Supplementary Materials

Water-Soluble Dicationic Deuteroporphyrin Derivative for Antimicrobial PDT: Singlet Oxygen Generation, Passive Carrier Interaction and Nosocomial Bacterial Strains Photoinactivation

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1. Synthesis of PS

The target compound (Comp. **5)** was prepared by the four-stage synthesis procedure presented in Figure 1. The starting 13(3),17(3)-dimethyl ester of deuteroporphyrin-IX (Comp. **3**) was obtained from natural hemin (Comp. **1**) using the Schumm devinylation reaction followed by reductive demetalation and esterification of the macroheterocycle [66]. The water-soluble derivative of PS **3**, 13(3),17(3)-*bis*-N-(2-N',N',N'-trimethylammoniaethyl iodide) amide of deuteroporphyrin-IX (Comp. **5**), was synthesized by a two-stage synthesis, including aminolysis with N,N-dimethylethylenediamine and further quaternization of the two tertiary amino groups of Comp. **4** formed in the reaction with methyl iodide (Fig. S1). The yield of compound **5** from PS **3** was 70%.

Dicationic chlorin, 3(1),3(2)-*bis*-(N,N,N-trimethylammoniomethyl iodide)-13(1)-N'-methylamide-15(2),17(3)-dimethyl ester of chlorin e_6 (Comp. **6**), was synthesized as recommended in paper [39].

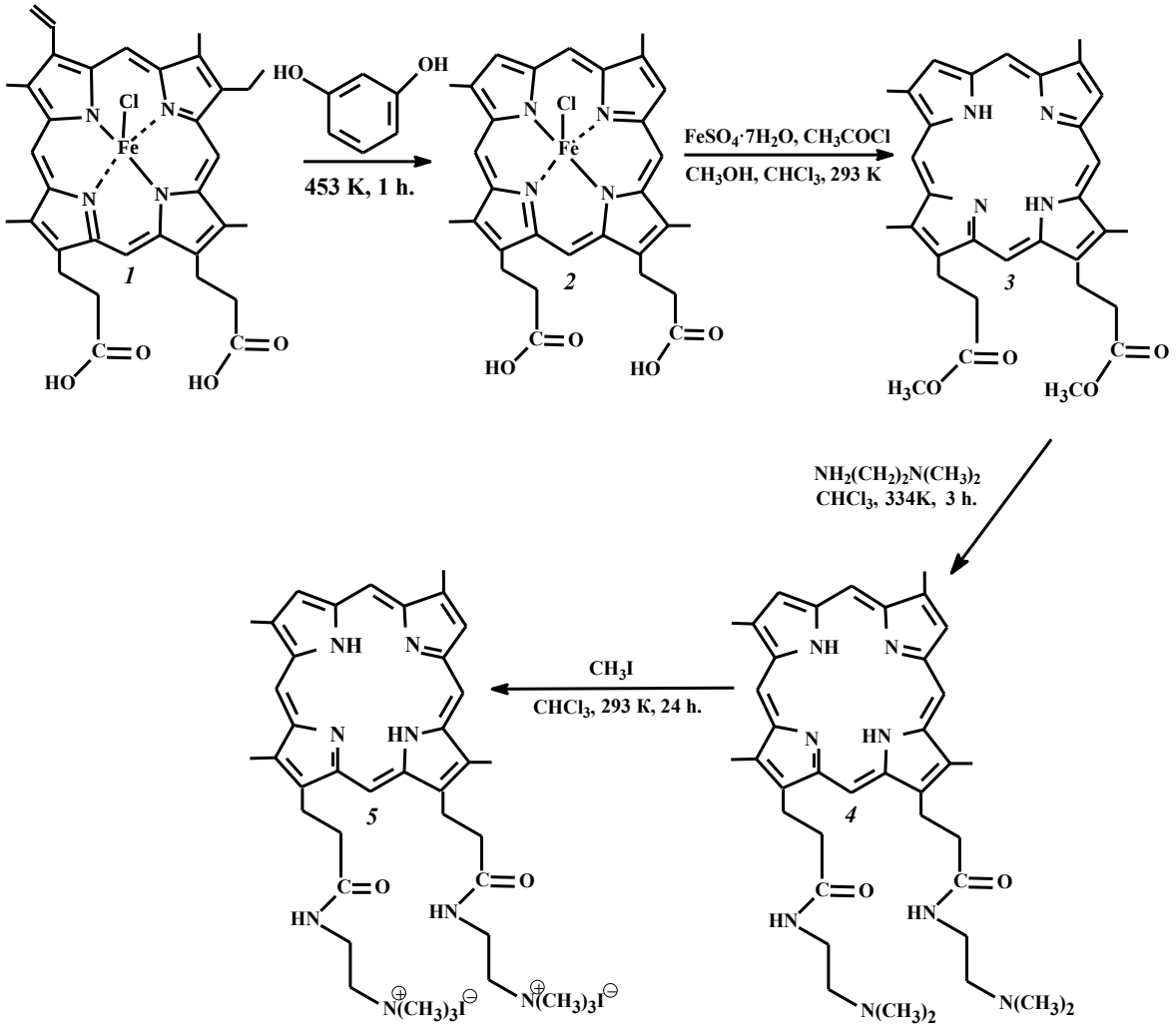

Figure S1. Synthesis of 13(3),17(3)-*bis*-N-(2-N',N',N'-trimethylammonioethyliodide) amide of deuteroporphyrin-IX (Comp. **5**) from hemin (Comp. **1**).

1.1. 13(3),17(3)-Dimethyl ester of deuteroporphyrin-IX (Comp. **3**)

At the first stage of the synthesis, 1 g of protohemin-IX (Comp. **1**, 1.54 mmol) was triturated with 3 g of resorcinol ($C_6H_6O_2$) and the mixture was alloyed for 1 h at 453 K. After cooling, the intermediate was extracted with diethyl ether until a colorless extract was obtained. Then, the solvent was evaporated, and the solid residue of deuterohemin-IX (Comp. **2**, Fig. 1) was dried. At the next stage, 1 g (1.62 mmol) of the deuterohemin-IX powder (Comp. **2**) and 2.5 g of $FeSO_4 \times 7H_2O$ were suspended in a mixture of 150 ml of methanol and 150 ml of chloroform. A total of 7.5 ml of acetyl chloride was added to the suspension dropwise so that the temperature did not exceed 293 K, then the mixture was stirred continuously for 24 h. The reaction mixture was filtered to remove the unreacted deuterohemine and iron(II) sulfate, the filtrate was diluted with chloroform (50 ml) and washed once in a separating funnel with a 10% ammonia solution and then several times with distilled water. The solvent was evaporated and the porphyrin was exposed to chromatographic purification using Al_2O_3 (activity grade II) and a mixture of chloroform and methanol (100:1 volume ratio) as the appropriate eluent. The eluate was evaporated to a minimum volume and 13(3),17(3)-dimethyl ester of deuteroporphyrin-IX (Comp. **3**) was precipitated with methanol. The yield was 0.54 g (1.01 mmol), 63%. The spectral characteristics of Comp. **3** are presented in Figures 2 and 3.

Mass spectrum MALDI-TOF: m/z (%): 539.2492 (100) $[M]^+$.

^{1}H NMR ($CDCl_3$): δ (ppm) 10.35, 10.06, 10.01, 9.95 (4s, 1H each, H^5, H^{10}, H^{15}, H^{20}); 8.89, 8.85 (2 s, 1H each, H^3, H^8); 4.32 (m, 4H, 13-,17-$\underline{CH_2}CH_2COOCH_3$); 3.47, 3.44, 3.34, 3.30 (4s, 3H each, 2-, 7-, 12-, 18- CH_3); 3.26, 3.25 (2s, 3H each, 13-,17-$CH_2CH_2COO\underline{CH_3}$); 3.17 (m, 4H, 13-, 17-$CH_2\underline{CH_2}COOCH_3$); -3.14 (br.s., 2H, 21,23-NH).

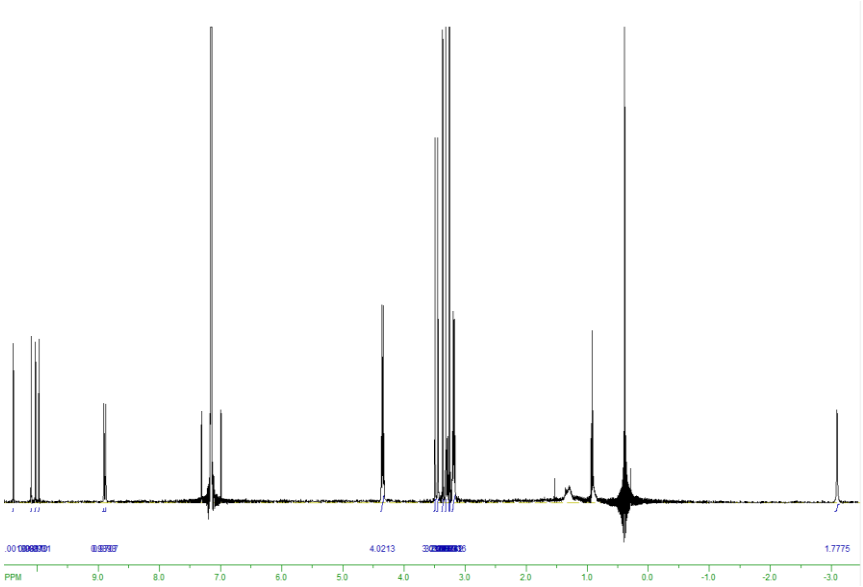

Figure S2. ^{1}H NMR spectrum of 13(3),17(3)-dimethyl ester of deuteroporphyrin-IX (Comp. **3**) in $CDCl_3$.

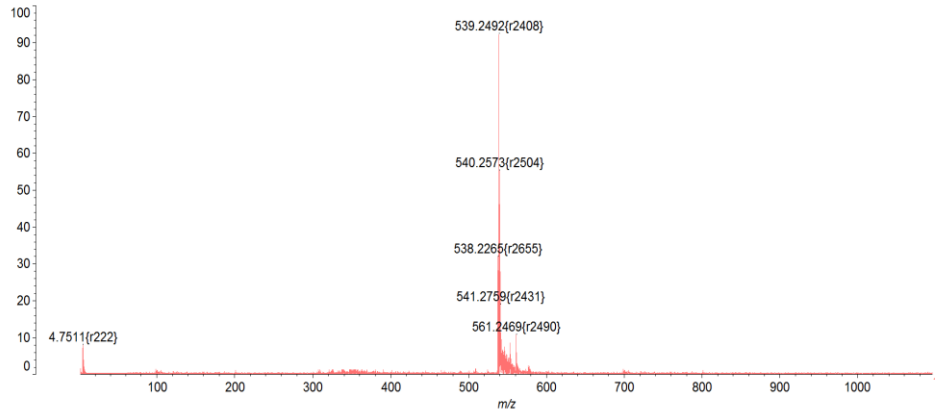

Figure S3. Mass spectrum (MALDI-TOF) of 13(3),17(3)-dimethyl ester of deuteroporphyrin-IX (Comp. **3**). DHB was used as a matrix.

1.2. 13(3),17(3)-*bis*-N-(2-N',N'-Dimethylaminoethyl) amide of deuteroporphyrin-IX (Comp. **4**)

A mixture of 7 ml (64.3 mmol) of N,N-dimethylethylenediamine and 0.55 g (1 mmol) of the 13(3),17(3)-dimethyl ester of deuteroporphyrin-IX (Comp. **3**) was dissolved in 10 ml of $CHCl_3$ and was refluxed for 3 h (Scheme), then was diluted with distilled water and neutralized with a dilute solution of AcOH to reach p*H* = 7. The solvent was evaporated, and then the final compound was purified by column chromatography on silica with a mixture of acetone and CCl_4 in the volume ratio of 1:40. The solution was evaporated, and the precipitate was filtered and dried in vacuo. The yield was 0.619 g (0.95 mmol), 93%. The spectral characteristics of Comp. **4** are presented in Figures 4 and 5.

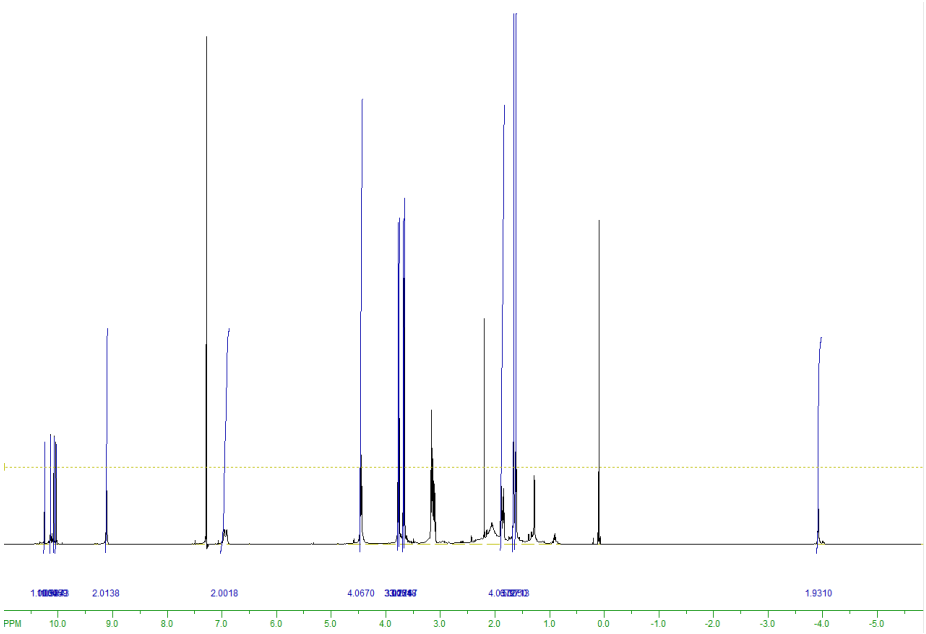

Figure S4. ^{1}H NMR spectrum of 13(3),17(3)-*bis*-N-(2-N',N'-dimethylaminoethyl) amide of deuteroporphyrin-IX (Comp. **4**) in $CDCl_3$.

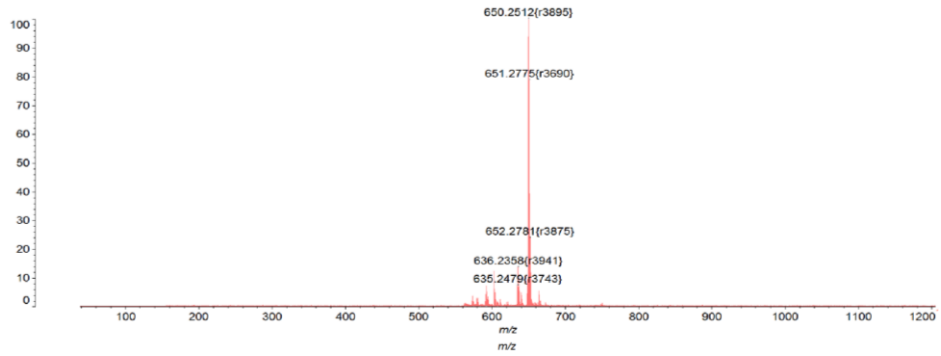

Figure S5. Mass spectrum (MALDI-TOF) of 13(3),17(3)-*bis*-N-(2-N',N'-dimethylaminoethyl) amide of deuteroporphyrin-IX (Comp. **4**). DHB was used as a matrix.

Mass spectrum MALDI-TOF: m/z (%) = 650.2512 (100) $[M]^+$.

^{1}H NMR ($CDCl_3$): δ (ppm) 10.25, 10.14, 10.08, 10.04 (4s, 1H each, *ms*-H); 9.11, 9.10 (2s, 1H each, H^3, H^8); 6.93 (m, 2H, 13(3)-,17(3)-N<u>H</u>$CH_2CH_2N(CH_3)_2$); 4.46, 3.12 (2t, 4H each, 13-,17-<u>$(CH_2)_2$</u>$CONH(CH_2)_2N(CH_3)_2$, J = 7.2 Hz); 3.78, 3.76, 3.68, 3.66 (4s, 3H each, 2, 7, 12, 18-CH_3); 3.11 (t, 4H, 13(3)-,17(3)-NH<u>CH_2</u>$CH_2N(CH_3)_2$, J = 5.8 Hz); 1.89, 1.85 (2t, 2H each, 13(3)-,17(3)-$NHCH_2$<u>CH_2</u>$N(CH_3)_2$, J = 6.2 Hz); 1.66, 1.62 (2s, 6H each, 13(3)-,17(3)-$NHCH_2CH_2N($<u>CH_3</u>$)_2$); -3.91 (br.s., 2H, 21,23-NH).

1.3. 13(3),17(3)-*bis*-N-(2-N',N',N'-Trimethylammoniomethyl iodide) amide of deutero-porphyrin-IX (Comp. **5**)

A total of 3 ml (48.19 mmol) of methyl iodide was added to a solution of 30 mg (0.05 mmol) of 13(3),17(3)-*bis*-N-(2-N',N'-dimethylaminoethyl)amide of deuteroporphyrin-IX (Comp. **4**) in 2 ml of $CHCl_3$ (Scheme). The reaction was carried out for 24 h under stirring at room temperature. Chloroform was added to the reaction mixture and the precipitate formed was filtered using a Schott filter. The final compound was washed with $CHCl_3$, then with hexane and dried under vacuo. The yield was 33 mg (0.035 mmol), 76%. The spectral characteristics of Comp. **5** are presented in Figures 6 and 7.

Mass spectrum MALDI-TOF: m/z (%) = 932/2 = 466.4119 (100) $[(M\text{-}2I^-+I_2)/2]^+$, 410.3652 (65) $[(M\text{-}2I^-+DHB\text{-}CH_3^++H^+)/2]^+$.

^{1}H NMR (CH_3OH d_4): δ (ppm) 10.61, 10.49, 10.48, 10.42 (4s, 1H each, *ms*-5,10,15,20-H); 9.02, 8.98 (2s, 1H each, H^3,H^8); 4.08, 2.81, (2t, 4H each, 13-,17-<u>$(CH_2)_2$</u>$CONH(CH_2)_2N^+(CH_3)_3$, J = 6.3 Hz); 3.25, 3.24, 3.16, 3.09 (4s, 3H each, 2,7,12,18-CH_3); 2.71, 2.55 (2t, 4H each, 13(3),17(3)-<u>$(CH_2)_2$</u>-$N^+(CH_3)_3$), J = 6.2 Hz); 2.32 (s, 18H, -$N^+(CH_3)_3$).

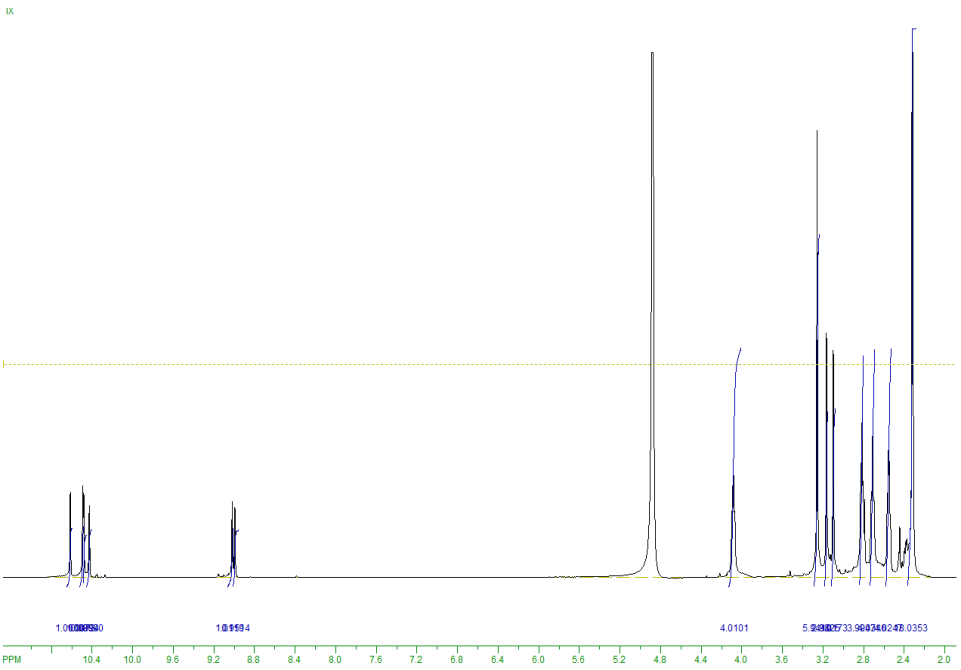

Figure S6. ^{1}H NMR spectrum of 13(3),17(3)-*bis*-N-(2-N',N',N'-trimethylammoniomethyl iodide) amide of deuteroporphyrin-IX (Comp. **5**) in CD_3OD.

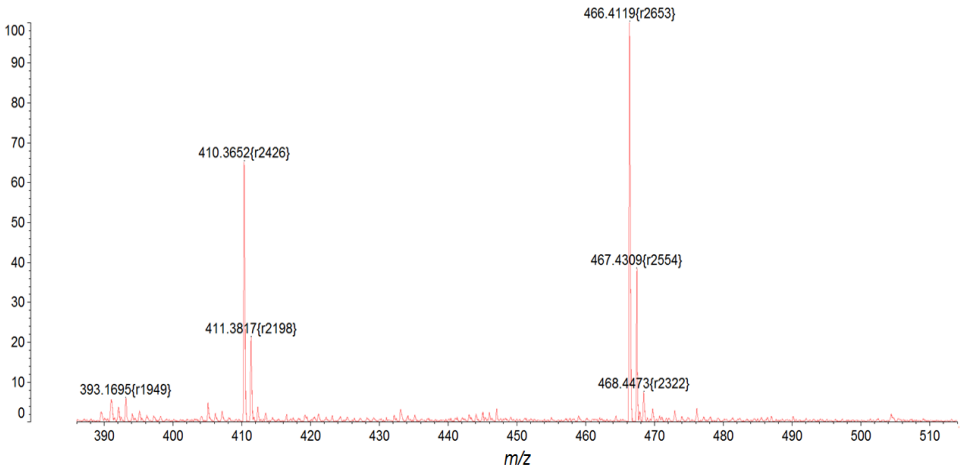

Figure S7. Mass spectrum (MALDI-TOF) of 13(3),17(3)-*bis*-N-(2-N',N',N'-trimethylammoniomethyl iodide) amide of deuteroporphyrin-IX (Comp. **5**). DHB was used as a matrix.

2. Physical chemical experiments

Since the formation of PS aggregates at the therapeutic concentration negatively affects the generation of singlet oxygen and membrane transport, its prevention is one of the most important tasks in the development of a new generation of the photosensitizers. In this regard, further studies were aimed at studying the interaction of the macrocyclic PS with the potential carrier—the nonionic surfactant Tween 80.

Table S1. Parameters of the absorption and fluorescence spectra for Comps. **5, 6**

PS	*m*, mol/kg	Solvent	λ_{Soret}* (lgε)	Absorption λ_{IV}(lgε)	λ_{III}(lgε)	λ_{II}(lgε)	λ_{I}(lgε)	Fluorescence λ_1*	$\Delta\lambda_{st}$*
Comp. **5**	7.3×10^{-6}	EtOH	395(5.06)	496(4.17)	530(4.08)	565(4.02)	620(3.90)	622	2
	7.3×10^{-6}	H_2O	391(4.55)	497(3.83)	532(3.76)	557(3.73)	610(3.64)	613	3
	7.3×10^{-6}	H_2O-Tween80 (m=7.3×10^{-4})	394(5.08)	496(4.22)	530(4.12)	567(4.08)	620(3.95)	622	2
Comp. **6**	7.3×10^{-6}	EtOH	393(5.12)	496(4.09)	522(3.48)	605(3.68)	655(4.67)	666	11
	7.3×10^{-6}	H_2O	393(4.99)	497(3.89)	522(3.40)	602(3.53)	652(4.43)	661	9
	7.3×10^{-6}	H_2O-Tween80 (m=7.3×10^{-4})	395(5.14)	496(4.16)	524(3.59)	604(3.73)	657(4.66)	660	3

* The spectral bands maxima (λ) and Stokes shifts ($\Delta\nu_{st}$) values are given in nanometers.

Table S2. Size distribution of aggregates in aqueous solution: Comp. **5**, m_{PS} = 0.0175 mmol/kg, Comp.**6**, m_{PS} = 0.789 mmol/kg.

Comp. **5**		Comp. **6**	
d, nm	*I*, %	*d*, nm	*I*, %
28.21	0	43.82	0
32.67	0	50.75	0
37.84	0	58.77	0.2
43.82	0	68.06	0.9
91.28	0	78.82	2.1
105.7	0	91.28	3.7
122.4	0	105.7	5.5
141.8	0	122.4	7.3
164.2	0.2	141.8	8.8
190.1	3	164.2	9.8
220.2	8	190.1	10.4
255	13.3	220.2	10.4
295.3	17	255	9.8
342	17.8	295.3	8.8
396.1	15.6	342	7.4
458.7	11.2	396.1	5.8
531.2	6.1	458.7	4.2
615.1	2	531.2	2.7
712.4	0	615.1	1.5
825	0	712.4	0.7
955.4	0	825	0.2
1106	0	955.4	0
43.82	0	1106	0

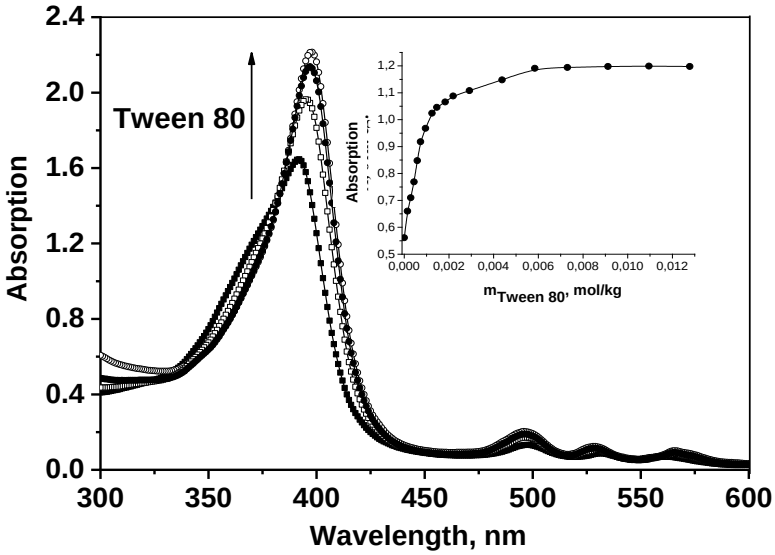

Figure S8. The UV-VIS spectra of Comp. **5** (m_{PS} = 7.3×10^{-5} mol/kg) in water and aqueous solutions of Tween 80 at room temperature. Curves from bottom to top show a gradual increase in the concentration of Tween 80: (■) - 0, (□) - 7.3×10^{-4} (●) - 1.5×10^{-3} and (○) - 1.3×10^{-2} mol/kg, respectively. Inset: dependence of the optical density of the solution of Comp. **5** on the surfactant concentration at λ=410 nm.

Table S3. Experimental values of the titration curves of aqueous solutions of Comp. **5** (m=7.3×10^{-6} mol/kg) with Tween 80 solution.

$m_{\text{Tween 80}}$, mol/kg	$A_{410\text{ nm}}$
0	0.05
3.69·10^{-5}	0.085
7.38·10^{-5}	0.105
1.01·10^{-4}	0.115
1.28·10^{-4}	0.121
1.81·10^{-4}	0.131
2.35·10^{-4}	0.133
2.89·10^{-4}	0.134
3.43·10^{-4}	0.135
3.97·10^{-4}	0.136
4.50·10^{-4}	0.137
5.14·10^{-4}	0.137
5.51·10^{-4}	0.137

Table S4. Dependence of PS **5** fluorescence intensity in Tween 80 solutions on potassium iodide concentration.

$m_{Tween\ 80}=6.5\times10^{-5}$ mol/kg	
m(KI), mol/kg	*F*
0	71.48
0.073	45.12
0.143	35.84
0.209	27.84
0.273	22.92
0.333	17.64
0.391	13.36
0.447	13.54
0.551	11.21
0.647	8.92
0.736	7.58
0.895	6.36
1.033	5.55
1.154	4.47

3. Microbiological investigations

Standard microbiological methods [44] were used to study the dark and photoinduced toxicity of the porphyrin dicationic PS **5** towards the following opportunistic pathogens in vitro—*Enterobacter cloacae*, *Escherichia coli* and *Pseudomonas aeruginosa.* All the nosocomial antibiotic-resistant strains of Gram-negative bacteria were carefully grown in the clinical laboratory of the Ivanovo regional clinical hospital. The microbiological studies of the PS cyto- and photocytotoxicity were carried out in certified laboratories of the Department of Microbiology and Research Center of Ivanovo State Medical Academy.

PS solutions were prepared in bidistilled water or in aqueous solutions of Tween 80 or Trilon B (Na_2H_2Edta). The final solution was then homogenized on a Sonopuls ultrasonic homogenizer (Bandelin electronic GmbH & Co., Germany).

Cultures of test strains were grown on beef infusion agar slant in an incubator overnight. The cell density was adjusted to the 5 McFarland standard in sterile saline (), which corresponds to approximately 5×10^8 CFU/ml. The inoculum dose of 2×10^7 CFU/ml from the stock standard suspension was prepared by diluting. To simulate aPDT, 0.5 ml of the tested microorganisms were added to 4-well plates and incubated in the dark for 0.5 h at room temperature with 0.5 ml of various photosensitizer solutions. After incubation half of the samples were irradiated with red light. Then all the plates were kept for 24 h at 310 K. To confirm the bactericidal effect in the samples studied, the latter were seeded from all the wells onto Petri dishes with a solid nutrient medium to count the CFU number. Some results of the microbiological experiments are presented in Table S5.

Modeling of microorganism photoinactivation in aqueous solutions was carried out in a dark place at room temperature by irradiating plates using a special LED source of visible light (BMC, Belarus) with adjustable radiation power and water cooling. The maximum radiation power of the LED panel used was ~ 0.2 W/cm^2; the illuminated surface area was up to 100 cm^2; the wavelength range of the incident light used for the porphyrin PS was 620±60 nm. The radiation power, the sample distance (~ 10 cm) and the exposure time were selected so as to give a radiation dose of 40 or 80 J/cm^2 during a typical therapeutic procedure (~10-15 min) applied in clinics. The radiation dose from the calibrated LED panel was measured with an Argus-03 radiation power meter (Russia).

Table S5. In vitro inactivation of nosocomial Gram-negative pathogens (m_{PS} = 0.0001 mol/kg)

	Darkness	**Dose 40 J/cm^2**	**Dose 80 J/cm^2**
	Escherichia coli hemolytic		
PS+0.5 wt. % Tween 80			

References:

[66] Koifman, O.I.; Askarov, K.A.; Berezin, B.D.; Enikolopyan, N.S. Natural sources of porphyrins. Methods for the isolation and modification of natural porphyrins. In *Porphyrins: structure, properties, synthesis;* Enikolopyan, N.S., Ed.; Nauka: Moscow, Russia, 1985; pp. 175–204 (in Russian).