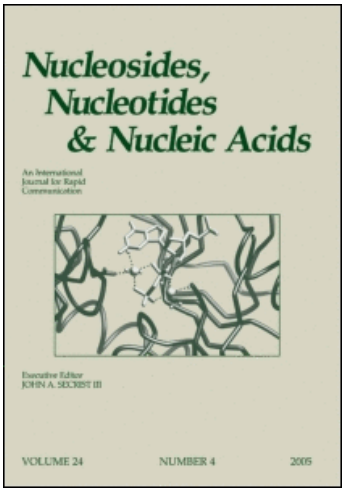

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Synthesis and Properties of mRNA Cap Analogs Containing Phosphorothioate Moiety in 5',5'-Triphosphate Chain

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SYNTHESIS AND PROPERTIES OF mRNA CAP ANALOGS CONTAINING PHOSPHOROTHIOATE MOIETY IN 5′,5′-TRIPHOSPHATE CHAIN

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▫ *Nucleosides and oligonucleotides with an oxygen replaced by sulfur atom are an interesting class of compounds because of their improved stability toward enzymatic cleavage by nucleases. We have synthesized several dinucleotide mRNA cap analogs containing a phosphorothioate moiety in the α, β, or γ position of 5′,5′-triphosphate chain [m^7Gp(s)ppG, m^7Gpp(s)pG, and m^7Gppp(s)G]. These are the first examples of the biologically important 5′mRNA cap analogs containing a phosphorothioate moiety, and these compounds may be useful in a variety of biochemical and biotechnological applications. Incorporation of a sulfur atom in the α or γ position within the dinucleotide cap analog was achieved using $PSCl_3$ in a nucleoside phosphorylation reaction followed by coupling the phosphorothioate of nucleoside with a second nucleotide. Synthesis of cap analogs with the phosphorothioate moiety in β position was performed using an organic phosphorothioate salt in a coupling reaction with an activated nucleotide. The structures of newly synthesized compounds was confirmed using MS and ^1H and ^{31}P NMR spectroscopy. We present here the results of preliminary studies on their interaction with translation initiation factor eIF4E and enzymatic hydrolysis with human and nematode DcpS scavengers.*

Keywords mRNA Cap Analogs, Phosphorothioate, eIF4E, DcpS

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INTRODUCTION

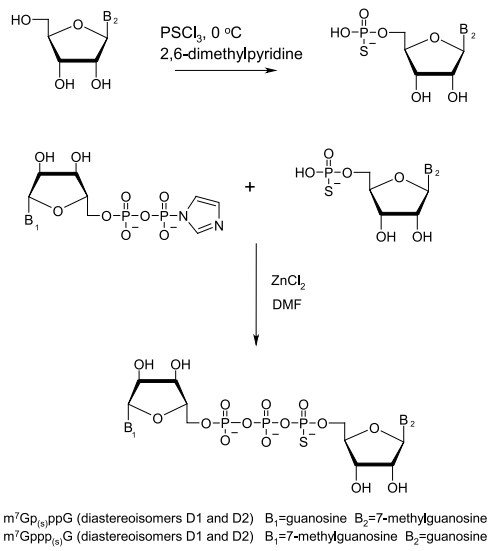
One of the most important functions of 5′mRNA cap structure is its involvement in initiation of protein translation.[1,2] Cap structure is recognized by protein initiation factor eIF4E.[3] Many tumors express very high levels of eIF4E and this may be a critical factor in disease progression.[4] It has been established in in vitro studies that synthetic cap analogs are effective inhibitors of protein biosynthesis due to their competition with capped mRNA for binding with eIF4E.[5,6] Unfortunately, many enzymes responsible for the hydrolysis of pyrophosphate bonds (for example ecto-NTPdiphosphohydrolase or ecto-nucleotide Pyrophosphatase/diesterase) are present inside the cell as well as on the cell membrane surface.[7] This makes the in vivo application of cap analogs as potential drugs problematic. In these studies we have synthesized and evaluated a novel class of cap analogs containing a phosphorothioate moiety in the triphosphate bridge. This modification of nucleotides is known to increase their stability and protects against many hydrolytic enzymes. We report here the synthesis of three cap analogs containing phosphorothioate moiety in α, β, or γ position of triphosphate bridge. In all cases, the final step of the synthesis led to a mixture of two diastereoisomers. Proteins that interact with the mRNA cap were shown to interact differentially with these diastereoisomers. We predict that this modification of the triphosphate bridge of cap analogs may produce compounds that are useful tools for in vivo assays. The synthetic approaches reported here should be applicable for preparation of many desirable nucleoside oligophosphates modified within the oligophosphate chain.

RESULTS AND DISCUSSION

Synthesis of Dinucleotide Cap Analogs Containing Sulfur in α [$m^7Gp_{(s)}ppG$] or γ [$m^7Gppp_{(s)}G$] Position of Triphosphate Bridge

Guanosine 5′O-phosphorothioate was synthesized using the method based on O-thiophosphorylation of guanosine with $PSCl_3$ in triethyl phosphate. Application of a basic catalyst like 2,6-dimethylpyridine significantly improved the yield of the reaction.[8] The obtained guanosine 5′O-phosphorothioate (as triethylammonium salt) was directly consumed in the subsequent coupling reaction in dimethylformamide solution in the presence of $ZnCl_2$, with imidazole activated 7-methylguanosine 5′-diphosphate to produce $m^7Gppp_{(s)}G$ (Scheme 1).

A similar procedure was applied for synthesis of $m^7Gp_{(s)}ppG$ containing sulfur in the α position. Steps for this synthesis included 5′-thiophosphorylation of 7-methylguanosine with $PSCl_3$ in trimethyl phosphate, isolation of 7-methylguanosine 5′-phosphorothioate as a triethylammonium salt, and finally its coupling with guanosine 5′-diphosphate imidazolide. The coupling reaction depicted in Scheme 1 could proceed by attack of 5′-O-phosphorothioate ion via oxygen or sulfur atom. That could lead to two products, one possessing sulfur in non-bridging position and the second with sulfur between the two phosphorus atoms. ^{31}P NMR studies

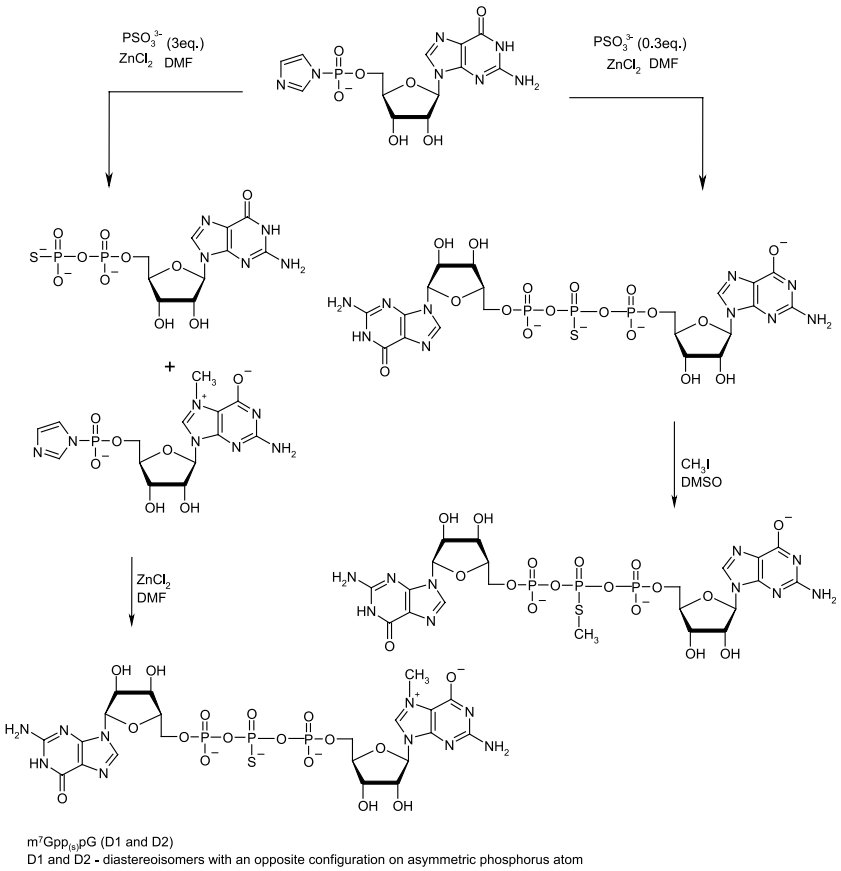


SCHEME 1 Synthesis of m7Gp(S)ppG and m7Gppp (S)G diastereoisomers.

demonstrated only one product. However, the product containing a P–S–P bond may rapidly decompose during the chromatographic separation. Activated nucleoside diphosphate was coupled with nucleoside 5′O-phosphorothioate as a triethylammonium salt in DMF using $ZnCl_2$ as a catalyst. The activation of nucleoside diphosphate (as imidazolide) was performed using high molar excess of imidazole and Ph_3P/2,2′-dithiodipiridine system. The final products, $m^7Gp_{(s)}ppG$ and $m^7Gppp_{(s)}G$, were obtained as diastereoisomeric mixtures. Separation of the mixtures by HPLC (RP column) gave two pure diastereoisomers D1 (diastereoisomer with shorter $Rt_{RP\ column}$) and D2 in both cases. However, at this time we are not able to assign an absolute configuration on the phosphorus atoms to the individual diastereoisomers. ^{31}P NMR spectra of $m^7Gp_{(s)}ppG$ showed the following δ values: P_α + 43.494 ppm, P_β − 23.999 ppm, P_γ − 11.355 ppm, and for $m^7Gppp_{(s)}G$: P_α − 11.678 ppm, P_β − 24.150 ppm, P_γ + 43.481 ppm (vs. 10% H_3PO_4 in D_2O as a external standard). A detailed description of ^1H and ^{31}P NMR spectra will be published elsewhere.

Synthesis of Dinucleotide Cap Analogs Containing Sulfur in Position β [$m^7Gpp_{(s)}pG$] of Triphosphate Bridge

In the first approach we decided to synthesize symmetrical $Gpp_{(s)}pG$ in one pot reaction and then to use an equimolar amount of methyl iodide for methylation of N-7 position of guanosine. This approach has been successfully adopted for synthesis of m^7GpppG, although doubly methylated compound has been also observed.[9] We synthesized $Gpp_{(s)}pG$ in excellent yield coupling of GMP imidazolide with phosphorothioate/TEA salt in 2:1 ratio in DMF. Unfortunately, the



SCHEME 2 Synthesis of m7Gpp(S)pG diastereoisomers.

methylation of $Gpp_{(s)}pG$ gave an undesired product, being exclusively methylated on the sulfur atom (Scheme 2). This result indicates that the phosphorothioate must be protected with a suitable protecting group that is easy removable under conditions mild enough to maintain the 7-methylguanine moiety not destroyed prior to methylation. Refinement of this approach is in progress.

The second approach to the synthesis of $m^7Gpp_{(s)}pG$ is depicted in Scheme 2. Guanosine 5′O-(1-thiodiphosphate) (abbr. GDP β-S) was first synthesized from guanosine 5′monophosphate imidazolide using two molar excess of triethylammonium phosphorothioate. Decomposition of GDP β-S to GMP was observed to occur at room temperature thus product purification was performed at 4°C. Obtained GDP β-S was then coupled with imidazole activated 7-methylguanosine 5′-monophosphate. The yield of this step was moderate (20%) mainly due to contamination of m^7GMP-Im with m^7GMP, which led to m^7Gppm^7G in the imidazolide reaction. $m^7Gpp_{(s)}pG$ was obtained as a mixture of diastereoisomers and we are presently trying to separate them by HPLC in order to obtain pure

TABLE 1 Binding Free Energies (G°) and Equilibrium Association Constants (K_{as}) for the Interaction of Mouse eIF4E(28-217) with Dinucleotide Cap Analogs at 20°C

Cap analog	K_{as} (μM^{-1})[a]	$\Delta G°$ (kcal/mol)
m^7GpppG	9.9 ± 0.2	−9.34 ± 0.01
$m^7Gp_{(s)}ppG$-D1	30.6 ± 0.5	−10.04 ± 0.01
$m^7Gp_{(s)}ppG$-D2	9.7 ± 0.4	−9.36 ± 0.02
$m^7Gppp_{(s)}G$-D2	12.4 ± 0.3	−9.51 ± 0.01

[a]Fluorescence titration were performed in 50 mM HEPES/KOH (pH 7.2), 0.1 mM KCl, 0.5 mM EDTA and 1 mM DTT, K_{as} were determined using equation from Niedzwiecka et al.[10]

diastereoisomers D1 ($Rt_{RP\ column}$ = 5.88 min) and D2 ($Rt_{RP\ column}$ = 6.25 min). The NMR spectra were determined for the mixture of $m^7Gpp_{(s)}pG$: D1 and D2. The ^{31}P NMR spectrum of D1 and D2 showed the same δ values (P_α and P_γ − 12.301 ppm, P_β + 30.034 ppm) and in the ^1H NMR spectrum (400 MHz) some signals from D1 and D2 are shifted by ∼0.02 ppm (e.g., H1′ in G and m^7G). MALDI-MS spectrum (negative mood) recorded for the mixture of diastereoisomers showed m/z = 816.977 (M − 1) being in good agreement with calculated value.

Binding Affinity of Cap Analogs for eIF4E

To determine how the substitution of phosphorothioate moiety for the phosphate influences the binding interaction of these cap analogs with eIF4E, we used the fluorescence titration method[10] to measure and compare the binding affinity of mouse eIF4E(28-217) for the three newly synthesized dinucleotide cap analogs: $m^7Gp_{(s)}ppG$-D1, $m^7Gp_{(s)}ppG$-D2 and $m^7Gppp_{(s)}G$-D2, to the standard cap analog, m^7GpppG. The observed values of K_{as} for $m^7Gp_{(s)}ppG$-D2 and $m^7Gppp_{(s)}G$-D2 do not differ considerably with K_{as} for m^7GpppG (Table 1). However, $m^7Gp_{(s)}ppG$-D1 binds eIF4E with a 3-fold higher affinity (Table 1). This may be due to preferences of eIF4E for a peculiar cap conformation or formation of additional intermolecular contacts ($\Delta\Delta G° \sim 0.7$ kcal/mol) between cap and eIF4E.

Hydrolytic Susceptibilities to DcpS Scavengers

Enzymatic hydrolysis of the three selected phosphorothioate caps was examined with human and nematode DcpS. These proteins catalyze cleavage of free cap dinucleotide or the cap on short mRNA fragments producing m^7GMP and 5′-diphosphate terminated cap or mRNA products.[11] Cap cleavage reactions (carried at 30°C in 50 mM Tris HCl pH 7.9 containing 30 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$ and 1 mM DTT) were evaluated by fluorescence and HPLC methods. $m^7Gp_{(s)}ppG$-D1 and $m^7Gp_{(s)}ppG$-D2 were resistant to cleavage by both enzymes. For $m^7Gppp_{(s)}G$-D2, hydrolysis was observed using both the human and nematode DcpS enzymes with a rate comparable to that observed for the standard cap analog m^7GpppG.

REFERENCES

1. Furuichi, Y.; Shatkin, A.J. mRNA caps: past and prospects. Adv. Virus Res. **2000**, *55*, 135–184.
2. von der Haar, T.; Gross, J.D.; Wagner, G.; McCarthy, J.E. The mRNA cap-binding protein eIF4E in post-transcriptional gene expression. Nat. Struct. Mol. Biol. **2004**, *11*(6), 503–511.
3. Gingras, A.-C.; Raught, B.; Sonenberg, N. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Ann. Rev. Biochem. **1999**, *68*, 913–963.
4. Berkel, H.J.; Turbat-Herrera, E.A.; Shi, R.H.; de Benedetti, A. Expression of the translation initiation factor eIF4E in the polyp-cancer sequence in the colon. Cancer Epidemiol. Biomark. Prev. **2001**, *10*, 663–666.
5. Cai, A.; Jankowska-Anyszka, M.; Centers, A.; Chlebicka, L.; Stepinski, J.; Stolarski, R.; Darzynkiewicz, E.; Rhoads, R.E. Quantitative assessment of mRNA cap analogs as inhibitors of in vitro translation. Biochemistry **1999**, *38*, 8538–8547.
6. Jemielity, J.; Fowler, T.; Zuberek, J.; Stepinski, J.; Lewdorowicz, M.; Niedzwiecka, A.; Stolarski, R.; Darzynkiewicz, E.; Rhoads, R.E. Novel "anti-reverse" cap analogs with superior transnational properties. RNA **2003**, *9*, 1108–1122.
7. Gendaszewska-Darmach, E.; Maszewska, M.; Zaklos, M.; Koziolkiewicz, M. Degradation of extracellular nucleotides and their analogs in HeLa and HUVEC cell cultures. Acta Biochim. Pol. **2003**, *50*, 973–984.
8. Moran, J.R.; Whitesides, G.M. A practical enzymatic synthesis of (S_P)-adenosine 5′-O-(1-thiotriphosphate) ((S_P)-ATP-α-S). J. Org. Chem. **1984**, *49*, 704–706.
9. Stepinski, J.; Bretner, M.; Jankowska, M.; Felczak, K.; Stolarski, R.; Wieczorek, Z.; Cai, A.; Rhoads, R.E.; Temeriusz, A.; Haber, D.; Darzynkiewicz, E. Synthesis and properties of P1,P2-, P1,P3- and P1,P4-dinucleoside di-, tri- and tetraphosphate mRNA 5′-cap analogues. Nucleosides Nucleotides **1995**, *14*, 717–721.
10. Niedzwiecka, A.; Marcotrigiano, J.; Stepinski, J.; Jankowska-Anyszka, M.; Wyslouch-Cieszynska, A.; Dadlez, M.; Gingras, A.-C.; Mak, P.; Darzynkiewicz, E.; Sonenberg, N.; Burley, S.K.; Stolarski, R. Biophysical studies of eIF4E cap-binding protein: recognition of mRNA 5′ cap structure and synthetic fragments of eIF4G and 4E-BP proteins. J. Mol. Biol. **2002**, *319*, 615–635.
11. Cohen, L.S.; Mikhli, M.; Friedman, C.; Jankowska-Anyszka, M.; Stepinski, J.; Darzynkiewicz, E.; Davis, R.E. Nematode m^7GpppG and $m_3^{2,2,7}$GpppG decapping: activities in ascaris embryos and characterization of *C. elegans* scavenger DcpS. RNA **2004**, *10*, 1609–1624.