Synthesis, Characterisation and Biological Properties of a Silver Helicate Which Displays Enhanced Activity Towards Methicillin-resistant *Staphylococcus aureus* (MRSA).

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Supplementary Information.

General Information.

Chemicals were purchased and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a 600MHz Bruker Avance Neo. Mass spectra were obtained on an Agilent 6210 TOF MS for the organic species with the metal complexes run on a Bruker MicroQTOF LC. CAUTION: perchlorate salts are potentially explosive and should be treated with due care. Those complexes described below which were isolated as perchlorates were only prepared in small amounts (5 – 10 mg) and we had no problems with them.

Synthesis

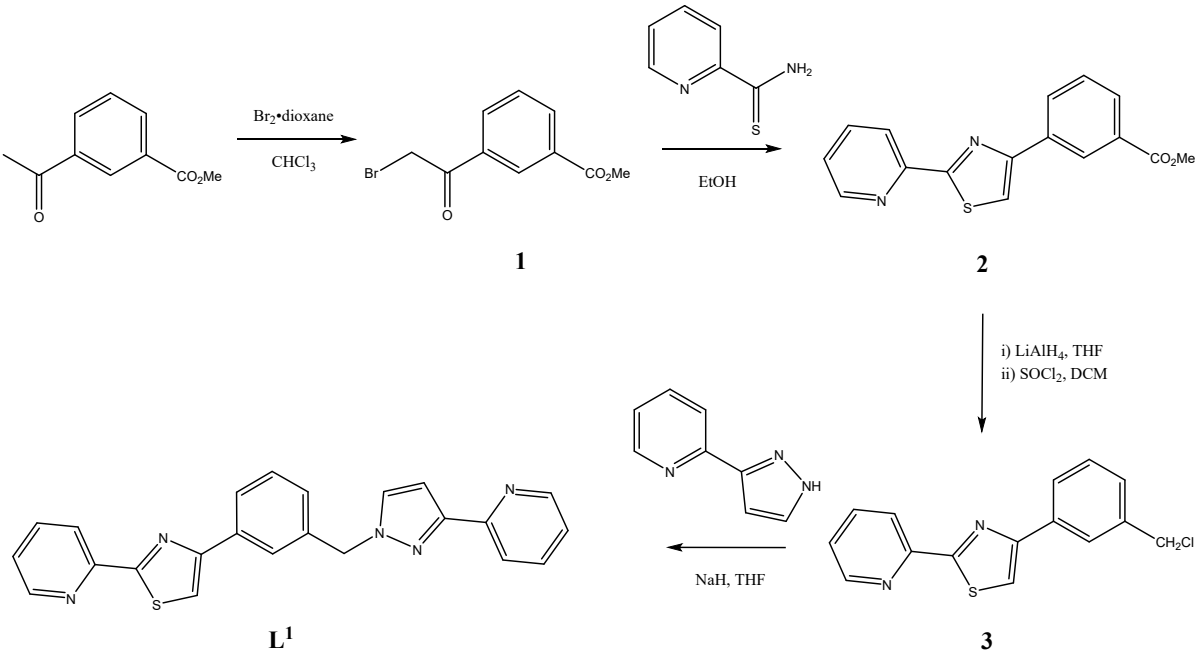


Figure S1. Synthesis of ligand **L**.

Synthesis of methyl 3-(2-bromoacetyl)benzoate **1**.

To a solution of methyl 3-acetylbenzanoate (0.49 g, 2.75 mmol) in $CHCl_3$ (15 mL) at 60 ºC was added dioxane dibromide (0.76 g, 3.07 mmol) slowly over a period of 1 h. The reaction was followed by TLC (SiO_2, DCM) and once the majority of the starting material had been consumed the reaction was cooled, extracted with $NaHCO_3$(aq), dried ($MgSO_4$) and evaporated. Purification by column chromatography (SiO_2, DCM) gave pure methyl 3-(2-bromoacetyl)benzoate **1** as a colourless solid (0.48g, 68%).^{1}H NMR [600 MHz, $CDCl_3$]: δ (ppm) 8.64 (s,1H), 8. 30 (dt, *J* = 7.77, 1.42 Hz, 1H), 8.21 (dt, *J* = 7.86, 1.52 Hz, 1H), 7.62 (t, *J* 7.79 Hz, 1H), 4.51 (s, 2H), 3.99 (s, 3H). ^{13}C NMR [150 MHz, $CDCl_3$]: δ (ppm) 190.6, 165.0, 134.7, 134.2, 133.0, 131.0, 130.0, 129.2, 52.5, 30.7.

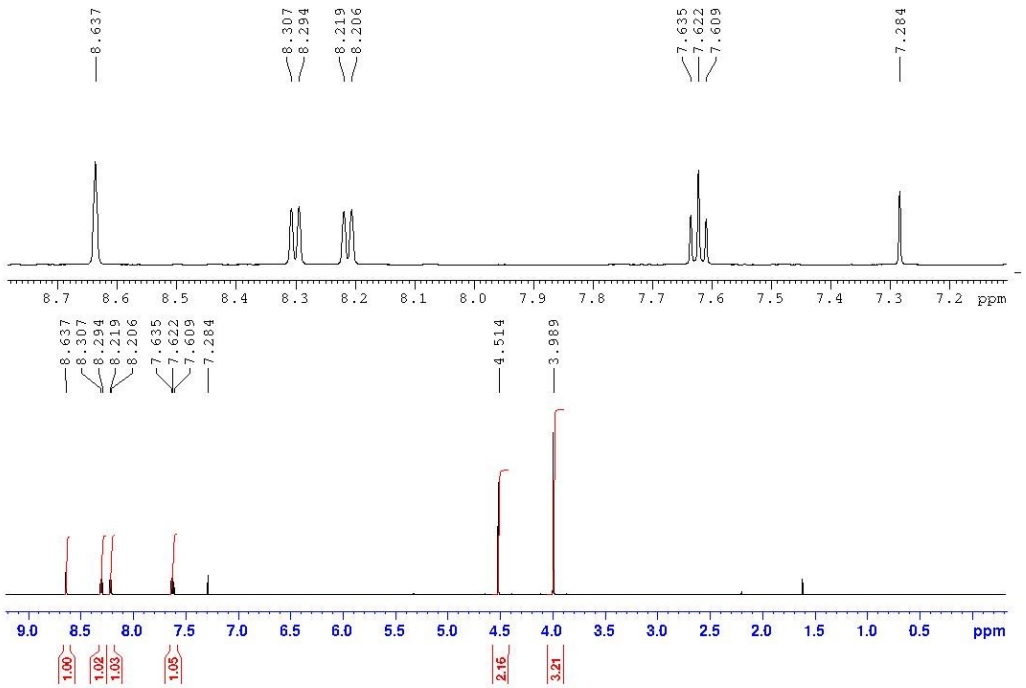


Figure S2. ^{1}H NMR (600 MHz, $CDCl_3$) of methyl 3-(2-bromoacetyl)benzoate **1**.

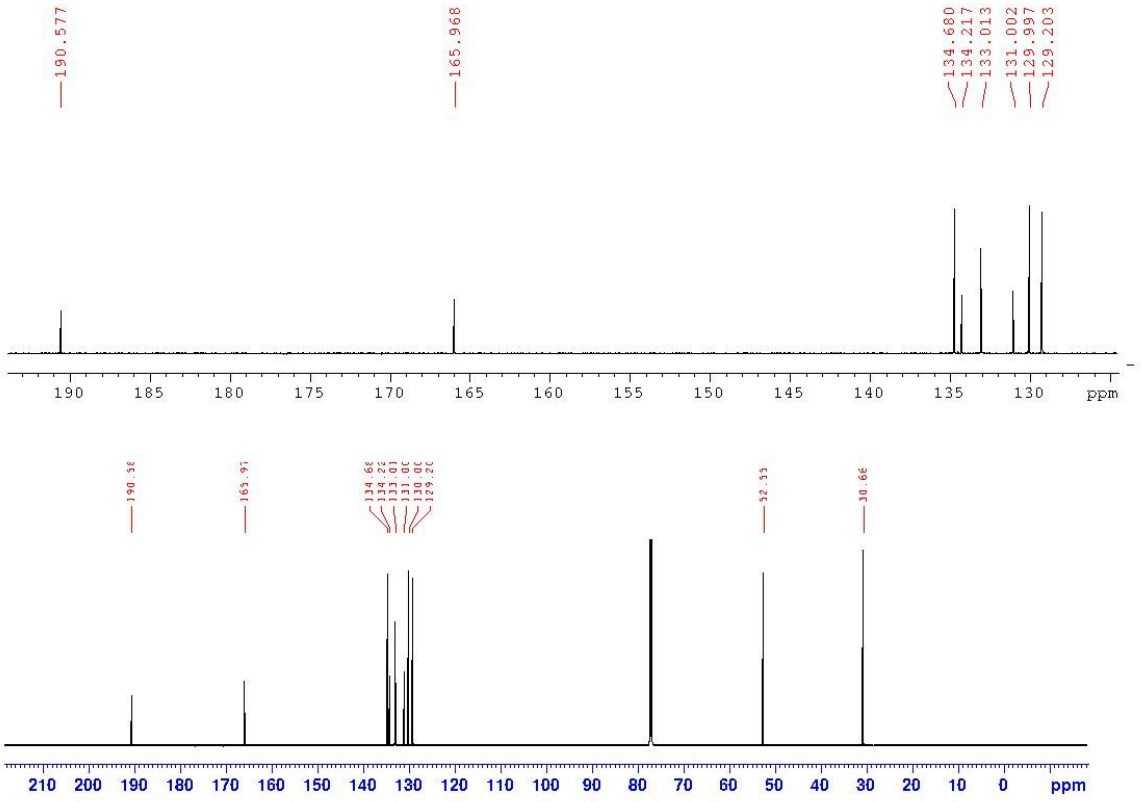


Figure S3. ^{13}C NMR (150 MHz, $CDCl_3$) of methyl 3-(2-bromoacetyl)benzoate **1**.

Synthesis of **2**.

To a solution of methyl 3-(2-bromoacetyl)benzoate **1** (0.46 g, 1.78 mmol) in EtOH (20 mL) was added pyridine-2-thioamide (0.26 g, 1.88 mmol) and the reaction heated at 50 ºC for 2 h. After this time the reaction was allowed to cool and during which time a pale-yellow precipitate was formed. This was isolated by filtration and partitioned between $NaHCO_3$(aq) (5 mL) and DCM (20 mL) and the organic phase was separated, dried ($MgSO_4$) and evaporated to give **2** as a pale yellow solid (0.37 g, 70 %). The resulting pyridyl-thiazole containing species **2** was sufficiently pure to be used in subsequent reactions. ^{1}H NMR [600 MHz, $CDCl_3$]: δ (ppm) 8.66 (m (overlap),2H), 8.38 (d, J = 7.92 Hz, 1H), 8.25 (d, J = 7.80 Hz, 1H), 8.04 (d, J = 7.76, 1H), 7.84 (td, J = 7.80, 1.69, 1H), 7.72 (s, 1H), 7.56 (t, J = 7.78, 1H), 7.36 (ddd, J = 6.03, 4.87, 1.01 Hz, 1H), 3.99 (s, 3H). ^{13}C NMR [150 MHz, $CDCl_3$]: δ (ppm) 169.2, 167.0, 155.6, 151.3, 149.5, 137.1, 134.8, 130.8, 130.7, 129.2, 128.9, 127.4, 124.7, 119.9, 116.1, 52.3. HR ESI-MS found 297.0697 $C_{16}H_{12}N_2O_2S$ requires 297.0692 (error 1.68 ppm).

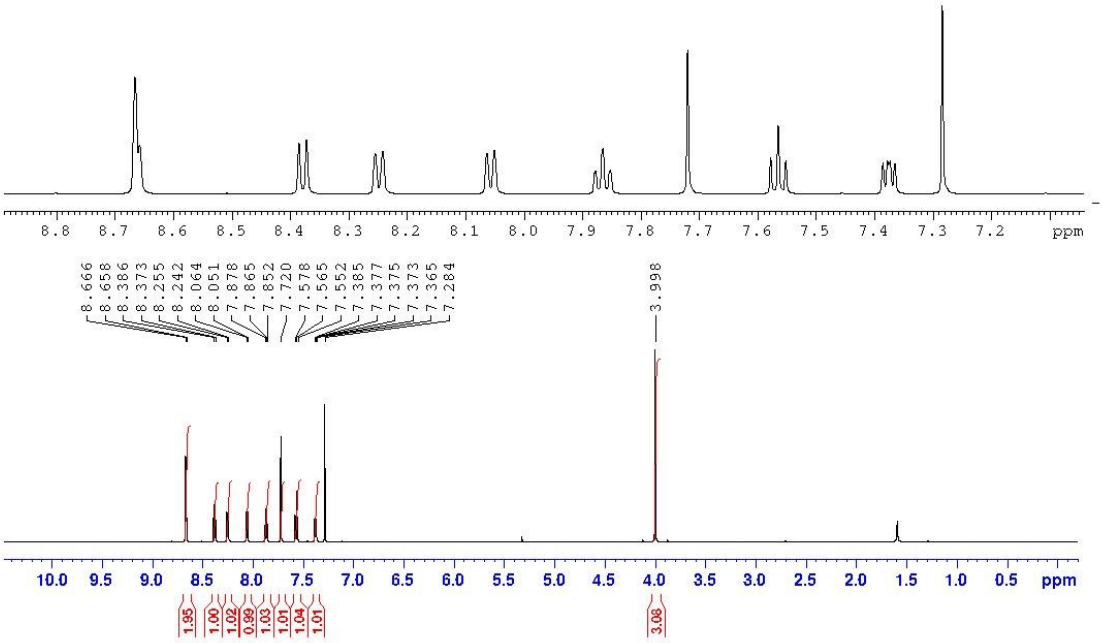


Figure S4. ^{1}H NMR (600 MHz, $CDCl_3$) of **2**.

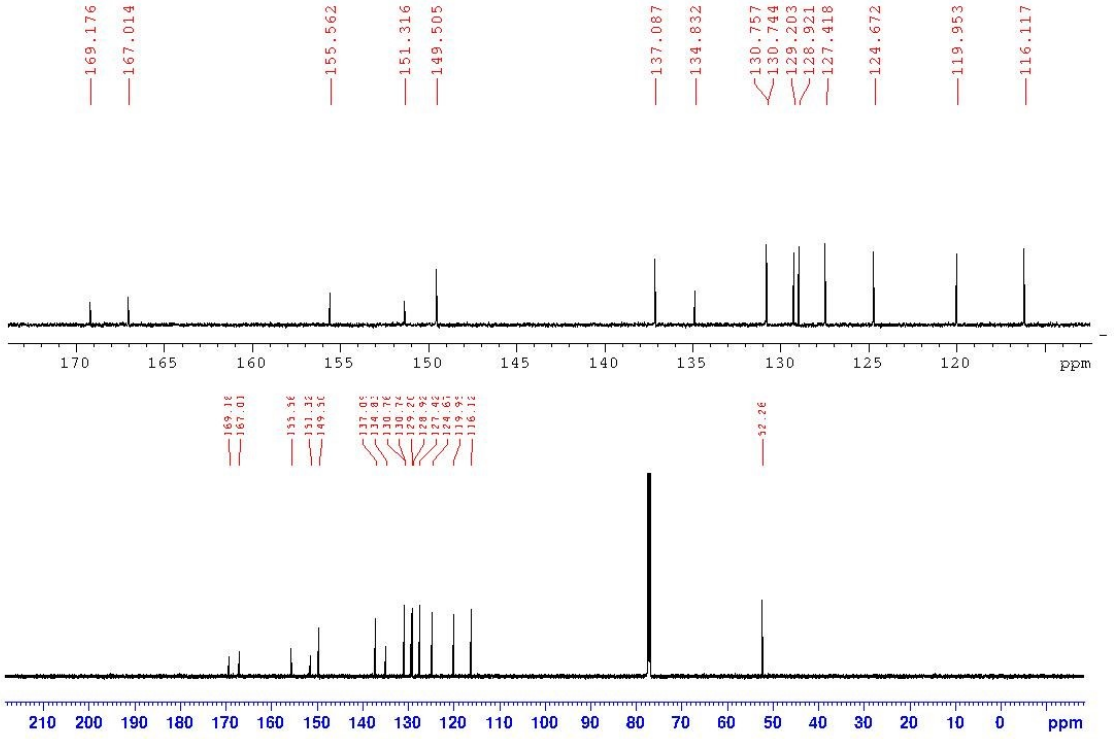


Figure S5. ^{13}C NMR (150 MHz, $CDCl_3$) of **2**.

Synthesis of **3**.

To an ice cooled solution of **2** (0.28 g, 0.94 mmol) in anhydrous THF (20 mL) under a nitrogen atmosphere was added a solution of $LiAlH_4$ in THF (2.4 M, 0.78 ml, 1.9 mmol). Upon addition the reaction was allowed to warm to room temperature and stirred for a further hour. To this was then added ethyl acetate (5 mL) and the reaction stirred for 30 min after which time EtOH (1 mL) was added dropwise. The solution was then evaporated and saturated brine added (10 mL) added and extracted with DCM (3 x 20 mL), dried ($MgSO_4$) and evaporated. The resulting solid was then dissolved in anhydrous DCM (20 mL), cooled to 0 ºC and $SOCl_2$ (0.2 mL, 0.83 mmol) added and the stirred reaction was slowly allowed to warm to room temperature. After stirring for 2 hrs a solution of $NaHCO_3$ (saturated, 10 mL) was added and the reaction stirred for 30 min. The layers were separated and the organic phase dried with $MgSO_4$ and evaporated. Purification was achieved by column chromatography (SiO_2, 1% MeOH in DCM) giving **3** as a pale yellow solid (0.16 g 59 %). ^1H NMR [600 MHz, $CDCl_3$]: δ (ppm) 8.66 (d, J = 4.56, 1H), 8.36 (d, J = 7.84, 1H), 8.06 (s,1H), 7.95 (d, J = 7.68, 1H), 7.85 (td, J = 7.76, 1.58, 1H), 7.65 (s, 1H), 7.46 (t, J = 7.66, 1H), 7.42 (d, J = 7.76, 1H), 7.37 (ddd, J = 5.79, 4.83, 1.0 Hz, 1H), 4.69 (s, 2H). ^{13}C NMR [150 MHz, $CDCl_3$]: δ (ppm) 169.0, 156.0, 151.4, 150.0, 138.1, 137.1, 135.0, 129.2, 128.4, 126.6, 126.3, 124.6, 119.9, 115.8, 46.2. HR ESI-MS found 287.0413 $C_{15}H_{11}ClN_2S$ requires 287.0404 (error 3.14 ppm).

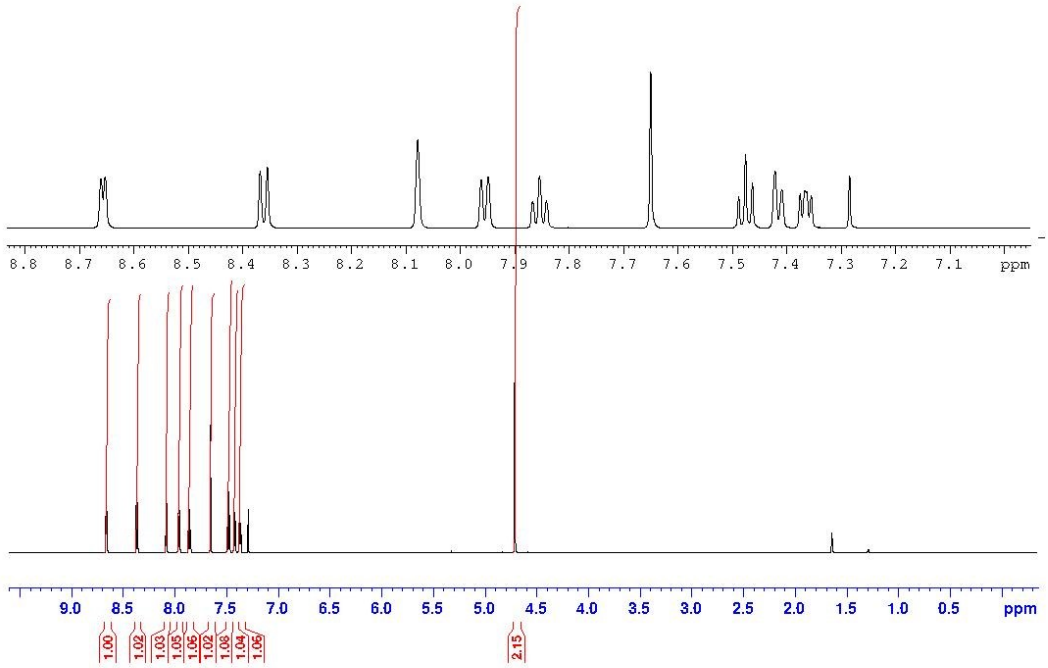


Figure S6. ^{1}H NMR (600 MHz, $CDCl_3$) of **3**.

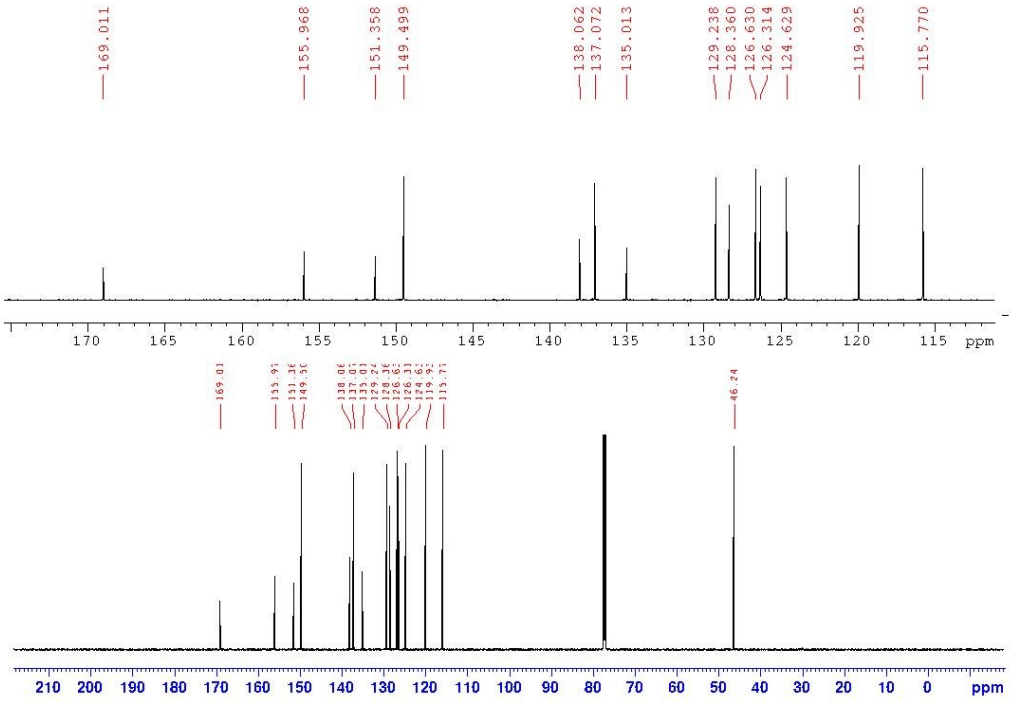


Figure S7. ^{13}C NMR (150 MHz, $CDCl_3$) of **3**.

Synthesis of ligand **L**.

To a two-necked 50 ml RB flask charged with 2-(1H-pyrazol-3-yl)pyridine (0.09 g, 0.62 mmol) and NaH (60% suspension in oil, 0.03 mg, 0.75 mmol) under an atmosphere of nitrogen was added anhydrous THF (20 mL) and subsequently a solution of **3** (0.16 g, 0.56 mmol) in anhydrous THF (10 mL) was added and the reaction heated at 60 ºC for 24 h. The reaction was then cooled and MeOH (5 mL) added and allowed to stir for 30 min. The solvent was then removed by evaporation and DCM (30 mL) added and extracted with aqueous $NaHCO_3$ (10 mL). The organic phase was then separated, dried with $MgSO_4$ and evaporated to give the crude ligand **L** which was purified by column chromatography (Al_2O_3, 1% MeOH in DCM) giving pure **L** as a colourless solid (0.18 g, 82 %).^{1}H NMR [600 MHz, $CDCl_3$]: δ (ppm) 8.68 (d, *J* = 4.62, 1H), 8.65 (d, *J* = 4.51, 1H), 8.32 (d, *J* = 8.0, 1H), 7.95 (m (overlap), 3H), 7.84 (td, *J* = 7.76, 1.78, 1H), 7.74 (td, *J* = 7.76, 1.79, 1H), 7.61 (s, 1H), 7.48 (d, *J* = 2.26, 1H), 7.45 (t, *J* = 7.78, 1H), 7.35 (ddd, *J* = 5.74, 4.87, 0.83, 1H), 7.24 (d, *J* = 7.60, 1H), 7.19 (ddd, *J* = 6.01, 4.89, 1.12, 1H), 6.95 (d, *J* = 2.40 Hz, 1H), 5.48 (s, 2H). ^{13}C NMR [150 MHz, $CDCl_3$]: δ (ppm) 169.0, 156.0, 152.3, 151.8, 151.7, 151.3, 149.5, 137.1, 136.9, 136.5, 135.1, 130.9, 129.4, 127.56, 129.1, 125.9, 124.6, 122.3, 120.1, 119.9, 115.8, 104.9, 56.3. HR ESI-MS found 396.1279 $C_{23}H_{17}N_5S$ requires 396.1277 (error 0.50 ppm).

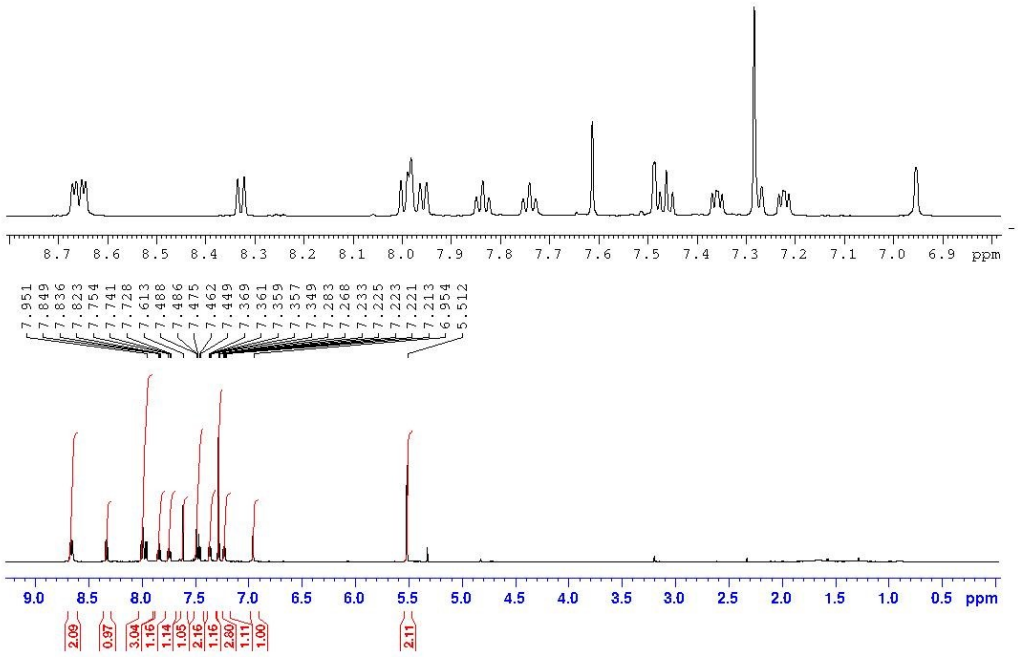


Figure S8. ^{1}H NMR (600 MHz, $CDCl_3$) of ligand **L**.

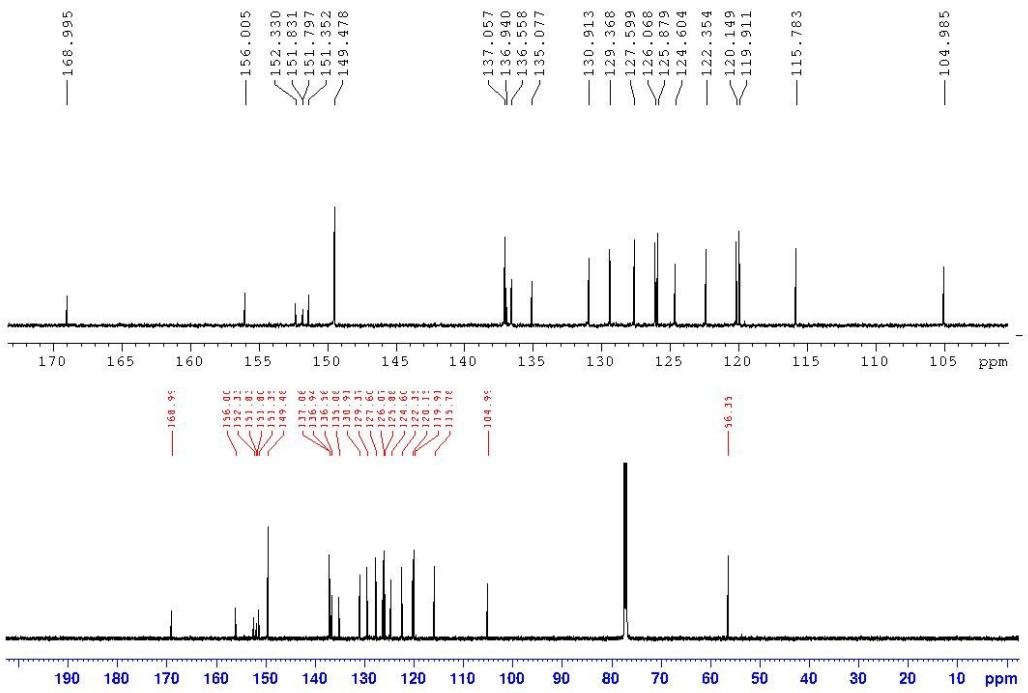


Figure S9. ^{13}C NMR (150 MHz, $CDCl_3$) of ligand **L**.

Synthesis and analysis of the silver complexes.

To a solution of either $AgClO_4$ (15.7 mg, 0.08 mmol) or AgOTf (19.5 mg, 0.08 mmol) in MeCN (2 mL) was added to ligand **L** (30 mg, 0.08 mmol) and the suspension sonicated until complete dissolution. This was then filtered and exposed to either an atmosphere of Et_2O (for the perchlorate salt) or diisopropyl ether (for the triflate salt). In both cases a colourless crystalline material was deposited over a period of a week and this solid was used for all analysis and biological experiments. Yield $[Ag_2\mathbf{L}_2](ClO_4)_2$ 31 mg (68 %). Found: C, 45.74; H, 2.71; N, 11.60 %; $C_{46}H_{34}N_{10}S_2Ag_2Cl_2O_8$ requires C, 45.83; H, 2.84; N, 11.62%. Yield $[Ag_2\mathbf{L}_2](OTf)_2$ 22 mg (44 %). Found: C, 43.85; H, 2.43; N, 10.71 %; $C_{48}H_{34}N_{10}S_4Ag_2F_6O_6$ requires C, 44.18; H, 2.63; N, 10.73%.

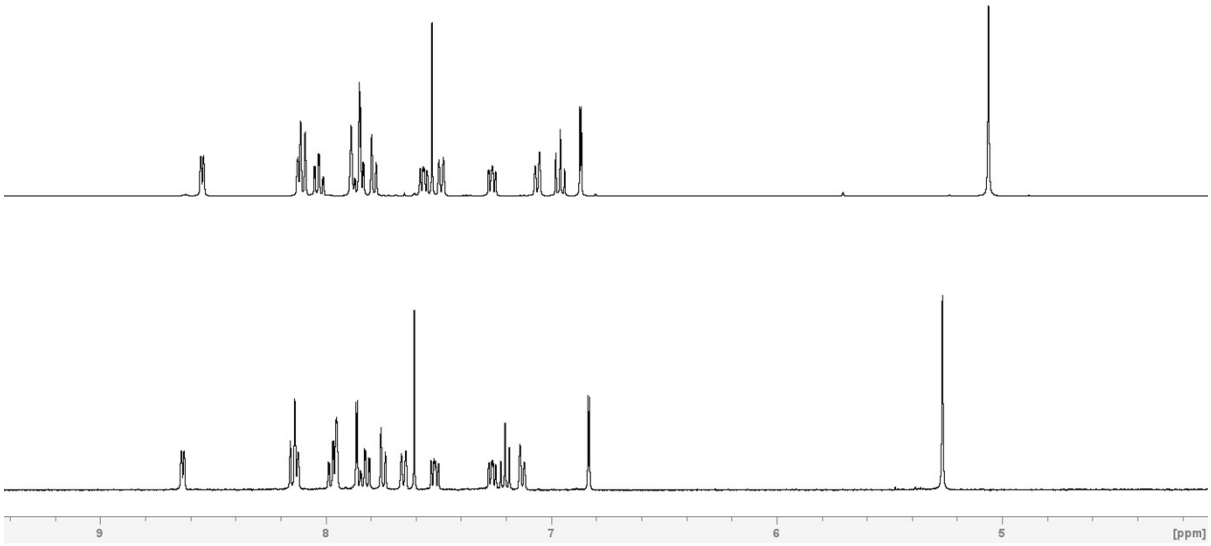
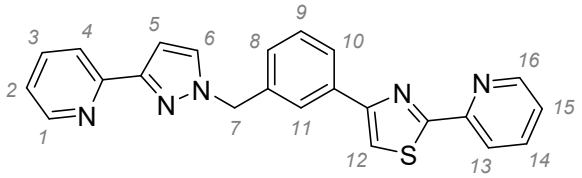


Figure S10. Selective regions of the ^{1}H NMR (CD_3CN, 600 MHz) of $[Ag_2\mathbf{L}_2](ClO_4)_2$ (top) and $[Ag_2\mathbf{L}_2](OTf)_2$ (bottom).



$[Ag_2\mathbf{L}_2](ClO_4)_2$ δ_H (CD_3CN, 400 MHz): 5.24 (s, 2H, 7-H_2), 6.81 (d, 1H, J = 2.4 Hz, 5-H), 7.11 (dt, 1H, J = 7.6, 1.4 Hz, 8-H), 7.18 (t, 1H, J = 7.7 Hz, 9-H), 7.24 (ddd, 1H, J = 7.5, 5.0, 1.3 Hz, 2-H), 7.49 (ddd, 1H, J = 7.6, 4.9, 1.2 Hz, 15-H), 7.59 (s, 1H, 12-H), 7.63 (dt, 1H, J = 7.7, 1.5 Hz, 10-H), 7.72 (dt, 1H, J = 8.0, 1.1 Hz, 4-H), 7.81 (td, 1H, J = 7.8, 1.8 Hz, 3-H), 7.84 (d, 1H, J = 2.4 Hz, 6-H), 7.92 – 7.97 (m, 2H, 11-H & 13-H), 8.09 – 8.14 (m, 2H, 1-H & 14-H), 8.60 – 8.63 (m, 1H, 16-H).

$[Ag_2\mathbf{L}_2](OTf)_2$ δ_H (CD_3CN, 400 MHz): 5.06 (s, 2H, 7-H_2), 6.87 (d, 1H, J = 2.5 Hz, 5-H), 6.96 (t, 1H, J = 7.7 Hz, 9-H), 7.06 (dt, 1H, J = 7.6, 1.2 Hz, 8-H), 7.26 (ddd, 1H, J = 7.4, 5.0, 1.4 Hz, 2-H), 7.49 (dt, 1H, J = 7.7, 1.4 Hz, 10-H), 7.53 (s, 1H, 12-H), 7.57 (ddd, 1H, J = 7.5, 4.9, 1.3 Hz, 15-H), 7.79 (dt, 1H, J = 8.0, 1.1 Hz, 4-H), 7.83 – 7.88 (m, 2H, 3-H & 6-H), 7.89 (t, 1H, J = 1.8 Hz, 11-H), 8.03 (td, 1H, J = 7.7, 1.7 Hz, 13-H), 8.08 – 8.13 (m, 2H, 1-H & 14-H), 8.53 – 8.56 (m, 1H, 16-H).

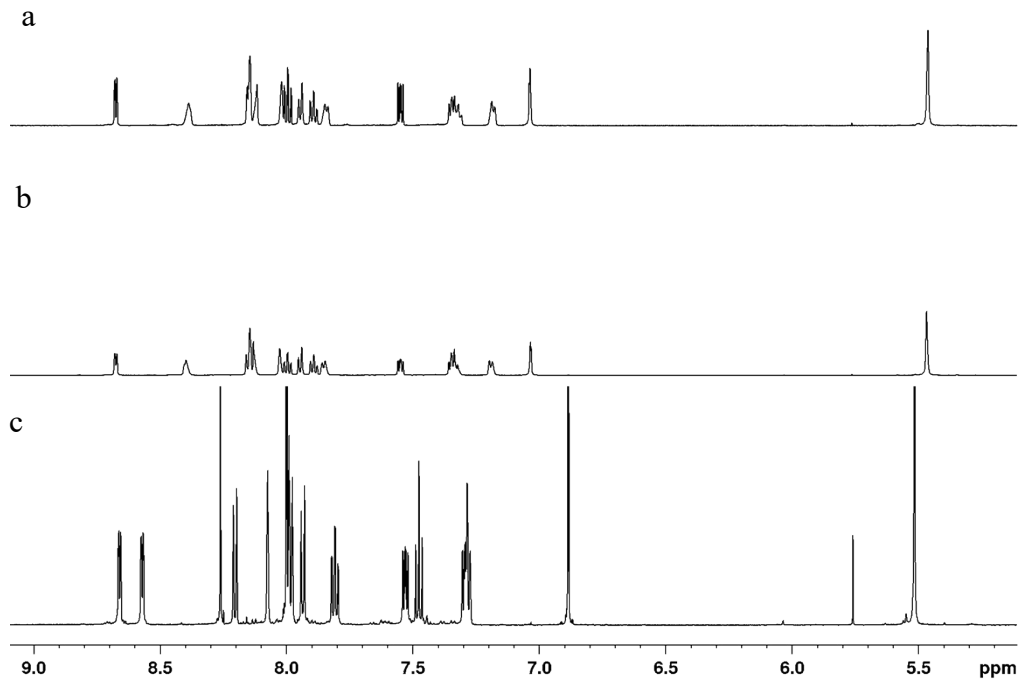


Figure S11. Selective regions of the ^{1}H NMR ($(CD_3)_2SO$, 600 MHz) of a) a *d*^{6}-DMSO solution of $[Ag_2\mathbf{L}_2](ClO_4)_2$ after exposure to natural light for 5 days, b) freshly prepared $[Ag_2\mathbf{L}_2](ClO_4)_2$ and c) ligand **L** in the same solvent. No precipitation of silver particulates was observed either in the dark or upon exposure to light.

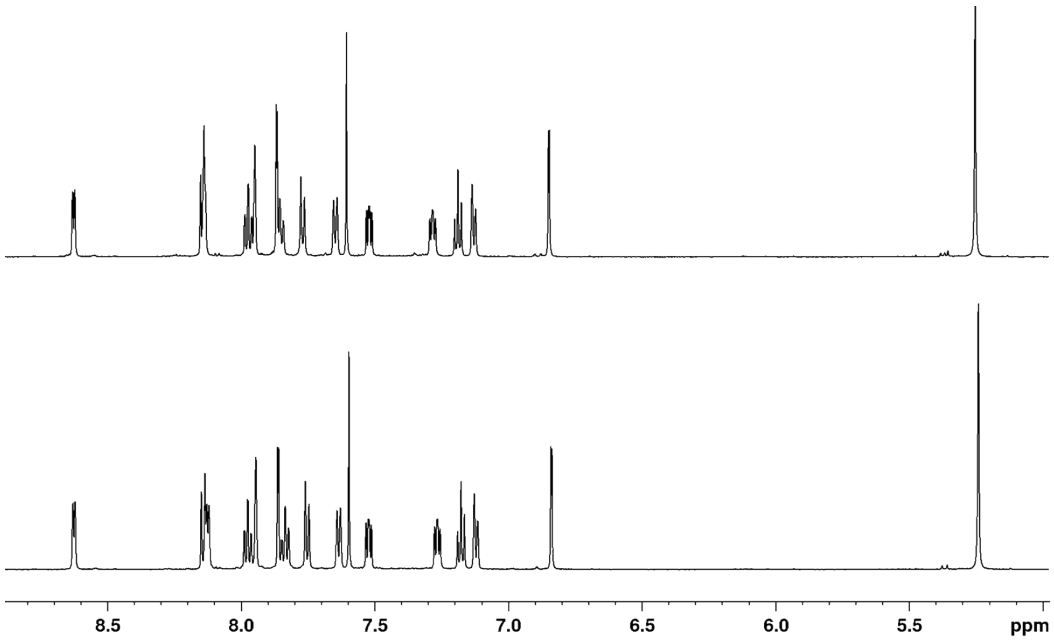


Figure S12. Selective regions of the ^{1}H NMR (CD_3CN, 600 MHz) of freshly prepared $[Ag_2\mathbf{L}_2](ClO_4)_2$ (top) and after exposure to natural light for 5 days (bottom). No change in the 1H NMR was observed nor precipitation of silver particulates.

Crystallography

Single crystal X-ray diffraction data was collected at 150(2) K on a Bruker D8 Venture diffractometer equipped with a graphite monochromated Mo(Kα) radiation source and a cold stream of N_2 gas. Solutions were generated by conventional heavy atom Patterson or direct methods and refined by full-matrix least squares on all F^2 data, using SHELXS-97 and SHELXL software respectively.[1] Absorption corrections were applied based on multiple and symmetry-equivalent measurements using SADABS.[2]

The structure of $[Ag_2\mathbf{L}_2](ClO_4)_2$ contained substitutional disorder of an MeCN and a molecule of Et_2O and these were modelled using the *PART* instruction in the least squares refinement. The bond lengths of these solvent molecules were restrained using the *DFIX* instruction and the thermal ellipsoids were restrained using *DELU* and *SIMU*. $\{[Ag\mathbf{L}](OTf)\}_\infty$ was refined without any notable issues.

Compound	$\{[Ag\mathbf{L}](OTf)\}\infty$	$[Ag_2\mathbf{L}_2](ClO_4)_2 \cdot 0.5Et_2O \cdot 0.5MeCN$
Formula	$C_{24}H_{17}AgF_3N_5O_3S_2$	$C_{25}H_{17}AgClN_{5.46}O_{4.54}S$
M	652.41	641.90
Crystal system	Monoclinic	Monoclinic
Space group	P2(1)/c	C2/c
a (Å)	11.8934(3)	19.933(4)
b (Å)	22.7852(6)	16.961(3)
c (Å)	9.4540(2)	15.528(4)
α (˚)	90	90
β (˚)	81.832(17)	100.411(12)
γ (˚)	90	90
V (Å^3)	2500.99(11)	5163(2)
Z	4	8
ρ_{calc} (Mg cm^{-1})	1.733	1.652
F(000)	1304	2572
Crystal dimensions (mm)	0.11, 0.15, 0.22	0.09, 0.18, 0.21
Reflections	26798	27707

measured		
Range	$2.381 \leq \theta \geq 30.534^{\circ}$	$2.747 \leq \theta \geq 33.144^{\circ}$
hkl range indices	$-16 \leq h \geq 16$, $-29 \leq k \geq 32$, $32 \leq l \geq -13$	$-16 \leq h \geq 30$, $-19 \leq k \geq 26$, $-23 \leq l \geq 23$
N^{o} independent reflections	7611	9789
Reflections with $I > 2\sigma(I)$	6905	7373
R_{int}	0.0215	0.0339
Final R_1 values	0.0325	0.0492
Final $wR(F^2)$ values	0.0719	0.1419
Final R_1 values (all data)	0.0369	0.0674
Final $wR(F^2)$ values (all data)	0.0739	0.1605
GOF	1.109	0.988
Refined parameters	343	381
Restraints	0	45
Largest peak and hole (e Å^{-3})	1.114 , -0.603	1.853, -1.363
CCDC Number	2516768	2516767

Cell lines and chemosensitivity studies

Mammalian cell lines for *in vitro* chemosensitivity testing were all low passage, short tandem repeat (STR)- authenticated human cell lines purchased from the American Cell Culture Collection (ATCC) with the exception of HCT116 $53^{+/+}$ human colorectal carcinoma cells which were a gift from Professor Bert Vogelstein.[S3] Cell lines were cultured in antibiotic-free cell culture media as recommended by ATCC additionally supplemented with 5% fetal calf serum, 2 mM L-glutamine and 1 mM sodium pyruvate. Chemosensitivity was performed as described in Allison *et. al.,*[S4] with reconstitution of $[Ag_2\mathbf{L}_2](OTf)_2$ in fresh DMSO on the day of testing (100 mM stock concentration) followed by its further dilution in cell culture media and with a final DMSO concentration of 0.1 % for all tested concentrations and the solvent control. Cell lines were seeded in 96 well plates a minimum of 24 h before drug treatment to allow cells to firmly adhere to the plate with ARPE-19 cells left to reach confluency before

drug addition. All cell lines were exposed to $[Ag_2\mathbf{L}_2](OTf)_2$ by direct media replacement followed by continuous exposure for 4 days (in the dark) with quantification of chemosensitivity by the MTT assay on day 4. 20 µL (5 mg/mL) MTT was added to 200 µL of media in wells and cells were incubated at 37 ^{0}C for a further 4 h. Formed formazan crystals were dissolved in 150 µL DMSO and absorbances were determined using a plate reader at 540 nm. Dose response curves were constructed, and the concentration required to reduce cell growth by 50 % (IC_{50}) were determined with calculation of the *in vitro* selectivity index by dividing the mean IC_{50} against ARPE-19 cells by the mean IC_{50} for the tested cancer cell line.

Antimicrobial Susceptibility Testing

Bacterial strains

Uropathogenic *Escherichia coli* (CFT073 (also designated as ATCC 700928)), *Pseudomonas aeruginosa* (PAO1-LAC (also designated as ATCC 47085) and *Staphylococcus aureus* (NCTC 12493; a methicillin-resistant *S. aureus* isolate (MRSA)) were utilised in this study. The strains were routinely cultured in Muller Hinton broth (MHB) at 37 °C with shaking for 18 h and inoculated onto Muller Hinton agar (MHA) (Merck, UK) at 37 °C for 18 h. MHA and MHB were prepared according to the manufacturer's instructions.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) of the $[Ag_2\mathbf{L}_2](OTf)_2$ complex was determined using the standard CLSI (Clinical and Laboratory Standards Institute) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) broth microdilution methods, as described by Wiegand *et al*., 2008.[S5] The $[Ag_2\mathbf{L}_2](OTf)_2$ complex was dissolved in 100 % dimethyl sulfoxide (DMSO) to prepare a 20 mM stock solution. Subsequently, a 2 mM working solution was prepared in MHB. A single bacterial colony was inoculated into 5 mL of MHB and incubated overnight at 37 °C, with shaking at 180 rpm. The overnight culture was adjusted to an $OD_{600\,nm}$ of 0.1 (*ca.*1×10^8 CFU mL^{-1}) and subsequently diluted 1:100 in MHB to obtain an inoculum of 1×10^6 CFU mL^{-1}. Two-fold serial dilutions of the complex working solution were performed directly in 96-well round-bottomed microplates starting from 2 mM. An equal volume of the bacterial suspension was then added to each well, resulting in a final inoculum of *ca.* 5×10^5 CFU mL^{-1} and a corresponding two-fold dilution of the complex. The highest concentration of the $[Ag_2\mathbf{L}_2](OTf)_2$ helicate tested in this study was 1 mM in 5 % DMSO. Each plate included growth (positive) control wells (bacteria without cryptand), sterility controls (MHB only) and solvent control (bacteria and DMSO at corresponding concentrations). Plates were incubated at 37 °C for 18 h in the dark. The MIC was recorded as the lowest complex concentration showing no visible bacterial growth compared to the growth control. All assays were performed in triplicate with three independent biological replicates ($n = 3$).

Minimum bactericidal concentrations (MBCs) were determined by subculturing aliquots from MIC plates after the 24 h incubation period, onto MHA plates using a 96 well replicator plater. Plates were incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration

showing no visible colonies after incubation. All assays were performed in triplicate with three independent biological replicates (n = 3).

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was used to visualise cell phenotypic changes following exposure to the silver complex and silver triflate analogues. Following 24 h of incubation (as conducted in the MIC assays), 10 µL of the supernatant from relevant conditions (MIC and above) were added to sterile silicon wafers, dried for 1 h at room temperature, and fixed in 4 % v/v glutaraldehyde for 24 h at 4˚C. The samples were then rinsed in sterile deionised water and subjected to an ethanol gradient from 10 % to 30 %, 50 %, 70 %, 90 % and 100 % v/v absolute ethanol with samples being submerged at each gradient step from 10 min before being transferred to the next sequential step in the ethanol gradient as per Slate *et. al*.[S6] and Butler *et. al*.[S7] Samples were then dried by desiccation over 24 h, sputter coated with a 5 nm gold/palladium conductive layer (Leica EM ACE200) prior to imaging using a Zeiss Crossbeam 350 Focussed Ion Beam – Scanning Electron Microscope with an accelerating voltage of 2 kV via a secondary electron detector.

References

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