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Synthesis, Photophysics and Potential Antifungal Activity of Benzo[*a*]phenoxazines †

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Abstract: The synthesis and full characterisation of new hydroxyl benzo[*a*]phenoxazinium chlorides with *N*-(di)propyl and/or *N*-isopropyl groups at 5- and 9-positions are described. Photophysical studies carried out in ethanol and water revealed strong absorbance and significant fluorescence emission up to 676 nm. The antifungal activity was assessed against *Saccharomyces cerevisiae* and the results provide clues to direct further studies.

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

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1. Introduction

Fungal infections represent a significant public health issue, especially due to the rising number of immunocompromised patients and the growing incidence of drug-resistant fungal strains. Although traditional antifungal treatments are effective, they often suffer from drawbacks such as toxicity, a narrow range of activity, and the potential for resistance. Consequently, there is a crucial need to discover new antifungal agents with innovative mechanisms of action [1–4]. In this regard, benzo[*a*]phenoxazines have surfaced as promising candidates [5–9].

Benzo[*a*]phenoxazines are a class of heterocyclic compounds known for their diverse biological activities, including antimicrobial, anticancer, and antiviral properties. Their unique structure, characterized by a fused aromatic system, imparts distinctive electronic and photophysical properties, making them attractive for various biomedical applications [10–20].

Given the interest of our research team in this type of compounds [5–9,11–20], the present work is focused on the synthesis of three hydroxyl benzo[*a*]phenoxazinium chlorides di- or mono-substituted with (iso)propyl groups at the amines of 5- and 9-positions of the polycyclic system. The photophysical characterization, and evaluation of the potential antifungal activity of the synthesized compounds were performed and are discussed.

2. Results and Discussion

The precursors of the target benzo[*a*]phenoxazine derivatives, namely the nitrosophenol hydrochlorides, were prepared by nitrosation of 3-(dipropylamino)phenol or 3-(propylamino)phenol with sodium nitrite in the presence of hydrochloric acid. In addition, 5-aminonaphthalen-2-ol was *N*-alkylated with 1-bromopropane or 2-bromopropane in ethanol to give the other required precursor.

Condensation of 5-(dipropylamino)-2-nitrosophenol or 2-nitroso-5-(propylamino)phenol with 5-(propylamino)naphthalen-2-ol or 5-(isopropylamino)naphthalen-2-ol, with hydrochloric acid, in ethanol, gave the corresponding hydroxyl benzo[*a*]phenoxazinium chlorides **1a–c** as blue solids in yields up to 39% (Figure 1). The full characterization of these compounds was carried out by the usual analytical techniques.

The ^{1}H NMR spectra of compounds **1a–c** showed the methyl groups as triplets, multiplets or a doublet (**1c**) (δ 1.06–1.50 ppm), the adjacent methylene protons as sextets or multiplets (δ 1.71–1.93 ppm), methylene protons adjacent to the nitrogen atoms as triplets or multiplets (δ 3.30–3.70 ppm), and the proton of tertiary carbon as quintet (**1c**), in addition to the aromatic protons (δ 6.78–8.33 ppm). In the case of ^{13}C NMR spectra, it was possible to confirm the presence of the methyl (δ 11.20–22.08 ppm) and methylene (δ 46.28–54.46 ppm) carbons of (iso)propyl groups, as well as the aromatic carbons of benzo[*a*]phenoxazinium core (δ 93.06–162.97 ppm).

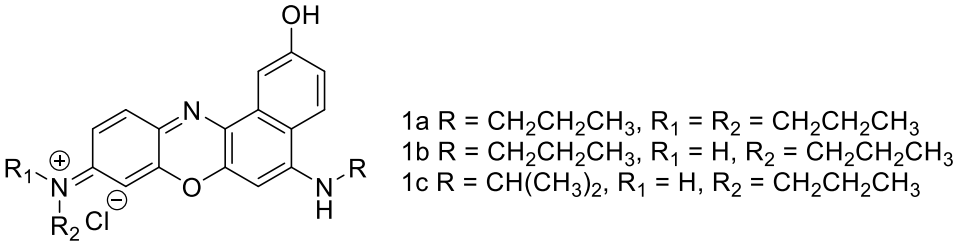

Figure 1. Structures of hydroxyl benzo[*a*]phenoxazinium chlorides **1a–c**.

The photophysical study of the benzo[*a*]phenoxazinium chlorides **1a–c** was based on absorption and emission spectra of 10^{-6} M solutions in ethanol and water. Oxazine 1 was used as standard (Φ_F = 0.11, in ethanol) with excitation at 590 nm for the determination of the relative fluorescence quantum yields (Φ_F). Table 1 shows a summary of the results.

In ethanol and water, maximum absorption wavelengths (λ_{abs}) for all compounds were found in the range 612–634 nm, with molar extinction coefficients (ε) between 17,460 and 44,613 $M^{-1}cm^{-1}$. The maximum emission wavelengths (λ_{emi}) lie in the range of 644–676 nm at excitation of 590 nm, with moderate Stokes' shifts ($\Delta\lambda$, 23–42 nm).

Table 1. Photophysical data of compounds **1a–c** in ethanol and water (λ_{exc} 590 nm).

Compound	**1a**	**1b**	**1c**
Ethanol			
λ_{abs} (nm)	621	618	620
ε ($M^{-1}cm^{-1}$)	37,906	37,982	44,613
λ_{emi} (nm)	644	644	645
Φ_F	0.59	0.56	0.54
$\Delta\lambda$ (nm)	23	26	25
Water			
λ_{abs} (nm)	634	616	612
ε ($M^{-1}cm^{-1}$)	17,460	19,055	31,086
λ_{emi} (nm)	676	650	651
Φ_F	0.10	0.28	0.27
$\Delta\lambda$ (nm)	42	34	39

In ethanol, λ_{abs} values are very similar for the three compounds, although higher for **1a**. On the other hand, in water there is a bathochromic shift in **1a** of 18 and 22 nm compared to **1b** and **1c**, respectively. This occurs essentially due to the di-alkylation of the amine in the 9-position, as noted earlier [14]. The ε values decrease for all compounds

when switching from ethanol to water, which may be related to differences in the solubility of the compounds in the two solvents.

Regarding the λ_{emi}, in ethanol, it is practically the same for the three compounds. Nevertheless, in water and as occurred in the λ_{abs} values, compound **1a** registers a bathochromic shift of 26 and 25 nm in relation to **1b** and **1c**.

The fluorescence quantum yields (Φ_F) are in the range 0.10 to 0.59, with the best values for all compounds in ethanol (0.54–0.59). The decrease in Φ_F in water, although it occurs for the three compounds, is more significant in the case of **1a** (0.10) than in **1b** (0.28) and **1c** (0.27). However, the values obtained in water continue to be very interesting given the limited number of fluorophores with emission at high wavelengths and above with considerable solubility in aqueous media.

The normalized absorption and emission spectra of the benzo[*a*]phenoxazinium chlorides **1a–c** in ethanol and water are shown Figures 1 and 2, respectively.

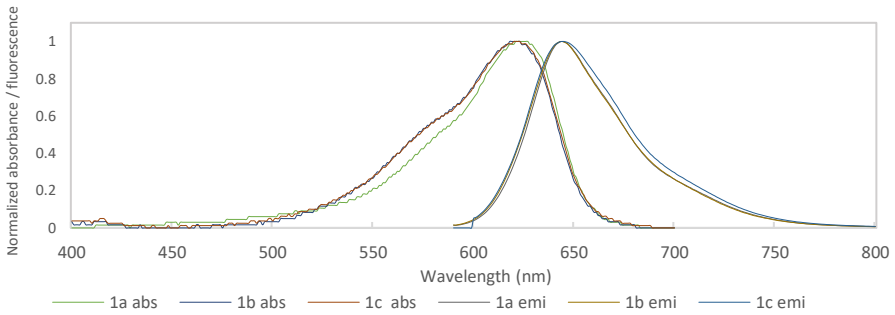

Figure 1. Normalized absorbance and emission spectra of compounds **1a–c** in ethanol.

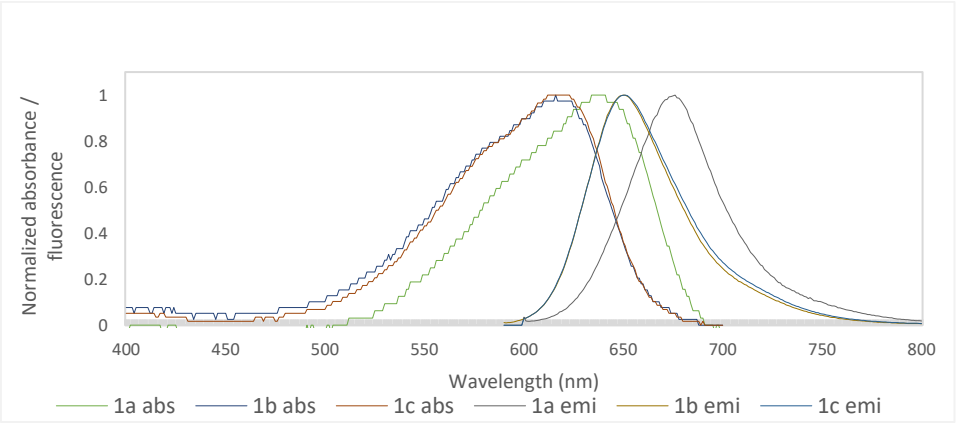

Figure 2. Normalized absorbance and emission spectra of compounds **1a–c** in water.

Benzo[*a*]phenoxazinium chlorides **1a–c** were evaluated for antifungal activity against *Saccharomyces cereviseae* PYCC 4072. The minimum concentration of each compound at which the yeast growth was inhibited by ≥80%, indicated as the Minimum Inhibitory Concentration (MIC) value, was superior to 200 μM for all the compounds. These results suggest that the hydroxyl group in position 2 of the polycyclic system decrease the potential antifungal activity of benzo[*a*]phenoxazines, since the presence of (di)propyl groups in the amines at positions 5 and 9 of the polycyclic system when this hydroxyl group is not present, are usually associated with low MIC values [7,13]. This fact stands out in the non-hydroxylated benzo[*a*]phenoxazinium chloride analogous to compound **1a**, with a MIC value of 1.56 [7]. However, the effect of the hydroxyl group on the antifungal activity of benzophenoxazine derivatives needs to be further evaluated with a higher number of compounds to allow conclusions about the structure-activity relationship.

3. Experimental

3.1. Typical Procedure for the Preparation of Compounds **1a**–**c** *(Illustrated for* **1a***)*

To a solution of 5-(dipropylamino)-2-nitrosophenol hydrochloride (0.125 g, 5.69×10^{-4} mol) in ethanol (2 mL), concentrated hydrochloric acid (5×10^{-2} mL) was added followed by 5-(propylamino)naphthalen-2-ol (0.057 g, 2.84×10^{-4} mol), and the resulting solution was refluxed for 15 h. The reaction progress was monitored by TLC (dichloromethane/methanol 98:2). After evaporation of the solvent and column chromatography purification on silica gel (mixtures of increasing polarity of dichloromethane/methanol as the eluent), *N*-(2-hydroxy-5-(propylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)-*N*-propylpropan-1-aminium chloride **1a** was obtained as a blue solid (0.049 g, 39%). $\upsilon_{máx}$ (solid) 3400, 3200, 2872, 1640, 1592, 1557, 1457, 1428, 1332, 1168, 1125, 1099, 1034, 917, 822, 754 cm^{-1}. δ_H (CD_3OD, 400 MHz) 1.06 (t, *J* = 7.2 Hz, 6H, $N(CH_2CH_2CH_3)_2$), 1.12 (t, *J* = 7.2 Hz, 3H, $NHCH_2CH_2CH_3$), 1.71–1.84 (m, 4H, $N(CH_2CH_2CH_3)_2$), 1.87–1.93 (m, 2H, $NHCH_2CH_2CH_3$), 3.60 (t, *J* = 8 Hz, 4H, $N(CH_2CH_2CH_3)_2$), 3.70 (t, *J* = 7.2 Hz, 2H, $NHCH_2CH_2CH_3$), 6.87 (broad s, 2H, H-6 and H-8), 7.20–7.30 (m, 2H, H-10 and H-11), 7.84 (d, *J* = 9.2 Hz, 1H, H-3), 8.23 (broad s, 2H, H-4 and H-1) ppm. δ_C (CD_3OD, 100.6 MHz) 11.20 ($N(CH_2CH_2CH_3)_2$ or $NHCH_2CH_2CH_3$), 11.46 ($N(CH_2CH_2CH_3)_2$ or $NHCH_2CH_2CH_3$), 11.74 ($N(CH_2CH_2CH_3)_2$ or $NHCH_2CH_2CH_3$), 20.69 ($N(CH_2CH_2CH_3)_2$), 21.76 ($N(CH_2CH_2CH_3)_2$), 23.26 ($NHCH_2CH_2CH_3$), 50.48 ($NHCH_2CH_2CH_3$), 54.46 ($N(CH_2CH_2CH_3)_2$), 93.51 (C-8), 97.13 (C-6), 110.09 (C-1), 115.80 (C-10), 117.05 (Ar-C), 120.14 (C-11), 126.35 (Ar-C), 130.35 (C-4), 133.67 (Ar-C), 135.22 (C-3), 135.60 (Ar-C), 149.30 (Ar-C), 153.41 (Ar-C), 155.56 (C-9), 159.65 (C-5) 162.97 (C-2) ppm.

3.2. Procedure for Antifungal Activity Tests

The Minimum Inhibitory Concentration of growth for benzo[*a*]phenoxazinium chlorides **1a**-**c** was determined through the broth microdilution method for the antifungal susceptibility testing of yeasts (M27-A3, CLSI—Clinical and Laboratory Standards Institute). The incubation of cells was carried out at 30 °C in RPMI 1640 medium, buffered to pH 7.0 with 0.165 M morpholenepropanesulfonic acid (MOPS) buffer. The initial cell concentration was 2.25×10^3 cells/mL. Stock solutions of the benzo[*a*]phenoxazinium chlorides **1a**–**c** were prepared in DMSO and a final dilution was performed in an RPMI 1640 medium (DMSO concentrations of 0.5% per well). After 48 h of incubation the optical density of the cultures was read using a microplate photometer. MIC values were considered as the lowest concentration of drug that resulted in a growth inhibition over 80%, when compared to the growth control containing 0.5% DMSO. All the drug concentrations were tested in triplicate and in two independent experiments.

4. Conclusions

New hydroxyl benzo[*a*]phenoxazinium chlorides were successfully synthetized and spectroscopically characterized. Studies of photophysical properties in ethanol and water revealed that these compounds showed fluorescence with λ_{emi} in the range of 644 to 683 nm, and fluorescent quantum yields up to 0.59, being the highest values in ethanol for compound **1a**, with *N*-dipropyl group at 9-position, and in water for compound **1b**, with only one propyl group at 9-amine position. In terms of activity, it was found that the presence of the hydroxyl group in position 2 of the polycyclic system reduces biological activity compared to non-hydroxylated analogues. However, the low toxicity of the new benzo[*a*]phenoxazinium chlorides can be beneficial for their applications as fluorescent markers in biological applications, given their interesting photophysical properties and solubility in aqueous media.

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