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ORGANIC SYNTHESIS AND INDUSTRIAL ORGANIC CHEMISTRY

Improvement of Ways to Obtain Ethidium Bromide and Synthesis of Ethidium Ethyl Sulfate, a New Fluorescent Dye for Detection of Nucleic Acids

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Abstract—Two methods for synthesis of ethidium bromide, a fluorescent dye widely used in molecular biology, were improved. An analog of ethidium bromide, ethidium ethyl sulfate, was synthesized.

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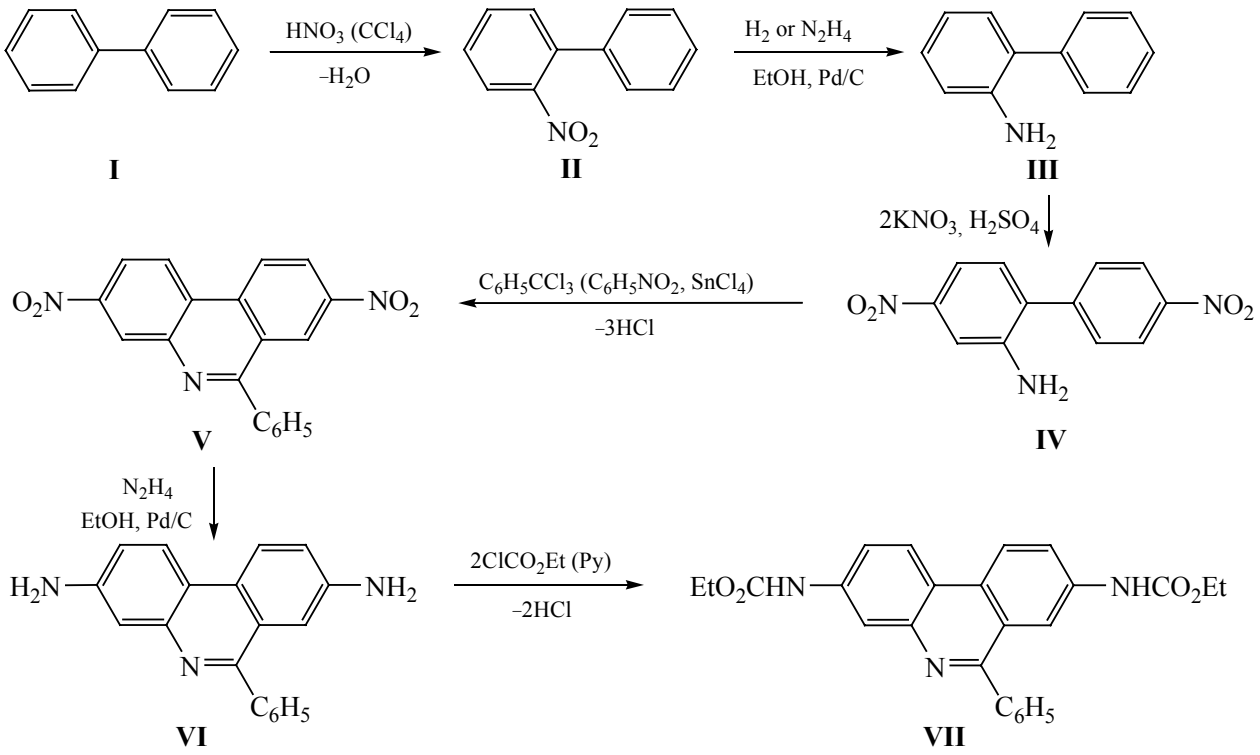
Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) is a fluorescent dye widely used in molecular biology, gene engineering, and diagnostic medicine [1–3]. When exposed to UV light, it fluoresces an orange color (590 nm), with the intensity of this emission increasing many times (by a factor of 20–25) upon binding of this dye to double-stranded DNA and RNA [4], which enables detection of nucleic acid in cells and tissues. Modern methods of fluorescent microscopy with ethidium bromide can detect DNA in amounts on the order of 2 ng in a sample [5].

Ethidium bromide is not manufactured in Russia. To improve the known patented techniques for synthesis of ethidium bromide, namely to simplify, diminish the cost, and raise the yield of this method, we studied the scheme of synthesis of ethidium bromide from diphenyl (**I**) as the starting compound. The chain of transformations leads to two key half-products, 3,8-dinitro-6-phenylphenanthridine (**V**) and 3,8-dicarboethoxyamino-6-phenylphenanthridine (**VII**) (Scheme 1).

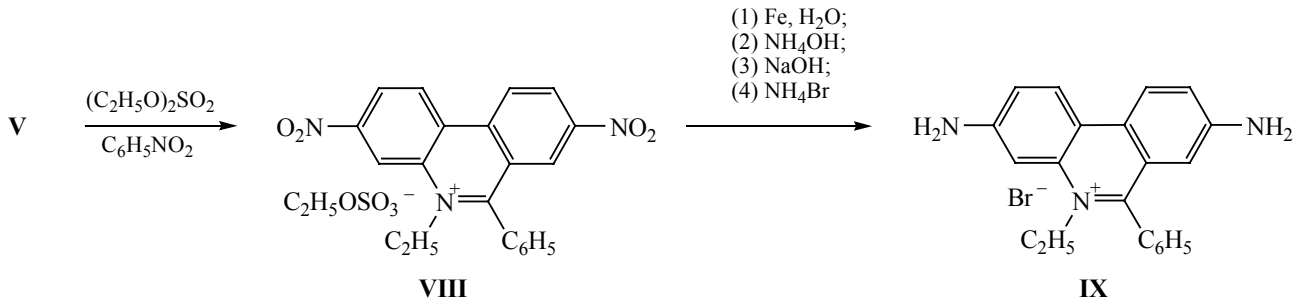
The known method [6] for synthesis of ethidium bromide is illustrated by Scheme 2:

The first stage of this method is quaternization of 3,8-dinitro-6-phenylphenatridine (**V**) (2,7-dinitro-9-phenylphenantridine according to outdated nomenclature) with diethyl sulfate. In this stage, the role of solvent was played by nitrobenzene, further removed by hydrodistillation. The patent gives no elemental analysis data or any characteristics for crystalline quaternization product synthesized in 24% yield, named 2,7-dinitro-9-phenyl-10-ethylphenanthridinium ethosulfate [3,8-dinitro-5-ethyl-6-phenylphenanthridinium ethyl sulfate (**VIII**)]. In the second stage, the thus obtained crystalline product was dissolved in boiling water and reduced with iron under boiling. Further, iron(II) hydroxide was precipitated with an excess amount of ammonia, the mixture was heated until this hydroxide coagulated, and the excess of iron and the hydroxide were removed by filtration. Then, a concentrated solution of sodium hydroxide was added to the reaction mixture. The mixture was extracted with *n*-butanol and the extract was washed with water and treated with an ammonium bromide solution. A hydrodistillation of butanol and treatment of the residue with a (1 : 1 (v/v) methanol–water mixture produced in 84% yield a product named 2,7-diamino-9-phenyl-10-ethylphananthridiunim bromide [3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (**IX**)] hemihydrate. Elemental analysis data or any characteristics were not presented for this product, either. According to the patent, the yield of ethidium bromide was 20% in terms of 3,8-dinitro-6-phenylphenanthridine (**V**).

Scheme 1.



Scheme 2.



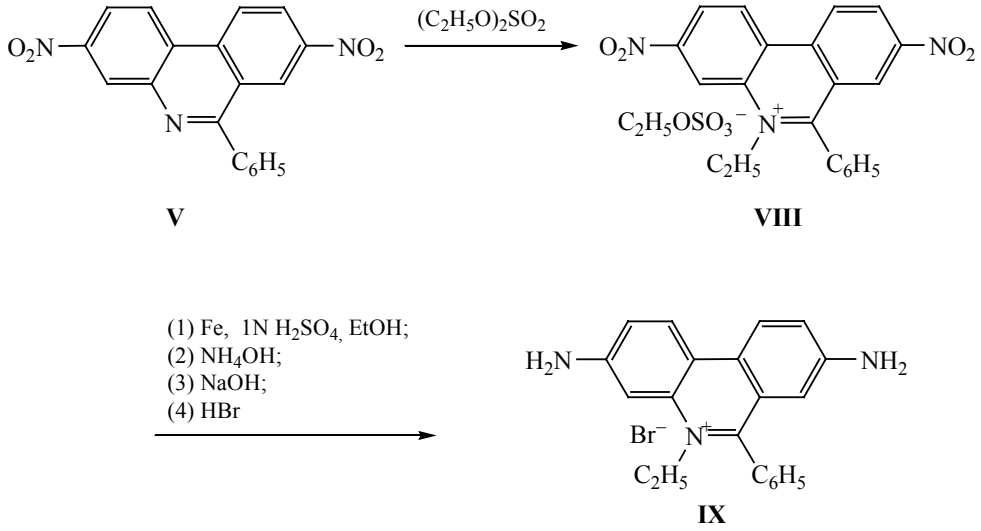
A disadvantage of this method is that nitrobenzene is used as solvent in the first stage (quaternization) and its removal is necessary for isolation of 3,8-dinitro-5-ethyl-6-phenylphenanthridinium ethyl sulfate (**VIII**), the half-product for the subsequent stage in which ethidium bromide is synthesized, which makes the process more expensive, and the synthesis duration longer. Another disadvantage is that water is used to dissolve ethyl sulfate (**VIII**) in its reduction with iron. Under these conditions, hydrolysis of the ethyl sulfate to give difficultly soluble by-products leads to a decrease in the yield and purity of the target ethidium bromide in subsequent processing stages. One more disadvantage is that ammonium bromide is used in the final stage of synthesis, which requires removal of its excess amount in the stage of isolation of the target product.

The synthesis pathway of ethidium bromide, reported here, is shown in Scheme 3.

Ethidium bromide was produced by quaternization of 3,8-dinitro-6-phenylphenanthridine with an excess amount of diethyl sulfate, which also served as solvent. After half-product **VIII** was isolated, the excess amount of diethyl sulfate was regenerated by vacuum distillation. As a result, the yield of half-product **VIII** could be substantially raised (from 24 to 74%).

Another improvement of the scheme for synthesis of ethidium bromide was that a mixture of 1 N sulfuric acid

Scheme 3.



and 95% ethanol was used in the stage of reduction of ethyl sulfate **VIII** with powdered iron, which precluded formation of difficultly soluble products in hydrolysis of compound **VIII**.

The use of hydrobromic acid in the final stage of synthesis made it possible to exclude ammonium bromide.

The improvements mentioned above resulted in that the yield of ethidium bromide increased from 20 [6] to 42% [in terms of 3,8-dinitro-6-phenylphenanthridine (**V**)].

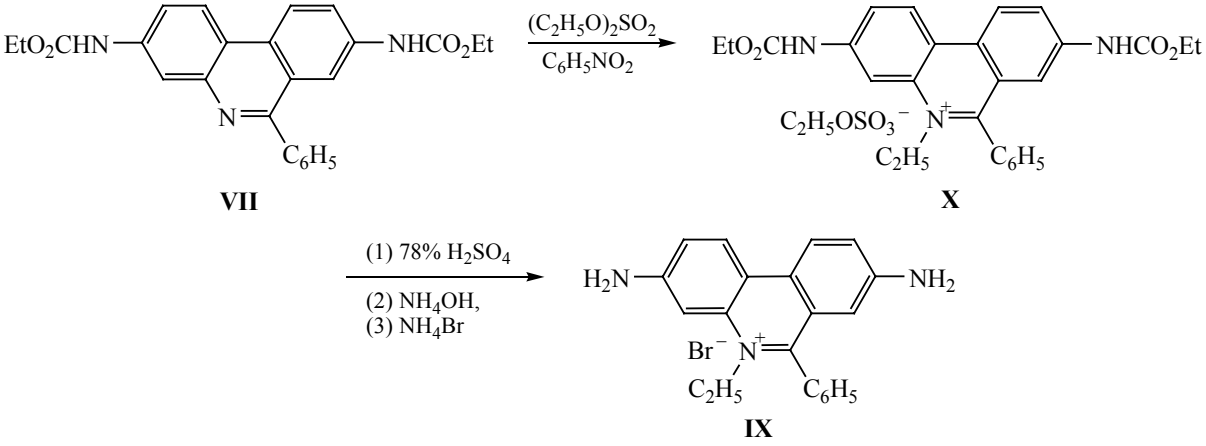
Thus, the method we developed is simpler and less expensive than that described in the patent [6] and enables a substantial increase in the yield of ethidium bromide. The production technology was implemented at the Pilot chemical shop, Vorozhtsov Institute of Organic Chemistry in Novosibirsk, its main concepts are described in the patent [8].

In studies aimed to develop a technique for synthesis of ethidium bromide, we obtained its analog, 3,8-diamino-5-ethyl-6-phenanthridinium ethyl sulfate (ethidium ethyl sulfate) (**XI**). Possibly, this previously undescribed compound may prove to be a substitute of ethidium bromide.

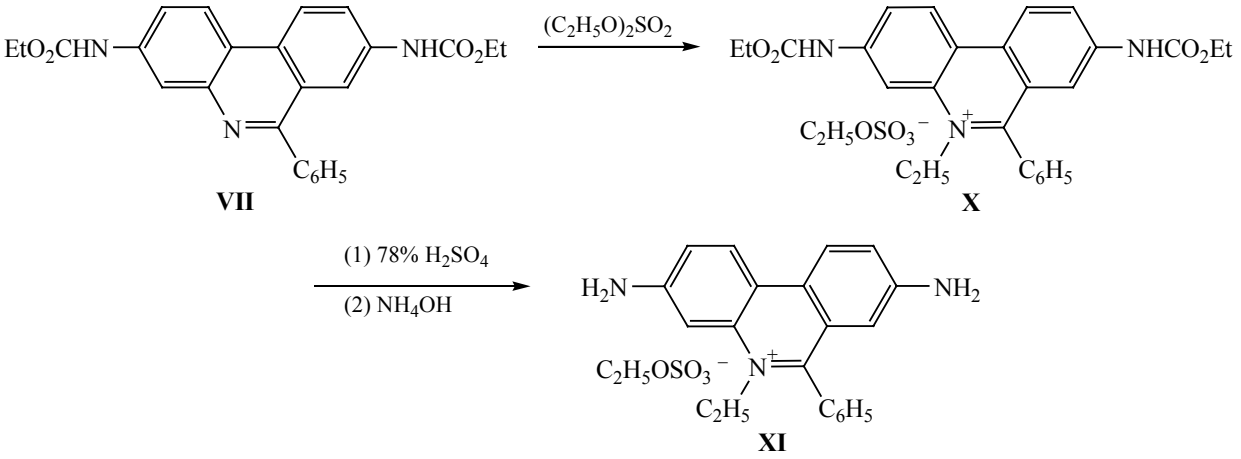
The synthesis of compound **XI** is based on the known method for synthesis of ethidium bromide **IX** [7], presented in Scheme 4.

In quaternization of 3,8-dicarboethoxyamino-6-phenylphenanthridine (**VII**) (2,7-biscarboethoxy-9-

Scheme 4.



Scheme 5.



phenanthridine by outdated nomenclature) with diethyl sulfate, nitrobenzene was used as solvent and then was removed by separation of the solid quaternization product, 3,8-dicarboethoxyamino-6-phenylphenanthridinium ethyl sulfate (**X**), upon treatment of the reaction mixture with ether. In the second stage of synthesis, the raw ethyl sulfate (**X**) was subjected to hydrolysis in 78% sulfuric acid at 135°C to remove carboethoxy groups [7]. The mixture of hydrolysis products was diluted with icy water and neutralized with a solution of ammonium hydroxide. To replace the anion in the resulting salt of 2,7-diamino-9-phenyl-10-ethylphenanthridinium, the mixture was treated with an excess amount of ammonium bromide. The subsequent purification of the product obtained produced ethidium bromide, whose yield is not specified, but elemental analysis data presented for nitrogen are in agreement with the empirical formula.

To make the process less expensive and diminish the synthesis duration of compound **X**, we replaced nitrobenzene with diethyl sulfate serving as both reagent and solvent.

The structure of the quaternization product **X** was confirmed on the basis of elemental analysis data and ^{1}H NMR, IR, and electronic absorption (EAS) spectra. 3,8-Dicarboethoxyamino-6-phenylphenanthridinium sulfate (**X**) is formed in 87% yield.

When performing the second stage of synthesis of ethidium bromide by the method described in the patent [7], we found that, on dilution of the mixture of hydrolysis products with icy water and its neutralization with ammonium hydroxide, *n*-butanol extracts 3,8-diamino-5-ethyl-6-phenylphenanthridinium ethyl sulfate (**XI**). The structure of this compound was confirmed using elemental analysis data, ^{1}H NMR spectrum similar to that of ethidium bromide, and IR and electronic absorption spectra. The fluorescence spectra (FS) of aqueous solutions of these to compounds are nearly identical. The yield of ethyl sulfate (**XI**) is 61% in terms of ethyl sulfate (**X**). Apparently, product **XI** is an intermediate in the second stage of synthesis of ethidium bromide by the method [7] and contains an ethidium cation bound to an anion serving as an intermediate component in the given synthesis.

3,8-Diamino-5-ethyl-6-phenylphenanthridinium ethyl sulfate **XI** was synthesized by Scheme 5.

We made a comparative characterization of the new fluorescent dye, ethidium ethyl sulfate **XI**, and the conventionally used ethidium bromide **IX** for DNA detection. We found nearly equal sensitivities of these dyes in detection of amplificated DNAs formed in the polymerase chain reaction (PCR). Thus, the new compound can be used to replace ethidium bromide in determination of nucleic acids. The material on synthesis of ethidium ethyl sulfate possessing a valuable property to enhance the fluorescence intensity in exposure to UV light as a result of binding to nucleic acids is presented in the patent [10].

EXPERIMENTAL

The IR spectra were recorded on a Vector 22 spectrometer in pellets with KBr; electronic absorption spectra, on a Specord UV-VIS spectrophotometer (10^{-4} N solutions in ethanol); fluorescence spectra, on

a Varian Cary Eclipse fluorescent spectrophotometer (excitation at wavelengths λ_{max} = 247, 333, and 486 nm; excitation of 10^{-4} N solutions in H_2O). The melting points were determined on a Kofler apparatus. ^1H NMR spectra were recorded on a Bruker AC 200 instrument (^1H at 200.13 MHz) for solutions at 25°C with resonant stabilization by the deuterium signal of a solvent: $CDCl_3$, acetone-d_6, or DMSO-d_6. The chemical shifts (ppm) were measured relative to signals of inner standards: $CHCl_3$, acetone, and DMSO (δ_{int} 7.24, 2.04, and 2.50 ppm, respectively).

The content of the main substance in the product formed upon reduction of 2-nitrodiphenyl (**II**) to 2-aminodiphenyl (**III**) on a Tsvet 162 chromatograph (flame-ionization detector; 5% SKTFV-803 on NAW-DMCS chromaton; grain size 0.20–0.25 μm; 300 × 0.3 cm column; column temperature 150–300°C; heating rate 11 deg min^{-1}; evaporator temperature 270°C; carrier-gas: nitrogen at a flow rate of 30 ml min^{-1}, hydrogen at 30 ml min^{-1}, and air at 15 l h^{-1}).

The content of main substances was determined by HPLC on a Milikhrom-1 analytical liquid chromatograph (Nauchpribor Production Association, Orel). The determination conditions were as follows: 2 × 75 mm steel column, column working pressure 5.2 MPa, temperature ~20°C, eluent flow rate 100 μl min^{-1}, UV detector (290 nm), and time constant 0.3 s. Solution samples (4–6 μl) were analyzed with a concentration of a substance being analyzed of 3–5 mg ml^{-1}. Samples of 2-amino-4,4'-dinitrodiphenyl (**IV**) and 3,8-dinitro-6-phenylphenanthridine (**V**) were analyzed with Lichrospher RP18 sorbent and methanol : water = 9 : 1 (v/v) eluent. The eluted volumes of 2-amino-4,4'-dinitrodiphenyl (**IV**) and 3,8-dinitro-6-phenylphenanthridine (**V**) were 199 and 366 μl, respectively. Samples of 3,8-dinitro-5-ethyl-6-phenylphenanthridinium ethyl sulfate (**VIII**) were analyzed with Separon C18 sorbent and methanol : 0.1 N H_2SO_4 = 8 : 2 (v/v) eluent. The eluted volumes of 3,8-dinitro-6-phenylphenanthridinium and 3,8-dinitro-6-phenylphenanthridinium cations were 187 and 770 μl, respectively. Samples of 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (**IX**) were analyzed with Separon C18 sorbent and acetonitrile : 0.1 N H_2SO_4 = 4 : 6 (v/v) eluent. The eluted volume of the 3,8-diamino-5-ethyl-6-phenylphenanthridinium cation was 179 μm, and the eluted volumes of admixtures, 180, 233, and 393 μl.

We used freshly distilled solvents and reagents of chemically pure and analytically pure grades.

2-Nitrodiphenyl (**II**) produced by nitration of diphenyl (**I**) [GOST (State Standard) 13487–78] by the known method [11] had bp 160–166°C (0.5 kPa) and mp 36–38°C and contained 96% main substance according to GLC data. Published data: bp 178–179°C (13 Torr) [11] and mp 36–38°C [12].

2-Aminodiphenyl (**III**) was produced by catalytic hydrogenation of 2-nitrodiphenyl in ethanol (2.94 MPa, IKT-3-20 catalyst [13] containing 5% Pd/C) [14] or by treatment of 2-nitrodiphenyl with hydrazine hydrate in ethanol at 60°C with the same catalyst by the general method for reduction of nitro aromatic compounds [15]. The yields of amine **III** were 97 and 89%, respectively. The product had bp 130–132°C (0.4 kPa) and mp 47–48°C and contained, according to GLC data, 96% main substance. Published data [16]: bp 145–148°C (5 Torr) and mp 47–48°C.

2-Amino-4,4'-dinitrodiphenyl (IV) synthesized by method [17] (cf. [18]) had mp 205–206°C (with decomposition, from dioxane) and contained, according to HPLC data, 95% main substance. Published data: mp 206°C (from 2-ethoxyethanol) [17], mp 209.5–210.0°C (from acetone) [18].

3,8-Dinitro-6-phenylphenanthridine (V) was synthesized by the method described in [19]. To crystallize the product, the mixture was allowed to stay at 20°C for 16 h and then a finely crystalline precipitate was filtered off and washed with methanol. Without being dried, it was subjected to distillation with steam, with nitrobenzene completely removed. The precipitate from still bottoms was separated by filtration and dried at 60–80°C. Dinitro compound **V** was obtained in 73% yield, mp 272–274°C (with decomposition); the content of the main substance was 98% according to HPLC data. Published data [19]: mp 273–274°C.

Diethyl sulfate obtained using the procedure described in [20] had bp 93–95°C (0.6 kPa); published data [20]: bp 93–95°C (5 Torr).

3,8-Diamino-6-phenylphenanthridine (VI). In contrast to the results of the study [21], in which dinitro compound **V** was reduced with powdered iron in an acid medium, we used hydrazine hydrate as reducing agent (cf. [15]) and IKT-3-20 catalyst [13], which simplified the reduction process.

A mixture of 2.17 g (6.28 mmol) of 3,8-dinitro-6-phenylphenanthridine (**V**), 10.1 ml of ethanol, 0.100 g of IKT-3-20 catalyst, and 2.0 ml of 95% hydrazine

hydrate (39.8 mmol N_2H_4) was heated with reflux under agitation to 75–80°C for 1 h. Evolution of a gas and dissolution of the nitro compound was observed. By the end of keeping, the gas evolution became substantially weaker. The catalyst was filtered off and washed with the minimum amount of ethanol. Ethanol was evaporated from the combined alcoholic filtrates, the residue was extracted with chloroform, the extract was dried with $MgSO_4$, the solvent was evaporated, and the residue was dried in a vacuum. The resulting foamy and tarry product was ground with pentane for crystallization and then pentane was evaporated to give 1.70 g (95%) of 3,8-diamino-6-phenylphenanthridine (**VI**), mp 192–194°C (with decomposition). Published data [21]: yield 91%, mp 197–198°C (from chloroform). ^{1}H NMR spectrum (5% solution in $CDCl_3$, δ, ppm, *J*, Hz): 3.82 br.s (4H, two NH_2 groups); 7.01 d.d (1H, *J* = 9 and *J* = 2, H-2 or H-9); 7.1–7.2 m (2H, H-9 or H-2 and H-4 or N-7); 7.37 d (1H, *J* = 2, H-7 or H-4); 7.4–7.6 m (3H) and 7.6–7.7 m (2H) (C_6H_5); 8.25 and 8.30, both d (each 1H, *J* = 9, H-1, H-10). IR spectrum, ν, cm^{-1} (KBr): 710, 820, 1250, 1490, 1630. UV spectrum (MeOH), λ_{max}, nm (log ε): 211 (4.44), 274 (4.69), and 408 (3.63). Published 1H NMR spectrum [22] mostly corresponds to that presented above; some distinctions are apparently due to differences in the solvents used [$CDCl_3$ vs. $(CD_3)_2SO$].

3,8-Dicarboethoxyamino-6-phenylphenanthridine (VII) synthesized by the method described in [21] had mp 138–139°C (indistinct). Published data [21] for the low-melting crystalline modification: mp 138°C (indistinct). ^{1}H NMR spectrum (1% solution in acetone-d_6), δ, ppm, *J*, Hz): 1.21 and 1.28, both t (each 3H, *J* = 7, 2 CH_3 groups); 4.14 and 4.20, both q (each 2H, *J* = 7, 2 CH2 groups); 7.5–7.6 m (3H) and 7.7–7.8 m (2H) (C_6H_5); 7.91 and 8.12, both d.d (each 1H, *J* = 9 and *J* = 2, H-2, H-9); 8.33 and 8.37, both d (each 1H, *J* = 2, H-4, H-7); 8.54 and 8.69, both d (each 1H, *J* = 9, H-1, H-10); 9.07 s (2H, 2 NH groups). The ^{1}H NMR spectrum corresponds to the spectra reported in [23, 24]. IR spectrum, ν, cm^{-1} (KBr): 710, 1080, 1230, 1300, 1330, 1390, 1430, 1490, 1530, 1560, 1600, 1630; 1730, and 1740 (C=O). UV spectrum (EtOH), λ_{max}, nm (log ε): 215 (4.48), 280 (4.79), and 375 (3.64).

3,8-Dinitro-5-ethyl-6-phenylphenanthridinium ethyl sulfate (VIII). A mixture of 121 g (0.351 mol) of 3,8-dinitro-6-phenylphenanthridine (**V**) and 221 ml (260 g, 1.69 mol) of diethyl sulfate was heated under agitation to a temperature of 165°C and kept at 165 ± 2°C for 1.5 h. A dark-color solution was formed and a gas evolved. The mixture was allowed to stay for 16 h at 20°C. Then, 150 ml of absolute ether was added, the ether layer was decanted, and the residue was treated with 2 × 75 ml of ether. To the remaining residue was added 330 ml of 95% ethanol and the mixture was thoroughly ground, The resulting finely crystalline precipitate was filtered off and washed on a filter with 100 ml of 95% ethanol. Without being dried, it was charged into the paper cartridge of a Soxhlet apparatus and extracted with 4 × 250 ml of 95% ethanol, each time with boiling for 8 h. Needle-like crystals were filtered off from combined alcoholic extracts allowed to stay at 0 to +5°C for 16 h and were dried at 120–130°C to constant weight. A 130-g portion (74%) of ethyl sulfate **VIII** was obtained in the form of cloudy eroded needles with mp 203–205°C (with decomposition, sealed capillary). The content of the main substance was 98% according to HPLC data. Found (%): C 55.49, H 4.18, N 8.38, S 6.75. $C_{23}H_{21}N_3O_8S$. Calculated (%): C 55.30, H 4.25, N 8.41, S 6.42.

^{1}H NMR spectrum (5%- solution in acetone-*d*6, δ, ppm, *J*, Hz): 0.93 and 1.76 both t (each 3H, *J* = 7, CH_2 groups of C_2H_5O and C_2H_5N fragments, respectively); 3.55 and 5.27 both q (each 2H, *J* = 7, CH_2 groups of the above-mentioned moieties, respectively); 7.8–8.0 m (3H) and 8.1–8.2 m (2H) (C6H5); 8.43 d (1H, *J* = 2.5, H-7 and H-4); 8.68 d.d (1H, *J* = 9 and *J* = 2, H-2 or H-9); 8.86 d.d (1H, *J* = 9 and *J* = 2.5, H-9 or H-2); 9.38 and 9.42, both d (each 1H, *J* = 9, H-1, H-10); 9.44 d (1H, *J* = 2, H-4 or H-7). IR spectrum, ν, cm^{-1} (KBr): 580, 720, 750, 780, 800, 1020, 1225, 1250, 1350, 1390, 1525, 1550, 1620. UV spectrum (EtOH), λ_{max}, nm (log ε): 210 (4.47), 260 (4.20), and 325 (4.22).

Ether, ethanol, and 147 g of diethyl sulfate with bp 95–97°C (0.4 kPa) were regenerated from ether and alcoholic decantates and mother liquors. A 27-g portion (22%) of the starting 3,8-dinitro-6-phenylphenanthridine (**V**) was regenerated from the paper cartridge of the Soxhlet apparatus upon its drying at 120–130°C. With this regenerated starting compound taken into account, the yield of ethyl sulfate **VIII** was 96%.

3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide (ethidium bromide) (IX). Into an agitated mixture of 49.0 g (0.0981 mol) of ethyl sulfate **VIII** in 40 ml of a 1 N solution of sulfuric acid (with 0.2 mol H_2SO_4) was poured 150 ml of 95% ethanol and

then 91.1 g (1.63 g-at) of powdered iron was added in 5-g portions. An exothermic reaction was observed, with the mixture colored to become dark red. On being agitated for 30 min, the mixture was boiled with reflux for 1 h, cooled to 25°C, and 100 ml of 25% ammonia (with 1.33 mol NH_3) was added. A bulky precipitate was formed; the mixture was brought to boiling, the precipitate was filtered off and washed with 2 × 50 ml of hot water (70–90°C). To the filtrate cooled to 25°C and combined with washing water was added dropwise under agitation a freshly prepared and cooled to 25°C solution of 115 g of NaOH in 115 ml of water (with 2.875 mol NaOH). The mixture was extracted with *n*-butanol (6 × 100 ml). The extract was filtered off to remove trace amounts of a slurry, 12.5 ml of 46.5% hydrobromic acid (with 0.105 mol HBr) was added to the filtrate, and then *n*-butanol and water were evaporated from the mixture at 60–70°C (4 kPa). The residual amounts of *n*-butanol were removed by repeated evaporation upon addition of 3 × 50 ml of water. The dark tarry residue was dissolved in 160 ml of methanol under boiling with reflux, cooled to a temperature of 0 to +5°C and allowed to stay for 16 h. The resulting precipitate was filtered off and extracted in a Soxhlet apparatus with methanol (2 × 250 ml) (boiling each time for 8 h). Dark red crystals were filtered off from combined extracts stored at 0 to +5°C. Their drying at 105–110°C (0.4 kPa) yielded 22.0 g (57%) of ethidium bromide (**IX**) in the form of a dark red powder with mp 261–264°C (with decomposition). Published data: mp 260–262°C (with decomposition) [25], mp 248–249°C (with decomposition) [21]. According to HPLC data, the content of the main substance was 97%. A TLC analysis (Kieselgel 60 F254 plates from Merck, system acetone : methanol : water : 0.1 N HCl = 3 : 3.5 : 2.5 : 1 by volume) yielded a value R_f = 0.78 for ethidium bromide. Found (%): C 63.51, H 5.07, N 10.57, Br 19.53. $C_{21}H_{20}BrN_3$. Calculated (%): C 63.96, H 5.12, N 10.66, Br 20.26.

^{1}H NMR spectrum (1% solution in DMSO-d_6, δ, ppm, *J*, Hz): 1.40 t (3H, *J* = 7, CH_3); 4.47 q (2H, *J* = 7, CH2); 5.93 and 6.36, both br.s (each 2 H, 2 CH_2 groups); 6.27 d (1H, *J* = 2, H-7); 7.3–7.4 m (2H, H-2, H-4); 7.53 d.d (1H, *J* = 9 and *J* = 2, H-9); 7.6–7.8 m (5H, C6H5); 8.58 and 8.68, both d (each 1H, *J* = 9, H-10 and H-1, respectively). The signals in the ^{1}H NMR spectrum were assigned by analogy with the data from [9]. IR spectrum, ν, cm^{-1} (KBr): 845, 1260, 1320, 1410, 1470, 1500, 1630 corresponds to the spectra reported in [9, 26]. EAS (MeOH), λ_{max}, nm (log ε): 215 (4.62), 295 (4.80),and 528 (3.85) corresponds to the spectrum reported in [27].

3,8-Dicarboethoxyamino-5-ethyl-6-phenylphenanthridinium ethyl sulfate (X). A mixture of 1.48 g (3.45 mmol) of 3,8-dicarboethoxyamino-6-phenylphenanthridine (**VII**) and 2.59 g (16.8 mmol) of diethyl sulfate was heated to 160–165°C for 30 min. A high-intensity crystallization in the originally formed solution was observed. On being cooled, the mixture was ground with 10 ml of dry ether, and the product formed was filtered off and washed with dry ether (3 × 3 ml) and dry acetone (3 × 3 ml). A 1.74-g portion (87%) of ethyl sulfate (**X**) was formed, decomposition point 200–220°C (without melting). Found (%): C 59.47, H 5.97, N 7.31, S 5.50. $C_{29}H_{33}N_3O_8S$. Calculated (%): C 59.67, H 5.71, N 7.20, S 5.49.

^{1}H NMR spectrum (5% solution of DMSO-d_6, δ, ppm, *J*, Hz): 1.09, 1.18, and 1.30, all t (each 3H, *J* = 7, three CH_3 groups of three C_2H_5O fragments); 1.51 br.t (3H, *J* = 7, CH3 group of C_2H_5N fragment); 3.73, 4.06, and 4.22, all q (each 2H, *J* = 7, three CH_2 groups of three C_2H_5O fragments); 4.63 br.q (2H, *J* = 7, CH_2 group of C_2H_5N fragment); 7.7–7.9 m (6H, H-4 or H-7 and C6H5); 8.02 and 8.18, both d.d (each 1H, *J* = 9 and *J* = 2, H-2, H-9); 8.72 d (1H, *J* = 2, H-7 or H-4); 8.95 and 9.01, both d (each 1H, *J* = 9, H-1, H-10); 10.24 and 10.51, both s (each 1H, 2 CH groups). IR spectrum, ν, cm^{-1} (KBr): 720, 730, 760, 780, 930, 1030, 1080, 1090, 1100, 1160, 1190, 1240, 1320, 1350, 1380, 1410, 1490, 1540, 1570, 1590, 1610, 1630, 1730 (C=O). EAS (EtOH), λ_{max}, nm (log ε): 217 (4.54), 285 (4.80), and 425 (3.82).

3,8-Diamino-5-ethyl-6-phenylphenanthridinium ethyl sulfate (ethidium ethyl sulfate) (XI). A solution of 1.00 g (1.74 mmol) of ethyl sulfate **X** in 2.79 ml of 78% sulfuric acid (with 37.8 mmol H2SO4) was heated to 135–140°C for 1 h under agitation. Gas evolution was observed in the resulting dark color solution; by the end of the keeping, the solution became lighter, and the gas evolution, noticeably weaker. The mixture was cooled and poured into 2.5 ml of icy water. A 10-ml portion of a 25% ammonia solution (133 mmol NH_3) was added dropwise under agitation, with a dark red tarry precipitate formed. The mixture was twice extracted with 4 ml of *n*-butanol each time. After the extraction the aqueous layer became nearly colorless. A finely crystalline dark red precipitate was filtered off from the *n*-butanolic extract allowed to stay at 0 to +5°C for 16 h; the precipitate was dried in air and recrystallized from 5.2 ml of hot water to give 0.48 g

(61%) of ethidium ethyl sulfate (**XI**), mp 250–252°C (with decomposition). Found (%): C 62.90, H 5.87, N 9.65, S 7.30. $C_{23}H_{25}N_3O_4S$. Calculated (%): C 62.84, H 5.74, N 9.56, S 7.29.

^{1}H NMR spectrum 5% solution in DMSO-d_6, δ, ppm, *J*, Hz): 1.10 t (3H, *J* = 7, CH_3 of the ethyl sulfate group); 1.40 br.t (3H, *J* = 7, CH_3 group of C_2H_5N fragment); 3.74 q (2H, *J* = 7, CH_2 group of ethyl sulfate group); 4.47 br.q (2H, *J* = 7, CH_2 group of C_2H_5N fragment); 5.93 and 6.36, both br.s (each 2H, $2CH_2$ groups); 6.25 d (1H, *J* = 2, H-7); 7.3–7.4 m (2H, H-2, H-4); 7.51 d.d (1H, *J* = 9 and *J* = 2, H-9); 7.7–7.8 m (5H, C_6H_5; 8.60 and 8.65, both d (each 1H, *J* = 9, H-10 and H-1, respectively). The signals in the ^{1}H NMR spectrum were assigned by analogy with the data of [9] for ethidium bromide. IR spectrum, ν, cm^{-1} (KBr): 720, 790, 840, 930, 1030, 1070, 1170, 1190, 1220, 1240, 1280, 1330, 1410, 1480, 1490, 1510, 1640. EAS (MeOH), λ_{max}, nm (log ε): 216 (4.61), 293 (4.81), and 528 (3.81). FS (H2O): λ_{max} 619 nm. For ethidium bromide from Fluka, FS (H_2O): λ_{max} 619 nm.

Results of tests of the detection sensitivity of a double-stranded DNA with ethidium bromide and ethidium ethyl sulfate suggest that ethidium ethyl sulfate is suitable for determination of PCR products in their electrophoretic analysis and is less expensive, compared with ethidium bromide, because its synthesis eliminates need to expend reagents and time for replacement of the intermediate anion with the bromine anion and isolate ethidium bromide.

CONCLUSIONS

(1) Two methods for synthesis of ethidium bromide were improved. The technological scheme of one of these methods was implemented at Pilot chemical shop, Vorozhtsov Institute of Organic Chemistry in Novosibirsk.

(2) An analog of ethidium bromide, ethidium ethyl sulfate, was synthesized. This is a new fluorescent dye suitable for analysis for double-stranded DNA and, in particular, for analyses for products of the practically important polymerase chain reaction.

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