



Synthesis and Hybridization Properties of the Conjugates of Oligonucleotides and Stabilization Agents—II†

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Abstract—New pyranone derivatives having tri- or pentamethylenamine linker functions were synthesized. These derivatives were covalently attached through the 5'-phosphoramidate linkage to heptanucleotide pd(CCAAACA). Complementary complexes of the octanucleotide pd(TGTTTGGC) and above oligonucleotide conjugates were tested for their thermodynamic response. The T_m data and thermodynamic parameters for complex formation have demonstrated the ability of chromone (γ -pyrone) and coumarin (α -pyrone) derivatives to stabilize strongly 7-mer/8-mer complementary complex, most likely through the stacking interaction of the pyran aromatic system with the neighboring nucleotide bases. The effect of chromone (or coumarin) derivatives on the stability of the oligonucleotide complexes ($\Delta\Delta G$ at 37 °C ranged from -1.0 to -1.7 kcal/mol) was shown to be comparable to the effect of one nucleotide base pair and similar to the effect ($\Delta\Delta G$ at 37 °C ranged from -1.5 to -2.0 kcal/mol) found for acridine-oligonucleotide conjugates served in this study as a reference. © 1997 Elsevier Science Ltd.

Introduction

An overall interest in oligonucleotides (including their derivatives and analogues) has been growing in the scientific community because of their potential as therapeutic agents in treating all kinds of infectious diseases, genetic disorders, and cancer.^{1–7} Whether they are used in the so-called 'antisense', 'antigenic', or 'anticode' strategies, the hybridization properties of the oligonucleotides with the complementary sequences of the target nucleic acids are of fundamental importance.

One of the perspective approaches to improve the hybridization ability (as well as nuclease resistance) of the oligonucleotides is a simple covalent attachment of the different stabilizing agents (SA), usually aromatic organic molecules. The nature of the SA and the length of the linker are fundamental in determining the stability of complementary complexes. Several polycyclic aromatic groups such as acridine,^{8–10} phenazine,^{11,12} ethidium,^{13,14} or psoralen^{15,16} derivatives, have been attached to one or both end(s) of the oligonucleotide.

We previously demonstrated that several compounds from the pyranone family are considered to be promising SA.¹⁷ Thus melting temperature data obtained for model 10-mer/20-mer and 14-mer/20-mer complexes have indicated that an effect of the covalently linked α - and γ -pyrones on the stabilization

of complementary oligonucleotide complexes is comparable with the stabilizing effect of well known and recognized acridines. However, in Part 1 of this work,¹⁷ the quantitative analysis of the melting curves was not performed due to complications resulting from the presence of the internal hairpin formed by the 20-mer oligonucleotide used in the study.

Here we report a synthesis of different chromone-(coumarin)-hepta-nucleotide conjugates, the evidence of formation of the complexes with complementary octanucleotide and the evaluation of thermodynamic parameters from the analysis of the melting temperature experiments. All experiments were performed on the model 7-mer/8-mer duplex, successfully tested in several studies^{14,18} for its thermodynamic response. This complex does not exhibit any kind of complications due to hairpin formation or self-association of the oligomers. The presence of the extra flanking nucleoside in the complementary complex is known to contribute to the stability of the complex and would allow the aromatic system of SA to intercalate between the flanking base and neighboring base pair.^{19,20} In addition the advantage of relatively short oligonucleotide complexes lies in more a pronounced effect of SA on the complex stability. Therefore one is able to estimate more precisely the changes in T_m values and in thermodynamic parameters. The quantitative evaluation of thermodynamic parameters for the oligonucleotide complexes of the pyrone conjugates should help in a better understanding of the role of the chemical structure of SA on the nature of stabilization effects in such complexes.

†For part I, see ref 17.

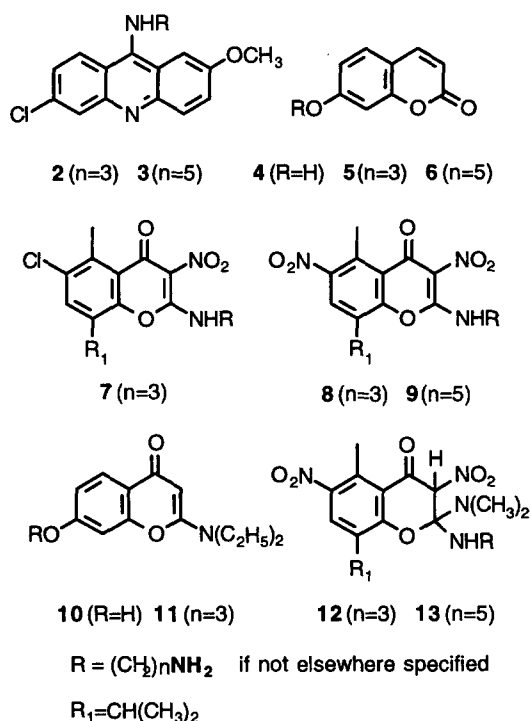
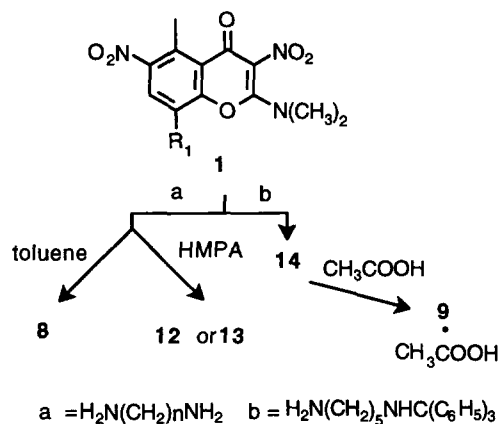


Chart 1.

Results and Discussion

On the basis of the results obtained in our previous work,¹⁷ and particularly because of the comparable stabilization abilities of chromone 7 and the acridine 2 (the most studied SA), we selected the γ -pyrone derivatives 8, 9, and 11 along with the acridine derivatives 2 and 3 and the chromanones 12 and 13.

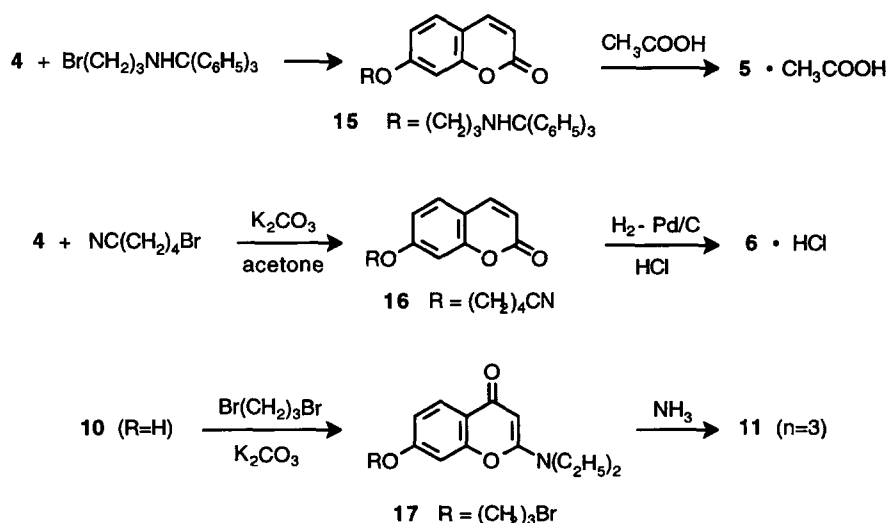
The synthesis of aminoderivatives 8, 12, and 13 was accomplished in one step applying our refined method,²¹ which involves the amine exchange in 2-amino-3-nitrochromones. In particular, compounds



Scheme 1.

1, reacting with 1,3-diamino-propane or 1,5-diamino-pentane in hexamethylphosphoramide (HMPA) at room temperature, gave the addition compounds 12 and 13, respectively, while reacting at 110 °C in toluene gave 8. Although 12 and 13, which come from the nucleophilic attack of the amine on the chromone 1, were also present in the reaction mixture at 110 °C, the pure compound 8 was readily isolated by silica gel chromatography. Compound 9 was obtained in good yield by hydrolysis of the corresponding *N*-trityl derivative 14, which comes from the nucleophilic substitution of 1 with the *N*-trityl-1,5-diaminopentane (Scheme 1).

Coumarins 5 and 6 were prepared following the routes sketched in Scheme 2. Compound 5 was obtained by hydrolysis with acetic acid of the corresponding protected precursor 15, which was in turn synthesized from 7-hydroxycoumarin 4 ($R = H$) and the tritylated bromopropylamine.²² The pentamethylenderivative 6 was in preference obtained by reduction of the cyano derivative 16, easily prepared from 4 ($R = H$) and 5-bromovaleronitrile.



Scheme 2.

The chromone **11** was prepared by treating the bromomethylene derivative **17** with ammonia, which was achieved by reaction of **10** and 1,3-dibromopropane.

Finally, the synthesis of compound **3** was carried out according to the method¹⁷ we followed for compound **2**. All new compounds were characterized by their elemental analyses and by IR and ¹H NMR spectra (see Experimental). The model duplex used in this study is shown in Figure 1.

All conjugates were attached to the 5'-end of the 7-mer oligonucleotide according to previously described methods^{23,24} with some modifications (see Experimental). The major one included the use as a catalyst of *N,N*-dimethylaminopyridine (DMAP) instead of *N*-methyl-imidazole recommended in the 'classical approach'.²⁵ In our case, DMAP generally led to an increased yield of the oligonucleotide conjugates and facilitated their recovery from the reaction mixture. However, in some cases the conjugate's yields seem to be sensitive to the position of the linker group in the chromone (coumarin) moiety.

All oligonucleotide conjugates were isolated and purified with 50–70% yield by reverse-phase HPLC, the homogeneity of the chromatographic fractions was controlled by the diode array detector. As expected, the reversed-phase retention times for all conjugates were higher than that for original 7-mer.

The proof of chemical structures of prepared oligonucleotide conjugates was based on their absorbance spectra. For all conjugates studied it was shown that their spectra contained both oligonucleotide and SA ultraviolet and visible bands conjugate and each spectrum can be represented by superposition of spectra of unmodified 7-mer and SA (see Fig. 2 as an example for compound **12**). Some noticeable differences (for free SA and conjugate) in the position of bands and in their intensities are mainly due to the different solvents used (for free SA, ethanol; for conjugates, water).

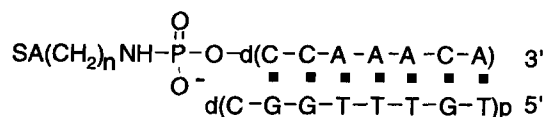


Figure 1.

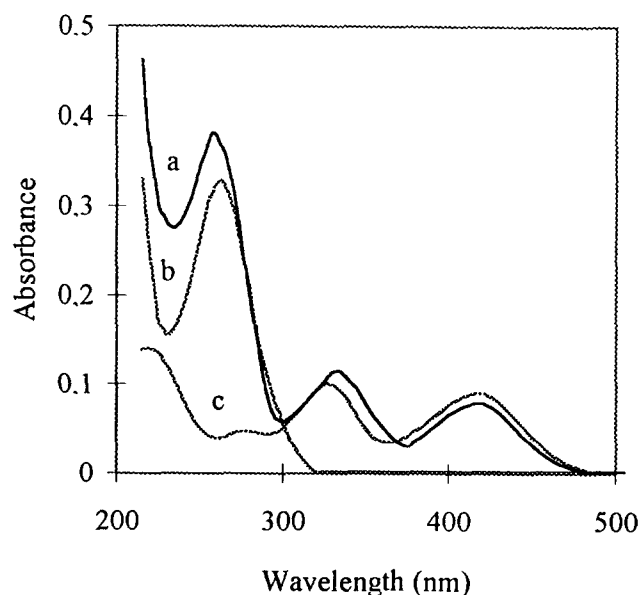


Figure 2. UV spectra of **12*** (a), UM (b) and **12** (c).

Table 1 summarizes the thermodynamic parameters evaluated for complexes of different 7-mer conjugates with the complementary 8-mer. The parameters were calculated from optical melting curves in the assumption of an 'all-or-nothing' model.²⁶

The results show that all chromone and coumarin 7-mer conjugates (except of **12*** and **13***) exhibit a higher affinity to the complementary 8-mer as compared to the parent 7-mer/8-mer complex. The stabilization effect ($\Delta\Delta G$, at 37 °C) ranged from -1.0 to -1.7 kcal/mol, that is comparable with an effect of one A–T base pair. A slightly higher stabilization effect is observed for acridine conjugates (**2*** and **3***). Similar results on the

Table 1. Melting temperature data and thermodynamic parameters for the complex formation of d(CCAAACA) and its conjugates^a with pd(IGTTTGGC)

ODN-SA	UM ^b	2*	3*	5*	6*	7*	8*	9*	11*	12*	13*
Linker, methylene groups (n)	—	3	5	3	5	3	3	5	3	3	5
T _m (± 0.2) (°C)	23.0	33.5	36.6	32.9	30.9	31.9	32.1	34.9	31.0	23.4	23.6
ΔT_m^c	—	10.5	13.6	9.9	7.9	8.9	9.1	11.9	8.0	0.4	0.6
$-\Delta G$ (0.05); 37 °C (kcal/mol)	5.1	67	7.2	6.6	6.1	6.3	6.4	6.8	6.2	5.2	5.2
$-\Delta\Delta G^c$	—	1.6	2.1	1.5	1.0	1.2	1.3	1.7	1.1	0.1	0.1
$-\Delta H$ (± 1) (kcal/mol)	44	51	52	48	54	—	—	57	52	—	45
$-\Delta\Delta H^c$	—	7	8	4	10	—	—	13	8	—	1
$-\Delta S$ (± 3) (kcal/mol K)	125	142	146	134	156	—	—	160	147	—	128
$-\Delta\Delta S^c$	—	17	21	9	31	—	—	35	22	—	3

Buffer used: 0.2 M NaCl, 0.01 M Na₂HPO₄, 0.1 M EDTA, pH 7.0; concentration of each oligomer: 6.7×10^{-6} M.

^aTo easily recognize the conjugates, number 2* means 3' ACAAACCP-SA (2) and so on.

^bUnmodified duplex.

^cThe difference between ΔT_m (or ΔG , or ΔH , or ΔS) values for modified and unmodified complexes.

same 7-mer/8-mer and relative (7-mer/12-mer) model systems were obtained earlier for phenazinium and ethidium conjugates.^{12,14}

The analysis of the data (Table 1) shows that the variations in the chemical structure of α - and γ -pyrone conjugates have influenced the stability of oligonucleotide complexes. Thus, α -pyrones with a linker group at position 7 exhibit a slightly higher stabilizing effect on the complexes than the respective γ -pyrones containing the same linker groups (compare data for conjugates **5*** and **11***). On the other hand, among γ -pyrones, a slight advantage in the stabilization seems to be obvious when the linker group is attached at position 2 (conjugate **8***) instead of 7 (conjugate **11***). Changes of the substituents at positions 3, 5, and 8 of the chromone residue cause only small variations in the stability of respective oligonucleotide complexes. The most pronounced effect is observed when a partial disruption of the aromatic system of chromone occurred. It results in a complete loss of the stabilization effect (compare T_m and ΔG data for conjugates **8*** or **9*** with **12*** or **13***, respectively).

Recently, a synthesis of the 3'-conjugate of 11-mer oligonucleotide and 4-trifluoromethyl, 7- ω -propylaminocoumarin was reported.²⁷ Melting temperature measurements of its complex with 30-mer oligonucleotide target did not show any increase in the T_m value as compared to unmodified 11-mer/30-mer complex. The reasons for discrepancy between data obtained here and in cited paper is unclear. Generally, in all cases here the effect of stabilization of complexes (as compared to parent 7-mer/8-mer complex) is reflected in the substantial increase of ΔH of the complex formation (Table 1). Comparison of the enthalpy changes for parent 7-mer/8-mer and respective complexes for conjugates **5***, **6***, **9***, and **11*** leads to $\Delta\Delta H$ values ranged from -4 to -13 kcal/mol. The changes of entropy values (ΔS) for complexes studied are negative ($\Delta\Delta S$ values range from -9 to -35 cal/mol K). They 'work' in the opposite direction (as compared to enthalpy term) decreasing the overall stabilizing effect of SA on the complex formation.

These values are comparable with $\Delta\Delta H$ values (-7 to -23 kcal/mol) and $\Delta\Delta S$ values (-17 to -62 cal/mol K) found for different complexes of phenazine oligonucleotide conjugates.¹² In several separate studies^{19,20,28,29} it has been demonstrated that a stacking interaction of aromatic system of phenazine and adjacent G-C base pair in 7-mer/8-mer complex is a main structural event to be responsible for the stabilization effect. (Also, a specific hydration pattern in minor and major grooves of the above duplex was observed).²⁹ One might expect that similar stacking interactions exist for pyranone derivatives. However, a detailed physical chemical study is required to verify this suggestion.

A number of conclusions can be reached regarding the influence of the length of a linker group on the complex

stability. While the length of linker group ($n = 3$ or $n = 5$) has a noticeable effect on the complex stability (see Table 1), no simple correlation (between the linker length and stability of the complex) is observed. On the contrary, with two examples available (**2*** and **3***, **5*** and **6***), a correlation between the length of a linker group and ΔH or ΔS seems obvious. Thus, for complexes with a longer linker group ($n = 5$) the changes of enthalpy (see $\Delta\Delta H$ values in Table 1) are higher than those for complexes with a shorter one ($n = 3$). One may conclude that one position of the SA aromatic system over the base pair is more favorable for stacking interaction in the complexes with longer linker group. The respective changes of entropy (compare ΔS values) for complexes with linkers $n = 3$ and $n = 5$ exhibit the opposite behaviour, this result could be interpreted as a higher cost for conformational restrictions of the SA in complexes with pentamethylene linker chain versus the ones with trimethylene linker chain.

The resulting stabilization effect of the linker length seems to be dependent on the chemical structure of the conjugate. The shorter linker group ($n = 3$) has the advantage for α -pyrones (compare data for conjugates **5*** and **6***), while the longer linker function ($n = 5$) is preferred for γ -pyrones (conjugates **8*** and **9***). However, as has been already demonstrated for other conjugates (phenazine, acridine, and ethidium), the optimal length of the linker may be specific for particular chemical structure of the pyranone and also on the nucleotide sequence of oligonucleotide complex (see review 6).

Conclusion

The experimental data quantitatively support the previous observations¹⁷ that the derivatives of pyranone family are powerful stabilizing agents for oligonucleotide duplexes. These conjugates may also be considered as potential photoreactive groups for modification of the oligo and polynucleotides. While the introduction of different substituents in the α - and γ -pyrone aromatic systems does not significantly alter the hybridization abilities of the oligonucleotide conjugate, it provides a convenient way to control a photosensitivity and photochemical reactivity of the conjugate residue. Future experiments on a sequence specific photomodification in the oligonucleotide duplexes should identify the most perspective photoreactive compounds among the SAs and chromone derivatives with an enhanced stabilization ability.

Experimental

General

Melting points for compounds were determined with a Fisher-Johns apparatus and are uncorrected. The IR spectra were recorded in potassium bromide disks on a Perkin-Elmer 398 IR spectrophotometer. The 1H NMR spectra were obtained on a Hitachi Perkin-Elmer R 600

(60 MHz) and on a Varian Gemini 200 (200 MHz) spectrometers with TMS as the internal standard ($\delta = 0$). NMR signals are quoted as singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), multiplet (m), and broad (br). The purity of the compounds was checked by TLC on silica gel 60-F254 precoated plates and the spots were located under UV light or by iodine vapor or with ninhydrine. Elemental analyses were performed in the Microanalysis Laboratory of the Institute of Pharmaceutical Sciences (Genoa) on a Carlo Erba 1106 Elemental Analyzer. UV absorption spectra of ODNs and ODNsAs were recorded with a Shimadzu UV-2100 UV-vis spectrophotometer.

HPLC

The liquid chromatograph was a Perkin-Elmer Series 4 (Norwalk, CT, U.S.A.) equipped with a Rheodine 7125 (Berkeley, CA, U.S.A.) injector valve with 20 μ L or 1 mL loop (for analytical or preparative purpose, respectively). 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 exchange chromatography was performed on a stainless steel column (250 $\times$ 10 mm) filled by us with Partisil-10 SAX (Whatman, U.S.A.). The reverse-phase column was a stainless steel column (250 $\times$ 10 mm) filled by us with 10 mm LiChrosorb RP18 (Merck, Germany).

Synthesis of stabilizing agents

6-Chloro-2-methoxy-9-(5-aminopentylamino)-acridine hydrochloride. The mixture of 6,9-dichloro-2-methoxyacridine (0.50 g, 1.80 mmol), 1,5-diaminopentane (1.84 g, 18 mmol) and 20 mL of hexamethylphosphoramide or dimethylformamide (DMF) was heated at 100 °C for 2 h. The solution was poured into cold water and the precipitate collected, filtered on a silica gel column (100% ethanol), and the collected fractions were evaporated under reduced pressure. The resulting solid was dissolved in methanol and treated with HCl gas. The yellow precipitate was filtered, crystallized from ethanol to give the pure 3·HCl (yield 0.5 g, 67%), mp 268–70 °C (dec); IR (KBr, cm^{-1}) 3250, 1630, 1610, 1570; ^1H NMR ($\text{DMSO}-d_6$) δ 1.72–1.98 (m, 6H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 2.80 (m, 2H, CH_2), 4.00 (s, 3H, OCH_3), 4.14 (m, 2H, CH_2), 7.44–8.21 (m, 5H, aromatic), 8.48 (d, 1H, aromatic), 10.00 (br s, 1H, NH, deuterium oxide exchangeable), 14.25 (br s, 3H, NH_3 , deuterium oxide exchangeable); Anal. calcd for $\text{C}_{19}\text{H}_{22}\text{N}_3\text{OCl}\cdot 2\text{HCl}$: C, 54.76; H, 5.80; N, 10.08. Found: C, 54.60; H, 5.72; N, 10.25.

2-[(3-Aminopropyl)amino]-8-isopropyl-5-methyl-3,6-dinitro-4H-1-benzopyran-4-one (8). A warm solution of 2-(dimethylamino)-8-isopropyl-5-methyl-3,6-dini-

tro-4H-1-benzopyran-4-one (1.0 g, 2.83 mmol) in toluene (30 mL) was added dropwise to a solution of 1,3-diaminopropane (0.21 g, 2.83 mmol) in toluene (15 mL) heated at 110 °C. This reaction mixture was allowed to stir at the same temperature for 1 h, then evaporated under reduced pressure. The resulting solid was dissolved in chloroform and coevaporated with silica gel (2 g) under reduced pressure. The silica gel was applied to the top of a silica gel column, which was then eluted with ethyl acetate giving 8 as yellow crystals from isopropyl alcohol (yield 0.35 g, 35%), mp 165 °C (dec); IR (KBr, cm^{-1}) 1750 (CO), 1640, 1570–1550 (NO_2) cm^{-1} ; ^1H NMR (CDCl_3) δ 1.40 (d, 6H, CH_3 isopropyl), 2.20 (t, 2H, CH_2), 2.40 (s, 3H, CH_3), 3.30 (m, 1H, CH isopropyl), 3.60 (m, 4H, CH_2), 3.65 (m, 1H, NH), 7.80 (s, 1H, H-7), 11.00 (s, 2H, NH_2 , deuterium oxide exchangeable); Anal. calcd for $\text{C}_{16}\text{H}_{20}\text{N}_4\text{O}_6$: C, 52.74; H, 5.53; N, 15.38. Found: C, 52.50; H, 5.54; N, 15.08.

2-[(3-Aminopropyl)amino]-2-(dimethylamino)-2,3-dihydro-8-isopropyl-5-methyl-3,6-dinitro-4H-1-benzopyran-4-one (12). A solution of 2-(dimethylamino)-8-isopropyl-5-methyl-3,6-dinitro-4H-1-benzopyran-4-one (0.50 g, 1.49 mmol) was allowed to stir at room temperature for 24 h in the presence of 1,3-diaminopropane (0.11 g, 1.49 mmol) in 15 mL of HMPA, then poured into cold water. The solid, precipitated from the water, was crystallized twice from ethanol/water to give 12 as pure yellow crystals (yield 0.28 g, 46%), mp 205–207 °C; IR (KBr, cm^{-1}) 1750 (CO), 1640, 1570–1550 (NO_2); ^1H NMR ($\text{DMSO}-d_6$) δ 1.40 (d, 6H, CH_3 isopropyl), 1.90 (m, 2H, CH_2), 2.30 (s, 3H, CH_3), 2.80 (m, 3H, CH_2 and CH isopropyl), 3.20 (br s, 7H, $(\text{CH}_3)_2\text{NH}$ and H-3), 3.60 (m, 2H, CH_2), 4.00 (m, 1H, NH deuterium oxide exchangeable), 7.80 (s, 1H, H-7), 8.20 (s, 2H, NH_2 , deuterium oxide exchangeable); Anal. calcd for $\text{C}_{18}\text{H}_{27}\text{N}_5\text{O}_6\cdot 2\text{H}_2\text{O}$: C, 48.53; H, 7.01; N, 15.72. Found: C, 48.83; H, 7.06; N, 15.85.

2-[(5-Aminopentyl)amino]-2-(dimethylamino)-2,3-dihydro-8-isopropyl-5-methyl-3,6-dinitro-4H-1-benzopyran-4-one (13). A warm solution of 1,5-diaminopentane (2.89 g, 28.3 mmol) was added dropwise to a warm solution of 2-(dimethylamino)-8-isopropyl-5-methyl-3,6-dinitro-4H-1-benzopyran-4-one (1.0 g, 2.83 mmol) in toluene (30 mL) and heated at 110 °C for 1 h. After cooling to room temperature, the solvent was evaporated under reduced pressure. The resulting solid was dissolved in chloroform and coevaporated with silica gel (2 g) under reduced pressure. The silica gel was applied to the top of a silica gel column, which was then eluted with ethanol containing traces of triethylamine, giving 13 as yellow crystals (yield 0.8 g, 57%), mp 178 °C (dec) from acetonitrile and then ethanol/acetone; IR (KBr, cm^{-1}) 3300, 2900, 1640, 1550–1570 (NO_2); ^1H NMR ($\text{DMSO}-d_6$) δ 1.40 (d, 6H, CH_3 isopropyl), 1.50 (m, 4H, CH_2CH_2), 1.7 (m, 2H, CH_2), 2.40 (s, 3H, CH_3), 2.60 (br s, 7H, $(\text{CH}_3)_2\text{NH}$ and H-3), 3.40 (m, 1H, CH isopropyl), 3.60 (m, 4H, NHCH_2 and CH_2NH_2), 6.40

(s, 2H, NH₂ deuterium oxide exchangeable), 8.05 (s, 1H, aromatic), 10.10 (s, 1H, NH deuterium oxide exchangeable); Anal. calcd for C₂₀H₃₁N₅O₆·2H₂O: C, 50.73; H, 7.45; N, 14.79. Found: C, 50.80; H, 7.09; N, 14.79.

2-[(N₅-tritylamino)pentyl]amino]-8-isopropyl-5-methyl-3,6-dinitro-4H-1-benzopyran-4-one (14). The mixture of **1** (1.0 g, 2.98 mmol), *N*-trityl-1,5-diaminopentane (1.50 g, 4.35 mmol) and 20 mL of acetonitrile was refluxed for 1 h. After cooling, removal of the solvent afforded a yellow solid. After recrystallization from ethanol, a pure **14** was obtained (yield 1.2 g, 63%), mp 202–203 °C; IR (KBr, cm⁻¹) 3300, 2900, 1640, 1600, 1200; ¹H NMR (CDCl₃) δ 1.20 (d, 6H, CH₃ isopropyl) 1.4 (m, 6H, CH₂), 2.2 (t, 2H, trityl-NHCH₂), 2.7 (t, 2H, CH₂NH), 2.85 (s, 3H, CH₃), 4.50 (m, 1H, CH isopropyl), 4.90 (s, 1H, NH) 7.18 (m, 3H, 4-trityl), 7.27 (m, 6H, 3,5-trityl), 7.48 (m, 6H, 2,6-trityl), 7.90 (s, 1H, H-7), 10.20 (s, 1H, NH); Anal. calcd for C₃₇H₃₈N₄O₆: C, 70.02; H, 6.03; N, 8.83. Found: C, 69.90; H, 6.03; N 8.82.

2-[(5-Aminopentyl)amino]-8-isopropyl-5-methyl-3,6-dinitro-4H-1-benzopyran-4-one acetate. Compound **14** (1.20 g, 1.90 mmol) was added to a solution of H₂O:CH₃COOH (1:3), stirred for 2 h at 60 °C. Removal of the solvent under reduced pressure afforded a white solid, which was stirred with ether for 6 h and filtered to give the pure **9-CH₃COOH** (yield 0.50 g, 58%), mp 210–12 °C; IR (KBr, cm⁻¹) 3300, 2900, 1640, 1600, 1200; ¹H NMR (DMSO-*d*₆) δ 1.20 (d, 6H, CH₃ isopropyl) 1.4 (m, 6H, CH₂), 2.2 (t, 2H, NHCH₂), 2.4 (s, 3H, CH₃ acetate), 2.7 (t, 2H, CH₂NH₃⁺), 2.85 (s, 3H, CH₃), 4.50 (m, 1H, CH isopropyl), 4.90 (s, 1H, NH), 5.70 (m, 3H, NH₃⁺) 8.10 (s, 1H, H-7); Anal. calcd for C₂₀H₂₈N₄O₈: C, 53.09; H, 6.24; N, 12.38. Found: C, 53.40; H, 6.03; N, 12.82.

7-[(3-(*N*-tritylamino)propoxy)-2H-1-benzopyran-2-one (15). The mixture of 7-hydroxycoumarin (0.50 g, 3.08 mmol), NaOH (0.13 g, 3.08 mmol) and 3-bromo-*N*-(trityl)propanamine (2.30 g, 6 mmol) in 40 mL of freshly distilled DMF was stirred at room temperature for 5h. The solution was poured into water, adjusted to pH 6 with 1 M HCl, then extracted with dichloromethane. The organic layer was washed several times with water, dried over sodium sulfate, and evaporated to dryness. Product **15** was purified on silica gel with a toluene:ethyl acetate (60:40 v/v) eluent as a white solid (yield 1.3, g, 91%), mp 180–181 °C; IR (KBr, cm⁻¹) 3300, 1710, 1610; ¹H NMR (CDCl₃) δ 1.80 (s, 1H, NH), 2.32 (m, 4H, CH₂CH₂), 4.2 (t, 2H, OCH₂), 6.4 (d, 1H, H-3), 7.01 (m, 2H, H-6,8), 7.35 (m, 15H, trityl), 7.70 (d, 1H, H-5), 8.20 (d, 1H, H-4); Anal. calcd for C₃₁H₂₇NO₃: C, 80.49; H, 6.10; N 3.01. Found: C, 80.39; H, 5.90; N, 3.00.

7-(3-Aminopropoxy)-2H-1-benzopyran-2-one acetate. To a solution of H₂O:CH₃COOH (1:3) was added **15** (1.0 g, 2.1 mmol), stirring 2 h at 60 °C. Removal of the solvent under reduced pressure afforded a white solid

stirred with ether for 6 h and filtered to give **5-CH₃COOH** (yield 0.35 g, 58%), mp 159–160 °C; IR (KBr, cm⁻¹) 3500, 3000, 1710, 1630; ¹H NMR (CF₃COOD) δ 2.38 (s, 3H, CH₃), 3.6 (m, 4H, CH₂CH₂), 4.42 (t, 2H, OCH₂), 6.3 (d, 1H, H-3), 7.32 (s, 2H, H-6,8), 7.7 (d, 1H, H-5), 8.2 (d, 1H, H-4); Anal. calcd for C₁₄H₁₇NO₅: C, 59.29; H, 6.25; N, 4.85. Found: C, 59.50; H, 6.16; N, 4.93.

5-[(2-Oxo-2H-1-benzopyran-7-yl)oxy]pentanonitrile (16). The mixture of 7-hydroxycoumarin (0.81 g, 5 mmol), 5-bromovaleronitrile (0.90 g, 5.5 mmol) and anhydrous potassium carbonate (0.90 g, 5.5 mmol) in 40 mL of freshly distilled DMF was heated at 90 °C for 2 h. After cooling, the solution was poured into cold water and the resulting white solid filtered. After recrystallization from ethanol (yield 1.0 g, 82%), mp 93–95 °C; IR (KBr, cm⁻¹) 2210, 1720, 1630; ¹H NMR (CDCl₃) δ 2.1 (m, 4H, CH₂CH₂), 2.48 (t, 2H, CH₂CN), 4.1 (t, 2H, OCH₂), 6.4 (d, 1H, H-3), 6.8 (m, 2H, H-6,8), 7.4 (m, 2H, H-4,5); Anal. calcd for C₁₄H₁₃NO₃: C, 69.11; H, 5.39; N, 5.76. Found: C, 68.95; H, 5.38; N, 5.72.

7-(5-Aminopentoxo)-2H-1-benzopyran-2-one hydrochloride. A solution of **16** (0.50 g, 2 mmol) in 50 mL of ethanol was stirred in a hydrogen atmosphere in the presence of Pd/C 10% (100 mg) for 8 h at room temperature in a Parr apparatus. The catalyst was removed by filtration and the solution concentrated to give a residue which was crystallized from ethanol to give **6-HCl** as white crystals, (yield 0.3 g, 58%), mp 159–160 °C; IR (KBr, cm⁻¹) 3400, 3000, 1720, 1610; ¹H NMR (CDCl₃) δ 2.1 (m, 4H, CH₂CH₂), 2.8 (t, 2H, CH₂), 3.90 (t, 2H, OCH₂), 4.3 (m, 2H, CH₂NH₃⁺), 6.4 (d, 1H, H-3), 6.8 (m, 2H, H-6,8), 7.4 (m, 2H, H-4,5), 7.90 (m, 3H, NH₃⁺); Anal. calcd for C₁₄H₁₈NO₃Cl·2H₂O: C, 52.58; H, 6.93; N, 4.38. Found: C, 52.33; H, 6.85; N, 4.35.

7-(3-Bromopropoxy)-2-diethylamino-4H-benzo[*b*]-pyran-4-one (17). The mixture of 2-diethylamino-7-hydroxy-4H-benzo[*b*]pyran-4-one (1.0 g, 4.7 mmol), 1,3-dibromopropane (1.10 g, 15 mmol), and anhydrous potassium carbonate (1 g) in 20 mL of anhydrous acetone was refluxed for 24 h. After cooling, removal of the solvent afforded an oil that was stirred together with water and extracted with chloroform. The organic extracts were dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The residue was purified on silica gel and eluted first with petroleum ether 40–60 °C and second with petroleum ether:ethyl acetate (80:20 v/v). The second eluted gave **17** as pure white crystals, (yield 0.8 g, 48%). After recrystallization from ethyl acetate, mp 130–131 °C; IR (KBr, cm⁻¹) 3060, 2970, 2930, 1630; ¹H NMR (CDCl₃) δ 1.22 (t, 6H, CH₃), 2.32 (q, 2H, OCH₂CH₂CH₂Br), 3.45 (q, 4H, CH₂), 3.62 (t, 2H, CH₂Br), 4.18 (t, 2H, OCH₂), 5.38 (s, 1H, H-3), 6.81 (s, 1H, H-8), 6.90 (d, 1H, H-6), 8.10 (d, 1H, H-5); Anal. calcd for C₁₆H₂₀NO₃Br: C, 54.25; H, 5.69; N, 3.95. Found: C, 54.01; H, 5.88; N, 3.80.

7-(3-Aminopropoxy)-2-diethylamino-4H-benzo[b]pyran-4-one (11). The solution of 3-bromopropyl derivative **17** (1.0 g, 3.65 mmol) in 10 mL of methanol was allowed to stir at 40 °C for 48 h 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 hydrochloride of **11**, which was filtered and washed with ethyl ether. The residue was soluted in water and precipitated adding a saturated solution of NaHCO₃ to give **11** as a white solid (yield 0.4 g, 35%), mp 118–120 °C; IR (KBr, cm⁻¹) 3400, 3060, 2970, 2930, 1630; ¹H NMR (DMSO-*d*₆), δ 1.20 (t, 6H, CH₃), 2.05 (q, 2H, OCH₂CH₂CH₂NH₂), 3.00 (t, 2H, CH₂NH₂), 3.50 (q, 4H, CH₂), 4.20 (t, 2H, OCH₂), 4.70 (or s, 2H, NH₂), 5.25 (s, 1H, H-3), 7.0 (d, 1H, H-8), 7.05 (d, 1H, H-6), 7.90 (d, 1H, H-5); Anal. calcd for C₁₆H₂₂N₂O₃: C, 66.18; H, 7.64; N, 9.65. Found: C, 66.01; H, 7.54; N, 9.49.

Synthesis of oligonucleotides

The oligonucleotides were synthesized in solution, deprotected, and purified following our previous routes.^{23,24} Each oligonucleotide in this preparation showed a single peak either with an anion exchange column or with a reversed phase column (see HPLC).

Synthesis of conjugates of oligonucleotides with stabilizing agents

General procedure.¹⁷ Three μ L of a 8% water solution of cetyltrimethylammonium bromide was added to a solution of the lithium salt of the completely deblocked ODN (5AU at 260 nm) dissolved in 50 μ L of water and this mixture was then centrifuged. An amount of 1 μ L 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 over P₂O₅. A solution of this compound in 60 μ L of dry DMSO, 0.010 g of triphenylphosphine, 0.010 g of dipyridyldisulfide, 0.005 g of *N,N*-dimethylaminopyridine was stirred for 10 min and then 0.002 g of the aminoderivative (**2**, **3**, **5**, **6**, **7**, **8**, **9**, **11**, **12**, or **13**) and 2 μ L of anhydrous triethylamine were added. After stirring for 1 h at room temperature, the solution was precipitated with 1 mL of 2% LiClO₄ in acetone. After centrifugation, the supernatant was eliminated and the precipitate dissolved in 50 μ L of 3 M LiClO₄ and treated with 1 mL of 2% LiClO₄ 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 in water and a precisely measured aliquot is taken off to measure the absorbance at 260 nm.²⁴ Then the solution was precipitated with 2% LiClO₄ in acetone to give an ODN conjugate (**2***, **3***, **5***, **6***, **7***, **8***, **9***, **11***, **12***, or **13***).

Thermal denaturation of complexes

Preparation of samples. Aqueous solutions of appropriate concentrations of ODNs were prepared by diluting a concentrated solution of the ODN or the ODN-SA according to molar extinction coefficients at 260 nm at 20 °C. Extinction coefficients were calculated according to results of total PDE-hydrolysis³⁰ and were as follows (260 nm): 8-mer 70200; 7-mer 66800. The extinction coefficients for the ODN-SA were estimated as a sum of respective values for each oligomer and stabilizing agent. Aqueous solutions of ODN or ODN-SA were mixed with concentrated buffer solutions. In all cases a final composition of buffer solution was: 0.16 M NaCl, 0.01 M Na₂HPO₄, 0.1 mM EDTA, pH 7.0. Concentration of each oligonucleotide chain was 1.6×10^{-5} M.

Melting curves. Optical melting curves were obtained with the use of a home-made apparatus developed on the base of UV detector of liquid chromatograph Milichrom (Orel, Russia) connected to PC computer. Volume of the optical cell was 2 μ L, 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 s. 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.

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