Supporting Information

Ruthenium based antimicrobial theranostics – using nanoscopy to identify therapeutic targets and resistance nechanisms in Staphylococcus aureus

Kirsty L. Smitten,*[a,b] Simon D. Fairbanks,[a] Craig C Robertson,[a] Jorge Bernardino de la Serna,[c,d] Simon J. Foster,[b] Jim A. Thomas*[a]

[a]Department of Chemistry, University of Sheffield, Sheffield S10 2TN, U.K.

[b]Florey Institute and Department of Molecular Biology and Biotechnology, University of Sheffield S10 2TN, U.K.

[c]National Heart and Lung Institute, Faculty of Medicine, Imperial College London, South Kensington Campus, London SW7 2AZ, U.K..

[d] Research Complex at Harwell, Rutherford Appleton Laboratory, Central Laser Facility, United Kingdom Research and Innovation, OX11 0FA, U.K

TABLE OF CONTENTS

S1. Synthesis and Chemical Methods
S1a. [Ru(5-methyl-1,10-phenanthroline)$_2$Cl]
S1b. [{Ru(5-methyl-1,10-phenanthroline)$_2$}(tpphz)][PF$_6$]$_4$
S1b. Anion metathesis
S1c. Determination of LogP

S2. Microbiological and Microscopy Methods
S2a. Bacterial strain information and general growth procedures
S2b. Determination of Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs)
S2c. Cytotoxicity MTT assay
S2d. IC$_{50}$ values
S2e. Time-kill assays
S2f. Uptake of 4^{4+} by *E. coli* and determination of cellular ruthenium and iron levels
S2g. Membrane damage ATP release assays
S2h. Single stain microscopy
S2i. NHS ester-405 counterstain microscopy
S2j. NHS ester-488 counterstain microscopy
S2k. DAPI counterstain microscopy
S2l. HADA counterstain microscopy
S2m. Transmission electron microscopy
S2n. BacLight^{TM} membrane polarisation assay
S2o. X-ray crystallography

S3. Supplementary Data
S3a. Summary of crystallographic data and structure refinement
S3b. ORTEP plot of the X-ray crystallographic structure of [{Ru(TMP)$_2$}$_2$(tpphz)]$^{4+}$
S3c. Comparison of LogP and MIC
S3d. *S. aureus* growth curves with and without DMSO
S3e. Time-kill kinetic assays
S3f. Uptake experiment – initial rates of uptake comparison
S3g. Uptake experiment CFU/mL count and time-kill kinetics
S3h. DNA damage assay
S3i. ATP release assay
S3j. NHS-ester 405 co-staining experiment
S3k. NHS-ester 488 co-staining experiment
S3l. HADA Pearson's colocalisation constants

S4. Instrumentation
S4a. Microscopy
S4b. Photochemistry
S4c. Mass Spectrometry
S4d. NMR Spectroscopy
S4e. Electron Microscopy
S4f. Flow cytometer
S4g. X-ray crystallography

S1. Chemistry Methods

Complexes **1-4**$^{4+}$ were synthesised by methods previously described by the Thomas group.[1]

S1a. [Ru(5-methyl-1,10-phenanthroline)$_2$Cl$_2$]

$RuCl_3.3H_2O$ (0.67 g, 2.56 mmol), LiCL (0.67 g, 15.95 mmol) and 5-methyl-1,10-phenanthroline (1 g, 5.15 mmol) were heated in DMF (8 mL) for 8 hours under reflux. The reaction mixture was cooled to room temperature and acetone (100 mL) added, this was stored at 4°C for 16 hours. The dark purple precipitate was washed with water and ethanol and dried *in vacuo*. Mass = 0.97 g (1.75 mmol, 68.1 % yield). ES-MS m/z (%): 542.9 (60) $[M-Cl]^+$, 552.9 (100) $[M-Cl]^+$+CO.

S1b. [{Ru(5mp)$_2$}$_2$(tpphz)][PF_6]$_4^{4+}$

[Ru(5mp)$_2$Cl$_2$] (0.9 g, 1.67mmol) and tpphz (0.263 g, 0.68 mmol) were added to a 1:1 solution of ethanol and water (50 mL). The solution was heated at reflux for 12 hours under nitrogen. After completion the reaction mixture was cooled to room temperature and stored at 4 °C for 16 hours. The red solution was filtered and the ethanol removed by rotary evaporation. A saturating amount of NH_4PF_6 was added; this caused the formation of a dark red precipitate. The precipitate was collected by filtration, washed with water and recrystallized in acetonitrile by addition of diethyl ether. The product was dried *in vacuo* and purified on an alumina column, using the following solvent system: was 95% MeCN, 3% dH_2O and 2% KNO_3. Mass 0.91 g (0.47 mmol, 68.7 % yield). ^{1}H NMR (MeCN-d_6) δ (splitting integration): 2.56 (s, 2H), 7.38 (m, 16H), 8.04 (dd, 4H), 8.12 (dd, 4H), 8.50 (dd, 4H), 8.91 (m, 12H). ES-MS; m/z (%): 827 (25) $[M-2PF_6]^{2+}$, 503 (15) $[M- 3PF_6]^{3+}$, 342 (18) $[M-4PF_6]^{4+}$. Accurate mass analysis $C_{76}H_{52}N_{14}[^{102}Ru]^{24+}$ Calculated 340.8711. Found 340.8714. Elemental analysis for [{Ru(5mp)$_2$}$_2$(tpphz)](PF$_6$)$_4$.4H$_2$O, $C_{76}H_{60}F_{24}N_{14}O_4P_4Ru_2$: Calcd C; 45.27, H; 3.00, N; 9.73. Found: C; 45.45, H; 3.16, N; 9.21.

S1c. Anion metathesis

The hexafluorophosphate salt of each complex was dissolved in the minimum volume of acetone and a saturated solution of tetrabutylammonium chloride in acetone added. The resultant precipitated chloride salt was collected by filtration, washed with cold acetone and dried in vacuo.

S1d. Determination of LogP

Relative lipophilicities or partition coefficients of **1**$^{4+}$ - **5**$^{4+}$ across l-octanol and H_2O (log$P_{oct/wat}$) were determined via the shake-flask method.[5]Aqueous stocks of **1**$^{4+}$ - **5**$^{4+}$ were prepared at 200 and 300 μg mL^{-1} were added to equal volumes of 1-octanol that had been pre-saturated with H_2O for 36 h. After incubation with shaking at 37 °C for 24 h, the 1-octanol and aqueous phases were recovered and the relative distribution of each compound was determined by UV-vis absorbance spectroscopy at 430 – 455 nm.

S2. Microbiology and Microscopy Methods

S2a. Bacterial strain information and general growth procedures

Microbiological studies were conducted with: (1) wild-type laboratory strain *Staphylococcus aureus* SH1000; (2) techoic acid and lipotechoic acid knockout strains dltA and tarO (3) mprF mutant strain. Bacteria were routinely grown under aseptic, aerobic conditions in autoclave-sterilised culture medium at 37 °C. Lysogeny Broth (LB) (Formedium), Tryptic Soy Broth (TSB) (Sigma-Aldrich) and Chemically Defined Minimal Medium (CDM). CDM solution 1: $Na_2HPO_4.2H_2O$ (7 g), KH_2PO_4 (3 g), L-Aspartic acid (0.15 g), L-Alanine (0.1 g), L-Arginine (0.1 g), L-Cysteine (0.05 g), Glycine (0.1 g), L-Glutamic Acid (0.15 g), L-Histidine (0.1 g), L-Isoleucine (0.15 g), L-Lysine (0.1 g), L-Leucine (0.1 g), L-Methionine (0.1 g), L-Phenylalanine (0.1 g), L-Proline (0.15 g), L-Serine (0.1 g), L-Threonine (0.15 g), L-Tryptophan (0.1 g), L-Tyrosine (0.1 g) and L-Valine (0.15 g), dissolved in 700 mL of distilled water. CDM solution 2 (makes 1000x working concentration): Biotin (0.02 g), Nicotinic acid (0.4 g), D-Pantothenic acid (0.4 g), Thiamine HCl (0.4 g), Pyridoxal HCl (0.8 g), Pyridoxamine di-HCl (0.8

g), Riboflavin (0.4 g) dissolved in 140 mL of distilled water. CDM solution 3: Adenine sulphate (0.02 g), Guanine HCl (0.02 g) dissolved in 50 mL of 0.1 M HCl. CDM solution 4 $CaCl_2.6H_2O$ (0.01 g) and $(NH_4)_2Fe(SO_4)_2.6H_2O$ (0.006 g) dissolved in 50 mL of 0.1 M HCl. CDM solution 5: Glucose (10 g) and $MgSO_4.7H_2O$ (0.5 g) dissolved in 100 mL of distilled water. CDM media (1L): solution 1 (700 mL), 1x solution 2 (100 mL), solution 3 (50 mL), solution 4 (10 mL) and solution 5 (100 mL). The pH was adjusted to 7.4 by the addition of NaOH. All media was sterilised in an autoclave.

Prior to experiments, bacterial starter cultures were prepared by inoculating TSB with a single colony of bacteria and then grown for overnight at 37 °C with shaking for 16 – 18 h. Starter cultures were washed once and resuspended in the appropriate growth medium for each experiment. For short-term storage, bacterial stocks were maintained on nutrient agar plates at 4 °C for 2 – 3 weeks. For long-term storage, strains were stored as cell suspensions in 30 % (v/v) LB 70 % (w/v) glycerol at – 70 °C.

S2b. Determination of Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs)

MICs and MBCs of $\mathbf{1}^{4+}$ - $\mathbf{5}^{4+}$ and oxacillin (control) were determined via the standard broth-dilution method in 96-well microtitre plates in either TSB or CDM. The MIC of each compound was evaluated using 2-fold increasing concentrations of each compound between 2 - 512 μg mL^{-1} against a bacterial inoculum of 10^7 – 10^9 colony forming units per mL (CFU mL^{-1}), corresponding to an optical density at 600 nm (OD_{600}) of 0.05 – 0.075. Plates were incubated at 37 °C for 20 h. After this time, the level of turbidity in each well was used to determine the extent bacterial cell growth in the presence of the compounds. The minimal concentration of compound that did not permit bacterial growth was determined to be the MIC. For MBC determination, 10 μL samples of each well were then transferred to nutrient agar plates and further incubated at 37 °C. The lowest concentration of compound in which no CFU were observed after plating was determined to be the MBC. MIC/MBCs were determined from 3 independent biological repeats.

S2c. Cytotoxicity MTT assays

Yellow MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) is reduced to a purple formazan product in the mitochondria of living cells by reductase enzymes. The reduction of MTT can only occur within active mitochondria, therefore the amount of formazan produced is directly related to the number of viable cells in the sample. In this experiment the amount of formazan was quantified by absorbance between 500-600 nm, the toxicity of the compound was deduced by comparison of formazan content for cells exposed to the compound and those in the untreated control.

S2d. IC_{50} values

Cell cultures were grown on 24 or 48 well plates and allowed to grow for 24 hours. Cell cultures were then treated with solutions of the Ru(II) complex at various concentrations (10% PBS: 90% medium) for the given incubation time in triplicate. Solutions were removed and the cells incubated with 0.5 mg ml-1 MTT dissolved in PBS for 30-40 minutes. The formazan product was eluted using 200 μl/well of acidified propan-2-ol, 150 μl of this was transferred to a 96 well plate and the absorbance quantified by spectrophotometer (540 nm, referenced at 640 nm). An average absorbance for each concentration was obtained and cell viability was determined as a percentage of untreated negative control wells (10% PBS: 90% medium). Using Sigmaplot 11.0 software, a 3 parameter sigmoidal curve was used to fit each data set ($R2 > 0.97$ for each fit) and the IC50 value (the concentration corresponding to 50% viability) calculated by interpolation.

S2e. Time-kill assays

SH1000 was grown in TSB to early exponential phase, at OD: 0.3-0.4 in three separate cell suspensions, oxacillin, $\mathbf{4}^{4+}$ and $\mathbf{5}^{4+}$ were added at 5 x MIC. Immediately prior to compound addition, and up to 180 minutes thereafter, culture growth (turbidity at OD_{600}) and viability (CFU mL^{-1}) were measured. Cell viability was determined via the standard viable counts method, which is based on the ability of a single viable bacterial cell to form a colony when grown on agar plates. 10-fold serial dilutions of cell culture samples were prepared in sterile PBS and then 3 x 10 μL of each dilution was spotted onto nutrient agar plates. Plates were then incubated overnight at 37 °C to permit colony growth and cell viability was determined as the average number of counted CFU mL^{-1}. Time-kill assays were performed as three independent biological repeats.

S2f. Uptake of **4**$_{4+}$ by *S. aureus* and determination of cellular ruthenium and iron levels

Uptake and cellular accumulation of **4**$_{4+}$ by *S. aureus* SH1000 was determined by measuring bacterial cell metal content by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) as follows. *S. aureus* SH1000 cultures were grown to mid-exponential phase in LB broth, washed in PBS and then resuspended in PBS +/- 20 mM glucose to approximately 10^8-10^9 CFU mL^{-1}. **4**$_{4+}$ was added to cells at MIC concentration and then 20 mL samples of culture were harvested at 5, 10, 20 and 60 min after **4**$_{4+}$ addition. Samples of non-**4**$_{4+}$ treated cells were also taken for comparison. Samples were centrifuged at 5,000 x *g* for 20 min at 4 °C to obtain cell pellets and the supernatant containing unbound extracellular **4**$_{4+}$ was discarded. The resultant cell pellets were then washed twice in 0.5 % (v/v) Aristar nitric acid to remove loosely bound residual **4**$_{4+}$. To prepare cell material for ICP-AES, cell pellets were resuspended in 0.5 mL Aristar nitric acid (69 % (w/v)) and then placed in a sonicator bath for 30 min to completely dissolve cells. The resulting digest was then diluted to a final volume of 5 mL with diluted nitric acid and then samples were analysed on a Spectro CirosCCD (Spectro Analysis) Inductively-Coupled Plasma-Atomic Emission Spectrophotometer. Levels of Ru and Fe in the samples were determined by a calibration curve using multi-element standard solutions containing 0.1, 0.2, 5 and 10 mg L^{-1} Ru and Fe.

S2g. Membrane damage ATP release assays

Loss of *S. aureus* membrane integrity following **4**$_{4+}$ exposure was determined by leakage of ATP, an intracellular marker, from *S. aureus* cells.[2] Levels of extracellular ATP of *S. aureus* grown with or without **4**$_{4+}$ were determined as follows. *S. aureus* SH1000 cells were grown to mid-exponential phase in TSB and then **4**$_{4+}$ (0 – 48 μM) was added to cultures. Immediately prior to, and at time-intervals after **4**$_{4+}$ addition, samples of cell culture were harvested by centrifugation at 15, 000 x *g* for 5 min at 4 °C to remove cells. Supernatants were recovered and retained at – 20 °C for ATP analysis. ATP analysis was conducted using the bioluminescence-based Molecular Probes' ATP Determination kit (Invitrogen, Fisher Scientific). The levels of ATP in supernatants were derived via a standard curve of ATP standards from 1 nM - 1 μM. Luminescence measurements of ATP standards and culture supernatants were measured in duplicate on a Lumat$_3$ Luminometer (Berthhold Technologies, UK).

S2h. Single stain microscopy

S2h(1). Preparing the culture

An overnight culture of bacteria (SH1000) was grown in TSB. The cells were washed (x2) and resuspended in CDM. The cells were diluted to OD_{600}-0.05 and grown to OD-0.3-0.4 in CD (50 mL). 1 mL of culture was harvested, and the compound added at MIC concentration. 1 mL cultures were then harvested at 5, 10, 20 and 60 minutes. The bacteria were pelleted (centrifuge, 14,000 RPM, 90 seconds), and supernatant removed. The pellet was suspended in fixant (16% paraformaldehyde in PBS, 500 μL) and Milli-Q water (500 μL) and placed on a rotary wheel at room temperature for 30 minutes. The samples were washed in PBS (x3) and water (x1).

S2h(1). Preparing the slides

Coverslips were sonicated in 1M KOH for 15 minutes, then coated in polylysine solution for 30 minutes. The pellets were suspended in 5 μL of a SlowFade Gold Antifade Mountant (Thermofisher). The suspension was mounted onto the slide and the coverslip placed on top. Slides were imaged using the structured illumination (SIM) microscope. Imaging was done using the 1514 immersion oil, and mol_probes microscope setting. A phase contrast image was taken (DIC) and the 450 nm laser used to excite the compound, luminescent images were collected in the A568 channel. OMX SI reconstruction was performed on images. Images were processed and analyzed using FIJI Image J software – the SIM plugin was used to perform a 16-bit conversion.

S2i. NHS ester-405 counterstain microscopy

Initial pellets were grown and fixed as before. After fixing the sample was washed with PBS. Cells were resuspended in PBS (250 μL), Cells were grown to an OD of 0.4 and then incubated with NHS-ester 405 (50 μg/mL) for 5 minutes on a rotary wheel at room temperatures. Cells were washed and fixed as before. Following fixing the pellet was resuspended in TSB containing **4**$_{4+}$ at MIC concentration and 1 mL cultures were harvested at 5, 10, 20 and 60 minutes. Cells were pelleted (14,000 RPM, 90 seconds) and washed with PBS (3 washes). Cell suspensions were mounted as previous. Laser 405 nm, emission filter DAPI. OMX SI reconstruction and OMX

SI image registration was performed on the images, and the images were processed and analysed using the FIJI Image J software.

S2j. NHS ester-488 counterstain microscopy

Initial pellets were grown and fixed as before. After fixing the sample was washed with PBS. Cells were resuspended in PBS (250 µL), NHS-ester 488 was added (50 µg/mL). Samples were incubated on a rotary wheel at room temperature for 5 minutes. Cells were pelleted (14,000 RPM, 90 seconds) and washed with PBS (3 washes). Cell suspensions were mounted as previous. Laser 488 nm, emission filter FITC. OMX SI reconstruction and OMX SI image registration was performed on the images, and the images were processed and analysed using the FIJI Image J software

S2k. DAPI counterstain microscopy

Initial pellets were grown and fixed as before. After fixing the sample was washed with PBS. Cells were suspended in 300 µL of DAPI (300 nM) in PBS. Samples were incubated on a rotary wheel at room temperature for 5 minutes. Cells were pelleted (14,000 RPM, 90 seconds) and washed with PBS (3 washes). Cell suspensions were mounted as previous. Laser 405 nm, emission filter DAPI. OMX SI reconstruction and OMX SI image registration was performed on the images, and the images were processed and analysed using the FIJI Image J software.

S2l. HADA counterstain microscopy

SH1000 cells were grown to an OD of 0.3-0.4. 1 mL samples were incubated with HADA (5 µL). Samples were incubated on a rotary wheel at room temperature for 5 minutes. Cells were pelleted (14,000 RPM, 90 seconds) and washed with PBS (3 washes). Cells were resuspended in dH_2O and incubated with TMP (24 µM) for 0, 5, 20 and 60 minutes. Cells were washed with PBS and mounted as previous. Laser 405 nm, emission filter DAPI. OMX SI reconstruction and OMX SI image registration was performed on the images, and the images were processed and analysed using the FIJI Image J software.

S2m. Transmission electron microscopy

Cell pellets up until fixation were prepared in the same way as the single-stain super-resolution microscopy samples. The cells were fixed using 3% glutaraldehyde. Cells were dehydrated using a series of ethanol washes (70 – 100% ethanol) and TEM samples sectioned in Araldite resin by microtome. Samples were examined on a FEI Tecnai instrument operating at 80 kV equipped with a Gatan 1 K CCD camera. Images were processed and analyzed using FIJI Image J software.

S2n. BacLight™ membrane polarisation assay

Membrane polarisation was studied using the Mol Probes BacLight™ polarisation kit.[10] Overnight cultures of SH1000 were grown as previously described. Cells were grown to the exponential phase (OD: 0.4) in CDM at 37.5 °C. One solution of bacteria was treated with $\mathbf{4}^{4+}$ (4 µM) and incubated at 37.5 °C for 60 minutes. The other solution was the untreated control. OD was measured and cells were diluted in PBS to a final concentrations of 10^6 CFU/mL. Four flow cytometry tubes were prepared to sample. A,B: 1 mL of the cells treated with $\mathbf{4}^{4+}$ and the unstained control were incubated with 10 µL of $DiOC_2(3)$ (3 mM) for 30 minutes in the dark in separate flow cytometry tubes. The membrane polarisation positive control tube was prepared with 10 µL of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (500 µM) 10 µL of $DiOC_2(3)$ (3 mM), the cells were incubated for 30 minutes in the dark before reading. In addition a fourth control was prepared in the absence of $DiOC_2(3)$ on $\mathbf{4}^{4+}$ stained cells. Readings were taken on an LSR II flow cytometer and data analysed using the FlowJo softwate.

S2o. X-ray crystallography

X-ray quality crystals of the [{Ru(TMP)$_2$}$_2$tpphz].4PF$_6$ were grown using vapor diffusion of a concentrated solution of the [{Ru(TMP)$_2$}$_2$tpphz].4PF$_6$ in pure nitromethane. Slow evaporation of the diethyl ether anti-solvent produced ruby red crystals suitable for structure determination. The intensity data was obtained on either a Bruker Kappa Apex-II CCD or Bruker Kappa Apex-II diffractometer operating with a MoKα sealed-tube X-ray source, at 110 K. The reflections were corrected for absorption via empirical methods (SADABS) based upon symmetry-equivalent reflections combined with measurements at varied azimuthal angles.[3,4] The crystal structure was solved and refined against F2 values using ShelXT for solution and ShelXL for refinement through the Olex2 program.[4-7] Non-hydrogen atoms were refined anisotropically and hydrogen atoms were placed in calculated geometries and refined utilizing a riding model and isotropic displacement parameters.

S3. Supplementary data

S3a. A summary of crystallographic data and structure refinement of [{Ru(TMP)$_2$}$_2$(tpphz)].4PF$_6$

Identification code	iaj675s_0m
Empirical formula	$C_{88}H_{76}F_{24}N_{14}P_4Ru_2$
Formula weight	2111.64
Temperature / K	110.0
Crystal system	Triclinic
Space group	P-1
a / Å	13.885(2)
b / Å	17.841(3)
c / Å	22.499(4)
α / °	83.990(2)
β / °	73.932(2)
γ / °	85.868(2)
Volume / Å^3	5321.1(16)
Z	2
ρ_{calc} / gcm^{-3}	1.318
μ / mm^{-1}	0.432
F(000)	2132.0
Crystal size / mm^3	0.34 × 0.12 × 0.08
Radiation	MoKα (λ = 0.71073)
2θ Range for Data Collection / °	3.696 to 55.362
Index Ranges	-18 ≤ h ≤ 18, -23 ≤ k ≤ 23, -29 ≤ l ≤ 29
Reflections Collected	90591
Independent Reflections	24621 [Rint = 0.0993, Rsigma = 0.1223]
Data / Restraints / Parameters	24621/0/1205
Goodness-of-fit on F$_2$	1.026
Final R indexes [I>=2σ (I)]	R1 = 0.0910, wR2 = 0.2262
Final R indexes [all data]	R1 = 0.1711, wR2 = 0.2806
Largest diff. peak/hole / e Å^{-3}	1.97/-1.07

S3b. ORTEP plot of the X-ray crystallographic structure of [{Ru(TMP)$_2$}$_2$(tpphz)].4PF$_6$

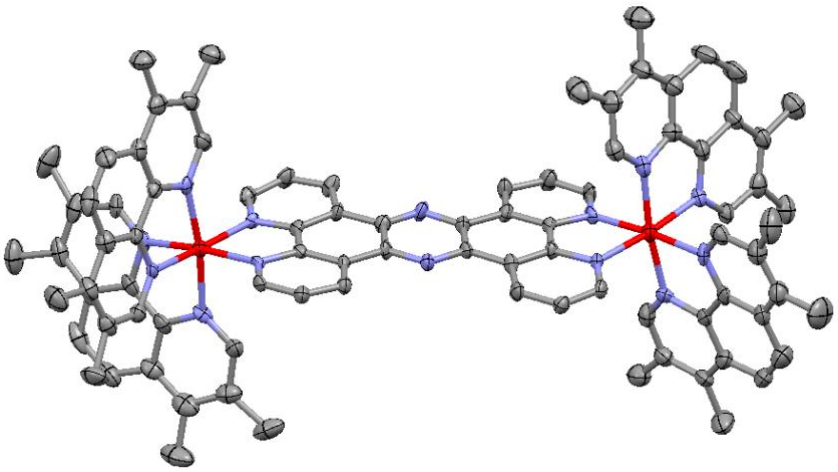

Figure 1| ORTEP plot of the X-ray crystallographic structure of [{Ru(TMP)$_2$}$_2$tpphz]. with thermal ellipsoids indicating 50% probability. The PF_6 Counter ions are omitted for clarity.

S3c. Comparison of LogP and MIC

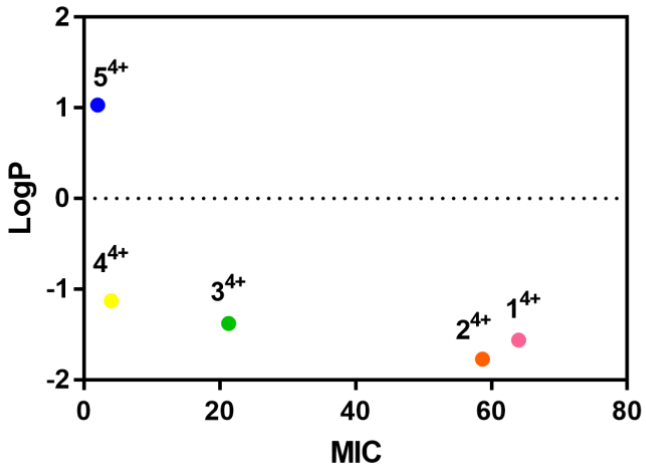

Figure 2| Comparison between the logP of complexes **1**$^{4+}$-**5**$^{4+}$ determined by the shake-flask method, and the MIC (µM) values determined in chemically defined media.

S3d. *S. aureus* growth curves with and without DMSO

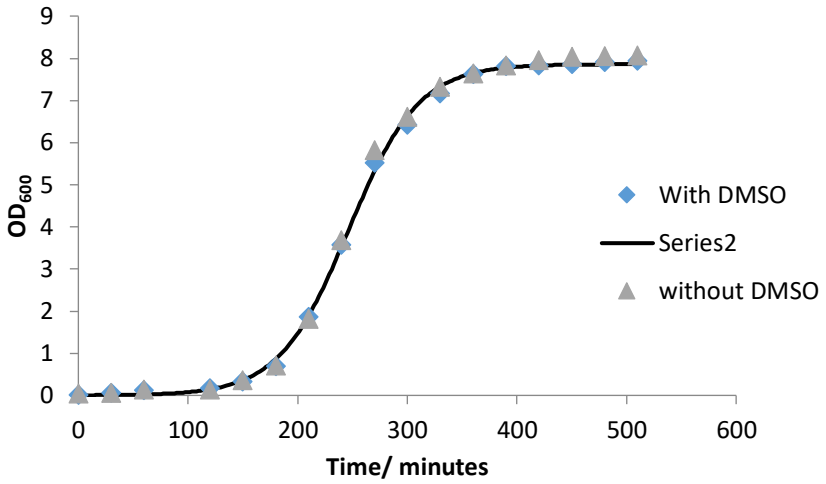

Figure 3| Growth of *S. aureus*, SH1000 in the absence (**grey**) and presence (**blue**) of 5 % dimethyl sulfoxide, DSMO. Growth curve was conducted in MH-II media incubated with shaking at 37.5 °C and bacterial cell proliferation was measured by OD_{600}.

S3e. Time-kinetic assays for **4**$^{4+}$, **5**$^{4+}$ and oxacillin with *S. aureus*, SH1000 cells.

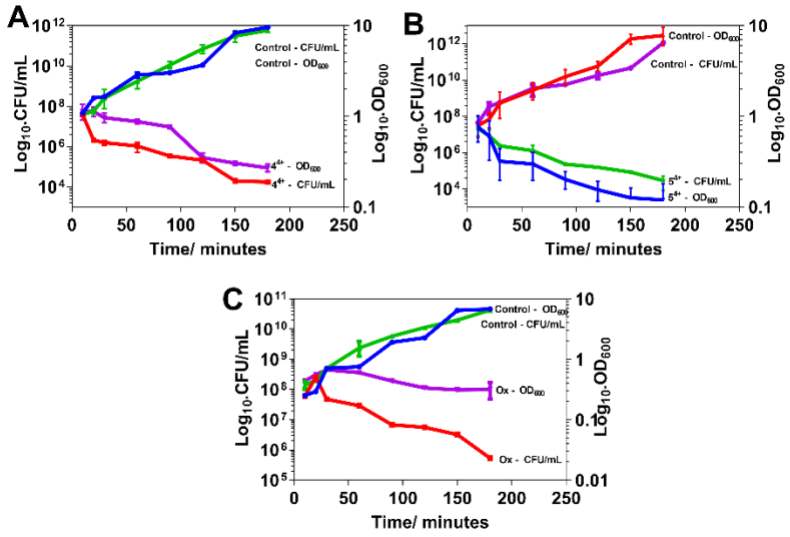

Figure 4| Time-kill kinetic assays**.** Dose-dependent killing of *S. aureus* SH1000 by **4**$^{4+}$(**A**), **5**$^{4+}$ (**B**) and oxacillin (**C**) *in vitro*. Each complex was added at 5 times the MIC in TSB. Killing was determined by monitoring the number of colony forming units (CFU) per mL at time intervals up to 6 h post treatment. Cell proliferation was determined by monitoring the optical density at 600 nm. Error bars represent three independent biological repeats ± standard deviation (SD).

S3f. Uptake experiment – initial rates of uptake comparison in the absence of glucose

Time/ min	Rate/ µM min^{-1}	P value	P significance
5	2.42 x 10^{-17}		
10	1.07 x 10^{-17}	0.0018	**
20	6.90 x 10^{-18}	<0.0001	****
60	2.98 x 10^{-18}	0.0014	**

Figure 5| Comparison between the rates of uptake (µM/min) of $\mathbf{4}_{4+}$by *S. aureus* SH1000 cells in the absence of glucose. Statistical t-tests were conducted to determine the significant difference relative to initial uptake at 5 minutes

S3g. Uptake experiment CFU/mL count and time-kill kinetics

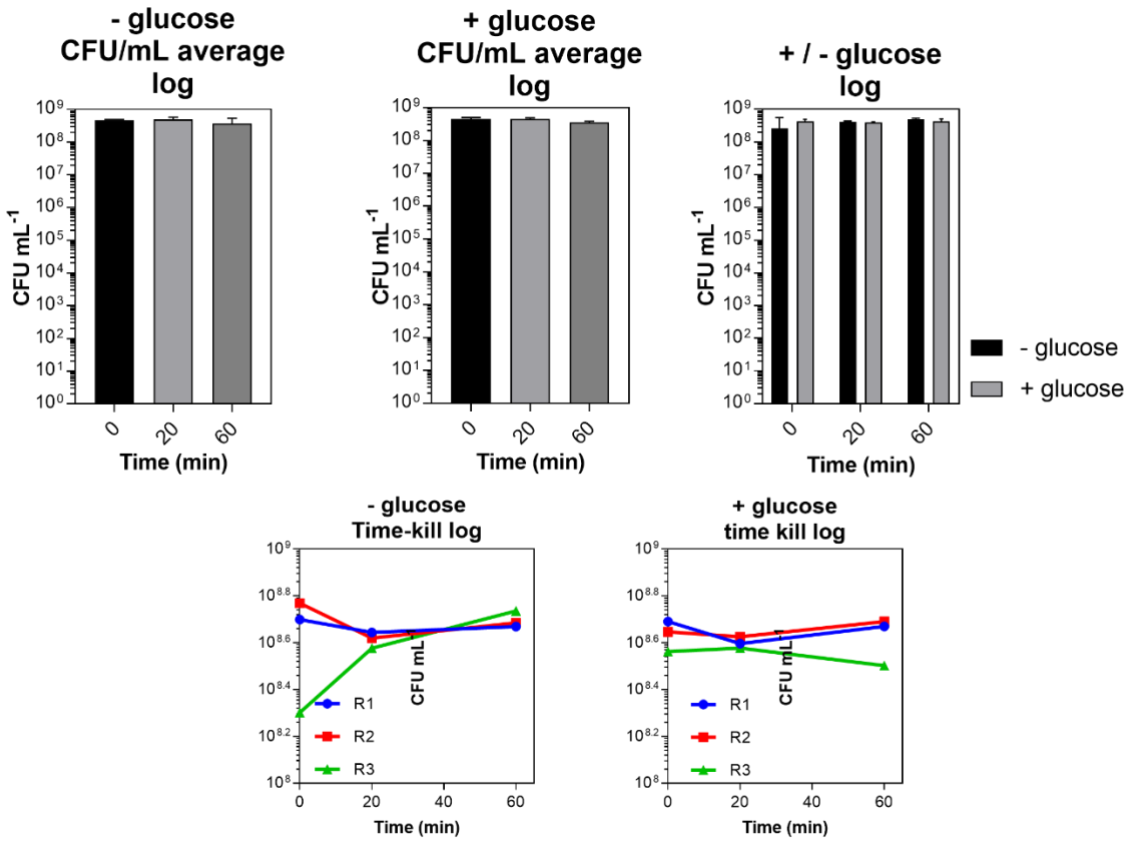

Figure 6| SH1000 CFU/mL counts from samples taken at 0,20 and 60 minutes in the presence and absence of glucose. Data is also shown as a time-kill kinetics curve.

S3h. DNA damage assay

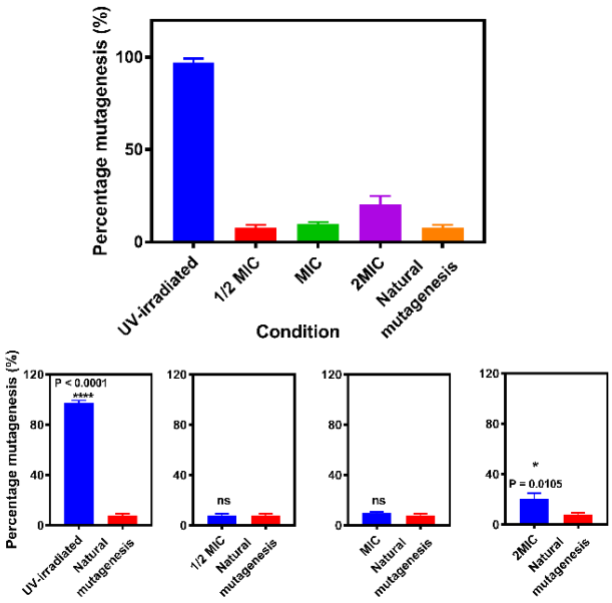

Figure 7. Ames test the fluctuation method. Percentage mutagenesis was measured by treated cells with $\mathbf{4}_{2+}$ (0-2MIC) and incubating with histidine, with UV-irradiated cells as a positive control, and natural mutagenesis as a negative control (A). T-tests were performed to determine the statistical difference between treated conditions and natural mutagenesis (B). Bromocresol purple indicator was added, and the percentage colour change from purple to yellow measured after incubation for 48 hours. A positive control with cells irradiated with UV-light is added for comparison.

S3i. ATP release assay

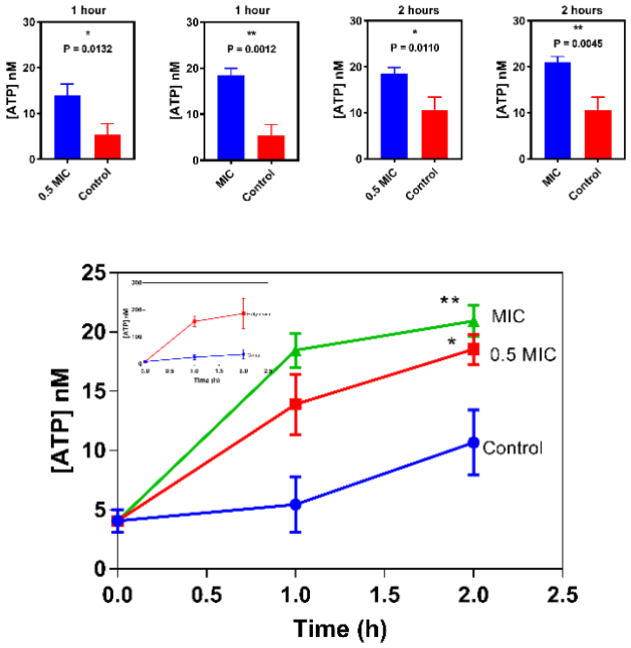

Figure 8| ATP release assay. Top: Determination of $\mathbf{4}_{4+}$-induced ATP released from *S. aureus* SH1000 cells with statistical difference t-test calculations. Bottom: Extracellular [ATP] (nM) quantified with recombinant luciferase and D-luciferin, with ATP released measured on a luminometer for samples exposed to 0 (control), 24, and 48 μM (MIC) of $\mathbf{4}_{4+}$ over a period of two hours. *-significant difference beween control and treated conidition. Error bars represent three biological repeats ± SD. Insert graph shows cells treated with 4 μg/mL of polymyxin

S3j. NHS-ester 405 co-staining experiment

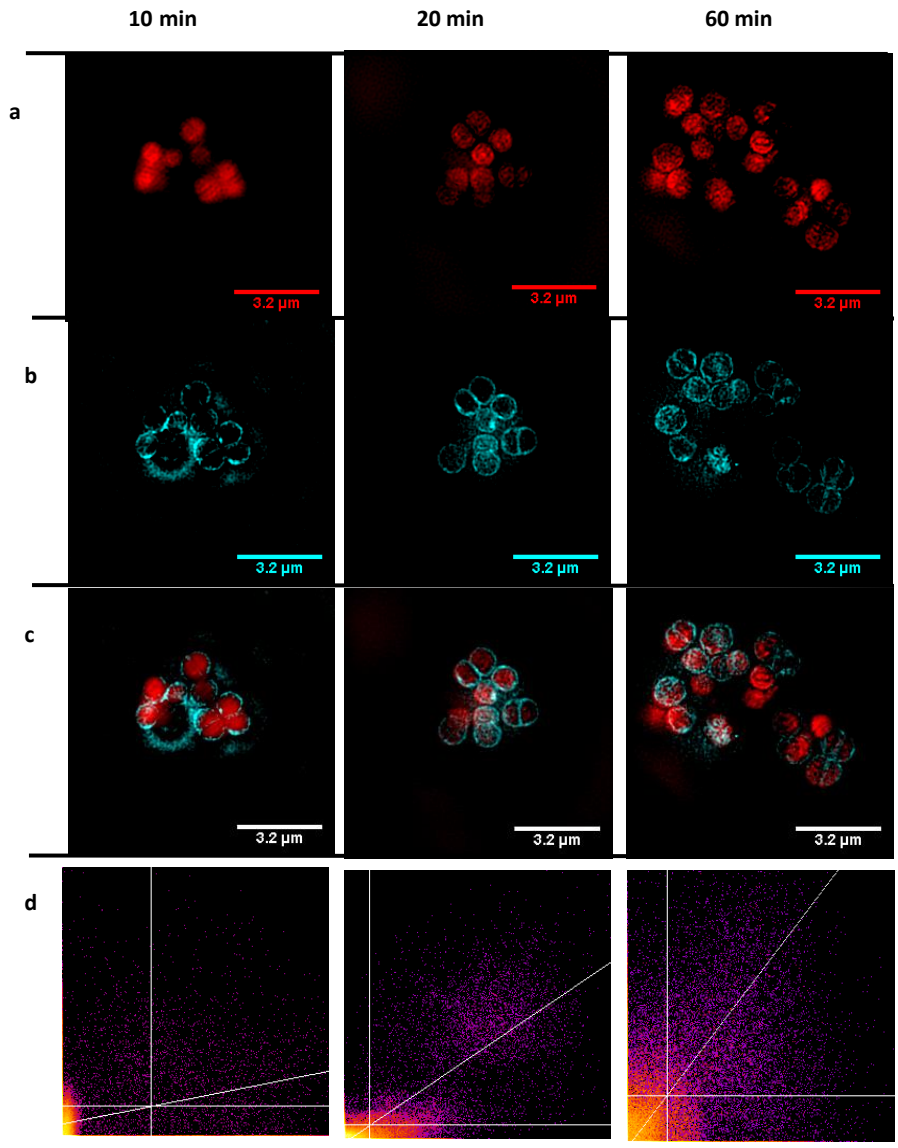

Figure 9| Super-resolution microscopy SIM - imaging. Localization of **4**$^{4+}$ and NHS-Ester 405 in *S. aureus* SH1000 cells visualized through SIM at 5 min, 20 min, and 60 min. Cells imaged using the emission of **4**$^{4+}$ on excitation at 450 nm using A568 filter (**a**), emission of NHS-Ester 405 using the DAPI filter (**b**), overlay image (**c**). Cells were incubated for 5-minutes with NHS-ester, washed with PBS and incubated with with 4 µM **4**$^{4+}$. Following this cells were washed with PBS before fixing with paraformaldehyde (16%). Colocalisation tests were conducted, scatterplots/cytofluorograms are shown (**d**) and Pearson's correlation constants Table 6| Pearson's colocalisation coefficients at each time point for NHS-ester and 4^{4+}.

Pearson's colocalisation coefficients at each time point for NHS-ester and 4^{4+}

Time/min	Pearson's colocalisation coefficient	Correlation
10	0.225	Small
20	0.514	Strong
60	0.426	Medium

S3k. NHS-ester 488 co-staining experiment

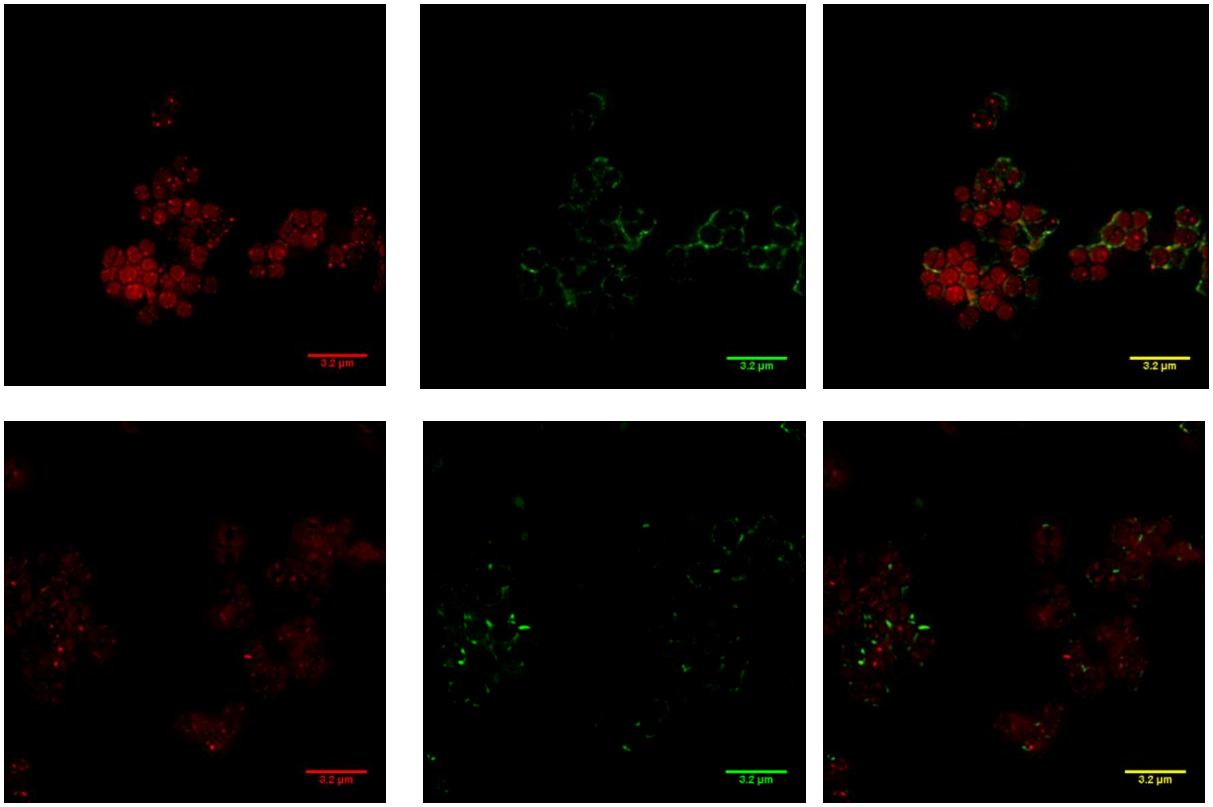

Figure 10| Super-resolution micrscopy SIM - imaging. Localization of **4**$^{4+}$ and NHS-Ester 488 in *S. aureus* SH1000 cells visualized through SIM at 20 min, and 60 min. Cells imaged using the emission of **4**$^{4+}$ on excitation at 450 nm using A568 filter (**a**), emission of NHS-Ester 488 using the FITC filter (**b**), overlay image (**c**). Cells were incubated for 5-minutes with NHS-ester, washed with PBS and incubated with with 4 μM **4**$^{4+}$. Following this cells were washed with PBS before fixing with paraformaldehyde (16%).

S3l. HADA pearson's colocalisation constants.

Time/ min	Pearson's colocalisation coefficient	Correlation
10	0.157	small
20	0.147	small
60	0.35	medium

S4. Instrumentation

S4a. Microscopy

SIM microscopy: images were taken on a DeltaVision/GE OMX optical microscope (version 4) for structured illumination at the Wolfson Microscope Facility in the University of Sheffield. Images were reconstructed using DeltaVision OMX SoftWoRx 6.0 software. All images were analysed using Fiji and Image J software.

HyVolution and STED microscopy: Initial pellets were grown, fixed and mounted as previously discussed. Imaging was done on a commercial LEICA SP8 3X gSTED SMD confocal microscope (Leica Microsystems, Manheim, Germany), with capability of also performing high-resolution microscopy via Hyvolution. The STED nanoscope is equipped with 3 depletion lines and it is also equipped with a 3D STED additional phase plate to obtain higher spatially resolved images in XY and Z (enhanced axial super-resolution). The excitation laser beam consisted of a pulsed (80MHz) super-continuum white light laser (WLL). For a cleaner emission, the excitation lines had a clean-up notch filter (NF) in the optical pathway. Images were taken using the pulsed White Light Laser (WLL) line excitation at 475 nm, and recording the emission between 600- 650 nm. The pulsed STED images were taken with a pulsed 775 nm depletion Laser, again in every case the respective NF were in place. The objective employed was a Leica 100x/1.4 NA oil objective. The pinhole was set at one Airy unit. The gated HyD detectors were set with the gated option on and the temporal gated selected was from 2 to 6.5ns. For the 3D STED images, the depletion lasers were split in two, the second vortex was set at 65%. For the Hyvolution mode, the pinhole was set at 0.5 Airy units. Every image was acquired in the Leica HyD photon counting detectors.

The Hyvolution technology, developed by Leica microsystems with the deconvolution Huygens software company (SVI, Netherlands), employs a reduced pinhole size at the detection pathway for the image acquisition. The image is later deconvoluted using the Huygens software package. The deconvolution software uses the raw data information from the Leica acquisition files for an optimal deconvolution, for 3D objects we employed a theoretical PSF, as it automatically counteracts the possible aberrations caused for in-depth imaging. The single plane images were deconvolved with a theoretical calculated PSF provided by the software and by an experimentally calculated PSF from fluorescent beads, in both cases no apparent difference was observed. Image analysis and deconvolution of STED images resolved the localisation and distribution of the organometallic dye at the bacteria. As expected the images had low intensity counts; to maximise signal-to-noise we deconvolved the images using a commercially available software (Huygens package software, SVI, Netherlands). To quantify the background level of noise we used either an automated quantification provided by the software or a manual by means of computing the averaged background intensity from regions outside the cell. We obtained better results with the manual process. For the deconvolution we used 40 iterations, a signal to noise ratio of 15, and the classical maximum likelihood estimation method provided by the software. The quantification of the resolution obtained at specific position on the bacteria was done employing the full width at half maxi- mum (FWHM) method. The figures have been developed employing the free open source software Inkscape. The surface rendered images were obtained by Huygens Professional, LAX software (Leica SP8) to generate the surface rendered 3D volumetric STED images, and the orthogonal views, the rest of the images have been post processed with Fiji (ImageJ; NIH).

S4b. Photochemistry

Absorption spectra at 200-800 nm for the compounds were obtained with a Cary 50 Scan UV-vis-NIR Spectrophotometer, double beam mode (spectral band width = 2 nm), medium scan speed – 600 nm.

Emission spectra and DNA binding studies for the compound were conducted on the Jobin Yvon Hariba Group FluoroMax®-3 Fluorimeter. DNA binding data was fitted using Origin Software.

UV-Vis absorbance spectroscopy for LogP values were conducted via a SpectraMax M2 Microtitre Plater Reader (Molecular Devices UK).

Turbidity measurements were performed on a Jenway Cary 7350 Spectrophotometer.

S4c. Mass Spectroscopy

All Mass Spectroscopy was conducted at the Mass Spectroscopy Facility at the University of Sheffield

Mass spectra and accurate mass for the compounds were conducted using a Krato MS80 mass spectrometer working in positive ion mode.

Uptake experiment data was collected using Spectro CirosCCD (Spectro Analysis) Inductively-Coupled Plasma-Atomic Emission Spectrophotometer.

S4d. NMR Spectroscopy

^{1}H NMR spectra were taken on a Bruker AV400 machine and a Bruke AVIIIHD400 machine, both working in Fourier transform mode. All spectra were analysed using "Mestrenova" software.

S4e. Electron Microscopy

Samples were examined on a FEI Tecnai instrument operating at 80 kV equipped with a Gatan 1 k CCD Camera.

S4f. Flow cytometry

Samples were examined on a BD SLRII flow cytometer. Measurements are performed using SPHERO™ Rainbow Calibration Particles, RCP-30-5A.

S4g. X-ray crystallography

Intensity data was obtained on either a Bruker Kappa Apex-II CCD or Bruker Kappa Apex-II diffractometer.

References

1. Smitten, K. L; Southam, H. M; Bernardino de la Serna, J; Gill, M. R; Jarman, J. P; Smythe, C. G. W; Poole, R. K; Thomas, J. A. Using Nanoscopy to Probe the Biological Activity of Antimicrobial Leads That Display Potent Activity Against Pathogenic, Multidrug Resistant, Gram-Negative Bacteria. *ACS Nano*. 2019.
2. A. J. O'Neill, A. J, K. Miller, B. Oliva, I. Chopra.,*J. Antimicrob. Chemother*. **2004**, *54* (6), 1127
3. Bruker. *SADABS*; Bruker Axs Inc.: Madison, Wisconsin, USA, 2016.
4. Krause, L.; Herbst-Irmer, R.; Sheldrick, G. M.; Stalke, D. Comparison of silver and molybdenum microfocus X-Ray sources for single-crystal structure determination. *J. Appl. Crystallogr.* **2015**, *48* (1), 3–10. https://doi.org/10.1107/S1600576714022985.
5. Sheldrick, G. M. SHELXT - Integrated space-group and crystal-structure determination. *Acta Crystallogr. Sect. A Found. Crystallogr.* **2015**, *71* (1), 3–8. https://doi.org/10.1107/S2053273314026370.
6. Sheldrick, G. M. Crystal structure refinement with SHELXL. *Acta Crystallogr. Sect. C Struct. Chem.* **2015**, *71* (1), 3–8. https://doi.org/10.1107/S2053229614024218.
7. Dolomanov, O. V.; Bourhis, L. J.; Gildea, R. J.; Howard, J. A. K.; Puschmann, H. OLEX2: A Complete structure solution, refinement and analysis program. *J. Appl. Crystallogr.* **2009**, *42* (2), 339–341. https://doi.org/10.1107/S0021889808042726.