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Synthesis and Complementary Complex Formation Properties of Oligonucleotides Covalently Linked to New Stabilizing Agents.

Alessandro Balbi*, Enzo Sottofattori, Teresa Grandi, Mauro Mazzei

Institute of Pharmaceutical Sciences, Viale Benedetto XV, 3 - Genoa (Italy)

Tatyana V. Abramova, Sergej G. Lokhov, Alexander V. Lebedev*

Institute of Bioorganic Chemistry, Novosibirsk (Russia)

Abstract: *Oligodeoxyribonucleotides of different lengths have been prepared and linked to new stabilizing agents related to the coumarin family. These ODNSAs (OligoDeoxyriboNucleotides with Stabilizing Agents) were tested against acridine connected oligomers of the same sequence. Melting temperature experiments demonstrated that all ODNSAs formed complexes of increased stability with complementary sequences of deoxyribo-20-mer. The order of stability of duplexes showed that the coumarins stabilize the complexes more than the acridine and the chromone derivatives.*

INTRODUCTION

Oligonucleotide derivatives containing attached polycyclic aromatic groups such as acridine[1-4], phenazine[5,6], ethidium[7-9] and others are desirable compounds in "antisense" and "antigene" strategies[10-12] due to their ability to form specific complexes of increased stability[1-5,7,9] with complementary oligo- and polynucleotides. Oligonucleotides bearing the above stabilizing agents (SA) are also shown to enhance penetration of oligodeoxyribonucleotide (ODN) into the cells and nuclei through the membrane[13-15] and improve endonuclease resistance[8,16-18]. When a stabilizing agent is linked to an ODN, this new compound is expected to selectively recognize the complementary nucleic acid base sequence and to bind itself in a stronger way to this complementary sequence than the one not containing SA. Therefore, the free energy change to unfold the complex of ODNSA conjugate with complementary sequence has to be higher than the respective complex of simple ODNs.

The overall stabilizing effect of SA on complementary complex formation was shown to depend on the nature of SA and on the nature and the length of the linker function[2-5]. At this point it is difficult to clearly estimate which role is played by the SA itself and the linker group. Thus, simple modifications of the ODNs were sufficient to improve, for example, nuclease resistance[19]. The influence of the linker structure on the stability of the complementary complexes was shown to depend on the oligonucleotide length and seems to be more pronounced for relatively short oligomers (up to 4-8 nucleotide residues)[2-4].

In order to construct nucleic acid-binding substances with base specificity and high binding activity, we started to study a family of molecules in which ODNs are covalently linked to new stabilizing agents related to coumarin derivatives.

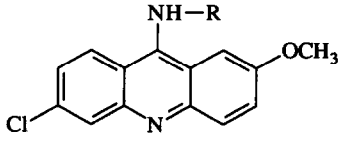
RESULTS and DISCUSSION

Compounds of the coumarin family are attractive candidates for attachment to oligodeoxyribonucleotides to serve as stabilizing agents. For this purpose, we chose two coumarin derivatives (a tricyclic α-pyrone **2** and a simple coumarin **3**), and a γ-pyrone derivative **5** which is correlated with the coumarin family.

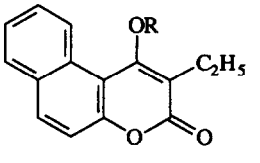
The accurate evaluation of the binding ability of ODNSAs in a complementary complex can be carried out by comparing the stability of these complexes with that found in a respective acridine ODN derivative. The acridine residue is known to be the most studied SA and has been employed in a number of publications [1-4,12]. Therefore, for comparative reasons, in all experiments the acridine derivative **1** was used as an SA reference.

Literature[20] gives compound **1** as synthesized from 6,9-dichloro-2-methoxyacridine and 1,3-diaminopropane without comprehensive indications. We now describe the method and give the spectra and analytical data.

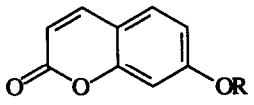
The bromomethylene derivatives **2b** and **3b** were prepared following general procedures[21] from the benzocoumarin **2a** (R=H) synthesized by us[22] and the commercially available 7-hydroxycoumarin **3a** (R=H). The above **2b** and **3b** treated with ammonia gave the desired aminomethylene derivatives **2** and **3**.



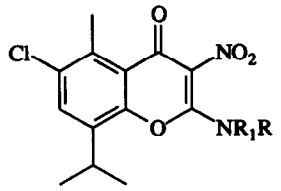
1 R=$CH_2CH_2CH_2NH_2$



2 R=$CH_2CH_2CH_2NH_2$
2a R=H
2b R=$CH_2CH_2CH_2Br$

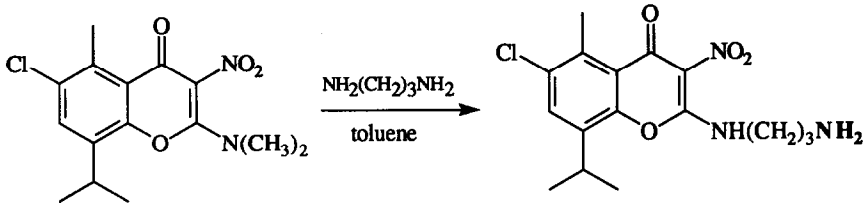


3 R=$CH_2CH_2CH_2NH_2$
3a R=H
3b R=$CH_2CH_2CH_2Br$



4 R_1=R=CH_3
5 R_1=H, R=$CH_2CH_2CH_2NH_2$

The chromone (benzopyran-4-one) **5** was directly synthesized from the 6-chloro-2-(dimethylamino)-8-isopropyl-5-methyl-3-nitro-4H-1-benzopyran-4-one **4**[23] and 1,3-diaminopropane in toluene. The mechanism of this reaction has already been explained for similar compounds[24] : the addition of 1,3-diaminopropane at the double bond 2-3 occurs initially, then the elimination of the dimethylamine yields **5**.



4 **5**

All new compounds were characterized by their elemental analyses and by IR and ^1H NMR spectra (see experimental).

The 5'-phosphorylated ODNs were synthesized in solution following our previous routes[25,26]. The reaction of the amino compounds **1-3**, **5** with the ODNs-phosphate was performed using the mixture of triphenyl phosphine and 2,2-dipyridyl-disulphide in the presence of N-methylimidazole as a catalyst[27] (see fig. 1).

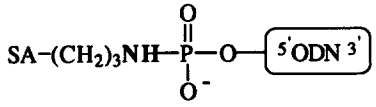


Fig. 1. Structure of the ODN linked to an SA

The following compounds **6-13** (see Table I) were prepared in the above way from **14** and **15** which were designed as complementary sequences to the 20-mer **16**.

All the oligonucleotide conjugates were purified by preparative HPLC on the reverse-phase column and the purity of the peak was checked by the diode array detector.

The retention times of these derivatives on the reverse-phase column (Table I) suggested that compounds **10** and **12** should better penetrate the membranes as seen in literature[14].

Table I. *Oligodeoxyribonucleotide derivatives prepared.*

Compound	antisense sequence (5' → 3')	stabilizing agent	HPLC RT*
6	dp(CCCAGGTGTGCGAA)	**1**	23.50
7	dp(CAGGTGTGCG)	**1**	21.40
8	dp(CCCAGGTGTGCGAA)	**2**	23.60
9	dp(CAGGTGTGCG)	**2**	20.55
10	dp(CCCAGGTGTGCGAA)	**3**	24.00
11	dp(CAGGTGTGCG)	**3**	22.00
12	dp(CCCAGGTGTGCGAA)	**5**	23.90
13	dp(CAGGTGTGCG)	**5**	21.00
14	dp(CCCAGGTGTGCGAA)	none	17.45
15	dp(CAGGTGTGCG)	none	15.40
16	dp(ACGTTCGCACACCTGGGTGC)	none	21.60

* Reverse-phase HPLC retention time.

A thermal stability of the complexes of the above ODNs and their conjugates with **16** are herewith described (see Table II, fig. 2). The 20-mer forms an internal hairpin complex with five base pairs and Tm of 66°C, which complicates the analysis of the melting temperature (Tm) data obtained for complexes of the oligomers **6-15** with 20-mer **16**.

Table II

Melting temperature data on the complexes of 20-mer with 10-mer, 14-mer and their conjugates (1:1)

Stabilizing agent	–	**1**	**2**	**3**	**5**
Complex with 10-mer	45	52	60	61	50
Complex with 14-mer	64	65	67	67	65

Buffer used: 0.2M NaCl, 0.01M Na_2HPO_4,0.1M EDTA, pH 7.0 - *Concentration of all oligomers*: $6.7 \cdot 10^{-6}$M

In most cases, however, it was possible to evaluate accurate or appropriate Tm values. Both 10-mer and 14-mer oligonucleotides were able (to different extents) to compete with the hairpin structure of 20-mer and to form along with it a duplex with Tm values of 45° and 64°C, respectively. Conjugation of all the above stabilizing agents to oligomers **14** or **15** increased the stability of the complementary complexes. The comparison of Tm values for complexes **16** + **14**, **16** + **9** and **16** + **11** allowed us to conclude that the stability of these complexes is nearly equal (the differences do not exceed 3°C, Table II). It means that covalent attachment of stabilizing agents **2** or **3** to 10-mer is almost equivalent to the increase of duplex length by three-four complementary base pairs or to the change of free energy of the complex formation by approximately -6 Kcal/mol (at 37°C). These stabilising effects exceed more than twice those of ethidium[9], acridine[4] or phenazine[5] dyes. There are no data regarding polycyclic aromatic groups linked to oligonucleotides having higher stabilizing effects on the duplex structure. The analysis of all the data presented in Table II allowed us to evaluate that the effect of the stabilizing agents on the Tm values falls in sequence: chromone ~ acridine ~ coumarin ~ benzocoumarin.

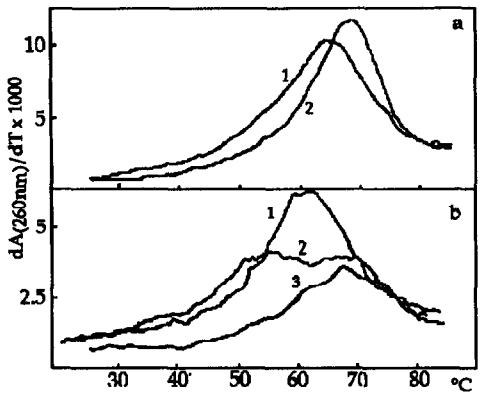


Figure 2. First derivatives of the melting curves for the complexes:
(a) 16+ 6 (curve 1) and 16+ 8 (curve 2);
(b) 16+ 9 (curve 1), 16+ 7 (curve 2) and hairpin complex of 16 (curve 3).

Two transitions (or a single broad transition) reflected the superposition of the melting of the internucleotide complex and a 20-mer intranucleotide hairpin complex were detected for the **16 + 7** complex (fig. 2[b]), as well as for complexes **16 + 13, 16 + 14** and **16 + 15** (data not shown), while single narrower transitions with high hyperchromicity (included both interchain and intrachain transitions) were observed for complexes **16 + 9** (fig. 2[a]) and **16 + 11** (data not shown). All 14-mer derivatives show single transition melting curve (see fig. 2[a]). While the contribution of a linker group is obviously important [2-5], it was difficult at this time tosuggest a general rule for this choice except by way of experimental verification. In our case the length of the linker function was probably not optimal for each stabilizing agent tested. At least for acridine-ODN derivatives the -$(CH_2)_5$- linker led to more stable complexes than -$(CH_2)_3$- linker[2-4]. In the case of phenazine-ODN derivatives -$(CH_2)_2$- and -$(CH_2)_4$- linkers are preferable compared to -$(CH_2)_3$- and -$(CH_2)_5$- linkers[5]. The influence of a linker length on the stabilizing effect was shown to be significant for relatively short ODN derivatives[2,3], and was found to have almost vanished in the case of longer oligomers[4]. It has, in fact, been observed that the linker length influenced rather weakly the stability of the complexes (about 2-3°C) of the oligomers longer than 10-mer[4,6].

CONCLUSION

The experimental data presented here testify that the family of coumarin stabilizing agents linked to ODNs increase complexation abilities of oligomers more than the acridine and chromone residues and are therefore expected to have a potential application on the gene regulation and expression. Suitably substituted coumarin with absorbance at wavelengths higher than 300 nm may also be considered as potentially reactive groups for specific photomodification of oligo and polynucleotide sequences.

EXPERIMENTAL

General

Melting points were determined with a Fisher-Johns apparatus and are uncorrected. The IR spectra were recorded in potassium bromide disks on a Perkin-Elmer 398 spectrophotometer. The following abbreviations were used to designate the shape of the IR bands: s = strong, l = large, sh = shoulder, br = broad. The ^{1}H NMR spectra were obtained on a Hitachi Perkin-Elmer R 600 (60 MHz) spectrometer with TMS as the internal standard (δ=0). NMR signals are quoted as singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), multiplet (m), broad (br). The purity of the compounds was checked by TLC on silica gel 60-F254 precoated plates and the spots were located in UV light or by iodine vapor or with ninidrine. Elemental analyses were performed in the Microanalysis Laboratory of our Institute on a Carlo Erba 1106 Elemental Analyzer. UV absortion spectra of ODNs and ODNSAs were recorded with a Shimadzu UV-2100 UV-visible spectrophotometer.

HPLC

The liquid chromatograph was a Perkin-Elmer Series 4 (Norwalk, CT, USA) equipped with a Rheodine 7125 (Berkeley, CA, USA) injector valve with 20 µl or 1 ml loop (respectively for analytical or preparative purpose). The diode array detector was a Perkin-Elmer LC-235. Retention times, peak areas and UV spectra were recorded on Perkin-Elmer LCI-100 integrator.

The anionic exchanger was a stainless steel column (25 cm x 10 mm) filled by us with Partisil-10 SAX (Whatman, USA). The reverse-phase column was a stainless steel column (25 cm x 10 mm) filled by us with 10 µm LiChrosorb RP18 (Merck, Germany).

Synthesis of Stabilizing Agents

6-Chloro-2-methoxy-9-(3-aminopropylamino)-acridine **1**

The mixture of 6,9-dichloro-2-methoxyacridine (1g, 3.59 mmol), 1,3-diaminopropane (2.66g, 35.9 mmol) and 30 ml of hexamethylphosphoramide was heated at 80°C for 2h. The solution was poured into cold water and extracted with chloroform. Removal of the solvent *in vacuo* afforded a viscous oil which was soluted in methanol and treated with HCl gas. The yellow precipitate was filtered, crystallized from ethanol, soluted in water and precipitated with 2M NaOH. The new yellow precipitate was filtered, washed with water, dried and finally crystallized from ethyl acetate to give the pure **1** (0.91g, 80%), m.p. 102-3°C; IR (cm^{-1}) 3250 l s, 1630, 1610, 1570; ^{1}H NMR (DMSO-d_6) δ 1.88 (q, 2H, CH_2), 2.80 (t, 2H, CH_2NH_2), 3.40-4.10 [m, 6H, $NHCH_2$ (t, 3.80) + OCH_3 (s, 4.00) + NH and NH_2 hidden by the superimposed triplet and singlet], 7.20-8.58 (m, 6H, H-arom.); Anal Calcd for $C_{17}H_{18}N_3OCl$: C, 64.66; H, 5.75; N, 13.31; Cl, 11.23; Found: C, 64.60; H, 5.72; N, 13.25; Cl, 11.12.

1-(3-bromopropoxy)-2-ethyl-3H-naphtho[2,1-b]pyran-3-one **2b**

The mixture of 3.3 mmol (0.8g) of 2-ethyl-1-hydroxy-3H-naphtho[2,1-b]pyran-3-one **2a** [22], 0.45g of anhydrous K_2CO_3, 9 mmol (1.82g) of 1,3-dibromopropane and 10ml of anhydrous acetone was refluxed for 24h. After cooling, removal of the solvent afforded an oil which was stirred vigo-

rously with water and extracted with chloroform. The chloroform extracts were dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography using silica gel and eluted first with petroleum ether 40°-70°C and secondly with petroleum ether-ethyl acetate (8:2 v/v). The second eluted gave **2b** (0.72 g, 60%) as pure white crystals. After recrystallization from ethanol, m.p. 124°C; IR (KBr, cm^{-1}) 1705; ^1H NMR ($CDCl_3$) δ 1.28 (t, 3H, CH_2CH_3), 2.60 (m, 4H, CH_2CH_3 and CH_2), 3.73 (t, 2H, CH_2Br), 4.08 (t, 2H, OCH_2), 7.30-8.12 (m, 5H, H-5-9), 9.00 (d, 1H, H-10); Anal Calcd for $C_{18}H_{17}O_3Br$: C, 59.85; H, 4.74; Br, 22.12; Found: C, 59.95; H, 4.80; Br, 22.23.

7-(3-bromopropoxy)-2H-1-benzopyran-2-one **3b**

Using the above procedure, from 5 mmol (0.81g) of 7-hydroxy-2H-1-benzopyran-2-one, **3b** (0.72g, 51%) was finally obtained; m.p. 108°C from ethyl acetate-ligroin (1:3) [lit.[28] m.p. 108°C].

General procedure for the preparation of the 3-aminopropoxy-derivatives (**2-3**)

A solution of 1 mmol of the 3-bromopropylderivative (**2b** or **3b**) in methanol (30 ml) was allowed to stir at 40°C for 48h under a flow of ammonia. The volatiles were removed under reduced pressure and the residue was dissolved in methanol. A saturated solution of hydrogen chloride was added to afford the hydrochlorides of **2-3** which were filtered and washed with ethyl ether. The residue was soluted in water and precipitated adding a saturated solution of $NaHCO_3$ to give the title products as white solids:

1-(3-aminopropoxy)-2-ethyl-3H-naphtho[2,1-b]pyran-3-one **2**

0.12g, 40%; m.p. 145-6°C from ethyl acetate; IR (cm^{-1}) 3400 l, 1710; ^1H NMR ($CDCl_3$) δ 1.28 (t, 3H, CH_2CH_3), 2.55 (m, 4H, CH_2CH_3 and CH_2), 3.20 (t, 2H, CH_2NH_2), 4.10 (t, 2H, OCH_2 and NH_2 partially hidden by the superimposed triplet), 7.32-8.20 (m, 5H, H-5-9), 9.00 (d, 1H, H-10). Anal Calcd for $C_{18}H_{19}NO_3$: C 72.71, H 6.44, N 4.71; Found: C, 72.69, H 6.40, N 4.70.

7-(3-aminopropoxy)-2H-1-benzopyran-2-one **3**

0.13g, 59%; m.p. 164-5°C from methanol; IR (cm^{-1}) 3400 br, 1730, 1710, 1615; ^1H NMR (DMSO-d_6) δ 2.20 (m, 2H, CH_2), 3.08 (br t, 2H, CH_2NH_2), 4.24 (t, 2H, OCH_2), 6.22 (d, 1H, H-3), 7.01 (m, 2H, H-6,8), 7.71-8.08 (dd, 4H, H-5, H-4, NH_2 hidden by the superimposed doublets). Anal Calcd for $C_{12}H_{13}NO_3$; C 65.74, H 5.98, N 6.39; Found: C 65.71, H 5.91, N 6.36.

2-[(3-aminopropyl)amino]-6-chloro-8-isopropyl-5-methyl-3-nitro-4H-1-benzopyran-4-one **5**

A warm solution of 6-chloro-2-(dimethylamino)-8-isopropyl-5-methyl-3-nitro-4H-1-benzopyran-4-one[23] (0.5g, 1.54 mmol) in toluene (15 ml) was added dropwise to a solution of 1,3-diaminopropane (0.14g, 1.85 mmol) in toluene (15 ml) heated at 110°C. This reaction mixture was allowed to stir at the same temperature for 1h, then evaporated under reduced pressure. The resulting solid was dissolved in chloroform and co-evaporated with silica gel (1g) under reduced pressure. The silica gel was applied to the top of a silica gel column which was then eluted with cyclohexane-ethyl acetate (7:3 v/v) giving the title compound **5** as yellow crystals (0.3g, 55%), m.p. 137°C from the same solvent mixture. The next elution with ethyl acetate gave 0,1g of a white solid (still unidenti-

fied); IR (cm^{-1}) 3300 s, 3230, 1620 sh, 1608, 1585; ^1H NMR ($CDCl_3$) δ 1.22 [d, 6H, $CH(CH_3)_2$], 2.07 (m, 2H, CH_2), 2.22 (s, 3H, CH_3), 3.18 (m, 1H, CH), 3.52 (m, 4H, CH_2NH_2 + $NHCH_2$), 4.1 (br s, 2H, NH_2), 7.15 (s, 1H, H-7), 9.50 (br s, 1H, NH). Anal Calcd for $C_{16}H_{20}N_3O_4Cl$; C 54.32, H 5.70, N 11.88, Cl 10.02; Found: C 54.30, H 5.69, N 11.85, Cl 9.98.

Synthesis of Oligonucleotides

The oligonucleotides were synthesized in solution following our previous routes[25, 26]. Then, the fully protected oligonucleotides were deblocked and purified by the same procedure as for the purification of unmodified oligonucleotides. Each oligonucleotide in this preparation showed a single peak either with an anionic exchange column or with a C-18 column (see *HPLC*).

Synthesis of Oligonucleotides Linked to Stabilizing Agents

General procedure

3µl of a 8% water solution of cetyltrimethylammonium bromide was added to a solution of the lithium salt of the completely deblocked ODN (5AU) dissolved in 50µl of water and this mixture was then centrifuged. 1 µl of the former solution was then added and the mixture again centrifuged. The procedure was repeated until no more precipitate was observed. The supernatant was eliminated and the residue was dried *in vacuo* overnight on P_2O_5. A solution of this compound in 60µl of dry DMSO, 0.010g of triphenylphosphine, 0.010g of dipyridyldisulfide, 8µl of 1-methylimidazole was stirred for 10 min. and then 0.002g of the aminoderivative **1-3**, **5** and 2µl of anhydrous triethylamine were added. After strirring for 1h at room temperature, the solution was precipitated with 1 ml of 2% $LiClO_4$ in acetone. After centrifugation, the supernatant was eliminated and the precipitate dissolved in 50 µl of 3 M $LiClO_4$ and treated with 1 ml of 2% $LiClO_4$ in acetone. The residue (lithium salt) was dissolved in 1 ml of water, purified by reverse-phase HPLC, collected and evaporated under reduced pressure. The final residue was dissolved and a precisely measured aliquot is taken off to measure the absorbance at 260 nm[26]. Then the solution was precipitated with 2% $LiClO_4$ in acetone to give the linked ODNs **6-13**.

Thermal Denaturation of Complexes:

Preparation of samples for thermal denaturation experiments

Aqueous solutions of appropriate concentrations of ODNs were prepared by diluting a concentrated solution of the ODN or the ODNSA according to molar extinction coefficients at 260 nm at 20°C. Extinction coefficients were calculated according to results of total PDE-hydrolysis[20] and were as follows (260 nm x 10): 20-mer 173; 14-mer 124; 10-mer 87. The extinction coefficients for the ODNSA were estimated as a sum of respective values for each oligomer and stabilizing group. Aqueous solutions of ODN or ODNSA were mixed with concentrated buffer solutions. In all cases a final composition of buffer solution was: 0.2 M NaCl, 0.01 M Na_2HPO_4 , 0.1 mM EDTA, pH 7.0. Oligonucleotide chain concentration was $6.7x10^{-6}$ M.

Melting curves

Optical melting curves were obtained with the use of a home-made apparatus developed on the base of spectrophotometric detector of liquid chromatograph Milichrom (Orel, Russia) connected to PC computer. Volume of the optical cell was 2 μl, the cell path length was 1.2 mm. Temperature of the optical cell was monitored using thermostat-connected water jacketed cell holder (rate of temperature change was 0.5°C/min.) and controlled by Cu-Constantane thermocouple calibrated with an accuracy of 0.1°C. Thermocouple was connected to PC through digital voltmeter SH-1516 (Russia). All the data (absorbance and temperature) were collected by the PC. Each experimental value of optical density was the integral of the signal for 10 seconds. A total of 500-600 points were collected for each melting curve. The corrections caused by the water heat volume change were added to the final melting curve profiles.

REFERENCES

1. Asseline, U.; Toulmé, F.; Thuong, N. T.; Delarue, M.; Montenay-Garestier, T.; Hélène , C. *Embo J.* **1984**, *3*, 795-800.
2. Asseline, U.; Delarue, M.; Lancelot, G.; Toulmé, F.; Thuong, N. T.; Montenay-Garestier, T.; Hélène, C. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 3297-3301.
3. Asseline, U.; Thuong, N. T.; Hélène, C. *J. Biol. Chem.* **1985**, *260* 8936-8941.
4. Stein, C. A.; Mori, K.; Loke, S.L.; Subasinghe, C.; Shinozuka, K.; Cohen, J. S.; Nechers, L. M. *Gene* **1988**, *72*, 333-341.
5. Lokhov, S. G.; Podyminogin, M. A.; Sergeev, D. S.; Silnikov, V. N.; Kutyavin, I. V.; Shishkin, C. V.; Zarytova, V.F. *Bioconjugate Chem.* **1992**, *3*, 414-419.
6. Kutyavin, I. V.; Podyminogin, M. A.; Bazhina, Yu. N.; Fedorova, O. S.; Knorre, D. G.; Levina, A. S.; Mamaev, S. V.; Zarytova, V. F. *FEBS Lett.* **1988**, *238*, 35-38.
7. Benimetskaya, L. Z.; Bulychev, N. V.; Kozionov, N. V.; Koshkin, A. A.; Lebedev, A. V.; Novozhilov, S. Yu.; Stokman, M. I. *Biopolymers* **1989**, *28*, 1129-1147.
8. Koshkin, A. A.; Lebedev, A. V.; Ryte, A. S.; Vlassov, V. V. *Nucleosides Nucleotides* **1991**, *10*, 541-542.
9. Koshkin, A. A.; Lokhov, S. G.; Mamaev, S. V.; Vlassov, V. V.; Lebedev, A. V. *J. Molecular Recognition*; submitted.
10. Wickstrom, E. Ed.*Prospects for Antisense Nucleic Acids Therapy of Cancer and AIDS;* Wiley-Liss, New York, N. Y., 1991, 269 pp.
11. Cohen, J.S. Ed; Oligonucleotides. Antisense Inhibitors of Gene Expression. In *Topics in Molecular and Structural Biology*, vol. 12; Macmillan Press, London, UK, 1989, 255 pp.
12. Hélène, C.; Toulmé J.J. *Biochim. Biophis. Acta* **1990**, 1049, 99-125.
13. Verspieren, P.; Cornelissen A.W.C.A.; Thuong N.T.; Hélène C.; Toulmé J.J. *Gene* **1987**, *61*, 307-315.
14. Butorin, A.S.; Boiziau, C.; Le Doan, T.; Toulmé, J.J.; Hélène, C. *Biochimie*, **1992** *74*, 485-489.
15. Cazenave, C.; Chevrier, M.; Thuong, N.T.; Hélène, C. *Nucl. Acids Res.* **1987**,*15*, 10507-10521.

16. Aktor, S.; Kole, R.; Juliano, R.L. Gene Regulation; Biology of Antisense RNA and DNA. In *Raven Press Ser. Mol. Cell. Biol.*; Vol. 1 ; Erikson, R.P.; Izant, J.G. Eds.; Raven Press, New York 1992, 364 pp.
17. Vlassov, V.V.; Yakubov, L.A. Antisense Inhibition of Gene Expression. In *Oligonucleotides;* Cohen, J.S. Ed. Macmillan, London 1991, 243 pp.
18. McShan, W.M.; Rossen, R.D.; Laughter, A.H.; Trial, J.; Kessler, D.J.; Zendegui, J.G.; Hogan, M.E.; Orson, F.M. *J. Biol. Chem.* **1992**, *267*, 5712-5721.
19. Hinrichsen, R.D.; Fraga, D.; Reed, M.W. *Proc. Natl. Acad. Sci USA* **1992**, *89*, 8601-8605.
20. Lown, I.W; Joshua, A.W. *J. Chem. Soc., Chem. Commun.* **1982**, *22*, 1298-1300.
21. Thuong, N. T.; Asseline, U. Oligonucleotides attached to intercalators, photoreactive and cleavage agents. In *Oligonucleotides and Analogues. A Practical Approach;* Eckstein, F. Ed.; Oxford University Press, New York, 1991; pp 283-306.
22. Balbi, A.; Roma, G.; Di Braccio, M.; Ermili, A. *Il Farmaco Ed. Sc.* **1978**, *33*, 807-821.
23. Balbi, A.; Roma, G.; Mazzei, M.; Ermili, A. *Il Farmaco Ed. Sc.* **1983**, *38*, 784-793.
24. Balbi, A.; Ermili, A.; Mazzei, M.; Roma, G.; Di Braccio, M. *Il Farmaco Ed. Sc.* **1984**, *39*, 863-875.
25. Abramova, T.V.; Komarova, N.I.; Mundus, D.A.; Pereboeva, O.S. *IZV. Sib. Otd. Akad. Nauk, ser. Chim nauk,* (URSS) **1990**, 5, 45-51.
26. Mazzei, M.; Balbi, A.; Grandi, T.; Sottofattori, E.; Garzoglio, R.; Abramova,T. V.; Ivanova, E. M. *Il Farmaco* **1993**, *in press.*
27. Zarytova, V.F.; Godovikova, T.S.; Kutyavin, I.V.; Khalimskaya, L.M. Synthesis, Structure, Metabolism and Activity. In *Biophosphate and Their Analogues;* Bruzik, K.S.; Stec, W.I. Eds.; Elsevier Science Publishers, Amsterdam, 1987; 149-164.
28. Antonello, C.; Marciani Magno, S.; Gia, O.; Carlassare, F.; Baccichetti, F.; Bordin, F. *Il Farmaco Ed. Sc.* **1979**, *34*, 139-156.

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