Electronic Supplementary Information

A Bivalent Cationic Dye Enabling Selective Photo-Inactivation against Gram-Negative Bacteria

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Experimental Section

Materials: Crystal Violet (**CV**) was purchased from Sigma-Aldrich and recrystallized from methanol. Monopiperazine modified **CV** (**MPCV**) was prepared following our published results.[10] Sodium dodecylsulphate (SDS), ethylene diamine tetraacetic acid (EDTA), sucrose, magnesium acetate and lysozyme were purchased from J&K. *Bacillus subtilis* (*B.subtilis*), *Streptococcus thermophilus* (*S. thermophilus*), *Staphylococcus aureus (S. aures), Pseudomonas aeruginosa* (*P.aeruginosa*) and *Escherichia coli* (*E. coli*) were obtained from Antimicrobial Test Center, TIPC, CAS.

Instruments and methods: UV-Vis absorption spectra were recorded on a Shimadzu UV-2450 spectrophotometer. EPR experiments were carried out at room temperature on a Bruker-ESP-300E spectrometer at 9.8 GHz, X-band with 100 Hz field modulation. Samples were injected quantitatively into quartz capillaries and illuminated in the cavity of the EPR spectrometer with a 100 W mercury lamp. Zeta potentials were measured in PBS suspensions on a Malvern Zetasizer 3000HS (Malvern Instruments Ltd.).

Cellular binding/uptake: Suspensions of bacterial cells (~10^8 cells mL^{-1}) in PBS (5 mM, pH = 7.4) were incubated with **CV** or **MPCV** (5 or 10 μM) at 37 °C for 30 min in the dark. Then, the bacterial cells were centrifuged at 8000 rpm for 10 min. The obtained pellets were either directly lysed by 3 mL SDS aqueous solution (2%) and shaken overnight at 4 °C and then subjected to UV-visible absorption measurement, or were repeatedly suspended in PBS (5 mM, pH = 7.4) and then centrifuged for several times before finally lysed and spectrophotometrically determined.

Intracellular distribution: The subcellular distribution of **CV** or **MPCV** was evaluated after fractionation of treated bacterial cells. A pellet of 2×10^8 treated cells was re-suspended in 2 mL Tris-HCl (0.05 M, pH 7.2) solution containing, 0.5 M sucrose, 0.01 M magnesium acetate and 1 mg lysozyme (for G+ bacteria) or 2 mL Tris-HCl (0.05 M, pH 6.8) solution containing 0.3 M sucrose, 0.01 M EDTA and 1 mg lysozyme (for G- bacteria). After 30 min (for G+) or 90 min (for G-) incubation at 37 °C with shake, the cell suspensions were centrifuged at 6000 rpm for 10 min. The pellets containing either spheroplasts or the protoplasts were re-suspended in 4 mL SDS aqueous solution (2%), while the supernatant containing the cell wall were diluted by 2 mL SDS aqueous solution (4%). The UV-visible absorption spectra of the resulting solutions were determined to estimate the intracellular distribution of dye molecules.

CFU reduction: The photodynamic antimicrobial properties of the examined dyes were determined by incubation with bacterial cell suspensions (~10^8 cells mL^{-1}) for 30 min in the dark at 37 °C and then exposed to an irradiation of ≥ 550 nm for 30 min (the total light dose was ca. 25.2 J cm^{-2}). For dark toxicity, the examined dyes were incubated with bacterial cell suspensions for 60 min in the dark at 37 °C. The treated bacterial samples were diluted in PBS and were spread on 3 M Petrifilm Count Plate and incubated at 37 °C for 48 h. The number of colony-forming units (CFU) was counted by a Shineso G6 Colony Counter. The data shown are mean values ± standard deviation of three independent experiments. Statistical significance was analyzed using a two-tailed unpaired Student's *t* test. ** represents a significance level of $P < 0.01$.

MIC measurement: The bacteria inocula were adjusted to a turbidity equivalent to that of a 0.5 McFarland standard and diluted to a final concentration of ~1.5×10^5 CFU mL^{-1}. Compounds were dissolved and serially diluted in nutrient broth (*B. subtilis, S. aures, E. coli, P. aeruginosa*) and MRS broth (*S. thermophilus*) in sterile 96-well flat-bottom plates to a final volume of 20 μL in each well. A 180 μL portion of inocula was added to each well, making a final concentration range (0.5 μM ~ 32 μM) of the compounds tested. Then the bacteria were incubated for 30 min at 37 °C and exposed to an irradiation of ≥ 550 nm for 30 min (the total light dose was ca. 25.2 J cm^{-2}). After irradiation, MIC values were recorded after 20 h of incubation at 37 °C.

AFM measurement: A 10 μL portion of the test bacterial suspension was deposited on clean thin mica pieces and left for 10 min for absorption. The absorbed bacteria were fixed by 2.5% aqueous glutaraldehyde at 4 °C for 2 h and then washed three times with ultrapure water, dried in air. An AFM (Bruker multimode 8) was used in PeakForce tapping mode and operated in air. Silicon nitride tips (SCANASYST-AIR, Park Bruker) were employed in all AFM measurements. The data obtained were processed by a microcomputer equipped on this microscope.

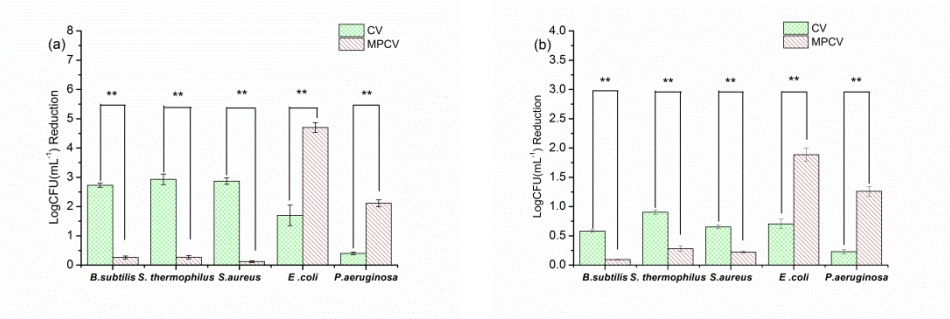

Figure S1. Photodynamic inactivation of *B. subtilis, S. thermophilus, S. aureus, E. coli and P. aeruginosa cells* by 5 μM **CV** and **MPCV**. (a) The samples containing bacteria and photosensitizer were incubated in the dark at 37 °C for 15 min and then exposed to illumination for 30 min; (b) the samples containing bacteria and photosensitizer were incubated in the dark at 37 °C for 30 min and then exposed to illumination for 15 min. The data shown are mean values ± standard deviation of three independent experiments, ** means $P < 0.01$.

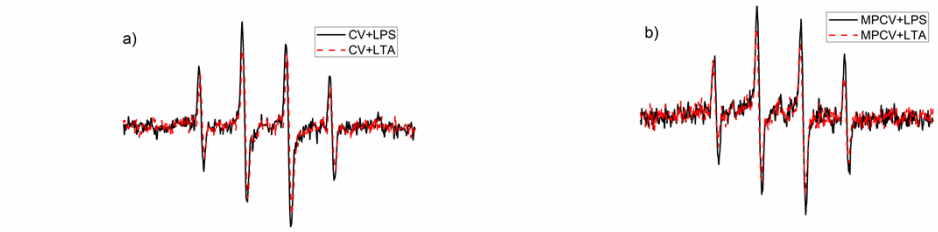

Figure S2. EPR spectra of **CV** (a) and **MPCV** (b) (0.5 mM) upon irradiation by a 100 W high pressure mercury lamp in the presence of DMPO and LPS/LTA (0.1 M) in PBS (5 mM, pH = 7.4).

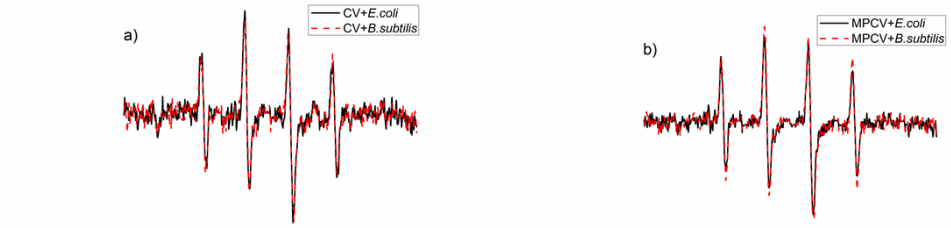

Figure S3. EPR spectra of **CV** (a) and **MPCV** (b) (0.5 mM) upon irradiation by a 100 W high pressure mercury lamp in the presence of DMPO and *E.coli/B.subtilis*(~10^8 cells/mL) in PBS (5 mM, pH = 7.4).

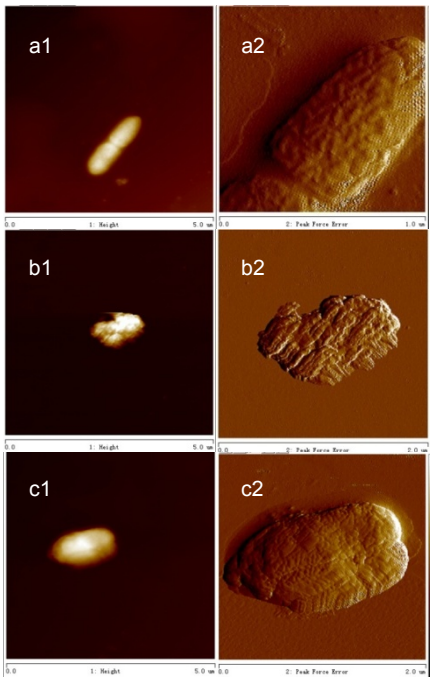

Figure S4. AFM images of untreated *B.subtilis* cells (a1, a2) and treated by 5 mM **CV** (b1, b2) or **MPCV** (c1, c2) and illumination. a2, b2 and c2 are respectively the amplified photos of a1, b1 and c1.

Table S1. MIC values of **CV** and **MPCV** after 30 min incubation and 30 min illumination. (The data shown are mean values ± standard deviation of three independent experiments).

Bacteria Strains	**CV**	**MPCV**
B. subtilis	2.3	10.7
S. thermophilus	1.3	8.0
S. aures	2.0	13.3
E. coli	2.0	0.6
P. aeruginosa	6.7	1.7

Table S2. Log CFU reduction of the examined bacterial cells by **CV** or **MPCV** at 5 or 10 μM in the dark (The data shown are mean values ± standard deviation of three independent experiments).

Bacteria Strains	**CV**		**MPCV**	
	5 μM	10 μM	5 μM	10 μM
B. subtilis	0.12±0.05	0.62±0.04	0.01±0.01	0.15±0.02
S. thermophilus	0.21±0.03	0.52±0.01	0.02±0.01	0.14±0.03
S. aures	0.10±0.02	0.43±0.06	0.03±0.01	0.10±0.03
E. coli	0.09±0.02	0.23±0.04	0.02±0.01	0.17±0.01
P. aeruginosa	0.01±0.01	0.09±0.03	0.03±0.02	0.18±0.03

Table S3. Binding/uptake of **CV** and **MPCV** after 30 min incubation with bacterial cells and then 0-3 times washing (The data shown are mean values ± standard deviation of three independent experiments).

Bacteria Strains	Washing	Binding/uptake (nmol/10^6 cells)	
		CV	**MPCV**
B. subtilis	0	1.92±0.08	2.26±0.03
	1	0.93±0.02	1.32±0.05
	3	0.27±0.04	0.68±0.01
S. thermophilus	0	1.90±0.05	2.03±0.06
	1	0.89±0.07	1.15±0.09
	3	0.24±0.03	0.53±0.05
S. aures	0	1.98±0.05	2.15±0.07
	1	0.97±0.03	1.29±0.02
	3	0.21±0.06	0.64±0.01
E. coli	0	1.59±0.07	1.85±0.05
	1	0.84±0.03	0.97±0.01
	3	0.24±0.02	0.48±0.01
P. aeruginosa	0	0.23±0.01	0.87±0.02
	1	0.18±0.01	0.63±0.01
	3	0.09±0.01	0.38±0.03