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Polymyxin-Based Photosensitizer for the Potent and Selective Killing of Gram-Negative Bacteria

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Supporting Information

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Abbreviations

Fmoc: fluorenylmethoxycarbonyl

Boc: tert-butyloxycarbonyl

EG: ethylene glycol

DIC: *N,N'*-Diisopropylcarbodiimide

HSPyU: dipyrrolidino(*N*-succinimidyloxy)carbenium hexafluorophosphate

DMF: dimethylformamide

DIPEA: *N,N*-diisopropylethylamine

TFA: trifluoroacetic acid

DCM: dichloromethane

DMSO: dimethylsulfoxide

1. Chemistry

A. General methods

Chemicals used in this work were purchased from Sigma Aldrich, Merck, Acros, AppliChem, and Fisher Scientific. Commercially available reagents were used without further purification. NMR spectra were recorded at 298 K in deuterated solvents using a Bruker AVA500 spectrometer operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C. Chemical shifts are reported in ppm and are referenced to residual non-deuterated solvent. Normal phase column chromatography was carried out on silica gel 60 (230–400 mesh). Analytical HPLC was performed on an Agilent Technologies 1100 modular HPLC system coupled to a multiwavelength and PL-ELSD-1000 detector and equipped with a Phenomenex Kinetex® 5 µm XB-C18 100 Å LC Column (50 × 4.6 mm) with a flow rate of 1 mL/min. Method: A gradient of H_2O/CH_3CN (95/5) to H_2O/CH_3CN (5/95) with 0.1% HCOOH, over 6 min, holding at 95% CH_3CN for 4 min, with ELSD or 600 nm detection. Semi-preparative RP–HPLC was performed on an Agilent 1100 system equipped with a Zorbax Eclipse XDB-C18 reverse-phase column (250 x 9.4 mm, 5 µm) with a flow rate 2.0 mL/min and eluting with 0.1% HCOOH in H_2O (A) and 0.1% HCOOH in CH_3CN (B), with a gradient of 5 to 95% B over 25 min and an additional isocratic period of 5 min. UV/Vis spectra were obtained with a BioTek SynergyHT plate reader using 96-well plates with a typical volume of 100 µL. Fluorescence spectra were measured on a Shimadzu RF-6000 spectrofluorophotometer using a quartz cuvette (10 mm). Electrospray ionisation mass spectrometry (ESI–MS) analysis were carried out on an Agilent Technologies LC/MSD Series 1100 quadrupole mass spectrometer (QMS) in ESI mode. MALDI TOF MS were run on a Bruker Ultraflextreme MALDI TOF/TOF with a matrix of sinapic acid (10 mg/mL) in H_2O/CH_3CN/TFA (50/50/0.1).

B. Synthesis of MB-PMX

The polymyxin fragment H_2N-PMX(*Boc*)$_4$ and MB-COOH were synthesized according to our previously published studies.[1 2]

Amide coupling: A solution of *Fmoc*-NH-$(EG)_2$-CH_2COOH (387 mg, 1.0 mmol, 2 equiv.) and DIC (156 µL, 1.0 mmol, 2 equiv.) in DMF (2.5 mL) was stirred for 1 min and added to H_2N-PMX(*Boc*)$_4$ (684 mg, 0.5 mmol) in DMF (4 mL) and the mixture was stirred for 3 hours. Solvents were evaporated under reduced pressure and the crude mixture was purified by column chromatography (dichloromethane:methanol 9:1) to afford *Fmoc*-NH-$(EG)_2$-CH_2-CO-NH-PMX(*Boc*)$_4$ (756 mg, 87%). LC-MS (ESI) $[M+H]^+$ 1730.8; HPLC (ELSD detection): 6.71 min.

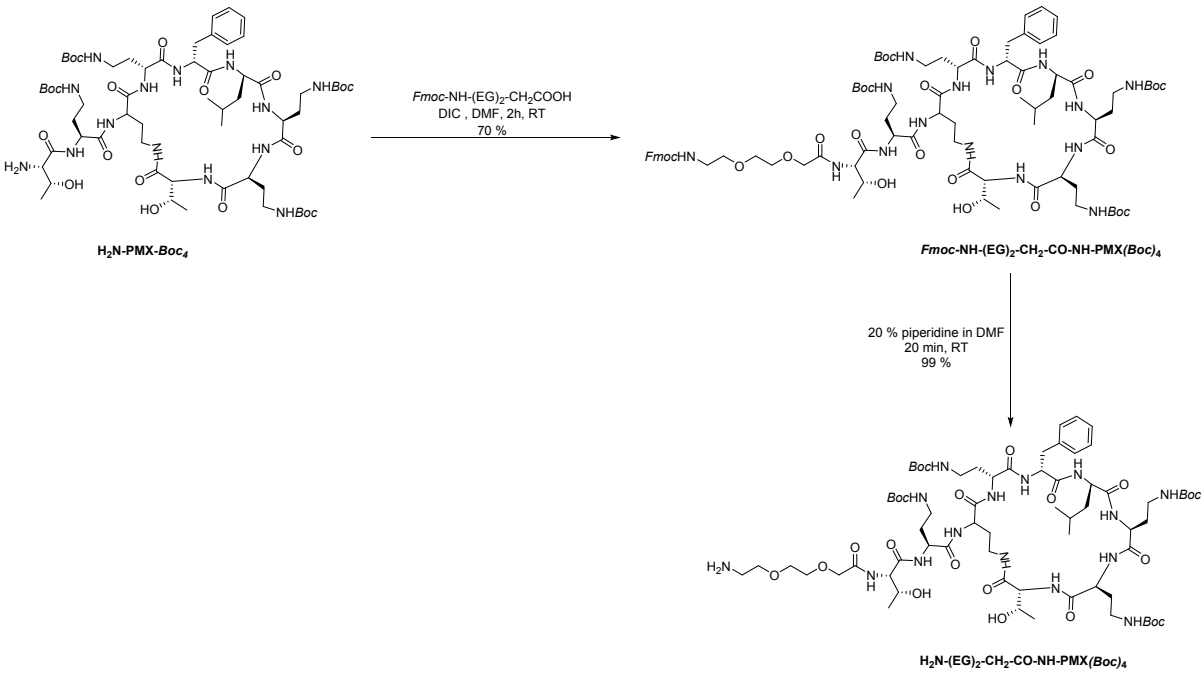
***Fmoc deprotection*:** A solution of *Fmoc*-NH-$(EG)_2$-CH_2-CO-NH-PMX(*Boc*)$_4$ (100 mg, 0.058 mmol) in 20% piperidine in DMF (2 mL) was stirred for 20 min. The solvent was removed *in vacuo*. The remaining residue was washed with diethyl ether (3 x 15 mL) and the resultant white solid was

dried under vacuum to afford H_2N-$(EG)_2$-CH_2-CO-NH-PMX(*Boc*)$_4$ (146 mg, 83%), which was used immediately for the next step. LC-MS (ESI) $[M+H]^+$ 1508.8; HPLC (ELSD detection): 4.43 min.

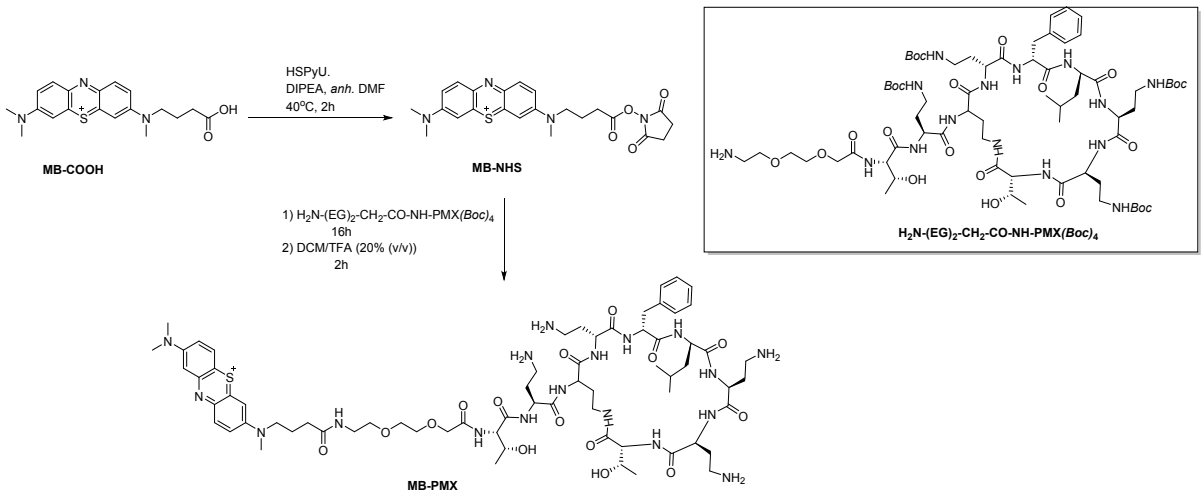
Addition of the photosensitiser: MB-COOH (9.2 mg, 0.026 mmol), HSPyU (10.6 mg, 0.026 mmol, 1 equiv.) and DIPEA (13.5 µL, 0.078 mmol, 3 equiv.) were dissolved in anhydrous DMF (1.5 mL). The reaction mixture was stirred at 40°C for 2 hours. Completion of activation was monitored by analytical RP-HPLC. To the reaction mixture the H_2N-$(EG)_2$-CH_2-CO-NH-PMX(*Boc*)$_4$ (39 mg, 0.026 mmol, 1 equiv.) was added and the reaction was stirred overnight. The solvent was evaporated under vacuum, the residue washed with diethyl ether (3 x 15 mL) and the product collected by centrifugation. The product was used without further purification.

Deprotection of the Boc groups: The Boc_4-protected MB-PMX was treated with TFA-DCM (20% (v/v)) for 2 hours. Solvent was evaporated under reduced pressure and the resultant residue washed with diethyl ether (3 x 15 mL). The dark blue residue was dissolved in water/acetonitrile (1:1 (v/v)) solution and purified by RP-HPLC. The fractions were collected, freeze-dried and the title compound MB-PMX obtained as dark blue solid, (15 mg, 40% (two steps)). LC-MS (ESI) $[M+H]^+$ 1446.7 HPLC (detection at 600nm) $t_{R(analytical)}$=2.4 min, HPLC (detection at 600nm) $t_{R(semi-prepl)}$=13.2 min, MALDI calc. for $C_{68}H_{106}N_{18}O_{15}S^+$ $[M+H]^+$: 1446.77507; found: 1446.77995.

C. Synthetic Schemes



Scheme S1. Synthetic route to H_2N-(*EG*)$_2$-CH_2-CO-NH-PMX*(Boc)*$_4$



Scheme S2. Synthetic route to MB-PMX

D. Characterization of MB-PMX

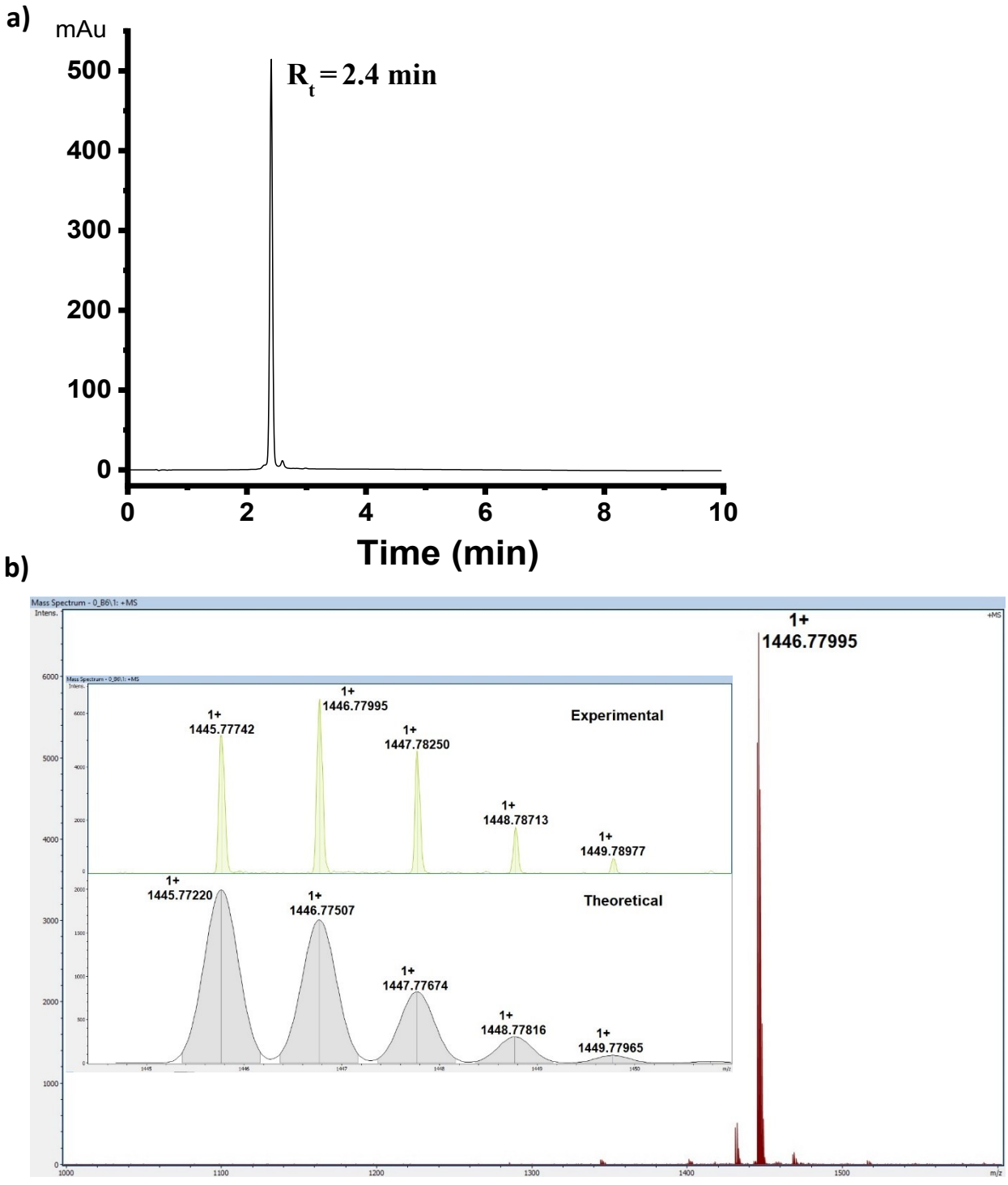


Fig. S1. a) HPLC trace of MB-PMX (detection at 600 nm); **b)** MALDI spectra of MB-PMX. (inset: experimental (top) vs theoretical (bottom)).

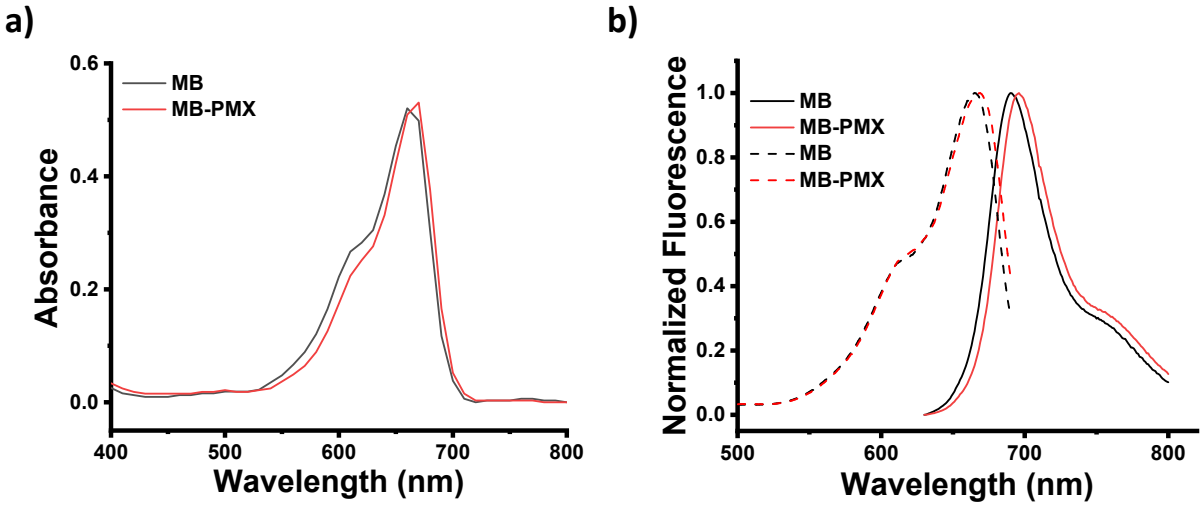


Fig. S2. a) Absorbance and **b)** Excitation (dashed line) and emission (solid line) spectra of MB and MB-PMX (10 µM in Saline (0.9%)).

E. Photo-stability Study of MB-PMX

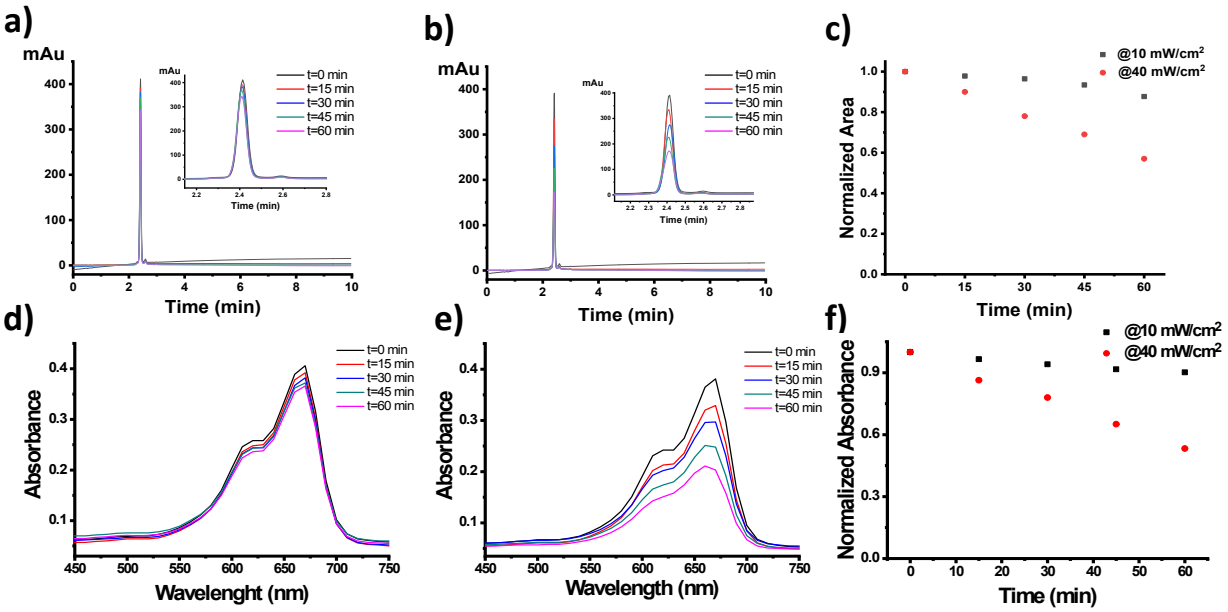


Fig. S3. Determination of the photo-stability of MB-PMX. **a)** & **b)** HPLC traces (detection at 600 nm) and **d)** & **e)** Absorbance spectra of MB-PMX (50 µM) after illumination at 630 nm, 10 mW/cm^2 or 40 mW/cm^2 over 60 mins. **c)** & **f)** Normalized HPLC (detection at 600 nm) and absorbance (λ_{abs}= 670 nm) after illumination.

F. Determination of $^1O_2$ and ROs generation

Determination of Singlet Oxygen Quantum Yield: To calculate the relative quantum yield of MB-PMX diphenyl 1,3-diphenylisobenzofuran (DPBF) was used as a trap molecule.[3] Methylene blue was used as reference photosensitiser which had a quantum yield of 0.5 in methanol.[4] Stock solutions of MB-PMX (0.5 mM) and DPBF were prepared (2 mM) in methanol and DMSO, respectively.

To air saturated methanol both compounds were added at a 1 to 10 molar ratio (5 µM MB-PMX and 50 µM DPBF) and kept in the dark. 1.0 mL aliquots of the MB-PMX/DPBF mixture was irradiated (630 nm, 1.5 mW/cm^2) and at different time points and absorbances at 415 nm were recorded. The singlet oxygen quantum yield of MB-PMX was calculated using the equation below:

$$\Phi_{\Delta\ (MB\text{-}PMX)} = \Phi_{\Delta\ (MB)} \times m_{(MB\text{-}PMX)} / m_{(MB)} \times F_{(MB)} / F_{(MB\text{-}PMX)} \times PF_{(MB)} / PF_{(MB\text{-}PMX)}$$

Φ_Δ	Quantum yield
m	Slope of decrease in absorbance of DPBF at 415 nm
f	Absorption correction factor
PF	Absorbed photonic flux

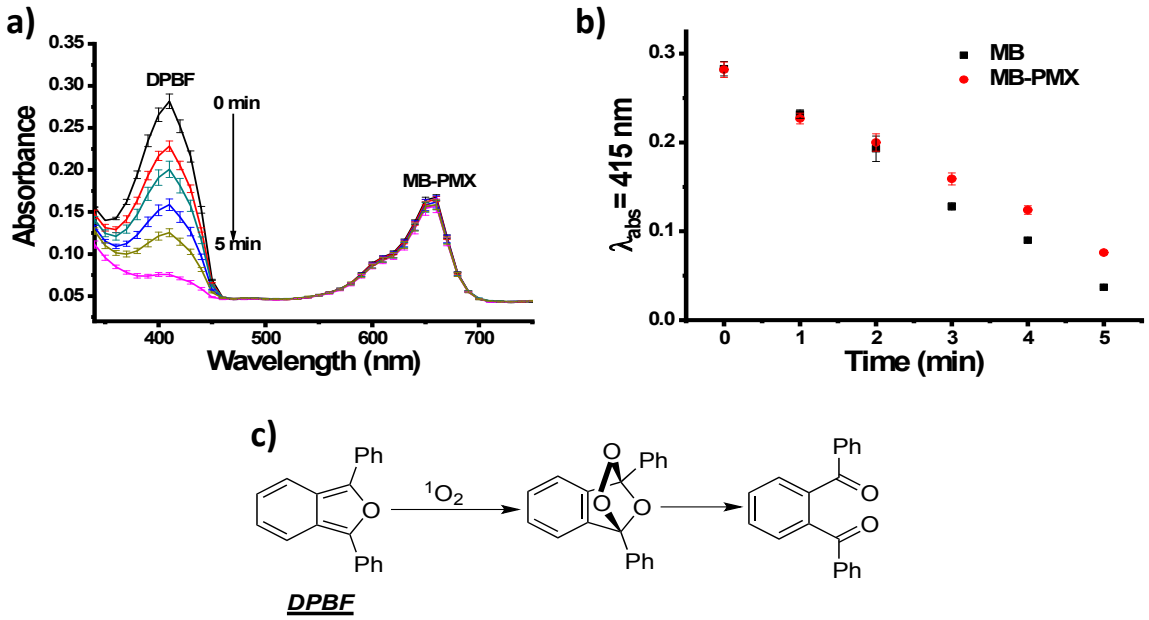


Fig. S4. Determination of the singlet oxygen efficiency of MB-PMX. **a)** Absorbance spectra of MB-PMX (5 µM) and DPBF (50 µM) mixtures in methanol at different irradiation times. **b)** Decrease in DPBF (50 µM) absorbance at 415 nm in the presence of MB (black) (5 µM) or MB-PMX (red) (5 µM) at different irradiation times (630 nm, 1.5 mW/cm^2). **c)** Singlet oxygen trap mechanism of DPBF.

Determination of the ROS generation ability of MB-PMX: The ability of MB-PMX to generate ROS (other than singlet oxygen) was investigated using dihydrorhodamine 123 (DHR 123) as a sensor.[5] The mixture of MB-PMX (10 µM) and dihydrorhodamine 123 (10 µM) in water were irradiated (630 nm, 1.5 mW/cm^2) at different time points and the increase in fluorescence emission at 526 nm was monitored (λ_{ex}= 500 nm).

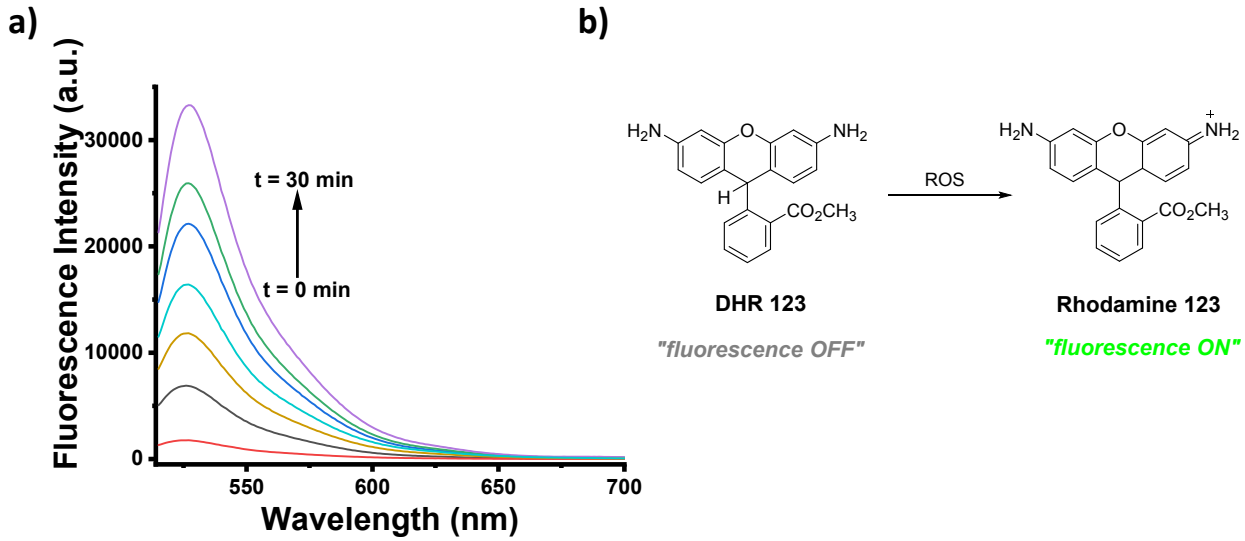


Fig. S5. Determination of ROS generation efficiency of MB-PMX. **a)** Fluorescence spectra of DHR 123 (10 µM) in the presence of MB-PMX (10 µM) and the generation of Rhodamine 123 via different irradiation times in water (630 nm, 1.5 mW/cm^2) **b)** ROS trap reaction of DHR 123.

Killing efficiency of MB-PMX, MB and/or PMX-B:

The antibacterial PDT agent, MB-PMX, demonstrated enhanced killing efficacy compared to the independent killing efficiency of MB and PMX-B. The rational behind this enhanced efficacy is due to the localised effect of the PDT agent and there are two main results: (i). In solution the methylene blue has a generic concentration (e.g. 10 µM). When immobilised onto the bacteria surface this is dramatically increased (many order of magnitude); (ii). The generation of the singlet oxygen from methylene blue in solution will give singlet oxygen that is distributed across the entire volume of the solution – so its chances of killing bacteria are low. With the MB-PMX probe the singlet oxygen is generated in close proximity to the bacteria – so much more likely to kill.

In addition, we expected similar electrostatic interaction efficiency of polymyxin-B as MB-PMX as both have the same penta-cationic polycyclic targeting group. Polymyxin-B is a known lipopeptide antibiotic and displays antimicrobial activity against Gram-negative bacteria. However, the application dose and time plays significant role in its killing efficacy, with literature reported MICs for polymyxin B being <2 µg mL^{-1} (1.5 µM) over a 16-24h incubation period. [6]

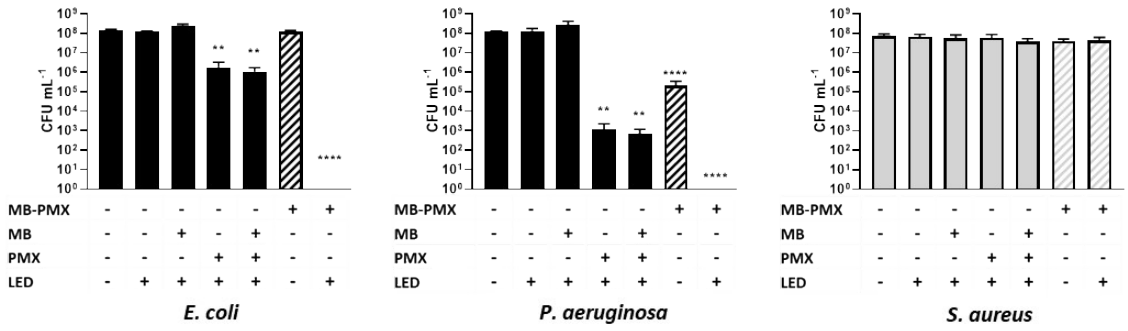
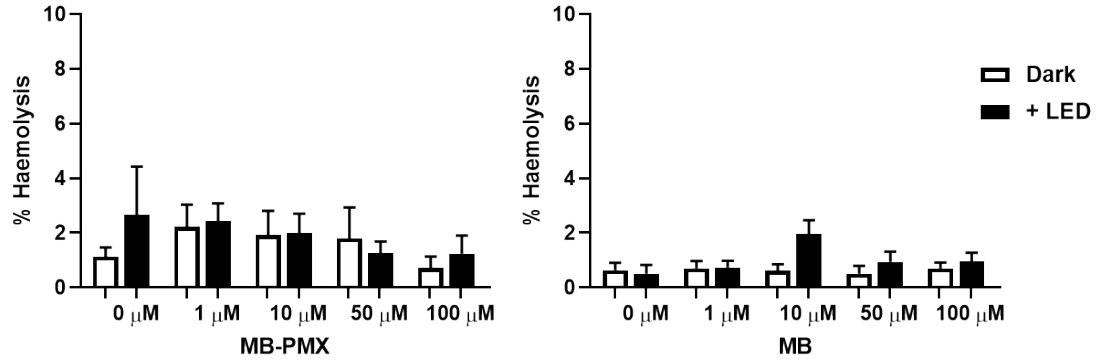
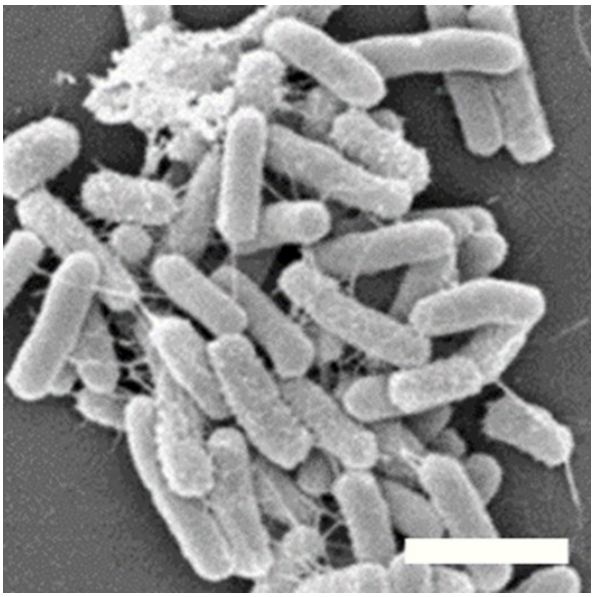


Fig. S6. Colony forming units (CFU) of Gram-negative (black bars) and Gram-positive (grey bars) bacteria with 10 µM MB and/or 10 µM PMX-B (solid bars) or MB-PMX (10 µM, dashed bars), following 10 min illumination at 630 nm, 10 mW/cm^2. No LED and/or no photosensitiser served as controls. Error bars show s.e.m, analysed by student t-test with comparison to bacteria-only control: ****P< 0.0001; **P<0.001. n ≥ 3 repeats.



Haemolysis assay:

Fig. S7. Red blood cell stability (haemolysis assay) following PDT treatment in the presence of excess MB-PMX or MB with 10 min illumination at 630 nm (black bars). Error bars show s.e.m. N = 3.



E. coli Biofilm

Fig. S8. Representative SEM image of *E. coli* biofilm grown and treated by PDT with MB-PMX. Microcolony formation and deposition of extracellular matrix are visible. Scale bar = 2 μm.

2. Biology

A. Ethical Approval

All experiments using human samples were performed following approval from the appropriate regional ethics committee (AMREC), reference 15/HV/013, following informed consent.

B. Data plotting and Statistics

Data plotting and statistical analyses were performed using Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Where appropriate, analyses were performed using the student's *t*-test or one-way ANOVA. Unless otherwise stated error bars show standard error of the mean (s.e.m).

C. Bacterial Growth Conditions

Bacteria used within this study were *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (clinical isolate J3284) and *Staphylococcus aureus* (ATCC 25923). Single colonies were inoculated into Lysogeny broth (LB) and incubated at 37 ^{o}C for 16 h with constant agitation (Sciquip Incushake Midi). Cultures were subcultured to an OD_{595} of 0.1 (Biotech Photometer) and incubated until mid-log phase (OD_{595} of 0.4-0.6). Where appropriate, cultures were serially diluted and plated onto solid LB agar and incubated at 37 °C overnight for determining numbers of colony forming units.

D. PDT of Planktonic Bacteria

Following growth in liquid culture as described above, bacteria with an OD_{595} 0.5 were harvested and washed in 0.9% NaCl (Baxter) by centrifugation at 12470 x *g* (Sigma 1-14 Microfuge). Cultures were incubated with 10 µM MB-PMX, methylene blue (MB) and/or polymyxin-B (PMX-B) as appropriate in the dark for 10 min at room temperature. Cultures were subsequently washed thrice, as described above, and either kept in the dark, or placed under the red LED (Thor Labs M62513 625nm 700mW (min) mounted LED 1000mA) for 10 min, 10 mW/cm^2. Every experiment involved three controls; no photosensitiser (PS) and no light), a light control (light only with no PS) and a dark control (PS only, no illumination). Phototoxicity was determined by CFU plating. N = 4 (performed in singlet).

E. Porcine Skin Model

The porcine skin model was adapted from Maisch et al[7] as follows; porcine skin samples were retrieved from the back and belly of five independently euthanized domestic pigs. Bristles were removed and the skin was cleaned using distilled water and 70% ethanol. The adipose tissue beneath the dermis was removed, prior to storage at -80 ^{o}C until needed.

On the day of the experiment the skin sample was carefully defrosted and equal sized pieces were removed using a biopsy punch (8 mm diameter) (Agar Scientific Biopunch 8.0 mm 15111-40). These

were submerged in 70% ethanol to reduce the number of resident bacteria (99.99 %, confirmed by CFU plating) and then submerged in saline. The skin pieces were then mounted in LB agar in one petri dish per experimental condition, allowing for two replicates per condition.

E. coli was prepared as described above. Each skin biopsy was inoculated with 5 µL of bacterial suspension (5 x 10^6 CFU mL^{-1} in PBS), followed by incubation at 37 °C for 60 min. To the infected skin, 5 µL of either 10 µM or 50 µM of MB-PMX was added and allowed to dry. The samples were placed under the red LED (as described above) at 40 mW/cm^2 for 2 h. Four experimental controls were carried out for each experiment, dark control (PS with no LED), light control (no PS with LED), no LED and no PS and no bacteria control (skin with no bacteria and no PS).

Following the experimental procedures, the skin punch was homogenised (3 x10 s, 300 rpm) (Precellys Hard Tissue Homogenising CK28 P000911-LYSKO-A, Precellys 24 Lysis and Homogenization) then placed onto ice. The resultant suspension was serially diluted for CFU plating. N = 5 (in duplicate).

F. Biofilm model

The method was adapted from Musken *et al* [8]. *E. coli* was cultivated as above and diluted to an OD_{595} of 0.005 in Muller Hinton Broth (Sigma 70192-500G). 200 µL of suspension was added to the wells of a 96 flat bottomed well plate (Corning Incorporated Costar 3595). Plates were incubated 18-24 h at 37^{o}C. Each biofilm was washed three times with saline to remove planktonic bacteria. Saline was then replaced with either 10 µM or 50 µM of MB-PMX as appropriate and the plate was placed under the red LED (as described above, 40 mW/cm^2) for up to 2 h. Three controls carried out for each experiment; a light control (biofilm and LED only with no PS), a control with no PS and no LED and a dark control (PS in dark). Each biofilm was removed by vigorous pipetting and serially diluted for CFU plating. N = 3 (in duplicate).

G. SEM Protocol

Biofilms were prepared as described above into an 8 well slide chamber slide (Ibidi 80821). Following 18-24 h incubation, the samples were fixed in a solution of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 2 h. They were then washed in 3 x 10 min changes of 0.1 M sodium cacodylate buffer. Samples were then post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 45 min. A further 3 x 10 min washes were performed in 0.1 M sodium cacodylate buffer. Dehydration under graded concentrations of acetone (50%, 70%, 90%, and 3 x 100%) for 10 min each was followed by critical point drying using liquid carbon dioxide. After mounting on aluminium stubs with carbon tabs attached, the specimens were sputter coated with 20 nm gold palladium and viewed using a Hitachi S-4700 scanning electron microscope.

H. TEM protocol

For Transmission Electron Microscopy, samples were fixed in 3% glutaraldehyde in 0.1 M Sodium Cacodylate buffer, pH 7.3, for 2 hours then washed with three 10-minute changes of 0.1 M Sodium Cacodylate. Specimens were then post-fixed with 1% Osmium Tetroxide in 0.1 M Sodium Cacodylate for 45 minutes, then washed with three 10-minute changes of 0.1 M Sodium Cacodylate buffer. These samples were then dehydrated in 50%, 70%, 90% and 100% ethanol (X3) for 15 minutes each, then in two 10-minute changes in Propylene Oxide. Samples were then embedded in TAAB 812 resin. Sections, 1µm thick were cut on a Leica Ultracut ultramicrotome, stained with Toluidine Blue, and viewed in a light microscope to select suitable areas for investigation. Ultrathin sections, 60 nm thick were cut from selected areas, stained in Uranyl Acetate and Lead Citrate then viewed in a JEOL JEM-1400 Plus TEM. Representative images were collected on a GATAN OneView camera.

I. Haemolysis assay

Freshly retrieved human erythrocytes[9] were adjusted to 0.7% (vol/vol) in saline (0.9% NaOH) and incubated with 0-100 µM MB-PMX as appropriate for 10 min, 37 °C under dark conditions. Following this, samples were either kept in the dark, or placed under the red LED (Thor Labs M62513 625nm 700mW (min) mounted LED 1000 mA) for 10 min, 10 mW/cm^2. Non-lysed erythrocytes were pelleted by micro-centrifugation, 400 x *g* for 5 min and the supernatant was retained for absorbance analysis at 415 nm using a microplate reader (BioTek Synergy H1). Samples were prepared in duplicate.

% haemolysis was calculated from interpolation of a standard curve. The standard curve was produced by complete lysis of controlled standards by probe-sonication. Standards were prepared by 2-fold dilutions of a 0.7 % blood solution (100 % lysis) to 0.01 % blood solution (0.78 % lysis), and absorbance was measured at 415 nm. All standards were produced in duplicate. The experiment was independently repeated thrice.

3. References

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