

Full Research Paper

Synthesis, Characterization and Metal Ion Detection of Novel Fluoroionophores Based on Heterocyclic Substituted Alanines

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Received: 19 September 2007 / Accepted: 27 September 2007 / Published: 3 October 2007

Abstract: The synthesis of new fluorescent probes containing the thiophene and benzoxazole moieties combined with an alanine residue is described. The resulting highly fluorescent heterocyclic alanine derivatives respond via a quenching effect, with paramagnetic Cu(II) and Ni(II) metal ions and with diamagnetic Hg(II), as shown by the absorption and steady-state fluorescence spectroscopy studies. The formation of mononuclear or dinuclear metal complexes was postulated based on the presence of the free carboxylic acid as binding site and also with the interaction with the donor atoms in the chromophore. Interaction with other important biological metal ions such as Zn(II), Ca(II) and Na(I) was also explored.

Keywords: Benzoxazole; Thiophene; Amino acids; Fluorescence; Fluorescent probes.

1. Introduction

In the last few years various 2-substituted benzoxazole derivatives were studied extensively for their antitumor, antiviral, antimicrobial activities as non-nucleoside topoisomerase 1 poison, HIV-1 reverse transcriptase and/or DNA gyrase inhibitors [1]. Other applications include *in vivo* probes for positron emission tomography (PET) and single-photon emission computed tomography for early detection of amyloid plaque formation and for visualization of brain dopamine D₃ receptors [2-4].

Derivatives containing the benzoxazole or the thiophene nucleus combined with amino acid moieties have also important biological applications such as potent inhibitors of human cystein proteases, as ligands of the NMDA receptor, in molecular recognition, as biomarkers or biosensors [5-8]. Owing to their excellent optical properties and high lipophilicity, it is possible for benzoxazole compounds to be used as sensing materials. Therefore, several authors described recently the application of benzoxazole derivatives as fluorescent and/or colorimetric sensors for metals, anionic species and pharmaceutical analysis [9-17].

The interesting biological activity and photophysical properties of thiophene and benzazole derivatives lead us to the synthesis of alanine derivatives containing these heterocyclic nuclei [18-24]. The thienyl-benzoxazole amino acid derivatives can be incorporated into peptide chains and as such used as energy donors in conformational studies of peptides by means of fluorescence or be used as fluorescence markers. To our knowledge this is the first time that a fluorescent sensor containing the thiophene and benzoxazole fluorophores combined with the alanine residue selectively deprotected is described.

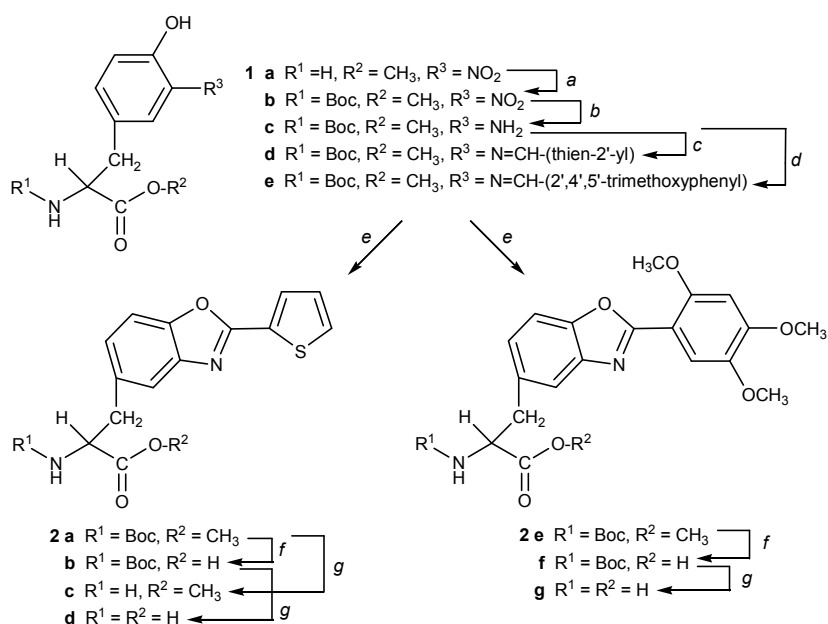
As a part of our ongoing research, in the present work we have combined the ability of amino acids for metal ion chelation with the strong fluorescence properties of the 2-substituted benzoxazole moiety. Thus, we were interested in studying the influence of both potential chelating units incorporated: *i*) the amino acid residue, bearing an amino and carboxylic acid terminal groups, partially or totally deprotected and *ii*) the aromatic chelating unit in the chromophore, with two possibilities for chelation: a NS or OS donor sets. The presence of two or more coordination centres in these compounds can lead to the design of interesting supramolecular structures for biological and environmental applications.

2. Results and Discussion

2.1. Synthesis

Starting from commercially available 3-nitro-L-tyrosine, reaction with thionyl chloride in methanol yielded the corresponding methyl ester **1a**. Subsequent reaction with *t*-butyl pyrocarbonate resulted in the *N*-Boc protected derivative **1b**. The nitro group in this compound was reduced to the amino group, by catalytic hydrogenation, yielding compound **1c** [25], which was condensed with 2-formylthiophene, in order to obtain the imino derivative **1d**. By reaction with lead tetraacetate (LTA) in DMSO [26], compound **1d** was oxidised to the [2-(2'-thienyl)benzoxazol-5-yl] alanine derivative **2a**.

Compound **2a** was then selectively deprotected at its C- and N-terminus, yielding the corresponding *N*-Boc protected compound **2b** and the methyl ester derivative **2c**, as well as the fully deprotected alanine derivative **2d**. By using similar procedures, compound **1c** was reacted with 2,4,5-trimethoxybenzaldehyde, yielding imine **1e**. Oxidation of this compound yielded [2-(2',4',5'-trimethoxyphenyl)benzoxazol-5-yl] alanine derivative **2e** [27]. Selective removal of the protecting groups resulted in the corresponding *N*-Boc protected **2f** and the fully deprotected **2g** alanine derivatives (Scheme 1). Compounds **2e-g** based on trimethoxyphenyl were prepared in order to confirm the involvement of the sulphur atom at the thiophene ring in the coordination process. All the compounds were obtained in good to excellent yields (Table 1) and were completely characterised by the usual techniques (MS, IR, UV-vis, ¹H and ¹³C NMR spectroscopy).



Scheme 1. Synthesis of tyrosine derivatives **1a-e** and alanine derivatives **2a-g**. *Reagents and conditions:* a) $(Boc)_2O$, NaOH 1 M aq solution, rt, 2 days; b) 1,4-cyclohexadiene, Pd/C, MeOH, reflux, 24h; c) 2-formylthiophene, EtOH, rt, 5 days; d) 2,4,5-trimethoxybenzaldehyde, EtOH, rt, 3 days; e) **1d** or **1e**, LTA, DMSO, rt, 3 days; f) NaOH 1 M aq solution, dioxane, rt, 3h; g) trifluoroacetic acid/dichloromethane, 1:1, rt, 2h.

Table 1. Synthesis data of tyrosine derivatives **1d-e** and alanine derivatives **2a-g**.

Compd	Yield (%)	IR (cm^{-1})	1H NMR (δ , ppm)
1d	98	3375, 1754	8.76 (N=CH) 6.90-6.95 (H-2), 7.05 (H-5)
1e	97	3430, 1741	9.01 (N=CH) 6.88-6.94 (H-2), 6.99 (H-5)
2a	56	3358, 1734	7.48 (H-4), 7.45 (H-7)
2b	84	3357, 1752	7.63 (H-4), 7.48 (H-7)
2c	74	3310, 1741	7.56-7.59 (H-4), 7.49 (H-7)
2d	54	3413, 1620	7.93-7.96 (H-4), 7.62-7.67 (H-7)
2e	65	---	7.50 (H-4), 7.45 (H-7)
2f	82	3340, 1761	7.66 (H-4), 7.50 (H-7)
2g	44	3400, 1618	7.95(H-4), 7.60 (H-7)

2.2. Photophysical study

The absorption and emission spectra of benzoxazolyl-alanines **2a-d** bearing a thiophene heterocycle and trimethoxy-phenyl alanine derivatives **2e-g** in absolute ethanol solution at 298 K were performed. The absorption and emission bands of the 2-substituted benzoxazole derivatives were centered at 315-336 and 393-398 nm, respectively. The fluorescence quantum yields for compounds **2a-g** were

determined and the derivatives **2a-d** with a thiophene moiety were found to be strongly emissive ($0.66 < \Phi_F < 0.80$) while compounds **2e-g** displayed much lower quantum yields ($0.26 < \Phi_F < 0.47$) (Table 2). As can be seen by the results, the selective deprotection at the amino and carboxylic groups of the amino acid residue had only a minor influence on the fluorescence quantum yields. No solvatochromic effect in the absorption spectra was observed in absolute ethanol/water (1:1), absolute ethanol, acetonitrile, dioxane and cyclohexane for all compounds studied.

Table 2. UV-vis and fluorescence data for alanine derivatives **2a-g**.

Compound	UV-vis		Fluorescence	
	λ_{\max} (nm)	λ_{em} (nm)	Φ_F	Stokes' shift (nm)
2a	315	394	0.80	79
2b	316	393	0.66	77
2c	315	394	0.77	79
2d	315	393	0.76	78
2e	334	396	0.47	62
2f	334	395	0.44	61
2g	336	398	0.26	62

Compounds **2a-g** are amino acid derivatives, which in solution are present in zwitterionic form, and during our studies their zwitterionic nature was taken into account. All of our studies were performed in absolute ethanol and/or in water-absolute ethanol mixtures and the measured pH value was always between 5-6, at which the zwitterionic form predominates. At this pH value, the amine group in alanine derivatives is protonated and the carboxylic group is deprotonated, being available for coordination with the metal ions studied, Cu(II), Hg(II) and Ni(II), in these conditions.

2.3. Spectrofluorimetric titrations and metal sensing effect

In order to evaluate the sensor ability of systems **2a-g** in solution towards Zn(II), Hg(II), Cu(II), Ni(II), Ca(II) and Na(I) in low acidic or neutral conditions, UV-vis and fluorescence studies were performed.

2.3.1. Protonation effects

In the presence of increasing amount of HBF_4 , only compounds **2a** and **2f**, in acetonitrile or absolute ethanol were slightly affected. The maximum of absorption at 315 nm (**2a**) and 334 nm (**2f**), showed a 4 nm red shift upon acid addition. For compound **2f**, a new band centered at 380 nm was observed when a larger amount of acid was added (30 to 190 equivalents). As previously reported for related quinoxalinyll derivatives [15], compounds **2b-d** and **2e,g** did not reveal any significant change in the absorption spectrum upon acid addition in absolute ethanol and absolute ethanol/water (1:1).

With further addition of acid, a decrease in the intensity of the fluorescence emission was observed. This quenching may be a result of protonation on the benzoxazole nitrogen atom.

2.3.2. Deprotonation effects

Addition of bases such as potassium hydroxide, triethylamine and tetramethylammonium hydroxide solutions to ligands **2b**, **2d**, **2f** and **2g** in ethanolic solutions (with a free carboxylic acid group) were performed.

The maximum of absorption at 315 nm for **2a**, and 334 nm for **2f**, was slightly affected upon addition of 100 equivalents of tetrabutylammonium hydroxide solution. With further addition of base (more than 500 equivalents), a 50% quenching effect in fluorescence emission was observed. This effect can be attributed to a photoinduced electron transfer (PET) process from the NH₂ deprotonated group to chromophore. At this point, the pH value was 10-11 and addition of Hg(II) ions to a basic solution of ligand **2d** led to the precipitation of a solid (hydroxide complex), thus preventing complexation studies in basic conditions.

2.3.3. Metal sensing effects

Compounds **2a** and **2e**, protected at amino and carboxylic terminals, did not show any change in the ground (absorption) and excited (emission) states after the addition of Na(I), Ca(II), Zn(II), Cu(II) and Ni(II) cations. In the titration of **2a** with a Hg(CF₃SO₂)₂ ethanolic solution, the intensity of fluorescence was reduced in 30% after the addition of 30 equivalents of metal ion, while the spectrum of trimethoxy-phenyl alanine derivative **2e** was not affected by Hg(II) addition. This result can be due to the involvement of the S atom in complexation.

Selective deprotection of the carboxylic terminal in **2a** and **2e**, gave compounds **2b** and **2f**. Both systems responded to the presence of Cu(II), Ni(II) and Hg(II), while addition of increasing amounts of Na(I) and Ca(II) did not change either the absorption and emission spectra. A rising CHEQ effect (Chelation Enhancement of the Quenching) [28-29] was observed in the cases of Cu(II), Ni(II) and Hg(II). This CHEQ effect is commonly observed in polyamine ligands containing aromatic fluorophores, and can be attributed to an energy transfer quenching of the π* emissive state through low-lying metal-centre unfilled d-orbitals for Cu(II), and to an intersystem crossing mechanism due to the heavy atom effect for Hg(II) [29]. While the quenching of fluorescence is predominantly connected with the nature of metal ion, the ligand fluorescence enhancement could be result from changes of the geometry between the steady and excited states, or be also induced by the ligand. A very small CHEF effect (Chelation enhancement of the Fluorescence Emission) was observed only when compound **2b** was titrated with Zn(II) [30-31].

Although Cu(II) and Ni(II) affected only the excited state of the chromophore in both aforementioned ligands **2b** and **2f**, Hg(II) affected the ground and excited states when ligand **2b**, bearing a thiophene unit, was used. This result suggests that the metal ion interacts with both chelating units present in the compound: the carboxylic acid group of the amino acid residue and the S atom of the thiophene heterocycle [32]. For ligand **2b**, complexation constants using the SPECFIT/32 program [33] were obtained only for the interaction with Hg(II). The results suggested that two metal ions are coordinated to the ligand. In the case of ligand **2f** all metal complexation studies showed values that

agree with 2:1 metal to ligand molar ratio for Hg(II) and 1:1 metal to ligand ratio for Cu(II) and Ni(II) (Table 3).

Table 3. Complexation constants for alanine derivatives **2b-d** and **2f-g** with Cu(II), Ni(II) and Hg(II) in absolute ethanol.

Compd.	Metal complex	Log K	M.L.H ^a
2b	2bHg	5.01 ± 4.0E-02	1.1.0
		9.37 ± 7.0E-02	2.1.0
2d	2dHg	7.78 ± 2.0E-02	2.1.0
		9.77 ± 2.0E-02	2.1.0
2f	2fHg	7.09 ± 8.0E-02	2.1.0
2f	2fCu	6.22 ± 2.0E-02	1.1.0
2f	2fNi	6.67 ± 6.0E-02	1.1.0
2g	2gCu	6.10 ± 6.0E-02	1.1.0
2g	2gNi	10.26 ± 8.0E-02	2.1.0

^aM.L.H means Metal. Ligand. proton ratio

In Figure 1 is represented the absorption (A) and emission (B) spectra of compound **2b** in the presence of increasing amount of Hg(II). The increase of Hg(II) ion concentration in absolute ethanol solution caused the decrease in the absorbance in the range of 280-340 nm, and increase in absorption between 340-390 nm. A sharp isosbestic point at $\lambda = 330$ nm was detected (Fig. 1A). After the addition of small molar ratio equivalents of metal ions (0.25 to 1 equivalent) a small effect in absorption and emission was observed. By increasing the metal-ligand molar ratio, strong changes in absorption and emission were observed (inset Figure 1B).

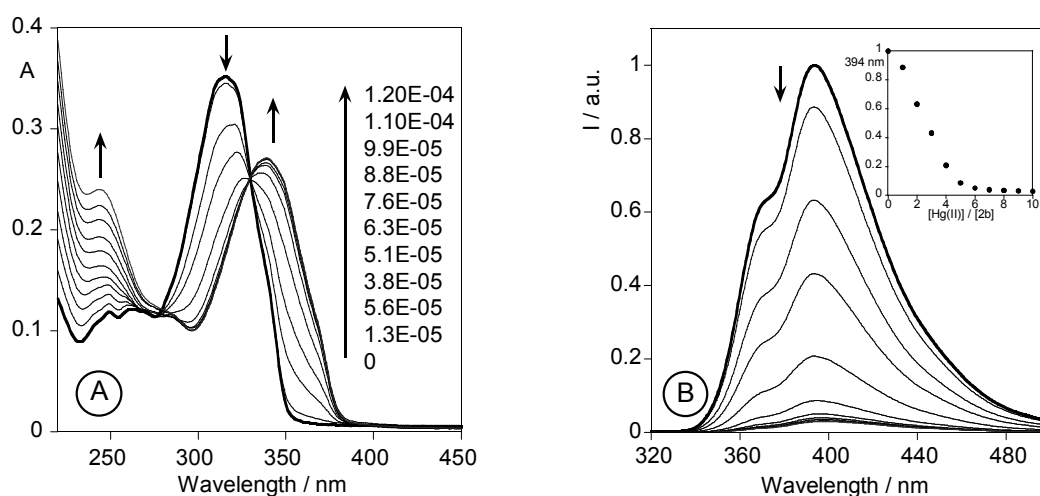


Figure 1. Spectrophotometric titration (A) and fluorimetric titration (B) of an ethanolic solution of **2b** with a standard solution of Hg(CF₃SO₂)₂ in absolute ethanol ([**2b**] = 1.30E-5 M, T = 298 K, λ_{exc} = 316 nm. Inset: normalized emission at 394 nm).

The absorption and emission spectra of compound **2b** with increasing amount of Cu(II) is represented in figure 2. It can be seen that while the emission was strongly affected by metal ion complexation, no major changes were seen in the absorption. These results did not allow us to calculate the stoichiometry of complexes with Cu(II) and Ni(II), formed in the ground state. Probably the formation of the 1:1 M:L metal complexes has relatively low values for the binding constants. Increasing the metal ion concentration forces the formation of dinuclear species. Due to the spectral response, complexation probably takes place firstly at the carboxylic acid group of the aminoacid residue, farther from the chromophore, and the second metal ion complexation occurs at the donor atoms present in the chromophore unit. As observed before, interaction of ligand **2b** with Hg(II), Ni(II) and Cu(II) also caused a strong CHEQ effect, suggesting the interaction with these metal ions with the chromophore.

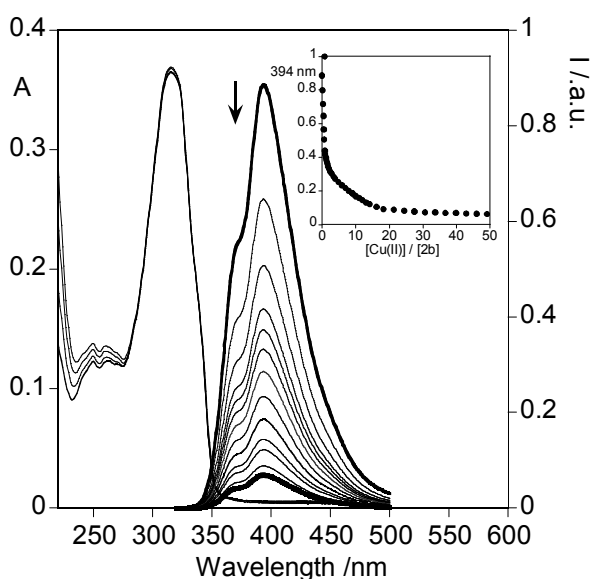


Figure 2. Spectrophotometric titration and fluorimetric titration of an ethanolic solution of **2b** with a standard solution of $\text{Cu}(\text{CF}_3\text{SO}_2)_2$ in absolute ethanol. ($[\mathbf{2b}] = 1.30\text{E-}5$ M, $T = 298$ K, $\lambda_{\text{exc}} = 316$ nm. Inset: normalized emission at 394 nm).

The complexation of ligand **2f** with Hg(II), Cu(II) and Ni(II) in absolute ethanol can be seen in Figures 3 and 4, respectively. The amount of metal ion necessary to quench the fluorescence emission was higher than that used for the thiophenic ligand **2b**. In the case of compound **2f**, fifty equivalents were used, in contrast with the six equivalents of Hg(II) needed to quench ligand **2b**. These results suggest lower complexation constants for compound **2f**. In the cases of Ni(II) and Cu(II), 5 equivalents of metal ion were enough in all cases to reduce the intensity of emission in 80%.

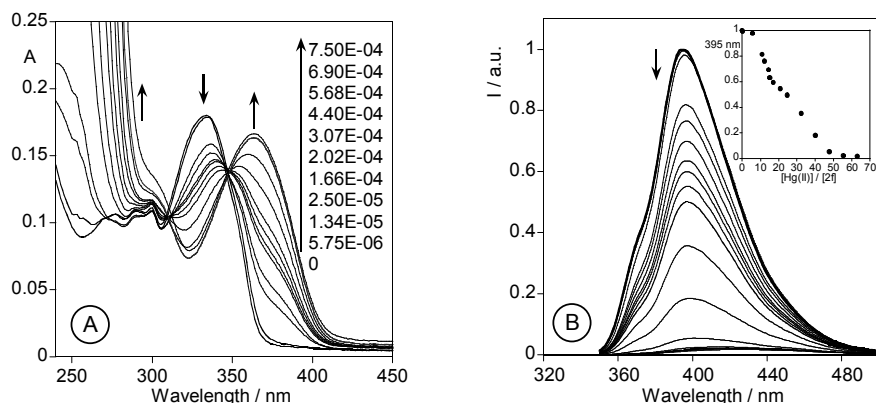


Figure 3. Spectrophotometric titration (A) and fluorimetric titration (B) of an ethanolic solution of **2f** with a standard solution of $\text{Hg}(\text{CF}_3\text{SO}_2)_2$ in absolute ethanol. ($[\text{2f}] = 1.00\text{E-}5$ M, $T = 298$ K, $\lambda_{\text{exc}} = 334$ nm. Inset: normalized emission at 395 nm).

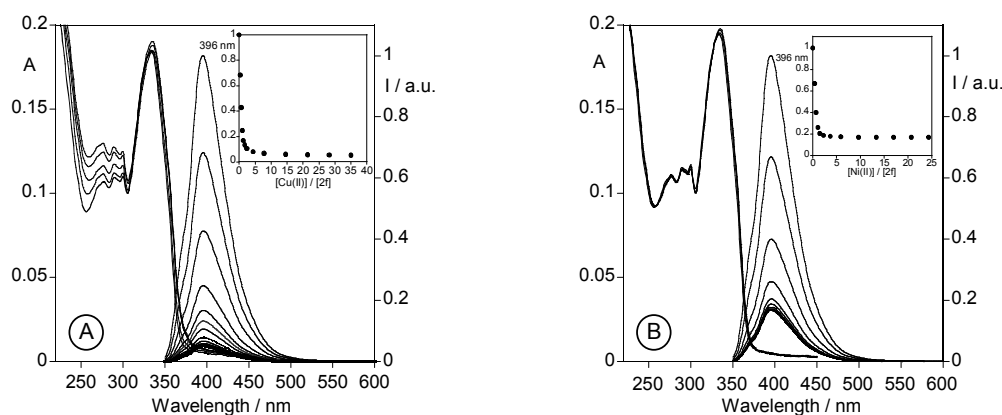


Figure 4. Spectrophotometric titration and fluorimetric titration of an ethanolic solution of **2f** with a standard solution of $\text{Cu}(\text{CF}_3\text{SO}_2)_2$ (A) and $\text{Ni}(\text{BF}_4)_2$ (B) in absolute ethanol. ($[\text{2f}] = 1.00\text{E-}5$ M, $T = 298$ K, $\lambda_{\text{exc}} = 334$ nm. Insets: normalized emission at 396 nm in both cases).

Selective deprotection at the amino terminal of the amino acid gave compound **2c** with a free NH_2 group. Due to the zwitterionic form of compound **2c** studied ($\text{pH} = 5\text{-}6$) in which the amine group is protonated, NH_3^+ , a strong decrease in the coordination ability in absolute ethanol and absolute ethanol/water (1:1) was observed when compared with ligand **2b**, with a free carboxylic acid group. After addition of $\text{Na}(\text{I})$, $\text{Ca}(\text{II})$, $\text{Zn}(\text{II})$, and $\text{Ni}(\text{II})$, only minor changes were observed in the absorption and emission spectra. In the case of $\text{Hg}(\text{II})$ and $\text{Cu}(\text{II})$ a decrease in the intensity of the fluorescence emission in 40% and 30%, respectively, was observed when a metal to ligand molar ratio of 30:1 was achieved.

Total deprotection of both amino and carboxylic acid terminals in alanines **2a** and **2e** gave compounds **2d** and **2g** in the form of free amino acid residues. In our conditions, the zwitterionic form was present in solution. Although higher solubility in water would be expected, ligands **2d** and **2g**

were sparingly soluble in water probably due to the heteroaromatic moiety present at the ligands. Therefore, the complexation studies were carried out in acetonitrile, absolute ethanol and/or water-absolute ethanol mixtures, in order to compare the results for all ligands studied. Once again, no interaction was observed upon complexation with Na(I), Ca(II) and Zn(II) for both ligands, **2d** and **2g**, in the absorption and emission spectra.

In figure 5 is represented the Hg(II) titration with ligand **2d**, showing that both, ground and excited states, are affected by the complexation and an isosbestic point at 325 nm is observed. In contrast, interaction of Hg(II) with compound **2g** affected only the excited state, and in this case the intensity of the emission was reduced in 30%. This result is comparable with the results obtained for compounds **2a** and **2b**, suggesting the importance of the sulphur atom for the sensing of this metal ion, due to its role on the complexation. The values for the complexation constants were calculated and are presented in Table 3.

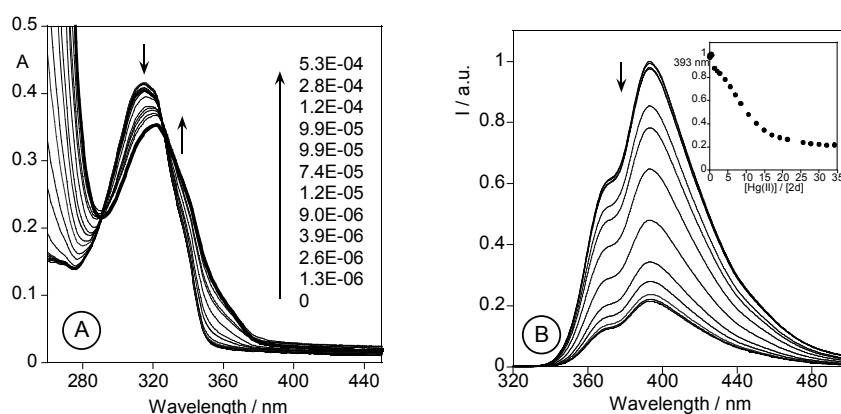


Figure 5. Spectrophotometric titration (A) and fluorimetric titration (B) of an ethanolic solution of **2d** with a standard solution of Hg(CF₃SO₂)₂ in absolute ethanol. ([**2d**] = 1.00E-5 M, T = 298 K, λ_{exc} = 315 nm. Inset: normalized emission at 393 nm).

Cu(II) and Ni(II) complexation are represented in figure 6A and 6B for ligand **2d**, and in figures 7A and 7B for ligand **2g**. Stronger interaction with Cu(II) was observed in both ligands. Five and two Cu(II) equivalents were enough to totally quench the fluorescence emission of **2d** and **2g**, while 40 and 10 equivalents of Ni(II), respectively, were necessary to quench the fluorescence emission to 90%. A dinuclear complex could be postulated by the global log K calculated using the SPECFIT32 programme in the case of Ni(II), and a mononuclear species was postulated for Cu(II) (see table 3). A schematic drawing of the proposed coordination process is displayed in Scheme 2. Firstly, the metal ion binds through the carboxylic acid at the alanine residue with small change in the fluorescence emission, followed by another metal ion interaction at the coordinative site present in the chromophore, which strongly affects the emission spectrum.

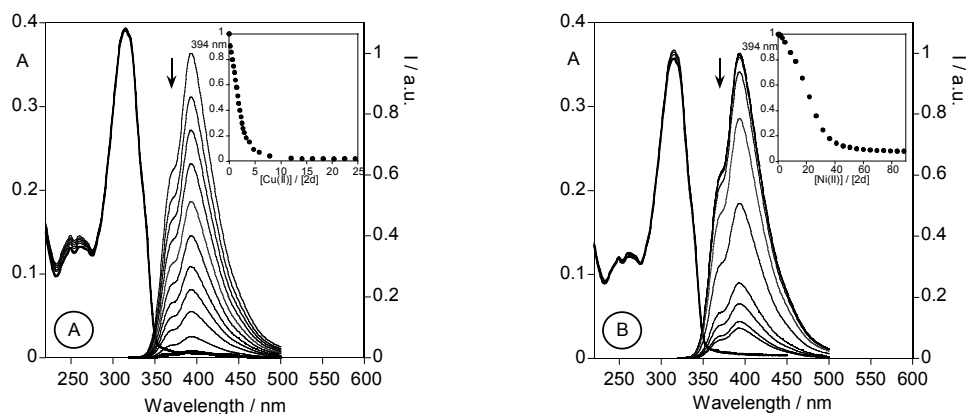


Figure 6. Spectrophotometric titration and fluorimetric titration of an ethanolic solution of **2d** with a standard solution of $\text{Cu}(\text{CF}_3\text{SO}_2)_2$ (A) and $\text{Ni}(\text{BF}_4)_2$ (B) in absolute ethanol. ($[\mathbf{2d}] = 1.80\text{E-}5 \text{ M}$, $T = 298 \text{ K}$, $\lambda_{\text{exc}} = 315 \text{ nm}$. Insets: normalized emission at 394 nm in both cases).

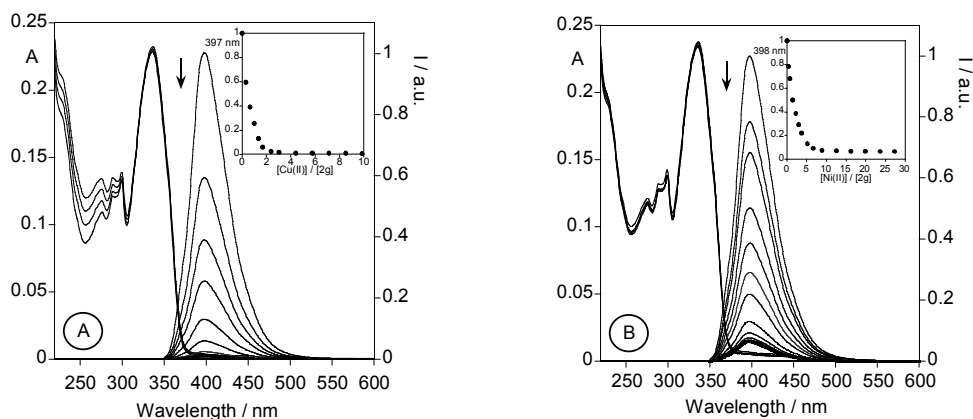
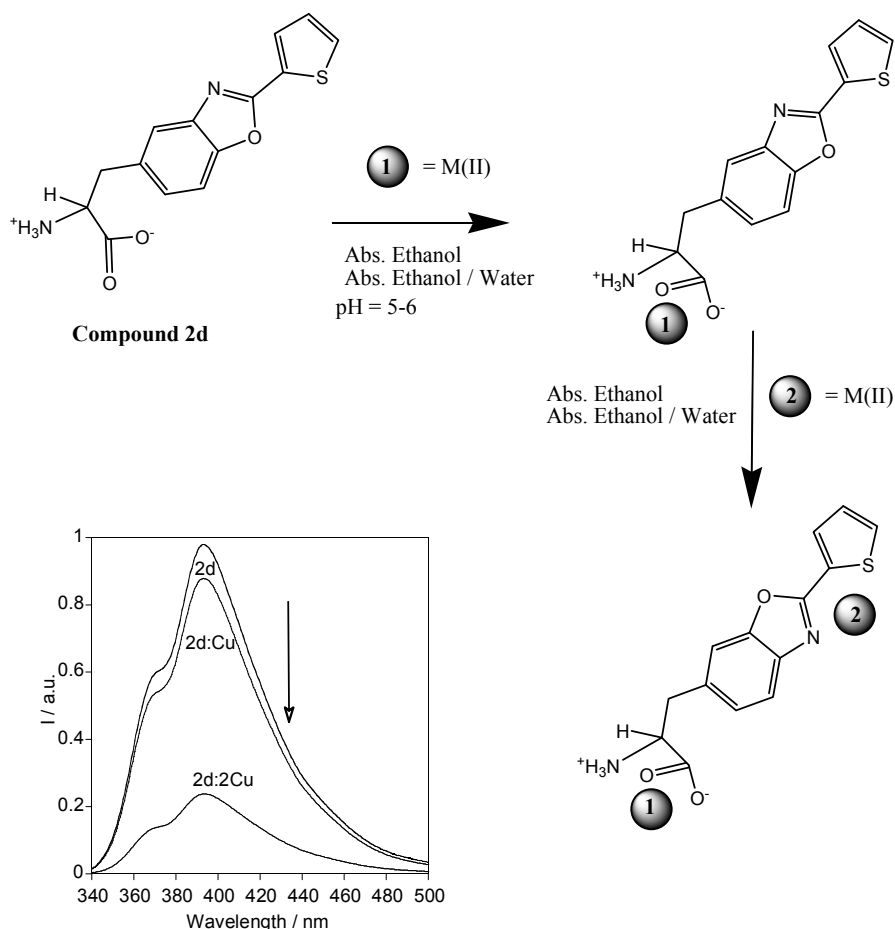


Figure 7. Spectrophotometric titration and fluorimetric titration of an ethanolic solution of **2g** with a standard solution of $\text{Cu}(\text{CF}_3\text{SO}_2)_2$ (A) and $\text{Ni}(\text{BF}_4)_2$ (B) in absolute ethanol. ($[\mathbf{2g}] = 1.00\text{E-}5 \text{ M}$, $T = 298 \text{ K}$, $\lambda_{\text{exc}} = 336 \text{ nm}$. Inset: normalized emission at 397 and 398 nm, respectively).



Scheme 2. Schematic representation of the complexation mechanism proposed for alanine **2d** upon complexation with Cu(II), Ni(II) and Hg(II). Fluorescence spectra of **2d** in the presence of one and two equivalents of Cu(II) in absolute ethanol.

2.4. Conclusions

The synthesis of new highly fluorescent probes containing the thiophene and benzoxazole moieties combined with an alanine residue was achieved by simple procedures in excellent yields.

The resulting heterocyclic alanine derivatives respond with a quenching effect to the presence of Cu(II), Ni(II) and Hg(II), as shown by the absorption and steady-state fluorescence spectroscopy studies. Selective deprotection of the carboxylic acid group increased the coordination ability, while that deprotection of the amino group reduced notably this effect. The sensor effect takes place by both coordinative sites, for systems **2b** and **2d**, involving first the terminal carboxylic acid group of the amino acid, and finally the donor atoms present in the chromophores. The synthetic, photophysical and metal ion sensing properties displayed by these compounds showed that they are promising candidates as fluorescent probes and also for sensory applications.

3. Experimental Section

3.1. Synthesis general

All melting points were measured on a Gallenkamp melting point apparatus and are uncorrected. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel 60F₂₅₄) and spots were visualised under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230-240 mesh). IR spectra were determined on a Perkin Elmer FTIR-1600 using KBr discs. UV/visible spectra were run on a Shimadzu UV/2501PC or a Perkin Elmer lamda-35 spectrophotometers. ¹H NMR spectra were recorded on a Varian 300 spectrometer in CDCl₃ or DMSO-*d*₆ at 300 MHz at 25 °C. All chemical shifts are given in ppm using $\delta_{\text{H}} \text{Me}_4\text{Si} = 0$ ppm as reference and *J* values are given in Hz. ¹³C NMR spectra were run in the same instrument at 75.4 MHz using the solvent peak as internal reference. Assignments were made by comparison of chemical shifts, peak multiplicities and *J* values and were supported by spin decoupling-double resonance and bidimensional heteronuclear HMBC and HMQC correlation techniques. Mass spectrometry analyses were performed at the “C.A.C.T.I. -Unidad de Espectrometria de Masas”, University of Vigo, Spain. Elemental analyses were carried out on a Leco CHNS 932 instrument. Fluorescence spectra were collected using a Perkin Elmer LS45.

3-Nitro-L-tyrosine methyl ester hydrochloride (1a): Thionyl chloride (0.65 mL, 8.85×10^{-3} mol) was added drop wise with stirring to methanol (10 mL), cooled in an ice bath, followed by the addition of 3-nitrotyrosine (2.00 g, 8.85×10^{-3} mol). The solution was heated at 40 °C for 5 h. The solvent was evaporated under reduced pressure, yielding a yellowish green solid (2.44 g, quant.). M.p. > 300 °C [lit. [25] data not quoted]. ¹H NMR (DMSO-*d*₆): $\delta = 3.11$ (d, *J* = 6.3 Hz, 2H, β CH₂), 3.71 (s, 3H, CH₃), 4.30 (t, *J* = 6.6 Hz, 1H, α H), 7.15 (d, *J* = 8.4 Hz, 1H, H-5), 7.38 (dd, *J* = 2.1 and 8.4 Hz, 1H, H-6), 7.78 (d, *J* = 2.1 Hz, 1H, H-2), 8.55 (br s, 3H, ⁺NH₃), 11.08 (br s, 1H, OH) ppm. ¹³C NMR (DMSO-*d*₆): $\delta = 34.26$ (β CH₂), 52.75 (CH₃), 52.94 (α C), 119.36 (C5), 125.46 (C1), 126.15 (C2), 136.30 (C3), 136.59 (C6), 151.51 (C4), 169.24 (C=O) ppm. IR (KBr): $\nu = 3388$ (br), 3204, 2856, 1746, 1635, 1589, 1544, 1493, 1445, 1390, 1318, 1241, 1064, 990 cm⁻¹.

***N*-tert-Butyloxycarbonyl-3-nitro-L-tyrosine methyl ester (1b):** Compound **1a** (1.52 g, 5.5×10^{-3} mol) was added to 1,4-dioxane (11 mL), water (5.5 mL) and sodium hydroxide 1 M aqueous solution (5.5 mL), with stirring in an ice bath. *t*-Butyl pyrocarbonate (1.36 g, 1.1 eq, 6.05×10^{-3} mol) was added and the mixture stirred at room temperature for 2 days. The volume was reduced by half in a rotary evaporator under reduced pressure and ethyl acetate was added to cover the aqueous layer. The mixture was acidified by addition of KHSO₄ 1 M aqueous solution, until acidic pH with congo red paper, and extracted with ethyl acetate (3 × 10 mL), followed by washing of the organic layer with water (3 × 10 mL). The organic layer was dried with anhydrous magnesium sulphate and evaporated under reduced pressure, resulting a yellow solid (1.68 g, 90%). M.p. 97.3-98.4 °C [lit. [25] 90-91°C]. ¹H NMR (CDCl₃): $\delta = 1.43$ (s, 9H, C(CH₃)₃), 2.95-3.04 (m, 1H, β CH₂), 3.12-3.22 (m, 1H, β CH₂), 3.76 (s, 3H, CH₃), 4.54-4.60 (m, 1H, α H), 5.07 (d, *J* = 6.6 Hz, 1H, NH), 7.10 (d, *J* = 8.4 Hz, 1H, H-5), 7.37 (dd, *J* = 1.8 and 8.4 Hz, 1H, H-6), 7.87 (d, *J* = 1.8 Hz, 1H, H-2), 10.50 (s, 1H, OH) ppm. ¹³C

NMR (CDCl₃) δ = 28.21 (C(CH₃)₃), 37.23 (β CH₂), 52.51 (CH₃), 54.18 (α C), 80.27 (C(CH₃)₃), 120.09 (C5), 125.25 (C2), 128.62 (C1), 133.28 (C3), 138.65 (C6), 154.13 (C4 and C=O Boc), 171.69 (C=O ester) ppm. IR (KBr): ν = 3335, 3023, 3007, 2984, 2955, 2936, 1732, 1685, 1629, 1578, 1536, 1439, 1324, 1221, 1162, 1061, 994, 838 cm⁻¹.

***N*-tert-Butyloxycarbonyl-3-amino-L-tyrosine methyl ester (1c)**: Compound **1b** (1.00 g, 2.94×10^{-3} mol), 1,4-cyclohexadiene (0.5 mL, 1.8 eq, 5.29×10^{-3} mol) and Pd/C (147 mg) were dissolved in methanol (15 mL) and refluxed for 24h. The solvent was evaporated and the residue submitted to column chromatography with silica gel (eluent: CHCl₃/MeOH, 98:2). The fractions were combined and the product was obtained as a light brown solid (0.92 g, 98%). M.p. 52.0-52.9 °C [lit. [25] data not quoted]. ¹H NMR (CDCl₃): δ = 1.43 (s, 9H, C(CH₃)₃), 2.92 (d, J = 4.8 Hz, 2H, β CH₂), 3.74 (s, 3H, CH₃), 4.47-4.54 (m, 1H, α H), 5.05 (d, J = 8.4 Hz, 1H, NH), 6.37 (dd, J = 2.1 and 8.1 Hz, 1H, H-6), 6.53 (d, J = 2.1 Hz, 1H, H-2), 6.64 (d, J = 8.1 Hz, 1H, H-5) ppm. ¹³C NMR (CDCl₃) δ = 28.27 (C(CH₃)₃), 37.64 (β CH₂), 52.21 (CH₃), 54.57 (α C), 80.11 (C(CH₃)₃), 115.30 (C5), 117.33 (C2), 119.89 (C6), 128.32 (C1), 134.65 (C3), 143.33 (C4), 155.36 (C=O Boc), 172.78 (C=O ester) ppm. IR (KBr): ν = 3376, 3005, 2955, 1738, 1694, 1615, 1520, 1505, 1454, 1441, 1393, 1368, 1288, 1251, 1217, 1164, 1059, 1022, 912, 861, 799, 758 cm⁻¹. MS (FAB): m/z (%) = 311 (M⁺ + H, 41), 310 (M⁺, 100), 307 (20), 255 (59), 254 (47), 211 (57), 193 (27) 155 (24), 154 (83). HRMS (FAB): calcd. for C₁₅H₂₃N₂O₅ 311.1607, found 311.1568.

***N*-tert-Butyloxycarbonyl-3-[(thien-2'-ylmethylene)amino]-L-tyrosine methyl ester (1d)**: Compound **1c** (0.54 g, 1.74×10^{-3} mol) was stirred with 2-formylthiophene (0.16 mL, 1.76×10^{-3} mol) in absolute ethanol (5 mL) for 5 days. The solvent was evaporated and the crude residue was used in the next step without further purification, as light yellow oil (0.68 g, 98%). ¹H NMR (CDCl₃): δ = 1.42 (s, 9H, C(CH₃)₃), 2.98-3.10 (m, 2H, β CH₂), 3.72 (s, 3H, CH₃), 4.52-4.60 (m, 1H, α H), 5.02 (d, J = 7.80 Hz, 1H, NH), 6.90-6.95 (m, 2H, H-2 + H-6), 7.05 (br s, 1H, H-5), 7.14-7.17 (m, 1H, H-4'), 7.51-7.55 (m, 2H, H-3' + H-5'), 8.76 (s, 1H, N=CH) ppm. ¹³C NMR (CDCl₃) δ = 28.28 (C(CH₃)₃), 37.84 (β CH₂), 52.23 (CH₃), 54.55 (α C), 79.99 (C(CH₃)₃), 114.99 (C2), 116.54 (C5), 127.56 (C1), 127.99 (C4'), 129.46 (C6), 130.82 (C3' or C5'), 132.59 (C3' or C5'), 134.98 (C3), 142.73 (C2'), 149.88 (CH), 151.15 (C4), 155.09 (C=O Boc), 172.41 (C=O ester) ppm. IR (KBr): ν = 3375, 2981, 2930, 1754, 1682, 1617, 1602, 1513, 1500, 1430, 1392, 1367, 1295, 1253, 1217, 1165, 1047, 1025, 855, 814 cm⁻¹. MS (FAB): m/z (%) = 405 (M⁺ + H, 40), 350 (22), 349 (100), 216 (33). HRMS (FAB): calcd. for C₂₀H₂₅N₂O₅S 405.1484, found 405.1488.

***N*-tert-Butyloxycarbonyl-3-[(2',4',5'-trimethoxyphenylmethylene)amino]-L-tyrosine methyl ester (1e)**: Compound **1c** (0.365 g, 1.18×10^{-3} mol) was stirred with 2,4,5-trimethoxybenzaldehyde (0.231 g, 1.18×10^{-3} mol) in absolute ethanol (5 mL) for 3 days. The solvent was evaporated and the residue was used in the next step without further purification as brown oil (0.56 g, 97%). ¹H NMR (CDCl₃): δ = 1.52 (s, 9H, C(CH₃)₃), 2.97-3.05 (m, 2H, β CH₂), 3.88 (s, 3H, OCH₃), 3.93 (s, 3H, OCH₃), 3.97 (s, 3H, OCH₃), 4.50-4.58 (m, 1H, α -H), 5.00 (d, J = 8.4 Hz, 1H, NH), 6.53 (s, 1H, H-3'), 6.88-6.94 (m, 2H, H-2 + H-6), 6.99 (br s, 1H, H-5), 7.63 (s, 1H, H-6'), 9.01 (s, 1H, N=CH) ppm. ¹³C NMR (CDCl₃) δ = 28.40 (C(CH₃)₃), 38.02 (β CH₂), 52.27 (CH₃), 54.65 (α C), 80.06 (C(CH₃)₃), 100.12

(C3'), 106.33 (C1'), 115.11 (C2), 115.71 (C6'), 116.33 (C5), 127.71 (C1), 129.76 (C6), 135.12 (C3), 135.79 (C5'), 144.76 (C4'), 149.79 (CH), 151.18 (C4), 152.06 (C2'), 155.01 (C=O Boc), 172.23 (C=O ester) ppm. IR (KBr): $\nu = 3430$ (br), 2956, 2927, 2854, 1741, 1708, 1611, 1596, 1510, 1466, 1439, 1412, 1366, 1340, 1280, 1210, 1165, 029, 811 cm^{-1} . MS (FAB): m/z (%) = 489 ($\text{M}^+ + \text{H}$, 53), 434 (31), 349 (100). HRMS (FAB): calcd. for $\text{C}_{25}\text{H}_{33}\text{N}_2\text{O}_8$ 489.2238, found 489.2252.

***N*-tert-Butyloxycarbonyl [2-(thien-2'-yl)benzoxazol-5-yl]-L-alanine methyl ester (2a)**: The crude imine **1d** (0.70 g 1.74×10^{-3} mol) and lead tetraacetate (1.17 g, 2.64×10^{-3} mol) were stirred at room temperature in DMSO (5 mL) for 3 days. The mixture was poured over water and extracted with ethyl acetate (3×10 mL). After drying with anhydrous magnesium sulphate and evaporation of the solvent, the resulting brown oil was submitted to column chromatography with silica gel (eluent: dichloromethane). The product was isolated as a light yellow solid (0.39 g, 56%). M.p. 106.6-107.8 °C. ^1H NMR (CDCl_3): $\delta = 1.42$ (s, 9H, $\text{C}(\text{CH}_3)_3$), 3.15-3.28 (m, 2H, β CH_2), 3.73 (s, 3H, CH_3), 4.61-4.67 (m, 1H, αH), 5.04 (d, $J = 7.8$ Hz, 1H, NH), 7.10 (dd, $J = 1.8$ and 8.4 Hz, 1H, H-6), 7.17-7.20 (m, 1H, H-4'), 7.45 (d, $J = 8.4$ Hz, 1H, H-7), 7.48 (d, $J = 1.8$ Hz, 1H, H-4), 7.56 (dd, $J = 1.2$ and 5.1 Hz, 1H, H-5'), 7.90 (dd, $J = 1.2$ and 3.9 Hz, 1H, H-3') ppm. ^{13}C NMR (CDCl_3) $\delta = 28.24$ ($\text{C}(\text{CH}_3)_3$), 38.27 (β CH_2), 52.29 (CH_3), 54.57 (αC), 80.00 ($\text{C}(\text{CH}_3)_3$), 110.26 (C7), 120.31 (C4), 126.32 (C6), 128.24 (C4'), 129.50 (C2), 129.97 (C3'), 130.31 (C5'), 132.79 (C5), 142.28 (C3a), 149.59 (C7a), 155.04 (C=O Boc), 159.41 (C2'), 172.08 (C=O ester) ppm. IR (KBr): $\nu = 3358$, 2982, 2928, 1734, 1693, 1573, 1526, 1475, 1438, 1369, 1289, 1244, 1172, 1052, 1021, 857 cm^{-1} . UV/Vis (ethanol, nm): λ_{max} (ϵ) = 315 (2.13×10^4). MS (FAB): m/z (%) = 403 ($\text{M}^+ + \text{H}$, 73), 348 (23), 347 (100), 243 (29), 215 (27), 185 (21). HRMS (FAB): calcd. for $\text{C}_{20}\text{H}_{23}\text{N}_2\text{O}_5\text{S}$ 403.1328, found 403.1324. $\text{C}_{20}\text{H}_{22}\text{N}_2\text{O}_5\text{S}$: calcd. C 59.69, H 5.51, N 6.96, S 7.97; found C 59.44, H 5.63, N 6.69, S 7.66.

***N*-tert-Butyloxycarbonyl [2-(thien-2'-yl)benzoxazol-5-yl]-L-alanine (2b)**: Compound **2a** (0.062 g, 1.54×10^{-4} mol) was dissolved in 1,4-dioxane (1 mL), in an ice bath, and sodium hydroxide 1 M aqueous solution (0.23 mL, 2.3×10^{-4} mol, 1.5 eq) was added drop wise. The mixture was stirred at room temperature for 3h. The pH was adjusted to 2-3 by addition of KHSO_4 1 M aqueous solution and extracted with ethyl acetate (3×10 mL). After drying with anhydrous magnesium sulphate and evaporation of the solvent, the residue was triturated with diethyl ether and a off-white solid was obtained (0.050 g, 84%). M.p. 153.5-154.0 °C. ^1H NMR (CDCl_3): $\delta = 1.46$ (s, 9H, $\text{C}(\text{CH}_3)_3$), 3.30-3.36 (m, 2H, β CH_2), 4.68-4.73 (m, 1H, αH), 5.17 (d, $J = 7.5$ Hz, 1H, NH), 7.16-7.22 (m, 2H, H-6 + H-4'), 7.48 (d, $J = 8.4$ Hz, 1H, H-7), 7.57 (dd, $J = 1.2$ and 5.1 Hz, 1H, H-5'), 7.63 (d, $J = 2.1$ Hz, 1H, H-4), 7.93 (dd, $J = 1.2$ and 3.9 Hz, 1H, H-3') ppm. ^{13}C NMR (CDCl_3) $\delta = 28.31$ ($\text{C}(\text{CH}_3)_3$), 37.63 (β CH_2), 54.46 (αC), 80.14 ($\text{C}(\text{CH}_3)_3$), 110.34 (C7), 120.03 (C4), 127.05 (C6), 128.43 (C4'), 128.67 (C2), 130.73 (C3'), 130.84 (C5'), 133.24 (C5), 141.26 (C3a), 149.35 (C7a), 155.31 (C=O Boc), 159.49 (C2'), 174.66 (COOH) ppm. IR (KBr): $\nu = 3357$, 3113, 2998, 2929, 1752, 1691, 1569, 1527, 1479, 1418, 1371, 1327, 1281, 1252, 1213, 1168, 1051, 856 cm^{-1} . UV/Vis (ethanol, nm): λ_{max} (ϵ) = 316 (2.73×10^4). MS (FAB): m/z (%) = 389 ($\text{M}^+ + \text{H}$, 57), 333 (45), 185 (100). HRMS (FAB): calcd. for $\text{C}_{19}\text{H}_{21}\text{N}_2\text{O}_5\text{S}$ 389.1171, found 389.1173.

[2-(Thien-2'-yl)benzoxazol-5-yl]-L-alanine methyl ester (2c): Compound **2a** (0.103 g, 2.56×10^{-4} mol) was stirred in a trifluoroacetic acid/dichloromethane solution (1:1, 1 mL) at room temperature for

2h. The solvent was evaporated, the solid residue dissolved in pH 8 aqueous buffer solution and extracted with ethyl acetate (3×10 mL). After drying with anhydrous magnesium sulphate and evaporation of the solvent, the product was isolated as a light yellow solid (0.054 g, 74%). M.p. 80.7–81.4 °C. ^1H NMR (CDCl_3): $\delta = 1.46$ (s, 9H, $\text{C}(\text{CH}_3)_3$), 2.98–3.06 (m, 1H, β CH_2), 3.17–3.24 (m, 1H, β CH_2), 3.74 (s, 3H, CH_3), 3.77–3.82 (m, 1H, αH), 7.17–7.22 (m, 2H, H-6 + H-4'), 7.49 (d, $J = 8.4$ Hz, 1H, H-7), 7.56–7.59 (m, 2H, H-5' + H-4), 7.91 (dd, $J = 1.5$ and 3.9 Hz, 1H, H-3') ppm. ^{13}C NMR (CDCl_3) $\delta = 40.77$ (β CH_2), 52.07 (CH_3), 55.92 (αC), 110.25 (C7), 120.18 (C4), 126.33 (C6), 128.24 (C4'), 129.51 (C2), 129.97 (C3'), 130.31 (C5'), 133.85 (C5), 142.33 (C3a), 149.49 (C7a), 159.41 (C2'), 175.12 (C=O ester) ppm. IR (KBr): $\nu = 3310, 2925, 1741, 1590, 1478, 1415, 1325, 1260, 1063, 855$ cm^{-1} . UV/Vis (ethanol, nm): $\lambda_{\text{max}} (\epsilon) = 315 (3.07 \times 10^4)$. MS (FAB): m/z (%) = 303 ($\text{M}^+ + \text{H}$, 100), 215 (20). HRMS (FAB): calcd. for $\text{C}_{15}\text{H}_{15}\text{N}_2\text{O}_3\text{S}$ 303.0803, found 303.0803.

[2-(Thien-2'-yl)benzoxazol-5-yl]-L-alanine (2d): Compound **2b** (0.100 g, 2.58×10^{-4} mol) was stirred in a trifluoroacetic acid/dichloromethane solution (1:1, 1 mL) at room temperature for 2h. The solvent was evaporated, the solid residue dissolved in pH 8 aqueous buffer solution and extracted with ethyl acetate (3×10 mL). After drying with anhydrous magnesium sulphate and evaporation of the solvent, the product was isolated as a light brown solid (0.040 g, 54%). M.p. 238.9–239.9 °C. ^1H NMR (DMSO-d_6): $\delta = 2.94$ –3.02 (m, 1H, β CH_2), 3.23–3.30 (m, 1H, β CH_2), 3.46–3.51 (m, 1H, αH), 7.28–7.31 (m, 2H, H-6 + H-4'), 7.62–7.67 (m, 2H, H-7 + H-5'), 7.93–7.96 (m, 2H, H-3' + H-4) ppm. ^{13}C NMR (DMSO-d_6) $\delta = 30.40$ (β CH_2), 53.01 (αC), 109.92 (C5'), 119.40 (C7), 126.57 (C6), 128.50 (C4'), 129.72 (C2), 130.03 (C4), 131.38 (C3'), 132.34 (C5), 141.36 (C3a), 151.70 (C7a), 159.50 (C2'), 180.83 (C=O) ppm. IR (KBr): $\nu = 3413, 3072, 2940, 1620, 1572, 1548, 1479, 1407, 1346, 1312, 1287, 1269, 1217, 1195, 1160, 1049, 1024, 1005, 872, 853$ cm^{-1} . UV/Vis (ethanol, nm): $\lambda_{\text{max}} (\epsilon) = 315 (2.17 \times 10^4)$. MS (FAB): m/z (%) = 289 ($\text{M}^+ + \text{H}$, 30), 278 (22), 277 (21), 186 (71). HRMS (FAB): calcd. for $\text{C}_{14}\text{H}_{13}\text{N}_2\text{O}_3\text{S}$ 289.0646, found 289.0647.

***N*-tert-Butyloxycarbonyl-3-[2-(2',4',5'-trimethoxyphenyl)benzoxazol-5-yl]-L-alanine methyl ester (2e)**: The crude imine **1e** (0.470 g, 9.63×10^{-4} mol) and lead tetraacetate (0.640 g, 1.44×10^{-3} mol, 1.5 eq.) were stirred at room temperature in DMSO (5 mL) for 3 days. The mixture was poured over water and extracted with ethyl acetate (3×10 mL). After drying with anhydrous magnesium sulphate and evaporation of the solvent, the resulting brown oil was submitted to column chromatography with silica gel (eluent: chloroform). The product was isolated as light brown oil (0.304 g, 65%) [lit. [27] M.p. 137–140 °C]. ^1H NMR (CDCl_3): $\delta = 1.43$ (s, 9H, $\text{C}(\text{CH}_3)_3$), 3.22 (d, $J = 4.2$ Hz, 2H, β CH_2), 3.74 (s, 3H, CH_3), 3.97 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 4.02 (s, 3H, OCH_3), 4.62–4.66 (m, 1H, $\alpha\text{-H}$), 5.01 (d, $J = 8.1$ Hz, 1H, NH), 6.66 (s, 1H, H-3'), 7.08 (dd, $J = 1.8$ and 8.4 Hz, 1H, H-6), 7.45 (d, $J = 8.4$ Hz, 1H, H-7), 7.50 (d, $J = 1.8$ Hz, 1H, H-4) 7.65 (s, 1H, H-6') ppm. UV/Vis (ethanol, nm): $\lambda_{\text{max}} (\epsilon) = 334 (1.41 \times 10^4)$. MS (FAB): m/z (%) = 489 ($\text{M}^+ + \text{H}$, 100), 488 (21), 433 (14), 300 (38), 154 (14). HRMS (FAB): calcd. for $\text{C}_{25}\text{H}_{33}\text{N}_2\text{O}_8$ 489.2237, found 489.2238.

***N*-tert-Butyloxycarbonyl-3-[2-(2',4',5'-trimethoxyphenyl)benzoxazol-5-yl]-L-alanine (2f)**: Compound **2e** (0.060 g, 1.23×10^{-4} mol) was dissolved in 1,4-dioxane (1 mL), in an ice bath, and sodium hydroxide 1 M aqueous solution (0.19 mL, 1.9×10^{-4} mol, 1.5 eq) was added drop wise. The

mixture was stirred at room temperature for 3h. The pH was adjusted to 2-3 by addition of KHSO_4 1 M aqueous solution and extracted with ethyl acetate (3×10 mL). After drying with anhydrous magnesium sulphate and evaporation of the solvent, the residue was triturated with diethyl ether and a brown solid was obtained (0.048 g, 82%). M.p. 135.5-137.0 °C. ^1H NMR (CDCl_3): δ = 1.45 (s, 9H, $\text{C}(\text{CH}_3)_3$), 3.30-3.34 (m, 2H, β CH_2), 3.94 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 4.64-4.68 (m, 1H, α -H), 5.12 (d, J = 8.4 Hz, 1H, NH), 6.62 (s, 1H, H-3'), 7.18 (dd, J = 1.5 and 8.4 Hz, 1H, H-6), 7.50 (d, J = 8.4 Hz, 1H, H-7), 7.60 (s, 1H, H-6'), 7.66 (s, 1H, H-4) ppm. ^{13}C NMR (CDCl_3) δ = 28.36 ($\text{C}(\text{CH}_3)_3$), 37.67 (β CH_2), 54.46 (α C), 80.20 ($\text{C}(\text{CH}_3)_3$), 100.35 ($\text{C}3'$), 106.43 ($\text{C}1'$), 110.35 ($\text{C}7$), 114.95 ($\text{C}6'$), 120.15 ($\text{C}4$), 126.96 ($\text{C}6$), 128.90 ($\text{C}2$), 133.34 ($\text{C}5$), 135.93 ($\text{C}5'$), 141.18 ($\text{C}3\text{a}$), 145.02 ($\text{C}4'$), 149.54 ($\text{C}7\text{a}$), 152.26 ($\text{C}2'$), 155.40 ($\text{C}=\text{O}$ Boc), 174.48 (COOH) ppm. IR (KBr): ν = 3340, 3108, 2988, 2930, 1761, 1684, 1569, 1527, 1420, 1377, 1343, 1265, 1250, 1178, 1049, 850 cm^{-1} . UV/Vis (ethanol, nm): λ_{max} (ϵ) = 334 (1.75×10^4). MS (FAB): m/z (%) = 473 ($\text{M}^+ + \text{H}$, 34), 472 (M^+ , 16), 417 (16), 307 (17), 195 (21), 155 (27), 154 (100). HRMS (FAB): calcd. for $\text{C}_{24}\text{H}_{29}\text{N}_2\text{O}_8$ 473.1924, found 473.1935.

3-[2-(2',4',5'-Trimethoxyphenyl)benzoxazol-5-yl]-L-alanine (2g): Compound **2f** (0.035 g, 7.41×10^{-5} mol) was stirred in a trifluoroacetic acid/dichloromethane solution (1:1, 1 mL) at room temperature for 2h. The solvent was evaporated, the solid residue dissolved in pH 8 aqueous buffer solution and extracted with ethyl acetate (3×10 mL). After drying with anhydrous magnesium sulphate and evaporation of the solvent, the product was isolated as an off-white solid (0.012 g, 44%). M.p. 251.6-253.3 °C. ^1H NMR ($\text{DMSO}-d_6$): δ = 2.94-3.02 (m, 1H, β CH_2), 3.23-3.30 (m, 1H, β CH_2), 3.46-3.51 (m, 1H, α H), 4.05 (s, 3H, OCH_3), 4.11 (s, 3H, OCH_3), 4.14 (s, 3H, OCH_3), 7.28-7.31 (m, 2H, H-6 + H-3'), 7.60 (d, J = 8.4 Hz, 1H, H-7), 7.65 (s, 1H, H-6'), 7.95 (br s, 1H, H-4) ppm. ^{13}C NMR ($\text{DMSO}-d_6$) δ = 30.28 (β CH_2), 53.11 (α C), 101.30 ($\text{C}3'$), 105.99 ($\text{C}1'$), 115.34 ($\text{C}6'$), 119.43 ($\text{C}7$), 126.35 ($\text{C}6$), 129.82 ($\text{C}2$), 130.06 ($\text{C}4$), 132.55 ($\text{C}5$), 135.41 ($\text{C}5'$), 141.34 ($\text{C}3\text{a}$), 145.13 ($\text{C}4'$), 151.57 ($\text{C}7\text{a}$), 152.25 ($\text{C}2'$), 180.88 ($\text{C}=\text{O}$) ppm. IR (KBr): ν = 3400, 3052, 2954, 1618, 1570, 1490, 1350, 1325, 1307, 1250, 1198, 1163, 1057, 1030, 1001, 853 cm^{-1} . UV/Vis (ethanol, nm): λ_{max} (ϵ) = 336 (2.29×10^4). MS (FAB): m/z (%) = 373 ($\text{M}^+ + \text{H}$, 30), 372 (M^+ , 43), 278 (100), 186 (51). HRMS (FAB): calcd. for $\text{C}_{19}\text{H}_{21}\text{N}_2\text{O}_6$ 373.1401, found 372.1395.

3.2. Spectrofluorimetric titrations

The linearity of the fluorescence emission vs. concentration was checked in the concentration range used (10^{-4} - 10^{-6} M). A correction for the absorbed light was performed when necessary. All spectrofluorimetric titrations were performed as follows: stock solutions of compounds **2a-g** (*ca.* 10^{-3} M) were prepared in the corresponding solvents (absolute ethanol, acetonitrile, dioxane or cyclohexane, all of UVA-sol or HPLC grade) and used in the preparation of titration solutions by appropriate dilution. Titration of compounds **2a-g** was carried out by addition of microliter amounts of standard solutions of the ions in acetonitrile or absolute ethanol. Protonation and deprotonation studies were performed by addition of HBF_4 , methanesulphonic acid, triethylamine and tetrabutylammonium hydroxide. $\text{Cu}(\text{CF}_3\text{SO}_3)_2$, $\text{Ni}(\text{BF}_4)_2$, $\text{Zn}(\text{CF}_3\text{SO}_3)_2$, $\text{Hg}(\text{CF}_3\text{SO}_3)_2$, $\text{Ca}(\text{ClO}_4)_2$ and NaNO_3 were purchased from Alfa Aesar and used as received. Luminescence quantum yields were measured

using as standard a solution of 1-naphthylamine in cyclohexane (0.1 M) ($\Phi_F = 0.46$) [34], and were corrected for different refractive indexes of solvents. In all fluorimetric measurements the optical density did not exceed 0.1 [35].

Acknowledgements

Thanks are due to Foundation for Science and Technology (Portugal) for financial support through Centro de Química (UM) and REQUIMTE (UNL) through project PTDC/QUI/66250/2006 and PhD grant to E. Oliveira (SFRH/BD/35905/2007).

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