N-(5-Amino-9*H*-benzo[*a*]phenoxazin-9-ylidene)propan-1-aminium chlorides as antifungal agents and NIR fluorescence probes

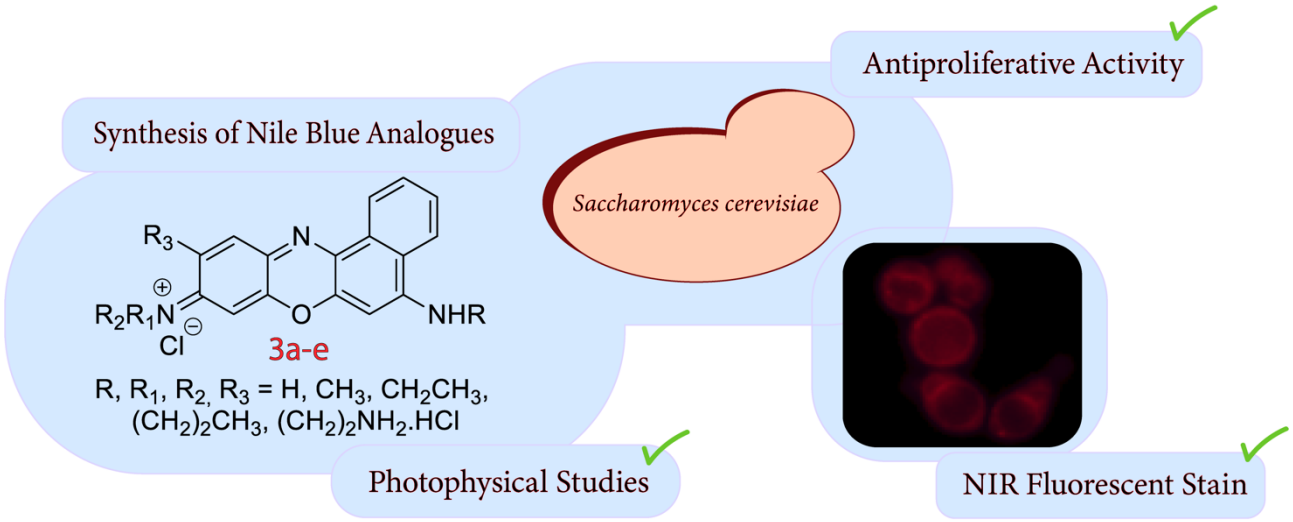
Rui P. C. L. Sousa[a,b,#], João C. C. Ferreira[a,b,c,#], Maria João M. F. Sousa[b,c] and M. Sameiro T. Gonçalves[a]

[a]*Centre of Chemistry,* [b]*Centre of Molecular and Environmental Biology,* [c]*Institute of Science and Innovation for Bio-Sustainability, University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal*

[#] *These authors contributed equally to this work.*

msameiro@quimica.uminho.pt

Graphical Abstract



Abstract: The search for benzo[*a*]phenoxazines, Nile Blue derivatives, with high antifungal activity and cell labelling capacity based on our previously published works in this type of compounds, led us to the design of compounds with specific substituents in the polycyclic system. Thus, in the present work, four new benzo[*a*]phenoxazinium chlorides, possessing at the 5-position amino or (3-aminopropyl) amino groups and at the 9-position propylamino or dipropylamino groups, were synthesized. Another analogue, with (3-aminopropyl) amino group at 5-position, ethyl amino group at 9-position and a methyl group at 10-position of the polycyclic system was also synthesized for

comparison in the studies performed. Fundamental photophysics (absorption and fluorescent emission) was carried out in absolute ethanol, water, and other aqueous solutions of different pH values, relevant for the potential biological applications of these compounds. The antiproliferative activity of the synthesized benzo[*a*]phenoxazinium chlorides was determined using *Saccharomyces cerevisiae* PYCC 4072 and the microdilution method described for antifungal susceptibility tests in yeast. All compounds revealed antifungal activity, being the most active the one possessing an amino group at 5-position and an aminopropyl group at 9-position. The potential as fluorescent probes were evaluated by fluorescence microscopy, using *S. cerevisae* as a model system of eukaryotic cells, and it was found that the benzo[*a*]phenoxazinium chlorides stained the cells with preferential accumulation that seems to appear at the vacuolar membrane and/or the perinuclear membrane of the endoplasmatic reticulum.

Keywords: Nile Blue; benzo[*a*]phenoxazines; NIR probes; antifungal drugs; fluorescent probes.

1. Introduction

Small fluorescent molecules have emerged as essential tools for contemporary analytical methodologies applied in the biosciences field [1–3]. In this context, a suitable fluorescent probe must present high affinity for specific labeling of biological targets, high molar extinction coefficient and quantum yield values, good stability against photobleaching as well as be designed to absorb and emit at longer wavelengths (650 - 1000 nm), where background interference caused by the biological material is minimal [3–5]. The cationic polycyclic benzo[*a*]phenoxazinium chlorides, often refered as Nile Blue derivatives, have been recognized as good examples, as they strongly emit fluorescence in the near-infrared (NIR) region, have both high photostability and molar absorption, modest stoke shifts and a compact structure able to be functionalized, giving the versatility to create molecules that can function as non-covalent or covalent binding probes [6,7,16–25,8,26,9–15].

Besides their great fluorophore characteristics, oxazine heterocycles, such as benzo[*a*]phenoxazinium chlorides, have had a greater impact in life sciences, as they have been shown to possess pharmaceutical properties [8,9], which has driven their study as antimicrobial [25–27] and antitumor agents [28]. Phenoxazine derivatives have been reported for their use as photosensitizers in photodynamic therapy [7,29], promising drugs for malaria [30], antiviral [31], antifungal [32–34], and antibacterial [35].

The search for benzo[*a*]phenoxazine derivatives with particular substituents in the polycyclic system possessing simultaneously great antifungal activity and cell staining capacity that aggregate all the knowledge acquired in previously published works in this type of compounds, led us to the design and synthesis of four new benzo[*a*]phenoxazinium chlorides, possessing at the 5-position the amino or (3-aminopropyl) amino groups and at the 9-position the propylamino or dipropylamino groups. Fundamental photophysical studies were performed for the four new compounds together with another analogue, with (3-aminopropyl) amino group at 5-position, ethylamino group at 9-position and a methyl group at 10-position. The five compounds were evaluated for their antifungal activity as well as staining potential using the yeast *Saccharomyces cerevisiae* as a model system of eukaryotic cells, and it was found that they displayed antiproliferative activity, with MIC values dependent on their substituents. Fluorescence microscopy studies showed that benzo[*a*]phenoxazinium chlorides stained the cells with preferential accumulation on the vacuolar membrane and/or on the perinuclear membrane of the endoplasmic reticulum.

2. Experimental section

2.1. Synthesis general

Melting points were measured on a Stuart SMP3 melting point apparatus. TLC analysis was carried out on 0.20 mm thick precoated silica plates (Macherey-Nagel), and spots were visualized under UV light on a CN-6 camera. Chromatography on silica gel was carried out on Acros Organics 60 (0.035-0.070 mm). IR spectra were determined on a BOMEM MB 104 spectrophotometer. Samples

were prepared in 1% KBr pellets. UV-Vis-NIR absorption spectra (200-800 nm) were obtained using Shimadzu UV/3101PC spectrophotometer and fluorescence spectra with Fluoromax-4 spectrofluorometer. NMR spectra were obtained on a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C. All chemical shifts are given in ppm using δ_H Me_4Si = 0 ppm as reference and *J* values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities and *J* values, and were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. Mass spectrometry analysis were performed at the "C.A.C.T.I. - Unidad de Espectrometria de Masas", at University of Vigo, Spain. All commercial reagents and solvents were used as received.

2.2. General procedure for the synthesis of benzo[*a*]phenoxazinium chlorides 3a-d

To a solution of the corresponding nitrosophenol hydrochloride (**1a**,**b**) in methanol (3 mL), concentrated hydrochloric acid was added followed by naphthalen-1-amine **2a** or N^1-(naphthalen-1-yl)propane-1,3-diamine hydrobromide **2b** and the resulting solution was refluxed. The progress of the reaction was monitored by TLC (mixtures of dichloromethane/methanol). After evaporation of the solvent and column chromatography purification on silica gel (mixtures of increasing polarity of dichloromethane/methanol as the eluent), the corresponding benzo[*a*]phenoxazinium chloride (**3a-d**) was obtained.

2.2.1 ***N*-(5-Amino-9*H*-benzo[*a*]phenoxazin-9-ylidene)propan-1-aminium chloride 3a**. The reaction between 2-nitroso-5-(propylamino)phenol hydrochloride **1a** (0.408 g, 1.88×10^{-3} mol, 2 eq.), concentrated hydrochloric acid (0.724 mL) and **2a** (0.135 g, 9.4×10^{-4} mol, 1 eq.) (reflux time: 24h) gave compound **3a** as a blue solid (0.157 g, 49%). mp > 300ºC. R*f* = 0.41 (dichloromethane/methanol 9:1). FTIR (KBr 1%): υ_{max} 3415, 3204, 3039, 2968, 1663, 1631, 1590, 1553, 1529, 1484, 1468, 1434, 1352, 1286, 1186, 1144, 1119, 1091, 1060, 1013, 977, 853, 792 cm^{-1}. ^{1}H NMR 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, $NHCH_2CH_2\mathit{CH_3}$), 3.51 (t, J = 7.6 Hz, 2H, $NH\mathit{CH_2}CH_2CH_3$), 6.98 (s, < 2H, H-6 and H-8), 7.26 (d, J = 9.2 Hz, 1H, H-10), 7.89-7.92 (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. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz) 11.53 ($NHCH_2CH_2\mathit{CH_3}$), 23.53 ($NHCH_2\mathit{CH_2}CH_3$), 46.16 ($NH\mathit{CH_2}CH_2CH_3$), 98.74 (C-6 and C-8), 113.13 (C-10), 124.61 (Ar-C), 125.08 (C-4), 126.10 (C-1), 130.49 (Ar-C), 131.61 (C-3), 132.74 (C-11), 133.29 (Ar-C), 134.26 (C-2), 144.51 (Ar-C), 152.46 (2×Ar-C), 153.08 (C-9), 164.59 (C-5) ppm. HRMS: m/z (ESI): Found $[M+1]^+$: 304.1441; $C_{19}H_{18}N_3O$ requires $[M+1]^+$: 304.1444.

2.2.2. *N*-(5-((3-Aminopropyl)amino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)propan-1-aminium chloride hydrobromide 3b. 2-Nitroso-5-(propylamino)phenol hydrochloride **1a** (0.816 g, 3.76×10^{-3} mol, 10 eq.), concentrated hydrochloric acid (1.447 mL) and **2b** (0.106 g, 3.8×10^{-4} mol, 1 eq.) were refluxed for 7h. Compound **2b** was once again added (0.053 g, 1.9×10^{-4} mol, 0.5 eq.) and the mixture was refluxed for more 18h. The product was obtained as a blue solid **3b** (0.053 g, 19%). mp > 300ºC. R*f* = 0.57 (dichloromethane/methanol 7:3). FTIR (KBr 1%): υ_{max} 3429, 2966, 2923, 2854, 1660, 1630, 1588, 1549, 1531, 1465, 1349, 1321, 1285, 1184, 1128, 1013, 774 cm^{-1}. ^{1}H NMR δ_H (CD_3OD, 400 MHz) 1.06 (t, J = 7.4 Hz, 3H, $NHCH_2CH_2\mathit{CH_3}$), 1.77 (sext, J = 7.2 Hz, 2H, $NHCH_2\mathit{CH_2}CH_3$), 2.22-2.30 (m, 2H, $NHCH_2\mathit{CH_2}CH_2NH_2{\cdot}HBr$), 3.19 (t, J = 7.4 Hz, 2H, $NHCH_2CH_2\mathit{CH_2}NH_2{\cdot}HBr$), 3.51 (t, J = 7.4 Hz, 2H, $NH\mathit{CH_2}CH_2CH_3$), 3.94 (t, J = 7.0 Hz, 2H, $NH\mathit{CH_2}CH_2CH_2NH_2{\cdot}HBr$), 7.18 (s, <2H, H-6 and H-8), 7.27 (d, J = 9.2 Hz, 1H, H-10), 7.87 (m, 2H, H-3 and H-11), 7.95 (t, J = 7.6 Hz, 1H, H-2), 8.50 (d, J = 8.0 Hz, 1H, H-4), 8.90 (d, J = 7.6 Hz, 1H, H-1) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz) 11.59 ($NHCH_2CH_2\mathit{CH_3}$), 23.55 ($NHCH_2\mathit{CH_2}CH_3$), 26.56 ($NHCH_2\mathit{CH_2}CH_2NH_2{\cdot}HBr$), 38.35 ($NHCH_2CH_2\mathit{CH_2}NH_2{\cdot}HBr$), 42.97 ($NH\mathit{CH_2}CH_2CH_2NH_2{\cdot}HBr$), 46.49 ($NH\mathit{CH_2}CH_2CH_3$), 94.14 (C-8), 95.18 (C-6), 113.76 (C-10), 124.49 (C-4), 124.68 (Ar-C), 125.58 (C-1), 130.83 (Ar-C), 131.37 (C-3), 132.16 (Ar-C), 132.64 (C-11), 133.41 (C-2), 135.62 (Ar-C), 152.57 (Ar-C), 152.60 (Ar-C), 158.35 (C-9), 160.20 (C-5) ppm. HRMS: m/z (ESI): Found $[M+1]^+$: 361.2018; $C_{22}H_{25}N_4O$ requires $[M+1]^+$: 361.2023.

2.2.3 *N*-(5-Amino-9*H*-benzo[*a*]phenoxazin-9-ylidene)-*N*-propylpropan-1-aminium chloride 3c. The reaction between 5-(dipropylamino)-2-nitrosophenol hydrochloride **1b** (0.363 g, 1.4×10^{-3} mol, 2 eq.), concentrated hydrochloric acid (0.539 mL) and **2a** (0.100 g, 7×10^{-4} mol, 1 eq.) (reflux time: 12h) gave compound **3c** as a blue solid (0.124 g, 46%). mp = 288.0-289.7 °C. R*f* = 0.48 (dichloromethane/methanol 9:1). FTIR (KBr 1%): υ_{max} 3437, 3031, 2961, 2900, 2869, 1666, 1641, 1585, 1548, 1484, 1425, 1382, 1366, 1333, 1294, 1238, 1191, 1171, 1144, 1114, 1062, 1040, 1008, 923, 858, 818, 779 cm^{-1}. ^1H NMR δ_H (CD_3OD, 400 MHz) 1.07 (t, *J* = 7.6 Hz, 6H, $N(CH_2CH_2\mathit{CH_3})_2$), 1.80 (sext, *J* = 7.6 Hz, 4H, $N(CH_2\mathit{CH_2}CH_3)_2$), 3.62 (t, *J* = 8.0 Hz, 4H, $N(\mathit{CH_2}CH_2CH_3)_2$), 6.89 (s, 1H, H-6), 6.90 (d, *J* = 2.4 Hz, 1H, H-8), 7.27 (dd, *J* = 9.4 and 2.4 Hz, 1H, H-10), 7.85 (t, *J* = 8.4 Hz, 1H, H-3), 7.88 (d, *J* = 9.6 Hz, 1H, H-11), 7.97 (t, *J* = 8.0 Hz, 1H, H-2), 8.34 (d, *J* = 8.0 Hz, 1H, H-4), 8.94 (dd, *J* = 8.0 and 0.8 Hz, 1H, H-1) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz) 11.41 ($N(CH_2CH_2\mathit{CH_3})_2$), 21.76 ($N(CH_2\mathit{CH_2}CH_3)_2$), 54.53 ($N(\mathit{CH_2}CH_2CH_3)_2$), 97.23 (C-8), 97.69 (C-6), 116.64 (C-10), 124.19 (Ar-C), 124.73 (C-4), 125.68 (C-1), 130.88 (C-3), 131.41 (Ar-C), 133.39 (Ar-C), 133.59 (C-2), 134.11 (C-11), 135.29 (Ar-C), 149.62 (Ar-C), 153.18 (Ar-C), 156.17 (C-9), 163.04 (C-5) ppm. HRMS: m/z (ESI): Found $[M+1]^+$: 346.1919; $C_{22}H_{24}N_3O$ requires $[M+1]^+$: 346.1914.

2.2.4 *N*-(5-((3-Aminopropyl)amino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)-*N*-propylpropan-1-aminium chloride hydrobromide 3d. 5-(Dipropylamino)-2-nitrosophenol hydrochloride **1b** (0.726 g, 2.8×10^{-3} mol, 10 eq.), concentrated hydrochloric acid (1.078 mL) and **2b** (0.079 g, 2.8×10^{-4} mol, 1 eq.) were refluxed for 12h. Compound **2b** was once again added (0.026 g, 9.3×10^{-5} mol, 0.33 eq.), and the mixture was refluxed for more 18h. The product **3d** was obtained as a blue solid (0.035 g, 18%). mp = 243.2-245.0 °C. R*f* = 0.87 (dichloromethane/methanol 7:3). FTIR (KBr 1%): υ_{max} 3412, 3201, 2958, 2928, 2865, 1639, 1588, 1548, 1533, 1498, 1436, 1384, 1334, 1290, 1240, 1168, 1126, 1009, 923, 865, 824, 778 cm^{-1}. ^1H NMR δ_H (CD_3OD, 400 MHz) 1.10 (t, *J* = 7.6 Hz, 6H, $N(CH_2CH_2\mathit{CH_3})_2$), 1.81 (sext, *J* = 7.6 Hz, 4H, $N(CH_2\mathit{CH_2}CH_3)_2$), 2.20-2.32 (m, 2H,

149 $NHCH_2CH_2CH_2NH_2{\cdot}HBr$), 3.24 (t, *J* = 7.6 Hz, 2H, $NHCH_2CH_2CH_2NH_2{\cdot}HBr$), 3.61 (t, *J* = 7.2 Hz,
150 4H, $N(CH_2CH_2CH_3)_2$), 3.84 (t, *J* = 7.2 Hz, 2H, $NHCH_2CH_2CH_2NH_2{\cdot}HBr$), 6.72 (d, *J* = 2.4 Hz, 1H,
151 H-8), 6.86 (s, 1H, H-6), 7.23 (dd, *J* = 9.6 and 2.8 Hz, 1H, H-10), 7.61-7.66 (m, 2H, H-3 and H-11),
152 7.78 (t, *J* = 8.0 Hz, 1H, H-2), 8.27 (d, *J* = 8.4 Hz, 1H, H-4), 8.54 (d, *J* = 8.0 Hz, 1H, H-1) ppm. ^{13}C
153 NMR δ_C (CD_3OD, 100.6 MHz) 11.54 ($N(CH_2CH_2CH_3)_2$), 21.95 ($N(CH_2CH_2CH_3)_2$), 27.68
154 ($NHCH_2CH_2CH_2NH_2{\cdot}HBr$), 38.49 ($NHCH_2CH_2CH_2NH_2{\cdot}HBr$), 42.63 ($NHCH_2CH_2CH_2NH_2{\cdot}HBr$),
155 54.76 ($N(CH_2CH_2CH_3)_2$), 94.51 (C-6), 97.20 (C-8), 117.55 (C-10), 124.16 (C-4), 124.19 (Ar-C),
156 125.17 (C-1), 130.61 (C-3), 131.94 (Ar-C), 131.98 (Ar-C), 132.87 (C-2), 133.97 (Ar-C), 134.06 (C-
157 11), 149.34 (Ar-C), 152.57 (Ar-C), 156.26 (C-9), 158.60 (C-5) ppm. HRMS: m/z (ESI): Found
158 $[M+1]^+$: 403.2490; $C_{25}H_{31}N_4O$ requires $[M+1]^+$: 403.2492.
159
160 **2.3. Photophysical studies**
161 Photophysical properties of benzo[*a*]phenoxazinium chlorides **3a-e** were determined in ethanol,
162 ethanol acidified with TFA, water and aqueous solutions at different pH values (3, 5 and 7.4), with
163 concentration between 10^{-7} and 10^{-5} M. These last solutions were prepared using boric acid, citric
164 acid and sodium phosphate buffers, or phosphate-saline buffer PBS (pH 7.4).
165 Fluorescence was measured at an angle of 90° to excitation incident radiation in quartz cells. The
166 excitation wavelength was 590 nm for all compounds. The area below the fluorescence spectrum
167 curve was determined, allowing the relative fluorescence quantum yield (Φ_F) of the test compound
168 to be calculated using Oxazine 1 as standard (Φ_F = 0.11 in ethanol [36]).
169
170 **2.4. Antifungal activity assays**
171 Minimum Inhibitory Concentration (MIC) of growth for compounds **3a**-**e** was determined using a
172 broth microdilution method for the antifungal susceptibility testing of yeasts (M27-A3, CLSI –
173 Clinical and Laboratory Standards Institute). The yeast *Saccharomyces cerevisiae* PYCC 4072 was
174 used as a model organism. Cells were incubated at 30 ºC 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.

2.5. Evaluation as fluorescent probes

Saccharomyces cerevisiae W303-1A was grown on YEPD (1% yeast extract, 2% peptone, 2% glucose) agar plates. A sample of this culture was used to prepare a cell suspension that was incubated overnight at 30°C and 120 rpm, in liquid YEPD, until it reached an optical density of approximately 0.8 at 640 nm. Aliquots of this culture were collected and incubated with the respective benzo[*a*]phenoxazinium chloride (12.5 μM) in PBS at 30 °C for two hours. Cells were centrifuged at 3000 rpm for 5 minutes, rinsed two times in PBS and resuspended in 30 μL of PBS. The samples were analyzed on an Olympus BX6F2 fluorescence microscope, with appropriate filter cubes: U-FDICT (differential interference contrast), U-FYW (Far-Red), with a 60x oil immersion objective. All treatment conditions were performed in three independent experiments and the images presented are representative of the results obtained.

3. Results and discussion

3.1. Synthesis of benzo[*a*]phenoxazinium chlorides 3a-d

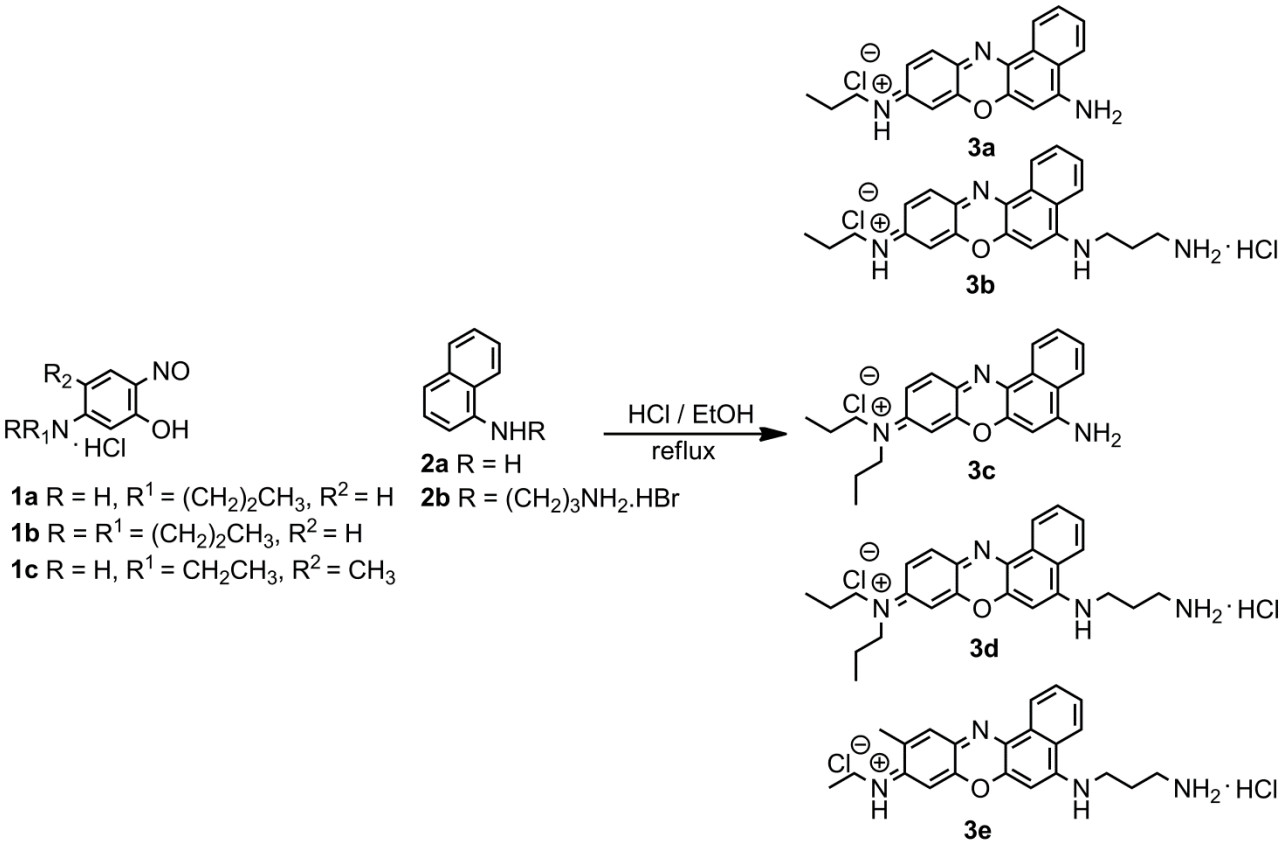
The synthesis of benzo[*a*]phenoxazinium chlorides **3a-d** started with the preparation of precursors **1a**,**b** and **2b**. 2-Nitroso-5-(propylamino)phenol hydrochloride **1a** and 5-(dipropylamino)-2-nitrosophenol hydrochloride **1b** were prepared by reaction of 3-(propylamino)phenol and 3-(dipropylamino)phenol with sodium nitrite in acid solution under ice cold conditions [32,37]. N^1-

(Naphthalen-1-yl)propane-1,3-diamine hydrobromide **2b** was obtained by the alkylation of naphthalen-1-amine **2a** with 3-bromopropan-1-amine hydrobromide in ethanol under reflux conditions [25].

The reaction of nitrosophenol precursors **1a**,**b** with naphthalen-1-amine **2a** or its derivative **2b** in an acidic medium afforded the corresponding benzo[*a*]phenoxazinium chlorides **3a-d** (Scheme 1). Thus, reaction between nitrosophenol **1a** with precursors **2a** or **2b** in ethanol, in the presence of concentrated hydrochloric acid, and after silica gel column chromatography purification gave *N*-(5-amino-9*H*-benzo[*a*]phenoxazin-9-ylidene)propan-1-aminium chloride **3a** or *N*-(5-((3-aminopropyl)amino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)propan-1-aminium chloride hydrobromide **3b**, which are *N*-monoalkylated at 9-amino position, possessing an amino group or propane-1,3-diamine hydrobromide at 5-position of the polycyclic systems, respectively. Starting from nitrosophenol **1b** and using the same precursors **2a** or **2b**, in similar conditions, *N*-(5-amino-9*H*-benzo[*a*]phenoxazin-9-ylidene)-*N*-propylpropan-1-aminium chloride **3c** or *N*-(5-((3-aminopropyl)amino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)-*N*-propylpropan-1-aminium chloride hydrobromide **3d**, with identical substitution to **3a** and **3b**, respectively, at 5-position, but *N*-dialkylated at 9-amino position were obtained.

Also, by reaction of 5-(ethylamino)-4-methyl-2-nitrosophenol **1c** and precursor **2b**, *N*-(5-((3-aminopropyl)amino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride hydrobromide **3e** was synthesized, which possesses at 5-position the propane-1,3-diamine hydrobromide like **3b** and **3d**, but the 9-position *N*-monosubstituted with ethyl group and at 10-position the methyl group. Spectroscopic data confirmed the structure of compound **3e** and is in according with those previously published [22]. It should be noted that this compound was synthesized for use in photophysical, antifungal and staining studies in comparison with new derivatives obtained in the present work.

Compounds **3a-d** were obtained as blue solids and were fully characterized by high resolution mass spectrometry, IR and NMR (^{1}H and ^{13}C) spectroscopy.



Scheme 1 - Synthesis of benzo[*a*]phenoxazinium chlorides **3a-e**.

FTIR spectra of compounds **3a-d** showed the bands corresponding to amine groups (3437-3201 cm^{-1}) and C-N bond of the central oxazine ring (1641-1630 cm^{-1}). The ^{1}H NMR spectra of the four new compounds showed the characteristic aromatic signals of benzo[*a*]phenoxazinium protons, H-1 to H-4, H-6, H-8 and H-11 (δ 6.72-8.96 ppm). The terminal methyl groups at 9-amino position appeared as triplets or multiplets (δ 1.04-1.12 ppm), adjacent methylene protons as sextets or multiplets (δ 1.70-1.81 ppm) and methylene protons adjacent to the nitrogen atoms as triplets (δ 3.45-3.62 ppm). Spectra of compounds **3b** and **3d** show the presence of the aminopropyl groups, with the central methylenic groups, $NHCH_2\mathit{CH_2}CH_2NH_2{\cdot}HBr$, as multiplets ($\delta$ 2.20-2.32 ppm), the adjacent methylenic groups, $NHCH_2CH_2\mathit{CH_2}NH_2{\cdot}HBr$ (δ 3.20-3.24 ppm) and $NH\mathit{CH_2}CH_2CH_2NH_2{\cdot}HBr$ (δ 3.84-3.87 ppm), as triplets. The ^{13}C NMR spectra showed the aromatic carbons of benzo[*a*]phenoxazinium core (δ 94.14-164.62 ppm). Signals of propyl groups at 9-amino

positions of di-alkylated compounds **3c**,**d** 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 **3a**,**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 propylamino group at 5-amino position appeared at δ 26.56-27.68 ($NHCH_2\mathit{CH_2}CH_2NH_2{\cdot}HBr$), δ 38.35-38.49 ppm ($NHCH_2CH_2\mathit{CH_2}NH_2{\cdot}HBr$) and δ 42.63-42.97 ppm ($NH\mathit{CH_2}CH_2CH_2NH_2{\cdot}HBr$).

3.2. Photophysical studies of benzo[*a*]phenoxazinium chlorides 3a-e

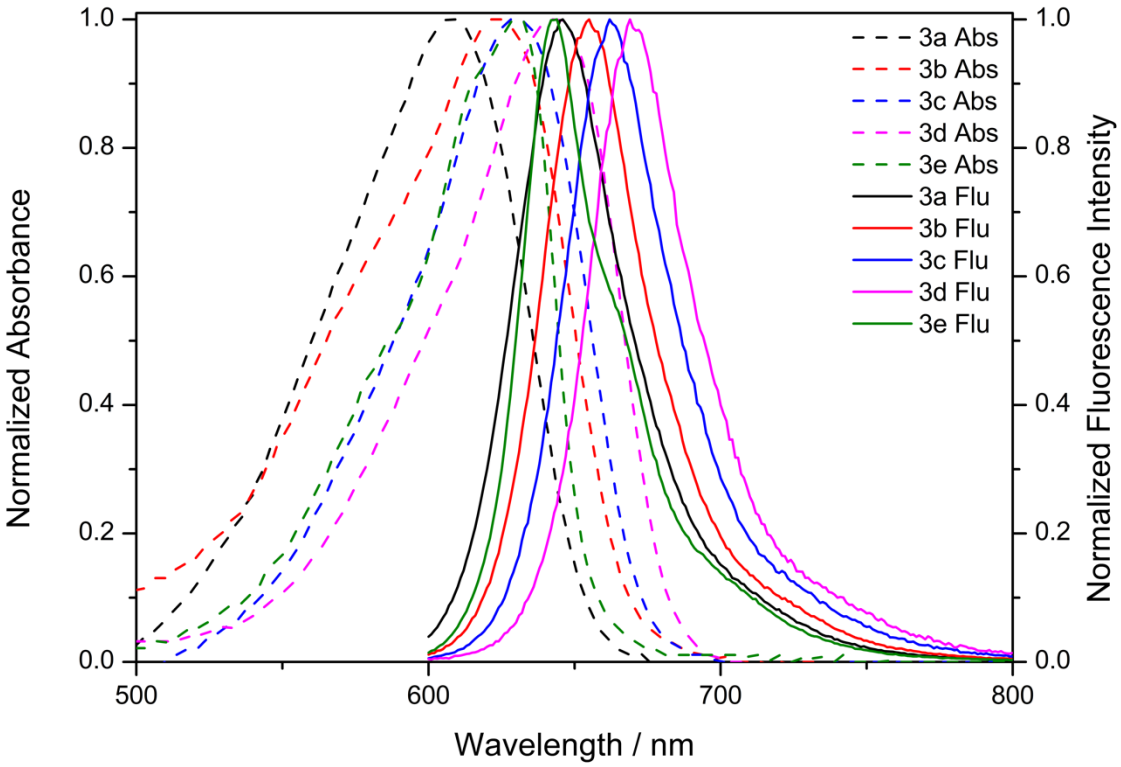
Photophysical studies of benzo[*a*]phenoxazinium chlorides **3a-e** were carried out in dry ethanol, water and aqueous solutions of different pH values, chosen according to potential biological applications of the compounds. The maximum absorption (λ_{abs}) and emission (λ_{emi}) wavelengths for each compound in each solvent were obtained, as well as the molar extinction coefficient (in logarithmic scale, log ε), the relative fluorescence quantum yield (Φ_F) and the Stokes shifts ($\Delta\lambda$). The relative fluorescence quantum yields were determined using Oxazine 1 as a standard (Φ_F = 0.11 in ethanol) at 590 nm excitation. The results obtained are summarized in Table 1.

Table 1 – Photophysical data for benzo[*a*]phenoxazinium chlorides **3a-e**.

Compound		**3a**	**3b**	**3c**	**3d**	**3e**
Ethanol	λ_{abs} (nm) | log ε	609 | 4.75	621 | 4.10	629 | 4.83	637 | 4.09	627 | 4.10
		489 | 4.40	492 | 4.38		512 | 4.03	530 | 3.40
	λ_{emi} (nm) | Φ_F	647 | 0.49	649 | 0.30	661 | 0.16	669 | 0.19	643 | 0.54
	$\Delta\lambda$ (nm)	38	28	32	32	16
Acidified ethanol	λ_{abs} (nm) | log ε	609 | 4.82	622 | 4.51	629 | 4.83	639 | 4.60	627 | 4.15
	λ_{emi} (nm) | Φ_F	646 | 0.47	655 | 0.35	662 | 0.20	669 | 0.18	644 | 0.58
	$\Delta\lambda$ (nm)	37	33	33	30	17
Water	λ_{abs} (nm) | log ε	608 | 4.48	619 | 4.19	637 | 4.68	647 | 4.18	624 | 3.70
	λ_{emi} (nm) | Φ_F	653 | 0.10	659 | 0.09	679 | 0.02	680 | 0.02	651 | 0.21
	$\Delta\lambda$ (nm)	45	40	42	33	27
pH 3	λ_{abs} (nm) | log ε	611 | 4.55	620 | 4.36	637 | 4.71	648 | 4.52	625 | 4.02
	λ_{emi} (nm) | Φ_F	654 | 0.11	658 | 0.14	674 | 0.03	673 | 0.05	651 | 0.37
	$\Delta\lambda$ (nm)	43	38	37	25	26
pH 5	λ_{abs} (nm) | log ε	610 | 4.53	622 | 4.33	638 | 4.75	650 | 4.56	625 | 4.06
	λ_{emi} (nm) | Φ_F	654 | 0.13	660 | 0.11	675 | 0.03	682 | 0.03	651 | 0.33
	$\Delta\lambda$ (nm)	44	38	37	32	26
pH 7.4	λ_{abs} (nm) | log ε	610 | 4.55	621 | 4.24	639 | 4.80	648 | 4.53	627 | 3.90
	λ_{emi} (nm) | Φ_F	656 | 0.12	658 | 0.12	675 | 0.02	683 | 0.03	651 | 0.24
	$\Delta\lambda$ (nm)	46	37	36	35	24

Benzo[*a*]phenoxazinium chlorides can be found in their cationic (acid) or neutral (basic) form. In ethanol, it is possible to observe for compounds **3a**, **3b**, **3d** and **3e** their basic form, a band in the range of 489-530 nm, even though for most cases their acid form is predominant (Figures S1 and S2 in Supplementary Material). In order to obtain only the acid form of the compounds, trifluoroacetic acid (TFA) was added to ethanolic solutions (acidified ethanol), resulting in the disappearance of the basic form and an increase in log ε values, as it was previously observed by the authors for other benzo[*a*]phenoxazinium chlorides [20].

From the absorption data, in acidified ethanol, a bathochromic shift of 20 nm (**3c**/**3a)** and 17 nm (**3d**/**3b**) is shown for compounds **3c** and **3d** in comparison with **3a** and **3b**. This indicates that the presence of two alkyl chains in 9-amino position of the heterocyclic system is correlated to an increase of maximum absorption wavelengths, which is in agreement with previous observations for compounds of this type [12]. Furthermore, compounds **3b** and **3d**, containing an aminopropyl group at 5-amino position, also show a bathochromic shift by comparison with compounds **3a** and **3c (**13 nm, **3b/3a**; 10 nm, **3d/3c)**, which possess a hydrogen atom at the same position. Therefore, this reinforces the fact that λ_{abs} is affected not only by the substitution at 9-amino position, but also at 5-amino position of the benzophenoxazinium core. In Figure 1 the absorption spectra for the five compounds, **3a**-**e**, in acidified ethanol are presented.



277

278 Figure 1 – Normalized of absorption and emission spectra of benzo[*a*]phenoxazinium chlorides **3a-**
279 **e** in acidified ethanol.

280

281 For biological applications, photophysical studies in aqueous solutions are particularly relevant.
282 Therefore, absorption studies in water (pH~5.5) and aqueous solutions of different biologically
283 relevant pH values (pH=3, 5 and 7.4) were carried out. In water, a decrease in log ε values for the
284 five compounds was observed. A bathochromic shift occurred for 9-position di-alkylated
285 compounds **3c**,**d** in comparison with the mono-alkylated ones **3a**,**b**, thus following the same trend
286 as in ethanol solutions. A broader band is observed in water due to the presence of H aggregates
287 (Figure 2). These aggregates practically do not have fluorescence, resulting in a decrease of
288 emission in water compared with ethanol.

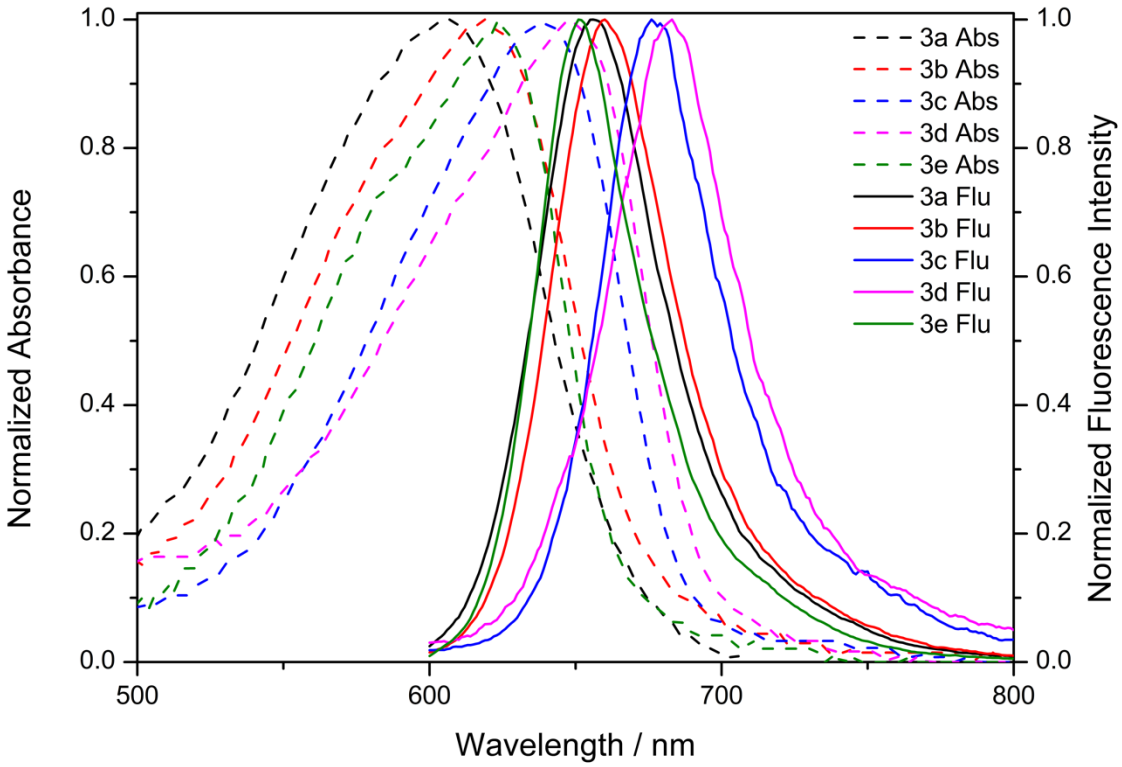


Figure 2 – Normalized spectra of absorption and emission of benzo[*a*]phenoxazinium chlorides **3a-e** in water.

In aqueous solutions at different pH values, it is shown that no relevant differences occur for λ_{abs} values, which are in the range 610-650 nm, compared to water results (λ_{max} 608-647 nm). The log ε values (3.90-4.80) are lower than those for acidic ethanol solutions (log ε 4.15-4.83). In Figure 3 the absorption spectra of compounds **3a-e** in aqueous solutions of pH 7.4 are shown. Spectra of all compounds at pH 3 and 5 are also presented in Figures S3 and S5, respectively, in Supplementary Material. Regarding compound **3e** with an aminopropyl group at 5-amino position, mono-alkylated at 9-amino position and bearing the methyl group at 10-position of the polycyclic system, the λ_{abs} values in ethanol, water and aqueous solutions with variable pH are always superior to those of the 9-amino mono-alkylated compound with the same substitution at 5-position (compound **3d**), which is probably due to the presence of the methyl substitution **3e.** The log ε value is inferior in comparison with **3b** (Table 1, Figures 1-3, S1-S6).

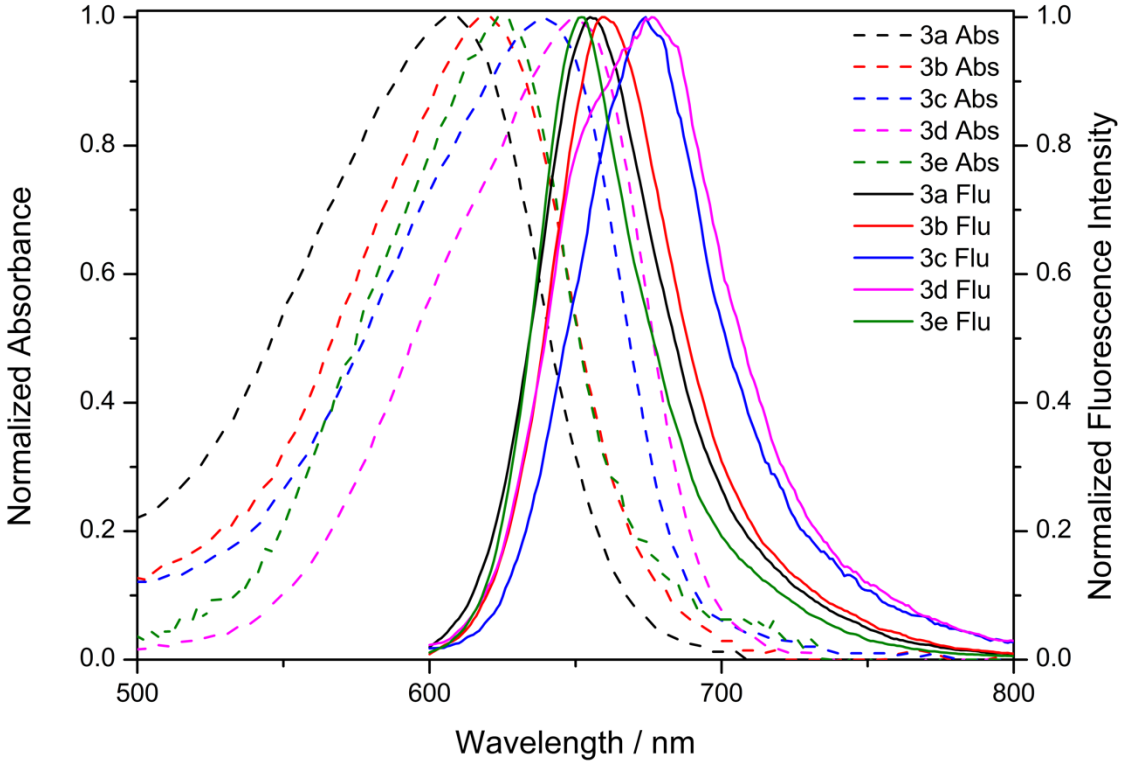


Figure 3 – Normalized spectra of absorption and emission of benzo[*a*]phenoxazinium chlorides **3a-e** in aqueous solution of pH=7.4.

From the emission data, no relevant differences are observed between ethanol and acidified ethanol data. The λ_{emi} values, as λ_{abs} values, are shown to be higher for di-alkylated compounds **3c**,**d** (661-669 nm) comparing to mono-alkylated compounds **3a**, **3c** and **3e** (643-655 nm) (Figure 1). Furthermore, compounds **3c** and **3d** present lower Φ_F values (0.16-0.20). Stokes' shifts are shown to be lower for compound **3e** (16-17 nm) in comparison whit the moderate values displayed for the other four compounds (28-38 nm).

In water, Φ_F values are significantly lower for all the compounds (0.02-0.21), and as in ethanol studies, lower values are related to di-alkylated compounds. The λ_{emi} values show a small bathochromic shift for all the compounds in comparison with ethanol results, especially for di-alkylated compounds (λ_{emi} 679-680 nm) (Figure 2).

The λ_{emi} values are similar in the aqueous solutions at different pHs (Figures 3, S4 and S6 in Supplementary Material). A clear difference between mono-alkylated and di-alkylated compounds

can be seen (Figures 3, S4 and S6 in Supplementary Material). The Φ_F values are also lower than those in ethanol solutions, as expected due to the higher solubility of the compounds in an organic solvent.

3.3. Antifungal activity of benzo[*a*]phenoxazinium chlorides 3a-e

Considering the previously reported antifungal activity of several benzo[*a*]phenoxazines [25–27,32,33], the five compounds synthesized were used in antiproliferative studies against the yeast *S. cerevisiae* PYCC 4072 by determination of MIC (Minimum Inhibitory Concentration) values using a broth microdilution method for antifungal activity testing. MIC represents the minimum concentration of the used compound in which 80% or higher of cell growth is inhibited. All the samples were diluted in DMSO, with the final concentration of this solvent in the growth medium being 0.5%. Control assay showed that this concentration of DMSO does not change the cell growth. RPMI-1640 media was used and the final concentration of each compound was between 3.125 and 100 μM. MIC and theoretically predicted log *P* values, an measure of compounds' hydrophobicity estimated by calculating the partition between membranes and aqueous media, are shown in Table 2.

Table 2 - MIC values of benzo[*a*]phenoxazinium chlorides **3a-e** against the yeast *S. cerevisiae* PYCC 4072.

Compound	**3a**	**3b**	**3c**	**3d**	**3e**
MIC (μM)	6.25	25	25	25	100
log *P*	1.64	1.09	1.70	1.15	0.96

The design of the synthesized compounds took into account the results of previous work showing that a propyl group as a substituent at 9-amino position improved antifungal activity compared to the combination of an ethyl group in this position and a methyl group at 10-position (**3e**) [32]. This can be corroborated by these results since compound **3e** presents the higher MIC value of all tested

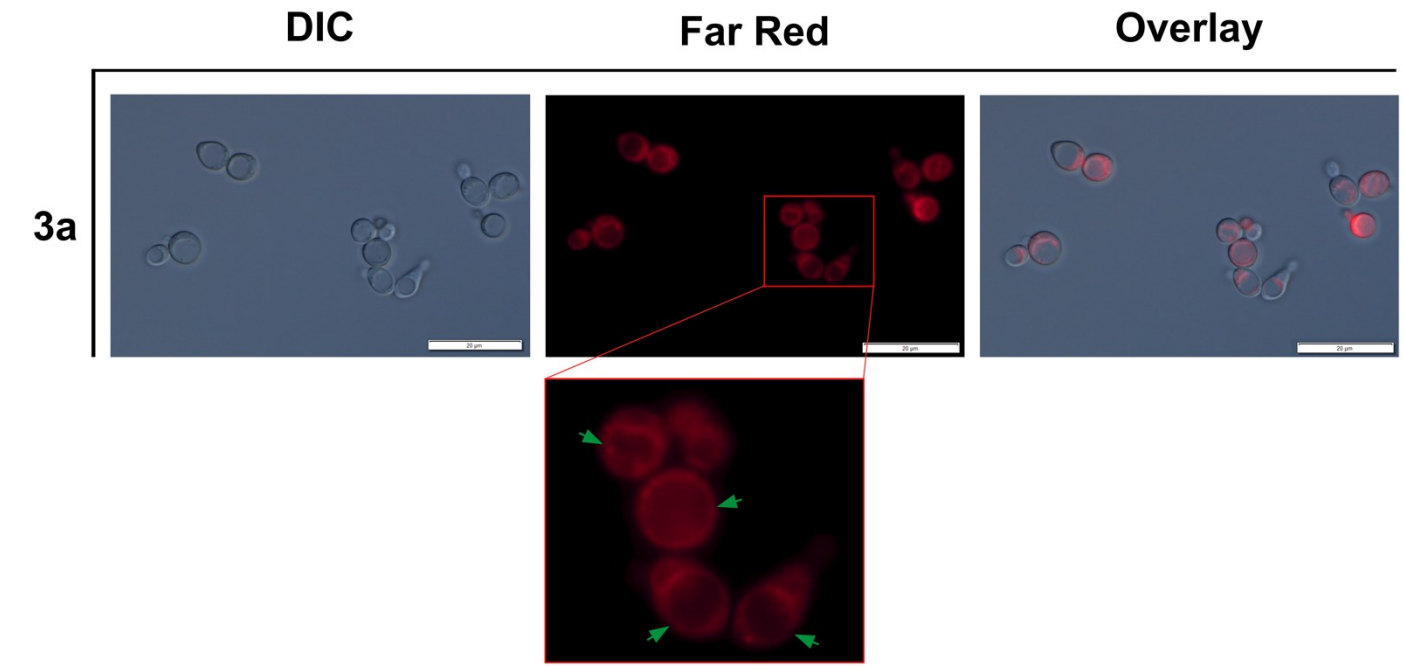
compounds (100 μM). Furthermore, previous work also suggested that di-alkylation at the 9-amino position improved antifungal activity comparing to mono-alkylation [32]. However, in the present work compound **3a** (only one alkyl chain at 9-amino position) shows a lower MIC value (6.25 μM) than analogues (25 μM), suggesting that biological activity may relate to the combination of all substituents and no correlation between MIC value and the number of alkyl chains at 9-amino position can be established. No correlation between MIC values and log *P* values is established either.

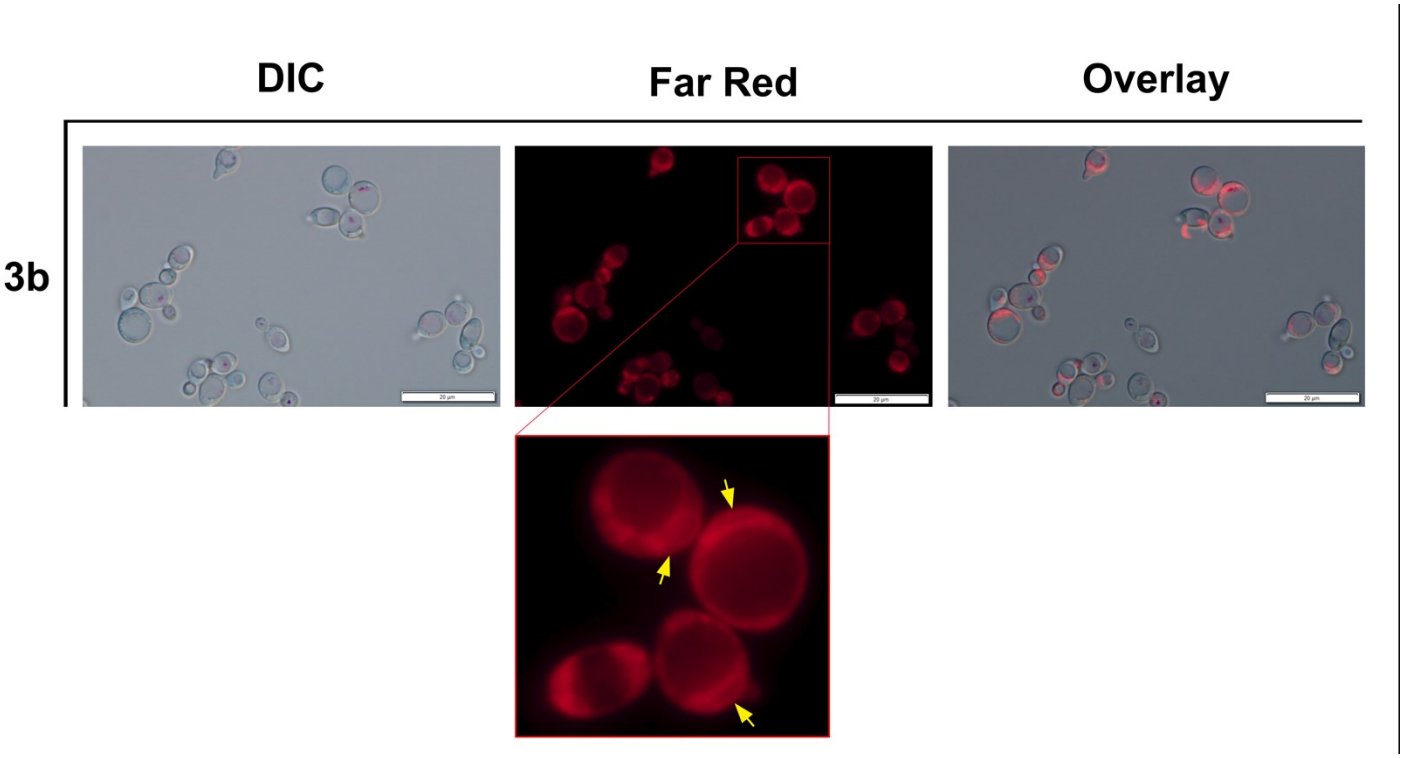
3.4. Evaluation as fluorescent stains for cells

In order to evaluate the application of the synthesized fluorochromophores for live-cell imaging experiments, the intracellular distribution of benzo[*a*]phenoxazinium chlorides **3a**-**e** was assessed by differential interference contrast (DIC) and fluorescence microscopy.

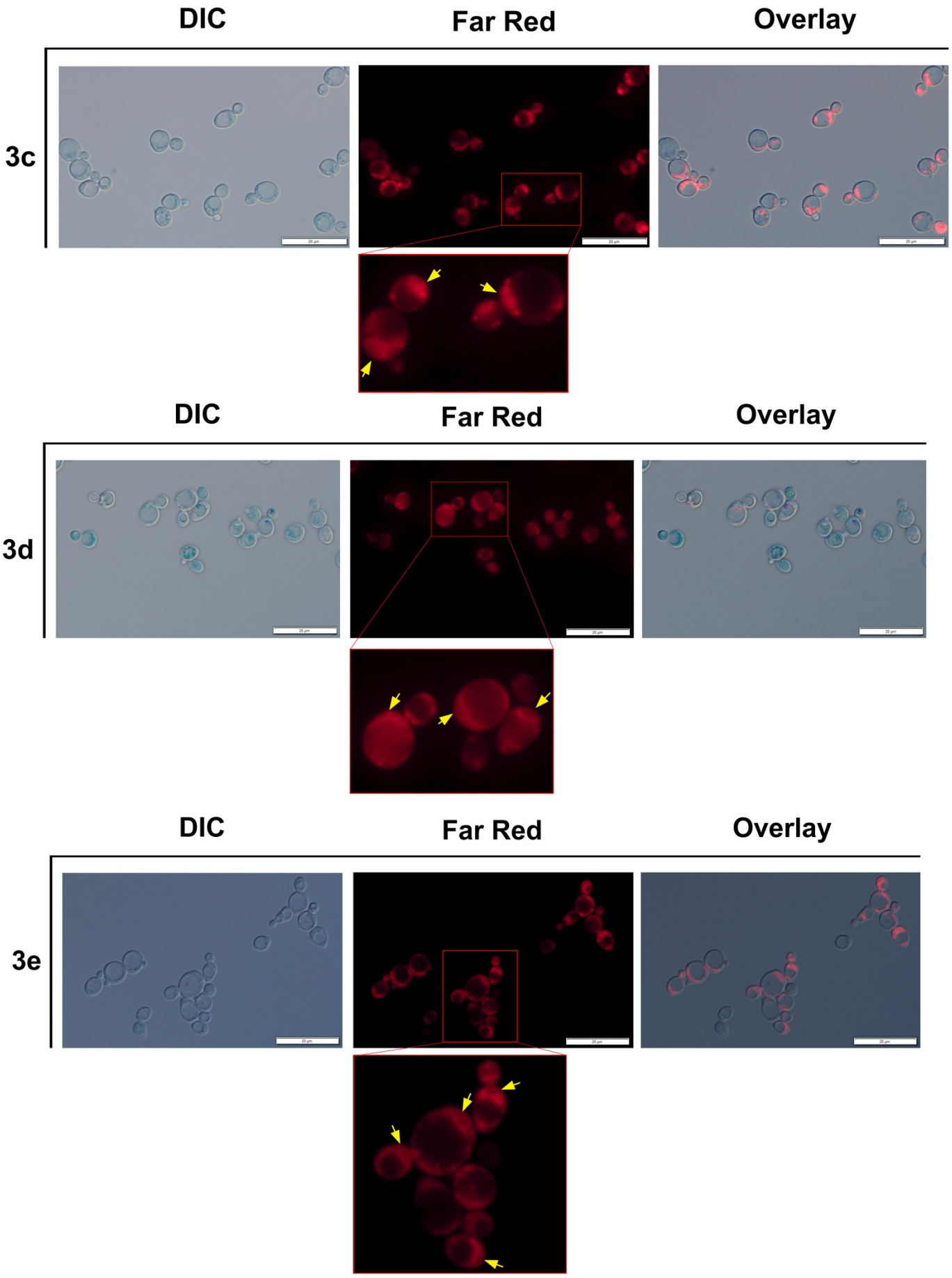
S. cerevisiae W303-1A cells were incubated for two hours with each compound at a concentration of 12.5 μM. This incubation resulted in their accumulation in the cells, as it was detected near infrared fluorescence emission, upon excitation with the far red filter setup for all the compounds (Figure 4). Furthermore, it is possible to observe the accumulation of compounds **3b**-**c** in the DIC images (Figure 4), as these compounds showed the ability to color the cells in blue. However, a direct correlation has not been observed between the ability to color the cells and the fluorescence emission of the compounds, this is evident in the case of **3c**. Although this compound was the one that stained the cells with highest intensity, it did not translate into a higher fluorescence intensity in comparison with the remaining compounds, which is in accordance with the photophysical data since compound **3c** is the one with the highest molar extinction coefficient, but with the lowest fluorescence quantum yield, in aqueous solutions. In previous studies it has been observed that benzo[*a*]phenoxazinium chlorides fluorescence in the cells accumulates preferential on the vacuolar membrane and/or on the perinuclear membrane of the endoplasmic reticulum [27]. Looking more closely for the far-red images of compounds **3a**-**e**, it is possible to identify similar phenotypes as the

ones previously reported [27]. The staining experiments with compound **3a**, reveal a preferential accumulation of the compound in the region of vacuolar membrane (green arrow) (Figure 4). In the case of compounds **3b-e** the accumulation seems to occur at the perinuclear membrane of endoplasmic reticulum (yellow arrows), but also on other non-identified mainly spherical structures (Figure 4).





375



376 Figure 4 – Intracellular distribution of **3a-e**. Differential interference contrast (DIC) and Far Red

fluorescence microscopy images of W303-1A cells after incubation with **3a**-**e** (12.5 μM). Samples were stained in PBS at 30 °C 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.

4. Conclusion

Four new benzo[*a*]phenoxazinium chlorides, Nile Blue analogues, having as substituents only amino or (3-aminopropyl) amino groups at the 5-position and a propylamino or dipropylamino groups at the 9-position were synthesized. Another analogue, with (3-aminopropyl) amino, ethyl amino and methyl groups at 5-, 9- and 10-positions of the polycyclic system was also synthesized for comparison in the studies performed. These compounds displayed absorption and emission maxima in the range 608-650 nm and 643-683 nm, respectively, with fluorescence quantum yields up to 0.58, in absolute dry ethanol, acidified ethanol, water and other aqueous solutions at pH values of 3, 5 and 7.4. It was found that all benzo[*a*]phenoxazinium chlorides revealed inhibitory activity against the yeast *Saccharomyces cerevisiae* PYCC 4072 used as a eukaryotic model organism. The best activity was obtained with compounds having amino and propylamino groups at 5- and 9-positions, respectively, with a MIC value of 6.25 μM. Fluorescence microscopy studies showed that all benzo[*a*]phenoxazinium chlorides stained the cells, with accumulation that seems to appear preferential at the level of vacuolar membrane and/or on the perinuclear membrane of the endoplasmic reticulum, as previously reported for similar compounds.

Overall, the results suggest that benzo[*a*]phenoxazinium chlorides with particular substituents in the polycyclic system can be very promising as potential antifungals and fluorescent probes for cell staining, motivating future research work where these capacities can be valued.

Acknowledgments

Thanks are due to Fundação para a Ciência e Tecnologia (FCT) and FEDER (European Fund for Regional Development)-COMPETE-QRENEU for financial support through the research centres

CQ/UM (UID/QUI/0686/2019 and UIDB/00686/2020) and CBMA (PEst-OE/BIA/UI4050/2019 and UID/BIA/04050/2020), as well as a PhD grant to J.C.F. (SFRH/BD/133207/2017). The NMR spectrometer Bruker Avance III 400 is part of the National NMR Network (PTNMR) and are partially supported by Infrastructure Project No 022161 (co-financed by FEDER through COMPETE 2020, POCI and PORL and FCT through PIDDAC).

References

1 T. Liu, J. Ning, B. Wang, B. Dong, S. Li, X. Tian, Z. Yu, Y. Peng, C. Wang, X. Zhao, X. Huo, C. Sun, J. Cui, L. Feng and X. Ma, *Anal. Chem.*, 2018, **90**, 3965–73.

2 B. M. White, Y. Zhao, T. E. Kawashima, B. P. Branchaud, M. D. Pluth and R. Jasti, *ACS Cent. Sci.*, 2018, **4**, 1173–1178.

3 M. Sameiro T. Gonçalves, *Chem. Rev.*, 2009, **109**, 190–212.

4 M. S. T. Gonçalves, *Advanced Fluorescence Reporters in Chemistry and Biology I: Fundamentals and Molecular Design*, 2010, 27–64.

5 J. V. Frangioni, *Curr. Opin. Chem. Biol.*, 2003, **7**, 626–34.

6 L. Yuan, W. Lin, K. Zheng, L. He and W. Huang, *Chem. Soc. Rev.*, 2013, **42**, 622–61.

7 S. S. Mishra and U. Subuddhi, *J. Photochem. Photobiol. B Biol.*, 2014, **14**, 67–75.

8 J. Jose, Y. Ueno and K. Burgess, *Chem. - A Eur. J.*, 2009, **15**, 418–423.

9 V. Martinez and M. Henary, *Chem. - A Eur. J.*, 2016, **22**, 13764–13782.

10 V. H. J. Frade, M. S. T. Gonçalves and J. C. V. P. Moura, *Tetrahedron Lett.*, 2005, **46**, 4949-4952.

11 V. H. J. Frade, M. S. T. Gonçalves and J. C. V. P. Moura, *Tetrahedron Lett.*, 2006, **47**, 8567–8570.

12 V. H. J. Frade, M. S. T. Gonçalves, P. J. G. Coutinho and J. C. V. P. Moura, *J. Photochem. Photobiol. A Chem.*, 2007, **185**, 220–230.

13 V. H. J. Frade, P. J. G. Coutinho, J. C. V. P. Moura and M. S. T. Gonçalves, *Tetrahedron*,

2007, **63**, 1654–1663.

14 V. H. J. Frade, S. A. Barros, J. C. V. P. Moura and M. S. T. Gonçalves, *Tetrahedron Lett.*, 2007, **48**, 3403–7.

15 V. H. J. Frade, S. A. Barros, J. C. V. P. Moura, P. J. G. Coutinho and M. S. T. Gonçalves, *Tetrahedron*, 2007, **63**, 12405–18.

16 C. M. A. Alves, S. Naik, P. J. G. Coutinho and M. S. T. Gonçalves, *Tetrahedron*, 2009, **65**, 10441–10452.

17 C. M. A. Alves, S. Naik, P. J. G. Coutinho and M. S. T. Gonçalves, *Tetrahedron Lett.*, 2011, **52**, 112–116.

18 S. Naik, C. Alves, P. Coutinho and M. S. Gonçalves, *European J. Org. Chem.*, 2011, **13**, 2491–2497.

19 A. D. G. Firmino and M. S. T. Gonçalves, *Tetrahedron Lett.*, 2012, **53**, 4946–4950.

20 B. R. Raju, A. D. G. Firmino, A. L. S. Costa, P. J. G. Coutinho and M. S. T. Gonçalves, *Tetrahedron*, 2013, **69**, 2451–2461.

21 B. R. Raju, S. Naik, P. J. G. Coutinho and M. S. T. Gonçalves, *Dyes Pigm.*, 2013, **99**, 220–227.

22 B. R. Raju, A. M. F. Garcia, A. L. S. Costa, P. J. G. Coutinho and M. S. T. Gonçalves, *Dyes Pigm.*, 2014, **110**, 203–213.

23 B. R. Raju, M. M. T. Carvalho, M. I. P. S. Leitão, P. J. G. Coutinho and M. S. T. Gonçalves, *Dyes Pigm.*, 2016, **132**, 204–212.

24 B. R. Raju, M. S. T. Gonçalves and P. J. G. Coutinho, *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.*, 2017, **171**, 1–9.

25 V. H. J. Frade, M. J. Sousa, J. C. V. P. Moura and M. S. T. Gonçalves, *Tetrahedron Lett.*, 2007, **48**, 8347–8352.

26 V. H. J. Frade, M. J. Sousa, J. C. V. P. Moura and M. S. T. Gonçalves, *Bioorganic Med. Chem.*, 2008, **16**, 3274–3282.

457 27 M. I. P. S. Leitão, B. Rama Raju, N. M. F. S. A. Cerqueira, M. J. Sousa and M. S. T.
458 Gonçalves, *Bioorg. Chem.*, 2020, **98**, 103730.
459 28 T. Shimamoto, A. Tomoda, R. Ishida and K. Ohyashiki, *Clin. Cancer Res.*, 2001, **7**, 704–
460 708.
461 29 M. Lopes, C. T. Alves, B. Rama Raju, M. S. T. Gonçalves, P. J. G. Coutinho, M. Henriques
462 and I. Belo, *J. Photochem. Photobiol. B Biol.*, 2014, **141**, 93–99.
463 30 Y. Mizukawa, J. F. Ge, A. Bakar Md, I. Itoh, C. Scheurer, S. Wittlin, R. Brun, H. Matsuoka
464 and M. Ihara, *Bioorganic Med. Chem.*, 2014, **22**, 3749–52.
465 31 L. I. Kozlovskaya, G. Andrei, A. A. Orlov, E. V. Khvatov, A. A. Koruchekov, E. S. Belyaev,
466 E. N. Nikolaev, V. A. Korshun, R. Snoeck, D. I. Osolodkin, E. S. Matyugina and A. V.
467 Aralov, *Antiviral Res.*, 2019, **163**, 117–124.
468 32 M. I. P. S. Leitão, B. R. Raju, S. Naik, P. J. G. Coutinho, M. J. Sousa and M. S. T.
469 Gonçalves, *Tetrahedron Lett.*, 2016, **57**, 3936–3941.
470 33 B. R. Raju, M. I. P. S. Leitão, M. J. Sousa, P. J. G. Coutinho and M. S. T. Gonçalves, *Dyes*
471 *Pigm.*, 2020, **173**, 107870.
472 34 K. Mickevičienė, R. Baranauskaitė, K. Kantminienė, M. Stasevych, O. Komarovska-
473 Porokhnyavets and V. Novikov, *Molecules*, 2015, **20**, 3170–3189.
474 35 M. T. Chhabria and M. H. Jani, *Eur. J. Med. Chem.*, 2009, **44**, 3837–44
475 36 R. Sens and K. H. Drexhage, *J. Lumin.*, 1981, **25**, 709–712.
476 37 M. L. Crossley, R. J. Turner, C. M. Hofmann, P. F. Dreisbach and R. P. Parker, *J. Am.*
477 *Chem.*, 1952, **74**, 578–584.
478