Synthesis and supramolecular self-assembly of phenothiazine functionalized by carboxyphenyl fragments*

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Dispersions of non-toxic compounds characterized by an intense absorbance in the near-IR range find use in photodynamic therapy as efficient antibacterial and anticancer agents. An approach to the preparation of aqueous dispersions of nanoparticles with controlled morphology is described. 3,7-Bis(arylamino)phenothiazin-5-ium derivatives containing the ester and carboxylic groups were synthesized for the first time by the oxidative addition of methyl anthranilate to phenothiazin-5-ium tetraiodide with subsequent hydrolysis of the ester groups. The association with 3-phenylimino-7-phenylaminophenothiazine was studied for the synthesized bis(carboxyl)phenothiazinium derivative. The obtained 1 : 1 associate is characterized by the bathochromic shift. The nanoprecipitation of the obtained nanoassociate in a methanol—water system was studied, and the morphology of the prepared dispersions was characterized: 3-phenylimino-7-phenylaminophenothiazine forms nanofiber structures, bis(carboxyl) derivative forms spherical particles, and the two-component associate forms facet-shaped particles.

Key words: phenothiazine, synthesis, methyl anthranilate, self-assembly, supramolecular associates, nanoprecipitation.

The vigorous publication activity observed in the recent years in the field of synthesis and study of new organic oligo- and polyaromatic compounds is caused by the whole series of practically important materials based on these substances. For example, the electric and photoelectric properties of the organic semiconductors[1] are attractive for electronics, since they allow one to produce materials characterized by a low weight, flexibility, resistance to deformations,[2] and intense absorption in the near-IR range,[3] which is important for manufacturing flexible electronics and semitransparent solar cells. Polyaromatic materials and compounds intensely absorbing in the long-wavelength range, which results in the development of photochemical and photothermal processes, are especially interesting for the application in medical practice. The radiation of the near-IR range is characterized by the high penetrating ability toward biological tissues without their damage.[4] At present, the photoactive antimicrobial,[5] antibacterial,[6] and anticancer[7] drugs based on organic oligo- and polyaromatic compounds attract increased attention of the researchers, since this trend provides prospects of surmounting resistance to antiobiotics produced by bacteria. In addition, photoactivity makes it possible to use the indicated materials directly in the area of incision during surgery[5] and for localized therapy of cancer tumors.[7,8]

Nowadays porphyrins are the most frequently used photodynamic agents.[8] The phenothiazine derivatives should be distinguished among other compound-competitors applied in this area as the most synthetically available.[5] The dialkylaminophenothiazine derivatives (structural analogs of Methylene Blue) are mainly presented in the literature as new antimicrobial agents and remedies for DNA footprinting.[9,10] The introduction of arylamino groups into the phenothiazine structure instead of dialkylamino groups significantly decreases the solubility of the synthesized derivatives in aqueous media but increases the absorption intensity in the near-IR range.[11] The increased absorption in the near-IR range characteristic of these derivatives is explained by the steric influence of the relatively bulky aromatic fragments on packing of the phenothiazine derivatives in aqueous media. For example, it is shown for the phenothiazine derivative containing *N*-methylphenylamine groups that the presence of the aromatic fragments decreases the aggregation at the concentrations lower than 10^{-3} mol L^{-1}. It should be mentioned that the aggregation decreases the yield of singlet oxygen and, therefore, decreases the efficiency of photodynamic therapy.[12] The photoactive nanoparticles based on porphyrins[13,14] and some phenothiazine derivatives[15] have already been described in several publications,

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whereas the studies in the field of preparation of similar nanoparticles from structural analogs of Methylene Blue are absent. In this work, we studied the formation of dispersions of the water-insoluble phenothiazine derivatives during nanoprecipitation and proposed a new approach to their stabilization. The formation of binary associates, in which the phenothiazine derivative containing carboxyl groups acts as an acid and the deprotonated phenothiazine derivative acts as a base, was demonstrated. This approach made it possible to enhance the stability of the dispersions formed upon nanoprecipitation.

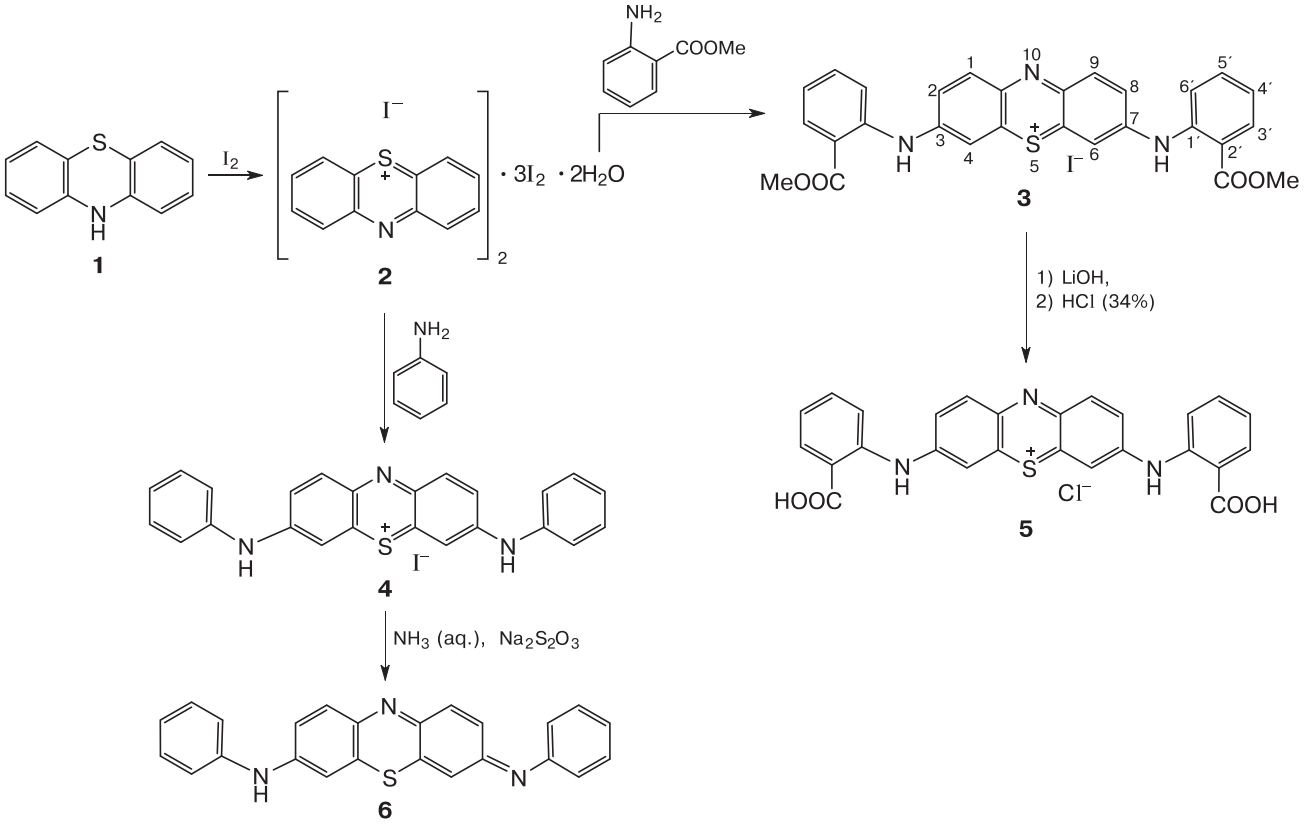
Results and Discussion

Synthesis of the phenothiazine derivatives containing ester and carboxyl groups. The present structural variety of the Methylene Blue analogs remains restricted from the viewpoint of supramolecular chemistry, and their application as complementary blocks of supramolecular associates is a nontrivial task. The synthesis of the arylaminophenothiazine derivatives is described in several publications, which also show the possibility of preparing mono- and bis(arylamino) derivatives[11] and their use in supramolecular ensembles with cyclophanes[16] and electroconducting polymers[17] and as components of the electrochemical sensors.[18] The fused aromatic systems bearing phenothiazine fragments find use in organoelectronics.[19] In spite of significant advances in the modern chemistry, the problem of functionalizing the phenothiazin-5-ium platform by proton-donor/proton-acceptor fragments for targeted self-assembly of related nanomaterials is far from solution.

Compound **3** containing the ester fragment was synthesized by the oxidative addition of methyl anthranilate to phenothiazin-5-ium (**2**) in methanol (Scheme 1). The hydrolysis of derivative **3** in a THF—water mixture by lithium hydroxide gave compound **5**. 3-Phenylamino-7-phenyliminophenothiazine (**6**) was synthesized using a known procedure by the oxidative addition of aniline to phenothiazin-5-ium tetraiodide to give salt **4** and subsequent deprotonation and removal of the iodide anion with a mixture of ammonia and sodium thiosulfate.[20,21]

The structures and compositions of compounds **3** and **5** were confirmed by the data of ^{1}H and ^{13}C NMR spectroscopy, IR spectroscopy, mass spectrometry, and elemental analysis. It should be mentioned that the electronic and spatial structures of the carboxyl (**5**) and ester (**3**) derivatives are close, which is observed in the similar chemical shifts of the $^{13}C\{H\}$ NMR spectra. The ^{1}H and ^{13}C NMR spectra of compound **5** exhibit no signal corresponding to the methyl group. The chemical shifts of

Scheme 1



the carbon atoms of the carboxyl (δ 167.2, compound **5**) and methoxycarbonyl (δ 165.8, compound **3**) groups differ insignificantly. The IR spectra contain the characteristic sets of absorption bands corresponding to vibrations of the phenothiazinium fragments (compound **3**: 1581, 1489, and 1380 cm^{-1}; compound **5**: 1578, 1496, and 1377 cm^{-1}). The positions and shapes of the absorption bands of the C(O)—O fragment in the IR spectra of compounds **3** and **5** are different being 1687 cm^{-1} for compound **5** and 1680 cm^{-1} for compound **3**.

Study of the association and nanoprecipitation of compounds 5 and 6. Since we pioneered in synthesizing the structural analog of Methylene Blue containing carboxyl groups, it seemed of interest to study the interaction of derivative **5** with deprotonated derivative **6**. Similar systems were not described earlier, and it is necessary to study them for the application of supramolecular chemistry approaches for the development of related associates and dispersions. Based on the fact of the presence of two carboxyl groups in compound **5** and taking into account that compound **6** is protonated with the equimolar amount of the acid, the formation of associates with different stoichiometries with the molar ratios of compounds **5** and **6** of 1 : 1 or 1 : 2 can be assumed. The method of isomolar series in methanol was used for the determination of the stoichiometries of the formed associates: the intensity of the absorption band of the binary associate in methanol at 660 nm corresponded to the 1 : 1 stoichiometry (Fig. 1, Scheme 2). This conclusion is confirmed by ^{1}H NMR spectroscopy data: the largest changes in the ^{1}H NMR spectra compared to the ^{1}H NMR spectra of the starting compounds were observed at the equimolar ratio of compounds **5** and **6** for the total concentration of compounds **5** and **6** in DMSO-d_6 equal to 1 mmol L^{-1}. Thus, the NMR spectroscopic data also confirm the formation of the 1 : 1 complex.

The ratio **5** : **6** = 1 : 1 was chosen for the nanoprecipitation of the compounds based on the data obtained.

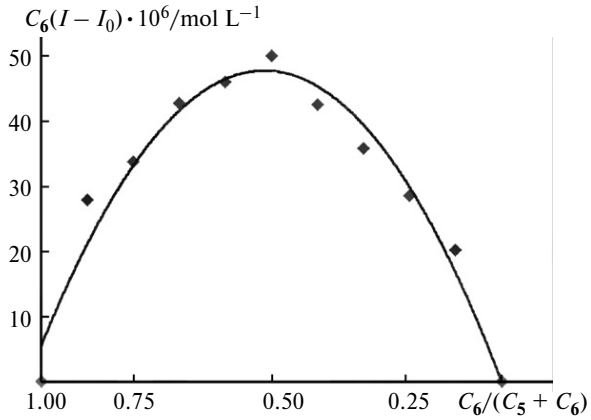
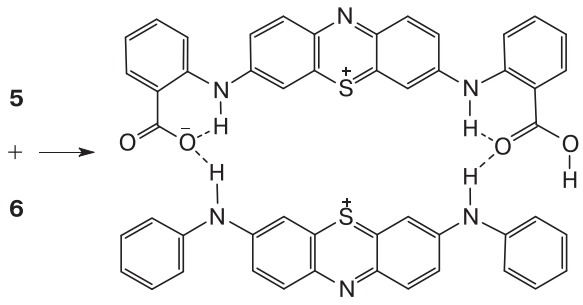


Fig. 1. Isomolar series plot for compounds **5** and **6** in methanol according to the UV spectroscopy data. The total concentration of compounds **5** and **6** in the mixtures is 10^{-5} mol L^{-1}.

Scheme 2



According to the dynamic light scattering data (Fig. 2), true solutions of compounds **5** and **6** and a mixture of compounds **5** and **6** are formed in methanol at a concentration of 10^{-4} mol L^{-1}. It is known that nanoprecipitation is a convenient approach for the preparation of dispersions of nanoparticles. The nanoprecipitation of compounds **5** and **6** and their associate was conducted from solutions in methanol into water under ultrasonication, and the concentration of the resulting dispersions was 10^{-5} mol L^{-1}. The dispersions of submicronic particles were formed, and the data on the hydrodynamic diameter and polydispersity of the obtained particles are presented in Table 1.

The obtained dispersions were studied by scanning electron microscopy (SEM) (Fig. 3). According to the

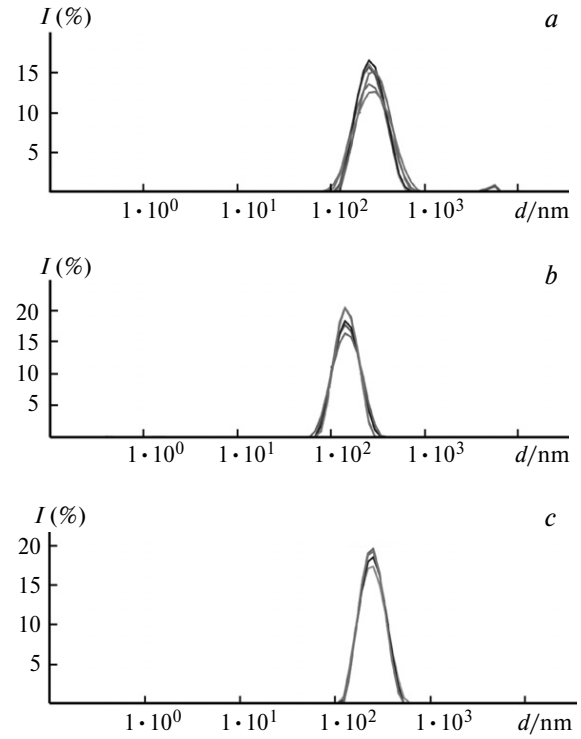


Fig. 2. Size distributions of the particles obtained by the nanoprecipitation (methanol—water) of compounds **5** (*a*), **6** (*b*), and a 1 : 1 mixture of compounds **5** and **6** (*c*) (DLS data).

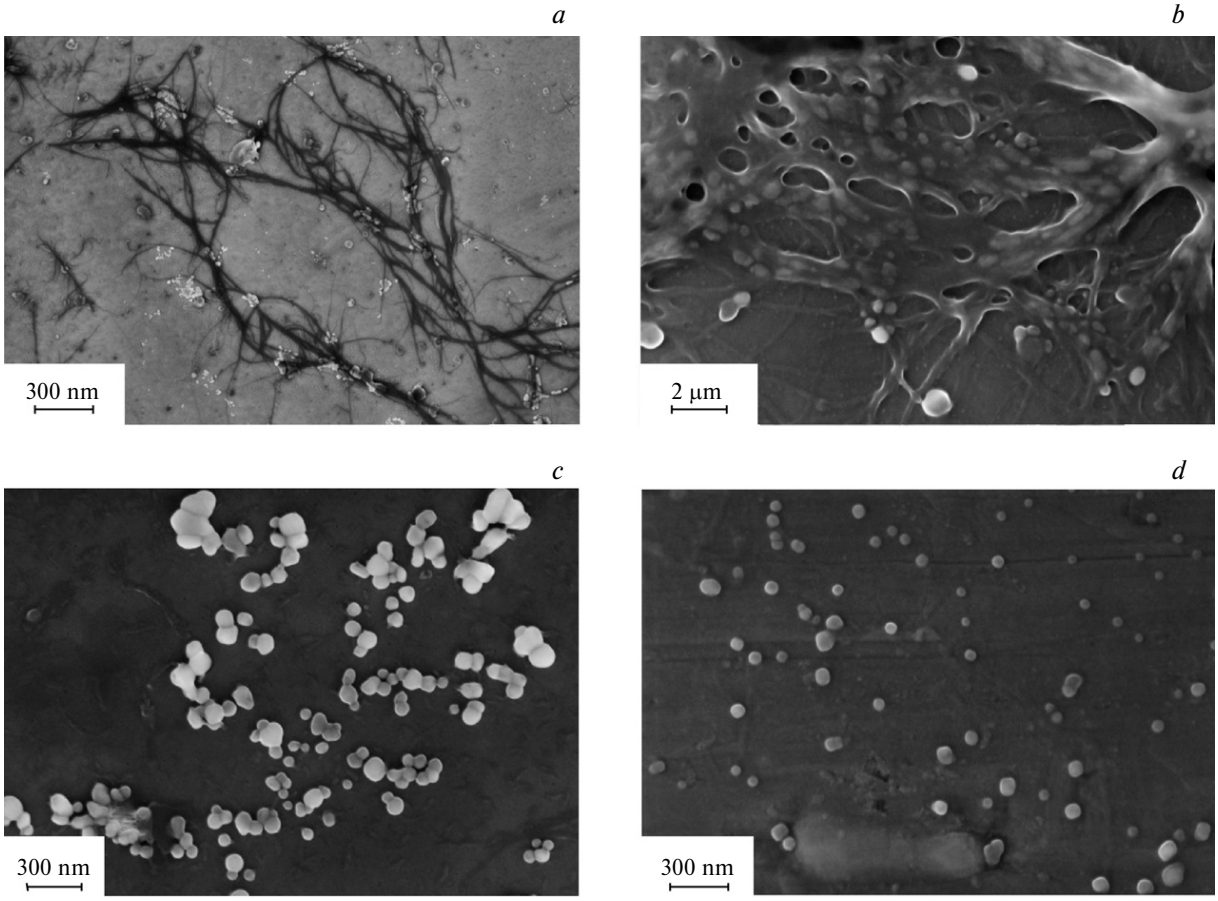


Fig. 3. SEM images of the particles obtained by the nanoprecipitation of compounds **6** (*a*, *b*), **5** (*c*), and a 1 : 1 mixture of compounds **5** and **6** (*d*).

SEM images, nanoprecipitation of 3-phenylimino-7-phenylaminophenothiazine (**6**) gives filament structures, whereas spherical nanoparticles are formed upon the nanoprecipitation of carboxyl derivative **5**.

According to the SEM images, the combination of two compounds **5** and **6** into a binary associate results in changes in the morphology of the particles obtained by nanoprecipitation. Unlike the nanoparticles obtained by the nanoprecipitation of one-component solutions of compounds **5** and **6**, the nanoprecipitation of their mixture gave faceted (close to the cubic shape) nanoparticles.

Table 1. Dynamic light scattering data for dispersions of compounds **5** and **6** and their equimolar mixture ($C = 10^{-5}$ mol L^{-1})

Compound	*d*/nm	PDI
5	140±1	0.07±0.02
6	260±3	0.16±0.02
Mixture of **5** and **6**	237±1	0.06±0.02

Note: d is the average hydrodynamic diameter, and PDI is the polydispersity index.

3,7-Bis(arylamino)phenothiazin-5-ium derivatives containing ester and carboxyl groups were synthesized for the first time and characterized. The association of the synthesized bis(carboxyl) derivative in water in the presence of 7-phenylamino-3-phenyliminophenothiazine was studied, and the formation of 1 : 1 associates was shown. The formation of two-component acid-base associates of the structural analogs of Methylene Blue makes it possible to change the morphology of particles formed by nanoprecipitation from a solution in methanol to water. The nanoprecipitation of 7-phenylamino-3-phenylimino-phenothiazine affords nanofibers, whereas spherical particles are formed by the nanoprecipitation of the phenothiazine derivative containing antranilic acid fragments. The combination of these compounds in the binary associate allows one to obtain faceted nanoparticles.

Thus, the possibility of synthesizing stable aqueous dispersions based on the bis(carbonyl)phenothiazine derivatives was shown. The developed nontrivial approach to the stabilization of water-insoluble arylaminopheno-

thiazine derivatives can be applied for the preparation of agents for photothermal therapy, which intensively absorb in the near-IR range.

Experimental

Phenothiazine (99%, Alfa-Aesar), I_2 (reagent grade), chloroform (reagent grade), methanol (reagent grade), methyl anthranilate (99%, Acros Organics), and sodium thiosulfate (anhydrous, 98%, Sigma-Aldrich) were used. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer at the working frequencies 400.0 and 100.0 MHz, respectively. Chemical shifts were determined relative to the signals of residual protons of the deuterated solvent (DMSO-d_5). The concentration of the examined solutions was 10 mmol L^{-1}. The proposed assignments of the signals in the NMR spectra were made by the simulation of the spin-spin systems. The SEM images of the particles were obtained using a Carl Zeiss Auriga Cross Beam microscope on the silicon support surface. Dispersions of colloidal particles with a concentration of 10^{-5} g mL^{-1} were deposited onto the support surface and dried for 1 h in a vacuum desiccator.

IR attenuated total reflectance (ATR) spectra were recorded in the wave number range 400—4000 cm^{-1} on a Spectrum 400 FT-IR spectrometer (Perkin Elmer): the resolution was 1 cm^{-1}, 64 scans were acquired, and the acquisition time was 16 s. Mass spectra were obtained on an AmazonX mass spectrometer (Bruker Daltonik GmbH, Germany) with an ionic trap (electrospray ionization mode). The measurements were performed in the positive ion detection mode in the m/z range from 70 to 3000. The voltage on the capillary was −3500 V. Nitrogen at 250 °C served as a nebulizer gas with a flow rate of 10 L min^{-1}. A methanol—water (7 : 3, vol/vol) system with a flow rate of 0.2 mL min^{-1} was used as an eluent (Agilent 1260 chromatograph, USA). The analyzed sample was dissolved in methanol to a concentration of 10^{-6} g L^{-1}. A sample was injected through a Rheodyne 7725 injector (Rheodyne, USA). The sample volume was 20 μL. The TrapControl 7.0 software (Bruker Daltonik GmbH, Germany) was used for mass spectrometer controlling and data collecting. The data were processed using the DataAnalysis 4.0 SP4 program (Bruker Daltonik GmbH, Germany). The particle sizes in the dispersion were determined by dynamic light scattering (DLS) on a Zetasizer Nano ZS instrument (Malvern) equipped with a He—Ne laser (4 mV, wavelength 633 nm, scattered light detection angle 173°) with the automatic determination of the position of measuring inside cells. The prolonged treatment with ultrasound was conducted using an Elmasonics S30H ultrasonic bath. The samples were dispersed with ultrasound on a Sonics Vibracell VCX 500 instrument using a stepped microtype (diameter 3 mm) immersed into a mixture of the solvent and compound insoluble in this solvent. Dionized ultrapure water (specific resistance >18.0 MOhm cm at 25 °C) was obtained from distilled water on a Millipore-Q system.

Phenothiazin-5-ium tetraiodide (**2**)[20] and compounds **4**[21] and **6**[22] were synthesized by known procedures.

3,7-Bis((2-(methoxycarbonyl)phenyl)amino)phenothiazin-5-ium iodide (3). A solution of methyl anthranilate (0.584 g, 3.86 mmol) in methanol (20 mL) was added to a suspension of phenothiazin-5-ium tetraiodide (**2**) (0.3 g, 0.425 mmol), and the mixture was vigorously stirred at room temperature for 24 h. The obtained intensively colored solution was filtered, and the precipitate was collected and washed with methanol. The yield of compound **3** (m.p. 198 °C) was 106 mg (40%). Found (%): C, 53.94; H, 3.56; I, 20.35; N, 6.74; S, 5.14. Calculated (%): C, 53.98; H, 3.57; I, 20.31; N, 6.72; S, 5.17. ^{1}H NMR, δ: 10.97 (s, 2 H, NH); 8.13 (d, 2 H, H(1), H(9), J = 9.2 Hz); 8.03 (d, 2 H, H(3′), J = 7.9 Hz); 7.78 (dd, 2 H, H(4′), J = 7.6 Hz, J = 7.9 Hz); 7.67 (d, 2 H, H(6′), J = 7.8 Hz); 7.57 (d, 2 H, H(2), H(8), J = 9.2 Hz); 7.52 (dd, 2 H, H(5′), J = 6.2 Hz, J = 7.9 Hz); 7.49 (s, 2 H, H(4), H(6)), 3.80 (s, 6 H, COOMe). ^{13}C NMR, δ: 165.8 (C(O)O), 152.4 (C(2′)), 139.0, 137.3, 137.1, 135.5, 134.2, 131.7, 127.4, 126.1, 124.6, 122.5, 107.3, 52.6 (CH_3). IR (ATR), ν/cm^{-1}: 1687 (C(O)—O), 1581, 1489, 1380 (phenothiazinium fragment), 1132 (C—N). MS (ESI), m/z (I_{rel} (%)): 496.3 (100) $[M + H]^+$.

3,7-Bis((2-(carboxyl)phenyl)amino)phenothiazin-5-ium chloride (5). Lithium hydroxide monohydrate (0.24 g, 10 mmol) and THF (20 mL) were added to compound **3** (0.62 g, 1 mmol), and then water (2 mL) was added with vigorous stirring. The mixture was refluxed for 8 h. The solvent was evaporated on a rotary evaporator, concentrated hydrochloric acid was added to the residue, and the obtained mixture was vigorously stirred at room temperature for 10 h. The precipitate formed was filtered off and washed with 2 *M* HCl. The yield of compound **5** (m.p 250 °C) was 353 mg (70%). Found (%): C, 61.97; H, 3.60; Cl, 7.03; N, 8.34; S, 6.36. Calculated (%): C, 62.01; H, 3.61; Cl, 7.01; N, 8.31; S, 6.31. ^{1}H NMR, δ: 8.10 (d, 2 H, H(1), H(9), J = 8.1 Hz); 8.04 (d, 2 H, H(3′), J = 8.1 Hz); 7.73 (dd, 2 H, H(4′), J = 8.1 Hz, J = 7.3 Hz); 7.63 (d, 2 H, H(2), H(8), J = 8.1 Hz); 7.57 (d, 2 H, H(6′), J = 8.1 Hz); 7.51 (s, 2 H, H(4), H(6)); 7.46 (dd, 2 H, H(5′), J = 8.1 Hz, J = 7.8 Hz). ^{13}C NMR, δ: 167.2 (C(O)O), 152.1 (C(2)), 138.9, 137.6, 137.1, 135.3, 133.9, 132.0, 127.1, 125.4, 125.1, 122.6, 107.5. IR (ATR), ν/cm^{-1}: 1680 (C(O)O), 1578, 1496, 1377 (phenothiazinium fragment), 1127 (C—N). MS (ESI), m/z (I_{rel} (%)): 468.2 (100) $[M + H]^+$.

Nanoprecipitation of compounds 5 and 6 and their binary associate. Solutions of compounds **5** and **6** in methanol with concentrations of 10^{-3} mol L^{-1} were prepared for nanoprecipitation. Each obtained solution (100 μL) was added to water (10 mL) during ultrasonication to a residual volume of 10 mL.

Study of the stoichiometry of an associate of compounds 5 and 6 by spectrophotometry. Solutions of compounds **5** and **6** with concentrations of 10^{-5} mol L^{-1} were mixed in volume ratios of 4 : 1, 3 : 1, 2 : 1, 3 : 2, 1 : 1, 2 : 3, 1 : 2, 1 : 3, and 1 : 4. Electronic absorption spectra were recorded in quartz cells, and the solution volume for measurements was 3 mL. The plot of the isomolar series was constructed by the absorption band at 660 nm.

Study of the stoichiometry of the association of compounds 5 and 6 by ^{1}H NMR spectroscopy. Solutions were prepared by the dissolution of compounds **5** and **6** in the NMR tubes in the molar ratios of 3 : 1, 2 : 1, 1 : 1, 1 : 2, and 1 : 3. The amounts corresponded to the total concentrations of **5** and **6** equal to 10^{-3} mol L^{-1}.

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