Supplementary Information

for

Sceptrin-Au Nano-Aggregates (SANA) for Overcoming Drug-resistant Gram-negative Bacteria

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Materials and Methods

General information

All chemical reagents were purchased from Sigma Aldrich (USA), TCI (Japan), Alfa Aesar (USA), and Acros Organics (USA). The sources of the bio-metals are $FeCl_2$, $AuCl_3$, $CuCl_2$, $NaCl_2$, and $CoCl_2$. Media of cation-adjusted Mueller-Hinton broth (CA-MHB) and tryptic soy broth (TSB) were purchased from BD/BBL (USA) for the bacteria cultivation. Ribospin™ II Kits for RNA extraction were purchased from GeneAll (South Korea). Oligonucleotide primers for this study were synthesized from Cosmogenetech (South Korea). BioFACT™ 2× OneStep qRT-PCR Master Mix for qRT-PCR was purchased from BioFACT (South Korea). 20× SFCgreen® I Dye for interchelating signals was purchased from BioFACT (South Korea). The 96-well microtiter plate (round bottom, Product No. 34296) and conical tube (15 mL and 50 mL, Product No. 50115 and 50050) were purchased from SPL Life Sciences (Rep. of Korea). Each measurement was repeated three times, and all experiments were performed in duplicate.

Bacteria strain and culture

All strain-related studies were conducted in certified biosafety level (BSL) facilities at Kyung Hee University Medical Center (Seoul, Republic of Korea). All strain-related information has been listed in Table 1. Strains of drug-sensitive bacteria (control strain) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and Culture Collection of Antibiotic-Resistant Microbes (CCARM, Seoul, Republic of Korea). Strains of drug-resistant bacteria were obtained from Asan Medical Center (ASM, Seoul, Republic of Korea). All bacterial strains were stored in skimmed milk and frozen at –80 °C. The bacterial strains were subcultured twice in cation-adjusted Mueller Hinton broth (CA-MHB) for 24 h at 37 °C before the minimum inhibitory concentration (MIC) analysis and the time- or concentration-dependent study.

UV-Vis spectroscopic method

UV/Vis absorption spectra were obtained using a spectrophotometer (Agilent Technologies Cary 8454, US). The quartz cuvette (Hellma Analytics, Jena, Germany) has an internal volume of 1 mL for the absorbance measurement. All UV-vis spectrum were measured using a shutter speed of 1/150, a wavelength interval of 1.0 μm, an integration time of 0.5 sec, and minimum intensity of 4122 cts (220–350 nm) and 1277 cts (350–500 nm). The absorbance spectra of sceptrin and **SANA** was measured after 1 h incubation at 37 °C. The concentration of sceptrin and **SANA** was 10 μM in deinoized water (DI.H_2O).

SANA characterization

The hydrodynamic size and zeta-potential of sceptrin and **SANA** was measured using Malvern Instruments Zetasizer Nano ZS90 (Worcester-shire, UK). The measurement was carried out with particles dispersed in DI H_2O. The morphology of nanoparticles was characterized by transmission electron microscopy (Tecnai, G2 F30ST, FEI Company, OR, USA). Attenuated total reflection Fourier transform infrared (FT-IR) spectroscopy was collected on a Thermo Scientific Nicolet™ iS™ 5 FT-IR spectrometer instrument. Matrix-assisted laser desorption/ionization time-of-flight mass

spectrometer (MALDI-TOF/TOFTM 5800 system, AB SCIEX) at the Institute for Basic Science (IBS, Rep. of Korea) was used for the mass analysis.

Quantum chemical calculation

The geometry optimization of molecules was performed using the density functional theory (DFT) method with the APFD functional and the 6-31G(d)/LANL2DZ-ECP basis[1] set as implemented in the Gaussian 16 package. 6-31G(d) basis set was used for H, C, O, N, and Br atoms, and LANL2DZ ECP basis set was used for Au atom. The vibrational frequency of all the optimized structures was calculated to ensure that the optimized structures of molecules have no imaginary vibrational frequencies.

Binding analysis of sceptrin with Au^{3+}

i) Job plot analysis: A solution (5 mM) of sceptrin in DMSO and Au^{3+} (gold(III) chloride hydrate) solution in DI.H_2O was prepared as a stock solution. Au^{3+} solution was added to 10 μM of sceptri for the final concentration of 2.5 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM, and 100 μM, respectively. Then, the molar ratio was adjusted to 0.09–0.80. The absorption measurement was conducted after 1 h incubation at 37 °C.

ii) Determination of the binding constant: The extent of the binding between sceptrin and Au^{3+} ion was calculated from an experimental plot of the absorption data using the Benesi-Hildebrand relation (Eq. S1). A: intermediate absorption value (at 600 nm), A_0: absorption value in the absence of Au^{3+} (at 600 nm), A_f: absorption value at the saturation point of sceptrin with Au^{3+} (at 600 nm), K_b: Binding constant.

$$\text{Eq. S1 -------- } \text{Log}\,[(A-A_0)/(A_f-A_0)] = \text{Log}\,[Au^{3+}] + \text{Log}\,K_b$$

Cytotoxicity analysis

The cytotoxicity of Au^{3+}, sceptrin, and **SANA** was evaluated within the bEnd.3 cell lines (endothelial cell) using Cell Counting Kit-8 (CCK-8, Dojindo Molecular Tech. Inc, Japan) according to the manufacturer's protocols. The cells (1×10^4 cells per well) were seeded into 96-well plates and incubated for 24 h at 37 °C in a humidified 5% CO_2 incubator. The media was then refilled with culture media (component: 89% DMEM, 10% FBS, and 1% PS). The cells were treated with concentrations of diluted Au^{3+}, sceptrin, and **SANA**, respectively. The cells were incubated for 24 h. Next, Au^{3+}, sceptrin, and **SANA** were removed by washing in PBS (pH 7.4, 2 times) followed by the serum-free media change. CCK-8 solution (10 μL, 10× working concentration) in a serum-free media was added to each well of a 96-well plate, and the cells were incubated for 2 h at 37 °C. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Multiskan FC, Thermo Fisher, MA, US). The percentage of the cell cytotoxicity was calculated using the formula below (Eq. S2). OD: optical density.

$$\text{Eq. S2 ------- Cell viability (\%)} = (\text{Mean OD of sample} \times 100) / (\text{Mean OD of the control group})$$

Hemolysis test for cytotoxicity

The blood was obtained from the hearts of the mice anesthetized with isofluorane. The red blood cells (RBCs) were extracted by centrifugation at 4 °C (3000 rpm, 3 min) washing in 1× cold PBS (2 times). The concentrations of the test (Au^{3+}, sceptrin, and **SANA**) were 50, 100, and 200 µM. In brief, the Au^{3+}, sceptrin, and **SANA** were treated with purified RBCs (8% working concentration, v/v, in cold 1× PBS). [Positive control: 0.1% (working concentration) Triton X-100]. The mixture was incubated in a shaking incubator (300 rpm, 37 °C) for 1 h. Then, the mixture was centrifuged at 3000 rpm at 4 °C, and the supernatant was measured under absorption at 492 nm.

Hemolytic activity

To investigate the effect of **SANA** on hemolytic activity, the hemolytic test was performed as mentioned in the experiment section with some modifications. The bacteria were cultured in LB broth overnight, inoculated in fresh LB broth with 0, 1/4 MIC, 1/2 MIC, MIC of **SANA**, and then incubated for 16 h at 37 °C at 150 rpm. The bacterial cultures were collected by centrifugation at 10,000 × g for 5 min, washed three times in cold PBS (pH 7.4), and resuspended in fresh PBS (pH 7.4). The preparation of mice blood cells was mentioned in the hemolysis test of the experimental section above. The prepared RBCs were resuspended in PBS (pH 7.4) diluted to a final concentration of 8%. A 100 µL of the aliquot of resuspended bacteria culture was mixed with 900 µL of 8% RBCs in an Eppendorf tube and incubated for 4 h at 37 °C. Finally, the mixtures were centrifuged at 3000 rpm at 4 °C, and the supernatant was measured under absorption at 450 nm.

Protein leakage assay

To investigate the protein leakage by antibiotic effect of **SANA** on CRPA, the protein leakage was measured using Pierce™ BCA Protein Assay Kit. Briefly, the bacteria were cultured in LB broth overnight, 1% (v/v)-inoculated into fresh LB broth, and then incubated for 20 min at 37 °C at 150 rpm. After 20 min, the bacterial cultures were treated with 1/2 MIC of **SANA**. At the each point (1 h= 0.05 and 5 h= 0.83) of OD_{600}, the bacterial cultures were spin-down by centrifugation at 10,000 × g for 5 sec, and the supernantant was used to measure the protein leakage. The supernantant was 5×-diluted before the measurement, and reacted with BCA solution for 15 min. Amount of protein was calculated by calibration curve based on bovine serum albumin following the Instrument protocol. The measurement was performed under the absorbance at 550 nm.

RNA extraction

To extract bacterial RNA, we used Ribospin™ II Kits. The steps were performed following the manufacturer's instructions. The bacterial membranes were broken chemically by adding lysis buffer along with centrifugation and vortexing. After the lysis and purification steps, RNA was eluted to 60 µL. Extracted RNA was stored at -80 °C until downstream analysis.

Real-time PCR

We performed quantitative reverse transcription PCR (qRT-PCR) to compare the expression level of the extracted bacterial mRNA. The primers used are listed in Table S2. The qRT-PCR procedures were

modified from the CFX96™ real-time PCR Instrument protocol (Bio-rad, USA). Briefly, 2 µL of isolated RNA was amplified in a total volume of 20 µL containing BioFACT™ 2× OneStep qRT-PCR Master Mix, 20× SFCgreen® I Dye, 500 mM of each oligonucleotide primer, and DI H_2O. The following thermal profile was used for the qRT-PCR: 30 min reverse transcription at 50 °C; 15 min pre-denaturation at 95 °C; 40 cycles of 20 s denaturation at 95 °C, and 40 s annealing and extension at 60 °C. The intercalating dye signals from the amplified products were acquired using CFX96™ real-time PCR Instrument.

SEM imaging

The bacteria were harvested by centrifuging (3000 rpm, 30 min) when OD600 of bacteria reached 1.0. To observe the SEM, the samples were platinum (Pt)-coated by negative staining (EM Ace600, Leica, Germany). Thickness: 30 nm. The critical point drying (CPD) was performed in Leica CPD300 (Germany) for 1.5 h. The prepared samples were evaluated by SEM.

Supporting Figures

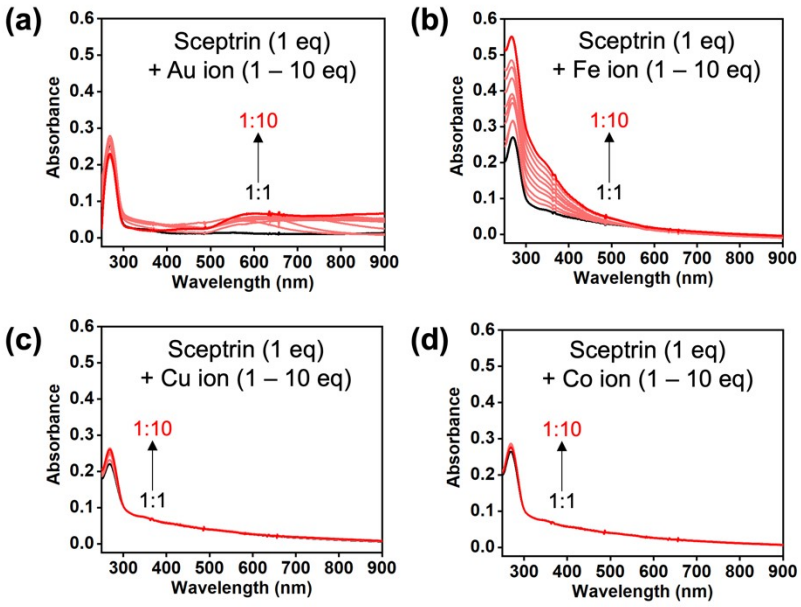

Figure S1. Absorption spectra of sceptrin in the presence of metal ions (a) $AuCl_3$, (b) $FeCl_2$, (c) $CuCl_2$, (d) $CoCl_2$ within DI H_2O. The measurement was conducted after 1 h incubation at 37 °C. The concentration of sceptrin was 10 µM. Tests were conducted in duplicate to obtain the measurements.

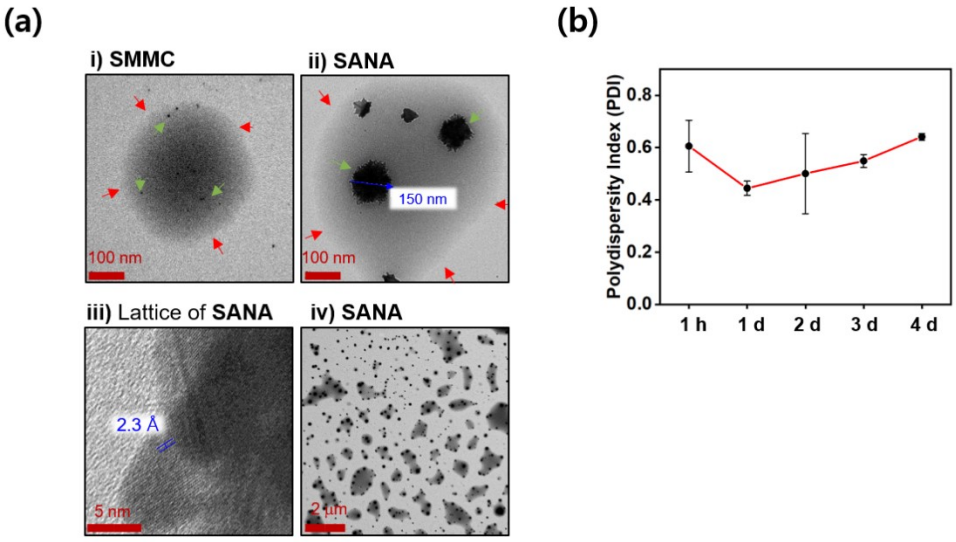

Figure S2. (a) TEM images coated by 1% (w/v) phosphotungstic acid (PTA) solution. i) SMMC. ii) Small-scale view of **SANA**. iii) Lattice of **SANA**. iv) Low magnification view of **SANA**. Red-color arrow: organic region. Green-color arrow: Au cluster region. (b) The polydispersity index (PDI) of **SANA** for 4 days in DI. H_2O. The error bar represents the mean ± S.D.

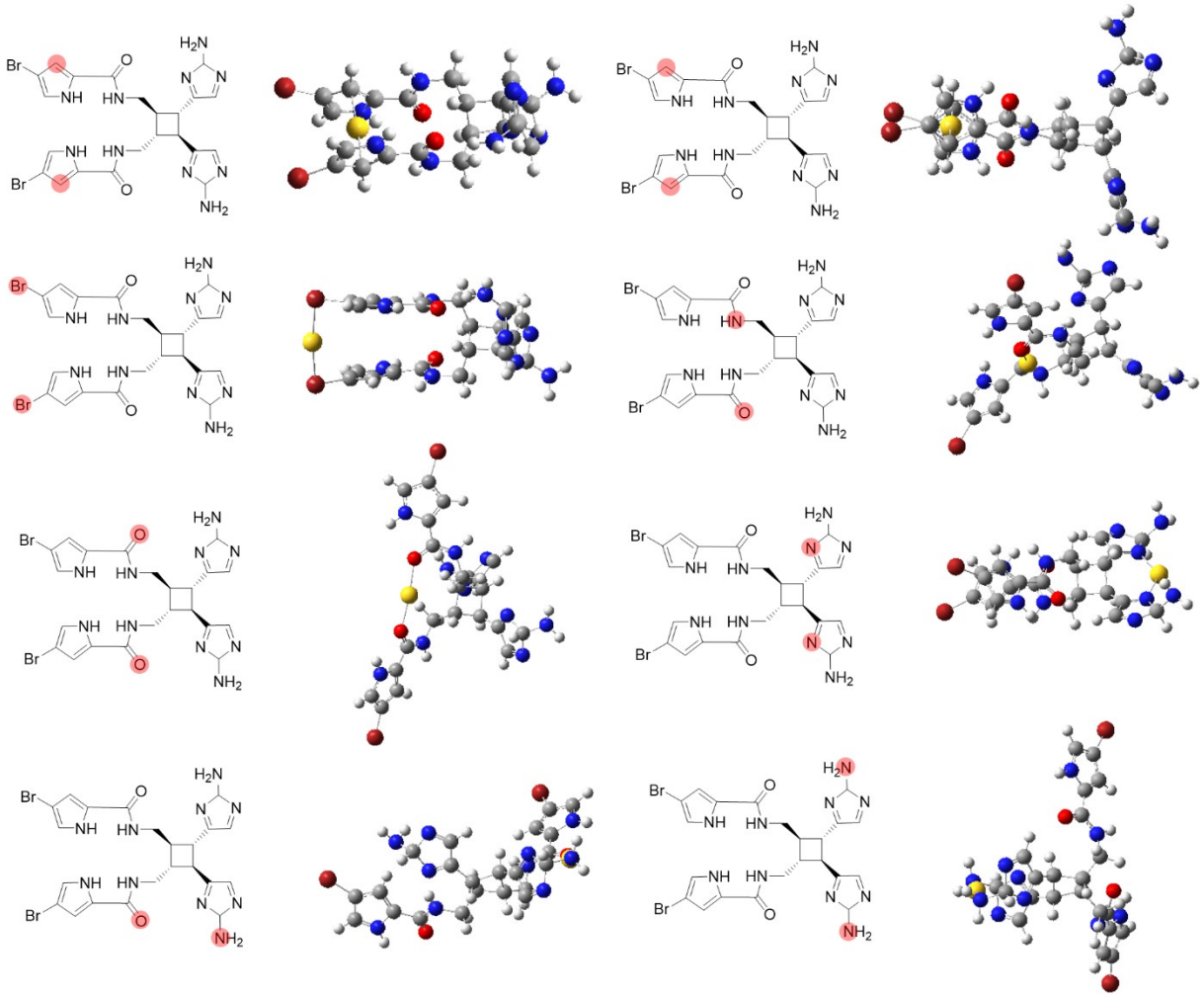

Figure S3. DFT-optimized geometries of Au-Sceptrin complexes. Coordinated atoms toward Au ion (Au^{3+}) are indicated by a red circle.

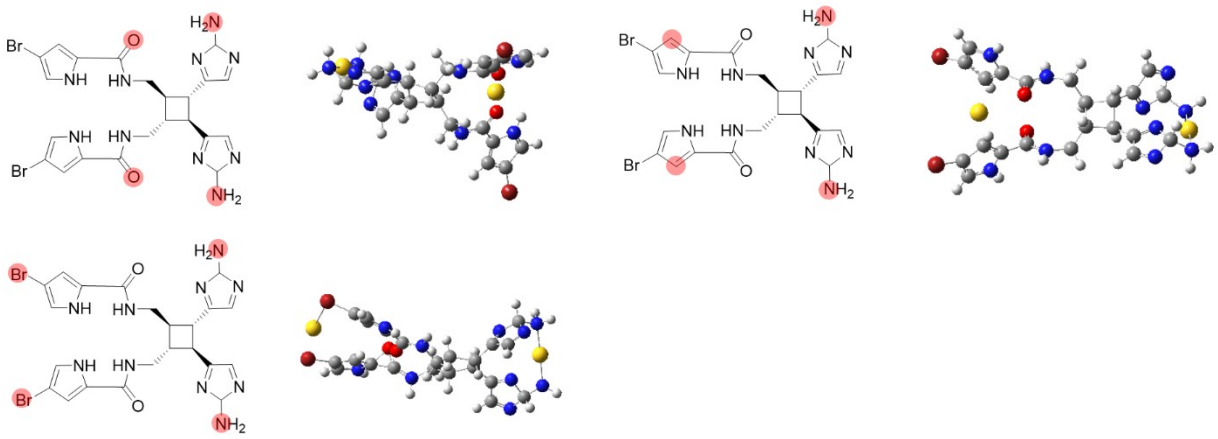

Figure S4. DFT-optimized geometries of 2Au-Sceptrin complexes. Coordinated atoms toward Au ion (Au^{3+}) are indicated by a red circle.

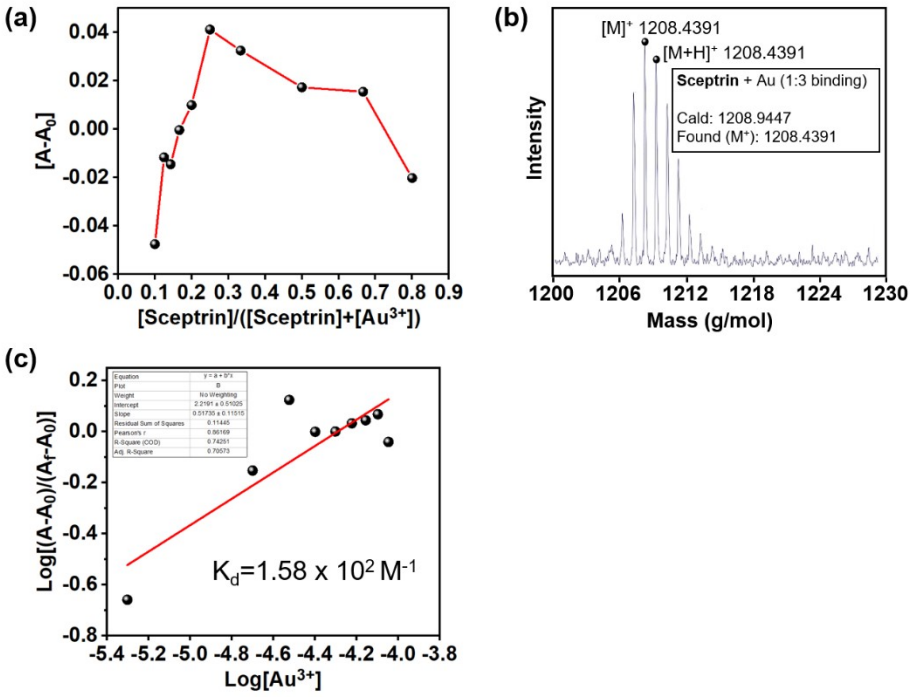

Figure S5. (a) Job-plot analysis. A: intermediate absorption value, A_0: absorption value (at 268 nm) in the absence of Au^{3+}. (b) MALDI-TOF/MS analysis of the complex of sceptrin and Au^{3+}. Matrix: 10 mg/mL of 2,5-Dihydroxybenzoic acid (DHB) in MeOH. Ratio (Matrix/**SANA**) was 5:1 in MeOH. (c) Benesi-Hildebrand relation plot. A: absorption value (at 600 nm) at the intermediate, A_0: absorption value (at 600 nm) in the absence of Au^{3+}, A_f: absorption value (at 600 nm) at the saturation point of sceptrin with Au^{3+}. The measurement was conducted after 1 h incubation at 37 °C.

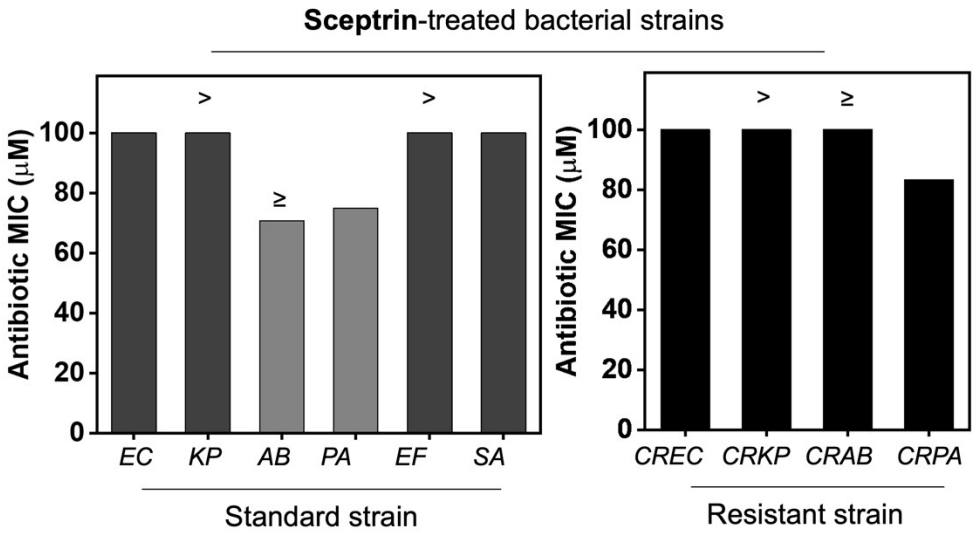

Figure S6. Minimum inhibitory concentration (MIC) assay results of sceptrin against 10 types of bacterial strains (EC, KP, AB, PA, EF, SA, CREC, CRKP, CRAB, and CRPA). Sceptrin was serially diluted two-fold in a 96-well round-bottom microplate at different concentrations ranging from 0.39 μM to 200 μM. Tests were conducted in quadruplicate to obtain the measurements. Incubation time: 24 h.

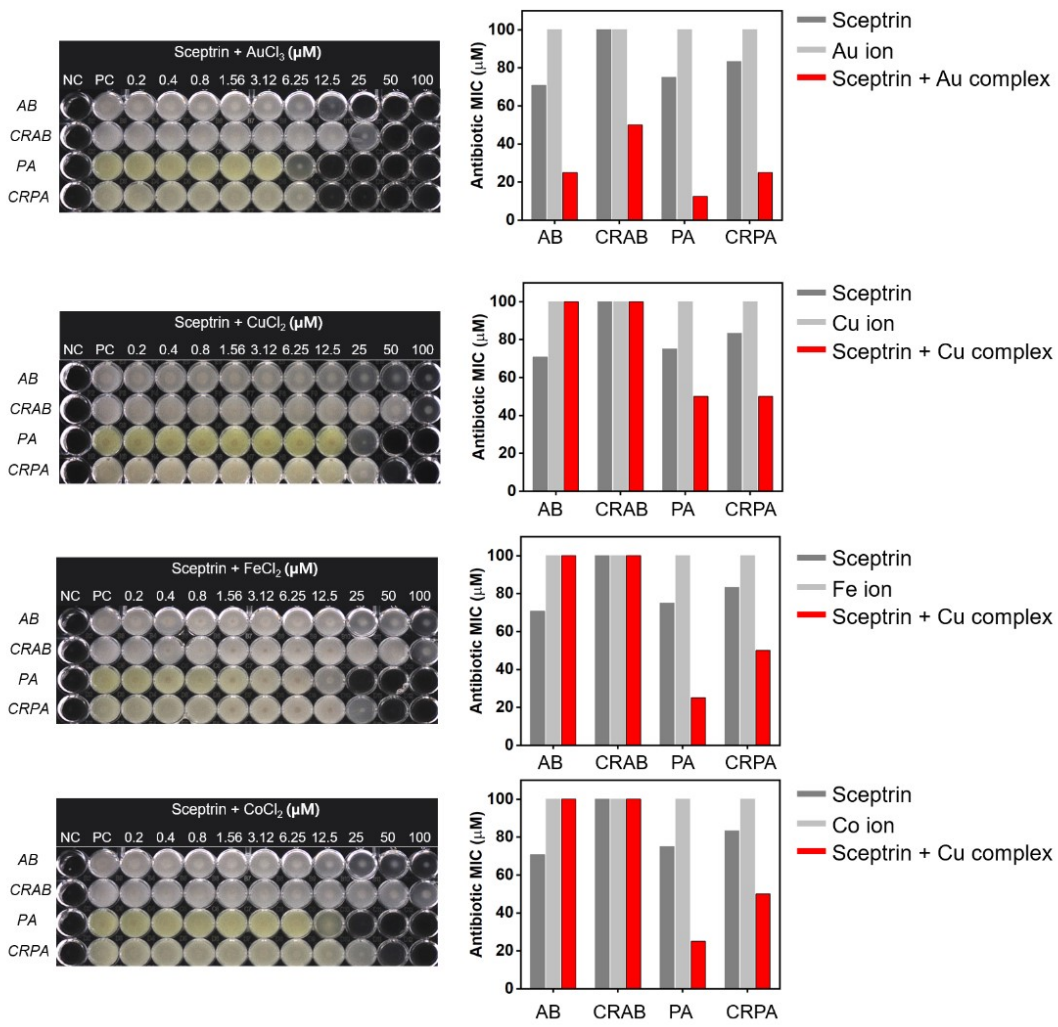

Figure S7. Minimum inhibitory concentration (MIC) assay results of metal ion ($AuCl_3$, $CuCl_2$, $FeCl_2$, and $CoCl_2$), sceptrin, and sceptrin + metal ion complex. Left: Photos of the 96-well round bottom microplate. Right: MIC assay results of metal ion, sceptrin, and sceptrin + metal ion against 4 types of bacterial strains (AB, CRAB, PA, and CRPA). The metal ion, sceptrin, and sceptrin + metal ion complex was serially diluted (1/2×) in a 96-well round-bottom microplate at different concentrations ranging from 0.39 μM to 200 μM. The measurement was conducted with an independent quadruple test. Incubation time: 24 h.

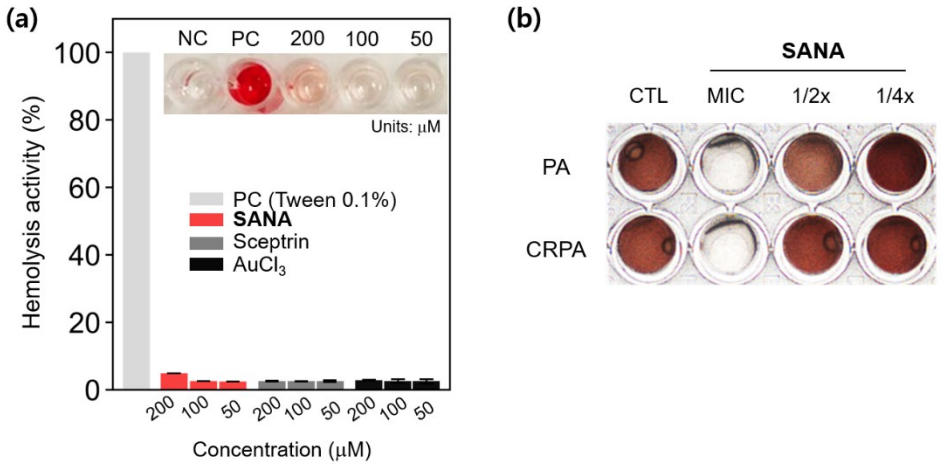

Figure S8. (a) Hemolysis assay of $AuCl_3$ (50, 100, and 200 μM), sceptrin (50, 100, and 200 μM), and **SANA** (50, 100, and 200 μM). Y-axis: hemolysis activity. The activity was measured by absorbance of solution at 492 nm. Inset photo: 8% (*v/v*, 1× PBS) red blood cells (RBCs) on 96-well plate after treatment of PC (0.1% Tween), **SANA** (50, 100, and 200 μM). The measurement was conducted with an independent triplicate. (b) Photo of blood containing RBCs after treatment of CTL (DI H_2O) and **SANA** (1/4×, 1/2×, and 1× MIC). MIC: 12.5 μM. NC: negative control. PC: positive control. CTL: control. Incubation time: 4 h.

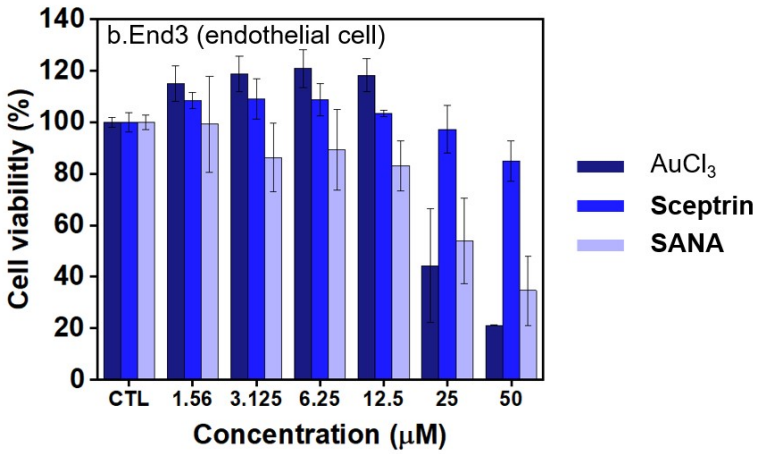

Figure S9. Cytotoxicity assay of $AuCl_3$, sceptrin, and **SANA** in b.End3 (Endothelial cell). Incubation time: 24 h (37 ˚C). CCK-8 assay kit was used to confirm the cytotoxicity. The error bar represents mean ± S.D. (standard deviation) with a two-way ANOVA test.

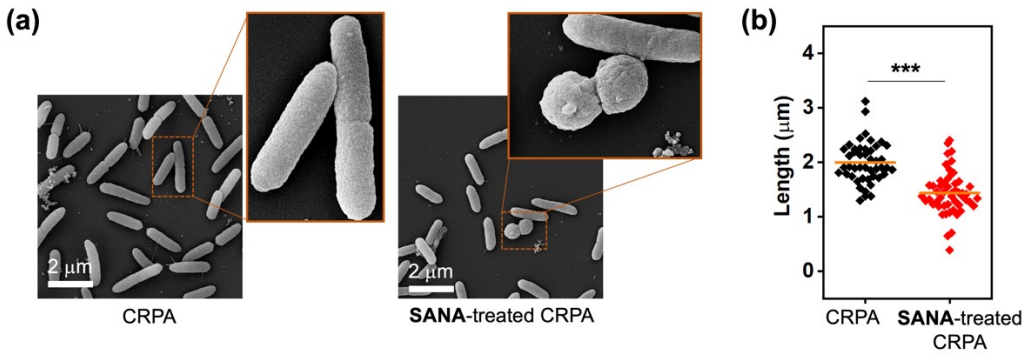

Figure S10. Scanning electron microscope (SEM; MERLIN, Carl-Zeiss, Germany) image analysis of CRPA strains after treatment of **SANA** (1× MIC) after 6 h incubation at 37 °C. Accelerating voltage: 10 kV, I probe: 190 pA. (a) SEM images. Scale bar = 2 μm. Inset figure: ROI-enlarged images. (b) Plotting for length (μm) of CRPA (n = 52) with/without treatment of **SANA**. Differentiating cells were not counted. The orange line is the average value. The data were evaluated for statistical significance using an unpaired t-test (***$p < 0.001$).

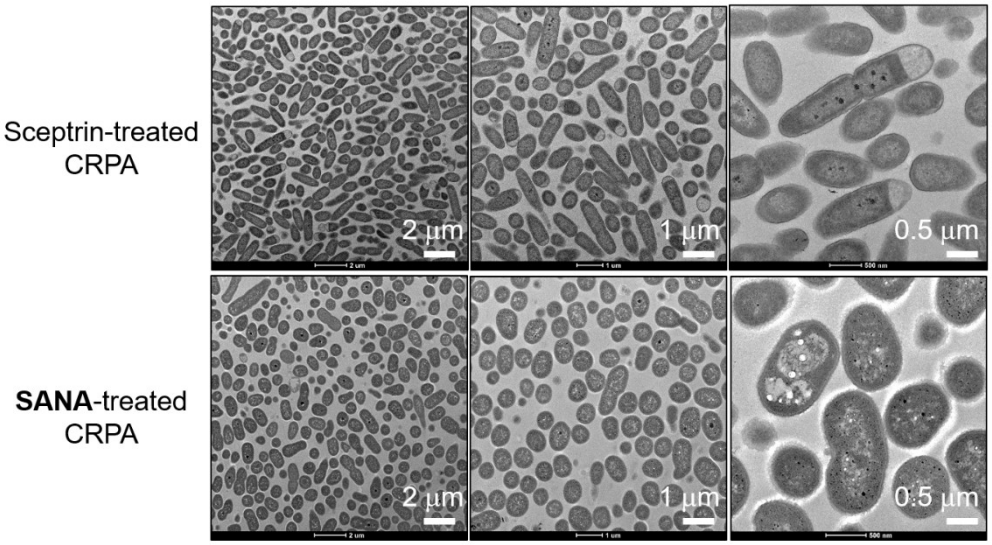

Figure S11. TEM images of CRPA strains after treatment of sceptrin (1× MIC) and **SANA** (1× MIC) after 6 h incubation at 37 °C. Culture media: tryptic soy broth (TSB; BD Difco, Product No. 211825, Franklin Lakes, NJ, USA).

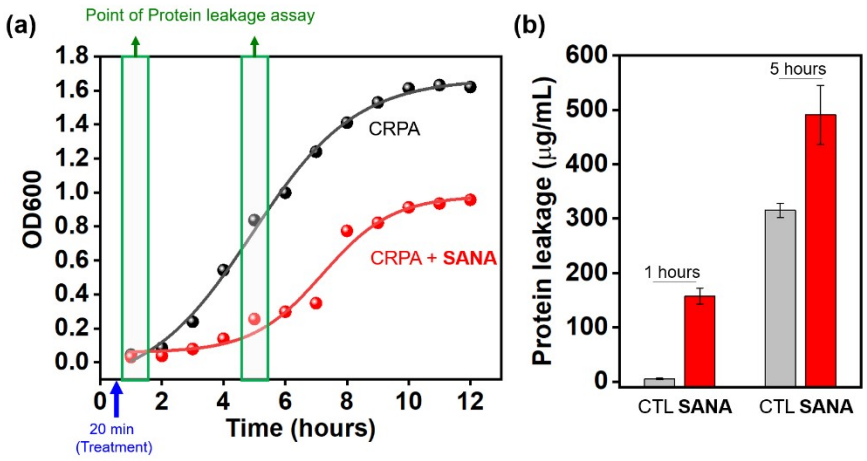

Figure S12. (a) Bacterial growth curve (CRPA) with/without treatment of **SANA** (1/2× MIC). The time point for the treatment of **SANA** is marked by a blue arrow. (b) Protein leakage assay at the time point of CRPA after 1% (v/v) inoculation. The time point is based on the green region of panel (a).

Supporting Tables

Table S1. Minimum inhibitory concentration (MIC) values. (a) the inhibitory concentration of biofilm formation against Acinetobacter baumannii (AB), Carbapenem-resistant Acinetobacter baumannii (CRAB), Pseudomonas aeruginosa (PA), and Carbapenem-resistant Pseudomonas aeruginosa (CRPA). The above tests were performed using (b) metal ion, sceptrin, and (c) sceptrin + metal complex. The biofilm inhibition rate (compare with non-treated control group) was calculated using the biofilm biomass in bacteria treated with the compounds. Control (untreated strain): biofilm biomass in untreated bacteria. Units: μM. The MIC values refer to the Clinical and Laboratory Standards Institute, 2016 (CLSI) guidelines.

Units: μM	**AB**[a]	**CRAB**[a]	**PA**[a]	**CRPA**[a]
Sceptrin (only)	70.8	>100	75	80.3
[b]$AuCl_3$ (only)	>100	>100	100	100
[c]Sceptrin + $AuCl_3$ (complex; **SANA**)	25	50	12.5 ([a]12.4)	25 ([a]12.4)
[b]$CuCl_2$ (only)	>100	>100	100	100
[c]Sceptrin + $CuCl_2$ (complex)	>100	>100	50	50
[b]$CoCl_2$ (only)	>100	>100	100	100
[c]Sceptrin + $CoCl_2$ (complex)	>100	>100	25	50
[b]$FeCl_2$ (only)	>100	>100	100	100
[c]Sceptrin + $FeCl_2$ (complex)	>100	>100	25	50

Table S2. Primers' sets for qRT-PCR.

Strain	Targets	Sequence (5' → 3')
Pseudomonas aeruginosa	*algD*	Forward: CAT CTT TGG TTT GGG CTA TGT
		Reverse: AGA TGA ACG ATA CGT CGG AGT
	mreB	Forward: GCC AAT ACC CTT ATT TAT GTG C
		Reverse: CTC GTG AAC CTT GTT GAT GAA G
	rhlR	Forward: GCT TCG ATT ACT ACG CCT ATG G
		Reverse: CGT AGT TCT GCA TCT GGT ATC G
	16S rRNA	Forward: GAG TTT GAT CMT GGC TCA G
		Reverse: WTT ACC GCG GCT GCT GG

References

1 Y. Yang, M. N. Weaver and K. M. Merz, Jr., *J Phys Chem A*, 2009, **113**, 9843-9851.