Supplementary Information

Multifunctional Divalent Vancomycin: The Fluorescent Imaging and Photodynamic Antimicrobial Properties for Drug Resistant Bacteria

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Experimental Section

General: Chemical reagents and solvents were used as received from commercial sources unless otherwise stated. UV-vis spectra were recorded in a 5-mm path quartz cell on a Beckman coulter DU800 spectrometer. Fluorescence spectroscopic studies were carried out on Varian Cary Eclipse Fluorescent Spectrometer. Fluorescence imaging was acquired with a confocal fluorescence microscope (Nikon Eclipse TE2000-E), using a super high pressure mercury lamp (Nikon, TE2-PS100W) with an excitation filter: 535/50nm and emission filter: 610/75nm. High performance liquid chromatography (HPLC) was performed on a reverse-phase column with a Shimadzu HPLC system. Analytical reverse-phase high performance liquid chromatography (RP-HPLC) was performed on Alltima C-18 column (250×3.0mm) at a flow rate of 1.0 mL/min and semi-preparative RP-HPLC was performed on the similar C-18 column (250×10mm) at a flow rate of 3 mL/min. HPLC elution employed linear gradients of [0.1% trifluoroacetic acid (TFA) in water (solution A)] and [0.1% TFA in acetonitrile (solution B)]. The linear gradient started from 70% solution A and 30% solution B, changed to 68.5% solution A and 31.5% solution B in 18 min, and to 0% solution A and 100% solution B in the following 12 min, and then to 70% solution A and 30% solution B in the next 5 min. 1H NMR spectra were obtained on a 300 MHz Bruker Advance in DMSO-*d6*. MALDI-MS spectrometric analyses were performed at the Mass Spectrometry Facility of School of Biological Sciences, Nanyang Technological University, Singapore.

Synthesis of monovalent Van-porphyrin 3a: Vancomycin hydrochloride (52.8 mg, 35.9 μmol, 1.04 equiv.) and porphyrin **2** (20.8 mg, 35.1 μmol, 1.00 equiv.)[1] were dissolved in 2 mL of dry dimethyl sulfoxide (DMSO). The mixture was cooled to 0 °C, and *O*-benzotriazol-1-yl-*N, N, N', N'* tetramethyluronium hexafluorophosphate (HBTU) (38.0 mg, 100.2 μmol, 2.85 equiv.) in 1 mL of dry *N, N*-dimethylformamide (DMF) was added, followed by *N, N*-diisopropylethylamine (DIEA) (0.06 mL, 344 μmol, 9.8 equiv.). The mixture was allowed to rise to room temperature and stirred overnight. The reaction was quenched by adding dropwise 20 mL of acetone. A deep purple solid was precipitated, filtered out and was washed once by 5 mL of acetone. The crude product was purified by reversed-phase HPLC (RP-HPLC) to give 37.4 mg (18.5 μmol) of pure product (yield: 52.9%). ^{1}H-NMR (300 MHz, DMSO-*d6*): 10.28 (br s), 10.22 (s), 10.27 (s), 9.43 (br s), 9.12(br s), 8.68 (br s), 8.60 (br s), 8.18 (s), 7.97 (s), 7.79 (d, 8.6Hz), 7.50-7.55 (overlapped), 7.28-7.40 (overlapped), 6.82 (s), 6.62 (s), 6.45 (s), 6.09 (br s), 5.81 (br s), 5.57 (s), 5.39 (s), 5.27 (s), 5.18 (br s), 4.90 (br s), 4.70 (d, 5.7Hz), 4.30-4.38 (overlapped), 4.03-4.14 (overlapped), 3.70 (m), 2.88 (s), 2.69 (s), 2.38 (s), 2.26 (m), 1.91 (s), 1.70 (m), 1.54 (m), 1.35 (s), 1.10 (m), 0.85-0.95 (overlapped). MALDI-ToF-MS: The peaks at m/z 2025 correspond to M^+, respectively.

Synthesis of divalent Van-porphyrin 3b: Vancomycin hydrochloride (106.5 mg, 71.7 μmol, 2.04 equiv.) and **2** (20.8 mg, 35.1 μmol, 1.00 equiv.) were dissolved in 2 mL of dry dimethyl sulfoxide

(DMSO). The mixture was cooled to 0 °C, and *O*-benzotriazol-1-yl-*N, N, N', N'* tetramethyluronium hexafluorophosphate (HBTU) (38.0 mg, 100.2 µmol, 2.85 equiv.) in 1 mL of dry *N, N*-dimethylformamide (DMF) was added, followed by *N, N*-diisopropylethylamine (DIEA) (0.06 mL, 344 µmol, 9.8 equiv.). The mixture was allowed to rise to room temperature and stirred overnight. The reaction was quenched by adding dropwise 20 mL of acetone. A deep purple solid was precipitated, filtered out and was washed once by 5 mL of acetone. The crude product was purified by reversed-phase HPLC (RP-HPLC) to give 64.9 mg (18.8 µmol) of pure product (yield: 53.6%). ^{1}H-NMR (300 MHz, DMSO-*d6*): 8.98 (br s), 8.69 (br s), 7.94 (s), 7.67 (br s), 7.54 (br, s), 7.28 (d, 8.4 Hz), 6.92 (d, 8.0 Hz), 6.80 (d, 8.1 Hz), 6.64 (br s), 6.54 (s), 6.43 (s), 6.37 (s), 5.96 (br s), 5.78 (br s), 5.62 (br s), 5.39 (br s), 5.23 (br s), 5.18 (br s), 4.93 (br m), 4.66 (d, 5.7 Hz), 4.23 (overlapped), 4.06 (br, m), 3.80 (br m), 3.69 (overlapped), 3.27 (br s), 3.04-2.88 (multiple), 2.63 (s), 2.28-2.10 (overlapped), 1.91-1.69 (overlapped), 1.61-1.56 (multiple), 1.30 (s), 1.12 (t, 13.6 Hz), 0.94 (d, 6.4Hz), 0.88 (d, 5.5 Hz). MALDI-ToF-MS: calc. M^+= 3455.3, obsvd., the peaks at m/z 3455 correspond to M^+, respectively.

In order to ascertain that the porphyrin is covalently bonded to Van, both **3a** and **3b** were effectively separated by reverse-phase HPLC and both molecules show characteristic retention times. On the other hand, a mixture consisting of porphyrin and Van shows only the retention times of the individual species. This result rules out the possibility of non-covalent adduct formation between Van and porphyrin. In addition, the MALDI-ToF-MS results clearly yield the precise mass of **3a** and **3b,** indicating the formation of covalently Van bonded porphyrin moieties.

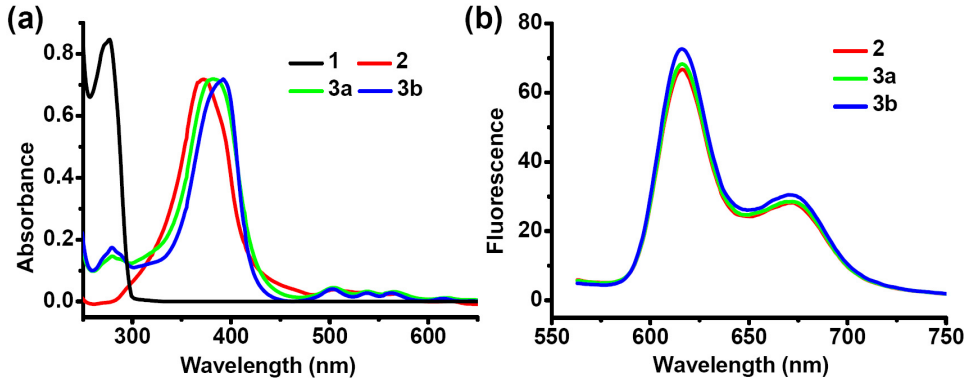

Figure S1. Absorption (a) and fluorescence (b) spectra of **1**, **2**, **3a** and **3b** in PBS (1% DMSO) at room temperature. (λ_{ex} = 530 nm).

UV-Vis and Fluorescence Detection: 2 mM stock solution (in DMSO) of Porphyrin (**2**), monovalent (**3a**) and divalent (**3b**) Van-porphyrin were diluted to 20 µM in 10 mM PBS, pH 7.2, containing 1.0% DMSO as co-solvent. 200 µM vancomycin was also prepared in 10 mM PBS, pH=7.2. 600 µl solution of each compound was added into a 5-mm path quartz cell and the UV-Vis spectra were recorded using a Beckman coulter DU800 spectrometer. Fluorescence spectroscopic studies were also performed using a Varian Cary Eclipse Fluorescence Spectrophotometer.[2]

Measurements of singlet oxygen ($^1O_2$) generation: Porphyrin (**2**), monovalent (**3a**) and divalent (**3b**) Van-porphyrin (10 µM) was mixed with 9, 10-anthracenediyl-bis(methylene) dimalonic acid (ABDA) (20 µM) in PBS buffer (10 mM, pH 7.2) and placed in quartz cuvette. The sample solutions were illuminated with white light (400 nm-800 nm) isolated from the emission of a Xenon lamp for 2 min and then the fluorescent emission of ABDA was measured at 431 nm when excited at 380 nm. The same sample solutions without light irradiation were used as control. The destruction of ABDA indicated the generation of singlet oxygen.[3]

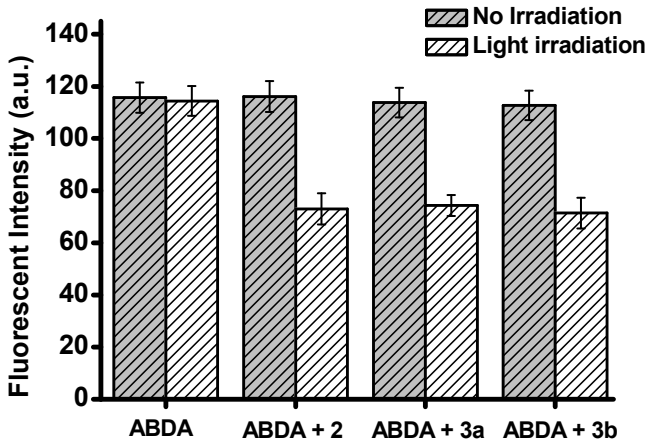

Figure S2. Fluorescent intensity of ABDA (20 μM) mixed with **2**, **3a** and **3b** (10 μM) in PBS buffer before and after light irradiation for 2 min. The destruction of ABDA indicated the generation of singlet oxygen. Excitation: 380 nm; Emission: 431 nm.

MIC test: A standard broth dilution method was used to determine the MICs.[4] Porphyrin (**2**), Van-porphyrin monovalent (**3a**) and divalent (**3b**) were dissolved in DMSO to obtain 4 mg/ml stock solution and 4 mg/ml vancomycin/H_2O stock solution was also prepared. A total of 250 μl of LB solution was added to a series of sterile test tubes, with an additional 218 μl added to the first one. 32 μl of a 4 mg/ml compound stock solution was added to the first test tube, and a series of 2-fold dilutions were prepared by transferring 250 μl to successive tubes. A 5-ml culture of three bacterial strains, *Bacillus subtilis* (ATCC 33677) *Enterococcus faecium* (ATCC 51559, Van A) and *Enterococcus faecalis* (ATCC 51299, Van B), was grown to an OD_{600} of 0.5 in LB medium. A 10 μl bacterial solution was added to each tube containing different concentration of compounds. The final concentration of bacterial strains was 10^5 colony forming units (CFU) per ml. The compound-treated cultures were incubated at 37°C for 24 h, and the OD_{600} was measured. The reported MICs were the lowest concentrations of compounds that prevented cell growth. Each measurement was performed in duplicate.

Table S1. The antibacterial activities (MIC) of vancomcyin and vancomycin-porphyrin conjugates.

Compds	MIC		
	B. subtilis (sensitive)	*E. faecium* (VanA)	*E. faecalis* (VanB)
2	1μM (1.5μg/ml)	>88.3μM (128μg /ml)	44.2 μM (64μg /ml)
3a	3μM (6μg/ml)	>15.8 μM (32μg /ml)[a]	>15.8 μM (32μg /ml)[a]
3b	2μM (7μg/ml))	>18.5 μM (64μg /ml)	>18.5 μM (64μg /ml)

[a]The MIC values of the substrate for bacterial strains VanA and VanB cannot be further determined due to the low solubility in LB medium.

Imaging test: Single colonies of *Escherichia coli* (ATCC 53868), *Bacillus subtilis* (ATCC 33677) *Enterococcus faecium* (ATCC 51559, Van A) and *Enterococcus faecalis* (ATCC 51299, Van B) on solid Luria-Bertani (LB) plates were transferred to 5ml of liquid LB culture medium and were grown at 37 ˚C for 12h. Bacteria were harvested by centrifuging (4000 rpm for 10 min) and washed with sterile

phosphate-buffered saline (PBS) three times. The supernatant was discarded and the remaining bacteria were re-suspended in PBS with an OD_{600} of 0.5. Then, 2 μM or 10 μM of porphyrin (**2**), monovalent Van-porphyrin (**3a**) and divalent Van-porphyrin (**3b**) were added to bacterial cells suspensions and incubated in the dark for 1 hr at 37 ˚C. After three times PBS washing, bacterial cells were spotted on glass slides and immobilized by the coverslips. Cell imaging tests were conducted with a Nikon Eclipse TE2000 Confocal Microscope. Images were captured with CFI VC 100 × oil immersed optics.

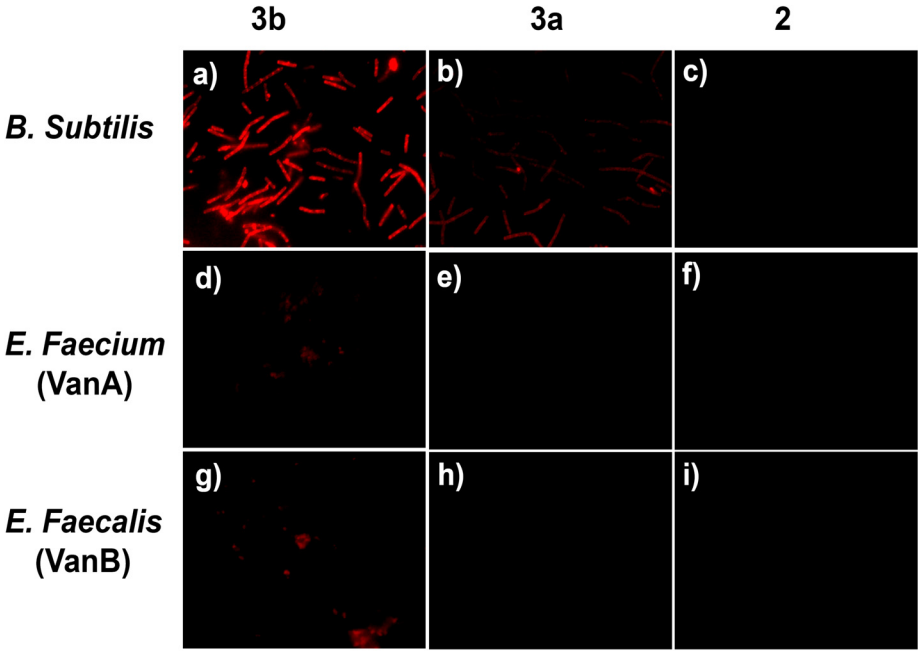

Figure S3. Fluorescent imaging of bacterial staining with Van-porphyrin derivatives. (a) - (c), *B. subtilis* loaded with 2 μM of **3b**, **3a**, and **2**, respectively; (d) - (f), *E. faecium* (VanA) loaded with 2 μM of **3b**, **3a**, and **2**; (g) - (i), *E. faecalis* (VanB) loaded with 2 μM of **3b**, **3a**, and **2**. Ex = 535/50nm; Em = 610/75nm.

Evaluation of photodynamic inactivation of bacterial strains:

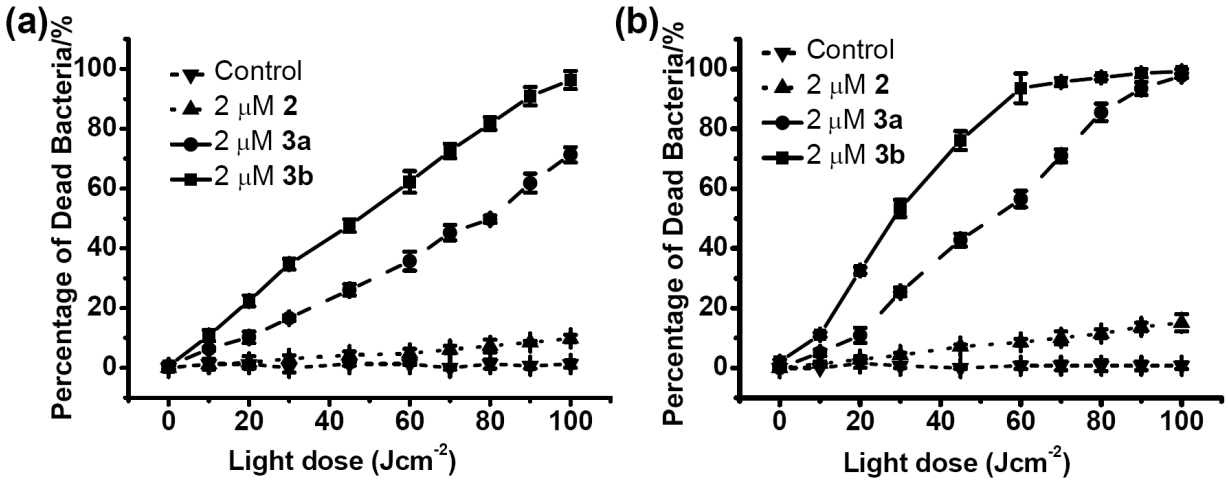

Figure S4. Light dose-dependent bacterial lethality towards different compounds **2**, **3a** and **3b**. (a): *E. faecium* (VanA); (b): *E. faecalis* (VanB). The concentration of all compounds was 2 μM. Bacteria treated with light illumination (without any photosensitizers) as control groups.

Photodynamic treatment was performed according to the methods previously described.[5] Four bacterial strains, vancomycin-susceptible Gram-positive *Bacillus subtilis* (ATCC 33677), vancomycin-resistant *Enterococcus faecium* (ATCC 51559, Van A) and *Enterococcus faecalis* (ATCC 51299, Van B) and Gram-negative *Escherichia coli* (ATCC 53868), were used to evaluate the photodynamic killing of porphyrin (**2**), Van-porphyrin monovalent (**3a**) and divalent (3**b**). A single colony of bacteria was transferred to 5ml of LB solution in the presence of 50 μg/ml ampicillin and was grown at 37 ˚C for 12h. Then bacterial solutions were centrifuged at 4000 rpm for 10 min at 4 ˚C. After washing with PBS three times, the bacteria were re-suspended in PBS with an OD_{600} of 0.5. Then, cells were incubated with different concentrations of **2**, **3a** and **3b** in the dark for 15 min at 37 ˚C. All samples were illuminated with white light (400 nm-800 nm) isolated from the emission of a Xenon lamp. The time of illumination was adjusted from 0 to 2 min, corresponding to the total light doses of 0 to 100 J/cm^2. Following irradiation, bacterial suspensions were centrifuged (4000 rpm for 10 min, at 4 ˚C) and the supernatant was removed. After that, bacterial pellet was suspended and serially diluted (6×10^4)-fold in PBS. A 100 μl portion of the diluted bacterial cells was spread on the solid LB agar plate and incubated for 16 hr at 37 ˚C. The colonies formed were counted. The percentage of dead bacteria was evaluated by dividing the number of colony-forming units (cfu) between the samples incubated with photosensitizers and the control without photosensitizers and light exposure treatment.

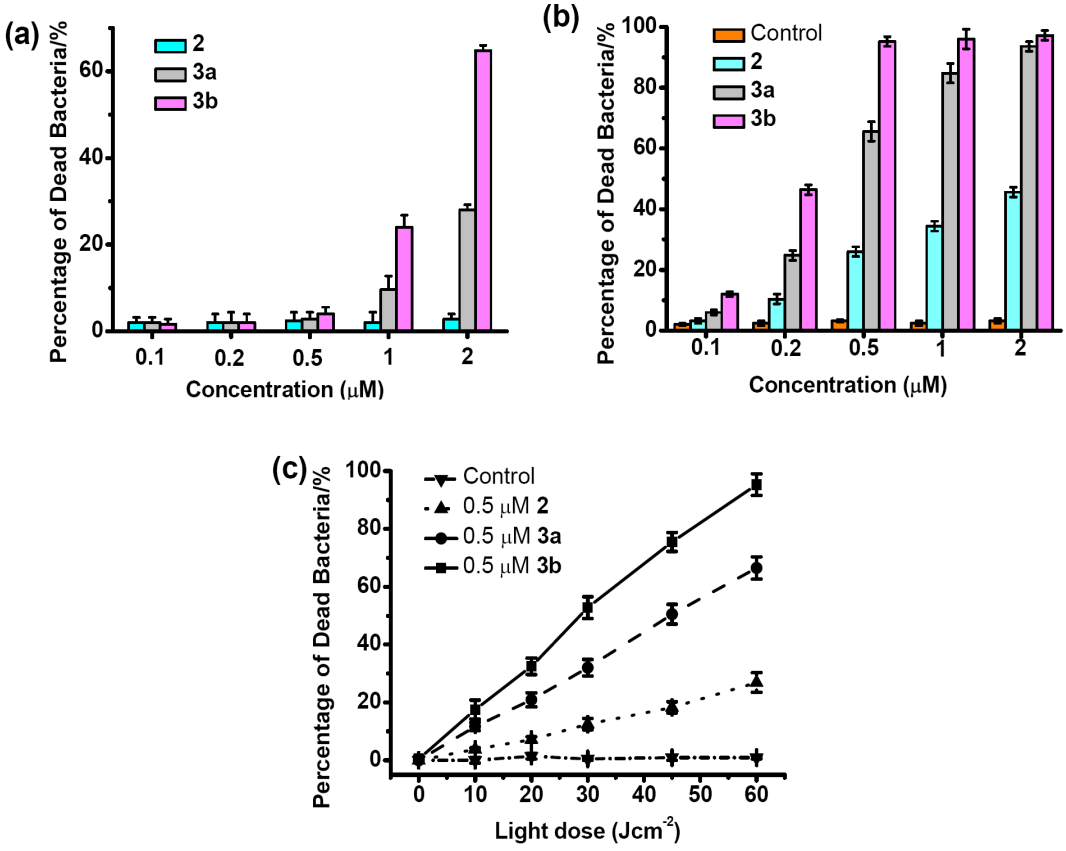

Figure S5. Photodynamic antibacterial activity towards *B. subtilis* in the presence of compounds **2**, **3a** and **3b**. (a) bacterial lethality with incubation of different concentration of compounds **2**, **3a** and **3b** in the dark. (b) bacterial lethality with incubation of different concentration of compounds **2**, **3a** and **3b** upon 60 J/cm^2 of white light illumination. (c) Light dose-dependent bacterial lethality with incubation of 0.5 μM **2**, **3a** and **3b** in the presence of different doses of white light irradiation. *B. subtilis* treated with light illumination without any compound incubation was used as control group.

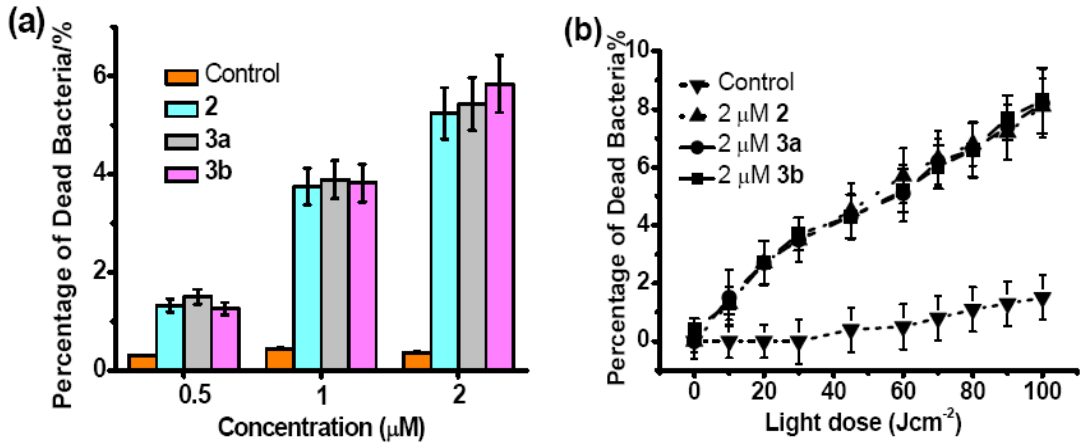

Figure S6. Photodynamic antibacterial activity towards *E. coli* in the presence of compounds **2**, **3a** and **3b**. (a) bacterial lethality with incubation of different concentration of compounds **2**, **3a** and **3b**. The white-light dose was 60 J/cm^2. Bacteria treated with compound **3b** but no light illumination as control groups. (b) Light dose-dependent bacterial lethality with incubation of 2 μM **2**, **3a** and **3b** in the presence of different doses of white light irradiation. *E. coli* treated with light illumination without any compound incubation was used as control group.

References:

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[5] Xing C.; Xu. Q.; Tang H.; Liu L. and Wang S. *J. Am. Chem. Soc.;* 2009, 131, 13117-13124.