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Enzymatically stable 5′ mRNA cap analogs: Synthesis and binding studies with human DcpS decapping enzyme

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Abstract—Four novel 5′ mRNA cap analogs have been synthesized with one of the pyrophosphate bridge oxygen atoms of the triphosphate linkage replaced with a methylene group. The analogs were prepared via reaction of nucleoside phosphor/phosphon-1-imidazolidates with nucleoside phosphate/phosphonate in the presence of $ZnCl_2$. Three of the new cap analogs are completely resistant to degradation by human DcpS, the enzyme responsible for hydrolysis of free cap resulting from 3′ to 5′ cellular mRNA decay. One of the new analogs has very high affinity for binding to human DcpS. Two of these analogs are Anti Reverse Cap Analogs which ensures that they are incorporated into mRNA chains exclusively in the correct orientation. These new cap analogs should be useful in a variety of biochemical studies, in the analysis of the cellular function of decapping enzymes, and as a basis for further development of modified cap analogs as potential anti-cancer and anti-parasite drugs.

1. Introduction

Eukaryotic messenger RNAs are modified at their 5′-ends by addition of a 7-methylguanosine attached by a 5′–5′ triphosphate bridge to the first nucleotide of the mRNA chain (Fig. 1). The cap structure plays a pivotal role in mRNA metabolism. It is required for mRNA processing, transport, translation, and is an important determinant in mRNA turnover.[1]

mRNA turnover plays an important role in the control of gene expression contributing to the control of mRNA levels in the cell.[2] There are two general pathways of eukaryotic mRNA degradation, both initiated by the shortening of mRNA 3′-polyadenyl tail. In 5′ to 3′ mRNA decay, the cap structure is cleaved by a specific pyrophosphatase Dcp1/Dcp2. This generates 7-methylGDP and an RNA with a 5′ exposed phosphate which is a substrate for 5′ to 3′ exonucleolytic decay by nuclease Xrn1. In the 3′ to 5′ pathway of mRNA decay, shortening of the poly(A)-tail of the RNA exposes the 3′ end of the RNA to a complex of 3′ to 5′ exonucleases, known as the exosome, which progressively degrade the mRNA. The products of this 3′ to 5′ RNA degradation are the monophosphates of the RNA and the mRNA cap. The free mRNA cap is hydrolyzed by a scavenger decapping enzyme known as DcpS. Current data suggest that the 5′ to 3′ pathway of mRNA degradation may be dominant in yeast, whereas at least in vitro the 3′ to 5′ pathway may be the major pathway in vertebrate cells.[2–4]

Sequence and mutagenesis analyses indicate that DcpS enzymes are members of the HIT family of pyrophosphatases containing a conserved histidine triad in the active site.[4] DcpS decapping enzymes catalyze the hydrolysis of capped dinucleotides (m^7GpppN) and short oligonucleotides (up to ~8 nucleotides) resulting from exosome mediated 3′–5′ mRNA decay. DcpS is unable to hydrolyze the cap structure linked to a long mRNA chain. This property protects functional mRNAs from degradation by DcpS. Crystallographic studies of DcpS in complex with m^7GpppG and m^7GpppA suggest that negative allosteric interactions between cap analogs and the enzyme are likely responsible for the inability of the enzyme to cleave the cap on a

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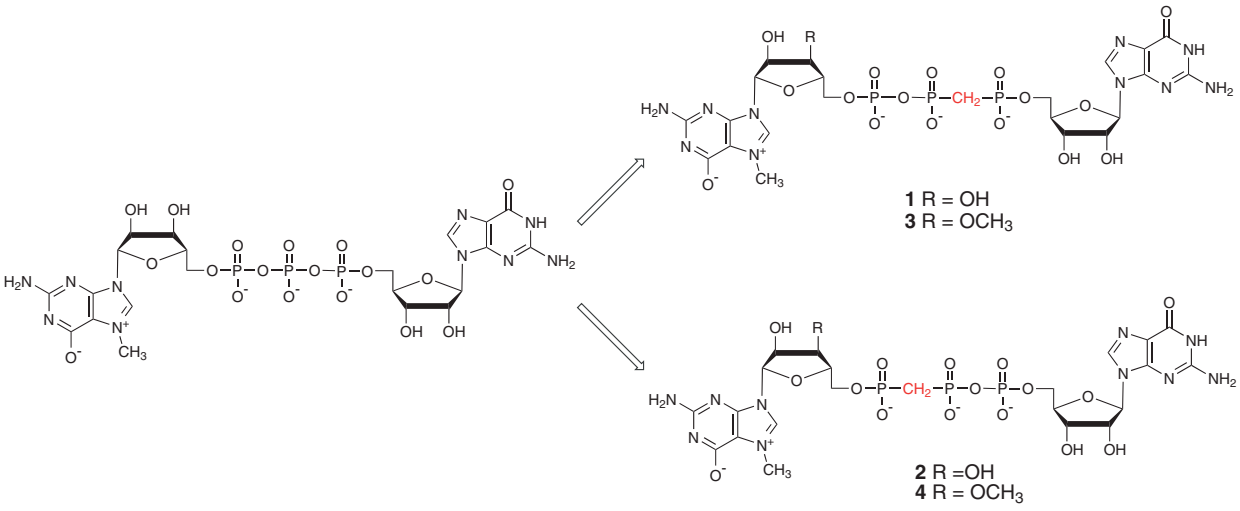


Figure 1. Design of the mRNA cap analogs containing a methylene group replacing the pyrophosphate bridge oxygen atom.

long mRNA chain.[5] DcpS cleaves the cap 5′–5′ pyrophosphate bond to release m^7GMP and ppN-. This contrasts with the RNA decapping enzyme Dcp2/Dcp1 which cleaves the cap on a long mRNA chain producing m^7GDP and pN-. DcpS cleavage proceeds through nucleophilic attack of His-277 on the γ-phosphate. Cap analogs resulting from 3′ to 5′ RNA degradation are predicted to be toxic to cells as they are inhibitors of a variety of cap-interacting proteins and could be misincorporated into nucleic acids. Many aspects of the activity and regulation of DcpS remain unknown. Furthermore, the contribution of 5′ to 3′ versus 3′ to 5′ decay in higher eukaryotes also remains unclear. Our group is interested in developing tools that will help provide a better understanding of the mechanism and role of both DcpS and Dcp2/Dcp1 decapping. One approach to the analysis of these decapping enzymes interaction with the cap is through the use of modified cap analogs that are resistant to hydrolysis of the triphosphate bridge. These resistant cap analogs can be used for biophysical studies of DcpS or Dcp2/Dcp1, as inhibitors in vitro and may enable the synthesis of mRNAs with enhanced stability. They also enabled us to initiate experiments aimed at understanding the relationships between mRNA translation and decapping in higher eukaryotes.

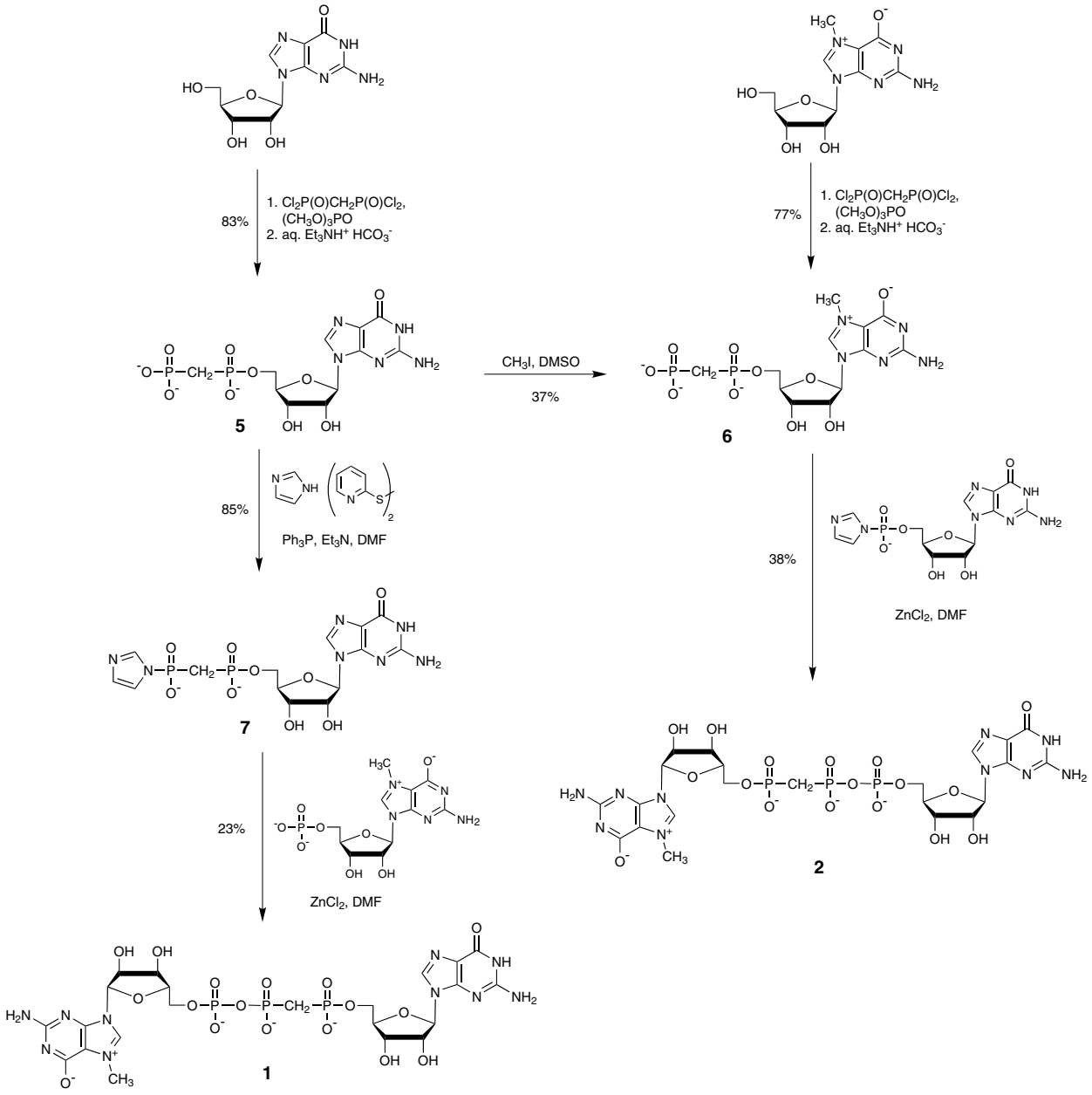
There are a number of approaches that might be applicable to make oligophosphates immune to hydrolysis by cellular enzymes including the use of phosphorothioates.[6–8] Another commonly used strategy is to replace a pyrophosphate bridge oxygen by a methylene group. The methylene group precludes any enzymatic cleavage due to the extreme stability of P–C bonds.[9–16] In a brief preliminary report, we have used the latter approach with the goal of producing analogs that retain high affinity for the DcpS and Dcp2/Dcp1 enzymes.[17] Here, we present a detailed description of their synthesis and susceptibility toward human DcpS enzyme.

2. Results and discussion

We prepared four different cap analogs (Fig. 1). In all of them, one oxygen atom of the pyrophosphate bridge is replaced with a methylene group. Compounds **1** and **2** are analogs of the standard cap structure. In **1**, CH_2 is placed between α- and β-phosphorus atoms. This is in a site that would not be cleaved by DcpS, but would be cleaved by Dcp2/Dcp1. On the contrary, the CH_2 of analog **2** is located between β- and γ-phosphates, directly next to phosphorus atom, which is subjected to the nucleophilic attack during the DcpS reaction (while not for Dcp2/Dcp1). Compounds **3** and **4** have a 3′*O*-methyl substituent in the 7-methylguanosine moiety. These compounds were designed to be used for orientation specific incorporation into the 5′ end of mRNAs during in vitro transcription.[18–20] Their incorporation is predicted to generate RNAs with a cap that is resistant to hydrolysis by cellular enzymes such as Dcp2/Dcp1. Cap analogs of this kind are referred as to Anti Reverse Cap Analogs (ARCA).[21,22]

Synthetic pathways leading to the methylene cap analogs **1** and **2** are depicted in Scheme 1. The key intermediates, guanosine 5′-bisphosphonate **5** and its 7-methyl counterpart **6**, were obtained using a novel methodology. We have generalized the Yoshikawa 5′ phosphorylation,[23] by replacing $POCl_3$ with methylenebis(phosphonic dichloride). This reaction proceeds with high 5′ regioselectivity, and after optimization the required products **5** and **6** could be obtained in good yields, 83% and 77%, respectively.[24] An attempt to convert compound **5** into **6**, by treatment with methyl iodide in DMSO, did not lead to satisfactory yield, due to poor solubility of **5** (in the form of either triethylammonium or tri-*n*-butylammonium salt) in the reaction media.

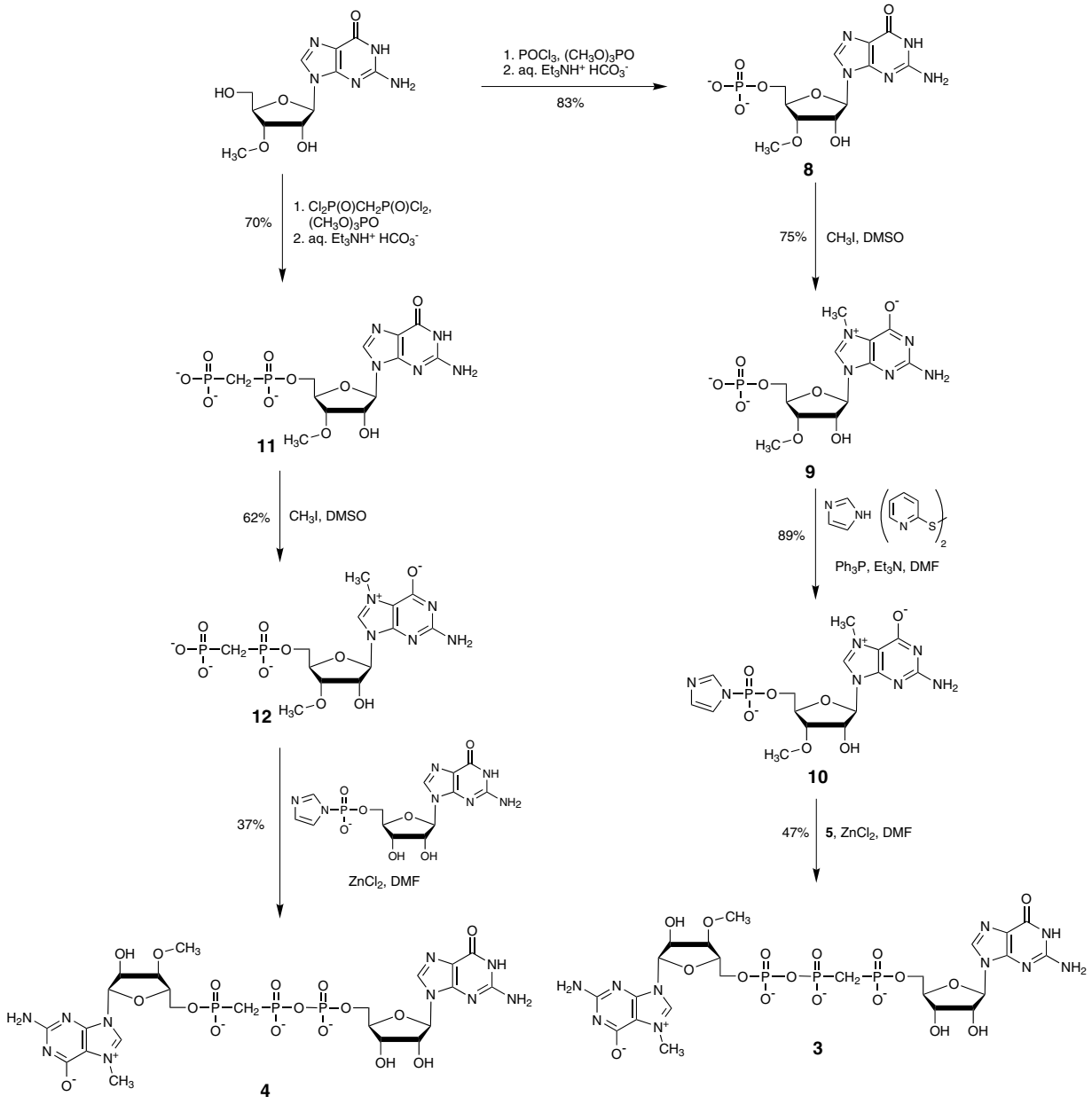
To introduce a single pyrophosphate bond of the phosphate–phosphonate 5′–5′ bridge, we used a well-developed and efficient two-step procedure. The sequence



Scheme 1. Synthesis of compounds **1** and **2**.

involves obtaining phosphor-1-imidazolidate in a reaction of the nucleotide with imidazole in the presence of 2,2′-dithiodipyridine, and triphenylphosphine, prior to coupling with the other nucleotide in DMF, using $ZnCl_2$ as Lewis acid catalyst.[21,22,25–27] This methodology enables the synthesis of linear oligophosphate chains as the imidazole is attached only to a terminal phosphorus most likely as a result of steric hindrance.[28] Also due to steric requirements, imidazole in the phosphor-1-imidazolidate is substituted only by a terminal phosphate from the other nucleotide during the coupling reaction. To our knowledge, this is the first report in which a phosphonate was activated this way or coupled with such an activated phosphate.

Treatment of compound **5** with imidazole, 2,2′-dithiodipyridine and triphenylphosphine led to formation of the expected imidazole derivative **7** in 85% yield. Compound **7** was reacted with 7-methylGMP[29] in the presence of $ZnCl_2$ to produce the cap analog **1** in 23% yield. The other desired final product **2** was obtained in the same manner by treating compound **6** with the imidazole derivative of GMP in 38% yield. These results are surprising because a phosphate group in phosphonates is normally more reactive during nucleophilic substitution than in phosphates.[30] We currently are not able to explain these interesting and unexpected results without additional studies. However, a practical conclusion can be drawn. It is better to obtain imidazole derivatives from phosphates and then couple them with phosphonates rather than the reverse. Unfortunately, this approach cannot be applied to the synthesis of **1** due to difficulties in obtaining 7-methylguanosine-5′-yl phosphor-1-imidazolidate.



Scheme 2. Synthesis of compounds **3** and **4**.

We synthesized ARCAs bearing a methylene group in the phosphate chain (Scheme 2). Thus, analog **4** was obtained in a similar way as **2**, but starting from 3′-*O*-methyl guanosine.[31] The second ARCA **3** was also obtained from 3′-*O*-methylguanosine in four steps. This compound was subjected to 5′-phosphorylation to yield the monophosphate **8**. Treating **8** with CH_3I produced the 7-methylated product **9**, which was converted into imidazole derivative **10** using a previously described procedure.[32] Compound **10** was transformed into **3** upon treatment with **5** in the presence of $ZnCl_2$.

The methylene-modified cap analogs **1**–**4** were subjected to digestion by human DcpS and the progress of reaction was monitored using HPLC and fluorescence methods. The results of these studies are summarized in Table 1. The cap analog **2**, containing a CH_2 group between β- and γ-phosphorus atoms in the DcpS cleavage site, was not hydrolyzed by hDcpS. The introduction of the CH_2 group instead of pyrophosphate oxygen into the second position, between α- and β-phosphorus atoms (**1**), did not protect the analog from hydrolysis, but caused a drastic decrease in the reaction rate. The

Table 1. The hDcpS hydrolysis of compounds **1**–**4** and equilibrium association constants (K_{as}) for complexes of hDcpS with methylene cap analogs resistant to enzymatic hydrolysis

Compound	Cap analog	hDcpS hydrolysis	$K_{as} \times 10^6$ [M^{-1}]
	m^7GpppG	Hydrolyzed	—
1	m^7GppCH_2pG	Hydrolyzed	—
2	m^7GpCH_2ppG	Resistant	234 ± 14
3	$m_2^{7,3'O}GppCH_2pG$	Resistant	37 ± 3
4	$m_2^{7,3'O}GpCH_2ppG$	Resistant	43 ± 4

calculated V_{max} values for the normal cap—m^7GpppG and **1** were 0.5 and 0.0125 μM/min, respectively. Therefore, the hydrolysis of **1** occurred 40-fold slower. The methylene caps with the additional CH_3 group at the 3′ position of the 7-methylguanosine moiety (**3** and **4**) were also totally resistant to cleavage by the human DcpS enzyme.

To examine the binding affinity of the resistant cap analogs to hDcpS, the fluorescence Time Synchronized Titration method was applied.[33] The association constants (K_{as}) (Table 1) of compounds **2**–**4** for hDcpS were calculated from the fluorescent titration curves (Fig. 2). The replacement of the pyrophosphate bridge oxygen within the triphosphate chain between β- and γ-phosphorus atoms (**2**) retains strong binding to DcpS enzyme as illustrated by the high K_{as} value. Weaker binding affinity is observed for two methylene ARCA type cap analogs **3** and **4**, resulting in about 5-fold decrease of K_{as} values (Table 1).

The reduced DcpS hydrolysis of compound **1** is surprising in that the substituted CH_2 group is located 3 atoms away from the phosphorus attacked during the reaction. The high value of K_{as} for **2** indicates a high affinity of hDcpS for this modified cap structure. These data indicate that the introduction of a methylene group into the triphosphate bridge does not dramatically affect the affinity of the protein for the analog and suggests the analog is likely to bind efficiently to DcpS. The observed significant decrease in the K_{as} for the ARCA methylene cap analogs **3** and **4** indicates that modifications in the ribose moiety of 7-methylguanosine decrease the binding affinity to hDcpS, probably as a result of the steric hindrance of the CH_3 substituents, as predicted from the active site structure.[5] This modification makes compound **3** stable to hydrolysis, despite the fact that **1** undergoes slow degradation.

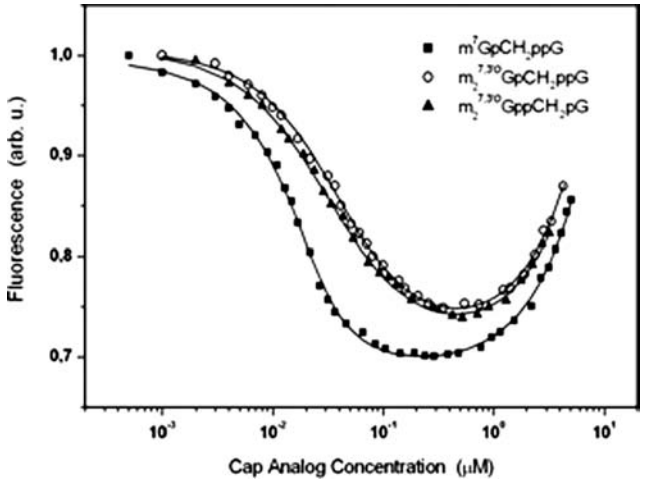


Figure 2. Fluorescent titration curves for methylene cap analogs resistant to cleavage by human DcpS. The shift of the titration curves toward the higher ligand concentration indicates the weaker binding between hDcpS and the respective cap analog. The increase of fluorescence intensity at high ligand concentration originates from the emission of the free cap analog.

For the non-hydrolysable analogs (**2**, **3**, and **4**) the inhibitory activities (IC_{50}) have been determined. IC_{50} for compound **2** was 2 μM, whereas for **3** and **4** the value of IC_{50} was over 20 μM. These results are consistent with the K_{as} values and support the above conclusions on the binding affinities toward hDcpS for those three cap dinucleotides.

3. Conclusions

The synthesis of four novel cap analogs containing a pyrophosphate bridge oxygen selectively replaced with a methylene group has been carried out. The compounds were prepared using phosphonylation of nucleosides with methylenebis(phosphonic dichloride) in Yoshikawa phosphorylation conditions and pyrophosphate bond formation from phosphor/phosphon-1-imidazolidate precursors. Three of the compounds are totally resistant to hydrolysis by human DcpS with one exhibiting reduced hydrolysis. The high association constant of hDcpS with one of the analogs indicates that the enzyme binding site is not highly restrictive toward the methylene modifications in the triphosphate bridge. However, addition of a 3′*O*-methyl substituent into the sugar of 7-methylguanosine moiety leads to a considerable decrease in the affinity of DcpS for the analog. The strong binding of analog **2** to the enzyme suggests that it will likely be an efficient inhibitor that may prove useful in future studies directed at understanding the biochemistry and functions of decapping by DcpS enzymes from different species.

Recently, we have shown that oligonucleotides capped with $m_2^{7,3'\text{-O}}Gpp_{CH_2}pG$ (compound **3**) were resistant to hydrolysis by recombinant human Dcp2 in vitro.[34] Accordingly, mRNAs capped with **3** but not **4** ($m_2^{7,3'\text{-O}}Gp_{CH_2}ppG$) were more stable in vivo, indicating the significant contribution of 5′ to 3′ pathway of mRNA degradation in mammalians. In the same study, we showed that the in vivo translational efficiency is strongly correlated with mRNA cap-dependent stability.[34]

4. Experimental

4.1. General

^1H, ^{31}P NMR chemical shifts δ are reported in ppm relative to their standard reference (^1H, TPS internal at 0.00 ppm; ^{31}P, H_3PO_4 external at 0.00 ppm). In the case of dinucleotides and phosphoro/phosphono-1-imidazolates, signals originating from 7-methylguanosine and imidazole moieties are indicated by subscripts m7G and im, respectively. Mass spectra (MS) were recorded in the negative electrospray mode and are reported in mass units (*m*/*z*). Ion exchange column chromatography was performed on DEAE–Sephadex (A-25/HCO_3^- form) using a linear gradient of triethylammonium bicarbonate (TEAB, obtained by acidification of aqueous triethylamine to pH 7.3 using CO_2) in water.

Fractions were collected, and product peaks (monitored at 260 nm) were pooled and evaporated to dryness with ethanol added repeatedly to remove the TEAB buffer. Analytical HPLC studies were carried out using a reverse-phase column (Supelco LC-18-T, 4.6×250 mm, 5 μm) with a linear gradient of methanol from 0% to 25% in aqueous 0.05 M $CH_3CO_2NH_4$, pH 5.9, over 15 min (detection at 260 nm). Elutions were conducted at room temperature with a 1.3 mL/min flow rate.

4.2. Trisodium P^1-(7-methyl)-guanosine-P^3-guanosine-α:β-methylene triphosphate (1)

Compound **7** (77 mg, 0.14 mmol), bis(triethylammonium) 7-methylguanosine monophosphate[29] (89 mg, 0.15 mmol), and $ZnCl_2$ (152 mg, 1.12 mmol) were stirred in anhydrous DMF (1.5 mL) at room temperature for 24 h. The mixture was poured into a cold solution of EDTA (0.5 g, 1.49 mmol) in water (20 mL) and neutralized to pH 7 by addition of $NaHCO_3$. The product was isolated by chromatography on DEAE–Sephadex using a 0–1.2 M gradient of TEAB. It was converted into the sodium salt using Dowex 50WX8/Na^+ form (27.4 mg, 23%). ^{1}H NMR (D_2O, 400 MHz): δ 8.05 (1H, s, H8), 5.92 (1H, d, H1′$_{m7G}$, $J_{1'-2'} = 3.0$ Hz), 5.81 (1H, d, H1′, $J_{1'-2'} = 6.0$ Hz), 4.69 (1H, t, H2′, $J_{2'-1',3'} = 6.0$ Hz), 4.54 (1H, dd, H2′$_{m7G}$, $J_{1'-2'} = 3.0$ Hz, $J_{2'-3'} = 4.7$ Hz), 4.49 (1H, dd, H3′$_{m7G}$, $J_{2'-3'} = 5.0$ Hz, $J_{3'-4'} = 3.7$ Hz), 4.44 (1H, t, H3′, $J_{2'-3',4'} = 5.5$ Hz), 4.37–4.31 (2H, m, H4′,4′$_{m7G}$), 4.28–4.19 (4H, m, H5′,5″,5′$_{m7G}$, 5″$_{m7G}$), 4.06 (3H, s, CH_3), 2.39 (2H, t, P–CH_2–P, $J = 20.4$ Hz). ^{31}P NMR (163 MHz): δ 28.45 (1P,β), 18.91 (1P, α), 0.19 (1P, γ). MS: calcd for $C_{22}H_{30}N_{10}O_{17}P_3$ 779. Found 799 $[M-H]^-$. HPLC t_R 6.3 min.

4.3. Trisodium P^1-(7-methyl)-guanosine-P^3-guanosine-β:γ-methylene triphosphate (2)

Compound **2** was obtained as a white powder (32.9 mg, 38%), starting from **6** (78 mg, 0.10 mmol) and sodium guanosin-5′-yl phosphor-1-imidazolidate[22] (52 mg, 0.12 mmol) using the same procedure as for **1**. ^{1}H NMR (D_2O, 400 MHz): δ 8.06 (1H, s, H8), 5.92 (1H, d, H1′$_{m7G}$, $J_{1'-2'} = 3.4$ Hz), 5.84 (1H, d, H1′, $J_{1'-2'} = 5.4$ Hz), 4.63 (1H, t, H2′, $J_{2'-1',3'} = 5.1$ Hz), 4.52 (1H, dd, H2′$_{m7G}$, $J_{1'-2'} = 3.4$ Hz, $J_{2'-3'} = 4.4$ Hz), 4.46 (1H, dd, H3′$_{m7G}$, $J_{2'-3'} = 3.9$, $J_{3'-4'} = 5.4$ Hz), 4.43 (1H, t, H3′, $J_{3'-2',4'} = 5.4$ Hz), 4.38–4.31 (2H, m, H4′,4′$_{m7G}$), 4.30–4.15 (4H, m, H5′,5″,5′$_{m7G}$, 5″$_{m7G}$), 4.07 (3H, s, CH_3), 2.38 (2H, t, P–CH_2–P, $J = 20.0$ Hz). ^{31}P NMR (163 MHz): δ 28.37 (1P, β), 19.42 (1P, γ), 0.55 (1P, α). MS: calcd for $C_{22}H_{30}N_{10}O_{17}P_3$ 779. Found 799 $[M-H]^-$. HPLC t_R 6.4 min.

4.4. Trisodium P^1-(7,3′-*O*-dimethyl-guanosine)-P^3-guanosine-α:β-methylene triphosphate (3)

Compound **3** was obtained as a white powder (32.9 mg, 47%), starting from **10** (37 mg, 0.08 mmol) and **5** (62 mg, 0.08 mmol) as described for **1**. ^{1}H NMR (D_2O, 400 MHz): δ 8.04 (1H, s, H8), 5.91 (1H, d, H1′$_{m7G}$, $J_{1'-2'} = 3.7$ Hz), 5.80 (1H, d, H1′, $J_{1'-2'} = 6.0$ Hz), 4.72 (1H, dd, H2′$_{m7G}$, $J_{1'-2'} = 4.0$ Hz, $J_{2'-3'} = 5.0$ Hz), 4.70 (1H, t, H2′, $J_{2'-1',3'} = 6.0$ Hz), 4.49 (1H, dd, H3′, $J_{2'-3'} = 6.0$ Hz, $J_{3'-4'} = 5.0$ Hz), 4.44 (1H, dd, H4′, $J_{3'-4'} = 4.7$ Hz, $J_{4'-5',5''} = 2.6$ Hz), 4.33–4.15 (5H, m, H4′$_{m7G}$,5′,5″, 5′$_{m7G}$, 5″$_{m7G}$), 4.12 (1H, t, H3′$_{m7G}$, $J_{3'-2',4'} = 5.0$ Hz), 4.08 (3H, s, CH_3N), 3.48 (3H, s, CH_3O), 2.39 (2H, t, P–CH_2–P, $J = 20.4$ Hz). ^{31}P NMR (163 MHz): δ 28.51 (1P, β), 18.93 (1P, α), 0.12 (1P, γ). MS: calcd for $C_{23}H_{32}N_{10}O_{17}P_3$ 813. Found 813 $[M-H]^-$. HPLC t_R 6.7 min.

4.5. Trisodium P^1-(7,3′-*O*-dimethyl-guanosine)-P^3-guanosine-β:γ-methylene triphosphate (4)

Compound **4** was obtained as a white powder (18.5 mg, 37%), starting from **12** (44 mg, 0.06 mmol) and sodium guanosin-5′-yl phosphor-1-imidazolidate[22] (29 mg, 0.07 mmol), and following the procedure described for **1**. ^{1}H NMR (D_2O, 400 MHz): δ 8.03 (1H, s, H8), 5.92 (1H, d, H1′$_{m7G}$, $J_{1'-2'} = 4.0$ Hz), 5.82 (1H, d, H1′, $J_{1'-2'} = 6.0$ Hz), 4.75 (1H, dd, H2′$_{m7G}$, $J_{1'-2'} = 4.0$ Hz, $J_{2'-3'} = 5.0$ Hz), 4.69 (1H, t, H2′, $J_{2'-1',3'} = 5.7$ Hz), 4.49 (1H, dd, H3′, $J_{2'-3'} = 5.4$ Hz, $J_{3'-4'} = 4.0$ Hz), 4.44 (1H, m, H4′$_{m7G}$), 4.33 (1H, m, H4′), 4.30–4.17 (4H, m, H5′,5″,5′$_{m7G}$, 5″$_{m7G}$), 4.15 (1H, t, H3′$_{m7G}$, $J_{3'-2',4'} = 5.0$ Hz), 4.08 (3H, s, CH_3N), 3.49 (3H, s, CH_3O), 2.39 (2H, t, P–CH_2–P, $J = 20.4$ Hz). ^{31}P NMR (163 MHz): δ 28.48 (1P, β), 18.58 (1P, γ), 0.29 (1P, α). MS: calcd for $C_{23}H_{32}N_{10}O_{17}P_3$ 813. Found 813 $[M-H]^-$. HPLC t_R 6.8 min.

4.6. Tris(triethylammonium) guanosin-5′-yl bisphosphonate (5)

A solution of methylenebis(phosphonic dichloride) (300 mg, 1.2 mmol) in trimethyl phosphate (10 mL) cooled to 0 °C was added to a suspension of guanosine (200 mg, 0.7 mmol) in trimethyl phosphate (10 mL) at 0 °C. The reaction mixture was stirred at 0 °C. After 1 h, 0.7 M aqueous TEAB (pH 7.0) was added. Chromatographic purification on DEAE–Sephadex, using a 0–1 M gradient of TEAB, gave **1** as a glassy solid (379 mg, 73%). ^{1}H NMR (D_2O, 400 MHz): δ 8.12 (1 H, s, H8), 5.92 (1H, d, H1′, $J_{1'-2'} = 5.6$ Hz), 4.76 (1H, dd, H2′, $J_{1'-2'} = 5.6$ Hz, $J_{2'-3'} = 5.0$ Hz), 4.55 (1H, dd, H3′, $J_{2'-3'} = 5.0$ Hz, $J_{3'-4'} = 5.1$ Hz), 4.33 (1H, dt, H4′, $J_{3'-4'} = 5.1$ Hz, $J_{4'-5',5''} = 3.3$ Hz), 4.17 (2H, dd, H5′,5″, $J_{4'-5',5''} = 3.3$ Hz, $J_{5',5''} - P = 5.7$ Hz), 2.14 (2H, t, P–CH_2–P, $J = 19.6$ Hz). ^{31}P NMR (163 MHz): δ 19.23 (1P, α), 15.67 (1P, β). MS: calcd for $C_{11}H_{16}N_5O_{10}P_2$ 440. Found 440 $[M-H]^-$. HPLC t_R 4.2 min.

4.7. Tris(triethylammonium) 7-methylguanosin-5′-yl bisphosphonate (6)

(A) Compound **6** was obtained as a glassy solid (85 mg, 77%) starting from 7-methylguanosine[35] (42 mg, 0.14 mmol) following the procedure described for **5**. (B) Methyl iodide (0.26 mL, 4.2 mmol) was added to a suspension of **5** (262 mg, 0.35 mmol) in DMSO (10 mL) and stirred at room temperature for 5 h. The mixture was poured into water (100 mL) and extracted

three times with diethyl ether (3×40 mL). Iodine remaining in the aqueous phase was reduced by addition of small amount of $Na_2S_2O_5$. After neutralization to pH 7 with $NaHCO_3$, residual ether dissolved in the water was removed by evaporation under vacuum. Chromatographic isolation on DEAE–Sephadex using a 0-0.9 M gradient of TEAB gave **6** as a glassy solid (98 mg, 37%). ^{1}H NMR (D_2O, 400 MHz): δ 6.07 (1H, d, H1′, $J_{1'-2'} = 3.3$ Hz), 4.68 (1H, dd, H2′, $J_{1'-2'} = 3.3$ Hz, $J_{2'-3'} = 4.7$ Hz), 4.53 (1H, dd, H3′, $J_{2'-3'} = 4.7$ Hz, $J_{3'-4'} = 5.4$ Hz), 4.40 (1H, dt, H4′, $J_{3'-4'} = 5.4$ Hz, $J_{4'-5',5''} = 2.0$ Hz), 4.31 (1H, ddd, H5′, $J_{4'-5'} = 2.0$ Hz, $J_{5'} - P = 4.3$ Hz, $J_{5'-5''} = 11.7$ Hz), 4.20 (1H, ddd, H5″, $J_{4'-5'} = 2.0$ Hz, $J_{5''} - P = 4.3$ Hz, $J_{5'-5''} = 11.7$ Hz), 4.13 (3H, s, CH_3), 2.21 (2H, t, P–CH_2–P, $J = 19.6$ Hz). ^{31}P NMR (163 MHz): δ 19.18 (1P, α), 15.11 (1P, β). MS: calcd for $C_{12}H_{18}N_5O_{10}P_2$ 454. Found 454 $[M-H]^-$. HPLC t_R 5.1 min.

4.8. Disodium guanosin-5′-yl (phosphon-1-imidazolidyl)-methylphosphonate (7)

Compound **5** (300 mg, 0.39 mmol), imidazole (138 mg, 1.95 mmol), 2,2′-dithiodipyridine (171 mg, 0.78 mmol), triethylamine (27 μL, 0.39 mmol), and triphenylphosphine (204 mg, 0.78 mmol) were added to anhydrous DMF (1.5 mL) and stirred overnight at room temperature. The heterogeneous mixture was centrifuged, the supernatant collected and treated with a solution of sodium perchlorate (478 mg, 3.9 mmol) in acetone (15 mL). After cooling for 3 h in the refrigerator, the mixture was centrifuged and the supernatant was discarded. The precipitate was washed twice with acetone and dried over P_2O_5 (177 mg, 85%). ^{1}H NMR (D_2O, 400 MHz): δ 8.17 (1H, s, $H2_{im}$), 8.09 (1H, s, H8), 7.38 (1H, s, $H4_{im}$), 7.17 (1H, s, $H3_{im}$), 5.92 (1H, d, H1′, $J_{1'-2'} = 5.2$ Hz), 4.45 (1H, m, H2′), 4.27 (1H, m, H3′), 4.20 (1H, m, H4′), 4.05 (2H, m, H5′,5″), 2.44 (2H, t, P–CH_2–P, $J = 19.2$ Hz). ^{31}P NMR (163 MHz): δ 14.82 (1P, α), 10.20 (1P, β). MS: calcd for $C_{14}H_{18}N_7O_9P_2$ 490. Found 490 $[M-H]^-$. HPLC t_R 4.8 min.

4.9. Sodium 7,3′-*O*-dimethylguanosin-5′-yl phosphor-1-imidazolidate (10)

Compound **10** was obtained as a yellowish powder (37 mg, 89%) starting from **9**[32] (49 mg, 0.08 mmol) and following the same procedure as for **7**. ^{1}H NMR (D_2O, 400 MHz): δ 7.91 (1H, s, $H2_{im}$), 7.24 (1H, s, $H4_{im}$), 7.07 (1H, s, $H3_{im}$), 6.01 (1H, m, H1′), 4.87 (1H, m, H2′), 4.41 (1H, m, H4′), 4.26 (1H, m, H5′), 4.11 (3H, s, CH_3N), 4.06 (1H, m, H3′), 4.05 (1H, m, H5″), 3.45 (3H, s, CH_3O). ^{31}P NMR (163 MHz): δ −7.80. MS: calcd for $C_{15}H_{19}N_7O_7P$ 440. Found 440 $[M-H]^-$. HPLC t_R 12.4 min.

4.10. Tris(triethylammonium) 3′-*O*-methylguanosin-5′-yl (phosphono)-methylphosphonate (11)

Compound **11** was obtained as a white powder (62 mg, 70%), starting from 3′*O*-methylguanosine[31] (36 mg, 0.12 mmol) and following the same procedure as for **5**. ^{1}H NMR (D_2O, 400 MHz): δ 8.17 (1H, s, H8), 5.91 (1H, d, H1′, $J_{1'-2'} = 6.4$ Hz), 4.92 (1H, dd, H2′, $J_{1'-2'} = 6.4$ Hz, $J_{2'-3'} = 5.1$ Hz), 4.43 (1H, m, H4′), 4.20 (1H, dd, H3′, $J_{2'-3'} = 5.1$ Hz, $J_{3'-4'} = 3.2$ Hz), 4.14 (2H, m, H5′,5″), 3.53 (3H, s, CH_3O) 2.17 (2H, t, P–CH_2–P, $J = 19.9$ Hz). ^{31}P NMR (163 MHz): δ 19.68 (1P, α), 16.32 (1P, β). MS: calcd for $C_{12}H_{18}N_5O_{10}P_2$ 454. Found 454 $[M-H]^-$. HPLC t_R 6.3 min.

4.11. Tris(triethylammonium) 7,3′-*O*-dimethylguanosin-5′-yl (phosphono)-methylphosphonate (12)

Compound **12** was obtained as a white powder (44 mg, 62%), starting from **11** (62 mg, 0.09 mmol), and following the same procedure as for **6B**. ^{1}H NMR (D_2O, 400 MHz): δ 6.09 (1H, d, H1′, $J_{1'-2'} = 3.7$ Hz), 4.88 (1H, t, H2′,$J_{1'-2'} = J_{2'-3'} = 3.7$ Hz), 4.51 (1H, m, H4′), 4.35–4.30 (1H, m, H5′), 4.23 (1H, m, H3′), 4.23–4.17 (1H, m, H5″), 4.16 (3H, s, CH_3N), 3.51 (3H, s, CH_3O), 2.22 (2H, t, P–CH_2–P, $J = 19.7$ Hz). ^{31}P NMR (163 MHz): δ 18.72 (1P, α), 14.84 (1P, β). MS: calcd for $C_{13}H_{20}N_5O_{10}P_2$ 468. Found 468 $[M-H]^-$. HPLC t_R 7.9 min.

4.12. Protein preparation

Human DcpS was expressed in *Escherichia coli* according to the procedure described previously.[36] The protein was stored at −80 °C in 20 mM Tris buffer, pH 7.5, containing 50 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 20% glycerol.

4.13. Enzymatic and fluorescence studies

The methylene cap analogs 1–4 were subjected to digestion with human DcpS at 30 °C, in 50 mM Tris buffer, pH 7.9, containing 30 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and 1 mM DTT. The concentration of cap analogs in reaction mixture was 10 μM. The progress of the reaction was monitored by analytical HPLC using the equipment described in Section 4.1, except application of linear gradient of methanol from 0% to 25% in aqueous 0.05 M $CH_3CO_2NH_4$, pH 5.9, over 15 min. The reaction products were identified by matching their retention time with the appropriate peaks of reference compounds.

The fluorimetric method has been applied to measure the hydrolysis rates of cap dinucleotides. Reactions were followed by recording the time-dependent increase of fluorescence intensity, caused by removal of intramolecular stacking as a result of enzymatic cleavage of the dinucleotides. The substrate concentration (c) at the time of hydrolysis (t) was calculated as

$$c = c_0(I_t - I_e)/(I_0 - I_e),$$

where I_t, I_0, and I_e are the emission intensities at time (t), at the start, and at the end of the reaction, respectively. The rate of hydrolysis was calculated by linear regression of substrate concentration versus time. For DcpS cleavage resistant cap analogs the inhibitory parameter IC_{50} has been determined. The IC_{50} values were obtained from plots of the inhibition ratio (v_i/v_0) versus inhibitor concentration, where v_0 is the initial velocity

of hydrolysis with no inhibitor added and v_i is the initial velocity in the presence of competitor. The measurements were performed for initial substrate (m^7GpppG) concentration 5 μM and five different competitor concentrations.

The binding affinity of hDcpS for methylene-modified cap analogs was determined by monitoring the quenching of intrinsic Trp fluorescence. The association constants were measured by means of fluorescence titration (time synchronized titration method).[33] The fluorescent experiments were performed on a LS-50B spectrofluorometer (Perkin Elmer Co.) in a quartz cuvette (Hellma) with optical path length of 4 mm for absorption and 10 mm for emission. All measurements were done at 20 °C, in 50 mM Tris buffer, pH 7.6, containing 200 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and 0.5 mM EDTA. hDcpS activity on different cap dinucleotides was carried out using 0.25 U/mL (one unit of the enzyme hydrolyzes 1 μmol of m^7GpppG per min. at 30 °C). Fluorescence intensity was observed at 340 nm (excitation at 280 nm) and corrected for sample dilution and for the inner filter effects.

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