Site-directed cleavage of DNA by an oligonucleotide conjugate with *o*-bromobenzoic acid in the presence of copper ions

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Site-directed cleavage of single- and double-stranded DNAs by an oligonucleotide conjugate with 5-[*N*-(3-aminopropyl)sulfamoyl]-2-bromobenzoic acid was investigated. When forming duplex complexes with a single-stranded DNA and triplex complexes with a double-stranded DNA, this conjugate cleaves DNA near the binding site in the presence of copper ions and free *o*-bromobenzoic acid. The efficacy and specificity of DNA cleavage by this conjugate and other oligonucleotide conjugates bearing tetracarboxyphthalocyanine Co^{II} and bleomycin A_5 as reactive groups were compared.

Key words: oligonucleotide conjugates, DNA cleavage, metal complexes, triplex DNA.

Chemical compounds capable of selective cleavage of DNA at definite nucleotide sequences are needed for the development of various fields of molecular biology and for elaboration of therapeutics acting on nucleic acids. These compounds can be obtained by attachment of reactive groups to oligonucleotides that react with particular nucleotide sequences of DNA.[1,2] The selection of reactive groups that could efficiently cleave or chemically modify DNA under physiological conditions is an important, yet unsolved, problem in the design of these compounds. Currently, a large number of metal complex groups able to induce the oxidative damage of DNA are known, including bleomycin—iron, phenanthroline—copper, and ascorbic acid—copper complexes.[3—6] These reagents cleave or modify DNA under aerobic conditions in the presence of reducing agents or donors of active oxygen; however, none of them modify DNA up to biologically significant levels.

Copper complexes are attractive as potential reactive groups for oligonucleotide conjugates, because copper ions are present in the cell in micromolar quantities, acting most often as enzyme cofactors.[7] Direct oxidation of biopolymers with oxygen in a solution is impossible because the O_2 molecule is a triplet in the ground state and its reaction with the substrate molecule is spin-forbidden. A way of overcoming this restriction is activation of oxygen through complexation with transition metals, for example, copper and iron. Transition metal ions catalyze the stepwise reduction of oxygen, which is accompanied by a change in the metal oxidation state $M^{n+1} \rightarrow M^n$ and gives active species able to damage DNA.[8,9] The feasibility of the design of a metal-dependent aromatic system that cleaves DNA in the absence of exogeneous reducing agents was exemplified by hydroxy salen complexes, which react with the redox system of copper, thus facilitating the spontaneous formation of Cu^{III}-containing oxidizing species involved in the DNA damage.[10]

We started the studies on the use of *ortho*-halogenobenzoic acids as potential reagents for DNA cleavage in the presence of Cu^{II} ions. With copper ions, benzoic acid derivatives form both monomeric complexes and complexes with two coordinated molecules of substituted benzoic acid; hence, they can be regarded as reactive groups for the design of binary systems based on oligonucleotides that may be potential catalysts of site-directed cleavage of DNA. Previously,[11] we have shown that the aromatic ring and the carboxy group are the essential elements for the effective metal-dependent cleavage of DNA. *o*-Bromobenzoic acid cleaves efficiently the plasmid DNA in the presence of Cu^{II} ions by an oxidative mechanism under the action of oxygen radicals formed in the specific DNA · Cu^{II} · *o*-bromobenzoic acid complex.[11]

The purpose of this work is to study the cleavage of double- and single-stranded DNA target by a conjugate of an oligonucleotide with 5-[*N*-(3-aminopropyl)sulfamoyl]-2-bromobenzoic acid in the presence of Cu^{II} ions and to compare the efficiency of this reagent with the efficiencies of some other oligonucleotide conjugates with metal complexes.

Results and Discussion

Among other ways, oligonucleotide derivatives can act on DNA through the formation of triplexes of an oligonucleotide with homopyrimidine—homopurine DNA sequences.[12,13] We chose a fragment of the promoter region of the gene *c-fos* containing a 14-mer polypurine sequence as the DNA target (Fig. 1, DNA II).[14] Oligo-

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nucleotides ON1 and ON2 were chosen in such a way that they formed pyrimidine- and purine-type triplexes, respectively, with the model DNA. Previously,[15] we demonstrated the possibility of specific modification of the aforementioned sequence within a reconstructed nucleosomal chain by an alkylating derivative of oligonucleotide ON1. A synthetic 20-mer oligonucleotide containing the same 14-mer sequence was used as a single-stranded target (DNA I). 5-[*N*-(3-Aminopropyl)sulfamoyl)-2-bromobenzoic acid (**1**) was attached by a phosphoramide bond to the 5′-end of oligonucleotide ON1 under conditions of the synthesis of the alkylating oligonucleotide derivatives[16] (see Fig. 1). It was shown that compound **1** cleaves plasmid DNA in the presence of Cu^{II} ions with an efficiency comparable to that observed with *o*-bromobenzoic acid.

Cleavage of a single-stranded DNA

Cleavage of a single-stranded 20-mer oligonucleotide (DNA I) incorporated in a complementary complex with a conjugate of oligonucleotide ON1 bearing an *o*-bromobenzoic acid (OBB) group was carried out in buffer T in the presence of 10 μM $CuSO_4$. The concentrations of the DNA target and the reagent were $1 \cdot 10^{-8}$ and 10^{-5} to 10^{-6} mol L^{-1}, respectively. The reaction products were analyzed by gel electrophoresis in 20% polyacrylamide gel (PAAG). It was found that no cleavage of the DNA target under the action of OBB conjugate in the presence of Cu^{II} ions occurred even after prolonged incubation.

It is known that *o*-bromobenzoic acid forms complexes in which the copper ion coordinates two molecules of the acid.[17] We suggested that the formation of such complexes is a prerequisite for the formation of a copper active site, which catalyzes the stepwise reduction of oxygen present in the solution to give radicals; hence, cleavage of DNA under the action of OBB conjugate requires the presence of an additional molecule of *o*-bromobenzoic acid in the active complex.

To check this assumption, we carried out the reaction in the presence of free *o*-bromobenzoic acid. Under these conditions, site-directed cleavage of DNA induced by the OBB conjugate (Fig. 2, lanes *12* and *13*) was found to take place at the G_{16} and G_{18} guanine sites. The kinetics of cleavage is shown in Fig. 3. After 22 h of incubation, the degrees of DNA cleavage at G_{18} and G_{16} were 2.9% and 2.6%, respectively. Further incubation did not result in a higher degree of cleavage at these sites, only products of nonspecific DNA degradation being accumulated.

We compared the efficiency of the OBB conjugate with the efficiency of a known[18] conjugate bearing a residue of a porphyrin analog, namely, cobalt(II) tetracarboxyphthalocyanine (Ptc)[18] able to modify DNA in an O_2—reducing agent system or in the presence of H_2O_2. The reaction of DNA with the Ptc conjugate was carried out in the presence of 2 *mM* H_2O_2 and the mixture was treated with piperidine. The data on cleavage of DNA I by the OBB- and Ptc-derivatives of oligonucleotide ON1 are presented in Fig. 2. It can be seen that cleavage by the Ptc conjugate takes place only in the presence of an oxidant, the specificity being higher than that observed with the OBB conjugate (Fig. 4, *a*). It is noteworthy that treatment of DNA with Ptc results in oxidation of heterocycles, which can be revealed only upon additional treatment with piperidine, whereas the reaction of the conjugate bearing *o*-bromobenzoic acid leads to direct cleavage of DNA. In addition, under the conditions used, a smaller degree of nonselective degradation of DNA is observed with the OBB conjugate than with the Ptc conjugate, 6% and 28%, respectively.

Cleavage of a double-stranded DNA

The double-stranded DNA fragment (see Fig. 1, DNA II), 152 base pairs (bp) long, representing a promoter region of the *c-fos* gene from −148 to +3, [^{32}P]-labeled at the 5′-end of one chain, was prepared by a polymerase chain reaction (PCR). The formation of triplexes by oli-

a

5′ GAAGGGGGGGGAAGCG$_{16}$CG$_{18}$CC 3′

DNA I

b

5′-GGGGCGCGCTTCCCCCCCCTTCCAGTTCCGCCCAGTGACGTAGGAAGTCCATCCATTCA-3′

3′-CCCCGCGCGAAGGGGGGGGGAAGGTCAAGGCGGGTCACTGCATCCTTCAGGTAGGTAAGT′-[^{32}P]

−100 −90 −80 −70 −60

DNA II

c

3′ CTTCCCCCCCCTTCp 5′

ON1

d

3′ pGAAGGGGGGGGGAAG 5′

ON2

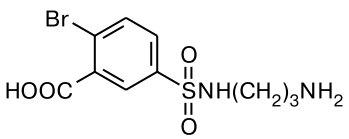

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Fig. 1. Nucleotide sequences of DNA I (*a*) and DNA II (*b*) and triplex-forming oligonucleotides ON1 (*c*) and ON2 (*d*).

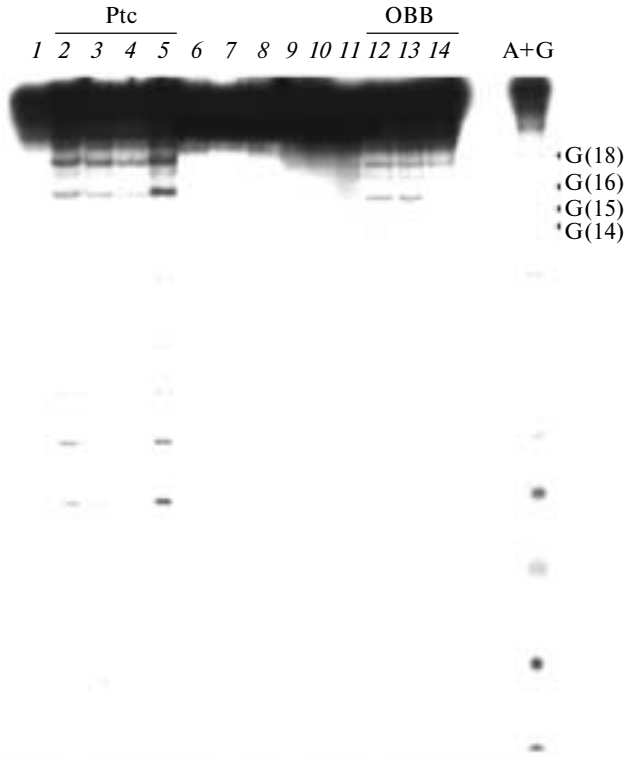

Fig. 2. Cleavage of DNA I by the Ptc and OBB conjugates: (*1*) DNA I, reference; (*2—6*) DNA I + Ptc in concentrations of 200 (*2*), 100 (*3*), 50 (*4*), 500 (*5*), and 0 μmol L^{-1} (*6*) in the presence of 2 *mM* H_2O_2 with subsequent treatment with piperidine; (*7*) DNA I + 200 *μM* Ptc without adding H_2O_2; (*8*) DNA I treated with piperidine; (*9*) DNA I + 100 *μM* *o*-bromobenzoic acid; (*10*) DNA I + 100 *μM* $CuSO_4$; (*11*) DNA I + 10 *μM* *o*-bromobenzoic acid and 10 *μM* $CuSO_4$; (*12—14*) DNA I + + 20 (*12*, *14*), 10 μmol L^{-1} OBB (*13*) and 10 (*12*, *13*) or 0 *μM* *o*-bromobenzoic acid (*14*). A+G is DNA I cleaved according to the Maxam—Gilbert method.

gonucleotides ON1 and ON2 with the target sequence within this DNA at pH 5.3 and 7.0, respectively, was preliminarily studied using alkylating derivatives of these oligonucleotides and by the footprinting technique using diethyl pyrocarbonate. The reaction with an alkylating derivative of oligonucleotide ON1 occurred at guanosine G_{-83} adjacent to the target sequence, which confirms the formation of a specific triple-stranded complex under the reaction conditions.[19]

We studied the reaction of DNA II with the OBB and Ptc conjugates and with the conjugate of oligonucleotide ON2 with antibiotic bleomycin A_5(Blm).[20—24] The autoradiogram of the separation of DNA II cleavage products by the OBB, Ptc, and Blm conjugates is shown in Fig. 5.

In the reaction with the OBB conjugate (see Fig. 5, lanes *13—15*), modification involves mainly the C_{-103}, G_{-83}, G_{-75}, and T_{-72} nucleotides, while in the case of phthalocyanine conjugate, these are G_{-109}, G_{-97}, C_{-81}, G_{-75}, and T_{-72} (see Fig. 5, lanes *3* and *5*). The degrees of

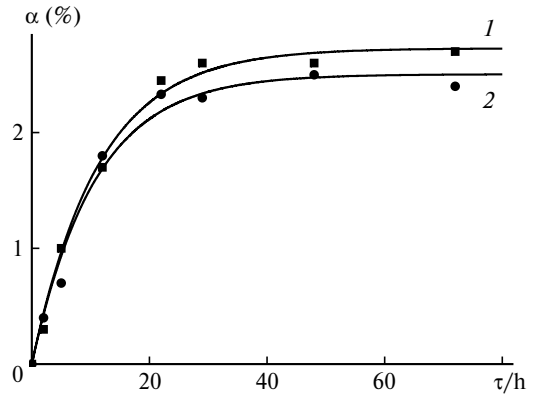

Fig. 3. Kinetics of DNA I cleavage by the OBB conjugate at the G_{18} (*1*) and G_{16} (*2*) sites. DNA I was incubated with the OBB conjugate (10^{-5} *M*) at 37 °C in the presence of 10 *μM* *o*-bromobenzoic acid and 10 *μM* $CuSO_4$. α is the degree of modification.

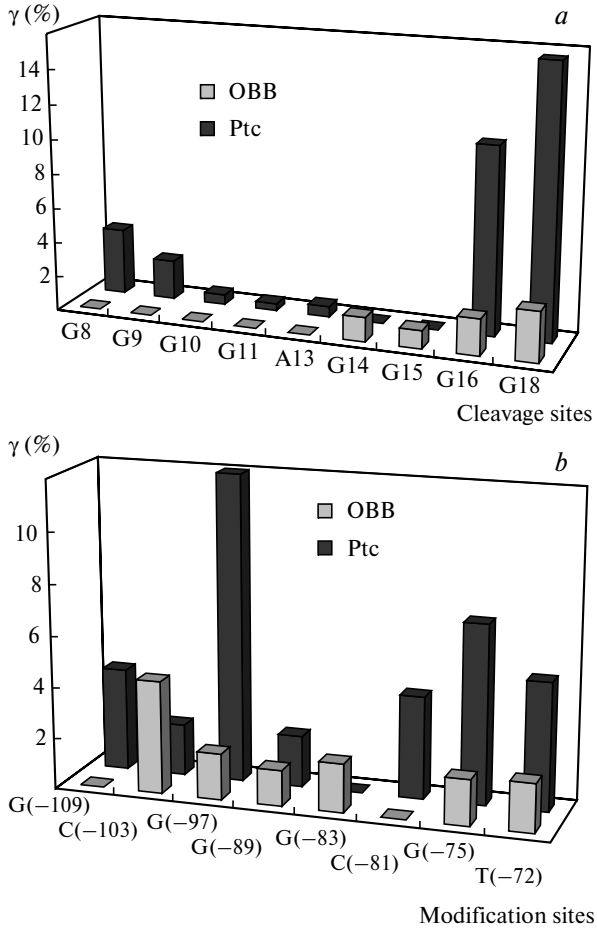

Fig. 4. Cleavage of DNA I (*a*) and DNA II (*b*) by the Ptc and OBB conjugates. DNA was incubated with $2 \cdot 10^{-4}$ (*a*) and 10^{-4} *M* Ptc in the presence of 2 *mM* H_2O_2 at 24 °C for 24 h; 10^{-5} *M* OBB in the presence of 10 *μM* *o*-bromobenzoic acid and 10 *μM* $CuSO_4$ at 37 °C for 24 h; γ is the degree of cleavage.

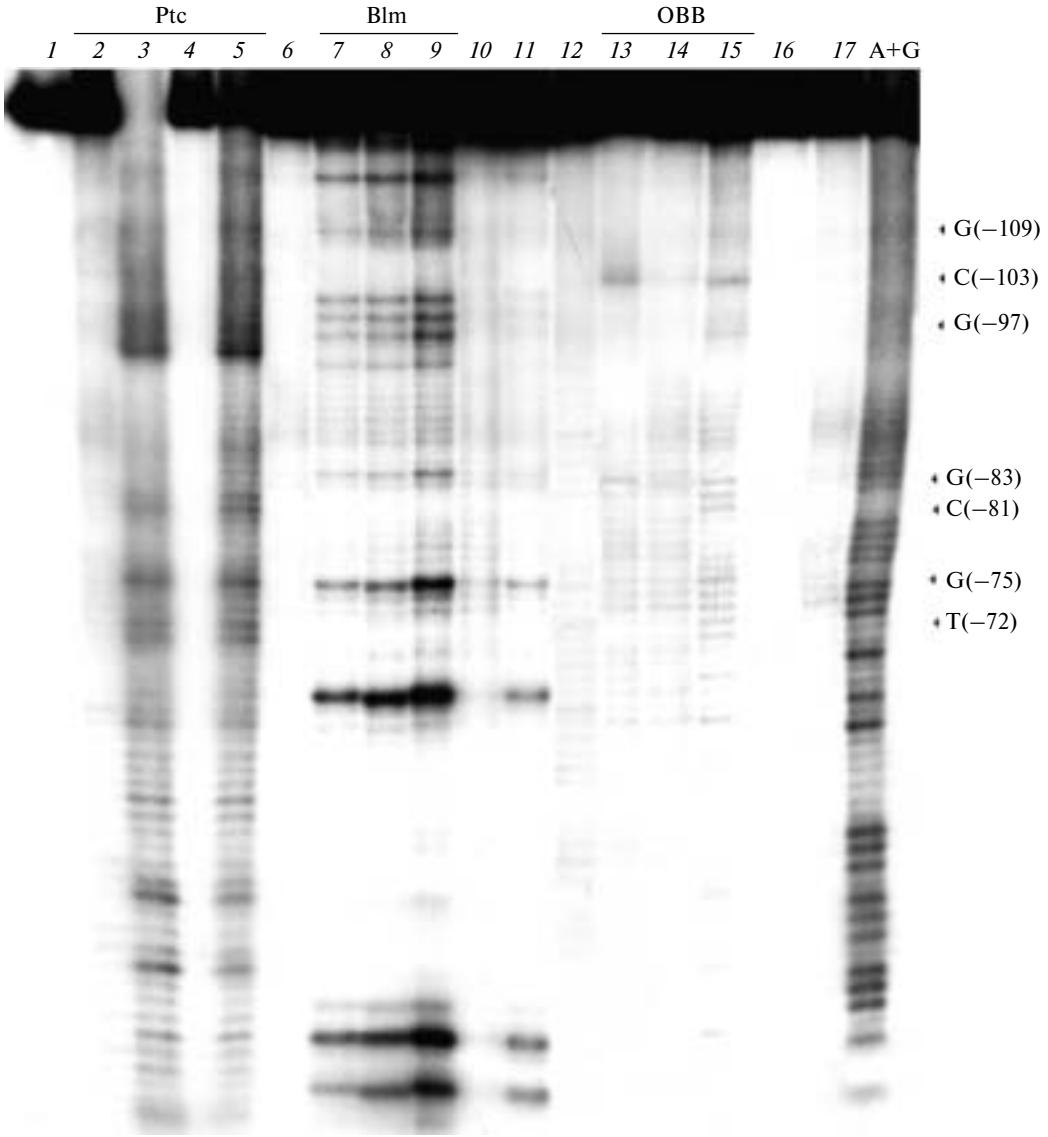

Fig. 5. Cleavage of DNA II by the Ptc, Blm, and OBB conjugates. (*1*) DNA II, reference; (*2—6*) DNA II + Ptc in concentrations of 500 (*2*), 200 (*3*), 100 (*4*), and 0 μmol L^{-1} (*5*) in the presence of 2 *mM* H_2O_2 (*3*, *5*, *6*) with subsequent treatment with piperidine; (*7—10*) DNA II + Blm in concentrations of 5 (*7*), 10 (*8*), 20 (*9*), and 0 μmol L^{-1} (*10*) in the presence of 10 *μM* $(NH_4)_2Fe(SO_4)_2$ and 5 *mM* 2-mercaptoethanol; (*11*) DNA II + 1 *μM* free bleomycin; (*12*) DNA II + 10 *μM* *o*-bromobenzoic acid and 10 *μM* $CuSO_4$; (*13*, *15*) DNA II + OBB in concentrations of 10 (*13*) and 20 *μM* (*15*) + 10 *μM* *o*-bromobenzoic acid + 10 *μM* $CuSO_4$; (*14*) DNA II + + 10 *μM* OBB + 10 *μM* $CuSO_4$; (*16*) DNA II + 100 *μM* *o*-bromobenzoic acid; (*17*) DNA II + 100 *μM* $CuSO_4$; A+G is DNA II cleaved by the Maxam—Gilbert method.

nonspecific degradation for the Ptc and OBB conjugates are 52% and 14%, respectively. Comparative diagrams of the efficiency of cleavage of a double-stranded DNA by these conjugates are presented in Fig. 4, *b*.

The presence of minor cleavage sites rather remote from the binding site of the conjugates is evidently due to the diffusion of some part of oxygen radicals formed in the solution.[25] In the case of both the OBB and Ptc conjugates, the modification involves predominantly the sites located not more than 20 nucleotides away from the reactive group; this is observed for groups that generate diffuse oxygen radicals.[26]

Since the pH optimum for the reaction of bleomycin with DNA is 7—9,[27] modification of DNA II was carried out using the Blm conjugate (with a bleomycin A_5 residue at the 3′-end), which forms a purine-type triplex with the same 14-mer DNA sequence at neutral pH values. The reaction of DNA II with the Blm conjugate was carried out for the reagent concentrations from 10^{-6} to $5 \cdot 10^{-6}$ mol L^{-1} and the DNA concentration of $2 \cdot 10^{-8}$ mol L^{-1}. It was

found that under these conditions the site-directed cleavage corresponding to the location of bleomycin is accompanied by cleavage at the GC and GT sequences, which are the same as the sites of DNA cleavage by free bleomycin (see Fig. 5, *cf.* lanes *7—9* and *11*). This pattern of cleavage suggests that in the presence of excess Blm with respect to the DNA target, the bleomycin conjugate is bound to the double-stranded DNA without formation of a triplex by the oligonucleotide moiety of the reagent. It is noteworthy that under experimental conditions, a decrease in the concentration of the oligonucleotide derivative results in a sharp decrease in the efficiency of triplex formation. In particular, for the concentration of the DNA target of 10^{-8} mol L^{-1} and the concentration of the Blm conjugate of 10^{-7} mol L^{-1}, no triplex formation was detected by either the gel retardation assay or DEPC-footprinting.

The results obtained show that oligonucleotide conjugates containing *o*-bromobenzoic acid residue as the reactive group can accomplish site-directed cleavage of both single-stranded and double-stranded DNA in the presence of free *o*-bromobenzoic acid and copper(II) ions. Cleavage by this reagent is less efficient than that with the oligonucleotide conjugates containing tetracarboxyphthalocyanine Co^{II} or bleomycin A_5 as the reactive groups; however, this does not require the presence of exogeneous reducing agents or hydrogen peroxide in the reaction mixture. In addition, it occurs more specifically and gives direct nicks.

Experimental

Materials and methods. Commercial T4 polynucleotide kinase (Fermentas, Lithuania), Taq-polymerase (Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences), deoxyribonucleoside 5′-triphosphates, acrylamide, *N*,*N*′-methylenebisacrylamide, urea, Tris, piperidine, 2-mercaptoethanol, EDTA, DMF, RNA-carrier (Sigma, USA), and γ-[^{32}P]-ATP with a specific activity of 1 PBq mol^{-1} (Biosan, Russia) were used.

Solutions were prepared using water purified on a MilliQ unit (Millipore, USA).

The oligonucleotides pd(CTTCCCCCCCCTTC), pd(GAAGGGGGGGGAAG), and d(GAAGGGGGGGGAAGCGCGCC) and the primers d(CCTGGGGCGTAGAGTTGA) and d(TGAAAATCTCGCCAAGCTTAT) were synthesized at the Novosibirsk Institute of Bioorganic Chemistry by the standard phosphoramidite method.

The following buffers were used: T (50 *mM* AcONa, pH 5.3, 150 *mM* NaCl, 10 *mM* $MgCl_2$), T1 (20 *mM* Tris, pH 7.0, 50 *mM* NaCl, 10 *mM* $MgCl_2$), B (10 *mM* Tris, pH 8.8, 50 *mM* KCl, 0.8% NP-40), and dye K (80% deionized formamide, 0.1% Xylene Cyanol, and 0.1% Bromophenol Blue).

Synthesis of 5-[*N*-(3-aminopropyl)sulfamoyl]-2-bromobenzoic acid (1). 2-Bromobenzoic acid was sulfochlorinated with chlorosulfonic acid by a known procedure,[28] and the resulting sulfonyl chloride was recrystallized twice from freshly distilled toluene, m.p. 153—154 °C (*cf.* Ref. 28: m.p. 151.5—154 °C), yield 75%. The sulfonyl chloride (0.11 g) was vigorously stirred with 3 g of crushed ice to produce a fine suspension, which was slowly added over a period of 1 h to a cooled solution of 1,3-diaminopropane (0.4 g) in 1 mL of water. The reaction mixture was stirred for 24 h at 24 °C, acidified with HCl to pH 3.5, and washed with chloroform (3×5 mL). The aqueous layer was separated and concentrated in air in a Petri dish. The oily residue was reprecipitated three times in the cold from 4 mL of hot ethanol, the product was passed through 5 g of silica gel using ethanol for elution, and the eluate was concentrated *in vacuo*. The residue was dissolved in 1 mL of methanol and precipitated with 2 mL of anhydrous ether, the solvent was decanted, and the precipitate was dried *in vacuo* over $CaCl_2$ to give 0.083 g of a paste-like product **1**, yield 60%; according to ^{1}H NMR data, the content of the principal substance was 97%. ^{1}H NMR (CD_3OD), δ: 1.40—1.80 (m, 2 H); 2.90—3.09 (m, 4 H); 7.79 (dd, 1 H, J = 8.4 Hz, J = 2.4 Hz); 7.91 (d, 1 H, J = 8.4 Hz); 8.19 (d, 1 H, J = 2.4 Hz). Found (%): C, 32.05; H, 3.67; N, 7.41; S, 8.47. $C_{10}H_{14}BrClN_2O_4S$. Calculated (%): C, 32.14; H, 3.78; N, 7.51; S, 8.59.

Reactive derivatives of oligonucleotides. The conjugate of oligonucleotide ON2 with bleomycin (Blm) was synthesized at the Laboratory for the Chemistry of Nucleic Acids (Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the RAS) by a known method.[29] The conjugate of oligonucleotide ON1 with cobalt tetracarboxyphthalocyanine (Ptc) was synthesized at the Laboratory for the Study of Biopolymer Modification of the same Institute by a method described previously.[18]

The conjugate of oligonucleotide ON1 with *o*-bromobenzoic acid was synthesized by a procedure similar to that used for the alkylating oligonucleotide derivative.[16] The OBB conjugate was separated from the starting oligonucleotide using HPLC on a Milikhrom chromatograph (Production Association Nauchpribor, Oryol) using a column with the Nucleosil 100-5 C-18 sorbent in aqueous methanol with 0 to 90% gradient of the concentration of MeOH containing 0.05 *M* $LiClO_4$.

[^{32}P]-Labeled DNA fragment, 152 bp (DNA II) was prepared by the PCR method. The reaction mixture for PCR (100 μL) contained buffer B, 3 μ*M* $MgCl_2$, 0.2 μ*M* of each of deoxyribonucleoside triphosphates, 1 μ*M* of the *fos*-CAT DNA plasmid,[30] 50 *pM* of each primer (one [^{32}P]-labeled), and 4 au of Taq DNA-polymerase. Amplification was carried out using an Hybaid Limited amplifier (UK) under the following conditions: 29 cycles; 95 °C, 1 min; 54 °C, 1 min; and 71 °C, 1 min. The amplification product was analyzed by electrophoresis in 7% PAAG, a strip corresponding to the length of 152 bp DNA was cut out and eluted into a buffer with the composition: 1 *mM* Tris, 1 *mM* H_3BO_3, pH 8.4, and 0.2 *mM* EDTA.

Oligonucleotides were 5′-[^{32}P]-labeled according to a standard protocol.[31]

Cleavage of DNA by the OBB conjugate was carried out in 20 μL of buffer solution T at a concentration of [^{32}P]-labeled DNA I (or DNA II) of $2\cdot10^{-8}$ mol L^{-1} and that of the OBB conjugate as indicated in the Figure captions, in the presence of 10—100 μ*M* $CuSO_4$ and 10—100 μ*M* *o*-bromobenzoic acid for 24 h at 37 °C. The reaction was quenched by adding 2 μL of 20 *mM* EDTA; RNA-carrier was added to a concentration of 1 mg mL^{-1}. The reaction mixtures were precipitated with 2% $LiClO_4$ in acetone and with ethanol, dissolved in 3 μL of the dye K solution, and analyzed in 20% and 8% PAAG under denaturing conditions for DNA I and DNA II, respectively.

Cleavage of DNA with oligonucleotide conjugate with bleomycin (Blm) was carried out in 20 μL of buffer solution T1 at a concentration of the [^{32}P]-labeled fragment (DNA II) equal to $2 \cdot 10^{-8}$ mol L^{-1} and concentrations of the Blm conjugate with oligonucleotide ON2 indicated in Figure captions, in the presence of 0.05 *M* 2-mercaptoethanol. The reaction was initiated by addition of a solution of $Fe(NH_4)_2(SO_4)_2$ in an argon-saturated bidistilled water up to a concentration of 10^{-5} mol L^{-1}. The reaction mixture was incubated for 5 h at 37 °C, the reaction was quenched by adding 2 μL of 20 *mM* EDTA, RNA-carrier was added to a concentration of 1 mg mL^{-1}, and the product was precipitated by ethanol and dissolved in 3 μL of the dye K solution. The cleavage products of DNA II were analyzed in 8% PAAG under denaturing conditions.

Cleavage of DNA with the oligonucleotide conjugate with cobalt tetracarboxyphthalocyanine (Ptc) was carried out in 20 μL of buffer solution T at a concentration of the [^{32}P]-labeled DNA I or DNA II equal to $2 \cdot 10^{-8}$ mol L^{-1} and the concentrations of the Ptc conjugate indicated in the Figure captions, in the presence of 2 *mM* H_2O_2 for 24 h at 24 °C. The reaction was quenched by adding 2 μL of 20 *mM* EDTA. Then RNA-carrier was added up to a concentration of 1 mg mL^{-1}, DNA or cleavage products were precipitated by a 2% solution of $LiClO_4$ in acetone or with ethanol, the precipitates were dissolved in 50 μL of 1 *M* piperidine, the solutions were kept for 40 min at 95 °C, and the products were precipitated again. The cleavage products were dissolved in 3 μL of the dye K solution and analyzed in 20% and 8% PAAG under denaturing conditions for DNA I and DNA II, respectively.

After completion of electrophoresis, the gels were transferred onto DEAE paper, dried using a Labconc device, and exposed with an Agfa film for 10—20 h at −40 °C. The gel autoradiograms were scanned and treated using a Gel-Pro Analyzer package (Media Cybernetics Inc., USA). The degree of modification of the DNA target was estimated as the ratio of the areas of peaks corresponding to the cleavage products to the total peak area corresponding to the products and the initial oligonucleotide.

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