10th International Electronic Conference on Synthetic Organic Chemistry (ECSOC-10). 1-30 November 2006. http://www.usc.es/congresos/ecsoc/10/ECSOC10.htm & http://www.mdpi.org/ecsoc-10/

[a040]

Benzo[*a*]phenoxazine dyes as new fluorescent labels of L-valine

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Abstract – Two fluorescent functionalised benzo[*a*]phenoxazinium chlorides were efficiently synthesised by condensation of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride with the corresponding *N*-substituted-naphthylamines. In order to evaluate their applicability as covalent probes for biomolecules, these heterocycles were coupled at the C-terminus of N-protected-L-valine through an ester or amide linkage. Studies of their photophysical properties revealed that all compounds absorbed and emitted at longer wavelengths with high fluorescent quantum yields.

1. Introduction

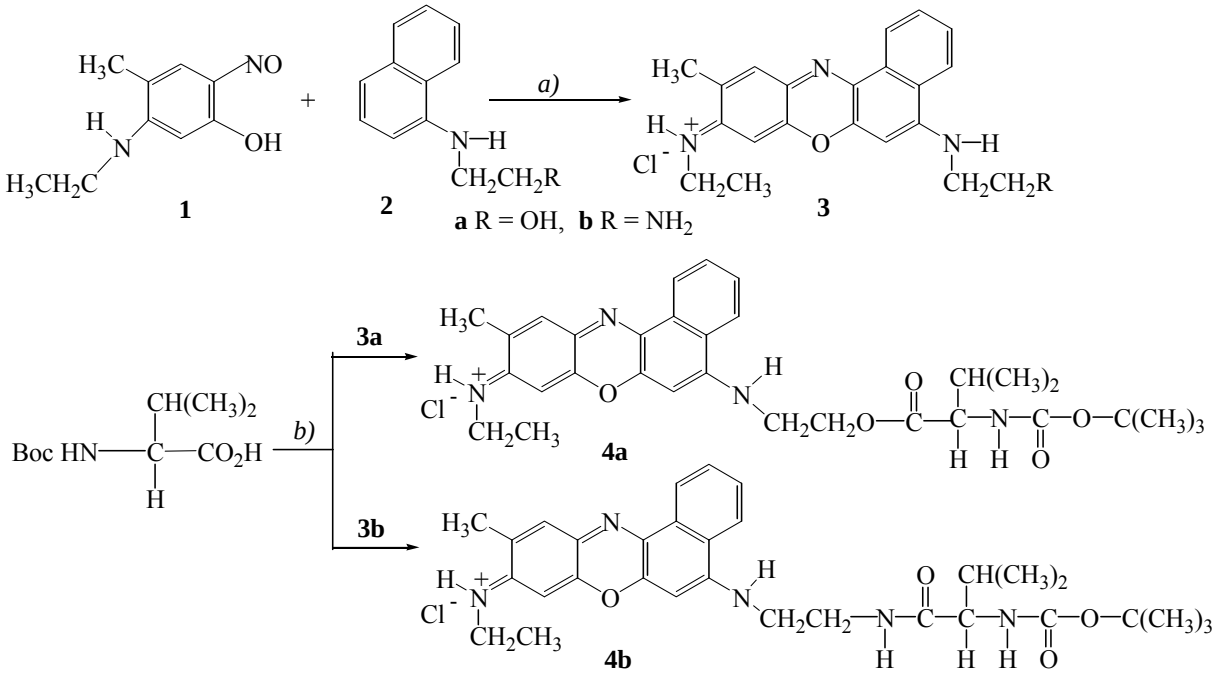
During the last decades many fluorescent dyes have been developed for labelling biomolecules.[1-2] Among the most interesting fluorophores are dyes with absorption and emission in longer wavelengths (600-1000 nm).[3] One important advantage of these compounds is related to the minimal background interference from biological material as well as their high sensitivity.

Bearing this in mind, and including our preliminary work,[4-6] we report the synthesis and characterisation of two fluorescent benzo[*a*]phenoxazinium chlorides and their use in the efficient covalent labelling of N-protected-L-valine. These compounds absorbed in the range 617 to 630 nm and fluoresced at about 644 nm.

2. Results and Discussion

Condensation of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride **1** with 3-(naphthalene-1-ylamino) ethanol **2a** or N^1-(naphthalene-1-yl) ethane-1,2-diamine **2b** in the presence of hydrochloric acid produced benzo[*a*]phenoxazinium chlorides **3a**,**b** in excellent yields (Scheme 1, Table 1). The required 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride **1** was synthesised using an usual procedure involving the treatment of 3-ethylamino-4-methylphenol with sodium nitrite in acid solution. Precursors **2a**,**b** were responsible for the functionality of the cationic dyes **3a**,**b** and were prepared by alkylation of 1-naphthylamine with 2-chloroethanol and 2-chloroethylamine, respectively (**2a**, 24% and **2b**, 71%).

In order to investigate the possibility of using oxazine derivatives as covalent labels in organic molecules of biological interest, the functionalised benzo[*a*]phenoxazinium chlorides **3a**,**b** synthesised were used in the derivatisation of the carboxylic acid function of N-protected-L-valine. Thus, coupling of *N*-*tert*-butyloxycarbonylvaline with the hydroxyl dye **3a** or the amine derivative **3b** with the aid of *N*,*N*'-dicyclohexylcarbodiimide (DCC) assisted by 1-hydroxybenzotriazole (HOBt) under standard conditions produced the labelled ester or amide conjugates **4a** (94%) and **4b** (89%), respectively (Scheme 1, Table 1).



Scheme 1. Synthesis of compounds **3a**,**b** and **4a**,**b**. *Reagents and conditions: a)* H^+, ethanol, reflux; *b)* DCC, HOBt, DMF, rt.

All compounds obtained were characterised by high resolution mass spectrometry, NMR (^{1}H and ^{13}C) and UV/ visible (compounds **3a**,**b** and **4a**,**b**) spectroscopy.

Table 1. Synthesis, UV/ visible and fluorescence data for compounds **3a**,**b** and **4a**,**b** in ethanol.

Compd	Yield [%]	UV/ vis	Fluorescence			Stokes' shift
		λ_{max} [nm] (ε)	λ_{exc} [nm]	λ_{em} [nm]	Φ_F	[nm]
3a	95	625 (26479)	590	644	0.44	54
3b	98	617 (22400)	590	643	0.27	53
4a	94	629 (22646)	590	643	0.32	53
4b	89	630 (27917)	590	643	0.35	53

Electronic absorption and emission spectra of 10^{-6} M solutions of benzo[*a*]phenoxazinium chlorides **3a**,**b** and labelled L-valine **4a**,**b** in degassed absolute ethanol were measured and the summarised data are presented in Table 1. The longest wavelength of maximum absorption of all compounds was located between 617 and 630 nm. When compounds **3a**,**b** were compared to the corresponding labelled derivatives **4a**,**b**, there was only a slight bathochromic shift (4 nm **3a**/ **4a** and 13 nm **3b**/ **4b**).

Regarding fluorescence properties, the quantum yields (Φ_F) were calculated using Oxazine 1 as a standard (Φ_F = 0.11 in ethanol).[7] For the determination of relative quantum yields, Oxazine 1 was excited at the wavelengths of maximum excitation found for each one of the compounds to be tested. Emission maxima for all compounds in ethanol was at about 644 nm and the Stokes' shifts were of ~ 54 nm. All compounds exhibited high levels of fluorescence, with Φ_F between 0.27 (**3b**) and 0.44 (**3a**).

Functionalised benzo[*a*]phenoxazinium chlorides were prepared through the usual procedure in excellent yields. Derivatisation with these fluorescent probes of N-protected-L-valine, representative of amino acids, were also achieved in excellent yields. Regarding these results, as well as the longer wavelength of absorption and emission maxima in connection with the high fluorescence and good fluorescent quantum yields of all compounds, these cationic dyes are suitable for fluorescent covalent labelling of organic molecules.

3. Experimental

Typical procedure for the synthesis of **3a**,**d** (described for **3a**): To a cold solution (ice bath) of 5-(ethylamino)-4-methyl-2-nitrosophenol hydrochloride **1** (173 mg; 9.63×10^{-4} mol) in ethanol (2 mL), **2a** (180 mg; 9.63×10^{-4} mol) and concentrated hydrochloride acid (5.0×10^{-2} mL) were added. The mixture was refluxed for 3 hours and 30 minutes and monitored by TLC (silica: dichloromethane/ methanol, 5.2:0.8). The solvent was removed under reduced pressure and the crude mixture was purified by dry chromatography using dichloromethane/ methanol, 5.5:0.5 as the eluent, *N*-(5-(2-hydroxyethylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene) ethanaminium chloride (**3a**) was obtained as a blue solid (317 mg, 95%). mp above 300 ºC. R_f = 0.45 (silica: dichloromethane/ methanol, 5.2:0.8). ^{1}H NMR (CD_3OD, 300 MHz): δ = 1.36 (broad s, 3 H, $NHCH_2CH_3$), 2.16 (s, 3 H, CH_3), 3.38 (broad s, 2 H, $NHCH_2CH_2$), 3.73 (broad s, 2 H, $NHCH_2CH_3$), 3.97 (broad s, 2 H, $NHCH_2CH_2$), 6.42 (s, 1 H, 8-H), 6.70 (s, 1 H, 6-H), 7.19 (s, 1 H, 11-H), 7.60-7.80 (s, 2 H, 2-H and 3-H), 8.12 (broad s, 1 H, 1-H), 8.41 (d, J = 6.9 Hz, 1 H, 4-H) ppm. ^{13}C NMR (CD_3OD, 75.4 MHz): δ = 14.17 ($NHCH_2CH_3$), 17.80 (CH_3), 39.77 ($NHCH_2CH_2$),

40.03 (NH*CH*$_2$CH$_3$), 60.98 (NH*CH*$_2$CH$_2$), 94.30 (C-6), 94.40 (C-8), 123.72 (C-1), 124.46 (C-4), 125.27 (C-10), 128.58 (Ar-C), 130.60 (C-3), 131.79 (Ar-C), 132.02 (Ar-C), 135.54 (C-2), 132.60 (C-11), 133.91 (Ar-C), 148.90 (Ar-C), 152.30 (Ar-C), 156.47 (C-9), 159.91 (C-5) ppm. HRMS (FAB): calcd for $C_{21}H_{22}N_3O_2$ [M^+]: 348.1712; found 348.1712.

Typical procedure for the synthesis of **4a**,**d** (described for **4a**): *N-tert*-Butyloxycarbonyl-L-valine, Boc-Val-OH (125 mg; 5.75×10^{-4} mol) was reacted with fluorophore **3a** (80 mg; 2.30×10^{-4} mol) in DMF by a standard DCC/ HOBt coupling. After evaporation of the solvent and dry chromatography using diclorometh ane/ methanol, 5.5:0.5 as the eluent, Boc-Val-OBza (**4b**) was obtained as a blue solid (118 mg, 94%). mp = 110-113 ºC. R_f = 0.67 (silica: diclorometh ane/ methanol, 5.2:0.5): ^{1}H NMR (CD_3OD, 300 MHz): δ = 0.90 (t, J = 6.9 Hz, 6 H, 2 × γ-CH_3 Val), 1.28-1.50 (m, 12 H, C(CH_3)$_3$ and NCH_2*CH*$_3$), 2.0-2.15 (m, 1 H, β-CH Val), 2.31 (s, 3 H, CH_3), 3.45-3.55 (m, 2 H, NHCH_2*CH*$_2$), 3.95-4.10 (m, 3 H, NH*CH*$_2$$CH_3$ and α-CH Val), 4.45-4.70 (m, 2 H, NH*CH*$_2$$CH_2$), 6.74 (s, 1 H, 8-H), 6.97 (s, 1 H, 6-H), 7.54 (s, 1 H, 11-H), 7.68-7.79 (m, 1 H, 3-H), 7.78-7.90 (1 H, m, 2-H), 8.25 (d, J = 7.5 Hz, 1 H, 1-H), 8.75 (d, J = 7.8 Hz, 1 H, 4-H), ppm. ^{13}C NMR (CD_3OD, 75.4 MHz): δ_C = 14.15 (NHCH_2*CH*$_3$), 17.78 (CH_3), 18.46 (γ-CH_3 Val), 19.56 (γ-CH_3 Val), 28.65 (C(*C*H_3)$_3$), 31.58 (β-CH Val), 39.85 (NHCH_2*CH*$_2$), 44.21(NH*CH*$_2$$CH_3$), 60.82 (α-CH Val), 63.48 (NH*CH*$_2$$CH_2$), 80.63 (*C*($CH_3$)$_3$), 94.12 (C-6), 94.50 (C-8), 123.66 (C-1), 124.44 (Ar-C), 125.38 (C-4), 129.35 (C-10), 130.64 (C-3), 132.28 (Ar-C), 132.59 (C-2), 132.71 (Ar-C), 132.84 (C-11), 133.70 (Ar-C), 149.41 (Ar-C), 152.50 (Ar-C), 157.03 (C-9), 158.26 (O*CO*C(CH_3)$_3$), 158.50 (C-5), 173.98 (CONH) ppm. HRMS (FAB): calcd for $C_{31}H_{39}N_4O_5$ [M^+]: 547.2920; found: 547.2920.

Acknowledgments

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

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