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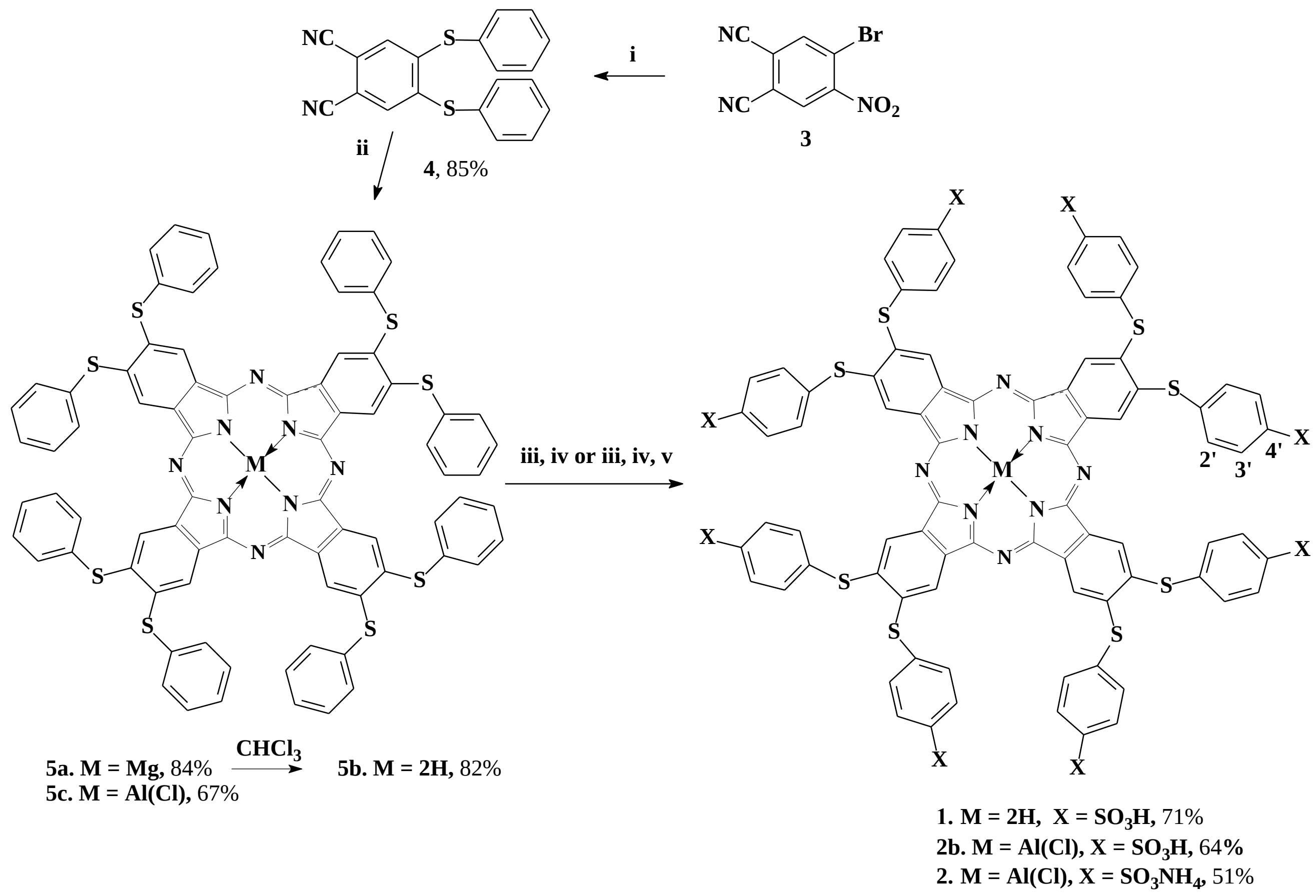
Aggregation of water soluble octaanionic phthalocyanines and their photoinactivation antimicrobial effect *in vitro*

Dmitry B. Berezin, Vladimir V. Makarov, Serafima A. Znoyko, Vladimir E. Mayzlish and Andrey V. Kustov

1. Materials

1.1 Synthetic schemes

Phthalocyanines (Pcs) **1,2** contained eight 4-sulfophenylsulfanyl groups at positions 2,3,9,10,16,17,23 and 24 of fused benzene rings of the macroheterocycle (MHC) were obtained according to Scheme S1.[1]



Scheme S1. *Reagents and conditions:* i, C_6H_5SH, DMF, 100°C, 12 h; ii, $Mg(OAc)_2 \cdot 4H_2O$ (or $AlCl_3$), 180-200°C, 2 h; iii, $SOCl_2$+HSO_3Cl, 25°C, 1 h; iv, H_2O, 100°C; v, NH_3+H_2O, 90-100°C.[1]

The nucleophilic substitution of halogen and nitro group in a molecule of 4-bromine-5-nitrophthalonitrile **3** for two sulfophenylsulfanyl fragments followed by high-temperature template condensation of the product **4** resulting in hydrophobic MHC **5** formation as a Mg- (*a*) or Al- (*b*) complex were performed. Compound **5***a* was treated with acidified $CHCl_3$ to release metal-free MHC **5***c*. Then the products **5***b* and **5***c* were consequently exposed to sulfochlorination followed by hydrolysis to the sulfonic acids yielding compounds **1** and **2***a*. Sulfonated MHC **2***a* was additionally neutralized with aqueous ammonia solution to reduce its hygroscopicity and to get compound **2**. Products required (comp. **1,2,2***a*) were identified with ^{1}H NMR, MS-, IR-, UV-Vis-spectra and elemental analysis. Their fluorescence spectra were extensively studied in a paper.[1]

*4-Bromo-5-nitrophthalonitrile (**3**)* was synthesized according to the known procedure [2]. M.p. 140–142 °C. Found,%: C 38.10; H 0.76; N 16.50. $C_8H_2BrN_3O_2$. Calculated,%: C 38.16; H 0.80; N 16.67.

*Bis-4,5-(phenylsulfanyl)phthalonitrile (**4**).*

2.52 g (0.01 mol) of comp. ***3*** and 2.2 g (2.36 ml, 0.02 mol) of thiophenol were dissolved in 50 ml of DMF, then a solution of 2.76 g (0.02 mol) of potassium carbonate in 7 ml of water was added. The reaction mixture was stirred at 80–85 °C for 9 h until the product precipitation. Then the target compound was filtered off, washed sequentially with 2-propanol and water, dried at 80–90 ° C.

Yield of ***4***: 85% (3.00 g). IR (ν/cm^{-1}): 2231 (ν^{CN}), 1609 (ν^{CS}), 1282 (ν^{CArH}), 691 (δ^{CSC}). MS, m/z: 345.72 $[M + H]^+$. Found, %: C 69.55; H 3.60; N 8.02; S 18.25. $C_{20}H_{12}N_2S_2$. Calculated,%: C 69.74; H 3.51; N 8.13; S 18.62.

Magnesium 2,3,9,10,16,17,23,24-*octaphenylsulfanyl phthalocyanine* (***5a***).

A mixture of 0.345 g (1 mmol) of di-4.5-(phenylsulfanyl)phthalonitrile ***4*** and 0.107 g (0.05 mmol) of magnesium acetate tetrahydrate was heated for 2 h at 180–185 °C. Then the reaction mixture was cooled, dissolved in chloroform and filtered. Column chromatography on silica gel was performed using toluene as the eluent.

Yield of ***5a***: 0.29 g (84%). IR (ν/cm^{-1}): 1577 ($\nu^{C\text{-}C}$), 1507 ($\nu^{C–N}$), 1257 (ν^{CArH}), 690 (δ^{CSC}), 645 (δ^{CH}). MS, m/z: 1402.86 $[M]^+$. Found,%: C 68.25; H 3.81; N 7.61; S 18.05. $C_{80}H_{48}MgN_8S_8$. Calculated, %: C 68.53; H 3.45; N 7.99; S 18.29.

2,3,9,10,16,17,23,24-*Octaphenylsulfanyl phthalocyanine* (***5b***).

0.1 g (0.7 mmol) of comp. ***5a*** was dissolved in 2 ml of chloroform, then chloroform was evaporated. The residue was chromatographed on silica gel using chloroform as eluent.

The yield of ***5b:*** 0.082 g (82%). MS, m/z: 1380.92 $[M]^+$. Found, %: C 69.51; H 3.80; N 8.00; S 18.25. $C_{80}H_{50}N_8S_8$. Calculated, %: C 69.64; H 3.65; N 8.12; S 18.59.

Aluminum 2,3,9,10,16,17,23,24-*octaphenylsulfanyl phthalocyanine* (***5c***).

A mixture of 0.345 g (1 mmol) di-4,5-(phenylsulfanyl)phthalonitrile **4** and 0.066 g (0.5 mmol) of anhydrous aluminum chloride was heated for 2 h at 190–200 ° C. Then the reaction mass was cooled, dissolved in chloroform, filtered, and column chromatography on silica gel was performed using chloroform as the eluent.

Yield of ***5c***: 0.24 g (67%). IR (ν/cm^{-1}): 1579 ($\nu^{C\text{-}C}$), 1506 ($\nu^{C–N}$), 1257 (ν^{CArH}), 690 (δ^{CSC}), 679 (δ^{CH}). MS, m/z: 1441.91 $[M+H]^+$. Found, %: C 66.15; H 3.76, N 7.42; S 17.55. $C_{80}H_{48}AlClN_8S_8$. Calculated, %: C 66.72; H 3.36; N 7.78; S 17.81.

2,3,9,10,16,17,23,24-O***cta-(4'-sulfophenylsulfanyl) phthalocyanine*** (***1***) ***and its complex with aluminum*** (***2a***).

0.2 Mmol of comp. ***5b*** (0.28 g) or ***3c*** (0.29 g) were added under vigorous stirring to a mixture of 2 ml (18 mmol) of chlorosulfonic acid and 2 ml (18 mmol) of thionyl chloride. The resulting mixture was stirred at 20 °C for 0.5–1 h, then poured onto ice treated with sodium chloride. The precipitate was collected and dried in a desiccator over concentrated sulfuric acid for 3 days. After that the intermediate compounds (sulfochlorides) were extracted from the reaction mixture with acetone, the solvent was evaporated. 0.2 g (0.1 mmol) of intermediate sulfochloride or its aluminum complex and 10 ml of water were placed in a porcelain dish and heated until complete dissolution, then water was removed. The resulting sulfonic acids ***1,2*** were subjected to liquid column chromatography on silica gel M60 (the eluent – water).

2,3,9,10,16,17,23,24-*Octa-(4'-sulfophenylsulfanyl) phthalocyanine* (***1***).

Yield of ***4a***: 0.17 g (71%). IR (ν/cm^{-1}): 3536 (ν^{OH}), 3150 (ν^{NH}), 1612 ($\nu^{C\text{-}C}$), 1523 ($\nu^{C–N}$), 1162 ($\nu^{S=O}$), 1281 (ν^{CArH}), 1011 (δ^{NH}), 722 (δ^{CH}), 692 ($\delta^{C–S–C}$). ^{1}H NMR (DMSO d_6), δ (ppm): 11.20 s (8H, SO_3H), 7.96 s (16H, H^1), 7.73 d (16H, H^3, J 8.2 Hz), 7.52 d (16H, H^2, J 8.2 Hz). MS, m/z: 2029.79 $[M + Li + 2H]^+$. Found, %: C 46.75; H 3.26; N 5.42; S 24.89. $C_{80}H_{50}N_8O_{24}S_{16}$. Calculated, %: C 47.56; H 2.49; N 5.55; S 25.39.

Aluminum 2,3,9,10,16,17,23,24-*octa-(4'-sulfophenylsulfanyl) phthalocyanine* (***2a***).

Yield of ***2a***: 0.17 g (64%). IR (ν/cm^{-1}): 3550 (ν^{OH}), 1614 ($\nu^{C\text{-}C}$), 1532 ($\nu^{C–N}$), 1164 ($\nu^{S= O}$), 1290 (ν^{CArH}), 712 (δ^{CH}), 692 ($\delta^{C–S–C}$). ^{1}H NMR (DMSO d$_6$), δ (ppm): 11.37 s (8H, SO$_3$H), 8.57 s (16H, H^1), 7.73 d (16H, H^3, J 8.2 Hz), 7.52 d (16H, H^2, J 8.2 Hz). MS, m/z: 2161.65 [M + 2Na]$^+$. Found ,%: C 45.95; H 3.06; N 5.24; S 24.34. $C_{80}H_{48}AlClN_8O_{24}S_{16}$. Calculated, %: C 46.18; H 2.33; N 5.39; S 24.65.

To reduce the hygroscopicity of MHC ***2a*** it was additionally neutralized with aqueous ammonia solution. 0.10 g (0.05 mmol) of comp. ***2a*** and 5 ml of 24% aqueous ammonia are placed in a porcelain dish. The mixture is heated until the solvent completely removed, then the product is extracted with DMF and subjected to column chromatography on silica gel M60, eluting with DMF.

*Aluminum (*2,3,9,10,16,17,23,24-*octa-(4'-sulfophenylsulfanyl) phthalocyanine ammonium salt* (***2***). Yield 0.082 g (80%). IR (ν/cm^{-1}):1615 ($\nu^{C\text{-}C}$), 1527 ($\nu^{C–N}$), 1160 ($\nu^{S=O}$), 1280 (ν^{CArH}), 723 (δ^{CH}), 693 ($\delta^{C–S–C}$). Found, %: C 42.85; H 3.45; N, 9.94; S 22.88. $C_{80}H_{72}AlClN_{16}O_{24}S_{16}$. Calculated,%: C 43.34; H 3.27; N, 10.11; S 23.14.

^{1}H NMR- and MS-spectra of pigments ***1,2*** are illustrated below (Fig. S1-S4).

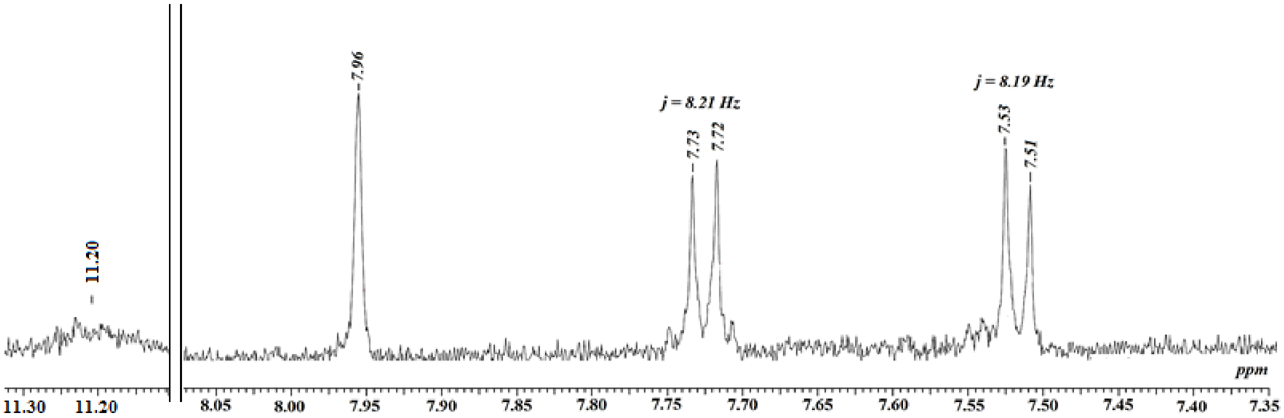


Fig. S1. ^{1}H NMR spectra of compound **1** (DMSO d$_6$)

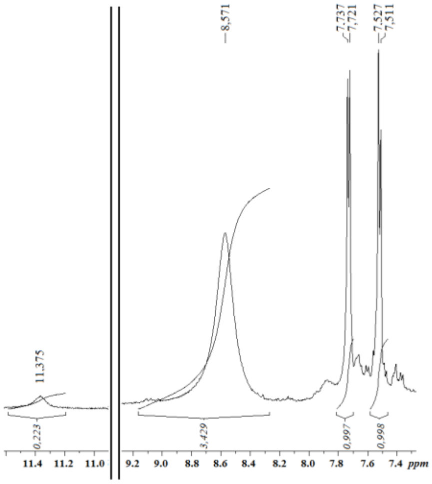


Fig. S2. ^{1}H NMR spectra of compound **2a** (DMSO d_6)

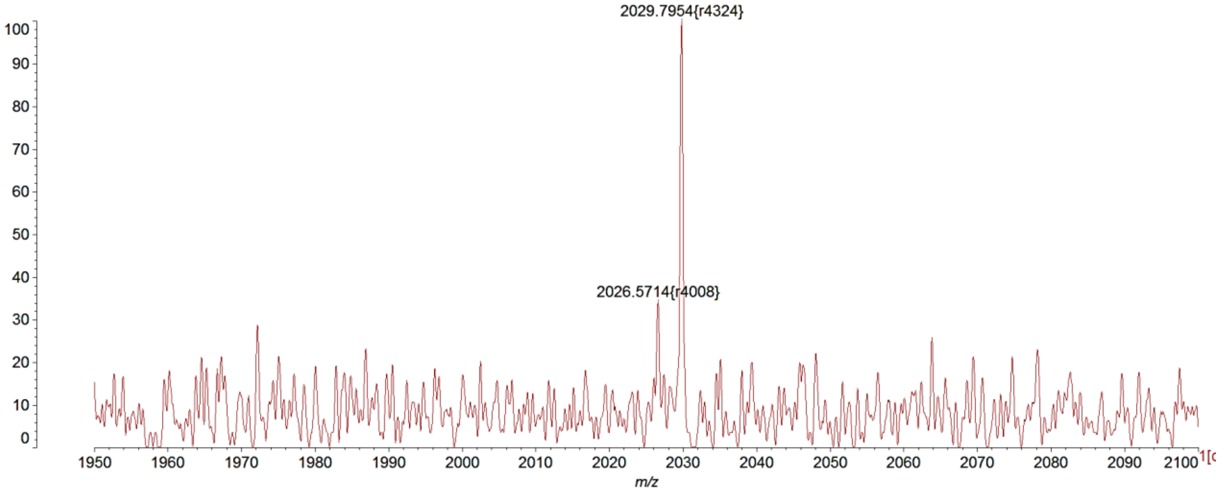


Fig. S3. MALDI MS spectra of compound **1**.

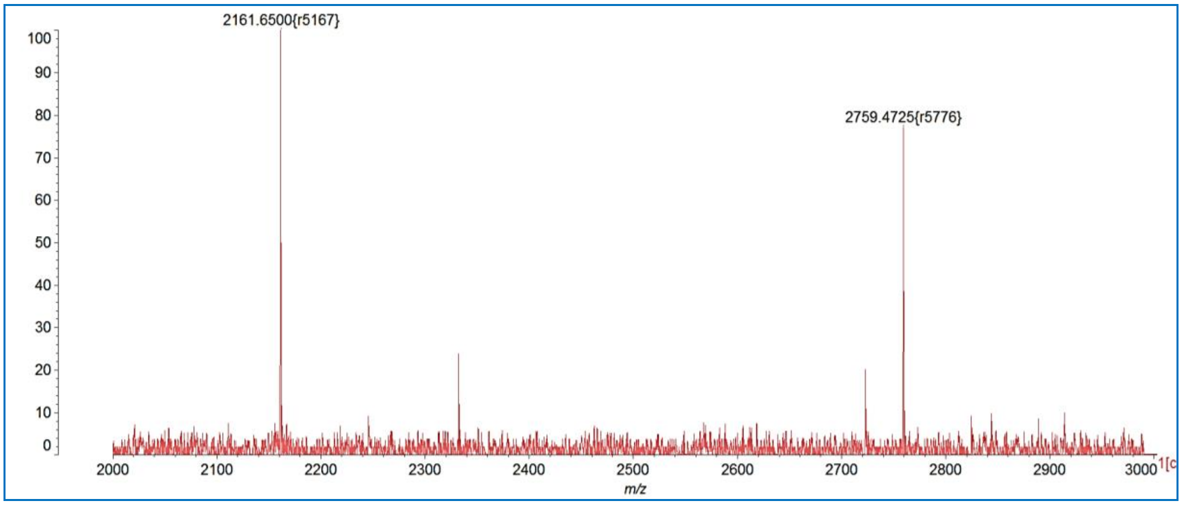


Fig. S4. MALDI MS spectra of compound **2a**.

1.2 Other materials

Sodium ethylenediamine tetraacetate ($Na_2H_2Edta\cdot 2H_2O$) and non-ionic surfactant Tween 80 both provided by Panreac (Chemical purity). Ethanol (Sigma Aldrich, >99.5) and N,N-dimethylformamide (Sigma Aldrich, >99.5) are used as supplied. Water was distilled twice. All solutions are prepared by weight. MHC solutions for the aggregation behavior study are filtered with the cellulose 0.46 μm filters and stored in a dark cool place for several days.

2. Apparatuses and methods

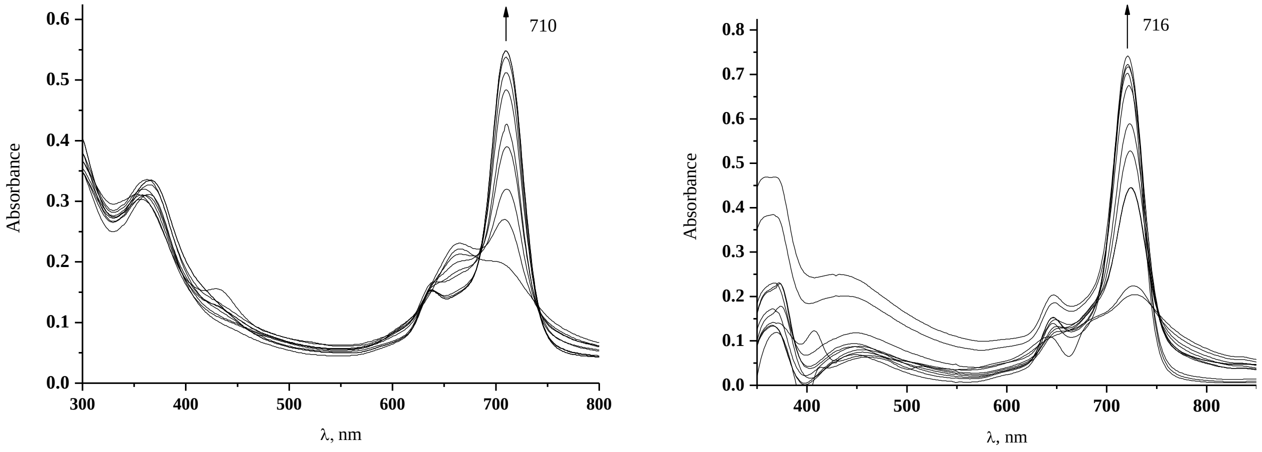
2.1 Measurements of the stationary spectra

^{1}H NMR spectra of solutions in $CDCl_3$ (comp. **5***a*-*c*) or DMSO d_6 (comp. **1,2*a***) are registered with the Bruker DRX-500 instrument. TMS is used as the internal standard for ^{1}H NMR measurements. MS-spectra were obtained with the MALDI FAB MS spectrometer Shimadzu AXIMA Confidence using 2,5-dihydroxybenzoic acid (DHB) as a matrix. UV–Vis spectra are registered at a room temperature with the Drawell G9 spectrophotometer (see Table S1). IR spectra are recorded on an Avatar 360 FT-IR ESP instrument in the region of 400–4000 cm^{-1} in thin films (chloroform, comp. **1**–**3**) or in KBr tablets (comp. **1,2**). Elemental analysis is performed on a CHNS-O FlashEA elemental analyzer. Before elemental analysis, all the samples are subjected to the heat treatment at 110 °C for 2 h.

Table S1. UV-Vis spectra of phthalocyanines ***1,2***

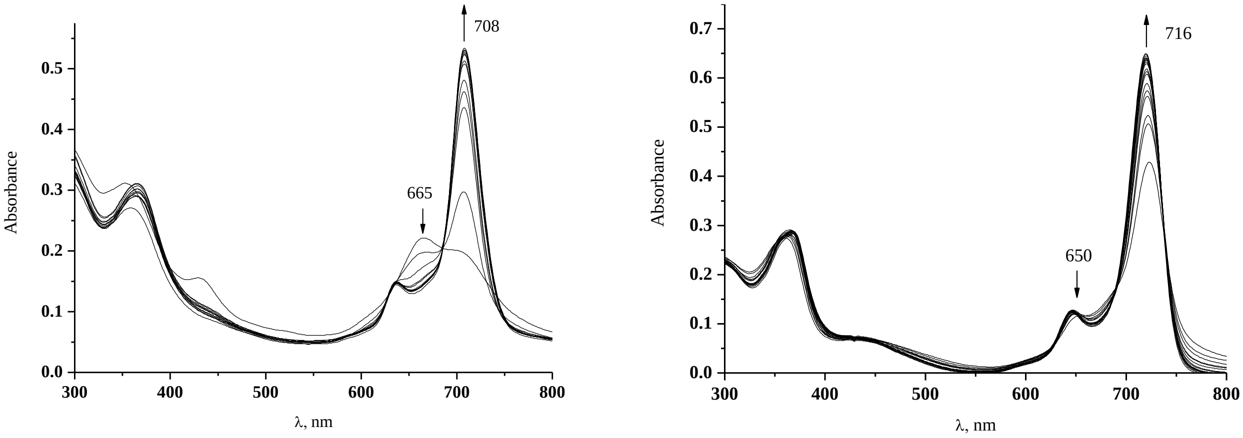
Compound	Solvent	Band positions			
		λ_4(lgε)	λ_3(lgε)	λ_2(lgε)	λ_1(lgε)
1	H_2O	353(4.63)	426(4.33)	665(4.48)	699(4.44)
	EtOH	357(4.59)	429sh(4.15)	658sh(4.37)	705(4.63)
	DMF	367(4.66)	422sh (4.31)	638(4.32)	710(4.88)
	1 wt% Tween 80 - H_2O	360(4.69)	422sh (4.29)	637(4.32)	708(4.87)
2	H_2O	360(4.62)	428sh (4.20)	650sh (4.29)	723(4.72)
	EtOH	339sh (4.62)	395sh (4.35)	662(3.98)	713(3.98)
	DMF	360(4.66)	424sh (4.35)	642(4.25)	716(4.80)
	1 wt% Tween 80 - H_2O	365(4.72)	430sh (4.36)	646(4.39)	716(4.89)

2.2. ***Spectrophotometric titrations.*** The stock solutions of comp. ***1,2*** are prepared by dissolving phthalocyanines in an appropriate amount of water, ethanol, DMF or their mixtures. Then, these solutions are diluted to reach the solute concentration of $7.3 \cdot 10^{-6}$ mol/kg for spectrophotometric measurements. Tween 80 is usually added before dilution. Each solution is placed into the spectrophotometer cell 10-15 minutes before to record UV-Vis spectra. The analytical wavelength for the phthalocyanine – surfactant interaction is chosen equal to 708-710 nm for comp. **1** and 716 nm for comp. **2**.



(a) *(b)*

Fig. S5. UV-Vis spectral changes of comp. **1** (*a*) ($m_{PS} = 7.3 \cdot 10^{-6}$ mol/kg) and comp. **2** (*b*) ($m_{PS} = 8.0 \cdot 10^{-6}$ mol/kg) at different components ratio in a solvent system H_2O – DMF.



(a) *(b)*

Fig. S6. UV-Vis spectral changes of comp. **1** (*a*) and comp. **2** (*b*) ($m_{PS} = 7.3 \cdot 10^{-6}$ mol/kg) depending on the molar excess of Tween 80.

The interaction of phthalocyanines **1,2** with Tween 80 micelles is quantitatively estimated using equation (1)[3]:

$$\lg[(A\text{-}A_0)/(A_{max}\text{-}A_0)]/(1\text{-}(A\text{-}A_0)/(A_{max}\text{-}A_0)) = \lg K_b + n\cdot\lg[m_T^m \text{-}n\ (m_{PS}\ (A\text{-}A_0)/(A_{max}\text{-}A_0))] \quad (1),$$

where K_b – equilibrium constant of the PS binding to Tween 80 molecules, n - the number of PS bounded surfactant molecules, $m_T^m = m_T$ - CMC - the molality of aggregated Tween 80 or the difference between its analytical concentration and CMC (critical micelle concentration, 1.5 10^{-5} M), m_{PS} – initial PS concentration, A_0, A_{max} and A – initial, final and current optical densities of PS in an aqueous Tween 80 solutions. The model parameters K_b and n are evaluated *via* a standard iteration mode.

2.3 *Dynamic light scattering (DLS)* measurements are performed with the Zetasizer Nano ZS apparatus (ZEN3600, Malvern Instruments) supplied with the laser with λ = 633 nm and the non-invasive backscatter technology (NIBS) of the optical registration system. The detector is located at the scattering angle of 173^0 to the incident light. We have analyzed only the particle size distribution obtained from the intensity of the laser light scattering.

DLS measurements of filtered solutions are carried out 8 to 10 days after the preparation of the solutions since the preliminary experiments have shown that for freshly prepared solutions the size of aggregates increases in time.[3]

2.4 Microbiological studies

2.4.1. Preparation of suspension of microbial cells

Gram-positive bacterium *Staphylococcus aureus* (6538-P ATCC=209-P FDA strain), Gram-negative bacterium *Escherichia coli* (M-17 strain) and yeast-like fungi *Candida albicans* (CCM 8261 ATCC 90028 strain) for modeling photodynamic inactivation *in vitro* are used. Daily cultures of test-strains are grown on microbiological agar mixed with brain-heart infusion broth as a nutrient medium. They are left in the shaking incubator at 37 ^{0}C overnight to allow adequate aeration. Then they are washed by an appropriate amount of physiological solution and diluted to achieve the concentration of 10^9 colony forming units per milliliter (CFU/ml), which is equal to 10 units according to the optical standard of turbidity. The sowing dose of $2\cdot10^7$ CFU/ml is prepared by a serial dilution of an initial suspension.

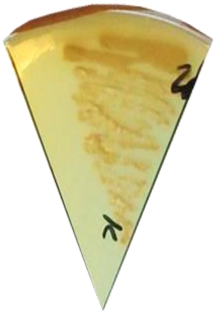
2.4.2 Photodynamic inactivation modeling

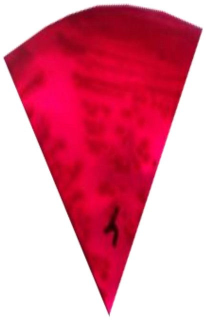
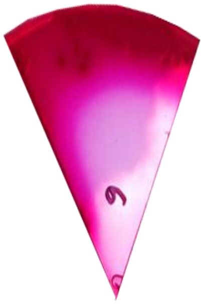
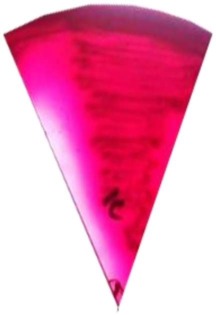
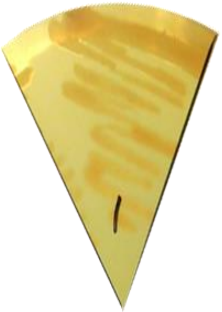
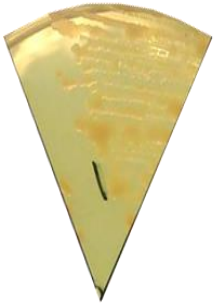
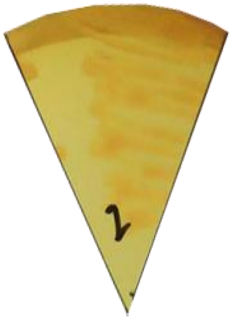
One-half of a milliliter of an aqueous pigment solution with an appropriate concentration containing Tween 80 and/or $Na_2H_2Edta\cdot2H_2O$[4] is added to each well of the 4-well plate equipped by a lid. Each well contains 0.5 ml of brain-heart infusion broth with an appropriate bacterial culture. After mixing and incubation in the dark during thirty minutes the plates are irradiated with the powerful light diode panel (OOO "BMC" (Belarus'), dimension of the light spot of 10×10 cm, the power density of

0.1 W/cm^2). The panel with λ=740±15 nm is used and the total light dose is chosen to be equal to 40 J/cm^2. The intensity of the light spot (power density) is measured by the Ophir Nova power meter. In order to construct a light dose–response curve with survival fraction the three aliquots of microbial cell suspension are obtained. The first aliquot is the original cell suspension (absolute control), the second one is the suspension with an added pigment solution kept in the dark and the third aliquot is the irradiated suspension after 30-minute incubation with an added pigment solution. All samples are incubated at 37 ^{0}C in the stationary incubator for 24-hours. Then test-cultures are sown with a sterile calibrated loop on Petri dishes contained the appropriate solid growth media - yolk-salt agar for Gram-positive cells, the Endo medium for Gram-negative cells or the Sabouraud medium for fungi. After the 24-h incubation the dishes are counted (see Table S2). The number of survived CFU in each aliquot of cell suspension is obtained by dividing the treatment CFU/ml by the CFU/ml in the original cell suspension (absolute control).

Table S2. Results of the antimicrobial PDT *in vitro* using archive strains and Pcs **1** and **2**.

Strain (sowing dose – 10^7 CFU)	Photosensitizer and the experimental conditions	No exposure (fragments of Petri dishes are presented)	After irradiation (740±15 nm, 40 J/см^2)
Staphylococcus aureus	Comp. **1**, 1 wt% Tween 80, $m_{PS} = 5 \cdot 10^{-4}$ mol/kg	 10^7 CFU	 25 CFU
	Comp. **2**, 1 wt% Tween 80, $m_{PS} = 5 \cdot 10^{-4}$ mol/kg	 10^7 CFU	 12 CFU

	Comp. **1**, 1 wt% Tween 80, $m_{PS} = 5 \cdot 10^{-5}$ mol/kg	 10^7 CFU	 10^7 CFU
	Comp. **1**, 1 wt% Tween 80, 0.1 wt% EDTA $m_{PS} = 5 \cdot 10^{-5}$ mol/kg	 10^7 CFU	 10^7 CFU
	Control	 10^7 CFU	 10^7 CFU
Escherichia coli	Comp. **1**, 1 wt% Tween 80, $m_{PS} = 5 \cdot 10^{-4}$ mol/kg	 10^7 CFU	 10^7 CFU
	Comp. **2**, 1 wt% Tween 80, $m_{PS} = 5 \cdot 10^{-4}$ mol/kg	 10^7 CFU	 10^7 CFU

	Comp. **1**, 1 wt% Tween 80, $m_{\mathrm{PS}} = 5 \cdot 10^{-5}$ mol/kg	 10^7 CFU	 10^7 CFU
	Comp. **1**, 1 wt% Tween 80, 0.1 wt% EDTA $m_{\mathrm{PS}} = 5 \cdot 10^{-5}$ mol/kg	 10^7 CFU	 25 CFU
	Control	 10^7 CFU	 10^7 CFU
Candida albicans	Comp. **1**, 1 wt% Tween 80, $m_{\mathrm{PS}} = 5 \cdot 10^{-4}$ mol/kg	 10^7 CFU	 10^7 CFU
	Comp. **2**, 1 wt% Tween 80, $m_{\mathrm{PS}} = 5 \cdot 10^{-4}$ mol/kg		

Comp. **1**, 1 wt% Tween 80, $m_{PS} = 5 \cdot 10^{-5}$ mol/kg	10^7 CFU 	10^7 CFU 
Comp. **1**, 1 wt% Tween 80, 0.1 wt% EDTA $m_{PS} = 5 \cdot 10^{-5}$ mol/kg	10^7 CFU 	10^7 CFU 
Control	10^7 CFU  10^7 CFU	10^7 CFU  10^7 CFU

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