



Cite this: *Org. Biomol. Chem.*, 2024, **22**, 7271

Chemistry of installing epitranscriptomic 5-modified cytidines in RNA oligomers

Anna Kuszczynska,  † Milena Bors,  Karolina Podskoczyj  and Grazyna Leszczynska  *

Studies of 5-hydroxymethylcytidine (hm⁵C), 5-formylcytidine (f⁵C) and 5-carboxycytidine (ca⁵C) modifications as products of the 5-methylcytidine (m⁵C) oxidative demethylation pathway in cellular mRNAs constitute an important element of the new epitranscriptomic field of research. The dynamic process of m⁵C conversion and final turnover to the parent cytidine is considered a post-transcriptional layer of gene-expression regulation. However, the regulatory mechanism associated with epitranscriptomic cytidine modifications remains largely unknown. Therefore, oligonucleotides containing m⁵C oxidation products are of great value for the next generation of biochemical, biophysical, and structural studies on their function, metabolism, and contribution to human diseases. Herein, we summarize the synthetic strategies developed for the incorporation of hm⁵C, f⁵C and ca⁵C into RNA oligomers by phosphoramidite chemistry, including post-synthetic C5-cytidine functionalization and enzymatic methods.

Received 2nd July 2024,
Accepted 15th August 2024
DOI: 10.1039/d4ob01098a
rsc.li/obc

1. Introduction

In addition to the four canonical nucleosides (A, C, G and U), cellular RNA molecules contain over 160 structurally distinct modified nucleosides.¹ Each modification yields specific properties, which can have a crucial impact on the RNA structure,

folding, stability, cellular localization and biological function.² Although most of the modifications have been found in transfer RNAs (tRNAs), recent advances in analytical and next-generation sequencing strategies have revealed an increasing number of modified nucleosides in other non-coding RNAs and coding messenger RNAs (mRNAs).^{3–7} Among others, N⁶-methyladenosine (m⁶A), 5-methylcytidine (m⁵C) and 5-hydroxymethylcytidine (hm⁵C) represent post-transcriptional, dynamic and reversible epitranscriptomic-type mRNA modifications linked to the regulation of gene expression.^{8–10}

Institute of Organic Chemistry, Faculty of Chemistry, University of Technology, 90-924 Lodz, Zeromskiego 116, Poland. E-mail: grazyna.leszczynska@p.lodz.pl
† Equal contribution.



Anna Kuszczynska

Anna Kuszczynska received her BSc (2021) and MSc (2022) degrees in chemistry from the Lodz University of Technology, Poland. Currently, she is pursuing her PhD under the supervision of Prof. Leszczynska. Her research interest encompasses the development of new methods for the synthesis of modified ribonucleosides and their incorporation into RNA oligomers. Her latest project focuses on the metabolism of 5-hydroxymethylcytidine (hm⁵C) in cancer cells.



Milena Bors

Milena Bors received her BSc and MSc degrees in chemistry from the Lodz University of Technology, Poland. Currently, she is pursuing her doctoral degree under the supervision of Prof. Leszczynska at the Department of Chemistry, Institute of Organic Chemistry. Her research primarily revolves around the development of innovative methodologies for the synthesis of modified nucleosides and the facile, robust, and orthogonal incorporation of these monomers into RNA oligomers to evaluate the role of epigenetic modifications in the translation process.



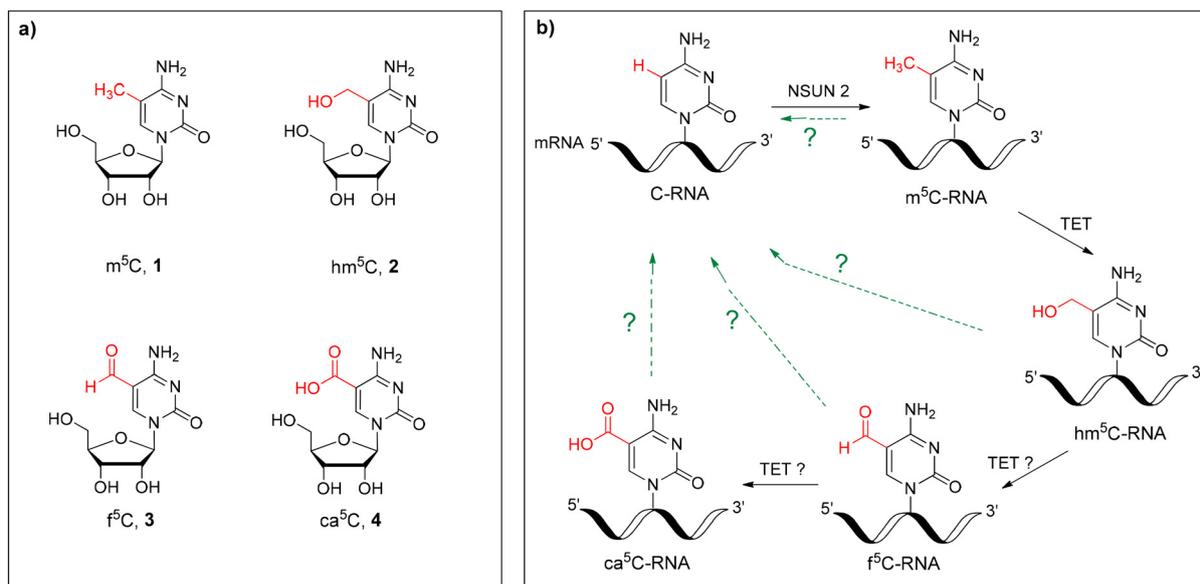


Fig. 1 (a) Chemical structures of 5-methylcytidine (m^5C , 1), 5-hydroxymethylcytidine (hm^5C , 2), 5-formylcytidine (f^5C , 3) and 5-carboxycytidine (ca^5C , 4); (b) cellular oxidation of m^5C -mRNA with the TET and hypothetical pathways of cytidine turnover (green arrows).

5-Methylcytidine (m^5C , 1, Fig. 1a) is one of the major RNA modifications identified in mRNA and non-coding RNAs, including tRNA, ribosomal RNA (rRNA), long non-coding RNA, small nuclear RNA, microRNA and enhancer RNA.^{1,4,11–13} Although m^5C modification is widespread in many RNA species in all three domains of life, its increased distribution has been reported in eukaryotic tRNA and mRNA sequences.^{11,12,14} In tRNA molecules, m^5C is known to stabilize the tRNA secondary structure and facilitate codon–anticodon pairing and tRNA aminoacylation.^{15,16} In addition, tRNA m^5C modification regulates the cellular stress response, potentially by controlling the translation rate.^{17,18} In mRNA, m^5C has been reported to regulate the nuclear-cytoplasmic trans-

port,¹⁹ mRNA stability,²⁰ splicing²¹ and translation.^{22,23} mRNA m^5C was also associated with bladder cancer and autoimmune diseases.^{24–26}

Over the last decade, it was demonstrated that m^5C undergoes *in vivo* oxidation to 5-hydroxymethylcytidine (hm^5C , 2) and 5-formylcytidine (f^5C , 3) (Fig. 1) in total RNA from organisms representing the three domains of life^{27,28} as well as in mammalian cells,^{29,30} including human and mouse embryonic stem cells.^{31,32} Similar to that in DNA,^{33,34} the ten–eleven translocation enzyme (TET) has been proven to catalyze the *in vitro* oxidation of m^5C -RNA to hm^5C as well as f^5C -RNA to 5-carboxycytidine (ca^5C , 4) (Fig. 1).^{29,35} Recently, the existence of four cytidines 1–4 has been confirmed in mammalian



Karolina Podskocznyj

nucleosides present in the anticodon domain on the structure and properties of tRNA molecules.

Karolina Podskocznyj received her PhD in chemistry from the Lodz University of Technology, Poland, in 2023. She is currently an Assistant Professor at the Institute of Organic Chemistry at the Lodz University of Technology, where she joined the research group of Prof. Leszczynska. Her research interests comprise the synthesis of modified nucleosides and RNA oligomers, as well as investigating the impact of modified



Grazyna Leszczynska

research focuses on the chemistry and physicochemical/structural properties of modified ribonucleosides and RNA.

Grazyna Leszczynska completed her postgraduate studies at the Lodz University of Technology (LUT) in 2000 and the Medical University of Lodz in 2010, specializing in organic chemistry and medical biotechnology, respectively. After receiving her PhD, she joined the group of Prof. A. Malkiewicz and Prof. E. Sochacka as an Assistant Professor. In 2019, she was appointed an Associate Professor and started independent



mRNA, including human colorectal carcinoma and hepatocellular carcinoma tissues.²⁷ The dynamic character of m⁵C, hm⁵C, f⁵C and ca⁵C epitranscriptomic modifications and the possible but unknown turnover pathway to the parent cytidine (green arrows, Fig. 1b) imply the regulatory roles of these modifications in biological processes.

Originally, hm⁵C was identified in rRNA isolated from wheat seedlings.³⁶ In mRNA, epitranscriptomic 5-hydroxymethylcytidine was found to increase translation and brain development in *Drosophila melanogaster*.³⁷ Recently, the regulatory function of hm⁵C has been discovered related to its contribution to the mRNA flexibility required for mouse embryonic stem cell differentiation.⁹ In contrast to hm⁵C, the biological significance of epitranscriptomic f⁵C and ca⁵C modifications remains unknown. The stable existence of 5-formylcytidine and 2'-O-methyl-5-formylcytidine (f⁵Cm) has been confirmed at the wobble position (first anticodon letter) of mammalian mitochondrial tRNAs^{Met} (mt-tRNA^{Met})³⁸ and cytoplasmic tRNA^{Leu},³⁹ respectively. 5-Formylcytidine increases the flexibility of the mt-tRNA^{Met} anticodon loop domain, facilitating the recognition of both purine-ending codons,^{40–42} and has been associated with several human diseases.^{43,44} Recently, f⁵C has also been detected in chromatin-associated RNA using a new, quantitative f⁵C-seq method.³²

To acquire more insight into the function of hm⁵C, f⁵C and ca⁵C in RNA, multidirectional and interdisciplinary studies are required. In this context, synthetically obtained modified RNA oligomers are valuable tools to elucidate the effect of modified nucleosides on the physicochemical and structural properties of RNA^{45–48} and to identify the proteins that might recognize and process epitranscriptomic cytidine marks.^{29,35} Despite the numerous benefits of modified RNA oligomers, e.g. site-specific positioning of modified nucleoside(s) and possibility of a large scale synthesis, incorporation of C5-hydroxymethyl-, formyl- or carboxyl-functionalized cytidines is challenging and troublesome. The major obstacles are selection of appropriate blocking groups for modified building blocks (particularly for electrophilic-type carboxyl and formyl residues⁴⁹) and appropriate modification of the standard phosphoramidite chemistry protocol.

In this review, we summarize the chemical methods for hm⁵C-, f⁵C- and ca⁵C-incorporation into RNA chains, including the preparation of modified monomer units and methods for C5-substituent installation. We focus on standard and post-synthetic phosphoramidite methods and *in vitro* enzyme-mediated transformations. In addition, the synthetic approaches to produce the epigenetically-modified DNA fragment are briefly discussed to highlight the difficulties in their implementation in RNA chemistry.

2. Chemical synthesis of modified RNA oligomers

Solid-phase phosphoramidite chemistry is the most common strategy for modified RNA synthesis.⁵⁰ It involves two

approaches: (1) the standard approach *via* the preparation of a modified phosphoramidite building block and its subsequent incorporation into the RNA chain (Scheme 1a) and (2) the post-synthetic RNA modification approach based on the selective chemical reaction of an easily convertible precursor oligonucleotide prepared by a standard procedure (Scheme 1b).

Post-synthetic conversions of RNA oligomers can be performed in the solid or liquid phase (Scheme 1b). The *solid-phase* approach involves a fully protected, support-linked oligoribonucleotide as a substrate. After conversion, the oligomer is subjected to a deprotection step and support cleavage. The most typical pattern of RNA deprotection involves a two-step procedure: (1) base deprotection of exoamino functions and phosphate residues with simultaneous support cleavage and (2) removal of 2'-protecting groups, e.g. desilylation when *tert*-butyldimethylsilyl (TBDMS) or triisopropylsilyloxymethyl (TOM) groups are present. If the Z → Y transformation is processed under alkaline conditions, the simultaneous removal of base-labile protecting groups and support cleavage are often observed. In this case, the post-synthetic reaction is carried out in one conversion–deprotection step.

Alternatively, the post-synthetic RNA modification can be performed in the liquid phase (Scheme 1b). The *solution* approach requires a fully deprotected oligoribonucleotide as a substrate. The hydrophilic character of RNA and the presence of free 2'-hydroxyl groups restrict the post-synthetic reaction conditions to polar solvents and mild basic conditions. The latter is required to prevent RNA cleavage or phosphate migration. Consequently, the number of organic reactions that can be used for post-synthetic transformation *in solution* is significantly reduced.

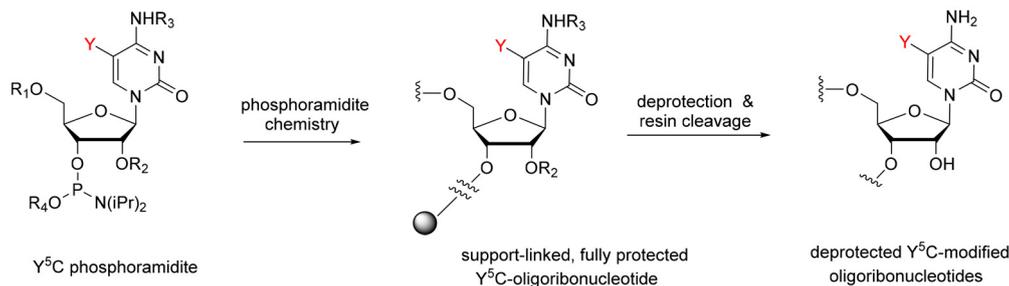
For successful solid-phase RNA synthesis, the phosphoramidite monomeric units must be protected with a combination of orthogonal R₁ transient, and R₂, R₃, and R₄ permanent protecting groups (Scheme 1). The standard protection strategy (Fig. 2) involves the R₁ acid-labile 4,4'-O-dimethoxytrityl (DMTr) group, the R₂ fluorolabile *tert*-butyldimethylsilyl (TBDMS) or triisopropylsilyloxymethyl (TOM) groups and two base-labile R₃ and R₄ protecting groups, acyl and β-cyanoethyl, respectively.

3. 5-Hydroxymethylcytidine (hm⁵C) modified RNA oligomers

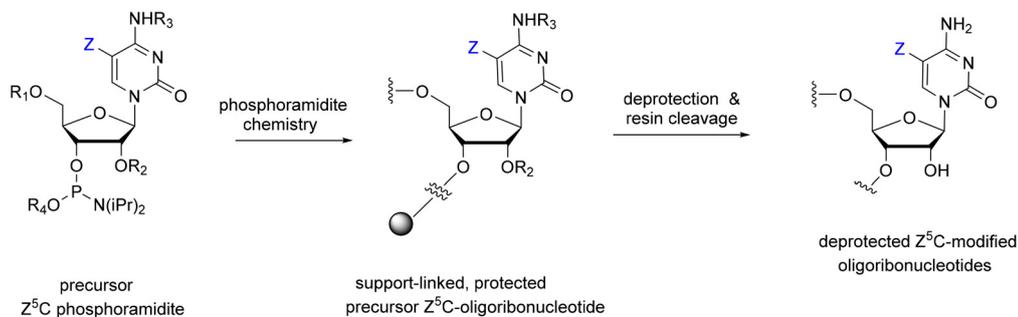
The solid-phase synthesis of hm⁵C-RNA is challenging because of the pseudobenzyllic positioning of the 5-hydroxymethyl group. The choice of the appropriate protection for –CH₂OH is crucial to avoid the attack of nucleophiles (e.g. ammonia or methylamine used during oligomer deprotection) on the pseudobenzyllic carbon and the formation of undesired side products.^{51,52} The problem worsens when –CH₂OH is protected by an ester type group, for instance, the use of sterically hindered pivaloyl ester (5-CH₂OPiv) almost exclusively promotes substitution of the –OPiv group with nitrogen nucleophiles.^{53,54}



a) Standard phosphoramidite chemistry



b) Post-synthetic modification approach

R₁-R₄ = protecting groups

Y = target modified group in cytosine

● = solid support

Z = reactive site of precursor phosphoramidite/oligomer

Scheme 1 Two approaches in phosphoramidite chemistry; (a) standard method and (b) post-synthetic method of RNA modification divided into two strategies: in the *solid phase* and *in solution*.

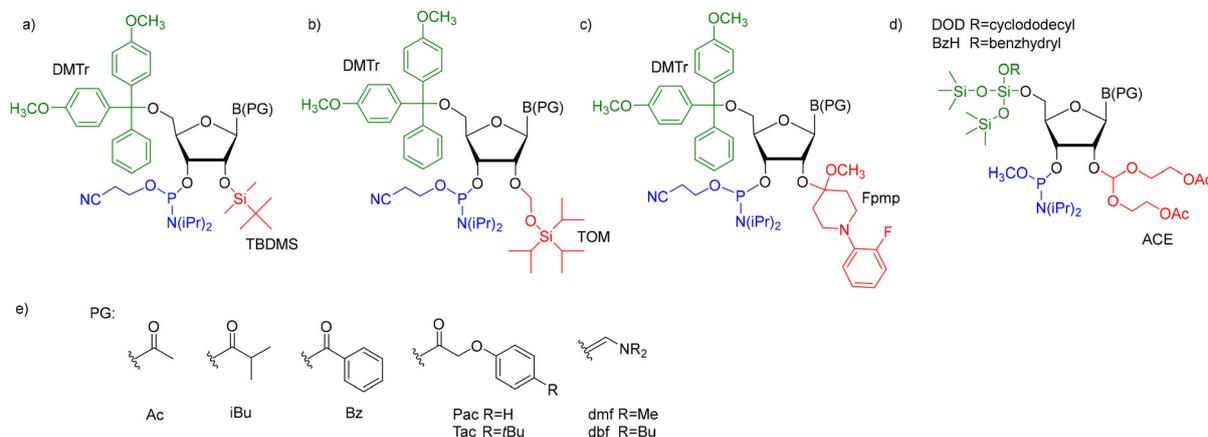


Fig. 2 The most common ribonucleoside phosphoramidite building blocks for solid-phase RNA synthesis: (a) 5'-O-DMTr-2'-O-TBDMS-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite; (b) 5'-O-DMTr-2'-O-TOM-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite; (c) 5'-O-DMTr-2'-O-Fpmp-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite; (d) 5'-O-DOD(BzH)-2'-O-ACE-3'-O-(methyl)-N,N-diisopropylphosphoramidite; and (e) commonly used nucleobase protecting groups.

To this day, two protection concepts of the hm⁵riboC phosphoramidite have been published (Fig. 3), both using the 5'-O-DMTr-2'-O-TBDMS-type ribose blockage. The first strategy uti-

lizes an acetyl (Ac) protecting group to mask both 5-hydroxymethyl and 4-exoamino functions (I, Fig. 3).^{45,55-57} In the second strategy, a combination of *tert*-butyldimethylsilyl



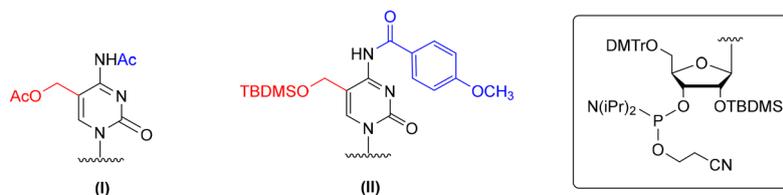


Fig. 3 Protection patterns of 5-hydroxymethylcytidine phosphoramidites, as reported in the literature.

(TBDMS) and *p*-methoxybenzoyl (*p*-MeOBz) groups was employed for 5-hydroxymethyl and *N*4-exoamino functions, respectively (II, Fig. 3).⁵⁸

Bisacetyl-protected hm⁵C phosphoramidite **13** was obtained by the Micura and Balasubramanian groups starting with 5-functionalized uridine or cytidine derivatives (Scheme 2a and b).^{45,55–57} The use of uridine derivatives, 5-hydroxymethyluridine (hm⁵U, **5**)^{55,56} and 5-methyluridine (m⁵U, **7**),⁵⁷ as substrates extends the synthetic route, since an additional U → C conversion step is required (Scheme 2a). On the other hand, both uridines are commercially available or easy to synthesize and their use eliminates the problem of *N*4-amino group reactivity observed in cytidine when the C5-side chain is installed. To synthesize hm⁵C phosphoramidite **13**, the 5-acetoxymethyl group was introduced by selective acetylation of 5-hydroxymethyluridine **5** (easily prepared from uridine) with acetic acid and catalytic amounts of trifluoroacetic acid^{55,56} or by radical bromination of m⁵U **7** and subsequent substitution with potassium acetate.⁵⁷ Although a significant drop in yield is observed when installing acetyl *via* the 5-CH₂Br derivative (42% yield of **8** → **9** conversion), the comparison of overall yields indicates its advantage over the first method starting with uridine due to the shorter synthetic route. 5-Acetylated product **9** was converted into 5-hydroxymethylcytidine **11** *via* substitution of the *O*4-(2,4,6-triisopropyl)-benzenesulfonyl derivative **10** with aq ammonia. Protection of the *N*4-exoamino function with the acetyl group, 2'-silylation and phosphitylation furnished bisacetylated hm⁵C phosphoramidite **13** in an overall yield of 3%^{55,56} or 6.4%⁵⁷ in eleven or eight steps, respectively. The relatively low yields of both synthetic routes result from a 50% decrease in the efficiency of the non-regioselective 2'-silylation reaction. This step was improved in the next method by temporarily protecting the ribose with a 3',5'-di-*tert*-butylsilylene group.

Therefore, in the second approach (Scheme 2b), bisacetylated hm⁵C-amidite **13** was synthesized starting with 5-iodination of ribose-protected cytidine **15**.⁴⁵ 5-Iodocytidine **16** offers rapid (2 step procedure) access to 5-hydroxymethylated cytidine **18** by Pd-catalyzed reductive carbonylation, followed by the reduction of 5-formylcytidine **17** with NaBH₄ under Luche conditions. Simultaneous protection of both the *N*4-amino and 5-hydroxymethyl groups with acetic anhydride furnished bisacetylated product **19**, which was converted into the desired hm⁵C phosphoramidite **13**. This methodology appears more effective and faster than those starting

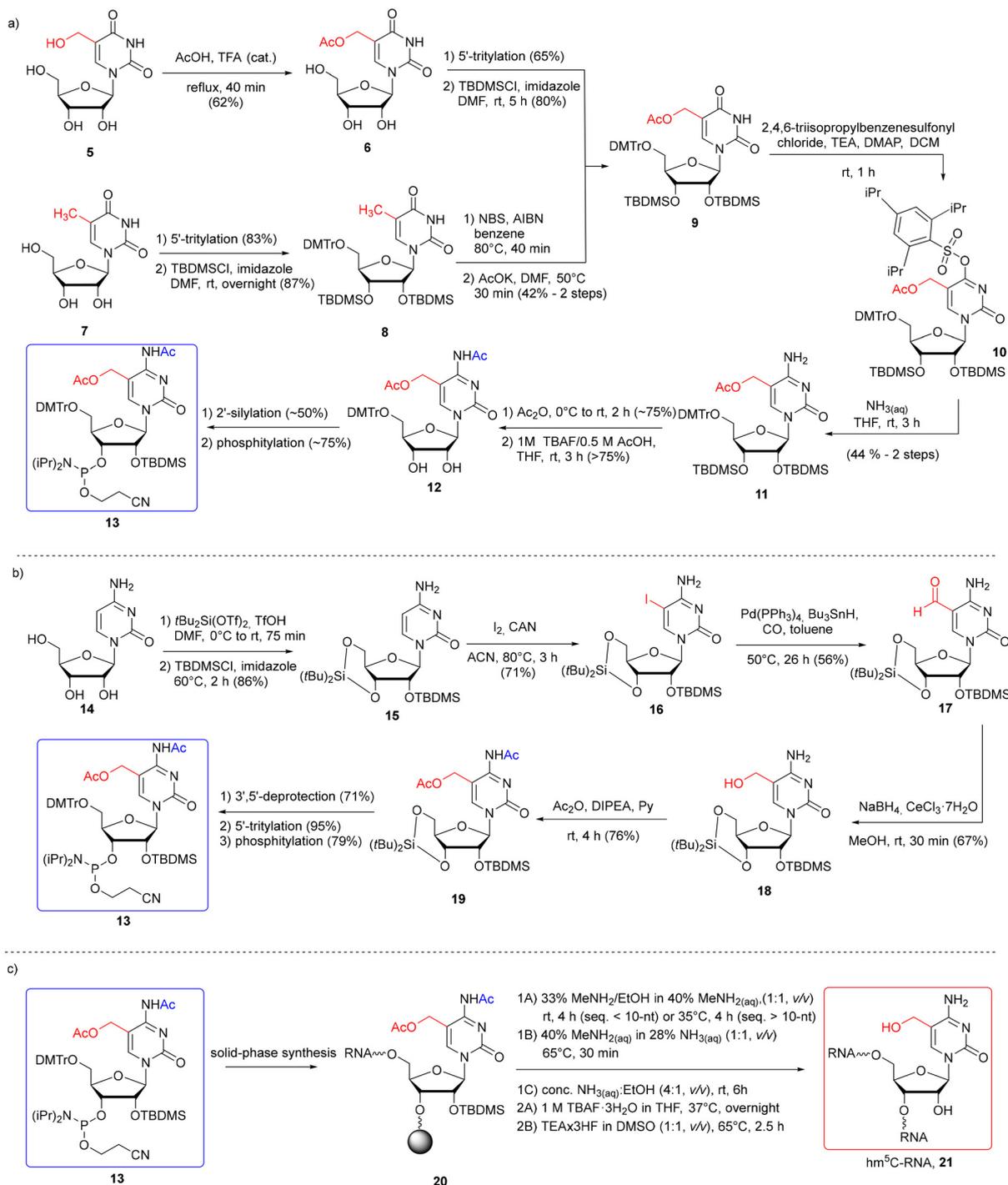
with uridines, providing an hm⁵C building block **13** in a 9% overall yield in eight steps.

Monomeric unit **13** was successfully incorporated into RNA oligomers **21** (Scheme 2c) as a single modification at one, two, and three positions of RNA oligomers according to the 2'-*O*-TOM^{55,56} or 2'-*O*-TBDMS⁵⁷ approach. The hm⁵C building block **13** was coupled within 6–10 min, with 80–98% coupling efficiency, based on a trityl assay. The resulting RNA **20** was subjected to simultaneous support cleavage and base labile group removal under three different conditions. Since it has been previously reported that 5-acetoxy-protected hm⁵dC-DNA undergoes partial substitution of 5-CH₂OAc → 5-CH₂NH₂ with aq ammonia at 50 °C,⁵⁹ Rimpl and colleagues employed more nucleophilic conditions offering fast and clean cleavage of base-labile groups: water–ethanol solution of methylamine (rt for shorter and 35 °C for longer RNA), or alternatively, aq methylamine–ammonia solution at 65 °C.^{45,55,56} Under both conditions, small amounts of side products (<15%) were detected, resulting from the methylamine attack on the pseudobenzyl carbon. Importantly, the use of the *N*4-acetyl group in hm⁵C monomeric unit **13** instead of the *N*4-benzoyl group prevented transamination at C4 by methylamine.^{60,61} As the third option, Tanpure and co-workers used a mixture of conc. NH_{3(aq)}–EtOH (4:1, v/v, rt, 6 h) for hm⁵C-RNA **20** deprotection.⁵⁷ In this case, no comments about side product formation were reported.

The problem of the benzylic nature of hm⁵C in solid-phase RNA synthesis was overcome by choosing the base-stable 5-CH₂OTBDMS protection.⁵⁸ The *N*4-exoamino function was protected with the *N*4-*p*-methoxybenzoyl (*p*-MeOBz) group since the standard *N*4-acetyl or benzoyl groups tend to be cleaved during solid-phase synthesis, yielding branched oligomers (additionally extended at the *N*4-amino site).⁵⁸ To synthesize the TBDMS-protected hm⁵C building block **23** (Scheme 3a), 5-hydroxymethylcytidine **18** (obtained by the reduction of 5-formylcytidine, see Scheme 2b) was treated with TBDMS chloride in DMF, in the presence of imidazole. The subsequent reaction with *p*-methoxybenzoyl chloride in pyridine furnished fully protected hm⁵C **22**, which was effectively converted into hm⁵C phosphoramidite **23** (note that the 5'-*O*-DMTr intermediate product was obtained using freshly prepared 4,4'-dimethoxytrityl triflate instead of the commonly used DMTrCl).

The building block **23** has been successfully incorporated into RNA strands (Scheme 3b) with an extended coupling time of 20 min. Support-linked oligomer **24** was treated with conc.



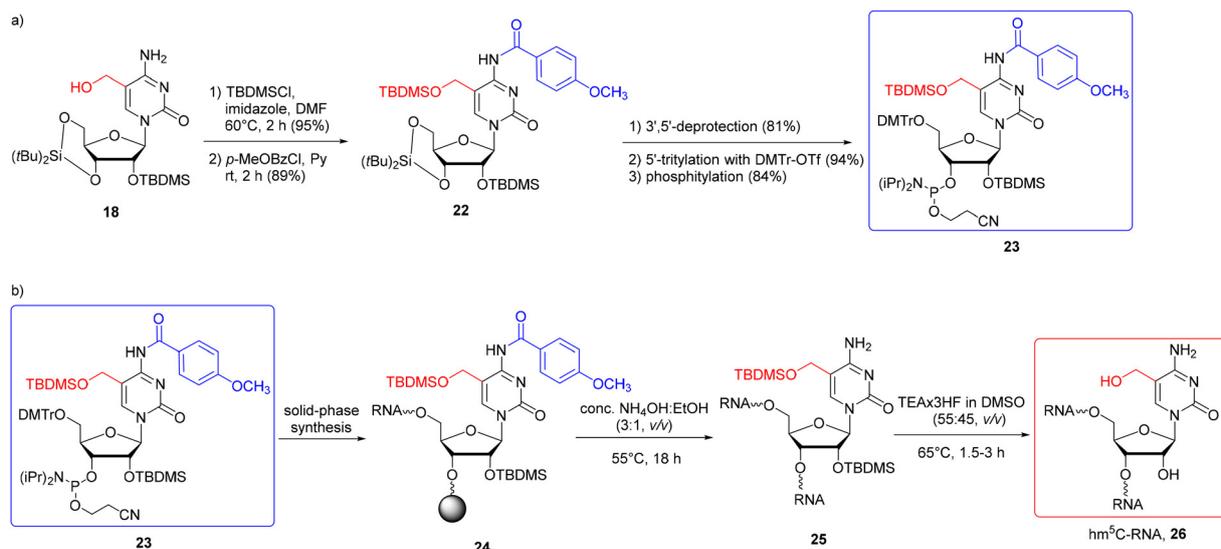


Scheme 2 a) Synthesis of hm^5C phosphoramidite with 5- CH_2OAc and $N4-Ac$ systems of protection with hm^5U 5 or m^5U 7 as the starting material;^{55–57} (b) synthesis of hm^5C phosphoramidite with 5- CH_2OAc and $N4-Ac$ systems of protection with cytidine 14;⁴⁵ (c) synthesis of hm^5C -RNA under different deprotection conditions (1A–1C refers to the alkaline deprotection step; 2A and 2B refer to desilylation).^{45,55–57}

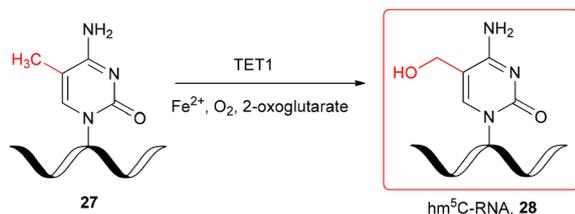
$NH_3(aq)$ -EtOH (3 : 1, v/v, 55 °C) to cleave the support and the base-labile protecting groups. Ammonolysis was prolonged to 18 h to achieve complete cleavage of the p -MeOBz group. Subsequent treatment of 25 with TEA-3HF in DMSO (55 : 45, v/v, 65 °C, 1.5–3 h) caused simultaneous deprotection of both 2'-OH and 5- CH_2OH groups.

The hm^5C -modified RNA oligomer was also obtained enzymatically, by the TET-mediated oxidation of chemically produced single-stranded m^5C -RNA 27 (Scheme 4) under *in vitro* conditions.²⁹ The recombinant catalytic domain of mouse TET1 protein was incubated with 11-nt m^5C -oligomer 27 in the reaction buffer containing Fe^{2+} and 2-oxoglutarate at 37 °C.





Scheme 3 a) Synthesis of hm^5C phosphoramidite with 5- CH_2 OTBDMS and N4-*p*-MeOBz protection systems; (b) synthesis of hm^5C -RNA.⁵⁸



Scheme 4 TET1-mediated formation of hm^5C -RNA under *in vitro* conditions.²⁹

The LC-MS monitoring of the TET1-mediated oxidation of m^5C -RNA revealed the formation of hm^5C -oligomer **28** after 3 min and almost complete conversion of m^5C -RNA **27** after 30–40 min.

Several protection concepts have been published for incorporation of 5-hydroxymethyl-2'-deoxycytidine (hm^5dC) into

DNA oligomers (Fig. 4), including 2-cyanoethyl^{62–65} and TBDMS^{66,67} protecting groups with N4-benzoyl or dbf (**III**, **IV**, and **V**, Fig. 4). The TBDMS group was also employed in combination with a triazolyl ring at the C4 position (**VI**, Fig. 4), which constitutes a precursor for post-synthetic 4-triazolyl → 4-NH₂ conversion by ammonia treatment.^{66,67} In addition to the ether-type protecting groups, an acetyl group representing an ester-type protection was employed in combination with N4-benzoyl (**VII**, Fig. 4).⁵⁹ Furthermore, an intrinsic cyclic carbamate was used to ensure simultaneous protection of both reactive centers of hm^5dC (**VIII**, Fig. 4).^{68–71}

Although the elaborated protection systems worked well for incorporation of hm^5dC amidite into DNA chains, their direct implementation in RNA synthesis is unlikely to succeed. For instance, carbamate^{68–71} and acetyl⁵⁹ removal (**VII** and **VIII**, Fig. 4) requires DNA incubation with NaOH_{aq} instead of standard aq ammonia to avoid the formation of amide side pro-

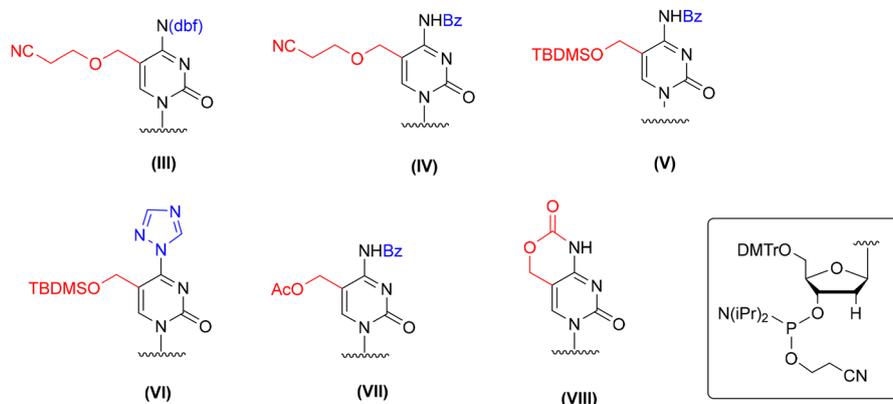


Fig. 4 Different protection patterns of 5-hydroxymethyl-2'-deoxycytidine phosphoramidite for application in the solid-phase synthesis of hm^5dC -DNA, as reported in the literature.



ducts. The 5-CH₂OCH₂CH₂CN (**III** and **IV**, Fig. 4)^{62–65,72} and 5-CH₂OTBDMS (**V** and **VI**, Fig. 4)^{66,67} protecting groups are superstable and their complete removal is achieved by prolonged DNA incubation with aq ammonia at 65–75 °C. All these conditions are too harsh to keep RNA intact. It is likely that partial deprotection of 2'-O-TBDMS groups will occur, resulting in the cleavage of phosphodiester linkages.^{73,74}

In the past, the hm⁵dC amidite blockage strategy with 5-CH₂OTBDMS and *N*4-benzoyl groups (**V**, Fig. 4)^{66,67} was employed in hm⁵C-RNA chemistry (see Scheme 3); however, the conditions of post-synthetic hm⁵C-RNA deprotection were adapted to ensure RNA stability.

An interesting alternative for hm⁵dC-DNA synthesis is the post-synthetic approach, based on the reduction of support-linked 5-formyl-2'-deoxycytidine-DNA (f⁵dC-DNA, **29**) (Scheme 5) with NaBH₄ under Luche conditions.⁶⁶ Hm⁵dC-DNA **30** obtained in 15 min was subjected to alkaline deprotection under two mild conditions: K₂CO₃ in MeOH/H₂O (rt, 2 h) or NH₃(aq) (rt, 2 h). Both conditions are potentially safe for RNA stability; however, the preparation of support-linked, formyl-protected f⁵C-RNA is still challenging (see the next section).

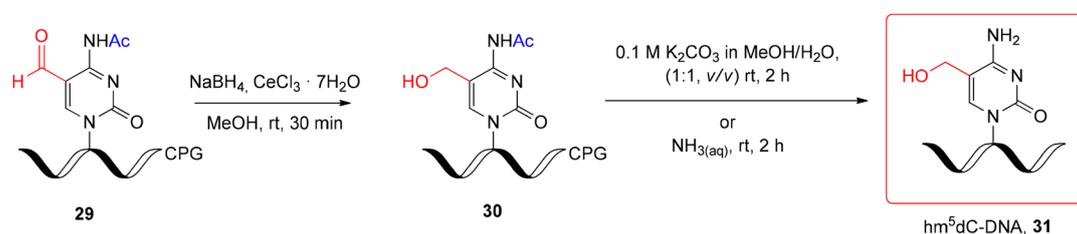
4. 5-Formylcytidine (f⁵C) modified RNA oligomers

The formyl group present in 5-formylcytidine (f⁵C) is susceptible to some conditions of solid-phase phosphoramidite synthesis, including acidic pH that facilitates *N*-glycosidic bond cleavage, oxidizers that promote -CHO oxidation and

ammonia/amine treatment (commonly used during alkaline oligomer deprotection), which leads to imine formation.^{62,66,69,75} Due to these restrictions, there is only one example of the successful use of formyl-protected f⁵C-phosphoramidite in f⁵C-RNA synthesis.⁴² To keep the formyl group intact, the 5'-O-silyl-2'-O-acetal system of f⁵C phosphoramidite protection was used (**IX**, Fig. 5). An alternative concept of f⁵C-RNA synthesis involved formyl-protected f⁵C in the 2'-O-TBDMS approach. Since commonly used formyl protecting groups are incompatible with phosphoramidite chemistry, only the cyclic γ -acetal group (**X** and **XI**, Fig. 5) has been described so far in the synthesis of f⁵C-RNA.^{57,58} A convenient alternative for the standard phosphoramidite method is the post-synthetic strategy approach based on oxidation of a precursor RNA containing 5-(1,2-diacetoxyethyl)cytidine (**XII**, Fig. 5).⁷⁶

The first attempt to incorporate f⁵C into an RNA chain was made by Lusic and co-workers.⁴² They employed formyl-protected f⁵C phosphoramidite – prepared as methyl diisopropylphosphoramidites, equipped with 5'-O-BzH-2'-O-ACE and *N*4-dbf protecting groups (**IX**, Fig. 5). This non-standard system of protection enables to avoid the acidic conditions during RNA synthesis (hazardous for the formyl group, but typical for the 5'-O-DMTr-2'-O-silyl approach). On the other hand, the mild acidic conditions applied in the final step of oligomer deprotection promote the removal of 2'-O-ACE groups but also the hydrolysis of any imine side products that may be formed in the prior reaction of the “free” formyl group with an amine.

To synthesize the monomeric unit **35** (Scheme 6a), 5-hydroxymethylcytidine **32** was selectively oxidized with RuO₂



Scheme 5 Post-synthetic reduction of support-linked f⁵dC-DNA and subsequent alkaline deprotection step.⁶⁶

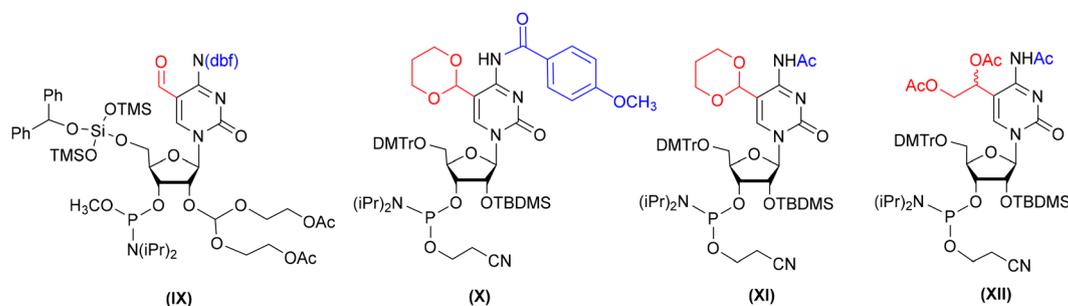
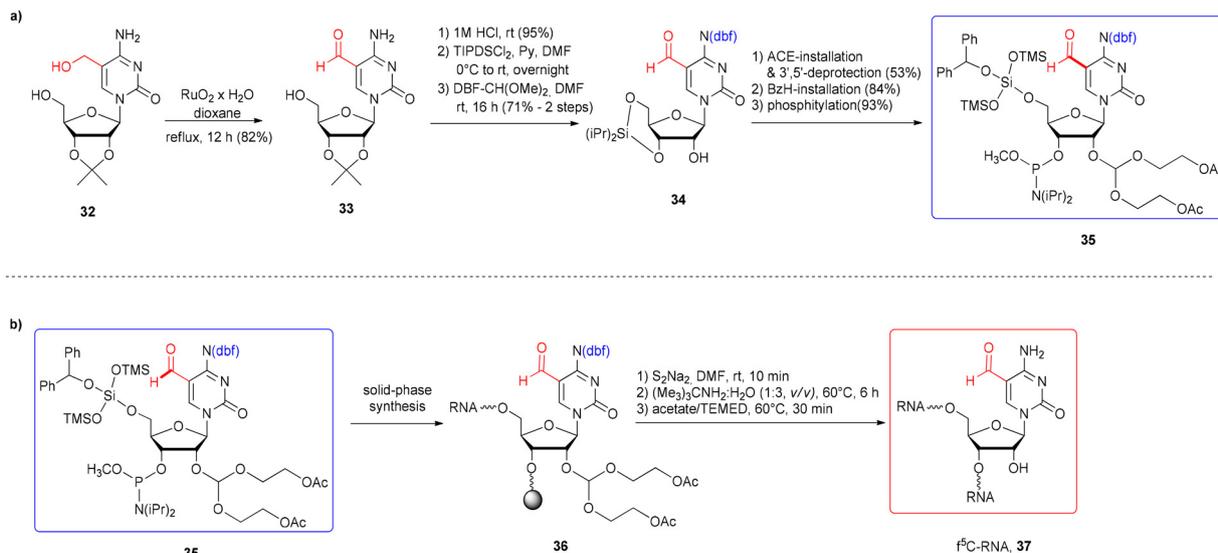


Fig. 5 Different protection patterns of 5-formylcytidine phosphoramidite (or the precursor unit) for the preparation of f⁵C-RNA oligomers using phosphoramidite chemistry, as reported in the literature.





Scheme 6 a) Synthesis of formyl-protected 5'-O-BzH-2'-O-ACE f⁵C phosphoramidite; (b) synthesis of f⁵C-RNA.⁴²

affording 5-formylcytidine **33**. Further steps involved the selective protection of *N*4-amino and 2'-OH functions, followed by phosphitylation.

5-Formylcytidine amidite **35** was introduced into the 17-nt RNA **36** by activation with 5-ethylthio-1*H*-tetrazole, with a 3.5 min coupling time (Scheme 6b). After 5'-O-BzH removal (TEA·3HF),⁷⁷ the oligomer **36** was subjected to a three-step deprotection procedure, involving release of phosphate groups by disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate (S₂Na₂), resin cleavage and removal of base-labile protecting groups with *tert*-butylamine–water solution (including acetyl groups from the 2'-O-ACE blockage) and finally, removal of acid-labile 2'-O-bis(2-hydroxyethoxy)methyl orthoesters with acetate/TEMED. Since the formation of imine adducts with the formyl group was considered, three alkaline deprotection conditions were tested: aq ammonia (rt, 24 h), methylamine (rt, 6 h) and *tert*-butylamine in water (1 : 3, v/v, 60 °C, 6 h). Only sterically hindered *tert*-butylamine provided the desired product with no *tert*-butylamine adducts.

To avoid problems with the potential reactivity of the aldehyde residue during f⁵C-RNA synthesis and deprotection, the formyl group was masked with a 1,3-dioxane residue (Scheme 7a).^{57,58} The cyclic γ -acetal formyl protection is able to survive the standard conditions of solid-phase synthesis in the 5'-O-DMTr-2'-O-TBDMS approach. To eliminate the risk of *N*4-Ac/Bz cleavage during oligomer synthesis, Michaelides and co-workers used *p*-MeOBz for the *N*4-exoamino function.⁵⁸ The acetal remains intact during the alkaline oligomer deprotection step, preventing the aldehyde from reacting with ammonia/amine. Cyclic acetal is removed under mild acidic conditions in the final deprotection step.

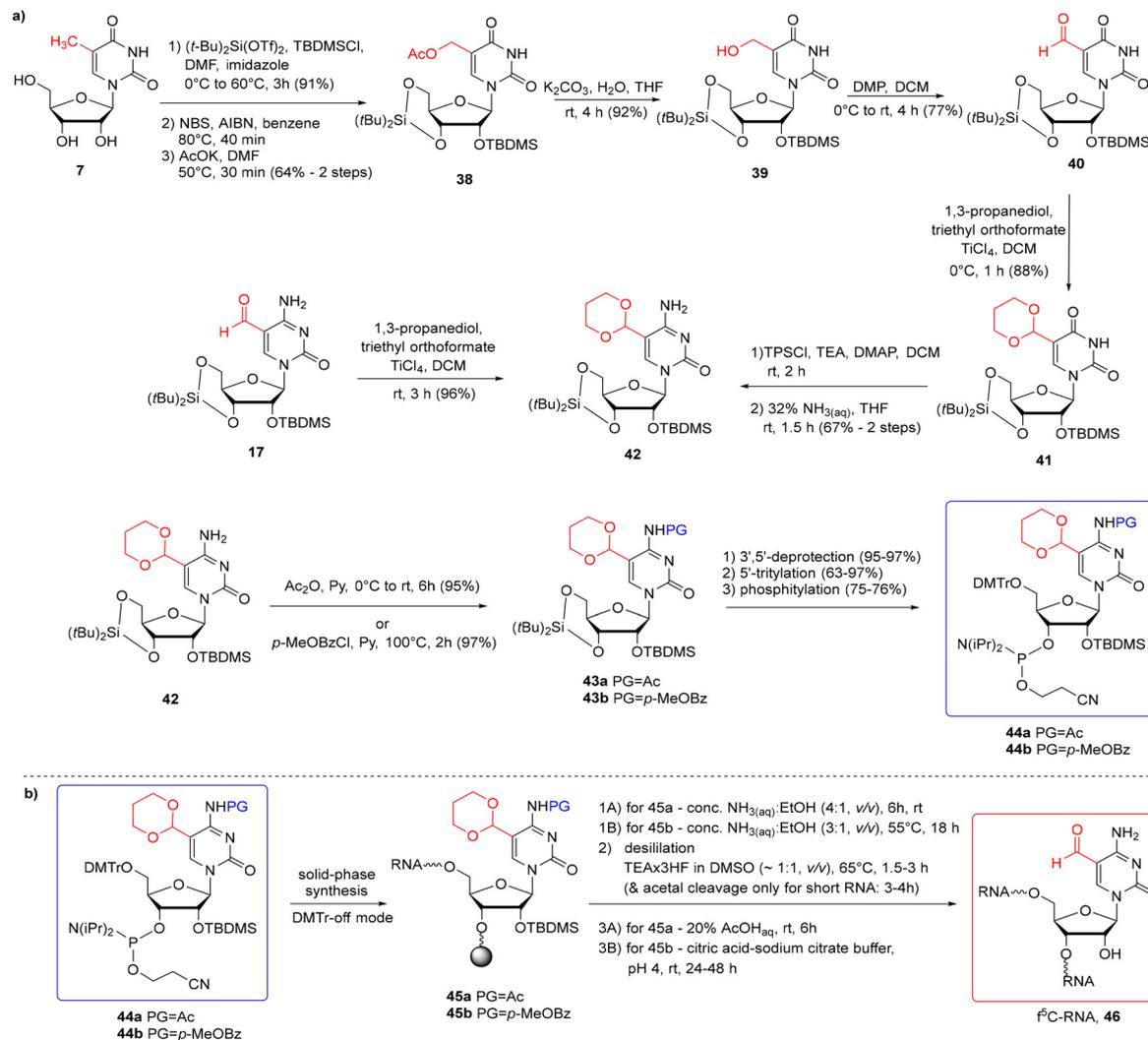
To introduce the cyclic γ -acetal protection, the 5-formyl-modified uridine **40** (Scheme 7a) or cytidine **17** was treated with propane-1,3-diol, in the presence of triethyl orthoformate

and TiCl₄ Lewis acid as an activator, affording **41**⁵⁷ and **42**,⁵⁸ respectively. 5-Formyluridine **17** was obtained by Pd-catalyzed reductive carbonylation of 5-iodocytidine (see Scheme 2a). To prepare the 5-formyluridine derivative **40**, 5-methyluridine **7** was converted to 5-hydroxymethylcytidine **39** through a three-step procedure: 5-bromination, nucleophilic substitution with potassium acetate and subsequent acetate ester hydrolysis. Next, 5-hydroxymethyluridine **39** was selectively oxidized to aldehyde **40** with Dess–Martin periodinane (DMP). Following the installation of the acetal group, protected uridine **41** was converted into cytidine **42** via the *O*4-(2,4,6-triisopropyl)benzenesulfonyl intermediate. Acetal-protected f⁵C **42** was then blocked at the *N*4-amino function by an acetyl⁵⁷ or *p*-MeOBz⁵⁸ group and further converted into 5'-O-DMTr-2'-O-TBDMS-protected phosphoramidite **44a/44b**.

Acetal-masked f⁵C phosphoramidite **44a/44b** was incorporated into RNA (Scheme 7b) with a coupling time of 10 min (ref. 57) or 20 min (ref. 58) and 80–90% coupling efficiency. The first two steps of oligonucleotide deprotection were performed under standard conditions: conc. NH_{3(aq)}–EtOH solution to cleave the support and the base-labile protecting groups, and TEA·3HF to remove the TBDMS groups. Since the desilylation solution is mildly acidic, prolonged incubation of short oligomers at 65 °C cleaved both 2'-O-TBDMS and acetal groups. For longer oligomers, an additional step of acetal deprotection was required by RNA incubation with citric acid–sodium citrate buffer at pH 4 (rt, 24–48 h)⁵⁸ or 20% AcOH_{aq} (rt, 6 h).⁵⁷ The orthogonal protection systems in f⁵C **44a** and hm⁵C **13** building blocks enabled the incorporation of both epitranscriptomic modifications into a single RNA strand according to the above-described protocol.⁵⁷

5-Formylcytidine-RNA oligomers were also prepared by the post-synthetic approach based on the oxidation of 5-diol-containing cytidine with sodium periodate (Scheme 8).⁷⁶ The 5'-O-





Scheme 7 a) Synthesis of $f^5\text{C}$ phosphoramidites with cyclic acetal and the $N4\text{-Ac-}^{57}$ or the $N4\text{-}p\text{-MeOBz-}^{58}$ protection system; (b) synthesis of $f^5\text{C}$ -RNA.

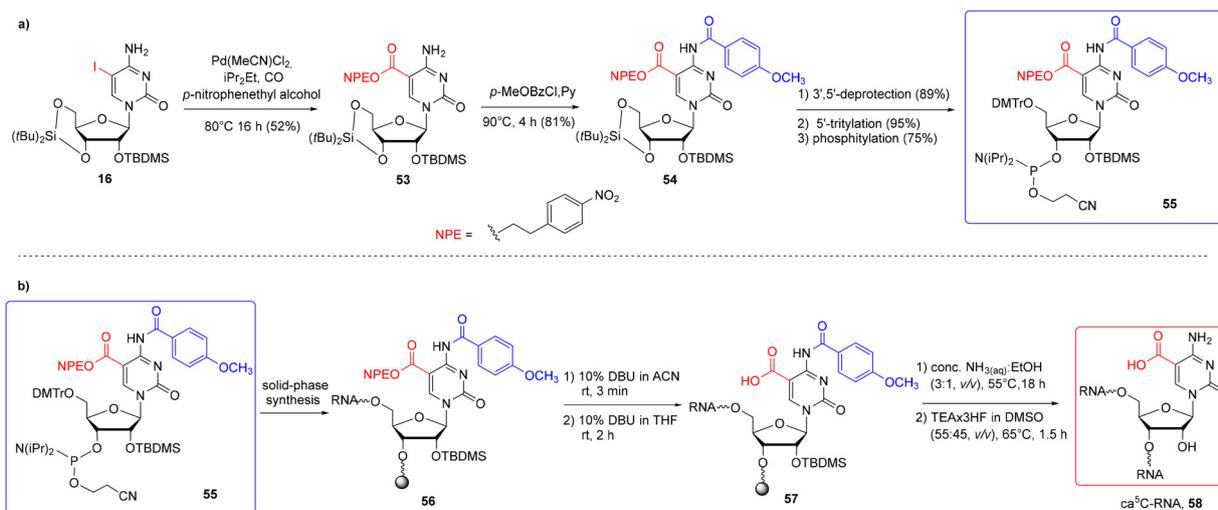
DMTr-2'-O-TBDMS-protected precursor phosphoramidite **49** offers a fully orthogonal system of protection as the C5-linked diol residue is protected with the acetyl groups.

The precursor building block **49** was synthesized as shown in Scheme 8a. 5-Iodocytidine **16** was subjected to a Pd-catalyzed Stille reaction, affording 5-vinylcytidine **47**. Treatment of **47** with OsO_4 in the presence of *N*-methylmorpholine-*N*-oxide, followed by acylation, gave the triacetylated product **48**. After selective removal of the di-*tert*-butylsilylene protection, the resulting nucleoside was tritylated and phosphitylated to furnish the 3'-*O*-phosphoramidite of 5-(1,2-diacetoxyethyl)-*N4*-acetylcytidine **49**. Phosphoramidite **49** was coupled twice with a 10 min coupling time, using the phosphoramidite chemistry on the CPG-Am(Pac) support (the use of a 2'-*O*-methylated nucleoside was necessary to preserve the 3'-terminal nucleoside against the C2'-C3' bond cleavage). After the synthesis, precursor RNA **50** (Scheme 8b) was subjected to alkaline deprotection consisting of the selective release of phosphate

groups under mild conditions (TEA-ACN, 1:1, v/v, rt, 20 min), followed by treatment with conc. $\text{NH}_3(\text{aq})$ -EtOH to remove the support and the remaining base-labile groups. Diol-containing oligomer **51** was desilylated with TEA-3HF/NMP (1:1, v/v, 65°C , 2.5 h) and purified by IE HPLC. The fully deprotected oligomer was quantitatively oxidized to $f^5\text{C}$ -RNA **52** with 0.05 M NaIO_4 used in a 50-fold molar excess at 0°C for 30 minutes. The excess of the oxidizing reagent was rapidly removed using a Sep-Pak cartridge.

The synthesis of $f^5\text{dC}$ -containing DNA was performed in several ways, including the incorporation of formyl-unprotected $f^5\text{dC}$ phosphoramidites (**XIII** and **XIV**, Fig. 6),^{64,66,69} acetal-protected cytidine (**XV**)^{70,71} and a C5-diol-functionalized precursor building block (**XVI**).⁷⁸ The latter two monomeric units were successfully adopted in $f^5\text{C}$ -RNA synthesis, although the protocols of alkaline deprotection were modified to avoid the risk of RNA degradation, e.g. prolonged incubation of DNA with conc. $\text{NH}_3(\text{aq})$ at 55°C was replaced with rapid





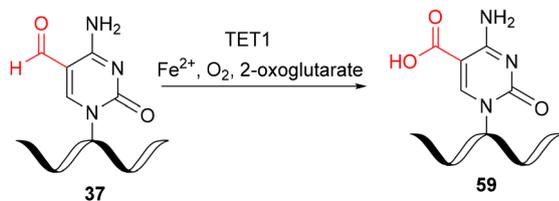
Scheme 9 a) Synthesis of ca^5C phosphoramidite **55**; (b) synthesis of ca^5C -RNA **58**.⁵⁸

dues, selective deprotection of the NPE group with 10% DBU in THF (rt, 2 h) and incubation of carboxyl-unprotected ca^5C -RNA **57** with conc. $NH_3(aq)$ -EtOH (3:1, v/v, 55 °C) to deprotect the exoamino functions and cleave the oligomer from the support. Oligomer desilylation finished the deprotection protocol providing the desired ca^5C -RNA **58**.

A slight modification of the above RNA synthesis method allowed the insertion of two other phosphoramidites hm^5C **23** and f^5C **44b** into a single RNA strand.⁵⁸ Since f^5C -phosphoramidite **44b** was protected with an acidic-labile acetal group, the aforementioned protocol of oligomer deprotection was extended with an additional step of RNA incubation with citric acid-sodium citrate buffer at pH 4 (rt, 24 h).

In 2017, the catalytic activity of mammalian *ten-eleven translocation methylcytosine dioxygenase 1* (TET1) was discovered for *in vitro* oxidation of 5-formylcytosine-modified RNA **37** to the 5-carboxycytidine derivative **59** (Scheme 10).³⁵ For this purpose, a synthetically prepared f^5C -RNA oligomer **37** (see Scheme 6) was incubated with the catalytic domain of the TET1 enzyme (expressed in insect cells) in a buffer containing 100 mM HEPES (pH 7.0), 50 mM NaCl, 1 mM 2-oxoglutarate, 2 mM ascorbic acid, 0.1 mM $Fe(NH_4)_2(SO_4)_2$, 1 mM DTT, 1 mM ATP, and 100 $\mu g\ ml^{-1}$ bovine serum albumin at 37 °C for 0.5 hours. The oligonucleotide was isolated by ultrafiltration and subjected to enzymatic digestion. In addition to the canonical nucleosides A, C, G and U, both f^5C and ca^5C were identified by UHPLC-MS analysis, demonstrating that the TET1 enzyme catalyzes the oxidative formation of ca^5C from f^5C in single stranded, double stranded and hairpin forms of RNA, albeit with low overall efficiency.

In the literature, several protection concepts for the incorporation of 5-carboxy-2'-deoxycytidine (ca^5dC) phosphoramidite into DNA oligomers (Fig. 7) have been published. In most cases, methyl (**XVII** and **XVIII**, Fig. 7)^{66,69,85} or ethyl (**XIX**, Fig. 7)⁷¹ esters were utilized to protect the carboxyl function of ca^5dC in combination with *N4*-Bz^{71,85} or *N4*-Ac groups.^{66,69} The alkyl esters were removed under hydrolytic conditions



Scheme 10 Preparation of ca^5C -RNA via TET1-mediated oxidation of f^5C -RNA under *in vitro* conditions.³⁵

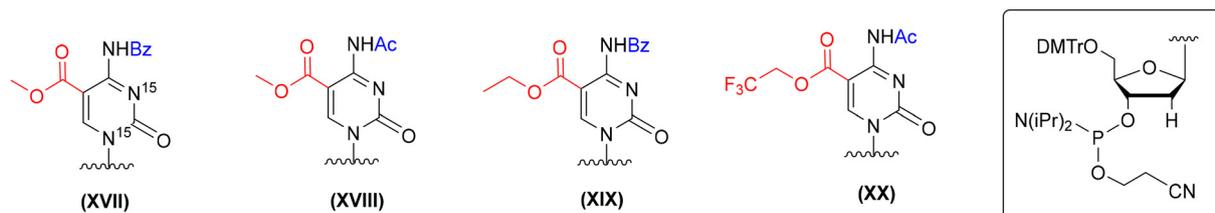
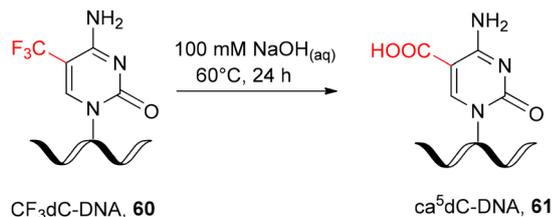


Fig. 7 Different protection patterns of ca^5dC phosphoramidite for the preparation of ca^5dC -DNA oligomers, as reported in the literature.





Scheme 11 Post-synthetic DNA modification with ca⁵dC.^{87,88}

with an aq solution of NaOH or K₂CO₃, since standard ammonolysis causes the formation of amide side products. Both hydrolytic conditions are harsh for RNA handling and could lead to the removal of the 2'-O-silyl protecting group and subsequent RNA degradation. The 5-trifluoroethyl ester of ca⁵dC-DNA (XX, Fig. 7) undergoes effective ammonolysis and aminolysis reactions,⁸⁶ but does not offer complete ester conversion to 5-carboxylic acid.⁶⁶

In addition, the post-synthetic strategy for ca⁵dC-DNA synthesis was reported *via* hydrolysis of 5-trifluoromethylcytidine-modified DNA **60** (CF₃dC-DNA, Scheme 11) as a precursor oligomer. Hydrolysis was performed *in solution* by prolonged incubation of fully deprotected, support-free oligonucleotide **60** with NaOH_{aq} at 60 °C.^{87,88} Although the 5-trifluoromethylcytidine ribonucleoside has been proven to react efficiently with various nucleophilic reagents, including NaOH_{aq},^{89,90} its direct implementation in RNA chemistry will lead to phosphodiester cleavage.

6. Summary

Since 5-hydroxymethyl-, 5-formyl- and 5-carboxycytidine are related to the poorly understood field of epitranscriptome science, future research needs to address the functional significance of these modifications. In this context, RNA solid-phase synthesis has become a key technology to produce RNAs with the target modification at any desired sequence position.

Numerous methods for the synthesis of oligonucleotides containing nucleophilic modifying groups, *e.g.* the hydroxymethyl group in hm⁵C/hm⁵dC, are known; their incorporation is usually achieved using standard protocols of phosphoramidite solid-phase synthesis. Therefore, no further efforts in this area should be expected.

In contrast, the preparation of oligonucleotides containing reactive electrophilic groups, *e.g.* carboxyl or aldehyde groups, is problematic because the commonly used protecting groups are incompatible with solid-phase conditions. The main problem concerns the alkaline deprotection conditions, which are difficult to change. An alternative methodology for installing troublesome modifying groups is the post-synthetic RNA modification approach. It seems that future work will focus on the selection of promising precursor monomeric units and post-synthetic reaction conditions that yield the desired RNAs.

Abbreviations

A	Adenosine
Ac	Acetyl
ACE	Bis(2-acetoxyethoxy)methyl
AIBN	Azobisisobutyronitrile
aq	Aqueous
ATP	Adenosine triphosphate
B	Base
Bz	Benzoyl
BzH	Benzhydryloxybis-(trimethylsilyloxy)silyl
C	Cytidine
ca ⁵ C	5-Carboxycytidine
ca ⁵ dC	5-Carboxy-2'-deoxycytidine
CAN	Ceric ammonium nitrate
conc.	Concentrated
CPG	Controlled pore glass
dbf	Dibutylaminomethylene
DBF-CH(OMe) ₂	<i>N,N</i> -Diisobutylformamidinedimethyl acetal
DBU	Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
dmf	Dimethylaminomethylene
DMF	Dimethylformamide
DMP	Dess–Martin periodinane
DMSO	Dimethyl sulfoxide
DMTr	4,4'-O-Dimetoxytrityl
DNA	Deoxyribonucleic acid
DOD	Bis(trimethylsilyloxy)cyclododecyloxysilyl
f ⁵ C	5-Formylcytidine
Fpmp	1-(2-Fluorophenyl)-4-methoxypiperidin-4-yl
G	Guanosine
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
hm ⁵ C	5-Hydroxymethylcytidine
hm ⁵ dC	5-Hydroxymethyl-2'-deoxycytidine
hm ⁵ U	5-Hydroxymethyluridine
HPLC	High-performance liquid chromatography
iBu	Isobutyryl
IE HPLC	Ion-exchange high-performance liquid chromatography
iPr	Isopropyl
LC	Liquid chromatography
Leu	Leucine
m ⁵ C	5-Methylcytidine
m ⁵ U	5-Methyluridine
m ⁶ A	<i>N</i> 6-Methyladenosine
Me	Methyl
Met	Methionine
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
mt	Mitochondrial
NBS	<i>N</i> -Bromosuccinimide
NMO	<i>N</i> -Methylmorpholine <i>N</i> -oxide



NMP	<i>N</i> -Methyl-2-pyrrolidone
NPE	2-(<i>p</i> -Nitrophenyl)ethyl
nt	Nucleotide
Pac	Phenoxyacetyl
PG	Protecting group
Py	Pyridine
RNA	Ribonucleic acid
rt	Room temperature
S ₂ Na ₂	Disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate
seq	Sequence
Tac	4-(<i>tert</i> -Butylphenoxy)acetyl
TBAF	Tetrabutylammonium fluoride
TBDMS	<i>tert</i> -Butyldimethylsilyl
<i>t</i> Bu	<i>tert</i> -Butyl
TEA	Triethylamine
TEA·3HF	Triethylamine trihydrofluoride
TEMED	<i>N,N,N',N'</i> -Tetramethylethylene diamine
TET	Ten–eleven translocation enzyme
Tf	Trifluoromethanesulfonate
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIPDSCl ₂	1,3-Dichloro-1,1,3,3-tetraisopropylidisiloxane
TMS	Trimethylsilyl
TMSE	2-(Trimethylsilyl)ethyl
TOM	Triisopropylsilyloxymethyl
TPS	(2,4,6-Triisopropyl)benzenesulfonyl
tRNA	Transfer RNA
U	Uridine
UHPLC	Ultra high-performance liquid chromatography
v/v	Volume per volume

Data availability

No primary research results, software or code have been included and no new data were generated or analyzed as part of this review.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This work was funded by the National Science Center, Poland, UMO-2021/43/B/ST4/01570 to GL.

This paper has been completed while the first authors (AK and MB) were Doctoral Candidates in the Interdisciplinary Doctoral School at the Lodz University of Technology, Poland.

References

- 1 A. Cappannini, A. Ray, E. Purta, S. Mukherjee, P. Boccaletto, S. N. Moafinejad, A. Lechner, C. Barchet,

- B. P. Klaholz, F. Stefaniak and J. M. Bujnicki, *Nucleic Acids Res.*, 2024, **52**, D239–D244.
- 2 M. Duechler, G. Leszczyńska, E. Sochacka and B. Nawrot, *Cell. Mol. Life Sci.*, 2016, **73**, 3075–3095.
- 3 W. V. Gilbert, T. A. Bell and C. Schaening, *Science*, 2016, **352**, 1408–1412.
- 4 J. Mathlin, L. Le Pera and T. Colombo, *Int. J. Mol. Sci.*, 2020, **21**, 4684.
- 5 L. Trixl and A. Lusser, *Wiley Interdiscip. Rev.: RNA*, 2019, **10**, e1510.
- 6 G. Guo, K. Pan, S. Fang, L. Ye, X. Tong, Z. Wang, X. Xue and H. Zhang, *Mol. Ther.–Nucleic Acids*, 2021, **26**, 575–593.
- 7 E. Destefanis, G. Avşar, P. Groza, A. Romitelli, S. Torrini, P. Pir, S. G. Conticello, F. Aguilo and E. Dassi, *RNA*, 2021, **27**, 367–389.
- 8 L. Shen, C.-X. Song, C. He and Y. Zhang, *Annu. Rev. Biochem.*, 2014, **83**, 585–614.
- 9 J. Lan, N. Rajan, M. Bizet, A. Penning, N. K. Singh, D. Guallar, E. Calonne, A. Li Greci, E. Bonvin, R. Deplus, P. J. Hsu, S. Nachtergaele, C. Ma, R. Song, A. Fuentes-Iglesias, B. Hassabi, P. Putmans, F. Mies, G. Menschaert, J. J. L. Wong, J. Wang, M. Fidalgo, B. Yuan and F. Fuks, *Nat. Commun.*, 2020, **11**, 4956.
- 10 I. A. Roundtree, M. E. Evans, T. Pan and C. He, *Cell*, 2017, **169**, 1187–1200.
- 11 J. E. Squires, H. R. Patel, M. Nousch, T. Sibbritt, D. T. Humphreys, B. J. Parker, C. M. Suter and T. Preiss, *Nucleic Acids Res.*, 2012, **40**, 5023–5033.
- 12 S. Edelheit, S. Schwartz, M. R. Mumbach, O. Wurtzel and R. Sorek, *PLoS Genet.*, 2013, **9**, e1003602.
- 13 Y. Motorin, F. Lyko and M. Helm, *Nucleic Acids Res.*, 2010, **38**, 1415–1430.
- 14 S. Shinoda, S. Kitagawa, S. Nakagawa, F.-Y. Wei, K. Tomizawa, K. Araki, M. Araki, T. Suzuki and T. Suzuki, *Nucleic Acids Res.*, 2019, **47**, 8734–8745.
- 15 Y. Motorin and M. Helm, *Biochemistry*, 2010, **49**, 4934–4944.
- 16 J. E. Squires and T. Preiss, *Epigenomics*, 2010, **2**, 709–715.
- 17 C. T. Y. Chan, M. Dyavaiah, M. S. DeMott, K. Taghizadeh, P. C. Dedon and T. J. Begley, *PLoS Genet.*, 2010, **6**, e1001247.
- 18 M. Schaefer, T. Pollex, K. Hanna, F. Tuorto, M. Meusburger, M. Helm and F. Lyko, *Genes Dev.*, 2010, **24**, 1590–1595.
- 19 X. Yang, Y. Yang, B.-F. Sun, Y.-S. Chen, J.-W. Xu, W.-Y. Lai, A. Li, X. Wang, D. P. Bhattarai, W. Xiao, H.-Y. Sun, Q. Zhu, H.-L. Ma, S. Adhikari, M. Sun, Y.-J. Hao, B. Zhang, C.-M. Huang, N. Huang, G.-B. Jiang, Y.-L. Zhao, H.-L. Wang, Y.-P. Sun and Y.-G. Yang, *Cell Res.*, 2017, **27**, 606–625.
- 20 Y. Yang, L. Wang, X. Han, W.-L. Yang, M. Zhang, H.-L. Ma, B.-F. Sun, A. Li, J. Xia, J. Chen, J. Heng, B. Wu, Y.-S. Chen, J.-W. Xu, X. Yang, H. Yao, J. Sun, C. Lyu, H.-L. Wang, Y. Huang, Y.-P. Sun, Y.-L. Zhao, A. Meng, J. Ma, F. Liu and Y.-G. Yang, *Mol. Cell*, 2019, **75**, 1188–1202.



- 21 J. I. Young, E. P. Hong, J. C. Castle, J. Crespo-Barreto, A. B. Bowman, M. F. Rose, D. Kang, R. Richman, J. M. Johnson, S. Berget and H. Y. Zoghbi, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 17551–17558.
- 22 T. P. Hoernes, N. Clementi, K. Faserl, H. Glasner, K. Breuker, H. Lindner, A. Hüttenhofer and M. D. Erlacher, *Nucleic Acids Res.*, 2016, **44**, 852–862.
- 23 Q. Li, X. Li, H. Tang, B. Jiang, Y. Dou, M. Gorospe and W. Wang, *J. Cell. Biochem.*, 2017, **118**, 2587–2598.
- 24 G. Guo, H. Wang, X. Shi, L. Ye, K. Yan, Z. Chen, H. Zhang, Z. Jin and X. Xue, *Front. Cell Dev. Biol.*, 2020, **8**, 430.
- 25 X. Chen, A. Li, B.-F. Sun, Y. Yang, Y.-N. Han, X. Yuan, R.-X. Chen, W.-S. Wei, Y. Liu, C.-C. Gao, Y.-S. Chen, M. Zhang, X.-D. Ma, Z.-W. Liu, J.-H. Luo, C. Lyu, H.-L. Wang, J. Ma, Y.-L. Zhao, F.-J. Zhou, Y. Huang, D. Xie and Y.-G. Yang, *Nat. Cell Biol.*, 2019, **21**, 978–990.
- 26 H. Song, J. Zhang, B. Liu, J. Xu, B. Cai, H. Yang, J. Straube, X. Yu and T. Ma, *Biomark. Res.*, 2022, **10**, 15.
- 27 W. Huang, M.-D. Lan, C.-B. Qi, S.-J. Zheng, S.-Z. Wei, B.-F. Yuan and Y.-Q. Feng, *Chem. Sci.*, 2016, **7**, 5495–5502.
- 28 S. M. Huber, P. van Delft, L. Mendil, M. Bachman, K. Smollett, F. Werner, E. A. Miska and S. Balasubramanian, *ChemBioChem*, 2015, **16**, 752–755.
- 29 L. Fu, C. R. Guerrero, N. Zhong, N. J. Amato, Y. Liu, S. Liu, Q. Cai, D. Ji, S.-G. Jin, L. J. Niedernhofer, G. P. Pfeifer, G.-L. Xu and Y. Wang, *J. Am. Chem. Soc.*, 2014, **136**, 11582–11585.
- 30 H.-Y. Zhang, J. Xiong, B.-L. Qi, Y.-Q. Feng and B.-F. Yuan, *Chem. Commun.*, 2016, **52**, 737–740.
- 31 M. Yu, G. C. Hon, K. E. Szulwach, C.-X. Song, L. Zhang, A. Kim, X. Li, Q. Dai, Y. Shen, B. Park, J.-H. Min, P. Jin, B. Ren and C. He, *Cell*, 2012, **149**, 1368–1380.
- 32 R. Lyu, K. Pajdzik, H. Sun, L. Zhang, L. Zhang, T. Wu, L. Yang, T. Pan, C. He and Q. Dai, *Isr. J. Chem.*, 2024, **64**, e202300111.
- 33 M. Tahiliani, K. P. Koh, Y. Shen, W. A. Pastor, H. Bandukwala, Y. Brudno, S. Agarwal, L. M. Iyer, D. R. Liu, L. Aravind and A. Rao, *Science*, 2009, **324**, 930–935.
- 34 S. Kriaucionis and N. Heintz, *Science*, 2009, **324**, 929–930.
- 35 M. Basanta-Sanchez, R. Wang, Z. Liu, X. Ye, M. Li, X. Shi, P. F. Agris, Y. Zhou, Y. Huang and J. Sheng, *ChemBioChem*, 2017, **18**, 72–76.
- 36 I. Rácz, I. Király and D. Lásztily, *Planta*, 1978, **142**, 263–267.
- 37 B. Delatte, F. Wang, L. V. Ngoc, E. Collignon, E. Bonvin, R. Deplus, E. Calonne, B. Hassabi, P. Putmans, S. Awe, C. Wetzels, J. Kreher, R. Soin, C. Creppe, P. A. Limbach, C. Gueydan, V. Kruys, A. Brehm, S. Minakhina, M. Defrance, R. Steward and F. Fuks, *Science*, 2016, **351**, 282–285.
- 38 J. Moriya, T. Yokogawa, K. Wakita, T. Ueda, K. Nishikawa, P. F. Crain, T. Hashizume, S. C. Pomerantz, J. A. McCloskey, G. Kawai, N. Hayashi, S. Yokoyama and K. Watanabe, *Biochemistry*, 1994, **33**, 2234–2239.
- 39 J. P. P. de Barros, G. Ketih, C. E. Adlouni, A. L. Glasser, G. Mack, G. Dirheimer and J. Desgrès, *Nucleic Acids Res.*, 1996, **24**, 1489–1496.
- 40 C. Takemoto, T. Ueda, K.-i. Miura and K. Watanabe, *Nucleic Acids Symp. Ser.*, 1999, **42**, 77–78.
- 41 C. Takemoto, L. L. Spremulli, L. A. Benkowski, T. Ueda, T. Yokogawa and K. Watanabe, *Nucleic Acids Res.*, 2009, **37**, 1616–1627.
- 42 H. Lusic, E. M. Gustilo, F. A. P. Vendeix, R. Kaiser, M. O. Delaney, W. D. Graham, V. A. Moye, W. A. Cantara, P. F. Agris and A. Deiters, *Nucleic Acids Res.*, 2008, **36**, 6548–6557.
- 43 N. Jonkhout, J. Tran, M. A. Smith, N. Schonrock, J. S. Mattick and E. M. Novoa, *RNA*, 2017, **23**, 1754–1769.
- 44 X. Wang, X. Jin and L. Cheng, *Isr. J. Chem.*, 2024, **64**, e202300178.
- 45 C. Rimpl, A. Lusser, E. Ennifar and R. Micura, *J. Org. Chem.*, 2017, **82**, 7939–7945.
- 46 M. Sierant, G. Leszczynska, K. Sadowska, A. Dziergowska, M. Rozanski, E. Sochacka and B. Nawrot, *Nucleic Acids Res.*, 2016, **44**, 10986–10998.
- 47 H. Sun, J. Sheng, A. E. A. Hassan, S. Jiang, J. Gan and Z. Huang, *Nucleic Acids Res.*, 2012, **40**, 5171–5179.
- 48 P. Szczupak, M. Sierant, E. Wielgus, E. Radzikowska-Cieciura, K. Kulik, A. Krakowiak, P. Kuwerska, G. Leszczynska and B. Nawrot, *Cells*, 2022, **11**, 1522.
- 49 A. V. Kachalova, E. M. Zubin and T. S. Oretskaya, *Russ. Chem. Rev.*, 2002, **71**, 1041–1059.
- 50 L. Flemmich, R. Bereiter and R. Micura, *Angew. Chem., Int. Ed.*, 2024, **63**, e202403063.
- 51 L. C. Sowers and G. P. Beardsley, *J. Org. Chem.*, 1993, **58**, 1664–1665.
- 52 N. Shakya, N. C. Srivastav, S. Bhavanam, C. Tse, N. Desroches, B. Agrawal, D. Y. Kunimoto and R. Kumar, *Bioorg. Med. Chem.*, 2012, **20**, 4088–4097.
- 53 K. Bartosik and G. Leszczynska, *Tetrahedron Lett.*, 2015, **56**, 6593–6597.
- 54 K. Bartosik, E. Sochacka and G. Leszczynska, *Org. Biomol. Chem.*, 2017, **15**, 2097–2103.
- 55 C. Rimpl and R. Micura, RNA Methylation: Methods and Protocols, in *Methods Mol. Biol.*, 2017, vol. 1562, pp. 295–302.
- 56 C. Rimpl and R. Micura, *Synthesis*, 2016, **48**, 1108–1116.
- 57 A. A. Tanpure and S. Balasubramanian, *ChemBioChem*, 2017, **18**, 2236–2241.
- 58 I. N. Michaelides, N. Tago, B. Viverge and T. Carell, *Chem. – Eur. J.*, 2017, **23**, 15894–15898.
- 59 M. de Kort, P. C. de Visser, J. Kurzeck, N. J. Meeuwenoord, G. A. van der Marel, W. Rüger and J. H. van Boom, *Eur. J. Org. Chem.*, 2001, 2075–2082.
- 60 R. A. Johnsson, J. J. Bogojeski and M. J. Damha, *Bioorg. Med. Chem. Lett.*, 2014, **24**, 2146–2149.
- 61 M. P. Reddy, N. B. Hanna and F. Farooqui, *Tetrahedron Lett.*, 1994, **35**, 4311–4314.
- 62 S. Tardy-Planechaud, J. Fujimoto, S. S. Lin and L. C. Sowers, *Nucleic Acids Res.*, 1997, **25**, 553–558.
- 63 A. S. Hansen, A. Thalhammer, A. H. El-Sagheer, T. Brown and C. J. Schofield, *Bioorg. Med. Chem. Lett.*, 2011, **21**, 1181–1184.
- 64 S. Xuan, Q. Wu, L. Cui, D. Zhang and F. Shao, *Bioorg. Med. Chem. Lett.*, 2015, **25**, 1186–1191.



- 65 K. Sugizaki, S. Ikeda, H. Yanagisawa and A. Okamoto, *Org. Biomol. Chem.*, 2011, **9**, 4176–4181.
- 66 Q. Dai and C. He, *Org. Lett.*, 2011, **13**, 3446–3449.
- 67 Q. Dai, C.-X. Song, T. Pan and C. He, *J. Org. Chem.*, 2011, **76**, 4182–4188.
- 68 M. Münzel, D. Globisch, C. Trindler and T. Carell, *Org. Lett.*, 2010, **12**, 5671–5673.
- 69 M. Münzel, U. Lischke, D. Stathis, T. Pfaffeneder, F. A. Gnerlich, C. A. Deiml, S. C. Koch, K. Karaghiosoff and T. Carell, *Chem. – Eur. J.*, 2011, **17**, 13782–13788.
- 70 A. S. Schröder, O. Kotljarova, E. Parsa, K. Iwan, N. Raddaoui and T. Carell, *Org. Lett.*, 2016, **18**, 4368–4371.
- 71 A. S. Schröder, J. Steinbacher, B. Steigenberger, F. A. Gnerlich, S. Schiesser, T. Pfaffeneder and T. Carell, *Angew. Chem., Int. Ed.*, 2014, **53**, 315–318.
- 72 D.-Z. Yang, Z.-Z. Chen, M. Chi, Y.-Y. Dong, S.-Z. Pu and Q. Sun, *Molecules*, 2022, **27**, 749.
- 73 S. A. Scaringe, C. Francklyn and N. Usman, *Nucleic Acids Res.*, 1990, **18**, 5433–5441.
- 74 T. Wu, K. K. Ogilvie and R. T. Pon, *Nucleic Acids Res.*, 1989, **17**, 3501–3517.
- 75 S. Schiesser, T. Pfaffeneder, K. Sadeghian, B. Hackner, B. Steigenberger, A. S. Schröder, J. Steinbacher, G. Kashiwazaki, G. Höfner, K. T. Wanner, C. Ochsenfeld and T. Carell, *J. Am. Chem. Soc.*, 2013, **135**, 14593–14599.
- 76 K. Podskoczyj, K. Kulik, J. Wasko, B. Nawrot, T. Suzuki and G. Leszczynska, *Chem. Commun.*, 2021, **57**, 12540–12543.
- 77 S. A. Hartsel, D. E. Kitchen, S. A. Scaringe and W. S. Marshall, Oligonucleotide Synthesis, in *Methods Mol. Biol.*, 2005, vol. 288, pp. 33–49.
- 78 N. Karino, Y. Ueno and A. Matsuda, *Nucleic Acids Res.*, 2001, **29**, 2456–2463.
- 79 M. Sundaram, P. F. Crain and D. R. Davis, *J. Org. Chem.*, 2000, **65**, 5609–5614.
- 80 A. C. Bajji, M. Sundaram, D. G. Myszka and D. R. Davis, *J. Am. Chem. Soc.*, 2002, **124**, 14302–14303.
- 81 A. C. Bajji and D. R. Davis, *J. Org. Chem.*, 2002, **67**, 5352–5358.
- 82 M. Eshete, M. T. Marchbank, S. L. Deutscher, B. Sproat, G. Leszczynska, A. Malkiewicz and P. F. Agris, *Protein J.*, 2007, **26**, 61–73.
- 83 G. Leszczynska, J. Pięta, K. Wozniak and A. Malkiewicz, *Org. Biomol. Chem.*, 2014, **12**, 1052–1056.
- 84 G. Leszczynska, P. Leonczak, A. Dziergowska and A. Malkiewicz, *Nucleosides, Nucleotides Nucleic Acids*, 2013, **32**, 599–616.
- 85 S. Schiesser, B. Hackner, T. Pfaffeneder, M. Müller, C. Hagemeyer, M. Truss and T. Carell, *Angew. Chem., Int. Ed.*, 2012, **51**, 6516–6520.
- 86 Y. Nomura, N. Haginoya, Y. Ueno and A. Matsuda, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 2811–2816.
- 87 Y. Ito, M. Matsuo, K. Yamamoto, W. Yamashita, T. Osawa and Y. Hari, *Tetrahedron*, 2018, **74**, 6854–6860.
- 88 Y. Ito, K. Yamamoto and Y. Hari, *Curr. Protoc. Nucleic Acid Chem.*, 2019, **78**, e91.
- 89 K. Podskoczyj, A. Klos, S. Drewniak and G. Leszczynska, *Org. Biomol. Chem.*, 2023, **21**, 2809–2815.
- 90 K. Podskoczyj, A. Kuszczynska, A. Dziergowska and G. Leszczynska, *Curr. Protoc.*, 2024, **4(2)**, e984.

