

Antiproliferative Activity of 2,3,6,7-Tetrahydro-1*H*-benzo[*a*]quinolizino[1,9-*hi*]phenoxazin-14(5*H*)-iminium Chloride Derivatives

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Abstract: Compounds based on 1,2,3,5,6,7-hexahydropyrido[3,2,1-*ij*]quinolone, trivially designated as julolidine, have been reported due to their biological interest, namely as inhibitors of the amyloid- β protein self-assembly (which is associated to the pathogenesis of Alzheimer's disease), as well as colorimetric and fluorometric probes. Taking this in account, in addition to the relevant biological importance of benzophenoxazinium salts such as Nile Blue derivatives, the present work evaluated the influence of the julolidine moiety in antimicrobial activity against *Saccharomyces cerevisiae* PYCC 4072 of newly synthesised fluorescent 2,3,6,7-tetrahydro-1*H*-benzo[*a*]quinolizino[1,9-*hi*]phenoxazin-14(5*H*)-iminium chlorides, in comparison with their analogues.

Keywords: benzo[*a*]phenoxazines, Nile Blue derivatives, julolidine derivatives, antimicrobial drugs.

1. Introduction

Polycyclic heteroaromatic compounds having the oxazine ring, namely phenoxazines, benzophenoxazines and their derivatives, with absorption and emission at longer wavelengths, presented various bioapplications as fluorescent probes.¹ These compounds also exhibited antiproliferative properties with potential applications as antitumor and antimicrobial agents, probably due to intercalation with DNA through the polycyclic planar system.²⁻⁴ An earlier study of structure-activity performed by our research group demonstrated the antiproliferative activity (antifungal) of several

benzo[*a*]phenoxazines and naphtho[2,3-*a*]phenoxazines possessing different combinations of substituents on the amine side groups.^{5,6} On the other hand, compounds based on the julolidine skeleton have been reported as inhibitors for the amyloid- β protein self-assembly, which is associated to the Alzheimer's disease, among other interesting applications in biological systems related to their interesting photophysical properties.⁷⁻¹⁰

Currently, our group is studying the photophysical properties of new benzophenoxazines fused with julolidine, in order to understand the effect of the replacement of a freely rotating *N*-substituent group by a rigid structure. The synthesis of a new benzophenoxazinium chloride **3a**, with the julolidine moiety and a *N*-(3-chloro)propyl group as a substituent at the 14-position was performed.

Furthermore, with the purpose of extending this work with julolidine-based compounds and in continuation of our research interests in the biological applications of Nile Blue derivatives, we evaluated their potential as antiproliferative agents. *Saccharomyces cerevisiae* was used as a model organism in the assessment of the activity of new benzophenoxazine derivatives, which was performed in comparison with other related compounds of this family.

2. Experimental

2.1. Procedure for the synthesis of compound 2.

To a solution of naphthalen-1-amine (0.500 g, 3.50×10^{-3} mol) in ethanol (3 mL), 1-bromo-3-chloropropane (0.379 mL, 3.85×10^{-3} mol) was added, and the resulting mixture was refluxed for 6 hours. The progress of reaction was monitored by TLC (*n*-hexane/ethyl acetate, 9:1). After completion of the reaction, the solvent was evaporated and the mixture was purified by column chromatography on silica gel using *n*-hexane and *n*-hexane/ethyl acetate, mixtures of increasing polarity, as the eluent. *N*-(3-Chloropropyl)naphthalen-1-amine **2** was obtained as violet oil (0.209 g, 27%). TLC (*n*-hexane/ethyl acetate, 9:1): $R_f = 0.66$. ¹H NMR (CDCl₃, 400 MHz): $\delta_H = 2.22$ (quint, $J = 6.4$ Hz, 2H, NHCH₂CH₂CH₂Cl), 3.51 (t, $J = 6.4$ Hz, 2H, NHCH₂CH₂CH₂Cl), 3.74 (t, $J = 6.4$ Hz, 2H, NHCH₂CH₂CH₂Cl), 4.41 (broad s, 1H, NH), 6.71 (d, $J = 7.6$ Hz, 1H, H-2), 7.36 (d, $J = 8.0$ Hz, 1H, H-4), 7.46 (t, $J = 8.0$ Hz, 1H, H-3), 7.49-7.58 (m, 2H, H-6 and H-7), 7.85 (d, $J = 8.4$ Hz, 1H, H-8), 7.90 (d, $J = 8.4$ Hz, 1H, H-5) ppm.

2.2. Procedure for the synthesis of compound 3a.

To a cold solution (ice bath) of 9-nitroso-1,2,3,5,6,7-hexahydropyrido[3,2,1-*ij*]quinolin-8-ol hydrochloride **1** (9-nitroso-8-hydroxyjulolidine hydrochloride) (0.335 g, 1.53×10^{-3} mol), in ethanol (4 mL), *N*-(3-chloropropyl)naphthalen-1-amine **2** (0.167 g, 7.64×10^{-4} mol), and concentrated hydrochloride acid (4.0×10^{-2} mL) were added. The mixture was refluxed for 24 hours, and monitored by TLC (dichloromethane/methanol, 9:1). After evaporation of the solvent and column chromatography purification on silica gel with dichloromethane and dichloromethane/methanol, mixtures of increasing polarity, as the eluent, 3-chloro-*N*-(2,3,6,7-tetrahydro-1*H*-benzo[*a*]quinolizino[1,9-*hi*]phenoxazin-14(5*H*)-ylidene)propan-1-aminium chloride **3a** was obtained as a blue solid (5%, 0.017 g). ^1H NMR (CD_3OD , 400 MHz): $\delta_{\text{H}} = 2.05\text{-}2.14$ (m, 4H, H-2 and H-6), 2.33 (quint t, $J = 6.4$ Hz, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{Cl}$), 2.83-2.92 (m, 2H, H-1), 2.95 (t, $J = 6.0$ Hz, 2H, H-7), 3.60-3.69 (m, 4H, H-3 and H-5), 3.78 (t, $J = 6.8$ Hz, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{Cl}$), 3.83 (t, $J = 6.4$ Hz, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{Cl}$), 6.73 (s, 1H, H-15), 7.33 (s, 1H, H-8), 7.71 (t, $J = 7.2$ Hz, 1H, H-12), 7.82 (t, $J = 7.6$ Hz, 1H, H-11), 8.21 (d, $J = 8.0$ Hz, 1H, H-13), 8.68 (d, $J = 7.6$ Hz, 1H, H-10) ppm. ^{13}C NMR (CD_3OD , 100.6 MHz): $\delta_{\text{C}} = 20.21$ (C-1), 20.46 (C-2), 21.56 (C-6), 28.53 (C-7), 32.47 ($\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{Cl}$), 42.71 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{Cl}$), 43.30 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{Cl}$), 52.06 (C-3), 52.56 (C-5), 93.28 (C-15), 106.75 (Ar-C), 123.44 (C-13), 124.01 (Ar-C), 124.97 (C-10), 129.22 (Ar-C), 129.89 (C-12), 130.14 (C-8), 131.24 (Ar-C), 132.02 (C-11), 132.26 (Ar-C), 133.76 (Ar-C), 144.78 (Ar-C), 151.73 (Ar-C), 152.82 (Ar-C), 156.92 (C-14) ppm.

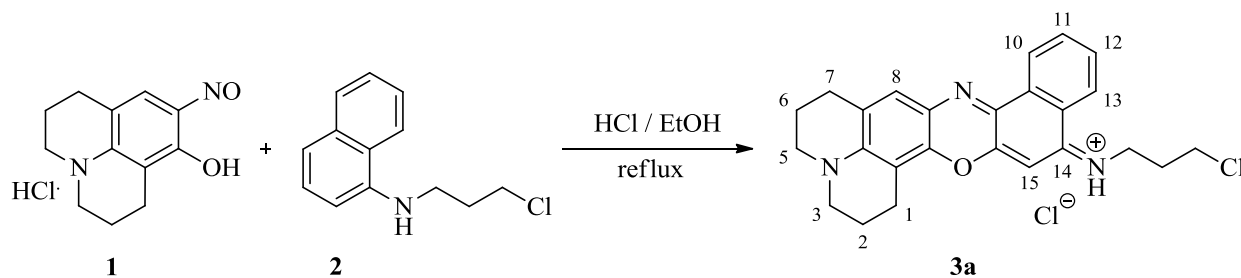
2.3. Antifungal activity tests.

Minimum Inhibitory Concentrations (MIC) of growth for the different compounds were 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 morpholene propanesulfonic 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 hours 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.

3. Results and discussion

Benzophenoxazinium chloride **3a** was synthesized by condensation of 9-nitroso-1,2,3,5,6,7-hexahydropyrido[3,2,1-*ij*]quinolin-8-ol hydrochloride **1** with *N*-(3-chloropropyl)naphthalen-1-amine **2** in acid media. Intermediate **2**¹¹ was obtained by alkylation of naphthalen-1-amine with 1-bromo-3-chloropropane in ethanol as the solvent, in moderate yield. The required 9-nitroso-8-hydroxyjulolidine hydrochloride **1** was obtained by nitrosation of 8-hydroxyjulolidine with sodium nitrite in the presence of hydrochloric acid, in a mixture of ethanol-water as the solvent.¹²

The cyclisation reaction of *N*-(3-chloropropyl)naphthalen-1-amine **2** with 9-nitroso-8-hydroxyjulolidine hydrochloride **1** occurred in the presence of concentrated hydrochloric acid under reflux conditions in ethanol. Purification by silica gel column chromatography gave 3-chloro-*N*-(2,3,6,7-tetrahydro-1*H*-benzo[*a*]quinolizino[1,9-*hi*]phenoxazin-14(5*H*)-ylidene)propan-1-aminium chloride **3a** as a blue solid in low yield (Scheme 1).



Scheme 1. Synthesis of benzophenoxazinium chloride **3a**

The ¹H NMR spectra showed the signals of aliphatic protons from the methylenic groups of substituent of positions 14, directly linked to the nitrogen atom NHCH₂ or to the chlorine atom CH₂Cl that appeared as triplets (δ 3.78 and 3.83 ppm, respectively), as well as the group closed to the same atoms, NHCH₂CH₂, showed as quintet (δ 2.33 ppm). The duos H-2/H-6 and H-3/H-5 are methylenic protons of the julolidine moiety, having both protons in each pair the same electronic environment and so they both appear in one signal each, as multiplets (δ 2.05-2.14 and 3.60-3.69 ppm, respectively). There was also the presence of protons of the methylenic groups directly linked to the aromatic ring at position 1 and 7, which appeared in different signals as a multiplet (δ 2.83-2.92 ppm, H-1) and a triplet (δ 2.95 ppm, H-7). In addition, spectra showed the expected aromatic protons of the polycyclic system, in particular H-15 (δ 6.73 ppm) and H-8 (δ 7.33 ppm), which appeared in the form of singlets.

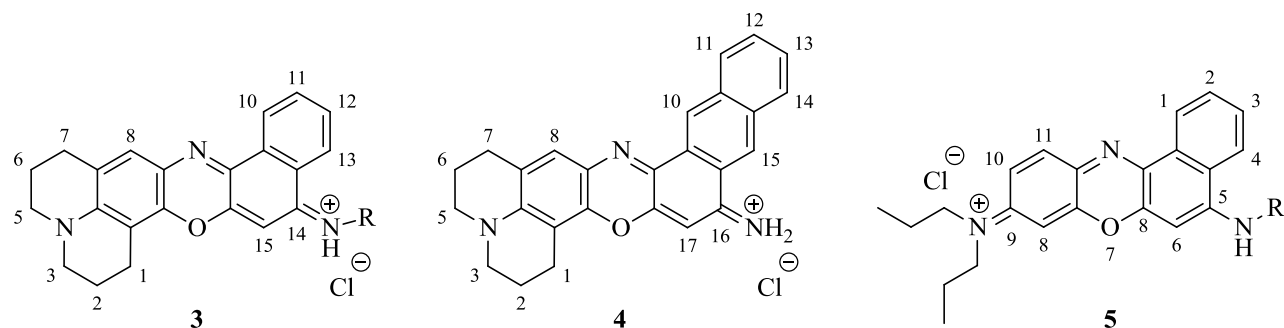
The ^{13}C NMR spectra showed the signals of the carbons of methylenic groups of substituent of position 14, directly linked to the nitrogen atom NHCH_2 or to the chlorine atom CH_2Cl (δ 42.71 and 43.30 ppm, respectively), as well as the group closed to the same atoms, NHCH_2CH_2 (δ 32.47 ppm). The methylenic carbons of the julolidine moiety also appeared before and after these last signals, δ 20.21-28.53 ppm (C-1/C-7, C-2/C-6) and δ 52.06-52.56 (C-3/C-5). The spectrum showed the expected aromatic carbons, in particular C-15 (δ 93.28 ppm) and C-8 (δ 130.14 ppm).

Along with other structurally related benzophenoxazinium chlorides **3b-d**, **4** and **5a,b** recently synthesised by our research group, compound **3a** was evaluated using a broth microdilution method for antifungal susceptibility testing of yeasts (M27-A3, CLSI). The Minimum Inhibitory Concentration values of growth obtained for *Saccharomyces cerevisiae* PYCC 4072, used as a reference organism, are shown in Figure 1.

Frade *et al.* demonstrated that the most active benzophenoxazinium had a chlorine atom or an ester group as terminals of substituents at 5-position of the polycyclic system.⁷ In the present study, both the presence of a rigid structure instead of a freely rotating *N*-substituent group and the substituent at 14- or 9-position showed to influence the activity of evaluated compounds. All dyes (excluding **3b**) exhibited an antiproliferative activity against the yeast *Saccharomyces cerevisiae* PYCC 4072 with MIC values between 25.0 and 0.78 μM .

Compound **3a** with a chloride atom as terminal of the propyl substituent at 14-position of the system displays the best activity against *S. cerevisiae*. Moreover, this compound is 8-times more active comparatively to its corresponding non-functionalized analogue **3c**, thus emphasising that the presence of this terminal is significant.

Regarding the effect of the presence of the julolidine structure, we can compare compound **3a** with the related unrestricted dye **5b** to observe the same increment in the activity observed for **3c**, i.e. an 8-times increase. The result for compound **4** revealed that extending the aromatic ring system, by replacing the benzene ring with fused naphthalene in the phenoxazine skeleton leads to an improved activity of the compounds (compare to compound **3d**).



Compound	R	MIC / μM	Log P
3a	$(\text{CH}_2)_3\text{Cl}$	0.78	3.23
3b	$(\text{CH}_2)_3\text{OH}$	400	1.99
3c	$(\text{CH}_2)_2\text{CH}_3$	6.25	5.60
3d	H	25.0	3.23
4	-	12.5	3.41
5a	$(\text{CH}_2)_2\text{CH}_3$	1.56	2.95
5b	$(\text{CH}_2)_3\text{Cl}$	6.25	2.95

Figure 1. Activity against *Saccharomyces cerevisiae* PYCC 4072 and log P values of benzophenoxazinium chlorides **3–5**.

A well-established measure of the lipophilicity of a compound is the logarithm of its partition coefficient between *n*-octanol and water, i.e. $\log (c_{\text{octanol}}/c_{\text{water}})$ or Log P , being used to stimulate drug partitioning into membranes.¹³ Drugs with higher values of Log P are more lipophilic, having lower affinity for an aqueous environment, like that existing in intracellular environment, but higher affinity for membranous systems, and therefore this property might influence the compound's absorption and permeation into cells.¹⁴ This physical parameter was predicted for all compounds under study using the online software Molinspiration property engine v2013.09,¹⁵ however there is not a specific correlation between the lipophilicity and the antimicrobial activity for the benzophenoxazine derivatives evaluated. In fact, compounds **3a**, **3d** and **4** are associated with the same or a very similar Log P (3.23 or 3.41) and show three quite different MIC values (0.78, 25.0 and 12.5, respectively).

4. Conclusions

New julolidine-based benzophenoxazinium chloride possessing the chloride atom as terminal in substituent at 14-amine position was successfully synthesised. Growth inhibition assays showed that this compound present the highest antifungal activity when compared to other derivatives of the same family, with or without the julolidine nucleus in their polycyclic system. All tested compounds exhibited activity against the yeast *Saccharomyces cerevisiae*. The results obtained show that the effect on the activity of the benzophenoxazine derivative is highly dependent on the substituent present at the 14-position (**3a-d** and **4**) or 5-position (**5a,b**). Further studies are required in order to better understand the effect of the julolidine structure in the biological activity of benzophenoxazinium chlorides.

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