

Benzo[*a*]phenoxazinium chlorides functionalized with chloride atoms and/or ester groups

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Abstract: With the aim of contributing to the development of fluorescent near-infrared (NIR) probes with applications in biomedicine, our research group is committed to the development of new water-soluble benzo[*a*]phenoxazine derivatives and the evaluation of their photophysical and biological potential. Herein we report the photophysical behaviour in anhydrous ethanol of four synthesised benzo[*a*]phenoxazinium chlorides, possessing the (3-chloropropyl)amino and/or (4-ethoxy-4-oxobutyl)amino groups at 5- and 9-positions of the polycyclic system.

Keywords: Benzo[*a*]phenoxazines, Nile Blue derivatives, NIR fluorescent probes.

1. Introduction

Fluorescent probes are recognised tools in the various fields, including in studies of biochemical processes, diagnosis of disease, and detection of hazardous compounds, among others. For biological and medicinal applications, fluorescent near-infrared probes are more adequate, due to the reduced interference with the self-fluorescence of the biological material, in addition to less harming effects induced by light at wavelengths longer than 600 nm.¹⁻⁴

Molecules possessing an oxazine ring as a part of a tetracyclic aromatic structure are called benzophenoxazines, and their derivatives having adequate substituents, namely the most well-known, Nile Blue, are associated with an absorbance and a fluorescence emission at longer wavelengths, belonging to the group of near-infrared fluorescence probes.⁵

Research in the design, synthesis and evaluation of the potential applications of compounds that can belong to this class of probes is required and still a very actual subject due to the challenging of developing more specific molecules and follow the advances of fluorescent techniques. As a result, several studies with different families of heterocyclic systems have been reported, and all are necessary and extremely important.

Recently, Wang *et al.* and Guan *et al.* reported cyanine derivatives capable of selectively sensing glutathione and cysteine.¹⁻³ These two thiol-based compounds are present in biological systems with both being detected at the same time by the NIR dyes.² On the other hand, Wang *et al.* achieved the enhancement of the fluorescence intensity of the NIR probe in presence of cysteine, while showing little fluorescence by interacting with glutathione.³ In 2015, Chai *et al.* reported a series of phosphorus-substituted rhodamines presenting phosphorous groups instead of the oxygen atom in traditional rhodamines. These new NIR probes enter animal cells both during *in vitro* and *in vivo* studies.⁴ A localized fluorescence signal was still detected 48 hours after the intramuscular injection in mice of one of these compounds, despite the decay in the fluorescence intensity.

As a continuation of the investigation of our research group,⁶⁻¹⁰ in this proceeding we intend to give a short contribution to the development of NIR probes, presenting the photophysical properties of four synthesized benzo[*a*]phenoxazinium chlorides functionalized with chloride atoms and/or ester groups. The experiments were performed in dry ethanol, in this medium acidified with trifluoroacetic acid (TFA) and basified with tetraethylammonium hydroxide (TEAH).

2. Results and discussion

Benzophenoxazinium chlorides **1–4** were synthesized by condensation of 5-((3-chloropropyl)amino)-2-nitrosophenol hydrochloride, ethyl 4-((3-hydroxy-4-nitrosophenyl)amino)butanoate hydrochloride or 5-(dipropylamino)-2-nitrosophenol hydrochloride with *N*-alkylated naphthalen-1-amine derivatives, namely *N*-(3-chloropropyl)naphthalen-1-amine or ethyl 4-(naphthalen-1-ylamino)butanoate in acid media (Figure 1). The required nitrosophenol hydrochlorides were obtained by nitrosation of the corresponding 3-aminophenol derivative with sodium nitrite in the presence of hydrochloric acid, in a mixture of ethanol-water as the solvent.¹¹

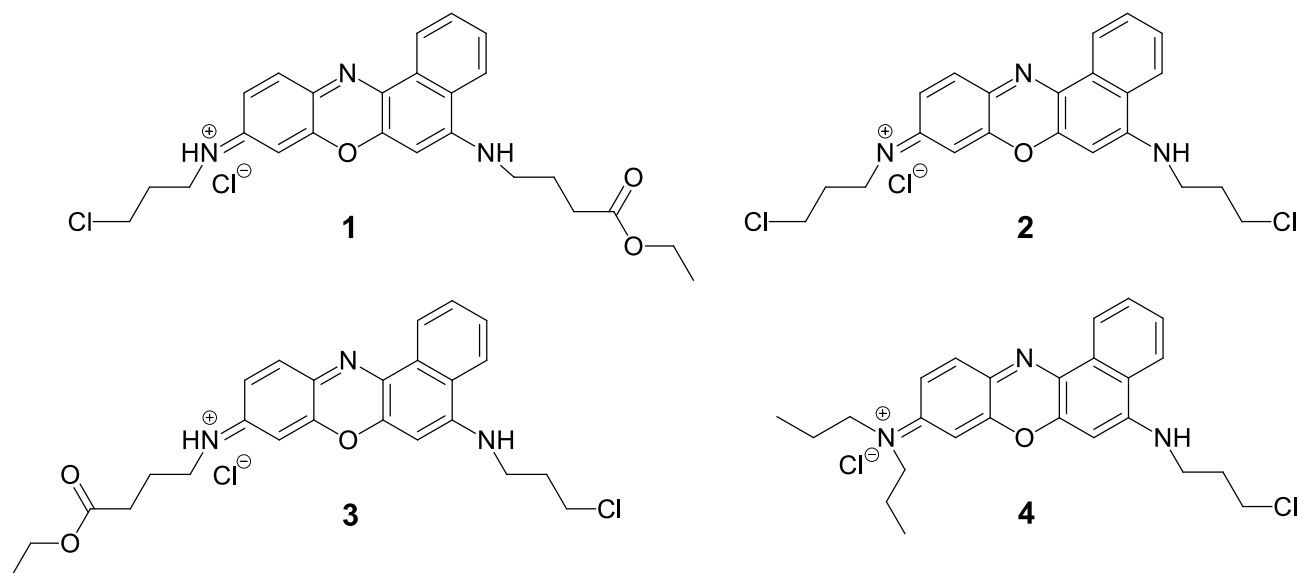


Figure 1. Structures of benzo[*a*]phenoxazininium chlorides **1–4**.

Electronic absorption and emission spectra of 4×10^{-6} M solutions in anhydrous ethanol were measured for synthesized benzo[*a*]phenoxazininium chlorides **1–4** (Table 1). The relative fluorescence quantum yields (Φ_F) were determined using Oxazine 1 as a standard ($\Phi_F = 0.11$ in ethanol).¹²

Table 1. Photophysical data of compounds **1–4** in dry ethanol acidified with TFA ($C_{\text{TFA}} = 4 \times 10^{-6}$ M; $\lambda_{\text{ex}} = 575$ nm).

	1	2	3	4
λ_{abs} (nm)	621	622	625	642
$\log \varepsilon$	4.73	4.66	4.80	4.94
λ_{em} (nm)	645	645	646	676
Φ_F	0.53	0.50	0.40	0.22
Δ (nm)	24	23	21	34

In our earlier investigation with similar type of compounds in reagent grade ethanol showed inconsistency and irreproducible data due to variations of water concentrations in different batches of ethanol. Hence, we prefer to use anhydrous ethanol following the drying procedure in literature.¹³

Previous studies on benzo[*a*]phenoxazininium chlorides showed that the photophysical properties in proton-accepting solvents is influenced by acid-base equilibria mainly located at the 5-amino position.^{14.}

¹⁵ In ethanol media the absorption spectra are dominated by an acidic form (AH⁺) and a 100 nm blue shifted neutral form (A).¹⁶ The fluorescence of the basic form is broad and centred at around 600 nm while the acid form (AH⁺) shows a band centred above 660 nm with a much higher quantum yield.¹⁶ The quantum yield reaches 0.4 when the 9-amino position is mono-alkylated and varies between 0.1–0.2 when it is di-alkylated.¹⁴⁻¹⁶

Table 1 shows that compound **4** has the greatest absorption and emission maxima. This is mainly due to the result of di-alkylation at 9-amino position of the heterocycle as previously observed.^{16, 17} Compounds **1** and **3** showed the highest fluorescence efficiency in ethanolic media and greater Stokes shift of 34 nm was registered for compound **3** (Figure 1).

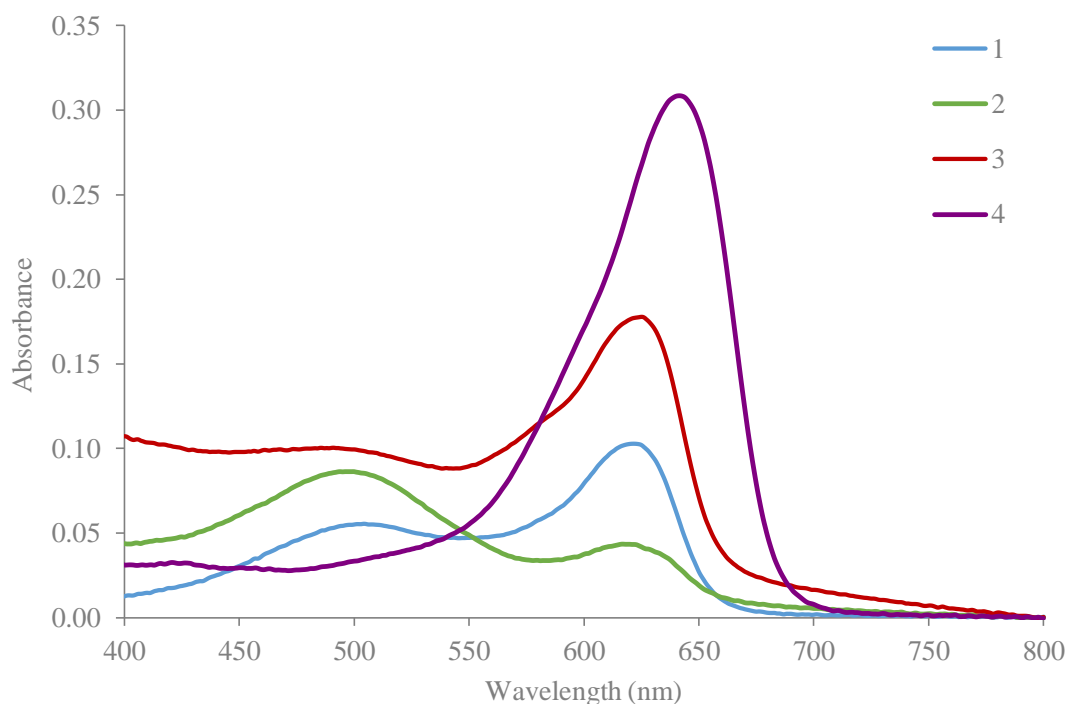


Figure 2. Absorbance spectra of compounds **1–4** in dry ethanol.

Figure 2 shows the absorption spectra for compounds **1–4** ($C = 4 \times 10^{-6}$ M) in dry ethanol. It was observed that dyes **1** and **3** display mostly acidic form with less basic form. On the other hand, the benzo[*a*]phenoxazininium chloride **2** possessing chloride atoms as terminals at 5- and 9-amino position presents mostly the basic form, twice the absorbance value in comparison with the acidic form. In case of

compound **4**, only the acidic form with absorbance maxima and bathochromic shift (20 nm) was observed with the disappearance of the band near 500 nm, which was seen for the other fluorophores.

Figure 3 displays the normalised emission spectra of compounds **1–4** ($C = 4 \times 10^{-6} \text{ M}$) excited at 470 nm and 575 nm in anhydrous ethanol. At 470 nm excitation, the basic form is mostly excited with little acidic form. It is evident from the emission spectra of compounds **3** and **4** the presence of a blue shoulder around 600 nm confirming the basic form. In contrast, dye **2** shows mostly the basic form with a hypsochromic shift in comparison to others with little acidic form at 470 nm excitation wavelength.

Additionally, the behaviour for the three compounds with a mono-alkylated amine at 9-position is in essence the same, being shown only one band for the emission and for the absorbance of dye **1** (Figures 4 and 5) which are representative of those obtained for compounds **2** and **3**. Figure 4 shows the absorption and emission spectra excited at 575 nm of compounds **1** and **4**. At 575 nm excitation only the acidic AH^+ form is excited as clearly evident. In acidified dry ethanol media the emission maxima is 645 nm and 676 nm for fluorophores **1** and **4**, respectively, with a bathochromic shift of 31 nm. Moreover, in these conditions a small bump around 550 nm indicates aggregation in solution.

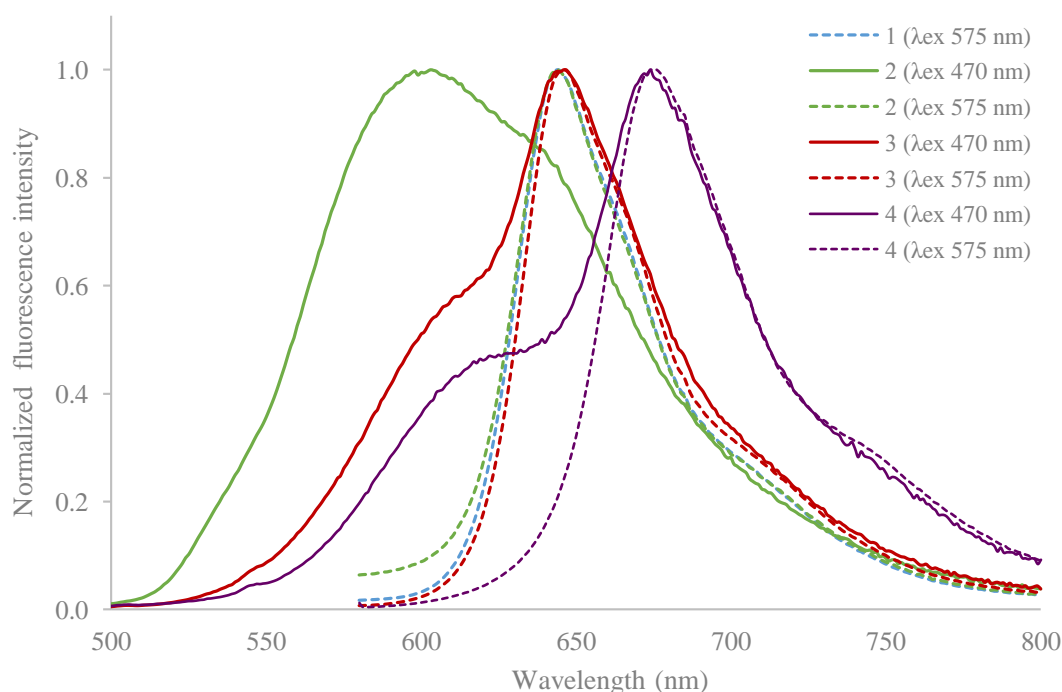


Figure 3. Normalized emission spectra excited at 470 nm and 575 nm of compounds **1–4** in dry ethanol.

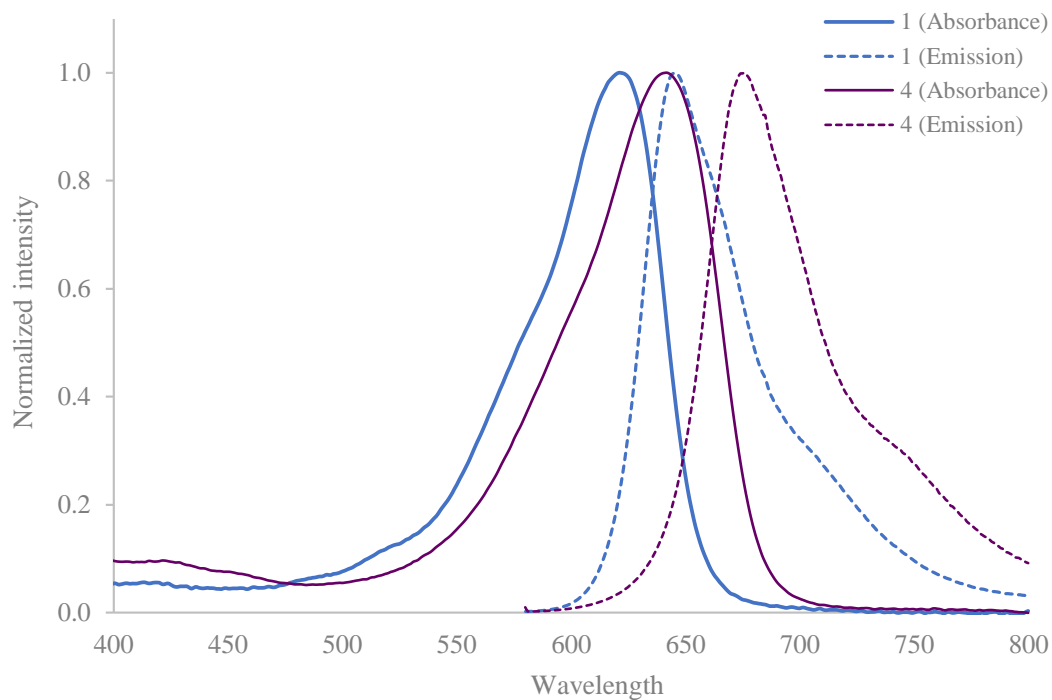


Figure 4. Normalized absorbance and emission spectra at 575 nm excitation of compounds **1** and **4** in acidified dry ethanol.

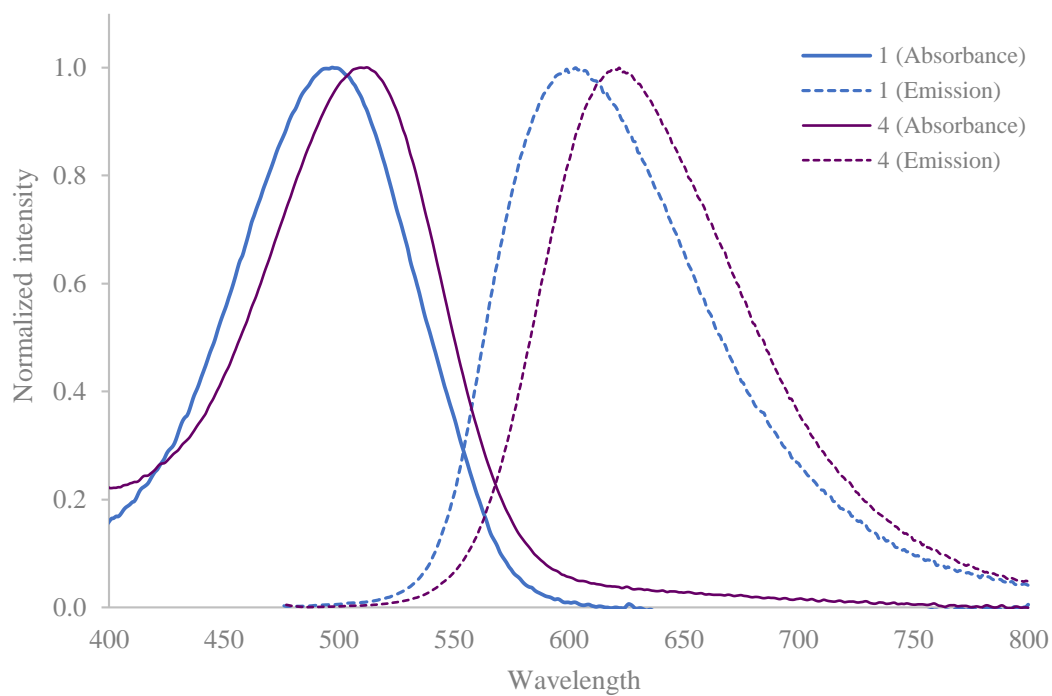


Figure 5. Normalized absorbance and emission spectra at 470 nm excitation of compounds **1** and **4** in basified dry ethanol.

To conclude, Figure 5 presents the normalized absorbance and emission spectra at 470 nm excitation of compounds **1** and **4**. At 470 nm excitation, the basic form is excited and it is seen that by the addition of TEAH it is possible to shift the equilibrium towards the basic form. Comparing compounds **1** and **4** in basified dry ethanol significant bathochromic shifts were observed in absorption (15 nm) and emission (17 nm). In particular the emission spectrum is broad for the dye **1** in comparison with the other fluorophore. Furthermore, in a basic medium the Stoke's shift is largely increased to ca. 100 nm, the only exception being dye **2** whose main absorption band in plain ethanol is already the correspondent to the basic form and so already there displays a Stoke's shift of a comparable magnitude.

4. Conclusions

Four new benzo[*a*]phenoxazinium chlorides were efficiently synthesized and preliminary photophysics of the acidic and basic forms were studied in dried ethanolic media, by the addition of either a strong acid or a strong base. Overall, the highest absorbance and emission was observed for compound **4**, which may due to the effect of di-alkylation at 9-amino position of the polycyclic system. Compound **2** possessing two chloride atoms as terminals at 5- and 9-position behaved the reverse way in comparison with **1** and **3**.

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