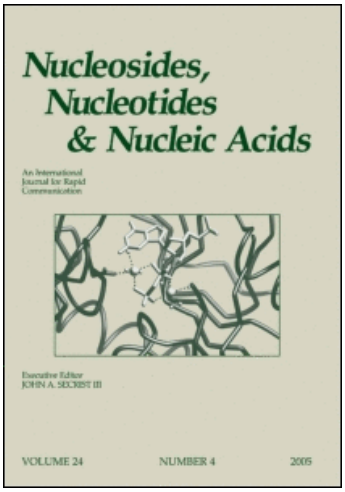

Nucleosides, Nucleotides and Nucleic Acids

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SYNTHESIS OF *LEISHMANIA* CAP-4 INTERMEDIATES, CAP-2 AND CAP-3

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□ *Synthesis of Leishmania mRNA 5′-cap analogs, $m^7Gpppm_2{}^6AmpAm$ (cap-2), and $m^7Gpppm_2{}^6AmpAmpCm$ (cap-3) is reported. Binding affinities of those cap analogs for LeishIF4E proteins were determined using fluorescence spectroscopy. Cap-3 showed similar affinity to LeishIF4Es compared to the mature trypanosomatids cap structure (cap-4).*

Keywords *Leishmania*; cap analogs; trypanosomatids

INTRODUCTION

Leishmania are trypanosomatids parasites that belong to the Kinetoplastida order. In trypanosomatids, the 5′-cap of mRNAs has a unique structure,[1] which includes base methylation on the first (A) and fourth (U) nucleotides, and 2′-*O*-methylations on the ribose moieties of the first four transcribed nucleotides, resulting in $m^7G(5')ppp(5')m_2{}^6AmpAmpCmpm^3Um\ldots$, where m preceding nucleobase denotes methyl group at the given positions of base and m following nucleobase denotes 2′-*O*-methyl

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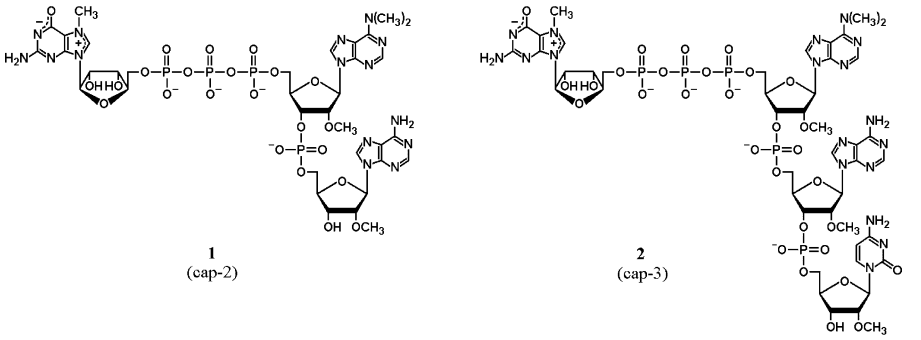

FIGURE 1 Structures of *Leishamnia* cap-2 and cap-3 intermediates.

group. This capped tetranucleotide fragment, denoted cap-4, has been recently synthesized and its binding affinity to *Leishmania* eIF4E homologue (LeishIF4E-1) was determined using fluorescence spectroscopy.[2,3] Here we report the synthesis of two cap-4 intermediates, $m^7Gppp m_2^6AmpAm$ (**1**) and $m^7Gppp m_2^6AmpAmpCm$ (**2**), assigned as "cap-2" and "cap-3", respectively (Figure 1). Our goal was to determine the binding affinities of those cap analogs to LeishIF4E proteins compared to the mature trypanosomatids cap structure.

RESULTS AND DISCUSSION

The synthesis was achieved by coupling of 5′-phosphorylated dimer (pm_2^6AmpAm, **4**) or trimer ($pm_2^6AmpAmpCm$, **5**) with an imidazole derivative of 7-methylguanosine diphosphate(**3**) in the presence of anhydrous zinc chloride as a promoter in dimethylformamide as a solvent[2,4] resulting in cap-2 and cap-3, respectively (Figure 2).

The dimer **4** was prepared by a multistep method "in solution" starting from 5′-*O*-dimethoxytrityl-N^6,N^6,2′-*O*-trimethyladenosine[2] and

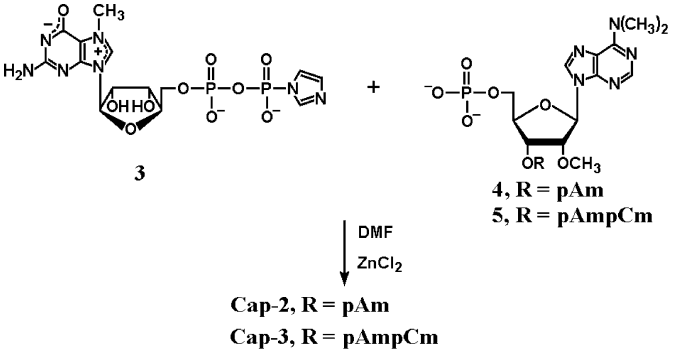

FIGURE 2 The final step for the synthesis of cap-2 and cap-3 by coupling of 7-methylguanosine 5′-diphosphate P^2-imidazolide with **4** and **5**, respectively.

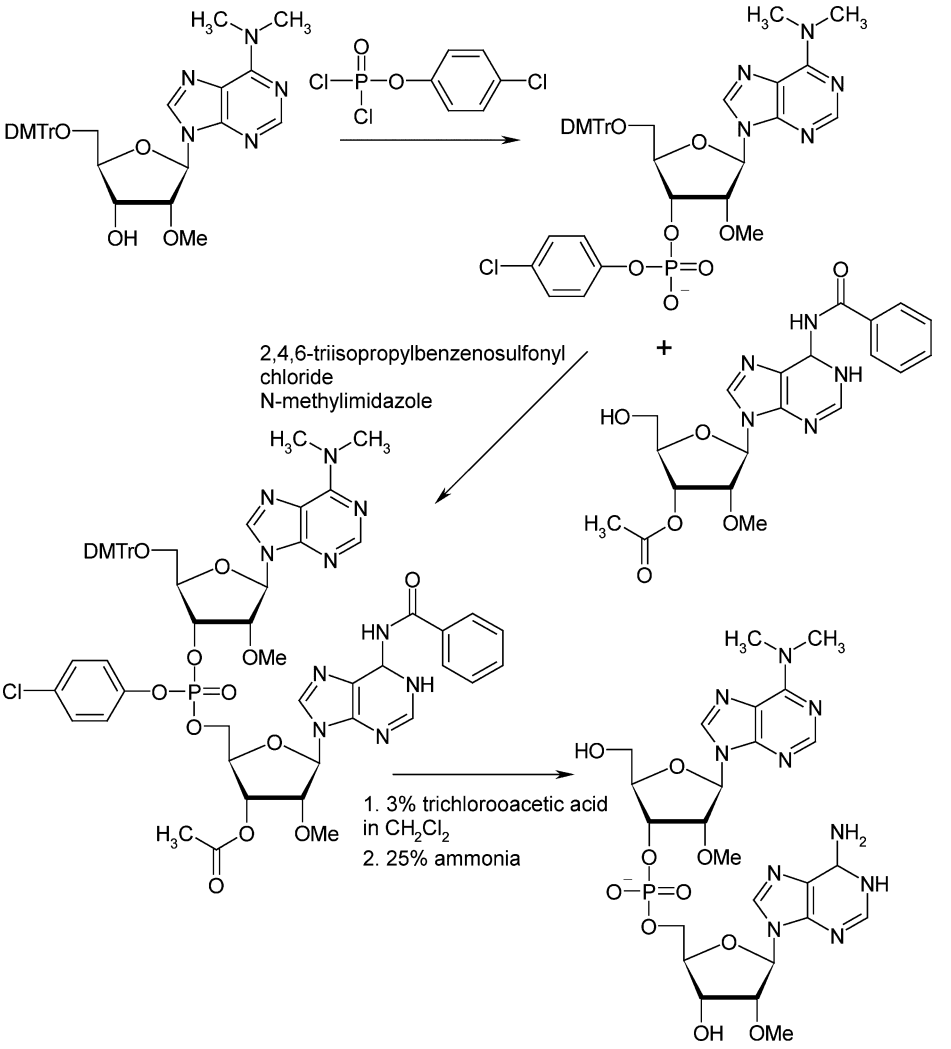

FIGURE 3 Synthesis of dinucleotide m_2^6 AmpAm.

using 4-chlorophenyl dichlorophosphate as 3′-*O*-phosphorylating agent[5] (Figure 3). Next steps of the synthesis included: (1) the reaction of 5′-*O*-dimethoxytrityl-N^6,N^6,$O^{2'}$-trimethyladenosine 3′-(*p*-chlorophenyl)phosphate with 3′-*O*-acetyl-N^6-benzoyl-2′-*O*-methyladenosine; (2) deprotection reaction with 3% trichloroacetic acid in dichloromethane; (3) deprotection with 25% ammonia in water; and (4) 5′-*O*-phosphorylation with phosphorus oxychloride in trimethylphosphate.

The trimer **5** was prepared by the phosphoramidite solid-phase method. Commercial 2′-*O*-methylcytidine linked to CPG solid support was the starting material, and subsequent couplings of adenosine,

TABLE 1 The association constants (K_{as}) for complexes of *Leishmania* eIF4Es and cap analogs[9]

Cap Analog	K_{as} (μM^{-1})				
	LeishIF4E-1	LeishIF4E-2	LeishIF4E-2 delta	LeishIF4E-3	LeishIF4E-4
$m^7Gpppm^6_2$ Am (cap-1)	0.08 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.08 ± 0.03
$m^7Gpppm^6_2$ AmpAm (cap-2)	0.07 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.23 ± 0.06
$m^7Gpppm^6_2$ AmpAmpCm (cap-3)	0.19 ± 0.03	0.24 ± 0.02	0.19 ± 0.03		
cap-4	0.25 ± 0.01	0.28 ± 0.04	0.22 ± 0.03	0.05 ± 0.02	0.77 ± 0.08

N^6,N^6,2′-*O*-trimethyladenosine and 2-cyanoethyl-3-(4,4′-dimetoxytrityloxy)-2,2-di(ethoxycarbonyl)propyl-1-N,N-diisopropyl phosphoramidite building blocks were done according to known procedures.[2,6,7]

The final products (cap-2 and cap-3) were isolated by preparative HPLC and their structures were confirmed by ^1H, ^{31}P NMR, and ESI mass spectrometry.

The new cap analogs were tested for their affinities for LeishIF4E-1 and other recently identified *Leishmania* eIF4E isoforms[8,9] by a fast and accurate time-synchronized fluorescence titration method, which is based on measuring the quenching of intrinsic Trp fluorescence of the proteins in response to binding of cap analog.[10] The association constants (K_{as}) for the complexes of *Leishmania* eIF4Es and the different cap analogs were determined. The binding affinities of cap-2 and cap-3 were compared to the affinities of cap-1 and cap-4 to *Leishmania* eIF4E isoforms.[2] Cap-3 showed similar although slightly lower affinity to LeishIF4Es compared to cap-4, whereas cap-2 bound to LeishIF4Es significantly weaker than cap-4. The results are shown in Table 1.

EXPERIMENTAL

Monitoring of the Chemical Synthesis

TLC analysis was performed on TLC plates coated with silicagel 60 F-254 (Merck, Darmstadt, Germany), developed by chloroform-methanol 9:1 v/v (A); chloroform-ethanol 9:1 v/v (B); chloroform-methanol 9.7:0.3 v/v (C).

Analytical HPLC was performed on a Spectra-Physics SP8800 apparatus, using a Supelcosil LC-18-T reverse phase column (4.6 × 250 mm, flow rate 1.3 mL/minute) with buffer A (0.05 M ammonium acetate at pH 5.9) and buffer B (methanol and buffer A, 1:1 v/v). Linear 0–100% gradient of buffer B in buffer A over 20 minutes.

^1H NMR and ^{13}C NMR spectra were run on a Varian UNITY-plus spectrometer at 400 and 100.56 MHz, respectively, at ambient temperatures and at concentrations of ca. 2 mg/mL (^1H NMR), and ca. 20 mg/mL

(^{13}C NMR) using tetramethylsilane (TMS) as the internal standard in $CDCl_3$ and sodium 3-trimethylsilyl-[2,2,3,3-D_4]-propinonate (TSP) in D_2O.

Mass spectra were recorded on a Micromass QToF 1 MS spectrometer using electrospray negative ionization.

Preparative HPLC

Preparative HPLC was performed on a Waters 600E Multisolvent Delivery System apparatus, using a Waters HR-C-18 reverse phase column (19 × 300 mm, flow rate 5.0 mL/minute). The mobile phase for this method was the same as for analytical HPLC (see above). Linear 0–100% gradient of buffer B in buffer A over 40 minutes.

Synthesis

N^6-Benzoyl-$O^{2'}$-methyladenosine.[11] $O^{2'}$-Adenosine (1 g, 3.5 mmol) was benzoylated in dry pyridine (10 mL) with 1.75 mL (2.1 g, 15 mmol) of benzoyl chloride for 1 hour at room temperature. The progress of the reaction was monitored by TLC ($Rf_{substrate} = 0.1$, $Rf_{product} = 0.6$ in A). The resulting solution was added into 100 mL of cold water and the insoluble product extracted with chloroform (3 × 100 mL). The chloroform extract was evaporated under reduced pressure to a gum and dissolved in a mixture of ethyl alcohol (45 mL) and pyridine (30 mL). The solution was treated with 15 mL of 2 M sodium hydroxide at room temperature for 5 minutes. An excess of pyridinium Dowex-50 ion exchange resin was then added to remove sodium ions, the resin was removed by filtration and the filtrate was concentrated under reduced pressure. Then water (50 mL) was added and resulting solution was extracted with diethyl ether (3 × 50 mL). Preparative HPLC purification gave N^6-benzoyl-$O^{2'}$-methyladenosine ($Rt_{substrate} = 10.95$ minutes, $Rt_{product} = 17.27$ minutes, 0.7 g, 1.8 mmol, yield 51%, m/z = 386.14 by ESI-MS).

N^6-Benzoyl-5′-dimethoxytrityl-O$^{2'}$-methyladenosine. A mixture of N^6-benzoyl-O$^{2'}$-methyladenosine (0.47 g, 1.2 mmol), 4,4′-dimethoxytrityl chloride (1.15 g, 3.4 mmol) and triethylamine (0.404 mL, 0.29 g, 2.9 mmol) in anhydrous pyridine (5 mL) was stirred for 3 hours at room temperature, and the progress of the reaction was monitored by TLC (B). The mixture was quenched with methanol (3 mL) and evaporated under reduced pressure at room temperature. The residual syrup was dissolved in chloroform (30 mL) and washed with two 23-mL portions of 1 M aqueous sodium hydrogen carbonate solution. The chloroform layer was dried over anhydrous sodium sulphate, filtered, and evaporated under reduced pressure. The residual pyridine was removed by coevaporation with three 3-mL portions

of toluene. The crude product was purified by column chromatography over silicagel eluting with a stepwise gradient system (0.25–2%) of methanol in dichloromethane to give N^6-benzoyl-5′-dimethoxytrityl-$O^{2'}$-methyladenosine (0.71 g, 1.03 mmoles, 85%, $Rf_{product} = 0.46$ in B, m/z = 688.3 by ESI-MS).

$O^{3'}$-Acetyl-N^6-benzoyl-$O^{2'}$-methyladenosine. A mixture of N^6-benzoyl-5′-dimethoxytrityl-$O^{2'}$-methyladenosine (0.483 g, 0.7 mmol) and acetic anhydride (0.079 mL, 0.84 mmol) in anhydrous pyridine (3.5 mL) was stirred overnight at room temperature. The progress of the reaction was monitored by TLC ($Rf_{substrate} = 0.3$, $Rf_{product} = 0.44$ in C). The reaction mixture was then added into 20 mL of dichloromethane and resulting solution was extracted with 1 M $NaHCO_3$ (3 × 10 mL). The organic layer was dried over anhydrous sodium sulphate, filtered, and evaporated to an oil under reduced pressure. Then 5 mL of 3% solution of trichloroacetic acid in dichloromethane (20 mL) was added, the mixture was kept at room temperature for 1 hour and extracted with water (3 × 30 mL). The organic layer was evaporated under reduced pressure. The resulting product was purified by column chromatography over silicagel and eluting with a stepwise gradient system (0.25–0.2%) of methanol in dichloromethane to give $O^{3'}$-acetyl-N^6-benzoyl-$O^{2'}$-methyladenosine (0.21 g, 0.49 mmol, yield 70%, m/z = 428.17 by ESI-MS).

5′-Dimethoxytrityl-N^6,N^6,$O^{2'}$-trimethyladenosine 3′-(*p*-chlorophenyl) monophosphate (Figure 3). The mixture of 5′-dimethoxytrityl-N^6,N^6,$O^{2'}$-trimethyladenosine[2] (0.1 g, 0.164 mmol) and *p*-chlorophenyl phosphodichloridate (0.133 mL, 0.2 g, 1.4 mmol) in dry pyridine was stirred for 0.5 hours. The progress of the reaction was monitored by TLC ($Rf_{substrate} = 0.4$, $Rf_{product} = 0.06$ in A). Then 20 mL of 1 M buffer TAEB (triethylammonium hydrogen carbonate) was added and the reaction mixture was cooled to 0°C for 10 minutes. After 10 minutes chloroform was added (20 mL) and the mixture was washed with TEAB buffer (0.1 M). The organic phase was evaporated under reduced pressure then coevaporated with toluene (3 × 5 mL) and dried in vacuo. 5′-Dimethoxytrityl-N^6,N^6,$O^{2'}$-trimethyladenosine 3′-(*p*-chlorophenyl)monophosphate was obtained as white solid (0.123 g, 0.15 mmol, yield 93%).

Protected Dinucleotide $m_2^6A^{pr}mpA^{pr}m$ (Figure 3) . 5′-Dimethoxytrityl-N^6, N^6,$O^{2'}$-trimethyladenosine 3′-(*p*-chlorophenyl)monophosphate (0.123 g, 0.15 mmol) and $O^{3'}$-acetyl-N^6-benzoyl-$O^{2'}$-methyladenosine (0.047 g, 0.11 mmol) were dried by coevaporation with anhydrous acetonitrile (20 mL). Then a solution of 2,4,6-triisopropylbenzenesulfonyl chloride (0.104 g, 0.347 mmol) and *N*-methylimidazole (0.058 g, 0.69 mmol) in 2 mL of

acetonitrile was added, and the mixture was allowed to react for 4 hours. The progress of the reaction was monitored by TLC ($Rf_{product} = 0.44$ in A). The crude product was purified by column chromatography (silicagel). Elution was performed with a step-wise gradient (0.25–2%) of methanol in chloroform, to give protected dinucleotide (0.09 g, 0.074 mmol, yield 67%, m/z = 1211.38 by ESI-MS).

Dinucleotide m_2^6AmpAm (Figure 3). To the protected dinucleotide (0.02 g, 0.016 mmol) 10 mL of 3% solution of trichloroacetic acid in dichloromethane was added and the mixture was stirred at room temparature for 1 hour and then extracted with water (3×20 mL). The organic layer was evaporated under reduced pressure and 25% ammonia (20 mL) was added. The reaction mixture was kept for 2 days at room temperature. The crude product was purified by HPLC ($Rt_{product}$ = 20.13 minutes., 5.7 mg, 0.008 mmol, yield 16%, m/z = 651.21).

Dinucleotide 5′-Monophosphate (pm_2^6AmpAm). A mixture of dinucleotide (m_2^6AmpAm, 0.017 g, 0.026 mmol) and $POCl_3$ (0.02 mL, 0.033 g, 0.2 mmol) in trimethyl phosphate (0.9 mL) was stirred for 1 hour at 0°C. The progress of the reaction was monitored by HPLC ($Rt_{substrate}$ = 20.13 minutes, $Rt_{product}$ = 16.20 minutes). Then water (3 mL) was added and mixture was neutralized (to pH = 7) with 0.7 M TEAB buffer. Crude product was purified by HPLC to give pm_2^6AmpAm (ammonium salt) (0.011 g, 0.015 mmol, yield 58%, m/z = 731.21 by ESI-MS).

Cap-2 ($m^7Gpppm_2^6$AmpAm; Figures 1 and 2). A mixture of ammonium salt of dinucleotide 5′-monophosphate (pm_2^6AmpAm; 0.011 g, 0.015 mmol), a sodium salt of P^2-imidazolide 7-methylguanosine 5′-diphosphate [Im(m^7GDP)] 0.027 g, 0.053 mmol), and $ZnCl_2$ (0.062 g, 0.46 mmol) in dimethylformamide (0.5 mL) was stirred for 2 days at room temperature. The progress of the reaction was monitored by HPLC ($Rt_{substrate}$ = 16.64 minutes, $Rt_{product}$ = 12.83 minutes). Then addition of a few drops of water quenched the reaction and EDTA was added until the reaction mixture turned clear. The crude product was then purified by HPLC (0.0063 g, 0.005 mmol, yield 37%, m/z = 1169.2 by ESI-MS; calculated molecular weight for cap-2: 1171.8).

^{1}H NMR (D_2O, δppm): 8.24 (1H, H2, s, adenine), 8.20 (1H, H2, s, N^6,N^6-dimethyladenine), 8.19 (1H, H8, s, adenine), 7.86 (1H, H8, s, N^6, N^6-dimethyladenine), 6.08 (1H, H1′, d, 7-methylguanosine, J = 3.99 Hz), 5.85 (1H, H1′, d, adenine), 5.81 (1H, H1′, d, N^6,N^6-dimethyladenine), 4.58-4.54 (3H, H2′, m, 7-methylguanosine, $O^{2'}$-methyladenosine, N^6,N^6,$O^{2'}$-trimethyladenosine), 4.54-4.40 (3H, H3′, m, 7-methylguanosine, $O^{2'}$-methyladenosine, N^6,N^6,$O^{2'}$-trimethyladenosine), 4.40-4.20 (9H, H4′ and

H5′, m, 7-methylguanosine, $O^{2'}$-methyladenosine, N^6,N^6,$O^{2'}$-trimethyladenosine), 3.97 (3H, N-CH_3, s, 7-methylguanosine), 3.54 (6H, s, O-CH_3, $O^{2'}$-methyladenosine and N^6,N^6,$O^{2'}$-trimethyladenosine), 3.47 (6H, s, N-CH_3, N^6,N^6-dimethyladenine).

^{31}P NMR (D_2O, δppm) 0.709 ApA, -11.207 P$\alpha\gamma$, -22.65 Pβ.

Solid Phase Synthesis[2]. The RNA trimer was synthesized on an Applied Biosystems 392 synthesizer using a 1 μm scale protocol with 10 minutes coupling for each step. The starting material, 5′-Dimethoxytrityl-*N*-benzoyl-2′-*O*-methylcytidine-3′-succinoyl-long chain alkylamino-CPG (Glen Research, Sterling, VA, USA) was placed in a standard reaction vessel and, successively, following building blocks were coupled for elongation of the ribonucleic chain: (1) 5′-dimethoxytrityl-2′-*O*-methyl-*N*-phenoxyacetyladenosine-3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (Glen Research); (2) 5′-Dimethoxytrityl-N^6,N^6,$O^{2'}$-trimethyladenosine-3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite[2]; and (3) 5′-phosphorylating reagent, 2-cyanoethyl-3-(4,4′-dimethoxytrityloxy)-2,2-di(ethoxycarbonyl)propyl-1-*N*,*N*-diisopropyl phosphoramidite (Glen Research).

Unprotected RNA Fragment (pm_2^6AmpAmpCm). A 5′-blocked RNA fragment was detritylated for 20 minutes with 3% trichloroacetic acid in dichloromethane. After 20 minutes the resin was washed with dichloromethane and 25% ammonia was added. The reaction mixture was kept overnight at 54°C. The crude product was purified by preparative HPLC ($Rt_{product}$ = 41.5 minutes) to give pm_2^6AmpAmpCm (ammonium salt; 1.32 mg, 1.2 μmol, m/z = 1050.29 by ESI-MS).

Cap-3 (m^7Gpppm_2^6AmpAmpCm; Figures 1 and 2). A mixture of ammonium salt of trinucleotide 5′-monophosphate (pm_2^6AmpAmpCm; 1.06 mg, 1.009 μmol), a sodium salt of P^2-imidazolide 7-methylguanosine 5′-diphosphate (Im-m^7GDP; 0.002 g, 3.4 μmol), and $ZnCl_2$ (0.003 g, 22 μmol) in dimethylformamide (0.150 mL) was stirred for 5 days at room temperature. The progress of the reaction was monitored by HPLC ($Rt_{substrate}$ = 16.73 minutes, $Rt_{product}$ = 14.33 minutes). Then EDTA (1,2-ethylenediaminetetraacetic acid) was added until the reaction mixture turned clear. The crude product was then purified by preparative HPLC ($Rt_{product}$ = 36 minutes, 0.006 g, 0.4 μmol, yield 39%, m/z = 1489.43 by ESI-MS). ESI-MS spectra (negative ionization): m/z 1489.4 (calculated molecular weight for cap-3: 1491.0).

^{1}H NMR (D_2O, δppm): 8.20 (1H, H8, s, adenine), 8.10 (1H, H8s, N^6,N^6-dimethyladenine), 8.08 (1H, H2, s, adenine), 7.78 (1H, H2, s, N^6,N^6-dimethyladenine), 7.60 (1H, H6, d, cytosine), 5.83 (1H, H1′, s, N^6,N^6,$O^{2'}$-trimethyladenosine), 5.99 (1H, H1′ s, 7-methylguanosine)

5.90 (1H, H1′, d, $O^{2'}$-methyladenosine, J = 3.6 Hz), 5.83 (1H, H1′, d, N^6,N^6,$O^{2'}$-trimethyladenosine), 5.71 (1H, H5 s, cytosine), 5.55 (1H, H1′ d, $O^{2'}$-methylcytidine), 4.52 (1H, H2′ m, $O^{2'}$-methyladenosine), 4.46 (1H, H2′ m, N^6,N^6,$O^{2'}$-trimethyladenosine), 4.31 (1H, H3′ m, N^6,N^6,$O^{2'}$-trimethyladenosine), 4.26 (1H, H3′ m, $O^{2'}$-methyladenosine), 4.22 (1H, H3′ m, $O^{2'}$-methylcytidine), 4.14 (1H, H2′ m, 7-methylguanosine), 4.40-4.0 (12H, H4′ and H5′ m, N^6,N^6,$O^{2'}$-trimethyladenosine, $O^{2'}$-methylcytidine, 7-methylguanosine, $O^{2'}$-methyladenosine), 3.99 (3H, N-CH_3 s, 7-methylguanosine), 3.80-3.60 (15H m, N-CH_3, N^6,N^6-dimethyladenine and O-CH_3 N^6,N^6,$O^{2'}$-trimethyladenosine, $O^{2'}$-methylcytidine, $O^{2'}$-methyladenosine), 3.73 (1H, H2′ m, $O^{2'}$-methylcytidine).

^{31}P NMR (D_2O, δppm) 10.022 pA, ApC, -0.024 P$\alpha\gamma$, -10.428 Pβ.

Fluorescence Spectroscopy

Fluorescence titration measurements were carried out with LS-50B spectrofluorometer (Perkin Elmer Co. Norwalk, CT, USA) in 50 mM Hepes pH 7.2, 100 mM NaCl, 1 mM EDTA, 1 mM DTT at 20°C using different protein concentrations, from 0.2 μM to 1 μM. The fluorescence intensity (excited at 295 nm and observed at 320 or 345 nm) was corrected, taking into account the sample dilution, the inner filter effect and the instability of protein fluorescence as described previously.[10]

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