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Synthesis and hybridization properties of the conjugates of oligonucleotides and stabilization agents. Part 3☆

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Abstract—New compounds having tri- or pentamethylenamine linker functions were synthesized. These derivatives were covalently attached through the 5′-phosphoramide 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 confirmed the ability of chromone (γ-pyrone) derivatives to stabilize strongly the 7-mer/8-mer complementary complex. Moreover, benzochromone (naphthopyrane) and, surprisingly, tetrahydropyrimidinethanone derivatives showed the capacity of stabilizing this 7-mer/8-mer complementary complex. The effect of all these compounds on the stability of the oligonucleotide complexes ($\Delta\Delta G$ at 37 °C ranged from −1.2 to −2.0 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, which served as a reference in this study.

1. Introduction

Since antisense oligodeoxynucleotide strategy was proposed for therapeutic use in 1978, major advances have been made in developing modified oligonucleotides (ODNs) whose potential use as therapeutic agents has been claimed in all kinds of infectious diseases, genetic disorders and cancer.[1–7] Whether they are used in the so-called 'antisense', 'antigene' or 'anticode' strategies, the hybridization properties of the ODNs with the complementary sequences of the target nucleic acids are of fundamental importance. Moreover, an increase of hydrophobicity and an efficient synthesis could be a valid strategy to increase cellular uptake, both favouring therapeutic use and lowering the end price of the potential drug.

One of the perspective approaches to improve the hybridization ability (as well as nuclease resistance) of the oligonucleotides is a simple covalent attachment of 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.

We previously demonstrated that several compounds from the pyranone family are considered to be promising SA.[8,9] In particular, compounds **4a**,**b** showed major stabilization abilities compared to other chromones and coumarins tested. They were comparable to the stabilizing effects of the well known acridines. Moreover, some showed interesting capabilities in inhibiting HIV-1 reverse transcriptase.[10]

In order to form conjugates with both enhanced stabilization ability and biological significance, we planned to synthesize new pyran derivatives having nitro, carbaldehyde and phenyl substituents. In particular, the benzo moiety could be a substituent in the chromone ring or fused to form a naphthopyrane ring.

Keywords: Oligonucleotides; Stabilization agents; Pyranone derivatives.
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† In memory of Enzo Sottofattori, who inspired this work.

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Benzo moiety was chosen to improve lipophilicity, while the nitro substituent was considered as a potential photoreactive group and the carbaldehyde moiety was introduced for its potential capability of inhibiting HIV-RT, as shown in previous works.[10,11]

This paper describes the synthesis of different (benzo)chromones (**4**–**7**, **11**), together with the new tetrahydropyrimidinethanones (**12**, **13**), the formation of the complexes with complementary octanucleotide and the evaluation of thermodynamic parameters from the analysis of the melting temperature experiments.

2. Chemistry

2.1. Synthesis of stabilizing agents

The synthesis of the intermediate and target compounds was performed by the reaction pattern shown in Schemes 1 and 2.

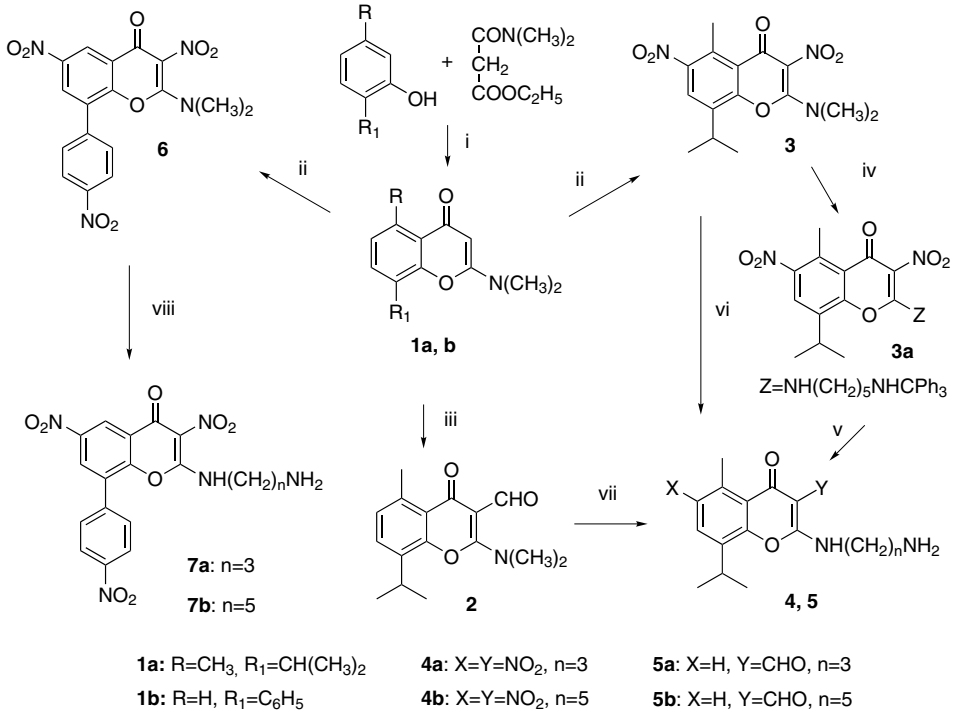
The starting molecules **1a**,**b** and **8** were obtained by following our well-established method from substituted phenols or 2-naphthol and *N*,*N*′-(dimethyl)malonamide in the presence of phosphorus oxychloride.[12,13] The reaction of **1a**,**b** with mixed nitric and sulfuric acid gave compounds **3** and **6** in excellent yields, which were, in turn, treated with propane(pentane)-1,3-(1,5)-diamine. The course of the amine exchange reaction from dimethylamino to α,ω-diaminoalkyl groups depends on the number of methylenes (3 or 5) and the substrate. Compounds **3** and **6** react smoothly with 1,3-diaminopropane to afford **4a** and **7a** in good yield and without side products. However, this procedure failed to synthesize **4b** and **7b**. A winning alternative strategy was that of using the protected reagent *N*-(triphenylmethyl)pentane-1,5-diamine. The amine exchange reaction proceeded efficiently yielding the protected derivatives, which gave the desired **4b** and **7b** by detritylation with acetic acid (**4b**) or by spontaneous loss of the trityl group during crystallization (**7b**).

Vilsmeier formylation of **1a** gave the 2-formylchromone **2**, which furnished **5a** and **5b** in accordance with the amine exchange method previously described.[14] The reaction is very sensitive to temperature, molar rate and solvents. Changing these parameters dramatically modifies the course of the reaction.

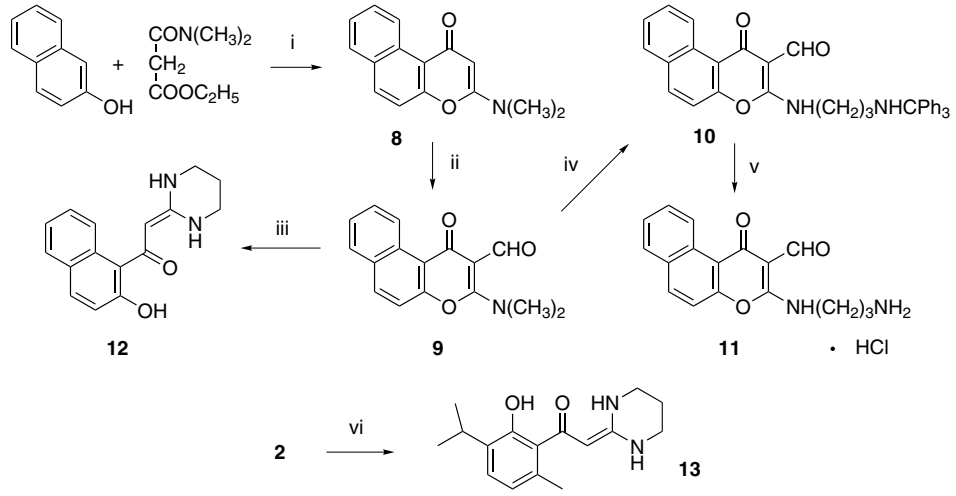
The reaction pathways depicted in Scheme 2 account for the formation of the formylbenzochromone derivative **11** and may give an explanation of the critical reaction. In fact, this compound was only obtainable via the protected derivative **10**, which was, in turn, easily obtained from **9** by amine exchange with *N*-(triphenylmethyl)propane-1,3-diamine at room temperature. Any other attempt to directly obtain **11** failed. The tetrahydropyrimidine derivative **12** was the main product, the yield of which depending only on temperature and molar rate. Following the experimental conditions that gave **12** in its highest yield, we were also able to obtain the tetrahydropyrimidine derivative **13** from chromone **2**.

2.2. Synthesis of oligonucleotides conjugates

All conjugars were attached to the 5′-end of the 7-mer oligonucleotide according to previously described



Scheme 1. Reagents and conditions: (i) $POCl_3$; (ii) HNO_3, H_2SO_4; (iii) *N*,*N*-dimethylformamide, $POCl_3$; (iv) *N*-(triphenylmethyl)pentane-1,5-diamine; (v) CH_3COOH–H_2O (3:1); (vi) propane-1,3-diamine; (vii) propane-1,3-diamine or pentane-1,5-diamine; (viii) propane-1,3-diamine or *N*-(triphenylmethyl)pentane-1,5-diamine.



Scheme 2. Reagents and conditions: (i) $POCl_3$; (ii) *N*,*N*-dimethylformamide, $POCl_3$; (iii) propane-1,3-diamine; (iv) *N*-(triphenylmethyl)propane-1,3-diamine; (v) HCl; (vi) propane-1,3-diamine.

methods[15,16] with some modifications (see Experimental). The major modification included the use of *N*,*N*-dimethylaminopyridine (DMAP) as a catalyst instead of *N*-methylimidazole, which is recommended in the 'classical approach'.[17] 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 yields seem to be sensitive to the position of the linker group in the (benzo)chromone moiety.

All oligonucleotide conjugates were isolated and purified by reverse phase HPLC, the homogeneity of the chromatographic fractions was monitored by the diode array detector.

3. Results and discussion

All experiments were performed on the model 7-mer/8-mer duplex, successfully tested in several studies[18–21] for its thermodynamic response.

The 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.[22]

The results show that all (benzo)chromone conjugates 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 −1.2 to −2.0 kcal/mol, which is comparable to an effect of one A–T base pair and is almost superimposable to that observed for acridine conjugates.[9] Similar results on the same 7-mer/8-mer and relative (7-mer/12-mer) model systems were obtained earlier for phenazinium and ethidium conjugates.[19,23]

The $\Delta\Delta G$ values found in this study differ significantly from an estimated value (~6 kcal/mol at 37 °C) reported in a past paper[8] for 10-mer/20-mer and 14-mer/20-mer oligonucleotide complexes, while they are comparable to the values reported in our previous work.[9] This inconsistency may be attributed to the presence of an internal hairpin formed by the 20-mer oligonucleotide used in the cited study.[8] In fact, the value of the stabilization effect of the conjugar is dependent on the specific ternary structure of the duplex (e.g., formation of internal hairpin

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

ODN-SA	UM[b]	**4a***	**4b***	**7a***	**7b***	**5a***	**5b***	**11***	**12***	**13***
Linker, methylene groups (*n*)	—	3	5	3	5	3	5	3	—	—
T_m (±0.2) (°C)	23.0	32.1	34.9	32.0	34.2	35.8	36.1	32.4	32.0	33.2
ΔT_m[c]	—	9.1	11.9	9.0	11.2	12.8	13.1	9.4	9.0	9.3
$-\Delta G$ (±0.05); 37 °C (kcal/mol)	5.1	6.4	6.8	6.3	6.7	6.9	7.1	6.5	6.3	6.7
$-\Delta\Delta G$[c]	—	1.3	1.7	1.2	1.6	1.8	2.0	1.4	1.2	1.6
$-\Delta H$ (±1) (kcal/mol)	44	51	58	51	57	51	56	48	51	51
$-\Delta\Delta H$[c]	—	7	14	7	13	7	12	4	7	7
$-\Delta S$ (±3) (kcal/mol K)	125	143	160	142	158	162	164	155	143	140
$-\Delta\Delta S$[c]	—	18	35	17	33	37	39	30	18	15

Buffer used: 0.16 M NaCl, 0.01 M Na_2HPO_4, 0.1 mM EDTA, pH 7.0; concentration of each oligomer: 1.6×10^{-5} M.

[a] To easy recognize the conjugates, number **4a*** means 3′ACAAACCp-SA (**4a**) and so on.

[b] Unmodified duplex.

[c] The difference between ΔT_m (or ΔG, or ΔH, or ΔS) values for modified and unmodified complexes.

structures and intercomplex associates). Moreover, the value of the effect may also depend on the nucleotide sequence of the duplex in the area of SA location.

The analysis of the data (Table 1) shows that changes of the substituents at positions **3**, **5**, **6**, **8** of chromone residue cause only small variations in the stability of respective oligonucleotide complexes. Furthermore, variations in the chemical structure from benzo to naphtho pyrone conjugars do not influence the stability of oligonucleotide complexes, which remain positive for all derivatives.

In general, in all cases, the effect of the 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 the parent 7-mer/8-mer and the respective complexes for conjugars **4a***, **4b***, **7a***, **7b***, **5b***, **11*** and **13*** leads to $\Delta\Delta H$ values, which ranged from −4 to −14 kcal/mol. These values are comparable to $\Delta\Delta H$ values (−9 to −23 kcal/mol) found in the complexes of different phenazine conjugates of the 7-mer with complementary 12-mer oligonucleotide.[23] Such similarities of the $\Delta\Delta H$ values is a good indication of the common mechanism of stabilization for both types of complexes (with phenazine and benzo(naphtho)chromone conjugates).

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 the 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. In general, for complexes with a longer linker group ($n = 5$) the changes of enthalpy (see $\Delta\Delta H$ and $\Delta\Delta G$ values in Table 1) are higher than those for complexes with a shorter one ($n = 3$). It may be concluded that one position of the conjugar 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, $\Delta\Delta S$ values) for complexes with linkers $n = 3$ and 5 exhibit the opposite behaviour, this result could be interpreted as a higher cost for conformational restrictions of the conjugar 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 conjugar. All γ-pyrones tested showed higher stability with the longer linker function ($n = 5$) than the shorter linker group ($n = 3$). On the other hand, the shorter linker group ($n = 3$) is advantageous for α-pyrones (coumarins).[8] However, as has already been demonstrated for other conjugars (phenazine, acridine, ethidium), the optimal length of the linker may be specific for the particular chemical structure of the pyranone and also on the nucleotide sequence of oligonucleotide complex (see review).[6]

Finally, it is worth noting that the tetrahydropyrimidinethanone derivatives **11** and **13**, in which a disruption of the aromatic system of (benzo)chromone occurred, also maintain a good stabilization effect. These two compounds deserve further experimental investigations to better understand their role as stabilization agents.

4. Conclusions

The experimental data quantitatively support the previous observations[8,9] that the conjugars of the pyranone family are powerful stabilizing agents for oligonucleotide duplexes. These conjugars may also be considered as potential photoreactive groups for modification of the oligo and polynucleotides. While the introduction of different substituents in a γ-pyrone aromatic system 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 conjugar residue. For this purpose, **7a**,**b** seem to be the most perspective photoreactive compounds to use in future experiments on a sequence specific photomodification in the oligonucleotide duplexes. On the other hand, compound **11**, due to its similarity to **5a**, is selected for its potential biological activity on interacting with HIV1-RT. Finally, the tetrahydropyrimidinethanone derivatives **12**–**13**, which have unexpectedly shown good stabilization capacity of the conjugates, could pave the way to the synthesis of new SAs with potential biological activities.

5. Experimental

5.1. General

Melting points of synthesized 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 ^{1}H 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), 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 vapour or with ninidrine. Elemental analyses were performed in the Microanalysis Laboratory of the Department of Pharmaceutical Sciences (Genoa) on a Carlo Erba 1106 Elemental Analyzer. UV absortion spectra of ODNs and ODN-SAs were recorded with a Shimadzu UV-2100 UV–vis spectrophotometer.

5.2. 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 exchange chromatography was performed on a stainless steel column (250 × 10 mm) filled by us with Partisil-10 SAX (Whatman, USA). The reverse-phase column was a stainless steel column (250 × 10 mm) filled by us with 10 mm LiChrosorb RP18 (Merck, Germany).

5.3. Synthesis of key intermediates

5.3.1. Compounds 1a,b and 8. Phosphorous oxychloride (0.15 mol) was added dropwise by stirring to 0.11 mol of *N*,*N*′-(dimethyl)malonamide which was contained in an ice bath-cooled flask protected from moisture with a calcium chloride drying tube. After the addition, the ice-bath was removed and the mixture was kept at room temperature for 30 min. A solution (a suspension for 2-naphthol) of a suitable phenol (0.10 mol) in 60 mL of chlorobenzene was added to the yellow solution, and the resulting mixture was heated at a temperature and for the time indicated for each compound. After cooling, a solution of 136 g of trihydrate sodium acetate in 350 mL of water was added and the mixture was heated by stirring at 80 °C for 90 min. The layers were separated and the aqueous phase was extracted twice with chloroform. The combined organic portions were washed with brine and evaporated under reduced pressure to yield a dark red oil. The oil was stirred at room temperature with 250 mL of 2 N NaOH and 80 mL of petroleum ether. The obtained solid was filtered, washed with water and crystallized, giving the following compounds.

5.3.1.1. 2-(Dimethylamino)-8-isopropyl-5-methylchromone (1a). From thymol at 110 °C for 5 h (yield 65%); mp 151–2 °C (ligroin).[12]

5.3.1.2. 2-(Dimethylamino)-8-phenylchromone (1b). From biphenyl-2-ol at 100 °C for 4 h (yield 60%); mp 175–6 °C (ligroin).[13]

5.3.1.3. 3-(Dimethylamino)-1*H*-naphtho[2,1-*b*]pyran-4(4*H*)-one (8). From 2-naphthol at 85 °C for 5 h (yield 71%); mp 190–1 °C (ethanol).[24]

5.3.2. Compounds 3 and 6. A solution of nitric acid ($d = 1.52$) in 5 mL of sulfuric acid was slowly added to a solution of 8.0 mmol of the suitable chromone in 15 mL of sulfuric acid at 0 °C by stirring. The resulting solution was kept at room temperature for 30 min then poured onto crushed ice. The resulting yellow solid was filtered, washed with water and crystallized giving the following compounds.

5.3.2.1. 2-(Dimethylamino)-8-isopropyl-5-methyl-3,6-dinitrochromone (3). From 24.0 mmol of nitric acid (yield 78%); mp 199–200 °C (ethyl acetate).[25]

5.3.2.2. 2-(Dimethylamino)-3,6-dinitro-8-(4′-nitrophenyl)chromone (6). From 36.0 mmol of nitric acid (yield 58%); mp 188–190 °C (toluene); IR *ν* 1710 (CO), 1640, 1600, 1580 (NO_2) cm^{-1}; ^1H NMR (DMSO-d_6): δ 3.03 (s, 6H, CH_3), 8.20 (s, 1H, arom H), 8.41 (s, 1H, arom H), 8.65 (d, 2H, arom H), 8.79 (d, 2H, arom H). Anal. Calcd for $C_{17}H_{12}N_4O_8$: C, 51.01; H, 3.02; N, 14.00. Found: C, 51.20; H, 3.00; N, 13.88.

5.3.3. Compounds 2 and 9. In a flask, cooled in an ice bath and protected from moisture by a calcium chloride tube, $POCl_3$ (0.92 g, 6.0 mmol) was added dropwise to 2 mL of *N*,*N*-(dimethyl)formamide. The resulting solution was stirred for 30 min at room temperature. A suspension of 4 mmol of a suitable pyranone (**1a**, **8**) in DMF (10 mL) was then added and heated at the temperature and time indicated for each compound. The mixture was then cooled and poured onto crushed ice. The solution was alkalinized with sodium carbonate giving a colourless solid which was collected, washed with H_2O, dried and crystallized with an appropriate solvent giving the following compounds.

5.3.3.1. 2-(Dimethylamino)-8-isopropyl-5-methyl-4-oxo-4*H*-1-benzopyran-3-carbaldehyde (2). From 2-(dimethylamino)-8-isopropyl-5-methyl-4*H*-1-benzopyran-4-one at 120 °C for 30 min (yield 85%); mp 134–5 °C (cyclohexane).[26]

5.3.3.2. 3-(Dimethylamino)-1-oxo-1*H*-naphtho[2,1-*b*]-pyran-2-carbaldehyde (9). From 3-(dimethylamino)-naphtho[2,1-*b*]pyran-1(1*H*)one at 95 °C for 90 min (yield 84%); mp 218–9 °C (EtOH).[27]

5.3.4. 2-{5-[(Triphenylmethyl)amino]pentylamino}-8-isopropyl-5-methyl-3,6-dinitro-4*H*-1-benzopyran-4-one (3a). The mixture of **3** (1.0 g, 2.98 mmol), *N*-(triphenylmethyl)pentane-1,5-diamino (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 **3a** was obtained (yield 1.2 g, 64%); mp 202–203 °C; IR *ν* 3300, 2900, 1640, 1600, 1200 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.20 (d, 6H, CH_3 isopropyl), 1.4 (m, 6H, CH_2), 2.2 (t, 2H, trityl-$NHCH_2$), 2.7 (t, 2H, CH_2NH), 2.85 (s, 3H, CH_3), 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 $C_{37}H_{38}N_4O_6$: C, 70.01; H, 6.03; N, 8.83. Found: C, 69.97; H, 5.99; N, 8.71.

5.4. Synthesis of stabilizing agents

5.4.1. 2-[(3-Aminopropyl)amino]-8-isopropyl-5-methyl-3,6-dinitro-4*H*-1-benzopyran-4-one (4a). A warm solution of 2-(dimethylamino)-8-isopropyl-5-methyl-3,6-dinitro-4*H*-1-benzopyran-4-one **3** (1.0 g, 2.98 mmol) in toluene (30 mL) was added dropwise to a solution of propane-1,3-diamine (0.22 g, 2.98 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 co-evaporated 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 **4a** as yellow

crystals from isopropylic alcohol (yield 0.36 g, 33%); mp 165 °C (dec); IR ν 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 $C_{16}H_{20}N_4O_6$: C, 52.74; H, 5.53; N, 15.38. Found: C, 52.54; H, 5.54; N, 15.08.

5.4.2. 2-[(5-Aminopentyl)amino]-8-isopropyl-5-methyl-3,6-dinitro-4*H*-1-benzopyran-4-one acetate (4b). Compound **3a** (1.20 g, 1.90 mmol) was added to a solution of H_2O/CH_3COOH (1:3), stirring 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 **4b**·CH_3COOH (yield 0.50 g, 58%); mp 210–12 °C; IR ν 3300, 2900, 1640, 1600, 1200 cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.20 (d, 6H, CH_3 isopropyl), 1.4 (m, 6H, CH_2), 2.2 (t, 2H, $NHCH_2$), 2.4 (s, 3H, CH_3 acetate), 2.7 (t, 2H, $CH_2NH_3^+$), 2.85 (s, 3H, CH_3), 4.50 (m, 1H, CH isopropyl), 4.90 (s, 1H, NH), 5.70 (m, 3H, NH_3^+), 8.10 (s, 1H, H-7). Anal. Calcd for $C_{20}H_{28}N_4O_8$: C, 53.09; H, 6.24; N, 12.38. Found: C, 52.81; H, 6.19; N, 12.21.

5.4.3. 2-[(3-Aminopropyl)amino]-8-isopropyl-5-methyl-4-oxo-4*H*-1-benzopyran-3-carbaldehyde (5a). A suspension of **2** (0.8 g, 2.90 mmol) and propane-1,3-diamine (0.21 g, 2.90 mmol) in toluene (8 mL) was kept at room temperature for 2 days, while stirring. The solvent was evaporated and the residue purified on silica gel and eluted first with cyclohexane–ethyl acetate (1:1 v/v) and second with cyclohexane–ethyl acetate (1:4 v/v). The second eluted gave 0.31 g (yield 35%) of **5a** as pale yellow crystals. Mp 110–112 °C (cyclohexane–ethyl acetate, 1:2). IR ν 3400, 2900, 1660, 1560, 1200 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.34 (d, 6H, CH_3 isopropyl), 2.30 (m, 2H, CH_2), 2.82 (s, 3H, CH_3), 3.70 (m, 5H, CH_2 and CH isopropyl), 7.20 (dd, 2H, arom H), 10.20 (s, 1H, CHO), 10.60 (s, 1H, NH, deuterium oxide exchangeable), 11.20 (s, 2H, NH_2, deuterium oxide exchangeable). Anal. Calcd for $C_{17}H_{22}N_2O_3$: C, 67.53; H, 7.33; N, 9.26. Found: C, 67.90; H, 7.35; N, 9.20.

5.4.4. 2-[(5-Aminopentyl)amino)]-8-isopropyl-5-methyl-4-oxo-4*H*-1-benzopyran-3-carbaldehyde (5b). Analogously to the preparation of **5a**, 0.8 g (2.90 mmol) of **2** and 0.30 g (2.90 mmol) of pentane-1,5-diamine gave, after purification on silica gel, **5b** as light brown crystals (yield 0.24 g, 25%). Mp 120–2 °C (cyclohexane–ethyl acetate, 1:1). IR ν 3400, 2900, 1660, 1560, 1200 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.30 (d, 6H, CH_3 isopropyl), 2.20 (m, 2H, CH_2), 2.80 (s, 3H, CH_3), 3.68 (m, 5H, CH_2 and CH isopropyl), 7.25 (dd, 2H, arom H), 10.20 (s, 1H, CHO), 10.58 (s, 1H, NH, deuterium oxide exchangeable), 11.15 (s, 2H, NH_2, deuterium oxide exchangeable). Anal. Calcd for $C_{19}H_{26}N_2O_3$: C, 69.06; H, 7.93; N, 8.48. Found: C, 69.46; H, 7.80; N, 8.31.

5.4.5. 2-[(3-Aminopropyl)amino]-3,6-dinitro-8-(4′-nitrophenyl)-4*H*-1-benzopyran-4-one (7a). A warm solution of propane-1,3-diamine (0.14 g, 1.85 mmol) in acetonitrile was added dropwise to a warm solution of **6** (0.6 g, 1.5 mmol) in acetonitrile (10 mL) and heated at 80 °C for 1 h. After cooling, the red precipitate was filtered off and crystallized from ethanol giving 0.3 g (yield 47%) of **7a** as red crystals. Mp 218 °C (ethanol). IR ν 3250, 1640, 1598, 1500 cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.80 (m, 2H, CH_2), 2.85 (m, 4H, CH_2), 3.65 (m, 1H, NH deuterium oxide exchangeable), 7.80 (d, 1H, arom H), 8.0 (d, 2H, arom H), 8.15 (d, 1H, arom H), 8.20 (d, 2H, arom H), 11.00 (s, 2H, NH_2 deuterium oxide exchangeable). Anal. Calcd for $C_{18}H_{15}N_5O_8$: C, 50.35; H, 3.52; N, 16.31. Found: C, 49.99; H, 3.71; N, 16.12.

5.4.6. 2-[(5-Aminopentyl)amino]-3,6-dinitro-8-(4′-nitrophenyl)-4*H*-1-benzopyran-4-one (7b). A solution of **6** (0.6 g, 1.5 mmol) and *N*-(triphenylmethyl)pentane-1,5-diamine (1.0 g, 2.9 mmol) in acetonitrile (20 mL) was refluxed for 1 h. The red precipitate, which formed after cooling, was filtered off and crystallized from ethanol giving the already deprotected **7b** as red crystals (yield 0.4 g, 58%). Mp 220–21 °C (ethanol). IR ν 3300, 2900, 1640, 1600, 1200 cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.40 (s, 6H, CH_2), 2.20 (t, 2H, NH–CH_2), 2.70 (t, 2H, CH_2), 3.65 (m, 1H, NH deuterium oxide exchangeable), 7.70 (d, 1H, arom H), 7.95 (d, 2H, arom H), 8.10 (d, 2H, arom H), 8.20 (d, 2H, arom H) 11.00 (s, 2H, NH_2 deuterium oxide exchangeable). Anal. Calcd $C_{20}H_{19}N_5O_8$: C, 52.52; H, 4.19; N, 15.31. Found: C, 52.88; H, 4.00; N, 15.09.

5.4.7. 3-[3-(Aminopropyl)amino]-1-oxo-1*H*-naphtho[2,1-*b*]-pyran-2-carbaldehyde hydrochloride (11·HCl). A solution of **9** (0.4 g, 1.5 mmol) and (1.5 mmol) of *N*-(triphenylmethyl)propane-1,3-diamine in 15 mL of toluene was stirred at rt for 3 h. Evaporation of the solvent and purification of the residue by column chromatography (SiO_2; AcOEt) yielded 0.65 g of crude 1-oxo-3-{3-[(triphenylmethyl)amino]-propylamino}-1*H*-naphtho[2,1-*b*]-pyran-2-carbaldehyde **10**, which was hydrolyzed with diluted HCl at 60 °C for 2 h. The H_2O was removed by co-evaporation with benzene and the residue crystallized with EtOH to give **11**·HCl (yield 0.3 g, 60%) as white crystals. Mp 210–213 °C (ethanol). IR ν 3400, 2900, 2000, 1660, 1630, 1610, 1560, 1490 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): δ 2.05 (2H, m, CH_2), 2.90 (2H, m, CH_2N), 3.72 (2H, m, CH_2N), 7.73 (3H, m, arom CH), 8.07 (1H, d, arom CH), 8.20 (3H, NH_3), 8.35 (1H, d, arom CH), 9.90 (1H, d, arom CH), 10.13 (1H, s, CHO), 10.25 (1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): δ 27.15 (CH_2), 36.46 (CH_2), 37.82 (CH_2), 100.67 (C), 114.48 (C), 117.46 (CH), 126.43 (CH), 126.54 (CH), 128.87 (CH), 129.26 (CH), 130.36 (C), 131.05 (C), 135.78 (CH), 154.36 (C), 163.05 (C), 177.94 (C), 188.74 (CHO). Anal. Calcd for $C_{17}H_{17}N_2O_3Cl$: C, 61.36; H, 5.15; N, 8.42; Cl, 10.65. Found: C, 61.00; H, 5.25; N, 8.31; Cl, 10.85.

5.4.8. 1-(2-Hydroxynaphthalen-1-yl)-2-(tetrahydropyrimidin-2(1*H*)-ylidene)ethanone (12). A suspension of **9** (0.2 g, 0.75 mmol) and propane-1,3-diamine (0.63 mL, 7.5 mmol) in toluene (5 mL) was stirred at 110 °C for 2 h. The solvent was evaporated and the residue purified by column chromatography (SiO_2; AcOEt/EtOH 8:2) to give 0.1 g (yield 50%) of **12** as brownish yellow-coloured

oil, which solidifies with Et_2O. Mp 226–7 °C (ethanol/ethyl acetate 1:4). IR ν 3320, 1620, 1580, 1510, 1460, 1380, 1370 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): δ 1.88 (t, 2H, CH_2), 3.33 (m, 4H, CH_2N), 5.05 (m, 1H, CH), 7.03 (d, 1H, arom CH), 7.27 (m, 1H, arom CH), 7.45 (m, 1H, arom CH), 7.76 (m, 2H, arom CH), 8.30 (d, 1H, arom CH), 9.25 (2H, NH), 13.12 (1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): δ 19.94 (CH_2), 37.72 (CH_2), 37.83 (CH_2), 85.36 (CH), 118.10 (C), 119.15 (CH), 122.48 (CH), 124.70 (CH), 126.40 (CH), 128.31 (C), 128.63 (CH), 130.71 (CH), 131.00 (C), 157.24 (C), 159.36 (C), 179.58 (C). Anal. Calcd for $C_{16}H_{16}N_2O_2$: C, 71.62; H, 6.01; N, 10.44. Found: C, 71.82; H, 6.11; N, 10.56.

5.4.9. 1-(2-Hydroxy-3-isopropyl-6-methylphenyl)-2-(tetrahydropyrimidin-2(1*H*)-ylidene)ethanone (13). Analogously to the preparation of **12**, 0.2 g (0.75 mmol) of 2-(dimethylamino)-8-isopropyl-5-methyl-4-oxo-4*H*-1-benzopyran-3-carbaldehyde **2** and propane-1,3-diamine (0.63 mL, 7.5 mmol) in toluene (5 mL) was heated at 110 °C for 3 h. After cooling, the yellow precipitate was filtered off and crystallized from AcOEt (yield 0.12 g, 58%). Mp 240 °C (AcOEt). IR ν 3320, 1620, 1580, 1510, 1460, 1380, 1370 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 1.19 (s, 3H, CH_3), 1.22 (s, 3H, CH_3), 1.6 (2H, NH), 2.00 (m, 2H, CH_2), 2.4 (s, 3H, CH_3), 3.38 (m, 5H, $2CH_2$, CH), 4.68 (s, 1H, CH), 6.62 (d, 1H, arom CH), 7.02 (d, 1H, arom CH), 11.25 (s, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): δ 20.79 (CH_2), 22.56 (CH_3), 23.10 ($2CH_3$), 26.91 (CH), 38.70 ($2CH_2$), 86.01 (CH), 121.77 (CH), 125.91 (C), 126.41 (CH), 132.60 (C), 133.58 (C), 155.97 (C), 159.99 (C), 184.02 (C). Anal. Calcd for $C_{16}H_{22}N_2O_2$: C, 70.04; H, 8.08; N, 10.21. Found: C, 69.89; H, 8.19; N, 10.32.

5.5. Synthesis of oligonucleotides

The oligonucleotides were synthesized in solution, deprotected and purified following our previous routes.[15,16] Each oligonucleotide in this preparation showed a single peak either with an anion exchange column or with a reversed phase column (see HPLC).

5.6. Synthesis of conjugates of oligonucleotides with stabilizing agents

Following the general procedure previously described,[8] 3 μL 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 mL of water and this mixture was then centrifuged. The former solution (1 μL) 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 P_2O_5. A solution of this compound in 60 μL 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 (**4a**,**b**, **7a**,**b**, **5a**,**b**, **11**, **12** or **13**) and 2 μL of anhydrous triethylamine were added. After stirring for 1 h 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 (yield 65%).[23] The final residue was dissolved in water and a precisely measured aliquot is taken off to measure the absorbance at 260 nm.[16] Then the solution was precipitated with 2% $LiClO_4$ in acetone to give an ODN conjugate (**4a***, **4b***, **7a***, **7b***, **5a***, **5b***, **11***, **12*** or **13***).

5.7. Thermal denaturation of complexes

5.7.1. 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-hydrolysis[28] 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 Na_2HPO_4, 0.1 mM EDTA, pH 7.0. Concentration of each oligonucleotide chain was 1.6×10^{-5} M.

5.7.2. 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 mL, 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 the 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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