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New highly fluorescent amino-acid derivatives Substituted 3-[2-(phenyl)benzoxazol-5-yl]-alanines: synthesis and photophysical properties

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Abstract

New derivatives of 3-[2-(phenyl)benzoxazol-5-yl]alanine (Box-Ala) substituted at position 2′ and 4′ with OH, OMe, NMe_2 groups were prepared. Fluorescence properties (spectra, quantum yields) of new compounds were determined and compared with parent molecules without amino acid moiety. It was established that fluorescence properties of known benzoxazoles and new ones are similar. In the case of 3-[2-(2′-hydroxyphenyl)benzoxazol-5-yl]alanine ((*o*-OH)Box-Ala), presence of many different tautomeric forms in the ground state were found depending on solvent used.

Keywords: Benzoxazole; 3-(Benzoxazol-5-yl)alanine derivatives; Fluorescence

1. Introduction

Benzoxazole, benzothiazole and their derivatives because of interesting properties have been objects of comprehensive studies in recent years. These compounds have been applied in numerous aspects of science and life. 5-Chloro-2-amino-benzoxazole displays quite high biological activities such as muscle-relaxant, fungicide [1,2]. Unsubstituted benzothiazole influences the expression of heptoic glutathione S-transferases Ya, Yb1, Yb2 and Yc2 [3], whereas 2-(4-amino-phenyl)benzothiazole shows potent inhibitory activity in vitro against a panel of human breast cancer cell lines MCF-7 and MDA 468 [4]. Moreover, structurally unique, bis(benzoxazole), in which the position 2 of the first benzoxazole molecule is bound to the position 4 of the second one, shows moderate cytotoxic activity against B16, Hela and P338 cells [5,6]. Benzoxazole with a nitrogen-containing heterocyclic substituent at the position 2 is active in isolated guinea pig ileum test [7]. Many scientific articles have focused on the absorption and emission properties of substituted 2-phenylbenzoxazoles because of their high quantum yields (QYs) [8–33]. The high fluorescence QYs of these compounds make them suitable as highly efficient UV dyes [12,13,21,22], scintillation counters [17] and optical fiber sensors [18]. The particular place among substituted 2-phenyl-benzoxazoles, 2-phenyl-benzothiazoles, 2-phenyl-imidazoles and 2-pyridine-imidazole belongs to derivatives substituted at position 2′ of phenyl ring or position 3′ of pyridyl ring by hydroxyl group because of excited state intramolecular proton transfer (ESIPT) occurring [23–55]. These heterocyclic compounds are generally colorless, but display red-shifted, dual fluorescence, which even moves into the near-infrared region for bis-2,5-(2′-benzoxazoyl)-hydroquinone [16,28]. Earlier studies using jet spectroscopy and semiempirical calculations [27,28,55], femtosecond emission spectroscopy [26], transient absorption spectroscopy and ab initio calculations [30] clearly established that the large Stokes-shifted fluorescence band observed for 2-(2′-hydroxyphenyl)benzoxazole (HBO) was connected with the presence of keto-tautomer, the tautomer produced on excitation (structure IV, Fig. 1), whereas the normal Stokes-shifted band was due to the enol form of molecule (structure II, Fig. 1). The very low fluorescence QYs of both bands are consistent with the observation that the ESIPT rate is very fast (60–100 fs) [26,43,44]. The ESIPT process is very fast because the energy barrier for this process is small or the process is even barrierless. In addition, the rate of internal conversion in the ESIPT process is high if the energy gap between tautomers level is not large. The fluorescence excitation spectrum indicates that these two species have the identical precursor.

The interesting photophysical properties of benzoxazole derivatives and their broad and fascinating biological

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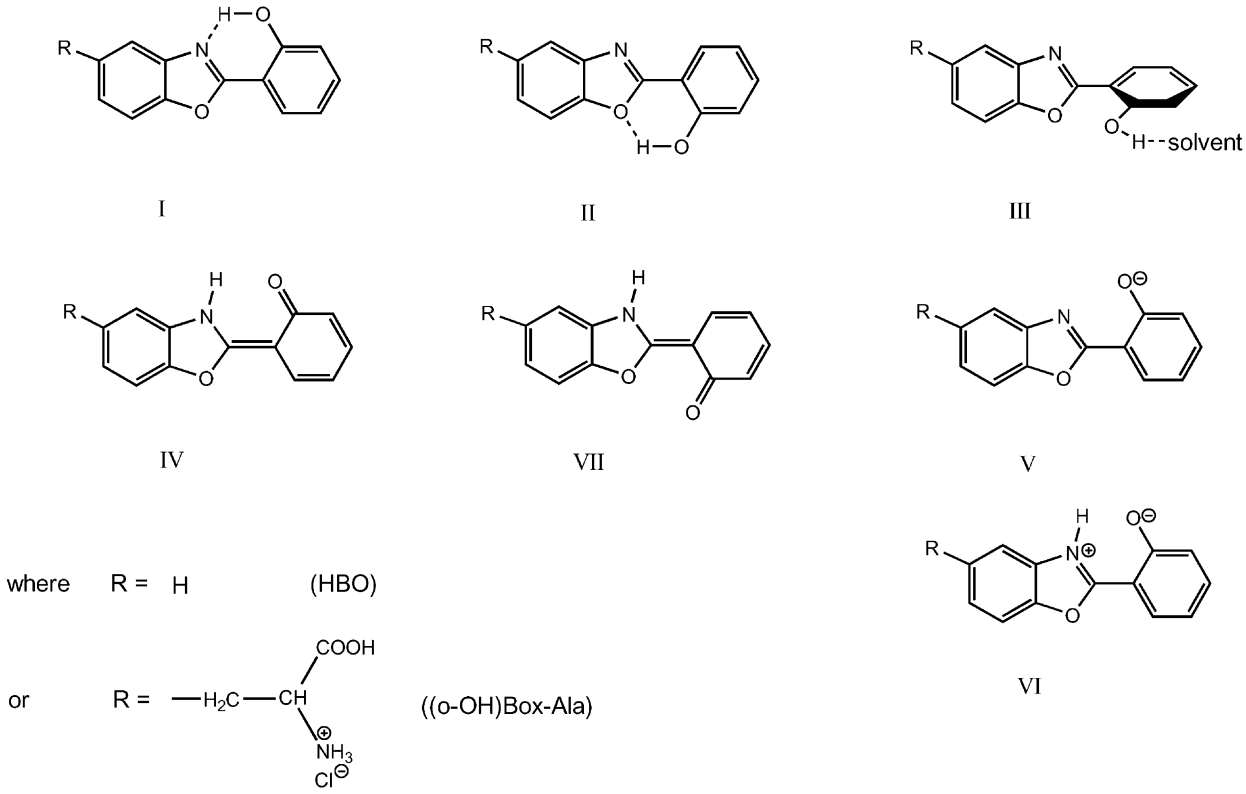

Fig. 1. Structure of different tautomers of HBO and 3-[2-(2′-hydroxyphenyl)benzoxazol-5-yl]alanine ((*o*-OH)Box-Ala).

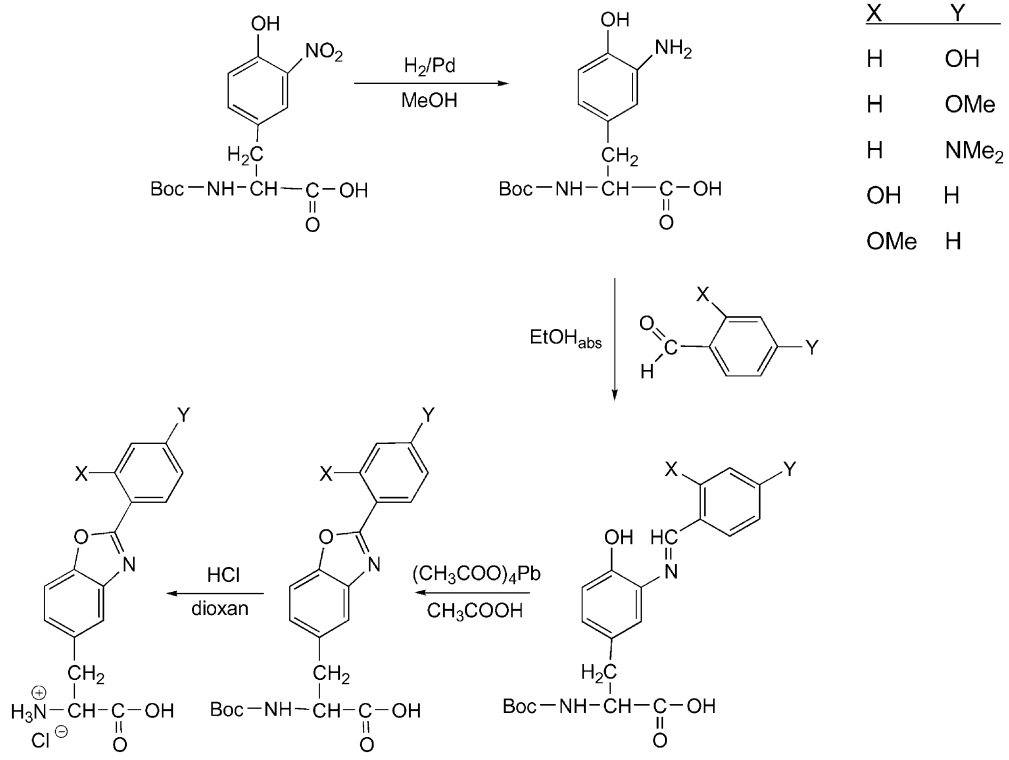

Fig. 2. Reaction scheme.

activity profiles led us to undertake synthesis of alanine derivatives containing substituted 2-phenylbenzoxazole moiety. Obtained amino acid derivatives, because of the presence of amino and carboxyl groups, can be incorporated into peptide chains and as such used as an energy donor in conformational studies of peptides by means of fluorescence. It can also be used as an energy donor in substrates working on the basis of intramolecular energy transfer in kinetic studies of enzymatic reactions (hydrolysis of substrates, estimation of inhibitor activities).

Benzoxazoles and their derivatives can be prepared using two general procedures. The first direct method (one-step process) is based on the reaction between appropriate benzoic acid or benzoyl chloride with *ortho*-aminophenol in polyphosphoric acid [56]. In the second method (two-step process), first, it is necessary to prepare Schiff base from *ortho*-aminophenol and an appropriate substituted benzaldehyde and then such prepared Schiff base is cyclized to benzoxazole moiety by means of oxidation [57–62]. The oxidation process can be realized also photochemically [59,60]. In our case, because of necessity of protection of amino group in amino acid moiety by labile Boc-protection and to avoid racemization process, studied compounds were obtained by a second method, i.e. by syntheses of appropriate Schiff base followed by cyclization using lead tetra-acetate ($Pb(OAc)_4$) in acetic acid (AcOH). The scheme of synthesis is presented in Fig. 2.

2. Experimental procedures

2.1. Synthesis

Boc-(3-nitro)tyrosine was prepared using (3-nitro)tyrosine (purchased from Fluka) according to a known procedure [63]. Boc-(3-nitro)tyrosine was reduced to Boc-(3-amino)-tyrosine by means of catalytic hydrogenation in methanol (H_2/Pd/C). Removal of catalyst and solvent from reaction mixture yielded Boc-(3-amino)tyrosine which was used, without any additional purification, in next step. The Schiff base was prepared according to the procedure described in literature [57].

An appropriate substituted benzaldehyde (1.1 mmol) in ethanol (15 ml) was added to a solution of Boc-(3-amino)tyrosine (1 mmol) in ethanol (15 ml). The mixture was refluxed for 5 min, cooled to room temperature and the solid product was removed by filtration. Recrystallization from ethanol gave the Schiff base with yield of about 85–90%.

Cyclization of Schiff base to benzoxazole structure was performed as described in literature [57]. The benzoxazoles were obtained by treatment of the corresponding Schiff base dissolved in AcOH with one molecular equivalent of $Pb(OAc)_4$. After a few minutes, the solvent was removed by evaporation and the desired compounds (alanine derivatives containing benzoxazole moiety at the side chain) were isolated from reaction mixture by means of column chromatography (silica gel 60, 0.040–0.063 mm, Merck). The typical mobile phase was a mixture of ethyl acetate and petroleum ether with small addition of acetic acid. The total yield of the reaction was about 10%. The Boc protection group was removed by treatment of 4 N HCl in dioxane. Final purifications of free amino acids were performed by reversed phase liquid chromatography (RP-HPLC) on C_{18} column. Mobile phase was a gradient running from water to 80% aqueous solution of acetonitrile with addition of 0.1% TFA.

2.2. Spectroscopic methods

Absorption spectra were recorded using a Perkin-Elmer Lambda-18 spectrophotometer. Fluorescence spectra were recorded applying a Perkin-Elmer LS-50B spectrofluorimeter. QYs were calculated using as a reference tryptophan in water (QY = 0.14) or quinine sulfate in 1 N H_2SO_4 (QY = 0.54). Solvents used in our studies: methanol (MeOH), propanol-2 (*i*-PrOH), dimethylformamide (DMF), acetonitrile (MeCN), dimethyl sulfoxide (DMSO), dichloromethane (DCM), tetrahydrofuran (THF) and water were spectroscopic or HPLC grade. The sample concentration was in the range 2×10^{-5}–5×10^{-5} M for the absorption measurements, whereas it was 10 times lower (2×10^{-6}–5×10^{-6} M) for fluorescence measurements. In this concentration range, no aggregation of the sample was observed.

3. Results and discussion

Absorption spectra of the obtained compounds in MeOH are presented in Fig. 3, except 3-[2-(2′-hydroxyphenyl)benzoxazol-5-yl]alanine ((*o*-OH)Box-Ala) which is discussed separately because of its unique photophysical properties. The spectra and band positions of all obtained compounds in MeOH are very similar to the spectra of the parent molecules (substituted 2-phenylbenzoxazoles without amino acid moiety) published in literature [13,40,46,47]. The molar absorption coefficients found for 3-{2-[4′-(*N*,*N*-dimethylamino)phenyl]benzoxazol-5-yl}-alanine ((*p*-NMe_2)Box-Ala), 3-[2-(4′-hydroxyphenyl)benzoxazol-5-yl]alanine ((*p*-OMe)Box-Ala) and 3-[2-(phenyl) benzoxazol-5-yl]alanine (Box-Ala) in MeOH are about 40 000 $dm^3\,mol^{-1}\,cm^{-1}$, whereas for 3-[2-(2′-methoxyphenyl)benzoxazol-5-yl]alanine ((*o*-OMe)Box-Ala) and 3-[2-(4′-hydroxyphenyl)benzoxazol-5-yl]alanine ((*p*-OH) Box-Ala) about 20 000 and 13 000 $dm^3\,mol^{-1}\,cm^{-1}$, respectively. In water, absorption bands of the studied compounds are slightly shifted to the short-wave region, while the molar absorption coefficients are substantially decreased to a value of about 12 000–14 000 $dm^3\,mol^{-1}\,cm^{-1}$ for (*p*-NMe_2)Box-Ala and about 9000 and 7000 $dm^3\,mol^{-1}\,cm^{-1}$ for (*o*-OMe)Box-Ala and for (*p*-OMe)Box-Ala and (*p*-OH)Box-Ala, respectively. Because of a very low solubility of Box-Ala in water, the measurement of absorption coefficient for this compound was performed in MeOH

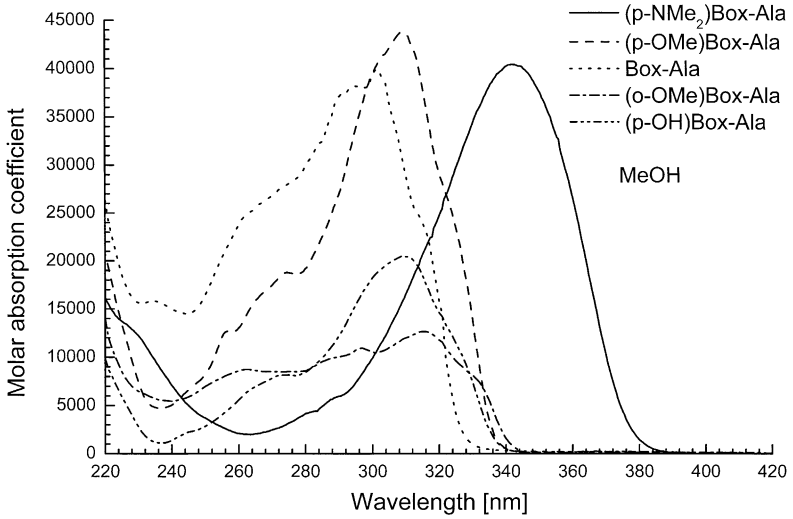

Fig. 3. Absorption spectra of Box-Ala, (*o*-OMe)Box-Ala, (*p*-OH)Box-Ala, (*p*-OMe)Box-Ala and (*p*-NMe_2)Box-Ala in MeOH.

only. The effect of water on the longest wavelength band, which has been attributed to a π–π^* state with charge transfer character [64], can be explained by the formation of strong hydrogen bond between solute and solvent. Such phenomenon was observed also by Dey and Dogra [29] for 2-(2′-hydroxyphenyl)benzothiazoles.

Fluorescence excitation spectra for all compounds studied, except (*o*-OH)Box-Ala, are very similar to the absorption spectra in both solvents: MeOH and water (figure not shown).

3.1. Emission spectra and fluorescence QY

Fluorescence spectra of studied compounds look like mirror reflection of absorption spectra (Fig. 4). A substituent at *para* position of the phenyl ring shifts the emission band to the longer wavelength. The maximum of emission is at 350–360 nm in both solvents, MeOH and water, except for (*p*-NMe_2)Box-Ala, for which the maximum of emission band is about 400 nm in MeOH and 410 nm in water. The same positions of the emission maxima were observed for the parent molecules [13,46]. Additionally, all studied derivatives, except (*p*-NMe_2)Box-Ala, exhibited the same weakly pronounced vibrational structure of emission spectra as the parent compounds [13,46]. The differences in positions of emission bands for (*p*-OH)- and (*p*-OMe)-substituted derivatives are very small. The highest shift of emission bands towards short wavelengths was observed for unsubstituted Box-Ala in MeOH.

Quantitative characteristics of the emission properties of substituted Box-Ala are presented in Table 1. The values of fluorescence QYs are quite large and are slightly higher in water than in MeOH. The observed large values of fluorescence QYs and their increase with solvent polarity are

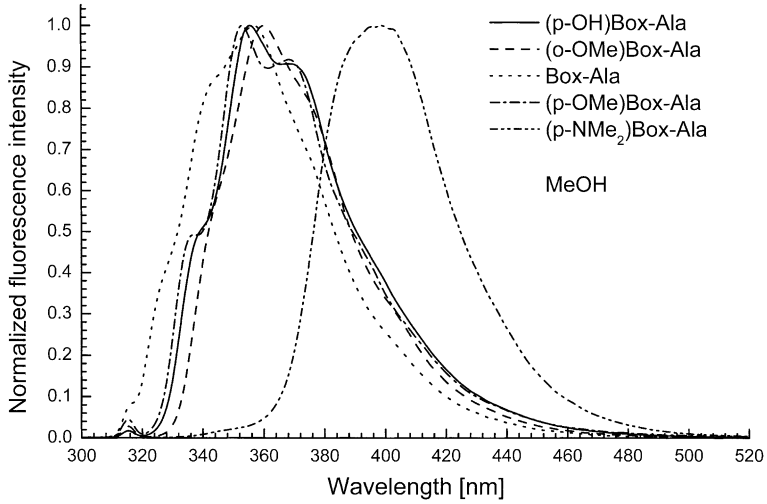

Fig. 4. Fluorescence spectra of Box-Ala, (*o*-OMe)Box-Ala, (*p*-OH)Box-Ala, (*p*-OMe)Box-Ala and (*p*-NMe_2)Box-Ala in MeOH.

Table 1
Fluorescence QYs of substituted 3-[2-(phenyl)benzoxazol-5-yl]alanines in different solvents

Compound	Solvent	
	MeOH	Water
Box-Ala	0.75	–
(*p*-OH)Box-Ala	0.81	0.87
(*p*-OMe)Box-Ala	0.75	0.88
(*o*-OMe)Box-Ala	0.65	0.67
(*p*-NMe_2)Box-Ala	0.86	0.92

in good agreement with data published by Kanegae et al. [13] for 2-phenylbenzoxazole derivatives, the compounds without amino acid moiety. The observed behavior of the emission properties of studied compounds suggest that the amino acid moiety of Box-Ala compounds influences only slightly the emission properties of the 2-phenylbenzoxazole chromophore.

3.2. Spectral properties of 3-[2-(2′-hydroxyphenyl)benzoxazol-5-yl]alanine

3.2.1. Absorption spectra

Absorption spectra of (*o*-OH)Box-Ala measured in different solvents are presented in Fig. 5. Additionally, the absorption spectrum of (*o*-OH)Box-Ala in MeOH with the addition of base (KOH) is incorporated in Fig. 5. The absorption spectra of that compound (shape and position of bands) in short-wave region are generally similar to published data for the compound without amino acid moiety [23,24,27,29,40,42–44,47,52,53]. The larger differences between absorption spectra of studied compound depending on the solvent used were observed in long-wave region. For most solvents, except MeCN and DCM, a band with maximum at 360–370 nm was observed and the position of this band maximum was dependent on solvent used, which was also observed for HBO by Woolfe et al. [43,44] and Cohen and Flavian [42]. In strong alkaline solution (MeOH/KOH) (Fig. 5), and in water at pH = 10 (Fig. 6), the structured absorption band at 320–340 nm, characteristic for HBO chromophore, disappeared, and a new long-wave band with a maximum at 360 nm was observed [47]. Additionally, the shape of the spectrum at 280–310 nm is changing and the band at 300 nm is becoming weaker, whereas the one at 290 nm is increasing. The long-wavelength absorption band of (*o*-OH)Box-Ala (at about 360–370 nm), according to Stroykov et al. [47], can be attributed to the absorption of monoanion present in a trace amount in the ground state (structure V, Fig. 1).

3.2.2. Emission spectra

Emission spectra of (*o*-OH)Box-Ala are characterized by dual fluorescence spreading over a very wide region, from 360 to about 600 nm. Fluorescence QYs are low, not exceeding 0.1 (Table 2) and depend on excitation wavelength. The dual fluorescence band and low QY observed for (*o*-OH)Box-Ala are in agreement with previously published data for HBO [23–25,27,29,43,44,47,53]. The intensity, shape and position of bands depend on solvent used [43,44,47,53]. In DCM (Fig. 7) and MeCN (Fig. 8), two emission bands of (*o*-OH)Box-Ala with maxima at 475 and 360 nm were observed (regardless of the excitation wavelength: 285 or 315 nm), indicating the possibility of proton transfer in the excited state. The emission band located at about 475 nm is attributed to the tautomeric species (“keto” form, structure IV in Fig. 1) in which the phenolic proton is transferred to the nitrogen atom. The short wave, normal Stokes-shifted emission with maximum at 360 nm is assigned to the excited form of the molecule in which the proton remains on the phenolic oxygen (structure II, Fig. 1). Woolfe et al. [43,44] as well as Das et al. [53] proposed

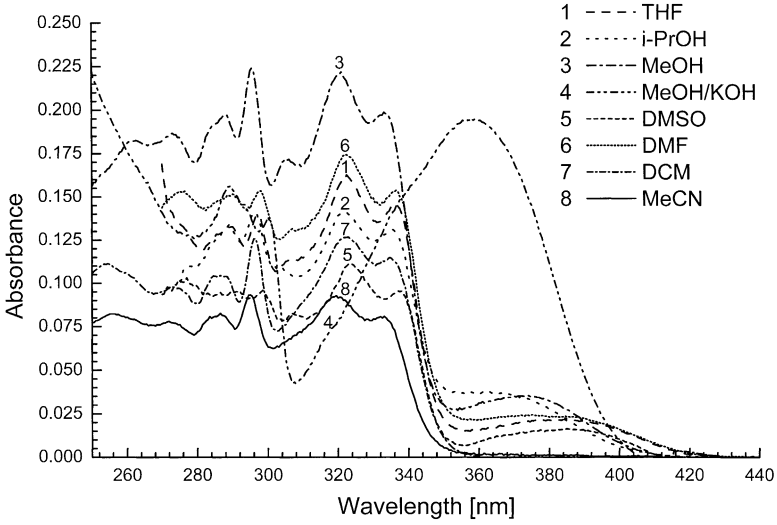

Fig. 5. Absorption spectra of (*o*-OH)Box-Ala in different solvents.

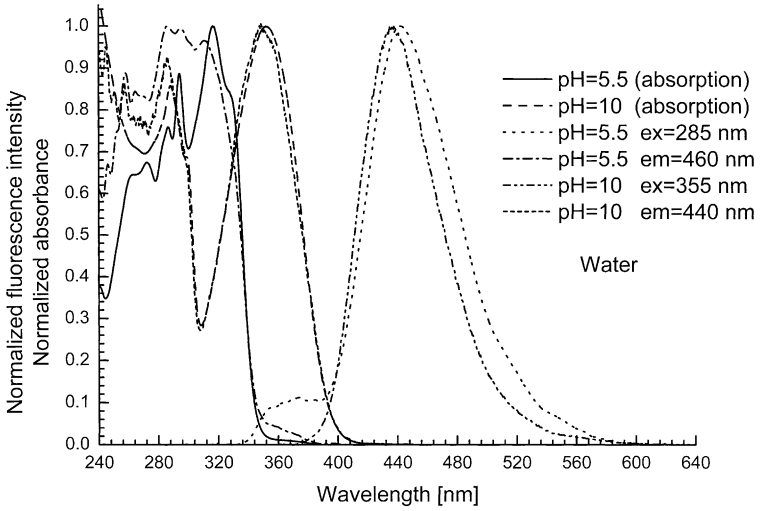

Fig. 6. Absorption, emission and fluorescence excitation spectra of (*o*-OH)Box-Ala in water at pH = 5.5 and 10, recorded at different excitation and observation wavelengths.

Table 2
Fluorescence QYs of 3-[2-(2′-hydroxyphenyl)benzoxazol-5-yl]alanine in different solvents at different excitation wavelengths

Solvent	Excitation wavelength (nm)		
	285	315	370
DCM	0.045	0.053	–
DMF	0.037	0.031	–
DMSO	0.078	0.077	0.594
i-PrOH	0.097	0.055	0.503
THF	0.065	0.022	0.595
MeCN	0.057	0.033	–
MeOH	0.089	0.028	0.518
MeOH/KOH	0.468	–	0.476

that the strongly solvated HBO (form III, Fig. 1) also contributes to the normal emission, because of intermolecular hydrogen bonding which inhibits the excited proton transfer process and favors the normal emission at the expense of the tautomer emission. The ratio of the intensity of both short- and long-wave emission bands in both above-mentioned solvents (DCM and MeCN) are clearly different. In DCM, the band attributed to the "keto" form dominates and the fluorescence excitation spectrum (λ_{em} = 500 nm) begins at about 350 nm and exhibits more pronounced vibrational structure. On the other hand, the fluorescence excitation spectrum observed at emission of 350 nm is shifted to shorter wavelength. In MeCN, the short-wave emission band is clearly dominating, with intensity about 10 times higher than the intensity of long-wave emission band at 475 nm. The fluorescence excitation spectrum observed

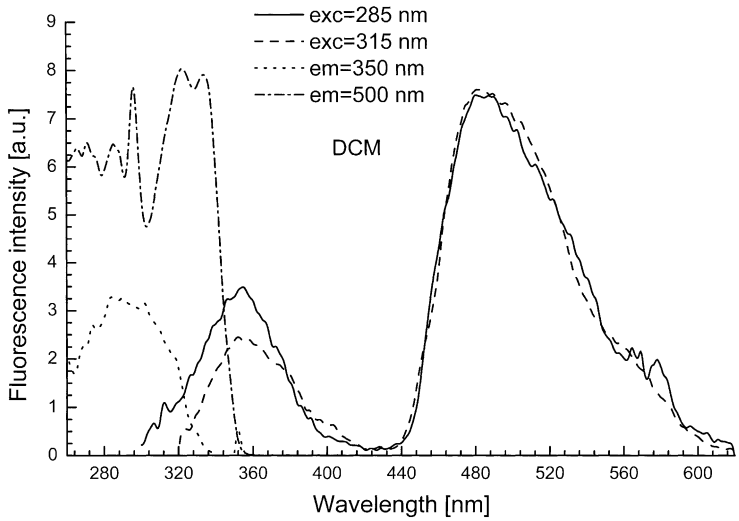

Fig. 7. Fluorescence (λ_{ex} = 285 or 315 nm) and fluorescence excitation spectra (λ_{em} = 350 or 500 nm) of (*o*-OH)Box-Ala in DCM.

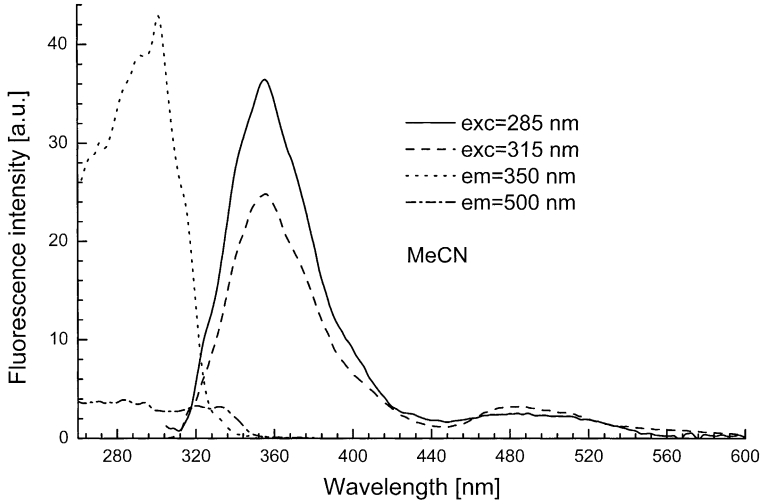

Fig. 8. Fluorescence (λ_{ex} = 285 or 315 nm) and fluorescence excitation spectra (λ_{em} = 350 or 500 nm) of (*o*-OH)Box-Ala in MeCN.

using emission of 350 nm displayed a distinct maximum in the 300–310 nm region, whereas the fluorescence excitation spectrum observed at emission 500 nm showed maximum at 340 nm (Fig. 8). The distinct domination of the normal short-wavelength band in the emission spectrum of (*o*-OH)Box-Ala in MeCN can be explained by the fact that the larger electric dipole moment of form II (Fig. 1) is stabilized better in a more polar solvent [53]. The opposite situation is true in non-polar solvent like in DCM.

In the remaining solvents used (DMF, DMSO, THF, MeOH and *i*-PrOH) capable of disrupting the intramolecular hydrogen bond, the ratio of short-wave to long-wave emission intensity is clearly larger in more polar solvents similar to what was observed by other authors for the parent molecule (HBO) [43,44,53]. On the other hand, distinct differences in the shape of the long-wave emission band between the published spectra of HBO [43,44,47,53] and the presently studied (*o*-OH)Box-Ala, in the above-mentioned solvents, are observed. In the emission spectrum of (*o*-OH)Box-Ala dissolved in DMF, apart from short-wave band with maximum at about 360 nm, the relatively intense band with maximum at about 440 nm and the shoulder at about 475 nm are observed (Fig. 9). A very similar shape of the emission spectrum of (*o*-OH)Box-Ala was also observed in THF, MeOH and *i*-PrOH. The relative intensities of these bands depend on the excitation wavelength used. The fluorescence QY dependence on the excitation wavelength, as well as the fact that the excitation spectra are different from one another in all solvents used confirms the existence of various tautomeric and/or rotameric species in equilibrium. As discussed above, the structure II is responsible for the short-wave emission band, whereas for the long-wave emission — structure IV (Fig. 1). A single, intense emission band for HBO, with maximum at

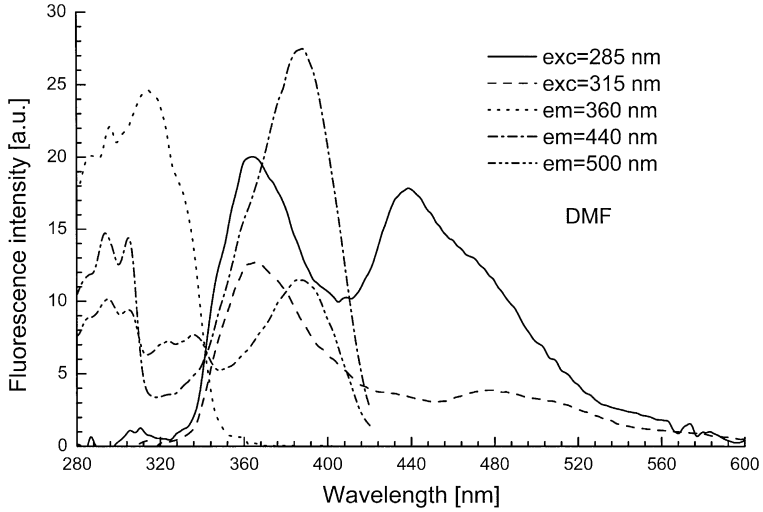

Fig. 9. Fluorescence (λ_{ex} = 285 or 315 nm) and fluorescence excitation spectra (λ_{em} = 360 or 440 or 500 nm) of (*o*-OH)Box-Ala in DMF.

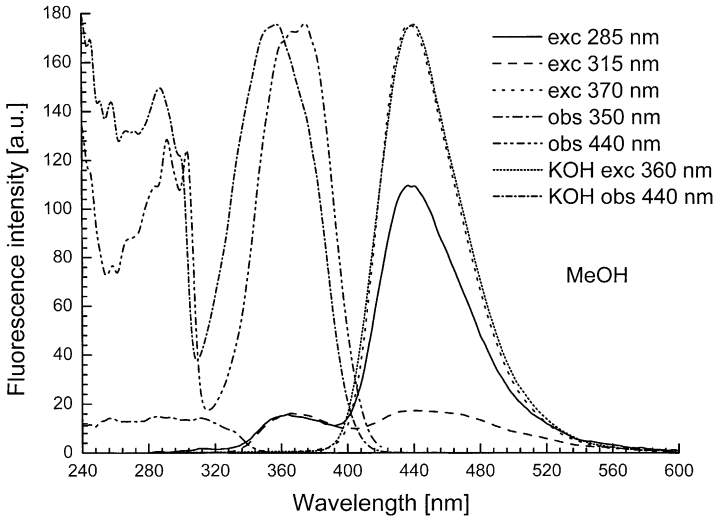

Fig. 10. Fluorescence and fluorescence excitation spectra of (*o*-OH)Box-Ala in MeOH and in alkaline methanolic solution recorded at different excitation and observation wavelengths.

440 nm, was observed by Woolfe et al. [43,44] as well as by Stroykov et al. [47] arising from excitation in the long-wave range (∼370 nm). This emission was attributed by Woolfe et al. [44] to the "*trans*-keto" form (structure VII, Fig. 1), while by Stroykov et al. [47] to the monoanion (structure V, Fig. 1). An internal twisting resulting *trans*-keto form has been observed in the H-atom transferred form of 2-(2′-hydroxyphenyl)benzothiazole [45,65], but not for HBO [45]. According to the works of Brewer et al. [65], and Guallar et al. [31], the *trans*-keto form have red-shifted emission in comparision to "*cis*-keto" form (structure IV, Fig. 1).

In alkaline methanolic solution of (*o*-OH)Box-Ala (Fig. 10), only one emission band is observed with maximum at about 440 nm, regardless of the excitation wavelength used. The fluorescence excitation spectrum recorded at 440 nm displayed two bands: one structureless with maximum at 355 nm and second one with vibrational structure in the range 260–300 nm. The fluorescence spectrum of (*o*-OH)Box-Ala in pure MeOH excited at 370 nm is identical with that recorded in alkaline methanolic solution, while the fluorescence excitation spectrum is different in shape and red-shifted (about 20 nm) in comparision to that recorded in alkaline solution. Taking these findings into account, the emission band with a maximum at about 440 nm can be attributed to the emission of monoanionic form (structure V, Fig. 1), as was suggested by Stroykov et al. [47] for HBO and found for 2-(2′-hydroxy-4′-methoxy-phenyl)benzothiazole by Dey and Dogra [29].

In aqueous solution, regardless of the wavelengths used for excitation (285 or 315 nm), two emission bands were observed; low intensity band at 350–400 nm and a 10 times more intense band with a maximum at 445 nm (Fig. 6). The first band can be attributed to the emission from a neutral form (structure II, Fig. 1), whereas the shape and position of the second band looks like the emission band of the monoanionic form of (*o*-OH)Box-Ala recorded in alkaline methanol solution (Fig. 10). The fluorescence spectrum of (*o*-OH)Box-Ala recorded in water at pH = 10 (monoanion, form V, Fig. 1) possesses only one feature blue-shifted by about 10 nm (maximum at about 435 nm) compared with that recorded for water at pH = 5.5.

The fluorescence excitation spectrum of (*o*-OH)Box-Ala monoanion recorded at the maximum of fluorescence intensity (440 nm) closely matches the absorption spectrum, whereas the fluorescence excitation spectrum of (*o*-OH)Box-Ala in water at pH = 5.5 recorded at 460 nm is sharply shifted towards short wavelength. The shape of this spectrum is also different than that of the monoanionic form. Also, the fluorescence excitation spectrum of (*o*-OH)Box-Ala in water at pH = 5.5 observed at 370 nm (not shown in Fig. 6) differs from the absorption spectrum. The differences of the spectra of (*o*-OH)Box-Ala in water recorded at different values of pH, as well as the differences in the shape and position of the absorption and fluorescence excitation spectra indicate that the zwitterion (form VI, Fig. 1) may be responsible for long-wavelength emission in water at pH = 5.5. Based on the steady-state and time-resolved fluorescence spectroscopy of HBO, Woolfe et al. [43] concluded that the more polar zwitterion should be favored in solvents of high dielectric constant, while less polar *cis*-keto form (IV) exists preferentially in lower dielectric constant solvents. Moreover, both of these forms (IV and VI) occur in equilibrium in the excited state. The existence of an excited state of the zwitterionic form of HBO was also suggested by LeGourrierec et al. [64].

Comparison of the data collected in Table 2 can lead to the conclusion that QYs of neutral and *cis*-keto forms (II and IV) are relatively lower than that of monoanionic form (V). Large fluorescence QY of monoanionic form, similar

to the values obtained for (*o*-OMe)Box-Ala, suggest lack of structural changes, such as H-transfer in the excited state.

4. Conclusion

The synthesized derivatives of alanine containing phenylbenzoxazole moieties have similar photophysical properties to the unsubstituted phenylbenzoxazole chromophore. All prepared and studied compounds, except derivative containing hydroxyl group at 2′ position of phenyl ring, displayed similar fluorescence properties. The high fluorescence QYs of the benzoxazole-modified alanines make them potentially very powerful energy donors in conformational studies of peptides by means of intramolecular Förster resonance energy transfer. Moreover, high overlap between absorption and emission spectra of Box-Ala derivatives makes them suitable also for determining intramolecular distances by means of donor–donor energy migration [66]. Additionally, the studied compounds have the virtue of high molar absorption coefficients and long-wavelength absorption, which can allow their selective excitation even in the presence of tryptophan.

The (*o*-OH)Box-Ala showed multiple fluorescence features characterized by low QY and covering a wide spectral region, possibly due to intramolecular proton transfer in the excited state. For this derivative, the emission behavior in different solvents revealed the presence of an amino acid moiety forming a hydrogen-bond network, a strong intermolecular hydrogen bond between phenolic hydroxyl group and solvent, which may have been the precursor of the monoanionic form created in the excited state. This was easily observed in the fluorescence spectrum of (*o*-OH)Box-Ala contrary to HBO (without amino acid moiety), even at short-wavelength excitation. In the case of aqueous solution at neutral pH, the fluorescence of (*o*-OH)Box-Ala is dominated by the zwitterionic form. The diversity of photophysical properties and the presence of dual fluorescence observed for (*o*-OH)Box-Ala make this compound potentially useful as a luminescence probe in protein studies as proposed by Kasha and coworkers [67,68] for compounds possessing intramolecular proton transfer in the excited state.

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