Synthesis and photophysical properties of *N*-(5-amino-2-substituted-10-methyl-9*H*-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-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride **1b** was obtained as a blue solid (0.041 g, 34%). Mp = 110.6-112.9 °C. R_f = 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}. ^1H NMR (CD_3OD, 400 MHz): δ 1.24-1.32 (3H, m, $CO_2CH_2CH_3$), 1.36-1.45 (3H, m, $NHCH_2CH_3$), 2.12 (2H, broad s, $OCH_2CH_2CH_2CO_2CH_2CH_3$), 2.15-2.24 (2H, m, $OCH_2CH_2CH_2CO_2CH_2CH_3$), 2.22 (3H, s, CH_3), 3.42-3.54 (2H, m, $NHCH_2CH_3$), 3.65 (2H, broad s, $OCH_2CH_2CH_2CO_2CH_2CH_3$), 4.14-4.26 (2H, m, $CO_2CH_2CH_3$), 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_3OD, 100.6 MHz): δ 14.22 ($NHCH_2CH_3$), 14.59 ($CO_2CH_2CH_3$), 17.77 (CH_3), 24.85 ($OCH_2CH_2CH_2CO_2CH_2CH_3$), 25.68 ($OCH_2CH_2CH_2CO_2CH_2CH_3$), 39.79 ($NHCH_2CH_3$), 44.80 ($OCH_2CH_2CH_2CO_2CH_2CH_3$), 68.74 ($CO_2CH_2CH_3$), 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 ($CO_2CH_2CH_3$) ppm. HRMS: m/z (ESI): calcd. for $C_{25}H_{28}N_3O_4$ $[M^+ +1]$ 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 60ºC 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): ν_{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}. ^1H NMR

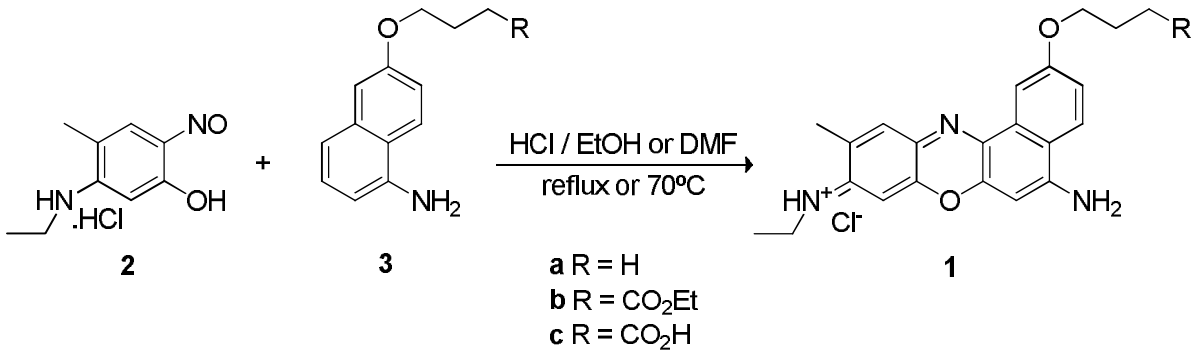
(DMSO-d_6, 300 MHz): δ 1.17 (3H, t, J = 7.2 Hz, $CO_2CH_2CH_3$), 1.95-2.08 (2H, m, $OCH_2CH_2CH_2CO_2CH_2CH_3$), 2.42-2.58 (2H, m, $OCH_2CH_2CH_2CO_2CH_2CH_3$), 4.00-4.18 (4H, m, $OCH_2CH_2CH_2CO_2CH_2CH_3$ and $CO_2CH_2CH_3$), 5.65 (2H, s, NH_2), 6.49 (1H, d, J = 6.9 Hz, H-6), 6.90-7.02 (2H, m, H-8 and H-3), 7.06-7.18 (2H, m, H-1 and H-7), 7.95 (1H, d, J = 9.3 Hz, H-4). ^{13}C NMR (DMSO-d_6, 75.4 MHz,): δ 14.11 ($CO_2CH_2CH_3$), 24.25 ($OCH_2CH_2CH_2CO_2CH_2CH_3$), 30.20 ($OCH_2CH_2CH_2CO_2CH_2CH_3$), 59.87 ($CO_2CH_2CH_3$), 66.33 ($OCH_2CH_2CH_2CO_2CH_2CH_3$), 105.82 (C-6), 106.85 (C-1), 114.58 (C-8), 115.85 (C-3), 117.98 (C-4a), 124.05 (C-4), 127.34 (C-7), 135.60 (C-8a), 144.66 (C-5), 156.09 (C-2), 172.58 ($CO_2CH_2CH_3$). HRMS: m/z (EI): calcd. for $C_{16}H_{19}NO_3$ [M^+] 273.1365; found 273.1371.

3. Results and Discussion

Benzo[*a*]phenoxazinium 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-propoxynaphthalen-1-amine **3a** was obtained by alkylation of 5-aminonaphthalen-2-ol with the 1-bromopropane using DMF as solvent and heating at 75ºC, in the presence of potassium carbonate. Reaction of 5-aminonaphthalen-2-ol with ethyl 4-bromobutanoate, in acetonitrile, heating at 60ºC 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-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride **1a** and *N*-(5-amino-2-(4-ethoxy-4-oxobutoxy)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride **1b**. In the preparation of *N*-(5-amino-2-(3-carboxypropoxy)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride **1c**, compound **2** reacted with intermediate **3c** also in an acidic medium, but using DMF as solvent and heating at 70 ºC. 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 (λ_{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 λ_{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, Φ_F = 0.11 in ethanol[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. [a]in nm.

	Ethanol				**Water**				**pH 7.4**			
Cpd	λ_{abs}[a]	λ_{em}[a]	Φ_F	$\Delta\lambda$[a]	λ_{abs}[a]	λ_{em}[a]	Φ_F	$\Delta\lambda$[a]	λ_{abs}[a]	λ_{em}[a]	Φ_F	$\Delta\lambda$[a]
1a	615	637	0.96	22	617	636	0.88	19	615	630	0.92	15
1b	628	642	0.66	14	622	649	0.64	27	615	652	0.25	37
1c	619	635	0.95	16	614	643	0.94	29	617	644	0.96	27

4. Conclusion

Three *N*-(5-amino-2-substituted-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chlorides, possessing propoxyl, 4-ethoxy-4-oxobutoxyl and 3-carboxypropoxyl 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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