Reversible Biotinylation of the 5′-Terminus of Oligodeoxyribonucleotides and Its Application in Affinity Purification

In *UNIT 4.2*, several methods for biotinylation of DNA are described (see structures 95b, 96, 101, 104a, 106, and 124a-c in that unit). Except for structure 124c, in which the biotin moiety can be removed nondestructively by UV irradiation, other biotinylation methods are irreversible. *UNIT 4.3* presents protocols for direct attachment of acridine and psoralen derivatives to the 5′-terminus of DNA via their phosphoramidites. In this unit, protocols are given for the preparation of two reversible biotinylation phosphoramidites for direct labeling of the 5′-terminus of DNA (see Basic Protocols 1 and 2 and Support Protocol 2) and their applications in NeutrAvidin-coated microsphere-mediated affinity purification of synthetic oligodeoxyribonucleotides (see Support Protocol 1). The DNAs synthesized using these two phosphoramidites feature a diisopropyl silyl acetal linkage between biotin and their 5′-termini; these linkages can be readily broken by fluoride ions. The first of the two phosphoramidites yields 5′-OH unmodified DNA upon treatment with HF/pyridine, while the second phosphoramidite yields 5′-phosphate DNA on treatment with HF/pyridine followed by aqueous methylamine.

CAUTION: All chemicals must be handled in a well-ventilated fume hood by individuals equipped with laboratory coats, safety glasses, and gloves.

REVERSIBLE BIOTINYLATION VIA A DIISOPROPYL SILYL ACETAL LINKER YIELDING 5′-OH DNA

BASIC PROTOCOL 1

This protocol describes the preparation of the reversible biotinylation phosphoramidite **S.3** (see Fig. 4.20.1), its coupling to the 5′-end of DNA on an automatic solid-phase synthesizer, and postsynthetic cleavage/deprotection to afford the biotinylated DNA **S.4** (see Fig. 4.20.1). A sample sequence is shown in **S.5**. Other appropriately protected deoxyribonucleosides, such as N^6-benzoyldeoxyadenosine, N^2-isobutyryldeoxyguanosine, and N^4-acetyldeoxycytosine, should be able to be biotinylated using the same procedure.

Materials

Biotinyl alcohol **S.1** (see Support Protocol 2 for preparation)
Imidazole (99%)
Argon and nitrogen gas
N,N-Dimethylformamide (DMF, anhydrous)
Diisopropylethylamine (DIEA)
Diisopropyldichlorosilane (Fluka)
Thymidine (99%+)
Ethyl acetate
5% (w/v) sodium bicarbonate ($NaHCO_3$)
Sodium sulfate (Na_2SO_4, anhydrous)
Chloroform ($CHCl_3$)
Methanol
40-μm silica gel (Baker)
TLC plates: silica gel on aluminum (60F-254, 200-μm thickness)
Methylene chloride (CH_2Cl_2, anhydrous)
2-Cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphoramidite (2-cyanoethyl-tetraisopropylphosphorodiamidite, 97%)

Contributed by Shiyue Fang and Donald E. Bergstrom
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1*H*-Tetrazole (99%+, sublimed)
Tetrahydrofuran (THF, 99%+)
5′-DMTr, 2-cyanoethyl phosphoramidite monomers:
5′-*O*-dimethoxytrityl-N^6-benzoyl-2′-deoxyadenosine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (dA^{Bz})
5′-*O*-dimethoxytrityl-N^2-isobutyryl-2′-deoxyguanosine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite ($dG^{i\text{-}Bu}$)
5′-*O*-dimethoxytrityl-N^4-acetyl-2′-deoxycytidine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (dC^{Ac})
5′-*O*-dimethoxytrityl-2′-deoxythymidine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (T)
Acetonitrile (CH_3CN, anhydrous)

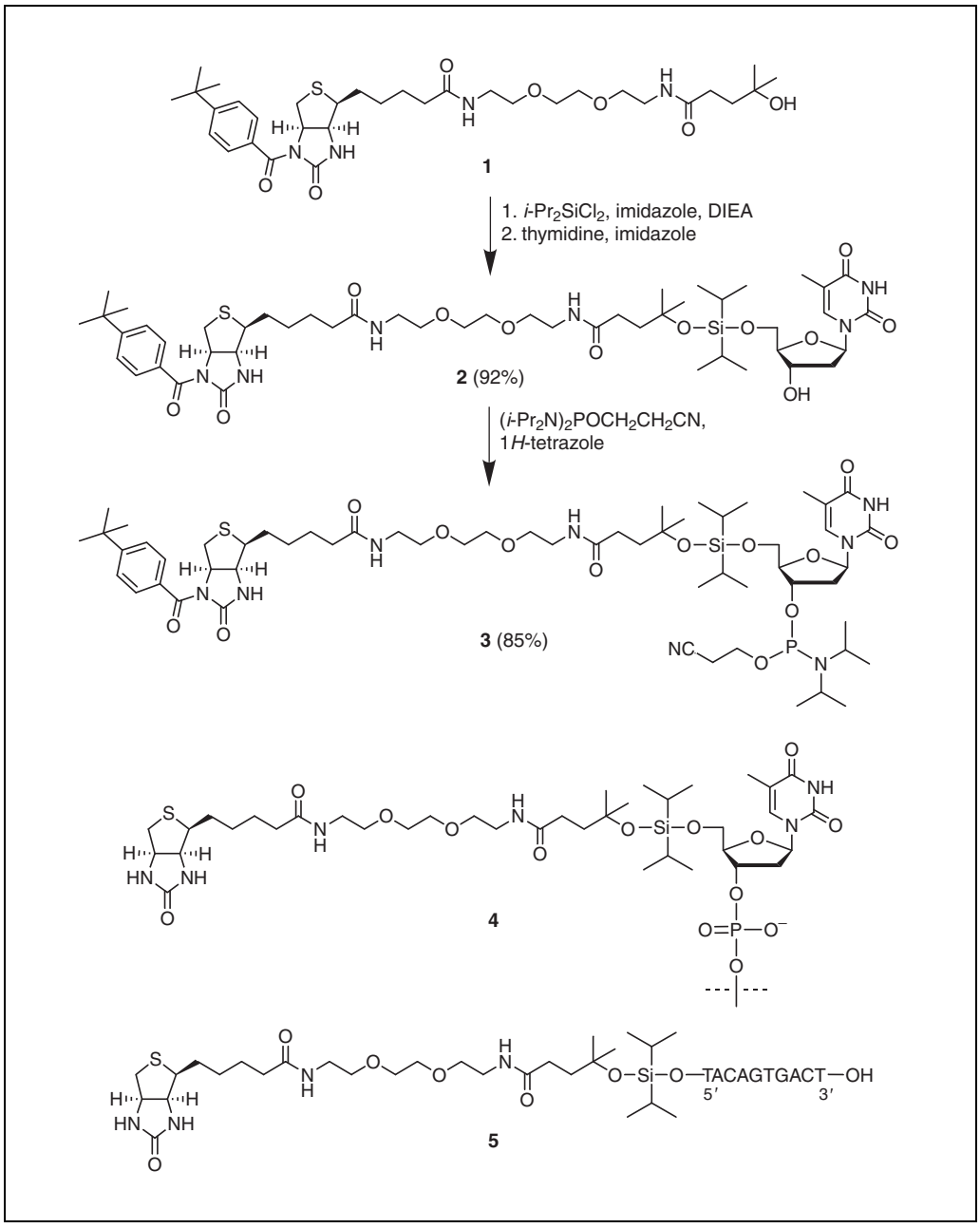

Figure 4.20.1 Preparation of reversible biotinylation phosphoramidite **S.3** and structures of biotinylated 5′-OH DNAs **S.4** and **S.5**.

~29% (v/v) ammonium hydroxide (NH_4OH)
~40% (v/v) methylamine (CH_3NH_2)

50-mL one-neck round-bottomed flasks (oven dried)
Vacuum pump
10-, 5-, 1-, 0.25-mL graduated glass syringes
20-G, 5-cm stainless steel needles
Septa (14/20, 24/40)
250-mL separatory funnel
Filter funnel and Whatman no. 1 filter paper
Rotary evaporator equipped with a water aspirator
Flash chromatography columns (4.5 × 12–cm, 3.0 × 12–cm)
UV lamp
5-mL screw-cap vials
Speedvac evaporator

Additional reagents and equipment for thin-layer chromatography (TLC, *APPENDIX 3D*), column chromatography (*APPENDIX 3E*), solid-phase automatic DNA synthesis (*APPENDIX 3C*) using classical phosphoramidite method (*UNIT 3.3*), and purification of oligonucleotides by SDS-PAGE (*UNIT 10.4*) and reversed-phase HPLC (*UNIT 10.5*)

Prepare biotinyl thymidine S.2

1. Dry 1.7 g (2.62 mmol) biotinyl alcohol **S.1** in a 50-mL one-neck round-bottomed flask (equipped with a magnetic stir bar) under vacuum overnight at room temperature.
2. Add 211 mg (3.1 mmol) imidazole and then flush the flask with an argon flow for 10 min.
3. Add 5 mL of dry DMF and 2.7 mL (15.5 mmol) DIEA with 10-mL and 5-mL glass syringes attached to 20-G stainless steel needles, respectively, and cool the mixture to 0°C.
4. Add 839 µL (4.65 mmol) diisopropyldichlorosilane with a 1-mL glass syringe in one portion. Stir the mixture at 0°C for 1 hr, then at room temperature for 4 hr on a magnetic stir plate.

   *The tertiary hydroxyl group in **S.1** substitutes one of the two chlorine atoms in diisopropyldichlorosilane; the other chlorine atom will be substituted by the primary hydroxyl group of thymidine in the next step.*

5. In another 50-mL oven-dried, one-neck round-bottomed flask, place 1.5 g (6.2 mmol) thymidine and 422 mg (6.2 mmol) imidazole. Seal the flask with a septum, flush with argon gas for ~10 min, and then add 5 mL dry DMF with a 20-G needle attached to a 10-mL glass syringe. Shake and dissolve the solids.
6. Cool the solution prepared in step 4 to 0°C. Add the solution prepared in step 5 with a 10-mL glass syringe and stir the mixture at 0°C for 4 hr.
7. Add 50 mL ethyl acetate and 50 mL of 5% $NaHCO_3$, transfer the mixture to a 250-mL separatory funnel, and separate the organic phase.
8. Extract the aqueous phase four times with 50 mL ethyl acetate.
9. Combine the organic phases, dry over ~2 g anhydrous Na_2SO_4, and filter off the solids using a filter funnel and Whatman no. 1 filter paper. Concentrate the filtrate to dryness on a rotary evaporator with a water aspirator.

10. Dissolve the residue in ~10 mL of 9:1 (v/v) $CHCl_3$/methanol and apply to the top of a 4.5 × 12–cm flash chromatography column (*APPENDIX 3E*) prepared with a slurry of 40-µm silica gel in the same solvent system. Elute the column with the same solvent system. Monitor the fractions by TLC (*APPENDIX 3D*).

11. Pool the fractions that contain the UV-active spot with an $R_f = 0.4$ (9:1 $CHCl_3$/methanol), concentrate to dryness on a rotary evaporator, and then dry under vacuum.

__S.2__ is obtained with an expected yield of 92% (2.49 g). ^{1}H NMR (CD_3OD, 500 MHz) δ 0.91-1.05 (m, 14H), 1.19 (s, 6H), 1.28 (s, 9H), 1.28-1.29 (m, 1H), 1.40-1.43 (m, 2H), 1.58-1.83 (m, 5H), 1.81 (s, 3H), 2.12-2.30 (m, 4H), 2.94-3.05 (m, 2H), 3.25-3.33 (m, 5H), 3.46-3.51 (m, 4H), 3.56-3.57 (m, 4H), 3.90-4.00 (m, 3H), 4.23 (dd, 1H, J = 4.5, 7.7 Hz), 4.38-4.40 (m, 1H), 5.13 (dd, 1H, J = 4.8, 7.7 Hz), 7.37 (d, 2H, J = 8.1 Hz), 7.44 (d, 2H, J = 8.4 Hz), 7.47 (s, 1H); ^{13}C NMR (CD_3OD, 500 MHz) δ 12.7, 14.5, 14.7, 18.3, 18.3, 18.3, 18.4, 26.8, 29.4, 29.5, 29.8, 30.2, 31.6, 32.1, 32.5, 35.8, 36.7, 38.8, 40.2, 40.3, 41.0, 41.5, 56.0, 56.6, 59.2, 64.0, 64.1, 70.5, 70.6, 71.3, 71.9, 74.8, 86.0, 88.5, 111.5, 125.5, 129.7, 133.6, 137.5, 152.2, 156.0, 158.0, 166.2, 171.7, 176.0, 176.2, 211.4.

Prepare biotinyl thymidine phosphoramidite S.3

12. Dry 220 mg (0.22 mmol) **S.2** in a 50-mL oven-dried, one-neck round-bottomed flask (equipped with a magnetic stir bar) under vacuum overnight at room temperature.

13. Flush the flask with an argon flow for ~5 min, then add (in order) 5 mL dry CH_2Cl_2 and 76 µL (0.23 mmol) of 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite with 10-mL and 250-µL, respectively, glass syringes. Stir the mixture at room temperature until it becomes a clear solution.

14. Add 15.4 mg (0.22 mmol) of 1*H*-tetrazole in three portions over a period of 1 hr. For each addition, open the neck slightly, quickly add with a spatula, and then stop the flask immediately to minimize the introduction of air into the flask.

15. Stir the reaction mixture for an additional 4 hr, and then concentrate to ~2 mL on a rotary evaporator.

16. Apply the suspension to the top of a 3.0 × 12–cm flash chromatography column prepared with a slurry of silica gel in 1:1 (v/v) $CHCl_3$/THF. Elute the column with the same solvent system and monitor the fractions by TLC.

17. Pool the fractions that contain the UV-active spot with an $R_f = 0.3$ (1:1 $CHCl_3$/THF), remove solvents on a rotary evaporator, and dry the product under vacuum.

__S.3__ is obtained as a white foam with an expected yield of 85% (225 mg). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 0.99–1.13 (m, 14H), 1.24 (d, 12H, J = 6.8 Hz), 1.28 (s, 6H), 1.36 (s, 9H), 1.36–1.90 (m, 8H), 1.90 (s, 3H), 2.21–2.39 (m, 6H), 2.75 (dt, 2H, J = 2.0, 5.8 Hz), 3.07–3.10 (m, 2H), 3.32–3.42 (m, 5H), 3.55–3.60 (m, 4H), 3.65 (s, 4H), 3.62–3.92 (m, 4H), 4.01–4.09 (m, 3H), 4.28–4.33 (m, 1H), 4.68–4.72 (m, 1H), 5.20–5.24 (m, 1H), 6.30 (t, 1H, J = 7.5 Hz), 7.49 (d, 4H, J = 7.8 Hz), 7.59 (s, 1H); ^{31}P NMR ($CDCl_3$, 250 MHz) δ 149.7, 149.8.

Couple phosphoramidite S.3 to 5′-end of oligodeoxyribonucleotide

18. Using the 5′-DMTr, 2-cyanoethyl phosphoramidites and following the classical phosphoramidite method (*UNIT 3.3*, *APPENDIX 3C*), synthesize the desired oligodeoxyribonucleotide on a DNA synthesizer. After completion, perform an additional detritylation step.

For the example in Figure 4.20.1 (__S.5__), the sequence 3′-TCAGTGACA-5′ was synthesized on a 1-µmol scale.

19. Place a solution of phosphoramidite **S.3** (0.1 M in dry acetonitrile) on the synthesizer. Carry out coupling by recycling a mixture of 0.1 mL phosphoramidite solution and 0.5 mL of 1*H*-tetrazole solution (0.45 M in dry acetonitrile) for 15 min.

20. Remove the excess coupling reagents and perform iodine oxidation according to the standard procedure (*UNIT 3.3*, *APPENDIX 3C*). Dry the product on CPG under nitrogen flow.

Cleave and deprotect biotinylated oligonucleotide

21. Place the oligonucleotide-bound CPG in a 5-mL screw-cap vial, add 500 µL of ~29% NH_4OH and 500 µL of ~40% methylamine, and heat the suspension to 65°C for 30 min.

22. Cool the vial to –20°C, remove the supernatant, and save. Wash the CPG three times with 500 µL water, keeping all washes. Combine the supernatant and water washes, and dry on a Speedvac evaporator.

23. Purify the biotinylated DNA by gel electrophoresis or reversed-phase HPLC according to procedures in *UNIT 10.4* and *UNIT 10.5*, respectively.

*The HPLC profile of crude biotinylated DNA **S.5** (see Fig. 4.20.1) is illustrated in Figure 4.20.2.*

REVERSIBLE BIOTINYLATION VIA A DIISOPROPYL SILYL ACETAL LINKER YIELDING 5′-PHOSPHATE DNA

BASIC PROTOCOL 2

This protocol describes the preparation of the reversible biotinylation phosphoramidites **S.7** (see Fig. 4.20.3), its coupling to the 5′-end of DNA on a solid-phase synthesizer, and postsynthetic cleavage/deprotection to afford the biotinylated DNA **S.8** (Fig. 4.20.3). A sample sequence is shown in **S.9**.

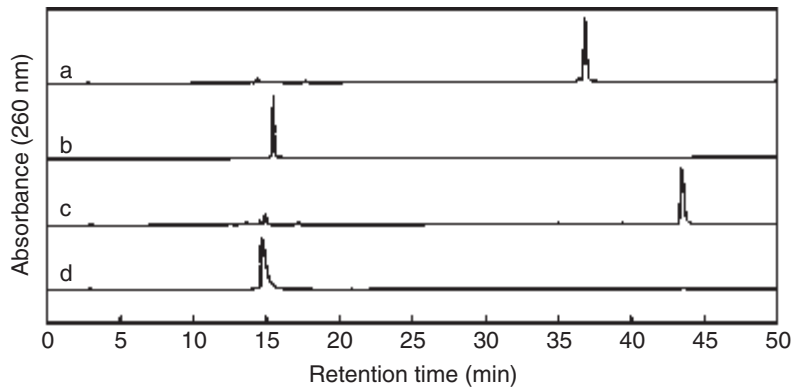

Figure 4.20.2 HPLC profiles of DNAs **S.5** (trace a), 5′-TACAGTGACT-3′ (trace b), **S.9** (trace c), and 5′-H_2O_3PO-ACAGTGACT-3′ (trace d) generated on a C18 reversed-phase column (100 Å, 250 × 4.6 mm, Varian Analytical Instruments), using a linear gradient of 0% to 45% solvent B (90% acetonitrile) in solvent A (0.1 M triethylammonium acetate, 5% acetonitrile) over 60 min at a flow rate of 1 mL/min by detecting the absorbance of DNA at 260 nm. For a detailed protocol for performing reversed-phase HPLC, refer to *UNIT 10.5*.

Materials

Biotinyl alcohol **S.1** (see Support Protocol 2 for preparation)
Imidazole (99%)
Argon and nitrogen gas
N,N-Dimethylformamide (DMF, anhydrous)
Diisopropylethylamine (DIEA)
Diisopropyldichlorosilane (Fluka)
Diethyl bis(hydroxymethyl)malonate (97%)
Methylene chloride (CH_2Cl_2)
5% (v/v) citric acid

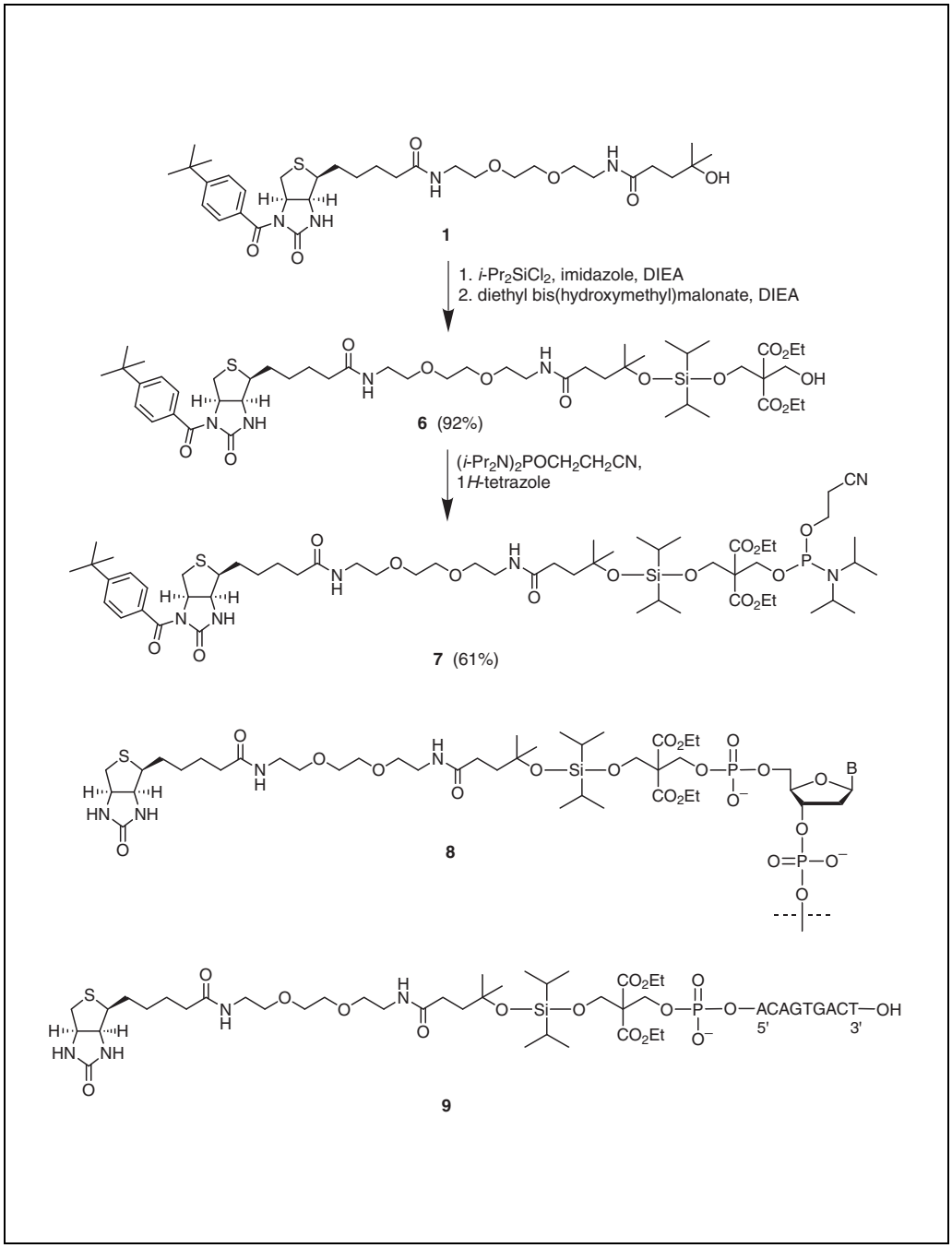

Figure 4.20.3 Preparation of reversible biotinylation phosphoramidite **S.7** and structures of biotinylated 5′-phosphate DNAs **S.8** and **S.9**.

Sodium sulfate (Na_2SO_4, anhydrous)
Chloroform ($CHCl_3$)
Methanol
40-µm silica gel (Baker)
TLC plates: silica gel on aluminum (60F-254, 200-µm thickness)
Acetonitrile (distilled over CaH_2)
2-Cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphoramidite (2-cyanoethyl-tetraisopropylphosphorodiamidite, 97%)
0.45 M 1*H*-tetrazole (99%+, sublimed) in acetonitrile
5% (w/v) sodium bicarbonate ($NaHCO_3$)
Tetrahydrofuran (THF, 99%+)
Triethylamine (TEA)

50-mL one-neck, round-bottomed flasks (oven dried)
Vacuum pump
20-G, 5-cm stainless steel needles
5-, 1-, and 0.5-mL graduated glass syringes
Septa (14/20, 24/40)
250-mL separatory funnels
Filter funnel and Whatman no. 1 filter paper
Rotary evaporator equipped with a water aspirator
Flash chromatography columns (3.0 × 12–cm, 3.0 × 10–cm)
UV lamp

Additional reagents and equipment for thin-layer chromatography (TLC, *APPENDIX 3D*), column chromatography (*APPENDIX 3E*), and incorporation of biotinylated phosphoramidite into oligonucleotide (see Basic Protocol 1)

Prepare biotin diethyl bis(hydroxymethyl)malonate conjugate S.6

1. Dry 605 mg (0.93 mmol) biotinyl alcohol **S.1** in a 50-mL oven-dried, one-neck, round-bottomed flask (equipped with a magnetic stir bar) under vacuum overnight at room temperature.
2. Add 63 mg (0.93 mmol) imidazole and then flush the flask with an argon flow for 10 min.
3. Add 2 mL of dry DMF and 498 µL (2.79 mmol) DIEA with a 20-G needle attached to a 5-mL and 1-mL glass syringe, respectively, and cool the mixture to 0°C.
4. Add 252 µL (1.40 mmol) diisopropyldichlorosilane with a 500-µL glass syringe in one portion. Stir the mixture at 0°C for 1 hr, then at room temperature for 4 hr on a magnetic stir plate.
5. In another 50-mL oven-dried, one-neck, round-bottomed flask, place 500 mg (2.20 mmol) diethyl bis(hydroxymethyl)malonate and 126 mg (1.86 mmol) imidazole. Seal the flask with a septum, flush with an argon flow for ~10 min, and then add 2 mL of dry DMF with a 5-mL glass syringe. Shake to dissolve the solids.
6. Cool the solution prepared in step 5 to 0°C. Add the reaction mixture obtained in step 4 with a 5-mL glass syringe very slowly (over at least a 30-min period) and stir the resulting solution at 0°C for 5 hr.
7. Add 30 mL CH_2Cl_2 and 50 mL of 5% citric acid, transfer the mixture to a 250-mL separatory funnel, and separate the organic phase.
8. Extract the aqueous phase four times with 30 mL CH_2Cl_2.
9. Combine the organic phases, dry over ~2 g anhydrous Na_2SO_4, and filter off the solids using a filter funnel and Whatman no. 1 filter paper. Concentrate the filtrate to dryness on a rotary evaporator with a water aspirator.

10. Dissolve the residue in ~8 mL of 19:1 (v/v) $CHCl_3$/methanol and apply to the top of a 3.0 × 12–cm flash chromatography column (*APPENDIX 3E*) prepared with a slurry of 40-µm silica gel in the same solvent system. Elute the column with the same solvent system. Monitor the fractions by TLC (*APPENDIX 3D*) using 9:1 (v/v) $CHCl_3$/methanol.

11. Pool the fractions that contain the UV-active spot with an $R_f = 0.5$ (9:1 $CHCl_3$/methanol), concentrate to dryness on a rotary evaporator, and then dry under vacuum.

*The product, (**S.6**), has the highest R_f value, and is the major UV-active spot in the reaction mixture under these TLC conditions. The expected yield is 92% (839 mg). ^1H NMR ($CDCl_3$, 500 MHz) δ 0.88-1.01 (m, 14H), 1.24-1.27 (m, 12H), 1.32 (s, 9H), 1.47-1.53 (m, 2H), 1.65-1.85 (m, 6H), 2.18-2.31 (m, 4H), 3.03-3.10 (m, 2H), 3.25 (dt, 1H, J = 7.4, 4.7 Hz), 3.38-3.50 (m, 4H), 3.54-3.56 (m, 4H), 3.60 (s, 4H), 4.14-4.27 (m, 9H), 5.22-5.24 (m, 1H), 6.37 (t, 1H, J = 5.5 Hz), 6.58 (t, 1H, J = 5.3 Hz), 7.39 (d, 2H, J = 8.5 Hz), 7.57 (d, 2H, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 13.4, 14.2, 17.7, 27.8, 29.8, 30.5, 31.3, 32.0, 35.1, 35.6, 38.4, 39.2, 39.5, 40.0, 55.2, 57.5, 61.6, 61.7, 61.8, 62.7,70.0, 70.1 (X 2), 70.3, 73.8, 124.7, 129.1, 131.9, 155.1, 156.3, 169.2, 170.2, 173.3, 174.2.*

Prepare biotin diethyl bis(hydroxymethyl)malonate conjugate phosphoramidite S.7

12. Dry 715 mg (0.72 mmol) **S.6** in a 50-mL oven-dried, one-neck round-bottomed flask (equipped with a magnetic stir bar) under vacuum overnight at room temperature.

13. Flush the flask with an argon flow for ~5 min, then sequentially add 2 mL dry acetonitrile and 264 µL (0.80 mmol) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite with a 5-mL and 500-µL glass syringe, respectively.

14. Add 1.68 mL (0.76 mmol) of 0.45 M 1*H*-tetrazole (in acetonitrile) with a 5-mL glass syringe, stir the mixture at room temperature for 3 hr, and then quench the reaction with 50 mL of 5% $NaHCO_3$.

15. Transfer the reaction mixture to a 250-mL separatory funnel, extract the mixture five times with 30 mL CH_2Cl_2, and combine the organic phases.

16. Dry over ~2 g anhydrous Na_2SO_4, filter off the solids, and concentrate the filtrate to dryness on a rotary evaporator.

17. Dissolve the residue in ~5 mL of 7:3:0.5 (v/v/v) $CHCl_3$/THF/TEA, and apply to the top of a 3.0 × 10–cm flash chromatography column prepared with a slurry of silica gel in the same solvent. Elute the column with the same solvent system and monitor the fractions by TLC.

18. Pool the fractions that contain the UV-active spot with an R_f = 0.5 (7:3:0.5 $CHCl_3$/THF/TEA), remove solvents on a rotary evaporator, and dry under vacuum.

***S.7** is obtained as a white foam with an expected yield of 61% (520 mg). ^1H NMR ($CDCl_3$, 250 MHz) δ 0.91-1.07 (m, 14H), 1.14-1.18 (m, 12H), 1.22-1.29 (m, 12H), 1.32 (s, 9H), 1.41-1.51 (m, 2H), 1.65-1.82 (m, 4H), 2.16-2.34 (m, 4H), 2.60 (t, 2H, J = 6.8 Hz), 3.04-3.06 (m, 2H), 3.21-3.26 (m, 1H), 3.40-3.48 (m, 4H), 3.52-3.61 (m, 8H), 3.76-3.82 (m, 2H), 4.05-4.25 (m, 11H), 5.19-5.22 (m, 1H), 6.28 (t, 1H, J = 5.5 Hz), 6.39 (t, 1H, J = 5.3 Hz), 7.39 (d, 2H, J = 8.0 Hz), 7.57 (d, 2H, J = 8.3 Hz); ^{31}P NMR ($CDCl_3$, 250 MHz) δ 166.36.*

Synthesize biotinylated DNA

19. Perform oligonucleotide synthesis, coupling of the biotinylated phosphoramidite **S.7**, and cleavage and deprotection of the biotinylated DNA (**S.8**) as described (see Basic Protocol 1, steps 18 to 23).

*For the example in Figure 4.20.3 (**S.9**), the sequence 3′-TCAGTGACA-5′ was synthesized on a 1-µmol scale prior to biotinylation. The HPLC profile of crude biotinylated **S.9** is illustrated in Figure 4.20.2.*

APPLICATION OF THE REVERSIBLE BIOTINYLATION METHOD IN AFFINITY PURIFICATION USING AVIDIN-COATED MICROSPHERES

This protocol describes the attachment of the reversibly biotinylated full-length DNAs **S.4** and **S.8** (see Figs. 4.20.1 and 4.20.3, respectively) to NeutrAvidin-coated microspheres, the removal of nonbiotinylated failure sequences resulting from inefficient coupling and other impurities, and the recovery of pure, unmodified DNAs **S.10** and **S.12** (see Fig. 4.20.4) from the solid microspheres.

Materials

UltraLink Immobilized NeutrAvidin (1000-Å pore size; 50- to 80-µm particle size; 0.08 µmol biotin/ml gel binding capacity; Pierce), prepared as 50% (v/v) slurry containing 0.02% (w/v) sodium azide
PBS (*APPENDIX 2A*)
Biotinylated DNA (see Basic Protocol 1 for **S.4** or Basic Protocol 2 for **S.8**)
Acetone (dried over anhydrous sodium sulfate)
Tetrahydrofuran (THF, distilled over sodium/benzophenone ketyl)
70:30 (v/v) hydrogen fluoride in pyridine (HF/pyridine)
Methoxytrimethylsilane
~40% (v/v) methylamine (CH_3NH_2; for 5′-phosphate DNA **S.8** only)
10- and 1.5-mL centrifuge tubes
Lyophilizer or Speedvac evaporator

1. Transfer 5 mL (for 1-µmol scale DNA synthesis) UltraLink Immobilized NeutrAvidin gel (i.e., 10 mL of 50% gel slurry) into a 10-mL centrifuge tube, centrifuge 30 sec at 2100 × *g*, room temperature, and remove the supernatant.

2. Resuspend the gel in 5 mL PBS, centrifuge 30 sec at 2100 × *g*, room temperature, and remove the supernatant. Repeat wash two additional times.

3. Dissolve 1 µmol biotinylated DNA in 2 mL PBS and transfer the solution to the centrifuge tube containing the NeutrAvidin gel. Wash the DNA tube two times with 0.5 mL PBS and add to the gel suspension. Incubate at room temperature for 1 hr with occasional gentle shaking.

4. Centrifuge the suspension 30 sec at 2100 × *g*, room temperature, and remove the supernatant. Wash and then dry the gel as follows:

   at least three times with 3 mL PBS
   three times with 3 mL water
   two times with 5 mL acetone
   three times with 5 mL THF.

5. Suspend the gel in 5 mL THF, add 300 µL of 70:30 (v/v) HF/pyridine with a 500-µL pipet, and incubate the suspension 1 hr at room temperature with occasional gentle shaking.

   *The fluoride ion cleaves the two Si-O bonds in **S.4** and **S.8** (see Fig. 4.20.4).*

   CAUTION: *HF reacts with glass and is toxic. The reaction must be performed in a plastic centrifuge tube in a well-ventilated fume hood.*

6. Add 3 mL methoxytrimethylsilane with a 1-mL pipet, incubate at room temperature for 10 min, and then centrifuge the mixture 30 sec at 2100 × *g* and discard the supernatant.

   Methoxytrimethylsilane quenches the excess HF, giving the volatile side products fluorotrimethylsilane and methanol (see Fig. 4.20.4).

7a. *For 5′-OH DNA (from **S.4**):* Collect pure DNA **S.10** by washing the gel six times with 0.5 mL water, saving each wash. Combine water washes and dry in a Speedvac evaporator or lyophilizer.

Expected recovery yield is >80%. The purity of the DNA can be checked by reversed-phase HPLC (UNIT 10.5). A typical profile for the short sequence 5′-TACAGTGACT-3′ is illustrated in Figure 4.20.2.

7b. *For 5′-phosphate DNA (from **S.8**):* Wash the gel with 1 mL THF, then add 3 mL of ~40% methylamine and incubate at room temperature for 30 min. Centrifuge the mixture 30 sec at 2100 × *g*, remove the supernatant, and wash the gel seven times with 1 mL water, saving each wash. Combine the supernatant and water washes, and dry the pure DNA **S.12** in a Speedvac evaporator or lyophilizer.

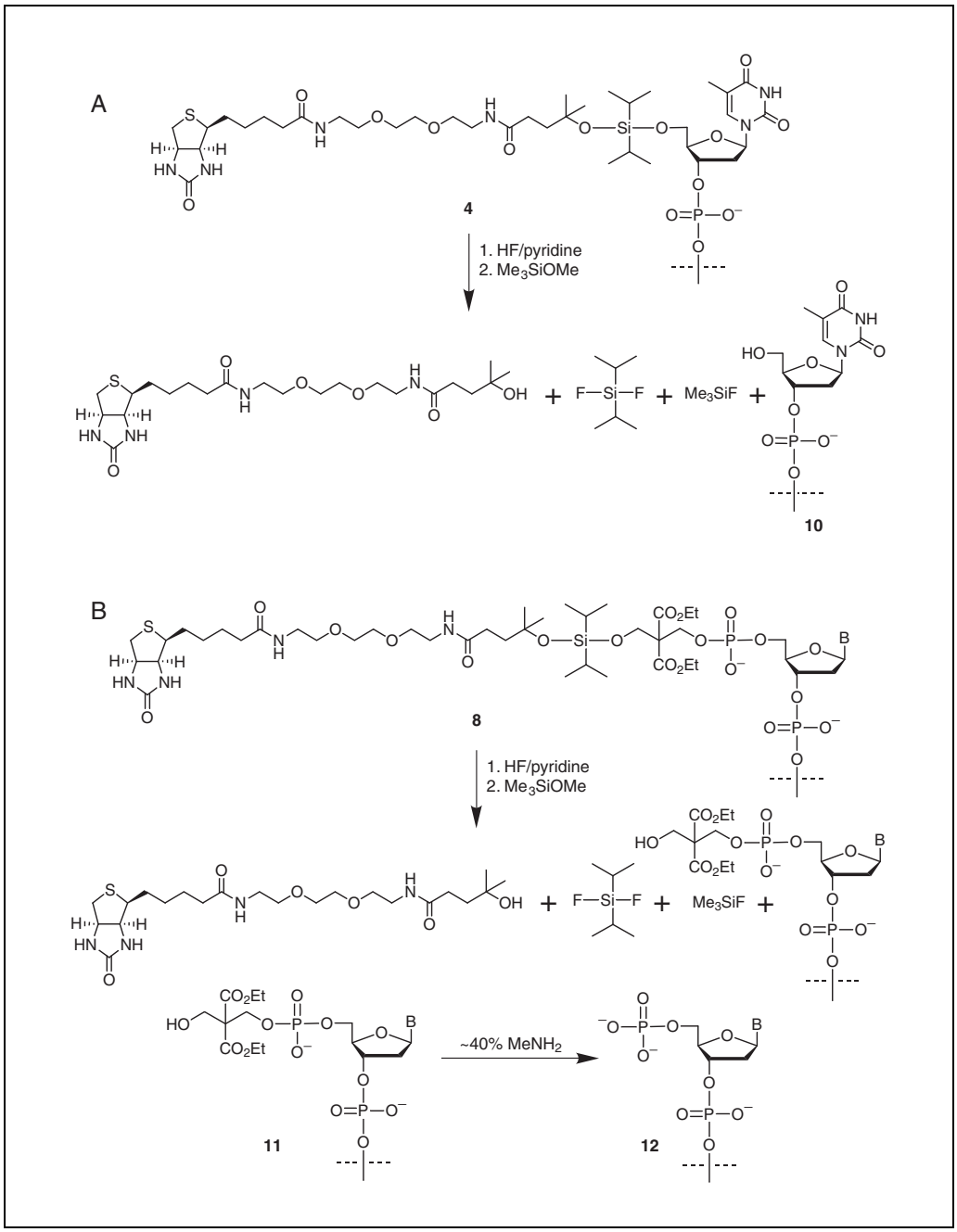

Figure 4.20.4 Removal of biotin from biotinylated DNAs **S.4** and **S.8** to generate unmodified 5′-OH DNA **S.10** and unmodified 5′-phosphate DNA **S.12**, respectively.

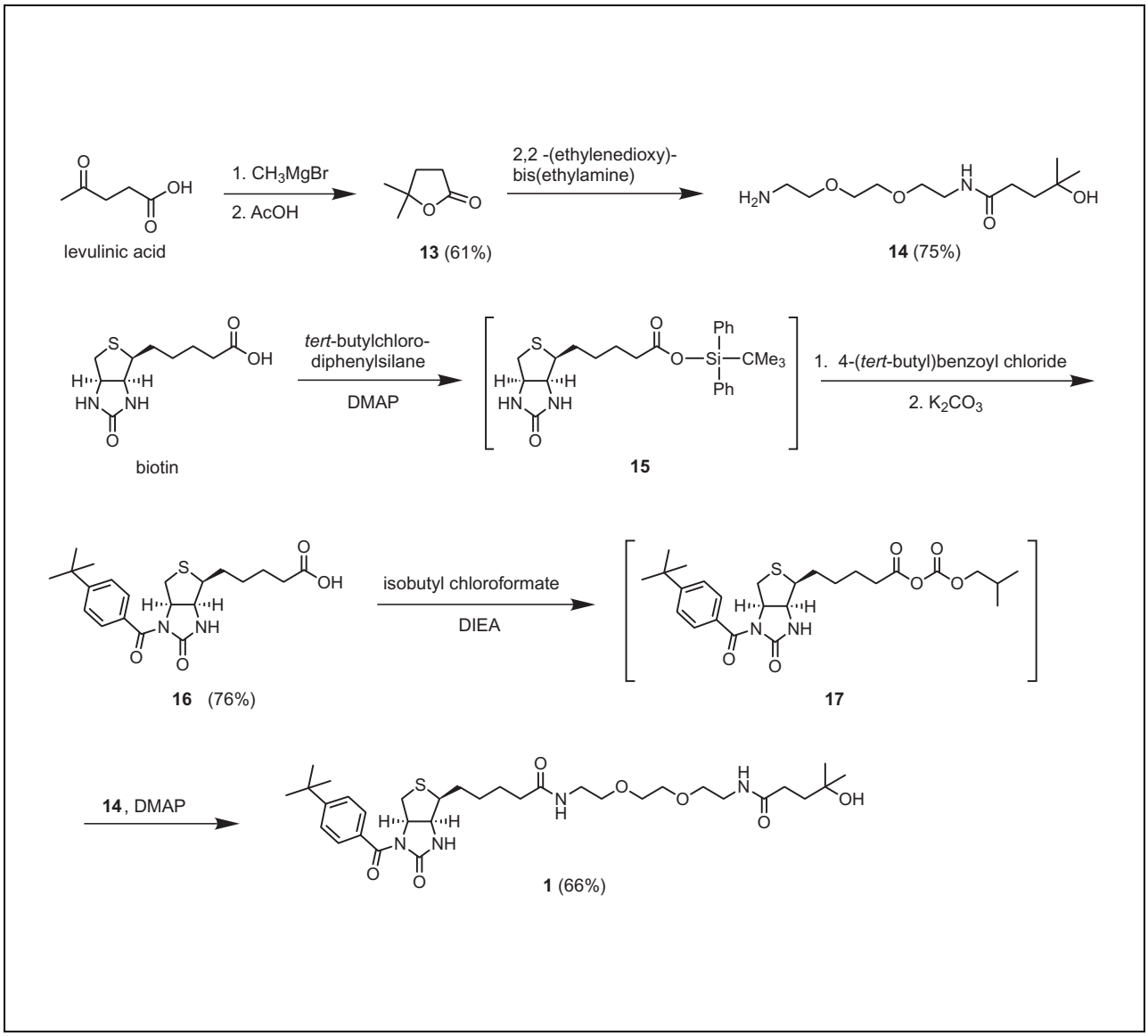

Figure 4.20.5 Preparation of the biotinyl alcohol **S.1**.

*Aqueous methylamine removes the 5′-tag in **S.11** to generate the 5′-phosphate DNA **S.12** (see Fig. 4.20.4). Expected recovery yield is >70%. The purity of the DNA can be checked by reversed-phase HPLC (UNIT 10.5). A typical profile for the short sequence 5′-H_2O_3PO-ACAGTGACT-3′ is illustrated in Figure 4.20.2.*

SYNTHESIS OF BIOTINYL ALCOHOL

SUPPORT PROTOCOL 2

The biotinyl alcohol **S.1** is required for the preparation of the reversible biotinylation phosphoramidites **S.3** and **S.7**. This protocol describes its preparation, which is shown in Figure 4.20.5.

Additional Materials *(also see Basic Protocol 1)*

Levulinic acid (98%)
Dry ice/acetone
3.0 M methyl magnesium bromide in ether
Acetic acid (99.7%+)
Magnesium sulfate ($MgSO_4$, anhydrous)

2,2′-(Ethylenedioxy)bis(ethylamine) (98%)
Ether
Triethylamine
Ninhydrin
Ethanol (anhydrous)
Biotin (AnaSpec)
4-(Dimethylamino)pyridine (DMAP)
Pyridine (distilled from CaH_2)
t-Butylchlorodiphenylsilane (98%)
4-*t*-Butylbenzoylchloride (98%)
Potassium carbonate (K_2CO_3)
5% citric acid
Isobutylchloroformate (98%)

100-, 20-, 10-, 5-, and 1-mL graduated glass syringes
1000-mL two-neck round-bottomed flask, oven dried
Condenser
500- and 250-mL separatory funnels
Aldrich short-path distillation apparatus
50-, 100- and 250-mL one-neck round-bottomed flasks, oven dried
Flash chromatography columns (4.5 × 30–cm and 4.5 × 10–cm)
Heat gun
Rubber septum
2- and 6-cm stainless steel needles

Prepare lactone S.13

1. Add 14 mL (300 mmol) levulinic acid and 300 mL THF with a 20-mL and 100-mL glass syringe, respectively, to a 1000-mL oven-dried, two-neck, round-bottomed flask equipped with a magnetic stir bar, connected to an argon flow through a condenser.
2. Cool the flask to −78°C with a slurry of dry ice in acetone. Slowly add 100 mL (300 mmol) of 3.0 M methyl magnesium bromide in ether with a 100-mL glass syringe. Stir the mixture for 3 hr while gradually warming to room temperature, then heat to 50°C overnight.

   This should form a light yellow solution.
3. Cool the solution to room temperature, add ~100 mL acetic acid with a disposable pipet, and stir the mixture for ~12 hr.

   At this stage, maintaining an anhydrous atmosphere is unnecessary.
4. Add 40 mL water and then remove volatile components (ether and THF) on a rotary evaporator.
5. Transfer the red residue to a 500-mL separatory funnel and extract three times with 100 mL (each extraction) CH_2Cl_2.
6. Combine the organic phases, dry over ~5 g anhydrous $MgSO_4$, and filter off the solids. Concentrate the filtrate on a rotary evaporator.
7. Distill the residue by means of a short-path distillation apparatus using a vacuum of ~0.6 mmHg. Collect the second fraction, which typically distills at 70°C (~0.6 mmHg).

   *The product 5,5-dimethyl-dihydrofuran-2-one (**S.13**) should be a colorless oil with an expected yield of 61% (9.5 g). ^1H NMR ($CDCl_3$, 250 MHz): δ 1.44 (s, 6H), 2.06 (t, 2H, J = 8.23 Hz), 2.63 (t, 2H, J = 8.3 Hz).*

Prepare amino alcohol S.14

8. Place 24.3 mL (166 mmol) 2,2′-(ethylenedioxy)bis(ethylamine), 9.5 g (83.2 mmol) **S.13**, and 10 mL water in a 100-mL one-neck, round-bottomed flask equipped with a condenser and a magnetic stir bar. Stir the mixture overnight at 90°C.

   This should give a light yellow solution.

9. Remove water and excess 2,2′-(ethylenedioxy)bis(ethylamine) by distilling at ~70°C at 0.6 mmHg under vacuum as in step 7.

10. Dissolve the light yellow oily residue in 15 mL of 5:2:2:1 (v/v/v/v) ether/methanol/acetonitrile/triethylamine and apply the solution to the top of a 4.5 × 30–cm flash chromatography column prepared using a slurry of silica gel in the same solvent mixture. Elute the column and monitor the fractions by TLC using the same solvent mixture. Detect the product by rinsing the TLC plate briefly in a solution of 0.5 g ninhydrin in 200 mL ethanol and heat with a heat gun until purple spots appear (~45 sec).

    The product should appear as a purple spot with an $R_f = 0.8$.

11. Pool the fractions containing the pure product, evaporate solvents on a rotary evaporator, and dry the light yellow oily product under vacuum.

    *The expected yield of the product (**S.14**) is 75% (16.3 g). ^{1}H NMR (CD_3OD, 500 MHz): δ 1.20 (s, 6H), 1.78 (t, 2H, J = 8.1 Hz), 2.35 (t, 2H, J = 7.5 Hz), 2.88 (t, 2H, J = 4.5 Hz), 3.89-3.42 (m, 2H), 3.54-3.57 (m, 4H), 3.62 (s, 4H); ^{13}C NMR (CD_3OD, 500 MHz): δ 28.8, 31.0, 38.5, 38.8, 40.8, 69.0, 69.3, 69.6, 69.7, 72.0, 174.0.*

Prepare N-t-butylbenzoylbiotin S.16

12. Place 2.44 g (10.0 mmol) biotin and 0.61 g (5.0 mmol) DMAP in a 250-mL oven-dried, one-neck, round-bottomed flask equipped with a magnetic stir bar and capped with a rubber septum. Connect the flask to an argon flow with a 2-cm stainless steel needle and flush the flask with argon for ~5 min.

13. Sequentially add 15 mL dry pyridine and 3.0 mL (15 mmol) *t*-butylchlorodiphenylsilane with a 20- and 5-mL glass syringe, repectively, and stir the mixture overnight at room temperature.

    *t-Butylchlorodiphenylsilane is used for temporary protection of the carboxylic acid group to form the intermediate **S.15**.*

14. Add 3.0 mL (15 mmol) 4-*t*-butylbenzoylchloride with a 5-mL glass syringe, stir the mixture for 3 hr, and then quench the reaction by adding 2 mL methanol.

15. Remove volatile components on a rotary evaporator. Dissolve the residue in a mixture of the following:

    12 mL THF
    6 mL methanol
    6 mL water
    6.9 g (50 mmol) K_2CO_3.

    Stir 30 min at room temperature.

    *This mild basic condition removes the t-butyldiphenylsilane group without affecting the 4-t-butylbenzoyl protecting group, to give **S.16**.*

16. Quench the reaction with 50 mL of 5% citric acid. Extract the mixture six times with 20 mL ethyl acetate. Combine the organic extracts, dry over ~2 g anhydrous $MgSO_4$, filter off the solids, and concentrate the filtrate to dryness on a rotary evaporator.

17. Dissolve the residue in ~4 mL $CHCl_3$, place the solution on top of a 4.5 × 10–cm flash chromatography column, and elute first with ~350 mL $CHCl_3$ and then with 19:1 (v/v) $CHCl_3$/methanol. Monitor the fractions by TLC using 9:1:10 (v/v/v) $CHCl_3$/methanol/ether as the solvent system.

18. Pool fractions that contain the UV-active spot with an $R_f = 0.5$ (9:1:10 $CHCl_3$/methanol/ether), concentrate to dryness on a rotary evaporator, and dry under vacuum.

*The expected yield of the product 1-(4-tert-butylbenzoyl)-biotin (**S.16**) is 76% (3.07 g). ^1H NMR (CD_3OD, 500 MHz) δ 1.15 (s, 9H), 1.27-1.63 (m, 6H), 2.14 (t, 2H, J = 7.4 Hz), 2.79-2.91 (m, 2H), 3.11-3.15 (m, 1H), 4.07 (dd, 1H, J = 4.6, 7.8 Hz), 4.98-5.00 (m, 1H), 7.25 (d, 2H, J = 8.5 Hz), 7.31 (d, 2H, J = 8.6 Hz); ^{13}C NMR (CD_3OD, 500 MHz) δ 26.1, 29.6, 29.9, 31.7, 34.8, 35.9, 38.9, 56.7, 59.3, 64.1, 125.7, 129.8, 133.8, 156.1, 158.2, 171.9, 177.6.*

19. Place 1.9 g (4.8 mmol) **S.16** in a 100-mL one-neck, round-bottomed flask equipped with a magnetic stir bar. Then place 292 mg (2.39 mmol) DMAP and 656 mg (2.5 mmol) **S.14** in a separate 50-mL one-neck, round-bottomed flask equipped with a stir bar. Dry both on a vacuum pump overnight at room temperature.

Prepare biotinyl alcohol S.1

20. Stop the neck of the flask containing **S.16** with a rubber septum. Flush the flask with an argon flow with two 6-cm stainless steel needles (one inlet and one outlet) for ~10 min and then remove the outlet needle.

21. Place the flask in an ice bath and sequentially add 4 mL dry DMF and 920 µL (5.28 mmol) DIEA with a 5-mL and 1-mL glass syringe, respectively. Let sit 10 min.

22. Add 684 µL (5.27 mmol) isobutylchloroformate with a 1-mL glass syringe and stir the mixture 1 hr at 0°C.

*This forms the intermediate **S.17**.*

23. Flush the flask containing DMAP and **S.14** (step 19) with an argon flow for 10 min, then add 4 mL dry DMF with a 5-mL glass syringe.

24. Very slowly add the solution of DMAP and **S.14** to the solution of intermediate **S.17** with a 10-mL glass syringe (at least over a 15-min period). After addition, stir the mixture 10 min at 0°C.

25. Quench the reaction with 2 mL water. Add 20 mL of 5% citric acid, transfer the mixture to a 250-mL separatory funnel, and extract six times with 30 mL (each extraction) ethyl acetate.

26. Pool the extracts, dry over ~2 g anhydrous Na_2SO_4, filter off the solids, and evaporate the filtrate on a rotary evaporator.

27. Dissolve the residue in ~10 mL of 19:1 (v/v) CH_2Cl_2/methanol and apply to the top of a 4.5 × 10–cm flash chromatography column prepared with a slurry of silica gel in the same solvent. Elute the column with the same solvent and monitor the fractions by TLC using a solvent system of 9:1 (v/v) CH_2Cl_2/methanol.

28. Collect the fractions that contain a UV-active spot with an $R_f = 0.5$ (9:1 CH_2Cl_2/methanol), pool these fractions, and evaporate to dryness on a rotary evaporator to give **S.1**.

*Under vacuum, biotinyl alcohol (**S.1**) can form a white foam. Expected yield is 66% (2.1 g). ^1H NMR (CD_3OD, 500MHz) δ 1.24 (s, 6H), 1.38 (s, 9H), 1.46-1.55 (m, 2H), 1.65-1.85 (m, 6H), 2.28 (t, 2H, J = 7.3 Hz), 2.34 (t, 2H, J = 7.7 Hz), 3.06-3.13 (m, 2H), 3.36-3.43 (m, 5H), 3.55-3.66 (m, 8H), 4.31 (t, 1H, J = 6.9 Hz), 5.22 (t, 1H, J = 6.6 Hz), 7.48 (d, 2H, J = 8.2 Hz), 7.54 (d, 2H, J = 8.1 Hz); ^{13}C NMR (CD_3OD, 500 MHz) δ 26.9, 29.3, 29.5, 29.9, 31.8, 32.4, 35.9, 36.8, 38.9, 40.3, 40.4, 40.4, 56.7, 59.3, 64.0, 70.7, 70.7, 70.9, 71.4, 79.6, 125.6, 129.8, 133.7, 156.1, 158.1, 171.8, 176.1, 176.6.*

COMMENTARY

Background Information

The strong noncovalent highly specific interaction between biotin and streptavidin or avidin (association constant = 10^{15}/M) has found numerous applications in areas such as immunology, cell biology, and molecular biology (McInnes and Symons, 1989). Consequently, many methods have been developed for biotinylation of various target molecules. Biotinylation of DNA has been achieved either enzymatically (Langer et al., 1981) or chemically. Site-specific chemical biotinylation of DNA can be performed manually after solid-phase synthesis, cleavage, and complete deprotection (Agrawal et al., 1986; Urdea et al., 1988; Gildea et al., 1990; De Vos et al., 1994) or automatically by the synthesizer during solid-phase synthesis (Pon, 1991; Neuner, 1996; Olejnik et al., 1996). For the latter method, which is more convenient to perform, biotinylation reagents that are compatible with DNA synthesis, cleavage, and deprotection conditions are required.

For some applications, releasing the target DNA from streptavidin or avidin may be necessary. For this purpose, cleavable linkers have been incorporated in between biotin and target DNA to avoid the harsh conditions required for breaking the biotin-streptavidin/avidin bonding. For example, a disulfide bond has been incorporated into the linker, which can be broken by reduction with dithiothreitol (Shimkus et al., 1985; Dawson et al., 1991). The drawback of this method is that part of the linker is left on the oligonucleotide, therefore modifying the oligonucleotide, which may not be further processed under biological and biochemical conditions. Another method is to incorporate an acid-labile linker in between biotin and DNA (Gildea et al., 1990). Although the DNA is not modified after cleavage from biotin, the synthesis of the reagent for biotinylation is complicated, and biotinylation is performed manually. A third method uses a photocleavable linkage between biotin and DNA (Olejnik et al., 1996). Using this method, biotinylation is performed automatically by the synthesizer during solid-phase synthesis and, after cleavage, unmodified DNA is obtained. The unsatisfactory feature of this method is that target DNA may be damaged by UV irradiation during photocleavage (Cadet and Vigny, 1990; Greenberg and Gilmore, 1994; Greenberg, 1995).

The biotinylation methods described in this unit use a fluoride-cleavable diisopropyl silyl acetal linkage between biotin and DNA (Fang and Bergstrom, 2003a,b). This method has several advantages over known methods. The biotinylation is automatically carried out by the synthesizer during solid-phase synthesis, no damage of target DNA during cleavage should occur, and, after cleavage, both unmodified 5′-OH (Fang and Bergstrom, 2003b) and unmodified 5′-phosphate DNA (Fang and Bergstrom, 2003a) can be obtained depending on which of the two phosphoramidites is used (see Figs. 4.20.1, 4.20.3, and 4.20.4).

One of the potential applications of this reversible biotinylation method is affinity purification of synthetic oligonucleotides mediated by biotin-avidin/streptavidin binding, as demonstrated in Support Protocol 1. The biotinylated full-length DNA can be readily attached to the NeutrAvidin-coated microspheres, nonbiotinylated failure sequences and other impurities can be removed by simply washing with buffer and water, and high-quality unmodified DNA can be obtained by simple incubation with fluoride ions (followed by aqueous amine treatment in the case of 5′-phosphorylated DNA; Guzaev et al., 1995) under mild conditions, followed by a water wash (see Fig. 4.20.4). Currently, synthetic DNA is generally purified by HPLC and/or gel electrophoresis, which are considered time consuming. DMTr-selective cartridge purification is convenient, but the quality of DNA thus obtained is less satisfactory (*UNIT 10.7*). The affinity purification method described in this unit is convenient, and high quality DNA can be obtained. It is anticipated that the advantages of this method will be more obvious when it is required for purification of very long DNA sequences and/or isolation of target DNA from very complex mixtures.

Critical Parameters and Troubleshooting

For the preparation of the two phosphoramidites (**S.3** and **S.7**, see Basic Protocols 1 and 2 and Support Protocol 2), many steps must be carried out under strict anhydrous conditions. When this is the case, make certain that glassware and reagents, especially solvents, are dry. Although some reactions can be performed in open air, the authors recommend all transformations be performed under an argon or nitrogen atmosphere.

The oligonucleotide to be biotinylated must be synthesized using acetyl-protected C rather than benzoyl-protected C; otherwise, transamination may occur during the aqueous CH_3NH_2/NH_4OH cleavage/deprotection (Reddy et al., 1994; Wincott et al., 1995).

When using the biotinylation method for affinity purification of synthetic oligonucleotides, make sure to handle the NeutrAvidin-coated microspheres gently. Vortexing of the gel should be avoided, as this may result in leaking of gel material and low recovery yield of full-length DNA.

Anticipated Results

Yields reported for each step during the preparation of the phosphoramidites **S.3** and **S.7** may vary depending on how carefully the reaction, work-up, and purification are performed. Since these reactions can be easily performed on a gram scale, it is not difficult to obtain sufficient amounts of **S.3** and **S.7** for biotinylating several batches of DNA. The yield for biotinylation of the 5′-end of DNA on solid support is >99% in each case. For affinity purification by a NeutrAvidin-coated gel, a recovery yield of full-length unmodified DNA can be >70% in both cases.

Time Considerations

The preparation of the phosphoramidites **S.3** or **S.7** requires ~13 days including drying glassware and reagents, setting up and working up reactions, and purifying and drying products. DNA synthesis, 5′-biotinylation, and postsynthetic cleavage/deprotection can be achieved in 1 or 2 days depending on the length of the sequence. Affinity purification can be carried out within 6 hr, but drying the product may require ~12 hr.

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