Exploring the potential of phosphotriazole 5' mRNA cap analogues as efficient translation initiators

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Supplementary Information

Content

Tables:

Table S1. Yields of syntheses of novel phosphotriazole dinucleotide cap analogues.

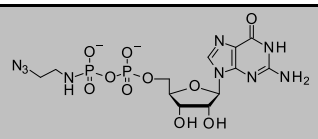
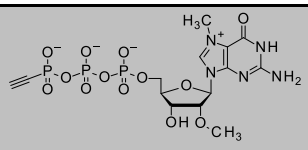
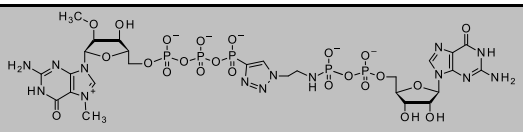
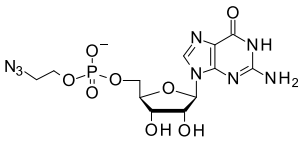
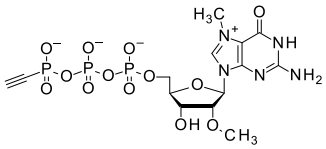
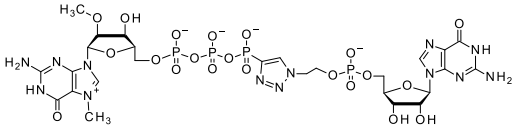
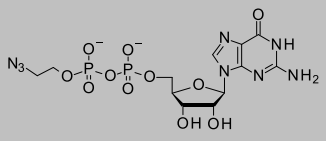
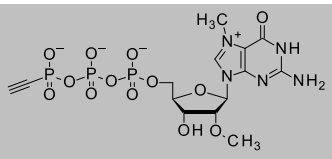
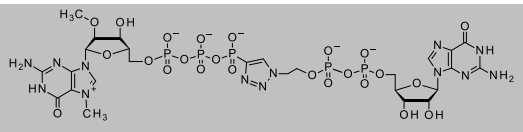
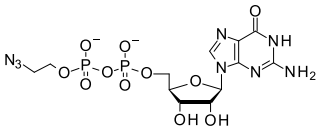
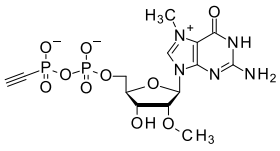
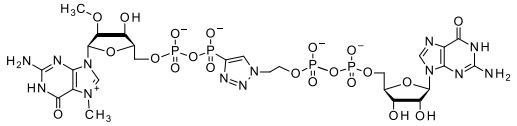
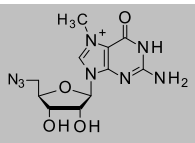
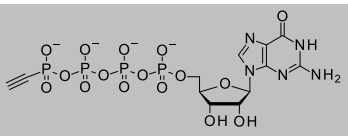
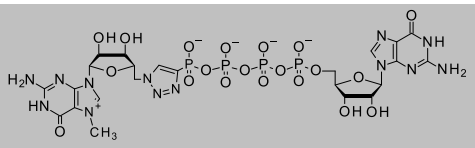
Azide-modified compound	Alkyne-modified compound	Product	HPLC yield
 **5**	 **4d**	 **1b**	>99%
 **6a**	 **4d**	 **1c**	>99%
 **6b**	 **4d**	 **1d**	>99%
 **6b**	 **4c**	 **1e**	>99%
 **9**	 **4e**	 **2b**	>99%

Table S2. pH-dependent stability of novel phosphotriazole cap analogues.

	Fraction remaining							
	Buffer pH 3		Buffer pH 5		Buffer pH 7		Buffer pH 9	
	4h	**24h**	**4h**	**24h**	**4h**	**24h**	**4h**	**24h**
$m_2^{7,2'\text{-}O}$Gppp-triazole-C_2H_4NHppG (**1b**)	81.0%	27.1%	100%	100%	100%	100%	99.5%	97.0%
$m_2^{7,2'\text{-}O}$Gppp-triazole-C_2H_4OpG (**1c**)	100%	100%	100%	100%	100%	100%	99.9%	98.8%
$m_2^{7,2'\text{-}O}$Gppp-triazole-C_2H_4OppG (**1d**)	100%	100%	100%	100%	100%	100%	98.9%	96.0%
$m_2^{7,2'\text{-}O}$Gppp-triazole-C_2H_4OppG (**1e**)	100%	100%	100%	100%	100%	100%	99.0%	95.5%

Table S3. Sequences of DNA and RNA oligonucleotides used in this work.

	Sequence
DNA1	ATACGATTTAGGTGACACTATAGAAGAAGCGGGCATGCGGCCAGCCATAGCCGATCA
DNA2	TGATCGGCTATGGCTGGCCGCATGCCCGCTTCTTCTATAGTGTCACCTAAATCGTAT
DNA3 (DNazyme10-23)[7]	TGATCGGCTAGGCTAGCTACAACGAGGCTGGCCGC
RNA1 (35nt)	GAAGAAGCGGGCAUGCGGCCAGCCAUAGCCGAUCA
RNA2 (25nt)	GAAGAAGCGGGCAUGCGGCCAGCCA

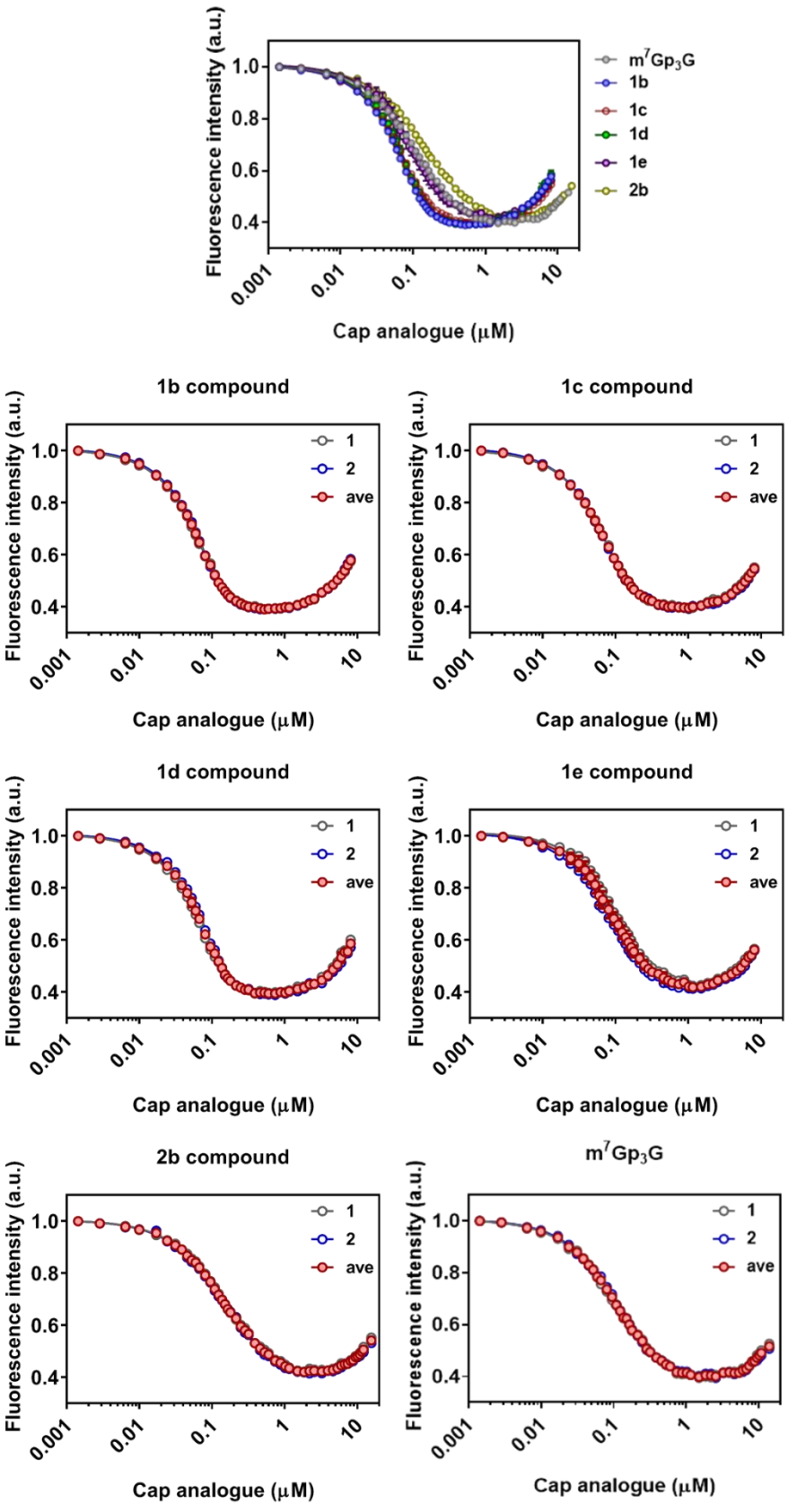


Figure S1. eIF4E fluorescence quenching titration curves for novel cap analogues and control m^7Gp_3G.

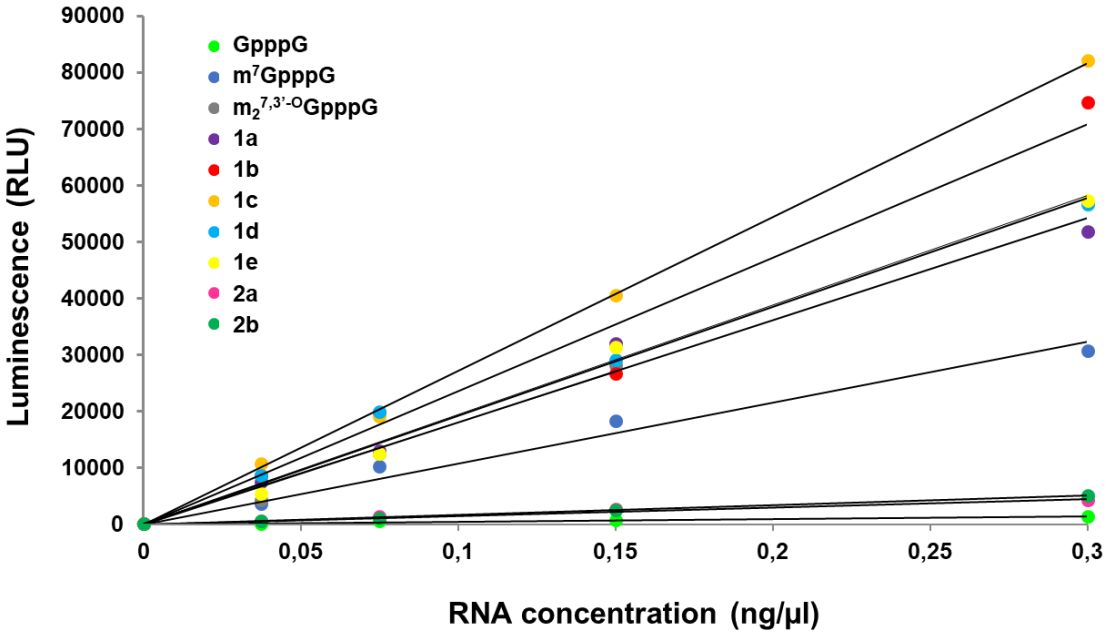


Figure S2. Luciferase activity resulting from translation of mRNAs capped with various cap analogues in rabbit reticulocyte lysate – results of a single experiment.

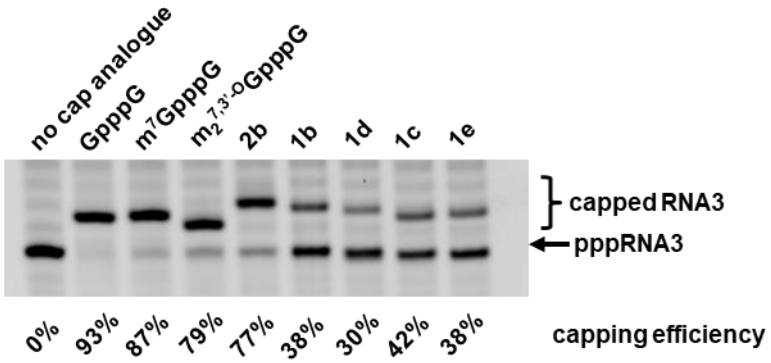


Figure S3. Co-transcriptional capping efficiency of triazole-modified cap analogues.

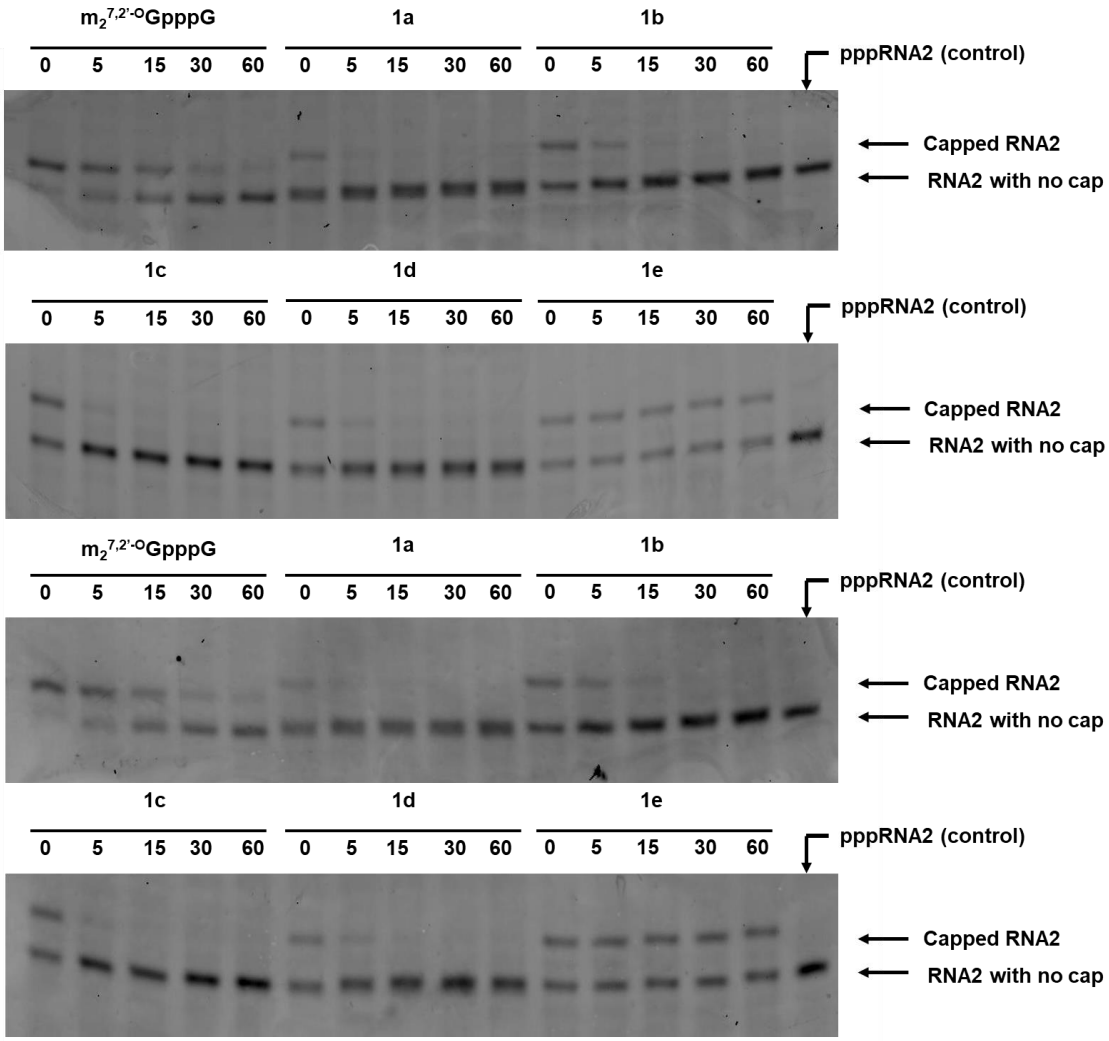


Figure S4. Susceptibility of short transcripts capped with novel phosphotriazole cap analogues to hDcp2-catalyzed decapping.

Experimental procedures:

1. General information

1.1 Starting materials and chemical reagents

All solvents and reagents were *synthesis grade* and used without further treatment, unless otherwise stated. Guanosine and guanosine 5'-monophosphate disodium salt were purchased from Carbosynth, 2'-O-methylguanosine from Sigma Aldrich. Dinucleotide analogues **1a**, **2a**,[1] mononucleotide analogues **4d**, **5**,[1] 5'-azido-5'-deoxy-7-methylguanosine **9**[1] and 2'-O-methylguanosine 5'-monophosphate[2] were synthesized as described previously.

1.2 Chromatography

1.2.1 Ion-exchange chromatography

The synthesized mononucleotide analogues (**4a**, **4c**, **4e**, **6a**, **6b**) were purified by ion-exchange chromatography on DEAE Sephadex A-25 (HCO_3^- form) column. After loading the column with reaction mixture and washing it with water, the products were eluted using different gradients of TEAB in deionized water: 0–0.6 M for nucleoside monophosphates, 0–0.8 M for nucleoside diphosphates, 0–1.0 M nucleoside triphosphates or 0–1.2 for nucleoside tetraphosphates. Fractions containing the desired product were collected together after RP HPLC and spectrophotometric analysis (at 260 nm). Evaporation under reduced pressure with repeated additions of 96% ethanol, then 99.8% ethanol and, at the end, MeCN resulted in isolation of nucleotide analogues as triethylammonium (TEA) salts.

1.2.2 Analytical and preparative reverse-phase (RP) HPLC

Both analytical and semi-preparative HPLC were performed on Agilent Technologies Series 1200 with UV-detection at 254 nm and fluorescence detection (Ex: 260 nm, Em: 370 nm). For chemical and enzymatic reactions, monitoring analytical HPLC was performed using Supelcosil LC-18-T column or Grace VisionHT C18-HL column (4.6 x 250 mm, 5 µm, flow rate 1.3 ml/min) with one of three different linear gradients of methanol in 0.05 M ammonium acetate buffer (pH 5.9): program A – gradient 0–25% of methanol in 15 min, program B – gradient 0–50% of methanol in 15 min, program C – gradient 0–50% of methanol in 7.5 min and then isocratic elution (50% of methanol) until 15 min. For pH-dependent degradation studies and reactions monitoring of different steps of ARCA analogues synthesis analytical HPLC was performed using Grace VisionHT C18-HL column (4.6 x 250 mm, 5µm, flow rate 1.3 ml/min) with linear gradient 0-25% of methanol in 0.05 M ammonium acetate buffer (pH 5.9) in 15 min. Semi-preparative RP HPLC was performed using Discovery RP Amide C-16 HPLC column (25 cm x 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). The products, after at least triple lyophilisation, were isolated as ammonium salts.

1.3 Yields and concentrations determination

The yields of mononucleotide analogues after ion-exchange purification and the concentrations of mono- and dinucleotide analogues solutions used for biophysical and biological experiments were determined on the basis of absorbance measurements performed at 260 nm in 0.1 M phosphate buffer pH 6.0 for 7-methylguanine mononucleotide analogues and in 0.1 M phosphate buffer pH 7.0 for dinucleotide analogues and guanine mononucleotide analogues. The quantities of obtained ion-exchange purified products were expressed as optical density miliunits (opt. mu = absorbance of the solution by volume in ml). For calculations of yields and concentrations following molar extinction coefficients [$M^{-1}cm^{-1}$] were employed: ε = 22600 (dinucleotides), ε = 11400 (m^7G mononucleotides), ε = 12080 (G mononucleotides). Concentrations of transcripts were determined using NanoDrop 2000c Spectrofotometer (Thermo Scientific).

1.4 NMR spectroscopy and mass spectrometry

The structure and purity of each final product were confirmed by high resolution mass spectrometry using negative or positive electrospray ionization (HRMS (-) ESI or HRMS (+) ESI) and ^1H NMR, ^{31}P NMR, gDQCOSY and gHSQCAD spectroscopy. Mass spectra were recorded on Thermo Scientific LTQ OrbitrapVelos spectrometer. NMR spectra were recorded on a Varian INOVA 500 MHz spectrometer equipped with a high stability temperature unit using 5 mm 4NUC probe, at 25 °C if not stated otherwise, at 500 MHz (^1H NMR) and 202 MHz (^{31}P NMR). The ^1H NMR and ^{31}P NMR chemical shifts were reported in ppm and referenced to respective internal standards: sodium 3-(trimethylsilyl)-2,2',3,3' tetradeuteropropionate (TSP) and 20% phosphorus acid in D_2O. Signals in ^1H NMR spectra of dinucleotides were assigned according to 2D NMR spectra (gDQCOSY, gHSQCAD). In ^{31}P signal assignment of dinucleotide cap analogues the phosphates were denoted analogously to $m^7Gp_\gamma p_\beta p_\alpha G$.

2. Chemical synthesis

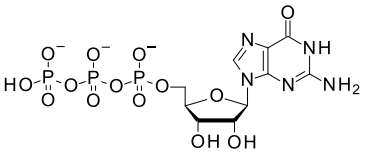
2.1 Synthesis of nucleotide imidazolide derivatives (3a, 3c, 3d):

2.1.1 Preparation of compounds for imidazole-activation

Preparation of triethylammonium (TEA) salts

The commercially available guanosine 5'-monophosphate (GMP) disodium salt and tetrasodium pyrophosphate were converted into triethylammonium forms by passing their aqueous solutions (ca. 1 g/20 ml) through Dowex 50 W x 8 cationite. The collected eluates were evaporated under reduced pressure with repeated additions of ethanol and acetonitrile to dryness yielding the nucleotide triethylammonium salt as a white solid and triethylammonium pyrophosphate as colorless oil.

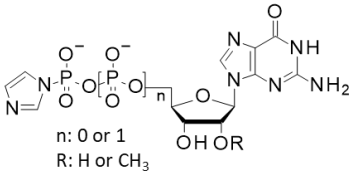
Synthesis of guanosine 5'-triphosphate (GTP)



Triethylammonium pyrophosphate (10.0 mmol) was suspended in 30 ml DMF in the presence of $ZnCl_2$ (1354 mg, 9.9 mmol) and stirred for ~5 min to obtain a solution. Then, GMP-Im (**3a**) (1000 mg, 24000 mOD, 2.0 mmol) along with a second portion of $ZnCl_2$ (1354 mg, 9.9 mmol) was added and the mixture was stirred for 1.5 h at room temperature. The reaction was stopped by 10-fold dilution with water and addition of $Na_2EDTA \cdot 2H_2O$ (7445 mg, 20.0 mmol) and $NaHCO_3$ (ca. 3722 mg, 44.3 mmol). The ion-exchange purification afforded triethylammonium salt of GTP (64%).

2.1.2 Imidazole-activation

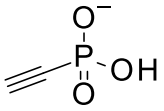
Synthesis of nucleotide imidazolides



Compounds **3a**, **3c**, **3d** were prepared according to Mukaiyama and Hashimoto.[3] In the synthesis of **3a** and **3d** an appropriate nucleotide (1 eq., TEA salt), imidazole (10 equiv.), 2,2'-dithiodipyridine (3 equiv.) were mixed in DMF (~ 2.5 ml/100 mg of nucleotide) before addition of triethylamine (3 equiv.) and triphenylphosphine (3 equiv.). The mixture was stirred for 6–8 h at room temperature. In case of **3c** double amounts of reagents were used because of problems with GTP dissolution and the reaction was performed overnight. The addition of a solution of anhydrous $NaClO_4$ (4 equiv.) in dry acetone (~ 8 volumes of DMF volume) resulted in precipitation of the product as sodium salt. The suspension was cooled at 4 °C and the precipitate was filtered off, washed repeatedly with cold, dry acetone and dried in vacuum over P_4O_{10}. Yields: 90–100 %.

2.2 Synthesis of C-phosphonate analogues

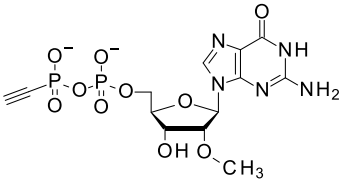
(10) 1-ethynyl C-phosphonate triethylammonium salt



Compound **10** was synthesized according to Wanat et al.[4] with minor modifications. Diethylchlorophosphate (3.5 ml, 24.5 mmol) was added by a syringe to a flask equipped with rubber septum and stirred at room temperature for about 20 min under the atmosphere of argon. The mixture-containing flask was cooled down to 0°C on ice/water bath and the argon flow was stopped. Then, 0.5 M ethynylmagnesium bromide solution in THF (50 ml, 25.0 mmol) was added gradually over 5 min after which the ice/water bath was removed. The reaction was stirred at room temperature for about 30 min and then quenched upon addition of 13 ml of saturated aqueous ammonium chloride. The resulting solution was concentrated under reduced pressure and subjected to extraction with ethyl acetate (6 x 25 ml). Organic fractions were collected, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by silica gel chromatography using gradient elution of 0-50% EtOAc in hexane yielding a yellowish oil which was then dried in vacuum over P_2O_5. 675 mg (4.2 mmol, 17%) of 1-ethynyl-ditehylphosphonate was obtained.

1-ethynyl-diethylphosphonate (675 mg, 4.2 mmol) was dissolved in 13.3 ml CH_2Cl_2 and 3.3 ml of trimethylsilyl bromide (TMS-Br) (3.81 g/ 25.0 mmol) and the solution was stirred at room temperature for 4 h. Then, another portion of TMS-Br (1.1 ml/ 1.27 g/ 8.3 mmol) was added and the stirring was continued for 2 h. As MS analysis showed only 50% conversion, yet another portion of TMS-Br (2.2 ml/ 2.53 g/ 16.0 mmol) was added and the mixture stirred overnight. Then, the mixture was evaporated under reduced pressure and 58 ml of trimethylamine was added along with 58 ml of DMF. The mixture was stirred at 90°C for 3.5 h. Afterwards, the mixture was washed with CCl_4 (6 x 20 ml). The resulting aqueous phase was evaporated under reduced pressure, dried in vacuum over P_2O_5 to afford 927 mg (3.0 mmol, 72%) of **10**.

(4a) β-C-(1-ethynyl) 2'-O-methylguanosine diphosphate ammonium salt

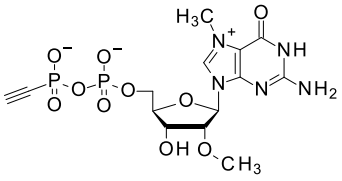


Triethylammonium 1-ethynyl C-phosphonate (1.1 mmol) was stirred in 3 ml DMF with $MgCl_2$ (134 mg, 1.4 mmol) until complete dissolution. Then, 2'-O-methylguanosine 5'-monophosphate

P-imidazolide sodium salt (4241 mOD, 0.35 mmol) along with second portion of $MgCl_2$ (134 mg, 1.4 mmol) were added and the mixture was stirred for 0.5 h at room temperature. The reaction was stopped by 10-fold dilution with water. The product was purified by ion-exchange chromatography on DEAE Sephadex A-25 and evaporated to dryness as described in General Information to afford 3647 mOD, (0.30 mmol, 86 %) of $m^{2'\text{-}O}GppC_2H$ triethylammonium salt. Additional HPLC purification of a fraction of obtained product gave $m^{2'\text{-}O}GppC_2H$ as ammonium salt.

^1H NMR (500 MHz, D_2O, 25 °C) δ_H: 8.26 (s, 1H, H8), 6.02 (d, *J* = 6.0 Hz, 1H, H1'), 4.69 (dd, *J* = 5.0, 3.3 Hz, 1H, H3'), 4.54 (dd, *J* = 6.0, 5.0 Hz, 1H, H2'), 4.37 (m, 1H, H4'), 4.24 (m, 2H, H5' and H5''), 3.48 (s, 3H, $m^{2'O}$), 3.18 (d, *J* = 12.8 Hz, 1H, C_2H); ^{31}P NMR (202 MHz, D_2O, 25°C) δ_P: -11.01 (bd, *J* = 22.0 Hz, 1P, Pα), -20.92 (m, 1P, Pβ); HRMS (-) ESI m/z found: 464.0379, calc. for: $C_{13}H_{16}N_5O_{10}P_2^-$ 464.0378.

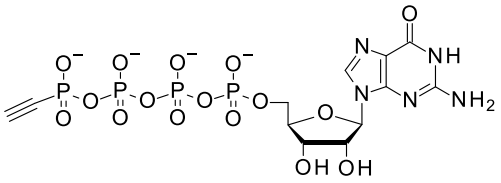
(4c) β-C-(1-ethynyl) 2'-O-N7-dimethylguanosine diphosphate ammonium salt



$m^{2'\text{-}O}GppC_2H$ (3100 mOD, 0.26 mmol) was dissolved at ~ 0.08 M concentration in ca. 0.5 mM aqueous CH_3COOH pH 4 (3.1 ml). Then, 12 portions of $(CH_3)_2SO_4$ (1 equiv. each, sum: 292 µl, 3.1 mmol) were added every 5 min to the mixture under vigorous stirring and the pH was maintained at 4 by adding 10% KOH if necessary. The stirring was continued at room temperature for 4h. The reaction was stopped by 10-fold dilution with water and organic-soluble compounds were removed by 3-time washing with diethyl ether. The pH of aqueous phase was then set to 7 by addition of solid $NaHCO_3$. The ion-exchange purification afforded 2180 mOD, (0.19 mmol, 74 %) of $m_2^{7,2'\text{-}O}GppC_2H$ triethylammonium salt. Additional HPLC purification of a fraction of obtained product gave $m_2^{7,2'\text{-}O}GppC_2H$ as ammonium salt.

^1H NMR (500 MHz, D_2O, 25 °C) δ_H: 6.16 (d, *J* = 3.5 Hz, 1H, H1'), 4.62 (t, *J* = 4.9, 5.5 Hz, 1H, H3'), 4.41 (dd, *J* = 4.9, 3.5 Hz, 1H, H2'), 4.40 – 4.38 (m, 1H, H4'), 4.36 (ddd, *J* = 11.8, 4.3, 2.4 Hz, 1H, H5'), 4.23 (ddd, *J* = 11.8, 5.2, 2.1 Hz, 1H, H5''), 4.13 (s, 3H, m^7), 3.60 (s, 3H, $m^{2'O}$), 3.19 (d, *J* = 12.5 Hz, 1H, C_2H); ^{31}P NMR (202 MHz, D_2O, 25°C) δ_P: -11.00 (dt, *J* = 22.1, 4.5 Hz, 1P, Pα), -20.80 (dd, *J* = 22.1, 12.5 Hz, 1P, Pβ); HRMS (-) ESI m/z found: 478.0537, calc. for: $C_{14}H_{18}N_5O_{10}P_2^-$ 478.0534.

(4e) δ-C-(1-ethynyl) guanosine tetraphosphate ammonium salt



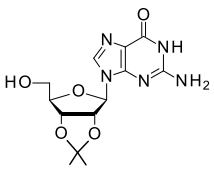
Triethylammonium 1-ethynyl C-phosphonate (0.80 mmol) was stirred in 4 ml DMF with $MgCl_2$ (95.5 mg, 1.0 mmol) until complete dissolution. Then, 2'-O-methylguanosine 5'-monophosphate P-imidazolide sodium salt (2700 mOD, 0.22 mmol) along with second portion of $MgCl_2$ (95.5 mg, 1.0 mmol) were added and the mixture was stirred for 2.5 h at room temperature. The reaction was stopped by 10-fold dilution with water. The product was purified by ion-exchange chromatography on DEAE Sephadex A-25 and evaporated to dryness as described in General Information to afford 2404 mOD, (0.20 mmol, 89 %) of $GppppC_2H$ triethylammonium salt. Additional HPLC purification of a fraction of obtained product gave $GppppC_2H$ as ammonium salt.

^1H NMR (500 MHz, D_2O, 25 °C) δ_H: 8.14 (s, 1H, H8), 5.93 (d, *J* = 6.5 Hz, 1H, H1'), 4.85(dd, *J* = 6.5, 5.2 Hz, 1H, H2'), 4.59 (dd, *J* = 5.2, 3.0 Hz, 1H, H3'), 4.40 – 4.35 (m, 1H, H4'), 4.28 (ddd, *J* = 11.5, 6.0, 3.4 Hz, 1H, H5'), 4.22 (ddd, *J* = 11.5, 4.9, 3.5 Hz, 1H), 3.18 (d, *J* = 12.9 Hz, 1H, H_{C2H}); ^{31}P NMR (202 MHz, D_2O, 25°C) δ_P: -10.58 (dt, *J* = 18.7, 6.0 Hz, 1P, Pα), -20.84 (dd, *J* = 18.6, 12.9 Hz, 1P, Pδ), -22.53 (t, *J* = 18.0 Hz, 1P, Pβ or Pγ), -23.04 (t, *J* = 18.0 Hz, 1P, Pβ or Pγ); HRMS (-) ESI m/z found: 609.9552, calc. for: $C_{12}H_{16}N_5O_{16}P_4^-$ 609.9548.

2.3 Synthesis of phosphoester analogues

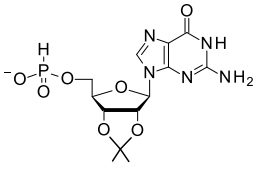
(6a) O-(2-azidoethyl) guanosine monophosphate ammonium salt $GpOC_2H_4N_3$

<u>Step 1: Synthesis of 2',3'-O,O-isopropylideneguanosine</u>



2',3'-O,O-isopropylideneguanosine was synthesized according to Warminski et al.[5] Guanosine (2.0 g, 7.1 mmol) was stirred with 2,2- dimethoxypropane (7.62 ml, 62.0 mmol) and p-toluenesulfonic acid monohydrate (1.77 g, 9.3 mmol) in 90 ml acetone at room temperature overnight. Then, the reaction was centrifuged, the resulting supernatant was partly evaporated under vacuum and the product was precipitated upon setting pH at 9 with 25% ammonia. The precipitate was filtered off, dried over P_2O_5 and directly used in the next step without crystallisation.

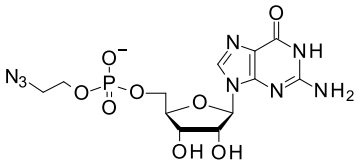
<u>Step 2: Synthesis of 2',3'-O,O-isopropylideneguanosine H phosphonate</u>



2',3'-O,O-isopropylideneguanosine (1.010 g, 3.1 mmol, 1 equiv.) was stirred with PCl_3 (312 µl, 3.6 mmol, 1.1 equiv.) in the presence of 15.2 ml of trimethyl phosphate at 0°C for 1 h. Then the mixture was diluted with approx. 150 ml of water and the pH was set to 7 using solid $NaHCO_3$. The ion-exchange purification afforded 17760 mOD, (1.5 mmol, 47 %) of 2',3'-O,O-isopropylideneguanosine H-phosphonate 2',3'-iPr-

GpH (**8**) triethylammonium salt.

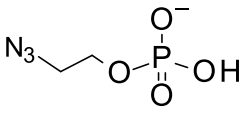
Step 3: Synthesis of O-(2-azidoethyl) guanosine monophosphate



To the flask containing 2'3'-iPr-GpH (3000 mOD, 0.25 mmol, 1 equiv.) BSA (607 µl, 2.5 mmol, 10 equiv.) and ACN (607 µl) were added. The mixture was stirred for 15 min after which TEA (102 µl, 0.744mmol, 3 equiv.) along with 2-azidoethanol (564 µl, 7.4 mmol, 30 equiv.) were added and the stirring was continued for 5 min. Next, iodine (126 mg, 0.50 mmol, 2 equiv.) was gradually added and the reaction was stirred for 7 days at 45 °C. After that, it was diluted with approx. 30 ml of water and the pH was set to 1.5 with conc. HCl. The resulting mixture was stirred at 60°C for 1.5 h and then the pH was set to 7 with solid $NaHCO_3$. The mixture was subjected to ion-exchange purification which afforded 1350 mOD (0.11 mmol, 45 %) of $GpOC_2H_4N_3$ triethylammonium salt. Additional HPLC purification of a fraction of obtained product gave $GpOC_2H_4N_3$ as ammonium salt.

^{1}H NMR (500 MHz, D_2O, 25 °C) δ_H: 8.20 (s, 1H, H8), 5.95 (d, *J* = 5.4 Hz, 1H, H1'), 4.81 (dd, *J* = 5.3, 5.4 Hz, 1H, H2', overlapped with HDO), 4.52 (dd, *J* = 5.2, 4.0 Hz, 1H, H3'), 4.34-4.36 (m, 1H, H4'), 4.20 – 4.08 (m, 2H, H5', H5''), 3.98 – 3.88 (m, 2H, CH_2-N_3), 3.46 – 3.36 (m, 2H, CH_2-O); ^{31}P NMR (202 MHz, D_2O, 25°C) δ_P: 0.94-1.05 (m, 1P, Pα); HRMS (-) ESI m/z found: 431.0626, calc. for: $C_{12}H_{16}N_8O_8P^-$ 431.0834.

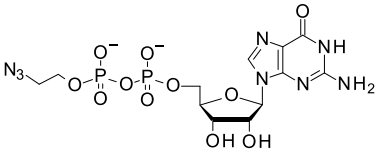
(7) O-(2-azidoethyl) phosphate ester cyclohexylammonium salt



H_3PO_3 (50 mg, 0.61 mmol, 1 equiv.) was added to a flask and dissolved in 2-azidoethanol (1.385 ml, 18 mmol, 30 equiv.). Then, TEA (253 µl, 1.8 mmol, 3 equiv.) was added and the mixture was stirred for 5 min. Next, iodine (308 mg, 1.2 mmol, 2 equiv.) was gradually added and the mixture was stirred for 96 h at room temperature. The product was precipitated upon addition of the mixture of 350 µl of cyclohexylamine in 15.8 ml acetone. The precipitate was filtered off, washed repeatedly with cold, dry acetone until it turned white and then dried in vacuum over P_2O_5.

^{1}H NMR (500 MHz, D_2O, 25 °C) δ_H: 3,92 (m, 2H, $H_{CH2\text{-}N3}$), 3.48 (m, 2H, $H_{CH2\text{-}O}$); ^{31}P NMR (202 MHz, D_2O, 25°C) δ_P: 4.10 (t, *J* = 6.2 Hz, 1P); HRMS (-) ESI m/z found: 166.0011, calc. for: $C_2H_5N_3O_4P^-$ 166.0023.

(6b) β-O-(2-azidoethyl) guanosine diphosphate ammonium salt $GppOC_2H_4N_3$



DMF (3.7 ml) and $MgCl_2$ (74.2 mg, 0.78 mmol, 4 equiv.) were added to a flask with dry O-(2-azidoethyl) phosphate ester cyclohexylammonium salt (**6**) (ca. 0.61 mmol, 3 equiv.) and the mixture was stirred at room temperature until complete dilution of the phosphate subunit. Then, guanosine 5'-monophosphate imidazolide (**2a**) (106 mg, 2353 mOD, 0.19 mmol, 1 equiv.) along with second portion of $MgCl_2$ (74.2 mg, 0.78 mmol, 4 equiv.) were added. The mixture was stirred at room temperature for 24 h. The reaction was then stopped by diluting with ten volumes of water and subjected to ion-exchange purification which afforded 2047 mOD, (0.17 mmol, 87 %) of $GppOC_2H_4N_3$ triethylammonium salt. Additional HPLC purification of a fraction of obtained product gave $GppOC_2H_4N_3$ as ammonium salt.

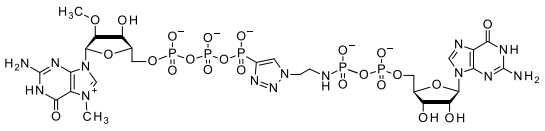
^{1}H NMR (500 MHz, D_2O, 25 °C) δ_H: 8.35 (s, 1H, H8), 5.97 (d, *J* = 5.5 Hz, 1H, H1'), 4.78 (dd, *J* = 5.1, 5.5 Hz, 1H, H2'), 4.53 (dd, *J* = 5.1, 3.9 Hz, 1H, H3'), 4.37 (dt, *J* = 3.9, 2.3 Hz, 1H, H4'), 4.30 – 4.18 (m, 2H, H5' and H5''), 4.06-4.09 (m, 2H, $CH_{2linker\ CH2\text{-}O}$), 3.48 (t, *J* = 5.0 Hz, 2H, $CH_{2linker\ CH2\text{-}N3}$); ^{31}P NMR (202 MHz, D_2O, 25°C) δ_P: -10.57- -10.30 (m, 2P, overlapped Pα and Pβ); HRMS (-) ESI m/z found: 511.0503, calc. for: $C_{12}H_{17}N_8O_{11}P_2^-$ 511.0498.

1.1 Synthesis of dinucleotide cap analogues

General procedure A (GP A): Synthesis of dinucleotide cap analogues containing triazole located between P-subunits

Aqueous solutions of an alkyne-containing nucleotide (1 equiv., 0.2-0.5 M) and an azide-containing nucleotide (1 equiv., 0.2-0.5 M) were mixed together followed by addition of H_2O and the mixture of H_2O:t-BuOH (1:1,v/v) (to the concentration of each analogue ca. 26-53 mM, final H_2O:t-BuOH (2-5:1, v/v)) and aqueous solutions of $CuSO_4{\cdot}5H_2O$ (0.4 equiv., 0.6-0.65 M) and sodium ascorbate (0.8 equiv., 1.2-1.3 M). The reaction was stirred at room temperature for 0.5-1 h and monitored by RP HPLC. Final concentrations of reagents are given in the detailed procedures below. When completed, the reaction was quenched by 5-fold dilution with water and addition of Na_2EDTA (ten equivalents of added $CuSO_4$) directly followed by semi-preparative RP HPLC purification.

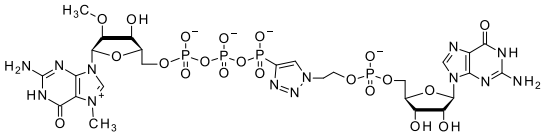
(1b) $m_2^{7,2'\text{-}O}$Gppp-t-C_2H_4NHppG



Obtained according to GP A from $m_2^{7,2'\text{-}O}GpppC_2H$ (377 mOD, 0.033 mmol, 50 mM) and $GppNHC_2H_4N_3$ (400 mOD, 0.033 mmol, 50 mM) while stirring with $CuSO_4$ (3.3 mg, 0.013 mmol, 20 mM) and sodium ascorbate (5.2 mg, 0.026 mmol, 40 mM) in 662 ml of H_2O:t-BuOH (2:1, v/v) for 0.5 h. After quenching the reaction with Na_2EDTA (48.4 mg, 0.13 mmol), the product was subjected to RP HPLC purification which afforded m^7Gppp-triazole-C_2H_4NHppG as ammonium salt.

^{1}H NMR (500 MHz, D_2O, 25 °C) δH: 9.13 (s, 1H, H8 m^7G), 8.24 (s, 1H, H8 G or $H_{triazole}$), 8.06 (s, 1H, H8 G or $H_{triazole}$), 6.08 (d, *J* = 3.1 Hz, 1H, H1' m^7G), 5.87 (d, *J* = 5.8 Hz, 1H, H1' G), 4.82 (ovelapped with HDO, 1H, H2' G), 4.59 (dd, *J* = 6.1, 4.9 Hz, 1H, H3' m^7G), 4.52 (dd, *J* = 5.3, 3.7 Hz, 1H, H3' G), 4.42 (t, *J* = 6.4 Hz, 2H, $H_{CH2\text{-}triazole}$), 4.35 – 4.26 (m, 4H, overlapped H2' m^7G, H4' G and m^7G, H5' G or m^7G), 4.25 – 4.13 (m, 3H, overlapped H5' G or m^7G, H5'' G and m^7G), 4.07 (s, 3H, m^7), 3.59 (s, 3H, $m^{2'O}$), 3.32 (dt, *J* = 12.6, 6.4 Hz, 2H, $H_{CH2\text{-}NH}$); ^{31}P NMR (202 MHz, D_2O, 25°C) δ_P: -1.36 – -1.72 (m, 1P, Pβ), -6.34 (d, *J* = 21.8 Hz, 1P, Pδ), -10.04 – -10.29 (m, 1P, Pα), -10.49– -10.65 (m, 1P, Pζ), -22.34 (dd, *J* = 21.8, 19.7 Hz, 1P, Pε); HRMS (-) ESI *m/z* found: 1069.0946, calc. for: $C_{26}H_{38}N_{14}O_{23}P_5^-$ 1069.0928.

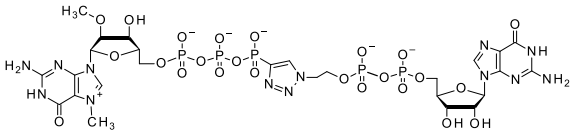
(1c) $m_2^{7,2'\text{-}O}$Gppp-t-C_2H_4OpG



Obtained according to GP A from $m_2^{7,2'\text{-}O}GpppC_2H$ (330 mOD, 0.029 mmol, 25 mM) and $GpOC_2H_4N_3$ (350 mOD, 0.029 mmol, 25 mM) while stirring with $CuSO_4$ (2.9 mg, 0.012 mmol, 10 mM) and sodium ascorbate (4.6 mg, 0.023 mmol, 20 mM) in 1.150 ml of H_2O:t-BuOH (3:1, v/v) for 0.5 h. After quenching the reaction with Na_2EDTA (44.7 mg, 0.12 mmol), the product was subjected to RP HPLC purification which afforded m^7Gppp-triazole-C_2H_4OpG as ammonium salt.

^{1}H NMR (500 MHz, D_2O, 25 °C) δH: 9.14 (s, 1H, H8 G or m^7G $H_{triazole}$), 8.31 (s, 1H, H8 G or m^7G or $H_{triazole}$), 8.05 (s, 1H, H8 G or m^7G $H_{triazole}$), 6.06 (d, J = 2.9 Hz, 1H, H1' m^7G), 5.83 (d, J = 5.8 Hz, 1H, H1' G), 4.70 (dd, J = 5.2, 5.8 Hz, 1H, H2' G), 4.65 (t, *J* = 5.2 Hz, 2H, $CH_{2\ linker\ CH2\text{-}N3}$), 4.57 (dd, J = 6.3, 4.9 Hz, 1H, H3' m^7G), 4.27-4.34 (m, 4H, overlapped H2' m^7G and H3' G, H4' G or H5' or H5'' m^7G), 4.27 – 4.22 (m, 1H, H4' G), 4.16-4.21 (m, 3H, overlapped $CH_{2\ linker\ CH2\text{-}O}$ and H4' G or H5' or H5'' m^7G), 4.06 (s, 3H, m^7), 3.87 (ddd, *J* = 11.6, 4.6, 2.9 Hz, 1H), 3.81 (ddd, *J* = 11.6, 5.2, 4.0 Hz, 1H), 3.60 (s, 3H, $m^{2'\text{-}O}$); ^{31}P NMR (202 MHz, D_2O, 25°C) δ_P: 0.65-0.67 (m, 1P, Pα), -6.73 (d, *J* = 22.2 Hz, 1P, Pγ), -10.49 – -10.69 (m, 1P, Pε), -22.36 (dd, *J* = 22.2, 18.7 Hz, 1P, Pδ); HRMS (-) ESI m/z found: 990.1097, calc. for: $C_{26}H_{36}N_{13}O_{21}P_4^-$ 990.11047.

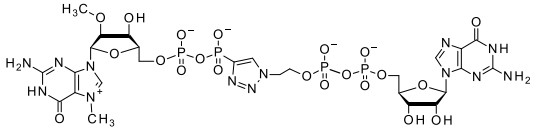
(1d) $m_2^{7,2'\text{-}O}$Gppp-t-C_2H_4OppG



Obtained according to GP A from $m_2^{7,2'\text{-}O}GpppC_2H$ (330 mOD, 0.029 mmol, 48 mM) and $GppOC_2H_4N_3$ (350 mOD, 0.029 mmol, 48 mM) while stirring with $CuSO_4$ (2.9 mg, 0.012 mmol, 19 mM) and sodium ascorbate (4.6 mg, 0.023 mmol, 38 mM) in 0.600 ml of H_2O:t-BuOH (5:1, v/v) for 1 h. After quenching the reaction with Na_2EDTA (44.7 mg, 0.12 mmol), the product was subjected to RP HPLC purification which afforded m^7Gppp-triazole-C_2H_4OppG as ammonium salt.

^{1}H NMR (500 MHz, D_2O, 25 °C) δH: 9.15 (s, 1H, H8 G or m^7G or $H_{triazole}$), 8.38 (s, 1H, H8 G or m^7G or $H_{triazole}$), 8.30 (s, 1H, H8 G or m^7G or $H_{triazole}$), 6.09 (d, *J* = 3.1 Hz, 1H, H1' m^7G), 5.93 (d, *J* = 5.3 Hz, 1H, H1' G), 4.77 (t, 1H, overlapped with HDO, H2' G), 4.66 (t, *J* = 5.4 Hz, 2H, $CH_{2linker\ CH2\text{-}N}$), 4.58 (dd, *J* = 6.0, 4.9 Hz, 1H, H3' m^7G), 4.48 (dd, *J* = 5.1, 4.0 Hz, 1H, H3' G), 4.39 – 4.26 (m, 6H, overlapped H2' m ^7G, $CH_{2linker\ CH2\text{-}O}$, H4'G, H4' m^7G, H5' G or m^7G), 4.11-4.23 (m, 3H, H4' m^7G, H5' G or m^7G, H5' and H5'' G or m^7G), 4.08 (s, 3H, m^7), 3.59 (s, 3H, $m^{2'\text{-}O}$); ^{31}P NMR (202 MHz, D_2O, 25°C) δ_P: -6.58 (d, *J* = 21.6 Hz, 1P), -10.08 – -10.87 (m, 3P, overlapped), -22.34 (t, *J* = 20.6 Hz, 1P, Pδ); HRMS (-) ESI m/z found: 1070.0792, calc. for: $C_{26}H_{37}N_{13}O_{24}P_5^-$ 1070.0768.

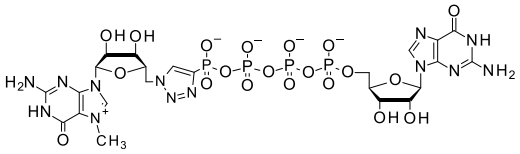
(1e) $m_2^{7,2'\text{-}O}$Gpp-t-C_2H_4OppG



Obtained according to GP A from $m_2^{7,2'\text{-}O}GppC_2H$ (330 mOD, 0.029 mmol, 48 mM) and $GppOC_2H_4N_3$ (350 mOD, 0.029 mmol, 48 mM) while stirring with $CuSO_4$ (2.9 mg, 0.012 mmol, 19 mM) and sodium ascorbate (4.6 mg, 0.023 mmol, 38 mM) in 0.600 ml of H_2O:t-BuOH (5:1, v/v) for 1 h. After quenching the reaction with Na_2EDTA (44.7 mg, 0.12 mmol), the product was subjected to RP HPLC purification which afforded m^7Gpp-triazole-C_2H_4OppG as ammonium salt.

^{1}H NMR (500 MHz, D_2O, 25 °C) δH: 9.13 (s, 1H, H8 G or m^7G or $H_{triazole}$), 8.31 (s, 1H, H8 G or m^7G or $H_{triazole}$), 8.09 (s, 1H, H8 G or m^7G or $H_{triazole}$), 6.06 (d, *J* = 3.0 Hz, 1H, H1' m^7G), 5.85 (d, *J* = 6.0 Hz, 1H, H1' G), 4.75 (dd, *J* = 5.1, 6.0 Hz, 1H, H2' G), 4.68 (t, *J* = 5.1 Hz, 2H, $CH_{2linker\ CH2\text{-}N}$), 4.53 (dd, *J* = 6.1, 4.9 Hz, 1H, H3 m^7G), 4.47 (dd, *J* = 5.1, 3.4 Hz, 1H, H3' G), 4.38 – 4.29 (m, 5H, overlapped overlapped H2' m^7G, $CH_{2linker\ CH2\text{-}O}$, H4'G, H4' m^7G), 4.26 (ddd, *J* = 11.9, 4.7, 2.6 Hz, 1H, H5' G or m^7G), 4.12-4.19 (m, 2H, overlapped H5' G or m^7G, H5'' G or m^7G), 4.05-4.10 (m, 1H, H5'' G or m^7G), 4.04 (s, 3H, m^7), 3.59 (s, 3H, $m^{2'\text{-}O}$); ^{31}P NMR (202 MHz, D_2O, 25°C) δ_P: -6.52 (d, *J* = 24.3 Hz, 1P), -10.70- -10.37 (m, 3P); HRMS (-) ESI m/z found: 990.1133, calc. for: $C_{26}H_{36}N_{13}O_{21}P_4^-$ 990.1105.

(2b) m^7G-t-ppppG



Aqueous solutions of $GppppC_2H$ (1 equiv.,424 mOD, 0.035 mmol, 50mM) and 5'-N_3-m^7G (1 equiv., 400 mOD, 0.035 mmol, 50mM) were mixed together followed by addition of H_2O and the mixture of H_2O:t-BuOH (1:1,v/v) (final H_2O:t-BuOH (2.6:1, v/v)) and aqueous solutions of $CuSO_4{\cdot}5H_2O$ (0.6 equiv.,5.3 mg, 0.021 mmol, 30mM) and sodium ascorbate (0.8 equiv.,8.4 mg, 0.042 mM,

60mM). The reaction was stirred at room temperature for 2 h and monitored by RP HPLC. After quenching the reaction with Na_2EDTA (78.2 mg, 0.21 mmol), the product was subjected to RP HPLC purification which afforded m^7G-triazole-ppppG as ammonium salt.

^1H NMR (500 MHz, D_2O, 25 °C) δH: 8.83 (bs, 1H, H8 G or m^7G or $H_{triazole}$), 8.40 (s, 1H, H8 G or m^7G or $H_{triazole}$), 8.29 (bs, 1H, H8 G or m^7G or $H_{triazole}$), 5.90 (d, *J* = 3.5 Hz, 1H, H1' m^7G), 5.86 (d, *J* = 5.7 Hz, 1H, H1' G), 4.97 – 4.86 (m, 2H, H5' and H5'' m^7G), 4.69 (dd, *J* = 5.1, 5.7 Hz, 1H, H2' G), 4.56 – 4.53 (m, 1H, H4' m^7G), 4.51 (dd, *J* = 5.1, 3.8 Hz, 1H, H3' G), 4.49 (dd, *J* = 5.4, 3.5 Hz, 1H, H2' m^7G), 4.34 (dd, *J* = 6.5, 5.4 Hz, 1H, H3' m^7G), 4.32 – 4.28 (m, 1H, H4' G), 4.25-4.17 (m, 2H, H5' and H5'' G), 4.08 (s, 3H, m^7); ^{31}P NMR (202 MHz, D_2O, 25°C) δ_P: -6.90 (d, *J* = 20.2 Hz, 1P, Pδ), -10.48 (dt, *J* = 17.2, 5.6 Hz, 1P, Pα), -22.12 – -22.75 (2P, overlapped Pβ and Pγ); HRMS (-) ESI m/z found: 932.0704, calc. for: $C_{23}H_{32}N_{13}O_{20}P_4^-$ 932.0686.

2. Stability studies

Analogues **1b-e** were incubated in sealed vials at 20 µM and at room temperature in four different buffers: 0.1 M formate buffer pH 3.0, 0.1 M acetate buffer pH 5.0, 0.1 M HEPES pH 7.0 and 0.1 M borate buffer pH 9.0. Samples of 50 µl for each condition were taken after 0, 2 and 24 h and analyzed by RP HPLC. The starting compound peak area was determined for each time-point and % of remaining cap was calculated in reference to the time-point 0 h.

3. Biological studies

3.1 Determination of association constants of eIF4E-analogue complexes

Association constants of triazole-modified cap analogues with eIF4E protein were determined using fluorescence quenching titration method as described previously.[6] Measurements were carried out in duplicates using a quartz cuvette with optical path length 4 nm for absorption and 10 nm for emission. Titration experiments were performed in 50 mM HEPES/KOH buffer pH 7.2 containing 100 mM KCl, 0.5 mM EDTA and 1 mM DTT at 20°C. During the titration experiments 1 µl volume of cap analogue solutions were added to the 1400 µl of 0.1 µM protein solution. The fluorescence of the protein was monitored at 337 nm with excitation at 280 nm. For data analysis fluorescence intensity correction on sample dilution and inner filter effect was applied. Association constants were calculated as averages by using theoretical dependence of fluorescence intensity on total cap concentration.

3.2 Incorporation of dinucleotide cap analogues at 5' end of transcripts

3.2.1 Synthesis of transcripts for capping efficiency determination

Prior to in vitro transcription annealing of a template was performed. **DNA1** and **DNA2** (20 µM each) were incubated in the presence of 4 mM Tris-HCl pH 8.0, 15 mM NaCl and 0.1 mM EDTA at 95 °C for 2 min and then at room temperature for 30 min. After 10-fold dilution the mixture was directly used for in vitro transcription.

In vitro transcription was performed on pre-annealed **DNA1** and **DNA2** (0.5 µM) with SP6 RNA polymerase (New England BioLabs) (1 U/µl) in the presence of 1x RNA Pol Reaction Buffer (New England BioLabs), 5 mM DTT, RiboLock RNase Inhibitor (ThermoFisher Scientific) (2.0 U/µl), 0.5 mM UTP, 0.5 mM ATP, 0.5 mM CTP, 0.125 mM GTP and 1.25 mM of appropriate triazole-modified dinucleotide cap analogue and reference GpppG, m^7GpppG and $m_2^{7,3'-O}$GpppG. The reaction mixture was incubated at 40 °C for 1.5 h followed by addition of TURBO Dnase (Ambion) (0.1 U/µl) and incubation at 37 °C for another 30 min. The reaction was quenched by addition of 2 µl of 0.5 M EDTA pH 8.0 and purified with RNA Clean&Concentrator-5 kit (Zymo Research) to afford capped **RNA1** strands.

To reduce heterogeneity, each capped **RNA1** (11.42 ng/µl, ca. 1 µM) was incubated in presence of 50 mM Tris-HCl pH 8.0, 50 mM $MgCl_2$, RiboLock RNase Inhibitor (ThermoFisher Scientific) (2.0 U/µl) and **DNA3** (1 µM) at 37°C for 1 h to afford **RNA2**.[7] The reaction was stopped by freezing in liquid nitrogen and directly analyzed by 15% PAGE.

Relative bands intensity was determined using CLIQS v1.0 program.

3.3 Translation studies

3.3.1 In vitro translation

mRNAs encoding *Firefly* luciferase were used for translation efficiency measurements in rabbit reticulocyte lysate. mRNAs were obtained by in vitro transcription performed on PCR product coding *Firefly* luciferase under the control of SP6 promoter (obtained from pGEN-luc (Promega) using primers: ATTTAGGTGACACTATAGAAGTACTGTTGGTAAAGCCACCATGGAAGACGCCAAAAACAT and TTACAATTTGGACTTTCCGCCCT) (5 ng/µl) with SP6 RNA polymerase (1 U/µl) (New England BioLabs) in the presence of 1x RNA Pol Reaction Buffer (New England BioLabs), 5 mM DTT, RiboLock RNase Inhibitor (ThermoFisher Scientific) (2.0 U/µl), 0.5 mM UTP, 0.5 mM ATP, 0.5 mM CTP, 0.125 mM GTP and 1.25 mM of appropriate triazole-modified dinucleotide cap analogue and reference GpppG, m^7GpppG and $m_2^{7,3'-O}$GpppG. The reaction mixture was incubated at 40 °C for 1.5 h followed by addition of TURBO Dnase (Ambion)

(0.1 U/µl) and incubation at 37 °C for another 30 min. The reaction was quenched by addition of 2 µl of 0.5 M EDTA pH 8.0 and purified with NucleoSpin RNA Clean-up XS kit (Macherey-Nagel) to afford luciferase-coding RNA strands capped with appropriate cap analogue.

For each capped luciferase-coding RNA four diluted solutions were prepared – 3.0 ng/µl, 1.5 ng/µl, 0.75 ng/µl, 0.375 ng/µl. Translation studies were performed using Rabbit Reticulocyte Lysate System (Promega). 9 µl of a mixture containing Rabbit Reticulocyte Lysate (4µl), Amino Acid Mixture Minus Leucine (0.05 µl of 1 mM solution), Amino Acid Mixture Minus Methionine (0.05 µl of 1 mM solution), potassium acetate (1.9 µl of 1 M solution), $MgCl_2$ (0.4 µl of 25 mM solution) and 2.4 µl of water was incubated at 30 °C for 1 h after which 1µl of appropriate luciferase-coding RNA solution was added and incubation of the reaction was continued at 37 °C for another hour. The reaction was stopped by freezing in liquid nitrogen.

Translation efficiency was determined using Luciferase Reporter System (Promega). The samples were defrosted just before the experiment. To every sample 50 µl of Luciferase Assay Reagent was added just before measurement of luminescence on Synergy H1 Microplate Reader (Bio Tek). The measurement were performed for every four samples independently due to low stability of luminescence signal. The results are presented as proportions between regression coefficients of linear relationships between capped luciferase-coding RNA concentration in translation reaction (300 pg/µl, 150 pg/µl, 75 pg/µl, 37.5 pg/µl) and corresponding luminescence signal.

3.3.2 Translation in vivo

3.3.2.1 Crude mRNA

mRNAs encoding *Firefly* and *Renilla* luciferase were used for translation efficiency measurements in cell culture and were synthetized by *in vitro* transcription with SP6 polymerase. pJET_luc_128A[8] and hRLuc-pRNA2(A)128[9], both digested with restriction enzyme AarI (ThermoFisher Scientifics) were used as a template to obtain *Firefly* and *Renilla* mRNA respectively. Typical *in vitro* transcription reaction (20 µl) was incubated in 40°C for 2 hours and contained: RNA Pol buffer, 1 U/µl SP6 polymerase or 1 U/µl T7 polymerase (New England BioLabs) to synthetized *Firefly* or *Renilla* mRNA, respectively, 1 U/µl RiboLock RNase Inhibitor, 0.5 mM ATP/CTP/UTP, 0.125 mM GTP, 1.25 mM cap analogue of interests and 5 µg/µl digested plasmid as a template. mRNA encoding *Renilla* luciferase was always capped with $m_2^{7,3'\text{-}O}$GpppG. Following 2-hour incubation, 1 U/µl DNase I (Ambion) was added and incubation was continued for 30 min in 37°C. Obtained mRNAs were purified with NucleoSpin RNA Clean-up XS (Macherey-Nagel). Quality of transcripts was checked on native 1.2% agarose gel whereas concentration was determined spectrophotometrically.

Human cervical carcinoma HeLa cells were grown in DMEM (Gibco) supplemented with 2 mM L-glutamine (Gibco), 10 % FBS (Sigma) and 1% penicillin/streptomycin (Gibco) at 5 % CO_2 and 37°C. In a typical experiment 24 hours before transfection, 10^4 cells were seeded in 100 µl medium without antibiotics per well of 96-well plate. Cells in each well were transfected for 1 hour using 0.3 µl Lipofectamine MessengerMAX Transfection Reagent (Invitrogen), 0.1 µg mRNA and 10 µl Opti-Mem (Gibco). mRNA used for transfection is a mixture of mRNA encoding *Firefly* luciferase (70 ng) with cap analogue of interest at its 5' end and mRNA encoding *Renilla* luciferase (30 ng) always capped with $m_2^{7,3'\text{-}O}$GpppG which serves as transfection efficiency control. After transfection, cells were washed with PBS and supplemented with fresh medium without antibiotics, and grown for the indicated times. Cell lysis was performed using Dual-Luciferase Reporter Assay System (Promega), then activities of *Firefly* and *Renilla* luciferases were measured sequentially with the Synergy H1 microplate reader. Obtained *Firefly* luminescence were normalized as before[8] in order to account transfection efficiency. Normalization operation was as follows: $F/R \cdot R_{AVG}$, where F – *Firefly* luminescence measured for chosen well, R – *Renilla* luminescence measured for the same well as F, R_{AVG} – average *Renilla* luminescence measured for all transfections at indicated time point. Normalized *Firefly* luminescence was depicted as a function of time. Total protein expression was calculated as integral of the line segments connecting obtained data points for normalized *Firefly* luminescence.

3.3.2.2 Capped mRNA

mRNAs encoding *Firefly* and *Renilla* luciferase were prepared as before (see section 3.3.2.1 crude mRNA). In order to obtain capped fraction of *in vitro* transcribed mRNAs, obtained transcripts were incubated with 10 U/µl 5'-polyphosphatase (Epicentre) in supplied reaction buffer supplemented with 1 U/µl RiboLock RNase Inhibitor for 30 min in 37°C. Following incubation mRNAs were phenol/chloroform extracted, ethanol precipitated and the recovered pellet was dissolved in deionized water. After that, extracted mRNAs were subjected to incubation with Xrn 1 (New England BioLabs) in supplied reaction buffer supplemented with 1 U/µl RiboLock RNase Inhibitor for 60 min in 37°C. After incubation mRNAs once again were phenol/chloroform extracted, ethanol precipitated and the recovered pellet was dissolved in deionized water. Capped mRNAs encoding *Firefly* and *Renilla* luciferase were used for transfection of HeLa cells as described in previous section (3.3.2.1 crude mRNA) with modification, 50 ng (35 ng of *Firefly* and 15 ng of *Renilla* mRNA) was used for transfection of each well of 96-well plate.

1.1 hDcp2-catalyzed decapping

The reaction mixture containing synthesized **RNA3** capped with $m_2^{7,2'\text{-}O}$GpppG or **1a-e** (for details of the synthesis see 3.2.2) was precipitated and the pellet was dissolved in water and incubated in the presence of 1x TURBO DNase Buffer (Ambion), RiboLock RNase Inhibitor (ThermoFisher Scientific) (2.0 U/µl) and TURBO Dnase (Ambion) (0.1 U/µl) at 37 oC for 30 min. The reaction was then

subjected to phenol-chloroform extraction followed by precipitation. The resulting RNA2 water solution was directly used for Dcp2-catalyzed decapping experiments.

Appropriate RNA2 was incubated in the presence of 50 mM NH_4Cl, 5 mM $MgCl_2$, 50 mM Tris-HCl pH 8.0, 0.01% IGEPAL CA-630 (Sigma Aldrich), 2 mM $MnCl_2$, 1 mM DTT and 7 nM hDcp2 at 37 °C. 5 µl aliquots of the reaction were collected after 5, 15, 30, 60 min of incubation or just after addition of the enzyme ("0 min" time point) and quenched with loading dye (5 M urea, 44% formamide, 20 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol) and directly analyzed by 15% PAGE.

4. References

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