Modification of the 5′ Terminus of Oligonucleotides for Attachment of Reporter and Conjugate Groups

Because of the base-pairing properties of oligomeric strands of DNA, which allow specific recognition of a particular base sequence by a complementary one in a single-stranded DNA or RNA target, such strands are used as nucleic acid probes for molecular biology, diagnostics in medicine, and as artificial regulators of gene expression according to the "antisense" strategy (Cohen, 1989; Hélène and Saison-Behmoaras, 1994). Recently, synthetic oligonucleotides have been used to regulate gene expression via the "antigene" approach upon hybridization with double-stranded DNA through Hoogsteen hydrogen-bonding interactions of an oligonucleotide third strand with the purine bases of the double helix (Thuong and Hélène, 1993; Soyfer and Potoman, 1996).

To improve the performance of oligonucleotides, various molecules have been attached to them to provide the following properties:

1. Easy detection with high sensitivity;
2. Increased affinity to complementary nucleic acids sequences;
3. Ability to induce irreversible modifications of the target sequences;
4. Capacity to recognize and permeate target cell membranes.

The structural, chemical, and physicochemical properties of the ligands used in these applications vary widely, and may range from very simple to very complex when a low-stability molecule, such as a protein, is involved. These different parameters must be taken into account when choosing the positions within each molecule that will be involved in the chemical reactions needed for their covalent linking. Care is essential when determining the best conjugation method for a chosen ligand, in order to preserve its chemical, physicochemical, and biochemical properties, without impairing the oligonucleotide's ability to bind specifically with the nucleic acid target.

As a general rule, it is necessary to insert a linker (L) between the ligand and the oligonucleotide to provide enough conformational freedom for the desired complex to form between the oligonucleotide-ligand conjugate and the nucleic acid or molecular target. The performance of the oligonucleotide-ligand conjugate depends on the attachment sites of the two entities as well as on the linker's nature and size. A ligand can be covalently attached to the oligonucleotide chain through the 5′ or 3′ end, the nucleobases, the sugars, or the internucleotide bridges. Using too short a linker may induce steric hindrance or prevent the ligand from adopting the most favorable functional conformation (Asseline et al., 1996); using too large a linker may impair cooperative binding between the oligonucleotide-ligand conjugate and the nucleic acid target. However, the nature and size of the linker are less important to small ligands and, in particular, to reporter groups.

This unit reviews the covalent attachment of various ligands to the 5′ end of oligonucleotides and the properties of these molecules. The synthesis of oligonucleotide-ligand conjugates involving the coupling of ligands to other oligonucleotidic sites will be presented elsewhere.

SYNTHESIS

The two major techniques used for the synthesis of oligodeoxynucleotides are the "phosphotriester" (Michelson and Todd, 1955; Reese and Shaffhill, 1968; Itakura et al., 1975) and "phosphite" (Letsinger and Lunsford, 1976; Beaucage and Caruthers, 1981; *UNIT 3.3*) methods. Although the first is more convenient for syntheses in solution, the second, using phosphoramidite intermediates, is the best one for solid-phase synthesis. Both methods involve very reactive intermediates requiring appropriate protection for the functional groups not engaged in the coupling reaction, and deprotection conditions that do not affect the integrity of the oligonucleotide chain. Because of these constraints, the phosphotriester and phosphite methods are essentially used to introduce simple and chemically stable functional groups or ligands to the 5′ end of oligonucleotides. In the case of ligands with poor chemical stability or complex structures, the best conjugation method involves coupling to unblocked oligomers that have 5′ ends functionalized with potent electrophilic or nucleophilic groups.

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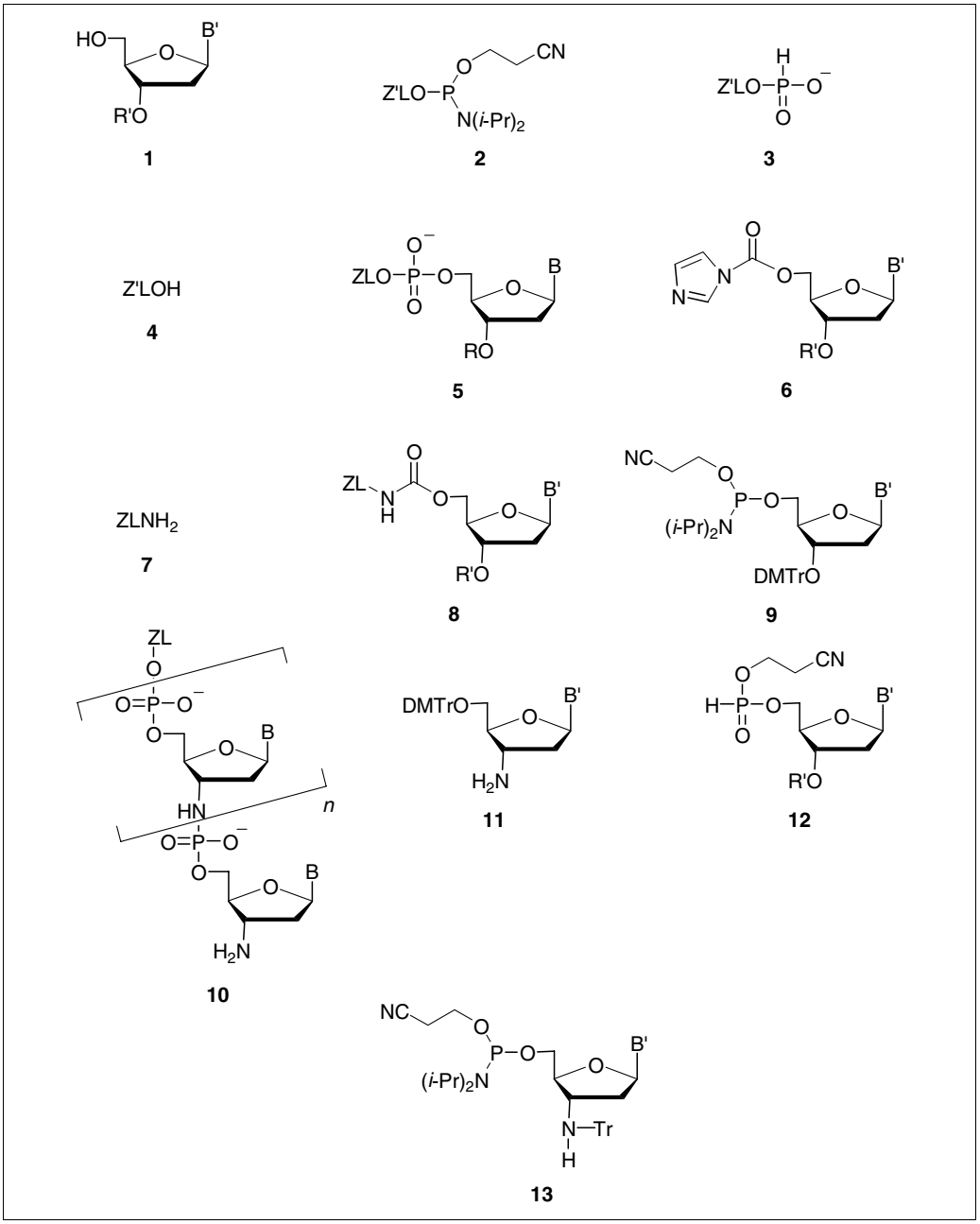

Figure 4.2.1 Functionalization of oligodeoxynucleotides via phosphite and phosphotriester derivatives. Abbreviations used in figures: B, nucleic base; B′, protected base; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DMTr, 4,4′-dimethoxytrityl; Fmoc, 9-fluorenylmethyl; L, linker; MMTr, monomethoxytrityl; Px,9-phenylxanthen-9-yl(pixyl); R, oligodeoxynucleotide; R′, protected oligonucleotide; TCEP, tris-(2-carboxyethyl)phosphine; Tr, trityl; Z, functional group or ligand; Z′, protected functional group or ligand.

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A protected oligonucleotide with a free hydroxyl group at the 5′ end immobilized on the support (**S.1**; Fig. 4.2.1) may easily be obtained by solid-phase synthesis using either nucleoside-3′-phosphoramidites or nucleoside-3′-*H*-phosphonates. The 5′-terminal hydroxyl can then be reacted with phosphoramidites (**S.2**), *H*-phosphonates (**S.3**), and 1,1′-carbonyldiimidazole.

Phosphoramidites and *H*-phosphonates often obtained from a molecule having a hydroxyl functionality (**S.4**), allow the direct introduction of a functional group or ligand (Z-L) via a phosphodiester bond (**S.5**) after oxidation and oligonucleotide deprotection. This synthetic route implies the preparation of "phophitylation reagents" and, if necessary, protection of the functional groups of the ligands not involved in the

coupling reaction. When using 1,1′-carbonyldiimidazole, it is possible to introduce functional groups or ligands bearing a primary amino functionality to the 5′-terminus of oligonucleotides, without the need to prepare intermediate reagents or protecting the functional groups of the ligands not involved in the formation of the oligonucleotide-ligand conjugates, provided these are less reactive than the ligands' primary amino group. The first step of this method is the formation of the carbonylimidazolide derivative (**S.6**) which then reacts with the primary amino functionality of ligand (**S.7**) to form the conjugate with a carbamate linkage (**S.8**) stable to the basic conditions used for oligonuleotide deprotection.

An alternative to this conjugation method is to use modified supports derivatized with the ligand to assemble the oligonucleotide chain in the (5′→3′)-orientation via 5′-phosphoramidites (**S.9**; Horne and Dervan, 1990). This strategy is particularly well adapted for the modification of the 5′ end of oligodeoxyribonucleotide (N3′→P5′)-phosphoramidates (**S.10**), whose synthesis is achieved in the (5′→3′)-orientation by coupling the 3′-aminonucleoside (**S.11**) with 5′-*H*-phosphonate (**S.12**; Chen et al., 1995) or with the 5′-phosphoramidites (**S.13;** McCurdy et al., 1997).

Addition of Functional Groups to the 5′ End of Oligonucleotides

Owing to the low reactivity of the 5′-hydroxyl functionality of oligonucleotides, its condensation with the functional group of ligands is not easily achieved without side reactions at the nucleic base level. Thus, the preparation of oligonucleotide-ligand conjugates from unblocked oligomers requires a reactive electrophilic or nucleophilic group at their 5′ end to react specifically, either directly or following activation, with a ligand bearing the appropriate functional group. Among the functional groups able to meet these requirements are the phosphoryl, thiophosphoryl, sufhydryl, amino, carboxylic, and *cis*-dihydroxyl groups.

5′-Terminal phosphate and phosphorothioate groups

5′-Phosphate (**S.14**; Fig. 4.2.2) or phosphorothioate (**S.15**) can be introduced either chemically during the oligonucleotide synthesis, or enzymatically on the 5′-hydroxyl group of an oligonucleotide chain through the use of T4 polynucleotide kinase, which transfers the γ-phosphoryl or thiophosphoryl of ATP (Eckstein, 1983) to the 5′-terminal hydroxy function of oligodeoxyribonucleotides or oligoribonucleotides.

The first chemical synthesis of dinucleotide 5′-phosphate (**S.16**) was achieved using the phosphotriester method in solution, by condensation of thymidine-5′-*O*-dibenzylphosphate (**S.17**) with 3′-acetyldeoxynucleotide (**S.18**) in the presence of dicyclohexylcarbodiimide followed by deblocking of the 3′-hydroxyl and the phosphate groups by alkaline hydrolysis and hydrogenolysis, respectively (Michelson and Todd, 1955).

The 5′-phosphate-containing oligonucleotides are easily prepared on solid phase by condensation of the phosphoramidite **S.19** (Uhlmann and Engels, 1986; Thuong and Chassignol, 1987), **S.20** (Uhlmann and Engels, 1986; Schwarz and Pfleiderer, 1987), **S.21** (Horn and Urdea, 1986), or **S.22** (Guzaev et al., 1985) with the 5′-terminal hydroxyl of the oligonucleotide bound to the support, followed by oxidation and deprotection steps.

The removal of the first ester group [2-cyanoethyl or 2-(4-nitrophenyl)ethyl] from the terminal phosphate triesters via β-elimination is achieved by treatment with concentrated aqueous ammonia, an approach similar to that used to deblock internucleotidic 2-cyanoethyl phosphate triesters. Deprotection of the second ester function of the terminal phosphate diesters, however, requires more drastic conditions, because of the increased electron density on the phosphodiester function making it a poorer leaving group. Although these phosphorylating methods are well adapted to oligodeoxyribonucleotides, they are difficult to use with oligoribonucleotides without inducing chain cleavage.

Because of the inherent hydrophobicity of reagent **S.22**, it facilitated purification of the appended oligomers by reversed-phase chromatography. This protective group can be removed by detritylation followed by ammonium hydroxide treatment. It is worth noting that among these four phosphorylating reagents only **S.19**, which can be purified by "molecular distillation," can be stored at −20°C for several months without decomposition (Thuong and Chassignol, 1987).

The preparation of oligonucleotides containing a phosphorothioate group at the 5′ end is similarly achieved, by replacing the iodine oxidation step with a sulfurization one using a solution of elemental sulfur solution in CS_2/pyridine (Horn and Urdea, 1986; Schwarz and Pfleiderer, 1987; Thuong and Chassignol, 1987). Since the original pioneering work, the sulfuriza-

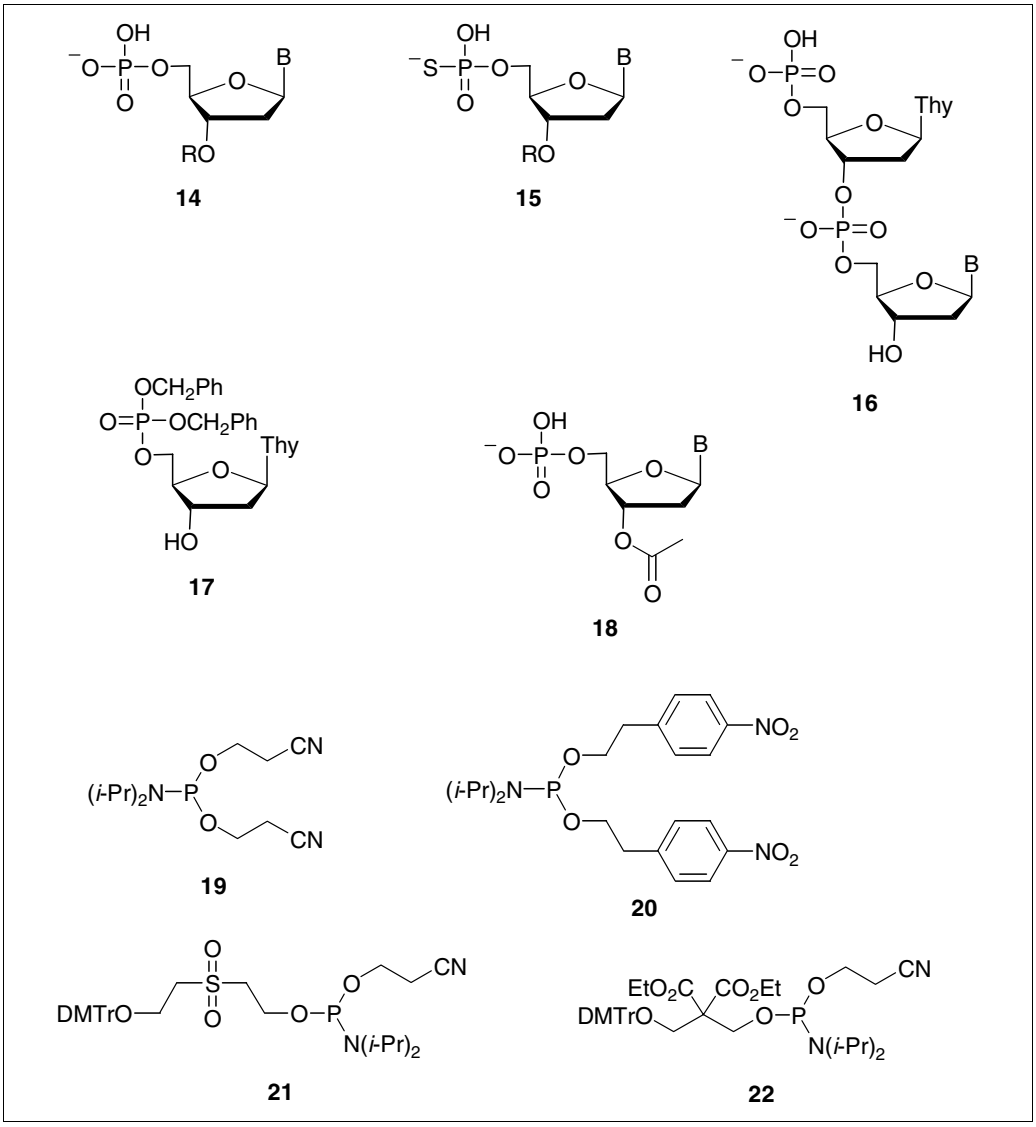

Figure 4.2.2 Incorporation of 5′-phosphate and 5′-phosphorothioate groups into oligodeoxynucleotides. Abbreviations: Thy, thymin-l-yl. See Figure 4.2.1 for additional definitions of functional group abbreviations.

tion step has been carried out using easy-to-handle and more reactive reagents such as 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Iyer et al., 1990), tetraethylthiuram disulfide (Vu and Hirschbein, 1991), or bis(*O*,*O*-diisopropoxyphosphinothioyl) disulfide (Stec et al., 1993).

5′-Thiol-containing oligonucleotides

The introduction of a sulfhydryl group to the 5′ termini of oligonucleotides has been achieved using modified nucleoside derivatives **S.23** (Fig. 4.2.3; Sproat et al., 1987a) or by using the nonnucleosidic derivatives **S.24** (Connolly, 1985), **S.25** (Kuijpers and van Boeckel, 1993), or **S.26** (Gao et al., 1995).

The *S*-trityl protecting group can ultimately be removed by treatment with excess $AgNO_3$, which must then be removed by precipitation with dithiothreitol (DTT). A simpler deblocking step can be accomplished by the use of the thioester derivative **S.25** and the disulfide-containing compound **S.26** upon incubation with aqueous ammonia and DTT, respectively. It is worth noting that the sulfhydryl group is easily oxidized by air at neutral or alkaline pH, so that oligomers obtained after purification are usually in the dimeric disulfide form. The free sulfhydryl must be liberated by reduction of the disulfide bridge just before conjugation. This reaction can be accomplished by treatment with DTT (Cleland, 1964) or tris-(2-carboxyethyl)-phosphine (TCEP; Burns et al., 1991).

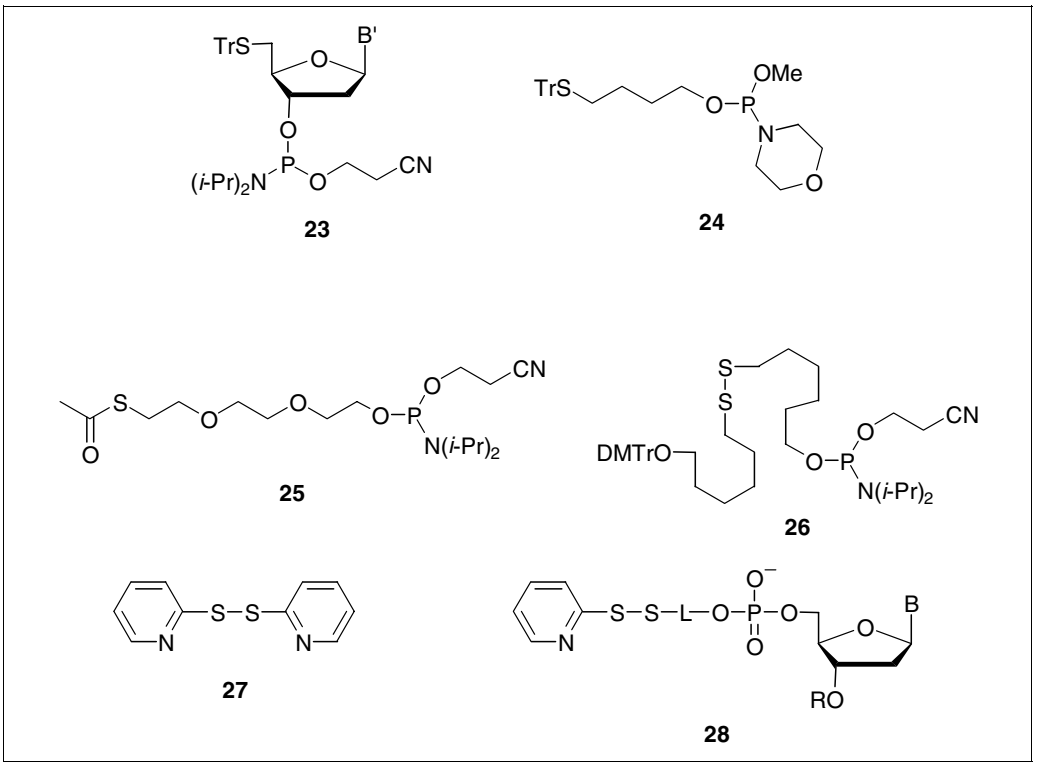

Figure 4.2.3 Incorporation of 5′-thiol groups into oligodeoxynucleotides. See Figure 4.2.1 for definitions of functional group abbreviations.

An interesting approach is to deblock the protected sulfhydryl group in the presence of 2,2′-dithiodipyridine (**S.27**; Py-SS-Py), which reacts with a free sulfhydryl to form the pyridinyldisulfide derivative **S.28** (Kuijpers and van Boeckel, 1993). This compound is air-stable and possesses an activated disulfide function that can either be used directly to achieve conjugation with thiol-containing compounds or easily reduced to the thiol form. During deprotection of oligonucleotides synthesized from β-cyanoethylphosphoramidites, acrylonitrile released by β-elimination of cyanoethyl phosphotriesters reacts easily with a free sulfhydryl group to give the β-cyanoethylthioether derivative, greatly decreasing the synthesis yield of the 5′-thiol-containing oligonucleotides. This drawback can be circumvented by deblocking the oligonucleotide using a mixture of concentrated aqueous ammonia, 2,2′-dithiopyridine, and phenol (Asseline and colleagues, unpub. observ.). Using this method large amounts of the 5′-pyridinyldisulfide-containing oligonucleotides **S.28** have been obtained both as phosphodiesters and phosphorothioates.

5′-Amino-containing oligonucleotides

Addition of a primary amino group to the 5′ end of oligonucleotides has been performed via 5′-azido-2′,5′-dideoxyribonucleoside phosphorochloridites such as **S.29** (Fig. 4.2.4; Mungall et al., 1975), 5′-masked amino-2′,5′-dideoxyribonucleoside phosphoramidites **S.30** (Smith et al., 1985) and **S.31** (Sproat et al., 1987b), or the non-nucleosidic phosphoramidite derivatives **S.32** (Coull et al., 1986), **S.33** (Connolly, 1987), and **S.34** (Connel et al., 1987).

The chlorophosphite **S.29** was used for the synthesis of 5′-amino-oligothymidylate (P3′→N5′)-phosphoramidate (**S.35**) in solution. At the end of the oligonucleotide-chain assembly, the azido group is easily reduced to an amino function by triphenylphosphine in the presence of ammonium hydroxide.

Alternatively, the phosphoramidites **S.30**, **S.31**, **S.32**, **S.33**, and **S.34** are condensed with the 5′-terminal hydroxyl of oligonucleotides bound to the support using standard conditions. In the case of the *N*-trifluoroacetyl-containing compounds **S.31**, **S.32**, **S.34**, and the *N*-9-fluorenylmethoxycarbonyl (Fmoc)-containing compound **S.30**, release of the amino group is achieved during the deprotection of the oligonucleotide chain by concentrated aqueous ammonia treatment. Deprotection of the tritylated derivative **S.33** requires acid treatment, which can be performed either while the oligonucleotide is still bound to the support or

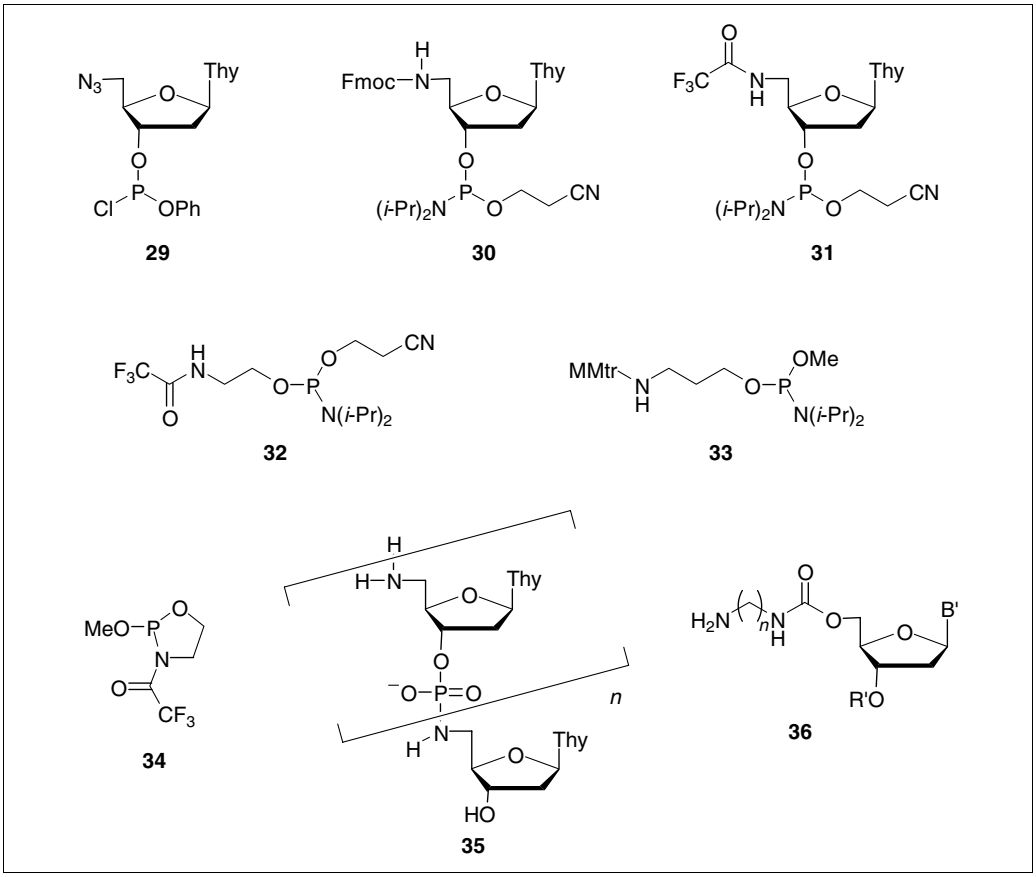

Figure 4.2.4 Incorporation of 5′-amino groups into oligodeoxynucleotides. See Figure 4.2.1 for definitions of functional group abbreviations.

after deblocking and purification of the tritylated oligonucleotide by reversed-phase chromatography.

Another method consists of condensing the 5′-hydroxyl group of protected oligomers bound to the support with 1,1′-carbonyldiimidazole (similar to **S.6**) and then reacting the resulting imidazolide derivative with an alkylenediamine to afford the corresponding aminoalkylated oligonucleotides **S.36** (Wachter et al., 1986).

5′-Carboxyl-containing oligonucleotides

5′-Carboxyl-containing oligonucleotides **S.37** (Fig. 4.2.5) or **S.38** were prepared, respectively, by condensation of the 5′-hydroxyl group of protected oligomers bound to a solid support with phosphoramidite (**S.39**; Kremsky et al., 1987), or by treatment with 1,1′-carbonyldiimidazole followed by reaction with an amino acid (Gotthikh et al., 1990).

5′-Diol-containing oligonucleotides

By analogy with the specific activation of the 3′-terminal *cis* diol of RNA by periodate oxidation, uridine was introduced at the 5′ end of oligonucleotides via a (5′-5′)-linkage (**S.40**; Fig. 4.2.6) generated from either the phosphodiester **S.41** (Agrawal et al., 1986) or the phosphoramidites **S.42** (Agrawal et al., 1986) and **S.43** (Kuijpers et al., 1993).

Conversion of one functional group to another

Numerous processes allowing the conversion of one functional group to another are available. The 5′-phosphate linkers can be coupled with diamines and with aminothiols in the presence of condensing agents to give the 5′-amino linker (Chu et al., 1983) and the 5′-sulfhydryl linkers (Chu and Orgel, 1988), respectively. The 5′-amino group reacts directly with iminothiolane (**S.44**; Fig. 4.2.7) to generate a sulfhydryl group via amidine bond formation (Jue et al., 1978). Heterobifunctional reagents such as succinimidyl 3-(2-pyridyldithio)-propionate (SPDP; **S.45**; Cumber et al., 1985), succinimidyl trans-4-(*N*-maleimidylmethyl)-cyclohexane-1-carboxylate (SMCC; **S.46**; Ghetie et al., 1990), succinimidyl (acetylthio)acetate (SATA; **S.47**; Julian et al., 1983;

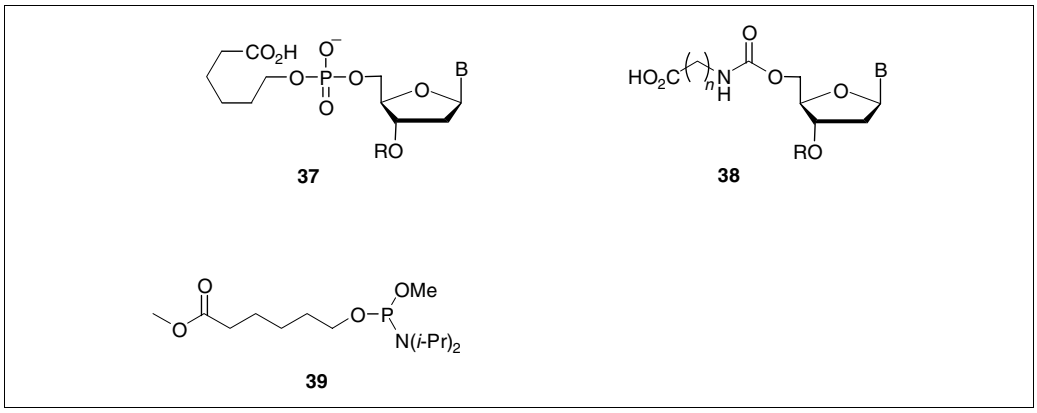

Figure 4.2.5 Incorporation of 5′-carboxyl groups into oligodeoxynucleotides. See Figure 4.2.1 for definitions of functional group abbreviations.

Ghetie et al., 1990), 4-[(succinimidyloxy)carboxyl]-α-methyl-α-(2-pyridyldithio)toluene (SMPT; **S.48**; Thorpe et al., 1987; Ghetie et al., 1990), succinimidyl 3-maleimidopropionate (**S.49**) succinimidyl 4-{[(iodoacetyl)amino]-methyl}-cyclohexane-1-carboxylate (SIAC; **S.50**; Haugland, 1989), succinimidyl iodoacetate (**S.51**), and 4-nitrophenyl iodoacetate (**S.52**) can be used to introduce various activated functional groups. Activated esters **S.45**-**S.52** react with primary amino groups to afford amide bonds. Alternatively, the pyridine disulfide derivatives **S.45** and **S.48** and the maleimido derivatives **S.46** and **S.49** react with the sulfhydryl groups to give disulfide and thioether derivatives, respectively. One of the two functional groups of these heterobifunctional reagents is used to couple the reagent to the oligonucleotide without affecting the other. The intact functional group is then reacted with selected ligands.

Covalent Attachment of Ligands to Oligonucleotides Functionalized at the 5′ End

Phosphorothioate, amino, thiol, phosphate, carboxylic, and *cis*-diol groups possess very different reactivities. The first three are nucleophiles that can be used directly for the conjugation reaction. Conversely, the last three groups, being weakly nucleophilic, must be activated to enable their reaction with, for example, amino functions. In a general way, conjugation reactions are often performed in aqueous medium or in a mixture of water and organic solvents in which oligonucleotides are soluble. However, strong solvation of nucleophilic groups such as oxygenated anions and amines by water often led to slow and poor yield conjugation reactions; in such solvents. Generally, sulfhydryl and thiophosphoryl groups are only slightly deactivated in water, but the formation of amide and phosphoramidate is more strongly disfavored.

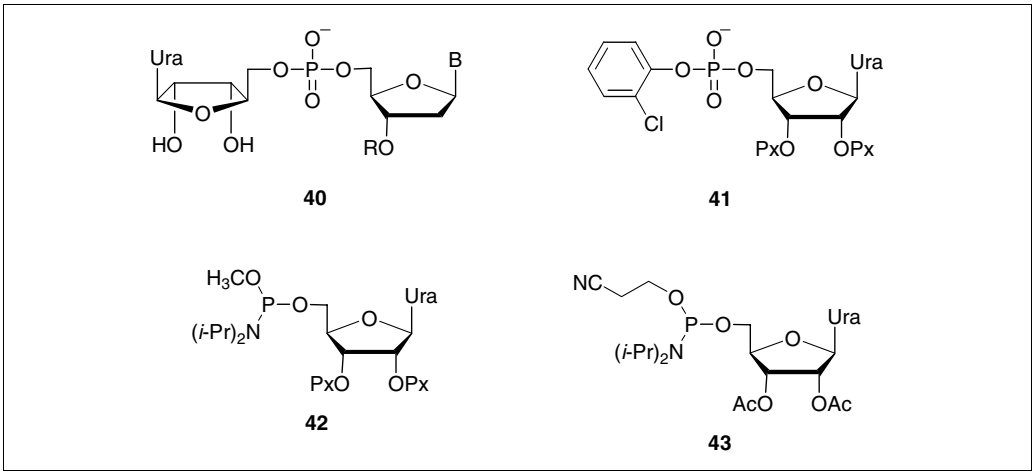

Figure 4.2.6 Some structures involved in the incorporation of 5′-diol groups into oligodeoxynucleotides. See Figure 4.2.1 for definitions of functional group abbreviations.

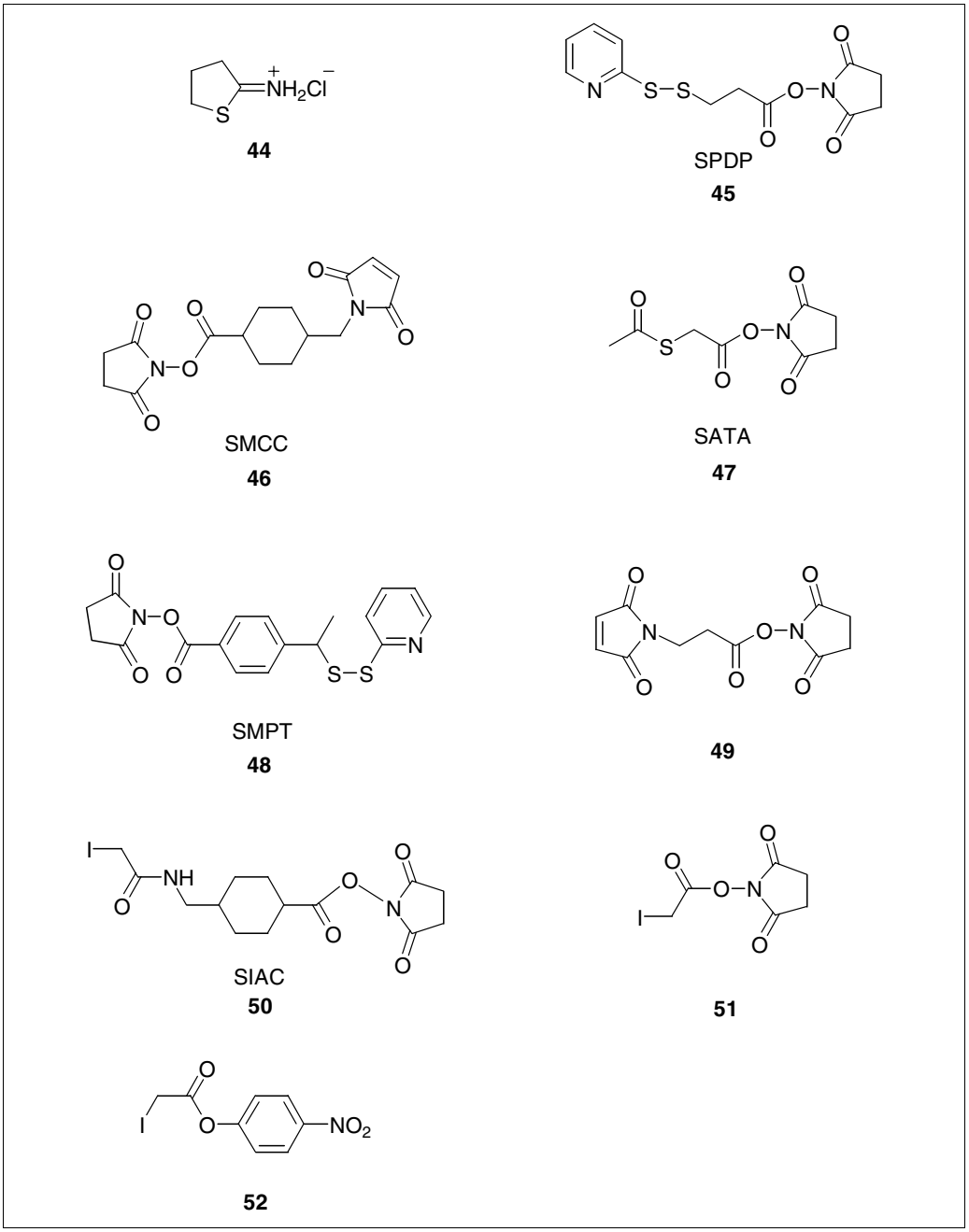

Figure 4.2.7 Heterobifunctional reagents for the conversion of one oligodeoxynucleotide 5′-functional group to another.

These drawbacks may be avoided by performing the coupling reactions in an organic medium. Solubilization of oligonucleotides in organic solvents can be achieved by exchange of phosphate counterions (NH_4^+, Et_3NH^+, Na^+, K^+) with lipophilic ammonium cations such as cetyltrimethylammonium (Zarytova et al., 1987), or by complexing the counterions (NH_4^+, Na^+, or K^+) with crown ethers (Odell, 1985; Thuong and Asseline, 1991) or Kryptofix (Dietrich et al., 1969). The latter method, easy to develop, generally gives good results. Possible organic solvents are methanol and dipolar aprotic solvents such as *N*,*N*-dimethylformamide (DMF) or dimethylsulfoxide (DMSO). It is worth noting that DMSO can oxidize thiols into disulfides (Tam et al., 1991) and hence is unsuitable for coupling reactions involving this functional group.

Via a 5′-terminal phosphate

Modification via the 5′-terminal phosphate is based on the specific activation of the phosphomonoester (which is much more nucleo-

phile than internucleotidic phosphodiesters) to give an electrophilic intermediate that can then react with nucleophilic derivatives such as alcohols or amines to give esters and phosphoramidates, respectively. This method, initially described by Gilham and Khorana in 1958, consisted of the condensation of 5′-*O*-tritylthymidine **S.53** (Fig. 4.2.8) with 3′-*O*-acetylthymidine-5′-phosphate **S.54** in the presence of dicyclohexylcarbodiimide (CDI) **S.55a** to give the dinucleoside monophosphate **S.56**. This method was then used to immobilize oligonucleotides on paper (Gilham, 1962). Due to the poor nucleophilicity of alcohols, this esterification reaction is not selective and leads to side reactions at the nucleic base level. Primary and secondary amines, which are much more nucleophilic than alcohols, selectively give phosphoramidate derivatives.

More recently this method has been optimized by the use of a water-soluble carbodiimide **S.55b** in the presence of a catalyst such as imidazole, *N*-methylimidazole, or 4-dimethylaminopyridine. The use of these particular amines leads to the reactive intermediates **S.57**, **S.58**, and **S.59**, which will react in situ with primary and secondary aliphatic amines. (In these intermediates the corresponding phosphate anion is not nucleophilic because one of its substituent group is electron withdrawing.)

These two strategies can be used to couple ligands that are not sensitive to condensing agents to short nucleic acid segments. The conversion of the decanucleotide-5′-phosphate group **S.60** (Fig. 4.2.9) to the aminated 5′-phosphoramidate **S.61** was performed by direct coupling of ethylenediamine and **S.60** in the presence of CDI, **S.55b**, and *N*-methylimidazole, or by reacting the phosphoimidazolide **S.57** with the diamine (Chu et al., 1983) in aqueous medium. Using this method, cystamine **S.62** (Chu and Orgel, 1988) and the orthophenanthroline (OP) derivative **S.63** (Fig. 4.2.9; Chen and Sigman, 1986) have been linked to oligodeoxyribonucleotides via a phosphoramidate linkage. The same method was also applied to the conjugation of psoralen (Pso) to oligodeoxyribonucleotides **S.64** and to oligodeoxyribonucleotide methylphosphonates **S.65** (Lee et al., 1988; Bhan and Miller, 1990). In the case of longer nucleic acid chains or ligands involving functional groups able to react with CDI, such as proteins, it is necessary to prepare and isolate the phosphoimidazolide derivative **S.57** prior to performing the coupling step (Chu et al., 1983; Chu and Orgel, 1988).

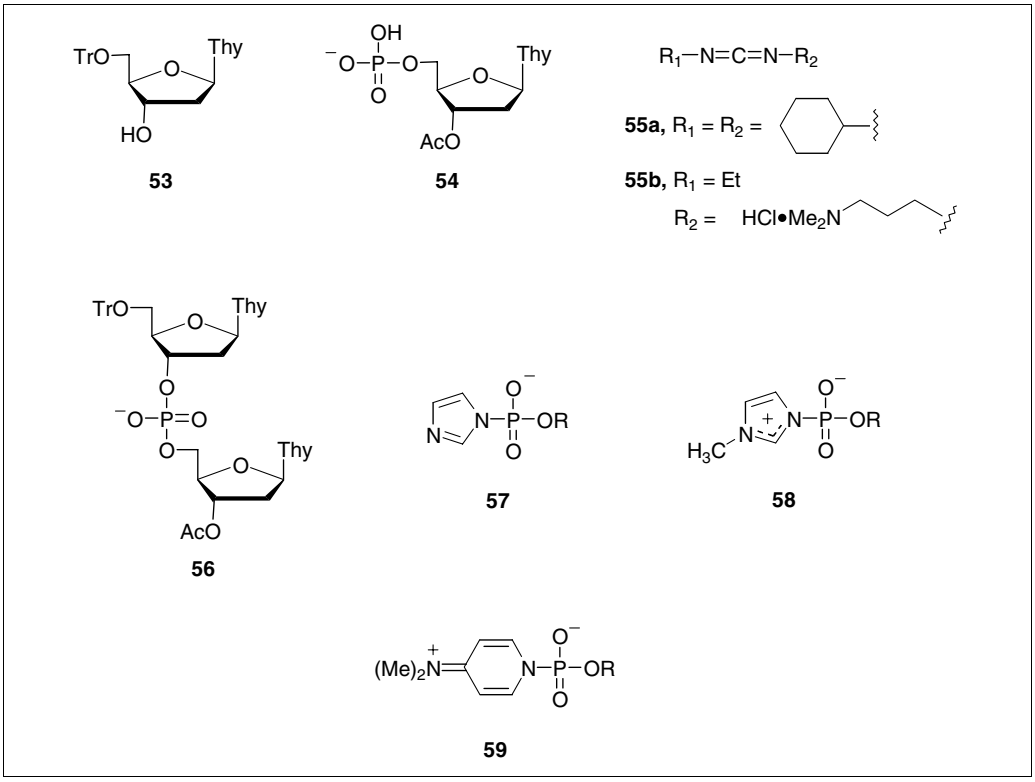

Figure 4.2.8 Modification of oligonucleotides via 5′-terminal phosphate groups. See Figure 4.2.1 for definitions of functional group abbreviations.

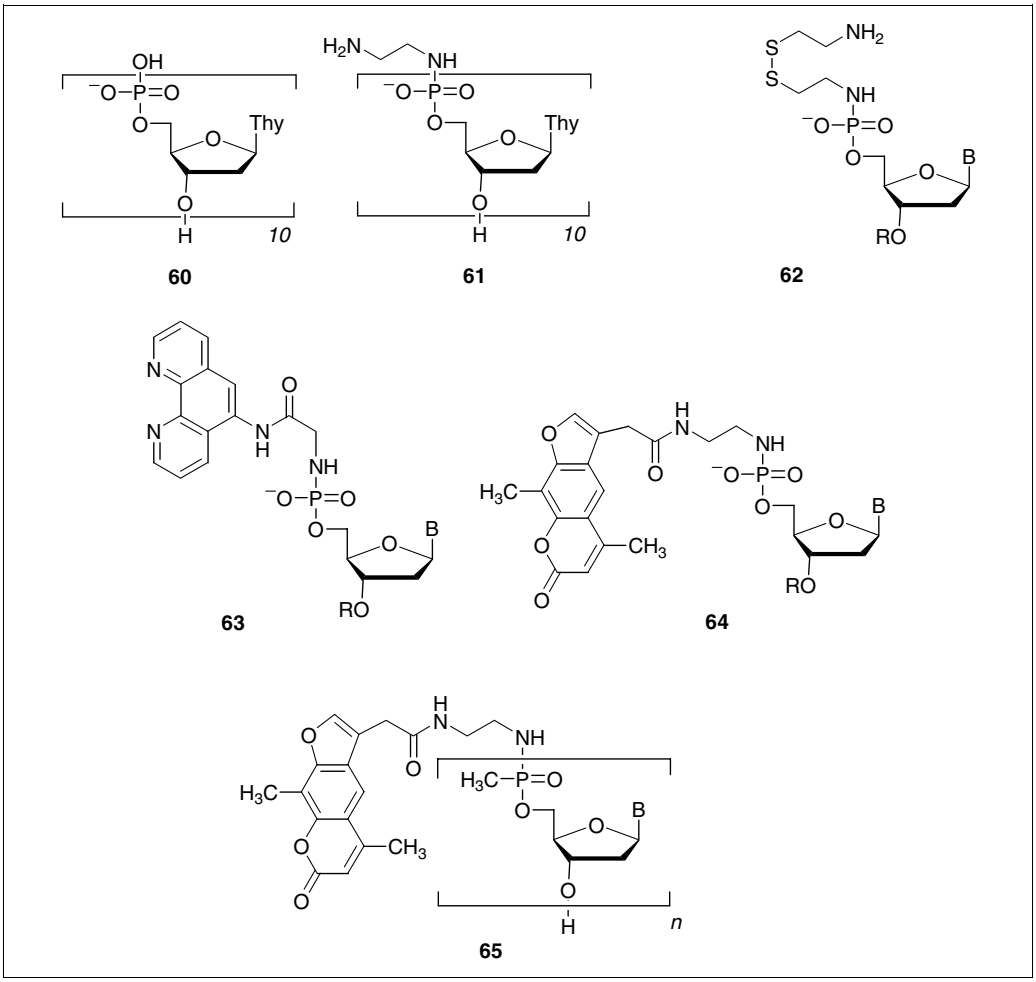

Figure 4.2.9 Conjugation of ligands to oligonucleotides via 5′-terminal phosphate groups. See Figure 4.2.1 for definitions of functional group abbreviations.

Carbodiimide, which has a tendency to react with the nucleic bases and particularly with thymine (Chu et al., 1983), can advantageously be replaced by the triphenylphosphine/2,2′-dipyridyldisulfide couple (Zarytova et al., 1987). Thus, when the cetyltrimethylammonium salt of oligonucleotides, *N*-methylimidazole or 4-dimethylaminopyridine (Zarytova et al., 1987) and the latter condensing reagent are mixed in DMF or DMSO, the zwitterionic derivatives **S.58** and **S.59**, respectively, are easily generated. These react with amino-containing compounds to form phosphoramidates. This method has been used to conjugate the sulfhydryl group **S.66** (Fig. 4.2.10; Boutorin et al., 1994), the coproporphyrin I chelate **S.67** (Fedorova et al., 1990), the bleomycin A5 **S.68** (Zarytova et al., 1993), and intercalating agents such as the phenazinium **S.69** (Lokhov et al., 1992), 2-methoxy-6-chloro-9-aminoacridine **S.70** (Balbi et al., 1994), the ethidium **S.71**, the azidoethidium **S.72** (Koshkin et al., 1994), the daunomycin **S.73** (Dikalov et al., 1991), and the benzopyridoindolium salt **S.74** (Silver et al., 1997) to the 5′-terminus of oligonucleotides.

Finally, it must be noted that ester bonds between 5′-phosphate-containing oligonucleotides and the free 3′-hydroxyl group of another oligonucleotide can easily be formed when using ligase and template oligonucleotide sequences.

Via a 5′-terminal phosphorothioate

The nucleophilicity of the phosphorothioate group allows easy coupling with electrophilic reagents. Oligonucleotides bearing thiophosphate groups at the 5′ ends (**S.15**) react specifically with halogenoalkyl derivatives to afford the corresponding 5′-phosphothioloester. For example, various intercalating agents such as acridine (**S.75**; Fig. 4.2.11), proflavin (**S.76**; Praseuth et al., 1988a), daunomycin (**S.77**; Garbesi et al., 1997), thiazole orange (**S.78**; N.N.T. and U.A., unpub. observ.), azidophenacyl (**S.79**; Praseuth et al., 1988b), pyrene (**S.80**;

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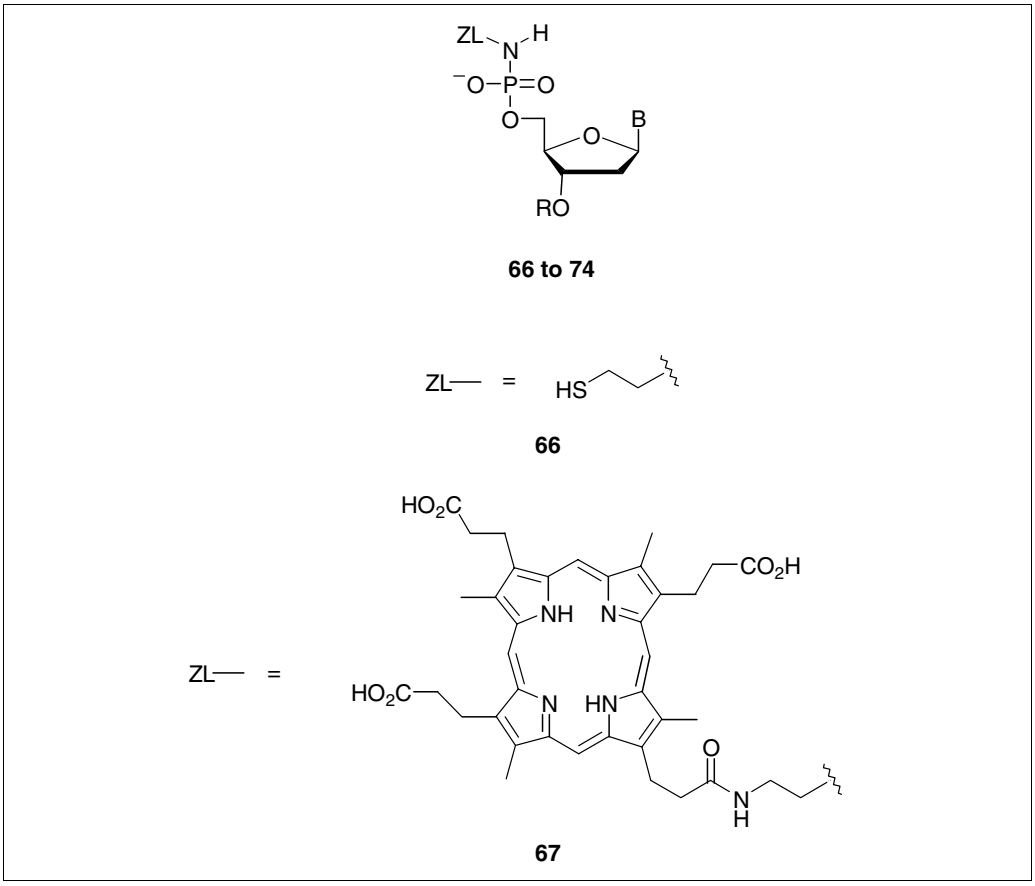

Figure 4.2.10 Attachment of ligands to oligonucleotides via 5′-terminal phosphate groups. See Figure 4.2.1 for definitions of functional group abbreviations.

Ebata et al., 1995), and the cyclopropapyrroloindole subunit of the antitumor antibiotic CC-1065 (**S.81**; Lukhtanov et al., 1997a,b) have been linked to oligonucleotides in this manner.

The kinetics and yields of these reactions depend on the nature of the ligands and the halogenoalkyl groups. In general, iodoacetamido derivatives [-NH-(CO)-CH_2-I] are more reactive than the corresponding iodoalkyl derivatives. The weakly reactive chloroalkyl derivatives can be easily converted to the corresponding iodoalkyl derivatives via a halogen exchange reaction with sodium iodide in acetone or acetonitrile. It is noteworthy that the coupling reaction of orthophenanthroline to oligonucleotides sometimes gives very low yields. In one such case, the formation of a 5′-monophosphate (**S.14**) and a dimer with a disulfide bridge (**S.82**; Fig. 4.2.12) was observed (Thuong and Asseline, 1991). These side reactions are probably caused by superoxide anion O_2^- and H_2O_2 generated by the presence of OP, thiophosphate, oxygen, and traces of divalent cations contained in the conjugation buffer. This is analogous to the mechanism proposed for the nucleolytic activity of the complex OP-Cu in the presence of thiol (Kuwabara et al., 1986; Hélène and Thuong, 1988). To prevent these side reactions it is necessary to eliminate traces of divalent cations contained in buffer solutions by chelation with, for example, Chelex 100 (Thuong and Asseline, 1991).

Formation of the 5′-phosphothioloester (**S.83**) can also be obtained by reaction of the 5′-phosphorothioate oligomer with the maleimido derivative of OP (Shimizu et al., 1994).

Another coupling reaction via the phosphorothioate group concerns the formation of the disulfides **S.84a** and **S.84b**, which is accomplished with ligands bearing a pyridinyldisulfide group (**S.85**; Chassignol and Thuong, 1998). One particularity of these conjugates is that oligonucleotide 5′-phosphorothioates can be regenerated by reduction of the disulfide linkages and, if necessary, transformed to the corresponding 5′-phosphate by oxidation.

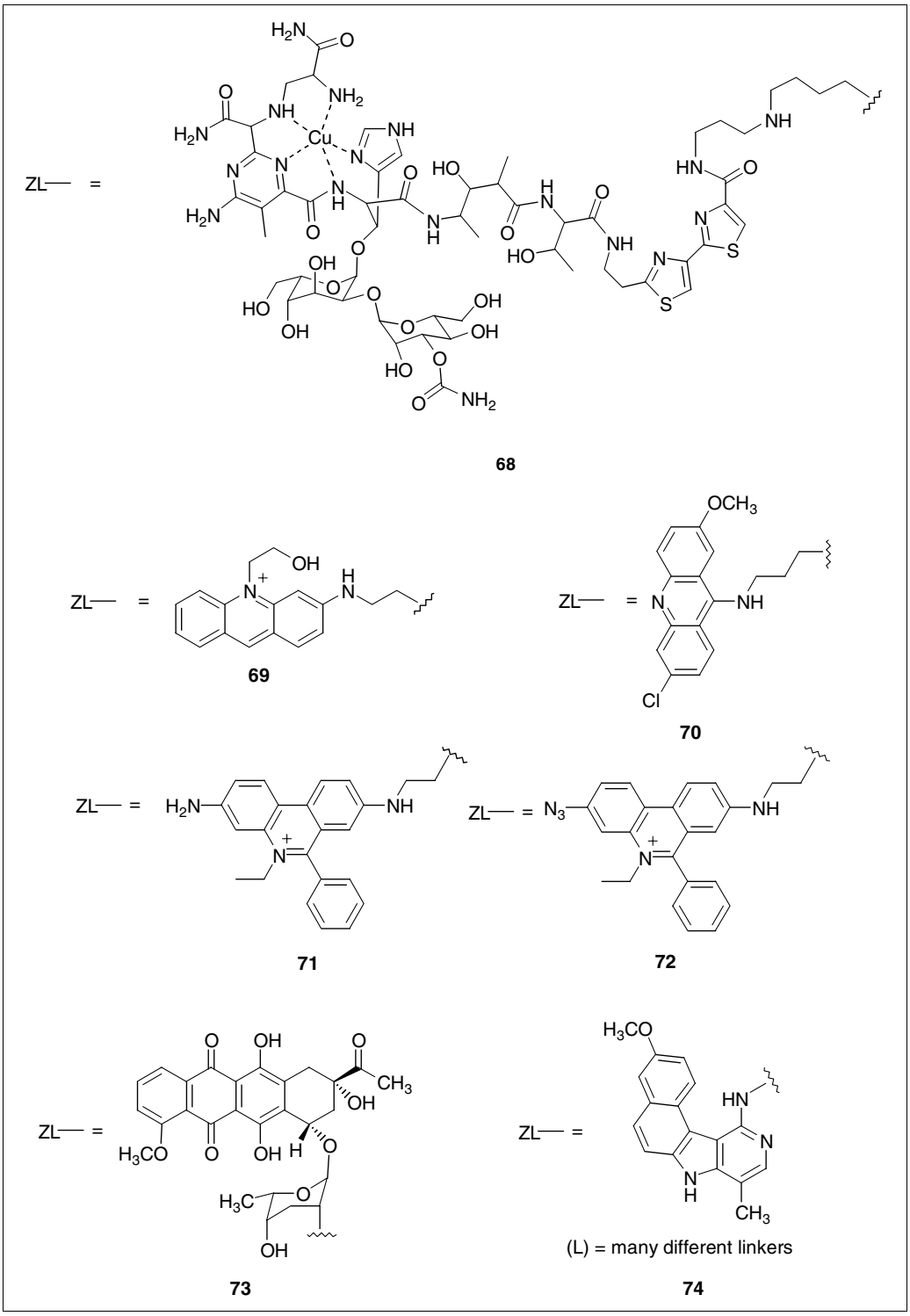

Figure 4.2.10 Continued

Via 5′-thiol oligonucleotides

The sulfhydryl group, which is most widely used in the modification of proteins, is also often employed for the preparation of oligonucleotide conjugates. However, the tendency to form symmetrical dimers through disulfide bridge formation requires DTT or TCEP reduction. To improve coupling efficiency the reaction should be performed in oxygen-free solution. Conjugation via thiol groups can lead to the formation of either a chemically stable thioether linkage or a disulfide bridge that can be reduced to give the starting thiols.

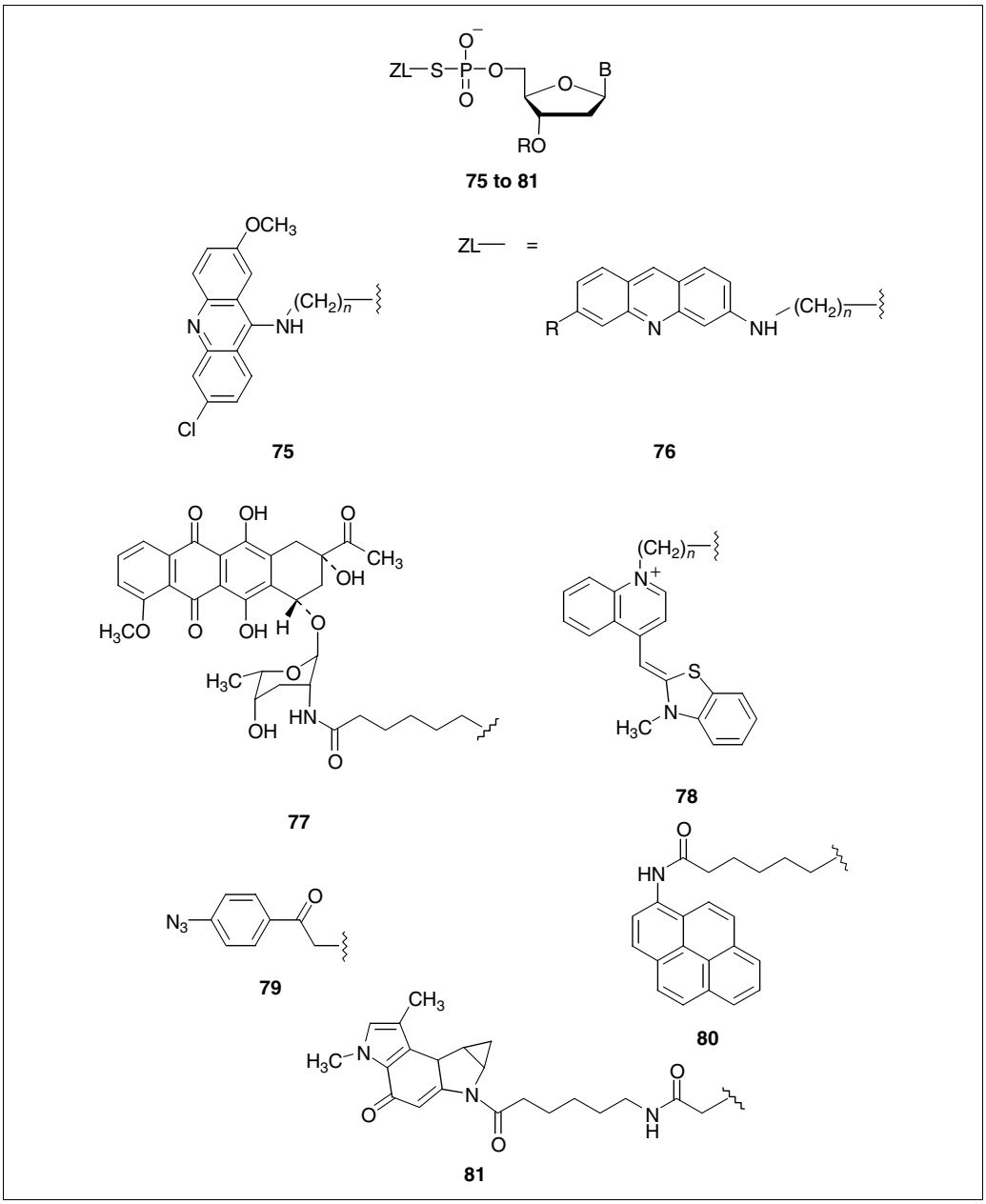

Figure 4.2.11 Conjugation of functonal groups to oligonucleotides via 5′-terminal phosphorothioate groups. See Figure 4.2.1 for definitions of functional group abbreviations.

The alkylation of thiol groups by iodoacetyl derivatives has been used to prepare the fluorescent conjugates derived from fluorescein (**S.86a**; Fig. 4.2.13; Ansorge et al., 1987) and *N*-(5-sulfo-1-naphtyl)ethylenediamine (**S.86b**; Connolly, 1985). This method has also been used to couple fullerene to the 5′-terminus of oligonucleotides (**S.87**; Boutorin et al., 1994).

Alternatively, the pyrenyl **S.88a** (Kumar et al., 1991) and antibody-oligonucleotide conjugates **S.88b** (Kuijpers et al., 1993), have been formed by adding oligonucleotide 5′ thiols to the corresponding maleimido-ligands.

5′-Thiol-containing oligonucleotides also react easily with tresyl-activated or epoxy-activated Sepharose to form thioether linkages (Blanks and McLaughlin, 1988). Air oxidation of two sulfhydryl-containing compounds gives a mixture of homo- and heterodimers. The synthesis of conjugates via formation of cleavable disulfide bridges implies the reaction of one of the thiol groups with 2,2′-dithiodipyridine to form the R-S-S-Pyr intermediate, which exchanges with the other thiol group to complete conjugation. The reaction of peptide, peroxidase, and human IgG with iminothiolane afforded with thiol deriva-

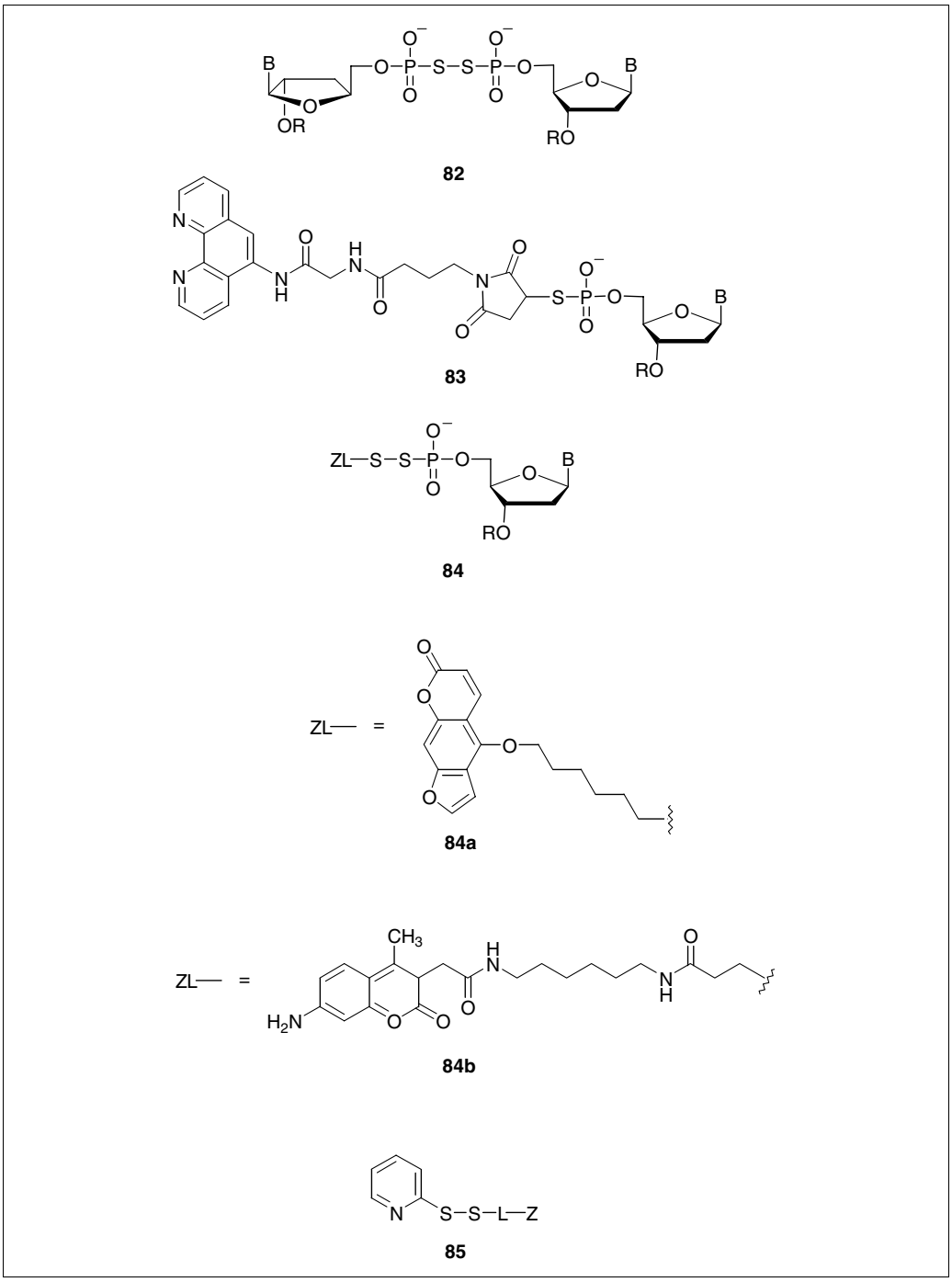

Figure 4.2.12 Incorporation of functional groups into oligonucleotides via 5′-terminal phosphorothioate groups. See Figure 4.2.1 for definitions of functional group abbreviations.

tives **S.89** (Fig. 4.2.14) condensation of these derivatives with the activated oligomers **S.90** gave the conjugates **S.91** (Chu and Orgel, 1988).

Modification of the 5′ Terminus of Oligonucleotides for Attachment of Reporter and Conjugate Groups

Via the 5′-amino group

The alkylamino groups easily react at pH ≥ 8 with electrophilic reagents to form stable linkages. Thus, fluorescein isothiocyanate (**S.92a**; Fig. 4.2.15) and tetramethylrhodamine isothiocyanate (**S.92b**) have been coupled to 5′-amino-oligonucleotides in aqueous solution to produce the thiourea derivatives **S.93a** and **S.93b** (Smith et al., 1985). The same reaction was used to link orthophenanthroline isothiocyanate **S.92c** to the 5′ end of oligonucleotides with (N3′→P5′)-linkages to afford **S.93c** (N.T.T. and U.A., unpub. observ.).

Certain acyl chlorides or anhydrides such as dansyl chloride or ethylenediaminetraacetic di-

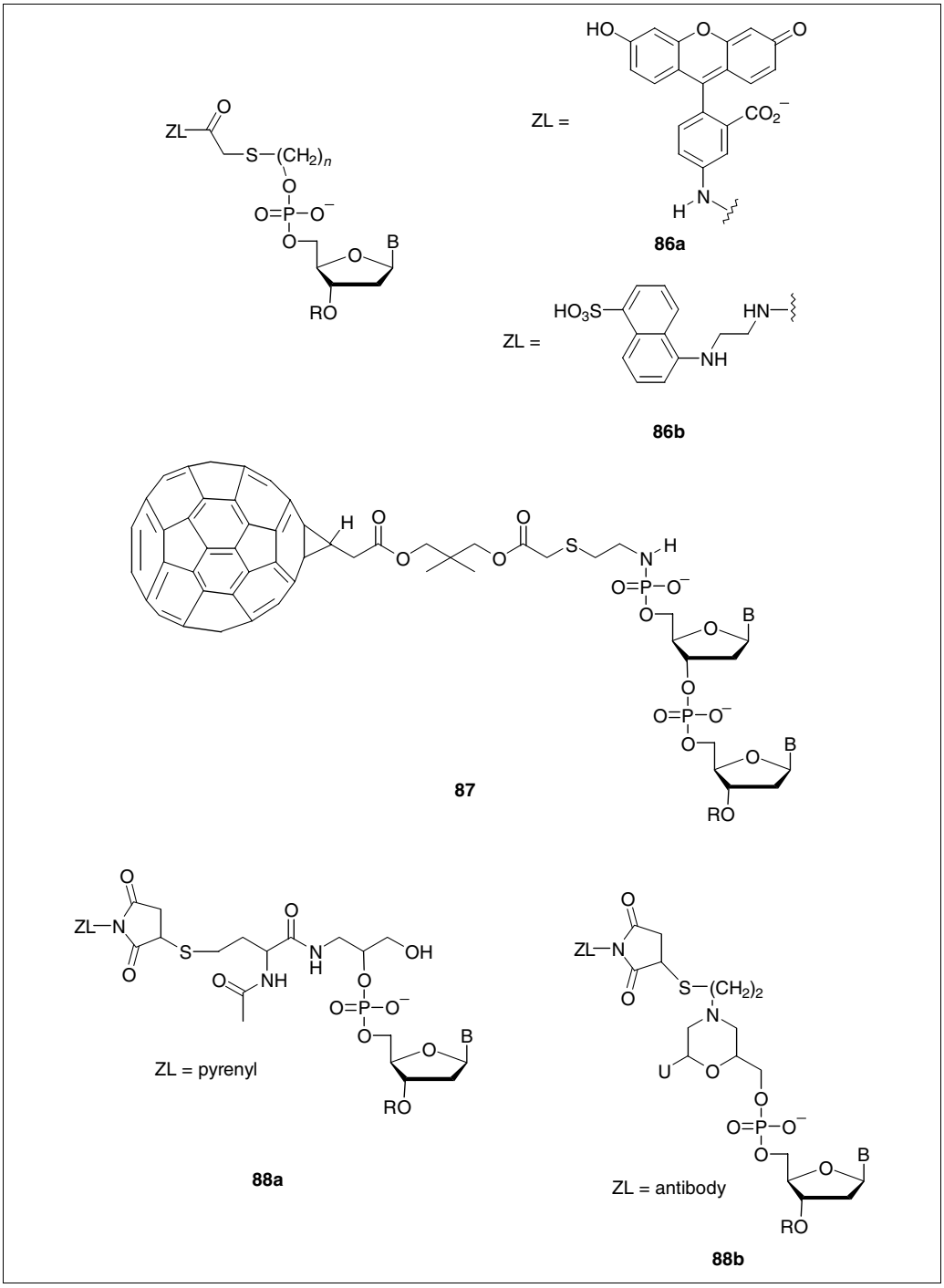

Figure 4.2.13 Conjugation of ligands to oligonucleotides via 5′-thiol groups. See Figure 4.2.1 for definitions of functional group abbreviations.

anhydride (EDTA), and activated esters such as biotin-4-nitrophenylester, *N*-hydroxysuccinimido-biotin, and the *N*-hydroxysuccinimide ester of bathophenanthroline have been used to prepare oligonucleotide conjugates **S.94** (Fig. 4.2.16), **S.95a-c**, **S.96**, and **S.97** through the formation of either sulfonamide **S.94** (Connolly, 1987) or amide bonds **S.95a** (Boutorin et al., 1984; Chu et al., 1985), **S.95b** (Agrawal et al., 1986), **S.95c** (Schubert et al., 1995), **S.96** (Wachter et al., 1986), and **S.97** (Bannwarth et al., 1988).

The 5′-arylazido-containing oligonucleotide **S.98** (Fig. 4.2.17) has been obtained by arylation of the 5′-terminal amino group of oligonucleotides with 2,4-dinitro-5-az-

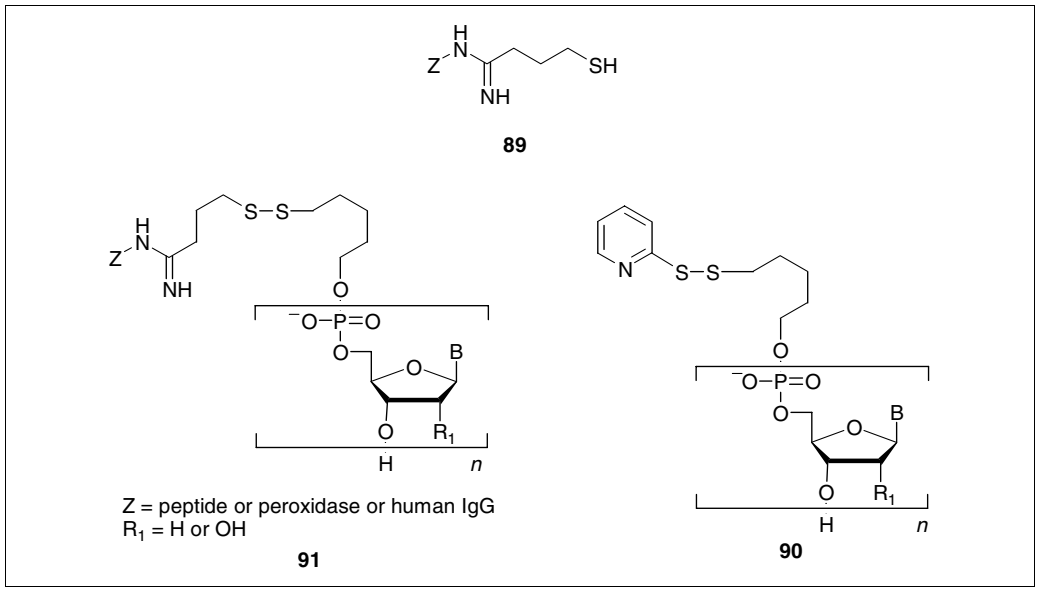

Figure 4.2.14 Incorporation of conjugate groups into oligonucleotides via 5′-thiol groups. See Figure 4.2.1 for definitions of functional group abbreviations.

idofluorobenzene (Grimautdinova et al., 1984). In the same way the phenazinium derivatives **S.99** were obtained by nucleophilic attack of 5′-amino alkylated oligonucleotides of the C2-halogenated heterocycle (Lokhov et al., 1992). 5′-Amino-containing oligonucleotides were also enzymatically coupled to a 2-methy-9-hydroxyellipticinium salt in the presence of horseradish peroxidase to give the oxazolopyridocarbazole derivative **S.100** (Mouscadet et al., 1994).

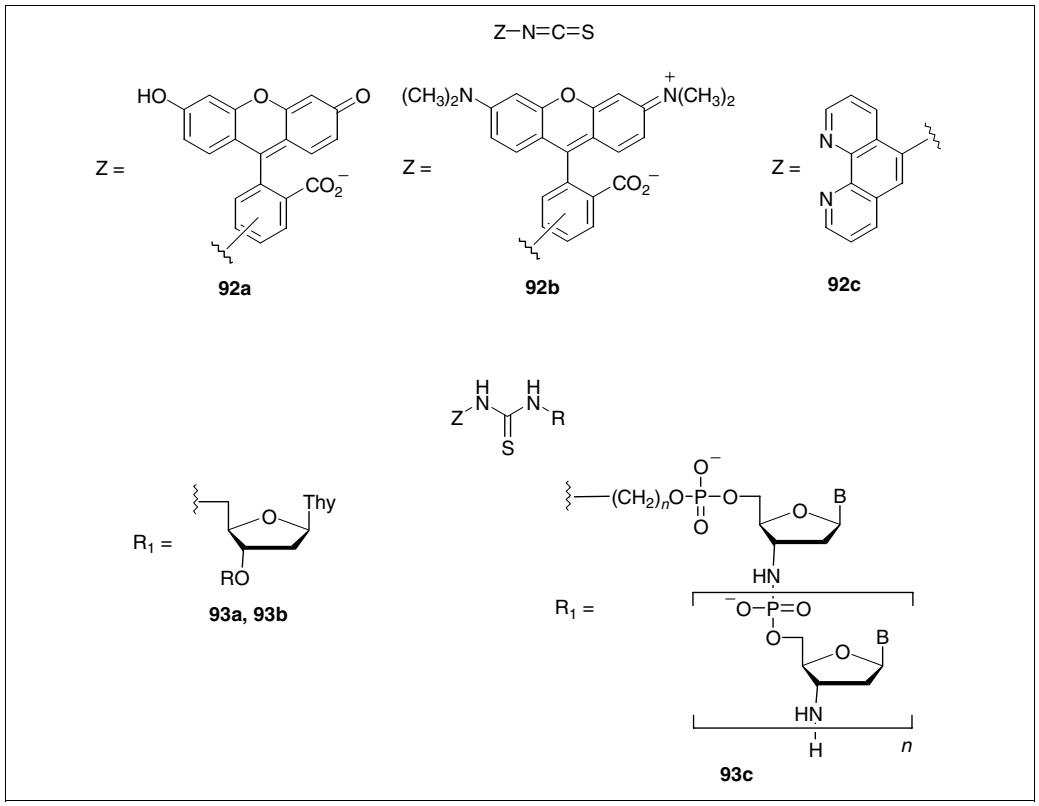

Figure 4.2.15 Attachment of reporter and conjugate groups to oligonucleotides via 5′-amino groups. See Figure 4.2.1 for definitions of functional group abbreviations.

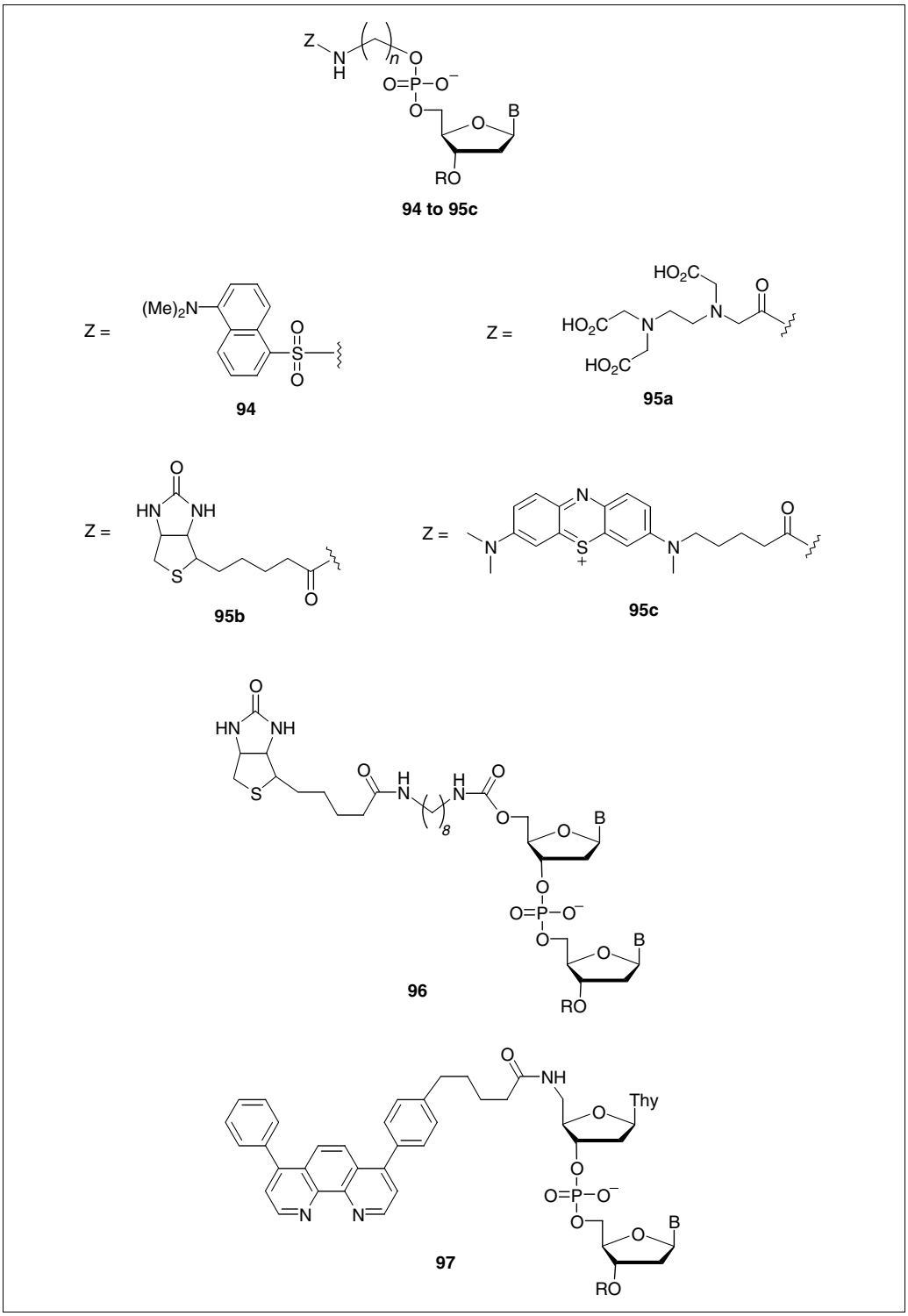

Figure 4.2.16 Conjugation of functional groups to oligonucleotides via 5′-amino groups. See Figure 4.2.1 for definitions of functional group abbreviations.

Via a 5′-carboxyl group

Like phosphates, carboxyl groups can be selectively activated and coupled to hydrazine or amine derivatives to give, for example, the biotin conjugate **S.101** (Fig. 4.2.18; Kremsky et al., 1987) and the daunomycin conjugate **S.102** (Gotthikh et al., 1990).

Via a 5′-terminal cis diol

The 2′- and 3′-hydroxyl functionalities of the ribose-containing oligomer **S.40**, upon periodate oxidation, can easily be coupled with the hydrazino or primary amino groups of the ligands to give the unstable 3,5-dihydroxymorpholine derivative **S.103** (Fig. 4.2.19). Sta-

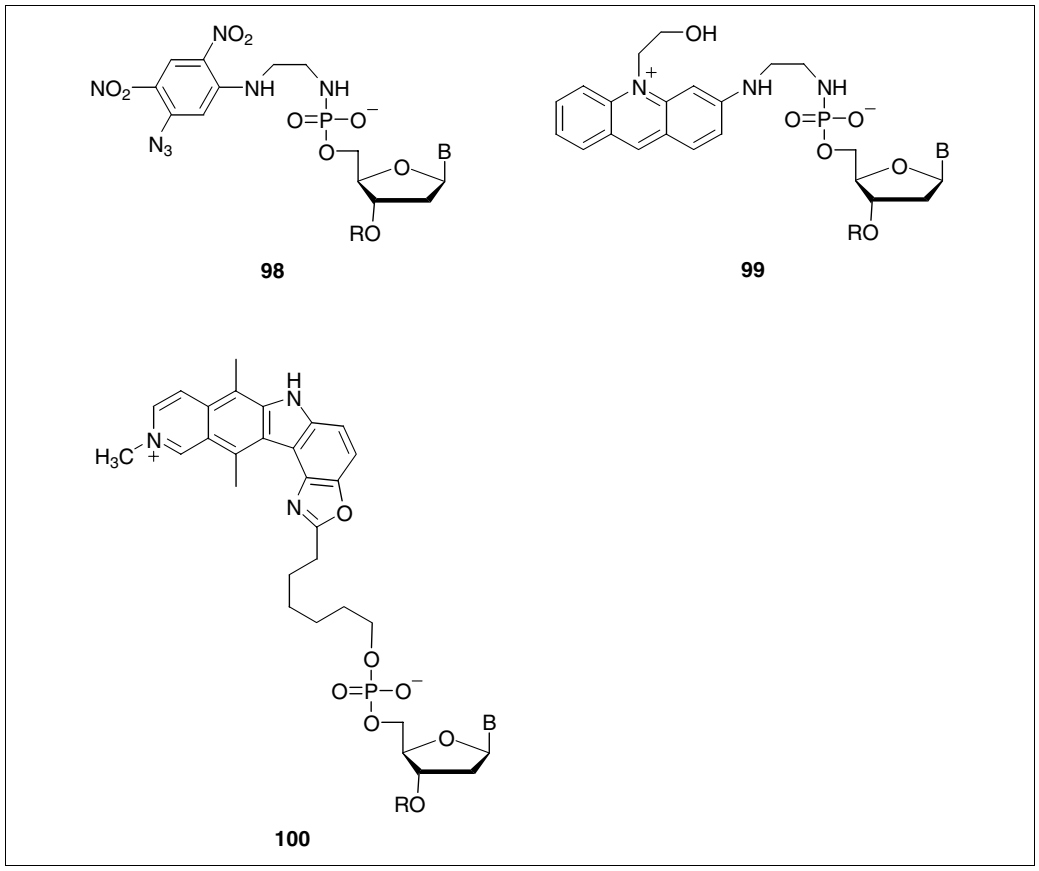

Figure 4.2.17 Introduction of functional groups to oligonucleotides via 5′-amino groups. See Figure 4.2.1 for definitions of functional group abbreviations.

ble morpholine derivatives **S.104** were obtained by reduction of **S.103** with $NaBH_4$ or $NaBH_3CN$. Using this method, biotin was coupled via its hydrazino derivative to afford **S.104a** (Agrawal et al., 1986). The ^{125}I-labeled tyramine derivative **S.104b** (Kuijpers and van Boeckel, 1993) was also prepared in a similar manner. Furthermore, periodate oxida-

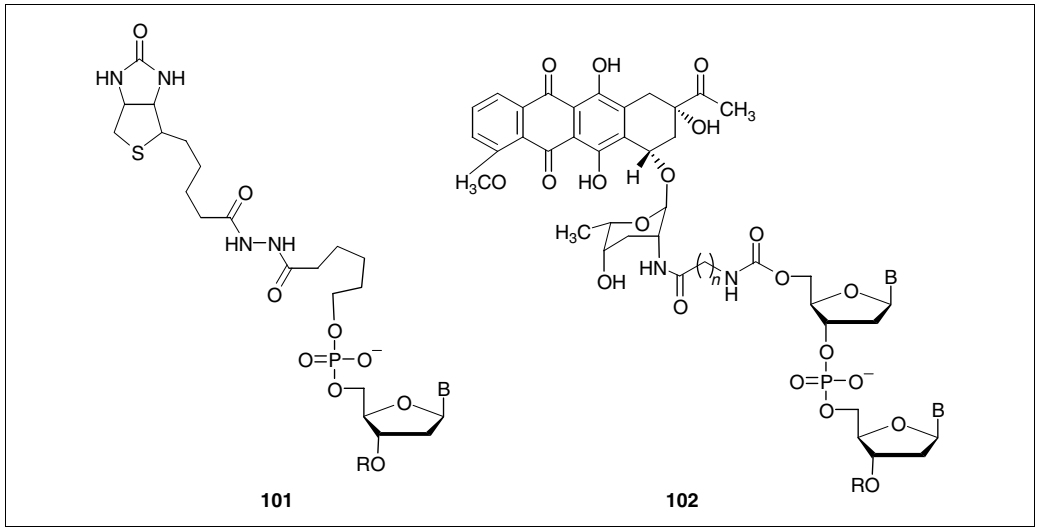

Figure 4.2.18 Conjugation of functional groups to oligonucleotides via 5′-carboxyl groups. See Figure 4.2.1 for definitions of functional group abbreviations.

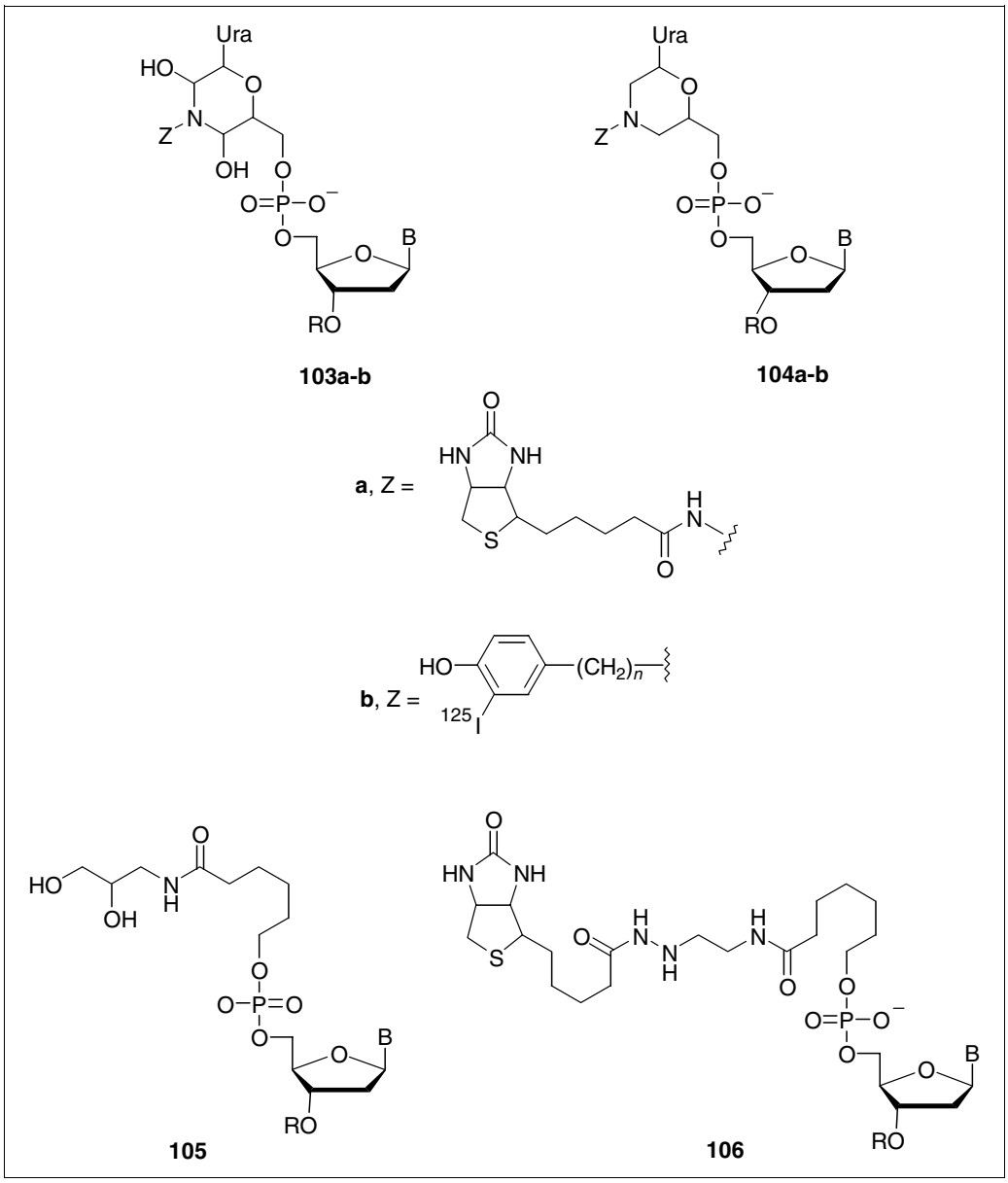

Figure 4.2.19 Attachment of functional groups to oligonucleotides via 5′-terminal *cis* diols. See Figure 4.2.1 for definitions of functional group abbreviations.

tion of the 5′-vicinal diol–containing oligonucleotide **S.105** (Fig. 4.2.16) followed by coupling with the hydrazinobiotin produced the conjugate **S.106** (Kremsky et al., 1987).

Direct Addition of Ligands to the 5′ End of Oligonucleotides

The synthesis of oligonucleotide conjugates derived from acridine, psoralen, and porphyrin was first achieved by the phosphotriester method in solution. Oligonucleotide-intercalator conjugates **S.107** (Fig. 4.2.20; Thuong et al., 1984) and **S.108** (Asseline and Thuong, 1988) were obtained by condensing oligonucleotides **S.109** with the hydroxylated ligands **S.110** and **S.111**, respectively. Similarly, the porphyrin-oligonucleotide conjugate **S.112** was synthesized from the protected oligomer **S.113** and the hydroxylated porphyrin **S.114** (Le Doan et al., 1986, 1987a) in the presence of a condensing reagent. Deprotection of the fully-protected oligonucleotides was achieved by successive treatment with the DBU salt of benzohydroxamic acid and concentrated ammonia or sodium hydroxide. Sodium hydroxide was used instead of aqueous ammonia for the *N*-deprotection of oligonucleotides covalently linked to acridine to avoid cleavage of the 9-aminoacridine linkage (Asseline et al., 1984; Thuong and Asseline, 1991).

Oligonucleotides bearing a ligand at the 5′ end are easily prepared on solid-phase by condensation of the terminal 5′-hydroxyl group with those phosphoramidite or H-phosphonate derivatives of ligands that are stable to the conditions required for synthesis. Many functional groups have been coupled to the 5′ terminus of oligonucleotides using these methods. Among them are intercalating agents, photoreactive groups, and labels.

The acridine and psoralen derivatives were first linked to the 5′ ends of oligonucleotides bound to a solid support by use of the phosphoramidites **S.115** (Fig. 4.2.21; Thuong and Chassignol, 1988) and **S.116** (Kurfürst et al., 1993; Dupret et al., 1994), respectively. The phosphoramidite derivatives of these compounds with different linkers **S.117** and **S.118** are now commercially available.

The pyrene group was also conjugated to the 5′ terminus of oligonucleotides using its phosphoramidites derivatives **S.119** and **S.120** (Korskun et al., 1992); likewise, fagaronine was linked to the 5′ ends of oligonucleotides via the parent phosphoramidite **S.121** (Chen et al., 1992).

Anthraquinone and sapphyrin were similarly attached to oligonucleotides from their H-phosphonate derivatives **S.122** (Lin and Matteucci, 1991) and **S.123** (Sessler et al., 1996), respectively.

Oligonucleotide-biotin conjugates **S.124a**, **S.124b**, and **S.124c** (Fig. 4.2.22) were prepared via phosphoramidites **S.125a**, **S.125b** (Cocuzza and Zagorsky, 1991) and **S.125c** (Olejnik et al., 1996). Conjugate **S.124c**, which has a photocleavable linker to enable the release of the oligonucleotide from the conjugate under UV irradiation at a wavelength of 300 to 350 nm.

Fluorescent oligonucleotide conjugates derived from fluorescein **S.126a** are easily obtained via phosphoramidite **S.127**. Replacing the hydrogen atoms of the fluorescein nucleus by chlorine leads to tetra- and hexachlorofluorescein derivatives **S.126b** and **S.126c**, the absorption and emission spectra of which are red shifted.

Designed to exploit energy-transfer techniques, oligomers labeled at their 5′ ends with two chromophores were developed as either linear structures, **S.128** (Fig. 4.2.23) and **S.129** (Ju et al., 1995, 1996), or hairpin structures, **S.130** (Fig. 4.2.24; Nazarenko et al., 1997). Fluorescence energy transfer is a dipole-dipole resonance interaction between two chromophores near to each other. One of these chromophores must be fluorescent and is called the "donor", whereas the other identified as the "acceptor" may or may not be fluorescent. A spectral overlap between the acceptor's excitation and donor's emission wavelengths, and a

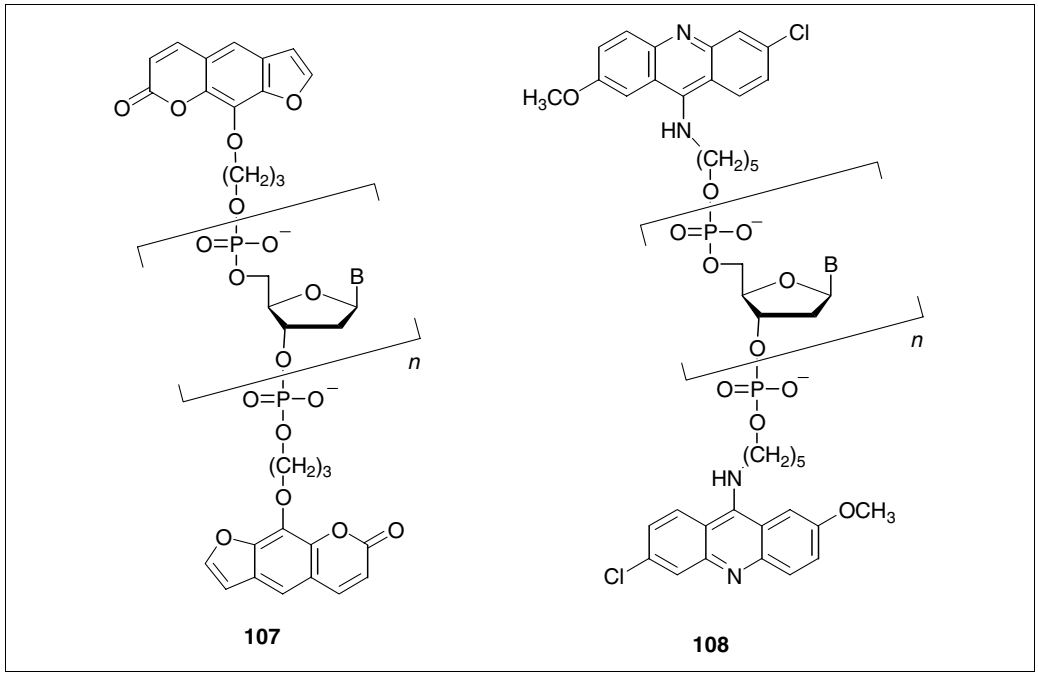

Figure 4.2.20 Direct addition of ligands to the 5′ ends of oligonucleotides by the phosphotriester coupling method. See Figure 4.2.1 for definitions of functional group abbreviations.

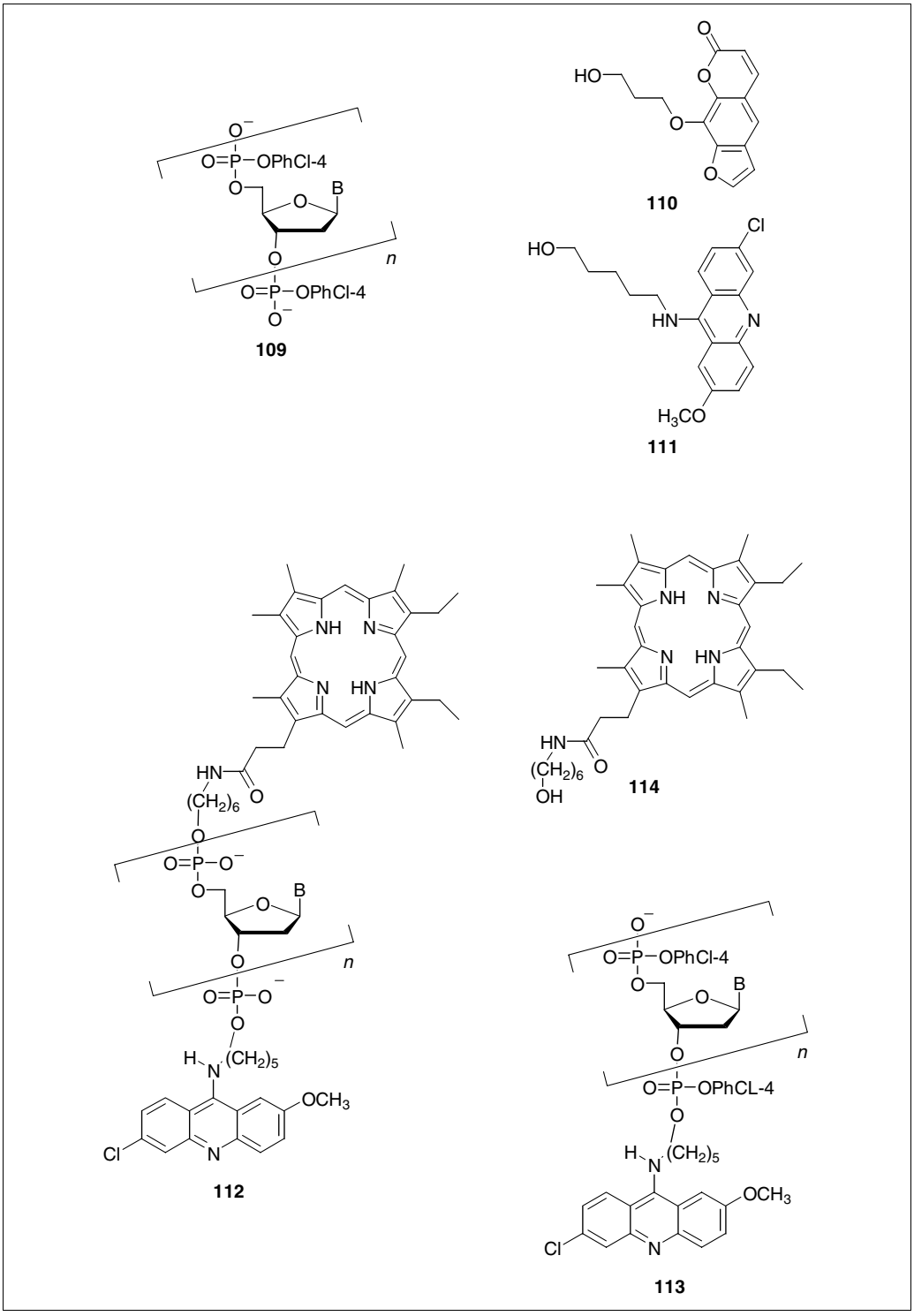

Figure 4.2.20 Continued

proper distance between the two entities are necessary for the donor to transfer energy, upon light excitation, to the acceptor. The efficiency of energy transfer is inversely proportional to the sixth power of the distance between the two chromophores.

In the case of the linear oligomers **S.128** and **S.129**, the donor was 5-carboxyfluorescein (FAM), whereas the acceptor was either FAM or its derivative 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein (JOE), or one of the two rhodamine derivatives 5-(and 6-)carboxy-*N,N,N′,N′*-tetramethyl rhodamine (TAMRA) and 5-(and 6-)carboxy-X-rhodamine (ROX; all available from Molecular Probes). Upon irradiation at 488 nm, the energy emitted by FAM

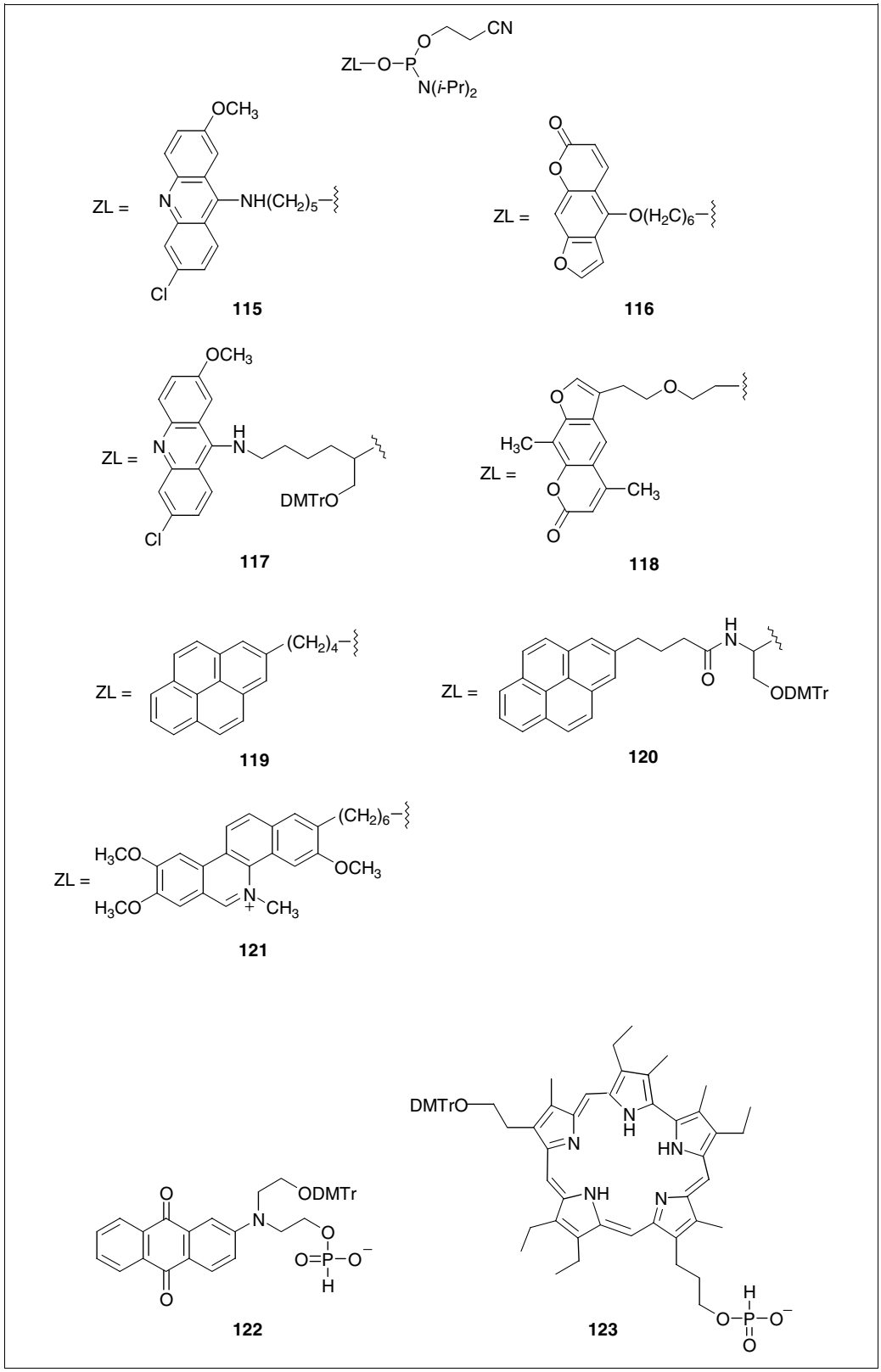

Figure 4.2.21 Direct addition of ligands to the 5′ end of oligonucleotides by the phosphoramidite coupling method. See Figure 4.2.1 for definitions of functional group abbreviations.

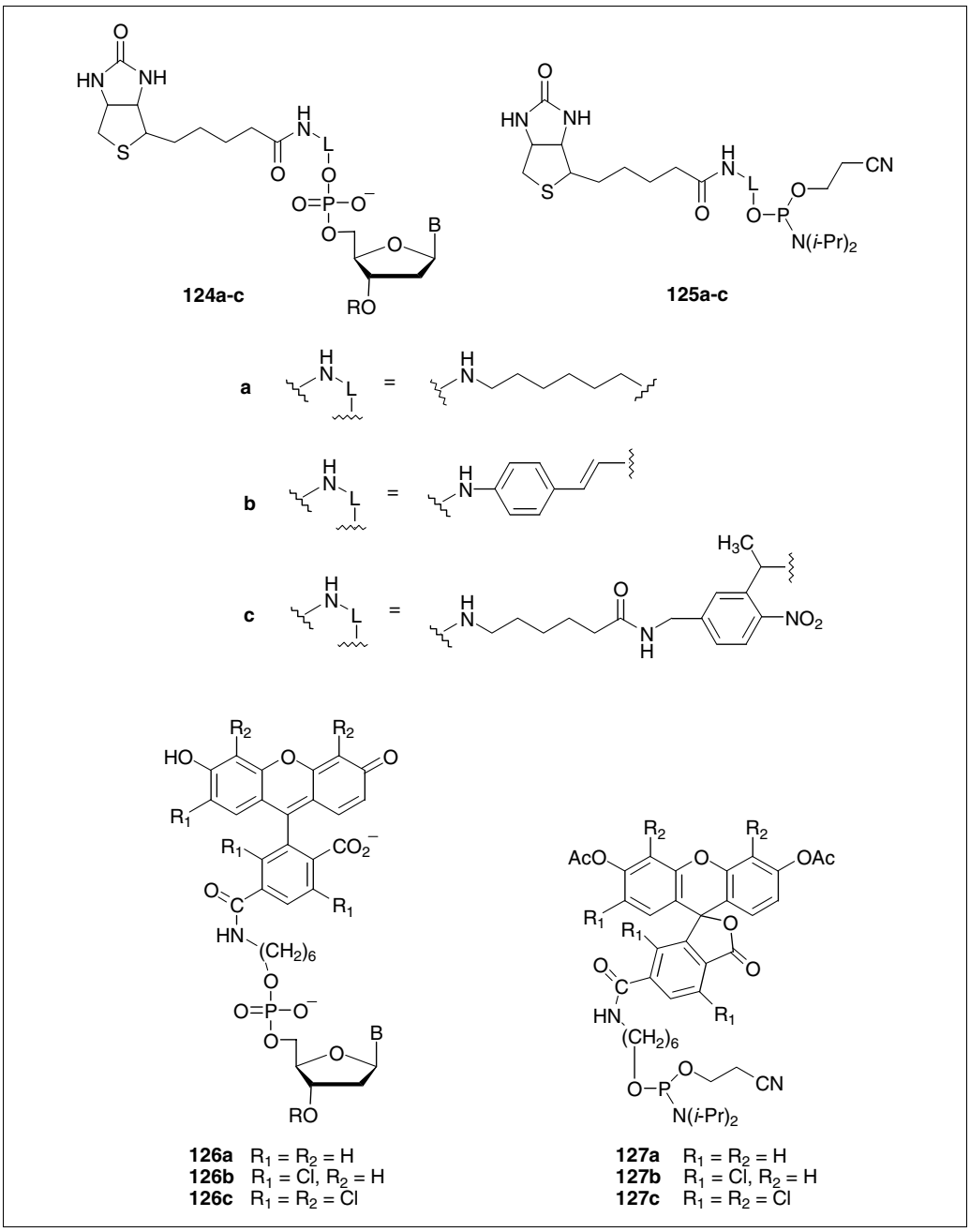

Figure 4.2.22 Additional examples of direct addition of ligands to the 5′ ends of oligonucleotides by the phosphoramidite coupling method. See Figure 4.2.1 for definitions of functional group abbreviations.

is transferred to the acceptors which in turn generated strong fluorescence at characteristic wavelengths. Typically, when FAM is separated from the acceptor by six consecutive 1′,2′-dideoxyribose phosphates, the emission spectra of **S.128a-d** showed a 2- to 12-fold enhancement in fluorescence intensity relative to that of the corresponding singly-labeled oligomers.

The hairpin structure **S.130** is functionalized with both the fluorescent dye FAM at the fluorescence quencher 4-[(4-(dimethylamino)-phenyl] azobenzoic acid (DABCYL). The hairpin keeps both chromophores in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer to the quencher (the energy is dissipated as heat). When the probe **S.130** encounters a complementary target molecule, it undergoes a spontaneous conformational change that causes the fluorophore and the quencher to move away from each other. Since the fluorophore is no longer in close proximity to the quencher, it fluoresces upon UV irradiation.

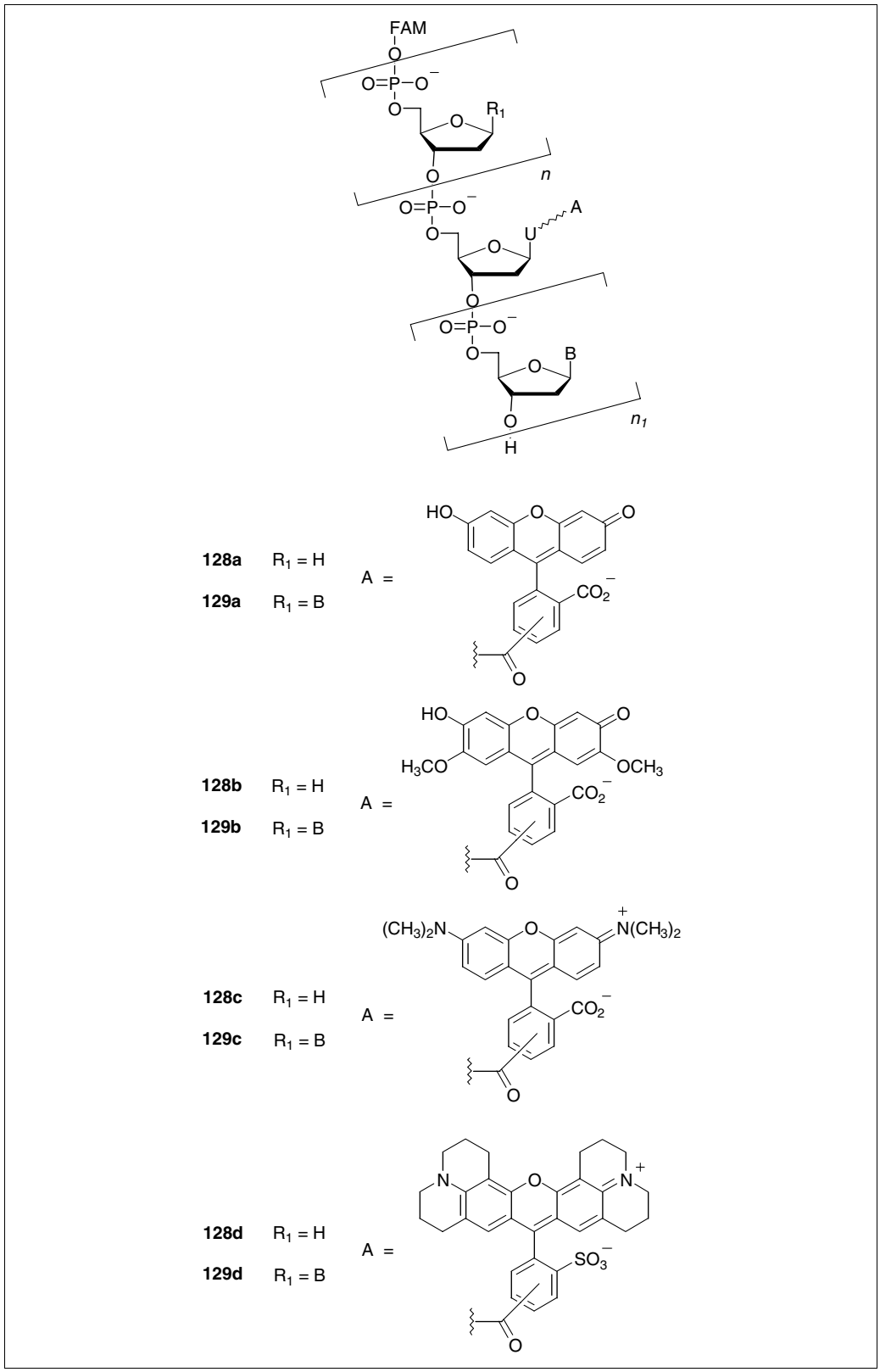

Figure 4.2.23 Direct addition of ligands to the 5′ terminus of oligonucleotides to permit sensitive fluorescence detection. Figure 4.2.1 for definitions of functional group abbreviations.

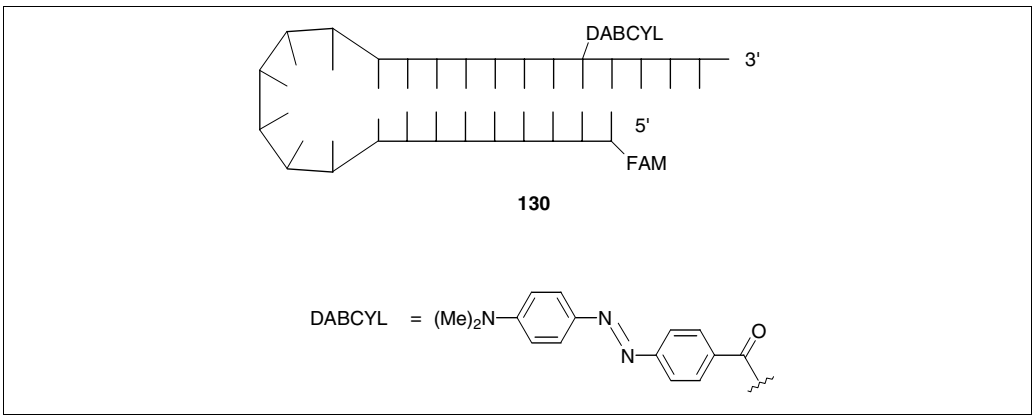

Figure 4.2.24 An additional example of direct addition of ligands to the 5′ ends of oligonucleotides to provide fluorescence detection.

Lastly, the 5′ terminus of oligonucleotides can be modified to tether other oligonucleotides through a linker and enable formation of triple-helices upon recognition of an oligopurine stretch on each limb of the conjugates **S.131** (Asseline and Thuong, 1994).

CHARACTERIZATION

Characterization of oligonucleotides bearing a ligand at the 5′ terminus can be achieved in different ways. The oligonucleotide-ligand conjugates can be purified by ion-exchange chromatography (Asseline et al., 1986; Thuong and Asseline, 1991); the retention time increases with the number of negative charges on the conjugate. For oligonucleotide conjugates with the same number of negative charges, separation can be performed by reversed-phase chromatography. Retention times of the conjugates are increased with the introduction of hydrophobic groups. The length and nature of the linkers used to join oligonucleotides and ligands also affect the chromatographic mobility of the conjugates. Purification of the oligonucleotide conjugates can also be achieved by polyacrylamide gel electrophoresis (PAGE).

After purification the nucleoside composition of each conjugate can be verified by hydrolysis with nucleases such as endonuclease P1 (from *Penicillium citrinum*), snake venom

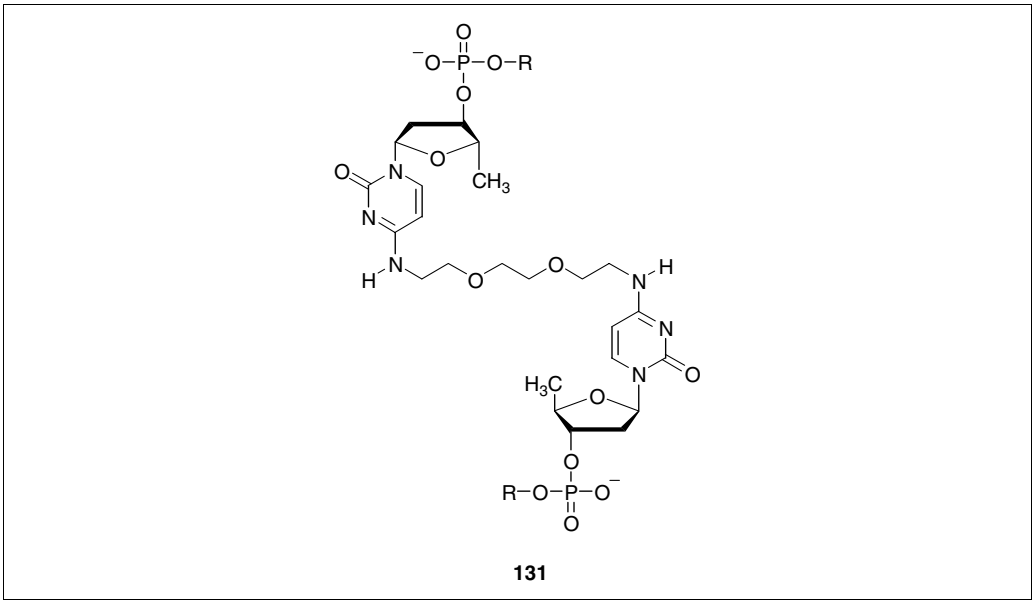

Figure 4.2.25 Conjugation of two third oligonucleotide strands for triple helical formation with double-stranded DNA targets via alternate strand recognition. See Figure 4.2.1 for definitions of functional group abbreviations.

phosphodiesterase (from *Crotalus durissus*), and alkaline phosphatase. The resulting hydrolysates are then analyzed by comparison with commercial nucleoside samples or previously characterized nucleosides. Such analysis is performed by TLC or preferably by reversed-phase HPLC. It should be pointed out that ligand attachment to the 5′ end of an oligonucleotide prevents hydrolysis of the conjugate by 5′-exonuclease. Thus, 5′-oligonucleotide conjugates should be resistant to digestion by calf spleen phosphodiesterase. Recent developments in mass spectrometry, namely matrix-assisted laser desorption/ionization time of flight (MALDI-TOF; *UNIT 10.1*) and electrospray ionization (ESI, *UNIT 10.2*) techniques have allowed molecular mass determination of derivatized oligonucleotides. Chromophore-oligonucleotide conjugates can also be characterized by UV/fluorescence spectroscopy and circular dichroism.

PROPERTIES

Numerous physicochemical, biochemical, and biological studies devoted to oligonucleotide-ligand conjugates have been widely reviewed. The discussion below will cover just a few observations on the hybridization properties of conjugates, followed by a description of a few specific properties and applications of 5′-derivatized oligonucleotides.

Formation of Double-Stranded Duplexes

Hybrid stabilities

The stability of hybrids formed between one oligonucleotide-ligand conjugate and its complementary nucleic acid sequence depends on factors, such as the nature of the ligand-linker combination and the site where the ligand is conjugated to the oligonucleotide chain. The importance of these parameters has been studied with oligonucleotide–intercalating agent conjugates. For example, phenazinium derivatives have been linked to the 5′ or 3′ end of oligonucleotides (Lokhov et al., 1992) as well as to the C-5 positions of deoxyuridine (Levina et al., 1993). Acridine has been conjugated to the 5′ and 3′ termini of oligonucleotides, the internucleotidic phosphodiesters, and the C-5 positions of deoxyuridine residues (Asseline et al., 1984, 1996). Ethidium derivatives have been attached to either the 5′ or 3′ ends of short oligonucleotide sequences (Koshkin et al., 1994). Results obtained with these different conjugates indicate that the attachment of the intercalating agents to the 5′ or 3′ ends of oligonucleotides via a phosphodiester or a phosphoramide linkage generally leads to the formation of more stable hybrids.

Specific properties

5′-Conjugated oligonucleotides with a free 3′-hydroxyl functionality are widely used as primers for polymerases. Thus, oligomers bearing a 5′-fluorescent label such as fluorescein or rhodamine were first used as primers for DNA sequencing by enzymatic methods to allow sensitive, fast fluorescence detection (Smith et al., 1985). The doubly labeled primers **S.128** and **S.129** recently introduced for sequencing not only increase detection sensitivity but allow detection at many wavelengths to simplify DNA sequencing readouts (Ju et al., 1995, 1996). The use of primers labeled with a fluorescent group and a quencher (like **S.130**) allows direct quantification of synthesized PCR fragments in which the fluorescence is restored during amplification (Nazarenko et al., 1997).

DNA fragments obtained by PCR from primers containing two adenines and a psoralen derivative at their 5′ end easily give photocross-linking products under irradiation at 360 nm with the thymines of complementary sequences. This “Chemi-clamp” system (Appligene-Oncor), enables very sensitive detection of point mutations contained in genes (Costes et al., 1993; Dupret et al., 1994) by the use of denaturing gradient (denaturing gradient gel electrophoresis, or DGGE; Børresen-Dale et al., 1998).

Formation of Triple-Helical Complex

Triple-helix stabilities

Oligonucleotides can bind to the major groove of DNA polypurine·polypyrimidine sequences to form triple helices. Recognition involves the formation of Hoogsteen or reversed-Hoogsteen hydrogen bonds between bases of the oligonucleotide third strand and the purines of the target DNA. This sequence-specific recognition of double-helical DNA was first described for pyrimidine oligonucleotides (Le Doan et al., 1987b; Moser and Dervan, 1987), then for oligonucleotides containing G and T (Cooney et al., 1988), G and A (Beal and Dervan, 1991; Pilch et al., 1991), and T, C, and G (Giovannangeli et al., 1992a). The attachment of an acridine derivative (Collier et al., 1991; Sun et al., 1989) to the 5′ end of a pyrimidine oligonucleotide was shown to strongly stabilize the triple-helical complex

formed with DNA by intercalation at the triplex-duplex junction. When attached to the 5′ end of a short oligopurine strand, oxazolopyridocarbazole was also shown to stabilize the triple-helical complex (Mouscadet et al., 1994). Another example of triple helix stabilization is demonstrated by conjugation of a daunorubicin derivative to the 5′ terminus of an oligopyrimidine oligonucleotide (Garbesi et al., 1997). Studies carried out with benzopyridoindole derivatives linked to C-5 of deoxyuridine located at either the 5′ or 3′ ends or at internal positions in the oligonucleotide chain, as well as on internucleotidic bridges, via phosphorothiolate or phosphoramidate linkages have shown that only the 5′ oligonucleotide conjugates induce triple-helical stabilization (unpub. observ.). Other results obtained with oligopyrimidine oligonucleotides linked to BePI, BgPI, BfPQ, and BhPQ derivatives through either the 5′ end or internal sites have shown that the 5′ end is the most favorable site for intercalator conjugation (Silver et al., 1997).

Irreversible modification of nucleic acid target sequences

The ability of oligonucleotides bearing an intercalating agent at their 5′ end to form stable triple-helical complexes with the DNA target by intercalation of the polycyclic ligand into the double-helix/triple-helix junction has been exploited to develop reactive oligomers able to induce irreversible localized modifications of the double-stranded DNA targets. Specifically, alkylating agents, cleaving reagents such as porphyrin or Cu-phenanthroline, and photocross-linking reagents have been conjugated to oligonucleotides to modify target nucleic acids.

Cross-linking. Many of the intercalating agents attached to oligonucleotides have been shown to cross-link target nucleic acids upon UVA or visible light irradiation. These include proflavin and its azido derivative (Praseuth et al., 1988a,b), ethidium and its azido derivative (Koshkin et al., 1994), and psoralen (Takasugi et al., 1991; Giovannangeli et al., 1992b). In the first two cases, cross-links can be converted to cleavage products upon alkaline treatment. In addition, photooxidation of guanine bases by singlet oxygen production resulted from the reaction of an excited proflavin with molecular oxygen. In the case of psoralen, a photochemical [2+2]-cycloaddition reaction involves the furan or pyrone ring of psoralen and the 5,6-double bond of thymine. The yield of cross-linked structures depends on the attachment site and the linker used to tether the psoralen to the oligonucleotide.

When the target is a DNA duplex, a psoralen derivative can be used to cross-link the two strands of the double helix when conjugated to the 5′ end of a pyrimidine triple-helix-forming oligonucleotide (Takasugi et al., 1991; Giovannangeli et al., 1992b). This reaction requires a 5′-TpA-3′ sequence at the junction between the duplex and triplex structures on the oligopurine-containing strand of the target duplex so that two thymines are properly located to form cyclobutane adducts with both the furan and the pyrone rings of a psoralen intercalated at the junction. Since the 5′-TpA-3′ sequence is self-complementary, one thymine is located in the duplex region on one strand and the other in the triplex region on the other duplex strand. The resulting product has psoralen linked to the three strands of the triplex structure (one covalent bond attaching psoralen to the oligonucleotide third strand and two photochemically generated bonds to thymines on the target DNA).

Recently, *N*-5-methylcyclopropapyrroloindole—a structural analog of cyclopropapyrroloindole (CPI), the reactive subunit of antibiotic CC-1065, which alkylates adenines at N3—was conjugated to the 5′ end of an oligonucleotide (Lukhtanov et al., 1997b). This conjugate has proven capable of alkylating the target DNA duplex region immediately adjacent to its double-stranded complementary binding region with which it forms a triple helix. The remarkable stability of the CPI alkylating function to side reactions in physiological buffers allows the reactive oligonucleotide conjugate to rapidly and efficiently cross-link to the DNA target.

Cleavage of the target DNA sequence. Cleaving reagents such as porphyrin, phenanthroline-Cu, or bleomycin-Cu have been linked to the 5′ ends of oligonucleotides in order to induce specific cuts on target nucleic acids. Among them orthophenanthroline (OP) used in the presence of Cu^{2+} and a reducing agent has proven to be the most efficient (Chen and Sigman, 1986, 1988; François et al., 1989). Using a 5′ oligonucleotide-OP conjugate bound to duplex DNA through a triple-helical structure, phenanthroline has been shown to intercalate at the triplex-duplex junction and induce cleavage of both DNA strands with high efficiency—up to 70% double-strand cleavage (François et al., 1989). Since OP is covalently linked to the oligonucleotide via its 5′ terminus, the oligonucleotide binds to the major groove of the DNA

duplex. OP then intercalates at the duplex-triplex junction within the minor groove. Therefore, Cu^{2+} chelation and the cleavage reaction take place in the minor groove even though recognition of the duplex sequence occurs in the major groove. The cleavage reaction was shown to be sequence specific. Using SV40 circular DNA (5000 bp) as a target, a single double-stranded cut was induced by an OP-oligonucleotide conjugate (François et al., 1989), providing a basis for sequence-specific artificial endonucleases.

Inhibition of gene expression via triple-helix formation

The synthesis of oligonucleotide-intercalator conjugates has paved the way for the development of new tools for molecular and cellular biology and for the design of new gene expression regulators. Oligonucleotides bearing an intercalator at their 5′ end have been used in the "antigene" strategy in which the target is double-helical DNA. The formation of a triple-helical complex within the promoter region of a selected gene may inhibit binding of transcription factors, thereby inhibiting transcription initiation (Grigoriev et al., 1992); this inhibition can be made irreversible when a psoralen conjugate is used in the presence of UV light (Grigoriev et al., 1993). Psoralen conjugates were also used as tools to demonstrate that DNA target sequences were accessible within the chromatin structure compressing genomic DNA in the cell nucleus (Giovannangeli et al., 1997).

An acridine-oligonucleotide conjugate was recently shown to arrest the elongation of an RNA transcript when involved in a triple-helical complex formed downstream of the transcription initiation site (Escudé et al., 1996). Unconjugated oligomer did not exhibit any effect on transcription elongation and demonstrated that the gain in stability provided by the intercalator was required for a biological response to be observed.

CONCLUSION

Numerous ligands have been covalently linked to the 5′ end of oligonucleotides. Coupling at this location is among the easiest to perform and generally produces conjugates with good hybridization properties. These conjugates are very useful in the applications of oligonucleotides in various fields, as they possess specific properties. The presence of the free 3′-terminal hydroxyl group allows their use as primers for polymerases, and the strong stabilization provided by intercalator ligands makes them efficient inhibitors of gene expression via triple-helix formation.

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