GRAPHICAL ABSTRACT

Bifunctionalised long-wavelength fluorescent probes for biological applications

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Bifunctionalised long-wavelength fluorescent probes for biological applications

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Abstract – The synthesis of benzo[*a*]phenoxazinium chlorides which are bifunctionalised in position 2 with 4-ethoxy-4-oxobutoxyl, 3-hydroxypropoxyl or 3-chloropropoxyl groups, and in position 9 with the (aminopropyl)amino group, was efficiently performed. The covalent labeling of valine was carried out by using one of the new fluorophores obtained. Photophysical studies in the homogeneous media of ethanol, distilled water and at simulated physiological conditions revealed that all the compounds absorbed and emitted from 610 to 651 nm.

Keywords: Benzo[*a*]phenoxazines; Nile Blue; Long-wavelength dyes; Covalent labelling; Fluorescent probes.

Detection based on the fluorescence ability of several compounds is an effective tool for the visualization and quantification of non- or weak fluorescent analytes due to its high sensitivity and simplicity.¹⁻³ In the case of biomolecules, the fluorescent detection of nucleic acids, peptides and proteins is frequently used in cell biology, biochemistry, proteomics, immunochemistry, flow cytometry and medical diagnostics.⁴⁻¹⁰

As peptides and proteins contain a broad variety of nucleophilic functional groups, namely thiols, carboxylic acids, amines, imidazole residues, phenolic and aliphatic hydroxyls, covalent labeling is frequently carried out by modifying a cysteine residue with appropriate thiol-reactive probes or through the reaction of their other groups with specific reagents.¹¹⁻¹⁴ Among the fluorophores used for protein and peptide labeling are coumarins, quinolones, chromones, fluoresceins, rhodamines, cyanines and benzophenoxazines.¹⁵⁻²¹

In order to avoid a possible interference with the measurement of the label fluorescence, which could happen in many biological samples and usually occurs in the blue or green regions of the spectrum, one should improve the sensitivity of detection in the case of

studies with biomolecules by using compounds with emission in the red or near-infrared.^{1-3,21}

Fluorescent derivatization has been considered to be one of the most sensitive methods for the determination of analytes at low concentrations. As a result, significant efforts have been directed at the development of new fluorescent labeling reagents or the modification of known fluorophores. Research in long-wavelength fluorophores continues to be relevant for biological purposes in order to obtain compounds with improved water solubility, functional groups, and enhanced fluorescence quantum efficiency, which tends to decrease dramatically with increasing emission wavelengths. Bearing all these facts in mind, and as a continuation of our previous research,²²⁻²⁵ the present work describes the synthesis of new benzo[*a*]phenoxazine derivatives possessing two functional groups, namely carboxylic ester, hydroxyl and the chlorine atom, in addition to the amine function. As a result, these compounds are capable of covalent linkage to bio(molecules), and can also connect to another entity. As a preliminary study, 3-amino-*N*-(2-(4-ethoxy-4-oxobutoxy)-5-(propylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)propan-1-aminium chloride **4b** was efficiently used in the derivatisation of L-valine at its carboxylic group. Evaluation of absorption and emission properties of all benzo[*a*]phenoxazines and the conjugate synthesised was performed in ethanol, at physiological pH and distilled water.

Alkylation of the hydroxyl group of 5-(propylamino)naphthalen-2-ol **1a**²⁶ with ethyl 4-bromobutanoate, 3-bromopropan-1-ol and 1-bromo-3-chloropropane, using cesium carbonate as a base, by heating at 60°C in acetonitrile, gave ethyl 4-((5-(propylamino)naphthalen-2-yl)oxy)butanoate **1b**, 3-((5-(propylamino)naphthalen-2-yl)oxy)propan-1-ol **1c** and 6-(3-chloropropoxy)-*N*-propylnaphthalen-1-amine **1d**, respectively.²⁷ Starting from 3-aminophenol and using 3-bromopropan-1-amine in ethanol under reflux conditions yielded 3-((3-aminopropyl)amino)phenol hydrobromide **2**.²⁸ After column chromatography purification or isolation by filtration from the reaction mixture (**2**), compounds **1b-d** and **2** were obtained as an oil (**1b**, 97%) or solids (**1c**, 96%; **1d**, 93%; **2**, 98%), and were characterised by high resolution mass spectrometry, IR and NMR (¹H and ¹³C) spectroscopy. Compound **2** was further reacted with sodium nitrite in an acid solution to give 5-((3-aminopropyl)amino)-2-nitrosophenol di-hydrochloride **3**.^{29,30}

Condensation of precursors **1a-d** with the nitrosophenol **3**, in the presence of hydrochloric acid, produced the target benzo[*a*]phenoxazinium chlorides **4a-d** (Scheme 1).³¹ Through purification by several washings with dichloromethane and mixtures of dichloromethane/methanol, these functionalised heterocycles were isolated as solids or an oily solid (**4b**) in good to excellent yields, and were fully characterised by the usual analytical techniques.

The covalent labelling of biomolecules with monofunctionalised benzo[*a*]phenoxazinium chlorides by the amine, or a terminal functional group of the substituent in 5-position of the tetracyclic fluorophore, was previously investigated by the authors.^{23,32} As a continuation of these studies, the present work also aims to investigate, for the first time, the possibility of using the 9-position, namely the terminal amino group, in the derivatisation of analytes. Thus, compound **4b** was bonded to the C-terminus of *N*-*tert*-butyloxycarbonyl-L-valine, as a simple model of biomolecules, by using *N,N'*-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt), under standard conditions (Scheme 1),³³ yielding the expected conjugate **5**, after purification by column chromatography.

<Scheme 1>

¹H NMR spectra of compounds **4a-d** and **5** showed signals of the aliphatic protons from the methylene and methyl groups (δ 1.03 to 4.30 ppm) of the substituents of positions 5 and 9, as well as the expected aromatic protons such as H-8 (δ 6.50-6.75 ppm), and H-6 (δ 6.47-6.80 ppm). In the case of conjugate **5**, protons corresponding to the valine residue appeared namely α -CH (δ 3.80-3.92 ppm), β -CH (δ 2.0-2.12 ppm), γ -CH₃ (δ 0.80-1.10 ppm), and the methyls of the *N*-*tert*-butyloxycarbonyl protecting group (δ 1.47 ppm).

¹³C NMR spectra of these compounds showed signals of the aliphatic carbons from the methylene and methyl groups (δ 11.72 to 68.78 ppm) of the substituents of positions 5 and 9, as well as the expected aromatic signals such as C-8 (δ 93.47-95.30 ppm) and C-6 (δ 92.94-95.53 ppm). In the conjugate **5**, carbons corresponding to the valine residue were observed, namely α -CH (δ 62.18 ppm), β -CH (δ 31.56 ppm), γ -CH₃ (δ 18.70 ppm), as well as the C(CH₃)₃ of the *N*-*tert*-butyloxycarbonyl protecting group (CH₃, 28.84 ppm; C(CH₃)₃, 80.61 ppm). The signals of the carbonyl groups of ester (**4b**, **5**; δ

~ 174.90 ppm), urethane (**5**, δ 157.74 ppm) and amide (**5**, δ 165.26 ppm) type were also shown in the spectra.

Electronic absorption studies of 10^{-6} or 10^{-5} M solutions of fluorophores **4a-d** and conjugate **5** in degassed absolute ethanol, simulated physiological conditions (pH 7.4³⁴) and distilled water were carried out (Table 1).

These compounds showed maximum absorption (λ_{abs}) in all the solvents studied at long wavelengths, namely in the range 610-625 nm. Considering the influence of the solvent in the values of λ_{abs} , it was found that from ethanol to aqueous solutions of distilled water and pH 7.4, there was a hypsochromic shift between 2 and 10 nm (**4b**, in water). The structural alterations relating to the ether substituents in the aromatic ring (**4b-d**) resulted in a bathochromic shift (with the exception of **4b** at pH 7.4 and distilled water), more notably the shift of 7 and 12 nm in **4b** and **5**, regarding **4a** in ethanol. It is also stressed the hypsochromic shift of 5 nm of **4b** in comparison with **4d** in distilled water. Additionally, the linkage of **4b** to valine resulted in a bathochromic shift in all solvents studied, which was higher in distilled water (8 nm).

<Table 1>

Furthermore, it was found that in ethanol and distilled water only compounds **4a** and **4c** showed aggregation, a trend that was less evident in water for both compounds (Figure 1). At pH 7.4, **4a** and **4c** continued to form H-aggregates, and the behavior of **4d** is identical to that for ethanol, with only a slight tendency for aggregation. Compound **4a** showed the largest aggregation, the maximum absorption value for the band corresponding to the monomer was only slightly higher than that of the dimer (Figure 2). In order to further investigate the concentration dependence, absorption spectra of these compounds in ethanol, distilled water and at pH 7.4 were run in concentrations between 2.0×10^{-6} to 3.0×10^{-5} M. It was found that in this range of concentrations, no significant variations in the dimerization occurred.

< Figure 1>

< Figure 2>

The evaluation of the fluorescence properties of benzo[*a*]phenoxazines **4a-d** and conjugate **5** in the UV/Vis/NIR using Oxazine 1 as a standard (relative fluorescence

quantum yield, $\Phi_F = 0.11$ in ethanol³⁵) was also performed using the same solvents as in the studies of absorbance, i.e., ethanol, aqueous solution at pH 7.4 and distilled water. The wavelengths of maximum emission, the Stokes' shifts, as well as the relative fluorescence quantum yields for all compounds were obtained and are presented in Table 1. All compounds were excited at 590 nm and displayed wavelengths of maximum emission (λ_{em}) between 641 and 651 nm (Figure 3). By comparing the values of λ_{em} in ethanol and aqueous solutions, it was found that a slight bathochromic shift occurred for all compounds, the highest value being that of compound **4a** at pH 7.4 (10 nm). As in the case of λ_{abs} , conjugate **5** displayed a bathochromic shift in λ_{em} , in all solvents, which was higher in distilled water (8 nm), when compared to the fluorophore precursor **4b**.

Regarding the Stokes' shift, all benzo[*a*]phenoxazinium chlorides showed the highest values in aqueous solutions (32-38 nm). The relative fluorescent quantum yields were located in the range 0.26 to 0.88; the best values were found in solutions of distilled water for compounds *O*-substituted **4b-d**, including conjugate **5** (showing the same value in ethanol). According to these data, it appears that Φ_F values are sensitive to pH, so compounds **4a-d** were investigated in aqueous solution of pH 3, 5, 8, 10 and 12 using appropriate buffer solutions³⁴ ($C = 3.0 \times 10^{-5} M$). The results showed that for all compounds the highest values occurred in the acid range, being superior to those obtained in distilled water. However, as it was expected,²¹ in the basic range there was a significant decrease in the relative fluorescent quantum yields, being these probes inadequate for labelling applications at pH 12. The observed variations can be influenced by the H-aggregation (detected in the absorption spectra), which is more effective at basic pH, resulting in lowest Φ_F values.²²

< Figure 3 >

Conclusion

In this work, bifunctionalised benzo[*a*]phenoxazinium dyes, possessing ester, hydroxyl and the chlorine atom as terminals in position 2, in addition to the amine group terminal in 9-position of the polycyclic system were obtained.

These cationic fluorophores, with absorption in ethanol, in physiologically simulated conditions and distilled water in the 610-620 nm range, were highly fluorescent and displayed a maximum emission wavelength between 642 and 651 nm. 3-Amino-*N*-(2-(4-ethoxy-4-oxobutoxy)-5-(propylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)propan-1-aminium chloride **4b** was used in the covalent labelling of valine, as a simple model of biomolecules, resulting in the corresponding bioconjugate with superior wavelengths of absorption and emission maxima regarding label **4b** and high fluorescence.

The preliminary results obtained in the present work suggest that the new 3-amino-*N*-(2-(un)functionalised-5-(propylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)propan-1-aminium chlorides are interesting candidates for further studies in the labelling of biomolecules, being the most effective fluorescence associated to aqueous buffer solutions in the acidic pH range.

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27. Typical procedure for the preparation of **1b-d** (described for **1b**): To a solution of 5-(propylamino)naphthalen-2-ol **1a**²² (0.04 g, 1.99×10^{-4} mol), in acetonitrile (2 mL), ethyl 4-bromobutanoate (0.032 mL, 2.17×10^{-4} mol) and cesium carbonate (0.317 g, 9.73×10^{-4} mol) were added, and the resulting mixture was heated at 60°C for 2h30min. The progress of the reaction was monitored by TLC (ethyl acetate/light petroleum 1:3). The excess of base was filtered, the solvent was evaporated and the crude mixture was purified by column chromatography on silica gel using ethyl acetate/light petroleum 1:3, as the eluent. Ethyl 4-((5-(propylamino)naphthalen-2-yl)oxy)butanoate **1b** was obtained as a light brown oil (0.061 g, 97%). R_f = 0.78 (ethyl acetate/light petroleum 1:3). FTIR (KBr 1%): $\nu_{\max}$ 3413, 3049, 2960, 2930, 2872, 1731, 1625, 1583, 1531, 1469, 1435, 1419, 1375, 1342, 1276, 1261, 1226, 1178, 1086, 1023, 977, 963, 937, 882, 852, 830, 800, 743, 727, 666. ¹H NMR (DMSO-*d*₆, 300 MHz): δ 0.97 (3H, t, J = 7.5 Hz, NHCH₂CH₂CH₃), 1.17 (3H, t, J = 7.2 Hz, CO₂CH₂CH₃), 1.60-1.75 (2H, m, NHCH₂CH₂CH₃), 1.95-2.07 (2H, m, OCH₂CH₂CH₂CO₂CH₂CH₃), 2.45-2.55 (2H, m, OCH₂CH₂CH₂CO₂CH₂CH₃), 3.12 (2H, t, J = 7.2 Hz, NHCH₂CH₂CH₃), 4.00-4.10 (4H, m, CO₂CH₂CH₃ and OCH₂CH₂CH₂CO₂CH₂CH₃), 6.02 (1H, broad s, NH), 6.33 (1H, d, J = 7.5 Hz, H-6), 6.92-7.02 (2H, m, H-8 and H-3), 7.12 (1H, d, J = 2.7 Hz, H-1), 7.19 (1H, t, J = 7.8 Hz, H-7), 8.07 (1H, d, J = 9.0 Hz, H-4). ¹³C NMR (DMSO-*d*₆, 75.4 MHz): δ 11.80 (NHCH₂CH₂CH₃), 14.10 (CO₂CH₂CH₃), 21.49 (NHCH₂CH₂CH₃), 24.26 (OCH₂CH₂CH₂CO₂CH₂CH₃), 30.20 (OCH₂CH₂CH₂CO₂CH₂CH₃), 45.06 (NHCH₂CH₂CH₃), 59.87 (CO₂CH₂CH₃), 66.34 (OCH₂CH₂CH₂CO₂CH₂CH₃), 101.17 (C-6), 107.17 (C-1), 114.36 (C-8), 115.75 (C-3), 118.12 (C-4a), 123.45 (C-4), 127.48 (C-7), 135.56 (C-8a), 144.32 (C-5), 156.13 (C-2), 172.58 (CO₂CH₂CH₃). HRMS: m/z (ED): calcd. for C₁₉H₂₅NO₃ [M⁺] 315.1834; found 315.1836.
28. Procedure for the preparation of 3-((3-aminopropyl)amino)phenol hydrobromide **2**: To a suspension of 3-aminophenol (0.50 g, 4.58×10^{-3} mol) in ethanol (3 mL), 3-bromopropan-1-amine (1.0 g, 4.58×10^{-3} mol) was added and the resulting mixture was refluxed for 5h40min, and monitored by TLC (dichloromethane/methanol 9.5:0.5 and

5:5). The precipitate formed was collected by vacuum filtration using dichloromethane and petroleum ether as solvent washing. After being dried under vacuum at room temperature overnight, compound **2** was obtained as a white solid (1.11 g, 98%). Mp = 268.7-269.4 °C. FTIR (KBr 1%): 3277, 3046, 2972, 2924, 1619, 1567, 1513, 1503, 1488, 1463, 1439, 1414, 1342, 1330, 1313, 1292, 1232, 1165, 1141, 1084, 1031, 997, 967, 921, 900, 878. ¹H NMR (DMSO-*d*₆, 300 MHz): δ 1.85-2.00 (2H, m, NHCH₂CH₂CH₂NH₂), 2.80-2.95 (2H, m, NHCH₂CH₂CH₂NH₂), 3.32 (2H, t, *J* = 7.5 Hz, NHCH₂CH₂CH₂NH₂), 6.74 (1H, d, *J* = 7.8 Hz, 4-H), 6.80 (2H, broad s, 2-H and 6-H), 7.25 (1H, t, *J* = 8.4 Hz, 5-H), 7.88 (3H, broad s, NH₂·HBr), 9.90 (1H, broad s, OH). ¹³C NMR (DMSO-*d*₆, 75.4 MHz): δ 23.89 (NHCH₂CH₂CH₂NH₂), 36.27 (NHCH₂CH₂CH₂NH₂), 46.54 (NHCH₂CH₂CH₂NH₂), 108.21 (C-2), 111.73 (C-6), 114.29 (C-4), 130.71 (C-5), 138.70 (C-3), 158.42 (C-1). HRMS: *m/z* (EI): calcd. for C₉H₁₄N₂O [M⁺] 166.1106; found 166.1105.

29. Procedure for the preparation of 5-((3-aminopropyl)amino)-2-nitrosophenol dihydrochloride **3**: To an ice-cold solution of the 3-((3-aminopropyl)amino)phenol hydrobromide **2** (0.066 g, 3.97×10⁻⁴ mol) in ethanol (2 mL), 2M hydrochloric acid (0.159 mL) was added and stirred during 5 min. The solution of sodium nitrite (0.03 g, 4.37×10⁻⁴ mol) in water (0.1 mL) was then added drop-wise within an interval of 5 min. The resulting mixture was stirred for 3h and monitored by TLC (dichloromethane/methanol, 2:8 and 5:5). After evaporation of the reaction, compound **3** was obtained as an orange oily solid (0.066 g), and was used in the following step without any purification.

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31. Typical procedure for the synthesis of compounds **4a-d** (described for **4b**): To a cold solution (ice bath) of 5-((3-aminopropyl)amino)-2-nitrosophenol dihydrochloride **3** (0.081 g, 3.49×10⁻⁴ mol), in ethanol (3 mL), ethyl 4-((5-(propylamino)naphthalen-2-yl)oxy)butanoate **1b** (0.055 g, 1.74×10⁻⁴ mol), and concentrated hydrochloride acid (3.58×10⁻³ mL) were added. The mixture was refluxed for 4h30min and monitored by TLC (dichloromethane/methanol 9.5:0.5). The solvent was removed under reduced pressure and the residue was washed several times with dichloromethane and dichloromethane/methanol 9.9:0.1 and 9.5:0.5. The 3-amino-*N*-(2-(4-ethoxy-4-oxobutoxy)-5-(propylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)propan-1-aminium chloride **4b** was obtained as an oily solid blue (0.063 g, 69%), *R*_f = 0.30

(acetonitrile/water/1M hydrochloride acid solution 40:5:1). FTIR (KBr): $\nu_{\max}$ 3414, 2960, 2927, 1732, 1616, 1590, 1549, 1465, 1380, 1302, 1261, 1226, 1178, 1127, 1035, 818, 665. ^1H NMR (CD_3OD , 400 MHz): δ 1.05-1.15 (3H, m, $\text{NHCH}_2\text{CH}_2\text{CH}_3$), 1.20-1.35 (3H, m, $\text{CO}_2\text{CH}_2\text{CH}_3$), 1.85 (4H, broad s, $\text{NHCH}_2\text{CH}_2\text{CH}_3$ and $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 2.00-2.25 (4H, m, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$ and $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$), 2.58 (2H, broad s, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 3.11 (2H, t, $J = 7.6$ Hz, $\text{NHCH}_2\text{CH}_2\text{CH}_3$), 3.40-3.65 (2H, m, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 4.13 (2H, broad s, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$), 4.15-4.30 (2H, m, $\text{CO}_2\text{CH}_2\text{CH}_3$), 6.47 (1H, s, H-6), 6.52 (1H, s, H-8), 7.00-7.10 (1H, m, H-3), 7.30-7.50 (2H, m, H-10 and H-11), 7.62 (1H, broad s, H-4), 8.06 (1H, s, H-1). ^{13}C NMR (CD_3OD , 100.6 MHz): δ 11.89 ($\text{NHCH}_2\text{CH}_2\text{CH}_3$), 14.67 ($\text{CO}_2\text{CH}_2\text{CH}_3$), 25.65 ($\text{NHCH}_2\text{CH}_2\text{CH}_3$), 26.53 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 27.74 ($\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$), 31.57 ($\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$), 37.94 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 38.70 ($\text{NHCH}_2\text{CH}_2\text{CH}_3$), 41.78 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 61.75 ($\text{CO}_2\text{CH}_2\text{CH}_3$), 68.75 ($\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$), 93.51 (C-6), 95.12 (C-8), 107.28 (C-4), 117.77 (C-Ar), 119.64 (C-3), 126.32 (C-1), 130.54 (C-Ar), 133.48 (C-10), 134.12 (C-11 and C-Ar), 134.49 (C-Ar), 148.79 (C-Ar), 152.52 (C-Ar), 157.14 (C-9), 158.69 (C-5), 162.70 (C-2), 174.90 ($\text{CO}_2\text{CH}_2\text{CH}_3$). HRMS: m/z (ESI): calcd. for $\text{C}_{28}\text{H}_{35}\text{N}_4\text{O}_4$ [$\text{M}^+ + 1$] 491.26528; found 491.26462.

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33. Procedure for the preparation of 3-(2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-*N*-(2-(4-ethoxy-4-oxobutoxy)-5-(propylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)propan-1-aminium chloride **5**: To a solution of *N*-*tert*-butoxycarbonyl valine (8.42×10^{-3} g, 3.87×10^{-5} mol) in dry DMF (0.5 mL) at 0 °C, 1-hydroxybenzotriazole (5.24×10^{-3} g, 3.87×10^{-5} mol) and triethylamine (6.45×10^{-3} mL, 4.65×10^{-5} mol) were added, followed by *N,N'*-dicyclohexylcarbodiimide (8.40×10^{-3} g, 4.07×10^{-5} mol) after stirring 10 minutes. After 10 minutes 3-amino-*N*-(2-(4-ethoxy-4-oxobutoxy)-5-(propylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)propan-1-aminium chloride **4b** (2.45×10^{-2} g, 4.65×10^{-5} mol) was added and the reaction mixture was stirred at room temperature during four days. The solvent was evaporated and the crude residue purified by column chromatography using dichloromethane/methanol, mixtures of increasing polarity, as the eluent, to give compound **5** as a blue solid (0.015 g, 43%). Mp. 90.0-91.4 °C. $R_f = 0.84$ (dichloromethane/methanol 8:2). FTIR (KBr 1%): $\nu_{\max}$

3359, 2967, 2932, 1702, 1645, 1592, 1548, 1494, 1463, 1421, 1390, 1366, 1302, 1258, 1163, 1129, 1102, 1041, 910, 821, 746. ^1H NMR (CD_3OD , 400 MHz): δ 0.80-1.10 (6H, m, $\gamma\text{-CH}_3$ Val), 1.11 (3H, t, $J = 6.8$ Hz, $\text{NHCH}_2\text{CH}_2\text{CH}_3$), 1.28 (3H, t, $J = 7.2$ Hz, $\text{CO}_2\text{CH}_2\text{CH}_3$), 1.47 (9H, s, $\text{C}(\text{CH}_3)_3$), 1.80-2.0 (4H, m, $\text{NHCH}_2\text{CH}_2\text{CH}_3$ and $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.10-2.12 (1H, m, $\beta\text{-CH}$ Val), 2.13-2.25 (2H, m, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$), 2.60 (2H, t, $J = 7.2$ Hz, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 3.35-3.45 (4H, m, $\text{NHCH}_2\text{CH}_2\text{CH}_3$ and $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$), 3.55-3.72 (2H, m, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 3.80-3.92 (2H, m, $\alpha\text{-CH}$ Val), 4.10-4.30 (4H, m, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$ and $\text{CO}_2\text{CH}_2\text{CH}_3$), 6.66 (1H, broad s, H-6), 6.73 (1H, broad s, H-8), 7.02 (1H, t, $J = 8.0$ Hz, H-3), 7.23-7.33 (2H, m, H-10 and H-11), 8.07 (1H, broad s, H-4), 8.18 (1H, s, H-1). ^{13}C NMR (CD_3OD , 100.6 MHz): δ 11.78 ($\text{NHCH}_2\text{CH}_2\text{CH}_3$), 14.61 ($\text{CO}_2\text{CH}_2\text{CH}_3$), 18.70 ($\gamma\text{-CH}_3$ Val), 23.21 ($\text{NHCH}_2\text{CH}_2\text{CH}_3$), 25.67 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 28.77 ($\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$), 28.84 ($\text{C}(\text{CH}_3)_3$), 31.56 ($\beta\text{-CH}$ Val), 37.85 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$ and $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$), 42.12 ($\text{NHCH}_2\text{CH}_2\text{CH}_3$ and $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 61.70 ($\text{CO}_2\text{CH}_2\text{CH}_3$), 62.18 ($\alpha\text{-CH}$ Val), 68.78 ($\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_2\text{CH}_3$), 80.61 ($\text{C}(\text{CH}_3)_3$), 93.47 (C-6 and C-8), 107.56 (C-4), 118.24 (C-3), 126.03 (C-1), 128.18 (C-Ar), 129.57 (C-Ar), 133.88 (C-11, C-Ar), 134.90 (C-10 and C-Ar), 142.91 (C-Ar), 153.23 (C-Ar), 157.74 (CONH), 158.06 (C-9), 159.19 (C-5), 163.11 (C-2), 165.26 (CONH), 174.87 ($\text{CO}_2\text{CH}_2\text{CH}_3$). HRMS: m/z (ESI): calcd. for $\text{C}_{35}\text{H}_{48}\text{N}_5\text{O}_5$ [$\text{M}^+ + 1$] 618.36500; found 618.36359.

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CAPTIONS

Table 1 – Absorbance and fluorescence data in ethanol, physiological pH and distilled water for compounds **4a-d** and **5**.

Figure 1 – Normalised absorbance spectra of compounds **4a-c** and **5** measured in ethanol ($C = 6.67 \times 10^{-6} - 3.00 \times 10^{-5}$ M).

Figure 2 – Normalised absorbance spectra of compounds **4a-c** and **5** measured at physiological pH ($C = 6.67 \times 10^{-6} - 3.00 \times 10^{-5}$ M).

Figure 3 – Normalised fluorescence spectra of compounds **4b** and **5** measured in ethanol and at physiological pH ($C = 6.67 \times 10^{-6} - 3.00 \times 10^{-5}$ M).

TABLES

Table 1.

Cpd	Ethanol				pH 7.4				Water			
	λ_{abs} (nm)	λ_{em} (nm)	Φ_{F}	Δ (nm)	λ_{abs} (nm)	λ_{em} (nm)	Φ_{F}	Δ (nm)	λ_{abs} (nm)	λ_{em} (nm)	Φ_{F}	Δ (nm)
4a	613	641	0.43	28	613	651	0.28	38	613	647	0.31	34
4b	620	642	0.60	22	612	646	0.26	34	610	644	0.64	34
4c	616	643	0.60	27	616	644	0.63	28	614	646	0.88	32
4d	617	643	0.32	26	614	648	0.27	34	615	648	0.65	33
5	625	645	0.54	20	616	650	0.28	34	618	651	0.54	33

FIGURES

Figure 1

Figure 2

Figure 3

SCHEMES

Scheme 1