Synthesis of RNA containing 5-hydroxymethyl-, 5-formyl-, and 5-carboxycytidine

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Abstract: *5-Hydroxymethyl-, 5-formyl-, and 5-carboxycytidine are new epigenetic bases (hmdC, fdC, cadC) that were recently discovered in the DNA of higher eukaryotes. The same bases (hmC, fC and caC) have now also been detected in mammalian RNA with a high abundance in mRNA. While for the DNA bases phosphoramidites (PAs) are available that allow the synthesis of xdC-containing oligonucleotides for deeper biological studies, the corresponding silyl protected RNA PAs for fC and caC have not yet been disclosed. Here we report novel RNA PAs for hmC, fC and caC that can be used in routine RNA synthesis. The new building blocks are compatible with the canonical PAs and also with themselves, which enables even the synthesis of RNA strands containing all three of these bases. The study will pave the way for detailed physical, biochemical and biological studies to unravel the function of these non-canonical modifications in RNA.*

Next to the four canonical nucleosides (A, C, G and U), RNA contains over 140 diverse non-canonical modifications.[1] These bases are often simple derivatives such as methylated versions of the canonical nucleosides.[2] Alternatively they may comprise more complex structures, as is the case with the hypermodified RNA bases.[3] The function of these bases is in many cases still unknown. Some of the modified nucleosides are essential for stabilizing the 3-dimentional structure of RNA.[4] Other nucleosides, particularly in tRNA, are needed to fine tune the translational process in order to avoid frameshifting. Furthermore, the presence of modified bases close to the anticodon stem loop of tRNAs influences the codon usage.[5] Modified bases are also found in DNA[1b] where mdC, hmdC (**d1**), fdC (**d2**) and cadC (**d3**) have attracted particular interest as bases that are dynamically generated and removed to regulate the transcriptional activity of genes (Fig. 1). These oxidized mdC derivatives are essential elements for the process of active DNA demethylation, which allows cells to remove transcription silencing mdCs from promoters.[6] In the past few years, mC,[2a] hmC, fC[7] as well as caC[8] have also been discovered in mRNA of mammalian cells. It is hypothesized that a dynamic regulation of these bases via writer, reader and eraser proteins, could also be occurring in mRNA which could in turn have implications for transcription and translation of genetic information at the RNA level. In fact, it was recently proven that ten-eleven translocation (TET) enzymes, which are known to oxidize the DNA base mdC, can also perform the oxidation of the RNA base mC to hmC, fC[7b] and even to caC.[9] This may establish a link between DNA and RNA epigenetics,[7b, 9] and perhaps implicate these modified RNA bases in the regulation of protein biosynthesis.[10] Overall, their functions are to a large extent unknown.

In order to elucidate the structural and functional consequences of hm(d)C **(d)1**, f(d)C, **(d)2** and ca(d)C, **(d)3** in RNA and DNA (Fig. 1) it is essential to develop PA building blocks and solid-phase procedures for the chemical synthesis of oligonucleotides with one or multiple incorporations of these bases at defined sites. Towards this goal, He,[11] Shao,[12] Brown[13] and our group[14] developed the corresponding DNA building blocks for hmdC, fdC and cadC (**d1**-**d3**). For RNA, Micura and co-workers reported an hmC (**1**) PA.[15] The Deiters group reported an fC (**2)** building block utilizing 5´-*O*-silyl-2´-*O*-acetal protected RNA synthesis[16] originally developed by Scaringe and Caruthers,[17] to investigate the properties of the modification in hmtRNA. These chemical achievements have allowed for a deeper investigation of the properties of hmC and fC in RNA.[18] PA building blocks for fC and caC that allow standard streamlined RNA synthesis are, however, not available and in addition it is so far not possible to orthogonally incorporate all three epigenetic bases in a single RNA strand such as ON1 (Fig. 1B). The synthesis of such oligonucleotides allows us to investigate highly modified macromolecules such as tRNA which contains multiple modifications in one strand.

Herein we report a new set of PAs for hmC (**1**), fC (**2**) and caC (**3**) that allow for the facile and robust incorporation of these bases in synthetic RNA using standard solid-phase synthesis and deprotection protocols. A fine-tuned global protecting group strategy enables the synthesis of RNA with one as well as multiple hmC, fC or caC incorporations. We envision that these new synthetic methods will enable the investigation of the unknown physical properties of RNA containing these bases as well as their implications in biological systems.

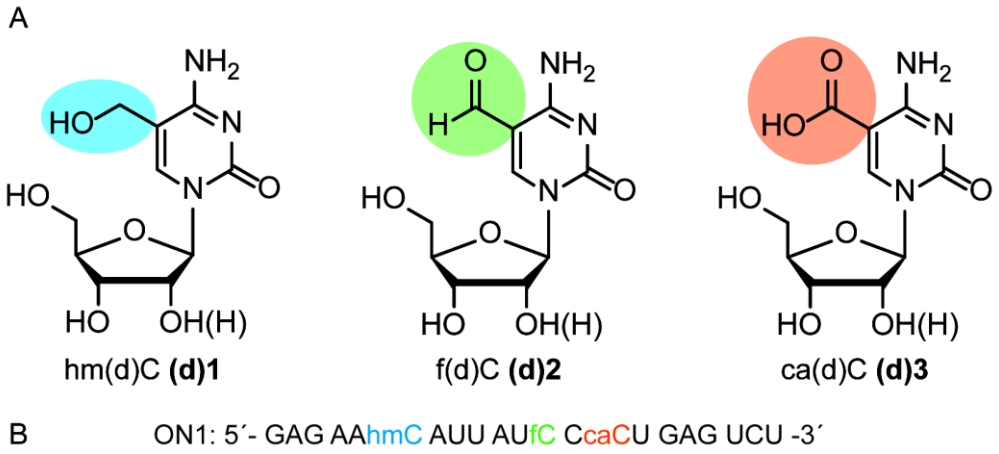


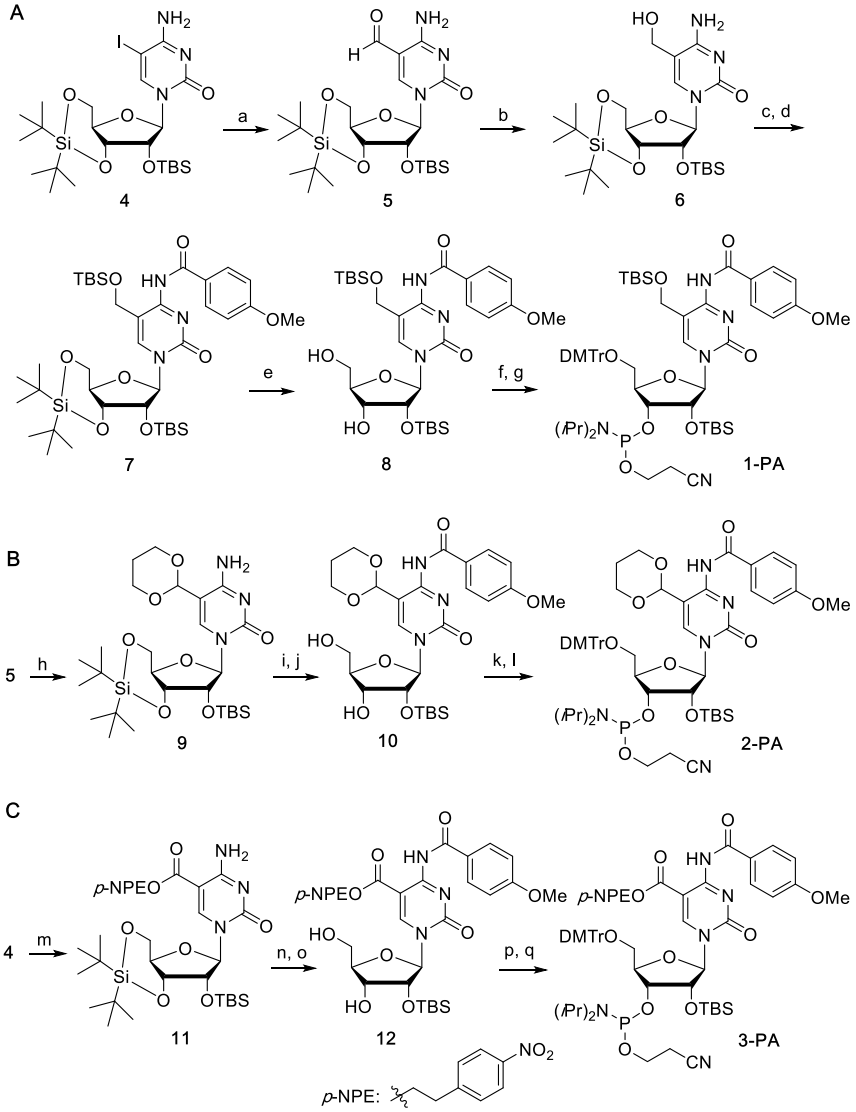
Figure 1. A) Depiction of the three modified RNA nucleosides hmC (**1**), fC (**2**) and caC (**3**) together with the corresponding DNA ones **d1**, **d2**, and **d3**. B) Sequence of an oligonucleotide (ON1) containing all three modifications.

Due to the sensitivity of RNA compared to DNA, a modified protecting group strategy to the one applied in our analogous DNA PA building blocks needed to be considered.[14] A typical deprotection protocol for the solid-phase RNA material involves its treatment with: (a) base (e.g. ethanolic ammonia) to cleave the strand from its solid support, deprotect the cyanoethyl groups on the phosphodiester linkage and remove the nucleobase protecting groups; and (b) a fluoride source (e.g. triethylamine trihydrofluoride ($NEt_3 \cdot 3HF$)), to remove the silyl protecting groups on the 2´-OH ribose position of the RNA. In our design, we attempted to incorporate protecting groups on our epigenetic bases that may be removed using these conditions, thus circumventing the need for a 3rd deprotection step. For all three bases, we chose to use the TBS group for the protection of the 2´-OH position of the ribose and the *p*-methoxybenzoyl group for the N^4 exocyclic amine.[14,19] In our development of the now commercially available fdC PA, this protecting group was vital in maintaining the integrity of the protected building block whilst at the same time being easily removed during the ammonolysis step. Alternative, more labile groups (acetyl, benzoyl) led to the undesired branching of the fdC-oligonucleotides during the solid-phase synthesis of DNA.

For the hmC building block **1-PA**, we chose to mask the 5-CH_2OH position with a silyl protecting group.[11] The benefit here, is that it protects the 5-CH_2 position from nucleophilic substitution during the basic deprotection step,[15a, 20] whilst at the same time circumventing the need for an additional one. Its removal can occur in parallel to that of the TBS on the ribose. The synthesis of the corresponding hmC building block is depicted in Scheme 1A. Starting from the fully protected 5-iodocytidine **4**, a Pd-catalyzed reductive carbonylation[20b] was used to afford the formylated nucleoside **5**. Treatment of **5** with $NaBH_4$ and sequential protection of the hydroxyl and amino functionalities with the aforementioned protecting groups, furnished compound **7** in an overall yield of 74% over 3 steps. Subsequently, using a short reaction time and a low temperature, we were able to deprotect the DTBS group in the presence of the primary and secondary TBS ethers to afford **8** in a high 81% yield.[21] Attempts to react the 5´-OH of the ribose sugar using DMTrCl were unsuccessful and resulted in the recovery of **8**. Instead, a solution of freshly synthesized DMTrOTf was required to realize this transformation in a fast and selective manner, affording the tritylated compound in 94% yield.[22] A final conventional phosphitylation yielded the PA building block **1-PA** in 84%.

For the fC building block **2-PA**, we chose to protect the aldehyde functionality using an acetal group similar to our previous reports on the analogous DNA PA.[14,19] This way, we aimed to avoid any potential liabilities due to the aldehyde's electrophilicity during the handling, synthesis and deprotection of the monomer and/or its corresponding oligonucleotide (e.g. concerns over the formation and reactivity of the corresponding imine during the deprotection). To achieve the synthesis of **2-PA**, we started by treating **5** with propanediol in the presence of $TiCl_4$ to afford acetal **9** in 96% yield. Similarly to the synthesis of **1-PA**, a sequential protection of the amino group followed by a selective DTBS deprotection, 5´-OH tritylation and 3´-OH phosphitylation successfully generated **2-PA** in 68% yield over 4-steps (Scheme 1B).

When considering the design of the caC building block **3-PA**, (Scheme 1C) we wanted to avoid the use of alkyl ester groups which were previously used successfully in corresponding DNA PAs.[23] These require hydrolysis with NaOH (aq) following the synthesis of the strand in order to avoid the formation of the 5-carboxamide side-product during the ammonolysis. We deemed these harsh conditions incompatible with RNA synthesis, as they could lead to the removal of the silyl protecting groups on the 2´-OH position and subsequently cleave the sensitive RNA. Instead, we chose to protect the carboxylic acid moiety as a *p*-nitrophenylethylester. This allows for the mild deprotection of the solid-phase material using DBU in THF at room temperature, prior to the ammonolysis, thus avoiding the formation of the 5-carboxamide. For its incorporation, we subjected the silyl protected iodocytidine **4** to a Pd-catalyzed esterification at high temperature, using molten 4-nitrophenethyl alcohol as the solvent to afford **11** in an acceptable 52% yield. Subsequently, **3-PA** was synthesized using the standard conditions optimized for the synthesis of **1-PA** and **2-PA**.



Scheme 1. Synthesis of the PA building blocks of hmC, fC and caC. Reagents and conditions: a) $Pd_2(dba)_3{\cdot}CHCl_3$, PPh_3, Bu_3SnH, CO, 60 °C, 17 h, 69%; b) $NaBH_4$, $CeCl_3$, MeOH, 0°C, 10 min, 88%; c) TBSCl, imidazole, DMF, 60 °C, 2 h, 95%; d) *p*-MeOBzCl, pyridine, r.t., 2 h, 89%; e) HF·pyridine, CH_2Cl_2, pyridine, 4 °C, 50 min, 81%; f) DMTrOTf, CH_2Cl_2, pyridine, r.t., 2.5 h, 94%; g) iPr$_2$NP(Cl)C$_2$H$_4$CN, iPr$_2$NEt, CH_2Cl_2, r.t., 2 h, 84% h) 1,3-propanediol, triethyl orthoformate, $TiCl_4$, CH_2Cl_2, r.t., 3 h, 96%; i) *p*-MeOBzCl, pyridine, 100 °C, 2 h, 97%; j) HF·pyridine, CH_2Cl_2, pyridine, 4 °C, 30 min, 97%; k) DMTrOTf, CH_2Cl_2, pyridine, r.t., 2 h, 97%; l) iPr$_2$NP(Cl)C$_2$H$_4$CN, iPr$_2$NEt, CH_2Cl_2, r.t., 3 h, 75%; m) $Pd(CH_3CN)Cl_2$, iPr$_2$Et, *p*-nitrophenethyl alcohol, CO, 80 °C, 16 h, 52%; n) *p*-MeOBzCl, pyridine, 90 °C, 4 h, 81%; o) HF·pyridine, CH_2Cl_2, pyridine, 4 °C, 30 min, 89%; p) DMTrOTf, CH_2Cl_2, pyridine, r.t., 21 h, 95%; q) iPr$_2$NP(Cl)C$_2$H$_4$CN, iPr$_2$NEt, CH_2Cl_2, r.t., 2 h, 75%.

To test the compatibility of the PAs with solid-phase synthesis, we sought to incorporate each modification individually in both 13mer and 21mer oligonucleotides using standard RNA synthesis and deprotection protocols. In order to ensure a high yielding synthesis, an extended 20 min coupling step was used for the special PAs. 28% NH_4OH in ethanol (75:25, v/v, 55 °C, 18 h) was used to cleave the oligonucleotides from the solid support and to deprotect the cyanoethyl groups of the phosphodiester linkage and N^4 amino protecting groups. For the removal of the TBS groups we used $NEt_3{\cdot}3HF$ solution in DMSO (55:45, v/v, 65 °C, 1.5-3 h). In some cases, an additional step was necessary in order to fully deprotect the special PAs, which is explained in detail in the following section. Table 1 lists the sequences of the synthesized oligonucleotides and the corresponding calculated and measured MALDI-TOF-MS values of the purified strands.

Table 1. List of synthesized ONs and the corresponding MS values.

	Sequence (5'-3')	Exact MS $[M-H]^-$	Found MS $[M-H]^-$
ON1	UCUGAGU**caC**C**fC**UAUUA**hmC**AAGAG	6754.9	6750.0
ON2	CCUAC**hmC**GCAUUAC	4047.6	4047.3
ON3	CCUAC**fC**GCAUUAC	4045.6	4048.3
ON4	CCUAC**caC**GCAUUAC	4061.6	4063.5
ON5	UCUGAGUCCCUAUUA**hmC**AAGAG	6682.9	6678.5
ON6	UCUGAGUCCCUAUUA**fC**AAGAG	6680.9	6676.5
ON7	UCUGAGUCCCUAUUA**caC**AAGAG	6696.9	6690.8

As per our design, we were pleased to note that a complete deprotection of the TBS protected hmC-containing ON2 and ON5 was possible using our aforementioned standard protocol. Removal of both the primary and secondary TBS groups was possible using $NEt_3 \cdot 3HF$, thus successfully avoiding an additional deprotection step.

Fig. 2A shows the crude HPL-chromatogram of ON2 before reverse-phase purification (for ON5, see SI: Fig. S2). The relatively clean trace allows for the facile isolation of the major peak which corresponds to the full length hmC-containing RNA strand as determined by MALDI-TOF-MS.

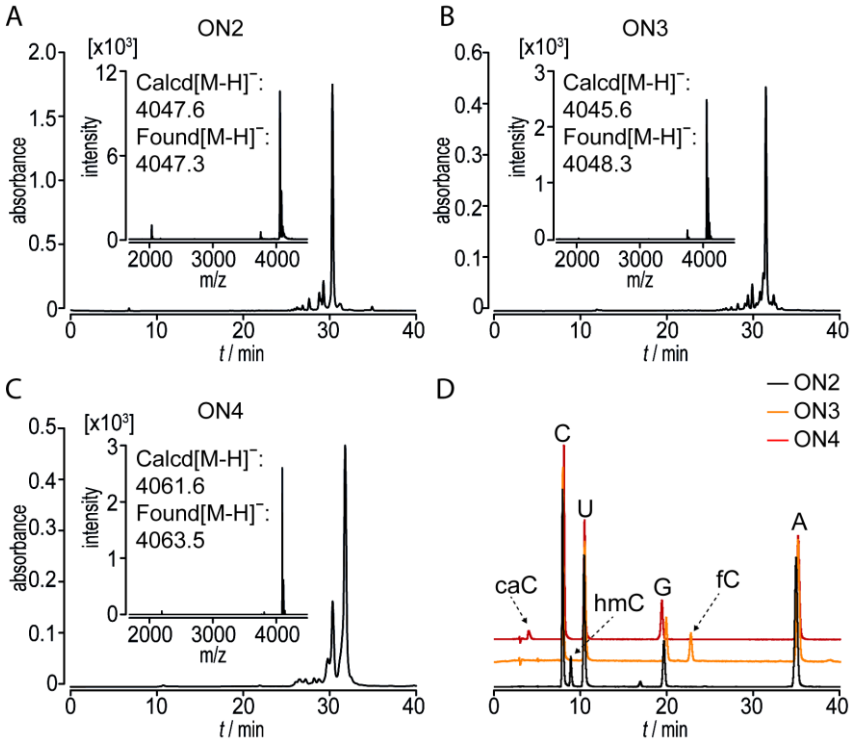


Figure 2. Synthesis of ON2-4 **A-C)** Crude HPL-chromatogram of ON2-4 and MALDI-TOF-MS data of the purified ON2-4 **D**) UPL-chromatogram of ON2-4 after the total digestion showing the presence of hmC, fC and caC in the corresponding ON without any byproduct.

For our fC-containing strand ON3, we were pleased to note the fortuitous deprotection of the acetal, by extending the reaction time of the $NEt_3 \cdot 3HF$ treatment to 3-4 h (Fig. 2B).[24] Although very practical for short sequences, longer oligonucleotides (e.g. ON6) required a further adjustment to the acetal deprotection. In these cases, we exposed the RNA strands to a third step using a citric acid-sodium citrate buffer (r.t., 24-48 h) at pH 4. This ensured the complete removal of the acetal group whilst maintaining the integrity of the RNA strand. Subsequent reverse-phase HPLC allowed for the isolation of the analytically pure oligonucleotide (for ON6, see SI: Fig. S2).

A more elaborate deprotection protocol was required for the caC-containing RNA strands (ON4 and ON7). We first treated the solid support with DBU in acetonitrile (10%, r.t., 1-3 min) to cleave the cyanoethyl group at the phosphodiester linkage. This step was performed to avoid the undesired conjugate addition of acrylonitrile onto the base moiety during the basic deprotection.[25] We then replaced the solution with DBU in THF (10%, r.t., 2 h) thus achieving the deprotection of the carboxyl group without the formation of the undesired 5-carboxamide. Subsequently, the strands were subjected to our standard conditions for the removal of the remaining groups. The RNA synthesis combined with the extended deprotection protocol, provided a relatively clean crude product prior to the final purification of the strands (Fig. 2C; for ON7, see SI: Fig. S2).

To prove the integrity of the special bases hmC, fC and caC following the synthesis and the optimized deprotection conditions, all ONs 2-4 were totally digested and the digests were analyzed by UHPLC-MS (Fig. 2D; SI: Fig. S6). In all cases the HPL-chromatograms were clean, containing solely 5 peaks corresponding to the 4 canonical bases plus one special base in each. The absence of additional signals excludes the possibility of degradation of the strands and confirms the complete removal of the protecting groups.

We finally wanted to investigate if the PAs and the chemistry are robust enough to prepare the first RNA strands containing all three special xC-bases (ON1). The synthesis was performed using the standard protocol with a 20 min coupling time for all hmC, fC and caC PAs.

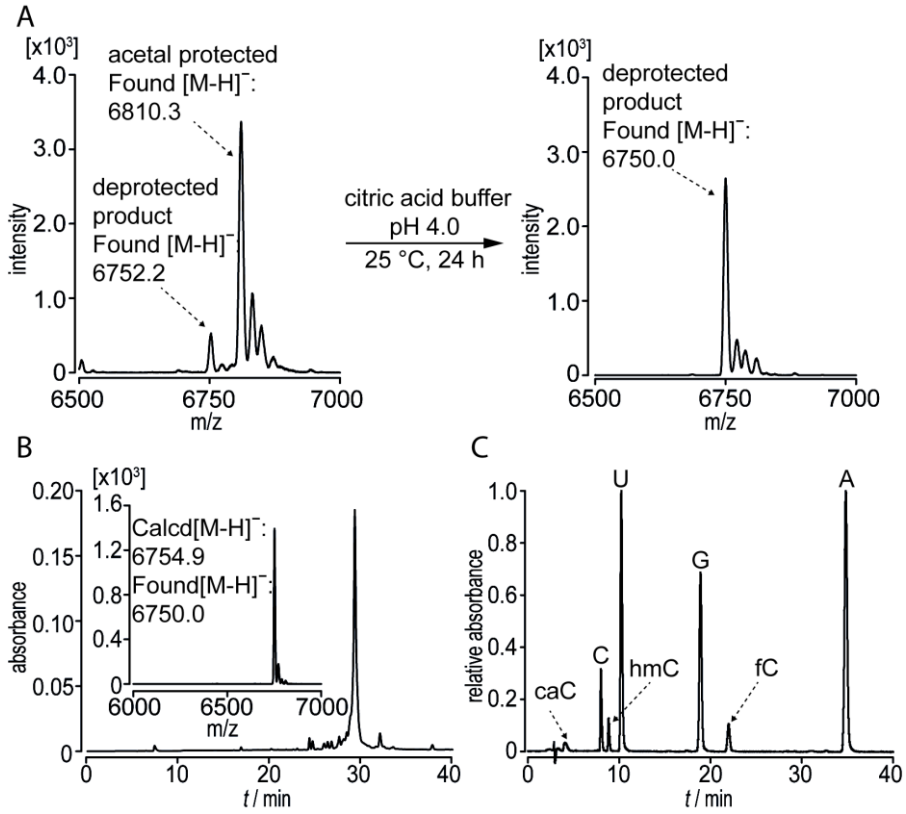


Figure 3. Synthesis of ON1. A) MALDI-TOF spectrum before and after the final citric acid cleavage step. B) Crude HPL-chromatogram of ON1 after full deprotection and the MALDI-TOF-MS data after purification. C) Total digestion data, showing the presence of hmC, fC and caC in ON1.

Following the synthesis, the solid support was treated with DBU in CH_3CN, then DBU in THF followed by 28% NH_4OH in ethanol. These steps sequentially removed the cyanoethyl groups, deprotected the carboxyl group of caC, and simultaneously cleaved ON1 from the solid support whilst removing all the amino protecting groups. The TBS groups on the 2´-OH position of the ribose and on hmC were subsequently cleaved using $NEt_3 \cdot 3HF$ in DMSO. A final treatment of the oligonucleotide with citric acid-sodium citrate buffer at pH 4 (r.t., 24 h) removed the acetal protecting group on fC providing the deprotected strand in a relatively clean manner according to the MALDI-TOF and HPLC analysis (Fig. 3A and 3B).[26] Finally, a total digest of this heavily functionalized RNA strand demonstrated that using our optimized deprotection protocol, all nucleosides, canonical and modified, were successfully incorporated and fully deprotected without the formation of by-products (Fig. 3C).
In summary, herein we report the development of three new PAs which allow for the facile, robust and orthogonal incorporation of the epigenetically relevant bases hmC, fC, caC into specific positions of synthetic RNA. Future investigations will focus on detailed biochemical and biophysical studies to identify the implications of these modifications to the properties and function of RNA. One can envision the design of nature mimicking oligonucleotide “baits” for the pull-down of RNA binding proteins from relevant cell-lines.[6f] Combined, these methods will pave the way to unraveling the regulatory implications the dynamic formation of these bases may have in gene expression at the RNA level.

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Keywords: RNA epigenetics, mRNA modifications, oligonucleotides, phosphoramidites, cytidine modifications

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[26] A negligible ≈2% acetal impurity was observed by MALDI-TOF-MS after 24 h which can be fully removed by extending the treatment to 48 h if deemed necessary (see SI: Fig. S5).