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Synthesis of ^{13}C - and ^{14}C -labeled dinucleotide mRNA cap analogues for structural and biochemical studies

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Abstract

Herein we describe the first simple and short method for specific labeling of mono- and trimethylated dinucleotide mRNA cap analogues with ^{13}C and ^{14}C isotopes. The labels were introduced within the cap structures either at the N7 for monomethylguanosine cap or N7 and N2 position for trimethylguanosine cap. The compounds designed for structural and biochemical studies will be useful tools for better understanding the role of the mRNA cap structures in pre-mRNA splicing, nucleocytoplasmic transport, translation initiation and mRNA degradation.

Keywords

Cap analogue; TMG cap; ^{13}C label; ^{14}C label; mRNA

Gene expression in eukaryotes is a complex process that involves several steps and requires numerous protein factors. One common RNA element involved in mRNA splicing, export, translation, and stability is the unusual structure at the 5' end of mRNA known as a 'cap'.¹ This unique structure consist of 7-methylguanosine linked via a 5',5'-triphosphate bridge to the first transcribed nucleotide (MMG cap). For many years, numerous studies have been conducted to describe the molecular mechanism of the interaction of the cap with eukaryotic initiation factor 4E (eIF4E) that is a key step in translation initiation. Structural requirements for the cap–eIF4E interaction were elucidated by biophysical and biochemical methods involving several chemically prepared cap analogues and subsequently confirmed and further defined by multidimensional nuclear magnetic resonance and X-ray crystallography.² In some cases, for example in nematodes such as *Caenorhabditis elegans* and *Ascaris suum*, in addition to regular MMG cap, an atypical hypermethylated form of the cap, with two additional methyl groups at the N2 position of 7-methylguanosine, is present (TMG cap).³ This cap is added to the 5' end of a large percentage of mRNAs by RNA trans-splicing of a short 22-nt spliced leader sequence (SL).⁴ In general, eIF4E proteins from higher eukaryotes are unable to effectively recognize and bind the TMG cap.^{2d,5} However, nematodes efficiently translate TMG-capped mRNAs.^{3b,6} Identification and exploration of five eIF4E isoforms in *C. elegans* provided evidence that the 4E isoforms vary in their ability to distinguish between MMG and TMG caps suggesting that eIF4E of these organisms may differ from that of other species.⁷ Apart from three *C. elegans* isoforms (IFE-1, -2, -5) which are able to bind MMG and TMG caps, other eIF4E orthologs recognizing the two mRNA caps have been described, for example *Ascaris suum* eIF4E-3^{6a}

and the sole *Schistosoma mansoni* eIF4E isoform.⁸ Biochemical, biophysical, theoretical, and structural data are available for different 4E proteins (*C. elegans*,⁹ *A. suum*,^{6a,10} *S. mansoni*,⁸ murine¹¹). Based on these data, models for binding the two caps have been proposed.^{6a,8–11} Recently the first crystal structure of *Ascaris* eIF4E-3 in complex with MMG and TMG cap has been published.¹⁰ While the data indicate how *Ascaris* eIF4E-3 interacts with the MMG and TMG cap, the exact coherent molecular mechanism describing the interaction of other isoforms binding either cap is not completely understood.

Herein we describe the synthesis of ¹³C and ¹⁴C labeled mono- and trimethylated cap analogues as tools that will facilitate analysis of the specificity of nematode eIF4E isoforms for the MMG and TMG caps and help to examine proteins interactions with the two caps in a more detailed way.

A well-defined synthetic pathway to prepare dinucleotide cap analogues involves obtaining imidazolide derivative of one nucleotide in a reaction with 2,2'-ditiopyridine and triphenylphosphine prior to coupling with a second nucleotide in the presence of an excess of double-charged ions (such as Zn²⁺ or Mn²⁺) in anhydrous or aqueous condition.¹² Nevertheless, for the synthesis of ¹³C/¹⁴C MMG and TMG caps, the procedure needed to be modified to minimize the number of steps with the radioactive materials and due to the cost of isotopically labeled substrates (¹³C or ¹⁴C methyl iodide). Our method to synthesize the MMG dinucleotide cap analogues labeled with either ¹³C or ¹⁴C was accomplished (Scheme 1) by direct methylation of the dinucleotide GpppG that was prepared by coupling of guanosine 5'-diphosphate (GDP) with an imidazolide derivative of guanosine 5'-monophosphate (imGMP).¹³ As GpppG is symmetric and can be methylated on both guanine residues, the procedure was optimized and evaluated using unlabeled methyl iodide and numerous reaction conditions (variation of reaction time, excess of methyl iodide, temperature) and HPLC (data not shown) to define conditions that prevent over methylation. The best results were obtained with twofold molar excess of ¹³CH₃I (Sigma–Aldrich) or ¹⁴CH₃I (ViTrax) at room temperature.¹⁴ The synthesis of ¹³C labeled MMG cap has been described previously¹⁵ but it was achieved by a longer two-step procedure.

In contrast to MMG cap, a TMG cap labeled with ¹³C and ¹⁴C isotopes required a multistep procedure (Scheme 2). To our knowledge this is the first report of such a synthesis. The first step required transformation of guanosine to the corresponding 2',3',5'-*O*-acetylguanosine using acetic anhydride (Ac₂O) in the presence of triethylamine (TEA) and *N,N*-(dimethylamino) pyridine (DMAP). The obtained derivative was further protected at the O6 position of guanosine using the Mitsunobu reaction with *p*-nitrophenylethanol (NPE) under anhydrous conditions. The product **3** was then converted to its 2-fluoro derivative **3** by performing non-aqueous diazotization and fluoridation at low temperature with *t*-butyl nitrite as the diazotizing agent and HF in pyridine as the fluoride source. The purified 2-fluoro nucleoside was then treated with a fourfold molar excess of (¹³CH₃)₂NH. As the dimethylamine labeled with ¹³C carbon can only be obtained as a hydrochloride, a nucleophilic substitution was performed in aqueous solution of DMSO/AcCN/H₂O/Et₃N in a temperature-controlled oil bath at 60 °C until the fluoronucleoside had completely disappeared, as evaluated by TLC analysis. After completion of the substitution reaction, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was added to the reaction mixture to remove the NPE group followed by the addition of a mixture of 0.5 M NaOH in THF/MeOH/NaOH_{aq} in order to complete acetyl deprotection.¹⁶ The nucleoside (**4**) was purified on silica gel and then phosphorylated using the Yoshikawa method with phosphorus oxide trichloride in trimethyl phosphate (**5**) and then methylated with fivefold excess of ¹³CH₃I in anhydrous DMSO (**6**). The ¹³C trimethylated guanosine 5'-monophosphate was further coupled with the imidazolide derivative of GDP (**7**) under anhydrous conditions (DMF) in the presence of

zinc ions.^{15,17} This route was chosen as it is routinely used by us for the preparation of unlabeled TMG cap analogues.

We chose to introduce the carbon-14 labeled methyl group into the N7 position of guanine as this reaction is relatively simple and with only one labeled methyl group in the cap moiety, leads to a relatively high specific activity of the cap. ¹⁴C TMG cap was obtained as described for the preparation of ¹³C labeled TMG cap except that the substitution step leading to *N*²,*N*²-dimethylguanosine was carried out with unlabeled dimethylamine. All four cap analogues were purified by ion-exchange chromatography on DEAE-Sephadex A-25 (HCO₃⁻ form), the relevant fractions collected, pooled, and evaporated repeatedly with cold ethanol. The fractions with both high UV absorbance and high specific activity were combined and the final specific activity of ¹⁴C labeled cap analogues was determined after lyophilization: 0.82 and 1.44 GBq/mmol for MMG and TMG cap, respectively. MMG and TMG cap analogues labeled with ¹⁴C and ¹³C isotopes were converted into sodium salts using Dowex 50 WX8 ion-exchange resin. The structure and homogeneity of final products were confirmed by HPLC, mass spectrometry, ¹H NMR, ³¹P NMR and ¹³C NMR. ¹H NMR spectra of ¹³C-labeled MMG and TMG caps revealed no signals corresponding to unlabeled methyl group (Fig. 1). All methyl groups in the spectra were ¹³CH₃ observed as a doublet with the expected large coupling constant. ¹³C labeling of **1** and **8** was also confirmed by a single-frequency proton decoupled ¹³C NMR spectra. Extremely strong signals from one (**1**) or three (**8**) methyl carbons with appropriate coupling pattern were also observed.

To evaluate the ¹⁴C cap analogues in a functional assay, we carried out decapping experiments (Fig. 2) using *A. suum* and *C. elegans* DcpS, the scavenger decapping enzymes. DcpS cleaves mono- as well as trimethylated cap regioselectively between β and γ phosphates of the 5',5'-triphosphate bridge to release m⁷GMP or m₃^{2,2,7}GMP and a downstream oligonucleotide. This simple experiment indicated that incubation of nematode DcpS with the labeled m⁷*GpppG or m₃^{2,2,7}*GpppG led to labeled m⁷*Gp and m₃^{2,2,7}*Gp cap-derived products, respectively, as illustrated by TLC and autoradiography (Fig. 2).

In conclusion we have developed a simple and short method for specific labeling of mono- and trimethylated caps with ¹³C and ¹⁴C isotopes. The compounds will be extremely useful as tools for NMR studies (¹³C), for monitoring chemical and enzymatic reactions (¹⁴C), or to synthesize RNA containing the ¹⁴C labeled cap structures by in vitro transcription.

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14. General procedure for a synthesis of m⁷*GpppG cap analogues labeled with ¹³C or ¹⁴C. GpppG was prepared by coupling of imidazolidine derivative of guanosine 5'-monophosphate (GMP) (21 mg, 0.05 mmol) with guanosine 5'-diphosphate (27 mg, 0.05 mmol) in anhydrous conditions in the presence of ZnCl₂ (50 mg, 0.37 mmol) as previously described. ¹³GpppG (20 mg, 0.02 mmol) was further methylated with appropriate methyl iodide, labeled with ¹³C or ¹⁴C (0.04 mmol) in anhydrous DMSO at room temperature for 2 h. The reaction mixture was poured into water and extracted three times with diethyl ether. Aqueous phase was purified on DEAE-Sephadex using a 0–0.8 M gradient of TEAB. Final products were obtained as colorless crystals ([¹³C] m⁷*GpppG, (1) 8.4 mg, 0.01 mmol, 52%, [¹⁴C] m⁷*GpppG, (2) 7.8 mg, 0.0096 mmol, 48%); [¹³C] m⁷*GpppG MS-ESI: *m/z* calcd. 804.1024, Found: 804.1183; ¹H NMR (D₂O, 500 MHz) δ 8.03 (s, 1H; H-8, G), 5.91 (d, 1H; H-1', m7G), 5.82 (d, 1H; H-1', G), 4.67 (t, 1H, H-2', G), 4.51–4.48 (m, 2H; H-2', m7G; H-3', G), 4.43–4.35 (m, 4H; H-3', m7G, H-4', G, H-4', m7G, H-5', G), 4.32–4.26 (m, 3H; H-5', H-5'', m7G; H-5'', G), 4.06 (d, 3H; 7-CH₃, J_{H,13C} = 145 Hz); ¹³C NMR (D₂O, 125 MHz, H1 decouple 500 MHz) δ 36.24; ³¹P NMR (D₂O, 202 MHz) δ –10.31 (1P, α), –10.40 (1P, γ), –21.91 (1P, β).
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16. N²-fluoro-2',3',5'-*O*-triacyl-*O*⁶-[2-(4-nitrophenyl)ethyl]inosine (3) The 2',3',5'-tri-*O*-acetylguanosine was prepared from guanosine using acetic anhydride in the presence of triethylamine and *N,N*-(dimethylamino) pyridine using a procedure modified from Nair et al. (*J. Am. Chem. Soc.*, **1987**, 109, 7223). In the next step a suspension of 2',3',5'-tri-*O*-acetylguanosine (2.37 g, 5.8 mmol), triphenylphosphine (2.28 g, 8.7 mmol) and 2-(4-nitrophenyl)ethanol (1.45 g, 8.7 mmol) in anhydrous toluene was stirred for 30 min and diisopropylazodicarboxylate (1.4 mL) was added dropwise over a period of 45 min. The reaction mixture was kept for 12 h at rt. The solvent was evaporated and the residual oil was purified by column chromatography on silica gel with chloroform to produce a pure product, 2',3',5'-tri-*O*-acetyl-*O*⁶-[2-(4-nitrophenyl)ethyl]guanosine, of yellowish crystals, 2.26 g (70%). ¹H NMR (200 MHz, CDCl₃) δ 8.17 (d, 2H), 7.72 (s, 1H), 7.49 (d, 2H), 6.05–5.91 (m, 2H), 5.87–5.75 (m, 1H), 4.73 (t, *J* = 6.7 Hz,

- 2H), 4.50–4.36 (m, 3H), 3.28 (t, 6.7 Hz, 2H), 2.14 (s, 3H), 2.09 (s, 3H), 2.08 (s, 3H); *m/z*: calcd for $C_{24}H_{26}N_6O_{10}(M+H)^+$: 559.1783, found: 559.1784. In the next step dry 2',3',5'-tri-*O*-acetyl-*O*⁶-[2-(4-nitrophenyl)ethyl]guanosine (1 g, 1.79 mmol) in polypropylene tube under nitrogen was dissolved in anhydrous pyridine (6.75 mL, 0.082 mol). The tube was placed in a dry ice/ acetonitrile cooling bath (–35 to –45 °C) and 70% HF/pyridine solution (12 mL, 0.42 mol) was added dropwise over a period of 5 min to 45% final HF. The reaction mixture was stirred for 15 min and *t*-butyl nitrite (0.54 mL, 4.5 mmol) was added. After 1 h, the reaction was quenched at 0 °C by slowly pouring the reaction mixture into an aqueous K_2CO_3 solution (28.5 g in 25 mL of water) and then extracted three times with ethyl acetate. The organic layers were collected, dried over anhydrous Na_2SO_4 and evaporated to dryness. Purification by column chromatography using as eluate 60:1 CH_2Cl_2 :MeOH gave 0.85 g (85%) of product (3); TLC silica gel, CH_2Cl_2 /MeOH, 60:1 R_F = 0.4; ¹H NMR (700 MHz, $CDCl_3$) δ 8.21–8.15 (m, 2H), 8.08 (s, 1H), 7.50 (d, 2H), 6.13 (d, *J* = 5.6 Hz, 1H), 5.82 (t, *J* = 4.9 Hz, 1H), 5.60–5.55 (m, 1H), 4.84 (t, *J* = 6.7 Hz, 2H), 4.46–4.42 (m, 2H), 4.38–4.36 (m, 1H), 3.32 (t, *J* = 6.7 Hz, 2H), 2.15 (s, 6H), 2.08 (s, 3H). *m/z*: calcd for $C_{24}H_{24}FN_5O_{10}(M+H)^+$: 562.1579, found: 562.1581. *Synthesis of ¹³C labeled m³,2,7*GpppG (8)* *N*²-fluoro-2',3',5'-*O*-triacetyl-*O*⁶-[2-(4-nitrophenyl)ethyl]inosine (3, 250 mg, 0.45 mmol) was dissolved in 2 mL of aqueous solution of DMSO/AcCN/H₂O/Et₃N and (¹³CH₃)₂NH (1.8 mmol) was added. The reaction mixture was stirred at 60 °C for 4 h until the fluoronucleoside completely disappeared. After completion of the substitution reaction, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was added to the reaction mixture followed by the addition of 2.75 mL a mixture of 0.5 M NaOH in THF/MeOH/NaOH_{aq} (5:4:2). The solvent was evaporated and the residual oil was purified by column chromatography on silica gel with CH_2Cl_2 /MeOH (6:1) to produce a pure product (4) (100 mg, 56%). *N*²*,*N*²*-dimethylguanosine (75 mg, 0.24 mmol) was in the next step phosphorylated (*Tetrahedron Lett.* **1967**, 8, 5065) with phosphorus trichloride oxide (100 μL) in trimethyl phosphate (2.35 mL) at 4 °C and methylated with fivefold excess of ¹³CH₃I (0.075 mL, 1.2 mmol) in anhydrous DMSO (0.5 mL). The reaction mixture was poured into water (5 mL) and extracted three times with diethyl ether. Aqueous phase was concentrated and applied on DEAE–Sephadex. The product was eluted using a 0–0.8 M gradient of TEAB. m³,2,7*GMP was obtained as a colorless crystals (6) (44 mg, 0.09 mmol, 60%). The ¹³C trimethylated guanosine 5'-monophosphate (6) (0.05 mmol, TEA salt) was further coupled with the imidazolide derivative of GDP (27 mg, 0.05 mmol) (7) in anhydrous DMF (0.5 mL) in the presence of zinc ions (50 mg, 0.37 mmol). The reaction mixture was poured into a solution of EDTA (110 mg, 0.46 mmol) in water (1.5 mL), neutralized to pH 7 by addition of 1 M TEAB and separated by ion-exchange chromatography on DEAE–Sephadex. Pure product (8) was obtained as plain crystals (29 mg, 0.035 mmol, 70%) ¹⁷ MS-ESI: *m/z*: calcd 834.1337, found 834.1749; ¹H NMR (D₂O, 500 MHz) δ 7.97 (s, 1H; H-8, G), 5.95 (d, 1H; H-1', m⁷G), 5.76 (d, 1H; H-1', G), 4.55–4.52 (m, 2H, H-2', G, m⁷G), 4.44–4.25 (m, 8H; H-2', H-3', H-4', H-5', H-5'', G, m⁷G), 4.07 (d, 3H; 7-CH₃, *J*_{H,13C} = 145 Hz), 3.10 (dd, 6H, N2-CH₃, *J*_{H,13C} = 140 Hz); ¹³C NMR (D₂O, 125 MHz, H1 decouple 500 MHz) δ 37.46, 36.14; ³¹P NMR (D₂O, 202 MHz) δ –10.31 (1P, α), –10.41 (1P, γ), –21.93 (1P, β).
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Figure 1. Portions of the ^1H NMR spectra of the synthesized ^{13}C -labeled MMG (A) and TMG (B) caps illustrating the characteristic $^{13}\text{CH}_3$ signals. (A) N7 ^{13}C methyl group of the MMG cap, $J_{\text{H-}^{13}\text{C}} = 145$ Hz (B) N7 ^{13}C methyl group, $J_{\text{H-}^{13}\text{C}} = 145$ Hz and two N2 ^{13}C methyl groups of the TMG cap, $J_{\text{H-}^{13}\text{C}} = 140$ Hz.

Figure 2.

DcpS hydrolysis of labeled cap analogues. DcpS reactions were carried out as previously described and the labeled substrates and products were separated by PEI-cellulose thin layer chromatography (TLC), plates developed in 0.45 M ammonium sulfate, and labeled substrate and products detected by autoradiography.¹⁸ The plates correspond to (A) ^{14}C -labeled MMG cap, (B) ^{14}C -labeled TMG cap; lane 1— ^{14}C cap analogue, lane 2— ^{14}C cap analogue treated with *C. elegans* DcpS (expressed and purified as described¹⁸), lane 3— ^{14}C cap analogue treated with *A. suum* DcpS (GenBank Accession number (ADB92583), expressed and purified as described¹⁸). *indicates the position of the label.

Scheme 1.

Synthesis of ^{13}C and ^{14}C MMG cap analogues. Reagents: (i) $^{13}\text{CH}_3\text{I}$ or $^{14}\text{CH}_3\text{I}$, DMSO.

R^1 and $R^2 = {}^{13}\text{CH}_3$ for ${}^{13}\text{C}$ labeled TMG cap (**8**)
 $R^1 = {}^{14}\text{CH}_3$ and $R^2 = \text{unlabelled } \text{CH}_3$ for ${}^{14}\text{C}$ labeled TMG cap (**9**)

Scheme 2.

Synthesis of ${}^{13}\text{C}$ and ${}^{14}\text{C}$ TMG cap analogues. Reagents and conditions: (i) (a) acetic anhydride, DMAP, Et_3N , AcCN, 4°C to rt, (b) NPE, PPh_3 , DIAD, toluene, rt, (c) HF/pyridine, tBuONO, pyridine, -40°C ; (ii) (a) $({}^{13}\text{CH}_3)_2\text{NH}$, DMSO/AcCN/ H_2O / Et_3N , 60°C or $(\text{CH}_3)_2\text{NH}$, DMSO, 60°C , (b) THF/MeOH/ NaOH_{aq} ; (iii) POCl_3 , trimethylphosphate, 4°C ; (iv) ${}^{13}\text{CH}_3\text{I}$ or ${}^{14}\text{CH}_3\text{I}$, DMSO, rt (v) ZnCl_2 , DMF.