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# Synthesis of short and long-wavelength functionalised probes: amino acids' labelling and photophysical studies

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**Abstract**—Fluorescent labelling of α-amino acids at their N or C terminals in the main and lateral chains at short and long wavelengths was carried out in different ways. The *N*-[3-(naphthalen-1-ylamino)propanoyl]amino acid methyl esters synthesised showed strong fluorescence in the visible region (~415 nm) of the electromagnetic spectrum. Condensation of these compounds with 5-diethylamino-2-nitrosophenol or 5-ethylamino-4-methyl-2-nitrosophenol produced the benzo[*a*]phenoxazine derivatives, with maximum emission wavelengths shifted to values higher than 644 nm. The synthesis of novel functionalised 5,9-diaminobenzo[*a*]phenoxazinium salts, by reaction of 5-ethylamino-4-methyl-2-nitrosophenol and *N*-substituted 1-naphthylamine and their use in the covalent labelling of the N or C terminals of valine, produced derivatives with long-wavelength emissions (644–653 nm). Photophysical studies using the synthesised compounds both in different solvents and in controlled pH were undertaken. Preliminary evaluation of photostability of the cationic polycyclic heterocycles in ethanol and water at physiological pH was also performed.


## 1. Introduction

Fluorescent probes for labelling of biomolecules have been widely used for analytical purposes in many areas of contemporary science. The label molecule may contain a reactive functional group for a covalent linkage to the analyte. Alternatively, a non-functionalised marker may possess a strong non-covalent interaction with the biomolecule for the formation of a stable complex. Despite the fact that the interactions resulting from the latter methodology are significantly less stable than the covalent attachment, it is also extremely important.

Covalent labelling at short wavelengths (<500 nm) of biomolecules has been achieved by using heterocycles, such as oxobenzopyranes,[1] oxobenzo[*f*]benzopyranes,[2] naphthofuranes,[3] oligothiophenes,[4] 4,7-phenanthroline-5,6-dione[5] and benzoxadiazoles.[6] The use of naphthalene derivatives, such as dansyl chloride[7,8] and naphthalene 2,3-dicarboxaldehyde,[9,10] has also been reported. Fluorescein[11] and rhodamine based labels[12] produced fluorescent bioconjugates in the range of about 500–600 nm. Examples of long-wavelength fluorophores (>600 nm) include squaraine,[13,14] cyanine[15,16] as well as benzo[*a*]phenoxazine heterocycles.[17,18]

Despite the interest of all these compounds, the use of labels that absorb and fluoresce in the red (600–700 nm) or near-infrared (>700 nm) regions of the electromagnetic spectrum is valuable. Considering the low interference from solvents and the biological matrix, the far-wavelength detection is the most advantageous.

Bearing this in mind and in connection with our interest in the synthesis, characterisation and application of fluorescent labels,[2,19,20] we have extended our preliminary work[21] related to the fluorescent derivatisation of biomolecules through the preparation of *N*-[3-(naphthalen-1-ylamino)propanoyl]amino acid methyl esters and their conversion into the corresponding benzo[*a*]phenoxazinium conjugates through condensation with 5-diethylamino-2-nitrosophenol or 5-ethylamino-4-methyl-2-nitrosophenol.

Maximum emission wavelengths were shifted from about 415 nm, in the former fluorescent derivatives, to values higher than 644 nm in the latter. Another approach for long-wavelength fluorescent labelling was also performed through the synthesis of novel functionalised 5,9-diaminobenzo[*a*]phenoxazinium salts through the reaction of 5-ethylamino-4-methyl-2-nitrosophenol with *N*-substituted 1-naphthylamine, and their use in the covalent labelling of

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the N or C terminals of valine. Photophysical characterisation of the synthesised compounds in different solvents and in controlled pH was undertaken. The last objective of this research was to study the behaviour of the cationic polycyclic heterocycles synthesised when exposed to ultraviolet and visible radiation. Thus, preliminary evaluation of their photostability at 350 and 419 nm was also carried out.

## 2. Results and discussion

3-(Naphthalen-1-ylamino)propanoic acid, Nap-OH (**1a**)[19] was synthesised by alkylation of 1-naphthylamine with methyl-3-bromopropionate, followed by hydrolysis (NaOH/1,4-dioxane) of the isolated ester intermediate—methyl 3-(naphthalen-1-ylamino)propanoate (**1b**). Compound **1a** was used in the covalent derivatisation of the α-amine group of L-alanine methyl ester (**2a**), by coupling with *N*,*N*′-dicyclohexylcarbodiimide (DCC) assisted by 1-hydroxybenzotriazole (HOBt) under standard conditions. To extend this research to other residues, a set of representative α-amino acid methyl esters, namely valine, glycine, phenylalanine and glutamic acid (protected at its carboxyl side-chain as methyl ester) were used in the same conditions (Scheme 1 and Table 1, entries 2–5). In addition to labelling amino acids at their *N*-terminus, the alternative acylation at a lysine ω-amine group was also investigated. Thus, the methyl ester of *N*-acetyl-L-lysine (**4**) was reacted with **1a** under the conditions reported above to give the expected fluorescent derivative **5** (Scheme 1 and Table 1, entry 6).

After purification by chromatography on silica gel, the corresponding conjugates **3a–e** and **5** were obtained as oils or oily solid (**3b**) in yields ranging from 85 to 98% (Table 1, entries 1–6) and were characterised by high resolution mass spectrometry, NMR ($^1$H and $^{13}$C) and IR spectroscopies.

The IR spectra of labelled compounds showed bands due to stretching vibrations of the carbonyl group from 1627 to 1651 cm$^{-1}$ (amide linkage) and from 1726 to 1750 cm$^{-1}$ (ester group).

**Table 1**. Synthesis of labelled amino acid derivatives **3a–e**, **5**, **8a–f** and **9**

| Entry | | Compound | Yield (%) |
|---|---|---|---|
| 1 | **3a** | Nap-Ala-OMe | 87 |
| 2 | **3b** | Nap-Val-OMe | 85 |
| 3 | **3c** | Nap-Gly-OMe | 90 |
| 4 | **3d** | Nap-Phe-OMe | 85 |
| 5 | **3e** | Nap-Glu(OMe)-OMe | 95 |
| 6 | **5** | Ac-Lys(Nap)-OMe | 98 |
| 7 | **8a** | Bfa-Ala-OMe | 96 |
| 8 | **8b** | Bfe-Ala-OMe | 57 |
| 9 | **8c** | Bfe-Val-OMe | 78 |
| 10 | **8d** | Bfe-Gly-OMe | 67 |
| 11 | **8e** | Bfe-Phe-OMe | 76 |
| 12 | **8f** | Bfe-Glu(OMe)-OMe | 68 |
| 13 | **9** | Ac-Lys(Bfe)-OMe | 88 |

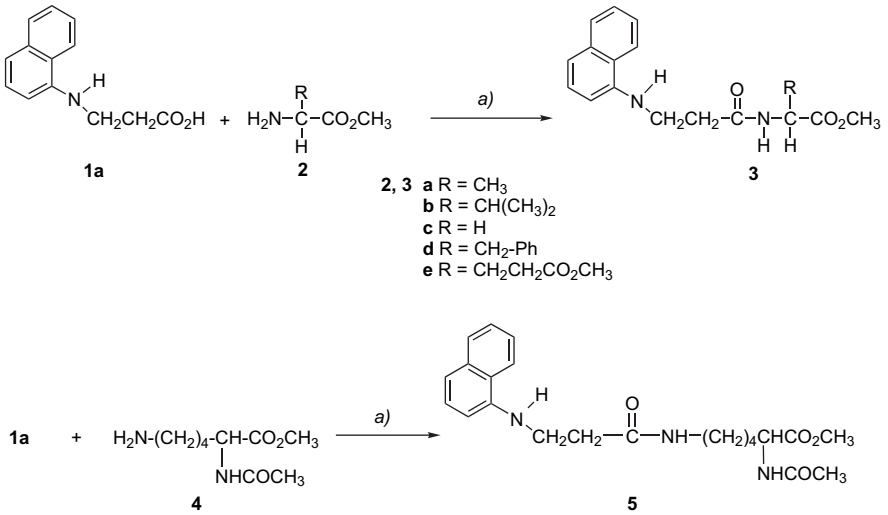
$^1$H NMR spectra showed signals of the amino acid residues, such as a singlet for the methyl ester protons (δ 3.69–3.74 ppm), a multiplet for the α-CH (δ 4.50–4.95 ppm, except for **3c**, which was a doublet at 4.03 ppm) and a doublet or broad singlet (**3c**) for α-NH (δ 6.24–6.78 ppm), in addition to the protons of the naphthalene moiety.

In $^{13}$C NMR, signals of amide type carbonyl were found from δ 170.24 to 172.17 ppm and those of the ester type occurred from δ 171.89 to 173.44 ppm.

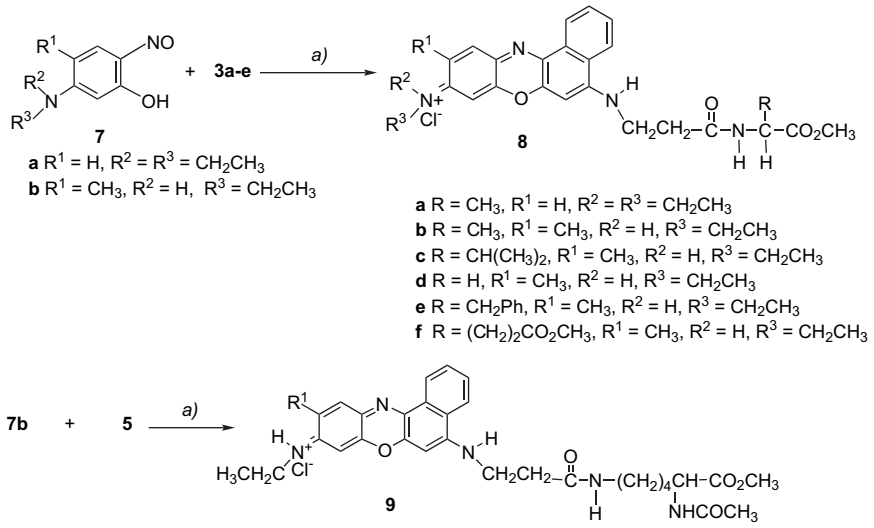
As was expected, and in accordance with the results obtained in the photophysical study, which will be discussed latter, labelled amino acid derivatives **3a–e** and **5** showed maximum absorbance and fluorescence inferior to 331 nm and at about 415 nm, respectively.

Considering the importance of long-wavelength fluorescent labelling, we decided to study the possibility of red shifting the fluorescence of the previously synthesised amino acid derivatives **3a–e** and **5** by using them in the preparation of the corresponding polycyclic systems based on the benzo[*a*]phenoxazine nucleus.

From the few synthetic methodologies for the preparation of benzo[*a*]phenoxazinium salts, which have been




**Scheme 1**. Synthesis of labelled amino acid derivatives **3a–e** and **5**. *Reagents and condition*: (a) DCC, HOBt, DMF, rt.




**Scheme 2**. Synthesis of benzo[*a*]phenoxazinium chlorides **8a–f** and **9**. *Reagents and condition*: (a) $H^+$/methanol, reflux.

reported,[22,17] the most applied synthetic protocol is condensation, in the presence of a strong mineral acid, of nitrosoanilines or nitrosonaphthylamines with the appropriate 1-naphthylamine, 3-aminophenol or 2-naphthol. Following this methodology, compounds **3a–e** and **5** were reacted with 5-diethylamino-2-nitrosophenol (**7a**) or 5-ethylamino-4-methyl-2-nitrosophenol (**7b**), using concentrated hydrochloric acid (Scheme 2). The required 5-alkylamino-2-nitrosophenol hydrochloride **7a** and **7b** was synthesised using the usual procedure[23] involving treatment of the corresponding 3-alkylaminophenol with sodium nitrite in an acid solution.

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*-ethyl-*N*-{5-[3-(1-methoxy-1-oxopropan-2-ylamino)-3-oxopropylamino]-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride (**8a**) is Bfa-Ala-OMe.

Reaction of the nitrosophenol **7a** with the alanine derivative **3a** (reflux in methanol) produced conjugate Bfa-Ala-OMe (**8a**). Starting from the nitroso compound **7b** and using derivative **3a** once again, Bfe-Ala-OMe (**8b**) was obtained. Through the comparison of fluorescent data obtained for these derivatives, which will be discussed later, it was concluded that 5-ethylamino-4-methyl-2-nitrosophenol (**7b**) produced the conjugate with superior fluorescence properties. As a result, precursor **7b** was used as the nitroso component in the cyclisation reactions with derivatives **3b–e** and **5** (Scheme 2).

After purification by dry chromatography, compounds **8a–f** and **9** were isolated as blue solids in yields ranging from 57 to 96% (Table 1, entries 7–13) and were fully characterised using the usual analytical techniques.

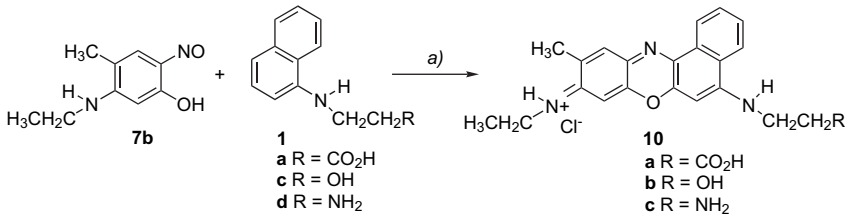
The IR spectra of labelled compounds showed bands due to the stretching vibrations of the carbonyl group from 1633 to 1659 $cm^{-1}$ (amide linkage) and from 1720 to 1746 $cm^{-1}$ (ester group), as happened in the case of compounds **3a–e** and **5**.

$^1H$ NMR spectra showed signals of the naphthalene ring and the amino acid residues (coming from derivatives **3a–e** and **5**), as well as the aliphatic and aromatic protons resulting from the introduction of the new unity. Thus, the protons of methyl groups ($R^1$) appeared as a singlet ($\delta$ 2.25–2.40 ppm) and the protons of the ethyl groups ($R^2$ and $R^3$), namely $CH_3$ as a multiplet (**8a**, **8e** and **9**), broad singlet (**8b**) or triplet (**8c**, **8d** and **8f**) ($\delta$ 1.23–1.60 ppm) and $CH_2$ as a multiplet or a broad singlet (**8b**) ($\delta$ 3.0–3.80 ppm). The aromatic protons, such as H-10 occurred as a double doublet ($\delta$ 6.97 ppm) (**8a**), H-8 as a singlet or doublet ($J$=2.4 Hz) ($\delta$ 6.18–6.62 ppm) and H-11 as a singlet or doublet (**8a**) ($\delta$ 7.36–7.77 ppm).

In $^{13}C$ NMR, we highlighted the signals of the aliphatic carbons of the heterocyclic, namely the methyl ($\delta$ 17.64–18.36 ppm) and ethyl groups ($CH_3$ $\delta$ 12.61–14.16 ppm and $CH_2$ $\delta$ 37.54–45.89 ppm), as well as the aromatic signals of C-6 ($\delta$ 92.81–94.03 ppm), C-8 ($\delta$ 93.24–95.60 ppm) and C-11 ($\delta$ 131.06–132.58 ppm). Signals of the carbonyl group were found from $\delta$ 170.65 to 171.97 ppm (amide type) and from $\delta$ 171.87 to 173.51 ppm (ester bond).

The design of new probes is determined by the spectral properties of the material to be marked. To obtain fluorophores with the adequate characteristics for the non-covalent or covalent labelling of biological material, as was purpose of this work, one of the following possibilities must be considered. The first is the use of labels with high lifetimes (>100 ns). In this case, the detection is initiated after a certain time of decay so as to eliminate the intrinsic fluorescence of the biological material. The other possibility is labelling using fluorophores possessing wavelengths of excitation and emission, which are considerably superior to that of the background. The auto-fluorescence of the biological material is particularly high at wavelengths inferior to 500 nm and reduced at wavelengths higher than 600 nm.

On the other hand, the number of fluorescent probes, which absorb and fluoresce in the region of 600–1000 nm is low




**Scheme 3**. Synthesis of benzo[*a*]phenoxazinium chlorides **10a–c**. *Reagents and conditions*: (a) $H^+$/DMF (70 °C, compound **10a**) or methanol (reflux, compounds **10b**,**c**).

and it is even lower in a suitable functional group for covalent linkage of the analyte.

Considering these facts, we decided to synthesise new functionalised benzo[*a*]phenoxazinium salts analogous to Nile Blue as well as to study the possibility of covalent bonding of these fluorophores with organic molecules of biological interest, using L-valine as a model.

Benzo[*a*]phenoxazinium chlorides **10a–c** were synthesised by condensation of 5-ethylamino-2-nitrosophenol hydrochloride **7b** with *N*-alkylated-naphthylamines **1a** and **1c**,**d** in the presence of hydrochloric acid; heating occurred at 70 °C (**10a**)[19] or reflux in ethanol (**10b** and **10c**) (Scheme 3). Intermediates **1c** and **1d** were prepared by alkylation of 1-naphthylamine with the appropriate chloro-derivative, 2-chloroethanol and 2-chloroethylamine monohydrochloride, respectively. Preparation of intermediate **1a** was previously described.

After dry chromatography purification compounds **1c** and **1d** were obtained as an oil (**1c**, 24%) or as a solid (**1d**, 71%), and they were characterised by high resolution mass spectrometry, NMR ($^1$H and $^{13}$C) and IR spectroscopies. The IR spectra showed strong stretching vibration bands for the hydroxyl and amine groups between 3055 and 3389 $cm^{-1}$.

In the $^1$H and $^{13}$C NMR spectra, signals of *N*-substituent protons occurred at $\delta$ 3.38–4.02 ppm and $\delta$ 39.62–61.13 ppm, respectively, in addition to the presence of naphthalene signals.

Dry chromatography purification or successive washes with solvents (**10c**) produced benzo[*a*]phenoxazinium chlorides **10a–c** as blue solids in yields ranging from 45 to 98% (Table 2). These polycyclic functionalised heterocycles were fully characterised by the usual analytical techniques.

$^1$H NMR spectra showed signals of the aliphatic protons of methyl groups, such as a singlet ($\delta$ 2.16–2.44 ppm) and ethyl groups, namely $CH_3$ as a triplet or a broad singlet (**10b**) ($\delta$ 1.36–1.43 ppm) and $CH_2$ as doublet (2×d) (**10a**), broad singlet (**10b**) or multiplet (**10c**) ($\delta$ 3.51–3.78 ppm), and $NCH_2$-$CH_2$ as a triplet or a broad singlet (**10b**) ($\delta$ 2.71–4.08 ppm).

**Table 2**. Synthesis of benzo[*a*]phenoxazinium chlorides **10a–c** and labelled amino acid derivatives **8c**, **12** and **13**

| Entry | | Compound | Yield (%) |
|---|---|---|---|
| 1 | **10a** | Bfe-OH | 45 |
| 2 | **10b** | Bfh-H | 95 |
| 3 | **10c** | Bfn-H | 98 |
| 4 | **8c** | Bfe-Val-OMe | 92 |
| 5 | **12** | Boc-Val-OBfh | 94 |
| 6 | **13** | Boc-Val-OBfn | 89 |

The highlighted aromatic protons are H-8 (singlet, $\delta$ 6.42–7.02 ppm), H-6 (singlet, $\delta$ 6.70–7.17 ppm) and H-11 (singlet or multiplet (**10c**), $\delta$ 7.19–7.96 ppm).

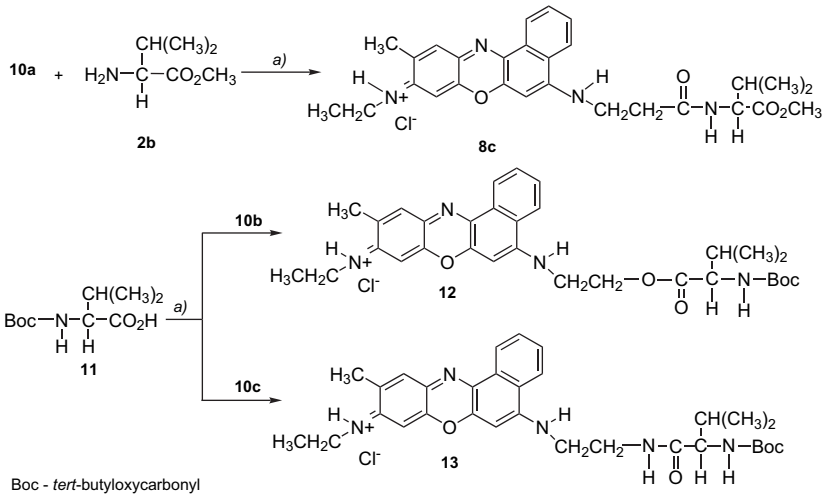
The $^{13}$C NMR spectra of compounds **10a–c** also confirmed the presence of the methyl group and the *N*-substituents, with signals assigned to the methyl groups ($\delta$ 17.77–18.47 ppm), ethyl groups ($CH_3$, $\delta$ 14.17–14.51 ppm and $CH_2$, $\delta$ 39.70 and 48.03 ppm) and $NCH_2CH_2$ ($\delta$ 34.93–60.98 ppm). Concerning the aromatic carbons, signals of C-8 ($\delta$ 93.92–94.60 ppm), C-6 ($\delta$ 94.40–95.17 ppm) and C-11 ($\delta$ 132.60–133.71 ppm) were highlighted. In the case of compound **10a**, a signal at $\delta$ 164.30 ppm was assigned to the carbonyl function.

Compounds **10a–c**, functionalised charged heterocycles, have the potential ability for covalent labelling applications, in addition to their non-covalent labelling capacity. In this work, the possibility of using these compounds in covalent labelling of biomolecules was carried out, using L-valine as a model.

Dyes **10a–c** were linked to the α-amine group of the L-valine methyl ester, H-Val-OMe (**2b**) or the carboxylic acid of *N*-*tert*-butyloxycarbonyl-L-valine Boc-Val-OH (**11**), by coupling with the aid of *N*,*N*′-dicyclohexylcarbodiimide (DCC) assisted by 1-hydroxybenzotriazole (HOBt), under standard conditions. Thus, reaction of the carboxylic acid dye Bfe-OH (**10a**) with H-Val-OMe (**2b**) produced the amide derivative Bfe-Val-OMe (**8c**), which was also obtained using the first labelling methodology described. When Boc-Val-OH (**11**) was coupled to the hydroxyl dye, Bfh-H (**10b**), the ester conjugate Boc-Val-OBfh (**12**) was isolated. Derivatisation of L-valine (Boc-Val-OH, **11**) with the amino fluorophore Bfn-H (**10c**) resulted in the fluorescent derivative Boc-Val-OBfn (**13**) (Scheme 4).

After dry chromatography purification, the L-valine conjugates **8c**, **12** and **13** were obtained as blue solids in yields ranging from 89 to 94% (Table 2) and were characterised by high resolution mass spectrometry, NMR ($^1$H and $^{13}$C) and IR spectroscopies. Data related to compound **8c** were previously discussed.

The IR spectra of conjugates **12** and **13** showed bands due to the stretching vibrations of the carbonyl group of the ester and the amide functions at 1743 $cm^{-1}$ and at about 1643 $cm^{-1}$, respectively.




**Scheme 4**. Synthesis of labelled amino acid derivatives **8c**, **12** and **13**. *Reagents and condition*: (a) DCC, HOBt, DMF, rt.

$^1$H NMR spectra of these compounds showed signals of the valine amino acid, such as a singlet for the methyl protons of the Boc group ($\delta$ 1.37 ppm), a multiplet ($\delta$ 3.95–4.10 ppm, **12**) or a doublet ($\delta$ 3.90 ppm, **13**) for the α-CH, as well as the protons of the label moiety. In $^{13}$C NMR, signals of the carbonyl were found at about $\delta$ 158 ppm (urethane type), $\delta$ 173.98 ppm (ester bond, **12**) and $\delta$ 176.36 ppm (amide type, **13**).

The electronic absorption spectra of $10^{-5}$ to $10^{-6}$ M solutions of label molecules, and the corresponding conjugates synthesised (**1a**, **3a–e**, **5**, **8a–f**, **9**, **10a–c**, **12** and **13**) in degassed absolute ethanol, were measured. Summarised data of this study are presented in Table 3. Regarding compounds **1a**, **3a–e** and **5**, which absorbed in the ultraviolet region of the electromagnetic spectrum, as was expected, the longest wavelength of maximum absorption ($\lambda_{max}$) being ~330 or 320 nm (**3d**, entry 5), with molar absorptivities between 3139 (**3b**, entry 3) and 10,682 $M^{-1}\,cm^{-1}$ (**3a**). Comparison of $\lambda_{max}$ values of derivatives **3a–e** (entries 2–6) and **5** (entry 7) with compound **1a** (entry 1) showed that the presence of the amino acid residues did not produce alteration in their value or was negligible (**1a**, 330 nm/**3d**, 320 nm).

**Table 3**. UV/visible data for compounds **1a**, **3a–e**, **5**, **8a–f**, **9**, **10a–c**, **12** and **13** in ethanol and water (pH 7.4)

| Entry | | Compound | Ethanol $\lambda_{abs}$ [nm] ($\varepsilon$) | Water (pH 7.4) $\lambda_{abs}$ [nm] ($\varepsilon$) |
|---|---|---|---|---|
| 1 | **1a** | Nap-OH | 330 (7000) | — |
| 2 | **3a** | Nap-Ala-OMe | 331 (10,682) | — |
| 3 | **3b** | Nap-Val-OMe | 330 (3139) | — |
| 4 | **3c** | Nap-Gly-OMe | 330 (5960) | — |
| 5 | **3d** | Nap-Phe-OMe | 320 (3596) | — |
| 6 | **3e** | Nap-Glu(OMe)-OMe | 330 (7442) | — |
| 7 | **5** | Ac-Lys(Nap)-OMe | 330 (7000) | — |
| 8 | **8a** | Bfa-Ala-OMe | 640 (22,115) | 650 (11,299) |
| 9 | **8b** | Bfe-Ala-OMe | 630 (23,558) | — |
| 10 | **8c** | Bfe-Val-OMe | 630 (39,815) | 625 (34,184) |
| 11 | **8d** | Bfe-Gly-OMe | 630 (32,283) | 625 (14,970) |
| 12 | **8e** | Bfe-Phe-OMe | 630 (40,945) | 625 (21,778) |
| 13 | **8f** | Bfe-Glu(OMe)-OMe | 630 (30,500) | 625 (24,500) |
| 14 | **9** | Ac-Lys(Bfe)-OMe | 630 (42,857) | 630 (27,439) |
| 15 | **10a** | Bfe-OH | 625 (26,364) | 625 (12,945) |
| 16 | **10b** | Bfh-H | 625 (26,479) | 619 (21,667) |
| 17 | **10c** | Bfn-H | 617 (22,400) | 620 (17,389) |
| 18 | **12** | Boc-Val-OBfh | 629 (22,646) | 626 (20,175) |
| 19 | **13** | Boc-Val-OBfn | 630 (27,917) | 627 (15,252) |

It is known that the photophysics of naphthylamines in aqueous solutions is strongly pH-dependent,[24] through acid–base equilibria both in the excited and ground states. In order to see if the amino acid substitution had some effect on the corresponding p$K_a$ values, some preliminary studies were undertaken with compound **3a** (solutions of methanol/buffers, 0.02:0.98). From the normalised fluorescence spectra, in Figure 1, it can be seen that only at pH=3 the protonated form started to appear through a structured emission to the blue side of the spectrum. In DMAN (*N*,*N*-dimethyl-1-aminonaphthalene) at pH=3, the fluorescence spectrum was dominated by the acidic form.[24] Thus, the p$K_a$ in the excited state of **3a** was much lower than the reported value of 2.5 for DMAN. This can be partly explained by the nearby carbonyl group, which, through hydrogen bond interaction, probably forms a six-membered ring with the amino group.

The transformation of derivatised amino acids **3a–e** and **5**, by condensation with the appropriate nitrosophenol, into

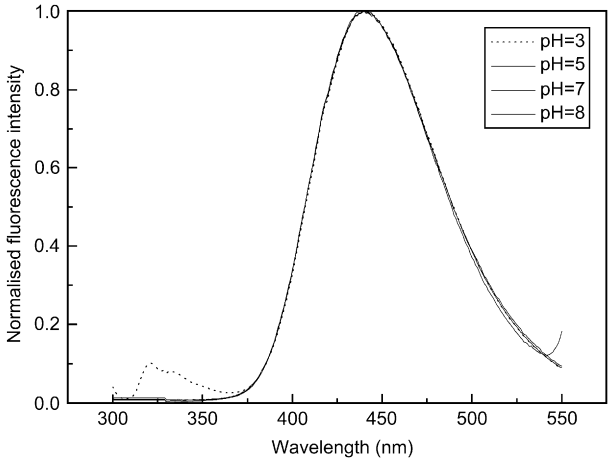



**Figure 1**. Normalised fluorescence intensity spectra of compound **3a** at different pH values.

the corresponding benzo[*a*]phenoxazine polycyclic derivatives **8a–f** and **9** shifted the maximum absorbance to 630 and 640 nm (**8a**), with molar absorptivities between 22,115 and 42,857 $M^{-1}\,cm^{-1}$.

The comparison of the maximum absorption wavelengths of the two groups of labelled amino acids **3a–e** and **5** (entries 2–7)/**8a–f** and **9** (entries 8–14), in Table 3, reveals that a bathochromic shift from 299 nm (**3a/8b** alanine) to 310 nm (**3d/8e** phenylalanine) has occurred. Thus, for example, it was possible to obtain derivatives of phenylalanine with absorption at 320 and 630 nm. Functionalised benzo[*a*]phenoxazinium salts **10a–c** also presented high maximum absorption wavelengths (617 nm—**10c** and 625 nm), as well as molar absorptivities from 22,400 (**10c**, entry 17) to 26,479 $M^{-1}\,cm^{-1}$ (**10b**, entry 16).

When compared to **10a–c** (entry 2, entries 15–17), the labelled derivatives of L-valine (**8c**, **12** and **13**, entries 10, 18 and 19) absorb at slightly longer wavelengths, the bathochromic shift is between 4 and 13 nm, the most accentuated being for compounds **10c/13** (617/630 nm).

In order to be closer to the biological environment, the absorption properties of all the above compounds (**8a–f**, **9**, **10a–c**, **12** and **13**) were also studied in water at physiological pH (pH 7.4, adjusted with HCl and NaOH). As was expected, compounds **1a**, **3a–e** and **5** were not soluble in water (pH 7.4) (Table 3).

Considering compounds **8a–f** and **9** (entries 8–14) in ethanol and water (pH 7.4), $\lambda_{max}$ values were equal (**9**) or only a slight alteration occurred (5 or 10 nm). Despite the fact that a red shift tendency was observed for compound **8a** (640 nm, in ethanol/650 nm, in water), a blue shift predominated in the other compounds (**8c–f**).

When compared to ethanol, in aqueous solution, the $\lambda_{max}$ of compounds **10a–c**, **12** and **13** (entries 15–19) showed no variation (**10a**) or only a slight hypsochromic (**10b**, **12** and **13**) or bathochromic shift (**10c**) occurred. Figure 2 shows normalised absorption spectra of compounds **10b**,**c**, **12** and **13** in water. The shoulder seen in the blue region was due to H-aggregate formation in water solutions.[19] It was possible to conclude that this aggregation increased from **10b** to **12** but decreased from **10c** to **13**.

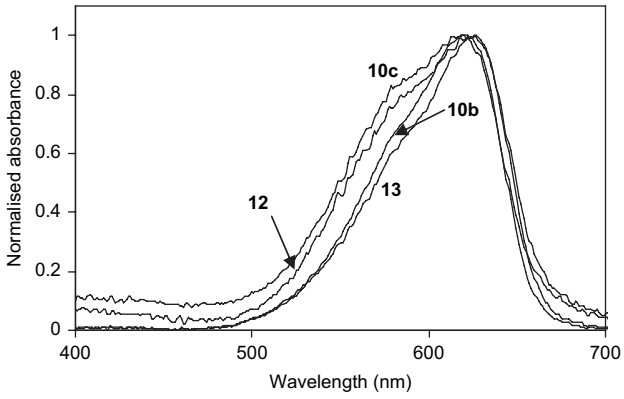



**Figure 2**. Normalised absorbance spectra of compounds **10b**,**c**, **12** and **13** in water (pH 7.4).

Studies of the fluorescent properties of compounds **1a**, **3a–e**, **5**, **8a–f**, **9**, **10a–c**, **12** and **13** were also carried out in ethanol and water (pH 7.4). Maximum excitation ($\lambda_{ex}$) and emission ($\lambda_{em}$) wavelengths, as well as fluorescence quantum yields ($\Phi_F$) for all compounds were obtained and are presented in Table 4. For the determination of $\Phi_F$, 9,10-diphenylanthracene ($\Phi_F$=0.95 in ethanol,[25] **1a**, **3a–e** and **5**) or Oxazine 1 were used as standards ($\Phi_F$=0.11 in ethanol,[26] **8a–f**, **9**, **10a–c**, **12** and **13**) and they were excited at the $\lambda_{ex}$ of each of the compounds tested. Compounds **1a**, **3a–e** and **5** (entries 1–7) exhibited emission maxima in the range of 414–417 nm, with high Stokes' shifts (84–95 nm) and $\Phi_F$ between 0.54 and 0.66, in ethanol.

**Table 4**. Fluorescence data for compounds **1a**, **3a–e**, **5**, **8a–f**, **9**, **10a–c**, **12** and **13** in ethanol and water (pH 7.4)

| Entry | | Compound | Ethanol | | | | Water (pH 7.4) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $\lambda_{ex}$[a] | $\lambda_{em}$[a] | $\Phi_F$ | DS[b] | $\lambda_{ex}$[a] | $\lambda_{em}$[a] | $\Phi_F$ | DS[b] |
| 1 | **1a** | Nap-OH | 330 | 417 | 0.54 | 87 | — | — | — | — |
| 2 | **3a** | Nap-Ala-OMe | 331 | 415 | 0.66 | 84 | — | — | — | — |
| 3 | **3b** | Nap-Val-OMe | 330 | 415 | 0.55 | 85 | — | — | — | — |
| 4 | **3c** | Nap-Gly-OMe | 330 | 417 | 0.55 | 87 | — | — | — | — |
| 5 | **3d** | Nap-Phe-OMe | 330 | 415 | 0.55 | 95 | — | — | — | — |
| 6 | **3e** | Nap-Glu(OMe)-OMe | 330 | 414 | 0.57 | 84 | — | — | — | — |
| 7 | **5** | Ac-Lys(Nap)-OMe | 330 | 417 | 0.54 | 87 | — | — | — | — |
| 8 | **8a** | Bfa-Ala-OMe | 600 | 672 | 0.18 | 32 | 580 | 684 | 0.13 | 34 |
| 9 | **8b** | Bfe-Ala-OMe | 590 | 644 | 0.39 | 14 | — | — | — | — |
| 10 | **8c** | Bfe-Val-OMe | 590 | 644 | 0.40 | 14 | 580 | 653 | 0.26 | 28 |
| 11 | **8d** | Bfe-Gly-OMe | 600 | 644 | 0.37 | 14 | 590 | 652 | 0.45 | 27 |
| 12 | **8e** | Bfe-Phe-OMe | 580 | 644 | 0.45 | 14 | 590 | 657 | 0.40 | 32 |
| 13 | **8f** | Bfe-Glu(OMe)-OMe | 600 | 644 | 0.48 | 14 | 600 | 652 | 0.16 | 27 |
| 14 | **9** | Ac-Lys(Bfe)-OMe | 590 | 645 | 0.32 | 15 | 590 | 654 | 0.46 | 24 |
| 15 | **10a** | Bfe-OH | 590 | 644 | 0.44 | 19 | 580 | 652 | 0.28 | 27 |
| 16 | **10b** | Bfh-H | 590 | 643 | 0.49 | 18 | 590 | 651 | 0.36 | 32 |
| 17 | **10c** | Bfn-H | 590 | 647 | 0.27 | 30 | 590 | 647 | 0.18 | 27 |
| 18 | **12** | Boc-Val-OBfh | 590 | 652 | 0.32 | 23 | 590 | 652 | 0.26 | 26 |
| 19 | **13** | Boc-Val-OBfn | 590 | 653 | 0.35 | 23 | 590 | 653 | 0.48 | 26 |

[a] Units in nm.
[b] Stokes' shift (nm).

Comparison of $\lambda_{em}$ and $\Phi_F$ values of derivatives **3a–e** and **5** with compound **1a** showed that the presence of the amino acid residues did not produce alteration or increased the $\Phi_F$ value, the highest being for derivative **3a** ($\Phi_F$=0.66).

Despite the importance of fluorescent derivatisation in the UV/visible region, the possibility of labelling at long wavelengths (>600 nm) is extremely interesting, mainly in biological applications. The fluorescent amino acid derivatives **8a–f** and **9** are included in the latter category, revealing $\lambda_{em}$ between 644 and 684 nm in ethanol and water (pH 7.4).

When compared to ethanol, in aqueous solutions at physiological pH, the $\lambda_{em}$ showed a red shift (8–13 nm) for all compounds. Despite their low Stokes' shifts, the best values occurred in aqueous solution (34 nm, **8a**). The highest $\lambda_{em}$ of the all dyes studied either in ethanol or water is related to compound **8a** (672 nm ethanol, 684 nm water).

Compounds **8a–f** and **9** exhibited moderate to high $\Phi_F$ in both solvents ($0.18<\Phi_F<0.48$, ethanol and $0.13<\Phi_F<0.46$, water).

Comparison of the maximum emission wavelengths of the two groups of labelled amino acids **3a–e** and **5/8a–f** and **9** reveals that a bathochromic shift from 173 nm (**5/9** lysine) to 257 nm (**3a/8a** alanine) has occurred. Thus, for example, it was possible to obtain alanine derivatives with emission at 415 and 672 nm.

Regarding fluorophores **10a–c** and the labelled L-valine **8c**, **12** and **13**, in ethanol and water (pH 7.4), $\lambda_{em}$ was located between 643 and 653 nm with low Stokes' shifts (14–32 nm), but superior in water (with the exception of compound **10c**). When compared to ethanol, in aqueous solutions at physiological pH, $\lambda_{em}$ showed no variation (**10c**, **12** and **13**) or a slight red shift (8–9 nm, **8c**, **10a** and **10b**) for all compounds.

Through the comparison of $\lambda_{max}$ values of labelled valine and fluorophores **10a–c**, it was verified that the presence of the amino acid residue did not produce a considerable alteration in their values, showing a bathochromic shift in pairs **10b/12** (643/652 nm) and **10c/13** (647/653 nm), in ethanol or in both solvents, respectively. Figure 3 shows the normalised fluorescence spectra of compounds **10b**,**c** and **13** in water (pH 7.4).

Regarding the $\Phi_F$ values of compounds **10a–c**, **8c**, **12** and **13** in the solvents used ($0.18<\Phi_F<0.49$), it was possible to conclude that the highest values were found in ethanol (with the exception of compound **13**, 0.35 ethanol/0.48 water).

By comparison of $\Phi_F$ values of labelled derivatives **8c**, **12** and **13** with compounds **10a–c** in both solvents, it was verified that there was a decrease in pairs **10a/8c** and **10b/12**, the most important being in the last case and in ethanol (**10b/12**, 0.49/0.32); in pair **10c/13** an increase occurred, which was higher in water (**10c/13**, 0.18/0.48). The higher quantum yield of compound **13** can be explained by the previously mentioned decrease in the formation of H-aggregates, which are non-fluorescent.

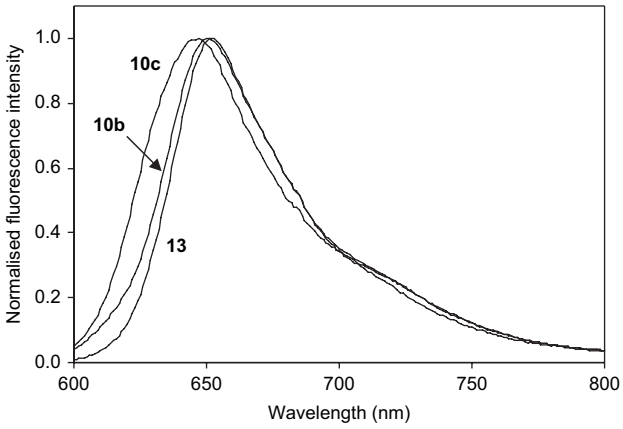



**Figure 3**. Normalised fluorescence intensity spectra of compounds **10b**,**c** and **13** in water (pH 7.4).

In order to further elucidate the photophysical alterations of these types of compounds upon coupling to amino acids, studies in various aqueous buffers at different pH as well as in various organic solvents were conducted. As in our previous study,[20] with the increase of pH, a slight increase of H-aggregate formation was observed as well as a tendency for the decrease of the maximum molar absorptivity (Table 5). While compound **10c** retained its pH-dependency upon amino acid linkage, the same behaviour was not observed for compound **10b**. For all compounds tested the fluorescence quantum yields did not vary significantly with pH.

**Table 5**. Variation of photophysical properties with pH of compounds **10b**,**c**, **12** and **13**

| pH | 10b | | 10c | | 12 | | 13 | |
|---|---|---|---|---|---|---|---|---|
| | $\varepsilon/\varepsilon^a$ | $\Phi_F/\Phi_F{}^b$ | $\varepsilon/\varepsilon^a$ | $\Phi_F/\Phi_F{}^b$ | $\varepsilon/\varepsilon^a$ | $\Phi_F/\Phi_F{}^b$ | $\varepsilon/\varepsilon^a$ | $\Phi_F/\Phi_F{}^b$ |
| 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 5 | 0.898 | 1.013 | 0.778 | 1.020 | 0.845 | 0.919 | 1.309 | 0.933 |
| 7 | 0.791 | 1.080 | 0.723 | 1.131 | 0.780 | 0.974 | 1.097 | 1.042 |
| 8 | 0.697 | 1.082 | 0.604 | 1.201 | 0.656 | 1.035 | 1.019 | 1.026 |

[a] $\varepsilon$ value at pH 3.
[b] $\Phi_F$ value at pH 3.

In a previous study,[19] we reported that, in organic solvents, the benzo[*a*]phenoxazine compounds are sensitive to the proton acceptance capability of the solvent through a deprotonation process. In relation to the cationic acidic form, the absorption and the fluorescence of the basic form appeared shifted to the blue approximately 100 and 50 nm, respectively, with a 10-fold decrease in quantum yield. The ratio of acid and basic forms thus depended on the solvent, but also on the substituent groups at the benzo[*a*]phenoxazine. The same behaviour was observed in compounds **10b**,**c**, **12** and **13**. Comparing **10c** and **13**, a very similar ratio of acidic and basic forms was detected in the studied solvents with compound **12** showing a much larger fraction of the basic form when compared to **10b** in the same solvent. As an example, in Figure 4, the absorption spectra in acetone are shown.

In order to confirm that the magnitude of the slight red shift of the normal cationic form absorption with the polarity of the solvent was maintained upon amino acid linkage, Lippert–Mataga plots[27] were constructed for the absorption

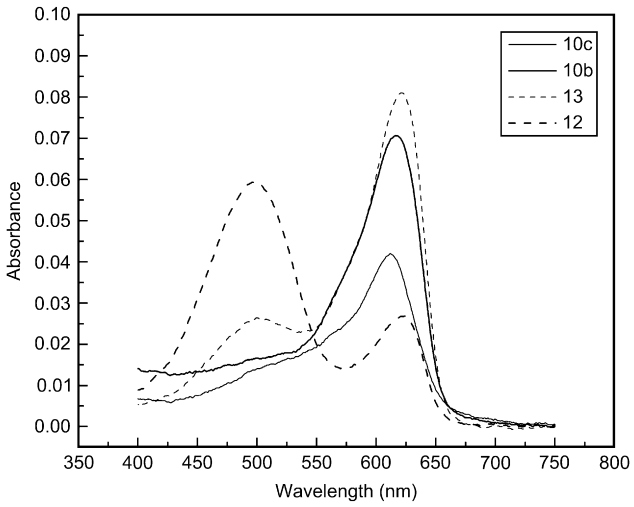



**Figure 4**. Absorbance spectra of compounds **10b**,**c**, **12** and **13** in acetone.

maximum wavenumber, $\tilde{\nu}_{abs}$ (Fig. 5). The use of absorption maxima instead of the usual difference between absorption and emission can be justified by the fact that the basic form was more separated from the cationic acidic one in absorption spectra than the corresponding emission. The expression used is given below:[27]

$$\tilde{\nu}_{abs} = -\frac{2}{hc}\vec{\mu}_g \cdot (\vec{\mu}_e - \vec{\mu}_g) a^{-3} \Delta f + \text{const}$$

where $\vec{\mu}_g$ and $\vec{\mu}_e$ are the ground state and excited state dipole moments, $a$ is the molecular radius and $\Delta f$ is the solvent orientation polarizability, which is given by:

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

where $\varepsilon$ and $n$ are, respectively, the solvents' dielectric constant and refractive index. The solvents used were water, methanol, acetone and dichloromethane in which the acidic form absorption was well resolved and separated from the basic form contribution. It was concluded that the variation in the dipole moment, $\Delta\vec{\mu} = \vec{\mu}_e - \vec{\mu}_g$, was the same for all compounds.

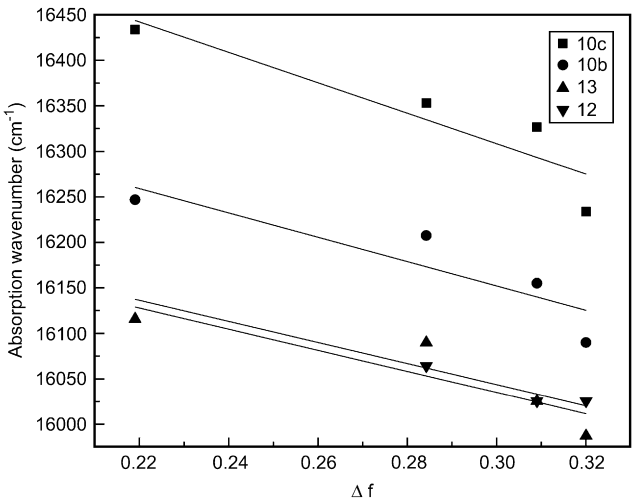



**Figure 5**. Lippert–Mataga plot of maximum absorption wavenumbers of compounds **10b**,**c**, **12** and **13**.

Long-wavelength fluorophores with high fluorescence efficiency and good stability are required in several applications. Bearing this in mind, our final purpose was the investigation of the photostability of fluorophores **10a–c**, in comparison with the commercial analogue Nile Blue, under irradiation in the ultraviolet and visible regions. In this study, solutions of compounds **10a–c** and Nile Blue, in ethanol or water at physiological pH ($C$=1×10$^{-5}$ M), were irradiated at 350 and 419 nm in a Rayonet RPR-100 reactor.

After 2 h of irradiation at 419 nm, in ethanol, the photostabilities of compounds **10a** and **10b** were similar to each other, comparable to the Nile Blue value and superior to that of compound **10c**. The values of the residual absorption were approximately 100% (**10a**,**b** and Nile Blue) and 80% (**10c**). Irradiation of the solutions for a long period of time (7 h) did not affect the photostabilities of compounds **10a**,**b** and Nile Blue, but this lowered the value of the residual absorption of fluorophore **10c** for 70%.

The photostabilities of these fluorophores by irradiation at 419 nm decreased in water (pH 7.4). After 2 h of irradiation, they presented residual absorption at about 48% (**10a**), 57% (**10b**) and 62% (**10c**). When the experiment was extended to 7 h, it was verified that the photostabilities decreased for all compounds, compound **10c** revealing the lowest value (32%, **10c**; 43%, **10a**,**b**).

Studies of the behaviour of compounds **10a–c**, in ethanol and water (pH 7.4), with irradiation at 350 nm, were also carried out. The results showed that, after 2 h of irradiation, the photostabilities of compounds **10a** and **10b** were similar to each other and, comparable to Nile Blue (≥97%), superior to that of compound **10c** in ethanol. Irradiation of solutions for 7 h did not significantly change the stability of fluorophores **10a**,**b** and Nile Blue (≥95%), and reduced the value of residual absorption of compound **10c** (66%).

In water, at physiological pH, irradiation at 350 nm showed a tendency for a decrease in the photostabilities, as previously occurred. Thus, after 2 h of irradiation, residual absorption of compounds **10a** and **10b** was the same (84%) and superior to that of compound **10c** (76%). The irradiation of solutions during 7 h reduced the photostabilities of all compounds, compounds **10a** and **10b** (61%) being similar to that of Nile Blue (66%) and inferior in the case of compound **10c** (46%).

In fact, by irradiation at 419 and 350 nm, compounds **10a** and **10b** presented photostabilities higher than those of compound **10c**, similar to that of Nile Blue, in both solvents studied.

The results obtained suggest that the photostabilities of the benzo[*a*]phenoxazinium salts studied are related with the solvent (ethanol or water at physiologic pH) as well as with the wavelength of irradiation.

There was also a relationship detected between the functional group of the lateral chain and the photostabilities of

these cationic dyes. The photostability experiments performed were only a preliminary study of the behaviour of the synthesised compounds. Further studies will be carried out for a better understanding of this correlation.

## 3. Conclusion

In this work, fluorescent conjugates of several α-amino acids were synthesised with absorption and fluorescence at low wavelengths (330 nm and 414–417 nm, respectively).

The efficient cyclisation of these compounds with an appropriate nitrosophenol generated the corresponding blue fluorescent derivatives possessing the benzo[*a*]phenoxazine nucleus, which presented maximum absorption in the region between 625 and 650 nm and strong fluorescence, with maximum emissions between 644 and 684 nm.

New water-soluble monofunctionalised benzo[*a*]phenoxazine derivatives were also synthesised and characterised. These were used in the efficient derivatisation of L-valine, through an ester or amide linkage, producing conjugates with absorption between 617 and 630 nm and emission between 644 and 653 nm, respectively.

Considering the obtained results, 3-(naphthalen-1-ylamino)-propanoic acid is a potential candidate for low-wavelength derivatisation, producing derivatives with a red shift in fluorescence for the region above 644 nm, after condensation with an appropriate nitrosophenol component. On the other hand, the new functionalised benzo[*a*]phenoxazine derivatives present the adequate structural and photophysical characteristics for their use as covalent and non-covalent labels of biomolecules.

## 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 precoated silica plates, 0.25 mm in thickness (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 at 25 °C. All chemical shifts are given in parts per million using $\delta_H$ $Me_4Si$=0 ppm as a reference and *J* values are given in Hertz. $^{13}$C NMR spectra were run on the same instrument at 75.4 MHz using the solvent peak as an internal reference. Assignments were carried out 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 spectrometry 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. Fluorescence spectra were collected using a Spex Fluorolog 1680 spectrometer.

Photostability studies were carried out using a Rayonet RPR-100 chamber reactor equipped with eight lamps of wavelengths of 350 and 419 nm (14 W each).

### 4.2. Synthesis of 3-(naphthalen-1-ylamino)propanoic acid, Nap-OH (1a)

To a solution of compound **1b**[19] (0.470 g, $2.05\times10^{-3}$ mol) in 1,4-dioxane (4 mL) 1 M NaOH (3.08 mL, $3.08\times10^{-3}$ mol) was added at low temperature. The solution was stirred at 0 °C for 6 h and acidified to pH 3 with 1 M $KHSO_4$. After extraction with chloroform and evaporation of the solvent, compound **1a** was obtained as a white solid (0.375 g, 85%). Spectroscopic data confirmed the expected structure and were in accordance with those previously obtained.[19]

### 4.3. General method for the synthesis of fluorescently labelled L-amino acids 3a–e and 5

3-(Naphthalen-1-ylamino)propanoic acid, Nap-OH (**1a**), was reacted with an amino acid methyl ester, in DMF, using a standard DCC/HOBt coupling. After evaporation of the solvent and chromatography on silica gel, the required derivative (**3a–e** and **5**) was obtained.

**4.3.1. *N*-[3-(Naphthalen-1-ylamino)propanoyl]alanine methyl ester, Nap-Ala-OMe (3a).** The product of reaction of Nap-OH (**1a**) (0.224 g, $1.04\times10^{-3}$ mol) with alanine methyl ester hydrochloride (**2a**) (0.208 g, $1.50\times10^{-3}$ mol) was chromatographed using chloroform as the eluent to give compound **3a** as a colourless oil (0.270 g, 87%). TLC (chloroform/methanol, 5.8:0.2): $R_f$=0.69. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$=1.38 (d, *J*=7.2 Hz, 3H, β-$CH_3$ Ala), 2.67 (t, *J*=6.2 Hz, 2H, $NHCH_2CH_2$), 3.66 (t, *J*=5.7 Hz, 2H, $NHCH_2CH_2$), 3.74 (s, 3H, $OCH_3$), 4.56–4.67 (m, 1H, α-CH Ala), 5.16 (br s, 1H, NH), 6.24 (d, *J*=6.6 Hz, 1H, α-NH Ala), 6.66 (d, *J*=7.5 Hz, 1H, 4-H), 7.27 (d, *J*=8.1 Hz, 1H, 2-H), 7.36 (t, *J*=7.5 Hz, 1H, 3-H), 7.40–7.50 (m, 2H, 6-H and 7-H), 7.76–7.82 (m, 1H, 5-H), 7.84–7.92 (m, 1H, 8-H). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=18.21 (β-$CH_3$ Ala), 35.04 ($NHCH_2CH_2$), 40.26 ($NHCH_2CH_2$), 48.03 (α-CH Ala), 52.46 ($OCH_3$), 104.56 (C-4), 117.79 (C-2), 120.18 (C-5), 123.80 (C-4a), 124.79 (C-6), 125.75 (C-7), 126.41 (C-3), 128.48 (C-8), 134.31 (C-8a), 142.85 (C-1), 171.49 (CONH), 173.44 ($CO_2CH_3$). IR (KBr 1%, $cm^{-1}$): $\nu$=3393, 3310, 3049, 2929, 2852, 1750, 1641, 1587, 1532, 1490, 1467, 1412, 1351, 1287, 1216, 1120, 983. HRMS (FAB): calcd for $C_{17}H_{20}N_2O_3$ [$M^+$]: 300.1474; found: 300.1484.

**4.3.2. *N*-[3-(Naphthalen-1-ylamino)propanoyl]valine methyl ester, Nap-Val-OMe (3b).** The product of reaction of Nap-OH (**1a**) (0.152 g, $7.07\times10^{-4}$ mol) with valine methyl ester hydrochloride (**2b**) (0.178 g, $1.06\times10^{-3}$ mol) was chromatographed using chloroform as the eluent to give compound **3b** as brownish oily solid (0.197 g, 85%). TLC (chloroform/methanol, 5.5:0.5): $R_f$=0.63. $^1$H NMR

($CDCl_3$, 300 MHz): $\delta$=0.85 (d, $J$=6.9 Hz, 3H, γ-$CH_3$ Val), 0.91 (d, $J$=6.9 Hz, 3H, γ-$CH_3$ Val), 2.08–2.20 (m, 1H, β-CH Val), 2.72 (dt, $J$=6.0 and 1.8 Hz, 2H, $NHCH_2CH_2$), 3.65 (t, $J$=6.0 Hz, 2H, $NHCH_2CH_2$), 3.72 (s, 3H, $OCH_3$), 4.17 (br s, 1H, N–H), 4.54–4.62 (m, 1H, α-CH Val), 6.38 (d, $J$=8.4 Hz, 1H, α-NH Val), 6.71 (d, $J$=7.2 Hz, 1H, 4-H), 7.29 (d, $J$=9.6 Hz, 1H, 2-H), 7.36 (t, $J$=7.5 Hz, 1H, 3-H), 7.40–7.50 (m, 2H, 6-H and 7-H), 7.76–7.84 (m, 1H, 5-H), 7.86–7.94 (m, 1H, 8-H). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=17.78 (γ-$CH_3$ Val), 18.90 (γ-$CH_3$ Val), 31.05 (β-CH Val), 35.21 ($NHCH_2CH_2$), 40.65 ($NHCH_2CH_2$), 52.19 ($OCH_3$), 57.13 (α-CH Val), 105.05 (C-4), 118.23 (C-2), 120.21 (C-8), 123.89 (C-4a), 124.91 (C-6), 125.82 (C-7), 126.38 (C-3), 128.51 (C-5), 134.32 (C-8a), 142.44 (C-1), 171.87 (CONH), 172.54 ($CO_2$ $CH_3$). IR (neat, $cm^{-1}$): $\nu$=3326, 3052, 2954, 2931, 2851, 1736, 1651, 1633, 1583, 1486, 1436, 1411, 1373, 1346, 1311, 1283, 1210, 1153, 1121, 1089, 1018, 892. HRMS (FAB): calcd for $C_{19}H_{24}N_2O_3$ [$M^+$]: 328.1787; found: 328.1796.

**4.3.3. *N*-[3-(Naphthalen-1-ylamino)propanoyl]glycine methyl ester, Nap-Gly-OMe (3c).** The product of reaction of Nap-OH (**1a**) (0.164 g, $7.63\times10^{-4}$ mol) with glycine methyl ester hydrochloride (**2c**) (0.143 g, $1.14\times10^{-3}$ mol) was chromatographed using dichloromethane/methanol, 5.8:0.2 as the eluent, to give compound **3c** as a brownish oil (0.196 g, 90%). TLC (dichloromethane/methanol, 5.5:0.5): $R_f$=0.53. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$=2.68 (t, $J$=6.0 Hz, 2H, $NHCH_2CH_2$), 3.64 (t, $J$=6.0 Hz, 2H, $NHCH_2CH_2$), 3.73 (s, 3H, $OCH_3$), 4.03 (d, $J$=5.4 Hz, 2H, $CH_2$ Gly), 6.45 (br s, 1H, α-NH Gly), 6.65 (d, $J$=7.5 Hz, 1H, 4-H), 7.26 (d, $J$=7.5 Hz, 1H, 2-H), 7.35 (t, $J$=7.5 Hz, 1H, 3-H), 7.40–7.50 (m, 2H, 6-H and 7-H), 7.72–7.81 (m, 1H, 5-H), 7.83–7.92 (m, 1H, 8-H). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=35.96 ($NHCH_2CH_2$), 40.27 ($NHCH_2CH_2$), 41.18 ($CH_2$ Gly), 52.39 ($OCH_3$), 104.63 (C-4), 117.92 (C-2), 120.20 (C-8), 123.82 (C-4a), 124.85 (C-6), 125.79 (C-7), 126.42 (C-3), 128.51 (C-5), 134.32 (C-8a), 142.80 (C-1), 170.32 (CONH), 172.11 ($CO_2CH_3$). IR (neat, $cm^{-1}$): $\nu$=3400, 3070, 2952, 2929, 2852, 1747, 1651, 1583, 1531, 1486, 1436, 1410, 1372, 1346, 1285, 1214, 1181, 1124, 1090, 1039, 984. HRMS (FAB): calcd for $C_{16}H_{18}N_2O_3$ [$M^+$]: 286.1317; found: 286.1309.

**4.3.4. *N*-[3-(Naphthalen-1-ylamino)propanoyl]phenylalanine methyl ester, Nap-Phe-OMe (3d).** The product of reaction of Nap-OH (**1a**) (0.086 g, $4.04\times10^{-4}$ mol) with phenylalanine methyl ester hydrochloride (**2d**) (0.129 g, $6.0\times10^{-4}$ mol) was chromatographed using chloroform as the eluent to give compound **3d** as a brownish oil (0.129 g, 85%). TLC (chloroform/methanol, 5.8:0.2): $R_f$=0.75. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$=2.61 (t, $J$=6.0 Hz, 2H, $NHCH_2CH_2$), 3.0–3.20 (m, 2H, β-$CH_2$ Phe), 3.55–3.65 (m, 2H, $NHCH_2CH_2$), 3.71 (s, 3H, $OCH_3$), 4.85–4.95 (m, 2H, α-CH Phe and NH), 6.35 (d, $J$=7.8 Hz, 1H, α-NH Phe), 6.62 (d, $J$=7.5 Hz, 1H, 4-H), 6.94–7.0 (m, 2H, 2-H and 6-H Phe), 7.06–7.16 (m, 3H, 3-H, 4-H and 5-H Phe), 7.28 (d, $J$=8.7 Hz, 1H, 2-H), 7.35 (t, $J$=7.5 Hz, 1H, 3-H), 7.41–7.50 (m, 2H, 6-H and 7-H), 7.80 (dd, $J$=7.2 and 2.1 Hz, 2H, 5-H and 8-H). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=35.05 ($NHCH_2CH_2$), 37.60 (β-$CH_2$ Phe), 40.16 ($NHCH_2CH_2$), 52.32 ($OCH_3$), 53.03 (α-CH Phe), 104.57 (C-4), 117.84 (C-2), 120.15 (C-5), 123.79 (C-4a), 124.81 (C-6), 125.75 (C-7), 126.39 (C-3), 127.02 (C-4 Phe), 128.47 (C-8, C-3 Phe and C-5 Phe), 129.05 (C-2 Phe and C-6 Phe), 134.30 (C-8a), 135.54 (C-1 Phe), 142.75 (C-1), 171.43 (CONH), 171.89 ($CO_2CH_3$). IR (neat, $cm^{-1}$): $\nu$=3374, 3324, 3061, 2929, 2851, 1731, 1651, 1582, 1530, 1500, 1430, 1410, 1371, 1346, 1283, 1217, 1177, 1121, 1089, 990. HRMS (FAB): calcd for $C_{23}H_{24}N_2O_3$ [$M^+$]: 376.1787; found: 376.1797.

**4.3.5. *N*-[3-(Naphthalen-1-ylamino)propanoyl]glutamic acid dimethyl ester, Nap-Glu(OMe)-OMe (3e).** The product of reaction of Nap-OH (**1a**) (0.150 g, $6.98\times10^{-4}$ mol) with glutamic acid dimethyl ester hydrochloride (**2e**) (0.222 g, $1.05\times10^{-3}$ mol) was chromatographed using chloroform as the eluent to give compound **3e** as a brownish oil (0.246 g, 95%). TLC (chloroform/methanol, 5.5:0.5): $R_f$=0.58. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$=0.82–2.00 and 2.10–2.21 (2×m, 2H, β-$CH_2$ Glu), 2.30–2.40 (m, 2H, γ-$CH_2$ Glu), 2.64 (t, $J$=6.0 Hz, 2H, $NHCH_2CH_2$), 3.53–3.59 (m, 2H, $NHCH_2CH_2$), 3.60 (s, 3H, $OCH_3$), 3.69 (s, 3H, $OCH_3$), 4.60–4.70 (m, 1H, α-CH Glu), 6.62 (d, $J$=7.2 Hz, 1H, 4-H), 6.78 (d, $J$=7.8 Hz, 1H, α-NH Glu), 7.25 (d, $J$=8.1 Hz, 1H, 2-H), 7.34 (t, $J$=7.5 Hz, 1H, 3-H), 7.40–7.46 (m, 2H, 6-H and 7-H), 7.77 (dd, $J$=8.5 and 3.6 Hz, 1H, 5-H), 7.88 (dd, $J$=9.0 and 2.7 Hz, 1H, 8-H). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=26.92 (β-$CH_2$ Glu), 29.91 (γ-$CH_2$ Glu), 35.02 ($NHCH_2CH_2$), 40.15 ($NHCH_2CH_2$), 51.58 (α-CH Glu), 51.69 ($OCH_3$), 52.41 ($OCH_3$), 104.39 (C-4), 117.62 (C-2), 120.16 (C-8), 123.71 (C-4a), 124.66 (C-7), 125.65 (C-6), 126.37 (C-3), 128.38 (C-5), 134.24 (C-8a), 142.86 (C-1), 171.93 ($CO_2CH_3$), 172.17 (CONH), 173.16 ($CO_2CH_3$ Glu main chain). IR (Nujol, $cm^{-1}$): $\nu$=3428, 3314, 2953, 2925, 2854, 1746, 1726, 1636, 1530, 1463, 1378, 1158. HRMS (FAB): calcd for $C_{20}H_{24}N_2O_5$ [$M^+$]: 372.1685; found: 372.1692.

**4.3.6. *N*-Acetyl-ω-[3-(naphthalen-1-ylamino)propanoyl]-lysine methyl ester, Ac-Lys(Nap)-OMe (5).** The product of reaction of Nap-OH (**1a**) (0.100 g, $4.65\times10^{-4}$ mol) with *N*-acetyl-lysine methyl ester hydrochloride (**4**) (0.175 g, $7.32\times10^{-4}$ mol) was chromatographed using chloroform as the eluent to give compound **5** as a brownish oil (0.182 g, 98%). TLC (chloroform): $R_f$=0.33. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$=1.21–1.35 (m, 2H, γ-$CH_2$ Lys), 1.40–1.70 (m, 4H, β-$CH_2$ and δ-$CH_2$ Lys), 1.98 (3H, s, $CH_3$), 2.61 (t, $J$=6.0 Hz, 2H, $NCH_2CH_2$), 3.15–3.30 (m, 1H, ε-$CH_2$ Lys), 3.61 (t, $J$=6.3 Hz, 1H, $NHCH_2CH_2$), 3.72 (s, 3H, $OCH_3$), 4.50–4.60 (m, 1H, α-CH Lys), 6.15 (br s, 1H, NH Lys lateral chain), 6.24 (d, $J$=7.8 Hz, 1H, α-NH Lys), 6.62 (d, $J$=7.5 Hz, 1H, 4-H), 7.24 (d, $J$=8.1 Hz, 1H, 2-H), 7.28 (t, $J$=7.5 Hz, 1H, 3-H), 7.40–7.48 (m, 2H, 6-H and 7-H), 7.74–7.80 (m, 1H, 5-H), 7.82–7.90 (m, 1H, 8-H). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=22.28 (γ-$CH_2$ Lys), 23.08 ($CH_3$), 28.58 (β-$CH_2$ Lys), 31.98 (δ-$CH_2$ Lys), 35.17 ($NHCH_2CH_2$), 38.80 (ε-$CH_2$ Lys), 40.39 ($NHCH_2CH_2$), 51.68 (α-CH Lys), 52.40 ($OCH_3$), 104.37 (C-4), 117.61 (C-2), 120.15 (C-8), 123.72 (C-4a), 124.80 (C-7), 125.78 (C-6), 126.46 (C-3), 128.50 (C-5), 134.28 (C-8a), 143.04 (C-1), 170.24 ($COCH_3$), 172.15 (CONH), 172.94 ($CO_2CH_3$). IR (Nujol, $cm^{-1}$): $\nu$=3326, 2954, 2925, 2854, 1726, 1627, 1463, 1377, 1311, 1260, 1088. HRMS (EI): calcd for $C_{22}H_{29}N_3O_4$ [$M^+$]: 399.2158; found: 399.2157.

### 4.4. General method for the synthesis of the fluorescently L-amino acids 8a–f and 9

To a cold solution (ice bath) of 5-(alkylamino)-2-nitrosophenol hydrochloride **7a**,**b** in methanol, compounds **3a**–**e** or **5** and concentrated hydrochloric acid ($5.0\times10^{-2}$ mL) were added. The mixture was refluxed during the time given below and monitored by TLC (chloroform/methanol). After evaporation of the solvent and dry chromatography on silica gel, the required derivative (**8a**–**f** and **9**) was obtained as a blue solid.

**4.4.1. *N*-Ethyl-*N*-{5-[3-(1-methoxy-1-oxopropan-2-ylamino)-3-oxopropylamino]-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride, Bfa-Ala-OMe (8a).** The product of the reaction of **7a** (0.087 g, $3.33\times10^{-4}$ mol) with **3a** (0.100 g, $3.33\times10^{-4}$ mol) (reflux time 6 h) was chromatographed with chloroform/methanol, 5.3:0.7 as the eluent, to give Bfa-Ala-OMe (**8a**) (0.152 g, 96%). Mp=101.2–103.2 °C. TLC (chloroform/methanol, 5.3:0.7): $R_f$=0.44. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$=1.23–1.45 (m, 9H, 2×$NCH_2CH_3$ and β-$CH_3$ Ala), 3.05–3.22 (m, 2H, $NHCH_2CH_2$), 3.55 (s, 3H, $OCH_3$), 3.56–3.65 (m, 6H, 2×$NCH_2CH_3$), 4.11 (t, $J$=6.6 Hz, 2H, $NHCH_2CH_2$), 4.40–4.52 (m, 1H, α-CH Ala), 6.62 (d, $J$=2.4 Hz, 1H, 8-H), 6.83 (s, 1H, 6-H), 6.97 (dd, $J$=9.3 and 2.4 Hz, 1H, 10-H), 7.77 (d, $J$=9.3 Hz, 1H, 11-H), 7.78–7.88 (m, 2H, 2-H and 3-H), 8.22 (d, $J$=7.2 Hz, 1H, α-NH Ala), 8.79 (dd, $J$=7.5 and 2.4 Hz, 1H, 1-H), 9.24 (d, $J$=7.2 Hz, 1H, 4-H). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=12.61 (2×$NCH_2CH_3$), 17.27 (β-$CH_3$ Ala), 34.67 ($NHCH_2CH_2$), 42.20 ($NHCH_2CH_2$), 45.89 (2×$NHCH_2CH_3$), 48.34 (α-CH Ala), 52.02 ($OCH_3$), 93.69 (C-6), 95.60 (C-8), 114.06 (C-10), 123.83 (C-1), 124.08 (Ar-C), 125.31 (C-4), 128.80 (Ar-C), 130.11 (C-3), 130.41 (Ar-C), 131.53 (C-2), 132.38 (C-11), 134.74 (Ar-C), 147.33 (Ar-C), 151.0 (Ar-C), 153.01 (C-9), 158.38 (C-5), 170.77 (CONH), 173.12 ($CO_2CH_3$). IR (KBr 1%, $cm^{-1}$): $\nu$=3437, 3224, 3055, 2963, 2927, 2854, 1740, 1640, 1588, 1547, 1497, 1455, 1438, 1384, 1329, 1277, 1258, 1197, 1165, 1127, 1074, 1013, 946. HRMS (FAB): calcd for $C_{27}H_{31}N_4O_4$ [$M^+$]: 475.2345; found: 475.2338.

**4.4.2. *N*-{5-[3-(1-Methoxy-1-oxopropan-2-ylamino)-3-oxopropylamino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride, Bfe-Ala-OMe (8b).** The product of the reaction of **7b** (0.0918 g, $5.1\times10^{-4}$ mol) with **3a** (0.300 g, $5.1\times10^{-4}$ mol) (reflux time 1 h and 30 min) was chromatographed with dichloromethane/methanol, 5.4:0.6 as the eluent, to give Bfe-Ala-OMe (**8b**) (0.133 g, 57%). Mp=above 300 °C. TLC (dichloromethane/methanol, 5.5:0.5): $R_f$=0.30. $^1$H NMR ($CD_3OD$, 300 MHz): $\delta$=1.41 (br s, 3H, $NCH_2CH_3$), 1.48 (d, $J$=7.2 Hz, 3H, β-$CH_3$ Ala), 2.39 (s, 3H, $CH_3$), 3.05 (br s, 2H, $NCH_2CH_2$), 3.28 (br s, 2H, $NCH_2CH_3$), 3.68 (s, 3H, $OCH_3$), 3.65–3.82 (m, 2H, $NCH_2CH_2$), 4.42–4.56 (m, 1H, α-CH Ala), 6.18 (s, 1H, 8-H), 6.35 (s, 1H, 6-H), 6.74 (br s, 1H, α-NH Ala), 7.37 (s, 1H, 11-H), 7.82 (br s, 2H, 2-H and 3-H), 8.32 (d, $J$=6.6 Hz, 1H, NH), 8.75 (br s, 1H, 1-H), 9.04 (br s, 1H, 4-H), 10.16 (br s, 1H, NH). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=13.87 ($NCH_2CH_3$), 17.42 (β-$CH_3$ Ala), 18.05 ($CH_3$), 34.21 ($NCH_2CH_2$), 38.79 ($NCH_2CH_3$), 41.98 ($NCH_2CH_2$), 48.46 (α-CH Ala), 52.21 ($OCH_3$), 92.83 (C-6), 93.27 (C-8), 123.4 (Ar-C), 124.03 (C-1), 125.31 (C-4), 127.22 (C-10), 129.98 (C-3), 130.18 (Ar-C), 130.54 (Ar-C), 131.11 (C-11), 131.67 (C-2), 133.42 (Ar-C), 146.92 (Ar-C), 150.51 (Ar-C), 154.29 (C-9), 157.24 (C-5), 170.65 (CONH), 173.17 ($CO_2CH_3$). IR (Nujol, $cm^{-1}$): $\nu$=3400, 2954, 2924, 2854, 1726, 1651, 1633, 1587, 1556, 1504, 1463, 1377, 1311. HRMS (FAB): calcd for $C_{26}H_{29}N_4O_4$ [$M^+$]: 461.2189; found: 461.2203.

**4.4.3. *N*-{5-[3-(1-Methoxy-3-methyl-1-oxobutan-2-ylamino)-3-oxopropylamino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride, Bfe-Val-OMe (8c).** The product of the reaction of **7b** (0.0423 g, $2.35\times10^{-4}$ mol) with **3b** (0.077 g, $2.35\times10^{-4}$ mol) (reflux time 11 h) was chromatographed with dichloromethane/methanol, 6.6:0.4 as the eluent, to give Bfe-Val-OMe (**8c**) (0.090 g, 78%). Mp=204.0–206.1 °C. TLC (dichloromethane/methanol, 5.5:0.5): $R_f$=0.88. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$=0.94 (d, $J$=6.9 Hz, 3H, γ-$CH_3$ Val), 0.99 (d, $J$=6.9 Hz, 3H, γ-$CH_3$ Val), 1.42 (t, $J$=7.2 Hz, 3H, $NHCH_2CH_3$), 2.12–2.26 (m, 1H, β-CH Val), 2.40 (s, 3H, $CH_3$), 3.05–3.16 (m, 2H, $NHCH_2CH_2$), 3.20–3.45 (m, 2H, $NHCH_2CH_3$), 3.57 (s, 3H, $OCH_3$), 3.72–3.85 (m, 2H, $NHCH_2CH_2$), 4.38–4.46 (m, 1H, α-CH Val), 6.26 (br s, 1H, NH), 6.27 (s, 1H, 8-H), 6.46 (s, 1H, 6-H), 7.51 (s, 1H, 11-H), 7.87 (t, $J$=8.7 Hz, 3H, 2-H, 3-H and α-NH Val), 8.78–8.90 (m, 1H, 1-H), 9.19 (br s, 1H, 4-H), 10.63 (br s, 1H, N–H). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=13.83 ($NHCH_2CH_3$), 18.09 ($CH_3$), 18.29 (γ-$CH_3$ Val), 19.11 (γ-$CH_3$ Val), 30.51 (β-CH Val), 34.28 ($NHCH_2CH_2$), 38.76 ($NHCH_2CH_3$), 42.13 ($NHCH_2CH_2$), 51.88 ($OCH_3$), 58.21 (α-CH Val), 92.81 (C-6), 93.24 (C-8), 124.0 (C-1), 125.51 (C-4), 127.41 (C-10), 129.87 (C-3), 130.25 (2×Ar-C), 130.55 (Ar-C), 131.06 (C-11), 131.66 (C-2), 133.29 (Ar-C), 146.92 (Ar-C), 150.40 (Ar-C), 154.34 (C-9), 157.16 (C-5), 171.10 (CONH), 172.09 ($CO_2CH_3$). IR (Nujol, $cm^{-1}$): $\nu$=3193, 2954, 2923, 2854, 1737, 1659, 1642, 1588, 1563, 1538, 1519, 1455, 1377, 1311, 1281, 1258, 1183, 1160, 1132, 1085, 1054. HRMS (FAB): calcd for $C_{28}H_{33}N_4O_4$ [$M^+$]: 489.2502; found: 489.2523.

**4.4.4. *N*-{5-[3-(2-Methoxy-2-oxoethylamino)-3-oxopropylamino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride, Bfe-Gly-OMe (8d).** The product of the reaction of **7b** (0.100 g, $5.59\times10^{-4}$ mol) with **3c** (0.160 g, $5.59\times10^{-4}$ mol) (reflux time 1 h) was chromatographed with chloroform/methanol, 5.5:0.5 as the eluent, to give Bfe-Gly-OMe (**8d**) (0.167 g, 67%). Mp=above 300 °C. TLC (dichloromethane/methanol, 5.5:0.5): $R_f$=0.34. $^1$H NMR ($CD_3OD$, 300 MHz): $\delta$=1.40 (t, $J$=7.2 Hz, 3H, $NHCH_2CH_3$), 2.25 (s, 3H, $CH_3$), 2.89 (br s, 2H, $NHCH_2CH_2$), 3.40–3.56 (m, 2H, $NHCH_2CH_3$), 3.73 (s, 3H, $OCH_3$), 3.93 (br s, 2H, $NHCH_2CH_2$), 4.04 (s, 2H, $CH_2$ Gly), 6.63 (s, 1H, 8-H), 6.78 (s, 1H, 6-H), 7.40 (s, 1H, 11-H), 7.62–7.84 (m, 2H, 2-H and 3-H), 8.16 (br s, 1H, 1-H), 8.50–8.68 (m, 1H, 4-H). $^{13}$C NMR ($CD_3OD$, 75.4 MHz): $\delta_C$=14.16 ($NHCH_2CH_3$), 18.36 ($CH_3$), 35.01 ($NHCH_2CH_2$), 39.81 ($NHCH_2CH_3$), 41.80 ($NHCH_2CH_2$), 41.93 ($CH_2$ Gly), 52.70 ($OCH_3$), 94.03 (C-6), 94.48 (C-8), 123.70 (Ar-C), 124.52 (C-1), 125.35 (C-4), 128.97 (Ar-C), 130.65 (C-3), 132.23 (C-10), 132.26 (Ar-C), 132.58 (C-11), 132.77 (C-2), 133.59 (Ar-C), 149.21 (Ar-C), 152.48 (Ar-C), 156.78 (C-9), 158.17 (C-5), 171.97 (CONH), 173.51 ($CO_2CH_3$). IR (Nujol, $cm^{-1}$): $\nu$=3366, 2954, 2924,

2854, 1739, 1643, 1588, 1557, 1519, 1463, 1455, 1377, 1311, 1160, 1133, 1007. HRMS (FAB): calcd for $C_{25}H_{27}N_4O_4$ [$M^+$]: 447.2032; found: 447.2037.

**4.4.5. *N*-{5-[3-(1-Methoxy-1-oxo-3-phenylpropan-2-ylamino)-3-oxopropylamino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride, Bfe-Phe-OMe (8e).** The product of the reaction of **7b** (0.144 g, $7.98\times10^{-4}$ mol) with **3d** (0.300 g, $7.98\times10^{-4}$ mol) (reflux time 1 h and 30 min) was chromatographed with chloroform/methanol, 5.8:0.2 as the eluent, to give Bfe-Phe-OMe (**8e**) (0.323 g, 76%). Mp=230.1–232.2 °C. TLC (dichloromethane/methanol, 5.5:0.5): $R_f$=0.56. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$=1.23–1.40 (m, 3H, NHCH$_2$*CH*$_3$), 2.40 (s, 3H, CH$_3$), 3.0 (br s, 2H, β-CH$_2$ Phe), 3.02–3.21 (m, 2H, NHCH$_2$*CH*$_2$), 3.22–3.40 (m, 2H, NH*CH*$_2$CH$_3$), 3.53 (s, 3H, OCH$_3$), 3.68–3.90 (m, 2H, NH*CH*$_2$CH$_2$), 4.70–4.80 (m, 1H, α-CH Phe), 5.96 (br s, 1H, α-NH Phe), 6.37 (s, 1H, 8-H), 6.55 (s, 1H, 6-H), 7.0–7.30 (m, 5H, 5×Ar-H Phe), 7.57 (s, 1H, 11-H), 7.80–7.95 (m, 1H, 2-H), 7.96–8.05 (m, 1H, 3-H), 8.80–8.90 (m, 1H, 1-H), 9.26 (s, 1H, 4-H), 10.73 (s, 1H, N–H). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=13.93 (NHCH$_2$*CH*$_3$), 17.86 (CH$_3$), 29.68 (β-CH$_2$ Phe), 34.43 (NHCH$_2$*CH*$_2$), 37.54 (NH*CH*$_2$CH$_3$), 38.77 (NH*CH*$_2$CH$_2$), 52.15 (OCH$_3$), 54.24 (α-CH Phe), 93.08 (C-6), 93.46 (C-8), 123.66 (Ar-C), 124.11 (C-1), 125.67 (C-4), 126.18 (Ar-C), 126.67 (C-10), 126.77 (C-4 Phe), 128.40 (C-3 and C-5 Phe), 129.19 (C-2 and C-4 Phe), 129.94 (Ar-C), 130.25 (C-3), 130.63 (Ar-C), 131.22 (C-11), 131.82 (C-2), 134.15 (C-1 Phe), 146.89 (Ar-C), 150.78 (Ar-C), 153.97 (C-9), 157.64 (C-5), 170.83 (CONH), 171.87 (*C*O$_2$CH$_3$). IR (KBr, cm$^{-1}$): $\nu$=3500, 2932, 2853, 1720, 1650, 1644, 1586, 1544, 1515, 1451, 1316, 1136, 1010. HRMS (FAB): calcd for $C_{32}H_{33}N_4O_4$ [$M^+$]: 537.2502; found: 537.2501.

**4.4.6. *N*-{5-[3-(1,5-Dimethoxy-1,5-dioxopentan-2-ylamino)-3-oxopropylamino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride, Bfe-Glu(OMe)-OMe (8f).** The product of the reaction of **7b** (0.077 g, $4.30\times10^{-4}$ mol) with **3e** (0.160 g, $4.30\times10^{-4}$ mol) (reflux time 3 h and 30 min) was chromatographed with chloroform/methanol, 5.3:0.7 as the eluent, to give Bfe-Glu(OMe)-OMe (**8f**) (0.156 g, 68%). Mp=above 300 °C. TLC (chloroform/methanol, 5.4:0.6): $R_f$=0.33. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$=1.40 (t, $J$=7.2 Hz, 3H, NHCH$_2$*CH*$_3$), 2.25 (br s, 2H, β-CH$_2$ Glu), 2.36 (s, 1H, CH$_3$), 2.42–2.52 (m, 2H, γ-CH$_2$ Glu), 3.0–3.20 (m, 2H, NHCH$_2$*CH*$_2$), 3.25–3.40 (m, 2H, NH*CH*$_2$CH$_3$), 3.59 (s, 3H, OCH$_3$ Glu), 3.61 (s, 3H, OCH$_3$ Glu), 3.70–3.80 (m, 2H, NH*CH*$_2$CH$_2$), 4.45–4.60 (m, 1H, α-CH Glu), 5.80 (br s, 1H, NH), 6.20 (s, 1H, 8-H), 6.38 (s, 1H, 6-H), 6.75 (br s, 1H, NH), 7.36 (s, 1H, 11-H), 7.75–7.85 (m, 2H, 3-H and 2-H), 8.45 (d, $J$=7.5 Hz, 1H, α-NH Glu), 8.71 (br s, 1H, 1-H), 8.98 (br s, 1H, 4-H). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=13.90 (NHCH$_2$*CH*$_3$), 16.57 (β-CH$_2$ Glu), 17.87 (CH$_3$), 30.23 (γ-CH$_2$ Glu), 34.31 (NHCH$_2$*CH*$_2$), 38.79 (NH*CH*$_2$CH$_3$), 42.01 (NH*CH*$_2$CH$_2$), 51.67 (α-CH Glu), 52.04 (OCH$_3$), 52.28 (OCH$_3$), 92.97 (C-6), 93.38 (C-8), 123.54 (Ar-C), 124.09 (C-1), 124.93 (C-4), 130.10 (Ar-C), 130.17 (C-3), 130.59 (C-10), 131.21 (Ar-C), 131.63 (C-11), 131.75 (C-2), 133.85 (Ar-C), 148.57 (Ar-C), 150.73 (Ar-C), 154.13 (C-9), 157.54 (C-5), 171.13 (CONH), 171.97 (*C*O$_2$CH$_3$), 173.0 (*C*O$_2$CH$_3$). IR (Nujol, cm$^{-1}$): $\nu$=3417, 2954, 2924, 2854, 1726, 1720, 1651, 1556, 1504, 1463, 1377, 1150, 1000. HRMS (FAB): calcd for $C_{29}H_{33}N_4O_6$ [$M^+$]: 533.2400; found: 533.2386.

**4.4.7. *N*-{5-[3-(5-Acetamido-6-methoxy-6-oxohexylamino)-3-oxopropylamino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride, Ac-Lys(Bfe)-OMe (9).** The product of the reaction of **7b** (0.063 g, $3.50\times10^{-4}$ mol) with **5** (0.140 g, $3.50\times10^{-4}$ mol) (reflux time 3 h) was chromatographed with dichloromethane/methanol, 5.3:0.7 as the eluent, to give Ac-Lys(Bfe)-OMe (**9**) (0.173 g, 88%). Mp=above 300 °C. TLC (chloroform/methanol, 5.2:0.8): $R_f$=0.51. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$=1.20–1.40 (m, 2H, γ-CH$_2$ Lys), 1.41–1.60 (m, 7H, β-CH$_2$ Lys, δ-CH$_2$ Lys and NHCH$_2$*CH*$_3$), 1.80 (s, 3H, COCH$_3$), 2.34 (s, 3H, CH$_3$), 3.0–3.40 (m, 6H, ε-CH$_2$ Lys, NHCH$_2$*CH*$_2$ and NH*CH*$_2$CH$_3$), 3.70 (s, 3H, OCH$_3$), 3.80–4.0 (m, 2H, NH*CH*$_2$CH$_2$), 4.40–4.50 (m, 1H, α-CH Lys), 6.19 (br s, 1H, NH Lys lateral chain), 6.34 (s, 1H, 8-H), 6.57 (br s, 1H, 6-H), 7.40–7.50 (m, 2H, 11-H and α-NH Lys), 7.83 (s, 2H, 2-H and 3-H), 8.34 (s, 1H, NH), 8.76 (s, 1H, 1-H), 9.03 (s, 1H, 4-H), 10.25 (br s, 1H, NH). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=13.97 (NHCH$_2$*CH*$_3$), 17.64 (CH$_3$), 22.53 (δ-CH$_2$ Lys), 28.58 (β-CH$_2$ Lys), 29.67 (γ-CH$_2$ Lys), 30.75 (CO*CH*$_3$), 34.41 (ε-CH$_2$ Lys), 38.39 (NH*CH*$_2$CH$_3$), 39.85 (NHCH$_2$*CH*$_2$), 42.44 (NH*CH*$_2$CH$_2$), 52.16 (OCH$_3$), 52.64 (α-CH Lys), 93.16 (C-6), 93.48 (C-8), 123.59 (Ar-C), 124.26 (C-1), 124.93 (C-4), 126.31 (C-10), 129.94 (C-3), 130.31 (Ar-C), 130.56 (Ar-C), 131.35 (C-11), 131.87 (C-2), 134.19 (Ar-C), 146.94 (Ar-C), 150.99 (Ar-C), 153.98 (C-9), 157.92 (C-5), 170.97 (CONH), 171.0 (*C*OCH$_3$), 173.17 (*C*O$_2$CH$_3$). IR (Nujol, cm$^{-1}$): $\nu$=3396, 2954, 2924, 2854, 1746, 1659, 1635, 1592, 1463, 1456, 1377, 1312, 1016. HRMS (FAB): calcd for $C_{31}H_{38}N_5O_5$ [$M^+$]: 560.2873; found: 560.2858.

### 4.5. Synthesis of the functionalised benzo[*a*]phenoxazinium salts 10a–c

**4.5.1. 2-(Naphthalen-1-ylamino)ethanol, Nap-H (1c).** To a solution of 1-naphthylamine (1.0 g, $6.99\times10^{-3}$ mol) in ethanol (2.5 mL), 2-chloroethanol (0.49 mL, $7.34\times10^{-3}$ mol) was added and the resulting mixture was refluxed for 20 h, and monitored by TLC (dichloromethane/methanol, 5.5:0.5). The solvent was removed under reduced pressure and the crude mixture was chromatographed with dichloromethane/methanol, 5.9:0.1 as the eluent, to give Nap-H (**1c**) as a brownish oil (0.309 g, 24%). TLC (chloroform/methanol, 5.5:0.5): $R_f$=0.84. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$=3.50 (t, $J$=5.1 Hz, 2H, NHCH$_2$*CH*$_2$), 4.02 (t, $J$=5.4 Hz, 2H, NH*CH*$_2$CH$_2$), 6.68 (d, $J$=7.2 Hz, 1H, 4-H), 7.29 (d, $J$=8.4 Hz, 1H, 2-H), 7.36 (t, $J$=7.5 Hz, 1H, 3-H), 7.42–7.50 (m, 2H, 6-H and 7-H), 7.78–7.84 (m, 1H, 8-H), 7.86–7.90 (1H, m, 5-H). $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta_C$=46.19 (NHCH$_2$*CH*$_2$), 61.13 (NH*CH*$_2$CH$_2$), 104.78 (C-4), 117.97 (C-2), 119.96 (C-5), 123.76 (C-4a), 124.85 (C-7), 125.80 (C-6), 126.46 (C-3), 128.65 (C-8), 134.30 (C-8a), 143.27 (C-1). IR (neat, cm$^{-1}$): $\nu$=3373, 3060, 2963, 2925, 2350, 2283, 1621, 1582, 1514, 1479, 1407, 1378, 1286, 1261, 1089.

**4.5.2. $N^1$-(Naphthalen-1-yl)ethane-1,2-diamine, Nan-H (1d).** The product of the reaction of 1-naphthylamine

(1.0 g, $6.99\times10^{-3}$ mol) with 2-chloroethylamine monohydrochloride (0.85 g, $7.34\times10^{-3}$ mol) (reflux time 26 h) was chromatographed with chloroform/methanol, mixtures of increasing polarity, as the eluent. The oil obtained was crystallised from dichloromethane to give Nan-H (**1d**) as a white solid (0.926 g, 71%). Mp=229.0–231.6 °C. TLC (chloroform/methanol, 6:1): $R_f$=0.16. $^1$H NMR ($CD_3OD$, 300 MHz): $\delta$=3.38 (t, $J$=5.1 Hz, 2H, $NHCH_2CH_2$), 3.64 (t, $J$=6.3 Hz, 2H, $NHCH_2CH_2$), 6.71 (d, $J$=7.2 Hz, 1H, 4-H), 7.27 (d, $J$=8.1 Hz, 1H, 2-H), 7.35 (t, $J$=7.2 Hz, 1H, 3-H), 7.40–7.50 (m, 2H, 6-H and 7-H), 7.75–7.82 (m, 1H, 8-H), 8.06–8.12 (m, 1H, 5-H). $^{13}$C NMR ($CD_3OD$, 75.4 MHz): $\delta_C$=39.62 ($NHCH_2CH_2$), 42.23 ($NHCH_2CH_2$), 105.15 (C-4), 118.90 (C-2), 122.18 (C-5), 125.45 (C-4a), 125.62 (C-7), 126.78 (C-6), 127.44 (C-3), 129.27 (C-8), 135.94 (C-8a), 144.44 (C-1). IR (KBr, cm$^{-1}$): $\nu$=3389, 3055, 2983, 2928, 2874, 1585, 1536, 1488, 1452, 1410, 1372, 1347, 1282, 1251, 1143, 1025, 948 cm$^{-1}$. HRMS (FAB): calcd for $C_{12}H_{14}N_2$ [M$^+$]: 186.1157; found: 186.1165.

**4.5.3. *N*-[5-(2-Hydroxyethylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride, Bfh-H (10b).** To a cold solution (ice bath) of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride (**7b**) (0.173 g, $9.63\times10^{-4}$ mol) in ethanol (2 mL), compound **1c** (0.180 g, $9.63\times10^{-4}$ mol) and concentrated hydrochloric acid ($5.0\times10^{-2}$ mol) were added. The mixture was refluxed for 3 h and 30 min and monitored by TLC (dichloromethane/methanol, 5.8:0.2). After evaporation of the solvent and dry chromatography on silica gel with dichloromethane/methanol, 5.5:0.5 as the eluent, Bfh-H (**10b**) was obtained as a blue solid (0.317 g, 95%). Mp=above 300 °C. TLC (dichloromethane/methanol, 5.2:0.8): $R_f$=0.45. $^1$H NMR ($CD_3OD$, 300 MHz): $\delta$=1.36 (br s, 3H, $NHCH_2CH_3$), 2.16 (s, 3H, $CH_3$), 3.38 (br s, 2H, $NHCH_2CH_2$), 3.73 (br s, 2H, $NHCH_2CH_3$), 3.97 (br s, 2H, $NHCH_2CH_2$), 6.42 (s, 1H, 8-H), 6.70 (s, 1H, 6-H), 7.19 (s, 1H, 11-H), 7.60–7.80 (m, 2H, 2-H and 3-H), 8.12 (br s, 1H, 1-H), 8.41 (d, $J$=6.9 Hz, 1H, 4-H). $^{13}$C NMR ($CD_3OD$, 75.4 MHz): $\delta_C$=14.17 ($NHCH_2CH_3$), 17.77 ($CH_3$), 39.77 ($NHCH_2CH_2$), 48.03 ($NHCH_2CH_3$), 60.98 ($NHCH_2CH_2$), 94.30 (C-8), 94.40 (C-6), 123.72 (C-1), 124.46 (Ar-C), 125.27 (C-4), 128.58 (C-10), 130.60 (C-3), 131.79 (Ar-C), 132.02 (Ar-C), 132.54 (C-2), 132.60 (C-11), 133.91 (Ar-C), 148.90 (Ar-C), 152.30 (Ar-C), 156.47 (C-9), 158.91 (C-5). IR (KBr, cm$^{-1}$): $\nu$=3500–3118, 2974, 2924, 1641, 1590, 1561, 1544, 1521, 1477, 1451, 1434, 1315, 1259, 1185, 1163, 1137, 1084, 1056, 1008. HRMS (FAB): calcd for $C_{21}H_{22}N_3O_2$ [M$^+$]: 348.1712; found: 348.1712.

**4.5.4. *N*-[5-(2-Aminoethylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride, Bfn-H (10c).** The product of the reaction of compound **7b** (0.136 g, $7.53\times10^{-4}$ mol) with **1c** (0.140 g, $7.53\times10^{-4}$ mol) (reflux time 1 h and 30 min), following the same procedure as described for the synthesis of compound **10b**, was successively washed with mixtures of increasing polarity of chloroform/*n*-hexane and dichloromethane, to give Bfn-H (**10c**) as a blue solid (0.256 g, 98%). Mp=above 300 °C. $^1$H NMR ($CD_3OD$, 300 MHz): $\delta$=1.43 (t, $J$=7.2 Hz, 3H, $NHCH_2CH_3$), 2.44 (s, 3H, $CH_3$), 3.48 (t, $J$=6.0 Hz, 2H, $NHCH_2CH_2$), 3.68–3.78 (m, 3H, $NHCH_2CH_3$), 4.08 (t, $J$=6.3 Hz, 2H, $NHCH_2CH_2$), 7.02 (s, 1H, 8-H), 7.17 (s, 1H, 6-H), 7.84–7.96 (m, 2H, 11-H and 3-H), 8.00 (t, $J$=7.8 Hz, 1H, 2-H), 8.48 (d, $J$=8.4 Hz, 1H, 1-H), 9.07 (d, $J$=8.4 Hz, 1H, 4-H). $^{13}$C NMR ($CD_3OD$, 75.4 MHz): $\delta_C$=14.51 ($NHCH_2CH_3$), 18.47 ($CH_3$), 40.53 ($NHCH_2CH_2$), 41.95 ($NHCH_2CH_2$), 43.01 ($NHCH_2CH_3$), 94.60 (C-8), 95.17 (C-6), 123.84 (Ar-C), 124.88 (C-1), 125.54 (C-4), 130.48 (C-3), 130.78 (C-10), 132.44 (Ar-C), 133.32 (C-2), 133.48 (Ar-C), 133.52 (Ar-C), 133.71 (C-11), 149.88 (Ar-C), 152.56 (Ar-C), 157.83 (C-9), 158.29 (C-5). IR (KBr, cm$^{-1}$): $\nu$=3452, 2931, 2853, 1642, 1615, 1588, 1557, 1508, 1472, 1438, 1400, 1315, 1393, 1193, 1163, 1135, 1006. HRMS (EI): calcd for $C_{21}H_{23}N_4O$ [M$^+$]: 347.1872; found: 347.1875.

### 4.6. General procedure for the synthesis of the fluorescently labelled L-valine 8c, 12 and 13

The carboxylic derivative (Bfe-OH **10a** or Boc-Val-OH **11**) was reacted with L-valine methyl ester hydrochloride H-Val-OMe (**2b**), Bfh-H (**10b**) or Bfn-H (**10c**) using a standard DCC/HOBt coupling. After evaporation of the solvent and dry chromatography, the required derivative Bfe-Val-OMe (**8c**), Boc-Val-OBfh (**12**) or Boc-Val-OBfn (**13**) was obtained.

**4.6.1. *N*-{5-[3-(1-Methoxy-3-methyl-1-oxobutan-2-ylamino)-3-oxopropylamino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride, Bfe-Val-OMe (8c).** The product of the reaction of Bfe-OH (**10a**) (0.030 g, $7.98\times10^{-5}$ mol) with valine methyl ester hydrochloride (**2b**) (0.020 g, $1.20\times10^{-4}$ mol) was chromatographed with dichloromethane/methanol, 5.5:0.5 as the eluent, to produce Bfe-Val-OMe (**8c**) as a blue solid (0.036 g, 92%). The experimental data confirmed the expected structure and were in accordance with that previously described (Section 4.4.3).

**4.6.2. *N*-{5-[2-(2-(*tert*-Butoxycarbonylamino)-3-methylbutanoyloxy)ethylamino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride, Boc-Val-OBfh (12).** The product of the reaction of *N*-*tert*-butyloxycarbonylvaline (0.050 g, $2.30\times10^{-4}$ mol) with Bfn-H (**10b**) (0.080 g, $2.30\times10^{-4}$ mol) was chromatographed with dichloromethane/methanol, 5.6:0.4 as the eluent, to give Boc-Val-OBfh (**12**) as a blue solid (0.118 g, 94%). Mp=110.0–113.0 °C. TLC (dichloromethane/methanol, 5.2:0.8): $R_f$=0.67. $^1$H NMR ($CD_3OD$, 300 MHz): $\delta$=0.90 (t, $J$=6.9 Hz, 6H, 2×γ-$CH_3$ Val), 1.37 (s, 9H, $C(CH_3)_3$), 1.38–1.50 (m, 3H, $NHCH_2CH_3$), 2.0–2.15 (m, 1H, β-CH Val), 2.31 (s, 3H, $CH_3$), 3.45–3.55 (m, 2H, $NHCH_2CH_2$), 3.95–4.10 (m, 3H, $NHCH_2CH_3$ and α-CH Val), 4.45–4.70 (2×m, 2H, $NHCH_2CH_2$), 6.74 (s, 1H, 8-H), 6.97 (s, 1H, 6-H), 7.54 (s, 1H, 11-H), 7.68–7.79 (m, 1H, 3-H), 7.80–7.90 (m, 1H, 2-H), 8.25 (d, $J$=7.5 Hz, 1H, 1-H), 8.75 (d, $J$=7.8 Hz, 1H, 4-H). $^{13}$C NMR ($CD_3OD$, 75.4 MHz): $\delta_C$=14.15 ($NHCH_2CH_3$), 17.78 ($CH_3$), 18.46 (γ-$CH_3$ Val), 19.56 (γ-$CH_3$ Val), 28.65 ($C(CH_3)_3$), 31.58 (β-CH Val), 39.85 ($NHCH_2CH_2$), 44.21 ($NHCH_2CH_3$), 60.82 (α-CH Val), 63.48 ($NHCH_2CH_2$), 80.63 ($C(CH_3)_3$), 94.12 (C-6), 94.50 (C-8), 123.66 (C-1), 124.44 (Ar-C), 125.38 (C-4), 129.35 (C-10), 130.64 (C-3), 132.28 (Ar-C), 132.59 (C-2), 132.71 (Ar-C), 132.84 (C-11), 133.70 (Ar-C), 149.41 (Ar-C), 152.50 (Ar-C), 157.03 (C-9), 158.26 ($CO_2C(CH_3)_3$),

158.50 (C-5), 173.98 ($CO_2CH_2$). IR (KBr, $cm^{-1}$): $\nu$=3250, 2974, 2933, 1743, 1708, 1642, 1591, 1561, 1545, 1522, 1430, 1317, 1258, 1185, 1164, 1143, 1087, 1011. HRMS (EI): calcd for $C_{31}H_{39}N_4O_5$ [$M^+$]: 547.2920; found: 547.2938.

**4.6.3. *N*-{5-[2-(2-(*tert*-Butoxycarbonylamino)-3-methylbutanamido)ethylamino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride, Boc-Val-OBfn (13).** The product of the reaction of *N*-*tert*-butyloxycarbonylvaline (0.025 g, $1.15\times10^{-4}$ mol) with Bfn-H (**10c**) (0.040 g, $1.15\times10^{-4}$ mol) was chromatographed with chloroform/methanol, 5.7:0.3 as the eluent, to give Boc-Val-OBfn (**13**) as a blue solid (0.056 g, 89%). Mp=100.0–103.0 °C. TLC (dichloromethane/methanol, 5.5:0.5): $R_f$=0.47. $^1$H NMR ($CD_3OD$, 300 MHz): $\delta$=0.96 (t, $J$=7.2 Hz, 6H, 2×γ-$CH_3$ Val), 1.37 (s, 9H, $C(CH_3)_3$), 1.38–1.50 (m, 3H, $NHCH_2CH_3$), 2.04–2.16 (m, 1H, β-CH Val), 2.28 (s, 3H, $CH_3$), 3.44–3.55 (m, 2H, $NHCH_2CH_2$), 3.61–3.80 (m, 4H, $NHCH_2CH_3$ and $NHCH_2CH_2$), 3.90 (d, $J$=6.3 Hz, 1H, α-CH Val), 6.66 (s, 1H, 8-H), 6.82 (s, 1H, 6-H), 7.33 (s, 1H, 11-H), 7.52 (d, $J$=8.1 Hz, 1H, 3-H), 8.50 (d, $J$=8.4 Hz, 1H, 2-H), 8.18 (d, $J$=7.8 Hz, 1H, 1-H), 8.86 (d, $J$=7.8 Hz, 1H, 4-H). $^{13}$C NMR ($CD_3OD$, 75.4 MHz): $\delta_C$=14.19 ($NHCH_2CH_3$), 17.81 ($CH_3$), 18.46 (γ-$CH_3$ Val), 19.76 (γ-$CH_3$ Val), 28.65 ($C(CH_3)_3$), 31.79 (β-CH Val), 39.08 ($NHCH_2CH_3$), 39.83 ($NHCH_2CH_2$), 45.85 ($NHCH_2CH_2$), 61.97 (α-CH Val), 80.60 ($C(CH_3)_3$), 93.88 (C-8), 94.47 (C-6), 123.75 (C-1), 124.31 (Ar-C), 125.33 (C-4), 129.02 (Ar-C), 129.42 (C-10), 130.67 (C-3), 132.12 (Ar-C), 132.55 (C-2), 132.73 (C-11), 133.76 (Ar-C), 149.16 (Ar-C), 152.37 (Ar-C), 156.79 (C-9), 158.02 ($CO_2C(CH_3)_3$), 158.30 (C-5), 176.36 (CONH). IR (KBr, $cm^{-1}$): $\nu$=3250, 2973, 2933, 1708, 1643, 1591, 1521, 1435, 1315, 1259, 1164, 1142, 1011. HRMS (EI): calcd for $C_{31}H_{40}N_5O_4$ [$M^+$]: 546.3080; found: 546.3066.

### 4.7. General procedure for the photofading of compounds 10a–c

Compounds **10a–c** were dissolved in ethanol or water (pH 7.4) with concentrations of $1\times10^{-5}$ M. The samples were irradiated at 350 or 419 nm in a Rayonet RPR-100 chamber reactor with 10 lamps. The photostabilities were expressed in terms of their remaining absorption (%) calculated from the change of absorption intensities at the absorption maxima before and after irradiation.

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