[29,30,34,35] On the other hand, some of the phosphorothioate analogs were both resistant to decapping and had higher affinity for eIF4E than for unmodified compounds. Consequently, these S-analogs were stable and efficient inhibitors of cap-dependent translation, and when introduced into mRNA produced transcripts with high translation efficiency and elongated half-lives in cells,[7,32] which had already been shown to be beneficial for mRNA-based immunotherapy.[27] Unfortunately, such non-bridging position modified analogs exist as a pair of two P-diastereomers, which usually differ in their biological activity and thus require tedious HPLC separations. This may be considered as a drawback of these compounds for medicinal applications.[26,27,30] We hoped that replacing a bridging oxygen by NH instead of CH_2 group, would also produce cap analogs that are resistant to enzymatic degradation, but since nitrogen is more electronegative than carbon, such substitution might not disturb the interaction of cap with eIF4E. In contrast to S-analogs, however, such substitution would not produce P-diastereomers. Hence, exploiting the imidodiphosphate modification could provide cap analogs sharing advantages, but free from drawbacks of phosphorothioate and (methylenebis)phosphonate ones.

2. Results

2.1. Chemical syntheses

The structures of synthesized cap analogs containing imidodiphosphate moiety (**1**–**8**) are depicted in Figure 1. Some of these analogs (**5**–**8**) were additionally modified by extending the 5′,5′-bridge from tri- to tetraphosphate to increase their affinity to eIF4E,[36] and some (**2**, **4** and **6**, **8**) by the presence of 2′-*O*-methyl group in the 7-methylguanosine moiety to ensure exclusively correct incorporation into mRNA during in vitro transcription (these are referred to as anti-reverse cap analogs, ARCA).[37]

The syntheses of analogs **1**–**8** are summarized in Schemes 1–3. The triphosphate series consists of four analogs (**1**–**4**) bearing NH substitutions at the α/β (**1**, **2**) or β/γ (**3**, **4**) positions, among which two (**2**, **4**) are ARCA-type (posses 2′-*O*-methyl group in the 7-methylguanosine moiety). The tetraphosphate series includes four compounds (**5**–**8**) bearing NH substitutions at either α/β (**5**, **6**) or γ/δ (**7**, **8**) positions, among which analogs **6** and **8** bear the ARCA modification as well.

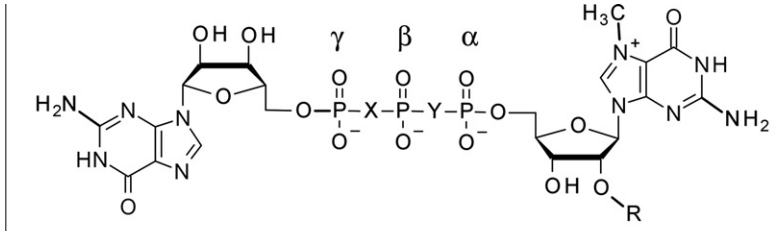
Synthesis of all cap analogs were based on the coupling of two mononucleotide subunits, one of which contained the imidodiphosphate modification and the other was activated as a *P*-imidazolide. A new pyrophosphate bond was formed as a result of nucleophilic attack of imidodiphosphate containing subunit on the terminal phosphorus of the second nucleotide. The reaction was mediated by divalent metal (Zn or Mg) chloride excess in either water or DMF. Generally, couplings in aqueous *N*-ethylmorpholine buffer in the presence of $MnCl_2$ or $MgCl_2$ led to the desired final products with very modest yields. Moreover, the reaction were slow (24–48 h) and degradation of *P*-imidazolides in aqueous conditions decreased their efficiencies (resulting in conversions into the desired products <40%). Hence, to avoid hydrolysis of *P*-imidazolides and possible subsequent side-reactions, the couplings were performed in DMF in the presence of anhydrous $ZnCl_2$ or $MgCl_2$. The presence of divalent metal ions in polar aprotic solvent has previously been found advantageous over aqueous conditions for efficient pyrophosphate bonds formation.[38,39]

Analogs bearing NH substitution at the α/β position (**1**, **2** and **5**, **6**) were obtained by coupling guanosine 5′-imidodiphosphate triethylammonium salt (**9**) with *P*-imidazolide derivative of an appropriate nucleoside 5′-mono- or diphosphate (**10**–**13**) in DMF; (Scheme 1). Excess $ZnCl_2$ was used as a coupling mediator except from analogs **1** and **2**, for which reactions proved to be more rapid in the presence of $MgCl_2$ (full completion within 1–2 h).

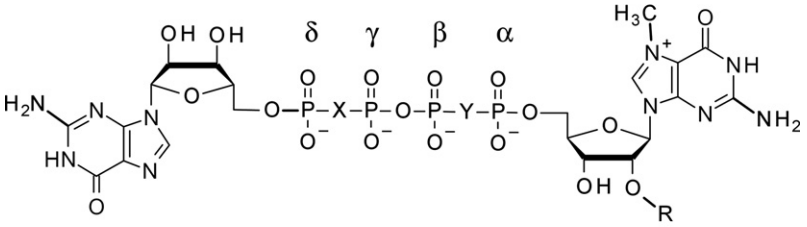
All reactions were monitored by reversed phase HPLC. The final conversions of guanosine 5′-imidodiphosphate into desired products exceeded usually 80% within several hours (Fig. 2a) and isolated yields after DEAE Sephadex ion exchange-purification ranged from 38% to 48%. The lower isolated yields compared to HPLC conversions were probably due to small scales at which these reactions were performed (~50 mg of each nucleotide) and consequently relatively large losses of material during ion-exchange chromatographies and salt conversions. Drawing from our experience, these yields can be improved by ~20% when performing an optimized large-scale synthesis (i.e., from 500 to 1000 mg of each nucleotide).

To obtain analogs having NH substitution at the β/γ (**3**, **4**) or γ/δ (**7**, **8**) position, couplings of 7-methylguanosine 5′-imidodiphosphate (**17**) or 7,2′-*O*-dimethylguanosine 5′-imidodiphosphate (**18**) with an appropriate *P*-imidazolide (**14** or **15**) were performed as depicted in Scheme 2. In most cases, $ZnCl_2$ served as a catalyst except for the synthesis of analog **3** where $MgCl_2$ was applied. These reactions were similarly efficient and fast as those for compounds modified at the α/β position (Fig. 2b) and were isolated with yields ranging from 24% to 44%.

The nucleoside 5′-imidodiphosphates 9 and **16** were obtained from appropriate nucleosides by reaction with dichlorophosphorylphosphorimidoyl trichloride in triethylphosphate at −8 °C followed by hydrolysis, that is, by adapting procedure for adenosine 5′-imidodiphosphate published by Tomasz et al. (Scheme 3).[40] This procedure proved to be regioselective to 5′-hydroxyls of both guanosine and 2′-*O*-methylguanosine and resulted in the desired compounds with satisfying yields (28% and 57%, respectively).

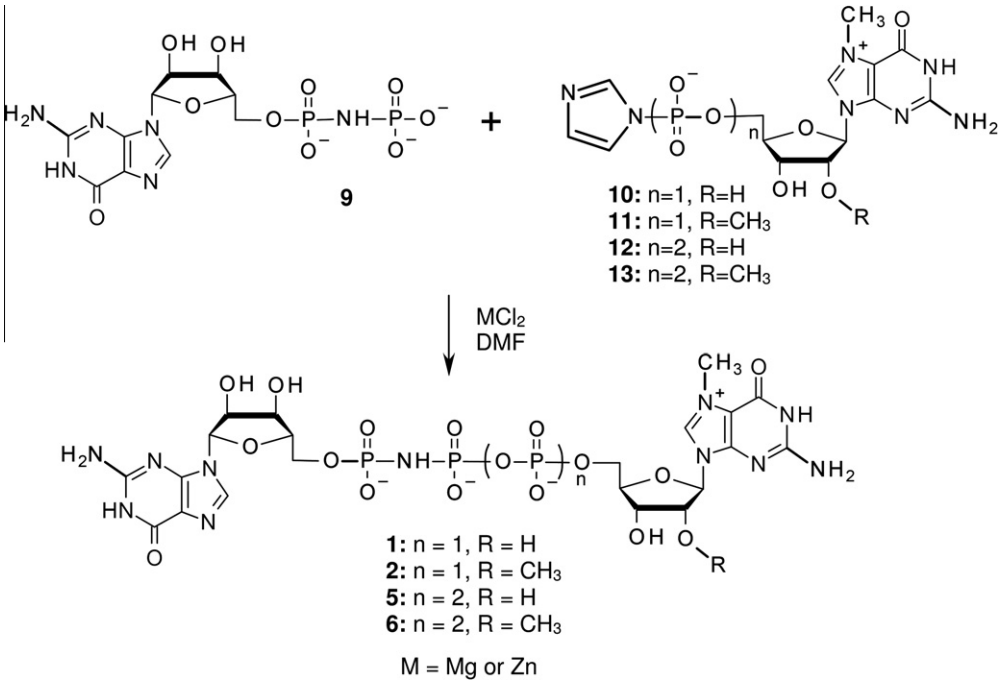


X = Y = O, R = H, m^7GpppG
X = Y = O, R= CH_3, $m_2^{7,2'\text{-}O}GpppG$
X = NH, Y = O, R = H, $m^7GppNHpG$ **(1)**
X = NH, Y = O, R= CH_3, $m_2^{7,2'\text{-}O}GppNHpG$ **(2)**
X = O, Y = NH, R = H, $m^7GpNHppG$ **(3)**
X = O, Y = NH, R= CH_3, $m_2^{7,2'\text{-}O}GpNHppG$ **(4)**



X = Y = O, R = H, $m^7GppppG$
X = Y = O, R= CH_3, $m_2^{7,2'\text{-}O}GppppG$
X = NH, Y = O, R = H, $m^7GpppNHpG$ **(5)**
X = NH, Y = O, R= CH_3, $m_2^{7,2'\text{-}O}GpppNHpG$ **(6)**
X = O, Y = NH, R = H, $m^7GpNHpppG$ **(7)**
X = O, Y = NH, R= CH_3, $m_2^{7,2'\text{-}O}GpNHpppG$ **(8)**

Figure 1. Structures of mRNA cap analogs **1**–**8** and their unmodified parent compounds used in this study.



9

10: n=1, R=H
11: n=1, R=CH_3
12: n=2, R=H
13: n=2, R=CH_3

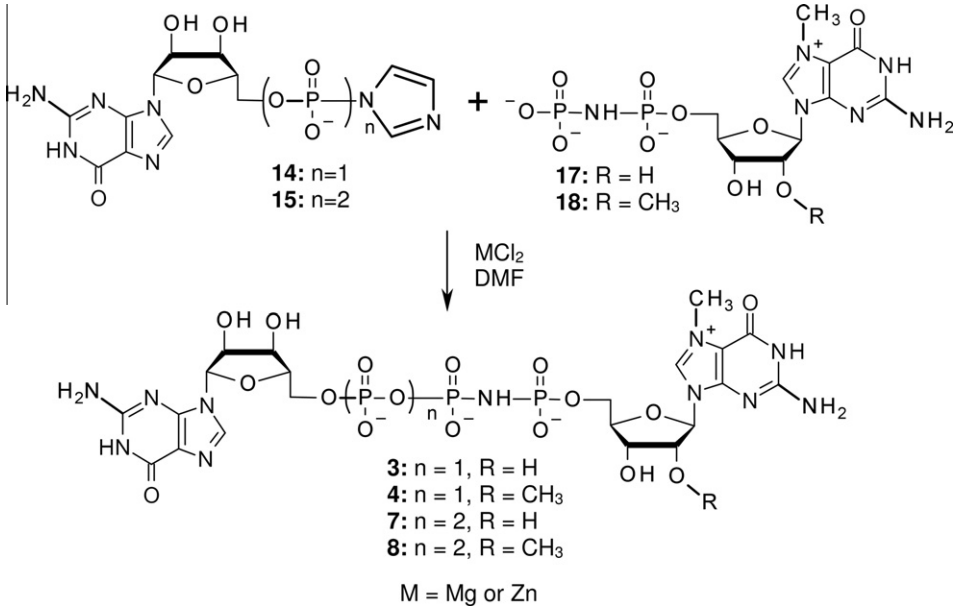
MCl_2
DMF

1: n = 1, R = H
2: n = 1, R = CH_3
5: n = 2, R = H
6: n = 2, R = CH_3

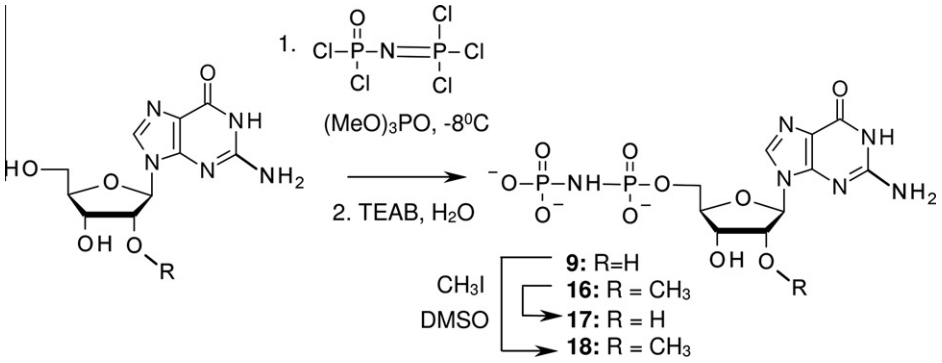
M = Mg or Zn

Scheme 1. Synthesis of NH-analogs modified at the α/β-position (**1**, **2**, **5**, **6**).

Therefore, we recognized it as more straightforward than the alternative method that employed coupling of 5′-*O*-tosylated nucleosides with tris(tetrabutylammonium) imidodiphosphate.[13] 7-Methylguanosine 5′-imidodiphosphate (**17**) and 7,2′-*O*-dimethylguanosine 5′-imidodiphosphate (**18**), were obtained from 9 and 16, respectively, by methylation with iodomethane in DMSO. Any decomposition or N-methylation of the imidodiphosphate group was not observed.



Scheme 2. Synthesis of NH-analogs modified at the β/γ and γ/δ-positions (**3**, **4**, **7**, **8**).



Scheme 3. Synthesis of nucleoside 5′-imidodiphosphates.

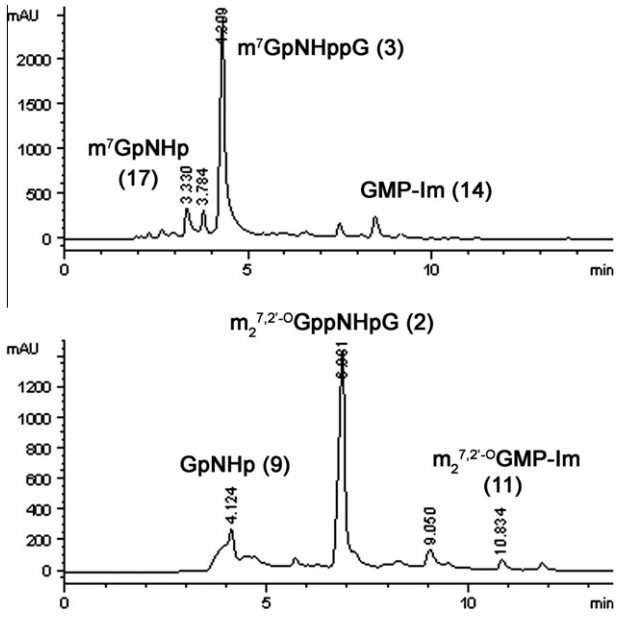


Figure 2. RP HPLC profiles from the reaction mixtures leading to cap analogs **3** and **2**.

Nucleoside mono- and diphosphates and their imidazolide derivates were prepared according to the well-established procedures described previously.[37] Yoshikawa's phosphorylation[41] was applied to obtain nucleoside 5′-monophosphates. *P*-imidazolides were obtained by employing imidazole, 2,2′-dithiodipyridine and triphenylphosphine in DMF and subsequent precipitation by $NaClO_4$ solution in acetone. Nucleoside 5′-diphosphates were obtained by coupling corresponding nucleoside 5′-monophosphate *P*-imidazolides with triethylammonium ortophosphate in DMF in the presence of $ZnCl_2$.

To complete the tetraphosphate series, we also attempted to obtain analogs modified at the β/γ position (e.g., m^7GppNHppG). This required preparation of appropriate nucleoside 5′-(β/γ-imido)triphosphates, which we tried to achieve by coupling nucleoside 5′-monophosphate *P*-imidazolides with triethylammonium salt of inorganic imidodiphosphate in DMF in the presence of either $MgCl_2$ or $ZnCl_2$. The reaction of GMP-Im with pNHp, analyzed by HPLC/MS(−)ES, proceeded quite smoothly to the desired guanosine β,γ-imidotriphosphate, in the presence of either metal chloride. However, the isolated yield was much lower than expected, even if the reaction work up was done maintaining pH not lower than 6 (NppNHp have been reported unstable in acidic pH[15]) and several products were eluted from ion-exchange DEAE Sephadex. MS(−)ES analysis revealed that the fraction containing

nucleoside 5′-imidotriphosphate was contaminated with nucleoside 5′-(β-amidodiphosphate) and 5′-imidotetraphosphate. When the guanosine 5′-imidotriphosphate obtained in this manner was reacted with **10** in DMF in the presence of $MgCl_2$ or $ZnCl_2$, the reaction did not proceed in the expected direction. Instead, a complex mixture of products was observed in the HPLC. MS analysis revealed the occurrence of further decomposition of reactant. Similar result was obtained when 7-methylguanosine 5′-imidiotriphosphate was synthesized in an analogous manner and subjected to coupling with **14**. Yount et al. 1971 have previously reported the decomposition of AppNHp, which they believed occurred during the work-up of fractions after ion-exchange chromatography, possibly due to triethylamine-catalyzed hydrolysis.[15] Therefore, we assume that the encountered problems were due to the instability of nucleoside 5′-imidotriphosphates in the reaction and purification conditions and obtaining cap analogs bearing NH substitution at the β/γ position require different synthetic approach.

The presence of imidodiphosphate moiety in all synthesized mono- and dinucleotides was confirmed by ^{31}P NMR (see Supplementary information). The ^{31}P signals of phosphorus nuclei bonded to nitrogen are shifted ~10 ppm upfield compared to the corresponding signals in unmodified cap analogs (Fig. 3). Moreover, the $^{2}J_{P-NH-P}$ coupling constants are in the range of 5–7 Hz compared to ~20 Hz for $^{2}J_{P-O-P}$.

2.2. Chemical stability of NH-analogs

As already mentioned, only few reports regarding the synthesis and properties of imidodiphosphate containing dinucleoside 5′,5′-oligophosphates have been published thus far. Therefore, before we performed biological evaluation of compounds **1**–**8**, we tested their chemical stability in aqueous solutions by means of HPLC. The analogs at ~5 mM concentrations were incubated at 25 °C in aqueous buffers of different pH (2, 6 or 8) and aliquots obtained at different time intervals were analyzed by RP HPLC as described in Section 5. No decomposition was observed after up to 2 h of incubation in all of these buffers, and less than 10% decomposition after 24 h. In neutral and mildly basic pH, the only products of decomposition were derived from 7-methylguanosine ring opening, but no products indicating triphosphate bridge cleavage were detected. In pH 2, small amounts (~10%) of products were observed after 24 h indicating cleavage of P–NH–P bond. Moreover, analogs **1**–**8** were stable at −20 °C for over 12 months both as solids and in frozen aqueous solutions.

2.3. Binding affinity of new analogs for eIF4E

eIF4E is a part of the translation initiation complex and is responsible for mRNA 5′ end recognition through cap binding during translation initiation.[18] Previous studies have shown that sufficiently high binding affinity for eIF4E is crucial for all applications of cap analogs related to mRNA translation and translation inhibition.[34] Therefore, determination of association constants for cap analogs-eIF4E complexes is a relatively simple yet informative test for assessing utility of new cap analogs in these applications.

The association constants (K_{AS}) for complexes of cap analogs **1**–**8** with eIF4E were determined by fluorescence titration as described previously.[42] The determined values along with K_{AS} for unmodified parent compounds are collected in Table 2. In the triphosphate series (analogs **1**–**4**), K_{AS} values were comparable to those for unmodified parent compounds (m^7GpppG and $m_2^{7,2'-O}$GpppG), ranging from 0.9 to 1.7-fold of their K_{AS}. The corresponding ranges for phosphorothioate analogs were ranging from 1.0 to 4.5[32] and for (methylenebis)phosphonate analogs 0.4–0.6.[35] Analogs modified at the γ/β position have higher affinities to eIF4E than those modified at the α/β position, which is opposite to the effect observed for O to CH_2 substitutions.

In agreement with previously published observations,[43] the additional phosphate moiety generally increased the binding affinity of cap analogs to eIF4E. In the NH-tetraphosphate series (analogs **5**–**8**), the K_{AS} values were comparable to the parent tetraphosphates (m^7GppppG and $m_2^{7,2'-O}$GppppG), ranging from 1.0 to 1.3-fold of their K_{AS}. The corresponding ranges for tetraphosphate series of CH_2- and S-analogs were 0.4–0.9 and 1.1–2.8-fold, respectively.[30,33]

2.4. Susceptibility to degradation by human Decapping Scavenger (hDcpS)

Cap structure is degraded in cells by at least three specific pyrophosphatases with different regioselectivity and substrate

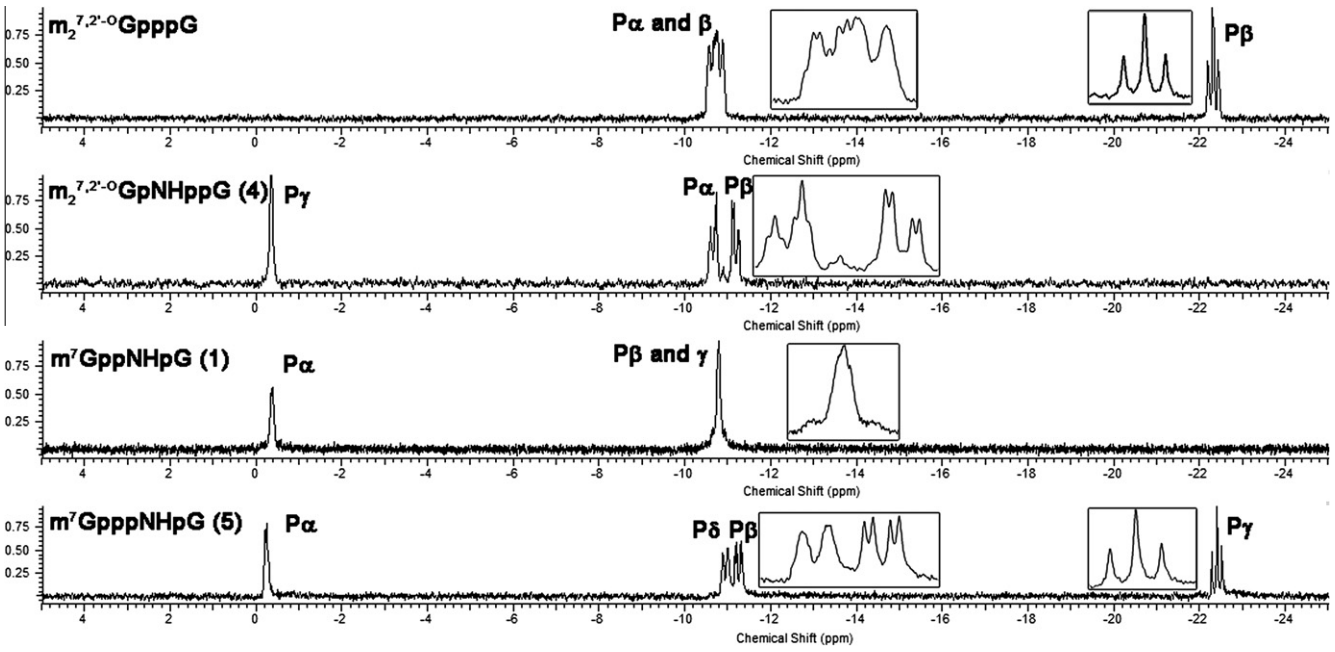


Figure 3. ^{31}P NMR spectra of selected cap NH-analogs compared to ^{31}P NMR spectrum of a representative phosphate-unmodified cap analog (D_2O, 25 °C, 162 MHz).

specificity.[20,24] The Decapping Scavenger (DcpS) is, at least in higher eukaryotes, the only cap-specific enzyme identified to date, able to cleave free dinucleotide caps and those placed on very short transcripts released after mRNA 3′→5′ degradation by exosome.[22] DcpS cleaves between β- and γ-phosphates of cap's triphosphate bridge, and reaction proceeds through attack of His277 on cap's γ phosphate.[23] Thus, the hydrolysis of m^7GpppG dinucleotide by DcpS leads to 7-methylguanosine 5′-monophosphate and GDP. We have previously found that tetraphosphate cap analogs such as m^7GppppG, which are not natural substrates for DcpS, are cleaved exclusively to 7-methylguanosine 5′-monophosphate and nucleoside 5′-triphosphate.[30]

The analogs **1**–**8** were incubated with human recombinant DcpS under the conditions described in Section 5 to qualitatively assess whether the O by NH substitution produces cap analogs resistant to decapping. The enzyme concentration was adjusted to provide complete degradation of m^7GpppG, used as a positive control, within ~15 min. The reaction progress was monitored by HPLC and an analog was considered as resistant to DcpS if it remained unhydrolyzed after two hours of incubation with the enzyme.

All analogs bearing NH group at the site of cleavage i.e. triphosphate analogs modified at the β/γ position (**3**, **4**) and tetraphosphates modified at the γ/δ (**7**, **8**) turned out to be resistant to DcpS (Fig. 4) Interestingly, analogs **1** and **2**, which do not contain modification directly at the cleavage site, but one phosphate next to it, were also less susceptible to DcpS. Analog **1** was hydrolyzed in only 20% after 90 min of incubation, whereas analog **2** was practically resistant to DcpS under these conditions. The tetraphosphates bearing NH group at the α/β position (**5**, **6**), that is, two phosphates away from the cleavage site, were hydrolyzed with rates similar to the unmodified counterparts (m^7GppppG and $m_2^{7,2'-O}$GppppG). Representative HPLC profiles from this assay are shown in Figure 4.

2.5. Binding affinities for hDcpS

For non-hydrolysable cap analogs (**2**–**4** and **7**–**8**), binding affinity constants (K_{AS}) to DcpS were also determined using fluorescence quenching titration, similar as in the case of eIF4E.[42,44] K_{AS} values, along with selected data previously reported for phosphorothioate and (methylenebis)phosphonate cap analogs, are summarized in Table 1. K_{AS} values for analogs modified at the β/γ position of triphosphate bridge with both imido (**3**) and methylene group[29] (m^7GpCH_2ppG) are comparable ($218 \pm 24\ \mu M^{-1}$ vs $234 \pm 14\ \mu M^{-1}$). The same pattern can be observed in the tetraphosphate series for analog modified at the γ/δ position (**7**) and corresponding methylene analog (m^7GpCH_2pppG),[30] however, the values are about 1.5-fold lower than that for the triphosphates due to the presence of additional phosphate group. As opposed to the NH-analogs and CH_2-analogs, dinucleotides containing non-bridging substitutions modification at the γ phosphate within triphosphate bridge, bind to DcpS with about 1.6-fold lower affinity (K_{AS}).

2.6. Inhibition of cap-dependent translation

One way of examining utility of a cap analog in mRNA translation-related studies and applications is performing an assay, in which dinucleotide cap analog competes with capped mRNAs for eIF4E binding thus, inhibiting their translation. Here, we determined the ability of non-ARCA compounds (**1**, **3**, **5** and **7**) to inhibit translation in rabbit reticulocyte lysate (RRL) programed with in vitro transcribed, ARCA-capped mRNA encoding firefly luciferase.

Two sets of experiments were performed, differing in that the analogs were either added to RRL together with reporter mRNA (experiment A) or incubated with lysate for 60 min before the

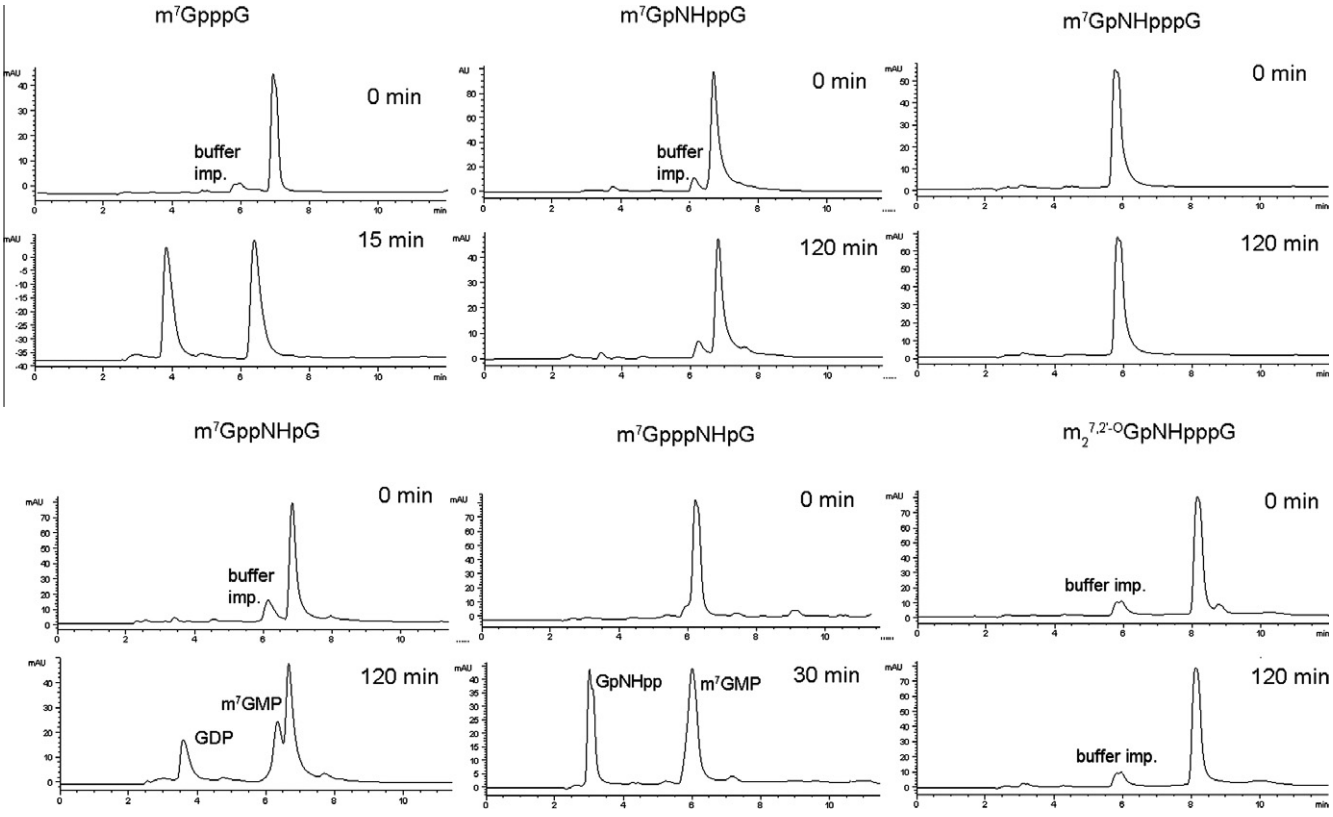


Figure 4. Representative RP HPLC profiles from DcpS susceptibility assay.

Table 1
Substrate properties for DcpS and determined binding affinities for DcpS and eIF4E for analogs **1**–**8**

No	Cap analog	K_{AS} eIF4E (μM^{-1})[a]	ΔG (kcal/mol)	Hydrolysis by hDcpS	K_{AS} hDcpS (μM^{-1})[b]	ΔG kcal/mol)[c]	R[d]
	m^7GpppG	12.5 ± 0.3	−9.51 ± 0.01	Yes	n.d.	—	>>1
	$m_2^{7,2'-O}$GpppG	10.8 ± 0.3	−9.42 ± 0.02	Yes	n.d.	—	—
	m^7GppppG	110.9 ± 6.0	−10.78 ± 0.03	Yes	n.d.	—	—
	$m_2^{7,2'-O}$GppppG	99.8 ± 6.0	−10.72 ± 0.04	Yes	n.d.	—	—
1	m^7GppNHpG	10.1 ± 0.2	−9.38 ± 0.01	Yes[e]	n.d.	—	—
2	$m_2^{7,2'-O}$GppNHpG	10.4 ± 0.2	−9.40 ± 0.01	No	12.8 ± 1.0	−9.53 ± 0.05	1.2
3	m^7GpNHppG	14.6 ± 0.7	−9.60 ± 0.03	No	218 ± 24	−11.07 ± 0.07	15
4	$m_2^{7,2'-O}$GpNHppG	18.5 ± 0.5	−9.74 ± 0.02	No	33.6 ± 1.7	−10.09 ± 0.03	1.8
5	m^7GpppNHpG	141.2 ± 12.2	−10.92 ± 0.05	Yes	n.d.	—	—
6	$m_2^{7,2'-O}$GpppNHpG	132.5 ± 1.9	−10.89 ± 0.01	Yes	n.d.	—	—
7	m^7GpNHpppG	112.3 ± 1.8	−10.79 ± 0.01	No	155 ± 12	−10.97 ± 0.05	1.4
8	$m_2^{7,2'-O}$GpNHpppG	125.0 ± 2.1	−10.85 ± 0.01	No	47.4 ± 2.6	−10.29 ± 0.03	0.38
	m^7GpCH_2ppG	6.3 ± 0.3[f]	−9.11 ± 0.03	No[g]	234 ± 14[g]	−11.21 ± 0.03	37.1
	m^7GpCH_2pppG	51.8 ± 0.8[h]	−10.34 ± 0.01	No[h]	160 ± 17[g,h]	−10.99 ± 0.06	3.1
	m^7Gp_SpppG (D1)	30.8 ± 0.5[i]	−10.04 ± 0.01	No[i]	146 ± 6[j]	−10.94 ± 0.02	4.7

[a] Determined in 50 mM Hepes/KOH (pH 7.2), 100 mM KCl, 0.5 mM EDTA, 2 mM DTT in 20 ± 0.2 °C.
[b] Determined in50 mM Tris/HCl (pH 7.6), 200 mM KCl, 1 mM DTT in 20 ± 0.2 °C.
[c] Calculated from the K_{AS} value according to the standard equation $\Delta G = -RT\ln K_{AS}$.
[d] $K_{AS(DcpS)}/K_{AS(eIF4E)}$ ratio.
[e] Very slow hydrolysis under experiment conditions.
[f] Data from Ref. 35.
[g] Data from Ref. 29.
[h] Data from Ref. 30.
[i] Data from Ref. 32.
[j] Data from Ref. 44.

reporter mRNA addition (experiment B) (Fig. S1). The mean IC_{50} values for NH-analogs and control cap analogs from multiple A and B experiments are summarized in Table 2.

Both analogs **1** and **3** were potent inhibitors of cap-dependent translation in experiment A. Analog **1** inhibited translation at a level similar to m^7GpppG, whereas analog **3**, (which has a slightly higher K_{AS} for eIF4E), was more potent, with about two-fold lower IC_{50} value. In the NH-tetraphosphate series (**5** and **7**) IC_{50} values were slightly lower than the triphosphate series and m^7GppppG. Analogs **1**, **3** and **7**, maintained their inhibitory properties after 60 min of pre-incubation in RRL system (exp. B). In contrast, analog **5**, m^7GpppG and m^7GppppG, become significantly less potent after pre-incubation. Notably, these results correlate well with the results form DcpS stability assay.

2.7. Incorporation into mRNA and mRNA translation efficiency

For the preparation of mRNAs by in vitro transcription only ARCA type analogs (**2**, **4**, **6**, **8**) were used, since they can be incorporated into 5′ end of transcripts exclusively in the correct orientation.[37,39] First, we investigated whether the NH-ARCAs can be incorporated into RNA chain by SP6 RNA polymerase. Short transcripts (5nt) capped with analogs **2**, **4**, **6** or **8** were transcribed from appropriate dsDNA template and analyzed by gel electrophoresis (Fig. S2a). We found that NH-ARCAs can efficiently compete with GTP to initiate the transcription and are incorporated into RNA 5′ end with efficiency comparable to ARCAs with unmodified polyphosphate bridge. Also full-length mRNAs capped with NH-ARCAs were obtained with similar efficiency and were of comparable quality to those bearing unmodified parent caps (Fig. S2b).

Table 2
Inhibition of translation by cap analogs in RRL system

Cap analog	IC_{50} exp A[a] (μM)	N	IC_{50} exp B[b] (μM)	N
m^7GpppG	8.30 ± 0.32	5	>20	4
m^7GppppG	5.0 ± 0.5[c]	—	>20[c]	—
m^7GppNHpG (1)	8.49 ± 0.54	2	5.97 ± 0.45	3
m^7GpNHppG (3)	3.97 ± 0.32	3	3.74 ± 0.39	3
m^7GpppNHpG (5)	3.52 ± 0.27	3	16.0 ± 0.7	3
m^7GpNHpppG (7)	2.62 ± 0.0	3	3.58 ± 0.52	2

[a] Inhibitor added to RRL together with mRNA.
[b] Inhibitor preincubated in RRL for 60 min before the addition of mRNA.
[c] Data from Ref. 30.

Next, we investigated how transcripts possessing these modified cap structures at their 5′ end undergo translation. Therefore, we programed rabbit reticulocyte lysate optimized for cap-dependent translation with differently capped mRNAs encoding firefly luciferase. Luciferase activity was measured by luminometry as a function of mRNA concentration after 60 min from translation start. The determined translation efficiencies were referred to control mRNA capped with m^7GpppG, for which translation efficiency was normalized to 1. mRNA with non-functional cap analog (ApppG) was used to estimate level of cap-independent translation. Relative translation efficiencies are summarized in Table 3. Although all NH-analogs were characterized by binding affinities to eIF4E not lower than unmodified cap structure, unexpectedly, translation efficiencies of mRNAs capped with triphosphate NH-ARCAs were lower than those of mRNAs capped with $m_2^{7,2'-O}$GpppG, being comparable to those for CH_2-ARCAs. Even more unexpectedly, the translational efficiencies of mRNAs capped with tetraphosphate NH-ARCAs were even lower, despite the fact that mRNAs capped with $m_2^{7,2'-O}$GppppG, $m_2^{7,2'-O}GpppCH_2pG$ and $m_2^{7,2'-O}GpCH_2pppG$ were translated more efficiently than mRNAs capped with their triphosphate counterparts.[30] In case of analog **6**, relative translation efficiency was the lowest, being lower even than for m^7GpppG-capped mRNA.

3. Discussion

The synthesis and evaluation of the new NH-cap analogs is a part of our project aimed at determining structure-activity relationship of mRNA 5′ end analogs variously modified within the 5′,5′-triphosphate bridge. The chemical syntheses developed for

Table 3
Relative translation efficiencies in rabbit reticulocyte lysate of mRNAs capped with anti-reverse imidodiphosphate cap analogs compared to mRNAs capped with corresponding unmodified and (methylenebis)phosphonate cap analogs

Cap analog on mRNA 5′ end	Relative translation efficiency	Cap analog on mRNA 5′ end	Relative translation efficiency
m^7GpppG	1	$m_2^{7,2'-O}GppNHpG$ (2)	1.11 ± 0.22
$m_2^{7,3'-O}GpppG$	1.47 ± 0.12	$m_2^{7,2'-O}GpNHppG$ (4)	1.13 ± 0.10
$m_2^{7,2'-O}GppppG$	1.56 ± 0.10	$m_2^{7,2'-O}GpppNHpG$ (6)	1.01 ± 0.10
ApppG	0.18 ± 0.05	$m_2^{7,2'-O}GpNHpppG$ (8)	0.73 ± 0.20
$m_2^{7,2'-O}GppCH_2pG$	1.25 ± 0.32[a]	$m_2^{7,2'-O}GpppCH_2pG$[b]	1.56 ± 0.10
$m_2^{7,2'-O}GpCH_2ppG$	1.10 ± 0.33[a]	$m_2^{7,2'-O}GpCH_2pppG$[b]	1.50 ± 0.20

[a] Data from Ref. 34.
[b] Data from Ref. 30.

these compounds are, in our opinion, straightforward and universal, thus may be adapted for various other NH-analogs of dinucleoside polyphosphates. The bridging imidodiphosphate modification (P–NH–P) not only shares some similarities with the (methylenebis)phosphonate moiety (P–CH_2–P), but also has some unique properties. Both NH- and CH_2-modified nucleotides are resistant to enzymatic cleavage and in both cases the enzymatic resistance arises from increased chemical stability of P–X–P bonds compared to P–O–P bond. Yount et al. 1971 reported that $AppCH_2p$ and AppNHp both have higher affinity for Ca^{2+} and Mg^{2+} than ATP, however, the bond lengths and geometry of the imidodiphosphate group are more similar to pyrophosphate than those of (methylenebis)phosphonate.[15] Higher electronegativity of nitrogen compared to carbon allows for the expectation that the charge distribution may be more similar to pyrophosphate in case of imidodiphosphate group, which is, for instance, reflected in terminal phosphate pK_a values for ATP (7.1), AppNHp (7.7) and $AppCH_2p$ (8.4). In contrast to CH_2 group, NH moiety has the ability to form H-bonds. These differences between imidodiphosphate and (methylenebis)phosphonate groups may be the cause of differences in affinities of NH- and CH_2-analogs for cap binding proteins.

3.1. Comparison of binding affinities to eIF4E and susceptibilities and binding affinities to DcpS

Both structural analysis (X-ray structures) and studies on interaction of modified cap analogs with murine eIF4E and human DcpS have shown previously that the cap-binding pockets and structural requirements for cap binding of these two proteins are significantly different. Both proteins interact with cap through an intermolecular π-stacking between tryptophan aromatic ring(s) and positively charged 7-methylguanine ring. Besides that, the most important interactions stabilizing the eIF4E-cap complex are hydrogen bonds and salt bridges between negatively charged phosphate bridge and positively charged side chains of lysine and arginine in a rather loose cap binding pocket.[42,45,46] On the other hand, the human DcpS cap-binding pocket,[22] appears to be tight and stabilized by larger number of interactions not only with the phosphate chain but also sugar moieties and both nucleobases, which suggests that spatial requirements may be crucial for specific cap recognition in the case of DcpS.

Taking these differences into account, it is not surprising that a particular chemical modification of the cap structure can have different, often even opposite, effects on binding affinity for the two proteins. For instance, it was previously found that extending the 5′,5′-bridge to tetraphosphate stabilizes the complex due to additional electrostatic interactions provided by the fourth phosphate group and increases the binding affinity for eIF4E to about ~10-fold. However, the same modification resulted in ~1.4 decrease in the affinity for hDcpS (e.g., compare K_{AS} values for m^7GpCH_2ppG, m^7GpCH_2pppG and analogs **3** and **7** in Table 1), probably as a result of steric hindrance caused by an additional phosphate group. The non-bridging S to O substitution in the cap's triphosphate bridge had either stabilizing or virtually no effect on the complex with eIF4E, whereas bridging methylene substitutions, either at α/β or β/γ position, had destabilizing effect. On the contrary, in the case of DcpS, the K_{AS} values for complexes with β/γ CH_2-substituted analogs were ~4-fold higher than those for complexes with γ-S-substituted corresponding compounds (compare with Table 1). Finally, eIF4E was not sensitive to even quite bulky modifications at the 2′- and 3′-hydroxyls of N^7-methylguanosine moiety,[37,39,47] whereas in the case of DcpS, the ARCA-type analogs, have significantly lower binding affinities than non-ARCA ones (e.g., analogs **4** and **8** vs **3** and **7** in Table 1).

Comparing the binding affinities of NH-analogs for eIF4E and DcpS to the affinities of previously investigated differently modified cap analogs, the general conclusions can be drawn that the imidodiphosphate moiety has little influence on binding of either of these proteins. Regarding the interaction with eIF4E, the NH-analogs do not resemble CH_2-analogs, but rather unmodified and phosphorothioate ones. For instance, the K_{AS} values for analogs **1**–**4** were either comparable or higher than those of m^7GpppG and $m_2^{7,2'-O}GpppG$ (Table 1). Similarly in the NH-tetraphosphate series, the K_{AS} values for cap-eIF4E complexes were comparable to those for $m^7GppppG$ and $m_2^{7,2'-O}GppppG$, rather than for corresponding CH_2-analogs (compare with Table 1).[30]

The K_{AS} values for cap-hDcpS complexes, however, were more similar to the corresponding values for (methylenebis)phosphonate analogs than phosphorothioate ones. The K_{AS} value of analog **3** ($m^7GpNHppG$, 218 ± 24 μM^{-1}) is high, being comparable to that of m^7GpCH_2ppG (234 ± 14 μM^{-1}) and not to that of m^7Gp_SppG (D1) or (D2) (~45 μM^{-1}), suggesting that the analog is non-hydrolysable, but well-fitting to the enzymes' binding site. This finding may be important for designing of DcpS inhibitors. The K_{AS} value for m^7GpppG–hDcpS complex has not been determined since the compound is rapidly hydrolyzed by the enzyme, nonetheless, some biochemical data indicate that cap binding by DcpS is much tighter than by eIF4E (e.g., DcpS can efficiently displace eIF4E from m^7GTP-Sepharose, but not vice versa).[23] Due to this, one can assume that m^7GpCH_2ppG and $m^7GpNHppG$ are bound by DcpS in a manner more similar to m^7GpppG than m^7Gp_SppG. Hence, in terms of binding to DcpS and eIF4E, $m^7GpNHppG$ is the enzymatically resistant analog most resembling m^7GpppG.

The other NH-analogs differ in K_{AS} values to eIF4E and DcpS, however, comparison to the previously published data (Table 1) clearly reveals that these differences are due to the presence of other modifications (additional phosphate, 2′-*O*-methylation of 7-methylguanosine) than due to imidodiphosphate modification. For instance, comparison of K_{AS} values for analogs **3** ($m^7GpNHppG$, K_{AS} = 218 ± 24 μM^{-1}) and **7** ($m^7GpNHpppG$, K_{AS} = 155 ± 12 μM^{-1}) reveals that the additional, fourth phosphate moiety decreases the affinity for DcpS in a very similar manner to that previously observed for the CH_2 substituted analogs (Table 1).[30]

As mentioned before, eIF4E and DcpS have different requirements for efficient cap binding. Since the NH-analogs are tightly bound by both eIF4E and DcpS, one can assume that the imidodiphosphate moiety, among all phosphate-modifications tested to date, is most similar to unmodified phosphate in terms of both electronic (important for eIF4E) and geometrical (important for DcpS) properties. Hence, the NH-analogs could be considered as best ligands for cap enzyme binding studies, where one requires a non-hydrolysable ligand with the binding pattern similar to the natural substrate. Whether this can be a general conclusion that can be applied to several different pyrophosphatases (or more generally proteins) is an issue that requires further studies, which are currently being pursued by our group.

3.2. Inhibition of cap dependent-processes by NH-analogs and potential medicinal implications

Both eIF4E and DcpS proteins are considered as therapeutic targets. eIF4E is a target for anticancer therapy, since it is overexpressed in many types of human cancers leading to increased translation levels of proteins responsible for tumor formation and metastasis.[25] It was also demonstrated that selective repression of eIF4E leads to the inhibition of tumor growth, being well tolerated by normal tissues.[48] Therefore, modified cap analogs are not only interesting research tools, but may also be employed to anticancer therapy as selective eIF4E inhibitors. In this work we tested imidodiphosphate cap analogs as inhibitors of translation in a model rabbit reticulocyte lysate (RRL) system. The NH-analogs are generally efficient inhibitors of translation in vitro with IC_{50} values 2–3-fold lower than for m^7GpppG (Table 3). This in contrast to previously reported CH_2-analogs, for which the corresponding values were 2–3-fold higher than that of m^7GpppG.[35] Importantly, the DcpS-resistant analogs maintained their inhibitory properties after 60 min preincubation in RRL (Table 1), showing a correlation between susceptibility to DcpS and stability towards RRL's hydrolytic machinery. This fact demonstrates the need of developing DcpS-resistant analogs when their use as inhibitors of in vivo translation is considered.

On the other hand, a growing interest in DcpS as a therapeutic target has also been observed.[49–51] Being localized in both cytoplasm and nucleus, with the ability to shuttle between these two compartments, DcpS can alter 5′-cap concentrations and availability. Therefore, it has been postulated to perform a possible general regulatory function.[49] Recent findings have revealed that DcpS is involved not only in mRNA turnover but also can modulate other cap-dependent processes such as first intron excision from pre-mRNA.[51] DcpS has also been identified as a therapeutic target for spinal muscular atrophy.[50] Chemically modified non-hydrolysable cap analogs are interesting in terms of detailed investigation of DcpS active center's binding preferences, which can help obtain tools allowing mRNA fate modulation in the cell. It is expected that cap analogs if introduced into cells would compete for various cap binding proteins including eIF4E and DcpS. Having a set of analogs varying in the relative affinity for the two proteins (compare Table 1, last column) one can select analogs that would be preferably bound by DcpS rather than eIF4E and vice versa, depending on the targeted goal. A broad range of analogs with varying affinities for the two proteins may also be useful for designing affinity resins for cap-binding proteins with particular binding specificity.

3.3. Translation efficiency

We have shown that ARCA-type NH-analogs are efficiently incorporated into mRNA, and then such mRNAs undergo translation. Unexpectedly, the translation efficiencies of these mRNAs were lower compared to mRNAs capped with unmodified ARCA analog, despite the facts that these analogs were efficiently bound by eIF4E both during fluorescent titration experiments and during in vitro translation inhibition assay. Moreover, in a related work,[52] susceptibility of $m_2^{7,2'-O}$GpNHpG and $m_2^{7,2'-O}$GpNHppG-capped mRNAs to Dcp2 (an mRNA dependent decapping enzyme) and their translational efficiencies in HeLa cells were tested. $m_2^{7,2'-O}$GpNHpG-capped RNA proved to be completely resistant to hDcp2 in vitro, but its translation efficiency in HeLa cells was lower compared to $m_2^{7,2'-O}$GpppG-capped RNA, which is susceptible to Dcp2.[52] The results from both in vitro and cell culture translation studies are difficult to explain without any further investigation. One could speculate that it may arise from the fact that mRNAs capped with NH-analogs are strongly bound by some other cap-binding protein present in the cells and cell lysates, which makes them inaccessible for translational machinery. However, we currently have no experimental evidence to support this.

4. Conclusion

The herein synthesized and characterized NH-analogs represent a novel class of enzymatically resistant, dinucleotide analogs of mRNA 5′ end. The NH bridging modification within the cap's triphosphate moiety, on the one hand, assures its enzymatic stability, and on the other, does not significantly alter interaction with DcpS and eIF4E cap-binding proteins. This makes NH-analogs potentially useful tools to elucidate mechanisms of various cellular processes without introducing changes into cap binding patterns. The NH-analogs are also potent and stable inhibitors of cap-dependent translation, which together with relatively straightforward method for their preparation described in this study, makes them promising candidates for in vivo studies. The mRNAs capped with NH-analogs prepared via in vitro transcription undergo translation in reticulocyte lysates, however, the process was not as efficient as expected on the basis of our current knowledge on the interdependence of cap-related processes and mRNA translation. This finding, underlines the need of further investigation into the roles of mRNA 5′ end and protein factors involved in its recognition.

5. Experimental section

5.1. General information

Synthesized nucleotides were purified by ion-exchange chromatography on DEAE-Sephadex A-25 (HCO_3^- form) column. A column was loaded with reaction mixture and washed thoroughly with excess of water to remove metal(II) salt/EDTA complex. Then, nucleotides were eluted using a linear gradient of triethylammonium bicarbonate (TEAB) in deionized water. After evaporation under reduced pressure with repeated additions of ethanol to decompose TEAB, compounds were 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 the case of any remaining impurities, purified further on semi-preparative RP HPLC. In the latter case, products, after repeated freeze-drying, were isolated as ammonium salts. Yields were calculated on the basis of either sample weight or (preferably) optical miliunits (opt.mu.) of the product. Optical unit measurements were performed in 0.1 M phosphate buffer (pH 7 or pH 6 for m^7G nucleotides) at 260 nm.

Analytical HPLC was performed on Agilent Tech. Series 1200 using Supelcosil LC-18-T HPLC 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

the same apparatus equipped with Discovery RP Amide C-16 HPLC column (25 cm × 21.2 mm, 5 μm, flow rate 5.0 mL/min) with linear gradients of acetonitrile 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, time of flight mass spectrometry using negative electrospray ionization (MS(−)ES TOF) and ^{1}H NMR and ^{31}P NMR spectroscopy. Intermediate products were characterized by low resolution MS(−)ES and NMR. Mass spectra were recorded on Micromass QToF 1 MS and AB Sciex API 3200 spectrometers. NMR spectra were recorded at 25 °C on a Varian UNITY-plus spectrometer (^{1}H NMR at 399.94 MHz and ^{31}P NMR at 161.90 MHz). ^{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. The raw NMR data were processed using ACD/Labs 12.0 Software.

5.2. Chemical syntheses

Dichlorophosphorylphosphorimidoyl trichloride was prepared as described previously,[40] with the exception that it was used in a liquid form for further reactions as problems with its crystallization occurred. Guanosine was purchased from US Biological and 2′-*O*-methylguanosine was prepared according to procedures described by Sugar and Kusmierek.[53] Synthesis of phosphorimidazolides **10**–**15** was performed as described previously.[37]

5.2.1. P1-(7-methylguanosin-5′-yl) P3-guanosin-5′-yl 1,2-imidotriphosphate; m^{7}GppNHpG (1)

Compound 10 (m^{7}GMP-Im, Na salt, 30 mg, 660 opt.mu, 0.058 mmol) and **9** (GpNHp, TEA salt, 30 mg, 490 opt.mu., 0.040 mmol) were mixed in anhydrous DMF (2.5 ml) followed by addition of anhydrous $MgCl_2$ (53 mg, 0.55 mmol). After 3 h, reaction was completed and quenched by addition of water (30 mL). Product was purified on DEAE-Sephadex, isolated as a glassy solid and converted to sodium salt on Dowex (15.0 mg, 0.017 mmol, 43%). MS(−)ES ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 8.07 (s, 1H, H8$_G$), 5.91 (d, 1H, $J_{1'-2'}$ = 3.0 Hz, H1′$_{m7G}$), 5.80 (d, 1H, $J_{1'-2'}$ = 6.0 Hz, H1′$_G$), 4.71 (t, 1H, $J_{1'-2'}$ = 6.0 Hz, $J_{2'-3'}$ = 5.5 Hz, H2′$_G$), 4.53 (dd, 1H, $J_{1'-2'}$ = 3.0 Hz, $J_{2'-3'}$ = 5.0 Hz, H2′$_{m7G}$), 4.49 (dd, 1H, $J_{2'-3'}$ = 5.0 Hz, $J_{3'-4'}$ = 2.0 Hz, H3′$_G$), 4.44 (dd, 1H, $J_{2'-3'}$ = 5.0 Hz, $J_{3'-4'}$ = 6.0 Hz, H3′$_{m7G}$), 4.38–4.27 (m, 4H, H4′$_{m7G}$, H4′$_G$, H5′$_{m7G}$, H5′$_{m7G}$), 4.17 (m, 2H, H5′$_G$, H5″$_G$), 4.04 (s, 3H, NC*H*$_3$); ^{31}P NMR (162 MHz, D_2O, 25 °C): δ [ppm]: -0.30 (m, 1P, P$_\alpha$), −11.00 (m, 2P, P$_\gamma$, P$_\beta$), HRMS (ESI$^-$) Calcd *m/z* for $C_{21}H_{29}N_{11}O_{17}P_3^-$ (M−H$^-$) 800.0961, found 800.0938.

5.2.2. P1-(7,2′-*O*-dimethylguanosin-5′-yl) P3-guanosin-5′-yl 1,2-imidotriphosphate; m$_2^{7,2'-O}$GppNHpG (2)

Compound 11 (m$_2^{7,2'-O}$GMP-Im, Na salt, 50 mg, 1100 opt.mu., 0.096 mmol) and **9** (GpNHp, TEA salt, 50 mg, 815 opt.mu., 0.067 mmol) were mixed in anhydrous DMF (2.5 ml) followed by addition of anhydrous $ZnCl_2$ (132 mg, 0.97 mmol). After 2 h, reaction was completed and quenched by addition of solution of EDTA (364 mg, 0.98 mmol) in water and adjusted to pH 6 with solid $NaHCO_3$. Product was purified on DEAE-Sephadex, obtained as a glassy solid (TEA salt, 860 opt.mu., 0.040 mmol, 60%) and converted into sodium salt on Dowex (Na salt, 30 mg, 0.032 mmol, 48%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 9.02 (s, 1H, H8$_{m7G}$), 8.06 (s, 1H, H8$_G$), 5.97 (br s, 1H, H1′$_{m7G}$), 5.80 (d, 1H, $J_{1'-2'}$ = 5.5 Hz, H1′$_G$), 4.68 (t, 1H, $J_{1'-2'}$ = $J_{2'-3'}$ = 5.5 Hz, H2′$_G$), 4.50 (m, 2H, H3′$_{m7G}$, H3′$_G$), 4.41–4.12 (m, 7H, H4′$_{m7G}$, H4′$_G$, H5′$_{m7G}$, H5′$_G$, H5″$_{m7G}$, H5″$_G$, H2′$_{m7G}$), 4.06 (s, 3H, NC*H*$_3$), 3.60 (s, 3H, OC*H*$_3$); ^{31}P NMR (162 MHz, D_2O, 25 °C): δ [ppm]: −0.53 (m, 1P, P$_\alpha$), −10.57 (m, 2P, P$_\gamma$, P$_\beta$), HRMS (ESI$^-$) Calcd *m/z* for $C_{22}H_{31}N_{11}O_{17}P_3^-$ (M−H$^-$) 814.1118, found 814.1097.

5.2.3. P1-(7-methylguanosin-5′-yl) P3-guanosin-5′-yl 2,3-imidotriphosphate; m^{7}GpNHppG (3)

Compound 14 (GMP-Im, Na salt, 30 mg, 720 opt.mu., 0.060 mmol) and **17** (m^{7}GpNHp, TEA salt, 30 mg, 452 opt.mu., 0.040 mmol) were mixed in anhydrous DMF (2.5 ml) followed by addition of anhydrous $MgCl_2$ (50 mg, 0.53 mmol). After 2 h, reaction was completed and quenched by addition of water (30 ml). The product was isolated by DEAE-Sephadex chromatography as a glassy solid and then further purified using RP HPLC. Obtained fractions were repeatedly lyophilized to give 15.2 mg of white powder (ammonium salt, 15.2 mg, 400 opt.mu., 0.018 mmol, 44%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 9.22 (s, 1H, H8$_{m7G}$), 8.03 (s, 1H, H8$_G$), 5.92 (d, 1H, $J_{1'-2'}$ = 3.5 Hz, H1′$_{m7G}$), 5.82 (d, 1H, $J_{1'-2'}$ = 6.0 Hz, H1′$_G$), 4.68 (m, 1H, $J_{1'-2'}$ = 6.0 Hz, $J_{2'-3'}$ = 5.0 Hz, H2′$_G$), 4.58 (dd, 1H, $J_{1'-2'}$ = 3.5 Hz, $J_{2'-3'}$ = 5.0 Hz, H2′$_{m7G}$), 4.48 (m, 2H, H3′$_{m7G}$, H3′$_G$), 4.39–4.27 (m, 4H, H4′$_{m7G}$, H4′$_G$, H5′$_{m7G}$, H5′$_G$), 4.27–4.21 (m, 2H, H5″$_{m7G}$, H5″$_G$), 4.07 (s, 3H, NC*H*$_3$); ^{31}P NMR (162 MHz, D_2O, 25 °C): δ [ppm]: −0.36 (m, 1P, P$_\gamma$), −10.65 (m, 1P, P$_\alpha$), −11.14 (m, 1P, P$_\beta$), HRMS (ESI$^-$) Calcd *m/z* for $C_{21}H_{29}N_{11}O_{17}P_3^-$ (M−H$^-$) 800.0961, found 800.0940.

5.2.4. P1-(7,2′-*O*-dimethylguanosin-5′-yl) P3-guanosin-5′-yl 2,3-imidotriphosphate; m$_2^{7,2'-O}$GpNHppG (4)

Compound 18 (m$_2^{7,2'-O}$GpNHp, TEA salt, 50 mg, 740 opt.mu., 0.065 mmol) and **14** (GMP-Im, Na salt, 43 mg, 1030 opt.mu, 0.085 mmol) were mixed in anhydrous DMF (2 ml) followed by addition of anhydrous $ZnCl_2$ (92 mg, 0.68 mmol). After 2 h, reaction was completed and quenched by addition of solution of EDTA (255 mg, 0.68 mmol) in water and adjusted to pH 6 with solid $NaHCO_3$. Product was purified on DEAE-Sephadex, obtained as glassy solid and converted into sodium salt on Dowex (Na salt, 22 mg, 0.025 mmol, 38%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 9.22 (s, 1H, H8$_{m7G}$), 8.09 (s, 1H, H8$_G$), 5.96 (d, 1H, $J_{1'-2'}$ = 3.0 Hz, H1′$_{m7G}$), 5.83 (d, 1H, $J_{1'-2'}$ = 6.0 Hz, H1′$_G$), 4.67 (dd, 1H, $J_{1'-2'}$ = 6.0 Hz, $J_{2'-3'}$ = 5.0 Hz, H2′$_G$), 4.57 (t, 1H, $J_{2'-3'}$ = $J_{3'-4'}$ = 5.5 Hz, H3′$_{m7G}$), 4.49 (dd, 1H, $J_{2'-3'}$ = 5.0 Hz, $J_{3'-4'}$ = 4.0 Hz, H3′$_G$), 4.38–4.15 (m, 7H, H2′$_{m7G}$, H4′$_{m7G}$, H4′$_G$, H5′$_{m7G}$, H5′$_G$, H5″$_{m7G}$, H5″$_G$), 4.09 (s, 3H, NC*H*$_3$), 3.59 (s, 3H, OC*H*$_3$); ^{31}P NMR (162 MHz, D_2O, 25 °C): δ [ppm]: −0.37 (m, 1P, P$_\gamma$), −10.64 (m, 1P, P$_\alpha$), −11.18 (m, 1P, P$_\beta$), HRMS (ESI$^-$) Calcd *m/z* for $C_{22}H_{31}N_{11}O_{17}P_3^-$ (M−H$^-$) 814.1118, found 814.1095.

5.2.5. P1-(7-methylguanosin-5′-yl) P4-guanosin-5′-yl 3,4-imidotetraphosphate; m^{7}GpppNHpG (5)

Compound 12 (m^{7}GDP-Im, Na salt, 53 mg, 900 opt.mu., 0.079 mmol) and **9** (GpNHp, TEA salt, 50 mg, 815 opt.mu., 0.067 mmol) were mixed in anhydrous DMF (3 mL) followed by addition of anhydrous $ZnCl_2$ (105 mg, 0.77 mmol). After 4 h reaction was completed and quenched by addition of solution of EDTA (287 mg, 0.77 mmol) and $NaHCO_3$ (144 mg, 1.71 mmol) in water. Product was purified on DEAE-Sephadex, isolated as a glassy solid (TEA salt, 870 opt. u., 0.041 mmol, 61%) and further purified on preparative HPLC yielding 32 mg of **5** (NH_4^+ salt, 0.034 mmol, 51%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 9.20 (s, 1H, H8$_{m7G}$), 8.15 (s, 1H, H8$_G$), 6.00 (d, 1H, $J_{1'-2'}$ = 3.5 Hz, H1′$_{m7G}$), 5.86 (d, 1H, $J_{1'-2'}$ = 6.0 Hz, H1′$_G$), 4.77 (dd, 1H, overlapped with HDO, $J_{1'-2'}$ = 6.0 Hz, $J_{2'-3'}$ = 5.0 Hz, H2′$_G$), 4.66 (dd, 1H, $J_{1'-2'}$ = 3.5 Hz, $J_{2'-3'}$ = 5.0 Hz, H2′$_{m7G}$), 4.56 (dd, 1H, $J_{2'-3'}$ = 5.0 Hz, $J_{3'-4'}$ = 3.0 Hz, H3′$_G$), 4.53 (dd, 1H, $J_{2'-3'}$ = 5.0 Hz, $J_{3'-4'}$ = 5.5 Hz, H3′$_{m7G}$) 4.42–4.16 (m, 6H, H4′$_{m7G}$, H4′$_G$, H5′$_{m7G}$, H5′$_G$, H5″$_{m7G}$, H5″$_G$), 4.09 (s, 3H, NC*H*$_3$); ^{31}P NMR (162 MHz, D_2O, 25 °C) δ −0.23 (d, 1P, $J_{\alpha,\beta}$ = 6.5 Hz, P$_\alpha$), −10.94 (d, 1P, $J_{\gamma,\delta}$ = 19.5 Hz, P$_\delta$), −11.25 (dd, 1P, $J_{\beta,\gamma}$ = 19.5 Hz, $J_{\alpha,\beta}$ = 6.5 Hz, P$_\beta$), −22.41 (t, 1P,

$J_{\beta,\gamma} = J_{\gamma,\delta}$ = 19.5 Hz, P_γ), HRMS (ESI^-) Calcd *m/z* for $C_{21}H_{30}N_{11}O_{20}P_4^-$ ($M-H^-$) 880.0625, found 880.0594.

5.2.6. P1-(7,2′-*O*-dimethylguanosin-5′-yl) P4-guanosin-5′-yl 3,4-imidotetraphosphate; $m_2^{7,2'-O}$GpppNHpG) (6)

Compound 13 ($m_2^{7,2'-O}$GDP-Im, Na salt, 53 mg, 850 opt.mu., 0.074 mmol) and **9** (GpNHp, TEA salt, 50 mg, 815 opt.mu, 0.067 mmol) were mixed in anhydrous DMF (3 mL) followed by addition of anhydrous $ZnCl_2$ (103 mg, 0.76 mmol). After 4 h reaction was completed and quenched by addition of solution of EDTA (282 mg, 0.76 mmol) and $NaHCO_3$ (141 mg, 1.68 mmol) in water. Product was purified on DEAE-Sephadex resin), isolated as a glassy solid (TEA salt, 713 opt.mu., 0.033 mmol, 50%) and converted into sodium salt on Dowex (Na^+ form) yielding 26 mg of **6** (Na salt, 0.027 mmol, 40%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 8.12 (s, 1H, $H8_G$), 6.06 (d, 1H, $J_{1'-2'}$ = 2.0 Hz, $H1'_{m7G}$), 5.84 (d, 1H, $J_{1'-2'}$ = 6.5 Hz, $H1'_G$), 4.79 (t, 1H, overlapped with HDO, $H2'_G$), 4.60 (t, 1H, $J_{2'-3'} = J_{3'-4'}$ = 5.5 Hz, $H3'_{m7G}$), 4.57 (dd, 1H, $J_{2'-3'}$ = 5.0 Hz, $J_{3'-4'}$ = 2.5 Hz, $H3'_G$), 4.43–4.15 (m, 7H, $H4'_{m7G}$, $H4'_G$, $H5'_{m7G}$, $H5''_{m7G}$, $H5'_G$, $H5''_G$, $H2'_{m7G}$), 4.10 (s, 3H, NCH_3), 3.61 (s, 3H, OCH_3); ^{31}P NMR (162 MHz, D_2O, 25 °C) δ [ppm] −0.40 (d, 1P, $J_{\alpha,\beta}$ = 6.5 Hz, P_α), −11.16 (d, 1P, $J_{\gamma,\delta}$ = 19.5 Hz, P_δ), −11.40 (dd, 1P, $J_{\beta,\gamma}$ = 19.5 Hz, $J_{\alpha,\beta}$ = 6.5 Hz, P_β), −22.52 (t, 1P, $J_{\beta,\gamma} = J_{\gamma,\delta}$ = 19.5 Hz, P_γ), HRMS (ESI^-) Calcd *m/z* for $C_{22}H_{32}N_{11}O_{20}P_4^-$ ($M-H^-$) 894.0781, found 894.0752.

5.2.7. P1-(7-methylguanosin-5′-yl) P4-guanosin-5′-yl 1,2-imidotetraphosphate; m^7GpNHpppG (7)

Compound 15 (GDP-Im, Na salt, 48 mg, 865 opt.mu, 0.071 mmol) and **17** (m^7GpNHp, TEA salt, 40 mg, 600 opt.mu., 0.053 mmol) were mixed in anhydrous DMF (3 mL) followed by addition of anhydrous $ZnCl_2$ (130 mg, 0.96 mmol). After 24 h, reaction was completed and quenched by addition of solution of EDTA (356 mg, 0.96 mmol) and $NaHCO_3$ (178 mg, 2.12 mmol) in water. Product was purified on DEAE-Sephadex, isolated as a glassy solid (TEA salt, 380 opt.mu., 0.018 mmol, 34%) and converted into sodium salt on Dowex (Na^+ form) yielding 13 mg of **5** (Na salt, 0.014 mmol, 24%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 9.32 (s, 1H, $H8_{m7G}$), 8.07 (s, 1H, $H8_G$), 6.00 (d, 1H, $J_{1'-2'}$ = 3.5 Hz, $H1'_{m7G}$), 5.85 (d, 1H, $J_{1'-2'}$ = 6.5 Hz, $H1'_G$), 4.77 (dd, 1H, $J_{1'-2'}$ = 6.5 Hz, $J_{2'-3'}$ = 6.0 Hz, $H2'_G$), 4.65 (dd, 1H, $J_{1'-2'}$ = 3.5 Hz, $J_{2'-3'}$ = 5.0 Hz, $H2'_{m7G}$), 4.56 (dd, 1H, $J_{2'-3'}$ = 6.0 Hz, $J_{3'-4'}$ = 3.0 Hz; $H3'_G$), 4.53 (t, 1H, $J_{2'-3'} = J_{3'-4'}$ = 5.0 Hz, $H3'_{m7G}$), 4.41 (m, 1H, $H4'_G$), 4.36 (m, 1H, $H4'_{m7G}$), 4.34–4.16 (m, 4H, $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.09 (s, 3H, NCH_3); ^{31}P NMR (162 MHz, D_2O, 25 °C) δ [ppm] −0.36 (m, 1P, P_δ), −11.11 (dt, 1P, $J_{\alpha,\beta}$ = 19.0 Hz, $J_{\alpha,\beta} = J_{\alpha,H5'}$ = 6.5 Hz, P_α), −11.45 (dd, 1P, $J_{\beta,\gamma}$ = 19.0 Hz, $J_{\gamma,\delta}$ = 6.5 Hz, P_γ), −22.64 (t, 1P, $J_{\alpha,\beta} = J_{\beta,\gamma}$ = 19.0 Hz, P_β), HRMS (ESI^-) Calcd *m/z* for $C_{21}H_{30}N_{11}O_2P_4^-$ ($M-H^-$) 880.0625, found 880.0597.

5.2.8. P1-(7,2′-*O*-dimethylguanosin-5′-yl) P4-guanosin-5′-yl 1,2-imidotetraphosphate; $m_2^{7,2'-O}$GpNHpppG (8)

Compound 15 (GDP-Im, Na salt, 40 mg, 815 opt.mu, 0.074 mmol) and **18** ($m_2^{7,2'-O}$**GpNHp,** TEA salt, 34 mg, 510 opt.mu., 0.045 mmol) were mixed in anhydrous DMF (3 ml) followed by addition of anhydrous $ZnCl_2$ (110 mg, 0.81 mmol). After 24 h, reaction was completed and quenched by addition of solution of EDTA (300 mg, 0.81 mmol) and $NaHCO_3$ (150 mg, 1.76 mmol) in water. Product was purified on DEAE-Sephadex and obtained as a glassy solid (TEA salt, 300 opt.mu. 0.014 mmol, 31%). Further purification was performed using preparative HPLC yielding in 12.0 mg of final compound (NH_4^+ salt, 0.013 mmol, 27%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 9.29 (s, 1H, $H8_{m7G}$), 8.10 (s, 1H, $H8_G$), 6.06 (d, 1H, $J_{1'-2'}$ = 3.0 Hz, $H1'_{m7G}$), 5.85 (d, 1H, $J_{1'-2'}$ = 6.0 Hz, $H1'_G$), 4.75 (dd, 1H, $J_{1'-2'}$ = 6.0 Hz, $J_{2'-3'}$ = 5.0 Hz, $H2'_G$), 4.60 (t, 1H, $J_{2'-3'} = J_{3'-4'}$ = 5.5 Hz, $H3'_{m7G}$), 4.54 (dd, 1H, $J_{2'-3'}$ = 5.0 Hz, $J_{3'-4'}$ = 3.0 Hz, $H3'_G$), 4.38–4.16 (m, 7H, $H3'_{m7G}$, $H3'_G$, $H4'_{m7G}$, $H4'_G$, $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.10 (s, 3H, NCH_3), 3.59 (s, 3H, OCH_3); ^{31}P NMR (162 MHz, D_2O, 25 °C) δ [ppm] −0.46 (m, 1P, P_δ), −11.11 (m, 2P, P_α, P_γ), −22.40 (t, 1P, $J_{\alpha,\beta} = J_{\beta,\gamma}$ = 20.0 Hz, P_β), HRMS (ESI^-) Calcd *m/z* for $C_{22}H_{32}N_{11}O_{20}P_4^-$ ($M-H^-$) 894.0781, found 894.0757.

5.2.9. P1-(guanosin-5′-yl) imidodiphosphate; GpNHp (9)

Guanosine (1 g, 3.53 mmol) was suspended in trimethyl phosphate (37.5 ml) and cooled to −8 °C. Then $Cl_3PNP(O)Cl_2$ (3.5 ml) was added under vigorous stirring. Reaction was maintained at −8 °C and after 2 h was stopped by addition of 0.7 M TEAB to pH 7 and diluted with water. The product was purified on DEAE-Sephadex and isolated as TEA salt (728 mg, 0.98 mmol, 28%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 8.32 (s, 1H, H8), 5.95 (d, 1H, $J_{1'-2'}$ = 5.0 Hz, H1′), 4.76 (m, 1H, overlapped with HDO, H2′), 4.54 (m, 1H, H3′), 4.35 (m, 1H, H4′), 4.14 (m, 2H, H5′, H5″), 3.20 (q, 12H, J = 7.4, $N(CH_2CH_3)_3$); 1.27 (t, 18H, J = 7.4, $N(CH_2CH_3)_3$). ^{31}P NMR (162 MHz, D_2O, 25 °C) δ [ppm] 0.96 (m, 1P, P_α), −0.64 (d, 1P, J = 5.5 Hz, P_β).

5.2.10. P1-(2′-*O*-methylguanosin-5′-yl) imidodiphosphate; $m^{2'-O}$GpNHp (16)

Compound 16 (1100 mg, 1.42 mmol, 57%) was obtained starting from $m^{2'-O}$G (740 mg, 2.50 mmol) following the same procedure as for **9**. 1H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 8.07 (s, 1H, H8), 5.88 (d, 1H, $J_{1'-2'}$ = 6.0 Hz, H1′), 4.63 (t, 1H, $J_{2'-3'} = J_{3'-4'}$ = 5.5 Hz, H3′), 4.41 (t, 1H, $J_{1'-2'}$ = 6.0 Hz, $J_{2'-3'}$ = 5.0 Hz, H2′), 4.22 (m, 1H, H4′), 3.94 (m, 2 H, H5′, H5″), 3.35 (s, 3H, OCH_3), 3.20 (q, 12H, J = 7.4, $N(CH_2CH_3)_3$); 1.27 (t, 18H, J = 7.4, $N(CH_2CH_3)_3$). ^{31}P NMR (162 MHz, D_2O, 25 °C) δ [ppm] 0.94 (m, 1P, P_α), −0.62 (d, 1P, $J_{\alpha\beta}$ = 5.5 Hz, P_β).

5.2.11. P1-(7-methylguanosin-5′-yl) imidodiphosphate; m^7GpNHp (17)

To **9** (GpNHp, 100 mg, 0.13 mmol) dissolved in DMSO (4 mL) CH_3I was added (90 μL, 1.47 mmol) and the mixture was stirred for 7 h at RT. Then, 40 mL of cold water was added and the solution was extracted 3–4 times with 20 ml portions of diethyl ether. The aqueous phase was neutralized with 1 M $NaHCO_3$ and purified by ion-exchange chromatography (DEAE-Sephadex resin). The product was obtained as TEA salt (40 mg, 0.07 mmol, 39%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 9.34 (s, 1H, H8), 6.07 (d, 1H, $J_{1'-2'}$ = 3.0 Hz; H1′); 4.68 (dd, 1H, $J_{1'-2'}$ = 3.0 Hz; $J_{2'-3'}$ = 5.0 Hz; H2′); 4.53 (dd, 1H, $J_{2'-3'}$ = 5.0 Hz, $J_{3'-4'}$ = 5.0 Hz; H3′), 4.40 (m, 1H, H4′); 4.27 (m, 1H, H5′), 4.15 (m, 1H, H5″); 4.12 (s, 1H, NCH_3); 3.20 (q, 12H, J = 7.4, $N(CH_2CH_3)_3$); 1.28 (t, 18H, J = 7.4, $N(CH_2CH_3)_3$). ^{31}P NMR (162 MHz, D_2O, 25 °C) δ [ppm] 0.90 (m, 1P, P_α), −0.80 (d, 1P, J = 5.5 Hz, P_β).

5.2.12. P1-(7,2′-*O*-dimethylguanosin-5′-yl) imidodiphosphate; $m_2^{7,2'-O}$GpNHp (18)

Compound **18** ($m_2^{7,2'-O}$**GpNHp,** TEA salt, 17.5 mg, 0.03 mmol, 10%) was obtained starting from 16 ($m^{2'-O}$GpNHp, TEA salt form, 170 mg, 0.31 mmol) and following the same procedure as for **17**. 1H NMR (400 MHz, D_2O, 25 °C): δ [ppm] 9.33 (s, 1H, H8), 6.17 (d, 1H, $J_{1'-2'}$ = 3.0 Hz, H1′), 4.63 (t, 1H, $J_{2'-3'} = J_{3'-4'}$ = 5.5 Hz, H3′), 4.38 (m, 2H, H2′, H4′), 4.27 (m, 1H, H5′), 4.15 (m, 1H, H5″), 4.12 (s, 3H, NCH_3), 3.60 (s, 3H, OCH_3), 3.20 (q, 12H, J = 7.4, $N(CH_2CH_3)_3$); 1.28 (t, 18H, J = 7.4, $N(CH_2CH_3)_3$). ^{31}P NMR (162 MHz, D_2O, 25 °C) δ [ppm] 0.92 (m, 1P, P_α), −0.78 (d, 1P, J = 5.5 Hz, P_β).

5.3. Chemical stability of cap analogs

Cap analogs were dissolved at 1 mM concentration either in 100 mM oxalate buffer (pH 2) or 100 mM ammonium acetate buffer (pH 6) or 100 mM TEAB buffer (pH 8). Samples were incubated

at 25 °C and analyzed by analytical RP HPLC (see general information) at different time points over a period of 24 h.

5.4. Biological assays

Binding affinity experiments were performed as described previously.[42] DcpS stability assay, translation inhibition experiments, mRNA transcription reaction and mRNA translation experiments were performed analogously to those described by Rydzik et al. 2009.[30] Short transcripts were synthesized analogously as described by Rydzik et al. 2009,[30] except that the reaction mixture additionally contained 0.1 mM GTP (to obtain molar ratio of cap analog to GTP in the reaction 10:1).

Acknowledgments

This work was supported by the Polish Ministry of Science and Higher Education (N N204 089438, IP 2010 020170, N N301 096339), and the Howard Hughes Medical Institute (55005604). The authors are grateful to the Laboratory of Biological NMR (Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, IBB PAS) for the access to the NMR apparatus and to Jacek Oledzki from the Laboratory of Mass Spectrometry (IBB PAS) for recording MS spectra.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.01.013. These data include MOL files and InChiKeys of the most important compounds described in this article.

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