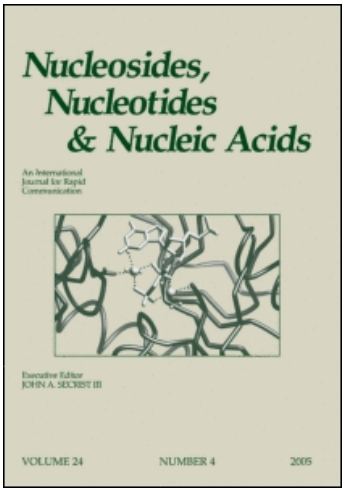

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I. I. Koukhareva[a]; A. V. Lebedev[a]
[a] TriLink BioTechnologies Inc., San Diego, California, USA

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Chemical Route to the Capped RNAs

I. I. Koukhareva and A. V. Lebedev*

TriLink BioTechnologies Inc., San Diego, California, USA

ABSTRACT

Eukaryotic and viral messenger RNAs contain a CAP structure that plays an important role in the initiation of translation and several other cellular processes that involve mRNAs. In this paper, we report a convenient chemical approach to the preparation of milligram quantities of short, capped RNA oligonucleotides, which overcomes some of the limitations of previous approaches. The method is based on the use of a reactive precursor, m^7GppQ [P^1-7-methylguanosine-5′-O-yl, P^2-O-8-(5-chloroquinolyl) pyrophosphate]. The precursor reacts smoothly with 5′-phosphorylated unprotected short RNA in the presence of $CuCl_2$ in organic media. The feasibility of this approach was demonstrated by the synthesis of the capped pentaribonucleotide m^7GpppGpApCpU. The synthesized capped oligonucleotide was isolated and purified by reverse phase and ion exchange HPLC with a final yield of 37%. The structure of the m^7GpppGpApCpU was confirmed by ^{31}P NMR, mass-spectrometry and enzymatic hydrolysis.

Key Words: mRNA; 5′-capped mRNA; 5′-capped oligoribonucleotide; m^7GpppG; Chemical synthesis of 5′-capped oligoribonucleotide.

*Correspondence: A. V. Lebedev, TriLink BioTechnologies Inc., 9955 Mesa Rim Road, San Diego, CA 92121, USA.

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INTRODUCTION

Eukaryotic and viral messenger RNAs[1–4] contain a CAP structure (m^7GpppG...) at the 5′ end where 7-methylguanosine is attached to the mRNA through a 5′-5′-triphosphate bridge. In vivo the CAP structure is attached to an mRNA transcript when it reaches a chain length of 25–30 nucleotides[5–7] by consecutive enzymatic reactions involving RNA 5′-triphosphatase, RNA guanylyltransferase and RNA guanine-7-methylase.[2–4,8] Extensive studies conducted over the past 30 years have revealed that the CAP structure plays an important role in processes leading to the initiation of peptide synthesis on the ribosome (transcript processing, transport, translation, turnover).[2–4,9]

Despite understanding the significance of the CAP structure in these cellular processes, reports of structural studies describing interactions between capped RNA oligonucleotides and different binding proteins, initiating factors, or ribosomal elements have been very limited.[10–12] These types of studies, which use techniques such as crystallography, 2D NMR, calorimetric studies, and stop-flow applications, require milligram quantities of pure compounds. Current synthetic methods, whether enzymatic or chemical, cannot easily produce capped RNAs in sufficient quantities for the investigations which are needed to uncover the structural details of binding interactions.

Typically, capped short and long RNAs are prepared enzymatically in small quantities by transcription reactions with T3, T7 or SP6 polymerases using the chemically synthesized dinucleotide m^7GpppG as a primer.[2] However, this approach results in the formation of two isomers of the capped RNAs with either natural (m^7GpppG...) or reverse (Gpppm^{7}G...) orientations that are present in ratios from 2:1 to 1:1.[13]

Recently, new CAP analogs have been designed as a strategy to eliminate formation of isomers. Substitution of 7-methylguanosine in the m^7GpppG for 2′- or 3′-deoxy analogs (or 2′- or 3′-methoxy analogs) of 7-methylguanosine,[14–16] when used as a primer for in vitro synthesis, resulted in production of capped RNAs only in the natural orientation. Another enzyme, gene 4 primase, uses m^7GpppA as a substrate in DNA-dependent synthesis of short RNA templates apparently exclusively in the natural direction.[10] It was also demonstrated that synthetic RNA oligonucleotides containing 5′-diphosphate[17] or 5′-triphosphate[18] groups can be converted enzymatically to the desired capped oligomers. In general, the enzymatic approaches are suitable for small-scale synthesis (i.e., micrograms) of capped RNAs.

However, for the preparation of significant quantities of capped RNA oligonucleotides a versatile all-chemical route is needed. There have been several methods reported in the past 20 years for the synthesis of capped RNAs (Refs. [19–28] see Table 1). Generally, all approaches may be divided in two major schemes for the attachment of the cap structure to the RNA molecule. The most widely used approach relies on the activation of phosphate function of m^7G-containing precursor and the other method uses activation of the terminal 5′-phosphate function of the RNA oligonucleotide.

In most cases activation of the m^7G-precursor was achieved through formation of an imidazolide of the 5′-phosphomonoester or pyrophosphate 5′-monoester derivative of m^7G (Im-pm^{7}G or Im-ppm^{7}G, respectively). The reaction is facilitated by catalysis with metal ions such as Mn^{2+}, Mg^{2+}, Ca^{2+} (for aqueous solutions) or Ag^{+}, Cu^{2+} (for non-aqueous media). Typically, in aqueous media the reaction requires 6–10 days for completion, and in organic solution the reaction proceeds 5–10 times faster.

Table 1. Chemically synthesized capped RNA oligonucleotides.

Capped RNA oligonucleotide sequence	Activated molecule	m^7G-precursor[a]	Starting RNA[b]	Catalyst	Conversion %[c]	Isolated yield %[d] (scale)	Ref.
New Procedure							
m^7GpppGACU	m^7G precursor	m^7GppQ	U/P	$CuCl_2$	80%	37% (1.5 μmole)	This work
Published							
m^7GpppAUG	m^7G precursor	ArS-ppm^{7}G (S, B)	T, B	$AgNO_3$/imidazole	40–50%	8% (0.5 μmole)	[19,20]
m^7GpppAUGACC	m^7G precursor	ArS-ppm^{7}G (S, B)	T, B	$AgNO_3$/imidazole	40–50%	5% (0.25 μmole)	[19,20]
m^7GpppGUAUUA	5′-Im-pRNA	ppm^{7}G (S, B)	T	Not used	> 50%	16% (0.08 μmole)	[21–23]
m^7GpppGUAUUAAUA	5′-Im-pRNA	ppm^{7}G (S, B)	T	Not used	NR	5% (0.2 μmole)	[21,24]
m^7GpppGUA	m^7G precursor	ArS-ppm^{7}G (S, B)	T	$AgNO_3$/imidazole	NR	11% (0.41 μmole)	[23]
m^7GpppGUAUUA	m^7G precursor	ArS-ppm^{7}G (S, B)	T	$AgNO_3$/imidazole	NR	2% (0.06 μmole)	[23]
m^7GpppAAAAAA	m^7G precursor	Im-ppm^{7}G	U/P	$MnCl_2$	49%	NR (NR)	[25]
m^7GpppACACUUGCUUU	m^7G precursor	Im-ppm^{7}G	U/P	$MnCl_2$	35%	NR (NR)	[25]
$m_3^{2,2,7}$GpppAmUmA	$m_3^{2,2,7}G$ precursor	Im-pm$_3^{2,2,7}$G	M	Not used	NR	40% (8.8 umole)	[26]
$m_3^{2,2,7}$GpppAmUmA	$m_3^{2,2,7}G$ precursor	Im-pm$_3^{2,2,7}$G (S)	M, T	Not used	30–40%	20% (NR)	[27]
$m_3^{2,2,7}$GpppAmUmAm	5′-Im-pRNA	ppm^{7}G	M	$ZnCl_2$	NR	NR (NR)	[28]

[a]B–N2-base protected 7-methylguanosine, S–sugar protected.
[b]T–2′-THP protected nucleoside(s), B–base protected nucleoside(s), M–2′-OMe nucleosides, U/P–unprotected oligomer.
[c]Percentage of starting RNA oligomer converted to capped RNA oligomer (estimated from HPLC profile).
[d]Isolated yield of capped RNA oligomer (NR–not reported).

Activation of 5′-phosphate function of RNA was carried out by converting to a 5′-phosphoroimidazole derivative using coupling reagents (such as N,N-carbonyldiimidazole or triphenyl phosphine/α,α-dipyridyl-disulfide). For reactions in organic media, RNA oligonucleotides containing acid labile 2′-protecting groups compatible with CAP structure and RNA backbone (or 2′-O-Methyl RNA derivatives) were used. The activated RNA oligomer was then reacted with pp-m^7G and once the reaction was completed the 2′-protecting groups were removed by treatment with 0.01M HCl (pH 2). Those syntheses required an additional deprotection step and an extensive chromatography purification was needed to isolate pure products. The overall yield of this process was quite low.

Despite numerous methods developed for preparing capped RNAs, the substantial amounts needed for detailed investigations are still not readily available. On the other hand, a large scale chemical synthesis of m^7GpppG was described ten years ago by Hata and co-workers.[23] The key intermediate P^1-guanosine-5′-O-yl, P^2-O-8-(5-chloroquinolyl) pyrophosphate (GppQ) was reacted without isolation in the presence of $CuCl_2$ with pm^7G to give the desired product, m^7GpppG. It was later shown that similar reaction of isolated and purified P^1-7-methylguanosine-5′-O-yl, P^2-O-8-(5-chloroquinolyl) pyrophosphate (m^7GppQ) with pA produced m^7GpppA with a moderate yield.[10] It was logical to approach the synthesis of the capped RNA oligonucleotides using a reaction of active precursor (m^7GppQ) with a purified 5′-phosphorylated unprotected RNA oligonucleotide. Here, a convenient all-chemical approach to the synthesis of short, capped RNA oligonucletides in quantities sufficient for many structural studies is described.

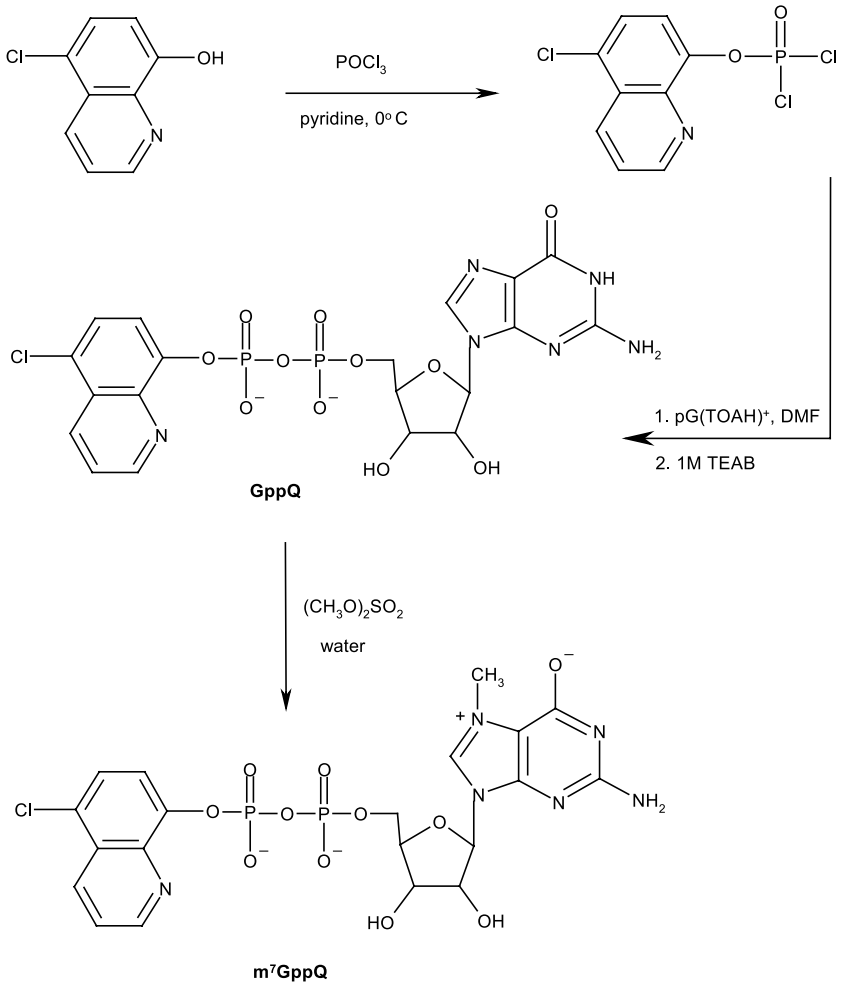
RESULTS

First, we tried a different procedure for preparation of m^7GppQ. The treatment of 5-chloro-8-hydroxyquinoline in pyridine with an excess of $POCl_3$[29–31] leads to formation of the reactive intermediate, presumably 5-chloroquinolyl-8-dichlorophosphate (Scheme 1). The latter compound without isolation was reacted with the mono-tri-n-octylammonium salt of guanosine 5′-monophosphate to give P^1-guanosine-5′-O-yl, P^2-O-8-(5-chloroquinolyl) pyrophosphate (GppQ) in 30% yield after isolation by DEAE anion-exchange chromatography.

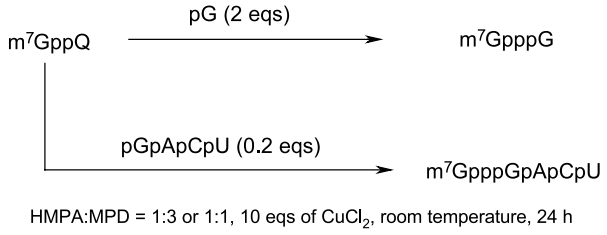
The GppQ was further treated with dimethyl sulfate in water for several hours to produce m^7GppQ. It was isolated after purification with reverse phase chromatography with 67% yield as a triethylammonium salt. The overall yield of 20% was comparable to 35% yield obtained in Ref. [10] where a different synthetic route for preparation of m^7GppQ was used.

To test the efficiency of the m^7GppQ prepared in this work we synthesized the simplest capped RNA derivative, m^7GpppG (Scheme 2), by reacting m^7GppQ with pG in the presence of $CuCl_2$ in the mixture of hexamethylphosphoramide (HMPA) and 1-methyl-2-pyrrolidinone (MPD) (1:3, v/v). HPLC analysis (not presented) of the reaction showed the main product formed was identical to an authentic sample of m^7GpppG.

However, the feasibility of the above approach for the synthesis of capped RNA oligonucleotides by a reaction of m^7GppQ with a purified 5′-phosphorylated



Scheme 1. Chemical synthesis of m^7GppQ.



Scheme 2. Chemical synthesis of m^7GpppG and m^7GpppGpApCpU.

unprotected RNA oligonucleotide had to be proven. One significant issue was the unknown solubility of RNA oligomers in HMPA-MPD organic media in the presence of $CuCl_2$. Also, the ability of phosphodiester bonds of unprotected RNA oligomers to withstand exposure to $CuCl_2$ in HMPA-MPD organic media for prolonged periods of time (hours or days) was not known. This was a considerable concern given the well-known ability of metals to catalyze RNA hydrolysis.[32]

These questions were answered in the following experiment. A 14-mer RNA oligonucleotide was chosen as a model for stability studies to represent intermediate length oligonucleotides. The 14-mer was first converted to the cetyl-triethylammonium (CTMAH +) salt using a published procedure[33] in order to ensure solubility at a concentration of at least 1 mM at room temperature. The 24 h treatment of the 14-mer with 200 molar excess of $CuCl_2$ in HMPA-MPD (1:1) revealed some degradation of phosphodiester linkages (Fig. 1). However, at this point we considered the level of

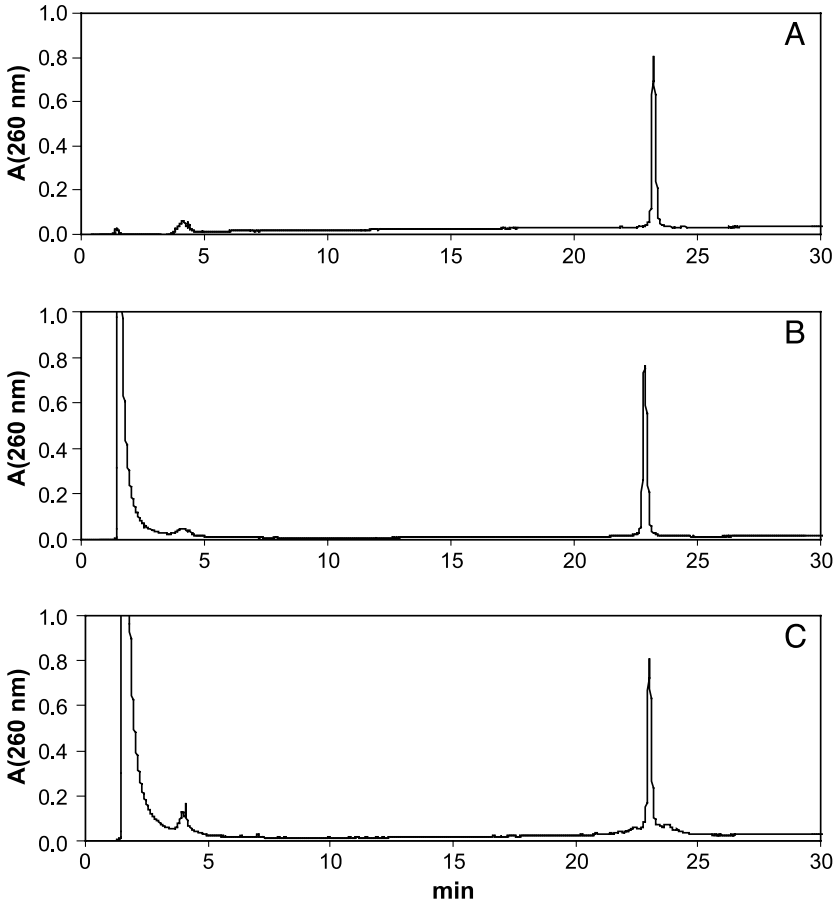


Figure 1. Anion-exchange HPLC analyses of 14-mer RNA (1 mM) dissolved in HMPA:MPD (1:1) mixture in the absence of $CuCl_2$ **(A)** and after incubation in the presence of 200 mM $CuCl_2$ for 2 hours **(B)** and 24 hours **(C)**.

RNA degradation to be acceptable, especially since a much lower concentration (and excess) of $CuCl_2$ would be used in the actual capping reaction (see below).

Finally, a short 4-mer RNA containing all four common RNA nucleosides (A, G, C and U) was chosen to evaluate the capping reaction with m^7GppQ in the presence of $CuCl_2$. The choice of the length and structure of the model 4-mer RNA and the scale of the synthesis was dictated by the necessity to simplify the isolation of the final product and confirm its structure. pGpApCpU was synthesized on the solid support using standard phosphoramidite chemistry. The oligonucleotide was deprotected and purified by reverse phase HPLC and DEAE Sephadex A25 anion-exchange chromatography and isolated as a triethylammonium salt.

To our surprise, the triethylammonium salt of pGpApCpU was soluble in the HMPA-MPD (1:1) mixture in the presence of $CuCl_2$. Therefore, conversion of the 4-mer RNA to the $CTMAH^+$ salt was not necessary. The results of the reaction of the m^7GppQ and pGpApCpU (see Scheme 2) are illustrated in the HPLC chromatograms (Fig. 2). Analysis of the reaction mixture after 2 hours revealed a formation of the new compound, presumably m^7GpppGpApCpU, which had an HPLC retention time approximately 1 minute longer then that of the starting material, pGpApCpU. The m^7GpppGpApCpU became the major oligonucleotide component of the mixture after the reaction proceeded for 23 hours. At this point the mixture was diluted with water, and m^7GpppGpApCpU was isolated, and purified by reverse phase and ion-exchange

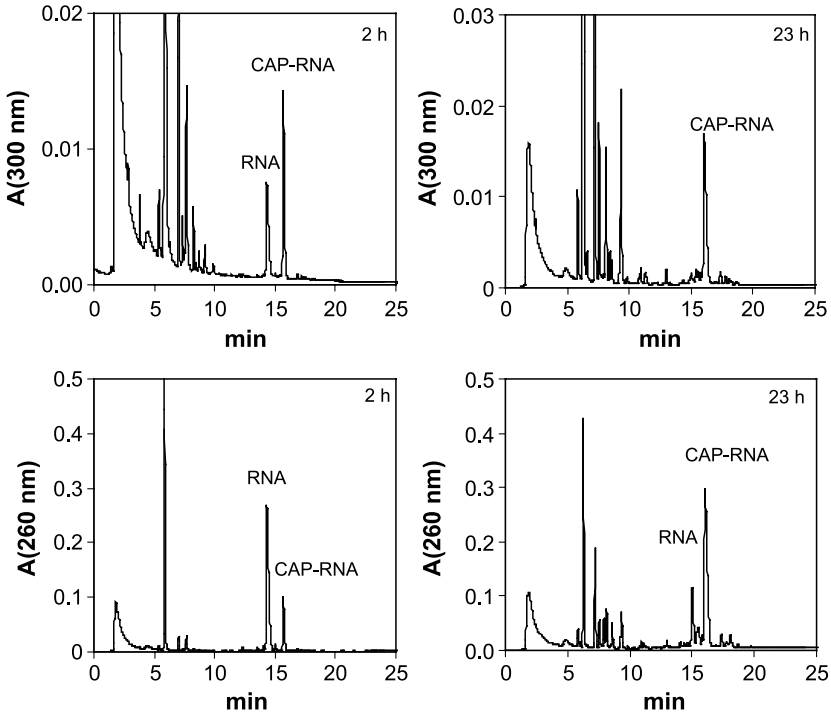


Figure 2. Synthesis of m^7GpppGpApCpU (CAP-RNA): anion-exchange HPLC analyses of the reaction mixture at 2 and 23 hours (detection at 260 or 300 nm).

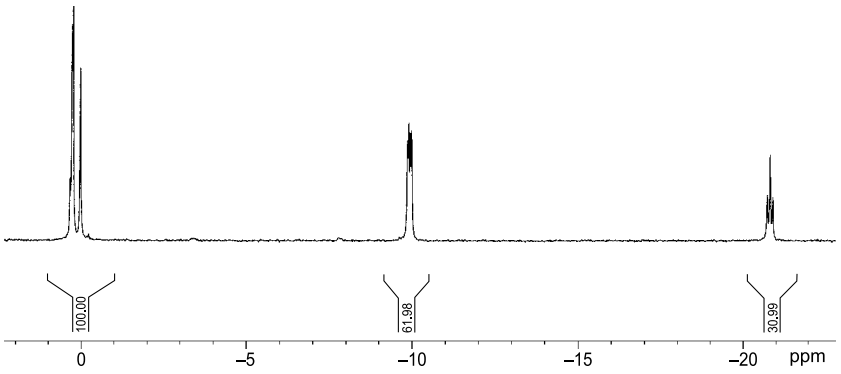


Figure 3. ^{31}P-{^{1}H} NMR spectrum of m^{7}GpppGpApCpU in D_2O.

HPLC. Based on the quantity of the starting 4-mer RNA oligonucleotide, a final yield of 37% (m^{7}GpppGpApCpU) was obtained.

The structure of isolated m^{7}GpppGpApCpU was confirmed using four different methods of analysis. First, while the starting pGpApCpU does not have significant absorbance at > 300 nm, absorbance of the m^{7}GpppGpApCpU at 300 nm is relatively high, indicating the presence of m^{7}G (at pH 10, the $A_{300\ nm}/A_{260\ nm}$ ratios are 0.029 and 0.136 for pGpApCpU and m^{7}GpppGpApCpU respectively; see Fig. 2). Second, the MALDI mass-spectrum of the purified product confirmed the molecular mass. Third, the ^{31}P NMR spectrum (Fig. 3) contains all expected phosphorus resonances. Finally, the enzymatic hydrolysis of m^{7}GpppGpApCpU gave the expected ratio of all nucleotides products (pC:pU:pA:m^{7}GpppG) in the correct proportion (not shown). The successful enzymatic hydrolysis also serves as evidence that no noticeable isomerization of the phosphodiester internucleotide bonds (3′–5′ to 2′–5′) took place during the synthesis and isolation of m^{7}GpppGpApCpU.

DISCUSSION

The first chemical syntheses of capped RNA oligomers were performed by Sekine and co-workers by the reaction of P^{1}-5′-O-(7-methylguanosine), P^{2}-S-(4-methoxyphenyl) pyrophosphate with 5′-phosphorylated/2′-THP protected RNA oligonucleotides in the presence of silver nitrate and imidazole.[19,20] The reaction proceeded with up to 40–50% conversion efficiency. Following acidic treatment and purification, m^{7}GpppApUpG and m^{7}GpppApUpGpApCpC were isolated with 8% and 5% yields, respectively. Later some improvement in the yield of capping reaction was achieved when 5′-phosphorylated RNA oligonucleotide (2′-THP protected) was converted to respective 5′-phosphoroimidazolide and reacted with ppm^{7}G (N2 and 2′, 3′-protected).[21,22]

Sawai and co-workers achieved a similar efficiency of capping of RNA oligomers.[25] They prepared Im-ppm^{7}G and used it to react with deprotected and purified 5′-phosphorylated 6-mer and 11-mer oligoribonucleotides in aqueous solution in the presence of Mn^{2+} ions (reaction in the presence of Mg^{2+} or Ca^{2+} was less efficient). The process required 10-fold excess of the Im-ppm^{7}G over oligonucleotide

and continued for 6–10 days at 25–30°C. Conversions of 49% (6-mer) and 35% (11-mer) to the capped oligonucleotides were achieved. The yield was calculated by integration of the respective peaks of starting oligonucleotide and capped oligonucleotide from analytical HPLC. It was demonstrated that the hydrolysis of Im-ppm^{7}G in 250 mM tris-acetate buffer (pH 7.5) seems to be slow (half-life time was estimated to be 142 hours at 25°C). However, it remains unclear if the unavoidable hydrolysis of Im-ppm^{7}G is accelerated in the presence of Mn^{2+}.

Recently, Darzynkiewicz and co-workers reported the efficient Zn^{2+} catalysis of the reaction of Im-pm^{7}G or Im-ppm^{7}G with ppG or pG, respectively, in organic media (DMF).[14] They used this approach for preparation of m^{7}GpppAmUmAm although no details of the reaction conditions, time of the reaction and no isolated yield were reported.[28]

In this publication, we report a convenient and reliable method for the synthesis of milligram quantities of short, capped RNA oligonucleotides (the capping experiments on pGpApCpU were successfully repeated three times). The method relies on the use of stable compounds: fully deprotected 5′-phosphorylated RNA oligonucleotide and m^{7}GppQ. We suggest a simple and easy way to prepare the key m^{7}G-precursor, m^{7}GppQ. It was demonstrated that trialkylammonium and tetraalkylammonium salts of 5′-phosphorylated and fully unprotected short oligonucleotides are soluble enough in HMPA-MPD (1:1) media to support an efficient capping reaction. The level of RNA oligonucleotide degradation during capping reaction is noticeable but considered acceptable for short RNA oligonucleotides and no significant isomerization of internucleotide phosphodiester linkages occurred. Also, the opportunity to use the pre-synthesized, fully deprotected and purified RNA oligomers significantly facilitates the purification and isolation of the final capped RNA oligonucleotide. Activation of m^{7}GppQ with $CuCl_2$ proceeds without noticeable complications and the RNA capping reaction could be completed in 23 hours. The isolated yield of capped RNA after purification by reverse phase and ion exchange chromatography was nearly 37% (based on starting RNA oligonucleotide).

Work is currently in progress on the optimization of conditions of capping reaction for RNA oligonucleotides of the intermediate length (10–20 mers). Also, an easier and more convenient method of preparing m^{7}GppQ by reaction of 5-chloroquinolyl-8-dichlorophosphate with pm^{7}G similar to preparation of GppQ (see above) is in development.

MATERIALS AND METHODS

Guanosine 5′-monophosphate (free acid) was purchased from ChemImpex, copper (II) chloride (anhydrous) from Acros Organics. Polymer support, fully protected nucleoside 3′-phoshoramidite monomers and 5′-phosphorylating reagent were purchased from Glen Research. Other chemicals and solvents were purchased from Sigma-Aldrich or Fisher Scientific and used without further purification.

NMR spectra (^{1}H, ^{13}C and ^{31}P) were recorded on Bruker AMX 500 instrument (NuMega, San Diego, CA) in D_2O at 25°C. Mass spectra were recorded on Applied Biosystems's instrument Voyager DE (HT Laboratories, San Diego, CA). HPLC (preparative and analytical) were performed on Beckman Gold HPLC System equipped with diode array UV detector unit (Beckman Instruments). For anion exchange HPLC a

gradient of 1M LiCl in 25 mM Tris-base (pH 10.0) was used; for reverse phase HPLC a gradient of acetonitrile in 100 mM triethylammonium acetate (pH 7.2) was used.

Oligoribonucleotides were synthesized (GCC ACG CCC CGA UU in 0.2 μmole scale and pGACU in 15 μmole scale) on polymer support on Expedite 8909 (PerSeptive Biosystems) using standard manufacturer suggested protocols for RNA synthesis. The oligoribonucleotides were deprotected and removed from polymer support using standard concentrated ammonia/tetrabutylammonium fluoride deprotection procedure.[34] The crude 14-mer was purified with preparative PAGE (then eluted and ethanol precipitated as Na^+ salt). The 4-mer was isolated by preparative reverse phase HPLC in gradient of acetonitrile (0–50%) in 100 mM triethylammonium acetate and then purified by ion exchange DEAE Sephadex A25 chromatography in gradient of triethylammonium bicarbonate, pH 7.5 (0.02 M to 1.0 M). Oligonucleotides were at least 95% pure by analytical anion exchange and reverse phase HPLC.

Synthesis of m^7GppQ

Preparation of Mono-tri-n-octylammonium Salt of Guanosine 5′-Monophosphate

To a stirring suspension of guanosine 5′-monophosphate (908 mg; free acid; 2.5 mmol) in 5 mL of methanol added 1.08 mL of tri-n-octylamine (876 mg; 2.5 mmol). After 10 min of stirring at room temperature the clear solution was evaporated to white foam. The foam was dissolved in 6 mL of dry DMF at 50°C and evaporated again to white foam and put on high vacuum for 24 h. The foam was dissolved in 5 mL of dry DMF (to be used in next step).

Preparation of GppQ

A solution of 5-chloro-8-hydroxyquinoline (385 mg; 2.14 mmol) in 2 mL of anhydrous pyridine was added dropwise to a solution of phosphorus oxychloride (600 μL; 6.44 mmol) in 3 mL of anhydrous pyridine with stirring and cooling in an ice bath. The bath was removed and the mixture was stirred for 5 h at room temperature. The pyridinium chloride was filtered off and the filtrate was evaporated to a yellow oily residue. The obtained 5-chloroquinolyl-8-dichlorophosphate was dissolved in 5 mL of dry DMF. To this mixture was added the solution of guanosine 5′-monophosphate ($TOAH^+$ salt, 5mL in DMF) and 510 uL of tri-n-butylamine with stirring. After 10 min at room temperature the reaction was quenched with 5 mL of 1 M TEAB (pH 7.5). The mixture was evaporated down to an oily residue and then shaken with dichloromethane:water = 1:1 (v/v, 200 mL). The organic layer was washed twice with 100 mL of water; the aqueous layers were combined and loaded on column (2.6 × 90 cm) with Sephadex-A25 DEAE (bicarbonate form, equilibrated with water). The column was washed with 0.5 L of water and the product was eluted with a linear gradient of 0.0–1.0 M TEAB (2L). Fractions containing the product with purity higher then 90% (determined by anion exchange HPLC) were combined, evaporated to a white foam and put on high vacuum for 24 hrs. The isolated yield was 520 mg (30%). ^{31}P-{^1H} NMR (D_2O), δ: −10.79 (d) and −16.62 (d); J_{POP} 20.6 Hz. ^{13}C-{^1H} NMR (D_2O), δ: 157.04, 152.28, 149.89, 149.07, 145.09 (d), 138.76 (d), 136.13, 132.65, 125.57, 125.39, 124.06,

121.60, 117.00, 114.75, 86.26, 82.36 (d), 72.47, 69.11, 64.25 (d), 45.65 (CH_2; $TEAH^+$), 7.22 (CH_3; $TEAH^+$). Electrospray mass spectrum: 603 and 605 (3:1; $M - H^+$). UV (water): λ_{max} 239 nm; λ_{min} 222 nm; λ_{sh} 247, 275, 310 nm.

Methylation of GppQ

Dimethyl sulfate (8.6 μL, 90 μmol) was added to a solution of GppQ (73 mg, 103 μmol, $TEAH^+$ salt) in 300 μL of water and the reaction mixture was stirred at room temperature for 4 hrs. The m^7GppQ was isolated by preparative reverse phase HPLC on 25 × 100 mm C18 cartridge (Waters) in gradient of acetonitrile in 100 mM TEAA, pH 7.2 (0–50% over 50 min, 5 mL/min). Fractions containing m^7GppQ were evaporated to remove acetonitrile and the product was re-purified and de-salted on the same cartridge with a linear gradient of acetonitrile in water (0–50% over 20 min). Fractions containing the m^7GppQ were combined, evaporated to white foam and dried on high vacuum for 24 hrs. The isolated yield was 50 mg (67%). ^{31}P-{^1H} NMR (D_2O), δ: −10.65 (d) and −16.65 (d); J_{POP} 20.1 Hz. ^1H NMR (D_2O), δ: 8.819 (1H, dd); 8.463 (1H, dd); 7.573 1H, dd); 7.511 (2H, s); 5.747 (1H, d); 4.40–4.47 (3H, m); 4.342 (1H, m); 4.221 (1H, m); 3.633 (3H, s); 3.175 (6H, q, CH_2; $TEAH^+$); 1.256 (9H, t, CH_3; $TEAH^+$). Electrospray Mass spectrum: 617 and 619 (3:1; $M - H^+$); 619 and 621 (3:1; $M + H^+$). UV (water): λ_{max} 239, 257 and 284 nm; λ_{min} 224, 253, and 277 nm; λ_{sh} 317 nm.

Reaction of m^7GppQ with pG (Analytical Experiment)

Approximately 1 mg of pG ($TOAH^+$ salt; ∼ 2 μmol) was mixed with 2 equivalents of m^7GppQ ($TEAH^+$ salt) in 100 μL of HMPA:MPD = 1:3 (v/v) in 1.5 mL conical tube. Solid $CuCl_2$ (5.4 mg; 40 μmol) was added to the reaction mixture and the tube was vortexed at room temperature. The progress of the reaction (up to 40 h) was monitored by anion exchange HPLC (identification of m^7GpppG was performed by the comparison with authentic sample: commercial product, TriLink BioTechnologies, Inc.). Note: m^7GpppG is relatively stable (for at least 20 min) during anion exchange HPLC analysis in 25 mM Tris-base (pH 10.0).

Synthesis of m^7GpppGpApCpU

To a solution of pGpApCpU (163 O.D. at 260 nm, 4.1 μmol, $TEAH^+$ salt) in 650 μL of HMPA:MPD = 1:1 (v/v) was added solid m^7GppQ, (14.5 mg, 20 μmol, $TEAH^+$ salt) followed by solid $CuCl_2$ (6.0 mg, 44.6 μmol). The mixture was kept at room temperature on vortex and the progress of the reaction was monitored by anion exchange HPLC. After 24 hrs the mixture was poured into 100 mL of water and the resulting solution was purified immediately by reverse-phase HPLC on 25 × 100 mm C18 cartridge (Waters) in gradient of acetonitrile in 100 mM TEAA, pH 7.2 (0–30% over 50 min, 5 mL/min). Fractions containing m^7GpppGpApCpU were combined, evaporated to remove acetonitrile and the product was de-salted on the same cartridge in gradient of acetonitrile in water (0–30% over 50 min, 5 mL/min). m^7GpppGpApCpU was further HPLC purified using anion-exchange Resource Q 8 mL column (Pharmacia) in gradient of 1 M LiCl in water (0–25% over 40 min, 5 mL/min) and de-salted as above to give 69 O.D. (at 260 nm; ∼1.5 μmol). ^{31}P-{^1H} NMR (D2O), δ: 0.283 (1P, s), 0.252 (1P, s), 0.028 (1P, s); −9.895 (1P, d, J_{pop}

17.2 Hz); −9.941 (1P, d, J_{pop} 17.2 Hz); −20.822 (1P, t, J_{pop} 17.2 Hz). MALDI Mass-spectrum: 1744 ($M + H^+$).

Enzymatic Hydrolysis of m^7GpppGpApCpU with Mung Bean Nuclease

To a solution of m^7GpppGpApCpU in water (1.0 O.D. at 260 nm; 10 μL) was added 1.4 μL of MBN 10 × buffer (BioLabs, New England) and 3.0 μL of MBN enzyme (10 U/μL; BioLabs, New England). The mixture was incubated at 37°C and 2 μL aliquots were removed and analyzed by reverse phase HPLC. Hydrolysis of m^7GpppGpApCpU was completed in several hours. The peaks corresponding to nucleotides pA, pC, pU and m^7GpppG were identified by UV spectra and by co-injection with authentic samples.

Stability Study of 14-mer RNA Oligonucleotide in the Presence of $CuCl_2$

The 14-mer oligonucleotide GCC ACG CCC CGA UU (Na^+ salt) was converted to cetyltrimethylammonium ($CTMAH^+$) salt according to a published procedure.[28] The residue (7.3 O.D. at 260 nm, 58 nmole) was dried down on high vacuum for 12 hours and dissolved in 58 μL of the mixture HMPA:MPD = 1:1 (v/v) in 1.5 mL conical tube. Solid $CuCl_2$ (0.5 mg; 4 μmole) was added to a 20 μL aliquot of that solution and the mixture was vortexed at room temperature. The integrity of 14-mer was monitored by anion exchange HPLC (Fig. 1).

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REFERENCES

1. Furuichi, Y.; Miura, K. A blocked structure at the 5′ terminus of mRNA from cytoplasmic polyhedrosis virus. Nature **1975**, *253*, 374–375.
2. Shatkin, A.J. Capping of eukaryotic mRNAs. Cell **1976**, *19*, 645–653.
3. Shuman, S. Origins of mRNA identity: capping enzymes bind to the phosphorylated C-terminal domain of RNA polymerase II. Prog. Nucleic Acid Res. Mol. Biol. **1995**, *50*, 101–129.
4. Furuichi, Y.; Shatkin, A.J. Viral and cellular mRNA capping: past and prospects. Adv. Virus Res. **2000**, *55*, 135–184.
5. Coppola, J.A.; Field, A.S.; Luse, D.S. Promoter–proximal pausing by RNA polymerase II in vitro: transcripts shorter than 20 nucleotides are not capped. Proc. Natl. Acad. Sci. U. S. A. **1983**, *80*, 1251–1255.

6. Hagler, J.; Shuman, S. A freeze-frame view of eukaryotic transcription during elongation and capping of nascent mRNA. Science **1992**, *255*, 983–986.
7. Rasmussen, E.B.; Lis, J.T. In vivo transcriptional pausing and cap formation on three drosophila heat shock genes. Proc. Natl. Acad. Sci. U. S. A. **1993**, *90*, 7923–7927.
8. Furuichi, Y.; Muthukrishnan, S.; Tomasz, J.; Shatkin, A.J. Mechanism of formation of reovirus mRNA 5′-terminal blocked and methylated sequence, m^7GpppGmpC. J. Biol. Chem. **1976**, *251*, 5043–5053.
9. Ro-Choi, T.S. Nuclear snRNA and nuclear function (discovery of 5′ cap structures in RNA). Crit. Rev. Eukaryot. Gene Expr. **1999**, *9*, 109–158.
10. Matsuo, H.; Takagi, T.; Kusakabe, T.; Buratowski, S.; Sekine, M.; Kyogoku, Y.; Moriguchi, T.; Wagner, G. Efficient synthesis of ^{13}C, ^{15}N-labelled RNA containing the cap structure m^7GpppA. J. Am. Chem. Soc. **2000**, *122*, 2417–2421.
11. Hodel, A.E.; Gershon, P.D.; Shi, X.; Wang, S.M.; Quiocho, F.A. Specific protein recognition of an mRNA cap through its alkylated base. Nat. Struct. Biol. **1997**, *4*, 350–354.
12. Hodel, A.E.; Gershon, P.D.; Quiocho, F.A. Structural basis for sequence-non-specific recognition of 5′-capped mRNA by a cap-modifying enzyme. Mol. Cell **1998**, *1*, 443–447.
13. Pasquinelli, A.E.; Dahlberg, J.E.; Lund, E. Reverse 5′ caps in RNAs made in vitro by phage RNA polymerases. RNA **1995**, *1*, 957–967.
14. Stepinski, J.; Waddell, C.; Stolarski, R.; Darzynkiewicz, E.; Roads, R.E. Synthesis and properties of mRNA containing the novel ''anti-reverse'' cap analogs 7-methyl(3′-O-methyl)GpppG and 7-methyl(3′-deoxy)GpppG. RNA **2001**, *7*, 1486–1495.
15. Peng, Z.-H.; Sharma, V.; Singleton, S.F.; Gershon, P.D. Synthesis and application of a chain-terminating dinucleotide mRNA cap analog. Org. Lett. **2002**, *4*, 161–164.
16. Jemielity, J.; Fowler, T.; Zuberek, J.; Stepinski, J.; Lewdorowicz, M.; Niedwiecka, A.; Stolarski, R.; Darzynkiewicz, E.; Rhoads, R.E. Novel ''anti-reverse'' cap analogs with superior translational properties. RNA **2003**, *9*, 1022–1108.
17. Brownlee, G.G.; Fodor, E.; Pritlove, D.C.; Gould, K.G.; Dalluge, J.J. Solid phase synthesis of 5′-diphosphorylated oligoribonucleotides and their conversion to capped m^7Gppp-oligoribonucleotides for use as primers for influenza A virus RNA polymerase in vitro. Nucleic Acids Res. **1995**, *23*, 2641–2647.
18. Olsen, D.B.; Benseler, F.; Cole, J.L.; Stahlhut, M.W.; Dempski, R.E.; Darke, P.L.; Kuo, L.C. Elucidation of basic mechanism and kinetic properties of influenza endonuclease using chemically synthesized RNAs. J. Biol. Chem. **1996**, *271*, 7435–7439.
19. Kohno, K.; Nishiyama, S.; Kamimura, T.; Sekine, M.; Hata, T.; Kumagai, I.; Miura, K. Chemical synthesis of capped RNA fragments and their ability to complex with eukaryotic ribosomes. Nucleic Acids Res. Symp. Ser. **1985**, *16*, 233–236.
20. Sekine, M.; Nishiyama, S.; Kamimura, T.; Osaki, Y.; Hata, T. Chemical synthesis of capped oligoribonucleotides, $m^7G^{5'}$pppAUG and $m^7G^{5'}$pppAUGACC. Bull. Chem. Soc. Jpn. **1985**, *58*, 850–860.
21. Iwase, R.; Tsukada, Y.; Sekine, M.; Hata, T.; Miura, K. Synthesis of capped oligoribonucleotides. Nucleic Acids Res. Symp. Ser. **1988**, *20*, 85–86.

22. Iwase, R.; Sekine, M.; Hata, T.; Miura, K. A new method for the synthesis of capped oligoribonucleotides by use of an appropriately protected 7-methylguanosine diphosphate derivative as a donor for the triphosphate bond formation. Tetrahedron Lett. **1988**, *29*, 2969–2972.
23. Sekine, M.; Iwase, R.; Hata, T.; Miura, K. Synthesis of capped oligoribonucleotides by use of protected 7-methyguanosine 5′-diphosphate derivatives. J. Chem. Soc., Perkin Trans. **1989**, *1*, 969–978.
24. Iwase, R.; Maeda, M.; Fujiwara, T.; Sekine, M.; Hata, T.; Miura, K. Molecular design of a eukaryotic messenger RNA and its chemical synthesis. Nucleic Acids Res. **1992**, *20*, 1643–1648.
25. Sawai, H.; Wakai, H.; Nakamura-Ozaki, A. Synthesis and reactions of nucleoside 5′-diphosphate imidazolide. A nonenzymatic capping agent for 5′-monophosphorylated oligoribonucleotides in aqueous solution. J. Org. Chem. **1999**, *64*, 5836–5840.
26. Sekine, M.; Kadokura, M.; Satoh, T.; Seio, K.; Wada, T.; Fisher, U.; Sumpter, V.; Luhrmann, R. Chemical synthesis of a 5′-terminal TMG-capped triribonucleotide $m_3^{2,2,7}G^{5'}$pppAmpUmpA of U1 RNA. J. Org. Chem. **1996**, *61*, 4412–4422.
27. Kadokura, M.; Wada, T.; Seio, K.; Moriguchi; Huber, J.; Lührmann, R.; Sekine, M. Solid-phase synthesis of a 5′-terminal TMG-capped trinucleotide block of U1 snRNA. Tetrahedron Lett. **2001**, *42*, 8853–8856.
28. Zuberek, I.; Wyslouch-Cieszynska, A.; Niedwiecka, A.; Dadlez, M.; Stepinski, J.; Augustyniak, W.; Gingras, A.C.; Zhang, Z.; Burley, S.K.; Sonenberg, N.; Stolarski, R.; Darzynkiewicz, E. Phosphorylation of elF4E attenuates its interaction with mRNA 5′ cap analogs by electrostatic repulsion: intein-mediated protein ligation strategy to obtain phosphorylated protein. RNA **2003**, *9*, 52–61.
29. Fukuoka, K.; Suda, F.; Suzuki, R.; Takaku, H.; Ishikawa, M.; Hata, T. Large scale synthesis of the cap part in messenger RNA using a new type of bifunctional phosphorylating reagent. Nucleosides Nucleotides **1994**, *13*, 1557–1567.
30. Fukuoka, K.; Suda, F.; Suzuki, R.; Takaku, H.; Ishikawa, M.; Hata, T. One-pot reaction for the synthesis of $m^7G^{5'}$pppG and $m^7G^{5'}$pppA by using an activatable bifunctional phosphorylating reagent. Tetrahedron Lett. **1994**, *35*, 1063–1066.
31. Takaku, H.; Yamaguchi, R.; Nomoto, T.; Hata, T. 5-Chloro-8-quinolyl group as high efficient phosphate protecting group for the synthesis of oligoribonucleotides. Tetrahedron Lett. **1979**, *20*, 3857–3860.
32. Butzow, J.J.; Eichhorn, G.L. Interactions of metal ions with polynucleotides and related compounds. IV. Degradation of polyribonucleotides by zinc and other divalent metal ions. Biopolymers **1965**, *3*, 95–107.
33. Balbi, A.; Sottofattori, E.; Grandi, T.; Mazzei, M.; Abramova, T.V.; Lokhov, S.G.; Lebedev, A.V. Synthesis and complementary complex formation properties of oligonucleotides covalently linked to new stabilizing agents. Tetrahedron **1994**, *50*, 4009–4018.
34. Hogrefe, R.I.; McCaffrey, A.M.; Borozdina, L.I.; McCampbell, E.S.; Vaghefi, M.M. Effect of excess water on the desilylation of oligoribonucleotides using tetrabutylammonium fluoride. Nucleic Acids Res. **1993**, *21*, 4739–4741.

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