Fluorescent Properties of Amino Acids Labeled with *ortho*-Aminobenzoic Acid

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ABSTRACT: *ortho*-Aminobenzoic acid (Abz) has been used as a convenient fluorescent donor group in internally quenched fluorescent peptides, which are employed as substrates for several proteolytic enzymes. As Abz is usually bound to the N-amino terminal of these peptides, it is of interest to investigate the Abz group fluorescent properties bound to different amino acids. We report in this article the optical absorption and fluorescent properties, in aqueous media, of Abz bound to the α-amino group of Ala, Gly, Leu, Ile, Val, Pro, Phe, Arg, Glu, Met, Asn, Tyr, and Trp, with monomethylamidated α-carboxyl group. In order to explore the origin of the drastic reduction of Abz attached to N^{α} amino group of prolyl-peptides, we also examined the fluorescence properties of Abz-$NHCH_3$, Abz-$N(CH_3)_2$, and Abz-pyrrolidine. Molecular dynamics simulation and NMR data indicated a lack of periplanarity of the Abz-dimethylamide, which could be the origin of low fluorescence quantum yield of Abz-prolyl-peptides.
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INTRODUCTION

Internally quenched fluorescent peptides have been synthesized and employed as substrates for protease assays. These peptides contain a fluorescent group (donor) at one end and a quencher group (acceptor) at the other end of the peptide, and the fluorescence increases with the progression of enzymatic hydrolysis due to the elimination of donor–acceptor intramolecular energy transfer. This subject was reviewed in its physical concept, chemical synthesis, and uses for protease studies.[1,2] *ortho*-Aminobenzoic acid (Abz), also named anthranilic acid, has a high quantum yield, and its size and structure are comparable to those of smaller natural amino acids. These structural characteristics reduce the potential influence of Abz on the substrate–protease interaction, which is a very desirable property for a fluorescent report group. Consequently, Abz has been largely used as a convenient donor group in internally quenched fluorescent peptides as substrates for several proteolytic enzymes.[1–8]

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We have previously observed a strong quenching of Abz fluorescence when the fluorophore is directly coupled to the N^{α} amino group of proline, preventing in this case its use as fluorescent probe.[9] A similar fluorescence reduction of *N*-methylanthranilic acid was observed when this compound was bound to the N^{α}-amino group of proline.[10]

In this work we performed a survey in the fluorescence properties of Abz bound to other natural amino acids in order to verify restrictions to the use of this group as a fluorescent probe attached to their N^{α}-amino group. These observations are relevant for design of fluorogenic peptides, looking for interference in the Abz fluorescence due to the nature of amino acid side chains, as well as to check if side reaction occurs as in the case of Abz-proline, where formation of pyrrolobenzodiazepine-5,11-dione was observed.[11,12] The characterization of optical parameters of Abz-amino acids is also of interest in order to establish the scope and limitations of the use of Abz as a fluorophore in the study of different properties of peptides, as for instance, end-to-end distances and interaction with lipids. In the present article, we report a study of the fluorescent properties of Abz bound to the N^{α}-amino group of Ala, Gly, Leu, Ile, Val, Pro, Phe, Arg, Glu, Met, Asn, Tyr, and Trp, with the α-carboxyl group monomethyl-amidated.

In order to explore the origin of the drastic reduction of Abz attached to Pro, we also examined the fluorescence properties of some Abz derivatives, namely, Abz-NH_2, Abz-$NHCH_3$, Abz-$N(CH_3)_2$, and Abz-pyrrolidine. These compounds have the amino component of the amide bond with Abz carboxyl group with none, one, and two methyl substitutions. They were chosen in order to verify if the origin of fluorescence quenching in Abz-Pro-peptides results from the double methyl substitution of proline α-amino group.

MATERIAL AND METHODS

Synthesis

N^{α}-Abz-Aminoacyl-Monomethyl Amide

These compounds were obtained starting from N^{α}-Boc amino acids, with the following protecting groups at side chain: tosyl for Arg, benzyl for Ser and Glu, and 2-bromobenzyloxycarbonyl for Tyr. Initially, the monomethyl amide of the amino acids was synthesized by the mixed anhydride procedure using isobutyloxycarbonyl chloride.[13] After treatment with neat trifluoroacetic acid (TFA), each amino acid was treated with Boc-Abz-*N*-hydroxysuccinimide generating Boc-Abz-aminoacyl-monomethyl amide. These compounds were treated with TFA or anhydrous HF and purified by HPLC till a homogeneity no lower than 97%. The synthesis of the compound corresponding to His was unsuccessful, even using tosyl at imidazole group and *O*-benzotriazol-1-yl-*N,N,N′,N′*-tetramethyluronium hexafluoroborate (TBTU) besides mixed anhydride as coupling procedures. Abz-NH_2, Abz-$NHCH_3$, Abz-$N(CH_3)_2$, and Abz-pyrrolidine were obtained by reaction of Boc-Abz-*N*-hydroxysuccinimide with NH_4OH, aqueous solution of methylamine and dimethylamine, and pyrrolidine, respectively, followed by treatment with TFA. The final deprotected compounds were purified by semipreparative HPLC using an Econosil C-18 column (10 μ, 22.5 $\times$ 250 mm) and a two-solvent system: (A) trifluoroacetic acid (TFA)/H_2O (1 : 1000) and (B) TFA/acetonitrile (ACN)/H2O (1 : 900 : 100). The column was eluted at a flow rate of 5 mL/min with a 10 (or 30)–50 (or 60)% gradient of solvent B over 30 or 45 min. Analytical HPLC was performed using a binary HPLC system from Shimadzu with a SPD-10AV Shimadzu UV–vis detector and a Shimadzu RF-535 fluorescence detector, coupled to an Ultrasphere C-18 column (5 μ, 4.6 $\times$ 150 mm) which was eluted with solvent systems A1 (H_3PO_4/H_2O, 1 : 1000) and B1 (ACN/H_2O/H_3PO_4, 900 : 100 : 1) at a flow rate of 1.7 mL/min and a 10–80% gradient of B1 over 15 min. The HPLC column eluates were monitored by their absorbance at 220 nm and by fluorescence emission at 420 nm following excitation at 320 nm.

All the compounds were characterized by mass spectrometry, which were obtained on a single quadrupole Finnigan SSQ10 spectrometer with a combined CI–EI ionization source. The compounds were dissolved in $CHCl_3$; all of the spectra were obtained using the electron impact (EI) method with the following conditions: ionization voltage, 70 eV; 300 μA ionizing current; source temperature 240°C.

Measurements

We employed the diode array HP spectrophotometer 8452 A for optical absorption measurements and steady state fluorescence experiments were performed in a Hitachi 3010 spectrofluorometer. We used the quantum yield of Abz in ethanol as a reference for the quantum yield of the Abz compounds in phosphate buffer. Intensities of fluores-

cence emission, obtained from the integration of the corrected spectra, were compared to that from Abz in ethanol. In the comparison, the intensities were normalized to a unitary value of absorbance at the wavelength of excitation. In the experiments, the absorbances at the excitation were always below 0.05, avoiding inner filter effects.

Time-resolved fluorescence experiments were made using a PTI spectrometer based on the stroboscopic optical boxcar technique.[14] The fluorescence lifetime measurements in the range between 1.0 to 400.0 ns can be determined to an accuracy and precision better than ±2%. In the PTI instrument a N_2 laser provided light pulses that pumped a dye, resulting in emission at 636 nm. A frequency doubling crystal generated light at 318 nm, that was used for the excitation of Abz compounds. Decay profiles were fitted to exponential curves, and the quality of the fit was judged through the statistical reduced-χ^2 and Durbin–Watson parameters and by inspection of the residue distribution.

Samples examined contained the compounds at concentration near $10^{-5}M$, in phosphate buffer 0.01*M* and pH 7.4. Experiments were conducted in the temperature of 22°C. Excitation wavelength was set at 310 nm in steady state measurements. For lifetime measurements the excitation and emission wavelengths were 318 and 420 nm, respectively.

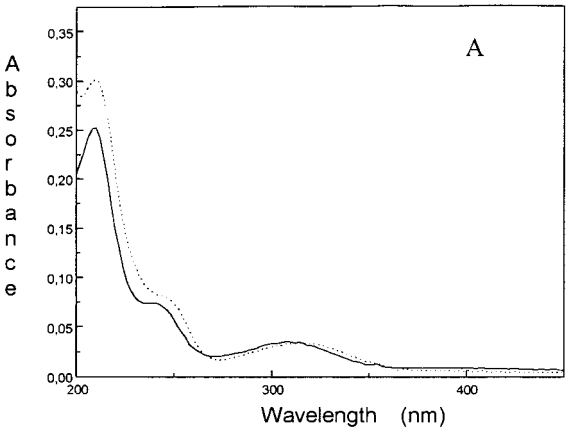
Molecular Modeling

The structures of Abz-NH(CH_3) and Abz-N(CH_3)$_2$, were obtained from a molecular dynamics simulation, performed using the Amber force-field (Insight II-95). The molecules were constructed using the builder module and MD-calculations were carried out first at 800°C, 10 psec, 10,000 steps and then at 400°C, 50 psec, 50,000 steps storing 1 structure every 500 steps in a history file for later examination. Every fifth structure from the history file of the two calculations was extracted. The lack of periplanarity of the dimethylamide is clearly observed and is the most probable reason for the lack of fluorescence in Abz-prolinyl-peptides and other Abz-dialkylamides.

RESULTS AND DISCUSSION

Representative optical absorption and fluorescence emission spectra of Abz derivatives and Abz-containing amino acids in aqueous media are presented in the Figure 1(A,B). Table I summarizes the results for wavelengths of maximum absorption and emission, fluorescence quantum yield, and fluorescence lifetime.

The spectral properties of the Abz compounds can be examined with respect to the optical absorption and fluorescence spectra of Abz. The bands located in 310 nm and 240 nm in the absorption spectrum of Abz (Fig. 1) are characteristic of the $^1A \rightarrow L_b$ and $^1A \rightarrow L_a$ transitions,



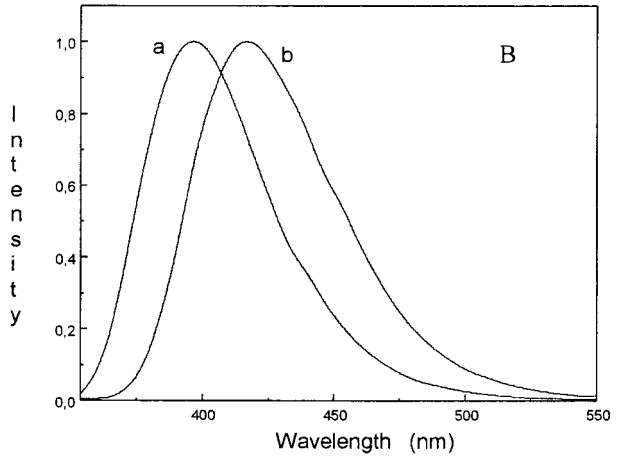


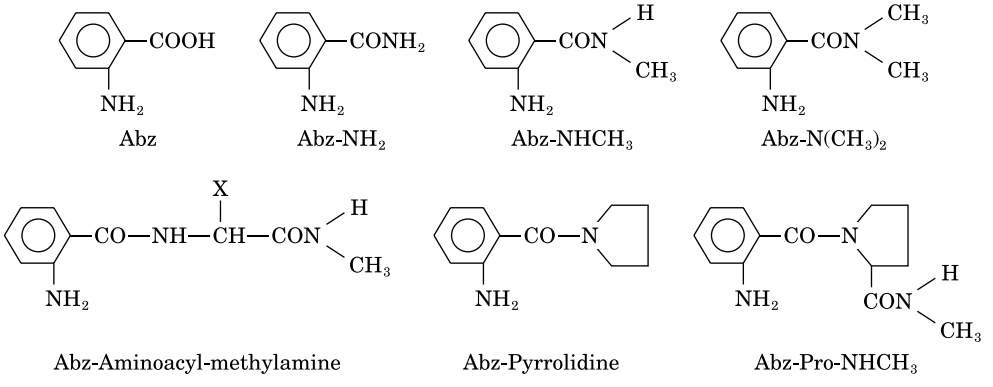
Figure 1. (A) Optical absorption spectra of *ortho*-aminobenzoic acid (Abz) (solid line) and Abz-Phe-$NHCH_3$ (dotted line) in phosphate buffer, 0.01*M*, pH 7.4, temperature 23°C. Concentrations $1 \times 10^{-5}M$. (B) Fluorescence emission spectra in phosphate buffer, 0.01*M*, pH 7.4, temperature 23°C, concentration $10^{-5}M$. (curve a) Abz, excitation 310 nm. (curve b) Abz-Leu-$NHCH_3$ $10^{-5}M$, excitation 315 nm. Intensities are normalized to have value 1.0 at the maximum emission.

Table I. Optical Absorption and Fluorescence Parameters for Compounds Derived from Abz, in Phosphate Buffer 0.01*M,* pH 7.4, Temp. 30°C

Compounds	λ_{max}^{abs} (nm)	λ_{max}^{em} (nm)	Φ	τ_f (ns)	τ_R (ns)
Abz	310	396	0.60	8.4 ± 0.1	14.0
Abz-NH_2	315	418	0.52	ND	ND
Abz-$NHCH_3$	310	414	0.58	7.5 ± 0.1	13.0
Abz-Ala-$NHCH_3$	314	417	0.52	7.1 ± 0.1	13.7
Abz-Ile-$NHCH_3$	314	417	0.58	7.7 ± 0.1	13.3
Abz-Phe-$NHCH_3$	314	418	0.47	7.5 ± 0.1	16.0
Abz-Gly-$NHCH_3$	314	416	0.46	7.8 ± 0.1	17.0
Abz-Leu-$NHCH_3$	314	416	0.48	8.4 ± 0.1	17.5
Abz-Val-$NHCH_3$	314	417	0.47	8.4 ± 0.1	17.8
Abz-Glu-$NHCH_3$	314	416	0.44	8.0 ± 0.2	18.2
Abz-Asn-$NHCH_3$	328	398	0.18	ND	ND
Abz-Arg-$NHCH_3$	316	415	0.35	8.1 ± 0.3	23.1
Abz-Met-$NHCH_3$	316	418	0.36	8.6 ± 0.2	23.8
Abz-Tyr-$NHCH_3$	314	419	0.33	7.9 ± 0.1	23.9
Abz-Trp-$NHCH_3$	312	419	0.03	2.5 ± 0.1	83
Abz-Pro-$NHCH_3$	290	365	0.02	ND	ND
Abz-$N(CH_3)_2$	290	436	0.02	<1.0	>50
Abz-Pyrrolidine	294	425	0.02	0.55 ± 0.01	25

λ_{max}^{abs}, wavelength of maximum absorption; λ_{max}^{em}, wavelength of maximum emission; Φ, quantum yield; τ_f, fluorescence lifetime; τ_R, radiative lifetime.

Chemical formulae:



respectively, commonly observed in derivatives of benzene.[15] Those transitions are observed in the Abz compounds studied here, as exemplified by the absorption spectrum of Abz-Phe-$NHCH_3$ shown in Figure 1.

The data presented in Table I, first column, show that the Abz compounds can be separated in two groups:

a. Abz-Pro-$NHCH_3$ and the related compounds, Abz-pyrrolidine and Abz-$N(CH_3)_2$, in which the band due to $^1A \rightarrow L_b$ transition is blue shifted to around 290 nm.
b. All others Abz-aminoacyl methylamides and the derivatives Abz-NH_2 and Abz-$NHCH_3$, in which the $^1A \rightarrow L_b$ transition is red shifted, to a position around 315 nm.

The fluorescence properties of the two groups are also distinct (Table I, columns 2 to 4). Using again Abz as a reference, we observed that there is no regular trend for the spectral position of the fluorescence bands from Abz-Pro-$NHCH_3$, Abz-$N(CH_3)_2$, and Abz-pyrrolidine, this last compound showing a very broad emission band. In the other group of compounds, fluorescence spectra are red shifted to around 415–418 nm. The data indicate that amidation of Abz originates spectral changes

similar to those observed in Abz-aminoacyl methylamide and in Abz-methylamide. From the results in Table I we calculated the Stokes shift between absorption and emission bands. For this group of compounds, a mean value of 7860 ± 160 cm^{-1} was obtained, a value significantly higher than the shift of 7010 cm^{-1} observed with the parent chromophore. The Stokes shift due to general effects of the solvent can be described by the Lippert model.[16] In this model, the shift between absorption and fluorescence bands can be attributed to changes in the electronic energy due to the interaction of the dipole moment of the chromophore (both in the ground and in the excited states) with the reactive field generated by the medium, which is treated as a dielectric continuum. For a fluorescent molecule in a given medium, the Stokes shift is proportional to the difference between the magnitudes of the dipole moment in the excited state and in the ground state. A possible explanation for the spectral changes observed in the Abz-aminoacyl methylamides, compared to the native Abz, could be an increase in the difference between the excited state and ground state dipole moments promoted by the amidation in the carboxyl group of the chromophore. Another possibility could be the occurrence of specific interactions of the amidated Abz with the solvent, and further studies are necessary to establish a definite explanation for the spectral modifications reported here.

The examination of the quantum yield of fluorescence allows us to identify the same two groups of compounds as mentioned above. The majority of the Abz compounds present quantum yield in the range between 0.33 to 0.58, meaning that they are highly fluorescent. Abz-Trp-$NHCH_3$ merits a particular observation: while its spectral characteristics are similar to the majority of Abz aminoacyl compounds, its quantum yield is very low, and similar to that of Abz-Pro-$NHCH_3$.

Table I presents also results from time resolved fluorescence experiments. The decay profiles of the Abz-aminoacyl methylamides and other Abz derivatives were best fitted to monoexponential curves, and lifetime values ranged from 7.1 to 8.6 ns., excepted the Abz-Trp-$NHCH_3$ whose lifetime was 2.5 ns. The compounds with the smallest quantum yields had lifetime values in the subnanosecond region, whose determinations were beyond the possibilities of the instrument utilized. Radiative lifetimes (τ_R) can be calculated from the experimental fluorescence lifetime (τ_f) and the quantum yield (ϕ) through $\tau_R = \tau_f/\phi$. We can identify three groups of compounds, in the first, comprising Abz, Abz-NH_2, Abz-Ala-$NHCH_3$, and Abz-Ile-$NHCH_3$, the quantum yield is near to 0.6 and radiative lifetimes are around 13.0 ns. The second group, with Abz-Phe-$NHCH_3$, Abz-Gly-$NHCH_3$, Abz-Leu-$NHCH_3$, Abz-Val-$NHCH_3$, and Abz-Glu-$NHCH_3$, have quantum yield circa 0.46 and radiative lifetime nearby 17.5 ns. In the third group we have Abz-Arg-$NHCH_3$, Abz-Met-$NHCH_3$, and Abz-Tyr-$NHCH_3$ with quantum yields and lifetime about 0.35 and 23.0 ns, respectively. The radiative lifetimes are related to the oscillator strength of the transitions, and the results are indicative of differences in the values of the electronic transition probabilities among the various Abz-aminoacyl methylamides. It seems thus, that differences in quantum yields of the compounds are originating not only from different pathways for nonradiative deactivation of the excited state, but also from the matrix element for the electronic transition between ground state and excited state. We could not see any direct correlation between the results and physicochemical properties of the aminoacids bound to Abz and the understanding of the results needs more detailed studies.

The optical properties of Abz-$N(CH_3)_2$, Abz-pyrrolidine, and Abz-Pro-$NHCH_3$ (Table I) are distinct from the others, namely, they present a blue shift of the transition $^1A \rightarrow L_b$, giving an absorption band displaced to circa 290 nm. In addition, these compounds have very low quantum yield. It can be noticed that, while the dimethyl amide derivative of Abz mimics properties of Abz-Pro-$NHCH_3$, the methyl amide derivative has optical properties that are similar to those observed in the others Abz-aminoacyl compounds. Thus, these Abz derivatives can be considered as simpler model for the different characteristics of Abz-aminoacyl methylamides. The double substitution of hydrogen for methyl group at the amide nitrogen in Abz-NH_2 is crucial to the modifications in the electronic structure of the chromophore, leading to the spectral changes and quenching of fluorescence observed in Abz-pyrrolidine and Abz-Pro-$NHCH_3$. An interesting observation comes from the comparison of the structures of Abz-$NH(CH_3)$ and Abz-$N(CH_3)_2$ obtained from a molecular dynamics simulation, as shown in Figure 2. The lack of periplanarity of the dimethylamide derivative is clearly observed and is one possible reason for the distinct spectroscopic characteristics that we verified in Abz-prolinyl-peptides and other Abz-dialkylamides, compared

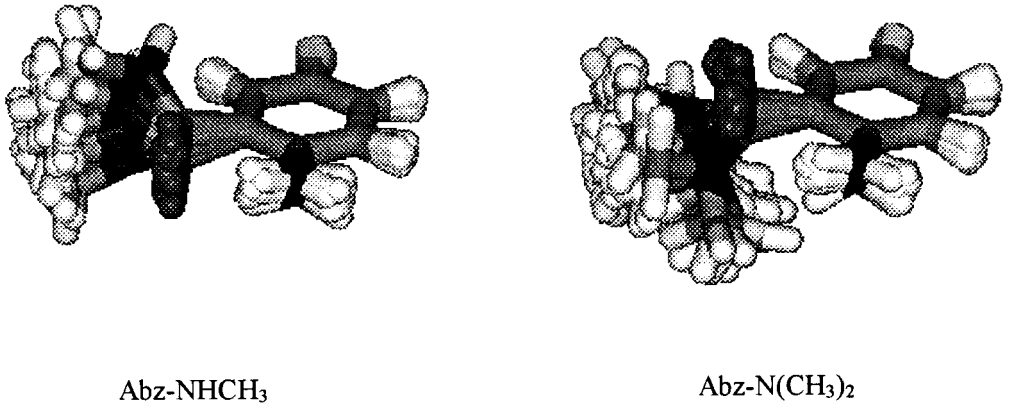


Figure 2. Structures of *ortho*-aminobenzoyl methylamide (Abz-$NHCH_3$) and *ortho*-aminobenzoyl dimethylamide [Abz-$N(CH_3)_2$], obtained from molecular dynamics simulation, performed using the Amber force-field (Insight II 95).

to the Abz-aminoacyl-peptides. This lack of periplanarity, associated to the absence of a hydrogen atom in the amide nitrogen, is probably involved with nonradiative routes for the deactivation of the excited state of the molecules.

Abz-$NHCH_3$ and Abz-$N(CH_3)_2$ were studied by 1D- and 2D-NMR spectroscopy on a Bruker 600 MHz spectrometer. Assignments were performed using COSY, ROESY, and carbon–proton correlated spectra. Both the ^{1}H-NMR and ^{13}C-NMR spectra were fully assigned (Table II) except for the amine protons in Abz-$NHCH_3$, which seem to exchange too fast with the solvent. The proton chemical shifts of Abz-$N(CH_3)_2$ are almost identical in polar DMSO and less polar $CHCl_3$. The carbon chemical shift values of carbon 1, 2, 3, 4, 5, and 6 of the aromatic ring in Abz-$N(CH_3)_2$ are as expected from calculation of substituent effects (ACD ^{1}H- and ^{13}C-NMR program package) and correspond also to that of aniline, where no hydrogen bond is possible. In contrast large changes in chemical shifts of 5–6 ppm, which can only be explained by the formation of a strong hydrogen bond are observed for Abz-$NHCH_3$. Carbon 1 is shielded and 2 and 4 are deshielded in accordance with the electron donation to carbon 1 through the hydrogen bond formation.

The proton chemical shift values of H3-6 in Abz-$N(CH_3)_2$ indicate that all the protons of the ring system are shielded compared to the calculated values (ACD program), whereas the values of Abz-$NHCH_3$ are normal. This observation is in agreement with an anisotrophic change of chemical shift values due to the lack of antiperiplanarity of the amide carbonyl group with the aromatic ring. The experimental data, therefore support the molecular modeling, demonstrating that in the Abz-dialkylamides the carbonyl group is not antiperiplanar and do not form a strong hydrogen bond to the aromatic *ortho*-amine, probably due to steric interaction between the methyl groups and H3 of the aromatic ring.

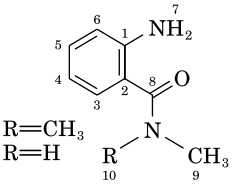
CONCLUSIONS

With exception of Abz-Pro-$NHCH_3$ and Abz-Trp-$NHCH_3$, all the other Abz-aminoacylamides studied in this work maintained the main fluorescence characteristics of the parent fluorophore, Abz, like the high quantum yield (above 0.33), the monoexponential decay of fluorescence and the fluorescence lifetime value near to 8.0 ns. Within this group of compounds there should be differences in the nonradiative pathways for the deexcitation of fluorescence and in the transition probabilities between ground and excited electronic states, leading to the observed differences in quantum yield. However, the differences among the Abz-aminoacyl compounds within this group are minor compared to the quenching of fluorescence occurring in Abz-Pro-$NHCH_3$ and Abz-Trp-$NHCH_3$. In the first, the quenching is similar to that occurring in Abz-dimethylamide, and in the second, the quenching possibly is involving interaction with the indol group. We observed previously that Abz is not a convenient probe in proteolitic assays when the first residue in the substrate, to which the fluorophore is bound, is proline.[9] In this work we demonstrated that a similar restriction applies to substrates having

Table II. ^{1}H-NMR and ^{13}C-NMR Spectra Assignments for AbzNHCH$_3$ and AbzN(CH$_3$)$_2$

AbzNHCH$_3$	^{1}H	^{13}C	n.O.e.	AbzN(CH$_3$)$_2$	^{1}H	^{13}C	n.O.e.
1		139.7		1		145.6	
2		121.4		2		120.0	
3	7.68	128.2	10	3	6.98	127.6	10
4	7.08	121.8		4	6.55	115.5	
5	7.40	131.8		5	7.07	129.7	
6	7.15	120.9		6	6.69	115.5	7
7	—[a]			7	5.11		6
8		167.8		8		169.8	
9	2.77	26	10	9	2.91	38.3	
10	8.61		3, 9	10	2.91	34.9	3

[a] Could not be determined probably due to solvent exchange. The spectra were recorded in d6 DMSO at 25°C.



the Trp residue as the first aminoacid in the primary sequence of the peptide.

All the others aminoacids examined in this work maintain the general fluorescence properties of the Abz. Thus, it is established that Abz can have a generalized use as fluorescent probe in peptide synthesis directed to the assay of proteolytic enzymes activity, when the aminoacid to which it is attached is one of those studied in the present work, with exception of proline and tryptophan. Furthermore, besides the application of the fluorophore in enzymology, examination of the fluorescence parameters of the compounds in the presence of interfaces with different polarities could give information about the possibility of the use of Abz as probes for peptide–lipid interactions. Such possibility is being demonstrated by experiments conducted in our laboratories with Abz bound to acidic (Glu), basic (Arg) or neutral (Phe) aminoacids in interaction with SDS vesicles.[17]

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