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Functionalised benzo[*a*]phenoxazine dyes as long-wavelength fluorescent probes for amino acids

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Abstract—A series of monoreactive functional benzo[*a*]phenoxazinium chlorides with a carboxyl, hydroxyl, amine or chloromethyl group were used as labels in the preparation of long-wavelength fluorescent amino acid bioconjugates. UV–visible and fluorescence studies of all compounds were carried out in ethanol and water at physiological pH. The absorption and emission of all compounds synthesised were in the range 555–640 nm and 632–681 nm, respectively.

1. Introduction

In recent years, the requirements for analytical determinations at increasingly lower levels of detection in various fields of science such as environmental studies, residue determination or pharmaceutical analysis have increased. This has shifted the research to the development of both new techniques and reagents.

Fluorescent labelling is one of the most commonly used methodologies for analytical purposes.[1–4] Although a large amount of fluorophores are commercially available, most dyes fluoresce in the visible region of the spectrum where the biological background also fluoresce, which can cause interference.[5] Thus, long-wavelength fluorophores (600–1000 nm) are preferred for biological applications.

The suitability of a fluorescent dye for the labelling of biomolecules depends on different factors related with their photophysical characteristics and the chemical compatibility with the respective application. Thus, the fluorophore must be chemically and photochemically stable in solution, as well as water-soluble. It should also have a considerable fluorescent quantum yield when bound to the analyte and excitation maxima accessible to simple sources such as laser diodes. In addition, a reactive functional group, such as a carboxyl, amine or hydroxyl group, is also an essential prerequisite for covalent labelling to the molecules.

Among the long-wavelength fluorophores are the oxazine derivatives, such as benzo[*a*]phenoxazinium salts, with an absorption higher than 600 nm and with strong red fluorescence.[6] Applications of oxazine fluorophores in life sciences are related to the labelling of small synthetic molecules and large biomolecules, such as proteins, antibodies or nucleotides in DNA studies.[7–12]

Amino acids are small molecules of extreme importance due to their presence in biological tissues and fluids. In most cases, monitorisation of these molecules is achieved only after the linkage of a chromophore or a fluorophore to the amine function or to the carboxylic group.[13,14]

Although there are few fluorescent probes, which absorb in the red or near-infrared region, and even fewer are available with a suitable functional group for covalent labelling of the analyte, the interest in their development still continues.

Bearing this in mind and, in connection with our interest in the synthesis and application of non-fluorescent and fluorescent reagents,[15–17] we have extended our preliminary work[18] by studying the application of several functionalised dyes of benzo[*a*]phenoxazine family to biomolecules. Derivatisation of L-valine and L-glycine amino acids at the carboxylic or amine function with benzo[*a*]phenoxazine fluorophores was achieved in good yields. Evaluation of absorption and emission properties of all compounds synthesised was carried out in ethanol and water at physiological pH. In order to investigate the sensitivity of the functionalised fluorophores to pH, photophysical studies under controlled pH were performed.

Keywords: Long-wavelength fluorescent dyes; Functionalised probes; Benzo[*a*]phenoxazine; Fluorescent labelling; Amino acids.
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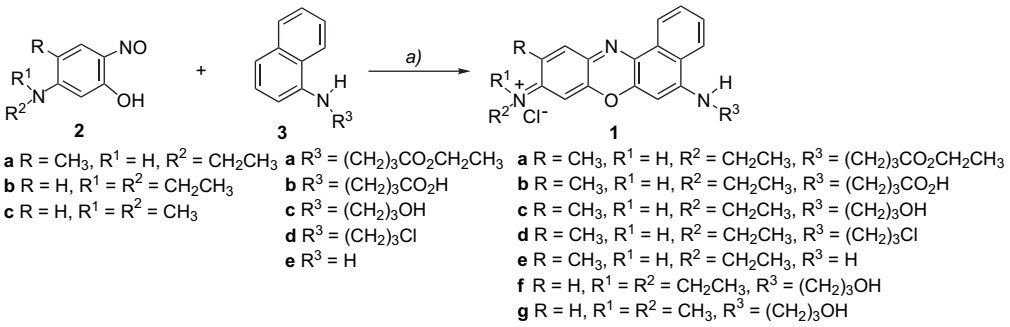


Scheme 1. Synthesis of benzo[*a*]phenoxazinium chlorides **1a–g**. Reagents and conditions: (a) H^+/ethanol, methanol (reflux) or DMF (70 °C).

2. Results and discussion

Benzo[*a*]phenoxazinium chlorides **1a–g** were synthesised by condensation of 5-alkylamino-2-nitrosophenol hydrochlorides **2a–c** with *N*-alkylated-naphthylamines **3a–e** in an acidic medium (Scheme 1). The required 5-alkylamino-2-nitrosophenol hydrochlorides **2a–c** were synthesised using the usual procedure[19] involving the treatment of the corresponding 3-alkylaminophenol with sodium nitrite in an acid solution. Intermediates **3a–e** were responsible for the functionality of the cationic dyes **1a–g**. Two of these, **3a** and **3c** were prepared by alkylation of 1-naphthylamine with the appropriate bromo derivative, ethyl-4-bromobutyrate and 3-bromo-1-propanol, respectively. Hydrolysis of the ester group of the intermediate **3a** (1 M NaOH/1,4-dioxane), yielded the corresponding 4-(naphthalen-1-ylamino)-butanoic acid **3b**. Treatment of compound **3c** with thionyl chloride, at room temperature, produced the chloro derivative **3d**.

After dry chromatographic purification or isolation by extraction (**3b**), these compounds were obtained as oils (**3a**, 46%; **3c**, 70% and **3d**, 31%) or as a solid material (**3b**, 98%) and were characterised by high-resolution mass spectrometry, IR and NMR (^{1}H and ^{13}C) spectroscopies. The IR spectra showed strong stretching vibration bands for the carbonyl group at 1728 (**3a**) and 1694 (**3b**) cm^{-1}, as well as bands for the hydroxyl group at 3418 (**3b**) and 3529–3109 (**3c**) cm^{-1}. In the ^{13}C NMR relevant signals at δ 173.8 (**3a**) and 179.5 (**3b**) ppm were assigned to the functional group.

Condensation of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride (**2a**) with functionalised precursors **3a** and **3c–e** in the presence of hydrochloric acid, refluxed in ethanol, gave the corresponding benzo[*a*]phenoxazinium chlorides **1a** and **1c–e**. In the preparation of compound **1b**, the nitroso intermediate **2a** reacted with 4-(naphthalen-1-ylamino)butanoic acid **3b** in an acidic medium, using DMF as a solvent and heating at 70 °C. The chloropropylamino derivative **1d** was also prepared by the treatment of dye **1c** with thionyl chloride, at room temperature, which proved to be more efficient than the above procedure involving the initial preparation of intermediate **3d**. In the same conditions reported above (reflux in ethanol or methanol/HCl), dyes **1f** and **1g** were, respectively, obtained by starting with nitrosophenol components **2b** and **2c** and reacting with precursor **3c**.

After purification by dry chromatography, cationic dyes **1a–g** were isolated as solid materials in moderate to excellent yields (Table 1) and were fully characterised by the usual analytical techniques.

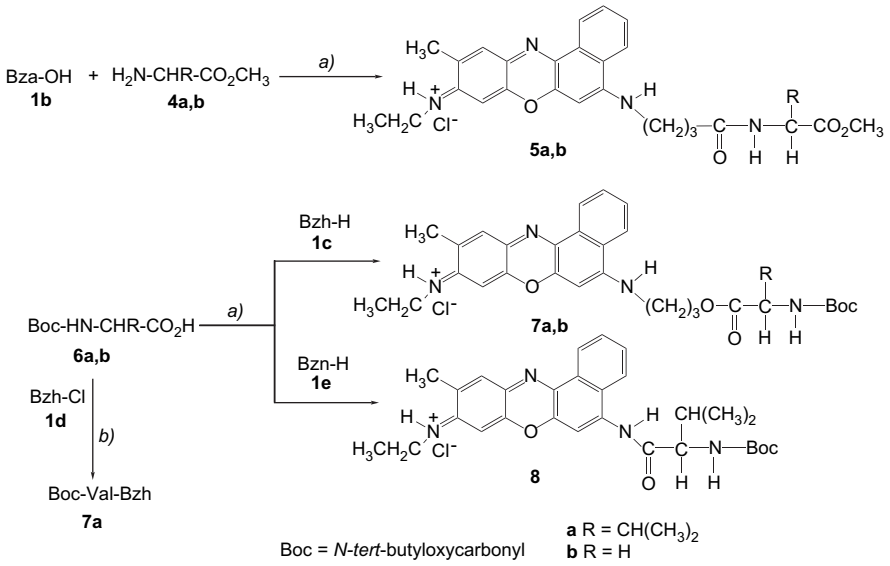
The IR spectral bands at 1727 cm^{-1} and from 3506 to 3200 cm^{-1} were assigned to the ester (**1a**) and the hydroxyl groups (**1b**, **1c**, **1f** and **1g**), respectively. The ^{13}C NMR of compounds **1a** and **1b** showed signals at δ 173.4 and 164.3 ppm, which confirmed the presence of the carbonyl function of the ester (**1a**) and the acid (**1b**). The fluorophores are designated in this report as a letter code so as to simplify the naming of the various amino acid fluorescent derivatives, for example, *N*-[5-(3-carboxypropylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride (**1b**) is Bza–OH.

In order to allow the covalent labelling of proteins or other biomolecules, the label must undergo an efficient bond formation to functional groups of the analyte, such as an amino group or a carboxylic acid. The functionalised dyes Bza–OH (**1b**), Bzh–H (**1c**) and Bzn–H (**1e**) were linked to the α-amine group of the L-valine methyl ester, H–Val–OMe (**4a**), and the L-glycine methyl ester, H–Gly–OMe (**4b**), or the carboxylic acid of *N*-*tert*-butyloxycarbonyl-L-valine, Boc–Val–OH (**6a**), and *N*-*tert*-butyloxycarbonyl-L-glycine, Boc–Gly–OH (**6b**), by coupling with the aid of *N*,*N*′-dicyclohexylcarbodiimide (DCC) assisted by 1-hydroxybenzotriazole (HOBt), under standard conditions. Thus, the reaction of carboxylic acid dye Bza–OH (**1b**) with L-valine and L-glycine methyl esters (**4a** and **4b**) produced the fluorescent amide derivatives Bza–Val–OMe (**5a**) and Bza–Gly–OMe (**5b**) (Scheme 2).

Table 1. Synthesis of benzo[*a*]phenoxazinium chlorides **1a–g** and labelled amino acid derivatives **5a**,**b**, **7a**,**b** and **8**

Entry	Starting materials		Compound		Yield (%)
1	**2a**	**3a**	**1a**	Bza–OEt	75
2	**2a**	**3b**	**1b**	Bza–OH	40
3	**2a**	**3c**	**1c**	Bzh–H	97
4	**2a**	**3d**	**1d**	Bzh–Cl	50/55[a]
5	**2a**	**3e**	**1e**	Bzn–H	98
6	**2b**	**3c**	**1f**	Bpe–H	77
7	**2c**	**3c**	**1g**	Bzm–H	82
8	**1b**	**4a**	**5a**	Bza–Val–OMe	75
9	**1b**	**4b**	**5b**	Bza–Gly–OMe	98
10	**1c**	**6a**	**7a**	Boc–Val–Bzh	65/60[b]
11	**1c**	**6b**	**7b**	Boc–Gly–Bzh	64
12	**1e**	**6a**	**8**	Boc–Val–Bzn	52

[a] Yield obtained using **1c** as precursor.
[b] Yield obtained by the reaction of **1d** with **6a** (KF, DMF, rt).



Scheme 2. Synthesis of labelled amino acid derivatives **5a**,**b**, **7a**,**b** and **8**. Reagents and conditions: (a) DCC, HOBt, DMF, rt; (b) KF, DMF, rt.

When Boc–Val–OH (**6a**) and Boc–Gly–OH (**6b**) were coupled to Bzh–H (**1c**), which presented the highest fluorescence quantum yield of the three hydroxyl derivatives obtained (**1c**, **1f** and **1g**), as will be discussed later, the labelled ester conjugates Boc–Val–Bzh (**7a**) and Boc–Gly–Bzh (**7b**) were obtained. Derivatisation of *N*-protected-L-valine **6a** with the amino dye Bzn–H (**1e**) resulted in the fluorescent valine derivative Boc–Val–Bzn (**8**). Labelled valine, Boc–Val–Bzh (**7a**) was also obtained by the reaction of Bzh–Cl (**1d**) and Boc–Val–OH (**6a**) with potassium fluoride,[20] in DMF, at room temperature (Scheme 2).

All labelled amino acids (**5a**,**b**, **7a**,**b** and **8**) were obtained as solid materials in yields ranging from 52 to 98% (Table 1) and were characterised by high-resolution mass spectrometry, NMR (^{1}H and ^{13}C), IR and UV–visible spectroscopies.

^{1}H NMR spectra of these compounds showed signals of the amino acid residues, such as a singlet for the methyl ester (at about δ 3.0 ppm, **5a**,**b**), a singlet for the methyl protons of the Boc group (δ 1.42–1.53 ppm, **7a**,**b** and **8**), a broad singlet or a duplet for the α-CH in valine derivatives (δ 3.73–4.54 ppm, **5a**, **7a** and **8**), a multiplet for glycine CH_2 (δ 3.60–4.0 ppm, **5b** and **7b**) and a broad singlet for α-NH (δ 4.30 and 6.00 ppm), as well as the protons of the label moiety. In ^{13}C NMR spectra, signals of the carbonyl were found from δ 166.60 to 168.10 ppm (amide type) and from δ 170.45 to 173.99 ppm (ester bond).

Electronic absorption spectra of 10^{-6} M solutions of benzo[*a*]phenoxazinium chlorides **1a**–**g** and labelled L-valine and L-glycine amino acids (**5a**,**b**, **7a**,**b** and **8**) in degassed absolute ethanol were measured. Summarised data of this are presented in Table 2. The longest wavelength maximum absorption (λ_{max}) of all compounds was located between 560 and 635 nm. When compared to **1b** (entry 2) and **1c** (entry 3), compounds **5a**,**b** (entries 8 and 9) and **7a** (entry 10) absorb at slightly longer wavelengths, the bathochromic shift was 10 and 5 nm, respectively.

Table 2. UV–visible data for compounds **1a**–**g**, **5a**,**b**, **7a**,**b** and **8** in ethanol and water (pH 7.4)

Entry	Compound		λ_{max} (nm) (ε) (ethanol)	λ_{max} (nm) (ε) (water, pH 7.4)
1	**1a**	Bza–OEt	630 (45,296)	625 (33,500)
2	**1b**	Bza–OH	620 (23,970)	620 (12,805)
3	**1c**	Bzh–H	625 (45,477)	620 (36,181)
4	**1d**	Bzh–Cl	625 (21,978)	620 (11,311)
5	**1e**	Bzn–H	620 (53,968)	—[a]
6	**1f**	Bpe–H	635 (46,535)	640 (48,066)
7	**1g**	Bzm–H	630 (33,913)	635 (29,908)
8	**5a**	Bza–Val–OMe	630 (13,400)	625
9	**5b**	Bza–Gly–OMe	630 (7294)	620
10	**7a**	Boc–Val–Bzh	630 (42,000)	625
11	**7b**	Boc–Gly–Bzh	615 (31,500)	615
12	**8**	Boc–Val–Bzn	560 (16,579)	555

[a] Compound insoluble in water (pH 7.4).

In order to be closer to the biological environment, the absorption properties of all the above compounds (**1a**–**g**, **5a**,**b**, **7a**,**b** and **8**) were also studied in water at physiological pH (pH 7.4, adjusted with HCl and NaOH). Labelled compounds were not completely soluble in water; as a result, only the maximum absorption wavelengths were presented (Table 2). Comparison of λ_{max} values in ethanol and water showed only a slight difference (~5 nm) or no variation occurred (**1b** and **7b**). A tendency of a blue shift was observed for R^1=H (compounds **1a**–**e** and **5a**,**b**, **7a**,**b** and **8**) and a red shift when R^1 is CH_3 or CH_2CH_3 (compounds **1f**,**g**). This can be explained by a higher solvent interaction of the molecule in its ground state when both nitrogen atoms are secondary. Another interesting feature is that compound **8** (entry 12) exhibited a distinctive absorption maximum near 560 nm. Besides other authors,[21] we[17] have also shown that this type of compounds forms non-fluorescent *H*-aggregates in aqueous solutions at a concentration of 10^{-5} M, with a corresponding blue shifted absorption of 560 nm, and that this aggregation does not occur in organic solvents. Thus, one possible explanation for the 560 nm band is an enhanced aggregation of compound **8** in water solution, which also occurs in ethanol medium. This aggregation could be induced by the more

hydrophobic nature of the valine residue, but a similar compound **7a** where the amino acid is further away from benzo[*a*]phenoxazine chromophore, does not show such enhanced aggregation effect. Only in compound **8** there is a conformation in which both nitrogen atoms are simultaneously in sp^2 hybridisation (the contribution of the zwitterionic amide form: $N^+{=}C{-}O^-$). This can originate better packing geometry of the aggregate due to increased planarity of the nitrogens in the chromophoric group. In the same zwitterionic amide form, there can also be an extra π conjugation between the chromophoric moiety and the amide group, which can influence the position of the absorption bands on its own.

Studies on the fluorescent properties of these compounds were also carried out in ethanol and water (pH 7.4) (Table 3). The fluorescence quantum yields (Φ_F) were calculated using Oxazine 1 as a standard (Φ_F=0.11 in ethanol).[22] For the determination of the relative quantum yields, Oxazine 1 was excited at the wavelengths of excitation of each one of the compounds tested. Emission maxima for all compounds in ethanol and water varied from 632 to 681 nm (Table 3).

When compared to ethanol, in aqueous solutions at physiological pH, the position of the maximum emission wavelength (λ_{em}) showed a bathochromic shift (2–14 nm) for all compounds (except for compound **7b**). The highest λ_{em} of all dyes studied either in ethanol or water is observed in hydroxyl benzo[*a*]phenoxazines **1f** and **1g** (669 nm in ethanol, 681 and 674 nm in water for **1f** and **1g**, respectively). Figure 1 shows the comparison of fluorescence spectra of compounds **1c** and **7a** in ethanol and water (pH 7.4).

All compounds exhibited moderate to high Φ_F in both solvents ($0.12<\Phi_F<0.55$, ethanol and $0.02<\Phi_F<0.44$, water). Comparison of Φ_F, in the three hydroxyl fluorophores synthesised **1c**, **1f** and **1g**, showed that heterocycle **1c** presented the highest value either in ethanol (**1c**, 0.41; **1f**, 0.17 and **1g**, 0.19) or in water (**1c**, 0.31; **1f**, 0.23 and **1g**, 0.14). Bearing in mind that our purpose was the investigation of the polycyclic oxazines as covalent probes, which required a significant Φ_F, compound **1c** was used as the hydroxyl representative for coupling with the amino acids.

Regarding the Φ_F values of the labelled amino acids **5a**,**b**, **7a**,**b** and **8** in the solvents used, it was possible to conclude

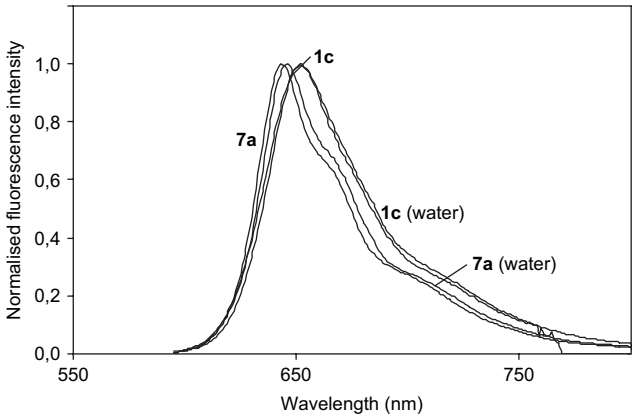


Figure 1. Normalised fluorescence spectra of compounds **1c** and **7a** in ethanol and water (pH 7.4).

that the highest values were found in ethanol ($0.12<\Phi_F<0.55$). In this solvent, the Φ_F increased after the linkage of benzo[*a*]phenoxazines **1b** and **1c** to the protected amino acid (**5b** and **7a**), the highest increase being for compound **1b** (**1b**, 0.34, entry 2/**5b**, 0.55, entry 9). However, in the case of water, this behaviour was not observed.

The enhanced aggregation effect of the amino acid substitution is confirmed by the fluorescence quantum yield measurement in water, as compounds **5a**,**b** and **7a**,**b** have lower Φ_F values than the corresponding starting materials (**1b–d**). The lowest Φ_F is observed for compound **8**, where the H-aggregation is more effective.

Compounds **1a–e** were also investigated in aqueous solution from pH 3 to 8 using appropriate buffer solutions.[23] The absorbance and fluorescence properties of 1.5×10^{-6} M buffer solutions of the studied compounds are reported in Table 4. Figure 2 also presents the typical spectral variations of normalised absorption with pH, for all compounds with the exception of **1d**.

Figure 2 shows an increase of the shoulder at 560 nm upon increase of pH. This observation is consistent with an increase in aggregation equilibrium constant with pH. From the inset of Figure 2 and from the data in Table 4, an overall decrease in the absorption spectra with an increase in pH is observed. This is not consistent with the aggregation process because one should see an increase in the 560 nm region

Table 3. Fluorescence data for compounds **1a–g**, **5a**,**b**, **7a**,**b** and **8** in ethanol and water (pH 7.4)

Entry	Compound		Ethanol			Water (pH 7.4)		
			λ_{exc} (nm)	λ_{em} (nm)	Φ_F	λ_{exc} (nm)	λ_{em} (nm)	Φ_F
1	**1a**	Bza–OEt	600	646	0.35	590	653	0.26
2	**1b**	Bza–OH	590	644	0.34	590	653	0.37
3	**1c**	Bzh–H	590	646	0.41	580	651	0.31
4	**1d**	Bzh–Cl	580	645	0.55	590	651	0.44
5	**1e**	Bzn–H	580	637	0.31	—	—	—
6	**1f**	Bpe–H	600	669	0.17	610	681	0.23
7	**1g**	Bzm–H	600	669	0.19	610	674	0.14
8	**5a**	Bza–Val–OMe	590	644	0.34	600	672	0.14
9	**5b**	Bza–Gly–OMe	600	643	0.55	600	652	0.15
10	**7a**	Boc–Val–Bzh	600	643	0.45	600	652	0.16
11	**7b**	Boc–Gly–Bzh	600	640	0.37	600	632	0.19
12	**8**	Boc–Val–Bzn	590	640	0.12	590	642	0.02

Table 4. Photophysical data of compounds **1a–e** in buffer solutions

pH	1a		1b		1c		1d		1e	
	$\varepsilon/\varepsilon^{a}$	$\Phi_F/\Phi_F{}^{b}$	$\varepsilon/\varepsilon^{a}$	$\Phi_F/\Phi_F{}^{b}$	$\varepsilon/\varepsilon^{a}$	$\Phi_F/\Phi_F{}^{b}$	$\varepsilon/\varepsilon^{a}$	$\Phi_F/\Phi_F{}^{b}$	$\varepsilon/\varepsilon^{a}$	$\Phi_F/\Phi_F{}^{b}$
3	1	1	1	1	1	1	1	1	1	1
5	1.17	0.734	0.930	0.906	0.970	1.02	0.817	1.04	0.828	0.994
7	0.997	0.717	0.776	—	0.865	1.04	0.731	1.02	0.784	0.968
8	0.972	0.720	0.684	0.861	0.815	1.02	0.583	1.07	0.663	0.997

[a] ε value at pH 3.
[b] Φ_F value at pH 3.

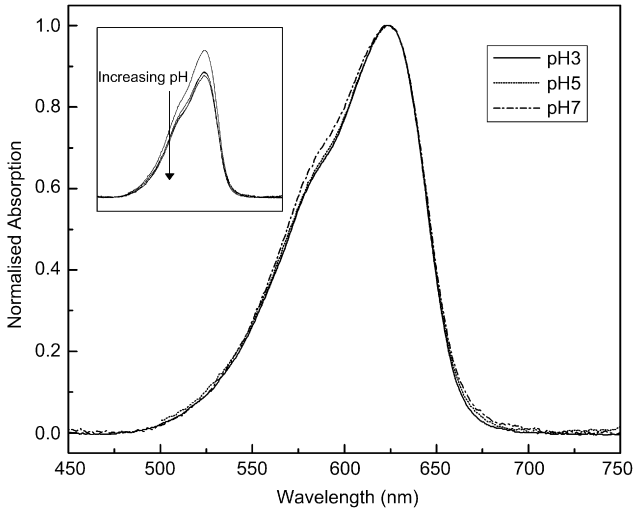


Figure 2. Normalised absorption spectra of compound **1a** at pH 3, 5 and 7.

with a corresponding decrease in the monomer (~600 nm) region. In this pH study, all the solutions were prepared using 150 μL of a concentrated methanol solution in 250 mL of buffer. The different solvent cages that occur in each pH buffer, in which methanol enrichments can occur, may induce different oscillator strengths for the studied electronic transition.

With the exception of compounds **1a** and **1b**, the Φ_F shows very little sensitivity to pH. The main expected effect is a decrease in Φ_F with increasing pH that corresponds to the observed increase in H-aggregation from absorption measurements (Fig. 2). The observed variations can also be influenced by the solvation cage composition through the kinetic constant (k_F) of the fluorescence process. On the one hand, the Strikler–Berg equation predicts a linear relation between k_F and the molar absorptivity (ε).[24] Thus, the observed decrease in absorptivity with increasing pH (Table 4) also predicts the decrease in the fluorescence quantum yield. On the other hand, the possible methanol enrichment of the solvent cage can lead to a higher Φ_F through the local environment effect, as Φ_F in alcohols[17] is higher than in water.

3. Conclusion

A series of functionalised benzo[*a*]phenoxazinium salts were synthesised in good to excellent yields by the corresponding condensation of 5-alkylamino-2-nitrosophenol hydrochloride and *N*-alkylated-naphthylamines. These dyes were covalently bonded to the amine or carboxylic function of L-valine and L-glycine amino acids through an amide or ester linkage.

The cationic character of the fluorophores synthesised is important for its use as probes in non-covalent staining of biomolecules. However, the possibility opened with this study of covalent bonding to biomolecules, such as proteins, makes these probes useful in biological imaging studies with the added advantage of emission in the long-wavelength range where scattering and background emission are much less important.

The small pH dependence of the photophysical properties (absorption and emission) of benzo[*a*]phenoxazinium salts **1a–g** makes these compounds unsuitable for pH probes. Nevertheless, these probes are sensitive to local environmental variations. This can be used, for example, in biological macromolecular conformational studies induced by pH gradients, where the probe is supposed to report only the local environmental variations avoiding the direct influence of the pH variation on the probe's photophysical properties.

4. Experimental

4.1. General

All melting points were measured on a Gallenkamp melting point apparatus and were uncorrected. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$) and spots were visualised under UV light. Dry 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, Nujol or neat samples. UV–visible spectra were run on a Hitachi U-2000 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian 300 spectrometer in $CDCl_3$ or CD_3OD solution at 300 MHz and 25 °C. All chemical shifts are given in parts per million using δ_H Me_4Si=0 ppm as reference and *J* values are given in hertz. ^{13}C 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 correlation HMBC and HMQC techniques. Mass spectrometric analyses were performed at the C.A.C.T.I.—Unidad de Espectrometria de Masas of the University of Vigo, Spain, on a Hewlett Packard 5989 A spectrometer for low-resolution spectra and an Autospec M spectrometer for high-resolution

mass spectra. Elemental analyses were carried out on a Leco CHNS 932 instrument. Fluorescence spectra were collected using a Spex Fluorolog 1680 spectrometer.

4.2. General method for the preparation of compounds 1a and 1c–g

To a cold solution (ice bath) of 5-alkylamino-2-nitrosophenol hydrochlorides **2a–c** in ethanol or methanol, *N*-alkylated-naphthylamine (**3a** and **3c**,**d**) or naphthylamine (**3e**) and concentrated hydrochloric acid (5.0×10^{-2} mL) were added. The mixture was refluxed for 3–9 h, and monitored by TLC (chloroform/methanol). After evaporation of the solvent and dry chromatography on silica gel, the required dyes (**1a** and **1c–g**) were obtained as a blue material.

4.2.1. *N*-[5-(4-Ethoxy-4-oxobutylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride, Bza–OEt (1a). The product of the reaction of **2a** (0.140 g, 7.78×10^{-4} mol) with **3a** (0.200 g, 7.78×10^{-4} mol) in ethanol (2 mL) was chromatographed with chloroform/methanol, 5.8:0.2 as the eluent, to give Bza–OEt (**1a**) (0.244 g, 75%). Mp=251.4–252.9 °C. TLC (chloroform/methanol, 6:1): R_f=0.62. ^{1}H NMR ($CDCl_3$, 300 MHz): δ=1.31 (t, J=6.3 Hz, 3H, OCH_2CH_3), 1.42 (t, J=6.3 Hz, 3H, $NHCH_2CH_3$), 2.16 (t, J=5.1 Hz, 2H, $NHCH_2CH_2CH_2$), 2.41 (s, 3H, OCH_3), 2.58 (t, J=6.3 Hz, 2H, $NHCH_2CH_2CH_2$), 3.30 (br s, 2H, $NHCH_2CH_3$), 3.70 (br s, 2H, $NHCH_2CH_2CH_2$), 4.19 (q, J=7.2 Hz, 2H, OCH_2CH_3), 6.0 (br s, 1H, NH), 6.31 (s, 1H, 8-H), 6.64 (s, 1H, 6-H), 7.49 (s, 1H, 11-H), 7.80–7.90 (m, 2H, 2-H and 3-H), 8.82 (d, J=9.3 Hz, 1H, 1-H), 9.15–9.30 (m, 1H, 4-H), 11.4 (br s, 1H, N–H). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C=13.9 ($NHCH_2CH_3$), 14.2 (OCH_2CH_3), 18.1 (CH_3), 23.5 ($NHCH_2CH_2CH_2$), 31.1 ($NHCH_2CH_2CH_2$), 38.7 ($NHCH_2CH_3$), 43.5 ($NHCH_2CH_2CH_2$), 60.7 (OCH_2CH_3), 92.8 (C-6), 93.3 (C-8), 123.9 (C-10), 124.1 (C-4), 125.6 (C-1), 126.5 (Ar–C), 129.5 (Ar–C), 130.2 (C-2), 130.6 (Ar–C), 131.1 (C-11), 131.6 (C-3), 134.1 (Ar–C), 146.7 (Ar–C), 150.9 (Ar–C), 153.8 (C-9), 157.5 (C-5), 173.4 ($CO_2CH_2CH_3$). IR (KBr 1%, cm^{-1}): ν=3230, 2963, 2927, 2855, 1727, 1642, 1592, 1563, 1544, 1520, 1451, 1436, 1313, 1295, 1261, 1185, 1163, 1135, 1089, 1017. HRMS (FAB) calcd for $C_{25}H_{28}N_3O_3$ [M^+]: 418.2131; found: 418.2128.

4.2.2. *N*-[5-(3-Hydroxypropylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride, Bzh–H (1c). The product of the reaction of **2a** (0.185 g, 5.97×10^{-4} mol) with **3c** (0.117 g, 5.82×10^{-4} mol) in ethanol (2 mL) was chromatographed with chloroform and chloroform/methanol, 5.5:0.5 as the eluent, to give Bzh–H (**1c**) (0.204 g, 97%). Mp=above 300 °C. TLC (chloroform/methanol, 6:1): R_f=0.69. ^{1}H NMR (CD_3OD, 300 MHz): δ=1.41 (t, J=6.3 Hz, 3H, $NHCH_2CH_3$), 2.11 (br s, 2H, $NHCH_2CH_2CH_2$), 2.39 (s, 3H, CH_3), 3.50–3.68 (m, 2H, $NHCH_2CH_2CH_2$), 3.82 (br s, 2H, $NHCH_2CH_2CH_2$), 3.87 (br s, 2H, $NHCH_2CH_3$), 6.92 (s, 1H, 8-H), 7.06 (s, 1H, 6-H), 7.76 (s, 1H, 11-H), 7.80–7.90 (m, 1H, 2-H), 7.96 (t, J=7.5 Hz, 1H, 3-H), 8.33 (br s, 1H, 1-H), 8.98 (d, J=7.5 Hz, 1H, 4-H). ^{13}C NMR (CD_3OD, 75 MHz): δ_C=14.2 ($NHCH_2CH_3$), 17.7 (CH_3), 32.2 ($NHCH_2CH_2CH_2$), 39.7 ($NHCH_2CH_2CH_2$), 43.3 ($NHCH_2CH_3$), 60.4 ($NHCH_2CH_2CH_2$), 93.9 (C-6), 94.5 (C-8), 123.6 (Ar–C), 124.7 (C-1), 125.5 (C-4), 128.7 (C-10), 130.8 (C-2), 132.0 (Ar–C), 132.4 (Ar–C), 132.7 (C-3), 132.8 (C-11), 134.3 (Ar–C), 149.2 (Ar–C), 152.8 (Ar–C), 156.6 (C-9), 158.6 (C-5). IR (KBr 1%, cm^{-1}): ν=3506, 3241, 1641, 1592, 1561, 1544, 1521, 1451, 1431, 1315, 1185, 1163, 1137, 1087, 1010. Anal. Calcd for $C_{22}H_{24}N_3O_2\cdot3.5HCl$ (490.07): C 53.92, H 5.66, N 8.58. Found: C 53.99, H 5.80, N 8.37.

4.2.3. *N*-[5-(3-Chloropropylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride, Bzh–Cl (1d). The product of the reaction of **2a** (0.016 g, 9.11×10^{-5} mol) with **3d** (0.020 g, 9.11×10^{-5} mol) in ethanol (1 mL) was chromatographed with dichloromethane and dichloromethane/methanol, mixtures of increasing polarity as the eluent, to give Bzh–Cl (**1d**) (0.018 g, 50%). Mp=above 300 °C. TLC (chloroform/methanol, 6:1): R_f=0.59. ^{1}H NMR (CD_3OD, 300 MHz): δ=1.41 (t, J=7.2 Hz, 3H, NCH_2CH_3), 2.28–2.40 (m, 5H, CH_3 and $NHCH_2CH_2CH_2Cl$), 3.53 (q, J=7.2 Hz, 2H, NCH_2CH_3), 3.80–3.90 (m, 4H, $NHCH_2CH_2CH_2Cl$ and $NHCH_2CH_2CH_2Cl$), 6.78 (s, 1H, 8-H), 6.88 (s, 1H, 6-H), 7.56 (br s, 1H, 11-H), 7.78 (t, J=7.8 Hz, 2H, 2-H), 7.86 (t, J=7.2 Hz, 1H, 3-H), 8.28 (d, J=7.8 Hz, 1H, 1-H), 8.79 (d, J=8.1 Hz, 1H, 4-H). ^{13}C NMR (CD_3OD, 75.4 MHz): δ_C=14.2 ($NHCH_2CH_3$), 17.8 (CH_3), 32.5 ($NHCH_2CH_2CH_2$), 39.8 ($NHCH_2CH_2CH_2$), 43.0 ($NHCH_2CH_2CH_2$), 43.2 ($NHCH_2CH_3$), 93.8 (C-6), 94.5 (C-8), 123.7 (C-4), 124.6 (C-10), 125.4 (C-1), 129.1 (Ar–C), 130.7 (C-2), 132.3 (Ar–C), 132.4 (Ar–C), 132.6 (C-3), 132.8 (C-11), 133.9 (Ar–C), 149.3 (Ar–C), 152.6 (Ar–C), 156.9 (C-5), 158.4 (C-9). IR (Nujol, cm^{-1}): ν=3300, 2956, 2924, 2854, 1642, 1592, 1563, 1546, 1520, 1503, 1461, 1455, 1377, 1316, 1187, 1162, 1135, 1012. HRMS (FAB) calcd for $C_{22}H_{23}N_3O^{35}Cl$ [M^+]: 380.1530; found: 380.1525. Calcd for $C_{22}H_{23}N_3O^{37}Cl$ [M^+]: 382.1500; found: 382.1503.

Compound Bzh–Cl (**1d**) was also prepared by the following procedure. Thionyl chloride (2.1×10^{-2} mL, 2.88×10^{-4} mol) was added to a solution of compound **1c** (0.020 g, 5.52×10^{-5} mol) in dichloromethane/chloroform, 2:1 (3.0 mL), and the reaction mixture was stirred at room temperature for 48 h. The solvent was removed under reduced pressure and the crude mixture was purified by dry chromatography (dichloromethane and dichloromethane/methanol, mixtures of increasing polarity). Bzh–Cl (**1d**) was obtained as a blue solid (0.012 g, 55%) and spectral data confirmed its structure.

4.2.4. *N*-(5-Amino-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride, Bzn–H (1e). The product of the reaction of **2a** (0.167 g, 5.97×10^{-4} mol) with **3e** (0.083 g, 5.82×10^{-4} mol) in ethanol (2 mL) was chromatographed with dichloromethane/methanol, 5.5:0.5 as the eluent, to give Bzn–H (**1e**) (0.174 g, 98%). Mp=above 300 °C. TLC (dichloromethane/methanol, 7:1): R_f=0.14. ^{1}H NMR (CD_3OD, 300 MHz): δ=1.40 (br s, 3H, $NHCH_2CH_3$), 2.36 (s, 3H, CH_3), 3.57 (br s, 2H, $NHCH_2CH_3$), 6.86 (br s, 2H, 6-H and 8-H), 7.69 (br s, 1H, 11-H), 7.84 (br s, 1H, 2-H), 7.96 (br s, 1H, 3-H), 8.32 (br s, 1H, 1-H), 8.93 (br s, 1H, 4-H). ^{13}C NMR (CD_3OD, 75.4 MHz): δ_C=14.2 ($NHCH_2CH_3$), 17.8 (CH_3), 39.7 (NCH_2CH_3), 94.5 (C-6), 97.2 (C-8), 124.1 (C-4), 124.6 (Ar–C), 125.5 (C-1), 128.4 (Ar–C), 130.7 (C-10), 131.7 (C-2), 132.9 (C-3), 133.1

(Ar–C), 133.2 (C-11), 134.5 (Ar–C), 149.2 (Ar–C), 152.7 (Ar–C), 156.7 (C-9), 162.1 (C-5). IR (Nujol, cm^{-1}): ν=3319, 2954, 2923, 2854, 1660, 1642, 1593, 1563, 1547, 1463, 1427, 1378, 1345, 1309, 1258, 1186, 1170, 1148, 1124, 1063, 1006. HRMS (FAB) calcd for $C_{19}H_{18}N_3O$ [M^+]: 304.1450; found: 304.1462.

4.2.5. *N*-Ethyl-*N*-[5-(3-hydroxypropylamino)-9*H*-benzo-[*a*]phenoxazin-9-ylidene]ethanaminium chloride, Bpe–H (1f). The product of the reaction of **2b** (0.084 g, 4.33×10^{-4} mol) with **3c** (0.087 g, 4.33×10^{-4} mol) in ethanol (4 mL) was chromatographed with chloroform/methanol, 5.6:0.4 as the eluent, to give Bpe–H (**1f**) (0.125 g, 77%). Mp=256.1–258.0 °C. TLC (chloroform/methanol, 6:1): R_f=0.37. ^{1}H NMR (CD_3OD, 300 MHz): δ=1.67 (t, J=6.0 Hz, 6H, 2×NHCH$_2$*CH*$_3$), 2.10 (br s, 2H, NHCH$_2$*CH*$_2$CH$_2$), 3.60–3.80 (m, 4H, 2×NH*CH*$_2$CH$_3$), 3.82 (br s, 4H, NHCH$_2$CH$_2$*CH*$_2$ and NH*CH*$_2$CH$_2$CH$_2$), 6.88 (s, 1H, 8-H), 6.95 (s, 1H, 6-H), 7.27 (d, J=7.5, 1H, 10-H), 7.80 (br s, 2H, 2-H and 11-H), 7.90 (br s, 1H, 3-H), 8.27 (br s, 1H, 1-H), 8.82 (d, J=7.5 Hz, 4-H). ^{13}C NMR (CD_3OD, 75.4 MHz): δ_C=13.0 (2×NHCH$_2$*CH*$_3$), 32.2 (NHCH$_2$*CH*$_2$CH$_2$), 43.4 (NH*CH*$_2$CH$_2$CH$_2$), 47.1 (2× NH*CH*$_2$CH$_3$), 60.4 (NHCH$_2$CH$_2$*CH*$_2$), 94.4 (C-6), 97.0 (C-8), 116.7 (C-10), 123.7 (C-1), 124.7 (Ar–C), 125.6 (C-4), 130.9 (C-2), 131.6 (Ar–C), 132.5 (Ar–C), 133.0 (C-3), 134.1 (C-11), 134.9 (Ar–C), 149.7 (Ar–C), 153.2 (Ar–C), 155.6 (C-9), 159.3 (C-5). IR (KBr 1%, cm^{-1}): ν=3500–3250, 1655, 1638, 1589, 1561, 1545, 1509, 1459, 1438, 1330, 1259, 1165, 1015. HRMS (FAB) calcd for $C_{23}H_{26}N_3O_2$ [M^+]: 376.2025; found: 376.2025.

4.2.6. *N*-[5-(3-Hydroxypropylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene]-*N*-methylmethanaminium chloride, Bzm–H (1g). The product of the reaction of **2c** (0.106 g, 5.27×10^{-4} mol) with **3c** (0.169 mg, 5.09×10^{-4} mol) in methanol (2 mL) was chromatographed with chloroform/methanol, 5.7:0.3 as the eluent, to give Bzm–H (**1g**) (0.148 g, 82%). Mp=above 300 °C. TLC (chloroform/methanol, 6:1): R_f=0.52. ^{1}H NMR ($CDCl_3$, 300 MHz): δ= 2.0–2.15 (m, 2H, NHCH$_2$*CH*$_2$CH$_2$), 3.16 (s, 6H, N(CH$_3$)$_2$), 3.72 (t, J=6.9 Hz, 2H, NH*CH*$_2$CH$_2$CH$_2$), 3.83 (t, J= 6.9 Hz, 2H, NHCH$_2$CH$_2$*CH*$_2$), 6.36 (s, 1H, 8-H), 6.61 (s, 1H, 6-H), 6.93 (dd, J=9.2 and 2.1 Hz, 1H, 10-H), 7.39 (d, J=9.3 Hz, 1H, 11-H), 7.68 (t, J=7.2 Hz, 1H, 2-H), 7.70 (t, J=7.5 Hz, 3-H), 8.02 (d, J=8.1 Hz, 1H, 1-H), 8.36 (t, J= 7.2 Hz, 4-H). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C=32.2 (NHCH$_2$*CH*$_2$CH$_2$), 41.2 (N(CH$_3$)$_2$), 43.5 (NH*CH*$_2$CH$_2$CH$_2$), 60.4 (NHCH$_2$CH$_2$*CH*$_2$), 94.3 (C-6), 96.9 (C-8), 116.3 (C-10), 123.7 (C-1), 124.3 (Ar–C), 125.3 (C-4), 130.8 (C-2 and Ar–C), 131.7 (Ar–C), 132.8 (C-3), 133.3 (C-11), 134.3 (Ar–C), 148.4 (Ar–C), 152.2 (Ar–C), 156.7 (C-9), 158.8 (C-5). IR (KBr 1%, cm^{-1}): ν=3500–3250, 1645, 1552, 1542, 1430, 1373, 1335, 1261, 1180, 1092. HRMS (FAB) calcd for $C_{21}H_{22}N_3O_2$ [M^+]: 348.1712; found: 348.1700.

4.2.7. *N*-[5-(3-Carboxypropylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride, Bza–OH (1b). To a solution of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride **2a** (0.132 g, 7.34×10^{-4} mol) in DMF (3 mL), 4-(naphthalen-1-ylamino)butanoic acid **3b** (0.168 g, 7.34×10^{-4} mol) and 12 M HCl (5.0×10^{-2} mL) were added and the mixture was heated at about 70 °C with stirring for 3 h. Evaporation of the solvent and purification by dry chromatography with chloroform/methanol, 5:1 as the eluent, produced Bza–OH (**1b**) (0.114 g, 40%). Mp= above 300 °C. TLC (chloroform/methanol, 5:1): R_f=0.59. ^{1}H NMR (CD_3OD, 300 MHz): δ=1.38 (br s, 3H, NCH$_2$*CH*$_3$), 2.0–2.35 (m, 2H, NHCH$_2$*CH*$_2$CH$_3$), 2.56 (br s, 2H, NHCH$_2$CH$_2$*CH*$_2$), 3.40–3.70 (2×m, 4H, NH*CH*$_2$CH$_2$CH$_2$ and NH*CH*$_2$CH$_3$), 6.50–6.80 (m, 2H, 8-H and 6-H), 7.41 (br s, 1H, 11-H), 7.60–7.90 (m, 2H, 2-H and 3-H), 8.20 (br s, 1H, 1-H), 8.65 (br s, 1H, 4-H). ^{13}C NMR (CD_3OD, 75.4 MHz): δ_C=14.2 (NHCH$_2$*CH*$_3$), 17.8 (CH$_3$), 34.9 (NHCH$_2$*CH*$_2$CH$_2$), 36.0 (NH*CH*$_2$CH$_2$CH$_2$), 39.7 (NH*CH*$_2$CH$_3$), 46.0 (NHCH$_2$CH$_2$*CH*$_2$), 93.9 (C-6), 94.4 (C-8), 124.1 (C-4), 124.7 (Ar–C), 125.4 (C-1), 128.5 (Ar–C), 130.8 (C-10), 131.7 (C-2), 132.2 (C-3), 132.6 (Ar–C), 132.7 (C-11), 134.3 (Ar–C), 149.1 (Ar–C), 152.6 (Ar–C), 156.5 (C-9), 158.4 (C-5), 164.3 (*C*O$_2$H). IR (Nujol, cm^{-1}): ν=3200–3500, 2954, 2924, 2854, 1646, 1592, 1563, 1546, 1519, 1461, 1377, 1308, 1263, 1185, 1162, 1135, 1084, 1011. HRMS (FAB) calcd for $C_{23}H_{24}N_3O_3$ [M^+]: 390.1818; found 390.1819.

4.3. Synthesis of compounds 3a–d

4.3.1. Ethyl 4-(naphthalen-1-ylamino)butanoate (3a). To a solution of 1-naphthylamine (2.0 g, 1.4×10^{-2} mol) in ethanol (5 mL), ethyl-4-bromobutyrate (2.10 mL, 1.47×10^{-2} mol) was added and the resulting mixture was refluxed for 15 h, and monitored by TLC (chloroform). The solvent was removed under reduced pressure and the crude mixture was purified by dry chromatography using chloroform and chloroform/*n*-hexane, 1:1 as the eluent. Compound **3a** was obtained as a brown oil (1.66 g, 46%). TLC (chloroform): R_f=0.75. ^{1}H NMR ($CDCl_3$, 300 MHz): δ=1.27 (t, J= 7.2 Hz, 3H, OCH$_2$*CH*$_3$), 2.0–2.20 (m, 2H, NCH$_2$*CH*$_2$CH$_2$), 2.54 (t, J=6.9 Hz, 2H, NCH$_2$CH$_2$*CH*$_2$), 3.36 (t, J=6.6 Hz, 2H, N*CH*$_2$CH$_2$CH$_2$), 4.18 (q, J=6.9 Hz, 2H, O*CH*$_2$CH$_3$), 4.55 (br s, 1H, NH), 6.62 (d, J=7.5 Hz, 1H, 4-H), 7.25 (d, J=8.4 Hz, 1H, 2-H), 7.37 (t, J=7.5 Hz, 1H, 3-H), 7.41–7.50 (m, 2H, 6-H and 7-H), 7.74–7.85 (m, 2H, 8-H and 5-H). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C=14.2 (OCH$_2$*CH*$_3$), 24.2 (NCH$_2$*CH*$_2$CH$_2$), 32.3 (NCH$_2$CH$_2$*CH*$_2$), 43.8 (N*CH*$_2$CH$_2$CH$_2$), 60.6 (O*CH*$_2$CH$_3$), 104.1 (C-4), 117.3 (C-2), 119.2 (C-5), 123.4 (C-8a), 124.7 (C-7), 125.7 (C-6), 126.6 (C-3), 128.6 (C-8), 134.3 (C-4a), 143.3 (C-1), 173.8 (*C*O$_2$CH$_2$CH$_3$). IR (neat, cm^{-1}): ν=3434, 3053, 2979, 2931, 1728, 1624, 1583, 1530, 1480, 1445, 1410, 1374, 1345, 1285, 1256, 1177, 1121, 1095, 1027. HRMS (FAB) calcd for $C_{16}H_{19}NO_2$ [M^+]: 257.1416; found: 257.1427.

4.3.2. 4-(Naphthalen-1-ylamino)butanoic acid (3b). To a suspension of ethyl 4-(naphthalen-1-ylamino)butanoate **3a** (0.110 g, 4.3×10^{-4} mol) in 1,4-dioxane (2.0 mL), 1 M NaOH (0.79 mL, 7.7×10^{-4} mol) was added. The solution was stirred at room temperature for 8 h and acidified to pH 2–3 with 1 M $KHSO_4$. The mixture was extracted with chloroform (4×15 mL) and the organic extracts were dried ($MgSO_4$) and evaporated to dryness giving compound **3b** as a pinkish solid (0.097 g, 98%). Mp=109.0–111.0 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ=2.06–2.20 (m, 2H, NCH$_2$*CH*$_2$CH$_2$), 2.58 (t, J=7.2 Hz, 2H, NCH$_2$CH$_2$*CH*$_2$), 3.37 (t, J=6.6 Hz, 2H, N*CH*$_2$CH$_2$CH$_2$), 6.63 (d, J=7.5 Hz, 1H, 4-H), 7.26 (d, J=7.8 Hz, 1H, 2-H), 7.37 (t, J=7.8 Hz,

1H, 3-H), 7.40–7.50 (m, 2H, 6-H and 7-H), 7.76–7.86 (m, 2H, 8-H and 5-H). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C=23.9 ($NCH_2\mathit{CH_2}CH_2$), 31.9 ($NCH_2CH_2\mathit{CH_2}$), 43.6 ($N\mathit{CH_2}CH_2CH_2$), 104.5 (C-4), 117.6 (C-2), 119.9 (C-5), 123.5 (C-4a), 124.7 (C-7), 125.7 (C-6), 126.5 (C-3), 128.6 (C-8), 134.3 (C-8a), 143.0 (C-1), 179.5 ($\mathit{C}O_2H$). IR (Nujol, cm^{-1}): ν=3418, 2954, 2924, 2854, 1694, 1651, 1581, 1526, 1463, 1455, 1378, 1349, 1290, 1220, 1126, 1093, 1036, 1015. HRMS (FAB) calcd for $C_{14}H_{15}NO_2$ [M$^+$]: 229.1103; found: 229.1102.

4.3.3. 3-(Naphthalen-1-ylamino)propanol (3c). The product of the reaction of 1-naphthylamine (2.0 g, 1.4×10^{-2} mol) with 3-bromo-1-propanol (1.33 mL, 1.47×10^{-2} mol) in ethanol (5 mL), according to the procedure described above for the preparation of compound **3a**, was chromatographed with chloroform and chloroform/methanol, 5.8:0.2 as the eluent, to produce compound **3c** as a pinkish oil (2.07 g, 70%). TLC (chloroform/methanol, 5.7:0.3): R_f=0.30. ^{1}H NMR ($CDCl_3$, 300 MHz): δ=1.96–2.10 (m, 2H, $NCH_2\mathit{CH_2}CH_2$), 3.10–3.50 (m, 1H, NH), 3.44 (t, J=6.0 Hz, 2H, $NCH_2CH_2\mathit{CH_2}$), 3.90 (t, J=5.7 Hz, 2H, $N\mathit{CH_2}CH_2\mathit{CH_2}$), 6.67 (d, J=7.0 Hz, 1H, 4-H), 7.28 (d, J=6.6 Hz, 1H, 2-H), 7.38 (t, J=7.5 Hz, 1H, 3-H), 7.43–7.51 (m, 2H, 6-H and 7-H), 7.78–7.86 (m, 2H, 8-H and 5-H). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C=31.2 ($NCH_2\mathit{CH_2}CH_2$), 42.5 ($NCH_2CH_2\mathit{CH_2}$), 61.8 ($N\mathit{CH_2}CH_2CH_2$), 104.7 (C-4), 117.6 (C-2), 120.0 (C-5), 123.5 (C-4a), 124.7 (C-7), 125.7 (C-6), 126.5 (C-3), 128.5 (C-8), 134.2 (C-8a), 143.3 (C-1). IR (neat, cm^{-1}): ν=3529–3109, 3046, 2953, 2914, 2846, 1624, 1581, 1524, 1468, 1406, 1368, 1337, 1274, 1249, 1205, 1174, 1124, 1061. HRMS (FAB) calcd for $C_{13}H_{15}NO$ [M$^+$]: 201.1154; found: 201.1157.

4.3.4. 3-(Naphthalen-1-ylamino)chloromethyl (3d). Thionyl chloride (0.015 mL, 1.99×10^{-4} mol) was added to a solution of compound **3c** (0.044 g, 2.19×10^{-4} mol) in dichloromethane (1 mL) and the reaction mixture was stirred for 22 h at room temperature. The solvent was removed under reduced pressure and the crude mixture was purified by chromatography with dichloromethane/*n*-hexane, 3:7 as the eluent, to produce compound **3d** as a colourless oil (0.015 g, 31%). TLC (chloroform/methanol, 4:6): R_f=0.71. ^{1}H NMR ($CDCl_3$, 300 MHz): δ=2.20–2.30 (m, 2H, $NCH_2\mathit{CH_2}CH_2$), 3.54 (t, J=6.9 Hz, 2H, $NCH_2CH_2\mathit{CH_2}$), 3.75 (t, J=6.0 Hz, 2H, $N\mathit{CH_2}CH_2\mathit{CH_2}$), 4.30–4.70 (br s, 1H, NH), 6.68 (d, J=7.5 Hz, 1H, 4-H), 7.26 (d, J=8.1 Hz, 1H, 2-H), 7.37 (t, J=7.5 Hz, 1H, 3-H), 7.42–7.50 (m, 2H, 6-H and 7-H), 7.78–7.86 (m, 2H, 8-H and 5-H). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C=31.7 ($NCH_2\mathit{CH_2}CH_2$), 41.3 ($NCH_2CH_2\mathit{CH_2}$), 49.2 ($N\mathit{CH_2}CH_2CH_2$), 104.4 (C-4), 117.6 (C-2), 119.7 (C-5), 123.4 (C-4a), 124.7 (C-7), 125.8 (C-6), 126.5 (C-3), 128.7 (C-8), 134.3 (C-8a), 142.9 (C-1). IR (neat, cm^{-1}): ν=3440, 3054, 2959, 2926, 1623, 1582, 1480, 1409, 1377, 1345, 1279, 1254, 1219, 1121. HRMS (FAB) calcd for $C_{13}H_{14}N^{35}Cl$ [M$^+$]: 219.0815; found 219.0811. Calcd for $C_{13}H_{14}N^{37}Cl$ [M$^+$]: 221.0785; found: 221.0795.

4.4. General method for the synthesis of fluorescently labelled L-amino acids 5a,b, 7a,b and 8

Carboxylic acid derivative Bza–OH (**1b**) was reacted with L-valine or L-glycine methyl ester hydrochloride **4a** and **4b**, respectively, in DMF by a standard DCC/HOBt coupling. After evaporation of the solvent and dry chromatography, the required derivatives Bza–Val–OMe (**5a**) and Bza–Gly–OMe (**5b**) were obtained. *N-tert*-Butyloxycarbonylvaline, Boc–Val–OH (**6a**) or *N-tert*-butyloxycarbonyl glycine, Boc–Gly–OH (**6b**) was reacted in the same conditions with fluorophores Bzh–H (**1c**) and Bzn–H (**1e**) to give compounds Boc–Val–Bzh (**7a**) and Boc–Gly–Bzh (**7b**) and Boc–Val–Bzn (**8**), respectively.

4.4.1. Bza–Val–OMe (5a). The product of the reaction of Bza–OH (**1b**) (0.043 g, 1.10×10^{-4} mol) with H–Val–OMe (**4a**) (0.042 g, 2.48×10^{-4} mol) was chromatographed using dichloromethane/methanol, 5.5:0.5 as the eluent, to produce Bza–Val–OMe (**5a**) (0.043 g, 75%). Mp=188.0–190.0 °C. TLC (dichloromethane/methanol, 5.5:0.5): R_f=0.39. ^{1}H NMR ($CDCl_3$, 300 MHz): δ=0.80–1.0 (m, 6H, γ-CH_3 Val), 1.40 (t, J=6.9 Hz, 3H, $NCH_2\mathit{CH_3}$), 1.90–2.0 (m, 1H, β-CH Val), 2.20–2.30 (m, 2H, $NHCH_2\mathit{CH_2}CH_2$), 2.36 (s, 3H, CH_3), 2.64 (t, J=5.7 Hz, 2H, $NH\mathit{CH_2}CH_2CH_2$), 3.02 (s, 3H, OCH_3), 3.30–3.50 (m, 2H, $NH\mathit{CH_2}CH_3$), 3.73 (br s, 1H, α-CH Val), 3.80–3.90 (m, 2H, $NHCH_2CH_2\mathit{CH_2}$), 6.0 (br s, 1H, α-NH Val), 6.46 (s, 1H, 8-H), 6.94 (s, 1H, 6-H), 7.47 (s, 1H, 11-H), 7.76–7.80 (m, 2H, 2-H and 3-H), 8.75–8.85 (m, 1H, 1-H), 9.0–9.10 (m, 1H, 4-H), 11.43 (br s, 1H, NH). ^{13}C NMR (CD_3OD, 75.4 MHz): δ_C=14.1 ($NHCH_2\mathit{CH_3}$ and γ-CH_3 Val), 17.7 (CH_3), 23.4 ($NHCH_2\mathit{CH_2}CH_2$), 30.6 ($NH\mathit{CH_2}CH_2CH_2$), 35.6 (OCH_3), 38.7 ($NH\mathit{CH_2}CH_3$), 44.6 ($NHCH_2CH_2\mathit{CH_2}$), 58.0 (α-CH Val), 93.7 (C-6 and C-8), 123.5 (Ar–C), 124.2 (C-4), 125.1 (Ar–C), 125.5 (C-1), 129.2 (C-10), 130.5 (C-2), 131.3 (C-11 and Ar–C), 131.6 (C-3), 135.2 (Ar–C), 146.8 (Ar–C), 151.3 (Ar–C), 153.4 (C-9), 158.3 (C-5), 166.6 (CONH), 172.6 ($\mathit{C}O_2CH_3$). IR (Nujol, cm^{-1}): ν=3408, 2954, 2924, 2854, 1742, 1639, 1592, 1463, 1377, 1318. HRMS (FAB) calcd for $C_{29}H_{36}N_4O_4$ [M$^+$]: 504.2737; found: 504.2718.

4.4.2. Bza–Gly–OMe (5b). The product of the reaction of Bza–OH (**1b**) (0.057 g, 1.46×10^{-4} mol) with H–Gly–OMe (**4b**) (0.055 g, 4.38×10^{-4} mol) was chromatographed using dichloromethane/methanol, 5.5:0.5 as the eluent, to produce the amide Bza–Gly–OMe (**5b**) (0.066 g, 98%). Mp=160.0–162.0 °C. TLC (dichloromethane/methanol, 5.5:0.5): R_f=0.56. ^{1}H NMR ($CDCl_3$, 300 MHz): δ=1.71 (br s, 3H, $NCH_2\mathit{CH_3}$), 2.02–2.21 (m, 2H, $NHCH_2\mathit{CH_2}CH_2$,), 2.36 (s, 3H, CH_3), 2.60–2.72 (m, 4H, $NHCH_2\mathit{CH_2}CH_2$ and $NH\mathit{CH_2}CH_2CH_2$), 3.07 (s, 3H, OCH_3), 3.40–3.60 (m, 2H, $NH\mathit{CH_2}CH_3$), 3.60–3.70 (m, 2H, CH_2 Gly), 3.90–4.10 (2H, m, $NHCH_2CH_2\mathit{CH_2}$), 6.0 (br s, 1H, α-NH Gly), 6.60 (1H, br s, 8-H), 7.0 (1H, br s, 6-H), 7.40 (1H, br s, 11-H), 7.70 (1H, br s, 3-H), 7.80 (1H, br s, 2-H), 8.40 (1H, br s, 1-H), 8.80 (1H, br s, 4-H), 11.60 (1H, br s, NH). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C=14.1 ($NHCH_2\mathit{CH_3}$ and CH_3), 24.2 ($NHCH_2\mathit{CH_2}CH_2$), 29.6 ($NH\mathit{CH_2}CH_2CH_2$), 33.3 (OCH_3), 40.0 ($NH\mathit{CH_2}CH_3$), 41.2 (CH_2 Gly), 47.4 ($NHCH_2CH_2\mathit{CH_2}$), 93.2 (C-6), 93.7 (C-8), 124.0 (Ar–C), 125.57 (C-4), 126.1 (Ar–C), 127.0 (C-1), 129.3 (C-10), 130.6 (C-2), 130.9 (C-3), 131.6 (C-11 and Ar–C), 139.9 (Ar–C), 144.8 (Ar–C), 151.3 (Ar–C), 151.5 (C-9), 157.0 (C-5), 166.7 (CONH), 173.6 ($\mathit{C}O_2CH_3$). IR (Nujol, cm^{-1}): ν=3400, 2954, 2924, 2854, 1742, 1657, 1463, 1377, 1302. HRMS (FAB) calcd for $C_{26}H_{29}N_4O_4$ [M$^+$]: 461.2189; found: 461.2194.

4.4.3. Boc–Val–Bzh (7a). The product of the reaction of Boc–Val–OH (**6a**) (0.020 g, 9.0×10^{-5} mol) with Bzh–H (**1c**) (0.039 g, 1.08×10^{-4} mol) was chromatographed using dichloromethane/methanol, mixtures of increasing polarity as the eluent, to produce the ester Boc–Val–Bzh (**7a**) (0.033 g, 65%). Mp=above 300 °C. TLC (dichloromethane/methanol, 5.5:0.5): R_f=0.45. ^{1}H NMR (CD_3OD, 300 MHz): δ=0.90–1.0 (m, 6H, γ-CH_3 Val), 1.47 (s, 3H, NCH_2CH_3), 1.40 (t, J=7.2 Hz, 2H, $NHCH_2CH_3$), 1.42 (s, 9-H, $C(CH_3)_3$), 2.0–2.20 (m, 1H, β-CH Val), 2.26 (t, J=6.3 Hz, 2H, $NHCH_2CH_2CH_2$), 2.34 (s, 3H, CH_3), 3.54 (q, J=7.2 Hz, 2H, $NHCH_2CH_3$), 3.80–3.90 (2H, m, $NHCH_2CH_2CH_2$), 3.95 (d, J=5.7 Hz, 1H, α-CH Val), 4.30–4.50 (2×m, 2H, $NHCH_2CH_2CH_2$), 6.79 (s, 1H, 8-H), 6.95 (s, 1H, 6-H), 7.61 (s, 1H, 11-H), 7.83 (d, J=8.4 Hz, 1H, 2-H), 7.92 (d, J=6.9 Hz, 1H, 3-H), 8.30 (d, J=8.1 Hz, 1H, 1-H), 8.84 (d, J=7.8 Hz, 1H, 4-H). ^{13}C NMR (CD_3OD, 75.4 MHz): δ_C=14.1 ($NHCH_2CH_3$), 17.6 (γ-CH_3 Val), 18.5 (γ-CH_3 Val), 28.7 ($C(CH_3)_3$), 28.9 (β-CH Val), 31.6 ($NHCH_2CH_2CH_2$), 39.7 ($NHCH_2CH_2CH_2$), 42.7 ($NHCH_2CH_3$), 60.9 ($NHCH_2CH_2CH_2$), 63.6 (α-CH Val), 80.5 ($C(CH_3)_3$), 94.1 (C-6), 94.5 (C-8), 118.7 (C-10), 123.7 (C-4), 124.9 (Ar–C), 125.7 (C-1), 129.0 (Ar–C), 130.8 (C-2), 132.8 (C-11 and Ar–C), 133.0 (C-3), 134.5 (Ar–C), 150.0 (Ar–C), 153.2 (Ar–C), 156.9 (C-9), 157.0 ($COC(CH_3)_3$), 158.8 (C-5), 174.0 (CO). IR (Nujol, cm^{-1}): ν=3375, 2954, 2924, 2854, 1717, 1610, 1592, 1563, 1537, 1513, 1458, 1397, 1377, 1366, 1259, 1243, 1326, 1306, 1291, 1213, 1193. HRMS (FAB) calcd for $C_{32}H_{41}N_4O_5$ [M^+]: 561.3077; found: 561.3073.

Boc–Val–Bzh (**7a**) was also prepared by the reaction of Bzh–Cl (**1d**) with Boc–Val–OH (**6a**), using the following procedure. To a solution Bzh–Cl (**1d**) (0.03 g, 7.88×10^{-5} mol) in DMF (2 mL), potassium fluoride (0.013 g, 2.24×10^{-4} mol) and Boc–Val–OH (**6a**) were added with stirring at room temperature. The reaction mixture was maintained in these conditions for 3 days and monitorised by TLC (chloroform/methanol, 5.5:0.5). The solvent was evaporated until dryness, and the residue was purified by dry chromatography using chloroform/methanol, 5.6:0.4 as the eluent. Boc–Val–Bzh (**7a**) was obtained as a blue solid (0.026 g, 60%) and the spectral data confirmed its structure.

4.4.4. Boc–Gly–Bzh (7b). The product of the reaction of Boc–Gly–OH (**6a**) (0.024 g, 1.38×10^{-4} mol) with Bzh–H (**1c**) (0.060 g, 1.66×10^{-4} mol) was chromatographed using dichloromethane/methanol, mixtures of increasing polarity as the eluent, to produce the ester Boc–Gly–Bzh (**7b**) (0.046 g, 64%). Mp=above 300 °C. TLC (dichloromethane/methanol, 5.5:0.5): R_f=0.50. ^{1}H NMR ($CDCl_3$, 300 MHz): δ=1.36 (t, J=6.9 Hz, 3H, NCH_2CH_3), 1.44 (s, 9-H, $C(CH_3)_3$), 2.10–2.21 (m, 2H, $NHCH_2CH_2CH_2$), 2.29 (s, 3H, CH_3), 3.38 (br s, 2H, $NHCH_2CH_3$), 3.70 (br s, $NHCH_2CH_2CH_2$, 2H), 3.87–4.0 (m, 4H, CH_2 Gly and $NHCH_2CH_2CH_2$), 4.30 (br s, 1H, α-NH Gly), 6.42 (s, 1H, 8-H), 6.50 (s, 1H, 6-H), 7.60–7.70 (m, 2H, 11-H and NH), 7.71–7.80 (m, 2H, 2-H and 3-H), 8.60–8.70 (m, H, 4-H and 1-H). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C=14.0 ($NHCH_2CH_3$), 28.3 ($C(CH_3)_3$), 29.7 ($NHCH_2CH_2CH_2$), 38.9 ($NHCH_2CH_2CH_2$), 41.4 (CH_2 Gly), 42.5 ($NHCH_2CH_3$), 62.5 ($NHCH_2CH_2CH_2$), 80.0 ($C(CH_3)_3$), 92.6 (C-6), 93.5 (C-8), 123.4 (C-10), 124.2 (C-4), 126.6 (C-1), 128.5 (Ar–C), 130.1 (Ar–C), 130.6 (C-2), 131.4 (C-11 and Ar–C), 131.7 (C-3), 133.8 (Ar–C), 147.1 (Ar–C), 151.0 (Ar–C), 154.2 (C-9), 156.0 ($COC(CH_3)_3$), 157.4 (C-5), 170.5 (CO). IR (Nujol, cm^{-1}): ν=3363, 2927, 2855, 1715, 1644, 1592, 1455, 1367, 1315, 1163, 1056, 1012. HRMS (FAB) calcd for $C_{29}H_{35}N_4O_5$ [M^+]: 519.2607; found: 519.2621.

4.4.5. Boc–Val–Bzn (8). The product of the reaction of Boc–Val–OH (**6a**) (0.021 g, 9.87×10^{-5} mol) with Bzn–H (**1e**) (0.030 g, 9.87×10^{-5} mol) was chromatographed using dichloromethane/methanol, 5.5:0.5 as the eluent, to produce Boc–Val–Bzn (**8**) (0.026 g, 52%). Mp=above 300 °C. TLC (dichloromethane/methanol, 5.5:0.5): R_f=0.32. ^{1}H NMR ($CDCl_3$, 300 MHz): δ=1.10 (d, J=6.3 Hz, 3H, γ-CH_3 Val), 1.15 (d, J=6.6 Hz, 3H, γ-CH_3 Val), 1.14 (br s, 3H, NCH_2CH_3), 1.53 (s, 9-H, $C(CH_3)_3$), 1.80–2.60 (2×m, 6H, β-CH Val, $NHCH_2CH_3$ and CH_3), 4.54 (br s, 1H, α-CH Val), 6.02 (br s, 2H, 8-H and 6-H), 7.80 (br s, 1H, 11-H), 8.80 (m, 2H, 3-H and 2-H), 9.0 (br s, 2H, 4-H and 1-H). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C=13.8 ($NHCH_2CH_3$), 18.2 (CH_3), 19.2 (γ-CH_3 Val), 28.3 ($C(CH_3)_3$), 30.2 (β-CH Val), 40.0 ($NHCH_2CH_3$), 61.7 (α-CH Val), 80.7 ($C(CH_3)_3$), 94.1 (C-6 and C-8), 123.4 (C-4), 124.2 (C-1), 124.4 (C-10), 128.9 (C-2), 130.3 (2×Ar–C), 130.5 (C-11), 132.2 (C-3), 136.7 (Ar–C), 152.8 (2×Ar–C), 154.9 (C-9), 156.8 (C-5), 158.9 ($COC(CH_3)_3$), 168.1 (CONH). IR (Nujol, cm^{-1}): ν=3326, 2954, 2924, 2854, 1693, 1642, 1590, 1555, 1504, 1462, 1455, 1377, 1366, 1318, 1281, 1255, 1164, 1139, 1087, 1072. HRMS (FAB) calcd for $C_{29}H_{35}N_4O_{54}$ [M^+]: 503.2658; found: 503.2663.

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References and notes

1. Sevestre, A.; Charmantray, F.; Hélaine, V.; Lásiková, A.; Hecquet, L. *Tetrahedron* **2006**, *62*, 3969.
2. Neuhaus, W.; Trzeciak, J.; Lauer, R.; Lachmann, B.; Noe, C. R. *J. Pharm. Biomed. Anal.* **2006**, *40*, 1035.
3. Barazzoni, A. M.; Clavenzani, P.; Chiocchetti, R.; Bompadre, G. A.; Grandis, A.; Petrosino, G.; Costerbosa, G. L.; Bortolami, R. *Res. Vet. Sci.* **2005**, *78*, 249.
4. Krayl, M.; Guiard, B.; Paal, K.; Voos, W. *Anal. Biochem.* **2006**, *355*, 81.
5. Haugland, R. P. *Handbook of Fluorescent Probes and Research Products*, 9th ed.; Molecular Probes: Eugene, OR, 2002.
6. Fabian, J.; Hartmann, H. *Light Absorption of Organic Colorants*; Springer: Berlin, 1980.
7. Westman, G.; Jennsiche, E.; Hamberger, A. Eur. Patent 104210A1, 2003.
8. Frangioni, J. V.; Onishi, S. U.S. Patent 082423A2, 2005.
9. Yan, X.; Miragila, S.; Yuan, P. M. U.S. Patent 6,140,500, 2000.
10. Simmonds, A.; Miller, J. N.; Moody, C. J.; Swann, E.; Briggs, M. S. J.; Bruce, I. Eur. Patent WO 97/29154, 1997.
11. Theodoropulos, S. U.S. Patent 4,873,318, 1989.
12. Briggs, M. S. J.; Bruce, I.; Miller, J. N.; Moody, C. J.; Simmonds, A. C.; Swann, E. *J. Chem. Soc., Perkin Trans. 1* **1997**, 1051.

13. Gatti, R.; Gioia, M. G.; Andreatta, P.; Pentassuglia, G. *J. Pharm. Biomed. Anal.* **2004**, *35*, 339.
14. Gatti, R.; Gioia, M. G.; Di Pietra, A. M. *Anal. Chim. Acta* **2002**, *474*, 11.
15. Fraga, S. M. B.; Gonçalves, M. S. T.; Moura, J. C. V. P.; Rani, K. *Eur. J. Org. Chem.* **2004**, *8*, 1750.
16. Piloto, A. M.; Fonseca, A. S. C.; Costa, S. P. G.; Gonçalves, M. S. T. *Tetrahedron* **2006**, *62*, 9258.
17. Frade, V. H. J.; Gonçalves, M. S. T.; Coutinho, P. J. G.; Moura, J. C. V. P. *J. Photochem. Photobiol.* **2006**. doi: 10.1016/j.jphotochem.2006.06.013.
18. Frade, V. H. J.; Gonçalves, M. S. T.; Moura, J. C. V. P. *Tetrahedron Lett.* **2006**, *47*, 8567.
19. Crossley, M. L.; Turner, R. J.; Hofmann, C. M.; Dreisbach, P. F.; Parker, R. P. *J. Am. Chem. Soc.* **1952**, *74*, 578.
20. Tjoeng, F. S.; Heavner, G. A. *Synthesis* **1981**, 897.
21. Nasr, C.; Hotchandani, S. *Chem. Mater.* **2000**, 1529.
22. Sens, R.; Drexhage, K. H. *J. Lumin.* **1981**, *24*, 709.
23. Perrin, D. D.; Dempsey, B. *Buffers for pH and Metal Ion Control*; Chapman and Hall: London, 1974.
24. Valeur, B. *Molecular Fluorescence—Principles and Applications*; Wiley-VCH: Weinheim, 2002.