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Phosphate-modified analogues of m^{7}GTP and m^{7}Gppppm^{7}G—Synthesis and biochemical properties

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ABSTRACT

The synthesis and biochemical properties of 17 new mRNA cap analogues are reported. Six of these nucleotides are m^{7}GTP derivatives, whereas 11 are 'two headed' tetraphosphate dinucleotides based on a m^{7}Gppppm^{7}G structure. The compounds contain either a boranophosphate or phosphorothioate moiety in the nucleoside neighbouring position(s) and some of them possess an additional methylene group between β and γ phosphorus atoms. The compounds were prepared by divalent metal chloride-mediated coupling of an appropriate m^{7}GMP analogue with a given P^1,P^2-di(1-imidazolyl) derivative. The analogues were evaluated as tools for studying cap-dependent processes in a number of biochemical assays, including determination of affinity to eukaryotic initiation factor eIF4E, susceptibility to enzymatic hydrolysis, and translational efficiency in vitro. The results indicate that modification in the phosphate chain can increase binding to cap-interacting proteins and provides higher resistance to degradation. Furthermore, modified derivatives of m^{7}GTP were found to be potent inhibitors of cap-dependent translation in cell free systems.

1. Introduction

A characteristic feature of the 5′ terminus of all eukaryotic mRNAs is the cap—a specific residue consisting of 7-methylguanosine attached to the first transcribed nucleotide via a 5′–5′ triphosphate bridge. The cap is involved in numerous aspects of mRNA metabolism, including its splicing, intracellular transport, translation and protection from degradation.[1,2] During the initiation of translation, the cap is specifically recognised by eukaryotic initiation factor 4E (eIF4E), which is overexpressed in many types of tumours.[3,4] Increased concentrations of eIF4E selectively stimulate translation of mRNAs essential for malignant transformation and metastasis.[4] Synthetic cap analogues, such as m^{7}Gp$_n$ or m^{7}Gp$_n$N, were shown to inhibit cap-dependent translation in vitro by competing with mRNA in the formation of a translation initiation complex with eIF4E,[1] making synthetic cap analogues potential candidates for translation inhibitors in anti-cancer therapy. However, one of difficulties in using unmodified cap analogues in vivo is their rapid enzymatic degradation, mediated mainly by Decapping Scavenger pyrophosphatase (DcpS).[5,6] We have recently shown that some chemical modifications of the oligophosphate chain within the cap structure can increase the affinity to eIF4E and confer resistance towards DcpS.[1,7–9] For instance, replacement of one of the γ-oxygens in m^{7}Gp$_\gamma$p$_\beta$p$_\alpha$G or α-oxygens in m^{7}GTP with either BH$_3$ or S generally stabilises the complex with eIF4E[10,11] and significantly decreases susceptibility to hydrolysis by DcpS. Inhibition studies in RRL (Rabbit Reticulocyte Lysate) have shown that a combination of these two features results in compounds that are potent and stable inhibitors of cap-dependent translation.[8,10–12]

In contrast to mononucleotide cap analogues, dinucleotide analogues can be incorporated in vitro into mRNA by bacterial[13] or viral[14] RNA polymerases. Such capped transcripts can find applications in biochemistry and gene therapy.[1] Our earlier studies clearly show that prepared in vitro transcripts bearing a structurally modified cap analogue could have better translational properties, which is caused by their higher affinity to eIF4E proteins and diminished susceptibility to degradation by Dcp1/2-mediated decapping.[15] One example could be m$^{7,2'\text{-}O}_2$Gpp$_S$pG, which provides considerable improvement in both stability and translational yield of mRNA; hence, it has been applied in gene therapy against cancer.[16]

Although previously synthesised compounds have been shown to be resistant to cap-specific degradation,[1,7,9–11,17,18] there is still

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field for improvement, for example, by protection against chemical degradation or unspecific enzymatic degradation by cellular or extracellular pyrophosphatases. To verify whether the biological properties of cap analogues can be further modulated, here, we synthesised and characterised a set of cap analogues **1**–**9** based on m^7GTP and $m^7Gppppm^7G$ structures, which were extensively modified within the oligophosphate bridge (Fig. 1). The m^7GTP analogues (compounds **1**–**5**) were modified in the phosphate chain at the α position by introduction of either a phosphorothioate or boranophosphate group, and, for compounds **2**–**4**, additionally at the β,γ-position with either a methylene or imido-group. Due to the presence of stereogenic α-phosphorous atom, compounds **1**–**5** were obtained as a mixture of two diastereomers: S_P, R_P (see Fig. 2A for details). The $m^7Gppppm^7G$ analogues (Fig. 1B, compounds **6**–**9**) were modified at the α and δ positions by the introduction of either a phosphorothioate or boranophosphate group, and, for compounds **7** and **9**, additionally at the β,γ-position with a methylene group. Since compounds **6**–**9** possess two stereogenic phosphorous atoms (α and δ), they were obtained as a mixture of three diastereomers: S_P–S_P, R_P–S_P and R_P–R_P (see Fig. 2 for details). Cap analogues **1**, **2**, **4** and **6**–**9** were successfully prepared and were not reported before. Hence, including diastereomers, 17 new compounds were synthesised in this work.

To assess how such extensive oligophosphate modifications influence properties of the cap, the analogues were evaluated as eIF4E binders, tested for susceptibility to specific (DcpS-mediated) and unspecific (blood serum) degradation in vitro, and some of them were evaluated as inhibitors of cap dependent translation in RRL. Additionally, $m^7Gppppm^7G$ and its phosphate-modified derivatives **6**–**9** can be incorporated into mRNA by in vitro transcription reactions to produce capped transcripts, $m^7Gppppm^7GpNp(Np)_n$. Due to the presence of two identical m^7G moieties, such analogues are often referred to as 'two-headed' and in contrast to analogues based on a m^7GpppG structure[19], always possess the 7-methylguanosine residue in distal position when incorporated into mRNA transcripts, thereby behaving similarly to so-called 'anti-reverse' cap analogues (ARCA) such as $m_2^{7,2'-O}GpppG$.[20] Therefore, we also incorporated some analogues, **6**–**9**, into mRNAs and tested for their translational efficiency in vitro.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis and characterisation

A number of synthetic strategies for the synthesis of sulphur-[21–24] or boron-containing[23,25–28] phosphate-modified nucleotides have been reported in the literature. However, only few of these methods are suitable for the preparation of 7-methylguanosine derivatives, since these compounds are susceptible to degradation at higher temperatures or extreme pH[11]. Mono- and dinucleotide cap analogues are usually synthesised in divalent metal chloride-mediated coupling reactions between nucleotide imidazolide derivatives, and appropriate inactivated nucleotides or other phosphate nucleophiles (e.g., phosphate or pyrophosphate).[11] This approach can also be applied for phosphate-modified nucleotides; however, with some limitations. For example, phosphorothioate nucleotide analogues can be used merely as nucleophiles, because their electrophilic activation with imidazole using standard protocols leads to decomposition with the loss of sulphur. To overcome this problem, especially for target compounds containing multiple phosphorothioate modifications, a reverse activation scheme can be applied. In the reverse approach an inorganic phosphate or pyrophosphate is activated with imidazole and then coupled with a non-activated nucleotide.[29–31] One example of such an approach has been applied to the synthesis of diuridine and diadenosine tetraphosphates and their α,δ-dithio analogues, by coupling 2 equiv of an appropriate nucleotide mono(thio)phosphate with 1 equiv of pyrophosphate P^1,P^2-di(1-imidazolyl) derivative.[29] To obtain nucleotides additionally containing a methylene bridge in the β,γ-position, activated phosphonate has also been developed. Here, we used a similar strategy to obtain bis(7-methylguanosine) tetraphosphates, and their α,δ-dithio and α,δ-diborano analogues optionally containing a β,γ-methylene group. Moreover, by optimising the reagent proportions we adapted the approach to the synthesis of phosphate-modified 7-methylguanosine triphosphate analogues (**1**–**5**).

P^1,P^2-di(1-imidazolyl) derivatives **10**–**13** were obtained from the corresponding phosphates **14**–**17** in the Mukaiyama–Hashimoto reaction (Scheme 1).[32] Preparation of **10**–**13** required a larger (20 equiv) excess of imidazole, dithiodipyridine and triphenylphosphine (5 equiv) in comparison to the synthesis of nucleotide phosphoroimidazolidates.[33] Compounds **10**–**13** were synthesised in good yields (>63%) and their purity was tested by ^{31}P NMR (Supplementary information). To optimise the synthesis of nucleoside triphosphates we performed reactions between either GMP or Gp_{BH3} (**18**) with **10** under conditions similar to previously described.[10] The reactions were carried out in DMF and were most efficient if at least an eightfold excess of Mg^{2+} was used. As determined by MS, the coupling reactions led initially to formation of imidazole derivatives of the desired nucleoside triphosphates, which had to be subsequently converted into **21**, **22** by hydrolysis (Scheme 2A). To avoid the formation of dinucleotide by-products, $Gp_{BH3}ppp_{BH3}G$ or $Gp_{BH3}pCH_2pp_{BH3}G$, the amount of a P^1,P^2-di(1-imidazolyl) derivative to nucleotide was optimised in

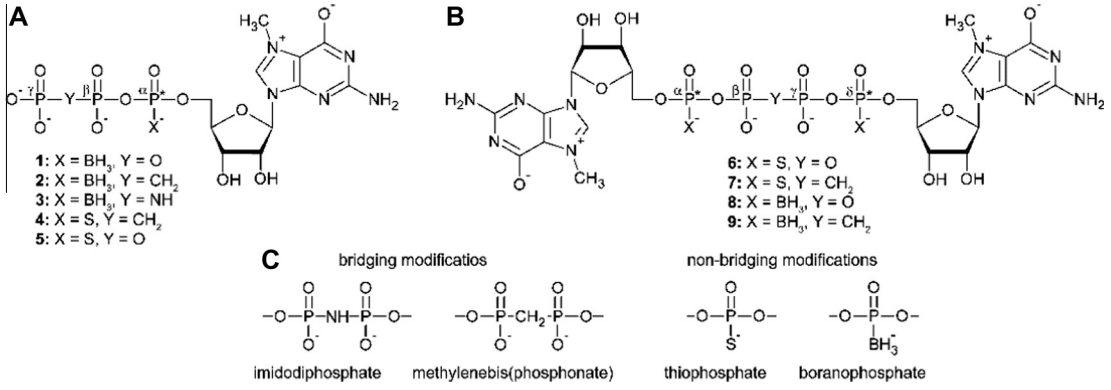


Figure 1. Structures of mono- and dinucleotide cap analogues modified within a phosphate chain. (A) Triphosphate mononucleotides, each compound exists as a mixture of two diastereomers. (B) Tetraphosphate dinucleotides, each compound exists as a mixture of three diastereomers. (C) Structures of the modifications introduced to compounds **1**–**9**. All cap analogues, except compound **3**, were successfully synthesised and characterised.

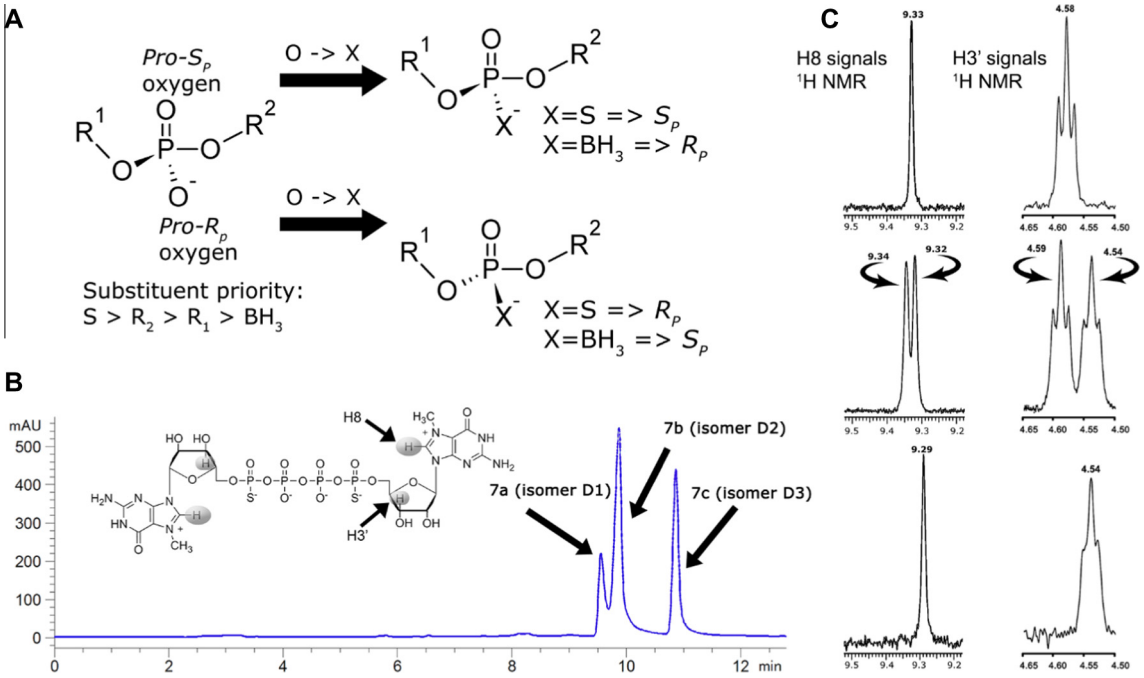
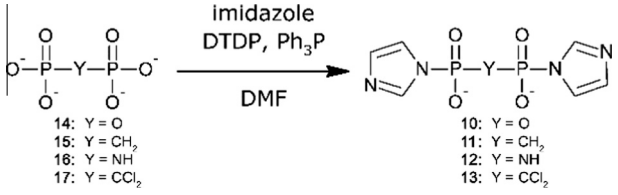


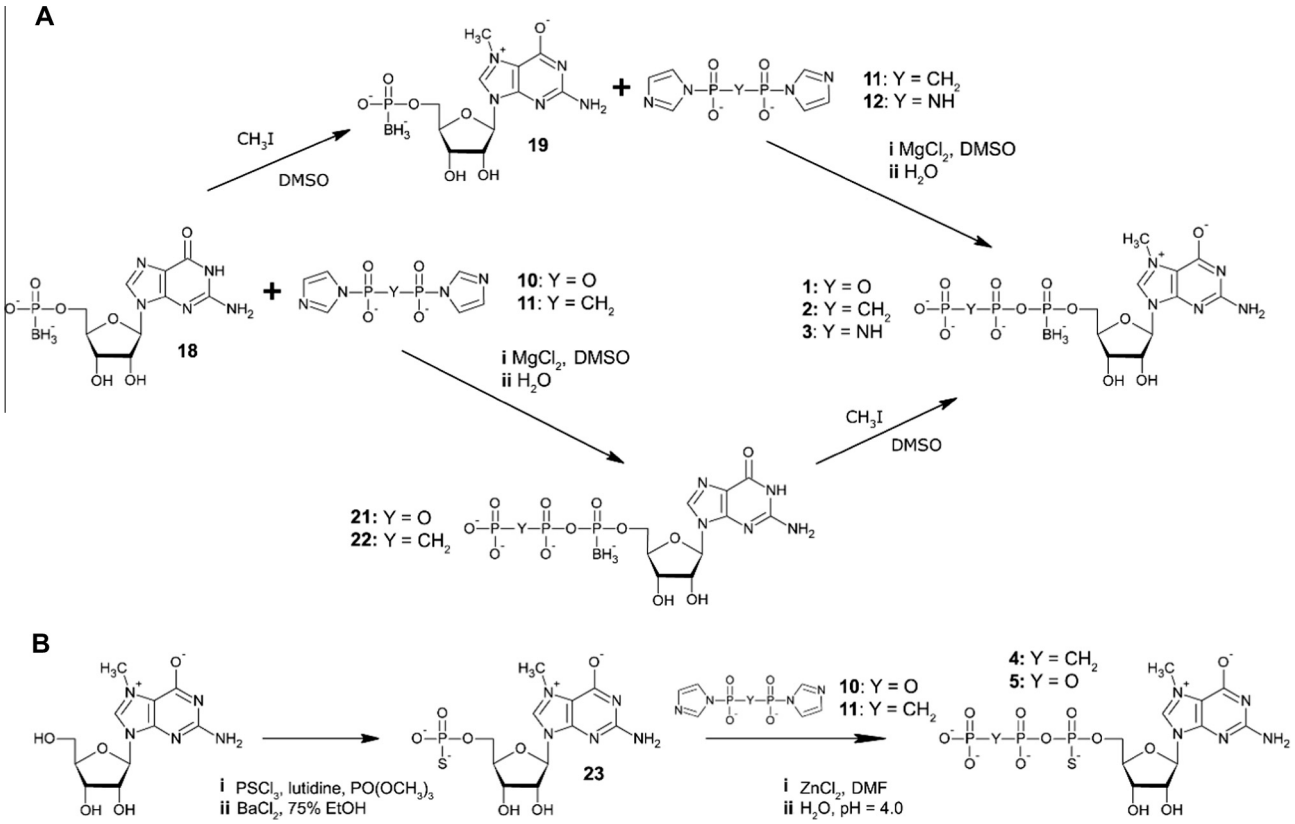
Figure 2. Stereochemistry of cap analogues. (A) Stereochemistry of a phosphorus atom in phosphorothioate and boranophosphate moiety. Substitutions of either O to BH_3 or O to S may result in P-diastereomerism. The same spatial arrangement of substituents around a stereogenic phosphorus centre for phosphorothioate and boranophosphate groups leads to different absolute configuration assignments (S_P and R_P), because of the different priority of BH_3 and S substituents according to CIP rules. (B) A representative HPLC chromatogram of a diastereoisomeric mixture of compounds **7a–c** after ion-exchange chromatography purification. (C) ^{1}H NMR spectra of compounds **7a** (upper), **7b** (middle), **7c** (lower) showing signals of H8 and H3′ protons—the chemical shift of the signals is dependent upon configuration of the phosphorus atom in the vicinity of 7-methylguanine moiety. Due to C_2-rotational symmetry in S_P–S_P and R_P–R_P stereoisomers, the corresponding protons of two nucleoside moieties are homotopic and equivalent in ^{1}H NMR. Conversely, in the R_P–S_P isomers, the corresponding protons from two nucleoside moieties are diastereotopic and consequently two sets of signals are observed.



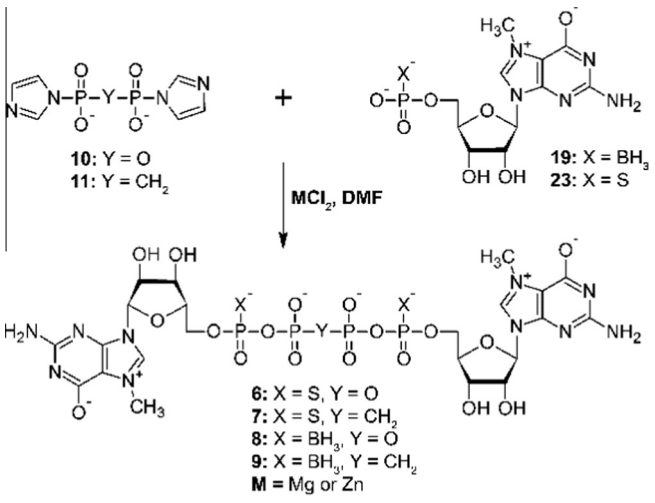
Scheme 1. Synthesis of P^1,P^2-di(1-imidazolyl) derivatives **10**–**13**.

the reaction. We found that the proportion 1:4 for a nucleoside monophosphate to an imidazole derivative was optimal, since only slight amounts of dinucleotides, $Gp_{BH3}pYpp_{BH3}G$ were observed in the reaction mixtures, and mononucleoside pentaphosphates were not observed. A greater excess of a P^1,P^2-di(1-imidazolyl) derivative results in the formation of nucleoside pentaphosphates as side products. After the optimisation, the desired products, GTP (**20**) or $Gp_{BH3}pp$ (**21**), were isolated in 65% and 44% yields, respectively. If compound **11** was used in the reaction with Gp_{BH3} (**18**) instead of **10**, the reaction proceeded significantly faster, requiring less than 5 h for completion, and affording $Gp_{BH3}pCH_2p$ (**22**) in a slightly lower isolated yield (39%). Nucleoside triphosphates **21** and **22** were subsequently methylated at the N^7 position using methyl iodide and the reaction yields were 40% and 31% for **1** and **2**, respectively. Since the terminal phosphate group in both compounds is prone to methylation, some undesired side products were detected by HPLC. To examine another synthetic route, compound **2** was also directly synthesised, starting from m^7Gp_{BH3} and **11**. Preparation of m^7Gp_{BH3} (**19**) was accomplished from Gp_{BH3} using CH_3I, as described before.[10] In the last step of the synthesis, preliminarily purified (by ion-exchange chromatography, IC) **1** and **2** were separated into pure diastereomers using semi-preparative RP-HPLC. The diastereomers were termed D1 and D2, according to their retention times. Compound **4** was prepared in an analogous way to compounds **1**–**2**, starting from m^7Gp_S (**23**; Scheme 2B). This starting material was synthesised via thiophosphorylation of m^7G in $(MeO)_3PO$ in the presence of 2,6-lutidine with some modification of the previously reported procedure, to facilitate the purification of the product.[8,34] Pure **23** was then coupled with **11** using $ZnCl_2$ as a catalyst and compound **4** was obtained with a 33% yield. Both diastereomers of **4** were then separated by semi-preparative HPLC. Using the same methodology, m^7Gp_Spp **5** was also prepared using **10**; however, the yield was lower than in the previously described one-pot procedure (20% vs 35%).[8]

Tetraphosphate analogues **6**–**9** were synthesised in a one-pot reaction from an appropriate m^7G monophosphate and P^1,P^2-di(1-imidazolyl) derivative (Scheme 3) using 0.75–1.0 equiv of a derivative per nucleoside monophosphate. These reactions were most efficient when a fourfold excess of divalent metal cations (Zn^{2+} for phosphorothioates and Mg^{2+} for boranophosphates) was used.[7,10,31] Since CH_3I can also methylate sulphur within the phosphorothioate moiety[35], compounds **6**, **7** were synthesised starting from m^7Gp_S (**23**). In order to avoid possible side products, namely m^7G triphosphates, only a 0.25 equiv of activated diphosphates was added to the reaction mixture at once. This operation was repeated 3–4 times until the HPLC chromatogram indicated the conversion of a nucleotide monophosphate into tetraphosphate. On the other hand, the synthesis of compounds **8** and **9**, containing a boranophosphate group, could also be attempted via methylation of a tetraphosphate dinucleotide. This alternative strategy was also tested (Scheme S2). Although the coupling of Gp_{BH3} (**18**) with P^1,P^2-di(1-imidazolyl) derivatives **10**, **11** to form the desired dinucleotides was efficient (69%–79% isolated yields), the subsequent methylations resulted in a mixture of mono- and dimethylated products and a partial decomposition of nucleotides which led to



Scheme 2. Synthesis of m^7GTP analogues modified in the phosphate chain. (A) Compounds **1**–**3** with a boranophosphate group. (B) Compounds **4**–**5** with a phosphorothioate group at α-position. In each case the initial product of the coupling is a highly reactive imidazole derivative which is subsequently hydrolysed to the desired product. In the case of boranophosphates, these imidazole derivatives are unstable and they are rapidly hydrolysed to the nucleotides; however, the derivatives of thiophosphates are considerably more stable (hydrolysis requires acidic pH and takes several hours to be finished).



Scheme 3. Synthesis of tetraphosphate cap analogues **6**–**9**.

deterioration in the overall yield of these syntheses (preparative yield was 14–24%). As such, compounds **8**–**9** were obtained by coupling from m^7Gp_{BH3} with **10** or **11** and purified using IC in satisfactory yields (26–48%). In the final step, diastereomers were separated using semi-preparative RP-HPLC. The diastereomers were termed D1–D3 according to their elution order on an RP column (see Fig. 2B for an example). The separation was successful in the case of all cap analogues except **6**, where isomers D1 and D2 did not separate well under any tested conditions.

Encouraged by the successful couplings with activated pyrophosphate and methylenebisphosphonate, we tried to apply P^1,P^2-di(1-imidazolyl) derivatives of imidodiphosphate **12** and dichloromethylenebisphosphonate **13** in the synthesis of m^7GTP analogues. A preliminary coupling reaction of **12** with AMP or GMP indicated the formation of the expected nucleoside triphosphates with satisfactory HPLC conversion (~50%). The reaction can be efficiently catalysed by either $MgCl_2$ or $ZnCl_2$. Earlier observations suggested that methylation of GppNHp is not efficient, leading to partial decomposition of the substrate with P–NH–P bond cleavage.[9] Hence, we decided to couple compound **12** with m^7GpBH_3 to avoid the methylation step at the stage of triphosphate. Coupling reactions involving **12** and either m^7GMP or m^7Gp_{BH3} conducted in DMF were unsuccessful and products were not detected even if the reaction time was prolonged to over 24 h. In contrast to reactions with AMP or GMP, neither $ZnCl_2$ nor $MgCl_2$ were able to catalyse the coupling. Since **12** was better soluble in DMSO, we decided to carry out the coupling in that solvent using $ZnCl_2$ as a catalyst. The coupling between m^7GMP and **12** was carried out over 60 h and HPLC measurements indicated that the reaction yield was above 60%. However, after purification by IC the yield was diminished to 12%, suggesting that **3** has low stability in aqueous solution. When compound **3** was separated to diastereomers by RP-HPLC, a significant fraction of **3** was decomposed during the procedure to a mixture of nucleoside diphosphates. Further studies by NMR and MS suggested the loss of the terminal phosphate group; however, other mechanisms of degradation could not be excluded. The stability of the synthesised nucleotides **1**–**3** was tested in aqueous buffers of various pH (2.2, 4.0, 7.0, 8.5).[36] In this study, no signs of degradation in the aforementioned conditions were found for compounds **1** and **2**, except when the buffer

was highly acidic (glycine buffer, pH = 2.2). However, for m^7GppNHp and both diastereomers of **3**, decomposition in either acidic or basic conditions was observed. For example, we found that both in acidic (glycine buffer, pH = 2.2) and alkaline solution (borane buffer, pH = 8.5) both diastereomers of **3** are prone to decomposition. The total loss of terminal phosphate in glycine buffer occurred within less than 16 h. Also, in neutral phosphate buffer (pH = 7.0) a significant (>50%) fraction of both diastereomers of **3** was decomposed after several hours and similar results were observed in pH 8.5. m^7GppNHp was also unstable in those conditions. Due to the mentioned instability of NH-containing mononucleotide cap analogues in aqueous solution, we decided not to conduct any biochemical or biophysical assays with these compounds.

2.1.2. Stereochemistry

The O to S and O to BH_3 substitutions in mono- and dinucleotide cap analogues may result in P-diastereoisomerism (Fig. 2A). Nucleoside triphosphates **1**–**5** possess a single stereogenic P-centre and consequently were obtained as an approximately equimolar mixture of two, S_P and R_P, diastereomers. Cap analogues **6**–**9** possess two stereogenic P-centres and were obtained as an approximately 1:2:1 mixture of three diastereomers, S_P–S_P, S_P–R_P, R_P–R_P, respectively. For all compounds, the formation of the diastereomers was observed when the syntheses were monitored by RP-HPLC and the analogues were termed D1, D2, D3 according to their elution order. Previous studies revealed that the biological properties of cap analogues may differ significantly depending on the P-centre configuration.[10,11] Therefore, we attempted the separation of compounds **1**–**9** into diastereomerically pure forms before biological studies. Nucleoside triphosphates **1**, **2** and **4**, **5**, as well as dinucleotide tetraphosphates **7**–**9**, were successfully separated, but compound **6** was separated only partially to pure D3 isomer and a mixture of diastereomers D1 and D2. Although the most straightforward way to assign the absolute configuration of a phosphorus centre is by crystallography, nucleotides are usually difficult to obtain in a crystalline form and thus indirect methods based on spectral analysis, susceptibility to enzymatic hydrolysis or stereochemical correlation with known compounds are often used. It has been previously shown that the absolute configuration of α-P-modified nucleotides and related compounds can be assigned by ^{1}H NMR spectroscopy, enzymatic studies, chemical correlation or their combinations.[37–40] A correlation between the RP-HPLC elution order, H8 proton shift and the absolute configuration has been reported for various purine NTPαS, NDPαS, NTPαBH_3, NDPαBH_3[41] and related compounds.[42] For example, the faster eluting diastereomers of ATPαS and ATPαBH_3 have less shielded H8 protons compared to corresponding slower eluting isomers, and the same stereochemistry (i.e., S_P and R_P configurations at α-phosphorus, respectively, see Fig. 2). We have recently found that in the case of boranophosphate 7-methylguanosine nucleotides, analysis of the H3′ chemical shifts is more useful for configuration assignment than H8 shifts.[10]

Here, to assign absolute P-configurations of D1, D2 and D3 stereoisomers, we used a combination of ^{1}H NMR data analysis and chemical correlation with compounds of previously determined stereochemistry. For different diastereoisomeric nucleotides, the largest differences in ^{1}H NMR spectra were observed for δ_H of H8 and H3′ protons (Fig. 2, Table 1). For phosphorothioate mono- and dinucleotides, the ^{1}H NMR data were in close agreement with previously reported findings and allowed unambiguous configuration assignment based on RP-HPLC and ^{1}H NMR (Table1, Fig. S1). For boranophosphate 7-methylguanosine mononucleotides (**1** and **2**), we correlated their configuration with corresponding guanosine nucleotides by single diastereomer N^7-methylation reactions (Figs. S4 and S5). We found that GTPαBH_3 D1 (R_P) has the same stereochemistry as m^7GTPαBH_3 D2, meaning that m^7GTPαBH_3 diastereomers are eluted from the RP-HPLC column in the opposite order to GTPαBH_3. A similar observation has previously been made for GDPαBH_3 D2.[10] Similarly, GTPαBH_3β,γ-CH_2 D1 has the same stereochemistry as m^7GTPαBH_3 D2 and, based on the ^{1}H NMR data ($\delta_{H3'}$) (Table 1),

Table 1
Representative ^{1}H NMR data for the discussed nucleotides

No.	Compound	Abs conf.	δ_{H8} (ppm)	$\Delta\delta_{H8}$[a] (ppm)	$\delta_{H3'}$ (ppm)	$\Delta\delta_{H3'}$[a] (ppm)
1a	$m^7Gp_{BH3}pp$ D1	S_P	9.22	−0.09	4.49	0.12
1b	$m^7Gp_{BH3}pp$ D2	R_P	9.13		4.61	
2a	$m^7Gp_{BH3}pCH_2p$ D1	S_P	9.21	−0.09	4.49	0.11
2b	$m^7Gp_{BH3}pCH_2p$ D2	R_P	9.12		4.58	
5a	m^7Gp_Spp D1[b]	S_P	9.36	0.05	4.63	0.06
5b	m^7Gp_Spp D2[b]	R_P	9.31		4.57	
4a	$m^7Gp_SpCH_2p$ D1	S_P	9.3	0.02	4.52	0.02
4b	$m^7Gp_SpCH_2p$ D2	R_P	9.28		4.5	
6a	$m^7Gp_Sppp_Sm^7G$ D1	S_P, S_P	9.33	0.04[c]	4.58	0.04[c]
6b	$m^7Gp_Sppp_Sm^7G$ D2	R_S, S_P	9.32, 9.29	0.03[c]	4.58; 4.55	0.03[c]
6c	$m^7Gp_Sppp\overset{7}{m}_SG$ D3	R_P, R_P	9.28		4.55	
7a	$m^7Gp_SpCH_2pp\overset{7}{m}_SG$ D1	S_P, S_P	9.33	0.04[c]	4.58	0.04
7b	$m^7Gp_SpCH_2pp_Sm^7G$ D2	R_S, S_P	9.34; 9.32		4.59; 4.54	
7c	$m^7Gp_SpCH_2pp_Sm^7G$ D3	R_P, R_P	9.29		4.54	
8a	$m^7Gp_{BH3}ppp_{BH3}m^7G$ D1	R_P, S_P	9.15; 9.10		4.59; 4.46	
8b	$m^7Gp_{BH3}ppp_{BH3}m^7G$ D2	R_P, R_P	9.08		4.61	
8c	$m^7Gp_{BH3}ppp_{BH3}m^7G$ D3	S_P, S_P	9.19		4.47	
9a	$m^7Gp_{BH3}pCH_2pp_{BH3}m^7G$ D1	R_P, S_P	9.17; 9.10		4.58; 4.46	
9b	$m^7Gp_{BH3}pCH_2pp_{BH3}m^7G$ D2	R_P, R_P	9.08		4.56	
9c	$m^7Gp_{BH3}pCH_2pp_{BH3}m^7G$ D3	S_P, S_P	9.15		4.46	
21a	$Gp_{BH3}pp$ D1[d]	R_P	8.19	0.02	4.62	0.08
21b	$Gp_{BH3}pp$ D2[d]	S_P	8.17		4.54	
22a	$Gp_{BH3}pCH_2p$ D1	R_P	8.20	0.02	4.60	0.08
22b	$Gp_{BH3}pCH_2p$ D2	S_P	8.18		4.52	

[a] Calculated as R_P–S_P difference for BH_3-analogs and S_P–R_P difference for S-analogs.
[b] Data from Ref. 8.
[c] For diastereomer D1 the difference was calculated using shifts from D3 and D1 spectra, and for the D2 diastereomer the difference was calculated from the D2 spectrum.
[d] Data from Ref. 10.

the absolute configuration of both nucleotides was assigned as R_P. In the case of dinucleotides, the R_P–S_P isomers could be easily identified based on the presence of two sets of signals corresponding to each nucleoside moiety (Fig. S3). In contrast, only one set of signals for S_P–S_P and R_P–R_P isomers is observed (Fig. 2). For S-analogues (**6** and **7**), R_P–S_P was the secondly eluted isomer (D2), whereas for BH_3-analogs (**8** and **9**) it was the first eluted isomer (D1).

Based on the ^{1}H NMR data of S-analogues **6**–**7**, the configuration of D1 isomers was assigned as S_P–S_P, whereas D3 diastereomers as R_P–R_P (Fig. 2**C**). On the other hand, diastereomers D2 of BH_3-analogues **8**–**9** have the configuration R_P–R_P and D3 diastereomers have the opposite configuration. These initial assignments were further confirmed by enzymatic susceptibility assay with a DcpS enzyme.

2.2. Biochemical properties

2.2.1. Binding affinities to eIF4E

Earlier studies on cap–eIF4E interactions indicated that the high affinity of a cap analogue to eIF4E is one of the requirements for efficient mRNA translation or translational inhibition. Therefore, measurement of association constants (K_{AS}) for eIF4E–cap complex formation is a useful preliminary test of new cap analogues in these applications.[15,43] The K_{AS} values were determined using fluorescence quenching titration (Table 2).

Mononucleotide cap analogues interact more strongly with eIF4E than their dinucleotide counterparts with the same structure of the polyphosphate bridge, which makes them promising translation inhibitors, especially as possible anticancer drugs.[44] One study examined interactions between eIF4E and m^{7}GTP phosphorothioate modified analogues[8] and the results indicated that compound **5**, m^{7}Gp$_S$pp, is a strong binder of eIF4E. Here, comparison between m^{7}GTP and its modified analogues showed that the introduction of a boranophosphate group can have either a stabilising or neutral effect on eIF4E binding. This effect is strongly correlated with stereochemistry at the stereogenic phosphorus, which is also in agreement with previous studies on boron-[10] and sulphur-containing[7,8] cap analogues. In each case, the stronger interacting isomer has the same spatial order of substituents. However, due to different CIP priority their absolute configurations are in opposition—R_P for boranophosphates and S_P for phosphorothioates. For both S- and BH_3-modified compounds the presence of an additional methylene group reduced the affinity to eIF4E. This result is reasonably consistent with previous studies, showing that methylene-modified cap analogues generally have lower K_{AS} then their unmodified parent compounds.[45] For analogues **1** and **2**, the D2 isomers have higher affinity to eIF4E than the D1 counterparts. This observation supports the claim that D1 isomers of boranophosphate m^{7}GTP analogues have the same stereochemistry at α phosphorus as the D2 diastereomers of phosphorothioate m^{7}GTP derivatives. The determined K_{AS} values also indicate that compound **4a** (D1) has an S_P configuration at α phosphorus, which is in agreement with previous reports, in which D1 diastereomers of **5** or m^{7}Gp$_S$ppG showed elevated binding to eIF4E.

The similarity in K_{AS} values for compounds **1a** and **1b** was unexpected, since in most cases the affinity of the more competitive binder is roughly two or threefold higher than the second diastereomer. [10] However, a better structural insight is necessary for understanding differences between m^{7}GTP derivatives **1**–**5** in their binding to eIF4E.

K_{AS} values for dinucleotide cap analogues **6**–**9** are shown in Table 3, together with the same data for several reference compounds (including m^{7}GpppG, m$_2^{7,2'-O}$GpppG, m^{7}Gpppm^{7}G, m^{7}Gppppm^{7}G).[46] Introduction of two thio- or boranophosphate groups to compounds **6**–**9** does not reduce their affinity to eIF4E (Table 3), compared to m^{7}Gppppm^{7}G, and generally has a stabilising effect on the complex. The stabilising effect is similar to the effect observed in the previously described m^{7}GpppG derivatives with a single modification in the γ position within the bridge.[7,10] Comparison of K_{AS} values in the following pairs, **6**–**7** and **8**–**9**, indicated a destabilising effect of the methylene group between β and γ position. For instance, in the case of D1 diastereomers of boranophosphate analogues, K_{AS} is diminished almost twofold after the introduction of a CH_2 group (77.7 ± 0.6 vs 41.3 ± 1.4 μM^{-1}). For other compounds, the difference was less noticeable. These findings are consistent with previous studies reporting that methylene bridge in the phosphate bridge decreases the affinity to eIF4E.[45,47] For all compounds except **9**, the stereochemistry of the phosphate chain have notable influence on binding to eIF4E. Similar to previous studies, we found that certain configurations at stereogenic phosphorus (assigned as *S* in the case of phosphorothioates and *R* in the case of boranophosphate) provide elevated affinity to that protein.[7,10,42]

2.2.2. Susceptibility to enzymatic degradation

A high stability of nucleotides is often beneficial for in vitro and, especially, in vivo applications, both for scientific and medicinal purposes. It has been suggested that the activity of DcpS is the main cause of cap analogues degradation after their introduction to a cell extract.[7,11,18,45] The susceptibility of novel m^{7}GTP and m^{7}Gppppm^{7}G analogues to human DcpS was tested in vitro using an HPLC-based assay. The assay conditions were adjusted to provide total degradation of the reference analogue, m^{7}GpppG, within 1 h.[8,10] It was found that, in contrast to m^{7}GpppG and m^{7}GTP, none of the modified m^{7}GTP or m^{7}Gppppm^{7}G analogues were susceptible to DcpS under those conditions. These observations are consistent with previous findings for m^{7}GpppG derivatives, which showed that substitution of the non-bridging oxygen atom

Table 2
Biochemical properties of mononucleotide cap analogues

No.	Cap analogue	K_{AS}(eIF4E) (μ M^{-1})	ΔG°(eIF4E) (kcal mol^{-1})	Susceptibility to DcpS	IC$_{50}$ A (μM)	IC$_{50}$ B (μM)
–	m^{7}GpppG	9.4 ± 0.4[a]	−9.35 ± 0.02	Yes	8.30 ± 1.00	25.00 ± 6.90
–	m^{7}GTP	124.0 ± 0.5[a]	−10.85 ± 0.03	Yes	2.36 ± 0.25	5.72 ± 2.33
–	m^{7}GppCH$_2$p	19.5 ± 1.4	−9.95 ± 0.07	n.d[b]	n.d	n.d
1a	m^{7}Gp$_{BH3}$pp (D1)	136.0 ± 5.0	−11.10 ± 0.02	No	4.12 ± 0.89	3.26 ± 0.76
1b	m^{7}Gp$_{BH3}$pp (D2)	148.5 ± 3.5	−11.42 ± 0.01	Slowly hydrolysed	1.19 ± 0.08	1.28 ± 0.30
2a	m^{7}Gp$_{BH3}$pCH$_2$p(D1)	18.5 ± 0.2	−9.74 ± 0.01	No	4.47 ± 0.24	3.22 ± 0.87
2b	m^{7}Gp$_{BH3}$pCH$_2$p(D2)	36.1 ± 0.5	−10.13 ± 0.01	No	2.55 ± 0.56	3.26 ± 0.92
4a	m^{7}Gp$_S$pCH$_2$p (D1)	85.9 ± 3.2	−10.82 ± 0.02	No	1.49 ± 0.12	1.24 ± 0.10
4b	m^{7}Gp$_S$pCH$_2$p (D2)	41.8 ± 1.8	−10.40 ± 0.03	No	3.68 ± 0.33	2.61 ± 0.21
5a	m^{7}Gp$_S$pp (D1)	371.0 ± 19.0[a]	−11.69 ± 0.03 [a]	No[a]	0.56 ± 0.08[a]	0.56 ± 0.02[a]
5b	m^{7}Gp$_S$pp (D2)	190.0 ± 7.0[a]	−11.39 ± 0.01 [a]	No[a]	1.44 ± 0.20[a]	1.30 ± 0.30[a]

[a] Data from Ref. 8.
[b] Not determined.

Table 3
Biochemical properties of studied dinucleotide cap analogues compared to similar compounds prepared in earlier studies

No.	Cap analogue	*KAS* (μM^{-1})	Susceptibility to DcpS	$\Delta G°$(eIF4E) (kJ mol^{-1})	Translational efficiency[g]
—	$m_2^{7,3'}$-OGpppG[a]	10.8 ± 0.3	Yes	−9.60 ± 0.03	1.50 ± 0.13
—	m^7GpppG[a]	9.4 ± 0.4	Yes	−9.51 ± 0.02	1.00
—	m^7Gpppm^{7}G[b]	5.0 ± 0.2	Yes	−9.14 ± 0.03	2.66 ± 0.64[h]
—	m^7Gppppm^{7}G[b]	49.4 ± 1.5	Yes	−10.49 ± 0.03	3.14 ± 0.24 [h]
—	m^7G_{pBH3}ppG D1[c]	9.6 ± 0.3	No	−9.52 ± 0.02	n.d
—	m7G_{pBH3}ppG D2[c]	17.3 ± 0.2	Yes	−9.87 ± 0.01	n.d
—	$m^7G_{pBH3}pm^7G$[d]	11.1 ± 0.2	No	−9.61 ± 0.01	1.50 ± 0.10
—	$m_2^{7,2'}$-OGpSpppG D1[e]	258.0 ± 6.0	No	−11.28 ± 0.01	2.15 ± 0.12
—	$m_2^{7,2'}$-OGpSpppG D2[e]	129.0 ± 4.0	No	−10.87 ± 0.02	2.59 ± 0.53
—	m^7GppCH2ppG[a]	38.4 ± 3.9	No	−10.35 ± 0.01	n.d
6a	m^7GpSpppSm^7G D1+D2	112.5 ± 6.1	n.d[f]	−10.80 ± 0.02	n.d
6b	m^7GpSpppmS^{7}G D3	61.3 ± 2.2	n.d	−10.45 ± 0.02	n.d
7a	$m^7GpSpCH_2ppmS^7$G D1	80.8 ± 2.5	n.d	−10.61 ± 0.02	1.86 ± 0.23
7b	$m^7GpSpCH_2ppSm^7$G D2	65.2 ± 4.8	No	−10.49 ± 0.05	1.56 ± 0.13
7c	$m^7GpSpCH_2ppSm^7$G D3	39.5 ± 0.8	n.d	−10.18 ± 0.02	1.12 ± 0.02
8a	$m^7G_{pBH3}ppp_{BH3}m^7$G D1	77.7 ± 0.6	No	−10.58 ± 0.1	n.d
8b	$m^7G_{pBH3}ppp_{BH3}m^7$G D2	69.2 ± 1.9	Yes	−10.52 ± 0.02	n.d
8c	$m^7G_{pBH3}ppp_{BH3}m^7$G D3	51.0 ± 0.6	No	−10.35 ± 0.02	n.d
9a	$m^7G_{pBH3}pCH_2pp_{BH3}m_7$G D1	41.3 ± 1.4	No	−10.21 ± 0.02	1.18 ± 0.12
9b	$m^7G_{pBH3}pCH_2pp_{BH3}m^7$G D2	41.9 ± 1.5	No	−10.23 ± 0.02	0.99 ± 0.17
9c	$m^7G_{pBH3}pCH_2pp_{BH3}m^7$G D3	36.4 ± 0.1	No	−10.13 ± 0.02	0.85 ± 0.14

[a] Data from Ref. 47.
[b] Data from Ref. 50.
[c] Data from Ref. 10.
[d] Data from Ref. 51.
[e] Data from Ref. 42.
[f] Not determined.
[g] Normalised to m^7GpppG.
[h] Data from Ref. 52.

adjacent to the γ (adjacent to m^7G) phosphorus atom may result in reduced susceptibility to DcpS. [8] Hence, replacement of an oxygen in the vicinity of m^7G by either an S or BH_3 group could provide resistance to DcpS-mediated cleavage. It has also been previously shown that in the presence of high concentrations of human DcpS, stereoselectivity towards BH_3-analogues of R_P configuration can be observed, that is, the R_P BH_3-analogues become slowly hydrolysed, whereas the S_P analogues remain resistant. Here, we used this observation to confirm the absolute configurations of BH_3-analogues determined by ^{1}H NMR. Analogues **1**, **2**, **8** and **9** were incubated with high DcpS concentrations, as described previously (Fig. S3).[10] Under these conditions, compound **1b** was slowly hydrolysed by DcpS to 7-methylguanosine monoboranophosphate (19% ± 2% of hydrolysis after 3 h). Compound **8b** was more readily hydrolysed to a mixture of 7-methylguanosine monoboranophosphate and compound **1b** (81% ± 2% of hydrolysis after 3 h). All other compounds remained completely resistant. This indicated that compound **1b** is of R_P configuration and **8b** is of R_P,R_P configuration. The resistance of other compounds of R_P configuration (**2b**, **9b**) can be explained based on the previous findings that the presence of the β,γ-methylene bridge additionally decreases the susceptibility to DcpS.[47]

Apart from testing resistance to DcpS, we aimed to examine the susceptibility of m^7GTP analogues to unspecific degradation in FBS medium (Fetal Bovine Serum). In that assay, an appropriate cap analogue was added to FBS medium diluted with PBS buffer and small amounts of the mixture were collected at several time points. Upon thermal deactivation, the samples were analysed via RP-HPLC to assess the stability and, in the case of degradation, determine the compound half-life in those conditions.[48,49] In the study, two different FBS concentrations were used (5% and 10%). We tested the stability of compounds **4** and **5**. m^7GTP was used as a positive control, since it is susceptible to degradation by pyrophosphatases. It was found that both diastereomers of compound **5** are stable at both FBS concentrations. In contrast, diastereomers of cap analogue **4** differ in their resistance towards degradation. Diastereomer D1 (**4a**) was, similar to **5**, resistant to hydrolysis. The D2 diastereomer (**4b**) was degraded to nucleoside monophosphate, albeit more slowly than m^7GTP (Fig. 3). We speculate that, owing to different stereochemistry at α phosphorus, diastereomer D1 can be recognised by unspecific intracellular nucleases and cleavage, whereas the D2 diastereomer might be unable to form a stable substrate–enzyme complex.

2.2.3. Inhibition of translation in vitro by mononucleotide analogues

m^7GTP analogues **1**–**2** and **4** were tested as inhibitors of cap-dependent translation using an in vitro assay in rabbit reticulocyte lysate (RRL).[8,9] The expression of exogenous ARCA-capped mRNA containing β-globin 5′UTR and firefly luciferase ORF was measured by luminometry. If a translation inhibitor is present in the system, the translation level of that reporter mRNA is lowered. By performing a series of experiments at different inhibitor concentrations one can determine the IC_{50} value. This experiment was conducted in two different conditions. In the first series (Experiment I, Fig. 4**A**), luciferase mRNA and the tested inhibitors were added to the reaction mixture simultaneously. In the second series (Experiment II), luciferase mRNA was added after a 60 min addition of a cap analogue. The rationale for employing two different conditions was to investigate whether prolonged incubation of an inhibitor could diminish its inhibitory potential. Some earlier reports showed that cap analogues which are not resistant to DcpS have considerably higher IC_{50} values if they are preincubated in the lysate prior to the addition of the reporter mRNA.[8,9]

If the conditions from Experiment I were applied, the most potent inhibitors were **1b** and **4a**, with IC_{50} values 1.19 ± 0.08 μM and 1.49 ± 0.12 μM, respectively (Table 2). These analogues also showed the highest affinity for eIF4E indicating a correlation between K_{AS} for eIF4E and the inhibitory potential in RRL.

Under conditions from experiment II (Fig. 4**B**), IC_{50} values of cap analogues **1**–**2** and **4**–**5**, in contrast to m^7GpppG or m^7GTP, were not diminished by incubation in RRL. Consequently, all these

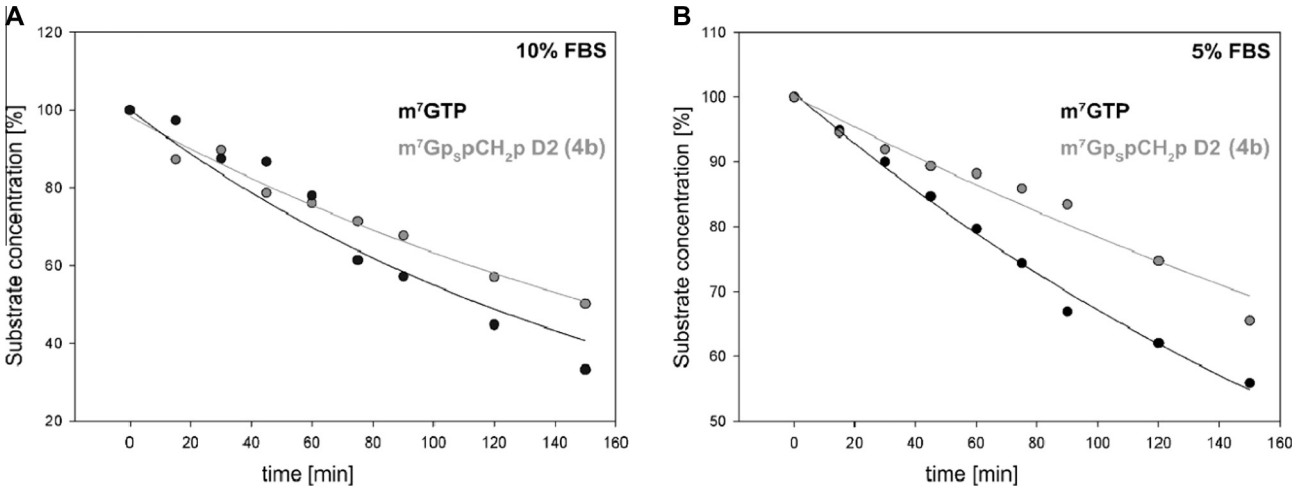


Figure 3. Representative results for FBS stability assay. (A) Decay of nucleoside triphosphates in 10% FBS. The estimated half-lives are 108 min for m^7GTP and 156 min for $m^7Gp_SpCH_2p$ D2 (**4b**). (B) Decay of nucleoside triphosphates in 5% FBS. The estimated half-lives are 190 min for m^7GTP and 295 min for **4b**.

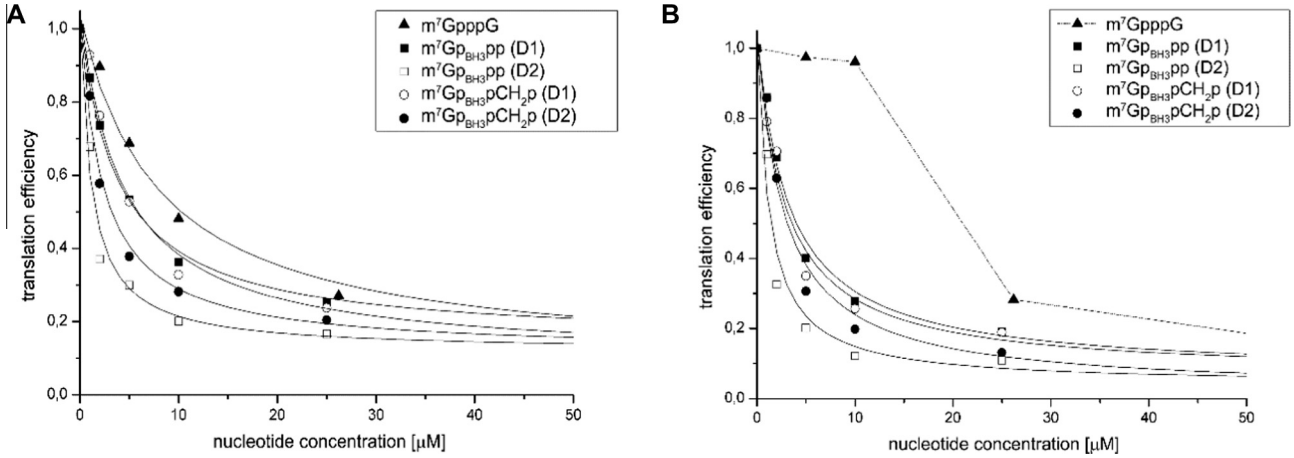


Figure 4. Inhibition of cap-dependent translation in rabbit reticulocyte lysate by mononucleotide cap analogues for borano-modified compounds **1** and **2**. (A) Results of experiments without 1 h preincubation. (B) Results of experiments with 1 h preincubation prior to mRNA addition. In these conditions, the inhibitory potential of m^7GpppG is significantly reduced, presumably due to DcpS-mediated hydrolysis to m^7GDP and GMP, which are poor inhibitors of translation.

analogues were much better inhibitors than m^7GpppG or m^7GTP under these conditions (Table 2). The comparison of IC_{50} values for compounds **1**, **2** and **4**, **5** shows that phosphorothioate m^7GTP derivatives display higher inhibitory potential than borano-modified compounds. For instance, IC_{50} values for stronger inhibiting diastereomers of m^7Gp_Spp (**5a**) and $m^7Gp_{BH3}pp$ (**1b**) are 0.56 ± 0.02 μM and 1.28 ± 0.30 μM, respectively. Furthermore we observed that the introduction of the β/γ methylene bridging group slightly reduced the inhibitory properties of m^7GTP analogues, which is correlated with their lowered affinity to eIF4E (compared to their counterparts with modifications only in the α position). Most importantly, all α-modified m^7GTP derivatives do not lose their inhibitory potential upon incubation in the lysate, which indirectly indicates their higher stability in intracellular environments compared to m^7GTP.

2.2.4. Dinucleotides as reagents for the preparation of capped mRNAs

Compounds **6**–**9** may act as functional mimics of ARCA analogues, since they are always correctly incorporated into RNA transcripts. Hence, selected of the compounds were evaluated as reagents for the preparation of capped mRNAs. We chose six compounds, **7a**–**c** and **9a**–**c**, and tested whether transcripts capped with these cap analogues display superior translational properties compared to transcripts containing unmodified ARCA analogues. The translation efficiency of luciferase-encoding RNA transcripts was measured in an RRL system (Table 3). All these transcripts are recognised by the translational machinery in vitro and their translation level is similar to either m^7GpppG or $m_2^{7,2'-O}GpppG$. Compounds with the same spatial configuration of the non-bridging modification (R_P for boranophosphates and S_P for phosphorothioates) bind tighter to eIF4E; hence, these RNAs capped with these diastereomers provide better translational properties. This is clearly visible in the case of compound **7**, since mRNAs capped with its S_P, S_P diastereomer (**7a**, D1) have considerably higher translational efficiency than those capped with R_P, R_P diastereomers (**7c**, D3). The relative translation efficiencies are (compared to m^7GpppG) 1.86 ± 0.23 and 1.12 ± 0.12, respectively (~1.6-fold reduction). On the other hand, the translational yields of compound **9** diastereomers are less divergent and oscillate near the yield of m^7GpppG. All investigated compounds are mimics of ARCA analogues with elevated affinity to translational machinery. Hence, one may expect higher translational yield than for $m_2^{7,2'-O}GpppG$ or $m^7Gppppm^7G$, which are 1.50 ± 0.13 and 3.14 ± 0.24, respectively. Our results can be partially explained by the less efficient capping of these compounds. Further studies on

the properties of RNAs capped with these compounds and their susceptibility to enzymatic degradation by Dcp1/2 complex are in progress.

3. Conclusions

Three m^7GTP and four m^7Gppppm^{7}G analogues in different stereoisomeric forms were obtained in this study. The compounds contain either phosphorothioate or boranophosphate modifications in the phosphate chain and some of them also possess an additional methylene group in a bridging position. Each of these compounds exists as a mixture of two or three diastereomers, which were separated by HPLC. A synthetic method relying upon the usage of activated pyrophosphate was adapted for the synthesis of all the mentioned nucleotides. The absolute configurations of stereogenic phosphorus atoms in the compounds were determined by a combination of chemical, spectroscopic and enzymatic methods. Introduction of a modified phosphate chain improved the biochemical properties of the cap analogues described here. All of them were tight binders of eIF4E translation initiation factor in vitro and showed high resistance to DcpS. Phosphorothioate mononucleotides also displayed elevated stability in FBS solution. The compounds were efficient and stable inhibitors of cap-dependent translation in vitro. Combination of these properties makes them promising tools in biochemical and medical studies on translation, mRNA degradation and other cellular processes. The dinucleotide cap analogues were functional mimics of previously synthesised ARCA analogues, and thus were tested as reagents for the preparation of capped RNAs to reveal superior translational properties.

4. Materials and methods

4.1. General procedures

Solvents and reagents were purchased from Sigma–Aldrich and used without any further treatment, unless otherwise stated. Intermediate products were separated by ion-exchange chromatography on a DEAE-Sephadex A-25 (HCO_3^- form) column using a linear gradient of triethylammonium bicarbonate (TEAB) in deionised water and after evaporation under reduced pressure with the addition of ethanol, isolated as triethylammonium (TEA) salts. Final products were purified using semi-preparative RP-HPLC and after a three-times repeated freeze-drying procedure they were isolated as ammonium salts. Yields were calculated either based on sample weight or optical units of the product. Optical measurements for m^7G mononucleotides were performed in 0.1 M phosphate buffer, pH = 6.0 at 260 nm, assuming $\varepsilon_{260} = 11400\ cm^{-1}\ M^{-1}$ for calculations. For guanine nucleotides, measurements were conducted in 0.1 M phosphate buffer, pH = 7 at 260 nm, assuming $\varepsilon_{260} = 12080\ cm^{-1}\ M^{-1}$. For dinucleotides, measurements were conducted in the same buffer, assuming $\varepsilon_{260} = 20000\ cm^{-1}\ M^{-1}$. Analytical HPLC was performed on an Agilent Tech. Series 1200 using a Supelcosil LC-18-T RP column (4.6 × 250 mm, flow rate 1.3 mL/min) with a linear gradient 0–50% of methanol in 0.05 M ammonium acetate buffer (pH = 5.9) for 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 an Agilent Tech. Series 1200 apparatus using a Discovery RP Amide C-16 HPLC column (25 cm × 21.2 mm, flow rate 5.0 mL/min) with a linear gradient 0%-30% 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 were confirmed by chromatography by RP-HPLC, mass spectrometry using negative electrospray ionisation (MS ESI$^-$) and 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 reference and ^{31}P NMR chemical shifts were reported to 20% phosphorus acid in D_2O as an external reference. Mass spectra were recorded on a Micromass QToF 1 MS spectrometer.

4.2. Chemical synthesis

Guanosine 5′-O-boranophosphate (**18**), 7-methylguanosine boranophosphate (**19**) and guanosine 5′-*O*-(1-P-borano)triphosphate triethylammonium salt, $Gp_{BH3}pp$ (**21**) were synthesised as described previously.[10]

4.2.1. 7-methylguanosine 5′-*O*-thiophosphate, triethylammonium salt (20)

To an ice-bath of a cooled and vigorously stirred suspension of 7-methylguanosine (830 mg, 2.79 mmol) in 12 cm^3 of trimethyl phosphate were added freshly distilled $PSCl_3$ (0.6 cm^3, 5.9 mmol) and 2,6-lutidine (0.67 cm^3, 8.69 mmol). The mixture was stirred at 0 °C until HPLC analysis revealed the complete disappearance of the substrate (4.5 h). Then, the reaction mixture was poured into a $BaCl_2$ (10.5 mg) solution in 105 cm^3 of cold deionised water. The mixture was titrated using triethylamine, until the pH reached 7.0 and 350 cm^3 of 96% ethanol was added to the solution and the mixture was cooled overnight to 4 °C. The resulting precipitate was centrifuged and the supernatant was removed. The pellet was washed once with cold 96% ethanol and the product was extracted three times from the pellet with cold deionised water (16 cm^3). The fractions were merged into one and the product was separated by ion-exchange chromatography on Sephadex resin using a linear TEAB gradient 0–0.6 M. The amount of 574 mg (42%, 1.16 mmol) of the product was isolated as a yellowish powder.

4.2.2. General procedure for P^1,P^2-di(1-imidazolyl) derivatives preparation

Pyrophosphate, or its analogue (triethylammonium salt, 1 equiv), was mixed with imidazole (20 equiv), 2′2′-dithiodipyridine (5 equiv), DMF (1 cm^3 per 1 mmol of bisphosphate) and triethylamine (2 equiv). The suspension was stirred for 30 min followed by the addition of triphenylphosphine (5 equiv) and left under stirring for 18 h. The product was precipitated as a sodium salt by pouring the reaction mixture into $NaClO_4$ (7 equiv) solution in ice-cold acetone (250 mM). The product was left for 4 h at 4 °C and centrifuged, the supernatant was discarded. The pellet was washed three times with cold acetone by resuspension/centrifugation.

4.2.3. Disodium P^1,P^2-di(1-imidazolyl)pyrophosphate (10)

Starting from pyrophosphate (triethylammonium salt, 1084 mg, 2.84 mmol) and after drying in vacuo over P_2O_5, 887 mg (97%, 2.75 mmol) of the product was isolated as a white powder.

4.2.4. Disodium P^1,P^2-di(1-imidazolyl)methylenebisphosphonate (11)

Starting from methylenediphosphonic acid (triethylammonium salt, 1075 mg, 2.84 mmol) and after drying in vacuo over P_2O_5, 572 mg (63%, 1.79 mmol) of the product was isolated as a white powder.

4.2.5. Disodium P^1,P^2-di(1-imidazolyl)imidodiphosphate (12)

Starting from imidodiphosphate (triethylammonium salt, 200 mg, 0.53 mmol) and after drying in vacuo over P_2O_5, 144 mg

(sodium salt, 84%, 0.33 mmol) of the product was isolated as a white powder; ^{31}P NMR (160 MHz, D_2O) δ 0.50 (s, 1P).

4.2.6. Disodium P^1,P^2-di(1-imidazolyl)dichloromethylenebisphosphonate (13)

Starting from dichloromethylenediphosphonic acid (triethylammonium salt, 500 mg, 1.12 mmol) and after drying in vacuo over P_2O_5, 311 mg (71%, 0.80 mmol) of the product was isolated as a white powder; ^{31}P NMR (160 MHz, D_2O) δ −11.28 (s, 1P).

4.2.7. General procedure for synthesis of nucleoside triphosphate 1–5, 20–22

4 equiv of an appropriate P^1,P^2-diimidazolyl derivative and 20 equiv of anhydrous $MgCl_2$ (or $ZnCl_2$ in the case of thiophosphates) were added to anhydrous DMF and vigorously stirred until both substrates had been dissolved. In the second step, 1 equiv of nucleoside monophosphate was added to the mixture and the reaction was performed until HPLC measurements indicated the formation of the product. Thereafter, the reaction was quenched via the addition of either MQ water or a solution of EDTA and $NaHCO_3$. Products were purified by ion-exchange chromatography on Sephadex resin using a linear gradient of TEAB 0–1.2 M and subsequently all diastereoisomers were separated via semi-preparative RP-HPLC.

4.2.8. Guanosine 5′-*O*-triphosphate, GTP (20)

Starting from 420 mg GMP (triethylammonium salt, 0.9 mmol) and 1180 mg of compound **10** (sodium salt, 3.6 mmol) and 700 mg of anhydrous $MgCl_2$, 7200 OD_{260} of the product was isolated as a white powder.

4.2.9. Guanosine 5′-*O*-(1-P-borano-2,3-methylene)triphosphonate, triethylammonium salt, $Gp_{BH3}pCH_2p$ (22)

P^1,P^2-di(1-imidazolyl)methylenebisphosphonate (572 mg, 1.79 mmol, sodium salt) and anhydrous $MgCl_2$ (850 mg, 8.95 mmol) were added to anhydrous DMF (18 cm^3) and the mixture was vigorously stirred until both substrates dissolved. Then, guanosine 5′-boranophosphate (**18**, 207 mg, 0.45 mmol, triethylammonium salt) was added to the mixture and the reaction was performed for 6 h. Then, the reaction was terminated by the addition of deionised water (200 cm^3) and separated by IC using a linear gradient of TEAB 0–1.4 M. The product was separated by IC using a linear gradient of TEAB 0–1.4 M. 145 mg (39%, 0.175 mmol) of the product was isolated as a yellowish powder. Diastereoisomers D1 and D2 (125 mOD) were then separated on semi-preparative RP-HPLC and, after freeze-drying, isolated as ammonium salts (D1: 45 mOD, 0.0044 mmol, 2.1 mg; D2: 35 mOD, 0.0035 mmol, 1.6 mg); MS ESI: [M]$^-$ found 518.04, calcd 518.04119; ^{1}H NMR: (400 MHz, D_2O) (D1) δ 8.19 (s, 1H), 5.95 (d, J = 6.0 Hz, 1H), 4.78 (under the HDO peak, 1H), 4.59 (dd, J = 4.8 Hz, J = 4.3 Hz, 1H), 4.36 (m, 1H), 4.30–4.14 (m, 2H), 2.34 (t, J = 20.3 Hz, 2H) (D2) δ 8.17 (s, 1H), 5.95 (d, J = 5.5 Hz, 1H), 4.80 (under the HDO peak, 1H), 4.52 (br s, 1H), 4.36 (m, 1H), 4.31–4.13 (m, 2H), 2.33 (t, J = 20.6 Hz, 2H); 8.19 (s, J = 8.0, J = 2.0 Hz, 1H), 5.95 (d, J = 6.0 Hz, 1H), 4.78 (under the HDO peak, 1H), 4.59 (dd, J = 4.8 Hz, J = 4.3 Hz, 1H), 4.36 (m, 1H), 4.30–4.14 (m, 2H), 2.34 (t, J = 20.3 Hz, 2H); ^{31}P NMR (160 MHz, D_2O) (D1) δ 82.49 (br s, 1P), 15.15 (dt, J = 19.8 Hz, J = 8.8 Hz, 1P), 8.57 (ddt, J = 21.5, J = 19.8 Hz, J = 8.8 Hz, 1P) (D2) δ 83.44 (br s, 1P), 14.72 (s, 1P), 7.96 (s, 1P).

4.2.10. 7-Methylguanosine 5′-*O*-(1-P-thio)triphosphate, ammonium salt, m^7Gp_Spp (5)

P^1,P^2-di(1-imidazolyl)pyrophosphate (300 mg, 0.94 mmol, sodium salt), anhydrous $ZnCl_2$ (500 mg, 3.67 mmol) and 7-methylguanosine 5′-thiophosphate (**23**, 90 mg, 0.188 mmol, triethylammonium salt) were added to anhydrous DMF (10 cm^3); the reaction was performed for 24 h. Then, the reaction was terminated via the addition of MQ water (100 cm^3) and the pH was adjusted to 2.0 using HCl. After 24 h, the pH was adjusted to 7.0 and the product was separated by IC using a linear gradient of TEAB 0–1.2 M. 51.2 mg (32%, 0.060 mmol) of the product was isolated as a yellowish powder.

4.2.11. 7-Methylguanosine 5′-*O*-(1-P-thio-2,3-methylene)triphosphonate, ammonium salt, $m^7Gp_SpCH_2p$ (4)

P^1,P^2-di(1-imidazolyl)methylenebisphosphonate (208 mg, 0.65 mmol, sodium salt), anhydrous $ZnCl_2$ (360 mg, 2.64 mmol) and 7-methylguanosine 5′-thiophosphate (**23**, 50 mg, 0.11 mmol, triethylammonium salt were added to anhydrous DMF (8 cm^3); the reaction was performed for 24 h. Then, the reaction was terminated via the addition of deionised water (80 cm^3) and the product was separated by ion-exchange chromatography using a linear gradient of TEAB 0–1.2 M. 61.6 mg (32%, 0.06 mmol) of the product was isolated as a yellowish powder. Diastereoisomers D1 and D2 were then separated on semi-preparative RP-HPLC and, after repeatedly freeze-drying, isolated as ammonium salts (D1: 6.5 mg, 0.01 mmol; D2: 9.5 mg, 0.015 mmol). MS ESI: [M]$^-$ found 549.99563, calcd 549.99692; ^{1}H NMR: (400 MHz, D_2O) (D1) δ 9.30 (s, 1H), 6.04 (d, J = 3.2 Hz, 1H), 4.68 (dd, J = 4.7 Hz, J = 3.2 Hz, 1H), 4.58 (dd, J = 5.3 Hz, J = 4.7 Hz 1H), 4.40 (m, 1H), 4.37–4.24 (m, 2H), 4.13 (s, 3H), 2.39 (t, J = 20.4 Hz, 2H) (D2) δ 9.28 (s, 1H), 6.08 (d, J = 3.2 Hz, 1H), 4.67 (dd, J = 4.6 Hz, J = 3.3 Hz, 1H), 4.50 (dd, J = 4.6, J = 5.5 Hz 1H), 4.40 (m, 1H), 4.36 (m, 1H), 4.29–4.27 (m, 1H) 4.16 (s, 3H), 2.38 (t, J = 20.2 Hz, 2H); ^{31}P NMR (160 MHz, D_2O) (D1) δ 43.63 (d, J = 32.3 Hz, 1P), 15.93 (br s, 1P), 9.17 (br s, 1P) (D2) δ 43.23 (d, J = 32.2 Hz, 1P), 15.43 (dt. J = 19.5 Hz, J = 8.3 Hz, 1P), 9.48 (ddt, J = 32.2, J = 19.5 Hz, J = 8.8 Hz, 1P).

4.2.12. 7-Methylguanosine 5′-*O*-(1-P-borano)triphosphate, ammonium salt, $m^7Gp_{BH3}pp$ (1)

$Gp_{BH3}pp$ (**21**, 104 mg, 0.127 mmol, triethylammonium salt) was dissolved in anhydrous DMSO (1.5 cm^3) and methyl iodide (0.075 cm^3, 0.1 mmol) was added. After 2 h, the reaction was quenched by the addition of MQ water (25 cm^3), the pH was adjusted to 6.0 using solid $NaHCO_3$. The reaction mixture was extracted three times with 4 cm^3 of diethyl ether. The product was separated by ion-exchange chromatography on Sephadex resin using a linear TEAB gradient of 0–1.4 M. 42.6 mg of the product (40%, 0.051 mmol, triethylammonium salt) was isolated as a white powder. Diastereoisomers D1 and D2 (34 mg) were then separated on semi-preparative RP-HPLC and, after repeatedly freeze-drying, isolated as ammonium salts (D1: 12 mg, 0.02 mmol; D2: 14 mg, 0.023 mmol). MS ESI: [M]$^-$ found 534.03556, calcd 534.03690; ^{1}H NMR: (400 MHz, D_2O) (D1) δ 9.22 (s, 1H), 6.08 (d, J = 3.6 Hz, 1H), 4.70 (dd, J = 4.3 Hz, J = 3.6 Hz, 1H), 4.49 (m, 1H), 4.45 (br s, 1H), 4.35–4.25 (m, 2H), 4.16 (s, 3H) (D2) δ 9.13 (s, 1H), 6.08 (d, J = 3.2 Hz, 1H), 4.70 (dd, J = 4.7 Hz, J = 3.6 Hz, 1H), 4.61 (dd, J = 5.3 Hz, J = 4.7 Hz, 1H), 4.42 (br s, 1H), 4.37–4.22 (m, 2H), 4.16 (s, 3H); ^{31}P NMR (160 MHz, D_2O) (D1) δ 83.65 (br s, 1P), −10.55 (d. J = 19.8 Hz, 1P), −22.65 (dd, J = 30.5 Hz, J = 20.4 Hz, 1P) (D2) δ 83.90 (br s, 1P), −10.38 (d, J = 19.0 Hz, 1P), −22.64 (dd, J = 28.3 Hz, J = 19.0 Hz, 1P).

4.2.13. 7-Methylguanosine 5′-*O*-(1-P-borano-2,3-methylene)-triphosphonate, ammonium salt, $m^7Gp_{BH3}pCH_2p$ (2)

$Gp_{BH3}pCH_2p$ (**22**, 140 mg, 0.171 mmol, triethylammonium salt) was dissolved in anhydrous DMSO (2.5 cm^3) and methyl iodide (0.115 cm^3, 0.156 mmol) was added. After 2 h, the reaction was quenched by the addition of deionised water (33 cm^3), the pH was adjusted to 6.0 using solid $NaHCO_3$. The reaction mixture

was extracted three times with 5 cm^3 of diethyl ether. The product was separated by IC on Sephadex resin using a linear TEAB gradient of 0–1.4 M. 44.2 mg of product (31%, 0.053 mmol, triethylammonium salt) was isolated as a white powder. Diastereoisomers D1 and D2 were then separated on semi-preparative RP HPLC and, after repeatedly freeze-drying, isolated as ammonium salts (D1: 12.2 mg, 0.02 mmol; D2: 16.5 mg, 0.027 mmol). MS ESI: $[M]^-$ found 532.05751, calcd 532.05763; ^1H NMR: (400 MHz, D_2O) (D1) δ 9.12 (s, 1H), 6.08 (d, J = 3.0 Hz, 1H), 4.69 (dd, J = 4.4 Hz, J = 3.0 Hz, 1H), 4.58 (m, 1H), 4.42 (m, 1H), 4.35–4.25 (m, 2H), 4.16 (s, 3H), 2.32 (t, J = 20.3 Hz, 2H) (D2) δ 9.21 (s, 1H), 6.08 (d, J = 3.3 Hz, 1H), 4.68 (dd, J = 4.5 Hz, J = 3.3 Hz, 1H), 4.49 (m, 1H), 4.44 (m, 1H), 4.37–4.22 (m, 2H), 4.16 (s, 3H), 2.34 (t, J = 20.3 Hz, 2H); ^{31}P NMR (160 MHz, D_2O) (D1) δ 82.49 (br s, 1P), 15.15 (dt. J = 20.3 Hz, J = 8.8 Hz, 1P), 8.57 (ddt, J = 30.0 Hz, J = 20.3 Hz, J = 8.8 Hz, 1P) (D2) δ 82.51 (br s, 1P), 15.17 (dt. J = 19.8 Hz, J = 8.6 Hz, 1P), 8.63 (ddt, J = 30.1 Hz, J = 20.3 Hz, J = 8.8 Hz, 1P).

4.2.14. General procedure for synthesis of $m^7Gppppm^7G$-analogues 6–9

0.5–0.75 equiv of an appropriate P^1,P^2-diimidazolyl derivative and 8 equiv of anhydrous $MgCl_2$ (or $ZnCl_2$ in the case of phosphorothioates) were added to anhydrous DMF and vigorously stirred until both substrates dissolved. In the second step, 1 equiv of nucleoside monophosphate was added to the mixture and the reaction was performed until HPLC measurements indicated the formation of the desired products. Then, the reaction was quenched via the addition of either MQ water or a solution of EDTA and $NaHCO_3$. The products were purified by ion-exchange chromatography on Sephadex resin using a linear gradient of TEAB 0–1.2 M and subsequently all diastereoisomers were separated via semi-preparative RP-HPLC.

4.2.15. 1*P*,4*P*-(7-methylguanosin-5′-yl) (2,3-methylene-,1,4-boranotetraphosphate), $m^7Gp_{BH3}pCH_2pp_{BH3}m^7G$ (9)

Method 1: Prepared from m^7Gp_{BH3} (**19**, 75 mg, 0.16 mmol, triethylammonium salt) and P^1,P^2-di(1-imidazolyl)methylenebisphosphonate (48 mg, 0.15 mmol, disodium salt) after 70 h. The diastereoisomer ratio was 8:3:4 (D1/D2/D3). HPLC yield was 91%. 57 mg (26%, 0.04 mmol, triethylammonium salt) of the product (white solid) was isolated. *Method 2:* 100 mg (0.079 mmol, TEA salt) of $Gp_{BH3}pCH_2pp_{BH3}G$ (**25**) was dissolved in 1 cm^3 anhydrous DMSO and 0.18 cm^3 (1.58 mmol; 20 equiv) of methyl iodide was added. The reaction was performed for 2 h, quenched by the addition of deionised water (15 cm^3) and the pH was adjusted to 7.0 using 1 M KOH water solution. The reaction mixture was extracted three times with 5 cm^3 of diethyl ether. After separation, 590 optical units of white solid (purity 45%, 14%, triethylammonium salt) were isolated. Purification of diastereoisomers: 590 optical units of the product (purity 45%) were purified on semi-preparative HPLC. After repeatedly freeze-drying, they were isolated as ammonium salts (D1: RT = 67.3 min, 4 mg; D2: RT = 68.7 min, mg; D3 RT = 71.2 min, 3.1 mg). ^1H (400 MHz, D_2O): (D1) δ 9.17 (s, 1H), 9.10 (s, 1H), 6.05 (d, J = 2.5 Hz, 1H), 6.04 (d, J = 3.2 Hz, 1H), 4.64 (m, 2H), 4.58 (m, 1H), 4.46 (m, 1H), 4.40 (m, 2H), 4.34–4.23 (m, 4H), 4.14 (s, 6H), 2.67 (t, J = 20.9 Hz, 2H), 0.37 (m, 6H) (D2) δ 9.08 (s, 2H), 6.05 (d, J = 2.5 Hz, 2H), 4.64 (dd, J = 4.3 Hz, J = 3.0 Hz, 2H), 4.56 (m, 2H), 4.40 (m, 2H), 4.30–4.24 (m, 4H), 4.12 (s, 6H), 2.53 (t, J = 20.8 Hz, 2H), 0.27 (m, 6H) (D3): ^1H (400 MHz, D_2O): δ 9.16 (s, 2H), 6.05 (d, J = 2.5 Hz, 2H), 6.01 (d, J = 2.5 Hz, 2H), 4.63 (m, 2H), 4.50–4.40 (m, 4H), 4.35–4.27 (m, 4H), 4.15 (s, 6H), 2.52 (t, J = 20.8 Hz, 2H), 0.36 (m, 6H). ^{31}P (160 MHz, D_2O): (D1) δ 79.23 (m, 2P), 3.68 (m, 2P) (D2) δ 79.23 (m, 2P), 3.68 (m, 2P) (D3) δ 82.59 (m, 2P), 7.74 (m, 2P).

4.2.16. 1P,4P-(7-methylguanosin-5′-yl) (1,4-boranotetraphosphate), $m^7Gp_{BH3}ppp_{BH3}m^7G$ (8)

Method 1: Prepared from m^7Gp_{BH3} (**19**, 75 mg, 0.16 mmol, triethylamine salt) and P^1,P^2-di(1-imidazolyl)pyrophosphate (48 mg, 0.15 mmol, disodium salt) after 70 h. 90 mg (0.033 mmol, 41%, triethylammonium salt) of the product (white solid) was isolated. The diastereisomer ratio was 8:3:4 (D1/D2/D3). *Method 2:* 105 mg (0.083 mmol, TEA salt) of the $Gp_{BH3}ppp_{BH3}G$ (**24**) was dissolved in 1 cm^3 anhydrous DMSO and 0.19 cm^3 (0.167 mmol; 20 equiv) of methyl iodide was added. The reaction was performed for 2 h, quenched by the addition of deionised water (15 cm^3) and the pH was adjusted to 7.0 using 1 M KOH water solution. The reaction mixture was extracted three times with 5 cm^3 of diethyl ether. After separation, 760 optical units of a white solid (TEA salt, purity 63%, 24% yield) were isolated. Purification of diastereomers: 760 optical units of the product (purity 63%) were purified on semi-preparative HPLC (0%–30% of acetonitrile in 180 min). After repeatedly freeze-drying, it was isolated as ammonium salts (D1: RT = 64.0 min, 4.6 mg; D2: RT = 65.6 min, 1.6 mg; D3: RT = 68.9 min, 4.2 mg). ^1H (400 MHz, D_2O): (D1) δ 9.15 (s, 1H), 9.10 (s, 1H), 6.04 (d, J = 3.0 Hz, 1H), 6.00 (d, J = 3.5 Hz, 1H), 4.66 (m, 1H), 4.63 (m, 1H), 4.59 (m, 1H), 4.46 (m, 1H), 4.41 (br s, 2H), 4.37–4.25 (m, 4H), 4.14 (s, 6H), 0.37 (m, 6H) (D2): δ 9.08 (s, 2H), 6.04 (d, J = 3.2 Hz, 2H), 4.68 (dd, J = 4.8 Hz, J = 3.2 Hz, 2H), 4.61 (m, 2H), 4.40 (m, 2H), 4.36–4.23 (m, 4H), 4.13 (s, 6H), 0.28 (m, 6H). ^{31}P (160 MHz, D_2O): δ 80.69 (m, 2P), −26.20 (d, J = 25.9 Hz, 2P). (D3) δ 9.19 (s, 1H), 5.99 (d, J = 3.2 Hz, 1H), 4.68 (dd, J = 4.8 Hz, J = 3.2 Hz, 2H), 4.63 (br s, 2H), 4.51–4.25 (m, 8H), 4.13 (s, 6H), 0.28 (m, 6H). ^{31}P (160 MHz, D_2O): (D1) δ 80.64 (m, 2P), −26.04 (d, J = 28.8 Hz, 2P) (D2) δ 80.64 (m, 2P), −26.04 (d, J = 28.8 Hz, 2P) (D3) δ 80.69 (m, 2P), −26.20 (d, J = 25.9 Hz, 2P).

4.2.17. 1*P*,4*P*-(7-methylguanosin-5′-yl) (1,4-thiotetraphosphate), $m^7Gp_Sppp_Sm^7G$ (6)

Prepared from m^7Gp_S (165 mg, 0.33 mmol, triethylammonium salt) and P^1,P^2-di(1-imidazolyl)pyrophosphate (48 mg, 0.15 mmol, disodium salt) after 45 h. 211 mg (0.16 mmol, 48%, triethylammonium salt) of the product (yellowish solid) was isolated. The diastereomer ratio was 3:8:4 (D1/D2/D3). 10 mg of the product was purified on semi-preparative HPLC to obtain pure diastereomers. After repeatedly freeze-drying, it was isolated as ammonium salts (D1+D2: RT = 58.2 min, 3.15 mg; D3: RT = 49.4 min, 1.4 mg). ^1H (400 MHz, D_2O): (D1/D2) δ 9.32 (s, 1H), 9.29 (s, 1H), 6.03 (m, 2H), 4.69 (m, 2H), 4.56 (m, 2H), 4.45–4.27 (m, 6H), 4.15 (s, 3H), 4.14 (s, 3H) (D3) δ 9.28 (s, 1H), 6.04 (d, J = 3.5, 2H), 4.69 (dd, J = 5.0, 3.5 Hz, 2H), 4.55 (m, 2H), 4.45–4.40 (m, 4H), 4.37–4.29 (m, 2H), 4.15 (s, 6H). ^{31}P (160 MHz, D_2O): (D1/D2) δ 44.11 (m, 2P), −23.40 (m, 2P) (D3) δ 40.27 (d, J = 14.7 Hz, 2P), −23.40 (m, 2P)

4.2.18. 1*P*,4*P*-(7-methylguanosin-5′-yl) (2,3-methylene-1,4-thiotetraphosphate), $m^7Gp_SpCH_2pp_Sm^7G$ (7)

Prepared from m^7Gp_S (**23**, 165 mg, 0.33 mmol, triethylammonium salt) and P^1,P^2-di(1-imidazolyl)methylenebisphosphonate (54 mg, 0.165 mmol, disodium salt) after 18 h. 162 mg (0.06 mmol, 37%, triethylammonium salt) of the product (yellowish solid) was isolated. 10 mg of the product was purified on semi-preparative HPLC to obtain pure diastereomers. After repeatedly freeze-drying, it was isolated as ammonium salts (D1: RT = 64.1 min. 1.0 mg; D2 RT = 68.7 min, 2.1 mg; D3: RT = 76.8 min, 1.3 mg). ^1H (400 MHz, D_2O): (D1) δ 9.33 (s, 2H), 6.07 (d, J = 3.3 Hz, 2H), 4.71 (m, 2H), 4.58 (m, 2H), 4.44–4.26 (m, 6H), 4.16 (s, 6H), 2.69 (t, J = 21.0 Hz, 2H). (D2) δ 9.34 (s, 1H), 9.32 (s, 1H), 6.07 (d, J = 3.3 Hz, 1H), 6.06 (d, J = 3.3 Hz, 1H), 4.70 (m, 2H), 4.59 (m, 1H), 4.59 (m, 1H), 4.54 (m, 1H), 4.45–4.25 (m, 6H), 4.17 (s, 3H), 4.15 (s, 3H), 2.69 (m, 2H) (D3) δ 9.29 (s, 2H), 6.05 (d, J = 3.0 Hz, 2H), 4.68 (m, 2H), 4.54 (m, 2H),

4.42 (m, 4H), 4.31 (m, 2H), 4.15 (s, 6H), 2.69 (t, J = 20.6 Hz, 2H). ^{31}P (160 MHz, D_2O): (D1) δ 42.34 (d, J = 32.2 Hz, 2P), 7.07 (m, 2P) (D2) δ 42.11 (m, 2P), 7.00 (m, 2P) (D3) δ 42.3 (m, 2P), 7.19 (m, 2P).

4.3. Biochemical studies

4.3.1. eIF4E fluorescence binding assay

Fluorescence titration measurements were conducted on an LS-55 spectrofluorometer (Perkin Elmer Co.), in 50 mM HEPES/KOH (pH 7.2), 100 mM KCl, 0.5 mM EDTA, and 1 mM DTT at 20.0 ± 0.3 °C. One millilitre aliquots of 1 μl of solutions with increasing concentrations of the cap analogue were added to 1.4 cm^3 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 the cap analogue to the experimental data points according to the equation described previously. The concentration of protein was fitted as a free parameter of the equilibrium equation rendering the fraction of protein capable of cap binding. 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 non-linear least-squares regression analysis was performed using ORIGIN 6.0 software (Microcal Software Inc., USA).

4.3.2. hDcpS stability assay

Human DcpS was expressed in *Escherichia coli*, purified according to previously described protocol[53] and stored at ~60 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 in the similar manner to that described in previous studies. An appropriate cap analogue at 40 μM concentration was treated with 200 nM hDcpS in 1400 μL of 50 mM TRIS buffer, pH = 7.9, 200 mM of KCl and 0.5 mM EDTA at 30 °C. After 10, 60 and 180 min, a 100 μL aliquot was collected from each reaction mixture and deactivated by freezing in liquid N_2. Collected samples were centrifuged (14000 rpm, 20 min) and subsequently analysed by analytical HPLC using a linear gradient of 0–25% methanol in 0.1 M KH_2PO_4, pH = 6.0, within 15 min and UV-detection at 260 nm.

4.3.3. FBS stability assay

For the serum stability assay, 1 mM stock solutions of compounds (**1**–**10**) in deionised water were prepared. Then, 50 μL of a respective stock solution, 750 μL of PBS buffer (pH = 7.2, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and 100 μL of FBS were mixed, so each solution contained 10% of FBS and the final compound concentration was 50 μM. The assay was performed at 37 °C, and after 15, 30, 45, 60, 75, 90, 120. 150, 180 and 240 min a 100 μL aliquot was collected from each reaction mixture and deactivated by incubation at 95 °C for 4 min. Collected samples were subsequently analysed without further treatment by analytical HPLC using a linear gradient of 0–25% methanol in 0.1 M KH_2PO_4, pH = 6.0, within 30 min and UV-detection at 260 nm. The substrate conversion for each compound was estimated using the equation:

$$S_{conv} = \frac{A_p}{A_p + A_s}$$

where A(p,s)—area under the products (p) or substrate (s) signal in the HPLC chromatogram. The obtained data were analysed using SigmaPlot 11.0 software (Systat Software Inc.).

4.3.4. In vitro translation and inhibition of translation in the RRL system

Inhibition of cap dependent translation in RRL (Flexi Rabbit Reticulocyte Lysate, Promega) by cap analogues, analysis of their stability in RRL and calculation of their IC_{50} values were performed as described previously.[8] A typical translation reaction was performed in 12.5 μl volume and contained: 56% concentration of reticulocyte lysate, mixture of amino acids (0.01 mM), RiboLock Ribonuclease Inhibitor (0.32 U/μl), luciferase mRNA, magnesium acetate and potassium acetate. Optimal conditions require 170 mM potassium acetate and 1.2 mM magnesium chloride. The luciferase activity was detected in a luminometer (Glomax, Promega) in the presence of Luciferase Assay Reagent (Promega)—2.5 μl of the translation reaction was mixed with 40 μl of the reagent and luminescence was measured for 10 s. Then in vitro translation reactions were performed in 12.5 μL volume for 60 min at 30 °C, in conditions suitable for cap-dependent translation. In the first series of experiments, m^7GpppG-capped luciferase mRNA and an inhibitor were added simultaneously to the mixture. In the second series, to analyse the stability of the studied cap analogues in the rabbit reticulocyte lysate translation system, each cap analogue was incubated in a translation mixture for 60 min at 30 °C and then luciferase mRNA was added to start the translation. Reactions were stopped by chilling on ice and the luciferase activity was measured in a luminometer (Glomax, Promega). Translation efficiency experiments were performed as described previously.[42] Shortly, capped and polyadenylated luciferase mRNAs were synthesised in vitro on a PCR-amplified dsDNA template containing: SP6 promoter sequence, 5′ UTR sequence of rabbit β-globin, luciferase ORF and a string of 31 adenosines. A typical in vitro transcription reaction mixture (40 ml) contained: SP6 transcription buffer (Fermentas), 0.7 mg of DNA template, 1 U/ml RiboLock Ribonuclease Inhibitor (Fermentas), 0.5 mM of each NTP and 0.1 mM GTP and 0.5 mM dinucleotide cap analogue (molar ratio cap analogue/GTP 5:1). The reaction mixture was preincubated at 37 °C for 5 min prior to addition of SP6 RNA polymerase (Fermentas) to final concentration 1 U/ml and reaction was continued for 45 min at 37 °C. After incubation, reaction mixtures were treated with DNase RQ1 (Promega) in transcription buffer for 20 min at 37 °C, the concentration of template DNA was 1U per 1 mg. RNA transcripts were purified using NucAway Spin Columns (Ambion), integrity of transcripts was checked on a nondenaturating 1% agarose gel and concentrations were determined by UV/VIS. A translation reaction in RRL was performed in 10 ml volume for 60 min 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 capped mRNA.

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Supplementary data

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References and notes

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