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Synthesis and fluorescence properties of side-chain carboxylated 5,9-diaminobenzo[*a*]phenoxazinium salts

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Abstract—The efficient synthesis of a series of novel side-chain carboxylated 5,9-diaminobenzo[*a*]phenoxazinium salts is described. The ring system was prepared by the reaction of 5-alkylamino-2-nitrosophenol hydrochlorides with the appropriate *N*-alkylated-naphthylamine. Evaluation of the visible and fluorescence properties of the cationic dyes was carried out in ethanol and water at physiological pH. In both solvents they showed intense visible absorption maxima in the range 500–638 nm (ethanol) and 625–650 (water), and fluoresced strongly, with fluorescence maxima from 612 to 669 nm (ethanol) and from 654 to 685 nm (water). A wide variation in fluorescence quantum yields is observed, ranging from 0.051 to 0.50 and 0.065 to 0.32 in ethanol and water, respectively.

Fluorescent chromophores have assumed increasing importance in life sciences, particularly for applications in detection, labelling, diagnosis and analysis.[1–5] Although there are many fluorescent dyes now commercially available, relatively few are long-wavelength light-emitting (600–1000 nm), which is an important requirement for many of these bio-applications, as in this region of the spectrum there is minimum interference from absorption scattering and from the natural auto fluorescence of biological molecules.[6]

Of the longer wavelength emitting dyes, oxazine derivatives such as benzophenoxazines and benzo[*a*]phenoxazines have seen a remarkable growth in research interest and technical importance. These cationic fluorophore systems have been used as biomarkers in histochemistry for nucleic acid detection,[7–9] for labelling proteins[10] and to study neuronal death in Parkinson's disease[11] and Alzheimer's disease[12] or functional peroxisome deficiency, as encountered in Zelleweger syndrome.[13]

Other examples include neuronal detection after cerebral ischaemia,[14] determination of enzymatic activity in the living cells,[15] bacterial identification[16] and erythroblast determination in blood by flow cytometry.[17]

Following our previous work with carboxylic acid derivative temporary markers in peptide chemistry,[18–20] we have now prepared new functionalised benzo[*a*]phenoxazinium salts. In order to explore eventual coupling of these fluorophores to biomolecules a carboxylic acid or ester (which can be hydrolysed) was chosen as the reactive functional group, and this was achieved by using the corresponding naphthyl carboxylic acid or ethyl ester precursor. We now report the efficient synthesis and characterisation of several side-chain carboxylated 5,9-diaminobenzo[*a*]phenoxazinium dyes **1**, which have the potential to provide longer wavelength emission. These compounds showed visible absorption in the 500–638 nm (ethanol) and 625–650 nm (water pH 7.4) region, and fluorescence with emission wavelength higher than 612 and 654 nm in ethanol and water, respectively.

5,9-Diaminobenzo[*a*]phenoxazinium dyes **1** were prepared by the reaction of a 5-alkylamino-2-nitrosophenol hydrochloride **2** with the appropriate *N*-alkylated-naphthylamine **3** in acidic medium (Scheme 1).[21] The required 5-alkylamino-2-nitrosophenol hydrochloride **2a**–**c** was synthesised by usual procedure involving treatment of the 3-alkylaminophenol with sodium nitrite in acidic solution. *N*-Alkyl-naphthylamines **3a**–**b** were prepared by alkylation of 1-naphthylamine with chloropropionic acid and 3-ethyl-3-bromopropionate, respectively.[22]

Keywords: Biomarkers; Benzo[*a*]phenoxazines; Long-wavelength fluorescence probes; Fluorescent dyes; Cationic dyes; Fluorophores.
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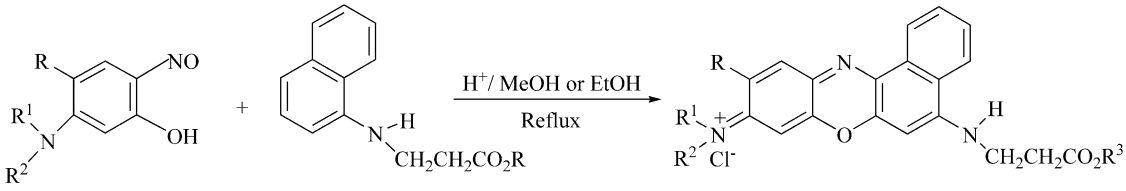

2a R = H, $R^1 = R^2 = CH_3$
2b R = H, $R^1 = R^2 = CH_2CH_3$
2c R = CH_3, R^1 = H , $R^2 = CH_2CH_3$

3a R = H
3b R = CH_2CH_3

1a R = H, $R^1 = R^2 = R^3 = CH_3$
1b R = H, $R^1 = R^2 = CH_3$, R^3 = H
1c R = H, $R^1 = R^2 = CH_2CH_3$, $R^3 = CH_3$
1d R = H, $R^1 = R^2 = CH_2CH_3$, R^3 =H
1e R = H, $R^1 = R^2 = CH_3$, $R^3 = CH_2CH_3$
1f R = CH_3, R^1 = H, $R^2 = R^3 = CH_2CH_3$

Scheme 1.

After purification by dry chromatography, these compounds were obtained as an off-white solid (**3a**, 48%) and as an oil (**3b**, 45%) and were characterised by high resolution mass spectrometry, IR and NMR (^1H and ^{13}C) spectroscopy. The presence of the carbonyl group was confirmed by IR, which showed a strong band at 1717 (**3a**) and 1727 (**3b**) cm^{-1}. In the ^{13}C NMR, signals at δ 164.4 (**3a**) and 172.5 (**3b**) ppm were assigned to the carbonyl carbon.

When compound **2a** was heated with **3a** in refluxing methanol, benzo[*a*]phenoxazinium chloride **1a** was isolated by dry chromatography purification as the major product (77%). Although esterification occurred, the carboxylic acid derivative (**1b**) was also obtained in 22%. Starting with 5-diethyl-2-nitrosophenol hydrochloride (**2b**) both dyes **1c** and **1d** were obtained in 64 and 7% yield, respectively. Reaction of compounds **2a** and **2c** with *N*-alkylated-naphthylamine **3b** in ethanol and under the same conditions described above led to benzo[*a*]phenoxazinium chlorides **1e** and **1f** in high yields (99%, **1e** and 90%, **1f**) (Table 1).

All dyes were obtained as blue materials and were fully characterised by elemental analysis or high resolution mass spectrometry, IR, NMR (^1H and ^{13}C) and visible spectroscopy.

The visible spectra of compounds **1a**–**f** in degassed absolute ethanol showed absorption peaks between 500 (**1a**) and 638 (**1c**) nm with ε values ranging from 12,075 (**1b**) to 43,030 (**1c**) (Table 1 and Fig. 1).

The fluorescence properties of the functionalised benzo[*a*]phenoxazinium salts **1a**–**f** measured in degassed absolute ethanol, using oxazine 1 as standard are summarised in Table 2. All compounds exhibit fluorescence, although in the case of **1a**, **1c** and **1e**, the quantum efficiency was rather low. However the presence of the carboxylic acid (compounds **1b** and **1d**) instead of the ester function (**1a** and **1c**) increased in two (**1a/1b**) or near five times (**1c/1d**) the fluorescence intensity. When 4-methyl-5-*N*-ethylamino-2-nitrosophenol was used a large increase in the fluorescence yield of the corresponding dye (**1f**, $\phi = 0.50$) was detected. The dyes reported showed large Stokes' shift (the lowest being 53 nm for **1f** and the highest 122 nm for **1e**). These results showing that the nature of the substituent groups on the benzo[*a*]phenoxazine moiety influences the fluorescence quantum yields as well as the wavelength of maximum emission. Emission spectra of dyes **1b**–**d** and **1f** measured in ethanol are presented in Figure 2.

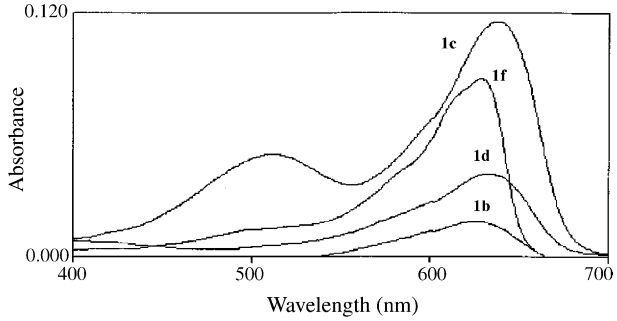

Figure 1. Absorbance spectra of compounds **1b**–**d** and **1f** measured in ethanol.

Having these facts in mind and further biological applications of these compounds, we also investigated their behaviour in water at physiological pH (pH 7.4). The high polarity of the aqueous solution leads to a bathochromic shift in both absorption (except for **1f**) and emission maxima as the largest change was in case of compound **1a** shifted from 500 nm (in ethanol) to 640 nm (in water) (absorption) and from 612 to 682 nm (emission) (Tables 1 and 2). All compounds exhibit fluorescence in water and the fluorescence quantum yield increased for compounds **1a**, **1c** and **1e**; **1f** was the most fluorescent moiety synthesised ($\phi = 0.32$) (Table 2).

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

Compound	Yield (%)	Vis λ_{max} (nm) (ε)[a]	Vis λ_{max} (nm) (ε)[b]
1a	77	500 (24,667)	640 (20,942)
1b	22	625 (12,075)	635 (14,063)
1c	64	638 (43,030)	650 (34,109)
1d	7	635 (22,458)	645 (24,219)
1e	99	633 (22,615)	640 (18,391)
1f	90	630 (29,798)	625 (57,895)

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

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

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	497	612	0.051	115	600	682	0.10	82
1b	588	669	0.110	81	600	682	0.094	82
1c	510	618	0.049	108	600	685	0.065	85
1d	588	666	0.225	78	600	684	0.080	84
1e	498	620	0.053	122	590	678	0.080	88
1f	590	643	0.50	53	600	654	0.32	54

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

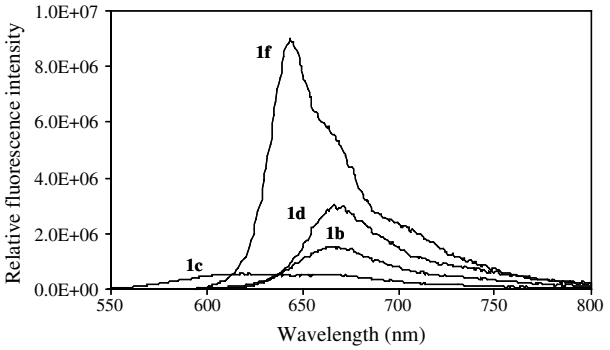

Figure 2. Fluorescence spectra of compounds **1b–d** and **1f** measured in ethanol.

Considering the high yields of the cyclisation reaction, the presence of a functional group, which gives the additional possibility of coupling these fluorophores to biomolecules by a covalent bond and also their water solubility, in connection with their longer wavelength of maximum visible absorption and emission fluorescence even in water at physiological pH, benzo[*a*]phenoxazines **1a–f** are expected to be good probes for biological applications.

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21. Typical procedure for the syntheses of **1a–f** (described for **1f**): To a cold solution (ice bath) of 4-methyl-5-*N*-ethylamino-2-nitrosophenol hydrochloride (144 mg; 8.23×10^{-4} mol) in ethanol (6 mL), ethyl-3-*N*-anthranylaminopropanoate (200 mg; 8.23×10^{-4} mol) and concentrated hydrochloride acid (5.0×10^{-2} mL) were added. The mixture was refluxed for 6 h and 30 min and monitored by TLC (silica: chloroform–methanol, 5.9:0.1). 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). Compound **1f** was obtained as a blue solid (297 mg; 90%). Mp 195.2–197.0 °C. R_f 0.48 (silica: chloroform–methanol, 6:1). UV–vis (EtOH): λ_{max} 630 (29,798) nm. FTIR (KBr, 1%): ν_{max} 3216, 2953, 1731, 1644, 1592, 1564, 1520, 1504, 1449, 1318, 1261, 1186, 1163, 1137, 1015, 801 cm^{-1}. ^1H NMR (CD_3OD, 300 MHz) δ 1.34 (6H, t J 7.2 Hz, OCH_2CH_3 and $NHCH_2CH_3$), 2.13 (3H, s, CH_3), 2.91 (1H, t J 6.6 Hz, $NHCH_2CH_2$), 3.30–3.40 (3H, m, $NHCH_2CH_3$ and NH), 3.78 (2H, t J 6.6 Hz, $NHCH_2CH_2$), 4.26 (1H, q J 7.2 Hz, OCH_2CH_3), 6.35 (1H, s, 8-H), 6.50 (1H, s, 6-H), 7.10 (1H, s, 11-H), 7.52–7.70 (2H, m, 2-H and 3-H), 8.05 (1H, d J 8.1 Hz, 1-H), 8.31 (1H, d J 7.2 Hz, 4-H) ppm. ^{13}C NMR (CD_3OD, 75 MHz): δ 14.2 ($NHCH_2CH_3$), 14.6 (OCH_2CH_3), 17.8 (CH_3), 34.0 ($NHCH_2CH_2$), 39.8 ($NHCH_2CH_3$), 41.7 ($NHCH_2CH_2$),

62.1 (OCH_2CH_3), 93.9 (6-C), 94.4 (8-C), 123.7 (1-C), 124.6 ($2 \times$ Ar–C), 124.9 (4-C), 128.3 (10-C), 130.4 (3-C), 131.4 (Ar–C), 131.6 (2-C), 132.3 (11-C), 133.7 (Ar–C), 148.4 (Ar–C), 151.5 (Ar–C), 156.1 (9-C), 157.8 (5-C), 172.9 ($CO_2CH_2CH_3$). The assignments were supported by HMBC and HMQC techniques. Anal. Calcd for $C_{24}H_{26}N_3O_3 \cdot 3.5HCl$: C, 54.17; H, 5.59; N, 7.89. Found: C, 53.78; H, 5.44; N, 7.70.

22. Typical procedure for the synthesis of **3b**: To a solution of 1-naphthylamine (2 g; 14.0 mmol) in ethanol (5 mL), 3-ethyl-3-bromopropionate (1.88 mL, 14.7 mmol) was added and the resulting mixture was refluxed for 11 h and monitored by TLC (silica: chloroform). The solvent was removed under reduced pressure and the crude mixture was purified by dry chromatography (silica: chloroform). Compound **3b** was obtained as a colourless oil (45%, 1.51 g). R_f 0.76 (silica: chloroform–methanol, 5.9: 0.1). FTIR (neat): ν_{max} 3434, 3052, 2981, 2937, 2905, 2871, 1727, 1625, 1583, 1530, 1483, 1444, 1410, 1374, 1347, 1315, 1284, 1252, 1214, 1188, 1120, 1094, 1049 cm^{-1}. ^1H NMR (CD_3Cl_3, 300 MHz) δ 1.31 (3H, t J 7.2 Hz, OCH_2CH_3), 2.78 (2H, t J 6.3 Hz, NCH_2CH_2), 3.64 (2H, t J 6.3 Hz, NCH_2CH_2), 4.19 (2H, q J 7.2 Hz, OCH_2CH_3), 4.91 (1H, br s, NH), 6.66 (1H, d J 7.5 Hz, 4-H), 7.29 (1H, d J 8.4 Hz, 2-H), 7.39 (1H, t J 7.5 Hz, 3-H), 7.42–7.52 (2H, m, 6-H and 7-H), 7.80–7.89 (2H, m, 8-H and 5-H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.1 (OCH_2CH_3), 33.5 (NCH_2CH_2), 39.6 (NCH_2CH_2), 60.6 (OCH_2CH_3), 104.4 (4-C), 117.7 (2-C), 119.9 (5-C), 123.6 (4a-C), 124.7 (7-C), 125.7 (6-C), 126.4 (3-C), 128.5 (8-C), 134.3 (8a-C), 142.8 (1-C), 172.5 ($CO_2CH_2CH_3$). The assignments were supported by HMBC and HMQC techniques. HRMS: m/z (FAB): calcd for $C_{15}H_{17}NO_2$ [M^+] 243.1255; found 243.1259.