Attachment of Reporter and Conjugate Groups to the 3′ Termini of Oligonucleotides

Applications of oligonucleotide conjugates encompass mechanistic and hybridization probes, antisense agents, and sensors. Consequently, methods for their synthesis constitute an active area of research. This unit focuses on methods for the preparation of oligonucleotides conjugated at their 3′ termini (Fig. 4.5.1, **S.1**). Conjugation at the 3′ terminus of an oligonucleotide has been noted as being less common than at other sites within oligonucleotides, because this position commonly serves as the site for covalent linkage to the solid phase synthesis support (Goodchild, 1990); however, the valuable physicochemical properties of 3′-oligonucleotide conjugates, such as their ability to stabilize nucleic acid hybridization complexes and to retard the activity of exonucleases, provides strong incentive to develop methods for their preparation.

The synthesis of 3′-oligonucleotide conjugates was included in previous reviews published during the past decade, and therefore this author will try to minimize redundancy. The reader is referred to these previous reviews for further information (Goodchild, 1990; Agrawal, 1994; Beaucage and Iyer, 1993; Fidanza et al., 1994). Methods for the synthesis of 3′-oligonucleotide bioconjugates will be divided into three general categories: preparation of modified supports and phosphoramidites, postsynthetic modification of deprotected oligonucleotides, and the recently developed method utilizing solution phase conjugation of protected oligonucleotides. The latter two methods require modified solid phase synthesis supports which release oligonucleotides containing the appropriate 3′-terminal functional group. Such supports are described briefly in this review. The variety of new supports that produce oligonucleotides containing 3′-hydroxyl termini are not included. Due to the growing importance of nucleopeptides and surface/polymer supported oligonucleotides, methods for the synthesis of these two general families of conjugates are presented separately. A representative, but not exhaustive, set of examples of each method is presented, and apologies are extended to any authors whose work is not cited.

Figure 4.5.1 Generic oligonucleotide 3′-conjugate.

MODIFIED SOLID PHASE SYNTHESIS SUPPORTS AND PHOSPHORAMIDITES

Modifying the 3′ termini of oligonucleotides with polymers such as poly-L-lysine or polyethylene glycol (PEG) is desirable in order to improve the pharmacological properties of antisense probes. 3′-PEG-derivatized oligonucleotides have been prepared from a specially designed solid phase synthesis support in which the PEG serves as a linker between the controlled-pore glass (CPG) support and the growing oligonucleotide (Fig. 4.5.2, **S.2**; Jaschke et al., 1993). By loading the PEG onto the solid support as its succinato ester, 3′-PEG-derivatized oligonucleotides are released under standard aqueous ammonium hydroxide deprotection conditions. Succinato derivatives of smaller molecules, such as cholesterol, have also been used to prepare 3′-oligonucleotide conjugates (Fig. 4.5.2, **S.3**; MacKellar et al., 1992).

A variety of solid phase supports have been prepared from a common amino derivative of glycerol (Nelson et al., 1992). The masked nucleophile was originally employed as it *N*-9-fluorenylmethoxycarbonyl (Fmoc)–protected derivatives as a means for introducing 3′-alkylamines into oligonucleotides (Nelson et al., 1989). In later studies, the support was modified to contain a longer butylamine linker in order to alleviate potential steric interactions between the conjugated moiety and the remainder of the oligonucleotide backbone (Fig. 4.5.3, **S.4** and **S.5**; Nelson et al., 1989). Recently, this modification was used for preparing double-dye-labeled oligonucleotides containing tetramethylrhodamine at their 3′ termini (Mullah and Andrus, 1997).

In each of the above examples a new solid phase synthesis support must be prepared for each 3′ reporter group or conjugate. Recently, a method that circumvents this procedure was reported (Nelson et al., 1997). The method employs a universal solid phase support (Fig. 4.5.4, **S.6**) which releases an intermediate 3′-

Contributed by Marc M. Greenberg
Current Protocols in Nucleic Acid Chemistry (2000) 4.5.1-4.5.19

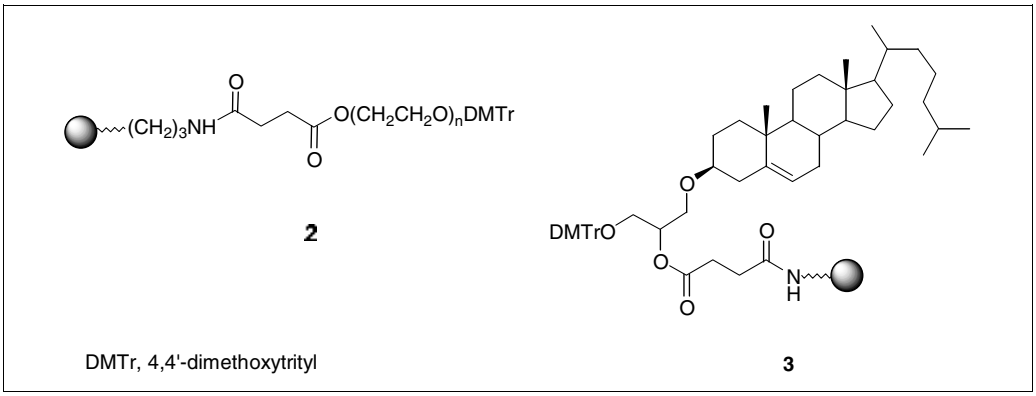


Figure 4.5.2 Solid phase supports for attaching polyethylene glycol (**S.2**) and cholesterol (**S.3**) to the 3′ termini of oligonucleotides.

tetrahydrofuranyl phosphodiester (Schwartz et al., 1995; Scheuer-Larsen et al., 1997). This intermediate undergoes subsequent cyclic phosphate formation and ultimately releases a 3′-hydroxyl oligonucleotide upon further alkaline treatment in a manner analogous to RNA hydrolysis. When a modified phosphoramidite is the first species coupled to the support, 3′-modified oligonucleotides containing hydroxyl termini are produced upon cleavage. Although the method still requires the preparation of the appropriate modified phosphoramidite, it has the advantage that the same amidite can be used for introducing the respective modification at other sites within the oligonucleotide. Hence, one does not need to synthesize both a modified support and a modified phosphoramidite. Furthermore, when used in conjunction with a more labile diglycolate linker in lieu of the standard succinato moiety, the oligonucleotides are cleaved using a mixture of *t*-butylamine, methanol, and water.

The dye-labeled oligonucleotides prepared by the above methods have been used for detecting a polymerase chain reaction (PCR) product in real time (Mullah et al., 1998). In an earlier oligonucleotide conjugate synthesis method, attomolar oligonucleotide detection limits were achieved using multiple nonradioactive labels at the 3′ termini (Haralambidis et al., 1990a,b). The method relied upon linear synthesis of a polyamide on CPG, followed by incorporation of a linker molecule, which allows one to then carry out standard oligonucleotide synthesis. This strategy is useful for preparing nucleopeptides (see below), but more relevant to the present discussion is its utility for preparing oligonucleotides containing multiple labels via conjugation to alkylamine side chains of the polyamide portion of the mole-

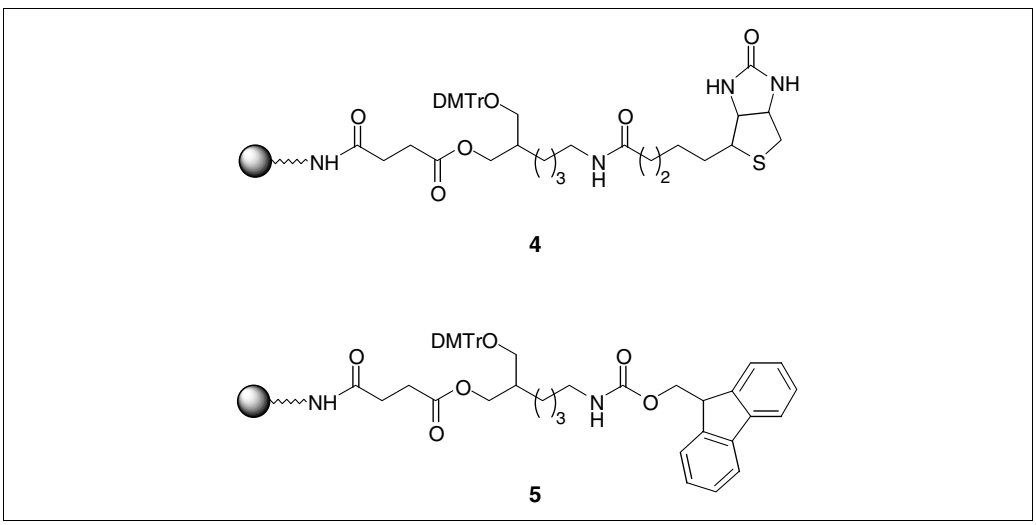


Figure 4.5.3 Solid support for conjugating biotin (**S.4**) or other conjugants (**S.5**) to oligonucleotides at their 3′ termini.

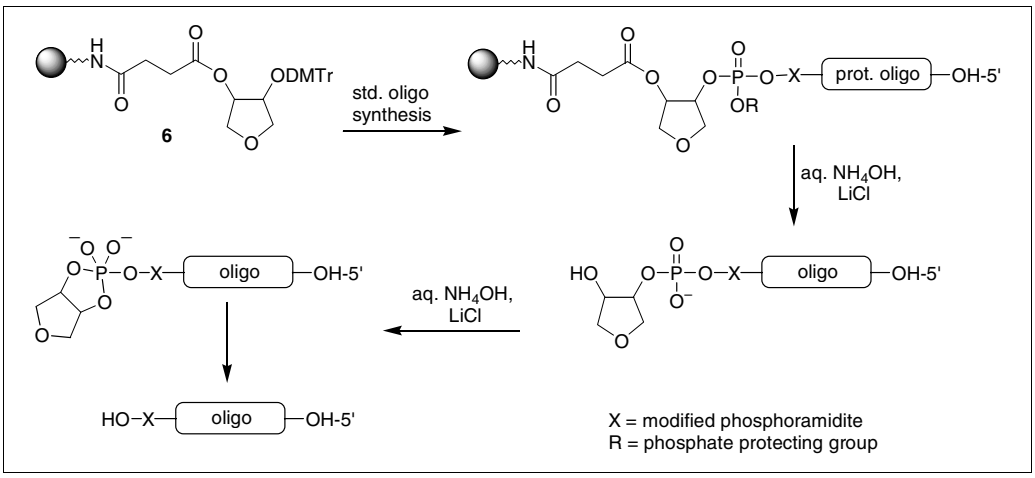


Figure 4.5.4 A universal support for preparing 3′-modified oligonucleotides.

cule. The labels can be incorporated prior to or after oligonucleotide synthesis, while the nucleic acid component is on the solid support or in solution.

In contrast, the thioester support (Fig. 4.5.5**, S.7**) enables introduction of conjugation as part of the main chain of the polymer (Hovinen et al., 1995). The thioester support was preceded by other versatile solid phase supports from which oligonucleotides containing a variety of 3′ termini could be prepared (Hovinen et al., 1993a,b, 1994); however, by utilizing a thioester linkage, more homogeneous products were obtained in good yields using a large excess of alkylamine nucleophiles (Hovinen et al., 1995).

SOLID PHASE SYNTHESIS SUPPORTS FOR PRODUCING 3′-FUNCTIONAL GROUPS SUITABLE FOR CONJUGATION

In order to carry out postsynthetic conjugation of oligonucleotides, the biopolymers must contain a suitable functional group at their 3′ termini. Utilization of the nucleophilicity of sulfur in conjugation chemistry has resulted in the preparation of numerous solid phase supports that release oligonucleotides containing thiols at their 3′ termini (e.g., Fig. 4.5.6, **S.8**; Asseline et al., 1992; Bonfils and Thuong, 1991; Gottikh et al., 1990; Gupta et al., 1990, 1991; Kumar, 1993a,b; Zuckerman et al., 1987). In some instances the initially cleaved 3′-thiol group is transformed into other functional groups that are also useful for preparing bioconjugates. These groups include 3′-phosphates, 3′-phosphorothioates, 3′-alkylamines, and 3′-alkyl carboxylic acids (Asseline et al, 1992; Gottikh et al., 1990; Gupta et al., 1991). 3′-Phosphorylated oligonucleotides can be obtained by a variety of supports, some of which are designed to be compatible with chemically unstable biopolymers (Gryaznov and Letsinger, 1992; Guzaev and Lönnberg, 1997). Preparation of oligonucleotides containing 3′-dialdehydes are readily prepared by utilizing a solid phase support containing a ribonucleoside, but this limits the structure of the tether between the oligonucleotide and 3′-electrophile. Consequently, supports designed to release alkyl aldehydes have been developed (e.g., Fig. 4.5.6, **S.9**; Urata and Akagi, 1993). This glyceryl support is designed to be universal, and to release a terminal, vicinal diol upon cleavage

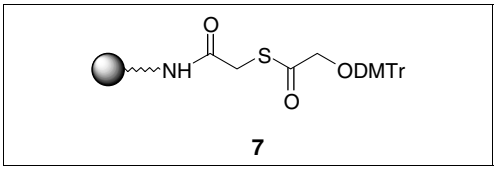


Figure 4.5.5 Thioester support for the preparation of 3′-modified oligonucleotides.

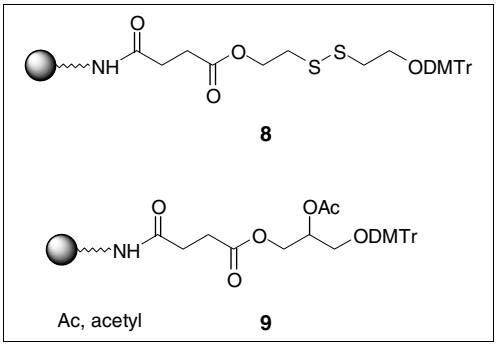


Figure 4.5.6 Supports for the release of oligonucleotides containing 3′-thiols (**S.8**) and 3′-aldehydes (**S.9**).

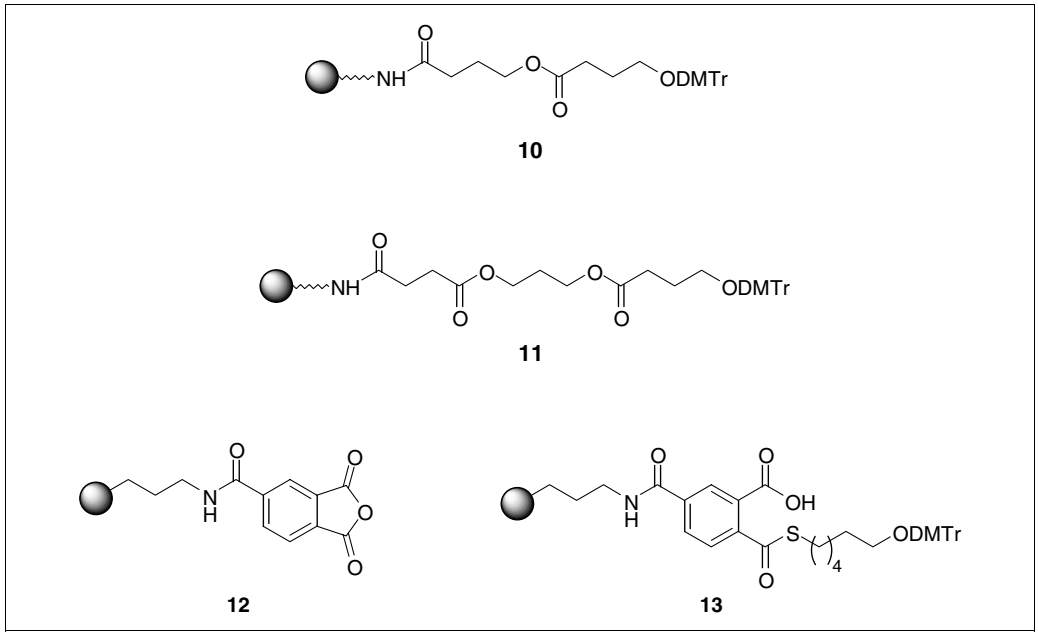


Figure 4.5.7 Supports for the release of oligonucleotides containing various 3′-functional groups.

under standard concentrated aqueous ammonia conditions. Subsequent periodate oxidation generates the desired 3′-aldehyde.

A versatile family of solid phase supports that enable one to introduce 3′-alkyl carboxylic acids, 3′-alkylamines, or 3′-alkyl thiols concomitantly with cleavage/deprotection of the oligonucleotides from the supports has also been reported (e.g., Fig. 4.5.7, **S.10** and **S.11**; Hovinen et al., 1993a,b, 1994, 1995). In some instances differentiation of the cleavage and deprotection steps can be achieved by employing a thioester-based support (Fig. 4.5.5, **S.7**). An alternative variation that utilizes this concept appeared recently (Lyttle et al., 1997). In this instance, a common solid phase support (Fig. 4.5.7, **S.12**) is modified prior to oligonucleotide synthesis with an appropriate dimethoxytritylated tether to produce a support (e.g., Fig. 4.5.7, **S.13**) that will release oligonucleotides containing the desired 3′-terminal functional group upon cleavage/deprotection. In most instances, oligonucleotides are cleaved from the above supports under conditions that also result in cleavage of the exocyclic amines and phosphate diester protecting groups. In some instances, transamination is a competitive process (Hovinen et al., 1995).

Orthogonal solid phase supports enable one to cleave oligonucleotides from the support without affecting the protecting groups throughout the biopolymer. These supports have proven invaluable in developing methods for bioconjugation using protected oligonucleotides. The first reported series of orthogonal solid phase supports utilized UV-irradiation to induce cleavage. Oligonucleotides containing 3′-hydroxyl groups (Greenberg and Gilmore, 1994; Venkatesan and Greenberg, 1996), 3′-alkyl carboxylic acids (Yoo and Greenberg, 1995), 3′-alkylamines (McMinn and Greenberg, 1996), and 3′-phosphates (Avino et al., 1996; Dell 'Aquila et al., 1997; McMinn et al., 1998; Fig. 4.5.8, **S.14** to **S.17**, respectively) were obtained using the *o*-nitrobenzyl photoredox reaction. More recently, Pd(0) labile supports that facilitate the release of 3′-phosphato, 3′-hydroxyl, and 3′-alkyl carboxylic acid containing oligonucleotides have been reported (Fig. 4.5.8, **S.18**, **S.19**, and **S.20**, respectively; Greenberg et al., 1998; Matray et al., 1997; Zhang et al., 1997).

POSTSYNTHETIC CONJUGATION

Conjugation of 3′-Alkylamines

The polyamide methods developed by Haralambidis (1990a,b) are compatible with conjugation to alkylamines in solution, following oligonucleotide deprotection. Common means of forming such conjugates involve in situ activation of carboxylic acids, or the use of the less reactive but more water tolerant *N*-hydroxysuccinimide esters (NHS-esters) or isothiocyanates.

Using a support similar to those developed by the Clontech group (Fig. 4.5.9, **S.21**), Thaden and Miller (1993a,b) prepared an oli-

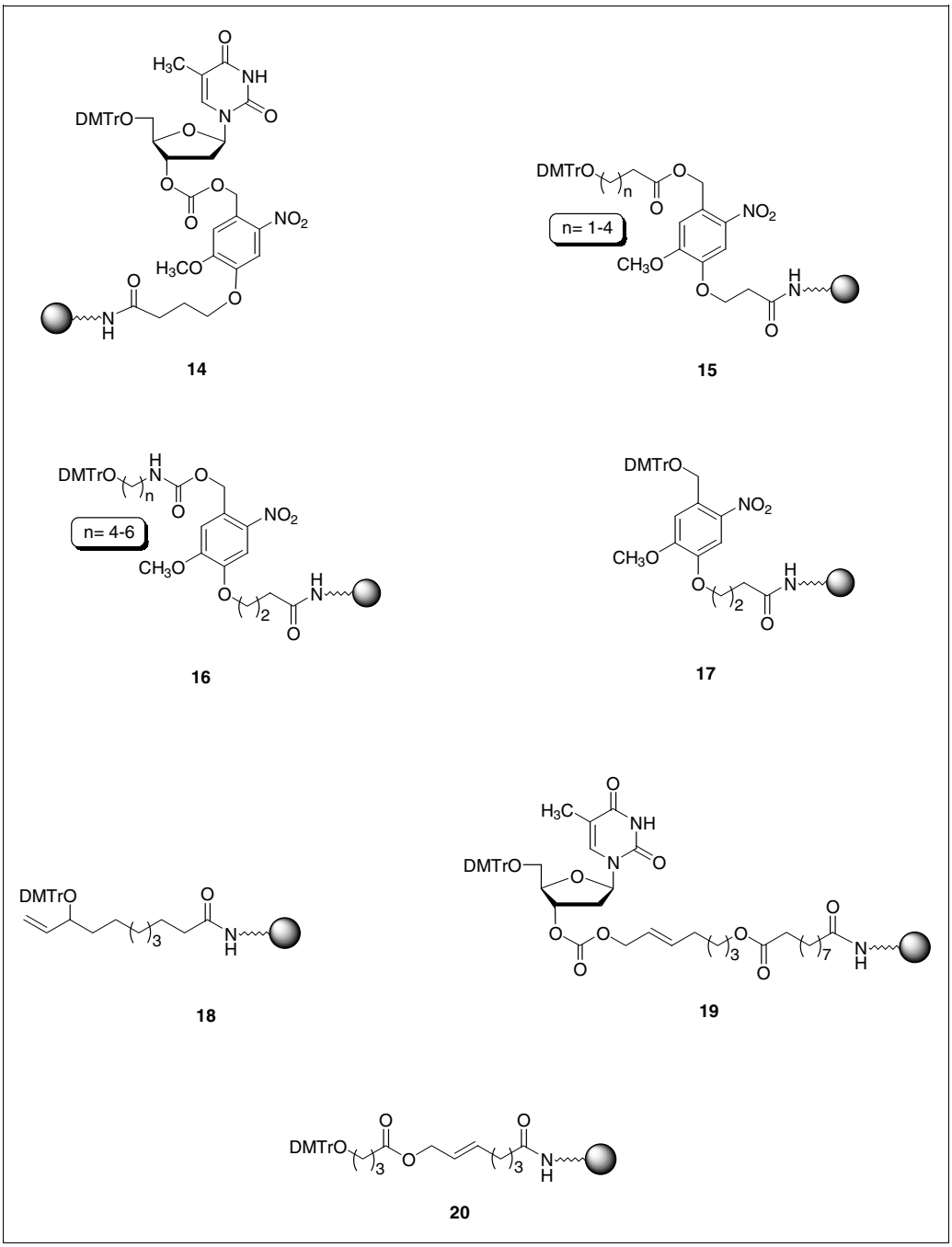
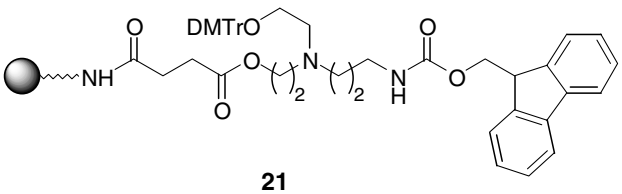
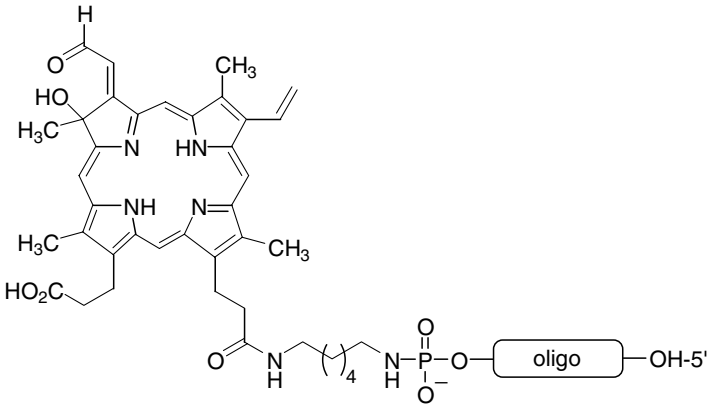


Figure 4.5.8 Photolabile and Pd(0)-labile orthogonal oligonucleotide synthesis supports.

gonucleoside methylphosphonate containing a 3′-rhodamine reporter group. Coupling is achieved with ~85% yield by reacting ten molar equivalents of rhodamine isothiocyanate with the biopolymer at room temperature for 18 hr (Thaden and Miller, 1993b). Oligonucleotides containing 3′-chlorin groups (Fig. 4.5.9, **S.22**) were prepared by activating the carboxylic acid on the chlorin component (Boutorine et al., 1996). The required alkylamine-containing oligonucleotide was prepared indirectly from the 3′-phosphorylated biopolymer via a redox condensation reaction with a diamine. The coupling proceeded in 30% to 40% conversion and, like the isothiocyanate example above, required several hours. In another example from the same contribution to the literature, the 3′-phosphate of the oligonucleotide was activated with a symmetrical dihydrazide, and the free hydrazide was condensed with a chlorin containing an aldehyde (Boutorine et al., 1996). The resulting hydrazone also was obtained in modest overall yield.



21



22

Figure 4.5.9 Introduction of dyes and porphyrins at the 3′ termini of oligonucleotides.

Conjugation of 3′-Alkyl Carboxylic Acids

Oligonucleotides containing 3′-alkyl carboxylic acids can be conjugated to amines following activation in situ with standard peptide coupling agents (Gottikh et al., 1990); however, one should note that these conditions can give rise to significant amounts of nonspecific covalent modification. One can obtain the requisite 3′-alkyl carboxylic acid directly from designed supports, or this functional group can be introduced postsynthetically. For example, daunomycin was linked to the 3′ terminus of an oligonucleotide (Fig. 4.5.10, **S.23**) via amide bond formation. The multistep process started from a disulfide support which yields 3′-phosphorylated oligonucleotides (Fig. 4.5.10, **S.8**; Gottikh et al., 1990). Activation of the phosphate group was followed by reaction with an amino acid. In this particular instance, activation/coupling with carbodiimide took place over 5 hr at 4°C, and yields were not reported.

Conjugation of 3′-Alkyl Thiols

Significant advantage has been taken of the nucleophilicity of sulfur for preparing oligonucleotide conjugates. As mentioned above, a variety of supports have been developed that result in the release of oligonucleotides containing 3′-alkyl thiols. Cleavage of 3′-alkyl thiolated oligonucleotides from these supports can be effected under conditions that do not remove the exocyclic amine and phosphate diester protecting groups; however, the thiol-containing oligonucleotides are typically cleaved/deprotected under reductive conditions in aqueous ammonia, and conjugated in aqueous solutions using the fully deprotected biopolymers. Excellent yields of sulfide linked conjugates are obtained by reacting thiolated oligonucleotides with α-halocarbonyl-containing conjugants (e.g., Fig. 4.5.11, **S.24**; Gupta et al., 1991).

3′-Oligonucleotide conjugates can also be prepared by reacting 3′-thiolated oligonucleotides with activated disulfides or maleimides (Gupta et al., 1991; Harrison and Balasubramanian, 1997; Kumar, 1993b). Reaction with a maleimide proceeds via the formation of a stable Michael adduct. In one approach, a variety of biologically useful conjugates was prepared in which the Michael acceptor (maleimide) was covalently attached to the 3′ terminus of the oligonucleotide, and the thiol-containing conjugant of interest was loaded on a solid support (Harrison and Balasubramanian, 1997). When preparing oligonucleotide conjugates containing 3′-disulfides, either the 3′-thiolated oligonucleotide or the conjugating species can be activated. Disul-

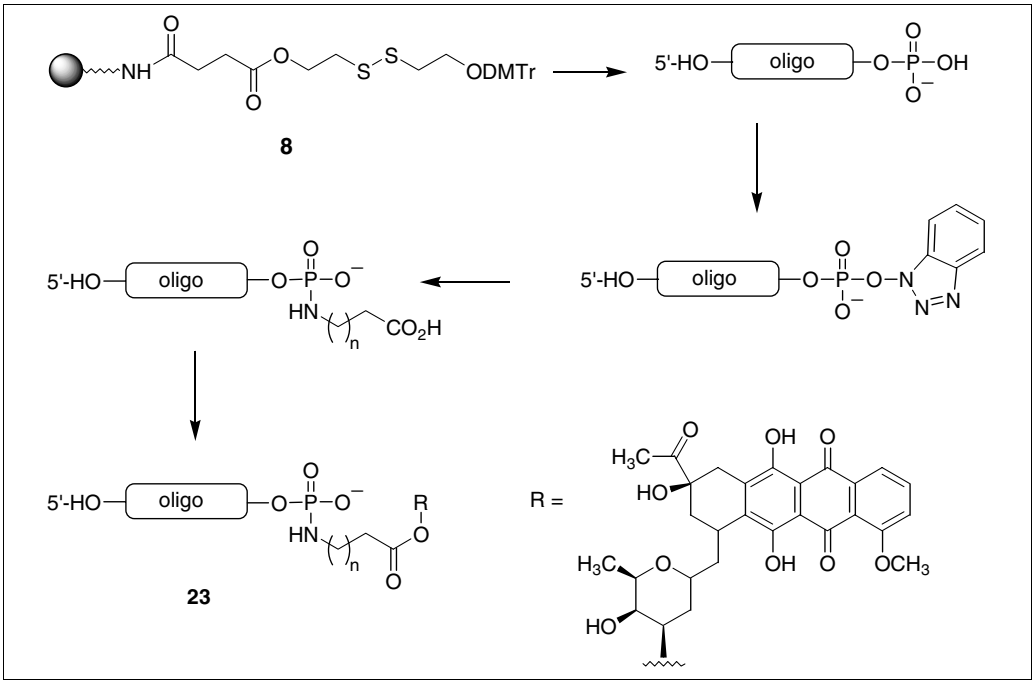


Figure 4.5.10 Postsynthetic modification of oligonucleotide 3′-carboxylic acids.

fide transfer has been used for conjugating oligonucleotides at their 3′ termini to small or large molecules and surfaces (Asseline et al., 1992; Gupta et al., 1991; Harrison and Balasubramanian, 1997; Kumar, 1993b; Zuckerman et al., 1987). For example, hybrid enzymes that hydrolyze RNA sequences specifically have also been prepared via disulfide exchange (Zuckerman et al., 1988; Zuckerman and Schultz, 1988).

Conjugation of 3′-Phosphorothioates

Conjugations at phosphorous atoms near the 3′ termini of oligonucleotides have been carried out by selectively oxidizing an *H*-phosphonate linkage with an appropriately substituted amine (Letsinger et al., 1989). The resulting phosphoramidate is carried through a conventional solid phase synthesis protocol using either phosphoramidite or *H*-phosphonate chemistry. Ammonolysis and chromatographic purification give a product containing >50% of the respective label. A more common approach to synthesizing 3′-oligonucleotide conjugates takes advantage of the nucleophilicity of phosphorothioates. In one example, 3′-phosphorothioate-containing oligonucleotides were prepared on a solid phase synthesis support (Fig. 4.5.6, **S.8**; Asseline et al., 1992). Following standard oligonucleotide synthesis and ammoniacal deprotection/cleavage under reducing conditions, the fully deprotected oligonucleotides were conjugated to halogenated substrates (Fig. 4.5.12). Conjugation to derivatives of daunomycin, fluorescein, and 1,10-phenanthroline (**S.25**) containing alkyl halide

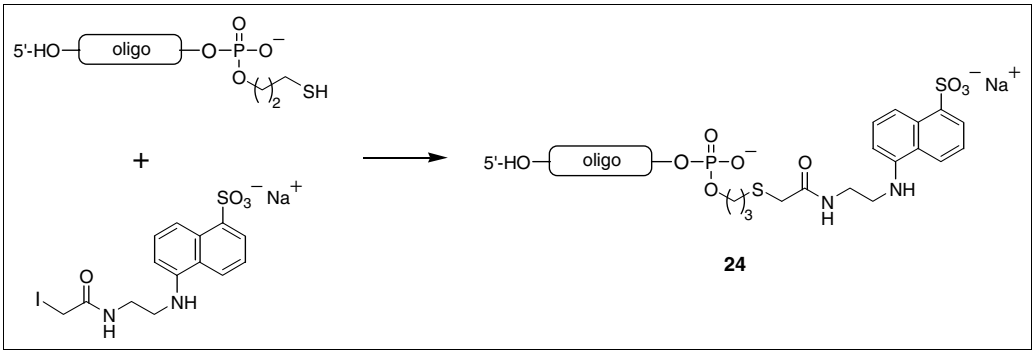


Figure 4.5.11 Postsynthetic modification of oligonucleotide 3′-thiols.

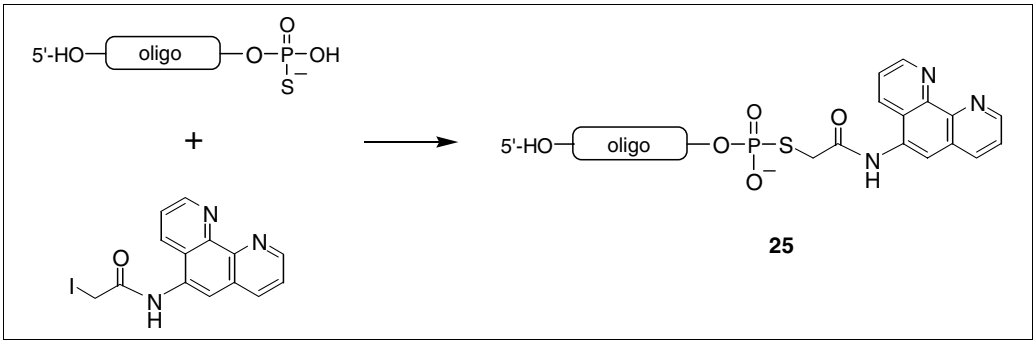


Figure 4.5.12 Postsynthetic modification of oligonucleotide 3′-phosphorothioates.

functional groups are believed to have proceeded in essentially quantitative yields over 24 hours in either protic organic solvents containing crown ethers to enhance biopolymer solubility, or mixtures of dimethylformamide and water. This strategy was utilized recently in studies on truncated derivatives of the potent antitumor antibiotics, CC-1065 and the duocarmycins, which alkylate deoxyadenosine at N-3 (Lukhtanov et al., 1996). The active pharmacophore, the cyclopropapyrroloindole, was covalently linked via an α-bromoacetamido linkage to a 3′-phosphorothioate in 50% to 60% yield.

Several reports of template-mediated oligonucleotide ligation involving 3′-phosphorothioate DNA have also appeared. Oxidative coupling of a 3′-phosphorothioate oligonucleotide to a 5′-phosphorothioated biopolymer is achieved rapidly (5 min) under mild conditions (0°C) using 1 μM $K_3Fe(CN)_6$ (Gryaznov and Letsinger, 1993a). Little or no coupling is detected under these conditions in the absence of a template. In contrast, ligation of a 5′-phosphorothioated oligonucleotide to a 3′-bromoacetamide-containing oligonucleotide proceeded in ~80% yield after 48 hr at 0°C in the absence of a template (Gryaznov and Letsinger, 1993b). As expected, the presence of a template, which increases the effective concentration of the reaction partners, significantly accelerated the reaction. Essentially quantitative yields of ligated product were obtained in only 20 minutes when a stoichiometric template was present. A distinct advantage of this latter coupling method is that the reaction does not require any exogenous condensing agents. In a subsequent investigation, autoligation was effectively carried out using reaction partners of the opposite polarity, 3′-phosphorothioate and 5′-bromoacetamide (Fig. 4.5.13; Gryaznov et al., 1994).

Conjugation to 3′-Aldehydes

Template-mediated synthesis has also been very useful for the conjugation of oligonucleotides containing 3′-aldehydes (Goodwin and Lynn, 1992; Zhan and Lynn, 1997). Aldehydes are attractive electrophiles with which to form oligonucleotide conjugates. Condensation with primary amines under reductive conditions provides secondary amines which are stable to acid and base. Solid supports that produce oligonucleotides containing 3′-aldehydes directly are unknown. Consequently, 3′-aldehydes are typically produced via periodate oxidation of a vicinal diol. A common practice for preparing 3′-oligonucleotide conjugates via aldehyde condensation takes advantage of incorporating a ribonucleoside at the 3′ terminus (Fig. 4.5.14; Leonetti et al., 1988). Periodate oxidation of the oligonucleotide containing a 3′-terminal ribonucleoside produces a dialdehyde which under reductive amination conditions generates a morpholine upon reaction with a primary amine. An often cited application of this method concerns the synthesis of poly-L-lysine conjugates of oligonucleotides (Leonetti et al., 1988, 1990). Haralambidis et al. (1994) have utilized this ability to introduce amino acids at the 3′ termini of oligonucleotides to enable attachment of a substituted benzaldehyde, which is then conjugated to an enzyme via reductive amination.

Condensation of a dialdehyde with a primary amine was also used recently in the segmental synthesis of a biologically active hammerhead ribozyme (Bellon et al., 1996). Linkage of the two segments was carried out in a loop region of the hammerhead ribozyme which had been shown to not be crucial for catalytic activity. One segment was synthesized so as to incorporate a 5′-alkylamine, while the other half contained a 3′-terminal uridine which served as the source of the dialdehyde. Follow-

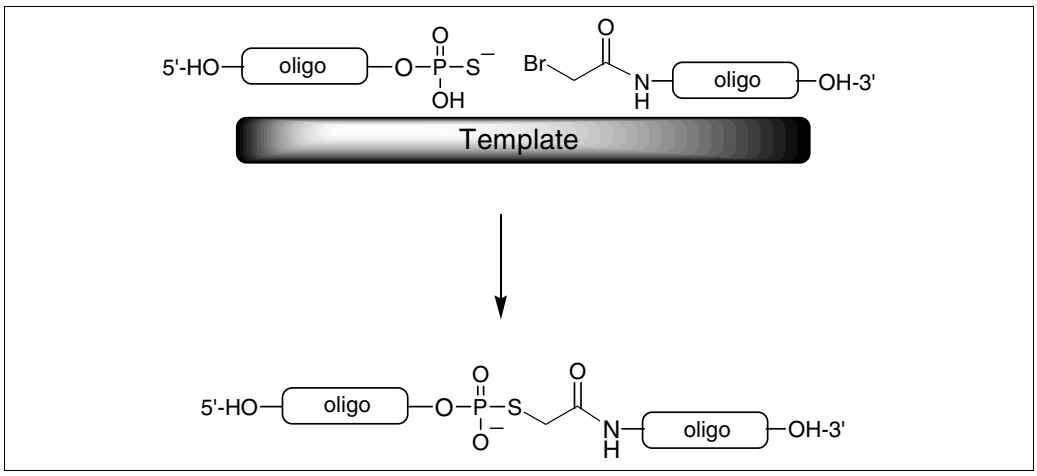


Figure 4.5.13 Template-mediated oligonucleotide coupling.

ing rapid and quantitative periodate oxidation, reductive condensation was carried out to 95% conversion over the course of seven days. In a more recent study, the electrophilic half of a ribozyme was synthesized on a glyceryl support (Fig. 4.5.6, **S.9**; Bellon et al., 1997; Urata and Akagi, 1993). In these experiments, conjugation of the two halves of a ribozyme within the loop II region of the unmodified hammerhead proceeded in as high as 81.2% yield

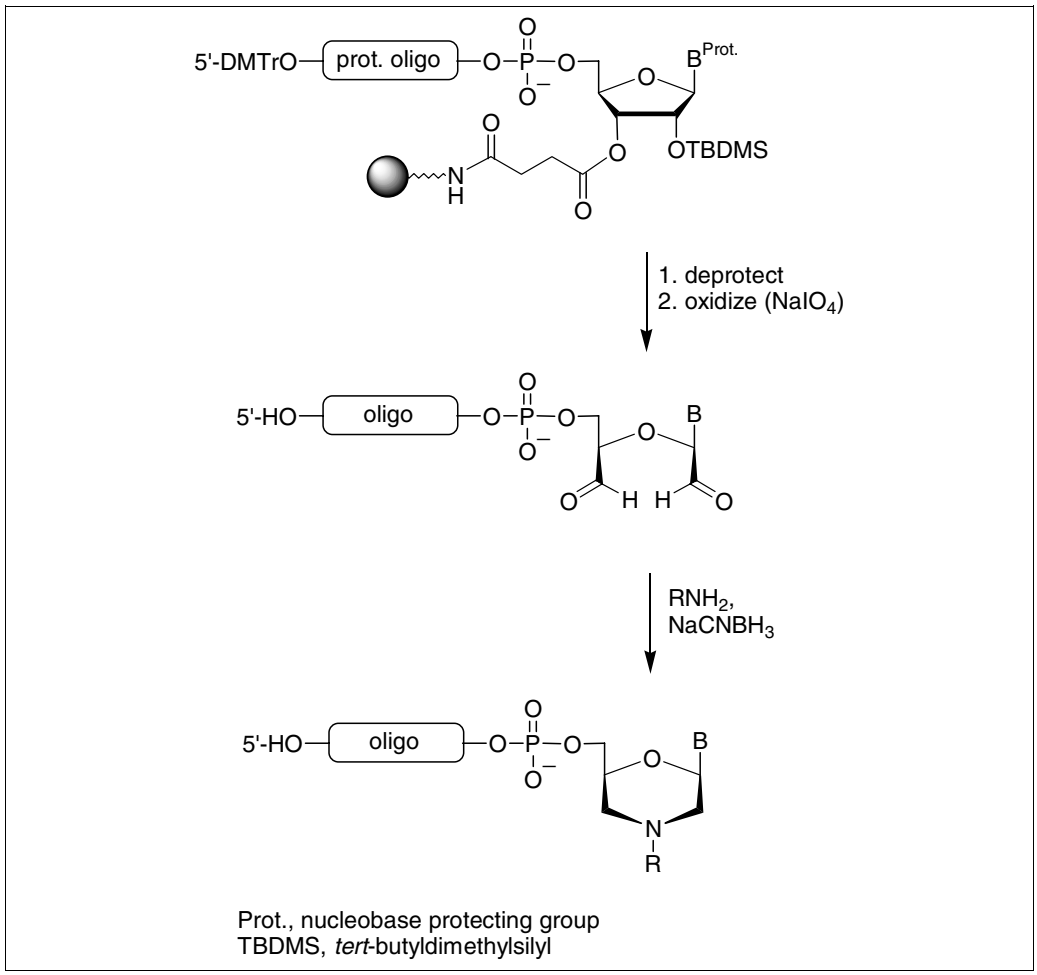


Figure 4.5.14 Postsynthetic modification of oligonucleotides by reductive amination.

(48 hr) when borane pyridine was employed as a reducing agent.

Solution Phase Conjugation of Protected Oligonucleotides

The need for orthogonal linkers that enable the removal of fully protected oligonucleotides from the support for further elaboration in solution was recognized by at least one leader in the field of oligonucleotide synthesis a number of years ago (Zon and Geiser, 1991). The first reports of conjugating protected oligonucleotides in solution appeared in 1997 (McMinn et al., 1997; also see McMinn and Greenberg, 1998, 1999). This new method for synthesizing oligonucleotide conjugates was made possible by the development of a family of orthogonal solid phase synthesis supports (Fig. 4.5.8, **S.14** to **S.20**). Using light or Pd(0), these supports enable one to release oligonucleotides containing 3′-alkylamines, 3′-alkyl carboxylic acids, or 3′-phosphate diesters which retain their exocyclic amine, phosphate diester, and 5′-hydroxyl protecting groups. One advantage of this method is that potential deleterious side reactions are eliminated by utilizing protected oligonucleotides. Nonspecific covalent modifications of unprotected oligonucleotides can be a significant problem when conjugating biopolymers (Bischoff et al., 1987; Erout et al., 1996; Ghosh and Musso, 1987; Lund et al., 1988). In addition, the rates at which conjugation reactions proceed are considerably faster than similar bond-forming reactions using unprotected oligonucleotides. An explanation for this acceleration is uncertain at this time, but may be related to the solvent conditions. Conjugation reactions of protected oligonucleotides are carried out in aprotic organic solvents, whereas unprotected oligonucleotides are often conjugated in aqueous solvents. Amide bond formation in aqueous solvents may be adversely affected by stronger solvation (hydrogen bonding) of the reactants, as well as a lower effective molarity of amines due to protonation.

The original report of solution phase conjugation of protected oligonucleotides utilized a redox condensation or a Mukaiyama reaction to activate carboxylic acids (Fig. 4.5.15; McMinn et al., 1997; Mukaiyama, 1976). The oligonucleotides contained 3′-alkylamines. During the course of developing the conjugation chemistry, it was discovered that the "fast deprotecting" amides used to protect deoxyadenosine and deoxyguanosine underwent transamidation with the 3′-terminal alkylamines (McMinn and Greenberg, 1997). Thus, phenoxyacetyl-protected phosphoramidites should not be used in conjunction with alkylamine modifiers. The observed transamidation is of general importance, because of the commercial availability of alkylamine modifiers for oligonucleotide synthesis. This problem was overcome by using isobutyryl exocyclic amine protecting groups for deoxyadenosine, deoxycytidine, and deoxyguanosine. Subsequently, a variety of biologically relevant reporter groups such as biotin and acridine were conjugated in excellent yields (88%) under mild reaction conditions (2 hr at 55°C) using only ten molar equivalents of carboxylic acids and activating reagents relative to oligonucleotide substrate. Only cholesterol coupled in <88% yield (i.e., 83%), and this may be attributed to difficulties in isolation of the very nonpolar conjugate. Optimization of the reaction process revealed that yields were not diminished by using as few as five molar equivalents of reagents relative to 3′-functionalized oligonucleotide, and were unaffected by increasing the length of the biopolymer (McMinn and Greenberg, 1998).

N-Protected tripeptides also served as suitable coupling partners. For example, conjugation of *N*-Fmoc-Gly-Gly-His proceeded in 89% yield (Fig. 4.5.16, **S.26**) and, in contrast to other methods, prior chemical elaboration of the tripeptide were not required in order to introduce this nuclease mimic (McMinn et al., 1997; Truffert et al., 1996). Coupling under the Mukaiyama conditions did result in some epimerization of the α-amino acid. This limitation was overcome using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) or 2-(1*H*-benzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as activating agents, with no detriment to the yields of the conjugates (McMinn and Greenberg, 1998). The greater ease of using PyBOP or HBTU also proved useful for the efficient synthesis of (5′,3′)-bis-conjugates (Fig. 4.5.16, **S.27**; Kahl et al., 1998).

Protected oligonucleotides containing 3′-alkylamines also proved to be suitable substrates for isocyanates. Nearly quantitative yields of alkyl aryl ureas (e.g., Fig. 4.5.17, **S.28**) were obtained upon reaction with ten molar equivalents of aryl isocyanates. Coupling of the less reactive alkyl isocyanate generated slightly lower yield (88%), despite using twice the number of equivalents of isocyanate. The ability to conjugate isocyanates to protected oligonucleotides containing 3′-alkylamines facilitated the synthesis of oligonucleotide-peptide conju-

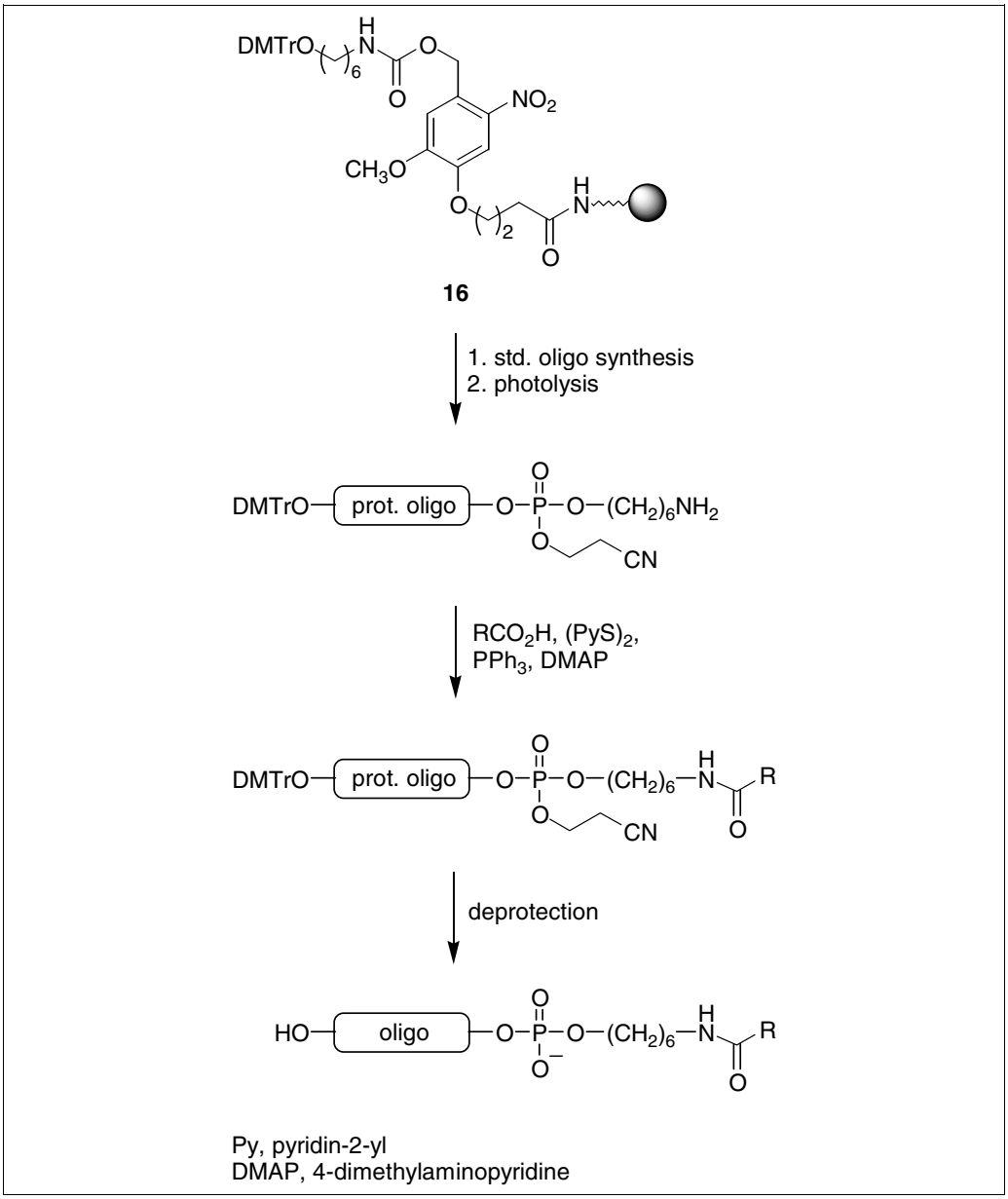
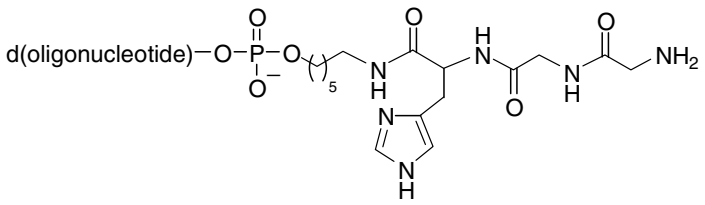


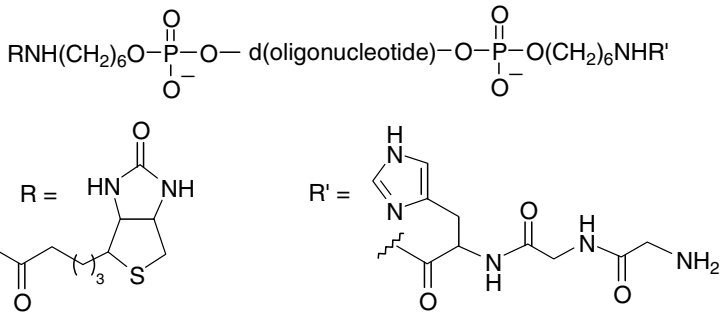
Figure 4.5.15 Solution phase conjugation of protected oligonucleotides.

gates in which the 3′ terminus of the oligonucleotide was coupled to the *N* terminus of a peptide (McMinn and Greenberg, 1998). Conjugate **S.29** (Fig. 4.5.17) was obtained in a 70% yield upon reaction of twenty molar equivalents of the respective isocyanate for 4 hr at 55°C; however, the method was limited with respect to the sequences of peptides that can be employed. For instance, peptides containing bulky side chains at their amino termini coupled poorly. The resulting conjugates contain the opposite topology between the oligonucleotide and peptide as that obtained from the amide-forming reaction described in Figure 4.5.18.

Conjugation of the 3′ termini of oligonucleotides to the amine terminus of tripeptides was achieved in a more general manner by using protected oligonucleotides containing 3′-alkyl carboxylic acids (Kahl and Greenberg, 1999). In general, conjugation of protected oligonucleotides containing 3′-alkyl carboxylic acids to primary alkyl amines proceeded equally efficiently as analogous reactions involving 3′-alkylamine-containing oligonucleotides. Furthermore, the amide-forming reaction was essentially unaffected by steric hindrance during coupling to the amino termini of peptides. Conjugation of 3′-alkyl-carboxylic-acid-containing oligonucleotides to the amino termini of peptides produces conjugates con-



26



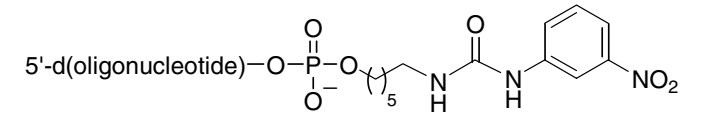
27

Figure 4.5.16 Oligonucleotide conjugates and bis-conjugates prepared from protected oligonucleotides.

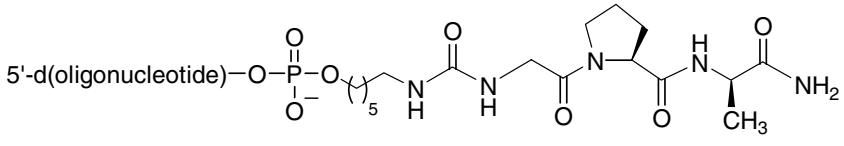
taining the same topology as the urea bond-forming reaction (Fig. 4.5.18). In contrast to the urea bond-forming method, oligonucleotide-peptide conjugates synthesized in this manner proceeded efficiently even when the *N*-terminal amino acid was sterically hindered. For instance, **S.30** (Fig. 4.5.18) was obtained in 95% yield via a PyBOP mediated coupling, whereas the analogous conjugate formed via coupling of an oligonucleotide amine and isocyanate proceeded in <20% yield (McMinn and Greenberg, 1998).

The Synthesis of Nucleopeptides

The importance of nucleopeptides and protein-nucleic acid interactions has provided the driving force behind several methods for synthesizing these bioconjugates. Consequently, many of the methods for preparing 3′-oligonucleotide conjugates described above have been applied to the synthesis of nucleopeptides. Recently, the method involving the solution phase coupling of protected oligonucleotides containing 3′-alkylamines to carboxyl-protected peptides was applied to the synthesis of a conjugate consisting of the operator site of the λ



28



29

Figure 4.5.17 Oligonucleotide conjugates prepared from protected substrates containing 3′-alkylamines.

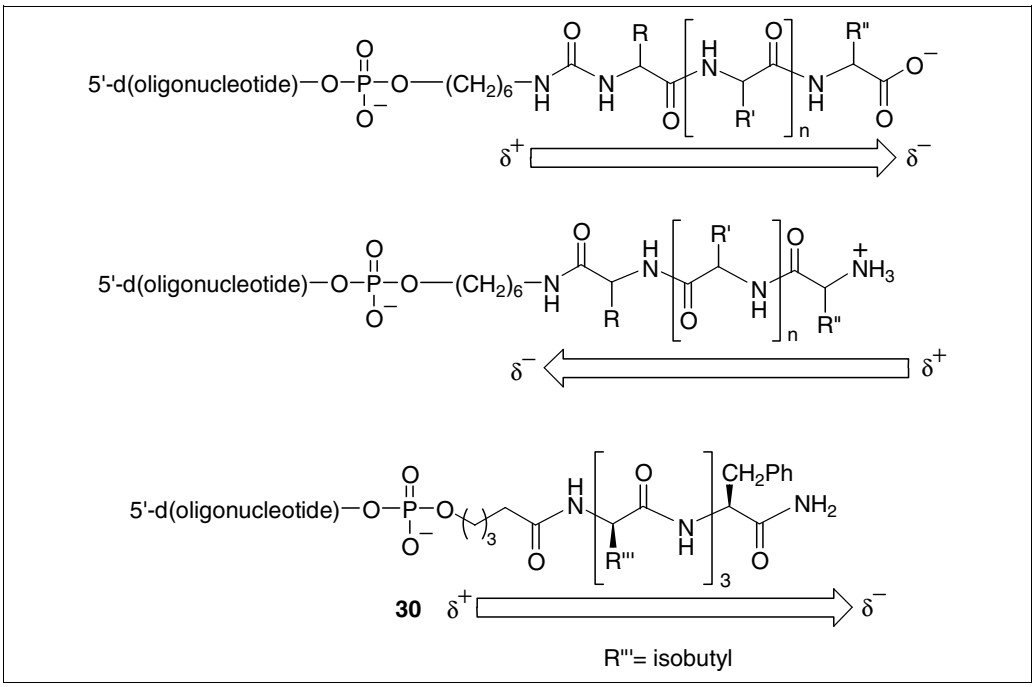


Figure 4.5.18 Topological control of nucleopeptide formation.

repressor helix-turn-helix protein and a modified version of the DNA-binding helix of λ repressor (McMinn and Greenberg, 1999). The desired bioconjugate (Fig. 4.5.19, **S.31**) was obtained in 72% yield using ten molar equivalents of peptide and PyBOP. Attempted synthesis of the related nucleopeptide (Fig. 4.5.19, **S.32**) containing the naturally occurring DNA-binding helix of λ repressor resulted in the isolation of **S.33** (Fig. 4.5.19) in 96% yield. It was proposed that **S.33** was formed via an *N*- to *O*-transacylation during the concentrated aqueous ammonia deprotection of the initially formed conjugate. The fact that **S.33** was not observed during the synthesis of **S.31** suggests that even slight steric hindrance prevents the undesired transacylation process.

Oligonucleotide conjugates of large proteins such as modified serum albumin have been prepared via disulfide exchange. In this study, a 3′-thiolated oligonucleotide obtained from a disulfide support was conjugated to a

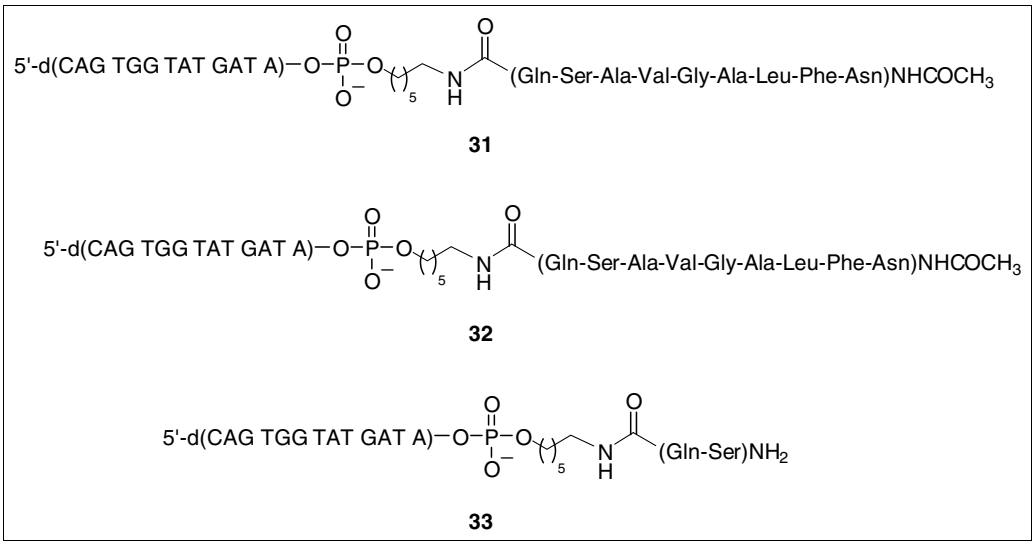
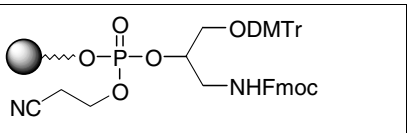


Figure 4.5.19 Solution phase synthesis of nucleopeptides.

neoglycoprotein which was derivatized through an alkylamine on its surface with *N*-succinimidyl 3-(2-pyridyldithio)propionate (Bonfils et al., 1992). Alternatively, 3′-thiolated oligonucleotides were conjugated to peptides that were derivatized with maleimides (Arar et al., 1993; Soukchareun et al., 1998). Bioconjugates can be formed in high yield by this method; however, disadvantages include susceptibility of the thiolated oligonucleotides to oxidation, and difficulties in reactions with highly charged peptides due to aggregation (Soukchareun et al., 1998). Nucleopeptide conjugates have also been prepared via displacement of a 3′-iodoacetamide-substituted oligonucleotide by a cysteine-containing peptide (Tung et al., 1995).

Two general methods have been developed for the synthesis of oligonucleotide-peptide conjugates on a single solid phase support. Bifunctional supports (e.g., Figs. 4.5.3 and 4.5.20, **S.5** and **S.34**, respectively) can be employed for the synthesis of peptides (using *N*-Fmoc chemistry) and oligonucleotides sequentially (Basu and Wickstrom, 1995; Juby et al., 1991). This approach was also used for the synthesis of peptide libraries in which the oligonucleotide component on the bead served as a tag for identifying the peptide sequence (Nielsen et al., 1993). Alternatively, nucleopeptides can be synthesized in a linear fashion on a single support in which the peptide is synthesized first, followed by coupling of a transitional linking molecule, and then automated oligonucleotide synthesis (de la Torre, 1994; Soukchareun et al., 1995; Truffert et al., 1994, 1996). Typically, the linking molecule contains a protected primary alcohol and a carboxylic acid (or activated ester) at the other terminus.

Finally, a convergent method for the synthesis of 3′-oligonucleotide-peptide conjugates was reported that takes advantage of a template-mediated transfer of a peptide from an intermediate thioester to a 3′-amine-containing oligonucleotide (Bruick et al., 1996).



34

Figure 4.5.20 Support for the linear synthesis of nucleopeptides.

Conjugation of Oligonucleotides to Surfaces Via their 3′ Termini

Usage of surface-bound oligonucleotides in applications such as sequencing by hybridization and as sensors has resulted in a variety of methods for covalently linking the biopolymers to surfaces (Drmanac et al., 1993). Methods have also been developed for in situ synthesis of oligonucleotides on surfaces (Caviani Pease et al., 1994; Chee et al., 1996; Cohen et al., 1997; Maskos and Southern, 1992; McGall et al., 1996; Southern et al., 1994); however, only methods which involve conjugation of a previously synthesized oligonucleotide to a surface will be discussed in this commentary. Furthermore, in keeping with the theme of this commentary, only those methods that involve coupling to the 3′ terminus of an oligonucleotide will be presented. As is the case for the synthesis of nucleopeptides, many of the methods for synthesizing bioconjugates catalogued above have been applied to the problem of preparing surface-bound oligonucleotides.

In one application directed towards detecting hybridization, silica surfaces are activated with terminal alkyl epoxides (Lamture et al., 1994). Oligonucleotides containing 3′-alkylamines are then conjugated to the surface via nucleophilic ring opening of the epoxide. In a related example, patterned DNA surfaces containing covalently bound oligonucleotides are also prepared via chemical modification of silica surfaces (Fig. 4.5.21; Chrisey et al., 1996a,b). The three step process for immobilizing the oligonucleotides begins with introducing an alkylamine by modifying the silica surface with the appropriate trialkoxysilane. Following derivatization of the alkylamines with a maleimide, 3′-thiolated oligonucleotides are conjugated to the surface. When combined with masks and existing photoresist technology, this chemistry is useful for preparing patterned surfaces on the micrometer scale. In another application directed towards developing sequencing by hybridization, oligonucleotides containing 3′-dialdehydes or 3′-alkylamines were coupled to acrylamide gels containing the complementary functionality via reductive amination (Timofeev et al., 1996).

Finally, an extremely sensitive method for detecting oligonucleotide hybridization was recently reported utilizing thiolated oligonucleotides (5′ and 3′) bound to gold particles (Elghanian et al., 1997; Storhoff et al., 1998). Hybridization to target oligonucleotides in solution mediates the formation of networked gold particle aggregates. Aggregates of the gold

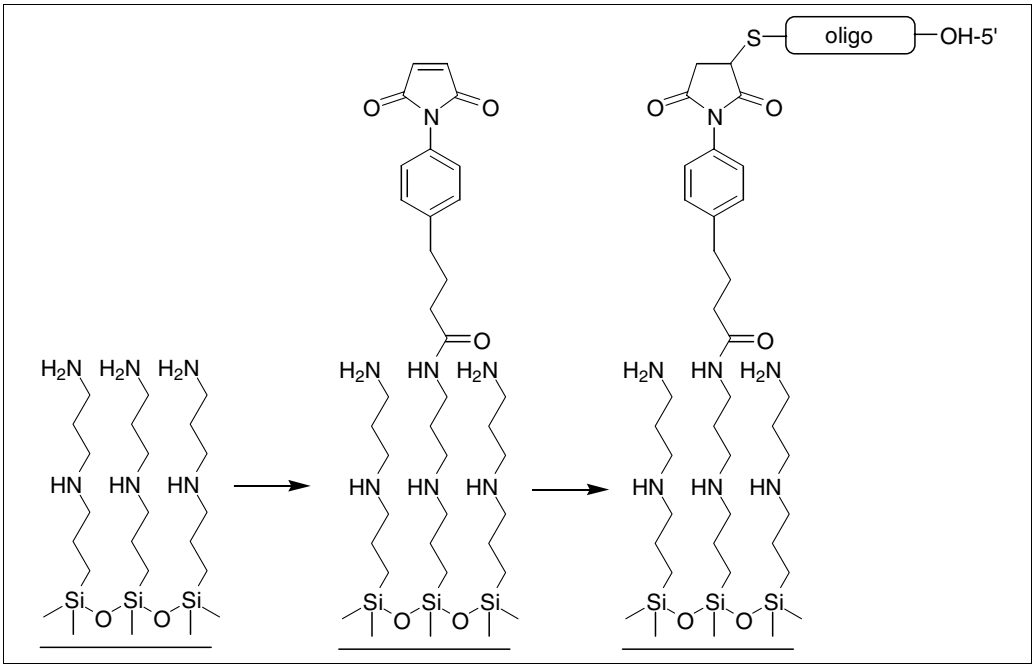


Figure 4.5.21 Modification of surfaces by conjugation of 3′-derivatized oligonucleotides.

particles result in readily measured color changes on as little as 10 femtomoles of material.

SUMMARY

Although the scope of this review was limited to the synthesis of oligonucleotide conjugates through their 3′ termini, it is clear that there has been significant activity during the past decade in the development of methods for the synthesis of oligonucleotide conjugates in general. As more oligonucleotide-based therapeutics enter the clinic, and other applications such as genome sequencing and gene function develop, the impetus for the development of oligonucleotide conjugate synthesis methods will certainly continue.

LITERATURE CITED

Agrawal, S. 1994. Functionalization of oligonucleotides with amino groups and attachment of amino specific reporter groups. *In* Protocols for Oligonucleotide Conjugates, Vol. 26: Synthesis and Analytical Techniques, pp. 93-120. Humana Press, Totowa, N.J.

Arar, K., Monsigny, M., and Mayer, R. 1993. Synthesis of oligonucleotide-peptide conjugates containing a KDEL signal sequence. *Tetrahedron Lett.* 34:8087-8090.

Asseline, U., Bonfils, E., Kurfurst, R., Chassignol, M., Roig, V., and Thuong, N. T. 1992. Solid-phase preparation of 5′,3′-heterobifunctional oligodeoxyribonucleotides using modified solid supports. *Tetrahedron* 48:1233-1254.

Avino, A., Garcia, R.G., Diaz, A., Albericio, F., and Eritja, R. 1996. A comparative study of supports for the synthesis of oligonucleotides without using ammonia. *Nucleosides Nucleotides* 15:1871-1889.

Basu, S. and Wickstrom, E. 1995. Solid phase synthesis of a D-peptide-phosphorothioate oligodeoxynucleotide conjugate from two arms of a polyethylene glycol-polystyrene support. *Tetrahedron Lett.* 36:4943-4946.

Beaucage, S.L. and Iyer, R.P. 1993. The functionalization of oligonucleotides via phosphoramidite derivatives. *Tetrahedron* 49:1925-1963.

Bellon, L., Workman, C., Scherrer, J., Usman, N., and Wincott, F. 1996. Morpholino-linked ribozymes: A convergent synthetic approach. *J. Am. Chem. Soc.* 118:3771-3772.

Bellon, L., Workman, C., Jarvis, T.C., and Wincott, F.E. 1997. Post-synthetically ligated ribozymes: An alternative approach to iterative solid-phase synthesis. *Bioconjugate Chem.* 8:204-212.

Bischoff, R., Coull, J.M., and Regnier, F.E. 1987. Introduction of 5′-terminal functional groups into synthetic oligonucleotides for selective immobilization. *Anal. Biochem.* 164:336-344.

Bonfils, E. and Thuong, N.T. 1991. Solid phase synthesis of 5′, 3′-bifunctional oligodeoxyribonucleotides bearing a masked thiol group at the 3′-end. *Tetrahedron Lett.* 32:3053-3056.

Bonfils, E., Depierreux, C., Midoux, P., Thuong, N.T., Monsigny, M., and Roche, A.C. 1992. Drug targeting: Synthesis and endocytosis of oligonucleotide-neoglycoprotein conjugates. *Nucl. Acids Res.* 20:4621-4629.

Boutorine, A.S., Brault, D., Takasugi, M., Delgado, O., and Helene, C. 1996. Chlorin-oligonucleotide conjugates: Synthesis, properties, and red light-induced photochemical sequence-specific DNA cleavage in duplexes and triplexes. *J. Am. Chem. Soc.* 118:9469-9476.

Bruick, R.K., Dawson, P.E., Kent, S.B.H., Usman, N., and Joyce, G.F. 1996. Template-directed ligation of peptides to oligonucleotides. *Chem. Biol.* 3:49-56.

Caviani Pease, A., Solas, D., Sullivan, E.J., Cronin, M.T., Holmes, C.P., and Fodor, S.P.A. 1994. Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc. Natl. Acad. Sci. U.S.A.* 91:5022-5026.

Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X.C., Stern, D., Winkler, J., Lockhart, D.J., Morris, M.S., and Fodor, S.P.A. 1996. Accessing genetic information with high-density DNA arrays. *Science* 274:610-614.

Chrisey, L.A., Lee, G.U., and O'Ferrall, C.E. 1996a. Covalent attachment of synthetic DNA to self-assembled monolayer films. *Nucl. Acids Res.* 24:3031-3039.

Chrisey, L.A., O'Ferrall, C.E., Spargo, B.J., Dulcey, C.S., and Calvert, J.M. 1996b. Fabrication of patterned DNA surfaces. *Nucl. Acids Res.* 24:3040-3047.

Cohen, G., Deutsch, J., Fineberg, J., and Levine, A. 1997. Covalent attachment of DNA oligonucleotides to glass. *Nucl. Acids Res.* 25:911-912.

de la Torre, B.G., Avino, A., Tarrason, G., Piulats, J., Albericio, F., and Eritja, R. 1994. Stepwise solid-phase synthesis of oligonucleotide-peptide hybrids. *Tetrahedron Lett.* 35:2733-2736.

Dell'Aquila, C., Imbach, J.-L., and Rayner, B. 1997. Photolabile linker for the solid-phase synthesis of base-sensitive oligonucleotides. *Tetrahedron Lett.* 38:5289-5292.

Drmanac, R., Drmanac, S., Strezoska, Z., Paunesku, T., Labat, I., Zeremski, M., Snoddy, J., Funkhouser, W.K., Koop, B., Hood, L., and Crkvenjakov, R. 1993. DNA sequence determination by hybridization: A strategy for efficient large-scale sequencing. *Science* 260:1649-1652.

Elghanian, R., Storhoff, J.J., Mucic, R.C., Letsinger, R.L., and Mirkin, C.A. 1997. Selective colorimetric detection of polynucleotides based on the distance-dependent optical properties of gold nanoparticles. *Science* 277:1078-1081.

Erout, M.-N., Troesch, A., Pichot, C., and Cros, P. 1996. Preparation of conjugates between oligonucleotides and *N*-vinylpyrrolidine/*N*-acryloxysuccinimide copolymers and applications in nucleic acids assays to improve sensitivity. *Bioconjugate Chem.* 7:568-575.

Fidanza, J.A., Ozaki, H., and McLaughlin, L.W. 1994. Functionalization of oligonucleotides by the incorporation of thio-specific reporter groups. *In* Protocols for Oligonucleotide Conjugates, Vol. 26: Synthesis and Analytical Techniques (S. Agrawal, ed.) pp. 121-143. Humana Press, Totowa, N.J.

Ghosh, S.S. and Musso, G.F. 1987. Covalent attachment of oligonucleotides to solid supports. *Nucl. Acids Res.* 15:5353-5372.

Goodchild, J. 1990. Conjugates of oligonucleotides and modified oligonucleotides: A review of their synthesis and properties. *Bioconjugate Chem.* 1:165-187.

Goodwin, J.T. and Lynn, D.G. 1992. Template-directed synthesis: Use of a reversible reaction. *J. Am. Chem. Soc.* 114:9197-9198.

Gottikh, M., Asseline, U., and Thuong, N.T. 1990. Synthesis of oligonucleotides containing a carboxyl group at either their 5′ end or their 3′ end and their subsequent derivatization by an intercalating agent. *Tetrahedron Lett.* 31:6657-6660.

Greenberg, M.M. and Gilmore, J.L. 1994. Cleavage of oligonucleotides from solid-phase supports using *o*-nitrobenzyl photochemistry. *J. Org. Chem.* 59:746-753.

Greenberg, M.M., Matray, T.J., Kahl, J.D., Dong, J.Y., and McMinn, D.L. 1998. Optimization and mechanistic analysis of oligonucleotide cleavage from palladium-labile solid-phase synthesis supports. *J. Org. Chem.* 63:4062-4068.

Gryaznov, S.M. and Letsinger, R.L. 1992. A new approach to synthesis of oligonucleotides with 3′ phosphoryl groups. *Tetrahedron Lett.* 33:4127-4128.

Gryaznov, S.M. and Letsinger, R.L. 1993a. Template controlled coupling and recombination of oligonucleotide blocks containing thiophosphoryl groups. *Nucl. Acids Res.* 21:1403-1408.

Gryaznov, S.M. and Letsinger, R.L. 1993b. Chemical ligation of oligonucleotides in the presence and absence of a template. *J. Am. Chem. Soc.* 115:3808-3809.

Gryaznov, S.M., Schultz, R., Chaturvedi, S.K., and Letsinger, R.L. 1994. Enhancement of selectivity in recognition of nucleic acids via chemical autoligation. *Nucl. Acids Res.* 22:2366-2369.

Gupta, K.C., Sharma, P., Sathyanarayana, S., and Kumar, P. 1990. A universal solid support for the synthesis of 3′-thiol group containing oligonucleotides. *Tetrahedron Lett.* 31:2471-2474.

Gupta, K.C., Sharma, P., Kumar, P., and Sathyanarayana, S. 1991. A general method for the synthesis of 3′-sulfhydryl and phosphate group containing oligonucleotides. *Nucl. Acid Res.* 19:3019-3025.

Guzaev, A. and Lönnberg, H. 1997. A novel solid support for synthesis 3′-phosphorylated chimeric oligonucleotides containing internucleosidic methyl phosphotriester and methylphosphonate linkages. *Tetrahedron Lett.* 38:3989-3992.

Haralambidis, J., Duncan, L., Angus, K., and Tregear, G.W. 1990a. The synthesis of polyamide--oligonucleotide conjugate molecules. *Nucl. Acids Res.* 18:493-499.

Haralambidis, J., Angus, K., Pownall, S., Duncan, L., Chai, M., and Tregear, G.W. 1990b. The preparation of polyamide-oligonucleotide probes containing multiple non-radioactive labels. *Nucl. Acids Res.* 18:501-505.

Haralambidis, J., Lagniton, L., and Tregear, G.W. 1994. The preparation of enzyme-labelled oligonucleotides by reductive amination. *Bioorg. Med. Chem. Lett.* 4:1005-1010.

Harrison, J.G. and Balasubramanian, S. 1997. A convenient synthetic route to oligonucleotide conjugates. *Bioorg. Med. Chem. Lett.* 7:1041-1046.

Hovinen, J., Gouzaev, A.P., Azhayev, A.V., and Lönnberg, H. 1993a. A new method to prepare 3′-modified oligonucleotides. *Tetrahedron Lett.* 34:5163-5166.

Hovinen, J., Guzaev, A., Azhayev, A., and Lönnberg, H. 1993b. Synthesis of 3′-functionalized oligonucleotides on a single solid support. *Tetrahedron Lett.* 34:8169-8172.

Hovinen, J., Guzaev, A., Azhayev, A., and Lönnberg, H. 1994. Novel solid supports for the preparation of 3′-derivatized oligonucleotides: introduction of 3′-alkylphosphate tether groups bearing amino, carboxy, carboxamido, and mercapto functionalities. *Tetrahedron* 50:7203-7218.

Hovinen, J., Guzaev, A., Azhayeva, E., Azhayev, A., and Lönnberg, H. 1995. Imidazole tethered oligodeoxyribonucleotides: Synthesis and RNA cleaving activity. *J. Org. Chem.* 60:2205-2209.

Jaschke, A., Furste, J.P., Dieter, C., and Volker, A.E. 1993. Automated incorporation of polythylene glycol into synthetic oligonucleotides. *Tetrahedron Lett.* 34:301-304.

Juby, C.D., Richardson, C.D., and Brousseau, R. 1991. Facile preparation of 3′ oligonucleotide-peptide conjugates. *Tetrahedron Lett.* 32:879-882.

Kahl, J.D. and Greenberg, M.M. 1999. Solution phase bioconjugate synthesis using protected oligonucleotides containing 3′-alkyl carboxylic acids. *J. Org. Chem.* 64:507-510.

Kahl, J.D., McMinn, D.L., and Greenberg, M.M. 1998. High-yielding method for on-column derivatization of protected oligodeoxynucleotides and its application to the convergent synthesis of 5′,3′-bis-conjugates. *J. Org. Chem.* 63:4870-4871.

Kumar, A. 1993a. A rapid solid phase method for the synthesis of 3′-thiol group containing oligonucleotides. *Nucleosides Nucleotides* 12:729-736.

Kumar, A. 1993b. A versatile solid phase method for the synthesis of masked 3′-thiol group containing oligonucleotides. *Nucleosides Nucleotides* 12:1047-1059.

Lamture, J.B., Beattie, K.L., Burke, B.E., Eggers, M.D., Ehrilch, D.J., Fowler, R., Hollis, M.A., Kosicki, B.B., Reich, R.K., Smith, S.R., Varma, R.S., and Hogan, M.E. 1994. Direct detection of nucleic acid hybridization on the surface of a charge coupled device. *Nucl. Acids Res.* 22:2121-2125.

Leonetti, J.P., Rayner, B., Lemaitre, M., Gagnor, C., Milhaud, P.G., Imbach, J.-L., and Lebleu, B. 1988. Antiviral activity of conjugates between poly(L-lysine) and synthetic oligodeoxyribonucleotides. *Gene* 72:323-332.

Leonetti, J.-P., Degols, G., and Lebleu, B. 1990. Biological activity of oligonucleotide-poly(L-lysine) conjugates: Mechanism of cell uptake. *Bioconjugate Chem.* 1:149-153.

Letsinger, R.L., Zhang, G., Sun, D.K., Ikeuchi, T., and Sarin, P.S. 1989. Cholesteryl-conjugated oligonucleotides: synthesis, properties, and activity as inhibitors of replication of human immunodeficieny virus in cell culture. *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556.

Lukhtanov, E.A., Podyminogin, M.A., Kutyavin, I.V., Meyer, R.B. Jr., and Gamper, H.B. 1996. Rapid and efficient hybridization-triggered crosslinking within a DNA duplex by an oligodeoxyribonucleotide bearing a conjugated cyclopropapyrroloindole. *Nucl. Acids Res.* 24:683-687.

Lund, V., Schmid, R., Rickwood, D., and Hornes, E. 1988. Assessment of methods for covalent binding of nucleic acids to magnetic beads, Dynabeads, and the characteristics of the bound nucleic acids in hybridization reactions. *Nucl. Acids Res.* 16:10861-10880.

Lyttle, M.H., Adams, H., Hudson, D., and Cook, R.M. 1997. Versatile linker chemistry for synthesis of 3′-modified DNA. *Bioconjugate Chem.* 8:193-198.

MacKellar, C., Graham, D., Will, D.W., Burgess, S., and Brown, T. 1992. Synthesis and physical properties of anti-HIV antisense oligonucleotides bearing terminal lipophilic groups. *Nucl. Acids Res.* 20:3411-3417.

Maskos, U. and Southern, E.M. 1992. Parallel analysis of oligodeoxyribonucleotide (oligonucleotide) interactions. I. Analysis of factors influencing oligonucleotide duplex formation. *Nucl. Acids Res.* 20:1675-1678.

Matray, T.J., Dong, J.Y., McMinn, D.L., and Greenberg, M.M. 1997. Synthesis of oligonucleotides containing 3′-alkylcarboxylic acids using a palladium labile oligonucleotide solid phase synthesis support. *Bioconjugate Chem.* 8:99-102.

McGall, G., Labadie, J., Brock, P., Wallraff, G., Nguyen, T., and Hinsberg, W. 1996. Light-directed synthesis of high-density oligonucleotide arrays using semiconductor photoresists. *Proc. Natl. Acad. Sci. U.S.A.* 93:13555-13560.

McMinn, D.L. and Greenberg, M. M. 1996. Novel solid phase synthesis supports for the preparation of oligonucleotides containing 3′-alkyl amines. *Tetrahedron* 52:3827-3840.

McMinn, D.L. and Greenberg, M.M. 1997. Synthesis of oligonucleotides containing 3′-alkyl amines using *N*-isobutyryl protected deoxyadenosine phosphoramidite. *Tetrahedron Lett.* 38:3123-3126.

McMinn, D.L. and Greenberg, M. M. 1998. Post-synthetic conjugation of protected oligonucleotides containing 3'-alkylamines. *J. Am. Chem. Soc.* 120:3289-3294.

McMinn, D.L. and Greenberg, M.M. 1999. Convergent solution-phase synthesis of a nucleopeptide using a protected oligonucleotide. *Bioorg. Med. Chem. Lett*. 9:547-550.

McMinn, D.L., Matray, T.J., and Greenberg, M.M. 1997. Efficient solution phase synthesis of oligonucleotide conjugates using protected biopolymers containing 3′-terminal alkyl amines. *J. Org. Chem.* 62:7074-7075.

McMinn, D.L., Hirsch, R., and Greenberg, M. M. 1998. An orthogonal solid phase support for the synthesis of oligonucleotides containing 3′-phosphates and its application in the preparation of photolabile hybridization probes. *Tetrahedron Lett.* 39:4155-4158.

Mukaiyama, T. 1976. Oxidation-reduction condensation. *Angew. Chem. Int. Ed. Engl.* 15:94-103.

Mullah, B. and Andrus, A. 1997. Automated synthesis of double dye-labeled oligonucleotides using tetramethylrhodamine (TAMRA) solid supports. *Tetrahedron Lett.* 38:5751-5754.

Mullah, B., Livak, K., Andrus, A., and Kenney, P. 1998. Efficient synthesis of double dye-labeled oligodeoxyribonucleotide probes and their application in a real time PCR assay. *Nucl. Acids Res.* 26:1026-1031.

Nelson, P., Frye, R.A., and Liu, E. 1989. Bifunctional oligonucleotide probes synthesized using a novel CPG support are able to detect single base pair mutations. *Nucl. Acids Res.* 17:7187-7194.

Nelson, P.S., Kent, M., and Muthini, S. 1992. Oligonucleotide labeling methods. 3. Direct labeling of oligonucleotides employing a novel, nonnucleosidic, 2-aminobutyl-1,3-propanediol backbone. *Nucl. Acids Res.* 20:6253-6259.

Nelson, P., Muthini, S., Kent, M.A., and Smith, T.H. 1997. 3′-Terminal modification of oligonucleotides using a universal solid support. *Nucleosides Nucleotides* 16:1951-1959.

Nielsen, J., Brenner, S., and Janda, K.D. 1993. Synthetic methods for the implementation of encoded combinatorial chemistry. *J. Am. Chem. Soc.* 115:9812-9813.

Scheuer-Larsen, C., Rosenbohm, C., Jorgensen, T.J.D, and Wengel, J. 1997. Introduction of a universal solid support for oligonucleotide synthesis. *Nucleosides Nucleotides* 16:67-80.

Schwartz, M.E., Breaker, R.R., Asteriadis, G.T., and Gough, G.R. 1995. A universal adapter for chemical synthesis of DNA or RNA on any single type of solid support. *Tetrahedron Lett.* 36:27-30.

Soukchareun, S., Tregear, G.W., and Haralambidis, J. 1995. Preparation and characterization of antisense oligonucleotide-peptide hybrids containing viral fusion peptides. *Bioconjugate Chem.* 6:43-55.

Soukchareun, S., Haralambidis, J., and Tregear, G. 1998. Use of *N*α-Fmoc-cysteine(*S*-thiobutyl) derivatized oligodeoxynucleotides for the preparation of oligodeoxynucleotide-peptide hybrid molecules. *Bioconjugate Chem.* 9:466-475.

Southern, E.M., Case-Green, S.C., Elder, J.K., Johnson, M., Mir, K.U., Wang, L., and Williams, J.C. 1994. Arrays of complementary oligonucleotides for analysing the hybridisation behaviour of nucleic acids. *Nucl. Acids Res.* 22:1368-1373.

Storhoff, J.J., Elghanian, R., Mucic, R.C., Mirkin, C.A., and Letsinger, R.L. 1998. One-pot colorimetric differentiation of polynucleotides with single base imperfections using gold nanoparticle probes. *J. Am. Chem. Soc.* 120:1959-1964.

Thaden, J. and Miller, P.S. 1993a. Automated synthesis of oligodeoxyribonucleoside methylphosphonates having [*N*-(3-aminoprop-1-yl)-*N*-(2-hydroxyethyl)-2-aminoethyl] phosphate or methylphosphonic acid at the 3′ end using a modified controlled pore glass support. *Bioconjugate Chem.* 4:395-401.

Thaden, J. and Miller, P.S. 1993b. Photoaffinity behavior of a conjugate of oligonucleoside methylphosphonate, rhodamine, and psoralen in the presence of complementary oligonucleotides. *Bioconjugate Chem.* 4:386-394.

Timofeev, E.N., Kochetkova, S.V., Mirzabekov, A.D., and Florentiev, V.L. 1996. Regioselective immobilization of short oligonucleotides to acrylic copolymer gels. *Nucl. Acids Res.* 24:3142-3148.

Truffert, J.-C., Lorthioir, O., Asseline, U., Thuong, N.T., and Brack, A. 1994. On-line solid phase synthesis of oligonucleotide-peptide hybrids using silica supports. *Tetrahedron Lett.* 35:2353-2356.

Truffert, J.-C., Asseline, U., Brack, A., and Thuong, N.T. 1996. Synthesis, purification and characterization of two peptide-oligonucleotide conjugates as potential artificial nucleases. *Tetrahedron* 52:3005-3016.

Tung, C.-H., Wang, J., Leibowtiz, M.J., and Stein, S. 1995. Dual-specificity interaction of HIV-1 TAR RNA with tat peptide-oligonucleotide conjugates. *Bioconjugate Chem.* 6:292-295.

Urata, H. and Akagi, M. 1993. A convenient synthesis of oligonucleotides with a 3′-phosphoglycolate and 3′-phosphoglycaldehyde terminus. *Tetrahedron Lett.* 34:4015-4018.

Venkatesan, H. and Greenberg, M. M. 1996. Improved utility of photolabile solid phase synthesis supports for the synthesis of oligonucleotides containing 3′-hydroxyl termini. *J. Org. Chem.* 61:525-529.

Yoo, D.J. and Greenberg, M. M. 1995. Synthesis of oligonucleotides containing 3′-alkyl carboxylic acids using universal, photolabile solid phase synthesis supports. *J. Org. Chem.* 60:3358-3364.

Zhan, Z.J. and Lynn, D.G. 1997. Chemical amplification through template-directed synthesis. *J. Am. Chem. Soc.* 119:12420-12421.

Zhang, X., Gaffney, B.L., and Jones, R.A. 1997. RNA synthesis using a universal, base-stable alkyl linker. *Nucl. Acids Res.* 25:3980-3983.

Zon, G. and Geiser, T.G. 1991. Phosphorothioate oligonucleotides: Chemistry, purification, analysis, scale-up and future directions. *Anti-Cancer Drug Des.* 6:539-568.

Zuckerman, R., Corey, D., and Schultz, P. 1987. Efficient methods for attachment of thiol specific probes to the 3′-ends of synthetic oligodeoxyribonucleotides. *Nucl. Acids Res.* 15:5305-5321.

Zuckerman, R.N. and Schultz, P.G. 1988. A hybrid sequence-selective ribonuclease S. *J. Am. Chem. Soc.* 110:6592-6594.

Zuckerman, R.N., Corey, D.R., and Schultz, P.G. 1988. Site-selective cleavage of RNA by a hybrid enzyme. *J. Am. Chem. Soc.* 110:1614-1615.

Contributed by Marc M. Greenberg
Colorado State University
Fort Collins, Colorado

Financial support of this work from the National Science Foundation (CHE-9732843), the CATI Center for RNA Chemistry, Dupont, and the Alfred P. Sloan Foundation is greatly appreciated.