Triazole-containing monophosphate mRNA cap analogs as effective translation inhibitors

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ABSTRACT

Synthetic analogs of the 5′ end of mRNA (cap structure) are widely used in molecular studies on mechanisms of cellular processes such as translation, intracellular transport, splicing, and turnover. The best-characterized cap binding protein is translation initiation factor 4E (eIF4E). Recognition of the mRNA cap by eIF4E is a critical, rate-limiting step for efficient translation initiation and is considered a major target for anticancer therapy. Here, we report a facile methodology for the preparation of N2-triazole-containing monophosphate cap analogs and present their biological evaluation as inhibitors of protein synthesis. Five analogs possessing this unique heterocyclic ring spaced from the m7-guanine of the cap structure at a distance of one or three carbon atoms and/or additionally substituted by various groups containing the benzene ring were synthesized. All obtained compounds turned out to be effective translation inhibitors with IC50 similar to dinucleotide triphosphate m7GpppG. As these compounds possess a reduced number of phosphate groups and, thereby, a negative charge, which may support their cell penetration, this type of cap analog might be promising in terms of designing new potential therapeutic molecules. In addition, an exemplary dinucleotide from a corresponding mononucleotide containing benzyl substituted 1,2,3-triazole was prepared and examined. The superior inhibitory properties of this analog (10-fold vs. m7GpppG) suggest the usefulness of such compounds for the preparation of mRNA transcripts with high translational activity.

Keywords: mRNA; cap-dependent translation; translation inhibitor; cap analog; click chemistry; 1,2,3-triazole

INTRODUCTION

All cellular eukaryotic mRNAs have at their 5′ end a unique m7GpppN cap structure (Fig. 1) composed of a 7-methylguanosine connected with the first transcribed nucleotide (N) via a 5′,5′-triphosphate bridge (Furuichi and Shatkin 2000). The cap plays a crucial role in several cellular processes, such as pre-mRNA splicing (Sharp 1994), transport of RNA from the nucleus to the cytoplasm (Lewis and Izaurralde 1997), protection of mRNA against degradation (Rhoads 1985), and translation initiation (Gingras et al. 1999). Over the years, chemically modified cap analogs have been used for biochemical, biophysical, and structural studies on mechanisms of the aforementioned processes (Niedźwiecka et al. 2002; Grudzien et al. 2004; Jankowska-Anyszka et al. 2011; Zienniak et al. 2013). So far, some of the most intense research has been directed toward the application of synthetic cap analogs, which has been focused on designing translation inhibitors that prevent binding of mRNA to elf4E. Interaction of the cap with the eukaryotic initiation factor 4E (eIF4E) is crucial and a rate-limiting step of protein synthesis (Sonenberg 1996; Rhoads 2009). Under physiological conditions, the cellular level of eIF4E is low, which constitutes one of the mechanisms enabling regulation of gene expression at the translational level. The idea of using cap analogs for therapeutic purposes emerged with reports that an elevated level of eIF4E is associated with tumor formation and progression in human malignancies and cancers of the breast, colon, bladder, prostate, lung, head, and neck (Clements and Bommer 1999; De Benedetti and Graff 2004; Mamane et al. 2004). As the cap is essential for efficient binding with the eIF4E protein, to date several structural features for the effectiveness of this interaction have been determined (Darzynkiewicz et al. 1985; Carberry et al. 1990; Marcotrigiano et al. 1997; Matsuo et al. 1997; Cai et al. 1999; Shen et al. 2001). It was shown that elements such as (1) the positive charge of the imidazole ring of guanosine, (2) the presence of at least two negative charges (or a negative charge and...
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FIGURE 1. mRNA cap structure.

the free electron pair) within the phosphate chain, and (3) the presence of at least one hydrogen atom on the amino group of the 7-methylguanosine should be preserved in order to synthesize an efficient translation inhibitor. To date, numerous modified cap analogs have been tested as inhibitors of cap-dependent translation in vitro by competing with mRNA for the binding site of eIF4E (Cai et al. 1999; Grudzien et al. 2004; Ghosh et al. 2005; Jia et al. 2010; Piecyk et al. 2012; Ziemniak et al. 2013). Unfortunately, due to their charged nature, in vivo application of such analogs is strictly limited. The first step to circumvent this problem might be designing nucleotide analogs with the least phosphate groups possible (mononucleotides monophosphates) that possess other, e. g., base modifications that provide an efficient level of inhibition. It was previously shown that a single substitution at the N2 position of the first (methylated) guanine moiety leads to increased translation inhibition (Cai et al. 1999). Based on these findings, we decided to introduce a new type of N2 substituents and evaluate the ability of obtained monophosphate analogs to inhibit the cap-dependent translation. We have chosen triazole-based groups as they not only exhibit excellent biological properties in the context of medicinal chemistry (Tron et al. 2008; Hein and Fokin 2010) but are also stable to oxidation and reduction and resistant to metabolic degradation (De las Heras et al. 1979). In this report, we describe the synthetic route for the preparation of N2-triazole-containing 7-methylguanosine-5'-monophosphates (mononucleotide cap analogs), and their biological evaluation as effective inhibitors of a cap-dependent translation. These types of compounds have not been described so far in the chemistry of mRNA cap analogs and have not been evaluated in vitro.

RESULTS AND DISCUSSION

Chemical synthesis

The triazole ring that was planned to be introduced within cap analogs can be easily generated by a copper (1) catalyzed version of the Huisgen 1,3 dipolar cycloaddition (CuAAC) between azides and terminal alkynes, which is one of the most popular “click chemistry” methodologies. This highly versatile reaction requiring mild conditions has gained over the past years enormous attention in pharmaceutical sciences and drug discovery (Solis et al. 1996; Johnson et al. 2008; Huang et al. 2013). During the past few years, a burst in the number of publications on the topic has occurred; likewise, in the area of nucleic acids, the CuAAC chemistry has been used to synthesize a number of modified nucleosides, nucleotides, and oligonucleotides for a broad range of applications (Ami and Fujimoto 2008; Seela and Sirivolu 2008; Seela et al. 2008; Amblard et al. 2009; El-Sagheer and Brown 2010). The widespread use of the CuAAC reaction is associated with the fact that both alkyne and azide functional groups can be incorporated into a wide range of compounds by several general methods. As a result of this, various strategies for the preparation of cap analogs containing 1,2,3-triazole substituents at the N2 position may be theoretically applied. Analyzing all technical aspects for the synthesis of such derivatives having this unique hetero-cyclic triazole ring (1) directly adjacent to the guanine ring, (2) spaced from the purine ring at a distance of one or three carbon atoms, or (3) additionally substituted by another aromatic ring such as benzene, we decided to explore whether a reliable efficient method(s) for their preparation could be found. Therefore, to synthesize guanosine analogs containing a triazole ring directly adjacent to the purine ring, we planned to introduce an azide group at the guanine N2 position through direct substitution of an appropriate intermediate (2) and subsequently perform the CuAAC reaction (Supplemental Scheme S1). Thus, guanosine was converted to its fully (ribose and O6 guanine) protected analog (1) through O-acylation and the Mitsunobu reaction, followed by introduction of the fluorine atom under anhydrous conditions with t-butylnitrite (tBuONO) as the diazotizing agent and HF as the fluoride source (Scheme 1; Piecyk et al. 2012). The obtained 2-fluoroinosine (2) was subjected to nucleophilic substitution with sodium azide. Unfortunately, the preparation of 2-azidoinosine was unsatisfactory, so we postulated that it might have been a consequence of spontaneous cyclization of azide substituted π-deficient nitrogen hetero-cycles to the corresponding fused tetrazole (Supplemental Fig. S1; Johnson et al. 1958; Temple et al. 1966a,b). It was previously speculated that some of the lower-yielding azide-alkyne ligations involving a C-2 azidopurine nucleoside could be due to the presence of the tetrazolyl tautomer (Amblard et al. 2009), and it was shown that the tetrazole-azide equilibrium depends on the temperature and the solvent polarity (Lakhman et al. 2010). Given these unsatisfactory results of obtaining 2-azidoinosine, we considered another route to triazole-containing cap analogs using the methodology shown in Scheme 2. We decided to perform a 1,3-dipolar cycloaddition reaction at the final stage of the synthesis using as substrates the methylated nucleotides possessing pent-4-ynyl...
or propargyl substituent at the N2 position and one of five selected azide derivatives (Fig. 2). For this, we used 2-fluorinosine (2) as a starting material and performed a substitution reaction with propargyl amine or pent-4-yn-1-amine, leading to 3a and 3b, respectively and their selective deprotection (Scheme 2). Subsequently, the N2-modified guanosine derivatives (3) were 5′-phosphorylated (Scheme 3) using the Yoshikawa method (Yoshikawa et al. 1967) with phosphorus oxide trichloride in trimethyl phosphate at 4°C (giving 4a–b) and further methylated at the N7 position of the guanine ring with CH3I in DMSO at RT, leading to compounds 5a–b. Purification of the analogs was done using ion-exchange chromatography on DEAE–Sephadex A-25 (HCO3− form). The obtained mononucleotides and selected azides (Fig. 2) were converted into triazole-containing analogs via a “click” reaction (Scheme 3). Due to the fact that the substrates (5a–b) and reaction products (6a–e) had exactly the same negative net charge, separation of the final products from the reaction mixtures using ion-exchange chromatography may have become problematic. In order to assure quantitative conversion of substrates into desired products, several trials were conducted to determine optimal reaction conditions such as solvent, copper ion concentration, reaction time, and temperature. Among all tested reaction parameters, DMF with a small amount of H2O ensuring complete dissolution of the reagents, a temperature of 50°C, and the concentration of copper ions 1.3 eq. in relation to the nucleotide turned out to be the most effective. Another important factor that allowed us to obtain efficiently triazole-containing cap analogs was usage as a coupling reaction catalyst Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (Chan et al. 2004). This polytriazolylamine ligand stabilized Cu(I) toward disproportionation and oxidation and, thus, enhanced its catalytic effect. The conducted experiments have shown that, regardless of the azide used, the reaction proceeded quantitatively when suitable coupling conditions, argon atmosphere, and TBTA were applied. In this manner, five new cap analogs containing the triazole ring at the N2 position (6a–e) (Table 1) were obtained and purified on ion-exchange DEAE–Sephadex resin. The structure and homogeneity of final products were confirmed by HPLC, MS, 1H NMR, and 31P NMR. For biological studies, compounds 6a–e were subsequently rechromatographed on a semipreparative reversed-phase HPLC column and isolated as ammonium salts.

Given these satisfactory results, we managed to develop a methodology that simplifies the synthesis, assures higher solubility of intermediates in solvents used in the “click” reaction, and eases product isolation and purification. Based on biological data described in the next paragraph, one of the mononucleotide cap analogs (6b) was converted into its dinucleotide counterpart (7). For the preparation of the desired compound, we used the well-known strategy in which the triphosphate bridge is created via a reaction between two mononucleotide units, one of which has been converted to a reactive imidazolide derivative (Kadokura et al. 1997). For this, in the first step, the imidazolide derivative of GDP (imGDP) was prepared through reaction with imidazole in the presence of 2,2′-dithiodipyridine and triphenylphosphine. Subsequently, the resultant imGDP was coupled with compound 6b in anhydrous conditions in the presence of zinc ions, giving dinucleotide 7 (Supplemental Scheme S2).

**Biology**

To evaluate the ability of newly synthesized triazole-containing monophosphates to inhibit cap-dependent translation in a cell-free translation system, the previously reported procedure was used (Kowalska et al. 2009). The chosen method,
although less direct than binding in a purified system, is preferred as it mimics competition between the cap analog and mRNA in a system containing all natural components. Briefly, experiments were performed in a micrococcal nuclease-treated rabbit reticulocyte lysate using an in vitro-transcribed, ARCA-capped, β-globin 5′ UTR containing mRNA encoding firefly luciferase to allow determination of protein synthesis by luminometry. Three standard cap analogs were used as controls in the experiments. m7GTP and m7GpppG were positive controls, as both are known to be effective translation inhibitors in various cell-free systems (Cai et al. 1999; Grudzien et al. 2004), and m7GMP was a negative control that has very little or no inhibitory capacity (Cai et al. 1999). It is well known that the largest determinants of inhibitory activity for m7G cap analogs are the phosphate groups (Cai et al. 1999). The strength of translation inhibition increases ∼20-fold for m7GpppG and ∼50-fold for m7GTP as compared to m7GMP.

In the present work, we reveal that synthesized triazole-containing monophosphate cap analogs efficiently inhibit translation similarly to m7GpppG. These results are promising in the context of designing new cap analogs that can be used as novel inhibitors of protein synthesis. We have observed that, in general, presence of a triazole ring within the N2 position of a guanine ring is sufficient to make a simple 5′-monophosphate cap analog a good translation inhibitor (Fig. 3). The determined IC50 values (Table 1) are relatively similar for all tested analogs; nevertheless, some trends can be definitely outlined.

The first explored factor was the distance between the triazole ring and the purine base and its influence on the ability of cap analogs to inhibit cap-dependent translation. Two (6b and 6e) out of five analogs were designed to differ only in the number of carbon atoms spacing both rings (one and three, respectively). Comparing IC50 values for compounds 6b and 6e, only a slight increase in inhibitory properties of the analog 6b vs. 6e was observed. This may suggest that the spacer length does not have a great impact on the effectiveness of cap analogs as translation inhibitors. A clear improvement of inhibitory properties was observed for compounds with the substituted triazole ring (6b–d) as compared to compound 6a. Interestingly, introduction of an additional oxygen hetero-atom close to the benzene ring affected considerably the ability of the cap analog to inhibit translation (about twofold for 6c and 6d vs. 6b). Introduction of further electron-donor substituents (e.g., methoxy) into the benzene ring did not decrease the IC50 value. Out of all tested monophosphate analogs, the best properties were observed for compounds 6c and 6d. Designated IC50 values for these compounds indicated that the strength of inhibition is similar to m7GTP which is known and used as a good translation inhibitor, and it is at least 10 times higher than for the corresponding 7-methylated monophosphate (m7GMP). The performed studies suggest that an appropriate substituent at the N2 position of guanine compensates for the absence of the two phosphate groups. As a consequence, the net negative charge of the cap analogs—a key factor related to the inability of compounds to cross the cell membrane—is decreased.

Taking all this into account, we decided to explore the effect of the N2 modification within the guanine ring on the inhibitory properties of the dinucleotide cap analog. This idea has appeared due to the fact that, in the case of analogs having various modifications at the N7 position, it was shown that, while the presence of a suitable substituent (e.g., benzyl or p-chlorobenzyl) caused increased (several fold) inhibitory properties of mononucleotide monophosphates, this effect was no longer visible for the corresponding dinucleotide triphosphates (Cai et al. 1999). To perform these studies, we
<table>
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<tr>
<th>N^*</th>
<th>Cap analogue structure</th>
<th>IC50 (µM)</th>
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<tr>
<td>6a</td>
<td><img src="image" alt="Structure" /></td>
<td>14.4 +/- 1.1</td>
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<td>6b</td>
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<td>10.5 +/- 1.3</td>
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<td>2.0 +/- 0.1</td>
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<td>6e</td>
<td><img src="image" alt="Structure" /></td>
<td>8.5 +/- 1.6</td>
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<tr>
<td>7</td>
<td><img src="image" alt="Structure" /></td>
<td>0.8 +/- 0.2</td>
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m^7GTP: 2.3 +/- 0.2
m^7GpppG: 8.6 +/- 0.7
m^7GMP: >50
decided to convert compound 6b into a dinucleotide (7) and test it analogously as monophosphates. It turned out that the obtained analog exhibited superior inhibitory properties (~10-fold vs. 6c and threefold vs. m7GTP). This observation emphasizes that the character of the modifications at the N7 and N2 positions is diverse and the influence of the N2 substituents on the ability of the cap analog to inhibit protein synthesis, as opposed to analogs modified at the N7 position, can still be beneficial for both mono- and dinucleotides.

In conclusion, the obtained analogs are good translation inhibitors and can be used as potential therapeutics in the future due to the fact that they possess a reduced number of phosphate groups, which may support their cell penetration. In addition to that, superior inhibitory properties for the examined dinucleotide analog suggest that this type of compound (N2 modified) may also be useful for a preparation of mRNA transcripts with high translational activity.

MATERIALS AND METHODS

Inhibition of cap-dependent translation in vitro in RRL

An inhibition assay of the cap-dependent translation in vitro in a micrococcal nuclease-treated RRL lysate (Flexi Rabbit Reticulocyte Lysate System, Promega) was performed as previously described (Kowalska et al. 2009).

A typical translation reaction contained: 56% of RRL lysate, 0.01 mM amino acids mixture, 0.32 units/μL RiboLock Ribonuclease Inhibitor (Fementas), magnesium acetate, potassium acetate, and m7GTP (up to 200 μM) inhibitors. The unmodified m7GMP (up to 200 μM) was used in the translation reactions in RRL at a final concentration 5 ng/μL. Optimal conditions for cap-dependent translation in vitro in the used batch of Flexi RRL lysate (3.2 mM concentration of endogenous Mg2+) were achieved at 0.6 mM concentration of magnesium acetate and 210 mM of potassium acetate. At these conditions, ApppG-capped luciferase RNA showed minimal translational activity that was insensitive to inhibition with the m7GpppG cap analog (Supplemental Fig. 2). Translation reactions were pre-incubated at 30°C for 60 min prior to addition of luciferase mRNA and appropriate dilution of a tested cap analog, and incubated further at 30°C. Reactions were stopped after 60 min after addition of mRNA by chilling on ice. The pre-incubation step was described in Kowalska et al. (2009) and was used in all of the further experiments that were performed in our lab. Using the same conditions allows comparison of data obtained for various cap analogs. To measure the luciferase activity, 2.5 μL of the translation reaction was mixed with 40 μL of Luciferase Assay Reagent (Promega), and a luminescence signal was detected in a luminometer (Glomax, Promega). The obtained data were used to determine IC50 values for inhibition of cap-dependent translation by cap analogs as previously described (Kowalska et al. 2009).

Chemical synthesis

All used reagents were purchased in the highest available purity from Sigma-Aldrich Chemical Co. and were used without any further treatment, except for the benzyl azides, which were prepared by treating the corresponding benzyl bromides with NaN3 in DMF. TLC analysis was performed on TLC plates coated with silicagel 60 F-254 (Merck), developed in methylene chloride-methanol-triethylamine 9:1:0.1 (v/v/v). Triethylamine and ammonium bicarbonate (TEAB) buffer was prepared by bubbling CO2 through an ice-cold aqueous solution of redistilled triethylamine. Intermediate nucleotides and final products were separated by ion-exchange chromatography on a DEAE-Sephadex A-25 (HCO3− form) using a linear gradient of TEAB buffer (pH 7.6). Fractions containing products were combined, evaporated under reduced pressure with several additions of ethanol, and isolated as

![FIGURE 3](image-url). Inhibition of m7GTP (E) and m7GpppG (F) by novel N2-substituted m7GMP analogs (A,B) 6b (●), 6a (○), 6c (△), 6d (□), and 6f (□). An example of inhibition by the dinucleotide cap analog 7 (★) derived from compound 6b is also shown (B). Standard unmodified compounds m7GTP (E) and m7GpppG (F) are included as controls. The figure shows fitted curves to the experimental data points (as described in Materials and Methods). The unmodified m7GMP (up to 200 μM) does not inhibit cap-dependent translation in the same experimental set-up.

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triethylammonium salts (TEA salts). HPLC was performed on a Knauer instrument using a Supelcosil LC-18-T RP column (4.6 × 250 mm, flow rate 1.0 mL min⁻¹) with a linear gradient of methanol from 0% to 50% (v/v) in 0.05 M ammonium acetate (pH 5.9). UV detection was performed at 254 nm. Exact mass measurements were recorded on a Micromass QToF spectrometer using electrospray detection was performed at 254 nm. Exact mass measurements were recorded on a Varian INOVA 200 and 400 MHz spectrometers using tetramethylsilane (TMS) as the internal standard in CDCl₃ and sodium 3-trimethylsilyl-2,2,3,3-D₄-propionate (TSP) in D₂O. 31P NMR spectra were recorded on a Varian INOVA 200 MHz.

**N²-Fluoro-2',3',5'-O-triacetyl-O²-[2-(4-nitrophenyl)ethyl]inosine (2)**

2',3',5'-tri-O-acetyl-O²-[2-(4-nitrophenyl)ethyl]guanosine (1.0 g, 1.79 mmol) was dissolved in anhydrous pyridine (6.75 mL, 0.082 mol) in a polypropylene tube under nitrogen atmosphere. The tube was cooled in a dry ice/acetonitrile bath (-35°C) and 70% HF/pyridine solution (12 mL, 0.42 mol) was added dropwise. The reaction mixture was stirred for 15 min, and HF/pyridine solution (12 mL, 0.42 mol) was added dropwise. The reaction mixture was stirred for 15 min, and t-butyl nitrite (0.54 mL, 4.5 mmol) was added. After 1 h, the reaction was quenched at 0°C by pouring the reaction mixture into an aqueous K₂CO₃ solution (28.5 g in 25 mL of water) and extracted three times with ethyl acetate. The organic layers were collected, dried over anhydrous sodium sulfate, and evaporated to dryness. Purification by column chromatography on silica gel using eluent 60:1 (v/v) CH₂Cl₂:MeOH gave 0.85 g (85%) of product; TLC silica gel, CH₂Cl₂:MeOH, 60:1 Rf = 0.4; ¹H NMR (700 MHz, CDCl₃) δ 8.21–8.15 (m, 2H, Ph), 8.12 (d, J = 4.7 Hz, 1H, H-1'), 4.69–4.66 (m, 6H, H-2', H-3'), 4.45–4.42 (m, 1H, H-4'), 4.37–4.34 (m, 1H, H-4'), 4.34–4.31 (m, 1H, H-5'), 4.08–4.03 (m, 1H, H-5'), 4.06 (s, 3H, N7-CH₃), 3.55–3.51 (t, 2H, NH₂-CH₂), 2.34–2.35 (t, 1H, CHC), 3.19–3.16 (t, 1H, CH₂C); m/z: (M+H)+: 562.1579, found: 562.1581.

**Procedure for the synthesis of N² methylated N2 modified 5'-monophosphates 5a–5b**

Phosphorus oxide trichloride (POCl₃) (1.1 mmol, 103 µL) and triethylamine (0.018 mol, 2.15 mL) were cooled to 4°C and added to the dried compounds 3a (0.31 mmol, 100 mg) or 3b (0.31 mmol, 109 mg). The reaction mixtures were stirred at 4°C for 3 h. The solutions were allowed to warm to RT, and 1.0 M aqueous TEAB was added to maintain the pH as neutral. Products were purified by ion-exchange chromatography on a DEAE-Sephadex A-25 column using a linear 0–1 M TEAB gradient to produce products 4a–4b as TEA salts. Compounds 4a and 4b were used directly for the methylation reaction. To the suspension of 0.28 mmol of non-nucleotide derivatives 4a (112 mg) or 4b (120 mg) in 1.5 mL anhydrous dimethylsulfoxide, methyl iodide (1.4 mmol, 87 µL) was added. The reaction mixtures were stirred at room temperature for 2 h and then were poured into the water (6 mL) and extracted several times with diethyl ether (~5 × 6 mL). The aqueous phases were purified by DEAE-Sephadex using a linear 0–0.8 M gradient of TEAB. Additionally, both nucleotides were purified on reversed-phase HPLC column. Products were lyophilized to yield: N²-(prop-2-ynyl)-7-methylguanosine-5'-monophosphate (5a); 0.185 mmol 59%, 78 mg; ¹H NMR (400 MHz, D₂O) δ 6.13 (d, J = 3.2 Hz, 1H, H-1'), 4.75–4.73 (m, 1H, H-2'), 4.48–4.45 (m, 1H, H-3'), 4.24–4.19 (m, 2H, NH₂-CH₂), 3.93–3.85 (m, 1H, H-4'), 4.45–4.42 (m, 1H, H-3'), 4.37–4.34 (m, 1H, H-4'), 4.23–4.18 (m, 1H, H-5'), 4.08–4.03 (m, 1H, H-5'), 4.06 (s, 3H, N7-CH₃), 3.55–3.51 (t, 2H, NH₂-CH₂), 2.34–2.35 (t, 1H, CHC), 3.19–3.16 (t, 1H, CH₂C); 3¹P NMR (283 MHz, D₂O) δ 1.742; m/z: (M+H)+: 444.0418; tR 15 min.

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**Procedure for the synthesis of mononucleotide cap analogs containing triazole ring ("click" reaction)**

Solution 1: 100 mg CuSO₄ × 5H₂O in 10 mL of water; Solution 2: 10 mg of sodium ascorbate in 200 mL of water.

To the suspension of mononucleotide derivatives 5a (0.0625 mmol, 26 mg) or 5b (0.0625 mmol, 27 mg) in anhydrous DMF (2 mL), solution 1 (80 µL), TBTB (0.00416 mmol, 2.2 mg), solution 2 (38 µL), and appropriate azide (0.125 mmol) (see Fig. 2) were
added and stirred under argon atmosphere overnight at 60°C. The next day reaction mixtures were terminated by the addition of EDTA (0.01 mmol, 4 mL) in 0.1 mL of water. Final products were purified by ion-exchange chromatography on a DEAE-Sephadex A-25 column using a linear 0–1 M TEAB gradient to produce compounds 6a–6c as TEA salts. Final nucleotides were additionally purified on a reversed-phase HPLC column. Compounds 6a–6c were lyophilized to yield:

N2-(((1H-1,2,3-triazol-4-yl)methyl)-7-methylguanosine-5′-monophosphate (6a): 24 mg, 86%, ammonium salt; 1H NMR (400 MHz, D2O) δ 7.89 (s, 1H, triazole), 6.07 (d, J = 3.3 Hz, 1H, H-1′), 4.73 (s, 2H, NHCH2), 4.56–4.58 (m, 2H, H-2′), 4.41–4.38 (m, 1H, H-3′), 4.35–4.32 (m, 1H, H-4′), 4.20–4.15 (m, J = 2.4 Hz, J = 4.3 Hz, J = 11.9 Hz, 1H, H-5′), 4.04–3.99 (m, J = 2.4 Hz, J = 5.0 Hz, J = 11.9 Hz, 1H, H-5′), 4.07 (s, 3H, N7-CH3); 31P NMR (162 MHz, D2O) δ 1.17; m/z: (M+H)+: 459.0882, τg 11 min.

N2-(((benzyl-1H-1,2,3-triazol-4-yl)methyl)-7-methylguanosine-5′-monophosphate (6b): 31 mg, 92%, ammonium salt; 1H NMR (400 MHz, D2O) δ 7.99 (s, 1H, triazole), 7.27–7.34 (m, 3H, Ph), 7.28–7.26 (m, 2H, Ph), 5.94 (d, J = 3.2 Hz, 1H, H-1′), 5.56 (s, 2H, NCH2Ph), 4.66 (s, 2H, NHCH2), 4.54–4.52 (m, 2H, H-2′), 4.38–4.36 (m, 1H, H-3′), 4.34–4.31 (m, 1H, H-4′) 4.21–4.17 (m, J = 2.4 Hz, J = 4.4 Hz, J = 12.0 Hz, 1H, H-5′), 4.02–4.05 (m overlapped with N7-CH3, 1H, H-5′), 4.03 (s, 3H, N7-CH3); 31P NMR (162 MHz, D2O) δ 0.61; m/z: (M+H)+: 549.1570, τg 16 min.

N2-((1-(3-(2,6-dimethoxyphenoxy)propyl)-1H-1,2,3-triazol-4-yl) methyl)-7-methylguanosine-5′-monophosphate (6c): 33 mg, 82%, ammonium salt; 1H NMR (400 MHz, D2O) δ 8.10 (s, 1H, triazole), 6.95 (t, J = 8.4 Hz, 1H, Ph), 6.47 (d, J = 8.4 Hz, 2H, Ph), 5.86 (d, J = 3.3 Hz, 1H, H-1′), 4.67–4.61 (m, 3H, H-2′, H-3′), 4.55 (m, 2H, NHCH2), 4.37–4.24 (m, 2H, H-3′, H-4′), 4.28–4.23 (m, 1H, H-5′), 4.09–4.05 (m, 1H, H-5′), 3.95 (s, 3H, N7-CH3), 3.87–3.72 (m, 2H, CH2O), 3.66 (s, 6H, OCH2), 2.38–2.32 (m, 2H, CH2CH2CH2); 31P NMR (162 MHz, D2O) δ 0.17; m/z: (M+H)+: 653.0789, τg 13 min.

N2-((1-(3-phenoxypropyl)-1H-1,2,3-triazol-4-yl)methyl)-7-methylguanosine-5′-monophosphate (6d): 32 mg, 88%, ammonium salt; 1H NMR (400 MHz, D2O) δ 8.00 (s, 1H, triazole), 7.05–7.01 (m, 2H, Ph), 6.80–6.75 (m, 1H, Ph), 6.51–6.48 (m, 2H, Ph), 5.88 (d, J = 2.9 Hz, 1H, H-1′), 4.65–4.62 (t, J = 6.1 Hz, 2H, NHCH2), 4.55 (s, 2H, NHCH2), 4.45–4.43 (m, 1H, H-2′, H-3′), 4.38–4.36 (m, 2H, H-3′, H-4′), 4.25–4.21 (m, 1H, H-5′), 4.09–4.04 (m, 1H, H-5′), 4.02 (s, 3H, N7-CH3), 3.87–3.72 (m, 2H, CH2O), 2.31–2.26 (m, 2H, CH2CH2CH2); 31P NMR (162 MHz, D2O) δ 0.70; m/z: (M+H)+: 593.1526, τg 15.3 min.

N2-(((1-(benzyl-1H-1,2,3-triazol-4-yl)propyl)-7-methylguanosine-5′-monophosphate (6e): 32 mg, 91%, ammonium salt; 1H NMR (400 MHz, D2O) δ 7.77 (s, 1H, triazole), 7.39–7.35 (m, 3H, Ph), 7.29–7.27 (m, 2H, Ph), 6.00 (d, J = 3.3 Hz, 1H, H-1′), 5.52 (s, 2H, CH2Ph), 4.61–4.58 (m, 1H, H-2′), 4.43–4.40 (m, 1H, H-3′), 4.35–3.33 (m, 1H, H-4′), 4.23–4.18 (m, 1H, H-5′), 4.08–4.03 (m, 1H, H-5′), 4.06 (s, 3H, N7-CH3), 3.43–3.39 (t, J = 6.5 Hz, 2H, NHCH2), 2.78–2.75 (t, J = 7.1 Hz, 2H, NHCH2CH2CH2), 2.04–1.92 (m, 2H, NHCH2CH2CH2); 31P NMR (162 MHz, D2O) δ 0.68; m/z: (M+H)+: 577.1842; τg 13 min.

Procedure for the synthesis of a dinucleotide cap analog (7)

P1′-N2″-(((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)-7-methylguanosine-5′-P3′-guanosine-5′-triphosphate (7): 5′-Methylguanosine 5′-diphosphate imidazolide (25.8 mg, 0.05 mmol) and ZnCl2 (33 mg, 0.24 mmol) were stirred in anhydrous DMF (0.7 mL) with compound 6b (33 mg, 0.06 mmol, TEA salt) at room temperature for 24 h. The reaction mixture was poured into a solution of EDTA (114 mg, 0.3 mmol) in water (1.5 mL) and neutralized to pH 7 by addition of 1 M TEAB. The product was separated from the reaction mixture by chromatography on DEAE-Sephadex using a 0–1.0 M gradient of TEAB. Compound 7 was obtained as colorless crystals (26 mg, 0.025 mmol, 42%, TEA salt). The final dinucleotide cap analogs were additionally purified on a reversed-phase HPLC column. Compound 7 was lyophilized to yield: 26 mg (0.025 mmol), 42%, ammonium salt; 1H NMR (400 MHz, D2O) δ: m/z 58.80 (s, 1H, triazole) 7.92 (s, 1H, H8[G]), 7.33–7.29 (m, 3H, Ph), 7.24–7.22 (m, 2H, Ph), 5.78 (d, J = 1H, J = 3.0 Hz, H-1′[m7G]), 5.72 (d, J = 6.1 Hz, 1H, H-1′[G]), 5.52 (s, 2H, PhCH2), 4.66–4.55 (m, 4H, H2′[G], H2′′[m7G], NHCH2), 4.42–4.20 (m, 8H, H3′[G], H3′′[m7G], H4′′[m7G], H4′′[G], H5′′[m7G], H5′′[G], H5′′′[m7G], H5′′′[G], 3.99 (s, 3H, N7-CH3); 31P NMR (162 MHz, D2O) δ = 11.64 (2P, Pαγ), 11.64 (2P, Pαγ), 11.64 (2P, Pαγ), 11.64 (2P, Pαγ), 11.64 (2P, Pαγ), 11.64 (2P, Pαγ), 11.64 (2P, Pαγ).
Triazole-containing cap analogs

Triazole-containing monophosphate mRNA cap analogs as effective translation inhibitors

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