Synthesis and photophysical properties of N-(5-amino-2-substituted-10-methyl-9H-benzo[a]phenoxazin-9-ylidene)ethanaminium chlorides

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Abstract: Fluorescent benzo[a]phenoxazinium chlorides possessing different substituents at position 2, namely propoxyl, 4-ethoxy-4-oxobutoxyl or 3-carboxypropoxyl groups, and at positions 5, 9 and 10 of the tetracyclic system, the amino, ethylamino and methyl groups, respectively, were synthesised in good to excellent yields. Absorption and emission studies carried out in ethanol, water and at simulated physiological conditions revealed that all compounds absorbed in the range 614–628 nm and emitted from 630 to 652 nm.

Keywords: Fluorescent probes; Benzo[a]phenoxazines; Nile Blue derivatives; Organic fluorophores.

1. Introduction

The use of fluorescent probes for detection of biological and organic molecules has increased in the last years due to their high sensitivity and ease of use compared to radiochemical methods.\(^1\) Interference with the measurement of the label fluorescence could occur in many biological samples, which display some fluorescence of their own, usually in the blue or green region of the spectrum. Consequently, for studies with biomolecules it is desirable to improve the sensitivity of detection by using dyes with absorption and fluorescence in the red or near infrared spectral region. Therefore, in this spectral regions it is possible the use of inexpensive and effective excitation sources, e.g. laser diodes.\(^2-4\)

In long-wavelength fluorophores some research continues to be required to obtain compounds with improved water solubility, functional groups for covalent staining, and enhanced fluorescence quantum efficiency, which tends to decrease dramatically with increasing wavelength of emission. As a continuation of our previous research,\(^5-8\) the present work aims to contribute to the variability of these type of fluorophores, with the synthesis of three new benzo[a]phenoxazine derivatives soluble in water and emitting in the near infrared region.
2. Experimental

2.1. Typical procedure for the synthesis of 1a-c (described for 1b): To a solution of 5-(ethylamino)-4-methyl-2-nitrosophenol hydrochloride 2 (0.111 g, 5.12×10^{-4} mol) in ethanol (3 mL), concentrated hydrochloric acid (5.3×10^{-3} mL) was added followed by the ethyl 4-((5-aminonaphthalen-2-yl)oxy)butanoate 3b (0.07 g, 2.56×10^{-4} mol). The reaction mixture was refluxed for 2h35min and monitored by TLC (dichloromethane/methanol, 9:1). After evaporation of the solvent and purification by column chromatography on silica gel with dichloromethane and dichloromethane/methanol, mixtures of increasing polarity, as the eluent, N-(5-amino-2-(4-ethoxy-4-oxobutoxy)-10-methyl-9H-benzo[a]phenoxazin-9-ylidene)ethanaminium chloride 1b was obtained as a blue solid (0.041 g, 34%). Mp = 110.6-112.9 °C. Rf = 0.42 (dichloromethane/methanol, 9:1). FTIR (KBr 1%): 3379, 3214, 2924, 1731, 1643, 1594, 1563, 1548, 1524, 1486, 1452, 1384, 1324, 1300, 1231, 1179, 1153, 1085, 1013, 960, 879, 816, 728, 665 cm^{-1}. \[^1\]H NMR (CD_{2}OD, 400 MHz): \(\delta^{1}1.24-1.32\) (3H, m, CO\(_2\)CH\(_2\)CH\(_3\)), 1.36-1.45 (3H, m, NHCH\(_2\)CH\(_3\)), 2.12 (2H, broad s, OCH\(_2\)CH\(_2\)CH\(_2\)CO\(_2\)CH\(_2\)CH\(_3\)), 2.15-2.24 (2H, m, OCH\(_2\)CH\(_2\)CH\(_2\)CO\(_2\)CH\(_2\)CH\(_3\)), 2.22 (3H, s, CH\(_3\)), 3.42-3.54 (2H, m, NHCH\(_2\)CH\(_3\)), 3.65 (2H, broad s, OCH\(_2\)CH\(_2\)CH\(_2\)CO\(_2\)CH\(_2\)CH\(_3\)), 4.14-4.26 (2H, m, CO\(_2\)CH\(_2\)CH\(_3\)), 6.63 (1H, s, H-8), 6.71 (1H, s, H-6), 7.18 (1H, d, \(J = 7.2\) Hz, H-3), 7.40 (1H, s, H-11), 7.91 (1H, broad s, H-1), 8.01 (1H, d, \(J = 7.6\) Hz, H-4) ppm. \[^{13}\]C NMR (CD\(_2\)OD, 100.6 MHz): \(\delta 14.22\) (NHCH\(_2\)CH\(_3\)), 14.59 (CO\(_2\)CH\(_2\)CH\(_3\)), 17.77 (CH\(_3\)), 24.85 (OCH\(_2\)CH\(_2\)CH\(_2\)CO\(_2\)CH\(_2\)CH\(_3\)), 25.68 (OCH\(_2\)CH\(_2\)CH\(_2\)CO\(_2\)CH\(_2\)CH\(_3\)), 39.79 (NHCH\(_2\)CH\(_3\)), 44.80 (OCH\(_2\)CH\(_2\)CH\(_2\)CO\(_2\)CH\(_2\)CH\(_3\)), 68.74 (CO\(_2\)CH\(_2\)CH\(_3\)), 92.97 (C-6), 94.98 (C-8), 107.12 (C-1), 118.02 (C-Ar), 119.85 (C-3), 125.75 (C-4), 128.28 (C-10), 131.25 (C-Ar), 132.61 (C-11), 133.86 (C-Ar), 134.48 (C-Ar), 149.00 (C-Ar), 152.73 (C-Ar), 156.50 (C-9), 158.26 (C-5), 162.73 (C-2), 174.87 (CO\(_2\)CH\(_2\)CH\(_3\)) ppm. HRMS: m/z (ESI): calcd. for C\(_{23}\)H\(_{28}\)N\(_3\)O\(_4\) [M\(^+\)+1] 434.20814; found 434.20810.

2.2. Typical procedure for the preparation of 3a-c (described for 3b): To a solution of 5-aminonaphthalen-2-ol (0.060 g, 3.77×10^{-4} mol) in acetonitrile (2 mL), ethyl 4-bromobutanoate (0.063 mL, 4.15×10^{-4} mol) and cesium carbonate (0.601 g, 1.84×10^{-3} 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-aminonaphthalen-2-yl)oxy)butanoate 3b was obtained as a rose solid (0.082 g, 80%). Mp = 61.5-63.0 °C. TLC (ethyl acetate/light petroleum 1:3): \(R_f = 0.55\). FTIR (neat): \(\nu_{\text{max}}\) 3456, 3376, 2963, 2937, 2870, 1733, 1625, 1586, 1517, 1467, 1451, 1385, 1377, 1318, 1293, 1274, 1249, 1213, 1050, 1029, 975, 921, 863, 842, 816, 780, 750, 666 cm^{-1}. \[^{1}\]H NMR
3. Results and Discussion

Benzo[a]phenoxazinum chlorides 1a-c were synthesised by condensation of 5-(ethylamino)-4-methyl-2-nitrosophenol hydrochloride 2 with a suitable 5-aminonaphthalen-2-ol derivative 3a-c in acidic media. The nitrosophenol 2 was obtained by the usual procedure involving treatment of the 3-(ethylamino)-4-methylphenol with sodium nitrite in the presence of hydrochloric acid.\(^9\)

The 6-propanaphthalen-1-amine 3a was obtained by alkylation of 5-aminonaphthalen-2-ol with the 1-bromopropane using DMF as solvent and heating at 75°C, in the presence of potassium carbonate. Reaction of 5-aminonaphthalen-2-ol with ethyl 4-bromobutanoate, in acetonitrile, heating at 60°C and using cesium carbonate as base resulted in ethyl 4-((5-aminonaphthalen-2-yl)oxy)butanoate 3b. Hydrolysis of the ethyl ester group of the intermediate 3b (1 M sodium hydroxide/1,4-dioxane), yielded the corresponding 4-((5-aminonaphthalen-2-yl)oxy)butanoic acid 3c. After column chromatographic purification or isolation by extraction (3c), compounds 3a-c were obtained as solid materials in good to excellent yields (65-90%), and were characterised by high-resolution mass spectrometry, IR and NMR (\(^1\)H and \(^13\)C) spectroscopies.

Cyclisation of nitrosophenol 2 with precursors 3a and 3b in the presence of concentrated hydrochloric acid refluxed in ethanol, gave N-(5-amino-2-propoxy-10-methyl-9H-benzo[a]phenoxazin-9-ylidene)ethanamium chloride 1a and N-(5-amino-2-(4-ethoxy-4-oxobutoxy)-10-methyl-9H-benzo[a]phenoxazin-9-ylidene)ethanamium chloride 1b. In the preparation of N-(5-amino-2-(3-carboxypropoxy)-10-methyl-9H-benzo[a]phenoxazin-9-ylidene)ethanamium chloride 1c, compound 2 reacted with intermediate 3c also in an acidic medium, but using DMF as solvent and heating at 70 °C. After purification by silica gel column chromatography compounds 1a-c were obtained as blue solids in yields of 77 (1a), 34 (1b) and 66% (1c) (Scheme 1), and were fully characterised by the usual analytical techniques.
Scheme 1. Synthesis of benzo[a]phenoxazinium chlorides 1a-c.

Electronic absorption spectra of $10^{-6}$ M solutions in degassed absolute ethanol and water were measured for the synthesised benzo[a]phenoxazinium chlorides 1a-c (Table 1). The absorption maxima ($\lambda_{abs}$) for all compounds are in the range 614-628 nm. It was found that in compounds 1b and 1c an hipsochromic shift (~ 6 nm) occurred from ethanol to water, and also in organic and aqueous medium these compounds displayed a bathochromic shift in comparison to 1a (13 nm, 1b).

Concerning the potential biological applications, the absorption properties were also studied in water at physiological pH (pH 7.4, adjusted with 0.2 M boric acid, 0.05 M citric acid and 0.1 M sodium phosphate). Comparison of $\lambda_{abs}$ values in water and at pH 7.4 revealed an hipsochromic shift for compounds 1a and 1b (7 nm, for 1b) at the latter solutions.

Evaluation of fluorescent properties of compounds 1a-c carried out in ethanol, water and at physiological pH, using Oxazine 1 as a standard (fluorescence quantum yield, $\Phi_F = 0.11$ in ethanol\textsuperscript{10}), and excitation at 590 nm showed emission maxima in the range 630-652 nm with fluorescence quantum yields of 0.25-0.96. For compounds 1b and 1c a batochromic shift (7-10 nm) in the emission maxima was observed in water and at pH 7.4 in comparison with ethanol.

Table 1. Photophysical data for compounds 1a-c in ethanol, water and at physiological pH. \textsuperscript{a}in nm.

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<th>Cpd</th>
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<th>$\lambda_{em}^{a}$</th>
<th>$\Phi_F$</th>
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<th>$\lambda_{abs}^{a}$</th>
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<th>$\Phi_F$</th>
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<td>637</td>
<td>0.96</td>
<td>22</td>
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<td>636</td>
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<td>630</td>
<td>0.92</td>
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4. Conclusion

Three \(\text{N-(5-amino-2-substituted-10-methyl-9H-benzo}[a]\text{phenoazin-9-ylidene)}\text{ethanaminium}
chlorides, possessing propoxy, 4-ethoxy-4-oxobutoxy and 3-carboxypropoxy groups at position 2
of the tetracyclic aromatic systems 1a-c were efficiently synthesised. These water soluble dyes
displayed high absorption and emission at longer wavelengths in ethanol, water and also at
simulated physiological conditions.

The cationic character of the fluorophores obtained is important for its use as probes in non-
covalent staining of biomolecules. However, the presence of a functional group, namely the
carboxylic acid or ester (which can be hydrolysed to carboxylic acid) provide them the additional
possibility of application in the covalent labeling of entities.

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