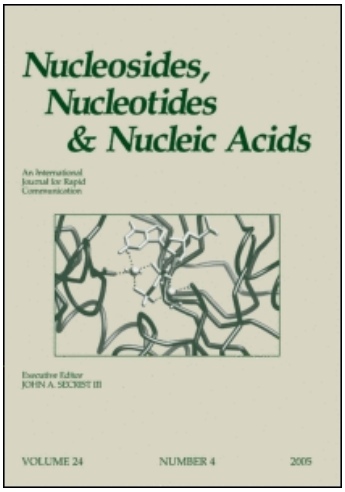

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Oligonucleotide Conjugates of Nile Red†

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ABSTRACT

Oligonucleotides containing long chain amine on uridine-2′-carbamate have been covalently modified with Nile Red, a hydrophobic long wavelength fluorescent benzophenoxazine dye. The fluorescence of the dye is quenched in oligonucleotide conjugates. Thermal denaturation studies show considerable interactions of Nile Red with DNA–DNA duplexes.

Key Words: Modified oligonucleotides; Nile Red; Interaction with ds DNA.

INTRODUCTION

The development of fluorescence methodologies and new dyes led to the rapid growth of molecular and physicochemical biology and related sciences. There are many useful techniques based on detection of fluorescently labeled nucleic acids.[1–4] On the other hand, fluorescent molecules are usually flat poly(hetero)aromatic

†In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

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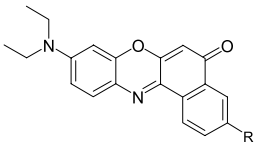
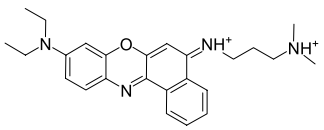
compounds, sometimes able to interact with ds DNA, e.g. by means of intercalation or groove binding.

Hydrophobic fluorescent dye Nile Red, 9-diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-5-one **1a**, is very sensitive to the local polarity (dielectric constant of the microenvironment) and can be used as a probe for hydrophobic surfaces in proteins.[5,6] In a polar environment Nile Red has a low fluorescence quantum yield, whereas in more hydrophobic environment its quantum yield increases and its emission maximum becomes progressively blue shifted.[7,8] The property was used for probing of non-polar sites on biomolecules (proteins[9,10] and lipids[11,12]). In water Nile Red has excitation maximum at 550 nm, emission maximum at 650 nm and the lifetime of the excited state is 2.8 ns.[13] To the best of our knowledge, fluorescent properties of Nile Red bound to DNA were never studied, although the preparation of oligonucleotide conjugates was reported once.[14] Interestingly, many chromophores structurally related to Nile Red are known as DNA binding agents. A number of the benzophenoxazine dye family members selectively stain nucleic acids and certain tumors and therefore may serve as anticancer agents[15] or to be used for staining of nucleic acids in solutions, in electrophoretic gels or other matrices,[16] in blotting experiments[16,17] and even in assays employing intact live cells (e.g. compound **2**[18]). The activity is ascribed to their ability to interact with double stranded nucleic acids and to inhibit the DNA-directed RNA synthesis.[19]

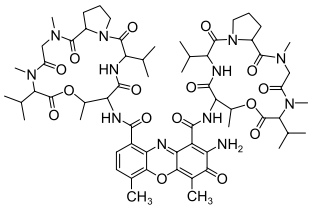
The most important related compounds are antibiotics actinomycins, e.g. actinomycin D (**3**, ActD). Its molecule contains two cyclic pentapeptides and planar phenoxazine chromophore. To date, two main models of ActD–nucleic acids interaction have been postulated. The first model is based on intercalation of the planar benzophenoxazine ring into the double helix. It is confirmed by high-resolution nuclear magnetic resonance (NMR) and molecular modeling data,[20,21] X-ray investigations[22,23] and competition dialysis experiments.[24] According to the second hypothesis, based on fluorescence experiments, ActD is binding into the minor groove of the duplex.[25] Moreover, evidences of ActD binding to a GpC site in a hairpin[26,27] or to a 5′-GXC/CYG-5′ sequence (where X/Y is G·C or T·A Watson–Crick base pair)[28] were obtained. In the latter case the central Watson–Crick X/Y base pairs are looped out and displaced by the ActD chromophore.



1a, R = H, Nile Red
1b, R = OCH_2COOH



2



3, Actinomycin D (ActD)

Taking into account these remarkable features of Nile Red and analogues, we hoped to obtain useful spectral data from the study of Nile Red–oligonucleotide conjugates. There are several examples of preparation of functionalized Nile Red derivatives.[14,29–33] The attachment of the alkoxy linker to the benzene ring of Nile Red does not cause significant spectral changes in absorbance and fluorescence of the dye. Unfortunately, the heterocyclic system of Nile Red is not stable enough to survive conventional oligonucleotide deprotection procedure (hot ammonolysis, conc. aq. NH_3, 55°C, 6 h). Therefore, the incorporation of Nile Red derivatives into oligonucleotides was achieved using a postsynthetic procedure.

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RESULTS AND DISCUSSION

Nile Red functional derivative, acid **1b**[30] was attached to oligonucleotides containing one well studied modification, a long chain amine on uridine-2′-carbamate.[34]

The Nile Red derivative has distinctive absorbance around 268 and 570 nm (Figure 1a). The chromophore decomposes during treatment with aqueous ammonia and 570 nm band disappears completely after several hours even at room temperature (Figure 1a). The time of the half-decomposition ($t_{1/2}$) of the dye at various temperatures

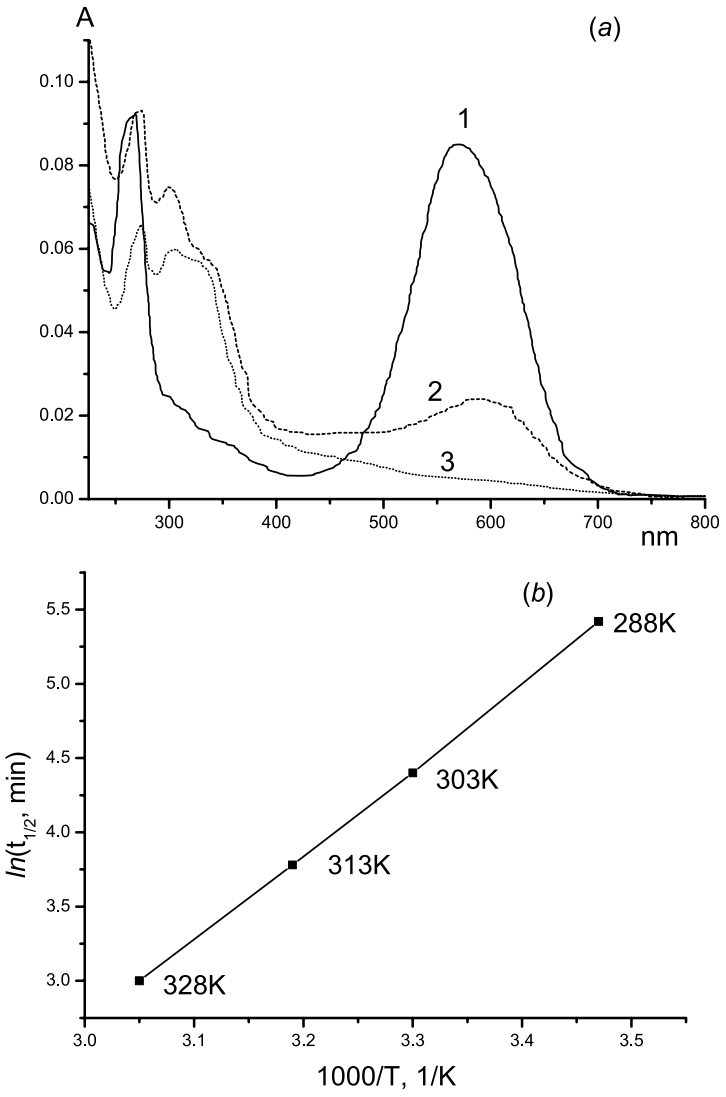


Figure 1. (*a*) The bleaching of the chromophore of the Nile Red acid **1b** in 25% aq. NH_3 at room temperature: the freshly prepared solution (*1*), the solution after 6 h (*2*) and 48 h (*3*). (*b*) The temperature dependence of the rate of the chromophore decomposition in 25% aq. NH_3.

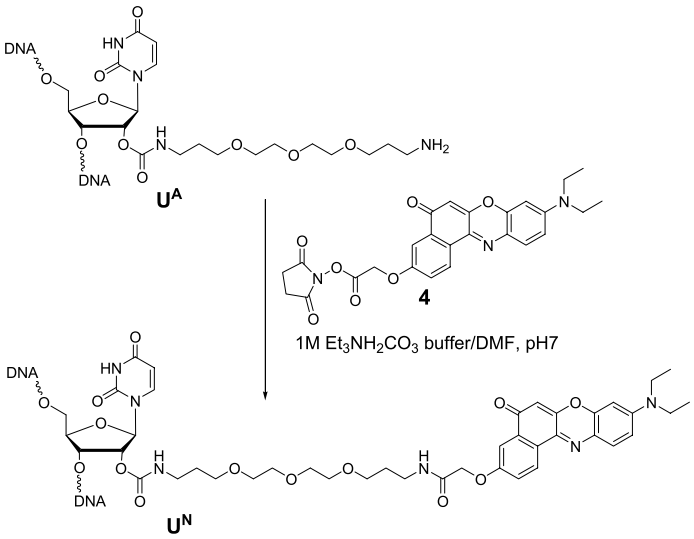
Table 1. Properties of uridine-2′-carbamate oligodeoxyribonucleotides.

Oligomer	Sequence, 5′ → 3′	MALDI-TOF, $[M + H]^+$, calc./found	RP-HPLC retention time, min[a]	Duplex with complementary DNA	
				T_m, °C	ΔT_m, °C[e]
ON1	ATTTGAGCCTGGGAG	4643.0/4643.8[b]	n.d.[c]		
ON2	*AUUUGAGCCUGGGAG* (RNA)	4830.7/4830.5[b]	n.d.[c]		
ON3	CTCCCAGGCU[d]CAAAT	4483.9/4482.2[b]	n.d.[c]	56.4[b]	–
ON4	CTCCCAGGCU^{A}CAAAT	4746.2/4744.3[b]	9.8	49.8[b]	−6.6
ON5	CTCCCAGGCU^{N}CAAAT	5120.6/5120.8	23.9	53.6	−2.8
ON6	CU[d]CCCAGGCTCAAAT	4483.9/4482.0[b]	n.d.[c]	56.2[b]	–
ON7	CU^{A}CCCAGGCTCAAAT	4746.2/4744.2[b]	10.9	52.6[b]	−3.6
ON8	CU^{N}CCCAGGCTCAAAT	5120.6/5121.3	22.0	55.4	−0.8

U^{A} and **U^{N}**—uridine-2′-carbamates, containing long chain amine and Nile Red, respectively (as depicted on Scheme 1).
[a]For HPCL conditions see Experimental section.
[b]Previously reported data. (From Ref. [34].)
[c]n.d.—Not determined.
[d]**U**—2′-deoxyuridine.
[e]The difference in T_m between modified and corresponding unmodified duplex.

was determined by absorbance at 570 nm and showed the pseudo-first order of the reaction (Figure 1b).

The oligonucleotide sequence chosen was DNA 15-mer CTCCCAGGCTCAAAT complementary to the sequence 22–36 of the *trans*-activation responsive region of the human immunodeficiency virus type 1 (HIV-1 TAR RNA).[35] 2′-Deoxyuridine or



Scheme 1. Oligonucleotide modification with Nile Red.

uridine-2′-carbamate with long-chain amine group ($\mathbf{U^A}$) were placed instead of internal thymidine (**ON3** and **ON4**) or of the thymidine close to the 5′-end (**ON6** and **ON7**).[34] Complementary unmodified oligodeoxyribonucleotide (**ON1**) and oligoribonucleotide (**ON2**) also were synthesized (Table 1). Oligonucleotides **ON4** and **ON7** were modified with activated Nile Red derivative (Scheme 1). The freshly prepared solution of *N*-hydroxysuccinimide ester **4** in DMF was used in 1M $Et_3NH_2CO_3$ buffer for the

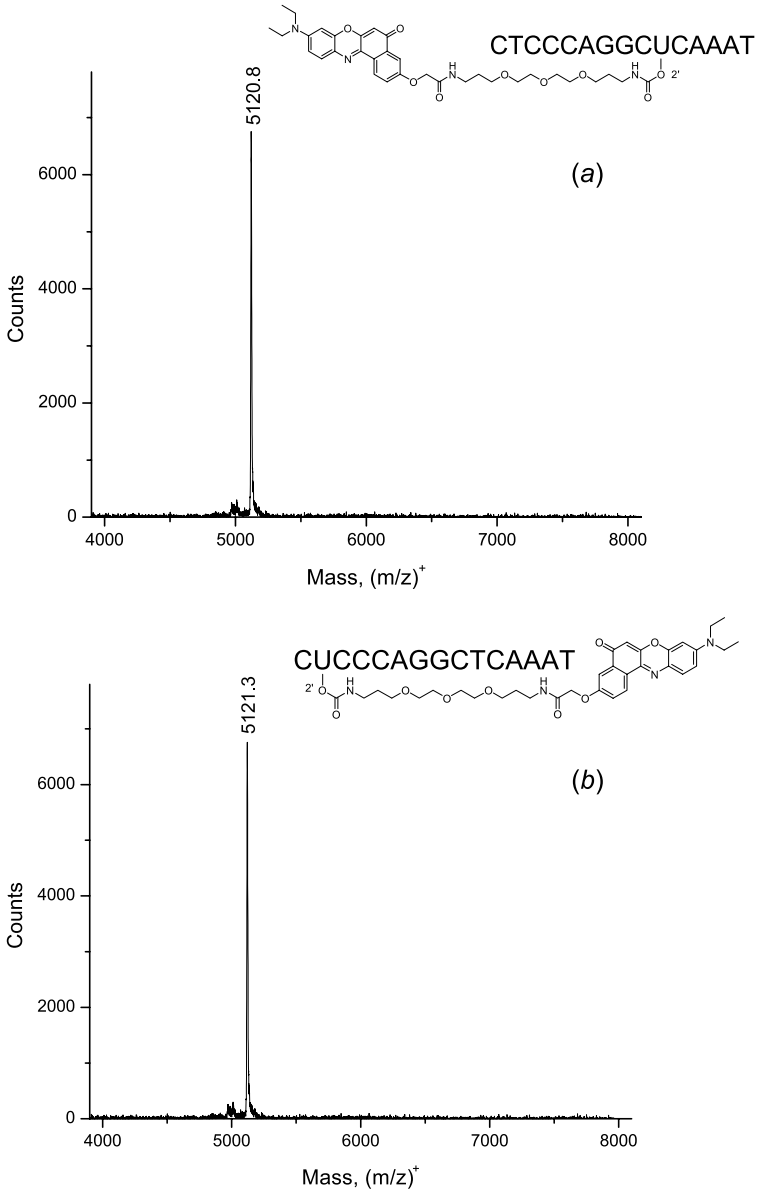


Figure 2. MALDI-TOF mass spectra of Nile Red-modified oligonucleotides **ON5** (a) and **ON8** (b).

reaction with oligonucleotides **ON4** and **ON7**. It is important to keep pH 7.5 for conjugation, because in case of pH > 8 the rapid hydrolysis of the hydroxysuccinimide ester **4** occurs. Conjugates **ON5** and **ON8** were isolated by PAGE, then purified by reversed phase HPLC and characterized by MALDI-TOF mass spectrometry (Table 1, mass spectra are given on Figure 2). The insertion of such bulky moieties into oligonucleotides reduces their mobility in gel (data not shown) and increases the retention time of conjugates on reverse-phase column (Table 1), obviously due to their hydrophobicity and considerable affinity to the reversed phase.

The attachment of the dye to an oligonucleotide was confirmed by the absorbance spectra of conjugates (Figure 3). There is an additional broad peak in the region 580–620 nm. The extinction maximum of the dye in the conjugates **ON5** and **ON8** is considerably red-shifted in comparison to the spectrum of the starting acid **1b**. A similar effect was observed for Nile Red in various liquids.[36] The effect originates in the shielding of the dye by nucleobases, which is more pronounced for conjugate **ON5**, containing an internal label (absorbance maxima are 595 nm for **ON8** and 600 nm for **ON5**).

As reported earlier, uridine-2′-carbamate modification dramatically destabilizes DNA–DNA duplexes.[34] The data of thermal denaturation studies show that Nile Red residue neutralizes the negative carbamate influence by ca 3°C when attached both in the internal as well as in near-terminal position of the duplex (Table 1, Figure 4). The increase of stability of 2′-carbamate-modified duplexes by hydrophobic planar polycyclic hydrocarbons attached through a short linker was already observed[34,37] and explained by the fluorophore binding in the grooves of DNA duplexes.

It is well known that Nile Red is an uncharged hydrophobic molecule whose fluorescence is strongly influenced by the polarity of the environment. From the structure of the dye, a substantial enhancement in the excited state dipole moment might be expected.[6] Not surprisingly, the fluorescence of the chromophore in polar solvents is considerably quenched in comparison with non-polar ones.[7,8] The intensity of fluorescence of conjugates **ON5** and **ON8** in aqueous solutions is quite moderate. The fluorescence of internal-labeled **ON5** is higher than for near-terminal **ON8**, and maximum emission of conjugate **ON5** is slightly blue shifted compared to **ON8** (data not shown). Upon hybridization of the conjugates with DNA or RNA the fluorescence is quenched further.

Thermal denaturation studies and the absence of significant changes in fluorescence properties of duplexes of modified oligonucleotides with complementary DNA and RNA can be considered as indirect evidence, that interaction of the dye with nucleic acid in double- and single-stranded DNA is not essentially different. It could mean, that intercalation of the dye in a duplex does not take place in this case. The chromophore may be stacked as a cover at the end of the duplex both in case of internal as well as near-terminal position of the dye. The long linker (4,7,10-trioxatridecamethylenene) allows chromophore to reach the end of duplex in the both cases. On the other hand, the fluorophore minor groove interaction should not be excluded.

To conclude, a method of oligonucleotide modification with hydrophobic benzophenoxazine dye Nile Red is described. Nile Red has positive effect on stability of DNA duplexes. The fluorescence of Nile Red is considerably quenched in aqueous solutions and in oligonucleotide conjugates. Thus, oligonucleotide conjugates of Nile

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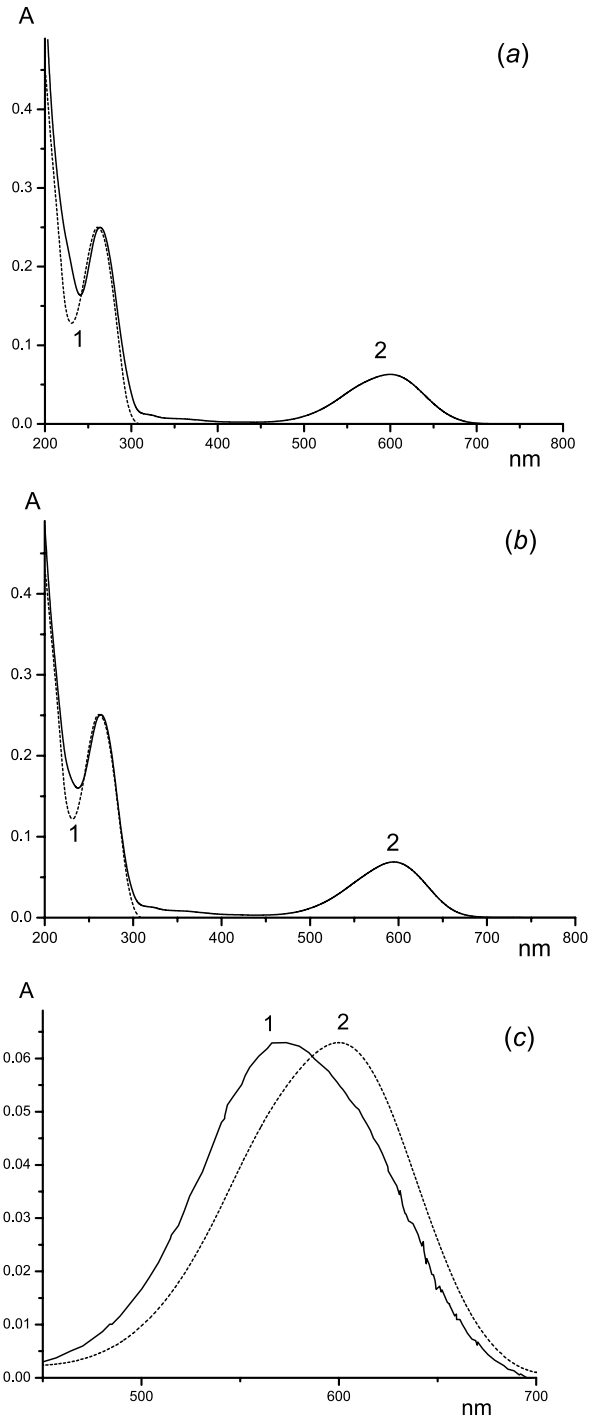

Figure 3. UV-spectra of oligonucleotides (*a*) **ON4** (*1*), **ON5** (*2*); (*b*) **ON7** (*1*), **ON8** (*2*) in water normalized at 260 nm; (*c*) Nile Red acid **1b** (*1*), oligonucleotide **ON5** (*2*) in water normalized at chromophore absorbance.

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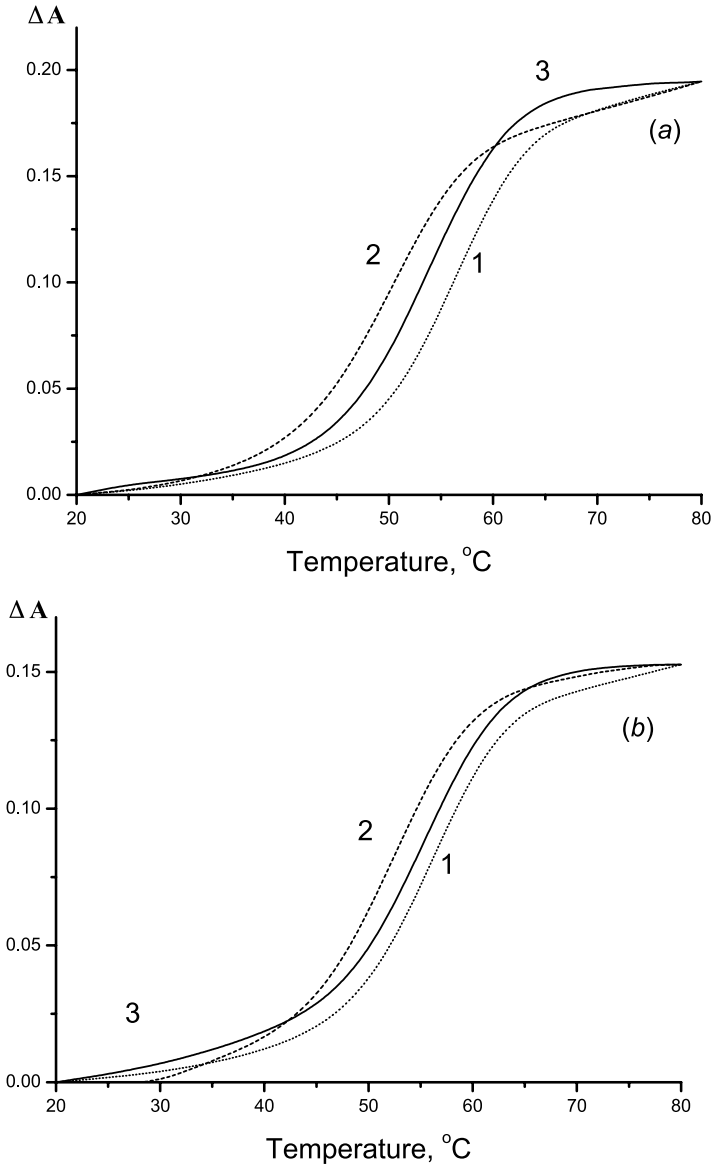

Figure 4. Melting curves of the modified oligonucleotides with complementary matrix **ON2** (*a*) **ON3** × **ON2** (*1*), **ON4** × **ON2** (*2*), **ON5** × **ON2** (*3*), (*b*) **ON6** × **ON2** (*1*), **ON7** × **ON2** (*2*), **ON8** × **ON2** (*3*). Conditions: hybridization buffer (see Experimental section), 0.5°C/min, concentration of each oligonucleotide $1 \cdot 10^{-6}$ M.

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Red are suitable candidates for testing in probing of nucleic acid–protein interactions on proteins containing hydrophobic domains.

EXPERIMENTAL SECTION

9-Diethylamino-5-oxo-5*H*-benzo[a]phenoxazin-2-yloxy acetic acid *N*-hydroxysuccinimide ester. The solution of 5*H*-3-carboxymethyloxy-9-diethylamino-benzo[a]-phenoxazin-5-one **1b**[30] (70 mg, 0.17 mmol), Et_3N (75 μL, 0.53 mmol) and di-(*N*-succinimidyl)carbonate (495 mg, 1.9 mmol) in DMF (7 mL) was stirred for 5 h at room temperature. The mixture was diluted with EtOAc (100 mL), washed with water (100 mL), 1% citric acid (5 × 70 mL), and water (100 mL), dried over Na_2SO_4, evaporated, and coevaporated with toluene (3 × 25 mL). The residue was chromatographed on silica gel column in step gradient (0 → 60%) of EtOAc in $CHCl_3$ (v/v). Fractions containing product were combined, evaporated, and the residue was dried in vacuo afford 21 mg (25%) of the desired product as a dark blue solid.

Synthesis of conjugates and spectral studies. Oligonucleotide synthesis was carried out on a ABI 380B DNA/RNA synthesizer in 1 μmole scale using 2′-deoxynucleoside phosphoramidite reagents obtained from Cruachem (Scotland) and standard synthetic procedures. Synthesized in 1 μmol scale aminomodified oligonucleotides **ON4** and **ON7** were isolated using PolyPak cartridges (Glen Research) and standard purification techniques and then modified using following procedure. Oligonucleotide was dissolved in water (945 μL) and 1M $Et_3NH_2CO_3$ buffer, pH 7 (135 μL), and freshly prepared crimson solution of 9-diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yloxy acetic acid *N*-hydroxysuccinimide ester (2.7 mg in 0.27 mL DMF) was added. The mixture was kept overnight at ambient temperature, diluted with 2M $LiClO_4$ (1.35 mL), extracted with 1-butanol (0.5 mL portions) until the volume of aqueous layer reaches 0.4 mL, and precipitated with acetone (1.6 mL). Modified oligonucleotides were purified using electrophoresis in 20% denaturing (7 M urea) polyacrylamide gel in tris-borate buffer, pH 8.3. Conjugates eluted from the gel were purified further by reversed phase HPLC using a RP-C18 SOTA column (4.5 × 250 mm) and UV detection at 254 nm, buffer A: 0.1M triethylammonuim acetate, buffer B: MeCN; flow rate 1 mL/min; **ON4** and **ON5**: the gradient of B in A: 5 → 15%, 10 min; 15 → 20% 10 min; 20 → 22% 20 min; **ON7** and **ON8**: 5%, 5 min; 5 → 12%, 10 min; 12 → 20% 20 min. Appropriate fractions were pooled, evaporated, dissolved in 1 M $LiClO_4$ (0.4 mL), and precipitated with acetone (1.6 mL).

Duplex stability studies were done in the hybridization buffer containing 100 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, 0.1 mM EDTA, pH 7.0 on a Perkin Elmer Lambda 40 UV/VIS Spectrometer with PTP 6 (Peltier Temperature Programmer), concentrations of oligonucleotides $1 \cdot 10^{-6}$ M. MALDI-TOF spectra were recorded on a Voyager DE system (Perseptive Biosystems) in positive ion mode using 1:1 (v/v) mixture of 2,6-dihydroxyacetophenone (40 mg/mL in MeOH) and aqueous diammonium hydrogen citrate (80 mg/mL) as a matrix premixed just before loading the samples onto a plate. Fluorescence spectra were recorded on a Perkin Elmer LS 50B Luminescence Spectrometer with an excitation wavelength 540 nm in hybridization buffer, concentrations of oligonucleotides $2 \cdot 10^{-7}$ M.

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