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General and Specific Solvent Effects in Optical Spectra of *ortho*-Aminobenzoic Acid

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We describe studies about solvent effects on the absorption and emission properties of *o*-aminobenzoic acid (*o*-Abz), interpreting the results within the framework of general and specific solute-solvent interactions. Measurements were performed in several solvents and analysis of the absorption and emission wavelengths were made based on Lippert's model for general solvent effects and on the use of different parameters to describe the ability of the solvent to promote specific interactions with the solute. We observed low sensitivity of the Stokes shift upon changes in the medium polarity, and large deviation from the linearity predicted by Lippert's equation when the solvents were characterized as Bronsted acid in the Kamlet-Taft π^* scale. Quantum yield and fluorescence lifetimes were best interpreted based on the AN+DN scale used to describe the electron donor/acceptor properties of the solvent. The results indicated that non-radiative deexcitation processes are favoured in solvents which promote the formation of intramolecular hydrogen bond, while interactions with electron acceptor solvents lead to enhancement of fluorescence.

KEY WORDS: Aminobenzoic acid; solvent effects; fluorescence; Stokes shift; hydrogen bond.

INTRODUCTION

ortho-Aminobenzoic acid (*o*-Abz) has been used as an extrinsic fluorescent probe for peptides. Abz high quantum yield, 0.59 in ethanol [1], small size, a structure comparable to those of natural amino acids, and the possibility of connecting the molecule to the N^{α}-amino group of peptides without any significant change in its spectroscopic characteristics [2], qualifies *o*-Abz as a good extrinsic fluorescent probe for peptides. The only exception was found when *o*-Abz was directly bound to proline, due to the formation of the non-fluorescent pyrrolobenzodiazepine-5,11-dione [3]. Internally quenched fluorogenic peptides have been used as efficient substrates in proteolitic enzymes [4,5] and *o*-Abz, forming a pair with the acceptor *N*-[2,4-dinitrophenyl]ethylenediamine (EDDnp), was a convenient donor group in the study of peptide sequences that were substrates for tissue kallikreins [6,7]. More recently, the Abz-EDDnp pair was used to investigate the conformational dynamics of bradykinin related peptides, using Förster resonance energy transfer (FRET) [8], and to study the interaction of heparin with internally quenched fluorogenic peptides derived from heparin-binding consensus sequence, kallistatin and anti-thrombin III [9].

Ray [10] reported an intensity increase and blue-shift of the *o*-Abz fluorescence emission in the presence of cetyltrimethylammonium bromide (CTAB) micelles. Properties of Abz-labeled amino acids and peptides were studied in aqueous medium and in the presence of sodium dodecyl sulfate (SDS) micelles [11] and it was verified that the intensity and wavelength of fluorescence emission changed due to interaction of the compounds with the amphiphilic aggregates. The sensitivity of *o*-Abz to the changes in its environment suggested that it could be used as a probe for the interaction between peptides and model membranes: the interaction of Abz-labeled

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bradykinin and fragments with DMPG lipid vesicles was reported, stressing the importance of the lipid phase for the peptide-membrane receptor interaction, a fact that may be relevant to the peptide biological activity [12].

An adequate use of the fluorophore as a probe for peptides or other biomolecules requires the knowledge of absorption and emission fundamental properties in different environments. We describe in this paper studies about solvent effects in the absorption and emission of *o*-Abz, interpreting the results within the framework of general and specific solvent effects. Measurements were performed in several solvents and analysis were made based on Lippert's model for the general effects and on the use of different parameters to describe the polarity of solvent and its ability to promote specific interactions like hydrogen bonding with the solute.

MATERIAL AND METHODS

o-Abz was purchased from Sigma Aldrich (Steinheim, Germany). Solvents were purchased from Merck (Darmstadt, Germany) and, except for acetone and dioxane (PA grade), they were spectroscopic grade and used without any further purification.

Absorption spectra were obtained with a Hewlett-Packard HP 8452A spectrophotometer. For steady state fluorescence measurements a Jobin-Yvon Spex Fluorolog 3 spectrofluorimeter and a SLM Aminco 8000 instrument have been employed. Time-resolved experiments were performed using an apparatus based on the time-correlated single photon counting method. The excitation source was a Tsunami 3950 Spectra Physics titanium-sapphire laser, pumped by a solid state Millenia Spectra Physics laser. The repetition rate of the 5 ps pulses was set to 800 KHz using the pulse picker Spectra Physics 3980. The laser was tuned to give output at 930 nm and a third harmonic generator BBO crystal (GWN-23PL Spectra Physics) gave the 310 nm excitation pulses that were directed to an Edinburgh FL900 spectrometer. The L-format configuration of the spectrometer allowed the detection of the emission at right angle from the excitation. The emission wavelength was selected by a monochromator, and emitted photons were detected by a refrigerated Hamamatsu R3809U microchannel plate photomultiplier. The FWHM of the instrument response function was typically 45 ps, determined with a time resolution of 6.0 ps per channel. Measurements were made using time resolution of 48 ps per channel. A software provided by Edinburgh Instruments was used to analyse the decay curves, and the adequacy of the multi-exponential decay fitting was judged by inspection of the plots of weighted residuals and by statistical parameters such as reduced chi-square.

Concentrated stock solutions of *o*-Abz (10^{-3} M) were prepared in the different solvents. Diluted solutions (around 5×10^{-5} M) were employed in the experiments. The same sample was used in the optical absorption, steady state fluorescence and time-resolved fluorescence measurements.

RESULTS

Optical Absorption and Fluorescence Emission Spectra

In the non polar solvent cyclohexane, characterized by low dielectric constant, the near-UV optical absorption band of *o*-Abz presented maximum intensity located around 340 nm, with slight red shift in benzene and dioxane (Table I). That absorption band was moderately blue shifted in solvents with intermediate polarity like ethanol and methanol, and strongly displaced in water (maximum at 310 m). There is not a direct correlation between solvent polarity and absorption wavelength, for in high dielectric constant solvents like acetonitrile and dimethyl sulfoxide (DMSO) the band was positioned in the same region as non polar solvents, while in trifluoroethanol (TFE), which has relatively low dielectric constant (27.68), a considerable shift to 320 nm was observed (Table I).

The emission band of *o*-Abz in the less polar solvent cyclohexane was located in 390 nm and was red-shifted in the other solvents (Table I). The more pronounced shifts were observed in ethanol and methanol (maximum emission at 410 nm) and in TFE (emission at 430 nm). Differently of the absorption band, in the most polar solvent (water) *o*-Abz presented fluorescence emission in 397 nm, in the same spectral region as non polar dioxane, benzene and acetonitrile.

Table I. Dieletric Constant Values (ε) for the Solvents, Spectral Position of Near-UV Optical Absorption (λ_{abs}) and Fluorescence Emission (λ_{em}) Bands and Stoke's Shift ($\Delta\nu$).

Solvent	ε	λ_{abs} (nm)	λ_{em} (nm)	$\Delta\nu$ (10^3 cm^{-1})
Cyclohexane	2.02	338	387	3.75
Dioxane	2.21	338	395	4.27
Benzene	2.28	340	394	4.03
Acetone	20.7	336	394	4.30
Ethanol	24.55	334	407	5.37
TFE	27.68	320	423	7.61
Methanol	32.66	334	406	5.31
Acetonitrile	37.5	336	397	4.57
DMSO	47.0	342	400	4.24
Water	78.3	310	397	7.07

Stokes Shift

As the polarity of the solvent affects both ground and excited electronic states of the solute, a better description of spectral displacement of absorption and emission bands is provided by the analysis of the Stokes shift dependence with the solvent. General effects of the solvent are described by considering the fluorophore as an electric dipole residing in a cavity of radius a in a continuous medium of uniform dielectric constant ε and refractive index n. Calculations were performed using the estimated value of 3.0 Åfor the cavity radius, based on the molecular dimensions of *o*-Abz. According to the model developed by Lippert [13] and Mataga [14] the shift between absorption and emission wavenumbers is given by the equation:

$$\bar{\nu}_a - \bar{\nu}_f = \frac{2\Delta f}{hca^3}(\mu^* - \mu)^2 + \text{const}$$

where h is the constant of Planck, c is the speed of light and μ and μ^* are the electric dipole moments of the fluorophore ground and excited states respectively. From the Lippert's equation it is expected a linear dependence between the Stokes shift represented by the difference in wavenumbers $\Delta\nu$ and the so-called orientation polarizability Δf given by

$$\Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}$$

The results that we obtained for the Stokes shift of *o*-Abz in different solvents are plotted in Fig. 1. A linear behavior according to the Lippert's equation can be observed in a range of orientation polarizability comprising the majority of solvents used in this work. However a significant deviation from linearity was verified for some of the solvents (ethanol, methanol, TFE and water), suggesting the occurrence of specific interactions with the fluorophore. The results of experiments with dioxane-water mixtures are presented in Fig. 2. In low water/dioxane ratios, the plot is approximately linear, and specific interactions with water predominate when the v/v ratio was above 50/50.

Empirical Parameters

The deviation in the linearity indicates the occurrence of specific effects in the interaction between *o*-Abz and solvent molecules, not included in Lippert's model. Empirical parameters have been proposed to characterize solvents, and in the π^* scale from Kamlet-Taft (Table II), the value 0.0 is ascribed to cyclohexane that is unable to stabilize electric charges or dipoles in the solute, and values above 1.0 are given to solvents like DMSO and water which promote such stabilization [15]. Complementary to the π^* scale are the α [16] and β [17] parameters (Table II) describing the Bronsted acid or Bronsted base character of the solvent, respectively.

For several solvents the Stokes shift of *o*-Abz increases linearly as a function of π^* (Fig. 3). However, deviation from linearity was verified when using ethanol, methanol, TFE or water as solvent. In the Kamlet-Taft

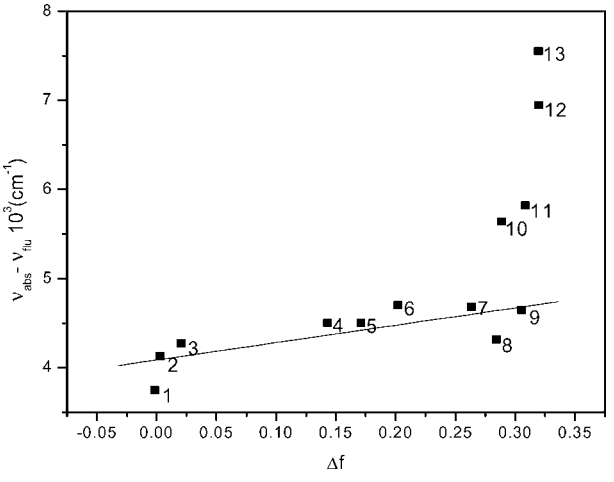

Fig. 1. Dependence of Stokes shift of *o*-Abz with orientation polarizability Δf in pure solvents. Fluorophore concentration 5×10^{-5} M, temperature 23°C. The solvents are: (1) cyclohexane, (2) benzene, (3) dioxane, (4) monochlorobenzene, (5) n-butyl-acetate (6) ethyl-acetate, (7) DMSO, (8) acetone, (9) acetonitrile, (10) ethanol, (11) methanol (12) TFE and (13) water.

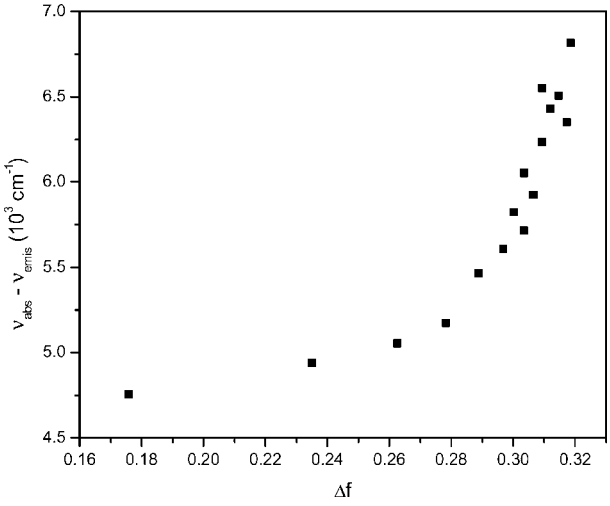

Fig. 2. Dependence of Stokes shift of *o*-Abz with orientation polarizability Δf in dioxane-water mixtures. Fluorophore concentration 5×10^{-5} M, temperature 23°C.

Table II. Parameters for the Solvents

Solvent	AN	DN	AN+DN
Cyclohexane	0.00	0.00	0.00
Dioxane	10.3	14.3	24.6
Benzene	8.2	0.1	8.3
Acetone	12.5	17.0	29.5
Ethanol	37.1	32.0	69.1
TFE	53.5	0.00	53.5
Methanol	41.3	30.0	71.3
Acetonitrile	18.9	14.1	33.0
DMSO	19.3	29.8	49.1
Water	54.8	18.0	72.8

Note: AN: electron acceptor; DN: electron donor.

scale, these solvents distinguish from the others (Table II) in presenting high values of α parameter, meaning that they have high Bronsted acid character (protic solvents). Reporting to Fig. 1, these are the solvents for which Lippert's model fails.

Another route to scale solvent effect was based on the capacity of the solvent to act as a Lewis acid or Lewis base, a donor or acceptor of electrons pairs. The AN number represents the electrophilic property of the solvent [18,19] and is related to the relative chemical shift of ^{31}P in triethylphosphine in the particular solvent, with hexane as a solvent reference. On the other hand, the DN number is representative of the basic (nucleophilic) property of the solvent molecule and is defined as the molar enthalpy for the reaction of the donor with $SbCl_5$ as reference acceptor [18,19]. Values for AN and DN parameters are presented in Table II, as well as the sum AN+DN, a term proposed

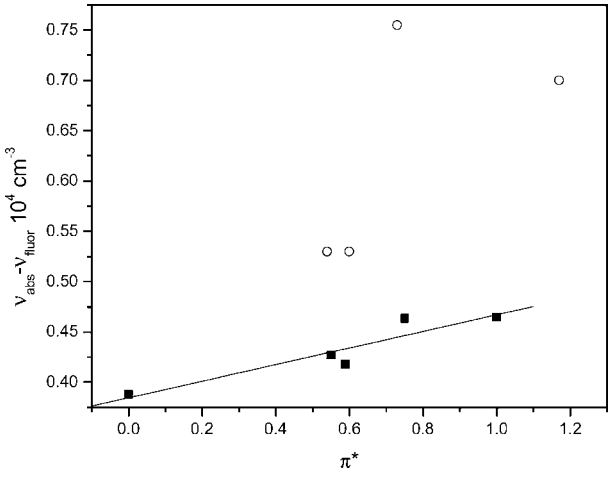

Fig. 3. Dependence of Stokes shift of *o*-Abz with π^* parameter of pure solvents. Fluorophore concentration 5×10^{-5} M, temperature 23°C. (■) Aprotic solvents; (□) Bronsted acid solvents.

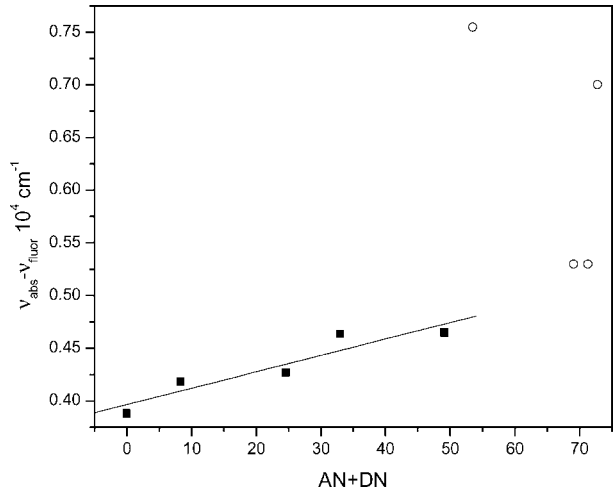

Fig. 4. Stokes shift of *o*-Abz as a function of AN+DN parameter. Fluorophore concentration 5×10^{-5} M, temperature 23°C. (■) Solvents with low AN values; (□) solvents with high AN values (electron acceptors).

to scale solvent effects, ranging from 0.0 (hexane) to 105.0 (trifluoroacetic acid) [18]. The plot of the Stokes shift as a function of the values AN+DN (Fig. 4) shows that also in this case deviation from linearity occurs for *o*-Abz in ethanol, methanol, TFE and water, that, differently from the other solvents, have high values of AN, which measures the electron acceptor properties of the solvent. Thus the above results allow us to say that *o*-Abz have specific interactions with solvents which are, both, electron acceptor and Bronsted acids.

Quantum Yield and Lifetime

The quantum yield of *o*-Abz in the different solvents (Table III) was calculated using the value of 0.59 in ethanol as reference [1] and lower quantum yield values were obtained in non polar solvents. Previous experiments

Table III. Quantum yield (ϕ), mean lifetime $\langle\tau\rangle$ and natural lifetime τ_n of *o*-Abz fluorescence

Solvent	ϕ	$\langle\tau\rangle$ (ns)	τ_n (ns)
Cyclohexane	0.29	5.7	19.7
Benzene	0.39	6.3	16.1
Acetone	0.31	Nd	Nd
Ethanol	0.59	7.6	12.9
TFE	0.27	5.4	20.0
Acetonitrile	0.56	6.9	12.3
DMSO	0.64	8.7	13.6
Water	0.60	8.7	14.5

Note: Nd: not determined.

performed with a lower resolution apparatus [2] were not able to detect the presence of a short lifetime component for *o*-Abz decay in water. In the present case, the decay profile of *o*-Abz in water was best fitted to a bi-exponential function, with lifetimes 8.9 and 1.8 ns. However, the contribution of the short component accounted for less than 3% of the total emission and the decay was dominated by the long lifetime component. In the other solvents we could observe a small contribution of a sub-nanosecond lifetime component and the decay was practically monoexpoential. To account for the presence of the short component, mean lifetimes were calculated from intensity weighted lifetimes according to $\langle\tau\rangle = \Sigma\alpha_i\tau_i^2/\Sigma\alpha_i\tau_i$ where τ_i are the lifetime components and α_i the corresponding pre-exponential factors. We can see (Table III) that the mean weighted lifetime ranged from 5.4 to 8.7 ns, depending on the characteristics of the solvent. Furthermore, the calculated natural radiative lifetime $\tau_n = \langle\tau\rangle/\phi$, that is equal to the inverse of the natural radiative decay rate k_r, decreases in the more polar solvents. Exceptions are TFE and water, which presents high natural radiative lifetime. High values of absorption extinction coefficient are expected when the natural radiative lifetimes are low.

DISCUSSION

The general effects of solvent upon the electronic states of the fluorophore are reflected in the linear part of the plots in Figs. 1 and 2, from which the difference between the excited state and ground state electric dipoles ($\mu^* - \mu$) could be extracted: 2.3 ± 0.4 D from the measurements in different solvents, and 3.0 ± 0.1 D from the experiments with dioxane/water mixtures. This last value is higher than the former possibly due to the contribution of specific effects promoted by water molecules, and they are relatively small, compared to those reported for probes like amino-aphtathalene-6-sulfonate derivatives which can amount to 40.0 D [13]. The optical absorption of *o*-Abz can be interpreted as originating from transitions from aniline charge transfer states to anti-bonding carboxy orbital [20]. Due to the electron acceptor character of the carboxy group in the excited state of *o*-Abz, there is a decrease in the electron density at the amino group. When *o*-Abz is in non polar solvents, an intramolecular hydrogen bond between the carboxy and amino groups can be promoted, originating an electron delocalization, decreasing the charge separation of those groups. A lowering of the dipole moment should result in low sensitivity of the Stokes shift upon changes in the solvent polarity, as observed in this work.

On the other hand, we observed deviations from linearity when the solvent presented properties of Bronsted acids, according to the Kamlet-Taft scale, or electron acceptor characteristics in the AN+DN scale. In solvents like TFE and water, hydrogen bond formation with the *o*-Abz amino or carboxy groups is effective. As observed in aniline [21], electron lone pairs (*l* orbitals) in *o*-Abz can form hydrogen bonds with protic solvents like alcohols and water. The hydrogen bonds then stabilize the ground state and causes a blue shift in the absorption band corresponding to $l - \pi^*$ transitions, compared to the values in non polar and non protic solvents. The relatively long lifetime of *o*-Abz allows the occurrence of solvent relaxation decreasing the minimum of the excited state energy profile so that the fluorescence emission is red shifted. In this way, the Stokes shift assumes values considerably higher than expected from the Lippert's equation for general effects.

Several compounds have been synthesized having the carboxy group from *o*-Abz covalently bound to the amino terminal of amino acids or peptides [2]. It is interesting to notice that those compounds present λ_{abs} around 315 nm and λ_{em} near to 415–420 nm, values close to observed for *o*-Abz in TFE. The results suggest the similarity in the electronic structures of *o*-Abz having the carboxy group, both covalently bound to amino acids, or making hydrogen bonding with TFE. In those compounds, general effects of solvent are relatively small, as revealed by the small spectral shift of absorption and emission bands in the presence of micelles or vesicles [11,12].

The best solvent parameter to describe a regular behavior of fluorescence lifetime and quantum yield is the AN+DN scale (Fig. 5). In environment unable to perform electron donation or electron acceptance, like cyclohexane, non-radiative processes of de-excitaiton can

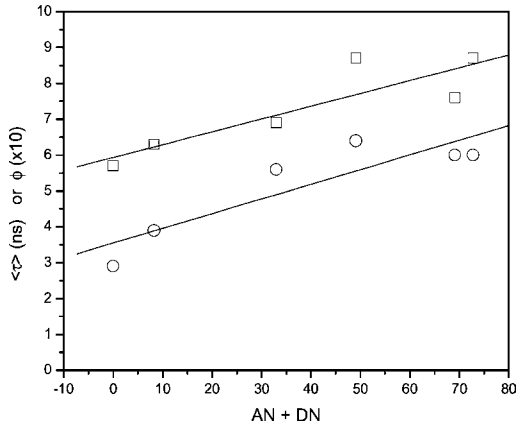

Fig. 5. Lifetime (□) and quantum yield (○) of *o*-Abz as a function of AN+DN parameter. Fluorophore concentration 5×10^{-5} M, temperature 23°C.

be promoted by intramolecular hydrogen bond, resulting in the low values for quantum yield and lifetime. It can be observed (Fig. 5) that higher values of quantum yields and fluorescence lifetimes are registered when the solvent presents high values of the AN+DN parameter, which reflects the electron donor and acceptor properties of the solvent. The results indicate that increasing the capability of the solvent to interact with the fluorophore by electron transfer mechanisms, the contribution of non-radiative processes to the decay of *o*-Abz excited state decreases, resulting in higher values of lifetime and quantum yield. An exception seems to be TFE where the quantum yield and lifetimes are low, despite of the strong electron acceptor character of the solvent. Quenching by TFE was already observed in *o*-Abz labelling the peptide bradykinin [8], and this is a point which deserves further attention.

It is also noteworthy that the extent of changes in quantum yield and lifetimes are not the same and, as a consequence, the natural radiative lifetime of *o*-Abz in different solvents is not constant. Its value is higher in non polar solvents, decreasing to numbers around 12.0 ns in the group of solvents with electron donor or acceptor properties. An indicated above, in TFE, the solvent with the most pronounced Bronsted acid/electron acceptor character, the natural radiative lifetime of *o*-Abz is quite distinct, around 20.0 ns. However, that value is close to those obtained for Abz-bradykinin or Abz-KKA in TFE [8,9].

CONCLUSIONS

The solvent dependence of *o*-Abz absorption and emission spectra reflects the changes in the energy levels of the fluorophore electronic structure, originated from general or specific interaction with the solvent. The use of Kamlet-Taft π^* scale indicate that Bronsted acid solvents promote hydrogen bond formation involving both amino and carboxy groups causing large effects in the wavelengths of absorption and emission of free *o*-Abz, which predominate over the general solvent effects. Thus the spectral properties of the fluorophore are quite informative about the environment, particularly in the aspects related to the formation of hydrogen bonds, both intramolecular or with the solvent, and can be extended to the formation of covalent bonds involving the carboxy group of *o*-Abz and ligands like amino acids and peptides.

Changes in the electronic structure that interfere with the transition probability, like wave function symmetries, lead to alterations in the natural lifetimes and are reflected in the solvent dependence of fluorescence lifetime and quantum yield. Based on the AN+DN scale, it is suggested that the formation of intramolecular hydrogen bond in non-polar environment promotes non-radiative deexcitation processes and that the excited state interaction with electron donor/acceptor solvents leads to increase in fluorescence lifetime.

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