Sensitive RNA Synthesis Using Fluoride-Cleavable Groups for Linking and Amino Protection

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Abstract: A chemical method suitable for the synthesis of RNAs containing modifications such as *N*4-acetylcytidine (ac4C) that are unstable under the basic and nucleophilic conditions used by standard RNA synthesis methods is described. The method uses the 4-((*t*-butyldimethyl**s**ilyl)**o**xy)-2-**m**ethoxybutanoyl (SoM) group for the protection of *exo*-amino groups of nucleobases and the 4-((*t*-butyldimethyl**s**ilyl)**o**xy)-2-((**a**minophosphaneyl)oxy)butanoyl (SoA) group as the linker for solid phase synthesis. RNA cleavage and amino deprotection are achieved using fluoride under the same conditions used for the removal of the 2′-OH silyl protecting groups. Using the method, a wide range of electrophilic and base-sensitive groups including those that play structural and regulatory roles in biological systems and those that are artificially designed for various purposes are expected to be able to be incorporated into any position of any RNA sequences. As a proof of concept, a 26-mer RNA containing the highly sensitive ac4C epitranscriptomic modification was successfully synthesized and purified with RP HPLC. MALDI MS analysis indicated that the ac4C modification is completely stable under the fluoride deprotection conditions. The sensitive RNA synthesis method is expected to be able to overcome the long lasting obstacle of accessing various modified sensitive RNAs to projects in areas such as epitranscriptomics, molecular biology and the development of nucleic acid therapeutics.

Introduction

Over 100 RNA modifications have been discovered in nature.[1] They play important roles in many biological systems. Some serve as an additional layer of regulation of various biological processes.[2-4] Some are indispensable components of functional RNA structures.[5-6] Errors related to them have been found to be the cause of many human diseases.[7-8] Among the modifications, many are sensitive to basic and nucleophilic conditions. Because standard chemical RNA synthesis methods use acyl groups such as acetyl, isobutyryl and benzoyl groups to protect the *exo*-amino groups of nucleobases, and use the succinyl ester linker to anchor RNA to the solid support, RNA deprotection and cleavage have to be carried out under strongly basic and nucleophilic conditions. As a result, standard RNA synthesis methods cannot be used to synthesize RNAs containing sensitive epitranscriptomic modifications as well as artificially designed sensitive functional groups.[9-11] An example of sensitive RNA modifications is *N*4-acetylcytidine (ac4C).[12-13] It was first discovered in tRNAs[14] and later rRNAs,[15] where it is typically static under normal biological conditions, and is required for the RNAs to adopt their 3D structures for normal function. More recently, it was also discovered in mRNAs[16] and even regulatory RNAs such as lncRNA,[17] where it is typically dynamic, and being dynamic is needed for its intended biological functions. Errors related to ac4C are

related to a wide range of human diseases such as various cancers,[12] cardiovascular diseases,[18] and aging.[19] A chemical method that is suitable for the synthesis of RNAs containing ac4C at any site without any sequence restrictions would open doors for projects aimed to answer a wide range of fundamental biological questions regarding ac4C. In addition, ac4C, as well as the non-natural ac2G and ac6A, can potentially increase nucleic acid duplex stability significantly.[20-22] A site-specific method for the synthesis of ac4C-containing RNAs would enable studies of their applications in antisense, siRNA and gRNA therapeutics. However, the ac4C modification cannot withstand the basic and nucleophilic conditions (e.g. 10 M methylamine in ethanol/water v/v 1:1[23]) used by standard RNA synthesis methods for deprotection and cleavage, and therefore standard methods are not suitable for the synthesis of RNAs containing ac4C.

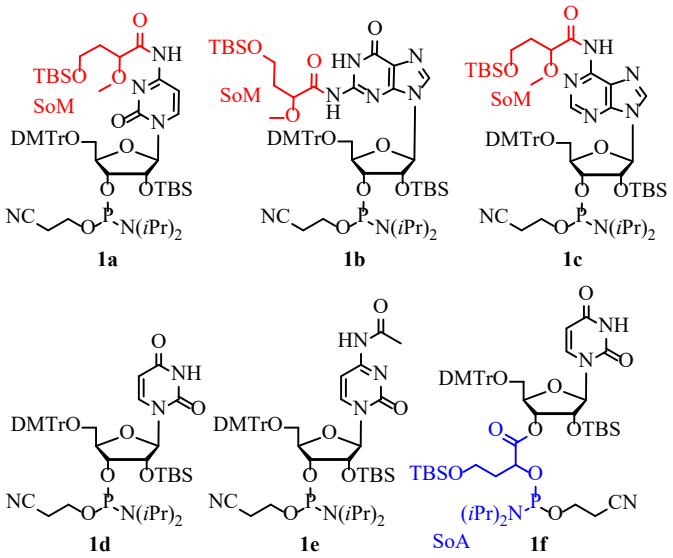
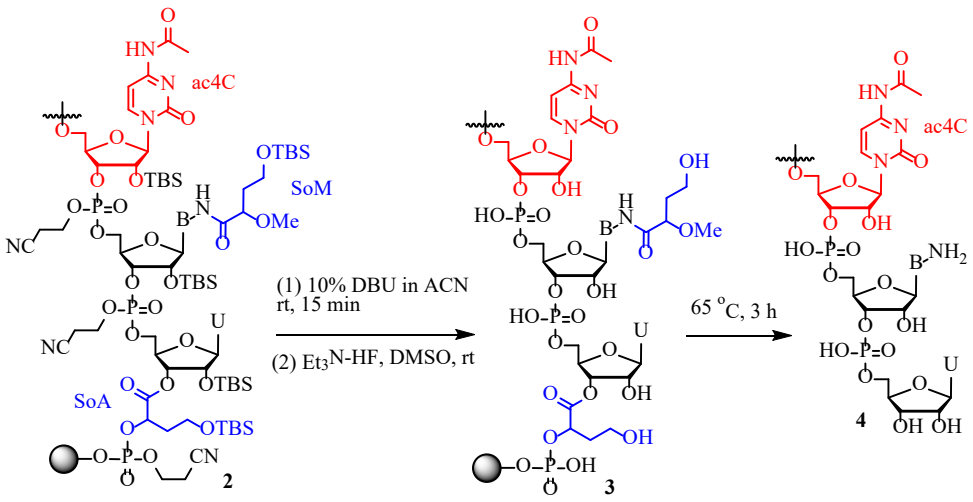


Figure 1. SoB-based phosphoramidites monomers and linker for sensitive RNA synthesis.

Significant efforts have been made to solve the ac4C-RNA synthesis problem but a satisfactory solution has not appeared. Using the *N*-cyanoethyl *O*-carbamate (*N*-ceoc) group, which can be removed under basic but non-nucleophilic conditions, for *exo*-amino protection, Meier and co-workers successfully synthesized ac4C-containing fragments of RNAs from the human 18S rRNA and $tRNA^{Ser}$.[21] Although a highly significant achievement, the method is still not ideal. Due to the use of a G phosphoramidite without amino protection, the method had to avoid the capping step, which may limit the length of RNAs that can be synthesized. In addition, the method uses a photocleavable linker to anchor RNA to solid support. Cleavage of RNA using UV may limit the scope of sensitive groups that can be incorporated into RNA, and the scope of sequences and the length of RNAs that can be synthesized. Other chemical methods that can potentially be used to synthesize sensitive RNAs such as ac4C-RNAs include those involving the use of palladium-deprotectable allyl and benzyl protecting groups,[24] and the use of phosphoramidites without amino protection.[25] However, these methods were only tested for DNA synthesis, and problems such as difficulty to remove palladium and branching may have discouraged researchers to test them for RNA synthesis. The fluoride-deprotectable ((*t*-butyl)(diphenyl)silyloxymethyl)benzoyl (SiOMB) and ((2-((triisopropylsilyl)oxy)benzyl)oxy)carbonyl (TBOC) groups were used for oligonucleotide synthesis[26-29] including two reports related to RNA synthesis,[30-31] but the success was limited. The oligonucleotides synthesized were short probably due to the instability of the protecting groups, and the method was not

tested for the synthesis of epitranscriptomically modified sensitive RNAs including ac4C-RNAs. Some of the above methods as well as a few others were summarized in a recent review.[10] Our research group reported the use of Dim and Dmoc groups, which can be deprotected under non-nucleophilic conditions by oxidation with $NaIO_4$ followed by K_2CO_3 at room temperature, for sensitive oligonucleotide synthesis,[32-37] but at this time, only sensitive DNAs have been synthesized. The study of these groups for RNA synthesis is still in progress. Besides chemical methods, enzymatic methods have been used for the synthesis of ac4C-RNAs as well, but these methods have limitations in efficiency, specificity, and sequence scope.[38]

Here, we report a sensitive RNA synthesis method using the 4-((*t*-butyldimethyl**s**ilyl)**o**xy)**b**utanoyl (SoB)-based groups 4-((*t*-butyldimethyl**s**ilyl)**o**xy)-2-**m**ethoxybutanoyl (SoM) group for *exo*-amino protection and 4-((*t*-butyldimethyl**s**ilyl)**o**xy)-2-((**a**minophosphaneyl)oxy)butanoyl (SoA) group as linker. The traditional 2-cyanoethyl (2-CE), *t*-butyldimethylsilyl (TBS), 4,4′-dimethoxytrityl (DMTr) groups are used for phosphate, 2′-OH and 5′-OH protection, respectively. The phosphoramidite monomers are **1a-c** as well as the commercially available **1d** (Figure 1). The SoA linker is introduced by phosphoramidite **1f**. The RNAs synthesized can be represented by **2** (Scheme 1). Deprotection and cleavage are achieved by removing the CE groups with DBU, followed by treating with Et_3N-HF, which simultaneously removes the SoM amino protecting groups and the TBS 2′-OH protecting groups, and cleaves the SoA linker (Scheme 1). The method can potentially be used for the synthesis of RNAs containing a wide ranges of sensitive group, but this paper is focused on ac4C. As a proof of concept, a 26-mer ac4C-containing RNA was synthesized. MALDI MS analysis indicated that the ac4C modification is completely stable under the deprotection and cleavage conditions.



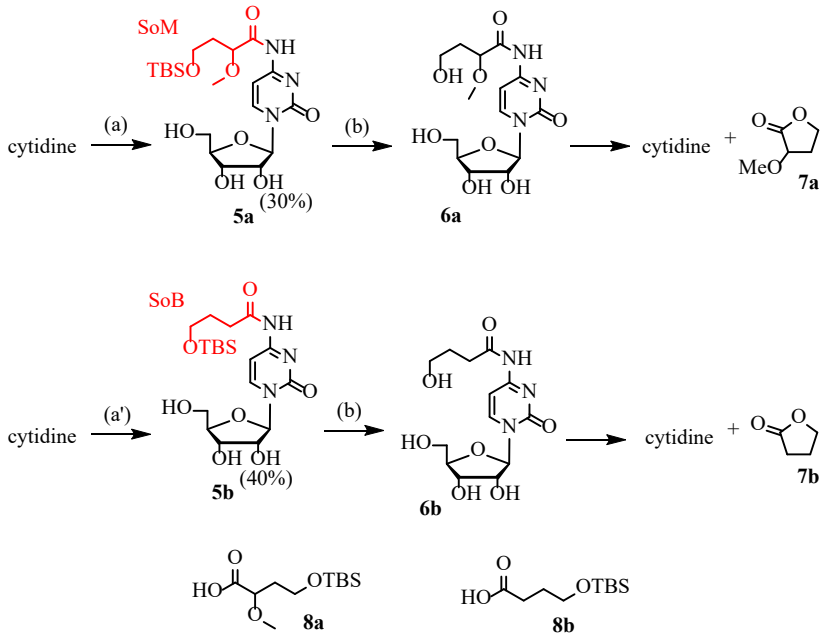
Scheme 1. Deprotection and cleavage of RNAs synthesized using SoM phosphoramidites and SoA linker. Incorporation of the ac4C modification into RNA is used to illustrate the compatability of the conditions with sensitive groups, but many other sensitive modications can also be incorporated using the SoM-based RNA synthesis method.

Results and Discussion

Design of SoB-based groups for sensitive RNA synthesis

The silyl-based protecting groups such as TBS and triisopropylsilyloxymethyl (Tom) are widely used in organic synthesis, mainly for the protection of hydroxyl groups. They are stable under many reaction

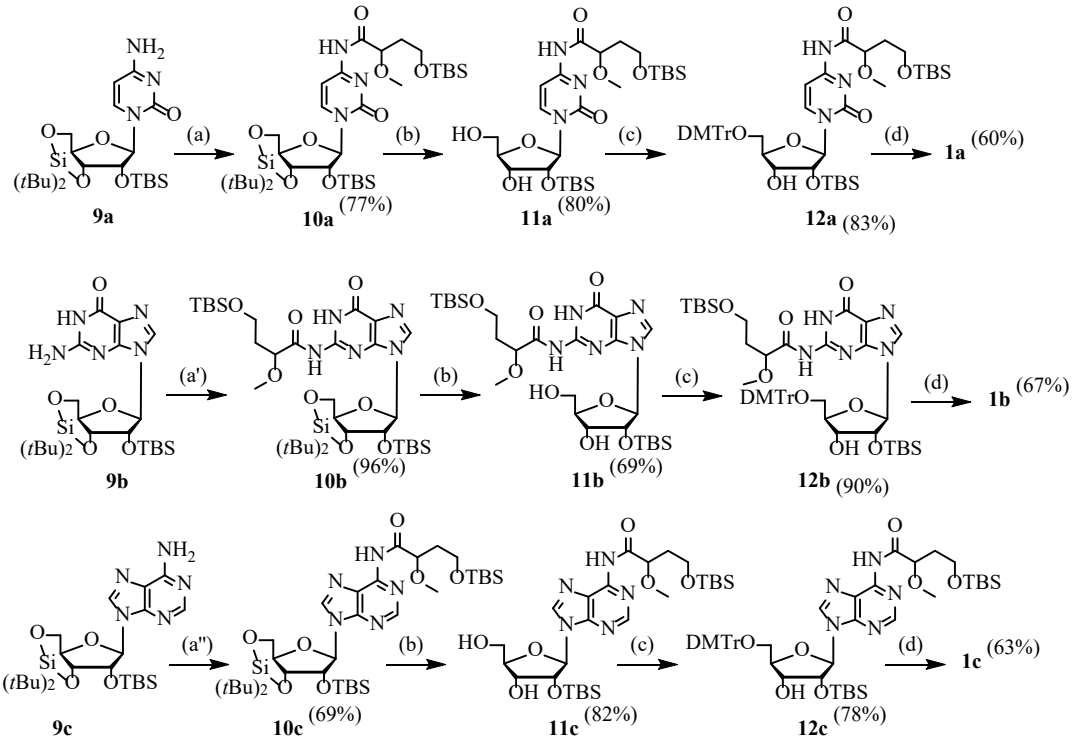
conditions including certain acidic and basic conditions, and can be removed with fluoride, which is compatible with almost all functionalities commonly found in organic compounds including electrophilic groups such as arylamides and esters. For RNA synthesis, because TBS and Tom are the standard groups for the protection of 2′-OH of nucleosides, if the *exo*-amino groups of nucleobases could also be protected with silyl-based groups, the resulting RNA synthesis method would not only be suitable for the synthesis of a wide range of sensitive RNAs but also simplify RNA deprotection, that is, removing both 2′-OH and *exo*-amino protecting groups with fluoride simultaneously. Unfortunately, silyl-based protecting groups are not suitable for the protection of amines due to the low stability of the N-Si bond in the presence of even very mild acids such as water.



Scheme 2: Synthesis of phosphoramidite monomers for sensitive RNA synthesis. Conditions: (a) Cytidine (1.1 eq), TMS-Cl (3.3 eq), pyridine, DCM, 0 °C to rt, 1 h; **8a** (1 eq), DIC (1.2 eq), DCM, rt, 5 min; then, mix the two solutions, rt, 6 h. (a') Cytidine (1 eq), TMS-Cl (3 eq), pyridine, DCM, 0 °C to rt, 1 h; **8b** (1.1 eq), DIC (2.2 eq), DCM, rt, 5 min; then, mix the two solutions, rt, 6 h. (b) Et_3N, Et_3N-3HF, THF; then, $MeOSiMe_3$; see Supporting Information for details.

With the above dilemma in mind, we envisioned that the SoM and SoB groups may be able to address the problem. To that end, we tested if the SoM and SoB protected cytidine, which are compounds **5a** and **5b** (Scheme 2), respectively, could be deprotected under non-nucleophilic conditions using fluoride. The hypothesis was that upon removal of the TBS group with fluoride, the intermediates **6a-b** would undergo intramolecular cyclization to release cytidine under neutral conditions driven by the formation of the 5-membered ring side products **7a-b**. For the model study, compounds **5a-b** were synthesized using **8a-b**, respectively (see Supporting Information for the synthesis of **8a-b**).[39-42] They were then treated with Et_3N-HF in the presence of excess Et_3N at various temperatures and times. TLC analysis of the reactions found that the TBS group in **5a-b** could be removed to give **6a-b** almost instantly even at 0 °C. Complete conversion of **6a** to cytidine could be achieved at 65 °C for 30 minutes, which are the conditions for the deprotection of 2′-OTBS in standard RNA synthesis methods. In contrast, complete conversion of **6b** to cytidine, however, needed harsher conditions. The fact that **6a** is easier than **6b** to deprotect may be due to

the electron-withdrawing effect of the methoxy group in SoM. In addition, the stereo effect of the methoxy group, which may potentially increase the frequency of collision between the hydroxyl oxygen and amide carbonyl carbon in **6a** that leads to the formation of **7a**, may also have contributed to the difference. Details of the studies are provided in Supporting Information (Figure S1). Based on the above observations, we decided to further study the suitability of SoM for sensitive RNA synthesis by synthesizing actual RNAs.



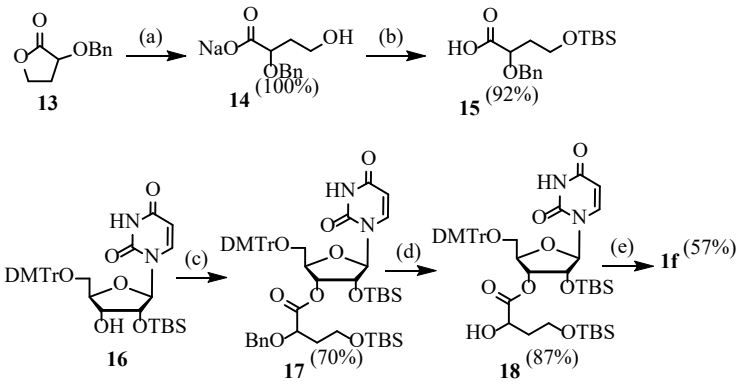
Scheme 3: Synthesis of phosphoramidite monomers for sensitive RNA synthesis. Conditions: (a) **8a** (1.1 eq), DMAP (0.25 eq), *p*TsOH (0.125 eq), DIC (1.4 eq), DCM, rt, 15 min; then, **9a** (1 eq), rt, 16 h. (b) **10a**, **b** or **c** (1 eq), pyridine-3HF (6 eq), DCM, 0 °C, 1 h. (c) **11a**, **b** or **c** (1 eq), DMTr-Cl (1.4 eq), pyridine, rt, 16 h. (d) **12a**, **b** or **c** (1 eq), diisopropylammonium tetrazolide (2.5 eq), 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiaidite (2.5 eq), 1*H*-tetrazole (2.5 eq), rt, 16 h. (a') The same as (a) except that **9b** was the starting material, and rt, 16 h was changed to reflux, 24 h. (a'') The same as (a') except that **9c** was the starting material, and DIC was changed to DCC.

Synthesis of SoM phosphoramidites

The synthesis of SoM phosphoramidite monomers is shown in Scheme 3. Compounds **9a-c** were synthesized using reported procedures.[43-45] Details are provided in the Supporting Information. Coupling of **9a-c** with **8a** using DIC or DCC as the activator gave compounds **10a-c** in good to excellent yields. The reactions were performed under similar conditions except that the amino group of **9a** was more reactive and the reaction could proceed at room temperature, while the amino groups of **9b-c** were less reactive, and higher temperatures were needed for the reactions to proceed with acceptable rates. Converting **10a-c** to **11a-c** were achieved using pyriding-3HF complex in DCM at 0 °C. Good yields could be obtained in all the cases. Under these conditions, the 2′-OTBS group was stable.[43] Installation of the 5′-ODMTr groups to give **12a-c**, and converting **12a-c** to the target SoM phosphoramidites **1a-c** were achieved under the conditions we used frequently in our lab for various projects.[33, 37]

Design and synthesis of SoA linker

In order for the SoM based method to be suitable for the synthesis of sensitive RNAs such as ac4C-RNAs, a linker that can be cleaved under mild non-nucleophilic conditions is required. In the literature, this was usually achieved using UV-cleavable 2-nitrobenzyl linkers.[21, 46] We used oxidatively cleavable Dmoc linker for the purpose, but only DNA synthesis has been demonstrated so far.[32-33] For the SoM based sensitive RNA synthesis method, we designed the SoA linker, the structure of which is illustrated in **1f** (Figure 1). The mechanism for linker cleavage is similar to that for the deprotection of SoM groups. Removal of the TBS group in SoA with fluoride exposes the hydroxy group, which attacks the ester intramolecularly to release the RNA. Formation of a 5-membered lactone provides the driving force for the reaction to proceed under neutral conditions. The phosphate group (pKa of $H_2PO_4^-$, 7.2) as illustrated in **3** (Scheme 1) was expected to provide even stronger electron-withdrawing effect than the methoxy group (pKa of MeOH, 15.5) in SoM for the lactonization reaction to occur, which is predictable based on related pKa values. Compared with UV-cleavable linkers as well as other linkers, the SoA linker has the advantage of cleavage at the same time as the deprotection of 2′-OTBS and *exo*-amino SoM groups. In addition, using SoA linker, there is no concern for RNA damage by UV or oxidation.



Scheme 4: Synthesis of linker phosphoramidite **1f**. Conditions: (a) **13** (1 eq), NaOH (1 eq), THF/H_2O. (b) **14** (1 eq), TBS-Cl (1.5 eq), imidazole (3 eq), DMF, 0 °C to rt, 16 h. (c) **15** (1.1 eq), DMAP (0.25 eq), *p*TsOH (0.125 eq), DIC (1.4 eq), DCM, rt, 15 min; then, **16** (1 eq), rt, 16 h. (d) **17** (1 eq), HCO_2NH_4 (20 eq), Pd/C, EtOH, reflux, 5 h. (e) **18** (1 eq), diisopropylammonium tetrazolide (5 eq), 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiaidite (5 eq), 1*H*-tetrazole 5 eq), rt, 16 h.

The synthesis of the SoA linker phosphoramidite **1f** is shown in Scheme 4. The SoB derivative **15** was easily prepared from compound **13**.[47] Reaction of **15** with **16** using DIC as the activator gave **17**. The benzyl group was then removed under catalytic hydrogenation conditions using ammonium formate as the hydrogen source and palladium on carbon as the catalyst. The DMTr protection was found stable under the conditions. Phosphitylation of **18** under standard conditions gave the target SoA linker phosphoramidite **1f** in acceptable yield.

RNA **19a** (8-mer): 5'-CCC CCC CU-3'
RNA **19b** (26-mer): 5'-CUGCac4CCAGGCUGCUGCCGCUGAUUUU-3'
RNA **19c** (26-mer): 5'-CUGCCCAGGCUGCUGCCGCUGAUUUU-3'

Figure 2. RNA sequences.

RNA synthesis

Although model deprotection studies using compounds **5a**-**b** indicated that the SoM group was suitable for amino protection for sensitive RNA synthesis, the efficiency of deprotection of SoM groups on actual RNA molecules was still uncertain. Therefore, once we synthesized the phosphoramidite **1a** and the linker **1f**, we decided to synthesize the 8-mer RNA **19a** (Figure 2) before synthesizing phosphoramidites **1b-c**. If SoM were found not ideal, alternative groups would have to be investigated. Because the fully protected RNA after solid phase synthesis was in the form of **2** and linker cleavage was to be carried out using fluoride (Scheme 1), the solid support had to be exposed to fluoride during cleavage. To avoid potential problems caused by the reactions between fluoride and controlled pore glass (CPG), the commercially available highly cross-linked polystyrene resin based solid support 1-dimethoxytrityloxy-2-*O*-dichloroacetyl-propyl-3-*N*-ureayl-polystyrene was used as the solid support. In principle, any support that is inert toward fluoride as well as other ingredients in the deprotection reagent with a hydroxy group or DMTr-protected hydroxy group can be used as the support.

The synthesis of RNA **19a** was carried out on a MerMade synthesizer at 1 μmol scale. The first nucleoside U at the 3′-end was introduced using phosphoramidite **1f**, which carries the SoA linkage. The synthesis was conducted under typical RNA synthesis conditions except that the coupling time for the first nucleoside was slightly longer. Detailed conditions are provided in the Supporting Information. At the end of the synthesis, the 5′-ODMTr group was kept. The fully protected RNA can be represented with **2** except that there was no ac4C in the sequence (Scheme 1). Deprotection and cleavage were achieved in two steps. In the first step, the 2-CE groups were removed with 10% DBU in ACN at room temperature. At this time, the RNA was still on the solid support, which made removal of deprotection side products and excess reagents simple. The support was washed with ACN. In the second step, the support was suspended in dry DMSO and heated at 65 °C briefly to ensure that the RNA molecules on the support were in full contact with the solvent. The TBS group was then removed with fluoride. To retain the 5′-ODMTr group for the purpose of RNA purification, excess triethylamine was added first, and then followed by Et_3N-3HF. Under these conditions, the silyl groups including those on the SoM and SoA groups were probably all removed, and a portion of the 2′-OTBS groups might have been deprotected as well. Assuming that all the silyl groups were removed, the RNA at this stage can be represented by **3** (except no ac4C). The mixture was then heated to 65 °C for 30 minutes, which are the typical conditions for the complete deprotection of 2′-OTBS groups in standard RNA synthesis methods. At this temperature, the remaining silyl groups, if any left, were removed, and the TBS-free SoM and SoA groups as represented in **3** were cyclized giving fully deprotected RNA **4** (no ac4C). RNA **19a** was purified using Glen-Pak^{TM} under conditions used by standard RNA synthesis methods. Details can be found elsewhere.[48] RP HPLC and MALDI MS analyses (see Supporting Information) indicated that the RNA could be cleaved from the support and the SoM groups could be completely removed under the non-nucleophilic conditions.

After confirming that the SoM protecting group and SoA linker can function as planned for RNA synthesis, we completed the synthesis of the remaining required phosphoramidites **1b**-**c**, and went ahead to synthesize ac4C-containing RNAs. The 26-mer RNA **19b** (Figure 2) was chosen as the target. This RNA was designed for the study of the effects of site-specific ac4C modification on mRNA translational

efficiency, results of which will be published when they become available. The RNA was synthesized under the same conditions as described for **19a**. The ac4C modification was introduced using standard commercially available phosphoramidite **1e** (Figure 1). Details are provided in the Supporting Information. The fully protected RNA can be represented with **2** (Scheme 1). Deprotection and cleavage were also carried out under the same conditions for **19a**. However, purification was conducted differently due to the difficulty of removal of unknown impurities using the Glen-Pak^{TM} method. The solution of 5′-DMTr-on otherwise fully deprotected crude RNA resulted from Et_3N-3HF treatment was quenched with excess Me_3SiOMe. After removing volatiles, the RNA was purified with trityl-on RP HPLC. The Me_3SiOMe quenching is important for preventing potential damage to HPLC column by fluoride. The crude trityl-on HPLC profile is given in Figure 3. As can be seen, the full-length sequence, which has a retention time of 35.7 minutes, is separated well from failure sequences at 12-25 minutes and other impurities at 39-55 minutes. The full-length sequence was collected, and detritylated with 80% acetic acid. After removing volatiles, the purified RNA was analyzed with RP HPLC. A single peak with a retention time of 17.3 minutes was given indicating that pure RNA could be obtained (Figure 3).

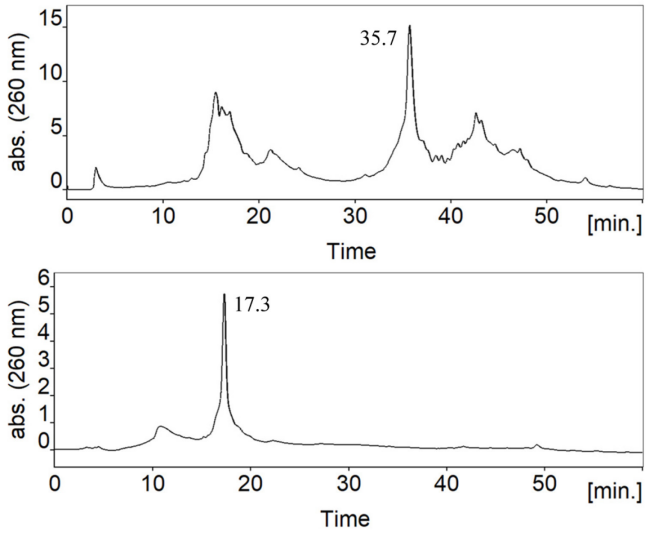


Figure 3. RP HPLC profiles of RNA **19b**. Top: Crude **19b**. The peak at 35.7 min is the full-length trityl-on RNA. Bottom: Purified **19b**. The peak at 17.3 min is the full-length trityl-off RNA.

The RNA was analyzed with MALDI MS. As shown in Figure 4, the molecular peak at *m/z* 8246 indicates that the acetyl group of the ac4C modification in RNA **19b** survived all the RNA deprotection and cleavage conditions, which include treating with 10% DBU in ACN at room temperature and Et_3N-3HF in DMSO at 65 °C (Scheme 1). To further confirm that RNA **19b** contains ac4C, the same batch of solid support carrying the un-deprotected RNA **19b** was subjected to the same deprotection, cleavage and purification conditions except that the DBU treatment was omitted and nucleobase deprotection was caried out under nucleophilic conditions using AMA (saturated NH_4OH and $MeNH_2$, v/v 1:1) at 65 °C. Under these conditions, the acetyl group of ac4C as well as the SoM groups and the SoA linker were all cleaved. After removal of 2′-OTBS groups under standard RNA deprotecting conditions, RNA **19c** (Figure 2) was given. MALDI MS analysis gave the molecular peak at *m/z* 8202, which corresponds to **19c** (Figure 4). A MALDI MS sample containing both **19b** and **19c** was then analyzed. As expected, two peaks were observed with *m/z* 8247 for **19b** and *m/z* 8206 for **19c** (Figure 4). The 41 mass difference between the two peaks

matched closely with the mass difference between ac4C and C. This further confirmed that the SoM method is suitable for the synthesis of ac4C-RNAs.

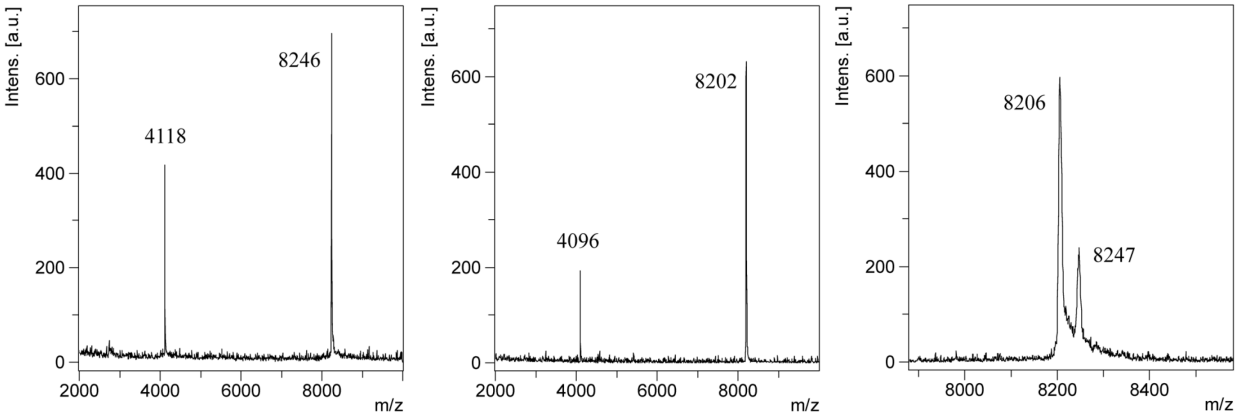


Figure 4. MALDI MS of RNAs. Left: RNA **19b**, calcd for $[M-H]^-$ *m/z* 8248, found 8246. Middle: RNA **19c**, calcd for $[M-H]^-$ *m/z* 8206, found 8202. Right: RNAs **19b** and **19c** mixed intentionally for analysis, found for **19b** $[M-H]^-$ *m/z* 8247, found for **19c** $[M-H]^-$ *m/z* 8206; the difference is 41, which matches the theoretical mass difference of 42 of an acetyl group between **19b** and **19c**.

Conclusion

In summary, a method suitable for the synthesis of the highly sensitive ac4C-containing RNAs has been developed. The method uses SoM for the protection of *exo*-amino groups of nucleobases, and SoA for linking RNA to solid support. Deprotection and cleavage can be achieved under non-nucleophilic conditions using fluoride. Because fluoride is compatible with a wide range of functional groups in organic molecules, the method is predicted to be suitable for the synthesis of RNAs containing many other sensitive groups as well. Many sensitive RNAs exist in nature. Malfunction of them is related to many human diseases. A method for chemical synthesis of these RNAs is expected to open doors to many projects aimed to answer a wide range of fundamental biological questions related to sensitive RNA modifications.

Supporting information

Experimental details, HPLC and MALDI MS images of RNAs, and NMR images of new compounds.

Acknowledgements

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