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Synthesis and Spectral Properties of a Hydrophobic Fluorescent Probe: 6-Propionyl-2-(dimethylamino)naphthalene†

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ABSTRACT: Environmentally sensitive fluorescent probes involve two groups, an electron donor and an electron acceptor, attached to an aromatic ring system, and maximal effects may be expected when these groups are as far apart as feasible. The synthesis, characterization, and spectroscopic properties of 6-propionyl-2-(dimethylamino)naphthalene (PRODAN), a compound that fulfills these conditions, are described. The maximum of emission is at 401 nm in cyclohexane solution and at 531 nm in water solution, indicating an increase of dipole moment of ~20 D units on excitation to the lowest singlet state. The effect of temperature upon the spectral distribution and the bandwidth of fluorescence of PRODAN in 1:1 complexes with albumin shows the existence of a dynamic relaxation process of the protein surroundings within the 2–4 ns of the fluorescence lifetime.

The red shift of the fluorescence spectrum with increasing polarity of the solvent environment has been the object of both theory and experiment (Oochika, 1954; Lippert, 1957; Bakhshiev, 1964). It is agreed that this red shift is dependent upon a large increase in dipole moment in the fluorescent state over that of the ground state, and it is possible to foresee qualitatively the possibility of such enhancement of the dipole moment on excitation for some aromatic structures. The fluorescence from the unsubstituted aromatic hydrocarbons may be expected to show little sensitivity to the environment on account of the high degree of symmetry of the ground and lowest singlet excited states (e.g., Platt, 1964). The attachment to the aromatic ring system of two groups which are respectively a good electron donor and a good electron acceptor results in a lowest excited state with an important charge-transfer character that results from loss of charge by the donor group and its gain by the acceptor. The most important potential donor is the amino or alkylamino group, and the best acceptors are probably the S=O and C=O groups. The attachment of these groups at different points of the aromatic ring structure produces similar, though not identical, results, and at present a wholly empirical approach to these secondary effects seems the most advisable course in planning the synthesis of suitable polarity-sensitive fluorescent molecules. However, other things being equal, we can expect strongest effects when the distance between donor and acceptor groups is a maximum. In a naphthalene derivative this condition is fulfilled when donor and acceptor groups are attached to the 2 and 6 positions of the rings.

This paper describes a general method for the preparation of 2-(dimethylamino)-6-acylnaphthalenes, substances which offer outstanding sensitivity of the fluorescence to the polarity of the environment, and reports some of the properties of one member of the family, 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN).[1]

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[1] Abbreviations used: PRODAN, 6-propionyl-2-(dimethylamino)-naphthalene; PROMEN, 6-propionyl-2-methoxynaphthalene; ANS, 8-anilino-1-naphthalenesulfonic acid; bis(ANS), 4,4′-bis(1-anilino-8-naphthalenesulfonic acid); HMPA, hexamethylphosphoramide.

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Table I: Spectral Properties of PRODAN Solutions

no.	solvent	absorption max (nm)	emission[c] max (nm)	Stokes shift (cm^{-1})	$\Delta\bar{\nu}_{1/2}$[b] (cm^{-1})	Δf[d]	τ[a] (ns)
1	cyclohexane	342	401	4302	2151	0.001	1.6; 1.8
2	benzene	355	421	4416	2154	0.002	3.3; 3.4
3	triethylamine	343	406	4523	2416	0.102	
4	chlorobenzene	254	430	4992	2738	0.143	
5	chloroform	357	440	5284	2694	0.185	
6	acetone	350	452	6448	2976	0.287	
7	dimethylformamide	355	461	6477	2723	0.276	4.0; 4.6
8	acetonitrile	350	462	6926	2901	0.304	
9	ethylene glycol	375	515	7249	3530	0.274	
10	propylene glycol	370	510	7419	2487	0.270	3.8; 3.6
11	ethanol	360	496	7616	2525	0.298	
12	methanol	362	505	8206	2849	0.308	
13	water	364	531	8640	2614	0.320	2.1; 2.4

[a] The first figure is the fluorescence lifetime measured by phase delay; the second figure is the lifetime measured by relative modulation. [b] Bandwidth of the fluorescence emission measured as an interval between wavenumbers of 50% emission. [c] Corrected for grating transmission and detector response. [d] Δf = orientational polarizability defined by eq 1.

Materials and Methods

The preparation of PRODAN takes advantage of the recently developed method of substitution of a methoxy group by a tertiary amine by employing the corresponding lithium amine derivative generated in hexamethylphosphoramide solutions in the presence of benzene (Cuvigny & Normant, 1971). 6-Acyl-2-methoxynaphthalenes have been routinely prepared by Friedel-Crafts synthesis (Fries & Schimmelschmidt, 1925; Haworth & Sheldrick, 1934), and the method has been systematized by Buu-Hoï (1949). 6-Propionyl-2-methoxynaphthalene was prepared following Buu-Hoï's directions. It was recrystallized from methanol in 85% yield, mp 111–111.5 °C. For conversion to the dimethylaminonaphthalene derivative, the technique of Cuvigny & Normant (1971) was employed in the following form. Gaseous dimethylamine was introduced in a mixture of 19 mL of HMPA and 20 mL of benzene dried over molecular sieve until 3.5 g (0.077 mol) was dissolved. Li wire (0.5 g, 0.07 mol) divided in small pieces was added under argon, and the suspension was magnetically stirred. Formation of the lithium amine began after a few minutes with the development of a deep red color and some warming. After dissolution of the lithium, which occurred in a few hours, 4 g of PROMEN (0.018 mol) was added at once. After a few minutes a sample of the reaction mixture in ethanol already showed yellow color and strong green fluorescence. The reaction, carried out at room temperature throughout, was followed spectrophotometrically by the progressive appearance of an absorption band in the region of 340–400 nm and the development of a trough at the PROMEN maximum (303 nm) in alcoholic solutions of samples of the reaction mixture. After 12–24 h the spectrum was stable with time and the reaction was terminated by pouring the reaction mixture on ice. The product was extracted with ether and, after evaporation of solvent, recrystallized twice from ethanol: yield 3.4 g (80% of theory); mp 139–140 °C. Calculated composition: C, 79.26; H, 7.54; N, 6.16. Found: C, 79.14; H, 7.35; N, 6.13. The same methods of preparation have been used to obtain the 6-acetyl, 6-phenacetyl, and 6-lauroyl derivatives of 2-(dimethylamino)-naphthalene. All of these compounds are very similar, if not identical, to PRODAN in their spectroscopic properties. Fluorescence measurements were carried out as follows. The emission spectra were obtained by means of the instrument described by Jameson et al. (1976), fluorescence polarization was obtained by the photometer described by Jameson et al. (1978), and fluorescence lifetimes were obtained by the cross-correlation method of Spencer & Weber (1969) by employing an SLM phase fluorometer.

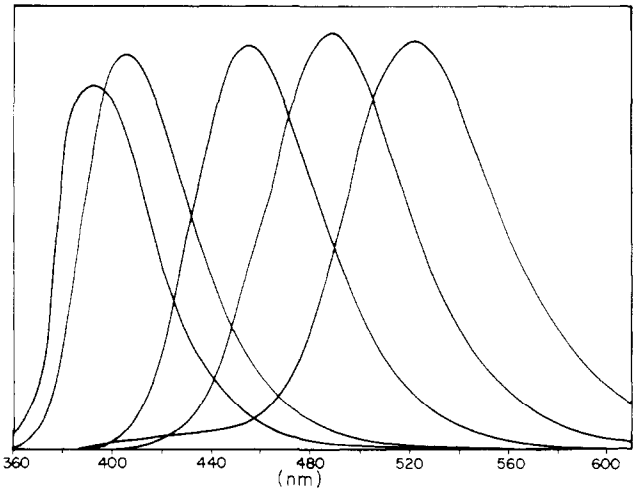

FIGURE 1: Technical fluorescence spectra of PRODAN solutions excited at 350 nm. The successive maxima correspond to cyclohexane, chlorobenzene, dimethylformamide, ethanol, and water. The heights do not reflect relative fluorescence yield.

Results

Absorption Spectrum of PRODAN. Ethanol solutions show a broad band with a maximum at 360 nm and a molar absorption coefficient of 18 400 cm^2 mM^{-1} and a deep minimum at 294 nm with a molar absorption of 3300 cm^2 mM^{-1}. From the molar absorption and the half-width of the S_0–S_1 transition (2525 cm^{-1}), the oscillator strength of this transition is estimated to be 0.45. The molar absorption coefficient of PRODAN in water solutions at 365 nm is somewhat smaller (14 500) with a wider bandwidth and a similar oscillator strength. The considerable oscillator strength is in good agreement with the fluorescence lifetimes of 2–4 ns observed in solutions with good fluorescence efficiency (Table I).

Fluorescence Properties of PRODAN. The solubility of PRODAN in media as different as cyclohexane and water is sufficient for spectroscopic studies. In Figure 1 a series of technical fluorescence spectra show the remarkable shifts with solvent polarity, the spectral maxima extending from 401 nm (corrected maximum for cyclohexane) to 531 nm (corrected maximum for water). The emission yield varies with the solvent, the maximum being found in solvents of medium polarity, like dimethylformamide, with a several-fold decrease in water and in cyclohexane. The fluorescence lifetimes, quoted for a few solvents in Table I, do not accurately parallel

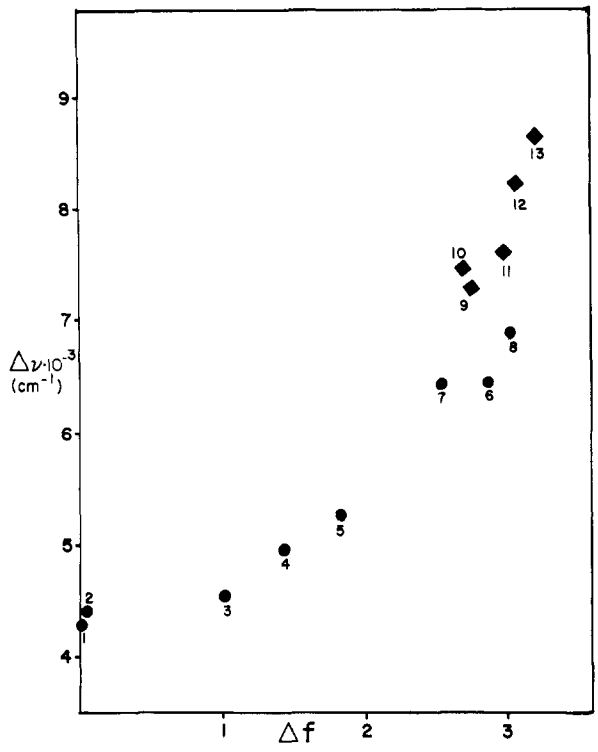

FIGURE 2: Lippert plot of corrected Stokes shift in wavenumbers against the orientational polarizability Δf of the solvents. Numbers below points identify the solvents of Table I.

the yield. The shift in solvent polarity, the most important fluorescence characteristic of PRODAN as regards its use as an environmental probe, will be discussed in relation to the dipole interaction theory of Lippert (1957). Figure 2 shows a plot of the Stokes shifts (absorption maximum − emission maximum) against the orientational polarizability of the solvent. This is defined by eq 1, where n is the refractive index

$$\Delta f = \frac{n^2 - 1}{2n^2 + 1} - \frac{D - 1}{2D + 1} \qquad (1)$$

of the solvent and D is its dielectric constant. Inspection of Table I shows that at similar values of Δf (compare ethanol and acetonitrile, both close to $\Delta f = 0.3$) the aprotic solvents produce considerably smaller Stokes shifts than those which can form a hydrogen bond by acting as proton donors. Similar effects have been previously noticed for other fluorophores (Lippert, 1957; Kawski & Bilot, 1964). A decision as to the real magnitude of this effect and its origin must await an examination of the emission shifts over a wide range of termperatures. For the moment we can give an estimate of the increase in dipole moment of PRODAN upon excitation to the fluorescent state. According to Lippert (1957)

$$\mu^* - \mu = \left(\frac{2a^3}{\Delta f} hc\Delta\bar{\nu}\right)^{1/2} \qquad (2)$$

where μ^* and μ are the dipole moments in the excited and ground state, respectively, a is the radius of the cavity scooped in the solvent to place the fluorophore, and $\Delta\bar{\nu}$ is the Stokes shift change upon a change in polarizability, Δf. The difference in Stokes shifts of the fluorescences from cyclohexane and water, respectively, amounts to 4338 cm^{-1} (Table I) while Δf changes by 0.32. From crystallographic data on naphthalene derivatives we can estimate the N to O distance in PRODAN as 8.4 Å, and with these figures $\mu^* - \mu = 20$ D. An estimate of the minimum value of $\mu^* - \mu$ may be obtained

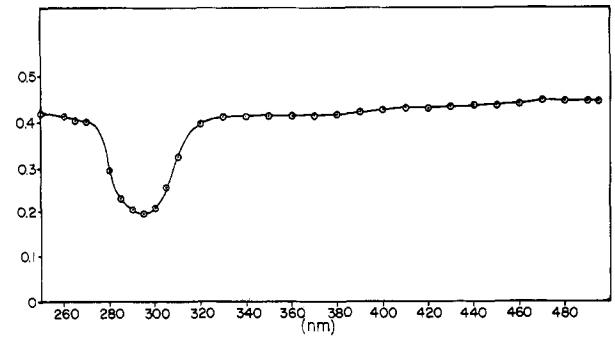

FIGURE 3: Fluorescence polarization spectrum of a glycerol solution of PRODAN at 20 °C. Abscissa is the wavelength of excitation; ordinate is the polarization owing to linearly polarized excitation.

independently by comparing the fluorescence spectral displacement in one solvent at two temperatures. The viscosity of the solvent at the lower temperature must be sufficiently high to impede all solvent relaxation during the fluorscence lifetime and sufficiently low at the higher temperature to permit readily the establishment of equilibrium between the Frank–Condon excited state and the relaxed state during the fluorescence lifetime. Propylene glycol at −50 and 20 °C comes close to fulfilling these conditions. The emission maximum in this solvent at 20 °C is at 510 nm, and at −50 °C it is at 458 nm; this gives a Stokes shift of 2226 cm^{-1}. If $\Delta f = 0.27$ at room temperature is introduced, eq 2 gives $\mu^* - \mu = 14.8$ D units. The difference of this figure with that obtained by comparison of the emissions in cyclohexane and water, 20 D units, arises from the residual orientation effects at low temperature because of the appreciable dipole moment of PRODAN in the ground state and is in reasonable agreement with an expectation of 5–7 D for this quantity. The dominance of the charge-transfer character of the S_0–S_1 transition is in agreement with the remarkable uniformity of the excitation–polarization spectrum, shown in Figure 3. Apart from a region close to 300 nm where a contribution from a transition normal to the charge-transfer transition decreases the limiting polarization, this is seen to be monotonous throughout with a value between 0.40 and 0.45.

Use of PRODAN as a Probe of Biological Environments. The dipole moment of the first singlet excited state of PRODAN is perhaps the largest observed in any fluorophore and provides for the use of PRODAN as a relaxation probe of various biological environments. One simple example will be discussed here. The solubility of PRODAN in water at room temperature is ~3.5 μM. Saturated solutions in water are readily obtained by stirring overnight a suspension of crystals and spinning or filtering the supernatant. Figure 4 shows the fluorescence spectra of the free probe in water and in water solutions 38–300 μM in bovine serum albumin. From the relative fluorescence enhancements we estimate the dissociation constant for the 1:1 molecular complex of PRODAN with albumin to be 1×10^{-5} M. Binding to albumin takes place with a fluorescence enhancement of about 3 and a blue shift of 2700 cm^{-1} which provides for easy separation of the fluorescence contributions from the free and bound probes (Torgerson et al., 1979). Comparison of the emission of albumin–PRODAN complexes at 1, 25, and 50 °C (Table II) shows a red shift with temperature characteristic of an environmental relaxation (Bakhshiev, 1964). Moreover, the half-width of the spectrum is 3306 cm^{-1} at 1 °C and 3480 cm^{-1} at 50 °C, values that exceed the half-width observed in pure solvents by several hundred wavenumbers and indicate an incomplete relaxation process of the protein surroundings,

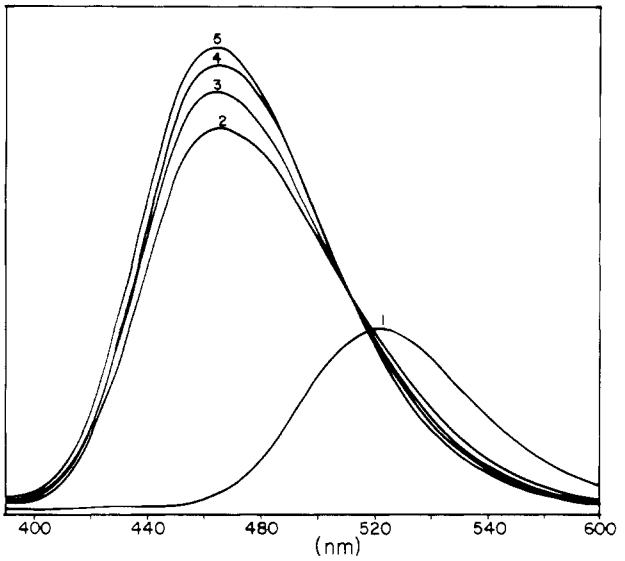

FIGURE 4: Fluorescence spectrum of PRODAN in water at 3 μM concentration. 2, 3, 4, and 5 are for the same PRODAN concentration and respectively 38, 75, 150, and 300 μM bovine serum albumin (excitation at 350 nm).

Table II: Fluorescence Spectral Characteristics of 1:1 Complexes of PRODAN with Bovine Serum Albumin in Water Solution

temp (°C)	corrected fluorescence max (nm)	bandwidth[a] (cm^{-1})
1	467	3306
25	469	3380
50	474	3480

[a] Defined as $\Delta\bar{\nu}_{1/2}$ in Table I.

conspicuously more extensive at 50 °C than at 1 °C. Observation of this process by direct decay methods (Ware et al., 1968; Brand & Gohlke, 1973) or by differential phase fluorometry (Weber, 1976) will be required to determine the kinetic constants of the relaxation process. These properties make PRODAN and in general the 6-acyl-2-(dimethylamino)naphthalenes valuable reagents in the study of processes of nanosecond relaxation of the protein matrix (Lakowicz & Weber, 1973; McCammon et al., 1977; Munro et al., 1978). Part of the usefulness of PRODAN in this respect is to be found in the absence of permanent charge, which makes it possible to eliminate contributions due to ionic interactions, as is the case with ANS and bis(ANS). A further advantage of PRODAN is the appreciable fluorescence yield of the free probe, which permits in many instances the separation of the contributions from two environments by analysis of the fluorescence spectrum alone. An example is given in the following paper (Torgerson et al., 1979) which describes the pressure effects of the inclusion complexes of PRODAN with poly-β-cyclodextrins.

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