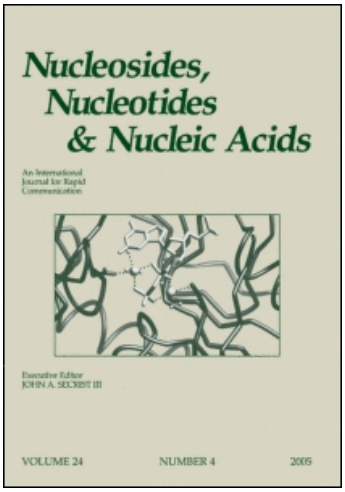

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Properties of Naphtho[2,1-B]pyrans Covalently Linked to Oligonucleotides

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PROPERTIES OF NAPHTHO[2,1-b]PYRANS COVALENTLY LINKED TO OLIGONUCLEOTIDES

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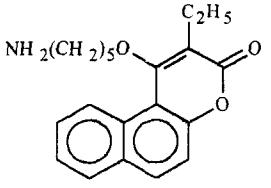
ABSTRACT - Two naphtho[2,1-b]pyrans (benzocoumarin **1** and benzochromone **2**) were covalently linked to a 7-mer ODN. Purification and hydrophobicity studies of the resulting conjugates **1*** and **2*** were performed by HPLC. Benzochromone conjugate **2*** was more hydrophobic than benzocoumarin conjugate **1***. Preliminary data on thermal stability and interaction with HIV reverse transcriptase showed that **1*** is slightly more efficient than **2***. This is probably due to the steric hindrance of the 3-diethylamino group in **2**.

INTRODUCTION

Oligonucleotides (ODNs) are interesting tools for therapeutic applications and research purposes [1]. In all cases, the formation of duplex and triplex helices could be favoured by the presence of structurally planar substances at 5'- and 3'-termini. Thus, ODNs covalently linked to polycyclic aromatic groups, such as acridine, phenazine, ethidium and others are interesting substances in antisense and antigene strategies, due to their ability to form specific complexes of increased stability with complementary oligo- and polynucleotides [2-4]. Moreover, the presence of stabilizing agents may promote more efficient blocking or delaying of the cleavage by nucleases [5].

We recently demonstrated that several compounds from the pyranone family (namely derivatives of coumarins and chromones) have an effect on the stabilization of complementary complexes comparable to the stabilizing effect of acridine [6,7]. Moreover,

we showed that ODNs modified with a benzopyranone group interact differently with HIV reverse transcriptase depending on the nature of the stabilizing agent [8].



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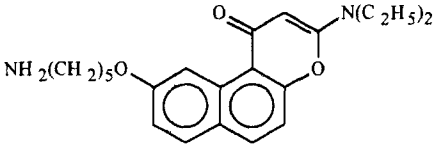


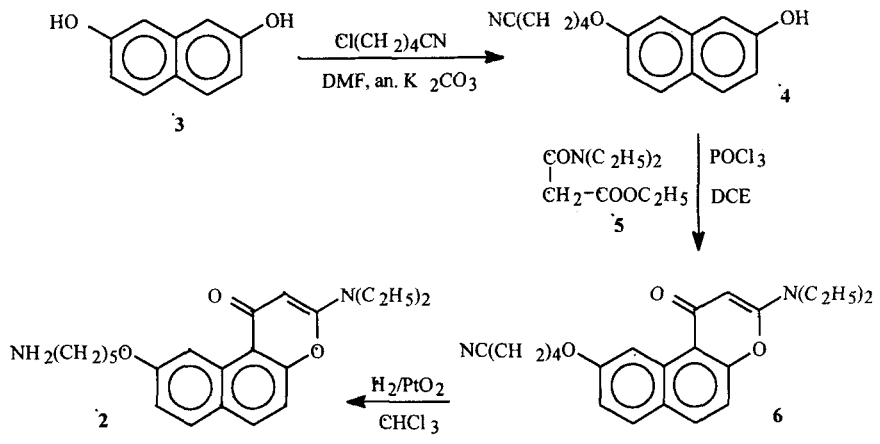
2

Due to our interest in 2-(dialkylamino)chromone chemistry and pharmacology [9-11], in the present work we compare some chemical, thermodynamic and biological properties of two new oligonucleotide conjugates having covalently linked tricyclic naphtho[2,1-b]pyrans. In particular, the planar heterocyclic derivatives used here refer to benzocoumarin (**1**) and benzochromone (**2**) systems.

CHEMISTRY

The 1-(5-aminopenthyloxy)-2-ethyl-3H-naphtho[2,1-b]pyran-3-one **1** was synthesized following reference 6. The 9-(5-aminopenthyloxy)-3-(diethylamino)-1H-naphtho[2,1-b]-pyran-1-one **2** was prepared following a pathway (see Scheme 1) very useful in forming pyran-4-ones carrying a dialkylamino group adjacent to the heteroatom [12-14]. First of all we need the suitable monosubstituted naphthol **4**. So the synthesis starts by reaction of 2,7-dihydroxynaphthalene **3** with 5-chlorovaleronitrile in N,N-dimethylformamide (DMF) in the presence of anhydrous potassium carbonate. The molar ratio between **3** and 5-chlorovaleronitrile was optimal for the monosubstitution. Compound **4** reacting with 3-(diethylamino)-3-oxo-propanoic acid ethyl ester **5** in the presence of phosphorus oxychloride in 1,2-dichloroethane resulted in the substituted 1H-naphtho[2,1-b]pyran-1-one **6**. The latter was dissolved in chloroform and reduced in the presence of PtO_2 as catalyst to give the aminoderivative **2**.

The structures of naphthol **4** and 1H-naphtho[2,1-b]pyran-1-ones **2** and **6** are in agreement with elemental analyses and spectral data. In particular, in compounds **2** and **6**,



SCHEME 1

the H-10 shows a signal at about 10.0 p.p.m. in ^{1}H-NMR spectra because of the deshielding effect of the carbonyl group in position 1. This fact is characteristic of such angular structures [12-14]. Also the low signal of the carbonyl group in IR spectra (1620 cm^{-1}) is useful in identifying the pyran-4-one structure [12-14].

The conjugation reaction of compounds **1** and **2** to the terminal 5' phosphate of 5'-pCCAAACA (**UM**) gave the modified ODNs **1*** and **2*** with the below specified yields. Final HPLC purification was performed on a C18-column. The modified ODNs were detected by UV spectra (in water) measuring the absorbance at 320 (**1***) and 340 (**2***) nm.

EXPERIMENTAL

Materials and Methods

Melting points were determined using a Fisher-Johns apparatus and are uncorrected. Microanalyses were carried out on a Carlo Erba 1106 elemental analyzer. ^{1}H-NMR spectra were performed on a Hitachi Perkin-Elmer R 600 (60 MHz) spectrometer using TMS as the internal standard (δ = 0). IR spectra were recorded on a Perkin-Elmer 398 spectrophotometer. The liquid chromatograph was a Perkin-Elmer Series 4 equipped with a Rheodyne 7125 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 x 10 mm) filled by us with Partisil-10 SAX (Whatman, USA). The reverse-phase column was a stainless steel column (250 x 10 mm) filled by us with 10 μm LiChrosorb RP18 (Merck, Germany).

Synthesis of 1H-naphtho[2,1-b]pyrans

7-(4-cyanobuthyloxy)-2-hydroxynaphthalene **4**

In a 250 ml flask, 5.4 g of 2,7-dihydroxynaphthalene **3** (33.7 mM) were dissolved in 25 ml of DMF. Then 3.8 ml of 5-chlorovaleronitrile (33.7 mM) and 5 g of anhydrous potassium carbonate were added and the mixture was heated at 105-110°C for 6 h, under stirring. At the end, the cooled mixture was poured onto crushed ice and the pH was set at 7. The solid which separated out was extracted with chloroform. The organic phase was counter-extracted with 2N sodium hydroxide and then the aqueous phase was acidified with (1:1) hydrochloric acid. The precipitate consisted in the monosubstituted naphthol **4**; crystallized from ethyl acetate/cyclohexane; 71% yield; m. p. 119-120°C. Anal. for $C_{15}H_{15}N\ O_2$: Calcd: C% 74.67, H% 6.27, N% 5.80; Found: C% 74.75, H% 6.29, N% 5.73; ^{1}H-NMR ($CDCl_3$) δ : 1.75-2.21 (OCH_2-CH_2-CH_2, m, 4), 2.29-2.72 (NC-CH_2, m, 2), 3.90-4.38 (OCH_2, m, 2), 5.45 (OH, broad s, 1), 6.75-7.92 (Aron.. H, m, 6).

9-(4-cyanobuthyloxy)-3-(diethylamino)-1H-naphtho[2,1-b]pyran-1-one **6**

In an ice bath-cooled flask, protected from moisture with a calcium chloride drying tube, 7.0 ml (76.5 mM) of phosphorus oxychloride were added dropwise under stirring to 10.3 g (55.0 mM) of 3-(diethylamino)-3-oxo-propanoic acid ethyl ester **5** [12]. After the addition, the mixture was removed from the ice bath and maintained at room temperature for 0.5 h. To the resulting yellow mixture, a solution of 7.91 g (50.0 mM) of 7-(4-cyanobuthyloxy)-2-hydroxynaphthalene **4** in 40 ml of 1,2-dichloroethane was added slowly under stirring. The reaction mixture was then heated for 5 h at reflux. After cooling, a solution of 68 g of sodium acetate trihydrate in 200 ml of water was added and the mixture was then heated for 1.5 h at 70°C. After cooling the organic phase was

removed and the aqueous one was extracted several times with chloroform. The pooled organic extracts were washed with water, dried and evaporated under reduced pressure to give a dark red oil. The oil was stirred at room temperature for 2 h together with 200 ml of 2N sodium hydroxide and 50 ml of light petroleum ether. The resulting solid was filtered off, washed with water and recrystallized from ethyl acetate; 48.1% yield; m. p. 139-140 C°. Anal. for $C_{22}H_{24}N_2O_3$: Calcd: C% 72.51, H% 6.64, N% 7.69; Found: C% 72.43, H% 6.70, N% 7.78; ^{1}H-NMR ($CDCl_3$) δ : 1.30 (CH_3, t, 6), 1.82-2.24 (OCH_2-CH_2-CH_2, m, 4), 2.35-2.70 (NC-CH_2, m, 2), 3.53 (N-CH_2, q, 4), 4.15-4.48 (OCH_2, m, 2), 5.54 (H-2, s, 1), 7.09-8.10 (Arom. H, m, 4), 9.75-9.90 (H-10, m, 1). I. R. (KBr) ν : 2220 cm^{-1} (CN), 1620 cm^{-1} (CO).

9-(5-aminopenthyloxy)-3-(diethylamino)-1H-naphtho[2,1-b]pyran-1-one **2**

A solution of 1 g of **6** in 30 ml of chloroform and 20 ml of methanol was hydrogenated at 60 p. s. i. in a Parr apparatus with 270 mg of Platinum oxide until gas uptake ceased (6 h). The catalyst was filtered off and the solvent was evaporated under reduced pressure. The white residue was the hydrochloride of **2**. The solid was treated with 2N sodium hydroxide obtaining a suspension. The suspension was extracted three times with chloroform and the organic extracts were evaporated under reduced pressure. The free base obtained was crystallized from ethyl acetate; 42% yield; m. p. 180-181°C. Anal. for $C_{22}H_{28}N_2O_3$: Calcd: C% 71.71, H% 7.66, N% 7.60; Found: C% 71.56, H% 7.72, N% 7.52; ^{1}H-NMR ($CDCl_3$) δ : 1.30 (CH_3, t, 6), 1.60-2.22 (OCH_2-CH_2-CH_2-CH_2, m, 6), 2.80-3.25 (NH_2-CH_2, m, 2), 3.53 (N-CH_2-CH_3, q, 4), 4.05-4.40 (OCH_2, m, 2), 4.58 (NH_2, qs, 2), 5.58 (H-2, s, 1), 7.06-8.11 (Arom. H, m, 4), 9.73 (H-10, qs, 1). I. R. (KBr) ν : 3400 and 3250 cm^{-1} (NH_2), 1620 cm^{-1} (CO).

Synthesis of unmodified ODNs

The oligonucleotide pCCAAACA (**UM**) and 8-mer pTGTTTGGC used as complementary target were synthesized in solution, deprotected and purified following our previous routes [6,7]. The final oligonucleotides showed a single peak either with an anion exchange column or with a reverse-phase column.

Synthesis of modified ODNs

3 µl of a 8% water solution of cetyltrimethylammonium bromide were added to a solution of the lithium salt of the completely deblocked ODN (5AU at 260 nm) dissolved in 50 µl of water and the resulting 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 over P_2O_5. A solution of this compound in 60 µl of dry DMSO, 10 mg of triphenylphosphine, 10 mg of dipyridyldisulfide, 5 mg of N,N-dimethylaminopyridine was stirred for 10 min. and then 2 mg of the aminoderivative (**1** or **2**) and 2 µl 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 in water and a precisely measured aliquot is taken off to measure the absorbance at 260 nm. Then the solution was precipitated with 2% $LiClO_4$ in acetone to give an ODN conjugate (**1*** or **2***).

Thermal Denaturation of Complexes

a) Preparation of samples: aqueous solutions of appropriate concentrations of ODNs were prepared by diluting a concentrated solution of the unmodified ODNs or the modified ODNs (**1***, **2***) according to molar extinction coefficients at 260 nm at 20°C. Extinction coefficients were calculated according to results of total PDE-hydrolysis and were as follows (260 nm): 8-mer 70200; 7-mer 66800. The extinction coefficients for the conjugate ODNs were estimated as a sum of respective values for each oligomer and stabilizing agent. Aqueous solutions of unmodified and modified ODNs 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.

b) 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 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.

Determination of kinetic parameters

Initial rates of polymerization reaction were measured in kinetic experiments. The Michaelis-Menten parameters, Km and Vmax, were determined according to Eisenthal and Cornish-Bowden direct linear plots.

Reverse transcriptase assay

The reaction mixture (50-100 ml) contained 50 mM Tris-HCl buffer pH 8.0, 5.0 mM $MgCl_2$, 80 mM KCl, 0.1 mM EDTA, 10 mM DTT, 50 mM dTTP, 1mCi [^{3}H] dTTP (56 Ci/mM). A saturating concentration of poly(A) was used: 1.5 A260 units/ml. The polymerization reaction was started by the addition of reverse transcriptase (5-20 nmol.).

RESULTS AND DISCUSSION

Table 1 shows the data concerning the attachment yields of the naphtho[2,1-b]pyranones **1** and **2** to the selected 7-mer pCCAAACA (**UM**) as well as the values of λmax for **UM**, **1*** and **2***. The choice of **UM** and of the complementary 8-mer ODN was made because their duplex does not exhibit any problem for hairpin formation or self-association [15]. Moreover, the presence of an extra flanking nucleotide in the complementary complex contributes to the stability of the duplex and allow insertion of the planar molecules **1** or **2** between the flanking nucleotide and the near base pair. In addition the use of short ODNs intensifies the effect of the stabilizing agent [7,16].

TABLE 1

ODN	yield (%)	λmax (nm)	RT (min)	Tm (°C)	ΔG (Kcal/mol)
UM	-	260	13.2	22.9	-5.1
1*	25	320	22.8	32.9	-6.6
2*	75	340	26.7	31.9	-6.3

Also in Table 1 the retention times (RTs) on reverse-phase column of **UM**, **1*** and **2*** are reported. Observing the RT values it is possible to deduce that **2*** is more hydrophobic than **1***. The marked differences in RTs and yields between the two modified ODNs are evident from these preliminary data and probably depend on the different positions of the linker. In this regard, previously synthesized chromones carrying the aminoalkoxy linker on the heterocyclic moiety (as the benzocoumarin **1**) demonstrated a low yield of conjugation reaction [6].

The second group of data in Table 1 refer to the stabilization of complexes between **UM**, **1*** or **2*** and the complementary 8-mer. From Tm values, and the corresponding ΔG values, we can observe that both covalently linked compounds **1** and **2** act as stabilizing agents but in these experiments the benzocoumarin **1** shows slightly more intense activity than benzochromone **2**. In this regard, we can assume that when **2** is present, the formation of the duplex and its stabilization could be hindered by the presence of dialkylamino group placed at the opposite side with respect to the linker. On the other hand, in the case of **1**, the opposite side with respect to the linker is free of substituents.

Also the interaction between HIV reverse transcriptase and oligonucleotide derivatives, containing **1** and **2**, was studied. Compound **1*** in low concentrations activated the polymerization, probably due to the interaction with the t-RNA-recognizing center of reverse transcriptase. In higher concentrations ($Km = 5x10^{-6}$) it inhibited reverse transcriptase, showing mixed inhibition towards the template-primer. In spite of being noncomplementary to the template, it served as a primer in the polymerization reaction, with the Km value 0.3 mkM. Compound **2*** when attached to noncomplementary oligonucleotide, did not show any of the above mentioned effects.

It is possible to conclude that in this comparison between **1** and **2** bound to the same ODN, the benzochromone **2** is endowed with an interesting hydrophobicity; however, the presence of the 3-diethylamino group would play an unfavourable role in duplex formation and stabilization due to its steric hindrance.

This research, dealing with oxygen containing heterocycles as stabilizing agents will be followed up with the synthesis of naphtho[2,1-b]pyran-1-ones carrying the linker on the pyranone moiety. In addition, the naphthalene moiety will be without substituents to promote better intercalation. In this regard, the dialkylamino group in position 3 of **2** seems to be susceptible to modification that would hopefully result in a more effective linker.

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