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Variation of Spectral Properties of Actinocin Derivatives due to Equilibrium Transformations*

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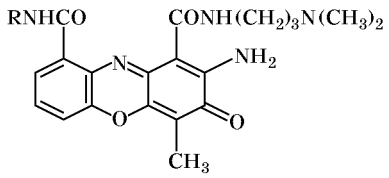
Abstract—Electron absorption, luminescence excitation, and emission spectra of 8-butylcabramoyl-, 8-cyclohexylcarbamoyl-, 8-dodecylcarbamoyl-, and 8-octadecylcarbamoyl-1-(3-dimethylaminopropylcarbamoyl)-2-amino-4-methyl-3*H*-phenoxazin-3-ones were studied in aqueous media with different pH values, in 2-propanol, and in octane. The compounds were presumed to exist as equilibrium mixtures of several forms. The highest fluorescence quantum yield was observed for alkaline aqueous solutions and solutions in nonpolar solvent where the predominant form is characterized by smaller number of intramolecular hydrogen bonds. A procedure was proposed for quantitative estimation of the overall donor–acceptor effect of the alkylamino groups in compounds existing in solution as equilibrium mixtures of several forms.

We previously studied the luminescence mechanisms of compounds capable of binding to nucleic acids without intercalating into the polynucleotide [1–4]. It was shown that enhanced fluorescence of such compounds is the result of both spatial stabilization due to formation of a planar structure and reduction of acceptor effect of terminal groups on the electronic density of the central fragment responsible for the luminescence properties. In continuation of these studies we turned to nucleic acid-intercalating compounds. In particular, a number of actinocin derivatives were examined, for actinocin can be regarded as chromophore of Actinomycine D which is a DNA-intercalating antibiotic [5, 6]. Taking into account our previous conclusions and the fact that the rigid planar phenoxazine core is the only fragment in their molecules capable of strong fluorescence, donor–acceptor effect of the terminal groups was presumed to be the main factor determining variation of luminescence properties of such compounds. Therefore, in the present work we examined 1,8-biscarbamoyl-2-amino-3*H*-phenoxazin-3-one derivatives **I**–**IV** differing by the length of the hydrocarbon chain in the radical attached to the 8-carbamoyl nitrogen atom; we thus were able to correlate spectral parameters of these compounds with their structure:



I–**IV**

I, R = $CH_3(CH_2)_3$; **II**, R = cyclohexyl; **III**, R = $CH_3(CH_2)_{11}$; **IV**, R = $CH_3(CH_2)_{17}$.

Figure 1 shows the absorption, fluorescence excitation, and fluorescence emission spectra of compounds **I**–**IV** in aqueous buffer with a low ionic strength and pH 8.0 (see also Table 1). The electron absorption spectra of **I**–**IV** are characterized by the presence of several bands in the ultraviolet (λ 190–400 nm) and visible regions (λ 400–600 nm) (Fig. 1a). In the fluorescence excitation spectra (Fig. 1b) only two maxima are observed in the region λ 190–400 nm.

In order to elucidate the reasons for such inconsistency between the absorption and fluorescence excitation spectra of compounds **I**–**IV** we examined their solutions in aqueous buffers in the pH range 6.0–9.0. It is known that at pH > 9 phenoxazine ring

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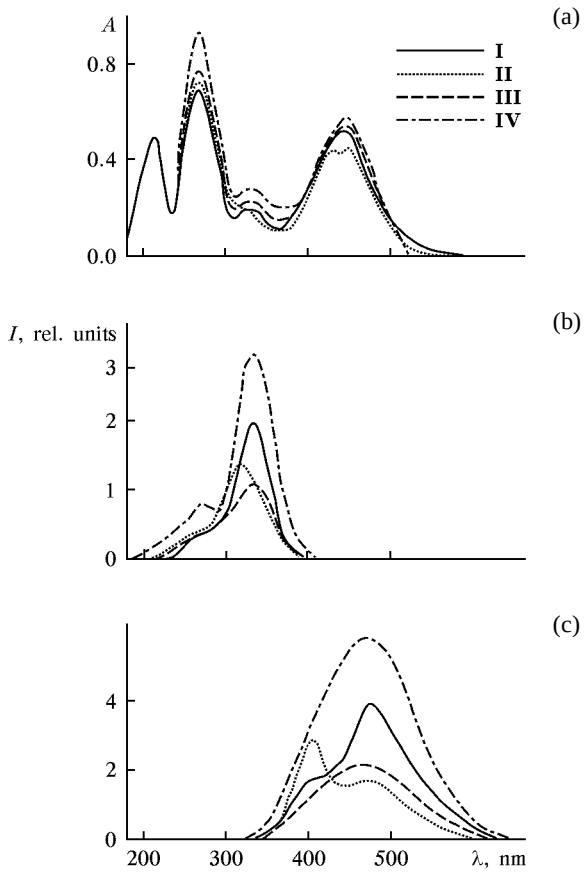


Fig. 1. (a) Electron absorption, (b) fluorescence excitation, and (c) fluorescence emission spectra of 2-amino-3*H*-phenoxazin-3-ones **I**–**IV** in aqueous buffer with pH 8.0.

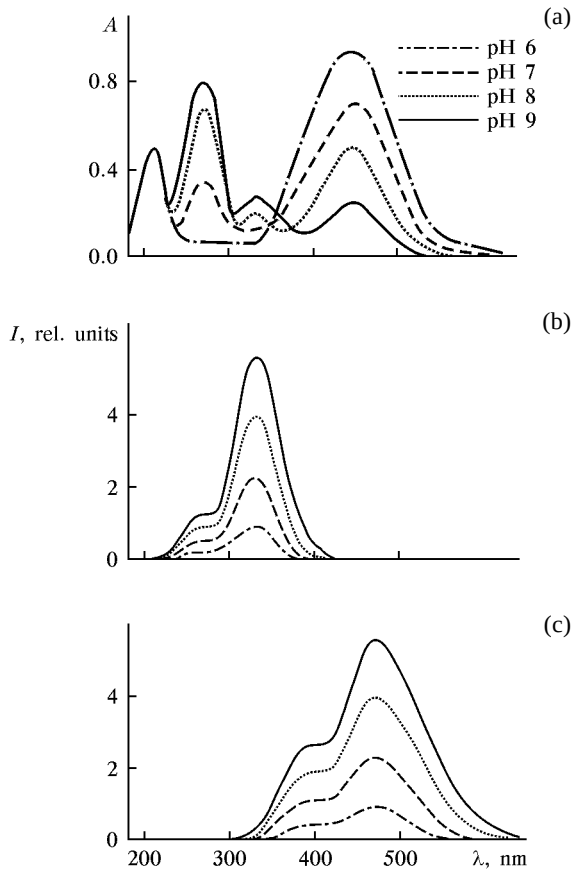


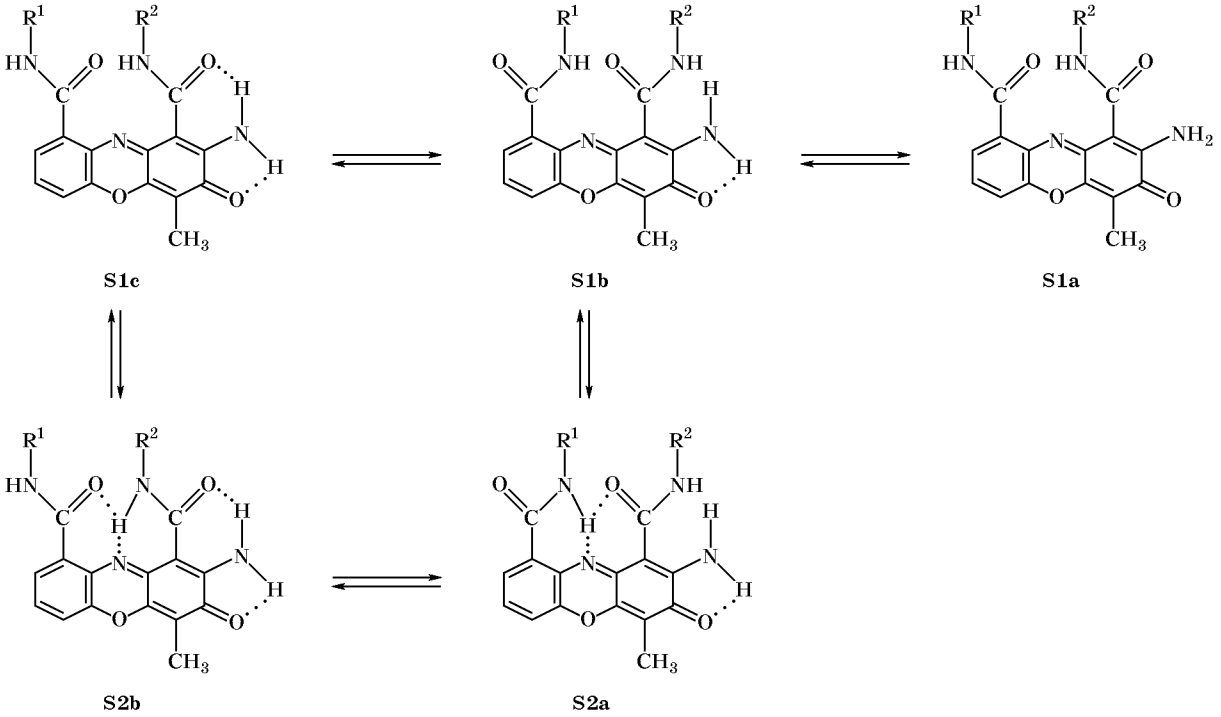
Fig. 2. (a) Electron absorption, (b) fluorescence excitation, and (c) fluorescence emission spectra of compound **I** in aqueous buffers with different pH values.

undergoes hydrolysis with irreversible cleavage of the C–O bond [7] or decomposition [8] and that at pH < 5 protonation of the nitrogen atom is possible [9]. The absorption maximum at λ 440 nm in the spectrum of **I** decreases, while the maxima at λ 270 and 330 nm decrease as pH rises. Simultaneously, the maxima at λ 270 and 330 nm in the fluorescence excitation spectrum and at λ 400 and 470 nm in the emission spectrum increase. Similar variations are observed in the spectra of compound **II**. Compounds **III** and **IV** show only one emission maximum at λ 450 nm (which can be regarded as a superposition of the fluorescence emission maximum typical of compound **I** with maxima at λ 400 and 470 nm; see below). Unlike phenoxazine derivatives **I**, **III**, and **IV**, the absorption spectrum of **II** is characterized by shift of the maximum at λ 330 nm to 315 nm. Finally, compound **II** shows two absorption maxima in the visible region (λ 430 and 450 nm), whereas in the spectra of **I**, **III**, and **IV** only one peak is present in that region at λ 440 nm.

The above data suggest that compounds **I**–**IV** in aqueous buffer solutions exist as equilibrium mixtures of at least two monomeric forms: **S1** and **S2**. The first of these (**S1**) predominates at higher pH values; it absorbs only in the UV region and gives rise to relatively strong fluorescence. The second form (**S2**) absorbs mainly in the visible region and exhibits no fluorescence properties. Probably, compounds **I**–**IV** can also exist in dimeric forms. However, the dimeric form, despite more extended hydrogen bond system, differs only slightly from monomeric **S2** form in spectral parameters (the maxima at λ 430 and 450 nm in the absorption spectrum of **II** are likely to arise from **S2** and dimeric form; see Fig. 1). Presumably, no larger associates were formed since the concentrations of **I**–**IV** were relatively low.

Monomeric forms **S1** and **S2** can differ by the degree of protonation of the nitrogen and oxygen atoms. However, it is more probable that these forms differ by the number of intramolecular hydrogen bonds (Scheme 1). The lack of fluorescence properties

Scheme 1



in **S2** may be explained by the presence of a large number of hydrogen bonds which reduce the electron density on the phenoxazine core. The red shift of the absorption band of **S2** (λ 430–450 nm against 315–330 nm for **S1**) can also be attributed to more planar structure of molecule **S2** due to intramolecular hydrogen bonding, which favors conjugation between the heterocycle and substituents. The ability of Actinomycine D analogs to form intramolecular hydrogen bonds like those shown in Scheme 1 was presumed in [10–13] on the basis of their NMR spectra.

Spectral parameters of phenoxazine derivatives **I**–**IV** in 2-propanol are given in Fig. 3 and Table 1. It is seen that in going from aqueous buffer to 2-propanol absorption maxima in the short-wave region (λ < 280 nm) are retained, while the maximum at λ 290–330 nm shifts by ~30 nm to the blue region. In addition, all compounds **I**–**IV** show in the visible region two absorption peaks with their maxima at 430 and 450 nm, which are likely to arise from **S2** and dimeric form. In aqueous buffer, two maxima were observed only for compound **II** (compounds **I**, **III** and **IV** showed only one maximum at λ 440 nm).

Unlike aqueous solutions, the fluorescence emission spectra of **I**V in 2-propanol contain three maxima at λ 350, 400, and 470 nm (Fig. 3c). Analogous structurization of emission spectra was also observed for other compounds with carbamoyl (RNHCO) substituents [4, 14]. We can conclude that the only fluorescence emission maximum of **III** and **IV** in aqueous buffer (λ 450 nm) is a superposition of emission maxima analogous to those found for compounds **I** and **II** at λ 400 and 470 nm.

Increase in the number of carbon atoms in the 8-substituent is accompanied by increase in hydrophobic properties. Compound **I** is readily soluble in water (ε = 80), and compounds **II** and **III** are readily soluble in 2-propanol (ε = 19); to obtain a solution of **IV** with a concentration greater than 100 μg/ml a medium with even lower dielectric constant is required (e.g., octane, ε = 2).

Comparison of the absorption and fluorescence spectra of **IV** in aqueous buffer (pH 7), 2-propanol, and octane (Fig. 4) showed the following. The ratio of the absorption maxima at λ_1 300–330 nm (**S1**) and λ_2 430–450 nm (**S2**) in 2-propanol (A_1/A_2 = 0.38) is greater than in aqueous buffer (0.14); however, the analogous ratio in octane (0.32) is also smaller than in 2-propanol. This fact may be regarded as an evidence that the main difference between **S1** and **S2** is the number of intramolecular hydrogen bonds rather than the degree of protonation. Otherwise, A_1/A_2 would change proportionally to dielectric constant ε of the solvent, so that in octane only one (nonprotonated) form of **IV** would be present. The observed pattern may be interpreted as follows. Taking into account the

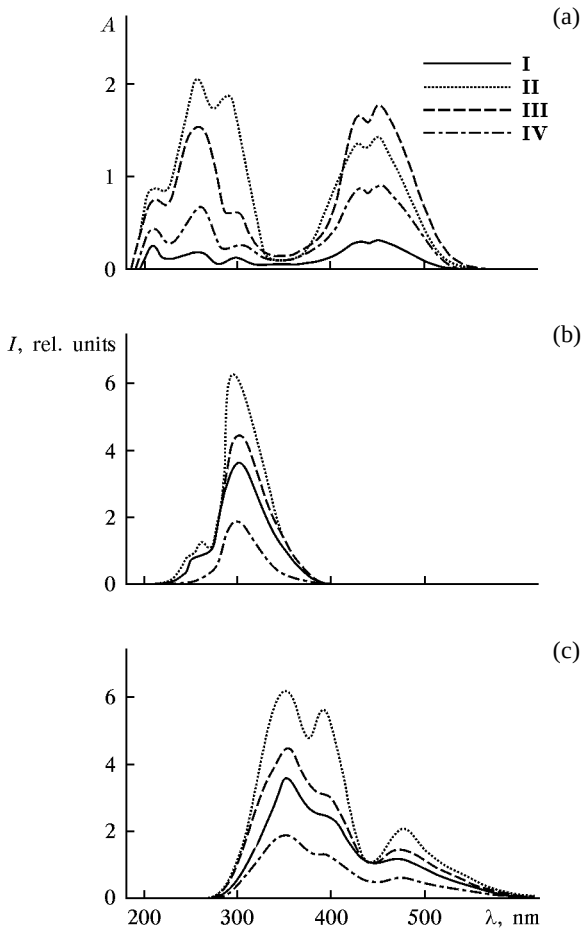


Fig. 3. (a) Electron absorption, (b) fluorescence excitation, and (c) fluorescence emission spectra of 2-amino-3*H*-phenoxazin-3-ones **I**–**IV** in 2-propanol.

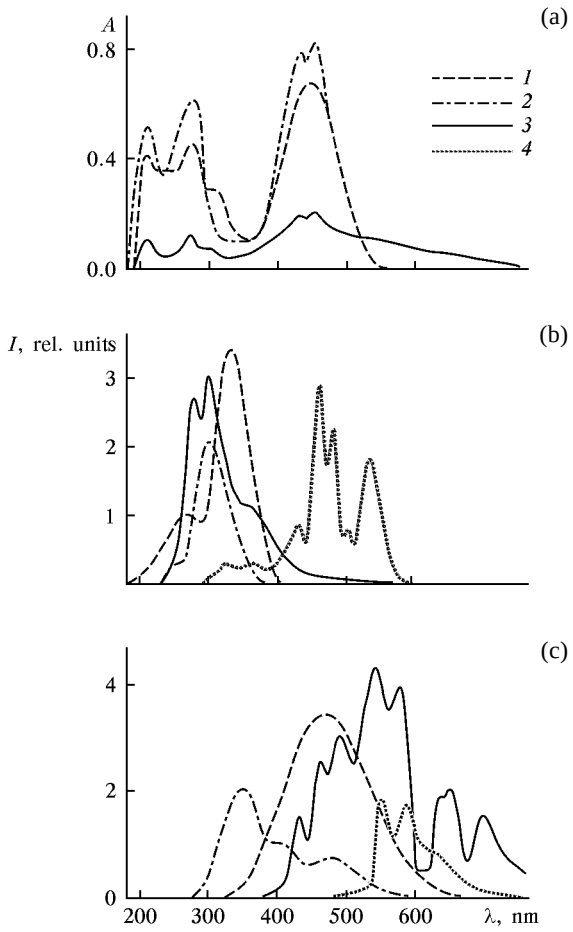


Fig. 4. (a) Electron absorption, (b) fluorescence excitation, and (c) fluorescence emission spectra of compound **IV** in (*1*) aqueous buffer (pH 7; λ_{exc} = 330 nm, λ_{em} = 470 nm), (*2*) 2-propanol (λ_{exc} = 300 nm, λ_{em} = 350 nm), and octane: (*3*) λ_{exc} = 300 nm, λ_{em} = 490 nm; (*4*) λ_{exc} = 460 nm, λ_{em} = 580 nm (scaled by a factor of 1/15).

ability of alcohols to form intermolecular hydrogen bonds with Actinomycine D [15], formation of intramolecular hydrogen bonds in **I**–**IV** is more favorable in water than in 2-propanol, and the equilibrium concentration of **S1** (and hence A_1/A_2) in 2-propanol should be greater than in water. Octane is incapable of forming intermolecular hydrogen bonds; therefore, the A_1/A_2 value for compound **IV** in octane should be smaller than in 2-propanol.

The absorption and especially fluorescence spectra of **IV** in octane are structurized even more strongly than in 2-propanol (Fig. 4). This might be expected, for less polar solvent should favor successful "separation" of electronic systems of different molecular fragments in fluorescing structure **S1** which is characterized by a small number of intramolecular hydrogen bonds. Moreover, compound **IV** in octane gives rise to one more fluorescing system: λ_{exc} 430, 460, 480, 500, and 530 nm; λ_{em} 540, 580, and 620 nm (Fig. 4b and 4c). Obviously, these maxima belong to associates in which polar groups are shielded from the solvent by aliphatic chains.

Figure 5 shows variation of the fluorescence quantum yield φ in aqueous buffer with pH 8.0 and 2-propanol with change in the number of carbon atoms in the 8-CONH-R substituent. In keeping with our previous conclusions [2–4] drawn for nucleotide-binding dyes, change in the luminescence properties in the series of compounds **I**–**IV** can be interpreted in terms of the mechanism of intramolecular donor–acceptor interaction between the heterocycle and the substituents. However, in [2–4] we took into account only one form of a compound in solution. In the case of compounds **I**–**IV**, at least two forms (**S1** and **S2**)

Table 1. Spectral parameters of compounds **I**–**IV** in aqueous buffer and 2-propanol

Parameter[a]	**I**	**II**	**III**	**IV**
Aqueous buffer (pH 8)				
$\lambda_{abs,1}$,* nm	210	210	210	210
$\lambda_{abs,2}$, nm	270	270	270	270
$\lambda_{abs,3}$, nm	330	315	330	330
$\lambda_{abs,4}$,* nm	–	430	–	–
$\lambda_{abs,5}$,* nm	440	450	440	440
$\lambda_{em,1}$, nm	400	400	–	–
$\lambda_{em,2}$, nm	470	470	450	450
$\varepsilon_1 \times 10^{-3}$	9.08	9.53	10.20	11.90
$\varepsilon_2 \times 10^{-3}$	6.84	5.92	7.10	7.60
$\mu \times 10^{-6}$	5.07	3.82	2.81	7.81
$\varphi \times 10^{4}$	9.25	8.05	4.95	12.85
2-Propanol				
$\lambda_{abs,1}$,* nm	210	210	210	210
$\lambda_{abs,2}$, nm	270	270	270	270
$\lambda_{abs,3}$, nm	300	290	300	300
$\lambda_{abs,4}$,* nm	430	430	430	430
$\lambda_{abs,5}$,* nm	450	450	450	450
$\lambda_{em,1}$, nm	350	350	350	350
$\lambda_{em,2}$, nm	400	400	400	400
$\lambda_{em,3}$, nm	470	470	470	470
$\varphi \times 10^{4}$	11.44	0.83	1.59	1.71
$\varphi_{i\text{-PrOH}}/\varphi_{H_2O}$	1.24	0.10	0.32	0.13

[a] λ_{abs} and λ_{em} stand for absorption and fluorescence emission maxima, respectively; ε_1 and ε_2 (l mol^{-1} cm^{-1}) are molar absorption coefficients corresponding to the greatest maxima in the ultraviolet ($\lambda_{abs,2}$) and visible region ($\lambda_{abs,5}$); μ (relative units, l mol^{-1} cm^{-1}) is the molar coefficient corresponding to the greatest maximum in the fluorescence excitation ($\lambda_{abs,3}$) and emission spectra ($\lambda_{em,2}$ in aqueous buffer and $\lambda_{em,1}$ in 2-propanol); φ is the fluorescence quantum yield corresponding to the greatest maxima in the fluorescence excitation and emission spectra; absorption maxima lacking in the fluorescence excitation spectra are marked with an asterisk.

should be considered; moreover, each form could give rise to several subforms (Scheme 1).

According to [5], increase in the number of carbon atoms in the R radical should lead to increase of electron density on the nitrogen and (to a lesser extent) oxygen atoms of the 8-CONH group in **I**–**IV**. This factor can affect the overall fluorescence quantum yield (with respect to all fluorescing **S1** subforms) in two ways: (1) by weakening the electron-acceptor power of the RNHCO group in the most actively fluorescing **S1a** state (φ increases) and (2) by favoring hydrogen bond formation with the nitrogen and oxygen atoms of the RNHCO group (as a result, the fraction of less actively fluorescing **S1b** and **S1c** states increases, and φ decreases). The joint action of these opposite factors is likely to determine the nonmonotonic change in φ with variation of C_R (Fig. 5). As follows from Fig. 5 and Table 1, the ratio of φ for aqueous buffer and 2-propanol is proportional to the number of carbon atoms in R, except for compound **II** in which the R group is cyclic rather than linear as in **I**–**III**.

Thus variation of spectral parameters of compounds **I**–**IV** is related to their existence in solution as equilibrium mixtures of several forms differing by the degree of donor–acceptor effect of the substituents on the heterocyclic core; the ratio of these forms depends on the pH value and solvent nature. The effect of a substituent in a molecular entity possessing fluorescence properties and existing in solution as an equilibrium mixture of *N* different forms can be expressed as follows (as applied to intramolecular donor–acceptor interaction):

$$\sigma_G = \Sigma \sigma_{Si} K_{Si}, \quad i = 1\text{–}N. \qquad (1)$$

Here, σ_{Si} are constants determining the effect of a substituent on the central entity of a compound in each of S*i* states; $K_{Si} = c_{Si}/c_G$ is the fraction of S*i* form (c_{Si} is the equilibrium concentration of S*i* form, and c_G is the overall concentration of a compound); and σ_G is a constant which determines the overall (with respect to all S*i* forms) effect of a substituent on the central fragment. The constants σ_{Si} depend only on structural factors, whereas K_{Si} strongly depends on the conditions; correspondingly, the overall constant σ_G also depends on the conditions.

To illustrate, let us calculate the parameters of compound **I** in aqueous buffers with different pH values. The experimental quantum yield φ_E changes with variation of pH (Table 2), indicating that at least two forms contribute to the resulting φ_E value; the ratio of these forms depends on the pH value. Assuming that the two forms are **S1** and **S2**, Eq. (1) can be written as follows:

$$\sigma_G = \sigma_{S1} K_{S1} + \sigma_{S2}(1 - K_{S1}); \qquad (2)$$

$$K_{S1} = c_{S1}/(c_{S1} + c_{S2}) = (A - A_0)/(A_{max} - A_0). \qquad (3)$$

Here, A is the absorbance of **I** at λ 270 nm (as noted above, only **S1** form absorbs in that region; see Fig. 2); $A_{max} = 0.9$, $A_0 = 0.05$ (the two latter values

were obtained from the titration curve of **I** at different pH values). Assuming $\sigma_{S1} = 1$, for $c_{S2} = 0$ we obtain $\sigma_G = \sigma_{S1}$ and $\varphi_G = \varphi_{S1}$, and for $c_{S1} = 0$, $\sigma_G = \sigma_{S2}$. Then, provided that $\varphi_G = a\,\sigma_G$, $a = \varphi_{S1}$ and hence

$$\varphi_G = \varphi_{S1}\,\sigma_G, \quad (4)$$

where φ_G is the overall quantum yield for the system under consideration, and φ_{S1} is the quantum yield for pure **S1** form.

By solving an additional system of two equations like

$$b_i = \varphi_{S1}\,d_i + \varphi_{S1}\,\sigma_{S2}(1 - d_i), \quad (5)$$

where $b_i = \varphi_{E,i}$ are the overall quantum yields, and $d_i = K_{S1i}$ are determined experimentally at different pH values), the remaining parameters of Eq. (3) can be calculated as follows:

$$\sigma_{S2} = (b_1\,d_2 - b_2\,d_1)/(b_2 - b_1 + b_1\,d_2 - b_2\,d_1); \quad (6)$$

$$\varphi_{S1} = b_1/(d_1 + \sigma_{S2} - \sigma_{S2}\,d_1); \quad (7)$$

$$\varphi_{S2} = \varphi_{S1}\,\sigma_{S2} \quad \text{[see Eq. (4)]}. \quad (8)$$

Substituting into Eqs. (6)–(8) b_i and d_i values calculated for compound **I** from the experimental data at pH 6 and 7 (Table 2) gives: $\sigma_{S1} = 1.0$, $\sigma_{S2} = 0.757$, $\varphi_{S1} = 1.105 \times 10^{-3}$, $\varphi_{S2} = 8.360 \times 10^{-4}$.

The calculated parameters can be verified by substituting them into Eqs. (3) and (4) to calculate the theoretical quantum yields φ_T. It is seen that the theoretical values differ from those determined experimentally at pH 8 and 9 (φ_E, Table 2). This means that the proposed model is insufficiently valid. The same follows from the clearly underestimated value of φ_{S2} calculated for **S2** form of compound **I**. This discrepancy can be treated as an additional evidence that the observed fluorescence of the compounds under study is the sum of fluorescence of not two (**S1** and **S2**, as in the case of absorption spectra) but greater number of equilibrium species (Scheme 1).

EXPERIMENTAL

Compounds **I**–**IV** were synthesized by the procedure reported in [16]. The products were chromatographically pure (chloroform–methanol–ammonia, 8 : 2 : 0.5, by volume; NH_3-saturated chloroform–methanol, 20 : 3, by volume). Their melting points and molar absorption coefficients did not change after two successive recrystallizations.

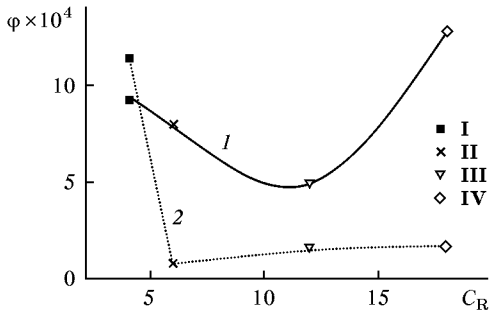


Fig. 5. Variation of the fluorescence quantum yields φ of compounds **I**–**IV** in (*1*) aqueous buffer with pH 8.0 and (*2*) 2-propanol with change in the number of carbon atoms C_R in the R radical.

The electron absorption spectra were recorded on a Beckman Model 35 spectrophotometer (Austria). The fluorescence measurements were performed using a Hitachi Model 850 spectrofluorimeter (Japan) (slit width 3 nm, scan rate 120 nm/min, response time 2 s, normal amplification of photoelectron multiplier). Unless otherwise stated, the fluorescence excitation spectra were recorded at a wavelength corresponding to the maximal emission, and the emission spectra were obtained at a wavelength corresponding to the maximal excitation. All spectra were recorded against background solution. Standard 1-cm square-section cells were used. The fluorescence spectra were corrected with the aid of a quantum counter on the basis of a standard alcoholic solution of Rhodamine B.

Aqueous buffer solutions contained 0.01 mol/l of NaCl, 0.01 mol/l of ethylenediaminetetraacetic acid disodium salt (Serva), and 0.01 mol/l of tris(hydroxymethyl)aminomethane (Serva); the pH was adjusted to a required value (6.0–9.0) by adding 2 M hydro-

Table 2. Calculated and experimental parameters of compound **I** in aqueous buffers with different pH values

Parameter[a]	pH 6	pH 7	pH 8	pH 9
$\varphi_E \times 10^4$	8.50	9.25	10.35	10.71
K_{S1}	0.05	0.33	0.53	0.79
σ_G	0.769	0.837	0.886	0.949
$\varphi_T \times 10^4$	8.50	9.25	9.79	10.49

[a] φ_E is the experimental fluorescence quantum yield of compound **I** at λ_{exc} = 330 nm and λ_{em} = 470 nm; φ_T is the theoretical fluorescence quantum yield; K_{S1} is the fraction of **S1** form (λ_{max} 270 nm) with respect to the overall concentration of **I**; and σ_G is a parameter characterizing the overall effect of substituents on the fluorescence properties of **S1** and **S2** (it is used in the calculation of φ_T).

chloric acid or 1 M aqueous sodium hydroxide. 2-Propanol (100%) and octane of cp grade were used; the other reagents were also of cp grade.

All measurement were performed at 20–25°C. The concentrations of compounds **I**–**IV** were 7.6×10^{-5} M. The molar absorption and fluorescence coefficients were determined, respectively, as $\varepsilon = A/c$ and $\mu = I/c$, where A and I are the optical density and fluorescence intensity, and c is the concentration. The fluorescence quantum yields φ were determined relative to a 1 M solution of quinine sulfate in 1 M sulfuric acid as reference ($\varphi_{st} = 0.55$) using formula (9) [4]:

$$\varphi = (\varphi_{st} A_{st} I)/(A I_{st}), \quad (9)$$

where A is the optical density corresponding to the greatest maximum in the fluorescence excitation spectrum, and I is the fluorescence intensity corresponding to the greatest maximum in the fluorescence emission spectrum.

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