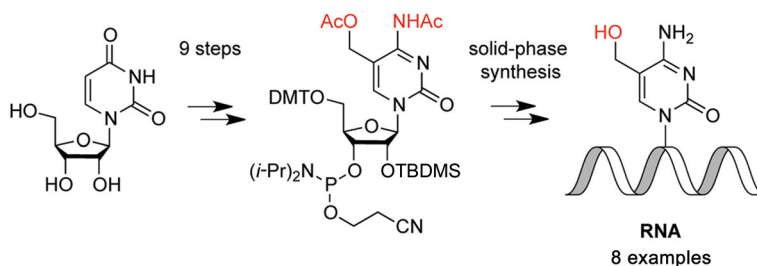


# Synthesis of 5-Hydroxymethylcytidine- and 5-Hydroxymethyluridine-Modified RNA

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**Abstract** We report on the syntheses of 5-hydroxymethyl-uridine [5hm(rU)] and -cytidine [5hm(rC)] phosphoramidites and their incorporation into RNA by solid-phase synthesis. Deprotection of the oligonucleotides is accomplished in a straightforward manner using standard conditions, confirming the appropriateness of the acetyl protection used for the pseudobenzyl alcohol moieties. The approach provides robust access to 5hm(rC/U)-modified RNAs that await applications in pull-down experiments to identify potential modification enzymes. They will also serve as synthetic probes for the development of high-throughput-sequencing methods in native RNAs.

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**Key words** nucleosides, oligonucleotides, modification, methylation, epigenetics

## Introduction

The nucleobase modification 5-methylcytosine (5mC) shows widespread occurrence in DNA and RNA.<sup>1</sup> Currently, the vast majority of efforts to understand the role of cytosine methylation, its function and its metabolism is focused on DNA because of its prominent role in epigenetics.<sup>2</sup> Epigenetic research seeks to describe cellular and physiological phenotypic-trait variations that are caused by external or environmental factors that switch genes on and off. DNA

methylation–demethylation is such a mechanism that triggers functionally relevant changes which determine how genes are expressed without altering the underlying DNA sequence, and thus generates dynamic modulations in the transcriptional profile of a cell.<sup>3</sup> While the molecular basis of cytosine methylation has been well studied over the decades, and a lot is known on the enzymatic installation of a methyl group by cytosine-5 methyltransferases involving the cofactors *S*-adenosyl methionine (SAM) or cobalamin (Cbl),<sup>4,5</sup> the demethylation of 5mC has been explored only recently in detail.<sup>6,7</sup> One of the main demethylation pathways of 5mC suggests 5-hydroxymethylcytosine (5hmC) to be the first degradation product of enzymatic oxidation by ketoglutarate-dependent hydroxylases of the ten-eleven-translocation (TET) family of proteins.<sup>8</sup>

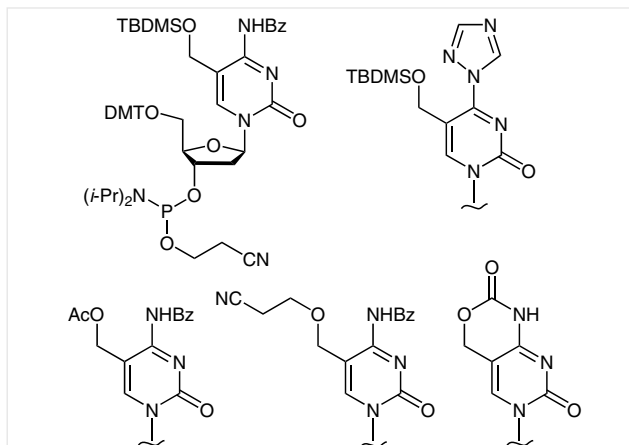
In RNA, 5mC is also prevalent,<sup>9–12</sup> however, little is known if this modification is dynamic and plays a role in post-transcriptional regulation.<sup>13</sup> In the emerging field of RNA epigenetics,<sup>14</sup> the question if a similar oxidative reversal pathway may also work for the 5mC modification in RNA has challenged several laboratories, but to the best of our knowledge, the native enzymes catalyzing such reactions in RNA have not yet been identified.<sup>15</sup>

The present work aims at generating synthetic RNA probes that contain 5-hydroxymethyluridine [5hm(rU)] and/or 5-hydroxymethylcytidine [5hm(rC)] modifications. Synthetic access to site-specifically modified 5hm(rC/U)-containing RNA provides the foundation for pull-down experiments which are needed to identify proteins that might specifically recognize and process these modifications in RNA.<sup>16,17</sup> Moreover, efficient access to 5hm(rU)- and/or 5hm(rC)-containing probes will also be invaluable to develop high-throughput-sequencing methods to identify these modifications in native RNA.<sup>18–20</sup>

## 2 Protection Strategies Reported for the Synthesis of 5hm(dC)-Modified DNA

On first glance, 5-hydroxymethylcytosine-modified nucleic acids appear to be easily accessible by chemical synthesis. However, this modification has been reported to be rather challenging for solid-phase approaches because of the observed  $S_N$  reactivity that originates from its pseudo-benzylic character.<sup>21,22</sup> With respect to oligodeoxynucleotide synthesis, several distinct protecting groups have been described in the literature for the 5-hydroxymethyl group of 5-hydroxymethyl-2'-deoxycytidine [5hm(dC)] (Figure 1). Among these are the 2-cyanoethyl,<sup>23,24</sup> acetyl,<sup>25</sup> and *tert*-butyldimethylsilyl (TBDMS) groups.<sup>26</sup> Additionally, the simultaneous protection of the 5-hydroxymethyl and the C4-NH<sub>2</sub> group as a cyclic carbamate has been described.<sup>27,28</sup> Although these approaches have their merits, they are also not entirely satisfying. For instance, post-synthetic removal of the cyanoethyl protecting group was reported to be troublesome with respect to completeness.<sup>23</sup> Also, not fully convincing was that carbamate deprotection required NaOH in methanol/water instead of the standard DNA deprotection (ammonolysis) to avoid aminocarbonyl- $N^4$  and 5-aminomethylcytosine byproducts.<sup>27</sup> When 5hm(dC) was protected with 5-CH<sub>2</sub>-OTBDMS together with  $N^4$ -benzoyl, the deprotection procedure came closest to standard DNA deprotection, however, extended reaction times and elevated temperatures had to be applied in order to demask the 5-CH<sub>2</sub>OH group completely and simultane-

ously with the base-labile protecting groups in concentrated aqueous ammonium hydroxide solution.<sup>26</sup>



**Figure 1** Different protection patterns of 5-hydroxymethyl-2'-deoxycytidine phosphoramidites for the solid-phase synthesis of 5hm(dC)-containing DNA that have been reported in the literature

## 3 Synthesis of 5-Hydroxymethylpyrimidine-Modified RNA

To the best of our knowledge, the synthesis of 5-hydroxymethylcytidine [(5hm(rC))] modified RNA has not yet been accomplished. To achieve this aim, we considered acetylation of both the 5-hydroxymethyl and the  $N^4$  exocyclic amino groups as a promising protection pattern for

### Biographical Sketches



**Christian Rimpl** was born in Zams (Austria) in 1986. He received his Magister degree in chemistry in 2012 from the Leopold-Franzens University in Innsbruck. In 2013, he was awarded a Marshall Plan Schol-

arship to conduct research at the University of New Orleans (USA). He returned to Innsbruck to successfully complete the teacher training program in chemistry and biology at the University of Innsbruck in 2014.

He is currently pursuing his Ph.D. degree in organic chemistry under the supervision of Ronald Micura. His research focuses on modified nucleosides in the context of RNA epigenetics.



**Ronald Micura** was born in 1970, and studied chemistry at the Johannes-Kepler University in Linz (Austria). He received his Ph.D. in the field of phycobilin pigments under the supervision of Karl Grubmayr in 1995. Immediately thereafter he joined the laboratory of Albert Eschen-

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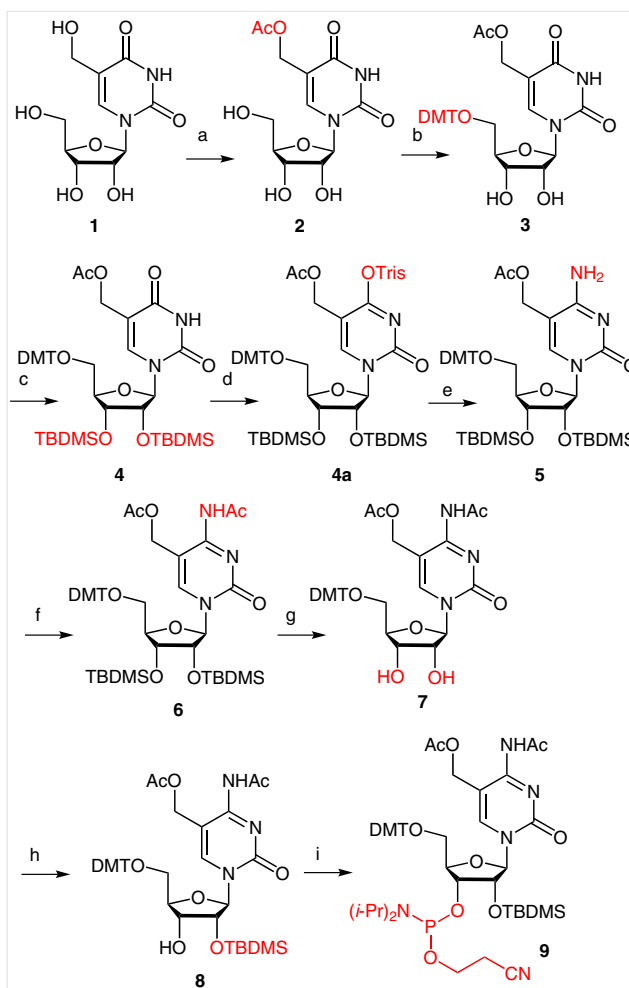
from the Austrian Academy of Science (OeAW) at the University of Linz. In 2004, he was appointed Professor of Organic Chemistry at the Leopold-Franzens University in Innsbruck (Austria). His research focuses on the chemistry, chemical biology, and biophysics of RNA.

building blocks that can be utilized in RNA solid-phase synthesis. At first sight, such a concept may be surprising since it was reported earlier that the incorporation of an *O*-acetylated and *N*<sup>4</sup>-benzoylated 5hm(dC) building block into DNA gave undesired benzamide byproducts that originated from nucleophilic displacement of the acetate moiety and migration of the benzoyl group during oligonucleotide deprotection with ammonium hydroxide.<sup>25</sup> However, in contrast to synthetic DNA, RNA is routinely deprotected by applying more nucleophilic conditions of methylamine in ethanol/water, or alternatively, of methylamine and ammonia in water. Under these conditions, RNA is deprotected very fast and cleanly; importantly, acetyl (instead of benzoyl) protection has to be used for the exocyclic *N*<sup>4</sup> amino group to avoid transamination at C4 by methylamine.<sup>29,30</sup> Considering all the above arguments, we set out to synthesize and evaluate the bisacetylated 5hm(rC) building block **9** for RNA solid-phase synthesis.

### 3.1 Synthesis of 5hm(rC) Phosphoramidite

We conceived the synthesis of 5hm(rC) phosphoramidite **9** from nucleoside **1** [5hm(rU)] as the starting point for two reasons (Scheme 1). First, access to 5hm(rU) **1** (or derivatives thereof) has been described,<sup>31–36</sup> and second, we considered it desirable to introduce a divergent synthetic pathway for phosphoramidites of both nucleosides 5hm(rU) and 5hm(rC). Hence, a uracil-into-cytosine conversion was envisaged, a transformation that is well established for various pyrimidine nucleoside modifications, with a wide range of conditions accepted.<sup>37–39</sup>

We started the synthesis with selective substitution of the hydroxy group of 5-hydroxy-methylated uridine 5hm(rU) **1** by acetic acid using a catalytic amount of trifluoroacetic acid (Scheme 1).<sup>31</sup> It should be pointed out that reaction times longer than 40 minutes resulted in additional acetylation of the ribose hydroxy groups. The monoacetylated derivative **2** was then transformed into the dimethoxy-tritylated compound **3**, followed by protection of the 2'- and 3'-hydroxy groups as TBDMS silyl ethers to yield nucleoside **4**. Next, reaction of **4** with 2,4,6-triisopropylbenzenesulfonyl chloride in the presence of triethylamine and DMAP in dichloromethane resulted in regioselective *O*<sup>4</sup>-tritylation. After work-up, the trisylated derivative **4a** could be used without further purification for the conversion into **5** upon treatment with aqueous ammonium hydroxide in THF. Acetylation of the amino function was achieved with acetic anhydride in pyridine to provide **6**, followed by cleavage of the 2'- and 3'-OTBDMS groups with TBAF (1 M) and acetic acid (0.5 M) in THF to give **7**. Selective silylation with TBDMSCl using silver nitrate according to Ogilvie<sup>40</sup> provided the 2'-OTBDMS-protected derivative **8** in 43% yield. To increase the overall yield, the undesired 3'-*O*-silylated regioisomer was treated with triethylamine in methanol,



**Scheme 1** Synthesis of 5hm(rC) phosphoramidite **9**. Reaction conditions: (a) TFA (0.0002 equiv), AcOH, reflux, 40 min, 62%; (b) DMT-Cl (1.2 equiv), DMAP (0.025 equiv), pyridine, 5 h, r.t., 65%; (c) TBDMSCl (3.0 equiv), imidazole (6.0 equiv), DMF, r.t., 5 h, 80%; (d) 2,4,6-triisopropylbenzenesulfonyl chloride (1.5 equiv), Et<sub>3</sub>N (10.0 equiv), DMAP (0.12 equiv), CH<sub>2</sub>Cl<sub>2</sub>, r.t., 1 h, no purification; (e) aq NH<sub>3</sub> (32%), THF, r.t., 3 h, 44% over 2 steps; (f) Ac<sub>2</sub>O (2.5 equiv), pyridine, 0 °C to r.t., 2 h, 76%; (g) 1 M TBAF/0.5 M AcOH, THF, r.t., 3 h, 75%; (h) AgNO<sub>3</sub> (1.6 equiv), TBDMSCl (1.6 equiv), pyridine (3.5 equiv), THF, r.t., 4 h, 43%; (i) CEP-Cl (2.0 equiv), DIPEA (4.0 equiv), 1-methylimidazole (0.5 equiv), CH<sub>2</sub>Cl<sub>2</sub>, r.t., 5 h, 79%.

with equilibration again generating a favorable distribution between the 2' and 3' regioisomers. Finally, phosphitylation was executed with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (CEP-Cl) in the presence of *N,N*-diisopropylethylamine in CH<sub>2</sub>Cl<sub>2</sub>. Starting with compound **1**, our route provides product **9** in a 3% overall yield in nine steps and with eight chromatographic purifications; in total, 0.5 gram of **9** was obtained in the course of this study.

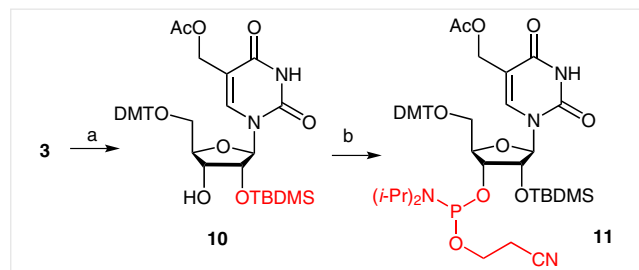
Concerning the overall synthetic strategy for phosphoramidite **9**, we mention that we did not consider 2'-*O*-tomylation of intermediate **3**, followed by 3'-*O*-acetylation and

U-to-C transformation.<sup>37–39</sup> The reason being that such a path would require selective cleavage of the temporary 3'-OAc group in the presence of the 5-AcOCH<sub>2</sub> and NHAc moieties, prior to transformation into phosphoramidite **9**. Another reason was that the yields for introducing the 2'-O-[(triisopropylsilyl)oxy]methyl (TOM) group on uridines are generally lower compared to the introduction of the 2'-OTBDMS group.<sup>40–42</sup>

Furthermore, we found that attempts to shorten the reaction sequence leading from compound **6** directly to **8** (by deprotection of one of the 2'-O/3'-OTBDMS groups and subsequent equilibration) suffered from good reproducibility, so that we recommend the here-elaborated path for reasons of robustness.

### 3.2 Synthesis of 5hm(rU) Phosphoramidite

As mentioned above, a divergent synthetic route was designed to allow access also to 5hm(rU) phosphoramidite building blocks. Starting from derivative **3** (Scheme 2), silylation with TBDMSCl using silver nitrate according to Ogilvie<sup>40</sup> yielded the 2'-OTBDMS-protected derivative **10** with remarkable regioselectivity over the 3'-O-silylated product (5:1). Phosphitylation was executed with CEP-Cl in the presence of *N,N*-diisopropylethylamine in THF. Starting from 5hm(rU) **1**, the corresponding phosphoramidite **11** was isolated in an overall yield of 14% over four steps with four chromatographic purifications; in total, 0.6 gram of **11** was obtained in the course of this study.

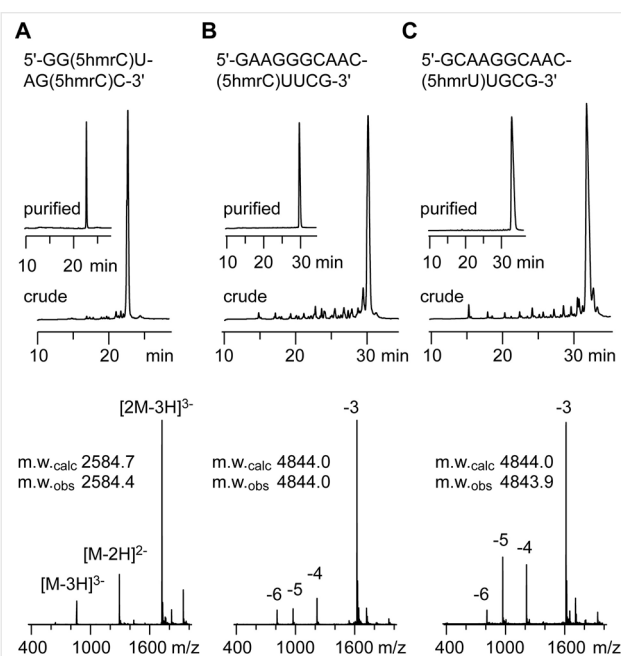


**Scheme 2** Synthesis of 5hm(rU) phosphoramidite **11**. Reaction conditions: (a) AgNO<sub>3</sub> (1.6 equiv), TBDMSCl (1.6 equiv), pyridine (3.5 equiv), THF, r.t., 4 h, 44%; (b) CEP-Cl (2.0 equiv), DIPEA (4.0 equiv), 1-methylimidazole (0.5 equiv), CH<sub>2</sub>Cl<sub>2</sub>, r.t., 5 h, 80%.

### 3.3 Synthesis of 5hm(rC)- and 5hm(rU)-Modified RNA

The solid-phase synthesis of RNA with site-specific 5hm(rC/U) modifications was performed following the TOM approach.<sup>41,43,44</sup> Coupling yields of the novel building block were higher than 98% according to the trityl assay. Cleavage of the synthetic RNA strands from the solid support and their deprotections were performed using methyl-

amine in ethanol/water, or alternatively methylamine/ammonia in water followed by treatment with TBAF in THF. Salts were removed by size-exclusion chromatography on a Sephadex G25 column. In general, the crude products gave a major product peak in anion-exchange (AE) HPLC analysis. RNA oligomers were purified by AE chromatography under strong denaturing conditions (6 M urea, 80 °C) (Figure 2). The molecular weights of the purified RNAs were confirmed by liquid chromatography–electrospray ionization (LC–ESI) mass spectrometry (MS). The synthesized RNAs containing 5hm(rC) and 5hm(rU) modifications are listed in Table 1.



**Figure 2** Characterization of 5hm(rC)- and 5hm(rU)-modified RNA. Anion-exchange HPLC traces (top) of: (A) 8 nt RNA, (B) 15 nt RNA, and (C) 15 nt RNA, and the corresponding LC–ESI mass spectra (bottom). HPLC conditions: Dionex DNAPac column (4 × 250 mm), 80 °C, 1 mL min<sup>-1</sup>, 0–60% buffer B in buffer A within 45 min; buffer A: Tris-HCl (25 mM), urea (6 M), pH 8.0; buffer B: Tris-HCl (25 mM), urea (6 M), NaClO<sub>4</sub> (0.5 M), pH 8.0. See the experimental for LC–ESI MS conditions

It is noteworthy that minor amounts of byproducts (<15% of the crude deprotected material) that originated from substitution reactions at the 5-acetyloxymethyl group by methylamine during RNA deprotection were found, as analyzed by LC–ESI mass spectrometry. However, these byproducts were easily separated from the desired product during anion-exchange and/or reversed-phase chromatography. In this context, deprotection using equal volumes of aqueous methylamine (40%) and saturated aqueous NH<sub>3</sub> solutions appeared slightly favorable over 'methylamine alone' conditions.

**Table 1** Selection of Synthesized RNAs with 5hm(rC), 5hm(rU) and 5m(rC) Modifications<sup>a</sup>

Sequence (5'→3')	Amount (nmol)	MW <sub>calcd</sub> (amu)	MW <sub>obsd</sub> (amu)
GU( <sup>hm</sup> C)ACC (6 nt)	280	1864.2	1863.9
GG( <sup>hm</sup> C)UAG( <sup>hm</sup> C)C (8 nt)	43	2584.6	2584.4
GAAGGGCAAC( <sup>hm</sup> C)UUCG (15 nt)	215	4844.0	4844.0
GCGAACCU( <sup>hm</sup> C)GGGUUCG (17 nt)	89	5487.3	5487.2
GU( <sup>hm</sup> U)ACC (6 nt)	354	1865.2	1865.0
GUC( <sup>hm</sup> U)AGAC (8 nt)	326	2539.6	2539.6
GCAAGGCAAC( <sup>hm</sup> U)UGCG (15 nt)	161	4844.0	4843.9
AUCUGCUUG( <sup>m</sup> C)( <sup>hm</sup> C)( <sup>m</sup> C)AUCGGGG( <sup>hm</sup> C)CGCGGAU (27 nt)	70	8704.2	8705.2

<sup>a</sup> (<sup>hm</sup>C) = 5-Hydroxymethylcytidine, (<sup>hm</sup>U) = 5-hydroxymethyluridine, (<sup>m</sup>C) = 5-methylcytidine.

## 4 Conclusions

We have elaborated the syntheses of 5-acetyloxy-methylated cytidine and uridine phosphoramidites and the incorporation of these building blocks into RNA by solid-phase synthesis. Importantly, we have demonstrated that subsequent deprotection of the oligoribonucleotides is straightforward using aqueous methylamine/ammonia solutions. Only very minor amounts of side products that resulted from the S<sub>N</sub> reactivity at the pseudobenzylic position of the 5-hydroxymethyl residues were observed. Consequently, this approach delivers high-quality 5hm(rC/U)-modified RNAs as valuable probes in the search for related modification enzymes and for the development of high-throughput-sequencing methods in the emerging field of RNA epigenetics.

All reactions were carried out under an argon atmosphere. Chemical reagents and solvents were purchased from commercial suppliers and were used without further purification. Organic solvents for reactions were dried overnight over freshly activated molecular sieves (3 Å). Analytical thin-layer chromatography (TLC) was carried out on Macherey-Nagel Polygram SIL G/UV<sub>254</sub> plates. Flash column chromatography was carried out on Sigma Aldrich silica gel 60 (70–230 mesh). <sup>1</sup>H, <sup>13</sup>C and <sup>31</sup>P NMR spectra were recorded on a Bruker DRX 300 MHz instrument. The chemical shifts are referenced to the residual proton signal of the deuterated solvents: CDCl<sub>3</sub> (7.26 ppm), DMSO-*d*<sub>6</sub> (2.49 ppm) for <sup>1</sup>H NMR spectra; CDCl<sub>3</sub> (77.0 ppm) or DMSO-*d*<sub>6</sub> (39.5 ppm) for <sup>13</sup>C NMR spectra. <sup>1</sup>H and <sup>13</sup>C NMR assignments were based on COSY and HSQC experiments. MS experiments were performed on a Finnigan LCQ Advantage MAX ion trap instrument.

### 5-Acetyloxymethyluridine (2)

5-Hydroxymethyluridine (**1**) (obtained by various routes as described in references 31–33) (3.3 g, 12 mmol) was suspended in glacial AcOH (100 mL). TFA (0.2 mL) was added and the mixture was refluxed for 40 min. AcOH was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 95:5 to 90:10). Although the product **2** can be contaminated with up to 10% of the 5'-O-acetylated regioisomer, it should be used

without further purification in the next step. This byproduct becomes easily separated from compound **3** by column chromatography (see below).

Yield: 2.4 g, 7.5 mmol (62%); white powder; *R*<sub>f</sub> = 0.7 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 75:25).

<sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 2.00 (s, 3 H, H<sub>3</sub>C-CO), 3.57 (m, 1 H, H(1)-C(5')), 3.63 (m, 1 H, H(2)-C(5')), 3.85 (m, 1 H, H-C(4')), 3.94–4.07 (m, 2 H, H-C(3'), H-C(2')), 4.68 (s, 2 H, H<sub>2</sub>C-C(5')), 5.08–5.10 (m, 2 H, HO-C(5'), HO-C(3')), 5.38 (d, *J* = 5.5 Hz, 1 H, HO-C(2')), 5.77 (d, *J* = 5.1 Hz, 1 H, H-C(1')), 8.08 (s, 1 H, H-C(6)), 11.49 (s, 1 H, H-N(3)).

<sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 21.24 (COCH<sub>3</sub>), 59.57 (CH<sub>2</sub>OH), 61.34 (C(5')), 70.33 (C(3')), 74.22 (C(2')), 85.46 (C(4')), 88.45 (C(1')), 108.86 (C(5)), 141.31 (C(6)), 151.06 (C(2)), 163.01 (C(4)), 170.83 (CO-CH<sub>3</sub>).

MS (ESI): *m/z* [M + H]<sup>+</sup> calcd for C<sub>12</sub>H<sub>16</sub>N<sub>2</sub>O<sub>8</sub>: 317.10; found: 316.93.

### 5-Acetyloxymethyl-5'-O-(4,4'-dimethoxytrityl)uridine (3)

5-Acetyloxymethyluridine (**2**) (2.4 g, 7.5 mmol) and DMAP (20 mg, 0.18 mmol) were dissolved in dry pyridine (10 mL). 4,4'-Dimethoxytrityl chloride (DMT-Cl) (3 g, 9 mmol) was added in two portions. The reaction was stirred for 5 h at r.t., and the progress monitored by TLC. The pyridine was removed under reduced pressure, the residue dissolved in CH<sub>2</sub>Cl<sub>2</sub> and extracted with 5% citric acid (2 ×), sat. aq. NaHCO<sub>3</sub> solution and sat. aq. NaCl solution. The organic phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and coevaporated twice with toluene and with CH<sub>2</sub>Cl<sub>2</sub>. The crude product was purified by column chromatography on silica gel (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 98:2–96:4).

Yield: 3 g, 4.8 mmol (65%); white foam; *R*<sub>f</sub> = 0.45 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 95:5).

<sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = 1.86 (s, 3 H, H<sub>3</sub>C-CO), 3.15–3.28 (m, 2 H, H<sub>2</sub>C-C(5')), 3.73 (s, 6 H, H<sub>3</sub>C-O), 3.97 (m, 1 H, H-C(4')), 4.07 (m, 1 H, H-C(3')), 4.16 (m, 1 H, H-C(2')), 4.29 (m, 2 H, H<sub>2</sub>C-C(5')), 5.17 (d, *J* = 5.7 Hz, 1 H, HO-C(3')), 5.50 (d, *J* = 5.4 Hz, 1 H, HO-C(2')), 5.79 (d, *J* = 4.6 Hz, 1 H, H-C(1')), 6.86–6.89 (m, 4 H, H-C(Ar)), 7.22–7.39 (m, 9 H, H-C(Ar)), 7.79 (s, 1 H, H-C(6)), 11.57 (s, 1 H, H-N(3)).

<sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = 21.02 (COCH<sub>3</sub>), 55.60 (2 × OCH<sub>3</sub>), 59.28 (CH<sub>2</sub>OH), 64.11 (C(5')), 70.55 (C(3')), 73.86 (C(2')), 83.45 (C(4')), 86.42 (t-C(DMT)), 89.28 (C(1')), 108.96 (C(5)), 113.84 (C(Ar)), 127.38 (C(Ar)), 128.30 (C(Ar)), 128.49 (C(Ar)), 130.30 (C(Ar)), 135.85 (C(Ar)), 141.53 (C(6)), 145.16 (C(Ar)), 150.95 (C(2)), 158.74 (C-OCH<sub>3</sub>(Ar)), 162.98 (C(4)), 170.55 (COCH<sub>3</sub>).

MS (ESI): *m/z* [M + Na]<sup>+</sup> calcd for C<sub>33</sub>H<sub>34</sub>N<sub>2</sub>O<sub>10</sub>: 641.20; found: 641.15.



**5-Acetyloxymethyl-5'-O-(4,4'-dimethoxytrityl)-2',3'-di-O-tert-butylidimethylsilyluridine (4)**

Compound **3** (3 g, 4.8 mmol) was dissolved in DMF (15 mL). Imidazole (1.95 g, 28.8 mmol) and TBDMSCl (2.16 g, 14.4 mmol) were added consecutively, and the solution was stirred at r.t. for 5 h. The excess of silyl reagent was quenched by slow addition of MeOH (10 mL). H<sub>2</sub>O (500 mL) was added and the mixture was extracted with EtOAc (3 × 200 mL). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> and the solvents evaporated. The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 2:1).

Yield: 3.4 g, 4.1 mmol (80%); white solid; *R*<sub>f</sub> = 0.6 (hexane/EtOAc, 50:50).

<sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = −0.08 (s, 3 H, H<sub>3</sub>C-Si), 0.00 (s, 3 H, H<sub>3</sub>C-Si), 0.03 (s, 6 H, H<sub>3</sub>C-Si), 0.74 (s, 9 H, (H<sub>3</sub>C)<sub>3</sub>-C), 0.84 (s, 9 H, (H<sub>3</sub>C)<sub>3</sub>-C), 1.83 (s, 3 H, H<sub>3</sub>C-CO), 3.23–3.35 (m, 2 H, H<sub>2</sub>-C(5')), 3.73 (s, 6 H, H<sub>3</sub>C-O), 3.99 (m, 1 H, H-C(4')), 4.07 (m, 1 H, H-C(3')), 4.16 (m, 1 H, H-C(2')), 4.29–4.42 (m, 2 H, H<sub>2</sub>-C(5)), 5.77 (d, *J* = 4.3 Hz, 1 H, H-C(1')), 6.86–6.89 (m, 4 H, H-C(Ar)), 7.22–7.39 (m, 9 H, H-C(Ar)), 7.82 (s, 1 H, H-C(6)), 11.62 (s, 1 H, H-N(3)).

<sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = −4.30 (4 × CH<sub>3</sub>-Si), 18.16 (2 × C(CH<sub>3</sub>)<sub>3</sub>), 21.02 (COCH<sub>3</sub>), 26.19 (6 × CH<sub>3</sub>-C-Si), 55.63 (2 × OCH<sub>3</sub>), 59.13 (CH<sub>2</sub>OH), 63.61 (C(5')), 72.16 (C(3')), 75.09 (C(2')), 83.89 (C(4')), 86.77 (t-C(DMT)), 88.90 (C(1')), 109.13 (C(5)), 113.84 (C(Ar)), 127.49 (C(Ar)), 128.30 (C(Ar)), 128.49 (C(Ar)), 130.30 (C(Ar)), 135.60 (C(Ar)), 140.93 (C(6)), 145.02 (C(Ar)), 150.84 (C(2)), 158.86 (C-OCH<sub>3</sub>(Ar)), 162.93 (C(4)), 170.55 (COCH<sub>3</sub>).

MS (ESI): *m/z* [M + Na]<sup>+</sup> calcd for C<sub>45</sub>H<sub>62</sub>N<sub>2</sub>O<sub>10</sub>Si<sub>2</sub>: 869.38; found: 869.40.

**5-Acetyloxymethyl-5'-O-(4,4'-dimethoxytrityl)-2',3'-di-O-tert-butylidimethylsilyl-O<sup>4</sup>-(2,4,6-triisopropylbenzenesulfonyl)uridine (4a)**

To a solution of compound **4** (3.4 g, 4.1 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (20 mL) was added DMAP (40 mg, 0.36 mmol) and Et<sub>3</sub>N (5.6 mL, 41 mmol). 2,4,6-Triisopropylbenzenesulfonyl chloride (1.8 g, 6.15 mmol) was added slowly, and the solution was stirred for 1 h at r.t. The reaction mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub>, washed with sat. NaHCO<sub>3</sub> solution, and the organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated. The crude product **4a** was obtained as a yellow foam and was used for the next step without further purification. An analytical sample of **4a** was purified by column chromatography on silica gel (hexane/EtOAc, 90:10–85:15).

*R*<sub>f</sub> = 0.5 (hexane/EtOAc, 75:25).

<sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = −0.14 (s, 3 H, H<sub>3</sub>C-Si), −0.07 (s, 3 H, H<sub>3</sub>C-Si), −0.04 (s, 3 H, H<sub>3</sub>C-Si), −0.03 (s, 3 H, H<sub>3</sub>C-Si), 0.67 (s, 9 H, (H<sub>3</sub>C)<sub>3</sub>-C), 0.77 (s, 9 H, (H<sub>3</sub>C)<sub>3</sub>-C), 1.20–1.25 (m, 18 H, (H<sub>3</sub>C)<sub>2</sub>CH), 1.85 (s, 3 H, H<sub>3</sub>C-CO), 2.95 (m, 1 H, (H<sub>3</sub>C)<sub>2</sub>CH), 3.24–3.44 (m, 2 H, H<sub>2</sub>-C(5')), 3.72 (s, 6 H, H<sub>3</sub>C-O), 3.95 (m, 1 H, H-C(4')), 4.07 (m, 1 H, H-C(3')), 4.16–4.42 (5 H, H-C(2')), 2 × (H<sub>3</sub>C)<sub>2</sub>CH, H<sub>2</sub>-C(5)), 5.66 (d, *J* = 2.9 Hz, 1 H, H-C(1')), 6.86–6.89 (m, 4 H, H-C(Ar)), 7.22–7.39 (m, 11 H, H-C(Ar)), 8.33 (s, 1 H, H-C(6)).

**5-Acetyloxymethyl-5'-O-(4,4'-dimethoxytrityl)-2',3'-di-O-tert-butylidimethylsilylcytidine (5)**

The crude compound **4a** (4.1 mmol) was dissolved in THF (50 mL) and treated with aq NH<sub>3</sub> (32%, 70 mL). The solution was stirred at r.t. for 3 h. The solvents were evaporated, and the mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> and washed with H<sub>2</sub>O. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and the solvents evaporated. The crude product was purified by column chromatography on silica gel (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 100:0–98:2).

Yield: 1.5 g, 1.8 mmol (44% over 2 steps); white foam; *R*<sub>f</sub> = 0.5 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 95:5).

<sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = −0.10 (s, 3 H, H<sub>3</sub>C-Si), −0.02 (s, 3 H, H<sub>3</sub>C-Si), 0.02 (s, 3 H, H<sub>3</sub>C-Si), 0.04 (s, 3 H, H<sub>3</sub>C-Si), 0.74 (s, 9 H, (H<sub>3</sub>C)<sub>3</sub>-C), 0.84 (s, 9 H, (H<sub>3</sub>C)<sub>3</sub>-C), 1.90 (s, 3 H, H<sub>3</sub>C-CO), 3.20–3.35 (m, 2 H, H<sub>2</sub>-C(5')), 3.73 (s, 6 H, H<sub>3</sub>C-O), 3.97 (m, 2 H, H-C(4')), H-C(3')), 4.22 (1 H, H-C(2')), 4.34–4.44 (m, 2 H, H<sub>2</sub>-C(5)), 5.81 (d, *J* = 4.0 Hz, 1 H, H-C(1')), 6.86–6.89 (m, 4 H, H-C(Ar)), 7.05 (s, 1 H, H(a)-N(4)), 7.22–7.41 (m, 9 H, H-C(Ar)), 7.52 (s, 1 H, H(b)-N(4)), 7.76 (s, 1 H, H-C(6)).

<sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = −4.30 (4 × CH<sub>3</sub>-Si), 18.20 (2 × C(CH<sub>3</sub>)<sub>3</sub>), 21.27 (COCH<sub>3</sub>), 26.20 (6 × CH<sub>3</sub>-C-Si), 55.61 (2 × OCH<sub>3</sub>), 60.83 (CH<sub>2</sub>OH), 63.74 (C(5')), 72.16 (C(3')), 75.76 (C(2')), 83.24 (C(4')), 86.65 (t-C(DMT)), 89.40 (C(1')), 101.26 (C(5)), 113.80 (C(Ar)), 127.44 (C(Ar)), 128.30 (C(Ar)), 128.49 (C(Ar)), 130.30 (C(Ar)), 135.77 (C(Ar)), 142.83 (C(6)), 145.05 (C(Ar)), 155.27 (C(2)), 158.86 (C-OCH<sub>3</sub>(Ar)), 164.78 (C(4)), 170.68 (COCH<sub>3</sub>).

MS (ESI): *m/z* [M + Na]<sup>+</sup> calcd for C<sub>45</sub>H<sub>63</sub>N<sub>3</sub>O<sub>9</sub>Si<sub>2</sub>: 868.40; found: 868.39.

**N<sup>4</sup>-Acetyl-5-acetyloxymethyl-5'-O-(4,4'-dimethoxytrityl)-2',3'-di-O-tert-butylidimethylsilylcytidine (6)**

A solution of compound **5** (1.5 g, 1.8 mmol) in dry pyridine (30 mL) was cooled to 0 °C under argon, treated with Ac<sub>2</sub>O (425 μL, 4.5 mmol), allowed to warm to r.t. and stirred for 2 h. The reaction was quenched by the addition of MeOH (2 mL), and the solvents were evaporated. The oily residue was diluted with CH<sub>2</sub>Cl<sub>2</sub>, and washed with 5% citric acid, H<sub>2</sub>O and sat. NaHCO<sub>3</sub> solution. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and the solvents were evaporated. The crude product was purified by column chromatography on silica gel (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 99.5:0.5–98:2).

Yield: 1.2 g, 1.36 mmol (76%); white foam; *R*<sub>f</sub> = 0.75 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 95:5).

<sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ = −0.12 (s, 3 H, H<sub>3</sub>C-Si), −0.03 (s, 3 H, H<sub>3</sub>C-Si), 0.05 (s, 3 H, H<sub>3</sub>C-Si), 0.10 (s, 3 H, H<sub>3</sub>C-Si), 0.68 (s, 9 H, (H<sub>3</sub>C)<sub>3</sub>-C), 0.86 (s, 9 H, (H<sub>3</sub>C)<sub>3</sub>-C), 1.81 (s, 3 H, H<sub>3</sub>C-CO), 2.25 (s, 3 H, H<sub>3</sub>C-CO-N(4)), 3.20–3.35 (m, 2 H, H<sub>2</sub>-C(5')), 3.73 (s, 6 H, H<sub>3</sub>C-O), 3.99 (m, 2 H, H-C(4')), 4.09 (m, 1 H, H-C(3')), 4.30 (1 H, H-C(2')), 4.38–4.61 (m, 2 H, H<sub>2</sub>-C(5)), 5.70 (d, *J* = 2.4 Hz, 1 H, H-C(1')), 6.86–6.89 (m, 4 H, H-C(Ar)), 7.25–7.41 (m, 9 H, H-C(Ar)), 8.13 (s, 1 H, H-C(6)), 10.08 (s, 1 H, H-N(4)).

<sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ = −4.80 to −3.56 (4 × CH<sub>3</sub>-Si), 18.20 (2 × C(CH<sub>3</sub>)<sub>3</sub>), 21.08 (COCH<sub>3</sub>), 25.49 (NCOCH<sub>3</sub>), 26.20 (6 × CH<sub>3</sub>-C-Si), 55.61 (2 × OCH<sub>3</sub>), 60.55 (CH<sub>2</sub>OH), 63.22 (C(5')), 71.19 (C(3')), 75.61 (C(2')), 82.91 (C(4')), 86.58 (t-C(DMT)), 91.53 (C(1')), 105.24 (C(5)), 113.80 (C(Ar)), 127.44 (C(Ar)), 128.30 (C(Ar)), 128.49 (C(Ar)), 130.30 (C(Ar)), 135.77 (C(Ar)), 144.92 (C(6)), 145.95 (C(Ar)), 154.35 (C(2)), 158.87 (C-OCH<sub>3</sub>(Ar)), 161.77 (C(4)), 170.45 (COCH<sub>3</sub>), 171.22 (NCO-CH<sub>3</sub>).

MS (ESI): *m/z* [M + Na]<sup>+</sup> calcd for C<sub>47</sub>H<sub>65</sub>N<sub>3</sub>O<sub>10</sub>Si<sub>2</sub>: 910.41; found: 910.45.

**N<sup>4</sup>-Acetyl-5-acetyloxymethyl-5'-O-(4,4'-dimethoxytrityl)cytidine (7)**

Compound **6** (1.2 g, 1.36 mmol) was treated with a solution (6 mL) of 1 M TBAF and 0.5 M AcOH in THF. The reaction mixture was stirred at r.t. for 3 h. The solvents were evaporated and the residue was coevaporated twice with CH<sub>2</sub>Cl<sub>2</sub>. The crude product was purified by column chromatography on silica gel (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 99:1–96:4).

Yield: 672 mg, 1.02 mmol (75%); white foam;  $R_f$  = 0.45 ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$ , 95:5).

$^1\text{H}$  NMR (300 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  = 1.87 (s, 3 H,  $\text{H}_3\text{C}-\text{CO}$ ), 2.24 (s, 3 H,  $\text{H}_3\text{C}-\text{CO}-\text{N}(4)$ ), 3.25–3.32 (m, 2 H,  $\text{H}_2-\text{C}(5')$ ), 3.73 (s, 6 H,  $\text{H}_3\text{C}-\text{O}$ ), 4.08 (m, 3 H,  $\text{H}-\text{C}(4')$ ),  $\text{H}-\text{C}(3')$ ),  $\text{H}-\text{C}(2')$ ), 4.38–4.61 (m, 2 H,  $\text{H}_2\text{C}-\text{C}(5)$ ), 5.13 (d,  $J$  = 3.5 Hz, 1 H,  $\text{HO}-\text{C}(3')$ ), 5.60 (d,  $J$  = 4.5 Hz, 1 H,  $\text{HO}-\text{C}(2')$ ), 5.78 (d,  $J$  = 1.9 Hz, 1 H,  $\text{H}-\text{C}(1')$ ), 6.86–6.89 (m, 4 H,  $\text{H}-\text{C}(\text{Ar})$ ), 7.25–7.41 (m, 9 H,  $\text{H}-\text{C}(\text{Ar})$ ), 8.08 (s, 1 H,  $\text{H}-\text{C}(6)$ ), 10.04 (s, 1 H,  $\text{H}-\text{N}(4)$ ).

$^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  = 21.12 ( $\text{COCH}_3$ ), 25.54 ( $\text{NCOCH}_3$ ), 55.60 ( $2 \times \text{OCH}_3$ ), 60.63 ( $\text{CH}_2\text{OH}$ ), 63.56 ( $\text{C}(5')$ ), 69.91 ( $\text{C}(3')$ ), 74.60 ( $\text{C}(2')$ ), 83.00 ( $\text{C}(4')$ ), 86.34 ( $t-\text{C}(\text{DMT})$ ), 91.59 ( $\text{C}(1')$ ), 105.20 ( $\text{C}(5)$ ), 113.85 ( $\text{C}(\text{Ar})$ ), 127.37 ( $\text{C}(\text{Ar})$ ), 128.33 ( $\text{C}(\text{Ar})$ ), 128.50 ( $\text{C}(\text{Ar})$ ), 130.30 ( $\text{C}(\text{Ar})$ ), 135.87 ( $\text{C}(\text{Ar})$ ), 136.11 ( $\text{C}(\text{Ar})$ ), 146.22 ( $\text{C}(6)$ ), 145.16 ( $\text{C}(\text{Ar})$ ), 154.33 ( $\text{C}(2)$ ), 158.74 ( $\text{C}-\text{OCH}_3(\text{Ar})$ ), 161.79 ( $\text{C}(4)$ ), 170.58 ( $\text{COCH}_3$ ), 171.22 ( $\text{NCOCH}_3$ ).

MS (ESI):  $m/z$  [ $\text{M} + \text{H}$ ] $^+$  calcd for  $\text{C}_{35}\text{H}_{37}\text{N}_3\text{O}_{10}$ : 660.26; found: 660.24.

#### ***N*<sup>4</sup>-Acetyl-5-acetyloxymethyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-tert-butylidimethylsilylcytidine (8)**

Compound **7** (672 mg, 1.02 mmol) was dissolved in dry THF (12 mL) and dry pyridine (300  $\mu\text{L}$ ) was added, followed by  $\text{AgNO}_3$  (270 mg, 1.6 mmol). The suspension was stirred for 30 min, then TBDMSCl (250 mg, 1.6 mmol) was added. Stirring was continued in the dark for 4 h until the starting material had been consumed as analyzed by TLC. The reaction mixture was filtered through Celite and the Celite bed washed with  $\text{CH}_2\text{Cl}_2$  (100 mL). The combined filtrates were evaporated, the residue redissolved in  $\text{CH}_2\text{Cl}_2$ , and then washed with aq 5% citric acid ( $2 \times$ ) and sat.  $\text{NaHCO}_3$  solution. The organic phase was dried over  $\text{Na}_2\text{SO}_4$ , filtered, and evaporated. The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 60:40–25:75).

Yield: 340 mg, 0.44 mmol (43%); white foam;  $R_f$  = 0.5 (hexane/EtOAc, 25:75).

$^1\text{H}$  NMR (300 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  = 0.05 (s, 3 H,  $\text{H}_3\text{C}-\text{Si}$ ), 0.07 (s, 3 H,  $\text{H}_3\text{C}-\text{Si}$ ), 0.85 (s, 9 H,  $(\text{H}_3\text{C})_3-\text{C}$ ), 1.84 (s, 3 H,  $\text{H}_3\text{C}-\text{CO}$ ), 2.22 (s, 3 H,  $\text{H}_3\text{C}-\text{CO}-\text{N}(4)$ ), 3.25–3.32 (m, 2 H,  $\text{H}_2-\text{C}(5')$ ), 3.71 (s, 6 H,  $\text{H}_3\text{C}-\text{O}$ ), 4.05 (m, 2 H,  $\text{H}-\text{C}(4')$ ),  $\text{H}-\text{C}(3')$ ), 4.17 (m, 1 H,  $\text{H}-\text{C}(2')$ ), 4.29–4.52 (m, 2 H,  $\text{H}_2\text{C}-\text{C}(5)$ ), 5.09 (d,  $J$  = 5.5 Hz, 1 H,  $\text{HO}-\text{C}(3')$ ), 5.71 (br, 1 H,  $\text{H}-\text{C}(1')$ ), 6.86–6.89 (m, 4 H,  $\text{H}-\text{C}(\text{Ar})$ ), 7.25–7.41 (m, 9 H,  $\text{H}-\text{C}(\text{Ar})$ ), 8.04 (s, 1 H,  $\text{H}-\text{C}(6)$ ), 10.02 (s, 1 H,  $\text{H}-\text{N}(4)$ ). The assignments of 2'-OTBDMS (vs 3'-OTBDMS) regioisomers were based on  $^1\text{H}$ ,  $^1\text{H}$  DQF COSY NMR experiments, unequivocally revealing the correlation between the  $\text{H}-\text{C}(3')$  and  $\text{HO}-\text{C}(3')$  (vs  $\text{H}-\text{C}(2')$  and  $\text{HO}-\text{C}(2')$ ) signals.

$^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  = –4.17, –4.36 ( $2 \times \text{CH}_3-\text{Si}$ ), 18.57 ( $\text{C}(\text{CH}_3)_3$ ), 21.12 ( $\text{COCH}_3$ ), 25.50 ( $\text{NCOCH}_3$ ), 26.33 ( $3 \times \text{CH}_3-\text{C}-\text{Si}$ ), 55.61 ( $2 \times \text{OCH}_3$ ), 60.63 ( $\text{CH}_2\text{OH}$ ), 63.21 ( $\text{C}(5')$ ), 69.49 ( $\text{C}(3')$ ), 76.75 ( $\text{C}(2')$ ), 82.78 ( $\text{C}(4')$ ), 86.36 ( $t-\text{C}(\text{DMT})$ ), 91.52 ( $\text{C}(1')$ ), 105.17 ( $\text{C}(5)$ ), 113.85 ( $\text{C}(\text{Ar})$ ), 127.40 ( $\text{C}(\text{Ar})$ ), 128.31 ( $\text{C}(\text{Ar})$ ), 128.50 ( $\text{C}(\text{Ar})$ ), 130.30 ( $\text{C}(\text{Ar})$ ), 135.78 ( $\text{C}(\text{Ar})$ ), 136.02 ( $\text{C}(\text{Ar})$ ), 145.16 ( $\text{C}(6)$ ), 145.80 ( $\text{C}(\text{Ar})$ ), 154.25 ( $\text{C}(2)$ ), 158.75 ( $\text{C}-\text{OCH}_3(\text{Ar})$ ), 161.82 ( $\text{C}(4)$ ), 170.52 ( $\text{COCH}_3$ ), 171.23 ( $\text{NCOCH}_3$ ).

MS (ESI):  $m/z$  [ $\text{M} + \text{H}$ ] $^+$  calcd  $\text{C}_{41}\text{H}_{51}\text{N}_3\text{O}_{10}\text{Si}$ : 774.95; found: 774.32.

#### ***N*<sup>4</sup>-Acetyl-5-acetyloxymethyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-tert-butylidimethylsilylcytidine 3'-O-(2-Cyanoethyl-*N,N*-diisopropylphosphoramidite) (9)**

Compound **8** (340 mg, 0.44 mmol) was dissolved in dry  $\text{CH}_2\text{Cl}_2$  (6 mL) under an argon atm. Next, DIPEA (312  $\mu\text{L}$ , 1.8 mmol), 1-methylimidazole (18  $\mu\text{L}$ , 0.22 mmol), and 2-cyanoethyl *N,N*-diisopropylchloro-

phosphoramidite (195  $\mu\text{L}$ , 0.88 mmol) were added to the solution using syringes. The reaction was monitored by TLC and the mixture stirred for 5 h at r.t. Then,  $\text{CH}_2\text{Cl}_2$  was added and the organic phase extracted with sat. aq  $\text{NaHCO}_3$  solution and brine. The organic phase was dried over  $\text{Na}_2\text{SO}_4$  and the solvent was evaporated. The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 50:50, 0.5%  $\text{Et}_3\text{N}$ ).

Yield: 330 mg, 0.34 mmol (79%); white foam;  $R_f$  = 0.4 (hexane/EtOAc, 25:75).

$^1\text{H}$  NMR (300 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  = 0.04 (s, 3 H,  $\text{H}_3\text{C}-\text{Si}$ ), 0.05 (s, 3 H,  $\text{H}_3\text{C}-\text{Si}$ ), 0.82 (s, 9 H,  $(\text{H}_3\text{C})_3-\text{C}$ ), 1.01–1.06 (d, 12 H,  $\text{H}_3\text{C}-\text{CH}$ ), 1.82 (s, 3 H,  $\text{H}_3\text{C}-\text{CO}$ ), 2.22 (s, 3 H,  $\text{H}_3\text{C}-\text{CO}-\text{N}(4)$ ), 2.23–2.70 (t, 2 H,  $\text{CH}_2\text{CN}$ ), 3.40–3.53 (m, 6 H,  $\text{H}_2-\text{C}(5')$ ),  $\text{CH}_2-\text{P}$ ,  $\text{H}_3\text{C}-\text{CH}$ ), 3.71 (s, 6 H,  $\text{H}_3\text{C}-\text{O}$ ), 4.06 (m, 2 H,  $\text{H}-\text{C}(4')$ ),  $\text{H}-\text{C}(3')$ ), 4.18 (m, 1 H,  $\text{H}-\text{C}(2')$ ), 4.38–4.54 (m, 2 H,  $\text{H}_2\text{C}-\text{C}(5)$ ), 5.77–5.87 (d, 1 H,  $\text{H}-\text{C}(1')$ ), 6.84–6.87 (m, 4 H,  $\text{H}-\text{C}(\text{Ar})$ ), 7.20–7.39 (m, 9 H,  $\text{H}-\text{C}(\text{Ar})$ ), 8.04 (s, 1 H,  $\text{H}-\text{C}(6)$ ), 10.02 (s, 1 H,  $\text{H}-\text{N}(4)$ ).

$^{31}\text{P}$  NMR (121 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  = 149.16, 149.45.

MS (ESI):  $m/z$  [ $\text{M} + \text{H}$ ] $^+$  calcd for  $\text{C}_{50}\text{H}_{69}\text{N}_5\text{O}_{11}\text{PSi}$ : 974.45; found: 974.29.

HRMS (ESI, FT-ICR):  $m/z$  [ $\text{M} + \text{H}$ ] $^+$  calcd for  $\text{C}_{50}\text{H}_{68}\text{N}_5\text{O}_{11}\text{PSi}$ : 974.4495; found: 974.4483.

#### **5-Acetyloxymethyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-tert-butylidimethylsilyluridine (10)**

Compound **3** (450 mg, 0.72 mmol) was dissolved in dry THF (5.6 mL), then dry pyridine (200  $\mu\text{L}$ , 2.5 mmol) was added, followed by  $\text{AgNO}_3$  (200 mg, 1.2 mmol). The suspension was stirred for 30 min, then TBDMSCl (185 mg, 1.2 mmol) was added. Stirring was continued in the dark for 4 h until the starting material had been consumed as analyzed by TLC. The reaction mixture was filtered through Celite and the Celite bed washed with  $\text{CH}_2\text{Cl}_2$  (100 mL). The combined filtrates were evaporated, the residue redissolved in  $\text{CH}_2\text{Cl}_2$ , and then washed with aq citric acid ( $2 \times$ ) and sat.  $\text{NaHCO}_3$  solution. The organic phase was dried over  $\text{Na}_2\text{SO}_4$ , filtered, and evaporated. The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 80:20–50:50).

Yield: 230 mg, 0.31 mmol (44%); white foam;  $R_f$  = 0.55 (hexane/EtOAc, 25:75).

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 0.16 (s, 3 H,  $\text{H}_3\text{C}-\text{Si}$ ), 0.17 (s, 3 H,  $\text{H}_3\text{C}-\text{Si}$ ), 0.96 (s, 9 H,  $(\text{H}_3\text{C})_3-\text{C}$ ), 1.87 (s, 3 H,  $\text{H}_3\text{C}-\text{CO}$ ), 2.70 (d,  $J$  = 4.4 Hz, 1 H,  $\text{HO}-\text{C}(3')$ ), 3.40–3.56 (m, 2 H,  $\text{H}_2-\text{C}(5')$ ), 3.82 (s, 6 H,  $\text{H}_3\text{C}-\text{O}$ ), 4.20 (m, 1 H,  $\text{H}-\text{C}(4')$ ), 4.28 (m, 1 H,  $\text{H}-\text{C}(3')$ ), 4.48 (m, 1 H,  $\text{H}-\text{C}(2')$ ), 4.06–4.32 (m, 2 H,  $\text{H}_2\text{C}-\text{C}(5)$ ), 6.04 (d,  $J$  = 5.0 Hz, 1 H,  $\text{H}-\text{C}(1')$ ), 6.87–6.89 (m, 4 H,  $\text{H}-\text{C}(\text{Ar})$ ), 7.28–7.41 (m, 9 H,  $\text{H}-\text{C}(\text{Ar})$ ), 7.91 (s, 1 H,  $\text{H}-\text{C}(6)$ ), 8.42 (s, 1 H,  $\text{H}-\text{N}(3)$ ).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  = –4.98, –4.59 ( $2 \times \text{CH}_3-\text{Si}$ ), 18.13 ( $\text{C}(\text{CH}_3)_3$ ), 20.79 ( $\text{COCH}_3$ ), 25.76 ( $3 \times \text{CH}_3-\text{C}-\text{Si}$ ), 55.36 ( $2 \times \text{OCH}_3$ ), 58.79 ( $\text{CH}_2\text{OH}$ ), 63.32 ( $\text{C}(5')$ ), 71.34 ( $\text{C}(3')$ ), 76.02 ( $\text{C}(2')$ ), 84.08 ( $\text{C}(4')$ ), 87.22 ( $t-\text{C}(\text{DMT})$ ), 88.46 ( $\text{C}(1')$ ), 109.99 ( $\text{C}(5)$ ), 113.50 ( $\text{C}(\text{Ar})$ ), 127.35 ( $\text{C}(\text{Ar})$ ), 128.34 ( $\text{C}(\text{Ar})$ ), 128.34 ( $\text{C}(\text{Ar})$ ), 130.25 ( $\text{C}(\text{Ar})$ ), 135.33 ( $\text{C}(\text{Ar})$ ), 135.46 ( $\text{C}(\text{Ar})$ ), 140.67 ( $\text{C}(6)$ ), 144.23 ( $\text{C}(\text{Ar})$ ), 150.00 ( $\text{C}(2)$ ), 158.91 ( $\text{C}-\text{OCH}_3(\text{Ar})$ ), 161.89 ( $\text{C}(4)$ ), 170.44 ( $\text{COCH}_3$ ).

MS (ESI):  $m/z$  [ $\text{M} + \text{Na}$ ] $^+$  calcd for  $\text{C}_{39}\text{H}_{48}\text{N}_2\text{O}_{10}\text{Si}$ : 755.30; found: 755.44.

### 5-Acetyloxymethyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-tert-butylidimethylsilyluridine 3'-O-(2-Cyanoethyl-N,N-diisopropylphosphoramidite) (11)

Compound **10** (230 mg, 0.30 mmol) was dissolved in dry  $\text{CH}_2\text{Cl}_2$  (3.5 mL) under an argon atm. Next, DIPEA (206  $\mu\text{L}$ , 1.2 mmol), 1-methylimidazole (12  $\mu\text{L}$ , 0.15 mmol), and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (133  $\mu\text{L}$ , 0.60 mmol) were added to the solution using syringes. The reaction was monitored by TLC and the mixture stirred for 5 h at r.t. Then,  $\text{CH}_2\text{Cl}_2$  was added and the organic phase extracted with sat. aq.  $\text{NaHCO}_3$  solution and brine. The organic phase was dried over  $\text{Na}_2\text{SO}_4$  and the solvent was evaporated. The crude product was purified by column chromatography on silica gel (hexane/EtOAc, 65:35, 0.5%  $\text{Et}_3\text{N}$ ).

Yield: 220 mg, 0.24 mmol (80%); white foam;  $R_f$  = 0.5 (hexane/EtOAc, 50:50).

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 0.11–0.16 (s, 3 H,  $\text{H}_3\text{C-Si}$ ), 0.91–0.93 (s, 9 H,  $(\text{H}_3\text{C})_3\text{-C}$ ), 1.00–1.20 (d, 12 H,  $\text{H}_3\text{C-CH}$ ), 1.82–1.84 (s, 3 H,  $\text{H}_3\text{C-CO}$ ), 2.35–2.68 (t, 2 H,  $\text{CH}_2\text{CN}$ ), 3.35–3.50 (m, 2 H,  $\text{H}_2\text{-C}(5')$ ), 3.59 (m, 4 H,  $\text{CH}_2\text{-P}$ ,  $\text{H}_3\text{C-CH}$ ), 3.82 (s, 6 H,  $\text{H}_3\text{C-O}$ ), 4.05 (m, 5 H,  $\text{H-C}(4')$ ,  $\text{H-C}(3')$ ,  $\text{H-C}(2')$ ,  $\text{H}_2\text{-C}(5)$ ), 6.01–6.11 (d, 1 H,  $\text{H-C}(1')$ ), 6.83–6.87 (m, 4 H,  $\text{H-C}(\text{Ar})$ ), 7.28–7.44 (m, 9 H,  $\text{H-C}(\text{Ar})$ ), 7.92–7.93 (s, 1 H,  $\text{H-C}(6)$ ), 8.13 (s, 1 H,  $\text{H-N}(3)$ ).

$^{31}\text{P}$  NMR (121 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 149.90, 151.33.

MS (ESI):  $m/z$  [ $\text{M} + \text{Na}$ ] $^+$  calcd for  $\text{C}_{48}\text{H}_{65}\text{N}_4\text{O}_{11}\text{PSi}$ : 955.40; found: 955.49.

HRMS (ESI, FT-ICR):  $m/z$  [ $\text{M} + \text{H}$ ] $^+$  calcd for  $\text{C}_{48}\text{H}_{65}\text{N}_4\text{O}_{11}\text{PSi}$ : 933.4229; found: 933.4224.

### RNA Solid-Phase Synthesis

Phosphoramidite chemistry was applied for automated RNA strand elongation using 2'-OTOM nucleoside building blocks (GlenResearch) and a polystyrene support (GE Healthcare, Custom Primer Support<sup>TM</sup>, 80  $\mu\text{mol/g}$ , PS 200) in combination with the new phosphoramidites **9** and **11**. All oligonucleotides were synthesized on an ABI 392 Nucleic Acid Synthesizer following standard methods: detritylation (2.0 min) with dichloroacetic acid/1,2-dichloroethane (4:96), coupling (6.0 min) with phosphoramidites/MeCN (0.1 M  $\times$  130  $\mu\text{L}$ ) and 5-benzylthio-1*H*-tetrazole/MeCN (0.3 M  $\times$  360  $\mu\text{L}$ ), capping (2  $\times$  20 sec, Cap A/Cap B = 1:1) with Cap A: DMAP in MeCN (0.5 M) and Cap B:  $\text{Ac}_2\text{O}/\text{sym-collidine}/\text{MeCN}$  (2:3:5), oxidation (1.0 min) with  $\text{I}_2$  (20 mM) in THF/pyridine/ $\text{H}_2\text{O}$  (35:10:5). The solutions of amidites and tetrazole, and MeCN were dried over activated molecular sieves (3 Å) overnight.

### Deprotection of 5-Hydroxymethylpyrimidine-Modified RNA

The solid support was removed and treated with  $\text{MeNH}_2$  in EtOH (33%, 0.65 mL) and  $\text{MeNH}_2$  in  $\text{H}_2\text{O}$  (40%, 0.65 mL) for 4 h at r.t. for short sequences (<10 nt), and for 4 h at 35 °C for longer sequences (>10 nt). Alternatively, the solid support was treated with  $\text{MeNH}_2$  in  $\text{H}_2\text{O}$  (40%, 0.65 mL) and  $\text{NH}_3$  in  $\text{H}_2\text{O}$  (28%, 0.65 mL) for 30 min at 65 °C. The supernatant was removed and the solid support was washed three times with EtOH/ $\text{H}_2\text{O}$  (1:1). The supernatant and the washings were combined with the deprotection solution of the residue and the whole mixture was evaporated to dryness. To remove the 2'-silyl protecting groups, the resulting residue was treated with tetrabutylammonium fluoride trihydrate ( $\text{TBAF} \cdot 3\text{H}_2\text{O}$ ) in THF (1 M, 1 mL) at 37 °C overnight. The reaction was quenched by the addition of triethylammonium acetate (TEAA) (1 M, pH 7.4, 1 mL). The volume of the solution was reduced and the solution was desalted with a size-exclusion column (GE Healthcare, HiPrep<sup>TM</sup> 26/10 Desalting; 2.6  $\times$  10 cm, Seph-

adex G25) eluting with  $\text{H}_2\text{O}$ , and the collected fraction was evaporated to dryness and dissolved in  $\text{H}_2\text{O}$  (1 mL). Analysis of the crude RNA after deprotection was performed by anion-exchange chromatography on a Dionex DNAPac<sup>®</sup> PA-100 column (4 mm  $\times$  250 mm) at 80 °C. Flow rate: 1 mL/min, eluent A: 25 mM Tris-HCl (pH 8.0), 6 M urea; eluent B: tris-HCl (25 mM) (pH 8.0),  $\text{NaClO}_4$  (0.5 M), urea (6 M); gradient: 0–60% B in A within 45 min and UV detection at 260 nm.

### Purification of 5-Hydroxymethylpyrimidine-Modified RNA

Crude RNA products were purified on a semipreparative Dionex DNAPac<sup>®</sup> PA-100 column (9 mm  $\times$  250 mm) at 80 °C with a flow rate of 2 mL/min. Fractions containing RNA were loaded on a C18 SepPak Plus<sup>®</sup> cartridge (Waters/Millipore), washed with 0.1–0.15 M ( $\text{Et}_3\text{NH}$ ) $^+\text{HCO}_3^-$  and  $\text{H}_2\text{O}$ , and eluted with  $\text{H}_2\text{O}/\text{MeCN}$  (1:1). RNA-containing fractions were evaporated to dryness and dissolved in  $\text{H}_2\text{O}$  (1 mL). Analysis of the quality of purified RNA was performed by anion-exchange chromatography under the same conditions as utilized for crude RNA; the molecular weight was confirmed by LC-ESI mass spectrometry. Yield determination was performed by UV photometric analysis of oligonucleotide solutions.

### Mass Spectrometry of 5-Hydroxymethylpyrimidine-Modified RNA

All experiments were performed on a Finnigan LCQ Advantage MAX ion trap instrument connected to an Amersham Ettan micro LC system. RNA sequences were analyzed in the negative-ion mode with a potential of –4 kV applied to the spray needle. LC: Sample (200 pmol RNA dissolved in 30  $\mu\text{L}$  of 20 mM EDTA solution; average injection volume: 30  $\mu\text{L}$ ), column (Waters XTerra<sup>®</sup>MS, C18 2.5  $\mu\text{m}$ ; 2.1  $\times$  50 mm) at 21 °C; flow rate: 30  $\mu\text{L}/\text{min}$ ; eluent A:  $\text{Et}_3\text{N}$  (8.6 mM), 1,1,1,3,3,3-hexafluoroisopropanol (100 mM) in  $\text{H}_2\text{O}$  (pH 8.0); eluent B: MeOH; gradient: 0–100% B in A within 30 min; UV detection at 254 nm.

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### Supporting Information

Supporting information for this article is available online at <http://dx.doi.org/10.1055/s-0035-1561220>.

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