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Synthesis and evaluation of 2′-*O*-allyl substituted dinucleotide cap analog for mRNA translation

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ABSTRACT

The first example of the synthesis and biological evaluation of a new analog containing 2′-OH modification on m^7G moiety, that is, $m^{7,2'\text{-}O\text{-}Allyl}GpppG$ is reported. The effect of the 2′-*O*-allyl substitution on cap analog has been evaluated with respect to its in vitro transcription by using T7 RNA polymerase, capping efficiency, and translational activity. The gel shift assay indicates that the new cap analog has 59% capping efficiency whereas the standard cap analog, m^7GpppG has a capping efficiency of 70%. The capping efficiency experiment clearly demonstrates that the new analog was a substrate for T7 RNA polymerase. The nature of the orientation has been determined by HPLC that reveals that the new analog incorporates exclusively in the forward orientation. It is noteworthy that the mRNA poly(A) capped with 2′-*O*-allyl substituted cap analog was translated ~1.7-fold more efficiently than the mRNA capped with standard cap analog. Based on the higher translational data compared to the standard cap analog, it is likely that the new analog may find application that utilize mRNA transfection such as protein production, anti-cancer immunization, and gene therapy.

1. Introduction

Capped mRNAs are those molecules that carry a cap structure $m^7G[5']ppp[5']N$ (where N is any nucleotide) at the 5′-end of the mRNA. The cap structure plays several important roles in mRNA metabolism, including mRNA splicing, localization, processing, nuclear transport, translation, and the protection of mRNA from unmethylated degradation.[1–9] Recently, their biological significance has been utilized in biotechnology that resulted in their use in gene therapy, protein production and anti-cancer immunization.[10–14] However, in vitro synthesis of capped mRNA remains challenging as the conventional method uses standard cap analog, m^7GpppG, as transcription initiator and results in two isomeric RNAs of the forms $m^7GpppG[pN]_n$ and $Gpppm^7G[pN]_n$ in approximately equal proportions depending upon the ionic conditions of the transcription reaction.[15,16] Only forward orientated sequences, that is, $m^7GpppG[pN]_n$ are translated, while the reverse form of capped mRNAs, that is, $Gpppm^7G[pN]_n$ are not recognized during the translational process. It was reasoned that the 3′-OH of both m^7G and G of the standard cap analog compete each other to act as an initiating nucleophile during the transcriptional elongation. To address this problem, anti-reverse cap analogs that carry 3′-OH modifications on m^7G moiety such as $m_2^{7,3'\text{-}O}GpppG$ and m^{7},3′dGpppG were used, in which case, the cap analogs were incorporated exclusively in the forward orientation.[17] In a subsequent study, it has been reported that cap analogs containing 2′-*O*-methyl modifications on m^7G moiety also incorporated solely in the forward orientation.[18] It is believed that the steric hindrance imparted by 2′-OH modification guides the cap analog for forward direction during the transcription.

In our continuous interest to identify novel modified cap analogs that give higher translational efficiency and longer half-life for mRNA,[19–21] we designed a novel cap analog of the form $m^{7,2'\text{-}O\text{-}Allyl}GpppG$, **5**. Since oligonucleotides containing 2′-*O*-allyl group exhibit higher target affinity and nuclease stability compared to similar sequences containing 2′-*O*-methyl group,[22] one can expect better transcriptional as well as translational properties of mRNA with 2′-*O*-ally group containing cap analog at its 5′-end.

2. Experimental

2.1. General

All commercial reagents and solvents used as such without further purification for the present study. 2-Aminoadenosine was purchased from Berry & Associates, Inc. MI, USA. ^1H NMR spectra were recorded in D_2O on a Bruker 400 MHz, ^{31}P NMR were recorded on a Bruker 162 MHz. Chemical shifts are reported in ppm, and signals are described as s (singlet), d (doublet), t(triplet), q (quartet), and m (multiplet). ESI mass spectra were recorded on a

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Applied Biosystems/Sciex MDX API 150 model and MALDI-TOF spectra were recorded on a Applied Biosystems Voyager DE-PRO model.

The HPLC based capping assay was carried out on a Waters Alliance 2695 HPLC equipped with 2996 PDA detector and Dionex DNAPac® PA200 (4 × 250 mm) column using 200 mM sodium perchlorate, pH 10.0 as a buffer. Gel shift assay was performed by using pTri β actin template and IVT reactions were performed by using linearized AmbLuc poly(A) DNA template by using the MEGAscript™ kit (Life Technologies Corporation). Purifications of the RNA from these transcription reactions were done by using the MEGAclear™ Kit (Life Technologies Corporation) as per manufacturer's protocol. Luminometer (POLARstar OPTIMA, BMG Labtech) in 96-well plates was used for luciferase assay readings as per manufacturer's protocol.

2.2. Chemistry

2.2.1. Synthesis of 2′-O-allyl guanosine 5′-monophosphate (2)

To a stirred solution of 2′-*O*-allyl guanosine **1** (1.0 g, 3.09 mmol) in 16 ml of trimethyl phosphate at 0 °C under argon atmosphere, was added phosphoryl trichloride (0.43 ml, 4.64 mmol) drop wise. After 2 h, the reaction was quenched with water and the pH of the solution was adjusted to 5.5. The crude product was chromatographed on a DEAE sepharose column using a linear gradient of 0–1 M TEAB to obtain the pure product. The desired fractions were pooled, evaporated on a rotavapor and dried in a desiccator over P_2O_5 to obtain 2′-O-allyl guanosine 5′-monophosphate **2** (0.88 g, 1.75 mmol, 56.5% yield) as a white solid. TEA salt of **1** was converted into sodium as a counter- ion and its spectral data was recorded. ^{1}H NMR (D_2O, 400 MHz) δ 8.18 (s, 1H), 5.99 (d, J = 6.4 Hz, 1H), 5.79 (m, 1H), 5.20–5.10 (m, 2H), 4.67–4.60 (m, 2H), 4.34 (m, 1H), 4.20 (dd, J = 12.8, 5.6 Hz, 1H), 4.12–3.98 (m, 3H); ^{31}P NMR (D_2O, 162 MHz) δ 4.41 (s, 1P); MS (*m/z*): 402 $[M-H]^-$.

2.2.2. Synthesis of $m^{7,2'\text{-}O\text{-}Allyl}$GMP (3)

To a stirred solution of 2′-O-allyl guanosine 5′-monophosphate **2** (0.55 g, 1.30 mmol) in water (volume: 12.96 ml) was slowly added glacial acetic acid dropwise to adjust the pH of the solution to 4. To this solution dimethyl sulfate (0.37 ml, 3.89 mmol) was added drop wise. During the addition of dimethyl sulfate, the pH of the solution was maintained at 3.8 ± 0.3 using 1 N NaOH solution. The progress of the reaction was monitored by IE HPLC (Hypersil SAX, 250 × 4.6 mm). After 5 h, the reaction mixture was extracted with ethyl acetate (4 × 50 mL) and the pH of the aqueous solution was adjusted to 5.5. Chromatography of this material on a DEAE sepharose column using a linear gradient of 0-1 M TEAB followed by evaporation of the pooled fractions containing the desired product, and dried in a desiccator over P_2O_5 to obtain $m^{7,2'\text{-}O\text{-}Allyl}$GMP **3** (0.42 g, 0.96 mmol, 73.8% yield) as a white solid. Data for **3**. ^{1}H NMR (D_2O, 400 MHz)) δ 6.21 (d, J = 2.0 Hz, 1H), 5.98 (m, 1H), 5.39 (d, J = 17.2 Hz, 1H), 5.29 (d, J = 10.4 Hz, 1H), 4.57–4.49 (m, 2H), 4.37 (m, 3H), 4.21 (m, 1H), 4.12 (s, 3H), 4.06 (m, 1H); ^{31}P NMR (D_2O, 162 MHz) δ 4.07 (s, 1P); MS (*m/z*): 417 $[M-H]^-$.

2.2.3. Synthesis of Im-GDP (4)

Im-GDP **4** was synthesized from commercially available sodium salt of GDP by following the reported procedure.[23] Sodium salt of GDP (2 g, 4.11 mmol) was passed through DEAE sephadex column and eluted with 0–1 M TEAB buffer to obtain the corresponding triethylamine salt. To a dried triethylamine salt of GDP (2.0 g, 3.69 mmol) was added triphenylphosphine (1.94 g, 7.37 mmol), 1,2-di(pyridin-2-yl)disulfane (1.63 g, 7.37 mmol), 1*H*-imidazole (1.25 g, 18.44 mmol) and 16 mL of DMF. To this solution triethylamine (0.54 ml, 4.06 mmol) was added and the solution was stirred at rt. After 15 h, the reaction mixture was precipitated with $NaClO_4$ in acetone. The precipitate was washed with acetone dried over P_2O_5 to obtain the final compound Im-GDP **4** (1.5 g, 2.92 mmol, 79% yield) as a fine white solid. The product was stored at −20 °C.

2.2.4. Synthesis of $m^{7,2'\text{-}O\text{-}Allyl}$GpppG (5)

Im-GDP **4** (0.11 g, 0.21 mmol) and $m^{7,2'\text{-}O\text{-}Allyl}$GMP **3** (0.1 g, 0.19 mmol) were taken in a round-bottomed flask and dried in vacuum over P_2O_5 for 60 min. Then, zinc(II) chloride (0.146 g, 1.07 mmol) was added followed by purging the flask with argon gas. To this flask was added 10 ml of DMF and the reaction mixture was stirred at 4 °C. The progress of the reaction mixture was monitored by IE HPLC. After 15 h, the reaction mixture was quenched with 10 ml of water containing EDTA (0.4 g, 1.07 mmol). The pH of the solution was adjusted to 5.5 with concentrated $NaHCO_3$. Chromatography of this material on a DEAE sepharose column using a linear gradient of 0–1 M TEAB followed by evaporation of the pooled fractions containing the desired product, and drying in a desiccator over P_2O_5 gave $m^{7,2'\text{-}O\text{-}Allyl}$GpppG **5** (0.1 g, 0.110 mmol, 58% yield) as a white solid. The purity of compound **5** was checked by analytical HPLC. ^{1}H NMR (D_2O, 400 MHz) δ 7.99 (s, 1H), 5.99–5.87 (m, 2H), 5.78 (d, J = 6.0 Hz, 1H), 5.37–5.23 (m, 2H), 4.63 (t, J = 5.6 Hz, 1H), 4.48–4.21 (m, 11H), 4.06 (s, 3H); ^{31}P NMR (D_2O, 162 MHz) δ −10.36 (d, J = 18.8 Hz, 1P), −10.44 (d, J = 21.4 Hz, 1P), −21.90 (t, J = 19.0 Hz, 1P); MS (*m/z*): 842 $[M-H]^-$.

2.3. Biology

2.3.1. Gel shift assay and HPLC based capping assay

The capping efficiency of modified cap **5** was compared with the standard cap m^7G[5′]ppp[5′]G. The pTri β-actin template was used in an in vitro transcription reaction omitting pyrimidine nucleotides, resulting in the termination of transcription after the first 7 coded nucleotides, all purines. Syntheses of the capped and uncapped oligoribonucleotides performed by using the MAXIscript™ kit (Life Technologies Corporation), following manufacturer's protocol. Typically, 20 μl of the transcription reactions contained the following final concentrations of components: linearized pTri β actin vector template, 25 ng/μl (0.5 μg total); ATP, 2 mM; GTP, 0.4 mM; standard cap or modified cap **5,** 1.6 mM each in separate reaction; reaction buffer, 1X; T7 RNA Polymerase-Plus™, 20 units/μl; and (α-^{32}P) ATP, 800 (Ci/mmol). In the control reaction, no cap analog was added. The transcription reactions were incubated at 37 °C for 2 h, after which the reaction mixtures (10 μl) were then applied to a 20% dPAGE gel. Radiation in the gel bands of interest were quantified by a phosphorimager (GE Healthcare). In order to determine the orientation of the incorporated cap analog the HPLC assay was developed. For this purpose, the above transcription reaction was scaled up 3X and the use of (α-^{32}P) ATP was eliminated from the reaction mixture. The remaining plasmid pTri β actin was hydrolyzed by adding 6 μl of turbo DNase to the reaction mixture, and further incubated at 37 °C for 15 min. The resulting crude reaction mixture was used as such for HPLC analysis.

2.3.2. Transcription reaction

T7 RNA polymerase transcription was performed by using the MEGAscript™ kit (Life Technologies Corporation). All transcription reactions were performed in a 20 μl final volume at the following final concentrations of components: linearized AmbLuc poly(A) DNA, (1.0 μg total); 1X reaction buffer; ATP, UTP, and CTP, 7.5 mM each; and 50 units/μl of T7 RNA polymerase. Additionally, GTP was present at 7.5 mM in the no-cap control; and in the reactions with cap analog included GTP was present at 1.5 mM while the cap analog (standard cap or modified cap **5**) was present at 6.0 mM. The transcription reactions were incubated at 37 °C for

2 h. In order to hydrolyze the remaining plasmid DNA, 1 μl of turbo DNase was added to the reaction mixture, and further incubated at 37 °C for 15 min. Purifications of the RNA from these transcription reactions were done by using the MEGAclear™ Kit (Life Technologies Corporation) as per manufacturer's protocol. The transcription yield of $m^{7,2'\text{-}O\text{-}Allyl}$GpppG **5** was comparable with the standard cap.

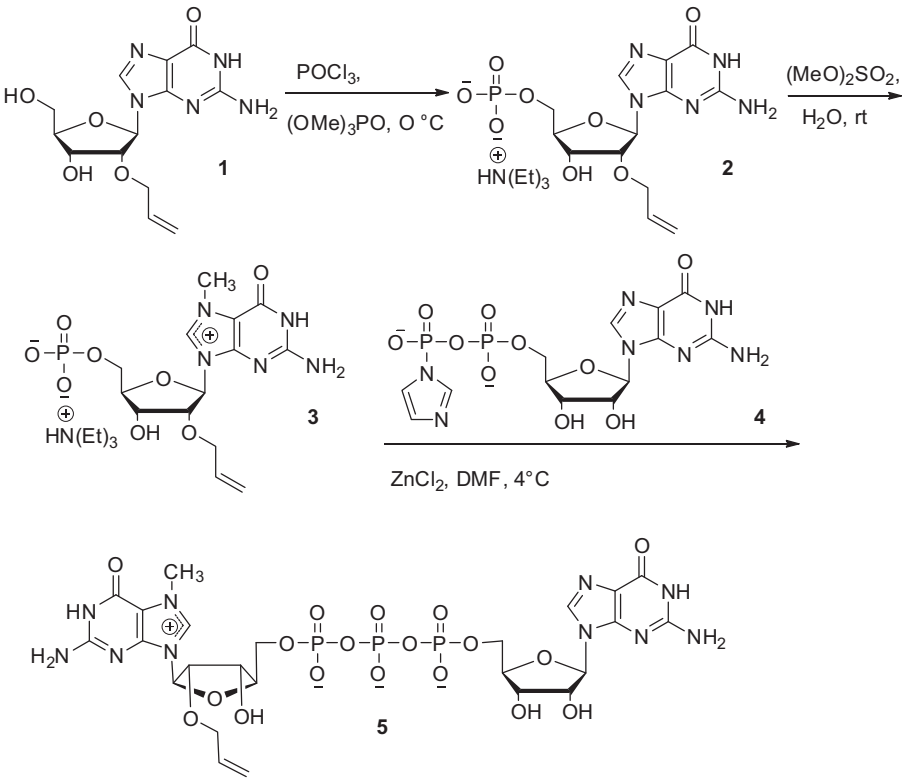
2.3.3. Luciferase assay

HeLa cells (~60,000/well in 24 well-plates) were seeded at least 12 h before transfection in growth medium without antibiotics. A complex of 5′-capped RNA was prepared by mixing 600 ng (2 μl) of RNA, 2.5 μl of TFX-20 (Promega), and 300 μl of serum-free DMEM in polystyrene tubes and incubated for 15 min at room temperature. After the incubation, media from the pre-plated HeLa cells was removed and 200 μl of the complex was added to each well. The plates were incubated for 1 h at 37 °C, and then 1 ml of pre-warmed media with serum was added. The transfected plates were incubated at 37 °C. Cells were harvested and lysed at 8, 16, 24, 32, and 40 h post-transfection. The cells were harvested and lysed by removing the media and adding 100 μl of 1X passive lysis buffer (Promega). The plate was mixed carefully to disrupt the cells and 10 μl of cell lysates from each transfections was mixed with 100 μl of luciferase substrate (Promega) and measured immediately on a luminometer (POLARstar OPTIMA, BMG Labtech) in 96-well plates. The fold induction of luciferase protein data was normalized to the control reaction, that is, the no cap, mRNA poly(A) transfection results.

2.3.4. Results and discussions

Several synthetic cap analogs have been described, which do not have the risk of reverse incorporation when co-transcriptionally attached, and do not compromise translation of the RNA. For example, a symmetrical 'two-way' cap variant of the format $m^7Gp(n)m^7G$, owing to their symmetry, are exclusively incorporated in the correct orientation.[24,25] Alternatively, substitution of one of the OH-groups in C3′ or C2′ position of the m^7G with OCH_3 overcomes reverse incorporation by completely blocking elongation at the methylated guanosine.[16,18] The respective dinucleotides $m_2{}^{7,2'\text{-}O}$GpppG and $m_2{}^{7,3'\text{-}O}$GpppG are therefore called 'Anti-Reverse Cap Analogs' or ARCA. In addition to these substitutions that block reverse incorporation, synthetic cap dinucleotides can be further modified to shape stability and translational characteristics of the respective RNA species. In continuation of our efforts[21] to test the potential beneficial effects additional chemical modifications could play, we have synthesized a 2′-*O*-allyl substituted dinucleotide cap analog, that is, $m^{7,2'\text{-}O\text{-}Allyl}$GpppG **5** and its compatibility with polymerase was studied. Literature survey shows that the precursor, 2′-*O*-allyl guanosine, required to synthesize the modified cap analog **5** can not be directly synthesized from guanosine as direct 2′-*O*-alkylation of guanosine always end up with a mixture of products the reason being guanosine base is susceptible to electrophilic attack at the O-6 and N-2 positions of the purine ring.[26] Hence, 2′-*O*-allyl guanosine was synthesized from 2-aminoadenosine in two steps as reported in the literature.[27] Thus, 2-aminoadenosine, a convenient precursor of guanosine, was alkylated with allyl bromide to give the 2′-*O*- and 3′-*O*-isomers which after treatment with adenosine deaminase in aqueous buffer at pH 7.5 gave 2′-*O*-allyl guanosine as a solid leaving the the unreacted 3′-*O*-isomer in the solution.

The synthesis of modified cap analog **5** from 2′-*O*-allyl guanosine is given in the Scheme 1. Thus, 5′-monophosphorylation of 2′-*O*-allyl guanosine under Yoshikawa condition with $POCl_3$ gave 2′-O-allyl guanosine 5′-monophosphate **2** in 57% yields. N^7-methylation of 2′-O-allyl guanosine 5′-monophosphate **2** with dimethyl sulfate at pH 4 gave the $m^{7,2'\text{-}O\text{-}Allyl}$GMP **3** as a white solid in 73% yields. Thus, sodium salt of GDP was converted into its corresponding triethylamine salt by passing through the DEAE sephadex column and eluted with TEAB buffer. The dried triethylamine salt of GDP was converted into Im-GDP with imidazole, aldrithiol, triphenylphosphine and triethylamine in 79% yield. The final coupling of



Scheme 1. Synthesis of modified cap analog **5**.

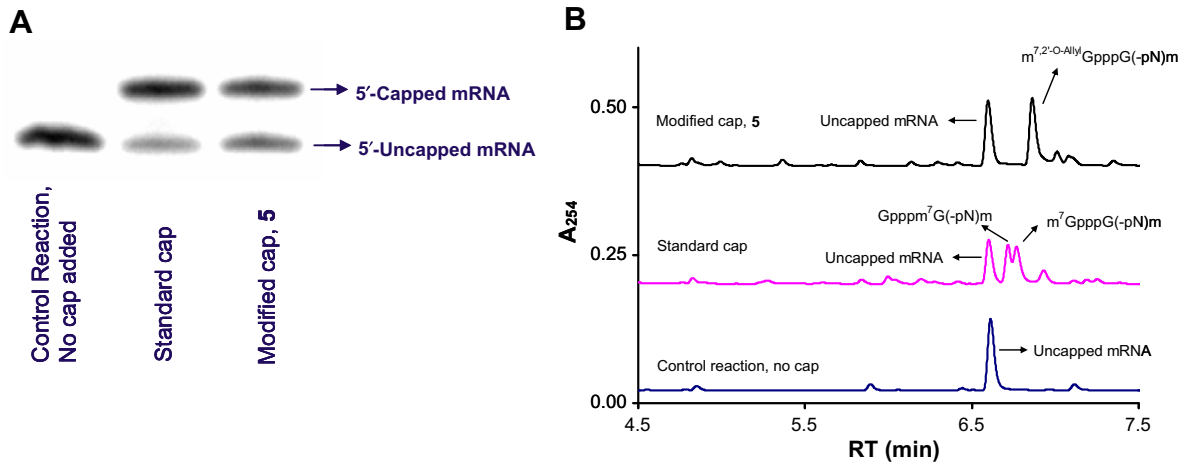


Figure 1. Capping efficiency (A) 20% dPAGE gel showing capping efficiency of standard cap and modified cap **5**. (B) Ion exchange HPLC analysis of forward and reverse orientation of 5′-capped mRNA by using modified cap **5** and standard cap analogs.

$m^{7,2'\text{-}O\text{-}Allyl}$GMP **3** with Im-GDP in the presence of zinc chloride as a catalyst gave the final compound, modified cap analog **5**, in 58% yield.

The capping efficiency of the modified cap analog **5** with respect to standard cap analog during transcription was measured by both gel shift assay (Fig. 1A) and IEX analytical HPLC assay (Fig. 1B). pTri β actin vector was used to generate transcripts of 6 nucleotides in length. For gel shift assay, the reactions were performed in the presence of (α-^{32}P) ATP to internally label the transcript. From the gel shift assay (Fig. 1A), it is clear that standard cap has a capping efficiency of 70%, while the modified cap analog **5** has a 59% capping efficiency. The capping efficiency is slightly higher because of the T7 RNA Polymerase-Plus™ enzyme (Life Technologies Corporation), which has single-base active-site mutation.

The slight lower capping efficiency of the modified cap **5** is likely due to 2′-*O*-allyl modification. The analytical HPLC (Fig. 1B) based capping efficiency experiment of modified cap analog **5** showed interesting results. RNA capped with standard cap analog showed three peaks, accounting for uncapped, forward and reverse capped RNAs. With the modified cap analog **5**, there were only two peaks, one for uncapped and another for exclusive singly directed capped RNA.

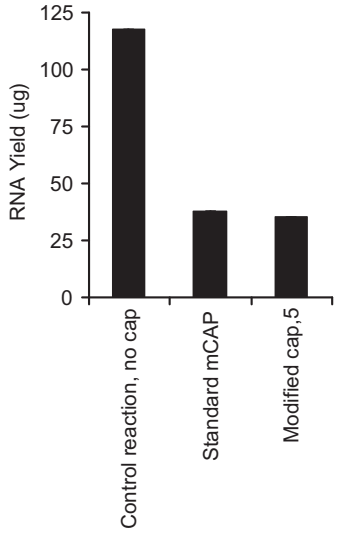


Figure 2. Yield of T7 RNA polymerase transcription reaction with standard cap and modified cap **5**. The yield of the IVT reactions was quantified by using Nanodrop at A260 nm wavelength.

The ability of modified cap analog **5** to be incorporated into 1.83 Kb mRNA was explored using AmbLuc poly(A) supercoiled plasmid (Life Technologies Corporation). Linearized plasmid, generated from the above plasmid by digestion with Blp 1 enzyme, was used as a template for in vitro transcription. The reaction was carried out in the presence of either modified cap analog **5**, or standard cap analog or no cap followed by the purification of the transcribed mRNAs using the MEGAclear™ Kit. The transcripts yield with T7 RNA polymerase indicates that modified cap analog **5** is a good substrate similar to standard cap analog (Fig. 2).

Next, the translational efficiency of modified cap analog **5** was determined with respect to standard cap analog by transfecting capped mRNA that was transcribed from AmbLuc poly(A) vector with T7 RNA polymerase into HeLa cells. Cells were harvested and lysed at different time points of post-transfection and the protein production was measured in terms of luciferase activity. Luciferase activity data was normalized to the control reaction wherein uncapped mRNA was used. After 16 h of post-transfection, the

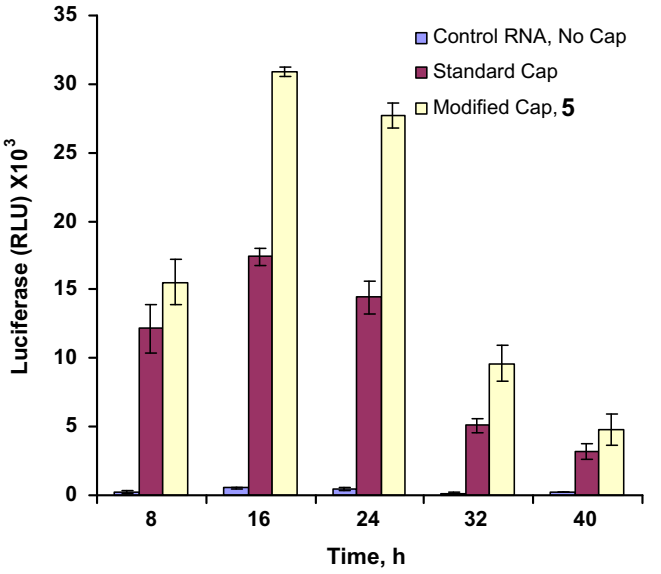


Figure 3. Translation efficiency of 5′-capped mRNA poly(A) from standard cap and modified cap **5**. The standard mCAP, and modified cap **5**, 5′-capped in vitro transcribed poly(A) tailed luciferase RNA(400 ng) were transfected into HeLa cells. The fold induction of luciferase protein data was normalized to the control reaction, that is, no cap, mRNA poly(A) transfection results.

luciferase activity of mRNA containing modified cap analog **5** was almost ~1.7-fold higher than that of mRNA containing standard cap analog. Both the longer life of modified cap analog **5** capped transcripts and the absence of nonfunctional (reverse-capped) capped transcripts during translation could be responsible for the higher translational efficiency of modified cap analog **5** capped mRNA (Fig. 3).

3. Conclusion

In conclusion, the synthesis of a new cap analog, $m^{7,2'\text{-}O\text{-}Allyl}GpppG$ **5**, that carries 2′-*O*-allyl guanosine modification is reported that generates exclusively forward-oriented capped mRNA during in vitro transcription. The new modified dinucleotide cap analog ($m^{7,2'\text{-}O\text{-}Allyl}GpppG$) was substrate for T7 RNA polymerase. Modified analog **5** also shows ~1.7-fold higher translational activity compared to standard cap analog that could be attributed to the presence of exclusive forward capped transcripts and their increased cellular stability. Considering the biological compatibility of 2′-*O*-allyl guanosine modified cap analog for both transcription and translational processes one can expect its application in protein production, anti-cancer and gene therapy.

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