Electronic Supplementary Material (ESI) for Chemical Communications

Enzyme Responsive Luminescent Ruthenium (II) Cephalosporin Probe for Intracellular Imaging and Photoinactivation of Antibiotics Resistant Bacteria

Supporting Information

*Qing Shao and Bengang Xing**

Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, 21 Nanyang Link, Singapore 637371

Content:

I. Synthesis, emission property and singlet oxygen productivity of BLRu

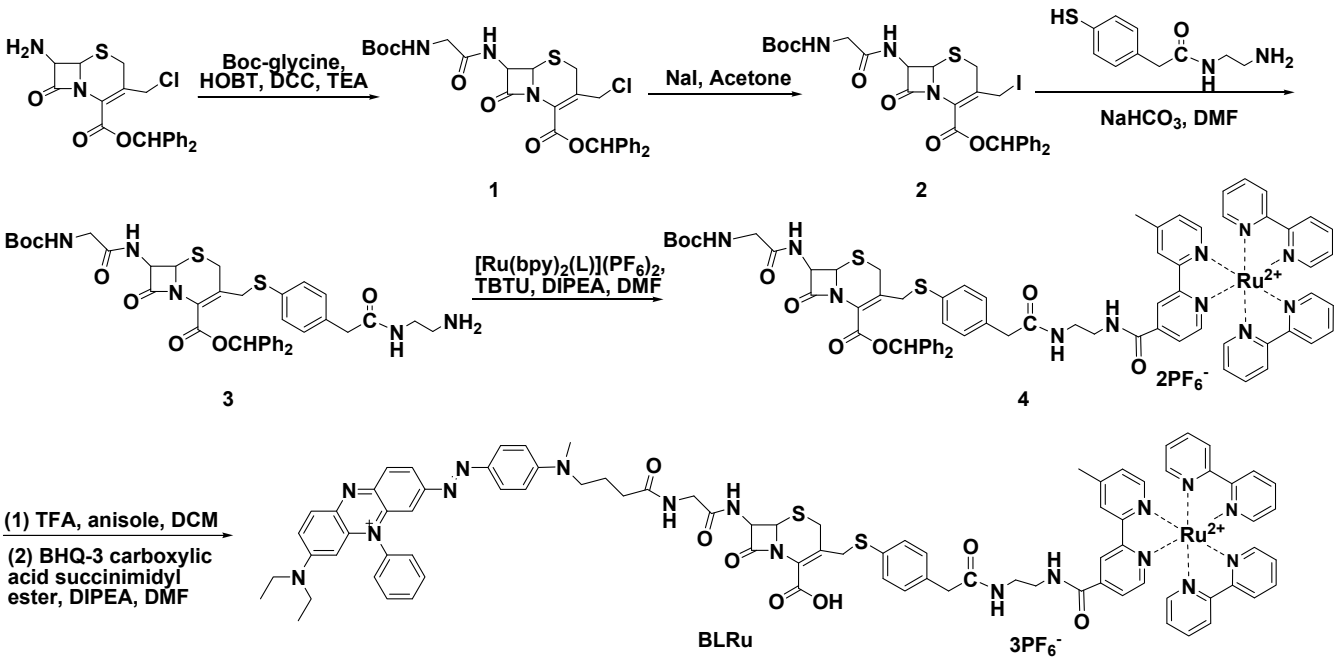
II. Enzymatic hydrolysis of BLRu by β-lactamase

III. Bacterial imaging and antimicrobial study

Experimental Section

General. BHQ-3 carboxylic acid succinimidyl ester was purchased from Bioserch technologies, CA, USA. ACLH was purchased from Ostuka Chemical Co. Ltd. Purified TEM-1 β-lactamase was obtained from Biologics Process Development, CA, USA. Other reagents were purchased from Aldrich. Bacterial strains were purchased from ATCC. NMR spectra were recorded on JEOL 400 MHz spectrometer. Mass spectra (MS) were measured with a Thermo LCQ Deca XP MAX for ESI. UV-vis spectra were recorded on a Beckman coulter DU800 spectrometer. Fluorescence spectroscopic studies were performed on a Varian Cary eclipse fluorescence spectrophotometer. Photo-irradiation experiments were performed at a fluence rate of 70 $mW \cdot cm^{-2}$ with a cool-light fiber optic illuminator provided by a 150W OSRAM Quartz Halogen bulb (400-900 nm).

I. Synthesis, emission property and singlet oxygen productivity of BLRu



Scheme S1 Synthesis of BLRu.

Synthesis of 1. TEA (140 ul, 1.00 mmol) was added to a stirred mixture of 7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid diphenylmethyl ester hydro-chloride (400 mg, 0.89 mmol) in 30 ml of

acetonitrile/dioxane (1:1). Then N-Boc-glycine (174 mg, 1.0 mmol) and HOBt (270 mg, 2.0 mmol) was added, followed by a solution of DCC (240 mg, 1.2 mmol) in 5 ml of DCM. The mixture was stirred at room temperature under nitrogen atmosphere for 10 hrs and concentrated. The residue was purified by column chromatography on silica gel with eluent EA:hexane = 1:1 to give 420 mg of white solid. Yield: 82.5%. ^{1}HNMR (400 MHz, $CDCl_3$) δ (ppm): 7.27-7.46 (m, 10H), 7.00 (s, 1H), 5.90 (dd, J = 5.04 Hz, 9.26 Hz, 1H), 5.03 (d, J = 5.04 Hz, 1H), 4.42 (d, J = 5.00 Hz, 2H), 3.80-3.95 (m, 2H), 3.66 (d, J = 18.32 Hz, 1H), 3.52 (d, J = 18.32 Hz, 1H), 1.48 (s, 9H); MS (ESI) m/z: 593.97, calculated for $[M+Na]^+$: 594.14.

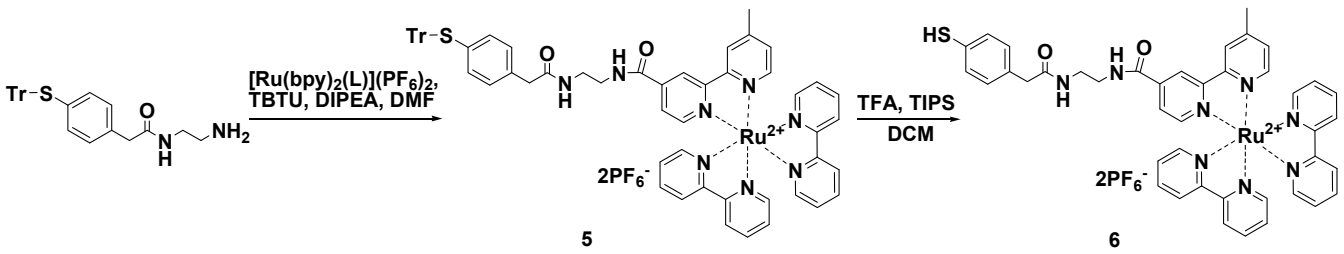
Synthesis of 2. Compound **1** (25 mg, 0.044 mmol) was dissolved in 10 ml acetone. Then sodium iodide (60 mg, 0.40 mmol) was added and the mixture was stirred at room temperature for 1 hr. After concentration, the residue was dissolved in 30 mL of EA and washed with 10% sodium thiosulfate (5 ml), water (5 ml) and brine (5 ml). The organic layer was dried over sodium sulfate and concentrated. The crude product was dried under vacuum and used directly without further purification.

Synthesis of 3. The iodide intermediate was dissolved in 0.4 ml of DMF and sodium bicarbonate (11 mg, 0.135 mmol) was added. Then a solution of *N*-(2-aminoethyl)-2-(4-mercaptophenyl)acetamide (21 mg, 0.1 mmol) in 0.1 ml of DMF was added by dropwise under nitrogen atmosphere. The mixture was stirred at room temperature for 2 hrs and purified by reverse-phase HPLC to collect 13 mg of white solid. Yield: 39.7%. ^{1}HNMR (400 MHz, MeOH-d4) δ (ppm): 7.25-7.44 (m, 10H), 7.14(dd, J = 8.24 Hz, 16.04 Hz, 4H), 6.77 (s, 1H), 5.68 (d, J = 5.04 Hz, 1H), 5.01 (d, J = 4.60 Hz, 1H), 4.12 (d, J = 13.28 Hz, 1H), 3.87 (d, J = 13.28 Hz, 1H), 3.70-3.80 (m, 4H), 3.40-3.61 (m, 6H), 1.45 (s, 9H); MS (ESI) m/z: 746.10, calculated for $[M+H]^+$: 746.26.

Synthesis of 4. $[Ru(bpy)_2(L)](PF_6)_2$ (L = 4'-methyl-2, 2'-bipyridine-4-carboxylic acid) (13 mg, 0.014 mmol) and compound **3** (10 mg, 0.013 mmol) were dissolved in 0.5 ml of DMF. Then TBTU (21 mg, 0.065 mmol) and DIPEA (5 µl, 0.028 mmol) were added under nitrogen atmosphere. The mixture was stirred at room temperature for 5 hrs and purified by reverse-phase HPLC to afford 12 mg yellow solid.

Yield: 56.2%. ^{1}HNMR (400 MHz, Acetone-d6) δ (ppm): 10.10 (m, 1H), 9.48 (d, J = 16.92 Hz, 1H), 9.00 (d, J = 13.72 Hz, 1H), 8.83-8.88 (m, 4H), 8.61 (m, 1H), 8.16-8.25 (m, 4H), 8.08-8.13 (m, 2H), 8.01-8.04 (m, 3H), 7.90 (m, 1H), 7.85 (dd, J = 3.44 Hz, 5.72 Hz, 1H), 7.51-7.61 (m, 6H), 7.25-7.43 (m, 8H), 7.20 (t, J = 7.80 Hz, 2H), 7.08 (d, J = 8.24 Hz, 1H), 7.02 (d, J = 8.24 Hz, 1H), 6.82 (d, J = 2.76 Hz, 1H), 6.38 (br, 1H), 5.81 (m, 1H), 5.09 (t, J = 4.12 Hz, 1H), 3.85-4.06 (m, 4H), 3.41-3.68 (m, 8H), 2.58 (d, J = 4.12 Hz, 3H), 1.42 (s, 9H); MS (ESI) m/z: 677.60, calculated for $[M\text{-}2PF_6^-]^{2+}$: 677.68.

Synthesis of BLRu. To a cooled (ice bath) solution of compound **4** (4 mg, 0.0024 mmol) in 1 ml DCM was added 200 µl TFA and 50 µl anisole. The mixture was stirred at this temperature for 2 hrs and the progress of reaction was monitored by HPLC. The solvent was removed at reduced pressure and the residue was redissolved in 300 µl DMF. Then BHQ-3 carboxylic acid succinimidyl ester (2.3 mg, 0.0029 mmol) and DIPEA (2.1 µl, 0.012 mmol) were added. The mixture was stirred at room temperature for 3 hrs and purified by reverse-phase HPLC to give 1.7 mg of dark blue product after lyophilization. Yield: 34.3%. ^{1}HNMR (400 MHz, DMSO-d6) δ (ppm): 9.14 (m, 1H), 9.12 (s, 1H), 8.84 (m, 6H), 8.41 (d, J = 9.16 Hz, 1H), 8.30 (t, J = 5.72 Hz, 1H), 8.13-8.21 (m, 7H), 8.00 (dd, J = 9.60 Hz, 2.28 Hz, 1H), 7.86-7.95 (m, 4H), 7.77 (m, 6H), 7.72 (m, 3H), 7.49-7.58 (m, 5H), 7.40 (d, J = 5.96 Hz, 1H), 7.10-7.30 (m, 6H), 6.87 (d, J = 9.16 Hz, 2H), 5.70 (d, J = 2.32 Hz, 1H), 5.65 (dd, J = 8.24 Hz, 4.82 Hz, 1H), 5.06 (m, 1H), 4.09 (m, 1H), 3.92 (m, 1H), 3.81-3.87 (m, 2H), 3.38 (s, 3H), 3.25 (m, 2H), 3.09 (s, 3H), 2.54 (s, 3H), 2.21 (t, J = 6.88 Hz, 2H), 1.79 (m, 2H), 1.23 (m, 4H), 0.98 (m, 3H); MS (ESI) m/z: 539.75, calculated for $[M\text{-}3PF_6^-]^{3+}$: 539.50.



Scheme S2 Synthesis of enzyme cleavage product **6**.

Synthesis of 5. $[Ru(bpy)_2(L)](PF_6)_2$ (12 mg, 0.013 mmol) and *N*-(2-aminoethyl)-2-(4-tritylmercapto-phenyl)acetamide (8.6 mg, 0.019 mmol) were dissolved in 0.5 ml of DMF. Then TBTU (21 mg, 0.065 mmol) and DIPEA (11 µl, 0.065 mmol) were added. The reaction was stirred overnight under nitrogen atmosphere and purified by reverse-phase HPLC to give 8 mg red powder. Yield: 45.5%. ^{1}HNMR (400 MHz, Acetone-d6) δ (ppm): 10.06 (br, 1H), 9.43 (s, 1H), 8.97 (s, 1H), 8.81-8.88 (m, 4H), 8.50 (br, 1H), 8.16-8.25 (m, 4H), 8.03-8.13 (m, 5H), 7.91 (d, J = 5.52 Hz, 1H), 7.88 (d, J = 5.96 Hz, 1H), 7.52-7.61 (m, 4H), 7.44 (d, J = 5.52 Hz, 1H), 7.31-7.34 (m, 6H), 7.19-7.26 (m, 9H), 7.02 (d, J = 8.24 Hz, 2H), 6.76 (d, J = 8.24 Hz, 2H), 3.55 (m, 2H), 3.31-3.37 (m, 4H), 2.58 (s, 3H); MS (ESI) m/z: 531.73, calculated for $[M\text{-}2PF_6^-]^{2+}$: 531.15.

Synthesis of 6. To a cooled solution of compound **5** (8 mg, 0.006 mmol) in 0.5 ml DCM was added 200 µl TFA and 50 µl TIPS. The mixture was stirred at this temperature for 1h and the solvent was removed at reduced pressure. The residue was purified by reverse-phase HPLC to give 4 mg red powder. Yield: 60.1%. ^{1}HNMR (400 MHz, Acetone-d6) δ (ppm): 10.10 (br, 1H), 9.51 (s, 1H), 9.02 (s, 1H), 8.89 (m, 4H), 8.72 (br, 1H), 8.15-8.24 (m, 4H), 8.03-8.11 (m, 5H), 7.88 (m, 1H), 7.85 (d, J = 5.96 Hz, 1H), 7.52-7.61 (m, 4H), 7.40 (d, J = 4.56 Hz, 1H), 7.28 (d, J = 8.24 Hz, 2H), 7.16 (dd, J = 1.84 Hz, 8.24 Hz, 2H), 3.55 (m, 2H), 3.47 (s, 2H), 3.36 (m, 2H), 2.54 (s, 3H); MS (ESI) m/z: 409.99, calculated for $[M\text{-}2PF_6^-]^{2+}$: 410.10.

Luminescence measurement.

A stock solution (5 mM, in DMSO) of BLRu was prepared and the absorption spectra were recorded at desired concentration adjusted by appropriate addition of the stock solution to phosphate buffered saline (PBS, pH 7.2). The emission spectra were obtained with excitation at 450 nm.

Singlet oxygen detection.

A solution of 9, 10-anthracenediyl-bis(methylene) dimalonic acid (ABDA) (30 µM in PBS) in the presence of each compound (including BLRu, $Ru(bpy)_3$, 5 µM) was illuminated with white light (400-

900 nm) for a certain amount of time (e.g. 5 min) and the corresponding fluorescence intensity of ABDA was measured at 431 nm with excitation at 380 nm. The same sample solutions without light irradiation were used as control. The destruction of ABDA indicated the generation of singlet oxygen.[1]

II. Enzymatic hydrolysis of BLRu by β-Lactamase

HPLC analysis of enzymatic reaction.

BLRu and TEM-1 Bla were incubated in PBS (pH 7.2) at 37 ˚C for 2 h. Then the mixture was subjected to HPLC analysis. The released product was compared with compound 6 and confirmed by ESI-MS.

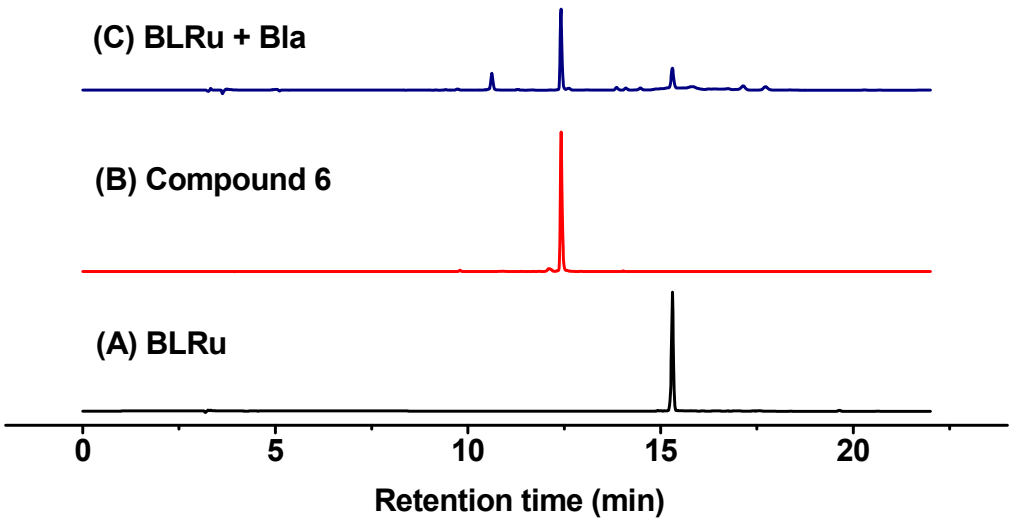


Fig. S1 HPLC analysis of BLRu enzymatic hydrolysis at 450 nm. (A) BLRu; (B) cleaved product 6 and (C) enzyme reaction of BLRu after incubation with β-lactamase at 37˚C for 2h. The formation of peak at 10.6 min was presumably due to the oxidation of released Ru-thiophenol complex.

Sensitivity of BLRu for the detection of Bla. Reaction mixtures (200 μL each) containing BLRu (10 μM) and TEM-1 Bla (0.01 to 10 nM) in PBS buffer (pH 7.2) were incubated at 37˚C for 2 h and subjected to luminescence measurement. In control experiments, BLRu was incubated with PBS buffer at 37˚C for 2 h. All the tests were performed in triplicate.

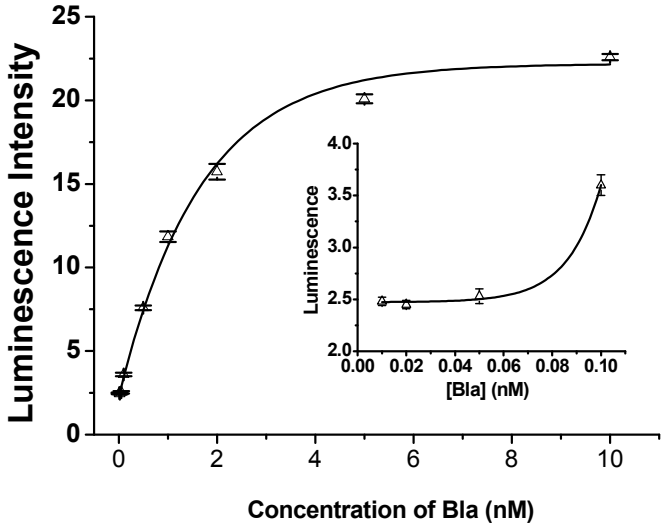


Fig. S2 Dependence of luminescence enhancement in enzyme concentration. Data between 0.01 nM to 0.1 nM was magnified in the inset curve.

III. Bacterial imaging and antimicrobial study

Susceptibility of different strains to BLRu and general antibiotics.

A standard broth dilution method was used to determine the MICs.[2] A 5 ml culture of the bacterial strains, *S. aureus* (ATCC 29213), *E. coli* DH5α (ATCC 53868), *B. cereus* (ATCC 13061) and methicillin-resistant *S. aureus* (MRSA, ATCC BAA39 and ATCC BAA44) was grown to an OD600 of 0.5 in LB medium. Then the culture was washed and resuspended in PBS buffer to 10^7 CFU/ml. A 10 μl bacterial solution was added to the sterile test tubes containing different concentration of Penicillin G, amoxicillin (from 0.5 mg/l to 1024 mg/l) or BLRu (from 0.5 μM to 120 μM) in a total of 1 ml LB solution. The final concentration of bacterial strains was 10^5 CFU/ml and cultures were incubated at 37 ˚C for 24 h. The OD600 was measured and the reported MICs were the lowest concentrations of compounds that prevented cell growth. Each measurement was performed in triplicate.

Table S1 The antibacterial activities (MICs) of penicillin G, amoxicillin and BLRu for bacterial strains

Compounds	*E. coli*	*S. aureus*	*B. cereus*	BAA39	BAA44
BLRu	>120 μM (246 mg/l)	≥ 80 μM (164 mg/l)	>120 μM (246 mg/l)	>120 μM (246 mg/l)	>120 μM (246 mg/l)
Penicillin G	48 μM (16 mg/l)	3 μM (1 mg/l)	3.06 mM (1024 mg/l)	1.53 mM (512 mg/l)	1.53 mM (512 mg/l)
Amoxicillin	11 μM (4 mg/l)	5 μM (2 mg/l)	1.40 mM (512 mg/l)	1.40 mM (512 mg/l)	1.40 mM (512 mg/l)

Imaging measurement of BLRu in bacterial strains.

Single colonies of penicillin G resistant *B. cereus* (ATCC 13061), two clinical isolates of methicillin-resistant *Staphylococcus aureus* MRSA (ATCC BAA39 and ATCC BAA44), one penicillin G susceptible *S. aureus* (non-MRSA, ATCC 29213) and control *E. coli* DH5α bacterial strains on solid Luria-Bertani (LB) plates were transferred to 5 ml 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 resuspended in PBS with an OD_{600} of 0.5. Then, BLRu (10 μM) was added to bacterial suspensions and incubated in the dark for 3 hrs at 37 ˚C. After PBS washing, bacterial cells were spotted on polylysine pretreated glass slides and immobilized by the coverslips. Cell imaging tests were conducted with a Nikon Eclipse TE2000 Confocal Microscope. Images were captured with 488 nm laser installation and CFI VC 100 × oil immersed optics.

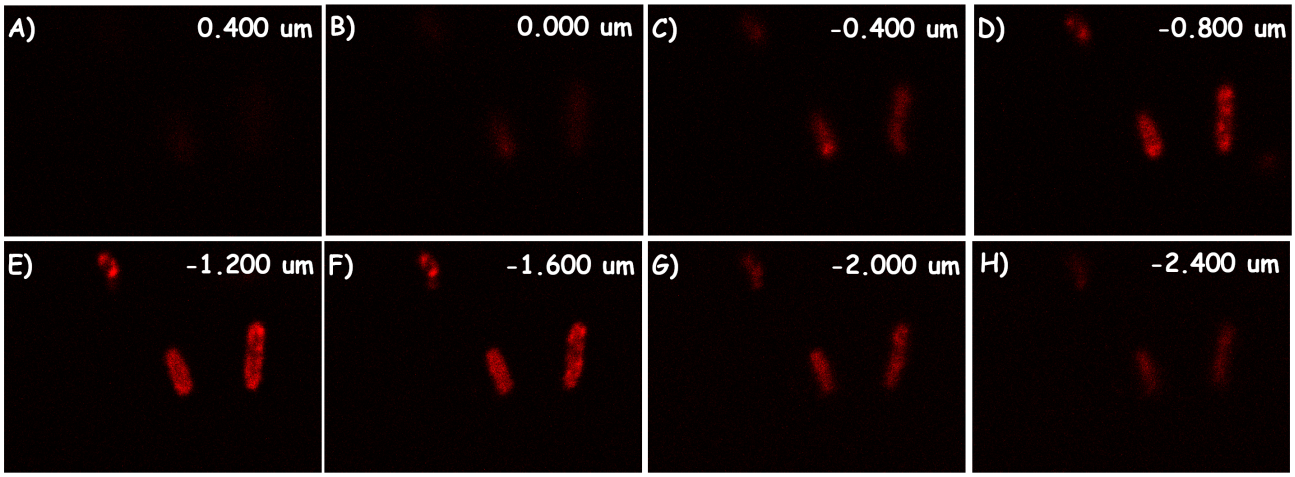


Fig. S3 Confocal microscopic imaging of BLRu (10 μM) distribution in *B. cereus* at different depths.

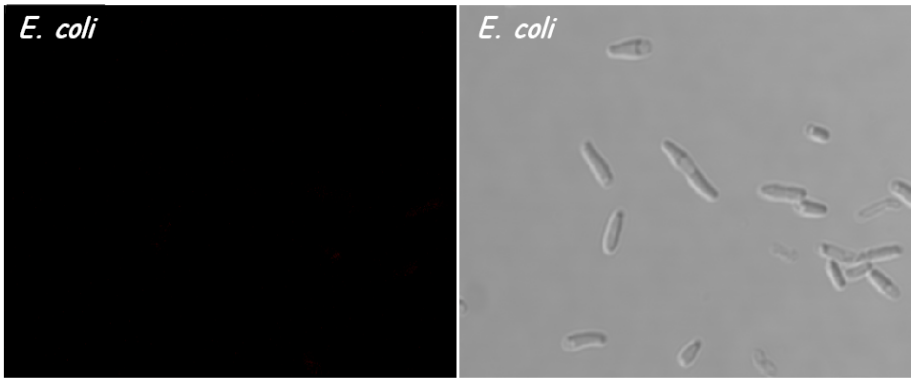


Fig. S4 Confocal microscopic (left) and differential interference contrast (right) images of *E. coli* incubated with BLRu (10 μM).

Detection of Bla in antibiotic resistant strains.

The different bacterial strains (10^6 to 2×10^8 CFU/ml) were lysed in 1 ml of PBS by sonication with a Vibra-Cell Sonics (3 × 10 min), pulsed at 70% max. BLRu (10 μM) was incubated with the bacterial lysates at 37 ˚C for 3h in the dark. Then the mixture was centrifuged (4000 rpm for 10 min) and the supernatant was collected for luminescence measurement. All the tests were performed in triplicate.

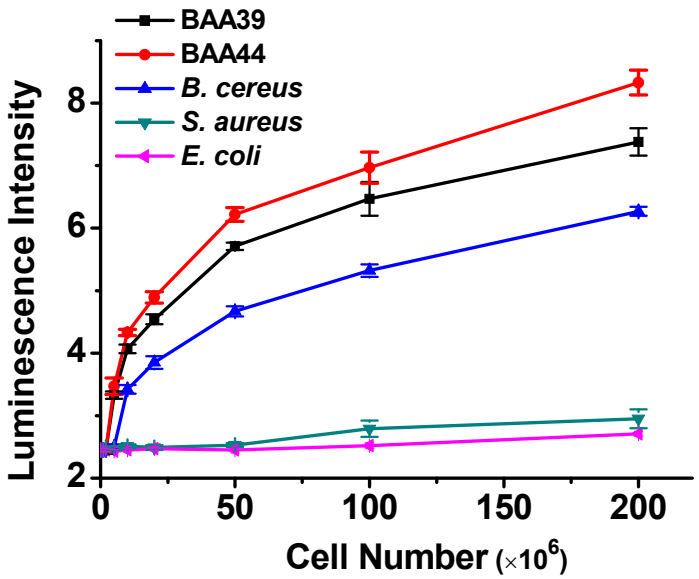


Fig. S5 BLRu hydrolysis in cell lysates of different bacterial strains.

Photodynamic antimicrobial chemotherapy

Photodynamic treatment was performed according to the methods previously described.[3] A single colony of bacteria was transferred to 5 ml of LB solution and grown at 37 ˚C for 12h. Then bacterial solutions were centrifuged at 4000 rpm for 10 min. After washing with PBS three times, the bacteria were re-suspended in PBS to 10^7 CFU/ml. Then, cells were incubated with different concentrations of BLRu or $Ru(bpy)_3$ in the dark for 3 h at 37 ˚C. All samples were illuminated with white light (400-900 nm) isolated from a cool-light fiber optic illuminator provided by a quartz-halogen bulb. The time of illumination was adjusted from 0 to 20 min, corresponding to the total light doses of 0 to 84 J/cm^2. Following irradiation, bacterial suspensions were serially diluted 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 bacterial survival was determined from the formed CFU on the solid LB agar plate and the control without photosensitizers and light exposure treatment. Photoinactivation of *E. coli* was also studied with the same method.

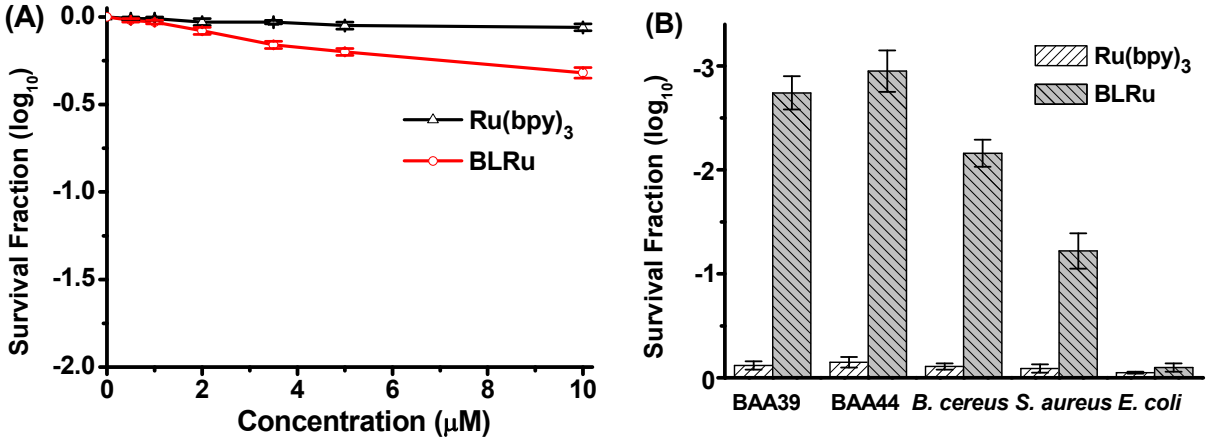


Fig. S6 Photodynamic antimicrobial chemotherapy. (A) Photoinactivation of *E. coli* with different BLRu and $Ru(bpy)_3$ concentration; (B) Comparison of bacterial survival upon photoinactivation in the presence of unmodified $Ru(bpy)_3$ (5 μM) or BLRu (5 μM). Light dose: 42 J/cm^2

Cellular affinity study of cephalosporin luminescent Ru(II) probe

The affinity study was performed according to the methods previously described.[4] In this study, *S. aureus* 29213 was selected as there was no probe hydrolysis in this non-resistant strain. Bacterial suspensions (10^8 CFU/ml, 1 ml) were incubated with BLRu or $Ru(bpy)_3$ (final concentration: 10 μM) in PBS buffer at 37˚C for 3h in the dark. Then the cultures were centrifuged at 4000 rpm for 10 min. After washing with PBS, the remaining bacterial cells were lysed in 10% SDS aqueous solution (1 ml) overnight at room temperature. The solution was ultrafiltered through a 0.2 μm membrane and the absorbance at 450 nm was measured. The reagent concentration was determined according to the calibration curve prepared from different concentrations of BLRu or $Ru(bpy)_3$ in 10% SDS solution, respectively. All the tests were performed in triplicate.

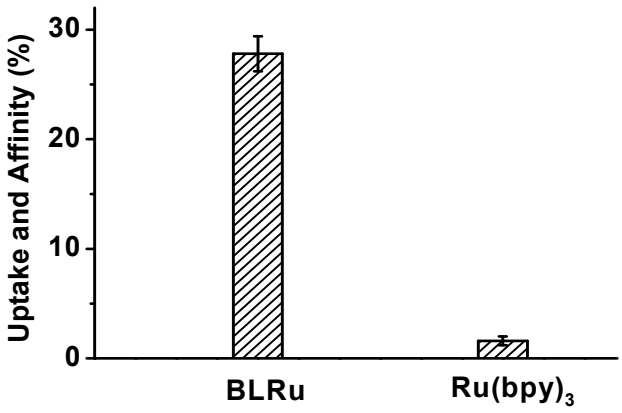


Fig. S7 Comparison of the affinity and uptake ability of ruthenium complexes in *S. aureus* ATCC 29213.

Reference:

(1) Qian, H. S.; Guo, H. C.; Ho, P. C. L.; Mahendran, R. and Zhang, Y. *Small,* 2009, 5, 2285-2290.

(2) Washington, J. A. (1996) in *Med. Microbiol.*, ed. Baron, S. (Univ. Texas Med. Branch, Galveston), p. 161

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

(4) Frimannsson, D. O.; Grossi, M.; Murtagh, J.; Paradisi, F. and O'Shea, D. F. *J. Med. Chem.*, 2010, 53, 7337-7343

(5) Zava, O.; Zakeeruddin, S. M.; Danelon, C.; Vogel, H.; Grätzel, M. and Dyson, P. J. *Chembiochem.,* 2009, 10, 1796-1800