

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

Bárbara S. G. Guimarães ¹, Maria João Sousa ^{2,3}  and M. Sameiro T. Gonçalves ^{1,*} 

¹ Centre of Chemistry (CQUM), Department of Chemistry, University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal; pg46790@alunos.uminho.pt

² Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal; mjsousa@bio.uminho.pt

³ Institute of Science and Innovation for Bio-Sustainability (IB-S), University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal

* Correspondence: msameiro@quimica.uminho.pt

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

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

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 their 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 our research team's interest in this type of compound [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 the 5 and 9 positions of the polycyclic system. A photophysical characterization of the synthesized compounds was carried out and their potential antifungal activity was evaluated.

2. Results and Discussion

The precursors of the target benzo[a]phenoxazine derivatives, namely the nitrosophenol hydrochlorides, were prepared via the 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 obtain the other required precursor.



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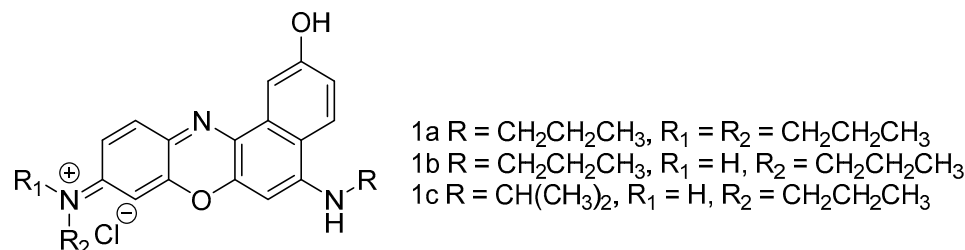
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The condensation of 5-(dipropylamino)-2-nitrosophenol or 2-nitroso-5-(propylamino)phenol with 5-(propylamino)naphthalen-2-ol or 5-(isopropylamino)naphthalen-2-ol, with hydrochloric acid, in ethanol, yielded the corresponding hydroxyl benzo[*a*]phenoxazinium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



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

The ¹H 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 a quintet (**1c**), in addition to the aromatic protons (δ 6.78–8.33 ppm). In the case of ¹³C 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 the benzo[*a*]phenoxazinium core (δ 93.06–162.97 ppm).

The photophysical study of the benzo[*a*]phenoxazinium chlorides **1a–c** was based on the absorption and emission spectra of 10^{−6} M solutions in ethanol and water. Oxazine 1 was used as the 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.

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

Compound	1a	1b	1c
<i>Ethanol</i>			
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
<i>Water</i>			
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 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 an excitation of 590 nm, with moderate Stokes' shifts ($\Delta\lambda$, 23–42 nm).

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

all compounds when switching from ethanol to water 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 all three compounds. Nevertheless, in water, as occurred for the λ_{abs} values, compound **1a** underwent a bathochromic shift of 26 and 25 nm in relation to **1b** and **1c**.

The fluorescence quantum yields (Φ_F) were in the range 0.10 to 0.59, with the best values obtained for all compounds in ethanol (0.54–0.59). The decrease in Φ_F in water, although present in all three compounds, was more significant in the case of **1a** (0.10) than in **1b** (0.28) and **1c** (0.27). However, the values obtained in water continued to be very interesting given the limited number of fluorophores with emissions at high wavelengths that showed 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 in Figures 1 and 2, respectively.

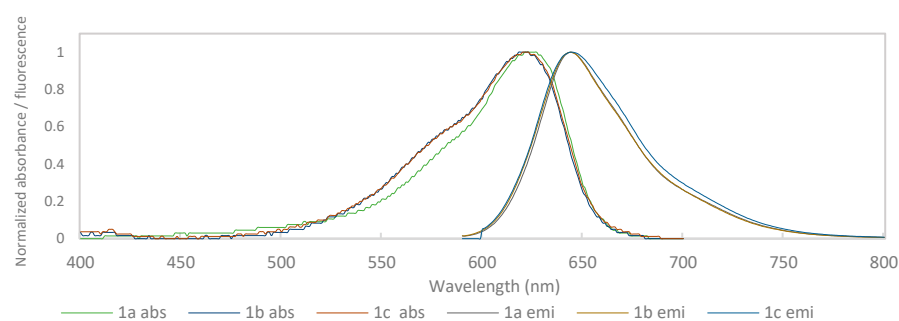


Figure 1. Normalized absorbance and emission spectra of compounds **1a–c** in ethanol.

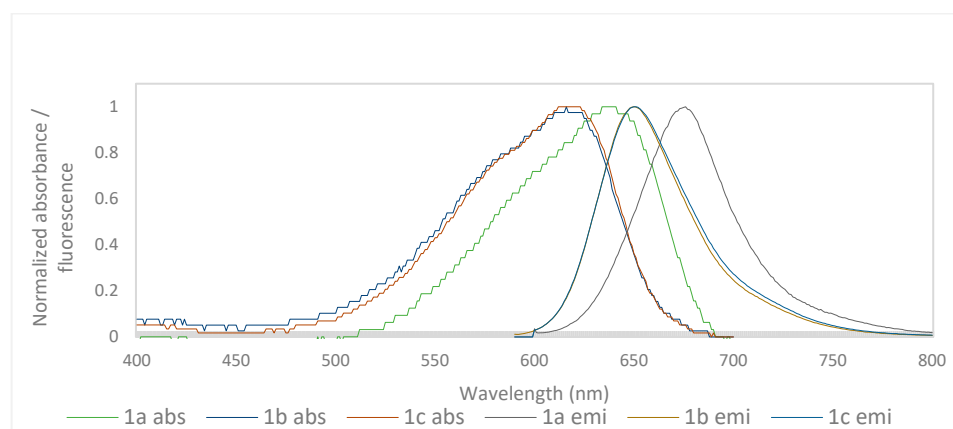


Figure 2. Normalized absorbance and emission spectra of compounds **1a–c** in water.

Benzo[*a*]phenoxazinium chlorides **1a–c** were evaluated to determine their antifungal activity against *Saccharomyces cerevisiae* PYCC 4072. The minimum concentration of each compound at which the yeast growth was inhibited by $\geq 80\%$, denoted 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 decreases 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 is 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 an 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 draw conclusions about the structure–activity relationship.

3. Experimental

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

Concentrated hydrochloric acid (5×10^{-2} mL) was added to a solution of 5-(dipropylamino)-2-nitrosophenol hydrochloride (0.125 g, 5.69×10^{-4} mol) in ethanol (2 mL), 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 via TLC (dichloromethane/methanol 98:2). After evaporation of the solvent and column chromatography purification on silica gel (mixtures with an increase in the polarity of dichloromethane/methanol as the eluent), *N*-(2-hydroxy-5-(propylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)-*N*-propylpropan-1-ammonium chloride **1a** was obtained as a blue solid (0.049 g, 39%). ν_{max} (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, $\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)_2$), 1.12 (t, $J = 7.2$ Hz, 3H, $\text{NHCH}_2\text{CH}_2\text{CH}_3$), 1.71–1.84 (m, 4H, $\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)_2$), 1.87–1.93 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_3$), 3.60 (t, $J = 8$ Hz, 4H, $\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)_2$), 3.70 (t, $J = 7.2$ Hz, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_3$), 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 ($\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)_2$ or $\text{NHCH}_2\text{CH}_2\text{CH}_3$), 11.46 ($\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)_2$ or $\text{NHCH}_2\text{CH}_2\text{CH}_3$), 11.74 ($\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)_2$ or $\text{NHCH}_2\text{CH}_2\text{CH}_3$), 20.69 ($\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)_2$), 21.76 ($\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)_2$), 23.26 ($\text{NHCH}_2\text{CH}_2\text{CH}_3$), 50.48 ($\text{NHCH}_2\text{CH}_2\text{CH}_3$), 54.46 ($\text{N}(\text{CH}_2\text{CH}_2\text{CH}_3)_2$), 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 to test the antifungal susceptibility 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 a 0.165 M morpholinopropanesulfonic 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 to be the lowest concentration of the 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 synthesized and spectroscopically characterized. Studies of their 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 of up to 0.59. The highest values were found in ethanol for compound **1a**, with the *N*-dipropyl group in the 9 position, and in water for compound **1b**, with only one propyl group found in the nine-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 could be beneficial to their use as fluorescent markers in biological applications given their interesting photophysical properties and solubility in aqueous media.

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