Fluorescent Conjugates: *p*H Stability, Dye-DNA Interaction and Biological Activity

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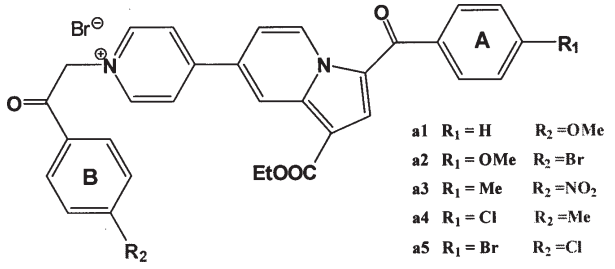
Spectral and biological properties of the new indolizinylpyridinium derivatives in the presence or absence of DNA were investigated. The interaction mechanism for binding the new fluorescent compounds to hsDNA was studied by UV-VIS spectrophotometry and fluorescence quenching technique.

Keywords: fluorescence, indolizinylpyridinium salts, DNA-dye complex

The quantitative analysis of nucleic acids is mandatory for molecular biology. Numerous strategies have been exploited to develop ligands which bind to and disrupt DNA functioning, DNA intercalators, and minor or major groove binding agents. Many organic dyes such as ethidium bromide [1], acridine orange [2], SYBR Green I [3], have been proved to be sensitive probes for DNA.

Many analytical methods have been used in the study of DNA binding or quantification, such as voltametry [4], chromatography [5], capillary electrophoresis [6] or UV-VIS, Raman spectroscopy, fluorescence [7,8]. Fluorescence analysis plays an important role in the study of DNA-binding [9,10].

In this paper, some recently synthesized indolizinyl-pyridinium derivatives [11] were used to investigate their interaction with DNA by spectral methods: UV-VIS spectrophotometry and fluorescence quenching technique. The electric transport of these compounds was already investigated, all compounds being found to behave as p-type semiconductors with polycrystalline structure [12]. The difference between these dyes concerns the substituents attached to heterocycles (scheme 1).



Scheme 1. Structure and substituents of 1-[2-(4-R_2-phenyl)-2-oxoethyl]-4-[[(1-ethoxycarbonyl)-(3-(4-R_1-benzoyl))]-indolizin-7-yl]-pyridinium bromides a1-**5**

Experimental part

Reagents

All chemicals were of analytical grade or better. Commercially prepared herring sperm DNA, hsDNA (Boehringer Mannheim, Germany) was dissolved in buffer, sodium acetate (Roth, Germany) 50 mM *p*H 4.5, at 2-3 mg/mL. Control plasmid pUC19, was prepared by transformation in DH5α (T7 Express Sampler, New England, BioLabs) and isolated using a Roti®- Prep Plasmid (Roth, Germany). These solutions were diluted to the corresponding concentration with the same buffer (for fluorescence and binding assays the buffer was daily prepared using a Steriflip® Filter Unit, Millipore). A dye stock solution (2 mM) was prepared by dissolving an appropriate amount of compound a in 10 mL DMF and stored in the dark. This solution was diluted 1:100 (20 μM final concentration) with buffer as working solution. The water which was used was doubly distilled.

Spectroscopic methods

All UV-Vis absorption spectra were recorded using a Libra single beam Spectrophotometer (Biochrom, UK) and quartz cuvettes (Hellma/Müllheim).

Fluorescence measurements

Steady state fluorescence measurements were conducted with a Modulus™ Microplate Multimode Reader (300 μL / well) equipped with a blue filter (excitation 490 nm; emission 510-570 nm) and the measurements were performed at 21°C. The dye concentration ranged from 2 to 20 μM depending on experiment.

Biological testing

One strain of *E. coli* (further called DH5α from a T7 Express Sampler, New England, BioLabs) was used in our experiments. Precultures of *E. coli* were incubated at 37°C with in liquid (LB) or solid medium (LB agar). For liquid culture, the dyes (only **a1** and **a4** have a good solubility in LB) were added 1:100 (final concentration 20 μM) or 1: 10 (final concentration 200 μM) to the media at OD_{580} = 0.2. Due to their low solubility, the dyes were added to the preheated (40-50°C) LB agar and the plates were poured using 5 mL medium per 55-mm plate. Alternatively, 50 μL solution (**a1** or **a4**; 2mM stock solution in DMF) was spreaded on the surface of LB plate. For lower dye concentration a slightly growth stimulation (about 10%) was observed. Intriguely, at dye higher concentration, a moderate inhibition (20-30%) on bacterial growth was seen.

Mathematical fit routines

All fit routines were executed with the program KaleidaGraph4 (Synergy Software). For *p*H dependence a variant of Dixon equation was used [13].

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Results and discussion

UV-VIS spectra of dyes

The UV-VIS spectra of the dyes were recorded over the range of λ between 200 and 800 nm, in aqueous solution. As seen in the figure 1, the absorbtion band of **a3** undergoes a bathochromic shift in comparison with **a1** and **a4**. This is quite trivial since the **a3** posses an extended heteroaryl-containing π-conjugated system. Moreover, **a3** has two small peaks in UV region. Spectrum of **a1** distinguishes by a single peak in the UV region (300 nm). In contrast, the other dyes have an extra peak in this region.

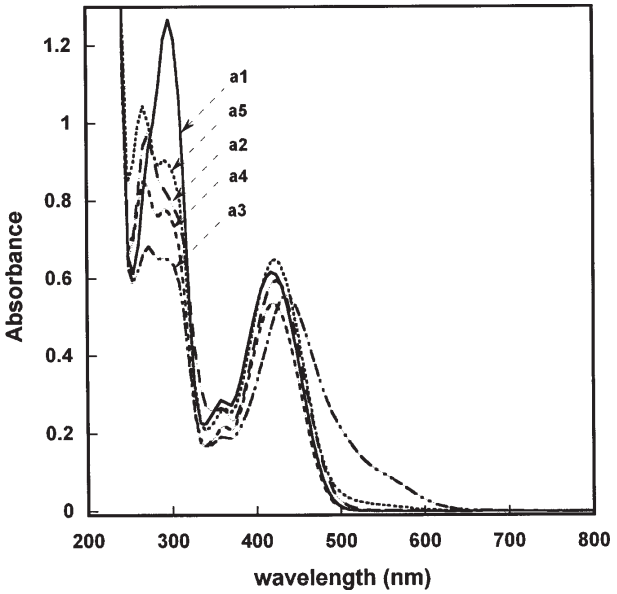

Fig. 1. UV-VIS absorption spectra of **a1** (——), **a2**(- - -), **a3**(. . . -), **a4** (- - - -) and **a5**(.....) (20 μM final concentration) in aqueous medium containing 1% DMF

The introduction of electron-donating methyl or methoxy group into the new inserted aromatic ring B results in small bathochromic shifts. The observed bathochromism upon introduction of electron donors, methyl, methoxy is in agreement with the theory. However, the bathochromic effects observed with our new synthesized compounds are similar with the shifts observed for precursors (exception: the precursor 2 is shifted to the highest wavelength; **a2** has a relatively lower absorbtion maximum compared with **a3** because it has a nitro group attached to the aromatic ring B).

*Effect of DNA on UV-VIS spectra of **a1***

The spectra were recorded by progressive addition of DNA solution to a1. The VIS spectra of the a1-DNA complex reflect a different dielectric constant of dye environment. DNA binding to **a1** leads to the hypsochromic shift (from 418 to 397 nm) of dye absorption maxima (fig. 2).

Moreover, in the UV region (350-360 nm) a hiperchromic effect was observed. This typical hyperchromic effect suggests that the DNA duble-helix structure is damaged due to the intercalation of **a1** to DNA.

The results suggest that an intercalative binding of **a1** to DNA phosphate backbone could be excluded.

Effect of pH on fluorescence

Some heterocycle compounds having with π-electron conjugated structures present strong fluorescence in aqueous solution. Our investigated dyes display a lower solubility and instability in weakly basic condition. Therefore, we investigate their fluorescence in acidic conditions. The effect of *p*H on dye fluorescence reached

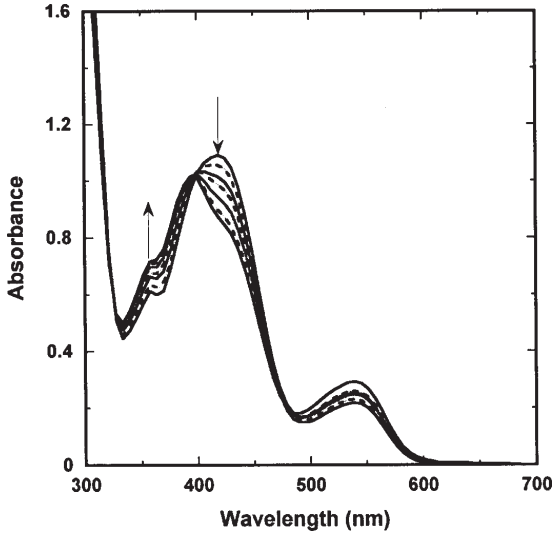

Fig. 2. The absorption spectra of **a1** in the absence and presence of hsDNA. Conditions: **a1** (40 μM) in DMF was titrated with 0.011, 0.055, 0.165, 0.385, 0.825, 1.265 μM hsDNA at 24 °C

a maximum in *p*H range 1.8-3.6. The fluorescence maxima were characteristic for each compound. Compound **a3**, a nitroderivate is distinguished by a higher blue fluorescence in high acidic condition while compound **a5** in lower acidic condition (fig.3).

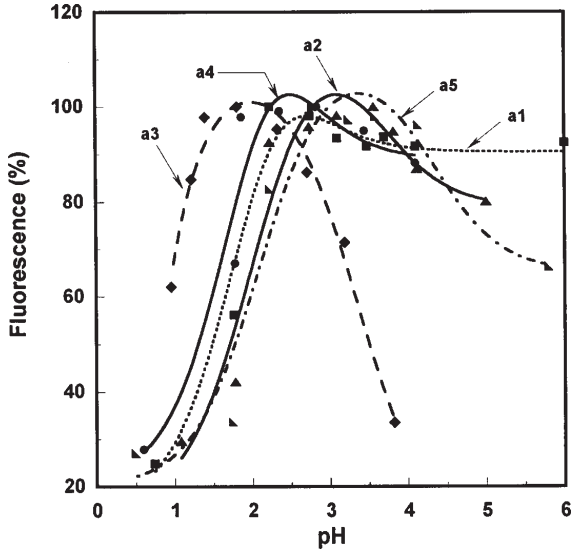

Fig. 3. pH dependence of dyes fluorescence in aqueous solution. Dye solutions were titrated with HCl. The fluorescence was measured in a 96 well plate using a blue filter (λ_{ex} = 490 nm; λ_{em} = 510 -570 nm). For a better comparison the values were normalized (the maximum fluorescence values corresponding to a1, a2, a3, a4 and a5 are 148467, 154747, 17902, 127036, 125836 – arbitrary units).The fits were made using Dixon equation [13]

The experimental results indicate that maxima fluorescence of those compounds varies as follow: **a2** > **a1** > **a5** or **a4** > **a3**. However, the fluorescence intensity is relatively higher in *p*H range 2.5-4.5. In general, at high *p*H values the solubility of dyes was very low and these compounds were sticked on *p*H electrode, this behaviour being attributed to the ylide formation.

Quenching of fluorescence by DNA

The preliminary investigation shows that addition of hsDNA or pUC19 would quench the fluorescence of **a1**. At lower DNA concentration (0-100 μM), the fluorescence decays linearly, then reaches a certain value (30% from initial fluorescence for **a2**, **a4** and **a5** and 50% for **a3** at

330 μg DNA/mL) and increases slowly at higher concentrations (fig. 4).

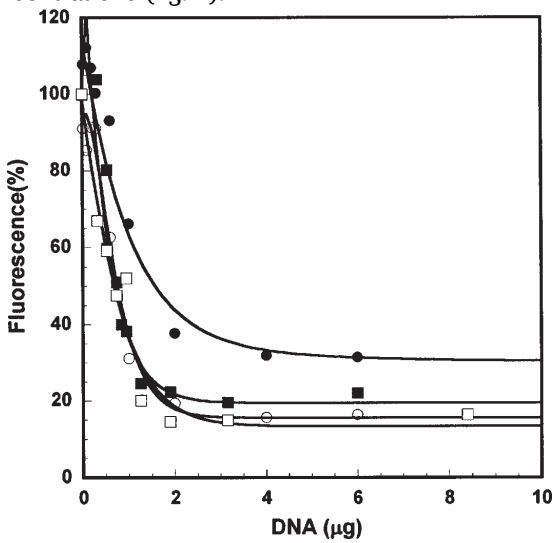

Fig. 4. Fluorescence quenching of **a1-5** at different DNA concentrations. The assay was performed in acetate 50 mM *p*H 4.5 (**a3** is unstable in this buffer). The dye (10 μM) was mixed with DNA and fluorescence was recorded as described before

Effect of dye concentration on fluorescence quenching

The fluorescence of **a1**-DNA system was investigated at different dye (**a1**) concentration. The fluorescence quenching extent has a maximum value at concentration of dye 6 μM (fig. 5).

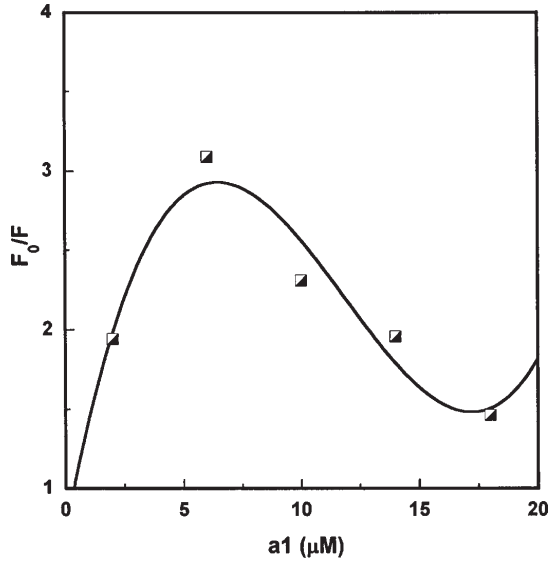

Fig. 5. The effect of **a1** concentration on quenched fluorescence. The DNA concentration was constant (6.25 μM) for all determinations (5 μg/ μL)

The disadvantage was that at this concentration values the measurement errors are quite big. Therefore, we used at higher dye concentration (10 μM) in all our experiments. At this concentration, the quenching extent is lower, but the accuracy is higher.

Effect of salt concentration

The fluorescence of **a1-DNA** system in the presence of sodium chloride (NaCl) was studied. Usually, the cations partially neutralize the negative charges of the DNA phosphate backbone. This class of ylides possess a quaternary cycloimmonium group which compete with the inorganic cations (in particularly with Na^+) and modify the intensity of fluorescence. In this experiment, it was found out that the salt concentration doesn't influence the fluorescence intensity (fig. 6). Thus, we could exclude the presence of electrostatic interaction.

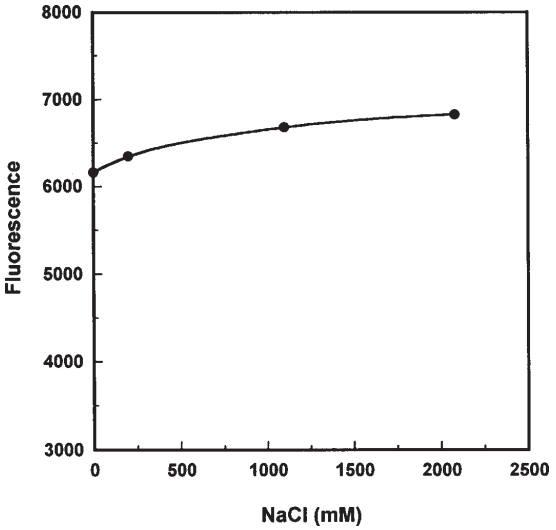

Fig. 6. Effect of salt concentration on **a1** fluorescence Conditions: **a1** (10 μM); buffer acetate 50 mM *p*H 4.5

KI quenching experiments

Potassium iodide (KI) and sodium nitrite ($NaNO_2$) are small compounds which posses quenching fluorescence properties. Initially, we used KI as a quencher, a salt which compete with DNA for **a1** fluorescence quenching. If the dye intercalates to DNA base pairs, this fluorescent molecule will be protected and consequently the quenching of the DNA-dye complex will be lowered. If the dye is bound outside the double stranded-DNA, this molecule will be exposed to the quencher (KI) and the quenching extent will be similar as in uncomplexed dye [14]. There are situations when the dye could interact with minor or major groove, and it will be partially protected by DNA, and iodide anion can partially quench its fluorescence.

Quenching behaviour of KI to **a1-DNA** system was investigated and the results are shown in figure 7. In the absence of DNA, the quenching extent was 7.4 times, while in the presence of DNA the quenching of **a1** was significantly lower (3.1 times).

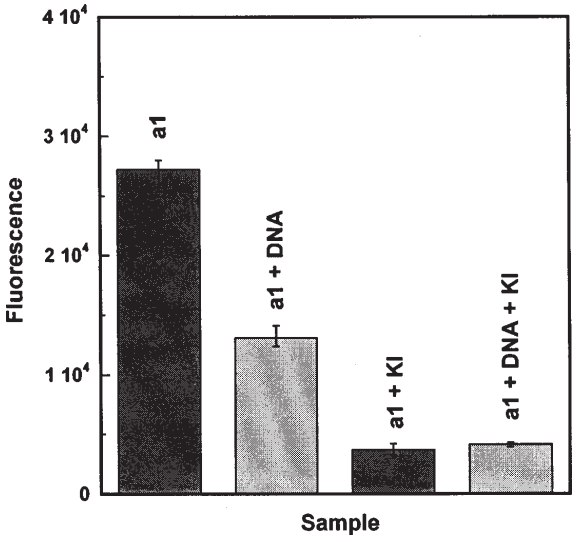

Fig. 7. The fluorescence quenching of KI for **a1** and **a1**-DNA systems Conditions: **a1** 10 μM, KI 20 mM, 5 μg hsDNA

As standard fluorescent dye we used ethidium bromide, a compound that bind tightly in the minor groove of DNA, and potassium bromide (KBr) or sodium nitrite ($NaNO_2$) as a small quencher. For standard, a moderate effect was observed when KI was used to quench its DNA partner. The most plausible explanation is that of ethidium iodine formation, and the consequence consists in a lowering of iodide anions concentration from solution. Replacement of KI with $NaNO_2$ was more efficient and the quenching extent was much higher. The both quenching experiments (using the standard and compound **a1**) lead to similar results. Therefore, we consider that the interaction mode of **a1** with DNA is groove binding mode.

Conclusions

In this study a new class of fluorescent compounds was used to study their spectral properties in the presence or absence of DNA. The interaction mechanism for binding of **a1** to hs DNA was studied by spectral methods and our results clearly suggest a similarly interaction way of **a1** and ethidium bromide with hsDNA. Compound **a1** is binding in the minor groove of DNA, displaying a lower fluorescence compared with free dye, and therefore its fluorescence was partially quenched.

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