Incorporation of Halogenoalkyl, 2-Pyridyldithioalkyl, or Isothiocyanate Linkers into Ligands

In *UNIT 4.3*, the direct incorporation of ligands to the 5′ ends of oligodeoxyribonucleotides via their phosphoramidite derivatives is described. A second strategy for the addition of ligands to the 5′ ends of oligodeoxyribonucleotides involves the introduction of appropriate functional groups to two unprotected reactants. Specific coupling of these reactants results in the formation of oligodeoxyribonucleotide conjugates. Many different oligodeoxyribonucleotide conjugates can be prepared starting with only one oligodeoxyribonucleotide synthesis, provided that the required amount for each conjugate is low. This method is particularly useful when (1) a low amount of the ligand is available, (2) the ligand is unstable under the conditions required for oligonucleotide deprotection, or (3) the poor solubility of the ligand in solvents usually used for oligonucleotide synthesis does not allow the preparation of its phosphoramidite or *H*-phosphonate derivatives. In this approach, functional groups such as amino, phosphate, phosphorothioate, thiol, and carboxyl, that are capable of reacting with functionalized ligands, are attached to the 5′ ends of oligodeoxyribonucleotides.

This unit describes the incorporation of linkers containing reactive groups such as halogenoalkyl, 2-pyridyldithioalkyl, or isothiocyanate into ligands. Halogenoalkyl (Asseline et al., 1992, 1996) and 2-pyridyldithioalkyl (Chassignol and Thuong, 1998) linkers can be reacted with either a phosphorothioate or a thiol group incorporated into the oligodeoxyribonucleotide, while the isothiocyanate can react with aminoalkylated oligodeoxyribonucleotides. These ligands are typically 2-methoxy-6-chloro-9-amino-acridine as an intercalator (see Basic Protocol 1), psoralen as a photo-cross-linking reagent (see Basic Protocol 2), phenanthroline-Cu as a cleaving reagent (see Basic Protocol 3), and thiazole orange as a label (see Basic Protocol 4). The addition of carboxyl, amino, phosporothioate, phosphate, and sulfhydryl functions to the 5′ ends of oligonucleotides, as well as methods for linking these functionalized oligonucleotides and ligands, are reported in *UNITS 4.9 & 4.10*.

Alternatively, many labels carrying functional groups that react with 5′-thiol, 5′-terminal phosphorothioate, and 5′-amino groups are commercially available. Heterobifunctional reagents, which allow coupling between two compounds following two successive specific reactions, are also available from commercial sources. The latter compounds, listed in *UNIT 4.2*, are very useful when conjugates with a well-defined linker between the oligodeoxyribonucleotide and the ligand are not required.

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

FUNCTIONALIZATION OF AN ACRIDINE DERIVATIVE WITH A BROMOALKYL LINKER

BASIC PROTOCOL 1

The synthesis of 2-methoxy-6-chloro-9-(ω-bromohexylamino)acridine **S.1e** (Fig. 4.8.1) is achieved by bromination of 2-methoxy-6-chloro-9-(ω-hydroxyhexylamino)acridine **S.1b** (discussed in *UNIT 4.3*; Fig. 4.3.1).

Contributed by Ulysse Asseline and Nguyen T. Thuong
Current Protocols in Nucleic Acid Chemistry (2001) 4.8.1-4.8.15

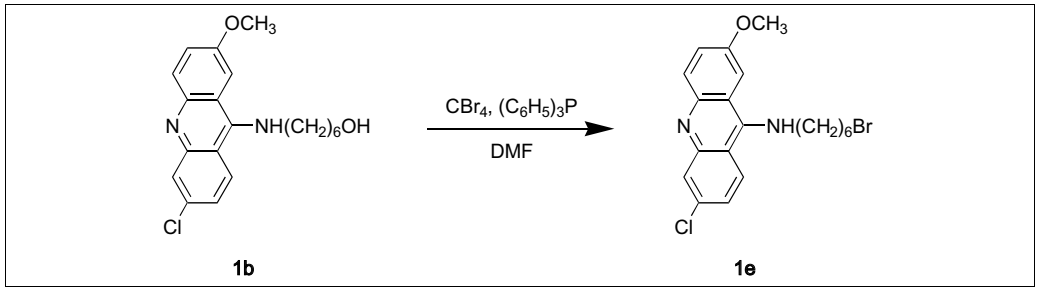

Figure 4.8.1 Bromination of an acridine derivative. DMF, *N*,*N*-dimethylformamide.

Materials

2-Methoxy-6-chloro-9-(ω-hydroxyhexylamino)acridine **S.1b** (*UNIT 4.3*)
Acetonitrile distilled from P_2O_5, stored over 3A molecular sieves
N,*N*-Dimethylformamide (DMF) redistilled in vacuo over ninhydrin, stored over 4A molecular sieves
Triphenylphosphine (Aldrich)
CBr_4 (Aldrich)
Dichloromethane (CH_2Cl_2) distilled from P_2O_5 and passed over basic aluminum oxide
Methanol, distilled or synthesis grade
Nitrogen source

10-mL round-bottom flask
Magnetic stirrer with Teflon stir bars
Vacuum pump (oil pump) capable of creating <0.1 mmHg pressure, with manifold and cold trap
Preparative 20 × 20–cm glass-backed silica TLC plates (2-mm thickness; Merck)
Short-wave UV light box
Mortar and pestle
1.5 × 15–cm chromatography column (empty)
2.5 × 7–cm Kieselgel 60F analytical TLC plates (Merck)

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

1. Place 138 mg (0.38 mmol) of 2-methoxy-6-chloro-9-(ω-hydroxyhexylamino)acridine **S.1b** into a 10-mL round-bottom flask, and dry by coevaporating three times with 5 mL acetonitrile.
2. Add 5 mL anhydrous DMF and stir with a Teflon stir bar and magnetic stirrer.
3. Add 105 mg (0.40 mmol) triphenylphosphine and 132 mg (0.40 mmol) CBr_4 to the acridine solution, and stir the reaction mixture overnight at room temperature.
4. Concentrate the solution to dryness using a vacuum pump.
5. Purify 450 mg of residue by preparative TLC (*APPENDIX 3D*) using 20 × 20–cm glass-backed silica TLC plates and 85:15 (v/v) CH_2Cl_2/methanol as the eluent. Dry the plate in a fume hood and visualize by UV shadowing.

   *Two distinct yellow bands should be visible. The upper one (which will also be the larger one if the yield is good) corresponds to **S.1e**; the band just below it corresponds to the residual starting material.*

CAUTION: *It is preferable to carry out steps 5 to 8 in a fume hood to avoid breathing solvent vapors and silica powder.*

6. Scrape off the silica gel band corresponding to **S.1e**. Grind the silica gel to a fine powder using a mortar and pestle.

7. Transfer the silica to an empty 1.5 × 15–cm chromatography column and elute with 100 mL of 65:35 (v/v) CH_2Cl_2/methanol under slight pressure of nitrogen until the yellow color almost disappears.

   The pressure should be adjusted so that the solvent elutes from the bottom of the column at ~3 to 5 drops/sec.

8. Perform analytical TLC on a 2.5 × 7–cm Kieselgel 60F plate using 85:15 (v/v) CH_2Cl_2/methanol (R_f **S1.b** = 0.49, R_f **S.1e** = 0.54).

   *When 8:2 (v/v) ethyl acetate/hexane is used as the eluent, the starting material **S.1b** and the product **S.1e** show an R_f of 0.1 and 0.38, respectively. The yield of **S.1e** is 60% (97 mg, 0.23 mmol). mp = 108°-110°C. ^{1}H-NMR ($CDCl_3$): δ: 1.55-1.56 (m, 4H, CH_2CH_2), 1.90 (m, 2H, CH_2), 1.99 (m, 2H, CH_2), 3.43 (t, 2H, J = 6.6 Hz, CH_2Br), 3.94-3.98 (m, 3H, CH_2N + NH), 4.0 (s, 3H, OCH_3), 7.13-7.86 (m, 5H, Acr), 8.00-8.04 (m, 1H, H-8 Acr). ^{13}C-NMR (DMSO-d_6): δ: 31.50, 33.32, 35.61, 38.22, 41.22, 55.14, 62.30, 109.04, 118.14, 121.42, 127.50, 129.96, 132.30, 134.15, 143.06, 145.80, 149.44, 152.30, 160.04, 161.75. Mass analysis. Electrospray ionization mass spectrometry (ESI-MS) polarity positive. Calcd. for $C_{20}H_{22}ClBrN_2O$: 420, 422, and 424; found: 421, 423, and 425 (M+H).*

FUNCTIONALIZATION OF A PSORALEN DERIVATIVE WITH AN IODOALKYL OR 2-PYRIDYLDITHIOALKYL LINKER

BASIC PROTOCOL 2

These syntheses are illustrated in Figure 4.8.2. The 5-(6-iodohexyloxy) derivative of psoralen **S.2f** is obtained by condensation of the hydroxyl derivative **S.2b** (*UNIT 4.3,* Fig. 4.3.1) with 1,6-diiodohexane in the presence of K_2CO_3, following a procedure adapted from the authors' previously published work (Takasugi et al., 1991). Replacement of the iodine atom of the 5-(6-iodohexyloxy) derivative **S.2f** by a 2-pyridyldithio group is achieved by a two-step procedure that differs from the one reported by Chassignol and Thuong (1998). First, a thioacetyl derivative **S.2g** is obtained by the reaction of **S.2f** with

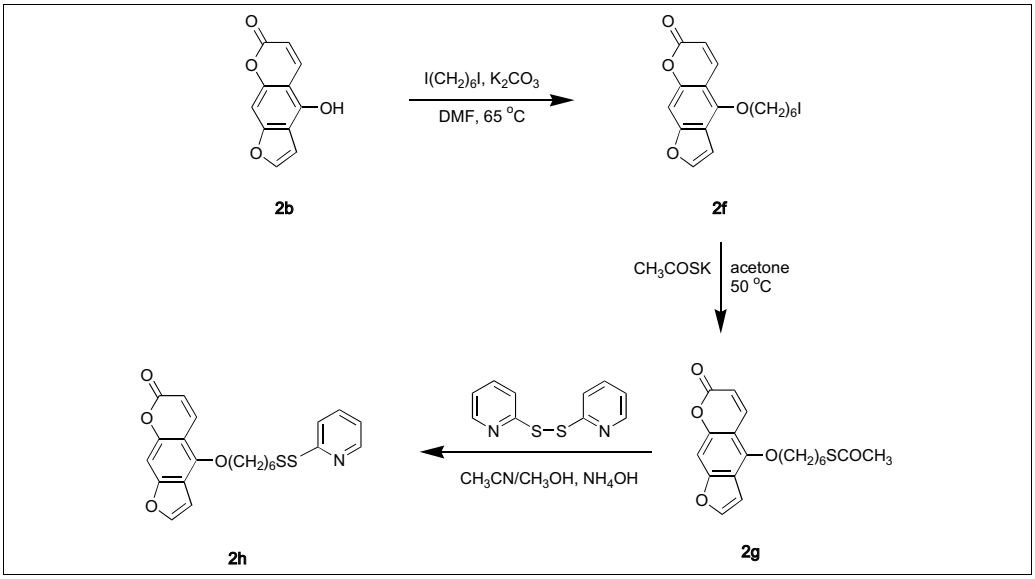

Figure 4.8.2 Functionalization of 5-hydroxypsoralen (**S.2b**) with an iodoalkyl linker (**S.2f**) or a 2-pyridyldithioalkyl linker (**S.2h**). DMF, *N,N*-dimethylformamide.

potassium thioacetate. The product **S.2g** is then hydrolyzed in situ by ammonia treatment and the resulting thiol derivative is reacted with 2,2′-dipyridyl disulfide to give the psoralen derivative **S.2h**.

Materials

5-Hydroxypsoralen **S.2b** (*UNIT 4.3*)
N,*N*-Dimethylformamide redistilled in vacuo over ninhydrin, stored over 4A molecular sieves
1,6-Diiodohexane (Aldrich)
Anhydrous potassium carbonate
Argon source
CH_2Cl_2
Distilled pentane (CE Instruments)
Distilled methanol
Nitrogen source
Potassium thioacetate (Aldrich)
Distilled acetone
Distilled ethyl acetate
Distilled hexane (CE Instruments)
Sodium sulfate (Aldrich)
2,2′-Dipyridyl disulfide (Aldrich)
Acetonitrile distilled from P_2O_5, stored over 3A molecular sieves
29% (v/v) aqueous ammonium hydroxide
2.5 mg/mL 2,6-dibromo-4-benzoquinone-*N*-chloroimine (DBPNC; Merck) in ethanol

100-mL and 25-mL round-bottom flasks and stoppers
Reflux condenser
$CaCl_2$ drying tube
Magnetic stirrer with temperature-controlled oil bath and Teflon stir bars
5-cm glass filter funnel (porosity 4)
Vacuum pump (oil pump) capable of creating <0.1 mmHg pressure, with manifold and cold trap
Kieselgel 60F chromatography column (e.g., 3-cm diameter, 50-cm height, 50 g silica gel; Merck)
2.5 × 7–cm analytical TLC plates (e.g., Kieselgel 60F plates, Merck)
Short-wave UV light box
Rotary evaporator with a water aspirator
Desiccator containing P_2O_5
7-cm filter funnel with filter paper
25-mL flask with stopper
Preparative 20 × 20–cm glass-backed silica gel TLC plates (2-mm thickness; e.g. Merck)
Mortar and pestle
1.5 × 15–cm chromatography column (empty)

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

Prepare 5-(6-iodohexyloxy)psoralen S.2f

1. Prepare a solution of 0.6 g (2.97 mmol) 5-hydroxypsoralen **S.2b** in 15 mL anhydrous *N*,*N*-dimethylformamide in a 100-mL round-bottom flask equipped with a reflux

condenser and a $CaCl_2$ drying tube. Stir reaction with a Teflon stir bar on a magnetic stirrer.

2. Add successively 5.4 mL (11.15 g, 33 mmol) 1,6-diiodohexane and 0.6 g (4.35 mmol) anhydrous potassium carbonate. Heat the stirred reaction mixture at 65°C for 4 hr in the dark under an argon atmosphere.

3. Allow the mixture to cool to room temperature and filter off the insoluble mineral salts by suction using a 5-cm glass filter funnel (porosity 4). Concentrate the filtrate to dryness using a vacuum pump.

4. Dissolve 14 g residue in 7 mL CH_2Cl_2 and purify on a 50-g Kieselgel 60F chromatography column eluting first with 200 mL of 50:50 (v/v) CH_2Cl_2/pentane to eliminate the excess diiodohexane, and then with increasing concentrations (0:100 to 4:96, v/v) of methanol in CH_2Cl_2. Perform chromatography under slight pressure of nitrogen.

   The pressure should be adjusted so that the solvent elutes from the bottom of the column at ~3 to 5 drops/sec.

5. Monitor fractions by analytical TLC using 2.5 × 7–cm Kieselgel 60F plates and 9:1 (v/v) CH_2Cl_2/methanol as the eluent. Visualize by UV shadowing and collect fractions containing pure product (R_f = 0.85).

6. Remove the solvent in a rotary evaporator with a water aspirator and wash the white solid **S.2f** with 2 mL pentane. Dry in a desiccator containing P_2O_5 for ≥3 to 4 hr.

   Yield 78% (0.96 g, 2.33 mmol). m.p = 90°-95°C. ^{1}H-NMR ($CDCl_3$): δ: 1.45-1.62 [m, 4H, $(CH_2)_2$], 1.81-1.94 [m, 4H, $(CH_2)_2$], 3.21 (t, 2H, J = 6.8 Hz,CH_2I), 4.45(t, 2H, J = 6.4 Hz, -CH_2OAr), 6.28 (d, 1H, J = 9.6 Hz, H-3 Pso), 6.94 (d, 1H, J = 2.45 Hz, H′-4 Pso), 7.1 (s, 1H, H-8 Pso), 7.58 (d, 1H, J = 2.5 Hz, H′-5 Pso), 8.16 (d, 1H, J = 9.8 Hz, H-4 Pso). ^{13}C-NMR (DMSO-d_6): δ: 15.13, 30.50, 35.36, 35.78, 38.97, 78.70, 99.44, 111.80, 112.29, 118.64, 119.22, 145.70 (d, J = 95 Hz), 152.15 (d, J = 145.5 Hz), 154.98, 158.34, 163.84, 166.32, 152.30, 160.04, 161.75.

Prepare 5-[6-(acetylthio)hexyloxy]psoralen S.2g

7. Place the following in a 25-mL round-bottom flask equipped with a reflux condenser:

   200 mg (0.48 mmol) 5-(6-iodohexyloxy)-psoralen **S.2f**
   65.8 mg (0.57 mmol) potassium thioacetate
   10 mL anhydrous acetone
   Heat at 50°C under magnetic stirring.

8. Monitor the reaction by analytical TLC and UV shadowing, using 3:1 (v/v) ethyl acetate/hexane as the eluent.

   *After 2 hr, the iodinated compound **S.2f** (R_f = 0.45) is completely transformed into the thiol ester **S.2g** (R_f = 0.34). The reaction can also be monitored using 96:4 (v/v) CH_2Cl_2/acetone (R_f **S.2f** = 0.72, R_f **S.2g** = 0.60).*

9. Remove the solid by gravity filtration through a 7-cm filter funnel fitted with filter paper.

10. Dilute the solution with 50 mL CH_2Cl_2 and wash the organic solution three times with 5 mL water.

11. Dry the organic phase over sodium sulfate and concentrate to dryness using a rotary evaporator with a water aspirator.

12. Stir residue with 2 mL hexane to obtain a solid.

Yield 93% (163 mg, 0.44 mmol). mp = 68°-70°C. ^{1}H-NMR ($CDCl_3$): δ: 1.42-1.70 (m, 6H, $(CH_2)_3$), 1.82-1.93 (m, 2H, CH_2), 2.34 (s, 3H, CH_3), 2.89 (t, 2H, J = 7.1 Hz, -CH_2S), 4.34 (t, 2H, J = 6.4 Hz, -CH_2OAr), 6.28 (d, 1H, J = 9.6 Hz, H-3 Pso), 6.94 (d, 1H, J = 2.4 Hz, H′-4 Pso), 7.13 (s, 1H, H-8 Pso), 7.58 (d, 1H, J = 2.3 Hz, H′-5 Pso), 8.15 (d, 1H, J = 9.6 Hz, H-4 Pso). ^{13}C-NMR (DMSO-d_6): δ: 31.07, 34.01, 34.50, 35.30, 36.76, 61.10, 78.70, 99.40, 111.80, 112.26, 118.60, 119.20, 145.67 (d, J = 87.5 Hz), 152.11 (d, J = 138 Hz), 154.98, 158.34, 163.86, 166.32, 201.51. Mass analysis. ESI-MS polarity positive. Calcd. for $C_{19}H_{20}O_5S$: 360; found: 361 (M+H).

Prepare 5-[6-(2-pyridyldithio)hexyloxy]psoralen S.2h

13. Place the following (in the order listed) in a 25-mL stoppered flask:

    150 mg (0.41 mmol) 5-[(6-acetylthio)hexyloxy]psoralen **S.2g**
    0.458 g (2.05 mmol) 2,2′-dipyridyl disulfide
    10 mL 50:50 (v/v) acetonitrile/methanol
    0.1 mL 29% aqueous ammonium hydroxide.

    Leave the mixture to react over 2 to 3 days at room temperature.

14. Monitor the reaction by analytical TLC and UV shadowing, using 3:7 (v/v) ethyl acetate/hexane as the eluent. Spray the TLC plate with 2.5 mg/mL DBPNC in ethanol and heat until color appears.

    *After 60 hr, the starting material **S.2g** (R_f = 0.48) is totally transformed into a new product (R_f = 0.36). 5-[6-(2-Pyridyldithio)hexyloxy]psoralen **S.2h** appears as a bright yellow-colored spot.*

15. Concentrate the solution under reduced pressure using a rotary evaporator with a water aspirator.

16. Purify 650 mg of residue by preparative TLC using 20 × 20–cm glass-backed silica gel TLC plates and 96:4 (v/v) CH_2Cl_2/acetone as the eluent. Elute plate two times. Visualize by UV shadowing at 365 nm.

    ***S.2h** can be seen as a pale blue band.*

    CAUTION: *It is preferable to carry out steps 16 to 18 in a fume hood to avoid breathing solvent vapors and silica powder.*

17. Scrape off the silica gel band corresponding to **S.2h**. Grind the silica gel to a fine powder using a mortar and pestle.

18. Transfer the silica to an empty 1.5 × 15–cm chromatography column and elute with 100 mL of 85:15 (v/v) CH_2Cl_2/acetone under slight pressure of nitrogen until the product is eluted (as monitored by TLC and UV shadowing).

    The pressure should be adjusted so that the solvent elutes from the bottom of the column at ~3 to 5 drops/sec.

    Yield = 86% (140 mg, 0.35 mmol). ^{1}H-NMR ($CDCl_3$): δ: 1.50-1.58 (m, 4H, $(CH_2)_2$), 1.72-1.78 (m, 2H, CH_2), 1.81-1.89 (m, 2H, CH_2), 2.81(t, 2H, J = 7.2 Hz, -CH_2-S-S), 4.43 (t, 2H, J = 6.4 Hz, -CH_2OAr), 6.27 (d, 1H, J = 9.8 Hz, H-3 Pso), 6.93 (d, 1H, J = 2.1 Hz, H′-4 Pso), 7.05-7.09 (m, 1H, Ar), 7.18 (s, 1H, H-8 Pso), 7.58 (d, 1H, J = 2.3 Hz, H′-5 Pso), 7.60-7.72 (m, 2H, Ar), 8.14 (d, 1H, J = 9.8 Hz, H-4 Pso), 8.45-8.47 (m, 1H, Ar). ^{13}C-NMR (DMSO-d_6): δ: 31.14, 33.59, 34.42, 35.36, 44.16 (t, J = 153 Hz), 78.70, 99.44, 111.82, 112.27, 118.62, 119.21, 125.43 (d, J = 116.5 Hz), 127.25, 143.92 (d, J = 116.5 Hz), 145.67 (d, J = 94.5 Hz), 152.11 (d, J = 138.5 Hz), 154.99, 155.68, 158.3, 163.85, 165.61, 166.32. Mass analysis. ESI-MS polarity positive. Calcd. for $C_{22}H_{21}NO_4S_2$: 427; found: 429 (M+H).

FUNCTIONALIZATION OF AN ORTHOPHENANTHROLINE DERIVATIVE WITH A BROMOALKYL LINKER OR AN ISOTHIOCYANATE GROUP

These syntheses are illustrated in Figure 4.8.3. The preparation of 5-(ω-bromohexanoamido)-1,10-phenanthroline **S.3c** was adapted from a previously reported two-step procedure (Thuong and Asseline, 1991). Reduction of 5-nitro-1,10-phenanthroline **S.3a** by ammonium sulfide leads to the 5-amino derivative **S.3b**, and acylation of the latter with 6-bromohexanoyl chloride gives the bromoalkyl derivative **S.3c**. Alternatively, the isothiocyanate derivative **S.3d** is obtained by treatment of the amino derivative **S.3b** with carbon disulfide in the presence of dicyclohexylcarbodiimide in pyridine.

Materials

20% (w/v) aqueous ammonium sulfide
Nitrogen source
5-Nitro-1,10-phenanthroline (**S.3a**; Aldrich)
Absolute ethanol, stored over 4A molecular sieves
Chloroform
Sodium sulfate, anhydrous (Aldrich)
Acetonitrile distilled from P_2O_5, stored over 3A molecular sieves (for **S.3c** only)
N,*N*-Diisopropylethylamine (Aldrich; for **S.3c** only)
6-Bromohexanoyl chloride (Aldrich; for **S.3c** only)
5% (v/v) aqueous $NaHCO_3$ (for **S.3c** only)
Dichloromethane (CH_2Cl_2) distilled from P_2O_5 and passed over basic aluminum oxide

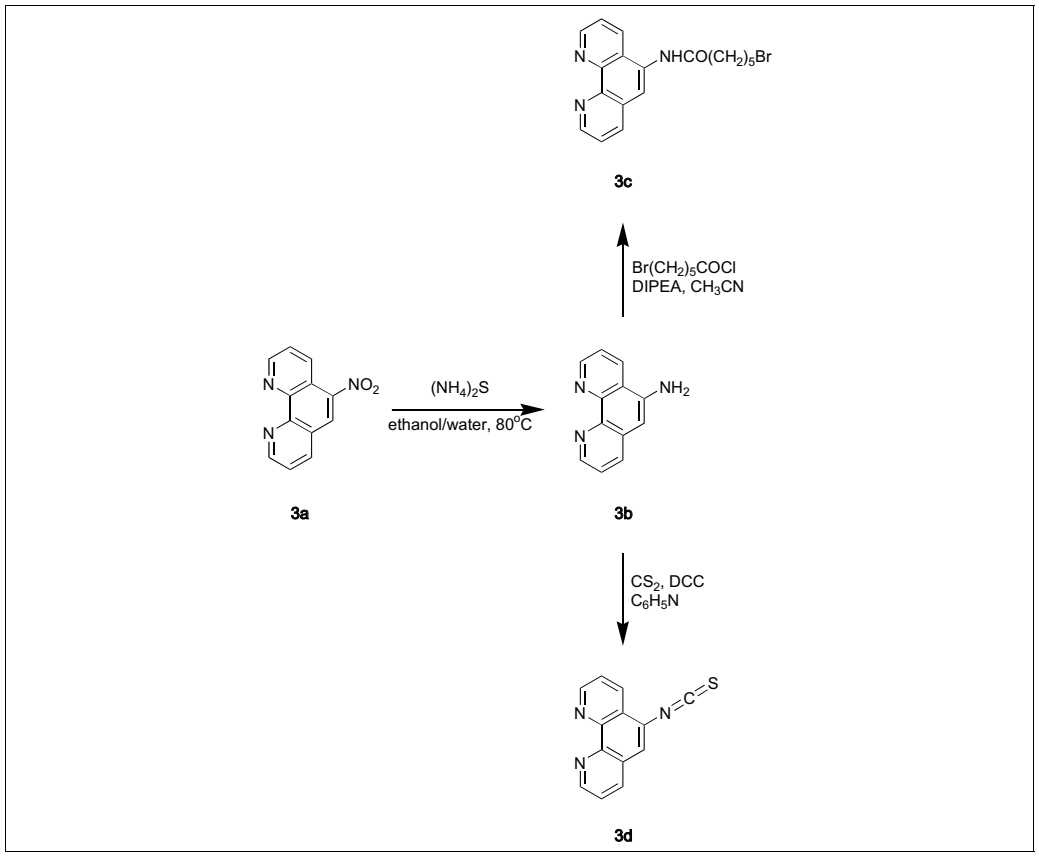

Figure 4.8.3 Derivatization of orthophenanthroline (**S.3a**) with a bromoalkyl linker (**S.3c**) or an isothiocyanate group (**S.3d**). DCC, 1,3-dicyclohexylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine.

Methanol
Pyridine redistilled from *p*-toluenesulfonylchloride, stored over 3A molecular sieves (for **S.3d** only)
1,3-Dicyclohexylcarbodiimide (Aldrich; DCC; for **S.3d** only)
CS_2 (Merck; for **S.3d** only)
Dioxane, freshly distilled (Aldrich; for **S.3d** only)

500-mL three-necked flask
Reflux condenser
100-mL dropping funnel
0.5-cm nitrogen inlet tube
Magnetic stirrer with temperature-controlled oil bath and Teflon stir bars
500-mL separatory funnel
7-cm filter funnel and filter paper
Rotary evaporator with water aspirator
5-cm glass filter funnel (porosity 4)
Desiccator containing P_2O_5
25-mL flask with rubber stopper
Neutral-activated chromatography column, 1.6-cm diameter, 50-cm height (e.g., 40 g of Kieselgel 60 or Aluminumoxid 90; Merck; for **S.3c** only)
2.5 × 7–cm analytical TLC plates (e.g., Kieselgel 60F or Aluminumoxid $60F_{254}$ neutral, Type E; Merck)
Short-wave UV light box
10-mL round-bottom flask with rubber septa and glass stoppers (for **S.3d** only)
Silica gel column, 1.5-cm diameter, 45-cm height (17 g Kieselgel 60; Merck; for **S.3d** only)

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

Prepare 5-amino-1,10-phenanthroline S.3b

1. Under an efficient fume hood, place 60 mL (0.17 mol) of 20% aqueous ammonium sulfide in a 500-mL three-necked flask equipped with a reflux condenser, a 100-mL dropping funnel, and a 0.5-cm nitrogen inlet tube. Heat the flask to 65°C under a nitrogen atmosphere.
2. Dissolve 1 g (4.48 mmol) of 5-nitro-1,10-phenanthroline (**S.3a**) in 40 mL boiling absolute ethanol and transfer this solution to the dropping funnel. Add this solution dropwise over 1 hr to the magnetically stirred 20% aqueous ammonium sulfide solution at 80°C.
3. Add another 25 mL of 20% aqueous ammonium sulfide and reflux for an additional hour.
4. Allow the stirred solution to cool to room temperature and extract four times with 70 mL chloroform.
5. Pool the chloroform extracts and back-extract two times with 20 mL water using a 500-mL separatory funnel.
6. Dry the organic solution over anhydrous sodium sulfate and remove the drying agent by gravity filtration through a 7-cm funnel fitted with filter paper. Concentrate the filtrate in a rotary evaporator with a water aspirator.
7. Dissolve 680 mg yellow residue in 20 mL boiling absolute ethanol, filter, and add 15 mL water to the filtrate. Stopper the flask and let stand 2 days at 2° to 5°C.

8. Collect the yellow crystals by suction using a 5-cm glass filter funnel (porosity 4) and dry in a desiccator containing P_2O_5 for at least one night.

Yield, 60% (520mg, 2.66 mmol). mp = 250°C (with decomposition at 255°-260°C). ^{1}H-NMR (DMSO-d_6): δ: ppm, 6.10 (b s, 2H, NH_2), 6.85 (s, 1H), 7.47-7.50 (m, 1H), 7.70-7.73 (m, 1H), 8.02-8.04 (m, 1H), 8.65-8.67 (m, 2H), 9.03-9.05 (m, 1H). ^{13}C-NMR (DMSO-d_6): δ: 107.98 (d, J = 153 Hz), 128.09 (d, J = 58.5 Hz), 128.38, 129.40 (d, J = 65.5 Hz), 136.79, 137.01, 138.95 (d, J = 109 Hz), 146.67, 148.89, 150.96, 152.37, 155.54 (d, J = 73 Hz). Mass analysis. ESI-MS polarity positive. Calcd. for $C_{12}H_9N_3$: 195; found: 197 (M+H).

Prepare 5-(ω-bromohexanoamido)-1,10-phenanthroline S.3c

9a. Dry 5-amino-1,10-phenanthroline **S.3b** (step 8) by coevaporating three times with 10 mL anhydrous acetonitrile.

10a. Combine the following with magnetic stirring in a 25-mL stoppered flask:

0.195g (1 mmol) 5-amino-1,10-phenanthroline **S.3b**
0.297 g (0.4 mL, 2.30 mmol) *N*,*N*-diisopropylethylamine
15 mL acetonitrile.

11a. Add dropwise, at room temperature, a solution of 0.245 g (0.176 mL, 1.15 mmol) of 6-bromohexanoyl chloride in 0.3 mL anhydrous acetonitrile using a syringe and needle inserted through the rubber stopper.

The mixture becomes homogeneous during the course of the addition.

12a. Add 5 mL of 5% aqueous $NaHCO_3$ and extract three times with 20 mL dichloromethane.

13a. Pool the organic extracts and back-extract three times with 8 mL water.

14a. Dry the organic phase over anhydrous sodium sulfate and remove the drying agent by gravity filtration through a funnel fitted with filter paper. Evaporate in a rotary evaporator with a water aspirator.

15a. Dissolve 750 mg yellow residue in 1 mL CH_2Cl_2 and purify on a 40-g neutral-activated chromatography column, eluting with (in order) 200 mL each 99:1, 98:2, and 97:3 (v/v) CH_2Cl_2/methanol. Perform chromatography under slight pressure of nitrogen.

The pressure should be adjusted so that the solvent elutes from the bottom of the column at ~3 to 5 drops/sec.

16a. Monitor by analytical TLC and UV shadowing, using Aluminumoxid 60F_{254} plates and 97:3 (v/v) CH_2Cl_2/methanol. Collect fractions containing pure product (R_f **S.3c** = 0.3).

Since the compound decomposes after a few weeks of storage at –20°C, it should be stored at –70°C.

Yield = 65% (0.230 g, 0.62 mmol). mp= 90°-95°C (decomposition). ^{1}H-NMR ($CDCl_3$) δ ppm, 1.60-1.92 (m, 6H, $(CH_2)_3$), 2.58 (m, 2H, $CH_2C(O)$), 3.43 (t, 2H, J = 6.4 Hz, CH_2Br), 7.24 (s, 1H), 7.58-7.59 (m, 2H), 7.90 (bs, 1H), 8.15-8.17 (m, 1H), 8.29-8.31 (m, 1H), 9.08-9.13 (m, 2H). ^{13}C-NMR (DMSO-d_6): δ: 30.58, 33.51, 38.26, 41.32, 41.97, 126.15, 129.10, 129.75, 130.86, 134.31, 137.88, 142.03, 149.94, 152.02, 155.43, 156.10, 178.48. Mass analysis. ESI-MS polarity positive. Calcd. for $C_{18}H_{18}N_3BrO$: 371 and 373; found: 372 and 374 (M+H).

Prepare 5-(1,10-phenanthroline) isothiocyanate S.3d

9b. Dry 5-amino-1,10-phenanthroline **S.3b** (step 8) by coevaporating two times with 5 mL anhydrous pyridine.

10b. Combine the following in a 10-mL round-bottom flask equipped with a stir bar:

97.6 mg (0.5 mmol) 5-amino-1,10-phenanthroline **S.3b**
412.6 mg (2 mmol) 1,3-dicyclohexylcarbodiimide
2.5 mL anhydrous pyridine
152.28 mg (126 µL, 2 mmol) CS_2.

11b. Stir the mixture at room temperature and monitor the reaction by analytical TLC on a Kieselgel 60F plate using 9:1 (v/v) CH_2Cl_2/methanol as the eluent. Visualize by UV shadowing.

*The starting material **S.3b** (R_f = 0.23; yellow spot by UV shadowing at 254 nm) is transformed into the isothiocyanate derivative **S.3d** (R_f = 0.54; blue spot). **S.3b** and **S.3d** are poorly resolved on TLC plates.*

12b. When the reaction is complete (usually 24 hr), remove pyridine and excess CS_2 by evaporation.

13b. Take up the residue with 3 mL freshly distilled dioxane. Stir the mixture for 5 min and filter off the insoluble material by gravity using a funnel fitted with filter paper. Concentrate the filtrate using a rotary evaporator with a water aspirator.

Dioxane should be freshly distilled to avoid peroxides.

14b. Purify 250 mg residue on a 17-g Kieselgel 60 column using the following eluents (in order): 100 mL CH_2Cl_2, 50 mL of 98:2 (v/v) CH_2Cl_2/methanol, and 100 mL of 95:5 (v/v) CH_2Cl_2/methanol. Perform chromatography under slight pressure (see step 15a).

Yield 76% (90 mg, 0.38 mmol). mp = 91°-93°C. ^{1}H-NMR ($CDCl_3$): δ: ppm, 7.27 (s, 1H), 7.54-7.57 (m, 1H), 7.63-7.66 (m, 1H), 8.11-8.13 (m, 1H), 8.57-8.59 (m, 1H), 9.06-9.07 (m, 1H), 9.18-9.19 (m, 1H). ^{13}C-NMR (DMSO-d_6): δ: 119.06, 129.68, 131.47, 134.79, 138.20, 141.84, 144.07, 148.14, 150.08, 151.45, 152.19, 155.11, 156.61. Mass analysis. ESI-MS polarity positive. Calcd. for $C_{13}H_7N_3S$: 237; found: 239 (M+H).

BASIC PROTOCOL 4

FUNCTIONALIZATION OF A THIAZOLE ORANGE DERIVATIVE WITH AN IODOALKYL LINKER

The preparation of iodooctylthiazole orange, illustrated in Figure 4.8.4, is achieved by a procedure adapted from the literature (Brooker et al., 1942; Benson et al., 1993). 3-Methyl-2-(methylthio)benzothiazolium iodide **S.4b** reacts with *N*-(8-iodooctyl)-4-methylquinolium iodide **S.4d** to give iodooctylthiazole orange **S.4e**.

Materials

3-Methyl benzothiazole-2-thione **S.4a**
Methyl iodide (Fluka)
Distilled methanol
Diethyl ether, anhydrous
1,8-Diiodooctane (Aldrich)
Dioxane, freshly distilled (Aldrich)
Lepidine (Aldrich)
CH_2Cl_2
Nitrogen source

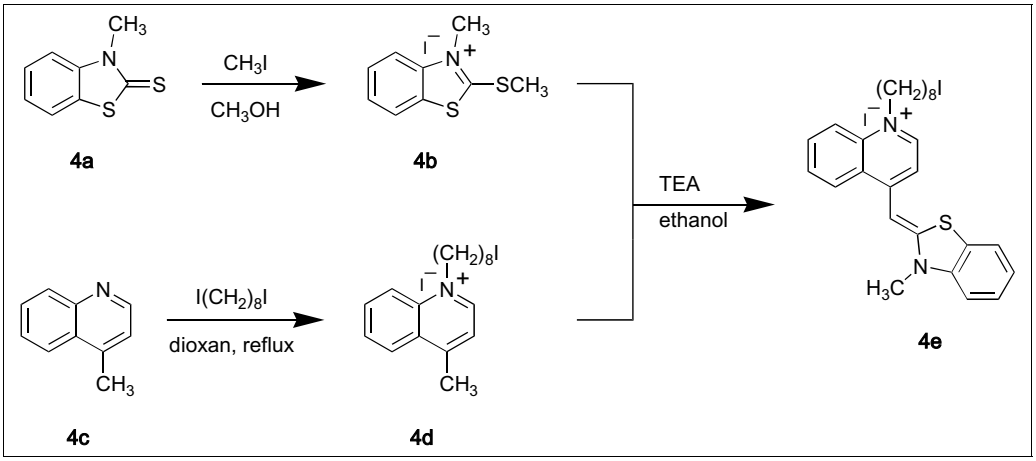

Figure 4.8.4 Thiazole orange derivative functionalized with an iodoalkyl linker (**S.4e**). TEA, triethylamine.

Ethanol, prewarmed (55°C)
Triethylamine (Merck)

100-mL and 10-mL round-bottom flask and rubber septa/glass stoppers
Magnetic stirrer with temperature-controlled oil bath and Teflon stir bars
Reflux condenser
5-cm glass filter funnel (porosity 4)
Desiccator containing P_2O_5
Two-necked round-bottom flask
10-mL dropping funnel
Silica gel chromatography columns: 35 g, 2.5-cm diameter, 45-cm height; and 25 g, 1.2-cm diameter, 40-cm height (e.g., Kieselgel 60, Merck)
2.5 × 7–cm analytical TLC plates (e.g., Kieselgel 60F, Merck)
Short-wave UV light box

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

Prepare 3-methyl-2-(methylthio)benzothiazolium iodide S.4b

1. Mix the following in a 100-mL round-bottom flask equipped a Teflon stir bar, magnetic stirrer, and reflux condenser:

   2.40 g (13.24 mmol) 3-methyl benzothiazole-2-thione **S.4a**
   4.25 g (1.86 mL, 30 mmol) methyl iodide
   30 mL distilled methanol.

2. Reflux the magnetically stirred reaction mixture for 3 hr.

   *When **S.4a** dissolves, a yellowish precipitate appears.*

3. Cool the reaction mixture in an ice bath and filter the precipitate by suction using a 5-cm glass filter funnel (porosity 4).

4. Wash the precipitate twice with 10 mL dry diethyl ether. Remove solvent using a rotary evaporator with a water aspirator, and then dry in a desiccator containing P_2O_5.

   CAUTION: *Diethyl ether can form peroxides. It is a highly flammable solvent that should be handled with care.*

Yield 95% (4.04 g, 12.5 mmol). mp = 152°-154°C (148°-149°C). R_f = 0.20 by TLC and UV shadowing using CH_2Cl_2/methanol (9:1, v/v) as eluent. ^{1}H-NMR (DMSO-d_6): δ ppm, 3.12 (s, 3H, SCH_3), 4.10 (s, 3H, NCH_3), 7.71 (t, 1H, J = 7.8 Hz), 7.82 (t, 1H, J = 7.8 Hz), 8.18 (d, J = 8 Hz, 1H), 8.40 (d, J = 8 Hz, 1H). ^{13}C-NMR (DMSO-d_6): δ: 24.37 (d, J = 116.5 Hz), 42.76 (d, J = 87.5 Hz), 121.96 (d, J =87.5 Hz), 130.21 (d, J = 58 Hz), 133.24 (d, J = 109 Hz), 134.48, 135.40 (d, J = 116.5 Hz), 148.77, 187.48. Mass analysis. ESI-MS polarity positive. Calcd. for $C_9H_{10}NS_2$: 196; found: 197 (M+H).

Prepare N-(8-iodooctyl)-4-methylquinolium iodide S.4d

5. Stir a solution of 9.15 g (4.97 mL, 25 mmol) of 1,8-diiodooctane in 10 mL of refluxing dioxane in a two-necked round-bottom flask equipped with a reflux condenser and a 10-mL dropping funnel. Add dropwise 0.69 mL (0.746 g, 5 mmol) lepidine **S.4c** over 30 min with continued heating, and maintaining heating (reflux) for an additional 3 hr.

6. Stop heating and stir mixture for 16 hr at room temperature.

7. Decant the resulting brown oil, wash with anhydrous diethyl ether, and dry under vacuum.

8. Purify on a 35-g silica gel chromatography column using 200 mL of 99:1 (v/v) CH_2Cl_2/methanol and then 400 mL of 98:2 (v/v) CH_2Cl_2/methanol as the eluents. Perform chromatography under slight pressure of nitrogen.

   The pressure should be adjusted so that the solvent elutes from the bottom of the column at ~3 to 5 drops/sec.

   *Yield 53% (1.35 g, 2.6 mol). Brown oil R_f **S.4d** = 0.35 by TLC and UV shadowing using 9:1 (v/v) CH_2Cl_2/methanol as eluent. ^{1}H-NMR (DMSO-d_6) δ ppm, 1.17-1.36 (m, 8H, $(CH_2)_4$), 1.68-1.73 (m, 2H, CH_2), 1.90-1.93 (m, 2H, CH_2), 2.99 (s, 3H, CH_3), 3.24 (t, 2H, J = 7 Hz, CH_2I), 4.99 (t, 2H, J = 7.8 Hz, NCH_2), 8.03-8.07 (m, 2H, Ar), 8.23-8.26 (m, 1H, Ar), 8.53-8.60 (m, 2H, Ar), 9.41-9.42 (m, 1H). ^{13}C-NMR (DMSO-d_6): δ: 7.36, 15.31, 25.96 (d, J = 153 Hz), 31.85, 33.83, 34.46, 35.60, 38.94, 63.02 (d, J = 131.5 Hz), 125.54, 128.73 (d, J = 116.5 Hz), 133.40, 135.20, 135.80 (d, J = 109.5 Hz), 141.5 (d, J = 80 Hz), 142.96, 154.53 (d, J = 138.5 Hz), 164.76. Mass analysis. ESI-MS polarity positive. Calcd. for $C_{18}H_{25}IN$: 382; found: 382 (M+H).*

Prepare 8-iodooctylthiazole orange S.4e

9. In a 10-mL round-bottom flask equipped with magnetic stirrer, dissolve 0.156 g (0.48 mmol) **S.4b** and 0.246 g (0.48 mmol) **S.4d** in 3 mL warm (55°C) absolute ethanol.

10. Add 20 µL (0.15 mmol) triethylamine and leave the red-colored mixture with magnetic stirring at room temperature. Monitor the reaction by analytical TLC using 9:1 (v/v) CH_2Cl_2/methanol as the eluent. Visualize by UV shadowing.

    *The spots corresponding to **S.4b** (R_f = 0.20) and **S.4d** (R_f = 0.31) disappear while a new red-colored spot appears (R_f = 0.46). The reaction is complete after ~15 min.*

11. Remove solvent using a rotary evaporator with a water aspirator, and dry compound in a desiccator containing P_2O_5.

12. Purify 450 mg residue on a 25-g silica gel chromatography column using 600 mL of 99:1 (v/v) CH_2Cl_2/methanol as the eluent. Perform chromatography under slight pressure of nitrogen.

    Yield 62% (0.208 g, 0.29 mmol). mp = 123°-124°C. R_f = 0.46 using 9:1 (v/v) CH_2Cl_2/methanol as the eluent. ^{1}H-NMR (DMSO-d_6) δ ppm, 1.28-1.45 (m, 8H, $(CH_2)_4$), 1.75-1.78 (m, 2H, CH_2), 1.85-1.88 (m, 2H, CH_2), 3.15 (t, 2H, J = 7.3 Hz, CH_2I), 3.99 (s, 3H, NCH_3), 4.99 (t, 2H, J = 7.5 Hz, NCH_2), 6.70 (s,1H), 7.24-7.25 (m, 1H), 7.27-7.32 (m,1H), 7.44-7.52 (m, 1H), 7.52 (d, J = 8Hz 1H), 7.62 (d, J = 8Hz 1H), 7.66-7.73 (m, 2H), 8.81(d, J = 8Hz, 1H),

8.90 (d, J = 8Hz, 1H). ^{13}C-NMR (DMSO-d_6): δ: 15.34, 31.95, 33.88, 34.52, 34.96, 35.88, 38.94, 40.10, 60.35, 94.33 (d, J = 145.5 Hz), 114.05, 119.21 (d, J = 116.5 Hz), 124.25, 129.11 (d, J = 116.5 Hz), 130.07, 130.47, 130.71, 132.07, 133.01 (d, J = 131 Hz), 134.47, 139.40, 143.24, 146.70, 150.55, 154.77, 166.26. Mass analysis. ESI-MS polarity positive. Calcd. for $C_{26}H_{30}IN_2S$: 529; found: 529 (M+H).

COMMENTARY

Background Information

The attachment of conjugate groups to the 5′ terminus of oligodeoxyribonucleotides can be achieved following two strategies. The first strategy is direct incorporation using phosphoramidites of ligands. The second strategy involves the coupling of an unblocked oligomer with the ligand via a specific reaction between the reactive groups present in both entities. This requires the functionalization of ligands with halogenoalkyl, 2-pyridyldithioalkyl, or isothiocyanate groups (described here) and the addition of amino, carboxyl, thiophosphate, phosphate, and thiol groups to the 5′ ends of oligodeoxyribonucleotides (*UNIT 4.9*).

The preparation oligonucleotide-ligand conjugates by coupling a functionalized ligand to an unprotected oligonucleotide carrying a suitable functional group offers many advantages over the direct incorporation of the ligands during the oligonucleotide synthesis. In particular, this method is very useful when only a small amount of ligand is available, when the ligand does not resist the chemical conditions required for oligonucleotide deprotection, or when its poor solubility in the solvents typically used for oligonucleotide synthesis does not allow preparation of phosphoramidite or *H*-phosphonate derivatives. Furthermore, provided that the required amount for each conjugate is low, this method is more convenient method than the direct method for obtaining many different oligodeoxyribonucleotide-ligand conjugates starting from only one oligodeoxyribonucleotide synthesis.

This unit describes the attachment of a halogenoalkyl and 2-pyridyldithioalkyl linker to a ligand for coupling with an oligodeoxyribonucleotide functionalized with either a 5′-thiophosphate or a 5′-pyridyldisulfide group. The use of a halogenoalkylated ligand yields a conjugate with a stable phosphothioloester or thioether linkage. The use of a 2-pyridyldithioalkylated ligand yields a conjugate with a disulfide bond that can be cleaved by the use of a reducing agent. The third option reported in this unit is the attachment of an isothiocyanate group to a ligand for coupling with an oligodeoxyribonucleotide that carries an aminoalkylated linker.

Procedures reported in this unit with acridine, psoralen, orthophenanthroline, and thiazole orange may be used to prepare other families of ligands. Using these procedures, the parameters of linkage between the oligodeoxyribonucleotide and the ligand—such as the size and nature of the linker used to connect the two entities—may be varied to prepare oligodeoxyribonucleotide-ligand conjugates with optimal properties. For many examples reported, the properties of the oligodeoxyribonucleotide-ligand conjugates are largely dependent on the geometry of the complex formed between the ligand and its target (Takasugi et al., 1991; Giovannangeli et al., 1992; Costes et al., 1993; Grigoriev et al., 1993). For example, the strongest stabilization is achieved when the ligand is an intercalator (Sun et al., 1989; Asseline et al., 1996; Giovannangeli et al., 1996), the best efficiency of cleavage when the ligand is a cleaving agent (François et al. 1989a,b), or effective cross-linking when the ligand is a reactive group (Takasugi et al., 1991).

Critical Parameters and Troubleshooting

Protection of flasks and columns from light throughout the steps of these protocols is recommended since acridine, psoralen, and thiazole orange derivatives are sensitive to light. This can be achieved by wrapping flasks and columns with aluminum foil. Purification steps must also be kept as short as possible.

In order to avoid low yields during purification, it is important to completely remove solvents such as dimethylformamide, pyridine, and ethanol, and to pack the columns with extreme care.

The use of nonanhydrous solvents can affect the yields obtained. Storing the products in hermetically sealed vials at –20°C in the dark is recommended. Under these conditions, good reactivity of these compounds is usually observed after months or even years of storage. The bromoalkylated phenanthroline derivative, however, may show significant degradation after a few months at –20°C.

Flasks and vials containing functionalized ligands should always be allowed to reach room temperature before being opened in order to

avoid moisture, which can cause sticky compounds (instead of solids) to be obtained. These sticky compounds are difficult to handle and have reduced stability over time.

R_f values given in this unit correspond to TLC analyses performed on 2.5 × 7–cm aluminum-backed sheets developed in jars (diameter 4.5 cm, height 8 cm) with caps, using the appropriate mixtures of solvents. The use of jars and plates with different sizes can give different R_f values.

Anticipated Results

The yields reported for the various steps can be different when the syntheses are performed at scales other than those described. Using the protocols provided here and starting with 138 mg of the hydroxyalkylated acridine derivative **S.1b**, 97 mg of the purified bromoalkylated derivative **S.1e** can be obtained.

In the case of the psoralen derivatives, starting with 600 mg of the 5-hydroxypsoralen **S.2b**, it is possible to obtain 970 mg of the iodoalkylated psoralen derivative **S.2f**. The latter (200 mg) is then used to prepare the acetylthio derivative **S.2g** (163 mg), which is then transformed into the 2-pyridyldithioalkylated compound **S.2h** (140 mg).

Starting with 1 g of 5-nitro-1,10-phenanthroline **S.3a**, 520 mg of the amino derivative **S.3b** can be obtained. Using 195 mg of the latter, it is possible to obtain 245 mg of the bromoalkylated derivative **S.3c**. Alternatively, starting with 97 mg of the amino derivative **S.3b**, 90 mg of the isothiocyanate derivative **S.3d** can be obtained.

In the case of the thiazole orange derivative preparation, starting with 2.4 g thione **S.4a**, 4.04 g of **S.4b** can be obtained; using 0.746 g of lepidine **S.4c**, it is possible to prepare 1.35 g of the iodoalkylated lepidine derivative **S.4d**. Reaction between 0.156 g of **S.4b** and 0.246 g of **S.4d** can give 0.208 g of thiazole orange **S.4e**.

Time Considerations

Provided that all reagents and materials required for each step are available, most of the procedures are simple and rapid. Preparation of compound **S.1e** requires 2 to 3 days while only 2 days are necessary for compound **S.2f**.

Compound **S.2g** can be obtained, starting from derivative **S.2f**, in 1 day; its transformation into **S.2g** requires 3 to 4 days. The time required for the preparation of compound **S.3b** is usually 3 days (including 2 days for the crystallization step), and the time required for the synthesis of **S.3c** and **S.3d** is 1 and 2 days, respectively. The synthesis of compounds **S.4b** and **S.4d** requires 1 and 2 days, respectively. Starting from **S.4b** and **S.4d**, compound **S.4e** is obtained in 1 day.

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The authors would like to express their appreciation to their past and present collaborators for their contribution to the development of various families of oligonucleotide-ligand conjugates. This work was supported by Rhône-Poulenc, the Agence Nationale de Recherches sur le SIDA and bio-Mérieux.