Synthesis of $N^4$-acetylated 3-methylcytidine phosphoramidites for RNA solid-phase synthesis

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Abstract
The growing interest in 3-methylcytidine (m3C) originates from the recent discoveries of m3C modified tRNAs in humans as well as its intensively debated occurrence in mRNA. Moreover, m3C formation can be catalyzed by RNA without the assistance of proteins as has been demonstrated for a naturally occurring riboswitch fold using the methylated form of its cognate ligand as cofactor. Additionally, new RNA sequencing methods have been developed to detect this modification in transcriptome-wide manner. For all these reasons, an increasing demand for synthetic m3C containing oligoribonucleotides is emerging. Their chemical synthesis relies on RNA solid-phase synthesis using phosphoramidite building blocks. Here, we describe a facile synthetic path towards $N^4$-acetylated 2′-O-TBDMS- and 2′-O-TOM m3C phosphoramidites to provide an optimal toolbox for solid-phase synthesis of m3C containing RNA.

Graphical abstract

Keywords Nucleosides · Nucleotides · Bioorganic chemistry · Solid-phase synthesis · RNA methylation

Introduction
More than 170 different nucleoside modifications have been identified in non-coding and coding RNAs [1–4]. These modifications influence formatively the cellular fate of RNAs by modulating their stabilities and functions. The most abundant class of RNA modifications is methylation which occurs in high structural diversity, including ribose 2′-O and almost any position of the four heterocyclic nucleobases, adenine, cytosine, guanine, and uracil. While nucleoside methylations are traditionally associated with tRNA, rRNA, and caps of mRNA, the recent discoveries on reversible mRNA methylation have opened a new realm of post-transcriptional gene regulation. In particular, $N^6$-methyladenosine (m6A) has been disclosed as a modification that eukaryotic cells utilize to tune mRNA metabolism and translation [5, 6]. More recently, 3-methylcytidine (m3C) has gained a lot of attention, because evidence for its occurrence in mRNA of mice and humans has been reported [7–9]. Notably, m3C was first discovered in 1963 in total RNA of yeast [10] and was later identified in the anticodon loop of eukaryotic tRNA where it impacts fold stability, ribosome-binding affinity and decoding activity of tRNA as well as mRNA processing [11–15]. Very recently, m3C has been identified as methylation product of preQ1 class I riboswitches possessing ribozyme (methyltransferase)
activity in vitro when m₆preQ₁ (2-amino-7-aminomethyl-6-methoxy-7-deazapurine) is provided as cofactor [16, 17]. Additionally, new RNA sequencing methods, AlkAniline-Seq [18] and HAC-seq [19], have been developed to detect m₃C modifications in transcriptome-wide manner. Furthermore, deoxyribozyme tools to detect m₃C, m₄C and m₅C have been selected in vitro and are able to distinguish the methylation position based on distinct kinetic signatures of their RNA catalyzed backbone cleavage reaction at the site of modification [20].

Recently, Mao et al. [21] reported the synthesis of N⁴-benzoyl-2'-O-tert-butylidemethysilyl(TBDMs)-3-methylcytidine (m₃C₁) (Scheme 1) [21]. Subsequently, the 5' hydroxyl group was masked as dimethoxytrityl ether using dimethoxytrityl chloride to furnish compound 2 [21]. Transient protection of the ribose OH groups as trimethylsilyl ether, followed by N⁴ acetylation using acetyl chloride, subsequent desilylation with methanol and aqueous workup delivered compound 3.

### Results and discussion

Our synthesis started from commercially available cytidine which was selectively methylated at position N3 by iodomethane to yield the corresponding hydroiodide salt of m₃C₁ (Scheme 1) [21]. Subsequently, the 5' hydroxyl group was masked as dimethoxytrityl ether using dimethoxytrityl chloride to furnish compound 2 [21]. Transient protection of the ribose OH groups as trimethylsilyl ether, followed by N⁴ acetylation using acetyl chloride, subsequent desilylation with methanol and aqueous workup delivered compound 3. Introduction of the 2'-O-TBDMs group was accomplished by treatment with tert-butylidemethysilyl chloride under basic conditions [24–26] resulting in a mixture of 2' and 3' regioisomers that was separated by column chromatography,
providing compound 4a. Alternatively, the 2′-O-TOM group was attached via in situ formation of a 2',3′-O-di-tert-butylstannylidene complex [30]. This cyclic intermediate was then treated with (trisopropylsiloxy)methyl chloride yielding a mixture of 2′ and 3′ regioisomers that was separated by column chromatography, providing compound 4b. Finally, phosphorylation was executed with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite under basic conditions. Starting with cytidine, our route provided nucleosides 5a and 5b in 21% and 25% overall yields in five steps and with four chromatographic purifications; in total, 0.5 g of 5a and 0.4 g of 5b were obtained in the course of this study.

The solid-phase synthesis of RNA with site-specific m^3C modifications (using the novel building blocks 5a and 5b) was performed following standard RNA synthesis protocols (see Supporting Information and references [24–29]). Coupling yields of the novel building blocks were higher than 98% according to the trityl assay. Cleavage of the oligonucleotides from the solid support and their deprotection were performed using aqueous ammonium hydroxide in ethanol followed by treatment with tetra-2-butylammonium fluoride in tetrahydrofuran. Salts were removed by size-exclusion chromatography on a Sephadex G25 column, and RNA sequences were purified by anion-exchange chromatography under denaturing conditions (for a typical example see Fig. 1A, left panel). The molecular weights of the purified oligoribonucleotides were confirmed by liquid-chromatography (LC) electrospray-ionization (ESI) mass spectrometry (MS) (Fig. 1A, right panel). Importantly, when aqueous CH₃NH₂ and NH₃ ('AMA' deprotection) was used for RNA deprotection, we obtained double methylated RNA as major product consistent with the trityl assay. Cleavage of the oligonucleotides from the solid support and their deprotection were performed using aqueous ammonium hydroxide in ethanol followed by treatment with tetra-2-butylammonium fluoride in tetrahydrofuran. Salts were removed by size-exclusion chromatography on a Sephadex G25 column, and RNA sequences were purified by anion-exchange chromatography under denaturing conditions (for a typical example see Fig. 1B, left panel) that was assigned to the transamminated m^3m^4C modified RNA by LC–ESI–MS (Fig. 1B, right panel).

**Conclusion**

The growing evidence for 3-methylated cytidines playing important roles in the life cycle of cellular RNA entails an increasing demand for synthetic m^3C modified oligoribonucleotides. These are needed for diverse applications ranging from simple RNA referencing to method developments aiming at advanced m^3C RNA sequencing approaches. The here presented synthesis of 2′-O-TBDM and 2′-O-TOM protected m^3C phosphoramidites 5a and 5b with N^4-acetyl protection of the nucleobase is practical and high-yielding. The novel building blocks are directly applicable in standard coupling cycles for RNA solid-phase synthesis. Importantly, it has to be taken into account that for RNA deprotection, transamination at the N^4-acylated m^3C nucleobase was observed if methylamine was applied. Therefore, RNA deprotection using ammonia is a requirement to accomplish the chemical synthesis of m^3C modified RNA in high quality.

More generally speaking, we point out that finetuning of protection groups in RNA solid-phase synthesis is needed to push the limits of accessible RNA lengths (> 50–60 nt). Thereby, the critical step is not the assembly of the RNA on the solid support but deprotection of the RNA, and hence, more labile acetyl protection of nucleobase exocyclic amino groups is preferred over benzoyl protection. In particular, an additional advantage for the application of N^4-acetyl-cytidine instead of N^4-benzoylcystidine building blocks is that transamination at C4 is avoided under standard RNA deprotection conditions which involve aqueous methylamine solutions. However, in the case of 3-methylcytidine building blocks, the expectation that N^4-acetyl protection...
also eliminates transamination did not fulfill in our hands; deprotection procedures based on ammonia instead of methylamine are required. Our findings, therefore, help to resolve contrasting reports in the literature upon suitable basic deprotection conditions for synthetic m^3C modified RNA [20, 21].

**Experimental**

Unless stated otherwise, all reactions were carried out under argon atmosphere using absolute solvents. Solvents and other reagents were purchased in highest quality from commercial suppliers (Sigma-Aldrich, Carbosynth, ChemGenes) and were used without further purification. $^1$H and $^{13}$C spectra were recorded on a Bruker DRX 400 MHz spectrometer. Chemical shifts (δ) are reported relative to tetramethylsilane (TMS) and were referenced to the residual signal of the deuterated solvent (CDCl$_3$: 7.26 ppm for $^1$H and 77.16 ppm for $^{13}$C). Signals were assigned according to $^1$H-$^1$H-COSY, $^1$H-$^{13}$C-HSQC, and $^1$H-$^{13}$C-HMBC experiments. Following abbreviations are used to describe observed multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet and br = broad. Diastereomeric protons which appear as distinct signals are marked with index a and b. Identity of synthesized compounds was further confirmed by high-resolution mass spectrometry experiments using a Thermo Scientific Q Exactive Orbitrap with an electrospray ion source. MS data were collected in the positive ion mode. Reaction progress was monitored via thin layer chromatography (TLC, Macherey–Nagel) with fluorescent indicator. Column chromatography was carried out on silica gel 60 (70–230 mesh).

3-Methylcytidine iodide (1, C$_{10}$H$_{16}$N$_2$O$_2$). In analogy to Ref. [21], Cytidine (1.70 g, 7.00 mmol) was suspended in 17.5 cm$^3$ N,N-dimethylformamide and treated with 0.87 cm$^3$ iodomethane (2.0 eq, 13.97 mmol). After 24 h, the solvent was removed under high vacuum and the residue was coevaporated three times with toluene. The solid was used in the next step without further purification. TLC (25% MeOH in CH$_2$Cl$_2$); $R_f$ = 0.60; HR-ESI–MS: $m/z$ calculated for [C$_{10}$H$_{16}$N$_2$O$_2$I]$^+$ ([M]$^+$) 258.1084, found 258.1079; $^1$H NMR (DMSO-d$_6$, 400 MHz): δ = 3.34 (3H, s, CH$_3$(N3)), 3.51 (1H, d, $^3$J$_{HH}$ = 12.74 Hz, CH$_a$(5)), 3.73 (1H, d, $^3$J$_{HH}$ = 10.78 Hz, CH$_b$(5)), 3.80 (2H, m, CH (DMT)), 3.90–3.96 (2H, t, $^3$J$_{HH}$ = 10.86 Hz, CH (1')), 4.18 (1H, m, CH (4')), 4.27 (1H, t, $^3$J$_{HH}$ = 4.18 Hz, CH (2')), 4.37 (1H, t, $^3$J$_{HH}$ = 5.11 Hz, CH (3')), 5.37 (1H, d, $^3$J$_{HH}$ = 8.08 Hz, CH (5)), 5.89 (1H, d, $^3$J$_{HH}$ = 3.80 Hz, CH (1')), 6.84, 7.21–7.31 (11H, m, aromat. CH (DMT)), 7.39–7.41 (3H, m, aromat. CH (DMT)), 7.51–7.61 (13H, m, aromat. CH (DMT), 8.78 ppm (13H, m, aromat. CH (DMT), 11.49 ppm (13H, m, aromat. CH (DMT)).

5’-O-(4,4’-Dimethoxytrityl)-methylcytidine (3, C$_{33}$H$_{35}$N$_3$O$_8$). Compound 2 (1.275 g, 2.28 mmol) was coevaporated trice with pyridine and was subsequently dried under high vacuum overnight. It was then dissolved in 28.3 cm$^3$ pyridine and was treated with 1.16 cm$^3$ chlorotrimethylsilane (4.0 eq, 9.11 mmol) over a period of 5 min. The reaction mixture was allowed to stir for 1 h followed by the dropwise addition of 0.19 cm$^3$ acetyl chloride (1.2 eq. 2.73 mmol) over 10 min. Stirring was continued for 4 h, followed by addition of 2 cm$^3$ methanol. Solvents were removed after an additional hour of stirring, and the residue was taken up in ethyl acetate. Extraction with saturated sodium bicarbonate solution and brine was followed by drying of the organic phase over sodium sulfate. After evaporation, the crude product was purified by column chromatography on...
silica gel (0–4% MeOH in CH₂Cl₂). Yield: 1.125 mg of 3 as a white foam (82%); TLC (5% MeOH in CH₂Cl₂): \( R_f = 0.47 \); HR-ESI-MS: \( m/z \) calculated for \([C_{33}H_{36}N_3O_8]^+ ([M + H]^+) 602.2497, found 602.2489; \( ^1H \) NMR (CDCl₃, 400 MHz): \( \delta = 2.20 \) (3H, s, OAc (N4)), 3.22 (1H, d, \( ^3J_{HH} = 4.94 \) Hz, OH (3')), 3.37 (3H, s, CH₃ (N3)), 3.40 (1H, d, \( ^2J_{HH} = 10.96 \) Hz, \( ^1^3J_{HH} = 3.19 \) Hz, CH (5')), 3.49 (1H, d, \( ^2J_{HH} = 10.98 \) Hz, \( ^3^3J_{HH} = 2.74 \) Hz, CH₅ (5'))). 3.80 (6H, s, 2 × OCH₃ (DMT), 4.26 (1H, m, CH (2')), 4.31 (1H, m, OH (2')), 4.36 (1H, d, \( ^3J_{HH} = 9.69 \) Hz, CH (3')), 5.81 (1H, d, \( ^3J_{HH} = 4.81 \) Hz, CH (3)), 5.94 (1H, d, \( ^3J_{HH} = 8.21 \) Hz, CH (5)), 6.83–6.85, 7.24–7.38 (13H, m, aromat. CH (DMT)), 7.62 (1H, d, \( ^3J_{HH} = 8.21 \) Hz, CH (6)) ppm; \( ^13C \) NMR (CDCl₃, 101 MHz): \( \delta = 27.29 \) OAc (N4), 29.74 CH₃ (N3), 55.37 2 × OCH₃ (DMT), 62.44 C (5'), 70.81 C (3'), 76.31 C (2'), 84.51 C (4'), 87.17 aromat. C (DMT), 91.61 C (1'), 97.65 C (5), 113.40, 127.25, 128.11, 128.22, 130.15, 130.20 aromat. C (DMT), 135.29 C (6), 135.39, 135.47, 144.26 aromat. C (DMT), 151.29 C (2), 152.26 C (4), 158.79, 158.81 aromat. C (DMT), 184.41 C = O (OAc N₃) ppm.

\( \text{N}^4\)-Acetyl-2'-O-tert-butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)-3-methylcytidine (4a, \( C_{48}H_{66}N_5O_9PSi \)) \n
Imidazole (2.0 eq. 180 ml, 123 mg) and 163 mg tert-butyldimethylsilyl chloride (1.2 eq. 1.08 mmol) were added consecutively to a solution of 542 mg compound 3 (0.90 mmol) in 5.4 cm³ N,N-dimethylformamide and stirred for 16 h. Then, solvents were removed, the residue was taken up in ethyl acetate and was washed extensively with brine. The organic layer was dried over sodium sulfate, was concentrated under high vacuum. The crude product was purified by column chromatography on silica gel (10–50% ethyl acetate in cyclohexane). Yield: 291 mg of 4a as a white foam (44%); TLC (1% MeOH in CH₂Cl₂): \( R_f = 0.42 \); HR-ESI-MS: \( m/z \) calculated for \([C_{48}H_{66}N_5O_9PSi]^+ ([M + H]^+) 716.3362, found 716.3353; \( ^1H \) NMR (DMSO-d₆, 400 MHz): \( \delta = 0.96–1.05 \) (21H, m, Si(CH(CH₃)₂)₃), 2.09 (3H, s, Ac (N4)), 2.31 (3H, s, CH₃ (N3)), 3.27 (2H, m, CH₃ (5')), 3.74 (6H, s, 2 × OCH₃ (DMT)), 4.00 (1H, m, CH (4')), 4.15 (1H, quartet, J = 11.02 Hz, CH (3')), 4.72 (1H, t, \( ^3J_{HH} = 5.32 \) Hz, CH (3')), 5.27 (1H, d, \( ^3J_{HH} = 5.20 \) Hz, CH₅ (3')), 4.93 (1H, d, \( ^3J_{HH} = 5.20 \) Hz, CH₅ (TOM)), 5.00 (1H, d, \( ^3J_{HH} = 5.17 \) Hz, CH₃ (TOM)), 5.29 (1H, d, \( ^3J_{HH} = 6.00 \) Hz, OH (3')), 5.91 (1H, d, \( ^3J_{HH} = 8.17 \) Hz, CH (5)), 5.94 (1H, d, \( ^3J_{HH} = 4.88 \) Hz, CH (1')), 6.88–6.90, 7.22–7.38 (13H, m, aromat. CH (DMT)), 7.59 (1H, d, \( ^3J_{HH} = 8.19 \) Hz, CH (6)) ppm; \( ^13C \) NMR (DMSO-d₆, 101 MHz): \( \delta = 11.35 \) Si(CH(CH₃)₂)₃, 17.58 Si(CH(CH₃)₂)₂, 27.01 Ac (N4), 29.29 CH₃ (N3), 55.01 2 × OCH₃ (DMT), 62.90 C (5'), 68.43 C (3'), 77.77 C (2'), 83.39 C (4'), 86.05 aromat. C (DMT), 87.66 C (1'), 88.48 CH₃ (TOM), 96.80 C (5), 113.23, 126.81, 127.75, 129.90, 129.72, 129.76, 135.20, 135.38 aromat. C (DMT), 136.21 C (6), 144.43 aromat. C (DMT), 149.72 C (2), 152.24 C (4), 158.14 aromat. C (DMT), 182.67 C = O (Ac N₃) ppm.

\( \text{N}^4\)-Acetyl-2'-O-tert-butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)-3-methylcytidine 3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (5a, \( C_{48}H_{66}N_5O_9PSi \)) Compound 4a (267 mg, 0.37 mmol) was dried under high vacuum overnight. It was then dissolved in 4.0 cm³ dichloromethane and consecutively treated with 0.26 cm³ N,N-diisopropylphosphorylamine (4.0 eq. 1.49 mmol) and 177 mg 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (2.0 eq. 0.75 mmol). After 20 h, the reaction mixture was diluted with dichloromethane and was washed with 5% sodium bicarbonate solution. The combined organic layers were dried over sodium sulfate and the crude product was purified by column chromatography on silica gel (10–30% ethyl acetate in cyclohexane + 1% Et₃N). Yield: 243 mg of 5a as ...
a white foam (71%); TLC (3% MeOH in CH2Cl2); Rf = 0.40 (both diastereomers); HR-ESI–MS: m/z calculated for [C30H57N5O6PSi]⁺ ([M + H]⁺) 988.5015, found 988.5001; ¹H NMR (CDCl3, 400 MHz): δ = 0.00–1.04, 1.14–1.17 (33H, m, Si(CH2)23), 2.12 (1H, s, Ac (N3)), 2.37 (1H, 2x t, JHH = 6.39, 6.44 Hz, CH2CN), 2.63 (1H, m, CH2CN), 3.34, 3.35 (3H, s, CH3 (N3)), 3.37 (1H, m, CH2 (5)), 3.50–3.71 (4H, m, CH2 (5)), 3.75–3.94 (2H, m, CH2 (5)), 5.86 (0.5H, d, JHH = 8.19 Hz, CH2 (5)), 6.13 (0.5H, d, JHH = 4.29 Hz, CH2 (5)), 6.15 (0.5H, d, JHH = 4.37 Hz, CH2 (5)), 6.62–6.85, 7.25–7.41 (13H, m, aromat. CH (DMT)); 7.59 (0.5H, d, JHH = 8.21 Hz, CH2 (6)), 7.66 (0.5H, d, JHH = 8.21 Hz, CH2 (6)) ppm; ³¹C NMR (CDCl3, 101 MHz): δ = 12.05, 12.07 Si(CH2)23), 17.91, 17.94 Si(CH2)23), 20.28 (d, JCP = 7.27 Hz, CH2CN), 20.51 (d, JCP = 6.54 Hz, CH2CN), 24.61, 24.68, 24.75 N(CH2CH2)2), 27.04, 27.28 Ac (N3), 29.71, 29.74 CH2 (N3), 43.29 (d, JCP = 13.08 Hz, N(CH2CH2)2), 43.46 (d, JCP = 12.35 Hz, N(CH2CH2)2), 55.35, 55.37 2x OCH3 (DMT), 57.96 (d, JCP = 19.61 Hz, POCH2), 58.96 (d, JCP = 16.61 Hz, POCH2), 61.99, 62.39 C (5), 70.32 (d, JCP = 13.81 Hz, C (3')), 77.77 (d, JCP = 4.36 Hz, C (2')), 78.40 (d, JCP = 3.46 Hz, C (2')), 83.14 (d, JCP = 2.91 Hz, C (4')), 83.29 (d, JCP = 2.91 Hz, C (4')), 83.73, 83.77 (d, JCP = 13.81 Hz, C (3')), 84.71, 84.75 (d, JCP = 19.61 Hz, POCH2), 85.16, 85.30 C (1'), 86.82, 87.14 amaromat. C (DMT), 87.20, 88.12 C (1'), 89.18, 89.40 CH2 (TOM), 97.64, 97.69 C (5), 113.37 aromat. CH (DMT), 117.47, 117.79 CN, 127.27, 128.10, 128.36, 128.45, 130.23–130.36 aromat. C (DMT), 135.26–135.74 (m, aromat. C (DMT), 138.21 C = O (Ac N3')) ppm; ³¹P NMR (CDCl3, 162 MHz): δ = 149.70, 149.97 ppm.

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