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Oligo(2'-*O*-methylribonucleotides) and Their Derivatives: IV. Conjugates of Oligo(2'-*O*-methylribonucleotides) with Minor Groove Binders and Intercalators: Synthesis, Properties, and Application

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Abstract—Triplex forming 3'-protected pyrimidine oligo(2'-*O*-methylribonucleotides) containing minor groove binders (MGB) and triplex specific intercalator, benzoindoloquinoline (BIQ), at the 5'-terminus were synthesized. The resulting conjugates formed stable complexes with a target dsDNA due to the simultaneous binding to the minor and major grooves and BIQ intercalation. The dissociation constants and the thermal stability were evaluated for the conjugate complexes with the model dsDNA corresponding to the polypurine tract (PPT) of the *nef* and *pol* genes from the HIV proviral genome. The conjugation of oligo(2'-*O*-methylribonucleotides) with MGB and BIQ was shown to increase the stability of the triple complexes with dsDNA at pH 7.2 and 37°C. Moreover, the intercalator accelerates the process of the complex formation. The dose-dependent arrest of the in vitro transcription was demonstrated when a 780-bp DNA fragment containing the polypurine tract was transcribed under the control of T7 promoter in the presence of different concentrations of conjugates of oligo(2'-*O*-methylribonucleotides) containing MGB and BIQ.

Keywords: oligo(2'-O-methylribonucleotides), conjugates, minor groove binder, intercalator, DNA triple helix, inhibition of in vitro transcription

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INTRODUCTION

Compounds capable of selectively binding to specific nucleotide sequences of double-stranded DNA (dsDNA) are used to study the structure and the function of genomic DNAs and the mechanisms of gene expression, as well as to develop dsDNA detection systems using sequence-specific probes. The ability of these compounds to selectively inhibit gene expression can be used to create a new generation of substances for the diagnostics and the treatment of cancer, viral, and genetic diseases. Triplex-forming oligonucleotides (TFO), which form a triple helix with genomic DNA and can, thereby, block transcription or modulate gene expression, are of particular interest [2, 3]. The use of TFO for targeting genomic DNA in cell systems faced a number of obstacles, namely the rapid degradation of oligodeoxy-ribonucleotides in cells, insufficient stability of triplex structures under physiological conditions, and poor penetration of oligonucleotides into living cells.

One of the approaches to improve the stability of oligonucleotides against nucleases is the introduction of modifying groups into their structure [3–5]. As triplex-forming oligonucleotides, we used oligo(2'-*O*-methylribonucleotides) having a number of advantages. Their synthesis is simple and efficient; they are resistant to nucleolytic degradation and form fairly strong triple helices with dsDNA [6, 7]. The main route of degradation of oligonucleotides in biological media is known to be the 3'-exonuclease cleavage [8]. The 2'-*O*-methylation prevents endonuclease degradation of oligoribonucleotides, but does not prevent

Abbreviations: BIQ, 6-[(3-aminopropyl)amino]-11-methoxy-13*H*-benzo[6,7]indolo[3,2-*c*]quinoline; C^m, 2'-*O*-methylribocytidine; DMAP, 4-*N,N*'-dimethylaminopyridine; γ, γ-aminobutyric acid; HEPES, (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid); Im, *N*-methylimidazole; MES, 2-(*N*-morpholino)ethanesulphonic acid; MGB, minor groove binder; N^m, 2'-*O*-methylribonucleoside; Py, *N*-methylpyrrole; $_{inv}T$, thymidine attached via 3'-3'-phosphodiester internucleotide bond ("inverted" thymidine); TEA, triethylamine; TFO, triplex-forming oligonucleotides; U^m, 2'-*O*-methyluridine.

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Table 1. Yields and nucleotide sequence of synthesized pyrimidine[1]

Code	Oligonucleotide (5'-3')	Yield, %[1]	Nucleoside content[2]
(III)	$U^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$	12	$C^m : U^m : T = 6.7 : 9.2 : 1.1$
(IV)	$pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$	8	$C^m : U^m : T = 6.8 : 9.0 : 1.3$
(V)	$pL_1pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$	9	$C^m : U^m : T = 6.8 : 9.0 : 1.3$
(VI)	$pL_2pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$	10	$C^m : U^m : T = 6.7 : 8.9 : 1.1$

C^m, 2'-*O*-methylribocytidine; U^m, 2'-*O*-methyluridine; L_1: -$(CH_2CH_2O)_3$-; L_2: -$(CH_2CH_2O)_6$-; ${}_{inv}T$, "inverted" thymidine.

[1] The yield after isolation by HPLC relative to the first nucleoside unit attached to the polymer support.

[2] According to data of RP HPLC of oligonucleotide hydrolysates obtained by the successive exhaustive hydrolysis with nuclease P1 and alkaline phosphatase.

their cleavage with exonucleases. To overcome this obstacle, oligo(2'-*O*-methylribonucleotides) were modified by the attachment to their 3'-terminus of "inverted" thymidine (${}_{inv}T$, i.e., thymidine attached through the 3'-3'-phosphodiester bond) [9, 10].

The introduction of triplex-specific intercalators in TFOs is used to enhance the stability of triplexes [11–15]. For example, it was shown that the attachment of intercalators of benzopyridoindole and benzopyridoquinoxaline series to an oligonucleotide leads to the significant increase in the stability of triplexes formed with these conjugates at pH close to neutral values [14, 15]. Of the intercalators most selective to triplex structures are benzoindoloquinolines (BIQ), particularly, 6-[(3-aminopropyl)amino]-11-methoxy-13*H*-benzo[6,7]indolo[3,2-*c*]quinoline [16–18]. The other way to enhance the triplex stability is the attachment of the DNA minor groove binder (MGB) to TFO [18–20]. MGB based on hairpin polyamides of *N*-methylimidazole and *N*-methylpyrrole are capable of sequence-specific binding to MGB regardless of pH and concentration of monovalent and divalent cations [21–23]. Conjugates of TFO and MGB, having properties of both components, are prospective selective tools for targeting genomic DNA. These conjugates provide an extension of the recognition site in a target DNA and, thereby, the increase in the specificity and stability of the complexes formed, which allows one to significantly reduce the effective conjugate concentration. Furthermore, the covalent attachment to oligonucleotides makes rather hydrophobic MGB more water soluble. Conjugates of oligo(2'-*O*-methylribonucleotides) containing one or two MGB residues at the 5'-end were synthesized and studied in our previous work [24].

We propose to use a combination of the advantages of three components (TFO, MGB, and intercalator) in the same conjugate. The goal of the presented work is to design conjugates stable in biological media composed of pyrimidine oligo(2'-*O*-methylribonucleotides), minor groove binders, and intercalators and to study their physico-chemical properties and biological effects in vitro. These conjugates should selectively bind to polypurine sites in dsDNA forming a triple helix due to the oligonucleotide component and to the minor groove of the double helix in the adjacent sequence due to MGB. The presence of the intercalator in the conjugate should additionally stabilize the resulting complex with dsDNA. We assume that these constructions can efficiently inhibit gene expression.

RESULTS AND DISCUSSION

When designing conjugates, we relied on the fact that the simultaneous binding of an oligonucleotide and ligand with the major and minor grooves of a target DNA, respectively, should be ensured by the linker with the length of at least 12 interatomic bonds [18, 19]. Oligoethylene glycols (starting with triethylene glycol) have all features of an optimal linker between an oligonucleotide and a ligand: enough length, hydrophilicity, and flexibility. We developed a simple method of the synthesis of 5'-oligoethyleneglycol-phosphate-containing oligonucleotides using the *H*-phosphonate chemistry.

For this purpose, the corresponding *H*-phosphonates of dimethoxytrityl-containing tri- and hexaethyleneglycols ((**IIa**) and (**IIb**), respectively) and *H*-phosphonate of sulfonyldiethanol (**IIc**) were synthesized by the reaction of dimethoxytrityl-containing derivatives

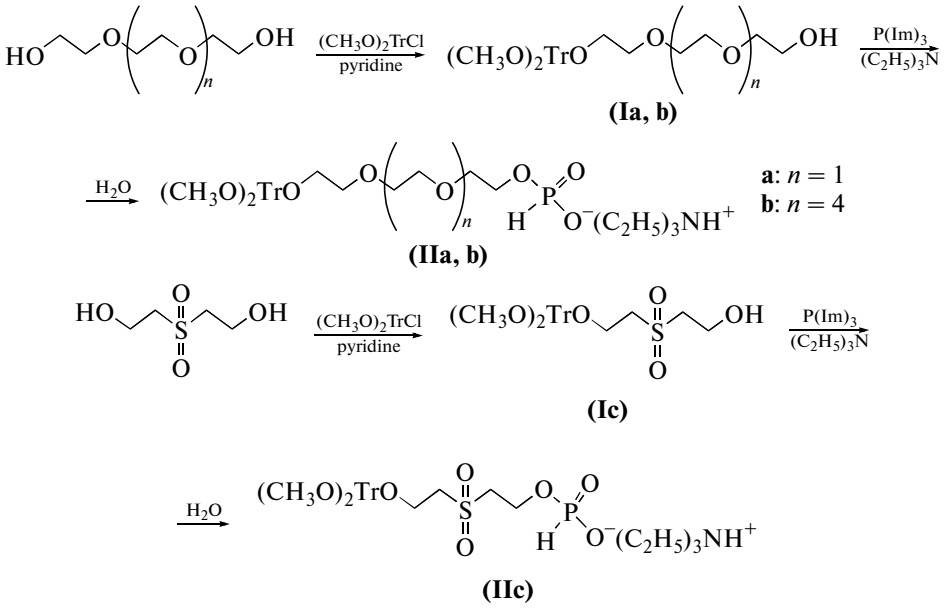
Table 2. Characteristics of the synthesized conjugates of oligo(2'-*O*-methylribonucleotides) with minor groove binders[1]

Code	Conjugate (5'-3')	Retention time during RP HPLC, min	Molecular weight[2]	
			Calculated, *m/z*	Experimental, *m/z**
(VIIa)	**(Py)$_3$-γ- (Py)$_3$-γ-NH-p*L*$_2$p**U^{m}U^{m}U^{m}U^{m}C^{m}U^{m}U^{m}U^{m}U^{m}C^{m}C^{m}C^{m}C^{m}C^{m}C^{m}U^m$_{inv}$***T***	13.8	6769.81	6765.28
(VIIb)	***BIQ*-(Py)$_3$-γ-(Py)$_3$-γ-NH-p*L*$_2$p**U^{m}U^{m}U^{m}U^{m}C^{m}U^{m}U^{m}U^{m}U^{m}C^{m}C^{m}C^{m}C^{m}C^{m}C^{m}U^m$_{inv}$***T***	18.4	7038.08	7038.80
(VIIIa)	**(Py)$_4$-γ- (Py)$_4$-γ-NH-p*L*$_1$p**U^{m}U^{m}U^{m}U^{m}C^{m}U^{m}U^{m}U^{m}U^{m}C^{m}C^{m}C^{m}C^{m}C^{m}C^{m}U^m$_{inv}$***T***	14.0	6881.90	6888.25
(VIIIb)	***BIQ*-(Py)$_4$-γ-(Py)$_4$-γ-NH-p*L*$_1$p**U^{m}U^{m}U^{m}U^{m}C^{m}U^{m}U^{m}U^{m}U^{m}C^{m}C^{m}C^{m}C^{m}C^{m}C^{m}U^m$_{inv}$***T***	20.3	7150.17	7155.07
(IXa)	**(Py)$_4$-γ-(Py)$_4$-γ-NH-p*L*$_2$p**U^{m}U^{m}U^{m}U^{m}C^{m}U^{m}U^{m}U^{m}U^{m}C^{m}C^{m}C^{m}C^{m}C^{m}C^{m}U^m$_{inv}$***T***	14.5	7014.06	7017.78
(IXb)	***BIQ*-(Py)$_4$-γ-(Py)$_4$-γ-NH-p*L*$_2$p**U^{m}U^{m}U^{m}U^{m}C^{m}U^{m}U^{m}U^{m}U^{m}C^{m}C^{m}C^{m}C^{m}C^{m}C^{m}U^m$_{inv}$***T***	21.2	7282.33	7287.78

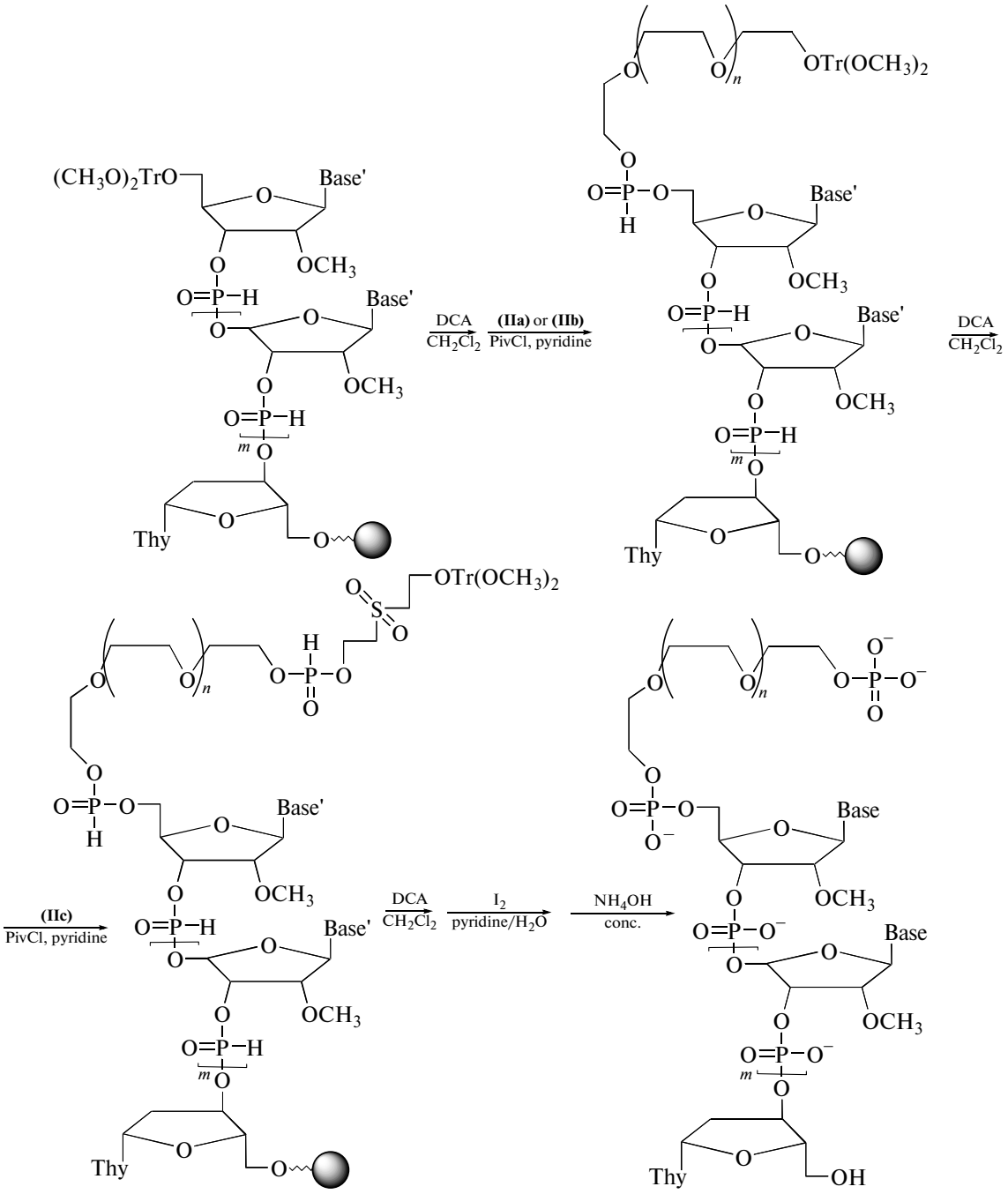
[1] ***BIQ***, 6-[(3-aminopropyl)amino]-11-methoxy-13*H*-benzo[6,7]indolo[3,2-*c*]quinoline; C^m, 2'-*O*-methylribocytidine; U^m, 2'-*O*-methyluridine; ***L*$_1$**, -(CH$_2$CH$_2$O)$_3$-; ***L*$_2$**, -(CH$_2$CH$_2$O)$_6$-; ***T*$_{inv}$**, "inverted" thymidine; p, phosphate group; the structure of the ligands is presented in Fig. 1.

* According to mass spectrometry data.

(**Ia**)–(**Ic**) [25, 26] with phosphorotriimidazolide, followed by the hydrolysis by analogy with the synthesis of nucleoside *H*-phosphonates [27] (Scheme 1). At the final stages of the solid-phase *H*-phosphonate synthesis, oligoethylene glycol *H*-phosphonate (**IIa**) or (**IIb**) and then sulfonyldiethanol *H*-phosphonate (**IIc**) were used resulting in pyrimidine oligo(2'-*O*-methylribonucleotides) (**V**) and (**VI**) with the phosphate-containing linkers of the different length at the 5'-end and "inverted" thymidine at the 3'-end (Scheme 2, Table 1). The resulting compounds were isolated by ion-exchange and reverse-phase HPLC.



Scheme 1.



Scheme 2.

During ion-exchange chromatography, the retention times for oligomers (**V**) and (**VI**) containing the 5'-oligoethyleneglycol-phosphate linkers were, as expected, higher than for 5'-phosphorylated oligomer (**IV**) of the same sequence. During reverse-phase HPLC, oligonucleotide (**VI**) containing the hexaethyleneglycol linker had the higher retention time value as compared to oligonucleotide (**V**) containing the triethyleneglycol linker (data not shown).

Based on 5'-oligoethyleneglycol-phosphate-containing oligo(2'-*O*-methylribonucleotides) (**V**) and (**VI**) with 3'-"inverted" thymidine, we synthesized a series of conjugates bearing either only the MGB residue (the previously described oligoamides NH_2-γ-$(Py)_3$-γ-

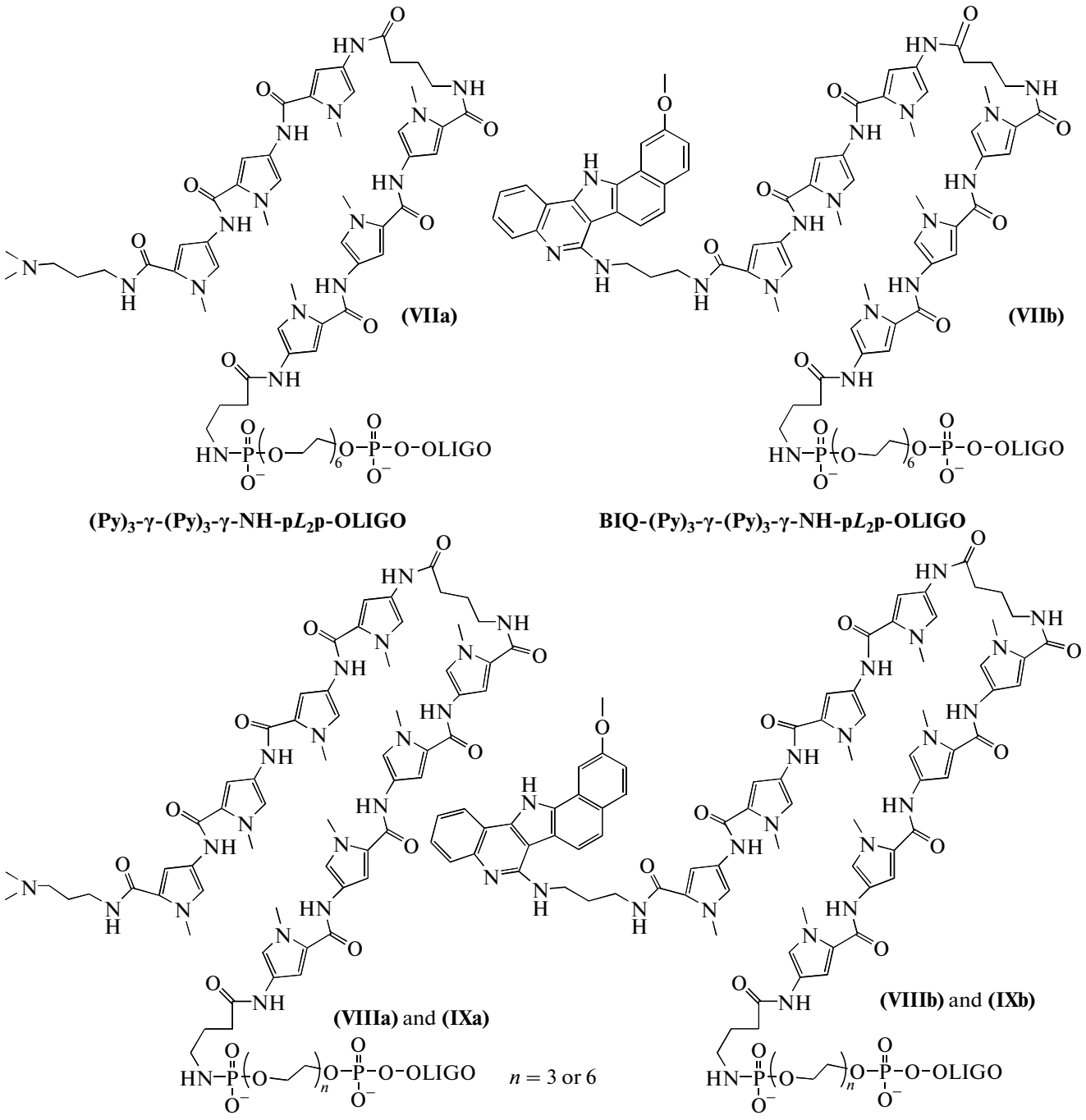
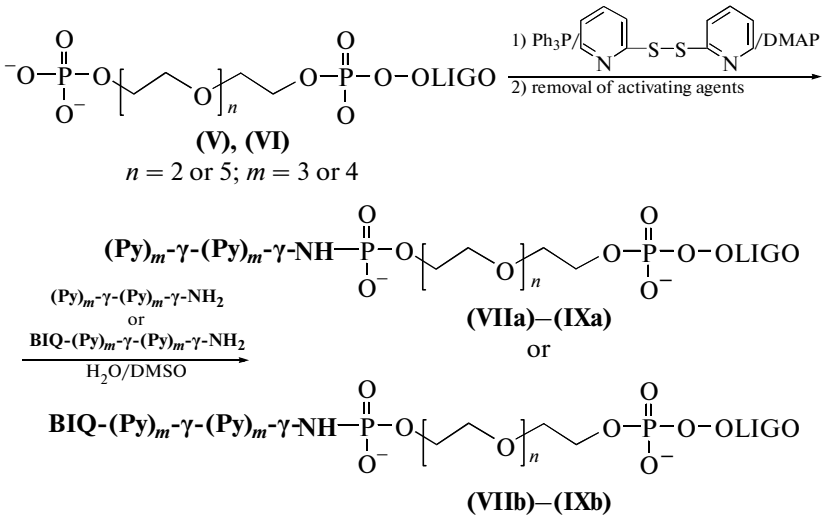


Fig. 1. Conjugates of oligo(2'-*O*-methylribonucleotides) with MGB and MGB-BIQ. **OLIGO**, 5'-$U^mU^mU^mU^mC^mU^mU^mU^m$-$U^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$.

$(Py)_3$-BIQ or NH_2-γ-$(Py)_4$-γ-$(Py)_4$-BIQ containing two fragments of 4-amino-1-methylpyrrole-2-carboxylic acid tri- and tetramer [30–32]), or the MGB residue and the intercalator; their structures have been confirmed. The structure of the resulting conjugates was confirmed by mass spectrometry and their characteristics are presented in Fig. 1 and Table 2.

The synthesis of the conjugates was carried out using the selective activation of the terminal phosphate of an unprotected oligonucleotide with the triphenylphosphine/dipyridyldisulfide pair in the presence of dimethylaminopyridine followed by the interaction of the activated oligonucleotide with the amino-containing ligand [28, 29] (Scheme 3). Previ-

ously described hexa- or octa(*N*-methylpyrrole)carboxamides [30–32] and the conjugates of BIQ with these carboxamides (NH_2-γ-$(Py)_3$-γ-$(Py)_3$-BIQ and NH_2-γ-$(Py)_4$-γ-$(Py)_4$-BIQ) were used as the amino-containing ligands.



Scheme 3.

The conjugates were isolated by RP HPLC. Their homogeneity was confirmed by gel-electrophoresis in denaturing PAAG. The increase of the number of the *N*-methylpyrrole units in the ligand from six (**VIIa**) to eight (**VIIIa**) led to the increase in the retention time of the corresponding conjugates during chromatography. As expected, the retention times for products (**VIIb**)–(**IXb**) containing the MGB residue and the intercalator exceeded those for conjugates (**VIIa**)–(**IXa**) containing only MGB. The yields of the products were 45–85%. The structure of the resulting derivatives was confirmed by ESI Q-TOF mass spectrometry. The electron spectra of these compounds contained the peaks at 260 nm and 310–320 nm corresponding to the absorption of the oligonucleotide part of the conjugate and the minor groove binder, respectively [24].

The sequence found in the *nef* and *pol* genes of the proviral human immunodeficiency virus containing the polypurine tract (16 bp) with the adjacent regions [33] was chosen as the target DNA. The ability of the synthesized conjugates of pyrimidine oligo(2'-*O*-methylribonucleotides) containing "inverted" thymidine to form triplexes was studied using the synthetic duplex (29 bp) containing the same pyrimidine sequence [24, 34]. The peculiarity of this fragment lies in the presence of five A • T pairs immediately adjacent to the polypurine region from the 5'-end (Fig. 2). These A • T pairs are an ideal target for the binding of the synthesized oligo(*N*-methylpyrrole)carboxamides. The schematic structure of the complex formed by the conjugate with the dsDNA target is shown in Fig. 2.

The formation of such triplexes was studied by both the gel retardation method and the thermal denaturation method by measuring optical absorption of triplexes at two wavelengths (260 and 330 nm) [35].

The process of the triplex formation between the conjugates and radioactively-labeled dsDNA over time was studied by the gel retardation method at pH 6.0. In all cases, we observed the formation of the complexes with lower electrophoretic mobility as compared to the initial dsDNA target. Figure 3 demonstrates, as an example, electrophoregrams of the complexes of conjugates (**VIIa**) and (**VIIb**) and oligonucleotide (**IV**) with dsDNA target. Under the given conditions, the complex is formed for about 3 h. The introduction of BIQ in the conjugate led to the acceleration of the process: the band of the duplex disappeared within a few minutes after mixing the components. In the case of conjugate (**VIIa**) containing the ligand, high molecular weight compounds of an unidentified nature were formed in addition to the triplex (Fig. 3).

The stability of triplexes consisted of the conjugates (**VIIa**), (**IXa**), (**VIIb**), and (**IXb**) and the dsDNA target was comparatively studied. Figure 4 demonstrates, as an example, radioautographs of gels obtained while studying the stability of the triplexes involving conjugate (**VIIa**) containing only the MGB residue and conjugate (**VIIb**) containing the MBG and BIQ residues. The dissociation constants of the triplexes at pH

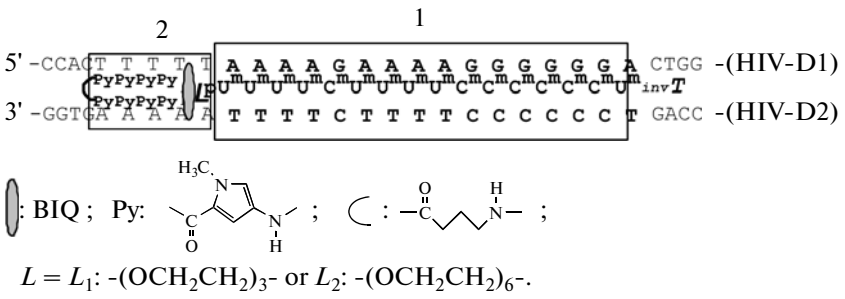


Fig. 2. Structure of triple complex between DNA duplex and the conjugate containing minor groove binder (octa(*N*-methylpyrrole)carboxamide) and intercalator BIQ. 1, polypurine region, 2, A/T binding site of oligo(*N*-methylpyrrole)carboxamide.

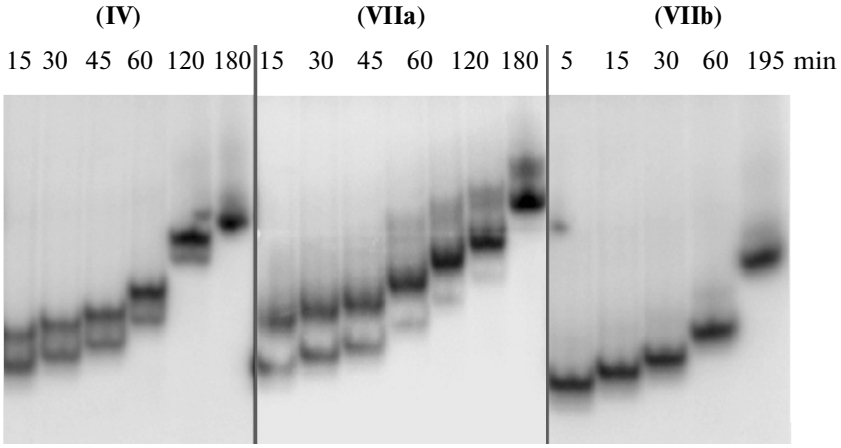


Fig. 3. Formation of triple complex between target DNA and oligonucleotide (**IV**) and conjugates (**VIIa**) and (**VIIb**). Native electrophoresis in 12% PAAG of ternary complexes between conjugates of 3'-"inverted" oligo(2'-*O*-methylribonucleotides) and dsDNA depending on time of incubation. Concentrations of DNA duplex (HIV-1) · (HIV-2) and the third strand are 60 nM and 2 μM, respectively; the incubation time of the components of the triplex before loading onto gel is indicated above the lanes. Buffer: 50 mM MES, pH 6.0, 50 mM NaCl and 5 mM $MgCl_2$; 10°C.

6.0 (10°C) and pH 7.2 (37°C) calculated from these data are presented in Table 3. The formation of the triplexes of the dsDNA target with oligonucleotides (**III**)–(**VI**) containing neither MGB nor BIQ was observed only at pH 6.0. The presence of oligoethyleneglycol phosphate at the 5'-end of oligonucleotides (**V**) and (**VI**) somewhat stabilized their triplexes with dsDNA. Conjugate (**VIIb**) containing the MGB and BIQ residues, as well as conjugate (**VIIa**) containing only the MGB residue formed complexes at both pH 6.0 and 7.2.

The attachment of one MGB residue to oligonucleotide (**VIIa**) stabilized the complex. The dissociation constant of this complex at pH 7.2 was eight times higher than at pH 6.0. Surprisingly, the introduction of BIQ in conjugates (**VIIb**) and (**IXb**) insignificantly increased the dissociation constant of the complex at pH 6.0, while at pH 7.2 the dissociation constant of the complex involving conjugate (**VIIb**) decreased by a factor of almost seven. These results confirm the participation of both components of the conjugates (oligonucleotide and MGB) in the recognition of the dsDNA target, with the ligand and the intercalator significantly stabilizing the triple complex under physiological conditions.

The stabilization of the triple complex with the MGB and intercalator residues was also shown by the thermal denaturation method. Differential curves of the thermal denaturation of the triplexes involving conjugates of oligo(2'-*O*-methylribonucleotides) with dsDNA were recorded at pH 6.0 (Fig. 5). An interesting feature of the melting curves is the absence, in most cases, of the pronounced peak corresponding to denaturation of the triple helix when the optical density was recorded at 260 nm, in contrast to the melting curve of the triplex formed with initial oligonucleotide (**VI**). However, the ternary complex was observed in all cases in the gel-retardation experiments. This means that, most likely, the melting of the triplex occurs simultaneously with the melting of the target duplex, and the triplex immediately dissociates into single-stranded fragments.

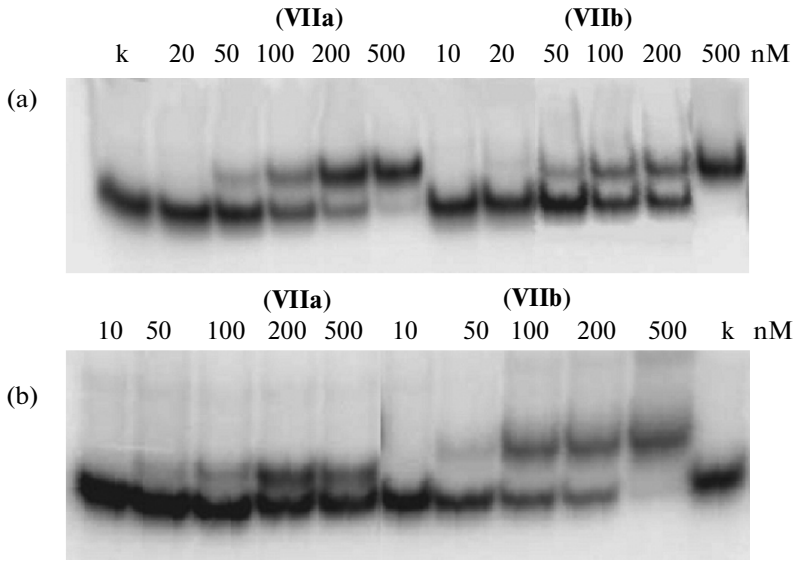


Fig. 4. Native gel-electrophoresis of triplexes between dsDNA and conjugates of 3'-"inverted"oligo(2'-*O*-methylribonucleotides) (**VIIa**) and (**VIIb**) in 12% PAAG. Concentration of DNA duplex is 60 nM; concentrations of the third strand are indicated above the lanes; 50 mM MES (pH 6.0), 50 mM NaCl and 5 mM $MgCl_2$, 10°C (a); 50 mM HEPES (pH 7.2), 50 mM NaCl, 5 mM $MgCl_2$, 37°C (b); k, control dsDNA.

It was previously shown [34] that the dissociation of the complex of MGB with dsDNA leads to the change in the absorption spectrum of the ligand, namely, to the decrease in the absorption at 330 nm. The negative peak in differential melting curves recorded at 330 nm evidences the interaction of the ligand with dsDNA. In the case of conjugate (**VIIb**) containing the ligand and the intercalator, the complete melting of the triple complex occurs at a temperature which is 8°C higher than that of the initial DNA duplex (Fig. 5). On the basis of these data, we assume that the conjugate containing MGB and BIQ stabilizes dsDNA and remains associated with it to the complete dissociation of the complex. Similar results were previously obtained in our study of thermal stability of the triplex formed from dsDNA and the conjugates of oligo(2'-*O*-methylribonucleotides) containing two MGB residues [24].

It should be noted that in control experiments, when unbound minor groove binders with or without

Table 3. Dissociation constants of triplexes of dsDNA with "3'-inverted" oligo(2'-*O*-methylribonucleotides) and their conjugates containing MGB and intercalator under different conditions[1]

Code	Oligonucleotide/conjugate	K_d, nM	
		pH 6.0, 10°C	pH 7.2, 37°C
(IV)	$pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$	308 ± 52	–
(V)	$\mathbf{p}L_1pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$	291 ± 35	–
(VI)	$\mathbf{p}L_2pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$	187 ± 30	n/d
(VIIa)	$(Py)_3$-γ-$(Py)_3$-γ-NH-$pL_2pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$	85 ± 40	600 ± 140
(VIIb)	*BIQ*-$(Py)_3$-γ-$(Py)_3$-γ-NH-$pL_2pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$	150 ± 60	90 ± 20
(IXa)	$(Py)_4$-γ-$(Py)_4$-γ-NH-$pL_2pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$	157 ± 70	–
(IXb)	*BIQ*-$(Py)_4$-γ-$(Py)_4$-γ-NH-$pL_2pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{inv}T$	218 ± 24	–

[1] ***BIQ,*** 6-[(3-aminopropyl)amino]-11-methoxy-13H-benzo[6,7]indolo[3,2-*c*]quinoline; C^m, 2'-*O*-methylribocytidine; U^m, 2'-*O*-methyluridine; L_1, $-(CH_2CH_2O)_3-$; L_2, $-(CH_2CH_2O)_6-$; $_{inv}T$, "inverted" thymidine; **p**, phosphate group. Dissociation constants of triplexes were evaluated by the gel retardation method at 10°C and pH 6.0 (50 mM MES, 50 mM NaCl, 5 mM $MgCl_2$) or at 37°C and pH 7.2 (50 mM HEPES, 50 mM NaCl, 5 mM $MgCl_2$).

* Concentration of duplex was 60 nM; concentration of TFO was varied from 10 nM to 500 nM; n/d, no complex was detected up to 5 mM of TFO.

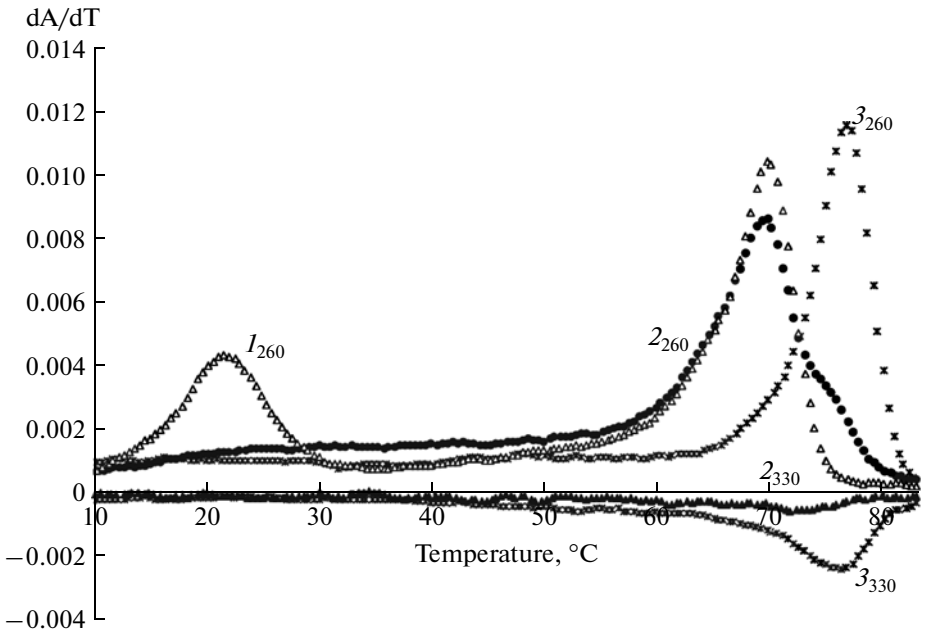


Fig. 5. First derivatives of thermal denaturation curves of triple complexes of dsDNA and oligo(2'-*O*-methylribonucleotide) (**VI**) and its conjugates. *1*, oligonucleotide (**VI**); *2*, conjugate (**VIIa**); *3*, conjugate (**VIIb**). Melting conditions: 10 mM sodium cacodylate, pH 6.0, 100 mM NaCl, 5 mM $MgCl_2$, [HIV-D1] = [HIV-D2] = 1.3×10^{-6} M, [conjugate] = 1.3×10^{-6} M. Rate of the temperature change was 0.1°C/min. UV absorption was registered at 260 and 330 nm.

intercalator BIQ were added to dsDNA or triplexes of dsDNA with the initial oligonucleotides, the change in the thermal denaturation curves was not observed.

Thus, we demonstrated that the conjugates of pyrimidine oligo(2'-*O*-methylribonucleotides) bearing 3'-"inverted" thymidine and the MGB and BIQ residues at the 5'-end can form the ternary complex with polypurine sequence of dsDNA of HIV-1 even at neutral pH and close to physiological temperatures. It was previously shown that TFO based on oligodeoxyribonucleotides and their modified analogues, as well as their conjugates with MGB, can efficiently inhibit RNA polymerase in the transcription complex [11, 36, 37]. Arrest of the enzyme at the site of the triplex formation leads to the suppression of RNA synthesis.

Analyzing the results obtained for conjugates (**VIIa**) and (**VIIb**), we came to the conclusion that in living cells two-component conjugates of oligo(2'-*O*-methylribonucleotides) and MGB (MGB-TFO) (**VIIa**)–(**IXa**) are unlikely to be effective inhibitors of transcription because their complexes with dsDNA are unstable at physiological pH. Three-component constructions BIQ-MGB-TFO (**VIIb**)–(**IXb**) can be more suitable candidates for this purpose.

The ability of the prepared conjugates of oligo(2'-*O*-methylribonucleotides) to inhibit transcription was studied in the in vitro system. The fragment of plasmid pSG-F47 after the cleavage with restrictase BspEI was transcribed in vitro under the control of promotor T7 (Fig. 6). This fragment contained the model polypurine tract in the transcribed region. The restriction site was located at a distance of 660 bp from the start of transcription, and the polypurine tract was at a distance of 332 bp from this site [33]. Thus, it is the percentage of the truncated 332-mer RNA transcript in the total pool of transcripts that was a measure of inhibition of transcription by the conjugate.

As expected, initial oligo(2'-*O*-methylribonucleotide) (**III**) and conjugates (**VIIIa**) and (**IXa**) containing one MGB residue very weakly inhibited transcription at 5 μM concentration. Conjugates (**VIIb**) and (**IXb**) containing both MGB and BIQ were the most efficient and stopped transcription at thepolypurine sequence even at nanomolar concentrations, with the inhibitory effect being dependent on the conjugate concentration. The length of the linker between the oligonucleotide and the ligand had almost no influence on the inhibition efficiency, although the inhibitory effect of conjugate (**IXb**) containing the hexaethyleneglycol linker was somewhat higher than that of conjugate (**VIIIb**) containing the triethyleneglycol linker. The inhibition amounted to 75–78% at 1 μM concentration of conjugates (**VIIIb**) and (**IXb**).

The efficient inhibition of transcription in vitro by conjugates of oligo(2'-*O*-methylribonucleotides) with minor groove binders and intercalators allows one to consider them as prospective specific agents aimed at dsDNA. The proposed approach to construct triplex-forming conjugates of oligo(2'-*O*-methylribonucleotides) stable in biological media may find application

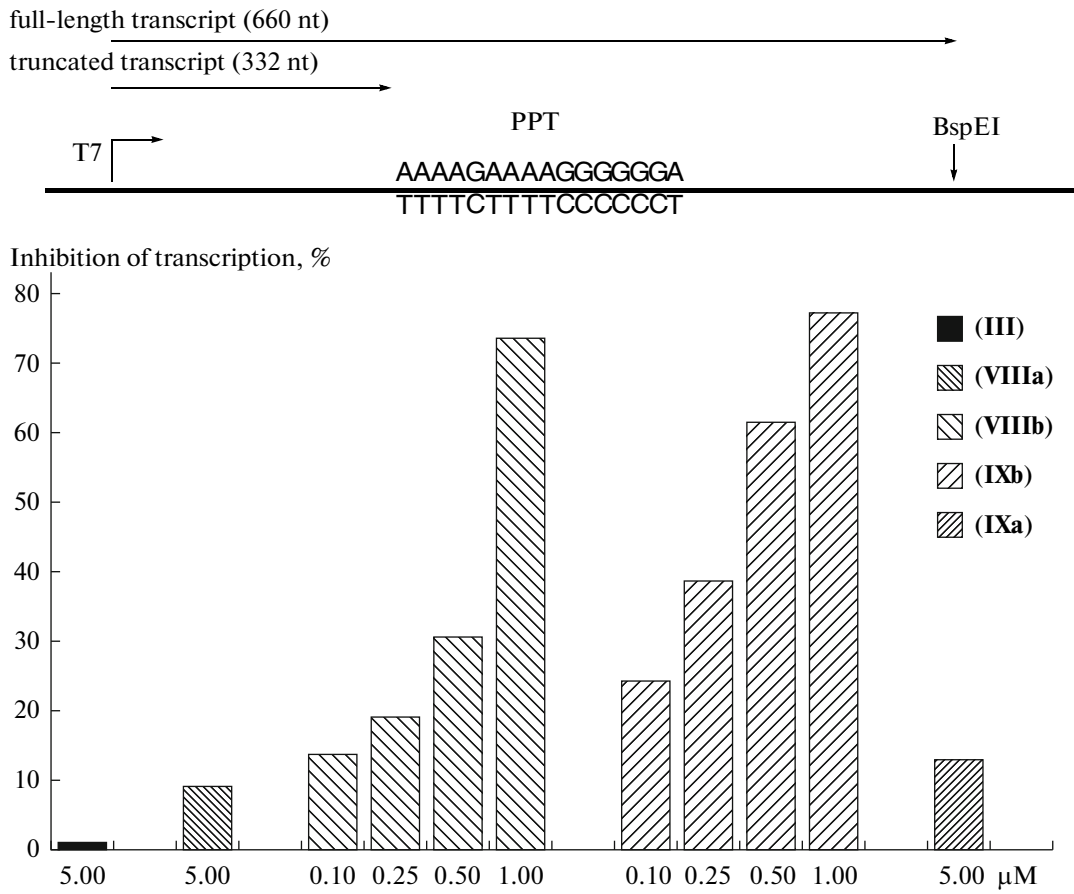


Fig. 6. Inhibition of transcription with conjugates of oligo(2'-*O*-methylribonucleotides) containing MGB and BIQ in vitro. The top panel shows the experimental system. Inhibition of transcription was evaluated by the content of the truncated transcripts in the total pool of RNA transcripts.

in designing therapeutic agents of a new generation for the treatment of cancer, viral, and genetic diseases, as well as to create highly specific probes for the detection of dsDNA.

EXPERIMENTAL

Materials

We used reagents and solvents from Sigma-Aldrich-Fluka (United States), Merck (Germany) and Reachim (Russia); radioactive [γ-^{32}P]ATP and [α-^{32}P]GTP were from Amersham Biosciences (United States). Absolute solvents were prepared by the standard methods [38].

The syntheses of *N*-protected 5'-*O*-dimethoxytrityl-2'-*O*-methylribonucleosides and their 3'-*H*-phosphonates [39], 3'-*O*-dimethoxytritylthymidine [40], and [2-[*O*-(4,4'-dimethoxytrityl]oxy]ethyl]sulfonylethanol [26] were carried out by the known methods. 6-[(3-Aminopropyl)amino]-11-methoxy-13*H*-benzo[6,7]indolo[3,2-*c*]quinoline (BIQ) prepared according to the previously described method [14, 15] was kindly provided by C.H. Nguyen and P. Schmitt (Institut Curie, France). Minor groove binders and Boc-protected derivatives **Boc-NH-γ-(Py)$_3$-γ-(Py)$_3$-COOH** and **Boc-NH-γ-(Py)$_4$-γ-(Py)$_4$-COOH** were prepared as described in [31, 32, 34]. Porous glass CPG-500 containing the anchor carboxyl groups (Theoretical Practice, Russia) was used as the polymer support for the solid-phase *H*-phosphonate synthesis of oligonucleotides. The attachment of *N*-acyl-5'-*O*-dimethoxytrityl-2'-*O*-methylribonucleosides and 3'-*O*-dimethoxytritylthymidine to the modified polymer was carried out as described in [41]. The capacity of the resulting nucleoside-bound polymers was 25–45 μmol/g for the nucleoside unit.

Oligodeoxyribonucleotides HIV-D1 (5'-CCACTTTTTAAAAGAAAAGGGGGG ACTGG) and HIV-D2 (5'-CCAGTCCCCCCTTTTCTTT-TAAAAAGTGG) were purchased from Eurogentec (Belgium). Oligo(2'-*O*-methylribonucleotides) (**III**) and (**IV**) were prepared according to the standard protocol for the solid-phase *H*-phosphonate synthesis (Table 1).

We used the following enzymes: nuclease P1 *Penicillium citrinum* (EC 3.1.30.1) (100 U/mL) (Sigma, United States), alkaline phosphatase *E. coli* (EC 3.1.3.1) (34 U/mg) (Biolar, Litvania), phosphodiesterase *Crotalus atros venom* (EC 3.1.4.1) (0.9 U/mL) (Sigma, United States), and T4 polynucleotide kinase (EC 2.7.1.78) (5000 U/mL) (Sibenzyme, Russia).

Methods

The analysis and identification of compounds were performed by TLC on DC-Alufolien Kieselgel 60 F_{254} plates (Merck, Germany). Adsorption chromatography was carried out using silica gel Kieselgel 60 (Merck, Germany).

^{1}H NMR spectra of the synthesized compounds were recorded on an NMR spectrometer Bruker AM400 in $(CD_3)_2SO$ or $CDCl_3$ using tetramethylsilane as a standard. ^{31}P NMR spectra of the synthesized compounds were recorded on an NMR spectrometer Bruker AM400 in a pyridine-acetonitrile mixture (1 : 1) using 85% H_3PO_4 as a standard.

Molecular masses of the oligonucleotide conjugates were evaluated by mass spectrometry with electrospray ionization on a quadrupole mass spectrometer Q-Star (Applied Biosystems, France) using the Q-TOF (ES Q-TOF MS) technique in the aqueous phase in the positive field (in the anion analysis mode).

Oligo(2'-*O*-methylribonucleotides) were isolated by preparative ion-exchange and reverse-phase HPLC on a Waters chromatograph (United States) on columns (4.6 × 250 mm) with Polysil-SA (Theoretical Practice, Russia) and LiChrosorb RP-18 (Merck, Germany), respectively. Elution during ion exchange chromatography was performed in a gradient of concentration of KH_2PO_4, pH 6.5 (0–0.3 M) in 30% acetonitrile for 100 min at a flow rate of 2 mL/min. Elution during RP HPLC was performed in a gradient of acetonitrile concentration (20–90%) in 0.05 M $LiClO_4$ for 30 min at a flow rate of 2 mL/min. Chromatographically pure oligonucleotides were precipitated as lithium salts.

The oligonucleotides and their derivatives were analyzed by RP HPLC on a Milichrom A-02 chromatograph (Econova, Russia) using a column (2 × 62 mm) with Nucleosil C-18 (5 μm, Macherey-Nagel, Germany) and a gradient of acetonitrile concentrations (0–80%) in 0.05 M $LiClO_4$ at a flow rate of 100 μL/min. Analytical RP HPLC of oligonucleotide hydrolysates was carried out in a gradient of concentrations of acetonitrile (0–50%) in 0.01 M NaOAc at 45°C. The analytical ion-exchange HPLC was carried out on a column (2 × 62 mm) with Partisil 10 SAX in a gradient of concentrations of KH_2PO_4 (0–0.3 M) in 30% acetonitrile at a flow rate of 100 μL/min.

Analytical and preparative RP HPLC of minor groove binders and oligonucleotide conjugates were performed on a 1100 Agilent Technologies system (United States) on a column (7.8 × 300 mm) with X-Terra C-18 (7 μm, Waters, United States). Oligocarboxamides were isolated by chromatography in a gradient of concentrations of acetonitrile (0–90%) in the presence of trifluoroacetic acid; oligonucleotides and their conjugates were subjected to chromatography in a gradient of concentrations of acetonitrile (5–40%) in 0.025 M NH_4OAc.

Absorption spectra were recorded on a Kontron 923 Uvikon Instrument (BioTek, United States). Radioactive gels were scanned on a PhosphorImager (Molecular Dynamics, United States) and treated with the ImageQuant program.

Molar absorption coefficients of oligonucleotides at 260 nm were calculated according to [42]. The values of molar absorption coefficients of 2'-*O*-methylribonucleotides were taken equal to those of corresponding ribonucleotides. Molar absorption coefficients of oligo(2'-*O*-methylribonucleotides) containing thymidine attached through the 3'-3'internucleotide bonds were taken equal to the sum of molar absorption coefficients of the corresponding oligo(2'-*O*-methylribonucleotides) and thymidine. Molar absorption coefficients of the conjugates with minor groove binders at 260 nm were taken equal to the sum of molar absorption coefficients of the corresponding oligonucleotides and the value of 36610 M^{-1} cm^{-1} for hexa(*N*-methylpyrrole)carboxamide or 51427 M^{-1} cm^{-1} for octa(*N*-methylpyrrole)carboxamide [43]. The contribution of BIQ to the molar absorption coefficients of conjugates at 260 nm was neglected.

***O*-(4,4'-Dimethoxytrityl)-tri- and hexaethyleneglycols (Ia) and (Ib)** were prepared starting from triethyleneglycol (0.6 mL, 3.75 mmol) or hexaethyleneglycol (0.95 mL, 3.75 mmol) and 4,4'-dimethoxytrityl chloride (1 g, 3 mmol) according to [25]. The monotritylated product with lower chromatographic mobility was isolated by silica gel chromatography in a linear gradient of concentrations of diethyl ether (0–5%) in CH_2Cl_2 with 0.2% TEA. The chromatographically pure product was evaporated to thick oil.

O-(4,4'-Dimethoxytrityl)-triethyleneglycol ***(Ia)***. Yield 65%. R_f 0.29 (diethyl ether). ^{1}H NMR ($CDCl_3$): 7.23–7.31 (13 H, m, aromatic protons), 6.77 (2 H, d, aromatic protons in *orto*-position to CH_3O group), 3.75 (6 H, s, CH_3O), 3.64 OCH_2CH_2O–), 1.97 (1H, t,–OH). *O-(4,4'-Dimethoxytrityl)hexaethyleneglycol* ***(Ib)***. Yield 50%. R_f 0.39 (diethyl ether). ^{1}H NMR ($CDCl_3$): 7.24–7.33 (13 H, m, aromatic protons), 6.79 (2 H, d, aromatic protons in *orto*-position to CH_3O group), 3.75 (6 H, s, CH_3O), 3.62 (24 H, t, OCH_2CH_2O–), 1.96 (1 H, t, -OH).

***H*-Phosphonates of *O*-(4,4'-dimethoxytrityl)-oligoethyleneglycols (IIa) and (IIb)** were prepared by

analogy with the synthesis of nucleoside *H*-phosphonates [27]. Imidazole (1.35 g, 19.9 mmol) was dissolved in 25 mL of abs. CH_3CN under argon. PCl_3 (0.54 mL, 6.2 mmol) and TEA (2.8 mL, 20 mmol) were quickly added under stirring to the resulting solution cooled to 0°C. Stirring was continued for 15 min, followed by the dropwise addition of *O*-(4,4'-dimethoxytrityl)-triethyleneglycol (**Ia**) (25 mL) or *O*-(4,4'-dimethoxytrityl)-hexaethyleneglycol (**Ib**) (1.37 mmol) in abs. CH_3CN for 30 min. The reaction was carried out under stirring for 90 min at room temperature. The reaction was monitored by TLC in a C_2H_5OH–CH_2Cl_2 mixture (4 : 6) containing 0.2% TEA. After the completion of the reaction, H_2O (15 mL) was added and the mixture was evaporated to dryness, the residue was dissolved in CH_2Cl_2 containing 0.2% TEA and extracted two times with 5% aqueous solution of NaCl and one time with water. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to oil. The reaction mixture was subjected to silica gel chromatography in a linear gradient of concentrations of C_2H_5OH (0–30%) in CH_2Cl_2 containing 0.2% TEA. The product with the lower mobility in TLC was collected and evaporated.

Products in the form of oil were dissolved in a small amount of CH_3CN, packed into tubes and dried at 1–2 mmHg over P_2O_5. The yields were 50–60%. ^{31}P NMR (pyridine-CH_3CN, 1 : 1): *O*-(4,4'-dimethoxytrityl)-triethyleneglycol-*H*-phosphonate (**IIa**): 4.77 (d, $J_{P\text{-}H}$ 933); *O*-(4,4'-dimethoxytrityl)-hexaethyleneglycol-*H*-phosphonate (**IIb**): 4.73 (d, $J_{P\text{-}H}$ 933).

***H*-Phosphonate of 2-[2-(4,4'-dimethoxytrityloxy)ethylsulfonyl]ethanol (IIc)** was prepared similar to the synthesis described above starting from [2-[*O*-(4,4'-dimethoxytrityl)oxy]ethyl]sulfonyl]ethanol (**Ic**). After silica gel chromatography, *H*-phosphonate(**IIc**) in the form of a thick oil was dissolved in a small amount of abs. CH_3CN, packed into tubes and dried at 1–2 mmHg over P_2O_5. The yield of (**IIc**) was 74%. ^{31}P NMR (pyridine/CH_3CN, 1 : 1): 2.63 ($J_{P\text{-}H}$ 607).

Synthesis of oligo(2'-*O*-methylribonucleotides) containing oligoethyleneglycol-phosphate at the 5'-end (IV)–(VI) was carried out according to the protocol of the solid-phase *H*-phosphonate synthesis [39]. The polymer support bearing 1.4 μmol of the attached 3'-*O*-dimethoxytritylthymidine was used for the synthesis of oligonucleotides containing "inverted" thymidine. Two last stages of the synthesis were the successive attachment of dimethoxytrityl-containing tri- or hexaethyleneglycol-*H*-phosphonate and [2-[*O*-(4,4'-dimethoxytrity)-loxy]ethyl]sulphonyl]ethanol-*H*-phosphonate. After the standard treatments, oligonucleotides were isolated by ion-exchange and RP HPLC. Oligonucleotides were precipitated with acetone containing 2% $LiClO_4$. The overall yield of oligonucleotides relative to the first nucleoside unit attached to the polymer support was 8–10%. The yields and characteristics of oligonucleotides (**IV**)–(**VI**) are presented in Table 1. The presence of the 5'-terminal phosphate was confirmed by the hydrolysis of the oligonucleotides with alkaline phosphatase followed by the analysis of the hydrolysate by analytical ion-exchange HPLC.

Analysis of nucleoside composition of oligo(2'-*O*-methylribonucleotides) (III-VI). The nucleoside composition of oligo(2'-*O*-methylribonucleotides) containing "inverted" thymidine was confirmed by the exhaustive hydrolysis with nuclease P1 (0.03 M NaOAc, pH 5.2, 1M $ZnSO_4$, 16 h, 37°C) and alkaline phosphatase (0.1M Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 4 h, 37°C) followed by the quantitative analysis of the hydrolysate by analytical RP HPLC. Nucleosides were identified by the retention time values on the column and the spectral ratios in comparison with specially prepared markers. The experimental ratios corresponded to the calculating values.

Conjugates of minor groove binders with 6-[(3-aminopropyl)amino]-11-methoxy-13*H*-benzo[6,7]indolo[3,2-*c*]quinoline (BIQ). 1-Hydroxybenzotriazole (5.1 mg, 0.033 mmol) and dicyclohexylcarbodiimide (7.5 mg, 0.03 mmol) were added to the solution of Boc-protected derivative of hexa- or octa(*N*-methylpyrole)carboxamide in the acid form (**Boc-NH-γ-(Py)$_3$-γ-(Py)$_3$-COOH** or **Boc-NH-γ-(Py)$_4$-γ-(Py)$_4$-COOH**) (0.03 mmol) in 0.5 mL of abs. DMF, and the mixture was stirred for 12 h at room temperature. The reaction was monitored by TLC in a $CHCl_3$–CH_3OH system (85 : 15). BIQ (11.1 mg, 0.03 mmol) and TEA (40 μL) were added to the reaction mixture, and the stirring was continued for another 12 h. The reaction mixture was evaporated, the dry residue was dissolved in 3 mL of a CF_3COOH–CH_2Cl_2 mixture (1 : 2) and was kept for 25 min at room temperature to remove the Boc protecting group. The reaction mixture was evaporated to dryness, the residue was dissolved in a minimal volume of DMF and the resulting solution was added in small portions to ethyl ether under stirring. The residue was washed with ethyl ether and dried, followed by its dissolution in water. The yields of the products isolated by RP HPLC were 55% for **NH$_2$-γ-(Py)$_3$-γ-(Py)$_3$-BIQ** and 61% for **NH$_2$-γ-(Py)$_4$-γ-(Py)$_4$-BIQ**.

^{1}H NMR ($(CD_3)_2SO$):

NH$_2$-γ-(Py)$_3$-γ-(Py)$_3$-BIQ: 13.66 (1 H, s, –NH, BIQ), 12.27 (1 H, s, –NH, BIQ), 9.90 (4 H, s, –CONH–), 9.89 (1 H, s, –CONH–), 9.88 (1 H, s, –ONH–), 8.69 (1 H, d, BIQ, *J* 8.0), 8.57 (1 H, d, BIQ, *J* 9.0), 8.30 (1 H, t, BIQ, *J* 8.0), 8.22 (1 H, d, BIQ, *J* 8.0), 8.18 (1 H, d, BIQ, *J* 8.0), 8.07–8.03 (2 H, m, BIQ), 7.88 (1 H, d, BIQ, *J* 9.0), 7.81 (1 H, t, BIQ, *J* 8.0), 7.71 (2 H, br.s, *NH$_2$*–$CH_2CH_2CH_2$–), 7.32 (1 H, d. *J* 1.7, –CH–, Py), 7.21–7.15 (5 H, m, –CH–, Py), 7.05 (1 H, d, *J* 1.7, –CH–, Py), 7.04 (1 H, d, *J* 1.7, –CH–, Py), 7.00 (1 H, d, J 1.7, –CH–, Py),

6.91 (1 H, d, *J* 1.7, –CH–, Py), 6.87 (1 H, d, *J* 1.7, –CH–, Py), 6.85 (1 H, d, *J* 1.7, –CH–, Py), 4.04 (3 H, s, $-OCH_3$), 3.91 (2 H, br. quartet, NH–CH_2–CH_2CH_2), 3.84 (3 H, s, $-NCH_3$), 3.83 (3 H, s, $-NCH_3$), 3.82 (6 H, s, $-NCH_3$), 3.79 (3 H, s, $-NCH_3$), 3.20 (2 H, quartet, *J* 6.0, –NH–CH_2–CH_2CH_2), 2.82 (2 H, quartet, *J* 6.0, NH–CH_2CH_2–CH_2), 2.55 (2 H, br.t, CH_2CH_2–CH_2–CO), 2.34 (2 H, t, *J* 6.0, CH_2CH_2–CH_2–CO), 2.27 (2 H, t, *J* 6.0, CH_2CH_2–CH_2–CO), 2.02 (2 H, br. quintet, *J* 6.0, CH_2–CH_2–CH_2), 1.85–1.75 (4 H, m, CH_2–CH_2–CH_2).

NH_2-γ-$(Py)_4$-γ-$(Py)_4$-BIQ: 13.61 (1 H, s, –NH, BIQ), 12.26 (1 H, s, –NH, BIQ), 9.92 (3 H, s, –CONH), 9.88 (3 H, s, –CONH), 9.83 (1 H, s, –CONH), 9.77 (1 H, s, –CONH), 8.70 (1 H, d, BIQ, *J* 8.0), 8.57 (1 H, d, BIQ, *J* 9.0), 8.30 (1 H, t, BIQ, *J* 8.0), 8.19 (1 H, d, BIQ, *J* 8.0), 8.17 (1 H, d, BIQ, *J* 8.0), 8.06–8.01 (2 H, m, BIQ), 7.89 (1 H, d, BIQ, *J* 9.0), 7.80 (1 H, t, BIQ, *J* 8.0), 7.70 (2 H, br.s, NH_2–$CH_2CH_2CH_2$–), 7.32 (1 H, d, *J* 1.7, –CH–, Py), 7.21–7.15 (7 H, m, –CH–, Py), 7.05–7.03 (3 H, m, –CH–, Py), 7.00 (1 H, d, *J* 1.7, –CH–, Py), 6.94 (1 H, d, *J* 1.7, –CH–, Py), 6.91 (1 H, d, *J* 1.7, –CH–, Py), 6.87 (1 H, d, *J* 1.7, –CH–, Py), 6.85 (1 H, d, *J* 1.7, –CH–, Py), 4.04 (3 H, s, $-OCH_3$), 3.91 (2 H, br. quartet, NH–CH_2–CH_2CH_2), 3.84 (15 H, s, $-NCH_3$), 3.82 (6 H, s, $-NCH_3$), 3.80 (3 H, s, $-NCH_3$), 3.21 (2 H, quartet, *J* 6.0, NH–CH_2–CH_2CH_2), 2.79 (2 H, quartet, *J* 6.0, NH–CH_2–CH_2CH_2), 2.55 (2 H, br.t, CH_2CH_2–CH_2–CO), 2.35–2.15 (4 H, m, CH_2CH_2–CH_2–CO), 2.02 (2 H, br. quintet, CH_2–CH_2–CH_2), 1.72 (2 H, br. quintet,CH_2–CH_2–CH_2), 1.72 (2 H, br. quintet, CH_2–CH_2–CH_2).

Conjugates of oligo(2'-*O*-methylribonucleotides) containing minor groove binder (VIIa)–(IXa) were synthesized according to protocols [28, 44]. Solutions of triphenylphosphine (6.8 mg 25 μmol) in 25 μL of abs. DMSO and 2,2'-dipyridyldisulfide (5.3 mg, 25 μmol) in 25 μL of abs. DMSO were added to the solution of oligonucleotide (**V**) or (**VI**) in the form of cetyltrimethylammonium salts (10–15 OU_{260}). The reaction mixture was stirred for 15 min at 25°C. The activated oligonucleotide was precipitated with 2% $LiClO_4$ in acetone, quickly washed with acetone, and dissolved in water (5 μL). The corresponding ligand (octa- or hexa(*N*-methylpyrrole)carboxamide) (1 μmol) in DMSO (100 μL) containing abs. TEA (5 μL) was added to the activated oligonucleotide, and the reaction mixture was kept for 2 h at room temperature, followed by the precipitation with 2% $LiClO_4$ in acetone and subsequent dissolution of the residue in water (100 μL). The resulting conjugates were isolated by RP HPLC in a gradient of concentrations of CH_3CN (5–40%) in 0.025 M ammonium acetate (pH 6.0) followed by precipitation in the form of sodium salts. The yields and characteristics of the synthesized oligonucleotide derivatives are presented in Table 2.

Conjugates of oligo(2'-*O*-methylribonucleotides) containing minor groove binder and 6-[(3-aminopropyl)amino]-11-methoxy-13*H*-benzo[6,7indolo[3,2-*c*] quinoline (BIQ) (VIIb)–(IXb) were synthesized as described above using as a ligand the conjugate of hexa- or octa(*N*-methylpyrrole)carboxamide with BIQ (**NH_2-γ-$(Py)_3$-γ-$(Py)_3$-BIQ** or **NH_2-γ-$(Py)_4$-γ-$(Py)_4$-BIQ**). The yields and characteristics of the synthesized oligonucleotide derivatives are presented in Table 2.

Thermal stability of triplexes. The thermal denaturation and renaturation of the complexes were performed according to [35] in 0.01 M cacodylate buffer, pH 6.0, containing 0.1 M NaCl and 5 mM $MgCl_2$. The concentrations of the target duplex and the third strand in each sample were 1–1.3 μM and 1.3–1.7 μM, respectively. The rate of the temperature change was 0.1°C/min, the absorption at 260, 330, and 580 nm was recorded every 200 s on a Kontron Uvikon 940 spectrophotometer (BioTek, United States) adjusted with nine thermostated 1-cm-path-length cuvettes. The melting curves were processed using the Kaleida-Graph and Microsoft Excel programs.

Dissociation constants of ternary complexes were evaluated by the gel retardation method according to [35] at pH 6.0 or 7.2 and at temperature of 10°C or 37°C varying the concentration of the triplex-forming oligonucleotide (10, 50, 100, 200, and 500 nM). Each sample (10 μL) containing 0.6 pmol of the 5'-^{32}P-labeled target in a 50 mM MES buffer, pH 6.0, containing 50 mM NaCl and 5 mM $MgCl_2$ or 50 mM HEPES, pH 7.2, containing 50 mM NaCl and 5 mM $MgCl_2$ was denatured at 90°C for 3 min, then slowly cooled to room temperature followed by the addition of the third strand to the needed concentration. The 5% solution (1 μL) of bromophenol blue and xylane cyanol FF in 50% glycerol was added, and the reaction mixture was incubated for 5–16 h at 10°C or 37°C. Electrophoretic separation of the products was carried out in 12% native PAAG at the corresponding temperature. Dissociation constants of the triplexes (K_d) were evaluated as described in [35].

Kinetic curves of triplex formation obtained by gel retardation method. The kinetic experiments were carried out by the gel retardation method at pH 6.0 and 10°C. The target 5'-[^{32}P]-dsDNA (60 nM) was heated in a buffer (50 mM MES, pH 6.0, containing 50 mM NaCl, 5 mM $MgCl_2$, 0.05% bromophenol, 0.05% xylene cyanol FF, and 10% glycerol) at 90°C for 3 min, slowly cooled to the room temperature followed by the addition of the third strand to 2 μM concentration. The reaction mixture was aliquoted after certain periods of time (0, 15, 30, 60, 120, and 180 min) and applied to 12% native PAGE. The data were processed using the ImageQuant and Sigma Plot 6.0 programs.

Inhibition of in vitro transcription with oligonucleotide conjugates was performed as described in [33,

36]. 780-mer fragment of plasmid pSG-F47 (0.5 μg, 10 nmol) containing the polypurine tract was added to 25 μL of the reaction mixture containing 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 2 mM spermidine, 4 mM dithiothreitol, 1 U/μL of RNase inhibitor, and the oligonucleotide or its conjugate to the concentration designated in Fig. 6. The final concentration of nucleoside triphosphates in the reaction mixture was 500 μM for ATP, CTP, and UTP, 100 μM for GTP, and 0.3 μM for [α-^{32}P]GTP. T7 RNA polymerase (25 AU) was added to the resulting mixture, and the transcription occurred for 10 min at 37°C. The reaction was stopped by precipitation with ethanol. The residue was dissolved in a loading buffer, and the transcription products were analyzed by electrophoresis in denaturing 6% PAAG.

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