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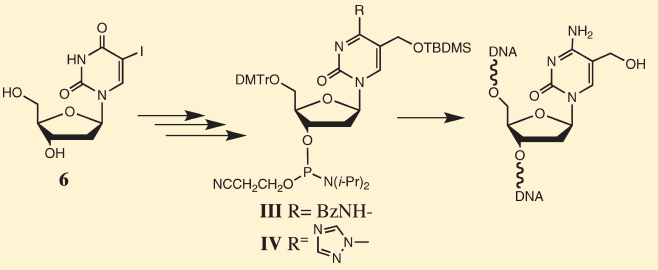
Syntheses of Two 5-Hydroxymethyl-2′-deoxycytidine Phosphoramidites with TBDMS as the 5-Hydroxymethyl Protecting Group and Their Incorporation into DNA

Qing Dai,*,† Chun-Xiao Song,† Tao Pan,‡ and Chuan He†

†Department of Chemistry and ‡Department of Biochemistry & Molecular Biology, The University of Chicago, 929 E. 57th St., Chicago, Illinois, 60637 United States

Supporting Information

ABSTRACT: 5-Hydroxymethylcytosine (5-hmC) is a newly discovered DNA base modification in mammalian genomic DNA that is proposed to be a major epigenetic mark. We report here the syntheses of two new versions of phosphoramidites **III** and **IV** from 5-iodo-2′-deoxyuridine in 18% and 32% overall yields, respectively, with TBDMS as the 5-hydroxyl protecting group. Phosphoramidites **III** and **IV** allow efficient incorporation of 5-hmC into DNA and a "one-step" deprotection procedure to cleanly remove all the protecting groups. A "two-step" deprotection strategy is compatible with ultramild DNA synthesis, which enables the synthesis of 5hmC-containing DNA with additional modifications.



5-Methylcytosine (5-mC) is an important DNA modification found in eukaryotes and is referred to as the fifth base besides dA, dC, dG, and dT. It constitutes ∼2−8% of the total cytosine in human genomic DNA and impacts a broad range of biological functions including gene expression, maintenance of genome integrity, parental imprinting, X-chromosome inactivation, regulation of development, aging, cancer, and so on.[1] In 2009, 5-hydroxymethylcytosine (5-hmC), an oxidized form of 5-mC, was discovered in substantial amounts in mammalian genome of certain cell types as the "sixth" base.[2] Recent studies have shown that 5-hmC is a widespread DNA modification in the brain tissues and stem cells, and its abundance is dependent on tissue type.[3] A group of Tet dioxygenases have been shown to utilize dioxygen to oxidize 5-mC to 5-hmC in the mammalian genome and display important functions in the maintenance and normal myelopoiesis of embryonic stem cells (ES cells).[2b,4] We have recently developed an efficient method to specifically label and pull down 5-hmC-containing DNA fragments for subsequent deep sequencing to reveal the genome-wide distribution of 5-hmC, which in mouse cerebellum appears to be enriched in a gene expression level-dependent manner.[5] All results so far suggested that 5-hmC is an important epigenetic modification.[6] In order to facilitate the biological study of 5-hmC in DNA, an efficient synthesis of 5-hmC-containing DNA as model substrates is a prerequisite. In addition, DNA with other labeling such as fluorophores or biotin is usually needed for biochemical characterizations, whose incorporation into DNA often requires or prefers the use of ultramild DNA synthesis. Therefore, it is also highly desirable to develop 5-hmC phosphoramidite building blocks that are compatible with ultramild DNA synthesis.

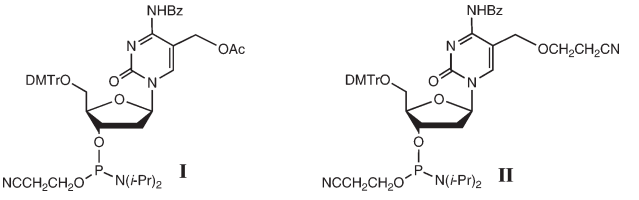


Figure 1. Two reported phosphoramidite building blocks of 5-hydroxymethyl-2′-deoxycytidine.

So far, two phosphoramidite building blocks (**I**[7] and **II**,[8] Figure 1) have been developed for the incorporation of 5-hmC into DNA. These methods are currently used in the synthesis of 5-hmC-containing DNAs for biological studies. However, both methods pose limitations in the postsynthetic removal of the protecting groups.

Phosphoramidite **I** uses Ac as the protecting group for the 5-hydroxymethyl (5-CH_2OH) group. After its incorporation into DNA, the postsynthetic treatment with NH_4OH generates an amide byproduct, which is produced by the S_N2 reaction of ammonia attacking the pseudobenzylic carbon with the OAc group as the leaving group to give an amine intermediate followed by migration of the benzoyl group.[7] To avoid the formation of this byproduct, strong base treatment (0.1 M NaOH in dioxane/H_2O) followed by ammonolysis is required. Phosphoramidite **II** with a cyanoethyl as the protecting group of 5-CH_2OH is more widely used since it is commercially available. However, the postsynthetic removal of the cyanoethyl group

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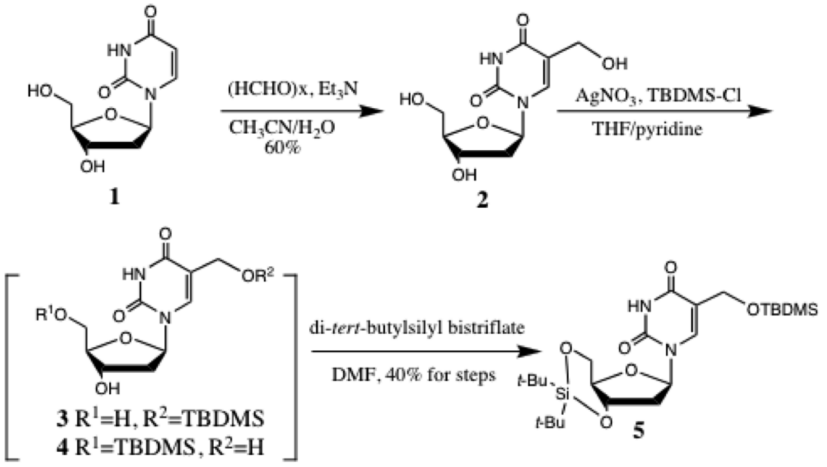
Scheme 1. Synthesis of Intermediate 5



turned out to be troublesome.[8] Treatment of synthetic DNA with NH_4OH at 65 °C for 60 h could not completely remove the protecting group. An additional treatment with stronger base (NaOMe in MeOH) is necessary. Obviously, neither of the phosphoramidites reported is suitable for synthesizing 5-hmC-containing DNA with additional base-labile modifications. Therefore, it is highly desirable to develop new 5-hmC phosphoramidites using protecting groups that can be cleanly removed under mild conditions. Herein we report the efficient syntheses of two 5-hmC phosphoramidite building blocks (**III** and **IV**) and demonstrated advantages in the removal of the protecting groups following oligo synthesis.

We first chose to synthesize phosphoramidite **III** with TBDMS and Bz as 5-CH_2OH and exocyclic amino protecting groups, respectively (Scheme 1). Unlike OAc at the pseudobenzylic position, *O*-TBDMS is not a good leaving group and thus will not result in the formation of the S_N2 byproduct during NH_4OH treatment. TBDMS can be readily removed using by fluoride treatment such as TBAF.

5-Hydroxymethyl-2′-deoxyuridine (5-hmdU, **2**) is commercially available but relatively expensive;[9] therefore, we chose to start from 2′-deoxyuridine (dU, **1**) and converted it to **2** in 60% yield (Scheme 1).[10] Both the 5- and 5′-hydroxyl of **2** are primary alcohols; however, the 5-hydroxyl is more reactive than 5′-hydroxyl since it is in the pseudobenzylic position.[11] Conte et al. reported that 5-CH_2OH of **2** could be selectively protected with TBDMS. When **2** was treated with $AgNO_3$ and TBDMS-Cl in the presence of pyridine and THF, a mixture of the desired **3** and its regioisomer **4** was obtained in a 4:1 ratio in 82% combined yield.[12] Unfortunately, these two isomers could not be resolved by silica gel chromatography using various eluent systems.[13] Instead we successfully resolved the two isomers by treating the mixture of **3** and **4** with di-*tert*-butylsilyl bistriflate in DMF. A cyclic six-membered ring was formed for **3** to generate **5**; in contrast, a cyclic silyl ring between 3′ and 5 hydroxyls of **4** could not form. Thus, compound **5** was obtained in pure form in 40% yield from **2** after silica gel chromatography (overall yield from **1** was 24%).

Although we were able to prepare the key intermediate **5** using the above method, several drawbacks such as the difficulty in purifying **2** due to its high polarity, the formation of the undesired regioisomer **4**, and the unsatisfactory overall yield prompted us to explore more efficient synthesis of **5**. Crouch et al. reported that 5-iodo-3′,5′-di-*O*-TBDMS-2′-deoxyuridine could be converted to the corresponding 5-formyl-dU analogue in 82% yield using standard Stille coupling conditions.[14] We reasoned that the reduction of the aldehyde analogue should generate the corresponding alcohol. Our new synthesis started from a commercial reagent, 5-iodo-2′-deoxyuridine (**6**, Scheme 2). We chose to protect the 3′ and 5′-hydroxyls with di-*tert*-butylsilyl group so that it can be selectively removed after 5-CH_2OH is protected by TBDMS. Thus, **6** was converted to **7** in 92% yield. Stille reaction generated the corresponding 5-formyl-dU analogue **8** in 85% yield. Reduction of **8** with $NaBH_4$ in the presence of $CeCl_3$ gave the corresponding 5-hmdC analogue **9** in 78% yield. Protection of the hydroxyl group with TBDMS provided **5** in 95% yield. The product for each step could be easily purified using low polarity eluents. The overall yield of **5** was improved to 58%.

To prepare 5-hmC phosphoramidite **III**, we next converted **5** into the 5-hmdC analogue by first treating **5** with $POCl_3$ and 1,2,4-triazole to generate a 4-triazolyl intermediate, followed by reacting with NH_4OH in dioxane to generate the amine **10** in 65% yield in two steps. Protection of the exocyclic amine provided **11** in 80% yield. Selective removal of the 3′,5′-silyl protecting group of **11** with HF in pyridine generated **12** in 84% yield. Selective protection of the 5′-OH of **12** with DMTr (**13**, 82%) and subsequent phosphitylation of the 3′-OH by standard procedure gave phosphoramidite **III** (87%). The synthesis entails 9 steps in 18% overall yield from **6**.

So far, all of the syntheses of phosphoramidite building blocks **I**, **II**, and **III** required a multiple-step transformation of converting 5-hmU derivatives to the corresponding 5-hmC analogues in two steps and an additional step to protect the resulting exocyclic amine with Bz. To simplify the synthesis, we chose to synthesize another version of phosphoramidite **IV** that bears 4-triazoylide instead of 4-BzNH. Since 4-triazolide can be converted simultaneously into 4-NH_2 during postsynthetic NH_4OH treatment, two steps of reaction could therefore be spared. The same strategy has been successfully used for synthesizing nucleotides containing 4-NH_2, 4-MeO, 4-EtO, 4-Me_2N-NH, and 4-SH analogues.[15] Besides allowing for the incorporation of 5-hmdC into DNA, phosphoramidite **IV** may also find applications in synthesizing DNA containing 4-substituted 5-hydroxymethylpyrimidine derivatives.

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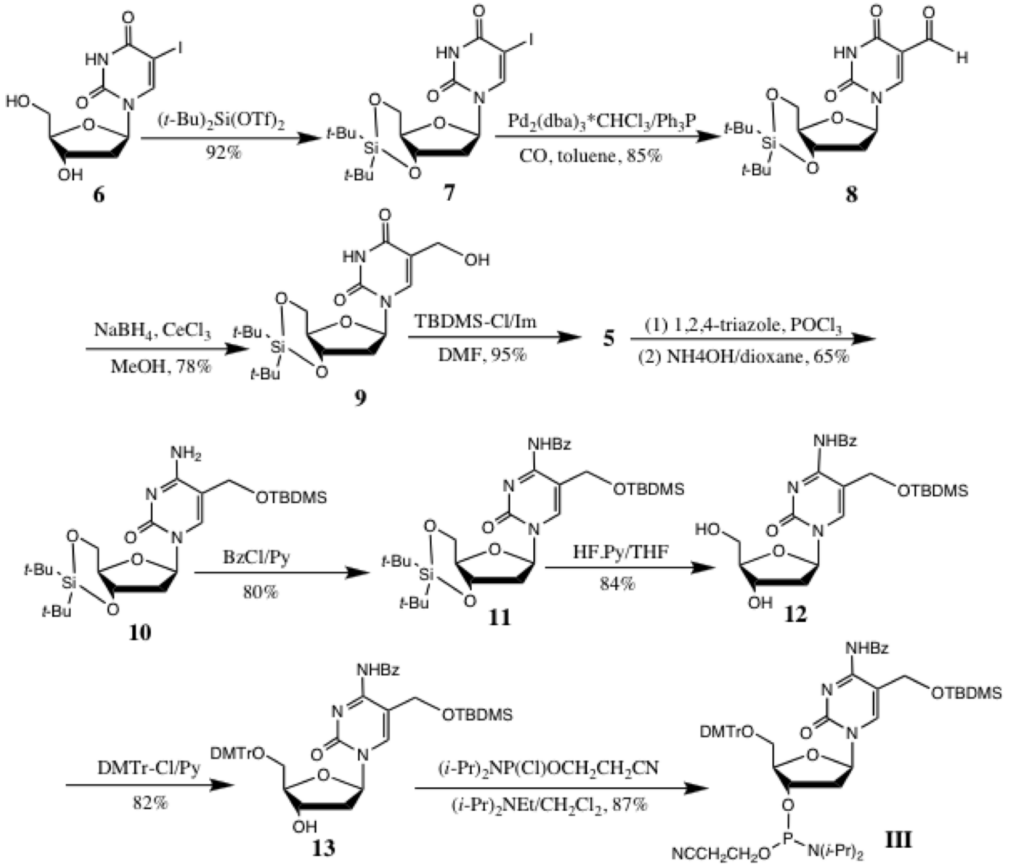
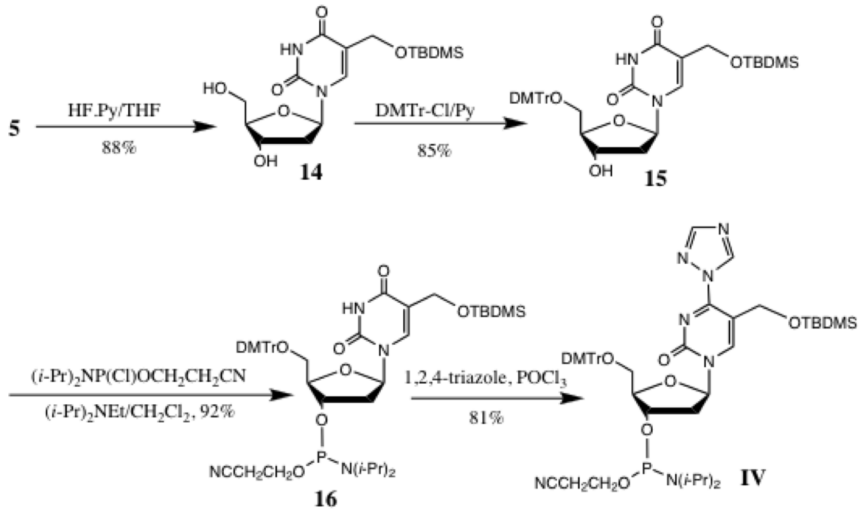
Scheme 2. Synthesis of Phosphoramidite III from 6



Scheme 3. Synthesis of Phosphoramidite IV



The synthesis of phosphoramidite **IV** started from intermediate **5** (Scheme 3). Selective removal of the 3′,5′-silyl protecting group of **5** with HF in pyridine generated **14** in 88% yield. Selective protection of the 5′-OH of **14** with DMTr (**15**, 85%) and subsequent phosphitylation of the 3′-OH gave phosphoramidite **16** in 92% yield. Phosphoramidite **16** was quantitatively converted to phosphoramidite **IV**, although it has a silylated hydroxymethyl group in the adjacent 5-position.[8a] Phosphoramidite **IV** was also stable during silica gel chromatography, and it was isolated in 81% yield. The synthesis entails 8 steps, and the overall yield from **1** reached 32%.

Phosphoramidites **III** and **IV** were then incorporated into a short model sequence 5′- TCXGA (X = 5-hmC) using ultramild reagents with the modified phosphoramidites using double

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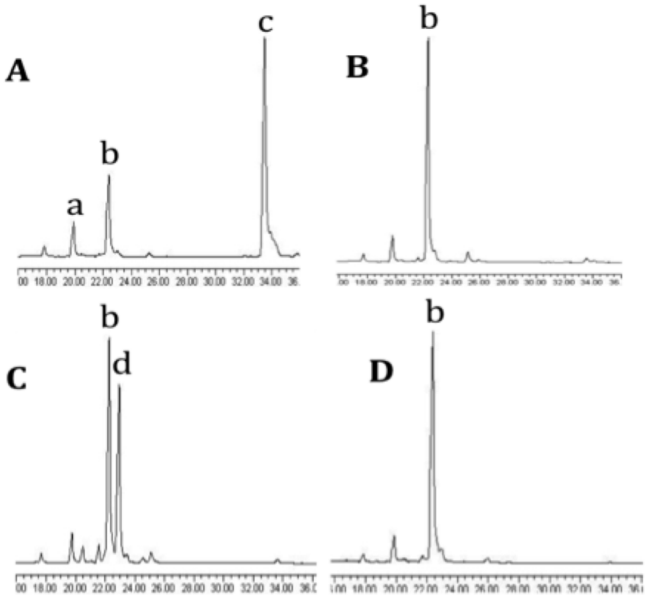

Figure 2. (A) **ODN-2** was deprotected by treatment of NH_4OH overnight at rt. Peak a is a failed sequence dimer, 5′-CT (Maldi ms $[MH]^+$ = 531.1); peak b is the fully deprotected 5mer 5′-GAXCT (Maldi ms $[MH]^+$ = 1493); peak c is the 5mer with TBDMS protecting group on 5hmC (Maldi ms $[MH]^+$ = 1607). (B) **ODN-2** was deprotected by treatment of NH_4OH overnight at 65 °C. (C) **ODN-3** was deprotected by treatment of NH_4OH overnight at 65 °C; peak d is the 5mer with cyanoethyl protecting group on 5hmC (Maldi ms $[MH]^+$ = 1546). (D) **ODN-2** was deprotected by treatment of 0.5 M NH_4F in MeOH overnight followed by NH_4OH treatment at rt for 2 h.

coupling to give oligodeoxynucleotides **ODN1** and **ODN2**, respectively. As comparion, phosphoramidite **II** from Glen Research was incorporated into the same sequence under the same conditions to give **ODN3**. After the postsynthetic treatment of **ODN2** with NH_4OH at room temperature overnight, reverse phase HPLC analysis (Figure 2A) showed that, besides DNA with a TBDMS protecting group (peak c, t_R = 34 min), over 20% of DNA without TBDMS group was also produced (peak b, t_R = 22 min). It is interesting to note that the 5-CH_2O-TBDMS group is much more labile to the NH_4OH treatment than the regular 2′-*O*-TBDMS protecting group in RNA synthesis, which can survive the overnight treatment at 55 °C. This observation is consistent with the enhanced reactivity of the 5-CH_2OH in **2** as indicated by selective protection of 5-CH_2OH by TBDMS or Ac against the 5′-hydroxyl.[11,12] The liability of the 5-CH_2O-TBDMS ether to the NH_4OH treatment suggested that it is possible to fully remove it from the oligo at elevated temperature so that the extra fluoride treatment might be spared. Indeed, when **ODN 2** was treated with NH_4OH at 65 °C for 16 h, HPLC analysis indicated that TBDMS was close to fully removed (Figure 2B). The same treatment of **ODN 1** also gave the same major desired product. In contrast, **ODN-3** only gave about 56% of the desired product, in addition to another 44% with the 5-cyanoethyl protecting group still on (Figure 2C), suggesting that the TBDMS is a superior protecting group to the cyanotheyl group.

The above one-step deprotection procedure is very convenient for the synthesis of DNA containing 5-hmC. For DNA containing additional labeling modifications that requires milder deprotection, a prior treatment with fluoride to remove the TBDMS group may allow the use of ultramild deprotection of the synthetic DNA. Thus, after treating the solid resin bearing newly synthesized **ODN-2** with NH_4F (0.5 M in MeOH) overnight at room temperature to remove the TBDMS group and rinsing with MeOH to get rid of excess NH_4F, the ultramild deprotection condition was applied (NH_4OH treatment at rt for 2 h). HPLC analysis showed that all of the protecting groups were cleanly removed and the desired product (peak b) was produced as the major product (Figure 2D). The same treatment of **ODN-1** also gave fully deprotected oligo. This is surprising because 4-benzoyl protecting group of cytidine is usually not compatible with ultramild deprotection conditions; probably the 5-CH_2OH facilitate the removal of benzoyl group in the presence of NH_4OH.

In summary, we have achieved the syntheses of two novel 5-hmC phosphoramidites **III** and **IV** with TBDMS as the 5-Hydroxymethyl protecting group from 5-iodo-2′-deoxyuridine in good overall yields (18% for **III** and 32% for **IV**). Complete removal of the TBDMS protection group was accomplished conveniently by a "one-step" procedure (overnight treatment with NH_4OH at 65 °C) or a "two-step" procedure (NH_4F treatment followed by the ultramild NH_4OH treatment). These two deprotection procedures are complementary. While the "one-step" procedure can be used conveniently for the synthesis of DNA with the 5-hmC modification, the "two-step" procedure is more suitable for ultramild synthesis of DNA containing 5-hmC and additional base-labile modifications. The application of the new phosphoramidite building blocks will greatly facilitate the biological study of 5-hmC in DNA. While the current work was going on, another 5-hmC phosphoramidite using a carbamate to protect simultaneously the amino and hydroxyl groups was reported.[16] The synthesis derives from 5-iodo-deoxycytidine and entails seven steps of reaction and gave the phosphoramidite in 7.5% overall yield. Although a significant advance compared to the commercial available phosphoramidite, the synthesis is still not compatible with ultramild deprotection conditions.

EXPERIMENTAL SECTION

3′,5′-*O*-Di-*tert*-butylsilyl-5-iodo-2′-deoxyuridine (7). To a cloudy solution of **6** (2.05 g, 5.79 mmol) in DMF (15 mL) at 0 °C was added di-*tert*-butylsilyl-bis(trifluoromethanesulfonate) (2.39 mL, 1.1 equiv). After 10 min of stirring at rt, imidazole (0.98 g, 2.5 equiv) was added, and the mixture was stirred at rt for 0.5 h. After removal of DMF under high vacuum, the residue was dissolved in ethyl acetate, washed with water, 5% $NaHCO_3$ solution, and brine, and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, the residue was purified by silica gel chromatography, eluting with 1−3% MeOH in CH_2Cl_2, to give **7** (2.63 g, 92%) as white foam. ^1H NMR (500 MHz) ($CDCl_3$) δ: 9.86 (br., 1H), 7.66 (s, 1H), 6.13 (m, 1H), 4.46 (m, 1H), 4.20 (m, 1H), 4.01 (m, 1H), 3.71 (m, 1H), 2.41 (m, 2H), 1.08 (s, 9H), 0.99 (s, 9H). ^{13}C NMR (125.8 MHz) ($CDCl_3$) δ: 160.2, 150.0, 144.2, 84.8, 78.3, 74.6, 69.0, 67.3, 39.0, 27.5, 27.2, 22.8, 20.2. HRMS calculated for $C_{17}H_{28}IN_2O_5Si$, $[MH^+]$ 495.0812 (calcd), 495.0807 (found).

3′,5′-*O*-Di-*tert*-butylsilyl-5-formyl-2′-deoxyuridine (8). To a solution of **7** (2.01 g, 4.06 mmol) in anhydrous THF (50 mL) in a flask with a self-contained glass coupling apparatus equipped with a pressure equalizing addition funnel were added triphenylphosphine (0.64 g, 0.6 equiv) and $Pd_2(dba)_3 \cdot CHCl_3$ (0.42 g, 0.40 mmol, 0.10 equiv). The apparatus was charged with 50 psi of CO and heated to 70 °C, and Bu_3SnH (1.19 mL, 1.05 equiv) was added slowly with a syringe within 1 h. After that, the mixture was kept stirring for 2 h at 70 °C. After cooled to rt, the small amount of solid was removed by filtration. The solvent of the filtrate was removed under reduced pressure, and the residue was

purified by silica gel chromatography, eluting with 2−3% MeOH in CH_2Cl_2, to give **8** (1.37 g, 85%) as pale foam. ^{1}H NMR (500 MHz) ($CDCl_3$) δ: 10.03 (s, 1H), 8.60 (br., 1H), 8.21 (s, 1H), 6.13 (m, 1H), 4.49 (m, 1H), 4.17 (m, 1H), 4.08 (m, 1H), 3.77 (m, 1H), 2.45 (m, 2H), 1.07 (s, 9H), 1.04 (s, 9H). ^{13}C NMR (125.8 MHz) ($CDCl_3$) δ: 186.7, 162.1, 149.7, 145.3, 112.1, 86.5, 79.6, 75.1, 68.1, 40.1, 28.4, 28.1, 23.6, 21.1. HRMS calcd for $C_{18}H_{29}N_2O_6Si$, [MH^+] 397.1795 (calcd), 397.1789 (found).

3′,5′-*O*-Di-*tert*-butylsilyl-5-hydroxymethyl-2′-deoxyuridine (9). To a solution of **8** (1.50 g, 3.79 mmol) in MeOH (40 mL) was added $CeCl_3.7H_2O$ (4.24 g, 3.0 equiv) under argon, and the mixture was cooled to 0 °C with an ice-bath. $NaBH_4$ (144 mg, 1.0 equiv) was added slowly within 15 min. After that, the ice-bath was removed, the mixture was stirred at rt for 0.5 h, and TLC showed that **8** was completely consumed. Silica gel (5.0 g) was added, and MeOH was removed under reduced pressure. The residue was purified by silica gel chromatography, eluting with 8−12% MeOH in CH_2Cl_2, to give **9** as pale foam (1.18 g, 78%). ^{1}H NMR (500 MHz) ($CDCl_3$) δ: 10.10 (br., 1H), 7.34 (s, 1H), 6.20 (t, J = 5.0 Hz, 1H), 4.34−4.45 (m, 3H), 4.21 (m, 1H), 4.01 (m, 1H), 3.68 (m, 1H), 2.36 (m, 2H), 1.06 (s, 9H), 1.00 (s, 9H). ^{13}C NMR (125.8 MHz) ($CDCl_3$) δ: 164.7, 151.1, 138.1, 115.3, 85.0, 79.0, 75.7, 68.2, 59.2, 39.6, 28.4, 28.1, 23.6, 21.1. HRMS calcd for $C_{18}H_{31}N_2O_6Si$, [MH^+] 399.1951 (calcd), 399.1946 (found).

3′,5′-*O*-Di-*tert*-butylsilyl-5-*tert*-butyldimethylsiloxymethyl-2′-deoxyuridine (5). To a solution of **9** (1.00 g, 2.51 mmol) in DMF (20 mL) were added TBDMS-Cl (454 mg, 1.2 equiv) and imidazole (427 mg, 2.5 equiv). The mixture was heated to 60 °C for 2 h under argon. DMF was then removed under high vacuum, and to the residue was added ethyl acetate (100 mL). The solution was washed with water, 5% $NaHCO_3$, and brine and dried over Na_2SO_4. After filtration, the solvent of the filtrate was removed under reduced pressure, and the residue was purified by silica gel chromatography, eluting with 1−2% MeOH in CH_2Cl_2, to give **5** (1.22 g, 95%) as white foam. ^{1}H NMR (500 MHz) ($CDCl_3$) δ: 9.69 (br., 1H), 7.33 (s, 1H), 6.30 (m, 1H), 4.39−4.43 (m, 3H), 4.20 (m, 1H), 3.99 (m, 1H), 3.68 (m, 1H), 2.36 (m, 2H), 1.05 (s, 9H), 1.01 (s, 9H), 0.93 (s, 9H), 0.08 (s, 6H). ^{13}C NMR (125.8 MHz) ($CDCl_3$) δ: 163.3, 151.2, 135.6, 116.1, 84.6, 78.9, 75.9, 68.2, 59.1, 39.6, 28.4, 28.1, 26.9, 23.6, 21.1, 19.2, −4.48, −4.49. HRMS calcd for $C_{24}H_{45}N_2O_6Si$, [MH^+] 485.3047 (calcd), 485.3041 (found).

3′,5′-*O*-Di-*tert*-butylsilyl-5-*tert*-butyldimethylsiloxymethyl-2′-deoxycytidine (10). To a solution of **5** (1.00 g, 2.51 mmol) in CH_3CN (20 mL) were added Et_3N (8.56 mL, 55.3 mmol, 22 equiv) and 1,2,4-triazole (3.47 g, 50.5 mmol, 20.0 equiv), and the mixture was cooled to 0 °C. $POCl_3$ (0.60 mL, 6.27 mmol, 2.5 equiv) was added, and the mixture was stirred at 0 °C for 0.5 h and at rt for 1 h. CH_2Cl_2 (100 mL) was added, and the mixture was washed with water, 5% $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated to dryness. The residue was dissolved in 1,4-dioxane (10 mL) and cooled to 0 °C, and NH_4OH (1 mL) was then added. The mixture was stirred at 0 °C for 1 h and then concentrated to dryness. The residue was purified by silica gel chromatography, eluting with 2−4% MeOH in CH_2Cl_2, to give **10** (835 mg, 65%) as white foam. ^{1}H NMR (500 MHz) ($CDCl_3$) δ: 7.28 (s, 1H), 6.17 (m, 1H), 4.36−4.46 (m, 3H), 4.06 (m, 1H), 3.97 (m, 1H), 3.71 (m, 1H), 2.36 (m, 2H), 1.02 (s, 9H), 0.98 (s, 9H), 0.86 (s, 9H), 0.06 (s, 6H). ^{13}C NMR (125.8 MHz) ($CDCl_3$) δ: 165.9, 156.3, 138.0, 106.5, 85.9, 79.0, 75.5, 68.5, 61.4, 40.3, 28.4, 28.1, 26.8, 23.6, 21.0, 19.2, −4.3. HMRS calcd for $C_{24}H_{46}N_3O_5Si_2$, [MH^+] 512.2976 (calcd), 512.2971 (found).

3′,5′-*O*-Di-*tert*-butylsilyl-5-*tert*-butyldimethylsiloxymethyl-*N*^{4}-benzoyl-2′-deoxycytidine (11). To a solution of **10** (800 mg, 1.56 mmol) in pyridine (12 mL) was added benzoyl chloride (0.22 mL, 1.87 mmol, 1.2 equiv) under argon. The mixture was stirred overnight at rt. MeOH (1 mL) was added to quench the reaction. After removal of the solvents under the reduced pressure, the residue was dissolved in CH_2Cl_2 (100 mL) and washed with water, 5% $NaHCO_3$, and brine. The organic phase was dried over Na_2SO_4 and concentrated to dryness. The residue was purified by silica gel chromatography, eluting with 1−2% MeOH in CH_2Cl_2, to give **11** (769 mg, 80%) as white foam. ^{1}H NMR (500 MHz) ($CDCl_3$) δ: 8.24 (m, 2H), 7.58 (s, 1H), 7.54 (m, 1H), 7.45 (m, 2H), 6.29 (m, 1H), 4.70 (m, 2H), 4.48 (m, 1H), 4.22 (m, 1H), 4.04 (m, 1H), 3.85 (m, 1H), 2.41 (m, 2H), 1.08 (s, 9H), 1.06 (s, 9H), 0.96 (s, 9H), 0.18 (s, 6H). ^{13}C NMR (125.8 MHz) (CD_3OD) δ: 158.4, 148.7, 137.9, 136.5, 133.6, 131.6, 130.8, 129.9, 129.1, 116.3, 85.3, 79.2, 75.7, 68.2, 59.4, 39.9, 28.4, 28.1, 27.0, 23.6, 21.1, 19.3, −4.36, −4.38. HRMS calcd for $C_{31}H_{50}N_3O_6Si_2$, [MH^+] 616.3238 (calcd), 616.3233 (found).

5-*tert*-Butyldimethylsiloxymethyl-*N*^{4}-benzoyl-2′-deoxycytidine (12). To a solution of **11** (720 mg, 1.17 mmol) in THF (20 mL) was added HF in pyridine (1.0 equiv) under argon, and the mixture was stirred at rt for 1 h. Silica gel (4.0 g) was added, and the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography, eluting with 3−7% MeOH in CH_2Cl_2, to give **12** (476 mg, 84%) as white foam. ^{1}H NMR (500 MHz) (acetone-d_6) δ: 8.26 (m, 2H), 8.15 (s, 1H), 7.56 (m, 1H), 7.47 (m, 2H), 6.34 (m, 1H), 4.73 (s, 2H), 4.52 (m, 1H), 4.03 (m, 1H), 3.80 (m, 1H), 2.39 (m, 1H), 2.30 (m, 1H), 0.97 (s, 9H), 0.18 (s, 6H). ^{13}C NMR (125.8 MHz) (acetone-d_6) δ: 159.7, 148.6, 139.6, 138.2, 133.3, 130.6, 129.0, 114.9, 89.1, 87.0, 72.3, 63.0, 59.4, 41.47, 26.5, 19.1, −5.0. HRMS calcd for $C_{23}H_{34}N_3O_6Si$, [MH^+] 476.2217 (calcd), 476.2211 (found).

5′-*O*-(4,4′-Dimethoxytrityl)-5-*tert*-butyldimethylsiloxymethyl-*N*^{4}-benzoyl-2′-deoxycytidine (13). To a solution of **12** (412 mg, 0.87 mmol) in pyridine (12 mL) was added DMTr-Cl (352 mg, 1.04 mmol, 1.2 equiv) under argon. The mixture was stirred overnight at rt. MeOH (1 mL) was added to quench the reaction. After concentrated to dryness, the residue was dissolved in CH_2Cl_2 (100 mL), and the mixture was washed with water, 5% $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated to dryness. The residue was purified by silica gel chromatography, eluting with 1−3% MeOH in CH_2Cl_2, to give **13** (553 mg, 82%) as white foam. ^{1}H NMR (500 MHz) (CD_3CN) δ: 8.24 (d, J = 8.0 Hz, 2H), 7.71 (s, 1H), 7.24−7.58 (m, 12H), 6.87 (m, 4H), 6.22 (m, 1H), 4.57 (m, 1H), 4.40 (m, 1H), 4.28 (m, 1H), 3.98 (m, 1H), 3.76 (s, 6H), 3.34 (m, 1H), 3.29 (m, 1H), 3.22 (m, 1H), 2.39 (m, 1H), 2.18 (m, 1H), 0.83 (s, 9H), 0.05 (s, 3H), 0.04 (s, 3H). ^{13}C NMR (125.8 MHz) (CD_3CN) δ: 159.7, 146.0, 139.3, 137.8, 136.9, 136.8, 133.5, 131.0, 130.9, 130.4, 129.2, 129.0, 128.9, 127.9, 114.1, 87.3, 86.8, 86.6, 71.9, 64.8, 59.5, 55.9, 40.8, 26.3, 18.9, −5.1. HRMS calcd for $C_{44}H_{52}N_3O_8Si$, [MH^+] 778.3524 (calcd), 778.3525 (found).

5′-*O*-(4,4′-Dimethoxytrityl)-5-*tert*-butyldimethylsiloxymethyl-*N*^{4}-benzoyl-2′-deoxycytidine 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (III). To a stirring solution of **13** (120 mg, 0.15 mmol) in CH_2Cl_2 (8 mL) were added 1-methylimidazole (3.40 mg, 41.5 μmol) and *N*,*N*-diisopropylethylamine (0.2 mL) under argon followed by 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (71.7 mg, 0.30 mmol). After the reaction mixture was stirred at room temperature for 0.5 h, CH_2Cl_2 (80 mL) was added. The mixture was washed with 5% aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated to dryness. The residue was purified by silica gel chromatography, eluting with 6−9% acetone in CH_2Cl_2 containing 0.2% Et_3N, to give **III** (131 mg, 87%) as white foam. ^{1}H NMR (500 MHz) (CD_3CN) δ: 8.30 (m, 2H), 7.31−7.72 (m, 13H), 6.87 (m, 4H), 6.18−6.28 (m, 1H), 4.62 (m, 1H), 4.40−4.50 (m, 2H), 4.15 (m, 1H), 3.76 (m, 6H), 3.65 (m, 4H), 3.32 (m, 2H), 2.65 (m, 2H), 2.54 (m, 2H), 2.28 (m, 1H), 2.16 (m, 1H), 1.17 (m, 9H), 1.05 (m, 3H), 0.84 (s, 9H), 0.04 (s, 6H). ^{31}P NMR (202.5 MHz) (CD_3CN) 148.44 ppm. HRMS calcd for $C_{53}H_{69}N_5O_9PSi$, [MH]$^+$ 978.4602 (calcd), 978.4611 (found).

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5-*tert*-Butyldimethylsiloxymethyl-2′-deoxyuridine (14). To a solution of **5** (440 mg g, 0.86 mmol) in THF (20 mL) was added HF in pyridine (1.0 equiv) under argon, and the mixture was stirred at rt for 1 h. Silica gel (4.0 g) was added and the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography, eluting with 3−7% MeOH in CH_2Cl_2, to give **14** (281 mg, 88%) as pale foam. ^{1}H NMR (500 MHz) (acetone-d_6) δ: 10.07 (br., 1H), 7.83 (s, 1H), 6.34 (m, 1H), 4.50 (s, 1H), 4.42 (s, 3H), 4.12 (m, 1H), 3.95 (m, 1H), 3.75 (m, 2H), 2.26 (m, 2H), 0.92 (s, 9H), 0.12 (s, 6H). ^{13}C NMR (125.8 MHz) (acetone-d_6) δ: 163.0, 151.3, 137.7, 114.7, 88.7, 85.9, 72.5, 63.2, 59.2, 41.0, 26.4, 19.0, −5.1. HRMS calculated for $C_{16}H_{29}N_2O_6Si$, [MH^+] 373.1795 (calcd), 373.1789 (found).

5′-*O*-(4,4′-Dimethoxytrityl)-5-*tert*-butyldimethylsiloxymethyl-2′-deoxyuridine (15). To a solution of **14** (260 mg, 0.70 mmol) in pyridine (8 mL) was added DMTr-Cl (284 mg, 0.84 mmol, 1.2 equiv) under argon. The mixture was stirred overnight at rt. MeOH (1 mL) was added to quench the reaction. The solvents were removed under reduced pressure, and the residue was dissolved with CH_2Cl_2 (100 mL). The mixture was washed with water, 5% $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated to dryness. The residue was purified by silica gel chromatography, eluting with 1−3% MeOH in CH_2Cl_2, to give **15** (400 mg, 85%) as pale foam. ^{1}H NMR (500 MHz) (CD_3CN) δ: 9.34 (br., 1H), 7.46 (m, 3H), 7.24−7.35 (m, 7H), 6.87 (m, 4H), 6.23 (m, 1H), 4.29 (m, 2H), 4.12 (m, 1H), 3.95 (m, 1H), 3.77 (s, 6H), 3.29 (m, 1H), 3.20 (m, 1H), 2.30 (m, 1H), 2.19 (m, 1H), 0.84 (s, 9H), 0.02 (s, 3H), 0.01 (s, 3H). ^{13}C NMR (125.8 MHz) (CD_3CN) δ: 163.3, 159.7, 151.3, 146.0, 137.6, 136.8, 131.0, 128.98, 128.90, 127.89, 118.3, 115.0, 114.1, 87.3, 86.0, 85.4, 72.1, 65.0, 59.0, 55.9, 40.5, 26.3, 18.9, 7.5. HRMS calculated for $C_{37}H_{47}N_2O_8Si$, [MH^+] 675.3102 (calcd), 675.3096 (found).

5′-*O*-(4,4′-Dimethoxytrityl)-5-*tert*-butyldimethylsiloxymethyl-2′-deoxyuridine 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (16). To a stirring solution of **15** (120 mg, 0.18 mmol) in CH_2Cl_2 (8 mL) were added 1-methylimidazole (3.40 mg, 41.5 μmol) and *N*,*N*-diisopropylethylamine (0.2 mL) under argon followed by 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (60 μL, 0.27 mmol, 1.5 equiv). After the reaction mixture was stirred at room temperature for 0.5 h, CH_2Cl_2 (80 mL) was added. The mixture was washed with 5% aqueous sodium carbonate and brine, dried over sodium sulfate, and concentrated to dryness. The residue was purified by silica gel chromatography, eluting with 6−9% acetone in CH_2Cl_2 containing 0.2% Et_3N, to give **16** (143 mg, 92%) as white foam. ^{1}H NMR (500 MHz) (CD_3CN) δ: 9.04 (br., 1H), 7.03−7.46 (m, 10H), 6.89 (m, 4H), 6.23 (m, 1H), 4.50 (m, 1H), 4.28 (m, 1H), 4.08 (m, 2H), 3.78 (s, 3H), 3.77 (s, 3H), 3.62 (m, 4H), 3.28 (m, 2H), 2.63 (m, 1H), 2.53 (m, 1H), 2.44 (m, 1H), 2.26 (m, 1H), 1.17 (m, 9H), 1.05 (d, 3H), 0.82 (s, 9H), 0.02 (s, 3H), 0.00 (s, 3H). ^{31}P NMR (202.5 MHz) (CD_3CN) 148.34, 148.27 ppm. HRMS calculated for $C_{46}H_{66}N_4O_9PSi$, [MH^+] 877.4337 (calcd), 877.4331 (found).

5′-*O*-(4,4′-Dimethoxytrityl)-5-*tert*-butyldimethylsiloxymethyl-4-triazolothymidine 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (IV). To a solution of **16** (110 mg, 0.13 mmol) in CH_3CN (5 mL) were added Et_3N (0.43 mL) and 1,2,4-triazole (174 mg), and the mixture was cooled to 0 °C. $POCl_3$ (30 μL) was added, and the mixture was stirred at 0 °C for 0.5 h and at rt for 1 h. Added another portion of $POCl_3$ (16 μL), and the mixture was stirred at rt for 5 h. CH_2Cl_2 (100 mL) was added, and the mixture was washed with water, 5% $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated to dryness. The residue was purified by silica gel chromatography, eluting with 6−10% acetone in CH_2Cl_2 containing 0.2% Et_3N, to give **IV** (94.5 mg, 81%) as white foam. ^{1}H NMR (500 MHz) (CD_3CN) δ: 9.18 (s, 1H), 8.50 (s, 0.5H), 8.38 (s, 0.5H), 8.15 (s, 1H), 7.24−7.42 (m, 9H), 6.87 (m, 4H), 6.18 (m, 1H), 4.92 (m, 1H), 4.55 (m, 2H), 4.22 (m, 1H), 3.75 (s, 6H), 3.73 (m, 4H), 3.40 (m, 2H), 2.66 (m, 1H), 2.56 (m, 1H), 2.25 (m, 2H), 1.17 (m, 9H), 1.07 (d, 3H), 0.79 (s, 9H), −0.04, −0.03 (s, 3H), 0.00 (s, 3H). ^{13}C NMR (125.8 MHz) (CD_3CN) δ: 159.7, 154.5, 154.5, 147.1, 147.0, 145.6, 136.6, 131.0, 131.0, 129.0, 128.9, 127.98, 127.95, 114.2, 89.9, 88.8, 89.0, 88.1, 88.0, 74.6, 74.4, 64.2, 60.9, 60.9, 59.5, 59.3, 55.9, 55.9, 44.1, 44.02, 44.00, 40.1, 40.1, 26.3, 26.2,, 25.0, 24.9, 24.8, 21.0, 18.6, −5.15, −5.16. ^{31}P NMR (202.5 MHz) (CD_3CN) 148.31, 148.24 ppm. HMRS calculated for $C_{48}H_{65}N_7O_8PSi$, [MH^+] 926.4402 (calcd), 926.4396 (found).

■ ASSOCIATED CONTENT

Supporting Information. Analytical data for compounds **5**, **7**−**13**, **III**, and **IV** including their ^{1}H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: daiqing@uchicago.edu.

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