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Disulfide bridge as a linker in nucleic acids' bioconjugation. Part I: An overview of synthetic strategies

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ABSTRACT

This 2-part article reviews methods of oligonucleotides functionalization with thiol tethers and their consecutive use in conjugation with other (macro)molecules via a disulfide bridge. This relatively inexpensive, robust and reversible method of conjugation of DNAs, RNAs and their analogs holds a prominent position in a modern biochemistry toolbox and therefore there is a wealth of literature on the subject. In part I methods of thiol/disulfide groups introduction into oligonucleotide strands have been systematized and discussed. A digest of conjugation methods is presented as well.

1. Introduction

This review, comprising two parts, focuses on oligonucleotide conjugates with biomolecules via a disulfide bond. Although nowadays it is a common strategy, offering convenient access to a plethora of multifunctional species, there are surprisingly few articles summarizing this topic, often treating it as yet another example of conjugation methodologies. Some of these deal with the chemical aspects of S—S conjugation (i.e.: Zatsepin et al. [1], Dolinnaya and Borisova [2], Zubin et al. [3]), whereas others describe the biological applications of such cross-linked entities (i.e.: Winkler [4], Gait [5], Norman and Verdine [6]). With this work we would like to provide a comprehensive summary and a long overdue update on this interesting topic, considering that many important results have been communicated during last 10–15 years. Moreover, it is our intention to treat the subject in a balanced manner: thoroughly describing the chemistry behind it (in Part I) and discussing applications thereof in studies of biomolecules (in Part II). For the sake of clarity, the terms "nucleic acids" and "oligonucleotides" will be used interchangeably in this review.

1.1. Thiol/disulfide chemistry

A disulfide bridge commonly refers to a covalent bond between two sulfur atoms formed by oxidation of two free thiols (—SH). Since thiols are highly reactive species, oxidation occurs even with atmospheric oxygen, but it can be greatly accelerated with oxidizing agents like H_2O_2 or molecular iodine [7]. Once formed, the disulfide bond is rather stable at ambient conditions (typical bond dissociation energy of ca. 60 kcal/mol [8]), although in the presence of a reducing agent in a weakly alkaline environment (i.e. pH > 8) it is readily cleaved back to free thiols, thus making the reaction reversible (Eq. (1A)). On the other hand, it has long been believed that disulfides exhibit only modest reactivity with oxidants. However, a recent study shows that selected disulfides react with multiple oxidants extremely rapidly, with a variation of even 10^4 in rate constants [9]. Five-membered ring disulfides have been evidenced as being particularly reactive as compared with acyclic (linear) disulfides or six-membered rings. This is consistent with selective oxidative damage observed for certain disulfides, including those in some proteins.

Moreover, disulfides are susceptible to thiol/disulfide exchange (interchange) reaction, where a free thiolate anion, due to its nucleophilicity, attacks and substitutes one of the sulfur atoms of an already existing disulfide bond (Eq. (1B)). Generally, this reaction is reversible at physiological pH and room temperature [10], however the equilibrium of the exchange strictly depends on the pKa value of each species and the pH of the reaction medium. In principle, the maximum rate of interchange in aqueous solution is achieved when the pH is approximately equal to the pKa of attacking thiol [11].

Typical reactions of disulfide bonds: reduction to free thiols (A) and thiol/disulfide exchange (B):

$$RS - SR' + 2H^+ + 2e^- \leftrightarrows RSH + R'SH \quad (1A)$$

$$RS - SR + R'S^- \leftrightarrows RS - SR' + RS^- \quad (1B)$$

The abovementioned reactions occur during many biochemical processes since disulfide bridges are ubiquitous structural features of proteins [12]. They play a key role in stabilizing the tertiary structures of proteins, disruption of which is strongly associated with loss of

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protein function and activity [13,14]. Furthermore, the thiol/disulfide exchange is the basis of action of an antioxidant tripeptide- glutathione [15]. In stark contrast, neither thiols nor disulfide bridges are found in native nucleic acids. Still, the manner in which the stability of the disulfide bond is controlled by changing the environment suggested that many interesting properties may be expected from oligonucleotides functionalized with moieties comprising sulfur derivatives, prompting scientists' efforts at obtaining such species.

2. Synthetic strategies and modification sites

A wide range of synthetic approaches available these days allows functionalization of DNA, RNA and their analogs: PNA (peptide nucleic acid, Fig. 1a), LNA [16] (locked nucleic acid) and PMO (phosphordiamidate morpholino oligomers, Fig. 1b) with thiols/disulfides.

Peptide nucleic acids (PNAs) are nucleic acid analogues that consist of uncharged and achiral polyamide backbone with standard nucleobases attached [17]. They have ability to bind to complementary DNA and RNA strands and resulting hybrid duplexes exhibit higher stability than native duplexes, i.e. melting temperatures of PNA-DNA or PNA-RNA duplexes are greatly increased [18] and they are resistant to enzymes such as peptidases, nucleases or proteases [19]. Another class of nucleic acid analogs that needs mentioning are phosphorodiamidite morpholino oligomers (PMOs or Morpholino)- species where the negatively charged phosphorodiester internucleoside linkage is replaced by the neutral phosphorodiamidate internucleoside linkage and the five-membered ring of (deoxy)ribose is replaced by the six-membered ring of morpholine [20]. Importantly, both PNAs and PMOs have been shown to display antisense activity which makes them excellent tools for gene function studies and potential therapeutic agents [21,22].

Functionalization with thiols/disulfides is carried out chemically either upon oligonucleotide synthesis or post-synthetically. By "oligonucleotide synthesis" we intend to understand conventional solid phase synthesis, using commonly established methods and building blocks (in most cases phosphoramidites and/or H-phosphonates and this is how they will be depicted in the majority of figures and schemes). Despite several other approaches having been developed over the years, the phosphoramidite method of oligonucleotides synthesis remains the most widely used, due to its overall low cost, simplicity and excellent yields of products. "Post-synthetic functionalization" will hereafter refer to reactions which take place either upon or after oligonucleotide's cleavage from the support and deprotection. This so to say "temporary" distinction is one approach that could be used to systematize the literature data on the subject.

Yet another criterion commonly used to discuss oligonucleotide modifications is "spatial" or "topological", i.e. focusing on exact position of thiol/disulfide incorporation in a oligonucleotide strand and resulting in 5' and 3' terminal modifications and internal modifications (Fig. 2a).

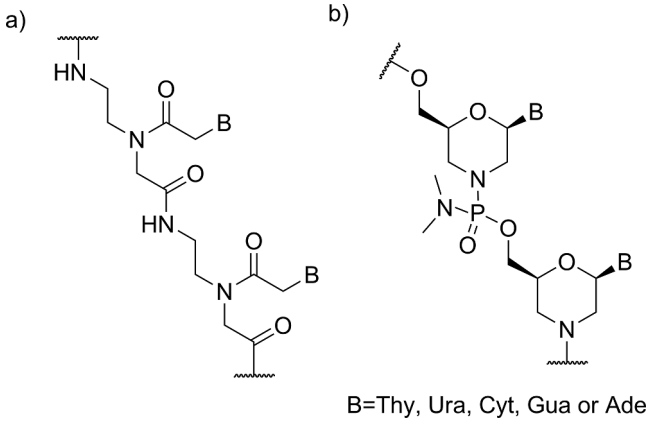

Fig. 1. Schematic structure of PNA (a) and PMO (b).

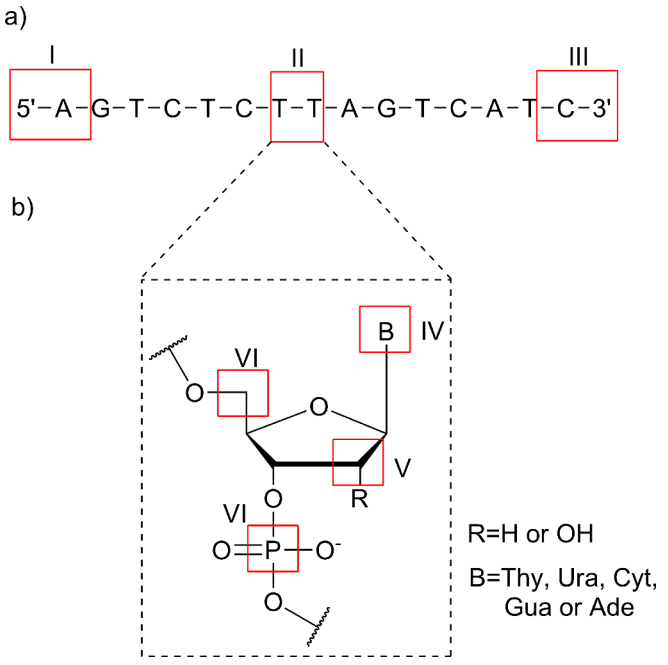

Fig. 2. Scheme representing possible modification sites of an exemplary oligonucleotide strand (a) and of an individual DNA/RNA nucleotide (b) with a thiol/disulfide tether. Indicative positions are marked with red boxes: I- 5′ end modifications; II- internal modifications; III- 3′ end modifications; IV- nucleobase modifications; V- sugar ring modifications; VI- internucleoside linkage/phosphate group modifications.

Internal modifications on a single nucleotide level can be further separated into modifications of sugar backbone, nucleobase or internucleoside linkage (Fig. 2b). Moreover, the exact position and structure (i.e. length, bulkiness, protecting group, additional functional groups) of thiol/disulfide tether depends heavily on intended application, so this subject can be also arranged from a "functional" point of view.

Conjugates of oligonucleotides with other species can be in turn systematized taking into account either topology (as head-on conjugates [23,24] or as side-on conjugates [25,26]) or nature of the cross-linked partner: other nucleic acid (including intrastrand cross-linking), peptide, protein and such.

In practice, clear separation of all these approaches is not always possible and desirable as it would lead to artificial and forced divisions. In this review we will therefore use these criteria interchangeably, in order to facilitate organization and understanding of the topic.

2.1. 5′ terminal strand modifications

The very first thiol modifiers for oligonucleotides were presented by Connolly et al. in 1985 in a form of S-trityl-O-methoxymorpholinophosphine alkyl mercaptans (Fig. 3a) [24].

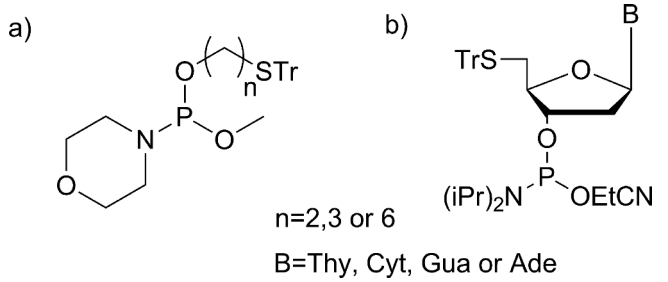

Fig. 3. Morpholinophosphine introduced by Connolly [24] (a) and 5′-S-trityl thiol-nucleoside phosphoramidite developed by Sproat et al. [27] (b).

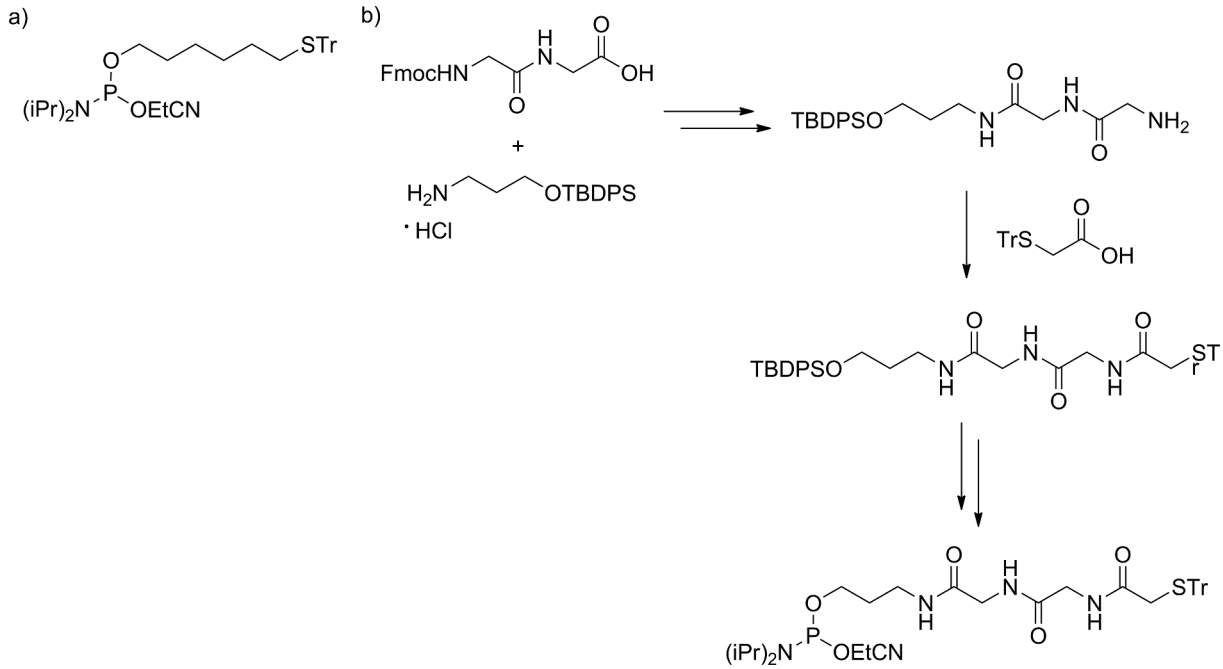

Fig. 4. Commercially available S-trityl-6-mercaptohexyl phosphoramidite (a). Synthesis of S-Trityl-(2-mercaptoacetyl-glycyl-glycyl)-3-aminopropyl phosphoramidite [30] (b).

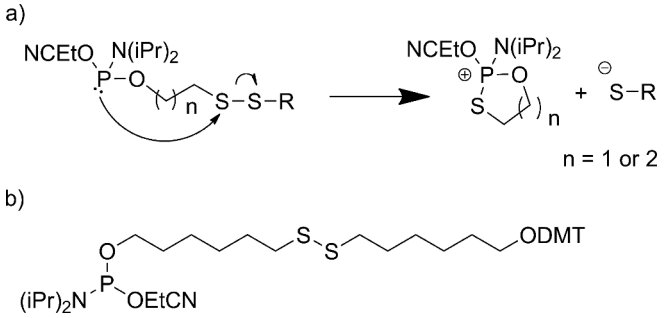

Fig. 5. Putative mechanism of disulfide linker-modified phosphoramidite decomposition via intramolecular cyclization [31] (a). C6 S—S thiol modifier (b).

Morpholinophosphine- building blocks were initially used in phosphoramidite oligonucleotide synthesis, however over the years O-(2-cyanoethyl)-N,N-diisopropylphosphoramidites took the lead in this method [27]. Connolly's compounds were primarily designed to attach thiol-specific fluorescent probes at the 5′ end of an oligonucleotide strand. Some years later, Sproat et al. developed a series of nucleoside phosphoramidites with a Tr-protected thiol moiety at 5′-deoxyribose position (Fig. 3b), which could be attached at the 5′ end of an oligonucleotide during the final step of synthesis and serve the same purpose [27]. Certain phosphoramidites of this kind are currently commercially available.

Because Connolly's approach is operationally much simpler than Sproat's (since it involves preparation of one universal reagent versus at least 4 different ones), it was perfected over the years and led to the creation of an S-trityl-6-mercaptopropyl and S-trityl-6-mercaptohexyl phosphoramidites [28], of which the latter is available from commercial suppliers and typically referred to as "thiol-modifier C6" [29] (Fig. 4a). It remains one of the most widely used, despite having the form of sticky, viscous oil. More recently, other related compounds have been reported, such as e.g. S-Trityl-(2-mercaptoacetyl-glycyl-glycyl)-3-aminopropyl phosphoramidite [30] (Fig. 4b) serving the same purpose and much more convenient to handle due to its crystalline nature.

In contrast, preparation of phosphoramidites which instead of protected thiol comprise a disulfide bridge suffers a major issue since the

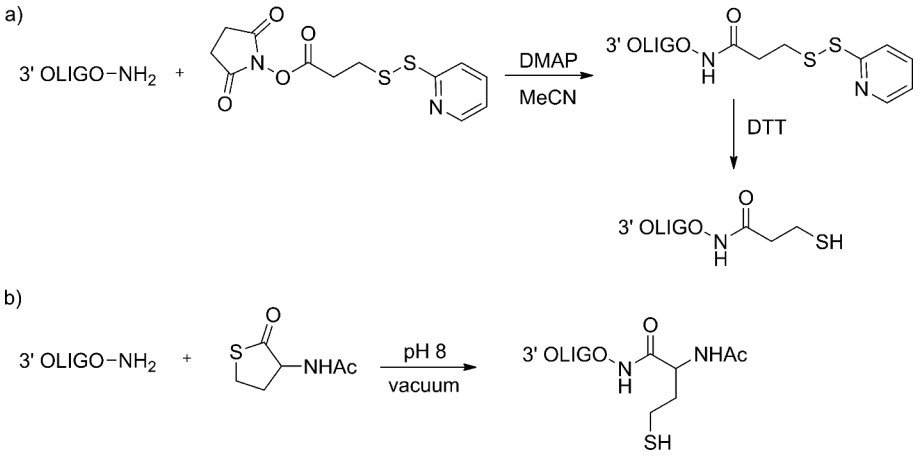

Fig. 6. 5′ thiol tethers introduced by Gaur et al. [32] (a) and Kumar et al. [33] (b).

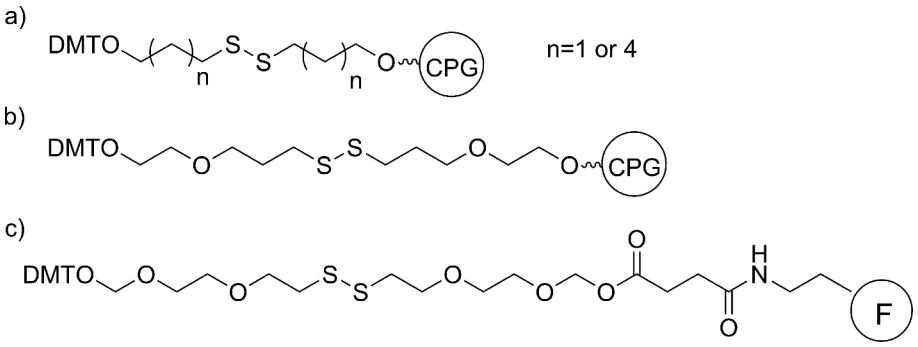

Fig. 7. Commercially available modified CPG solid supports functionalized with 3 or 6 carbon alkyl disulfide (a), 3-oxahexyl disulfide (b) linkers. Fractosil 500 support with a "disulfide arm" linker for 3' thiol-modified oligonucleotide synthesis [34] (c).

phosphite triester function can intramolecularly attack one of the sulfur atoms (Fig. 5a) [31]. Cyclization proceeds all the more readily if five- or six-membered rings are created, leading to decomposition. Introduction of long alkyl chains was therefore required to enhance the stability of disulfide-bearing phosphoramidite, resulting in creation of so-called "C6 S—S thiol modifier" (Fig. 5b).

The concept of 5′-thiol terminated nucleic acids gained interest rapidly and over the years post-synthetic methods of modifications have been established as well. Gaur et al. functionalized 5′-amino terminated oligonucleotide with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) [32] (Fig. 6a), while Kumar et al. exploited reaction between similar 5′-amino terminated oligonucleotide and *N*-acetyl-DL-homocysteine-thiolactone [33] (Fig. 6b). Whereas in the latter method reactive thiol species at oligonucleotide's 5′ terminus was obtained directly, in the former case additional step of reductive cleavage of disulfide bond was required. However, neither group reported attempts of bioconjugation of so-prepared modified oligonucleotides.

2.2. 3′ terminal strand modifications

Since the solid phase synthesis of oligonucleotides occurs in direction from the 3′ to the 5′ end, in order to functionalize the oligonucleotide on the 3′ terminus it is most convenient to possess the thiol (or any other modification, for that matter) incorporated directly onto the solid support. Nowadays solid supports pre-functionalized with 3 or 6 carbon disulfide spacers are commercially available and widely employed due to their handiness (Fig. 7a and b). Historically speaking though, there were a few other attempts that need to be mentioned.

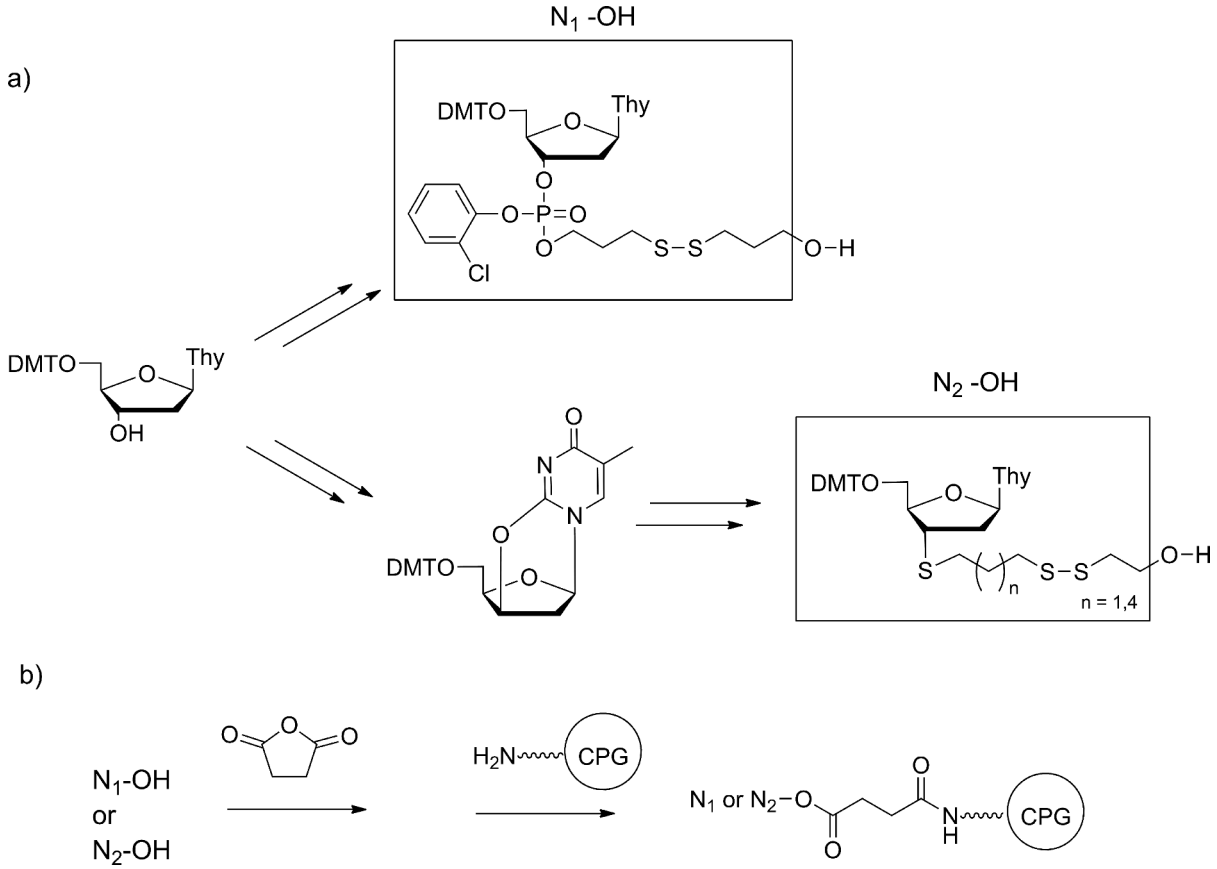

Fig. 8. Stepwise synthesis of CPG solid support functionalized with thiol bearing thymidine [23]. Synthesis of modified thymidine nucleosides (a) and their attachment to amino-CPG (b).

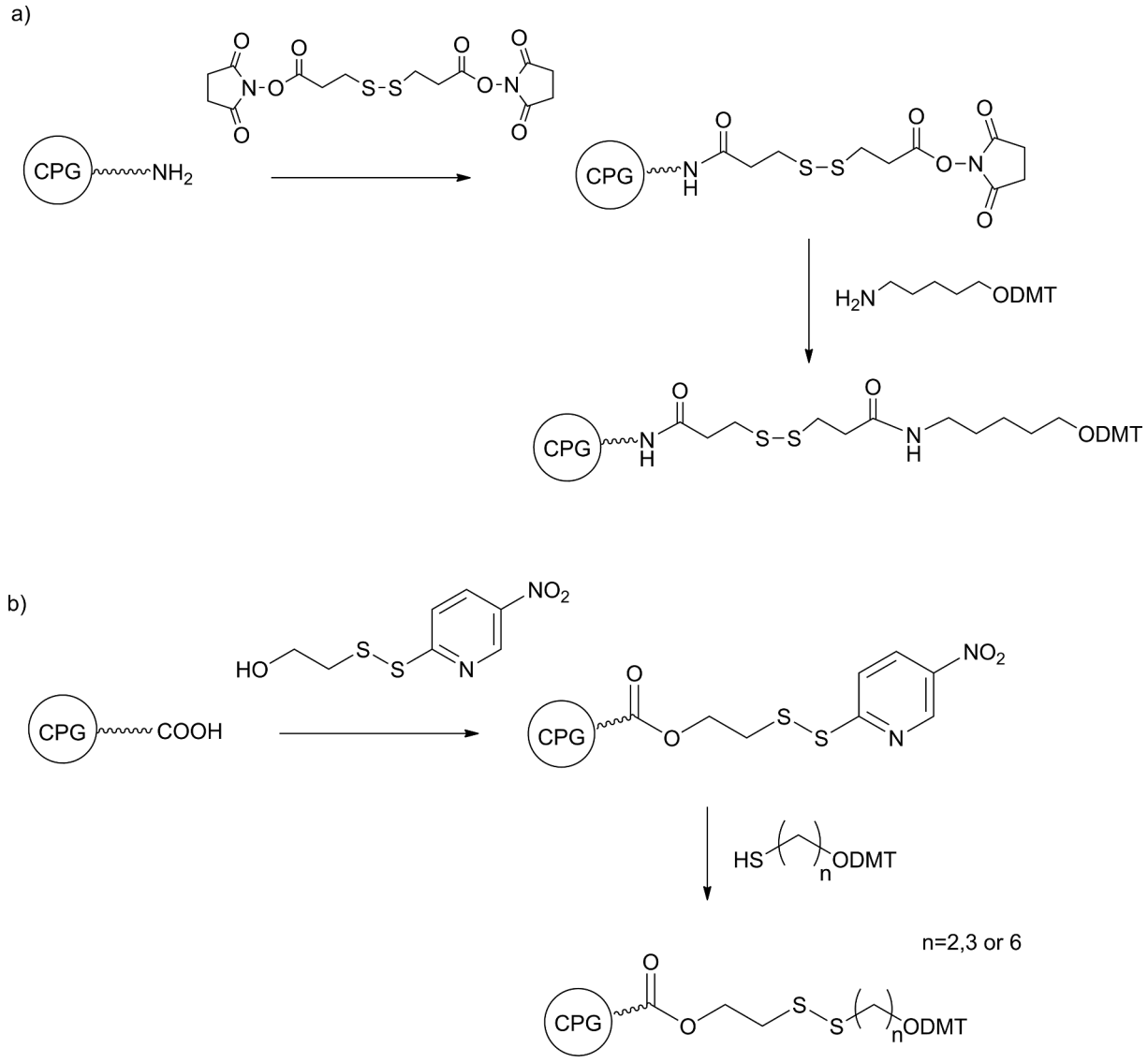

Fig. 9. Synthesis of universal supports with disulfide linkers by Gupta et al. [35,36].

Zuckermann et al. addressed this issue by preparing a CPG support functionalized with alkyldithioethanol thymidine derivatives [23]. Importantly, the thiol species was either attached directly to a sugar backbone in C3′ position or to a phosphorus center. Then the thiol-bearing nucleoside was succinylated and coupled to aminopropylated CPG (Fig. 8). Such modified supports were then used in standard phosphotriester or phosphoramidite syntheses.

The concept, however, seemed needlessly laborious and limiting (since every synthesized strand was bound to bear thymidine at 3′ end) and was soon simplified by Gupta et al. [35,36]. They have created a series of universal solid supports with disulfide linkages. The initial strategy was based on conjugation of 3,3′-dithiobis-(*N*-succinimidyl propionate) to 3′-aminopropylated CPG and subsequent attachment of 4,4′-dimethoxytrityloxypentylamine (Fig. 9a). Such modified support was ready to use in standard phosphoramidite synthesis. In another attempt, a CPG support was succinylated first and then conjugated with *S*-(2-thio-5-nitropyridyl)-2-mercaptoethanol and the product was subjected to thiol/disulfide exchange with O-DMT protected mercaptoalcohols of different carbon chain lengths (Fig. 9b). Oligonucleotides synthesized on both types of supports could be cleaved either under standard basic conditions (typically incubation in aqueous ammonia at

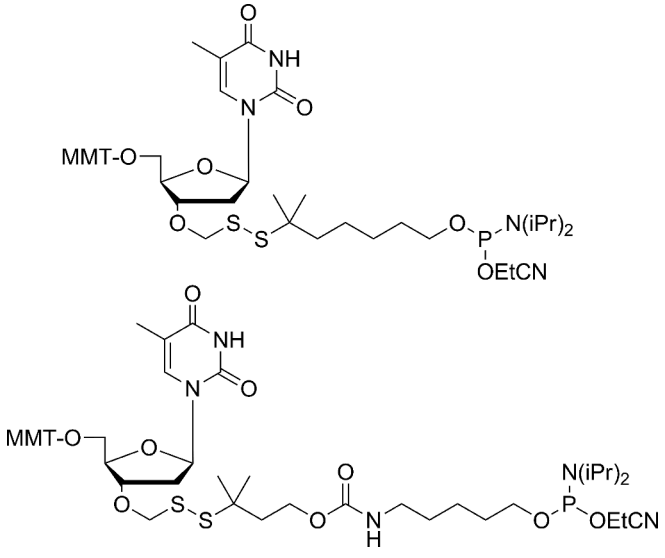

Fig. 10. Phosphoramidites obtained by Semenyuk and Kwiatkowski [31].

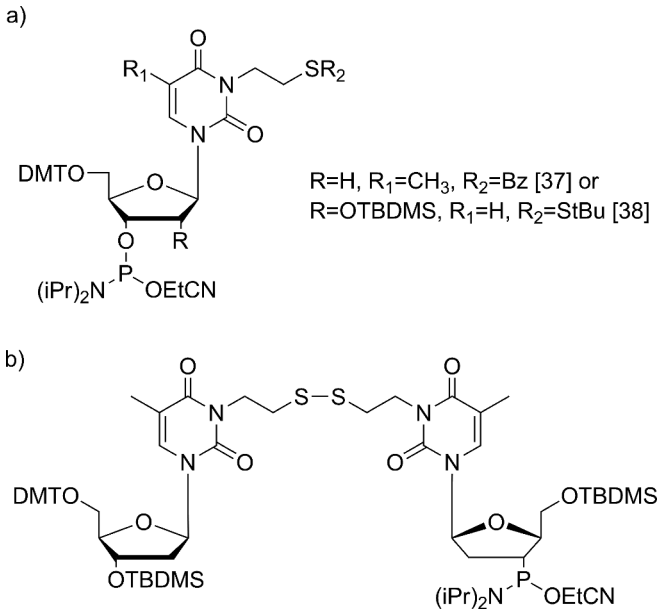

Fig. 11. N3-Bz-mercaptoethyl deoxythymidine [37] and N3-tBu-S-ethyldisulfide uridine [38] (a) and deoxythymidine dimer synthesized by Osborne and Ellington [39] (b).

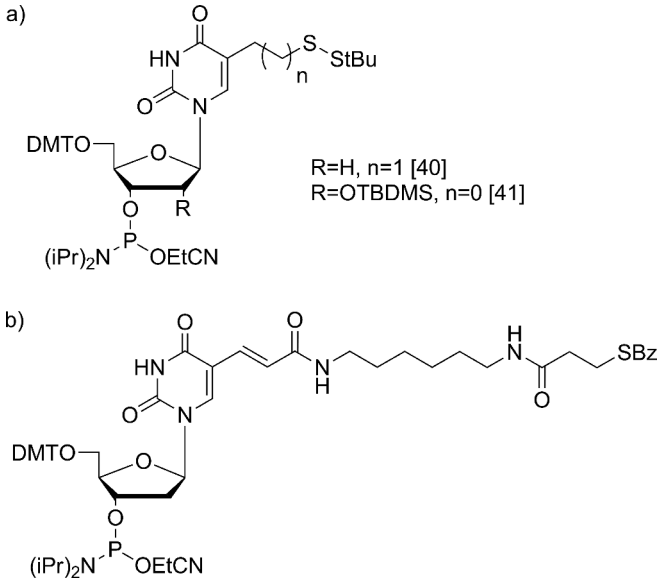

Fig. 12. C5-tBu-S-ethyldisulfide deoxyuridine [40] and C5-tBu-S-methyldisulfide uridine [41] (a). Commercially available S-Bz-Thiol Modifier C6-dT (b).

elevated temperatures) to yield a disulfide at the 3′ end or directly to the free thiol by reduction, e.g. with DTT.

Independently, Bonfils and Thuong developed another universal solid support [34]. They modified the aminopropyl Fractosil 500 solid support with a succinylated polyether linker called “disulfide arm” (Fig. 7c). Analogous to the case described above, this support could be used for standard synthesis of oligonucleotides, and the product cleaved afterwards either under basic conditions or by disulfide reduction.

In contrast, Semenyuk and Kwiatkowski synthesized deoxythymidine phosphoramidites bearing a disulfide-containing linker insusceptible to hydrolysis even in harsh basic conditions (Fig. 10) [31]. Incorporation of the tether in 3′ position enabled direct attachment to polystyrene support and its consecutive application in standard oligonucleotide synthesis. Its distinct ability to withstand basic hydrolysis allows efficient removal of failure sequences coming from cleavage of abasic (depurinated) sites and thus guarantees obtainment of high-purity synthesis products. Nevertheless, just as in Zuckermann support’s case, the use of this linker is severely limited to sequences possessing thymidine at 3′ end.

2.3. Internal strand modifications

2.3.1. Nucleobase modifications

In the early ‘90 s researchers focused on structural studies of conformationally restrained nucleic acid hairpins. Disulfide bridge between neighboring residues (either intra- or interstrand) was one of structural motifs exploited to stabilize conformations of DNA and RNA structures of interest. The concept entailed overcoming several challenges: thiol/disulfide tethers had to be first introduced synthetically onto nucleobases in appropriate positions, then phosphoramidites had to be prepared and incorporated into oligonucleotide strands upon solid state synthesis and finally efficient crosslink formation was required.

2.3.1.1. Pyrimidine nucleosides. In a pivotal study, a thymidine bearing Bz-mercaptoethyl tether at N3 position was designed, synthesized and attached at both ends of the oligonucleotide strand of interest (Fig. 11a) [37]. Thiol placement at N3 of pyrimidine was deliberately chosen as in a DNA duplex this position faces toward the center of the helix. After Bz deprotection and upon air oxidation liberated thiols formed an intramolecular disulfide bridge and hence stabilized the hairpin without disrupting the native geometry of the helix stem. A corresponding RNA building block: uridine phosphoramidite, with a *tert*-butyl-protected disulfide moiety (Fig. 11a) [38] was prepared and applied analogously. Later on, in order to increase the overall yield of disulfide cross-linking, Osborne and Ellington prepared a deoxythymidine dimer joined via disulfide bridge (Fig. 11b) that allowed synthesis of terminally pre-cross-linked dsDNA [39].

Another position in pyrimidine ring that has been of interest is C5. This is because modifications attached at C5 are located in the major groove of duplex B-DNA and do not disrupt Watson-Crick base pairing.

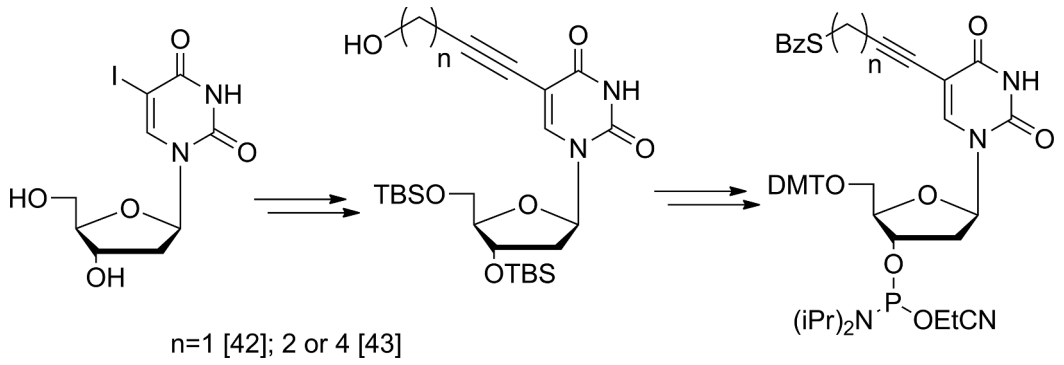

Fig. 13. C5-Bz-thiopropyl thymidine synthesized by Chaudhuri and Kool [42], and Prestinari and Richert [43].

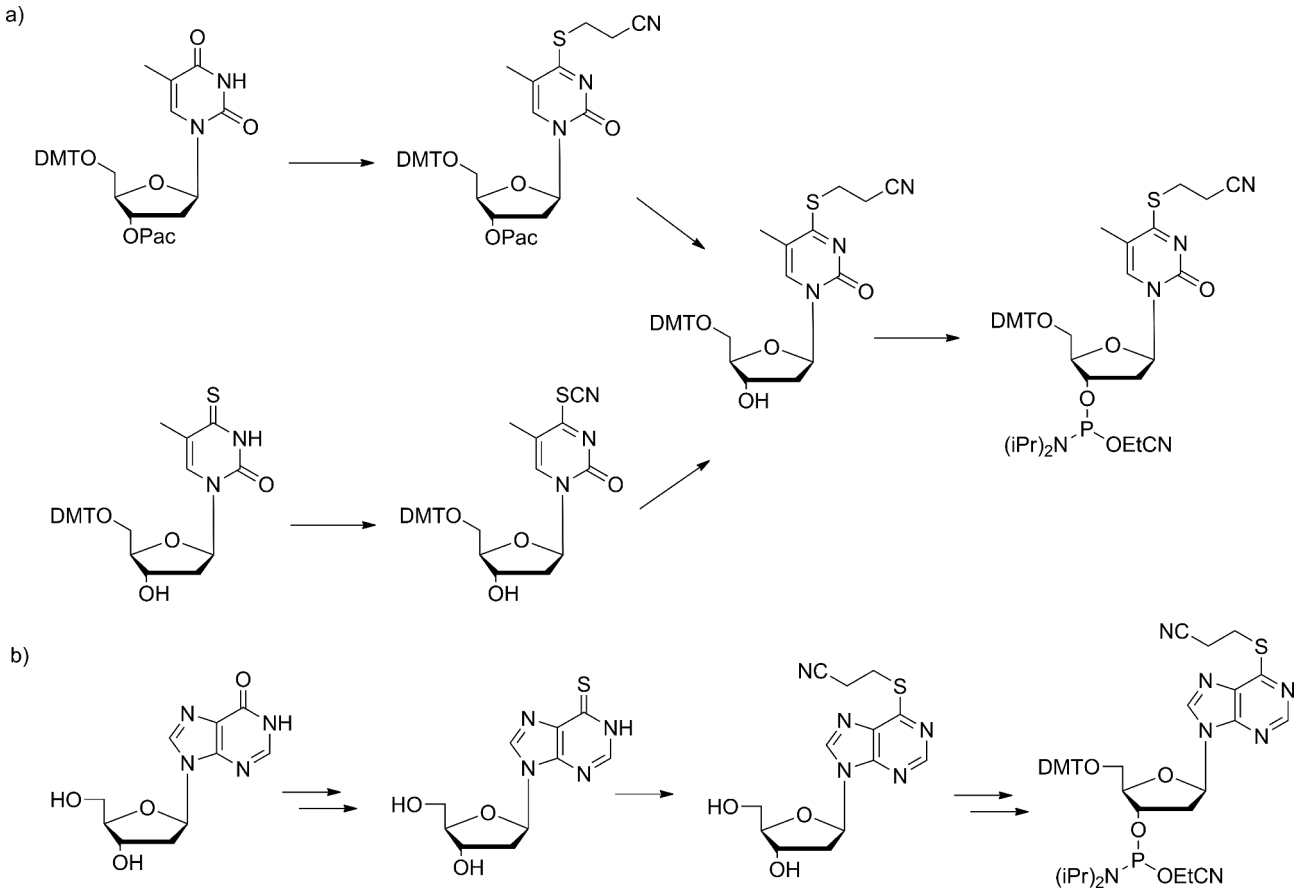

Fig. 14. Synthetic pathways to protected 6-thiodeoxythymidine [45,46] (a) and 4-thiodeoxyinosine [44] (b) phosphoramidites adapted by Milton et al. [47].

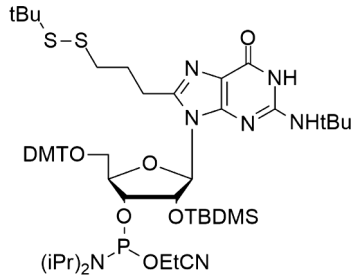

Fig. 15. C8-propyl-tert-butyl disulfide guanosine phosphoramidite synthesized by Gundlach et al. [48].

Alkylthiol homologs of deoxyuridine and uridine in a form of phosphoramidites have been proposed by Goodwin and Glick [40] and by Sun et al. [41], respectively (Fig. 12a). Similar compound, although with much longer linker, known under name "S-Bz-Thiol-Modifier C6-dT" is commercially available (Fig. 12b).

Chaudhuri and Kool stabilized non-canonical triplex DNA structure using disulfide cross-linking between two of the three oligonucleotide strands [42]. To this purpose, they synthesized 5-Bz-thiopropynyl-deoxyuridine phosphoramidite from 5 to iododeoxyuridine (Fig. 13) and incorporated it into DNA strands. After deprotection of thiol tethers a disulfide bond between two such species on opposite strands was formed. Similar phosphoramidites were obtained by Prestinari and Richert, who attached thiobutynyl and thiohexynyl linkers at C5 of deoxythymidine [43]. In this case however, when two modified nucleosides were placed adjacently in the oligonucleotide strand, an intrastrand disulfide cross-link was spontaneously formed.

Although methods of synthesis of cyanoethyl-protected 6-thiodeoxyinosine [44] and 4-thiodeoxythymidine [45,46] were established early, such species served mainly as intermediates in the synthesis of other nucleoside derivatives. Milton et al. used them to cross-link a self-complementary DNA sequence [47]. Incorporation of both 4-thio-dT (Fig. 14a) and 6-thio-dI (Fig. 14b) into an oligonucleotide strand, on penultimate positions from 3′ and 5′ termini, allowed the formation of a DNA duplex covalently stabilized through disulfide bridge.

2.3.1.2. Purine nucleosides. Much less attention has been given to thiol incorporation into purine nucleosides because it is synthetically more challenging. However, Gundlach et al. demonstrated viable synthetic route to C8-substituted thioalkylguanosines (Fig. 15) [48].

More recently, Hou et al. reported synthesis of (deoxy)guanosine phosphoramidite carrying a thiopropyl tether masked as *tert*-butyl disulfide at N2 [49,50] (Fig. 16). This modification has been designed for crosslinking from the minor groove of either DNA or RNA to proteins or other nucleic acids and as such it was successfully used for covalent binding and interaction studies between mutant reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1), DNA and nucleoside analog RT drug [51].

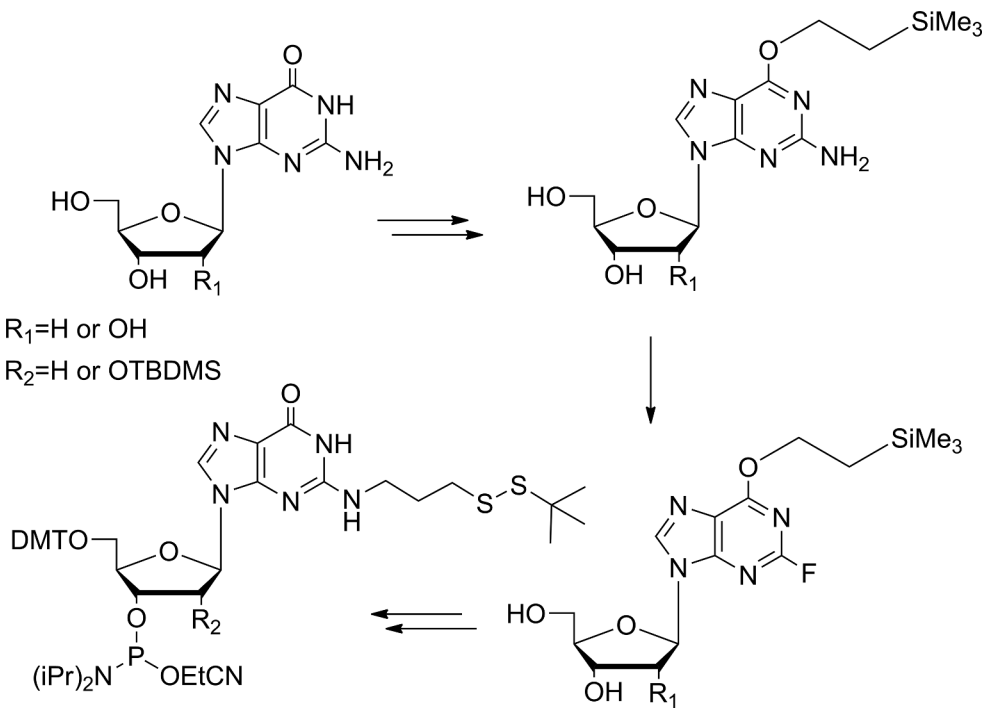

Fig. 16. N3-3-aminopropyl-tert-butyl disulfide guanosine phosphoramidite developed by Hou et al. [49].

2.3.1.3. Convertible nucleosides. All the above-mentioned modification methods rely on the preparation of phosphoramidite building blocks comprising a thiol linker prior to the oligonucleotide synthesis. In contrast, "convertible nucleoside" approach allows one-step post-synthetic functionalization of entire oligonucleotide strand with a wide range of linkers [52]. This elegant, versatile method was first proposed by Macmillan and Verdine in 1990 [53,54]. Initially applicable to pyrimidine nucleobases, namely deoxyuridine, it was quickly adapted to purines too (deoxyinosine) [55]. In the first step, phosphoramidites with good leaving groups on exocyclic functional groups of nucleobases (usually phenolates or triazoles) are prepared. The resulting products, e.g. O4-aryl-dU or O6-aryl-dI are incorporated in the sequence during the solid phase synthesis and the oligonucleotide is then cleaved from the support under standard conditions. Nucleophilic tethers of choice (aliphatic amines of adjustable carbon chain length and bearing various other functional groups) may be then anchored to a functionalized nucleobase upon aminolysis. This reaction requires elevated temperatures (55–65 °C) and up to 16 h to reach completion and yields oligonucleotides comprising N4-aminoalkyl-dC or N6-aminoalkyl-dA, respectively. Disulfide-modified sequences were obtained in this way when the cystamine and its homologs were used as nucleophilic linkers (Fig. 17a and b). Later on, post-synthetic method of functionalization of oligonucleotides comprising 2-fluorodeoxyinosine was introduced (Fig. 14c) [56,57] and over the time, analogous strategy was developed for modification of RNA nucleosides [52]. In turn, Ueno et al. obtained a 2'-deoxyuridine with 5-trifluoroethoxycarbonyl moiety, which could be functionalized, e.g. with SPDP in analogous, post-synthetic manner [58]. Nowadays, all DNA and RNA (except for U) building blocks for convertible nucleoside approach synthesis are available commercially.

2.3.2. Phosphate group modifications & other internucleoside linkages

In order to prepare 5′ thiol-modified oligonucleotides for head-on chemical ligation, Chu and Orgel first produced a deoxyoligonucleotide with a phosphate group at 5′ terminus and then converted it into a phosphoramidate by treatment with cystamine in the presence of *N*-methylimidazole and EDC (Fig. 18) [59]. If two DNA strands were functionalized in this fashion, the disulfide bond reduction with DTT followed by air oxidation resulted in head-on ligated sequences. Whenever a high specificity of ligation was required (to avoid the formation of a mixture of homo- and heterodimers), thiol exchange reaction was preferentially employed and, consequently, one of the DNA strands had to be converted into a 2,2′-dipirydyl disulfide derivative first. Oligonucleotides (both DNAs and RNAs) thiolated in such a manner were also shown to efficiently bind peptides and proteins (e.g. bradykinin) and functional small molecules like biotin [60,61].

Fidanza and McLaughlin noticed the potential limitations of this method and expanded it so as to obtain oligodeoxynucleotides that could be modified with a disulfide linker at virtually any position in the sequence via a semi-automatic approach [26]. In principle, the sequence is synthesized automatically using phosphoramidite chemistry until the desired site of modification is reached. Then *H*-phosphonate of an appropriate nucleoside is coupled manually to the existing oligomer, followed by oxidative coupling with cystamine, to yield a phosphoramidate bearing a disulfide tether (Fig. 19). Further elongation of such modified sequence is then possible on a synthesizer. It is noteworthy that, through attachment of a disulfide tether, the phosphorus atom becomes the center of chirality (with Rp and Sp stereoisomers). Such oligonucleotides, after reduction of the cystamine disulfide linker to a free thiol, were primarily used for attachment of thiol-specific probes and also to stabilize DNA duplexes.

Yet another approach relied on use of phosphorothioates (PTOs):

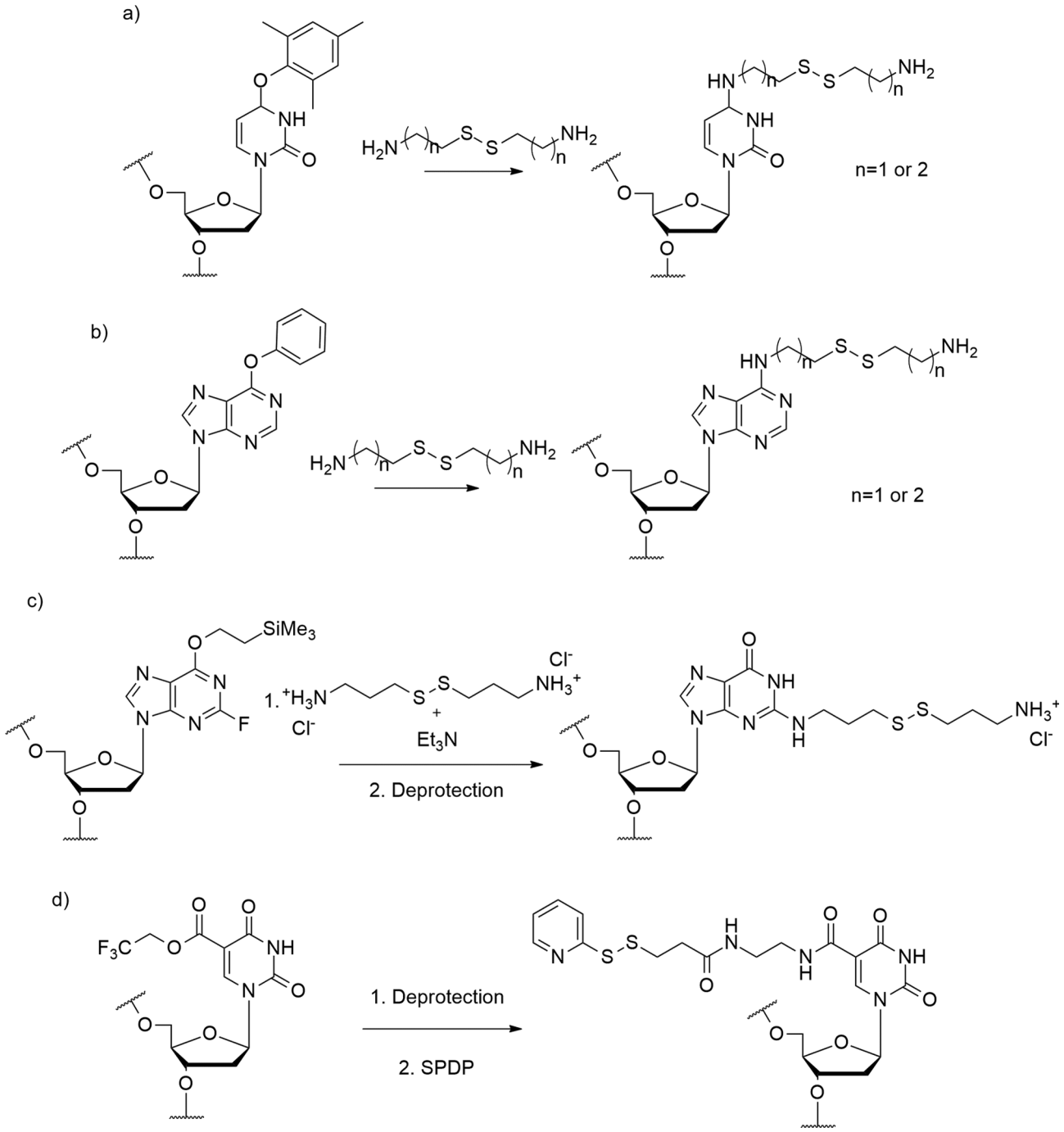

Fig. 17. Post-synthetic disulfide functionalization of oligonucleotides using convertible nucleosides: an O4-aryl-deoxyuridine [53] (a), an O6-aryl-deoxyinosine [55] (b), a 2-fluorodeoxyinosine [56] (c) and a 5-trifluoro-ethoxycarbonyl-2′-deoxyuridine [58] (d).

nucleic acid analogues in which one of the oxygens of the phosphate group is replaced with a sulfur atom. Phosphorothioates are mainly used as oligonucleotide derivatives which are more enzymatically resistant than native structures [62]. Although it is not their primary application, they can be used for cross-linking between two oligonucleotide strands if they are placed exclusively in terminal positions, thus yielding a single elongated sequence [63]. Moreover, there were reported attempts to use bisphosphorothioates of individual nucleosides as substrates for oligonucleotide analog synthesis via disulfide bond formation [64]. Unfortunately this approach was not successful, since bisphosphorothioates preferentially underwent intramolecular cyclization rather than desired oligomer formation (Fig. 20). Only when already prepared 5′-phosphorothioate poly-adenosine 12-mer was incubated with an excess of adenosine bisphosphorothioate in the presence of an oxidizing agent, an extension of the strand of up to 5 nucleotides was observed.

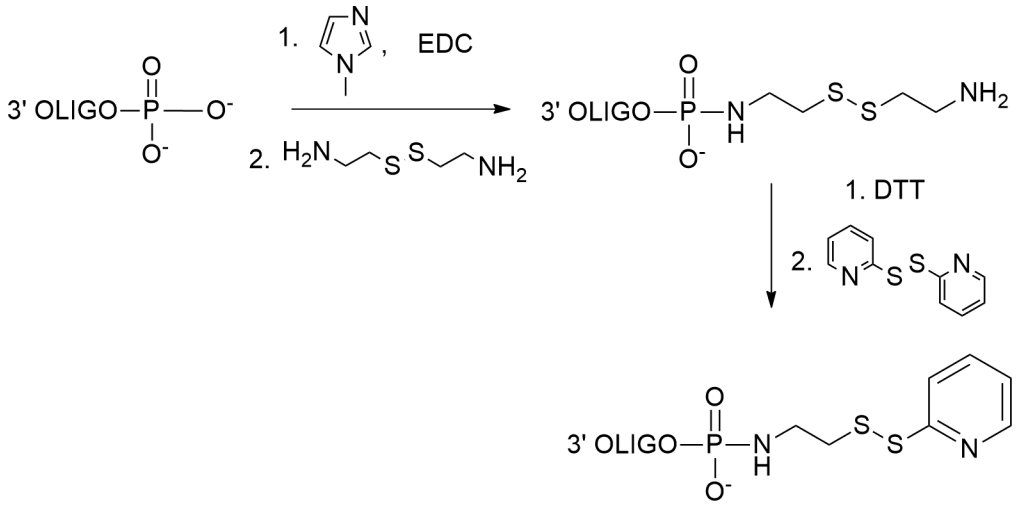

Fig. 18. Functionalization of 5′-phosphorylated oligonucleotide with cystamine and subsequent activation with 2,2′-dipyridyl disulphide [59].

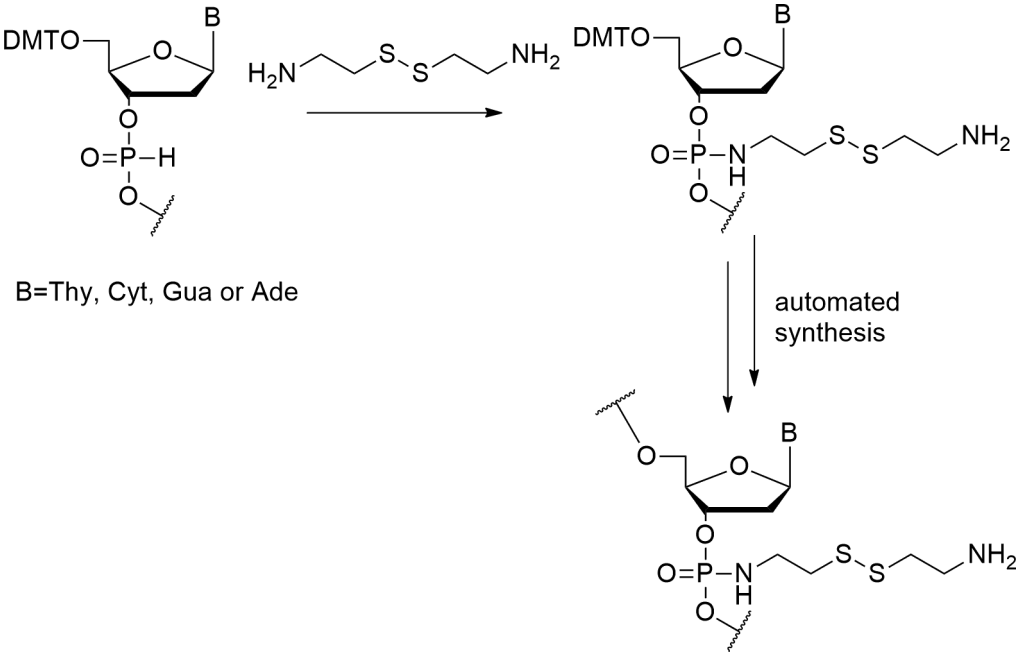

Fig. 19. Modification of a phosphorus center with cystamine introduced by Fidanza and McLaughlin [26].

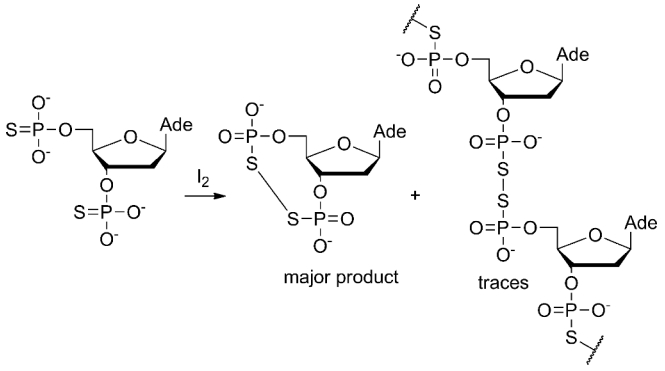

Fig. 20. Cyclization and oligomerization of adenosine bisphosphorothioate [64].

Metelev et al. pointed out that a disulfide bridge formed between 3′ terminal phosphorothioate of one DNA strand and 5′ terminal thiol group of the other would mimic the native phosphodiester moiety better (Fig. 21a) [65]. The resulting bond was referred to as a "pss-linkage" to stress the structural difference from the —P—S—S—P— bond between two phosphorothioates. Moreover, the authors experimentally confirmed the selectivity of thiolate nucleophilic attack during the thiol exchange reaction of the pss-linkage, with release of the more acidic thiol and retention of the less acidic one in the new disulfide (Fig. 21b) [66].

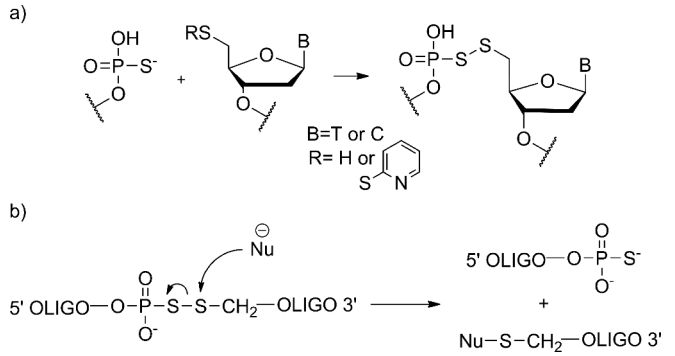

Fig. 21. Ligation of two oligonucleotide strands via disulfide bridge formation between terminal phosphorothioate and a C5-thiol (a) and mechanism of nucleophilic attack on the disulfide bond [65].

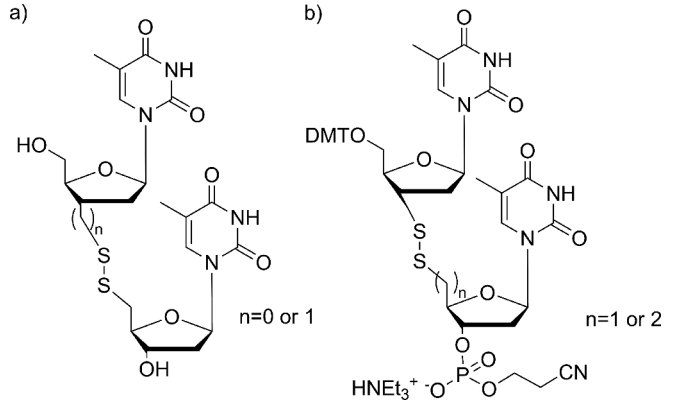

Fig. 22. Thymidine dinucleoside obtained by Witch and Cosstick [67] comprising a disulfide bond instead of phosphodiester group and corresponding dinucleoside phosphate synthesized by Hansen et al. [68].

In order to increase antisense oligonucleotides' resistance towards nucleases and affinity to RNA targets, Witch and Cosstick proposed to replace internucleoside phosphodiester groups with linkages containing disulfide bridges: non-ionic, non-chiral and insusceptible to hydrolysis [67]. Two thymidine dinucleosides were obtained (Fig. 22a) but no attempts at oligonucleotide synthesis were reported. Recently, Hansen et al. developed this concept further and presented analogous thymidine dinucleoside phosphate monomers (Fig. 22b) with 3′–5′ and 3′–6′ disulfide linkages replacing a phosphodiester which were readily incorporated into the DNA strand upon synthesis [68]. It is worth noticing that 3′–5′ disulfide linkages can be also obtained post-synthetically upon chemical ligation of 3′-thio- and 5′-thio-terminated sequences [69].

2.3.3. Sugar ring modifications

While several positions of the (deoxy)ribose ring are available for introduction of modifications, C2′ is the one most frequently employed because it offers the possibility of less interference with base pairing and/or stacking interactions as well as positioning the modifications in the minor groove of the duplex.

Initially, Hamm and Piccirilli attempted substitution of 2′ hydroxyl group of RNA by a thiol [70]. The synthesis of appropriately protected phosphoramidites: 2′-deoxy-2′-mercaptocytidine and 2′-deoxy-2′-mercaptouridine (Fig. 23) was presented as well as their incorporation into oligonucleotides. As long as thiol groups remained masked as 2′-tritylthio or 2′-(2-pyridyldithio) derivatives, oligonucleotides were stable in solution. Otherwise, they tended to undergo slow decomposition via intramolecular S_N attack by sulfur on the adjacent 1′-carbon of the ribose, cleaving the heterocyclic base. No results of bioconjugation were reported.

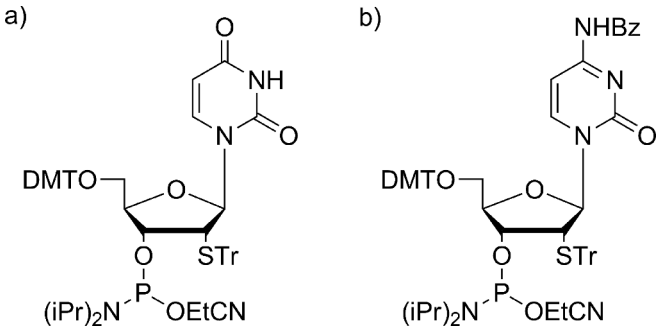

Fig. 23. 2′-deoxy-2′-S-Tr-mercapto uridine (a) and 2′-deoxy-2′-S-Tr-mercapto cytidine (b) obtained by Hamm and Piccirilli [70].

In turn, Manoharan et al. suggested attachment of thioether linkages to sugar rings at C2′. It was assumed that once introduced into the oligonucleotide sequence, they could increase the lipophilicity thereof (and in consequence improve cellular delivery), allow conjugation with thiol-reactive species and aid generation of constrained tertiary structures of nucleic acids via adjustment of the length of alkyl chain. On that account a 2′-O-(*S*-trityl-hexylthio)adenosine phosphoramidite was prepared (Fig. 24a) [71,72]. In the following years synthetic routes to phosphoramidites of cytidine [73] and guanine [48] were presented (Fig. 24b). Finally, in the early 2000 s' Jin et al. synthesized a complete set of RNA building blocks bearing such thioether linkages (Fig. 24a) [74]. Fujita et al. developed a method to detect the helix bending of DNA by the formation of a disulfide bond [75]. They reasoned that tritylthioether side chain at 2′ upper position of the sugar ring should point toward the complementary strand across the major groove of bent helix and participate in interstrand disulfide bond formation. For that reason a 2′-O-(4-mercaptobutyl)arabinofuranose phosphoramidite (Fig. 24c) was designed, prepared and incorporated into DNA strands.

2′-*O*-alkyldithiomethyl constitutes a particular case of thioether linkage as it can be treated as a temporary biolabile masking of a 2′-OH functionality (also referred to as a "protected protecting group" in literature). Although 2'-*O*-modifications confer efficient resistance to nucleases of oligonucleotides, they usually decrease activity of small interfering RNAs (siRNAs) and ribozymes. Introduction of 2'-O biolabile groups like e.g. ones comprising disulfide bridge helps to overcome low activity issues. In presence of intracellular glutathione, the disulfide of 2'-O-alkyldithiomethyl is cleaved, forming an unstable thiohemiacetal derivative which subsequently decomposes into native RNA (Fig. 25a)

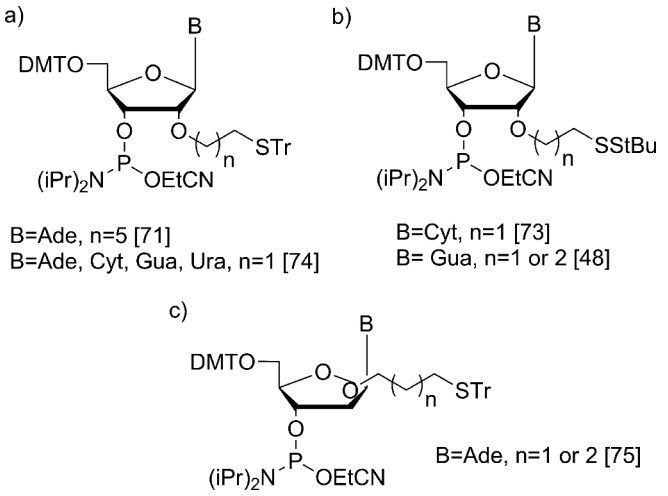

Fig. 24. Phosphoramidites bearing a 2′-O-thiol modifications synthesized by Manoharan et al. [71] and Jin et al. [74] (a), Goodwin et al. [73] and Gundlach et al. [48] (b), and phosphoramidite arabino-analogue by Fujita et al. [75] (c).

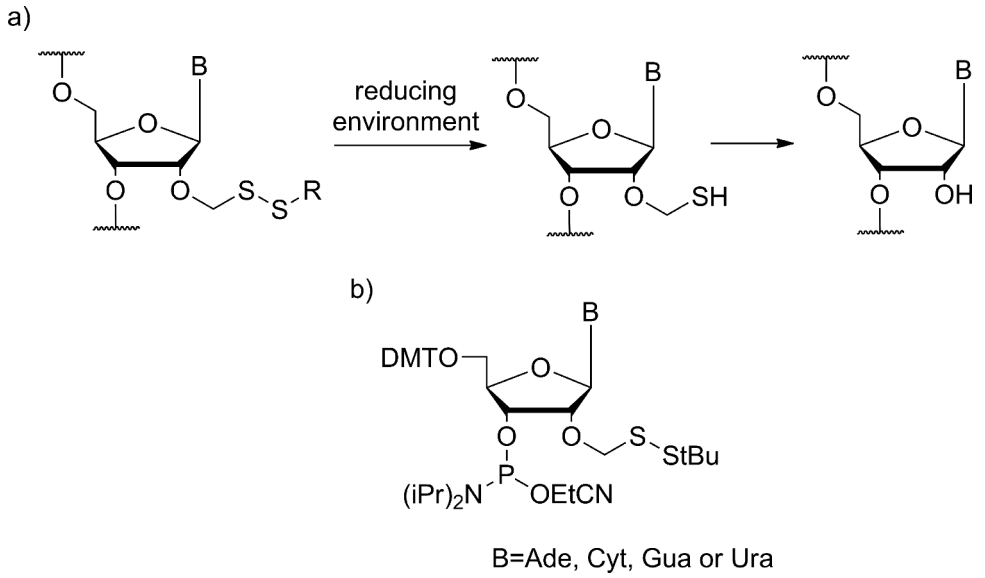

Fig. 25. Decomposition of 2′-O-alkyldithiomethyl moiety [76] (a) and 2′-O-tBu-dithiomethyl modified phosphoramidite obtained by Semenyuk et al. [77] (b).

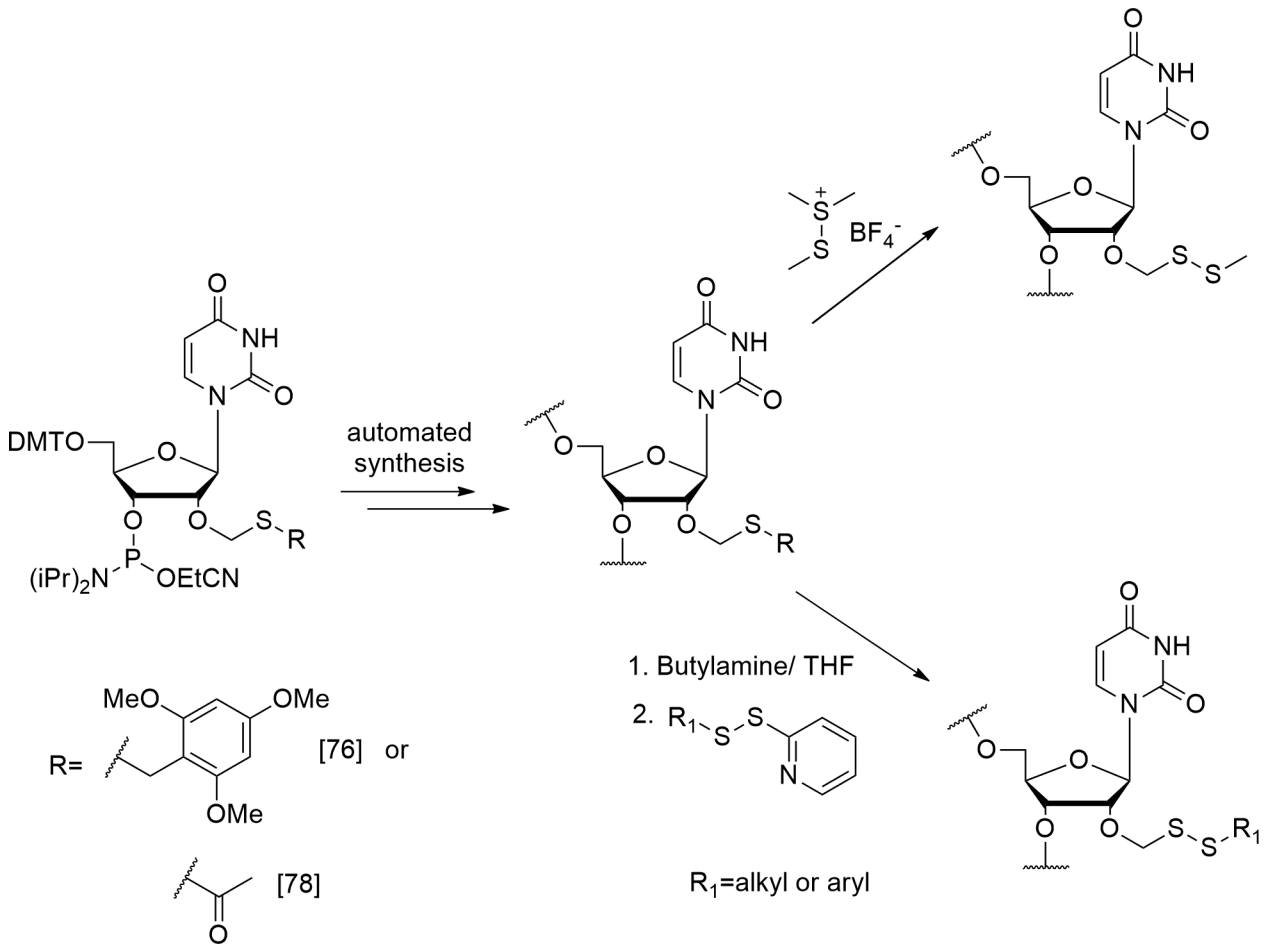

Fig. 26. Post-synthetic approaches to functionalization of oligonucleotides with 2′-O-alkyldithiomethyl tethers presented by Ochi et al. [76] (top) and Biscans et al. [78] (bottom).

[76]. Synthesis of all four phosphoramidite building blocks of this kind with 2'-*O*-*tert*-butyldithiomethyl (DTM) protection was first reported by Semenyuk et al. (Fig. 25b) [77]. These compounds were stable in solid state yet once dissolved in acetonitrile tended to decompose within 12–24 h. This instability was attributed to intramolecular attack of trivalent phosphorus on the disulfide bond.

More recently, Ochi et al. [76] and Biscans et al. [78] developed post-synthetic approaches for the synthesis of oligonucleotides with 2′-*O*-alkyldithiomethyl linkages. In the former case 2′-*O*-2,4,6-trimethoxybenzylthiomethyl (TMBTM) uridine phosphoramidite unit is prepared

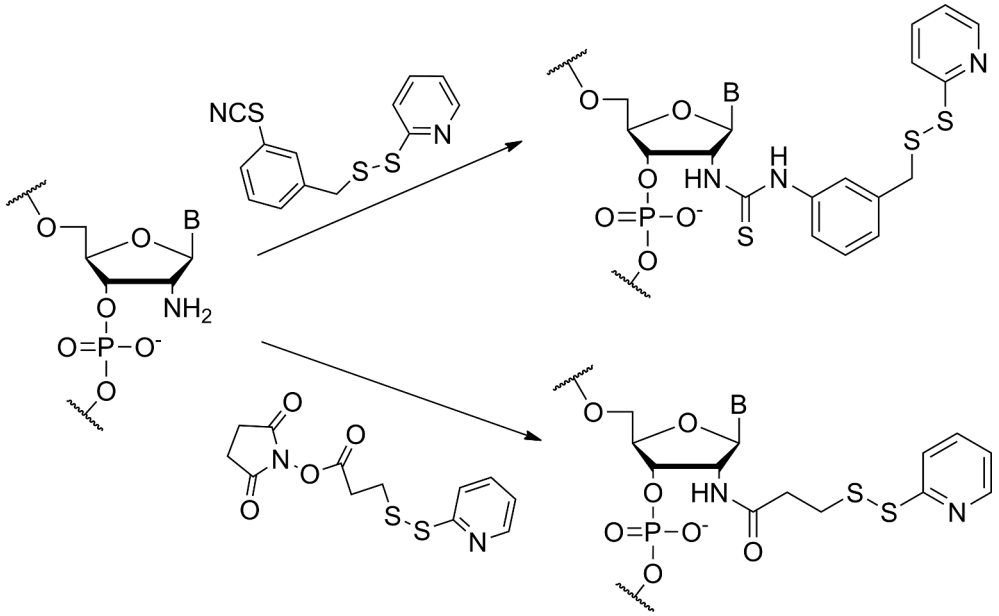

Fig. 27. Post-synthetic modifications of 2′-aminoribose position of a nucleotide: 2′ thiourea derivative by Sigurdsson et al. [81] (top) and 2′-O-(S-pirydyl-2-dithio) ethylamide derivative by Cohen and Cech [82] (bottom).

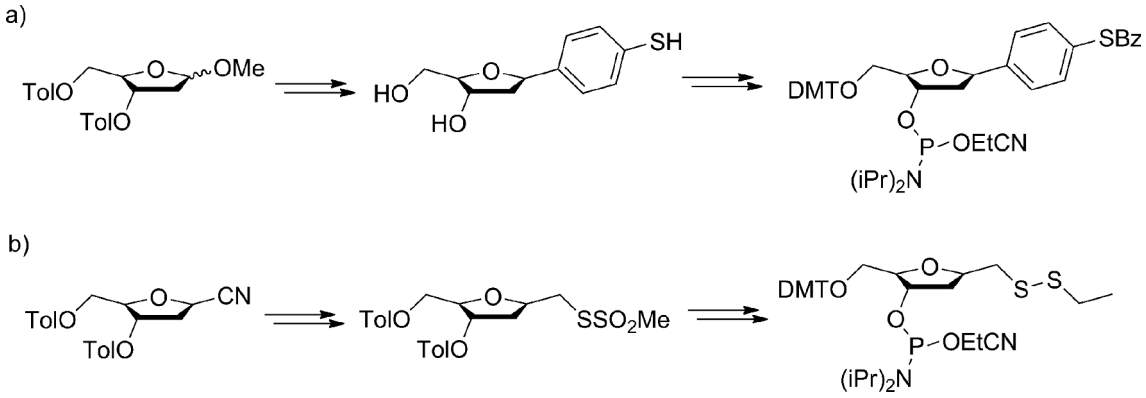

Fig. 28. Simplified synthesis of C1′-p-thiophenol derivatized deoxyribose phosphoramidite (a) [83] and C1′-methylene disulfide phosphoramidite (b) [84].

and introduced into the strand. After deprotection and cleavage of the product from the support, in the final step TMBTM is converted into the methyldithio group by treatment with dimethyl(methylthio)sulfonium tetrafluoroborate (DMTSF, Fig. 26 top). In the latter case, 2′-*O*-acetylthiomethyl uridine phosphoramidite serves as a building block. Treatment of synthesized oligonucleotide with butylamine in THF converts acetylthio groups into thiolate anions that undergo a thiol–disulfide exchange with an alkyldisulfanylpyridine (RSSPy) derivative. This approach allows functionalization of oligonucleotides with various polar or lipophilic groups. In addition, when 2′-*O*-acetylthiomethyl uridine units were placed in neighboring positions in the loop of an RNA hairpin and disulfide bridge between them was formed and conformationally locked the hairpin, increasing its thermal stability and nuclease resistance (Fig. 26 bottom) [79].

Other post-synthetic methods of thiol linkers introduction in C2′ position have been developed for studying structure and dynamics of large RNAs, since so-positioned tethers point towards the outside of the RNA helix [80]. In the structural studies of the hammerhead ribozyme, Sigurdsson et al. prepared two ribozymes in which two nucleotides (either C or U) carrying 2′-amino groups were reacted with disulfide-bearing isothiocyanates yielding thiourea derivatives (Fig. 27 top) [81]. S—S bridges were then reduced to free thiols and re-oxidized to cross-link modified nucleotides. For study of thermal motions dynamics in the role of catalytic RNA molecules, Cohen and Cech also used 2′-amino modification [82]. Their concept, though, was much simpler compared to that of Sigurdsson: 2′-amino nucleosides were derivatized post-synthetically with maleimido-disulfide pirydyl ester to yield 2′-*O*-(*S*-pirydyl-2-dithio)ethylamide tether (Fig. 27 bottom). When modifications in two RNA strands were positioned precisely across the minor groove of the helix, cross-linking between them occurred within minutes, but when they were separated by eight base pairs no cross-linking at all was observed. In this way conformational flexibility of the helix was verified.

Another interesting concept was developed by Hanato et al. who synthesized phosphoramidite equipped with a *p*-thiophenol attached at C1′ position instead of a native nucleobase (Fig. 28a) and incorporated it into the DNA sequence using standard methods [83]. Following similar strategy, Shigdel and He synthesized C1′-methylenedisulfide deoxyribose phosphoramidite (Fig. 28b) [84].

Other non-native analogues of nucleosides with tailored properties were developed along the years. Baba et al. obtained so called bridged nucleic acids (BNAs), where building blocks were equipped with reducible disulfide bridge between C2′ and C4′ sugar ring carbon atoms (Fig. 29) [85]. Conformation of sugar rings of such nucleosides is completely restricted, which in principle may be beneficial for certain biological applications such as enzyme inhibition or enhancement of

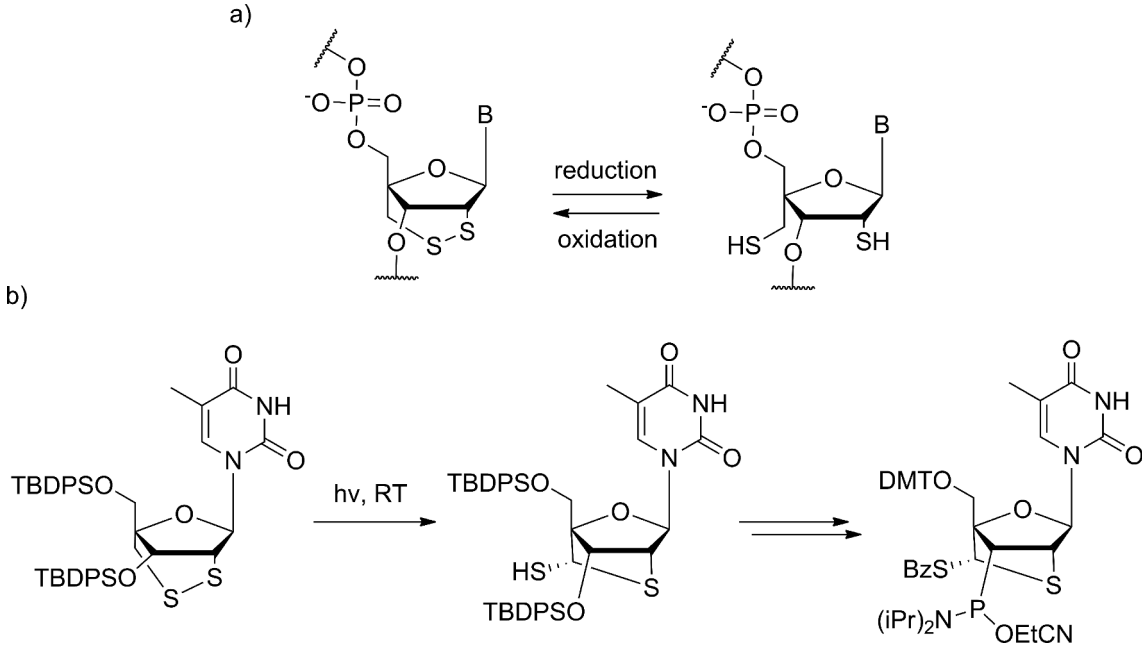

Fig. 29. Reversible redox reaction of a BNA nucleotide [85] (a) and preparation of a building block for conjugations at 6′ thiol [87] (b).

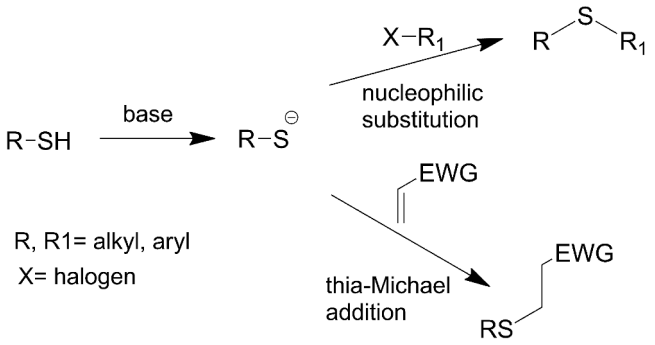

Fig. 30. Examples of adverse reactions thiols and thiolates may undergo.

complementary strand binding [86]. As a starting material the authors used D-glucose, which after over 15 reaction steps yielded desired product: C2′-C4′ disulfide BNA monomers. Interestingly, when subjected to photoirradiation, these nucleosides were converted into C6′-thiol bearing building blocks [87]. Oligonucleotides synthesized with these monomers within the sequence easily underwent conjugation with a variety of hydrophilic molecules. Moreover, they exhibited higher affinity to complementary DNA strands as well as increased resistance against 3′-exonucleases than phosphorothioate oligonucleotides.

3. Conjugation methods

Although there are only two reaction pathways that lead to disulfide bond formation, there is a multiplicity of conjugation methods of oligonucleotides bearing a disulfide or thiol tether with other species. The choice of the procedure depends mainly on the specific application, on material availability and to some extent on the preferences of the scientist. However, it is still possible to enunciate some general principles. Considering that -SH groups are reactive species, they usually require preliminary protection (indicated as −S−PG) or masking as disulfides (indicated as −S−S−R), in order to avoid adverse side reactions (Fig. 30) upon oligonucleotides synthesis, cleavage from the support and nucleobases/modifications deprotection.

Typical thiol protecting groups are for example benzoyl (Bz), *tert*-butyl (tBu), trityl (triphenylmethyl, Tr) and its modifications: DMT (4,4′-dimethoxytrityl) and MMT (4-monomethoxytrityl) [88]; fluorenylmethyloxycarbonyl (Fmoc); 2,4,6-trimethoxybenzyl (Tmob) [89]; acetamidomethyl (Acm) [90] and allyloxycarbonylaminomethyl (Alloc) [91]. They may be conveniently grouped taking into account deprotection conditions (see Table 1).

Table 1
Summary of most common protecting groups for thiol modifications of nucleic acids and their cleavage conditions.

Protecting group	Cleavage conditions
Tr, MMT, DMT, Tmob	Acidic (e.g. aqueous TFA or TCA)
Bz, Fmoc	Basic (e.g. aqueous NH_4OH)
S−S, *t*Bu	Reducing (e.g. DTT, TCEP)
Alloc	Pd-catalyzed
Acm	Thallium (III) trifluoroacetate in DMF

Base-labile thiol protecting groups are hardly ever used in oligonucleotide synthesis. This may seem odd at first glance because their simultaneous removal with standard protecting groups of nucleobases would significantly streamline the synthetic process. Nevertheless, free thiols would be then prone to undergo thia-Michael addition with excess of acrylonitrile (product of β–cyanoethyl elimination reaction, see Fig. 28bottom) and be effectively consumed. Disulfide masking or *t*Bu protection seem the most convenient, since they allow entirely orthogonal deprotection of the thiol under reducing conditions. There is a variety of reducing agents that can be used for this purpose, dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) being the most widespread, mainly because they act rapidly when applied in excess to the modified nucleic acid [92,93]. E.g. a 10-fold excess of either of these at pH > 8 results in complete reduction of the disulfide within 1 h.

Since S−S conjugation between thiol-functionalized

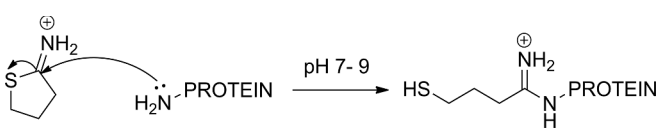

Fig. 31. Thiolation of a primary amine group with 2-iminothiolane [59].

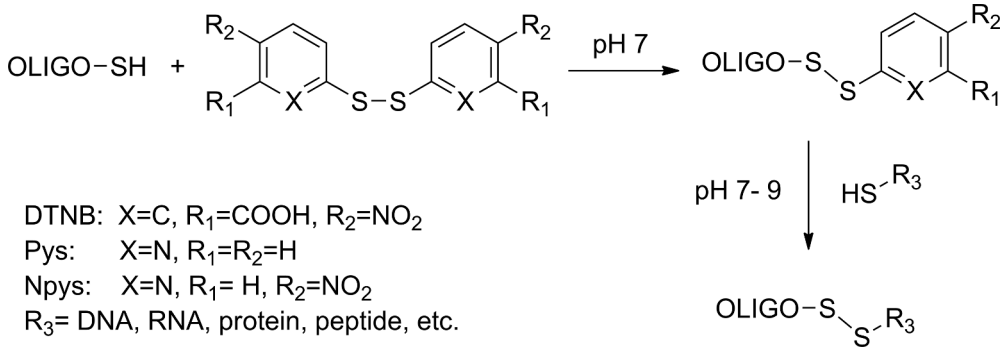

Fig. 32. Activation of a thiol group of oligonucleotide with small-molecule organic disulfide and consecutive conjugation with other thiol-bearing species via thiol-disulfide exchange.

oligonucleotides and peptides/proteins requires presence of (free) thiols in both partners, the latter are selected so as to possess easily-accessible cysteine residues. In case these are lacking in the wild type proteins, appropriate mutant proteins are prepared by means of genetic manipulations [57,94]. Yet another solution involves conjugation of primary amine of *N*-terminal amino acids or side chains with 2-iminothiolane (Traut's reagent), to yield free thiols (Fig. 31).

Upon reoxidation liberated thiols form mixtures of homo- and heterodimers. Activating one of the thiols prior to conjugation is therefore a common practice to increase selectivity, efficiency and rate of heterodimer formation [95,96]. "Activation" refers to the thiol/disulfide interchange reaction with a small-molecule organic disulfide. In case of S—S cross-linking of oligonucleotides usually 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) [95], 2,2′-Dipyridyl disulfide (Pys, Aldrithiol®) [97] or 2,2′-Dithiobis(5-nitropyridine) (Npys) [29] (Fig. 32) are used. Such formed disulfide adducts now possess a good leaving group and readily undergo nucleophilic substitution by a free thiol at neutral pH [98].

There is no universal method established for conjugation of species prepared as mentioned above. Whereas the only common denominator is aqueous environment of the reaction, literature abounds with combinations of buffers, salts and other additives. Since pH upon conjugation should be maintained between 7 and 9 to favor disulfide formation, buffers that are typically used include Tris-HCl [57,99], TE (Tris-HCl and EDTA) [98,100], sodium phosphate buffer [37,101] and TEAA [96,102] Furthermore, salts can be added to the reaction mixture to enhance conjugation rate and efficiency and those are typically NaCl or $MgCl_2$ at final concentration of 100 mM [103,104]. When working with highly cationic peptides and oligonucleotides with negatively charged backbones, an aggregation and precipitation may occur during the conjugation reaction. This can be avoided e.g. by using triethylammonium trifluoroacetate buffer [105] or strong denaturing agents like e.g. formamide [106].

Both thiol oxidation and thiol/disulfide interchange can be conducted under aerobic conditions at room temperature, which typically results in product formation within a couple of hours [38,96], but it is not uncommon to carry out the reaction for as long as overnight, 24 h or even for a couple of days, often at 4 °C [93,101]. HPLC analysis or analytical PAGE of the crude could serve for detection of conjugates and assessment of reaction's progress. Conjugation can be quenched by lowering pH below 6.5 [93,104] or addition of urea [107], however this step does not seem to be necessary prior to product isolation and purification.

All published conjugation strategies require several time- and resource-consuming steps, often leading to loss of material and/or poor yields. That is why efforts are underway to simplify the synthetic protocols, e.g. by combining thiol deprotection and activations steps via either direct substitution of the trityl protection by the nitropyridine sulfenyl group [108] in acidic environment or of the benzoyl group by 2,2′-dipyridyl disulfide in basic environment [87] (Fig. 33).

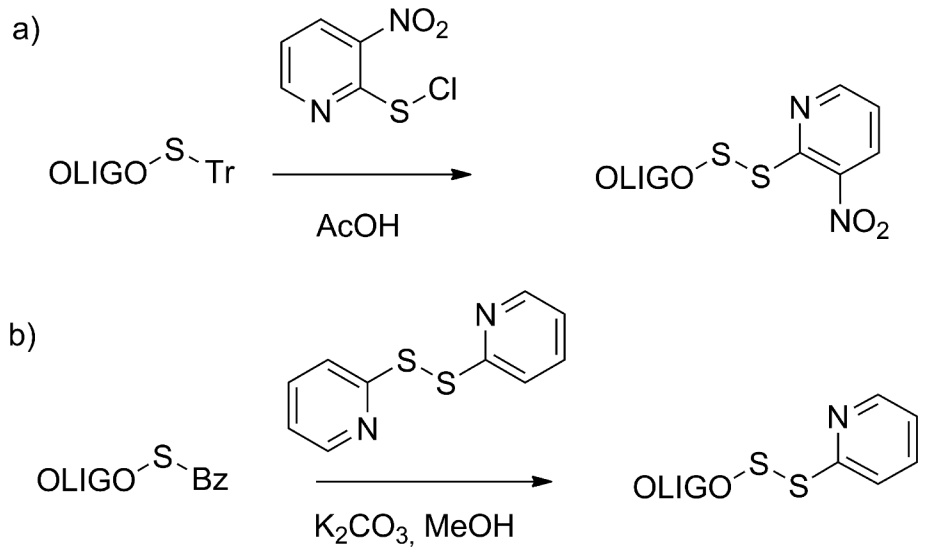

Fig. 33. Direct substitution of protecting groups with activating agents: substitution of trityl group with nitropyridine sulfenyl group [108] (a) and of benzoyl group with 2, 2′-dipirydyl disulphide [87] (b).

4. Summary

Among many oligonucleotide cross-linking strategies such as via maleimide chemistry, NHS ester, psoralen or click chemistry, use of disulfide chemistry stands out as a unique solution: operationally simple, robust and efficient. In addition, adjusting the site of modification and structure of the linker allows control of spatial arrangement and accommodation of the cross-link. What is more, disulfide bonds are biocompatible and non-toxic as well as undergo reduction inside the cells [109,110], which makes S−S conjugates promising therapeutic agents [1,4].

Over the years, a lot of distinct methods of nucleic acid functionalization with thiols/disulfides were devised. Some of them were found to be unnecessarily complex and disproportionately labour-intensive when compared to effects they allowed to achieve. Others were turned into successful commercial products. It may be thus safely stated that from the synthetic point of view, the field is pretty well covered by now. The focus has inevitably shifted towards structural and mechanistic studies of nucleic acid-biomolecule conjugates [93,111,112] and therapeutic applications thereof [113–115] which will be the subject of Part II of this review.

Acknowledgement

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References

[1] T.S. Zatsepin, J.J. Turner, T.S. Oretskaya, M.J. Gait, Curr. Pharm. Des. 11 (2005) 3639–3654.
[2] N.G. Dolinnaya, O.A. Borisova, Mol. Biol. 34 (2000) 790–803.
[3] E.M. Zubin, E.A. Romanova, T.S. Oretskaya, Russ. Chem. Rev. 71 (2002) 239–264.
[4] J. Winkler, Ther. Deliv. 4 (2013) 791–809.
[5] M.J. Gait, Cell. Mol. Life Sci. 60 (2003) 844–853.
[6] G.L. Verdine, D.P.G. Norman, Annu. Rev. Biochem. 72 (2003) 337–366.
[7] D. Schilter, Nat. Rev. Chem. 1 (2017) 1–2.
[8] I.V. Koval', Russ. Chem. Rev. 63 (2007) 735–750.
[9] M. Karimi, M.T. Ignasiak, B. Chan, A.K. Croft, L. Radom, C.H. Schiesser, D.I. Pattison, M.J. Davies, Sci. Rep. 6 (2016) 1–12.
[10] R. Singh, G.M. Whitesides, Chem. Sulphur-Containing Funct. Groups (1993) 633–658.
[11] G.V. Lamoureux, G.M. Whitesides, J. Org. Chem. 58 (1993) 633–641.
[12] R.J. Huxtable, The Biochemistry of Sulfur, Springer, Boston, MA, 1986, pp. 199–268.
[13] S.F. Betz, Protein Sci. 2 (1993) 1551–1558.
[14] H.F. Gilbert, Methods Enzymol. 251 (1995) 8–28.
[15] R.P. Szajewski, G.M. Whitesides, J. Am. Chem. Soc. 102 (1980) 2011–2026.
[16] S.K. Singh, A.a. Koshkin, J. Wengel, P. Nielsen, Chem. Commun. (1998) 455–456.
[17] P. Nielsen, M. Egholm, R. Berg, O. Buchardt, Science (80-.) 254 (1991) 1497–1500.
[18] R. Bischoff, J.M. Coull, A.E. Regnier, Anal. Biochem. 164 (1987) 336–344.
[19] V.V. Demidov, V.N. Potaman, M.D. Frank-Kamenetskil, M. Egholm, O. Buchard, S.H. Sönnichsen, P.E. Nlelsen, Biochem. Pharmacol. 48 (1994) 1310–1313.
[20] P.L. Iversen, Antisense Drug Technology, second ed., CRC Press, 2006, pp. 375–389.
[21] J. Hanvey, N. Peffer, J. Bisi, S. Thomson, R. Cadilla, J. Josey, D. Ricca, C. Hassman, M. Bonham, K. Au, A. Et, Science (80-.) 258 (1992) 1481–1485.
[22] H. Knudsen, P.E. Nielsen, Nucleic Acids Res. 24 (1996) 494–500.
[23] R. Zuckermann, D. Corey, P. Schultz, Nucleic Acids Res. 15 (1987) 5305–5321.
[24] B.A. Connolly, P. Rider, Nucleic Acids Res. 13 (1985) 4485–4502.
[25] H. Gao, M. Yang, A.F. Cook, Nucleic Acids Res. 23 (1995) 285–292.
[26] J.A. Fidanza, L.W. McLaughlin, J. Org. Chem. 57 (1992) 2340–2346.
[27] B.S. Sproat, B. Beijer, P. Rider, P. Neuner, Nucleic Acids Res. 15 (1987) 4837–4848.
[28] N.D. Sinha, R.M. Cook, Nucleic Acids Res. 16 (1988) 2659–2670.
[29] R. Eritja, A. Pons, M. Escarcellar, E. Giralt, F. Albericio, Tetrahedron 47 (1991) 4113–4120.
[30] Z. Kupihár, Z. Schmél, Z. Kele, B. Penke, L. Kovács, Bioorganic Med. Chem. 9 (2001) 1241–1247.
[31] A. Semenyuk, M. Kwiatkowski, Tetrahedron Lett. 48 (2007) 469–472.
[32] R.K. Gaur, P. Sharma, K.C. Gupta, Nucleic Acids Res. 17 (1989) 4404–4404.
[33] A. Kumar, S. Adwani, H. Dawar, G.P. Talwar, Nucleic Acids Res. 19 (1991) 4561.
[34] E. Bonfils, N.T. Thuong, Tetrahedron Lett. 32 (1991) 3053–3056.
[35] K.C. Gupta, P. Sharma, S. Sathyanarayana, P. Kumar, Tetrahedron Lett. 31 (1990) 2471–2474.
[36] K.C. Gupta, P. Sharma, P. Kumar, S. Sathyanarayana, Nucleic Acids Res. 19 (1991) 3019–3026.
[37] G.D. Glick, J. Org. Chem. 56 (1991) 6746–6747.
[38] J.T. Goodwin, G.D. Glick, Tetrahedron Lett. 35 (1994) 1647–1650.
[39] S.E. Osborne, A.D. Ellington, Bioorganic Med. Chem. Lett. 6 (1996) 2339–2342.
[40] J.T. Goodwin, G.D. Glick, Tetrahedron Lett. 34 (1993) 5549–5552.
[41] S. Sun, X.-Q. Tang, A. Merchant, J. Org. Chem. 61 (1996) 5708–5709.
[42] N.C. Chaudhuri, E.T. Kool, J. Am. Chem. Soc. 117 (1995) 10434–10442.
[43] C. Prestinari, C. Richert, Chem. Commun. 47 (2011) 10824–10826.
[44] M.J. Robins, G.L. Basom, Can. J. Chem. 51 (1973) 3161–3169.
[45] T.T. Nikiforov, B.A. Connolly, Tetrahedron Lett. 32 (1991) 2505–2508.
[46] T.T. Nikiforov, B.A. Connolly, Tetrahedron Lett. 33 (1992) 2379–2382.
[47] J. Milton, B.a. Connolly, T.T. Nikiforov, R. Cosstick, J. Chem. Soc. Chem. Commun. (1993) 779.
[48] C.W. Gundlach, T.R. Ryder, G.D. Glick, Tetrahedron Lett. 38 (1997) 4039–4042.
[49] X. Hou, G. Wang, B.L. Gaffney, R.A. Jones, Nucleosides Nucleotides Nucleic Acids 28 (2009) 1076–1094.
[50] X. Hou, G. Wang, B.L. Gaffney, R.A. Jones, Curr. Protoc. Nucleic Acid Chem. (2010) 1–23.
[51] K. Das, R.P. Bandwar, K.L. White, J.Y. Feng, S.G. Sarafianos, S. Tuske, X. Tu, A.D. Clark, P.L. Boyer, X. Hou, B.L. Gaffney, R.A. Jones, M.D. Miller, S.H. Hughes, E. Arnold, J. Biol. Chem. 284 (2009) 35092–35100.
[52] C.R. Allerson, S.L. Chen, G.L. Verdine, J. Am. Chem. Soc. 119 (1997) 7423–7433.
[53] A.M. MacMillan, G.L. Verdine, J. Org. Chem. 55 (1990) 5931–5933.
[54] A.M. Macmillan, G.L. Verdine, Tetrahedron 47 (1991) 2603–2616.
[55] A.E. Ferentz, G.L. Verdine, J. Am. Chem. Soc. 113 (1991) 4000–4002.
[56] D.A. Erlanson, L. Chen, G.L. Verdine, J. Am. Chem. Soc. 115 (1993) 12583–12584.
[57] E.N. Peletskaya, P.L. Boyer, A.A. Kogon, P. Clark, H. Kroth, J.M. Sayer, D.M. Jerina, S.H. Hughes, J. Virol. 75 (2001) 9435–9445.
[58] Y. Ueno, A. Nakagawa, A. Matsuda, Nucleosides Nucleotides Nucleic Acids 17 (1998) 283–289.
[59] B.C.F. Chu, L.E. Orgel, Nucleic Acids Res. 16 (1988) 3671–3691.
[60] B.C.F. Chu, F.R. Kramer, L.E. Orgel, Nucleic Acids Res. 14 (1986) 5591–5603.
[61] T.-P. Wang, N.C. Ko, Y. Su, E. Wang, S. Severance, C. Hwang, Y.T. Shih, M.H. Wu, Y. Chen, Bioconjug. Chem. 23 (2012) 2417–2433.
[62] F. Eckstein, Antisense Nucleic Acid Drug Dev. 10 (2000) 117–121.
[63] S.M. Gryaznov, R.L. Letsinger, Nucleic Acids Res. 21 (1993) 1403–1408.
[64] T. Wu, L.E. Orgel, J. Mol. Evol. 32 (1991) 274–277.
[65] V.G. Metelev, O.A. Borisova, E.M. Volkov, T.S. Oretskaya, N.G. Dolinnaya, Nucleic Acids Res. 29 (2001) 4062–4069.
[66] V.G. Metelev, E.A. Kubareva, O.V. Vorob'eva, A.S. Romanenkov, T.S. Oretskaya, FEBS Lett. 538 (2003) 48–52.
[67] E.M. Witch, R. Cosstick, Tetrahedron Lett. 38 (1997) 6745–6748.
[68] D.J. Hansen, I. Manuguerra, M.B. Kjelstrup, K.V. Gothelf, Angew. Chemie Int. Ed. 53 (2014) 14415–14418.
[69] V. Patzke, J.S. McCaskill, G. von Kiedrowski, Angew. Chemie Int. Ed. 53 (2014) 4222–4226.
[70] M.L. Hamm, J.A. Piccirilli, J. Org. Chem. 62 (1997) 3415–3420.
[71] M. Manoharan, L.K. Johnson, K.L. Tivel, R.H. Springer, P.D. Cook, Bioorg. Med. Chem. Lett. 3 (1993) 2765–2770.
[72] M. Manoharan, K.L. Tivel, B. Ross, P. Dan Cook, Gene 149 (1994) 147–156.
[73] J.T. Goodwin, S.E. Osborne, E.J. Scholle, G.D. Glick, J. Am. Chem. Soc. 118 (1996) 5207–5215.
[74] S. Jin, C.V. Miduturu, D.C. McKinney, S.K. Silverman, J. Org. Chem. 70 (2005) 4284–4299.
[75] M. Fujita, S. Watanabe, M. Yoshizawa, J. Yamamoto, S. Iwai, PLoS One 10 (2015) 1–17.
[76] Y. Ochi, O. Nakagawa, K. Sakaguchi, S. Wada, H. Urata, Chem. Commun. 49 (2013) 7620.
[77] A. Semenyuk, A. Földesi, T. Johansson, C. Estmer-Nilsson, P. Blomgren, M. Brännvall, L.A. Kirsebom, M. Kwiatkowski, J. Am. Chem. Soc. 128 (2006) 12356–12357.
[78] A. Biscans, S. Rouanet, J.J. Vasseur, C. Dupouy, F. Debart, Org. Biomol. Chem. 14 (2016) 7010–7017.
[79] F. Gauthier, F. Beltran, A. Biscans, F. Debart, C. Dupouy, J.J. Vasseur, Org. Biomol. Chem. 16 (2018) 3181–3188.
[80] W. Saenger, Principles of nucleic acid structure, Springer-Verlag, 1984.
[81] S.T. Sigurdsson, T. Tuschl, F. Eckstein, RNA 1 (1995) 575–583.
[82] S.B. Cohen, T.R. Cech, J. Am. Chem. Soc. 119 (1997) 6259–6268.
[83] A. Hatano, S. Makita, M. Kirihara, Tetrahedron 61 (2005) 1723–1730.
[84] U.K. Shigdel, C. He, J. Am. Chem. Soc. 130 (2008) 17634–17635.
[85] T. Baba, T. Kodama, T. Imanishi, S. Obika, Nucleic Acids Symp. Ser. 53 (2009) 107–108.
[86] T. Baba, T. Kodama, K. Mori, T. Imanishi, S. Obika, Chem. Commun. 46 (2010) 8058.
[87] K. Mori, T. Kodama, T. Baba, S. Obika, Org. Biomol. Chem. 9 (2011) 5272–5279.
[88] C.B. Reese, Tetrahedron 34 (1978) 3143–3179.
[89] M.C. Munson, C. Garcia-Echeverria, F. Albericio, G. Barany, J. Org. Chem. 57 (1992) 3013–3018.
[90] D. Veber, J. Milkowski, S. Varga, R. Denkewalter, R. Hirschmann, J. Am. Chem. Soc. 94 (1972) 5456–5461.
[91] A. Malanda Kimbonguila, A. Merzouk, F. Guibé, A. Loffet, Tetrahedron 55 (1999) 6931–6944.
[92] G.T. Hermanson, Functional Targets for Bioconjugation (2013).

[93] J.E. Corn, J.M. Berger, Structure 15 (2007) 773–780.
[94] D. Corey, P. Schultz, Science (80-.) 238 (1987) 1401–1403.
[95] S.A. Wolfe, A.E. Ferentz, V. Grantcharova, M.E.A. Churchill, G.L. Verdine, Chem. Biol. 2 (1995) 213–221.
[96] J.J. Turner, D. Williams, D. Owen, M.J. Gait, Curr. Protoc. Nucleic Acid Chem., 2006, Chapter 4, Unit 4.28.
[97] E. Vivès, B. Lebleu, Tetrahedron Lett. 38 (1997) 1183–1186.
[98] M. Dirin, E. Urban, B. Lachmann, C.R. Noe, J. Winkler, Future Med. Chem. 7 (2015) 1657–1673.
[99] C. He, G.L. Verdine, Chem. Biol. 9 (2002) 1297–1303.
[100] A.E. Ferentz, T.A. Keating, G.L. Verdine, J. Am. Chem. Soc. 115 (1993) 9006–9014.
[101] H. Gao, M. Yang, R. Patel, A.F. Cook, Nucleic Acids Res. 23 (1995) 2025–2029.
[102] M. Antopolsky, E. Azhayeva, U. Tengvall, S. Auriola, I. Jääskeläinen, S. Rönkkö, P. Honkakoski, A. Urtti, H. Lönnberg, A. Azhayev, Bioconjug. Chem. 10 (1999) 598–606.
[103] S.E. Osborne, R.J. Cain, G.D. Glick, J. Am. Chem. Soc. 119 (1997) 1171–1182.
[104] S.E. Osborne, J. Völker, S.Y. Stevens, K.J. Breslauer, G.D. Glick, J. Am. Chem. Soc. 118 (1996) 11993–12003.
[105] G. Ferenc, Z. Kupihár, Z. Kele, L. Kovács, Nucleosides Nucleotides Nucleic Acids 24 (2005) 1059–1061.
[106] J.J. Turner, A.A. Arzumanov, M.J. Gait, Nucleic Acids Res. 33 (2005) 27–42.
[107] D.A.P. Krummel, O. Kent, A.M. MacMillan, S. Altman, J. Mol. Biol. 295 (2000) 1113–1118.
[108] F. Maurel, F. Debart, F. Cavelier, A.R. Thierry, B. Lebleu, J.J. Vasseur, E. Vivès, Bioorganic Med. Chem. Lett. 15 (2005) 5084–5087.
[109] E.P. Feener, W.-C. Shen, H.J.-P. Ryser, J. Biol. Chem. 265 (1990) 18780–18785.
[110] J. Yang, H. Chen, I.R. Vlahov, J.-X. Cheng, P.S. Low, Proc. Natl. Acad. Sci. 103 (2006) 13872–13877.
[111] S.G. Sarafianos, A.D. Clark, S. Tuske, C.J. Squire, K. Das, D. Sheng, P. Ilankumaran, A.R. Ramesha, H. Kroth, J.M. Sayer, D.M. Jerina, P.L. Boyer, S.H. Hughes, E. Arnold, J. Biol. Chem. 278 (2003) 16280–16288.
[112] A. Banerjee, W.L. Santos, G.L. Verdine, Science (80-.) 311 (2006) 1153–1157.
[113] M. Pooga, U. Soomets, M. Hällbrink, A. Valkna, K. Saar, K. Rezaei, U. Kahl, J.X. Hao, X.J. Xu, Z. Wiesenfeld-Hallin, T. Hökfelt, T. Bartfai, Ü. Langel, Nat. Biotechnol. 16 (1998) 857–861.
[114] T.C. Chu, J.W. Marks, L.A. Lavery, S. Faulkner, M.G. Rosenblum, A.D. Ellington, M. Levy, Cancer Res. 66 (2006) 5989–5992.
[115] S. El-Andaloussi, H.J. Johansson, T. Holm, Ü. Langel, Mol. Ther. 15 (2007) 1820–1826.