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Mechanisms of Variation in Fluorescent Properties of bis-Benzimidazole Dyes

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Abstract – Spectral properties of a series of externally binding substrate-specific bis-benzimidazole dyes that were either bound or not bound to DNA were studied. Feasible mechanisms of variation in fluorescent properties of the dyes under study were considered depending on their chemical structure. Contribution of the rotational diffusion and the donor–acceptor interactions to the prediction and explanation of fluorescent properties of the substrate-specific dyes were discussed.

Key words: DNA, dyes, bis-benzimidazoles

Two hypothetical mechanisms have been recently suggested to account for the variations in fluorescent properties of externally binding dyes (DNA ligands, in particular) upon their specific interaction with substrates. First, the more developed hypothesis is based on the fact that only sufficiently rigid flat conjugated electron systems can fluoresce intensively [1, 2]. If the dye molecule contains fragments that are able to fluoresce actively but do not possess sufficient rigidity, then, not being bound to the substrate, it fluoresces weakly in aqueous solutions due to the high probability of nonradiative transitions from the excited state to the ground state due to the rotational diffusion of its fluorophores. In the case of specific interaction with the substrate, the molecule under consideration transforms into a flatter state and stabilizes. These processes exclude vibrational motions. Therefore, the fraction of radiative transitions and, consequently, the quantum yield for such a molecule should increase and, in the limit, should depend only on its excitability, which is determined by the number and character of potentially fluorogenic fragments.

The second hypothetical mechanism, along with these features of the ligand dye, takes into account its active, usually terminal groups, which can affect the π-electron system of its molecule and, therefore, inhibit fluorescence in the unbound state. It is assumed [3 - 5] that, upon interaction with DNA fragments, the inhibiting effect of these groups on the π-electron system of the dye molecule weakens, and the dye recovers its fluorescent properties. Such terminal groups appear to play the predominant role in the variation of the fluorescent features of monocompounds such as commercial fluorophores: DAPI, DIPI, etc. [3, 5 - 8]. However, their effect should also be taken into account for bis-compounds that contain two nuclei capable of its own active fluorescence (e.g., benzimidazoles [9]), but that are involved in the united conjugated electron system. The electron-donor groups should slightly increase the fluorescence of the dye in the unbound state, and electron-acceptor groups, on the contrary, should additionally inhibit it.

Thus, the study of limitations for the applicability of these two mechanisms to various systems is particularly needed in order to create the actual model for the explanation of variations of fluorescent properties of various substrate-specific dyes depending on their structure. Such a model can be useful for the search for optimal structures of new fluorescent probes for nucleic acids and other substrates.

Therefore, we performed a comparative study of the spectral properties of a series of bis-benzimidazole dyes:

2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(4-methyl-1-piperazyl)-benzimidazole (Hoechst 33258) (**I**),

2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-piperazyl)-benzimidazole (**II**),

2-[2-(4-ethoxyphenyl)-6-benzimidazolyl]-6-(4-methyl-1-piperazyl)-benzimidazole (Hoechst 33342) (**III**),

1,4-bis[6-(4-methyl-1-piperazyl)-2-benzimidazolyl]-benzene (**IV**), and

1,4-bis[6-(3-dimethylaminopropylcarbamoyl)-2-benzimidazolyl]benzene (**V**).

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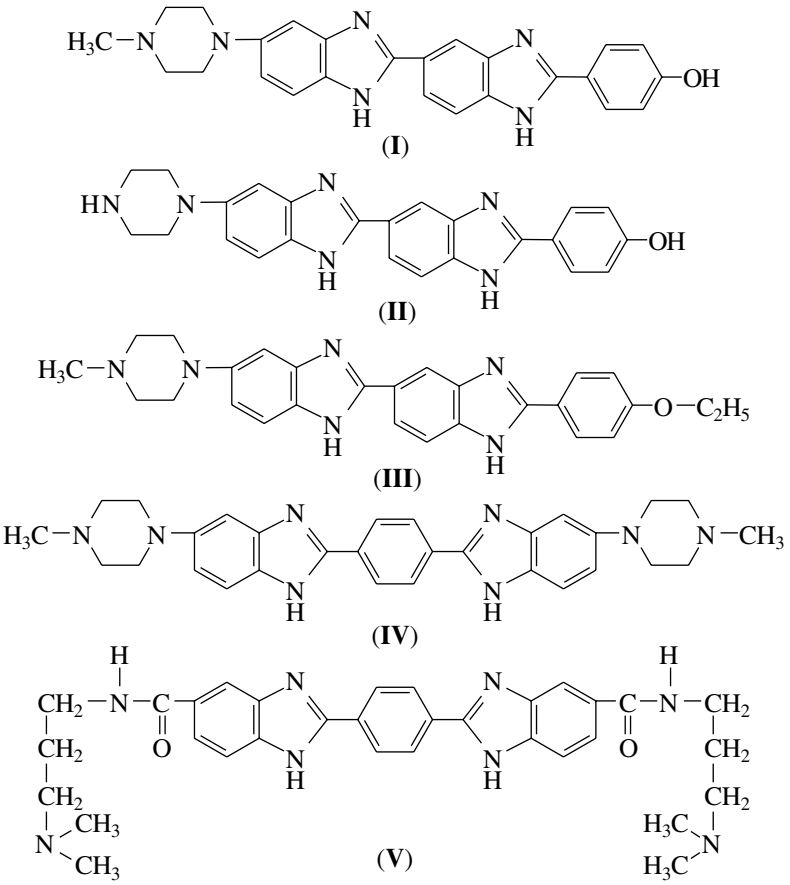


The maxima of absorption and fluorescence and quantum yields of the listed bis-benzimidazoles in the presence of various amounts of DNA in a buffer solution and in a 50% solution of sucrose, in which the rotational diffusion in such compounds is inhibited [10] are represented in the table.[2] The data given show that each of the discussed hypothetical mechanisms cannot by itself adequately explain the variations in the fluorescent properties of all the dyes under consideration.

The lower quantum yield in the absence of DNA for compound (**IV**) (φ_0) as compared with compound (**I**) can be accounted for by the less rigid bond of the benzimidazole rings because of the phenyl ring located between them. An increase in the φ_0 value for compound (**V**) in comparison with (**IV**) appeared to be a result of the interaction of its long and branched hydrophilic terminal groups with water. Such an interaction can, to some extent, reduce the probability of rotational diffusion of the molecule of this dye and, therefore, stabilize its planar state.

In principle, the same mechanism can explain the more than two-fold change in the zero quantum yield (φ_0) for the series of compounds (**I**) - (**III**). It seems that the bulky methyl group at the piperazine substituent of compound (**I**) can, to some extent, decrease the probability of rotational diffusion for its molecule in comparison with compound (**II**). The presence of an additional ethoxy group at the phenyl terminal substituent of compound (**III**) can reduce this probability to a greater extent.

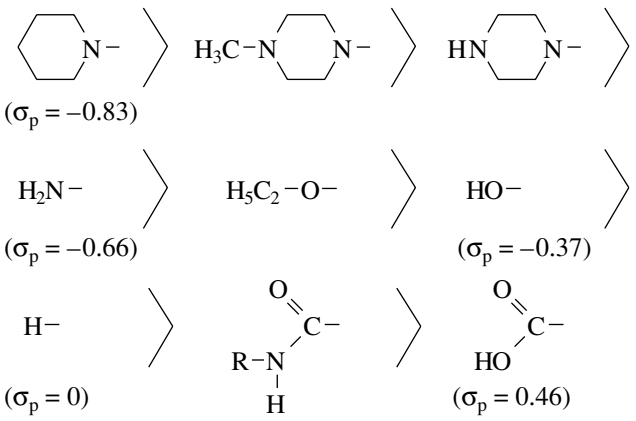
Along with this, the fluorescence of the dyes under consideration should be determined, first, by the interaction of the two benzimidazole fragments of the molecule. They are bound to one another more rigidly in compounds (**I**) - (**III**) than in compounds (**IV**) and (**V**) due to the occurrence in the latter compounds the intermediate phenylene bridge. Consequently, one can assume that rotational diffusion in the two latter compounds should be less affected by the terminal substituents. Second, the effect of methyl and ethyl terminal substituents on the rotational diffusion of benzimidazole fragments in compounds (**I**) - (**III**) can be additionally weakened since the bond between them is indirect. Third, the bulky and hydrophobic terminal substituents hinder the stabilization of the dye molecule through the

[2] All dyes under study have an additional absorption maximum in the range 260 - 280 nm with lower molar extinction both in the standard buffer and 50% sucrose solution. Due to the complex character of the terminal substituents, dye (**V**) exhibits the fine structure of emission spectra with maxima at 380, 400, 420, and 450 nm. An explanation of the possible reasons for the appearance of such a structure of the spectra is in [11], in which bis-benzimidazole dyes with similar terminal substituents were studied. In the table the data are given only for the main maxima of absorption and fluorescence of the compounds studied.

formation of hydrogen bonds with water molecules by the N atom of piperazine or the O atom of ethoxy group. As a result, we have an ambiguity in the explanation of the changes that are introduced by the terminal substituents into the rigidity of the planar structure of the molecules considered.

We propose that considering the data obtained in terms of different donor–acceptor activity of the terminal substituents is more adequate. For instance, compound (**III**), whose terminal groups have the highest electron-donor activity in the series of the compounds, has the most intensive fluorescence in the unbound state in solution. Compound (**I**), whose hydroxyl group is a weaker donor of electrons than ethoxy group of compound (**III**), has an accordingly lower quantum yield. Compound (**II**), whose piperazine terminal group is a weaker electron donor in respect to the aromatic nucleus of its molecule than the methylpiperazine group in compound (**I**), has, correspondingly, an even lower φ_0.

Note that to evaluate the relative electron-donor activity of the terminal substituents, we used well-known data. First, the nitrogen atom bound to an aromatic system with a single σ-bond is a stronger electron donor for it than the oxygen atom. Second, if such atoms have the opportunity to compensate for the donated electron density through their substituents (hydrocarbon, for example), their electron-donor capacity increases. On the contrary, when such substituents accept the division of the electron density, the corresponding electron-donor capacity of the atom with an unshared electron pair decreases. Third, the carboxylic group is a rather strong acceptor of the electron density, and the amino group bound to it can only slightly reduce the electron-acceptor capacity of it. Therefore, we can build the following series of terminal substituents, whose electron-donor activity with respect to the aromatic ring decreases from left to right and the electron-acceptor activity, on the contrary, increases when $\sigma_p > 0$.



where σ_p is the Hammett constant.

However, for dyes (**I**), (**IV**), and (**V**) the consideration based on the mechanism of donor–acceptor interactions leads to results opposite of those observed. In fact, according to this mechanism, the fluorescence of dye (**IV**) in the unbound state should be higher than that of dye (**I**), because the interaction of benzimidazole groups, inhibiting the fluorescence, is not reduced in dye (**I**) by intermediate phenylene group as for dye (**IV**), and the hydroxy group is a weaker donor of electron density than methylpiperazine group. Zero quantum yield, φ_0, for dye (**V**) should be lower than that for dye (**IV**), because dimethylaminopropylcarbamoyl substituents are acceptors, and methylpiperazine substituents are donors of electron density.

Variatio in the spectral properties of fluorescent dyes in dependence on the medium composition and on the presence of DNA

Dye	(**I**)	(**II**)	(**III**)	(**IV**)	(**V**)
λ_0^{abs}*	345	345	350	360	327
λ_0^{fl}	497	495	500	553	400
$\varphi_0 \times 10^{2}$**	4.3	3.2	6.8	2.1	26.7
λ_c^{abs}	352	350	354	368	340
λ_c^{fl}	474	474	475	516	400
φ_c/φ_0	7.0	6.9	6.4	8.4	2.0
λ_{max}^{abs}	353	350	355	370	346
λ_{max}^{fl}	455	455	455	500	400
φ_{max}/φ_0	41.6	39.6	39.1	38.8	13.8
E***	85.2	84.7	85.8	80.4	92.2
R***	14.8	15.3	14.2	19.6	7.8
E/R	5.76	5.54	6.04	4.10	11.8

* λ^{abs} and λ^{fl}, nm, are the maxima of the absorption and fluorescence of the dyes studied; subscripts 0, *c* and max belong to the values measured in a standard buffer, in a 50% solution of sucrose in a standard buffer, and in the buffer at the maximum ratio of the concentrations of DNA and the dye, respectively.

** φ_0, φ_c, and φ_{max} are relative quantum yields of fluorescence of the dyes studied in a standard buffer, in a 50% solution of sucrose in a standard buffer, and in buffer at the maximum ratio of the concentrations of DNA and the dye, respectively.

*** *E* and *R*, %, were the relative contributions of donor-acceptor interaction of the substituents with the aromatic ring and of the rotational diffusion, respectively, to fluorescence of the dyes upon their specific binding with DNA.

Thus, as is obvious from the given data, the first mechanism describes correctly the results in the series of dyes (**I**), (**IV**), and (**V**). On the contrary, in the series of dyes (**I**) - (**III**), the variations in the fluorescent properties are better described by the second donor–acceptor mechanism. In addition, an increase in the quantum yield from φ_0 to φ_{max} upon interaction with DNA cannot be explained in terms of "clean" processes of rotational diffusion even for the first series of dyes (**I**), (**IV**), and (**V**).

Similar results were obtained in the study of the relative variation in the fluorescence of dyes (**I**) - (**V**) in a 50% solution of sucrose, which inhibits rotational

diffusion. The values of absorption and emission maxima and relative quantum yields (φ_c/φ_0) were intermediate between those obtained in the standard buffer in the unbound state and those obtained upon saturation with polynucleotide. The ratio of the contributions of donor–acceptor interaction (*E*) and rotational diffusion (*R*) in the variation of the fluorescence of the dyes under study changed along with their zero quantum yields (φ_0). The occurrence of both these contributions for all the dyes under consideration, as well as the character of the change in the ratio *E*/*R*, confirm the dual character of their coupling with DNA.

Thus, it is evident that both mechanisms of the variation in fluorescent properties of the dyes under consideration upon their specific interaction with DNA have limitations in their applicability. In order to predict the relative variation in the fluorescent properties of substrate-specific dyes depending on their chemical structure, one should take into account two factors. First, the steric conformity of the dye molecule to the substrate and the possibility of the specific interaction between them are important. For instance, dye (**V**), whose long and branched terminal radicals can inhibit such an interaction, has quite a low relative quantum yield in the presence of DNA (see the table). It seems that the dye molecule cannot intercalate into the narrow minor groove of DNA at the distance sufficient for the formation of hydrogen bonds with AT pairs. As was shown earlier [4, 7, 12, 13], only this type of interaction is necessary for the strong specific interaction of externally-bound fluorescent dyes with polynucleotide. Second, the structure of potentially fluorogenic dye fragments should be taken into account. In our case, the similar structure of these fragments (two benzimidazole rings) results in the closeness of the φ_{max}/φ_0 values for dyes (**I**) - (**IV**).

Despite the similarity in structures of the fluorescing fragments in the molecules of the dyes compared, the differences in rigidity and planarity of these molecules are of importance. If there are such differences [as, e.g., for dyes (**I**), (**IV**), and (**V**)], one should primarily apply the first mechanism to predict the relative fluorescence of these compounds. If the dyes compared have the same degree of rigidity and planarity (as, e.g., dyes (**I**) - (**III**) considered in this work or monocompounds considered earlier in [3, 5 - 7]), their fluorescent properties should be predicted according to the mechanism of the intermolecular donor–acceptor interaction. Evidently, the latter mechanism is applicable not only to rigid flat electron systems.

As mentioned above, a 50% sucrose solution inhibits the rotational diffusion of the dyes studied. The results of the investigation of the dyes in this solution indicate the dual character of their interaction with DNA, which depends slightly on the initial degree of the planarity of their molecules. For instance, while the φ_0 value for compounds (**IV**) and (**V**) differs 12.7 times due to the different degree of rigidity of the planar structure of their molecules, the *E*/*R* ratio for them changes only 2.9 times. Moreover, the relative contribution of donor–acceptor interaction (*E*) in variation in fluorescence for all the compounds under study was 4 - 11 times higher than the contribution of rotational diffusion (*R*). These results also indicate that donor–acceptor interaction makes a substantial contribution to the mechanism of the variation in fluorescence of dyes, similar to those studied in this work, upon their specific binding with polynucleotides.

Thus, if a dye molecule does not have a sufficiently rigid and flat structure [which, in particular, can occur upon increasing the molecular length or the number of axes around which rotation of the fluorescing fragments is possible, as, e.g., by passing from monocompounds to bis-compounds or from dyes (**I**) - (**III**) to dyes (**IV**) and (**V**)], it seems that the mechanism of the rotational diffusion plays the predominant role in the variation of its fluorescent properties. If the structure of the dye molecule is rigid and sufficiently planar, the donor–acceptor mechanism plays the main role. This mechanism should also be taken into account when the molecules of the dyes compared, even those with insufficient rigidity, slightly differ in their capacity for rotational diffusion [as in the case of compounds (**I**) - (**III**)], or have a single fluorescing fragment (as for the monocompounds considered in [3, 5 - 7]).

Hence, to evaluate the fluorescent properties of substrate-specific dyes, one should take into account both of the mechanisms considered. Such an approach allows one more adequately to formulate the requirements for the chemical structure of newly synthesized fluorescent probes both for nucleic acids and, probably, for other substrates.

EXPERIMENTAL

Dyes (**I**) (Hoechst 33258) and (**III**) (Hoechst 33342) were purchased from Serva (Germany). Compounds (**II**), (**IV**), and (**V**) were synthesized at the Department of Molecular Biotechnology of St. Petersburg Institute of Technology [14, 15]. Calf thymus DNA (Serva, Germany) (58% AT-pairs; average molecular mass per nucleotide of 326 Da, molar extinction coefficient, ε_{260} 6600 M^{-1} cm^{-1}) was used as a substrate. In order to homogenize DNA and decrease the light scattering, the DNA was processed preliminarily by ultrasound in an UZDN-2 apparatus for 15 s at current 0.3 A and resonance frequency 22 kHz (the average mass of the nucleotide fragments of the DNA molecule was 3500 Da).

All measurements were performed at a constant value of dye concentration $C_{dye} = 6.42 \times 10^{-7}$ M and various values of the concentration of oligonucleotide at 20 - 25°C. The substances were assayed in a buffer: 0.01 M NaCl, 0.01 M Na_2EDTA, and 0.01 M Tris (pH 7.4). The rather high ionic strength could, naturally, slightly change the φ_0 value, in particular, due to the different values of the charge at the terminal groups

of the dyes studied. The sucrose solution was prepared by adding one weight part of sucrose (analytical grade) to one volume part of the standard buffer.

The absorption spectra of the solutions studied were recorded on a Beckman 35 spectrophotometer (Austria). The fluorescence measurements were carried out on a Hitachi 850 spectrofluorimeter (Japan). The bandwidth was 3 nm for excitation and emission slits, the scan rate was 120 nm/min, response time was 2 s, photomultiplier attenuation was normal. Excitation spectra were recorded at λ_{max}^{tl}, and emission spectra were recorded at λ_{max}^{ex}.

The absorption and fluorescence spectra for the compounds studied essentially were not overlapped, and therefore, the calculation of the quantum yield ratios φ_{DNA}/φ_0 and φ_c/φ_0 was made from the peak values of absorption and fluorescence of the compounds at the corresponding wavelengths [5, 16, 17] using the equation

$$\varphi_2/\varphi_1 = (I_2/A_2)/(I_1/A_1),$$

where I_2, I_1 and A_2, A_1 were the values of the fluorescence intensity and optical density of the compounds studied in states 2 and 1. The fluorescence spectra were corrected using an alcohol solution of Rhodamine B as a quantum counter according to the manual to a Hitachi 850 spectrofluorimeter.

The φ_0 values were determined from equation:

$$\varphi_0 = (I_0/A_0) \times 10^{-4},$$

where I_0 and A_0 were the values of the fluorescence intensity and the optical density in the standard buffer in the absence of DNA. Evidently, the φ_0 value obtained differed from the characteristic zero quantum yield of the dye studied by a certain constant k. However, the relative value of the zero quantum yields φ_0^2/φ_0^1 for the compounds under study was retained. Such an approach seemed appropriate, because in this work we considered mainly variations in the ratios, not in absolute values.

The relative contributions of the rotational diffusion (R, %) and donor–acceptor interaction (E, %) in the variation of the fluorescence of the compounds under study upon their interaction with DNA were determined as follows:

$$R = (\varphi_c/\varphi_0 - 1)/(\varphi_{max}/\varphi_0 - 1) \times 100; \quad E = 100 - R.$$

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