Reversible DNA Ligation

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Synthesis, Dynamic Combinatorial Chemistry, and PCR Amplification of 3′–5′ and 3′–6′ Disulfide-linked Oligonucleotides**

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Abstract: *Disulfide dithymidines linked 3′–5′ or 3′–6′ were synthesized and incorporated into oligonucleotides through a combined phosphotriester and phosphoramidite solid-phase oligonucleotide synthesis approach. The disulfide links are cleaved and formed reversibly in the presence of thiols and oligonucleotides. This link was shown to be sequence-adaptive in response to given templates in the presence of mercaptoethanol. The artificial 3′–5′ and 3′–6′ disulfide link was tolerated by polymerases in the polymerase chain reaction (PCR). By using sequencing analysis, we show that single mutations frequently occurred randomly in the amplification products of the PCR.*

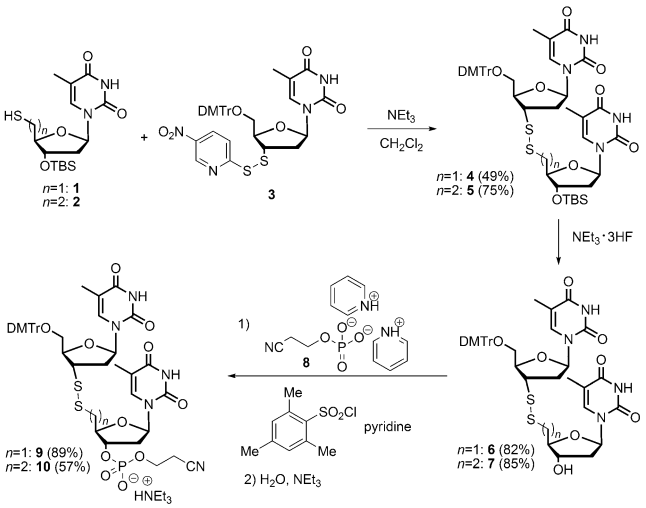
The disulfide bond is common in nature, in which it contributes significantly to the tertiary structure and stability of extracellular proteins.[1] Disulfides are known to exist in dynamic exchange equilibria in the presence of thiolate, a process known to alter the structure of proteins,[2] and are commonly exploited within the field of dynamic combinatorial chemistry to generate novel ligands and receptors.[3–7] Disulfide-linked dinucleosides have previously been synthesized by Witch et al.[8] Only recently was the disulfide linkage incorporated into oligonucleotides as an internucleosidic linkage.[9] Patzke et al. utilized a DNA-templated reaction between an activated disulfide and a thiol to generate a fast ligation reaction. We set out to study if disulfide-linked oligonucleotides can exist in dynamic equilibria that would form different products under the influence of different templates.

It has been argued that in early evolution it would be advantageous for pre-oligonucleotides to have reversible backbone linkages that could be formed and cleaved in the absence of enzymes, and synthetic models of such systems have, for example, included the imine,[10] the glyoxylate,[11] and the boronic ester[12] linkage. An alternative approach employing reversible linkages was devised by Ura et al.[13] They utilized a preformed peptidic backbone with reversible thioester linkages to the nucleobases, and in this manner they were able to exchange the nucleobases in response to given templates.

Applications of reversibly linked oligonucleotides could include formation of randomized libraries of oligonucleotides. If the artificial backbone is tolerated by polymerases, such libraries could hereafter generate peptide libraries by phage display.[14,15] If the randomized sequences are made up of stable nucleotide trimers, stop codons could principally be omitted.[16] We have therefore also investigated the behavior of the disulfide-linked oligonucleotides toward polymerases by employing these as templates in a primer extension reaction.

Two different disulfide linkages were designed; the 3′–5′ linkage contains the same carbon skeleton as natural nucleosides, whereas the 3′–6′ linkage contains the same number of atoms in the internucleosidic linkage as natural nucleosides (Scheme 1). Patzke et al. modeled the 3′–5′ disulfide link and found that the internucleotidic geometry is comparable with the natural phosphodiester link.[9]

The thiols **1** and **2**, and the disulfide **3** were synthesized from thymine by modified literature procedures (see the Supporting Information, SI).[17–21] The coupling of thiols, **1** and **2**, with pyridyl disulfide, **3**, was achieved with triethylamine in CH_2Cl_2 over few minutes, resulting in the protected disulfide dithymidines, **4** and **5**, in up to 75% yield. Triethylamine



Scheme 1. Formation of the disulfide-linked dinucleoside and subsequent preparation of the 2-cyanoethylphosphate esters. DMTr = dimethoxytrityl.

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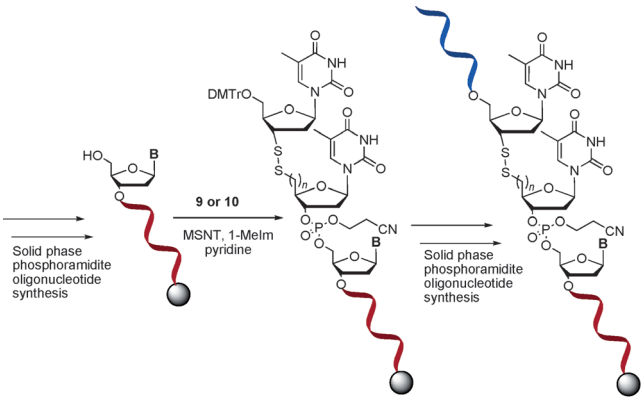
trihydrofluoride facilitated the removal of the *tert*-butyldimethylsilyl (TBS) protecting group in 82–85% yield.

Incorporation of the disulfide dithymidines into oligonucleotides was attempted by standard phosphoramidite chemistry. Preparation of the phosphoramidite of **7** was, however, unsuccessful apparently due to reduction of the disulfide moiety by P^{III} in the phosphoramidite, as was evident from ^{31}P NMR measurements. This is surprising, because disulfide linkers are commonly employed in phosphoramidite-based solid-phase synthesis.[22] Instead we turned to the phosphotriester method for oligonucleotide synthesis, in which the monomer contains P^{V}, and therefore would not be prone to undesired redox reactions. However, the phosphotriester method typically involves 2-chlorophenyl protection groups for the phosphate moiety, but this protection group requires oximate reagents in the final deprotection.[23,24] We therefore decided to employ the 2-cyanoethyl protection group. The resulting intermediate is the same as that obtained from the phosphoramidite coupling cycle; therefore, only one final deprotection step is required. The 2-cyanoethyl-protected phosphate monomers could thus be prepared by a mesitylene sulfonyl chloride mediated condensation of the respective 3′ alcohol and pyridinium 2-cyanoethylphosphate. Oligonucleotides bearing the disulfide-linked backbone modification could hereafter be synthesized as shown in Scheme 2. We synthesized six different oligonucleotides (ODN-1–ODN-6, Table 1).



Scheme 2. Oligonucleotide synthesis employing a mixed phosphoramidite and phosphotriester strategy for the incorporation of disulfide dithymidines **16** and **17**. MSNT = 1-(2-mesitylensulfonyl)-3-nitro-1*H*-1,2,4-triazol; 1-MeIm = 1-methylimidazole.

Since the oligonucleotides contain a disulfide group in the backbone at one single position, we expected that the oligonucleotides could be site-specifically cleaved under reductive conditions to yield two thiol-terminated oligonucleotides. This was indeed the case, as shown in Figure 1. Treatment of the disulfide-linked oligonucleotides with an excess of tris(2-carboxyethyl)phosphine (TCEP), dithiothreitol (DTT), or mercaptoethanol (ME) resulted in two shorter oligonucleotides of the expected molecular size as seen by denaturing polyacrylamide gel electrophoresis (PAGE).

Inspired by the concepts of dynamic combinatorial chemistry of disulfides, we speculated that it may be possible

Table 1: Sequences of the oligodeoxyribonucleotides synthesized containing the 3′–5′ or 3′–6′ disulfide linkage.

Name	Sequence
ODN-1 (aSSb)	d(CTTTCATCTTACTTATCTACATCCCGACCGC TCGT$_{6'SS}$TTAACCGCAACCTCACCATACAACC)
ODN-2 (cSSd)	d(TCTCACCTCACCATCCCTACTACACTAACT$_{6'SS}$TCG CGAGGCATTATCACACTACACTTCACACTTCACATCA)
ODN-3	d(GTTCCGCCAACTTACGACCGCTCGT$_{6'SS}$TCGC GAGGCATGCCCTCAGGACCAGCACGGATAAC)
ODN-4	d(AACCCTACACTAACT$_{6'SS}$TCGCGAGG CATGCCCTCAGGACCAGCACGGATAAC)
ODN-5	d(GTTCCGCCAACTTACGACCGCTCGT$_{5'SS}$TCGC GAGGCATGCCCTCAGGACCAGCACGGATAAC)
ODN-6	d(AACCCTACACTAACT$_{5'SS}$TCGCGAGG CATGCCCTCAGGACCAGCACGGATAAC)

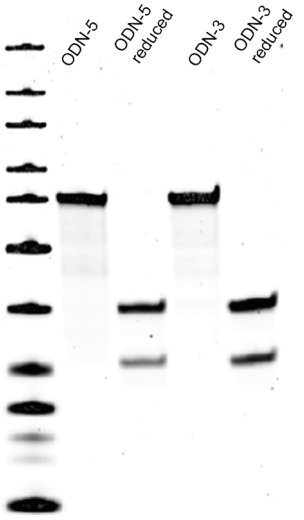


Figure 1. Denaturing PAGE analysis of the reductive cleavage of 3′–5′ or 3′–6′ disulfide-linked oligonucleotides.

to bring these disulfide-linked oligonucleotides into a dynamic equilibrium under thermodynamic control.[4] In this manner it should be possible to favor the formation of a certain oligonucleotide, if a complementary oligonucleotide is present in the solution. To investigate this concept, the oligonucleotides ODN-1 (aSSb) and ODN-2 (cSSd) were brought into dynamic exchange equilibrium by the addition of ME, as illustrated in Figure 2. The dynamic equilibrium was investigated by denaturing PAGE, without a template, and with the sequences complementary to the central regions surrounding the disulfide linkage a′b′, c′d′, a′d′, c′b′ (ODN-11–ODN-14, SI).

In the presence of ME the oligos are cleaved as expected (Figure 2, lanes 2, 4, and 6). In the presence of templates and ME, it is observed that the resulting sequence of the oligonucleotides adapts a sequence complementary to the template. Control experiments confirm that in the absence of ME this is not observed. ME is present in 10000-fold excess (2.5 mM) and the disulfides present are a mixture of all mixed disulfides of a, b, c, d, and in particular of ME. However, in the presence of ME and template a′b′, we observe complete

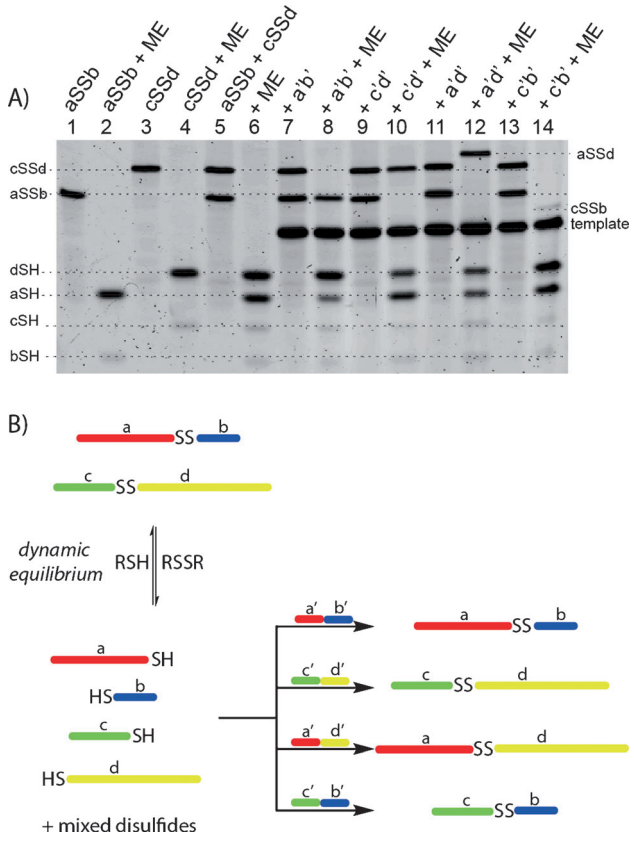


Figure 2. A) Denaturing PAGE analysis of mercaptoethanol (ME)-mediated dynamic equilibration of disulfide-linked oligonucleotides in the presence of various templates. In all cases experiments were performed with ODN-1 (aSSb) and ODN-2 (cSSd). B) The dynamic equilibrium and the template-induced equilibrium shift.

disappearance of cSSd, whereas a band for the formation of aSSb is observed (Figure 2, lane 8). In the presence of ME and c′d′ we observe disappearance of aSSb and appearance of aSH and HSb, whereas the band for cSSd appears (Figure 2, lane 10). The same templating effect is observed for the crossover products aSSd and cSSb when the respective templates are applied. As observed in Figure 2, lane 12, the presence of ME and template a′d′ leads to the formation of aSSd and the amounts of aSH or dSH are greatly reduced. In the presence of ME and c′b′ the last and shorter crossover product cSSb appears as a new weak band (Figure 2, lane 14). It is therefore evident that the various templates induce a thermodynamically controlled equilibrium shift to form sequences complementary to the given template. We believe the template-directed formation of disulfide-linked oligonucleotides is mediated by the template because the product is thermodynamically highly favored by hybridization to the template. This dynamic equilibration was observed at ME concentrations down to 250 μM. At lower concentrations no reaction took place, which is probably caused by significantly lower rates of the bimolecular reactions.

For the analysis of potential combinatorial libraries of oligonucleotides containing one or more disulfides in the backbone it is crucial that the disulfide linkage can be read by polymerases for amplification and sequencing. The ability of two DNA polymerases to read the disulfide linkage was assessed through primer extension reactions with three different primers (ODN-7–ODN-9, SI) for the oligonucleotides ODN-4 and ODN-6 containing the 3′–6′ and 3′–5′ disulfide linkage, respectively. The primers were designed to bind at different distances relative to the disulfide of the oligonucleotide, with primer 1 binding directly next to the disulfide modification, and primer 2 and 3 binding 5 and 10 bases, respectively, downstream of the disulfide. Primer extension was hereafter attempted utilizing two different polymerases; the Q5 polymerase and the Taq polymerase. In all cases we observed an amplicon of the expected molecular size with primers 2 and 3, whereas primer 1 only yielded a significant amount of amplicon in the case of the 3′–5′ linked ODN-6 with the Q5 polymerase. In the remaining cases either no amplicon was observed or only inefficient amplification was observed.

The results shown for primer extension of ODN-6 containing the 3′–5′ disulfide linkage shows that the polymerase can indeed read through the artificial disulfide backbone (Figure 3). Almost identical results were observed for primer extension of ODN-4 containing the 3′–6′ disulfide linkage (SI). For both oligonucleotides it is also observed that the polymerase does not bind efficiently to the oligonucleotide when the primer is extended from the position of the disulfide. These experiments do, however, not give insight into the nature of the amplicon. It has previously been

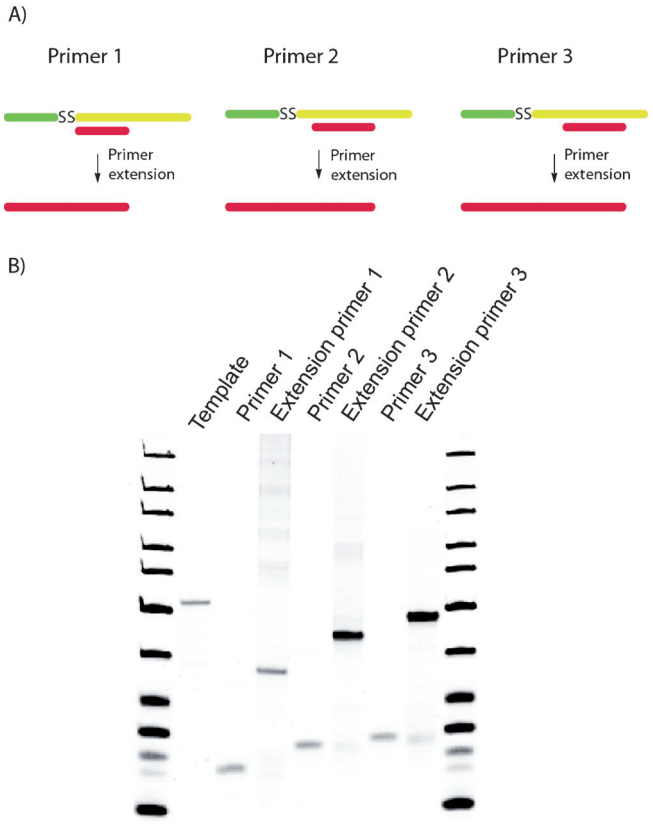


Figure 3. A) The primer extension setup with ODN-6 as template and three different primers. B) Denaturing PAGE analysis of primer extension using the Q5 polymerase.

observed, that for some artificial backbones, the polymerase skips a nucleoside around the unnatural linkage,[25] whereas for other backbones the nucleosides are amplified as if they were completely natural oligonucleotides.[26] The polymerase chain reaction (PCR) amplicons were therefore analyzed by DNA sequencing. For this purpose, we utilized oligonucleotides ODN-3 and ODN-5 to be able to design a set of primers, ODN-9 and ODN-10, binding to the oligonucleotide distant to the disulfide linkage. Cloning into plasmids and replication in *E. coli* resulted in plasmids with the PCR product inserted that could be analyzed by DNA sequencing. For each disulfide modification and polymerase, ten colonies were sequenced. For 39 out of 40 clones we observed that the two thymidines surrounding the disulfide were indeed amplified as two thymidines. We did, however, also observe a relatively high rate of point mutations occurring during the PCR amplification (SI). In particular, nonsystematic deletions of nucleotides in the sequence surrounding the disulfide modification were observed. Insertions or substitutions of nucleotides were also observed, although not at a frequency as high as for the deletions. The two different linkages present in ODN-3 (3′–6′) and ODN-5 (3′–5′) displayed similar error rates. One clone, in which the two thymidines surrounding the disulfide linkage were amplified as one thymidine, was therefore ascribed to such a nonsystematic mutation. In the context of the application of such experiments for in vitro evolution, these point mutations could in fact prove beneficial, as these disulfide-linked oligonucleotides would most certainly exhibit significantly faster evolution than naturally occurring oligonucleotides.

We have developed a synthetic method that allows for the incorporation of disulfide-linked dinucleoside monomers into oligonucleotides and the disulfide-linked oligonucleotides display an interesting new class of reversibly backbone-linked oligonucleotides. Whether the oligonucleotides are linked 3′–5′ or 3′–6′ does not seem to alter the properties significantly. The sequence of such oligonucleotides is able to adapt to a given template, and the disulfide moiety is tolerated by DNA polymerases although with a relatively high mutation rate of the sequence surrounding the disulfide linkage. The methodology potentially allows for the incorporation of several disulfide linkages in one sequence, which would enable the formation of complex libraries of disulfides.

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Communications

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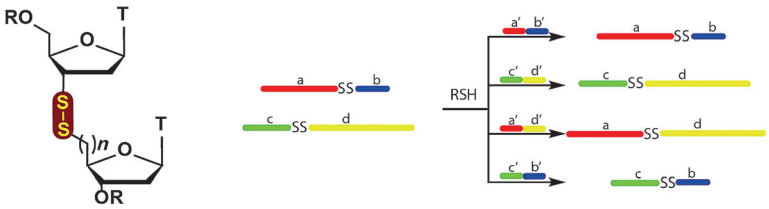
Synthesis, Dynamic Combinatorial Chemistry, and PCR Amplification of 3′–5′ and 3′–6′ Disulfide-linked Oligonucleotides



Internucleosidic 3′–5′ and 3′–6′ disulfide linkages allows cleavage and template-directed formation of desired disulfides in the presence of mercaptoethanol. The artificial disulfide backbone is tolerated by polymerases and the sequences can be amplified by polymerase chain reaction.