Synthesis and Purification of N^3-Methylcytidine (m^3C) Modified RNA Oligonucleotides

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This protocol describes a step-by-step chemical synthesis approach to prepare N^3-methylcytidine (m^3C) and its phosphoramidite. The method for synthesizing m^3C starts from commercially available cytidine, and proceeds via N^3-methylation in the presence of MeI, which generates the N^3-methylcytidine (m^3C) nucleoside, followed by the installation of several protecting groups at sites that include the 5'-hydroxyl group (4,4'-dimethoxytrityl protection), the 4-amino group (benzoyl protection), and the 2'-hydroxyl group (*tert*-butyldimethylsilyl, TBDMS, protection). Standard phosphoramidite chemistry is applied to prepare the final m^3C phosphoramidite for solid-phase synthesis of a series of RNA oligonucleotides. © 2021 Wiley Periodicals LLC.

Basic Protocol 1: Synthesis of N^3-methylcytidine (m^3C) and its phosphoramidite
Basic Protocol 2: Automated synthesis of m^3C modified RNA oligonucleotides

Keywords: N3-methylcytidine • phosphoramidite • RNA modification • solid-phase synthesis

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INTRODUCTION

RNA molecules display striking functional and structural diversity using four canonical ribonucleotides (adenosine, guanosine, cytidine, and uridine) and a wealth of post-transcriptional modifications (Nachtergaele & He, 2017; Vilfan et al., 2013). To date, more than 170 RNA modifications have been discovered in all domains of life, since the initial discovery of RNA modification over five decades ago (Holley, Apgar et al., 1965; Holley, Everett et al., 1965). In addition to the nucleobases, modifications are also found on the phosphate backbone and ribose moiety (Basanta-Sanchez, Temple, Ansari, D'Amico, & Agris, 2015; Boccaletto et al., 2017; Machnicka et al., 2013; Cantara et al., 2010; Wu et al., 2020). Many studies suggest that RNA modifications play pivotal roles in cell functions under both normal and disease-associated conditions as well as biological processes such as circadian rhythms, embryonic stem cell differentiation, meiotic progression, temperature adaptation, stress response, and

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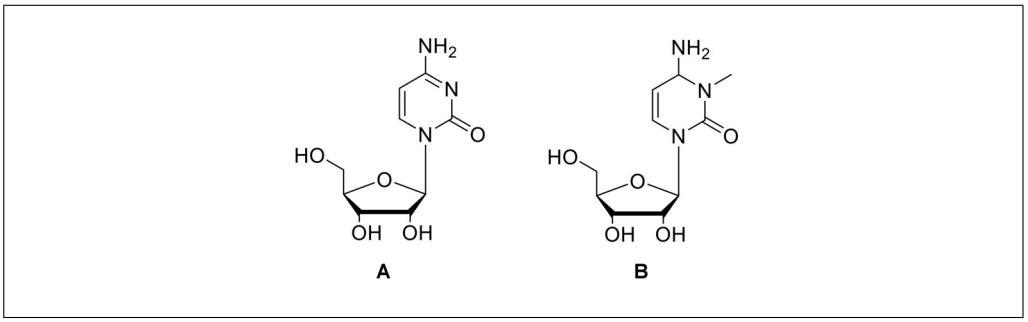

Figure 1 Chemical structures of native C (**A**) and m^3C(**B**).

tumorigenesis, among others (Machnicka et al., 2013). RNA modification, collectively termed the epitranscriptome, is mediated and regulated by systems of reader-writer-eraser enzymes (Roundtree, Evans, Pan, & He, 2017). On account of this, the field of modification enzymes represents a new frontier and research hotspot in RNA molecular biology. RNA-protein complexes are very likely to be crucial targets for antiviral and anticancer drug discovery (Jiang, Crews, Holm, & Jamieson, 2017). Excitingly, several chemical biology tools and high-throughput strategies have recently emerged to identify the sites of RNA methylation in pro- and eukaryotes as well as archaea (Hori, 2014; Nachtergaele & He, 2018; Roundtree et al., 2017; Mongan, Emes, & Archer, 2019; Sergiev, Aleksashin, Chugunova, Polikanov, & Dontsova, 2018; Song & Yi, 2017; Clark, Evans, Dominissini, Zheng, & Pan, 2016). RNA methylation has been demonstrated to be involved in and to regulate numerous biological functions and pathological processes. For instance, N^6-methyladenosine (m^6A), adenosine that is methylated at the N^6 position, is well known as the most abundant epigenetic modification in eukaryotic mRNAs (Guo et al., 2021). This modification plays a role in multiple critical biological functions, affecting mRNA splicing, export, stability, translation, and miRNA biogenesis (Song & Yi, 2017; Desrosiers, Friderici, & Rottman, 1974; Zaccara, Ries, & Jaffrey, 2019). Furthermore, researchers have also detected RNA methylation in viral RNA, and this may become a vital tool to knock down viral gene expression, with promising therapeutic potential (Wu, 2019; Chen, Li, Song, Xue, & Xu, 2019; Ciuffi, 2016; Lichinchi et al., 2016). N^3-methylcytidine (m^3C, Fig. 1) was found in many eukaryotic organisms after its first discovery in *Saccharomyces cerevisiae* (Clark et al., 2016; Cozen et al., 2015; D'Silva, Haider, & Phizicky, 2011, Han, Marcus, D'Silva, & Phizicky, 2016; Iwanami & Brown, 1968; Noma et al., 2011; Olson et al., 1981). The m^3C residue mostly appears at position 32 of the tRNA anticodon stem-loop (ASL) (Luthra et al., 2019). It can impact the tRNA structure, change the decoding specificity during translation, and provide determinants for selectivity and specificity in aminoacylation (Menezes et al., 2011). Formation of the m^3C modification in tRNA is catalyzed by the tRNA-modifying enzymes Trm140 and Trm141 (also known as METTL2 and METTL6). In addition, METTL8, a member of the methyltransferase-like protein family that methylates 3-methylcytidine (m^3C), is found as a key factor in the R-loop regulating methyltransferase complex (Zhang et al., 2020). The m^3C modification has also been discovered in human and mouse mRNA (Xu et al., 2017). In viral RNA, m^3C exclusively exists in ZIKV, HCV, and DENV virions, as well as in the cells infected by these viruses (Xu et al., 2017). The eraser alkylation repair homolog 3 (ALKBH3) has demethylation activity to remove the methyl group in the m^3C residue, and consequently affects the secondary structure of tRNA and RNA degradation (McIntyre et al., 2018). Not surprisingly, ALKBH3 expression promotes tumor formation by up-regulating protein synthesis, thereby heralding m^3C's paramount role in the etiology of various cancers (Ougland et al., 2004). Overall, m^3C modification on the target mRNA can impact a wide range of biological functions of cells. Despite the efforts to discover and detect m^3C, its fundamental properties and physiological functions remain elusive. This article reports the chemical synthesis of the m^3C phosphoramidite

building block and its incorporation into RNA oligonucleotides via solid-phase synthesis. Its aims are to synthesize, purify, and biochemically characterize the m^3C-modified RNA oligonucleotide as per our recent publication (Mao et al., 2020).

SYNTHESIS OF N^3-METHYLCYTIDINE (m^3C) AND ITS PHOSPHORAMIDITE

BASIC PROTOCOL 1

Iodomethane (MeI) is used to initially methylate the commercially available cytidine (**1**, Fig. 2) to produce m^3C nucleoside **2**. Selective protection of the 5′-hydroxyl group with dimethoxytrityl (DMTr) yields compound **3**, followed by protection of the N^4 position with benzoyl to yield compound **4**, which then undergoes a further protection step at the 2′-OH position with *tert*-butyldimethylsilyl (TBDMS) to result in compound **5**. This essential product is then subjected to a regular phosphitylation reaction to yield compound **6**, the final phosphoramidite building block for solid-phase synthesis of oligonucleotides with incorporated m^3C.

Materials

Cytidine (**1**; Alfa Aesar, 99%, CAS Number: 65-46-3)
N,N-Dimethylformamide (DMF, Aldrich, anhydrous, 99%, CAS Number: 68-12-2)
Argon source
Iodomethane (MeI, Alfa Aesar, ≥99%, CAS Number: 74-88-4)
Toluene (Aldrich, anhydrous, 99.8%, CAS Number: 100-88-3)
Acetone (VWR, ≥99.5% ACS, CAS Number: 67-64-1)
Hexane (mixture of isomers, VWR Chemicals BDH®, ≥98.5% ACS, CAS Number: 110-54-3)
Pyridine (Aldrich, anhydrous, 99.8%, CAS Number: 110-86-1)
4,4′-Dimethoxytrityl chloride (DMTrCl, Aldrich, ≥97.0%, CAS Number: 40615-36-9)
Dichloromethane (DCM, Aldrich, anhydrous, ≥99.8%, contains 40-150 ppm amylene as a stabilizer, CAS Number: 75-09-2)

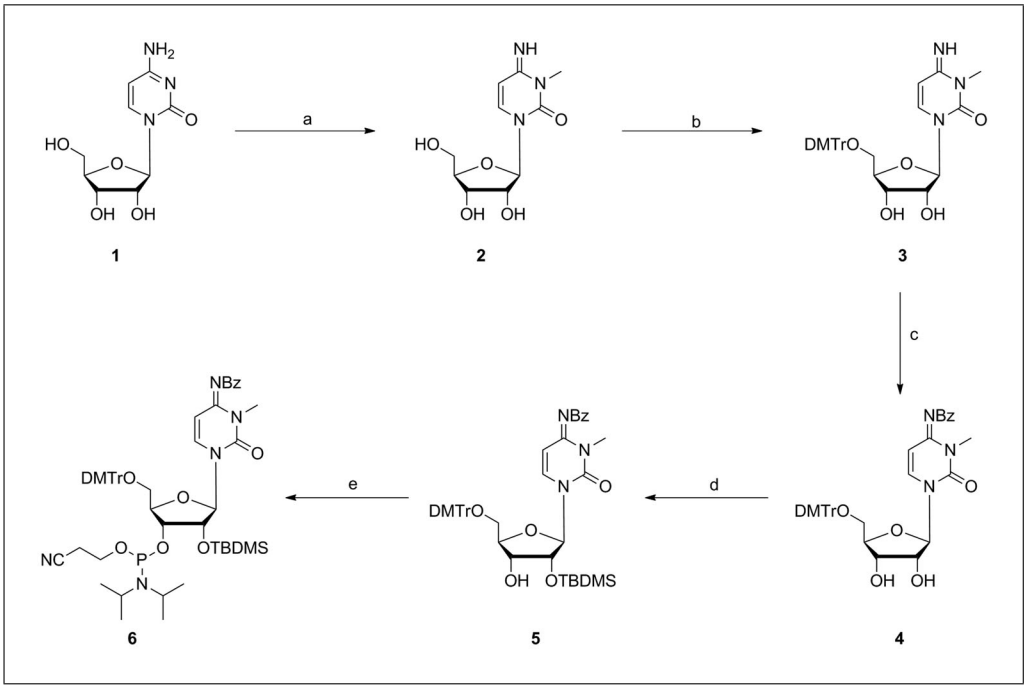

Figure 2 Synthesis of N^3-methyl-cytidine phosphoramidite **6**. Reagents and conditions: (a) MeI, DMF; (b) DMTrCl, Py; (c) TMSCl, Py; BzCl; (d) TBDMSCl, imidazole, DMF; (e) $(i\text{-}Pr_2N)_2P(Cl)OCH_2CH_2CN$, $(i\text{-}Pr)_2NEt$, 1-methylimidazole, DCM.

Sodium thiosulfate ($Na_2S_2O_3$; Aldrich, CAS Number: 7772-98-7)
Aqueous, saturated sodium bicarbonate ($NaHCO_3$, CAS Number: 144-55-8)
Brine: aqueous, saturated sodium chloride (NaCl, , CAS Number: 7440-23-5)
Sodium sulfate (Na_2SO_4; Aldrich, ACS reagent, anhydrous, CAS Number: 7757-82-6)
Methanol (MeOH, VWR Chemicals BDH®, ≥99.8% ACS, CAS Number: 67-56-1)
Dichloromethane (DCM, Macron Fine Chemicals™, ≥99.9%, CAS Number: 75-09-2)
Silica gel (Aldrich, technical grade, pore size 60 Å; 230-400 mesh particle size, 40 to 63 μm particle size)
Triethylamine (Aldrich, ≥99.5%, CAS Number: 121-44-8)
Trimethylsilyl chloride (TMSCl, Aldrich, purified by redistillation, ≥99%, CAS Number: 75-77 4)
Benzoyl chloride (BzCl, Alfa Aesar, ≥99%, CAS Number: 98-88-4)
Ammonia solution (Aldrich, 7 N in methanol, CAS number: 7664-41-7)
Ethyl acetate (EtOAc, Macron Fine Chemicals™, ≥99.5%, CAS Number: 141-78-6)
tert-Butyldimethylsilyl chloride (TBDMSCl, Aldrich, > 97%, CAS Number: 18162-48-6)
Imidazole (Alfa Aesar, 99%, CAS Number: 288-32-4)
N,N-Diisopropylethylamine (DIPEA; Aldrich, ≥99%, CAS Number: 7087-68-5)
2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (Aldrich, 97.0%, CAS Number: CAS Number: 89992-70-1)
1-Methylimidazole (Aldrich, 99%, CAS Number: 616-47-7)

25-, 100-, and 500-ml round-bottom flasks
Magnetic stirrer and stir bar
Rubber septum
Lyophilizer
Rotary evaporator
Vacuum oil pump
Filtering funnel
Filter paper
Separatory funnels
22 × 457–mm silica gel chromatography columns

Additional reagents and equipment for performing thin-layer chromatography (TLC; see Current Protocols article: Meyers & Meyers, 2008) and column chromatography (see Current Protocols article: Meyers, 2001)

Synthesis of 3-N-methyl-cytidine (2)

1. Transfer 4.86 mg (20 mmol) of cytidine **1** in an oven-dried 250-ml one-neck round-bottom flask.
2. Place a stir bar into the same flask.
3. Add 50 ml of argon-protected DMF to the same flask while stirring.
4. Add 2.5 ml of MeI (40 mmol) to the reaction mixture and introduce an inert atmosphere in the flask by flushing with argon gas for 2-3 min.
5. Keep stirring the reaction at room temperature for 24 hr.
6. Evaporate the DMF using a rotary evaporator under reduced pressure.
7. Evaporate the residue twice with 100 ml toluene using a rotary evaporator under reduced pressure.
8. After evaporation, dissolve the residue in 20 ml of acetone.

9. Add 50 ml of hexane to the above solution and keep the resulting mixture at –20°C for 1 hr.
10. Filter the precipitant with a filter funnel under vacuum.
11. Wash the precipitate twice with a cold mixture of 1:1 (v/v) acetone:hexane. Use a total of 50 ml of the mixture for this step.
12. Dry the resulting product under vacuum to give compound **2** (3.6 g, 14 mmol, 70% yield): yellow solid.

    ^{1}H NMR (500 MHz, DMSO-d6) δ 9.79 (br, 1H), 9.15 (br, 1H), 8.31 (d, J = 7.5 Hz, 1H), 6.20 (d, J = 8.0 Hz, 1H), 5.70 (d, J = 3.5 Hz, 1H), 5.51 (br, 1H), 5.17 (br, 1H), 4.05-4.03 (m, 1H), 3.95-3.89 (m, 2H), 3.73 (dd, J = 2.5, 12.5 Hz, 1H), 3.60 (dd, J = 2.5, 12.0 Hz, 1H), 3.35 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.4, 148.1, 142.1, 94.5, 91.2, 85.1, 74.6, 69.0, 60.2, 31.2. HRMS (ESI-TOF) $[M+H]^+$ = 258.1090 (calc. 258.1090). Chemical formula: $C_{10}H_{15}N_3O_5$.

Synthesis of 1-(5′-O-4,4′-dimethoxytrityl-beta-D-ribofuranosyl)-3-N-methyl-cytidine (3)

13. Transfer 3.5 g (13.6 mmol) of compound **2** into a 250-ml one-neck round-bottom flask.
14. Place a stir bar into the same flask.
15. Dissolve the starting material in 40 ml dry pyridine with magnetic stirring.
16. Add 6.9 g of DMTrCl (20.4 mmol) to the same flask and introduce an argon atmosphere into it.
17. Keep stirring at room temperature overnight.
18. Using a separatory funnel, extract the organic layer with 200 ml of DCM and subsequently wash twice, each time 100 ml of 5% aqueous $Na_2S_2O_3$, 100 ml of saturated aqueous $NaHCO_3$, and 100 ml of brine.
19. Dry the organic layer using anhydrous sodium sulfate and filter off the drying agent.
20. Concentrate the solvent using a rotary evaporator under reduced pressure.
21. Purify the residue using column chromatography (10 g of silica gel and 2%-10% methanol/dichloromethane; see Meyers, 2001).

    Silica gel is treated with 1% triethylamine in DCM (20 ml) for 1 hr and dried under reduced pressure before loading. All eluents contain 1% triethylamine.

    TLC $R_f = 0.2$ (10% methanol in dichloromethane).

    The resulting product, ***1-(5′-O-4,4′-dimethoxytrityl-beta-D-ribofuranosyl)-3-N-methyl-cytidine*** (**3**), is obtained in 43% yield (3.9 g, 5.9 mmol) as a white solid.

    ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61(d, J = 8.0 Hz, 1H), 7.39-7.36 (m, 2H), 7.28-7.16 (m, 7H), 6.82-6.79 (m, 4H), 5.86 (d, J = 3.2 Hz, 1H), 5.76 (d, J = 7.2 Hz, 1H), 4.36-4.30 (m, 2H), 4.18 (m, 1H), 3.73 (d, 6H), 3.48-3.40 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.9, 158.6, 149.4, 149.37, 144.5, 135.5, 135.3, 130.2, 128.2, 128.0, 113.3, 98.2, 90.9, 86.9, 83.5, 74.9, 69.9, 62.5, 55.3, 30.3. HRMS (ESI-TOF) $[M+H]^+$ = 560.2390 (calc. 560.2397). Chemical formula: $C_{31}H_{33}N_3O_7$.

Synthesis of 1-(5′-O-4,4′-dimethoxytrityl-beta-D-ribofuranosyl)-4-N-benzoyl-3-N-methyl-cytidine (4)

22. Co-evaporate compound **3** (2.3 g, 4.2 mmol) twice, each time with 50 ml pyridine using a rotary evaporator under reduced pressure.
23. Transfer compound **3** into a 250-ml one-neck round-bottom flask.

24. Place a stir bar into the same flask.
25. Dissolve the compound **3** in 40 ml dry pyridine with magnetic stirring.
26. Add 2.1 ml of TMSCl (16.8 mmol) to the same flask and stir the mixture at room temperature for 1 hr.
27. Add 0.84 ml of BzCl (5.04 mmol) to the same flask and stir the resulting solution for 4 hr at room temperature.
28. Add 10 ml of water to the same flask and stir the mixture for 5 min.
29. Mix 15 ml of aqueous ammonia (15.8 M) into the mixture and stir for 15 min at room temperature.
30. Evaporate the mixture to dryness using a rotary evaporator under reduced pressure.
31. Purify the residue using 10 g of silica gel by column chromatography (20%-50% ethyl acetate/dichloromethane).

    Silica gel is treated with 1% triethylamine in DCM (20 ml) for 1 hr and dried under reduced pressure before loading. All eluents contain 1% triethylamine.

    TLC $R_f = 0.4$ (50% EtOAc in CH_2Cl_2).

    The resulting product, ***1-(5′-O-4,4′-dimethoxytrityl-beta-D-ribofuranosyl)-4-N-benzoyl-3-N-methyl-cytidine*** (**4**), is obtained in 82% yield (2.3 g, 3.47 mmol) as a white solid.

    ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15-8.12 (m, 2H), 7.64 (d, J = 8.4 Hz, 1H),7.56-7.51 (m, 1H), 7.46-7.42 (m, 2H), 7.35-7.33 (m, 2H), 7.29-7.18 (m, 8H), 6.84-6.80 (m, 4H), 6.25 (d, J = 8.4 Hz, 1H), 5.82 (d, J = 3.6 Hz, 1H), 4.37-4.26 (m, 3H), 3.78 (d, 6H), 3.58 (s, 3H). 3.48 (dd, J = 2.8 Hz, 10.8 Hz, 1H), 3.38 (dd, J = 3.2 Hz, 10.8 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.4, 158.7, 151.1, 144.1, 135.8, 135.7, 135.4, 135.2, 132.5, 130.1, 130.0, 129.7, 128.2, 128.1, 128.0, 127.1, 113.31, 113.30, 98.2, 91.5, 87.1, 84.3, 76.2, 70.5, 62.2, 55.2, 30.0. HRMS (ESI-TOF) $[M+H]^+$ = 664.2646 (calc. 664.2659). Chemical formula: $C_{38}H_{37}N_3O_8$.

Synthesis of 1-(2′-O-tert-butyldimethylsilyl-5′-O-4,4′-dimethoxytrityl-beta-D-ribofuranosyl)-4-N-benzoyl-3-N-methyl-cytidine (5)

32. Transfer 1.3 g of compound **4** (2 mmol) into a 125-ml one-neck round-bottom flask.
33. Place a stir bar into the same flask.
34. Dissolve compound **4** in 12 ml dry pyridine with magnetic stirring.
35. Add 362 mg of TBDMSCl (2.4 mmol) to the same flask with magnetic stirring.
36. Add 272 mg imidazole (2.4 mmol) to the same flask with magnetic stirring.
37. Keep stirring at room temperature overnight.
38. Use 200 ml EtOAc to extract the organic layer, then wash twice, each time using 100 ml brine.
39. Dry the organic using anhydrous sodium sulfate and filter off the drying agent.
40. Concentrate the solvent using a rotary evaporator under reduced pressure.
41. Purify the residue using 10 g of silica gel by column chromatography (20%-50% ethyl acetate/hexane).

    Silica gel is treated with 1% triethylamine in DCM (20 ml) for 1 hr and dried under reduced pressure before loading. All eluents contain 1% triethylamine.

TLC $R_f = 0.6$ (hexane:ethyl acetate = 1:1).

The resulting product, ***1-(2′-O-tert-butyldimethylsilyl-5′-O-4,4′-dimethoxytrityl-beta-D-ribofuranosyl)-4-N-benzoyl-3-N-methyl-cytidine*** (**5**), is obtained in 39 % yield (600 mg, 0.77 mmol) as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15-8.12 (m, 2H), 7.82 (d, J = 8.5 Hz, 1H), 7.55-7.50 (m, 1H), 7.46-7.42 (m, 2H), 7.38-7.35 (m, 2H), 7.29-7.25 (m, 6H), 7.22-7.16 (m, 1H), 6.85-6.82 (m, 4H), 6.08 (d, J = 8.0 Hz, 1H), 5.94 (d, J = 2.5 Hz, 1H), 4.38-4.33 (m, 1H), 4.29-4.27 (m, 1H), 4.09-4.06 (m, 1H), 3.78 (d, J = 1.0 Hz, 6H), 3.55 (s, 3H), 3.57-3.46 (m, 2H), 0.94 (s, 9H), 0.24 (s, 3H), 0.18 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.3, 158.7, 155.7, 150.3, 144.1, 136.0, 135.9, 135.4, 135.2, 132.4, 130.1, 130.0, 129.7, 128.20, 128.19, 128.0, 127.2, 98.1, 89.8, 87.1, 83.3, 76.8, 69.8, 61.9, 55.2, 30.0, 25.8, 18.1, -4.5, -5.2. HRMS (ESI-TOF) $[M+H]^+$ = 778.3527 (calc. 778.3524). Chemical formula: $C_{44}H_{51}N_3O_8Si$.

Synthesis of 1-[2′-O-tert-butyldimethylsilyl-3′-O-(2-cyanoethyl-N,N-diisopropylamino)phosphoramidite-5′-O-(4,4′-dimethoxytrityl-beta-D-ribofuranosyl)]-4-N-benzoyl-3-N-methyl-cytidine (6)

42. Transfer 600 mg of compound **5** (0.77 mmol) into a 125-ml one-neck round-bottom flask.
43. Place a stir bar into the same flask.
44. Dissolve compound **5** in 13 ml dry DCM with magnetic stirring and introduce an argon atmosphere into the flask.
45. Add 0.38 ml of DIPEA (3.08 mmol) to the same flask with magnetic stirring.
46. Add 0.23 ml of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.54 mmol) to the same flask with magnetic stirring.
47. Keep stirring at room temperature overnight.
48. Use 5 to 10 ml of water to quench the reaction and wait for 5 min.
49. Use 200 ml of EtOAc to dilute the solution, and subsequently wash it twice, each time with 100 ml brine.
50. Dry the organic layer using anhydrous sodium sulfate and filter off the drying agent.
51. Concentrate the solvent using a rotary evaporator under reduced pressure.
52. Purify the residue using 10 g of silica gel by column chromatography (20%-50% ethyl acetate/hexane).

    Silica gel is treated with 1% triethylamine in DCM (20 ml) for 1 hr and dried under reduced pressure before loading. All eluents contain 1% triethylamine.

    TLC $R_f = 0.6$ (hexane:ethyl acetate = 1:1).

    The residue product, ***1-[2′-O-tert-butyldimethylsilyl-3′-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite-5′-O-(4,4′-dimethoxytrityl-beta-D-ribofuranosyl)]-4-N-benzoyl-3-N-methyl-cytidine*** (**6**), is obtained in 66% yield (500 mg, 0.51 mmol) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15-8.12 (m, 2H), 7.90-7.19 (m, 13H), 6.84-6.80 (m, 4H), 6.16-5.91 (m, 2H), 4.38-3.91 (m, 3H), 3.78 (s, 6H), 3.61-3.36 (m, 7H), 2.67-2.37 (m, 2H), 2.06-2.04 (m, 1H), 1.29-1.14 (m, 12H), 1.02-0.98 (m, 3H), 0.93-0.9 (m, 9H), 0.19-0.13 (m, 6H). ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.97, 148.94. HRMS (ESI-TOF) $[M+H]^+$ = 978.4561 (calc. 978.4602). Chemical formula: $C_{53}H_{68}N_5O_9PSi$.

AUTOMATED SYNTHESIS OF m^3C MODIFIED RNA OLIGONUCLEOTIDES

Basic Protocol 2 describes the 1.0-μmol-scale solid-phase synthesis of m^3C modified RNA oligonucleotides using the automated Oligo-800 DNA synthesizer. The synthesis uses ultra mild–protected RNA phosphoramidites. These phosphoramidites use N^6-benzoyl, N^4-acetyl, N^2-isobutyryl for adenine, cytosine, and guanine protection, respectively. The DMTr-off, free 5′-OH m^3C oligos are obtained using a regular four-step synthesis cycle that includes detritylation, activation-coupling, capping, and oxidation on a controlled pore glass (CPG) solid support. All amidites are dissolved in anhydrous acetonitrile under argon atmosphere, including during the synthesis. The synthesis cycle begins with removing the DMT group using 3% trichloroacetic acid in DCM. The deep orange-colored cation produced during detritylation determines the detritylation efficiency, and the trityl monitor visually shows the success of each detritylation throughout the synthesis. The activation-coupling step uses 0.07 M regular and 0.1 M modified amidites with the 5-ethylthio-1H-tetrazole solution in acetonitrile as the activator. Acetic anhydride and 16% *N*-methylimidazole mixed in THF act as a capping agent, and 0.02 M I_2 in THF/Py/H_2O solution completes the oxidation step. After final detritylation, the oligos are removed from the solid support using ammonium hydroxide solution and methylamine mixture, followed by TBDMS removal using triethylamine trihydrogen fluoride (Et_3N•3HF).

Materials

Controlled pore glass (CPG, 1000 Å, ChemGenes Corporation)

RNA phosphoramidites: rA-CE, rG-CE, Ac-rC-CE, U-CE-0.07 M (ChemGenes Corporation, cat. no.: rA-CE- ANP-5671, rG-CE- ANP-5673, rC-CE- ANP-6676, U-CE- ANP-5674)

3-Methylcytosine (m^3C) phosphoramidite, 0.1 M (compound **6** from Fig. 2, synthesized as in Basic Protocol 1)

Acetonitrile (CH_3CN; Aldrich, anhydrous, CAS Number: 75-05-8), to wash the column during each step in the synthesizer

Acetonitrile (CH_3CN; ChemGenes Corporation, anhydrous, cat. no.: RN-1447), for preparing amidites

Argon source

3% Trichloroacetic acid in dichloromethane (ChemGenes Corporation, cat. no.: RN-1462)

5-Ethylthio-1H-tetrazole (ETT) in acetonitrile solution (0.25 M) (ChemGenes Corporation)

I_2 (0.02 M) in THF/Py/H_2O solution (ChemGenes Corporation, cat. no.: RN-1455)

CapA (acetic anhydride in THF, cat. no.: RN-1458) and CapB (16% *N*-methylimidazole in THF, cat. no.: RN-7776) solutions (ChemGenes Corporation)

Ammonium hydroxide solution (Aldrich, 28.0-30.0% NH_3 basis, CAS Number: 1336-21-6)

Methylamine (Aldrich, 40 wt. % in H2O, CAS Number: 74-89-5)

Dimethyl sulfoxide (DMSO, Aldrich, anhydrous, ≥99.9%, CAS Number: 67-68-5)

Triethylamine trihydrofluoride ($Et_3N \cdot 3HF$; Aldrich, 98%, CAS Number: 73602-61-6)

3 M sodium acetate, pH 5.5

Ethanol (PHARMCO, anhydrous, ACS grade, CAS Number: 64-17-5.)

Buffer A: 20 mM Tris·HCl, pH 8.0

Buffer B:1.25 M NaCl in 20 mM Tris·HCl, pH 8.0.

RNase-free water

2.0 M Triethylammonium acetate (TEAAc) buffer, pH 7.0 (to prepare 100 ml of the buffer, combine 26.8 ml of triethylamine, 11.4 ml of acetic acid, and 61.9 ml of deionized water, and adjust the pH accordingly)

Oligo-800 DNA synthesizer (BIOSSET Ltd.)
Oven dried amber glass bottles (for preparing phosphoramidites)
13-mm syringe filter with 0.2-μm nylon membrane (Life Sciences)
Empty twist synthesis columns (1 μmol, Glen Research)
Screw-cap tubes or vials
Microcentrifuge tubes
Refrigerated microcentrifuge
HPLC system with the detector at 260 nm
Ion-exchange HPLC column: Dionex DNASwift™ (Thermo Scientific)
Lyophilizer
Nanodrop spectrophotometer
Sep-Pak C18 columns for desalting
Burette stand

Additional reagents and equipment for polyacrylamide gel electrophoresis of oligonucleotides (see Current Protocols article: Ellington & Pollard, 2001)

Synthesis, deprotection, and purification

1. Pack the columns with CPG beads and dissolve 0.07 M regular RNA and 0.1 M m^3C phosphoramidite in anhydrous acetonitrile under argon atmosphere.
2. Perform 1-μmol DMTr-off automated solid-phase synthesis cycle in oligo-800 DNA synthesizer (also see introduction to Basic Protocol 2).
3. Remove the column from the synthesizer after completion and slowly introduce a vacuum to dry the beads.
4. Collect all the beads in a screw-cap tube and add 800 μl of 1:1 mixture of ammonium hydroxide and methylamine solution (AMA). Keep it at 65°C for 50 min.
5. Collect the supernatant in a new 2-ml tube, wash the beads twice with 200 μl of AMA, and transfer it to the 2-ml tube.
6. Using a SpeedVac evaporator, dry the RNA completely.
7. Add 100 μl of DMSO to the dry residue and vortex for 2 min, then add 125 μl of $Et_3N \cdot 3HF$ to the tube, mix the solution, and keep the tube at 65°C for 2.5 hr.
8. Keep the fluoride-treated sample at –20°C for 1 min to let it cool down, and add 25 μl of 3 M sodium acetate, pH 5.5.
9. Add 1 ml of ethanol, vortex for 1 min, and keep the tube at −80°C overnight to precipitate.
10. Centrifuge the tubes 20 min at 14,000 × *g*, 4°C, remove the supernatant and re-dissolve the pellet in 200 μl of water for HPLC purification.
11. Use ion-exchange HPLC with Dionex DNASwiftTM column to purify the oligonucleotides. Buffer A is 20 mM Tris·HCl (pH = 8), and buffer B contains 20 mM Tris·HCl and 1.25 M NaCl (pH = 8). Use a linear gradient from 100% buffer A to 70% buffer B in 20 min and elute the oligos at 1 ml/min flow rate.

Lyophilize the collected fractions overnight, then desalt with Sep-Pak C18 columns and re-concentrate the samples.

Desalting

12. Attach Sep-Pac C18 desalting column to a burette stand and keep a 2-ml tube below the column.
13. To wash the column, add 1.0 ml of 100% acetonitrile, use vacuum to push the solution out, and collect it in the tube.

14. Repeat the above step.
15. Add 1.0 ml of RNase-free water and follow the same procedure as in step 13.
16. Repeat the above step.
17. Activate the column by adding 1.0 ml of 2.0 M TEAAc buffer (pH = 7.0), push the buffer out slowly from the column using the vacuum, and collect it in a 2-ml tube.
18. In the prewashed and activated column, add the RNA, use gravity to drain the solution, and use 2-ml autoclaved vials to collect all the eluents.
19. Wash the column twice with 1 ml of RNase-free water and elute the solutions slowly.
20. Add 1 ml of 50% acetonitrile to recover RNA from the column and quickly elute the solution.
21. Repeat the above step.
22. Analyze the samples from steps 20 and 21 in a Nanodrop spectrophotometer and validate the RNA.
23. Check the quality of the oligonucleotides by running a 15% polyacrylamide (PAGE) gel before combining the samples from the two 50% acetonitrile washes.

COMMENTARY

Background Information

The N^3 position of cytidine is involved in an H-bond with N^1H of G in a standard Watson-Crick base pair. Accordingly, the introduced methylation is expected to alter the base-pairing preferences and possibly reduce C:G base-pairing fidelity. We hypothesized that methylation at the N^3 position of cytidine might increase base mispairing and affect codon-anticodon pairing. Thus, we set out to uncover the base-pair stability and specificity of modified RNA to better understand the function of N^3 methylation and the regulatory mechanisms. Thermal denaturation studies of modified RNA strands show dramatically decreased thermal stability compared to their native counterparts. Mismatched duplexes involving m^3C also show lower melting temperatures (e.g., m^3C:A, m^3C:U, and m^3C:C) compared to duplexes with unmodified mispairs. Interestingly, the RNA duplex containing the m^3C:G pair displays a melting temperature that is similar to those of other mismatch pairs, i.e., C:A, C:U, and C:C, indicating that this modification decreases base-pairing discrimination and fidelity. Moreover, the results of molecular dynamics simulations are consistent with our experimental results and provide more detailed structural insights on m^3C base pairing (Mao et al., 2020).

Critical Parameters

Phosphoramidite synthesis is sensitive to the presence of water/moisture, so it is vital to maintain an anhydrous environment throughout the reaction and during solid-phase synthesis. The synthesis reagents must be in an inert atmosphere, and it is advisable to use fresh reagents, especially during the synthesis of modified long RNA oligonucleotides. Before performing column chromatography to purify any tritylated compound, the packed silica gel should be treated with 1%-3% triethylamine, and all eluents should contain 1%-3% triethylamine, due to the acid-liable nature of the DMTr group. Because of the instability of the DMTr group, we highly recommend performing rotary evaporation using a water bath temperature below 30°C. Due to the same nucleophilicity and site selectivity issue between the two -OH groups, a relatively low yield of compound **5** is observed. However, the byproduct (the 3′-O-TBDMS analog) is also helpful for constructing the 2'-5'-linked RNA strands. After the final phosphoramidite building block is dissolved in acetonitrile, a sterile syringe filter should be used to obtain a clear, light-yellow solution for the following solid-phase synthesis.

Understanding Results

The 3-methylcytosine (m^3C) phosphoramidite and the corresponding modified RNA oligonucleotides can be synthesized efficiently with this protocol. The incorporation yields of this modified m^3C could be similar to the those with commercially available native phosphoramidites, depending on the

quality and purity of the final phosphoramidite building block. The purity of the modified RNA strands is confirmed by reversed-phase HPLC purification and analysis. The final modified RNA strands can be leveraged for future studies examining the structures and functions of this important RNA modification. In addition, the modified residue can also be incorporated into siRNA or microRNA, as well as CRISPR RNA, as potential molecular tools and RNA therapeutics with enhanced functions.

Time Considerations

Preparation of N^3-methylcytidine (m^3C) phosphoramidite is relatively straightforward and requires about 3 weeks. Modified RNA oligonucleotide synthesis, purification, and analysis require about 2 weeks.

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Author Contributions

Johnsi Mathivanan: investigation, writing original draft, writing review and editing; **Jinxi Du**: investigation, writing original draft, writing review and editing; **Song Mao**: data curation, investigation, methodology, writing review and editing; **Ya Ying Zheng**: methodology, writing review and editing; **Jia Sheng**: conceptualization, data curation, funding acquisition, methodology, project administration, resources, 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 request.

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