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Synthesis and spectral–luminescent studies of novel 4-oxo-4,6,7,8-tetrahydropyrrolo[1,2-*a*]thieno[2,3-*d*]pyrimidinium styryls as fluorescent dyes for biomolecules detection

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Abstract

A series of novel 4-oxo-4,6,7,8-tetrahydropyrrolo[1,2-*a*]thieno[2,3-*d*]pyrimidinium and 5-oxo-1,2,3,5-tetrahydropyrrolo[2,1-*b*]quinazolinium styryl dyes were synthesized. For preparing of studied dyes the standard method of styrylcyanines synthesis was modified. Spectral–luminescent properties of obtained dyes in free state and in the presence of nucleic acids and BSA were studied. It was shown that *p*-dimethylaminostyryls based on 4-oxo-4,6,7,8-tetrahydropyrrolo[1,2-*a*]thieno[2,3-*d*]pyrimidinium with aliphatic substituents in 2 and 3 positions demonstrated RNA-binding preference. These dyes in the presence of RNA significantly enhance emission intensity and could be used as RNA-specific fluorescent probes. Besides, the fluorescence emission after two-photon absorption of dye–RNA complexes in buffer solutions was measured.

Keywords: Styryl dyes; Nucleic acids detection; Fluorescent probes; Two-photon excitation

1. Introduction

Owing to their unique physico-chemical properties styrylcyanines are successfully applied in various biomedical techniques [1–3]. At present styryl dyes are known to be among the most sensitive probes for unspecific fluorescent detection of proteins in the presence of sodium dodecyl sulfate [1]. Styrylcyanines were also proposed as DNA-specific fluorescent probes [3–6]. Besides, these dyes were suggested to be used in microfluorescence cytology for cell imaging, including whole blood components visualization, due to their ability to penetrate through cell membranes [1–3]. Recently, styrylcyanine dyes were described as fluorescent compounds with high values of two-photon absorption cross-section, and thus could be used in a number of multidisciplinary areas, particularly in multiphoton fluorescence imaging, three-dimensional optical data storage, laser technique, optical sensor protection and photodynamic therapy [7–11].

Earlier the novel styrylcyanines containing imidazo[1,2-*a*]pyridinium moiety were synthesized, and their spectral–luminescent properties were evaluated as well. The moderate intrinsic fluorescence was found for the majority of the studied styryl dyes [12]. Afterwards, a series of homodimer *p*-dimethylaminostyryls based on pyridinium, benzoxazolium, benzothiazolium and 1,3,3-trimethyl-3*H*-indolium residues that are connected with aliphatic linker as potential probes for DNA fluorescent detection were studied. It was revealed that being virtually non-fluorescent, these homodimer dyes selectively interact with dsDNA and demonstrate significant fluorescence intensity enhancement [5,6].

As a continuation of these studies, in the present work we synthesized a series of novel styryl dyes based on the

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4-oxo-thieno[2,3-*d*]pyrimidinium and a convenient method for their synthesis was developed. Thereto the commonly used method of styrylcyanines preparation was improved by using high-boiling solvent, *n*-butanol. We aimed to study spectral–luminescent characteristics of the styrylcyanines both in free state and in the nucleic acids and BSA presence and the influence of various substituents in 2 and 3 positions of dye molecule on these properties of dyes. Besides this, efficiency of two-photon excitation (TPE) of the novel dyes fluorescence was studied.

2. Results and discussion

2.1. Synthesis of styrylcyanines

General scheme of the synthesis of Stp-1–Stp-5 dyes (Fig. 1) is represented in Scheme 1. Derivatives of 2-aminothiophene (**I**) were prepared using the Gewald reaction from ethyl cyanoacetate, sulfur and corresponding ketones (2-butanone, cyclohexanone, 4-*tert*-butylcyclohexanone and 4′-methylacetophenone) according to Ref. [13]. 4,6,7,8-tetrahydropyrrolo[1,2-*a*]thieno[2,3-*d*]pyrimidin-4-ones (**II**) were obtained by interaction between (**I**) and 2-pyrrolidinone (Procedure 1) [14]. Further treatment of the obtained heterocycle with methyl iodide (for Stp-5 with ethyl iodide) in dioxane gave the corresponding quarternary salts (**III**) (Procedure 2). For obtaining the dyes Stp-1–Stp-5 the mixture of corresponding quaternary salt and *p*-dimethylaminobenzaldehyde was refluxed in *n*-butanol in the presence of piperidine (Procedure 3). We failed to synthesize styryl dyes according to the standard condensation procedure in ethanol, and while using the high-boiling solvent *n*-butanol this reaction proceeded in less than 1 h.

To obtain the Sbp dye the similar scheme of the synthesis was applied (Scheme 1). Reaction of ethyl anthranilate with 2-pyrrolidinone gave 1,2,3,5-tetrahydropyrrolo[2,1-*b*]quinazolin-5-one (Procedure 1), and by heating of this heterocycle with methyl iodide the quaternary salt was obtained. To get the dye Sbp the mixture of corresponding quaternary salt and *p*-dimethylaminobenzaldehyde was refluxed in *n*-butanol (Procedure 3). Stp-6 dye was obtained by interaction between (II) (R1, R2 = Me) and *p*-dimethylaminobenzaldehyde according to Ref. [15].

2.2. Spectral properties of free dyes

Spectral–luminescent characteristics of styryl dyes in methanol and aqueous buffer are presented in Table 1. The studied dyes have wide absorption spectral band, which is typical for styryls, with maxima in methanol situated between 477 and 484 nm, except for the dyes Stp-6 (408 nm) and Stp-5 (main maximum at 416 nm with the shoulder at 481 nm). The values of molar extinction coefficients are in the range from $1.9 \times 10^4\ M^{-1}\,cm^{-1}$ to $3 \times 10^4\ M^{-1}\,cm^{-1}$. For the dyes Stp-1–Stp-4 the positions of absorption spectra maxima in buffer are shifted, as compared to those for dyes in methanol, to the short-wavelength region up to 6 nm, while for Sbp styryl the long-wave shift in 3 nm was observed (Fig. 2). The changes in the shape of spectra also were observed for the Stp-6 dye; its absorption maximum hypsochromically shifts on 11 nm, and also the long-wave shoulder at 453 nm appears (Fig. 3). For the Stp-5 dye in aqueous buffer, besides the main band with maximum at 412 nm, additional long- (449 nm) and short-wavelength (394 nm) shoulders were noticed. The presence of several bands in Stp-5 absorption spectrum could be due to the dye ability to form aggregates with different number of monomers and/or monomers' packing structure. Even in small concentrations dye Stp-5 forms aggregates, which were stable to high temperatures.

The excitation maxima position of the studied dyes is shifted to the long-wavelength spectral region up to 8 nm in methanol and 20 nm in buffer relatively to those in absorption spectra. For the majority of styryls fluorescence spectra maxima in methanol are situated in the range of 563–567 nm (for Stp-6 at 502 nm), and the positions of emission maxima in buffer are insignificantly shifted to the long-wavelength region and thus located between 575 and 584 nm (for Stp-6 the main maximum is situated at 557 nm with shoulder at 599 nm)

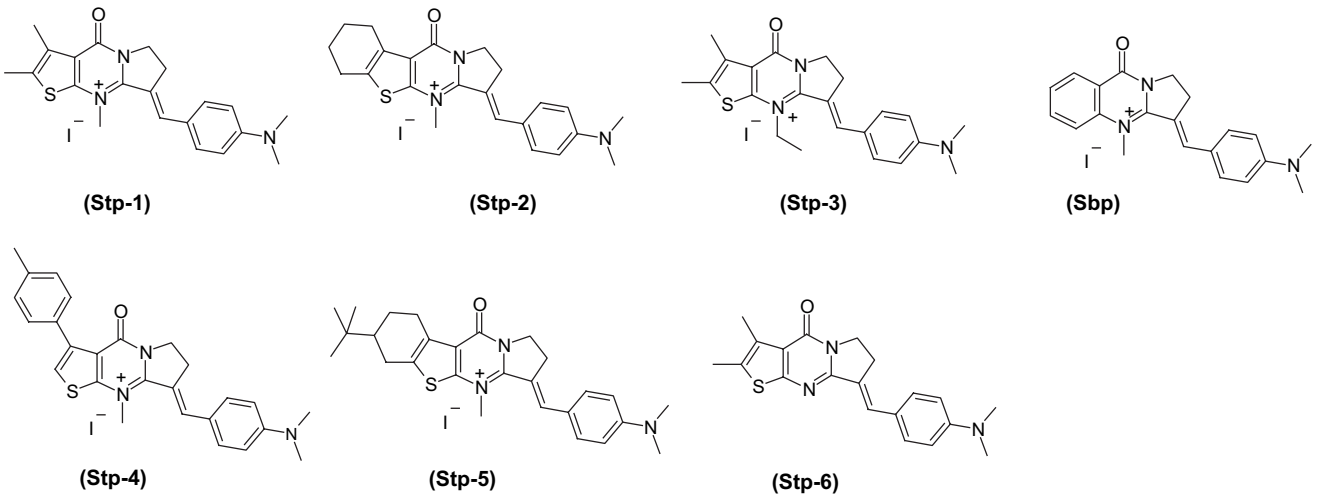
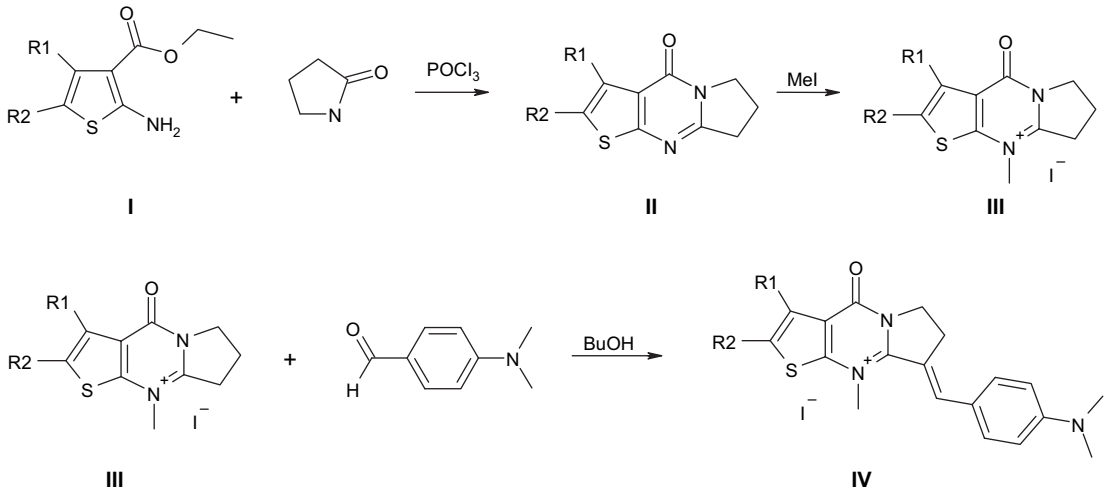


Fig. 1. Structures of novel styryl dyes.



Scheme 1. Synthesis of styryl dyes.

(Fig. 4). Stokes shifts values for the dyes are large (up to 104 nm), that is typical for styryls. For the majority of studied dyes the low fluorescence intensity values were observed, being in the range of 3.3–4.5 a.u. (arbitrary units) in methanol and 1.5–2.9 a.u. in buffer. The exception is the uncharged styryl Stp-6, for which emission intensity values in buffer and in methanol amount to 24.2 and 16.2 a.u., respectively.

2.3. Spectral properties of the dyes in the presence of DNA and RNA

Characteristics of absorption, excitation and emission spectra of the studied dyes in the presence of nucleic acids are summarised in Table 2. Absorption maxima of the studied dyes (except the dye Stp-6) in nucleic acid containing solution are shifted to long-wavelength region up to 8 nm relative to the corresponding maxima of free styryls in buffer and are situated between 477 and 491 nm. For the majority of styryls the shapes of absorption bands remain practically unchanged (Fig. 2). The exception was Stp-6 dye, whose absorption maximum in the presence of DNA was shifted to the short-wave region on 5 nm. Moreover, in the absorption spectra of this styryl in complexes with DNA no spectral shoulders were observed, whereas long-wavelength ones were noticed both for the free dye in buffer and in the presence of RNA. Besides this, for the styryl Stp-5 in the presence of DNA the absorption bands intensities redistribution was observed, namely intensity increasing of the short-wavelength band (at 397 nm) and decreasing of the long-wavelength one (at 452 nm), as well as the appearing of the shoulder situated at 517 nm.

For the presented dyes in nucleic acid complexes excitation maxima are strongly bathochromically shifted relatively to the absorption maxima (up to 47 nm in the presence of DNA and up to 67 nm in the presence of RNA). The shift values in the presence of nucleic acids significantly exceed those observed for free styryls.

For the Stp-1–Stp-5 and Sbp styryl dyes emission maxima in the presence of DNA/RNA are situated between 583 and 596 nm (Fig. 4). It should be noticed that the fluorescence

Table 1
Spectral–luminescent characteristics of styryl dyes in methanol and buffer

Name	Free dye							
	In methanol				In buffer			
	λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	I_m (a.u.)	λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	I_0 (a.u.)
Stp-1	477	485	564	4.3	472	487	575	2.2
Stp-2	480	488	566	4.5	474	493	578	2.9
Stp-3	480	486	563	3.7	474	492	576	2.4
Stp-4	484	491	567	4.2	483	503	583	2.3
Stp-5	416				394[a]			
	481[a]	490	565	3.3	412	490	576	1.5
					449[a]			
Stp-6	408	411	502	24.2	397	389	557	16.2
					453[a]		599[a]	12.4[a]
Sbp	484	492	564	3.8	487	496	584	2.0

λ_{abs}, λ_{ex}, λ_{em} – maximum wavelengths of absorption, fluorescence excitation and emission spectra; I_m – flourescent intensity of the dye in methanol; I_0 – dye intrinsic fluorescent intensity.
[a] Band manifested as shoulder.

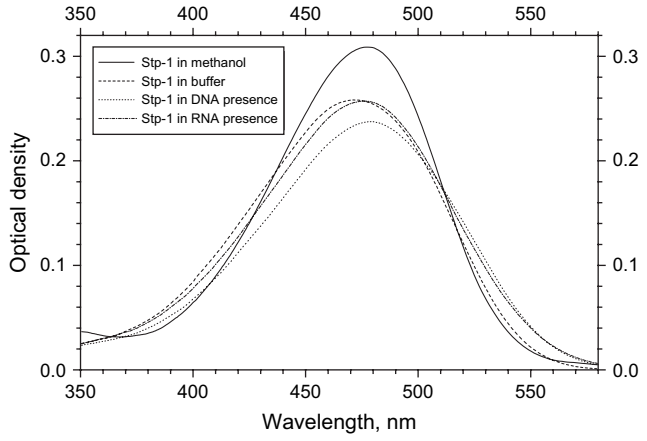


Fig. 2. Absorption spectra of Stp-1 dye in methanol, buffer, and also in the presence of DNA and RNA.

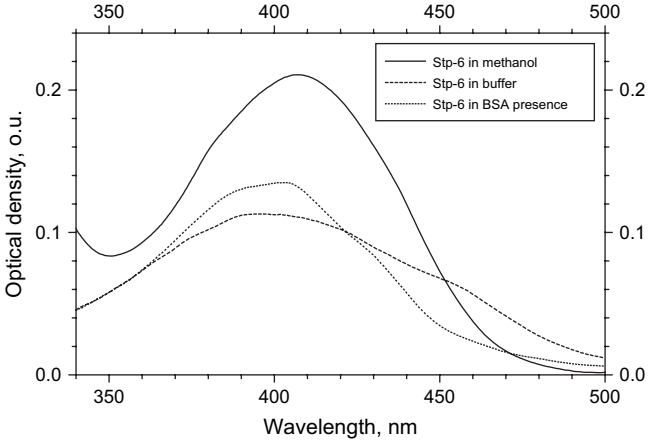


Fig. 3. Absorption spectra of Stp-6 dye in methanol, buffer, and in the presence of BSA.

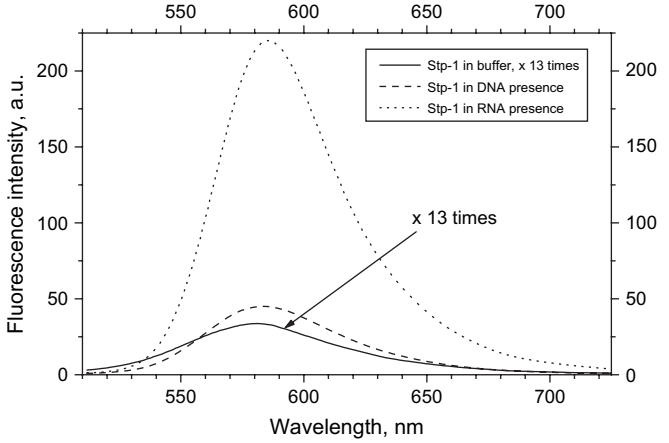


Fig. 4. Profiles of fluorescence spectra of dye Stp-1 in unbound state and in the presence of nucleic acids. The low-intensive spectrum of free dye is multiplied in 13 times (a.u., arbitrary units).

intensity increase in the presence of RNA significantly surpassed those values for dye–DNA complexes. For comparison, I^{DNA}/I_0 values were not large (13.1–26.5 times), whereas studied dyes emission upon RNA binding increased in 61.7–133 times. The only exception was Sbp dye based on quinazolinium heterocycle, for which the fluorescence intensity enhancement in the presence of DNA and RNA is almost similar and amounts to 20 and 23 times, respectively. Hence the styryl dyes based on thieno[2,3-*d*]pyrimidin-4-one, as opposed to dyes based on quinazolinium heterocycle, demonstrate the RNA-specificity.

Moreover, the dyes with aliphatic substituents in 2 and 3 positions of 4-oxo-4,6,7,8-tetrahydropyrrolo[1,2-*a*]thieno[2,3-*d*] pyrimidinium appear to be more RNA-selective and possess higher value of fluorescence intensity increase in complexes with RNA, than the ones with aromatic substituent.

Emission spectra maxima for styryl dye Stp-6 in the presence of nucleic acids are situated at 554 and 519 nm in case of DNA and RNA, respectively. It should be admitted, that this styryl showed the lowest value of the fluorescence intensity among the studied dyes in both free state and in the presence of nucleic acids.

2.4. Spectral properties of the dyes in the presence of BSA

Spectral characteristics of the dyes in the presence of BSA are represented in Table 2. Absorption spectra maxima of the studied dyes in the presence of BSA are located mostly at the same spectral region as corresponding absorption maxima for free styryls, namely between 472 and 487 nm; while for the dye Stp-6 this maximum is situated at 402 nm (Fig. 3).

For the styryl dyes Stp-1–Stp-5 and Sbp excitation maxima positions in the presence of BSA are shifted to the long-wavelength region relatively to the corresponding absorption maxima (up to 44 nm). Fluorescence maxima of these dyes are situated between 570 and 580 nm. Concerning Stp-6, emission maxima position for this dye in complex with BSA is shifted to the short-wavelength region in 77 nm, whereas in the case of nucleic acids these shifts were equal to 3 nm for DNA and 38 nm for RNA.

The fluorescence intensity enhancement of the studied dyes in the presence of BSA is between 1.5 and 9.2 times thus being insignificant. Moreover, emission intensity for the majority of styryls in the presence of BSA does not exceed 13.8 a.u. The exception is the uncharged styryl Stp-6, which demonstrates both high fluorescence intensity value (1168 a.u.) and noticeable emission increasing (I^{BSA}/I_0) value (72 times).

2.5. Two-photon excitation study

As it was mentioned above, the styryl dyes Stp-1, Stp-2, Stp-3, and Stp-5 in the presence of RNA demonstrate the highest fluorescence intensity enhancement and significant preference to RNA as compared to DNA is observed. Hence these dyes are considered to be applicable for the specific detection of RNA. On the other hand, the styryl dyes typically could be efficiently excited by the two-photon excitation. Thus for the dyes Stp-1, Stp-2, Stp-3, and Stp-5 the fluorescent properties of the dye–RNA complexes upon two-photon excitation by YAG:Nd^{3+} laser with wavelength 1064 nm were studied.

For the dyes Stp-1, Stp-2, Stp-3, and Stp-5 in the presence of RNA the intensive fluorescence spectra were registered under the two-photon excitation (Fig. 5). For Stp-2, Stp-3 and Stp-5 dyes the position of fluorescence maxima upon two-photon excitation is close to this of the single-photon excited emission spectra. In the case of styryl dye Stp-1, the 19 nm long-wavelength shift of TPE fluorescence spectra maximum wavelength relatively to the SPE emission maximum was observed. The intensity of TPE fluorescence of studied styryl–RNA complexes is of the same order as the one in ethanol solution of Rhodamine 6G that was measured as the reference dye. Therefore we believe that the investigated styrylcyanines are promising to be applied as fluorescent probes for RNA detection upon TPE.

3. Conclusions

1. For the first time, a series of styrylcyanines based on 4-oxothieno[2,3-*d*]pyrimidinium and 5-oxo-quinazolinium

Table 2
Spectral–luminescent characteristics of styryl dyes in nucleic acids and BSA presence

Name	In the presence of DNA					In the presence of RNA					In the presence of BSA				
	λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	I^{DNA} (a.u.)	I^{DNA}/I_0	λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	I^{RNA} (nm)	I^{RNA}/I_0	λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	I^{BSA} (a.u.)	I^{BSA}/I_0
Stp-1	480	525	585	44.4	20	477	544	587	235	99	472	505	572	5.3	2.4
Stp-2	481	528	587	52.4	18	480	542	588	234	80.7	473	517	571	11	3.8
Stp-3	480	526	583	31.5	13.1	481	545	589	250	104	474	508	573	5.6	2.3
Stp-4	488	534	591	61	26.5	488	547	593	142	61.7	485	518	572	12	5.2
Stp-5	397					396									
	452[a]	531	585	37	24.7	415	544	588	170	133		511	570	13.8	9.2
	517[a]					449[a]									
Stp-6	392	393	554	17.4	1.07	394	428	519	21.3	1.31	402	400	480	1168	72
						449[a]									
Sbp	491	531	591	40.1	20	488	548	596	46.1	23	487	502	580	2.9	1.5

λ_{abs}, λ_{ex}, λ_{em} – maximum wavelengths of absorption, fluorescence excitation and emission spectra; I_0 – dye intrinsic fluorescence intensity; $I^{(DNA/RNA/BSA)}$ – dye fluorescence intensity in the presence of DNA/RNA/BSA; I^{DNA}/I_0 (I^{RNA}/I_0, I^{BSA}/I_0) – enhancement of dye fluorescence intensity in the presence of DNA/RNA/BSA.

[a] Band manifested as shoulder.

heterocyclic system were synthesized. Spectral–luminescent properties of obtained dyes in free state and in the presence of DNA, RNA and BSA were studied.

2. Fluorescence intensity of the 4-oxo-thieno[2,3-*d*]pyrimidinium dyes in RNA complexes is up to 8 times higher than that in the corresponding complexes with DNA. The styryls that demonstrated the brightest emission in the presence of RNA (Stp-1, Stp-2 and Stp-3) could be proposed as fluorescent dyes for specific RNA detection.
3. In opposite to the styryl dyes containing charged 4-oxo-thieno[2,3-*d*]pyrimidinium heterocycle having strong RNA preference, styryl based on 5-oxo-quinazolinium heterocycle demonstrate the same fluorescence intensity in DNA and RNA complexes.
4. Fluorescence intensity of the 3-aryl substituted 4-oxo-4,6,7,8-tetrahydropyrrolo[1,2-*a*]thieno[2,3-*d*]pyrimidinium dye Stp-4 in the presence of RNA was considerably lower than that of dyes with aliphatic substituents in 2 and 3 position. The dye Stp-5 containing bulky aliphatic substituent demonstrated increased tendency to aggregates' formation when compared to other dyes.
5. Dye Stp-6 in the presence of BSA demonstrated bright fluorescence (1168 a.u.) and 72-fold emission intensity increase, while the presence of nucleic acids slightly changes its intrinsic fluorescence.
6. Fluorescence spectra after two-photon absorption of the 1064 nm radiation of YAG:Nd^{3+} 20 ns pulsed laser were obtained for dyes Stp-1, Stp-2, Stp-3, Stp-5 in the presence of RNA. Investigated styrylcyanines are perspective to be successfully used as probes for RNA detection upon TPE.

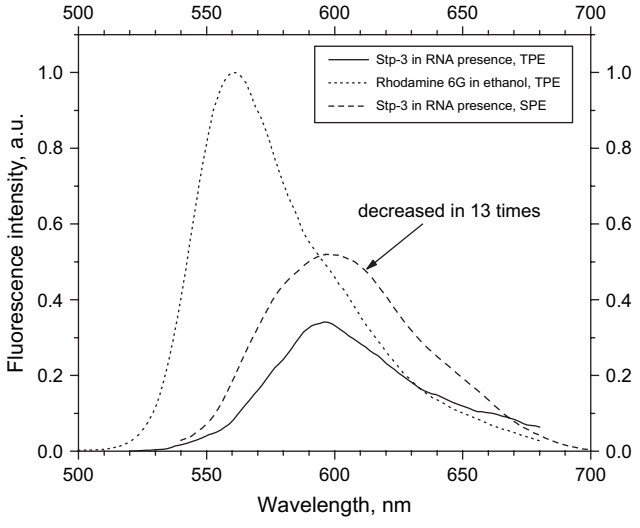


Fig. 5. Fluorescence spectra of styryl Stp-3 in the presence of RNA upon SPE and TPE, and of Rhodamine 6G upon TPE. Single-photon excitation is at 532 nm, two-photon excitation at 1064 nm. The intensity of SPE spectrum is decreased in 13 times.

4. Experimental

The styryl dimer dyes were synthesized according to the procedures described below. Structures of obtained compounds were confirmed with ^{1}H NMR and element analysis.

4.1. Procedure 1: preparation of heterocycles

To the solution of 0.04 mol of (**I**) and 0.044 mol of 2-pyrrolidinone in 30 ml of absolute dichloroethane under cooling 4 ml of $POCl_3$ was added. A reaction mixture was refluxed for 20 min, after cooling, 5 g of sodium acetate dissolved in 30 ml of water was added and was refluxed for 20 min. Organic layer was separated and aqueous layer was extracted with dichloroethane. Both extracts were washed with water, dichloroethane was vacuum stripped and precipitate was recrystallized from alcohol.

4.2. Procedure 2: preparation of quaternary salts of heterocycles

A mixture of 0.001 mol of substituted 4,6,7,8-tetrahydropyrrolo[1,2-*a*]thieno[2,3-*d*]pyrimidin-4-one (**II**) and 0.1 ml (0.0016 mol) of methyl iodide in 3 ml of dioxane was refluxed for 8 h. After cooling, the product was filtered and washed

with diethyl ether. Quaternary salts were used without further purification.

4.3. Procedure 3: preparation of styryl dyes

A mixture of 0.001 mol of quaternary salt (**III**), 0.0011 mol of *p*-dimethylaminobenzaldehyde and 5 drops of piperidine in 4 ml of *n*-buthanol was heated for 1 h. The precipitate was filtered off, washed with isopropanol and ether and then crystallized from methanol.

4.4. Spectroscopic studies

Absorption spectra were obtained with Specord M 40 spectrophotometer (Carl Zeiss, Germany). Fluorescence excitation and emission spectra were obtained with a Cary Eclipse fluorescence spectrophotometer (Varian, Australia). Spectroscopic measurements were performed in standard quartz cells (1×1 cm). All measurements were carried out at room temperature. The ^{1}H NMR spectra were recorded in DMSO-d_6 using the ''Varian'' (300 MHz) instrument with TMS as an intrinsic standard; coupling constants are quoted in Hz.

Two-photon excited fluorescence measurements were carried out with the usage of YAG:Nd^{3+} laser generating 15 ns pulses at a repetition rate about 6 Hz. Fluorescence light was detected at right angle as it passed the telecentric system of lenses and the entrance slit (2 mm) of the monochromator (Cherney–Turner scheme with the grid 600 lines/mm). Behind the exit slit (2 mm) of monochromator the light was directed to the photomultiplier tube, then the signal was amplified and measured. For the measurement of the single-photon excited fluorescence spectra the second harmonic generator (efficiency of conversion 10%) was used. Both single- and two-photon excited fluorescence spectra were corrected for the sensitivity of registration system.

4.5. Preparation of stock solutions

The 2×10^{-3} M dye stock solutions were prepared by dilution of the dye in DMFA. Total DNA from salmon testes, total yeast RNA, and BSA were purchased from Sigma. Stock solutions of nucleic acids (DNA, RNA) and BSA were prepared by dissolving them in 0.05 M Tris–HCl buffer (pH 8.0). The concentrations of nucleic acids and BSA in stock solutions were 6×10^{-3} M base pairs (bp) for DNA, 1.2×10^{-2} M bases for RNA, and 0.2 mg/ml for BSA.

4.6. 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 dye–DNA (or RNA) complexes were prepared by mixing an aliquot of the dye stock solution and an aliquot of DNA/RNA stock solution in a buffer. Working solutions of dyes in the presence of BSA were prepared by adding the dye stock solution in protein stock solution. The concentrations of dye, DNA, RNA and BSA in working solutions were equal to 5×10^{-6} M, 6×10^{-5} M bp, 1.2×10^{-4} M bases and 0.2 mg/ml, respectively. For the two-photon measurements the concentration of styryls in working solutions was 1.5×10^{-5} M; RNA concentration was 3.6×10^{-4} M bases; the concentration of Rhodamine 6G in working solutions was 5×10^{-6} M.

4.7. ^{1}H NMR spectra and CHN characteristics of synthesized dyes

4.7.1. 8-[1-(4-Dimethylaminophenyl)methylidene]-2,3,9-trimethyl-4-oxo-4,6,7,8-tetrahydropyrrolo[1,2-a]thieno[2,3-d]-pyrimidin-9-ium iodide (Stp-1)

Yield: 74%; m.p. (dec.): 218–220 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 2.43 (3H, s), 2.48 (3H, s), 3.06 (6H, s), 3.32 ($2H + H_2O$, m), 4.17–4.27 (5H, m), 6.85 (2H, d, $J = 8.3$), 7.61 (2H, d, $J = 8.3$), 7.87 (1H, s). Anal. calcd. for $C_{21}H_{24}IN_3OS$: C, 51.12; H, 4.90; N, 8.52. Found: C, 51.23; H, 4.95; N, 8.58.

4.7.2. 1-[1-(4-Dimethylaminophenyl)methylidene]-11-methyl-5-oxo-1,2,3,5,6,7,8,9-octahydrobenzo[4,5]thieno[2,3-d]pyrrolo[1,2-a]pyrimidin-11-ium iodide (Stp-2)

Yield: 68%; m.p.: 242–244 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 1.83 (4H, m), 2.89 (4H, m), 3.07 (6H, s), 4.15–4.34 (5H, m), 6.85 (2H, d, $J = 8.1$), 7.62 (2H, d, $J = 8.1$) 7.89 (1H, s). Anal. calcd. for $C_{22}H_{26}IN_3OS$: C, 53.18; H, 5.04; N, 8.09. Found: C, 53.41; H, 4.98; N, 8.17.

4.7.3. 8-[(E)-1-(4-Dimethylaminophenyl)methylidene]-9-ethyl-2,3-dimethyl-4-oxo-4,6,7,8-tetrahydropyrrolo-[1,2-a]thieno[2,3-d]pyrimidin-9-ium iodide (Stp-3)

Yield: 71%; m.p. (dec.): 210–213 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 1.67 (3H, t, $J = 8.0$), 2.43 (3H, s), 2.48 (3H, s), 3.07 (6H, s), 3.27–3.42 ($2H + H_2O$, m), 4.19 (2H, t, $J = 7.3$), 4.61 (2H, d, $J = 7.2$), 6.86 (2H, d, $J = 8.8$), 7.57–7.63 (3H, m). Anal. calcd. for $C_{22}H_{26}IN_3OS$: C, 52.07; H, 5.16; N, 8.28. Found: C, 51.85; H, 5.19; N, 8.15.

4.7.4. 8-[(E)-1-(4-Dimethylaminophenyl)methylidene]-9-methyl-3-(4-methylphenyl)-4-oxo-4,6,7,8-tetrahydropyrrolo-[1,2-a]thieno[2,3-d]pyrimidin-9-ium iodide (Stp-4)

Yield: 78%; m.p.: 235–236 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 2.36 (3H, s), 3.08 (6H, s), 3.34 ($2H + H_2O$, m), 4.20 (2H, t, $J = 6.8$), 4.31 (3H, s), 6.87 (2H, d, $J = 8.3$), 7.25 (2H, d, $J = 7.8$), 7.41 (2H, d, $J = 7.8$), 7.63 (2H, d, $J = 8.8$), 7.73 (1H, s), 7.93 (1H, s). Anal. calcd. for $C_{26}H_{26}IN_3OS$: C, 56.22; H, 4.72; N, 7.56. Found: C, 56.47; H, 4.77; N, 7.42.

4.7.5. 8-(tert-Butyl)-1-[(E)-1-(4-dimethylaminophenyl)-methylidene]-11-methyl-5-oxo-1,2,3,5,6,7,8,9-octahydrobenzo-[4,5]thieno[2,3-d]pyrrolo[1,2-a]pyrimidin-11-ium iodide (Stp-5)

Yield: 80%; m.p. (dec.): 165 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 0.94 (9H, s), 1.29 (1H, s), 1.53 (1H, s), 2.06 (1H, d, $J = 12.7$), 2.54–2.71 (2H, m), 2.95 (1H, br d, $J = 16.1$),

3.19 (1H, br d, $J = 17.0$), 3.32 (2H, br t), 4.20 (2H, d, $J = 8.3$), 4.23 (3H, s), 6.86 (2H, d, $J = 8.3$), 7.6 (2H, d, $J = 8.0$), 7.87 (1H, s). Anal. calcd. for $C_{27}H_{34}IN_3OS$: C, 56.35; H, 5.95; N, 7.30. Found: C, 56.41; H, 6.02; N, 7.46.

4.7.6. 8-[(E)-1-(4-Dimethylaminophenyl)methylidene]-2,3-dimethyl-4,6,7,8-tetrahydropyrrolo[1,2-a]thieno[2,3-d] pyrimidin-4-one (Stp-6)

Yield: 69%; m.p.: 288–289 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 3.32 (3H, s), 2.37 (3H, s), 2.96 (6H, s), 3.12–3.22 ($2H + H_2O$, m), 4.07 (2H, t, $J = 7.2$), 6.74 (2H, d, $J = 8.7$), 7.43 (2H, d, $J = 8.7$), 7.45 (1H, s). Anal. calcd. for $C_{20}H_{21}N_3OS$: C, 68.35; H, 6.02; N, 11.96. Found: C, 68.46; H, 6.09; N, 12.11.

4.7.7. 1-[(E)-1-(4-Dimethylaminophenyl)methylidene]-10-methyl-5-oxo-1,2,3,5-tetrahydropyrrolo[2,1-b]quinazolin-10-ium iodide (Sbp)

Yield: 83%; m.p.: 238–240 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 3.07 (6H, s), 3.31 (2H, t, $J = 6.5$), 4.21–4.29 (5H, m), (6.87, d, $J = 9.3$), 7.63 (2H, d, $J = 8.8$), 7.77 (1H, t, $J = 7.3$), 7.98 (1H, s), 8.10 (2H, m), 8.28 (1H, d, $J = 8.0$). Anal. calcd. for $C_{21}H_{22}IN_3O$: C, 54.91; H, 4.83; N, 9.15. Found: C, 55.04; H, 4.88; N, 9.22.

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