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Synthesis and spectral properties of long-wavelength fluorescent dyes

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Abstract

Benzo[*a*]phenoxazinium salts were synthesised by reacting 5-alkylamino-2-nitrosophenol hydrochloride with *N*-alkylated-naphthylamine in good to excellent yields. Photophysical properties of these fluorophores with emphasis in solvent effects were studied. Remarkable shifts in the absorption and emission maximum have been observed as a function of polarity and proton accepting capability of the solvents. The influence on the fluorescence quantum yield was also studied.

Keywords: Near-infrared dyes; Fluorescence probes; Oxazine derivatives; Benzo[*a*]phenoxazines; Solvent effect

1. Introduction

The fluorometric detection method has been widely used in medical area and diagnostics as well as in environmental analysis and material sciences. To make this method more useful, advances in instrumentation along with the synthesis of new fluorescent reagents are of extreme importance. In recent years, many fluorescence compounds have been reported to label biomolecules. However, only a few are long-wavelength absorbing and fluorescent dyes. The development and use of these type of fluorophores are valuable, because the background autofluorescence from biological samples, typically in the blue or green region of the spectrum, could interfere in the measurement of the label emission. Therefore, it is desirable to enhance the sensitivity of detection by using fluorophores with absorption and emission in the red or near-infrared spectral region.

Long-wavelength excitation and emission probes have been synthesised [1–3] and used in protein labelling [4], chromatography studies [5], measurements in blood [6] and DNA analysis [7]. Among these reagents, oxazine derivatives have been reported in several spectroscopic investigations because of their wide use as a dye laser in the range 600–900 nm. They are also used as standards for fluorescence measurements, and in biological stains [8,9]. These studies showed that the absorption and emission properties of this family of compounds are affected by the solvent physical and chemical characteristics. Benzophenoxazines and benzo[*a*]phenoxazines have been used in various biomedical applications [10–13], and also as biomarkers for nucleic acid detection [14,15], and protein labelling [16]. Despite their importance for bioassays purposes, fewer have reactive groups, such as carboxylic function.

With this in mind and the fact that only dyes having a suitable functional group, which will react with the analyte, appropriate for covalent labelling, we prepared new functionalised benzo[*a*]phenoxazininium salts. Thus, 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. To extend our previous work [17], we now report the efficient synthesis and characterisation of several side-chain substituted 5,9-diaminobenzo[*a*]phenoxazinium dyes **1**. Slight variations in the side-chain substituents can have marked effects on the photophysic behaviour of these chromophores. The effects were studied using the set of new benzo[*a*]phenoxazininium

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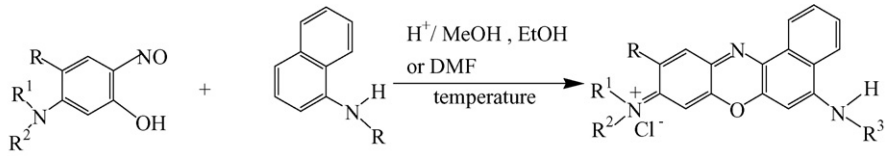

2a R = H, $R^1 = R^2 = CH_3$
2b R = H, $R^1 = R^2 = CH_2CH_3$
2c R = H, R^1 = H , $R^2 = (CH_2)_5CH_3$
2d R = CH_3, R^1 = H , $R^2 = CH_2CH_3$

3a R = $(CH_2)_2CO_2H$
3b R = $(CH_2)_2CO_2CH_2CH_3$
3c R = $(CH_2)_2CO_2CH_3$
3d R = Ph
3e R = H

1a R = H, $R^1 = R^2 = CH_3$, $R^3 = (CH_2)_2CO_2CH_3$
1b R = H, $R^1 = R^2 = CH_3$, $R^3 = (CH_2)_2CO_2H$
1c R = H, $R^1 = R^2 = CH_3$, $R^3 = (CH_2)_2CO_2CH_2CH_3$
1d R = H, $R^1 = R^2 = CH_3$, R^3 = Ph
1e R = H, $R^1 = R^2 = CH_3$, R^3 = H
1f R = H, $R^1 = R^2 = CH_2CH_3$, $R^3 = (CH_2)_2CO_2CH_3$
1g R = H, $R^1 = R^2 = CH_2CH_3$, $R^3 = (CH_2)_2CO_2H$
1h R = H, R^1 = H, $R^2 = (CH_2)_5CH_3$, R^3 = Ph
1i R = CH_3, R^1 = H, $R^2 = CH_2CH_3$, $R^3 = (CH_2)_2CO_2H$
1j R = CH_3, R^1 = H, $R^2 = CH_2CH_3$, $R^3 = (CH_2)_2CO_2CH_2CH_3$
1l R = CH_3, R^1 = H, $R^2 = CH_2CH_3$, $R^3 = (CH_2)_2CO_2CH_3$
1m R = CH_3, R^1 = H, $R^2 = CH_2CH_3$, R^3 = Ph

Scheme 1.

derivatives synthesised. Thus, spectral properties, i.e. absorption and fluorescence, emphasising on the solvent influence were also studied.

2. Results and discussion

2.1. Synthesis

Among the few synthetic methodologies for the synthesis of benzo[*a*]phenoxazinium salts that have been reported in the literature [18], we decided to prepare benzo[*a*]phenoxazinium chlorides **1** by the reaction of 5-alkylamino-2-nitrosophenol hydrochloride **2a–d**, with *N*-alkylated-naphthylamine **3a–e** in acidic medium (Scheme 1). The required 5-alkylamino-2-nitrosophenol hydrochloride **2a–d** was synthesised by usual procedure involving treatment of the corresponding 3-alkylaminophenol with sodium nitrite in acid solution. 3-Alkylaminophenols used were commercial reagents, except in case of 3-hexylaminophenol which was obtained by refluxing 3-aminophenol with 1-bromohexane in ethanol by the usual procedure.

N-alkyl-naphthylamines **3a–c** were prepared by alkylation of 1-naphthylamine with chloropropionic acid, 3-ethyl-3-bromopropionate and 3-methyl-3-bromopropionate, respectively. After purification by dry chromatography, compounds **3a–c** were obtained as solid (**3a**) and oils (**3b** and **3c**) in yields ranging from 45 to 55%, and were characterised by high resolution mass spectrometry, IR and NMR (^{1}H and ^{13}C) spectroscopy. The presence of the carbonyl group was confirmed by IR, which showed a strong band at 1717 (**3a**), 1727 (**3b**) and 1731 cm^{-1} (**3c**). In the ^{13}C NMR, signals at δ 164.4 (**3a**) and about 173.0 ppm (**3b** and **3c**) were assigned to the functional group. Compounds **3d** and **3e** used were commercial reagents.

When 5-alkylamino-2-nitrosophenol hydrochloride (**2a**) reacted with 3-(naphthalen-1-ylamino) propanoic acid (**3a**) in the presence of hydrochloric acid, reflux in 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%. Reaction of **2a** with the ethyl 3-(naphthalen-1-ylamino) propanoate (**3b**), in ethanol, gave **1c** in a quantitative yield (99%). In the preparation of dyes **1d** (76%) and **1e** (66%) the nitrosophenol component used was also **2a** which reacted with *N*-phenyl-1-naphthylamine (**3d**) and 1-naphthylamine (**3e**), respectively.

Starting with 5-(diethylamino)-2-nitrosophenol hydrochloride (**2b**) and **3a**, reaction in methanol, both dyes **1f** (64%) and **1g** (7%) were obtained. The reaction between 5-(hexylamino)-2-nitrosophenol (**2c**) and **3d** yielded compound **1h** in 46%.

To avoid the possibility of esterification, compound **1i** (45%) was prepared by reaction of 5-(ethylamino)-4-methyl-2-nitrosophenol hydrochloride (**2d**) with propanoic acid derivative **3a**, using DMF as solvent and heating at 70 °C. Reaction of the same nitroso precursor (**2d**) with the alkylated naphthylamines **3b** and **3c** under reflux in ethanol gave benzo[*a*]phenoxazinium salts **1j** and **1l** in high yields (90%, **1j**; 75%, **1l**) (Table 1). Dye **1m** was prepared in 54% yield by reaction of compounds **2d** and **3d** using the same conditions reported above.

Although compounds **1d** and **1e** were reported before [19–23], we decided to prepare them, for comparison with the new fluorophores, at the same conditions, in the absorption and fluorescence studies. In addition their full characterisation was also presented, which from the knowledge of the authors, had never been reported.

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

The IR shows bands due to the ester group for compounds **1a**, **1c**, **1f**, **1j** and **1l** between 1723 (**1c** and **1l**) and 1738 cm^{-1} (**1a**). In ^{13}C NMR, signals from δ 172.0 (**1c**) to δ 173.3 ppm (**1l**) and from δ 164.3 (**1i**) to δ 169.3 ppm (**1g**) also confirmed the presence of the carbonyl function of the ester (**1a**, **1c**, **1f**, **1j** and **1l**) and the acid (**1b**, **1g** and **1i**). The ^{1}H and ^{13}C NMR spectra of derivatives **1d**, **1h** and **1m** showed the expected signals due

Table 1
Synthesis and visible data of benzo[*a*]phenoxazinium dyes **1a–m**

Product (no.)	Reflux time (h)	Yield (%)	λ_{max} (nm) (ε, $10^4\,M^{-1}cm^{-1}$)[a]	λ_{max} (nm) (ε, $10^4\,M^{-1}\,cm^{-1}$)[b]
1a	4	77	500 (3.5)	640 (2.1)
1b	4	22	625 (1.2)	635 (1.4)
1c	7	99	633 (2.3)	640 (1.8)
1d	8.5	76	515 (4.4)	[c]–
1e	2	66	625 (2.9)	[c]–
1f	7	64	638 (4.3)	650 (3.4)
1g	7	7	635 (2.2)	645 (2.4)
1h	4.5	46	520 (2.0)	[c]–
1i	14[d]	45	625 (2.6)	625 (1.3)
1j	6.5	90	630 (3.0)	625 (5.8)
1l	6	75	615 (3.8)	620 (2.1)
1m	7	54	523 (3.4)	[c]–

[a] Spectra were measured in absolute ethanol.
[b] Spectra were measured in water (pH 7.4).
[c] Compounds insoluble in water (pH 7.4).
[d] Reaction temperature was 70 °C.

to the additional phenyl ring at 6.96–7.44 and 117.7–130.2 ppm, respectively.

2.2. Absorption and emission studies

Electronic absorption spectra of 10^{-6} M solutions of compounds **1a–m** in absolute ethanol showed absorption peaks between 500 (**1a**) and 638 nm (**1f**) with ε values ranging from 1.2×10^4 (**1b**) to $4.4 \times 10^4\,M^{-1}\,cm^{-1}$ (**1d**) (Table 1). Bearing in mind further biological applications of these compounds, we also investigated their behaviour in aqueous medium. In aqueous solutions at physiological pH (pH 7.4, adjusted with HCl and NaOH), the position of the absorption maximum shows a huge bathochromic shift for compound **1a** which changes from 500 nm (in ethanol) to 640 nm (in water) (Table 1). This fact could be explained by a greater stabilization of the excited state energy relative to that of the ground state with increasing polarity, resulting in a decrease of the HOMO–LUMO energy separation. The observed increase (~140 nm) seems too large and we note that not all compounds show maximum absorbance near 500 nm in ethanol (only **1a**, **1d**, **1h** and **1m**) which now appears in the 600 nm region.

In water, charged dyes are known to aggregate due to the high dielectric constant of water reducing the electrostatic repulsion. The oxazine dyes are known to form H-aggregates [24]. These types of dimers have an absorption band to the blue of the monomer and have a very low fluorescence quantum yield. The differences observed in absorption maximum can then be due to variations of dimerization equilibrium constants with the solvent. In order to test this hypothesis we measured absorption spectra of compound **1a** at different concentrations (Fig. 1) in several solvents.

We confirm the formation of H-aggregates in water with a separation of ~40 nm between the absorption maxima of the monomer and the dimer. The extent of this dimerization depends on the substituents of the benzo[*a*]phenoxazine compounds studied in this work (Fig. 2). As H-aggregates are non-fluorescent we can obtain a pure monomer spectra from the excitation spectra.

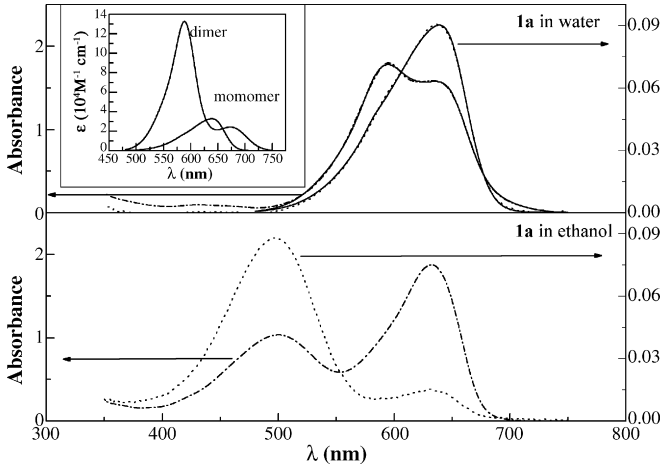

Fig. 1. Absorption spectra of **1a** at 2.75×10^{-6} M (dotted line) and 5×10^{-5} M (dash-dotted line) concentration, in water (pH 7.4) (A) and in ethanol (B). Solid lines in A are the fitted spectra. The inset in A plots the monomer molar absorptivity and half the dimer molar absorptivity.

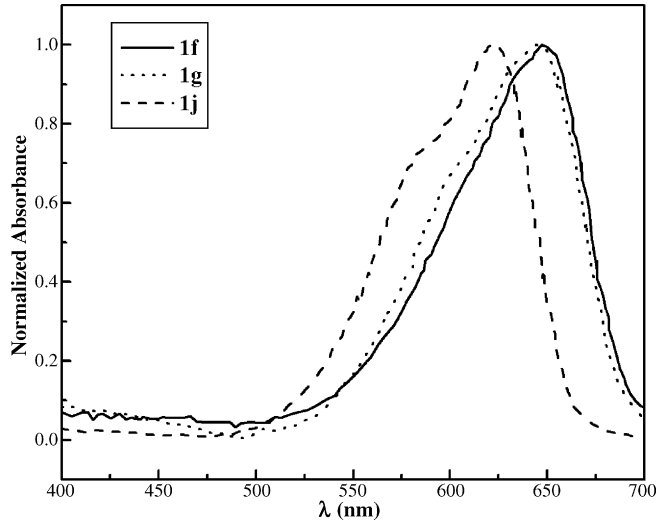

Fig. 2. Absorption spectra of **1f** at 1.3×10^{-6} M, **1g** at 1.5×10^{-6} M and **1j** at 2.3×10^{-6} M in water (pH 7.4).

Table 2
The absorption spectra data of compounds **1a**, **1c**, **1f**, **1g**, **1j** and **1m** in various solvents

Solvent	1,4-Dioxane	Chloroform	Ethyl acetate	Dichloromethane	Acetone	Ethanol	Methanol	Acetonitrile	DMF	Water
ε[a]|π^*	2.20|0.55	4.80|0.58	6.08|0.55	8.93|0.82	21.0|0.71	25.3|0.54	33.0|0.6	36.6|0.75	38.25|0.88	80.1[b]|1.09
$\beta\|\alpha$	0.37|0	0|0.44	0.45|0	0|0.3	0.48|0.08	0.77|0.83	0.62|0.93	0.31|0.19	0.69|0	0.18|1.17
1a										
$\lambda_1\|\lambda_2$	485|–	497|619	489|–	505|629	492|–	500|634	500|629	493|639	503|–	–|640
$\varepsilon_1\|\varepsilon_2$	1.0|–	1.1|1.1	1.3|–	1.2|3.4	3.5|–	3.5|0.6	0.2|2.0	3.2|0.3	6.3|–	–|2.1
Concentration	5.9	3.3	7.2	3.9	4.5	2.7	5.0	2.8	3.2	1.9
1c										
$\lambda_1\|\lambda_2$	487|–	531|616	489|611	503|638	494|–	–|633	–|629	495|636	501|–	–|640
$\varepsilon_1\|\varepsilon_2$	1.3|–	0.5|1.7	1.5|0.3	0.8|2.2	1.5|–	–|2.3	–|2.2	2.2|0.4	1.1|–	–|1.8
Concentration	3.0	4.4	7.0	1.9	4.7	2.8	4.0	2.8	3.5	1.7
1f										
$\lambda_1\|\lambda_2$	497|602	|628	499|621	514|635	505|–	512|638	–|637	507|640	511|	–|650
$\varepsilon_1\|\varepsilon_2$	1.8|0.6	|5.7	2.1|0.6	1.1|3.7	3.1|–	1.9|4.3	–|4.6	1|1.6	2.9|	–|3.4
Concentration	8.9	4.3	4.8	4.5	4.2	9.9	4.6	14.9	5.0	1.3
1g										
$\lambda_1\|\lambda_2$	489|–	–|626	494|–	539|634	505|–	–|635	–|632	524|628	511|	–|645
$\varepsilon_1\|\varepsilon_2$	1.2|–	–|2	2.1|–	0.4|0.6	1.4|–	–|2.2	–|2.2	1|0.5	1.1|	–|2.4
Concentration	3.1	2.3	1.9	3.8	3.1	5.6	4.1	1.7	1.9	3.2
1j										
$\lambda_1\|\lambda_2$	495|590	505|610	495|–	–|620	496|–	–|630	–|625	–|620	510|–	–|625
$\varepsilon_1\|\varepsilon_2$	2.2|0.8	1.4|1.9	0.9|–	–|2.8	3.4|–	–|3	–|6.8	–|2.5	2.8|–	–|5.8
Concentration	2.4	1.5	4.9	2.0	2.9	4.8	4.8	2.1	4.2	2.3
1m										
$\lambda_1\|\lambda_2$	502|–	506|–	509|–	510|–	514|–	523|–	527|634	517|–	527|–	–|–
$\varepsilon_1\|\varepsilon_2$	2.7|–	3.4|–	2.2|–	4.8|–	3.8|–	3.4|–	3.1|1.4	3.1|–	9.1|–	–|–
Concentration	3.3	5.1	2.5	2.0	3.2	2.0	13.7	2.8	2.6	

Units: Concentration, μM; λ_1 and λ_2 nm; ε_1 and ε_2, $10^4 M^{-1}\,cm^{-1}$. π^*, β and α—Kamlet–Taft solvents parameters [25].
[a] Dielectric constant.
[b] Value for distilled water.

In order to estimate the dimerization equilibrium constant, we can simultaneously fit both absorption spectra, at high and low concentrations, by using the following equations:

$$A(\lambda) = \alpha \mathrm{Ex}(\lambda) f_M C + \frac{\varepsilon_D(\lambda)}{2}(1 - f_M)C$$

$$k_D = \frac{1 - f_M}{2C f_M^2}$$

where k_D is the dimerization equilibrium constant, C the dye concentration and f_M the mole fraction of monomer, Ex(λ) represents the excitation spectrum, α a proportionality constant and $\varepsilon_D(\lambda)$ the dimmer absorption spectrum taken as a sum of three Gaussian functions.

In Fig. 1a the solid lines represent the result of this fitting procedure. A value of $k_D = 2.09 \times 10^3\ M^{-1}$ was then obtained for the dimerization equilibrium constant. This estimate is in accordance with a recent k_D determination of an oxazine derivative where double lysine labelling was used in order to obtain a pure dimer absorption spectrum [33]. From the described fitting procedure we could also obtain the dimer absorption spectrum which is plotted in the inset of Fig. 1a.

For other solvents, not only is the wavelength separation of the two band much greater (~100 nm), but the weight of the blue band also increased upon dilution (Fig. 1). This is a clear indication that no dimerization occurred, but rather an interaction with the solvent (possibly with the labile hydrogen atoms in the amine groups), which increased when diluted. This effect can be explained by the influence of concentration in the ionization equilibrium of weak acids ($HA \rightarrow H^+ + A^-$). If the acid/base ionization constant is 10^{-5} M the ratio $[A^-]/[HA]$ goes from 2.7 to 0.56 when the HA concentration increases from 5 to 50 μM.

In order to further elucidate this solvent interaction, absorption spectra of compounds **1a**, **1c**, **1f**, **1g**, **1j** and **1m** were run in another eight solvents of different polarity and proton donor ability, such as 1,4-dioxane, chloroform, ethyl acetate, dichloromethane, acetone, methanol, DMF and acetonitrile. The wavelength maxima (λ_{max}) and molar absorptivities (ε) of these compounds are listed in Table 2. Some representative spectra are shown in Fig. 3.

We observed one main absorption in the 500 nm region and another near 600 nm. The weight of these two bands depends not only on the solvent but also on the substituents of the benzo[*a*]phenoxazine moiety. Also in Table 2 we have included the Kamlet–Taft solvent parameters [25] π^*, β and α. Parameter π^* takes into account the solvent capability of stabilizing ionic solutes (dipolarity/polarizability). Parameter β evaluates the ability of a solvent to accept a proton (donate an electron pair) in a solvent to solute hydrogen bond. Parameter α evalu-

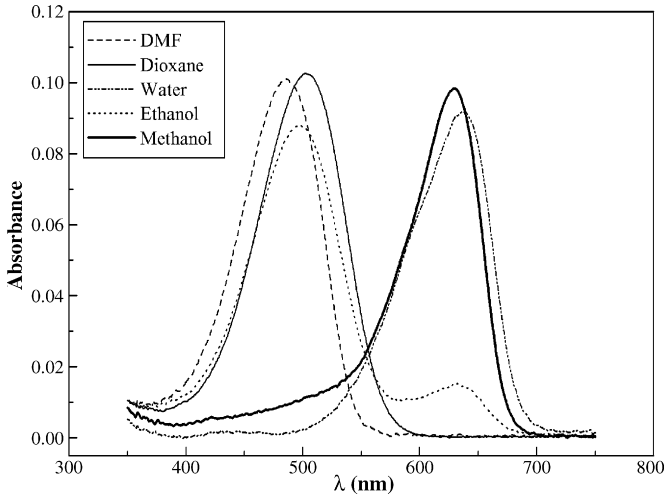

Fig. 3. Absorption spectra of **1a** at 5.0×10^{-6} M in DMF, 1,4-dioxane, water (pH 7.4), ethanol and methanol.

Table 3
Fluorescence data for compounds **1a–m** in absolute ethanol

Product (no.)	Fluorescence			Stokes' shift (nm)
	λ_{exc} (nm)	λ_{em} (nm)	Φ_F	
1a	497	612	0.051	115
1b	588	669	0.110	81
1c	498	620	0.053	122
1d	515	643	0.0017	128
1e	590	661	0.19	71
1f	510	618	0.049	108
1g	588	666	0.225	78
1h	520	622	0.0022	102
1i	590	644	0.44	54
1j	590	643	0.50	53
1l	590	643	0.49	55
1m	521	–	–	–

ates the ability of a solvent to donate a proton (accept an electron pair) in a solvent to solute hydrogen bond.

We observed that for solvents with high hydrogen bond accepting capability (acetone, DMF, dioxane and ethyl acetate) we can see the predomination of the blue (∼500 nm) absorption band. We can conclude that the solvent interacts with benzo[*a*]phenoxazinium dyes by either accepting a hydrogen bond, or by completely removing the amine proton thus originating the corresponding base. Douhal [26] showed that for Nile Blue (NBA: benzo[*a*]phenoxazinium salt with R^1=R^2=CH_2CH_3 and R^3=H) the hydrogen bond interaction results in a ∼5 nm blue shift, and the complete removal of a proton from the amine (forming Nile Blue base, NBB) results in the formation of a new band shifted ∼100 nm to the blue. We can then assign the band near 500 nm to the basic form of the studied compounds.

Chloroform and dichloromethane are the only solvents that cannot accept a proton. But in some compounds the band corresponding to the basic form is observed. This can be explained by the presence of trace amount of water in these solvents that can then accept a proton from the red absorbing acid form of these dyes. Douhal [26] also observed a small fluorescence from NBB in a solution of NBA in neat 1-chloronaphthalene, which decreased upon drying of the solvent.

In both DMF and acetone only the basic form is observed for all compounds. This is due to the strong proton accepting nature of these solvents.

Coming from ethanol to water the proton accepting capability decreases while the proton donor strength increases. In accordance with these variations we observe that the basic form is only important in ethanol and completely vanishes in water. Note that in methanol the absorbance spectrum shows a tail to the blue which is completely absent in water solutions (see Fig. 3).

The R^1, R^2 and R^3 substituents also affect the relative amount of basic and acid forms as can be seen from Table 2. This probably occurs through inductive/chemical effects on the acidity of the amino group of the benzo[*a*]phenoxazinium derivatives. For example, in compounds **1a** and **1c** we observe that only an extra methylic group in the R^3 ester substituent decreases the acidity of the amino group leading to the appearance of acid form in ethyl acetate. Using geometry optimisation with semi-empirical quantum methods within the PM3 framework by ArgusLab software, we may conclude that the resonance structure with a double bond in the N atom with the R^3 substituent establishes a hydrogen bond with the ester group (N···H distance of 1.02 Å and H···O distance of 1.83 Å). The slightly stronger electron donating capability of the C_2H_5 group in the ester leads to a stronger hydrogen bond and a corresponding reduced acidity of the amine. Thus, the absorbance spectrum of these oxazine dyes can be tuned by the type of substituents and are very sensitive to the proton accepting capability of the solvent.

In the case of compound **1a** and **1f** which differ only in an extra methylene group in R^1 and R^2 positions, the inductive effect in the proton on the other side of the molecule is expected to be very small. Yet we observe that the dominant form in the absorption spectrum changes from basic to acid in ethanol solutions. This variation can be mainly due to a duplication of concentration from **1a** to **1f**. If the acid/base dissociation constant is 4×10^{-6} M the ratio $[A^-]/[HA]$ goes from 1.47 to 0.9 when the HA concentration increases from 5 to 10 μM.

Compounds **1j** and **1m** have two labile protons but the absorbance characteristics are not much different from those observed for the other compounds which only have one removable proton.

The fluorescent properties of benzo[*a*]phenoxazinium salts **1a–m** measured in absolute ethanol, using Oxazine 1 as standard (fluorescence quantum yield, $\Phi_F = 0.11$ [27] in ethanol) are summarised in Table 3.

When the basic form is dominant (compounds **1a**, **1c**, **1d**, **1f**, **1h** and **1m**) the fluorescence occurs with a large Stokes shift (102–128 nm) with emission maximum in the 612–643 nm region. When the acid form is more important (compound **1b**, **1e**, **1g**, **1i**, **1j** and **1m**) the emission maximum occurs in 643–669 nm with a smaller Stokes' shift (53–81 nm). Douhal [26] observed a similar behaviour for Nile Blue. Thus, we assigned the emissions in the 612–643 and 643–669 nm regions to the basic and acid forms of benzo[*a*]phenoxazinium dyes, respectively.

As the Stokes' shift of the basic form is larger than the acid one we can say that the protonated form interacts less with the

Table 4
Fluorescence data for compounds **1a–c**, **1f**, **1g** and **1i–l** in water (pH 7.4)

Product (no.)	Fluorescence			Stokes' shift (nm)
	λ_{exc} (nm)	λ_{em} (nm)	Φ_F	
1a	600	682	0.10	82
1b	600	682	0.094	82
1c	590	678	0.080	88
1f	600	685	0.065	85
1g	600	684	0.080	84
1i	580	652	0.28	72
1j	600	654	0.32	54
1l	580	650	0.19	70

Compounds **1d**, **1e**, **1h** and **1m** were insoluble in water (pH 7.4).

solvent than the deprotonated one. We can also see that the Φ_F of the basic form is one order of magnitude lower than the acid one. Thus, the stronger interaction of the basic form with the solvent seems to introduce an enhanced non-radiative deactivation of the excited state.

When R^3 is a phenyl group (compounds **1d**, **1h** and **1m**) the absorption maximum of the basic form shifts 10 nm to the red (Table 1) and Φ_F decreases another order of magnitude (Table 3). When the R group is changed from H to CH_3 we observe a ~4 times increase in the fluorescence quantum yield (**1b/1i**). It seems that electron donating groups in the R position enhance the fluorescence of these compounds while electron donating groups in the R^3 position originate a quenching effect.

Fluorescence spectra of water-soluble compounds were also measured in water (pH 7.4) and the results are reported (Table 4).

In aqueous media only the acid form of all benzo[*a*]phenoxazinium compounds is observed. For those compounds that were excited in ethanol in the basic form (**1a**, **1c**, **1d** and **1f**) an increase of quantum yield was observed in water as the acid form is more fluorescent than the basic one (see Table 3). For the other compounds we can see that the acid form seems less fluorescent in water than in ethanol. This can be the result of the formation of non-fluorescent H-aggregates in aqueous media. This phenomena still occurs in the diluted solutions used in fluorescent studies as is apparent from Fig. 1.

Fluorescent molecules, whose emission spectra and Φ_F are markedly sensitive to solvent properties, are widely used as reporter probes for investigating chemical, biochemical and biological phenomena [28,29].

Having these fact in mind, a fluorescence study was carried out with the most interesting synthesised fluorescent compound (**1j**), in solvents of different polarity (Table 5, Fig. 4).

Considering only the acid form emission we noticed that in acetonitrile the Φ_F is one order of magnitude lower than in the other solvents. Among the latter, the lowest fluorescence quantum yield occurred in water. This can again be explained by the residual dimerization process that produces a small fraction of dark H-aggregates. In the remaining solvents (chloroform, ethyl acetate and ethanol) the Φ_F increased with parameter α (see Table 2). The main internal deactivation pathway of oxazines

Table 5
The fluorescence data of compounds **1j** in various solvents

Solvent	λ_{exc} (nm)	λ_{em} (nm)	Φ_F	Stokes' shift (nm)
Chloroform	580	629	0.44	49
Ethyl acetate	590	644	0.41	54
Acetone	510	598	0.0145	88
Ethanol	590	643	0.50	53
Acetonitrile	590	644	0.038	54
DMF	490	611	0.023	121
Water (pH 7.4)	600	654	0.32	54

depends on its substituents and mainly determined by either the N–H vibrations or internal rotations of the *N*-alkyl groups [28]. In the case of oxazine 720, which is compound **1j** with an C_2H_5 group instead of a $(CH_2)_2CO_2CH_2CH_3$ in the R^3 position, the principal deactivation process is the *N*-alkyl rotation [8]. Thus, the observed variation of Φ_F with solvent can be determined by the influence of viscosity on rotation, or/and by an intermolecular process between **1j** and solvent molecules in the solvation sphere. The highest viscosity occurs in ethanol but it is in this solvent that the Φ_F is greater. Thus, we conclude that there is a deactivation of the excited state by an intermolecular process with solvent molecules. This process seems to be more important when the H-bond donating/electron density acceptance power of the solvent decreases. For Nile Blue, Kobayashi et al. [31] reported a high fluorescence quenching by DMA (dimethylaniline) through a photoinduced electron transfer from DMA to Nile Blue. So, we can say that the solvent deactivation of excited state benzo[*a*]phenoxazinium derivatives occur by an electron transfer process from the solvent molecules. This process can also explain the low Φ_F observed with compounds with phenyl group in R^3 position, as it can donate electron density. Also the very low Φ_F observed in acetonitrile can be explained by a very efficient electron transfer from acetonitrile to excited benzo[*a*]phenoxazinium molecules.

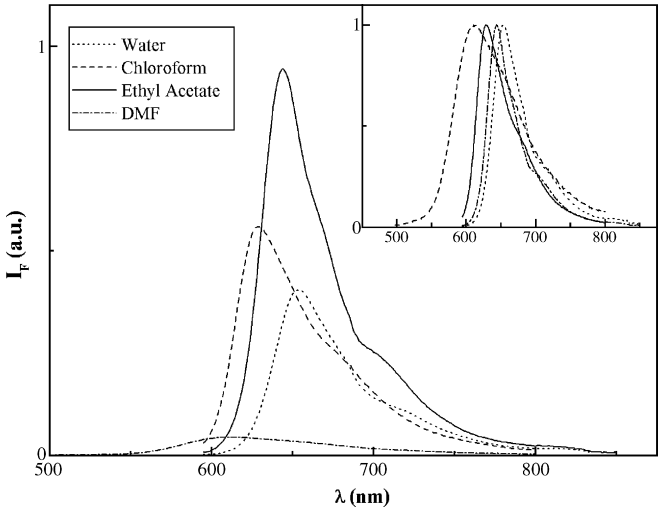

Fig. 4. Fluorescence spectra of **1j** at 2.3×10^{-6} M in water (pH 7.4), at 1.5×10^{-6} M in chloroform, at 4.9×10^{-6} M in ethyl acetate and at 4.2×10^{-6} M in DMF.

3. Conclusion

A series of new water-soluble long-wavelength benzo[*a*]phenoxazinium salts were synthesised. Studies of their absorption and emission properties in different solvents showed a relationship between their behaviour and the substituent groups on the dye nucleus. It was found that in water the photophysical behaviour is dominated by the acid form of the dye that can produce non-fluorescent H-aggregates depending on the substituent groups. In other solvents, the acid and basic forms coexist depending on the proton accepting nature of the solvent. The latter is one order of magnitude less fluorescent than the former. Small changes in the substituents have a marked effect on the acid/base equilibria, with intramolecular hydrogen bond making an important contribution. Our data also give some indication that one of the possible radiationless desactivation processes of the acid form excited state involves a photoinduced electron transfer process from solvent molecules. The results presented suggest good prospects for using of these cationic fluorophores as fluorescent probes in various applications, such as proton induced biological processes, photoinduced electron transfer studies, as well as local pH measurement.

4. Experimental

4.1. Synthesis

4.1.1. General

All melting points are uncorrected and they were measured on a Gallenkamp melting point apparatus. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$) and spots were visualised under UV light. Dry chromatography was carried out on Merck Kieselgel (230–240 mesh). Light petroleum refers to the fraction boiling within the range (40–60 °C). IR spectra were determined on a Perkin-Elmer FTIR-1600. ^{1}H NMR spectra were recorded on a Varian 300 spectrometer in 5% $CDCl_3$, DMSO-d_6 (DMSO) or CD_3OD solution at 25 °C. All chemical shifts are given in δ (ppm) using δ_H $Me_4Si = 0$ as reference and J values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities and J values. ^{13}C NMR spectra were run in the same instrument but at 75.4 MHz using the solvent peak as internal reference. Mass spectrometry analyses were performed at the C.A.C.T.I.—Unidad de Espectrometria de Masas of the University of Vigo, Spain, on a Hewlett Packard 5989 A spectrometer for low resolution spectra and a VG Autospec M spectrometer for high resolution mass spectra. Elemental analyses were carried out on a Leco CHNS 932 instrument. 3-(Dimethylamino)-phenol, 3-(diethylamino)-phenol, 3-(ethylamino)-4-methylphenol and *N*-phenyl-1-naphthylamine were commercial products. 3-Hexylaminophenol was prepared by alkylation of the 3-aminophenol with 1-bromohexane by the usual procedure. The required 5-alkylamino-2-nitrosophenol hydrochlorides **2a**–**d** were prepared by a standard method involving treatment of the corresponding 3-alkylaminophenol with sodium nitrite in acid solution.

4.1.2. General method for preparation of compounds *1a–h* and *1j–m*

To a cold solution (ice bath) of 5-alkylamino-2-nitrosophenol hydrochloride (**2a**–**d**) in methanol or ethanol, 1.0–1.54 equivalents of naphthalene compound (**3a**–**e**) and concentrated hydrochloride acid (5.0×10^{-2} mL) were added. The mixture was refluxed during 2–8.5 h (Table 1), and monitored by TLC (chloroform/methanol, 5.9:0.1). After evaporation of the solvent, dry chromatography on silica gel, and recrystallisation from chloroform/*n*-hexane, the required dye (**1a**–**h** and **1j**–**m**) was obtained as a blue material.

4.1.2.1. N-(5-(3-methoxy-3-oxopropylamino)-9H-benzo[a] phenoxazin-9-ylidene)-N-methylmethanaminium chloride (1a). The product of the reaction of **2a** (94.4 mg, 5.69×10^{-4} mol) with **3a** (187 mg, 8.70×10^{-4} mol) in methanol (2 mL) was chromatographed with chloroform/methanol (1:1) as the eluent to give the dye **1a** (165 mg, 77%). mp above 300 °C. TLC (chloroform/methanol, 6:1): $R_f = 0.64$. IR (KBr 1%): $\nu = 3442$, 2921, 1738, 1715, 1639, 1593, 1561, 1496, 1477, 1461, 1428, 1382, 1367, 1332, 1284, 1224, 1177, 1145, 1116, 1069, 1002 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.10$ (t, $J = 4.6$ Hz, 2H, $NHCH_2CH_2$), 3.19 (s, 6H, $N(CH_3)_2$), 3.72 (s, 3H, OCH_3), 4.07 (br s, 2H, $NHCH_2CH_2$), 6.40 (s, 1H, 8-H), 6.69 (s, 1H, 6-H), 6.82 (dd, $J = 9.0$ and 2.1 Hz, 1H, 10-H), 7.60 (d, $J = 7.2$ Hz, 1H, 11-H), 7.68–7.78 (m, 2H, 2-H, 3-H), 8.62–8.70 (m, 1H, 1-H), 9.09 (br s, 1H, 4-H) ppm. The assignments were supported by spin decoupling-double resonance. ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 34.8$ ($NHCH_2CH_2$), 40.5 ($N(CH_3)_2$), 44.0 (NCH_2CH_2), 51.8 (OCH_3), 95.1 (C-6), 96.5 (C-8), 110.9 (C-10), 123.8 (C-1), 125.3 (C-4), 126.6 (2 × Ar-C), 129.2 (Ar-C), 130.5 (C-3), 130.6 (C-2), 130.8 (C-11), 139.6 (Ar-C), 146.4 (2 × Ar-C), 153.0 (C-9), 157.8 (C-5), 172.7 (CO_2CH_3) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS (FAB): calcd. for $C_{22}H_{22}N_3O_3$ [M$^+$] 376.1661; found 376.1646. Although compound **1a** was the major product, compound **1b** was also isolated in this preparation.

4.1.2.2. N-(5-(2-carboxylethylamino)-9H-benzo[a]phenoxazin-9-ylidene)-N-methylmethanaminium chloride (1b). Compound **1b** was obtained (45 mg, 22%) in the same preparation of compound **1a**. TLC (chloroform/methanol, 8:2): $R_f = 0.47$. IR (KBr 1%): $\nu = 3407$–3000, 2928, 1630, 1593, 1556, 1538, 1429, 1335, 1292, 1180, 1060 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): $\delta = 2.71$ (s, 6H, $N(CH_3)_2$), 2.84 (br s, 2H, NCH_2CH_2), 4.00 (br s, 2H, NCH_2CH_2), 6.89 (br s, 1H, 8-H), 7.02 (br s, 1H, 6-H), 7.24–7.34 (m, 2H, 10-H, 11-H), 7.78–7.88 (m, 1H, 2-H), 7.93 (t, $J = 7.2$ Hz, 1H, 3-H), 8.41 (d, $J = 7.8$ Hz, 1H, 1-H), 8.86 (d, $J = 7.2$ Hz, 1H, 4-H) ppm; ^{13}C NMR (75.4 MHz, CD_3OD): $\delta = 35.4$ ($NHCH_2CH_2$), 41.2 (NCH_2CH_2), 47.8 ($N(CH_3)_2$), 95.0 (C-6), 97.2 (C-8), 111.1 (C-10), 120.7 (C-1), 124.1 (C-4), 125.6 (Ar-C), 126.2 (Ar-C), 129.8 (C-3), 131.1 (C-2), 131.5 (Ar-C), 133.1 (C-11), 133.8 (Ar-C), 149.3 (Ar-C), 149.6 (Ar-C), 153.8 (C-9), 157.3 (C-5), 167.5 (CO_2H) ppm. HRMS (FAB): calcd. for $C_{21}H_{20}N_3O_3$ [M$^+$] 362.1505; found 362.1500.

*4.1.2.3. N-(5-(3-ethoxy-3-oxopropylamino)-9H-benzo[a]phenoxazin-9-ylidene)-N-methylmethanaminium chloride (**1c**).* The product of the reaction of **2a** (44 mg, 2.65×10^{-4} mol) with **3b** (50 mg, 2.06×10^{-4} mol) in ethanol (2 mL) was chromatographed using chloroform/methanol (5.5:0.5) as the eluent to give the dye **1c** (79.5 mg, 99%). mp above 300 °C. TLC (chloroform/methanol, 6:1): $R_f = 0.52$. IR (KBr 1%): $\nu = 3432$, 3206, 2927, 1723, 1641, 1591, 1561, 1536, 1476, 1460, 1430, 1382, 1335, 1294, 1179, 1133, 1112, 1010 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 1.26$ (t, $J = 6.9$ Hz, 3H, $CO_2CH_2CH_3$), 3.17 (t, $J = 7.2$ Hz, 2H, NCH_2CH_2), 3.29 (s, 6H, $N(CH_3)_2$), 4.12–4.42 (m, 4H, $CO_2CH_2CH_3$, NCH_2CH_2), 6.60 (d, $J = 2.7$ Hz, 8-H), 6.91 (s, 1H, 6-H), 6.99 (dd, $J = 9.3$ and 2.4 Hz, 1H, 10-H), 7.79 (d, $J = 9.3$ Hz, 11-H), 7.86 (t, $J = 7.2$ Hz, 2H, 2-H, 3-H), 8.80 (d, $J = 7.95$ Hz, 1H, 1-H), 9.41 (d, $J = 7.8$ Hz, 1H, 4-H) ppm. The assignments were supported by spin decoupling-double resonance. ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 29.7$ ($CO_2CH_2CH_3$), 33.4 (NCH_2CH_2), 40.6 (NCH_2CH_2), 41.0 ($N(CH_3)_2$), 61.0 ($CO_2CH_2CH_3$), 93.6 (C-6), 96.0 (C-8), 114.2 (C-10), 124.1 (C-1), 126.1 (C-4), 127.5 (2× Ar-C), 128.8 (Ar-C), 130.6 (C-3), 132.0 (C-2), 132.1 (C-11), 135.2 (Ar-C), 146.9 (Ar-C), 151.4 (Ar-C), 154.7 (C-9), 158.8 (C-5), 172.0 ($CO_2CH_2CH_3$) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS (FAB): calcd. for $C_{23}H_{24}N_3O_3$ [M^+] 390.1818; found 390.1805.

*4.1.2.4. N-methyl-N-(5-(phenylamino)-9H-benzo[a]phenoxazin-9-ylidene)methanaminium chloride (**1d**).* The product of the reaction of **2a** (300 mg, 1.81×10^{-3} mol) with **3d** (590 mg, 2.71×10^{-3} mol) in methanol (5 mL) was chromatographed with chloroform/methanol (5.8:0.2) as the eluent to give the dye **1d** (500 mg, 76%). mp 278.8–280.1 °C. TLC (chloroform/methanol, 5.8:0.2): $R_f = 0.40$. IR (Nujol): $\nu = 2954$, 2923, 2854, 1642, 1601, 1588, 1558, 1523, 1461, 1376, 1366, 1320, 1202, 1132, 1114, 1005 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 3.05$ (s, 6H, $N(CH_3)_2$), 6.28 (d, $J = 3.0$ Hz, 1H, 8-H), 6.33 (s, 1H, 6-H), 6.56 (dd, $J = 9.0$ and 2.7 Hz, 1H, 10-H), 6.96 (d, $J = 7.2$ Hz, 2H, 2′-H, 6′-H), 7.13 (t, $J = 7.5$ Hz, 1H, 4′-H,), 7.40 (d, $J = 7.8$ Hz, 2H, 3′-H, 5′-H), 7.52 (d, $J = 9.0$ Hz, 1H, 11-H), 7.60–7.70 (m, 2H, 2-H, 3-H), 8.58–8.63 (m, 2H, 1-H, 4-H) ppm. The assignments were supported by spin decoupling-double resonance. ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 44.3$ ($N(CH_3)_2$), 94.0 (C-6), 95.6 (C-8), 108.8 (C-10), 111.7 (C-1′), 120.8 (C-2′, C-6′), 123.8 (C-4′), 125.0 (C-1, C-4), 129.0 (C-3′, C-5′), 130.0 (C-2, C-3), 130.1 (C-11), 131.4 (Ar-C), 132.7 (Ar-C), 142.9 (Ar-C), 146.2 (Ar-C), 148.5 (2 × Ar-C), 152.0 (C-9), 156.8 (C-5) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS (FAB): calcd. for $C_{24}H_{20}N_3O$ [M^+] 366.1606; found 366.1615.

*4.1.2.5. N-(5-amino-9H-benzo[a]phenoxazin-9-ylidene)-N-methylmethanaminium chloride (**1e**).* The product of the reaction of **2a** (87.0 mg, 5.24×10^{-4} mol) with **3e** (75 mg, 5.24×10^{-4} mol) in methanol (2 mL) was chromatographed with dichloromethane/methanol (5:1) as the eluent to give the dye **1e** (99.7 mg, 66%). mp above 300 °C. TLC (dichloromethane/methanol, 6:1): $R_f = 0.72$. IR (oil): $\nu = 3330$, 2954, 2923, 2854, 1640, 1588, 1553, 1483, 1463, 1426, 1395, 1378, 1365, 1331, 1306, 1201, 1178, 1145, 1129, 1117, 1056, 1041, 1004 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): $\delta = 3.38$ (s, 6H, $N(CH_3)_2$), 6.91 (s, 1H, 8-H), 6.96 (1H, d, $J = 2.7$ Hz, 6-H), 7.31 (dd, $J = 8.7$ and 2.7 Hz, 1H, 10-H), 7.84–7.98 (m, 2H, 2-H and 11-H), 7.98 (dt, $J = 7.8$ and 1.2 Hz, 1H, 3-H), 8.37 (d, $J = 7.5$ Hz, 1H, 1-H), 9.0 (d, $J = 7.8$ Hz, 1H, 4-H) ppm. The ^{13}C NMR (75.4 MHz, CD_3OD): $\delta = 41.0$ ($N(CH_3)_2$), 96.1 (C-6), 97.5 (C-8), 112.0 (C-10), 125.0 (C-1), 126.2 (C-4), 127.6 (2 × Ar-C), 130.0 (Ar-C), 131.5 (C-3), 132.0 (C-2), 132.3 (C-11), 140.2 (Ar-C), 147.4 (2 × Ar-C), 154.0 (C-9), 158.2 (C-5) ppm. HRMS (FAB): calcd. for $C_{18}H_{16}N_3O$ [M^+] 290.1293; found 290.1290.

*4.1.2.6. N-ethyl-N-(5-(3-methoxy-3-oxopropylamino)-9H-benzo[a]phenoxazin-9-ylidene)ethanaminium chloride (**1f**).* The product of the reaction of **2b** (135 mg, 6.98×10^{-4} mol) with **3a** (200 mg, 9.30×10^{-4} mol) in methanol (2 mL) was chromatographed with chloroform/methanol (5.5:0.5) as the eluent to give the dye **1f** (180 mg, 64%). mp 202.3–204.1 °C. TLC (chloroform/methanol, 5:2): $R_f = 0.91$. IR (KBr 1%): $\nu = 3414$, 3184, 2975, 1734, 1639, 1588, 1547, 1454, 1439, 1384, 1330, 1276, 1257, 1165, 1129, 1112, 1074, 1020 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 1.32$ (t, $J = 7.2$ Hz, 6H, $N(CH_2CH_3)_2$), 3.11 (t, $J = 6.9$ Hz, 2H, $NHCH_2CH_2$), 3.54–3.59 (q, $J = 6.9$ Hz, 4H, $N(CH_2CH_3)_2$), 3.71 (s, 3H, OCH_3), 4.10 (t, $J = 6.9$ Hz, 2H, $NHCH_2CH_2$), 6.53 (s, 1H, 8-H), 6.77 (s, 1H, 6-H), 6.89 (d, $J = 8.7$ Hz, 1H, 10-H), 7.69 (d, $J = 9.3$ Hz, 1H, 11-H), 7.72–7.80 (m, 2H, 2-H, 3-H), 8.66–8.76 (m, 1H, 1-H), 9.16 (br s, 1H, 4-H) ppm. ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 12.6$ ($(N(CH_2CH_3)_2$), 33.6 ($NHCH_2CH_2$), 41.5 (NCH_2CH_2), 45.7 ($N(CH_2CH_3)_2$), 51.8 (OCH_3), 93.7 (C-6), 95.6 (C-8), 113.2 (C-10), 123.8 (C-1), 125.6 (Ar-C), 125.8 (C-4), 128.0 (Ar-C), 130.1 (Ar-C), 130.5 (C-3), 131.3 (C-2), 132.0 (C-11), 136.0 (Ar-C), 147.1 (Ar-C), 150.7 (Ar-C), 152.5 (C-9), 158.2 (C-5), 172.1 (CO_2CH_3) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS (FAB): calcd. for $C_{24}H_{26}N_3O_3$ [M^+] 404.1974; found 404.1963.

Although compound **1f** was the major product, compound **1g** was also isolated in this preparation.

*4.1.2.7. N-(5-(2-carboxylethylamino)-9H-benzo[a]phenoxazin-9-ylidene)-N-ethylethanaminium chloride (**1g**).* Dye **1g** was obtained (20 mg, 7.0%) in the same preparation of compound **1f**. mp above 300 °C. TLC (chloroform/methanol, 1:1): $R_f = 0.60$. IR (KBr 1%): $\nu = 3418$, 3235, 2970, 2927, 1639, 1586, 1546, 1454, 1437, 1383, 1328, 1276, 1257, 1208, 1166, 1130, 1075, 1012 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): $\delta = 1.33$–1.50 (m, 6H, $N(CH_2CH_3)_2$), 2.80–2.95 (m, 2H, $NHCH_2CH_2$), 3.07 (m, 4H, $N(CH_2CH_3)_2$), 3.60–3.85 (br s, 2H, $NHCH_2CH_2$), 6.83 (br s, 1H, 8-H), 7.25 (br s, 1H, 6-H), 7.65 (br s, 1H, 10-H), 7.74 (br s, 1H, 11-H), 7.80–8.0 (m, 2H, 2-H, 3-H), 8.80 (br s, 2H, 1-H, 4-H) ppm. ^{13}C NMR (75.4 MHz, CD_3OD): $\delta = 13.0$ ($N(CH_2CH_3)_2$), 30.8 ($NHCH_2CH_2$), 40.2 (NCH_2CH_2), 47.1 ($N(CH_2CH_3)_2$), 96.0 (C-6), 97.0 (C-8), 111.2 (C-10), 123.9 (C-1), 124.7 (C-4), 125.5 (Ar-C), 129.9 (Ar-C), 130.9 (C-3), 131.6 (Ar-C), 132.4 (C-2), 132.9 (C-11), 134.9 (Ar-C), 149.6

(Ar-C), 153.1 (Ar-C), 155.6 (C-9), 159.0 (C-5), 169.3 (CO_2H) ppm. HRMS (FAB): calcd. for $C_{23}H_{23}N_3O_3$ [M^+] 389.1739; found 389.1752.

*4.1.2.8. N-(5-(phenylamino)-9H-benzo[a]phenoxazin-9-ylidene)-hexan-1-aminium chloride (**1h**).* The product of the reaction of **2c** (156 mg, 7.03×10^{-4} mol) with **3d** (236 mg, 1.08×10^{-3} mol) in methanol (2 mL) was chromatographed with chloroform/methanol (5.8:0.2) as the eluent to give the dye **1h** (136 mg, 46%). mp above 300 °C. TLC (chloroform/methanol, 5.6:0.4): $R_f = 0.67$. IR (Nujol): $\nu = 3399$, 3064, 2924, 2954, 1613, 1557, 1505, 1463, 1377, 1271, 1188, 1156, 1113, 1081, 1002 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 0.78$–1.0 (m, 3H, CH_3), 1.20–1.42 (m, 6H, $NHCH_2CH_2(CH_2)_3CH_3$), 1.46–1.72 (m, 2H, $NHCH_2CH_2$), 3.14 (t, $J = 6.0$ Hz, $NHCH_2$), 6.20 (s, 1H, 8-H), 6.34 (s, 1H, 6-H), 6.50 (d, $J = 9.6$ Hz, 1H, NH), 6.98 (d, $J = 7.5$ Hz, 2H, 10-H, 2′-H or 6′-H), 7.14 (t, $J = 7.5$ Hz, 6′-H or 2′-H), 7.34–7.44 (m, 4H, 11-H, 4′-H, 3′-H, 5′-H), 7.60–7.70 (m, 2H, 2-H, 3-H), 8.55–8.65 (m, 2H, 1-H, 4-H) ppm. ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 14.0$ (CH_3), 22.6 ($NH(CH_2)_2(CH_2)_3$), 26.7 ($NH(CH_2)_2(CH_2)_3$), 29.1 ($NHCH_2CH_2$), 31.5 ($NH(CH_2)_2(CH_2)_3$), 43.6 ($NHCH_2$), 96.6 (C-6), 98.5 (C-8), 111.7 (C-1′), 120.8 (C-2′, C-6′), 123.5 (C-4′), 123.9 (C-1), 125.0 (C-4), 129.0 (C-10), 130.0 (C-3′), 130.1 (C-11), 130.3 (C-5′, Ar-C), 131.3 (C-2, C-3), 133.5 (Ar-C), 143.6 (2 × Ar-C), 148.4 (Ar-C), 150.1 (Ar-C), 156.5 (C-9), 156.8 (C-5) ppm.

*4.1.2.9. N-(5-(3-ethoxy-3-oxopropylamino)-10-methyl-9H-benzo[a]phenoxazin-9-ylidene) ethanaminium chloride (**1j**).* The product of the reaction of **2d** (148 mg, 8.23×10^{-4} mol) with **3b** (200 mg, 8.23×10^{-4} mol) in ethanol (6 mL) was chromatographed with chloroform/methanol (5.8:0.2) as the eluent to give the dye **1j** (394 mg, 90%). mp195.2–197.0 °C. TLC (chloroform/methanol, 6:1): $R_f = 0.48$. IR (KBr 1%): $\nu = 3216$, 2953, 2922, 1731, 1644, 1592, 1564, 1520, 1504, 1449, 1318, 1261, 1186, 1163, 1137, 1015 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): $\delta = 1.34$ (t, $J = 7.2$ Hz, 6H, OCH_2CH_3, NCH_2CH_3), 2.13 (s, 3H, CH_3), 2.91 (t, $J = 6.6$ Hz, 2H, $NHCH_2CH_2$), 3.30–3.40 (m, 3H, $NHCH_2CH_3$, NH), 3.78 (t, $J = 6.6$ Hz, 2H, $NHCH_2CH_2$), 4.26 (q, $J = 7.2$ Hz, 2H, OCH_2CH_3), 6.35 (s, 1H, 8-H), 6.50 (s, 1H, 6-H), 7.10 (s, 1H, 11-H), 7.52–7.70 (m, 2H, 2-H, 3-H), 8.05 (d, $J = 8.1$ Hz, 1H, 1-H), 8.31 (d, $J = 7.2$ Hz, 1H, 4-H) ppm. ^{13}C NMR (75.4 MHz, CD_3OD): $\delta = 14.2$ ($NHCH_2CH_3$), 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 (C-6), 94.4 (C-8), 123.7 (C-1), 124.6 (2 × Ar-C), 124.9 (C-4), 128.3 (C-10), 130.4 (C-3), 131.4 (Ar-C), 131.6 (C-2), 132.3 (C-11), 133.7 (Ar-C), 148.4 (Ar-C), 151.5 (Ar-C), 156.1 (C-9), 157.8 (C-5), 172.9 ($CO_2CH_2CH_3$) ppm. The assignments were supported by HMBC and HMQC techniques. $C_{24}H_{26}N_3O_3 \cdot 3.5HCl$ (532): calcd. C 54.17, H 5.59, N 7.89; found C, 53.78; H, 5.44; N 7.70.

*4.1.2.10. N-(5-(3-methoxy-3-oxopropylamino)-10-methyl-9H-benzo[a]phenoxazin-9-ylidene) ethanaminium chloride (**1l**).* The product of the reaction of **2d** (79 mg, 4.37×10^{-4} mol) with **3c** (100 mg, 4.37×10^{-4} mol) in ethanol (2 mL) was chromatographed with dichloromethane/methanol (5.6:0.4) as the eluent to give the dye **1l** (127.5 mg, 75%). mp above 300 °C. TLC (dichloromethane/methanol, 5.5:0.5): $R_f = 0.48$. IR (oil): $\nu = 3193$, 2954, 2924, 2854, 1723, 1642, 1589, 1563, 1553, 1537, 1519, 1503, 1461, 1455, 1377, 1316, 1264, 1227, 1162, 1138, 1057, 1012 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): $\delta = 1.41$ (t, $J = 7.2$ Hz, 3H, NCH_2CH_3), 2.38 (s, 3H, CH_3), 2.97 (t, $J = 6.6$ Hz, 2H, $NHCH_2CH_2$), 3.55, 3.59 (2 × d $J = 7.2$ Hz, 2H, $NHCH_2CH_3$), 3.76 (s, 3H, OCH_3), 4.01 (t $J = 6.6$ Hz, 2H, $NHCH_2CH_2$), 4.60 (broad s, 1H, NH), 6.90 (s, 1H, 8-H), 7.04 (s, 1H, 6-H), 7.74 (s, 1H, 11-H), 7.83 (t, $J = 6.9$ Hz, 1H, 2-H), 7.94 (t, $J = 7.2$ Hz, 1H, 3-H), 8.36 (d, $J = 8.4$ Hz, 1H, 1-H), 8.96 (d, $J = 7.5$ Hz, 1H, 4-H) ppm. ^{13}C NMR (75.4 MHz, CD_3OD): $\delta = 14.2$ ($NHCH_2CH_3$), 17.8 (CH_3), 33.6 ($NHCH_2CH_2$), 39.8 ($NHCH_2CH_3$), 41.7 ($NHCH_2CH_2$), 52.6 (OCH_3), 93.9 (C-6), 94.5 (C-8), 123.7 (C-1), 124.5 (2 × Ar-C), 125.2 (C-4), 128.9 (C-10), 130.6 (C-3), 132.2 (Ar-C), 132.5 (C-2), 132.7 (C-11), 133.7 (Ar-C), 149.1 (Ar-C), 152.2 (Ar-C), 156.7 (C-9), 158.0 (C-5), 173.3 (CO_2CH_3) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS (FAB): calcd. for $C_{23}H_{24}N_3O_3$ [M^+] 390.1818; found 390.1814.

*4.1.2.11. N-(5-(phenylamino)-10-methyl-9H-benzo[a]phenoxazin-9-ylidene)ethanaminium chloride (**1m**).* The product of the reaction of **2d** (159.9 mg, 8.88×10^{-4} mol) with **3d** (300 mg, 1.37×10^{-3} mol) in ethanol (6 mL) was chromatographed with chloroform/methanol (5.9:0.1) as the eluent to give the dye **1m** (181 mg, 54%). mp above 300 °C. TLC (chloroform/methanol, 5.8:0.2): $R_f = 0.44$. IR (KBr 1%): $\nu = 3428$, 2958, 2914, 2858, 1631, 1593, 1587, 1575, 1545, 1518, 1474, 1337, 1293, 1286, 1249, 1099, 1011 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 1.33$ (t, $J = 6.9$ Hz, 3H, $NHCH_2CH_3$), 2.17 (s, 3H, CH_3), 3.16–3.30 (m, 2H, $NHCH_2CH_3$), 3.96 (br s, 1H, NH), 6.24 (s, 1H, 8-H), 6.32 (s, 1H, 6-H), 6.96 (d, $J = 7.2$ Hz, 2H, 2′-H, 6′-H), 7.12 (t, $J = 7.5$ Hz, 1H, 4′-H), 7.32–7.44 (m, 3H, 3′-H, 5′-H, 11-H), 7.70–7.60 (m, 2H, 2-H, 3-H), 8.56–8.66 (m, 2H, 1-H, 4-H) ppm. ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 14.5$ ($NHCH_2CH_3$), 16.8 (CH_3), 38.3 ($NHCH_2CH_3$), 95.0 (C-6), 98.3 (C-8), 118.5 (C-1′), 120.8 (C-2′, C-6′), 123.3 (C-4′), 123.9 (C-1), 125.0 (C-4), 129.0 (C-10), 129.9 (C-3′), 130.0 (C-11), 130.2 (C-5′, Ar-C), 131.4 (C-2, C-3), 133.1 (Ar-C), 143.2 (Ar-C), 145.3 (Ar-C), 148.7 (Ar-C), 148.8 (Ar-C), 152.0 (C-9), 156.5 (C-5) ppm. The assignments were supported by HMBC and HMQC techniques. $C_{25}H_{22}N_3O$ (380): calcd. for C 78.92, H 5.83, N 11.05; found C, 78.77; H, 5.57; N 11.04.

*4.1.2.12. N-(5-(2-carboxylethylamino)-10-methyl-9H-benzo-[a]phenoxazin-9-ylidene)-ethanaminium chloride (**1i**).* To a solution of **2d** (33 mg, 1.86×10^{-4} mol) in DMF (2 mL) compound **3a** (40 mg, 1.86×10^{-4} mol) and 12 M HCl (0.2 mL) were added and the mixture was heated at about 70 °C for 14 h with stirring. Evaporation of the solvent and purification by dry chromatography with dichloromethane/methanol (1:1) as the eluent gave compound **1i** (31.5 mg, 45%). mp above 300 °C.

TLC (dichloromethane/methanol, 5:1): $R_f = 0.79$. IR (oil): $\nu = 3500$–3250, 2954, 2925, 2854, 2778, 1655, 1618, 1577, 1545, 1510, 1353, 1271, 1243, 1199, 1170, 1065, 1023 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): $\delta = 1.40$ (t, $J = 7.2$ Hz, 3H, NCH_2CH_3), 2.34 (s, 3H, CH_3), 2.71 (t, $J = 6.0$ Hz, 2H, $NHCH_2CH_2$), 3.51, 3.56 (2 × d, 2H, $NHCH_2CH_3$), 3.92 (t, $J = 6.6$ Hz, 2H, $NHCH_2CH_2$), 6.82 (s, 1H, 8-H), 6.98 (s, 1H, 6-H), 7.64 (s, 1H, 11-H), 7.81 (t, $J = 7.2$ Hz, 3-H), 7.91 (d, $J = 7.8$ Hz, 1H, 2-H), 8.28 (d, $J = 8.1$ Hz, 1H, 1-H), 8.87 (1H, d $J = 7.8$ Hz, 4-H) ppm. ^{13}C NMR (75.4 MHz, CD_3OD): $\delta = 14.2$ ($NHCH_2CH_3$), 17.8 (CH_3), 34.9 ($NHCH_2CH_2$), 36.0 ($NHCH_2CH_2$), 39.7 ($NHCH_2CH_3$, 93.9 (C-8), 94.4 (C-6), 124.1 (C-1), 124.7 (Ar-C), 125.4 (C-4), 128.5 (1 × Ar-C), 130.8 (C-10), 131.7 (C-3), 132.2 (C-2), 132.6 (Ar-C), 132.7 (C-11), 134.3 (Ar-C), 149.1 (Ar-C), 152.6 (Ar-C), 156.5 (C-9), 158.4 (C-5), 164.3 (CO_2H) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS (FAB): calcd. for $C_{22}H_{23}N_3O_3$ [M^+] 377.1739; found 377.1736.

*4.1.2.13. 3-(Naphthalen-1-ylamino)propanoic acid (**3a**).* To a suspension of 1-naphthylamine (2 g, 14 mmol) in destilated water (6.2 mL), an aqueous 6 M NaOH solution (2.8 mL, 17 mmol) and chloropropionic acid (1.52 mL, 14 mmol) were added and the resulting mixture was refluxed for 8 h, and monitored by TLC (chloroform). The solvent was removed under reduced pressure and the crude mixture was purified by dry chromatography (chloroform/methanol, mixtures of increasing polarity) to give compound **3a** as an off-white solid (48%, 1.44 g). mp 131.0–133.1 °C. TLC (chloroform/methanol, 5.5:0.5): $R_f = 0.73$. IR (KBr 1%): $\nu = 3378$, 2923, 1717, 1583, 1525, 1477, 1434, 1409, 1315, 1224, 1212, 1119 cm^{-1}. ^1H NMR (300 MHz, DMSO): $\delta = 2.66$ (t, $J = 7.2$ Hz, 2H, $NHCH_2CH_2$), 3.43 (t, $J = 6.9$ Hz, 2H, $NHCH_2CH_2$), 6.14 (br s, 1H, NH), 6.51 (d, $J = 7.8$ Hz, 1H, 4-H), 7.11 (d, $J = 8.1$ Hz, 1H, 2-H), 7.28 (t, $J = 7.8$ Hz, 1H, 3-H), 7.33–7.46 (m, 2H, 6-H, 7-H), 7.74 (d, $J = 8.4$ Hz, 1H, 8-H), 8.10 (d, $J = 8.3$ Hz, 1H, 5-H), 12.2 (br s, 1H, OH) ppm. The assignments were supported by spin decoupling-double resonance. ^{13}C NMR (75.4 MHz, DMSO): $\delta = 24.3$ (NCH_2CH_2), 30.1 (NCH_2CH_2), 94.0 (C-4), 106.7 (C-2), 112.6 (C-5), 114.1 (C-4a), 115.0 (C-7), 116.6 (C-6), 117.8 (C-3), 119.0 (C-8), 125.0 (C-8a), 134.8 (C-1), 164.4 (CO_2H) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS (EI): calcd. for $C_{13}H_{13}NO_2$ [M^+] 215.0946; found 215.0944.

*4.1.2.14. Ethyl 3-(naphthalen-1-ylamino)propanoate (**3b**).* To a solution of 1-naphthylamine (2 g, 14 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 (chloroform). The solvent was removed under reduced pressure and the crude mixture was purified by dry chromatography (chloroform). Compound **3b** was obtained as a colourless oil (45%, 1.51 g). TLC (chloroform/methanol, 5.9:0.1): $R_f = 0.76$. IR (film): $\nu = 3434$, 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 (300 MHz, $CDCl_3$): $\delta = 1.31$ (t, $J = 7.2$ Hz, 3H, OCH_2CH_3), 2.78 (t, $J = 6.3$ Hz, 2H, NCH_2CH_2), 3.64 (t, $J = 6.3$ Hz, 2H, NCH_2CH_2), 4.19 and 4.24 (q, $J = 7.2$ Hz, 2H, OCH_2CH_3), 4.91 (br s, 1H, NH), 6.66 (d, $J = 7.5$ Hz, 1H, 4-H), 7.29 (d, $J = 8.4$ Hz, 1H, 2-H), 7.39 (t, $J = 7.5$ Hz, 1H, 3-H), 7.42–7.52 (m, 2H, 6-H, 7-H), 7.80–7.89 (m, 2H, 8-H, 5-H) ppm. ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 14.1$ (OCH_2CH_3), 33.5 (NCH_2CH_2), 39.6 (NCH_2CH_2), 60.6 (OCH_2CH_3), 104.4 (C-4), 117.7 (C-2), 119.9 (C-5), 123.6 (C-4a), 124.7 (C-7), 125.7 (C-6), 126.4 (C-3), 128.5 (C-8), 134.3 (C-8a), 142.8 (C-1), 172.5 ($CO_2CH_2CH_3$) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS (FAB): calcd. for $C_{15}H_{17}NO_2$ [M^+] 243.1259; found 243.1255.

*4.1.2.15. Methyl 3-(naphthalen-1-ylamino)propanoate (**3c**).* The product of reaction of 1-naphthylamine (2 g; 14 mmol) with methyl-3-bromopropionate (1.60 mL, 14.7 mmol) in methanol (5 mL), according to the procedure described above for compound **3b** was chromatographed with dichloromethane/*n*-hexane (4.7:1.3) as the eluent to give compound **3c** as a brown oil (55%, 1.60 g). TLC (dichloromethane/methanol, 5:1): $R_f = 0.46$. IR (film): $\nu = 3436$, 3052, 3008, 2952, 2850, 1731, 1625, 1583, 1530, 1484, 1461, 1436, 1409, 1370, 1347, 1318, 1284, 1197, 1174, 1121, 1092, 1016 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 2.79$ (t, $J = 6.3$ Hz, 2H, NCH_2CH_2), 3.65 (t, $J = 6.0$ Hz, 2H, NCH_2CH_2), 3.75 (s, 3H, OCH_3), 6.67 (d, $J = 7.2$ Hz, 1H, 4-H), 7.30 (d, $J = 8.1$ Hz, 1H, 2-H), 7.39 (t, $J = 7.5$ Hz, 1H, 3-H), 7.44–7.51 (m, 2H, 6-H, 7-H), 7.80–7.88 (m, 2H, 8-H, 5-H) ppm. ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 33.3$ (NCH_2CH_2), 39.6 (NCH_2CH_2), 51.8 (OCH_3), 104.4 (C-4), 117.7 (C-2), 119.9 (C-5), 123.6 (C-4a), 124.8 (C-7), 125.7 (C-6), 126.4 (C-3), 128.6 (C-8), 134.3 (C-8a), 142.8 (C-1), 173.0 (CO_2CH_3) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS (EI): calcd. for $C_{14}H_{15}NO_2$ [M^+] 229.1103; found 229.1101.

4.2. Spectroscopic measurements

Absorption spectra were recorded either in a Shimadzu UV-3101PC UV–vis–NIR spectrophotometer or in a Hitachi U-2000 spectrophotometer. Fluorescence measurements were performed using a Spex Fluorolog 212 spectrofluorimeter. Fluorescence spectra were corrected for the instrumental response of the system.

All solutions were prepared using spectroscopic grade solvents or Milli-Q grade water. The fluorescence quantum yields (Φ_s) were determined using the standard method (Eq. (1)) [32]. Oxazine 1 in ethanol was used as reference, $\Phi_r = 0.11$ [30].

$$\Phi_s = \left[\frac{(A_r F_s n_s^2)}{(A_s F_r n_r^2)}\right] \Phi_r \quad (1)$$

where A is the absorbance at the excitation wavelength, F the integrated emission area and n the refraction index of the solvents used. Subscripts refer to the reference (r) or sample (s) compound.

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