Conjugation of 5′-Functionalized Oligodeoxyribonucleotides with Properly Functionalized Ligands

This unit describes a collection of methods for the chemical attachment of reporter groups and various conjugate groups (e.g., intercalators, photoreactive agents, cleaving agents) to the 5′ terminus of oligodeoxyribonucleotides. Two strategies can be used. The first (described in *UNIT 4.3*) involves the direct addition of the ligand to the 5′ end of the oligodeoxyribonucleotide via its phosphoramidite or *H*-phosphonate derivative. This approach works well when there is a sufficient amount of the ligand and when its solubility and chemical stability are compatible with the conditions required for the preparation of the phosphoramidite or *H*-phosphonate derivative. In addition, the ligand should be stable under the conditions used for the coupling step and for the complete deprotection of the oligodeoxyribonucleotide, including its release from the support.

The second strategy involves the incorporation of appropriate functional groups into both the ligand and the 5′ terminus of the oligodeoxyribonucleotide. Specific coupling of these reactants results in the formation of oligodeoxyribonucleotide conjugates. Using this strategy, several oligodeoxyribonucleotide conjugates can be prepared from only one oligodeoxyribonucleotide synthesis provided that the required amount for each conjugate is low. In this approach, appropriate functional groups on the ligand and oligonucleotide must be matched so that a conjugation reaction can be performed. This unit delineates methods used for the following coupling reactions: 5′-carboxylated oligonucleotides with aminoalkylated ligands (see Basic Protocol 1); 5′-aminoalkylated oligonucleotides with ligands that have been functionalized with an isothiocyanate or *N*-hydroxysuccinimidyl group (see Basic Protocol 2); oligonucleotide 5′-phosphorothioates with ligands functionalized with halogenoalkyl, 2-pyridyldithio, or iodoacetamidyl groups (see Basic Protocol 3); oligonucleotide 5′-phosphates with ligands functionalized with amino groups (see Basic Protocol 4); and 5′-mercaptoalkylated oligonucleotides with ligands functionalized with halogenoalkyl, 2-pyridiyldithio, or iodoacetamidyl groups (see Basic Protocol 5). The initial incorporation of these functional groups into the ligand and oligonucleotide has been described in *UNITS 4.8 & 4.9*, respectively.

The ligands described in this unit are the intercalator 2-methoxy-6-chloro-9-aminoacridine, the photoreactive group psoralen, the cleaving reagent phenanthroline-Cu, and the label thiazole orange (*UNIT 4.8*). 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 reactions with a thiol function and a primary amine, are also available from commercial sources. The latter compounds, listed in *UNIT 4.2*, are very useful when conjugates between the oligodeoxyribonucleotide and the ligand do not require a well-defined linker.

The procedures reported in this unit are for 10- to 25-mer oligodeoxyribonucleotides derivatized at their 5′ termini by various ligands, and require that the sequences under consideration will not form self-associated complexes such as G-tetrads. Such oligonucleotide structures require a specific procedure that is not discussed in this unit. Purification can be improved by performing ion-exchange chromatography at pH 12 when the obligonucleotide-ligand conjugate is stable at the pH. Another solution to prevent the formation of G-tetrads is to partially replace 2′-deoxyguanosine with 7-deaza-2′-deoxyguanosine (Raymond et al., 1996) or with 7-chloro-7-deaza-2′-deoxyguanosine (Aubert et al., 2001).

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

NOTE: When purifying and handling phosphorothioate- or phenanthroline-containing oligodeoxyribonucleotides, it is necessary to mix solutions, buffers, and solvents with Chelex 100 resin in order to remove divalent cations.

BASIC PROTOCOL 1

CONJUGATION OF 5′-CARBOXYLATED OLIGODEOXYRIBONUCLEOTIDES TO AMINOALKYLATED LIGANDS

The conjugation of oligodeoxyribonucleotides containing a carboxyl group at the 5′ terminus with the primary amino function of a ligand (or with an amine-like function such as a hydrazine derivative) is accomplished in the presence of carbodiimide. It generally requires a large excess of both ligand and carbodiimide (100 to 500 eq). Usually the oligodeoxyribonucleotide is solubilized in buffer and the ligand is added to the solution in amine-free dimethylformamide (DMF) dimethylsulfoxide (DMSO). The procedure is illustrated in Figure 4.10.1, using the hydrazine derivative of Lucifer Yellow (LY) as an example. The coupling reaction is performed at pH 5.5 to 6 in order to prevent the modification of the nucleobase. At this pH, the hydrazine derivative remains reactive. When the coupling reaction is complete, as determined by thin-layer chromatography (TLC; *APPENDIX 3D*), the conjugate is isolated from the reaction mixture. Different strategies can be used depending on the solubility of the ligand in the solvent or mixture of solvents used to perform the conjugation reactions. A general procedure for analysis, purification, and characterization of conjugates is given for 10- to 25-mer non-self-associating oligodeoxyribonucleotides derivatized at the 5′ end by a variety of ligands.

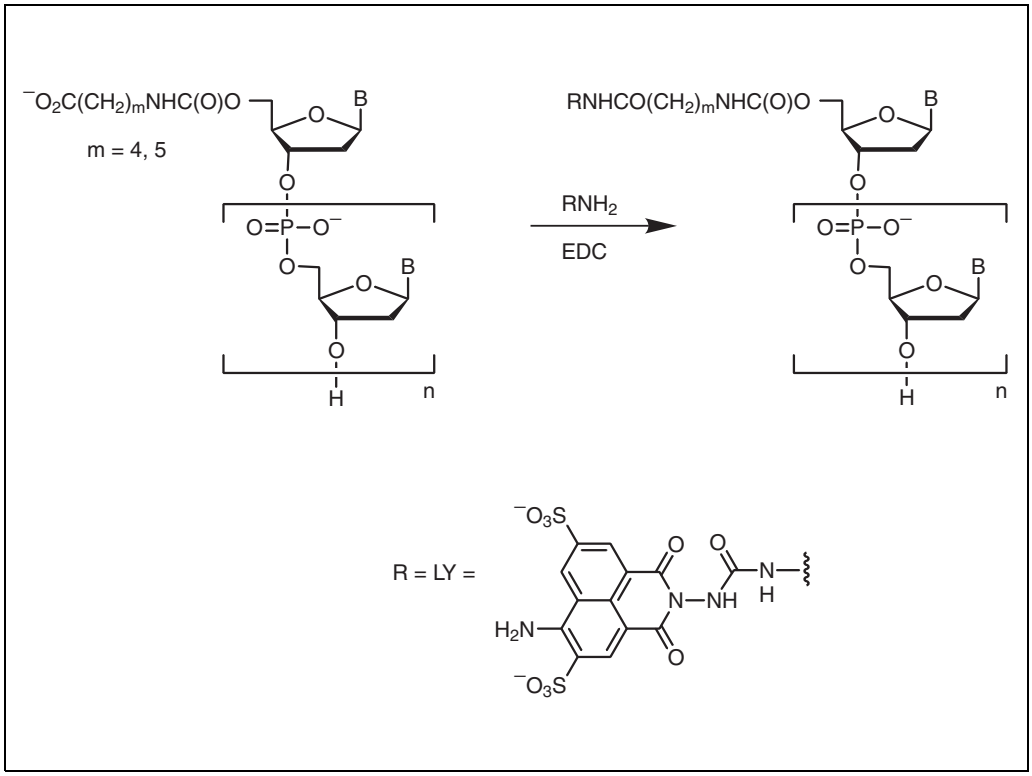

Figure 4.10.1 Conjugation of 5′-carboxylated oligodeoxyribonucleotides with aminated ligands. Abbreviations: B, base (i.e., thymine, cytosine, adenine, or guanine); EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; LY, Lucifer Yellow (R).

Materials

Purified 5′-carboxylated oligodeoxyribonucleotide (*UNIT 4.9*)
0.5 M pyridine buffer, pH 5.5 to 6 (use pyridine redistilled from *p*-toluenesulfonyl chloride and stored over 3A molecular sieves; adjust pH with HCl)
10 to 20 mg/mL aminoalkylated ligand (*UNIT 4.8*; e.g., Lucifer Yellow hydrazine derivative) dissolved in water, dimethylsulfoxide (DMSO), or *N*,*N*-dimethylformamide (DMF; redistilled in vacuo over ninhydrin)
1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC)
80:20 or 50:50 (v/v) dichloromethane (CH_2Cl_2)/methanol
55:10:20 or 55:10:30 (v/v/v) isopropanol/NH_4OH/H_2O
Sephadex G-10, G-15, or G-25 resin (Pharmacia; for H_2O-soluble ligands)
0.5 M triethylammonium acetate (TEAA), pH 7
Dichloromethane or ethyl acetate (for very lipophilic ligands)
4% (w/v) $LiClO_4$ in distilled acetone, or *n*-butanol (for ligands of medium lipophilicity)
Mobile phase A: 5% (v/v) acetonitrile in 0.1 M aqueous TEAA, pH 7, all HPLC grade
Mobile phase B: 80% (v/v) acetonitrile in 0.1 M aqueous TEAA, pH 7, all HPLC grade

2-mL vial equipped with Teflon-faced septum
Analytical silica gel TLC plates (e.g., 5554 Kieselgel 60F plates; Merck)
5-mL column
UV lamp and viewing box
Rotary evaporator with water bath and vacuum pump
High-performance liquid chromatography (HPLC) system, including:
  Chemically inert syringes with replaceable needles
  Reversed-phase column: 125-mm × 4-mm, 5-µm Lichrospher 100 RP18 (Merck), CC Nucleosil 100-5 C18 (125/4; Macherey-Nagel), or polystyrene reversed-phase (PRP-1; Hamilton)
  Multiwavelength detector capable of measuring UV-Vis absorption between 230 and 600 nm
Spectrophotometer
Lyophilizer

Additional reagents and equipment for thin-layer chromatography (TLC; *APPENDIX 3D*) and purification and characterization of oligonucleotide-acridine conjugates (*UNIT 4.3*)

NOTE: All buffered solutions used for HPLC purifications should be filtered through a 0.22- or 0.45-µm disposable filter.

Couple ligand to oligonucleotide

1. In a 2-mL vial equipped with a Teflon-faced septum and magnetic stir bar, dissolve 5 OD_{260} units of purified 5′-carboxylated oligodeoxyribonucleotide in 0.1 mL of 0.5 M pyridine/HCl buffer, pH 5.5 to 6.
2. Add 0.1 mL of 10 to 20 mg/mL aminoalkylated ligand and 10 mg EDC. Stir 3 to 4 hr at room temperature.

   Water-soluble ligands should be prepared in water. Non-water-soluble ligands should be prepared in amine-free DMF or DMSO.

   EDC is a water soluble form of carbodiimide and is used to form amide bonds.

Evaluate coupling efficiency

3. Monitor the coupling reaction by thin-layer chromatography (TLC; *APPENDIX 3D*) on analytical silica gel plates, eluting first with either 80:20 or 50:50 (v/v) dichloromethane/methanol, and then with 55:10:20 or 55:10:30 (v/v/v) isopropanol/NH_4OH/H_2O.

   The Lucifer yellow conjugate is directly visible as a yellow spot. In most cases, the main excess chromophore travels to the top of the TLC plate while the conjugated oligodeoxyribonucleotide moves slightly faster (i.e., higher R_f) than the starting oligomer.

   When monitoring the coupling of Lucifer Yellow with the oligonucleotide $HOOC(CH_2)_5$-NH-CO-d[CTCTCGCACCCATCTCTC], elute the TLC plate directly with 55:10:20 (v/v/v) isopropanol/NH_4OH/H_2O (not with dichloromethane/methanol). The Lucifer Yellow excess has an R_f of 0.62, while the starting oligodeoxyribonucleotide and the conjugated product have R_f values of 0.31 and 0.34, respectively.

   The coupling reaction can also be monitored by reversed-phase HPLC using the procedures described below. In most cases, the oligodeoxyribonucleotide-ligand conjugate exhibits a longer retention time than that of the unconjugated oligomer.

Recover oligodeoxyribonucleotide-ligand conjugate

4a. *For water-soluble ligand (e.g., Lucifer Yellow):* Remove excess ligand by gel filtration using a 5-mL column of Sephadex G-10, G-15, or G-25 resin, depending on the size of the oligodeoxyribonucleotide. Collect 0.5-mL fractions and monitor for presence of conjugate by spotting 3 µL on TLC plates. Visualize by UV shadowing at 254 nm.

4b. *For very lipophilic ligand:* Concentrate the reaction mixture to dryness under reduced pressure using a rotary evaporator with a water bath and a vacuum pump. Dissolve the residue in water or 0.5 M TEAA, pH 7. Remove excess ligand by extraction with organic solvents such as dichloromethane or ethyl acetate.

4c. *For medium-lipophilicity ligand:* Precipitate conjugate by adding 10 vol of 4% $LiClO_4$ in distilled acetone or *n*-butanol. Vortex 1 min and centrifuge 5 min at 2000 × *g*, room temperature. Decant supernatant and resuspend pellet in 200 µL water. Perform gel filtration as in step 4a.

   Precipitation can also be performed using n-butanol prechilled at –30°C.

Purify and characterize conjugate

5. Preequilibrate a 125-mm × 4-mm, 5-µm Lichrospher 100 RP18 RP-HPLC column for at least 15 min with mobile phase A.

6. Before analysis and purification, run a blank linear gradient from 100% mobile phase A to 100% mobile phase B at a flow rate of 1 mL/min over 80 min.

   Repeat this step between analyses and purifications of samples having different DNA sequences or modifications.

7. Analyze sample by RP-HPLC using the flow rate and gradient in step 6. Detect the presence of oligodeoxyribonucleotide-ligand conjugates by measuring UV absorption at 254 or 260 nm.

   This method allows easy separation of underivatized or 5′-functionalized oligodeoxyribonucleotides from oligodeoxyribonucleotide-ligand conjugates, which are, in most instances, more lipophilic. In most cases, RP-HPLC using a Lichrospher 100 RP18 column or a CC Nucleosil 100-5 C18 column is efficient for purifying the conjugate, and these columns give nearly equivalent results. A PRP-1 column can be used when these columns are not efficient, as in the case of oligonucleotide-orthophenanthroline conjugates. Detection is usually performed where the oligonucleotide absorbs light (254 or 260 nm) and at a second wavelength where the ligand absorbs light (usually in the visual range). It is also possible to record the UV-Vis spectrum corresponding to each peak of the chromatogram between 230 and 600 nm.

Purification of conjugates can also be performed by ion-exchange chromatography (e.g., for oligonucleotide-orthophenanthroline conjugates; see Basic Protocol 2). Ion-exchange chromatography that requires a desalting step should only be used when RP-HPLC is not successful. Conjugates can also be purified by preparative polyacrylamide gel electrophoresis (PAGE; APPENDIX 3B). The conjugates have migrate more slowly through the gel than the starting oligonucleotides.

For all compounds described in this unit, except for oligodeoxyribonucleotide-phenanthroline conjugates, UV detection can be achieved at any wavelength at which the ligand absorbs UV light.

The steepness of the gradient used must be chosen empirically to afford the best chromatographic separation between the oligonucleotide conjugate and the 5′-functionalized oligonucleotide precursor. Usually a rate of increase of 1% acetonitrile per minute is used for RP-HPLC.

8. After determining the system that gives the best separation, purify the remaining crude 5′-modified oligonucleotide by preparative reversed-phase or ion-exchange chromatography.

   Alternatively, purify directly on an analytical column when the coupling reaction is achieved on a small scale (i.e., ~5 OD units).

9. Purify and characterize as reported in *UNIT 4.3* (steps 3 to 8 of Support Protocol), but analyze the purified conjugate by RP-HPLC.

   After purification, UV absorption spectra of purified oligodeoxyribonucleotide-ligand conjugates show absorptions that are characteristic to the ligand (acridine, psoralen, pyrene, fluorescein, thiazole orange).

10. Determine the extinction coefficient (ε) of the oligodeoxyribonucleotide-ligand conjugate.

    It is generally accepted that the ε value of the oligodeoxyribonucleotide-ligand conjugate at a given wavelength is the sum of the ε values of the oligodeoxyribonucleotide and the ligand at the same wavelength.

11. Measure the OD_{260} to determine the yield of the oligodeoxyribonucleotide-ligand conjugate.

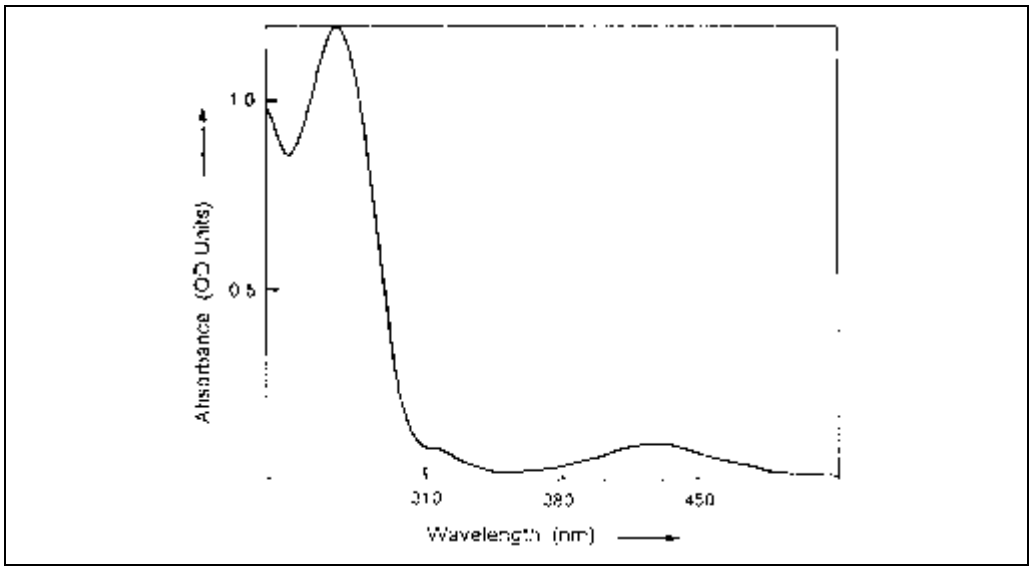

Figure 4.10.2 UV-visible spectrum of the Lucifer Yellow–oligodeoxyribonucleotide conjugate LY-NH-CO$(CH_2)_5$-NH-CO-d[CTCTCGCACCCATCTCTC] recorded in water between 230 and 520 nm.

12. Lyophilize the conjugate and store at −20°C (stable for one to many years).

Nuclease digestion of the conjugates and RP-HPLC analysis of the resulting monomers can also be performed to verify that base modifications did not occur during coupling. Moreover, these methods confirm the structure of oligodeoxyribonucleotide-ligand conjugates. Additional characterization can be made by matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) mass spectrometry.

Following conjugation of Lucifer Yellow with $HOOC(CH_2)_5$-NH-CO-d[CTCTCGCACCCATCTCTC], analyses performed on a 125-mm × 4-mm, 5-μm Lichrospher 100 RP18 column using a linear gradient of 5% to 42.5% acetonitrile in 0.1 M TEAA (100% to 50% mobile phase A versus B) over 50 min at a flow rate of 1 mL/min give a retention time of 15 min 59 sec for the product LY-NH-$CO(CH_2)_5$-NH-CO-d[CTCTCGCACCCATCTCTC], compared to 15 min 25 sec for the starting oligonucleotide. Detection was achieved at both 260 and 420 nm. Figure 4.10.2 shows the UV-visible spectrum of the LY-oligodeoxyribonucleotide conjugate in water.

Mass analysis: ESI polarity negative, LY-NH-$CO(CH_2)_5$-NH-CO-d[CTCTCGCACCCATCTCTC]. Calcd. for $C_{189}H_{240}N_{61}O_{120}P_{17}S_2$: 5875 Da; found 5874 ± 2 Da (M–H).

BASIC PROTOCOL 2

CONJUGATION OF 5′-AMINOALKYLATED OLIGODEOXYRIBONUCLEOTIDES TO LIGANDS FUNCTIONALIZED WITH AN ISOTHIOCYANATE OR *N*-HYDROXYSUCCINIMIDYL GROUP

Whereas 5′-aminoalkylated oligodeoxyribonucleotides are conjugated with carboxylated ligands in the presence of a water-soluble carbodiimide, activated ligands such as pentafluorophenyl or *N*-hydroxysuccinimidyl esters or isothiocyanate derivatives are often used in a wide variety of solvents. The coupling step can be performed in a mixture of aqueous buffer and organic solvents (e.g., DMF, DMSO), or in pure organic solvents. In the latter case, oligodeoxyribonucleotides with lipophilic counter-ions such as cetyltrimethylammonium or triethylammonium are used to facilitate solubilization. The procedure is illustrated in Figure 4.10.3. When the coupling reaction is performed in organic solvent, a tertiary amine is added to ensure that the amino function on the oligodeoxyribonucleotide is unprotonated. This protocol describes the conjugation of 5′-aminoalkylated oligodeoxyribonucleotides with orthophenanthroline (OP) isothiocyanate, Oregon Green (OR) isothiocyanate, and the *N*-hydroxysuccinimidyl ester of 1-pyrene butanoic acid. Purification procedures are similar to those described in Basic Protocol 1. The optimal purification procedure must be determined empirically.

Materials

Purified 5′-aminoalkylated oligodeoxyribonucleotide (*UNIT 4.9*)
0.5 M sodium bicarbonate ($NaHCO_3$) buffer, pH 9.5
5 to 10 mg/mL functionalized ligand (select one) in *N*,*N*-dimethylformamide (DMF):
  Orthophenanthroline (OP) isothiocyanate (*UNIT 4.8*, structure 3d)
  N-Succinimidyl ester of 1-pyrene butanoic acid (Molecular Probes)
  Oregon Green (OR) 5- and 6-isothiocyanate (mixed isomers; Molecular Probes)
Ninhydrin
25 mM Tris·Cl, pH 7 (*APPENDIX 2A*), containing 10% (v/v) methanol:
  without NaCl and
  with 1 M NaCl (for ion-exchange with a Mono Q column) or
  with 1.5 M NaCl (for ion-exchange with a DEAE column)
2-mL vial equipped with Teflon-faced septum
Mono Q HR 5/5 or HR 10/10 (Pharmacia; for OP isothiocyanate ion-exchange HPLC)

100-mm × 10-mm, 8-µm DEAE (Waters; for OP isothiocyante ion-exchange HPLC)

HR 10/10 column packed with Lichroprep PR 18 (Art 13900, Merck) or Sephadex G-10 or G-25 (for desalting)

Additional reagents and equipment for TLC (see Basic Protocol 1 and *APPENDIX 3D*) and for isolating, purifying, and characterizing the final conjugate (see Basic Protocol 1)

Perform and monitor coupling reaction

1. In a 2-mL vial equipped with a Teflon-faced septum and magnetic stir bar, dissolve 5 OD_{260} units of purified 5′-aminoalkylated oligodeoxyribonucleotide in 0.2 mL of 0.5 M sodium bicarbonate buffer, pH 9.5.

   Oligonucleotides are typically purified as triethylammonium salts, in which case 10- to 12-mers are soluble in pure DMSO. Otherwise, counter-ion exchange can be performed to

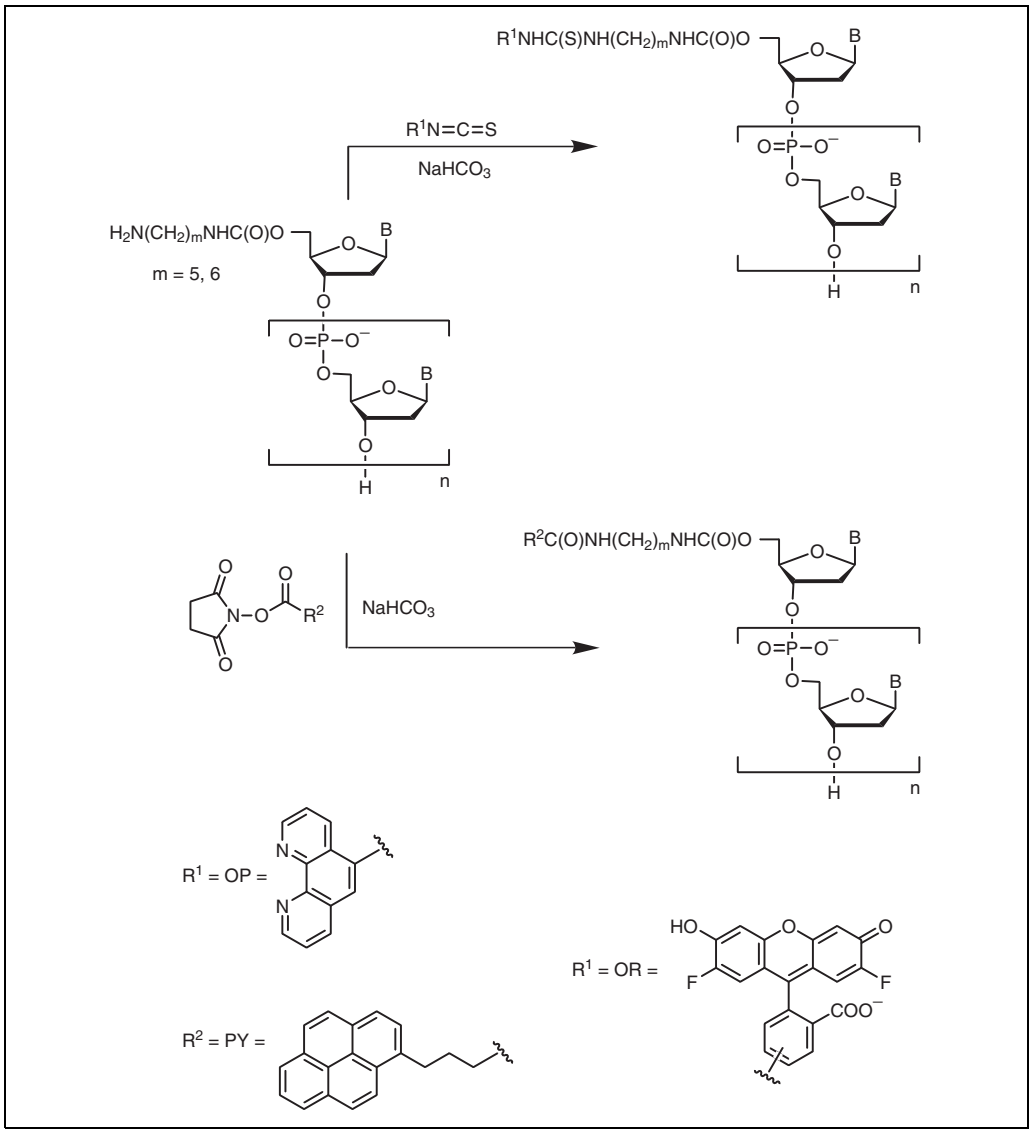

Figure 4.10.3 Conjugation of 5′-aminoalkylated oligodeoxyribonucleotides with ligands functionalized with isothiocyanate or *N*-hydroxysuccinimidyl groups. Abbreviations: OP, orthophenanthroline; OR, Oregon Green; PY, 1-pyrenepropyl.

prepare cetyltrimethylammonium salts using a procedure adapted from Zarytova et al. (1987). Start with 5 to 10 OD_{260} units of oligonucleotide in a 100-μL volume and add 10 vol of 4% $LiClO_4$ in acetone. Vortex 2 min and then centrifuge 5 min at 2000 × g, room temperature. Wash pellet two to three times with distilled acetone to remove the $LiClO_4$. Dry pellet and dissolve in 20 to 30 μL deionized water. Add 3 μL of 8% hexadecyltrimethylammonium bromide, vortex, and centrifuge again. Dry the pellet.

2. Add 0.2 mL of 5 to 10 mg/mL functionalized ligand in DMF. Stir the mixture at room temperature overnight.

3. Monitor the reaction by TLC (*APPENDIX 3D*) using the conditions described above (see Basic Protocol 1, step 3).

The presence of OP, pyrene, and their corresponding oligodeoxyribonucleotide conjugates can be easily visualized on a TLC plate by irradiation at 254 or 365 nm. At 254 nm, unmodified and conjugated oligonucleotides can be visualized as grey or black spots, depending on their concentration. At 365 nm, OP-oligonucleotide conjugates appear as slightly fluorescent spots, and pyrene-oligonucleotide conjugates appear as gold fluorescent spots.

OR and its oligodeoxyribonucleotide conjugates can be directly visualized on the TLC plate as yellow-green spots. After elution and drying of the TLC plate, the presence of the remaining 5′-amino-containing oligodeoxyribonucleotide can be detected as a grey or black spot by irradiation at 254 nm, or as a pink spot by spraying with ninhydrin and heating the plate.

Recover, purify, and characterize conjugate

4a. *For orthophenanthroline:* Recover, purify, and characterize conjugate as described above (see Basic Protocol 1, steps 5 to 12) using the appropriate modifications below.

a. *Reversed-phase HPLC:* Use a PRP-1 column with a linear gradient from 100% to 50% mobile phase A versus B over 50 min, and a flow rate of 1 mL/min for both purification and evaluation of efficiency.

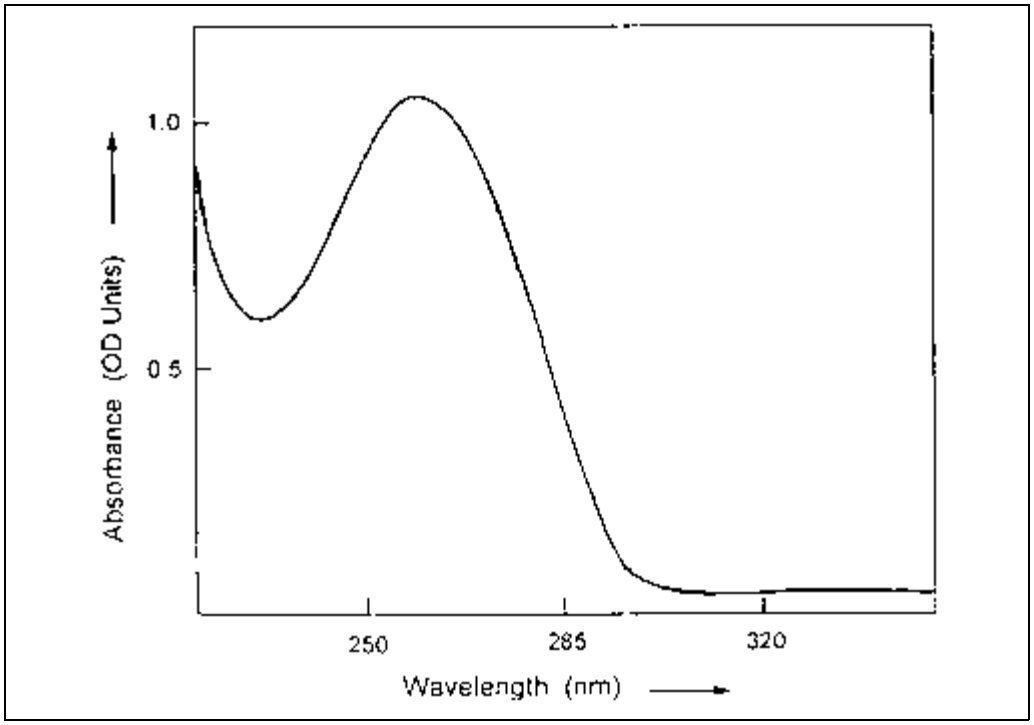

Figure 4.10.4 UV spectrum of the orthophenanthroline-oligodeoxyribonucleotide conjugate OP-NH-C(S)-NH$(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA] recorded in water between 230 and 350 nm.

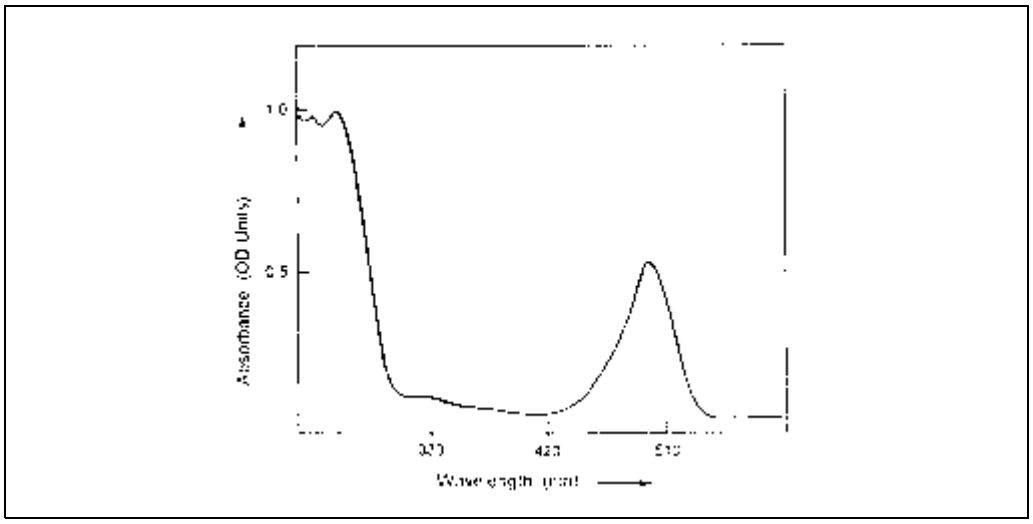

Figure 4.10.5 UV-visible spectrum of the Oregon Green–oligodeoxyribonucleotide conjugate OR-NH-C(S)-NH(CH_2)$_5$-NH-CO-d[CCGCTTAATACTGA] recorded in water between 230 and 600 nm.

b. *Ion-exchange HPLC using a Mono Q column:* Use a Mono Q HR 5/5 or 10/10 column, a linear gradient of 1 M NaCl (0% to 60%) in 25 mM Tris·Cl, pH 7, containing 10% methanol over 40 min, and a flow rate of 1 mL/min (HR 5/5) or 4 mL/min (HR 10/10). After purification, desalt the conjugate using an HR 10/10 column packed with Lichroprep RP18, Sephadex G-10, or G-25, and eluting with water.

c. *Ion-exchange HPLC using a DEAE column:* Use a 100-mm × 10-mm, 8-µm DEAE column, a linear gradient of 1.5 M NaCl (0% to 60%) in 25 mM Tris·Cl, pH 7, containing 10% methanol over 60 min, and a flow rate of 1 mL/min. After purification, desalt the conjugate using an HR 10/10 column packed with Lichroprep RP18, Sephadex G-10, or G-25, and eluting with water.

For RP-HPLC of OP isothiocyanate coupled to the oligonucleotide H_2N-(CH_2)$_5$-NH-CO-d[CCGCTTAATACTGA], analysis on an RP18 column using the conditions described above (see Basic Protocol 1) yields a higher retention time than that expected for oligomer conjugates exhibiting similar lipophilicity; however, peaks are poorly resolved. Therefore, analysis and purification of these conjugates are performed on a PRP-1 column. A retention time of 16 min 10 sec is observed for the conjugate OP-NH-C(S)-NH(CH_2)$_5$-NH-CO-

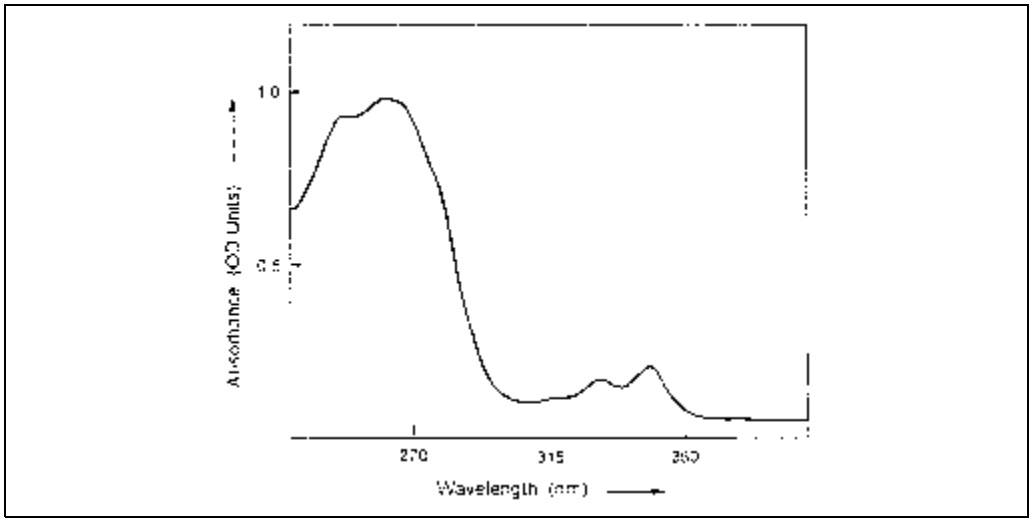

Figure 4.10.6 UV-visible spectrum of the pyrene-oligodeoxyribonucleotide conjugate PY-C(O)-NH-(CH_2)$_6$-NH-CO-d[CCGCTTAATACTGA] recorded in water between 230 and 400 nm.

d[CCGCTTAATACTGA] compared to a retention time of 12 min 30 sec for the starting oligonucleotide $H_2N(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA].

When the same conjugate and starting oligonucleotide are analyzed by ion exchange on a Mono Q HR 5/5 column using a linear gradient of 1 M NaCl (20% to 60%) in 25 mM Tris·Cl, pH 7, with 10% methanol over 40 min at a flow rate of 1 mL/min, a retention time of 19 min 30 sec is obtained for the conjugate compared to 17 min 30 sec for the starting oligodeoxyribonucleotide.

The coupling of the OP derivative to the oligodeoxyribonucleotide does not induce a significant change in the absorption spectrum. Figure 4.10.4 shows the UV spectrum of OP-NH-C(S)-NH$(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA] in water.

Denaturing polyacrylamide gel electrophoresis allows resolution of the coupling product from the unreacted oligodeoxyribonucleotide. The mobility of the conjugate in the gel is more retarded than that of the starting oligodeoxyribonucleotide. The difference between the electrophoretic mobilities is similar to that observed between $H_2N(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA] and d[CCGCTTAATACTGA], shown in Figure 4.9.6.

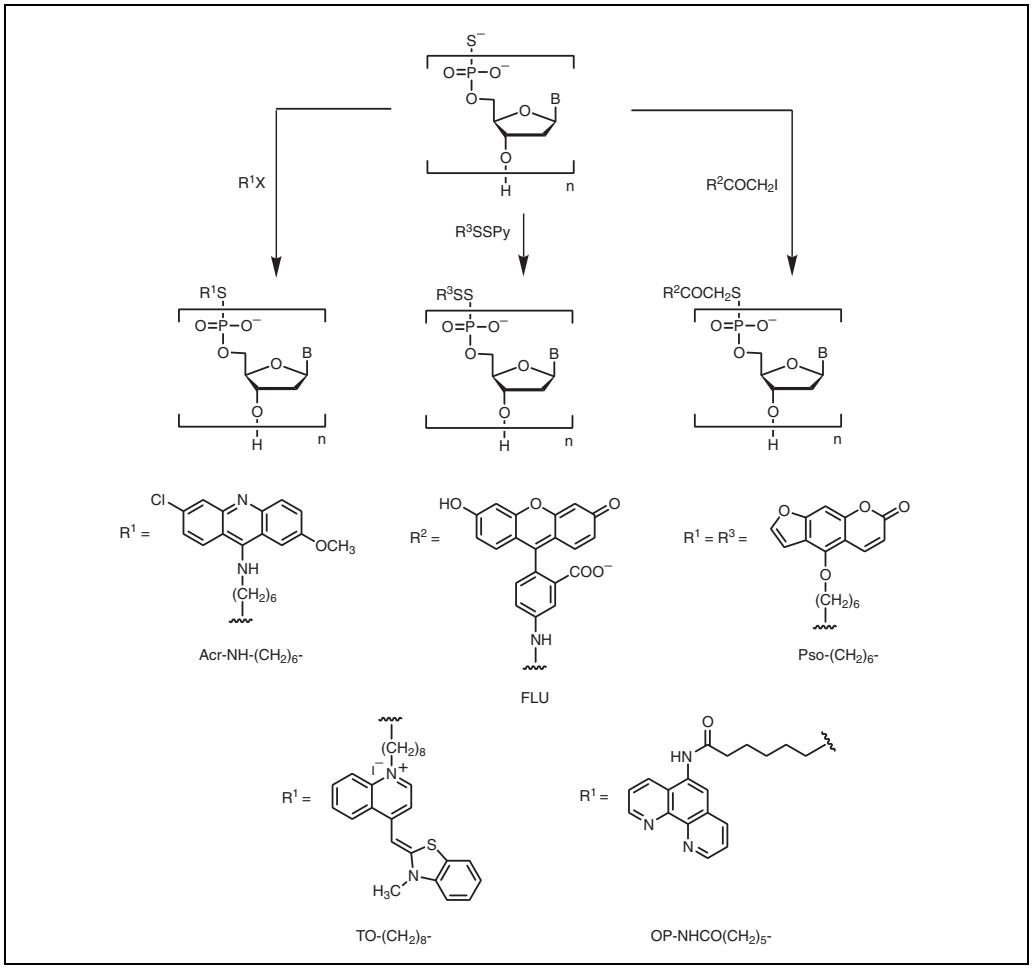

Figure 4.10.7 Conjugation of oligodeoxyribonucleotide 5′-phosphorothioates with halogenoalkylated ligands (left), an iodoacetamidylated ligand (right), and a 2-pyridyldithioalkylated ligand (center). For halogenoalkylated ligands, R^1 = Acr-NH-$(CH_2)_6$- (*UNIT 4.8*, structure 1e); Pso-$(CH_2)_6$- (*UNIT 4.8*, structure 2f), OP-C(O)-$(CH_2)_5$- (*UNIT 4.8*, structure 3c); and TO-$(CH_2)_8$- (*UNIT 4.8*, structure 4e). For the iodoacetamidylated ligand, R^2 = FLU. For the 2-pyridyldithioalkylated ligand, $R^3 = P_{50}$-$(CH_2)_6$- (*UNIT 4.8*, structure 2h). Abbreviations: Acr, acridine; FLU, fluorescein; OP, orthophenanthroline; Pso, psoralen; P_Y, 2-pyridyl; TO, thiazole orange.

4b. *For Oregon Green and N-hydroxysuccinimidyl ester of pyrene:* Recover, purify, and characterize conjugate as described above (see Basic Protocol 1, steps 5 to 12).

For coupling of Oregon Green (OR) 5- or 6-isothiocyanate with oligonucleotide H_2N-$(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA], detection at both 260 and 500 nm yields two peaks for the conjugate OR-NH-C(S)-NH$(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA], with retention times of 19 min 5 sec and 20 min 46 sec, corresponding to the two geometrical isomers of OR. The starting H_2N-$(CH_2)_6$-NH-CO-d[CCGCTTAATACTGA] is eluted with a retention time of 14 min. Figure 4.10.5 shows the UV-visible spectrum of the OR-oligodeoxyribonucleotide conjugate in water.

Mass analysis: ESI polarity negative, OR-NH-C(S)-NH-$(CH_2)_5$-NH-CO-d[CCGCTTAATACTGA]. Calcd. for $C_{163}H_{194}N_{53}O_{88}P_{13}SF_2$: 4776 Da; found 4477 ± 1 Da (M–H).

For coupling of the N-hydroxysuccinimidyl ester of pyrene (PY) with oligonucleotide $H_2N(CH_2)_6$-NH-CO-d[CCGCTTAATACTGA], detection at both 260 and 350 nm gives a retention time of 35 min 42 sec for the conjugate PY-NH-C(O)-NH$(CH_2)_6$-NH-CO-d[CCGCTTAATACTGA], compared to 14 min for the starting oligonucleotide. Figure 4.10.6 shows the UV-visible spectrum of the PY-oligodeoxyribonucleotide conjugate in water.

Mass analysis: ESI polarity negative, PY-C(O)-NH-$(CH_2)_6$-NH-CO-d[CCGCTTAATACTGA]. Calcd. for $C_{163}H_{201}N_{52}O_{84}P_{13}$: 4636 Da; found 4636 Da (M–H).

CONJUGATION OF OLIGODEOXYRIBONUCLEOTIDE 5′-PHOSPHOROTHIOATES TO LIGANDS FUNCTIONALIZED WITH HALOGENOALKYL, 2-PYRIDYLDITHIO, OR IODOACETAMIDYL GROUPS

BASIC PROTOCOL 3

The derivatization of oligodeoxyribonucleotide 5′-phosphorothioates, illustrated in Figure 4.10.7, is achieved over a wide pH range (5 to 8) with ligands carrying iodoacetamidyl, halogenoalkyl, or 2-pyridyldithio groups. The conjugation reaction can be performed in water, aqueous DMF, or aqueous methanol. The reaction can also be performed in pure methanol in the presence of crown ethers to facilitate solubilization of the oligodeoxyribonucleotide salts (i.e., 18-crown-6 for potassium salts and 15-crown-5 for sodium salts).

Procedures are presented for (1) halogenoalkyl derivatives of acridine, psoralen, orthophenanthroline, and thiazole orange; (2) a 2-pyridyldithio derivative of psoralen; and (3) an iodoacetamido derivative of fluorescein. The halogenoalkyl derivatives are reacted with oligodeoxyribonucleotide 5′-phosphorothioates in methanol in the presence of crown ether. The 2-pyridyldithio derivative of psoralen requires minor changes to the conjugation procedure. The protocol for the iodoacetamido derivative is identical to that for the halogenoalkyl-containing ligands; however, since iodoacetamido derivatives are, in general, more reactive than the halogenoalkyl containing ligands, the coupling times are often shorter. Iodoacetamido derivatives of numerous ligands are commercially available. The reader can also refer to Asseline et al. (1996) for preparation of an iodoacetamido derivative of acridine.

Materials

Purified lyophilized oligodeoxyribonucleotide 5′-phosphorothioates (Na^+ or K^+ salt; *UNIT 4.9*)
12.5 mg/mL 15-crown-5 or 18-crown-6 (Aldrich) in methanol
Functionalized ligand (select one):
  Halogenoalkylated acridine, psoralen, orthophenanthroline, or thiazole orange (*UNIT 4.8*, structures 1e, 2f, 3c, and 4e, respectively)
  2-Pyridyldisulfide psoralen (*UNIT 4.8*, structure 2h)
  Iodoacetamidylated fluorescein (Aldrich)
Methanol (for halogenoalkylated ligands and iodoacetamidylated fluorescein)
Dichloromethane
0.5 M sodium or potassium phosphate buffer, pH 7 (*APPENDIX 2A*)
Sephadex G-10 or G-15 column
0.1 M triethylammonium acetate (TEAA) buffer, pH 7

2-mL vial equipped with a Teflon-faced septum
30° to 35°C water bath (optional; for halogenoalkylated ligands)
UV-Vis spectrophotometer

Additional reagents and equipment for TLC (see Basic Protocol 1and *APPENDIX 3D*) and for isolating, purifying, and characterizing the final conjugate (see Basic Protocol 1)

NOTE: In order to prevent chelation of phosphorothioates and phenanthrolines attached to oligodeoxyribonucleotides, all solvents and buffer solutions used for their purification must be passed through a column of Chelex 100 resin to remove divalent cations.

Prepare oligodeoxyribonucleotide 5′-phosphorothioate

1. In a 2-mL vial equipped with a Teflon-faced septum and magnetic stir bar, dissolve 5 OD_{260} units of purified lyophilized oligodeoxyribonucleotide 5′-phosphorothioate (sodium salt or potassium salt) in 0.4 mL of 12.5 mg/mL 15-crown-5 (for sodium salt) or 18-crown-6 (for potassium salt) in methanol.

Perform conjugation

For halogenalkylated derivatives:

2a. Dissolve 10 eq halogenoalkylated acridine, psoralen, orthophenanthroline, or thiazole orange in a minimal volume of methanol (enough to dissolve the ligand). Add to the oligodeoxyribonucleotide and seal the vial. For the psoralen derivative, also add 0.2 mL dichloromethane and vortex 20 or 30 sec to fully dissolve.

3a. Stir the mixture at room temperature or at 30° to 35°C.

The conjugation is usually completed after a few hours (5 to 8 hr), but a longer reaction time is sometimes required (e.g., 24 hr).

4a. Monitor the reaction by TLC using the conditions described above (see Basic Protocol 1, step 3).

Acridine- and thiazole orange–oligodeoxyribonucleotide conjugates can be easily visualized on TLC plates as yellow and red spots, respectively. Psoralen and orthophenanthroline conjugates can be visualized by irradiation at 254 nm (grey or black spots) or 365 nm (pale fluorescent spots). The presence of unreacted oligodeoxyribonucleotide 5′-thiophosphates can be detected by spraying with DBPNC and heating; the 5′-thiophosphate-containing oligodeoxyribonucleotides give a pink spot.

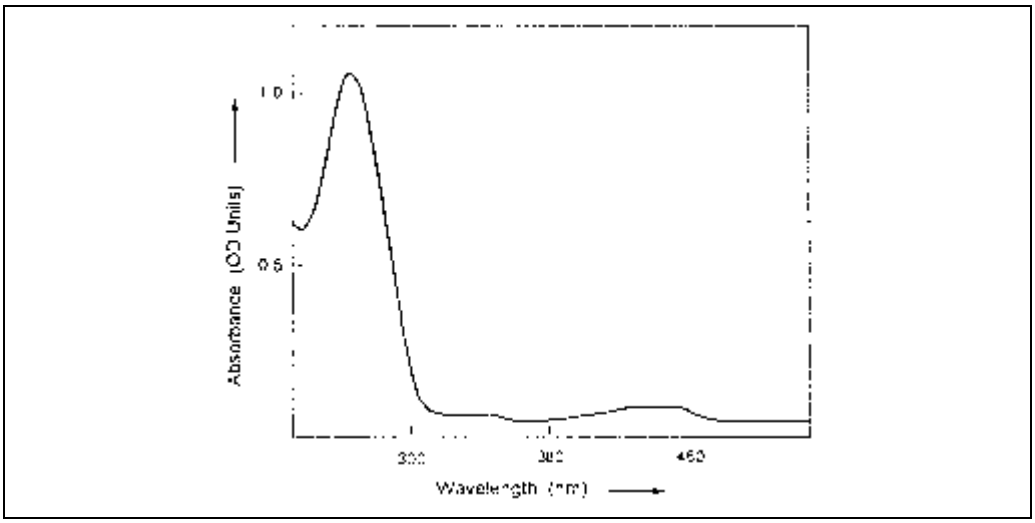

Figure 4.10.8 UV-visible spectrum of the acridine-oligodeoxyribonucleotide conjugate Acr-NH$(CH_2)_6$-S-p-d[CCGCTTAATACTGA] recorded in water between 230 and 530 nm.

For 2-pyridydisulfide psoralen derivative:

2b. Dissolve 5 eq 2-pyridyldisulfide psoralen derivative in 0.2 mL dichloromethane. Add to the oligodeoxyribonucleotide and seal the vial. Vortex for 20 or 30 sec to completely solubilize the mixture.

3b. Stir the mixture at room temperature for 3 to 4 hr.

4b. Monitor the reaction by TLC (step 4a).

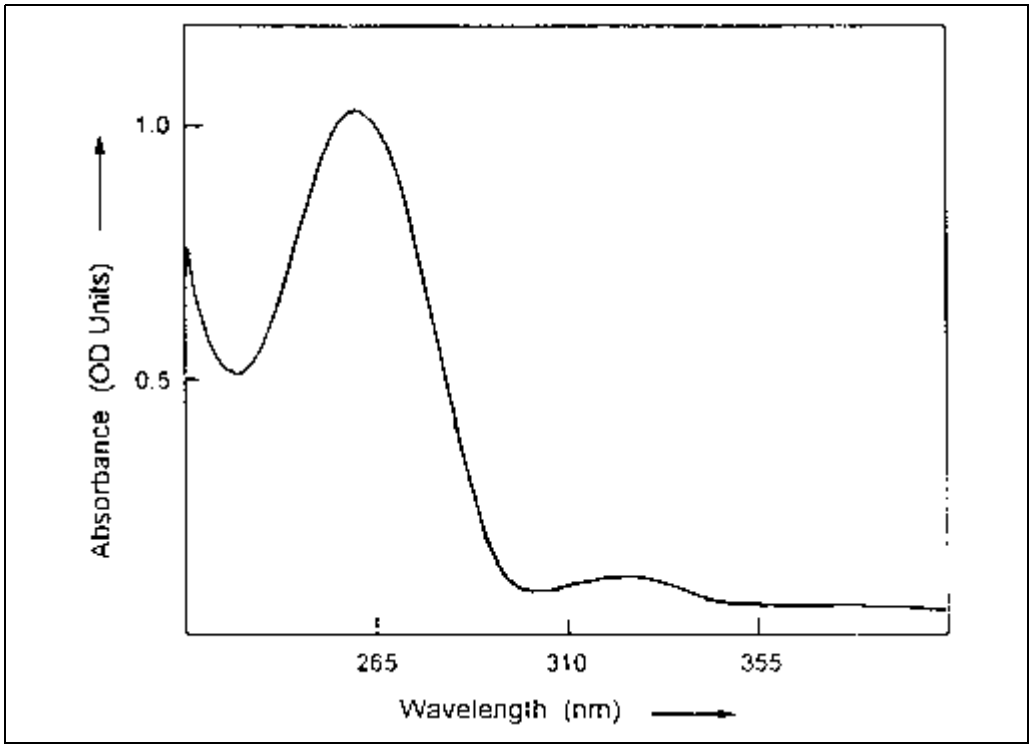

Figure 4.10.9 UV-visible spectrum of the psoralen-oligodeoxyribonucleotide conjugate Pso-$(CH_2)_6$-S-p-d[CCGCTTAATACTGA] recorded in water between 220 and 400 nm.

For iodoacetamido fluorescein derivative:

2c. Dissolve 10 eq iodoacetamidylated fluorescein in a minimal volume of methanol (enough to dissolve the ligand). Add to the oligodeoxyribonucleotide and seal the vial.

3c. Stir the mixture at room temperature or at 30° to 35°C.

The conjugation is usually completed after a few hours (5 to 8 hr), but a longer reaction time is sometimes required (e.g., 24 hr).

4c. Monitor the reaction by TLC (step 4a).

Fluorescein conjugates can be visualized directly on the TLC plate as yellow spots.

Analyze, purify, and characterize conjugate

5. When the reaction is complete or nearly complete, add 0.4 mL of 0.5 M sodium or potassium phosphate buffer, pH 7.

6. Extract three times with 1 mL dichloromethane each to remove the bulk of the excess ligand.

7. Remove all remaining ligand by gel filtration on a Sephadex G-10 or G-15 column, eluting with water or 0.1 M TEAA buffer, pH 7. Monitor elution by detection at 260 nm using a UV-Vis spectrophotometer.

The size of the column depends on the scale of the synthesis. Usually a 5-mL column is used.

8. Analyze, purify, and characterize the oligodeoxyribonucleotide-ligand conjugates as described above (see Basic Protocol 1, steps 5 to 12).

For halogenoalkylated acridine derivative: *The conjugate has a retention time of 19 min 32 sec, compared to 13 min 7 sec for the unreacted oligodeoxyribonucleotide 5′-thiophosphate. In addition to detection at 260 nm, a second detection at 425 nm confirms the presence of acridine. Figure 4.10.8 shows the UV-visible spectrum of the acridine-oligodeoxyribonucleotide conjugate in water. Mass analysis: ESI polarity negative, Acr-NH(CH_2)$_6$-S-p-d[CCGCTTAATACTGA]. Calcd. for $C_{156}ClH_{197}N_{52}O_{85}P_{14}S$: 4659 Da; found 4657 ± 2 Da (M–H).*

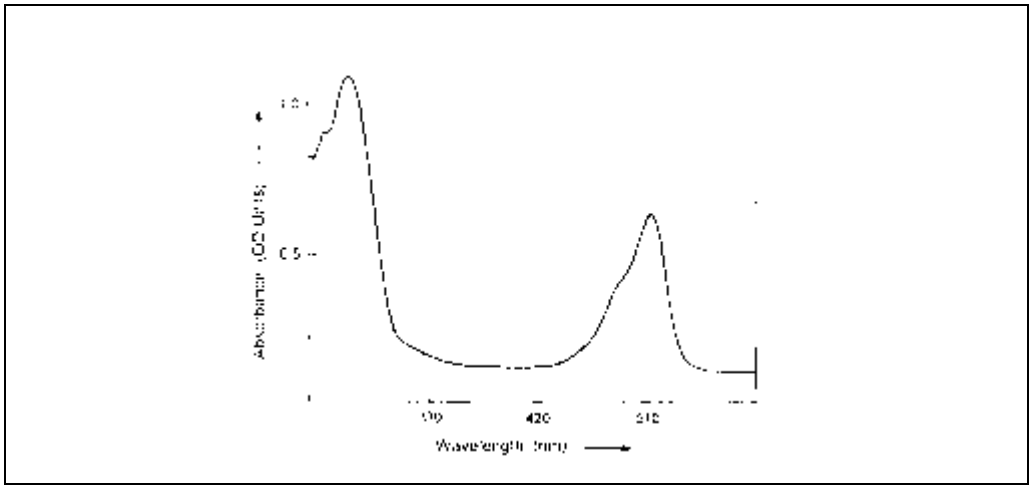

Figure 4.10.10 UV-visible spectrum of the thiazole orange–oligodeoxyribonucleotide conjugate TO-(CH_2)$_8$-S-p-d[CCGCTTAATACTGA] recorded in water between 230 and 600 nm.

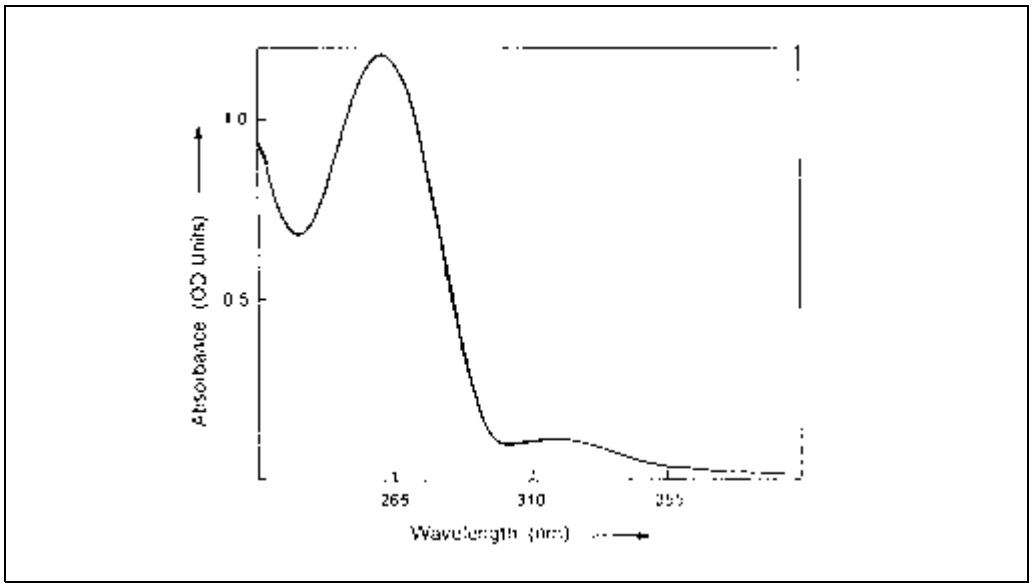

Figure 4.10.11 UV-visible spectrum of the psoralen-oligodeoxyribonucleotide conjugate Pso-$(CH_2)_6$-S-S-p-d[CCGCTTAATACTGA] recorded in water between 220 and 400 nm.

For halogenoalkylated psoralen derivative: *The conjugate has a retention time of 25 min 19 sec. In addition to detection at 260 nm, a second detection at 340 nm confirms the presence of psoralen. Figure 4.10.9 shows the UV-visible spectrum of the psoralen-oligodeoxyribonucleotide conjugate recorded in water. Mass analysis: ESI polarity negative, Pso-$(CH_2)_6$-S-p-d[CCGCTTAATACTGA]. Calcd. for $C_{153}H_{192}N_{50}O_{88}P_{14}S$: 4602 Da; found 4603 ± 1 Da (M–H).*

For halogenoalkylated thiazole orange derivative: *The conjugate has a retention time of 26 min 19 sec. In addition to detection at 260 nm, a second detection at 510 nm confirms the presence of thiazole orange. Figure 4.10.10 shows the UV-visible spectrum of the thiazole orange–oligodeoxyribonucleotide conjugate in water. Mass analysis: ESI polarity negative, TO-$(CH_2)_8$-S-p-d[CCGCTTAATACTGA]. Calcd. for $C_{162}H_{205}N_{52}O_{84}P_{14}S$: 4719 Da; found 4720 ± 1 Da (M–H).*

For halogenoalkylated orthophenanthroline derivative: *As previously reported (see Basic Protocol 2), the phenanthroline-oligonucleotide conjugates are poorly eluted on an RP18 reversed-phase HPLC column. Thus, this conjugate is analyzed and purified as described in Basic Protocol 2 (step 4a, substep a) for the coupling of the orthophenanthroline isothiocyanate derivative with a 5′-aminoalkylated oligodeoxyribonucleotide. Analysis on*

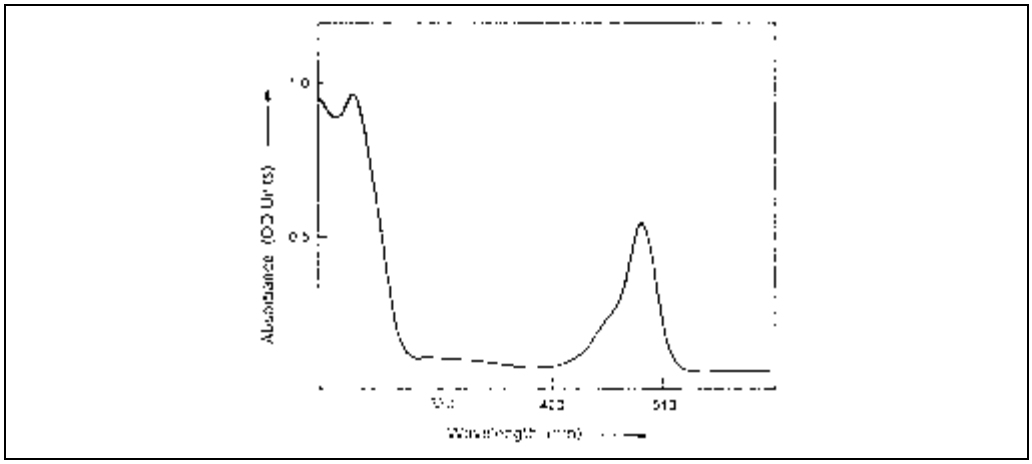

Figure 4.10.12 UV-visible spectrum of the fluorescein-oligodeoxyribonucleotide conjugate FLU-NH-C(O)-CH_2-S-p-d[CCGCTTAATACTGA] recorded in 0.1 M sodium bicarbonate buffer, pH 9, between 230 and 600 nm.

a PRP-1 column using a gradient from 5% to 42.5% CH_3CN in 0.1 M TEAA, pH 7, over 50 min at a flow rate of 1 mL/min gives a retention time of 15 min 24 sec for the conjugate OP-NH-C(O)-$(CH_2)_5$-S-p-d[CCGCTTAATACTGA], compared to 11 min 21 sec for the starting oligonucleotide 5′-thiophosphate. As in Basic Protocol 2, this coupling of orthophenanthroline halogenoalkyl derivative with the oligodeoxyribonucleotide 5′-thiophosphate does not induce significant changes in the absorption spectrum.

For 2-pyridyldisulfide psoralen: *Analysis under these conditions gives a retention time of 28 min 7 sec for the conjugate. As in the case of the halogenoalkyl derivative of psoralen, additional detection at 340 nm confirms the presence of psoralen. Figure 4.10.11 shows the UV-visible spectrum of the psoralen-oligodeoxyribonucleotide conjugate (via a disulfide bond) in water. Mass analysis: ESI polarity negative, Pso-$(CH_2)_6$-S-S-p-d[CCGCTTAATACTGA]. Calcd. for $C_{153}H_{192}N_{50}O_{88}P_{14}SS$: 4634 Da; found 4633 ± 1 Da (M–H).*

***For 5′-iodoacetamidylated fluorescein*:** *The conjugate of the 5′-iodoacetamidylated fluorescein (isomer 5; FLU) with S-p-d[CCGCTTAATACTGA] has a retention time of 17 min 18 sec using an RP18 reversed-phase HPLC column under the conditions described above. Figure 4.10.12 shows the UV-visible spectrum of the fluorescein-oligodeoxyribonucleotide conjugate in 0.1 M sodium hydrogen carbonate buffer, pH 7. Mass analysis: ESI polarity negative, FLU-NH-C(O)-CH_2-S-p-d[CCGCTTAATACTGA]. Calcd. for $C_{158}H_{189}N_{51}O_{90}P_{14}S$: 4707 Da; found 4708 Da (M–H).*

Ion-exchange for halogenoalkylated ligand-oligodeoxyribonucleotide conjugates: *Analysis and purification of these conjugates can also be performed on a Mono Q or DEAE ion-exchange column using the eluents and conditions described above (see Basic Protocol 2, step 4a, substeps b and c). The conjugate is retained more than the oligonucleotide 5′-thiophosphate because of the hydrophobicity of the ligand. For example, using a Mono Q column under the conditions described in Basic Protocol 2, the conjugate OP-NH-C(O)-$(CH_2)_5$-S-p-d[CCGCTTAATACTGA] is eluted at 28 min 30 sec, compared to 26 min for the starting oligodeoxyribonucleotide 5′-phosphorothioate.*

PAGE of halogenoalkylated ligand-oligodeoxyribonucleotide conjugates: *Denaturing polyacrylamide gel electrophoresis (UNIT 10.4) allows the resolution of the conjugate OP-NH-C(O)-$(CH_2)_5$-S-p-d[CCGCTTAATACTGA] from unreacted S-p-d[CCGCTTAATACTGA]. The electrophoretic mobility of the conjugate on the gel is retarded when compared to that of the oligodeoxyribonucleotide 5′-thiophosphate. A third band with weak intensity and lower electrophoretic mobility relative to the other two bands is also observed. This band may correspond to the dimer of the oligodeoxyribonucleotide 5′-phosphorothioate. Mass analysis: ESI polarity negative, OP-NH-C(O)-$(CH_2)_5$-S-p-d[CCGCTTAATACTGA]. Calcd. for C154H193N53O85P14S: 4609 Da; found 4610 ± 1 Da (M-H).*

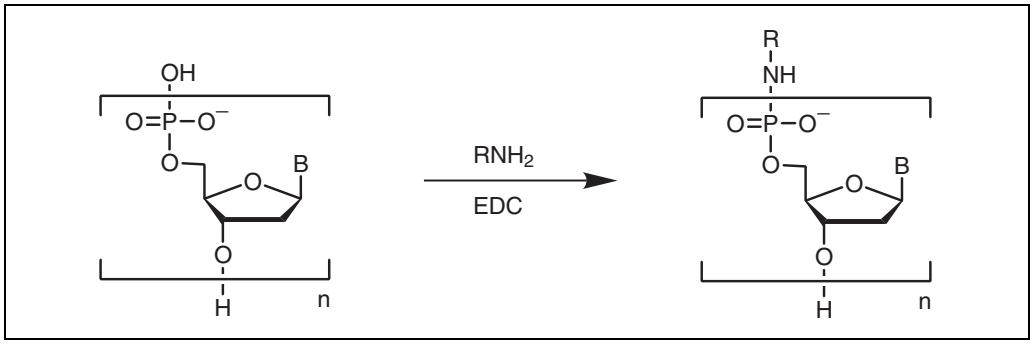

Figure 4.10.13 Condensation reaction of oligodeoxyribonucleotide 5′-phosphates with aminated compounds. R, $H_2N(CH_2)_5CH_2$-.

CONJUGATION OF OLIGODEOXYRIBONUCLEOTIDE 5′-PHOSPHATES TO LIGANDS FUNCTIONALIZED WITH AMINO GROUPS

The conjugation of oligodeoxyribonucleotides carrying a phosphate group at the 5′ terminus with the primary amino function of a ligand is accomplished in the presence of carbodiimide. It generally requires a large excess of both ligand and carbodiimide (100 to 500 eq). Usually the oligodeoxyribonucleotide is solubilized in a buffer and the ligand is added to the solution in amine-free DMF. The procedure is illustrated in Figure 4.10.13 and exemplified by the coupling of 1,5-diaminopentane with oligodeoxyribonucleotide 5′-phosphates.

Materials

Purified oligodeoxyribonucleotide 5′-phosphate (*UNIT 4.9*)
0.5 M aqueous 1,5-diaminopentane, pH 4.5
1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC)
4% (w/v) $LiClO_4$ in acetone
Mobile phase A: 0.1 M triethylammonium acetate buffer pH 7, containing 5% CH_3CN
Mobile phase B: 0.1 M triethylammonium acetate (TEAA) buffer, pH 7 containing 80% CH_3CN
2-mL vial equipped with a Teflon-faced septum
HPLC system (also see Basic Protocol 1)
Column: 150-mm × 3-mm, 5-µm RP8 glass cartridge system (Merck)

Additional reagents and equipment for TLC (see Basic Protocol 1 and *APPENDIX 3D*) and for isolating, purifying, and characterizing the final conjugate (see Basic Protocol 1)

1. In a 2-mL vial equipped with a Teflon-faced septum and magnetic stir bar, dissolve 5 OD_{260} units of purified oligodeoxyribonucleotide 5′-phosphate in 0.2 mL of 0.5 M aqueous 1,5-diaminopentane, pH 4.5.
2. Add 10 mg water-soluble EDC and incubate with stirring at room temperature for 4 to 5 hr.
3. Divide the reaction mixture between two microcentrifuge tubes.
4. Add 1 mL of 4% (w/v) $LiClO_4$ in acetone to each tube. Vortex for at least 1 min and centrifuge for 5 min at 2000 × *g*, room temperature. Discard supernatant and dissolve the residue in 0.1 mL water. Repeat three times.
5. Analyze condensation product by reversed-phase HPLC using a 150-mm × 3-mm, 5-µm RP8 glass cartridge system with a gradient from 100% to 50% mobile phase A versus B, over 50 min at a flow rate of 0.5 mL/min.

   Using these conditions, the condensation product H_2N-$(CH_2)_5$-NH-p-d[TTCTCCCCCGCTTA] shows one peak with a retention time of 15 min 34 sec, compared to 14 min 45 sec for the oligodeoxyribonucleotide 5′-phosphate. Mass analysis: ESI polarity negative, H_2N-$(CH_2)_5$-NH-p-d[TTCTCCCCCGCTTA]. Calcd. for $C_{138}H_{189}N_{43}O_{88}P_{14}$: 4289 Da; found 4289 ± 1 Da (M–H).

   The presence of the primary amino function at the end of the linker can be verified after TLC elution by spraying the plate with ninhydrin solution and heating.

BASIC PROTOCOL 5

CONJUGATION OF 5′-MERCAPTOALKYLATED OLIGODEOXYRIBONUCLEOTIDES TO LIGANDS FUNCTIONALIZED WITH HALOGENOALKYL, IODOACETAMIDYL, OR 2-PYRIDYLDITHIO GROUPS

This coupling reaction can be performed in buffer (pH 7 to 9), in a mixture of buffer and DMF, or directly in DMF using oligodeoxyribonucleotides converted to their cetyltrimethylammonium salts. Solutions must be degassed by bubbling with argon or nitrogen. Halogenoalkylated, iodoacetamidylated, and 2-pyridyldithioalkylated derivatives of various ligands are conjugated with an oligodeoxyribonucleotide bearing a masked mercaptoalkylated linker at its 5′ end. The procedures are illustrated in Figure 4.10.14.

The coupling of halogenoalkylated thiazole orange with HS-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] is performed in situ by treatment of C_5H_5N-S-S-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] with the reducing agent Tris-(2-carboxyethyl)phosphine (TCEP). The coupling of iodoacetamidyl ligands with 5′-mercaptoalkylated oligodeoxyribonucleotides is performed using the same procedure.

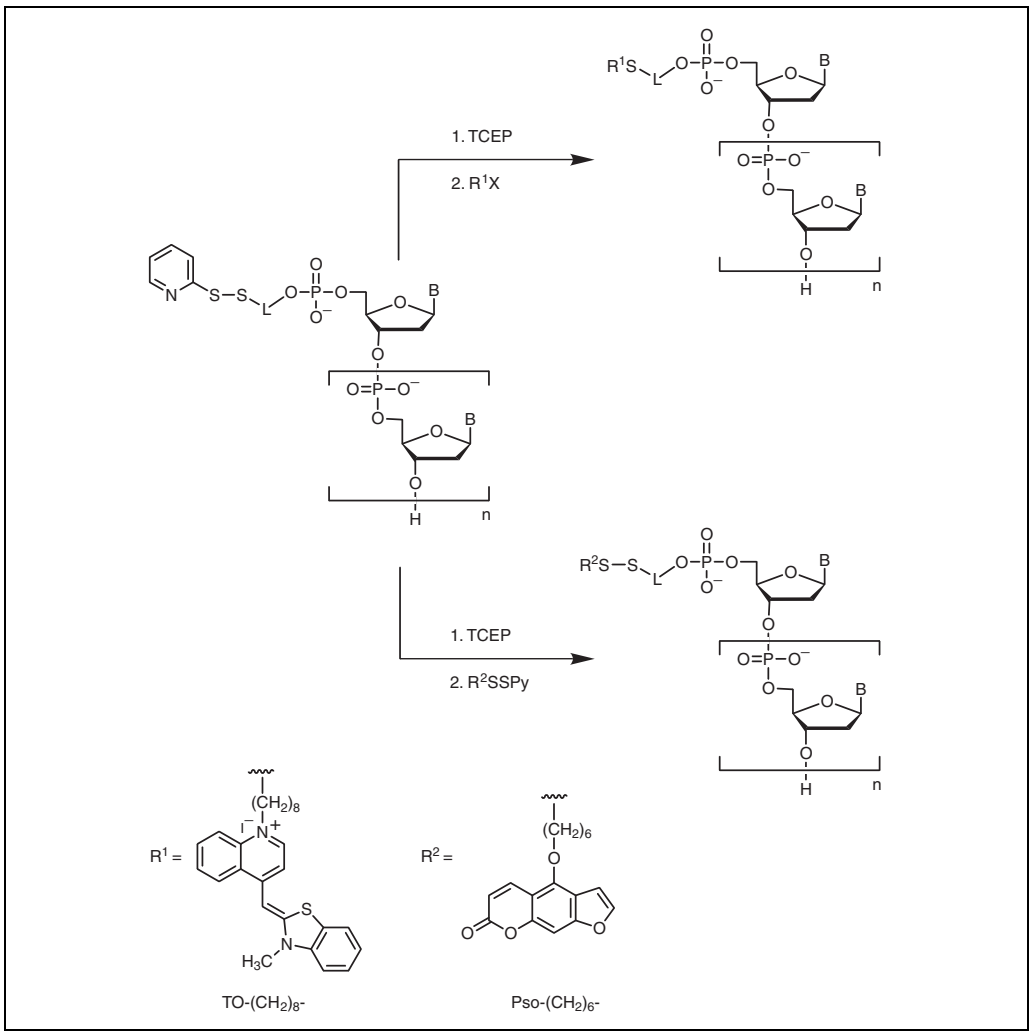

Figure 4.10.14 Conjugation of oligodeoxyribonucleotides bearing a 5′-(2-pyridyldithioalkylated) linker with a halogenoalkylated ligand (top) or a 2-pyridyldithioalkylated ligand (bottom). R^1 = TO-$(CH_2)_8$- (*UNIT 4.8*, structure 4e); R^2 = Pso-$(CH_2)_6$- (*UNIT 4.8*, structure 2h). Abbreviations: TCEP, Tris-(2-carboxyethyl)phosphine hydrochloride; X, I; Py, 1-pyridyl; L, $CH_2CH_2OCH_2CH_2OCH_2CH_2$.

The coupling of 2-pyridyldithioalkylated ligands with 5′-(2-pyridyldithioalkylated)-oligodeoxyribonucleotides is quite similar, but in this case the amount of TCEP must be strictly controlled in order to prevent the cleavage of the product, which also contains a disulfide bridge. This protocol describes the condensation of C_5H_5N-S-S-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] with 2-pyridyldithioalkylated psoralen.

Conjugate Halogenoalkylated and Iodoacetamidylated Ligands to 5′-Mercaptoalkylated Oligodeoxyribonucleotides

Materials

Purified 5′-(2-pyridyldithioalkylated) oligodeoxyribonucleotide (*UNIT 4.9*)
30 mM sodium bicarbonate buffer, pH 9
Dimethylformamide (DMF)
Nitrogen or argon gas
Tris-(2-carboxyethyl)phosphine (TCEP)
Halogenoalkylated or iodoacetamidylated ligand (e.g., halogenoalkylated thiazole orange; *UNIT 4.8*, structure 4e)
Diisopropylethylamine (optional)
2-mL vial equipped with a Teflon-faced septum

Additional reagents and equipment for TLC (see Basic Protocol 1 and *APPENDIX 3D*) and for isolating, purifying, and characterizing the final conjugate (see Basic Protocol 1)

1a. In a 2-mL vial equipped with a Teflon-faced septum and magnetic stir bar, dissolve 10 OD_{260} units of purified 5′-(2-pyridyldithioalkylated) oligodeoxyribonucleotide in 0.15 to 0.3 mL of the desired solvent—i.e., 30 mM sodium bicarbonate buffer, pH 9, buffer/DMF, or DMF.

The solvent must be chosen empirically. The resulting reaction mixture must be homogeneous.

2a. Degas the solution by bubbling argon or nitrogen gas through it for 10 min.

3a. Dissolve 2 or 3 eq TCEP in a minimal volume (2 to 3 µl) of water and add to the degassed oligonucleotide solution. Leave 30 min at room temperature.

4a. Dissolve 5 to 20 eq halogenoalkylated or iodoacetamidylated ligand in 0.15 to 0.3 mL DMF and add to the degassed oligonucleotide solution. If the conjugation reaction is performed in DMF without buffer, also add 5 µl diisopropylethylamine.

5a. Stir the mixture at room temperature or at 30° to 35°C for 3 to 4 hr. Monitor reaction by TLC (see Basic Protocol 1, step 3).

The thiazole orange conjugate is readily visible on the TLC plate as a red spot.

6a. Analyze, purify, and characterize the conjugate as described for condensation of halogenoalkylated ligands with oligodeoxyribonucleotide 5′-thiophosphates (see Basic Protocol 1, steps 5 to 12).

Analysis of the coupling reaction between the thiazole orange (TO) derivative and C_5H_5N-S-S-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] is performed on an RP18 (5% to 42.5% of CH_3CN over 50 min in 0.1 M TEAA, pH 7 at a flow rate of 1 mL/min) reversed-phase HPLC column using the conditions described in Basic Protocol 3. The conjugate TO-$(CH_2)_8$-S-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] elutes with a retention time of 24 min 25 sec. A second peak at 18 min 11 sec corresponds to either the thiol-containing oligodeoxyribonucleotide HS-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] or its (5′-5′)-dimer derivative 3′-d[CTCTCTACCCACGCTCTC]-p-CH_2CH_2-$(OCH_2CH_2)_2$-S-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC]. The presence of thiazole orange in the conjugate is

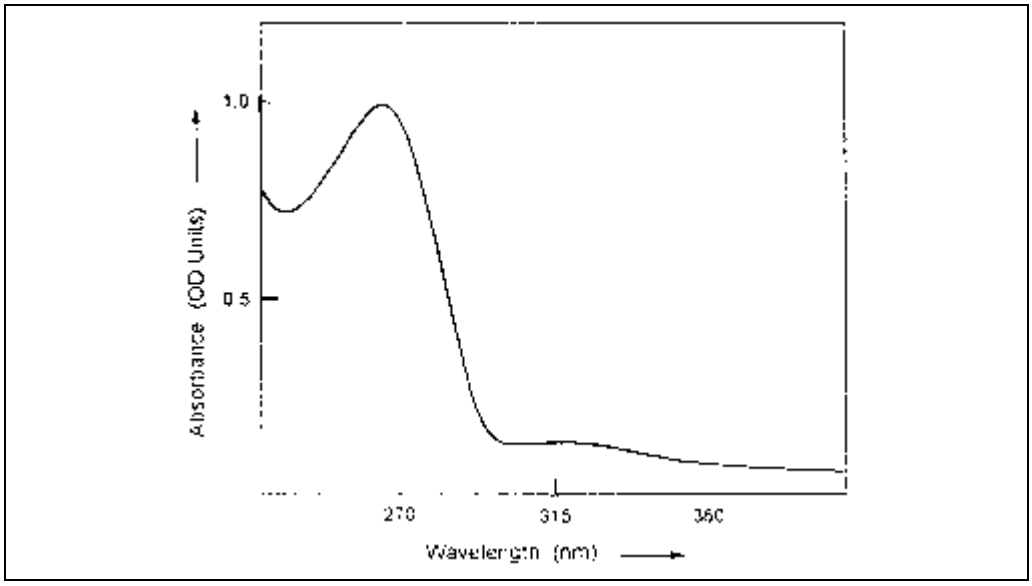

Figure 4.10.15 UV-visible spectrum of the psoralen-oligodeoxyribonucleotide conjugate Pso-$(CH_2)_6$-S-S-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] recorded in water between 230 and 400 nm.

detected at 260 and 510 nm. Mass analysis: ESI polarity negative, TO-$(CH_2)_8$-S-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC]. Calcd. for $C_{203}H_{264}N_{57}O_{114}S_2P_{18}$: 5948 Da; found 5955 ± 3 Da (M–H).

Conjugate 2-Pyridyldisulfide-Containing Ligands With 5′-Mercaptoalkylated Oligodeoxyribonucleotides

Materials

Purified 5′-(2-pyridyldithioalkylated) oligodeoxyribonucleotides (*UNIT 4.9*)
30 mM sodium bicarbonate buffer, pH 9
Nitrogen or argon gas
Tris-(2-carboxyethyl)phosphine (TCEP)
2-Pyridyldithioalkylated ligand (e.g., 2-pyridyldisulfide derivative of psoralen; *UNIT 4.8*, structure 2f)
Dimethylformamide (DMF)
2-mL vial equipped with a Teflon-faced septum

Additional reagents and equipment for TLC (see Basic Protocol 1 and *APPENDIX 3D*) and for isolating, purifying, and characterizing the final conjugate (see Basic Protocol 1)

1b. In a 2-mL vial equipped with a Teflon-faced septum and magnetic stir bar, dissolve 10 OD_{260} units of purified 5′-(2-pyridyldithioalkylated) oligodeoxyribonucleotide in 0.2 mL of 30 mM sodium bicarbonate buffer, pH 9.

2b. Degas the solution by bubbling argon or nitrogen through it for 10 min.

3b. Dissolve ≤2 eq TCEP in a minimal volume (2 to 3 µl) of water and add to the degassed oligonucleotide solution. Leave 90 min at room temperature.

4b. Dissolve 1.2 eq 2-pyridyldithioalkylated ligand in 360 µl DMF and add to the degassed oligonucleotide solution.

5b. Stir the mixture at room temperature for 4 hr. Monitor reaction by TLC (see Basic Protocol 1, step 3).

The psoralen conjugate can be visualized as a pale blue fluorescent spot by irradiation at 365 nm.

6b. Analyze, purify, and characterize the conjugate as described for condensation of halogenoalkylated ligands with oligodeoxyribonucleotide 5′-thiophosphates (see Basic Protocol 1, steps 5 to 12).

Analysis of the coupling reaction between the psoralen derivative and the oligonucleotide HS-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] is performed on an RP18 reversed-phase HPLC column using the conditions described above (see Basic Protocol 1). The conjugate Pso-$(CH_2)_6$-S-S-CH_2CH_2-$(OCH_2CH2)_2$-p-d[CTCTCGCACCCATCTCTC] has a retention time of 35 min 38 sec. A second peak with a retention time of 18 min 11 sec corresponds to either the thiol-containing oligodeoxyribonucleotide HS-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC] or its (5′-5′)-dimer derivative 3′-[CTCTCTACCCACGCTCTC]-p-CH_2CH_2-$(OCH_2CH_2)_2$-S-S-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC]. The psoralen-containing conjugate can be detected at 260 and 320 nm. The UV-visible spectrum of the psoralen-oligodeoxyribonucleotide conjugate in water is shown in Figure 4.10.15. Mass analysis: ESI polarity negative, Pso-$(CH_2)_6$-S-CH_2CH_2-$(OCH_2CH_2)_2$-p-d[CTCTCGCACCCATCTCTC]. Calcd. for $C_{194}H_{249}N_{55}O_{118}S_2P_{18}$: 5852 Da; found 5852 ± 2 Da (M–H).

COMMENTARY

Background Information

The methods reported in this unit describe the conjugation of ligands containing halogenoalkyl, iodoacetamidyl, and 2-pyridylthioalkyl linkers with oligodeoxyribonucleotides functionalized with either a 5′-thiophosphate or a 5′-pyridyldithio group. When using halogenoalkylated or iodoacetamidylated ligands, the conjugates obtained have irreversible phosphothioester or thioether linkages. In contrast, the conjugates obtained from 2-pyridylthioalkylated ligands have phosphodisulfide or disulfide bonds that can be cleaved by the use of a reducing agent such as TCEP or dithiothreitol (DTT). These reactions, which proceed without activation, can be performed either in organic solvents (using a crown ether to solubilize the oligonucleotides) or in a mixture of organic solvents and aqueous buffer. The conjugation of oligodeoxyribonucleotides carrying aminoalkylated linker is illustrated by examples using isothiocyanate or *N*-hydroxysuccinimidyl derivatives of ligands. In these cases, the amino function must be unprotonated and the reaction is carried out in the presence of buffer. The coupling of 5′-modified oligonucleotides with phosphate and carboxylated linker with nucleophilic ligands such as primary amine or hydrazino derivatives requires the use of a coupling reagent.

Procedures reported in this unit may be used to prepare oligodeoxyribonucleotides covalently linked via their 5′ ends to a wide variety of molecules such as labels, intercalating agents, and reactive compounds, the properties of which are reported in *UNIT 4.2.* In many cases, the properties of the oligodeoxyribonucleotide-ligand conjugates are largely dependent on the geometry of the complex formed between the ligand and its target. The linkage between the oligodeoxyribonucleotide and the ligand (i.e., the size and nature of the linker) may be varied to optimize the properties of oligodeoxyribonucleotide-ligand conjugates.

The conjugation methods presented in this unit can be used to prepare oligonucleotide conjugates composed of natural β-deoxyribonucleosides, phosphorothioate oligodeoxyribonucleotides, α-D-deoxyribonucleosides or 2′-*O*-methylribonucleosides, and many other modified oligonucleotides. The methods reported for conjugating 5′-modified oligodeoxyribonucleotides with appropriately functionalized ligands are also valid for coupling these ligands with the same functional groups incorporated into other positions of the oligonucleotides (i.e., phosphate thiophosphate, thiolcarboxyl, and amino at the 3′ end); or thiol carboxyl, and amino attached via a linker to the 2′ position of the sugar, nucleobase, or internucleotide phosphate. Ligands functionalized with an iodoacetamidyl group can also be reacted with internucleotide thiophosphate groups (Asseline et al., 1996). The methods reported in *UNIT 4.9* for introducing functional groups at the 5′ ends of oligodeoxyribonucleotides are compatible in most cases with the presence of a second functional group at another position in the oligodeoxyri-

bonucleotide. It is thus possible to prepare bis-derivatized oligonucleotides with two different ligands (Aubert et al., 2000).

The conjugation of functionalized ligands to unprotected oligonucleotides carrying suited functional groups offers many advantages over the direct incorporation of the ligands during oligonucleotide synthesis (*UNIT 4.3*). In particular, this method is very useful when a limited amount of the ligand is available, when the ligand does not resist the chemical conditions required for oligonucleotide deprotection, or when it is only weakly soluble in the solvents needed for oligonucleotide synthesis, thus preventing the preparation of a phosphoramidite or *H*-phosphonate derivative. Furthermore, the purification of oligonucleotide-ligand conjugates, and particularly those involving two ligands, can be easier using this method, because the oligonucleotides are purified prior to their coupling with ligands, which is not the case when ligands are incorporated during oligonucleotide chain elongation before deprotection. Lastly, as long as only small amounts of conjugate are required, this method is the most convenient one for obtaining many different oligodeoxyribonucleotide-ligand conjugates starting from only one oligodeoxyribonucleotide synthesis.

Critical Parameters

For TLC analyses of conjugation reactions, the reservoir containing the solvent mixture (including concentrated ammonia) must be well-saturated. It is better to prepare it at least half a day before use.

Before loading HPLC columns, organic impurities, such as excess ligands and coupling reagents, should be extracted from crude oligodeoxyribonucleotide solutions with organic solvents or removed by gel filtration. The oligodeoxyribonucleotide solutions must then be filtered through a 0.45-μm disposable filter to remove any particulates and prevent the clogging of columns.

An important parameter is the choice of the solvents used to perform the conjugation reactions since oligodeoxyribonucleotides are soluble in water and in buffer solutions, while most ligands are not. A second important parameter is the choice of the method used to recover the oligonucleotide-ligand conjugate prior to its purification by chromatography. When working with a new ligand, it is critical to experiment with different methods on an analytical scale in order to chose the best one.

When working with acridine-containing oligodeoxyribonucleotides, the pH of all solutions must be kept below 7 or above 9.5 in order to prevent the cleavage of the bond between the acridine ring and the linker.

The coupling of halogenoalkylated orthophenanthroline derivatives with oligodeoxyribonucleotide 5′-thiophosphates sometimes gives low product yields. Liquid chromatography analysis (Thuong and Asseline, 1991) shows, in addition to the starting oligodeoxyribonucleotide 5′-thiophosphate and the expected oligodeoxyribonucleotide-phenanthroline conjugate, the presence of two other products identified as the (5′-5′)-oligodeoxyribonucleotide phosphorothioate dimer obtained by disulfide bond formation, and the corresponding oligodeoxyribonucleotide 5′-phosphate. The formation of these side-products can be explained by an oxidoreduction reaction triggered by the simultaneous presence of thiophosphate, oxygen, orthophenanthroline, and divalent cations. It is important to note that the characterization of orthophenanthroline-oligonucleotide conjugates by mass spectrometry is often difficult.

Oligodeoxyribonucleotides containing a thiol function require degassing of solvents and buffer solutions in order to remove oxygen and any oxidizing reagents, which might lead to the formation of a dimer via disulfide bond formation.

Under the conditions described in these protocols, reactivity of oligonucleotide and ligand functional groups with nucleobases should not be problematic. For 5′-carboxylated oligonucleotides, amide linkages should not form with the exocyclic amino function of the nucleobase at the recommended pH (5.5 to 6), as it has been reported that, under these conditions, nucleobases are not modified at pH ≤6 (Ivanovskaya et al., 1987). Ligands functionalized with isothiocyanate or *N*-hydroxysuccinimide ester groups should also not react with the primary amino function of the nucleobase, which is less reactive than the primary aliphatic amino function at the linker extremity. Succinimidyl esters have a high selectivity for reaction with aliphatic amines. Aromatic isothiocyanates have also been reported to selectively react with aliphatic primary amines (Smith et al., 1985). Finally, aminoalkylated compounds should not form phosphoramidate linkages with internucleotidic phosphodiesters, which are less reactive than the terminal phosphomonoester group. This modification has been reported to be highly selective, and internucleotidic phos-

phates are not affected (Ivanovskaya et al., 1987).

Oligonucleotide-ligand conjugates should be lyophilized and stored in tightly closed vials at −20°C. The ligands should be protected from light at all times, since they are sensitive to light (*UNIT 4.8*).

Under these conditions, they are stable for a year or more. RP-HPLC analyses of four oligonucleotide-ligand conjugates (Figs. 4.10.2, 4.10.8, 4.10.9, 4.10.10) that were stored as aqueous solutions for 18 months in the dark at –20°C showed no detectable degradation.

Anticipated Results

The yields obtained for the conjugates are good, typically exceeding 80% for reactions involving 5′-thiophosphate-, 5′-amino-, and 5′-thiol-containing oligonucleotides. The yields for 5′-carboxylated and 5′-phosphate oligonucleotides are more variable, ranging from 50% to 80%. The yield of recovered oligonucleotide-ligand conjugate after purification and desalting depends on the nature of the ligand and on the scale of the synthesis. When working on a micromolar scale, it is possible to obtain 20 to 25 OD_{260} units of the acridine- or psoralen-oligonucleotide conjugate. Purification of 5′-oligonucleotide conjugates by ion-exchange chromatography leads to lower recovery yields because of the requirement for an additional desalting step. The thiazole orange–oligodeoxyribonucleotide conjugate is also obtained in good yield (6 OD_{260} units are recovered from 10 OD_{260} units of the 5′-modified oligodeoxyribonucleotide starting material); however, when reactions are performed with only 4 to 5 OD_{260} units of 5′-modified oligodeoxyribonucleotides, the amount of purified oligonucleotide-ligand conjugate obtained is ~1.5 to 2 OD_{260} units.

Time Considerations

Provided that all reagents and materials required for each step are available, most of the procedures described herein are simple and rapid. The time required to perform each protocol—including purification, characterization, and lyophylization—is ~1 week.

Literature Cited

Asseline, U., Bonfils, E., Dupret, D., and Thuong, N.T. 1996. Synthesis and binding properties of oligonucleotides covalently linked to an acridine derivative: New study of the influence of the dye attachment site. *Bioconjugate Chem.* 7:369-379.

Aubert, Y., Bourgerie, S., Meunier, L., Mayer, R., Roche, A.-C., Monsigny, M., Thuong, N.T., and Asseline, U. 2000. Optimized synthesis of phosphorothioate oligodeoxyribonucleotides substituted with a 5′-protected thiol function and a 3′-amino group. *Nucl. Acids Res.* 28:818-825.

Aubert, Y., Perrouault, L., Hélene, C., Giovannangeli, C., and Asseline, U. 2001. Synthesis and properties of triple helix-forming oligodeoxyribonucleotides containing 7-chloro-7-deaza-2′-deoxyguanosine. *Bioorg. Med. Chem.* 9:1617-1624.

Ivanovskaya, M.G., Gottihk, M.B., and Shabarova, Z.A. 1987. Modification of Oligo(poly)nucleotide Phosphomonoester groups in aqueous solutions. *Nucleosides-Nucleotides* 6:913-934.

Raymond, F., Asseline, U., Roig, V., and Thuong, N.T. 1996. Synthesis and characterization of O^6 modified deoxyguanosine-containing oligodeoxyribonucleotides for triple helix formation. *Tetrahedon* 52:2047-2064.

Smith, L.M., Fung, S., Hunkapiller, M.W., Hunkapiller, T.J., and Hood, L.E. 1985. The synthesis of oligonucleotides containing an aliphatic amino group at the 5′ terminus: Synthesis of fluorescent DNA primers for use in DNA analysis. *Nucl. Acids. Res.* 13:2399-2412.

Thuong, N.T. and Asseline, U. 1991. Oligodeoxyribonucleotides attached to intercalators, photoreactive and cleavage agents. *In* Oligodeoxyribonucleotides and Analogues: A Practical Approach (F. Eckstein, ed.) pp. 283-308. IRL Press, Oxford.

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