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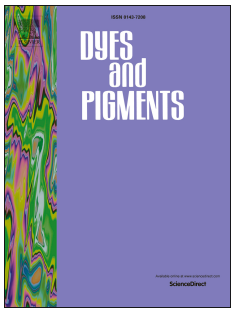

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Photodynamic self –disinfecting surface using pyridinium phthalocyanine

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ABSTRACT

We have synthesized novel phthalocyanine with four pyridyl substituents connected to α-phthalo-positions via direct C-C bond. The Zn complex and tetracationic derivatives of phthalocyanine were also synthesized and the dyes were impregnated into filter paper to prepare photoactive antimicrobial surface. The photodynamic antimicrobial efficacy of the dyed paper samples was evaluated by a simple and fast setup using bioluminescent microbes. *Escherichia coli* and *Acinetobacter baylyi* ADP1 strains carrying bacterial luciferase genes were used in the screening experiment. The most efficient compound, tetracationic zinc derivative **8,** was investigated further. The compound was highly water soluble, had high molar absorptivity and exhibited good adhesion to the filter paper without leaching into the solution. The singlet oxygen quantum yield of tetracationic zinc derivative **8** in water was found out to be 30 ± 20 %. According to the cell viability assay test performed on *E. coli* wild type in solution, the molecule had similar or better photo toxicity as the reference photosensitizer, tetrakis (1-methyl-pyridinium-4-yl)porphyrin (TMPyP). Antimicrobial efficacy of the dye **8** on photoactive surface was studied by live cell assessment through colony forming unit (CFU) counting. The colored surface demonstrated 3 log reduction in CFU against *E. coli* and *A. baylyi* ADP1 just after 1 h of illumination with the white light of low intensity.

KEYWORDS: Antimicrobial • Self-disinfecting surface •Pyridinium phthalocyanine • Singlet oxygen • Photodynamic antimicrobial chemotherapy

1. INTRODUCTION

One of the major challenges of the 21st century is how to prevent the spread of life threatening epidemics. Nosocomial or Healthcare associated infections (HAI) account for the major source of transmission of infectious disease. Contaminated surfaces play a significant role in the spread of

microbes. The contamination leads to the formation of biofilms, which facilitate microbial proliferation.[1–6] Together with the emergence of drug resistant bacteria, the risk is multiplied several times.[7–13] The so called "ESKAPE"- pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* strains) survive almost any individual antibiotic treatment.[14] Hence, in order to control the transmission of the pathogenic microorganism, new approaches are required.

One such approach is photodynamic antimicrobial chemotherapy (PACT), which has been found to be effective against drug resistant bacteria and biofilms. [15–19] The term "photodynamic reaction" was introduced by Hv Tappeiner for inactivation of microbes by dyes in the presence of light; he also demonstrated the involvement of oxygen in the process.[20,21] Inactivation is achieved via the oxidative action of singlet oxygen produced by an organic dye upon light irradiation. Since the singlet oxygen can diffuse in liquids and air, as well as through cell wall, [22,23] its applications are extended in the preparation of photoactive self-disinfecting surfaces such as coatings, films, polymers, paints for controlling microbial contamination [24–33], and to water sanitation.[34] Whether or not an organic dye is suitable for the purpose, depends first of all on the quantum yield of singlet oxygen generation, on extinction coefficient, photo- and thermal stability and appropriate wavelength absorptions. Additional properties, such as dark toxicity, redox potentials of excited states, lipophilicity and ionization degree must also be taken into consideration while selecting the photosensitizer. [35–39]

Many organic dyes such as methylene blue, toluidine blue O, acridine, rose bengal, and various macrocyclic structures are capable of generating singlet oxygen.[23,40,41] In particular porphyrins, such as haematoporphyrin and protoporphyrin and their various derivatives are excellent photosensitizers for treatment of microbes and malignant cells.[42,43] Complexes of phthalocyanines and napthalocyanines also exhibit considerable photobiological activity against tumors.[44] The level of photo toxicity is strongly related to the size, charge, and hydrophobicity balance of the dye

molecule.[16,39] It was found that cationic photosensitizers have higher activity than anionic or neutral ones against both Gram-negative and Gram-positive bacteria.[45–48] Such difference can be explained by the fact that even though photosensitizers are not required to penetrate into the cells, the electrostatic interaction between cationic dye and poly-anionic lipopolysaccharide layer of the cell wall structure of the bacteria may result in its destabilization and thus facilitates the subsequent photosensitization. [33,49,50] On the other hand, testing the photosensitizers is a long and time-consuming procedure. To the best of our knowledge, there is no fast and simple procedure for screening of potential photoactive molecules; this fact considerable restricts their development.

Inspired by the above observations, we synthesized novel tetrakis(α-phthalo-pyridyl) substituted phthalocyanine, its zinc complex and their tetracationic salts, which can serve as a potent photosensitizer for antimicrobial treatment. In our present work, a photoactive self-disinfecting surface was prepared by immobilizing the photosensitizer on to the filter paper by a simple technique. The surface hence prepared was found to be stable without any leaching into water. We also propose a simple and fast method to evaluate the antimicrobial efficacy of the surface using bioluminescent microbes. In addition, photo inactivation of the surface was also confirmed by conventional CFU counting method using two different microbes. However, we have also tested the phototoxic action of the dye in solutions.

2. MATERIALS AND METHODS

2.1. General Methods.

Reagents and solvents were purchased from TCI Europe, Sigma Aldrich Co. or from VWR and were used without further purifications unless otherwise mentioned. Purification of the products was carried out either by column chromatography on Silica gel 60 or Silica gel 100 (Merck) or on preparative TLC plates (Merck) coated with neutral aluminum oxide 60 F_{254}. NMR spectra were recorded using Varian

Mercury 300 MHz spectrometer using TMS as internal standard. HRMS measurements were done with Waters LCT Premier XE ESI-TOF bench top mass spectrometer. Lock-mass correction (leucine enkephaline as a reference compound), centering and calibration were applied to the raw data to obtain accurate mass. UV-Vis absorption spectra were recorded using Shimazu spectrophotometer.

2.2. Synthesis.

The compounds 3-hydroxyphthalonitrile **1**, 2,3-dicyanophenyl trifluoromethanesulfonate **2** and 4-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl) pyridine **3** were synthesized according to the literature procedure.[51,52] The synthetic route for compounds **2-8** were described in scheme 1.

2.2.1. Synthesis of 3-(pyridin-4-yl) phthalonitrile 4

Pyridine boronate ester **3** (120 mg, 0.628 mmol), triflate phthalonitrile **2** (174 mg, 0.628 mmol), $PdCl_2(dppf)\bullet DCM$ (26 mg, 0.0314 mmol), K_3PO_4 (400 mg, 1.884 mmol) was dissolved in a mixture of 7.5 ml water and 7.5 ml of toluene, and was heated at vigorous stirring at 90 °C for 2 h. The reaction mixture was extracted with $CHCl_3$ and washed with brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure to yield 105 mg of a crude product. The pure product **4** was isolated by re-precipitating from $CHCl_3$/ hexane. Yield 75 mg, 60 %.

MS (ESI-TOF): $[M+H]^+$ calcd for $C_{13}H_7N_3^+$, 206.0718; found, 206.0719. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 8.83 (d, *J* = 5.86 Hz, 2 H), 7.93-7.77 (m, 3 H), 7.50 (d, *J* = 5.86 Hz, 2 H); ^{13}C NMR (75 MHz, CDCl3, 25 °C, TMS): δ = 150.65, 144.35, 143.77, 133.72, 133.26, 123.03, 117.81, 115.24, 114.54 ppm.

2.2.2. Synthesis of 1, 8, 15, 22-tetra(pyridin-4-yl)-29H,31H-phthalocyanine 5

Freshly cut lithium shots (57 mg, 8.212 mmol) were dissolved in n-butanol (5.7 ml) at 90 °C under argon atmosphere. The reaction mixture was allowed to cool to room temperature and pyridine phthalonitrile **2** (80 mg, 0.3898 mmol) was added to the above solution under argon atmosphere. The

mixture was stirred at 90 °C for 18 h. The product was extracted with $CHCl_3$ and washed with water several times until the pH of the aqueous layer was neutral. The organic layer was evaporated under reduced pressure to get a crude residue. The residue was washed with acetonitrile and purified by column chromatography (neutral alumina, 1% EtOH in $CHCl_3$) to yield free base phthalocyanine **5** (40 mg, 50 %).

MS (ESI-TOF): $[M+H]^+$ calcd for $C_{52}H_{30}N_{12}^+$, 823.2795; found, 823.2832. ^1H NMR (300 MHz, $CDCl_3$, TMS): δ = 9.01-8.95 (m, 5 H), 8.86-8.84 (m, 2 H), 8.60-8.59 (m, 6 H), 8.55-8.53 (m, 1 H), 8.35-8.33 (m, 1 H), 8.28-8.26 (m, 2 H), 8.11-8.08 (m, 1 H), 8.02-7.96 (m, 3 H), 7.86-7.80 (m, 8 H), 7.67-7.61 (m, 5 H), 7.57-7.51 (m, 7 H), 7.44-7.42 (m, 2 H), 1.65 (br, 1 H), 2.21 (br, 1 H). UV/Vis (CHCl3): λ (ε) = 715 (100000), 680 nm (87037), 650 nm (31481), 615 nm (20370 $Lmol^{-1}cm^{-1}$).

2.2.3. Synthesis [1,8,15,22-tetra(pyridin-4-yl)-29H,31H-phthalocyaninato(2-)-κ4N29,N30,N31,N32]zinc 6

Tetrapyridinyl phthalocyanine free base **5** (12 mg, 0.0146 mmol) was dissolved in $CHCl_3$ (1.5 ml) and $ZnOAc•2H_2O$ (12 mg, 0.0547 mmol) in 120 µl H_2O was added into it. The reaction mixture was stirred at 60 °C for 2 h. The product was extracted with $CHCl_3$ (20 ml) and washed with water (25 ml x 3), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to yield crude residue. The product was purified with column chromatography. (Neutral alumina, 10 % EtOH in $CHCl_3$) and later washed with diethyl ether and acetonitrile to yield a pure compound **6** (11 mg, 85 %).

MS (ESI-TOF): $[M+H]^+$ calcd for $C_{52}H_{28}N_{12}Zn^+$, 885.1929; found, 885.1970. ^1H NMR (300 MHz, DMSO D_6, TMS): δ = 9.52-9.50 (m, 1 H), 9.10-9.06 (m, 2 H), 8.62 (br, 8 H), 8.45 (br, 2 H), 8.31 (br, 2 H), 8.16-8.14 (m, 1 H), 7.78-7.58 (m, 19 H). UV/Vis (CHCl3): λ (ε) = 695 (113077), 634 nm (24615 $Lmol^{-1}cm^{-1}$).

2.2.4. Synthesis of [1,8,15,22-tetra(pyridin-4-yl)-29H,31H-phthalocyaninato(2-)-κ4N29,N30,N31,N32]zinc 6 (direct method)

A mixture of pyridine phthalonitrile **4** (77 mg, 0.3752 mmol) and anhydrous zinc acetate (84.67 mg, 0.4615 mmol) in dimethylaminoethanol (DMAE, 810 µl) was heated at reflux at 140 °C for 12 h. The reaction mixture was cooled to room temperature and the product was precipitated by adding a mixture of MeOH/ H_2O (9:1). The green solid was filtered and washed with methanol to yield the product **6** (80 mg, 96 %).

MS (ESI-TOF): $[M+H]^+$ calcd for $C_{52}H_{28}N_{12}Zn^+$, 885.1929; found, 885.1904

2.2.5. Synthesis of 4,4',4'',4'''-(29H,31H-phthalocyanine-1,8,15,22-tetrayl)tetrakis(1-methylpyridinium) tetraiodide 7

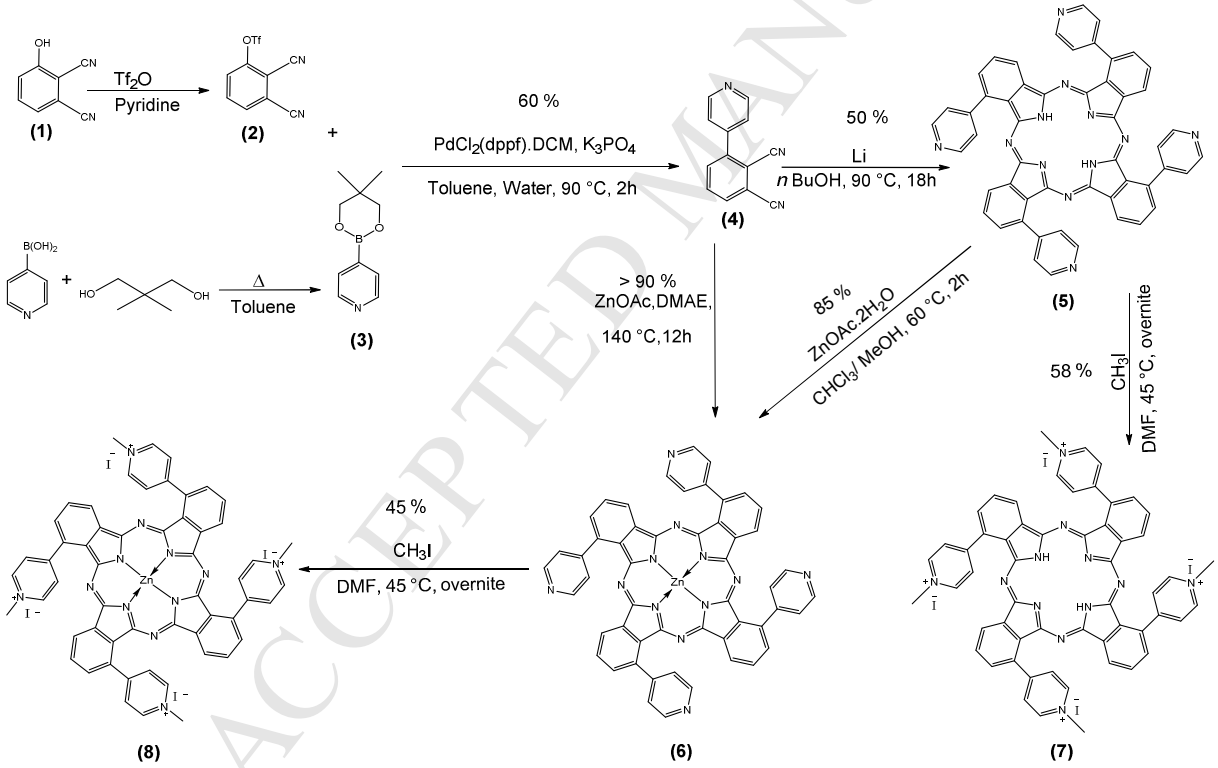
Phthalocyanine **5** (15 mg, 0.0182 mmol) was dissolved in DMF (3 ml) and methyl iodide (1 ml, 16.0642 mmol) was added into solution. The reaction mixture was stirred at 45 °C for 18 h. The reaction mixture was cooled in an ice bath and product was precipitated with diethyl ether (15 ml). The solid was filtered, washed with diethyl ether several times and later with acetone/diethyl ether to yield pure product **7** (14.8 mg, 58.32 %).

MS (ESI-TOF): $[M-4I]^{4+}$ calcd for $C_{56}H_{38}N_{12}{}^{4+}$, 220.5914; found, 220.5889; $[M-3I]^{3+}$ calcd for $C_{56}H_{39}IN_{12}{}^{3+}$, 336.4233; found, 336.4204. ^{1}H NMR (300 MHz, DMSO D6, TMS): δ = 9.69-9.55 (m, 5 H), 9.14-9.07 (m, 8 H), 8.77-8.69 (m, 6 H), 8.46-8.32 (m, 9 H), 4.88-4.61 (m, 12 H). UV/Vis (DMF): λ (ε) = 702 (141250), 635 nm (28750 $Lmol^{-1}cm^{-1}$).

2.2.6. Synthesis of {4,4',4'',4'''-(29H,31H-phthalocyanine-1,8,15,22-tetrayl-κ4N29,N30,N31,N32)tetrakis[1-methylpyridiniumato(2-)]}zinc(4+) tetraiodide 8

Tetrapyridyl phthalocyanine zinc **6** (20 mg, 0.0225 mmol) was dissolved in DMF (3 ml), and methyl iodide (1 ml, 16.0642 mmol) was added to solution. The reaction mixture was stirred at 45 °C for 18 h. The reaction mixture was cooled in an ice bath and product was precipitated by adding diethyl ether (15 ml). The solid was filtered and washed several times with diethyl ether and later with mixture of acetone/ H_2O (1:1) to yield the product **8** (15 mg, 45.71 %).

MS (ESI-TOF): $[M-4I]^{4+}$ calcd for $C_{56}H_{36}N_{12}Zn^{4+}$, 236.0698; found, 236.0692; $[M-3I]^{3+}$ calcd for $C_{56}H_{37}IN_{12}Zn^{3+}$, 357.0612; found, 357.0617. ^{1}H NMR (300 MHz, DMSO D6, TMS): δ = 9.77-9.70 (m, 1 H), 9.95-9.54 (m, 5 H), 9.21-9.04 (m, 8 H), 8.76 (br, 3 H), 8.62 (br, 2 H), 8.46-8.35 (m, 6 H), 8.25-8.19 (m, 1 H), 4.85-4.59 (m, 12 H). UV/Vis (DMF): λ (ε) = 692 (128500), 625 nm (22000 $Lmol^{-1}cm^{-1}$).



Scheme 1. Synthetic route for the preparation of pyridine substituted phthalocyanine.

2.3. Singlet oxygen measurement.

Singlet oxygen kinetics were monitored in aqueous solution via time-correlated multi-photon counting (TCMPC) at 1270 ± 15 nm, the characteristic singlet oxygen luminescence wavelength. For sample

149 excitation, a LMD-405D diode laser (Omikron-Laserage, Rodgau-Dudenhofen, Germany) was used:
150 excitation wavelength 405 nm, pulse width 120 ns, channel width 20 ns, average power 1.2 W, duration
151 of measurement 60 s. A TCMPC-1270 Singlet Oxygen Luminescence Detection System by SHB
152 Analytics (Berlin, Germany) was used for luminescence signal detection. Singlet oxygen quantum
153 yields were determined indirectly from fitting the luminescence signal and using TMPyP, optical
154 density adjusted for the excitation wavelength, as reference.[41] Fits of the data were conducted
155 following the standard bi-exponential model for singlet oxygen kinetics and an additional mono-
156 exponential phosphorescence term for TMPyP.[53] The goodness of the fit is indicated by the reduced
157 χ^2 -test.

158 **2.4. Antimicrobial tests.**

159 **2.4.1. Screening test on dyed paper.**

160 The efficiency of dyes was screened by conducting antimicrobial test with bioluminescent bacterial
161 strains *Escherichia coli* (XL1-Blue, Stratagene, USA) pBAV1C-T5-lux and *Acinetobacter baylyi*
162 ADP1 (DSM 24193) carrying plasmid pBAV1C-T5-lux, The plasmid was constructed by replacing *gfp*
163 with *lux* in pBAV1C-T5-GFP. [54] The lux operon was cloned from the pBAV1K-T5-lux plasmid,
164 kind gift from Ichiro Matsumura (Addgene plasmid # 55800)[55] using standard BioBrick cloning.
165 Whatman 1 filter papers (area 12.25 cm^2) were soaked in solutions of dyes **5**-**8** (0.9 mg dye in 200 µl
166 solvent) and allowed to dry out. After drying, three discs 0.5 cm in diameter were cut from each dyed
167 paper and pasted on the LA agar gel plate (15 g/l agar, 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) in
168 a 3×4 grid. As shown in Figure 1, each column contains 4 disks with dyes **5**-**8**, while the rows contain
169 disks of the same dye. A dark screen with a square hole was placed over the agar plate in such a way
170 that, one column of the four dyes was under the dark area while the two other columns would be
171 accessible for light. Blank control samples were prepared by cutting neat uncolored filter papers of the

same size and placing them in the dark and illuminated areas of LA agar plate. Background luminescence arising from the setup was recorded (Xenogen IVIS Lumina, Caliper Life Sciences, USA). Microbial strains were inoculated in 5 ml of LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) containing 0.5% glucose and 25 µg/ml of chloramphenicol and incubated at 30 °C 300 rpm. After overnight cultivation, 100 µl of the culture was diluted with 5 ml of LB medium containing 0.5% glucose and 25 µg/ml of chloramphenicol and incubated for 3 h at 30 °C 300 rpm. Microbial solution thus prepared, was pipetted over the paper discs (5 µl per disk). To check the influence of filter paper on the bioluminescence of bacteria the microbial solution was pipetted as well straight on the agar in the dark region of the plate. Luminescence of the plate with microbes deposited was recorded by IVIS and the plate was subjected to illumination. The whole plate was placed inside solar simulator (Luzchem, Canada) and the light intensity was adjusted to 18 $mW \cdot cm^{-2}$ by lifting the plate up/down. Two filters [KG3 band pass with 315–750 nm transmittance and YG-17 filter with transmittance > 485 nm] were placed over the square hole to remove the infrared and UV radiation. After 1 hour of illumination, the luminescence was measured again, and the antimicrobial efficacy of dyes was compared.

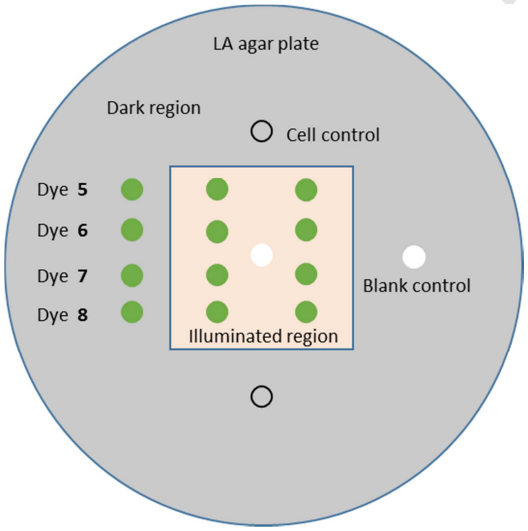


Figure 1. Schematic diagram of the setup for screening dyes.

2.4.2. Cell viability assay.

For relative cell viability tests a resazurin assay was used.[56] Two different sample sets with *E. coli* wild type (ATCC 25922) cell suspension in PBS (approx. 4×10^8 cfu/ml) were incubated for 2 hours under standard ambient temperature in the dark and under low white-light illumination conditions (fluence rate of 8 ± 2 mW·cm^{-2}). First set: 200 µl cell suspension on M9-minimal-agar substrate in 24-well-plates. Second set: 1 ml cell suspension without any agar in 24-well-plates. A photosensitizer concentration of 5 µM was used. After addition of the resazurin reactant (for each well 900 µl of 0.05 g/l Resazurin sodium salt, Sigma-Aldrich, Germany) all samples were incubated for another 4 hours in the dark under gentle stirring. The relative viability was then determined from resorufin fluorescence using a VICTOR^3 plate reader, PerkinElmer Inc., USA. Per sample, three wells were used measuring each well nine times.

2.4.3. Determination of optimal dye loading on filter paper.

The filter papers with different dye loading of were prepared in the following way. First four solutions of phthalocyanine **8** (1 mg, 0.5 mg, 0.25 mg and 0.1 mg) in 200 µl milli Q water was prepared in 4 different vials. Whatman 1 filter paper of size 3.5 cm x 3.5 cm (12.25 cm^2) soaked in the solution containing 1 mg dye gives 0.081 mg/cm^2 dye loading. The filter paper of same size soaked in 0.5 mg dye solution gives loading of 0.04 mg/cm^2. Similarly, 0.25 mg dye solution and 0.1 mg dye solution gives dye loading of 0.02 mg/cm^2 and 0.008 mg/cm^2 respectively. A filter paper of same size without dye was kept as a control.

Antimicrobial efficiency of the filter papers was confirmed by live cell assessment through CFU counts using microbe *Acinetobacter baylyi* ADP1 (ATCC 33305). Microbial strain was inoculated in a solution of 5 ml of LB medium and supplemented with 1 % glucose at 30 °C and 300 rpm overnight. The overnight-cultivated solution (100 µl) was diluted with 5 ml of LB medium and 1 % glucose and

211 shaken (300 rpm) at 30 °C for 3 h. The optical density of culture was measured at 600 nm. The
212 microbial solution was centrifuged for 5 minutes at 6500 rpm and the LB medium was decanted out
213 from the vial. The residual microbes were suspended in 5 ml of PBS (phosphate-buffered saline)
214 buffer. Circular discs (cut from filter papers of different dye loading and control paper) were placed in
215 the wells of a microplate and microbial solution (25 µl) was pipetted over the disks. The microplate
216 was illuminated in the solar simulator for 1 hour. UV and IR radiations were cut off using a
217 combination of KG3 band pass filter (315-750 nm transmittance) and YG-17 filter (transmittance > 485
218 nm) and the overall light intensity kept at 18 $mW \cdot cm^{-2}$. After 1 h of illumination, the microbes were
219 extracted from wells with 975 µl of LB medium and serial dilutions (up to two times) were made from
220 each extract. The dilutions were then plated on LA agar plates and incubated at 30 °C overnight. The
221 number of colonies grown on the agar plate were counted and CFUs per milliliter were calculated from
222 it and the filter paper with optimal dye loading was determined from it.

223 **2.4.4. Determination of antimicrobial efficacy of dyed-filter paper.**

224 The *E. coli* MG1655 (*E. coli* Genetic Resources at Yale) and *Acinetobacter baylyi* ADP1 (ATCC
225 33305) strains were used in determining antimicrobial efficacy. The cultivations and resuspensions
226 were carried out as described above. Two sets of paper discs (original and duplicate), with
227 phthalocyanine **8** and an uncoloured blank control, were placed in the wells of a microplate and
228 microbial solution (25 µl) was pipetted over the disks. The microplate was illuminated in the solar
229 simulator for 1 hour. UV and IR radiations were cut off using a combination of KG3 band pass filter
230 (315-750 nm transmittance) and YG-17 filter (transmittance > 485 nm) and the overall light intensity
231 kept at 18 $mW \cdot cm^{-2}$. Dark control samples were prepared by depositing microbial medium over dyed
232 and uncoloured disks and keeping the microplate in dark for 1 h at room temperature inside the laminar
233 hood. After 1 h of illumination or incubation, the microbes were extracted from wells with 975 µl of

LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) and serial dilutions (up to 10^{-6}) were made from each extract. The dilutions were then plated on LA agar plates and incubated at 30 °C overnight. The number of colonies grown on the agar plate was counted and CFUs per milliliter were calculated to determine the antimicrobial efficacy.

3. RESULTS AND DISCUSSION

3.1. Synthesis.

Photodyanamic antimicrobial activities of pyridine substituted phthalocyanines are already known.[57,58] However most of these compounds describe substitution in beta-phthalo position via oxy or thio-bridge. In the present work, we give a first example of direct C-C link between the α-phthalo position and pyridine unit. Novel pyridine-containing phthalocyanine and its zinc complex were synthesized according to Scheme 1. The triflate phthalonitrile **2** was prepared from 3- hydroxy phthalonitrile **1** which in turn synthesized from commercially available 3-nitro phthalonitrile by following the literature procedures reported elsewhere.[51]-[52] Pyridine phthalonitrile **4** was prepared with 80 % yield by coupling pyridine boronate ester **3** and triflate phthalonitrile **2**. It should be mentioned that the coupling reaction between commercially available pyridine boronic acid and triflate phthalonitrile **2** did not produce reasonable yield of pyridine phthalonitrile. Therefore, pyridine boronic acid was converted into boronate ester **3** by reacting with neopentyl glycol. When the reaction was accomplished in the presence of molecular sieves, the ester **3** precipitated in 1,4- dioxane at room temperature and its separation from the molecular sieves was difficult. Therefore, we used the azeotropic distillation in the synthesis and obtained boronic ester **3** with high yield (ca. 80 %). Free base phthalocyanine **5** was prepared by the tetramerization of pyridine phthalonitrile **4** with 50 % yield. The zinc complex **6** was synthesized by reacting free base phththalocyanine **5** with zinc acetate in a mixture of chloroform and methanol with a yield of around 85 %. Nontheless, direct synthesis of zinc complex **6** produced better overall yield than converting free base phthalocyanine **5**.[58]

The free base **5** and phthalocyanines zinc complex **6** were converted into cationic salts **7** and **8** respectively by methylation with iodomethane in DMF. High-resolution MS spectrometry was used to identify the molecules. The 0.25 Da separation between the peaks in the MS signals confirmed the tetra cationic charge of the molecule. However, since the substance was a mixture of regioisomers, NMR spectra were rather broad and difficult to interpret (see SI).

UV-visible absorption spectra were measured in chloroform (for **5** and **6**) and DMF (**7** and **8**) and shown in figure 2. The Q band peak for free base phthalocyanine **5** was split into two when compared to corresponding zinc phthalocyanine **6**.[59] However, broadening of the peaks after methylation for the free base cationic phthalocyanine **7** indicates the aggregation.

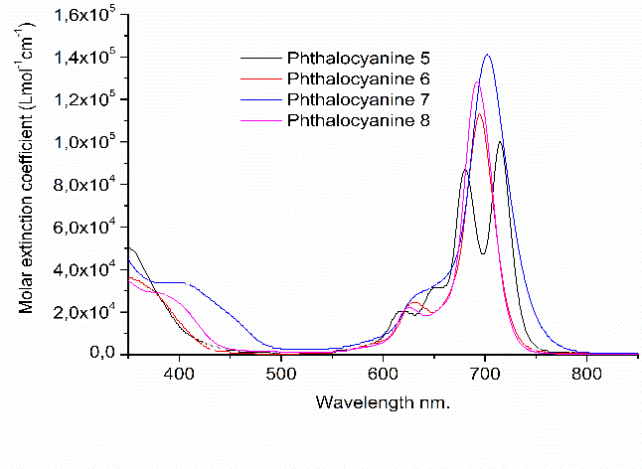


Figure 2. UV-visible absorption spectra of phthalocyanines.

Overall, we synthesized a novel phthalocyanine with pyridyl substitutuentsat α phthalo positions through direct C-C linkage. Cationic tetra salts were found to be soluble in water, ethanol, could be easily precipitated into a solid, and gave a clear mass-spectrum, which suggests good degree of quaternization. Integrals of ^{1}H-NMR signals support a good purity of the obtained salt, though the signals are broad indeed.

3.2. Singlet oxygen quantum yield.

Quantitative measurement of singlet oxygen for cationic zinc phthalocyanine **8** was done in water (shown in figure 3). The singlet oxygen quantum yield of phthalocyanine **8** was calculated to be 30 ± 20 % by comparing the phosphorescence signal intensity at 1270 nm of with that of TMPyP as reference with a quantum yield of 74 %. This value was reasonably good since water was known to quench the singlet oxygen. [60] However, cationic pyridine free base phthalocyanine **7** did not produce any signal for singlet oxygen in water. Most probable reason may be the aggregation of the molecule in water as previously explained in the discussion of UV- visible absorption spectra.

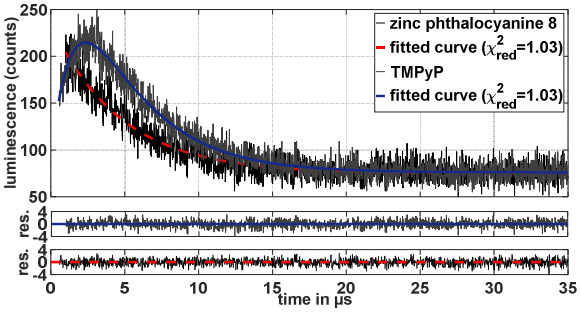


Figure 3. Time resolved singlet oxygen measurement of TMPyP and cationic zinc phthalocyanine 8. The optical density of TMPyP is adjusted for the excitation wavelength at 405 nm. Pearson residuals illustrate the goodness of the fit (reduced chi-squared below 1,01 for both fits). No distinct signal for cationic free base phthalocyanine 7 could be observed.

3.3. Screening of dyes' efficiency.

In our search for surfaces with photodynamic antimicrobial effect, we decided to identify the most efficient dye from the set of synthesized phthalocyanines, by comparing its antimicrobial capability on a solid support. In particular, the dyes **7** and **8** were highly soluble in water and readily adsorbed to the filter paper to form stable and permanent color. The leaching of the dyes to the medium was tested in by sonicating a piece of the filter paper impregnated with cationic phthalocyanines (**7** and **8**) in 3 ml of milli Q water for 30 minutes. The UV visible absorption measurement of the resultant water sample did not show any indication of the dye in the water nor the color of the filter paper faded out after 30 minutes of sonication. Moreover, even an overnight incubation of filter paper in milli Q water at room

294 temperature did not induce leaching of the dye into water. Leaching was observed only after acidifying
295 the extraction water down to pH 2. In this case, phthalocyanine tetra salts were obviously extracted into
296 water, however not completely.
297 As we had four dyes to test, we needed a simple and rapid way of evaluation of the phototoxic effect.
298 One possibility to identify the efficient photosensitizer was to use bioluminescent bacteria as reporter
299 cells. The bioluminescence-based screening method has been applied in multiple studies[61] as it
300 allows a growth-independent and sensitive monitoring of toxic effects of different agents on the cells.
301 The intensity of bioluminescence arising from the bacteria on a surface is directly related to the
302 metabolic status of the bioluminescent bacteria.[58] In other words, when an efficient dye effectively
303 inactivates bioluminescent microbes, we expect a sharp decrease in the signal intensity. The
304 bioluminescence arising from surface of filter paper soaked with bioluminescent *E. coli* cell solution
305 was recorded before and after illumination as shown in Figure 4a. As expected, surfaces with cationic
306 derivatives **7** and **8** were more effective against *E. coli*. Moreover, cationic zinc derivative **8** had shown
307 higher efficacy than free base phthalocyanine **7**. It should be noted that the bioluminescense from the
308 paper with dye **8** incubated in the dark region of the plate was also reduced. However, this decrease
309 may not necessarily arise from the dark toxicity of the substance, but rather can be attributed to the
310 photo toxicity induced by the stray light, since the incubation was done in the same plate.

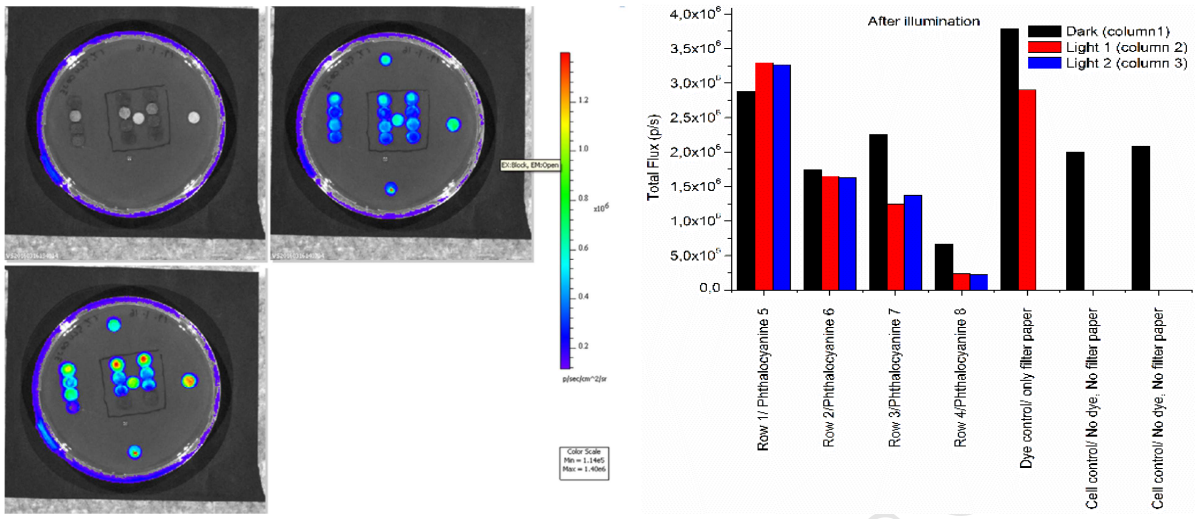


Figure 4. (a) Bioluminescent images of E. coli (carrying pBAV1C-T5-lux plasmid) on surface, before and after illumination (clock wise direction: background image, before illumination, after illumination). (b) Graph showing the antibacterial activity of the phthalocyanine phthalocyanine after illumination for 1 h with light of intensity 18 mW·cm^{-2} and wavelength 485-750 nm. The data and graphs for back ground luminescence and before illumination were shown in supplementary information.

Similar test was conducted using filter paper soaked with more resistant bacteria - bioluminescent *Acinetobacter baylyi* ADP1 carrying plasmid pBAV1C-T5-lux. In this case, the phthalocyanines (**6**, **7** and **8**) showed much activity (Figure 5). However, the cationic zinc derivative was far more efficient in inactivation of microbes. This time also the signal from the filter paper incubated in the dark region was absent probably due to the stray light exposure. These experiments concluded that tetra cationic derivates of pyridine phthalocyanines (**7** and **8**) are more efficient dyes compared to the neutral ones. The extra cationic charges of the molecules might had played an important role in binding the gram negative bacteria towards the surface of the filter paper there by ensuring a better photodynamic inactivation.

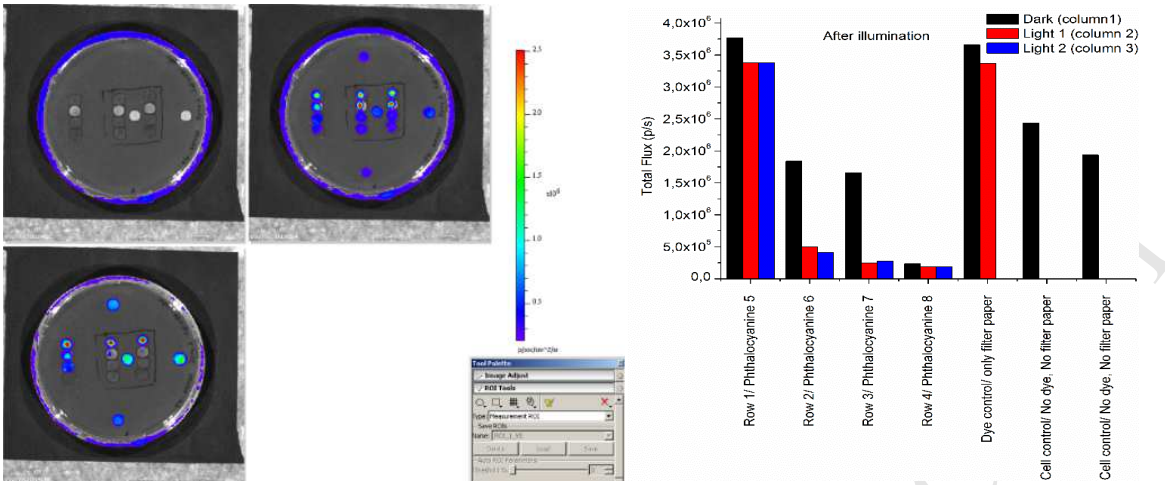


Figure 5. (a) Bioluminescent images of *Acinetobacter baylyi* ADP1 (carrying plasmid pBAV1C-T5-lux) on surface, before and after illumination (clock wise direction: background image, before illumination, after illumination). (b) Graph showing the antibacterial activity of the phthalocyanine after illumination for 1 h with light of intensity 18 mW·cm^{-2} and wavelength 485-750 nm. The data and graphs for back ground luminescence and before illumination were shown in supplementary information.

3.4. Cell viability assay.

In order to understand the antimicrobial efficacy of the photosensitizers directly in a medium without substrate support, the cell viability assay of both cationic derivatives (**7** and **8**) were tested in M9- agar medium and PBS suspension. The results concluded that cationic zinc phthalocyanine **8** was highly phototoxic towards *E. coli* wild type upon 2 h of illumination. The antimicrobial efficacy of the compound was found to be superior to that of the reference photosensitizer tetrakis(methylpyridinum iodide) porphyrin TMPyP. However, the free base cationic phthalocyanine **7** had comparable phototoxicity to that of reference photosensitizer. The results are presented in Figure 6.

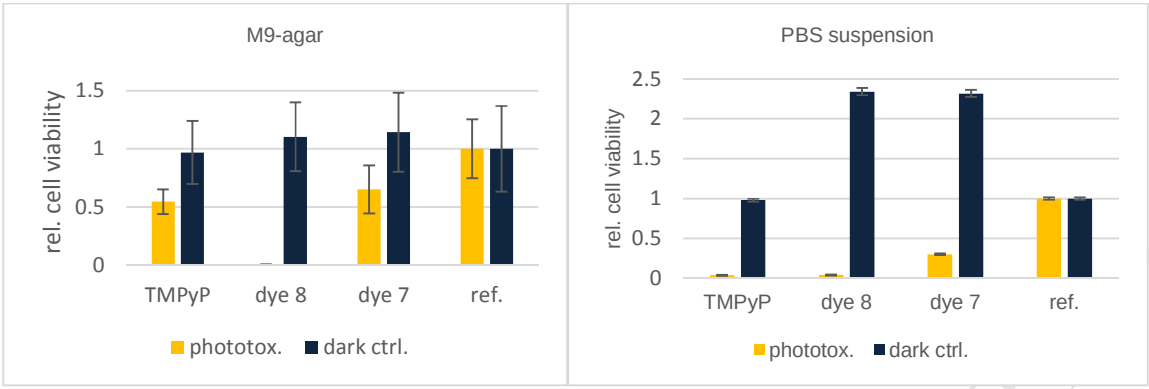


Figure 6. Relative cell viability was measured on M9-Agar (minimal medium for E. coli) and directly in PBS suspension. The values are normalized with the reference (bacteria without any photosensitizers) fluorescence. The photosensitizer concentration is 5 µM. Error bars result from standard deviation and error propagation. Phototox.: illuminated samples, dark ctrl.: dark controls

3.5. Live cell assessment through colony forming unit (CFU) counting.

All the above-mentioned tests pointed out that cationic zinc derivative **8** was best among the set of phthalocyanines synthesized. Therefore, antimicrobial efficacy of the filter paper impregnated with phthalocyanine **8** was determined by CFU counting. In order to control the growth of microbes during the illumination experiment and on serial dilution, the LB medium was replaced with PBS before the deposition on the filter paper. It must be mentioned that the paper impregnated with the photosensitizer **8** was highly toxic towards both *E. coli* and *A. baylyi*. If the dye loading was higher than 0.008 mg/cm^2, no any single bacterial colony could be found on LA plates plated with the microbial extracts from the illuminated filter papers even after overnight incubation. (Table:1)

Table 1. Photoinactivation of *A. baylyi* under illumination.

Dye loading	Number of Colonies	Number of colonies
	1st dilution	2nd dilution
0.08 mg/cm^2	0	0
0.04 mg/cm^2	0	0
0.02 mg/cm^2	0	0
0.008 mg/cm^2	20	1
Control	Too many to count	Too many to count

Hence, the filter paper with dye loading 0.008 mg/cm^2 was found to be optimal for activity testing and was used for further experiments. The optical densities of the microbial solutions measured before deposition on the filter paper was 0.2 and 0.1 for *E. coli* and *A. baylyi* respectively. Thus, the higher number of colonies of *E. coli* compared to *A. baylyi* grown after plating agree with the absorbance values. We have found that photo inactivation against *E. coli* was as high as 2.7 log reduction in CFU, whereas the phototoxic effect against *A. baylyi* demonstrated 3.4 log reduction in CFU (Figure 7).

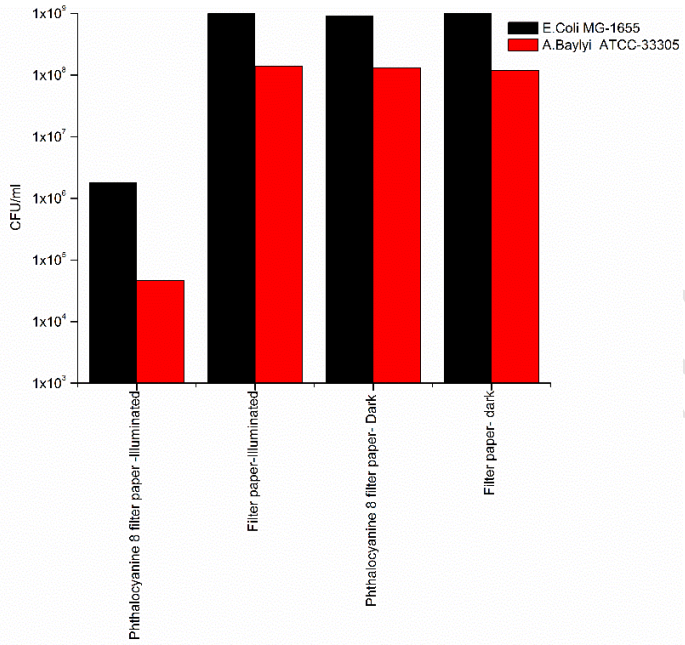


Figure 7. Antimicrobial efficacy of filter paper dyed with phthalocyanine 8 against *E. coli* and *A. baylyi* after illumination for 1 h with light of intensity 18 mW·cm^{-2} and wavelength 485-750 nm.

These values are very well comparable with the best results reported in literature for the dyes immobilized on similar surfaces. Ringot et al. prepared photoactive cotton fabrics by covalently grafting anionic, neutral, and cationic amino porphyrins on cotton fabric via 1,3,5-triazine linker (dye load 18 mg/g of substrate). When subjected to light irradiation of 0.16 mW/cm^2 for 24 h (total light dose 13.8 J/cm^2), the cationic fabric exhibited 100 % photo inactivation against gram-positive bacteria (*Staphylococcus aureus*) while did not show any activity against gram-negative bacteria (*E.coli*).[62] Porphyrin- grafted filter paper through 1,3,5-triazine linker was prepared by Mbakidi et al. The

substrate demonstrated antimicrobial activity of 4 and 2 log decrease in CFU against both *S.aureus* and *E.coli* respectively under same illumination condition as mentioned above.[63] In these experiments the dye load was 19 mg/g of substrate (0.03 µmol/mg, MW=672). Similarly Carpenter et al. was able to achieve photo inactivation of 4 log CFU reduction against different types of bacterial strains using porphyrin linked covalently to cellulose paper with the dye load ca. 8 mg/g (12.4 nmol/mg, MW=672), and with the illumination intensity of 65 ± 5 mW/cm^2 for 30 minutes (total light dose 117 J/cm^2).[64] In our case, similar activity was achieved with the white light dose 64.8 J/cm^2 (1 h at 18 mW/cm^2)and the dye load of 1.2 mg/g of substrate (0.008 mg/cm^2, paper density 68.8 g/m^2, figure S16) prepared by a simple method without any complex chemical modifications.

4. CONCLUSIONS

Novel phthalocyanine with pyridine substitution at α- position, its zinc complexes and cationic derivatives were synthesized in high yield. The dyes exhibited good dyeing ability on filter paper, good stability against leaching, and good photostability. We have elaborated a fast and simple screening setup for testing the photodynamic antimicrobial substances using bioluminescent bacteria *E. coli* and *A. baylyi*. We applied the method to study the antimicrobial efficacy of self-disinfecting surfaces prepared from the dyes. The tetracationic derivatives were found to be the most efficient. Cell viability assay in M9 agar medium and PBS suspension clearly demonstrated the superior photo toxicity of cationic zinc derivative of pyridine phthalocyanine **8**. Finally, the antimicrobial activity using the filter paper dyed with the photosensitizer **8** was studied by CFU counting method. We have achieved 2.7 log CFU reduction against *E. coli* and 3.4 log CFU reduction against *A. baylyi*, respectively, which is comparable with the best results reported to date.

Further study using different metal complexes of pyridine phthalocyanine and substrates for immobilization of the photosensitizer are under progress.

ACKNOWLEDGEMENTS

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APPENDIX A. Supporting information

Supporting information contains the spectra of the compounds synthesized (4-8), the graphs for background luminescence and before illumination for screening experiment using bioluminescent *E. coli* and *A. baylyi*, and the data for CFU counting.

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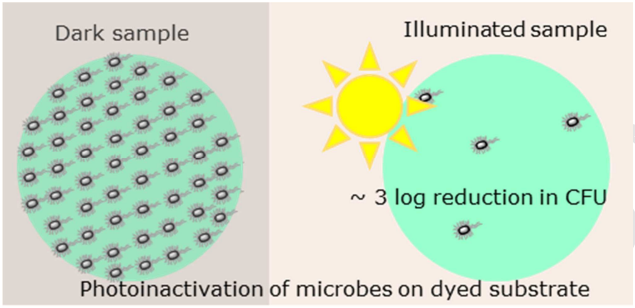
GRAPHICAL ABSTRACT



Highlights

- Novel water-soluble phthalocyanines substituted at α- phthalo position by pyridine units via direct C-C bond were synthesized.
- Fast and simple method to screen the antimicrobial efficacy of dyes was evaluated.
- The paper impregnated with dyes demonstrated the photoantimicrobial effect as strong as 3 log decrease in CFU against *E.coli* and *A.baylyi* upon 1 h irradiation with low intesity white light.