# Modification of the 5′ Terminus of Oligodeoxyribonucleotides for Conjugation with Ligands

The chemical attachment of reporter and various conjugate groups (e.g., intercalators as well as photoreactive and cleaving agents) to the 5′ terminus of oligodeoxyribonucleotides can be achieved following two strategies. The first involves the direct addition of a ligand to the 5′ end of the oligodeoxyribonucleotide via its phosphoramidite or *H*-phosphonate derivative. This strategy has been described in *UNIT 4.3*. The second strategy involves the incorporation of appropriate functional groups into both the ligand and the 5′ terminus of an oligonucleotide. Specific coupling of these reactants results in the formation of oligodeoxyribonucleotide conjugates. Using the second strategy, many different oligodeoxyribonucleotide conjugates can be prepared starting from only one oligodeoxyribonucleotide, provided that the amount required for each conjugate is low. In this approach, functional groups (such as amino, phosphate, phosphorothioate, thiol, carboxyl, and *cis*-diol) that are capable of undergoing specific reactions with selected ligand functional groups are added to the 5′ end of oligodeoxyribonucleotides. Examples of incorporation of halogenoalkyl, 2-pyridyldithioalkyl, or isothiocyanate linkers into ligands have been reported in *UNIT 4.8*. This unit describes methods used in the authors' laboratory for the preparation of oligodeoxyribonucleotides carrying the following 5′-terminal functional groups: carboxyl and amino (see Basic Protocol 1), phosphorothioate and phosphate (see Basic Protocol 2), and masked thiol (see Basic Protocol 3; see Alternate Protocols 1 and 2). The functional groups are incorporated upon completion of oligonucleotide chain assembly prior to deprotection and purification. Another strategy—adding functional groups after deprotection and purification—has been described elsewhere (Grimm et al., 2000). The coupling of these functionalized oligonucleotides with the reactive functions incorporated into the ligands reported in *UNIT 4.8*, and finally the purification and characterization of the oligodeoxyribonucleotide-ligand conjugates, is described in *UNIT 4.10*.

*CAUTION*: All chemicals must be handled in a fume hood by personnel equipped with a laboratory coat, glasses, and gloves.

*NOTE*: For each family of 5′-oligonucleotide modifications, a sample of detritylated oligodeoxyribonucleotide bound to the support is deprotected and purified separately using the general conditions described in *APPENDIX 3C*. These samples are used as references for chromatography and polyacrylamide gel electrophoresis analyses.

## ADDITION OF A CARBOXYLATED OR AMINOALKYLATED LINKER TO THE 5′ END OF OLIGODEOXYRIBONUCLEOTIDES

*BASIC PROTOCOL 1*

The addition of carboxylated or aminoalkylated linkers at the 5′ end of oligodeoxyribonucleotides is achieved via a two-step procedure adapted from Gottikh et al. (1990) and Wachter et al. (1986), respectively. The different steps involved are illustrated in Figure 4.9.1. In these procedures, the 5′-hydroxyl group of an oligonucleotide bound to a support (**S.1**) is activated by treatment with carbonyldiimidazole followed by reaction with either an amino acid (aminovaleric acid or aminocaproic acid) or a bis amine (1,5-diaminopentane or 1,6-diaminohexane). After deprotection and cleavage from the support, the oligodeoxyribonucleotides containing carboxylated or aminoalkylated linkers (**S.3** and **S.2**, respectively) are purified by chromatography.

**Contributed by Ulysse Asseline and Nguyen T. Thuong**
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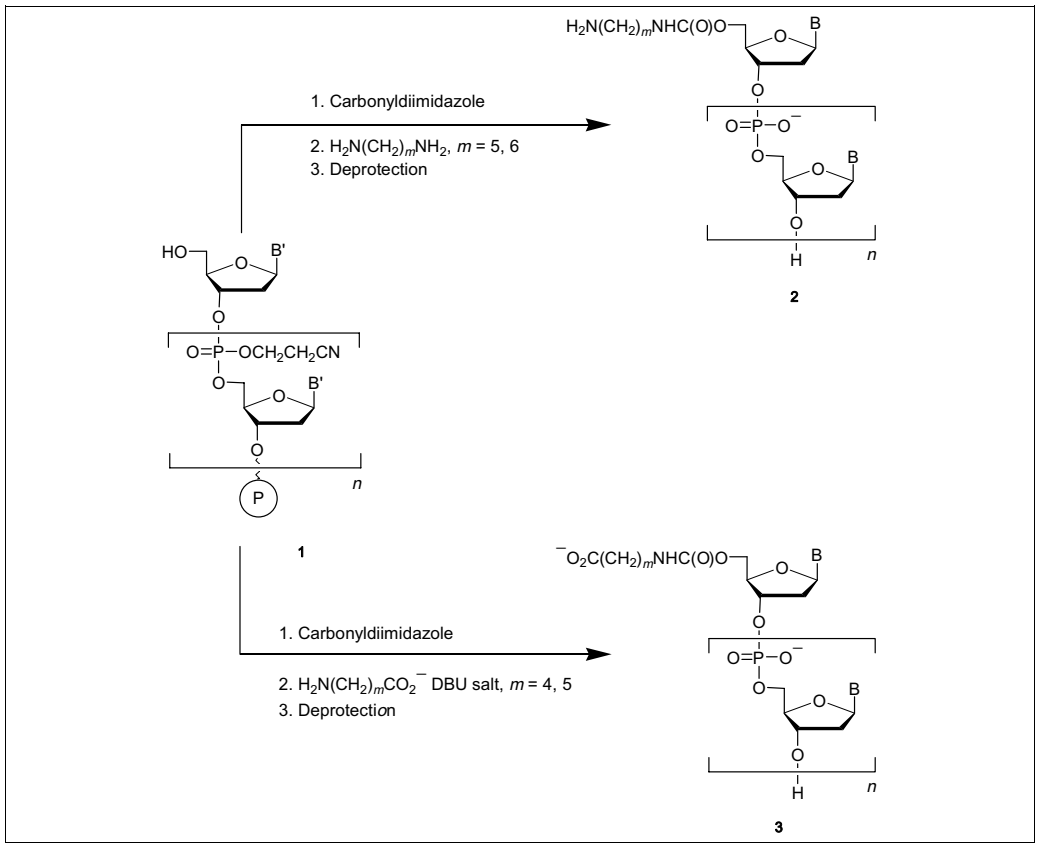


**Figure 4.9.1** Addition of a carboxyl or amino function to the 5′ end of an oligodeoxyribonucleotide. B, adenine, cytosine, guanine, or thymine; B′, thymine or any *N*-protected nucleobase; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; P, controlled-pore glass.

### *Materials*

5′-Detritylated oligodeoxyribonucleotide bound to a controlled-pore glass (CPG) support
Nitrogen gas
40 mg/mL anhydrous 1,1′-carbonyldiimidazole in anhydrous dioxane
Anhydrous dioxane
Amino acid or bis amine (select one):
- 10 mg/mL 5-aminovaleric acid or 6-aminocaproic acid, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) salt (Aldrich), in pyridine
- 12 mg/mL 1,5-diaminopentane or 1,6-diaminopentane in pyridine

Pyridine, redistilled from *p*-toluenesulfonyl chloride, stored over 3A molecular sieves
Acetonitrile ($CH_3CN$), DNA synthesis grade, stored over 3A molecular sieves, and HPLC grade
Concentrated ammonium hydroxide (25%)
Ethyl acetate, distilled
1.5 M NaCl
25 mM Tris·Cl, pH 7 (*APPENDIX 2A*), containing 10% (v/v) distilled HPLC-grade methanol
1 M triethylammonium acetate (TEAA) buffer, pH 7 (stock solution)

8-mL vial with septum and screw cap
22-G hypodermic needle
Desiccator containing $P_2O_5$ and KOH
50° and 55°C ovens or water baths

0.45-µm filter attached to a disposable syringe
UV spectrophotometer
Rotary evaporator with water bath and a water aspirator
Ion-exchange chromatography system and column (select one):
Mono Q HR 5/5 or HR 10/10 column (Amersham Pharmacia Biotech)
DEAE column (8 µm, 100 × 10 mm; Waters)
High-performance liquid chromatograph (HPLC) equipped with multiwavelength detector and reversed-phase column (select one):
Lichrospher 100 RP 18 column (5 µm; 125 mm × 4 mm; Merck)
Lichrospher 100 RP 18 column (10 µm; 250 mm × 10 mm; Merck)
Delta-Pak $C_4$ column (5 µm, 100 Å; 150 × 3.9 mm; Waters)
Lyophilizer

Additional reagents and equipment for analytical and preparative ion-exchange chromatography and reversed-phase HPLC (RP-HPLC), and for sample purification (*UNIT 4.3*)

***Activate 5′-hydroxyl group***

1. Place 1 µmol of 5′-detritylated oligodeoxyribonucleotide bound to a CPG support in an 8-mL vial with a septum. Push a 22-G hypodermic needle through the septum and place the vial in a desiccator containing $P_2O_5$ and KOH. Keep under vacuum overnight.
2. Fill the desiccator with nitrogen before opening it.
3. Add 500 µL of 40 mg/mL anhydrous 1,1′-carbonyldiimidazole in anhydrous dioxane under nitrogen and allow to react for 25 min with occasional shaking.
4. Remove excess 1,1′-carbonyldiimidazole solution with a syringe and wash the support with 1 mL anhydrous dioxane.

***Add linker***

5. Add 500 µL of one of the following solutions and incubate 3 to 4 hr with occasional shaking by hand.

   10 mg/mL 5-aminovaleric acid or 6-aminocaproic acid (DBU salt) in pyridine (for **S.3**)
   12 mg/mL 1,5-diaminopentane or 1,6-diaminopentane in pyridine (for **S.2**).

***Deprotect and remove from support***

6. Remove liquid with a syringe. Wash support twice with 1 mL of 2:1 (v/v) pyridine in DNA synthesis–grade $CH_3CN$, and twice with 1 mL $CH_3CN$.
7. Add 5 mL concentrated ammonium hydroxide, seal the vial with a screw cap, and heat 1 hr at 50°C.
8. Cool the ammoniacal solution to room temperature.

   *The solution should be cooled before opening the vial for safety and to prevent spillage and loss of product.*

9. Discard the support, transfer the ammoniacal solution to a new vial, and incubate for an additional 6 hr at 55°C.
10. Cool solution to room temperature and evaporate to dryness under reduced pressure using a rotary evaporator with a water aspirator.

11. Solubilize crude material with 6 mL water and extract three times with 4 mL ethyl acetate. Discard the organic phase.

12. Filter solution using a 0.45-μm filter attached to a disposable syringe. Wash filter with 0.3 mL water and add wash to filtrate.

13. Dilute 25 μL crude deprotected oligonucleotide solution with 975 μL water and record the UV spectrum between 220 and 400 nm using a UV spectrophotometer. Determine absorbance at 260 nm and multiply by 40 (the dilution factor) to determine the concentration of the crude oligonucleotide solution.

***Analyze quality of synthesis***

14. Analyze by ion-exchange chromatography and RP-HPLC. Set the instrument for detection at 260 nm. Preequilibrate columns with starting buffer for ≥15 min. Run a blank gradient before analysis and purification, and between analyses and purifications of samples with different sequences or different modifications. Analyze the unmodified oligonucleotide sequence for comparison.

    a. *For ion-exchange chromatography:* Use a Mono Q HR 5/5 or HR 10/10 column or a DEAE column with a linear gradient of 1.5 M NaCl (0% to 100%) in 25 mM Tris·Cl, pH 7, containing 10% (v/v) methanol. Elute at a flow rate of 1 mL/min with an HR 5/5 column or DEAE column, or at 4 mL/min with an HR 10/10 column.

    b. *For RP-HPLC:* Use a Lichrospher 100 RP 18 column with a linear gradient of HPLC-grade $CH_3CN$ (5% to 80%) in 0.1 M TEAA buffer, pH 7, at a flow rate of 1 mL/min. Alternatively, use a semipreparative Lichrospher 100 RP 18 column at a flow rate of 4 mL/min, or a Delta-Pak $C_4$ column at a flow rate of 0.8 mL/min.

    *The gradients must be chosen to afford the best separation. Usually 1 M NaCl is increased at 1% per minute for ion-exchange chromatography, and $CH_3CN$ is increased at 0.5% to 1% per minute for RP-HPLC.*

    *Characterization data can be found following step 17.*

***Purify sample***

15. Using the system determined to give the best separation, purify the remaining crude 5′-modified oligonucleotide by preparative reversed-phase or ion-exchange chromatography.

    *Because preparative chromatography columns do not have the resolution of analytical columns, the product peak should be fractionated to obtain good purification.*

16. Proceed with sample purification as reported in *UNIT 4.3* (steps 3 to 8 of the Support Protocol). Measure the $OD_{260}$ to determine the yield of 5′-modified oligonucleotide.

17. Lyophilize the modified oligonucleotide and store up to two years at –20°C in a tightly sealed vial.

    ***Characterization of carboxylated oligodeoxyribonucleotides:***

    *Yield: 25 $OD_{260}$ units.*

    *The best separation was obtained by ion-exchange chromatography on a Mono Q column (HR 50 × 5 mm) using a linear gradient of NaCl (0.2 to 0.5 M over 40 min) in 25 mM Tris·Cl, pH 7, containing 10% methanol at a flow rate of 1 mL/min. Retention times were 22 min for $^-OOC(CH_2)_4$-NH-CO-d[CTCTCGCACCCATCTCTC] and $^-OOC(CH_2)_5$-NH-CO-d[CTCTCGCACCCATCTCTC], and 21 min for d[CTCTCGCACCCATCTCTC]. Ion-exchange analysis of a crude oligonucleotide derivatized with aminovaleric acid is shown in Figure 4.9.2.*

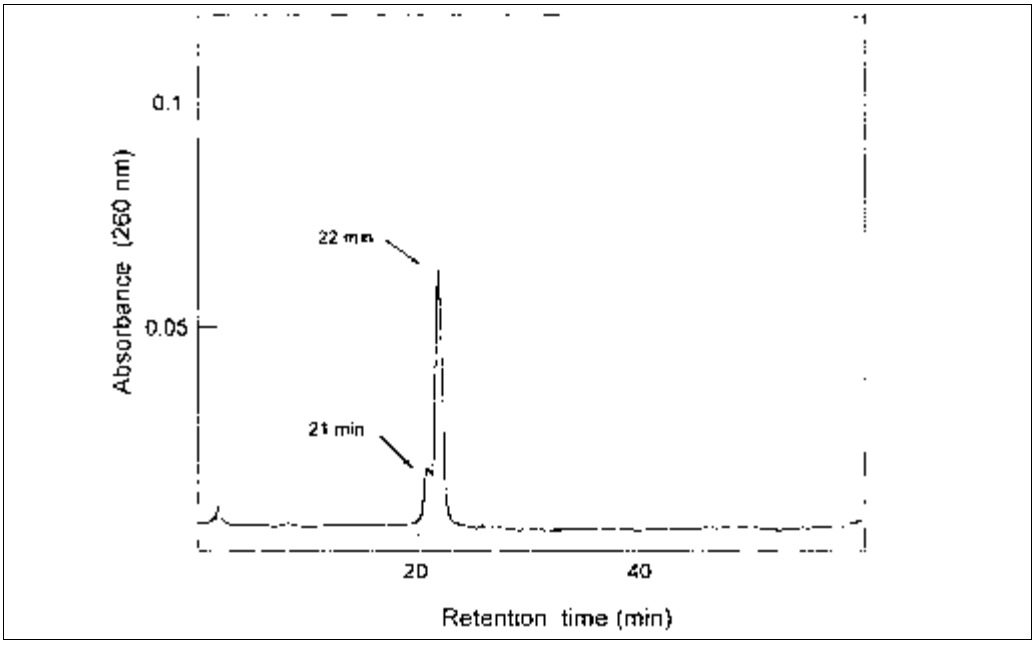


**Figure 4.9.2** Ion-exchange chromatography analysis of crude $HOOC(CH_2)_4$-NH-CO-d[CTCTCGCACCCATCTCTC] (see Basic Protocol 1) on a Mono Q column.

*RP-HPLC of carboxylated oligonucleotides on a Lichrospher 100 RP 18 (5 μm; 125 × 4 mm) column using a gradient of $CH_3CN$ (10% to 30% over 50 min) in 0.1 M TEAA buffer, pH 7, at 1 mL/min gives retention times of 11 min 25 sec for $^-OOC(CH_2)_4$-NH-CO-d[CTCTCGCACCCATCTCTC], 13 min 13 sec for $^-OOC(CH_2)_5$-NH-CO-d[CTCTCGCACCCATCTCTC], and 9 min 40 sec for d[CTCTCGCACCCATCTCTC].*

*RP-HPLC performed on a Delta-Pak $C_4$ (5 μm, 100 Å, 150 × 3.9 mm) column with a linear gradient of $CH_3CN$ (5% to 35% over 60 min) in 0.1 M TEAA buffer, pH 7, at 0.8 mL/min gives retention times of 12 min 10 sec for $^-OOC(CH_2)_5$-NH-CO-d[CTCTCGCACCCATCTCTC], and 11 min 8 sec for d[CTCTCGCACCCATCTCTC]. Figure 4.9.3 shows a chromatogram obtained for a mixture of purified $^-OOC(CH_2)_5$-NH-CO-d[CTCTCGCACCCATCTCTC] and d[CTCTCGCACCCATCTCTC].*

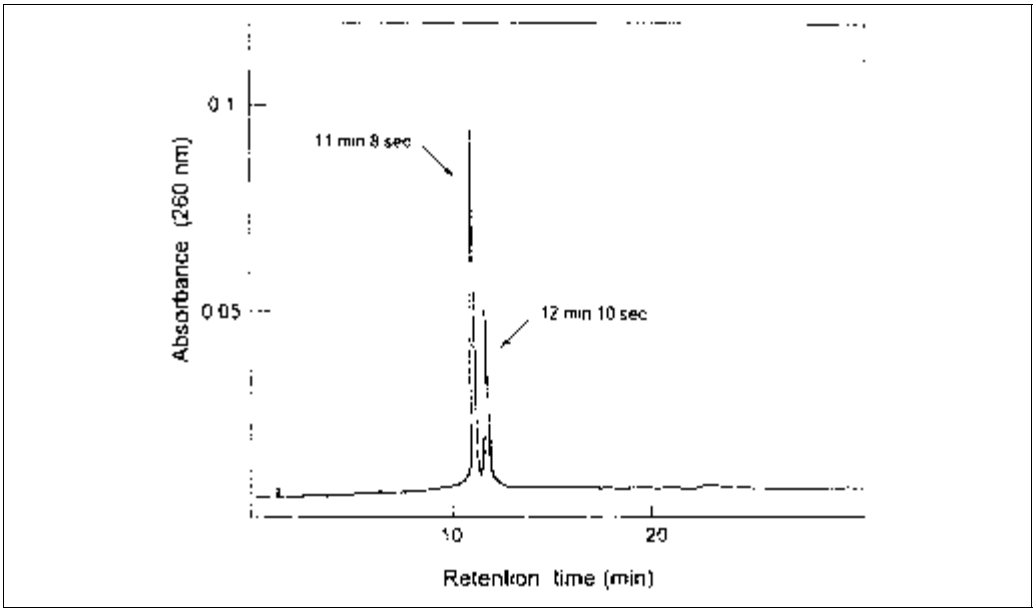


**Figure 4.9.3** RP-HPLC analysis on a Delta Pak $C_4$ column of a purified mixture of $HOOC(CH_2)_5$-NH-CO-d[CTCTCGCACCCATCTCTC] and d[CTCTCGCACCCATCTCTC] (see Basic Protocol 1).

*Mass analysis. ESI-MS polarity negative. $HOOC(CH_2)_5$-NH-CO-d[CTCTCGCACCCATCTCTC]. Calcd. for $C_{177}H_{233}N_{56}O_{112}P_{17}$: 5461 Da; found: 5460 ± 3 Da (M-H). $HOOC(CH_2)_4$-NH-CO-d[CTCTCGCACCCATCTCTC] Calcd. for $C_{176}H_{231}N_{56}O_{112}P_{17}$: 5447 Da; found: 5446 Da (M-H).*

*Denaturing 20% PAGE does not allow easy separation of 5′-modified oligonucleotides with carboxylated linkers from the corresponding unmodified oligonucleotide.*

***Characterization of aminoalkylated oligodeoxyribonucleotides:***

*Yield: 25-30 $OD_{260}$ units.*

*Ion-exchange chromatography of 5′-aminoalkylated oligonucleotides on a DEAE column (8 μm, 100 × 10 mm) using a linear gradient of NaCl (0 to 0.6 M over 60 min) in 25 mM Tris·Cl, pH 7, containing 10% methanol at 1 mL/min gives retention times of 44 min 54 sec for $H_2N(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA] and $H_2N(CH_2)_6$-NH-CO-d[CCGCTTAATACTGA], and 45 min 48 sec for d[CCGCTTAATACTGA]. Figure 4.9.4 shows a chromatogram obtained for crude $H_2N(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA].*

*RP-HPLC on a Lichrospher 100 RP 18 column using the conditions described above gives retention times of 10 min 25 sec for $H_2N(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA], 10 min 54 sec for $H_2N(CH_2)_6$-NH-CO-d[CCGCTTAATACTGA], and 9 min 42 sec for d[CCGCTTAATACTGA].*

*RP-HPLC on a Delta-Pak $C_4$ column (5 μm, 100 Å, 150 × 3.9 mm) with a linear gradient of $CH_3CN$ (5% to 35% over 60 min) in 0.1 M TEAA buffer, pH 7, at 0.8 mL/min gives retention times of 11 min 40 sec for $H_2N(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA], 12 min 21 sec for $H_2N(CH_2)_6$-NH-CO-d[CCGCTTAATACTGA], and 11 min 15 sec for d[CCGCTTAATACTGA]. Figure 4.9.5 shows a chromatogram for a mixture of purified $H_2N(CH_2)_6$-NH-CO-d[CCGCTTAATACTGA] and d[CCGCTTAATACTGA].*

*Denaturing 20% PAGE (APPENDIX 3B) allows easy separation of 5′-modified oligonucleotides with aminoalkylated linkers from the corresponding unmodified oligonucleotide (Fig. 4.9.6).*

*Mass analysis. ESI-MS polarity negative. $H_2N(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA]. Calcd. for $C_{142}H_{185}N_{52}O_{83}P_{13}$: 4351 Da; found: 4351 ± 1 Da (M-H). $H_2N(CH_2)_6$-NH-CO-d[CCGCTTAATACTGA]. Calcd. for $C_{143}H_{187}N_{52}O_{83}P_{13}$: 4365 Da; found: 4365 ± 1 Da (M-H).*

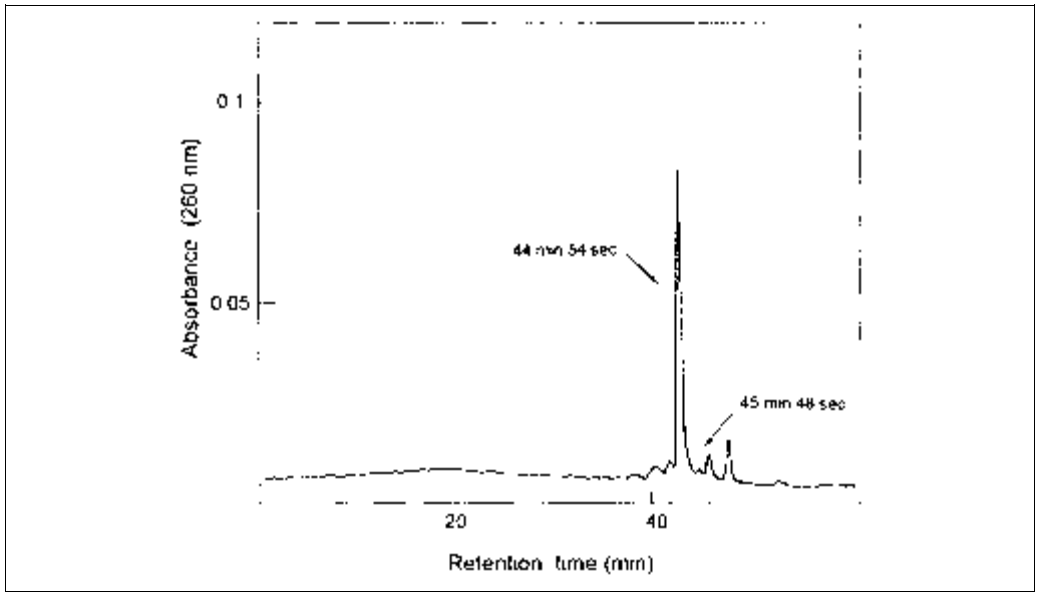


**Figure 4.9.4** Ion-exchange chromatography on a DEAE column of crude $H_2N$-$(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA] (see Basic Protocol 1).

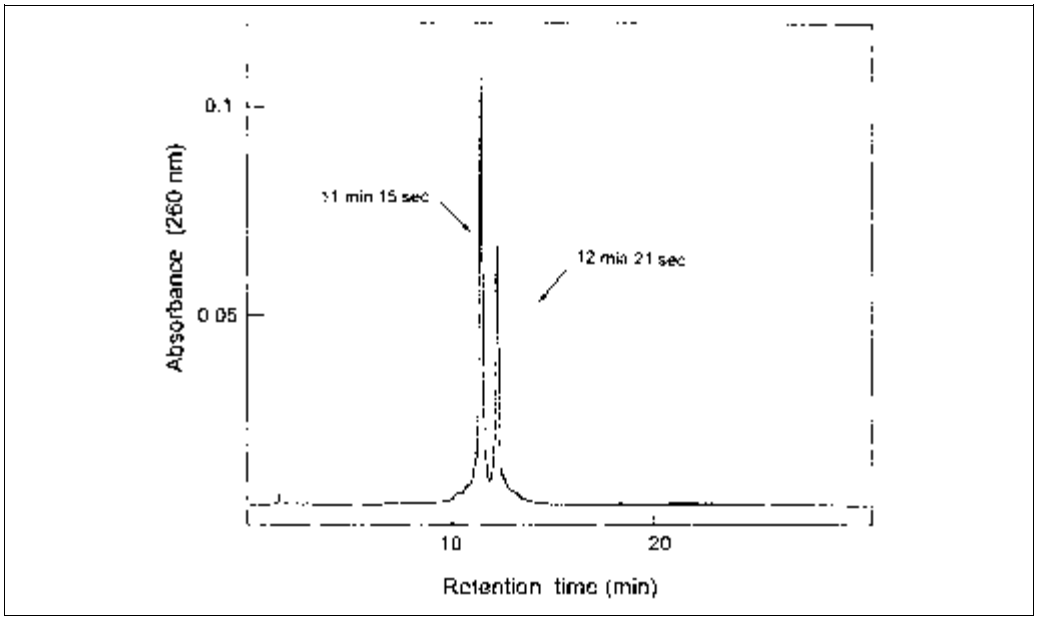


**Figure 4.9.5** RP-HPLC on a Delta Pak $C_4$ column of a purified mixture of $H_2N$-$(CH_2)_6$-NH-CO-d[CCGCTTAATACTGA] and d[CCGCTTAATACTGA] (see Basic Protocol 1).

## ADDITION OF A PHOSPHOROTHIOATE OR PHOSPHATE GROUP TO THE 5′ TERMINUS OF OLIGODEOXYRIBONUCLEOTIDES

*BASIC PROTOCOL 2*

The phosphorothioate group is incorporated at the 5′ end of oligodeoxyribonucleotides via a two-step procedure involving the coupling of bis-(2-cyanoethyl)-diisopropylamidophosphite with the 5′-terminal hydroxyl function of an oligodeoxyribonucleotide bound to a support (**S.4**), followed by a sulfurization step, as seen in the bottom of Figure 4.9.7. The phosphate group is obtained by replacing the sulfurization step with a standard aqueous iodine oxidization (top). After deprotection and cleavage from the support, the 5′-phosphorothioate-containing (**S.6**) or 5′-phosphate-containing (**S.5**) oligodeoxyribonucleotide is purified by liquid chromatography. The procedure was adapted from a previously described procedure (Thuong and Asseline, 1991).

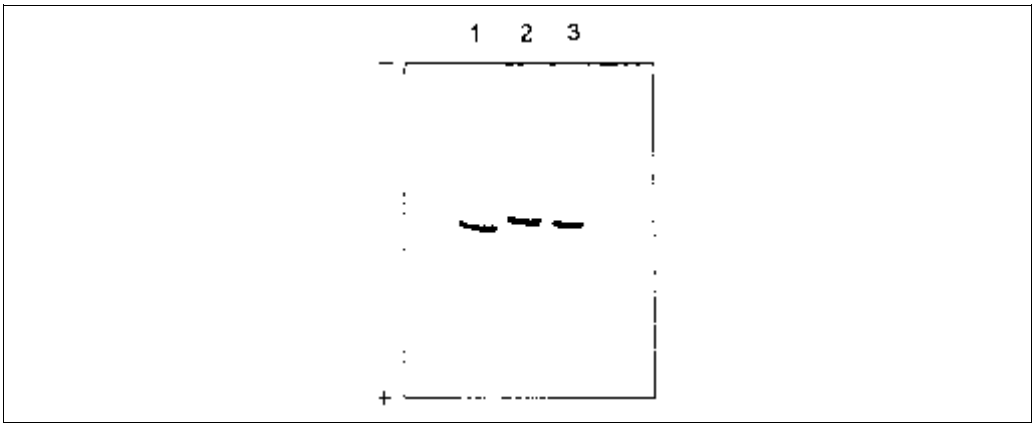


**Figure 4.9.6** PAGE analysis of d[CCGCTTAATACTGA] (lane 1), $H_2N$-$(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA] (lane 2), and $H_2N$-$(CH_2)_6$-NH-CO-d[CCGCTTAATACTGA] (lane 3; see Basic Protocol 1).

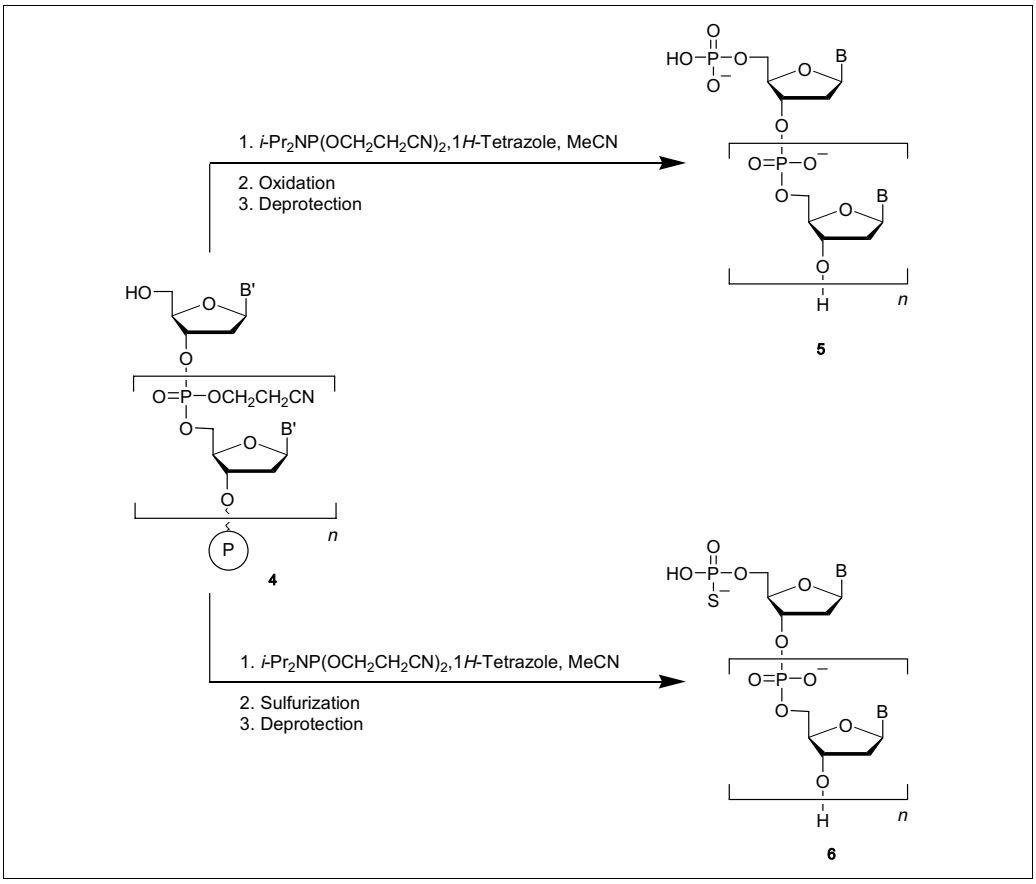


**Figure 4.9.7** Addition of a phosphorothioate or phosphate group to the 5′ end of an oligodeoxyribonucleotide.

### *Materials*

Diisopropylethylamine, distilled from KOH
2-Cyanoethanol
Diethyl ether dried over sodium wires
Nitrogen source
*N*,*N*′-Diisopropylphosphoramidous dichloride (Aldrich)
Argon atmosphere
5′-Detritylated oligodeoxyribonucleotide bound to a controlled-pore glass (CPG) support
0.5 M tetrazole in anhydrous $CH_3CN$
Anhydrous $CH_3CN$
10 mg/mL Beaucage reagent in anhydrous $CH_3CN$ *or* 100 mg/mL tetraethylthiuram disulfide in anhydrous $CH_3CN$ (optional; for sulfurization)
Iodine solution (same composition as for DNA synthesis; optional; for oxidation)
Concentrated ammonium hydroxide ($NH_4OH$; 25%)
Dithiothreitol (DTT; optional)
Ethyl acetate, distilled
Isopropanol
2.5 mg/mL 2,6-dibromo-4-benzoquinone-*N*-chloroimine (DBPNC; Prolabo) in ethanol

1-liter three-neck round-bottom flask
Dropping funnel
Reflux condenser with a calcium chloride drying tube

Gas inlet adapter
Glass filter (porosity 4)
Rotary evaporator with a water aspirator and a water bath
Falling-film distillation head (Aldrich) or other vacuum distillation apparatus
50° and 55°C ovens or water baths
Kieselgel 60F plates for analytical TLC (Merck)

Additional reagents and equipment for automated oligonucleotide synthesis (*APPENDIX 3C*), thin-layer chromatography (TLC; *APPENDIX 3D*), and purification and characterization of the product (see Basic Protocol 1)

***Prepare bis-(2-cyanoethyl)diisopropylamidophosphite***

1. Place 70 mL (51.6 g, 0.40 mol) diisopropylethylamine and 25.6 g (0.36 mol) 2-cyanoethanol in 300 mL dry diethyl ether in a three-neck 1-liter round-bottom flask equipped with a dropping funnel, a reflux condenser with a calcium chloride drying tube, and a gas inlet adapter connected to a nitrogen source. Cool to 0°C while stirring vigorously with a magnetic stir bar or by mechanical stirring.
2. Add 36 g (33.18 mL, 0.18 mol) *N*,*N*-diisopropylphosphoramidous dichloride to 50 mL dry diethyl ether. Add dropwise, under an argon atmosphere, to the solution in step 1 using a dropping funnel.
3. Allow the stirred mixture to warm to room temperature for 1 hr.
4. Remove the precipitated salt by vacuum filtration with a glass filter and wash it with 100 mL dry diethyl ether. Concentrate filtrate by evaporation under reduced pressure using a rotary evaporator with a water aspirator.
5. Purify the residue by vacuum distillation.

   *An apparatus can be fashioned in the laboratory, or a falling-film distillation head can be used.*

   *Yield 60% (29.3 g, 0.108 mol). $^{1}$H-NMR ($CDCl_3$), δ: 1.19 [d, 12H, J = 6.8 Hz, $(CH_3)_2CH$]; 2.64 [t, J = 6.2 Hz, $(OCH_2CH_2CN)$]; 3.55-3.68 [m, 2H, $(CH_3)_2CH$]; 3.78-3.94 [4H, m, $OCH_2CH_2CN$)]. $^{31}$P-NMR ($CDCl_3$), δ: 150.2 ppm. $^{13}$C-NMR (DMSO-$d_6$), δ: 25.25, 29.78, 48.06 (d, J = 36.5 Hz), 63.84 (d, J = 73 Hz), 124.36. Mass analysis. ESI-MS, polarity positive. Calcd. for $C_{12}H_{22}N_3O_2P$: 271; found: 272 (M+H).*

   *The purified product can be stored up to 1 year at –20°C without loss of coupling efficiency. However, when the reaction is performed on a small scale, bis-(2-cyanoethyl)diisopropyl-amidophosphite is purified by chromatography on a silica gel column using 4:1 (v/v) diethyl ether/hexane containing 2% to 3% triethylamine as the eluent, and the fractions are monitored by TLC using the same eluent (pre-elute the plate before analysis). After treating the plate with DBPNC (step 13), a pale-colored spot appears ($R_f$ = 0.6). When purified this way, the product shows loss of coupling efficiency after 3 to 4 months of storage.*

   *Bis-(2-cyanoethyl)diisopropylamidophosphite is now commercially available from Chemgenes.*

***Couple to oligonucleotide***

6. Place 0.1 M bis-(2-cyanoethyl)diisopropylamidophosphite in anhydrous $CH_3CN$ on the synthesizer. React a fully 5′-detritylated oligodeoxyribonucleotide bound to a CPG support for 5 min at a flow rate of 1 mL/min by recycling a mixture containing:

   0.1 mL 0.1 M phosphoramidite in anhydrous $CH_3CN$
   0.5 mL 0.5 M tetrazole in anhydrous $CH_3CN$
   0.1 mL $CH_3CN$.

Perform this reaction twice. For synthesizers without a recycle program, perform a double coupling step.

*Alternatively, bis-(2-cyanoethyl)diisopropylamidophosphite can be manually added to the 5′ terminus of oligonucleotides bound to a solid support according to the method described for the phosphoramidites 1c and 2d in UNIT 4.3.*

***Perform sulfurization/oxidation***

7a. *For sulfurization:* Treat product with 1 mL of 10 mg/mL Beaucage reagent in anhydrous $CH_3CN$ added at a rate of 1 mL/min, or with 15 mL of 100 mg/mL tetraethylthiuram disulfide in $CH_3CN$ added at 1 mL/min.

7b. *For oxidation:* Perform automated iodine oxidation step.

*Either the sulfurization or the oxidation step can be performed manually.*

***Deprotect and remove from support***

8. Add 5 mL concentrated ammonium hydroxide and 100 eq (15 mg) DTT to the oligodeoxyribonucleotide 5′-bis-(2-cyanoethyl)phosphorothioate and heat 1 hr at 55°C. For the oligodeoxyribonucleotide 5′-bis-(2-cyanoethyl)phosphate, omit the DTT.

*DTT is used to prevent formation of an acrylonitrile adduct on the thiophosphate group.*

9. Cool the ammoniacal solution to room temperature.

*The solution should be cooled before opening the vial for safety and to prevent spillage and loss of product.*

10. Discard the support, transfer the ammoniacal solution to a new vial, and incubate for an additional 7 hr at 50°C.

11. Cool the ammoniacal solution and evaporate it to dryness under reduced pressure using a rotary evaporator and a water aspirator.

12. Solubilize the crude material with 6 mL water and extract four times with 4 mL ethyl acetate for the 5′-phosphorothioate, or three times for the 5′-phosphate.

13. For medium-sized oligodeoxyribonucleotides (10- to 25-mers), verify the presence of the phosphorothioate group by analyzing the crude deprotected oligodeoxyribonucleotide by analytical TLC (APPENDIX 3D) using isopropanol/concentrated $NH_4OH/H_2O$ as eluent in varying proportions depending on the length and base composition of the oligodeoxyribonucleotide. Spray the plate with 2.5 mg/mL DBPNC in ethanol and heat until color appears.

*Typically, 65:9:15 (v/v/v) isopropanol/$NH_4OH/H_2O$ is suitable for ≤10-mers, and 55:10:25 or 55:10:35 is suitable for 10- to 20-mers. Phosphorothioate-containing oligodeoxyribonucleotides give a pink-colored spot on the TLC plate after treatment with DBPNC and heating. (Eluent mixture must be prepared at least a half-day before use.)*

***Purify and characterize***

14. Purify and characterize the 5′-phosphorothioate- or 5′-phosphate-containing oligodeoxyribonucleotide as described (see Basic Protocol 1, steps 12 to 17).

*Ion-exchange chromatography of 5′-phosphorothioate- and 5′-phosphate-containing oligodeoxyribonucleotides using the conditions described above with a DEAE column gives retention times of 49 min 24 sec for sp-d[CCGCTTAATACTGA] (53 min 36 sec for oligonucleotide disulfide), 47 min 36 sec for p-d[TTCTCCCCCGCTTA], 46 min 12 sec for d[TTCTCCCCCGCTTA], and 45 min 48 sec for d[CCGCTTAATACTGA]. Figure 4.9.8*

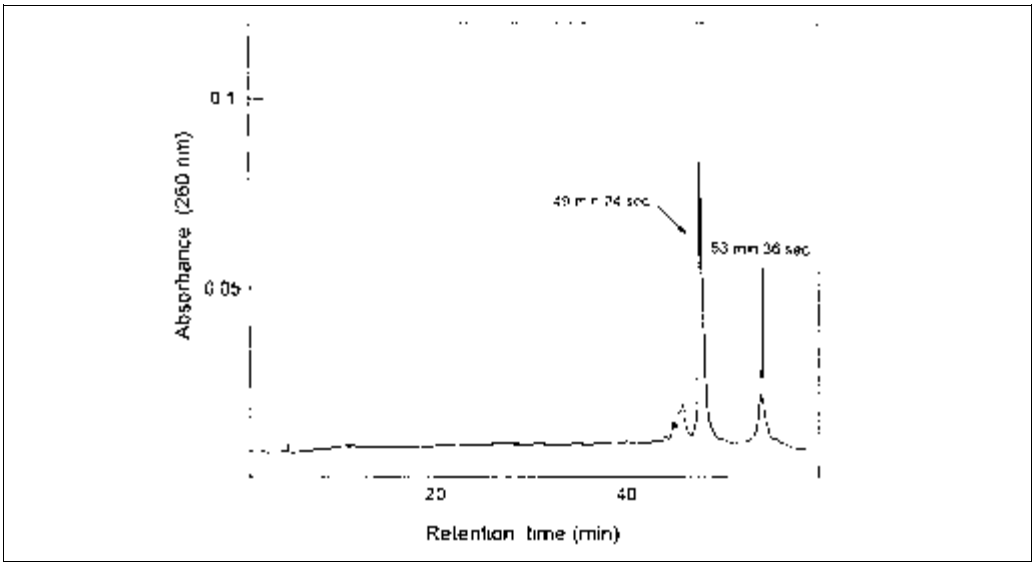


**Figure 4.9.8** Ion-exchange chromatography on a DEAE column of crude sp-d[CCGCTTAATACTGA] (see Basic Protocol 2).

*shows the chromatogram of crude sp-d[CCGCTTAATACTGA], and Figure 4.9.9 shows the chromatogram of crude p-d[TTCTCCCCCGCTTA].*

*Denaturing 20% PAGE does not allow easy separation of oligonucleotide 5′-thiophosphates and 5′-phosphates from the corresponding unmodified oligonucleotide. In the case of 5′-thiophosphate oligonucleotides, however, the presence of a weak band with a mobility corresponding to that of a 30-mer confirmed that the peak observed at 53 min 36 sec is probably the chimeric oligonucleotide disulfide 3′-[AGTCATAATTCGCC]-ps-sp-d[CCGCTTAATACTGA]-3′. The 5′-thiophosphate oligonucleotide can be recovered by treatment of the chimera with DTT.*

*RP-HPLC on a Lichrospher RP 18 column using the conditions described above gives retention times of 9 min for sp-d[CCGCTTAATACTGA], 9 min 42 sec for d[CCGCTTAATACTGA], 10 min 30 sec for p-d[TTCTCCCCCGCTTA], and 10 min 50 sec for d[TTCTCCCCCGCTTA].*

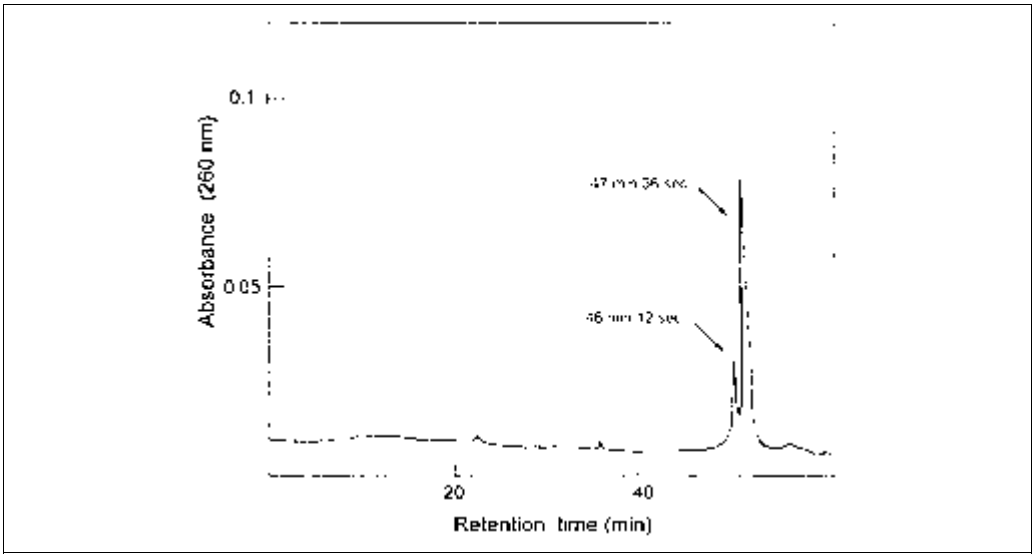


**Figure 4.9.9** Ion-exchange chromatography on a DEAE column of crude p-d[TTCTCCCCCGCTTA] (see Basic Protocol 2).

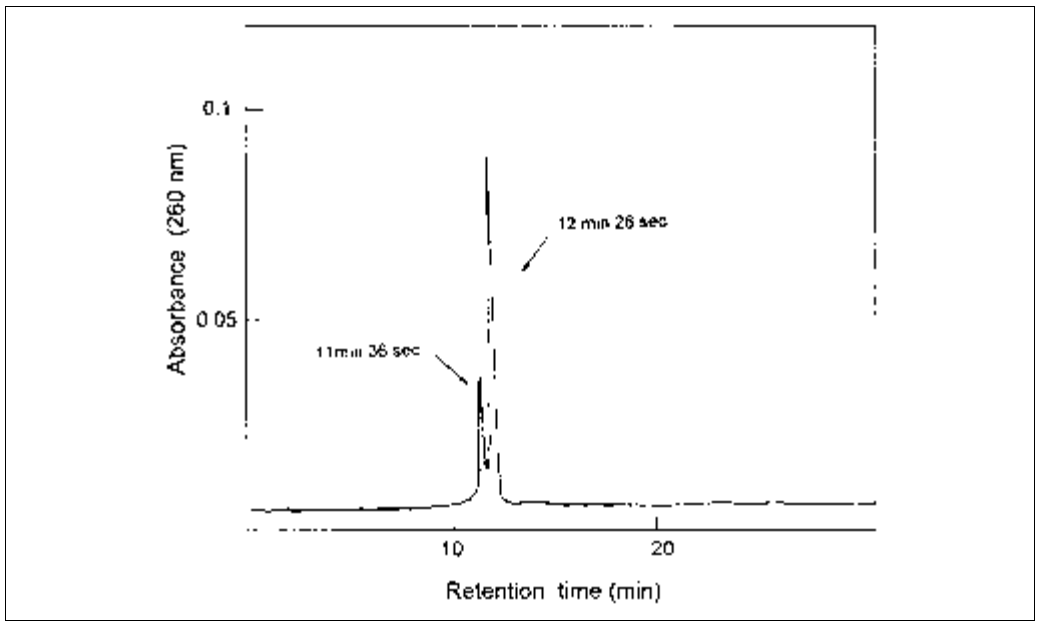


**Figure 4.9.10** RP-HPLC on a Delta Pak $C_4$ column of a purified mixture of p-d[TTCTCCCCCGCTTA] and d[TTCTCCCCCGCTTA] (see Basic Protocol 2).

*RP-HPLC on a Delta-Pak $C_4$ column (5 µm, 100 Å, 150 × 3.9 mm) using a linear gradient of $CH_3CN$ (5% to 16 % by volume over 30 min) in 0.1 M TEAA buffer, pH 7, at 0.8 mL/min gives retention times of 10 min 40 sec for sp-d[CCGCTTAATACTGA], 11 min 15 sec for d[CCGCTTAATACTGA], 11 min 36 sec for p-d[TTCTCCCCCGCTTA], and 12 min 26 sec for d[TTCTCCCCCGCTTA]. Figure 4.9.10 shows a chromatogram obtained for a mixture of purified p-d[TTCTCCCCCGCTTA] and d[TTCTCCCCCGCTTA].*

*Mass analysis. ESI-MS polarity negative. sp-d[CCGCTTAATACTGA]. Calcd. for $C_{136}H_{175}N_{50}O_{84}P_{14}S$: 4319 Da; found: 4318 Da (M-H). Yield: 35-40 $OD_{260}$ units.*

*Mass analysis. ESI-MS polarity negative. p-d[TTCTCCCCCGCTTA]. Calcd. for $C_{133}H_{176}N_{41}O_{89}P_{14}$: 4206 Da; found: 4205 ± 1 Da (M-H). Yield: 35-40 $OD_{260}$ units.*

***BASIC PROTOCOL 3***

## ADDITION OF A MASKED THIOL GROUP TO THE 5′ TERMINUS OF OLIGODEOXYRIBONUCLEOTIDES USING AN *S*-DIPHENYLPHOSPHINATE PHOSPHORAMIDITE

Two strategies can be used to add a masked thiol group to the 5′ end of an oligodeoxyribonucleotide. This protocol presents the first strategy, which is illustrated on the left of Figure 4.9.11. In this approach, an *S*-diphenylphosphinate phosphoramidite (**S.7a**) is added to the 5′ terminus of a support-bound oligodeoxyribonucleotide. This gives a 5′ thiol group masked with an alkali-labile protecting group (**S.7b**). Addition of 2,2′-dithiodipyridine during oligonucleotide deprotection converts the released thiol function to a 2-pyridyldisulfide group (**S.10**). This strategy can also be performed using an *S*-acetyl phosphoramidite (**S.8a**) as described in Alternate Protocol 1. The second strategy (right side of Fig. 4.9.11) uses a tritylated alkyl disulfide-containing linker (**S.9a**) and is described in Alternate Protocol 2.

**Modification of the 5′ Terminus of Oligodeoxyribonucleotides for Conjugation with Ligands**

The preparation of the *S*-diphenylphosphinate derivative **S.7a** is included in the steps below and is illustrated in Figure 4.9.12. The synthesis of **S.7a** is achieved in four steps from the commercial methyldiphenylphosphinite **S.11**. Sulfurization of **S.11** gives the methyldiphenylthionophosphinate **S.12**, which is then demethylated by treatment with trimethylamine. The resulting tetramethylammonium salt **S.13** reacts with 2-[2-(2-io-

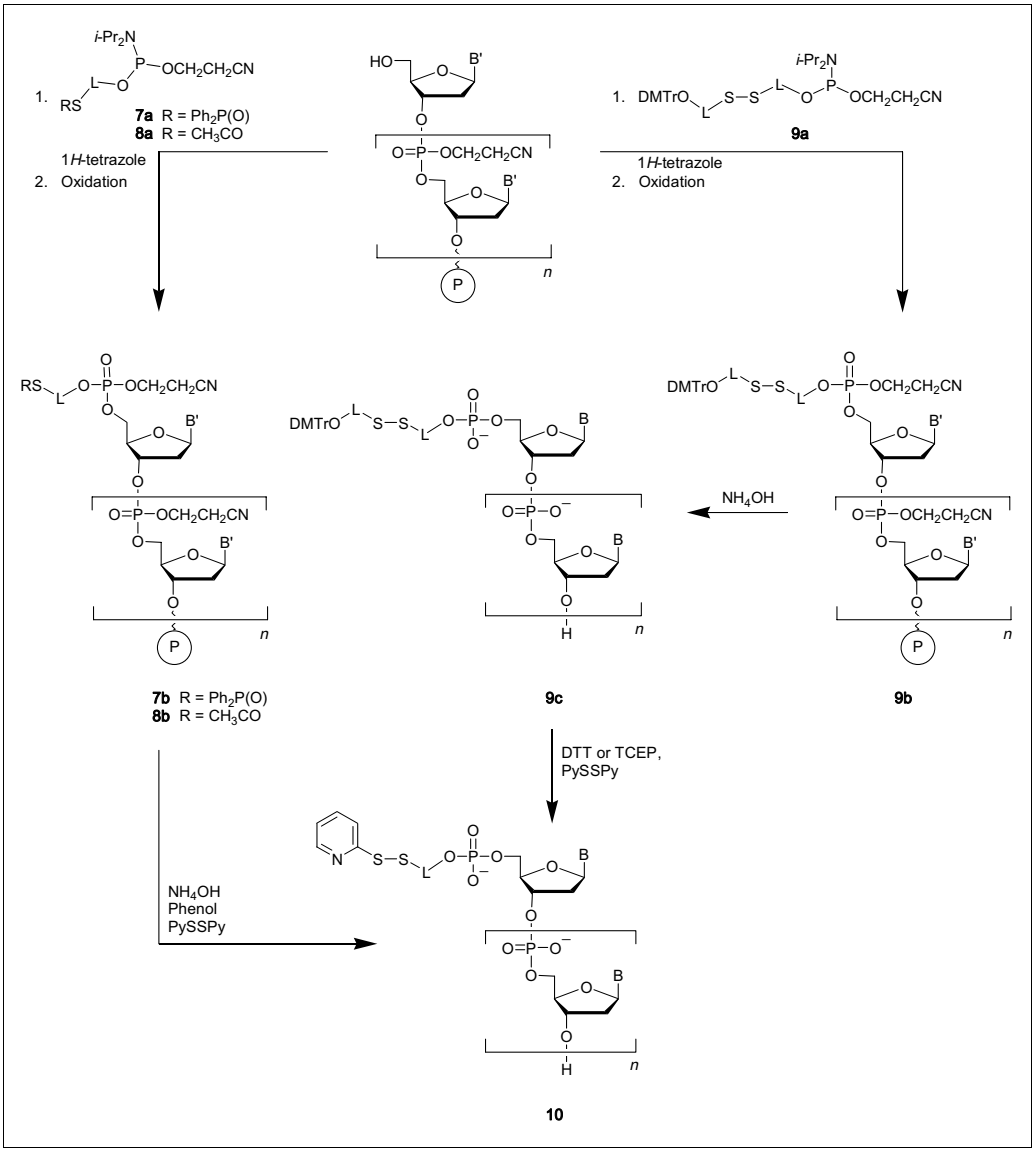


**Figure 4.9.11** Addition of a masked thiol group to the 5′ end of an oligodeoxyribonucleotide. DTT, dithiothreitol; L, $CH_2CH_2OCH_2CH_2OCH_2CH_2$; PySSPy, 2,2′-dipyridyldisulfide; TCEP, tris(2-carboxyethyl)phosphine hydrochloride.

doethoxy)ethoxy]ethanol to give 2-{2-[2-(*S*-diphenylphosphinate)ethoxy]ethoxy}ethanol **S.14**. 2-[2-(2-Iodoethoxy)ethoxy]ethanol is obtained by treatment of 2-[2-(2-chloroethoxy)ethoxy]ethanol (**S.15**; Fig. 4.9.16) with NaI in acetone in the presence of $NaHCO_3$. The alcohol **S.14** is then treated with diisopropylethylamine and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite to give the phosphoramidite **S.7a**.

### *Materials*

Methyldiphenylphosphinite (**S.11**; Aldrich)
Toluene
Elemental sulfur ($S_8$)
Hexane, distilled
Ethyl acetate, distilled
Triethylamine, distilled from KOH
2.5 mg/mL 2,6-dibromo-4-benzoquinone-*N*-chloroimine (DBPNC; Prolabo) in ethanol

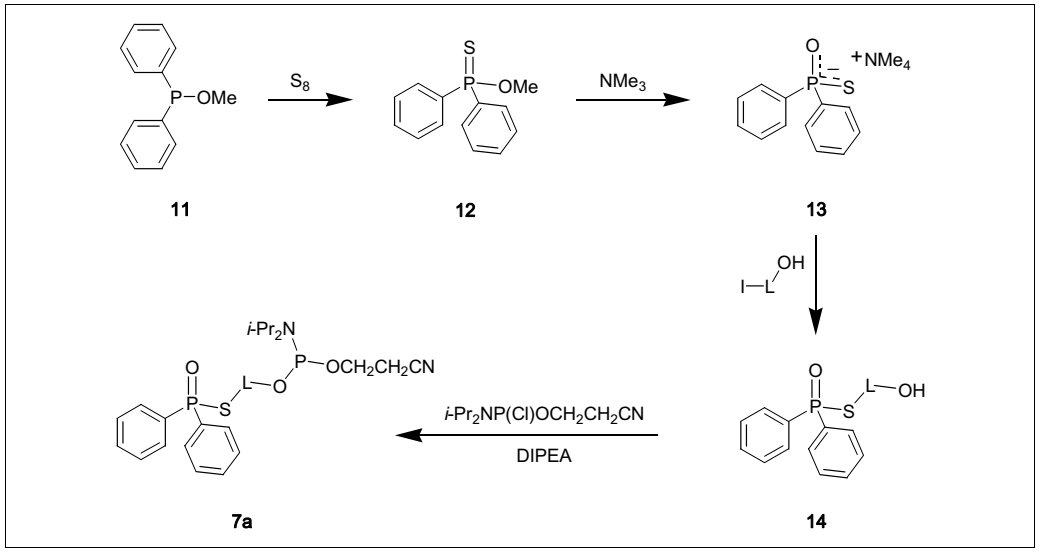


**Figure 4.9.12** Preparation of the *S*-diphenylphosphinate phosphoramidite derivative **S.7a**. DIPEA, diisopropylethylamine; L, $CH_2CH_2OCH_2CH_2OCH_2CH_2$.

Celite 521 (Aldrich)
20% (w/v) trimethylamine/acetonitrile
Diethyl ether, dried over sodium wires, ice cold and room temperature
Dichloromethane ($CH_2Cl_2$) distilled over $P_2O_5$ and passed through a column of basic alumina
Methanol, distilled
2-[2-(2-Chloroethoxy)ethoxy]ethanol (**S.15**; Fig. 4.9.16; Aldrich)
NaI
Sodium bicarbonate ($NaHCO_3$)
Acetone, anhydrous
Anhydrous acetonitrile ($CH_3CN$)
Diisopropylethylamine, distilled from KOH
2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (Aldrich)
Nitrogen gas
10% (w/v) aqueous sodium carbonate ($Na_2CO_3$)
Saturated aqueous NaCl, ice cold
Sodium sulfate, anhydrous
5′-Detritylated oligodeoxyribonucleotide bound to a controlled-pore glass (CPG) support
2,2′-Dithiodipyridine
Phenol
Concentrated ammonium hydroxide
1 M triethylammonium acetate (TEAA) buffer, pH 7 (stock solution)
Tris-(2-carboxyethyl)phosphine (TCEP), hydrochloride

250-mL three-necked round-bottom flask
Reflux condenser
Magnetic stirrer with heating element
Kieselgel 60F plates for analytical TLC (Merck)
Rotary evaporator with a water aspirator
Calcium chloride drying tube

3 × 50–cm chromatography column containing 40 g silica gel (e.g., Kieselgel 60; Merck) and 1.6 × 45–cm column containing 25 g silica gel
10-mL vial and stoppers
Separatory funnel
Spectrophotometer

Additional reagents and equipment for thin-layer chromatography (TLC; *APPENDIX 3D*); column chromatography (*APPENDIX 3E*); direct addition of an acridinyl phosphoramidite (*UNIT 4.3*); and analysis, purification, and characterization of product (see Basic Protocol 1)

***Prepare diphenylthiophosphinate salt S.13***

1. Place 3 g (13.8 mmol) methyldiphenylphosphinite **S.11** and 60 mL toluene in a 250-mL three-necked round-bottom flask equipped with a reflux condenser and a magnetic stir bar. Add 883 mg (27.6 mmol) $S_8$ slowly to the stirring solution at room temperature.

   *The reaction is slightly exothermic.*

2. Heat suspension at 50°C and monitor reaction by analytical TLC (*APPENDIX 3D*) and UV shadowing using 90:10:4 (v/v/v) hexane/ethyl acetate/triethylamine as eluent. Spray the plate with 2.5 mg/mL DBPNC in ethanol and heat until color appears.

   *The sulfur-containing compound **S.12** ($R_f$ = 0.43) gives a pink-colored spot on the TLC plate after treatment with DBPNC. The starting material **S.11** ($R_f$ = 0.70) appears as a white spot.*

3. After the reaction is complete (1 to 2 hr), allow reaction mixture to cool to room temperature, filter through celite 521, and concentrate under reduced pressure using a rotary evaporator with a water aspirator.

   *Yield 85% (2.92 g, 11.78 mmol). White solid. $^{31}$P-NMR ($CDCl_3$), δ: 85.85 ppm. $^{1}$H-NMR ($CDCl_3$), δ: 3.75 (d, 3H, J = 12.2 Hz, $OCH_3$), 7.41-7.53 (m, 6H, Ar), 7.81-7.91 (m, 4H, Ar). $^{13}$C-NMR (DMSO-$d_6$), δ: 56.81, 134.44, 136.21, 137.70. Mass analysis. ESI-MS polarity positive. Calcd. for $C_{13}H_{13}OSP$: 248; found: 249 (M+H).*

4. Add 2 g (8.05 mmol) methyldiphenylthionophosphinate **S.12** to 50 mL of 20% trimethylamine/acetonitrile in a 100-mL flask equipped with a stirring bar. Stopper the flask and stir 2 to 3 days at room temperature, monitoring the reaction by analytical TLC and UV shadowing, with 9:1 (v/v) hexane/ethyl acetate as eluent.

   *A white precipitate appears ($R_f$ = 0) and the starting material ($R_f$ = 0.40) disappears. The reaction can be faster if performed at higher temperature (50°C for 10 hr) in a sealed reactor.*

5. Filter precipitate by vacuum filtration and wash with 5 mL ice-cold diethyl ether. Analyze **S.13** by TLC using 85:10:5 (v/v/v) $CH_2Cl_2$/methanol/triethylamine as eluent ($R_f$ = 0.72).

   *Yield 95% (2.35g, 7.65 mmol). $^{31}$P-NMR (DMSO-$d_6$), δ: 52.36 ppm. $^{1}$H-NMR (DMSO-$d_6$), δ: 3.06 (s, 12H, $NMe_4$), 7.16-7.20 (m, 6H, Ar), 7.70-7.76 (m, 4H, Ar). $^{13}$C-NMR (DMSO-$d_6$), δ: 59.85, 132.34, 133.42, 135.97. Mass analysis. ESI-MS polarity positive. Calcd. for $C_{12}H_{10}OSP$: 233; found: 235 (M+H).*

***Prepare 2-[2-(2-iodoethoxy)ethoxy]ethanol***

6. Place the following in a 250-mL round-bottom flask equipped with a reflux condenser and magnetic stirring bar:

4 g (24 mmol) 2-[2-(2-choroethoxy)ethoxy]ethanol **S.15**
14.96 g (100 mmol) NaI
8.36 g (100 mmol) $NaHCO_3$
140 mL anhydrous acetone.

7. Heat at 60°C until the reaction is complete. Monitor reaction by analytical TLC using 9:1 (v/v) $CH_2Cl_2$/methanol as eluent.

   *After one night (5 to 6 hr) the starting chlorinated material ($R_f$ = 42) is transformed into the iodinated compound ($R_f$ = 0.56).*

8. Remove solid by filtration and evaporate solvent under reduced pressure using a rotary evaporator with a water aspirator.

9. Precipitate excess NaI by adding 8 mL $CH_2Cl_2$. Remove solid by filtration and evaporate solvent under reduced pressure. Repeat for a total of four times with 8 mL $CH_2Cl_2$ and two times with 10 mL diethyl ether.

   *Take care to use diethyl ether free of peroxides.*

   *Yield 83% (4.94 g, 19.9 mmol). Mass analysis ESI-MS polarity positive. Calcd. for $C_6H_3IO_3$: 260; found: 261 (M+H).*

   *This compound can be prepared in advance and stored for months at –20°C.*

***Prepare S-diphenylphosphinate derivative 7a***

10. Place 1 g (3.25 mmol) diphenylthiophosphinate salt **S.13**, 1.01 g (3.9 mmol) 2-[2-(2-iodoethoxy)ethoxy]ethanol, and 30 mL anhydrous $CH_3CN$ in a 50-mL flask equipped with a magnetic stirring bar, reflux condenser, and a calcium chloride drying tube. Allow reaction to proceed 15 hr at 50°C.

11. Allow the mixture to cool, remove solid by filtration, and concentrate the solution to dryness.

12. Purify the residue **S.14** (2 g) on a 3 × 50–cm column containing 40 g silica gel (*APPENDIX 3E*), using 100 mL each of 0% to 5% (v/v) methanol in $CH_2Cl_2$ in 1% increments. Elute under slight pressure of nitrogen.

    *The pressure must be adjusted until the solvent elutes from the bottom of the column at ~3 drops/sec.*

    *Yield 70% (0.834g, 2.27 mmol). $^1$H-NMR ($CDCl_3$), δ: 2.95-3.03 (2H, m, $CH_2S$), 3.58-3.63 (6H, m, $CH_2O$), 3.68-3.74 (4H, m, $CH_2O$), 7.45-7.58 (6H, m, Ar), 7.85-7.92 (4H, m, Ar). $^{31}$P-NMR ($CDCl_3$), δ: 45.40 ppm. $^{13}$C-NMR (DMSO-$d_6$), δ: 33.77, 65.64, 75.05, 77.78, 134.42, 136.35, 138.02. Mass analysis. ESI-MS polarity positive. Calcd. for $C_{18}H_{23}O_4SP$: 366; found: 367 (M+H).*

13. Dry **S.14** by three rounds of coevaporation with 10 mL anhydrous $CH_3CN$ and leave under vacuum overnight.

14. Add 0.317 g (0.426 mL, 2.45 mmol) diisopropylethylamine to a solution of 0.30 g (0.82 mmol) **S.14** in 5 mL $CH_2Cl_2$. Cool in an ice-water bath.

15. Add 0.23 mL (1.02 mmol) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite dropwise to the cold solution via a syringe under a nitrogen atmosphere.

16. Monitor reaction by analytical TLC and by UV shadowing, using 70:30:4 (v/v/v) ethyl acetate/hexane/triethylamine as eluent. Pre-elute the plate once with eluent, and then load 1 to 2 µl reaction mixture and elute.

*After 15 to 20 min the starting material ($R_f$ = 0.15) is completely transformed into compound **S.7a** ($R_f$ = 0.31). TLC using 95:5 (v/v) ethyl acetate/triethylamine gives $R_f$ = 0.33 for the starting material and $R_f$ = 0.70 for the phosphoramidite derivative.*

17. When the reaction is complete (15 to 20 min), dilute the reaction mixture with 45 mL ethyl acetate and wash the organic phase twice with 3 mL of 10% aqueous $Na_2CO_3$, and twice with 3 mL ice-cold saturated aqueous NaCl solution, using a separatory funnel.

18. Dry organic phase over 15 g sodium sulfate and evaporate to dryness under reduced pressure.

19. Purify the oily residue **S.7a** (0.8 g) on a 1.6 × 45–cm column containing 25 g silica gel, using 95:5 (v/v) ethyl acetate/triethylamine as eluent. Elute under slight pressure of nitrogen. Store up to two years (or more) at −20°C under argon.

    *The pressure must be adjusted until the solvent elutes from the bottom of the column at ~2 drops/sec.*

    *Yield 75% (0.50 g, 0.61 mmol). $^{31}$P-NMR ($CDCl_3$), δ: 39.13 and 144.11 ppm. $^1$H-NMR ($CDCl_3$), δ: 1.13-1.16 (m, 12H, $[CH(CH_3)_2]_2$), 2.59-2.62 (m, 2H, $CH_2CN$), 2.93-2.98 (m, 2H, $CH_2SP$), 3.51-3.65 (m, 8H, $CH_2O$ + $CH_2OP$), 3.65-3.69 (m, 1H), 3.70-3.83 (m, 2H, $CH_2OP$), 3.66-3.70 (m, 1H, C(H)), 7.43-7.47 (m, 4H, Ar), 7.50-7.52 (m, 2H, Ar), 7.83-7.88 (m, 4H, Ar). $^{13}$C-NMR (DMSO-$d_6$), δ: 25.25, 29.79, 33.77, 47.87, 63.64, 67.80, 75.00, 75.92, 134.42, 135.51, 138.00. Mass analysis. ESI-MS polarity positive. Calcd. for $C_{27}H_{40}N_2O_5SP_2$: 566; found: 568 (M+H).*

***Add masked thiol function to oligonucleotide***

20. Couple the phosphoramidite **S.7a** to the 5′-terminal hydroxyl of a fully protected oligodeoxyribonucleotide bound to a CPG support according to the method described for direct addition of an acridinyl phosphoramidite (*UNIT 4.3*, steps 15a to 17b of Basic Protocol 1).

***Deprotect and remove from support***

21. Place 1 µmol of the oligodeoxyribonucleotide bound to the support in a 10-mL vial and add successively:

    26 mg (0.118 mmol, 120 eq) 2,2′-dithiodipyridine
    17 mg (0.179 mmol, 10 eq/phosphate) phenol
    3 mL methanol
    3.5 mL concentrated ammonium hydroxide.

    Allow to stand at room temperature for 60 hr.

22. Remove support by vacuum filtration. Wash support successively with 1 mL concentrated ammonium hydroxide, 1 mL water, 1 mL methanol. Pool the original filtrate and the three washes and concentrate them under reduced pressure.

23. Add 6 mL water to the residue and extract four times with 4 mL ethyl acetate to remove organic contaminants.

24. Analyze, purify and characterize the product **S.10** as described (see Basic Protocol 1, steps 12 to 17). Store up to two years (or more) at −20°C in a tightly sealed container.

    *Yield: 35-40 $OD_{260}$ units.*

    *Ion-exchange chromatography on a DEAE column using conditions described in Basic Protocol 1 gives retention times of 52 min for $C_5H_5N$-S-S-$CH_2CH_2$-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC], and 50 min 36 sec for d[CTCTCGCACCCATCTCTC]. Figure 4.9.13 shows a chromatogram obtained for crude $C_5H_5N$-S-S-$CH_2CH_2$-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC].*

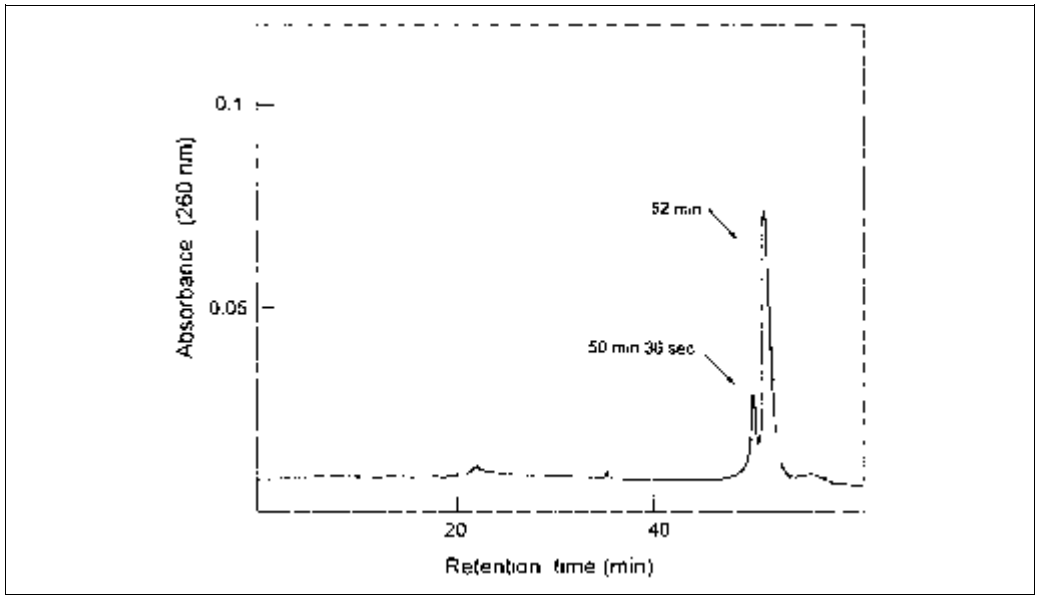


**Figure 4.9.13** Ion-exchange chromatography on a DEAE column of crude $C_5H_5N$-S-S-$CH_2CH_2$-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] (see Basic Protocol 3).

*RP-HPLC on a Lichrospher RP 18 column using conditions described in Basic Protocol 1 gives retention times of 15 min 14 sec for $C_5H_5N$-S-S-$CH_2CH_2$-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC], and 9 min 40 sec for d[CTCTCGCACCCATCTCTC]. See Figure 4.9.14.*

*Mass analysis. ESI-MS polarity negative. Calcd. for $C_{182}H_{238}N_{56}O_{114}P_{18}S_2$: 5652 Da; found: 5651 Da (M-H).*

*The presence of the pyridyldisulfide group at the 5′ end of the oligomer can be detected by reductive cleavage of the disulfide bridge with dithiothreitol or tris-(2-carboxyethyl)phosphine, as described below. The 2-pyridinethione that is released can be detected spectrophotometrically at 343 nm (Carlsson et al., 1978).*

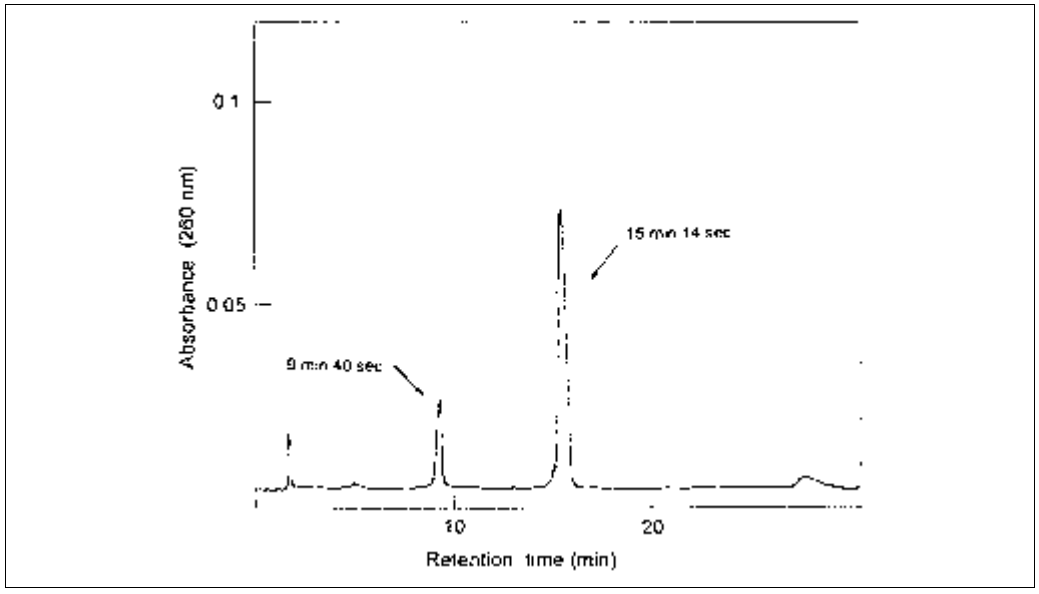


**Figure 4.9.14** RP-HPLC on a Lichrospher RP 18 column of crude $C_5H_5N$-S-S-$CH_2CH_2$-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] (see Basic Protocol 3).

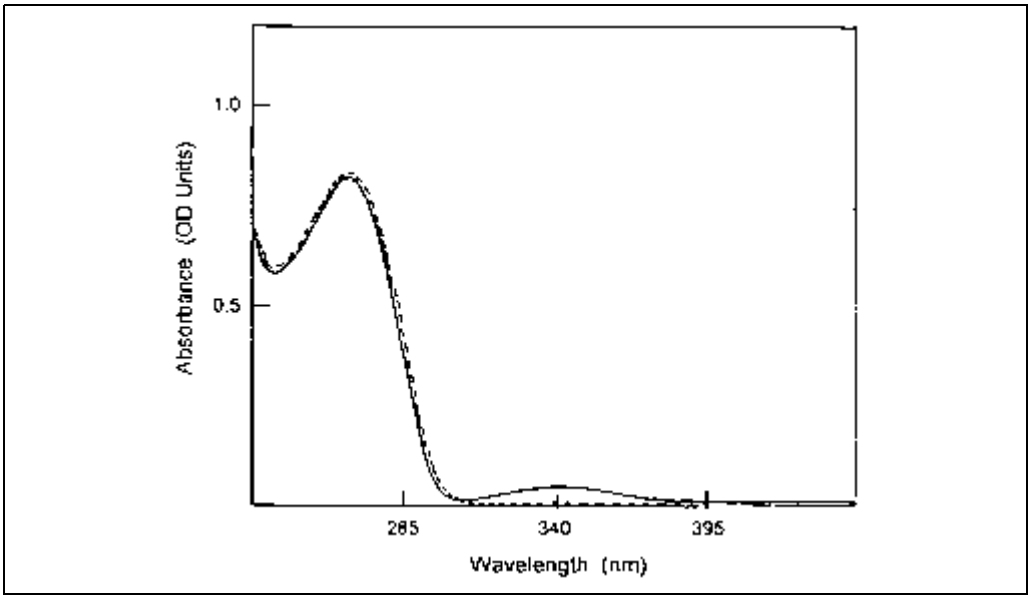


**Figure 4.9.15** Absorption spectra of a solution of $C_5H_5N$-S-S-$CH_2CH_2$-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] before (broken line) and after (solid line) reduction of the disulfide bridge (see Basic Protocol 3).

***Verify presence of pyridyldisulfide group***

25. Prepare an oligonucleotide solution in 0.1 M TEAA buffer, pH 7, that gives an absorbance of ~0.5 or 1 $OD_{260}$. Record the absorption spectrum of this solution between 220 and 450 nm using the same buffer as a reference.

26. Add 10 eq TCEP (dissolved in 5 µl water) to both the sample and reference. Wait 30 min and record the absorption spectrum again.

*A new absorption band is observed, corresponding to the 2-pyridinethione released ($\lambda_{max}$ = 343 nm). Knowing the* ε *value of the oligonucleotide at 260 nm, the absorbance ratio at 260 and 343 nm allows one to determine the quantity of 2-pyridyldisulfide group present in oligonucleotide* ***S.10****. Figure 4.9.15 illustrates results from this procedure applied to the oligonucleotide $C_5H_5N$-S-S-$CH_2CH_2$-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC].*

## ADDITION OF A MASKED THIOL GROUP TO THE 5′ TERMINUS OF OLIGODEOXYRIBONUCLEOTIDES USING AN *S*-ACETYL PHOSPHORAMIDITE

***ALTERNATE PROTOCOL 1***

This protocol is a modification of the strategy in Basic Protocol 3 (Fig. 4.9.11, left side) that adds an *S*-acetyl phosphoramidite **S.8a** to the 5′ terminus of the support-bound oligodeoxyribonucleotide. The alcohol **S.16**, which is required for the preparation of **S.8a** (Fig. 4.9.16), is obtained in one step by reaction of 2-[2-(2-chloroethoxy)ethoxy]ethanol **S.15** with potassium thioacetate. This procedure is a shortened version of that described by Kuijpers and Van Boeckel (1993). The synthesis can also be started with 2-[2-(2-iodoethoxy)ethoxy]ethanol, which is prepared as described in Basic Protocol 3. However, **S.15** is commercially available and thus more convenient.

***Additional Materials*** *(also see Basic Protocol 3)*

Potassium thioacetate
1.6 × 45–cm column containing 20 g silica gel (e.g., Kieselgel 60; Merck)

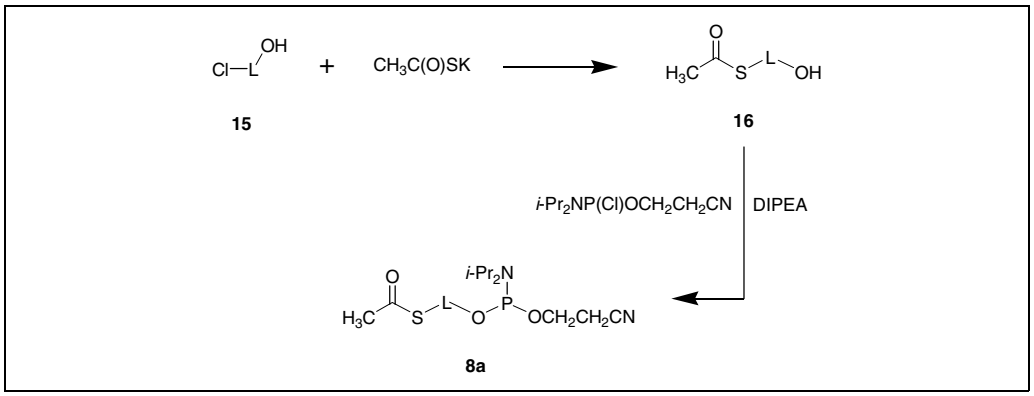


**Figure 4.9.16** Preparation of the *S*-acetyl phosphoramidite derivative **S.8a**. DIPEA, diisopropylethylamine; L, $CH_2CH_2OCH_2CH_2OCH_2CH_2$.

### *Synthesize 2-[2-(2-acetylthioethoxy)ethoxy]ethanol S.16*

1. Add 2 g (1.72 mL, 11.86 mmol) 2-[2-(2-chloroethoxy)ethoxy]ethanol **S.15** to 2 g (17.5 mmol) potassium thioacetate and 35 mL anhydrous acetone in a 25-mL flask equipped with a reflux condenser and a magnetic stirring bar.
2. Heat the stirred mixture 12 hr at 50°C.
3. Allow the mixture to cool to room temperature.
4. Filter off the white precipitate and concentrate the filtrate under reduced pressure.
5. Purify the residue **S.16** (2.4 g) on a 3 × 50–cm column containing 40 g silica gel (*APPENDIX 3E*) using a gradient of 98:2 to 9:1 (v/v) ethyl acetate/methanol. Elute under slight pressure of nitrogen.

   *The pressure must be adjusted until the solvent elutes from the bottom of the column at ~2 to 3 drops/sec.*

6. Perform analytical TLC (*APPENDIX 3D*) using 8:2 (v/v) ethyl acetate/hexane as eluent. Spray plate with 2.5 mg/mL DBPNC in ethanol and heat until the color appears.

   *A pale yellow spot ($R_f$ = 0.41) appears on the TLC plate after treatment with DBPNC.*

   *Yield 80% (1.97g, 9.48 mmol). $^1$H-NMR ($CDCl_3$): δ: 3.73 (2H, m, $CH_2OH$), 3.70-3.55 (8H, m, 4($CH_2O$)), 3.11 (2H, t, $CH_2S$). $^{13}$C-NMR (DMSO-$d_6$), δ: 34.49, 35.80, 36.72, 66.43, 75.12, 75.88, 78.56, 201.33. Mass analysis. ESI-MS polarity positive. Calcd. for $C_8H_{16}O_4S$: 208; found: 209 (M+H).*

### *Prepare S-acetyl phosphoramidite derivative S.8a*

7. Dry 0.208 g (1 mmol) **S.16** by three rounds of coevaporation with 5 mL anhydrous $CH_3CN$ and leave under vacuum overnight.
8. Add 0.387 g (0.522 mL, 3 mmol) diisopropylethylamine to a solution of 0.208 g (1 mmol) **S.16** in 5 mL $CH_2Cl_2$. Cool in an ice-water bath.
9. Add 0.30 mL (1.3 mmol) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite dropwise to the cold solution via syringe under a nitrogen atmosphere.
10. Monitor reaction by analytical TLC using 80:20:4 (v/v/v) hexane/ethyl acetate/triethylamine as eluent. Pre-elute the TLC plate once with the eluent, then load 1 to 2 µl reaction mixture and elute.

    *After 15 min, the starting material ($R_f$ = 0.38) is completely transformed into compound* ***S.8a*** *($R_f$ = 0.70).*

11. When the reaction is complete, dilute the reaction mixture with 45 mL ethyl acetate and wash the organic phase twice with 3 mL of 10% aqueous $Na_2CO_3$ and then twice with ice-cold saturated aqueous NaCl using a separatory funnel.

12. Dry the organic phase over 15 g sodium sulfate and evaporate it to dryness under reduced pressure.

13. Purify the residue **S.8a** (0.45g) on a 1.6 × 45–cm column containing 20 g silica gel using 80:15:5 (v/v/v) hexane/ethyl acetate/triethylamine as eluent. Elute under slight pressure of nitrogen. Store up to two years (or more) at −20°C under argon.

    *The pressure must be adjusted until the solvent elutes from the bottom of the column at ~2 to 3 drops/sec.*

    *Yield 75% (0.306 g, 0.75 mmol). $^{31}$P-NMR ($CDCl_3$), δ: 150.63 ppm. $^1$H-NMR ($CDCl_3$), δ: 1.15-1.19 (m, 12H, $[CH(CH_3)_2]_2$), 2.33 (s, 3H, $CH_3$), 2.64 (t, 2H, J = 6.56 Hz, $CH_2CN$), 3.08 (t, 2H, J = 6.40 Hz, $CH_2S$) 3.57-3.72 (m, 12H, $CH_2O$), 3.74-3.84 (m, 2 CH). Mass analysis. ESI-MS polarity positive. Calcd. for $C_{17}H_{33}O_5SN_2P$: 408; found: 409 (M+H).*

***Add masked thiol function to oligonucleotide***

14. Couple the phosphoramidite **S.8a** to the oligonucleotide and then deprotect, purify, and characterize the product **S.10** as described (see Basic Protocol 3, steps 20 to 26).

    *Yield: 40-50 $OD_{260}$ units.*

## ADDITION OF A MASKED THIOL GROUP TO THE 5′ TERMINUS OF OLIGODEOXYRIBONUCLEOTIDES USING A DISULFIDE PHOSPHORAMIDITE DERIVATIVE

***ALTERNATE PROTOCOL 2***

The second strategy for adding a masked thiol group to the 5′ end of an oligodeoxyribonucleotide is shown in the right side of Figure 4.9.11. The masked thiol is incorporated using a tritylated alkyl disulfide–containing linker (**S.9a**), which allows full deblocking of the oligodeoxyribonucleotide but not the thiol function. The 4,4′-dimethoxytrityl (DMTr) group makes it possible to monitor the incorporation yield of the linker, and facilitates the purification of the 5′-derivatized oligodeoxyribonucleotide.

The phosphoramidite **S.9a** is obtained via a four-step procedure (Fig. 4.9.17) starting from of 2-[2-(2-acetylthioethoxy)ethoxy]ethanol **S.16**, which was prepared in Alternate Protocol 1. First, alkali treatment removes the acetyl group to release a thiol-containing linker (**S.17**), which dimerizes in contact with oxygen in the air (**S.18**). The dimerized linker is

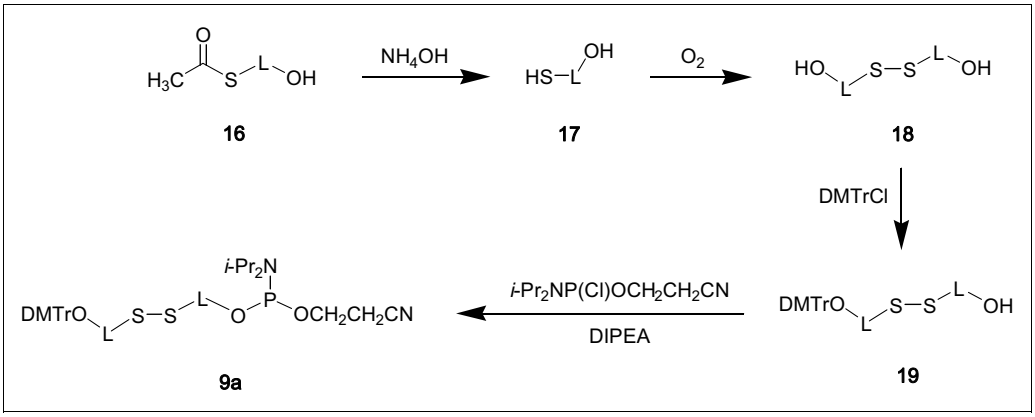


**Figure 4.9.17** Preparation of the tritylated disulfide phosphoramidite derivative **S.9a**. DIPEA, diisopropylethylamine; DMTr, 4,4′-dimethoxytrityl; L, $CH_2CH_2OCH_2CH_2OCH_2CH_2$.

monotritylated (**S.19**) and then phosphinylated to give the phosphoramidite **S.9a**. This procedure is a shortened version of that described by Bonfils and Thuong (1991).

***Additional Materials*** *(also see Basic Protocols 1, 2, and 3)*

2-[2-(2-Acetylthioethoxy)ethoxy]ethanol **S.16** (see Alternate Protocol 1)
5% ammonium hydroxide
Anhydrous pyridine
Dimethoxytrityl chloride (DMTr·Cl)
10% perchloric acid
$MgSO_4$
80% (v/v) acetic acid
Ethanol
1 M triethylammonium acetate (TEAA) buffer, pH 6 (stock solution)
Dithiothreitol (DTT)
Methanol, HPLC grade

1.6 × 45–cm chromatography column containing 20 g silica gel (e.g., Kieselgel 60; Merck)
10-mL gel-filtration column
Lyophilizer

Additional reagents and equipment for gel-filtration chromatography

***Prepare dihydroxylated disulfide S.18***

1. Solubilize 2-[2-(2-acetylthioethoxy)ethoxy]ethanol **S.16** (1 g, 4.80 mmol) in 2 mL of 5% ammonium hydroxide. Magnetically stir the resulting thiol **S.17** and allow to dimerize to the disulfide **S.18** upon contact with air.

2. Monitor reaction by analytical TLC (*APPENDIX 3D*) using 9:1 (v/v) $CH_2Cl_2$/methanol as eluent. Spray with 2.5 mg/mL DBPNC in ethanol and heat until the color appears.

   *The starting material ($R_f$ = 0.54) appears as a pale-yellow colored spot on the TLC plate after treatment with DBPNC. The thiol derivative **S.17** ($R_f$ = 0.50) appears as a brown-colored spot, and its disulfide **S.18** ($R_f$ = 0.34) as an intense yellow spot.*

3. When the reaction is complete (usually 24 hr), concentrate the mixture and purify the residue (1 g) by flash chromatography (*APPENDIX 3E*) on a 3 × 50–cm column containing 40 g silica gel using increasing concentrations of methanol in $CH_2Cl_2$ (0% to 6%) as eluent. Elute under slight pressure of nitrogen. Monitor elution by TLC.

   *The pressure must be adjusted until the solvent elutes from the bottom of the column at ~2 to 3 drops/sec.*

   *Yield: 40% (0.633 g, 1.92 mmol).*

***Prepare tritylated disulfide S.19***

4. Dry 0.7 g (2.09 mmol) **S.18** by three rounds of coevaporation with 10 mL anhydrous pyridine and keep it stirring in 5 mL anhydrous pyridine under a nitrogen atmosphere.

5. Cool solution to 5° to 10°C and add 0.18 g (0.53 mmol) DMTr·Cl.

6. Allow solution to warm to room temperature.

7. Monitor reaction by analytical TLC using 9:1 (v/v) $CH_2Cl_2$/methanol as eluent. Spray the plate with 10% perchloric acid.

*Upon spraying the plate with perchloric acid, two orange-colored spots are obtained, corresponding to the monodimethoxytritylated compound* ***S.19*** *($R_f$ = 0.65) and the bis-dimethoxytritylated compound ($R_f$ = 0.95).*

8. When the reaction is complete (6 to 8 hr), remove the solvent by evaporation under reduced pressure.

9. Dilute the residue with 10 mL $CH_2Cl_2$ and wash twice with 3 mL saturated aqueous NaCl and then twice with 3 mL water using a separatory funnel.

10. Dry the organic layer over $MgSO_4$ and concentrate it to dryness.

11. Purify the residue **S.19** (0.85 g) by chromatography on a 3 × 50–cm column containing 40 g silica gel using increasing concentrations of methanol in $CH_2Cl_2$ (0% to 4%) as eluent. Elute under slight pressure of nitrogen. Monitor elution by TLC.

*The pressure must be adjusted until the solvent elutes from the bottom of the column at ~2 to 3 drops/sec.*

*Yield 75% (0.250 g, 0.397 mmol). $^1$H-NMR ($CDCl_3$), δ: 2.80-2.92 (m, 4H, $CH_2S$), 3.23 (t, J = 5.18 Hz, 2H), 3.58-3.80 (m, 18H, $CH_2O$), 3.82 (s, 6H, $OCH_3$), 6.80-7.47 (m, 13H, Ar). $^{13}$C-NMR (DMSO-$d_6$): δ: 35.14, 40.79, 43.40, 54.07, 60.48, 65.70, 68.35, 74.13, 75.20, 75.51, 77.83, 90.79, 118.61, 132.08, 133.23, 135.15, 141.28, 150.49, 163.51. Mass analysis. ESI-MS polarity positive. Calcd. for $C_{33}H_{44}O_8S_2$: 632; found 633 (M+H).*

***Prepare tritylated disulfide phosphoramidite derivative S.9a***

12. Dry 0.30 g (0.47 mmol) **S.19** by three rounds of coevaporation with 6 mL anhydrous $CH_3CN$ and leave under vacuum overnight.

13. Add 0.183 g (0.248 mL, 1.42 mmol) diisopropylethylamine to a solution of 0.30 g (0.47 mmol) **S.19** in 4 mL $CH_2Cl_2$. Cool in an ice-water bath.

14. Add 0.137 mL (0.61 mmol) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite dropwise to the cold solution via a syringe under a nitrogen atmosphere.

15. Monitor reaction by analytical TLC using 50:50:4 (v/v/v) ethyl acetate/hexane/triethylamine as eluent. Pre-elute the TLC plate once with the eluent, then load 1 µl reaction mixture and elute. Visualize by UV shadowing and trityl detection.

*After 20 min the starting material ($R_f$ = 0.15) is completely tranformed into the phosphoramidite* ***S.9a*** *($R_f$ = 0.59).*

16. When the reaction is complete, dilute reaction mixture with 30 mL ethyl acetate and wash the organic phase twice with 3 mL of 10% aqueous $Na_2CO_3$ and then twice with 3 mL ice-cold saturated aqueous NaCl using a separatory funnel.

17. Dry the organic phase over sodium sulfate and concentrate it to dryness under reduced pressure.

18. Purify the oily residue **S.9a** (0.4 g) on a 1.6 × 45–cm column containing 20 g silica gel using 50:50:4 (v/v/v) ethyl acetate/hexane/triethylamine as eluent. Elute under slight pressure of nitrogen. Store up to two years (or more) at −20°C under argon.

*Yield 75% (0.293 g, 0.352 mmol). $^{31}$P-NMR ($CDCl_3$), δ: 150.48 ppm. $^1$H-NMR ($CDCl_3$), δ: 1.16-1.24 (m, 12H, $[CH(CH_3)_2]_2$), 2.80 (t, 2H, J = 6 Hz, $CH_2CN$), 3.20-3.24 (m, 4H, $CH_2S$), 3.58-3.83 (m, 20H, $CH_2O$, $CH_2OP$ +2 CH), 3.75 (s, 6H, $OCH_3$), 6.80-7.47 (m, 13H, Ar). Mass analysis. ESI-MS polarity positive. Calcd. for $C_{42}H_{62}O_9S_2N_2P$: 831; found: 832 (M+H).*

***Couple masked thiol function to oligonucleotide***

19. Couple tritylated disulfide phosphoramidite **S.9a** to the 5′-terminal hydroxyl of a fully protected oligodeoxyribonucleotide bound to a CPG support according to the

method described for direct addition of an acridinyl phosphoramidite (*UNIT 4.3*, steps 15a to 17b of Basic Protocol 1).

*Detritylating a small amount of the support-bound oligonucleotide allows one to verify the coupling efficiency and, if necessary, to perform a second coupling to improve the yield.*

20. Deprotect the oligodeoxyribonucleotide **S.9b** (Fig. 4.9.11) as described for the deprotection of the oligodeoxyribonucleotides with 5′-bis-(2-cyanoethyl)phosphate groups (see Basic Protocol 2, steps 8 to 11, of this unit) but without DTT in step 8.

21. Analyze, purify, and characterize the tritylated disulfide-containing oligodeoxyribonucleotide **S.9c** as described (see Basic Protocol 1, steps 11 to 17).

*Yield: 40-50 $OD_{260}$ units.*

*Ion-exchange chromatography on DEAE using conditions described in Basic Protocol 1 gives retention times of 52 min 12 sec for DMTrO$CH_2CH_2$-($OCH_2CH_2)_2$-S-S-$CH_2CH_2$-($OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCCTC] and 50 min 36 sec for d[CTCTCGCACCCATCTCTC]. Figure 4.9.18 shows a chromatogram obtained for crude DMTrO$CH_2CH_2$-($OCH_2CH_2)_2$-S-S-$CH_2$ $CH_2$-($OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC].*

*RP-HPLC on a Lichrospher RP 18 column using a linear gradient of $CH_3CN$ (5% to 50% over 60 min) in 0.1 M TEAA buffer, pH 7, at 1 mL/min gives retention times of 32 min 26 sec for DMTrO$CH_2CH_2$-($OCH_2CH_2)_2$-S-S-$CH_2CH_2$-($OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] and 12 min for d[CTCTCGCACCCATCTCTC]. Figure 4.9.19 shows a chromatogram obtained for crude DMTrO$CH_2CH_2$-($OCH_2CH_2)_2$-S-S-$CH_2CH_2$-($OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC]. An additional peak with a retention time of 13 min 40 sec has been identified as the detritylated oligonucleotide HO$CH_2CH_2$-($OCH_2CH_2)_2$-S-S-$CH_2CH_2$-($OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC].*

*Mass analysis. ESI-MS polarity negative. HO$CH_2CH_2$-($OCH_2CH_2)_2$-S-S-$CH_2CH_2$-($OCH_2CH_2)_2$- p-d[CTCTCGCACCCATCTCTC]. Calcd. for $C_{182}H_{247}N_{55}O_{117}S_2P_{18}$: 5699; found: 5697 ± 4 (M-H).*

*Sometimes a significant amount of detritylation occurs when handling the functionalized oligonucleotide. In this case, it is best to detritylate prior to purification by adding 4 mL of 80% acetic acid to the tritylated oligonucleotide. After 20 to 30 min incubation at room temperature, the acetic acid is removed by evaporation under reduced pressure. The residue*

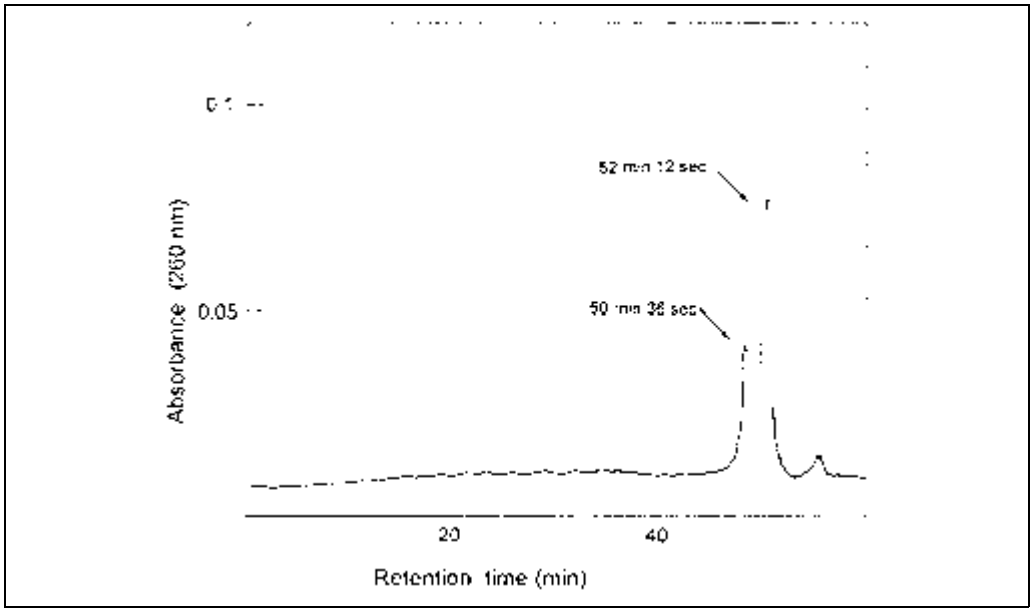


**Figure 4.9.18** Ion-exchange chromatography on a DEAE column of crude DMTrO-$CH_2CH_2$-($OCH_2CH_2)_2$-S-S-$CH_2CH_2$-($OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] (see Alternate Protocol 2).

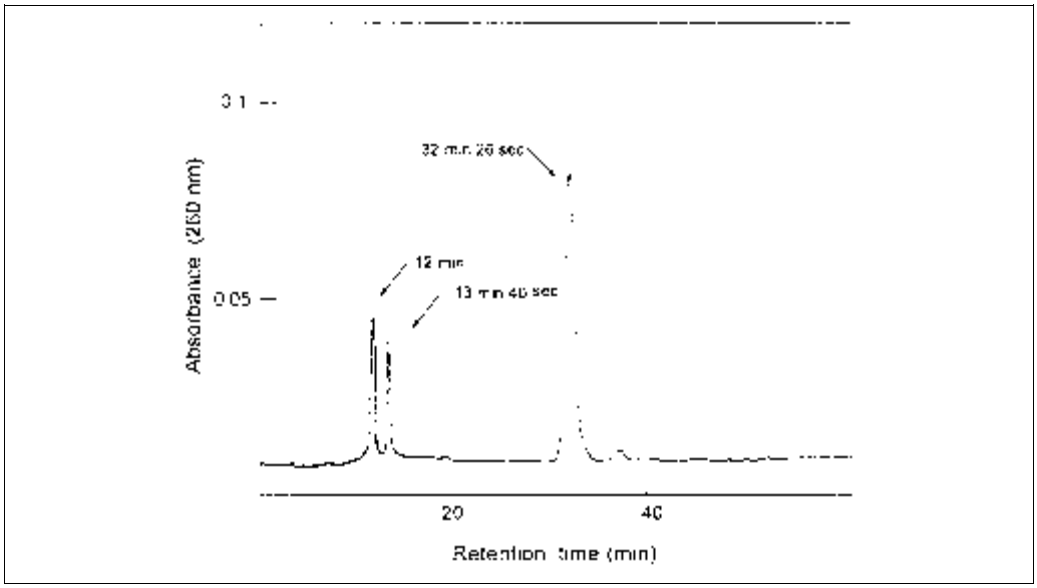


**Figure 4.9.19** RP-HPLC on a Lichrospher RP 18 column of crude DMTrO-$CH_2CH_2$-$(OCH_2CH_2)_2$-S-S-$CH_2CH_2$-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] (see Alternate Protocol 2).

*is then coevaporated three times with 4 mL ethanol, dissolved in 4 mL water, and extracted three times with 4 mL ethyl acetate. The purity of the detritylated oligonucleotide is verified by RP-HPLC using a linear gradient of $CH_3CN$ (0% to 30% over 60 min) in 0.1 M TEAA, pH 7, at 1 mL/min. If required, further purification is performed by RP-HPLC. When the modified oligodeoxyribonucleotide is purified at the tritylated step, detritylation should be performed before storage to avoid loss of trityl, resulting in a mixture of products. When transformation into oligomer **S.10** is performed immediately after purification, the tritylated compound can be used. The chromatography data given above is for the tritylated compound; mass data is for detritylated product.*

### *Transform disulfide-containing oligodeoxyribonucleotide into oligomer S.10*

22. Prepare a solution of 10 $OD_{260}$ units **S.9c** in 1 mL of 0.05 M TEAA buffer, pH 6.

23. Add 30 eq DTT or 3 eq TCEP and monitor cleavage of the disulfide bridge by RP-HPLC on an RP 18 column using the conditions described above (see Basic Protocol 1, step 14b).

    *The thiol-containing oligodeoxyribonucleotide HS-$CH_2CH_2$-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] is eluted first (11 min 30 sec) while the oligonucleotide containing the disulfide linker, $HOCH_2CH_2$-$(OCH_2CH_2)_2$-S-S-$CH_2CH_2$-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC], is eluted ~2 min later (13 min 25 sec).*

24. When the reaction is complete (overnight), extract excess DTT five times using 1 mL ethyl acetate.

    *It is not necessary to remove the excess TCEP.*

25. Load aqueous solution on a 10-mL gel-filtration column and elute with 0.05 M TEAA buffer, pH 6. Collect oligodeoxyribonucleotide in a solution containing 20 eq of 2,2′-dithiodipyridine in 2 mL HPLC-grade methanol. Monitor the elution of the oligonucleotide spectrophotometrically at 260 nm.

    *The choice of Sephadex G-10 or G-25 depends on the molecular weight of the oligodeoxyribonucleotide. The oligonucleotide is usually eluted in the void volume (~3 to 4 mL).*

26. Allow eluent/dithiodipyridine/methanol mixture to incubate for 5 to 6 hr.

27. Remove methanol by evaporation, and extract excess 2,2′-dithiodipyridine four times using 2 mL ethyl acetate.

28. Verify formation of 2-pyridyldisulfide by RP-HPLC on RP 18 column using the conditions described (see Basic Protocol 1, step 14b).

*The 2-pyridyldithioalkylated oligodeoxyribonucleotide **S.10** has a longer retention time (15 min 14 sec) than that of the mercaptoalkylated oligonucleotide (11 min 30 sec).*

29. Purify the 5′-terminal 2-pyridyldithioalkylated oligodeoxyribonucleotide **S.10** by RP-HPLC using the same column and conditions used for analysis.

*Purification can be achieved in three or four runs.*

30. Remove organic solvent by evaporation and remove buffer by three consecutive lyophilizations. Store up to two years at −20°C in a tightly sealed container.

*Yield 60%. Mass analysis. ESI-MS polarity negative. Calcd. for $C_{182}H_{238}N_{56}O_{114}P_{18}S_2$: 5652 Da; found: 5651 Da (M-H).*

## COMMENTARY

### Background Information

This unit reports methods for the preparation of modified oligodeoxyribonucleotides containing 5′-terminal carboxyl, amino, phosphorothioate, phosphate, or masked thiol groups. These 5′-modified oligonucleotides may be used to prepare oligodeoxyribonucleotides covalently linked to a wide variety of molecules such as labels, intercalating agents, and reactive compounds, a number of which are reported in *UNIT 4.10*. The methods developed in this unit can be used to prepare 5′-modified oligodeoxyribonucleotides built with natural β-deoxyribonucleosides, phosphorothioate phosphodiesters (Aubert et al., 2000), unnatural α-D-deoxyribonucleosides (Kurfürst et al., 1993), or 2′-*O*-methyldeoxyribonucleosides. Given that oligodeoxyribonucleotides can be purchased fully protected and bound to a support, 5′-end modification can be performed manually. When oligonucleotide synthesis is performed in the 5′→3′ orientation as in the case of N3′→P5′ phosphoramidates, 5′-derivatization can be achieved using a support bearing either the ligand or the masked functional group.

These methods can also be adapted to oligonucleotides containing modified nucleosides, but it must be verified that the conditions required for deprotection are compatible with the stability of the modified nucleosides. For oligoribonucleotides and modified oligonucleotides that are sensitive to basic conditions, it is recommended to introduce 5′-phosphate and 5′-thiophosphate groups enzymatically (Eckstein, 1983).

Alternate methods reported by Grimm et al. (2000) use the properties of a terminal phosphomonoester group for the introduction of linkers ending with amino, carboxyl, or sulfhydryl functions into unprotected oligonucleotides. These methods are mainly limited to the introduction of either one functional group at one end or two identical functional groups at both ends of the oligonucleotide. In contrast, the methods reported in this unit, including the incorporation of masked functional groups prior to deprotection, allow the incorporation of a wide combination of functional groups into oligonucleotides for specific subsequent coupling of various ligands. Masked amine, sulfhydryl, and carboxyl can be introduced via a linker to various sites of the oligonucleotide chain: base, sugar, internucleotidic phosphate, and 3′ terminus (*UNIT 4.5*), as well as phosphate and thiophosphate at the 3′ terminus or thiophosphate at one or many internucleotidic positions during the assembly steps; these groups are released during deprotection. Amine and masked thiol or thiophosphate are compatible as well as masked thiol and amine in the phosphorothioate series (Aubert et al., 2000). The procedure for incorporation of a thiophosphate at the 5′ terminus can be modified to introduce a masked thiol group at the 3′ terminus by using a modified support (Bonfils and Thuong, 1991).

Using the *S*-acetyl-containing linker **S.16**, synthesis of 25-mer oligodeoxyribonucleotide in the phosphorothioate series has been performed at a 10-µmol scale with very good yields (70 $OD_{260}$ units per µmol).

### Critical Parameters and Troubleshooting

The success of these procedures is dependent on a number of critical parameters. The methods reported include either carbonyldiimidazole activation or the use of phosphoramidite chemistry, both of which are highly water sensitive. For these reasons, strictly anhydrous

solvents and solutions should be used as well as dry starting material. Precautions should be taken to exclude moisture, as well as oxygen in the case of the phosphoramidite derivatives. Phosphoramidites such as **S.7a**, **S.8a**, **S.9a**, and bis-(2-cyanoethyl)diisopropylamidophosphite, as well as most of the intermediate compounds used for their preparation, should be stored in tightly closed flasks or vials at –20°C under argon atmosphere. Cold flasks and vials should always be allowed to reach room temperature before they are opened, and vials should remain open as little as possible.

To avoid low yields of oligonucleotides containing 5′-terminal functional groups, extreme care should be taken when using a syringe to remove solutions from vials containing the support-bound oligonucleotide in order to avoid unnecessary loss. After modification of the 5′ termini, it is recommended to deprotect an aliquot of the oligonucleotide conjugates to verify the efficiency of the reaction. In the case of the addition of the tritylated disulfide phosphoramidite **S.9a**, the coupling yield can be evaluated by quantitative trityl analysis performed as reported in *UNIT 3.2*. If the yield of 5′-modification is not sufficient, it is possible to perform an additional coupling step with the appropriate phosphoramidite derivatives. After 26 months storage of the tritylated disulfide phosphoramidite **S.9a** under the conditions described above, 5′-modification of an oligonucleotide has been performed on a µmol scale (by using 20 eq of **S.9a**) with 90% to 95% yield as determined by trityl quantification. This result indicates that, although the compound contains both a disulfide linkage and a tricoordinated phosphorus, it is unlikely to self-destruct to a large extent. This behavior differs from that of TCEP used to reduce the disulfide bond, probably for the following reasons: (1) the reducing properties of the phosphoramidite derivatives are weaker than those of the TCEP derivative; (2) the reductive cleavage of the disulfide linkage by TCEP requires the presence of one water molecule (Burns et al., 1991) and it is unlikely that 1 eq of water can be present in the stock of phosphoramidite **S.9a**; (3) the oligoethylene glycol linker can adopt a crown ether–like structure that prevents close proximity of the disulfide linkage and the tricoordinated phosphorus.

After deprotection and before loading chromatography columns, organic impurities are extracted from crude oligodeoxyribonucleotide solutions with organic solvents. Oligodeoxyribonucleotide solutions must be filtered through a 0.45-µm disposable filter to remove any particulates and prevent the clogging of analytical chromatography columns. In order to prevent chelation of phosphorothioate oligodeoxyribonucleotides, all solvents and buffer solutions used for their purification must be passed through a column of Chelex 100 resin (Bio-Rad) to remove divalent cations.

The crude oligodeoxyribonucleotides obtained from these protocols are purified by ion-exchange chromatography on Mono Q (Amersham Pharmacia Biotech) and DEAE (Waters) columns. After a desalting step, the purity of these oligonucleotides is verified on a Lichrospher 100 RP column. When purified by ion-exchange chromatography, oligodeoxyribonucleotides containing 5′-functional groups are, in most cases, sufficiently pure for subsequent coupling reactions with selected ligands. These purification procedures are suitable for 10- to 25-mers. They are not suitable for oligonucleotides that can form self-associated complexes such as G-tetrads.

Purification of tritylated compounds such as the 5′-tritylated disulfide-containing oligonucleotide **S.9c** or the 2-pyridyldithioalkylated oligonucleotide **S.10** can easily be carried out not only with a reversed-phase column but also with an ion-exchange column such as DEAE (Waters). In the latter case, the presence of a lipophilic group allows good separation between the full-length 5′-modified and 5′-unmodified oligodeoxyribonucleotides. For 5′-sulfur-containing oligodeoxyribonucleotides, purification by RP-HPLC on a Lichrospher RP 18 column using a low gradient of $CH_3CN$ in TEAA buffer, pH 7, allows separation of 5′-mercaptoalkylated and 5′-(2-pyridyldithioalkylated) oligodeoxyribonucleotides. For 5′-carboxyalkylated or 5′-aminoalkylated oligodeoxyribonucleotides, the presence of the 5′-functional group can be verified by RP-HPLC by comparison with unmodified oligodeoxyribonucleotides, which are eluted more quickly than their 5′-derivatized counterparts. The presence of the amino function on 5′-aminoalkylated oligodeoxyribonucleotides can be verified by TLC using ninhydrin. For oligodeoxyribonucleotide 5′-phosphates, the use of a Delta-Pak $C_4$ or RP 18 HPLC column allows separation of unmodified oligodeoxyribonucleotides from 5′-phosphate-containing oligomers. Purification can be difficult when working with oligonucleotides containing particular sequences, e.g., stretches of G. The best solution may be to replace the difficult se-

quences, e.g., by replacing dG with 7-deaza-2′-dG (Raynaud et al., 1996).

Nuclease digestion of the conjugates and RP-HPLC analysis of the resulting monomers ascertain full removal of the protecting groups and base composition. Additional characterization can be made by matrix-assisted laser desorption/ionization MS (MALDI-MS) and electrospray ionization MS (ESI-MS). After purification and characterization, oligonucleotides should be lyophilized and stored in tightly closed vials at −20°C. Under these conditions, they are stable up to two years (or more).

## Anticipated Results

Protocols reported in this unit are given for syntheses at a 1-µmol scale. When purification requires only ion-exchange chromatography or RP-HPLC, yields of 25 to 45 $OD_{260}$ units can be expected for oligonucleotides of medium size (12- to 16-mers). The 5′-modifications of oligonucleotides reported in this unit have also been performed at a 10-µmol scale with similar yields.

## Time Considerations

Provided that all reagents and materials required for each step are available, most of the procedures are simple and rapid. When all the phosphoramidite derivatives are ready to use, the time required to prepare one oligonucleotide modified at its 5′ terminus with a carboxylated or aminoalkylated linker, a phosphate, a thiophosphate, or a masked thiol group (including purification and characterization) is ~1 week. The time required for the preparation of the *S*-acetyl phosphoramidite derivative **S.8a** and the tritylated disulfide phosphoramidite derivative **S.9a** is ~3 days. The preparation of the *S*-diphenylphosphinate phosphoramidite derivative **S.7a** requires 1 week or more.

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Contributed by Ulysse Asseline and Nguyen T. Thuong
Centre de Biophysique Moléculaire, CNRS
Orléans, France



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