

# Evaluation of Fluorescent Staining Capacity of Two New Nile Blue Analogues <sup>†</sup>

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**Abstract:** Benzophenoxazine fluorophores, including derivatives of Nile Blue, one of the best known in this family of compounds, have been used as histological stains due to their optical properties in the NIR region and stability. Given their potential, the synthesis of two new Nile Blue derivatives with different substituents on the amines of the positions 5 and 9 was performed, with the introduction of a sulfonamide group in one of the compounds. Photophysical properties of the compounds were evaluated in acidified ethanol and aqueous solution at physiological pH. The fluorescent staining capacity was evaluated by staining *Saccharomyces cerevisiae* with the compounds, followed by fluorescence microscopy.

**Keywords:** benzo[*a*]phenoxazines; Nile Blue derivatives; NIR fluorescent probes; fluorochromophores; *Saccharomyces cerevisiae*

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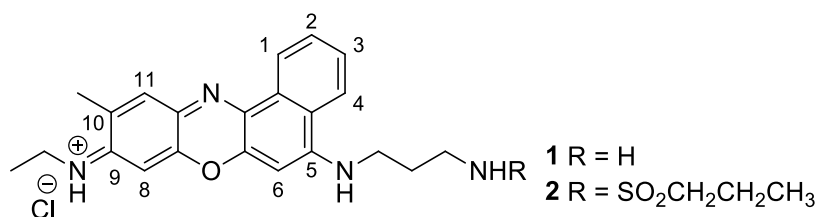
## 1. Introduction

Small fluorescent molecules have emerged as essential tools for contemporary analytical methodologies applied in the biosciences field [1]. In this field, benzo[*a*]phenoxazines, that include Nile Blue, the best known of this family of compounds, present a set of features that suits them as good fluorescent probes for biological applications [2–4]. They emit fluoresce in the near-infrared (NIR) region of the spectrum, a region that does not interfere with the natural fluorescence of biological compounds. Furthermore, they are characterized by presenting high photostability, molar absorption molar coefficients and modest Stokes shifts, great features for fluorescent applications [5,6]. As such, they have been used in covalent and non-covalent labeling of amino acids, proteins, DNA, among other biological material [7–10].

Considering all these facts, the synthesis of two new benzo[*a*]phenoxazinium chlorides possessing one ethyl group at 9-amino position and a methyl group at 10-position, with a propylamino or a 3-(propylsulfonamido)propylamino group at the 5-amino position, was carried out. Photophysical properties in ethanol acidified with trifluoroacetic acid (TFA) and in aqueous solution at physiological pH were measured. The fluorescent staining pattern was evaluated in staining *Saccharomyces cerevisiae* with the compounds, followed by fluorescence microscopy analysis.

## 2. Results and Discussion

Benzo[*a*]phenoxazininium chlorides **1** and **2** were synthesized by condensation of 5-(ethylamino)-4-methyl-2-nitrosophenol hydrochloride with *N*<sup>1</sup>-(naphthalen-1-yl)propane-1,3-diamine hydrobromide or *N*-(3-(naphthalen-1-ylamino)propyl)propane-1-sulfonamide, respectively. Nitrosophenol hydrochloride was obtained by nitrosation of the 3-(ethylamino)-4-methylphenol with sodium nitrite in the presence of hydrochloric acid. The two benzo[*a*]phenoxazininium chlorides **1** and **2** were obtained as blue solids in 44 and 56% yields, respectively, and characterized by the usual analytical techniques (Figure 1).



**Figure 1.** Structures of benzo[*a*]phenoxazininium chlorides **1** and **2**.

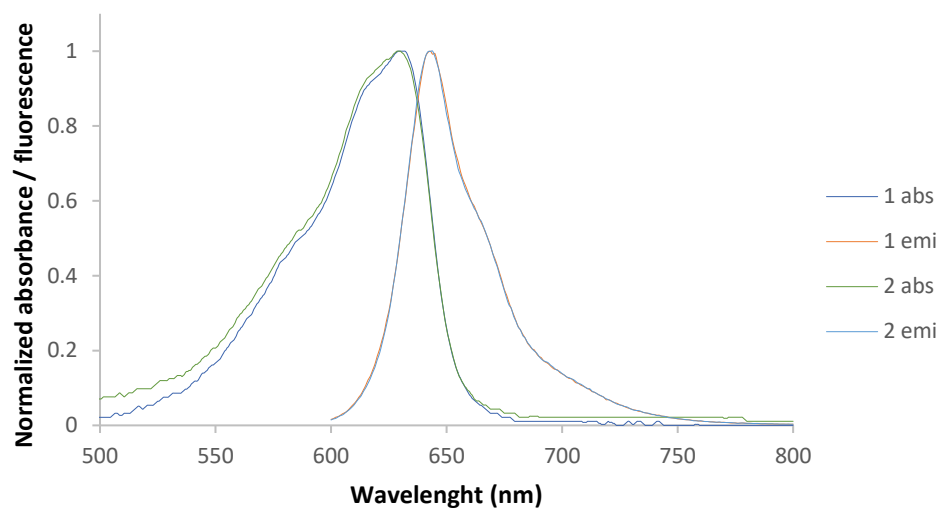
Photophysical properties of benzo[*a*]phenoxazininium chlorides **1** and **2** were evaluated through absorption and emission spectra of 10<sup>-6</sup> M solutions in ethanol acidified with TFA and aqueous solution at physiological pH. The relative fluorescence quantum yields ( $\Phi_F$ ) were determined using Oxazine 1 as a standard ( $\Phi_F = 0.11$  in ethanol) at a wavelength of excitation ( $\lambda_{exc}$ ) of 590 nm. Results are presented in Table 1.

**Table 1.** Photophysical data of compounds **1** and **2** in acidified ethanol and aqueous solutions at pH 7.4 ( $\lambda_{exc}$  590 nm).

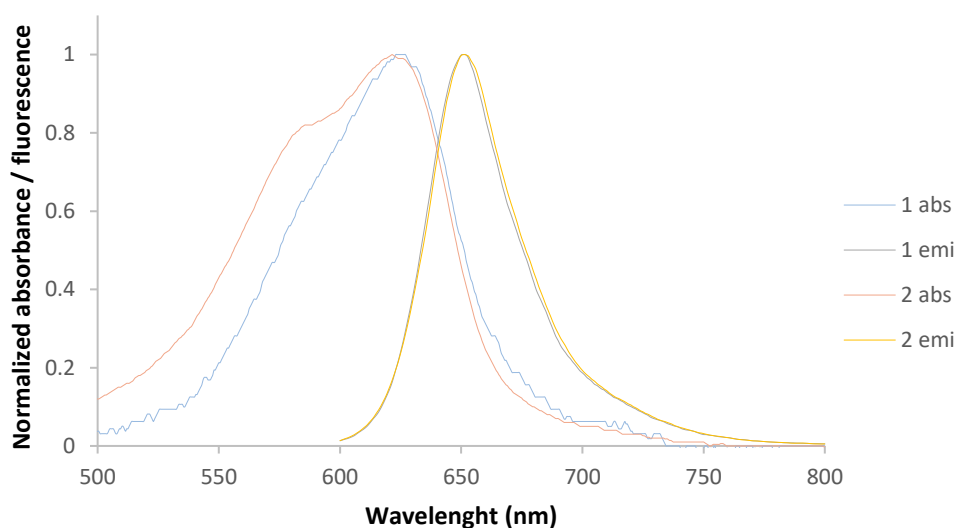
Compound	<b>1</b>	<b>2</b>
<i>Log P</i>	0.96	2.20
<i>Acidified ethanol</i>		
$\lambda_{abs}$ (nm)	627	627
$\text{Log } \epsilon$ (M <sup>-1</sup> cm <sup>-1</sup> )	5.17	4.60
$\lambda_{emi}$ (nm)	644	643
$\Phi_F$	0.58	0.58
$\Delta\lambda$ (nm)	17	16
pH 7.4		
$\lambda_{abs}$ (nm)	627	621
$\text{Log } \epsilon$ (M <sup>-1</sup> cm <sup>-1</sup> )	3.90	4.40
$\lambda_{emi}$ (nm)	651	649
$\Phi_F$	0.24	0.20
$\Delta\lambda$ (nm)	24	28

In acidic ethanol and pH 7.4 maximum absorption wavelengths ( $\lambda_{abs}$ ) for both compounds is 621 or 627 nm, with molar extinction coefficients in its logarithmic form ( $\text{Log } \epsilon$ ) between 3.90 and 5.17. The maximum emission wavelengths ( $\lambda_{emi}$ ) were found to be in the range of 643–651 nm at excitation of 590 nm, with moderate Stokes' shifts ( $\Delta\lambda$  16–28 nm). In general, it is clear that in ethanol both compounds show higher values for  $\text{Log } \epsilon$  and for  $\Phi_F$ , which is expected. It can also be seen that, even though  $\lambda_{abs}$  is similar in both solvents,  $\lambda_{emi}$  is higher in water, which leads to higher Stokes' shifts.

Figures 2 and 3 show normalized absorption and emission spectra of the two benzo[*a*]phenoxazininium chlorides **1** and **2** in acidified ethanol and aqueous solution at physiological pH, respectively.



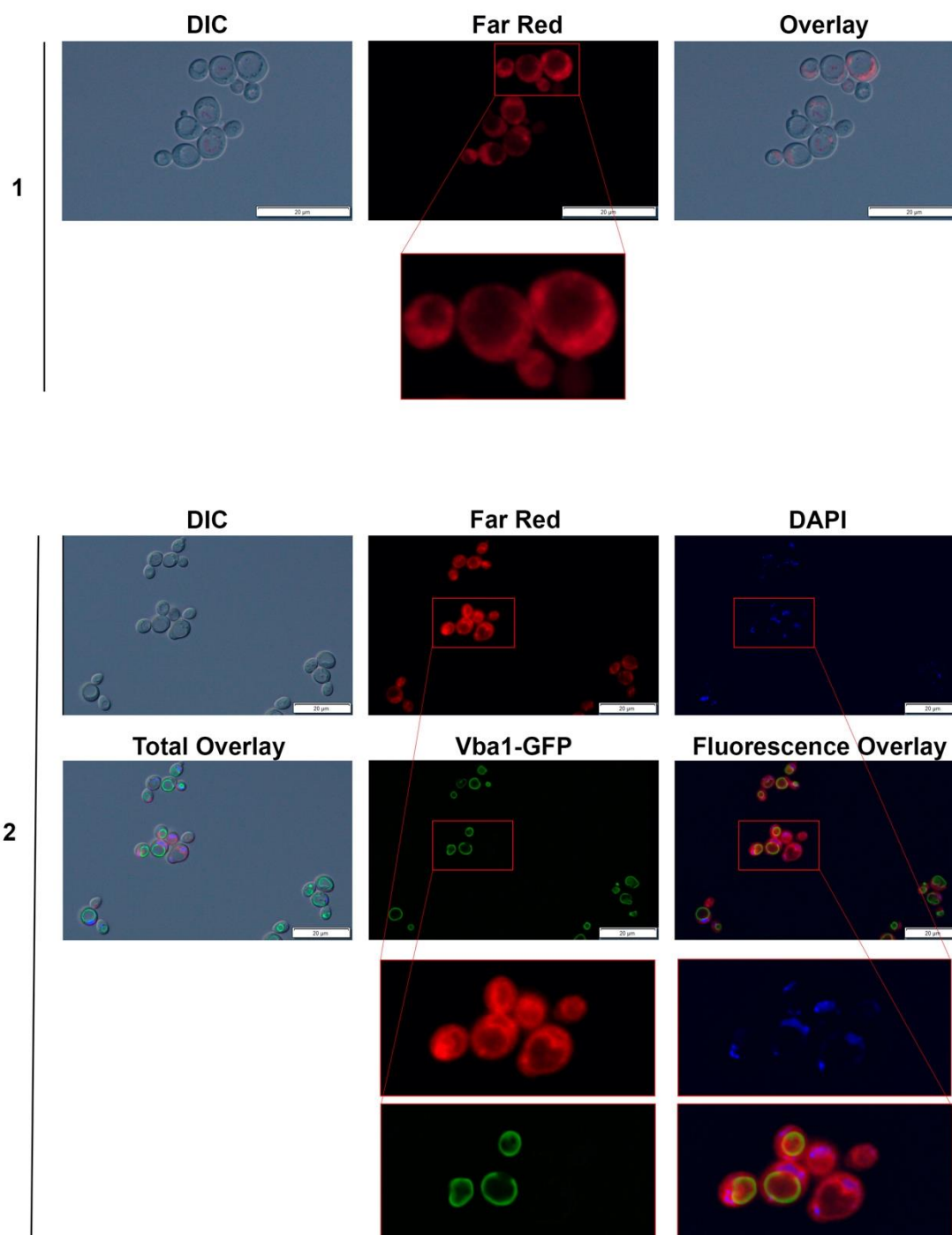
**Figure 2.** Normalized absorption and emission spectra of compounds **1** and **2** in acidified ethanol.



**Figure 3** Normalized absorption and emission spectra of compounds **1** and **2** in aqueous solution at physiological pH.

In order to evaluate the fluorescent staining capacity of the compounds, the intracellular distribution of the benzo[*a*]phenoxazinium chlorides **1** and **2** was assessed by fluorescence microscopy.

*S. cerevisiae* W303-1A cells were incubated with 12.5  $\mu$ M of **1** and **2** for two hours and observed on the fluorescence microscopy. It was observed that the incubation resulted in the accumulation of both the compounds within the cells, as it was detected near infrared fluorescence emission from the cells cytoplasmatic material upon excitation with a far-red filter setup. However, it was noticed that compound **1** presented a non-so much specific fluorescence staining of the cell (Figure 4), in contrast with compound functionalized with the sulfonamide group **1** (data not show) which led to a more specific pattern.



**Figure 4.** Compounds **1** and **2** intracellular distribution. Differential interference contrast (DIC) and Far Red fluorescence microscopy images of W303-1A cells after incubation with **1** (12.5 µM). DIC, Far Red, Green and Blue fluorescence microscopy images of W303-1A pDF01-VBA1-YEGFP cells after incubation with **2** (12.5 µM) co-stained with DAPI. Samples were stained in PBS at 30 ° for two hours and visualized by epifluorescence microscopy with a 60x oil immersion objective. Green arrows indicate compound accumulation on vacuolar membrane and yellow arrows indicate compound accumulation on the perinuclear membrane of endoplasmic reticulum.

In previous studies, it has been observed that compounds of this class stained with a greater specificity the vacuolar membrane and/or on the perinuclear membrane of the endoplasmic reticulum [11]. As such, in order to identify if the fluorescence staining pattern of compound **2** exhibit the same phenotype, *S. cerevisiae* W303-1A1 pDF01-VBA1-YEGFP cells (expressing a vacuolar protein

VBA1 fused with a GFP, which allow the identification of the vacuolar membrane) were incubated with 12.5  $\mu\text{M}$  of **2**, for two hours, and co-stained the cells with DAPI, to observe de nuclei, that is surrounded by the endoplasmic reticulum. Upon this incubation it was possible to identify by co-localization analysis, that the compound **2** accumulates at the vacuolar membrane (green arrows) and at the perinuclear membrane of the endoplasmic reticulum (yellow arrows) (Figure 5).

### 3. Experimental

#### 3.1. Typical Procedure for the Preparation of benzo[a]phenoxazinium Chlorides (Illustrated for **2**)

To a solution of 5-(ethylamino)-4-methyl-2-nitrosophenol hydrochloride (0.071 g,  $3.80 \times 10^{-4}$  mol, 1.5 eq.) in methanol, concentrated hydrochloric acid (0.118 mL) and *N*-(3-(naphthalen-1-ylamino)propyl)propane-1-sulfonamide (0.067 g,  $2.19 \times 10^{-4}$  mol, 1 eq.) were added and the resulting solution was refluxed for 20 h. The progress of the reaction was monitored by TLC (dichloromethane/methanol 9:1). After evaporation of the solvent and column chromatography purification on silica gel (mixtures of increasing polarity of dichloromethane/methanol as the eluent), compound **1** was obtained as a blue solid (0.044 g, 48%). mp = 141.2–143.5 °C. R<sub>f</sub> = 0.57 (dichloromethane/methanol 9:1). FTIR (KBr 1%):  $\nu_{\text{max}}$  3380, 3290, 2975, 2931, 2873, 1641, 1592, 1563, 1544, 1450, 1385, 1313, 1257, 1186, 1140, 1087, 1008, 965, 893, 822, 782  $\text{cm}^{-1}$ . <sup>1</sup>H RMN  $\delta_{\text{H}}$  (CD<sub>3</sub>OD, 400 MHz) 1.05 (t, *J* = 7.6 Hz, 3H, N<sub>2</sub>SO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 1.32–1.39 (m, 3H, NHCH<sub>2</sub>CH<sub>3</sub>), 1.81 (sext, *J* = 7.6 Hz, 2H, N<sub>2</sub>SO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 2.05–2.15 (m, 2H, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N<sub>2</sub>SO<sub>2</sub>), 2.35 (s, 1H, CH<sub>3</sub>) 3.02–3.08 (m, 2H, N<sub>2</sub>SO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 3.20–3.30 (m, 2H, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N<sub>2</sub>SO<sub>2</sub>), 3.50–3.60 (m, 2H, NHCH<sub>2</sub>CH<sub>3</sub>), 3.80–3.87 (m, 2H, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N<sub>2</sub>SO<sub>2</sub>), 6.90 (s, 1H, H-8), 7.03 (s, 1H, H-6), 7.75 (s, 1H, H-11), 7.82 (t, *J* = 7.6 Hz, 1H, H-3), 7.92 (t, *J* = 7.6 Hz, 1H, H-2), 8.34 (d, *J* = 8.0 Hz, 1H, H-4), 8.96 (d, *J* = 8.0 Hz, 1H, H-1) ppm. <sup>13</sup>C RMN  $\delta_{\text{C}}$  (CD<sub>3</sub>OD, 100.6 MHz) 13.21 (N<sub>2</sub>SO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 14.12 (NHCH<sub>2</sub>CH<sub>3</sub>), 17.61 (CH<sub>3</sub>), 18.39 (N<sub>2</sub>SO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 30.21 (NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N<sub>2</sub>SO<sub>2</sub>), 39.71 (NHCH<sub>2</sub>CH<sub>3</sub>), 41.32 (NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N<sub>2</sub>SO<sub>2</sub>), 42.78 (NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N<sub>2</sub>SO<sub>2</sub>), 54.44 (N<sub>2</sub>SO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 94.03 (C-6), 94.52 (C-8), 123.68 (C-4), 124.82 (Ar-C), 125.59 (C-1), 128.97 (Ar-C), 130.80 (C-3), 132.44 (Ar-C), 132.69 (C-2), 132.73 (C-11), 132.93 (Ar-C), 134.53 (Ar-C), 149.64 (C-10), 153.15 (Ar-C), 156.92 (C-9), 158.71 (C-5) ppm.

#### 3.2. Procedure for Fluorescence Staining.

*Saccharomyces cerevisiae* W303-1A and W303-1 pDF01-VBA1-YEGFP were grown respectively on YEPD (1% yeast extract, 2% peptone, 2% glucose) and synthetic complete medium SC (2% glucose, 0.5% ammonium sulfate, 0.7% yeast nitrogen base w/o amino acids, 0.2% dropout mix, 0.01% histidine and tryptophan, 0.02% leucine) agar plates. A sample of the cultures was used to prepare cell suspensions that were incubated overnight at 30°C and 120 rpm, in liquid YEPD and SC, until reach an optical density of approximated 0.8 at 640 nm. An Aliquot of W303-1A culture was collected and incubated with 12.5  $\mu\text{M}$  of **1** and **2** in PBS at 30 °C for two hours. In the same way, the W303-1 pDF01-VBA1-YEGFP strain was incubated with 12.5  $\mu\text{M}$  of **2**.

Cells were centrifuged at 3000 rpm for 5 min, rinsed two times in PBS and resuspended in 30  $\mu\text{L}$  of PBS. W303-1 pDF01-VBA1-YEGFP cells were also incubated with DAPI (final concentration 10  $\mu\text{g}/\text{mL}$ ), to allow the visualization of the nuclei. The samples were then analyzed on an Olympus BX6F2 fluorescence microscope, with appropriate filter cubes: U-FDICT (differential interference contrast), U-FYW (Far-Red), U-FUNA (blue), U-GFP (Green), with a 60 $\times$  oil immersion objective. All treatment conditions were performed in three independent experiments and the images presented are representative of the results obtained.

### 4. Conclusions

Two new benzo[a]phenoxazinium chlorides were successfully synthesized. Photophysical studies in acidic ethanol and aqueous solution at physiological pH showed that compounds display fluorescence with  $\lambda_{\text{emi}}$  between 643 and 651 nm, and fluorescent quantum yields up to 0.58 in acidified ethanol. The two compounds were able to stain the cells. The compound functionalized with the

sulphonamide group **2** exhibited a better and more specific fluorescence staining pattern, with preferential accumulation on the vacuolar membrane and perinuclear membrane of the endoplasmic reticulum. A result seems to be associated with the polarity of the compounds, as compound **2** is considerably more nonpolar, higher *Log P* value (2.20), than **1**, *Log P* (0.96).

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**Conflicts of Interest:** The authors declare no conflict of interest.

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