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Spectroscopic study of squaraines as protein-sensitive fluorescent dyes

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Abstract

A series of new symmetrical and asymmetrical squaraines were synthesised and efficiency of their use as fluorescent probes for the specific detection of proteins was studied. Spectral-luminescent properties of the squaraines were measured in presence of bovine serum albumin (BSA), human serum albumin (HSA), ovalbumin, avidin from hen egg white (AVI), lysozyme, and trypsin. All investigated squaraines show considerable (in 24–190 times) emission increase in the presence of BSA. At the same time the fluorescent response of the studied dyes in the presence of other albumins is significantly lower – emission enhances up to 24 times. The 3-oxo-substituted indolenine dye **9(74)** demonstrates sufficient fluorescence increasing value and emission intensity level in the presence of BSA as well as of HSA and ovalbumin. Dyes containing *N*-carboxyalkyl group demonstrate sufficient emission enhancement (up to 16 times) and noticeable fluorescent signal in the presence of avidin from hen egg white. Squaraines slightly increase or even decrease their emission intensity in the presence of hydrolases lysozyme or trypsin.

Keywords: Squaraines; Fluorescent probes; Synthesis; Proteins detection

1. Introduction

Fluorescence detection of proteins at long-wavelength excitation is widely used for biomedical application due to such benefits of near-infrared-based methods as possibility to use of non-expensive diode lasers operating at 635–650 nm [1] and decreased autofluorescence from biomolecules beyond 600 nm [2]. Squarylium dyes are suitable for these purposes owing to their unique physico-chemical properties namely effective light absorption in the visible and near-infrared (NIR) regions [3], sharp and intensive fluorescence [4] and photoconductivity [5]. Since the squaraines contributed greatly to various areas of optoelectronic fields [6], these dyes are also successfully used as long-wavelength fluorescent probes and labels in biological assay techniques [7,8].

Squaraines derived from different heterocyclic methylene bases can be used as labels and probes for selective biomolecules detection because of easiness of their structure functionality. One approach to structural modification consists in introduction of substituents into the aromatic ring or the N-atom of the heterocyclic moiety. We have already realised other approach which lied in an introduction of different groups in the squaric ring. This leads to red-shift in absorption and fluorescence spectra, but the influence of such substituents on the spectral properties of the dye in various solvents and in presence of proteins was not investigated [9].

Welder et al. [3] described spectral properties of 3-oxo-substituted squaraines in presence of various proteins and concluded that both the symmetrical and unsymmetrical squarylium dyes showed enhancement of fluorescence intensity upon noncovalent interactions with proteins, however, symmetrical dye interacted strongly with HSA, β-lactoglobulin A and trypsinogen whereas unsymmetrical one showed markedly greater binding affinity to HSA and BSA. Furthermore,

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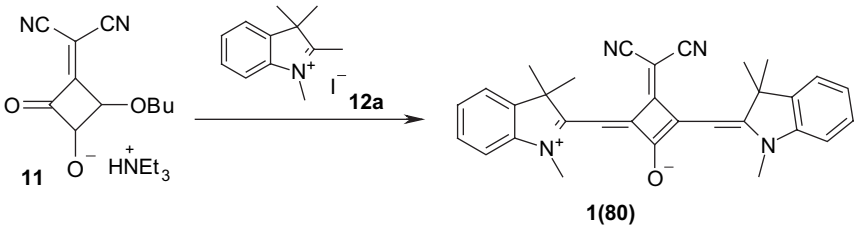


Scheme 1.

Terpetschnig et al. [10] studied spectral characteristics of 10 squarylium dyes in free form and when bound to BSA and reached a conclusion that the most suitable protein probes for use in biomedical applications were the symmetrical indolenine-based squaraines which displayed the highest (28-fold) fluorescence intensity increase upon proteins binding.

In this paper we synthesise a series of symmetrical and unsymmetrical squaraine dyes **1**, **3**–**10** (Schemes 1–4) and evaluate them for detection of a variety of proteins such as bovine serum albumin (BSA), human serum albumin (HSA) ovalbumin, avidin from hen egg white (AVI), and hydrolases such as trypsine and lysozyme. The influence of the dye molecules' structures on selectivity towards certain protein is studied.

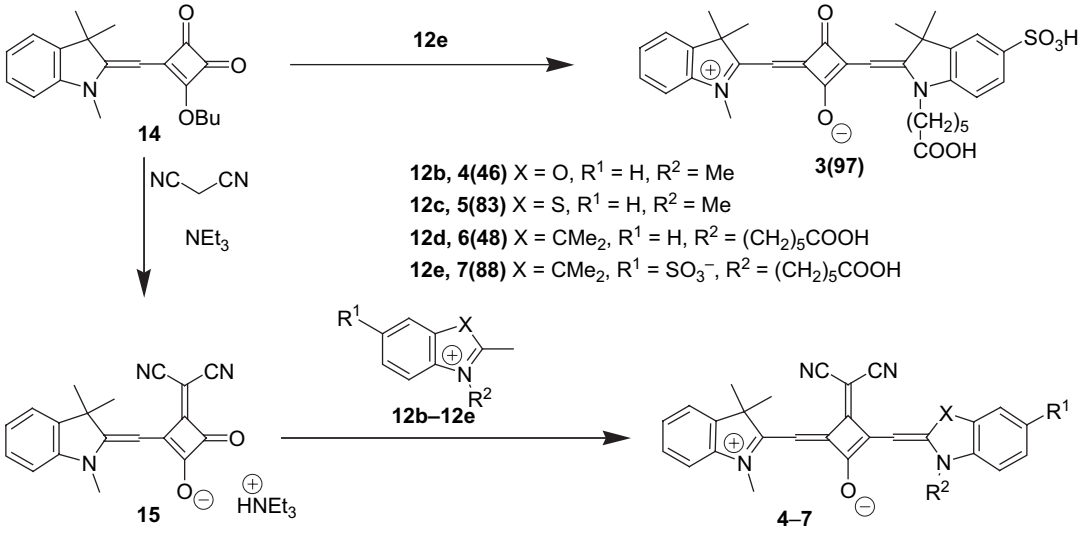
2. Experimental

The progress of the chemical reactions and the purity of products were monitored by TLC (Sorbfil TLC Plates) and ^{1}H NMR. IR spectra were recorded using KBr pellets on a Specord M80 spectrophotometer. ^{1}H NMR spectra were measured on Varian Mercury-VX-200 (200 MHz) spectrometers in DMSO-d_6 using TMS as an internal standard. FAB mass spectra were taken on a SELMI MI-1201E (Ukraine) instrument using 3-nitrobenzylalcohol (NBA) as a matrix.

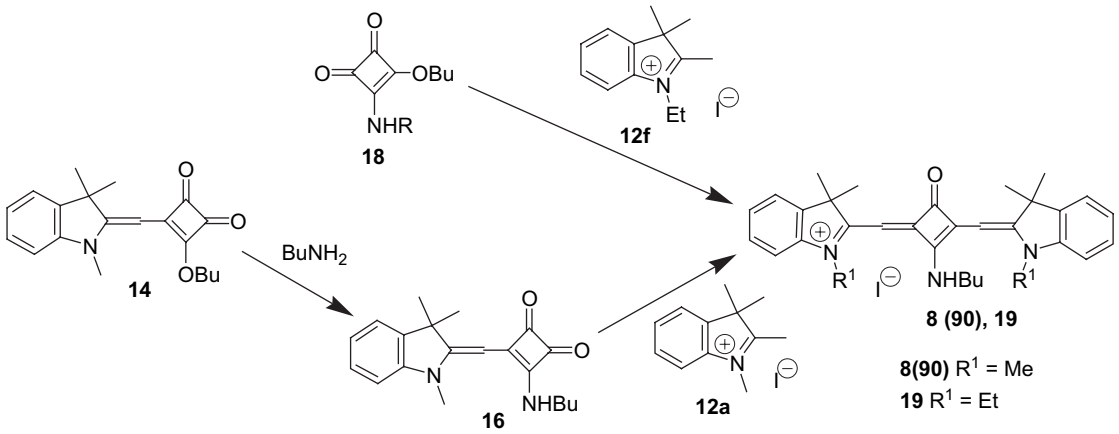
2.1. Materials

Anhydrous dimethylformamide (DMF) distilled under reduced pressure, methanol and 0.05 M Tris–HCl buffer (pH 8.0) were used as solvents. Bovine serum albumin (BSA), human serum albumin (HSA) ovalbumin, avidin from hen egg white (AVI), lysozyme and trypsin were purchased from Sigma–Aldrich (USA).

3-Dicyanomethylene-2-(3-methyl-2,3-dihydro-1,3-benzoxazol-2-ylidenmethyl)-4-(1,3,3-trimethyl-3*H*-2-indoliumylmethylene)-1-cyclobuten-1-olate **4**(**46**), 3-dicyano-methylene-2-(3-methyl-2,3-dihydro-1,3-benzothiazol-2-ylidenmethyl)-4-(1,3,3-trimethyl-3*H*-2-indoliumylmethylene)-1-cyclobuten-1-olate **5**(**83**), 3-oxo-2-(1,3,3-trimethyl-2,3-dihydro-1*H*-2-indolylidenmethyl)-4-(1,3,3-trimethyl-3*H*-2-indoliumylmethylene)-1-cyclobuten-1-olate **9**(**74**) [11], triethylammonium 2-butoxy-3-dicyanomethylene-4-oxo-1-cyclobuten-1-olate (**11**) [12], quaternized indolenines, 2-methyl-1,3-benzoxazole and 2-methyl-1,3-benzothiazole (**12a**–**12e**) [1,2,13,14], 1,3,3-trimethyl-2-methylene-5-nitroindoline (**13**), 3-butoxy-4-(1,3,3-trimethyl-2,3-dihydro-1*H*-2-indolylidenmethyl)-3-cyclobutene-1,2-dione (**14**) [15,16], and triethylammonium 3-dicyanomethylene-4-oxo-2-(1,3,3-trimethyl-2,3-dihydro-1*H*-2-indolylidenmethyl)-1-cyclobuten-1-olate (**15**) [9] were synthesised according to the corresponding literature procedure.



Scheme 2.



Scheme 3.

2.2. Synthesis

2.2.1. 3-Dicyanomethylene-2-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-4-(1,3,3-trimethyl-3H-2-indoliumylmethylene)-1-cyclobuten-1-olate ***1(80)***

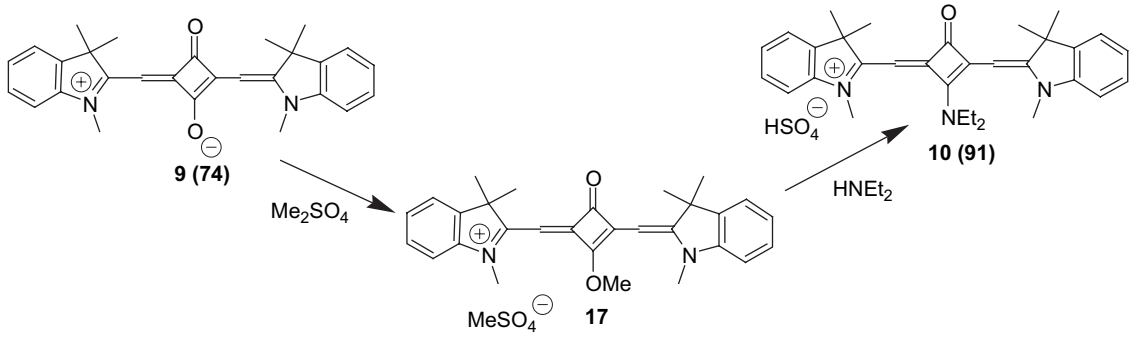
A mixture of 500 mg (1.66 mmol) of 1,2,3,-tetramethyl-3*H*-indolium iodide (**12a**) and 265 mg (0.83 mmol) of triethylammonium 2-butoxy-3-dicyanomethylene-4-oxo-1-cyclobuten-1-olate (**11**) was refluxed in 20 ml of 1-butanol–pyridine mixture (1:1 v/v) for 12.5 h. The solvent was removed under reduced pressure by a rotary evaporator. The residue was purified by column chromatography (Silica gel 60, chloroform) to give product **1**(**80**). Yield: 180 mg (46%) as the green crystals with gold lustre; R_f 0.47 (Sorbfil, chloroform–methanol, 50:1 v/v); Analysis: N, 11.73 $C_{31}H_{28}N_4O$ requires N, 11.86%; ^{1}H NMR (DMSO-d_6), δ, ppm: 7.54 (2H, d, 7.4 Hz, arom. H), 7.47–7.37 (4H, m, arom. H), 7.32–7.18 (2H, m, arom. H), 6.30 (2H, s, C*H*), 3.61 (6H, s, NCH_3), 1.68 (12H, s, (CH_3)$_2$); FAB-MS (NBA) *m*/*z* 472 (M^+), 473 (MH^+); IR (KBr) 2228 (CN), 2208 (CN), 1724, 1624, 1604 cm^{-1}.

2.2.2. 2-[1-(5-Carboxypentyl)-3,3-dimethyl-5-sulfo-2,3-dihydro-1H-2-indolylidenmethyl]-3-oxo-4-(1,3,3-trimethyl-3H-2-indoliumylmethylene)-1-cyclobuten-1-olate ***3(97)***

A mixture of 300 mg (0.92 mmol) of 3-butoxy-4-(1,3,3-trimethyl-2,3-dihydro-1*H*-2-indolylidenmethyl)-3-cyclobutene-1,2-dione (**14**) and 500 mg (0.92 mmol) of 1-(5-carboxypentyl)-2,3,3-trimethyl-3*H*-5-indoliumsulfonate (**12e**) containing 35% of KBr (after synthesis) was refluxed in 15 ml of pyridine for 8 h. The solvent was removed under reduced pressure and the residue was dissolved in 30 ml of a 0.1 M potassium hydroxide solution, acidified with 1 M HCl, and the precipitate was isolated and purified by column chromatography (Silica gel 60 RP-18, 0–40% methanol–water) to give title product. Yield: 135 mg (24%) of **3**(**97**); ^{1}H NMR (DMSO-d_6), δ, ppm: 7.66 (1H, s, arom.), 7.60 (1H, d, 8.0 Hz, arom.), 7.53 (1H, d, 7.4 Hz, arom.), 7.45–7.30 (2H, m, arom.), 7.25 (1H, d, 8.4 Hz, arom.), 7.30–7.09 (1H, m, arom.), 5.79 (2H, s, C*H*), 4.16–4.02 (2H, m, NCH_2), 3.59 (3H, s, NCH_3), 2.21 (2H, t, 7.0 Hz, CH_2COOH), 1.69 (6H, s, (CH_3)$_2$), 1.68 (6H, s, (CH_3)$_2$), 1.82–1.29 (6H, m).

2.2.3. 4-[1-(5-Carboxypentyl)-3,3-dimethyl-3H-2-indoliumylmethylene]-3-dicyanomethylene-2-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-1-cyclobuten-1-olate ***6(48)***

A mixture of 150 mg (0.36 mmol) of triethylammonium 3-dicyanomethylene-4-oxo-2-(1,3,3-trimethyl-2,3-dihydro-1*H*-2-indolylidenmethyl)-1-cyclobuten-1-olate (**15**) and 140 mg (0.40 mmol) of 1-(5-carboxypentyl)-2,3,3-trimethyl-3*H*-indolium bromide (**12d**) were heated under reflux in a mixture of 20 ml of a 1-butanol–toluene (1:1 v/v) for 6 h. The solvent was removed under reduced pressure by a rotary evaporator. The residue was purified by column chromatography (Silica



Scheme 4.

gel 60, 0–3% methanol–chloroform) to give product **6**(**48**) (120 mg, 58%), R_f 0.49 (Sorbfil, chloroform–methanol, 5:1 v/v); ^{1}H NMR (DMSO-d_6), δ, ppm: 11.95 (1H, br s, COOH), 7.55 (2H, d, 7.3 Hz, arom. H), 7.48–7.33 (4H, m, arom. H), 7.32–7.17 (2H, m, arom. H), 6.31 (2H, s, C*H*), 4.03 (2H, t, 6.4 Hz, NCH_2), 3.60 (3H, s, NCH_3), 2.19 (2H, t, 7.0 Hz, CH_2COOH), 1.68 (12H, s, (CH_3)$_2$), 1.80–1.29 (6H, m).

*2.2.4. 2-[1-(5-Carboxypentyl)-3,3-dimethyl-5-sulfo-2,3-dihydro-1H-2-indolylidenmethyl]-3-dicyanomethylene-4-(1,3,3-trimethyl-3H-2-indoliumylmethylene)-1-cyclobuten-1-olate (triethylammonium salt) **7**(**88**)*

A mixture of 150 mg (0.36 mmol) of triethylammonium 3-dicyanomethylene-4-oxo-2-(1,3,3-trimethyl-2,3-dihydro-1*H*-2-indolylidenmethyl)-1-cyclobuten-1-olate (**15**) and 200 mg (0.39 mmol) of 1-(5-carboxypentyl)-2,3,3-trimethyl-3*H*-5-indoliumsulfonate (**12e**) containing 31% of KBr was refluxed in 20 ml 1-butanol–toluene (1:1 v/v) mixture for 5 h. After cooling, the solvent was removed under reduced pressure and the raw product was purified by column chromatography (Silica gel 60 RP-18, 0–50% methanol–water) to give 46 mg (17%) of product **7**(**88**). ^{1}H NMR (DMSO-d_6), δ, ppm: 7.72 (1H, s, arom.), 7.64 (1H, d, 8.5 Hz, arom.), 7.55 (1H, d, 7.9 Hz, arom.), 7.47–7.36 (2H, m, arom.), 7.35–7.21 (2H, m, arom.), 6.32 (2H, s, C*H*), 4.03 (2H, t, 7.1 Hz, NCH_2), 3.62 (3H, s, NCH_3), 3.10 (6H, q, 14.4, 7.0 Hz, N(CH_2CH$_3$)$_3$), 2.18 (2H, t, 7.0 Hz, CH_2COOH), 1.69 (12H, s, (CH_3)$_2$), 1.83–1.33 (6H, m), 1.19 (9H, t, 7.3 Hz, N(CH$_2$CH_3)$_3$).

*2.2.5. 2-[2-Butylamino-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclobutenylidenmethyl]-1,3,3-trimethyl-3H-indolium iodide **8**(**90**)*

To a suspension of 300 mg (0.92 mmol) of 3-butoxy-4-(1,3,3-trimethyl-2,3-dihydro-1*H*-2-indolylidenmethyl)-3-cyclobutene-1,2-dione (**14**) in 10 ml of ethanol were added 120 μl (1.22 mmol) of *n*-butylamine and this mixture was refluxed for 30 min. The solvent was removed under reduced pressure and crystallized product **16** (290 mg, 97%) was used without further purification. R_f 0.30 (Sorbfil, chloroform–methanol, 100:1 v/v); Analysis for **16**: N, 8.96 $C_{20}H_{24}N_2O_2$ requires N, 8.64%; ^{1}H NMR (DMSO-d_6), δ, ppm: 8.43 (1H, br s, NH), 7.33 (1H, d, 7.3 Hz, arom. H), 7.23 (1H, td, 1.1, 7.6 Hz, arom. H), 7.00 (1H, d, 7.9 Hz, arom. H), 6.96 (1H, t, 7.3 Hz, arom. H), 5.48 (1H, s, C*H*), 3.63 (2H, q, 6.7, 13.2 Hz, NHCH_2), 3.33 (3H, s, NCH_3), 1.68–1.49 (2H, m, CH_2), 1.58 (6H, s, (CH_3)$_2$), 1.48–1.24 (2H, m), 0.92 (3H, t, 7.3 Hz, CH_3). A mixture of 150 mg (0.46 mmol) of **16** and 150 mg (0.50 mmol) of 1,2,3,3-tetramethyl-3*H*-indolium iodide (**12a**) was refluxed in 20 ml of a 1-butanol–toluene mixture (1:1 v/v) for 12 h. The solvent was removed by a rotary evaporator. The residue was column purified (Silica gel 60, 0–1% methanol–chloroform) to give product **8**(**90**). Yield: 50 mg (18%); R_f 0.56 (Sorbfil, chloroform–methanol, 85:15 v/v); Analysis: N, 6.84 $C_{32}H_{38}IN_3O$ requires N, 6.92%; ^{1}H NMR (DMSO-d_6), δ, ppm: 9.08 (1H, t, 5.5 Hz, NH), 7.56 (2H, t, 7.0 Hz, arom. H), 7.49–7.35 (4H, m, arom. H), 7.35–7.16 (2H, m, arom. H), 6.03 (1H, s, C*H*), 5.79 (1H, s, C*H*), 3.78 (2H, t, 7.0 Hz, NHCH_2), 3.73 (3H, s, NCH_3), 3.65 (3H, s, NCH_3), 1.85–1.61 (2H, m, CH_2), 1.68 (12H, s, (CH_3)$_2$), 1.60–1.40 (2H, m), 0.99 (3H, t, 7.3 Hz, CH_3); FAB-MS (NBA) *m/z* 480 (M–I$^-$); IR (KBr) 1748 (w), 1708 (w), 1632, 1608, 1556 cm^{-1}.

*2.2.6. 2-[2-Diethylamino-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclobutenylidenmethyl]-1,3,3-trimethyl-3H-indolium hydrogensulfonate **10**(**91**)*

To the 0.5 g (1.18 mmol) of squaraine **9**(**74**), which was dissolved in 10 ml of chloroform, was added dimethyl sulfate (5.5 ml, 58.00 mmol). The obtained mixture was then heated at reflux for 7.5 h. The solvent was removed by a rotary evaporator and 200 ml of diethyl ether was added to the residue and scratched. Then the ether solution was decanted and raw product was purified by flash chromatography (Silica gel 60, 1–15% methanol–chloroform) to give 200 mg of **17** and 140 mg of starting dye **9**. ^{1}H NMR (DMSO-d_6) for **17**, δ, ppm: 7.60 (2H, d, 7.2 Hz, arom. H), 7.55–7.39 (4H, m, arom. H), 7.32 (2H, t, 7.0 Hz, arom. H), 5.87 (2H, s, C*H*), 4.68 (3H, s, OCH_3), 3.73 (6H, s, NCH_3), 3.39 (3H, s, CH_3OSO$_3^-$), 1.65 (12H, s, (CH_3)$_2$). One hundred milligrams (0.18 mmol) of dye **17** was dissolved in 10 ml of dichloromethane. Two millilitres of diethylamine solution (approximately one drop per millilitre CH_2Cl_2) was added dropwise to the stirred mixture while the starting dye is present (TLC monitoring). The solvent was then evaporated and the residue was column purified (Silica gel 60, 1–15% methanol–chloroform) to give the title dye **10**(**91**). Yield: 68 mg (65%); R_f 0.42 (Sorbfil, chloroform–methanol, 85:15 v/v); Analysis: N, 7.47; S, 5.62 $C_{32}H_{39}N_3O_5S$ requires N, 7.27, S, 5.55%; ^{1}H NMR (DMSO-d_6), δ, ppm: 7.56 (2H, d, 7.3 Hz, arom. H), 7.50–7.38 (4H, m, arom. H), 7.35–7.22 (2H, m, arom. H), 5.71 (2H, s, C*H*), 3.90–3.70 (4H, m, NCH_2), 3.68 (6H, s, NCH_3), 1.61 (12H, s, (CH_3)$_2$), 1.43 (6H, t, 7.2 Hz, N(CH$_2$CH_3)$_3$).

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 DMF. Stock solutions of proteins (BSA, HSA, ovalbumin, AVI, lysozyme and trypsin) were prepared by their dissolving in 0.05 M Tris–HCl buffer (pH 8.0). Their concentrations in stock solutions were equal to 0.2 mg/ml.

2.4. Preparation of working solutions

Working solutions of free dyes were prepared by dilution of the dye stock solution in either buffer or methanol. Working solutions of dyes in presence of proteins were prepared by adding the dye stock solution in proteins stock solution. The concentrations of dye and proteins in working solutions amounted to 5×10^{-6} M and 0.2 mg/ml, respectively. All working solutions were prepared immediately before the experiments.

2.5. Spectroscopic measurements

Absorption spectra were recorded on a spectrophotometer Specord M40 (Carl Zeiss, Germany). Fluorescence excitation and emission spectra were taken on a fluorescence spectrophotometer Cary Eclipse (Varian, Australia). Spectroscopic measurements were performed in standard quartz cells (1 × 1 cm). All the measurements were carried out at room temperature.

3. Results and discussion

3.1. Synthesis

The synthesis of squaraine dyes **1**, **3**–**10** is shown in Schemes 1–4. Symmetrical 3-dicyanomethylene-substituted dye **1** was synthesised by condensation of the triethylammonium 2-butoxy-3-dicyanomethylene-4-oxo-1-cyclobuten-1-olate (**11**) with two equivalents of 1,2,3,3-tetramethyl-3*H*-indolium iodide (**12a**) under reflux in a 1-butanol–pyridine (1:1 v/v) or 1-butanol–toluene (1:1 v/v) mixture (Scheme 1).

Unsymmetrical squaraine dyes were synthesised by a multistep reaction. In the first stage 3-butoxy-4-(1,3,3-trimethyl-2,3-dihydro-1*H*-2-indolylidenmethyl)-3-cyclobutene-1,2-dione (**14**) was prepared by the procedure described in Refs. [15,16]. The **14** was reacted with the quaternized indolenine **12e** in boiling pyridine to give 3-oxo-substituted dye **3**, or alternatively with malononitrile and triethylamine in alcohol at room temperature to give triethylammonium 3-dicyanomethylene-4-oxo-2-(1,3,3-trimethyl-2,3-dihydro-1*H*-2-indolylidenmethyl)-1-cyclobuten-1-olate (**15**) [9]. Then **15** was condensed under reflux in a 1-butanol–toluene (1:1 v/v) mixture with the appropriate benzoxazole (**12b**), benzothiazole (**12c**) or indolenine (**12d**, **12e**) quaternized salts to 3-dicyanomethylene-substituted dyes **4**–**7** (Scheme 2).

Kim et al. [17] have recently suggested to synthesise amino-squaraines (squaric ring oxygen is substituted by an alkylaminogroup) by a reaction of monoalkylamino-squarates **18** with 1-ethyl-2,3,3-trimethylindolenium iodide (**12f**) in a boiling 1-butanol–benzene (4:1 v/v) mixture (Scheme 3). The yield was only 15%. This method allows synthesising only symmetrical squaraines **19**.

We synthesised amino-squarylium dye **8** by refluxing of mono-squaraine **16** with indolenine **12a** in a 1-butanol–toluene mixture (1:1 v/v) for 12 h. This method affords to synthesise not only symmetrical but also unsymmetrical dyes. The mono-squaraine **16** was synthesised by reflux of **14** with *n*-butylamine in ethanol.

Alternatively amino-squarylium dyes can be obtained by methylation of squaraine **9** with dimethyl sulfate in chloroform [13] followed by nucleophilic substitution of the squarate methoxy group with a primary or secondary amine. According to this method diethylamino-substituted dye **10**(**91**) was obtained with good yield. It is worth mentioning that the methoxy group substitution was accompanied by demethylation of the methyl sulfate counter ion (Scheme 4). This fact was confirmed by ^{1}H NMR and element analysis for product **10**(**91**).

Squarylium dye **10** is better soluble in aqueous media as compared to **8**. While spectral properties of amino-squaraine **8**(**90**), which contain iodine as counter ion, cannot be measured in aqueous solutions, squaraine **10**(**91**) containing hydrogen-sulfate counter ion is soluble enough to provide the spectral measurements.

3.2. Spectroscopic characterisation of free dyes in methanol and buffer

Spectroscopic characteristics of series of 3-oxo- and 3-dicyanomethylene substituted squarylium dyes in methanol and aqueous buffer are presented in Table 1. In methanol solutions only one absorption band within the 500–750 nm region was observed for studied squaraines. The positions of absorption maxima situated between 622 and 698 nm.

In aqueous buffer noticeable changes in the absorption spectra of the squaraines occurred. For 3-oxo-substituted indolenine-based squaraines (**74**, **90**, **91**, and **97**) and 3-dicyanomethylene

Table 1
Spectral characteristics of squaraines in methanol, aqueous buffer, and in presence of BSA

Dye	Methanol	Buffer				In presence of BSA				
	λ_{abs} (nm)	λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	I_0 (a.u.)	λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	I^{BSA} (a.u.)	I^{BSA}/I_0
80	661	636 685[a]	690	700	11	637[a] 680	684	698	502	46
48	665	616[a] 646	654	669	84	630[a] 678	689	708	4246	51
88	670	617[a] 656	661	675	199	672	683	696	2850	24
46	625	605 653[a]	622	634	9	601[a] 647	654	671	1723	191
83	665	676	688	702	1	676	686	702	121	121
74	622	617	621	629	125	630	637	646	6500	52
97	630	620	632	643	269	625	646	655	6573	24
90	640	636	640	652	43	643	661	669	2991	70
91	654	649	650	676	9	650	679	688	1255	139

λ_{abs}, absorption maxima; λ_{ex}, excitation wavelengths; λ_{em}, fluorescence maxima; I_0 (I^{BSA}), fluorescence intensity of dye in buffer (and in presence of BSA).
[a] Absorption spectra shoulders.

benzothiazole-based dye **83**, "monomer" bands could be clearly distinguished in aqueous solutions. In buffer solutions absorption maxima for these dyes were shifted to the short-wavelength region by 5–11 nm as compared to their methanol solutions. In absorption spectra of indolenine and benzoxazole-based 3-dicyanomethylene-substituted dyes (**80**, **48**, **88** and **46**) measured in buffer intensive short-wavelength bands, corresponding to H-aggregates, appeared (Fig. 1). For dyes **80** and **46** these new bands were more intensive than that for the monomer forms.

Emission of the dyes was excited at the wavelength of corresponding monomer absorption maxima. Fluorescence spectra maxima of studied squaraines in buffer were between 629 and 702 nm, such red-shifted emission spectra are typical for 3-oxo- and 3-dicyanomethylene-substituted squarylium dyes (Fig. 2) [10]. All the dyes in aqueous buffer show weak fluorescence. The highest emission intensities were found for indolenine-based squaraines containing sulfo (**88**) and *N*-carboxyalkyl groups (**97**) as the substituents.

3.3. Spectroscopic characterisation of squarylium dyes in the presence of various proteins

Spectroscopic properties of studied squaraines in the presence of proteins are represented in Tables 1 and 2. For the 3-dicyanomethylene-substituted indolenine and benzoxazole-based dyes **80**, **48**, **88**, and **46** interaction with protein causes redistribution of intensities of bands that is attributed to aggregate and monomer forms (Fig. 1). Thus in absorption spectra of these dyes the ''monomer'' band becomes more pronounced and for the dye **88** aggregation band even disappeared. Similarly to buffer, in absorption spectra of squaraines **83**, **97**, **90,** and **91** only one band, corresponding to the monomer absorption maxima, was observed. Emission of studied squarylium dyes was excited on the wavelength of corresponding monomer absorption maxima. The presence of proteins resulted in red-shift of fluorescence maxima up to 47 nm as compared to the free dye in buffer (Fig. 2).

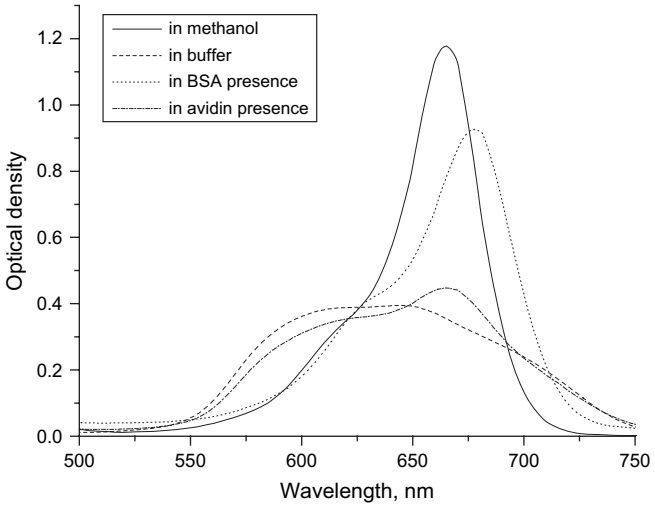


Fig. 1. Absorption spectra of 5×10^{-6} M of dye **48** in methanol, buffer, and also in the presence of BSA (0.2 mg/ml) and avidin (0.2 mg/ml).

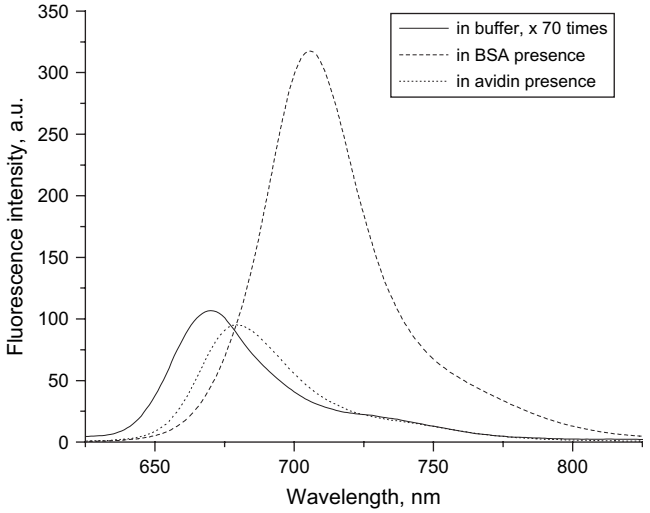


Fig. 2. Profiles of fluorescence spectra of squarylium dye **48** (5×10^{-6} M) and in presence of proteins (0.2 mg/ml BSA and 0.2 mg/ml avidin). The low-intensive spectrum of free dye is multiplied in 70 times (a.u., arbitrary units). Fluorescence was excited at 600 nm.

3.3.1. BSA

Spectral characteristics of the squaraines in the presence of BSA are presented in Table 1. For the studied squarylium dyes fluorescence intensity is 24–191-fold enhanced in presence of BSA (Fig. 2). The most pronounced emission increase was observed for the dyes **46**, **83** and **91**. Majority of studied squaraines showed bright fluorescence but top emission intensity values (6500 and 6573 a.u.) was noticed for the 3-oxo-substituted indolenine-based squarylium dyes, in particular for **74** and **97** squaraines. It is worth mentioning that emission intensities were significantly higher for 3-oxo- than for corresponding 3-dicyanomethylene-substituted squarylium dyes (compare **74** and **80**, **97** and **88** in Table 1). Furthermore, about of 50-fold fluorescence intensity increase upon interaction with BSA was noticed for all the 3-oxo-substituted indolenine-based squarylium dyes, which makes them an attractive tool for selective protein detection.

3.3.2. HSA, ovalbumin and avidin

Selected spectral properties of the squaraines in presence of albumins (HSA, ovalbumin) and avidin are shown in Table 2. Behaviour of the dyes in the presence of these proteins is similar to that in presence of BSA. Thus the main attention is given just to intensity of the fluorescent response and selectivity of studied squaraines to different proteins. Interaction of squarylium dyes with HSA, ovalbumin and AVI resulted in lesser fluorescent response than that in case of BSA. In presence of these proteins fluorescence intensity increased up to 24 times.

The dyes **74** and **88** demonstrated in complexes with HSA both bright fluorescence (about 1200–1400 a.u.) and emission increase in HSA presence up to 10 and 7.3 times, correspondingly. For unsymmetrical 3-dicyanomethylene-substituted dye **83** the highest emission increase in the presence of HSA up to

Table 2
Spectral characteristics of squaraines in presence of HSA, ovalbumin, AVI, lysozyme and trypsin

Dye	In HSA presence		In ovalbumin presence		In avidin presence		In lysozyme presence		In trypsin presence	
	λ_{em} (nm)	I^{HSA} (a.u.)	λ_{em} (nm)	I^{OVA} (a.u.)	λ_{em} (nm)	I^{AVI} (a.u.)	λ_{em} (nm)	I^{LYZ} (a.u.)	λ_{em} (nm)	I^{TRYP} (a.u.)
80	691	245	690	120	686	13	696	12	694	5
48	698	368	692	159	681	1318	690	40	670	47
88	698	1455	697	841	688	2473	692	268	677	261
46	654	112	656	70	647	154	641	14	631	9
83	689	31	694	12	683	8	691	4	697	2
74	645	1228	647	879	634	170	631	140	631	133
97	653	1273	650	410	650	1787	649	487	641	323
90	667	434	667	546	663	82	663	68	652	45
91	683	131	681	144	681	15	676	11	675	10

λ_{em}, maximum of fluorescence emission band; I^{HSA} (I^{OVA}, I^{AVI}, I^{LYZ}, I^{TRYP}), fluorescence intensity of dye in presence of HSA (ovalbumin, avidin, lysozyme, trypsin).

44 times was observed, but fluorescence intensity of this dye is not enough to use it in detection systems.

Only two of the studied dyes 3-oxo-substituted **74** and **90** in complexes with ovalbumin demonstrated both moderate fluorescence intensity (880 and 546 a.u.) and noticeable emission increase. The top emission increase (up to 16.7 times) was observed for symmetrical indolenine-based squaraines **80**, **90** and **91** but fluorescence intensity of these dyes remained quite low. Attention should be drawn to the fact that fluorescence enhanced greater for symmetrical indolenine-based squaraines with non-substituted heterocycle in complexes with HSA or ovalbumin than for squaraines containing substituents in the heterocycle or *N*-carboxyalkyl chain.

Only squarylium dyes **48**, **88** and **97** containing *N*-carboxyalkyl group gave intensive fluorescent response to the presence of avidin from hen egg white (Fig. 2). In case of these dyes emission intensity reached 1318–2473 a.u., whereas for other squaraines fluorescence intensity was less than 170 a.u. Furthermore, only for above-mentioned squarylium dyes emission intensity increased in avidin presence by 7–16 times, whereas other dyes demonstrated comparatively low fluorescence intensity enhancement (up to two times). Though considerable emission increase observed for unsymmetrical dye **46** (up to 16.7 times) its fluorescence intensity in complexes was quite low.

3.3.3. Lysozyme and trypsin

The squaraine dyes slightly response to the presence of hydrolases lysozyme or trypsin. The top emission intensity increase reached only 3.2 and 1.8 times in cases of lysozyme and trypsin, respectively. Moreover, for the 3-dicyanomethylene-substituted dye **48** the fluorescence intensity decreases as compared to aqueous buffer solutions being admitted in presence of lysozyme as well as for other 3-dicyanomethylene-substituted dyes **80**, **48** and **46** in the presence of trypsin.

4. Conclusions

1. The series of novel symmetric and asymmetric 3-oxo- and 3-dicyanomethylene squaraines containing indolenine, benzothiazole and benzoxazole moiety was synthesised. Spectral-luminescent properties of these dyes in unbound state and in the presence of various proteins were studied.
2. Studied squaraines demonstrate considerable emission increase (up to 190 times) in the presence of BSA. Fluorescence enhancing of the dyes in the presence of other albumins (HSA and ovalbumin) is significantly lower (up to 24 times).
3. Non-substituted symmetrical indolenine-based squaraines in complexes with HSA or ovalbumin show greater enhancement of fluorescence as compared to analogues containing substituents in heterocyclic moiety or *N*-carboxyalkyl group. Symmetrical 3-oxo- indolenine-based squaraine 74 demonstrates sufficient fluorescence increasing (up to 10 times) in the presence of HSA and ovalbumin and forms bright-fluorescent complexes with these proteins.
4. Squarylium dyes containing *N*-carboxyalkyl group give noticeable fluorescent response to the presence of avidin from hen egg. It could be proposed that introduction of carboxyalkyl group in the dye molecule could increase the affinity of dye to AVI.
5. In the presence of hydrolases lysozyme or trypsin studied squaraines either insignificantly increased (up to 3 times) or even decreased their fluorescence intensity.

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