

MODIFIED OLIGONUCLEOTIDES: Synthesis and Strategy for Users

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ABSTRACT

Synthetic oligonucleotide analogs have greatly aided our understanding of several biochemical processes. Efficient solid-phase and enzyme-assisted synthetic methods and the availability of modified base analogs have added to the utility of such oligonucleotides. In this review, we discuss the applications of synthetic oligonucleotides that contain backbone, base, and sugar modifications to investigate the mechanism and stereochemical aspects of biochemical reactions. We also discuss interference mapping of nucleic acid–protein interactions; spectroscopic analysis of biochemical reactions and nucleic acid structures; and nucleic acid cross-linking studies.

The automation of oligonucleotide synthesis, the development of versatile phosphoramidite reagents, and efficient scale-up have expanded the application of modified oligonucleotides to diverse areas of fundamental and applied biological research. Numerous reports have covered oligonucleotides for which modifications have been made of the phosphodiester backbone, of the purine and pyrimidine heterocyclic bases, and of the sugar moiety; these modifications serve as structural and mechanistic probes. In this chapter, we review the range, scope, and practical utility of such chemically modified oligonucleotides. Because of space limitations, we discuss only those oligonucleotides that contain phosphate and phosphate analogs as internucleotidic linkages.

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SYNTHETIC METHODS

Solid-Phase Chemical Synthesis

The ease of customizing reaction cycles in automated, solid-phase DNA synthesizers has allowed for efficient and site-specific introduction of chemical modifications in oligodeoxynucleotides. In the past decade, significant progress has also been made toward developing novel synthetic and deprotection strategies for oligoribonucleotide synthesis. In this section we outline the solid-phase synthetic process. Details of other synthetic methods are discussed in subsequent sections.

For the synthesis of oligodeoxynucleotide, automated solid-phase synthesizers make use of 5'-protected deoxynucleosides anchored to a controlled pore glass support at the 3'-end via a chemically cleavable linker. A series of programmed reactions sequentially couple deoxynucleoside phosphoramidites to the support-bound nucleoside. This approach circumvents the need to purify synthetic intermediates at each step of the reaction cycle, thereby saving enormous amounts of time. Finally, base hydrolysis is used to cleave the linker from the anchored nucleoside to yield a fully deprotected oligomer (1, 2). A detailed description of the synthetic process and related chemistry is beyond the scope of this chapter and is described elsewhere (3). Similarly, oligoribonucleotide synthesis is also performed on solid supports through the use of appropriately protected phosphoramidites. Continued development of 2'-OH protecting groups, the use of efficient activators, and purification methods have greatly improved RNA synthesis (4, 5). Despite these advances, RNA synthesis

still remains less efficient, necessitating further improvement in the synthetic methodology (6).

Commercial availability of phosphoramidites of suitably protected natural and nonnatural nucleosides, such as 2'-substituted-2'-deoxy- and 2'-*O*-methyl nucleosides, terminal nonradioactive reporter groups, and functionalized alkyl tethers for postsynthetic modifications, have greatly added to the convenience of solid-phase synthesis. The phosphoramidite approach, developed for the synthesis of oligonucleotides containing a phosphodiester backbone, can also be used to synthesize phosphorothioates by replacing the oxidation step with sulfurization (7). The solid-phase synthesis of other modified backbones, such as phosphorodithioates (8) or methylphosphonates (9), however, requires special nucleoside amidites.

The key features of automated solid-phase oligonucleotide synthesis include ease of operation, efficiency, and reproducibility. Despite these advantages, this method is difficult to use for the synthesis of long oligonucleotides (100 or more nucleotides) in high yields. In such cases, it is more convenient to use the enzymatic ligation approach, in tandem with chemical synthesis, to give an oligomer of desired length.

Enzymatic Ligation

The enzymatic approach to ligate short oligonucleotides offers an attractive route for the synthesis of long deoxynucleic and ribonucleic acids. These reactions are catalyzed by DNA or RNA ligases that promote intermolecular ligation of the 5' and 3' termini of oligonucleotides through the formation of a phosphodiester bond.

In the case of DNA ligase, two oligodeoxynucleotides, one bearing a 5'-phosphoryl donor group and another with a free 3'-hydroxyl acceptor, constitute the substrate requirement. It is essential that the phosphorylated and the hydroxyl termini-bearing oligonucleotides be present either on homo-(DNA:DNA) or hetero-duplexes (DNA:RNA) (10, 11). Oligonucleotides bearing terminal fluorescent and chemiluminescent labels can also act as substrates for DNA ligase. Such modified oligomers have been successfully employed to study cystic fibrosis gene mutations in hybridization-ligation assays (12, 13).

T4 RNA ligase catalyzes the formation of a phosphodiester bond between a 5'-phosphoryl donor and a free 3'-hydroxyl acceptor group of two single-stranded oligoribonucleotides. It accepts a variety of oligonucleotide substrates for ligation. With the use of this enzyme, nucleoside analogs can be site-specifically introduced in oligoribonucleotides at the ligation junction (10). The use of this enzyme makes possible the introduction of modifications in oligoribonucleotides that are longer than those accessible by chemical synthesis alone. This methodology has been used to introduce nonstandard nucleosides in

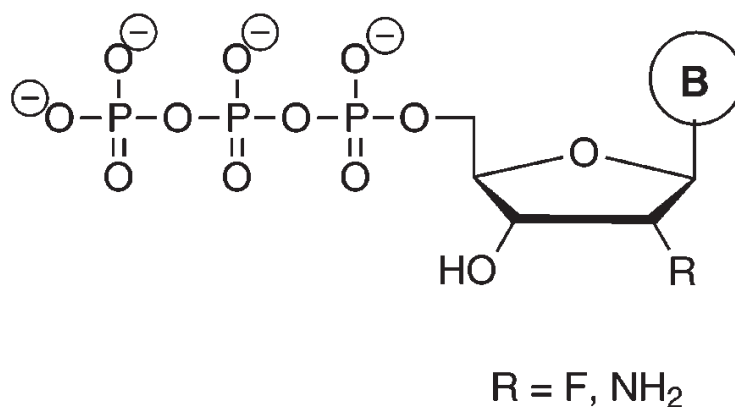
oligoribonucleotides (14, 15), for fluorescent labeling of tRNA and oligodeoxynucleotides (16), for DNA strand ligation within a duplex (17), and for the synthesis of mRNA and tRNA segments (18, 19). In general, the use of chemical synthesis in conjunction with enzymatic ligation provides a more efficient route to the synthesis of long oligoribonucleotides compared to the use of solid-phase chemical synthesis alone.

Two novel approaches for the ligation of oligoribonucleotides have been described. In the first method, two oligoribonucleotide fragments are annealed to a complementary oligodeoxynucleotide (cDNA) bridge and then ligated through use of T4 DNA ligase (20). With this ligation strategy, modified RNA substrates are synthesized, allowing study of the importance of the 2'-hydroxyl group in nuclear pre-mRNA splicing. It was demonstrated that T4 DNA ligase efficiently incorporated 2'-deoxy or 2'-*O*-methyl nucleotides immediately adjacent to the splice site of pre-mRNA substrates, with a high degree of acceptor specificity. In another method, two oligoribonucleotide fragments are annealed to a partially complementary oligodeoxynucleotide splint and then ligated through use of T4 RNA ligase (21). The DNA splint used in the latter method was designed to lack base-pairing nucleotides in the vicinity of the ligation site, thus providing single-stranded 5'-phosphoryl and 3'-hydroxyl termini for enzymatic ligation.

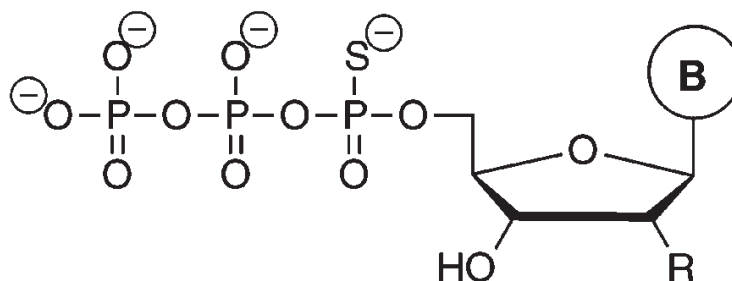
Enzymatic Incorporation of Nonnatural Nucleotides

The enzymatic synthesis of oligonucleotides containing modified bases, sugars, or phosphate groups is a viable alternative to chemical synthesis. Enzymatic incorporation of modified nucleoside triphosphates, with T7 or a similar phage RNA polymerase, is particularly well suited for template-directed transcription reactions, provided that they are good substrates for the polymerase. This requirement limits the number of modified nucleotides that can be used in transcription reactions; thus only a few modifications can be enzymatically introduced into oligoribonucleotides. For oligodeoxynucleotides, these modifications must be made by chain extension of a primer annealed to a template, through use of a DNA polymerase such as the Klenow fragment of *Escherichia coli* DNA polymerase I.

Among internucleotidic linkages, phosphorothioate is the most common enzymatically introduced modification, as all of the four nucleoside α -thiotriphosphates are good substrates for DNA and RNA polymerases (Figure 1; 22). In phosphorothioates, substitution of one of the nonbridging oxygen atoms with sulfur introduces chirality at the phosphorus center. Only the *Sp*-diastereomer of nucleoside α -thiotriphosphates is a good substrate for the polymerase. The enzymatic incorporation proceeds with inversion of configuration at phosphorus, resulting in the formation of the *Rp* internucleotidic linkage. Therefore,



2'-Modified nucleoside 5'-triphosphates



Nucleoside 5'- α -thiotriphosphates

Figure 1 Modified nucleotides for transcription.

enzymatic synthesis is limited to the incorporation of Rp-phosphorothioate linkage (22). This methodology has been applied to the synthesis of all Rp-phosphorothioate transcripts (23) and oligodeoxynucleotides through use of either a modified T7 DNA polymerase (24) or Taq DNA polymerase (25). The applications of phosphorothioate modification are discussed later in this chapter.

2'-Deoxynucleoside triphosphates were the first sugar-modified analogs to be examined as substrates for T7 RNA polymerase (26). However, in their presence, transcription was aborted 30% of the time. Recently, the use of Mn^{2+} instead of Mg^{2+} , in carefully controlled ratios with respect to the ribonucleotide triphosphates, was shown to permit more efficient incorporation of these analogs

(27). This report also describes the incorporation of 2'-*O*-methyl nucleotides, which are not incorporated under normal transcription conditions. Efficient incorporation of deoxynucleoside triphosphates can also be achieved by a mutant T7 RNA polymerase, without the requirement for special transcription conditions (28). 2'-Amino-2'-deoxynucleoside triphosphates are good substrates for the wild-type enzyme. As an example, synthesis of luciferase transcripts 2500 nt in length has been achieved (Figure 1; 29). However, corresponding 2'-fluoro-2'-deoxy analogs are less readily incorporated (29). The mutant T7 RNA polymerase improves the incorporation of the 2'-fluoro derivatives (30).

T7 RNA polymerase normally initiates transcription with a guanosine triphosphate, but it can also accept other guanosine derivatives such as guanosine 5'-monophosphorothioate (31), guanosine 5'- β -thiotriphosphate (32), or guanosine 5'- γ -thiotriphosphate (33–35) for initiation. Applications of terminal phosphorothioates are discussed in later sections.

Postsynthetic Modification of Oligonucleotides

Conjugation of oligonucleotides to other biological macromolecules, to nonradioactive reporter groups, to spin labels, and to cross-linking agents has necessitated the development of postsynthetic modification procedures. The chemical strategies for oligonucleotide conjugation have been reviewed (36).

The modification of either the 3'- or the 5'-terminus is a convenient method for equipping an oligonucleotide with a reactive aminoalkyl or a mercaptoalkyl group. Introduction of these functionalities in an oligomer can be achieved either through use of appropriate commercial phosphoramidite reagents or by solution-phase chemistry. In the case of solution-phase modification, a diamino compound such as ethylenediamine or cystamine can be reacted with the 5'-phosphate group in the presence of a water-soluble carbodiimide. This reaction results in the formation of an oligonucleotide containing a reactive functional group at the 5'-terminal. The introduction of a 5'-terminal phosphorothioate group that can be used as a nucleophile is easily achieved through use of ATP γ S and polynucleotide kinase (33). Alternatively, a 3'-terminal phosphorothioate can be introduced through use of 2'-deoxycytidine-5'-phosphate 3'-phosphorothioate (pdCpS) and RNA ligase (16).

Such oligonucleotide conjugates have been used extensively for a number of applications, which include cellular delivery of antisense oligonucleotides, synthesis of artificial nucleases, and hybridization probes for biological detection. Examples include conjugation of oligonucleotides to viral fusogenic peptides (37, 38), staphylococcal nuclease and other enzymes (39, 40), neoglycopeptides (41), phospholipids (42), thiolated polysaccharides (43), vitamin E (44), lipophilic groups such as cholesterol (45–47), and nonradioactive detection labels (48–50).

INTERNUCLEOTIDE LINKAGE MODIFICATION

Phosphorothioates

The exchange of one nonbridging oxygen atom with a sulfur atom in the phosphorothioate modification is a conservative change, as the negative charge of the phosphate group is retained and the size of the sulfur atom is only slightly larger than the oxygen atom. However, this exchange makes the phosphorus center chiral; therefore, two diastereomers of the phosphorothioate internucleotide linkage exist (Figure 2). The chirality of the phosphorus center permits the determination of stereospecificity and the stereochemical course of reactions occurring at phosphorus. In addition, sulfur is a “soft” atom that coordinates preferentially with “soft” metal ions, whereas oxygen is a “hard” atom that coordinates to “hard” metal ions. Therefore, use of phosphorothioates also permits the identification of metal-ion binding sites in the biological systems. The phosphorothioate internucleotidic linkage is considerably more stable than the phosphodiester bond toward nucleases, and this feature makes it useful for cell culture and in vivo studies. The literature on nucleoside phosphorothioates up to 1984 has been reviewed by Eckstein (22).

Oligonucleotide phosphorothioates can be synthesized either by chemical synthesis, with use of a sulfurizing reagent in the oxidation step, or by enzymatic incorporation. Chemical synthesis produces a mixture of diastereomers, which in some cases can be separated, while the enzymatic incorporation invariably results in the formation of the Rp-diastereomeric internucleotidic linkage (7, 22, 23, 51).

ANALYSIS OF THE STEREOCHEMICAL COURSE OF ENZYMATIC REACTIONS Site-specific incorporation of phosphorothioates has been used to study the stereochemical course of many nucleotidyl-transfer reactions (22). These studies have been extended to investigate reaction mechanisms involving restriction enzymes such as *EcoRI* (52) and *EcoRV* (53), DNA integration (54), and site-specific DNA recombination (55). All of these reactions proceed with an inversion of configuration as a result of a single nucleophilic substitution.

The chirality of the P-S bond in phosphorothioates can be used to identify oxygen atoms responsible for or involved in the interaction of specific phosphate groups with proteins. Such studies have been conducted extensively by the incorporation of Rp and Sp linkages at several positions in the recognition sequences for *EcoRI* (56) and *EcoRV* (57) restriction endonucleases. The latter study also identified several phosphate groups, besides those at the cleavage site, as important for catalysis.

The stereochemical course of ribozyme-catalyzed reactions such as RNA cleavage by the group I intron (58, 59), pre-mRNA splicing (60, 61), and group II

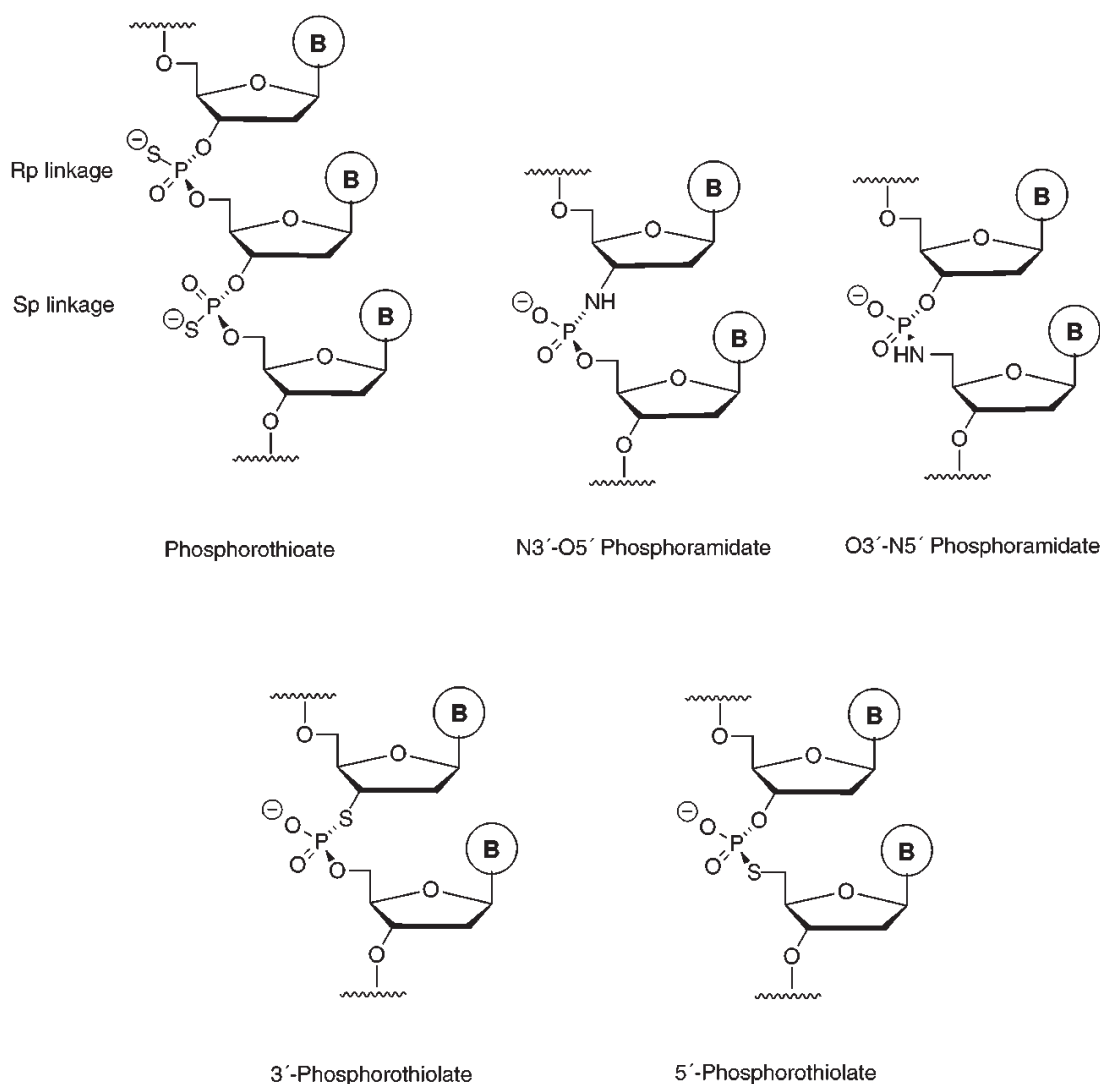


Figure 2 Modified oligonucleotide internucleotidic linkages.

intron self-splicing (62, 63) have been studied with the use of phosphorothioates as mechanistic probes. All of these reactions proceed with an inversion of configuration of phosphorus present at the cleavage site. A comparison of the stereochemical requirements for the phosphorothioates in the individual steps allows for the establishment of similarities for different splicing systems. This analysis emphasizes a close relationship between pre-mRNA splicing and the group II splicing reaction (63, 64). Inversion of configuration at the phosphorus center has also been reported for the hammerhead ribozyme-mediated catalysis (65, 66), indicating an in-line mechanism that is not obvious from the X-ray structures (67, 68). In another study, phosphorothioate substitution in one of the two strands involved in bacteriophage lambda recombination showed a strong bias in the choice of strands to initiate crossing-over (69).

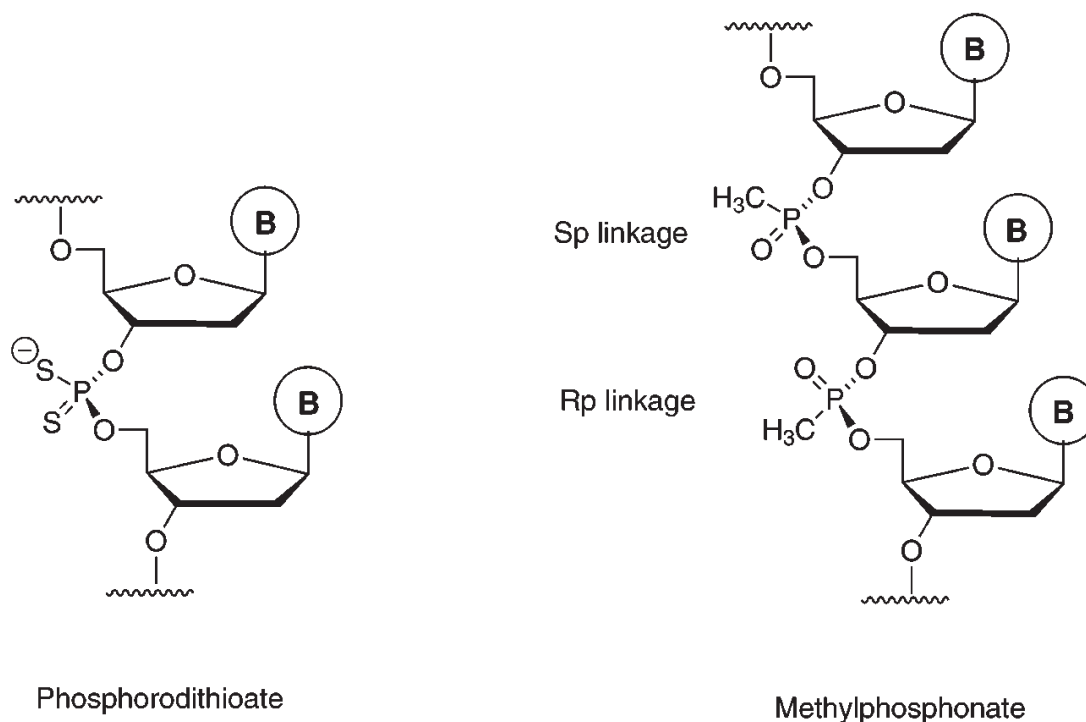


Figure 2 (Continued)

A thorough and critical discussion of how to treat rate differences observed between phosphate and phosphorothioate substrates in enzyme-catalyzed reactions has been outlined (70). From a purely chemical viewpoint, reactivity of the phosphorothioate group should be only marginally lower than that of the phosphate diester. One study involving mechanistic investigation of the polymerase activity of Klenow DNA polymerase (71) and another study investigating the interaction of GTP γ S with transducin- α (72) have pinpointed the source of the rate difference. It was suggested that steric clashes in the transition state because of the larger van der Waals radius of sulfur, rather than the electronegativity difference, result in slowing the reaction rate for the phosphorothioates.

PHOSPHOROTHIOATE INTERFERENCE Phosphorothioate interference studies have been widely employed to identify the role of specific phosphate groups in biochemical reactions. These studies exploit the change in binding specificity when sulfur is substituted for oxygen. Thus, any observed interference by phosphorothioates should identify specific phosphate groups important for recognition. For example, binding of a metal ion to a specific phosphate group might be altered if it is replaced by a phosphorothioate, making it easier to identify the exact position of the phosphate group involved in metal ion binding. Although the reasons for phosphorothioate interference may not always

be readily understood, it is still possible to assess the mechanistic contribution of specific phosphate groups.

For interference studies with oligoribonucleotides, phosphorothioates are generally incorporated randomly by transcription. The enzymatic incorporation results in the formation of an Rp phosphorothioate linkage, and from an observed interference, the mechanistic role of the pro-Rp oxygen atom of the replaced phosphate group can be identified. The success of this method depends on the actively interfering species being separable from the inactive ones, so that the precise positions of phosphorothioate incorporation can be determined. The latter can be accomplished by iodine footprinting (73, 74). A comparison of the cleavage patterns allows one to identify positions of interference with the reaction or interaction being studied.

A study using phosphorothioate interference has identified 44 phosphate groups important for Mg^{2+} ion binding in the group I intron reaction (75, 76). However, for most of these positions the cleavage reaction could be restored or rescued with Mn^{2+} ions. This metal ion coordinates to both oxygen and sulfur atoms, whereas Mg^{2+} ion coordinates only to oxygen atoms (77, 78). Thus, the restoration of catalytic activity indicates that the pro-R oxygen atoms are coordinated to the metal ion in the splicing reaction.

The phosphorothioate interference analysis and footprinting with iodine can also be used as a probe for the identification of phosphate groups involved in RNA-protein interaction. This methodology was first applied to study the affinity of an oligoribonucleotide to the R17 coat protein (79). This method was extended to probe the interaction of *E. coli* tRNA^{Ser} with its cognate aminoacyl synthetase. The results suggested that the contact with the tRNA variable loop might be located on the characteristic long extended arm of the synthetase (75). With one exception, all of these contacts were later confirmed by the X-ray crystal structure analysis of the related *Thermus thermophilus* complex (80). Results with the yeast tRNA^{Asp} system were also consistent with the interaction of the identity elements of the tRNA with the synthetase protein (81). Phosphorothioate footprinting has also been used to investigate the interaction between tRNA^{Phe} transcripts from *T. thermophilus* and its synthetase. In this case, iodine footprinting revealed a complex and novel pattern of tRNA^{Phe}-synthetase interaction (82).

The phosphorothioate protection strategy has also been used to study mitochondrial group I intron RNA splicing, which involves interaction with tyrosyl-tRNA synthetase (83). These results have aided the construction of a model that suggests that the recognition of the synthetase occurs by a tRNA-like structure in the group I intron (84). Similarly, tRNA interaction with the ribosome A and P sites was also investigated to map the essential "identity elements" involved in ribosome-tRNA recognition (85). Four phosphate groups in the RNase P RNA

were identified as important for an intramolecular pre-tRNA cleavage reaction (86). Besides the 4 positions identified in this study, 12 additional phosphate positions were found to be important for the binding of the pre-tRNA (87). Phosphorothioate substitution at the cleavage site of the pre-tRNA resulted in a 1000-fold reduction of the cleavage rate for both diastereomers, but rate inhibition of the Rp diastereomer could be largely restored by Cd^{2+} , a soft metal ion (88). In yet another study, phosphorothioate substitutions of the *pro*-Rp oxygens in a pre-tRNA transcript revealed that direct coordination of Mg^{2+} ion to the *pro*-Rp oxygen of the scissile bond is required for RNase P catalysis (89).

Recently, a “nucleotide analog interference mapping” approach has been applied to identify the functional groups essential for group I intron activity (90). Modified group I intron constructs were prepared through the substitution of inosine α -thiotriphosphate for GTP in the transcription reactions. Iodine-mediated phosphorothioate cleavage (73) was used to identify the positions of essential guanosine residues. This assay successfully mapped almost every exocyclic amino group of guanosine residues essential for the catalytic activity of the group I intron ribozyme.

Rp-phosphorothioate interference has also identified three phosphate groups, besides the group at the cleavage site, as important for catalysis by the hammerhead ribozyme (91). The importance of one of these sites, where a metal ion is coordinated to the *pro*-R oxygen of the A9 phosphate, has been confirmed by X-ray structural analyses (67,68). Knöll and coworkers have also used synthetic hammerhead ribozymes, containing a mixture of phosphorothioate diastereomers, for mechanistic investigations (92). They identified interference by the Sp isomer at A6 and U16.1. A metal-ion rescue could be observed with the Rp isomer at A9, which complements the observation of metal ion coordination to this site as determined by the X-ray structure. In a more recent X-ray structure, a Mg^{2+} ion has been found coordinated to the *pro*-R oxygen of the phosphate group at the cleavage site (93). These results are consistent with the cleavage of the Rp phosphorothioate in the presence of Mn^{2+} ion and that of the Sp-diastereomer in the presence of Mg^{2+} ion (66,94). The phosphate groups required for the self-cleavage reaction by the extended hammerhead ribozyme, present in satellite 2 transcripts, has been studied by phosphorothioate interference analysis (95). This approach has also been used to map the phosphate oxygens critical for the hepatitis delta virus (HDV) ribozyme self-cleavage reaction. A set of phosphate groups and their *pro*-Rp oxygen atoms were identified as being important for the ribozyme activity. In particular, positions 0, 1, and 21 were deduced as the most critical for the ribozyme catalysis (96).

As is the case with other chemical cleavage methods, caution should be exercised when interpreting phosphorothioate-iodine footprinting results obtained

with RNA-RNA and RNA-protein complexes. An observed protection might not be due entirely to direct nucleic acid-macromolecule interaction but may be due partly to induced conformational changes in the nucleic acid, which can bury the phosphorothioate groups accessible to iodine cleavage. Conversely, phosphorothioates can also become hyperreactive by a similar mechanism.

NUCLEASE STABILITY Phosphorothioate modification increases nuclease stability of oligonucleotides. This fact was first demonstrated in cell culture experiments with interferon-inducing polynucleotides (97). This property is particularly important when oligonucleotides are employed for in vivo therapeutic purposes such as the antisense oligonucleotide-mediated inhibition of gene expression. The applications of antisense phosphorothioate oligonucleotides were reviewed recently (98).

Resistance of phosphorothioate-modified oligonucleotides to exonuclease III has allowed for the delineation of the borders of the thymine dimer removal by the human nucleotide excision nuclease by filling the resulting gap through polymerization with the four dNTP α S nucleotides (99). Surprisingly, analysis of the excision-repair mechanism with UV-sensitive rodent cell lines, through use of a phosphorothioate patch assay, shows that such cells have normal incision, excision, and repair steps (100). In the case of the Klenow fragment, 3'-exonuclease hydrolysis differs more than 100-fold for a phosphorothioate substitution when compared to the phosphate group (101). Similarly, introduction of a single 3'-phosphorothioate also inhibits degradation of oligonucleotide primers by the 3'-exonuclease activity of the thermostable DNA polymerases *Vent* and *Pfu* (102), an important consideration for the use of such primers in polymerase chain reactions.

Nuclease resistance of phosphorothioate internucleotide linkages, particularly to the restriction enzyme *Nci* I, is the basis of an oligonucleotide-directed, site-specific mutagenesis procedure (103, 104). Interestingly, to confer complete nuclease resistance, the position 5' to the cleavage site also has to be a phosphorothioate, a phenomenon very similar to that observed for the enzymes *Ban* II and *EcoRV* (105).

The isothermal single-strand displacement amplification (SDA) method, originally developed for the detection of DNA from pathogens of the *Mycobacterium* genus, is based on the resistance of phosphorothioates to the restriction endonuclease *Hinc* II (106). This methodology has been extended to mimic the rolling-circle replication of single-stranded phages (107).

PHOSPHOROTHIOATE DIESTERS AS NUCLEOPHILES The sulfhydryl anion of internucleotidic phosphorothioates can act as an efficient nucleophile for the

site-specific attachment of reporter groups, such as photoaffinity agents (108) and fluorescent dyes (109), to oligodeoxynucleotides via alkylation reactions. The resulting triesters are very stable below pH 7 but are rapidly hydrolyzed under alkaline conditions. However, the rate of hydrolysis is remarkably slow for double-stranded oligonucleotides (109). Such triester formation is not possible for phosphorothioate-substituted oligoribonucleotides because the 2'-hydroxyl group readily reacts to form a cyclic 2',3'-phosphate by displacing the reporter group via an intramolecular reaction. A similar chemical approach is the basis for the determination of the position of phosphorothioate groups in oligodeoxyribonucleotides by using iodoethanol (73) or iodine for oligoribonucleotides (74).

MERCURY ION INTERACTION As mentioned before, phosphorothioate monoesters interact strongly with mercury (II), and this property has been used to purify modified oligonucleotides by mercury-affinity techniques. In contrast, phosphorothioate diesters, which are present in the internucleotidic linkage, interact poorly with mercury (II), as evident by the lack of retention of thiolated tRNA on mercury (II)-polyacrylamide gels (33). Surprisingly, a recent study demonstrated that a mercury (II) complex could be prepared using a phosphorothioate-containing oligonucleotide and $\gamma\delta$ -resolvase. This complex was used further for X-ray structural analysis (110). A similar structural study has been reported for a phosphorothioate oligodeoxynucleotide and yeast RAP1 protein complex (111). However, this method might not be general because nonspecific interactions with protein sulfhydryl groups may cause interference.

TERMINAL PHOSPHOROTHIOATES FOR COUPLING REACTIONS Oligonucleotides with a phosphorothioate at the termini can be prepared by chemical synthesis (112, 113), by enzymatic thiophosphorylation at the 5' end with ATP γ S (33, 114), by attachment of pdCpS with RNA ligase to the 3' end of oligoribonucleotides (16), or by initiating transcription with guanosine derivatives such as GMPS (31), GTP β S (32), and GTP γ S (33, 35). Terminal phosphorothioate groups in oligonucleotides have been used for a variety of purposes, such as (a) the separation of oligonucleotides or transcripts on mercury gels or columns (32, 33, 35), (b) reaction with haloacetyl derivatives of fluorescent dyes (16) and bromoacetyl agarose for the affinity purification of DNA-binding proteins (115), (c) reaction with photoaffinity agents (31), (d) reaction with intercalators (112), (e) the template-directed coupling of two oligonucleotides (113, 116), and (f) cross-linking a 5'-phosphorothioate oligonucleotide to its complementary sequence by reacting it with trans-platinum(II)diamine dichloride (114).

NH- AND S-BRIDGING PHOSPHATE INTERNUCLEOTIDIC LINKAGES Another series of oligonucleotides have been prepared through the replacement of either the 3'- or the 5'-bridging oxygen of the phosphodiester internucleotidic linkage with sulfur or nitrogen. Oligodeoxynucleotides containing N3'-O5'phosphoramidate or N5'-O3'phosphoramidate linkages where either the 3'- or the 5'-oxygen is respectively exchanged with nitrogen possess interesting biophysical and biochemical properties (Figure 2). The amidate linkage in N3'-P5' (117) or N5'-P3' phosphoramidates (118, 119) can be selectively cleaved by mild acidic treatment. However, N5'-O3' (120) and N3'-O5' phosphoramidate oligodeoxynucleotides are resistant to various nucleases and have high binding affinity for RNA (121). These properties make them suitable for antisense studies even though they do not invoke the RNaseH cleavage mechanism (122).

Oligodeoxynucleotides with 3'-S- or 5'-S-phosphorothiolate internucleotide linkage, where the bridging oxygen at position 3'- or 5'- is replaced by sulfur, have also generated considerable interest (Figure 2). These linkages are susceptible to Ag^+ , Hg^{2+} and iodine-mediated chemical cleavage (123, 124). However, the 3'-S-phosphorothiolate linkage is stable to the action of restriction endonuclease *EcoRV* (124) and is refractory to the corresponding methylase (125). Interestingly, topoisomerase I and lambda integrase protein can cleave a 5'-S-phosphorothiolate linkage in an oligodeoxynucleotide by forming a covalent enzyme intermediate with a 5'-thio-nucleoside leaving group. In contrast, the reverse reaction with a 5'-thio-nucleoside as the attacking nucleophile, to accomplish topoisomerase-mediated ligation, is inhibited (126). This observation is not surprising because sulfur is a poor nucleophile at phosphorus and requires a good leaving group to form the P-S bond (127).

Oligonucleotides with such linkages have also been used to probe the mechanism of ribozyme catalysis. In the group I intron ribozyme-catalyzed transesterification reaction, cleavage of a substrate containing a 3'-S-phosphorothiolate internucleotidic substitution was about 1000-fold slower in the presence of Mg^{2+} ion when compared to an unmodified phosphodiester substrate (128). However, in the presence of soft metal ions such as Mn^{2+} and Zn^{2+} , the cleavage activity was restored. This observation indicates a possible role of metal ions in stabilizing the negative charge on 3'-oxygen or sulfur in the transition state. Recently, the presence of a second metal ion involved in group I ribozyme catalysis was demonstrated through the use of 3'-(thioinosyl)-(3'→5')-uridine (IspU) dinucleotide as a substrate (129). In a reaction mimicking the exon ligation step, the IspU substrate required a second, thiophilic metal ion for optimum activity. Based on these observations, it was proposed that a second metal ion that activates the 3'-hydroxyl group of guanosine is involved in the first step of splicing.

The mechanism of the hammerhead ribozyme-catalyzed reaction has also been probed with 5'-S-phosphorothiolates. One group has used this modification

at the cleavage site in a DNA substrate (130). Because there was no appreciable thio effect from changing the metal ion from Mg^{2+} to Mn^{2+} , the authors concluded that there is no metal ion coordination with the 5'-oxygen of the leaving group in the transition state. Contradicting arguments were presented by another group, which used an all-RNA substrate for similar experiments (131). The RNA 5'-S-phosphorothiolate substrate was cleaved almost 100 times faster than the unmodified substrate in the presence of low concentrations of Mg^{2+} ions. It was reasoned that attack at the 2'-OH is rate-limiting for the modified substrate, whereas the departure of the leaving group is rate-limiting for the unmodified one. Rate differences observed for the hydrolysis of 3'- and 5'-S-phosphorothiolate substrates with different metal ions should be interpreted with caution because the metal ion-dependent hydrolysis of simple dinucleotides is not well understood (132).

Phosphorodithioates

In the phosphorodithioate linkage, both of the nonbridging oxygens of the phosphodiester backbone are replaced by sulfur atoms (Figure 2; 133–135). Such modified biopolymers are isostructural and isopolar to the natural nucleic acid backbone, and unlike phosphorothioates, they retain the achiral nature of the phosphorus atom.

Stability studies with phosphorodithioate oligonucleotides have demonstrated resistance to a variety of nucleases. The effect was most prominent when the dithioate modification was introduced at alternating internucleotide positions (136). However, introduction of phosphorodithioate linkages in an oligonucleotide reduces its binding to a complementary target, induces secondary structures in single-stranded oligomers (137), and enhances binding of modified oligonucleotides to proteins (138). Despite these potential drawbacks, phosphorodithioate-modified antisense oligonucleotides have been shown to interfere with the expression of *erbB-2* mRNA associated with breast cancer (139), to inhibit HIV-1 reverse transcriptase activity (140), and to induce B-cell proliferation and differentiation (141).

Methylphosphonates

The methylphosphonate internucleotidic linkage is nonionic in contrast to the polyanionic natural phosphodiester backbone (Figure 2). Solid-phase synthesis of methylphosphonate oligomers requires nucleoside methylphosphoramidites, and following the synthesis, the oligomers require special deprotection chemistry (9). Akin to the phosphorothioate linkage, replacing one nonbridging oxygen with the methyl group introduces chirality at the phosphorus center. Efforts have been made to devise new stereospecific methods for the synthesis of Rp-methylphosphonate linkage to improve the binding properties

of the modified oligomers (142, 143). Also determined was a high-resolution NMR structure of an alternating, chirally pure, Rp-methylphosphonate chimeric oligodeoxynucleotide hybridized to an RNA target (144).

Methylphosphonate oligomers have been shown to hybridize sequence-specifically to complementary nucleic acid targets. Examples for biological effects include antisense inhibition of herpes simplex virus replication (145), inhibition of human collagenase IV mRNA expression (146), and triplex-directed inhibition of chloramphenicol acetyltransferase mRNA expression in cell cultures (147).

In a study on deformations caused by DNA bending, several methylphosphonate linkages were introduced to create a neutral patch in the anionic phosphodiester backbone. Electrophoretic experiments revealed that the modified DNA bends toward the neutral surface (148, 149). Electrophoretic gel retardation analysis with Oct-2 POU and Oct-2 POU_{HD} proteins and 14-mer duplex targets containing diastereomeric methylphosphonate linkages was used to map phosphate groups essential for protein–nucleic acid interaction (150). This interference method relies on the fact that the introduction of methylphosphonate linkages preserve B-DNA conformation while abolishing electrostatic contacts (151). Methylphosphonate linkages were also incorporated in a four-way DNA junction. The results support the notion that charge-charge interactions influence the structure of the Holliday junction (152).

BASE MODIFICATIONS

The heterocyclic ring of purine and pyrimidine bases provides hydrogen-bonding functional groups in nucleic acids. Carefully designed base analogs, when introduced into oligonucleotides, can provide information on the importance of specific functional groups in natural bases. In interpreting results obtained with base analogs, even a subtle change in the analog can have a dramatic effect because of the change in size, electronic distribution, nucleoside sugar conformation, tautomeric structure, or functional group pK_a values. Representative structures of several modified bases are presented in Figure 3, and their use is described in subsequent sections. Base analogs possessing unusual spectroscopic properties or a potential for cross-linking are discussed in appropriate sections.

Purine Modifications

The imine nitrogen at position 7 of purine nucleosides is located in the major groove of B-form DNA and can act as a potential hydrogen bond acceptor site in the recognition processes. For example, this nitrogen atom is used as a hydrogen bond acceptor in Hoogsteen base pairing. 7-Deazapurine nucleosides, which lack the N-7 nitrogen atom, are therefore valuable probes for studying the

role of N-7 nitrogens in recognition. These analogs can easily be incorporated in oligonucleotides, either by enzymatic means (153) or by automated solid-phase synthesis (154, 155). Because of altered base stacking, oligonucleotides containing 7-deazapurine analogs destabilize DNA duplex slightly, and the effect is sequence dependent (156).

The interaction of the *trp* repressor with the *trp* *EDCBA* operon was studied through the use of 7-deazaadenine and 7-deazaguanine to validate the importance of four purine N-7 nitrogens implicated in recognition (157). Other studies also have used these analogs to investigate protein-DNA (158, 159) and restriction enzyme-DNA interactions (125, 160, 161).

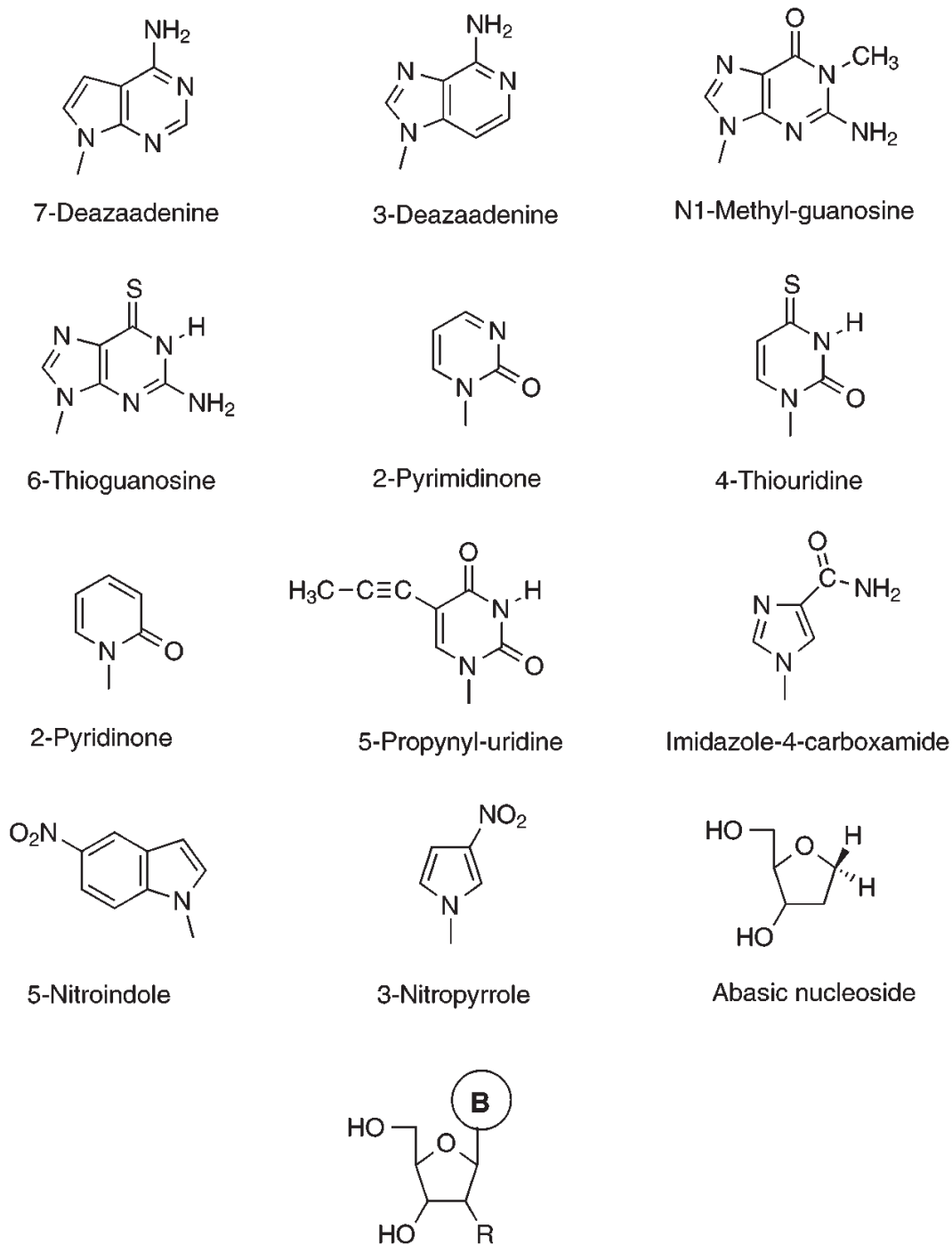
The role of purine N-7 nitrogens in the folding of G-rich telomere sequences was investigated through the use of 7-deazaadenine and 7-deazaguanine substitutions (162). G-rich oligonucleotide sequences possess a high propensity to form tetraplexes through hydrogen bonding between the N-7 nitrogen of one guanine and the N-2 hydrogen of another. The effect of 7-deaza substitutions on tetraplex formation was studied by gel electrophoretic mobility shift experiments and guanine- and adenine-specific chemical footprinting reactions. Based on these experiments, the role of guanine N-7 nitrogen in stabilizing tetraplexes by specific hydrogen bond formation was confirmed.

Other examples that have exploited 7-deazapurine analogs are studies of DNA-DNA contacts in homologous recombination (163), the role of tertiary interactions and self-cleavage of hepatitis delta virus ribozyme (164), and tertiary structure mapping of the retinoblastoma gene (165).

The N-3 nitrogen of purines is located in the minor groove of DNA and is not directly involved in base pairing through hydrogen bond formation. However, 3-deazaadenosine substitution in the hammerhead ribozyme considerably reduces its catalytic efficiency, which indicates an important role for the N-3 nitrogen atom in the reaction (166). Similarly, when 3-deazaadenosine was substituted in the recognition sequence of *EcoRV* methylase, the enzymatic reaction was inhibited, thus implying a role for N-3 nitrogen in protein-DNA recognition (167).

Another purine analog, N-1-methylguanosine, when substituted in the core region of the hammerhead ribozyme, completely inhibits its catalytic activity (168). This analog has also been incorporated into a tRNA^{Asp}, where it abolished mischarging (169).

2-Aminopurine is often used for fluorescence measurements, which are discussed further in a subsequent section. Other analogs such as purine, isoguanine, 2,6-diaminopurine, 6-thioguanine, and hypoxanthine have been used as probes for the identification of purine nucleosides essential for protein-DNA interaction (167, 170–172) and RNA catalysis (166, 173–175) and to estimate the contribution of the hydrophobic interaction in duplex stability (176). A more



R : OCH₃ , 2'-O-Methylnucleoside
 SH , 2'-Thionucleoside
 OCH₂CH=CH₂ , 2'-O-Allylnucleoside

Figure 3 Base and sugar modifications.

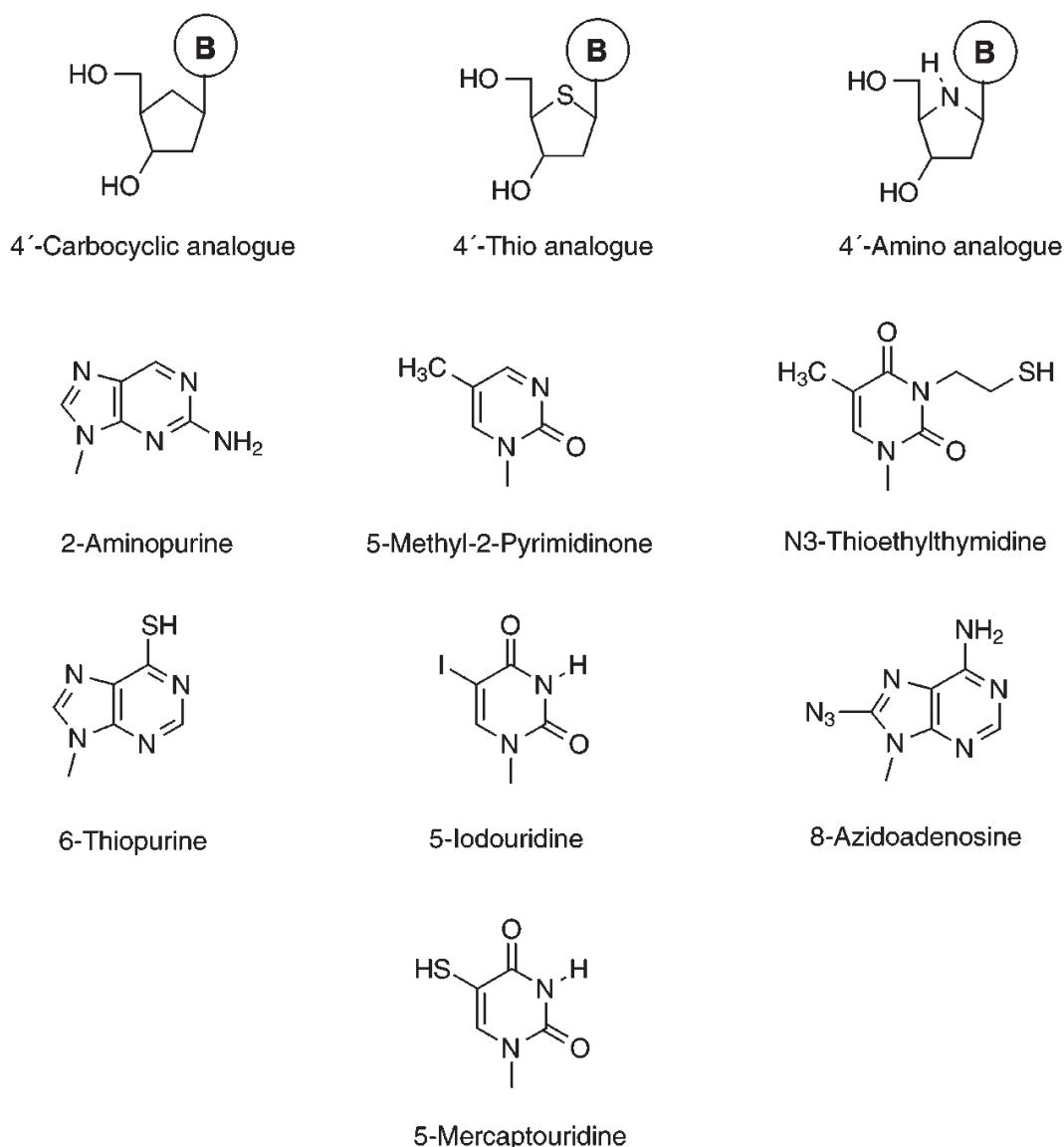


Figure 3 (Continued)

detailed account of purine and pyrimidine nucleoside analogs and their utility in delineating biochemical mechanisms can be found elsewhere (177, 178).

Pyrimidine Modifications

2-Pyrimidinone and 4-thiouridine ribonucleosides have been used to assess the participation of conserved pyrimidine bases in making hydrogen-bonding contacts in the catalytic core of hammerhead ribozyme (179). This study provided evidence for a magnesium ion-binding site in the catalytically active ribozyme. 2- and 4-pyridinone ribonucleosides have also been used to study the structure-function relationship of the hammerhead ribozyme (180).

The role of conserved deoxycytidine residues in template recognition by bacteriophage T7 primase was studied with oligonucleotides containing 2-pyrimidinone and 5-methyldeoxycytidine bases (181). The importance of the hydrogen-bonding framework for protein-DNA recognition in conjunction with other nucleoside substitutions was demonstrated.

Oligonucleotides containing a propynyl substituent at the C-5 position of pyrimidines possess high binding affinity toward complementary RNA targets and display a gene-specific antisense effect (182, 183). When such modified oligonucleotides additionally contain phosphorothioates, they can be used to target low and high copy messages with equal efficiency (184). Remarkably, a sequence-specific inhibition of gene expression has been observed with a C-5 propyne antisense phosphorothioate as short as a heptanucleotide (185).

Universal Bases

Natural nucleobases display exquisite selectivity in recognizing complementary bases. A universal base can be defined as an analog that can substitute for any of the four natural bases in oligonucleotides without significantly impairing the duplex stability. In general, universal base analogs use aromatic ring stacking, instead of specific hydrogen bonds, to stabilize a duplex. However, universal recognition by imidazole-4-carboxamide nucleoside has been attributed to specific hydrogen-bonding contacts (186).

The efficiency of oligonucleotide primers containing multiple substitutions of 5-nitroindole (187, 188) and 3-nitropyrrole (189, 190) has been studied and compared in DNA sequencing and in polymerase chain reactions. These analogs can be used at primer sites corresponding to degenerate base positions and specifically where the sequence data is incomplete. The use of imidazole-4-carboxamide nucleoside in oligonucleotide templates results in nucleotide misincorporation during PCR amplification, and it has been suggested that this property can be further used to generate mutant gene libraries (186).

Depending on the length of the oligonucleotides, a true mismatch in a DNA hybrid is often difficult to detect. A recent study used 3-nitropyrrole-containing oligonucleotide probes to analyze either a PCR-amplified or an allele-specific PCR-amplified polymorphic region of the HLA-DRB locus by artificial mismatch hybridization (191). The presence of 3-nitropyrrole in a hybridization probe created an artificial mismatch, and it provided an excellent discrimination between perfectly matched and mismatched duplexes when compared to conventional hybridization methods.

Abasic Nucleosides

Deletion of the purine or pyrimidine bases from the deoxyribose or ribose sugar moiety gives rise to abasic nucleosides. These analogs preserve the sugar hydroxyl groups; therefore, any biochemical effect observed from their

use results directly from the loss of the heterocyclic ring. A deoxy-abasic nucleoside was used to replace both dA and T in a dA/T base pair to study the binding of *EcoRV* DNA methyltransferase to its recognition sequence (171). Such a substitution is expected to disrupt hydrogen bonding and was therefore used to assess the role of base pairing to protein-binding affinity.

Substitution of abasic ribonucleosides in the hammerhead ribozyme significantly impairs its catalytic activity (192). However, it was possible to restore the activity of four ribozymes by a simple addition of the missing base. This finding indicated that the ribozyme structure can be engineered to create a binding site for small ligands. Other studies using ribo-abasic residue include the recognition of branch-point adenosine in the Group II introns (175); the identification of essential base residues in loop B of the hairpin ribozyme (193); the importance of the stem-loop II; and the importance of conserved U residues in the catalytic core of hammerhead ribozyme (194, 195).

SUGAR MODIFICATION

The 2'-deoxyfuranose ring in oligodeoxynucleotides can adopt C_{3'}-endo and C_{3'}-exo as two major, interchangeable conformations, whereas the C_{3'}-endo sugar ring conformation is maintained in oligoribonucleotides (196). The electronegativity of the 2'-substituent has a profound effect on the sugar conformation, and as a result of such substitution, the population of C_{3'}-endo conformer increases with an increase in the electronegativity. Several sugar modifications, primarily at the 2'-position, have been described, and there has been renewed interest in oligoribonucleotides containing such modifications to generate nuclease-resistant RNA aptamers and ribozymes.

Sugar-modified analogs have been used extensively to study ribozymes and RNA aptamers to probe the importance of specific 2'-hydroxyl groups in catalysis and to provide resistance against nucleases. The 2'-hydroxyl modifications introduced to probe hammerhead ribozyme catalysis include deoxy (197, 198); fluoro, amino (199, 200); methoxy, allyloxy (201); and 2'-C-allyl substituents (202). 2'-Deoxy- or 2'-O-methyl sugar modifications have been introduced to identify the positions of 2'-hydroxyl groups necessary for the hairpin ribozyme activity (203). Interactions involving 2'-hydroxyl groups, and the importance of having a ribose moiety at the cleavage site in *Tetrahymena* ribozyme reaction, have been studied through the use of 2'-deoxy- and 2'-fluoro-substituted nucleosides (204–207). 2'-Deoxy substitution has also been used to investigate the role of active-site 2'-hydroxyl groups in group II intron molecular recognition and catalysis (208). Recently, incorporation of 2'-deoxy-2'-mercaptocytidine into an oligonucleotide, via suitably protected phosphoramidite, has been reported (209). However, enzymatic incorporation of 2'-mercapto group-containing modified nucleotides remains to be established.

2'-Fluoro- and 2'-difluoro-2'-deoxyuridine-containing oligonucleotides have been used to investigate the mechanism of human G/T glycosylase (210). The modified oligonucleotides resisted the excision repair activity of the enzyme and resulted in the formation of a tight-binding DNA-glycosylase complex. Based on these observations, a rationale for preferential repair of deaminated CpG sites was presented.

When a variety of selection protocols were employed, high-affinity nucleic acid aptamers were isolated. These aptamers can recognize a wide range of molecular targets such as antibiotics, amino acids, nucleoside triphosphates, cofactors, organic dyes, porphyrins, and proteins (211). Improved chemical and enzymatic stability is essential for the development and use of RNA-based aptamers in therapeutics and diagnostics. The substitution of 2'-fluoro- and 2'-aminopyrimidines in RNA imparts resistance to nucleases (200). Moreover, these modifications can be introduced easily during aptamer selection because the corresponding nucleotide triphosphates are good substrates for T7 RNA polymerase (29, 30). When these analogs were used, nuclease-resistant aptamers were isolated against the basic fibroblast growth factor (212), human thyroid-stimulating hormone (213), a monoclonal antibody that recognizes the main immunogenic region of the human ACh receptor (214), and the keratinocyte growth factor (215).

2'-*O*-methyl-modified, biotinylated oligoribonucleotides have been used as antisense probes for mapping U2 snRNP-pre-mRNA interactions and for studying structure and function of U4/U6 snRNPs (216, 217). Modified nucleosides such as 2'-deoxy and 2'-*O*-methyl have also been placed at either the 3'- or the 5'-splice site of a nuclear pre-mRNA to aid in understanding chemical steps involved in the spliceosomal assembly (20). In vitro studies revealed that the presence of the 2'-hydroxyl group is more important at the 3'-splice site for the second step in the splicing reaction, which results in ligation of two exons with a concomitant release of an intron.

Posttranscriptional nuclear processing of most eukaryotic mRNAs involves 3'-end polyadenylation, which is suggested to confer stability to the transcript. A highly conserved hexameric-sequence AAUAAA, present in the transcript, acts as a polyadenylation signal by binding a specificity factor protein. 2'-*O*-methyl ribose-modified nucleosides were chemically substituted at all nucleotide positions in synthetic RNA substrates, including the signal sequence, to aid in the study of the polyadenylation reaction in vitro (218). A single 2'-*O*-methyl substitution, within the AAUAAA sequence, inhibited the polyadenylation and binding of the specificity factor. Oligoribonucleotides with substitutions at any position, except the 3'-terminal adenosine, were efficiently polyadenylated.

Another sugar modification involves replacement of the O-4' furanose ring oxygen with a methylene group to give carbocyclic analogs. Such sugar-modified analogs of thymidine and 5-methyl-2'-deoxycytidine have been incorporated in oligodeoxynucleotides to allow study of their effect on triplex formation (219). The mechanism of action of the MutY protein of *E. coli*, which deletes mismatched deoxyadenosine residues from a DNA substrate, has been studied through use of the carbocyclic analog of 2'-deoxyadenosine (220). The proposed mechanism of excision involves an enzyme-catalyzed attack of water on the C-1' carbon to displace the base from the duplex, followed by the opening of the furanose ring to give an acyclic structure. In accordance with the proposed mechanism, it was found that DNA substrates containing carbocyclic dA analog, designed to prevent the ring opening step, were not excised by the MutY protein. Ribocarbocyclic analogs of cytidine and adenosine have been chemically incorporated in the hammerhead ribozyme to confer RNase resistance to these ribozyme constructs (221).

The O-4' ring oxygen can also be replaced by sulfur to result in 4'-thiofuranose modification, and oligonucleotides containing such modification have been used to probe protein-DNA interactions such as in the *EcoRV* restriction endonuclease and methylase systems (125, 222). In one study, modified deoxynucleosides possessing an inverted C4' configuration or capable of forming C4' adducts were incorporated in DNA templates to investigate structural the requirement of the Klenow fragment of *E. coli* DNA pol I (223). The polymerase halts transiently at inversions while adduct-forming modifications completely inhibit the DNA synthesis.

The furanose ring oxygen has also been replaced with a pyrrolidine ring. This modified base has been incorporated in a 25-mer to study mechanism-based inhibition of an *E. coli* DNA repair enzyme, 3-methyladenine DNA glycosylase II (224).

NONRADIOACTIVE LABELS OF OLIGONUCLEOTIDES

Fluorescent Labels

BASE ANALOGS 2-Aminopurine (2AP), a fluorescent nucleoside analog, has been used extensively to detect changes in oligonucleotide conformation (225). It can substitute for adenosine in base pairing with thymidine without distorting the double helix. The absorption and excitation maximum for 2AP is at 330 nm and has an emission at 380 nm. The quantum yield of 2AP fluorescence, when substituted in oligonucleotides, depends on the degree of base stacking (226). Therefore, any change in its fluorescence is a sensitive indicator of structural perturbations in the modified oligonucleotide and provides an insight

into the dynamics of such processes. Temperature-dependent conformational changes in oligonucleotides (227) and the dynamics of mismatched base pairs in oligodeoxynucleotides have been followed in the monitoring of change in fluorescence intensity of 2AP (228). Several reports have described the use of 2AP-containing oligonucleotides to study base-pairing interactions in DNA polymerase 3'-5'-exonuclease proofreading (229–231), kinetics of nucleotide incorporation with the Klenow fragment and T4 DNA polymerase, (232, 233), and the helicase-catalyzed unwinding of duplex DNA (234). In one study, Mg^{2+} ion-induced conformational perturbations in the hammerhead ribozyme tertiary structure were followed by the monitoring of change in fluorescence of 2AP. The data were used to determine the affinity constants for Mg^{2+} ion binding (235).

A fluorescent pteridine analog, 3-methyl-8-(2-deoxy- β -D-ribofuranosyl) isoxanthopterin, has been introduced as a guanosine mimic for mechanistic studies (236). The fluorescence of this analog, when incorporated into an oligodeoxynucleotide identical to the U5 terminal sequence of HIV-1, increases with 3'-processing reaction of integrase. This analog extends the repertoire of fluorescent nucleosides, even though its base-pairing properties are not well documented. 5-Methyl-2-pyrimidinone has been reported as an alternative to 2AP, but it has not found wider application, presumably because of the lack of base-pairing capabilities (237).

Fluorescent reporter groups have also been attached to the 5-position of deoxyuridine, via aminoalkyl linkers, to follow oligonucleotide-protein interactions. One such study with template/primer oligonucleotides, where a fluorescent dansyl probe was appended at various nucleotide positions from the 3'-end of the primer, indicated that the Klenow polymerase remains in contact with the template/primer up to six nucleotides from the 3'-end (238). Similarly, contacts between a 42-mer oligonucleotide and the *E. coli* regulatory protein TyrR were also identified through the use of such nucleoside analogs (239).

A fluorescent derivative has also been attached to 5-sulfhydryl-deoxyuridine triphosphates for polymerization into a template/primer for the determination of dissociation constants of DNA and the dNTPs for the Klenow fragment of *E. coli* DNA polymerase I (240).

NONBASE ATTACHMENT Fluorescent dyes have been attached to oligodeoxynucleotides by the reaction of internucleotidic phosphorothioates with 5-iodoacetamido-eosin and -fluorescein (241). Distances calculated from fluorescence resonance energy transfer (FRET) studies that used the fluorescently tagged oligonucleotides were in reasonable agreement with B-DNA helical structure.

A variety of reporter groups have been attached sequence-specifically to oligodeoxynucleotides by the oxidation of an internucleotidic H-phosphonate

in presence of cystamine to form a phosphoramidate linkage (242). Reduction of cystamine results in a free sulfhydryl group that can act as a nucleophile to react with thiol-specific reporter groups. However, this modification results in a pair of diastereomers that might have to be separated for an accurate interpretation of the spectroscopic data.

Fluorescent dyes have also been attached to the sugar of dideoxynucleoside triphosphates to act as chain terminators in fluorescence-based DNA sequencing (243). In one study, two dyes were attached to the sequencing primer, with a common donor at the 5'-end, and to four different acceptors at a thymidine within the primer, which facilitate DNA sequencing with the use of FRET measurements (244). Recently, guanosine-specific fluorescence quenching of 3'-fluorescein-labeled oligoribonucleotides was used to determine rate constants for substrate binding, cleavage, and dissociation of the hairpin ribozyme (245). There is considerable literature on fluorescent dyes attached at the termini of oligonucleotides, particularly for FRET studies; this literature has been reviewed elsewhere (225).

Electron Spin Resonance Probes

Electron paramagnetic resonance (EPR) is an elegant method of monitoring global tumbling and internal motion of oligonucleotides. A nitroxide spin-label probe has been attached to the 5-position of deoxyuridine, via a mono- or diacetylene tether, to form a complementary base pair with an adenosine (246). A quinolonyl nitroxide analog has base paired with 2-aminopurine nucleoside (247). This derivative seems to be particularly attractive because the motion of the spin label can be directly correlated to that of the base. A nitroxide spin label has also been introduced at the phosphate backbone, via a phosphoramidate sulfhydryl tether, but EPR measurements were not reported (242).

Resonance Raman Spectroscopy

The UV-absorbance maximum at 330–340 nm of 4-thiothymidine facilitates assignment of the bands in the resonance Raman spectrum to a specific nucleotide. Raman spectroscopy was performed on a complex of *EcoRV* endonuclease with an oligodeoxynucleotide containing 4-thiothymidine, which represents the enzyme recognition sequence (248). The observations were found to be consistent with the distortion of the DNA as seen in the X-ray structure. Such distortion was not observed with a nonsubstrate oligonucleotide. These results document the utility of this analog for its use in Raman spectroscopic studies.

NUCLEIC ACID CROSS-LINKING

Chemically modified oligonucleotides are extremely useful in fixing conformations by introducing cross-links in DNA, RNA, or nucleic acid–protein complexes. Site-specific cross-linking permits the determination of the proximity

of nucleotides or amino acids because the cross-linking would not be possible at distances greater than the length of the spacer. Such reagents are usually attached to either the base or the sugar moiety, and in most cases, the sulfhydryl group is used for introducing cross-links.

Chemical Cross-Links

A versatile strategy for the cross-linking of DNA and RNA oligomers results from the recently described convertible nucleoside approach (249–251). In this method, uridine and inosine derivatives possessing good leaving groups at the 4 or 6 position, respectively, are incorporated site-specifically in oligonucleotides. Subsequent reaction of the convertible nucleoside, at the oligonucleotide level, with a symmetrical ω , ω -dithiobis(alkylamine), results in the formation of cytidine and adenosine derivatives bearing an alkyl disulfide tether. Reduction of the disulfide linkage results in the formation of a free sulfhydryl group, which can be reoxidized to form a cross-link. For example, this approach has been applied to form adenine-adenine cross-links at 5'-AT-3' sequences in the major groove and guanine-guanine cross-links at 5'-GC-3' sequences in the minor groove of oligodeoxynucleotides. Such an interstrand cross-link mimics a bent DNA structure and binds to a high mobility group protein, thereby supporting the view that this protein recognizes bent DNA (252). An interstrand disulfide bridge has been utilized to prevent DNA strand separation in the DNA-(cytosine-5) methyltransferase-catalyzed reaction (253). It was suggested that reduction in the rate of methylation of the cross-linked oligonucleotide was due to the extrusion of the cytosine to an extrahelical position in the normal substrate oligonucleotide. Oligoribonucleotides can also be cross-linked in this manner, as exemplified by the formation of an intramolecular disulfide in an RNA ministem loop (254).

In another approach, a mercaptoalkyl group is attached to the N-3 of a pyrimidine base, such as in N-3-thioethylthymidine (255). When incorporated at the 3'-end and 5'-end of two oligonucleotides, the sulfhydryl group can be oxidized, thus linking two oligonucleotides through the formation of disulfide loops. The cross-link imparts greater thermal stability and a unique conformation. Mercaptoethyl groups can also be attached to C5 of pyrimidines and oxidized to the disulfides, as described for the cross-linking of triple helices and tRNA (256, 257). Interstrand disulfide cross-linking can also be obtained through the use of 6-mercaptapurine and 4-thiothymidine bases in an oligodeoxynucleotide without the employment of tether (258).

Cross-linked duplexes have been prepared to allow the study of the mechanism of DNA polymerases through the introduction of an aziridinyl group at the 4-position of a cytidine or at position 6 of guanosine, which can react with the 4-amino group of a mismatched cytidine in the complementary strand to link

two cytidines or a 2,6 diaminopurine and cytidine, respectively, by an ethylene bridge (259, 260).

In some systems, however, cross-linking via base modification is undesirable. Therefore, attachment of the cross-linking reagent via the sugar moiety is preferred. This approach has been used to discriminate between two models of the hammerhead ribozyme, one based on X-ray crystallographic analysis and the other based on FRET results (261). In this method, the nucleotides to be linked were replaced by the corresponding 2'-amino nucleotides, which were then reacted with 2-pyridyl 3-isothiocyanatobenzyl disulfide. After reduction, an intrastrand disulfide was reintroduced by oxidation, and the catalytic activity of cross-linked ribozymes was determined. Recently, more reactive aliphatic isocyanates have been used for the cross-linking of the hairpin ribozyme as part of an effort to build a first 3-D model (262; DJ Earnshaw, B Masquida, S Müller, STh Sigurdsson, F Eckstein, et al, manuscript in preparation). This method has been further extended to introduce interstrand disulfide cross-links, by a sulfhydryl exchange reaction, in a group I ribozyme (264). The cross-linked ribozymes were catalytically competent, and this observation reveals interesting dynamic properties of large catalytic RNAs.

Alternatively, 2'-hydroxyl groups have also been derivatized with ethylthio groups to introduce intra- and interhelical disulfide cross-links in tRNA^{Phe} (256).

Photoaffinity Cross-Linking

BASE CROSS-LINKS Natural nucleosides can be photoactivated to form new bonds under favorable distance conditions. A good example is the formation of a cross-link between a cytidine and a uridine residue, which was observed upon irradiation in yeast tRNA^{Phe} and was found to be consistent with the X-ray structure (265). Another example is described for the hairpin ribozyme, in which a uridine and a guanosine in a loop were cross-linked in high yields (266).

Usually, the cross-linking yields obtained with the natural nucleotides are low. Moreover, the wavelength required for activation is around 260 nm, which is not specific for a particular nucleotide. Thus, analogs that are more reactive and absorb at a higher wavelength are usually preferred for photoactivated cross-linking. 4-Thiothymidine and -uridine, 5-bromo- and -iodouridine, and 6-thioguanosine meet this requirement and are also able to form Watson-Crick base pairs. The most frequently used analogs are 4-thiouridine and 4-thiothymidine, which absorb at 331 nm and can be irradiated between 300 and 400 nm for activation (267). The first intrastrand RNA site-specific cross-link with 4-thiouridine was achieved upon irradiation of yeast tRNA^{Val}, which contains 4-thiouridine as a natural constituent (268). In one study, 4-thiouridine was incorporated by enzymatic methods in oligoribonucleotides. Depending on its position, cross-links were observed to ribosomal proteins and 16S RNA (269).

The chemical synthesis of oligodeoxy- and oligoribonucleotides containing 4-thiopyrimidine and 6-thiodeoxyguanosine requires special protection of the sulfhydryl group (267, 270). The *EcoRV* restriction endonuclease and modification methylase have been investigated with oligonucleotides containing these analogs (267). These analogs could be cross-linked to the enzymes with higher yields for 4-thiothymidine compared to 6-thiodeoxyguanosine. Unfortunately, oligonucleotides without the recognition sequences were also cross-linked as a result of nonspecific binding.

4-Thiouridine has been incorporated at specific positions in an adenovirus pre-mRNA, through the Moore and Sharp ligation approach (20), to identify contacts to snRNAs during splicing reaction (271). Cross-links were observed with a loop sequence in U5 snRNA and with an invariant sequence of U6. Also investigated through use of a similar strategy is strand-specific cross-linking between 4-thiouridine-modified human tRNA^{Lys} with HIV-1 reverse transcriptase subunits p66 and p51 (272). 4-Thiouridine was used to cross-link small nuclear ribonucleoprotein particles U11 and U6atac to the 5'-splice site of an AT-AC intron found in metazoan genes (273).

6-Thioinosine and 4-thiouridine have been used as photoaffinity probes to study hammerhead and hairpin ribozyme conformations (274). Multiple cross-links were found in both of the cases, and the results were difficult to explain on the basis of models. It was concluded that these ribozymes can adopt multiple conformations, making it difficult to identify catalytically competent conformations by this approach alone.

5-Bromo- and 5-iodouridine, which can be activated at 308 and 325 nm, respectively, are also convenient photoaffinity labels, as exemplified by the cross-linking of an RNA transcript and an oligodeoxynucleotide containing these modified nucleosides to the bacteriophage R17 coat protein (275, 276). Protein photodamage was considerably reduced with the iodo derivative. A telomeric DNA containing 5-bromodeoxyuridine has been cross-linked to the *Oxytricha* telomeric protein (277). Two of the three cross-links were traced to the tyrosine residues in the hydrophobic region, suggesting that intercalation plays a major role in protein-telomeric DNA binding.

The 5-position of uridine and deoxyuridine can be conveniently functionalized, without hydrogen bonding interaction being affected, to attach various nonradioactive probes. In one of the first reports, biotin was covalently attached at the 5-position of thymidine or uridine triphosphate for incorporation into DNA or RNA, respectively, for a subsequent streptavidin-affinity isolation (278). Many biotin phosphoramidites are commercially available for solid-phase chemical coupling to oligonucleotides. A similar approach uses incorporation of probes such as digoxigenin (DIG) or fluorescein instead of biotin. For photo-cross-linking, azido-nitrobenzoate has been coupled to the 5'-position,

via an amino linker, and the nucleotide has been incorporated chemically into oligonucleotides to allow study of the contact with the Klenow fragment (279), T4 DNA polymerase (280), and *E. coli* DNA polymerase III (281).

In one study, multiple 5-methyleneaminouridine triphosphate residues were introduced in oligoribonucleotides by T7 RNA polymerase transcription of synthetic DNA templates (282). The free amino group in modified uridine was postsynthetically derivatized, at the RNA level, with an aziriny group-containing reagent. The photo-cross-linking results with the aziriny-uridine derivative and 6-thioguanosine were used to study the binding of radiolabeled mRNA analogs to *E. coli* ribosomes. Precise cross-link positions were determined by a combination of ribonuclease H and T₁ digestion and by primer extension analysis.

8-Azidoadenosine and -guanosine are frequently used for the photoaffinity labeling of enzymes (283,284). Due to incompatibility of the azido group with phosphoramidite-based chemical synthesis, these analogs are not suitable for automated synthesis. However, enzymatic methods have been used to incorporate 8-azidoadenosine into the acceptor stem of tRNAs to probe its interaction with ribosomes (285). 5-Azido-uridine and -deoxyuridine triphosphates, with maximum absorption at around 300 nm, are also suitable for photoactivated cross-linking and are substrates for RNA and DNA polymerases. However, affinity labeling studies have not been reported with these analogs (286).

5-Sulphydryluridine triphosphate has been enzymatically incorporated into RNA, followed by subsequent derivatization with azidophenacyl bromide for cross-linking to several RNA polymerases (287).

TERMINAL CROSS-LINK Posttranscriptional reaction of a 5'-terminal guanosine phosphorothioate is a versatile method to attach a photoactivatable azidophenacyl group to the transcripts. Upon irradiation, intra- and intermolecular cross-links in RNase P RNA were obtained in the absence or presence of pre-tRNA (288). In another study, RNA containing a 5'-terminal phosphorothioate was derivatized with an azidophenacyl group to determine the tertiary structure of the ribonuclease P ribozyme-substrate cross-linked complex (289). A similar cross-linking study was also used to identify "neighbors" in the group I intron ribozyme (290).

METAL DERIVATIVES A solid-phase synthesis of an oligodeoxynucleotide that was site-specifically platinated, employing a cisplatin-derivatized deoxyguanosine 3'-H-phosphonate monomer, has been described (291). In another study, 2'-O-methyl oligoribonucleotides were postsynthetically modified with transplatin to give a 1,3-intrastrand cross-linked oligomer (292). When hybridized with

complementary RNA strands, intrastrand platinated oligomers trigger the formation of specific interstrand cross-links. The utility of transplatin-modified oligoribonucleotides was demonstrated through the introduction of cross-links in Ha-ras mRNA and through concomitant inhibition of cell proliferation.

CONCLUSIONS

Chemically modified oligonucleotides have been immensely helpful in understanding mechanistic and stereochemical aspects of numerous biochemical reactions and processes. The imagination and ability of chemists will ensure that tailor-made nucleoside analogs will be synthesized to answer specific questions in the future. There is, however, a real challenge to widen the arsenal of nucleotide analogs that can be enzymatically incorporated into nucleic acids. Novel strategies, such as mutant polymerases, will have to be devised to achieve this goal.

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