The 4-Methylthio-1-Butyl Group for Phosphate/Thiophosphate Protection in Oligodeoxyribonucleotide Synthesis

UNIT 3.11

This unit provides a detailed preparation of deoxyribonucleoside phosphoramidites functionalized with a 4-methylthio-1-butyl group for P(III) protection, and describes their use in the solid-phase synthesis of DNA oligonucleotides. Specifically, the efficiency of these phosphoramidites in the synthesis of a 20-mer DNA oligonucleotide and its phosphorothioated analog is demonstrated. The thermolytic cleavage of the 4-methylthio-1-butyl protecting group from these oligonucleotides, at neutral pH or under the standard basic conditions used for nucleobase deprotection, proceeds through a cyclodeesterification mechanism with the concomitant formation of a cyclic sulfonium salt (Cieślak et al., 2004). The 4-methylthio-1-butyl protecting group is distinct from previously studied thermolytic groups (*UNITS 2.7 & 3.9*; Grajkowski et al., 2001) in terms of structural simplicity and ease of deprotection. Thus, this unit describes an attractive method for preparation of alkylation-free oligonucleotides for potential therapeutic applications, while bringing closer to reality the implementation of a "heat-driven" process for oligonucleotide synthesis on microarrays.

BASIC PROTOCOL

PREPARATION OF 4-METHYLTHIO-1-BUTYL DEOXYRIBONUCLEOSIDE PHOSPHORAMIDITES AND THEIR USE IN SOLID-PHASE DNA SYNTHESIS

The 4-methylthio-1-butyl group for phosphate/thiophosphate protection can be thermolytically cleaved from oligonucleotides at a lower temperature and at a faster rate than the 4-oxopentyl (*UNIT 3.9*; Wilk et al., 2001), 3-(*N-tert*-butylcarboxamido)-1-propyl (*UNIT 3.9*; Wilk et al., 2002), and 2-(*N*-formyl,*N*-methyl)aminoethyl (Grajkowski et al., 2001) groups under neutral conditions. Moreover, the 4-methylthio-1-butyl group can also be removed from oligonucleotides under conditions (NH_4OH, 10 hr at 55°C) that are not suitable for removal of 3-(*N-tert*-butylcarboxamido)-1-propyl and 2-(*N*-formyl,*N*-methyl)aminoethyl groups. As illustrated in Figure 3.11.1, this protocol outlines a general method for the preparation of deoxyribonucleoside phosphoramidites (**S.3a-d**) from commercial 5′-*O*- and *N*-protected deoxyribonucleosides (**S.1a-d**), *O*-(4-methylthio-1-butyl)-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (**S.2**; see Support Protocol), and 1*H*-tetrazole. Procedures for the use of **S.3a-d** in solid-phase DNA synthesis and for the analysis of synthetic oligonucleotides are also described.

Materials

- Protected deoxyribonucleosides (**S.1a-d**; Chem-Impex International):
  - 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxythymidine
  - N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine
  - N^6-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyadenosine
  - N^2-isobutyryl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyguanosine
- Argon source
- Anhydrous dichloromethane (CH_2Cl_2; Aldrich)
- *O*-(4-Methylthio-1-butyl)-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (**S.2**; see Support Protocol)
- 1*H*-Tetrazole, sublimed
- Triethylamine (TEA; Aldrich)
- Benzene (Aldrich)

Contributed by Jacek Cieślak, Andrzej Grajkowski, Victor Livengood, and Serge L. Beaucage
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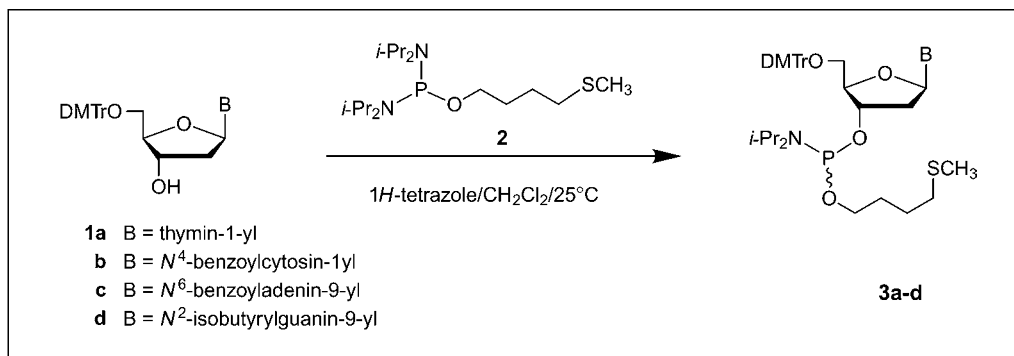

Figure 3.11.1 Preparation of deoxyribonucleoside phosphoramidites **S.3a-d**. DMTr, 4,4′-dimethoxytrityl; *i*-Pr, isopropyl. Adapted and reprinted from Cieślak et al. (2004) with permission from the American Chemical Society.

Silica gel (60 Å, 230 to 400 mesh; Merck)
Hexane (−20°C)
Dry ice/acetone bath
Reagents recommended for automated solid-phase oligonucleotide synthesis (Applied Biosystems and/or Glen Research):
  Standard 2-cyanoethyl deoxyribonucleoside phosphoramidites (T, C^{Bz}, A^{Bz} and $G^{i\text{-}Bu}$)
  Activator solution: 1*H*-tetrazole in acetonitrile
  Oxidation solution: 0.02 M iodine in THF/pyridine/water
  Cap A solution: acetic anhydride in THF/pyridine
  Cap B solution: 1-methylimidazole in THF
  Deblocking solution: trichloroacetic acid in dichloromethane
Acetonitrile
3*H*-1,2-Benzodithiol-3-one-1,1-dioxide (Glen Research)
Concentrated ammonium hydroxide (NH_4OH; Fisher Scientific)
2 M triethylammonium acetate (TEAA) buffer, pH 7.0 (Applied Biosystems)
80% acetic acid (AcOH)
Loading buffer: 1:4 (v/v) 10× TBE electrophoresis buffer (*APPENDIX 2A*) in formamide containing 2 mg/mL bromphenol blue
20 × 40–cm, 7 M urea/20% polyacrylamide gel (*UNIT 10.4* and *APPENDIX 3B*)
Staining buffer: 1:5:20:0.1 (v/v/v/v) formamide/isopropyl alcohol/ddH_2O/3.0 M Tris·Cl, pH 8.8
1 mg/mL Stains-All (Aldrich) in formamide
1.0 M Tris·Cl, pH 9.0 (*APPENDIX 2A*)
1.0 M $MgCl_2$ (Sigma)
Snake venom phosphodiesterase (SVPD, *Crotalus adamanteus*; Sigma)
Bacterial alkaline phosphatase (BAP, *E. coli*; Sigma)

50-, 100-, and 250-mL round-bottom flasks
Rubber septa for 14/20- and 24/40-glass joints
Vacuum desiccator
High-vacuum oil pump
1- and 10-mL glass syringes
5-mm NMR tube
Rotary evaporator connected to a vacuum pump
2.5 × 20–cm disposable Flex chromatography columns (Kontes)
2.5 × 7.5–cm EMD TLC plates precoated with a 250-µm layer of silica gel 60 F_{254}
Lyophilizer
392 DNA/RNA synthesizer (Applied Biosystems)
1-mL Luer-tipped syringes

4-mL screw-cap glass vials
Heating block (VWR)
25 cm × 4.6–mm Supelcosil LC-18S HPLC column (5-μm; Supelco)
1.5-mL microcentrifuge tubes
37°C water bath

Additional reagents and equipment for thin-layer chromatography (TLC; *APPENDIX 3D*), column chromatography (*APPENDIX 3E*), automated DNA synthesis (*APPENDIX 3C*), DNA quantification by UV spectrophotometry (*UNIT 10.3*), reversed-phase HPLC (*UNIT 10.5*), and polyacrylamide gel electrophoresis (PAGE; *UNIT 10.4 & APPENDIX 3B*)

Prepare deoxyribonucleoside phosphoramidites

1. Dry 2.2 mmol of a suitably protected deoxyribonucleoside (**S.1a-d**) by exposure to high vacuum for 2 hr at 25°C in a 50-mL round-bottom flask. Add a stir bar and seal flask with a rubber septum.

2. Under an argon atmosphere, add 10 mL anhydrous dichloromethane using a 10-mL glass syringe and ∼700 mg (∼2 mmol) *O*-(4-methylthio-1-butyl)-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (**S.2**) via a 1-mL glass syringe. Stir the solution with a magnetic stirrer.

3. While stirring the solution under a positive pressure of argon, remove the rubber septum from the flask and quickly add 112 mg (1.6 mmol) sublimed 1*H*-tetrazole using a spatula. Seal the flask with the rubber septum.

   Other activators such as ethylthio-1H-tetrazole and dicyanoimidazole could theoretically be used, but have not been tested.

4. Carefully transfer ∼0.5 mL of the reaction mixture by syringe to a dry 5-mm NMR tube. Monitor the progress of the reaction in the NMR tube by ^{31}P NMR spectroscopy. Upon completion of the reaction, as seen by the disappearance of **S.2**, return the NMR sample to the main reaction mixture before proceeding with the next step.

   *The ^{31}P NMR signal corresponding to **S.2** (δ_P 121 ppm) disappears within 2 hr at ambient temperature and thus indicates complete phosphinylation of **S.1a-d**.*

5. Add 1 mL triethylamine and immediately concentrate the reaction mixture to a syrup using a rotary evaporator connected to a vacuum pump.

6. Suspend the crude product in ∼3 mL of 9:1 (v/v) benzene/triethylamine and apply the suspension to a 2.5 × 20–cm disposable Flex chromatography column containing ∼20 g silica gel that has been equilibrated in 9:1 (v/v) benzene/triethylamine (*APPENDIX 3E*).

7. For A, C, and T derivatives, elute the column with 9:1 (v/v) benzene/triethylamine and collect 6-mL fractions. For the G derivative, elute with 45:45:10 (v/v/v) benzene/dichloromethane/triethylamine.

8. Analyze fractions by TLC (*APPENDIX 3D*) on a 2.5 × 7.5–cm EMD silica gel 60 F_{254} TLC plate using 9:1 (v/v) benzene/triethylamine as the eluent. Pool appropriate fractions and rotoevaporate under reduced pressure (∼20 mmHg) until a white amorphous solid is obtained.

   *TLC analysis of diastereomeric **S.3a-d** shows two tight spots. The R_f of each pair of spots ranges between 0.1 and 0.6 when 9:1 (v/v) benzene/triethylamine is used as the eluent. For the G derivative using 45:45:10 benzene/dichloromethane/triethylamine, the R_f is ∼0.3.*

9. Dissolve the solid in ∼3 mL benzene and add the solution to ∼100 mL vigorously stirred cold (−20°C) hexane in a 250-mL round-bottom flask.

10. Allow the suspension to settle and carefully decant off most of the supernatant.

11. Rotoevaporate the wet material to dryness under reduced pressure and then dissolve in ~10 mL benzene.

12. Transfer the solution to a 100-mL round-bottom flask, freeze in a dry ice/acetone bath, and lyophilize under high vacuum to afford triethylamine-free **S.3a-d** as white powders in yields ranging from 72% to 85%.

*5′-O-(4,4′-Dimethoxytrityl)-3′-O-[(N,N-diisopropylamino)(4-methylthio-1-butyloxy)] phosphinyl-2′-deoxythymidine **S.3a**: ^{31}P NMR (121 MHz, $CDCl_3$): δ 147.6, 147.9 Fast-atom bombardment high-resolution mass spectrometry (FAB-HRMS): anal. calcd. for $C_{42}H_{56}N_3O_8PS$ $(M+Cs)^+$ 926.2580, found 926.2537.*

*N^4-Benzoyl-5′-O-(4,4′-dimethoxytrityl)-3′-O-[(N,N-diisopropylamino)(4-methylthio-1-butyloxy)]phosphinyl-2′-deoxycytidine **S.3b**: ^{31}P NMR (121 MHz, $CDCl_3$): δ 147.5, 147.9. FAB-HRMS: anal. calcd. for $C_{48}H_{59}N_4O_8PS$ $(M+Cs)^+$ 1015.2846, found 1015.2870.*

*N^6-Benzoyl-5′-O-(4,4′-dimethoxytrityl)-3′-O-[(N,N-diisopropylamino)(4-methylthio-1-butyloxy)]phosphinyl-2′-deoxyadenosine **S.3c**: ^{31}P NMR (121 MHz, $CDCl_3$): δ 147.3, 147.7. FAB-HRMS: anal. calcd. for $C_{49}H_{59}N_6O_7PS$ $(M+Cs)^+$ 1039.2958, found 1039.2996.*

*N^2-Isobutyryl-5′-O-(4,4′-dimethoxytrityl)-3′-O-[(N,N-diisopropylamino)(4-methylthio-1-butyloxy)]phosphinyl-2′-deoxyguanosine **S.3d**: ^{31}P-NMR (121 MHz, $CDCl_3$): δ 147.4, 147.8. FAB-HRMS: anal. calcd. for $C_{46}H_{61}N_6O_8PS$ $(M+Cs)^+$ 1021.3064, found 1021.3011.*

It is common practice to mix cesium iodide, potassium iodide, and/or sodium iodide with a sample to be analyzed by FAB-HRMS. The accuracy in mass determination can be better ascertained through the association of cesium $(M+Cs)^+$, potassium $(M+K)^+$, or sodium $(M+Na)^+$ with the parent ion. The accurate mass is not always easy to determine as a $(M+H)^+$ ion when the sample is dissolved in the traditional glycerol matrix in the absence of metallic salts.

Synthesize oligonucleotides

13. Perform a 0.2-µmol scale solid-phase synthesis of both d(ATCCGTAGCTAAGGTCATGC) and its phosphorothioate analog using an Applied Biosystems 392 DNA/RNA synthesizer in “trityl-on” mode according to the manufacturer’s recommendations (also see *APPENDIX 3C*). Perform parallel syntheses employing exclusively commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites to serve as reference standards for comparing the overall quality of the synthetic oligonucleotides.

*As in the case of 2-cyanoethyl deoxyribonucleoside phosphoramidites, **S.3a-d** are used as 0.1 M solutions in dry acetonitrile. All ancillary reagents required for the automated preparation of oligonucleotides were purchased from Applied Biosystems and/or Glen Research and used as recommended by the manufacturer(s).*

The synthetic cycle recommended for the preparation of phosphorothioated DNA oligonucleotides differs from the conventional cycle used for the synthesis of unmodified oligonucleotides in that the “capping step” is performed after the oxidative sulfurization reaction (Iyer et al., 1990). The sulfur transfer reaction is effected by treatment with 0.05 M 3H-1,2-benzodithiol-3-one-1,1-dioxide in acetonitrile using a wait step of 30 sec.

Cleave oligonucleotide from support

14. Using two 1-mL Luer-tipped syringes, one attached to each end of the synthesis column, push back and forth 0.5 mL of concentrated NH_4OH three times through the column every 10 min over a 30-min period. Transfer the solution to a 4-mL screw-cap glass vial. Push back and forth 0.5 mL of fresh NH_4OH for 1 min through the synthesis column and pour the wash into the glass vial.

Complete oligonucleotide deprotection

15. Place the tightly capped vial containing each oligonucleotide solution in a heating block pre-heated to 55°C ± 2°C and continue heating at this temperature for 10 hr. Cool each sample to room temperature.

16. Determine the concentration of the oligonucleotide by UV spectrophotometry at 260 nm (UNIT 10.3).

Analyze oligonucleotide by RP-HPLC

17. Analyze ~0.25 OD_{260} of each oligonucleotide on a 5-µm Supelcosil LC-18S HPLC column (UNIT 10.5) using a linear gradient of 1% acetonitrile/min starting from 0.1 M triethylammonium acetate, pH 7.0, at a flow rate of 1 mL/min for 40 min. Hold the gradient isocratically for 20 min.

RP-HPLC profiles of oligonucleotides synthesized from ***S.3a-d*** *and conventional 2-cyanoethyl deoxyribonucleoside phosphoramidites are presented in Figure 3.11.2. The two peaks for the PS-oligonucleotides are caused by the presence of a terminal 5′-DMTr group, which facilitates the hydrophobic resolution of two populations of diastereomers (R_P-rich and S_P-rich). The resolution of these diastereomeric populations is dependent*

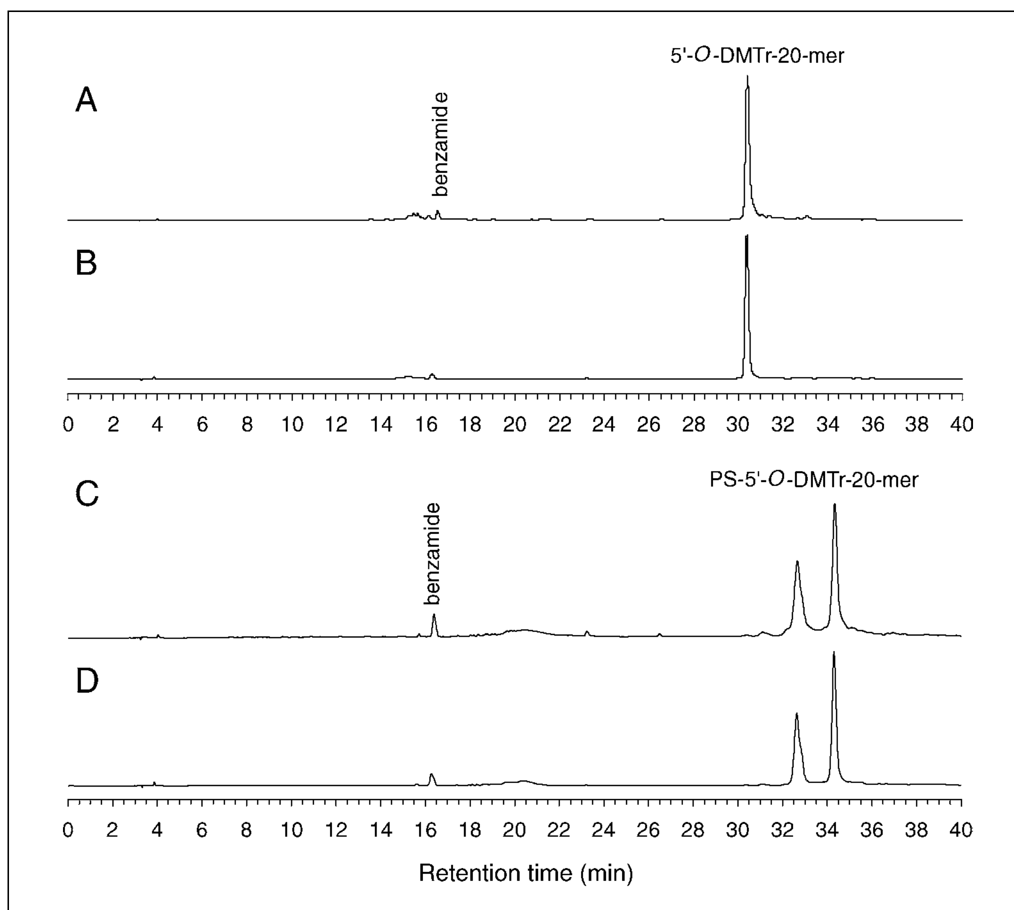

Figure 3.11.2 RP-HPLC chromatograms of crude 5′-*O*-DMTr-d(ATCCGTAGCTAAGGTCATGC) and 5′-*O*-DMTr-d($A_{PS}T_{PS}C_{PS}C_{PS}G_{PS}T_{PS}A_{PS}G_{PS}C_{PS}T_{PS}A_{PS}A_{PS}G_{PS}G_{PS}T_{PS}C_{PS}A_{PS}T_{PS}G_{PS}C$). (**A**) 5′-*O*-DMTr-20-mer and (**C**) PS-5′-*O*-DMTr-20-mer synthesized via phosphoramidites **S.3a-d** and deprotected as described in the Basic Protocol. (**B**) 5′-*O*-DMTr-20-mer and (**D**) PS-5′-*O*-DMTr-20-mer synthesized from commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites and deprotected as described in Basic Protocol, steps 14 and 15. RP-HPLC analyses were performed as described in Basic Protocol, step 17. DMTr, 4,4′-dimethoxytrityl; PS, phosphorothioate diester. Adapted and reprinted from Cieślak et al. (2004) with permission from the American Chemical Society.

on the sequence and size of the oligomer and will completely vanish upon removal of the 5′-DMTr group under acidic conditions. This phenomenon is not well understood, although it has consistently been observed in several laboratories.

Analyze oligonucleotide by PAGE

18. Pipet 0.25 OD_{260} of each oligonucleotide solution into a 1.5-mL microcentrifuge tube. Add 100 µL of 80% AcOH. After 30 min, evaporate to dryness using a Speedvac.

19. Add 10 µL of loading buffer. Vortex well and then centrifuge 5 sec at 14,000 × *g*.

20. Load the sample in a 2-cm-wide well of a 20 × 40–cm, 7 M urea/20% polyacrylamide gel (*UNIT 10.4* and *APPENDIX 3B*). Electrophorese at 350 V until the bromphenol blue dye travels 75% of the length of the gel.

21. Dismantle the gel apparatus and immerse the gel in 250 mL staining buffer containing 10 mL of 1 mg/mL Stains-All in formamide. Agitate the gel for ∼1 hr in the dark.

22. Discard the staining solution and rinse the gel three times with 250 mL distilled water.

23. Expose the gel to natural light until the purple background disappears and photograph the blue bands against a white background.

    A photograph of the gel is presented in Figure 3.11.3.

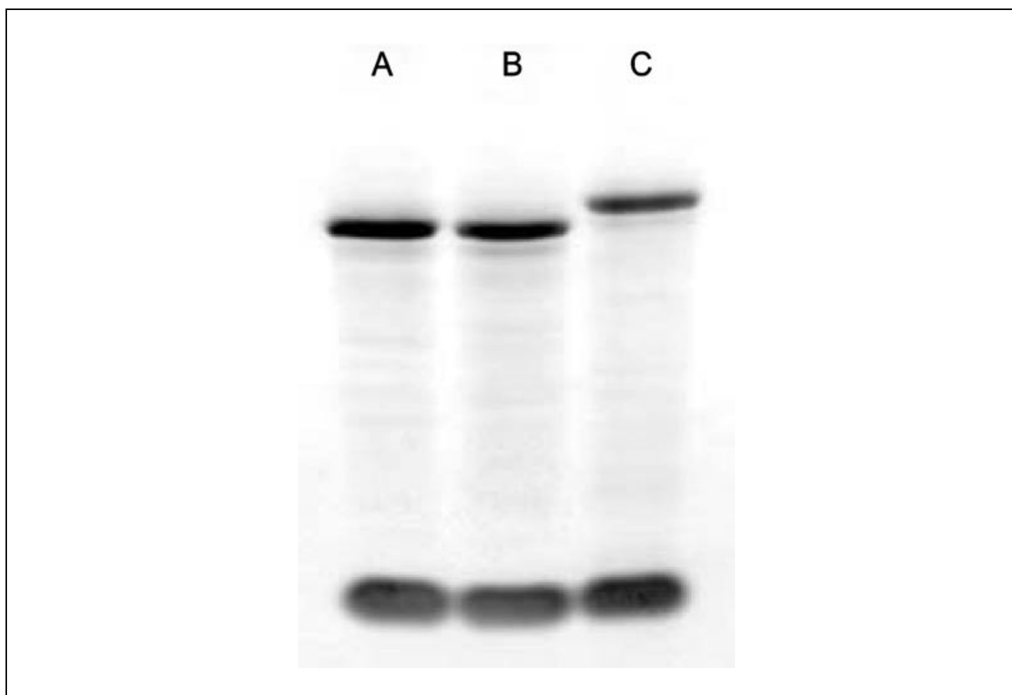

Figure 3.11.3 PAGE analysis of d(ATCCGTAGCTAAGGTCATGC) and its phosphorothioated analog under denaturing conditions (7 M urea, 1× TBE buffer, pH 8.3). (**A**) Crude oligomer synthesized from commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites. (**B**) Crude oligomer synthesized from phosphoramidites **S.3a-d**. (**C**) Crude phosphorothioated oligomer synthesized from **S.3a-d**. All were deprotected as described in Basic Protocol, steps 14, 15, and 18. Unmodified oligonucleotides are visualized as blue bands and fully thioated oligonucleotides as purple bands upon staining the gel with Stains-All. Bromphenol blue is used as a marker and shows as a large band at the bottom of each lane. Adapted and reprinted from Cieślak et al. (2004) with permission from the American Chemical Society.

Characterize oligonucleotide by enzyme hydrolysis

24. Pipet 1 OD_{260} of each oligonucleotide solution into a 1.5-mL microcentrifuge tube. Add 100 µL of 80% AcOH. After 30 min, evaporate to dryness using a Speedvac.

25. Add 6 µL of 1.0 M Tris·Cl, pH 9.0, 8 µL of 1.0 M $MgCl_2$, and 75 µL water. Mix well.

26. Add 5 µL (0.015 U) SVPD and 6 µL (0.7 U) BAP and heat 16 hr in a 37°C water bath (also see *UNIT 10.6*).

27. Heat deactivate the enzyme 3 min at 90°C. Centrifuge 5 min at 14,000 × *g*, 25°C.

28. Analyze a 50-µL aliquot of the digest by RP-HPLC as in step 17.

The RP-HPLC profile of the enzyme hydrolysates is shown in Figure 3.11.4.

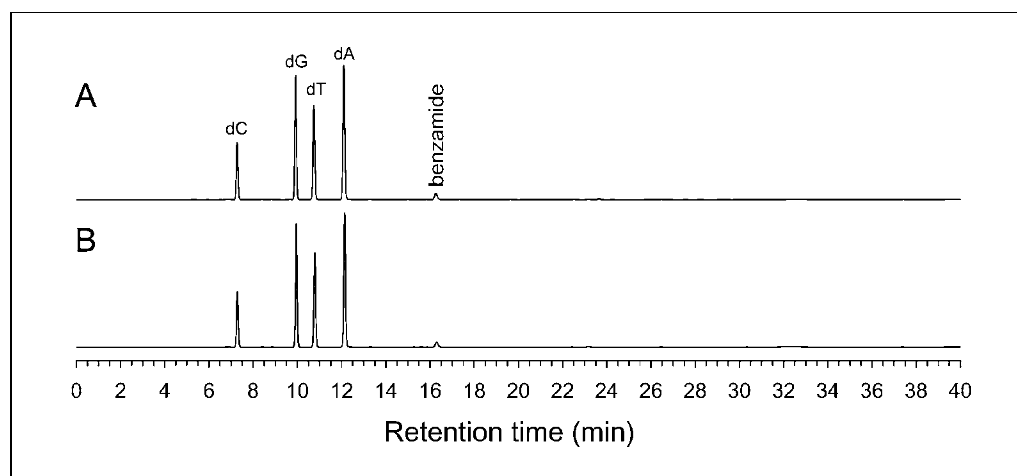

Figure 3.11.4 RP-HPLC analysis of crude d(ATCCGTAGCTAAGGTCATGC) after digestion with SVPD and BAP (37°C, 16 hr). Hydrolysates were made from (**A**) a 20-mer synthesized using phosphoramidites **S.3a-d** and (**B**) a 20-mer synthesized using commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites. Both were deprotected as described in Basic Protocol, steps 14 and 15. RP-HPLC analyses were performed as described in Basic Protocol, step 17. Identities of the RP-HPLC peaks are confirmed through co-injection with authentic 2′-deoxyribonucleosides. Adapted and reprinted from Cieślak et al. (2004) with permission from the American Chemical Society.

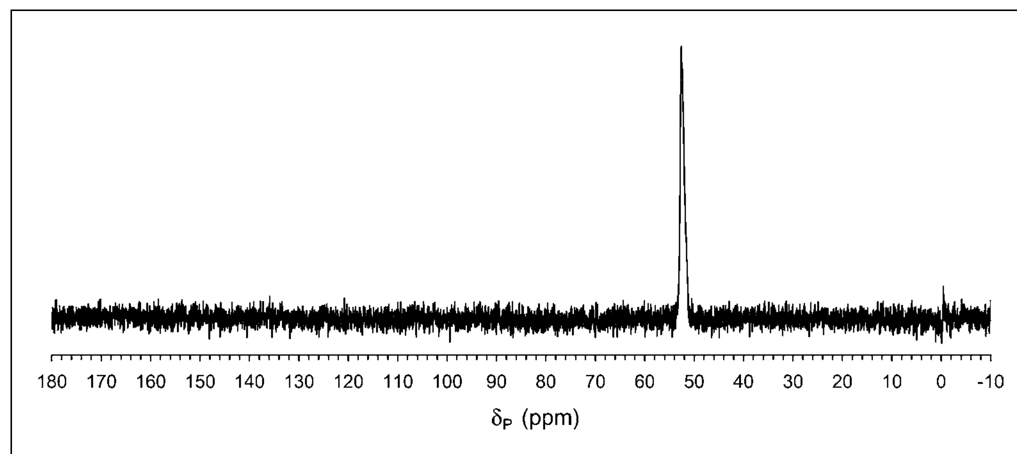

Figure 3.11.5 121 MHz ^{31}P NMR spectrum of crude d($A_{PS}T_{PS}C_{PS}C_{PS}G_{PS}T_{PS}A_{PS}G_{PS}C_{PS}T_{PS}A_{PS}A_{PS}G_{PS}G_{PS}T_{PS}C_{PS}A_{PS}T_{PS}G_{PS}$C) in NH_4OH. The crude 20-mer was prepared from **S.3a-d** and deprotected as described in Basic Protocol, steps 14 and 15. Adapted and reprinted from Cieślak et al. (2004) with permission from the American Chemical Society.

Analyze phosphorothioated oligonucleotide by ^{31}P NMR spectroscopy

29. Analyze the crude phosphorothioated oligonucleotide (synthesized using **S.3a-d** and deprotected as described in steps 14 and 15) in NH_4OH by ^{31}P NMR spectroscopy.

    A ^{31}P NMR spectrum of phosphorothioated d(ATCCGTAGCTAAGGTCATGC) is shown in Figure 3.11.5. The crude oligomer appears as a broad singlet at ~52 ppm.

SUPPORT PROTOCOL

PREPARATION OF *O*-(4-METHYLTHIO-1-BUTYL)-*N,N,N′,N′*-TETRAISOPROPYLPHOSPHORODIAMIDITE

This protocol delineates the preparation of bis(*N*,*N*-diisopropylamino)chlorophosphine (**S.4**) in situ from the reaction of phosphorus trichloride and *N*,*N*-diisopropylamine, and its condensation with 4-methylthio-1-butanol (**S.5**) to produce the phosphorodiamidite **S.2** (see Fig. 3.11.6).

Additional Materials *(also see Basic Protocol)*

Phosphorus trichloride (Aldrich)
N,*N*-Diisopropylamine (Aldrich)
4-Methylthio-1-butanol (Aldrich)
Drierite, 8 mesh (Aldrich)

10-µm stainless-steel frit (20 × 10–mm o.d.; Kontes)
3-mm-i.d. Teflon canula

1. To 100 mL of dry benzene in an oven-dried 250-mL round-bottom flask, add by syringe through a rubber septum 1.75 mL (20 mmol) of freshly distilled phosphorus trichloride under a dry argon atmosphere.

2. Cool the stirred solution to 5°C using an ice bath. Add by syringe 17.6 mL (140 mmol) anhydrous *N*,*N*-diisopropylamine over a period of 30 min under an argon atmosphere.

3. 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.4**) is complete.

    *The progress of the reaction is monitored by ^{31}P NMR spectroscopy. The formation of **S.4** (singlet, 135.5 ppm, downfield relative to a phosphoric acid external standard) is essentially complete (>96%) within 72 hr.*

4. Add by syringe 2.7 mL (22 mmol) of 4-methylthio-1-butanol (**S.5**) to the suspension. Stir for 2 hr at 25°C under a positive pressure of argon.

    *^{31}P NMR spectrum of reaction mixture shows a singlet at 118.5 ppm indicating the near quantitative formation of **S.2**.*

5. Using argon pressure, filter the suspension through a 10-µm stainless steel frit (20 × 10–mm o.d.) coupled to a 3-mm-i.d. Teflon canula into a 250-mL round-bottom flask. Wash the salt with 40 mL of dry benzene and resume filtration. To release gas pressure, pierce a 20-G needle through the rubber septum of the receiving flask.

6. Rotoevaporate the filtrates under reduced pressure to afford **S.2** as an oil.

    *Crude O-(4-methylthio-1-butyl)-N,N,N′,N′-tetraisopropylphosphorodiamidite (**S.2**) can be used for the phosphinylation of **S.1a-d** without further purification. ^{1}H NMR (300 MHz, C_6D_6): δ 3.56 (m, 2H), 3.53 (sept, J = 6.9 Hz, 2H), 3.49 (sept, J = 6.9 Hz, 2H), 2.30 (m, 2H), 1.80 (s, 3H), 1.63 (m, 4H), 1.23 (d, J = 6.9 Hz, 12H), 1.19 (d, J = 6.9 Hz, 12H). ^{13}C NMR (75 MHz, C_6D_6): δ 15.2, 24.0, 24.1, 24.7, 24.8, 26.2, 31.1 (d, J_{PC} = 9.6 Hz), 34.2, 44.6, 44.7, 64.1 (d, $^2J_{PC}$ = 21.5 Hz). ^{31}P NMR (121 MHz, C_6D_6): δ 118.5.*

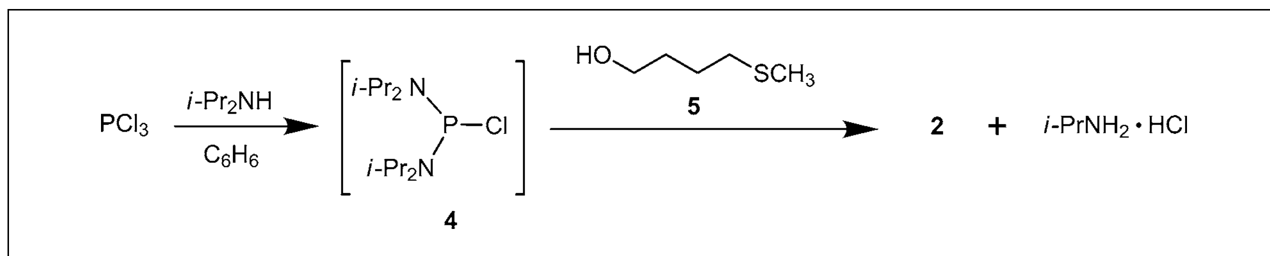

Figure 3.11.6 Preparation of *O*-(4-methylthio-1-butyl)-*N,N,N′,N′*-tetraisopropylphosphorodiamidite (**S.2**). Adapted and reprinted from Cieślak et al. (2004) with permission from the American Chemical Society.

COMMENTARY

Background Information

The thermolabile 4-methylthio-1-butyl phosphate/thiophosphate-protecting group for DNA oligonucleotides has been investigated for its potential application to oligonucleotide synthesis on diagnostic microarrays and to the large-scale preparation of alkylation-free therapeutic oligonucleotides. With the advent of DNA microarrays as powerful diagnostic tools in clinical medicine (Rhodes and Chinnaiyan, 2002; Guo, 2003), the development of thermolytic groups for 5′-hydroxyl (Chmielewski et al., 2003) and phosphate (Grajkowski et al., 2001; Wilk et al., 2001, 2002; Cieślak and Beaucage, 2003) protection toward the implementation of a “heat-driven” method (Grajkowski et al., 2003) for the synthesis of DNA oligonucleotides on planar glass surfaces is being investigated. Thermolabile 5′-/3′-hydroxyl- and phosphate-protecting groups are designed for rapid and efficient release from oligonucleotides when heated under neutral conditions. Such properties eliminate the use of harsh chemicals that are typically required for iterative oligonucleotide chain extension and final oligonucleotide deprotection. These chemicals, however, may damage the glass surface and result in oligonucleotide leaching, which would lead to a poorer performance of microarrays in terms of diagnostic sensitivity.

In contrast, the large-scale preparation of oligonucleotides for therapeutic applications has challenged the suitability of 2-cyanoethyl deoxyribonucleoside phosphoramidites (Sinha et al., 1984) for such syntheses, given the potent DNA-alkylating properties of acrylonitrile (Wilk et al., 1999a,b) that is generated under the conditions used for nucleobase deprotection. DNA alkylation can permanently compromise the affinity of therapeutic oligonucleotides for their mRNA targets. To circumvent such a limitation, a number of deoxyribonucleoside phosphoramidites functionalized with phosphorus-protecting groups that would prevent formation of DNA-alkylating species during oligonucleotide deprotection have been reported. These groups include the 4-[(*N*-methyl,*N*-(2,2,2-trifluoroacetyl)amino]butyl group (Wilk et al., 1999a,b; *UNIT 2.7*) and the 4-oxopentyl group (Wilk et al., 2001; *UNIT 3.9*), which can easily be removed from oligonucleotides by standard treatment with concentrated ammonium hydroxide or pressurized ammonia gas (Boal et al., 1996). The removal of these phosphate/thiophosphate-protecting groups occurred through an intramolecular cyclodeesterification reaction with the concomitant formation of side-products that were inoccuous to the nucleobases of DNA oligonucleotides (Wilk et al., 1999a,b; 2001). Deoxyribonucleoside phosphoramidites functionalized with phosphorus-protecting groups exhibiting thermolytic properties were also reported. Groups such as 2-(*N*-formyl,*N*-methyl)aminoethyl (Grajkowski et al., 2001) and 3-[(*N*-*tert*-butyl)carboxamido]-1-propyl (Wilk et al., 2002) were cleaved from *N*-deprotected oligonucleotides upon heating at temperatures up to 90°C in an aqueous buffer at neutral pH. Under these conditions, the removal of these phosphate/thiophosphate-protecting groups was achieved within 3 hr and 1 hr, respectively. The thermolytic cleavage of the 3-[(*N*-*tert*-butyl)carboxamido]-1-propyl group is consistent with the participation of the amidic carbonyl group in an intramolecular cyclodeesterification reaction resulting in the release of a phosphate/thiophosphate diester as its 2-*tert*-butyliminotetrahydrofuran salt (*UNIT 3.9*; Wilk et al., 2002). The spatial arrangement of the amidic carbonyl group relative to the phosphate/thiophosphate leaving group is crucial in that it determines the thermolytic deprotection kinetics of the protecting group.

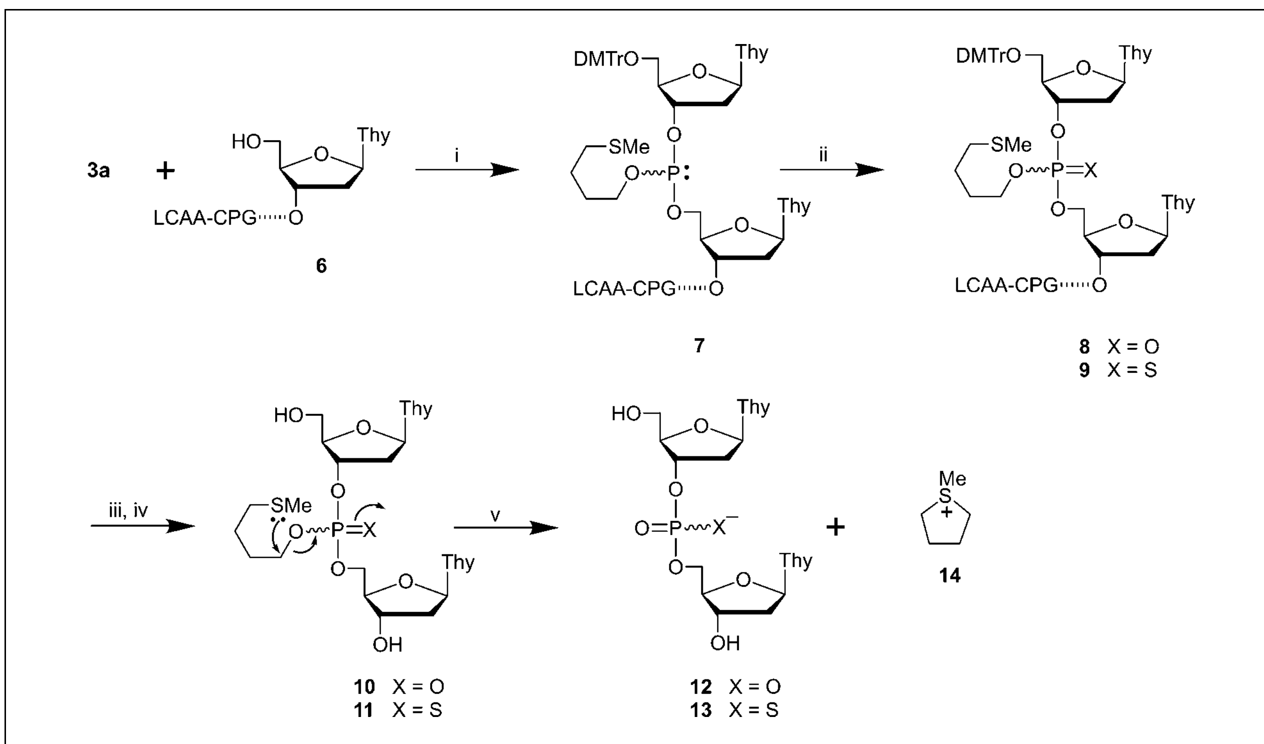

Figure 3.11.7 Proposed mechanism for the cleavage of the 4-methylthio-1-butyl group under thermolytic conditions at neutral pH. Reagents and conditions: (i) 0.45 M 1*H*-tetrazole in MeCN, 1 min; (ii) 0.02 M I_2 in THF/pyridine/water or 0.05 M 3*H*-benzodithiol-3-one-1,1-dioxide in MeCN, 3 min; (iii) 3% TCA in CH_2Cl_2, 1 min; (iv) $MeNH_2$ gas (2.5 bar), 3 min; (v) 0.1 M TEAA, pH 7.0, 55°C, 30 min. LCAA-CPG, succinyl long-chain alkylamine controlled-pore glass; TCA, trichloroacetic acid; Thy, thymin-1-yl; TEAA, triethylammonium acetate; THF, tetrahydrofuran. Adapted and reprinted from Cieślak et al. (2004) with permission from the American Chemical Society.

Indeed, the thermolytic deprotection of the homologous 3-[(*N-tert*-butyl)carboxamido]-1-butyl and 2-(*N-tert*-butylcarboxamido)ethyl thiophosphate-protecting group is, respectively, 25-fold and >2000-fold slower than that of the 3-[(*N-tert*-butyl)carboxamido]-1-propyl group under identical conditions (Wilk et al., 2002). Thus, it is critically important to consider the spatial relationship between the nucleophilic function and the phosphate/thiophosphate leaving group when designing novel thermolytic groups.

These findings prompted the search for phosphate/thiophosphate-protecting groups that would be removed under milder thermolytic conditions in either the presence or absence of ammonium hydroxide. The selection of the 4-methythio-1-butyl group was based on its structural simplicity and its potential compliance with the spatial requirements for optimal thermolytic deprotection kinetics, assuming an intramolecular cyclodeesterification process. The preparation of deoxyribonucleoside phosphoramidites functionalized with the 4-methylthio-1-butyl group for P(III) protection (**S.3a-d**) began with the synthesis of the phosphinylating reagent **S.2**, which was achieved upon reaction of the chlorophosphine **S.4** with commercial 4-methylthio-1-butanol (**S.5**; see Support Protocol). Condensation of commercial nucleosides (**S.1a-d**) with crude **S.2** in the presence of 1*H*-tetrazole afforded the deoxyribonucleosides phosphoramidites **S.3a-d** (see Basic Protocol).

The thermolytic properties of the 4-methylthio-1-butyl group were evaluated using a dinucleotide model. Specifically, the reaction of phosphoramidite **S.3a** with thymidine covalently attached to long-chain alkylamine controlled-pore glass (LCAA-CPG, **S.6**) and 1*H*-tetrazole gave the dinucleoside phosphite triester **S.7** (Fig. 3.11.7). Exposure of **S.7** to an iodine solution or to 3*H*-1,2-benzodithiol-3-one-1,1-dioxide in MeCN produced the support-bound dinucleoside phosphotriester **S.8** or **S.9**, respectively (Cieślak et al., 2004). Removal of the 5′-*O*-DMTr group under acidic conditions followed by release of the dinucleotide from LCAA-CPG upon treatment with methylamine gas afforded **S.10** or **S.11** as the major product. RP-HPLC analysis of crude **S.10** revealed the presence of the corresponding dinucleoside phosphodiester **S.12**

to the extent of ∼10%, which is indicative of the facile removal of the 4-methylthio-1-butyl phosphate protecting group. Heating either **S.10** or **S.11** in 0.1 M triethylammonium acetate (pH 7.0) for 30 min at 55°C, resulted in complete cleavage of the 4-methylthio-1-butyl group to give the dinucleoside phosphodiester **S.12** or **S.13** in near quantitative yield (>99%) on the basis of RP-HPLC analysis of the thermolytic deprotection reaction (Cieślak et al., 2004). Alternatively, treatment of **S.8** or **S.9** with concentrated NH_4OH for 30 min at room temperature, followed by heating the ammoniacal solution for 2 hr at 55°C, produced comparable results.

As shown in Figure 3.11.7, the thermolytic cleavage of the 4-methylthio-1-butyl group from **S.10** or **S.11** proceeds through an intramolecular cyclodeesterification reaction with the production of a sulfonium salt (**S.14**). To confirm the formation of **S.14**, the solid-phase-linked dinucleoside phosphotriester **S.8** was suspended in 0.1 M NaCl in D_2O and heated for 30 min at 55°C. Analysis of the supernatant by ^1H NMR spectroscopy (Fig. 3.11.8A) revealed signals identical to those recorded for **S.14** (Fig. 3.11.8B), which was also prepared as its chloride salt from the reaction of tetrahydrothiophene with methyl chloroformate (Byrne and Lafleur Lawter, 1986). These data convincingly demonstrate the participation of the 4-methylthio function in the thermolytic phosphate/thiophosphate deprotection of **S.10** or **S.11** (Cieślak et al., 2004).

The use of the 4-methylthio-1-butyl group for phosphate protection in solid-phase oligonucleotide synthesis was adequate only if the conventional 0.02 M iodine solution in THF/pyridine/H_2O was selected as the oxidant for iteratively converting phosphite to phosphate triester throughout oligonucleotide synthesis. The use of *tert*-butyl hydroperoxide or (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine instead of iodine resulted in the partial conversion of the 4-methylthio-1-butyl group to its 4-methylsulfinyl-1-butyl congener, which could

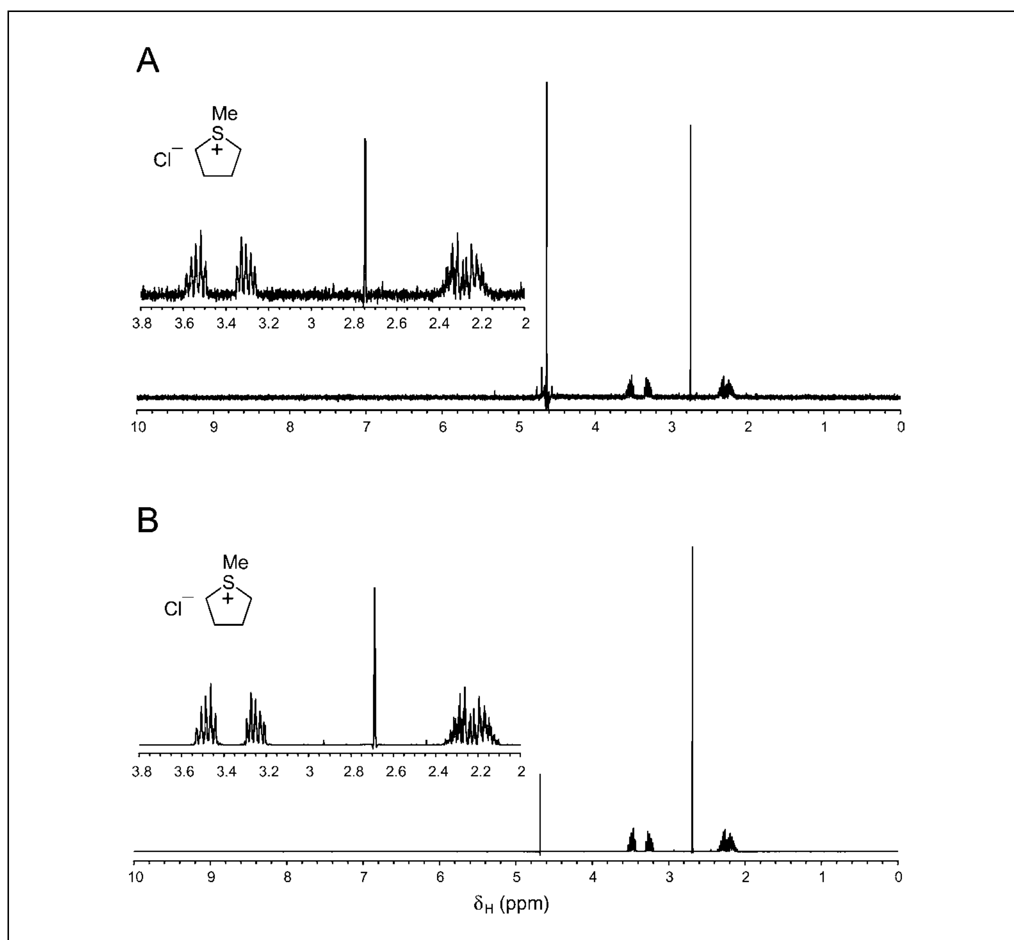

Figure 3.11.8 300 MHz ^1H NMR spectra of **S.14** (as its chloride salt in D_2O). (**A**) **S.14** produced by thermolytic deprotection of **S.8** in D_2O containing 0.1 M NaCl. (**B**) **S.14** prepared by reaction of tetrahydrothiophene with methyl chloroformate. Adapted and reprinted from Cieślak et al. (2004) with permission from the American Chemical Society.

not be thermolytically cleaved from the parent dinucleoside phosphotriester even upon heating for 3 hr at 90°C in a neutral buffer (Cieślak et al., 2004). These results further validate the participation of the 4-methylthio function in the thermolytic phosphate/thiophosphate deprotection of **S.10** or **S.11** through an intramolecular cyclodeesterification process.

The deoxyribonucleoside phosphoramidites **S.3a-d** were successfully used in the small scale (0.2-μmol) solid-phase synthesis of d(ATCCGTAGCTAAGGTCATGC) and its phosphorothioated analog. Following release from the CPG support, the thermolytic cleavage of the 4-methylthio-1-butyl protecting group from crude oligonucleotides was performed along with complete removal of nucleobase-protecting groups by treatment with concentrated NH_4OH for 10 hr at 55°C. Neither nucleobase alkylation (see Fig. 3.11.4) nor significant phosphorothioate desulfurization were detected under these conditions.

To further substantiate the absence of nucleobase alkylation under conditions that would simulate millimole-scale oligonucleotide deprotection, deoxyribonucleosides and their *N*-protected counterparts were heated in concentrated NH_4OH for 10 hr at 55°C in the presence or absence of a representative concentration of the sulfonium salt **S.14** (Cieślak et al., 2004). RP-HPLC analyses of each reaction mixture did not show significant nucleobase modifications under these conditions (Fig. 3.11.9). Exposure of *O*,*O*-diethyl thiophosphate to **S.14** under concentration conditions similar to those prevailing in large-scale oligonucleotide synthesis did not show, on the basis of ^{31}P NMR data, desulfurization of the phosphorothioate model in concentrated NH_4OH over a period of 10 hr at 55°C. Typically, no conversion of *O*,*O*-diethyl thiophosphate (δ_P ∼55 ppm) to *O*,*O*-diethyl phosphate (δ_P ∼2 ppm) was detected (see Fig. 3.11.10). Altogether, the results reported therein indicate that the 4-methythio-1-butyl group is suitable for the large-scale preparation of therapeutic oligonucleotides as well as for the microscale synthesis of oligonucleotides on diagnostic microarrays.

Critical Parameters and Troubleshooting

The phosphorodiamidite **S.2** is prepared using the bis(*N*,*N*-diisopropylamino)chlorophosphine intermediate **S.4** (see Support Protocol, Fig. 3.11.6) in a manner similar to that reported for *N*,*N*,*N*′,*N*′-tetraisopropyl-*O*-[3-(*N*-*tert*-butylcarboxamino)-1-propyl]phosphorodiamidite (*UNIT 3.9*, Figure 3.9.5). Readers are referred to the Critical Parameters and Troubleshooting section of *UNIT 2.7*, which addresses issues pertaining to the preparation of phosphorodiamidites. The formation of **S.4** must be carefully monitored by ^{31}P NMR spectroscopy to ensure complete exhaustion of dichloro(*N*,*N*-diisopropylamino)phosphine

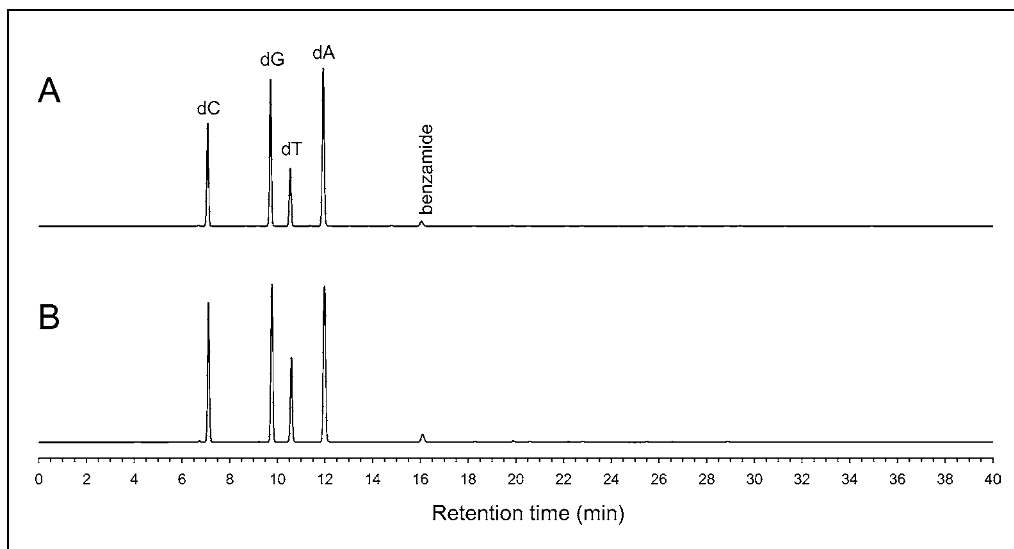

Figure 3.11.9 RP-HPLC analysis of the DNA-modifying properties of **S.14**. (**A**) Chromatogram of the reaction of T, dC, dA, dG, N^4-Bz-dC, N^6-Bz-dA, and N^2-*i*-Bu-dG with **S.14** in NH_4OH under conditions simulating large-scale oligonucleotide deprotection. (**B**) Chromatogram of an identical reaction in the absence of **S.14**. Chromatographic conditions for RP-HPLC analyses are as described in *UNIT 3.9*. Adapted and reprinted from Cieślak et al. (2004) with permission from the American Chemical Society.

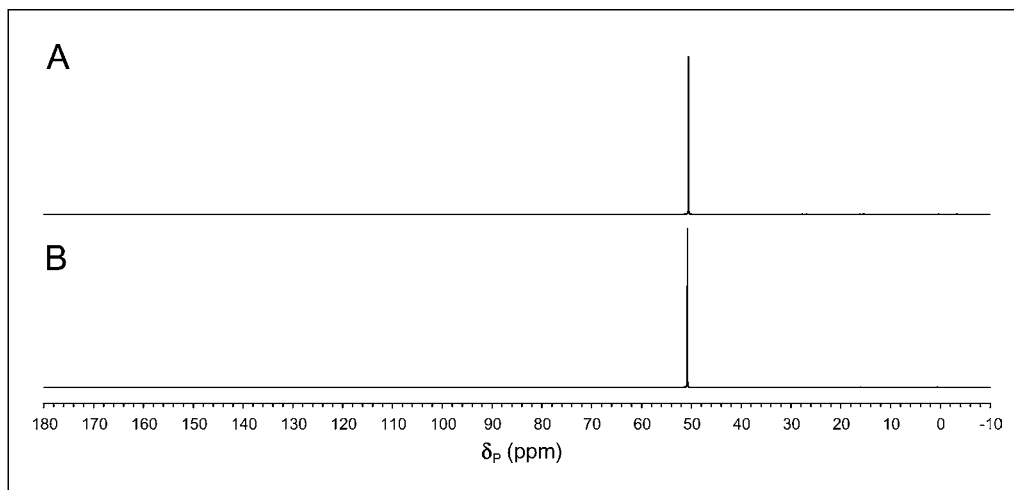

Figure 3.11.10 ^{31}P NMR analysis (121 MHz) of the phosphorothioate desulfurization properties of **S.14**. (**A**) Reaction of *O,O*-diethyl thiophosphate (potassium salt) with **S.14** under conditions simulating large-scale oligonucleotide deprotection in concentrated NH_4OH. (**B**) Potassium salt of *O,O*-diethyl thiophosphate in concentrated NH_4OH. Adapted and reprinted from Cieślak et al. (2004) with permission from the American Chemical Society.

(δ_P ~169 ppm in C_6D_6) prior to adding 4-methylthio-1-butanol.

It is recommended that the deoxyribonucleoside phosphoramidites **S.3a-d** be precipitated from cold hexane following silica gel purification. This precipitation step removes a hydrolyzed phosphinylating agent that often co-elutes with the desired phosphoramidite monomers.

To ensure optimal coupling efficiency of individual deoxyribonucleoside phosphoramidites during solid-phase oligonucleotide synthesis, a number of parameters including exclusion of residual triethylamine and moisture must be taken into consideration. These issues are also addressed in *UNIT 2.7*.

When using **S.3a-d** in solid-phase oligonucleotide synthesis, oxidants such as *tert*-butyl hydroperoxide and (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine should be avoided during chain assembly.

Anticipated Results

The synthesis of deoxyribonucleoside phosphoramidites **S.3a-d** from phosphorodiamidite **S.2** is performed essentially as recommended by Barone et al. (1984). The coupling efficiency of silica gel–purified and triethylamine-free phosphoramidites **S.3a-d** is comparable to that of commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites.

Time Considerations

The preparation of the phosphorodiamidite **S.2** takes 3 to 4 days, whereas synthesis and purification of each of the deoxyribonucleoside phosphoramidites **S.3a-d** require 1 to 2 days to complete.

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Contributed by Jacek Cieślak, Andrzej Grajkowski, and Serge L. Beaucage
Food and Drug Administration
Bethesda, Maryland

Victor Livengood
National Institutes of Health
Bethesda, Maryland