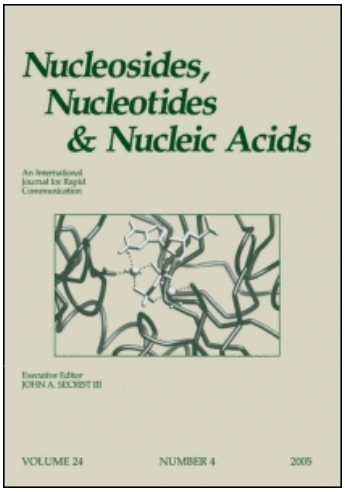


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# Synthesis and Biochemical Properties of Novel mRNA 5' Cap Analogs Resistant to Enzymatic Hydrolysis

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# SYNTHESIS AND BIOCHEMICAL PROPERTIES OF NOVEL mRNA 5′ CAP ANALOGS RESISTANT TO ENZYMATIC HYDROLYSIS

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▫ *A series of new dinucleotide cap analogs with methylene groups replacing oxygens within the pyrophosphate moieties have been synthesized. All the compounds were resistant to the human scavenger decapping hydrolase, DcpS. Binding constants of the modified caps to eIF4E are comparable to those obtained for $m^7$GpppG. This suggests these methylene modifications in the pyrophosphate chain do not significantly affect cap-binding at least for eIF4E. These cap analogs are also good inhibitors of in vitro translation. mRNAs capped with novel analogs were translated similarly to the mRNA capped with the parent $m^7$GpppG.*

**Keywords** Cap Structure, Bisphosphonates, DcpS, eIF4E, Translation

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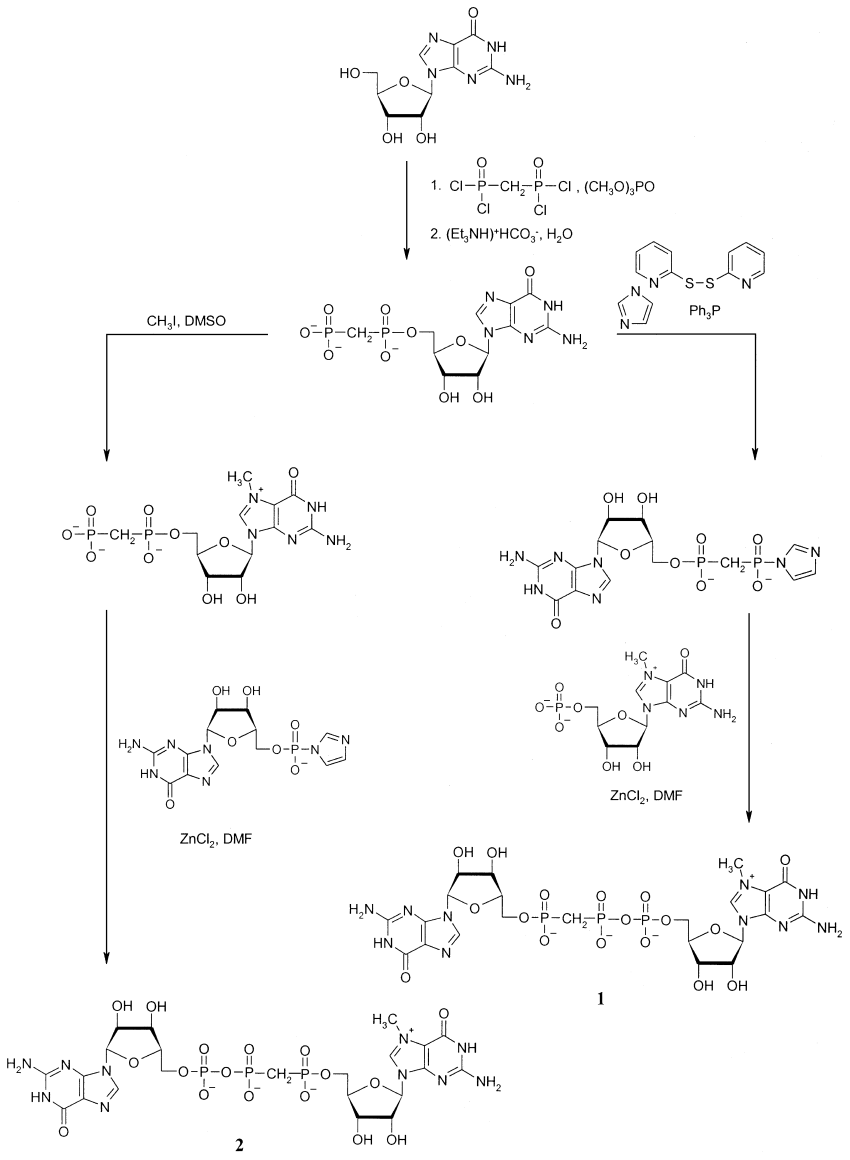
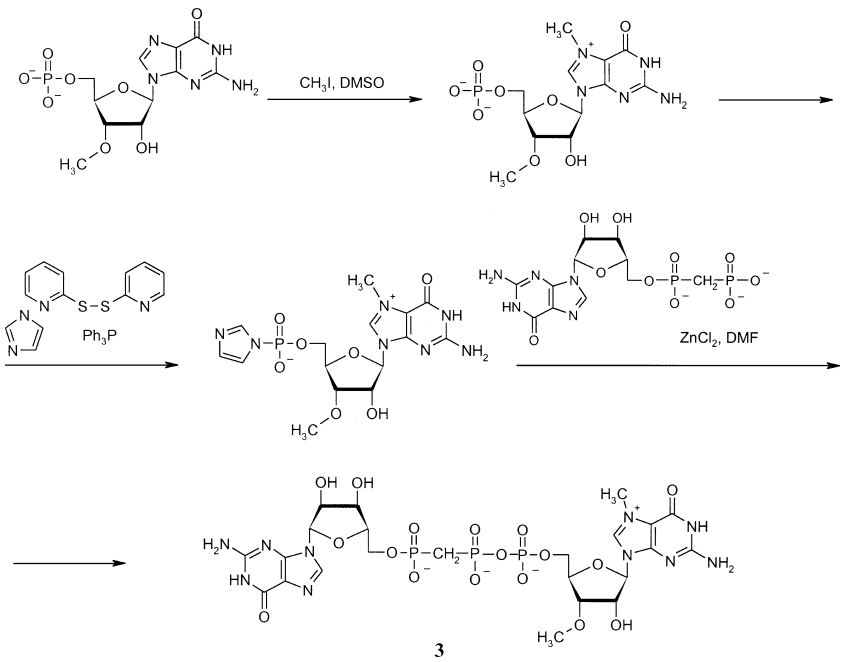
## INTRODUCTION

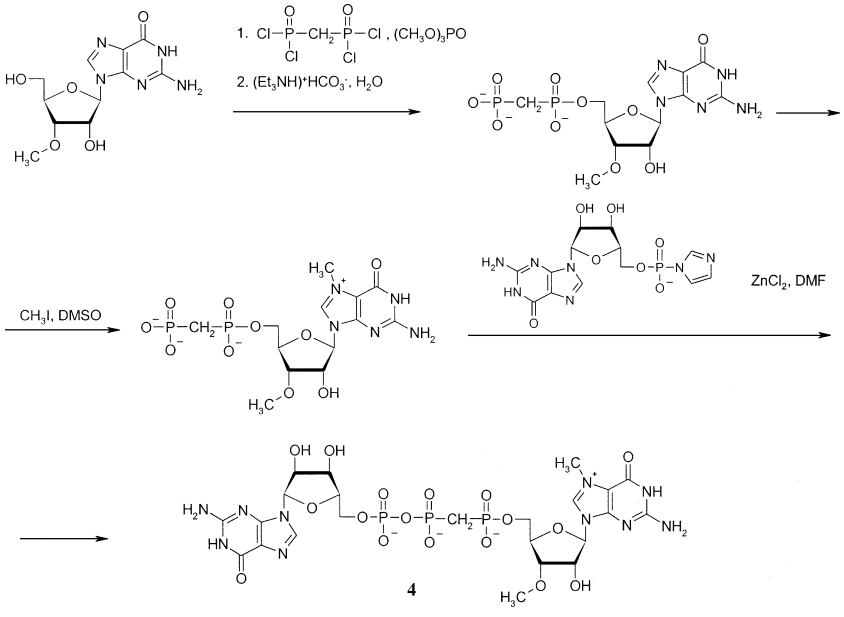
mRNA 5′ cap structures play pivotal roles in many biological processes. Among the most important is its binding to translational initiation factor eIF4E at an early step in initiation of translation.[1] The cap also protects mRNA from 5′-3′ exonucleolytic degradation. There are several enzymes responsible for degradation of capped mRNAs, thereby affecting steady-state mRNA levels, and hence, gene




**SCHEME 1** Synthesis of **1** and **2**.




**SCHEME 2** Synthesis of **3**.




**SCHEME 3** Synthesis of 4.

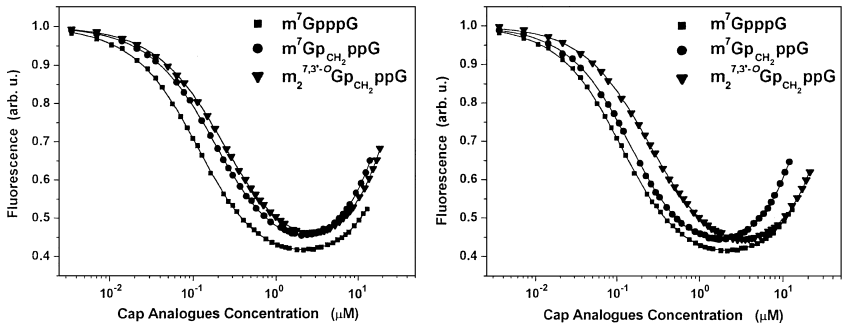



**FIGURE 1** Fluorescence titration curves for binding of the cap analogs to eIF4E. Measurements were performed as described previously by Niedzwiecka et al.[6]

expression.[2] Cap analogs such as $m^7$GpppG are known to be efficient inhibitors of translation. Dinucleotide cap analogs resistant to chemical and/or enzymatic degradation are expected to be more efficient inhibitors due to their higher stability compared with conventional caps, especially in an in vivo setting where all degradative enzymes are present. This property would be important for potential antitumor drug based on inhibition of cap-dependent translation, since many cancer cells exhibit elevated levels of eIF4E.[3]

Four new dinucleotide cap analogs with methylene groups replacing oxygen atoms within the pyrophosphate moieties have been synthesized: **1**, **2**, **3**, and **4**. The latter two are anti reverse cap analogs (ARCAs), which are exclusively incorporated, with the appropriate orientation during in vitro mRNA synthesis.[4]

These new compounds have been subjected to a series of biophysical and biochemical studies. The results of these studies indicate mRNAs capped with the novel methylene cap dinucleotides may be useful for in vivo studies of translation and mRNA degradation.

## RESULTS

### Chemical Synthesis

Schemes 1, 2, and 3 illustrate the synthetic paths for obtaining the described cap analogs. The key reaction was coupling of nucleoside monophosphate and

**TABLE 1** Summarized Biophysical and Biochemical Properties of the Novel Methylene Cap Analogs

| | Cap analog | $K_{as}$ ($\mu M^{-1}$) | Relative translational efficiency |
|---|---|---|---|
| | $m^7$GpppG | 11.5 ± 0.3 | 1.00 |
| **1** | $m^7$GppC$H_2$pG | 8.6 ± 0.4 | 0.89 ± 0.13 |
| **2** | $m^7$GpC$H_2$ppG | 6.3 ± 0.3 | 0.77 ± 0.14 |
| | $m_2^{7,3'O}$GpppG | 7.4 ± 0.1 | 1.88 ± 0.40[8] |
| **3** | $m_2^{7,3'O}$GppC$H_2$pG | 4.41 ± 0.2 | 1.25 ± 0.32 |
| **4** | $m_2^{7,3'O}$GpC$H_2$ppG | 4.65 ± 0.03 | 1.10 ± 0.33 |

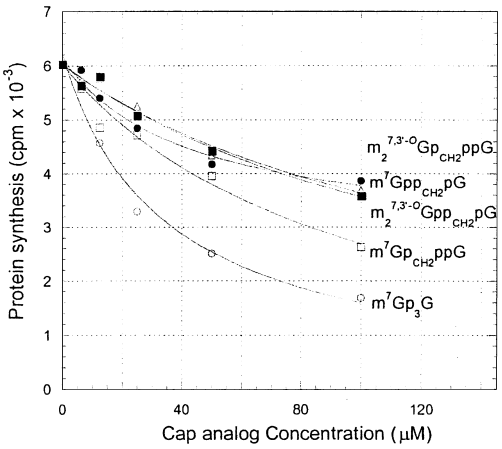



**FIGURE 2** Inhibition of protein translation by the methylene cap analogs.



nucleoside methylenebis(phosphonate) leading to pyrophosphate bond formation. The reaction was performed by activation of one of the fragments with imidazole, followed by coupling with the second nucleotide carried out in DMF, in a presence of $ZnCl_2$.[5] The appropriate fragments for coupling were prepared using guanosine and 3′-O-methyl guanosine as the starting material. A new method was developed for the synthesis of nucleoside 5′-methylenebis(phosphonate)s, $NpCH_2p$. It consists of reacting *unprotected* nucleoside with methylenebis(phosphonic dichloride), followed by hydrolysis. The structures of the final products and the intermediates were confirmed by $^1H$ and $^{31}P$ NMR spectroscopy and ES MS spectrometry. Preparation of dinucleotides with 5′,5′-triphosphate bond analog of type $NppCH_2pN'$ represents new, efficient, and general entry to this class of compounds.

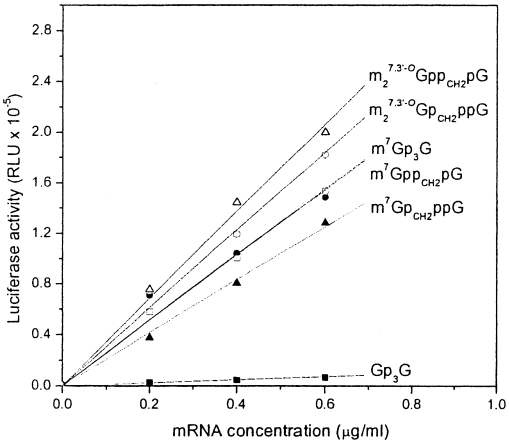



**FIGURE 3** Translational efficiency of mRNAs capped with the methylene cap analogs.

### Affinity for eIF4E

eIF4E protein possesses eight conserved tryptophan residues located in cap-binding slot. Interaction of the protein with a cap analog leads to quenching of the protein intrinsic fluorescence. Quenching as a result of cap-binding was measured by means of time-synchronized titration[6] (Figure 1). The observed association constants determined for the methylene cap analogs were lower, but comparable to those obtained for their parent compounds, $m^7GpppG$ and $m_2^{7,3'O}GpppG$ (Table 1).

### Inhibition of In Vitro Translation

One criterion for assessing cap analog interaction with the translational machinery is inhibition of protein synthesis as measured by in vitro translation.[7] We tested the new methylene cap analogs and the reference standards ($m^7GpppG$ and $m_2^{7,3'O}GpppG$) for their ability to inhibit translation of a capped luciferase mRNA in a rabbit reticulocyte lysate system (Figure 2). The methylene cap analogs showed inhibitory properties similar to the reference standards and the data are in a relatively good agreement with the binding constants measured in vitro: $m^7GpCH_2ppG$ and $m^7GppCH_2pG$ are 2–3-fold weaker inhibitors than $m^7GpppG$ and the ARCAs are even more weaker.

### Translational Efficiency of RNAs Capped with Novel Cap Analogs

We also measured the translational efficiency in vitro of the transcripts capped with the novel cap analogs. Translation reactions were conducted under conditions where luciferase production was linear both with time and mRNA concentration. Translational efficiency relative to $m^7GpppG$-capped mRNA was calculated as described previously.[8] The results for all analogs are shown in Figure 3 and the calculated relative translational efficiencies are listed in Table 1. We found that mRNAs capped with novel cap analogs were translated very similarly to mRNAs capped with $m^7GpppG$ what qualifies them as suitable candidates for in vivo studies.

### Resistance to DcpS Scavenger Decapping Enzyme

DcpS hydrolyzes the residual cap structure of short mRNA fragments that are 3′ to 5′ decay of an mRNA. This enzyme is responsible for much of the decapping activity in mammalian cells.[9] A standard dinucleotide cap analog $m^7GpppG$ is degraded by DcpS to $m^7GMP$ and GDP.

We examined the ability of the human and nematode DcpS proteins to hydrolyze the four novel methylene cap analogs. Notably, all methylene cap analogs were resistant to human and nematode DcpS protein hydrolysis based on detection of hydrolysis products by HPLC analysis.

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