Direct Attachment of Conjugate Groups to the 5′ Terminus of Oligodeoxyribonucleotides

The attachment of conjugate groups (intercalating and photoreactive groups) to the 5′ terminus of oligodeoxyribonucleotides can be achieved using either of two strategies. This unit presents one strategy, the direct incorporation of ligands during oligodeoxyribonucleotide synthesis using phosphoramidite derivatives. The second strategy, coupling of an unblocked oligomer with a ligand via a specific reaction between the reactive groups present in both entities, will be presented in future units. The protocols delineated below cover the direct addition of an intercalator, 2-methoxy-6-chloro-9-aminoacridine (see Basic Protocol 1), and a photo-cross-linking reagent, psoralen (see Basic Protocol 2), to the 5′ end of oligonucleotides via their phosphoramidite derivatives. These procedures require the following steps: the incorporation of hydroxylated linkers into ligands, the preparation of the phosphoramidite derivatives, and the addition of the latter to the 5′ ends of protected oligonucleotides bound to the support. After deprotection, the oligodeoxyribonucleotide conjugates are purified and characterized (see Support Protocol). Properties of a number of conjugates synthesized by these procedures are discussed in *UNIT 4.2*.

CAUTION: All chemicals must be handled in a fume hood by individuals equipped with lab coats, glasses, and gloves.

DIRECT ADDITION OF ACRIDINE DERIVATIVES TO THE 5′ END OF OLIGODEOXYRIBONUCLEOTIDES

BASIC PROTOCOL 1

This strategy (illustrated in Figs. 4.3.1, 4.3.2, and 4.3.3) involves the covalent attachment of a linker carrying a hydroxyl function to the acridine derivative **S.1b**, and the preparation of the acridinyl phosphoramidite derivative **S.1c**. The acridinyl phosphoramidite derivative can be coupled either automatically or manually to the 5′-terminal hydroxyl group of oligodeoxyribonucleotides bound to solid supports. After deprotection, the acridine-oligodeoxyribonucleotide conjugate **S.1d** is purified and characterized as described in the Support Protocol.

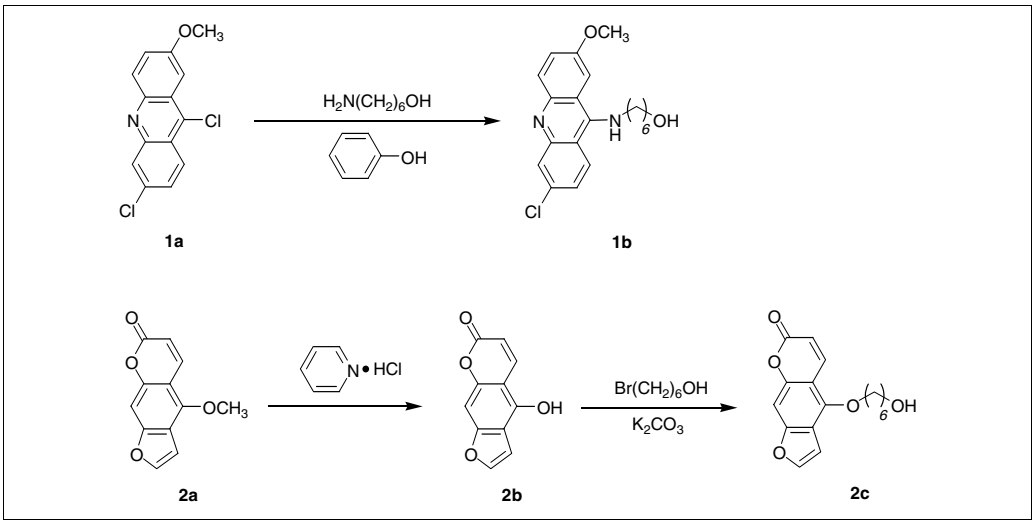

Figure 4.3.1 Synthesis of acridine and psoralen derivatives functionalized with hydroxylated linkers.

Contributed by Ulysse Asseline and Nguyen T. Thuong
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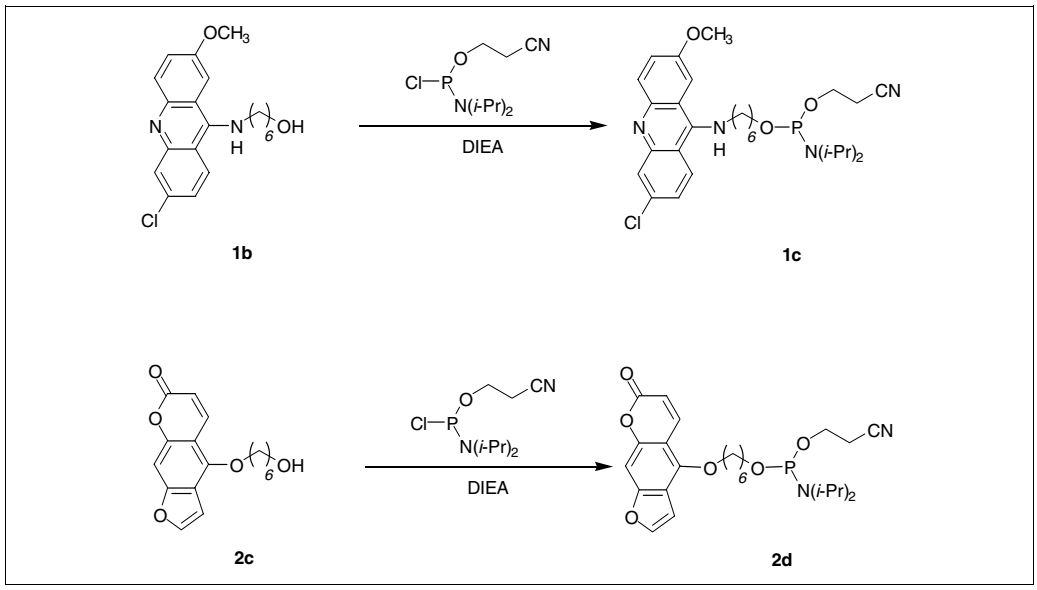

Figure 4.3.2 Synthesis of the phosphoramidite derivatives of acridine and psoralen ligands.

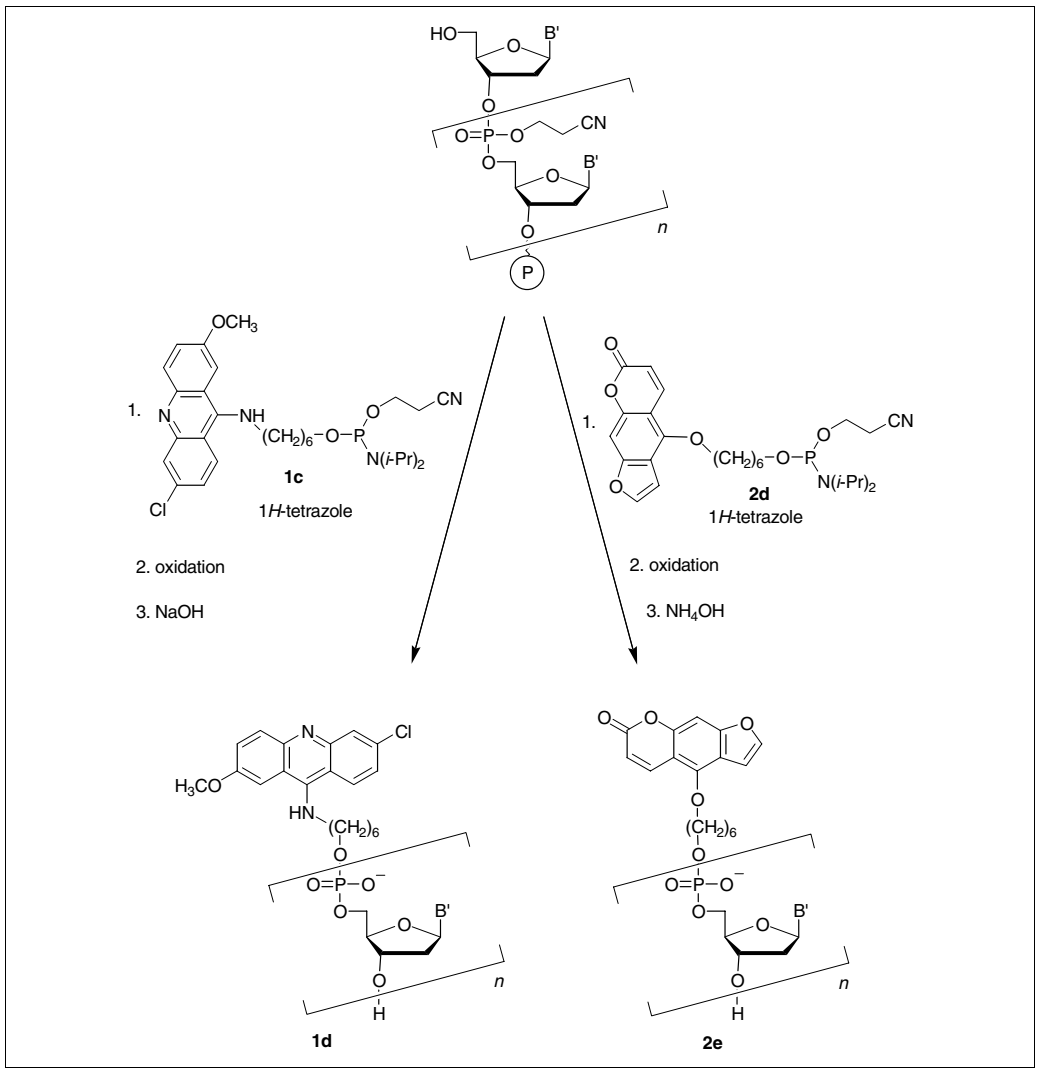

Figure 4.3.3 Direct addition of ligands to the 5′ terminus of oligodeoxyribonucleotides.

Materials

6-Amino-1-hexanol
6,9-Dichloro-2-methoxyacridine
Phenol
Dichloromethane (CH_2Cl_2), distilled from P_2O_5 and passed over activated, basic aluminum oxide
Distilled methanol (MeOH)
2 M sodium hydroxide (NaOH)
Acetonitrile (CH_3CN), HPLC grade
2,6-Dibromo-4-benzoquinone-*N*-chloroimine (DBPNC; Prolabo)
Ethanol, distilled
P_2O_5
Acetonitrile (CH_3CN), DNA synthesis grade, anhydrous and stored over 3-Å molecular sieves
N,*N*-Diisopropylethylamine (DIEA), distilled from KOH
2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphite
Ethyl acetate, distilled
Hexane, distilled
Triethylamine (Et_3N), distilled from KOH
10% (w/v) Na_2CO_3
Saturated NaCl (brine)
$MgSO_4$
Oligodeoxyribonucleotide bound to solid supports, such as controlled-pore glass (CPG; *UNIT 3.1*), synthesized by classic phosphoramidite chemistry (1-µmol scale; *UNIT 3.3* and *APPENDIX 3C*)
DNA synthesis reagents recommended by the synthesizer's manufacturer
0.4 M sodium hydroxide (NaOH) in 50:50 (v/v) MeOH/H_2O
Dowex 50 resin, pyridinium form (Aldrich)

Round-bottomed flasks (various sizes) with rubber septa and glass stoppers
Drying tube containing $CaCl_2$
Reflux condenser
Analytical thin-layer chromatography (TLC) setup and silica-gel plates: e.g., Merck 5554 Kieselgel 60F plates, including UV lamp for detection
Desiccator
Beakers, various sizes
Silica gel for column chromatography: Merck 9387 Kieselgel 60 or Merck 7734 Kieselgel 60
Chromatographic column (3 cm × 45 cm)
Argon
Nitrogen gas
Spectrophotometer
Rotary evaporator with water bath
Chemically inert syringes with replaceable needles
Funnel
Separatory funnels (various sizes)
Filter (porosity 4)
Vials and Teflon-faced septa
Disposable filters for syringes and for filtration of HPLC buffers
Liquid chromatography apparatus equipped with multiwavelength detector
Melting-point apparatus

Synthesize 2-methoxy-6-chloro-9-(ω-hydroxyhexylamino)-acridine S.1b

1. Place 840 mg (7.18 mmol) 6-amino-1-hexanol, 1 g (3.59 mmol) 6,9-dichloro-2-methoxyacridine, and 2.5 g phenol in a 25-mL round-bottomed flask equipped with a magnetic stir-bar, a reflux condenser, and a calcium chloride drying tube. Heat 80 to 90 min at 100°C.

2. Perform TLC analysis on a silica-gel plate using 90:10 (v/v) CH_2Cl_2/MeOH as the eluent to check that the reaction is complete. Dilute an aliquot 10-fold with MeOH and load 1 µl on the TLC plate.

   This should show the disappearance of 6,9-dichloro-2-methoxyacridine (R_f = 0.9) and the formation of a new compound (R_f = 0.18).

3. Allow the mixture to cool, dilute it with 2.5 mL MeOH, and add the solution dropwise to a magnetically stirred 2 M NaOH solution (20 mL contained in a 100-mL beaker). Maintain stirring for 15 min.

4. Filter the yellow precipitate using a porosity 4 filter and wash it with water until neutral. Crystallize twice from 20:80 (v/v) H_2O/MeOH.

   Expected yield: 80% (1.03 g, 2.87 mmol).

5. Analyze the product by TLC using silica-gel plates and 75:25 (v/v) CH_2Cl_2/MeOH as eluent. Dilute 1 to 2 mg in 100 µl MeOH and use 1 µl for spotting.

   *In addition to 2-methoxy-6-chloro-9-(ω-hydroxyhexylamino)-acridine (**S.1b**, R_f = 0.58),TLC analysis will show three side products, identified as the starting material 6,9-dichloro-2-methoxyacridine (R_f = 0.85), 2-methoxy-6-chloro-9-acridone (R_f = 0.81), and an unidentified third product (R_f = 0.39). These three products do not react during subsequent steps and are easily removed from the conjugates during purification.*

   Reversed-phase HPLC as reported in Asseline et al., 1986 can also be used.

   Purity should be ~95%.

   Characterize the product by other analytical methods as desired. Melting point: 152° to 153°C.

   ^{1}H-NMR (DMSO-d_6): δ 1.15-1.40 (m, 6H, $CH_2CH_2CH_2$), 1.60-1.75 (m, 2H, CH_2), 3.20-3.33 (m, 3H, CH_2N + NH), 3.61-3.75 (m, 2H, CH_2OH), 3.90 (s, 3H, OCH_3), 4.20-4.28, (m, 1H), 7.25-7.88 (m, 5H, Acr), 8.25-8.38 (m, 1H, H_8 Acr).

   Mass analysis: I.S. Polarity positive. Calculated for $C_{20}H_{23}ClN_2O_2$: M+H= 359.18 and 361.18. Found 359.5 and 361.5.

6. Check for the absence of sodium phenolate by TLC analysis using the conditions described in step 5. Place plate in fume hood and dry using a hair dryer. Spray the TLC plate with a solution of 50 mg 2,6-dibromo-4-benzoquinone-*N*-chloroimine (DBPNC) in 20 mL ethanol and heat with a TLC drying plate or in an oven at 100°–130°C for several minutes.

   Phenol will give a blue-colored spot.

Prepare [2-methoxy-6-chloro-9-(ω-hexylamino)-acridinyl]-(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite, S.1c

7. Place 300 mg (0.83 mmol) 2-methoxy-6-chloro-9-(ω-hydroxyhexylamino)acridine, **S.1b**, in a 25-mL round-bottomed flask and dry it by three rounds of coevaporation with anhydrous acetonitrile (3 × 5 mL).

8. Place a stir bar in the flask and stopper it with a septum. Push a needle through the septum and leave the flask in a P_2O_5-containing desiccator under vacuum overnight.

9. Fill the desiccator with nitrogen before opening it.

10. Under argon atmosphere and magnetic stirring, add with a syringe 6 mL anhydrous CH_3CN, 0.32 mL DIEA (235 mg, 1.82 mmol), and 0.2 mL 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphite (216 mg, 0.91 mmol) at room temperature.

11. Let reaction proceed 30 min. Monitor its progress by TLC on a silica-gel plate using 50:50:4 (v/v/v) ethyl acetate:hexane:Et_3N as eluent. Pre-elute the TLC plate once with the eluent, then load 1 µl of the reaction mixture and elute.

    *After 30 min the starting material **S.1b** (R_f = 0.10) is transformed into two new compounds, a main product (≈80%; R_f = 0.50) and a side-product (≈15%; R_f = 0.65). Trace amounts of the starting material are also observed.*

12. Add 20 mL CH_2Cl_2 and wash the organic layer with 3 mL of 10% (w/v) aqueous Na_2CO_3 and then 3 mL of saturated NaCl (brine). Decant the organic phase, dry it over $MgSO_4$, and concentrate it to dryness.

13. Apply the residue, diluted with 2 to 3 mL anhydrous CH_3CN to a silica-gel chromatography column (3 cm × 45 cm, containing 60 g silica gel) and elute with 500 mL of 50:50:4 (v/v/v) ethyl acetate:hexane:Et_3N. Monitor the fractions by TLC analysis using 50:50:4 (v/v/v) ethyl acetate:hexane:Et_3N as eluent, and collect the main product (R_f = 0.50).

14. Pool the fractions containing the pure product, and remove the solvent under reduced pressure. Coevaporate the residue twice using 8 mL anhydrous CH_3CN each time.

    *Expected yield: 65% (300 mg, 0.54 mmol) of a yellow oil. Purified acridinyl phosphoramidite **S.1c** can be stored at −20°C for a few months and should be reconstituted in anhydrous acetonitrile just prior to use (add a few pearls of 3-Å molecular sieves and wait ~30 min before using the solution).*

    ^{31}P-NMR ($CDCl_3$): δ 143.52 ppm.

    ^{1}H-NMR ($CDCl_3$): δ 1.09-1.20 (m, 12H-[C(H)(CH_3)$_2$]$_2$), 1.35-1.50 (m, 2H, CH_2), 1.56-1.68 (m, 4H, CH_2CH_2), 1.70-1.83 (m, 2H, CH_2), 2.61 (t, 2H, J = 6.34 Hz, CH_2CN), 3.20-3.33 (m, 3H, CH_2N + NH), 3.50-3.87 (m, 6H, CH_2OP, (2 CH)), 3.97 (s, 3H, OCH_3), 7.17-8.10 (m, 6H, Acr)

Couple the acridinyl phosphoramidite S.1c to 5′ end of oligodeoxyribonucleotide chain

Automated coupling:

15a. When employing standard solid-phase DNA oligonucleotide synthesis cycles (1-µmol scale) according to the classical phosphoramidite method, perform an additional detritylation step.

16a. Place a 0.1 M solution of the acridinyl phosphoramidite **S.1c** in anhydrous CH_3CN on the synthesizer. Carry out coupling by recycling a mixture of 0.1 mL of the acridinyl phosphoramidite, 0.5 mL of 0.4 M 1*H*-tetrazole in anhydrous CH_3CN, and 0.1 mL anhydrous CH_3CN for 5 min. Repeat.

    For synthesizers without a recycle program, perform a double coupling step. For optimal coupling yields, changes in phosphoramidite concentration may be required depending on the synthesizer used.

17a. Remove the excess phosphoramidite and tetrazole, and perform an iodine oxidation reaction according to the standard procedure (*UNIT 3.3, APPENDIX 3C*).

Manual coupling:

15b. When performing standard solid-phase oligodeoxyribonucleotide synthesis (1-μmol scale) according to the classical phosphoramidite method, place the oligonucleotide bound to the support in a short (4-mL) vial stoppered with a septum. Push a needle through the septum and leave the vial in a desiccator containing P_2O_5 under vacuum overnight.

The oligonucleotide must have a free 5′-hydroxy group.

16b. Fill the desiccator with nitrogen before opening it. With a syringe, add a mixture of phosphoramidite **S.1c** (0.15 mL of a 0.15 M solution in anhydrous CH_3CN) and 1*H*-tetrazole (0.5 mL of a 0.5 M solution in anhydrous CH_3CN) to the support-linked oligonucleotide. Leave the mixture for 10 min, shaking gently by hand from time to time.

17b. Remove the excess reagents with a syringe and perform an oxidation reaction by adding 1 mL of an aqueous iodine solution that is normally used on the synthesizer. Remove the iodine solution after 1 min and wash the support extensively with CH_3CN.

Deprotect the acridine-oligodeoxyribonucleotide conjugates

18. Treat the support bearing the acridine-oligodeoxyribonucleotide conjugates with 5 mL of 0.4 M NaOH in 50:50 (v/v) MeOH/H_2O at room temperature.

19. After 1 to 2 hr, remove the solid support by filtration. Wash it twice with 0.5 mL water. Maintain the yellow solution at room temperature until deprotection is complete.

The time required for oligonucleotide deprotection depends on the base composition of the sequence and on the nature of the protective groups used to protect the nucleic bases. Removal of the isobutyryl group from guanine residues requires ~30 to 35 hr under these conditions.

20. Neutralize the solution (containing the oligonucleotide conjugate **S.1d**) by adding Dowex 50 ion-exchange resin until the pH of the solution reaches 6. Remove the resin by filtration and wash it with water until the resin becomes pale yellow in color.

21. Concentrate the solution under vacuum to remove MeOH. Add ~10 mL water, then extract the aqueous solution three times with 3 mL ethyl acetate each time. Concentrate the aqueous solution to ~3 to 4 mL.

22. Filter the solution through a 0.45-μm disposable filter to remove any particulates and prevent the clogging of HPLC columns. Purify and characterize the oligonucleotide-acridine conjugate (see Support Protocol).

SUPPORT PROTOCOL

PURIFICATION AND CHARACTERIZATION OF OLIGONUCLEOTIDE-ACRIDINE CONJUGATES

The following general procedure is applicable to the purification of 10- to 20-mer oligodeoxyribonucleotides-acridine conjugates, assuming that the sequences under consideration are not likely to form self-associated complexes such as G-tetrads. Should this be the case, a specific purification procedure—not provided in this unit—must be used. Purification can by improved by performing ion-exchange chromatography at pH 12 (Raynaud et al., 1996). Another solution to prevent the formation of G tetrads is to partially replace 2'-deoxyguanosine with 7-deaza-2′deoxyguanosine (Raynaud et al., 1996). Generally, the crude oligodeoxyribonucleotide conjugates obtained from the protocol described above are purified by ion-exchange chromatography. After a desalting step, the

purity of the conjugates is verified on a reversed-phase column; in most cases this will show that sufficient purity has been achieved. Alternatively, reversed-phase chromatography allows easy purification of oligodeoxyribonucleotide-acridine conjugates when acridine is bound to the 5′ end of oligonucleotides (making the conjugates more lipophilic than the underivatized oligonucleotides). Sometimes the conjugate can be insufficiently pure and a second purification is required. Usually it is performed using the system that was not used for the first purification (i.e., a second purification step can be made using reversed-phase after a first purification by ion-exchange chromatography and vice versa). In all cases, oligodeoxyribonucleotide-acridine conjugates are detected during analysis and purification by measuring UV absorption at 254 or 260 nm and at 425 nm (only the acridine moiety of the conjugate absorbs light at that wavelength).

Materials

0.01 bis-Tris, pH 6 *or* 0.01 M NaH_2PO_4, pH 6.8 (*APPENDIX 2A*), each containing 10% or 10% HPLC-grade methanol (MeOH)
Sodium chloride (NaCl)
0.025 M Tris·Cl, pH 7 (*APPENDIX 2A*)
Acetonitrile (CH_3CN)
1 M triethylammonium acetate (TEAA) buffer, pH 7 (stock solution; see recipe)
0.01 M Tris·Cl, pH 8 (*APPENDIX 2A*)
1 U snake venom phosphodiesterase (3′ exonuclease)
10 µg alkaline phosphatase

Ion-exchange columns: Mono Q HR 5/5 or HR 10/10 (Amersham Pharmacia Biotech) or DEAE (Waters)
Reversed-phase columns: e.g., Lichrospher 100 RP 18 (5 µm, 125 × 4 mm, *or* 10 µm, 250 ×10 mm; Merck) or CC Nucleosil 100-5 C18 (125/4) column (Macherey-Nagel)
Desalting columns: HR 10/10 columns (Amersham Pharmacia Biotech) packed with Lichroprep RP 18 (Merck) or Sephadex G-10 or G-25 resin

1. Analyze the crude oligonucleotide-acridine conjugate solution via chromatography by one of the following procedures, generally loading 10 to 20 µl of the crude solution onto the column:

   a. Ion-exchange chromatography with Mono Q HR 5/5 or HR 10/10 column: Use a linear gradient of 0 to 100% 1 M NaCl in 0.01 M bis-Tris, pH 6, containing 10% or 20% MeOH, *or* 0 to 100% 1.5 M NaCl in 0.01 M NaH_2PO_4, pH 6.8, containing 10% or 20% MeOH, with a flow rate of 1 mL/min (HR 5/5) or 4 mL/min (HR 10/10).

   b. Ion-exchange chromatography with DEAE column (100 × 10 mm) : Use a linear gradient of 0 to 100% 1.5 M NaCl in 0.025 M Tris·Cl, pH 7, containing 10% MeOH, with a flow rate of 1 mL/min.

   c. Reversed-phase chromatography with Lichrospher 100 RP 18 column (5 µm, 125 × 4 mm; *or* 10 µm, 250 × 10 mm): Use a linear gradient of CH_3CN (5% to 80%) in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 mL/min (5-µm column) or 4 mL/min (10-µm column).

   d. Reversed-phase chromatography with CC Nucleosil 100-5 C18 (125/4) column: Use a linear gradient of CH_3CN (5% to 80%) in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 mL/min.

   The gradients used must be chosen so as to afford the best separation. Usually an increasing concentration of 1% of 1 M or 1.5 M NaCl per minute is used for ion-exchange chromatography and an increasing concentration of ~1% CH_3CN per minute is used for reversed-phase chromatography.

2. Purify the remaining oligonucleotide-acridine conjugate by preparative reversed-phase or ion-exchange chromatography column.

   Use any type of column that was described in step 1, according to the recommended conditions. Usually 1 µmol of crude oligonucleotide-acridine conjugates can be purified in two or three runs on Mono Q HR 10/10 column. On preparative reversed-phase columns, reasonable purification can be achieved in a single run, but it is better to perform two. Alternatively, purification can be achieved on analytical columns by performing five or more runs.

3. Pool the fractions containing the conjugate and remove the organic solvents by evaporation under reduced pressure.

4. If ion-exchange purification was used in step 2, desalt the conjugate using appropriate desalting column. If reversed-phase purification was used, remove triethylammonium acetate by three consecutive lyophilizations. Resuspend the sample in 1 to 2 mL water between lyophilizations.

5. Analyze the oligonucleotide-acridine conjugate by ion-exchange chromatography when the purification was performed by reversed-phase, or by reversed-phase when the purification was achieved by ion-exchange, using the columns and parameters described in step 1.

   For example, analyze on a Lichrospher 100 RP 18 (5 µm; 125 mm × 4 mm) column from Merck using a linear gradient of CH_3CN (5% to 35% over 40 min) in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 mL/min. Expected retention time is 28 min 18 sec for the acridine-oligonucleotide conjugate Acr$(CH_2)_6$pd5[T_3C_2T C_2TCT].

   After purification, UV spectra of oligodeoxyribonucleotide-acridine conjugates show absorptions characteristic to the ligand. A typical spectrum of an oligonucleotide-acridine conjugate is shown in Figure 4.3.4 (left). In addition, nuclease digestion of the conjugates and reversed-phase HPLC analysis of the resulting monomers can be used to ascertain full removal of the protective groups and base composition.

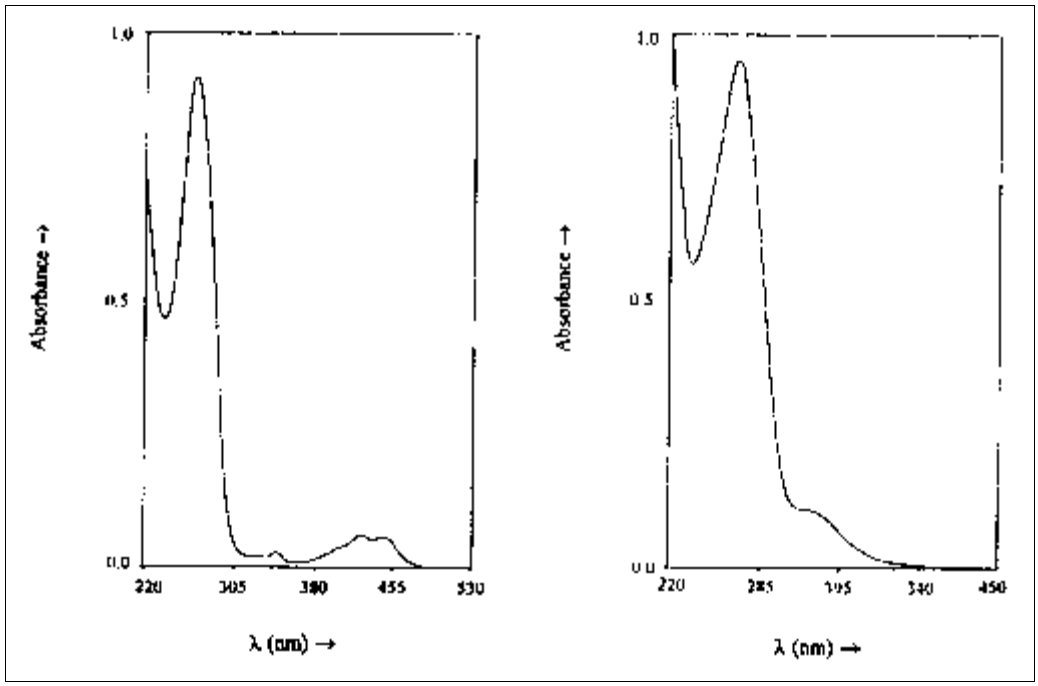

Figure 4.3.4 Absorption spectra of Acr$(CH_2)_6$pd[T_3C_2T C_2TCT] (left) and Pso$(CH_2)_6$pd[T_4 $^{5\text{-Me}}C_2T^{5\text{-Me}}CT^{5\text{-Me}}C_3T^{5\text{-Me}}CT$] (right) recorded in water.

6. Hydrolyze the oligonucleotide-acridine conjugate **S.1d** with nucleases by placing the following in a 1.5-mL microcentrifuge tube:

   ~1 OD unit conjugate **S.1d** in 10 µl H_2O
   50 µl 0.01 M Tris·Cl, pH 8
   1 U snake venom phosphodiesterase
   10 µg alkaline phosphatase.

   Incubate 2 to 3 hr at 37°C.

7. Inactivate the enzyme by heating the mixture at 90°C for 2 min.

8. Analyse the hydrolysate by reversed-phase HPLC: e.g., using a Lichrospher 100 RP 18 column with a gradient of CH_3CN (0% for 10 min, then 0 to 24% CH_3CN over 30 min, then 24% to 56% CH_3CN over 20 min) in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 mL/min.

   *These conditions permit the separation of natural nucleosides and a variety of modified nucleosides. Identification can be made by comparison with authentic nucleosides. For example: $Rt_{dC} \approx 5$ min, $Rt_{5\text{-}MedC} \approx 10$ min, $Rt_T \approx 13$ min, $Rt_{dG} \approx 14$ min 30 sec, $Rt_{dA} \approx 17$ min 30 sec. Under these conditions the retention time of the acridine-linker derivative **S.1b** is ~51 min. Prolonged incubation at pH 8 induces degradation of **S.1b**.*

   Additional characterization can be made by MALDI and ESI mass spectrometry (UNITS 10.1 & 10.2). Acr$(CH_2)_6$pd[$T_3C_2T\ C_2TCT$] has been characterized by MALDI-TOF using 2,4,6-trihydroxyacetophenone as matrix in the presence of diammonium L-tartrate. Quantification of oligonucleotide-acridine conjugates can be made by using $\varepsilon = 8850\ M^{-1}\ cm^{-1}$ at $\lambda = 425$ nm (Asseline and Thuong, 1988). Usually when the synthesis is performed at the micromole scale ~15 to 20 OD units of pure oligonucleotide-acridine conjugate 15-mer with mixed bases is obtained.

DIRECT ADDITION OF PSORALEN DERIVATIVES TO THE 5′ END OF OLIGODEOXYRIBONUCLEOTIDES

BASIC PROTOCOL 2

This strategy (illustrated in Figs. 4.3.1, 4.3.2, and 4.3.3) involves the attachment of a hydroxylated linker to the psoralen ligand. Specifically, 5-methoxypsoralen **S.2a** is demethylated to give the 5-hydroxypsoralen **S.2b**. The latter is reacted with 6-bromohexanol in the presence of K_2CO_3 (following a procedure adapted from that previously reported by Kurfürst et al., 1993) to give the psoralen derivative **S.2c**. Phosphitylation of **S.2c** affords **S.2d** (in another procedure adapted from the same reference). The phosphoramidite derivative **S.2d** is then condensed with the 5′-terminal hydroxyl of oligodeoxyribonucleotides bound to solid supports. This conjugation is completed by the deprotection, purification, and characterization of the psoralenyl-oligonucleotide conjugates **S.2e**.

Materials

5-Methoxypsoralen (**S.2a**)
Pyridine hydrochloride
$CaCl_2$
Dichloromethane (CH_2Cl_2), distilled from P_2O_5 and passed over activated, basic aluminum oxide
Methanol (MeOH), distilled
P_2O_5
N,*N*-Dimethylformamide, redistilled under vacuum over ninhydrin and stored over 4-Å molecular sieves
6-Bromo-1-hexanol
Potassium carbonate, anhydrous

Ethyl acetate
Pyridine, anhydrous
N,*N*-Diisopropylethylamine (DIEA), distilled from KOH
Sodium sulfate, anhydrous
2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphite
Triethylamine (Et_3N)
10% (w/v) aqueous sodium carbonate ($NaHCO_3$)
Cold saturated aqueous NaCl
Acetonitrile (CH_3CN), DNA synthesis grade, anhydrous and stored over 3 Å-molecular sieves
Oligodeoxyribonucleotides bound to solid supports, such as controlled-pore glass (CPG; *UNIT 3.1*), synthesized by classic phosphoramidite chemistry (1-µmol scale)
DNA synthesis reagents recommended by synthesizer manufacturer
Concentrated (25%) aqueous ammonia

25-mL round-bottomed flask
Reflux condenser
Drying tube
Nitrogen gas
Oven
Analytical thin-layer chromatography (TLC) setup and silica-gel plates: e.g., Merck 5554 Kieselgel 60F plates, including UV lamp for detection
Filter funnel, porosity 4
Desiccator
Argon
Silica gel for column chromatography: Merck 9387 Kieselgel 60 or Merck 7734 Kieselgel 60
Chromatographic columns: (3 cm × 50 cm) and (1.5 cm × 40 cm)

Prepare 5-(hydroxypsoralen), S.2b

1. Place 1 g (4.64 mmol) 5-(methoxypsoralen) **S.2a** and 4.27 g (37 mmol) pyridine hydrochloride in a 25-mL round-bottomed flask equipped with a reflux condenser and a calcium chloride drying tube, under a nitrogen atmosphere.

2. Verify that pyridine hydrochloride is at the bottom of the flask and heat the mixture 90 min at 180°C. Monitor the reaction by TLC using 90:10 (v/v) CH_2Cl_2/MeOH as eluent.

   *The starting material **S.2a** (R_f = 0.9) will be transformed into **S.2b** (R_f = 0.50).*

3. Allow the mixture to cool to room temperature. Solubilize the reaction mixture with 3 mL MeOH and slowly pour the solution in 50 mL cold water while stirring magnetically to precipitate **S.2b**. Filter the solid over a filter funnel (porosity 4) and dry it over P_2O_5 in a desiccator for 2 days. Analyze **S.2b** again by TLC.

   *The R_f value of **S.2b** is 0.50 when 90:10 (v/v) CH_2Cl_2/MeOH is used as eluent, and 0.65 when 50:50 (v/v) CH_2Cl_2/ethyl acetate is the eluent. Expected yield: 95% (885 mg, 4.38 mmol).*

   Characterize the product by appropriate analytical methods. Melting point: 280°C.

   ^{1}H-NMR (DMSO-d_6): δ 3.34 (br s, 1H), 6.23 (d, 1H, J = 9.7 Hz, C_3 Pso), 7.14 (S, 1H, C_8 Pso), 7.18 (d, 1H, J = 2.2 Hz, C_4·Pso), 7.89 (d, 1H, J = 2.3 Hz, C_5·Pso), 8.23 (d, 1H, J = 9.5 Hz, C_4 Pso).

   Mass analysis. I.S. polarity positive. Calculated for $C_{11}H_6O_4$: M+H=203.0. Found 203.0.

Prepare 5-(6-hydroxyhexyloxy)-psoralen, S.2c

4. Place a solution of 5-hydroxypsoralen **S.2b** (0.6 g, 2.97 mmol) in 10 mL anhydrous *N*,*N*-dimethylformamide in a 25-mL round-bottomed flask equipped with a reflux condenser and a calcium chloride drying tube. Add successively 1.16 mL 6-bromo-1-hexanol (1.61 g, 8.91 mmol) and 0.61 g anhydrous potassium carbonate (4.45 mmol).

5. Heat the flask at 65°C in the dark under an argon atmosphere and stir for 4 hr.

6. Allow the mixture to cool to room temperature and remove insoluble salts by filtration, then concentrate the filtrate to dryness under reduced pressure.

7. Purify the residue (2.5 g) on a silica-gel column (3 cm × 50 cm, 70 g silica) using increasing concentrations of MeOH in CH_2Cl_2 (1:100 to 3:97, v/v):

   200 mL of 1% MeOH in CH_2Cl_2;
   200 mL of 2% MeOH in CH_2Cl_2;
   200 mL of 2.5% MeOH in CH_2Cl_2;
   and 200 mL of 3% MeOH in CH_2Cl_2.

8. Monitor the fractions by silica-gel TLC using 90:10 (v/v) CH_2Cl_2/MeOH or 50:50 (v/v) CH_2Cl_2/ethyl acetate as eluent. Collect the fractions containing the pure product and remove the solvent under reduced pressure.

   *The R_f value of **S.2c** is 0.43 when 90:10 (v/v) CH_2Cl_2/MeOH is the eluent, and 0.45 when 50:50 (v/v) CH_2Cl_2/ethyl acetate is the eluent.*

   Expected yield: 75% (672 mg, 2.22 mmol). Melting point: 100°C.

   ^{1}H-NMR ($CDCl_3$): δ 1.38-1.60 (m, 6H), 1.82-1.95 (μ, 2H, XH_2), 3.60-3.71 (m, 2H, CH_2OH), 4.45 (t, 2H, J = 6.4 Hz, -CH_2OAr), 6.27 (d, 1H, J = 9.7 Hz, C_3 Pso), 6.94 (d, 1H, J = 2.3 Hz, C_4 Pso), 7.26 (s, 1H, C_8), 7.58 (d, 1H, J = 2.4 Hz, C_5 Pso), 8.15 (d, 1H, J = 9.5 Hz, C_4 Pso)

   Mass analysis. I.S. polarity positive. Calculated for $C_{17}H_{18}O_5$: M+H=303.0. Found 303.0.

Prepare [5-(ω-hexyloxy)-psoralenyl]-(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite S.2d

9. Place 0.30 g 5-(6-hydroxyhexyloxy)-psoralen (1 mmol; **S.2c**) in a 25-mL round-bottomed flask, dry it by coevaporation with anhydrous pyridine and leave under vacuum for at least 12 hr.

   See Basic Protocol 1, steps 7, 8 and 9, for procedure.

10. Under a nitrogen atmosphere and magnetic stirring at room temperature add using a syringe 6 mL dichloromethane, 0.35 mL DIEA (0.258 g, 2 mmol) and then dropwise 0.29 mL 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphite (0.307 mg, 3 mmol). Leave 20 min at room temperature. Monitor the reaction by silica-gel TLC using 95:5 (v/v) ethyl acetate/Et_3N as eluent. Pre-elute the TLC plate with eluent before spotting the samples.

11. Shake the solution twice with 2 mL of 10% (w/v) aqueous Na_2CO_3 and once with 2 mL of a cold saturated aqueous NaCl solution. Dry the organic layer over anhydrous sodium sulfate and concentrate it to dryness.

12. Purify the residue (500 mg) on a silica-gel column (1.5 cm × 40 cm, 20 g silica) using 95:5 (v/v) ethyl acetate/Et_3N as eluent. Monitor the fractions by silica-gel TLC using 95:5 (v/v) ethyl acetate/Et_3N as eluent.

   *The R_f value of **S.2d** is 0.87.*

13. Pool the fractions containing the pure product. Remove the solvents under reduced pressure and coevaporate with anhydrous CH_3CN.

Expected yield: 65% (326 mg, 0.65 mmol) of a colorless oil. Purified psoralenyl phosphoramidite ***S.2d*** *can be stored at −20°C for a few months and should be reconstituted in anhydrous acetonitrile just prior to use (add a few pearls of 3-Å molecular sieves and wait ~30 min before using the solution).*

^{31}P-NMR ($CDCl_3$): δ 149.47 ppm

^{1}H-NMR ($CDCl_3$): δ 1.12-1.23 (m, 12H -[C(H)(CH_3)$_2$]$_2$), 1.38-1.70 (m, 6H, (CH_2)$_3$), 1.82-1.85 (m, 2H, CH_2), 2.60 (t, 2H, J = 6. Hz, CH_2CN), 3.52-3.73 (m, 4H, CH_2O, [C(H)]$_2$), 3.74-3.90 (m, 2H, CH_2OP), 4.45 (t, 2H, J = 6.33 Hz, -CH_2OAr), 6.27 (d, 1H, J = 9.8 Hz, C_3Pso), 6.94 (d, 1H, J = 2 Hz, $C_{4'}$Pso), 7.13 (s, 1H, C_8), 7.58 (d, 1H, J = 2.3 Hz, $C_{5'}$Pso), 8.15 (d, 1H, J = 9.8 Hz, C_4Pso).

Couple the psoralenylphosphoramidite S.2d to the 5′-end of oligodeoxyribonucleotides

Automated coupling:

14a. After standard solid-phase DNA oligonucleotide synthesis (1-µmol scale) according to the classical phosphoramide method, perform an additional detritylation step.

15a. Place a 0.1 M solution of the psoralenyl phosphoramidite **S.2d** in anhydrous CH_3CN on the synthesizer. Carry out coupling by recycling a mixture of 0.1 mL of the psoralenyl phosphoramidite, 0.5 mL of 0.4 M 1*H*-tetrazole in anhydrous CH_3CN, and 0.1 mL anhydrous CH_3CN for 5 min. Repeat.

For synthesizers without a "recycle" program, perform a double coupling step. For optimal coupling yields changes in amidite concentration may be required, depending on the synthesizers used.

16a. Remove the excess phosphoramidite and tetrazole, and perform an aqueous iodine oxidation reaction according to the standard procedure.

Manual coupling:

14b. When performing standard solid-phase oligodeoxyribonucleotide synthesis (1-µmol scale) according to the classical phosphoramidite method, place the oligonucleotide bound to the support in a short (4-mL) vial stoppered with a septum. Push a needle through the septum and leave the vial in a desiccator containing P_2O_5 under vacuum overnight.

The oligonucleotide must have a 5′-hydroxy group.

15b. Fill the desiccator with nitrogen before opening it. Using a syringe, add a mixture of phosphoramidite **S.2d** (0.15 mL of a 0.15 M solution in anhydrous CH_3CN) and 1*H*-tetrazole (0.5 mL of a 0.5 M solution in anhydrous CH_3CN) to the support-linked oligonucleotide. Leave the mixture at room temperature for 10 min, shaking gently by hand from time to time.

16b. Remove the excess reagents with a syringe and perform an oxidation reaction by adding 1 mL iodine solution that is normally used on the synthesizer. Remove the iodine solution after 1 min and wash the support extensively with CH_3CN.

Deprotect oligodeoxyribonucleotide-psoralen conjugates

17. Add 5 mL concentrated 25% aqueous ammonia to a vial containing the psoralen-oligodeoxyribonucleotide conjugate bound to the support and leave it 1 hr at 55°C.

18. Discard the support and leave the ammonium hydroxide solution an additional 6 hr at 55°C.

19. Evaporate the ammoniacal solution to dryness.

20. Solubilize the crude material **S.2e** with 12 mL water and extract three times with 3 mL ethyl acetate each time. Reduce the volume of the solution to ~3 to 4 mL under reduced pressure.

21. Purify and characterize the oligodeoxyribonucleotide-psoralen conjugates (see Basic Protocol 1; the conjugates can be detected at 260 and 320 nm).

For example analysis performed on a Lichrospher 100 RP 18 (5 μm; 125 mm × 4 mm) column using a linear gradient of CH_3CN (5% to 35% over 40 min) in 0.1 M aqueous triethylammonium acetate, pH 7, at a flow rate of 1 mL/min indicates a retention time of 30 min 16 sec for the psoralen-oligonucleotide conjugate $Pso(CH_2)_6pd[T_4^{5\text{-}Me}C_2T^{5\text{-}Me}\text{-}CT^{5\text{-}Me}C_3T^{5\text{-}Me}CT]$.

*As for oligonucleotide-acridine conjugates, base composition can be verified by nuclease degradation (same protocol). In this case reversed-phase analysis of hydrolysates performed under the conditions described for the oligonucleotide-acridine conjugates indicates a retention time of 53 min 36 sec for **S.2c**. Quantification of oligonucleotide- psoralen conjugates can be made by using $\varepsilon \approx 10\ 000\ M^{-1}\ cm^{-1}$ at $\lambda = 320$ nm (unpub. observ.). When the synthesis is performed at the micromole scale, ~15 to 20 OD units of pure oligonucleotide-psoralen conjugates 15-mer with mixed bases are obtained. The absorption spectrum of the psoralen-oligonucleotide conjugate $Pso(CH_2)_6pd[T_4^{5\text{-}Me}C_2T^{5\text{-}Me}\text{-}C\ T^{5\text{-}Me}C_3T^{5\text{-}Me}CT]$ is represented in Figure 4.3.4 (right).*

REAGENTS AND SOLUTIONS

1M triethylammonium acetate (TEAA) buffer, pH 7 (stock solution)

In a fume hood, add successively to a 1 liter beaker (equipped with a stirring bar) distilled water (810 mL), triethylamine (134 mL) and glacial acetic acid (57 mL). Mix thoroughly and adjust to pH 7 by adding either a few drops of triethylamine or a few drops of glacial acetic acid.

COMMENTARY

Background Information

Two strategies can be used to attach ligands to the 5′ end of oligodeoxyribonucleotides. This unit describes one of these strategies, which involves the direct addition of a ligand to the 5′ end of an oligodeoxynucleotide during its initial synthesis via the phosphoramidite approach. Although methods have been developed for derivatizing the 5′ terminus of oligodeoxyribonucleotides in homogenous solution (Thuong et al., 1984, 1987; Asseline and Thuong, 1984; Asseline et al., 1988), only conjugation reactions on solid supports are reported in this unit (Thuong and Chassignol 1988, Dupret et al., 1994; Asseline et al., 1996). This strategy is applicable when the ligand is available in sufficient amounts, and its solubility and chemical stability are compatible with the conditions required for the preparation of its phosphoramidite or H-phosphonate derivatives. In addition, the ligand should be stable under the conditions required for the coupling reaction and to those conditions used for complete deprotection of the oligodeoxynucleotide chain and its release from the support.

The second strategy, not covered here, involves the incorporation of appropriate functional groups into two unprotected reactants. The coupling of these functionalized reactants results in the formation of oligodeoxyribonucleotide conjugates. Various oligodeoxyribonucleotide conjugates can be prepared using this strategy from only one oligodeoxyribonucleotide synthesis, provided that the required amount for each conjugate is low.

Future units will describe the coupling of a number of ligands (intercalators, photoreactive and cleavage reagents, and labels) to oligodeoxyribonucleotides post-synthetically. These ligands are typically 2-methoxy-6-choro-9-

aminoacridine as an intercalator, psoralen as a photo-cross-linking reagent, and phenanthroline-Cu as a cleavage reagent. More recently, the authors have used thiazole orange as a reporter group. This strategy involves the incorporation of linkers carrying suitable functional groups into ligands and the addition of amino, phosphorothioate, phosphate, or sulfhydryl functions to the 5′ end of oligonucleotides, as well as coupling methods for linking both entities.

Alternatively, many reporter groups bearing functional groups that will react with 5′-thiol, 5′-terminal phosphorothioate, and 5′-amino groups are available from commercial sources. Heterobifunctional reagents, which allow reaction with a thiol function and with the primary amino function of various ligands, are also available commercially. These reagents, listed in *UNIT 4.2*, are very useful when there is no need for conjugates to have a well-defined linker between the oligodeoxyribonucleotide and the ligand.

The coupling methods developed in this unit and future units can be used to prepare oligodeoxyribonucleotide conjugates composed of natural β-deoxyribonucleosides (Thuong and Chassignol 1988, Dupret et al., 1994, Asseline et al., 1996), unnatural α-D-deoxyribonucleosides (Kurfürst et al., 1993), or 2′-*O*-methylribonucleosides (unpub. observ.). When the chain assembly is performed in the usual (3′→5′) orientation, the modifying steps (i.e., direct incorporation of the ligand or the masked 5′-terminal functional group) are carried out at the end of the chain assembly just prior to the deprotection step. Given that oligodeoxyribonucleotides can be purchased fully protected and bound to the support, 5′-end modification can be performed manually.

Procedures reported in this unit may be used to prepare oligodeoxyribonucleotides covalently linked via their 5′ end to molecules such as intercalating agents, and other reactive compounds the properties of which are described in *UNIT 4.2*. In many examples, oligodeoxyribonucleotide conjugates properties, such as strong complex stabilization when the ligand is an intercalator (Asseline et al., 1984, 1996, Sun et al., 1989; Giovannangeli et al., 1996) or best cleavage or cross-linking efficiency when the ligand is a reactive group, 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). Using these procedures linkage parameters between the oligodeoxyribonucleotide and the ligand, such as the size and nature of the linker connecting the two entities, may be varied to prepare oligodeoxyribonucleotide-ligand conjugates exhibiting optimal properties for specific experimental needs.

Critical Parameters and Troubleshooting

In the case of acridine-containing oligodeoxyribonucleotides, the use of ammonia for the deprotection step must be avoided since it induces cleavage of the C9-N bond between the acridine and the linker. Therefore, a 0.4 N sodium hydroxide solution in 50:50 (v/v) H_2O/MeOH is recommended. It is also recommended that the oligonucleotide-acridine conjugate solutions be stored between pH 6 and 7. Storage at pH values between 7 and 9 leads to considerable degradation. Protection of flasks and columns from light during the different steps of these protocols is recommended because acridine and psoralen derivatives are light-sensitive. In any case, purification steps must be as short as possible. It is always possible that the conditions required for deprotection of the conjugates may be incompatible with the stability of some modified nucleoside incorporated into the sequence of interest. To avoid obtaining a low yield of oligonucleotide-ligand conjugates, it is possible to deprotect an aliquot of the conjugates to verify the coupling efficiency of the amidite derivatives. If the coupling efficiency is insufficient, it is possible to perform additional coupling of the ligand. At the micromole scale it is usual to obtain about 20 OD units of pure conjugates when working with mixed DNA sequences. In some cases, when working with oligonucleotides containing sequences, inducing self associated structure, the purification step can be particularly challenging. In such cases, it is advisable to purify the acridine-oligonucleotide conjugates on a Mono-Q column at pH 12. In the case of sequences containing stretches of dG, the better way to solve the problem is to partially replace the dGs with 7-deaza-2′-deoxyguanosines (Raynaud et al., 1996).

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 300 mg of the acridine-linker derivative **S.1b**, 300 mg of the purified acridine phosphoramidite **S.1c** can be obtained. This can be used to

synthesize acridine-oligodeoxyribonucleotide conjugates **S.1d**. Usually 15 to 20 OD units of purified conjugates **S.1d** can be expected when the synthesis is performed at the µmol scale.

In the case of the psoralen derivatives, starting with 310 mg of the psoralen-linker derivative **S.2c** it is possible to obtain 325 mg of the purified psoralen phosphoramidite derivative **S.2d**. The latter is then used to prepare psoralen-oligodeoxyribonucleotide conjugates **S.2e**. When DNA synthesis is performed at the µmol scale, the yields obtained for conjugates **S.2e** (15 to 20 OD units) are similar to those obtained for the preparation of the conjugates **S.1d.**

Time Considerations

Providing all reagents and materials required for each step are available, most of the reactions are simple and rapid. Preparation of compounds **S.1b** and **S.2b** requires 3 days including the drying step, while only two days are necessary to obtain compound **S.2c**. The time required for the preparation of phosphoramidite derivatives **S.1c** or **S.2d** is one day provided that the ligand-linker derivatives have been dried the previous day and left under vacuum in a desiccator overnight. Oligonucleotides chain assemblies and the addition of the ligand to the 5′-end of the oligonucleotide can be performed the same day when the ligand is added directly with the synthesizer. Otherwise the manual coupling requires leaving the support bearing the oligonucleotide under vacuum overnight. The deprotection step for psoralen-oligodeoxyribonucleotide conjugates **S.2e** takes a few hours while the duration of the deprotection step for acridine-oligodeoxyribonucleotide conjugates **S.1d** can be extended to almost two days. The time required for the purification of the ligand-oligonucleotide conjugates **S.1d** and **S.2e** can vary depending on the sequences. When two successive purification steps are necessary the time can be from five to ten days including rounds of lyophilization (overnight) or desalting and additional analyses. Characterization of the purified conjugates **S.1d** and **S.2e** can be achieved in three or four days.

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