Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron Letters
Tetrahedron Letters 47 (2006) 8567–8570

Synthesis of fluorescent water-soluble functionalised benzo[*a*]phenoxazinium salts

Vânia H. J. Frade, M. Sameiro T. Gonçalves* and João C. V. P. Moura

Centro de Química, Universidade do Minho, Gualtar, 4710-057 Braga, Portugal

Received 7 July 2006; revised 20 September 2006; accepted 22 September 2006
Available online 17 October 2006

Abstract—In order to develop long-wavelength functionalised oxazine dyes with good water solubility and high photostability for biological applications, a series of novel side-chain functionalised benzo[*a*]phenoxazinium salts were synthesised and characterised. These polycyclic cationic compounds showed strong fluorescence in ethanol and water (pH 7.4), with an emission wavelengths higher than 637 nm, as well as high quantum yields and moderate Stokes' shifts. The photostability of the fluorophores synthesised, under irradiation at 419 nm, was good to excellent in ethanol and moderate in water at physiological pH.

1. Introduction

In recent years, the interest in the development of monofunctional, water-soluble long-wavelength fluorochromophores for the detection of biological and organic molecules has dramatically increased.[1] The sensitivity, simplicity and selectivity of fluorescence-based techniques make them particularly attractive compared to radiochemical methods.

Among the long-wavelength absorbing and emitting fluorophores are rhodamine,[2,3] squaraine,[4,5] cyanine[6,7] and oxazine[8–10] dyes. Excitation in the high wavelength region is particularly interesting for in vitro and in vivo cellular and molecular biology studies, because of reduced cell damage.[11] In addition, the presence of a functional group in the dye is fundamental for covalent labelling of analytes. The chemical and photochemical stability of chromophores is also an important prerequisite for their use as probes in biological analysis.

Bearing this in mind, and in connection with our preliminary work concerning the preparation of carboxylated 5,9-diaminobenzo[*a*]phenoxazinium salts,[12,13] we now present the efficient synthesis of new side-chain functionalised 5,9-benzo[*a*]phenoxazinium salts. These cationic dyes resulted from the cyclisation of 5-(ethylamino)-4-methyl-2-nitrosophenol hydrochloride with N-substituted-naphthylamine or 1-naphthylamine. All compounds exhibit visible absorption in the 620–630 nm region (in ethanol or water, pH 7.4) and show strong fluorescence from 637 to 646 nm (in ethanol) and at about 653 nm (in water, pH 7.4). Preliminary evaluation of the photostability of the cationic polycyclic heterocycles synthesised, in ethanol and water at physiological pH, was carried out.

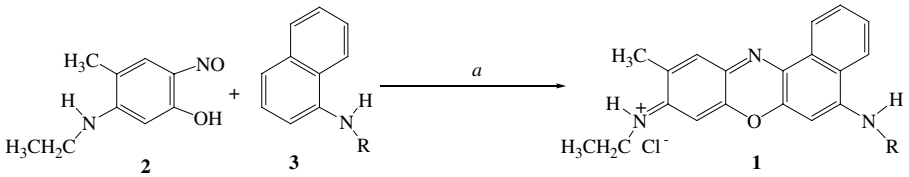
The synthesis of functionalised 5,9-diaminobenzo[*a*]-phenoxazinium chlorides **1** started with the preparation of the nitroso intermediate **2** and the N-substituted-naphthylamines **3** (except in the case of compound **3d**). The required 5-(ethylamino)-4-methyl-2-nitrosophenol hydrochloride **2** was synthesised by the usual procedure involving the treatment of the 3-ethylamino-4-methylphenol with sodium nitrite in an acid solution.[14]

Alkylation of 1-naphthylamine with ethyl-4-bromobutyrate, followed by dry chromatography purification produced ethyl-4-(naphthalen-1-ylamino)butanoate **3a** (46%).[15] Hydrolysis of the ester group of this compound (1 M NaOH/1,4-dioxane), yielded the corresponding 4-(naphthalen-1-ylamino)butanoic acid **3b** in a quantitative scale.

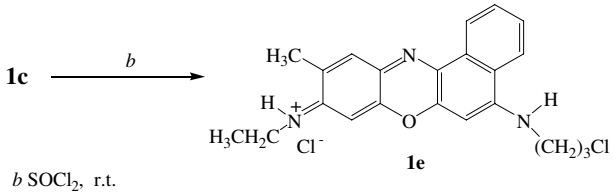
Condensation of the nitrosophenol **2** with the intermediate **3a**, in boiling ethanol and hydrochloric acid, yielded the corresponding carboxylated benzo[*a*]phenoxazinium chloride **1a**.[16] When compound **2** reacted

Keywords: Near-infrared dyes; Functionalised probes; Benzo[*a*]phenoxazine; Fluorescent label; Fluorochromophores.
* Corresponding author. Fax: +351 253 678 986; e-mail: msameiro@quimica.uminho.pt

0040-4039/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2006.09.133



a i) H^+/ EtOH (reflux) for compounds **1a**, **1c** and **1d**; ii) H^+/ DMF (70 °C) for compound **1b**.



Scheme 1.

with 4-(naphthalen-1-ylamino)butanoic acid **3b**, in acidic medium using DMF as a solvent and heating at 70 °C, the (carboxypropylamino)-benzo[*a*]phenoxazinium derivative **1b** was obtained (Scheme 1). After dry chromatography purification, cationic dyes **1a**,**b** were isolated as solid materials in 75% (**1a**) and 40% (**1b**) yields (Table 1).

Bearing in mind the synthesis of a fluorochromophore with a hydroxyl group instead of carboxyl, the functionalised intermediate **3c** was prepared and used in the cyclisation together with compound **2**. Thus, 1-naphthylamine was alkylated with 3-bromo-1-propanol and after purification by dry chromatography, 3-(naphthalen-1-ylamino)propanol **3c** was obtained in 70%. By reacting the nitrosophenol **2** with this intermediate, following the same procedure as described above for the preparation of dye **1a**, the desired hydroxypropylamino benzo[*a*]phenoxazinium salt **1c** was isolated in an excellent yield (97%). Using 1-naphthylamine and the nitroso compound **2**, the amino derivative **1d** was also prepared (80%) (Scheme 1, Table 1).

In the synthesis of the chloropropylaminobenzo[*a*]phenoxazinium chloride **1e**, compound **1c**, used as precursor, was treated with thionyl chloride, at room temperature.

All compounds were fully characterised by elemental analysis or high resolution mass spectrometry, IR, NMR (^{1}H and ^{13}C) and visible spectroscopies. In compounds **1a** and **1b**, the ^{13}C NMR spectra showed signals at δ 173.4 (**1a**) and 164.3 (**1b**) ppm due to the presence of the carbonyl carbon, and IR of compound **1a** also presented a band at 1727 cm^{-1}, related to the ester group. The IR of compounds **1c** and **1d** showed the bands expected, due to the hydroxyl (3506 and 3241 cm^{-1}) and amino functions (3319 cm^{-1}), respectively.

Table 1. Yields and visible data for compounds **1a**–**e**

Compound	Yield [%]	Vis[a] λ_{max} [nm] (ε)	Vis[b] λ_{max} [nm] (ε)
1a	75	630 (45,296)	625 (33,500)
1b	40	620 (23,970)	620 (12,805)
1c	97	625 (45,477)	620 (36,181)
1d	80	620 (53,968)	—
1e	55	625 (21,978)	620 (11,311)

[a] Spectra were measured in absolute ethanol.
[b] Spectra were measured in water (pH 7.4).

The visible absorption spectra of 3×10^{-6} M solutions of compounds **1a**–**e** in degassed absolute ethanol showed absorption peaks at 620 nm (**1b**,**d**), 625 nm (**1c**,**e**) and 630 nm (**1a**) with ε values ranging from 21,978 (**1e**) to 53,968 (**1d**) (Table 1).

The fluorescent properties of these compounds measured in the same solvent, using oxazine **1** as standard, are summarised in Table 2. All the compounds exhibit high levels of fluorescence, with quantum yields between 0.31 (**1d**) and 0.55 (**1e**) and showed a moderate Stokes' shift (the highest 57 for **1d**).

Considering the water solubility of the cationic dyes (**1a**–**c** and **1e**) synthesised, as well as the importance of having fluorescent long-wavelength probes for biological applications, their visible and fluorescent properties were studied in water at physiological pH. The wavelengths of maximum absorption (λ_{max}), excitation (λ_{ex}) and emission (λ_{em}) and also fluorescence quantum yields of compounds **1a**–**e** in water (pH 7.4) are shown in Tables 1 and 2. The maximum absorption of these compounds in water was at about 620–625 nm, which was similar to λ_{max} in ethanol. However, in the fluorescence emission, a slight bathochromic shift occurred in water when compared to ethanol. Thus, maximum emission wavelengths were at about 653 nm (in water) and 637 to 646 nm (in ethanol). All compounds also exhibited high levels of fluorescence in water, with quantum yields ranging from 0.26 (**1a**) to 0.44 (**1e**) and moderate Stokes' shifts (61–71 nm), yet superior to ethanol (46–57 nm).

Table 2. Fluorescence data for compounds **1a–e**

Compd	Fluorescence[a]			Stokes' shift [nm]	Fluorescence[b]			Stokes' shift [nm]
	λ_{exc} [nm]	λ_{em} [nm]	ϕ		λ_{exc} [nm]	λ_{em} [nm]	ϕ	
1a	600	646	0.35	46	590	653	0.26	63
1b	590	644	0.34	54	590	653	0.37	63
1c	590	646	0.41	56	580	651	0.31	71
1d	580	637	0.31	57	—	—	—	—
1e	580	645	0.55	53	590	651	0.44	61

[a] Spectra were measured in absolute ethanol.
[b] Spectra were measured in water (pH 7.4).

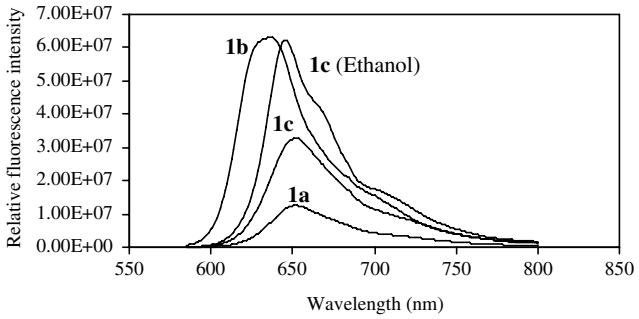


Figure 1. Fluorescence spectra of compounds **1a–c** measured in water (pH 7.4) and/or in ethanol.

Figure 1 shows the comparison between the emission spectra of compounds **1c** in ethanol and water (pH 7.4) as well as the emission spectra of compounds **1a** and **1b** in water.

One of the major limitations in the complete use of long-wavelength dyes for several applications is the short number of fluorophores with high fluorescence efficiency and good stability.

Since our final purpose is that of the investigation of the photostability of these fluorophores, we decided to evaluate their behaviour under irradiation in the visible region. In this study, solutions of compounds **1a–e** in ethanol or water at physiological pH (1×10^{-5} M) were irradiated at 419 nm in a Rayonet RPR-100 reactor.[17] The photostabilities of compounds **1a–e**, in ethanol, after seven hours of irradiation were in the order of **1e** > **1d** > **1c** > **1a** > **1b** and the remaining absorption varied from ~98% (**1e**) to ~80% (**1b**). In water, there was a decrease in the photostability values: compounds **1a** (~48% of remaining absorption) and **1e** (~23% of remaining absorption) were the most and least stable, respectively. These results suggested that there was a relationship between the functional group of the side chain and the photostability of these benzo[*a*]phenoxazine dyes. Further studies will be carried out for a better comprehension of this correlation.

2. Conclusion

In this work, 5,9-diaminobenzo[*a*]phenoxazinium dyes **1a–e**, containing different functional groups, were synthesised in good to excellent yields. These water-soluble cationic dyes, with absorption in the 620–630 nm range, were highly fluorescent and revealed a maximum emission wavelength between 637 and 653 nm. Their photostability in ethanol (good to excellent) and water (moderate), in connection with the above results, strongly suggests that these oxazine derivatives constitute promising fluorescent probes in biological applications.

Acknowledgements

We thank the Fundação para a Ciência e Tecnologia (Portugal) for its financial support to the Centro de Química (Universidade do Minho).

References and notes

1. Haugland, R. P. *Handbook of Fluorescent Probes and Research Products*, 9th ed.; Molecular Probes: Eugene, OR, 2002.
2. Matveeva, E. G.; Gryczynski, Z.; Lakowicz, J. R. *J. Immunol. Methods* **2005**, *302*, 26–35.
3. Waggoner, A. *Curr. Opin. Chem. Biol.* **2006**, *10*, 62–66.
4. Arun, K. T.; Ramaiah, D. *J. Phys. Chem. A* **2005**, *109*, 5571–5578.
5. Jyothish, K.; Avirah, R. R.; Ramaiah, D. *Org. Lett.* **2006**, *8*, 111–114.
6. Pham, W.; Medarova, Z.; Moore, A. *Bioconjugate Chem.* **2005**, *16*, 735–740.
7. Shindy, H. A.; El-Maghraby, M. A.; Eissa, F. M. *Dyes Pigments* **2006**, *68*, 11–18.
8. Marmé, N.; Habl, G.; Knemeyer, J.-P. *Chem. Phys. Lett.* **2005**, *408*, 221–225.
9. Westman, G.; Jennsiche, E.; Hamberger, A. Eur. Pat. 104210A1, 2003.
10. Frangioni, J. V.; Onishi, S. U.S. Pat. 082423A2, 2005.
11. Weissleder, R.; Ntziachristos, V. *Nat. Med.* **2003**, *9*, 123–128.
12. Frade, V. H. J.; Gonçalves, M. S. T.; Moura, J. C. V. P. *Tetrahedron Lett.* **2005**, *46*, 4949–4952.
13. Frade, V. H. J.; Gonçalves, M. S. T.; Coutinho, P. J. G.; Moura, J. C. V. P. *J. Photochem. Photobio. A* **2006**, doi:10.1016/j.jphotochem.2006.06.013.
14. *Synthesis of compound* **2**: Sodium nitrite (1.81 g; 26 mmol) was added in portions, with stirring, to a cold solution (0 °C) of 3-ethylamino-4-methylphenol (3.78 g; 25 mmol) in concentrated HCl (15 mL). The reaction mixture was left stirring for 1 h with external cooling. The precipitate was filtered off, washed with cold 1 M HCl and dried in vacuum. 5-(Ethylamino)-4-methyl-2-nitrosophenol hydrochloride **2** was obtained as a brown solid (4.05 g, 90%) and was used in the following reaction without purification. Crossley, M. L.; Turner, R. J.; Hofmann, C.

M.; Dreisbach, P. F.; Parker, R. P. *J. Am. Chem. Soc.* **1952**, *74*, 578–584.

15. *Typical procedure for the synthesis of compounds* **3a** *and* **3c** *(described for* **3c***):* To a solution of 1-naphthylamine (2 g; 14.0 mmol) in ethanol (5 mL), 3-bromo-1-propanol (1.33 mL; 14.7 mmol) was added and the resulting mixture was refluxed for 8 h and monitored by TLC (silica: chloroform–methanol, 5.8:0.2). The solvent was removed under reduced pressure and the crude mixture was purified by dry chromatography (silica: chloroform and chloroform–methanol, 5.8:0.2). 3-(Naphthalen-1-ylamino) propanol **3c** was obtained as a pinkish oil (2.07 g, 70%). R_f 0.30 (silica: chloroform–methanol, 5.7:0.3). FTIR (neat): ν_{max} 3529–3109, 3046, 2953, 2914, 2846, 1624, 1581, 1524, 1468, 1406, 1368, 1337, 1274, 1249, 1205, 1174, 1124, 1061 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 1.96–2.10 (2H, m, $NCH_2CH_2CH_2$), 3.10–3.50 (1H, m, NH), 3.44 (2H, t, *J* 6.0 Hz, $NCH_2CH_2CH_2$), 3.90 (2H, t, *J* 5.7 Hz, $NCH_2CH_2CH_2$), 6.67 (1H, d, *J* 7.0 Hz, 4-H), 7.28 (1H, d, *J* 6.6 Hz, 2-H), 7.38 (1H, t, *J* 7.5 Hz, 3-H), 7.43–7.51 (2H, m, 6-H and 7-H), 7.78–7.86 (2H, m, 8-H and 5-H) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ 31.2 ($NCH_2CH_2CH_2$), 42.5 ($NCH_2CH_2CH_2$), 61.8 ($NCH_2CH_2CH_2$), 104.7 (4-C), 117.6 (2-C), 120.0 (5-C), 123.5 (4a-C), 124.7 (7-C), 125.7 (6-C), 126.5 (3-C), 128.5 (8-C), 134.2 (8a-C), 143.3 (1-C) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS: *m*/*z* (FAB): calcd for $C_{13}H_{15}NO$ [M^+] 201.1154; found 201.1157.

16. *Typical procedure for the synthesis of* **1a**, **1c** *and* **1d** *(described for* **1c***):* To a cold solution (ice bath) of 5-(ethylamino)-4-methyl-2-nitrosophenol hydrochloride **2** (185 mg; 5.97×10^{-4} mol) in ethanol (2 mL), 3-(naphthalen-1-ylamino) propanol **3a** (117 mg; 5.82×10^{-4} mol) and concentrated hydrochloride acid (5.0×10^{-2} mL) were added. The mixture was refluxed for 3 h and 30 min and monitored by TLC (silica: chloroform and chloroform–methanol, 6:1). The solvent was removed under reduced pressure and the crude mixture was purified by dry chromatography (silica: chloroform–methanol, 5.5:0.5). *N*-(5-(3-hydroxypropylamino)-10-methyl-9*H*-benzo[*a*]-phenoxazin-9-ylidene) ethanaminium chloride **1c** was obtained as a blue solid (204 mg, 97%). Mp above 300 °C. R_f 0.69 (silica: chloroform–methanol, 6:1). FTIR (KBr, 1%): ν_{max} 3506, 3241, 1641, 1592, 1561, 1544, 1521, 1451, 1431, 1315, 1185, 1163, 1137, 1087, 1010 cm^{-1}. ^1H NMR (CD_3OD, 300 MHz): δ 1.41 (2H, t, *J* 6.3 Hz, $NHCH_2CH_3$), 2.11 (2H, br s, $NHCH_2CH_2CH_2$), 2.39 (3H, s, CH_3), 3.50–3.68 (2H, m, $NHCH_2CH_2CH_2$), 3.82 (2H, br s, $NHCH_2CH_2CH_2$), 3.87 (2H, br s, $NHCH_2CH_3$), 6.92 (1H, s, 8-H), 7.06 (1H, s, 6-H), 7.76 (1H, s, 11-H), 7.80–7.90 (1H, m, 2-H), 7.96 (1H, t, *J* 7.5 Hz, 3-H), 8.33 (1H, br s, 1-H), 8.98 (1H, d, *J* 7.5 Hz, 4-H) ppm. ^{13}C NMR (CD_3OD, 75 MHz): δ 14.2 ($NHCH_2CH_3$), 17.7 (CH_3), 32.2 ($NHCH_2CH_2CH_2$), 39.7 ($NHCH_2CH_2CH_2$), 43.3 ($NHCH_2CH_3$), 60.4 ($NHCH_2CH_2CH_2$), 93.9 (6-C), 94.5 (8-C), 123.6 (Ar–C), 124.7 (1-C), 125.5 (4-C), 128.7 (10-C), 130.8 (2-C), 132.0 (Ar–C), 132.4 (Ar–C), 132.7 (3-C), 132.8 (11-C), 134.3 (Ar–C), 149.2 (Ar–C), 152.8 (Ar–C), 156.6 (9-C), 158.6 (5-C) ppm. The assignments were supported by HMQC technique. Anal. Calcd for $C_{22}H_{24}N_3O_2 \cdot 3.5HCl$: C, 53.92; H, 5.66; N, 8.58. Found: C, 53.99; H, 5.80; N, 8.37.

17. *Photofading of compounds* **1a**–**e***:* Compounds **1a**–**e** were dissolved in ethanol or water (pH 7.4) with concentrations of 1×10^{-5} M. The samples were irradiated at 419 nm, in a Rayonet RPR-100 chamber reactor with 10 lamps. The photostabilities were expressed in terms of the remaining absorption (%) calculated from the change of absorption intensities at the absorption maximum before and after irradiation.