Cleavage of a DNA fragment by a binary oligonucleotide reagent

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The possibility of specific cleavage of a single-stranded DNA fragment due to cooperative action of two oligonucleotide derivatives bearing chemical groups (at the 3′-phosphate and 5′-thiophosphate ends, respectively) located close to each other in a complementary complex is demonstrated.

Key words: oligonucleotide derivatives, specific cleavage of single-stranded DNA, binary oligonucleotide reagent.

One method of affecting the hereditary system of a cell is the antisense approach[1] in which oligonucleotides or oligonucleotide derivatives react with a nucleic acid (NA), which is a target. In particular, this is done using oligonucleotide derivatives capable of DNA cleavage at a specified site.

Oligonucleotide derivatives that cleave DNA have now been described; they carry reactive groups, *e.g.*, EDTA residue (as a complex with Fe^{2+} in the presence of dithiothreitol),[2] phenanthroline residue (as a complex with Cu^{2+} in the presence of β-mercaptopropionic acid),[3] ellipticine residue (on exposure to light),[4] Fe^{3+} porphyrin residue (on treatment with the Lys—Trp—Lys tripeptide),[5] and bleomycin (in the presence of Fe^{2+} ions and 2-mercaptoethanol).[6] The progress in this field is related to the preparation of oligonucleotide derivatives that efficiently cleave DNA without additional treatment. New opportunities could be provided by the use of binary oligonucleotide reagents (BORs) self-activated upon the formation of perfect complementary complexes with the DNA target.[7] Previously, this approach was proposed for site-directed chemical modification of nucleic acids. The method involves two oligonucleotide derivatives bearing chemical groups with low reactivity toward the NA target at the 3′- and 5′-end, respectively. The oligonucleotide moieties of the derivatives are complementary to neighboring stretches of the NA target. When the oligonucleotide derivatives occur in a proper complementary complex with the NA target, self-ligation of the chemical groups takes place, resulting in a more reactive group. As a result, this group modifies the NA target. The potential of this approach was demonstrated using two 16-mer oligonucleotide derivatives, namely, derivative **1**, bearing a 4-carbonylhydrazidophenyl group, and derivative **2**, bearing a 3-*N*-(2-chloroethyl)-*N*-(*p*-formylphenyl)aminopropylamino group (R^2 group) at the 3′- and 5′-ends, respectively. Under conditions used normally in the antisense approach, chemical ligation of these derivatives took place only in those cases where they were included in a complementary complex. Modification (through covalent attachment) of a 48-mer polydeoxyribonuclotide with the BOR was several times as great as that by either of its components, even when the complex contained the oligonucleotide precursor of the second component.[7]

The purpose of this work is to design another version of binary oligonucleotide reagent to affect DNA. Derivative **2** studied previously has aromatic alkylating 2-chloroethylamino and aldehyde groups, *i.e.*, it is bifunctional as regards the reaction with DNA and, therefore, it has been used as the base to which an appropriate second oligonucleotide* component was selected.

The chemical synthesis of reactive groups for modification of nucleic acids is rather cumbersome; in addition, one cannot predict the efficiency of their action in binary oligonucleotide systems. A way of solving this problem may be screening of properties of the corresponding oligonucleotide derivatives. We proceeded from imidazole and cystamine derivatives, which we had available at the moment. It is known that imidazolides give no covalent products with aromatic aldehydes, whereas oligonucleotide derivatives with an imidazole group at the end efficiently cleave RNA.[8] We meant to find out whether the imidazole group combined with the R^2 group, forced to be closely located, would exert a cooperative influence on DNA. It was supposed that 3′-cystaminophosphooligonucleotide and 3′-mercaptoethylaminophosphooligonucleotide, readily prepared from it, can

* Only oligodeoxyribonucleotides and their derivatives were used in the study; therefore, the prefix d (deoxy) was omitted in the designation.

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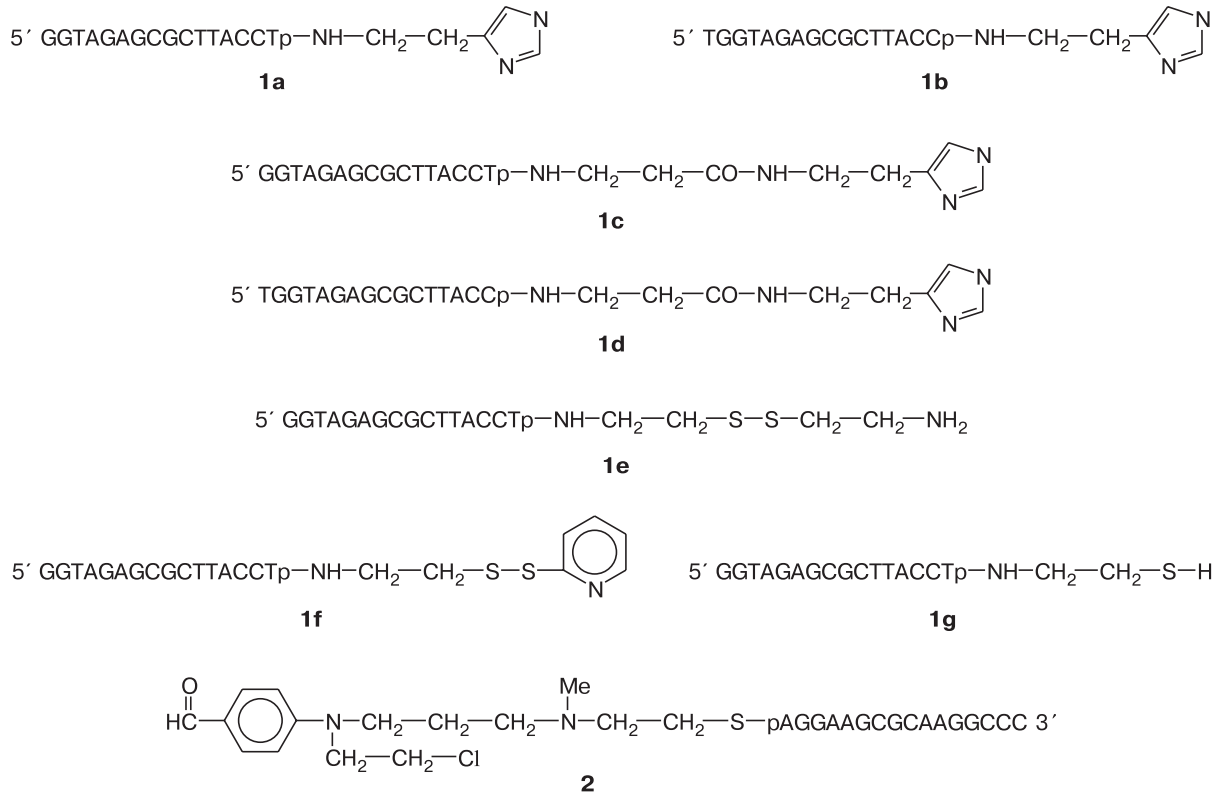


either be covalently bonded to the aromatic aldehyde group of derivative **2**, when located closely to it in a complementary complex, and thus activate the alkylating function, or cooperatively affect DNA without self-ligation.

Derivatives **1a—d** were synthesized by a known method[9] from the corresponding oligonucleotide 3′-phosphates, histamine, and 3-amino-*N*-[2-(1*H*-imidazol-4-yl)ethyl]propionamide. There was no need to confirm the product structure, as the synthesis of these oligonucleotide conjugates by this method[9] has been described previously.[10] The degree of conversion of the initial compounds with respect to oligonucleotide was 69% (**1a**), 81% (**1b**), 72% (**1c**), and 80% (**1d**); therefore, products **1a—d** were tested in the binary system without further purification. The oligonucleotide moieties of 16-mer derivatives **1a**, **1c** and **1b**, **1d** are pairwise identical. These derivatives, together with derivative **2** and the DNA target form so-called nick- or gap-type complexes (in the former case, derivatives **1** and **2** are arranged in the complex with DNA end-to-end, while in the second case, they are located on the DNA matrix with a gap equal to one nucleotide unit). Thus, the reactivity of the pair of reactive groups is tested in two different orientations.

The synthesis of derivatives **1e—g** is described in the Experimental. The electrophoretic analysis of the products of synthesis of derivatives **1e,f** after labeling at the 5′-end of the oligonucleotide moiety is presented in Fig. 1, that for **1g**, in Fig. 2 (lane *26*). The synthesis of these oligonucleotide conjugates has been reported[11] and the products have been characterized by polyacrylamide gel electrophoresis (PAGE). In the synthesis of 3′-cystaminophosphooligonucleotide in the previous study,[11] carbodiimide was used as the condensing reagent. The relative electrophoretic mobilities of the starting oligonucleotide and derivatives **1e,f** correspond to those presented there. The degree of conversion with respect to the oligonucleotide in the synthesis of derivative **1e** was 84%, that for **1f** was 81%, and that for **1g**, was 80%; therefore, they were used without separation from the starting oligonucleotide. Derivatives **1g** were prepared from derivative **1e** as described previously[11] immediately prior to the introduction into the modification reaction, because 3′-mercaptoethylaminophosphooligo-

Fig. 1. Electrophoretic analysis of the 5′-[^{32}P]-products of synthesis of derivatives **1e,f**. Lane *1*, mixed disulfide **1f**; lane *2*, 3′-cystaminophosphooligonucleotide **1e**. The arrows mark the major products. The lower band corresponds to the starting oligonucleotide.

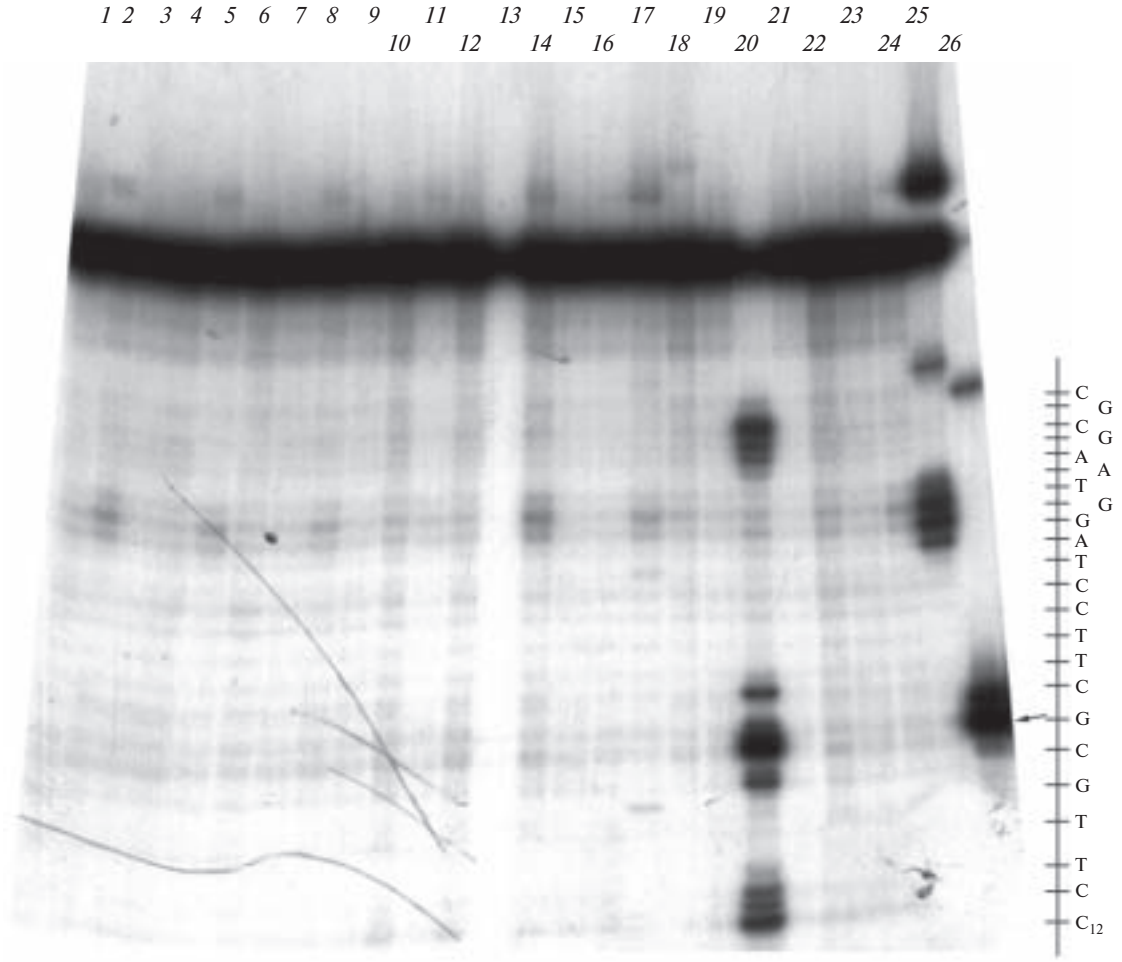


Fig. 2. Electrophoretic analysis of the modification products of the 5′-[^{32}P]-48-mer polynucleotide with oligonucleotide derivatives: (*1*) **2**; (*2*) **1c** + **2**; (*3*) **1c**; (*4*) **1c** + oligonucleotide precursor of derivative **2**; (*5*) **1a** + **2**; (*6*) **1a**; (*7*) **1a** + oligonucleotide precursor of derivative **2**; (*8*) **1d** + **2**; (*9*) **1d**; (*10*) **1d** + oligonucleotide precursor of derivative **2**; (*11*) **1b** + **2**; (*12*) **1b**; (*13*) **1b** + oligonucleotide precursor of derivative **2**; (*14*) **1e** + **2**; (*15*) **1e**; (*16*) **1e** + oligonucleotide precursor of derivative **2**; (*17*) **1f** + **2**; (*18*) **1f**; (*19*) **1f** + oligonucleotide precursor of derivative **2**; (*20*) **1g** + **2**; (*21*) **1g**; (*22*) **1g** + oligonucleotide precursor of derivative **2**; (*23*) without derivatives; (*24*) **2**; (*25*) **2** activated by sodium borohydride; (*26*) analysis of the reaction mixture of labeling of compound **1g**: the arrow marks [^{32}P]-**1g**, the starting 3′-cystaminophosphooligonucleotide is located above, and the upper band is the corresponding disulfide. The line on the right shows the positions on the gel of the products of the Maxam—Gilbert cleavage[12] of the given polynucleotide (sequencing cleavage).

nucleotides are readily oxidized by atmospheric oxygen even during simple operations.

The modification of the DNA target with BOR is shown in Scheme 1. The results of electrophoretic analysis of the products of modification of the [^{32}P]-48-mer polynucleotide with the presumptive BOR and with its components are presented in Fig. 2. It can be seen that in the case of derivative **2** in combination with imidazolide oligonucleotide derivatives **1a**, **1b**, **1c**, and **1d** (lanes *5*, *11*, *2*, and *8*, respectively), the alkylating capacity of the group R^2 is slightly higher than that in the nonactivated derivative **2** (lanes *1* and *24*); this shows itself as a minor increase in the amount of addition products of oligonucleotide derivative **2** to the DNA target (they have a lower mobility than the DNA target and the same as the product of addition of activated derivative **2**, see lane *25*, and further in the text). The slight increase in the alkylating capacity is also confirmed by a slight enhancement of the cleavage of the DNA target at nucleotide residues G26, G27 for derivatives **1a**, **1c**, and **1d** (derivatives **1c** and **1d** are the nick and gap modifications, respectively). Similar activation of the alkylating capacity of the R^2 group is found on treatment with derivatives **1e** and **1f** (lanes *14* and *17*, respectively). Thus, derivatives **1a**—**d** and **1e**—**f** exert a slight activating effect on derivative **2**. A combination of derivatives **1g** and **2** (lane *20*) gives rise to products of modification of the DNA target, which includes substantial (60%) cleavage of the DNA target without additional treatment, directly under the reaction conditions. The cleavage sites are localized in

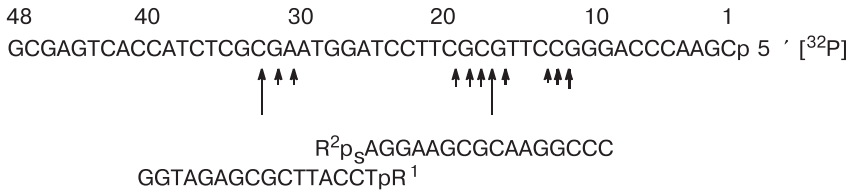


Fig. 3. Schematic representation of the major sites of cleavage of the 5′-[^{32}P]-48-mer polynucleotide with BOR.

such a way that the products form three groups, each group containing one major product. The percentages in the groups with respect to the whole radioactive material in the lane *20* amount to 17, 29, and 14% (from the top down). In the cases where derivative **1g** alone is used (lane *21*) or derivative **1g** is used with oligonucleotide precursor of derivative **2** (lane *22*) (or derivative **1g** with the 5′-phosphorothioate precursor of derivative **2**, the data are not given), no substantial cleavage of the DNA target is observed. Other combinations of oligonucleotide derivatives and their precursors did not cause noticeable cleavage of DNA target (see also above). Among them, the maximum cleavage is observed for lane *14* (derivatives **2** and **1e**). It should be noted that it corresponds to 1/20 of the cleavage in lane *20* or to 3% of the DNA target. As an additional verification, lane *25* shows the result of alkylation of the DNA target with derivative **2** activated by reduction of its aldehyde group by sodium borohydride.[13] It is significant that the major product is the product of addition of the oligonucleotide derivative to the DNA target (lane *25*, a radioactive band above the polynucleotide), whose ratio to the cleavage products in the same lane is 3 : 2. The main cleavages in lane *25* refer to G26 and G27.[13] In view of the foregoing together with our results,[13] concerned with the Maxam-Gilbert cleavage of a 48-mer polynucleotide (presented schematically in Fig. 2), the cleavage products found in lane *20* were analyzed in more detail. The following specific features were found: in the lower group of products, cleavage mainly involves the neighboring cytidine residues with preference of C12; there is also a product with an intermediate mobility (*i.e.*, a product whose electrophoretic mobility is not a multiple of one nucleotide unit); in the medium group, the main site is cytidine residue C17, and a less distinct product with an intermediate mobility is located in the close vicinity of this residue (due to its close position on the gel to the product of cleavage at C17, this product can be seen only in the initial autoradiograph); the cleavage product at C19 is equally significant; those at G16 and G18 are weaker; in the upper group, the cleavage product at C32 is the major one, and two weaker products, at G31 and A30, are also present. Comparison of the results in lanes *20* and *25* demonstrates that in the former case, cleavage of the DNA target not only involves sites different from those affected in the case of cleavage caused by alkylation by activated derivative **2** but also involves predominantly other nucleotide residues of DNA, namely, cytidine. The primary sites of cleavage of the [^{32}P]-target by derivative **1g** coupled with derivative **2** are sketched in Fig. 3. They are located in three regions involved in the complementary complex of the target with BOR, rather far from one another. This cleavage cannot be explained by alkylation of the DNA target by the R^2 group activated by the second BOR component, because alkylation with borohydride-activated derivative **2**, in particular, within a BOR[7] (having oligonucleotide derivative bearing a 4-carbonylhydrazidophenyl group as the second component) gives the products of reagent addition to the DNA target as the major products. In addition, insignificant amounts of products of the DNA cleavage at the site located in the immediate vicinity of the alkylating group are also detected.[7] Analysis of the hypothetical secondary structures of the DNA target and the structures of its possible complexes with derivatives **1g** and **2** gave rise to the structure shown in Fig. 4. The formation of a nearly cross-shaped structure cannot be ruled out, the more so, because in this case, the sites of cleavage of the DNA target prove to be much closer to each other than in the

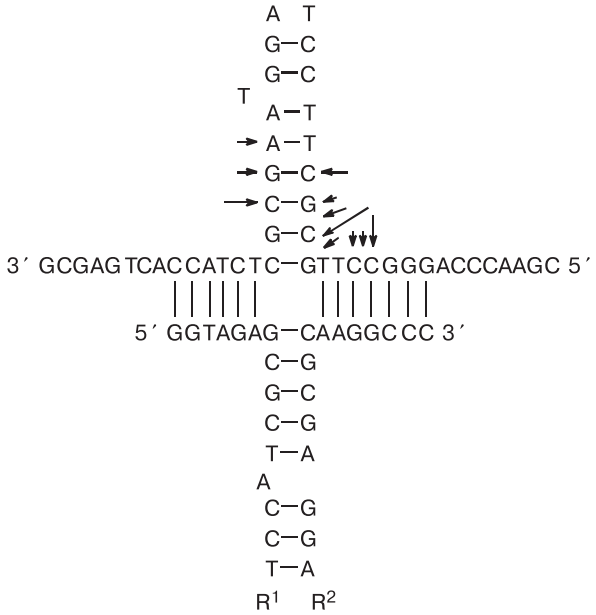
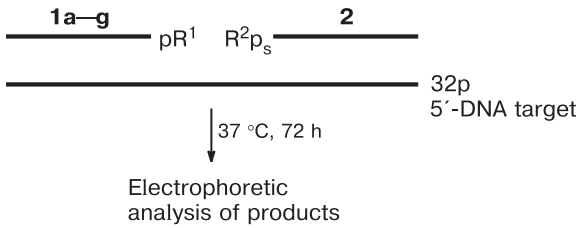


Fig. 4. Plausible secondary structure of the complex of the DNA target with BOR.

linear structure. It appears probable that the DNA target and BOR components, *viz.*, derivatives **1g** and **2** (with 3′-mercaptoethylamino and 3-*N*-(2-chloroethyl)-*N*-(*p*-formylphenyl)aminopropylamino groups), produce a structure shaped like a cross, which forms an active site, and this results in the cleavage of the DNA target. The efficiency of cleavage attests indirectly to a mechanism of action other than alkylation. The possibility of formation of chemical ligation products of derivatives **1g** and **2** in the absence and in the presence of a DNA target was studied using [^{32}P]-labeled derivative **1g** and analyzing the reaction mixtures by gel electrophoresis in denaturing PAAG. No ligation products were detected (the data are not shown). This may imply that either ligation does not occur or its products are unstable against electrophoresis or thermal denaturation (100 °C, 1 min), which is carried out in a standard formamide-containing loading buffer prior to electrophoresis.

Scheme 1



Thus, we demonstrated slight enhancement of the alkylating capacity of derivative **2** by compounds of various chemical natures: **1a—d**, **1e**, and **1f**. Partially, this might be due to the influence of oligonucleotide derivatives **1** as oligonucleotide effectors.[14] This effect is rather insignificant compared to the overall effect of derivatives **2**+**1g**, which has a different mechanism. It is surprising that the cleavage induced by this couple involves, most of all, cytidine residues and, besides, gives additional products with intermediate electrophoretic mobility. Elucidation of the mechanism of the discovered phenomenon is of indubitable interest.

Three distinctive features are inherent in a binary oligonucleotide reagent:[7] activation through self-ligation in a proper complementary complex with the DNA target, the absence of ligation products outside a proper complementary complex with DNA, and a higher modifying capacity compared to that of the initial oligonucleotide derivatives. The reagent prepared in this study complies with the second and third items. It also meets, to some extent, the first condition because it is activated and, apparently, the activation is a result of complementary interactions "proper" for this DNA target. Thus, the BOR concept proposed previously has been extended in this study.

Further investigation of these structures opens up new prospects for addressed modification of nucleic acids.

Experimental

Commercial histamine, cystamine dihydrochloride, 2,2′-dipyridyl disulfide, triphenylophosphine, 4-dimethylaminopyridine (DMAP), sodium borohydride, dimethylformamide, cetyltrimethylammonium bromide (Aldrich, USA); dithiothreitol (DTT; Fluka AG, Germany); *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) (Sigma, USA); T4 phage polynucleotide kinase, T4 phage DNA ligase, adenosine-5′-[γ-thio]triphosphate (Boehringer Mannheim, Germany); Sephadex G-50 Superfine (Pharmacia, Sweden); and [γ-^{32}P]-ATP (Amersham, UK) were used. *N*-Methyl-*N*,*N*′-bis(2-chloroethyl)-*N*′-(*p*-formylphenyl)trimethylenediamine, whose synthesis and characterization have been described previously,[15] was kindly provided by A. A. Gall and G. V. Shishkin (Novosibirsk Institute of Bioorganic Chemistry of the Siberian Branch of the RAS).

An analog of carnosine, 3-amino-*N*-[2-(1*H*-imidazol-4-yl)ethyl]propionamide, was synthesized, characterized,[16] and kindly provided by V. N. Silnikov (Novosibirsk Institute of Bioorganic Chemistry of the Siberian Branch of the RAS).

Oligodeoxyribonucleotides were kindly provided by V. P. Kumarev and V. F. Kobzev (Institute of Cytology and Genetics of the Siberian Branch of the RAS). The cetrimonium salts of 3′-phosphooligonucleotides were prepared by adding a 20 *mM* aqueous solution of cetyltrimethylammonium bromide (CETAB) to an aqueous solution of an oligonucleotide. The first portion of the CETAB solution was half of the calculated amount, and every subsequent portion was decreased twofold, until precipitation was no longer observed; the mixture was centrifuged and dried by evaporation with three portions of anhydrous acetonitrile.

The polydeoxyribonucleotide target was assembled by ligation of three 16-mer oligodeoxyribonucleotides by the T4 phage DNA ligase, isolated by electrophoresis in denaturing PAAG, and labeled as described previously.[13]

Gel electrophoresis of oligo- and polynucleotide products was carried out in denaturing 20% PAAG. To estimate the degree of transformation of oligonucleotide derivatives, modification products, and cleavage, the autoradiographs of polyacrylamide gels containing the products under analysis were scanned in the transmission mode, and the integral optical densities were calculated by the standard method using the GelProAnalyzer program (Media Cybemetics, USA).

In isolation and in experiments with oligonucleotide derivatives, buffer solutions were degassed by being purged with helium.

The other reagents used in the work were at least high-purity grade commercial preparations.

The synthesis of 3′-cystaminophosphooligonucleotide (1e) was carried out by a known procedure.[9] The cetrimonium salt of 3′-phosphooligonucleotide (10 nmol) was dissolved in anhydrous DMF, and Ph_3P (2 mg), $(PyS)_2$ (1.7 mg), and DMAP (1.9 mg) were added. After dissolution of the components, the reaction mixture was incubated for 45 min at ~20 °C. The DMAP derivative of 3′-phosphooligonucleotide was precipitated by adding 750 μL of a 2% solution of $LiClO_4$ in acetone

and isolated by centrifuging. The precipitate was quickly dried *in vacuo*, dissolved in 200 μL of a 0.25 *M* aqueous solution of cystamine (brought to pH 9.0 by adding a 0.02 *M* solution of NaOH), and incubated for 8 h at ~20 °C. The product was isolated by gel filtration on a column with Sephadex G-50 (20 mL) with UV detection ($A_{260/280}$) using an Ob´-4 microspectrophotometer. Fractions containing the polymeric product were combined and concentrated on a rotary evaporator at a bath temperature of 30—35 °C. The residue was dissolved in water, the optical density A_{260} was measured, and the oligonucleotide concentration was brought to 100 μmol L^{-1}. The yield of the total oligonucleotide material was 80%. The products were analyzed by electrophoresis after labeling with [γ-^{32}P]-ATP and the T4 phage polynucleotide kinase (see Fig. 1).

Synthesis, isolation, and analysis of 3´-imidazolidooligonucleotides (1a—d) was carried out similarly to the procedure described above. The yields of the total oligonucleotide material were 66 (**1a**), 67 (**1c**), 75 (**1b**), and 77% (**1d**).

The preparation of 3´-mercaptoethylaminophosphooligonucleotide (1g) was carried out as described previously.[11] The reaction mixture containing 75 *μM* 3´-cystaminophosphooligonucleotide and 12 *mM* DTT in the 30 *mM* Tris HCl (pH 7.2)—3 *mM* EDTA system was incubated for 3 h at ~20 °C and the product was isolated by gel filtration on a column with Sephadex G-50, the sample volume to the column volume ratio being 1 : 30. The optical density A_{260} was measured. The yield of the total oligonucleotide material was 75%. Subsequent electrophoretic analysis of the product after labeling with T4 polynucleotide kinase and [γ-32P]-ATP showed a 80% degree of conversion of the initial 3´-cystaminophosphooligonucleotide into 3´-mercaptoethylaminophosphooligonucleotide with about 5% of the product of oxidation of 3´-mercaptoethylaminophosphooligonucleotide to the corresponding disulfide under labeling and analysis conditions (see Fig. 2, lane *26*).

Synthesis of mixed 2-thiopyridine and 3´-mercaptoethylaminophosphooligonucleotide disulfide (1f). The synthesis was carried out in a similar way.[11] A 200-μL mixture containing 25 *μM* 3´-cystaminophosphooligonucleotide and 5 *mM* DTT in a 10 *mM* Tris HCl (pH 7.2)—1 *mM* EDTA mixture was incubated for 1.5 h at 25 °C. Then 27 μL of the 0.5 *M* Tris HCl (pH 7.2)—1 *mM* EDTA system and 928 μL 3 *mM* 2,2´-dipyridyl disulfide were added, and the mixture was incubated for 3 h. The product was isolated by gel filtration on a column with Sephadex G-50 followed by evaporation. After dissolution, the optical density A_{260} was measured. The yield of the total oligonucleotide material was 78%. The products were labeled by ^{32}P and analyzed by electrophoresis (see Fig. 1).

Derivative **2** was prepared by the procedure described in our previous publication.[17]

Modification of the 48-mer polydeoxyribonucleotide by BOR. The reaction mixture contained [^{32}P]-polynucleotide (0.4 *μM*), derivative **1a—g** (one of them or their precursors) (1 *μM*), and derivative **2** (or its precursor) (1 *μM)* in a 10 *μM* HEPES—KOH (pH 7.3)—0.5 *mM* EDTA mixture. The product was analyzed by electrophoresis after 72 h of incubation at 37 °C (see Fig. 2).

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