Squaraine dyes derived from indolenine and benzo[*e*]indole as potential fluorescent probes for HSA detection and antifungal agents

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ABSTRACT

Four squaraine dyes derived from 2,3,3-trimethylindolenine and 1,1,2-trimethyl-1*H*-benzo[*e*]indole with different combinations of barbituric groups attach to the central ring, having ester groups and alkyl chains in the nitrogen atoms of heterocyclic rings were synthesized. These dyes were fully characterized and their photophysical behavior was studied in ethanol and phosphate-buffered saline solution. Absorption and emission bands between 631 and 712 nm were detected, with the formation of aggregates in aqueous media, which is typical of this class of dyes. Tests carried out with 1,3-diphenylisobenzofuran allowed us to verify the ability of the dyes to produce singlet oxygen. The interaction of synthesized dyes with human serum albumin (HSA) was also evaluated, being demonstrated a linear correlation between fluorescence intensity and protein concentration. The antifungal potential of the dyes against the yeast *Saccharomyces cerevisiae* was evaluated using a broth microdilution assay. In order to test the photosensitizing capacity of the synthesized dyes, tests were carried out in the dark and with irradiation, using a custom-built light-emitting diode that emits close to the absorption wavelength of the studied dyes. The results showed that the interaction of dyes with HSA and the antifungal activity depends on the different structural modifications of the dyes.

Keywords: Squaraine dyes; Singlet oxygen; Photostability; Photodynamic activity; Fluorescence probes; Human Serum Albumin; Antifungal activity

INTRODUCTION

The first synthetic organic dye was discovered by William Perkin in 1865, which led to the beginning of the production of dyes on a global scale [1, 2]. The color emitted by these compounds results from the ability to absorb light in the visible and near-infrared (NIR) range, the presence of a chromophore group in their structure, the existence of a conjugated π-system and the presence of a resonance structure [3]. The rigid planar structure of most organic dyes reduces energy losses from non-radiative processes, causing these compounds to exhibit significant fluorescence [4].

Initially, organic dyes were applied essentially in the textile industry, for dyeing fabrics and skins and also for coloring other objects [5]. Over the years, its applications have become more and more varied, having come to be used in dye-sensitized solar cells [6], in organic light-emitting diode [7] and in field-effect transistors [8]. Various organic molecules are also used in biomedical applications ranging from diagnosis to treatment of a particular pathology [9].

Squaraine dyes are one family of functional organic dyes that due to their appealing photophysical and photochemical characteristics have received significant attention in recent decades. These compounds have in their general structure two electron donor groups on both sides of the squaric acid core as an electron acceptor group [10, 11]. This zwitterionic structure, together with the rigidity and planarity conferred by the central ring, lead the squaraine dyes to present absorption in the visible to NIR range, high molar extinction coefficients, good photoconductivity, good chemical/photochemical stability, moderate fluorescence quantum yields, and long fluorescence lifetime [12-15].

There are several applications in which these compounds can be used, from sensitizers in solar cells [16, 17], and in photovoltaic devices [18, 19], to applications aimed at biological areas such as fluorescent probes for the detection of biomolecules [20-22] and fluorescence

bioimaging [23, 24]. In addition, the application of these dyes as photosensitizers in photodynamic therapy (PDT) has been reported, and their efficacy *in vivo* and *in vitro* in anti-cancer and antimicrobial treatment has been proved [25-28].

The use of squaraine dyes as fluorescent probes for the detection and quantification of biomolecules is well known. Recently, studies have focused on the detection of bovine serum albumin (BSA) and human serum albumin (HSA), using squaraine dyes, verifying that these dyes change their fluorescence patterns when in the presence of these proteins, showing, in most cases, a linear relationship between the fluorescence intensity emitted and the amount of protein present [29-34]. The results obtained revealed a promising future for the possible application of squaraine dyes in standardized fluorescent methods for the detection of these proteins. However, structural improvements are still required to promote some parameters in order to make the dyes ideal for the considered use.

Given the importance of these dyes previously mentioned and in order to find new potential structural alternatives, in this work is reported the synthesis of four squaraine dyes and performed the evaluation of their interaction with HSA and thus determine its effectiveness in detecting and quantifying this protein. The antiproliferative activity of squaraine dyes using *Saccharomyces cerevisiae* yeast as a biological model was also assessed. The minimum inhibitory concentration (MIC) of the dyes was determined, in the dark and with irradiation, using a LED system, in order to verify if the compounds exhibited better activity after photoactivation.

All compounds tested were shown to interact with HSA, and also showed considerable antifungal activity, with some of the compounds improving their effectiveness after irradiation.

MATERIALS AND METHODS

Synthesis general: All the reagents and solvents used in the synthesis process, including 2,3,3-trimethylindolenine (**1a**), 1,1,2-trimethyl-1*H*-benzo[*e*]indole (**1b**), 3-bromopropionic acid (2), 3,4-dihydroxycyclobut-3-ene-1,2-dione (**4**), barbituric acid (**9a**), thiobarbituric acid (**9b**) were purchased from commercial suppliers and used without further purification; the intermediate 3,4-dibutoxycyclobut-3-en-1,2-dione (**7**) was prepared according to the literature procedure [35].

All reactions were monitored by thin-layer chromatography (TLC) on aluminium plates with 0.25 mm of silica gel (Merck 60 F254). Melting points (m.p.) were measured in a hot plate binocular microscope apparatus (URA Technic, Oporto, Portugal) and were uncorrected. Absorption spectra were recorded on a Lambda 25 UV/Vis spectrophotometer (Perkin Elmer, USA) in the spectral range 500-900 nm, at room temperature. Emission spectra were performed using a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, USA), with excitation and emission slits of 10 nm and an excitation wavelength of 580 nm. All the spectroscopic measurements were performed in a quartz cuvette with a 1 cm path length. The infrared (IR) spectra were recorded on a IRAffinity-1S FTIR spectrophotometer (Shimadzu, Kyoto, Japan) using KBr pellets, at room temperature in the 4000-500 cm^{-1} range by averaging 64 scans at a spectral resolution of 2 cm^{-1}. The bands were described as s (strong), m (medium) or w (weak). The proton nuclear magnetic resonance (^{1}H NMR) and carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were obtained at 298.15 K on a NMR Brucker Avance III 400 spectrometer operating at 9.4 T, observing ^{1}H at 400.13 MHz or on a NMR Brucker Avance III 600 spectrometer operating at 14.09 T observing ^{1}H at 600.10 MHz and ^{13}C at 150.91 MHz. The solutions were prepared in hexadeuterodimethyl sulfoxide (DMSO-d_6) and deuterated chloroform ($CDCl_3$) and the chemical shifts are expressed as δ (ppm) relative to tetramethylsilane (internal standard) or to residual solvent signals; the coupling constant (J) values are given in hertz (Hz). Splittings

were described as s (singlet), d (doublet), t (triplet), qt (quintet), st (sextet), m (multiplet), bs (broad singlets), bt (broad triplet). The assignments of the carbon were made based on DEPT 135 spectra. High resolution electrospray ionization time-of-flight mass spectra (HRESI-TOFMS) were recorded using a microTOF (focus) Brucker Daltonics spectrometer (C.A.C.T.I. at the University of Vigo, Spain).

Synthesis of 1-(2-carboxyethyl)-2,3,3-trimethyl-3H-indol-1-ium bromide (3a). The quaternary ammonium salt **3a** was synthesized using a similar procedure to that described by Boto *et al.* [36]. A mixture of 2,3,3-trimethylindolenine (**1a**) (1.108 g, 6.95 mmol) and 3-bromopropionic acid (**2**) (1.064 g, 6.95 mmol) was heated at 80˚C, under stirring, for 1 hour. After cooling to room temperature, methanol was added to ensure complete solubilization of the resulting solid. The mixture was cooled on an ice bath to allow the product to precipitate by the addition of CH_2Cl_2. After successive washings with CH_2Cl_2 the light pink precipitate was collected and dried under reduced pressure. Yield: 51 %; m.p.: 163-167˚C (dec.); IR υ_{max} (KBr): 3460 (s, OH), 3028 (w, Ar-CH), 2982 (w, CH), 2929 (w, CH), 1718 (s, C=O), 1622 (m, C=C), 1603 (m, C=C), 1589 (m, C=C), 1476 (m), 1457 (m), 1409 (m), 1364 (m), 1331 (w), 1299 (w), 1271 (m), 1233 (m), 1224 (m), 1123 (w), 1047 (w), 1007 (w), 934 (w), 915 (w), 884 (w), 840 (w), 817 (w), 785 (m) cm^{-1}; ^1H NMR (600.10 MHz, DMSO-d_6) δ: 8.00-7.97 (1H, m, ArH), 7.85-7.82 (1H, m, ArH), 7.63-7.59 (2H, m, ArH), 4.66 (2H, t, J = 6.9, $NC\underline{H}_2CH_2COOH$), 2.98 (2H, t, J = 6.9, $NCH_2C\underline{H}_2COOH$), 2.86 (3H, s, CCH_3), 1.52 (6H, s, $C(CH_3)_2$) ppm; ^{13}C NMR (150.91 MHz, DMSO-d_6) δ: 197.95, 171.51, 141.77, 140.82, 129.35 (ArCH), 128.92 (ArCH), 123.49 (ArCH), 115.58 (ArCH), 54.28 ($\underline{C}(CH_3)_2$), 43.58 ($N\underline{C}H_2CH_2COOH$), 31.14 ($NCH_2\underline{C}H_2COOH$), 21.90 ($C(\underline{C}H_3)_2$), 14.44 ($C\underline{C}H_3$) ppm; HRESI-TOFMS m/z: 232.1332 $[M]^+$ ($C_{14}H_{18}NO_2$ calc. 232.1327).

Synthesis of 1-(2-carboxyethyl)-2,3,3-trimethyl-1H-benzo[e]indol-1-ium bromide (3b). The quaternary ammonium salt **3b** was synthesized using a similar procedure to that described for **3a**. A mixture of 1,1,2-trimethyl-1*H*-benzo[*e*]indole (**1b**) (1.720 g, 8.22 mmol) and 3-bromopropionic acid (**2**) (1.257 g, 8.22 mmol) was heated at 120˚C, under stirring, for 1.30 hours. After cooling to room temperature, methanol was added to ensure complete solubilization of the formed solid. The mixture was cooled on an ice bath to allow the product to precipitate by the addition of CH_2Cl_2 and diethyl ether. After successive washings with CH_2Cl_2 the light pink precipitate was collected and dried under reduced pressure. Yield: 30 %; m.p.: 105-107 ˚C; IR υ_{max} (KBr); 3400 (s, OH), 3067 (w, Ar-CH), 2972 (w, CH), 1729 (s, C=O), 1637 (m), 1617 (m), 1584 (m), 1522 (m), 1466 (m), 1405 (m), 1342 (m), 1278 (w), 1253 (w), 1227 (w), 1020 (w), 929 (w), 873 (m), 824 (m), 792 (m), 776 (m) cm^{-1}; ^{1}H NMR (600.10 MHz, DMSO-d_6) δ: 8.36 (1H, d, J = 8.4, ArH), 8.27 (1H, d, J = 9.0, ArH), 8.21 (1H, d, J = 8.4, ArH), 8.17 (1H, d, J = 8.4, ArH), 7.78 (1H, t, J = 7.5, ArH), 7.72 (1H, t, J = 7.5, ArH), 4.78 (2H, t, J = 6.9, NC$\underline{H}_2$CH$_2$COOH), 3.05 (2H, t, J = 6.9, NCH$_2$C$\underline{H}_2$COOH), 2.97 (3H, s, CCH_3), 1.75 (6H, s, $C(CH_3)_2$ ppm; ^{13}C NMR (150.91 MHz, DMSO-d_6) δ: 197.77, 171.52, 138.33, 136.81, 133.00, 130.64 (ArCH), 129.72 (ArCH), 128.42 (ArCH), 127.26 (ArCH), 127.20, 123.40 (ArCH), 113.44 (ArCH), 55.60 ($\underline{C}(CH_3)_2$), 43.83 (N$\underline{C}$H$_2$CH$_2$COOH), 31.38 (NCH$_2\underline{C}$H$_2$COOH), 21.48 (C($\underline{C}$H$_3$)$_2$), 14.26 (C$\underline{C}$H$_3$) ppm; HRESI-TOFMS m/z: 282.1489 $[M]^+$ ($C_{18}H_{20}NO_2$ calc. 282.1480).

Synthesis of 4-[(1-(2-butoxycarbonylethyl)-3,3-dimethyl-3H-indol-1-ium-2-yl)methylene]-2-[(1-(2-butoxycarbonylethyl)-3,3-dimethylindolin-2-ylidene)methyl]-3-oxocyclobut-1-en-1-olate (5a). Dye **5a** was synthesized from a reaction of the quaternary salt 1-(2-carboxyethyl)-2,3,3-trimethy-3*H*-lindol-1-ium bromide (**3a**) (0.400 g, 1.28 mmol) and squaric acid **4** (0.073 g, 0.640 mmol) in a mixture (1:1 v/v) of *n*-butanol/toluene (10

mL), stirred for 6 h at reflux in a Dean-Stark apparatus. Cold distilled water was added to the reaction mixture and after separation by decantation, the organic layer was dried with anhydrous Na_2SO_4 and the solvent removed under reduced pressure. After successive recrystallizations from CH_2Cl_2/MeOH/petroleum ether/diethyl ether, light blue crystals were recoiled and dried under reduced pressure. Yield: 17 %; m.p.: 156-157 ºC; IR υ_{max} (KBr): 3049 (w, Ar-CH), 2958 (m, CH), 2932 (m, CH), 2871 (w, CH), 1727 (m, C=O), 1596 (s), 1507 (s), 1497 (s), 1454 (m), 1428 (m), 1393 (w), 1355 (m), 1300 (m), 1199 (m), 1164 (m), 1096 (m), 1057 (m), 1020 (m), 962 (m), 912 (m), 847 (w), 785 (m) cm^{-1}; ^1H NMR (600.10 MHz, $CDCl_3$) δ: 7.35 (2H, d, J = 7.2, ArH), 7.31 (2H, t, J = 7.5, ArH), 7.14 (2H, t, J = 7.2, ArH), 7.08 (2H, d, J = 7.8, ArH), 5.93 (2H, s, CH=C), 4.34 (4H, bs, $OC\underline{H}_2(CH_2)_2CH_3$), 4.05 (4H, t, J = 6.6, $NC\underline{H}_2CH_2COO(CH_2)_3CH_3$), 2.82 (4H, t, J = 7.5, $NCH_2C\underline{H}_2COO(CH_2)_3CH_3$,), 1.78 (12H, s, $C(CH_3)_2$), 1.54 (4H, qt, J = 7.0, $OCH_2C\underline{H}_2CH_2CH_3$), 1.31 (4H, st, $OCH_2CH_2C\underline{H}_2CH_3$), 0.89 (6H, t, J = 7.5, $O(CH_2)_3C\underline{H}_3$ ppm; ^{13}C NMR (150.91 MHz, $CDCl_3$) δ:182.22, 180.93, 170.88, 170.16, 142.08, 127.99 (ArCH), 124.12 (ArCH), 122.48 (ArCH), 109.68 (ArCH), 86.93 ($\underline{C}$H=C), 65.25 (CH_2), 49.51 $\underline{C}(CH_3)_2$, 39.40 (CH_2), 31.75 (CH_2), 30.57 (CH_2), 27.25 $C(\underline{C}H_3)_2$, 19.17 (CH_2), 13.78 ($N(CH_2)_5\underline{C}H_3$) ppm; HRESI-TOFMS m/z: 652.3507 $[M]^+$ ($C_{40}H_{48}N_2O_6$ calc. 652.3487).

Synthesis of 2-[(1-(2-butoxycabonylethyl)-1,1-dimethyl-2H-benzo[e]indol-2-ylidene)methyl]-4-[(1-(2-butoxycabonylethyl)-1,1-dimethyl-1H-benzo[e]indol-3-ium-2yl)methylene]-3-oxocyclobut-1-en-1-olate (5b). Dye **5b** was synthetized from a reaction of the quaternary salt 3-(2-carboxyethyl)-1,1,2-trimethyl-1*H*-benzo[*e*]indol-3-ium bromide (**3b**) (0.427 g, 1.18 mmol) and squaric acid **4** (0.067 g, 0.591 mmol) in a mixture (1:1 v/v) of *n*-butanol/toluene (10 mL), stirred for 7 h at reflux in a Dean-Stark apparatus. After work-up in the same way as dye **5a**, the obtained residue was successively recrystallized from

CH_2Cl_2/diethyl ether/petroleum ether. Light blue crystals were recoiled and dried under reduced pressure. Yield: 59 %; m.p.: 117-118 ˚C; IR υ_{max} (KBr): 3035 (w, Ar-CH), 2957 (m, CH), 2930 (m, CH), 2868 (m, CH), 1735 (m, C=O), 1595 (m), 1526 (m), 1488 (s), 1455 (m), 1430 (m), 1286 (m), 1253 (m), 1187 (m), 1097 (m), 1042 (m), 1014 (m), 930 (m), 837 (w), 806 (w) cm^{-1}; ^1H NMR (600.10 MHz, $CDCl_3$) δ: 8.20 (2H, d, J = 8.4, ArH), 7.89 (2H, d, J = 7.8, ArH), 7.87 (2H, d, J= 9.0, ArH), 7.57 (2H, t, J = 7.5, ArH), 7.42-7.39 (4H, m, ArH), 6.00 (2H, s, CH=C), 4.47 (4H, bs, $OC\underline{H}_2(CH_2)_2CH_3$), 4.05 (4H, t, J = 6.6, $NC\underline{H}_2CH_2COO(CH_2)_3CH_3$) 2.90 (4H, t, J = 7.2, $NCH_2C\underline{H}_2COO(CH_2)_3CH_3$), 2.07 (12H, s, $C(CH_3)_2$), 1.52 (4H, qt, J = 6.8, $OCH_2C\underline{H}_2CH_2CH_3$), 1.28 (4H, st, $OCH_2CH_2C\underline{H}_2CH_3$), 0.84 (6H, t, J = 7.5, $O(CH_2)_3C\underline{H}_3$ ppm; ^{13}C NMR (150.91 MHz, $CDCl_3$) δ:182.44, 179.38, 171.46, 170.89, 139.25, 134.36, 131.48, 129.84 (ArCH), 129.82 (ArCH), 128.79, 127.44 (ArCH), 124.51 (ArCH), 122.67 (ArCH), 110.31 (ArCH), 86.67 ($\underline{C}H=C$), 65.26 (CH_2), 51.36 $\underline{C}(CH_3)_2$, 39.52 (CH_2), 32.01 (CH_2), 30.55 (CH_2), 26.95 $C(\underline{C}H_3)_2$, 19.13 (CH_2), 13.70 ($N(CH_2)_5\underline{C}H_3$) ppm; HRESI-TOFMS m/z: 752.3820 $[M]^+$ ($C_{48}H_{52}N_2O_6$ calc. 752.3811).

Synthesis of 3-hexyl-1,1,2-trimethyl-1H-benzo[e]indol-3-ium iodide (6). The quaternary ammonium salt **6** was synthesized using a procedure similar to that described by Pardal *et al.* [37] A solution of 1,1,2-trimethyl-1*H*-benzo[*e*]indole (**2**) (4.036 g, 19.0 mmol) and 1-iodohexane (8.54 mL, 57 mmol) in acetonitrile was stirred under reflux for 10 days. After cooling, diethyl ether was added to allow the product precipitation and the desired quaternary salt collected by filtration under reduced pressure and washed several times with diethyl ether. Yield: 90 %; m.p.: 164-165 ˚C; IR υ_{max} (KBr): 3055 (w, Ar-CH), 3011 (w, Ar-CH), 2967 (w, CH), 2943 (w, CH), 2924 (m, CH), ,1634 (w), 1614 (w), 1580 (m), 1522 (w), 1470 (s), 1445 (m), 1375 (w), 1368 (w), 1339 (w), 1217 (w), 1196 (w), 1161 (w), 1130 (w), 1040 (w), 1024 (w), 995 (w), 939 (w), 907 (w), 889 (w), 864 (m), 827 (s), 789 (w) cm^{-1}. ^1H NMR

(600.10 MHz, $CDCl_3$) δ: 8.13-8.06 (3H, m, ArH), 7.77-7.67 (3H, m, ArH), 4.82 (2H, t, J = 7.8, $NC\underline{H}_2(CH_2)_4CH_3$), 3.20 (3H, s, CCH_3), 1.99 (2H, qt, J = 7.7, $NCH_2C\underline{H}_2(CH_2)_3CH_3$), 1.90 (6H, s, $C(CH_3)_2$), 1.50 (2H, qt, J = 7.3, $N(CH_2)_2C\underline{H}_2(CH_2)_2CH_3$), 1.42-1.28 (4H, m, $N(CH_2)_3(C\underline{H}_2)_2CH_3$), 0.89 (3H, t, J = 7.0, $N(CH_2)_5C\underline{H}_3$) ppm; ^{13}C NMR (150.91 MHz, $CDCl_3$) δ: 195.21, 138.29, 137.25, 133.79, 131.57 (ArCH), 130.16 (ArCH), 128.77 (ArCH), 127.94, 127.94 (ArCH), 127.74 (ArCH), 122.95 (ArCH), 112.56 (ArCH), 56.02 ($\underline{C}(CH_3)_2$), 50.50 ($N\underline{C}H_2(CH_2)_4CH_3$), 31.29 ($NCH_2\underline{C}H_2(CH_2)_3CH_3$), 28.23 ($N(CH_2)_2\underline{C}H_2(CH_2)_2CH_3$), 26.55 ($N(CH_2)_3\underline{C}H_2CH_2CH_3$), 22.83 ($C(\underline{C}H_3)_2$), 22.44 ($N(CH_2)_4\underline{C}H_2CH_3$), 16.98 ($C\underline{C}H_3$), 13.97 ($N(CH_2)_5\underline{C}H_3$) ppm.

Synthesis of 3-butoxy-4-[(3-hexyl-1,1-dimethyl-2H-benzo[e]indol-2-ylidene)methyl]cyclobut-3-ene-1,2-dione (8). Precursor **8** was synthesized using a procedure similar to that described by Lima *et al*. [26]. A solution of the quaternary ammonium salt **6** (1.151 g, 2.73 mmol) and dibutylsquarate (**7**) (0.618 g, 2.73 mmol) in ethanol (20 mL) in the presence of triethylamine (1.515 mL, 10.9 mmol) was stirred for 15 min. and left to rest overnight at room temperature. Cold distilled water and CH_2Cl_2 were added to the reaction mixture and after separation by decantation, the organic layer was dried with anhydrous Na_2SO_4 and the solvent removed under reduced pressure. The obtained residue was purified by successive recrystallizations from CH_2Cl_2/petroleum ether. Yellow crystals were recoiled and dried under reduced pressure. Yield: 53 %; m.p.: 91-92 ˚C; IR υ_{max} (KBr): 3074 (w), 2957 (m, Ar-CH), 2935 (m, Ar-CH), 2871 (m, Ar-CH), 1767 (s, C=O), 1715 (s, C=O), 1626 (w), 1588 (m), 1545 (s), 1518 (m), 1471 (w), 1442 (w), 1421 (m), 1339 (m), 1305 (m), 1259 (w), 1208 (w), 1184 (m), 1140 (w), 1119 (w), 1096 (w), 1047 (w), 1017 (w), 949 (m), 892 (w), 859 (w), 825 (w), 815 (w), 792 (w), 785 (w) cm^{-1}; ^{1}H NMR (600.10 MHz, $CDCl_3$) δ: 8.09 (1H, d, J = 8.4, ArH), 7.87 (1H, d, J = 8.0, ArH), 7.84 (1H, d, J = 8.8,

ArH), 7.54 (1H, t, J = 7.4, ArH), 7.34 (1H, t, J = 7.4, ArH), 7.23 (1H, d, J = 8.8, ArH), 5.45 (1H, s, CH=C), 4.91 (2H, t, J = 6.8, $OC\underline{H}_2(CH_2)_2CH_3$), 3.92 (2H, t, J = 7.4 , $NC\underline{H}_2(CH_2)_4CH_3$), 1.93-1.86 (8H, m, $OCH_2C\underline{H}_2CH_2CH_3$ + $C(C\underline{H}_3)_2$), 1.79 (2H, qt, J = 7.5, $NCH_2C\underline{H}_2(CH_2)_3CH_3$), 1.54 (2H, st, $OCH_2CH_2C\underline{H}_2CH_3$), 1.44 (2H, qt, J = 7.3, $N(CH_2)_3C\underline{H}_2CH_2CH_3$), 1.39-130 (4H, m, $N(CH_2)_3(C\underline{H}_2)_2CH_3$), 1.02 (3H, t, J = 7.4, $O(CH_2)_3C\underline{H}_3$), 0.89 (3H, t, J = 6.8, $N(CH_2)_5C\underline{H}_3$) ppm; ^{13}C NMR (150.91 MHz, $CDCl_3$) δ: 192.92, 187.38, 187.34, 173.22, 170.35, 139.95, 132.59, 130.92, 129.85 (ArCH), 129.68 (ArCH), 128.73, 127.32 (ArCH), 123.89 (ArCH), 122.34 (ArCH), 109.87 (ArCH), 80.98 ($\underline{C}$H=C), 73.85 (CH_2), 49.95 ($\underline{C}(CH_3)_2$), 43.24 (CH_2), 32.33 (CH_2), 31.56 (CH_2), 26.82 ($C(\underline{C}H_3)_2$), 26.77 (CH_2), 26.74 (CH_2), 22.62 (CH_2), 18.89 (CH_2), 14.09 ($O(CH_2)_3\underline{C}H_3$), 13.87 ($N(CH_2)_5\underline{C}H_3$) ppm. HRESI-TOFMS m/z: 446.2690 $[M+H]^+$ ($C_{29}H_{36}NO_3$ calc. 446.2696).

Synthesis of triethylammonium 2-[(3-hexyl-1,1-dimethyl-2H-benzo[e]indol-2-ylidene)methyl]-4-oxo-3-(2,4,6-trioxotetrahydro-5-pyrimidinyliden)cyclobut-1-en-1-olate (10a). The semisquaraine **10a** was prepared by a reaction of a suspension of **8** (0.519 g, 1.17 mmol), barbituric acid (**9a**) (0.522 g, 4.08 mmol) and triethylamine (0.161 mL, 1.17 mmol) in ethanol (15 mL). The mixture was stirred under reflux for 9 h and then was cooled on an ice bath to allow the product to precipitate by the addition of diethyl ether. The resulting residue was washed with diethyl ether, filtered under reduced pressure, dried in a vacuum pump and used in the next step without further purification.

Synthesis of triethylammonium 2-[(3-hexyl-1,1-dimethyl-2H-benzo[e]indol-2-ylidene)methyl]-4-oxo-3-(2,6-dioxo-4-thioxohexahydro-5-pyrimidinyliden)cyclobut-1-en-1-olate (10b). The semisquaraine **10b** was prepared by a reaction of a suspension of **8** (0.680 g, 1.53 mmol), thiobarbituric acid (**9b**) (0.440 g, 3.05 mmol) and triethylamine (0.211 mL,

1.53 mmol) in ethanol (20 mL). The mixture was stirred under reflux for 6 h and then was cooled on an ice bath to allow the product to precipitate by the addition of diethyl ether. The resulting residue was washed with diethyl ether, filtered under reduced pressure, dried in a vacuum pump and used in the next step without further purification.

Synthesis of 2-[(3-hexyl-1,1-dimethyl-2H-benzo[e]indol-2-ylidene)methyl)]-4-[(3-hexyl-1,1-dimethyl-1H-benzo[e]indol-3-ium-2-yl)methylene]-3-(2,4,6-trioxotetrahydro-5-pyrimidinylidene)cyclobut-1-en-1-olate (11a). The squaraine dye **11a** was prepared by reaction from the semisquaraine dye **10a** (0.494 g, 0.822 mmol) and the quaternary ammonium salt **6** (0.572 g, 1.36 mmol) in *n*-butanol (15 mL), stirred for 10 h at reflux. The reaction mixture then was cooled on an ice bath to allow the product to precipitate by the addition of diethyl ether. The obtained residue was purified by alumina column chromatography (2% MeOH/CH_2Cl_2). Dark green crystals were recoiled and dried under reduced pressure. Yield: 18 %; m.p.: 261-263 ºC (dec.); IR υ_{max} (KBr): 3442 (w, NH), 3193 (w, ArCH), 3072 (w, ArCH) 2954 (m, CH), 2930 (m, CH), 2857 (m, CH), 2351 (w), 1720 (m, C=O),1710 (m), 1603 (m), 1576 (m), 1525 (m), 1489 (s), 1481 (s), 1454 (s), 1435 (m), 1336 (m), 1249 (m), 1208 (m), 1178 (m), 1152 (m), 1153 (m), 1124 (m), 1072 (m), 1014 (m) 981 (w), 933 (m), 891 (w), 829 (w), 800 (w), 779 (w), 745 (w) cm^{-1}; ^1H NMR (600.10 MHz, DMSO-d_6) δ: 10.00 (2H, s, NH, change with D_2O), 8.30 (2H, d, J = 8.4, ArH), 8.05 (4H, t, J = 7.5, ArH), 7.74 (2H, d, J = 8.4, ArH), 7.65 (2H, t, J = 7.8, ArH), 7.50 (2H, t, J = 7.5, ArH), 6.56 (2H, s, CH=C), 4.20 (4H, bt, J = 6.6, $NC\underline{H}_2(CH_2)_4CH_3$), 1.98 (12H, s, $C(CH_3)_2$), 1.78 (4H, qt, J = 7.5, $NCH_2C\underline{H}_2(CH_2)_3CH_3$), 1.38 (4H, qt, J = 7.2, $N(CH_2)_2C\underline{H}_2(CH_2)_2CH_3$), 1.32-1.22 (8H, m, $N(CH_2)_3(C\underline{H}_2)_2CH_3$), 0.81 (6H, t, J = 6.9, $N(CH_2)_5C\underline{H}_3$) ppm; ^{13}C NMR (100.6 MHz, DMSO-d_6) δ: 179.89, 175.68, 172.55, 169.71, 163.01, 139.32, 134.03, 131.24, 129.94 (ArCH), 129.77 (ArCH), 127.66 (ArCH), 124.68 (ArCH), 122.55 (ArCH), 111.65 (ArCH),

93.35 (CH=C), 86.72, 50.60 ($\underline{C}(CH_3)_2$), 43.96 ($N\underline{C}H_2$), 30.70 (CH_2), 26.61 (CH_2), 25.96 ($C(\underline{C}H_3)_2$), 25.66 (CH_2), 21.98 (CH_2), 13.78 ($N(CH_2)_5\underline{C}H_3$) ppm. HRESI-TOFMS m/z: 775.4218 $[M+H]^+$ ($C_{50}H_{55}N_4O_4$ calc. 775.4214).

Synthesis of 2-[(3-hexyl-1,1-dimethyl-2H-benzo[e]indol-2-ylidene)methyl)]-4-[(3-hexyl-1,1-dimethyl-1H-benzo[e]indol-3-ium-2-yl)methylene]-3-(4,6-dioxo-2-thioxotetrahydropyrimidinylidene)cyclobut-1-en-1-olate (11b). The squaraine dye **11b** was prepared by reaction from the semisquaraine dye **10b** (0.598 g, 0.970 mmol) and 6 (0.408 g, 0.970 mmol) in a mixture (1:1 v/v) of *n*-butanol/toluene (20 mL), stirred for 4 h at reflux. The reaction mixture was quenched with cold distilled water, and the organic layer, after separation by decantation, dried with anhydrous Na_2SO_4 and the solvent removed under reduced pressure. The obtained residue was purified by alumina column chromatography (2% MeOH/CH_2Cl_2). Dark green crystals were recoiled and dried under reduced pressure. Yield: 9%; m.p.: 255-257 ºC (dec.); IR υ_{max} (KBr): 3440 (w, NH), 3069 (w, ArCH), 2928 (w, CH), 2855 (w, CH), 1734 (m, C=O), 1578 (m), 1524 (m), 1483 (s), 1454 (s), 1433 (s), 1348 (w), 1333 (w), 1287 (s), 1250 (m), 1207 (m), 1179 (m), 1119 (s), 1065 (m), 1015 (m), 978 (w), 932 (w), 891 (w), 826 (w), 804 (w), 781 (w) cm^{-1}; ^1H NMR (400.13 MHz, DMSO-d_6) δ: 11.26 (2H, s, NH, change with D_2O), 8.30 (2H, d, J = 8.8, ArH), 8.06 (2H, d, J = 6.0, ArH), 8.04 (2H, d, J = 5.6, ArH), 7.75 (2H, d, J = 9.2, ArH), 7.65 (2H, t, J = 7.6, ArH), 7.50 (2H, t, J = 7.4, ArH), 6.44 (2H, s, C$\underline{H}$=C), 4.20 (4H, t, J = 7.0, $NC\underline{H}_2(CH_2)_4CH_3$), 1.98 (12H, s, $C(C\underline{H}_3)_2$), 1.78 (4H, qt, J = 7.2, $NCH_2C\underline{H}_2(CH_2)_3CH_3$), 1.38 (4H, qt, J = 7.1, $N(CH_2)_2C\underline{H}_2(CH_2)_2CH_3$), 1.33-1.21 (8H, m, $N(CH_2)_3(C\underline{H}_2)_2CH_3$), 0.82 (6H, t, J = 7.0, $N(CH_2)_5C\underline{H}_3$) ppm; ^{13}C NMR (100.6 MHz, DMSO-d_6) δ: 180.11, 175.54, 174.62, 172.88, 169.70, 160.84, 139.24, 134.15, 131.30, 129.96 (ArCH), 129.76 (ArCH), 127.67 (ArCH), 124.75 (ArCH), 122.55 (ArCH), 111.68 (ArCH), 93.19 (CH=C), 89.05, 50.66 ($\underline{C}(CH_3)_2$),

44.04 (N<u>C</u>H_2), 30.68 (CH_2), 26.59 (CH_2), 25.85 (C(<u>C</u>H_3)$_2$), 25.63 (CH_2), 21.94 (CH_2), 13.79 (N(CH_2)$_5$<u>C</u>H_3) ppm. HRESI-TOFMS m/z: 791.3989 $[M+H]^+$ ($C_{50}H_{55}N_4O_3S$ calc.791.3978).

Photophysical measurements: Some photophysical properties of the synthesized dyes were determined in ethanol and in phosphate-buffer (PB, pH 7.3). Phosphate buffer (PB, 0.05 M, pH 7.3) was prepared by dissolving $Na_2HPO_4 \cdot 7H_2O$ (4.540 g) and NaH_2PO_4 (1.130 g) in 1.0 L of milipore water.

The fluorescence quantum yield (Φ_F) was determined according to the equation 1 [29], using zinc phthalocyanine as reference (Φ_F = 0.17 in dimethylformamide (DMF)) [38].

$$\Phi_F = \Phi_{F(ZnPh)} \left(\frac{F_{Dye}}{F_{(ZnPh)}}\right) \left(\frac{A_{ZnPh}}{A_{Dye}}\right) \left(\frac{n^2_{media}}{n^2_{DMF}}\right) \quad (1)$$

where $\Phi_{F(ZnPh)}$ is the quantum yield of zinc phthalocyanine, F represent the integral areas of the emission spectra, A the absorbances and n the refractive indices of DMF and the solvent where the dye under examination is dissolved. The subscripts *ZnPh* and *Dye* refers to the reference zinc phthalocyanine and the dye under study, respectively.

Photostability analysis: From stock solutions of dyes in dimethyl sulfoxide (DMSO) at 1 mM, working solutions of each studied dye at a concentration of 25 µM were prepared by diluting them in PB. The solutions were transferred to a standard quartz cell (1 cm path length) and irradiated continuously during 20 minutes with a 150 W lamp with an emission in the UV/Vis range. Every 1-minute absorption spectra between 500-800 nm were recorded using a Cary 50 Bio spectrophotometer.

Qualitative evaluation of singlet oxygen ($^1O_2$) generation: Solutions of each squaraine dye, methylene blue (MB) (6.7×10^{-4} M) and 1,3-diphenylisobenzofuran (DPBF) (1 mM) were prepared in DMSO and subsequently diluted in PB (pH 7.3) or DMSO to obtain working solutions of 20 μM and 0.1 mM, for dyes and DPBF, respectively. Solutions were added in quadruplicate to a 96-well microplate and irradiated using an LED system with emission at 660 nm (described below). After periods of 5 s of irradiation up to 60 s, every 10 s up to 200 s, every 20 s up to 400 s, and every 50 s up to 650 s, absorbance readings at 410 nm (maximum absorption wavelength of the singlet oxygen indicator) were taken using a Thermo Scientific Multiskan GO microplate spectrophotometer. The experiments were carried out in DMSO and PB and the qualitatively analysis of the singlet oxygen production ability of the dyes was achieved by subtracting the absorbance at the 410 nm wavelength of the dyes in each solvent from the absorbance of the dyes incubated with DPBF.

Dye/ HSA interaction study: To investigate the interaction of the synthesized squaraine dyes with HSA, stock solutions of each dye in dimethylformamide (DMF) with a concentration of 6.7×10^{-4} M were prepared. The HSA solution at 14 μM were prepared by dissolving the protein in PB (0.05 M, pH 7.3). The working solutions were prepared by adding the dye stock solutions to the protein stock solutions, to obtain a dye concentration of 2.0 μM and a protein concentration between 0 and 3.5 μM. All solutions were prepared immediately before the experiments start. After 1 hour of incubation an emission spectrum was collected using a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, United States of America), operating with an excitation wavelength (λ_{exc}) of 580 nm and an excitation and emission slit of 10 nm and 20 nm respectively for dyes **11a**, **11b** and **5b**, and an excitation and emission slit of 5 nm and 10 nm for dye **5a**.

Binding and sensing parameters determination: From the data obtained in the dye/HSA interaction assays, the binding parameters were determined. Binding constants (K_b) for each dye were determined according to equation 2 (Benesi–Hildebrand equation) [39]:

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{max}} + \left(\frac{1}{K_b \Delta F_{max}}\right)\left(\frac{1}{[P]}\right) \quad (2)$$

where $\Delta F = F_x - F_0$, $\Delta F_{max} = F_\infty - F_0$, and F_0, F_x and F_∞ are the fluorescence intensities of dyes in the absence of protein, at a certain concentration, and at a concentration of complete interaction, respectively, and $[P]$ is the protein concentration.

Dissociation constant (K_d) and the Hill coefficient (n_H)were calculated based on equation 3 [40]:

$$log\frac{Q}{1-Q} = n_H log[P] - logK_d \quad (3)$$

where $Q = F/F_{max}$, is the fractional binding saturation, fraction of sites occupied with the ligand, $[P]$ is the protein concentration.

Sensing parameters as detection limit (DL), quantification limit (QL) and sensitivity (S) were also calculated from fluorescence protein assays and based in equation 4 and 5 [31]:

$$DL = \frac{3\sigma}{k} \quad (4)$$

$$QL = \frac{10\sigma}{k} \quad (5)$$

where σ is the standard deviation of blank, k is the slope between the fluorescence intensity *versus* protein concentration.

Biological activity assays: Minimum Inhibitory Concentration (MIC) of growth for the synthesized dyes **5a**,**b** and **11a**,**b** was determined against *Saccharomyces cerevisiae strain* PYCC 4072 using a broth microdilution method for the antifungal susceptibility assessment (M27-A3, CLSI–*Clinical and Laboratory Standards Institute*) [41]. PYCC 4072 cells were grown in YPD agar plates and a fresh culture was prepared for each experiment. The cells were cultivated in 96-well plates after dilution in Roswell Park Memorial Institute (RPMI) 1640 medium, buffered to pH 7.0 with 0.165 M morpholenepropanesulfonic acid (MOPS) buffer, in order to present an initial concentration of 2.25×10^3 cells/ mL. The stock solutions of the synthesized squaraine dyes were prepared in dimethyl sulfoxide (DMSO) at the concentration of 10 mM and a final dilution was carried out in RPMI 1640 medium (DMSO concentration of 0.5% per well, v/v), before each experiment. After adding the dye solutions, the microplates were subjected to two different conditions: without irradiation (protected from ambient light) and under 30 minutes of irradiation with an LED system centered at 640 or 660 nm, chosen according to the maximum dye absorption wavelength in RPMI medium, being subsequently incubated at 30 °C for 48 hours. Growth was assessed by measuring the absorbance at 640 nm in a microplate photometer (Molecular Devices SpectraMax Plus). The obtained values allowed the determination of MICs, which corresponds to the lowest concentration of dye that causes a growth inhibition of >80 %, when compared to a control. Five concentrations of each dye were tested, each in triplicate in at least two independent experiments.

LEDs systems: Two led systems with wavelength centered at 640 and 660 nm were used. The device with emission peak at 640 nm, measured using the Ocean Optics HR4000CG CCD spectrometer, were constructed using aluminium gallium indium phosphide (AlGaInP) and aluminium gallium phosphide (AlGaP) LEDs respectively, with clear epoxy lenses of 5 mm diameter and viewing angle of 30°. The radiant flux was measured to be P=7.3 ± 0.4 mW, operating at 20 mA using an UDP Instruments S350 Optometer coupled with a UDT Instruments S5124A sensor and a S2575 integrating sphere. For a 30-minute exposure time the fluence is 13.1 J/cm^2.The device was placed over the 96-well plates, with the LEDs facing the cells, and each LED illuminated a single well. The light system with a wavelength of 660 nm uses LEDs manufactured by Kingbright (model: L-53SRC-F) and are GaAlAs based emitters, with water clear lens type with the diameter of 5 mm and viewing angle of 30°. The radiant flux and irradiance were measured using a Thorlabs PM100USB power meter coupled to a S120C calibrated head, with an applied forward DC current of 20 mA and a distance between LED and detector of 1.5 cm. The measured value was 3.8 ± 0.3 mW for the radiant flux and 5.4 ± 0.5 mW/cm^2 for the irradiance. For a 30-minute exposure time the fluence for this LED system is 9.7 J/cm^2. A CCD spectrometer from Ocean Optics (model HR4000CG) was used to measure the spectral emission. Using the spectra data obtained for 10 LEDs, representative of the emitters assembled in the system; the spectral emission peak was found to be at 653 ± 1 nm, with a full width at half maximum (FWHM) of 21.1 ± 0.2 nm.

RESULTS AND DISCUSSION

Synthesis of squaraine dyes 5a,b and 11a,b

Four squaraine dyes derived from indolenine and benzo[*e*]indole containing an ester group or alkyl chains at the nitrogen atoms of heterocyclic rings, and groups derived from barbituric

and thiobarbituric acid in the central four-membered ring were successfully synthesized and their synthesis are schematically represented in Schemes 1 and 2, respectively. According to the knowledge of the authors, and as far as they were able to ascertain, dyes **5a**,**b** and **11a**,**b** are new, so their synthesis and complete spectroscopic characterization are detailed here for the first time.

The synthesis of dyes **5a** and **5b** (Scheme 1) begins with the preparation of quaternary ammonium salts 1-(2-carboxyethyl)-2,3,3-trimethyl-3*H*-indol-1-ium bromide (**3a**) or 1-(2-carboxyethyl)-2,3,3-trimethyl-1*H*-benzo[*e*]indol-1-ium bromide (**3b**) through the reaction of 2,3,3-trimethylindolenine (**1a**) or 1,1,2-trimethyl-1*H*-benz[*e*]indole (**1b**) with 3-bromopropionic acid (**2**). Then, the quaternary salts are subjected to a condensation reaction with squaric acid (**4**) in the presence of *n*-butanol and toluene at reflux using a Dean-Stark apparatus. During the condensation reaction, esterification of the chains occurred, which was confirmed by the analysis of ^{1}H and ^{13}C NMR spectra of dyes.

<Scheme 1>

The barbiturate squaraine dyes **11a** and **11b** (Scheme 2) were synthesized using a multistep procedure similar to the previously described by some of us [31, 32] for that type of dyes. In a first step and through an alkylation reaction between 1,1,2-trimethyl-1*H*-benz[*e*]indole (**1b**) and an excess of iodohexane in presence of acetonitrile, the quaternary ammonium salt **6** was obtained, which in turn reacting with dibutylsquarate (**7**), resulted from the reaction of squaric acid with *n*-butanol at reflux, allowed to obtain the monosubstituted intermediate **8**. The later, was reacted with the respective barbiturate derivative (**9a**,**b**) in the presence of ethanol and triethylamine giving rise to semisquaraines **10a** and **10b** that were used in the next step without prior purification. The final dyes **11a**,**b** were obtained by a condensation reaction between the intermediates **10a**,**b** and the quaternary ammonium salt **6** in a *n*-butanol/toluene (1:1 v/v) mixture and using a Dean-Stark system. In this way, the desired

dyes were obtained in the form of dark blue (**5a**,**b**) and dark green (**11a**,**b**) solids, with low and moderate yields, being subsequently subjected to respective characterization through standard spectroscopic methods. All the spectra are shown in the supporting material (Fig. S1-33).

By analyzing the ^{1}H NMR spectra it is possible to verify that dye **5a** presents the aromatic protons of the heterocyclic ring, in the form of duplets and triplets at δ 7.08-7.35 ppm, while dye **5b** present the signals at δ 7.42-8.20 ppm, in the form of duplets, triplets and a multiplet. Squaraine dyes **11a** and **11b**, where the difference between them is based only on the presence of an oxygen and sulfur atom attached to the barbiturate group, present the aromatic protons signals at exactly the same shift, at δ 7.50-8.30 ppm. Table 1 summarizes the most relevant ^{1}H and ^{13}C signals. The methine protons of this class of dyes emerge in the form of singlets at approximately δ 6.00 ppm. For **5a**, this signal appears at δ 5.93 ppm, however when we replace the heterocycles derived from indolenine to heterocycles derived from benzo[*e*]indole (**5b**), the signal appears at a slightly higher chemical shift (δ 6.00 ppm). In the case of **11a** and **11b**, both derived from benzo[*e*]indole, the signal appears at a higher chemical shift compared to dyes **5a** and **5b**, which should be caused by the presence of the barbiturate group. Dye **11a**, whose signal arises at δ 6.56 ppm, and **11b** with the signal arise at δ 6.44 ppm, show a small difference in chemical deviations, which in this case may be justified by the greater electronegativity of the oxygen atom present in the molecule of dye **11a**. It is important to mention that, as expected, the signals of the methine groups only present a single signal, which is in accordance with the symmetrical character of the molecules presented.

The methylene protons bound to the nitrogen (NCH_2-), appear at δ 4.05 ppm for **5a**,**b**, while for **11a**,**b** this signal appears at δ 4.20 ppm. This shift can be justified by the fact that dyes **5a**,**b** have an ester group on the *N*-alkyl chain. The terminal methyl groups present in this

same chain emerged thereabout δ 0.87 ppm for compound **5a**,**b** and about δ 0.82 ppm for **11a**,**b**. Once again, the chain esterification in dyes **5a**,**b** will be the justification for this slight deviation observed.

The ^{13}C NMR spectra exhibited aromatic carbons in the form of four signals at δ 109.68-127.99 ppm, for dye **5a** and six signals at δ 110.31-129.84 ppm, at δ 111.66-129.94 ppm and at δ 111.68-129.96 ppm, for dye **5b** and **11a**,**b**, respectively. The existence of a single signal at about δ 90 ppm, related to the methine carbons (CH=C) confirms the symmetrical character of the synthesized dyes. The IR spectra of dyes **5a**,**b** and **11a**,**b** showed a signal near 1700 cm^{-1} resulting from asymmetric stretching vibrations of carbonyl groups present in all the dyes. In IR spectra of dyes **11a**,**b** the band relative to the stretching vibrations of N-H bonds of the barbituric group is observed near to 3400 cm^{-1}.

<Scheme 2>

<Table 1>

Photophysical studies

The fundamental photophysical studies of synthesized dyes are carried out in ethanol and PB (pH 7.3) and are presented in Figure 1 A-D and Table 2. The maxima absorption wavelength (λ_{abs}) of dyes **5a**,**b** and **11a**,**b** in both solvents are located in a range of 631-682 nm and the maximum emission wavelength (λ_{em}) lies between 636-728 nm, with low to moderate Stokes shifts ($\Delta\lambda$, 3–46 nm). The lowest absorption and emission values are assigned to dye **5a** in the two solvents tested. By comparing the results obtained for dye **5a** and **5b** it is possible to verify that the fusion of a benzene ring in the heterocyclic bases leads to a bathochromic shift in the maximum absorption wavelengths of 32 nm in ethanol and 49 nm in PB. Dyes **5b** and

11a, **b** shows very similar absorption and emission wavelengths despite their structural differences. In PB these three dyes have the same absorption wavelength (682 nm) while in ethanol the difference is 5 nm for **11a** and 4 nm for **11b** when compared to dye **5b**. It is possible to conclude that the barbituric groups present in dyes **11a** and **11b** slightly affect the absorption wavelengths, which are more affected by the introduction of the group derived from benzo[*e*]indole. The molar extinction coefficients vary in the range 3.37×10^4- 3.86×10^5 M^{-1} cm^{-1}, with dye **5a** showing the highest values in both solvents, followed by **5b**, **11b** and lastly the dye **11a**. The high molar absorptivity ($\varepsilon > 1\times10^5$ M^{-1} cm^{-1}) in organic solvent reveals the strong absorption of the four synthesized dyes at longer wavelengths (> 630 nm), which is an advantage for the use of these dyes as fluorescence probes.

The relative fluorescence quantum yields ranged from 10.3% to 80.7% in EtOH to <1%–6.3% in PB. Dye **5a** presents the highest fluorescence quantum yield in both solvents. The lowest value in EtOH is attributed to the dye **11a** (10.3%) and in PBS is assigned to dye **11b** (<1%). Comparing **5a** and **5b** dyes it is possible to verify that the structural difference between them leads to a decrease of 68 % in the fluorescence quantum yield in EtOH and 86 % in PB. For **11a** and **11b** dyes the difference in the values obtained in EtOH is almost insignificant, while in PBS there is a decrease of about 50 %, resulting from the replacement of an oxygen atom with a sulfur atom.

<Figure 1>

<Table 2>

Through the observation of Figure 1 it is possible to verify that in aqueous media the four dyes tend to form aggregates, which is evidenced by the presence of two slightly overlapping bands, one of which is relative to the monomer and the other to the formed aggregates. This trend is already widely reported in the literature [42-45], and is associated with the low solubility of squaraine dyes in aqueous media. The aggregates can be classified into *J*-

aggregates and *H*-aggregates, which is related to the type of alignment of the transition dipole moments on adjacent molecules [46, 47]. One way to determine the formed aggregates type is through the use of Triton X-100. This non-ionic surfactant will attenuate the formation of aggregates allowing to obtain an absorption spectrum in which the most evident band corresponds to the monomer band. The analysis of Figure 2 confirms the presence of *H*-aggregates for the four dyes since the solid lines related to the Triton X-100 tests reveal that the band at the higher wavelength is attributed to the monomer and the band with a blue shift of 38, 47, 45, 46 nm for dyes **5a**,**b** and **11a**,**b**, respectively, corresponds to the band characteristic of the *H*-aggregates. The type of aggregates formed also allows us to conclude that the dye molecules establish covalent bonds, acquiring a side-by-side orientation with crossed dipoles [48, 49]. This aggregation behavior of the dyes also justifies the low molar extinction coefficients obtained as well as the low fluorescence quantum yields.

One of the squaraine dyes family characteristics is their photoactivation capacity, being necessary an efficient light source with a wavelength that falls within the absorption range of the dyes in the medium to be used. To build the most appropriate LED system for cell irradiation, spectra of the synthesized dyes in RPMI media was recorded (Figure 1 E). The absorption pattern is similar to that observed in PB, having broader absorption bands also associated with the formation of aggregates, with the monomer band appearing at a slightly longer wavelength.

<Figure 2>

Photostability analysis

Photostability refers to the effect that light has on a substance. Light can cause the photodegradation of a drug and eventually lead to the loss or alteration of its active principle, to the reduction of its potency and effectiveness and also to the formation of degradation

products of high toxicity, causing adverse effects at the biological level [50-52]. Monitoring this parameter is extremely important in order to ensure that the cytotoxic effect produced is caused only by the molecule and not by products from photodegradation [53].

To assess photostability, solutions of the synthesized dyes were prepared in DMSO with a concentration of 1 mM and then diluted in PB in order to obtain a concentration of 25 μM. Each solution was irradiated with a 150W lamp with emission in the UV/vis region for 20 minutes, with an absorption spectrum being obtained every minute.

All dyes showed a photostability lower than that observed for MB (Figure 3). Dye **11b** showed high photostability, with a pattern similar to that obtained with MB blue, followed by compound **5a** which showed a decrease in this parameter of about 20% at the end of 20 minutes of irradiation. Compounds **5b** and **11a** showed poor light- stability, being degraded almost entirely, resulting from this photodegradation process the loss of the compounds' ability to emit coloration, called photofading.

Comparing dyes **5a** and **5b** it is possible to conclude that the dye derived from 1,1,2-trimethyl-1*H*-benz[*e*]indole has a poor stability to this physical agent while the indolenine derivative has a good response to light. These are in agreement with previously reported studies, which demonstrate that indolenine derivatives generally have good photostability [54, 55]. Structurally, dyes **11a** and **11b** differ only in the presence of an oxygen atom and a sulfur atom, respectively. However, the results revealed that the presence of the sulfur atom confers a high photostability to the dye while the oxygen atom makes the dye very photounstable.

<Figure 3>

Generation of singlet oxygen

Dye's ability to generate singlet oxygen has been qualitatively evaluated in comparison with MB using DPBF. DPBF is a probe which, when react with singlet oxygen, loses its extended π-electron system, forming a *o*-dibenzoylbenzene derivative, a compound without capacity to absorb Vis light [56, 57]. The decomposition of DPBF can be followed by monitoring the absorbance at about 410 nm, proceeding simultaneously to irradiation with a LED system. The DPBF assay was qualitatively performed in DMSO and PB in order to mimic the physiological conditions that dyes were subjected in the biological tests. The decrease in the absorption of DPBF in DMSO as a function of irradiation time (Figure 4A) confirmed the excellent singlet oxygen production capacity of MB [58], with a complete degradation of the DPBF after about 40 s of irradiation. In DMSO, all the dyes evaluated showed an activity lower than MB, with compound **5b** presenting the best result, with a degradation of DPBF of about 50%, after 650 s of irradiation. The other dyes led to an DPBF absorbance decrease of about 30%. A marked decrease in absorbance was observed in the first 20 s of irradiation caused by dye **11a**, and then this decrease was less accentuated in the remaining irradiation time. Comparing dyes **5a** and **5b** it is possible to verify that in DMSO the introduction of heterocyclic bases derived from benzo[*e*]indole is advantageous in terms of singlet oxygen production. Dyes **11a** and **11b** showed a similar result demonstrating that replacing an oxygen atom with a sulfur atom does not cause significant changes.

In aqueous medium, the results revealed that the solvent used interferes with the ability to produce singlet oxygen (Figure 4B). In this medium, MB showed a lower photosensitizing capacity, being surpassed by dye **5a**, which led to a degradation of about 80% of the DPBF. The superior oxygen production capacity of squaraine dyes compared to MB has already been reported by other authors [26, 55, 59]. Dyes **5a** and **11a**,**b** did not undergo significant changes in their activity, showing a similar behavior in DMSO and in PB after 650 s of irradiation. However, it is important to mention that in PB, at 100 s of irradiation, a

degradation of about 20% of the DPBF was observed for dyes **5b** and **11b**, while in DMSO, after the same irradiation time, the degradation value was about 10%.

<Figure 4>

HSA interaction studies

Human serum albumin is the major protein in human blood plasma [60]. This protein plays an essential role in the maintenance of several metabolic processes, such as the regulation of plasma oncotic pressure, the decrease in the activity of some toxins, the control of the antioxidant properties of the plasma, the transport of some drugs, among others [61, 62]. Changes in the concentration of HSA in biofluids such as saliva, urine and serum are generally associated with serious disease states such as liver damage, kidney failure, diabetes and cardiovascular diseases, so methods that allow its rapid detection and quantification are of extreme relevance in clinical diagnosis [63-65].

The interaction of synthesized dyes with HSA was evaluated through the application of a protocol that allows to increase the concentration of protein (0-3.5 μM) and maintain the concentration of dye (2 μM).

As previously mentioned, the synthesized dyes, as well as other dyes of the same family already reported in other studies [31, 32], tend to form non-fluorescent aggregates in an aqueous medium. This fact leads the synthesized dyes to present, in buffer solution, a very low fluorescence intensity. However, after addition of HSA, a significant increase in fluorescence intensity is observed, which reveals that a dye-protein complex is formed in which interactions are established through hydrophobic, electrostatic and hydrogen bonds [66].

By analyzing Figure 5, it is possible to verify that the four studied dyes increase their fluorescence intensity due to the interaction with the protein and this variation is directly

644 proportional to the protein concentration, presenting a linear correlation, with a square of
645 correlation coefficient (R^2) very close to unity (Inset graphs in Figure 5).

646 Dye **5a** showed the most expressive response with a 43-fold increase in fluorescence
647 intensity, followed by dye **5b** with a 15-fold increase. Despite demonstrating an almost
648 insignificant increase in fluorescence emission, dyes **11a**,**b** also show a linear variation
649 between fluorescence intensity and protein concentration.

650 Emission spectra in the absence and presence of 3.5 μM of protein (Figure S34) reveal
651 that shifts in emission wavelengths occur upon addition of protein. Dye **5a** undergoes a
652 bathochromic shift of 15 nm, while other dyes show a hypsochromic shift of 43 nm, for dyes
653 **5b** and **11a**, and 51 nm for dye **11b**. These deviations prove the existence of strong
654 interactions between the dyes and the protein.

655 <Figure 5>

656 As previously discussed, dyes show low fluorescence quantum yields due to their high
657 tendency to form non-fluorescent aggregates in aqueous media. However, after interaction
658 with HSA there is an increase in the value of this parameter, visible in all tested dyes (Table
659 3), that suggesting the formation of a SQ-BSA/ HSA fluorescent complex. The increase is
660 more pronounced for **5a**, which increases the fluorescence quantum yield to a value greater
661 than 100%. The remaining dyes showed a not so pronounced increase, but which reveal the
662 existence of an interaction between the dyes and the biomolecule used.

663 <Table 3>

664

665 As reported for other squaraine dyes [32, 67], the fluorescence quantum yields of the dyes
666 in PB presenting similar values and in the same order of magnitude, are insignificant when
667 compared to the fluorescence quantum yields after interaction with HSA. After the

668 interaction with the protein, variations in the results obtained are verified as a consequence
669 of the different structural variations that in turn lead to different dye-protein interactions.
670 Comparing the dyes derived from indolenine (**5a**) and benzo[*e*]indole (**5b**) with an ester
671 group in the *N*-alkyl chains it is verified that the introduction of a ring in the heterocyclic
672 bases does not favor the interaction of the dye with the HSA. By comparing the dyes **11a**,**b**
673 with their indolenine-derived counterparts [31], it is possible to verify that, once again, the
674 benzo[*e*]indole-derived dye did not prove to be advantageous in terms of increased
675 fluorescence intensity after interaction with HSA. These results are in agreement with other
676 published studies that proved that dyes derived from indolenine are the most suitable for use
677 as fluorescent probes [30, 54, 68, 69].

678 Using equation 2, the Benesi-Hildebrand graphs depicted in Fig. 6 were plotted. The
679 visibly non-linear trend observed for dyes **11a** and **11b** suggests a 1:2 complexation between
680 fluorophore and protein. In contrast, dyes **5a** and **5b** show a good linear correlation which
681 may indicate a 1:1 complexation. Based on the same graphs, the binding constants were also
682 determined (Table 3), with values with an order of magnitude of 10^5 being obtained for all
683 dyes, which is speculative of the occurrence of an intercalative binding between the dye and
684 the protein [70]. The K_b values obtained are very close to each other indicating that all dyes
685 have similar affinity for HSA.

686 <Figure 6>

687

688 An attempt was made to determine the dissociation constant and the Hill constant through
689 equation 3. The graphs obtained (Fig. 7) reveal a non-linear trend for all evaluated dyes. As
690 already reported in a previously published article by some of us, this fact is due to the
691 different affinity of the dyes for the different binding sites of the protein. More simply, the
692 dye first binds to the site with the highest affinity and will only bind to other sites after the

693 first one is saturated, always in order of affinity [40]. In these cases, it is described that an
694 estimation of these constants for the highest affinity binding site can be performed using the
695 end of the non-linear plot for small values of HSA concentration while using the other end
696 of the plot (of HSA concentrations plus high) the constant value for the site of least affinity
697 is obtained [71]. The interactions between the dye and the protein are of greater complexity,
698 which would lead to obtaining non-coherent values because variations in the value of the
699 constants can occur due to changes in the interaction between the different binding sites and
700 the dye after binding and for this reason these parameters have not been determined.
701 Although it is not possible to determine these constants, this fact allows for a better
702 understanding of the type of interactions that occur between the dye and the HSA.

703 <Figure 7>

704

705 The linearity observed between fluorescence intensity and HSA concentration (Inset graph
706 in Figure 5) allow the determination of parameters such as detection limit, quantification
707 limit and sensitivity (Table 3), essential for the validation of a quantitative method. Based
708 on the results obtained, it is possible to verify that dyes **5a**,**b** have lower values than those
709 obtained for compounds **11a**,**b** which indicates that they will have a more effective response
710 to variations in the concentration of HSA, bringing together better conditions for their
711 application as fluorescent probes for the detection of the protein under study.

712 <Table 4>

713

714 **Biological activity of squaraine dyes 5a**,**b and 11a**,**b**

715 Using the yeast *Saccharomyces cerevisiae* PYCC 4072 strain as a model organism and a
716 broth microdilution method [72, 73], the potential antifungal activity investigation of the

717 synthesized dyes **5a**,**b** and **11a**,**b** was carried out. Table 4 shows the values of the MIC in the
718 two experimental conditions tested and the logarithms of the partition coefficient of the
719 synthesized dyes (LogP)**,** which were theoretically predicted [74]. The tested dyes exhibited
720 antiproliferative activity with MIC values of 50 and 100 μM, with exception of **5a** (MIC >
721 100 μM), which could not be tested at higher concentrations than 100 μM due to its limited
722 solubility. Dye **5a** has a MIC > 100 μM whereas for dye **5b** this value is 50 μM, which allows
723 us to conclude that the substitution of the heterocyclic bases derived from indolenine with
724 bases derived from benzo[*e*]indole increases the antifungal capacity. With regard to dyes **11a**
725 and **11b**, it can be seen that the presence of a group derived from barbituric or thiobarbituric
726 acid does not cause any change in the antifungal activity of the dyes, since both dyes have
727 an equal MIC value (100 μM).
728 In order to increase the antifungal capacity of the synthesized dyes, tests were carried out
729 using a radiation system with an appropriate wavelength. Considering the absorption
730 wavelength of the dyes in RPMI, a LED system with emission at 640 nm was used for **5a**
731 and a system with emission at 660 nm in the tests of dyes **5b**, **11a** and **11b**. Dye **5a** did not
732 show any response to irradiation while **5b** and **11a**,**b** improve their antifungal response when
733 irradiated for 30 minutes before incubation. Dye **11a** showed the most significant decrease
734 in MIC value, going from 100 μM to 25 μM. Dye **5b** showed the best antifungal activity in
735 the tests performed in the absence of light and when subjected to the radiation reduced its
736 MIC value by half, presenting the same value as that obtained for dye **11a** (25 μM).
737 Irradiation also improved the performance of dye **11b** which lowered its MIC value to half
738 that obtained in the dark. The calculated Log P of the dyes, ranged from 1.70 to 5.96 and
739 corresponds to an estimated measure of the dye's hydrophobicity. Dyes with smaller Log P
740 values are more soluble in water while dyes with higher values have greater affinity for
741 cellular membrane systems. The low Log P value obtained for **5a** is compatible with its weak

742 antifungal activity. It was expected that the greater biological activity would be attributed to
743 dye **11b**, however, this was not verified, which may be associated with the deficient solubility
744 of the dye in an aqueous medium that constitutes the cellular environment, as already
745 reported by Lima *et al.* [26].

746 <Table 5>

747 It is important to mention that three tested dyes showed an MIC value equal or lower than
748 the fluconazole and miconazole, two reference antifungal compounds, with MIC´s of 50 and
749 100 μM, respectively [75].

750

751 # CONCLUSIONS

752 Four squaraine dyes with different modifications at the level of the central ring derived from
753 squaric acid and the *N*-chains were successfully synthesized. Fundamental photophysics
754 characterization of these dyes revealed absorption and emission bands in the visible and near
755 infrared region (631-728 nm). In ethanol the bands are narrow and intense, however when in
756 aqueous media there is a widening of the absorption bands which is an indicator of the typical
757 aggregation tendency of this polymethine dyes. A simple test using Triton X-100 showed
758 that the four dyes form *H*-aggregates, which reveals that in aqueous media the dyes
759 molecules are oriented in a side-by-side position.
760 The interaction studies with HSA showed that all dyes, in response to the interaction with
761 HSA, increased their fluorescence intensity, with this response showing an increase
762 proportional to the protein concentration. The best response was given by dye **5a** in which it
763 was possible to observe a 43-fold increase in fluorescence emission. Binding constants were
764 also determined which, due to their high value, allow us to conclude that there are strong
765 interactions between the synthesized dyes and HSA. The values of the sensing parameters

validating the possible use of these dyes, specially dyes **5a** and **5b**, as a method to detect and quantify of the protein under study.

Regarding the studies carried out with *Saccharomyces cerevisiae*, MIC values between 50 - >100 μM were obtained in the dark tests. After using the irradiation system, in order to photoactivate the dyes, it was found that dyes **5b** and **11a**,**b** decrease in this value, demonstrating better antifungal activity when irradiated. All tested dyes exhibited considerable antiproliferative activity.

Tests using DPBF allowed to verify the ability of dyes to generate singlet oxygen. All dyes showed a good ability to generate this reactive oxygen species, however it was not possible to relate this parameter with the results obtained in terms of antifungal activity. The non-correlation of these results may be related to the different solubility of the dyes in the cell medium and in their distribution in different cell structures.

In general, regarding the possible application of dyes as fluorescent probes for the detection of HSA, dye **5a**, derived from indolenine, was the most promising one. In potential application as an antifungal agent, the best result without irradiation is attributed to compound **5b**. However, after photoactivation, dyes **5b** and **11a** had the same MIC value, with compound **11a** showing a greater increase in its antiproliferative capacity after irradiation.

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SUPPORTING MATERIAL

In the supporting material the ^{1}H NMR, ^{13}C NMR, DEPT-135 and HRESI-TOFMS spectra are presented, as well as emission spectra of the dyes in the presence and absence of HSA.

Figure S1. ^{1}H NMR spectrum of intermediate **3a** (600 MHz, DMSO-d_6, ppm).

Figure S2. ^{13}C NMR spectrum of intermediate **3a** (150.9 MHz, DMSO-d_6, ppm).

Figure S3. DEPT-135 spectrum of intermediate **3a** (150.9 MHz, DMSO-d_6, ppm).

Figure S4. HRESI-TOFMS spectrum of intermediate **3a.**

Figure S5. ^{1}H NMR spectrum of intermediate **3b** (600 MHz, DMSO-d_6, ppm).

Figure S6. ^{13C} NMR spectrum of intermediate **3b** (150.9 MHz, DMSO-d_6, ppm).

Figure S7. DEPT-135 spectrum of intermediate **3b** (150.9 MHz, DMSO-d_6, ppm).

Figure S8. HRESI-TOFMS spectrum of intermediate **3b.**

Figure S9. ^{1}H NMR spectrum of dye **5a** (600 MHz, $CDCl_3$, ppm).

Figure S10. ^{13}C NMR spectrum of dye **5a** (150.9 MHz, $CDCl_3$, ppm).

Figure S11. DEPT - 135 spectrum of dye **5a** (150.9 MHz, $CDCl_3$, ppm).

Figure S12. HRESI-TOFMS spectrum of dye **5a.**

Figure S13. ^{1}H NMR spectrum of dye **5b** (600 MHz, $CDCl_3$, ppm).

Figure S14. ^{13}C NMR spectrum of dye **5b** (150.9 MHz, $CDCl_3$, ppm).

Figure S15. DEPT-135 spectrum of dye **5b** (150.9 MHz, $CDCl_3$, ppm).

Figure S16. HRESI-TOFMS spectrum of dye **5b.**

Figure S17. ^{1}H NMR spectrum of intermediate **6** (400 MHz, $CDCl_3$, ppm).

Figure S18. ^{13}C NMR spectrum of intermediate **6** (100.6 MHz, $CDCl_3$, ppm).

Figure S19. DEPT-135 spectrum of intermediate **6** (100.6 MHz, $CDCl_3$, ppm).

Figure S20. ^{1}H NMR spectrum of intermediate **8** (400 MHz, $CDCl_3$, ppm).

Figure S21. ^{13}C NMR spectrum of intermediate **8** (100.6 MHz, $CDCl_3$, ppm).

Figure S22. DEPT-135 spectrum of intermediate **8** (100.6 MHz, $CDCl_3$, ppm).

Figure S23. HRESI-TOFMS spectrum of intermediate **8**.

Figure S24. ^{1}H NMR spectrum of dye **11a** (600 MHz, $CDCl_3$, ppm).

Figure S25. ^{1}H NMR spectrum of dye **11a** (400 MHz, DMSO-d_6 + D_2O, ppm).

Figure S26. ^{13}C NMR spectrum of dye **11a** (150.9 MHz, DMSO-d_6, ppm).

Figure S27. DEPT-135 spectrum of dye **11a** (150.9 MHz, DMSO-d_6, ppm).

Figure S28. HRESI-TOFMS spectrum of dye **11a**.

Figure S29. ^{1}H NMR spectrum of dye **11b** (400 MHz, DMSO-d_6, ppm).

Figure S30. ^{1}H NMR spectrum of dye **11b** (400 MHz, DMSO-d_6 + D_2O, ppm).

Figure S31. ^{13}C NMR spectrum of dye **11b** (150.9 MHz, DMSO-d_6, ppm).

Figure S32. DEPT-135 spectrum of dye **11b** (150.9 MHz, DMSO-d_6, ppm).

Figure S33. HRESI-TOFMS spectrum of dye **11b.**

Figure S34. Emission spectra of synthesized dyes **5a**,**b** and **11a**,**b**, A-D, respectively, in absence (solid lines) and presence (dash lines) of HSA at a concentration of 3.5 μM.

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TABLES

Table 1. Relevant ^{1}H and ^{13}C NMR spectra signals of **5a**,**b** and **11a**,**b**.

Dye	^{1}H NMR				^{13}C NMR
	C<u>H</u>=C	N<u>H</u>	NC<u>H</u>$_2$-	R-COO-C<u>H</u>$_2$-	<u>C</u>H=C
5a	5.93 (2H, s)	-	4.05	4.34 (4H, bs)	86.93
5b	6.00 (2H, s)	-	4.05	4.47 (4H, bs)	86.67
11a	6.56 (2H, s)	10.00 ((2H, s)	4.20	-	93.35
11b	6.44 (2H, s)	11.26 (2H, s)	4.20	-	93.19

Table 2. Photophysical data of dyes **5a**,**b** and **11a**,**b** in ethanol and PB (λ_{exc} 580 nm, excitation and emission slits 10 nm).

Dye	Solvent	λ_{abs} (nm)	λ_{em} (nm)	$\Delta\lambda$ (nm)	ε (M^{-1} cm^{-1})	Φ_F (%)
5a	EtOH	631	639	8	3.86×10^5	80.7
	PB	633	636	3	7.94×10^4	6.3
	RPMI	647	-	-	-	-
5b	EtOH	663	672	9	3.25×10^5	25.7
	PB	682	715	33	6.82×10^4	1
	RPMI	625/691	-	-	-	-
11a	EtOH	668	686	18	1.78×10^5	10.3
	PB	682	726	44	3.37×10^4	1.3
	RPMI	637/689	-	-	-	-
11b	EtOH	667	685	18	1.87×10^5	10.9
	PB	682	728	46	4.36×10^4	<1
	RPMI	632/687	-	-	-	-

Table 3. Absorption and fluorescence data of squaraine dyes **5a**,**b** and **11a**,**b** in PB, in the presence of HSA (3.5 μM).

Dye	HSA presence			
	λ_{abs} (nm)	λ_{em} (nm)	$\Delta\lambda$ (nm)	Φ_F (%)
5a	642	647	5	>100
5b	675	681	6	9.5
11a	686	690	4	7.7
11b	684	687	3	2.6

Table 4. Binding constant (K_b), detection limit (DL), quantification limit (QL) and sensitivity (S) obtained for interaction of squaraine dyes with HSA.

Dyes	K_b (M)	DL (nM)	QL (nM)	S (nM)
5a	3.00×10^5	128	427	1.63×10^5
5b	4.22×10^5	108	359	2.88×10^4
11a	4.38×10^5	202	673	4.36×10^3
11b	3.77×10^5	196	653	2.26×10^3

Table 5. Activity against *Saccharomyces cerevisiae* PYCC 4072 strain and Log P values of squaraine dyes **5a**,**b** and **11a**,**b**. MIC values are present in μM.

Dye	MIC (dark)	MIC (irrad)	Log P
5a	>100	>100	1.70
5b	50	25	4.02
11a	100	25	5.62
11b	100	50	5.96

SCHEMES

Scheme 1. Synthesis of squaraine dyes **5a** and **5b**. Conditions *i*) heated at 80 ˚C (**3a**) / 120 ˚C (**3b**); *ii*) *n*-butanol/toluene (1:1, v/v), Dean-Stark apparatus 6 h (**5a**) / 7 h (**5b**).

Scheme 2. Synthesis of barbiturate squaraine dyes **11a** and **11b**. Conditions: *i*) acetonitrile, reflux for 10 days; *ii*) *n*-butanol, reflux for 4 h; *iii*) Ethanol/triethylamine, r.t., overnight; *iv*) Ethanol/triethylamine, reflux 9 h (**10a**) / 6 h (**10b**), *v*) *n*-butanol, reflux 10 h (**11a**) / *n*-butanol/toluene (1:1, v/v), Dean-Stark apparatus, 4 h (**11b**).

FIGURE CAPTIONS

Figure 1. Absorption (solid lines) and emission spectra (dash lines) in ethanol (−) and PBS (−) of squaraine dyes **5a**,**b** and **11a**,**b**, A, B, C and D, respectively. E - Absorption spectra of synthesized dyes in RPMI.

Figure 2. Normalized absorption spectra of synthesized dyes **5a**,**b** and **11a**,**b** in PBS, in absence (dash lines) and in presence (solid lines) of Triton X-100.

Figure 3. Photostability evaluation of squaraine dyes **5a**,**b**, **11a**,**b** in PBS, using MB as standard reference. Dye solutions were irradiated continuously for 20 minutes with a 150 W lamp with an emission in the UV/Vis range and an absorption spectrum was recorded every minute.

Figure 4. Qualitative evaluation of the singlet oxygen generation capacity of dyes **5a**,**b**, **11a**,**b** in DMSO (**A**) and in PBS (**B**) using methylene blue (MB) as standard reference. Experiments were performed in quadruplicate and data are presented as mean ± standard deviation.

Figure 5. Fluorescence spectra of dyes **5a**,**b** and **11a**,**b** upon addition of increasing amounts of HSA (0-3.5 μM) in PB solution, after one hour of incubation. Inset: Plot of variation of maximum fluorescence intensity as a function of protein concentration with the respective

R^2. Data presented are relative to the mean ± standard deviation of three independent assay. λ_{exc}=580 nm, excitation and emission slits with 10 nm and 20 nm bandwidth, respectively for **5b** and **11a**,**b** and excitation and emission slits with 5 nm and 10 nm bandwidth for **5a**.

Figure 6. Benesi-Hildebrand plot obtained for the interaction of squaraine dyes **5a**,**b** and **11a**,**b** with HSA.

Figure 7. Hill's plots obtained for interaction of **5a**, **5b**, **11a** and **11b** with HSA.