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Solvent Effects in Optical Spectra of *ortho*-Aminobenzoic Acid Derivatives

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Abstract We investigated three amino derivatives of *ortho*-aminobenzoic or anthranilic acid (*o*-Abz): a) 2-Amino-benzamide (AbzNH$_2$); b) 2-Amino-*N*-methyl-benzamide (AbzNHCH$_3$) and c) 2-Amino-*N-N'*-dimethyl-bezamide (AbzNH(CH$_3$)$_2$), see Scheme 1. We describe the results of ab-initio calculations on the structural characteristics of the compounds and experimental studies about solvent effects in their absorption and steady-state and time-resolved emission properties. Ab-initio calculations showed higher stability for the rotameric conformation in which the oxygen of carbonyl is near to the nitrogen of *ortho*-amino group. The derivatives present decrease in the delocalization of π electron, and absorption bands are blue shifted compared to the parent compound absorption, the extent of the effect increasing from to Abz-NH$_2$ to Abz-NHCH$_3$ Abz-NH(CH$_3$)$_2$. Measurements performed in several solvents have shown that the the dependence of Stokes shift of the derivatives with the orientational polarizability follows the Onsager-Lippert model for general effects of solvent. However deviation occurred in solvents with properties of Bronsted acids, or electron acceptor characteristics, so that hydrogen bonds formed with protic solvents predominates over intramolecular hydrogen bond. In most solvents the fluorescence decay of AbzNH$_2$ and AbzNHCH$_3$ was fitted to a single exponential with lifetimes around 7.0 ns and no correlation with polarity of the solvent was observed. The fluorescence decay of AbzN(CH$_3$)$_2$ showed lifetimes around 2.0 ns, consistent with low quantum yield of the compound. The spectroscopic properties of the mono-amino derivative AbzNHCH$_3$ are representative of the properties presented by Abz labelled peptides and fatty acids previously studied.

Keywords Aminobenzoic acid derivatives ·
Anthranilic acid · Solvatochromism ·
Time resolved fluorescence · Stokes shift ·
Hydrogen bond

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Introduction

Internally quenched fluorogenic (IQF) peptides containing the fluorescent probe *ortho*-aminobenzoic acid (*o*-Abz), also known as anthranilic acid, have been used as efficient substrates in proteolitic enzyme assay, forming a pair with the acceptor N-[2,4-dinitrophenyl]ethylenedi-amine (EDDnp) [1, 2]. Spectral properties of *o*-Abz-labeled amino acids and peptides were studied in aqueous medium [3] and in the presence of micelles, and it was verified that wavelength and intensity of fluorescence emission changed due to interaction of the compounds with amphiphilic aggregates like cetyltrimethylammonium bromide (CTAB) [4] or sodium dodecyl sulfate (SDS) micelles [5]. The sensitivity of *o*-Abz emission to changes in the local environment allowed its use as a probe for studies about the interaction between Abz-labeled brady-kinin and fragments with DMPG vesicles [6] and recently it was reported the synthesis of the new lipophilic probe

2-Amino-N-hexadecyl-benzamide (Ahba), where o-Abz is bound to hexadecylamide, forming a fluorescent labeled alkyl chain. Examination of spectroscopic properties of Ahba demonstrated that it can be used as a probe for the surface region of micelles and phospholipid vesicles [7]. Despite of several applications of o-Abz bound to amino acids, peptides or hydrocarbon chain, many details of the structural arrangement and electronic properties of the compounds, as well as of changes promoted by interaction with the medium are not yet established.

In a sistematic investigation of spectral properties of o-Abz in various organic solvents, it was verified that the absorption and emission properties in different media reflect the changes in the electronic structure of the fluorophore, originated from general and specific interactions with the solvent [8]. The use of different polarity scales had shown that Bronsted acid solvent promotes hydrogen bond formation involving both amino and carboxy groups, causing large effects in the wavelengths of absorption and emission of o-Abz, which predominate over the general solvent effects. Interaction with protic solvents competes with formation of intramolecular hydrogen bond between amino and carboxyl groups observed in spectroscopic studies of anthranilic acid in supersonic jet [9]. Dimers of o-Abz were also investigated in supersonic jet [10] and it was shown that electronic excitation localizes on one of the monomeric units, as a consequence of the large geometry change associated with the excited state of anthranilic acid. For o-Abz and the related molecule p-Abz dissolved in several solvents, the emission was assigned to $\pi \rightarrow \pi^*$ transition involving the whole electronic system with a considerable intramolecular charge transfer (ICT) character, from the amino group to the carbonyl group [11, 12].

In o-Abz labelled amino acids and peptides or in the lipophilic probe Ahba, the fluorophore is covalently bound, through its carbonyl group, to amino groups of the reacting molecule (amino acid, peptide or alkyl chain). To understand the effects of that covalent binding on the spectroscopic properties of the cromophore, we investigated three simpler amino derivatives of o-Abz: a) 2-Amino-benzamide (Abz-NH₂); b) 2-Amino-N-methyl-benzamide (Abz-NHCH₃) and c) 2-Amino-N-N′-dimethyl-bezamide (Abz-NH(CH₃)₂), see Scheme 1. We describe in this paper the results of ab-initio calculations on the structural characteristics of the compounds and experimental studies about solvent effects in their absorption and emission properties, interpreting the results within the framework of general and specific solvent effects. Experiments were performed in several solvents and analysis was made based on Lippert's model for the general effects and on the use of different parameters to describe the ability of the solvent 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. Derivatives were prepared according to procedures previously described [3]. Diluted solutions (around 10^{-5} M) of o-Abz and derivatives in the different solvents were employed in the experiments and the same sample was used in the optical absorption, steady state fluorescence and time-resolved fluorescence measurements.

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 X 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, where the L-format configuration 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 75 ps, determined with a time resolution of 6.0 ps per channel. Measurements were made using time resolution of 12 ps per channel. A software provided by Edinburgh Instruments was used to analyse the decay curves, and the adequacy of the multiexponential decay fitting was judged by inspection of the
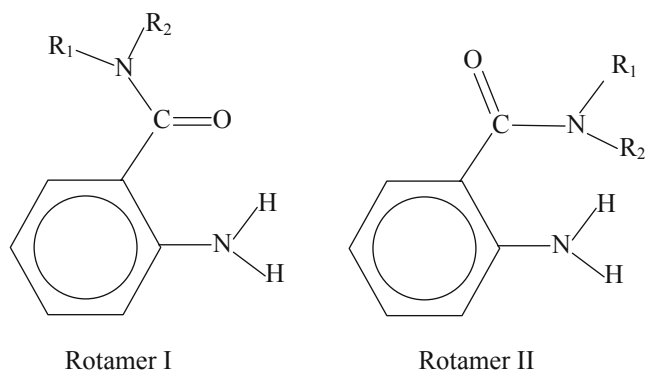
Scheme 1 AbzNH₂ : R₁=R₂=H; AbzNHCH₃ : R₁=H, R₂=CH₃ ; AbzN(CH₃)₂ : R₁=R₂=CH₃

plots of weighted residuals and by statistical parameters such as reduced chi-square.

General effects of the solvent on the electronic properties of the compounds were examined assuming the solvent as a continuous medium of uniform dielectric constant ε and refractive index n, and with orientation polarizability Δf given by

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

The interaction between chromophore and solvent was described considering the molecule as an electric dipole residing in a cavity of radius a. According to the model developed by Lippert [13] and Mataga [14] the difference between absorption and emission wavenumbers is given by

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

where h is the constant of Planck, c is the speed of light, μ and μ^* are, respectively, the electric dipole moments of the ground and first excited state of chromophore. From Lippert's equation, it is expected that the Stokes shift, represented by the difference in wavenumbers Δv, will be linearly dependent on the orientation polarizability Δf.

Ab-initio calculations were performed through density functional theory (DFT) using the Becke 3LYP functional with a 6–311-G+(d) basis set. Calculations were carried out on the ground electronic state of o-Abz and derivatives, using the Gaussian 03 suite of programs [15].

Results and discussion

Ab-initio calculations

Two lowest energy conformations of the neutral form of o-Abz were obtained, corresponding to rotamers I and II which differ by 180° rotation of carboxyl group (see Scheme 1; in o-Abz there is a carboxy group instead of NR_1R_2). The energy difference between the two conformations in o-Abz was 2.60 kcal/mol, rotamer I being the more stable. The geometrical parameters referring to atom-atom distances and bond and dihedral angles were similar to those previously reported from ab-initio calculations using Gaussian 98 [9] and semi-empirical calculations using AM1 program [11].

Results for amino derivatives of o-Abz are presented in Table 1. The calculations were performed for the two rotamers differing by 180° rotation of carboxyl group, and in all derivatives, rotamer I is more stable, by about 3 kcal/mol, compared to rotamer II. Like the parent compound o-Abz, in the derivatives the distance between carbonyl oxygen and nitrogen from the *ortho*-amino group is lower in rotamer I, indicating those rotameric conformations as preferential to stabilize hydrogen bond involving those oxygen and nitrogen atoms.

Optical absorption and fluorescence emission spectra

o-Abz absorbs in the UV region, and the nearest to visible band characteristically peaks around 335 nm in the less polar solvent cyclohexane. Compared to o-Abz, the absorption of amino benzamide derivatives $AbzNH_2$ and $AbzNHCH_3$ in the non-polar environment are slightly blue shifted, while absorption of $AbzNH(CH_3)_2$ is largely blue shifted (Table 2). It has been proposed the occurrence of intramolecular hydrogen bond in o-Abz between its amino and carboxyl groups [9], comparable to verified in N-methylanthranilic acid and N,N-dimethylanthranilic acid studied in crystal state and in solution [16, 17]. As a consequence of the intramolecular hydrogen bond, there is an electron delocalization which originates the band in 335 nm, absent in benzoic acid and *para*-Abz, which have maximum absorption in non-polar solvents at 283 and

Table 1 Distances (R) and internal angles (θ) calculated for rotamers of o-Abz derivatives

R(Å) / θ (°)	Abz-NH$_2$(I)	Abz-NH$_2$(II)	Abz-NH(CH$_3$)(I)	Abz-NH(CH$_3$) (II)	Abz-N(CH$_3$)$_2$ (I)	Abz-N(CH$_3$)$_2$ (II)
R(C-C$_a$)	1,48	1,50	1,49	1,50	1,50	1,50
R(C$_a$-O)	1,23	1,22	1,23	1,22	1,23	1,22
R(C$_a$-N)	1,37	1,38	1,37	1,37	1,37	1,38
R(C-N$_b$)	1,37	1,40	1,38	1,37	1,38	1,39
θ(C$_2$ -C$_1$ -C$_a$)	120,2	125,3	120,0	125,5	119,5	122,8
θ(O- C$_a$- N)	119,9	120,9	120,4	121,4	120,9	121,3
θ(C$_1$ - C$_2$ -N)	122,2	122,5	121,9	122,1	121,3	121,8
θ(C$_2$-C$_1$-C$_\alpha$-N)	161,57	22,9	−155,9	−25,5	−142,1	55,5
θ(C$_1$-C$_2$-N-H)	19,1	37,1	−21,7	−41,7	−29,4	−19,9
ΔE (kcal /mol)	0	3,08	0	3,13	0	3,85
μ(D)	2,03	4,22	1,98	4,28	2,16	4,75

319 nm, respectively [11]. In the derivatives of *o*-Abz studied here, binding of amino group to carboxy oxygen decreases the extent of intramolecular interaction between carbonyl oxygen and the *ortho*-amino group, decreasing the electron delocalization, and shifting the absorption band to lower wavelengths. As shown in Table 1, the effect of blue shift in absorption, compared to the parent compound, occurs in order of increasing extent, from to 2-Amino-benzamide (Abz-NH$_2$) to 2-Amino-*N*-methyl-benzamide (Abz-NHCH$_3$) and 2-Amino-*N*-*N*′-dimethyl-bezamide (Abz-NH(CH$_3$)$_2$).

Solvatochromism in the absorption band was observed as small red shift with increase in the solvent polarity: changing from cyclohexane ($\Delta f = 0.001$) to propylene carbonate ($\Delta f = 0.2861$), the maximum absorption of *o*-Abz goes from 335 nm 338 nm (Table 2). For AbzNH$_2$, the shift was from 328 nm to 332 nm, and for AbzNHCH$_3$, from 324 to 330 nm. However, anomalous behavior was observed in aqueous medium, and in buffer solution at pH 7.4, absorption bands peaked at 311 nm for *o*-Abz, 314 nm for AbzNH$_2$ and 309 for AbzNHCH$_3$ (Fig. 1). It is to be noticed that, as reported by Stalin and Rajendran [9], spectral properties of *o*-Abz in pH 7.0 are ascribed to anionic species in which the carboxyl group is ionized (NH$_2$COO$^-$), resulting in absorption at 310 nm and emission in 395 nm.

The emission of the amino derivatives in cyclohexane had maximum intensity around 380–385 nm and in the polar solvent propylene carbonate the emission peaks were red shifted by approximately 20 nm (Table 2). In addition, it was verified that in the high polar aqueous medium the emission band of the derivatives were further red shifted until circa 420 nm (Fig. 2). The emission of the *o*-Abz derivatives is characterized by a single broad band comparable to the emission of *o*-Abz, whose excited state behaviour is described as an intramolecular hydrogen-atom dislocation, due to differences in the relative positions of the amino and carboxyl groups in the ground and excited states [9]. An intramolecular charge transfer character where the donor group is the amino/aromatic moiety and the carboxy group is the acceptor was also proposed for the emission spectra of *o*-Abz in polar and non-polar solvents [11].

Absorption and emission in water/dioxane mixtures

The dependence of electronic transitions with the polarity of the medium, was examined in dioxane-water mixtures, whose orientation polarizability Δf ranged from 0.021 (for 0/100 water/dioxane volume ratio) to 0.320 (for 100/0 water/dioxane volume ratio). Absorption peaks in pure dioxane (at 335 nm, 328 nm and 326 nm for *o*-Abz, AbzNH$_2$ and AbzNHCH$_3$ respectively), changed in pure water to 310 nm, 316 nm and 312 nm for the fluorophores, in the same order. The emission spectra of *o*-Abz, AbzNH$_2$ and AbzNHCH$_3$ in pure dioxane showed maxima at 395 nm, 394 nm and 390 nm respectively, while in water buffered solution, the peaks located at 395 nm, 420 nm and 413 nm, respectively. In water/dioxane mixtures, the orientation polarizability Δf gradually increases with addition of water to dioxane and at low water/dioxane ratios, the plots of the Stokes shift in function of Δf are approximately linear. Above 50/50 water/dioxane volume ratio, the experimental data show significant deviation from the linearity (Fig. 3), indicating that specific interactions of the fluorophores with water molecules predominate over general effects of the solvent.

Stokes shift and specific effects on solvatochromism

Absorption and emission spectra were measured in several solvents characterized by different orientational polarizability (Table 3) and solvent-fluorophore interactions were examined through plots of Stokes shift versus Δf (Fig. 4). Beyond the orientational polarizability, empirical parameters have been proposed to characterize electron or proton donor/acceptor properties of solvents, like the π^* scale from Kamlet-Taft [18], where 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. Complementary to the π^* scale, the α and β parameters, shown in Table 3, describe the Bronsted acid or Bronsted base character of the solvent, respectively[19, 20]. For the amino derivatives of *o*-Abz, the Stokes shift in most of the solvents listed in Table 3 increases linearly as a

Table 2 Wavelength of absorption and emission of *o*-Abz and derivatives in solvents with different polarity

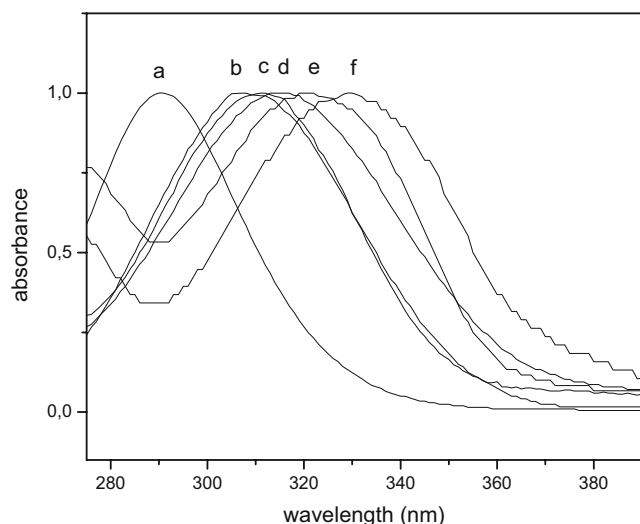| compound	λ_{absorb} (nm)			λ_{fluor} (nm)		
	cyclohexane	Propylene carbonate	Water[a]	cyclohexane	Propylene carbonate	Water[a]
o-Abz	335	336	311	386	405	395
Abz-NH$_2$	328	333	314	384	402	417
Abz-NHCH$_3$	324	327	309	380	398	411
Abz-N(CH$_3$)$_2$	305	305	291	385	408	424

[a] buffer pH 7

Fig. 1 Normalized absorption spectra; **a** AbzN(CH₃)₂ in buffer pH 7.4; **b** AbzNHCH₂ in buffer pH 7.4; **c** AbzN(CH₃)₂ in ciclohexane; **d** AbzNH₂ in buffer pH 7.4; **e** AbzNHCH₂ in ciclohexane; **f** AbzNH₂ in ciclohexane

Fig. 3 Stokes shift for 2-amino-benzamide in water-dioxane mixtures. Fluorophore concentration 5×10^{-5} M, temperature 23 $^{\circ}$ C

function of Δf or π^* (Fig. 4). However, deviation from linearity was verified when using ethanol, methanol, TFE or water as solvent. In the Kamlet-Taft scale, these solvents distinguish from the others (Table 3) by the high values of α parameter, meaning that they have high Bronsted acid character (protic solvents). In the solvent scale based on the capacity of the solvent to act as a donor or acceptor of electrons pairs (a Lewis acid or Lewis base, respectively), the AN number represents the electrophilic property of the solvent [21, 22]. The solvents where occurs deviation from linearity (ethanol, methanol, TFE and water) have high values of AN, which measures the electron acceptor
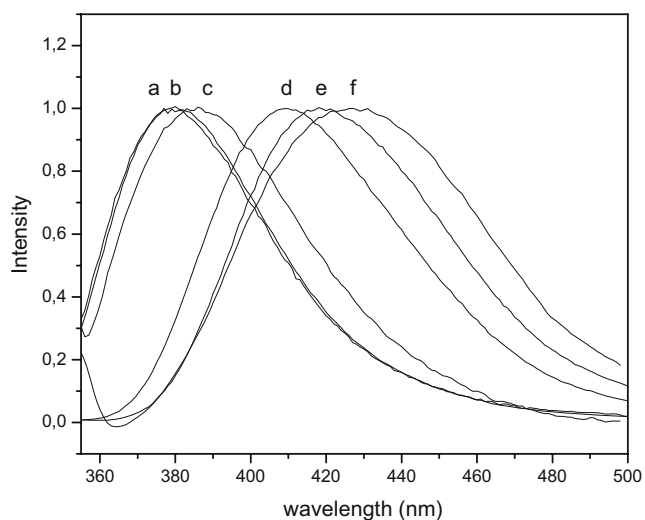

Fig. 2 Normalized emission spectra : **a** AbzNH₂ in ciclohexane; **b** AbzNHCH₂ in ciclohexane; **c** AbzN(CH₃)₂ in ciclohexane; **d** AbzNHCH₂ buffer pH 7.4; **e** AbzNH₂ buffer pH 7.4; **f** AbzN(CH₃)₂ buffer pH 7.4
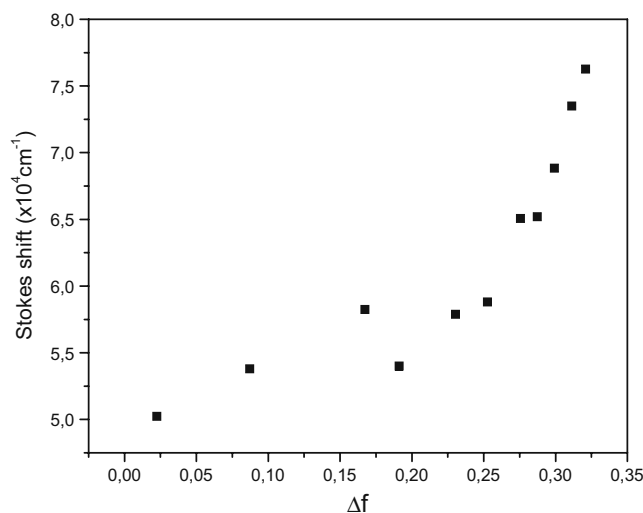
properties of the solvent. Thus, in solvents which are both electron acceptor and Bronsted acids, specific interactions like hydrogen bond formation with solvent molecules compete with intramolecular hydrogen bond.

In non polar aprotic solvents, the intramolecular hydrogen bond in the amino derivatives of *o*-Abz leads to electronic delocalization causing red shift in absorption and emission bands, compared to benzoic acid. The general effects of solvent upon the electronic states of the fluorophore are reflected in the linear part of the plots in Fig. 4, from which the difference between the excited state and ground state electric dipoles (μ^* - μ) could be extracted, according to equation 2. Values obtained were 2.7±0.8 D and 3.4±0.9 D for AbzNH₂ and AbzNHCH₃ respectively, comparable to reported for *o*-Abz [8].

Excited state decay

Decay profiles of *o*-Abz derivatives in different solvents were obtained by single photon time correlation technique (Fig. 5) and multi-exponential functions were employed to fit the experimental data. For most solvents the best fit was attained with a single exponential, with lifetime around 7.0 ns and in some cases a small contribution from a short lifetime (lower than 1.0 ns) was present, responding for less than 3% of the total emission. To fit the decay of Abz-NH₂ and Abz-NHCH₃ in acetonitrile, benzonitrile and dichloromethane, bi-exponential curves were necessary with significant contribution from both lifetimes. To account for the presence of more than one decay component, mean lifetimes were calculated from intensity weighted lifetimes according to $<\tau> = \sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i$ where τ_i is the i lifetime components and α_i the corresponding pre-exponential factor.

Table 3 Orientational polarizability, Kamlet-Taft π^* and proton donor α and proton acceptor β parameters

	Δf	π^*	α (donor)	β (acceptor)
methanol	0.3085	0.60	0.93	0.62
acetonitrile	0.3046	0.75	0.19	0.31
propyleno carbonate	0.2861	0.83	0.00	0.40
acetone	0.2841	0.71	0.08	0.48
N,N-dimethylformamide	0.2744	0.88	0.00	0.69
dimethyl sulfoxide	0.2630	1.00	0.00	0.76
dichloromethane	0.2171	0.82	0.30	0.00
cyclohexane	0.001	0.00	0.00	0.00
ethanol	0.289	0.54	0.83	0.77
1,4-dioxane	0.021	0.55	0.00	0.37
chloroform	0.1344	0.58	0.20	0.10
benzene	0.150	0.59	0.00	0.10
2,2,2-trifluoroethanol	0.320	0.73	1.51	0.00
water	0.320	1.09	1.17	0.18

We can see (Table 4) that in most solvents the mean weighted lifetime of o-Abz, AbzNH$_2$ and AbzNH(CH$_3$) ranged from 5.4 to 8.7 ns, and smaller values (between 2.0 and 4.0 ns) were observed for AbzNH$_2$ and AbzNH(CH$_3$) in benzonitrile, dichloromethane and benzene, cases in which the solvent promotes non radiative decay processes. On the other hand, the dimethyl amino derivative AbzN(CH$_3$)$_2$ showed lifetimes below 2 ns, in agreement with low intensity of fluorescence emission.

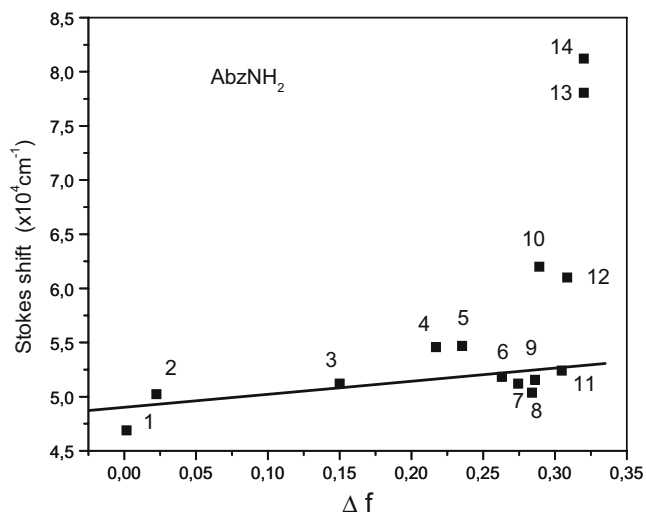It can be observed some distinction among lifetimes of excited states in different solvents, so that decays are relatively longer in more polar solvents, and there is a tendency for longer lifetimes to be associated to AbzNH$_2$. Fast decays are systematically observed for NH(CH$_3$)$_2$, the derivative that does not show the formation of intramolecular hydrogen bond. For the other two derivatives there is no clear distinction between results in protic or aprotic solvents, and both intramolecular hydrogen bond hydrogen bond with circumventing solvent molecules lead to similar rates for deexcitation by fluorescence emission. The lowest values of lifetimes of AbzNH$_2$ and AbzNH(CH$_3$) were observed in benzene, benzonitrile and dichloromethane, for which there is not hydrogen bond with solvent, suggesting predominance of processes involving internal rearrangement of the hydrogen bonded ring.
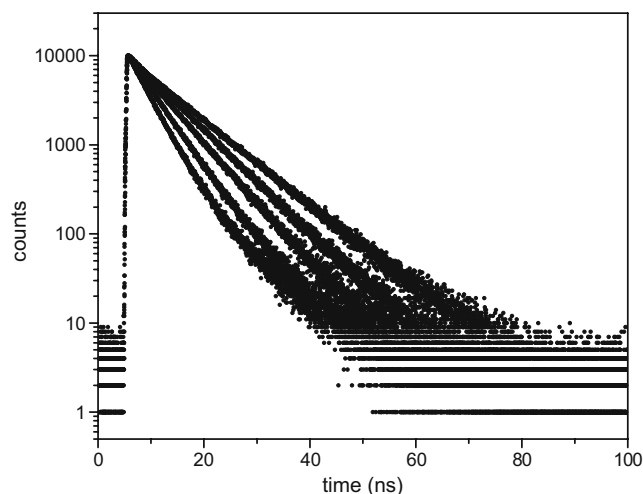

Fig. 4 Dependence of Stokes shift of 2-amino benzamide with orientation polarizability Δf in pure solvents. Fluorophore concentration 5×10^{-5} M, temperature 23 $^\circ$ C. Solid line was obtained from linear fit of non protic solvent data. (solvents are: (*1*) cyclohexane, (*2*) dioxane, (*3*) benzene, (*4*) dichloromethane, (*5*) cyanobenzene, (*6*) DMSO, (*7*) DMFA, (*8*) acetone, (*9*) propilenocarbonate, (*10*) ethanol, (*11*) acetonitrile, (*12*) methanol, (*13*) buffer pH 7.4 and (*14*) TFE)

Fig. 5 Fluorescence decay of AbzNH$_2$ in different solvents. From upper to lower: acetone, metanol, dmfa, dichloromethane, acetonitrile. Fluorophore concentration was 3×10^{-5} M, temperature 23 $^\circ$ C

Table 4 Intensity weighted mean lifetimes calculated from decay profiles obtained using time-correlated single photon techniques. Experiments were performed in different solvents using fluorophore concentration near to 3×10^{-5} M, and temperature 23 °C

	Δf	Abz	AbzNH$_2$	AbzNHCH$_3$	AbzNH(CH3)$_2$
água	0.32	8.70	6.68	7.32	2.81[a]
TFE	0.32	5.40	6.61	7.98	1.90
metanol	0.309	7.00	7.36	6.87	2.48
acetonitrilo	0.305	6.88	5.06 [a]	5.97[a]	1.18
etanol	0.289	7.50	7.33	6.75	2.21[b]
propilenocarbon	0.286	8.94	7.20	6.42	Nd
acetona	0.284	6.42	8.86	7.26	Nd
DMFA	0.274	7.65	6.33	6.33	Nd
DMSO	0.263	8.66	7.41	5.52	1.38
benzonitrilo	0.235	5.65	2.64[a]	4.16[a]	Nd
diclorometano	0.217	7.35	4.33[a]	2.01[a]	Nd
benzeno	0.150	6.30	4.31	2.77	1.67
clorofórmio	0.134	Nd	6.71	2.51	0.42[b]
dioxano	0.021	Nd	10.14	9.12	1.17[b]

Nd not determined

[a] best fit with bi-exponential function

[b] best fit with tri-exponential function

Conclusions

The amino derivatives of *ortho*-amino benzoic acid, AbzNH$_2$, AbzNH(CH$_3$) and AbzN(CH$_3$)$_2$ present a covalent bond between the carboxy oxygen and amine, methyl amine and dimethyl amine groups respectively. Ab-initio calculations showed higher stability for the rotameric conformation in which the remaining oxygen of carbonyl is near to the nitrogen of the *ortho*-amino group (rotamer I). The derivatives AbzNH$_2$ AbzNH(CH$_3$) maintained the ability of carbonyl oxygen to accept the hydrogen from nitrogen of amino benzyl group, forming the intramolecular hydrogen bond responsible for the large red shift in absorption and emission bands in non polar solvents, compared to benzoic acid. The intramolecular hydrogen bond does not occur in the dimethyl amine derivative AbzN(CH$_3$)$_2$.

Solvatochromism of derivatives was observed as small red shift in absorption and emission bands with increase in the solvent polarity. The emission is characterized by a single broad band comparable to the emission of *o*-Abz, whose excited state behaviour is described as an intramolecular hydrogen-atom dislocation, due to differences in the relative positions of the amino and carboxyl groups in the ground and excited states. The Stokes shift of *o*-Abz derivatives presented deviations from the linearity predicted by the Onsager-Lippert model when the solvent had properties of Bronsted acids, according to the Kamlet-Taft scale, or electron acceptor characteristics in the AN scale. As observed in *o*-Abz, electron lone pairs in the derivatives can form hydrogen bonds with protic solvents like alcohols and water, causing a blue shift in the absorption band.

In most of the solvents the fluorescence decay of the amino and monomethyl amino derivatives (AbzNH$_2$ and AbzNHCH$_3$) was fitted to a single exponential with lifetimes around 7.0 ns and no correlation with polarity of the solvent was observed. As reported for *o*-Abz, the emission of the derivatives has a charge transfer character where the donor group is the amino/aromatic moiety and the carboxy group is the acceptor and the process occurs both when intramolecular hydrogen bond was present (in non protic solvents) or when the fluorophore forms hydrogen bond with protic solvents. Bi-exponential decay and decreased lifetime was observed in acetonitrile, benzonitrile and dichloromethane. The fluorescence decay of the dimethyl amine derivative (AbzN(CH$_3$)$_2$ showed lifetimes around 2.0 ns, significantly lower than those of the other derivatives, and consistent with the low quantum yield of the compound. It was seen that that hydrogen bond, present in AbzNH$_2$ and AbzNHCH$_3$, is necessary to stabilize the excited state and decrease the rate of non-radiative decay processes.

The spectroscopic properties of the monoamino derivative AbzNHCH$_3$ are representative of the properties presented by Abz labelled peptides and fatty acids previously studied [5–7]. In aprotic solvents an intramolecular hydrogen bond between carbonyl oxygen and nitrogen from *ortho*-amino group is stabilized in the rotameric conformation corresponding to the lowest distance between those two atoms. In polar protic solvents the specific effect of hydrogen bond with solvent molecules is predominant over the general solvent effects. In addition, the small fluorescence intensity and the short lifetime of the diamino derivative AbzN(CH$_3$)$_2$ find correspondence with the observation that *o*-Abz bound to proline, where nitrogen bound to carbonyl oxygen is linked to two carbon atoms, also has small quantum yield and short lifetime decay [23].

References

1. Chagas JR, Portaro FC, Hirata IY, Almeida PC, Juliano MA, Juliano L, Prado ES (1995) Determinants of the unusual cleavage specificity of Lysyl-Bradykinin-Releasing kallikreins. Biochem J 306:63–69

2. Del Nery E, Chagas JR, Juliano MA, Prado ES, Juliano L (1995) Evaluation of the extent of the binding-site in human Kallikrein by synthetic substrates with sequences of Human Kininogen fragments. Biochem J 312:233–238

3. Ito AS, Turchiello RF, Hirata IY, Cezari MH, Meldal M, Juliano L (1998) Fluorescent properties of amino acids labeled with orthoaminobenzoic acid. Biospectroscopy 4:395–402. doi:10.1002/(SICI)1520-6343(1998)4:6<395::AID-BSPY4>3.0.CO;2-Y

4. Ray R (1993) Fluorescence quenching of o-Aminobenzoic acid by ethylene trithiocarbonate in aqueous micellar media. J. Photochem. Photobiol. A 76:115–120. doi:10.1016/1010-6030(93)80182-9

5. Turchiello RF, Lamy-Freund MT, Hirata IY, Juliano L, Ito AS (1998) Ortho-aminobenzoic acid as a fluorescent probe for the interaction betwen peptides and micelles. Biophys Chem 73:217–225. doi:10.1016/S0301-4622(98)00144-6

6. Turchiello RF, Lamy-Freund MT, Hirata IY, Juliano L, Ito AS (2002) *Ortho*-Aminobenzoic acid-labeled bradykinins in interaction with lipid vesicles Fluorescence study. Biopolymers 65:336–346. doi:10.1002/bip.10238

7. Marquezin CA, Hirata IY, Juliano L, Ito AS (2006) Spectroscopic characterization of 2-amino-N-hexadecyl-benzamide (AHBA), a new fluorescence probe for membranes. Biophys Chem 124:125–133. doi:10.1016/j.bpc.2006.06.002

8. Takara M, Ito AS (2005) General and specific solvent effects in optical spectra of *ortho*-aminobenzoic acid. J. Fluoresc. 15:171–177. doi:10.1007/s10895-005-2525-3

9. Southern CA, Levy DH, Florio GM, Longarte A, Zwier TS (2003) Electronic and infrared spectroscopy of anthranilic acid in a supersonic jet. J. Phys. Chem. A 107:40032–40040. doi:10.1021/jp027041x

10. Southern CA, Levy DH, Stearns JA, Florio GM, Longarte A, Zwier TS (2004) Spectroscopic consequences of localized electronic excitation in anthranilic acid dimer. J. Phys. Chem. A 108:4599–4609. doi:10.1021/jp0496093

11. Stalin T, Rajendiran N (2006) Itramolecular charge transfer associated with hydrogen bonding effects on 2-aminobenzoic acid. J. Photchem. Photobiol. A Chem. 182:137–150. doi:10.1016/j.jphotochem.2006.02.002

12. Stalin T, Rajendiran N (2006) Itramolecular charge transfer effects on 3-aminobenzoic acid. Chem Phys 322:311–322. doi:10.1016/j.chemphys.2005.09.002

13. Lakowicz, J.R.: *Principles of Fluorescence Spectroscopy*, 2nd edition, Kluwer Academic/Plenum Press, New York. For original references see Lippert, E., (1957), Z. Elektrochem, Ber. Bungenses. *Phys. Chem.* **61**, 962 or Lippert, E., (1961). Angew. Chem. **73**, 695 (1999)

14. Mataga N, Kaifu Y, Koizumi M (1956) Solvent Effects upon Fluorescence Spectra and the Dipolemoments of Excited Molecules. Bull Chem Soc Jpn 29(4):465–470. doi:10.1246/bcsj.29.465

15. Gaussian 03, Revision B.04, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman,J. A. Montgomery, Jr.,T. Vreven,K. N. Kudin,J. C. Burant,J. M. Millam,S. S. Iyengar,J. Tomasi,V. Barone,B. Mennucci,M.Cossi, G. Scalmani,N. Rega,G. A. Petersson,H. Nakatsuji,M. Hada,M. Ehara,K.Toyota,R. Fukuda,J. Hasegawa,M. Ishida,T. Nakajima,Y. Honda,O. Kitao,H. Nakai,M. Klene, X. Li,J. E. Knox,H. P. Hratchian,J. B. Cross,C. Adamo,J. Jaramillo,R.Gomperts,R. E. Stratmann,O. Yazyev,A. J. Austin, R. Cammi,C. Pomelli, J. W. Ochterski, P. Y. Ayala,K. Morokuma, G. A. Voth, P. Salvador, J.J. Dannenberg, V.G.Zakrzewski,S. Dapprich,A. D. Daniels,M. C. Strain,O. Farkas,D. K. Malick,A.D. Rabuck,K.Raghavachari, J.B. Foresman,J. V. Ortiz, Q. Cui,A. G. Baboul,S. Clifford, J. Cioslowski,B. B. Stefanov,G.Liu, A.Liashenko,P.Piskorz, I. Komaromi,R. L.Martin, D. J. Fox, T.Keith, M. A. Al-Laham,C. Y. Peng,A. Nanayakkara,M. Challacombe, P. M. W. Gill,B. Johnson, W. Chen,M. W. Wong,C. Gonzalez,and J. A. Pople, Gaussian Inc., Pittsburgh PA, 2003.

16. Tramer A (1969) Tautomeric and protolytic properties of *o*-aminobenzoic acids. I. ground electronic states. J. Mol. Struct. 4:313–325. doi:10.1016/0022-2860(69)80065-7

17. Tramer A (1970) Tautomeric and protolytic properties of *o*-aminobenzoic acids in their lower singlet and triplet states. J. Phys. Chem. 74:887–894. doi:10.1021/j100699a037

18. Kamlet MJ, Abboud JL, Taft RW (1977) The Solvatochromic Comparison Method. 6. The π* Scale of Solvent Polarities. J. Am. Chem. Soc 99(18):6027–6038. doi:10.1021/ja00460a031

19. Kamlet MJ, Taft RW (1976) The Solvatochromic Comparison Method. 2. The α-Scale of Solvent Hydrogen-Bond Donor (HBD) Acidities. J. Am. Chem. Soc. 98(10):2886–2894. doi:10.1021/ja00426a036

20. Kamlet, M.J., Taft, R.W.: The Solvatochromic Comparison Method. I. The β-Scale of Solvent Hydrogen-Bond Acceptor (HBA) Basicities. J. Am. Chem. Soc. **98**(2), 377–386 (1976) doi:10.1021/ja00418a009

21. Cilli EM, Oliveira E, Marchetto R, Nakaie CR (1996) Correlation between solvation of peptide-resins and solvent properties. J. Org. Chem. 61:8992–9000. doi:10.1021/jo9611632

22. Mataga N (1963) Solvent Effects on the absorption and Fluorescence Spectra of Naphthylamines and Aminobenzoic Acids. Bull Chem Soc Jpn 6(6):654–662. doi:10.1246/bcsj.36.654

23. Hirata IY, Cezari MHS, Boshcov P, Garrat RC, Ito AS, Spisni A, Franzoni L, Juliano L (1998) Reduction of o-Aminobenzoyl-proline fluorescence due to formation of pyrrolobenzodiazepine-5, 11-dione. Lett. Pept. Sci. 5:19–28