Deoxyribonucleoside Phosphoramidites

This unit provides methods for converting *N*- and 5′-*O*-protected deoxyribonucleosides to deoxyribonucleoside phosphoramidites, which are the building blocks in solid-phase DNA oligonucleotide synthesis. Deoxyribonucleoside phosphoramidites carrying the 2-cyanoethyl group for P(III)-protection are extensively used in oligonucleotide synthesis and are commercially available. The preparation of deoxyribonucleoside phosphoramidites bearing a different P(III)-protecting group will be described in this unit; phosphoramidites functionalized with the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl groups will be synthesized. Considering the importance of phosphordiamidite precursors in the preparation of deoxyribonucleoside phosphoramidites, two syntheses of these phosphinylating agents will be reported. These detailed methods provide a convenient and reliable approach to the synthesis of various deoxyribonucleoside phosphoramidites, assuming compatibility of the nucleosidic *N*- and 5′-*O*-protecting groups with the reagents used for such syntheses.

PREPARATION OF 5′-*O*-(4,4′-DIMETHOXYTRITYL)-3′-*O*-(*N*,*N*-DIISOPROPYLAMINO)-{4-[*N*-METHYL-*N*-(2,2,2-TRIFLUOROACETYL) AMINO]BUTOXY}PHOSPHINYL-2′-DEOXYRIBONUCLEOSIDES

BASIC PROTOCOL

This protocol describes a general method for the synthesis of deoxyribonucleoside phosphoramidites **S.3a-d** from commercially available *N*- and 5′-*O*-protected deoxyribonucleosides **S.1a-d** and *N,N,N′,N′*-tetraisopropyl-*O*-{4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl}phosphordiamidite **S.2** (see Support Protocol 1) in the presence of 1*H*-tetrazole. This approach to the preparation of deoxyribonucleoside phosphoramidites is illustrated in Figure 2.7.1.

Materials

5′-*O*-(4,4′-Dimethoxytrityl)-2′-deoxythymidine (**S.1a**; Chem-Impex International)
N^4-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine (**S.1b**; Chem-Impex International)
N^6-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyadenosine (**S.1c**; Chem-Impex International)
N^2-Isobutyryl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyguanosine (**S.1d**; Chem-Impex International)
Anhydrous methylene chloride (Aldrich)

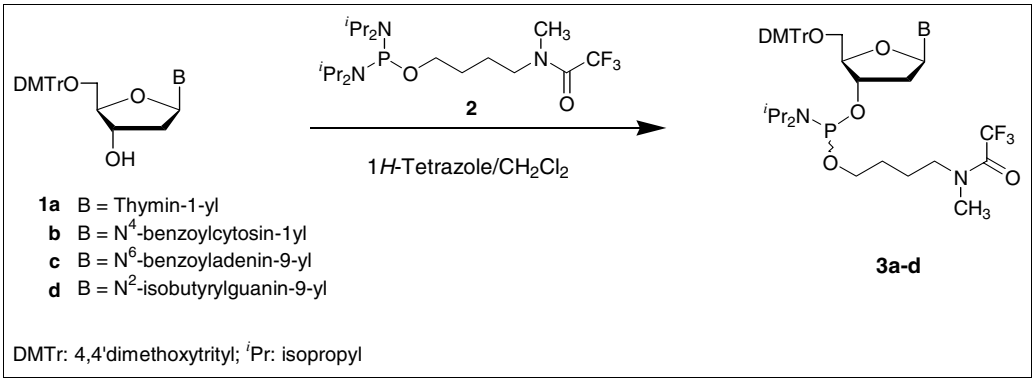

Figure 2.7.1 Preparation of deoxyribonucleoside phosphoramidites from *N*- and 5′-*O*-protected deoxyribonucleosides.

Contributed by Andrzej Wilk, Andrzej Grajkowski, Marcin K. Chmielewski, Lawrence R. Phillips, and Serge L. Beaucage

Current Protocols in Nucleic Acid Chemistry (2001) 2.7.1-2.7.12

N,N,N′,N′-Tetraisopropyl-*O*-{4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)-amino]butyl}phosphordiamidite (**S.2**; see Support Protocol 1)
Sublimed 1*H*-tetrazole (Aldrich)
9:1 (v/v) benzene/triethylamine (both available from Aldrich)
230- to 400-mesh silica gel 60Å (Merck)

50-mL three-necked round-bottom flask and rubber septa
15-mL powder addition funnel (Labglass)
Vacuum desiccator
Rotary evaporator
High vacuum pump
Dry argon gas cylinder
10-mL syringe
2.5 × 20–cm disposable Flex chromatography columns (Kontes)
Fraction collector

Additional reagents and equipment for thin-layer chromatography (TLC; *APPENDIX 3D*) and column chromatography (*APPENDIX 3E*)

1. Dry 2 mmol of a suitably protected deoxyribonucleoside (**S.1a-d**) in a 50-mL three-necked round-bottom flask for 2 hr at 25°C under high vacuum in a desiccator. Seal the flask with a 15-mL powder addition funnel containing 140 mg (2 mmol) dry 1-*H* tetrazole, and rubber septa.
2. Under an argon atmosphere, using a 10-mL syringe, add 10 mL anhydrous methylene chloride followed by 900 mg (2.1 mmol) *N,N,N′,N′*-tetraisopropyl-*O*-{4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl}phosphordiamidite **S.2** and stir with magnetic stir bar.
3. Add the 140 mg (2 mmol) 1*H*-tetrazole in the 15-mL powder addition funnel over a 0.5-hr period.
4. Monitor the progress of the reaction by TLC (*APPENDIX 3D*) using 9:1 benzene/triethylamine as an eluent.

   *Phosphinylation of suitably protected 2′-deoxynucleosides **S.1a-c** is usually complete within 1 hr at ambient temperature. For best results, phosphinylation of properly protected 2′-deoxyguanosine **S.1d** should be allowed to proceed for 12 hr at 25°C.*

5. Concentrate the reaction mixture to a foam using a rotary evaporator/vacuum pump system.
6. Suspend the crude product in a minimum amount (~3 mL) of 9:1 benzene/triethylamine and apply the suspension to a 2.5 × 20–cm disposable Flex column containing 40 g silica gel (*APPENDIX 3E*) that has been equilibrated in 9:1 benzene/triethylamine.
7. Elute the column with 9:1 benzene/triethylamine. Collect in a fraction collector, pool appropriate fractions, and concentrate using a rotary evaporator/vacuum pump system.

   *Each deoxyribonucleoside phosphoramidite **S.3a-d** is isolated as a white amorphous foam in yields ranging from 92% to 98%. Pure **S.3a-d** can be stored indefinitely at –20°C in sealed amber glass vials.*

   *5′-O-(4,4′-Dimethoxytrityl)-3′-O-(N,N-diisopropylamino)-{4-[N-methyl-N-(2,2,2-trifluoroacetyl)amino]butoxy}phosphinyl-2′-deoxythymidine **S.3a**: ^{31}P-NMR (121 MHz, C_6D_6): δ 143.3, 143.4, 143.66, 143.75. FAB-HRMS: calcd. for $C_{44}H_{56}F_3N_4O_9P$ $(M+Na)^+$ 895.3635, found 895.3599.*

N^4-Benzoyl-5′-O-(4,4′-dimethoxytrityl)-3′-O-(N,N-diisopropylamino)-{4-[(N-methyl-N-(2,2,2-trifluoroacetyl)amino]butoxy}phosphinyl-2′-deoxycytidine ***S.3b****: ^{31}P-NMR (121 MHz, C_6D_6): δ 143.82, 143.87, 143.92. FAB-HRMS: calcd. for $C_{50}H_{59}F_3N_5O_9P$ $(M+Na)^+$ 984.3900, found 984.3908.*

N^6-Benzoyl-5′-O-(4,4′-dimethoxytrityl)-3′-O-(N,N-diisopropylamino)-{4-[N-methyl-N-(2,2,2-trifluoroacetyl)amino]butoxy}phosphinyl-2′-deoxyadenosine ***S.3c****: ^{31}P-NMR (121 MHz, C_6D_6): δ 143.5, 143.6, 143.8, 144.0. FAB-HRMS: calcd. for $C_{51}H_{59}F_3N_7O_8P$ $(M+Na)^+$ 1008.4010, found 1008.3970.*

N^2-Isobutyryl-5′-O-(4,4′-dimethoxytrityl)-3′-O-(N,N-diisopropylamino)-{4-[N-methyl-N-(2,2,2-trifluoroacetyl)amino]butoxy}phosphinyl-2′-deoxyguanosine ***S.3d****: ^{31}P-NMR (121 MHz, C_6D_6): δ 142.9, 143.1, 143.5, 143.7. FAB-HRMS: calcd. for $C_{48}H_{61}F_3N_7O_9P$ $(M+Na)^+$ 990.4118, found 990.4099.*

PREPARATION OF *N,N,N′,N′*-TETRAISOPROPYL-*O*-{4-[*N*-METHYL-*N*-(2,2,2-TRIFLUOROACETYL)AMINO]BUTYL}PHOSPHORDIAMIDITE

SUPPORT PROTOCOL 1

4-[*N*-Methyl-*N*-(2,2,2-trifluoroacetyl)amino]butan-1-ol **S.4** (see Support Protocol 2; Fig. 2.7.2) is treated with phosphorus trichloride to generate the corresponding *O*-{4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl}phosphordichloridite **S.5**, which is then purified by vacuum distillation. Condensation of the phosphordichloridite **S.5** with *N,N*-diisopropylamine affords *N,N,N′,N′*-tetraisopropyl-*O*-{4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl}phosphordiamidite **S.2**, which can be purified further by silica gel chromatography. As shown in Figure 2.7.1, the phosphordiamidite **S.2** is required for the phosphinylation of *N*- and 5′-*O*-protected deoxyribonucleosides **S.1a-d** (see Basic Protocol).

Materials

Phosphorus trichloride (Aldrich), freshly distilled
Anhydrous acetonitrile (Aldrich)
4-[*N*-Methyl-*N*-(2,2,2-trifluoroacetyl)amino]butan-1-ol (**S.4**; see Support Protocol 2)
Anhydrous petroleum ether (Aldrich), freshly distilled from phosphorus pentoxide
Drierite, 8 mesh (Aldrich)
Anhydrous *N,N*-diisopropylamine (Aldrich)

250- and 1000-mL round-bottom flasks
25-mL pressure-equalizing dropping funnel
Vacuum distillation head 24/40 and appropriate thermometer
Three-way stopcock
Reflux condenser

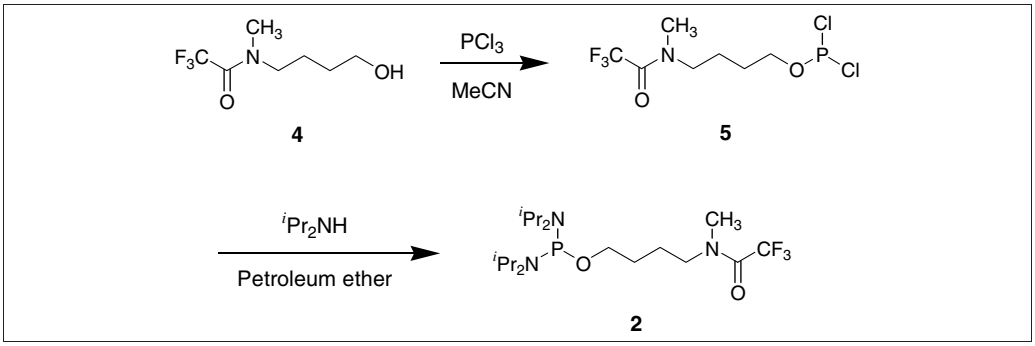

Figure 2.7.2 Preparation of *N,N,N′,N′*-tetraisopropyl-*O*-{4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl}phosphordiamidite.

Drying tube
Rubber septum
250-mL sintered glass funnel (coarse porosity)
Rotary evaporator/vacuum pump system

NOTE: Phosphordichloridites are very sensitive to moisture. Reaction yields depend on the dryness of the reaction conditions. It is recommended that all glassware be oven-dried overnight at 120°C. The dried glassware should then be cooled to ambient temperature under an inert gas atmosphere in a desiccator. Acetonitrile is refluxed over calcium hydride for ≥2 hr prior to distillation.

Prepare O-{4-[N-methyl-N-(2,2,2-trifluoroacetyl)amino]butyl}phosphordichloridite

1. Dissolve 6.8 g (50 mmol) freshly distilled phosphorus trichloride in 50 mL anhydrous acetonitrile in an oven-dried 250-mL round-bottom flask.
2. Place 8 g (40 mmol) of 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butan-1-ol **S.4** and 10 mL anhydrous acetonitrile in a 25-mL pressure-equalizing dropping funnel connected to a vacuum line via a three-way stopcock.
3. Add the **S.4** solution dropwise over a 2-hr period to the magnetically stirred phosphorus trichloride solution from step 1.

   CAUTION: During the course of the addition, a slight vacuum should be applied through the dropping funnel to maintain an internal pressure of ~750 mmHg. This lower-than-atmospheric pressure ensures the removal of gaseous hydrogen chloride that is being generated.
4. Stir the reaction mixture for an additional 3 hr at 40°C and ~40 mmHg.
5. Fractionally distill the remaining material under reduced pressure.

   *O-{4-[N-Methyl-N-(2,2,2-trifluoroacetyl)amino]butyl}phosphordichloridite **S.5** is a colorless liquid boiling at 110°C/0.1 mmHg. Yield 11 g (37 mmol, 92%). ^{1}H-NMR (300 MHz, C_6D_6): δ [1.01(bm) (33%) and 1.11 (bm) (67%) (4H)], [2.38 (bs) (67%) and 2.45 (bs) (33%), (3H)], [2.75 (m) (33%) and 2.88 (m) (67%) (2H)], 3.77 (m, 2H). ^{13}C-NMR (75 MHz, C_6D_6): δ 22.3, 23.9, 26.0, 26.3, 33.2, 33.5, 47.9, 48.0, 67.5 (d, $^{2}J_{PC}$ = 10.6 Hz), 67.7 (d, $^{2}J_{PC}$ = 10.6 Hz), 116.9 (q, $^{1}J_{CF}$ = 288 Hz), 117.0 (q, $^{1}J_{CF}$ = 288 Hz), 156.6 (q, $^{2}J_{CF}$ = 36.0 Hz), 156.7 (q, $^{2}J_{CF}$ = 36.0 Hz). ^{31}P-NMR (121 MHz, C_6D_6): δ 174.2, 174.3.*

   *Although **S.5** can be stored for a week at –20°C without significant decomposition, it is recommended to use **S.5** when freshly distilled.*

Prepare N,N,N′,N′-tetraisopropyl-O-{4 -[N-methyl-N-(2,2,2-trifluoroacetyl)amino]butyl}phosphordiamidite

6. Add 300 mL anhydrous petroleum ether and 11 g (37 mmol) ***S.5*** to an oven-dried 1000-mL round-bottom flask equipped with a reflux condenser fitted with a drying tube filled with Drierite.
7. Add in portions over a 15-min period a solution of 28 mL (200 mmol) anhydrous *N*,*N*-diisopropylamine in 100 mL anhydrous petroleum ether through the reflux condenser to the vigorously stirred phosphordichloridite solution.

   Large quantities of white precipitate (N,N-diisopropylammonium hydrochloride) are produced.
8. As the resulting suspension cools to ambient temperature, remove the reflux condenser and seal the flask with a rubber septum. Allow the reaction mixture to stir for an additional 48 hr at 25°C.

9. Filter off the precipitated *N*,*N*-diisopropylammonium hydrochloride salt using a 250-mL sintered glass funnel of coarse porosity.

10. Concentrate the filtrate on a rotary evaporator/vacuum pump system and keep under high vacuum for 12 hr.

*As prepared, compound **S.2** is sufficiently pure for phosphinylating properly protected deoxyribonucleosides. However, **S.2** can be purified by silica gel chromatography (APPENDIX 3E) using ~20 g of silica gel per gram of crude product, and 9:1 (v/v) benzene/triethylamine as the eluent. Fractions containing pure **S.2** are detected on analytical TLC plates (APPENDIX 3D) by staining with a saturated solution of silver nitrate in ethanol and then briefly heating the TLC plate at 50°C.*

*N,N,N′,N′-Tetraisopropyl-O-{4-[N-methyl-N-(2,2,2-trifluoroacetyl)amino]butyl}phosphordiamidite **S.2**. Yield 15 g (35 mmol, 95%). ^{1}H-NMR (300 MHz, C_6D_6): δ 1.21 (m, 24H), 1.40 (m, 4H), [2.41 (q, $^5J_{HF}$ = 1.6 Hz) (67%) and 2.50 (q, $^5J_{HF}$ = 0.7 Hz, (33%), (3H)], [2.89 (tq, J = 7.4 Hz, $^5J_{HF}$ = 1.0 Hz) (33%) and 3.04 (t, J = 6.9 Hz) (67%), (2H)], 3.43 (m, 2H), 3.50 (m, 4H). ^{13}C-NMR (75 MHz, C_6D_6): δ 23.2, 23.7, 23.8, 24.3, 24.5, 25.0, 28.3, 28.4, 28.5, 28.6 33.3, 33.5 (q, $^4J_{CF}$ = 4.2 Hz), 44.3, 44.5, 48.5, 48.7 (q, $^4J_{CF}$ = 4.2 Hz), 63.4 (d, $^2J_{CP}$ = 6.4 Hz), 63.7 (d, $^2J_{CP}$ = 6.4 Hz), 117.0 (q, $^1J_{CF}$ = 288 Hz), 117.1 (q, $^1J_{CF}$ = 288 Hz), 156.1 (q, $^2J_{CF}$ = 36 Hz). ^{31}P-NMR (121 MHz, C_6D_6): δ 117.9, 118.2. FAB-MS: calcd. for $C_{19}H_{39}F_3N_3O_2P$ $(M+H)^+$ 430, found 430.*

*Pure **S.2** can be stored indefinitely at –20°C in sealed amber glass vials.*

PREPARATION OF 4-[*N*-METHYL-*N*-(2,2,2-TRIFLUOROACETYL)AMINO]BUTAN-1-OL

SUPPORT PROTOCOL 2

As shown in Figure 2.7.3, 4-(*N*-methyl)aminobutan-1-ol **S.8** is prepared from the reaction γ-butyrolactone **S.6** with gaseous methylamine followed by reduction of the resulting amido alcohol **S.7** with lithium aluminum hydride. Chemoselective *N*-trifluoroacetylation is performed by condensing 4-(*N*-methyl)aminobutan-1-ol **S.8** with methyl trifluoroacetate. 4-[*N*-Methyl-*N*-(2,2,2-trifluoroacetyl)amino]butan-1-ol **S.4** is required in the preparation of *N*,*N*,*N*′,*N*′-tetraisopropyl-*O*-{4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)-amino]butyl}phosphordiamidite **S.2** (see Support Protocol 1).

Materials

γ-Butyrolactone (**S.6**; Aldrich)
Anhydrous gaseous methylamine (Aldrich)
Diethyl ether (Malinckrodt), freshly distilled from sodium
Drierite (Aldrich)
Lithium aluminum hydride powder (LAH, Aldrich)

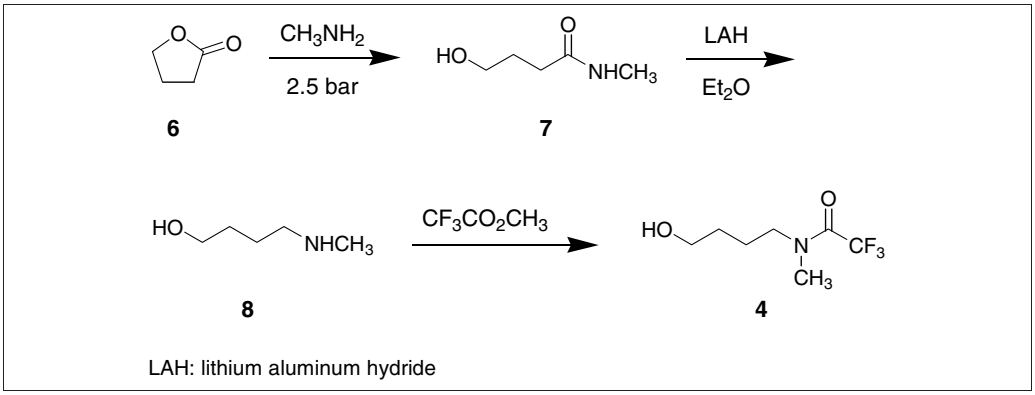

Figure 2.7.3 Preparation of 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butan-1-ol.

Triethanolamine (Aldrich)
Methyl trifluoroacetate (Aldrich)

250-mL pressure vessel (Barrskogen)
2-L three-necked round-bottom flask
Mechanical stirrer (Arrow)
Pressure-equalizing dropping funnel
Reflux condenser connected to a drying tube
Dry argon gas cylinder
250-mL sintered glass funnel (coarse porosity)
Rotary evaporator/water aspirator system
50-mL round-bottom flask

Prepare N-methyl-4-hydroxybutyramide

1. Place 17.2 g (199 mmol) γ-butyrolactone **S.6** in a 250-mL pressure vessel.
2. Evacuate the vessel to ~2 mmHg and fill it with anhydrous gaseous methylamine.
3. Stir the reaction mixture overnight under a positive pressure of methylamine (~2.5 bar).

   CAUTION: This reaction involves highly irritating gaseous methylamine under pressure. Ensure that the pressure vessel is tight and placed in a well-ventilated chemical fume hood. Use moist pH-indicator paper to check for leaks. Even trace amounts of strongly alkaline methylamine can be detected using this test.

4. Carefully release excess methylamine to the atmosphere of a well-ventilated chemical fume hood.
5. Evacuate the vessel and maintain the reaction product under vacuum (2 mmHg) for 1 hr.

   *N-Methyl-4-hydroxybutyramide **S.7** is isolated as a pure (>98%) crystalline mass (mp 32° to 34°C) in near quantitative yield (23.1 g, 197 mmol). ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.63 (dt, J = 7.6, 6.4 Hz, 2H), 2.10 (t, J = 7.6 Hz, 2H), 2.56 (d, J = 4.6 Hz, 3H), 3.37 (t, J = 6.4 Hz, 2H), 3.58 (b, ~1H), 7.75 (b, 1H). ^{13}C-NMR (75 MHz, DMSO-d_6): δ 25.6, 28.7, 32.2, 60.5, 173.1.*

   ***S.7** can be stored indefinitely at –20°C in sealed amber glass vials.*

Reduce N-methyl-4-hydroxybutyramide

6. Add 1 L diethyl ether to an oven-dried 2-L three-necked round-bottom flask equipped with a mechanical stirrer, a pressure-equalizing dropping funnel, and a reflux condenser connected to a drying tube filled with Drierite.
7. Add, under an argon atmosphere, 36 g (0.94 mol) lithium aluminum hydride powder.
8. Melt 58.5 g (0.50 mol) *N*-methyl-4-hydroxybutyramide **S.7** and place in the dropping funnel.
9. Add the neat supercooled liquid (**S.7**) to the mechanically stirred LAH suspension, dropwise, over a 2-hr period at 25°C.
10. Reflux the reaction mixture for 6 hr and stir for an additional 6 hr at 25°C.

    CAUTION: Diethyl ether is highly flammable. Lithium aluminum hydride is extremely sensitive to water; traces of water from the solvent and the environment will result in the release of highly flammable hydrogen gas. Consequently, steps 7 to 10 must be performed with the utmost care to exclude moisture from the reaction mixture. A flow of anhydrous argon gas through the reaction vessel greatly reduces the danger of fire.

11. Quench the reduction reaction while stirring by first adding 125 mL (0.94 mol) triethanolamine dropwise over a 1-hr period (Powell et al., 1986; Gardrat et al., 1990), followed by 50 mL (2.8 mol) water dropwise over a 1-hr period.
12. Stir the resulting slurry for 12 hr at 25°C.
13. Filter the slurry through a 250-mL sintered glass funnel (coarse porosity) and wash the solid cake three times with 200 mL diethyl ether.
14. Pool the etheral filtrates and evaporate on a rotary evaporator/water aspirator system. Distill the residue under reduced pressure.

    *The amino alcohol **S.8** (34.3 g ,0.33 mol, 67%) is obtained as a colorless liquid boiling at 47°C/0.25 mmHg. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.64 (m, 4H), 2.42 (s, 3H), 2.62 (dd, J = 6.0, 5.3 Hz, 2H), 3.57 (dd, J = 5.3, 4.6 Hz, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 28.3, 32.3, 35.7, 51.7, 62.5.*

    ***S.8** can be stored indefinitely at –20°C in sealed amber glass vials.*

Perform chemoselective trifluoroacetylation of 4-(N-methyl)aminobutan-1-ol

15. To 5 g (48 mmol) of 4-(*N*-methyl)aminobutan-1-ol **S.8** in a 50-mL round-bottom flask, add 7.8 g (60 mmol) methyl trifluoroacetate.
16. Stir the reaction mixture for 12 hr at 25°C.
17. Remove methanol and excess methyl trifluoroacetate by distillation at atmospheric pressure.
18. Distill the residue under reduced pressure.

    *4-[N-Methyl-N-(2,2,2-trifluoroacetyl)amino]butan-1-ol **S.4** is obtained as a slightly viscous colorless liquid (8.9 g, 45 mmol, 94%) boiling at 71°C/0.36 mmHg. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.56 (m, 2H), 1.70 (m, 2H), [3.00 (q, $^{5}J_{HF}$ = 0.7 Hz) (33%) and 3.14 (q, $^{5}J_{HF}$ = 1.6 Hz) (67%), (3H)], 3.46 (m, J = 7.4 Hz, 2H), 3.65 (t, J = 6.1 Hz, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 22.8, 24.7, 29.2, 29.3, 34.1, 34.6, 49.1, 49.4, 61.7, 61.8, 116.5 (q, $^{1}J_{CF}$ = 288 Hz), 116.6 (q, $^{1}J_{CF}$ = 288 Hz), 156.7 (q, $^{2}J_{CF}$ = 36.0 Hz), 156.8 (q, $^{2}J_{CF}$ = 36.0 Hz).*

    *Pure **S.4** can be stored indefinitely at –20°C in sealed amber glass vials.*

PREPARATION OF *N,N,N′,N′*-TETRAISOPROPYL-*O*-{2-[(*N*-FORMYL-*N*-METHYL)AMINO]ETHYL}PHOSPHORDIAMIDITE

ALTERNATE PROTOCOL

Depending on the protecting group used for P(III), the preparation of phosphordichloridites may be problematic and could complicate the synthesis of phosphordiamidites, which are required for the preparation of deoxyribonucleoside phosphoramidites. A number of phosphordichloridites have been reported to decompose violently when heated (Beaucage, 1993). This protocol describes the generation of bis(*N,N*-diisopropylamino)chlorophosphine **S.9** in situ from phosphorus trichloride and *N,N*-diisopropylamine, and its reaction with 2-(*N*-formyl-*N*-methyl)aminoethan-1-ol **S.10** to give the desired phosphordiamidite **S.11** (Fig. 2.7.4). The method thus alleviates the need of distilling potentially hazardous phosphordichloridites.

***Additional Materials** (also see Basic Protocol)*

2-(Methylamino)ethanol (Aldrich)
Ethyl formate (Aldrich)
Anhydrous benzene
Freshly distilled phosphorous trichloride
Anhydrous *N,N*-diisopropylamine
95:5 (v/v) anhydrous benzene/triethylamine
Toluene

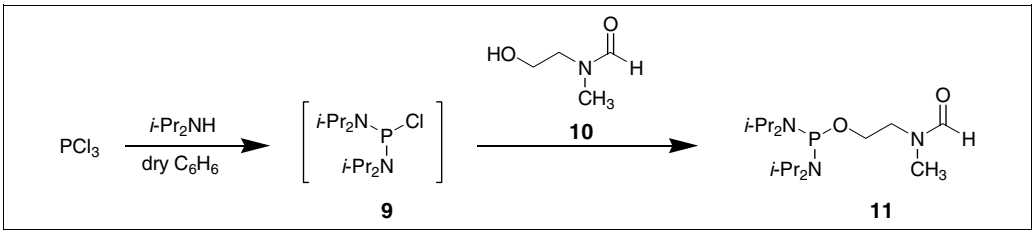

Figure 2.7.4 Preparation of *N,N,N′,N′*-tetraisopropyl-*O*-{2-[(*N*-methyl-*N*-formyl)amino]ethyl}-phosphordiamidite.

100- and 250-mL round-bottom flasks equipped with a reflux condenser
Heating mantle
60-mL sintered glass funnel (coarse porosity)
3 × 20–cm chromatography column (*APPENDIX 3E*)

Prepare 2-(N-formyl-N-methyl)aminoethan-1-ol

1. Place 51.0 g (0.68 mol) of 2-(methylamino)ethanol into a 250-mL round-bottom flask equipped with a reflux condenser, and cool to 5°C by immersion in an ice bath.

2. Add, in portions through the condenser, 75.0 g (1.01 mol) ethyl formate to the stirred amino alcohol over a 5-min period at 5°C.

   CAUTION: This reaction is exothermic.

3. Remove the ice bath and bring the solution to reflux for 1 hr using a heating mantle.

4. Distill the solution at atmospheric pressure to remove excess ethyl formate, and then carefully distill the remaining residue under high vacuum.

   2-(N-Formyl-N-methyl)aminoethan-1-ol ***S.10*** *was obtained as a clear colorless liquid (63.1 g, 0.61 mol, 90%) boiling at 120° to 122°C at 0.15 mmHg.* ^{1}*H-NMR (300 MHz, DMSO-*d_6*): δ [2.75 (s) and 2.94 (s, 30%) (3H)], 3.27 (m, 2H), 3.47 (m, 3H), [7.94 (s) and 7.99 (s, 30%) (1H)].* ^{13}*C-NMR (75 MHz, DMSO-*d_6*): δ 29.2, 34.9, 46.2, 51.2, 57.8, 57.9, 58.1, 58.2, 162.7, 163.0. EI-MS: calcd. for* $C_4H_9NO_2$ *(*$M^{\bullet+}$*) 103, found 103.*

   Pure ***S.10*** *can be stored indefinitely at –20°C in sealed amber glass vials.*

Prepare N,N,N′,N′-tetraisopropyl-O-{2 -[(N-formyl-N-methyl)amino]ethyl}phosphordiamidite

5. To an oven-dried 100-mL round-bottom flask containing 50 mL anhydrous benzene under a dry argon atmosphere, add by syringe through a rubber septum 876 µL (10 mmol) freshly distilled phosphorus trichloride.

6. Cool the stirred solution to 5°C by immersing in an ice bath and then, over a 30-min period, add 7.7 mL (55 mmol) of anhydrous *N,N*-diisopropylamine under argon using a 10-mL syringe.

7. Remove the ice bath and allow the stirred reaction to warm to 25°C under a positive pressure of argon until the formation of bis(*N,N*-diisopropylamino)chlorophosphine **S.9** is complete.

   The rate of the reaction is monitored by ^{31}*P-NMR spectroscopy; after ~48 hr, the presence of* ***S.9*** *(132.0 ppm downfield relative to a phosphoric acid external standard) is observed as the major (>96%) reaction product.*

8. Add 1.03 mL (10 mmol) of 2-(*N*-formyl-*N*-methyl)aminoethan-1-ol **S.10** to the suspension. Stir resulting mixture for 2 hr at 25°C under a positive pressure of argon.

   ^{31}P-NMR indicates that the reaction is essentially quantitative (~96%). Spectrum of the product in benzene-d_6 shows two singlets at 118.0 and 118.7 ppm.

9. Filter the suspension through a 60-mL sintered glass funnel (coarse porosity) and wash the collected salt with 20 mL anhydrous benzene.

10. Evaporate the filtrates under reduced pressure to an oil and dissolve with a minimum amount (~3 mL) of 95:5 anhydrous benzene/triethylamine.

11. Apply the viscous solution uniformly to the top of a 3 × 20–cm chromatography column (*APPENDIX 3E*) packed with 30 g of a 60 Å silica gel slurry in a solution of 95:5 benzene/triethylamine.

12. Elute the column isocratically with 95:5 benzene/triethylamine collecting 8-mL fractions.

13. Analyze the fractions by TLC (*APPENDIX 3D*) and pool the fractions that contain the product. Evaporate fractions containing the phosphordiamidite **S.11** to an oil on a rotary evaporator/vacuum pump system.

    *Using 95:5 benzene/triethylamine as the eluent, **S.11** has an R_f ~ 0.49. **S.11** is visible only by staining the TLC with silver nitrate as recommended in Support Protocol 1, step 10.*

14. Co-evaporate the oil four times with 10 mL toluene to remove traces of triethylamine. Leave under high vacuum for ≥3 hr.

    *N,N,N′,N′-tetraisopropyl-O-{2 -[(N-formyl-N-methyl)amino]ethyl}phosphordiamidite **S.11**. Yield: 2.43 g (7.3 mmol, 73%). ^{1}H-NMR (300 MHz, C_6D_6): δ [1.14 (d, J = 6.9 Hz), 1.16 (d, J = 6.7 Hz) 1.18 (d, J = 6.7 Hz) (24H)], [2.40 (s, 34%) and 2.64 (s, 66%) (3H)], 2.80 (t, J = 5.4 Hz, 2H), 3.43 (m, 4H), [3.29 (dt, J =5.4 Hz, J_{HP} = 6.6 Hz) and 3.60 (dt, J =5.4 Hz, J_{HP} = 6.6 Hz)(2H)], [7.82 (s, 34%) and 7.98 (s, 66%) (1H)]. ^{13}C-NMR (75 MHz, C_6D_6): δ 24.1, 24.2, 24.6, 24.7, 44.7, 44.9, 45.8 (d, $^2J_{CP}$ = 8.5 Hz), 50.4(d, $^2J_{CP}$ = 8.5 Hz), 61.3, 61.5, 61.9, 62.2, 161.9, 162.3. ^{31}P-NMR (121 MHz, C_6D_6): δ 118.0, 118.7. EI-MS: calcd. for $C_{16}H_{36}N_3O_2P$ ($M^{\bullet+}$) 333.2545, found 333.2528.*

    *Pure **S.11** can be stored indefinitely at –20°C in sealed amber glass vials.*

COMMENTARY

Background Information

Since its inception in the early 1980s, the phosphoramidite method for oligonucleotide synthesis (Beaucage and Caruthers, 1981; Beaucage and Iyer, 1992; *UNIT 3.3*) has been extensively used to produce polynucleotides (Pon et al., 1994) on solid supports. The method is most convenient when using the 2-cyanoethyl group for phosphate protection (Sinha et al., 1984) on small-scale syntheses of oligonucleotides. This group is eventually removed along with nucleobase-protecting groups during oligonucleotide deprotection by treatment with either concentrated ammonium hydroxide or pressurized ammonia gas (Boal et al., 1996). Under these conditions, 2-cyanoethyl groups undergo β-elimination, generating acrylonitrile as a side-product (Tener, 1961). Acrylonitrile is a potent carcinogen (Solomon et al., 1984), and alkylation of the nucleobase of nucleosides and oligonucleotides by this reagent is well documented (Chambers, 1965; Solomon et al., 1984; Prokopczyk et al., 1988; Crippa et al., 1993).

In an effort to obviate nucleobase modification during oligonucleotide deprotection at any scale level, an alternative to the 2-cyanoethyl group for phosphate protection is recommended. The use of the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)]aminobutyl group for phosphodiester protection in the synthesis of oligodeoxyribonucleotides was recently reported (Wilk et al., 1999). Polydeoxyribonucleotides carrying this phosphodiester protecting group are easily deprotected by treatment with concentrated ammonium hydroxide. Cleavage of

the 2,2,2-trifluoroacetyl groups is rate limiting, and is followed by rapid cyclo-deesterification of the resulting 4-(*N*-methyl)aminobutyl phosphotriesters to give the corresponding phosphodiesters with the concomitant formation of *N*-methylpyrrolidine (Fig. 2.7.5). When compared to the potential DNA-alkylating properties of acrylonitrile, 2,2,2-trifluoroacetamide and *N*-methylpyrrolidine, which are produced during oligonucleotide deprotection, are quite innocuous.

The high coupling efficiency of the deoxyribonucleoside phosphoramidites **S.3a-d** (Fig. 2.7.1), their stability in acetonitrile solutions, and the relative ease of 4-(*N*-methyl)aminobutyl phosphotriester deprotection prompted the authors to describe the preparation of these phosphoramidites in this unit as alternatives to the commercially available 2-cyanoethyl deoxyribonucleoside phosphoramidites. Given that the procedure for preparing phosphoramidites **S.3a-d** is similar to that of the 2-cyanoethyl phosphoramidites, it shall serve as a relevant model for the synthesis of deoxyribonucleoside phosphoramidites, which carry different P(III) protecting groups. In this context, depending on the group selected for P(III) protection, the method used for the preparation of either the phosphordiamidite **S.2** or the analogous *N,N,N′,N′*-tetraisopropyl-*O*-(2-cyanoethyl)phosphordiamidite may not be adequate. For example, the phosphordiamidite **S.11** (Fig. 2.7.4) could not be conveniently prepared from a phosphordichloridite precursor. Thus, the phosphordiamidite **S.11** was prepared from bis(*N*,*N*-diisopropylamino)chlorophosphine, which provides added versatility to the synthesis of phosphordiamidites as phosphinylating agents. The advantage of the 2-[(*N*-formyl-*N*-methyl)amino]ethyl group for oligonucleotide phosphodiester protection is that, unlike the 2-cyanoethyl group, it can be removed in the absence of base under neutral conditions (authors' unpub. observ.).

The use of (*N*,*N*-diisopropylamino)-*O*-alkylchlorophosphine (Sinha et al., 1984) in the preparation of deoxyribonucleoside phosphoramidites will not be discussed in this unit as these phosphinylating reagents are, because of their inherent reactivity, less stable to moisture than the corresponding phosphordiamidites, and are consequently much less convenient to handle.

Critical Parameters and Troubleshooting

Since 4-(*N*-methyl)aminobutan-1-ol **S.8** (see Support Protocol 2) is not commercially available, its synthesis has been studied for careful optimization. The lithium aluminum hydride reduction of the parent amido alcohol **S.7** is demanding in that it requires strict anhydrous conditions and a mechanical stirrer for optimal performance. In addition, quenching the reduction reaction by adding triethanolamine followed by water to destroy aluminum complexes (as recommended by Powell et al., 1986, and Gardrat et al., 1990), and carefully washing the solid aluminate cake with diethyl ether (see Support Protocol 2, steps 11 to 13) must be performed to ensure a maximum recovery of **S.8**.

The selection of acetonitrile as the solvent for converting 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butan-1-ol **S.4** to its phosphordichloridite **S.5** under lower than atmospheric pressure conditions (see Support Protocol 1) is required for optimal production of **S.5**. The use of other solvents will result in a considerably lower yield of **S.5**. Performing the reaction at atmospheric instead of reduced pres-

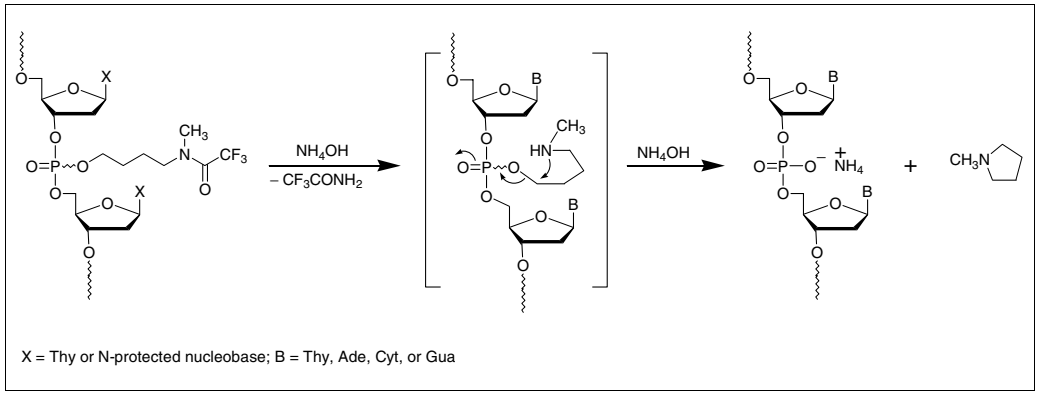

Figure 2.7.5 Deprotection of oligodeoxyribonucleotides carrying the 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate protecting group under standard basic conditions.

sure will also result in unwanted formation of side-products.

It is important to mention that the preparation of phosphordiamidites from the bis(*N*,*N*-diisopropylamino)chlorophosphine intermediate **S.9** (see Alternate Protocol) should be performed under strictly anhydrous conditions to minimize the formation of side-products resulting from hydrolysis. These side-products unfortunately co-elute with deoxyribonucleoside phosphoramidites during silica gel chromatography. Depending on the amount of contaminating side-products, the stoichiometry of deoxyribonucleoside phosphoramidites required for solid-phase oligonucleotide synthesis may be difficult to determine accurately. Furthermore, the chemical reactivity of the phosphorus-containing hydrolysis side-products is not known; these side-products may react with activated phosphoramidites and further decrease the effective concentration of the amidites.

Occasionally, deoxyribonucleoside phosphoramidites may not produce the expected coupling efficiency during conventional solid-phase oligonucleotide synthesis. A number of parameters may be responsible for such an apparent lack of reactivity. (1) Phosphoramidites may be contaminated with the triethylamine used with benzene as an eluent during purification on silica gel chromatography. Triethylamine stoichiometrically neutralizes 1*H*-tetrazole, the weak acid required for the activation of deoxyribonucleoside phosphoramidites in solid-phase oligonucleotide synthesis. Thus, it will prevent optimal coupling efficiency of the phosphoramidites to an extent corresponding to its concentration. It is therefore critical that the phosphoramidites be subjected to high-vacuum foaming after chromatographic purification to ensure complete removal of triethylamine contaminants. Alternatively, lyophilization of a frozen benzene solution of deoxyribonucleoside phosphoramidites is an efficient method for the removal of residual triethylamine. (2) Phosphoramidites may be contaminated with adventitious moisture upon storage, which will result in lower coupling efficiency. It is recommended that deoxyribonucleoside phosphoramidites be dried overnight under high vacuum in a desiccator containing phosphorus pentoxide as a drying agent. (3) The overall purity of deoxyribonucleoside phosphoramidites, as determined by ^{31}P-NMR spectroscopy, should be >95%; phosphoramidites contaminated with >15% of phosphorus-containing hydrolysis products invariably exhibit lower coupling efficiency when compared to that of pure deoxyribonucleoside phosphoramidites.

Anticipated Results

It should be emphasized that deviation from Support Protocol 1 in regard to preparing phosphordichloridite **S.5** with a solvent other than acetonitrile at atmospheric pressure will generally cause the yield of the product to drop to 50% to 60%. Caution should be exercised when attempting the distillation of unknown phosphordichloridites; a number of these compounds may decompose or even explode on heating (Beaucage, 1993). In this context, the preparation of phosphordiamidites from bis(*N*,*N*-diisopropylamino)chlorophosphine **S.9** generated in situ (see Alternate Protocol) is a much safer process and is easier to perform experimentally. Furthermore, the purification of phosphordiamidites on silica gel chromatography is milder than distillation to the phosphonylating reagents and safer to perform. Isolated yields of phosphordiamidites after silica gel chromatography range between 70% and 80% based on the starting amido alcohol **S.10**; it should be noted that formation of some hydrolysis side-products cannot be avoided, only minimized by careful attention to experimental detail.

The synthesis of deoxyribonucleoside phosphoramidites **S.3a-d** from phosphordiamidite **S.2** (see Basic Protocol) is performed essentially as recommended by Barone et al. (1984). More reaction time is required for the preparation of **S.3d** to optimize yields. It is possible that phosphinylation at O6 of guanine by **S.2** might occur and serve as a secondary, slower, phosphinylating path (Barone et al., 1984) for the nucleoside **S.1d**. The phosphoramidites **S.3a-d** are very soluble and stable in acetonitrile. For example, an acetonitrile solution of **S.3d**, unlike the corresponding 2-cyanoethyl phosphoramidite, does not precipitate upon storage exceeding 3 days. In fact, the coupling efficiency of phosphoramidites **S.3a-d** is essentially unchanged after being in acetonitrile solution for 1 week (Wilk et al., 1999).

Time Considerations

The preparation and purification of 4-(*N*-methyl)aminobutan-1-ol **S.8** can be accomplished in ~3 days. The conversion of **S.8** to the amido alcohol **S.4** can be done in one day, and the purified phosphordiamidite **S.2** can be obtained from **S.4** within 3 days. The synthesis and purification of deoxyribonucleoside phosphoramidites **S.3a-d** require ~2 days to com-

plete. Alternatively, the synthesis and purification of the amido alcohol **S.10** takes a half day, and its conversion to the purified phosphordiamidite **S.11** takes ~3 days.

Literature Cited

Barone, A.D., Tang, J.-T., and Caruthers, M.H. 1984. In situ activation of bis-dialkylaminophosphines—A new method for synthesizing deoxyoligonucleotides on polymer supports. *Nucl. Acids Res*. 12:4051-4061.

Beaucage, S.L. 1993. Oligodeoxyribonucleotides synthesis—Phosphoramidite approach. *In* Methods in Molecular Biology, Vol. 20: Protocols for Oligonucleotides and Analogs (S. Agrawal, ed.) pp. 33-61. Humana Press, Totowa, N.J.

Beaucage, S.L. and Caruthers, M.H. 1981. Deoxynucleoside phosphoramidite—A new class of key intermediates for deoxypolynucleotide synthesis. *Tetrahedron Lett*. 22:1859-1862.

Beaucage, S.L. and Iyer, R.P. 1992. Advances in the synthesis of oligonucleotides by the phosphoramidite approach. *Tetrahedron* 48:2223-2311.

Boal, J.H., Wilk, A., Harindranath, N., Max, E.E., Kempe, T., and Beaucage, S.L. 1996. Cleavage of oligodeoxyribonucleotides from controlled-pore glass supports and their rapid deprotection by gaseous amines. *Nucl. Acids Res.* 24:3115-3117.

Chambers, R.W. 1965. The chemistry of pseudouridine. IV. Cyanoethylation. *Biochemistry* 4:219-226.

Crippa, S., Di Gennaro, P., Lucini, R., Orlandi, M., and Rindone, B. 1993. Characterization of adducts of nucleic bases and acrylic-monomers. *Gazz. Chim. Ital*. 123:197-203.

Gardrat, C., Latxague, L., and Picard, J.P. 1990. A new synthesis of *dl*-5-vinyloxazolidine-2-thione, a natural antithyroid factor. *J. Het. Chem*. 27:811-812.

Grajkowski, A., Wilk, A., Chmielewski, M.K., and Beaucage, S.L. 2000, *unpublished results*.

Pon, R.T., Buck, G.A., Niece, R.L., Robertson, M., Smith, A.J., and Spicer, E. 1994. A survey of nucleic-acid services in core laboratories. *BioTechniques* 17:526-534.

Powell, J., James, N., and Smith, S.J. 1986. Lithium aluminum hydride reductions: A new hydrolysis method for intractable products. *Synthesis* 338-340.

Prokopczyk, B., Bertinato, P., and Hoffman, D. 1988. Synthesis and kinetics of decomposition of 7-(2-cyanoehtyl)guanine and *O*-6-(2-cyanoethyl)guanine, markers for reaction of acrylonitrile and 3-(methylnitrosamino)propionitrile with DNA. *Carcinogenesis* 9:2125-2128.

Sinha, N.D., Biernat, J., McManus, J., and Köster, H. 1984. Polymer support oligonucleotide synthesis. 18. Use of β-cyanoethyl-*N,N*,-dialkylamino/-*N*-morpholino phosphoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. *Nucl. Acids Res.* 12:4539-4557.

Solomon, J.J., Cote, I.L., Wortman, M., Decker, K., and Segal, A. 1984. In vitro alkylation of calf thymus DNA by acrylonitrile—Isolation of cyanoethyl adducts of guanine and thymine and carboxyethyl adducts of adenine and cytosine. *Chem.-Biol. Interactions* 51:167-190.

Tener, G.M. 1961. 2-Cyanoethyl phosphate and its use in the synthesis of phosphate esters *J. Am. Chem. Soc*. 83:159-168.

Wilk, A., Grajkowski, A., Phillips, L.R., and Beaucage, S.L. 1999. The 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl group as an alternative to the 2-cyanoethyl group for phosphate protection in the synthesis of oligodeoxyribonucleotides. *J. Org. Chem*. 64:7515-7522.

Contributed by Andrzej Wilk, Andrzej Grajkowski, Marcin K. Chmielewski, and Serge L. Beaucage
Food and Drug Administration
Bethesda, Maryland

Lawrence R. Phillips
National Cancer Institute
Frederick, Maryland