Volume 14 Number 10 1986 Nucleic Acids Research

Complementary addressed modification and cleavage of a single stranded DNA fragment with alkylating oligonucleotide derivatives

V.V.Vlassov, V.F.Zarytova, I.V.Kutiavin, S.V.Mamaev and M.A.Podyminogin

Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the Academy of Sciences of the USSR, Novosibirsk, 630090, USSR

Received 31 January 1986; Revised and Accepted 1 May 1986

ABSTRACT

A single stranded DNA fragment was modified with alkylating derivatives of oligonucleotides complementary to a certain nucleotide sequences in the fragment. The derivatives carried aromatic 2-chloroethylamino groups at their 3'- or 5'-terminal nucleotide residues. Some of the derivatives carried both alkylating group and intercalating phenazine group which stabilized complementary complexes. It was found that these oligonucleotide derivatives modify the DNA fragment in a specific way near the target complementary nucleotide sequences, and the DNA fragment can be cleaved at the alkylated nucleotides positions. Alkylating derivatives carrying phenazine groups were found to be the most efficient in reaction with the DNA fragment.

INTRODUCTION

Affinity modification of nucleic acids with reactive derivatives of oligonucleotides capable of complementary interactions with certain nucleotide sequences (complementary addressed modification) (1,2) was proposed as an approach to specific chemical modification of predetermined sequences in nucleic acids and for specific chemical modification of certain nucleic acids. Potential applications of this approach were reviewed in (3,4). Experiments on the complementary addressed modification of ribosomal RNA and denatured DNA with alkylating oligonucleotide derivatives have demonstrated high efficiency in reactions of these derivatives with polynucleotides (2,5). Identification of alkylated nucleotides in yeast $tRNA^{Val}$ modified with the derivative of oligonucleotide d(pCpG)pA carrying aromatic 2-chloroethylamino group at its 3'-terminal ribose has revealed the specific nature of the complementary addressed modification (6).

The most convenient models for investigation of the comple-

mentary addressed modification are single stranded ^{32}P-end-labeled DNA fragments (7,8). Analysis of the modified targets is simple in this case since DNA can be cleaved at positions of alkylated purines (9) and alkylated cytidine (10) and the cleavage products can be analysed by gel electrophoresis.

In this paper, we describe the complementary addressed modification of a 303 nucleotides long single stranded DNA fragment with oligonucleotide derivatives carrying aromatic 2-chloroethylamino groups.

MATERIALS AND METHODS

All chemicals were analytical grade from Serva (reagents for electrophoresis and reaction mixtures) and Merck (LiChrosorb RP-18). Enzymes were from Ferment, Vilnius, USSR.

Isolation of the single stranded DNA fragment

303 nucleotides long DNA fragment corresponding to a part of the tick borne encephalitis virus RNA sequence was cloned in M13 mp7 bacteriophage as described earlier (11). The fragment was cut off the single stranded recombinant phage DNA with Bam HI endonuclease (12) and 3'-end-labeled. Bam HI endonuclease digestion of the fragment was carried out for 2 h at 37°C in 0.01 M Tris HCl pH 7.5, 0.01 M $MgCl_2$, 0.05 M NaCl, 0.1 mM mercaptoethanol. 50 µl of the mixture contained 40 µg of the DNA and 100 units of the enzyme. Following incubation, the solution was supplemented with a mixture of α-^{32}P-labeled deoxyribonucleoside triphosphates at 0.1 mM, 50 µCi each and 5 units of Klenow fragment of E.coli DNA polymerase I. The mixture was incubated for 20 min at 20°C, supplemented with 5 µl of 50% glycerol containing marker dyes and layered onto a 4% polyacrylamide gel. After electrophoresis (2 h, 20 V/cm) and autoradiography the fragment was electroeluted on the dialysis membrane and precipitated with 2% $LiClO_4$ in acetone.

Synthesis of reactive oligonucleotide derivatives

Oligonucleotides and oligonucleotide derivatives carrying alkylating groups at their 3'-terminal ribose or at 5'-terminal phosphate were synthesized by the procedures described previously (13). Oligonucleotide derivatives were purified by HPLC on

LiChrosorb RP-18 columns using gradient of acetonitrile concentration (0-20%) in 0.05 M aqueous $LiClO_4$.

To prepare reactive oligonucleotide derivatives with intercalating groups, ethylenediamine was coupled to the 5'-terminal phosphate of oligonucleotides following the procedure described in (13), using the mixture of triphenylphosphine and 2,2'-dipyridyldisulfide as a condensation reagent, in the presence of 4-N,N-dimethylaminopyridine as a catalyst. Alkylating groupings were attached to the 3'-terminal ribonucleotide residue of the oligonucleotide derivatives as previously described (13). Phenazine groups were coupled to the aliphatic amino group of the prepared alkylating oligonucleotide derivatives as follows: oligonucleotide derivative ($2 \cdot 10^{-4}$mmol) was dissolved in 80 µl of aqueous solution containing 0.05 M N-(2-oxyethyl)phenazinium chloride and 0.1 M Na_2CO_3. The solution was incubated at 22°C for 8 min. 2% solution of $LiClO_4$ in acetone (1.5 ml) was added. The precipitated product was collected by centrifugation, redissolved in 60 µl of water and precipitated by the addition of 1.5 ml of 2% solution of $LiClO_4$ in acetone. The reprecipitation procedure was repeated 3 times and the oligonucleotide derivative was purified by HPLC. This stage of purification yielded chemically homogeneous derivatives. Yield ~80% (14).

Modification of the DNA fragment with alkylating oligonucleotide derivatives

DNA fragment was reacted with the oligonucleotide derivatives in 10 mM Tris HCl pH 7.6, 0.1 M NaCl, 1 mM EDTA ("buffer A"), at 37°C unless otherwise stated. Concentration of the fragment in reaction mixtures was $1.5 \cdot 10^{-8}$ M, concentrations of the oligonucleotide derivatives were $1 \cdot 10^{-8}$- $1 \cdot 10^{-4}$ M. Alkylation with derivatives of short oligonucleotides (6- and 8-mers) was carried out in "buffer A" at 25°C for 12 h or at 10°C for 72 h. Concentrations of the fragment and of the reagents were $1.5 \cdot 10^{-8}$ M and $5 \cdot 10^{-7}$ M, respectively. Reactions with oligonucleotide derivatives carrying phenazine groups were carried out in the "buffer A" at 35°C, 40°C and 50°C. Incubation times were 12 h, 6 h and 1.5 h, respectively. These incubation times guarantee >90% conversion of the aromatic 2-chloroethylamino

groups of the derivatives into high reactive ethyleneimmonium cations (15).

Alkylation reactions were terminated by adding 20 volumes of 2% $LiClO_4$ solution in acetone. DNA was collected by centrifugation, washed with ethanol and vacuum dried. To cleave the modified fragment at positions of alkylated purines, the fragment was dissolved in 30 µl of 1 M piperidine and incubated at 100°C for 15 min. After the incubation, DNA was precipitated with 20 volumes of 2% $LiClO_4$ in acetone. The precipitate was collected by centrifugation, twice washed with ethanol, vacuum dried and dissolved in formamide with marker dyes. The cleavage products were resolved by electrophoresis through 10% polyacrylamide 7 M urea gel (30 x 40 x 0.04 cm) at 50 V/cm for 3 h. After autoradiography, the radioactive bands were cut off the gel and counted by liquid scintillation.

RESULTS AND DISCUSSION

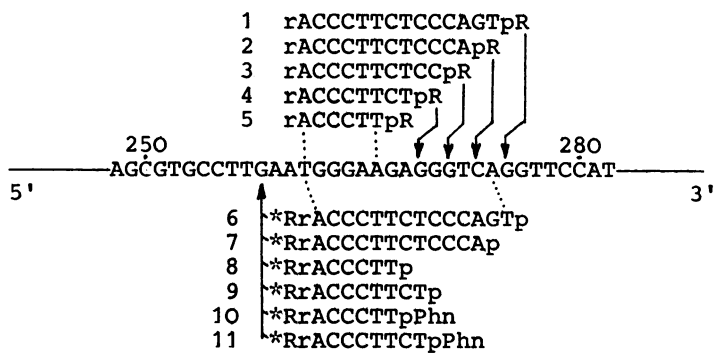
Fig.1 shows the nucleotide sequence of the DNA fragment (11) which was the target for complementary addressed modification in the present study. Oligonucleotides carrying reactive groups (Fig.2) are complementary to nucleotide sequence in this fragment (positions 261-274). They were expected to bind to this target sequence and to bring about chemical modification there. Computer analysis of potential secondary structures of the DNA fragment according to (16) and our data on cleavage of the fragment with S1 nuclease suggest that the target sequence is located in a single stranded region of its structure. According to these data, the only similar nucleotide sequence (positions 144-151) is in a hairpin region of the fragment and should be unavailable for oligonucleotide derivatives.

To investigate specificity of alkylation of the DNA fragment with oligonucleotide derivatives, ^{32}P-end-labeled DNA fragment was reacted with them at 37°C in "buffer A". The alkylated fragment was cleaved at positions of alkylated nucleotides by chemical treatment (9,10) and the cleavage products were resolved using gel electrophoresis. Results presented in Fig. 3 and results of experiments with reagents 4 and 9 at 20°C and 10°C (data not shown) demonstrate that the oligonucleotide derivatives

```
                                                                    50
GATCC GTCGA CCTGC AGGGG GGGGG GGGGG GGGGT TGCTC AGGGT GAGGC
                                                                   100
GGAAA AGAGT CGACC CAACC TTCCG CCGGC CGTCA CTGGC ACAGG CTGGA
                                                                   150
CAGCA AAAGG GCAGA TCACA GTGCT GGACA TGCAC CCAGG CTCTG GGAAG
                                                                   200
ACCCA CAGAG TCCTC CCGGA GCTCA TTCGC CAATG CATTG ACAGA CGCCT
                                                                   250
AAGGA CATTG GTGTT GGCCC CAACC CGTGT GGTGC TTAAG CAAAT GGAGC
                                                                   300
GTGCC TTGAA TGGGA AGAGG GTCAG GTTCC ATTCT CCTGC AGGTC GACGG AT*
```

Figure 1. Nucleotide sequence of the DNA fragment (11) which was the target for modification with alkylating oligonucleotide derivatives. Domain complementary to the oligonucleotide derivatives is underlined.

carrying alkylating groups at their 5'-terminal phosphates (reagents 1-4, Fig. 2) deliver the groups to the first nucleotides adjacent to the complementary sequences and modify them. Neigh-



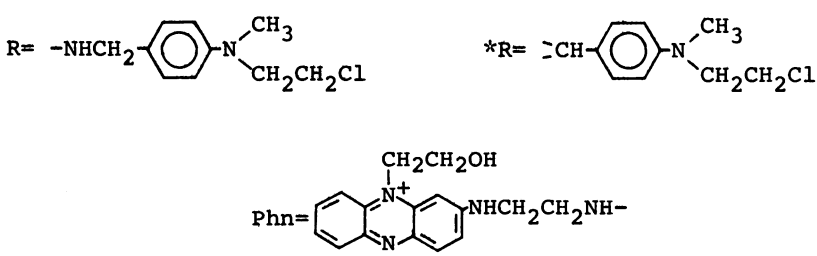


Figure 2. Alkylating oligonucleotide derivatives and the target DNA sequence. Arrows indicate sites of preferential attacks by the derivatives.

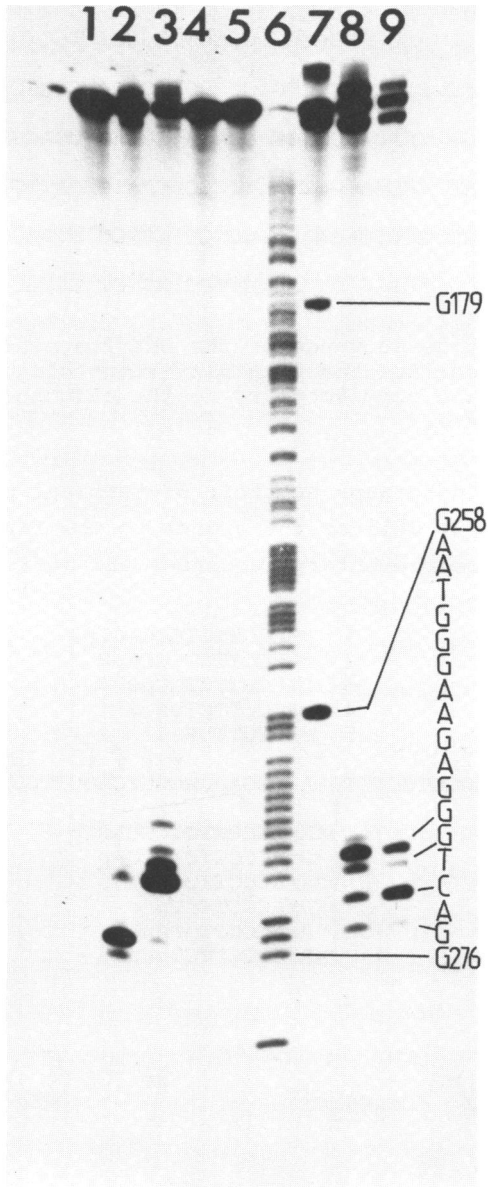


Figure 3. Autoradiogram of gel electrophoresis of the DNA fragment cleaved by alkylation with the oligonucleotide derivatives 1-6 and piperidine treatment. Alkylation of the fragment was carried out at 37°C for 1 h. Concentration of the fragment in reaction mixtures was $1.5 \cdot 10^{-8}$ M. Lane 1 is control, the fragment incubated under alkylation conditions without reagents, treated with piperidine. Lane 6 contains products of the (A+G)

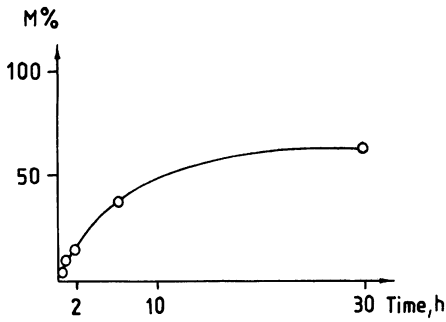
sequencing reaction (17). Lanes 2,3,4,5,7, and 8 are fragments produced by alkylation with reagents 1 ($5 \cdot 10^{-7}$ M), 3 ($6 \cdot 10^{-6}$ M), 4 ($5 \cdot 10^{-7}$ M), 5 ($5 \cdot 10^{-7}$ M), 6 ($6 \cdot 10^{-7}$ M), and 2 ($2 \cdot 10^{-6}$ M), respectively. Lane 9 is the same as the lane 8 except for an additional treatment with hydrazine (10) was included for cleaving at alkylated cytidine position.

bouring nucleotides are affected by these reagents to low extent. Piperidine treatment of the fragment modified with reagents 1,3, 4 results in cleavage of the fragment at alkylated purines positions. DNA fragment modified with reagent 2 at cytidine residue is stable under piperidine treatment (Fig. 3, lane 8). In this case, cleavage was achieved (Fig. 3, lane 9) by consequent treatments with hydrazine and piperidine (10). Crosslinked reagent 2 and the fragment are seen in Fig. 3, lanes 8 and 9, as an additional slow moving band above the band of the fragment.

Derivatives carrying alkylating groups at their 3'-terminal riboses (reagents 6 and 7, Fig. 3 and Fig. 7, respectively) deliver them to the third nucleotide counting from the 5'-terminus of the complementary sequence in the target fragment in accordance with earlier observations (6).

Reagents 1-4 modify the DNA fragment mainly in vicinity of the target sequence. No unspecific modification was observed even in experiments where the reagents were used in high concentrations (up to $3 \cdot 10^{-5}$ M). Some modification of the fragment in vicinity of its 5'-terminus (Fig. 3) which was most pronounced in the case of derivatives of long cytidine-rich oligonucleotides (reagents 2 and 6, Fig. 3, lanes 7-9) is obviously the result of binding of the oligonucleotide derivatives to the oligoguanylic sequence G17-G34 attached to the 5'-terminus of the DNA fragment while cloning. Location of the modified nucleotides within the oligoguanylic sequence was proved in special experiments with the 5'-^{32}P-end-labeled DNA fragment (data not shown).

Oligonucleotide derivatives carrying alkylating groups at their 3'-termini attack two regions in the DNA fragment: the target region and guanosine residue G179 which is 80 nucleotides far from target sequence, where there are no nucleotide sequences capable of binding the oligonucleotide derivatives. To explain this profound effect, one can assume cnly that G179 is juxtaposed



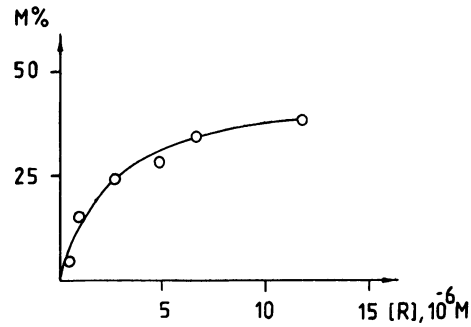


Figure 4

Figure 5

Figure 4. Effect of reaction time on splitting of the DNA fragment with reagent 3 at 37°C. M%, yield of the cleavage products, per cent.
Figure 5. Effect of the reagent 3 concentration R in the reaction mixture on cleaving of the DNA fragment. Reaction was carried out at 37°C for 1 h. M%, yield of the cleavage products, per cent.

with the target sequence in the three-dimensional structure of the fragment and is located in position where the reagents deliver their alkylating groups. Similar effect was observed when an attempt to accomplish complementary addressed modification of another single stranded DNA fragment had been made (7). Alkylating oligonucleotide derivative which was different from those used in the present experiments modified that fragment far from the expected position (7).

Modification of the DNA fragment with oligonucleotide derivatives was inhibited when 10-fold excess of unreactive oligonucleotides of the same sequence was added to reaction mixtures. Oligonucleotides of another sequences did not influence the reaction. Therefore, we conclude that the only source of specificity in reaction of oligonucleotide derivatives with the fragment is their binding to the complementary target sequence.

In accordance with expected stability of complementary complexes, alkylation of the DNA fragment at 37°C was observed in reaction mixtures containing $5 \cdot 10^{-7}$ M oligonucleotide derivatives 1-3 and 6. Under these conditions alkylating derivatives of octanucleotides and hexanucleotides did not react with the fragment, obviously due to their inability to form complementary complexes. At $5 \cdot 10^{-7}$ M concentration, octanucleotide derivatives

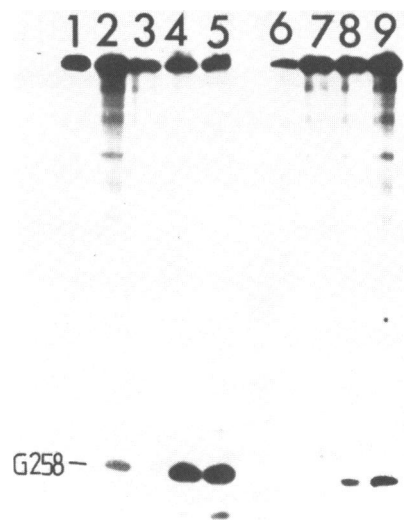


Figure 6. Autoradiogram of gel electrophoresis of the DNA fragment cleaved by alkylation with oligonucleotide derivatives 8-11 and piperidine treatment. In alkylation experiments concentration of the DNA fragment was $2 \cdot 10^{-8}$ M, concentration of oligonucleotide derivatives was $4 \cdot 10^{-6}$M. Lane 1 is control, the fragment incubated under alkylation conditions in the absence of reagents and treated with piperidine. Lanes 2,3,4, and 5 are fragments produced by alkylation with reagents 9,8,10, and 11, respectively, reaction was carried out at 40°C. Lanes 6,7,8, and 9 are fragments produced by alkylation with reagents 8,9,10, and 11, respectively, reaction was carried out at 50°C.

alkylated the fragment at temperatures below 25°C. Reaction with these derivatives was observed also at 37°C, at concentrations of the reagents higher than ~$4 \cdot 10^{-6}$ M. Derivatives of hexanucleotides 5 and 8 at concentration $5 \cdot 10^{-7}$ M were inefficient even at 10°C. Kinetics of the DNA fragment alkylation with reagent 3 and

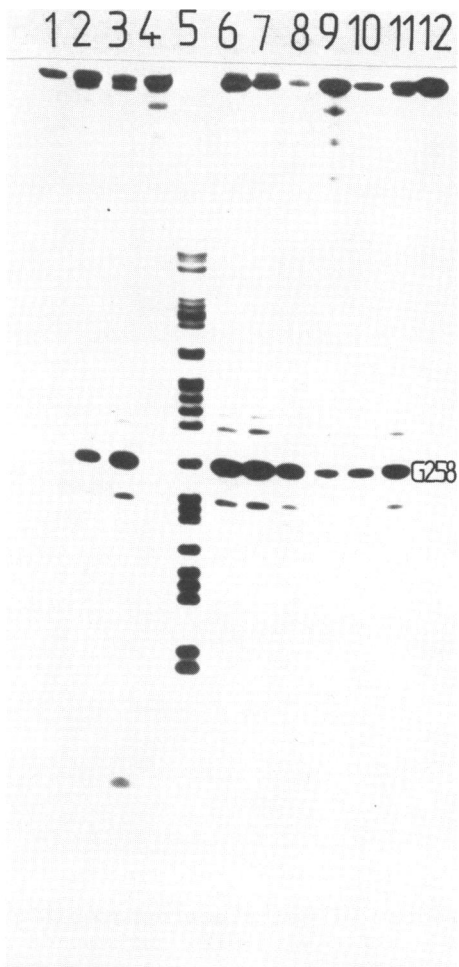


Figure 7. Autoradiogram of gel electrophoresis of the DNA fragment cleaved by alkylation with oligonucleotide derivatives 7-11 and piperidine treatment. Reaction was carried out at 37°C for 12 h. Concentration of the fragment was $2 \cdot 10^{-8}$M. Lane 12 is control, the fragment incubated under alkylation conditions without reagents and treated with piperidine. Lane 5 contains products of the (A+G) sequencing reaction (17). Lanes 1,2, and 3 are fragments produced by alkylation with reagent 10, concentrations $4 \cdot 10^{-6}$ M, $2 \cdot 10^{-5}$ M, and $1 \cdot 10^{-4}$ M, respectively. Lane 4 contains fragments produced by alkylation with reagent 8 ($1 \cdot 10^{-4}$ M). Lanes 6,7, and 8 are fragments produced by alkylation with reagent 11, concentrations $4 \cdot 10^{-6}$ M, $2 \cdot 10^{-5}$ M, and $1 \cdot 10^{-4}$ M, respectively. Lane 9 contains fragments produced by alkylation with reagent 9 ($1 \cdot 10^{-4}$ M). Lanes 10 and 11 are fragments produced by alkylation with reagent 7, concentrations $2 \cdot 10^{-5}$ M and $1 \cdot 10^{-4}$ M, respectively.

effect of the reagent concentration on the yield of the reaction are shown in Figs. 4,5.

Attachment of intercalating groups to oligonucleotides enhances binding of the oligomers to complementary sequences in polynucleotides (18,19). We have investigated the effect of attachment of an intercalating group to the alkylating oligonucleotide derivatives on their interaction with the DNA fragment. Data shown in Fig. 6 demonstrate that for a given chain length, the derivatives 10 and 11 carrying both alkylating and intercalating phenazine groups modify efficiently the DNA fragment at higher temperatures than those without intercalating groups. Thus, the derivatives of hexanucleotide 10 and octanucleotide 11 modify the fragment at 40°C much better as compared to the similar derivatives 9 and 8 (Fig. 6). Reagents 10 and 11 modify the fragment efficiently even at 50°C. It is seen (Fig.6) that attachment of the phenazine group to the oligonucleotide derivatives does not interfere with the specificity of reaction even in the cases where high reagent concentrations have been used (Fig. 7). When reagents were used in concentrations $1 \cdot 10^{-4}$M, extent of the fragment modification in vicinity of the target sequence approached 80-90%.

Results of the present study, data of other experiments with alkylating oligonucleotide derivatives (2,5,6) and oligonucleotide derivatives carrying DNA-cleaving groups EDTA·Fe(II) (20-22) demonstrate the possibility of complementary addressed modification of single stranded polynucleotides. Efficiency of specific fragmentation of single stranded DNA observed in our experiments was quite high, so one can propose modification with alkylating oligonucleotide derivatives for preparative cleaving of single stranded DNA at predetermined sites. Specificity of complementary addressed modification can be regulated by varying lengths of oligonucleotides carrying reactive groups. Coupling of appropriate intercalating groupings to reagents provides the possibility to accomplish complementary addressed modification by means of derivatives of short oligonucleotides at high temperatures and may allow to modify nucleic acids in the presence of agents interfering with complementary complex formation by usual oligonucleotide derivatives.

REFERENCES

1. Belikova, A.M., Zarytova, V.F. and Grineva, N.I. (1967) Tetrahedron Lett., 3557-3562.
2. Grineva, N.I. (1977) Biokhimiya 42, 370-374.
3. Summerton, J. (1979) J. Theor. Biol. 78, 77-99.
4. Knorre, D.G. and Vlassov, V.V. (1985) Prog. Nucl. Acids Res. and Mol. Biol. 32, 291-320.
5. Grineva, N.I. and Karpova, G.G. (1973) FEBS Lett. 32, 351-355.
6. Grineva, N.I., Karpova, G.G., Kuznetsova, L.M., Venkstern, T.V. and Bayev, A.A. (1977) Nucl. Acids Res. 4, 1609-1631.
7. Grachev, M.A. and Oshevsky, S.I. (1983) Dokl. Akad. Nauk SSSR 272, 1259-1262.
8. Vlassov, V.V., Gaydamakov, S.A., Gorn, V.V. and Grachev,S.A. (1985) FEBS Lett. 182, 415-418.
9. Maxam, A.M. and Gilbert, M. (1980) Methods Enzymol. 65, 499-560.
10. Kirckegaard, K., Buc, H., Spassky, A. and Wang, J.G. (1983) Proc. Natl. Acad. Sci. USA 80, 2544-2548.
11. Brosalina, E.B., Vlassov, V.V., Kutiavin, I.V., Mamaev, S.V., Pletnev, A.G. and Podyminogin, M.A. (1986) Bioorg. Khim. 12, 240-247.
12. Patton, J.R. and Chae, C.B. (1982) Anal. Biochem. 126, 231-234.
13. Knorre, D.G., Vlassov, V.V., Zarytova, V.F. and Karpova, G.G. (1985) Advances in Enzyme Regulation 23.
14. Zarytova, V.F., Kutiavin, I.V., Silnikov, V.N. and Shishkin, G.V. (1986) Bioorg. Khim. 12.
15. Vlassov, V.V., Grineva, N.I. and Knorre, D.G. (1969) Izv. Sib. Otd. Akad. Nauk SSSR 1, 104-109.
16. Kolchanov, N.A., Soloviev, V.V. and Zharkikh, A.A. (1963) Dokl. Akad. Nauk SSSR 273, 741-745.
17. Korobko, V.G., Grachev, S.A. and Kolosov, M.N. (1978) Bioorg. Khim. 4, 1281-1283.
18. Letsinger, R.L. and Schott, M.E. (1981) J. Am. Chem. Soc. 103, 7394-7396.
19. Asseline, U., Delarue, M., Lancelot, G.G., Toulme, F., Thuong, N.T., Motenay-Gavestier, T. and Helene, C. (1984) Proc. Natl. Acad. Sci. USA 81, 3297-3301.
20. Boutorin, A.S., Vlassov, V.V., Kazakov, S.A., Kutiavin, I.V. and Podyminogin, M.A. (1984) FEBS Lett. 172, 43-46.
21. Dreyer, G.B. and Dervan, P.B. (1985) Proc. Natl. Acad. Sci. USA 82, 968-972.
22. Chu, B.C.F. and Orgel, L.E. (1985) Proc. Natl. Acad. Sci. USA 82, 963-967.