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Preparation of DNA Containing 5-Hydroxymethyl-2'-Deoxycytidine Modification Through Phosphoramidites with TBDMS as 5-Hydroxymethyl Protecting Group

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Abstract

This unit describes procedures for preparation of two phosphoramidite building blocks **III** and **IV**, both containing a TBDMS as 5-CH₂OH-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. Methods are also presented for their incorporation into oligonucleotides by solid-phase synthesis, subsequent deprotection, and HPLC analysis.

Keywords

5-Hydroxymethylcytosine; DNA modification; oligodeoxyribonucleotide (ODN); phosphoramidite; solid-phase synthesis; genomic DNA; epigenetic; ultramild deprotection

INTRODUCTION

The discovery of 5-hydroxymethylcytosine (5-hmC) in the mammalian genome of certain cell types as the “sixth” base in 2009 (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009) has triggered tremendous studies in this area. 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; Tahiliani et al., 2009; Ito et al., 2010; Ko et al., 2010). All results so far have suggested that 5-hmC is an important epigenetic modification (Loenarz and Schofield, 2009). In order to facilitate the biological study of 5-hmC in DNA, an efficient synthesis of 5-hmC-containing DNA as a model substrate 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, which are compatible with ultramild DNA synthesis.

Before we started this work, two phosphoramidite building blocks (**I** and **II**, Fig. 4.47.1; Tardy-Planechaud et al., 1997; De Kort et al., 2001; Hansen et al., 2011) have been developed for the incorporation of 5-hmC into DNA (Fig. 4.47.1). These methods are currently used in the synthesis of 5-hmC-containing DNAs for biological studies. However, both methods pose limitations in the post-synthetic removal of the protecting groups.

Phosphoramidite **I** uses Ac as the protecting group for the 5-hydroxymethyl (5-CH₂OH) group. After its incorporation into DNA, the post-synthetic treatment with NH₄OH generates an amide byproduct, which is produced by the S_N2 reaction of ammonia attacking the

pseudobenzyl carbon with the OAc group as the leaving group to give an amine intermediate followed by migration of the benzoyl group (De Kort et al., 2001). To avoid the formation of this byproduct, strong base treatment (0.1 M NaOH in dioxane/H₂O) is required. Phosphoramidite **II** with a cyanoethyl as the protecting group of 5-CH₂OH is more widely used since it is commercially available. However, the post-synthetic removal of the cyanoethyl group turned out to be troublesome (Tardy-Planechaud et al., 1997). Treatment of synthetic DNA with NH₄OH at 65°C for 60 hr could not completely remove the protecting group. 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 demonstrate the advantages in the removal of the protecting groups following oligo synthesis.

We chose TBDMS as the 5-CH₂OH-protecting group for phosphoramidites **III** and **IV** for two reasons: (1) unlike OAc at the pseudobenzyl position, *O*-TBDMS is not a good leaving group and thus will not result in the formation of the S_N2 byproduct during NH₄OH treatment; (2) TBDMS may be readily removed using fluoride treatment such as ammonium fluoride.

CAUTION: Carry out all operations involving organic solvents and reagents in a well-ventilated fume hood, and wear protective glasses and gloves.

NOTE: Evaporation of solvents is carried out under reduced pressure at temperatures below 35°C.

PREPARATION OF KEY INTERMEDIATE 3',5'-O-di-*t*-BUTYLSILYL-5-*t*-BUTYLDIMETHYLSILOXYMETHYL-2'-DEOXYURIDINE (S.5)

To synthesize phosphoramidites **III** and **IV**, intermediate **S.5** is the key intermediate. We have tried several methods and found the route depicted in Figure 4.47.2 is the most efficient (Dai et al., 2011). 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 (Crouch and Eaton, 1994). 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 (**S.1**, Fig. 4.47.2). We chose to protect the 3' and 5'-hydroxyls with a di-*t*-butylsilyl group so that it could be selectively removed after 5-CH₂OH is protected by TBDMS. Thus, **S.1** was converted into **S.2** in 92% yield. The Stille reaction generated the corresponding 5-formyl-dU analogue **S.3** in 85% yield. Reduction of **S.3** with NaBH₄ in the presence of CeCl₃ gave the corresponding 5-hmdC analogue **S.4** in 78% yield. Protection of the hydroxyl group with TBDMS provided **S.5** in 95% yield. The product for each step could be easily purified using low-polarity eluents. The overall yield of **S.5** reaches to 58% from **S.1**.

Materials

5-Iodo-2'-deoxyuridine (Berry Associate)

N,N-Dimethylformamide (DMF, anhydrous, 99.8%, Sigma-Aldrich)

Argon

Di-*tert*-butylsilyl-bis(trifluoromethanesulfonate) (97%, Sigma-Aldrich)

Imidazole (>99%, Sigma-Aldrich)
Ethyl acetate (EtOAc; >99%, Fisher)
5% NaHCO₃ solution (see recipe)
Brine (see recipe)
Na₂SO₄
Silica gel (Sigma-Aldrich)
Methylene chloride (CH₂Cl₂; HPLC grade, Fisher)
Methanol (MeOH; HPLC grade, Fisher)
Toluene (99.8%, anhydrous, Sigma-Aldrich)
Triphenylphosphine (Ph₃P; 99%, Sigma-Aldrich)
Pd₂(dba)₃ (97%, Sigma-Aldrich)
Carbon monoxide (Airgas)
Tributyltin hydride (Bu₃SnH; 97%, Sigma-Aldrich)
Methanol (MeOH; anhydrous, 99.8%, Sigma-Aldrich)
Cerium(III) chloride heptahydrate (99.9%, Sigma-Aldrich)
Sodium borohydride (NaBH₄; >99.9%, Sigma-Aldrich)
t-Butyldimethylsilyl chloride (TBDMS-Cl; 97%, Sigma-Aldrich)
100-mL round-bottom flasks
Ice bath
Rotary evaporator connected to vacuum pump (delivering ~100 micro max vacuum), chilled by dry-ice
2 × 40-cm glass columns
Self-contained glass coupling apparatus equipped with a pressure-equalizing addition funnel (Chemglass)
Silicone oil bath (Fisher)
Additional reagents and equipment for thin-layer chromatography (*APPENDIX 3D*)

Prepare 3',5'-O-di-*t*-butylsilyl-2'-deoxyuridine (**S.2**)

- 1 Place 2.05 g (4.06 mmol) of 5-iodo-2'-deoxyuridine (**S.1**) in a 100-mL round-bottom flask.
- 2 Add 20 mL anhydrous DMF under argon to give a cloudy solution. Cool the mixture to 0°C using an ice bath.
- 3 Add 2.39 mL di-*tert*-butylsilyl-bis(trifluoromethanesulfonate) (1.1 eq.) dropwise (the cloudy solution turns clear). Keep the mixture stirring at 0°C for 10 min.
- 4 Remove the ice bath and allow the temperature of the reaction to reach room temperature.
- 5 Add 0.98 g imidazole (2.5 eq.) and stir the mixture at room temperature for 0.5 hr.

- 6 Evaporate DMF using a rotary evaporator connected to a vacuum pump.
- 7 Add 100 mL EtOAc to the residue and wash the solution with 50 mL deionized water, 50 mL of 5% NaHCO₃ solution, and 50 mL brine, dry over Na₂SO₄, and filter by gravity filtration (e.g., place a piece of Whatman filter paper on a conical funnel).
- 8 Evaporate the filtrate to dryness using a rotary evaporator to afford a crude residue.
- 9 Pack a 2 × 40-cm glass column 20-cm high with silica gel in CH₂Cl₂. Apply the crude residue dissolved in a minimum of CH₂Cl₂. Elute the desired product using 0% to 3% (v/v) HPLC-grade MeOH in CH₂Cl₂.
- 10 Monitor fractions by TLC (*APPENDIX 3D*) using 3% HPLC-grade MeOH in CH₂Cl₂ and combine fractions containing product. Remove solvents and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.2** as white foam.
- 11 Characterize the product, **S.2**, by ¹H NMR, ¹³C NMR, and HRMS.
3',5'-O-Di-*t*-butylsilyl-5-iodo-2'-deoxyuridine (**S.2**) Yield 2.63 g (92%); ¹H NMR (500 MHz) (CDCl₃) 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). ¹³C NMR (125.8 MHz) (CDCl₃) 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 C₁₇H₂₈IN₂O₅Si, [MH⁺] 495.0812 (calcd.), 495.0807 (found).

Prepare 3',5'-O-di-*t*-butylsilyl-5-formyl-2'-deoxyuridine (**S.3**)

- 12 Place 2.01 g 3',5'-O-di-*t*-butylsilyl-5-iodo-2'-deoxyuridine (5.79 mmol, **S.2**) in a 100-mL round-bottom flask with a self-contained glass coupling apparatus equipped with a pressure-equalizing addition funnel.
- 13 Add 50 mL anhydrous toluene, 0.64 g Ph₃P (0.6 eq.), and 0.42 g Pd₂(dba)₃ (0.40 mmol, 0.10 eq.).
- 14 Charge the apparatus with 50 psi of carbon monoxide (CO) and heat to 70°C in an oil bath.
- 15 Add 1.19 mL Bu₃SnH (1.05 eq.) slowly with a syringe within 1 hr. After that, keep stirring the mixture for 2 hr at 70°C and then cool to room temperature.
- 16 Filter to remove the small amount of solid.
- 17 Evaporate the filtrate to dryness using a rotary evaporator to afford a crude residue.
- 18 Pack a 2 × 40-cm glass column 20-cm high with silica gel in CH₂Cl₂. Apply the crude residue dissolved in a minimum of CH₂Cl₂. Elute the desired product using 0% to 3% (v/v) MeOH in CH₂Cl₂.
- 19 Monitor fractions by TLC (*APPENDIX 3D*) using 3% MeOH in CH₂Cl₂ and combine fractions containing product. Remove solvents and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.3** as white foam.
- 20 Characterize the product, **S.3**, by ¹H NMR, ¹³C NMR, and HRMS.

3',5'-O-Di-*t*-butylsilyl-5-formyl-2'-deoxyuridine (**S.3**) Yield 1.37 g (85%); ¹H NMR (500 MHz) (CDCl₃) 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). ¹³C NMR (125.8 MHz) (CDCl₃) 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 calculated for C₁₈H₂₉N₂O₆Si, [MH⁺] 397.1795, (calcd.), 397.1789 (found).

Prepare 3',5'-O-di-*t*-butylsilyl-5-hydroxymethyl-2'-deoxyuridine (**S.4**)

- 21 Place 1.50 g 3',5'-O-di-*t*-butylsilyl-5-formyl-2'-deoxyuridine (3.79 mmol, **S.3**) in a 100-mL round-bottom flask, add 40 mL anhydrous MeOH and 4.24 g CeCl₃·7H₂O (3.0 eq.) under argon.
- 22 Cool the mixture to 0°C using an ice bath and add 144 mg NaBH₄ (1.0 eq.) slowly (within 15 min).
- 23 Remove the ice bath and stir the reaction mixture at room temperature for 30 min.

TLC analysis shows that **S.8** is completely consumed with a slower new spot formed.
- 24 Add 5.0 g silica gel and remove the volatile under reduced pressure to give a residue.
- 25 Pack a 2 × 40-cm glass column 20-cm high with silica gel in CH₂Cl₂. Apply the crude residue on the top of the column. Elute the desired product using 0% to 4% MeOH in CH₂Cl₂.
- 26 Monitor fractions by TLC (*APPENDIX 3D*) using 4% MeOH in CH₂Cl₂ and combine fractions containing product. Remove solvents using a rotary evaporator and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.4** as white foam.
- 27 Characterize the product, **S.4**, by ¹H NMR, ¹³C NMR, and HRMS.

3',5'-O-Di-*t*-butylsilyl-5-hydroxymethyl-2'-deoxyuridine (**S.4**) Yield 1.18 g (78%). ¹H NMR (500 MHz) (CDCl₃) 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). ¹³C NMR (125.8 MHz) (CDCl₃) 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 calculated for C₁₈H₃₁N₂O₆Si, [MH⁺] 399.1951 (calcd.), 399.1946 (found).

Prepare 3',5'-O-di-*t*-butylsilyl-*t*-butyldimethylsiloxymethyl-2'-deoxyuridine (**S.5**)

- 28 Place 1.00 g 3',5'-O-di-*t*-butylsilyl-5-hydroxymethyl-2'-deoxyuridine (**S.4**) (2.51 mmol) in a 100-mL flask. Add 20 mL anhydrous DMF under argon.
- 29 Add 427 mg imidazole (2.5 eq.) and 454 mg TBDMS-Cl (1.2 eq.) and heat the mixture to 60°C in an oil bath for 2 hr under argon.
- 30 Evaporate DMF using a rotary evaporator connected to a vacuum pump to give an oily residue.
- 31 Add 100 mL EtOAc to the residue and wash the solution with 50 mL water, 50 mL 5% NaHCO₃ solution, and 50 mL brine, dry over Na₂SO₄, and filter.

- 32 Evaporate the filtrate to dryness using a rotary evaporator to afford a crude residue.
- 33 Pack a 2 × 40-cm glass column 20-cm high with silica gel in CH₂Cl₂. Apply the crude residue dissolved in a minimum of CH₂Cl₂. Elute the desired product using 0% to 2% (v/v) MeOH in CH₂Cl₂.
- 34 Monitor fractions by TLC (*APPENDIX 3D*) using 3% MeOH in CH₂Cl₂ and combine fractions containing product. Remove solvents and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.5** as white foam.
- 35 Characterize the product, **S.5**, by ¹H NMR, ¹³C NMR, and HRMS.
 3',5'-O-Di-*t*-butylsilyl-*t*-butyldimethylsiloxymethyl-2'-deoxyuridine (**5**): Yield, 1.22 g, (95%) as white foam. ¹H NMR (500 MHz) (CDCl₃) 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). ¹³C NMR (125.8 MHz) (CDCl₃) 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 calculated for C₂₄H₄₅N₂O₆Si, [MH⁺] 485.3047 (calcd.), 485.3041 (found).

PREPARATION OF PHOSPHORAMIDITE III

Phosphoramidite **III** is prepared from intermediate **S.5**, which is converted into the 5-hmdC analogue by first treating **S.5** with POCl₃ and 1,2,4-triazole to generate a 4-triazolyl intermediate, followed by reacting with NH₄OH in dioxane to generate the amine **S.6** in 65% yield in two steps. Protection of the exocyclic amine provided **S.7** in 80% yield. Selective removal of the 3',5'-silyl-protecting group of **S.7** with HF in pyridine generated **S.8** in 84% yield. Selective protection of the 5'-OH of **S.8** with DMTr (**S.9**, 82%) and subsequent phosphitylation of the 3'-OH by standard procedure gave phosphoramidite **III** (87%) (see Fig. 4.47.3). The synthesis entails 9 steps in 18% overall yield from **S.1**.

Materials

3',5'-*O*-di-*t*-butylsilyl-*t*-butyldimethylsiloxymethyl-2'-deoxyuridine (**S.5**; see Basic Protocol 1)
 Acetonitrile (CH₃CN)
 Triethylamine (Et₃N; Sigma-Aldrich)
 Imidazole
 1,2,4-Triazole (Chemgenes)
 Phosphorus (V) oxychloride (POCl₃; 99%, Sigma-Aldrich)
 Dichloromethane (CH₂Cl₂; anhydrous, 99.8%, Sigma-Aldrich)
 5% NaHCO₃ solution (see recipe)
 Brine (see recipe)
 Na₂SO₄
 1,4-Dioxane (99.8%, anhydrous, Sigma-Aldrich)
 Ammonium hydroxide (NH₄OH; 28% to 30%, Sigma-Aldrich)
 Silica gel (Sigma-Aldrich)

Methylene chloride (CH_2Cl_2 ; HPLC grade, Fisher)
Methanol (MeOH, HPLC grade, Fisher)
Pyridine (99.8%, anhydrous, Sigma-Aldrich) Benzoyl chloride (99%, Sigma-Aldrich)
Argon
Toluene
Tetrahydrofuran (THF; 99.8%, anhydrous, Sigma-Aldrich)
Hydrogen fluoride-pyridine (HF; Sigma-Aldrich)
DMTr-Cl (Chemgenes)
1-Methylimidazole (>99%, Sigma-Aldrich)
N,N-Diisopropylethylamine (99.5%, Sigma-Aldrich)
N,N-diisopropylamino cyanoethyl phosphoramidic-chloride (Chemgenes)
Acetone
100-mL flasks
Ice box
Rotary evaporator connected to vacuum pump (delivering ~100 micro max vacuum), chilled by dry ice
2 × 40-cm glass columns
1 × 40-cm glass columns
Additional reagents and equipment for TLC (*APPENDIX 3D*)

Prepare 3',5'-O-di-*t*-butylsilyl-5-*t*-butyldimethylsiloxymethyl-2'-deoxycytidine (S.6)

- 1 Place 1.00 g 3',5'-*O*-di-*t*-butylsilyl-*t*-butyldimethylsiloxymethyl-2'-deoxyuridine (2.51 mmol, **S.5**) in a 100-mL flask.
- 2 Add 20 mL CH_3CN , 8.56 mL Et_3N (55.3 mmol, 22 eq.), 427 mg imidazole (2.5 eq.), and 3.47 g of 1,2,4-triazole (50.5 mmol, 20.0 eq.).
- 3 Cool the mixture to 0°C using an ice box. Add 0.60 mL POCl_3 (6.27 mmol, 2.5 eq.) and stir the mixture at 0°C for 0.5 hr and at room temperature for 1 hr.
- 4 Add 100 mL CH_2Cl_2 and wash the mixture with 50 mL deionized water, 50 mL 5% NaHCO_3 solution, and 50 mL brine, dry over Na_2SO_4 , and filter by gravity filtration (e.g., place a piece of Whatman filter paper on a conical funnel).
- 5 Evaporate the filtrate to dryness using a rotary evaporator to afford a crude residue.
- 6 Dissolve the residue in 10 mL 1,4-dioxane (10 mL) and cool to 0°C. Add 1 mL NH_4OH and stir the mixture at 0°C for 1 hr and then concentrate to dryness.
- 7 Pack a 2 × 40-cm glass column 20-cm high with silica gel in HPLC-grade CH_2Cl_2 . Apply the crude residue dissolved in a minimum of CH_2Cl_2 . Elute the desired product using 0% to 4% (v/v) MeOH in CH_2Cl_2 .
- 8 Monitor fractions by TLC (*APPENDIX 3D*) using 4% MeOH in CH_2Cl_2 and combine the fractions containing product. Remove solvents using a rotary

evaporator and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.5** as white foam.

- 9 Characterize the product, **S.6**, by ^1H NMR, ^{13}C NMR, and HRMS.

3',5'-O-Di-*t*-butylsilyl-5-*t*-butyldimethylsiloxymethyl-2'-deoxycytidine (**S.6**): Yield, 835 mg, (65%). ^1H 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. HRMS calculated for $\text{C}_{24}\text{H}_{46}\text{N}_3\text{O}_5\text{Si}_2$, $[\text{MH}^+]$ 512.2976 (calcd.), 512.2971 (found).

Prepare 3',5'-O-di-*t*-butylsilyl-5-*t*-butyldimethylsiloxymethyl- N^4 -benzoyl-2'-deoxycytidine (S.7**)**

- 10 Place 0.80 g 3',5'-O-di-*t*-butylsilyl-5-*t*-butyldimethylsiloxymethyl-2'-deoxycytidine (1.56 mmol, **S.6**) in a 100-mL flask.
- 11 Add 12 mL pyridine followed by 0.22 mL benzoyl chloride (1.87 mmol, 1.2 eq.) under argon.
- 12 Stir the mixture overnight at room temperature, followed by the addition of 1 mL MeOH to quench the reaction.
- 13 Evaporate the reaction mixture to dryness using a rotary evaporator followed by co-evaporating twice with 20 mL toluene to afford a crude residue.
- 14 Dissolve the residue in 100 mL CH_2Cl_2 , wash with 50 mL deionized water, 50 mL of 5% NaHCO_3 , and 50 mL brine. Dry the organic phase over Na_2SO_4 and concentrate to dryness.
- 15 Pack a 2×40 -cm glass column 20-cm high with silica gel in HPLC-grade CH_2Cl_2 . Apply the crude residue dissolved in a minimum of H_2Cl_2 . Elute the desired product using 0% to 2% (v/v) MeOH in CH_2Cl_2 .
- 16 Monitor the fractions by TLC (*APPENDIX 3D*) using 2% MeOH in CH_2Cl_2 and combine the fractions containing product. Remove the solvents using a rotary evaporator and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.7** as white foam.
- 17 Characterize the product, **S.7**, by ^1H NMR, ^{13}C NMR, and HRMS.

3',5'-O-Di-*t*-butylsilyl-5-*t*-butyldimethylsiloxymethyl- N^4 -benzoyl-2'-deoxycytidine (**S.7**): Yield, 769 mg, (80%). ^1H 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 calculated for $\text{C}_{31}\text{H}_{50}\text{N}_3\text{O}_6\text{Si}_2$, $[\text{MH}^+]$ 616.3238 (calcd.), 616.3233 (found).

Prepare 5-*t*-butyldimethylsiloxymethyl- N^4 -benzoyl-2'-deoxycytidine (S.8**)**

- 18 Place 720 mg 3',5'-O-di-*t*-butylsilyl-5-*t*-butyldimethylsiloxymethyl- N^4 -benzoyl-2'-deoxycytidine (1.17 mmol, **S.7**) in a 100-mL flask.

- 19 Add 20 mL THF followed by HF in pyridine (1.0 eq.) under argon.
- 20 Stir the mixture at room temperature for 1 hr followed by addition of 4.0 g silica gel.
- 21 Evaporate to dryness using a rotary evaporator to afford silica gel–mixed residue.
- 22 Pack a 2 × 40–cm glass column 20-cm high with silica gel in CH₂Cl₂. Apply the crude residue and elute the desired product using 3% to 7% (v/v) MeOH in CH₂Cl₂.
- 23 Monitor the fractions by TLC (*APPENDIX 3D*) using 7% MeOH in CH₂Cl₂ and combine the fractions containing product. Remove the solvents and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.8** as white foam.
- 24 Characterize the product, **S.8**, by ¹H NMR, ¹³C NMR, and HRMS.
 5-*t*-Butyldimethylsiloxyethyl-N⁴-benzoyl-2'-deoxycytidine (**S.8**):
 Yield, 476 mg, (84%). ¹H NMR (500 MHz) (Aceton-d₆) 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). ¹³C NMR (125.8 MHz) (Aceton-d₆) 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 calculated for C₂₃H₃₄N₃O₆Si, [MH⁺] 476.2217 (calcd.), 476.2211 (found).

Prepare 5'-O-(4,4'-dimethoxytrityl)-5-*t*-butyldimethylsiloxyethyl-N⁴-benzoyl-2'-deoxycytidine (S.9**)**

- 25 Place 412 mg 5-*t*-butyldimethylsiloxyethyl-N⁴-benzoyl-2'-deoxycytidine (0.87 mmol, **S.8**) in a 100-mL flask.
- 26 Add 12 mL pyridine followed by 352 mg DMTr-Cl (1.04 mmol, 1.2 eq.) under argon.
- 27 Stir the mixture overnight at room temperature, followed by addition of 1 mL MeOH to quench the reaction.
- 28 Evaporate the solvent to dryness using a rotary evaporator followed by co-evaporating twice with 20 mL toluene to afford a residue.
- 29 Dissolve the residue in 100 mL CH₂Cl₂, wash with 50 mL deionized water, 50 mL of 5% NaHCO₃, and 50 mL brine. Dry the organic phase over Na₂SO₄ and concentrate to dryness.
- 30 Pack a 2 × 40–cm glass column 20-cm high with silica gel in CH₂Cl₂. Apply the crude residue and elute the desired product using 0% to 3% (v/v) MeOH in CH₂Cl₂.
- 31 Monitor the fractions by TLC (*APPENDIX 3D*) using 3% MeOH in CH₂Cl₂ and combine the fractions containing product. Remove the solvents using a rotary evaporator and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.9** as white foam.
- 32 Characterize the product, **S.9**, by ¹H NMR, ¹³C NMR, and HRMS.
 5'-O-(4,4'-Dimethoxytrityl)-5-*t*-butyldimethylsiloxyethyl-N⁴-benzoyl-2'-deoxy-cytidine (**S.9**): Yield, 553 mg, (82%). ¹H NMR (500

MHz) (CD₃CN) 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). ¹³C NMR (125.8 MHz) (CD₃CN) 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 calculated for C₄₄H₅₂N₃O₈Si, [MH⁺] 778.3524 (calcd.), 778.3525 (found).

Prepare 5'-O-(4,4'-dimethoxytrityl)-5-*t*-butyldimethylsiloxymethyl-N⁴-benzoyl-2'-deoxycytidine 3'-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (III)

- 33 Place 120 mg 5'-O-(4,4'-dimethoxytrityl)-5-*t*-butyldimethylsiloxymethyl-N⁴-benzoyl-2'-deoxycytidine (0.87 mmol, **S.9**) in a 100-mL flask.
- 34 Add 8 mL anhydrous CH₂Cl₂, 3.40 mg (41.5 μ mol) of 1-methylimidazole, and 0.2 mL *N,N*-diisopropylethylamine under argon, followed by the addition of 71.7 mg (0.30 mmol) *N,N*-diisopropylamino cyanoethyl phosphoramidic-chloride.
- 35 Stir the mixture at room temperature for 0.5 hr, followed by the addition of 80 mL CH₂Cl₂.
- 36 Wash the solution with 40 mL deionized water, 40 mL of 5% NaHCO₃, and 40 mL brine. Dry the organic phase over Na₂SO₄ and concentrate to dryness.
- 37 Pack a 1 $\times$ 40-cm glass column 20-cm high with silica gel in CH₂Cl₂. Apply the crude residue and elute the desired product using 6% to 9% acetone in CH₂Cl₂ containing 0.2% Et₃N.
- 38 Monitor the fractions by TLC (*APPENDIX 3D*) using 9% acetone in CH₂Cl₂ and combine the fractions containing product. Remove the solvents using a rotary evaporator and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.9** as white foam.
- 39 Characterize the product, **III**, by ¹H NMR, ³¹P NMR, and HRMS.
 5'-O-(4,4'-Dimethoxytrityl)-5-*t*-butyldimethylsiloxymethyl-N⁴-benzoyl-2'-deoxycytidine (**III**): Yield, 131 mg, (87%). ¹H NMR (500 MHz) (CD₃CN) 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). ³¹P NMR (202.5 MHz) (CD₃CN) 148.44 ppm. HRMS calcd. for C₅₃H₆₉N₅O₉PSi, [MH]⁺ 978.4602 (calcd.), 978.4611 (found).

PREPARATION OF PHOSPHORAMIDITE IV

So far, all 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-triazolide instead of 4-BzNH. Since 4-triazolide can be converted simultaneously into 4-NH₂ during post-synthetic NH₄OH treatment, two steps of the reaction could therefore be spared. The same strategy has been successfully used for

synthesizing nucleotides containing 4-NH₂, 4-MeO, 4-EtO, 4-Me₂N-NH, and 4-SH analogues (Xu et al., 1992). 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.

The synthesis of phosphoramidite **IV** starts from intermediate **S.5** (Fig. 4.47.4). Selective removal of the 3',5'-silyl protecting group of **S.5** with HF-pyridine generates **S.10** in 88% yield. Selective protection of the 5'-OH of **S.10** with DMTr (**S.11**, 85%) and subsequent phosphitylation of the 3'-OH by standard procedure gives phosphoramidite **S.12** (92%). Phosphoramidite **S.12** is quantitatively converted to phosphoramidite **IV** although it has a silylated hydroxymethyl group in the adjacent 5-position (Tardy-Planechaud et al., 1997). Phosphoramidite **IV** is also stable during silica gel chromatography and is isolated in 81% yield. The synthesis entails 8 steps and the overall yield from **S.1** reaches 32%.

Materials

3',5'-*O*-di-*t*-butylsilyl-*t*-butyldimethylsiloxymethyl-2'-deoxyuridine (**S.5**; see Basic Protocol 1)

Tetrahydrofuran (THF; 99.8%, anhydrous, Sigma-Aldrich)

Hydrogen fluoride-pyridine (HF; Sigma-Aldrich)

Silica gel (Sigma-Aldrich)

Methylene chloride (CH₂Cl₂; HPLC grade, Fisher)

Methanol (MeOH; HPLC grade, Fisher)

Pyridine (99.8%, anhydrous, Sigma-Aldrich)

DMTr-Cl (Chemgenes)

Argon

Toluene

5% NaHCO₃ solution (see recipe)

Brine (see recipe)

Na₂SO₄

Dichloromethane (CH₂Cl₂; anhydrous, 99.8%, Sigma-Aldrich)

1-Methylimidazole (>99%, Sigma-Aldrich)

N,N-Diisopropylethylamine (99.5%, Sigma-Aldrich)

N,N-diisopropylamino cyanoethyl phosphoramidic-chloride (Chemgenes)

Acetone

Triethylamine (Et₃N; Sigma-Aldrich)

Acetonitrile (CH₃CN; anhydrous, 99.8%, Sigma-Aldrich)

1,2,4-Triazole (Chemgenes)

Phosphorus (V) oxychloride (POCl₃; 99%, Sigma-Aldrich)

100-mL flasks

Rotary evaporator connected to vacuum pump (delivering ~100 micro max vacuum), chilled by dry ice

2 × 40-cm glass columns

1 × 40-cm glass columns

Additional reagents and equipment for TLC (*APPENDIX 3D*)

Prepare synthesis 5-t-butyldimethylsiloxymethyl-2'-deoxyuridine (**S.10**)

- 1 Place 440 mg 3',5'-*O*-di-*t*-butylsilyl-*t*-butyldimethylsiloxymethyl-2'-deoxyuridine (2.51 mmol, **S.5**) in a 100-mL flask.
- 2 Add 20 mL THF followed by HF-pyridine (1.0 eq.) under argon.
- 3 Stir the mixture at room temperature for 1 hr followed by addition of 3.0 g silica gel.
- 4 Evaporate to dryness using a rotary evaporator to afford silica gel-mixed residue.
- 5 Pack a 2 × 40-cm glass columns 20-cm high with silica gel in CH₂Cl₂. Apply the crude residue and elute the desired product using 0% to 7% (v/v) MeOH in CH₂Cl₂.
- 6 Monitor fractions by TLC (*APPENDIX 3D*) using 7% MeOH in CH₂Cl₂ and combine the fractions containing product. Remove and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.10** as white foam.
- 7 Characterize the product, **S.10**, by ¹H NMR, ¹³C NMR, and HRMS.
5-t-Butyldimethylsiloxymethyl-2'-deoxyuridine (**S.10**): Yield, 281 mg, (88%). ¹H NMR (500 MHz) (Aceton-d₆) 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). ¹³C NMR (125.8 MHz) (Aceton-d₆) 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 C₁₆H₂₉N₂O₆Si, [MH⁺] 373.1795 (calcd.), 373.1789 (found).

Prepare 5'-O-(4,4'-dimethoxytrityl)-5-t-butyldimethylsiloxymethyl-2'-deoxyuridine (**S.11**)

- 8 Place 260 mg 5-*t*-butyldimethylsiloxymethyl-2'-deoxyuridine (0.70 mmol, **S.10**) in a 100-mL flask.
- 9 Add 8 mL pyridine followed by 284 mg DMTr-Cl (0.84 mmol, 1.2 eq.) under argon.
- 10 Stir the mixture overnight at room temperature followed by addition of 1 mL MeOH to quench the reaction.
- 11 Evaporate the solvent to dryness using a rotary evaporator followed by co-evaporating twice with 20 mL toluene to afford a residue.
- 12 Dissolve the residue in 100 mL CH₂Cl₂, wash with 50 mL water, 50 mL of 5% NaHCO₃, and 50 mL brine. Dry the organic phase over Na₂SO₄ and concentrate to dryness.
- 13 Pack a 2 × 40-cm glass column 20-cm high with silica gel in CH₂Cl₂. Apply the crude residue and elute the desired product using 0% to 3% (v/v) MeOH in CH₂Cl₂.

- 14 Monitor fractions by TLC (*APPENDIX 3D*) using 7% MeOH in CH₂Cl₂ and combine the fractions containing product. Remove solvents using a rotary evaporator and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.9** as white foam.
- 15 Characterize the product, **S.11**, by ¹H NMR, ¹³C NMR, and HRMS.
- 5'-O-(4,4'-Dimethoxytrityl)-5-t-butyldimethylsiloxymethyl-2'-deoxyuridine (**S.11**): Yield, 400 mg, (85%). ¹H NMR (500 MHz) (CD₃CN) 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). ¹³C NMR (125.8 MHz) (CD₃CN) 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 C₃₇H₄₇N₂O₈Si, [MH⁺] 675.3102 (calcd.), 675.3096 (found).

Prepare 5'-O-(4,4'-dimethoxytrityl)-5-t-butyldimethylsiloxymethyl-2'-deoxyuridine 3'-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (S.12**)**

- 16 Place 120 mg 5'-O-(4,4'-dimethoxytrityl)-5-t-butyldimethylsiloxymethyl-2'-deoxyuridine (0.18 mmol, **S.11**) in a 100-mL flask.
- 17 Add 8 mL anhydrous CH₂Cl₂, 3.40 mg of 1-methylimidazole (41.5 μmol), and 0.2 mL *N,N*-diisopropylethylamine under argon, followed by addition of 60 μL *N,N*-diisopropylamino cyanoethyl phosphoramidic-chloride (0.27 mmol).
- 18 Stir the mixture at room temperature for 0.5 hr followed by addition of 80 mL CH₂Cl₂.
- 19 Wash the solution with 40 mL water, 40 mL of 5% NaHCO₃, and 40 mL brine. Dry the organic phase over Na₂SO₄ and concentrate to dryness.
- 20 Pack a 1 × 40-cm glass column 20-cm high with silica gel in CH₂Cl₂. Apply the crude residue and elute the desired product using 6% to 9% acetone in CH₂Cl₂ containing 0.2% Et₃N.
- 21 Monitor the fractions by TLC (*APPENDIX 3D*) using 9% acetone in CH₂Cl₂ and combine the fractions containing product. Remove solvents using a rotary evaporator and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **S.12** as white foam.
- 22 Characterize the product, **S.12**, by ¹H NMR, ³¹P NMR, and HRMS.
- 5'-O-(4,4'-Dimethoxytrityl)-5-t-butyldimethylsiloxymethyl-2'-deoxyuridine 3'-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (**S.12**): Yield, 143 mg (92%). ¹H NMR (500 MHz) (CD₃CN) 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). ³¹P NMR (202.5 MHz) (CD₃CN) 148.34, 148.27 ppm. HRMS calculated for C₄₆H₆₆N₄O₉PSi, [MH⁺] 877.4337 (calcd.), 877.4331 (found).

Prepare 5'-O-(4,4'-Dimethoxytrityl)-5-t-butyldimethylsiloxymethyl-4-triazolothymidine 3'-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (IV)

- 23 Place 110 mg 5'-O-(4,4'-dimethoxytrityl)-5-t-butyldimethylsiloxymethyl-2'-deoxyuridine 3'-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (**S.12**) in a 100-mL flask.
- 24 Add 5 mL CH₃CN, 0.43 mL Et₃N, and 174 mg 1,2,4-triazole. Cool the mixture to 0°C.
- 25 Add 30 µL POCl₃. Stir the mixture at 0°C for 0.5 hr and at room temperature for 1 hr. Add another portion of POCl₃ (16 µL) and stir the mixture at room temperature for 5 hr.
- 26 Add 100 mL CH₂Cl₂ and wash the mixture with 50 mL water, 50 mL of 5% NaHCO₃, and 50 mL brine. Dry over Na₂SO₄ and concentrate to dryness.
- 27 Pack a 1 × 40-cm glass column 20-cm high with silica gel in CH₂Cl₂. Apply the crude residue dissolved in a minimum of CH₂Cl₂. Elute the desired product using 6% to 10% acetone in CH₂Cl₂ containing 0.2% Et₃N.
- 28 Monitor the fractions by TLC (*APPENDIX 3D*) using 10% acetone in CH₂Cl₂ and combine the fractions containing product. Remove the solvents using a rotary evaporator and dry the resulting residue using a high-performance vacuum pump to afford analytically pure nucleoside **IV** as white foam.
- 29 Characterize the product, **IV**, by ¹H NMR, ¹³C NMR, ³¹P NMR, and HRMS.
 5'-O-(4,4'-Dimethoxytrityl)-5-t-butyldimethylsiloxymethyl-4-triazolothymidine 3'-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (**IV**): Yield, 94.5 mg (81%). ¹H NMR (500 MHz) (CD₃CN) 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). ¹³C NMR (125.8 MHz) (CD₃CN) 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. ³¹P NMR (202.5 MHz) (CD₃CN) 148.31, 148.24 ppm. HRMS calculated for C₄₈H₆₅N₇O₈PSi, [MH⁺] 926.4402 (calcd.), 926.4396 (found).

SOLID-PHASE SYNTHESIS OF DNA USING PHOSPHORAMIDITES III AND IV AND SUBSEQUENT DEPROTECTION

To optimize the coupling and deprotection conditions, we incorporated phosphoramidites **III** and **IV** into a short model sequence 5'-TCXGA (X = 5-hmC) using ultramild reagents to give oligodeoxynucleotides **ODN1** and **ODN2**, respectively. When using the standard protocol to do 1-µmol solid-phase synthesis, the DMTr cation release showed that the coupling efficiency of phosphoramidite **III** or **IV** was as efficient as that of wild-type phosphoramidites. Following the deprotection of **ODN1** and **ODN2**, we analyzed the coupling by running C18 reversed-phase HPLC with a gradient of 0% to 18% acetonitrile in 0.1 M TEAA within 30 min. The HPLC analysis showed that more dimers were formed (due to the failed coupling of phosphoramidite **III** or **IV**) than 3mer and 4mer (due to the failed coupling of wild-type phosphoramidites G or A), suggesting that the coupling yields of

phosphoramidite **III** and **IV** are not as good as wild-type phosphoramidites (phosphoramidite **IV** coupled better than **III**). To improve their coupling yields, we modified the coupling program so that phosphoramidite **III** or **IV** underwent double-coupling. For comparison, we also incorporated phosphoramidite **II** from Glen Research into the same sequence under the same conditions to give **ODN3**.

Following oligosynthesis, we first treated **ODN2** with concentrated NH_4OH at room temperature overnight. Reversed-phase HPLC analysis (Fig. 4.47.5A) showed that, besides DNA with a TBDMS-protecting group (peak c), over 20% of DNA without TBDMS group was also produced (peak b). 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 **S.2** as indicated by selective protection of 5- CH_2OH by TBDMS or Ac against the 5'-hydroxyl (Conte et al., 1992; Sowers and Beardsley, 1993). The liability of the 5- CH_2O -TBDMS ether to the NH_4OH treatment suggests 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 we treated **ODN2** with NH_4OH at 65°C for 16 hr, HPLC analysis indicated that TBDMS was almost fully removed (Fig. 4.47.5B). The same treatment of **ODN1** also gave the same major desired product. In contrast, **ODN3** only gave about 56% of the desired product, while another 44% with the 5-cyanoethyl protecting group still hung on (Fig. 4.47.5C), suggesting that the TBDMS is a superior protecting group to the cyanoethyl.

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 for the use of ultramild deprotection of the synthetic DNA. Thus, after treating the solid resin bearing newly synthesized **ODN2** 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 , we applied the ultramild deprotection condition (NH_4OH treatment at room temperature for 2 hr) to the resin. HPLC analysis showed that all the protecting groups were cleanly removed and the desired product (peak b) was produced as the major product (Fig. 4.47.5D). The same treatment of **ODN1** also gave fully deprotected oligo. This is surprising because 4-benzoyl protecting group of cytidine is usually not compatible with ultramild deprotection conditions; the 5- CH_2OH probably facilitates the removal of benzoyl group in the presence of NH_4OH .

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Brine

Add 200 g NaCl to a 1-L bottle and add deionized water until the volume is 1 L. Shake the bottle to dissolve the solid as much as possible. Use the supernatant as the brine.

NaHCO_3 solution, 5%

Add 50 g NaHCO_3 into a 1-L bottle and add deionized water until the volume is 1 L. Shake the bottle to dissolve the solid to give 5% NaHCO_3 solution.

COMMENTARY

Background Information

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% to 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, and cancer (Goll and Bestor, 2005; Klose and Bird, 2006; Reik, 2007; Weber and Schubeler, 2007; Gal-Yam et al., 2008). In 2009, 5-hydroxymethylcytosine (5-hmC), an oxidized form of 5-mC, was discovered in substantial amounts in the mammalian genome of certain cell types as the “sixth” base (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). Recent studies have shown that 5-hmC is a widespread DNA modification in brain tissues and stem cells, and its abundance is dependent on tissue type (Münzel et al., 2010a; Szwagierczak et al., 2010). 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 (Song et al., 2011; Moran-Crusio et al., 2011). In addition, it has been hypothesized that the TET protein-catalyzed conversion of 5-mC DNA to 5-hmC may represent the first step of multiple-step reactions for DNA demethylation (Wu and Zhang, 2010; Globisch et al., 2010; Dai and He, 2011). Further oxidation of 5-hmC in DNA may result in the formation of 5-formylcytosine (5-fC) DNA. It is possible that 5-fC is a transient intermediate, which can be quickly converted to 5-carboxylcytosine (5-caC) in vivo and cytosine by further decarboxylation (Radzicka and Wolfenden, 1995). Indeed, using the 5-hmC-containing DNA oligos prepared by the method in this unit together with the corresponding 5-fC and 5-caC-containing oligos we synthesized (Dai et al., 2011), we and our collaborators recently found strong evidences that 5-hmC can be further oxidized to 5-fC and 5caC by all the three Tet proteins. (He et al., 2011; Ito et al., 2011).

Compound characterization—Chemical characterization data are provided for all new compounds. NMR spectra were recorded on a Bruker 500 or Bruker 400 MHz NMR spectrometer. The ^1H , ^{13}C , and ^{31}P NMR chemical shifts are described as δ values in ppm relative to Me_4Si (for ^1H and ^{13}C) and 85% H_3PO_4 (for ^{31}P). High-resolution mass spectra (HRMS) were obtained from Notre Dame Mass Spectrometry & Proteomics Facility. Maldi-MS was recorded on a Voyager Biospectrometry Workstation with THAP as matrix.

Relevant work—While the current work was going on, another 5-hmC phosphoramidite using a carbamate to protect the amino and hydroxyl groups simultaneously was reported (Münzel et al. 2010b). The synthesis derives from 5-iododeoxycytidine 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. After this work, we found that DNA oligos containing 5-fC modification, which were prepared by ultramild DNA synthesis (Dai and He, 2011), could be quantitatively converted to the corresponding 5-hmC oligos by the reduction of NaBH_4 . It offered an alternative method for ultramild synthesis of DNA oligos containing 5-hmC modification.

Critical Parameters and Troubleshooting

The purity of all solvents and reagents is critical to obtaining high yields and pure compounds. Where specified, solvents must be anhydrous. Running reactions under an inert atmosphere, such as argon, is good laboratory practice. The syntheses described above

require understanding of and experience with common organic synthetic procedures. It is important that intermediates be pure for subsequent steps.

The transformation from **S.2** to **S.3** required 50 psi of CO in the reactor. We also tried the reaction in the 1 atm atmosphere of CO, but found there was a significant amount of 3',5'-*O*-di-*t*-butylsilyl-5-formyl-2'-deoxyuridine formed as a byproduct. The quality of tributyltin hydride is also important. It should be stored at 0°C. It may lose its reducing ability after storing at room temperature for a long time. Addition of tributyltin hydride too quickly may also result in the formation of the byproduct.

The transformation from **S.12** to **IV** is tricky. The excess amount of reagents, such as Et₃N and 1,2,4-triazole, is important, but adding too much POCl₃ may result in the decomposition of the resulting phosphoramidite **IV**. Use TLC to monitor the reaction until starting material **S.12** is completely consumed. Otherwise, phosphoramidite **IV** may be contaminated with **S.12**, which will result in the contamination of 5-hmU in the synthesized DNA oligo.

Anticipated Results

An experienced synthetic chemist should be able to obtain yields comparable to those reported (the overall yield for phosphoramidite **III** and **IV** is 18% and 32%, respectively), if the procedures are followed carefully. Less skilled researchers may require several attempts to master the reactions, but should eventually succeed, provided that the procedures are followed exactly.

Time Considerations

Each of the intermediates described in this unit can be prepared within 1 or 2 days. Synthesis of the phosphoramidite **IV** is by far the shortest (8 days) compared to that of **III** (10 days). We also use phosphoramidites **III** and **IV** for longer ODN synthesis (e.g., 32mer) and we did not encounter any problems. However, we prefer to use phosphoramidite **IV** since it is easier to prepare.

Solid-phase synthesis (1-μmol scale), deprotection, and purification of three to four 5-hmC ODN of 20 to 32 nt by HPLC can typically be achieved within 5 to 7 days.

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Figure 4.47.1.

Two reported phosphoramidite building blocks of 5-hydroxymethyl-2'-deoxycytidine.

Figure 4.47.2.
Synthesis of key intermediate **S.5**.

Figure 4.47.3.
Synthesis of phosphoramidite **III** from **S.5**.

Figure 4.47.4.
Synthesis of phosphoramidite **IV** from **S.5**.

Figure 4.47.5.

Reversed-phase HPLC analysis using C18 column with a gradient of 0% to 18% CH_3CN in 0.1 M TEAA from 6 to 36 min. (A) ODN2 was deprotected by treatment of NH_4OH overnight at room temperature. Peak a is a failed sequence dimer, 5'-CT ($[\text{MH}]^+ = 531$); peak b is the fully deprotected 5mer 5'-GAXCT ($[\text{MH}]^+ = 1493$); peak c is the 5mer with TBDMS-protecting group on 5hmC ($[\text{MH}]^+ = 1607$). (B) ODN2 was deprotected by treatment of NH_4OH overnight at 65°C. (C) ODN3 was deprotected by treatment of NH_4OH overnight at 65°C; peak d is the 5mer with cyanoethyl-protecting group on 5hmC ($[\text{MH}]^+ =$

1546). **(D)** ODN2 was deprotected by treatment of 0.5 M NH_4F in MeOH overnight, followed by NH_4OH treatment at room temperature for 2 hr.