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Synthesis, Thermodynamic Properties, and Crystal Structure of RNA Oligonucleotides Containing 5-Hydroxymethylcytosine

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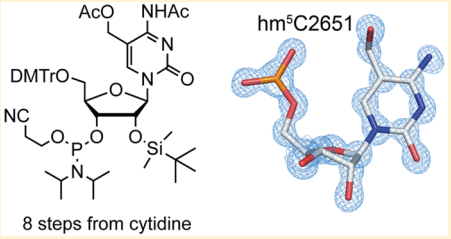
Supporting Information

ABSTRACT: 5-Hydroxymethylcytosine (hm^{5}C) is an RNA modification that has attracted significant interest because of the finding that RNA hydroxymethylation can favor mRNA translation. For insight into the mechanistic details of hm^{5}C function to be obtained, the availability of RNAs containing this modification at defined positions that can be used for in vitro studies is highly desirable. In this work, we present an eight-step route to 5-hydroxymethylcytidine (hm^{5}rC) phosphoramidite for solid-phase synthesis of modified RNA oligonucleotides. Furthermore, we examined the effects of hm^{5}rC on RNA duplex stability and its impact on structure formation using the sarcin-ricin loop (SRL) motif. Thermal denaturation experiments revealed that hm^{5}rC increases RNA duplex stability. By contrast, when cytosine within an UNCG tetraloop motif was replaced by hm^{5}rC, the thermodynamic stability of the corresponding hairpin fold was attenuated. Importantly, incorporation of hm^{5}rC into the SRL motif resulted in an RNA crystal structure at 0.85 Å resolution. Besides changes in the hydration pattern at the site of modification, a slight opening of the hm^{5}rC−G pair compared to the unmodified C−G in the native structure was revealed.



INTRODUCTION

5-Hydroxymethylcytosine (hm^{5}C) is a nucleobase modification occurring in DNA and RNA.[1,2] Although its role in DNA as a stable intermediate during oxidative demethylation of the dynamic epigenetic mark 5-methylcytosine (m^{5}C) has been investigated intensively,[3−7] little is known regarding the functions of m^{5}C and hm^{5}C in RNA. Recent studies have shown widespread occurrence of m^{5}C not only in tRNA and rRNA, where it stabilizes RNA secondary structures and affects translational efficiency,[8−14] but also in poly(A) RNA and noncoding RNA.[15−20] Moreover, it was shown that m^{5}C in RNA can be oxidized to hm^{5}C in vitro by methyldioxygenases of the ten-eleven translocation (TET) enzyme family,[21] and isotope-tracing experiments also proved in vivo that 5-hydroxymethylcytidine (hm^{5}rC) originates from 5-methylcytidine (m^{5}rC) in RNA.[22] These findings suggest that m^{5}rC may be turned-over in a way that is similar to m^{5}C oxidation in DNA. Delatte et al. recently demonstrated that hm^{5}rC is present in coding sequences of transcripts in *Drosophila melanogaster* and that hm^{5}rC can promote mRNA translation.[23] Because these findings establish hm^{5}rC as a component of the so-called "epitranscriptome",[24] future research needs to address the functional significance of this modification. In vitro assays, such as in vitro translation, or affinity purification strategies to identify hm^{5}rC "reader" proteins depend on access to readily generated RNA molecules bearing hm^{5}rC at defined positions. Thus, there is a need for fast and efficient protocols to produce hm^{5}rC building blocks for solid-phase synthesis of modified oligonucleotides. Here, we present an eight-step route to hm^{5}rC phosphoramidite, and we report thermodynamic and structural properties of hm^{5}rC-modified RNAs.

RESULTS AND DISCUSSION

Although hm^{5}C is chemically stable per se, it is a challenging modification when it comes to solid-phase synthesis of such modified nucleic acids. Because of the pseudobenzylic character of the 5-hydroxymethyl group, the choice of appropriate protection groups at the pyrimidine nucleobase is critical to avoid or minimize unwanted amino-substituted (S_N) by-products that can emerge during deprotection of the synthetic RNA under standard conditions involving ammonia and methylamine. This problematic matter is the reason why different synthetic approaches using various protection concepts are found in the literature for the solid-phase synthesis of hm^{5}dC-containing DNA.[25−28] Although most of these approaches have their merits, all of them require specific adaptations of standard methods for postsynthetic deprotection with respect to solvents, reagents, temperature, and reaction time. This is an even bigger issue for the postsynthetic deprotection of hm^{5}rC-containing RNA because of fast hydrolysis of its phosphate ester backbone under basic conditions. We have previously reported the first and still only synthesis of an hm^{5}rC building block for RNA solid-phase synthesis, namely, phosphoramidite **9**.[29] This building block that utilizes bisacetylation of exocyclic amino- and 5-

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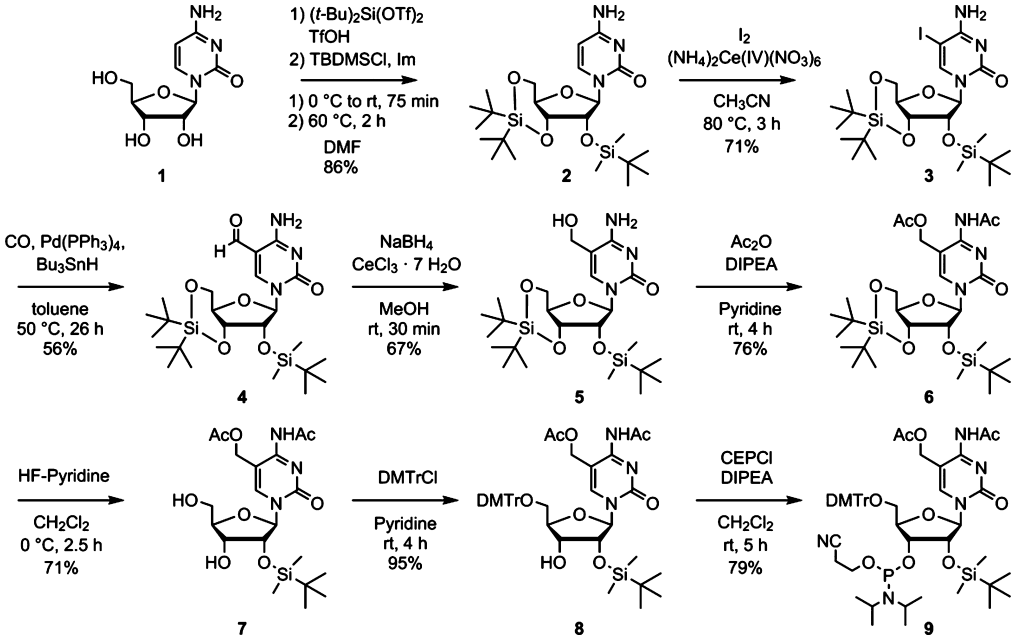
Scheme 1. Synthesis of hm^{5}rC Phosphoramidite 9[a]



[a]Abbreviations: di-*tert*-butylsilyl bis(trifluoromethanesulfonate) ((*t*-Bu)$_2$Si(OTf)$_2$); trifluoromethanesulfonic acid (TfOH); *tert*-butyldimethylsilyl chloride (TBDMSCl); *N*,*N*-dimethylformamide (DMF); *N*,*N*-diisopropylethylamine (DIPEA); 4,4′-dimethoxytriphenylmethyl chloride (DMTrCl); *N*,*N*-diisopropyl-chloro-phosphoramidite (CEPCl).

Table 1. Thermal and Thermodynamic Data for Unmodified and hm^{5}rC-Modified RNA

sequence (5′-3′)	T_m [°C]	$\Delta G°_{298}$ [kcal/mol]	$\Delta H°$ [kcal/mol]	$\Delta S°$ [cal/mol]
GGCUAGCC	60.3	−15.8	−77.5	207
GG(hm^{5}rC)UAGCC	63.8	−17.8	−91.1	246
GAAGGGCAACCUUCG	70.6	−12.7	−86.3	247
GAAGGGCAAC(hm^{5}rC)UUCG	73.6	−16.2	−99.6	280
GCGAACCUGCGGGUUCG	82.2	−10.6	−75.6	218
GCGAACCUG(hm^{5}rC)GGGUUCG	75.8	−9.7	−75.0	219

hydroxymethyl groups is very well suited for standard RNA solid-phase synthesis and deprotection; however, its preparation requires 11 steps (from uridine), several of which proceed with low yields.[29] Therefore, we set out to improve the synthesis of building block **9** and succeeded in the development of a faster and more convenient route to this phosphoramidite (Scheme 1).

Starting from commercially available cytidine, the ribose moiety was simultaneously protected with 3′,5′-*O*-di-*tert*-butylsilyl and 2′-*O*-*tert*-butylsilyl protecting groups in a one-pot procedure to provide compound **2** in high yield following a procedure published by Beigelman.[30] Then, treatment of **2** with iodine in the presence of ceric ammonium nitrate gave the corresponding 5-iodocytidine derivative **3**. The 5-formyl group of compound **4** was generated subsequently by a Stille reaction in good yield and further converted to the desired 5-hydroxymethyl group of compound **5** using reductive conditions (Luche) with $NaBH_4$ and $CeCl_3$.[26,27] Bis-acetylation of both the 5-hydroxymethyl and exocyclic amino group was performed with acetic anhydride to furnish compound **6**. Selective removal of the 3′,5′-*O*-di-*tert*-butyl silylether was accomplished with hydrogen fluoride (HF) in pyridine and gave nucleoside **7** in 70% yield, leaving the 2′-*O*-*tert*-butylsilyl group unaffected. Nucleoside **7** was then transformed into dimethoxytritylated compound **8** using 4,4′-dimethoxytriphenylmethyl chloride in pyridine without any additional base to avoid migration of the 2′-*O*-*tert*-butylsilyl group. Finally, phosphitylation was executed with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in the presence of *N*,*N*-diisopropylethylamine. Starting from cytidine, our route provides phosphoramidite **9** with 9.2% overall yield in eight steps and with seven chromatographic purifications; in total, 0.5 g of **9** was obtained in the course of this study. Compared to our previously published route,[29] which started from uridine and involved a hm^{5}U-to-hm^{5}C transformation, the overall yield was increased by a factor of 3.

To evaluate the impact of hm^{5}rC on base pairing stability, we synthesized three RNAs with a single modification in different sequence contexts and the corresponding unmodified references (Table 1) applying solid-phase RNA assembly and deprotection protocols as described previously.[29] Thermal denaturation experiments based on UV melting profile analysis[31,32] revealed a thermal stabilization (1.8 °C per hm^{5}rC) of an 8 bp self-complementary RNA duplex [5′-GG(hm^{5}rC)UAGCC]$_2$ in comparison to the unmodified counterpart [5′-GGCUAGCC]$_2$ (Table 1, Figure 1A). Furthermore, the 15 nt RNA hairpin 5′-GAAGGGCAA-(hm^{5}rC)CUUCG, comprising an extra-stable GNRA loop and hm^{5}C in the loop-closing base pair, exhibited an increase in thermal (T_m) and thermodynamic (ΔG) base pairing stabilities compared to those of the unmodified RNA 5′-GAAGGGCAACCUUCG (Table 1, Figure 1B). By contrast,

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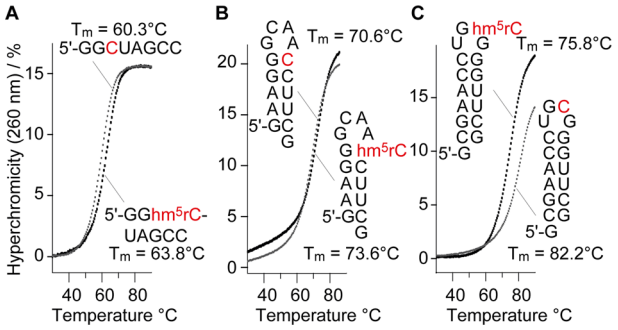

Figure 1. Melting profiles of hm^{5}rC-containing RNA and unmodified counterparts. Conditions: c_{RNA} = 8 μM; 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.0.

when C was replaced by hm^{5}rC in the extra-stable UNCG loop motif of the 17 nt RNA hairpin 5′-GCGAACCUGCGGGU-UCG, a decrease in base pairing stability was observed (Table 1, Figure 1C). Most likely, the decrease can be attributed to steric repulsion within the loop; the bulky 5-hydroxymethyl group likely interferes with the nonbridging phosphate oxygen of the second residue of the UUCG tetraloop.[33]

The importance of the sequence context for thermodynamic effects of hm^{5}C has also been found for DNA. Similar to what we show here for hm^{5}rC, hm^{5}dC affects thermodynamic stability in regular duplex structures of DNA only slightly, ranging from 1 to 2 °C of destabilization per modification to maximal 0.5 °C of stabilization in different sequence environments.[34−38]

Finally, we set out to examine the impact of hm^{5}rC on RNA structure using X-ray crystallography. To this end, we used the 27 nt fragment of the *E. coli* 23S rRNA sarcin−ricin loop (SRL).[39] This sequence is known to be a robust and well-behaved crystallization scaffold that can accommodate small modifications.[40,41] For the incorporation of hm^{5}rC, we deemed nucleotide C2651 appropriate, which forms a Watson−Crick base pair with G2669 in the regular A-form double helical region of this scaffold. Crystallization trials with hm^{5}rC2651-modified SRL RNA indeed provided crystals that diffracted to subatomic resolution (Table S1). X-ray structure determinations showed that the 5-hydroxymethyl group is well-defined in the electron density maps for the hm^{5}rC-modified RNA (Figures 2 and 3). Superimpositions of the hm^{5}C-modified RNA structure with the unmodified RNA showed a root-mean-square deviation (rmsd) of 0.17 Å (within the errors on coordinates of 0.12 Å). Overall, the structure revealed that the 5-hydroxymethyl group does not significantly affect the SRL RNA fold. However, a slight opening of the hm^{5}rC2651-G2669 base pair (Figure 2B) was visible in that the N4−O6 hydrogen bond increased from 2.95 to 3.4 Å, which is significantly above the rmsd of atomic positions. Interestingly, such an opening was not observed in hm^{5}C-G base pairs in DNA crystal structures observed at high resolution.[36,42] This difference might be attributed to a context dependency due to neighboring base pairs and to modifications of the hydration network due to the 5-hydroxymethyl group. Indeed, analysis of the RNA hydration pattern revealed some alterations in comparison with the unmodified SRL structure. The 5-hydroxymethyl group dislodges a water molecule involved in an interaction between phosphate 2650 and phosphate 2651 and takes part in the interactions with two water molecules in the RNA minor groove (Figure 3). These modifications might

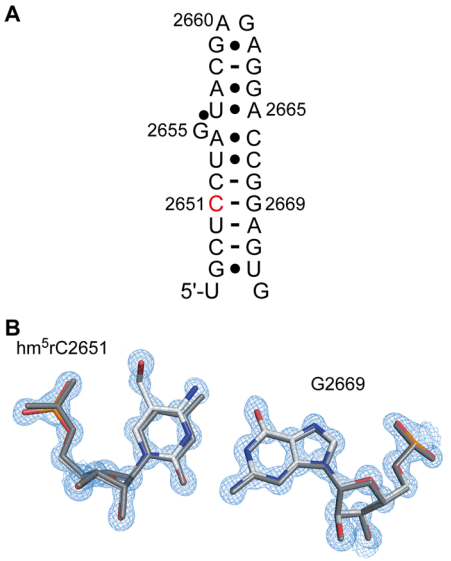

Figure 2. X-ray structure of RNA with a single 5-hydroxymethylcytosine modification at atomic resolution. (A) *E. coli* sarcin−ricin stem-loop (SRL) RNA used for crystallization; secondary structure; nucleosides that were modified are indicated in red. (B) Detailed view of the hm^{5}rC-G base pair. The 2Fo-Fc electron density map contoured at the 1.6 σ level is shown in light blue. The unmodified SRL structure (represented in dark gray) is superimposed with the hm^{5}rC-modified SRL structure, showing the slight opening of the hm^{5}rC-G base pair.

be responsible for the slight changes in melting temperatures compared to those of the unmodified RNA double helixes.

CONCLUSIONS

Starting from cytidine, we developed an eight-step synthesis of a hm^{5}rC phosphoramidite building block that was utilized in automated solid-phase synthesis to generate hm^{5}rC-modified RNA. Using this approach, we analyzed the impact of hm^{5}rC on the structural properties of a small RNA, the sarcin−ricin loop RNA, by X-ray crystallographic analysis. Compared to the unmodified Watson−Crick C-G base pair, we found slight base pair opening and changes in the hydration pattern in the presence of hm^{5}rC. These alterations may account for the differences in thermodynamic stability that we observed by UV melting profile analysis. Our findings have impact with respect to the growing demand for hm^{5}rC RNA probes to study this modification in biochemical and biological settings and additionally pave the way for synthetic access to the newest discovery of a related nucleoside modification, 2′-*O*-methyl-5-hydroxymethylcytidine (hm^{5}rCm), in RNA.[43]

EXPERIMENTAL SECTION

General Information. All reactions involving moisture- or air-sensitive reagents were carried out under an argon atmosphere. Reagents were purchased from commercial suppliers, and dry solvents were dried over activated molecular sieves (3 Å) prior to use. All solid compounds were dried under high vacuum for at least 5 h prior to use; no azeotropical drying was performed. Thin layer chromatography was performed on Macherey−Nagel Polygram SIL G/UV$_{254}$ plates. Column chromatography was carried out on silica gel 60 (70−230 mesh). All NMR experiments were recorded on a 300 MHz instrument. ^{1}H NMR chemical shifts (δ) were referenced to $CDCl_3$ (7.26 ppm) or DMSO-d_6 (2.49 ppm). ^{13}C NMR chemical shifts (δ) were referenced to $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.5 ppm). Assignments were based on ^{1}H,^{1}H-COSY and ^{1}H,^{13}C-HSQC

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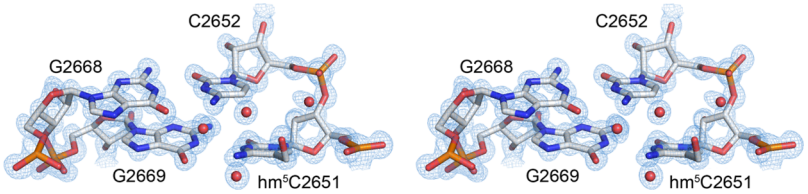

Figure 3. Cross-eye stereo view of the hm^{5}rC-G base pair showing some surrounding water molecules (represented as red spheres). The 2Fo-Fc electron density map contoured at the 1.6 σ level is shown in light blue. Two water molecules are involved in hydrogen bonds with the 5-hydroxymethyl group.

experiments. Mass spectrometric analysis (HRMS) of nucleoside derivatives was performed on an ESI TOF device. MS analysis of oligonucleotides was performed on a μLC-ESI quadrupole device.

2′-*O*-(*tert*-Butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilylene)-cytidine (2).[30] Cytidine **1** (2.0 g, 8.2 mmol) was dissolved in dry DMF (20 mL) and stirred at 0 °C. Then, TfOH (726 μL, 1.23 g, 8.2 mmol) was added. Subsequently, di-*tert*-butylsilyl bis(trifluoromethanesulfonate) (3.2 mL, 4.3 g, 9.8 mmol) was added dropwise. After 45 min, imidazole (2.8 g, 41 mmol) was added, and the reaction was warmed to rt over a period of 30 min. Then, *tert*-butyldimethylsilyl chloride (1.6 g, 10.6 mmol) was added in one portion, and the reaction was heated to 60 °C for 2 h. Subsequently, the reaction mixture was diluted with EtOAc (200 mL) and extracted twice with sat. $NaHCO_3$ (200 mL) and water (200 mL). The organic layer was dried over Na_2SO_4 and evaporated. The crude product was purified by SiO_2 column chromatography (1−4% MeOH in CH_2Cl_2) and gave compound **2** (3.5 g, 86%) as white foam. R_f = 0.4 (5% MeOH in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.14 (s, 3H, H_3C-Si), 0.20 (s, 3H, H_3C-Si), 0.93 (s, 9H, $(H_3C)_3$-C), 1.02 (s, 9H, $(H_3C)_3$-C), 1.04 (s, 9H, $(H_3C)_3$-C), 3.87 (dd, 1H, *H*-C(3′), J_1 = 5 Hz, J_2 = 4 Hz), 3.97 (t, 1H, *H*(*b*)-C(5′), J = 10 Hz), 4.15 (m, 1H, *H*-C(4′), J_1 = 5 Hz, J_2 = 4.5 Hz), 4.30 (d, 1H, *H*-C(2′), J = 4 Hz), 4.49 (dd, 1H, *H*(*a*)-C(5′), J_1 = 10 Hz, J_2 = 4.5 Hz), 5.68 (s, 1H, *H*-C(1′)), 5.88 (d, 1H, *H*-C(5), J = 7 Hz), 7.25 (d, 1H, *H*-C(6) J = 7 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ −4.5, −3.9 (2x CH_3-Si), 18.7, 20.7, 23.2 (3x $C(CH_3)_3$), 26.3, 27.4, 27.9 (9x CH_3-C-Si), 68.2 (*C*(5′), 74.8 (*C*(4′)), 75.7 (*C*(2′)), 76.3 (*C*(3′)), 95.0 (*C*(1′)), 95.6 (*C*(5)), 140.4 (*C*(6)), 155.9 (*C*(2)), 166.4 (*C*(4)); HRMS (ESI TOF) calcd for $C_{23}H_{43}N_3O_5Si_2$ [MH$^+$] 498.2814, found 498.2799.

2′-*O*-(*tert*-Butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilylene)-5-iodocytidine (3). Compound **2** (3.3 g, 6.6 mmol) and I_2 (1.25 g, 4.9 mmol) were dissolved in CH_3CN (50 mL). Then, ammonium cerium(IV) nitrate (5.4 g, 9.8 mmol) was added to the solution in one portion. The mixture was stirred for 3 h at 80 °C under reflux. Then, the solution was diluted with EtOAc (300 mL) and extracted with sat. $NaHCO_3$ (200 mL) and sat. $Na_2S_2O_3$ (200 mL) until the yellow color disappeared. After purification by silica gel column chromatography (33−66% EtOAc in cyclohexane), the product was dried under high vacuum to yield **3** (2.9 g, 71%) as a yellow foam. R_f = 0.15 (50% EtOAc in cyclohexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.15 (s, 3H, H_3C-Si), 0.23 (s, 3H, H_3C-Si), 0.94 (s, 9H, $(H_3C)_3$-C), 1.02 (s, 9H, $(H_3C)_3$-C), 1.04 (s, 9H, $(H_3C)_3$-C), 3.81 (dd, 1H, *H*-C(3′), J_1 = 5 Hz, J_2 = 4 Hz), 3.97 (t, 1H, *H*(*b*)-C(5′), J = 10 Hz), 4.22 (m, 1H, *H*-C(4′), J_1 = 5 Hz, J_2 = 4.5 Hz), 4.30 (d, 1H, *H*-C(2′), J = 4 Hz), 4.53 (dd, 1H, *H*(*a*)-C(5′), J_1 = 10 Hz, J_2 = 4.5 Hz), 5.65 (s, 1H, *H*-C(1′)), 7.65 (s, 1H, *H*-C(6)); ^{13}C NMR (75 MHz, DMSO-d_6) δ −4.5, −4.0 (2x CH_3-Si), 18.6, 20.5, 22.8 (3x $C(CH_3)_3$), 26.3, 27.4, 27.9 (9x CH_3-C-Si), 58.0 (*C*(5)), 67.6 (*C*(5′)), 74.4 (*C*(4′)), 74.6 (*C*(2′)), 75.8 (*C*(3′)), 95.1 (*C*(1′)), 148.6 (*C*(6)), 154.0 (*C*(2)), 164.5 (*C*(4)); HRMS (ESI TOF) calcd for $C_{23}H_{42}IN_3O_5Si_2$ [MH$^+$] 624.1780, found 624.1777.

2′-*O*-(*tert*-Butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilylene)-5-formylcytidine (4). Compound **3** (750 mg, 1.2 mmol) and $Pd(PPh_3)_4$ (140 mg, 0.12 mmol) were evaporated under high vacuum for 5 h in a flask with a gas valve inlet. Then, a balloon was filled with approximately 1 L of CO, and the flask was flushed with CO. Toluene (10 mL) was added, and the solution was heated to 50 °C. Tri-*n*-butyltin hydride (356 ul, 1.32 mmol) was added over a syringe pump at 15 μL/h. After the addition was complete, the solution was stirred for another 4 h at 50 °C under the CO atmosphere. After discharging the CO, the reaction mixture was diluted with CH_2Cl_2 (250 mL) and extracted with sat. $NaHCO_3$ (200 mL). Purification by SiO_2 column chromatography (25−60% EtOAc in *n*-hexane) gave product **4** (350 mg, 56%) as an off-white foam. R_f = 0.45 (50% EtOAc in *n*-hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.20 (s, 3H, H_3C-Si), 0.29 (s, 3H, H_3C-Si), 0.99 (s, 9H, $(H_3C)_3$-C), 1.06 (s, 18H, $(H_3C)_3$-C), 3.78 (dd, 1H, *H*-C(3′), J_1 = 5 Hz, J_2 = 4 Hz), 4.03 (t, 1H, *H*(*b*)-C(5′), J = 10 Hz), 4.34 (m, 1H, *H*-C(4′), J_1 = 5 Hz, J_2 = 4.5 Hz), 4.42 (d, 1H, *H*-C(2′), J = 4 Hz), 4.62 (dd, 1H, *H*(*a*)-C(5′), J_1 = 10 Hz, J_2 = 4.5 Hz), 5.75 (s, 1H, *H*-C(1′)), 6.28 (br s, 1H, *H*(*a*)-N(4)), 8.05 (s, 1H, *H*-C(6)), 8.17 (br s, 1H, *H*(*b*)-N(4)), 9.50 (s, 1H, *H*-CO); ^{13}C NMR (75 MHz, $CDCl_3$) δ −4.4, −3.9 (2x CH_3-Si), 18.6, 20.8, 23.2 (3x $C(CH_3)_3$), 26.3, 27.4, 27.9 (9x CH_3-C-Si), 68.2 (*C*(5′)), 75.2 (*C*(4′)), 75.6 (*C*(2′)), 76.2 (*C*(3′)), 94.9 (*C*(1′)), 105.8 (*C*(5)), 152.5 (*C*(6)), 153.1 (*C*(2)), 163.0 (*C*(4)), 187.4 (*C*HO); HRMS (ESI TOF) calcd for $C_{24}H_{43}N_3O_6Si_2$ [MH$^+$] 526.2763, found 526.2771.

2′-*O*-(*tert*-Butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilylene)-5-hydroxymethylcytidine (5). Compound **4** (350 mg, 0.67 mmol) and $CeCl_3{\cdot}7H_2O$ (740 mg, 2.0 mmol) were dissolved in MeOH (25 mL). Then, $NaBH_4$ (25.3 mg, 0.67 mmol) was added to the solution in one portion. The mixture was stirred for 30 min at rt. Then, a solution of sat. NH_4Cl (50 mL) was added to quench the reaction. Subsequently, the solution was extracted with EtOAc (200 mL) and sat. NH_4Cl (200 mL). After purification by SiO_2 column chromatography (4−6% MeOH in CH_2Cl_2), product **5** (235 mg, 67%) was dried under high vacuum. R_f = 0.40 (5% MeOH in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.15 (s, 3H, H_3C-Si), 0.21 (s, 3H, H_3C-Si), 0.97 (s, 9H, $(H_3C)_3$-C), 1.04 (s, 18H, $(H_3C)_3$-C), 3.69 (dd, 1H, *H*-C(3′), J_1 = 5 Hz, J_2 = 4 Hz), 3.95 (t, 1H, *H*(*b*)-C(5′), J = 10 Hz), 4.21 (m, 1H, *H*-C(4′), J_1 = 5 Hz, J_2 = 4.5 Hz), 4.28 (d, 1H, *H*-C(2′), J = 4 Hz), 4.35 (dd, 2H, H_2-C(5), J_1 = 37 Hz, J_2 = 13 Hz), 4.54 (dd, 1H, *H*(*a*)-C(5′), J_1 = 10 Hz, J_2 = 4.5 Hz), 5.47 (s, 1H, *H*-C(1′)), 6.57 (br s, 1H, *H*(*a*)-N(4)), 7.17 (s, 1H, *H*-C(6)), 8.89 (br s, 1H, *H*(*b*)-N(4)); ^{13}C NMR (75 MHz, $CDCl_3$) δ −4.5, −3.9 (2x CH_3-Si), 18.7, 20.7, 23.2 (3x $C(CH_3)_3$), 26.3, 27.4, 27.9 (9x CH_3-C-Si), 60.0 (CH_2-C(5)), 68.2 (*C*(5′)), 74.9 (*C*(4′)), 75.6 (*C*(2′)), 76.4 (*C*(3′)), 94.1 (*C*(1′)), 107.0 (*C*(5)), 137.9 (*C*(6)), 156.3 (*C*(2)), 165.5 (*C*(4)); HRMS (ESI TOF) calcd for $C_{24}H_{45}N_3O_6Si_2$ [MH$^+$] 528.2920, found 528.2927.

***N*^{4}-Acetyl-5-acetyloxymethyl-3′,5′-di-*O*-*tert*-butylsilyl-2′-*O*-*tert*-butyldimethylsilylcytidine (6).** Compound **5** (150 mg, 0.28 mmol) was dissolved in dry pyridine (4 mL), and DIPEA (50 μL, 0.56 mmol) and Ac_2O (250 μL, 2.6 mmol) were added to the solution. The solution was stirred for 4 h at rt. Then, the solution was diluted with CH_2Cl_2 (100 mL) and extracted twice with 5% citric acid (200 mL) and once with sat. $NaHCO_3$ (200 mL). Then, the organic layer was dried over Na_2SO_4 and evaporated. After purification by SiO_2 column chromatography (25−50% EtOAc in cyclohexane), product **6** (130 mg, 76%) was dried under high vacuum. R_f = 0.4 (50% EtOAc in cyclohexane); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.12 (s, 3H, H_3C-Si), 0.18 (s, 3H, H_3C-Si), 0.91 (s, 9H, $(H_3C)_3$-C), 0.99 (s, 9H, $(H_3C)_3$-C), 1.02 (s, 9H, $(H_3C)_3$-C), 1.99 (s, 3H, (H_3C)CO), 2.28 (s, 3H, (H_3C)CO), 3.98 (dd, 1H, *H*-C(3′), J_1 = 5 Hz, J_2 = 4 Hz), 4.05 (t, 1H, *H*(*b*)-C(5′), J = 10 Hz), 4.15 (m, 1H, *H*-C(4′), J_1 = 5 Hz J_2 = 4.5 Hz), 4.38 (d, 1H, *H*-C(2′), J = 4 Hz), 4.44 (dd, 1H, *H*(*a*)-C(5′), J_1 = 10 Hz, J_2 = 4.5 Hz), 4.96 (s, 2H, H_2-C(5)), 5.65 (s, 1H, *H*-C(1′)), 7.95 (s, 1H, *H*-C(6)), 10.00 (s, 1H, *H*-N(4)); ^{13}C NMR (75 MHz, DMSO-

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d_6) δ −4.1, −3.6 (2x CH_3-Si), 18.8, 20.8, 23.0 (3x $C(CH_3)_3$), 21.6 ($CO(CH_3)$), 26.0 ($CO(CH_3)$), 26.6, 27.6, 28.2 (9x CH_3-C-Si), 60.7 (CH_2-C(5)), 67.7 (C(5′)), 75.2 (C(4′)), 75.4 (C(2′)), 75.7 (C(3′)), 94.7 (C(1′)), 105.4 (C(5)), 146.5 (C(6)), 154.2 (C(2)), 162.1 (C(4)), 171.2 ($COCH_3$), 171.6 ($COCH_3$); HRMS (ESI TOF) calcd for $C_{28}H_{49}N_3O_8Si_2$ [MH^+] 612.3131, found 612.3119.

N^4-Acetyl-5-acetyloxymethyl-2′-*O*-*tert*-butyldimethylsilylcytidine (7). Compound **6** (150 mg, 0.24 mmol) was dissolved in dry CH_2Cl_2 (2 mL) and cooled to 0 °C. HF pyridine (25.5 μL, 0.98 mmol) was mixed with dry pyridine (150 μL), and the solution was added to the reaction mixture. The solution was stirred for 2.5 h at 0 °C. Then, it was diluted with CH_2Cl_2 (70 mL) and extracted with sat. $NaHCO_3$ (150 mL). Then, the organic layer was dried over Na_2SO_4 and evaporated. After purification by SiO_2 column chromatography (2−5% MeOH in CH_2Cl_2), product **7** (80 mg, 71%) was dried under high vacuum. R_f = 0.3 (5% MeOH in CH_2Cl_2); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.06 (s, 3H, H_3C-Si), 0.08 (s, 3H, H_3C-Si), 0.87 (s, 9H, $(H_3C)_3$-C), 2.01 (s, 3H, $(H_3C)CO$), 2.24 (s, 3H, $(H_3C)CO$), 3.65−3.80 (m, 2H, H_2-C(5′)), 3.95 (m, 2H, H-C(3′) + H-C(2′)), 4.11 (m, 1H, H-C(4′)), 4.85 (s, 2H, H_2-C(5)), 4.97 (d, 1H, HO-C(3′)), 5.24 (t, 1H, HO-C(5′)), 5.71 (s, 1H, H-C(1′)), 8.58 (s, 1H, H-C(5)), 10.05 (s, 1H, H-N(4)); ^{13}C NMR (75 MHz, DMSO-d_6) δ −4.1, −3.9 (2x CH_3-Si), 18.8 ($C(CH_3)_3$), 21.5 ($CO(CH_3)$), 25.7 ($CO(CH_3)$), 26.6 (3x CH_3-C-Si), 60.1 (C(5′)), 61.1 (CH_2-C(5)), 68.8 (C(3′)), 77.3 (C(4′)), 84.7 (C(2′)), 91.2 (C(1′)), 105.4 (C(5), 146.5 (C(6)), 154.6 (C(2)), 161.8 (C(4)), 171.1 ($COCH_3$), 171.5 ($COCH_3$); HRMS (ESI TOF) calcd for $C_{20}H_{33}N_3O_8Si$ [MH^+] 472.2110, found 472.2078.

N^4-Acetyl-5-acetyloxymethyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-*tert*-butyldimethylsilylcytidine (8).[29] Compound **7** (70 mg, 0.15 mmol) was dissolved in dry pyridine (3 mL). DMTr-Cl (80 mg, 0.23 mmol) was added, and the reaction was stirred for 4 h at rt. After TLC control showed completion of the reaction, the residual DMTr-Cl was quenched with MeOH (0.5 mL). Then, the reaction mixture was diluted with CH_2Cl_2 (50 mL) and extracted twice with 5% citric acid (100 mL) and once with sat. $NaHCO_3$ (100 mL). The organic layer was dried over Na_2SO_4 and evaporated. After purification by SiO_2 column chromatography (50% EtOAc in cyclohexane), product **8** (110 mg, 95%) was dried under high vacuum. R_f = 0.5 (75% EtOAc in cyclohexane); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.06 (s, 3H, H_3C-Si), 0.08 (s, 3H, H_3C-Si), 0.86 (s, 9H, $(H_3C)_3$-C), 1.85 (s, 3H, $(H_3C)CO$), 2.22 (s, 3H, $(H_3C)CO$), 3.25−3.32 (m, 2H, H_2-C(5′)), 3.71 (s, 6H, H_3C−O), 4.06 (m, 2H, H-C(4′), H-C(3′)), 4.17 (m, 1H, H-C(2′)), 4.29−4.52 (dd, 2H, H_2C-C(5)), 5.09 (d, 1H, HO-C(3′)), 5.72 (d, 1H, H-C(1′)), 6.85−6.88 (m, 4H, H-C(ar)), 7.24−7.36 (m, 9 H, H-C(ar)), 8.05 (s, 1H, H-C(6)), 10.02 (s, 1H, H-N(4)); ^{13}C NMR (75 MHz, DMSO-d_6) δ −4.4, −4.2 (2x CH_3-Si), 18.6 ($C(CH_3)_3$), 21.1 ($COCH_3$), 25.5 ($COCH_3$), 26.3 (3x CH_3-C-Si) 55.6 (2x OCH_3), 60.3 (CH_2-C(5)), 63.2 (C(5′), 69.5 (C(3′)), 76.8 (C(2′)), 82.8 (C(4′)), 86.4 (tert.C(DMT)), 91.5 (C(1′)), 105.2 (C(5)), 113.9 (C(ar)), 127.4 (C(ar)), 128.3 (C(ar)), 128.5 (C(ar)), 130.3 (2x C(ar)), 135.8 (C(ar)), 136.0 (C(ar)), 145.2 (C(6)), 145.8 (C(ar)), 154.3 (C(2)), 158.8 (C-OCH_3(ar)), 161.8 (C(4)), 170.5 ($COCH_3$), 171.2 ($COCH_3$); HRMS (ESI TOF) calcd for $C_{41}H_{51}N_3O_{10}Si$ [MNa^+] 796.3241, found 796.3218.

N^4-Acetyl-5-acetyloxymethyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-*tert*-butyldimethylsilyl cytidine-3′-*O*-2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (9).[29] Compound **8** (340 mg, 0.44 mmol) was dissolved in dry CH_2Cl_2 (6 mL). Simultaneously, DIPEA (312 μL, 1.8 mmol), 1-methylimidazole (18 μL, 0.22 mmol), and 2-cyanoethyl *N*,*N*-diisopropyl-chlorophosphoramidite (195 μL, 0.88 mmol) were added to the solution by syringes. The reaction was stirred at rt for 5 h. The reaction mixture was diluted with CH_2Cl_2 (250 mL) and extracted with sat. $NaHCO_3$ (250 mL). The organic layer was dried over Na_2SO_4, and the solvent was evaporated. The crude product was purified by SiO_2 chromatography (50% EtOAc in *n*-hexane, 0.5% TEA) and yielded **9** as a white foam (330 mg, 79%). R_f = 0.4 (75% EtOAc in *n*-hexane); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.04 (s, 6H, H_3C-Si), 0.82 (s, 9H, $(H_3C)_3$-C), 0.90−1.04 (m, 12H, H_3C-CH), 1.82 (s, 3H, $(H_3C)CO$), 2.22 (s, 3H, (H_3C)CO-N(4)), 2.50−2.72 (t, 2H, H_2C-CN), 3.40−3.53 (m, 6H, H_2-C(5′), CH_2-P, 2x HC-$(CH_3)_2$), 3.71 (s, 6H, H_3C−O), 4.06 (m, 2H, H-C(4′), H-C(3′)), 4.39 (m, 1H, H-C(2′)), 4.38−4.54 (m, 2H, H_2C-C(5)), 5.79−5.87 (d, 1H, H-C(1′)), 6.84−6.87 (m, 4H, H-C(Ar)), 7.24−7.37 (m, 9H, H-C(Ar)), 8.04 (s, 1H, H-C(6)), 10.03 (s, 1H, HN(4)). ^{31}P NMR (121 MHz, DMSO-d_6) δ 149.2, 149.5 ppm; HRMS (ESI, FT-ICR) calcd for $C_{50}H_{68}N_5O_{11}PSi$ [MH^+] 974.4495, found 974.4483.

RNA Solid-Phase Synthesis. Phosphoramidite chemistry was applied for automated RNA strand elongation using 2′-*O*-TOM nucleoside building blocks[48] (ChemGenes) and a polystyrene support (GE Healthcare, Custom Primer SupportTM, 80 μmol/g, PS 200 or 5G) in combination with phosphoramidite **9**. All oligonucleotides were synthesized on an ABI 392 Nucleic Acid Synthesizer with the following setup: detritylation (2.0 min) with dichloroacetic acid/1,2-dichloroethane (4:96), coupling (6.0 min) with phosphoramidites/MeCN (0.1 M × 130 μL), and 5-benzylthio-1*H*-tetrazole/MeCN (0.3 M × 360 μL), capping (2 × 20 s, 1:1 Cap A/Cap B) with Cap A: DMAP in MeCN (0.5 M) and Cap B: Ac_2O/*sym*-collidine/MeCN (2:3:5), oxidation (1.0 min) with I_2 (20 mM) in THF/pyridine/H_2O (7:2:1). The solutions of amidites and tetrazole and MeCN were dried over activated molecular sieves (3 Å) overnight.

Deprotection of 5-Hydroxymethylpyrimidine-Modified RNA.[49] The solid support was removed and treated with $MeNH_2$ in EtOH (33%, 0.65 mL) and $MeNH_2$ in H_2O (40%, 0.65 mL) for 4 h at rt for short sequences (<10 nt) and for 4 h at 35 °C for longer sequences (>10 nt). Alternatively, the solid support was treated with $MeNH_2$ in H_2O (40%, 0.65 mL) and NH_3 in H_2O (28%, 0.65 mL) for 30 min at 65 °C. The supernatant was removed, and the solid support was washed three times with EtOH/H_2O (1:1). The supernatant and the washings were combined with the deprotection solution of the residue, and the whole mixture was evaporated to dryness. For the 2′-silyl protecting groups to be removed, the resulting residue was treated with tetrabutylammonium fluoride trihydrate (TBAF·$3H_2O$) 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, HiPrepTM 26/10 Desalting; 2.6 × 10 cm, Sephadex G25) eluting with H_2O, and the collected fraction was evaporated to dryness and dissolved in H_2O (1 mL). Analysis of the crude RNA after deprotection was performed by anion-exchange chromatography on a Dionex DNAPac PA-100 column (4 mm × 250 mm) at 80 °C; injection: 200 pmol of crude RNA in 100 μL of H_2O; 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), $NaClO_4$ (0.5 M), urea (6 M); gradient: 0−60% B in A within 45 min and UV detection at 260 nm (Figure S1).

Purification of 5-Hydroxymethylpyrimidine-Modified RNA. Crude RNA products were purified on a semipreparative Dionex DNAPac PA-100 column (9 mm × 250 mm) at 80 °C with a flow rate of 2 mL/min; injection: 10−40 nmol of crude RNA in 100 μL of H_2O; eluent A: 25 mM Tris·HCl (pH 8.0), 6 M urea; eluent B: Tris·HCl (25 mM) (pH 8.0), $NaClO_4$ (0.5 M), urea (6 M); gradient (for target RNA between 25 and 30 nt in length): 30−45% B in A within 20 min; UV detection at 260 nm. Fractions containing RNA were loaded on a C18 SepPak Plus cartridge (Waters/Millipore), washed with 0.1−0.15 M $(Et_3NH)^+HCO_3^-$ and H_2O, and eluted with H_2O/MeCN (1:1). RNA-containing fractions were evaporated to dryness and dissolved in H_2O (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 photometrical 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 microLC system. RNA sequences were analyzed in negative-ion mode with a potential of −4 kV applied to the spray needle. LC: sample (200 pmol RNA dissolved in 30 μL of 20 mM EDTA solution; average injection volume: 30 μL), column (Waters XTerraMS, C18 2.5 μm; 2.1 × 50 mm) at 21 °C; flow rate: 30 μL/min; eluent A: Et_3N (8.6

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mM), 1,1,1,3,3,3-hexafluoroisopropanol (100 mM) in H_2O (pH 8.0); eluent B: MeOH; gradient: 0−100% B in A within 30 min; UV detection at 254 nm (Figure S2).

Thermal Denaturation Experiments. Solutions of the duplex and hairpin RNAs were prepared in 10 mM sodium phosphate buffer with 150 mM NaCl (pH 7) at concentrations of 2−32 μM. Thermal denaturation was performed on a Cary 300 UV−vis spectrophotometer equipped with a thermal controller. Heating and cooling cycles were performed from 0 to 90 °C three times at a rate of 0.5 °C/min and recorded at 260 nm.

Crystal Structure. A chemically synthesized 27-nucleotide RNA corresponding to the *E. coli* 23S rRNA sarcin−ricin loop (SRL) modified with 5-hydroxymethylcytosine at position 2651 was used for crystallization trials. RNA was dissolved in a buffer made with 1 mM Na_2EDTA (pH 8.0) and 10 mM Tris-HCl (pH 8.0) at 350 μM concentration. The RNA sample was heated at 55 °C for 10 min and cooled at 25 °C by switching off the heating block. Crystals were grown for 2 weeks at 20 °C by mixing 4 μL of RNA sample with 2 μL of a crystallization buffer made with 3.0 M ammonium sulfate, 10 mM magnesium chloride, 10 mM manganese chloride, and 50 mM potassium 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.0). Prior to data collection, crystals were cryoprotected for approximately 5 min in a reservoir solution containing 15% of glycerol, 3.4 M of ammonium sulfate, 10 mM magnesium chloride, 10 mM manganese chloride, and 50 mM potassium MOPS (pH 7.0), flash-frozen in liquid ethane. X-ray diffraction data were collected on the X06SA beamline at the SLS synchrotron. Data were processed with the XDS Package,[44] and the structure was solved by molecular replacement with MOLREP[45] using an unmodified SRL RNA as a search model (PDB ID: 3DVZ).[39] The structure was refined with the PHENIX package[46] with a reasonable free R factor despite merohedral twinning (Table S1). The model was built using Coot.[47] Coordinates have been deposited with the PDB database (ID: 5NQI).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.7b01171.

^{1}H and ^{13}C NMR spectra, HPLC traces of deprotected (crude and purified) hm^{5}rC-modified RNA, X-ray data collection, and crystallographic refinement statistics (PDF)

Crystallization data for 5hmC2651-SRL (CIF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Shen, L.; Song, C.-X.; He, C.; Zhang, Y. *Annu. Rev. Biochem.* **2014**, *83*, 585.
(2) Zhang, H.-Y.; Xiong, J.; Qi, B.-L.; Feng, Y.-Q.; Yuan, B.-F. *Chem. Commun.* **2016**, *52*, 737.
(3) Münzel, M.; Globisch, D.; Carell, T. *Angew. Chem., Int. Ed.* **2011**, *50*, 6460.
(4) Song, C.-X.; Szulwach, K. E.; Fu, Y.; Dai, Q.; Yi, C.; Li, X.; Li, Y.; Chen, C.-H.; Zhang, W.; Jian, X.; et al. *Nat. Biotechnol.* **2011**, *29*, 68.
(5) Zheng, G.; Fu, Y.; He, C. *Chem. Rev.* **2014**, *114*, 4602.
(6) Kriaucionis, S.; Heintz, N. *Science* **2009**, *324*, 929.
(7) Shukla, A.; Sehgal, M.; Singh, T. R. *Gene* **2015**, *564*, 109.
(8) Klagsbrun, M. *J. Biol. Chem.* **1973**, *248*, 2612.
(9) Helm, M. *Nucleic Acids Res.* **2006**, *34*, 721.
(10) Motorin, Y.; Helm, M. *Biochemistry* **2010**, *49*, 4934.
(11) Squires, J. E.; Preiss, T. *Epigenomics* **2010**, *2*, 709.
(12) Chow, C. S.; Mahto, S. K.; Lamichhane, T. N. *ACS Chem. Biol.* **2008**, *3*, 30.
(13) Nakano, S.; Suzuki, T.; Kawarada, L.; Iwata, H.; Asano, K.; Suzuki, T. *Nat. Chem. Biol.* **2016**, *12*, 546.
(14) Haag, S.; Sloan, K. E.; Ranjan, N.; Warda, A. S.; Kretschmer, J.; Blessing, C.; Hübner, B.; Seikowski, J.; Dennerlein, S.; Rehling, P.; et al. *EMBO J.* **2016**, *35*, 2104.
(15) Squires, J. E.; Patel, H. R.; Nousch, M.; Sibbritt, T.; Humphreys, D. T.; Parker, B. J.; Suter, C. M.; Preiss, T. *Nucleic Acids Res.* **2012**, *40*, 5023.
(16) Hussain, S.; Sajini, A. A.; Blanco, S.; Dietmann, S.; Lombard, P.; Sugimoto, Y.; Paramor, M.; Gleeson, J. G.; Odom, D. T.; Ule, J.; et al. *Cell Rep.* **2013**, *4*, 255.
(17) Khoddami, V.; Cairns, B. R. *Nat. Biotechnol.* **2013**, *31*, 458.
(18) Edelheit, S.; Schwartz, S.; Mumbach, M. R.; Wurtzel, O.; Sorek, R. *PLoS Genet.* **2013**, *9*, e1003602.
(19) Amort, T.; Soulière, M. F.; Wille, A.; Jia, X.-Y.; Fiegl, H.; Wörle, H.; Micura, R.; Lusser, A. *RNA Biol.* **2013**, *10*, 1003.
(20) Amort, T.; Rieder, D.; Wille, A.; Khokhlova-Cubberley, D.; Riml, C.; Trixl, L.; Jia, X.-Y.; Micura, R.; Lusser, A. *Genome Biol.* **2017**, *18*, 1.
(21) Fu, L.; Guerrero, C. R.; Zhong, N.; Amato, N. J.; Liu, Y.; Liu, S.; Cai, Q.; Ji, D.; Jin, S.-G.; Niedernhofer, L. J.; et al. *J. Am. Chem. Soc.* **2014**, *136*, 11582.
(22) Huber, S. M.; van Delft, P.; Mendil, L.; Bachman, M.; Smollett, K.; Werner, F.; Miska, E. A.; Balasubramanian, S. *ChemBioChem* **2015**, *16*, 752.
(23) Delatte, B.; Wang, F.; Ngoc, L. V.; Collignon, E.; Bonvin, E.; Deplus, R.; Calonne, E.; Hassabi, B.; Putmans, P.; Awe, S.; et al. *Science* **2016**, *351*, 282.
(24) Saletore, Y.; Meyer, K.; Korlach, J.; Vilfan, I. D.; Jaffrey, S.; Mason, C. E. *Genome Biol.* **2012**, *13*, 175.
(25) Tardy-Planechaud, S.; Fujimoto, J.; Lin, S. S.; Sowers, L. C. *Nucleic Acids Res.* **1997**, *25*, 553.
(26) Dai, Q.; Song, C.-X.; Pan, T.; He, C. *J. Org. Chem.* **2011**, *76*, 4182.
(27) Münzel, M.; Globisch, D.; Trindler, C.; Carell, T. *Org. Lett.* **2010**, *12*, 5671.
(28) de Kort, M.; de Visser, P. C.; Kurzeck, J.; Meeuwenoord, N. J.; Van Der Marel, G. A.; Rüger, W.; van Boom, J. H. *Eur. J. Org. Chem.* **2001**, *2001*, 2075.
(29) Riml, C.; Micura, R. *Synthesis* **2016**, *48*, 1108.
(30) Serebryany, V.; Beigelman, L. *Tetrahedron Lett.* **2002**, *43*, 1983.
(31) Marky, L. A.; Breslauer, K. J. *Biopolymers* **1987**, *26*, 1601.
(32) Petersheim, M.; Turner, D. H. *Biochemistry* **1983**, *22*, 256.
(33) Ennifar, E.; Nikulin, A.; Tishchenko, S.; Serganov, A.; Nevskaya, N.; Garber, M.; Ehresmann, B.; Ehresmann, C.; Nikonov, S.; Dumas, P. *J. Mol. Biol.* **2000**, *304*, 35.
(34) Münzel, M.; Lischke, U.; Stathis, D.; Pfaffeneder, T.; Gnerlich, F. A.; Deiml, C. A.; Koch, S. C.; Karaghiosoff, K.; Carell, T. *Chem. - Eur. J.* **2011**, *17*, 13782.
(35) Thalhammer, A.; Hansen, A. S.; El-Sagheer, A. H.; Brown, T.; Schofield, C. J. *Chem. Commun.* **2011**, *47*, 5325.
(36) Renciuk, D.; Blacque, O.; Vorlickova, M.; Spingler, B. *Nucleic Acids Res.* **2013**, *41*, 9891.
(37) Xu, B.; Devi, G.; Shao, F. *Org. Biomol. Chem.* **2015**, *13*, 5646.

DOI: 10.1021/acs.joc.7b01171
J. Org. Chem. 2017, 82, 7939−7945

(38) Szulik, M. W.; Pallan, P. S.; Nocek, B.; Voehler, M.; Banerjee, S.; Brooks, S.; Joachimiak, A.; Egli, M.; Eichman, B. F.; Stone, M. P. *Biochemistry* **2015**, *54*, 1294.
(39) Olieric, V.; Rieder, U.; Lang, K.; Serganov, A.; Schulze-Briese, C.; Micura, R.; Dumas, P.; Ennifar, E. *RNA* **2009**, *15*, 707.
(40) Fauster, K.; Hartl, M.; Santner, T.; Aigner, M.; Kreutz, C.; Bister, K.; Ennifar, E.; Micura, R. *ACS Chem. Biol.* **2012**, *7*, 581.
(41) Košutić, M.; Jud, L.; Da Veiga, C.; Frener, M.; Fauster, K.; Kreutz, C.; Ennifar, E.; Micura, R. *J. Am. Chem. Soc.* **2014**, *136*, 6656.
(42) Szulik, M. W.; Pallan, P. S.; Nocek, B.; Voehler, M.; Banerjee, S.; Brooks, S.; Joachimiak, A.; Egli, M.; Eichman, B. F.; Stone, M. P. *Biochemistry* **2015**, *54*, 2550.
(43) Huber, S. M.; van Delft, P.; Tanpure, A.; Miska, E. A.; Balasubramanian, S. *J. Am. Chem. Soc.* **2017**, *139*, 1766.
(44) Kabsch, W. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *66*, 133.
(45) Vagin, A.; Teplyakov, A. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *66*, 22.
(46) Adams, P. D.; Afonine, P. V.; Bunkóczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L.-W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; et al. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *66*, 213.
(47) Emsley, P.; Cowtan, K. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2004**, *60*, 2126.
(48) Pitsch, S.; Weiss, P. A.; Jenny, L.; Stutz, A.; Wu, X. *Helv. Chim. Acta* **2001**, *84*, 3773.
(49) Riml, C.; Micura, R. *Methods Mol. Biol.* **2017**, *1562*, 295.

DOI: 10.1021/acs.joc.7b01171
J. Org. Chem. 2017, 82, 7939−7945