Synthesis of N^4-Methylcytidine (m^4C) and N^4,N^4-Dimethylcytidine ($m^4{}_2C$) Modified RNA

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This article summarizes the protocols for phosphoramidite chemistry and solid phase synthesis of RNA oligonucleotides containing N^4-methylcytidine (m^4C) and N^4,N^4-dimethylcytidine ($m^4{}_2C$) residues for base-pairing, structural, and enzymatic activity studies. The two key m^4C and $m^4{}_2C$ phosphoramidite building blocks can be synthesized starting from the partially protected cytidine nucleosides, followed by solid-phase synthesis and HPLC purification of the modified target RNA oligonucleotides. These modified RNA strands are then prepared for base pairing stability, specificity, and structural studies using UV-melting temperature (T_m) measurements and X-ray crystallography. Functional studies are performed by reverse transcription assays in primer extension reactions employing different enzymes. © 2021 Wiley Periodicals LLC.

Basic Protocol 1: Chemical synthesis of m^4C phosphoramidite
Basic Protocol 2: Synthesis of $m^4{}_2C$ phosphoramidite
Basic Protocol 3: Synthesis and purification of m^4C and $m^4{}_2C$ containing RNA oligonucleotides

Keywords: base pairing • N^4,N^4-dimethylcytidine ($m^4{}_2C$) • N^4-methylcytidine (m^4C) • nucleic acids modification • thermostability • X-ray crystallography

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INTRODUCTION

Nucleic acid modifications have been extensively studied in all three domains of life for many decades. The existence of these modifications not only diversifies the appearance of RNAs but more importantly confers an additional layer of gene regulation in many biological processes (Ontiveros, Stoute, & Liu, 2019; Song & Yi, 2017). To date, scientists have discovered over 170 chemical modifications in cellular RNAs, with the majority having unknown functions (Boccaletto et al., 2018). These post-transcriptional modifications were initially considered as merely fine-tuning the structure and function of RNAs; however, emerging evidence has linked RNA modifications to various diseases such as neurological disorders and cancers (Jonkhout et al., 2017). Similar to DNA modifications known as epigenetic markers, these post-transcriptional RNA modifications are collectively called 'epitranscriptome' (Schwartz, 2016), which can be dynamically and reversibly regulated by specific corresponding enzymes termed 'reader' (translator), 'writer' (installer), and 'eraser' (demodifier). Indeed, the improved understanding of the

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modification-associated enzymes with linkages to human diseases has paved the way to the design and development of new RNA-based therapeutics.

Among all the naturally occurring modifications, methylation is considered the most abundant chemical modification affecting cellular processes and development (Ji & Chen, 2012; Romano, Veneziano, Nigita, & Nana-Sinkam, 2018). For example, N^6-methyladenosine (m^6A) is a well-studied and prevalent modification found in all higher eukaryotes in mRNA, tRNA, rRNA, and small nuclear RNA (snRNA), as well as long non-coding RNAs (Dominissini et al., 2012; Wang et al., 2014). Its associated enzymes were identified recently, including writer enzymes (METTL3 and METTL14), eraser enzymes or demethylases (FTO and ALKBH5), and reader enzymes (YTHDF1/2/3, IGF2BP1 and HNRNPA2B1) that bind m^6A for downstream activities (Lewis, Pan, & Kalsotra, 2017). Cumulative evidence has revealed the functional significance of m^6A in RNA stability, cellular metabolism, and, more importantly, the implications that these working enzymes have in many human diseases (Chen, Zhang, & Zhu, 2019). N^1-Methyladenosine (m^1A) is mainly found in tRNA and rRNA (Shima & Igarashi, 2020) and carries an additional methyl group that enables protonation of the base, thus affecting Watson-Crick base pairing, RNA secondary structure, and RNA-protein interactions through electrostatic effects (Roundtree, Evans, Pan, & He, 2017). 2′-O-Methylation (2′OMe) in rRNA plays important roles in translation (Liang, Liu, & Fournier, 2009). 5-Methylcytosine (m^5C) on mRNA has also been reported to play roles in nuclear export when bound to ALYREF/THOC4 factors. Thus, ALYREF is regarded as the bona fide m^5C reader. Moreover, m^5C in rRNA can influence protein translation, and inside mRNA it can enhance the stability of transcripts (Trixl & Lusser, 2019).

Unlike other methylated nucleotides, N^4-methylcytidine (m^4C) modification in RNA is less understood. It is highly conserved across species from bacteria to human in both cytoplasmic and mitochondrial rRNAs (Bohnsack & Sloan, 2018; Kimura & Suzuki, 2010). RsmH is the m^4C methyltransferase or writer enzyme responsible for the addition of the methyl group in rRNA of *Escherichia coli*. Moreover, m^4C can be further modified into N^4,N^4-dimethylcytidine ($m^4{}_2C$) by the same working enzyme together with S-adenosyl methionine (SAM), a typical source of the methyl moiety (Kimura & Suzuki, 2010). METTL15 has recently been identified as the methyltransferase to catalyze the formation of m^4C on human 12S ribosomal RNA in mitochondria. Studies have shown the significance of this modification in mitochondrial ribosomal biogenesis and translation, as well as in energy production processes (Van Haute et al., 2019). The N^4-methylated nucleobase directly participates in the Watson-Crick pairing. Therefore, this article includes a basic protocol intended to enable studies of the effects of one methyl group (m^4C) and two methyl groups ($m^4{}_2C$) on H-bonding between C and G. We recently demonstrated that the presence of these modifications can potentially modulate the transcription fidelity by affecting base-pairing stability and specificity (Mao et al., 2020).

The results obtained from the detailed protocols in this article will bring us closer to revealing the biochemical functions of m^4C and $m^4{}_2C$ modifications. Starting from the synthesis of modified m^4C and $m^4{}_2C$ phosphoramidites described in Basic Protocols 1 and 2, respectively, the reactions are carried out in stepwise fashion, each using specific conditions that are carefully monitored. Basic Protocol 3 then details the procedure for solid-phase synthesis of the modified RNA oligonucleotides, followed by HPLC purification of modified RNA strands. The final products are confirmed by MALDI-TOF MS. Finally, the conclusions of structural and functional studies of m^4C and $m^4{}_2C$ containing RNA oligonucleotides are presented in the Commentary.

CHEMICAL SYNTHESIS OF m^4C PHOSPHORAMIDITE

Phosphoramidite chemistry is the standard method for the synthesis of nucleic acid oligomers. Its high efficiency in the production of specific oligo sequences coupled with ease of incorporating modifications have made the study of gene regulation at the transcript level possible. Since the discovery of RNA modifications, their functional roles have received considerable attention, although m^4C and $m^4{}_2C$ have remained less explored. The synthesis of m^4C has been reported previously (Jun Lu, Koo, & Piccirilli, 2010), starting from the silylated uridine. As shown in Figure 1, 2,4,6-triisopropylbenzene sulfonyl chloride (TPSCl) was used to activate the C4 position, followed by treatment with methylamine solution to obtain compound **2**. Compound **3** is obtained by acetylation of **2** with acetic anhydride, and is subsequently desilyated by hydrogen fluoride in pyridine (HF·Py) to produce compound **4**. Compound **5** is obtained by selectively tritylating the 5′-position of **4** with 4,4′-dimethoxytrityl chloride (DMTrCl), and finally converted to m^4C phosphoramidite **6** for oligonucleotide solid-phase synthesis.

Materials

Compound **1** (1-(2 -O-tert-butyldimethylsilyl-3 ,5 -O-di-tert-butylsilyleneβ-D-ribofuranosyl)-uridine) (Fig. 1; see supporting information for the synthesis of compound 1 in Riml et al., 2017)

Triethylamine (Et_3N; Sigma-Aldrich, cat. no. T0886-1 L)

4-Dimethylaminopyridine (DMAP; Sigma-Aldrich, cat. no. 107700-100g)

Dichloromethane (DCM; Sigma-Aldrich, cat. no. 270997-1 L)

2,4,6-Triisopropylbenzenesulfonyl chloride (TPSCl; Sigma-Aldrich, cat. no. 119490)

Saturated aqueous sodium bicarbonate ($NaHCO_3$; Sigma-Aldrich, cat. no. S6014-1Kg)

Brine (saturated aqueous sodium chloride)

Sodium sulfate (Na_2SO_4; Sigma-Aldrich, cat. no. 7757-82-6)

Tetrahydrofuran (THF; Sigma-Aldrich, cat. no. 186562-1 L)

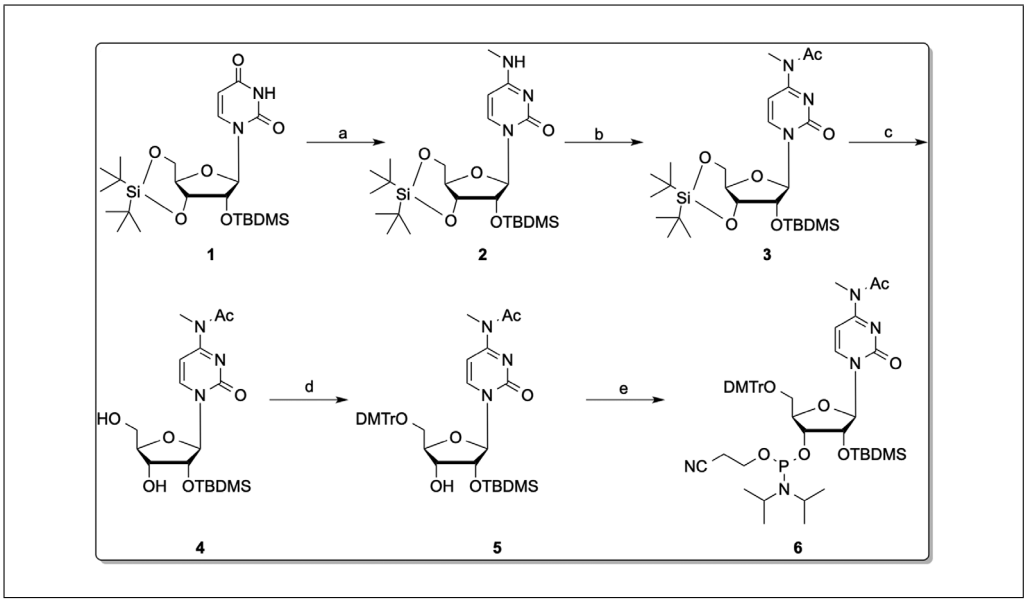

Figure 1 Synthesis of m^4C phosphoramidite **6**. Reagents and conditions: (**A**) (1) 2,4,6-triisopropylbenzenesulfonyl chloride, Et_3N, DMAP, CH_2Cl_2; (2) $MeNH_2$ (40% v/v aqueous solution), THF; (**B**) Ac_2O, Et_3N, DMAP, DCM (**C**) HF.Py, THF; (**D**) DMTrCl, Py; (**E**) $(i\text{-}Pr_2N)_2P(Cl)OCH_2CH_2CN$, $(i\text{-}Pr)_2NEt$, 1-methylimidazole, DCM.

Methylamine ($MeNH_2$) (Sigma-Aldrich, cat. no. 534102-250 ml)
Silica gel (particle size, 32-63 μm)
Methanol (MeOH; Sigma-Aldrich, cat. no. 179337)
N,*N*-Diisopropylethylamine (DIPEA; Sigma-Aldrich, cat. no. 387649-100 ml)
Acetic anhydride (Ac_2O; Sigma-Aldrich, cat. no. 108-24-7)
Argon (Ar) source
Hydrogen fluoride-pyridine (Sigma-Aldrich, cat. no. 184225)
Pyridine (Sigma-Aldrich, cat. no. 270970-2 L)
4,4′-Dimethoxytrityl chloride (TCI, cat. no. 40615-36-9)
Ethyl acetate (EA; Sigma-Aldrich, cat. no. 34858-4 L)
1-Methyl-1*H*-imidazole (Alfa Aesar, cat. no. 616-47-7)
2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (Sigma-Aldrich, cat. no. 89992-70-1)

Separatory funnels
Vacuum concentrator
Vacuum pump
Chromatography columns
TLC plates pre-coated with silica gel (SiliCycle, cat. no. TLG-R10014B-323)

Additional reagents and equipment for column chromatography (see Current Protocols article: Meyers, 2001), thin-layer chromatography (TLC; see Current Protocols article: Meyers & Meyers, 2008), nuclear magnetic resonance (NMR), and mass spectrometry (MS)

Synthesis of compound 2 (1-(2′-O-tert-butyldimethylsilyl-3′,5′-O-di-tert-butylsilylene-β-D-ribofuranosyl)-N^4 –methylcytidine) starting from compound 1

1. Dissolve 996 mg (2.0 mmol) of compound **1** in DCM (20 ml) containing Et_3N (0.66 ml, 4 mmol) and DMAP (24 mg, 0.1 mmol).
2. Add 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl, 788 mg, 2.6 mmol) to the solution at 0°C under Ar.
3. Stir the resulting solution at room temperature for 3 hr.
4. Dilute the mixture with 100 ml DCM and wash twice with 50 ml of saturated $NaHCO_3$ and twice with 50 ml brine using a separatory funnel.
5. Dry the organic layer by Na_2SO_4 and concentrate under vacuum to obtain yellow solid.
6. Add 20 ml THF and 10 ml of a 40% (v/v) aqueous solution of $MeNH_2$ to dissolve the solid product.
7. Stir the mixture at room temperature for 15 hr.
8. Purify the residue by using silica gel chromatography (see Current Protocols article: Meyers, 2001) with 25 g silica gel and a 0% to 10% gradient of MeOH/DCM as mobile phase to obtain compound **2**.

   • ∼60% yield (about 640 mg), 1.2 mmol white solid with the following parameters:
   • TLC $R_f = 0.4$ (DCM: MeOH = 20:1)
   • ^{1}H NMR (500 MHz, CDCl3) δ 7.28 (d, $J = 7.5$ Hz, 1H), 5.66 (s, 1H), 5.56 (s, $J =$ 7.0 Hz, 1H), 4.53-4.49 (m, 1H), 4.37-4.35 (m, 1H), 4.25-4.19 (m, 1H), 4.00-3.95 (t, $J = 10.0$ Hz, 1H), 3.87-3.83 (dd, $J = 4.5$, 9.5 Hz, 1H), 3.01 (d, $J = 4.5$ Hz, 3H), 1.03 (s, 9H), 1.02 (s, 9H), 0.94 (s, 9H), 0.25 (s, 3H), 0.16 (s, 3H)

Synthesis of compound 3 (N^4-acetyl-1-(2′-O-tert-butyldimethylsilyl-3′,5′-O-di-tert-butylsilylene-β-D-ribofuranosyl)-N^4-methylcytidine)

9. Dissolve 640 mg (1.2 mmol) of compound **2**, DIPEA (0.8 ml, 4.8 mmol), and DMAP (13 mg, 0.12 mmol) in DCM (15 ml).

10. Add 0.3 ml (2.4 mmol) of Ac_2O under Ar and stir the mixture at room temperature for 20 hr.

11. Dilute the solution with 100 ml DCM and wash twice with 50 ml of saturated $NaHCO_3$ and twice with 50 ml brine using a separatory funnel.

12. Purify the residue by using silica gel chromatography (see Current Protocols article: Meyers, 2001) with 25 g silica gel and a 0% to 10% gradient of MeOH/DCM as mobile phase to obtain compound **3**.

    • ∼90% yield (about 600 mg), 1.0 mmol white solid with the following parameters:
    • TLC $R_f = 0.7$ (DCM: MeOH = 20:1)
    • ^{1}H NMR (500 MHz, CDCl3) δ 7.63 (d, $J = 8.0$ Hz, 1H), 7.20 (d, $J = 8.0$ Hz, 1H), 5.71 (s, 1H), 4.55 (dd, $J = 5.0$, 9.0 Hz, 1H), 4.36 (d, $J = 4.0$ Hz, 1H), 4.32-4.26 (m, 1H), 4.00 (dd, $J = 9.5$, 10.5 Hz, 1H), 3.81 (dd, $J = 4.5$, 9.5 Hz), 3.48 (s, 3H), 2.41 (s, 3H), 1.03 (s, 9H), 10.2 (s, 9H), 0.96 (s, 9H), 0.27 (s, 3H), 0.18 (s, 3H)

Synthesis of compound 4 (N^4-acetyl-1-(2′-O-tert-butyldimethylsilyl-β-D-ribofuranosyl)-N^4-methylcytidine)

13. Dissolve 280 mg (0.5 mmol) of compound **3** in 5 ml THF at 0°C

14. Add a solution of hydrogen fluoride−pyridine complex (hydrogen fluoride, ∼70%; pyridine, ∼30%, commercially available from Sigma-Aldrich; 0.1 ml) in pyridine (0.6 ml) to the solution of compound **3**.

15. Incubate 1 hr at 0°C. Add 2 ml of pyridine after 1 hr when the reaction is complete.

16. Dilute the mixture with 50 ml DCM, wash the solution with 50 ml saturated $NaHCO_3$ in a separatory funnel, dry over Na_2SO_4, and evaporate.

17. Purify the residue by using silica gel chromatography (see Current Protocols article: Meyers, 2001) with 25 g silica gel and a gradient of 0% to 10% MeOH/DCM as mobile phase to obtain compound **4**.

    • ∼73% yield (about 150 mg), 0.36 mmol white solid with the following parameters:
    • TLC $R_f = 0.3$ (DCM:MeOH = 20:1)
    • ^{1}H NMR (400 MHz, CDCl3) δ 7.99 (d, $J = 7.6$ Hz, 1H), 7.19 (d, $J = 7.2$ Hz, 1H), 5.53 (s, 1H), 4.22-4.14 (m, 2H), 4.02-3.98 (m, 1H), 3.90 (m, 1H), 3.83-3.79 (m, 1H), 3.45 (s, 3H), 2.40 (s, 3H), 0.88 (s, 9H), 0.11-0.09 (d, 6H)

Synthesis of compound 5 (N^4-acetyl-1-(2′-O-tert-butyldimethylsilyl-5′-O-4,4′-dimethoxytrityl-5′-β-D-ribofuranosyl)-N^4-methylcytidine)

18. Dissolve 150 mg (0.36 mmol) of compound **4** in 5 ml dry pyridine.

19. Add 244 mg (0.72 mmol) of 4,4′-dimethoxytrityl chloride to compound **4** solution under Ar and stir at room temperature overnight.

20. Quench the solution with 1 ml of methanol and stir for another 5 min.

21. Dry the reaction mixture under vacuum.

22. Purify the residue by using silica gel chromatography (see Current Protocols article: Meyers, 2001) with 25 g silica gel and a gradient of 0% to 50% EA/DCM as mobile phase to obtain compound **5**.

IMPORTANT NOTE: *Silica gel is treated with 1% triethylamine and dried before loading. All eluents contain 1% triethylamine.*

- ~58% yield (about 150 mg), 0.21 mmol white solid with the following parameters:
- TLC $R_f = 0.4$ (DCM: EA = 1:1)
- ^{1}H NMR (400 MHz, CDCl3) δ 8.41 (d, $J = 7.6$ Hz, 1H), 7.43-7.40 (m, 2H), 7.33-7.30 (m, 6H), 7.28-7.23 (m, 1H), 6.86 (d, $J = 8.8$ Hz, 1H), 6.65 (d, $J = 7.6$ Hz, 1H), 5.87 (s, 1H), 4.41-4.34 (m, 1H), 4.30-4.28 (m, 1H), 4.15-4.07 (m, 1H), 3.81 (s, 6H), 3.61-3.58 (m, 1H), 3.55-3.51 (m, 1H), 3.42 (s, 3H), 2.39 (s, 3H), 0.94 (s, 9H), 0.35 (s, 3H), 0.21 (s, 3H)

Synthesis of compound 6 (N^4-acetyl-1-(2′-O-tert-butyldimethylsilyl-3′-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite-5′-O-4, 4′-dimethoxytrityl-5′-β-D-ribofuranosyl)-N^4 -methylcytidine)

23. Dissolve 150 mg (0.21 mmol) of compound **5** in 3 ml DCM.
24. Add DIPEA (0.14 ml, 0.8 mmol), 1-methyl-1*H*-imidazole (17 µl, 0.21 mmol), and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.1 ml, 0.4 mmol) to the solution of compound **5** and stir at room temperature overnight under Ar.
25. Dilute the mixture with 50 ml DCM and wash twice with 50 ml brine.
26. Dry the organic layer over Na_2SO_4, filter, and evaporate under reduced pressure.
27. Purify the residue by using silica gel chromatography (see Current Protocols article: Meyers, 2001) with 25 g silica gel and a gradient of 0% to 50% EA/DCM as mobile phase to obtain compound **6**.

IMPORTANT NOTE: *Silica gel is treated with 1% triethylamine and dried before loading. All eluents contain 1% triethylamine*

- ~73% yield (about 140 mg), 0.15 mmol white solid with the following parameters:
- TLC $R_f = 0.4$ (DCM: EA = 1:1)
- ^{1}H NMR (400 MHz, CDCl3) δ 8.53-8.41 (m, 1H), 7.47-4.26 (m, 9H), 6.87-6.83 (m, 4H), 6.54-6.34 (m, 1H), 5.88-5.79 (m, 1H), 4.36-4.30 (m, 2H), 3.81-3.80 (d, 6H), 3.75-3.64 (m, 2H), 3.56-3.46 (m, 3H), 3.41-3.37 (d, 3H), 2.40-2.37 (d, 3H), 1.30-1.08 (m, 12H), 0.93-0.91 (d, 9H), 0.29 (s, 3H), 0.16 (s, 3H)
- ^{31}P NMR (162 MHz, CDCl3) δ 150.60, 148.76
- HRMS (ESI-TOF) $[M+H]^+ = 916.4374$ (calc. 916.4446). Chemical formula: $C_{48}H_{66}N_5O_9PSi$.

BASIC PROTOCOL 2

SYNTHESIS OF $m^4{}_2C$ PHOSPHORAMIDITE

As shown in Figure 2, synthesis of $m^4{}_2C$ starts from the silylated cytidine. Dimethylation of compound **17** with methyl iodide in the presence of sodium hydride gives compound **8**. Desilylation of compound **8** with hydrogen fluoride is used to produce compound **9**. Tritylation of compound **9** at the 5′-position with 4,4′-dimethoxytrityl chloride (DMTrCl) affords compound **10**, which is then converted to $m^4{}_2C$ phosphoramidite for subsequent oligonucleotide solid-phase synthesis.

Materials

Compound **7** (2′-O-tert-butyldimethylsilyl-3′,5′-O-di-tertbutylsilylene-β-D-ribofuranosyl)-cytidine) (Fig. 2; see Uchiyama, Ogata, Oka, & Wada, 2011, for synthesis of compound 1 in cytosine)
Tetrahydrofuran (THF; Sigma-Aldrich, cat. no. 186562-1 L)
Sodium hydride (NaH, 60% dispersion in mineral oil; TCI, cat. no. 7646-69-7)
Argon (Ar) source
Iodomethane (MeI; Sigma-Aldrich, cat. no. 74-88-4)

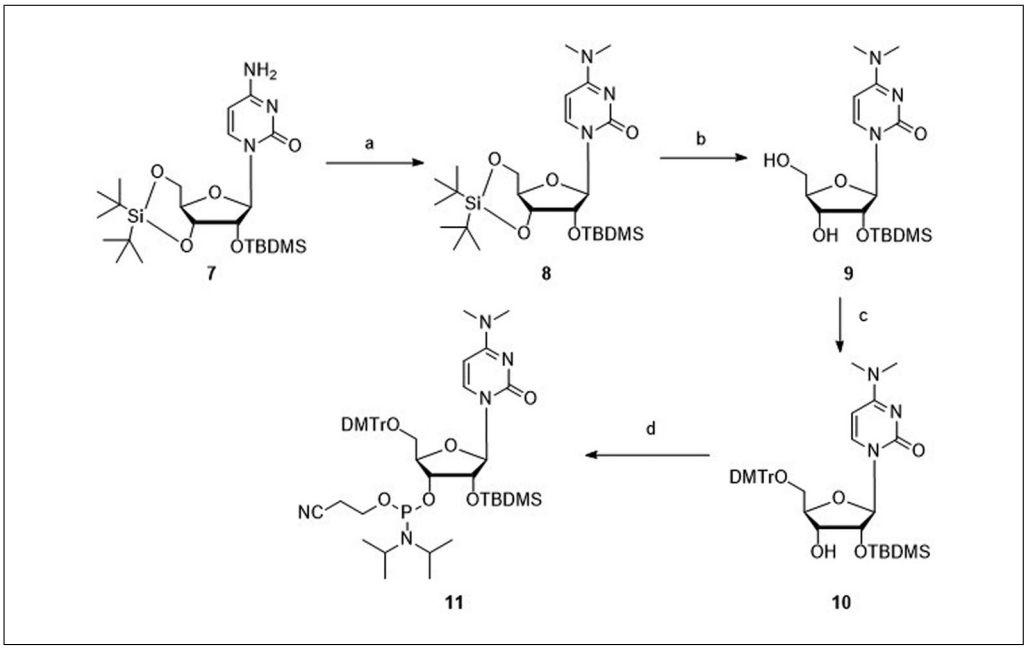

Figure 2 Synthesis of $m^4{}_2C$ phosphoramidite **11**. Conditions: (**A**) MeI, NaH, THF; (**B**) HF.Py, THF; (**C**) DMTrCl, Py; (**D**) $(i\text{-}Pr_2N)_2P(Cl)OCH_2CH_2CN$, $(i\text{-}Pr)_2NEt$, 1-methylimidazole, DCM.

Ethyl acetate (EA; Sigma-Aldrich, cat. no. 34858-4 L)
Sodium sulfate (Na_2SO_4; Sigma-Aldrich, cat. no. 7757-82-6)
Dichloromethane (DCM) (Sigma-Aldrich, cat. no. 270997-1 L)
Methanol (MeOH) (Sigma-Aldrich, cat. no. 179337)
Silica gel (particle size, 32-63 μm)
Hydrogen fluoride-pyridine (Sigma-Aldrich, cat. no. 184225)
Pyridine (Sigma-Aldrich, cat. no. 270970-1 L)
Saturated aqueous sodium bicarbonate ($NaHCO_3$; Sigma-Aldrich, cat. no. S6014-1Kg)
Brine (saturated aqueous sodium chloride)
Sodium sulfate (Na_2SO_4; Sigma-Aldrich, cat. no. 7757-82-6)
4,4′-Dimethoxytrityl chloride (TCI, cat. no. 40615-36-9)
N,N-Di-iso-propylethylamine (DIPEA; Sigma-Aldrich, cat. no. 387649-100 ml)
1-Methyl-1H-imidazole (Alfa Aesar, cat. no. 616-47-7)
2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (Sigma-Aldrich, cat. no. 89992-70-1)

Separatory funnels
Vacuum concentrator
Vacuum pump
Chromatography columns
TLC plates pre-coated with silica gel (SiliCycle, cat. no. TLG-R10014B-323)

Additional reagents and equipment for column chromatography (see Current Protocols article: Meyers, 2001), thin-layer chromatography (TLC; see Current Protocols article: Meyers & Meyers, 2008), nuclear magnetic resonance (NMR), and mass spectrometry (MS)

Synthesis of compound 8 (1-(2′-O-tert-butyldimethylsilyl-3′,5′-O-di-tert-butylsilylene-β-D-ribofuranosyl)-N^4,N^4-dimethylcytidine) starting from compound 7

1. Dissolve 1.5 g (3.0 mmol) of compound **7** in 30 ml of THF.

2. Add NaH (0.6 g, 15 mmol, 60% dispersion in mineral oil) in portions at 0°C over a 5-min time course to the solution of compound **7** under Ar.
3. Add MeI (0.75 ml, 12 mmol) after 15 min.
4. Stir the mixture for 24 hr at room temperature.
5. Quench the mixture with 50 ml water.
6. Extract in a separatory funnel three times, each time with 50 ml ethyl acetate.
7. Dry the organic layer with Na_2SO_4, filter, and evaporate under reduced pressure.
8. Purify the residue by using silica gel chromatography (see Current Protocols article: Meyers, 2001) with 25 g silica gel and a 0% to 10% gradient of MeOH/DCM as mobile phase to obtain compound **8**.

   • ~82% yield (about 1.3 g), 2.5 mmol white solid with the following parameters:
   • TLC $R_f = 0.3$ (DCM: MeOH = 20:1)
   • ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33 (d, $J = 7.5$ Hz, 1H), 5.77 (d, $J = 7.5$ Hz, 1H), 5.62 (s, 1H), 4.47 (dd, $J = 5.5$, 9.5 Hz, 1H), 4.33 (d, $J = 9.5$ Hz, 1H), 4.21–4.15 (m, 1H), 3.94 (dd, J = 9.0, 10.5 Hz, 1H), 3.83 (dd, J = 4.5, 9.5 Hz, 1H), 3.16 (s, 3H), 3.01 (s, 3H), 0.99 (s, 9H), 0.98 (s, 9H), 0.90 (s, 9H), 0.21 (s, 3H), 0.12 (s, 3H)
   • ^{13}C NMR (125 MHz, CDCl3) 163.5, 155.0, 139.7, 94.5, 91.2, 75.8, 75.2, 74.4, 67.9, 27.5, 27.0, 26.0, 22.7, 20.3, 18.2, –4.4, –4.8
   • HRMS (ESI-TOF) $[M+H]^+$ = 526.3130 (calc. 526.3132). Chemical formula: $C_{25}H_{47}N_3O_5Si_2$

Synthesis of compound 9 (1-(2′-O-tert-butyldimethylsilyl-beta-D-ribofuranosyl) -N^4,N^4-dimethylcytidine

9. Dissolve 1.3 g (2.5 mmol) of compound **8** in 20 ml THF at 0°C
10. Add a solution of hydrogen fluoride−pyridine complex (hydrogen fluoride, ~70%; pyridine, ~30%, commercially available from Sigma-Aldrich; 0.5 ml) in pyridine (3 ml) to the solution of compound **8**.
11. Add 7.5 ml pyridine after 1 hr at 0°C when the reaction is completed.
12. Dilute the mixture with 200 ml DCM and wash twice in a separatory funnel, each time with 100 ml of saturated $NaHCO_3$, then twice with 100 ml brine.
13. Dry the organic layer over Na_2SO_4 and evaporate.
14. Purify the residue by silica gel chromatography (see Current Protocols article: Meyers, 2001) with 25 g silica gel and a 0% to 10% gradient of MeOH/DCM as mobile phase to obtain compound **9**.

   • ~ 73% yield (about 700 mg), 1.8 mmol white solid with the following parameters:
   • TLC $R_f = 0.5$ (DCM: MeOH = 10:1)
   • ^{1}H NMR (500 MHz, CDCl3) δ 7.54 (d, $J = 7.5$ Hz, 1H), 5.81 (d, $J = 8.0$ Hz, 1H), 5.29 (d, $J = 4.5$ Hz, 1H), 4.88–4.85 (m, 1H), 4.84–4.80 (m, 1H), 4.10–4.08 (m, 1H), 3.89–3.86 (m, 1H), 3.73–3.68 (m, 1H), 3.14 (s, 3H), 3.02 (s, 3H), 0.83 (s, 9H), 0.03 (s, 3H), 0.01 (s, 3H).
   • ^{13}C NMR (125 MHz, CDCl3) δ 163.5, 155.5, 144.2, 96.1, 91.8, 85.9, 73.3, 70.9, 62.2, 25.7, 17.9, –4.8, –5.2.
   • HRMS (ESI-TOF) $[M+H]^+$ = 386.2111 (calc. 386.2111). Chemical formula: $C_{17}H_{31}N_3O_5Si$.

Synthesis of compound 10 (1-(2´-O-tert-butyldimethylsilyl-5´-O-4,4´-dimethoxytrityl-5´-β-D-ribofuranosyl)- N^4,N^4- dimethylcytidine

15. Dissolve 700 mg (1.8 mmol) compound **9** in 10 ml dry pyridine.
16. Add 1.25 g (3.6 mmol) of 4,4´-dimethoxytrityl chloride to the solution of compound **9** under Ar.
17. Stir the solution at room temperature overnight.
18. Quench the reaction with 1 ml methanol and stir for another 5 min.
19. Concentrate the reaction mixture to dryness under vacuum.
20. Purify the residue by silica gel chromatography (see Current Protocols article: Meyers, 2001) with 25 g silica gel and a 0% to 10% gradient of MeOH/DCM as mobile phase to obtain compound **10**.

    IMPORTANT NOTE: *Silica gel is treated with 1% triethylamine and dried before loading. All eluents contain 1% triethylamine*

- ~89% yield (about 1.1 g), 1.6 mmol white solid with the following parameters:
- TLC $R_f = 0.6$ (ethyl acetate)
- ^{1}H NMR (500 MHz, CDCl3) δ 8.13 (d, $J = 7.5$ Hz, 1H), 7.46–7.43 (m, 2H), 7.36–7.23 (m, 8H), 6.87–6.84 (m, 4H), 5.88 (d, $J = 1.0$ Hz, 1H), 5.34 (d, $J = 7.5$ Hz, 1H), 4.39–4.33 (m, 1H), 4.32–4.30 (m, 1H), 4.07–4.04 (m, 1H), 3.80 (s, 6H), 3.60 (dd, $J = 2.0$, 11.0 Hz, 1H), 3.51 (dd, $J = 3.0$, 11.5 Hz, 1H), 3.20 (s, 3H), 2.96 (s, 3H), 0.94 (s, 9H), 0.36 (s, 3H), 0.22 (s, 3H).
- ^{13}C NMR (125 MHz, CDCl3) δ 163.6, 158.63, 158.62, 155.4, 144.6, 140.8, 135.6, 135.4, 130.3, 130.2, 128.3, 128.0, 127.0, 113.24, 113.23, 90.9, 90.4, 86.9, 82.8, 76.6, 69.1, 61.5, 55.2, 25.9, 18.1, –4.3, –5.5.
- HRMS (ESI-TOF) $[M+H]^+ = 688.3415$ (calc. 688.3418). Chemical formula: $C_{38}H_{49}N_3O_7Si$.

Synthesis of compound 11 (1-(2´-O-tert-butyldimethylsilyl-3´-O-(2-cyanoethyl-N,N-diisopropylamino)phosphoramidite-5´-O-4,4´-dimethoxytrityl-5´-β-D-ribofuranosyl)- N^4,N^4- dimethylcytidine

21. Dissolve 225 mg (0.33 mmol) of compound **10** in 5 ml DCM.
22. Add 0.24 ml (1.32 mmol) *N,N*-di-iso-propylethylamine, 27 µl (0.33 mmol) 1-methyl-1H-imidazole, and 0.17 ml (0.66 mmol) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite to the solution of compound **10**.
23. Stir the resulting solution at room temperature overnight under Ar.
24. Quench the reaction with water and extract with ethyl acetate.
25. Dry the organic layer over Na_2SO_4 and evaporate the residue.
26. Purify by using silica gel chromatography (see Current Protocols article: Meyers, 2001) with 25 g silica gel and a 0% to 10% gradient of MeOH/DCM as mobile phase to obtain compound **11**.

    IMPORTANT NOTE: *Silica gel is treated with 1% triethylamine and dried before loading. All eluents contain 1% triethylamine.*

- ~68% yield (about 200 mg), 0.23 mmol white solid with the following parameters:
- TLC $R_f = 0.6$ (ethyl acetate)
- ^{1}H NMR (500 MHz, CDCl3) δ 8.24–8.22 (m, 1H), 7.48–7.45 (m, 2H), 7.37–7.22 (m, 9H), 6.86–6.83 (m, 4H), 5.77 (d, $J = 0.5$ Hz, 1H), 5.28 (d, $J = 8.0$ Hz, 1H), 4.33–4.23 (m, 3H), 3.79 (s, 6H), 3.74–3.73 (m, 1H), 3.65–3.42 (m, 5H), 3.18

(s, 3H), 2.93 (s, 3H), 2.38 (t, J = 6.5 Hz, 2H), 1.15 (s, 3H), 1.13 (s, 3H), 1.11 (s, 3H), 1.09 (s, 3H), 0.90 (s, 9H), 0.28 (s, 3H), 0.14 (s, 3H).

- ^{31}P NMR (202 MHz, CDCl3) δ 150.06, 148.89.
- HRMS (ESI-TOF) $[M+H]^+$ = 888.4490 (calc. 888.4497). Chemical formula: $C_{47}H_{66}N_5O_8PSi$.

BASIC PROTOCOL 3

SYNTHESIS AND PURIFICATION OF m^4C AND $m^4{}_2C$ CONTAINING RNA OLIGONUCLEOTIDES

Modified synthetic oligonucleotides have been developed into sequence-specific tools with broad therapeutic applications, and studies of nucleoside modifications have attracted significant interest. Oligonucleotides incorporating either m^4C or $m^4{}_2C$ are chemically synthesized on a 1.0-µmol scale using the Oligo-800 DNA solid-phase synthesizer. The entire system is protected under helium gas. All reagents used are standard solutions obtained from ChemGenes Corporation. The synthesis proceeds in the 3′ to 5′ direction and adds one nucleotide per synthesis cycle. The cycle consists of four main steps: detritylation, coupling, capping, and oxidation. Synthesis is performed on controlled pore glass (CPG-500) immobilized with the appropriate nucleoside through a succinate linker. At the beginning, the first 3′ end of the oligonucleotide is pre-attached to the resin and has a 5′-DMT protecting group (DMT = 4,4′-dimethoxytrityl). This trityl group must be removed before synthesis can proceed. Trichloroacetic acid in methylene chloride (3%) is used for 5′-detritylation. The m^4C and $m^4{}_2C$-phosphoramidite are dissolved in anhydrous acetonitrile to a concentration of 0.1 M. The coupling is carried out using 5-ethylthio-1H-tetrazole solution (0.25 M) in acetonitrile for 12 min, for both native and modified phosphoramidites. Acetic anhydride and 1-methylimidazole in tetrahydrofuran and pyridine are used for the capping step. I_2 solution in THF/Py/H_2O (0.02 M) is used as an oxidizing reagent. The oligonucleotide is prepared in the DMTr off form. After synthesis, the oligos are cleaved from the solid support and fully deprotected with ammonium hydroxide solution and methylamine at 65°C for 45 min. The solution is evaporated to dryness by Speed-Vac concentrator and desilylated using 125 µl triethylamine trihydrogen fluoride ($Et_3N \cdot 3HF$) in 100 µl DMSO at 65°C for 2.5 hr. When cooled down to room temperature, the RNA is precipitated by adding 0.025 ml of 3 M sodium acetate and 1 ml of ethanol at −80°C for 1 hr before being recovered by centrifugation and dried under vacuum. Oligonucleotides are purified by IE-HPLC and their identity checked by MALDI-TOF MS. The 31-mer RNA template oligonucleotides are purified on a preparative 20% denaturing polyacrylamide gel (PAGE).

Materials

m^4C phosphoramidite (Basic Protocol 1) and $m^4{}_2C$ phosphoramidite (Basic Protocol 2)

Protected RNA phosphoramidites (ChemGenes, rA-CE cat. no. ANP-5671, rG-CE cat. no. ANP-5673, Ac-rC-CE cat. no. ANP-6676, rU-CE cat. no. ANP-5674)

Acetonitrile (CH_3CN), HPLC-grade (Sigma-Aldrich, cat. no. 34851-4 L)

CPG 500 Å solid support (ChemGenes, cat. no. N-6103-05)

Argon

Reagents for solid-phase synthesis:

- CapA (acetic anhydride in THF; ChemGenes, cat. no. RN-1458) and CapB (16% *N*-methylimidazole in THF) (ChemGenes, cat. no. RN-7776) solutions
- 5-Ethylthio-1H-tetrazole (ETT) in acetonitrile solution (0.25 M) (ChemGenes, cat. no. RN-1466)
- 3% Trichloroacetic acid in dichloromethane (ChemGenes, cat. no. RN-1462)

Ammonium hydroxide (Sigma-Aldrich, cat. no. 221228-500 ml)

Dimethylsulfoxide (DMSO; Sigma-Aldrich, cat. no. D8418-100 ml)

Methylamine (Sigma-Aldrich, cat. no. 534102-250 ml)

Ion-exchange HPLC buffer A: 20 mM Tris·HCl, pH 8.0
Ion-exchange HPLC buffer B: 1.25 M NaCl in 20 mM Tris·HCl, pH 8.0
Triethylamine trihydrogen fluoride ($Et_3N·3HF$; Sigma-Aldrich, cat. no. 344648-25 g)
Sodium acetate (Invitrogen, cat. no. AM9740)
Ethanol (Fisher Scientific, cat. no. BP2818-500)

Automated solid-phase oligonucleotide synthesizer (Oligo 800 synthesizer; ASM-800ET DNA/RNA Synthesizer, Biosset Ltd., cat. no. 800-003-004)
Screw-cap tubes or vials
Heating block
Speed-Vac concentrator
Refrigerated centrifuge
Ion-exchange HPLC columns (analytical, 5 × 150 mm)
HPLC system with detector at 260 nm
Lyophilizer

Additional reagents and equipment for ion-exchange HPLC of nucleic acids (see Current Protocols article: Sinha & Jung, 2015), MALDI-TOF MS, and preparative denaturing PAGE

Solid-phase synthesis

1. Dissolve RNA phosphoramidites including m^4C and $m^4{}_2C$ in acetonitrile to a concentration of 0.1 M.

   We dissolve A, U, C, G, m^4C, and $m^{42}c$ protected phosphoramidites in separate vials. Depending on the sequence input, the synthesizer draws up the corresponding phophoramidite solution.

2. Assemble the column with two filters that hold the solid support CPG 500 Å in the center where the oligonucleotide synthesis takes place at a 1-μmol scale.

3. Perform automated solid-phase oligonucleotide synthesis for m^4C- and $m^4{}_2C$-containing RNAs employing the Oligo-800 DNA synthesizer in DMTr-off mode on a 1-μmol scale, using CPG 500 Å, with two frits acting as a filter to hold the CPG in the column where the oligonucleotide synthesis takes place.

   The synthesis lines are pre-filled with each individual phosphoramidite solution including A, T, C, G, and the modified moiety. With the success of the pressure check, the synthesis can proceed (Haruehanroengra et al., 2017).

4. After completion of the synthesis, purify RNAs and remove unused nucleobases and backbone protecting groups as described in the following steps.

5. Transfer the RNA product on the glass beads into a screw-cap tube or vial.

6. Cleave the oligos from the solid support and deprotect by adding 1:1 v/v ammonium hydroxide solution (28% NH_3 in H_2O) and methylamine (40% w/w aqueous solution) and incubating at 65°C for 45 min.

7. Transfer the supernatant to a new tube, leaving behind the glass beads.

8. Evaporate the solution to dryness using a Speed-Vac concentrator.

   This usually takes 3-4 hr without heating.

9. Dissolve the solid residue in 100 μl DMSO by vortexing. and desilylate by adding 125 μl triethylamine trihydrogen fluoride ($Et_3N·3HF$) solution, mix the solution thoroughly, and gently heat it at 65°C for 2.5 hr using heating block.

   A screw-cap vial is required during this heating step.

10. After heating, cool down the solution to room temperature and add 0.025 ml of 3 M sodium acetate and 1 ml of ethanol. Mix well with vortex and precipitate RNAs at −80°C for 1 hr.

11. Centrifuge 45 min at 15,000 × *g*, 4°C, for 45 min to recover the RNA. Dry the RNA completely under vacuum.

IE-HPLC purification of m^4C and $m^4{}_2C$ containing RNA oligonucleotides

12. Purify the RNA product directly by ion-exchange HPLC at a flow rate of 1 ml/min. Buffer A is 20 mM Tris·HCl, pH 8.0, and buffer B is 1.25 M NaCl in 20 mM Tris·HCl, pH 8.0, with a linear gradient from 100% buffer A to 70% buffer B in 20 min.

    An analytical C18 column can be applied for quality control by using the same eluent and linear gradient from 100% buffer A to 70% buffer B in 20 min.

13. Check the resulting oligos by MALDI-TOF MS; the 31-mer RNA template oligonucleotides were purified on a preparative 20% denaturing polyacrylamide gel (PAGE).

COMMENTARY

Background Information

The design and synthesis protocols provided in this work describe detailed methods to study the fundamental significance of m^4C and $m^4{}_2C$ in base-pairing stability and specificity in the context of RNA duplexes. This is just the beginning of our continuous effort to decipher the structural and functional relationships of these natural RNA modifications. The chemical synthesis of N^4-methylcytidine (m^4C) phosphoramidite was adapted from a literature procedure starting from the silylated uridine with high-yield product formation (Jun Lu et al., 2010). Synthesis of $m^4{}_2C$ could have been achieved using a convertible nucleoside approach (Guennewig, Stoltz, Menzi, Dogar, & Hall, 2012). However, our method of employing a phosphoramidite building block affords a more efficient and higher-quality approach to making these modified RNAs. Both phosphoramidite building blocks were capable of efficient coupling and were well compatible with solid-phase RNA synthesis conditions. Modified oligos were found to be stable during the deprotection process, and then purified by HPLC. Incorporation of these modifications into different RNA sequences was confirmed by ESI or MALDI-TOF-MS, and the coupling yield was found to be comparable to those of commercially available, native counterparts. We anticipate that the method provided here will be beneficial for further exploration of the explicit epigenetic functions of m^4C and $m^4{}_2C$ and their cellular regulatory mechanisms in the context of chaperone proteins. Analyses of both functional and structural aspects of m^4C and $m^4{}_2C$ within RNA transcripts require a reliable protocol to synthesize these modifications, such as the phosphoramidite approach employed here, to generate modified nucleoside building blocks and incorporate them into oligonucleotides. The strategies described here entail the design and reaction conditions needed for the synthesis of both m^4C and $m^4{}_2C$ phosphoramidites and the preparation and purification of m^4C- and $m^4{}_2C$-containing RNA oligonucleotides, and are easy to follow, producing reproducible results.

Critical Parameters and Troubleshooting

The design and chemical synthesis of m^4C and $m^4{}_2C$ phosphoramidites involves stepwise reactions with anhydrous solvents and argon protection for air-sensitive compounds. All solid reagents must be dried under high vacuum prior to use. All reactions require careful monitoring, and the reaction products need to be checked by analytical TLC on plates precoated with silica gel F254 (Dynamic Adsorbents), and visualized by UV light. Flash column chromatography is performed using silica gel with a particle size of 32-63 μm. The final products are phosphoramidite building blocks with specific protecting groups such as *tert*-butyldimethylsilyl (TBDMS) at the 2′-OH positions and 4,4′-dimethoxytrityl (DMTr) at the 5′-OH positions. Use only RNase-free water, tips, and tubes for RNA purification and crystallization, and thermodynamic studies of modified RNA oligonucleotides. Standard analytical assays include ^1H, ^{13}C, and ^{31}P NMR on a Bruker 400 spectrometer, and high-resolution ESI-MS. Please be advised that compound **2** in Figure 1 has low yield due

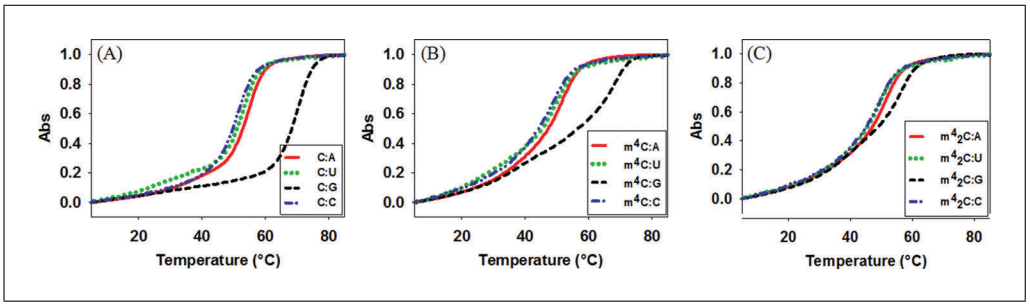

Figure 3 Normalized UV-melting curves of RNA duplexes. (**A**) Pairing of the native sequence 5′-GGACUCCUGCAG-3′ with matched and mismatched strands. (**B**) Pairing of the m^4C-modified sequence 5′-GGACU$\underline{m}^4\underline{C}$CUGCAG-3′ with matched and mismatched sequences. (**C**) Pairing of the $m^4{}_2C$-modified sequence 5′-GGACU$\underline{m}^4{}_2\underline{C}$CUGCAG-3′ with matched and mismatched sequences.

to the hydrolysis back to starting compounds when using $MeNH_2$ aqueous solution, and so $MeNH_2$ in THF would be better to increase the yield.

Understanding Results

With this protocol, the synthesized RNA oligonucleotides containing either m^4C or $m^4{}_2C$ are subjected to structural and functional studies. Thermal denaturation and base-pairing studies compare the native RNA duplex with the m^4C- and $m^4{}_2C$-modified ones (Fig. 3). The normalized UV-melting curves of the native sequence (5′-GGACUCCUGCAG-3′) as well as m^4C (5′-GGACUm^4CCUGCAG-3′)− and $m^4{}_2C$ (5′-GGACU$m^4{}_2$CCUGCAG-3′)−modified sequences paired with matched and mismatched counterpart strands show a decreased thermal stability for both m^4C- and $m^4{}_2C$-modified RNA duplexes. Specifically, m^4C decreases the T_m by 2.0°C, whereas $m^4{}_2C$ dramatically decreases the T_m by 15.5°C. These data suggest that one methyl group has a minor effect on the base-paring stability. However, the stability is drastically diminished by the addition of a second methyl group inside the RNA duplex.

The influence of m^4C and $m^4{}_2C$ on base pairing is further explored in the X-ray crystallographic studies of RNA duplexes containing these modifications. Using the self-complementary 10-mer $(CCGG\underline{C^*}GCCGG)_2$ as a model system, the structural data show that single methylation of cytosine does not disrupt Watson-Crick pairing, as N4 is still able to form an H-bond with O6 of G6 (Fig. 4). However, the base-pairing geometry changes significantly with the addition of a second methyl group in $m^4{}_2C$. Two methyl groups prevent N4 from participating in an H-bond to O6 of G6. As a result, only two H-bonds are formed, and the overall architecture shifts from a canonical Watson-Crick to a wobble-like pairing pattern, which could potentially affect RNA-protein recognition.

The molecular impact of m^4C and $m^4{}_2C$ is further investigated by employing reverse transcription studies in primer-extension reactions. For example, using DNA with 5′-end labeled FAM as primer and two 31-nt-long synthesized RNAs containing either m^4C or $m^4{}_2C$ at the replication site as templates (Fig. 5) in the presence of different base-pairing substrates together with AMV-RT (Avian Myeloblastosis Virus Reverse Transcriptase) or HIV-1 reverse transcriptase, the transcription yield is quantified by fluorescence gel images with single-nucleotide resolution. The results (Fig. 6) show that incorporation of dGTP is decreased from 48.4% of the native RNA (lane G in Fig. 6A) to 18.2% of m^4C RNA (lane G in Fig. 6B), and finally to 5% of $m^4{}_2C$ RNA (lane G in Fig. 6C). The full transcript product is observed and is comparable between the native and m^4C RNA templates (lane Nat vs. N). However, no full-length transcript is observed for $m^4{}_2C$ RNA template in the presence of all natural dNTPs (lane N). This suggests that single methylation can retain the DNA synthesis function. while the double methylation completely abolishes the reverse transcription process.

Time Consideration

The time needed for the chemical synthesis, purification, and confirmation of both the m^4C and $m^4{}_2C$ phosphoramidites is about a month. The NMR and mass spectrometry analyses take several hours with the correct parameters set up. The synthesis of modified RNA oligonucleotides, cleavage, deprotection, and purification take about a week.

Acknowledgment

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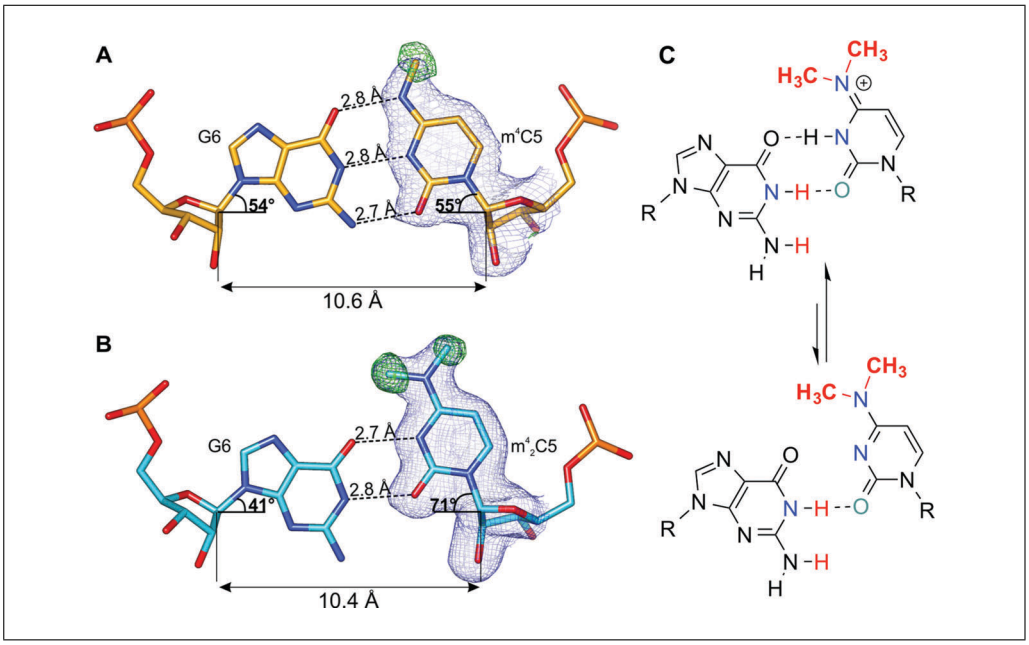

Figure 4 Pairing between m^4C and $m^4{}_2C$ and G. (**A**) The m^4C5:G6 pair in the CCGG(m^4C)GCCGG duplex. (**B**) The $m^4{}_2C5$:G6 pair in the CCGG($m^4{}_2C$)GCCGG duplex. Dashed lines indicate H-bonds; blue mesh represents 2Fo-Fc electron density (contoured at 1 σ) for m^4C and $m^4{}_2C$; and green mesh represents 'omit' Fo-Fc electron density (contoured at 3 σ) calculated only for methyl groups of modified nucleotides. (**C**) Two possible forms of the $m^4{}_2C5$:G6 pair.

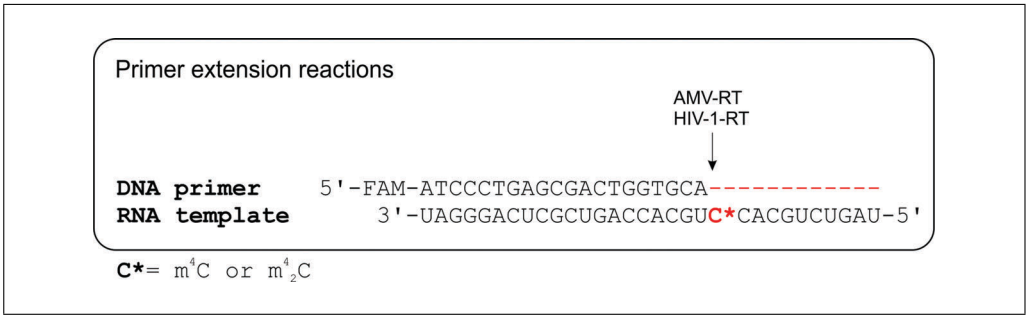

Figure 5 Primer extension reaction as the reverse transcription model.

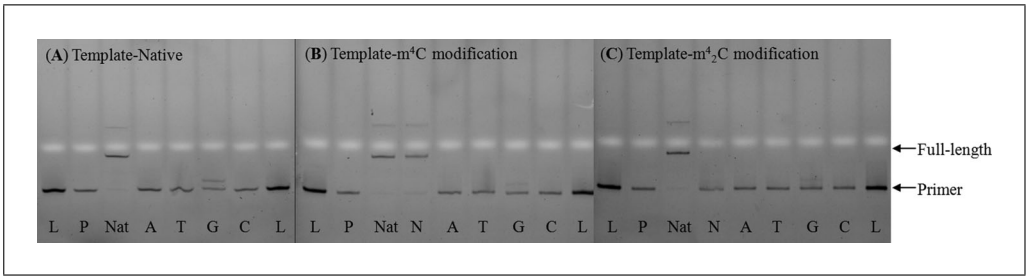

Figure 6 Fluorescent gel images of standing-start primer extension reactions with AMV-RT using (**A**) native, (**B**) m^4C-modified, and (**C**) $m^4{}_2C$-modified RNA strands as templates. Lanes: L, reference DNA 20-mer ladder; P, primer; Nat, natural template with all four dNTPs as positive controls in each gel; A, T, G, and C, reactions in the presence of the respective dNTP only; N, reactions in the presence of all four dNTPs.

Author Contributions

Ya Ying Zheng: data curation, writing original draft, writing review and editing; **Song Mao**: investigation, methodology, writing review and editing; **Jia Sheng**: conceptualization, funding acquisition, investigation, methodology, project administration, supervision, writing review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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