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Specific conjugation of DNA binding proteins to DNA templates through thiol–disulfide exchange

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Abstract The double-stranded oligodeoxyribonucleotides with single internucleotide disulfide linkages were successfully used for covalent trapping of cysteine containing protein. In particular, an efficient conjugation of DNA methyltransferase *Sso*II to sequence-specific decoys was demonstrated. The obtained results assume that synthetic oligodeoxyribonucleotides bearing a new trapping site can be used as new tools to study and manipulate biological systems.

Key words: Modified oligonucleotide; Conjugation; Thiol–disulfide exchange; Phosphoryldisulfide linkage; DNA methyltransferase

1. Introduction

The formation, reconstruction and dissociation of highly specific DNA–protein complexes are very important in regulating cellular processes. Synthetic oligonucleotides have a great potential as a universal (multi-purpose) tool in defining these processes as well as a potentially attractive therapeutic means. Synthetic double-stranded (ds) modified oligonucleotides bearing a recognition site of sequence-specific DNA binding proteins are among the most perspective compounds. These decoys could compete with cognate sequences in the genome for binding to the protein and serve as a bona fide antagonist [1,2]. In our investigations we are trying to design oligonucleotide reagents that contain a recognition site of the protein under study as well as a chemically active group specially introduced into the oligonucleotide sugar-phosphate backbone which provides a 'sewing together' (cross-linking) of functionally essential protein to decoy. The important feature is that cross-linking takes place after the formation of a highly specific protein–decoy complex that brings together the reactants: the nucleophilic amino acid side residue and the introduced electrophilic center of DNA. The perspectives of utilization of decoys with intercross-linking activity were demonstrated on DNA and RNA duplexes with the substituted pyrophosphate or acylphosphate internucleotide groups that have provided the specific and efficient covalent binding of proteins to decoys via reaction with nearby located lysine or histidine residue without additional activation and under physiological conditions [3–9]. Recently we synthesized new dsDNAs with internucleotide disulfide links potentially capable of covalent catching of DNA binding proteins through inner protein cysteine [10,11]. The following facts were taken into account upon the design of new cysteine-specific DNA reagents: (a) the ability of cysteine to employ its nucleophile at physiological pH is greater than that of other amino acid side residues [12]; (b) cysteine has a high propensity to contact the DNA backbone, its side chain is a weak donor and the bonding geometry suggests a possible hydrogen bond with the phosphate group [13]; (c) thiol–disulfide interchange is a unique reaction in organic chemistry: although it involves the cleavage and formation of a strong covalent bond, it occurs reversibly at room temperature in water at physiological pH [14,15]; (d) in the reduced state cysteine has a great tendency to be in DNA binding domains or active sites rather than on the surface of the protein globule.

In this paper we describe the first example of efficient conjugation of DNA methyltransferase *Sso*II to sequence-specific decoys which contain single phosphoryldithio linkage (**pss**) in place of the native phosphodiester bonds and where the new disulfide bond formation was chosen as the cross-linking chemistry.

2. Materials and methods

2.1. Chemicals and proteins

Reduced glutathione and Coomassie brilliant blue G 250 were purchased from Aldrich, N^{α}-acetyl-L-lysine from ICN Biochemicals (USA).

The recombinant (cytosine-5)-DNA-methyltransferase *Sso*II (M.*Sso*II), containing an amino-terminal His_6 sequence as an affinity tag and purified by a two-step procedure involving heparin–Sepharose and Ni-NTA–agarose chromatography [16], was a kind gift of Dr. A.S. Karyagina (Institute of Agricultural Biotechnology, Moscow, Russia).

2.2. Oligonucleotide synthesis

Oligodeoxyribonucleotides were synthesized on a DNA synthesizer (Applied Biosystems 380B) using standard phosphoramidite chemistry. The preparation of oligonucleotide components required for the ligation was performed as in [11].

2.3. Synthesis of *pss*-oligonucleotides

3 nmol of oligodeoxynucleotide-3′-phosphorothioate and 2.7 nmol

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Abbreviations: **pss**, phosphoryldithio linkage; M.*Sso*II, (cytosine-5)-DNA-methyltransferase from *Shigella sonnei*

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of 5′-deoxy-5′-(pyrid-2-yldithio)oligodeoxynucleotide were dissolved in 30 μl of 0.015 M sodium citrate buffer, pH 4.5–5.0, containing 0.15 M NaCl and 0.02 M $MgCl_2$. The mixture was incubated at 25°C during 30 min. Oligonucleotides were precipitated by 2% $LiClO_4$ in acetone and were analyzed by electrophoresis in 20% denaturing polyacrylamide gel followed by visualization in UV light. The modified oligonucleotides were eluted from gel slices by 10 mM Tris–HCl buffer, pH 7.6, containing 0.3 M NaCl and 1 mM ethylenediaminetetra acetate (EDTA).

2.4. Characterization of *pss*-oligonucleotides

2.4.1. Reaction with glutathione. 30 μl of 0.25 mM glutathione solution in buffer A (34 mM NaCl, 1 mM $MgCl_2$, 0.05 mM EDTA, 7.5 mM HEPES) with pH 8.0 was added to 2.5 nmol of **pss**-oligodeoxynucleotide. The mixture was incubated at 25°C during 1 h and analyzed as described in Section 2.3.

2.4.2. Reaction with N^{α}-acetyl-L-lysine. 30 μl of 8 mM N^{α}-acetyl-L-lysine solution in buffer A, pH 7.5, was added to 1.5 nmol of **pss**-oligodeoxynucleotide. The mixture was incubated at 25°C during 5 h and analyzed as described above.

2.5. Covalent binding of DNA containing single *pss* linkage with M.SsoII

80 pmol of duplexes (**II**)–(**IV**) were incubated with 160 pmol of M.*Sso*II in 20 μl of 0.05 M Tris–HCl buffer, pH 7.5, containing 0.15 M NaCl, 0.1 mM AdoHcy (*S*-adenosyl-L-homocystein) and 25% glycerol at 37°C during 12 h. After precipitation, before loading onto gel, the samples were dissolved in 0.06 M Tris–HCl buffer, pH 6.8, with 1% glycerol, and 2% sodium dodecyl sulfate (SDS) to disrupt non-covalent DNA–protein complexes. Further, the reaction products were analyzed by electrophoresis in 12% polyacrylamide gel with 0.1% SDS. The protein bands were visualized by Coomassie brilliant blue G 250 staining.

2.6. Cleavage of DNA–M.SsoII conjugates by thiol containing reagents

To the DNA–M.*Sso*II conjugate obtained as described in Section 2.5 1 μl of 10% aqueous SDS solution and 0.1 M dithiothreitol (DTT) or 2-mercaptoethanol to a final concentration of 10 mM were added. The resulting mixture was incubated for 1 h at 37°C and the products were separated as described in Section 2.5.

3. Results and discussion

The original chemical strategy of the introduction of non-native functional groups (functionalities) at the selected positions of the sugar-phosphate backbone of oligonucleotide duplexes was developed by Shabarova and coworkers [3,4]. Modified DNA or RNA duplexes were successfully used for specific covalent binding with restriction–modification enzymes [4,9], RNA recognizing TAT peptide [6] and transcription factors NF-κB [5,7] and HNF1 [8]. The chemical basis of intercross-linking was the reaction of introduced substituted pyrophosphate internucleotide linkages with nucleophilic groups of lysine or histidine. Continuing to develop the strategy, we are trying to widen the set of non-native functionalities that can be introduced into DNA without duplex perturbation to fix covalently DNA–protein complexes through the above-mentioned and other inner amino acids. In particular, we think that inner cysteine can be a good candidate for taking part in cross-linking. Unfortunately, the peculiarity of cysteine thiolate is that it rarely reacts with activated phosphorus (it does not react well with substituted pyrophosphate internucleotide linkages) but serves as a good nucleophile in the reaction of disulfide exchange. This property was used in different conjugate formations. In contrast to other research groups [17–19] that use the oligonucleotides with disulfide containing moieties tethered to heterocyclic bases, we have chosen the strategy of modifying the sugar-phosphate backbone by replacing native internucleotide phosphodiester bonds with disulfide containing linkages, taking into account that cysteine has a high propensity to contact the DNA backbone [13].

The following duplexes ((**I**)–(**V**)) were synthesized and used in the present study:

```
5′ ACGTTCCTGGCTATTGACTGC  3′      5′ ACGTTCCpssTGGCTATTGACTGC  3′
3′CTGCAAGGACCGATAACTGACGT 5′      3′CTGCAAGG---ACCGATAACTGACGT 5′
          (I)                               (II)

5′ ACGTTCCTGGCTApssTTGACTGC  3′    5′ ACGTTCCTGGCTATpssTGACTGC  3′
3′CTGCAAGGACCGAT---AACTGACGT 5′    3′CTGCAAGGACCGATA---ACTGACGT 5′
          (III)                             (IV)

5′  TCGGTTCCpssTGACTGC  3′
3′   GCCAAGG---ACTGA    5′
            (V)
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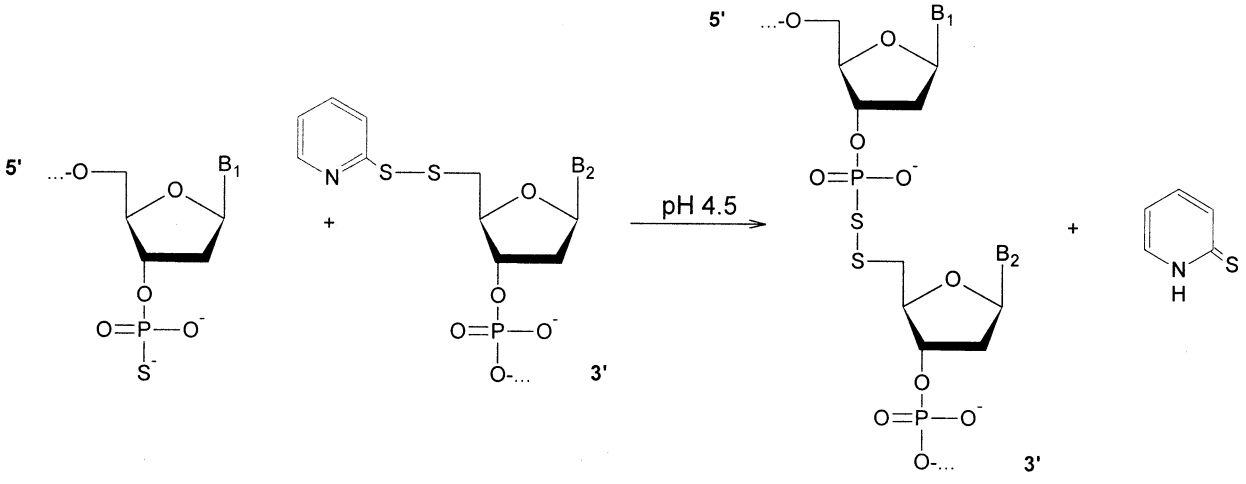
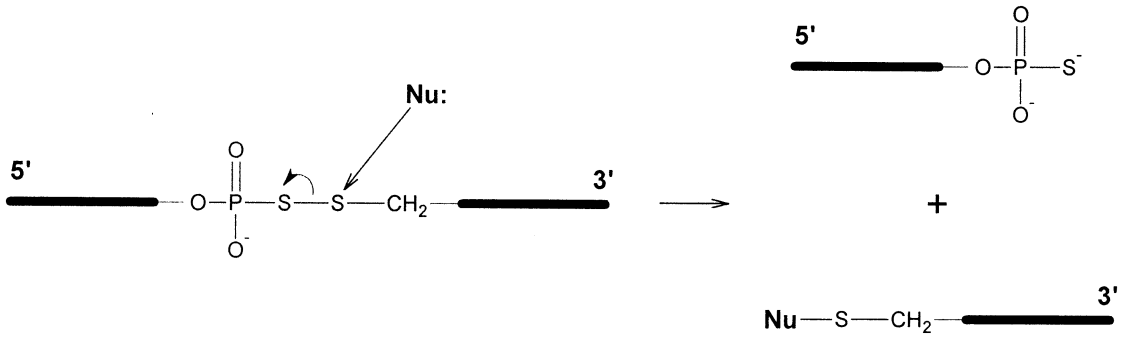
M.*Sso*II recognition site is underlined.

pss-Oligodeoxyribonucleotides have been synthesized by coupling of an oligonucleotide-3′-phosphorothioate and a 5′-mercaptooligonucleotide previously converted to a 2-pyri-



Scheme 1.



Scheme 2.

dyldisulfide adduct as described in [11] with some modifications and according to Scheme 1.

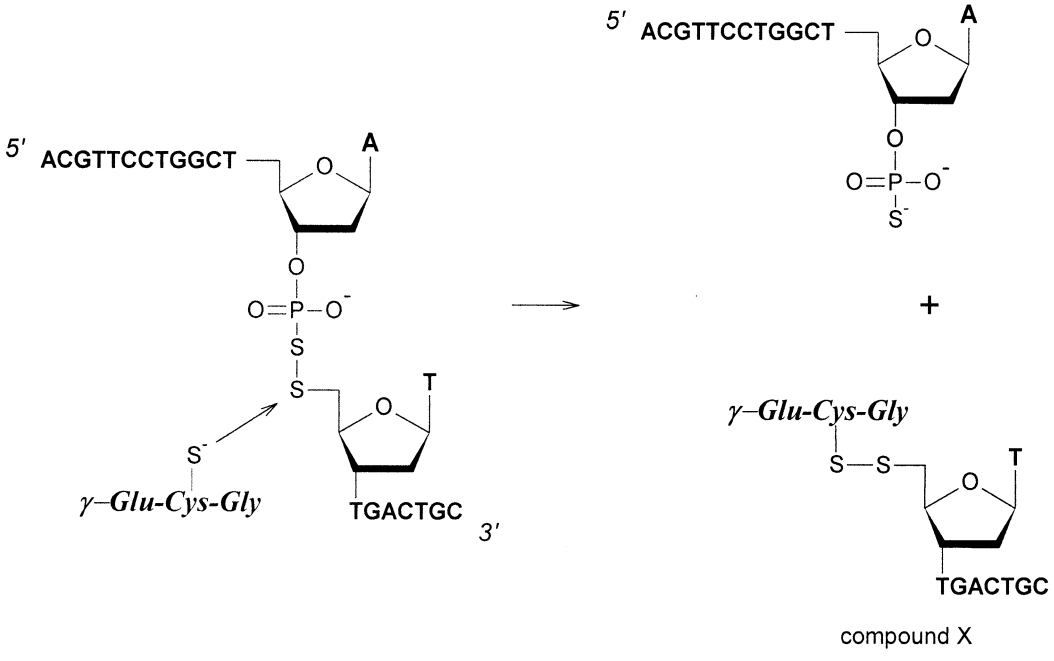
The modification included lowering the incubation mixture pH to 4.5–5.0, which allowed the reaction time to be decreased by up to 30 min. In the used conditions the yield of **pss**-oligonucleotides was usually 95–98%. The insertion of a single phosphoryldisulfide linkage leads to shade (2–3°C) thermal destabilization of corresponding duplexes. For example, T_m of the non-modified duplex (**I**) is about 65°C in 0.015 M sodium citrate buffer, pH 7.25, containing 0.15 M NaCl, and T_m of the modified duplexes (**III**) and (**IV**) is about 63°C and 62°C, respectively. Most likely the single substitution of the native phosphodiester bond in duplex (**I**) by phosphoryldisulfide linkage leads to a slight duplex perturbation.

A very important feature of the introduced disulfides is that they are mixed and asymmetrical and have two constituent thiols of different acidities. According to theory, in a general case the cleavage of disulfide R^1SSR^2 by nucleophilic thiolate RS^- occurs favorably with release of the more acidic thiol R^{1-}, and the less acidic R^2S group is retained in the new mixed disulfide ($RSSR^2$) [14]. In our case the acidities are very different and therefore it is the sulfur adjacent to the methylene group that must be attacked by nucleophilic thiolate (Scheme 2).

We have chosen a glutathione (γ-L-glutamyl-L-cysteinylglycine) as a model peptide with one inner cysteine and have shown that it really attacks **pss**-linkage as a theory predicts in ACGTTCCTGGCTA**pss**TTGACTGC and ACCTCGGAAAGT**pss**CCCCTCT as well as in duplexes formed by these oligonucleotides with complementary templates. The reaction of disulfide exchange goes quickly and efficiently (see Fig. 1), releasing oligodeoxyribonucleotide-3′-phosphorothioate (a good leaving group) and coupling to 5′-deoxy-5′-mercaptoconstituent (Scheme 3).

The structure of the peptide–oligonucleotide conjugates obtained was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass detection (for example, compound X has an observed mass peak 2721.52 which is very close to the expected mass 2721.97) and by counter-synthesis (the conjugates were synthesized from corresponding 5′-mercaptooligonucleotides and glutathione and had the same retention times upon high-performance liquid chromatography).

At the same time, **pss**-oligodeoxyribonucleotides were practically stable to N^{α}-acetyl-L-lysine, which was used as a very



Scheme 3.

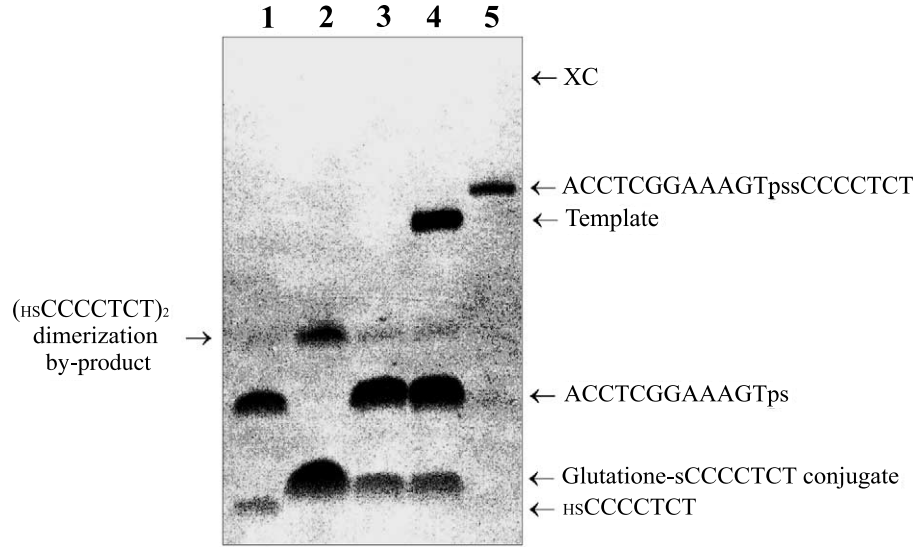


Fig. 1. The gluthatione treatment of ACCTCGGAAAGT**pss**CCCCTCT (lane 3) and duplex formed by this oligonucleotide and complementary template AGAGGGGACTTTCCGAG (lane 4). The following controls were used: a mixture of ACCTCGGAAAGTps and HSCCCCTCT (lane 1); a conjugate of HSCCCCTCT and gluthatione (lane 2) obtained by counter synthesis; ACCTCGGAAAGT**pss**CCCCTCT without treatment (lane 5). The position of the marker xylene cyanol (XC) is shown.

simplified model of inner protein lysine. These results have demonstrated that new dsDNA reagents with internucleotide phosphoryldithio linkages are potentially capable of covalent catching of DNA binding proteins through inner protein cysteine.

Further, we have studied the interaction of M.*Sso*II with duplexes (**II**)–(**V**), containing single internucleotide phosphoryldithio linkages. M.*Sso*II recognizes the following DNA site: 5′-...CCNGG...-3′, 3′-...GGNCC...-5′ (N–A, G, T, C), and methylates the inner cytosine at position C5. The presence of the DNA recognition site and cofactor *S*-adenosyl-L-methionine (AdoMet) or its metabolic product *S*-adenosyl-L-homocysteine (AdoHcy) are required for specific DNA–protein complex formation. M.*Sso*II contains just one cysteine in the position located in motif IV conserved for all (cytosine-5)-DNA-methyltransferases and involved in the catalytic process [20]. This cysteine residue is close to the DNA surface in the enzyme–substrate complex. The crucial role of cysteine in catalysis does not exclude its interaction with the sugar-phosphate DNA backbone during the search for the target deoxycytidine. We suggested that our modified substrates could be useful for elucidation of this question. The incubation of M.*Sso*II with **pss**-oligonucleotides in the presence of AdoHcy was performed. The formation of conjugates which were stable during the separation in SDS–PAGE (polyacrylamide gel electrophoresis) (Fig. 2), but unstable to DTT or 2-mercaptoethanol treatment, was observed (Fig. 3). Analyzing the results obtained we can draw some conclusions:

- the thiolate of inner M.*Sso*II cysteine attacks the sulfur adjacent to the methylene group as in the model experiments (just one conjugation product in each case and appropriate molecular weight band position on the gel were observed in Fig. 2);
- major conjugation (binding efficiency up to 50%) was obtained with duplex (**II**), which contains a modification in the recognition site near the cytosine to be methylated;
- minor conjugation (11% and 8%, respectively) was obtained with duplexes (**III**) and (**IV**), where the modification was located outside of the recognition site, as well as with control duplex (**V**) (8%), which does not contain the M.*Sso*II recognition site;

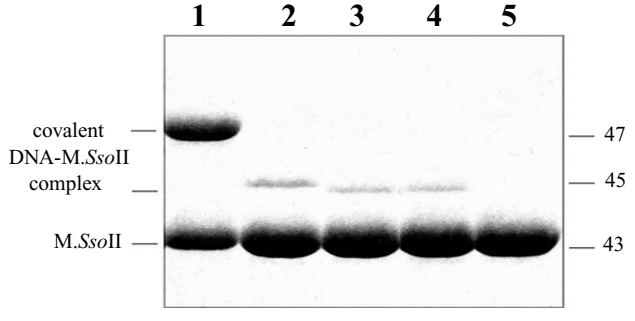


Fig. 2. Cross-linking of DNA duplexes and methyltransferase *Sso*II. Analysis of reaction mixtures by SDS–PAGE after incubation of duplexes (**II**)–(**V**) (lanes 1–4, respectively) with M.*Sso*II; lane 5, control M.*Sso*II. On the right, the molecular weights (kDa) of the protein and its conjugates with DNA are indicated.

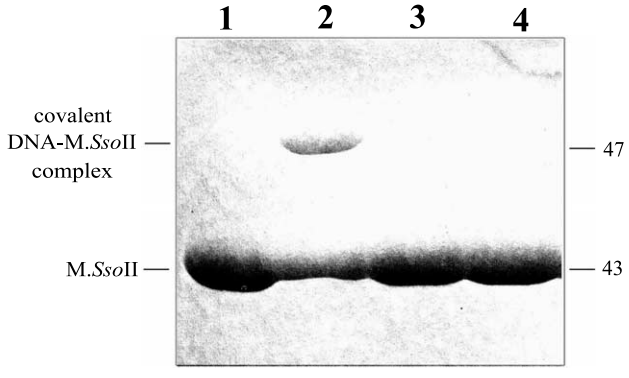


Fig. 3. The treatment of the conjugate of duplex (**II**) with M.*Sso*II (lane 2) by disulfide reducing agents: lane 3, by DTT; lane 4, by 2-mercaptoethanol; lane 1, control M.*Sso*II. On the right, the molecular weight (kDa) of the protein and its conjugate with DNA are indicated.

- the quantitative cleavage of conjugates by DTT or 2-mercaptoethanol treatment confirms the disulfide nature of intermolecular bond forming (Fig. 3).

At the same time, neither restriction endonuclease *Sso*II (which contains two cysteines) nor uracil-DNA-glycosylase (one cysteine) capable of unspecific interaction with DNA form conjugates with **pss**-oligonucleotides under study. Apparently, the efficient formation of the cross-linked product may signify that a cysteine residue of M.*Sso*II was located near the modified position on the DNA in the enzyme–duplex (**II**) complex. The covalent binding of the protein with duplexes (**III**)–(**V**) could also indicate that cysteine 142 of M.*Sso*II is involved in interaction with the DNA, not only at the catalytic step but also in the binding process.

To our knowledge the cross-linking of proteins to nucleic acids using the disulfide moiety introduced into the sugar-phosphate backbone has not been reported before.

The present study was aimed at widening the set of non-native functionalities that can be introduced into DNA without duplex perturbation to fix covalently DNA–protein complexes through inner amino acids. Using **pss** containing duplexes of different structure we are the first to demonstrate that cysteine from Pro-Cys catalytic dipeptide of (cytosine-5)-DNA-methyltransferases [21] may interact with the sugar-phosphate backbone of ligand DNA on the step of specific as well as (to a much lesser extent) unspecific binding. Our first results demonstrate that **pss**-oligonucleotides can be used as new tools to study and manipulate biological systems. The possibility of a flexible change of position and a type of oligonucleotide modification during its chemical synthesis opens a good perspective of defining the mechanisms of nucleic acid–protein interactions, including the determination of amino acids involved in the recognition step or catalysis and the creation of new inhibitors of DNA binding proteins.

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