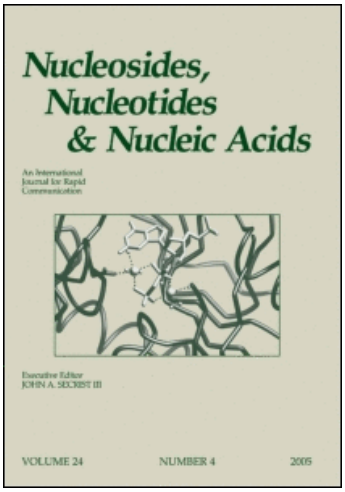

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NOVEL DINUCLEOSIDE 5′,5′-TRIPHOSPHATE CAP ANALOGUES. SYNTHESIS AND AFFINITY FOR MURINE TRANSLATION FACTOR eIF4E

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▫ *Chemical synthesis of a series of novel dinucleoside cap analogues, m^7GpppN, where N is formycin A, 3′-O-methylguanosine, 9-β-D-arabinofuranosyladenine, and isoguanosine, has been performed using our new methodology. The key reactions of pyrophosphate bonds formation were achieved in anhydrous dimethylformamide solutions employing the catalytic properties of zinc salts. Structures of the new cap analogues were confirmed by ^{1}H NMR and ^{31}P NMR spectra. The binding affinity of the new cap analogues for murine eIF4E(28–217) were determined spectroscopically showing the highest association constant for the analogue that contains formycin A.*

Keywords eIF4E, Fluorescence Titration, Cap Analogues, Formycin A, 3′-O-methylguanosine, Isoguanosine

INTRODUCTION

The mRNAs cap structure, comprising a 7-methylguanosine joined to the 5′ end of the mRNA *via* a 5′,5′ triphosphate linkage plays an essential role in many processes including initiation of translation, pre-mRNA splicing, mRNA stability, and intracellular transport of mRNA in eukaryotic cells.[1] A great variety of cap analogues have been synthesized and tested in vitro for their capacity to substitute for the native form of mRNA 5′ end.[2–5] In this regard, the synthetic cap analogues

The NMR and MS spectra were recorded in the Laboratories of Nuclear Magnetic Resonance and Mass Spectrometry, Institute of Biochemistry, and Biophysics Polish Academy of Sciences in Warsaw, respectively. The project was supported by the State Committee for Scientific Research P 04A 021 25, PBZ-KBN 059/T09/10, and by NIH FIRCA No. 1R03TW006446-01.

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turned out to be an invaluable tool for elucidating physiological processes on the way of gene expression.

In the present work we describe syntheses of new dinucleotide cap analogues with four different nucleosides, formycin A, 3′-O-methylguanosine, 9-β-D-arabinofuranosyladenine, and isoguanosine, linked by triphosphate to the terminal 7-methylguanosine, m^7GpppF, $m^7Gpppm^{3'O}G$, $m^7Gppp^{ara}A$, and $m^7Gppp^{iso}G$. The newly-synthesized analogues have been tested for the binding to truncated murine eIF4E (28–217) by measurements of the intrinsic protein fluorescence quenching in the titration experiments.[6] A motivation for discovering cap analogues with higher affinity for eIF4E is the potential of developing novel biochemical tools in anticancer therapies.

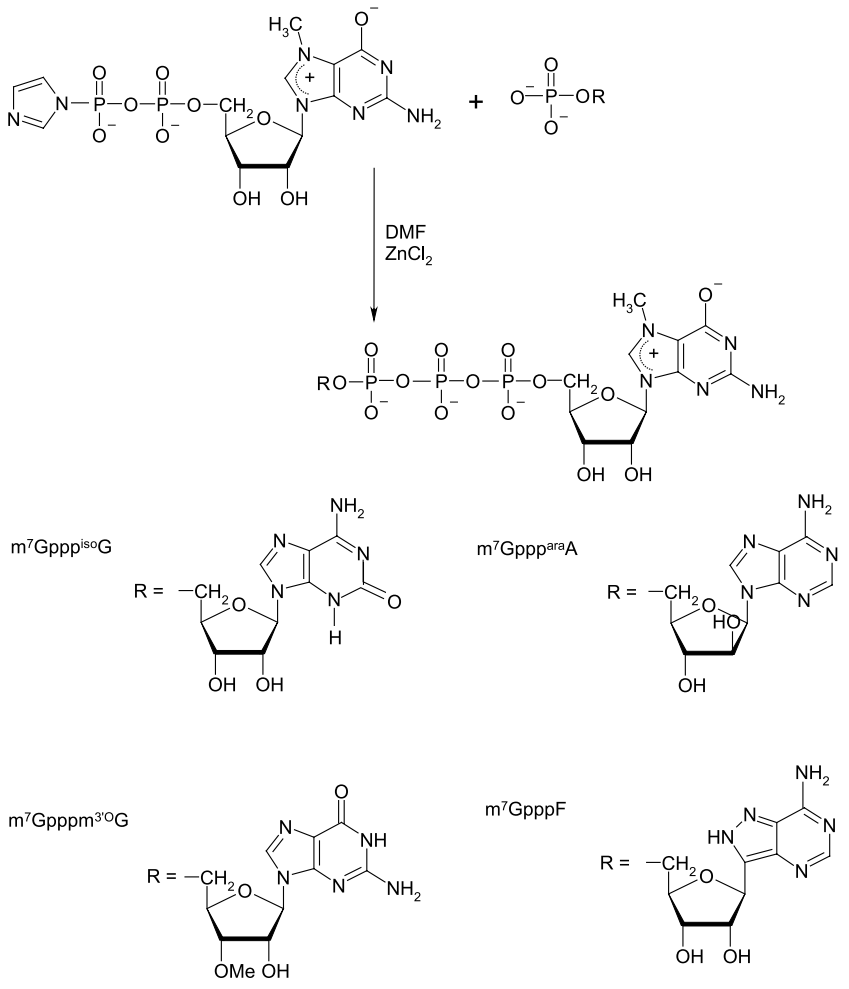

FIGURE 1 The synthesis and structures of the four new cap analogues.

RESULTS AND DISCUSSION

Synthesis of Cap Analogues

Synthesis of new cap analogues were carried out in dimethylformamide in the presence of zinc chloride, starting from 7-methylguanosine 5′-diphosphate P^2-imidazolide and appropriate nucleoside 5′-monophosphate (Figure 1), using methodology developed previously in our laboratory.[3,4] Formycin A 5′-monophosphate was a commercial product from Sigma. The other three monophosphates were prepared from corresponding nucleosides, *i.e.*, 3′-O-methylguanosine, 9-β-D-arabinofuranosyladenine and isoguanosine by Yoshikawa's phosphorylation procedure.[7] The final products were isolated from the reaction mixtures by ion exchange column chromatography (DEAE-Sephadex A-25, bicarbonate form) using linear gradient of triethylammonium bicarbonate (TEAB, 0–1.2 M, pH 7.5). Appropriate fractions were pooled and evaporated to dryness adding ethanol to facilitate TEAB decomposition. Thus obtained triethyl ammonium salts of the final products were converted to their sodium salts.

The structures of the four new cap analogues (Figure 1) were confirmed by mass spectrometry (Q-tof2 Micromass spectrometer) and NMR spectroscopy (Varian UNITYplus 400 MHz spectrometer). Values of the ^{1}H chemical shifts in ppm *vs.* internal sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]-propionate and of the ^{31}P chemical shifts *vs.* external H_3PO_4.

m^7Gppp^{iso}G, ^{1}H NMR (s, singlet, d, doublet, t, triplet, m, multiplet): m^7GH8 exchanged for ^{2}H; ^{iso}GH8 8.105 ppm (s), m^7GH m^7GN7CH_3 4.043 ppm (s), m^7GH1′ 5.909 ppm (d), ^{iso}GH1′ 5.810 ppm (d), m^7G and ^{iso}G H2′, H3′, H4′, H5′, H5″ 4.80–4.25 ppm (m); ^{31}P NMR: Pα and Pγ −12.20 ppm (m), Pβ −23.74 ppm (d).

m^7Gpppm$^{3'O}$G ^{1}H NMR: m^7GH8 exchanged for ^{2}H, $m^{3'O}$GH8 7.99 ppm (s), m^7GH m^7GN7CH_3, 4.034 ppm (s), $m^{3'O}$G3′OCH_3 3.524 ppm (s), m^7GH1′ 5.834

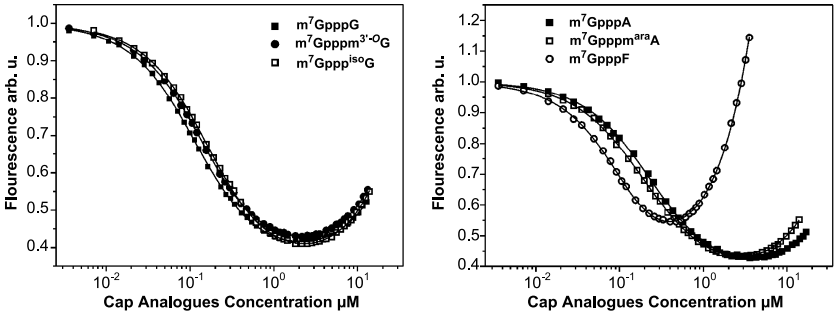

FIGURE 2 Fluorescence titration curves for binding of dinucleotide cap analogues to murine eIF4E(28–217). Fluorescence measurements were performed in 50 mM HEPES/KOH pH 7.2, 0.1 M KCl, 0.5 mM EDTA, and 1 mM DTT at 20°C by adding 1 µL of cap analogue solutions to 1400 µL of 0.1 µM eIF4E(28–217) obtained by reconstruction from inclusion bodies.[8] Protein fluorescence was excited at 280 nm and observed at 337 nm. The observed increasing fluorescence intensity at higher concentration of ligand originates from the emission of free cap analogues in solution.

TABLE 1 Binding Free Energies (ΔG°) and Equilibrium Association Constants (K_{as}) for Interaction of Murine eIF4E(28–217) with the Newly Synthesized Dinucleotide Cap Analogues at 20°C

Cap analogue	K_{as} (μM^{-1})[a]	ΔG° (kcal/mol)
m^7GpppG	11.03 ± 0.15	−9.441 ± 0.008
$m^7Gppp^{iso}G$	8.82 ± 0.08	−9.311 ± 0.005
$m^7Gpppm^{3'O}G$	9.73 ± 0.11	−9.368 ± 0.007
m^7GpppA	5.0 ± 0.2[b]	−8.980 ± 0.023
$m^7Gppp^{ara}A$	5.78 ± 0.07	−9.065 ± 0.007
m^7GpppF	21.7 ± 0.5	−9.835 ± 0.013

[a] K_{as} were determined as described previously by Niedzwiecka et al.[6]
[b] Data from Zuberek et al.[8]

ppm (d); $m^{3'O}$GH1′ 5.764 ppm (d); m^7G and $m^{3'O}$G H2′, H3′, H4′, H5′, H5″ 4.80–4.17 ppm (m); ^{31}P NMR: Pα and Pγ −12.20 ppm (m); Pβ −23.74 ppm (d);

$m^7Gppp^{ara}A$ ^{1}H NMR m^7GH8 exchaned for ^{2}H; ^{ara}AH8 and H2 8.282 ppm (s) and 8.175 ppm (s), m^7GN7CH_3 4.050 ppm (s), m^7GH1′ 5.894 ppm (d); ^{ara}AH1′ 6.231 ppm (d); m^7G and ^{ara}AH2′, H3′, H4′, H5′, H5″ 4.80–4.17 ppm (m); ^{31}P NMR: Pα and Pγ −12.20 ppm (m); Pβ −23.82 ppm (d);

m^7GpppF ^{1}H NMR: m^7GH8 and FH2 exchanged for ^{2}H, FH(?) 8.130 ppm (s), m^7GH m^7GN7CH_3 3.973 ppm (s), m^7GH1′ 5.833 ppm (d), FH1′ 5.163 ppm (d), m^7G and F H2′, H3′, H4′, H5′, H5″ 4.75–4.21 ppm (m); ^{31}P NMR: Pα −12.20 ppm (d), Pβ −24.27 ppm (t), Pγ −12.62 ppm (d).

Binding Affinity of Dinucleotide Cap Analogues for eIF4E

The binding affinity of murine eIF4E(28–217) for the new four dinucleotide cap analogs differing in the second nucleoside, $m^7Gppp^{iso}G$, $m^7Gpppm^{3'O}G$, $m^7Gppp^{ara}A$, and m^7GpppF was determined using quenching of intrinsic tryptophan fluorescence of eIF4E on titration with the analogue. Three out of eight conserved tryptophans, Trp56, Trp102, Trp166 (murine protein numeration), are located in the cap-binding slot of eIF4E.[6] Fluorescence titration data for the new cap analogues as well as for standard compounds, m^7GpppG and m^7GpppA, are shown in Figure 2. The equilibrium association constants derived by numerical fitting to the experimental data points[6] are presented in Table 1.

All cap analogues, with the exception of m^7GpppF, bind to eIF4E with similar affinities. Replacement of guanosine by isoguanosine results in insignificant change of the association constant, $K_{as} = 11.03 \pm 0.15 \times 10^6\ M^{-1}$ *vs.* $K_{as} = 8.82 \pm 0.08 \times 10^6\ M^{-1}$, respectively. Methylation of sugar moiety at position C3′ ($m^7GpppG \rightarrow m^7Gpppm^{3'O}G$) or replacement of ribose by arabinose ($m^7GpppA \rightarrow m^7Gppp^{ara}A$) have not influenced the eIF4E-cap interaction; the observed differences being only within 10% (Table 1). The cap analogue, in which the second fluorescencent base is formycin A (m^7GpppF), binds to eIF4E most effectively of all investigated dinucleotides. The K_{as} value for this analogue is about four-fold higher than that for

m^7GpppA and two-fold higher than for m^7GpppG. The corresponding energetic effect is about $\Delta\Delta G^\circ \cong 0.9$ kcal/mol (Table 1), typical for one hydrogen bond. This suggests that the formycin base is additionally stabilized in the cap binding pocket in comparison with adenine. Thus, m^7GpppF is a very interesting cap analogue for biochemical and therapy studies.

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