Dyes and Pigments 90 (2011) 41–47

Contents lists available at ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

Aza-substituted squaraines for the fluorescent detection of albumins

Kateryna D. Volkova [a], Vladyslava B. Kovalska [a,*], Mykhaylo Yu. Losytskyy [a], Lucinda V. Reis [b], Paulo F. Santos [b], Paulo Almeida [c], Daniel E. Lynch [d], Sergiy M. Yarmoluk [a]

[a] Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, 150 Zabolotnogo St., 03143 Kyiv, Ukraine
[b] Department of Chemistry and Centro de Química – Vila Real, Universidade de Trás-os-Montes e Alto Douro, 5001-801 Vila Real, Portugal
[c] Department of Chemistry and Unidade de I&D de Materiais Têxteis e Papeleiros, Universidade da Beira Interior, 6201-001 Covilhã, Portugal
[d] Exilica Limited, The Technocentre, Puma Way, Coventry CV1 2TT, UK

ARTICLE INFO

Article history:
Received 18 June 2010
Received in revised form
3 November 2010
Accepted 8 November 2010
Available online 18 November 2010

Keywords:
Squaraine dyes
Fluorescent detection
X-ray analysis
Albumins detection

ABSTRACT

Three benzothiazole substituted squaraines bearing hydroxyethylamino, aminoethylamino and 3-iodobenzylamino substituents on the cyclobutane unit have been synthesized and characterized. The structure of the hydroxyethylamino substituted analogue was determined by X-ray crystallography. The fluorescent properties of the new dyes plus those of seven previously reported aza-substituted squaraines were studied in both pure solution as well as in the presence of human, bovine and horse serum albumins, and ovalbumin. All of the dyes demonstrated low fluorescence emission levels in pure solution, and fluorescence intensity increases of up to 400 times in the presence of albumins. Dyes with an aminoethylamino and *N*,*N*-dimethylhydrazino substituents demonstrated good emission intensities in complexes with albumins, with fluorescence quantum yields of the latter dye reaching 0.18. The aminoethylamino substituted dye demonstrated a wide human serum albumin detection range (1.5 μg/mL to 20 mg/mL of protein). Studies of the dyes in the presence of model sodium dodecyl sulphate and bovine serum albumin – sodium dodecyl sulphate systems have shown that these squaraines do not give a considerable fluorescent response in the presence of denatured proteins or surfactant, with the exception of the 2-sulfoethylamino aza-substituted dye.

1. Introduction

Albumins are the major plasma proteins circulating in the bloodstream and are produced in the liver [1]. They play a significant role in the maintenance of colloid osmotic pressure and the binding of long-chain fatty acids, bile acids, bilirubin, haematin, calcium and magnesium. They also act as carriers for nutritional factors and drugs. Because serum albumin is a reliable prognostic indicator for morbidity and mortality, liver disease, kidney disease and malnutrition, analytical study methods are in high demand. Up to date fluorometric methods have been proposed for the detection and study of albumins. These have advantage over other methods because of their high sensitivity, selectivity and convenience [2,3]. Currently, commercially available fluorescent dyes such as eosin B and eosin Y [4], Albumin Blue [5] and Nano Orange are used for protein determination in solution. Interest in squaraine compounds has been recently renewed, due to their potential usefulness in a large number of technologically relevant fields such as NIR-emitting fluorescent probes. Squaraine dyes have been reported as efficient noncovalent labels for albumins [6], exhibiting high quantum yields when bound to these proteins [7].

Previously, we studied a series of benzothiazole and benzoselenazole squaraines as fluorescent probes for protein detection. For squaraine dyes with *N*-hexyl pendent groups, about a 100 to 540 fold fluorescence intensity increase upon albumin addition was observed. Amongst the series of dyes examined the aza-substituted compounds, possessing simple *N*-methylamino and *N*,*N*-diethylamino substituents on the central four-membered ring, displayed pronounced enhancement of emission intensity, observed in the presence of human (HSA) and bovine (BSA) serum albumins. Thus, benzothiazole dye **6** with a *N*,*N*-diethylamino substituent on the squaraine ring allows quantification of HSA in the range from 0.2 μg/mL to 500 μg/mL, which is comparable with commercially used dyes such as Coomassie Brilliant Blue and Pyrogallol Red Protein [8,9]. Since the presence of an amine moiety in the framework of the dye embodies a diversity of possible specific interactions between the dye and the protein, conditioning their mutual affinity, it seemed worthwhile to study the effect that differently substituted amine groups had on the fluorescent properties of aza-substituted squarylium dyes in the presence of proteins.

* Corresponding author. Tel./fax: +380 44 252 24 58.
E-mail address: v.b.kovalska@imbg.org.ua (V.B. Kovalska).

0143-7208/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dyepig.2010.11.005

As a continuation of these studies, we synthesized a range of new aza-substituted benzothiazole squaraine dyes (**2**, **3** and **10**, Fig. 1) and characterized them by IR, UV/Vis, ^{1}H NMR, ^{13}C NMR spectroscopy and HRFABMS (High Resolution Fast Atom Bombardment Mass Spectrometry). For dye **2**, crystals were obtained and the structure was solved and confirmed using X-ray crystallographic analysis. A series of ten new, as well as previously reported [10], aza-substituted benzothiazole and benzoselenazole squaraines (Table 1) was studied as probes for the detection of various albumins. The fluorescent properties of dyes when unbound and in the presence of human (HSA), bovine (BSA) and horse (HRSA) serum albumin, and ovalbumin (OVA) were characterized. The sensitivity of dyes to denatured proteins – BSA/SDS mixture – and thus their applicability for non-specific detection of proteins was also evaluated. The dependence between dye molecule structure and its selectivity towards certain protein was analyzed.

2. Materials and methods

2.1. Synthesis

All reagents were purchased from Sigma-Aldrich and were used without further purification. Solvents were of analytical grade. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm Al-backed silica gel plates (Merck 60 F_{254}). Melting points (M.p.) were measured in open capillaries on a Büchi-530 melting-point apparatus and are uncorrected. IR spectra were recorded on a Unicam Research Series FTIR spectrophotometer. UV/Vis spectra were performed on a Spectronic Genesys 2PC instrument. ^{1}H and ^{13}C NMR spectra were recorded on either Bruker ARX 300 or Bruker ARX 400 spectrometers; δ in ppm relative to $SiMe_4$ or to residual solvent signals. HRFABMS were obtained on a Micromass AutoSpec M spectrometer, operating at 70 eV, using a matrix of 3-nitrobenzyl alcohol (3-NBA).

2.1.1. 3-Hexyl-2-[3-(3-hexyl-3H-benzothiazol-2-ylidenemethyl)-2-(2-hydroxyethylamino)-4-oxocyclobut-2-enylidenemethyl] benzothiazol-3-ium trifluoromethanesulfonate (**2**)

To a solution of the *O*-methylsquaraine dye **1** [10] (0.43 g, 0.61 mmol) in anhydrous CH_2Cl_2 (10.0 mL), under N_2 atmosphere, was added an excess of 2-aminoethanol (0.048 mL, 0.79 mmol). The reaction mixture was stirred at r.t. for 30 min and then concentrated by partial removal of the solvent under reduced pressure. Addition of Et_2O yielded a solid, which was collected by filtration under reduced pressure and recrystallized from CH_2Cl_2/Et_2O. Yield: 68%. Purple crystals. M.p. 162.5–163.5 °C. UV/Vis (MeOH/CH_2Cl_2, 99/1) λ_{max} (log ε): 654 (5.30). IR (KBr) ν_{max}: 3463w, 2928w, 2858w, 1628w, 1568w, 1457s, 1354w, 1254s, 1155 m, 1028 m, 983w, 748w. ^{1}H NMR (400.13 MHz, DMSO-d_6) δ: 8.86 (1H, br s, N*H* or O*H*), 7.98 (1H, d, J = 7.7, Ar*H*), 7.92 (1H, d, J = 7.7, Ar*H*), 7.69 (1H, d, J = 8.2, Ar*H*), 7.64 (1H, d, J = 8.2, Ar*H*), 7.55–7.47 (2H, m, Ar*H*), 7.39–7.30 (2H, m, Ar*H*), 6.22 (1H, s, C*H*=C), 5.95 (1H, s, C*H*=C), 5.17 (1H, br s, N*H* or O*H*), 4.31–4.26 (4H, m, NC*H*$_2$(CH$_2$)$_4$CH$_3$), 3.68 (4H, br s, NHC*H*$_2$C*H*$_2$OH), 1.72 (4H, br s, NCH$_2$C*H*$_2$(CH$_2$)$_3$CH$_3$), 1.39–1.29 (12H, m, N(CH$_2$)$_2$(C*H*$_2$)$_3$CH$_3$), 0.85 (6H, br t, J = 6.7, N(CH$_2$)$_5$C*H*$_3$). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ: 173.6, 163.7, 161.0, 159.4, 157.1, 155.7, 140.6, 127.8, 127.6, 127.5, 124.9, 124.4, 122.9, 122.6, 113.4, 113.0, 87.2, 86.3, 60.7, 46.3, 45.5, 30.9, 30.8, 27.2, 26.8, 25.7, 22.0, 21.9, 13.8. HRFABMS (3-NBA): 588.2709 ([M-CF_3SO_3]$^+$, $C_{34}H_{42}N_3O_2S_2^+$; calc. 588.2718).

2.1.2. 2-[2-(Aminoethylamino)-3-(3-hexyl-3H-benzothiazol-2-ylidenemethyl)-4-oxocyclobut-2-enylidenemethyl]-3-hexylbenzothiazol-3-ium trifluoromethanesulfonate (**3**)

To a solution of the *O*-methylsquaraine dye **1** [10] (0.20 g, 0.28 mmol) in anhydrous CH_2Cl_2 (100.0 mL), under N_2 atmosphere, was added 1,2-diaminoethane (0.021 mL, 0.31 mmol). The reaction mixture was stirred at r.t. for 40 min. and then cooled in an ice-bath. Upon addition of Et_2O a solid precipitate, which was collected by filtration under reduced pressure and was recrystallized from CH_2Cl_2/MeOH/Et_2O. Yield: 84%. Purple crystals. M.p. 202.0 °C (dec.). UV/Vis (MeOH/CH_2Cl_2, 99/1) λ_{max} (log ε): 654 (5.26). IR (KBr) ν_{max}: 3480 w, 2929 w, 1633 w, 1562 w, 1511 w, 1427 s, 1357 m, 1238 s, 1155 m, 1133 m, 1027 m, 981 m, 827 w, 792 w, 748 w cm^{-1}. ^{1}H NMR (400.13, DMSO-d_6) δ: 7.99 (1H, d, J = 7.8, Ar*H*), 7.94 (1H, d, J = 7.8, Ar*H*), 7.71 (1H, d, J = 8.2, Ar*H*), 7.66 (1H, d, J = 8.2, Ar*H*), 7.56–7.49 (2H, m, Ar*H*), 7.40–7.32 (2H, m, Ar*H*), 6.18 (1H, s, C*H*=C), 5.91 (1H, s, C*H*=C), 4.37 (2H, br s, NHC*H*$_2$(CH$_2$)$_4$CH$_3$), 4.28 (2H, br s, NHC*H*$_2$(CH$_2$)$_4$CH$_3$), 3.67 (2H, t, J = 5.8, NHC*H*$_2$CH$_2$NH$_2$), 2.95 (2H, t, J = 5.8, NHCH$_2$C*H*$_2$NH$_2$), 1.73 (4H, br s, NCH$_2$C*H*$_2$(CH$_2$)$_3$CH$_3$), 1.39–1.29 (12H, m, N(CH$_2$)$_2$(C*H*$_2$)$_3$CH$_3$), 0.85 (6H, t, J = 6.6, N(CH$_2$)$_5$C*H*$_3$). ^{13}C NMR (100.62 MHz, DMSO-d_6) δ: 173.6, 163.4, 161.1 159.8, 157.1, 155.5, 140.6, 127.8, 127.7, 127.5, 125.4, 125.0, 124.6, 122.9, 122.6, 122.2, 119.0, 115.8, 113.5, 113.2, 86.6, 86.2, 46.4, 45.7, 30.9, 30.8, 27.2, 26.9, 25.7, 21.9, 13.8. HRFABMS (3-NBA): 587.2877 ([M-CF_3SO_3]$^+$, $C_{34}H_{43}N_4OS_2^+$; calc. 587.2878).

2.1.3. 3-Hexyl-2-[3-(3-hexyl-3H-benzothiazol-2-ylidenemethyl)-2-(3-iodobenzylamino)-4-oxocyclobut-2-enylidenemethyl] benzothiazol-3-ium iodide (***10***)

To a solution of the *O*-methylsquaraine dye **1** [10] (0.36 g, 0.50 mmol) and triethylamine (0.14 mL, 1.0 mmol) in anhydrous CH_2Cl_2 (20.0 mL), under N_2 atmosphere, was added an excess of 3-iodobenzylamine (0.33 mL, 2.5 mmol). The reaction mixture was stirred at r.t. for 3 h and then washed with cold water. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure. The resulting residue was dissolved in MeOH and to this solution was added an approximately equal volume of 14% aqueous KI. After about 2 h, the precipitated dye was collected by filtration, washed with water and

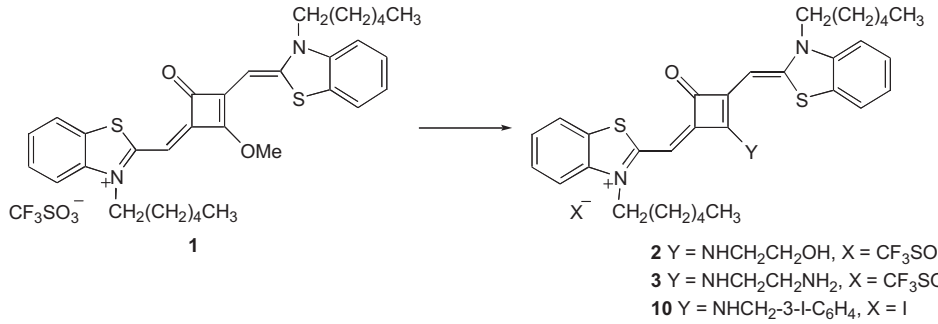
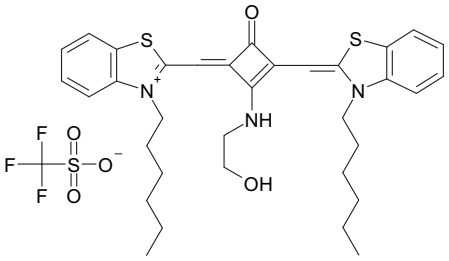
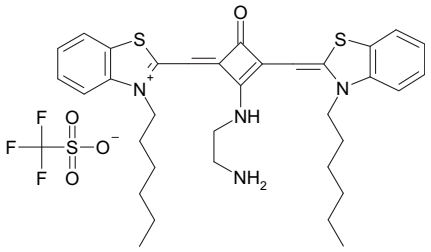


Fig. 1. Reagents and conditions. Dye **2**: $NH_2CH_2CH_2OH$, CH_2Cl_2, N_2, r.t.; dye **3**: $NH_2CH_2CH_2NH_2$, CH_2Cl_2, N_2, r.t.; dye **10**: 3-I-$C_6H_4CH_2NH_2$, Et_3N, CH_2Cl_2, N_2, r.t.

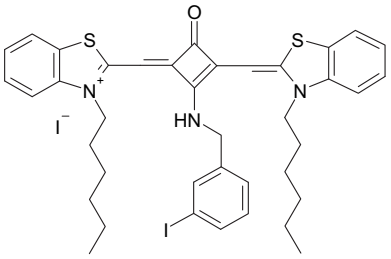
Table 1
Chemical structures of studied aza-substituted squaraine dyes.



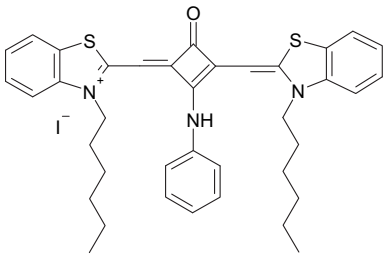
2



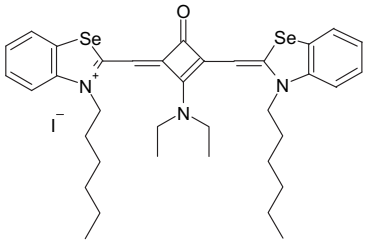
3



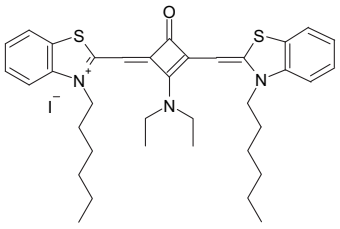
10



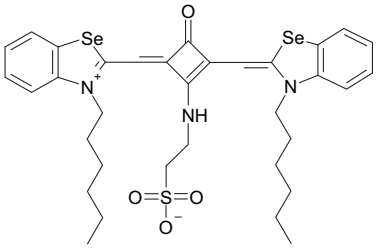
11



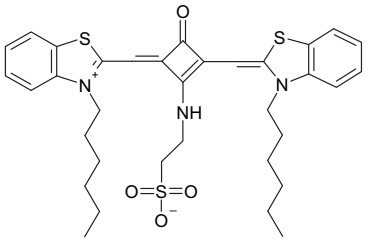
4



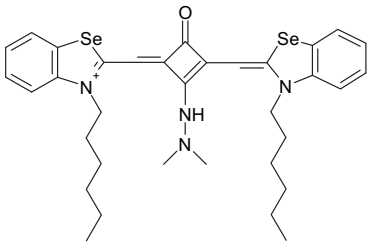
6



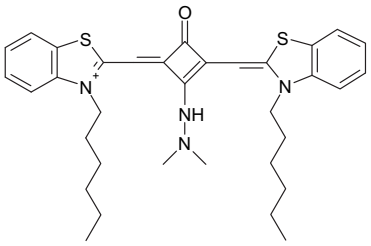
5



8



9



7

recrystallized from $CHCl_3$/MeOH/Et_2O. Yield: 67%. Blue crystals. M.p. 257.5 °C (dec.). UV/Vis (MeOH/CH_2Cl_2, 99/1) λ_{max} (log ε): 657 (5.37). IR (KBr) ν_{max}: 3130 w, 2962 w, 2920 w, 1634 w, 1562 m, 1458 s, 1431 s, 1353 m, 1238 s, 1178 m, 1155 m, 1134 m, 973 w, 806 w, 755 w. ^{1}H NMR (300.13 MHz, $CDCl_3$/DMSO-d_6) δ: 9.18 (1H, t, J = 6.1, N*H*, exchanging with D_2O), 7.90 (1H, d, J = 7.8, Ar*H*), 7.82–7.80 (2H, m, Ar*H*), 7.69 (1H, d, J = 7.8, Ar*H*), 7.61 (1H, d, J = 8.3, Ar*H*), 7.54–7.21 (7H, m, Ar*H*), 6.32 (1H, s, C*H*=C), 5.64 (1H, s,

C*H*=C), 4.90 (2H, d, $J = 6.1$, NHCH_2Ph, collapses to s with D_2O), 4.30 (2H, br t, $J = 7.0$, NCH_2(CH$_2$)$_4$CH$_3$), 4.14 (2H, br t, $J = 6.5$, NCH_2(CH$_2$)$_4$CH$_3$), 1.81–1.78 (2H, m, NCH$_2$(CH_2)$_4$CH$_3$), 1.48–1.20 (14H, m, NCH$_2$(CH_2)$_4$CH$_3$), 0.91–0.82 (6H, m, N(CH$_2$)$_5$CH_3). ^{13}C NMR (75.47 MHz, $CDCl_3$/DMSO-d_6) δ: 173.7, 163.4, 161.3, 159.6, 157.2, 155.5, 140.6, 140.4, 140.3, 136.2, 135.0, 130.6, 127.6, 127.4, 127.3, 125.6, 124.8, 124.3, 122.5, 122.2, 121.8, 113.1, 112.6, 95.0, 86.7, 86.3, 46.4, 45.8, 30.9, 30.8, 27.3, 26.7, 25.7, 21.9, 13.7. HRFABMS (3-NBA): 760.1856 ([M-I]$^+$, $C_{39}H_{43}IN_3OS_2^+$; calc. 760.1887).

2.1.4. X-ray crystallographic analysis

Single crystals were obtained by the slow evaporation of a dilute solution of dye **2** in $CHCl_3$. Diffraction data were collected on a Bruker SMART APEX2 CCD diffractometer using synchrotron radiation ($\lambda = 0.7848$ Å). The structure was solved by direct methods SHELX97, and refined by full-matrix least-squares calculations. Crystal data for dye **2**: $C_{35}H_{42}F_3N_3O_5S_3$, $Mw = 737.93$, triclinic, $P\bar{1}$, $Z = 2$, $a = 9.786(2)$, $b = 10.538(2)$, $c = 18.370(4)$ Å, $\alpha = 104.496(2)$, $\beta = 94.051(3)$, $\gamma = 104.868(2)^\circ$, $D_{calcd} = 1.397$ g cm^{-3}, $T = 120(2)$ K, $F(000) = 776$, $\mu = 0.274$ mm^{-1}, 7578 reflections were collected, 4800 unique ($R_{int} = 0.0507$), 2792 observed ($I > 2\sigma$ (I)), 453 parameters, $R_1 = 0.0767$, $wR_2 = 0.2142$. Crystallographic data (dye **2** (CCDC 754805) have been deposited at the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK.

2.2. Materials

Dimethyl sulfoxide (DMSO) and 0.05 M TRIS–HCl buffer (pH 8.0) were used as solvents. DMSO, TRIS–HCl, human serum albumin (HSA), bovine serum albumin (BSA), horse serum albumin (HRSA), ovalbumin (OVA) and SDS were purchased from Sigma–Aldrich (USA).

2.3. Preparation of stock solutions of dyes and proteins

The 2×10^{-3} M dye stock solutions were prepared by dilution of the dye in DMSO. Stock solutions of proteins (HSA, BSA, HRSA, OVA) and SDS were prepared by dissolving in 0.05 M TRIS–HCl buffer (pH 8.0). Proteins and SDS concentrations in stock solutions were equal to 3 μM and 0.05% w/w respectively.

2.4. Preparation of working solutions

Working solutions of free dyes were prepared by dilution of the dye stock solution in 0.05 M TRIS–HCl buffer. Working solutions of the dyes in the presence of proteins were prepared by addition of the dye stock solution to the protein stock solution. The concentrations of dye and proteins in working solutions amounted to 5×10^{-6} M and 3×10^{-6} M, respectively. All working solutions were prepared immediately before the experiments.

2.5. Spectroscopic measurements

Absorption spectra were recorded on a Specord M40 spectrophotometer (Carl Zeiss, Germany). Fluorescence excitation and emission spectra were taken on a Cary Eclipse fluorescence spectrophotometer (Varian, Australia). Spectroscopic measurements were performed in standard quartz cells (1×1 cm). The quantum yield value for dye **7** in the presence of HSA was determined using 3,3′-diethylthiadicarbocyanine iodide solution in ethanol as the reference (quantum yield value $\varphi_{DCI} = 0.35$) [11]. Namely, dye **7** in the presence of HSA in buffer and 3,3′-diethylthiadicarbocyanine iodide in ethanol solutions were taken in such concentrations that their optical density values were equal at 671 nm. Fluorescence of both solutions was excited at this wavelength, and the area below each spectrum (S_7 and S_{DCI} respectively) was calculated. Further, the fluorescence quantum yield of dye **7** in the presence of HSA was calculated as $\varphi_7 = \varphi_{DCI} \times S_7/S_{DCI}$.

The dynamic range and lower detection limit of HAS detection for benzothiazole squarilyum dye **3**, was determined by the titration of a fixed concentration (5×10^{-6} M) of the dye with the increasing amounts of HSA. The constant of dye **3** binding to HSA was estimated based on the aforementioned dependence of dye **3** (5×10^{-6} M) fluorescence intensity on the HSA concentration added (titration curve). The binding constant K_b is:

$$K_b = \frac{C_{bd}}{C_{fd} \times C_{fp}} \quad (1)$$

where C_{bd}, C_{fd} and C_{fp} are concentrations of bound dye, free dye and free dye-binding sites of the protein respectively. If we consider, for dye **3**, one binding site per protein globule and then regard the total protein concentrations C_p much exceeding the total dye concentrations C_d so that $C_p \approx C_{fp}$; and if the free dye fluorescence intensity I_f is negligibly small as compared to the fluorescence intensity in the presence of the protein I^{HSA} so that $C_{bd}/C_d = I^{HSA}/I_{max}$ (I_{max} is the saturation dye fluorescence intensity upon HSA addition); then equation (1) could be transformed to:

$$I^{HSA} = I_{max} \times \frac{K_b \times C_p}{1 + K_b \times C_p} \quad (2)$$

Thus if we approximate the points of the titration curve corresponding to HSA concentrations higher than 3×10^{-5} M (in HSA globules) with equation (2), the value of K_b (together with this of I_{max}) could be obtained as the approximation parameter. All measurements were carried out at room temperature.

3. Results and discussion

3.1. Synthesis of dyes

The aza-substituted squaraines **2**, **3** and **10** were prepared according to a previously established procedure [10,12] involving the nucleophilic substitution of the methoxy group of 3-hexyl-2-[3-(3-hexyl-3H-benzothiazol-2-ylidenemethyl)-2-methoxy-4-oxocyclobut-2-enylidenemethyl]benzothiazol-3-ium trifluoromethane sulfonate (dye **1**), easily accessible by methylation of the unsubstituted analogue with methyl triflate, by the appropriate amine (Fig. 1) [10]. Reaction with the weaker nucleophile 3-iodobenzylamine to give compound **10** required catalysis with triethylamine. The synthesis and structural characterization of squaraines **4**, **5**, **6**, **7**, **8**, **9** and **11** has already been described [10].

3.2. X-ray crystallographic analysis of dye 2

The X-ray crystal structure of dye **2** is shown in Fig. 2. In this structure, the whole molecule is crystallographically unique (Fig. 2a), caused by a disruption in the molecular symmetry due to the substitution of the hydroxyethylamino group for one of the squarate oxygen atoms. Another effect that this substitution has had on the squaraine molecule, which also disrupts the symmetry, is that the benzothiazole groups are pseudo-mirrored (*cis* conformation), as opposed to being inverted/*trans* conformation (as in the majority of symmetrical squaraines), to each other. The resultant O···S distances (indicated in Fig. 2b) are 3.031(8) Å (O1···S1) and 3.046(8) Å (O1···S2). As with the majority of other squaraine dyes, the squaraine unit in dye **2** is essentially flat with dihedral angles between the two phenyl rings and the squarate ring being 1.0(6) and 16.8(5)°, respectively. In terms of packing, dye **2** displays

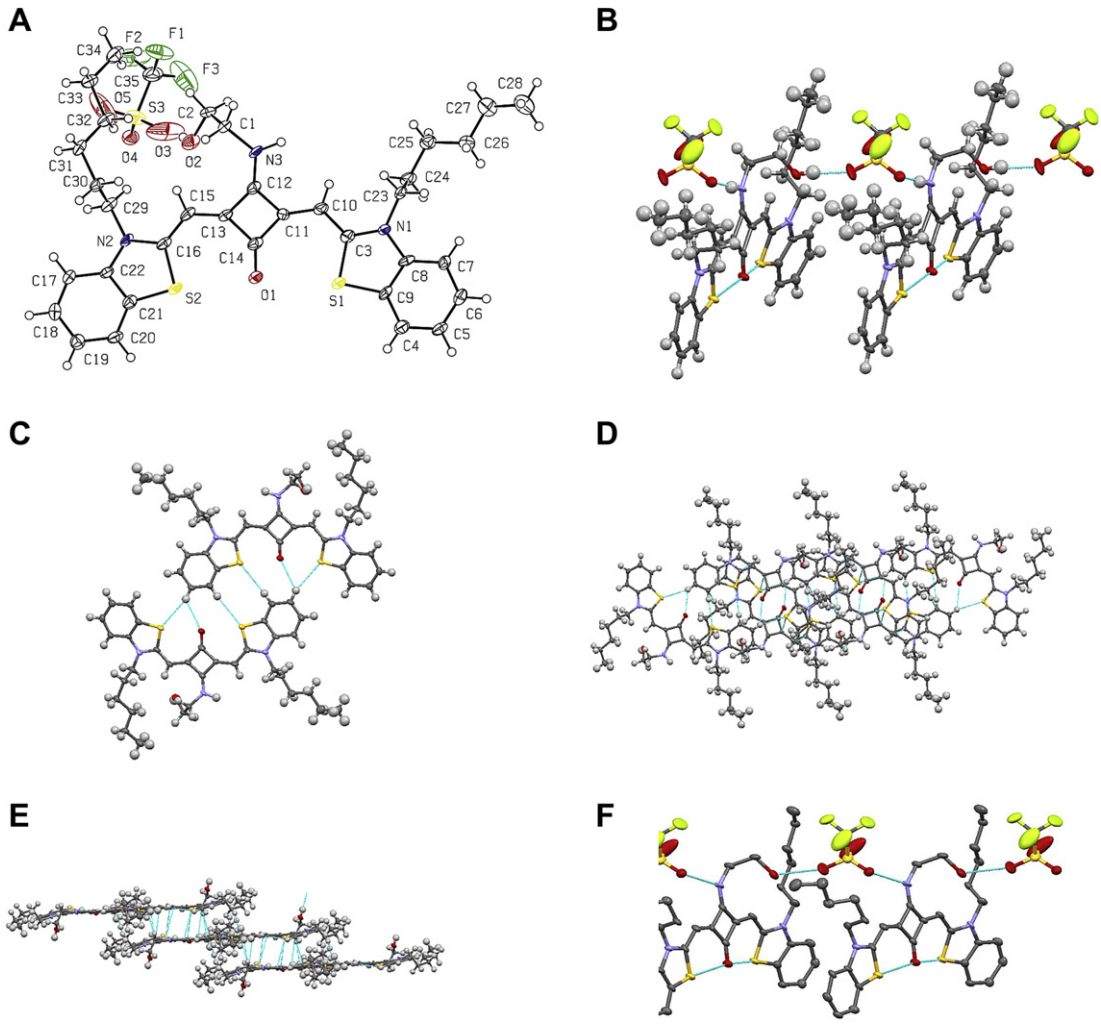


Fig. 2. X-ray crystallography of dye **2**. A – ORTEP; B – strong hydrogen bonding associations and O···S interactions; C – weak C–H···O/S interactions between the dimers [C4···S1 3.854(8) Å; C5···O1 3.337(8) Å; C5···S2 3.744(8) Å]; D – top-view (slipped-stack); E – side-view (slipped-stack), F- Hydrogen atoms have been removed for clarity.

a unique three-dimensional arrangement in that most squaraine structures adopt a slip-stacked charge-transfer column, with intermolecular distances between planes usually being ~ 3.4–3.9 Å, the squaraine units in dye **2** form dimers that are then slip-stacked as dimers (intermolecular distances > 3.4 Å) (shown in Fig. 2d and e). These dimers associate *via* weak C–H···O hydrogen-bonding interactions (shown in Fig. 2c), which is interesting because of the presence of the strong N–H and O–H hydrogen bond donors that only associate with the sulfonate oxygen atoms (shown in Fig. 2b) [Hydrogen bond distances: O2···O3 2.781(8) Å; N3···O4 2.921(7) Å], and not either the squarate oxygen atom or the benzothiazole sulfur atoms.

3.3. Spectral-luminescent properties of free dyes in aqueous buffer

The fluorescent characteristics of a series of benzothiazole and benzoselenazole aza-substituted squaraine dyes in aqueous buffer are presented in Table 2. The excitation wavelengths of the studied dyes in buffer reside in the range 651–695 nm, while the fluorescence emission maxima lie between 658 and 709 nm. Stokes shift values observed for the free squaraines are small – between 6 nm (dyes **2** and **3**) and 18 nm (dye **9**). All free squaraines demonstrated low intrinsic fluorescence intensity values (I_f) – up to 17 a.u. (arbitrary units). It should be noted that in the pairs of structurally-related benzothiazole/benzoselenazole dyes, the emission intensities are higher for benzothiazole squaraines. The fluorescence intensity of benzoselenazole squaraines does not exceed 1.4 a.u (dyes **4**, **5** and **9**). The lowest emission intensity among the benzothiazole squaraines was displayed by the dyes with aromatic substituents (3-iodobenzylamino (**10**) and anilino (**11**)) and a 2-sulfoethylamino fragment (**8**) in the squaraine ring – I_f values for these dyes are extremely low and do not exceed 1.2 a.u.

3.4. Spectroscopic characterization of squaraine dyes in the presence of various albumins

The spectral-luminescent properties of the studied aza-substituted squaraines in the presence of BSA (bovine serum albumin), HSA (human serum albumin), HRSA (horse serum albumin) and OVA (ovalbumin) are presented in Table 2. For all squaraines, studied in the presence of albumins, noticeable spectral changes were observed. For the majority of dyes, addition of the proteins resulted in the shift of excitation and emission maxima positions to the long-wavelength spectral region, of up to 35 nm. For benzoselenazole dyes **5** (with 2-sulfoethylamino fragment) and **11** (with anilino group), excitation and emission maxima were bathochromically shifted by up to 52 and 60 nm, respectively, relative to the corresponding spectra of the free dyes. For squaraines **8** and **10**,

Table 2
Characteristics of fluorescence excitation/emission spectra of studied aza-substituted squaraine dyes solutions in buffer, as well as in the presence of albumins.

Dye	In free form			In HSA presence				In BSA presence			In HRSA presence			In OVA presence		
	λ_{ex}, nm	λ_{em}, nm	I_f, a.u.	λ_{ex}, nm	λ_{em}, nm	I^{HSA} a.u.	I^{HSA}/I_f	λ_{em}, nm	I^{BSA} a.u.	I^{BSA}/I_f	λ_{em}, nm	I^{HRSA}, a.u.	I^{HRSA}/I_f	λ_{em}, nm	I^{OVA}, a.u.	I^{OVA}/I_f
2	652	658	5.1	676	683	386	75.7	684	269	52.7	683	343	67.3	682	276	54.1
3	652	658	12	672	682	623	51.9	681	387	32.3	682	660	55.0	680	408	34.0
4	695	709	0.7	712	718	280	400	717	212	303	718	221	316	714	257	367
5	680	693	1	726	730	16	16.0	745	60	60.0	739	43	43.0	734	16	16.0
6	665	675	5.2	687	695	318	61.2	698	237	45.6	699	1113	214	696	358	68.8
7	651	661	17	670	678	559	32.9	682	631	37.1	685	827	48.6	681	429	25.2
8	678	692	1.2	673	680	92	76.7	680	89	74.2	680	187	156	680	47	39.2
9	667	685	1.4	692	702	236	169	701	320	229	703	205	146	700	275	196
10	670	682	1	665	672	90	90.0	679	108	108	680	21	21.0	677	83	83.0
11	660	670	0.7	720	725	6.4	9.14	721	12	17.1	722	3.6	5.14	721	36	51.4

λ_{ex} (λ_{em}) – maximum wavelength of fluorescence excitation (emission) spectrum, I_f (I^{BSA}, I^{HSA}, I^{HRSA}, I^{OVA}) – emission intensity of dye in free state (in presence of bovine serum albumin, human serum albumin, horse serum albumin and albumin from chicken egg white respectively), a.u. — arbitrary units; concentrations of dyes and albumins in working solutions were 5×10^{-6} M and 0.2 mg/ml respectively; (I^{BSA}, I^{HSA}, I^{HRSA}, I^{OVA})/I_f – emission increasing of the dye in the presence of corresponding proteins.

spectral maxima were shifted to the short-wavelength region, by up to 12 nm. The Stokes shift values for the dyes in the presence of the albumins are in the range close to that of the free dyes, namely from 4 to 12 nm.

The majority of the squaraines, studied in the presence of albumins, demonstrated a good increase of emission intensity value, up to 400 times (dye **4**). But, because of the weak intrinsic fluorescence of the dyes, the overall fluorescence intensity level of the formed dye/albumin complexes could be characterized as from low to medium. The lowest emission intensity values, in the presence of proteins, were observed for dyes bearing 2-sulfoethylamino (**5** and **8**), *m*-iodobenzylamino (**10**) and anilino (**11**) substituents on the squarate ring, ranging from 3.6 to 187 a.u. The benzothiazole dye with a pendent diethylamino substituent (**6**) specifically forms a bright complex with HRSA (emission intensity 1113 a.u.), while its intensity in the presence of other proteins is 3–4 times lower. It should be noted that its benzoselenazole analogue dye **4** did not show a similar selectivity and displayed approximately the same emission intensity in the presence of all of the studied albumins.

Benzothiazole dyes **3** and **7** possessing aminoethylamino and *N*,*N*-dimethylhydrazino substituents, respectively, demonstrated high emission intensities in the presence of albumins (up to 827 a.u. for dye **7** in the presence of HRSA). Excitation profiles and emission spectra of dye **7** are presented in Fig. 3. In complexes with proteins, these dyes demonstrated a 25–56 fold increase in emission. The fluorescence quantum yield of dye **7** in the presence of HSA was estimated to be *ca.* 0.18. Due to these observations, benzothiazole squaraines **3** and **7** could be proposed for further studies as dyes for the detection and quantification of albumins.

The dynamic range and lower detection limit of HSA detection for benzothiazole squarylium dye **3** was determined via the titration of a fixed concentration of the dye (5×10^{-6} M) with increasing amounts of HSA. It was shown that the fluorescence intensity enhancement relates to the protein concentration over the wide range – 1.5 μg/mL to 20 mg/mL (Fig. 4). Based on the analysis of the titration curve, the binding constant of dye **3** to HSA was estimated to be $K_b = 2.5 \pm 0.5 \times 10^4$ M^{-1}; such a value lies in the range 10^4–10^6 M^{-1}, which is characteristic of ligands binding with high-affinity sites of HSA [13].

3.5. SDS and BSA/SDS mixture

Characteristics of excitation and fluorescence spectra of squaraine dyes studied in the presence of SDS and a BSA/SDS mixture are presented in Table 3. For the majority of dyes in the presence of SDS and the BSA/SDS mixture the excitation and emission maxima positions were red-shifted by 4–25 nm, relative to that in the simple dye buffer solution. Similarly as in the presence of albumins, in the presence of SDS and the BSA/SDS mixture, benzoselenazole

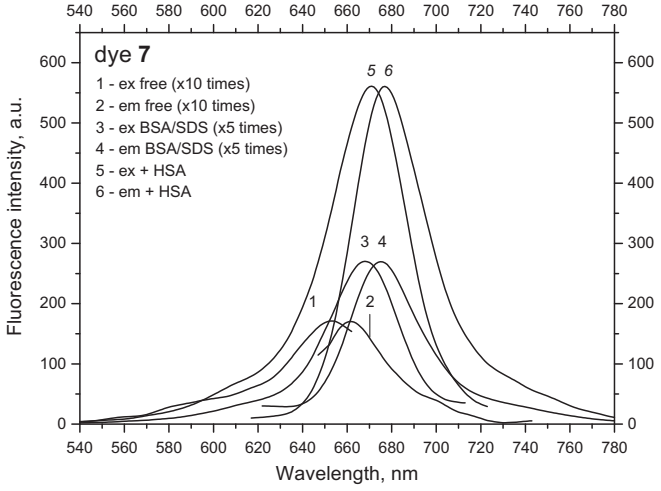


Fig. 3. Profiles of excitation and fluorescence spectra of dye **7** (5×10^{-6}) in the unbound state in 0.05 M TRIS–HCl buffer (pH 8.0), in presence of HSA and a BSA/SDS mixture. The low-intensity spectra of free dye and dye in presence of BSA-SDS are multiplied 10 times and 5 times respectively (a.u., arbitrary units). Proteins and SDS concentrations were 3 μM and 0.05% respectively.

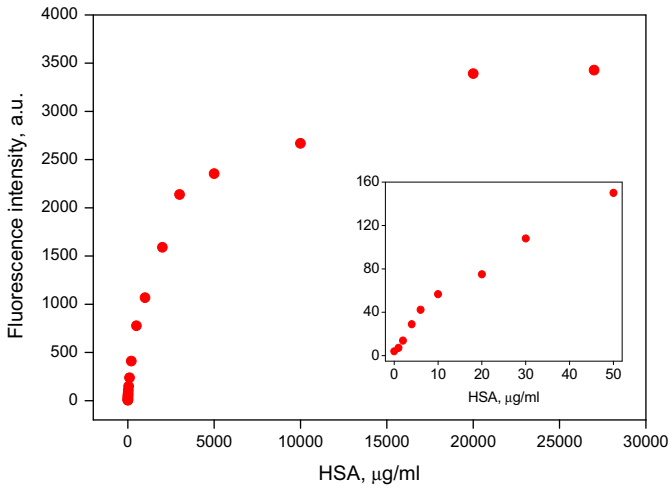


Fig. 4. Dynamic range of HSA detection for benzothiazole squaraine dye **3**. The fluorescence intensity of the dye was plotted against increased amounts of protein. The results demonstrate a broad detection range from 1.5 μg/ml to 20 mg/ml.

Table 3
Spectral-luminescent characteristics of studied aza-substituted squaraine dyes (5×10^{-6} M) solutions in the presence of SDS and BSA/SDS mixture.

Dye	In SDS presence				In BSA/SDS mixture presence			
	λ_{ex}, nm	λ_{em}, nm	I^{SDS}, a.u.	I^{SDS}/I_f	λ_{ex}, nm	λ_{em}, nm	$I^{BSA/SDS}$, a.u.	$I^{BSA/SDS}/I_f$
2	665	673	45	8.82	665	676	154	30.2
3	672	680	13	1.08	671	680	36.5	3.04
4	702	713	8	11.4	707	717	51	72.9
5	721	727	2	2.00	726	730	50	50.0
6	686	700	39	7.50	687	700	97	18.7
7	688	675	24	1.41	670	677	54	3.18
8	668	677	327	273	671	680	1842	1535
9	687	695	5.3	3.79	684	696	19	13.6
10	667	675	11.4	11.4	667	678	15	15.0
11	719	725	2	2.86	717	729	2	2.86

λ_{ex} (λ_{em}) – maximum wavelength of fluorescence excitation (emission) spectrum, I^{SDS} ($I^{BSA/SDS}$) – emission intensity of dye in presence of SDS (BSA/SDS mixture), a.u. – arbitrary units; concentration of SDS in working solution and in BSA/SDS solution was 0.05%; concentration of BSA was 0.2 mg/ml. I^{SDS}/I_f, and $I^{BSA/SDS}/I_f$ – emission increasing of the dye in the presence of correspondingly SDS and BSA/SDS.

dyes **5** and **11** demonstrated the largest shift of excitation and emission maxima (37–59 nm). For benzothiazole squaraines **7** and **10**, both excitation and emission maxima were shifted hypsochromically by 3–15 nm. Thus, the excitation and emission maxima of all the studied dyes were situated in the range 665–730 nm. The Stokes shifts for the dyes in the presence of SDS and the BSA/SDS mixture were between 6 and 14 nm.

In the presence of SDS (0.05% w/w), which is five times lower than its critical micelle concentration, the aza-substituted squaraines in this study increased their fluorescence intensity by up to 11.4 times. However, because of low intrinsic fluorescence intensity of the studied squaraines, the emission intensity of aza-substituted squaraines in the presence of SDS was quite low (about 2–45 a.u.). The only exception to this was the benzothiazole dye **8** bearing a 2-sulfoethylamino substituent on the squaraine ring for which its fluorescence intensity value in the presence of SDS was I^{SDS} ~327 a.u.

For the majority of the studied squaraines, the emission intensity enhancement in the BSA/SDS mixture, relative to that measured for the pure SDS ($I^{BSA/SDS}/I^{SDS}$), was rather small (up to 6.3 times); with the exception being dye **5**, which demonstrated an increase in emission of *ca*. 25 times. The brightest fluorescence in the presence of BSA/SDS ($I^{BSA/SDS}$ value of ~1842 a.u.) was observed for benzothiazole dye **8** having a 2-sulfoethylamino fragment, while for its benzoselenazole analogue **5** the emission intensity in the mixture with BSA/SDS was considerably lower – 50 a.u.

4. Conclusions

1. Three novel aza-substituted benzothiazole squaraines **2**, **3** and **10** were synthesized; their structures were characterized by HRFABMS, IR, UV/Vis, ^{1}H NMR, and ^{13}C NMR spectroscopy. Single crystals of dye **2** were obtained and the structure was solved using X-ray crystallographic analysis.
2. A series of new and previously described aza-substituted squaraine dyes was characterized in the presence of various albumins and denatured proteins (BSA/SDS mixture) using fluorescence spectroscopy. The studied dyes demonstrated a weak intrinsic fluorescent emission, while in the presence of albumins an increase in the emission intensity (up to 400 times) and shift of excitation and emission maxima of up to 60 nm occurred.
3. The aminoethylamino substituted dye **6** demonstrated the strongest fluorescent response and bright emission in complex with HRSA (emission intensity 1113 a.u.), while its fluorescence intensity level in the presence of other albumins is 3–4 times lower.
4. Benzothiazole dyes **3** and **7** with aminoethylamino and *N*,*N*-dimethylhydrazino subsituents demonstrated good emission intensities in complexes with albumins (up to 827 a.u) and sufficient increase in emission (up to 56 fold). The fluorescence quantum yield of dye **7** in the presence of HSA was estimated to be about 0.18. For dye **3** a wide HSA detection range (1.5 μg/mL to 20 mg/mL of protein) has been shown.
5. The studied squaraine dyes demonstrated a weak fluorescent response in the presence of SDS and a BSA/SDS mixture. The exception is the benzothiazole dye with a 2-sulfoethylamino substituent **8** that showed moderate fluorescence in the presence of SDS (347 a.u.), and bright emission (1842 a.u.) in the presence of the BSA/SDS mixture.

Acknowledgements

The authors are grateful to Fundação para a Ciência e a Tecnologia, Portugal, POCTI and FEDER for financial support (Project POCTI/32915/QUI/00), and also thank the EPSRC National Crystallography Service (Southampton University, England) and Synchrotron Service (Newcastle University, England).

References

[1] Arai K, Madison J, Shimizu A, Putnam FW. Point substitutions in albumin genetic variants from Asia. Proc Natl Acad Sci USA 1990;87:497–501.
[2] Chen Y, Yang J, Wang Z, Wu X, Wang F. Scopoletine as fluorescence probe for determination of protein. Spectrochim Acta A Mol Biomol Spectrosc 2006;66 (3):686–90.
[3] Kessler M, Wolfbeis OS. Method for detection and determination of human serum albumin United States Patent 5182214, 01/26/1993.
[4] Waheed AA, Rao KS, Gupta PD. Mechanism of dye binding in the protein assay using eosin dyes. J Anal Biochem 2000;287(1):73–9.
[5] Tseng WL, Chiu TC, Weng JM, Chang HT. Analysis of albumins, using albumin blue 580, by capillary electrophoresis and laserinduced fluorescence. J Liq Chrom Rel Technol 2001;24(19):2971–82.
[6] Jisha VS, Arun KT, Hariharan M, Ramaiah D. Site-selective binding and dual mode recognition of serum albumin by a squaraine dye. J Am Chem Soc 2006;128(18):6024–5.
[7] Nakazumi H, Colyer Ch L, Kaihara K, Yagi S, Hyodo Y. Red luminescent squarylium dyes for noncovalent HSA labeling. Chem Lett 2003;32:804–5.
[8] Volkova KD, Kovalska VB, Tatarets AL, Patsenker LD, Kryvorotenko DV, Yarmoluk SM. Spectroscopic study of squaraines as protein-sensitive fluorescent dyes. Dyes Pigments 2007;72:285–92.
[9] Volkova KD, Kovalska VB, Losytskyy MY, Bento A, Reis LV, Santos PF, et al. Studies of benzothiazole and benzoselenazole squaraines as fluorescent probes for albumins detection. J Fluoresc 2008;18(5):877–82.
[10] Reis LV, Serrano JP, Almeida P, Santos PF. The synthesis and characterization of novel, aza-substituted squarylium cyanine dyes. Dyes Pigments 2009;81:197.
[11] Dempster DN, Morrow T, Rankin R, Thompson GF. Photochemical characteristics of cyanine dyes. Part 1. 3,3′-diethyloxadicarbocyanine iodide and 3,3′-diethylthiadicarbocyanine iodide. J Chem Soc Faraday Trans II 1972;68: 1479–96.
[12] Reis LV, Serrano JPC, Almeida P, Santos PF. New synthetic approach to. aminosquarylium cyanine dyes. Synlett 2002;10:1617–20.
[13] Kragh-Hansen U, Chuang VTG, Otagiri M. Practical aspects of the ligand-binding and enzymatic properties of human serum albumin. Biol Pharm Bull 2002;25:695–704.