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Synthesis and application of 2′-fluoro substituted cap analogs

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Abstract

Design, synthesis and biological evaluation of 2′-fluoro substituted cap analogs, i.e. $m^{7,2'F}$G[5′]ppp[5′]G and $m^{7,2'F}$G[5′]ppp[5′]m^7G are described. Structures were confirmed by ^{1}H, ^{31}P, ^{19}F NMR and MS data. The effects of the 2′-fluoro substituted moiety from the normal and N^7 double methylated mCAP were evaluated with respect to their capping efficiency, in vitro T7 RNA polymerase transcription efficiency, and translation activity using cultured HeLa cells. Luciferase fusion protein production was monitored by measuring the luciferase activity. mRNA Poly(A) capped with 2′-fluoro substituted cap analogs, ($m^{7,2'F}$G[5′]ppp[5′]G) and ($m^{7,2'F}$G[5′]ppp[5′]m^7G) were translated ~2.4 and 2.5 fold more efficient, respectively, than mRNA capped with conventional m^7G[5′]ppp[5′]G.

Keywords

Capping efficiency; Translation efficiency; In vitro transcription; 5′ Capped RNA; HeLa Cells; Luciferase activity; 2′-Fluoro substituted cap analogs

In eukaryotes, the 5′-cap structure on mRNAs consists of a 7-methylguanosine that is linked via a triphosphate bridge to the 5′-end of the first transcribed nucleotide, resulting in m^7G(5′)ppp(5′)N, where N is any nucleotide. The mRNA cap plays an important role in gene expression. It protects the mRNAs from degradation by exonucleases, enables transport of RNAs from the nucleus to the cytoplasm, and participates in assembly of the translation initiation complex. The prevailing method for the in vitro synthesis of capped mRNA employs a preformed dinucleotide of the form m^7G[5′]ppp[5′]G (mCAP) as an initiator of transcription.[1–7] The drawback of mCAP analog is that the 3′ OH of either the G or m^7G can serve as the initiating nucleophile for transcriptional elongation leading to the synthesis of two isomeric RNAs of either forward or reverse form in approximately equal proportions depending upon the ionic conditions of the transcription reaction. The reverse form of capped mRNAs i.e. G[5′]pppm^7G[pN]^n will not be recognized during the translation process, while only forward orientated sequences i.e. m^7G[5′]pppG[pN]^n will be translated.[8] The recent literature reveals that the synthesis of two "anti-reverse" cap analogs (ARCAs) such as $m_2^{7,3'\text{-}O}$G[5′]ppp[5′]G and $m^{7,}$ 3′dG[5′]ppp[5′]G are exclusively incorporated only in the forward orientation because of modifications at the 3′ OH group from N^7-methylguanosine.[9,10] In a subsequent study, it

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is also reported that by chemical modification at either the 2′ or 3′ OH group of N^7-methylguanosine, the cap has incorporated solely in the forward orientation, even though the 2′ OH group does not participate in the phosphodiester linkage.[11] This observation has prompted us to investigate 2′ OH modifications of N^7-methylguanosine. Therefore, we designed and synthesized two novel cap analogs, $m^{7,\,2'\text{-}F}$G[5′]ppp[5′]G and $m^{7,\,2'\text{-}F}$ G[5′]ppp[5′]m^7G. In order to gain further information about the structure, properties, interactions, and the impact on translational efficiency by using the 2′-fluoro modification of N^7-methylguanosine in a cap analog, we have explored the chemically convenient and reproducible method for the synthesis of novel cap analogs by using the 2′-fluoro moiety. We have synthesized two 2′-fluoro substituted cap analogs, and evaluated their biological activity with respect to in vitro transcription and translation.

The reaction leading to the desired product formation of $m^{7,\,2'\text{-}F}$GpppG **7** is shown in Scheme 1. The monophosphorylation reaction of 2′-fluoro guanosine (Metkinen Chemistry, Kuusisto, Finland) **1** was carried out using $POCl_3$ and trialkyl phosphate that afforded the corresponding 2′-F GMP **2** with 87% yield.[12] The 2′-F GMP **2** was converted into the corresponding imdiazolide salt **3** using imidazole, triphenyl phosphine and aldrithiol with 81% yield.[13] Next, the resulting imidazolide salt **3** was further phosporylated in the presence of zinc chloride that furnished the corresponding 2′-F GDP **4** with 72% yield.[13] Notably, the methylation of **4** using dimethyl sulfate as the methylating agent under acidic conditions furnished highly regioselective N^7 methylation product **5** with 83% yield.[14,15] No other isomer was detected based on the proton and phosphorous NMR spectrum. Finally, the coupling reaction of **5** with ImGMP **6** in the presence of zinc chloride as the catalyst gave $m^{7,\,2'\text{-}F}$ GpppG **7** with 58% yield.[16] The structure of **7** was thoroughly characterized by ^{1}H, ^{13}P and ^{19}F NMR and mass spectroscopic techniques.[17] The formation of $m^{7,\,2'\text{-}F}$ Gpppm^{7}G is shown in Scheme 2. Treatment of **5** with m^7ImGMP **8** in the presence of zinc chloride as the catalyst furnished $m^{7,\,2'\text{-}F}$ Gpppm^{7}G **9** with 64% yield.[18]

In order to determine the capping efficiency of the new cap analogs i.e., compounds **7** and **9**, were next tested in an in vitro transcription system by using a pTri β actin vector from MAXIscript® Kit (Ambion, Inc.) and followed by a gel shift assay.[19] Under the reaction conditions, of the four NTPs, only ATP and GTP was used, while CTP and UTP were omitted from the transcription reaction. Due to this omission only the 5′ end was transcribed by T7 RNA polymerase, producing a transcript of only 6 nucleotides in length. During the transcription reaction, GTP was compensated along with compound **7** and **9** in presence of (α-^{32}P) ATP, while the control reaction was performed without any cap analog. The resulting transcription products (6 mer RNA) were analyzed by 20% denaturing polyacrylamide/8M urea gel. The outcome of the gel shift assay, shown in Fig. 1, indicates that due to the cap at the 5′ end, the capped RNAs migrated slower than uncapped RNA. These reactions were performed in triplicate and the capping efficiency was determined by quanitating the intensities of capped versus uncapped RNA by normalizing with the background intensity. From the gel shift assay, it was clear that conventional cap i.e. m^7G[5′]ppp[5′]G has a capping efficiency of 61%, while compound **7** has a 70% capping efficiency, and compound **9**, which has a N^7 methyl on both bases of the cap structure and a 2′ fluoro substitute has a 52% capping efficiency.

To study the ability of these cap structures to reconstitute translation in cells, compounds **7** and **9** were used in a transcription reaction.[20] The supercoiled plasmid, AmbLuc Poly(A) (Ambion, Inc.), contains a 60 base poly(A) tail and was digested with Blp 1 enzyme, resulting in a linearized plasmid for use in in vitro transcription. The in vitro transcription was performed with compound **7** and **9** along with conventional mCAP cap and a control reaction which contains no cap. The transcribed 5′ capped and uncapped mRNAs were purified by using the MEGAclear™ Kit (Ambion, Inc.). Transcripts produced with T7 RNA polymerase using compounds **7** and **9** cap analogs were of the predicted length (1.85 kb) and indistinguishable

in size and homogeneity as compared to those produced with either conventional cap or control reaction. Analysis of each transcribed mRNA with poly(A) tail was performed on an Agilent 2100 Bioanalyzer, which revealed that mRNAs were not degraded and were of similar size (data not shown). The AmbLuc Poly(A) vector will express the firefly luciferase mRNA, when transcribed with T7 RNA polymerase. The RNA product generated from this transcription reaction was next transfected. During in vitro translation, protein production was measured as luciferase activity by using luciferase assay reagent (Promega). In Fig. 2, the control reaction was a normal in vitro transcription reaction having final concentration of 7.5 mM of GTP, while the cap reactions had final concentrations of 1.5 mM of GTP and 6.0 mM of each of compounds **7**, **9** and conventional mCAP, in a separate reactions. The 5′ capped and uncapped luciferase RNAs were transfected into HeLa cells[21] to determine whether the presence of $m^{7,2'F}$ G in the cap structures of compounds **7** and **9** had any effect on translation. Cells were harvested and lysed at 5, 10, 15, 20, 25, 35, and 40 h post-transfection. Luciferase activity was measured during the in vitro translation reactions and was quantified by measuring the relative light units (RLU) in a luciferase assay. Luciferase activity data was normalized to the control reaction i.e., no cap, poly(A) transfection results. Comparison of capped luciferase RNAs with poly(A) tail luciferase expression at different time points after transfection from conventional mCAP, and $m^{7,2'F}$ G containing cap analogs **7** and **9** was illustrated in Fig. 3.

To summarize, we report the synthesis of two novel dinucleotide cap analogs that, when used in in vitro transcription protocols, allows the generation of 5′ capped and Poly(A) mRNA. Capping efficiency of the 2′-fluoro substituted cap analog **7** was 9% higher than the conventional mCAP, while compound **9** was 9% lower than the mCAP. The yield from the transcription reactions in the presence of compounds **7** and **9** was in agreement with conventional mCAP. The 2′-fluoro substituted cap analogs **7** and **9** are compatible with in vitro transcription and translation of luciferase mRNA. The higher translation activity obtained with the 2′-fluoro cap analogs may be attributed to synthesis of mRNAs in which the cap is correctly oriented in all molecules, and 2′-fluoro capped mRNAs could be less prone to degradation than standard capped mRNA thereby leading to an increase in the level of translation product. Indeed, the amount of translation of the 2′-fluoro capped mRNAs was increased ~2.4 to 2.5 fold as would be expected if all the caps are correctly oriented in contrast to conventional cap analog in which half of the product would be non-functional due to incorrect orientation of the cap. The presence of the 2′-fluoro group is not responsible for improved translation activity, but rather the increase in correctly capped mRNAs molecules.

Acknowledgments

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15. Synthesis of $m^{7, 2'F}$GDP **5**: To a stirred solution of 2′-F GDP **4** (0.75 g, 1.16 mmol) in 20.0 mL of water, acetic acid was added slowly to adjust the pH of the solution to 4.0. To this mixture, dimethyl sulfate (3.0 mL) was added drop wise over a period of 30 min. and the reaction mixture was allowed to stir at rt for 4 h. As the methylation proceeds, the pH drops down to around 2.0 and readjust the pH back to 4.0 using 1M NaOH solution. After 4h, the reaction mixture was extracted with $CHCl_3$ (3 × 50 mL) to remove unreacted excess dimethyl sulfate. The collected aqueous solution was adjusted to pH 5.5 and loaded on a DEAE Sephadex column. The desired product was eluted using a linear gradient of 0 – 1M TEAB and the fractions containing the product were pooled, evaporated and dried in vacuum desiccator over phosphorous pentoxide to give a fine white powder **5** (Yield 0.64 g, 83%). Data for **5**: ^{1}H NMR (D_2O, 400 MHz) δ 6.35 (d, *J* = 14.4 Hz, 1H), 5.56 – 5.42 (m, 1H), 4.69 (m, 1H), 4.43 (m, 2H), 4.25 (m, 1H), 4.12 (s, 3H), 3.21 (q, *J* = 7.2 Hz, 12H), 1.28 (t, *J* = 7.2 Hz, 18H); ^{31}P NMR (D_2O, 162 MHz) δ −9.32 (d, *J* = 19.1 Hz), −10.54 (d, *J* = 19.6 Hz); MS (m/z): 458 $[M+H]^+$.
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17. Typical procedure to make compound **7**. To a stirred solution of $m^{7, 2'-F}$GDP **5** (0.2 g, 0.3 mmol) and ImGMP **6** (0.19g, 0.45 mmol) in 10.0 mL dry DMF, zinc chloride (81 mg, 0.6 mmol) was added under nitrogen atmosphere and the reaction mixture was stirred at rt for 1 h. After 1 h, the reaction mixture was added to a solution of EDTA disodium (0.45 g, 1.2 mmol) in 100.0 mL of water at 0 ° C. The resulting aqueous solution was adjusted to pH 5.5 and loaded on a DEAE Sephadex column. The desired product was eluted using a linear gradient of 0 – 1M TEAB and the fractions containing the product were pooled, evaporated and concentrated to 10.0 mL TEA salt of **7**. The resulting 10.0 mL was passed through a Strata-X-AW column (Phenomenex) and washed with 10.0 mL water followed by 10.0 mL MeOH. Then, the desired compound was eluted with 15.0 mL of NH_4OH/MeOH/H_2O (2/25/73) and the collected solution was evaporated and dried to give a fine white powder **7**. (Yield: 0.15 g, 58%). Data for **7**. ^{1}H NMR (D_2O, 400 MHz) δ 8.02 (s, 1H), 6.14 (d, *J* = 14.4 Hz, 1H), 5.80 (d, *J* = 5.6 Hz, 1H), 5.41 – 5.27 (m, 1H), 4.63 – 4.47 (m, 3H), 4.39 – 4.24 (m, 6H), 4.03 (s, 3H); ^{31}P NMR (D_2O, 162 MHz) δ −10.4 (d, *J* = 16.8 Hz), −10.54 (d, *J* = 18.6 Hz), −21.95 (t, *J* = 19.3 Hz); ^{19}F NMR (D_2O, 376 MHz) δ −20.47 (m); MS (m/z): 803 $[M+H]^+$.
18. Compound **9**: ^{1}H NMR (D_2O, 400 MHz) δ 6.31 (d, *J* = 14.8 Hz, 1H), 6.02 (d, *J* = 3.2 Hz, 1H), 5.53 – 5.37 (m, 1H), 4.75 – 4.62 (m, 2H), 4.52 – 4.38 (m, 5H), 4.30 – 4.21 (m, 2H), 4.11 (s, 6H); ^{31}P NMR (D_2O, 162 MHz) δ −10.35 (d, *J* = 11.8 Hz), −10.50 (d, *J* = 10.0 Hz), −21.80 (t, *J* = 19.1 Hz); ^{19}F NMR (D_2O, 376 MHz) δ −20.51 (m); MS (m/z): 817 $[M]^+$.
19. Gel shift assay was performed by using MAXIscript kit (Ambion, Inc.) by following manufacturer's protocol. Typical 20 μL of T7 RNA polymerase transcriptions contained the following reagents at the final concentrations indicated: linearized pTri β actin vector template, 0.5 μg; ATP, 2 mM; GTP, 0.4 mM; compound **7** and **9** 1.6 mM each in separate reaction; 10X reaction buffer, 4X; T7 RNA polymerase, 50 units/μL; (α-^{32}P) ATP, 800 (Ci/mmol); and DEPC water. The control reaction was normal in vitro transcription reaction, in which 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).
20. T7 RNA polymerase transcription was performed by using MEGAscript kit (Ambion, Inc.) in 20 μL final volume, and contains the following reagents at the final concentrations indicated: linearized AmbLuc Poly(A) DNA, 1.5 μg; 1X reaction buffer; ATP, UTP, and CTP, 7.5mM each; while GTP was compensated with compound **7**, **9**, and conventional cap analog. GTP, 1.5mM; compound **7**, **9** or m^7GpppG cap analogs, 6.0 mM; and 50 units/μl of T7 RNA polymerase. 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. Purification of transcription reactions were done by using MEGAclear™ Kit (Ambion, Inc.) as per manufacturer's protocol.

21. 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 capped RNA was prepared by mixing 1.8 μg of RNA, 7.5 μL of Tfx™-20 Reagent (Promega), and 900 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 (400 ng) of the complex was added to three wells. 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 for different timepoints. Cells were harvested and lysed at 5, 10, 15, 20, 25, 35, and 40 h. The cells are harvested 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 were mixed with 100 μL of Luciferase Assay System substrate (Promega) and measured immediately on POLARstar OPTIMA Luminometer (BMG Labtech) in 96-well plates. The fold induction of luciferase protein data was normalized to the control reaction i.e. no cap, mRNA Poly(A) transfection results.

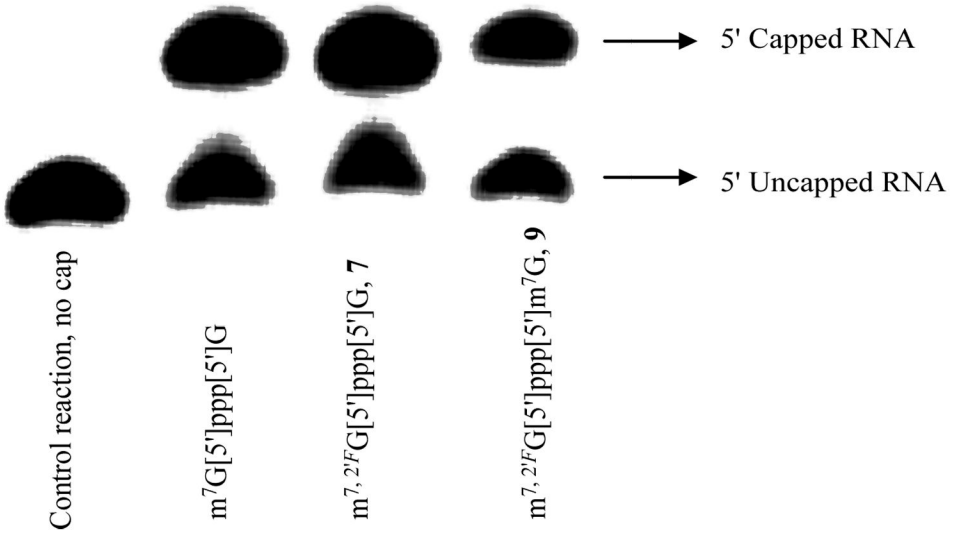


Figure 1.
20% dPAGE gel showing capping efficiency of compound **7** and **9** along with conventional cap analog. The control reaction was normal in vitro transcription reaction, in which no cap analog was added. Radiation in the gel bands of interest were quantified by a phosphorimager (GE Healthcare).[19]

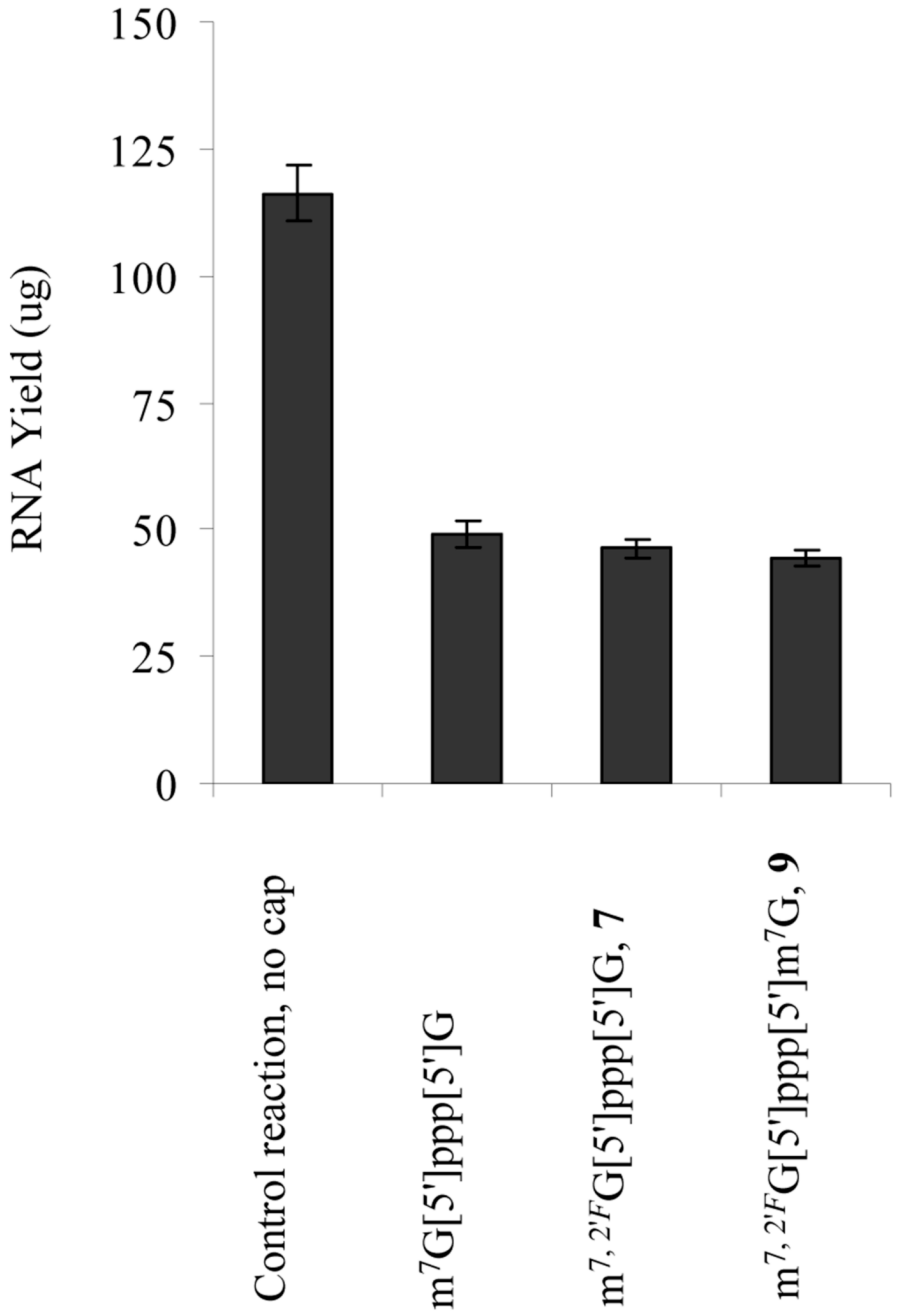


Figure 2.
Yield of T7 RNA polymerase transcription recation with compound **7** and **9**.[20]

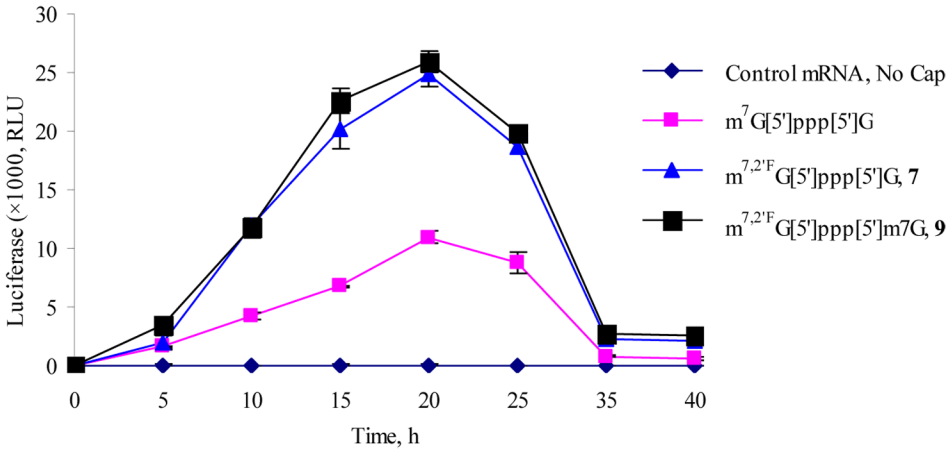
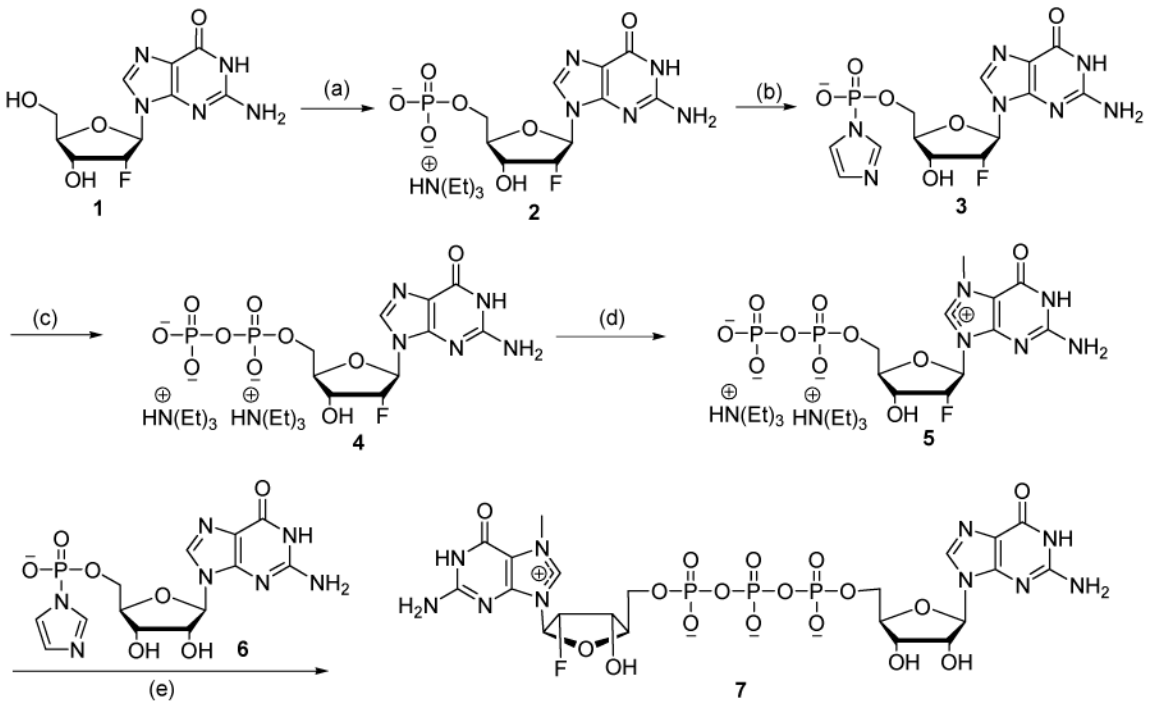


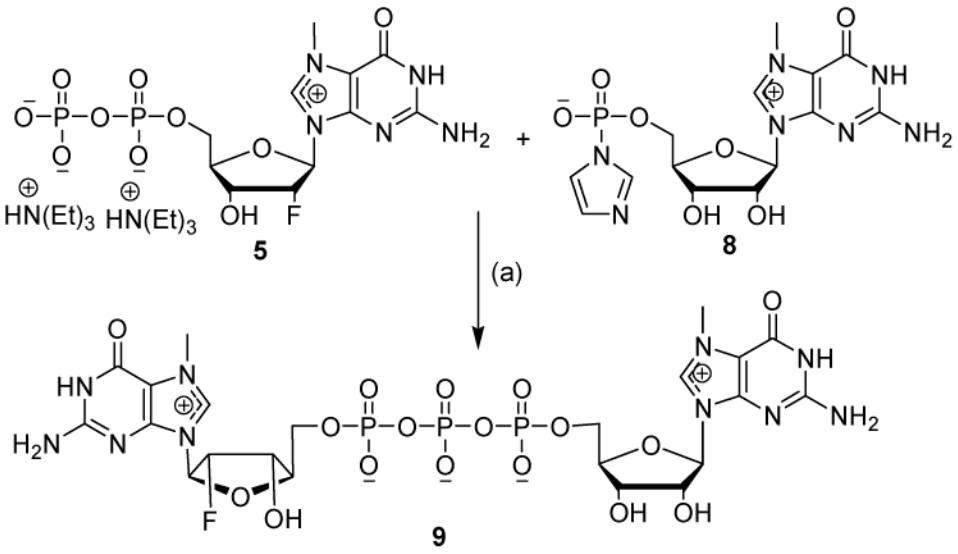
Figure 3.

Translation efficiency of compound **7** and **9** capped luciferase RNA Poly(A). Comparison of protein expression from conventional mCAP and 2′-fluoro containing compounds **7** and **9** capped luciferase RNAs Poly(A), at different time points after Transfection. The conventional mCAP, compound **7** and **9** capped in vitro transcribed Poly(A) tailed luciferase RNA (400 ng) was Transfected into HeLa cells. Cells were harvested and lysed at different time points. Luciferase activity was measured and plotted against transfection time. The fold induction of luciferase protein data was normalized to the control reaction i.e. no cap, mRNA Poly(A) transfection results.[21]



Scheme 1.

Reagents and conditions: (a) $POCl_3$, $(OMe)_3P$, O °C, 2 h; (b) imidazole, aldrithiol, PPh_3, DMF, rt, 5 h; (c) $(Et_3NH)_3PO_4$, DMF, rt, 3 h; (d) dimethyl sulfate, water, pH 4.0, rt, 4 h; (e) $ZnCl_2$, DMF, rt, 1 h.



Scheme 2.
Reagents and conditions: (a) $ZnCl_2$, DMF, rt, 6 h.