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New Nile Blue Derivatives as NIR Fluorescent Probes and Antifungal Agents †

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Abstract: The synthesis of four new Nile Blue derivatives with hydrogen, propyl and/or aminopropyl groups as substituents of the amines of 5- and 9-positions is described. Photophysical properties were evaluated in acidified ethanol and aqueous solution at physiological pH. Antifungal activity is also studied through the obtention of MIC values.

Keywords: benzo[*a*]phenoxazines; Nile Blue derivatives; NIR fluorescent probes; antifungal agents

1. Introduction

The development of new near-Infrared (NIR) fluorescent probes is a very important issue due to the wide range of applications [1–4]. These probes are an excellent choice to label biological material since its emission will not interfere with the natural fluorescence of biological compounds. Benzo[*a*]phenoxazinium salts, with Nile Blue being the best known, display fluorescence at around 600 nm and have been used as covalent and non-covalent fluorescent probes for amino acids, proteins and DNA, among other biological material [5–10]. In addition, applications as sensors or agents for photodynamic therapy (PDT) have been described [11,12]. Furthermore, medical applications of these compounds have been found, showing antifungal and antimalaria capacities [13–15].

Considering all these facts, the synthesis of four new benzo[*a*]phenoxazinium chlorides possessing one or two propyl groups at the 9-amino position and the aminopropyl group or a single hydrogen atom at the 5-amino position was carried out. Photophysical properties in ethanol acidified with trifluoroacetic acid (TFA) and in aqueous solution at physiological pH, as well as the antifungal activity of all these compounds were evaluated and are described.

2. Results and Discussion

Benzo[*a*]phenoxazinium chlorides **1a**,**b** and **2a**,**b** were synthesized by condensation of 5-(dipropylamino)-2-nitrosophenol hydrochloride or 5-(propylamino)-2-nitrosophenol hydrochloride with naphthalen-1-amine and N^1-(naphthalen-1-yl)propane-1,3-diamine hydrobromide. Nitrosophenol hydrochlorides were obtained by nitrosation of the 3-(dipropylamino)phenol or 3-(propylamino)phenol with sodium nitrite in the presence of hydrochloric acid.

The benzo[*a*]phenoxazinium chlorides **1a**,**b** and **2a**,**b** were obtained as blue solids in 18–49% yields (Figure 1). All compounds were fully characterized by the usual analytical techniques.

The [1]H NMR spectra exhibited aromatic protons of the polycyclic system (H-1, H-2, H-3, H-4, H-6, H-8, H-10 and H-12) at *δ* 6.86–8.96 ppm. The terminal methyl groups at the 9-amino position appeared as triplets or multiplets (*δ* 1.04–1.12 ppm), adjacent methylene protons as quintets or multiplets (*δ* 1.70–1.81 ppm) and methylene protons adjacent to the nitrogen atoms as triplets or multiplets (*δ* 3.45–3.62 ppm). Methylene protons of propylamino groups at the 5-amino position for compounds **1a** and **2a** appeared as triplets, multiplets or broad singlets at *δ* 2.20–2.32 ppm ($NHCH_2\mathit{CH_2}CH_2NH_2$·HBr), *δ* 3.20–3.24 ppm ($NHCH_2CH_2\mathit{CH_2}NH_2$·HBr) and *δ* 3.84–3.87 ppm ($NH\mathit{CH_2}CH_2CH_2NH_2$·HBr).

The [13]C NMR spectra showed the aromatic carbons of benzo[*a*]phenoxazinium core (*δ* 94.14–164.62 ppm). Methyl and methylene carbons of the propyl groups at the 9-amino position of di-alkylated compounds **1a**,**b** appeared at *δ* 11.41–11.54 ppm ($N(CH_2CH_2\mathit{CH_3})_2$), *δ* 21.76–21.95 ppm ($N(CH_2\mathit{CH_2}CH_3)_2$) and *δ* 54.53–54.76 ppm ($N(\mathit{CH_2}CH_2CH_3)_2$). There is a slight difference for mono-alkylated compounds **2a**,**b**, which showed the carbons of methyl groups at *δ* 11.52–11.59 ppm), adjacent methylene groups at *δ* 23.53–23.55 ppm, and methylenes adjacent to the nitrogen at *δ* 46.16–46.49 ppm. Methylene carbons of the propylamino group at the 5-amino position appeared at *δ* 26.56–27.68 ($NHCH_2\mathit{CH_2}CH_2NH_2$·HBr), *δ* 42.63–42.97 ($NH\mathit{CH_2}CH_2CH_2NH_2$·HBr) and *δ* 38.35–38.49 ppm ($NHCH_2CH_2\mathit{CH_2}NH_2$·HBr).

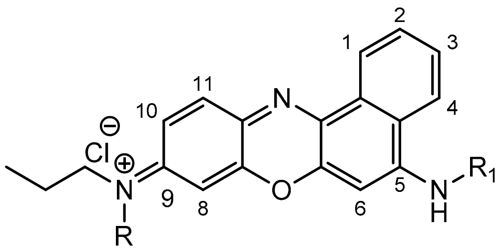

Figure 1. Structures of benzo[*a*]phenoxazinium chlorides **1a**,**b** and **2a**,**b**.

Photophysical properties of benzo[*a*]phenoxazinium chlorides **1a**,**b** and **2a**,**b** were evaluated through absorption and emission spectra of 10^{-6} M solutions in ethanol acidified with TFA and aqueous solution at physiological pH. The relative fluorescence quantum yields (Φ_F) were determined using Oxazine 1 as a standard (Φ_F = 0.11 in ethanol) at 590 nm excitation. Results are presented in Table 1.

Table 1. Photophysical data of compounds **1a**,**b** and **2a**,**b** in acidified ethanol and aqueous solution at pH 7.4 (λ_{exc} 590 nm).

Compound	**1a**	**1b**	**2a**	**2b**
Acidified ethanol				
λ_{abs} (nm)	639	629	622	609
ε ($M^{-1}cm^{-1}$)	39,920	67,500	32,301	65,800
λ_{emi} (nm)	669	662	655	646
Φ_F	0.18	0.20	0.35	0.47
$\Delta\lambda$ (nm)	30	33	33	37
pH 7.4				
λ_{abs} (nm)	648	639	621	610
ε ($M^{-1}cm^{-1}$)	33,622	62,425	17,250	35,093
λ_{emi} (nm)	683	675	658	656
Φ_F	0.03	0.02	0.12	0.12
$\Delta\lambda$ (nm)	35	36	37	46

In acidic ethanol and pH 7.4 maximum absorption wavelengths (λ_{abs}) for all compounds lie in the range 609–648 nm, with molar extinction coefficients (ε) between 17,250 and 67,500 $M^{-1}cm^{-1}$. The maximum emission wavelengths (λ_{emi}) were found to be in the range of 646–683 nm at an excitation wavelength of 590 nm, with moderate Stokes' shifts (Δ*λ*, 30–46 nm). In comparison, compounds **1a**,**b** displayed a bathochromic shift in both λ_{abs} (17–29 nm) and λ_{emi} (14–25 nm) in acidified ethanol and at physiological pH. This is mainly due to the di-alkylation at the 9-amino position as previously observed [16]. Furthermore, compounds **1a** and **2a**, with an aminopropyl at the 5-amino position, show also a bathochromic shift comparing to compounds **1b** and **2b**, which have a hydrogen atom at the same position. This indicates that the presence of an alkyl chain at the 5-amino position of the benzophenoxazinium core increases the maximum absorption wavelength.

Comparing data of λ_{emi} in ethanol and aqueous solution for all compounds a bathochromic shift is observed at pH 7.4 (3–14 nm). Fluorescence quantum yields are higher for compounds **2a**,**b** in both solvents, but decrease considerably at pH 7.4 (Φ_F 0.12) comparing to ethanol (Φ_F 0.35, **2a**; 0.47, **2b**).

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

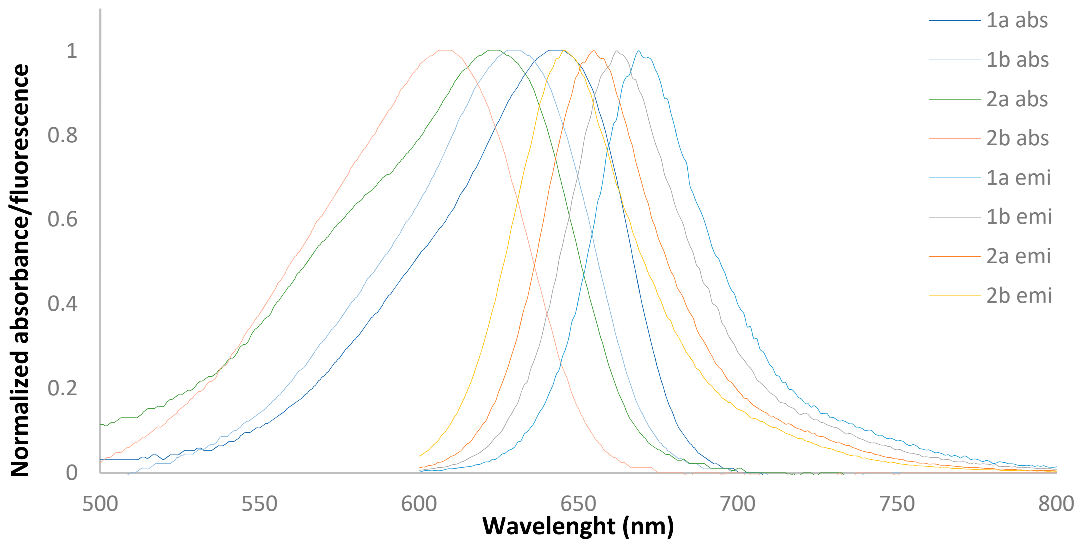

Figure 2. Normalized absorption and emission spectra of compounds **1a**,**b** and **2a**,**b** in acidified ethanol.

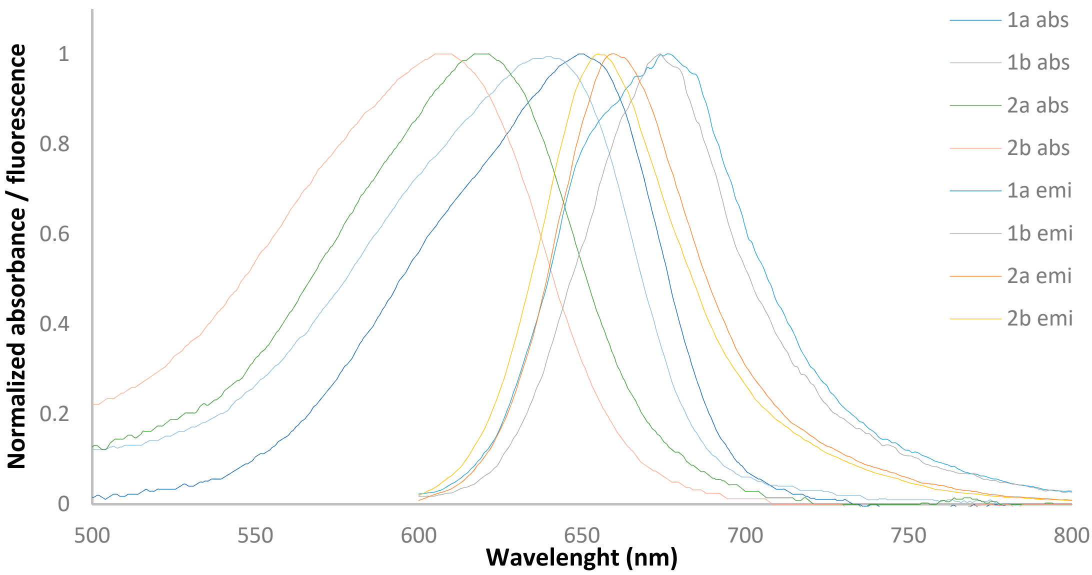

Figure 3. Normalized absorption and emission spectra of compounds **1a**,**b** and **2a**,**b** in aqueous solution at physiological pH.

Antifungal activity of benzo[*a*]phenoxazinium chlorides **1a**,**b** and **2a**,**b** was measured against *Saccharomyces cerevisiae* PYCC 4072. Minimum Inhibitory Concentration (MIC) values indicate the minimum concentration of each compound in which the yeast growth is inhibited by ≥80%. Log *P* is an estimated measure of the compounds' hydrophobicity by calculating the partition between membranes and aqueous media (Table 2).

Table 2. MIC values of compounds **1a**,**b** and **2a**,**b** against *Saccharomyces cerevisiae* PYCC 4072.

Compound	MIC (μM)	Log *P*
1a	25	1.15
1b	25	1.70
2a	25	1.09
2b	6.25	1.64

Compound **2b** have a MIC value of 6.25 μM, while the other three compounds have 25 μM. Previous work appeared to show di-alkylation at the 9-amino position improved antifungal activity comparing to mono-alkylation [14]. However, this work showed compound **2b** (only one alkyl chain at 9-position) has a lower MIC value than analogues, indicating that biological activity may relate to the combination of all substituents and no correlation between MIC value and the number of alkylic chains at the 9-amino position can be established. No correlation between MIC values and Log *P* values is established either.

3. Experimental

*Typical procedure for the preparation of compounds **1a,b** and **2a,b** (illustrated for **2b**)*

To a solution of 5-(propylamino)-2-nitrosophenol hydrochloride (0.408 g, 1.88×10^{-3} mol, 2 eq.) in methanol (3 mL), concentrated hydrochloric acid (0.724 mL) was added followed by naphthalen-1-amine (0.135 g, 9.4×10^{-4} mol, 1 eq.), and the resulting solution was refluxed for 24 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), *N*-(5-amine-9*H*-benzo[*a*]phenoxazin-9-ilidene)propane-1-aminium chloride was obtained as a blue solid (0.157 g, 49%). δ_H (CD_3OD, 400 MHz) 1.07 (t, *J* = 7.2 Hz, 3H, $NHCH_2CH_2\mathit{CH_3}$), 1.78 (sext, *J* = 7.2 Hz, 2H, $NHCH_2\mathit{CH_2}CH_3$), 3.51 (t, *J* = 7.6 Hz, 2H, $NH\mathit{CH_2}CH_2CH_3$), 6.98 (s, 2 H, H-6 and H-8), 7.26 (d, *J* = 9.4 Hz, 1H, H-10), 7.88–7.95 (m, 2H, H-3 and H-11), 8.02 (dt, *J* = 8.0 and 0.8 Hz, 1H, H-2), 8.39 (d, *J* = 8.0 Hz, 1H, H-4), 8.96 (dd, *J* = 8.0 and 0.8 Hz, 1H, H-1) ppm. δ_C (CD_3OD, 100.6 MHz) 11.52 ($NHCH_2CH_2\mathit{CH_3}$), 23.53 ($NHCH_2\mathit{CH_2}CH_3$), 46.16 ($NH\mathit{CH_2}CH_2CH_3$), 98.29 (C-8), 98.86 (C-6), 113.04 (C-10), 124.75 (Ar-C), 125.08 (C-4), 126.09 (C-1), 130.43 (Ar-C), 131.60 (C-3), 132.68 (C-11), 133.29 (Ar-C), 134.21 (C-2), 144.49 (Ar-C), 152.40 (2 × Ar-C), 153.01 (C-9), 164.62 (C-5) ppm.

Procedure for antifungal activity tests

Minimum inhibitory concentration of growth for the different compounds was determined using a broth microdilution method for the antifungal susceptibility testing of yeasts (M27-A3, CLSI—Clinical and Laboratory Standards Institute). Cells were incubated at 30 °C in RPMI 1640 medium, buffered to pH 7.0 with 0.165 M morpholenepropanesulfonic acid (MOPS) buffer. Initial cell concentration was 2.25×10^3 cells/mL. Stock solutions of the compounds were prepared in DMSO and a final dilution was carried out in an RPMI 1640 medium (DMSO concentrations of 0.5% per well). MIC values were determined using a microplate photometer, after 48 h of incubation, as the lowest concentration of drug that resulted in a growth inhibition over 80%, as compared to the growth observed in the control wells containing 0.5% DMSO. Each drug concentration was tested in triplicate and in two independent experiments.

4. Conclusions

Four 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 λ_{emi} between 646 and 683 nm, and fluorescent quantum yields up to 0.47, being the highest values related to compound with propyl and aminopropyl groups at 9- and 5-positions, respectively. All compounds revealed good antifungal activity, with benzo[*a*]phenoxazinium with the later combination of substituents presenting the best result, a MIC value of 6.25 μM.

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

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