Electronic Supplementary Information

(10 pages)

Co-crystallization of antibacterials with inorganic salts: paving the way to activity enhancement

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1. Synthesis and solid-state characterization

All reagents and solvents used in this work were purchased from Sigma-Aldrich and used without further purification.

1.1 Solution synthesis of PF·2H$_2$O. Commercially available PF·0.5H$_2$SO$_4$·xH$_2$O (76.25 mg) was dissolved in 5 mL of water and 23.65 mg of NaOH was added to the solution, yielding PF·2H$_2$O as a crystalline powder, which was filtered and washed several times with water. The experimental XRPD pattern of the product matched the one calculated on the basis of single crystal data available in the CSD (PROFLV[1]) (fig. ESI-1).

1.2 Solid-state synthesis. Complexes of PF (122.64 mg, 0.5 mmol) with CuCl (49.5 mg, 0.5 mmol) and AgNO$_3$ (84.94 mg, 0.5 mmol) were obtained by kneading equimolar quantities of the reagents for 120 minutes in a Retsch MM200 ball miller, operated at a frequency of 25 Hz, in the presence of 2-3 drops of acetone.

1.3 Slurry synthesis. PF·CuCl (**1a**), PF·AgNO$_3$ (**1b**), catena-(MV^{2+})tetrakis(μ_2-chloro)-di-copper(I) (**2a**, DMDPCU), and N,N'-Dimethyl-4,4'-bipyridinium tetrachloro-copper(II) (**2b**, MBPYCU), were all obtained via slurry in acetone (**1a** and **1b**) or water (**2a** and **2b**) at room temperature for 3-5 days.

1a: 122.64 mg - 49.5 mg, 2 mL acetone; **1b**: 122.64 mg - 84.94 mg, 2 mL acetone; **2a**: 128.58 mg - 49.5 mg, 2 mL of H$_2$O; **2b**: 128.58 mg – 67.23 mg, 2 mL of H$_2$O.

1.4 X-ray powder diffraction - phase identification. For phase identification purposes, room temperature X-ray powder diffraction (XRPD) patterns were collected on a PANalytical X'Pert PRO automated diffractometer equipped with an X'celerator detector in the 2θ range 3–50° (step size 0.011, time/step 50 s, VxA 40x40).

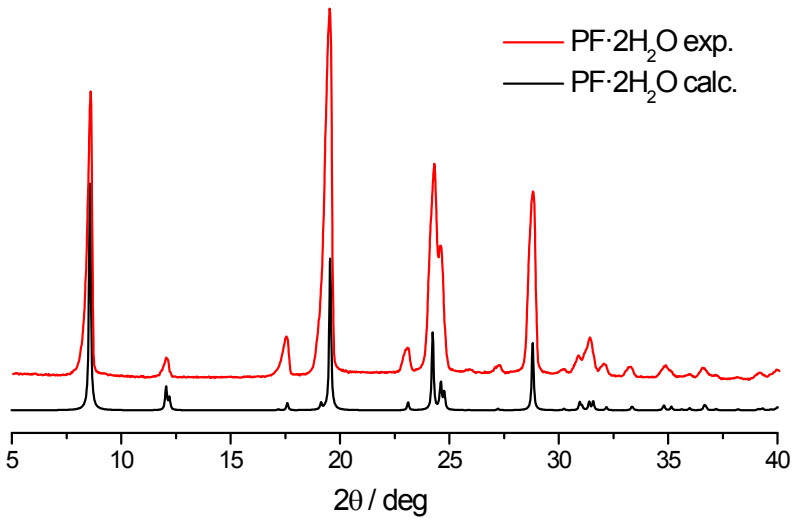


Fig. ESI-1. Comparison between the experimental XRPD pattern for PF·2H$_2$O (red line) and the pattern (black line) calculated on the basis of single crystal data from the CSD (PROFLV[1]) (black line).

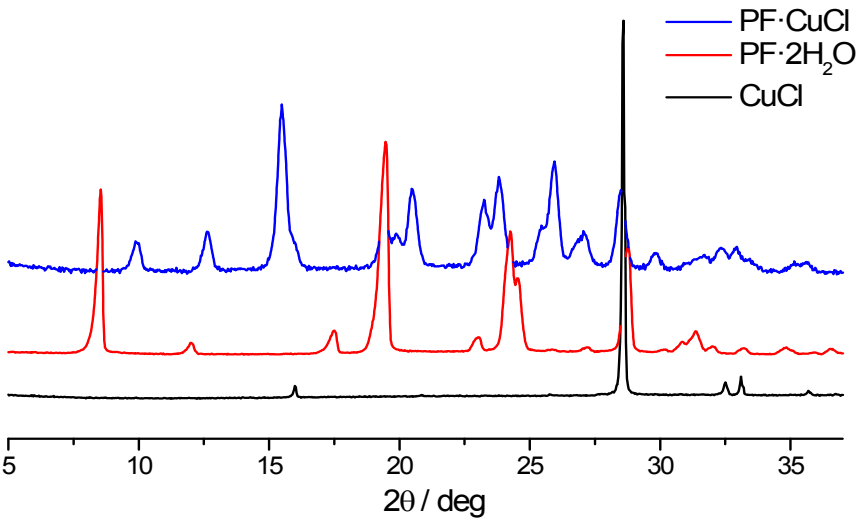


Fig. ESI-2. Comparison between the experimental XRPD pattern of PF·CuCl (blue line) and those of the starting materials PF·$2H_2O$ (red line) and CuCl (black line).

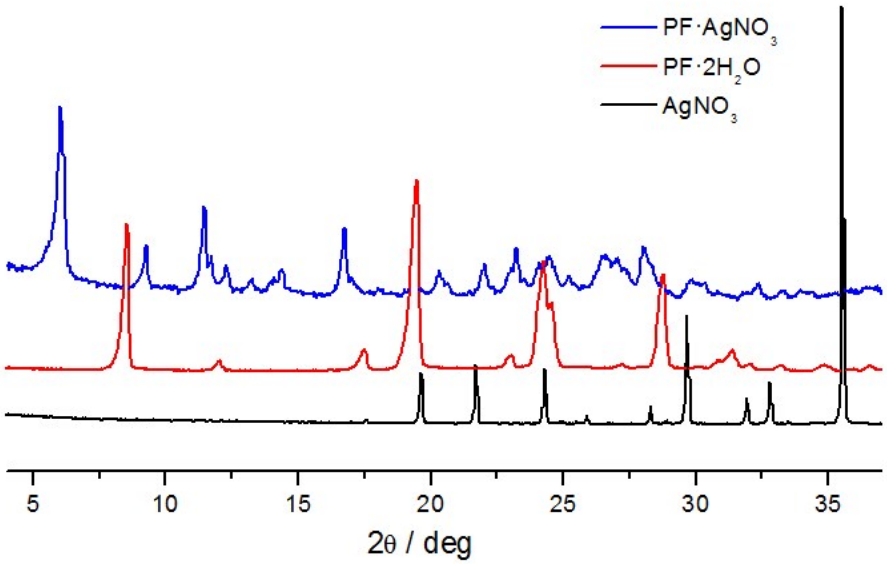


Fig. ESI-3. Comparison between the experimental XRPD pattern of PF·$AgNO_3$ (blue line) and those of the starting materials PF·$2H_2O$ (red line) and $AgNO_3$ (black line).

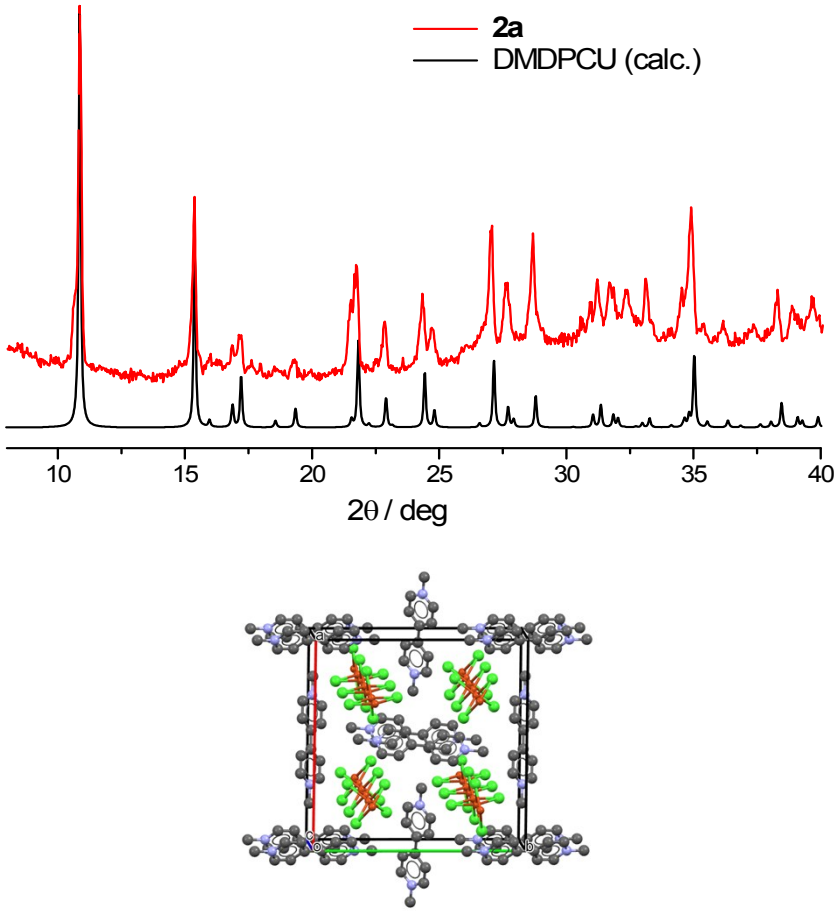
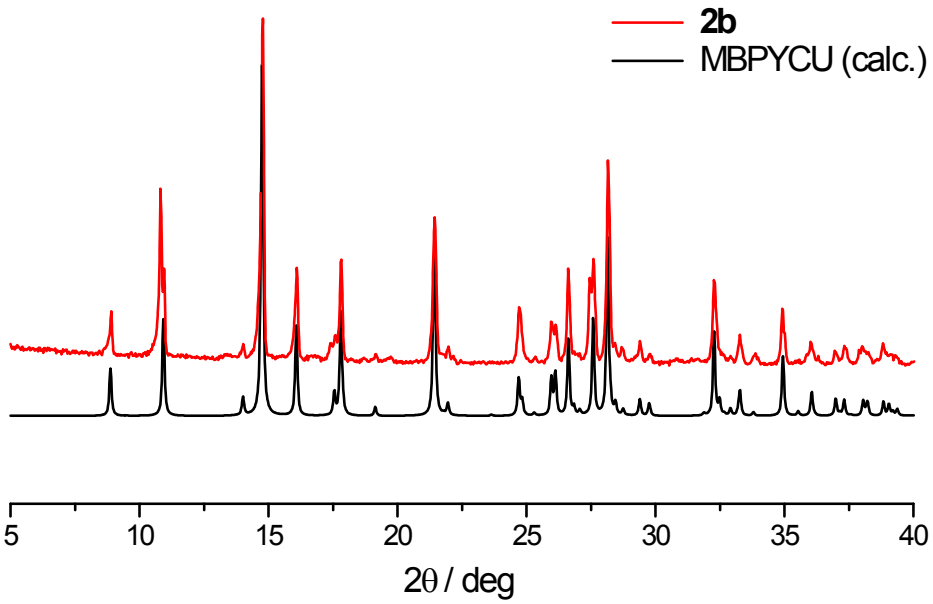


Fig. ESI-4. (top) Comparison between the experimental (red line) XRPD pattern for **2a** as obtained by slurry and the pattern (black line) calculated on the basis of single crystal data (DMDPCU[7]), and (bottom) the packing arrangement in crystalline **2a**.[7]



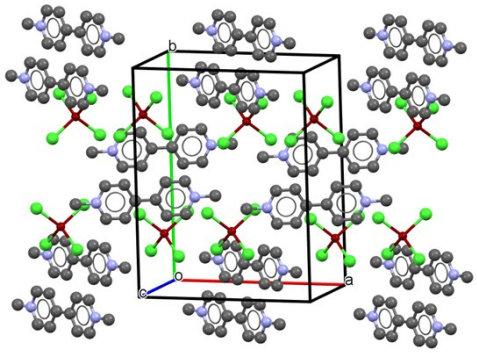


Fig. ESI-5. (top) Comparison between the experimental (red line) XRPD pattern for **2b** as obtained by slurry and the pattern (black line) calculated on the basis of single crystal data (MBPYCU[8]), and (bottom) the packing arrangement in crystalline **2b**.[8]

1.5 Structural characterization of PF·CuCl from powder data.

A room temperature X-ray powder diffraction (XRPD) pattern was collected on a PANalytical X'Pert PRO automated diffractometer with transmission geometry, equipped with Focusing mirror and Pixcel detector, in the 2θ range 5–70° (step size 0.0130°, time/step 118.32 s, VxA 40kV x 40mA). Powder diffraction data were analysed with the software X'Pert HighScore Plus[2] and unit cell parameters were found using the DICVOL4 algorithm.[3] Initially PF·CuCl was indexed in the monoclinic system with a unit cell volume of ~ 600 Å^3 and space group $P2_1$ by simulated annealing, performed with EXPO2014,[4] using Cu and Cl atoms, and one molecule of proflavine. Ten runs for simulated annealing trial were set, and a cooling rate (defined as the ratio T_n/T_{n-1}) of 0.95 was used. The Platon[5] ADDSYMM SHELX command was subsequently applied, and data were transformed into the orthorhombic space group $Cmc2_1$ with $PF_{0.5}{\cdot}Cu_{0.5}Cl_{0.5}$ in the asymmetric unit. A Rietveld refinement (fig. ESI-6) was subsequently performed, using new cell parameters, with TOPAS 5.0,[6] treating half of proflavine molecule as a rigid body and using a spherical harmonics model to describe the preferred orientation. Structural details are listed in table ESI-1.

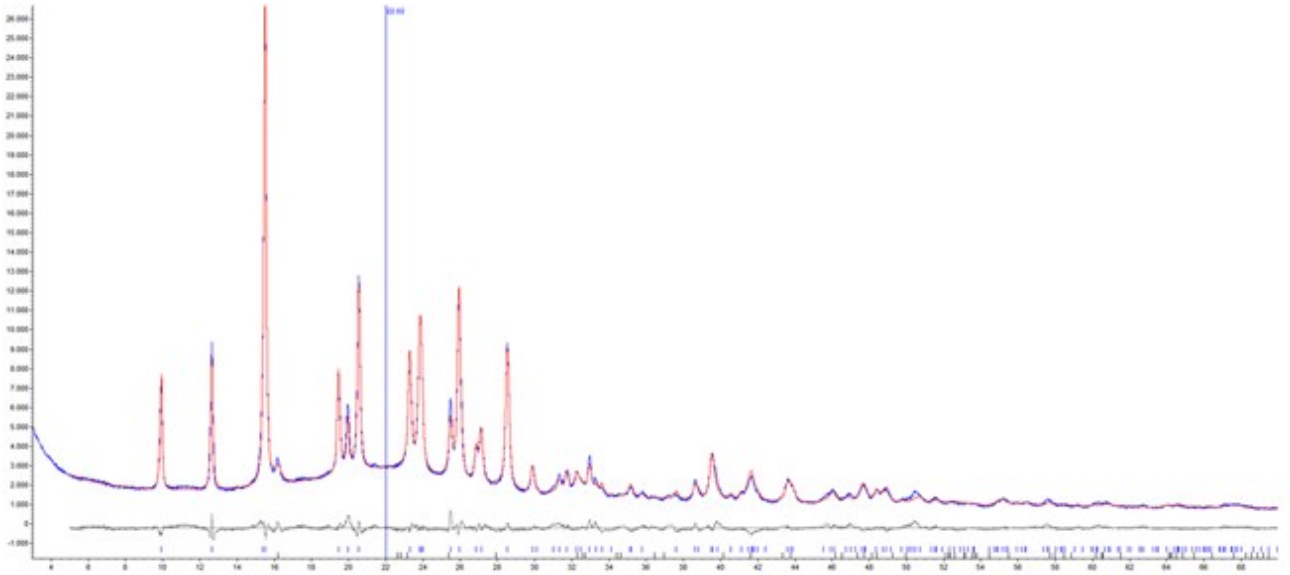


Fig. ESI-6. Rietveld analysis plot of PF·CuCl: In red the calculated pattern, in blue the experimental one, and in grey the difference plot.

Table ESI-1. Structural details for PF·CuCl.

	PF·CuCl
Formula	$C_{13}H_{11}ClCuN_3$
Fw (g mol^{-1})	308.25
Crystal system	Orthorhombic
Space group	$Cmc2_1$
Z, Z'	4, 0.5
a (Å)	13.98(1)
b (Å)	11.53 (1)
c (Å)	7.44 (1)
α (°)	90.0
β (°)	90.0
γ (°)	90.0
V (Å^3)	1199.8(1)
R_wp	5.45
R_p	3.81
R_exp	2.2
χ^2	2.47

Crystal data can be obtained free of charge from the Cambridge Crystallographic Data Centre via https://www.ccdc.cam.ac.uk and have been allocated the accession number **CCDC** 1964562 (*PF·CuCl*).

1.6 DSC and TGA

Differential Scanning Calorimetry. DSC measurements were performed for PF·$2H_2O$, PF·CuCl (**1a**) and PF·$AgNO_3$ (**1b**) with a Perkin–Elmer Diamond. The samples (3–5 mg) were placed in sealed aluminium pans, and heating was carried out at 5°C min^{-1}.

Thermogravimetric analysis. TGA measurements for PF·$2H_2O$, PF·CuCl (**1a**) and PF·$AgNO_3$ (**1b**) were performed using a Perkin-Elmer TGA7 in the temperature range 30-400 °C under an N_2 gas flow, at a heating rate of 5 °C min^{-1}.

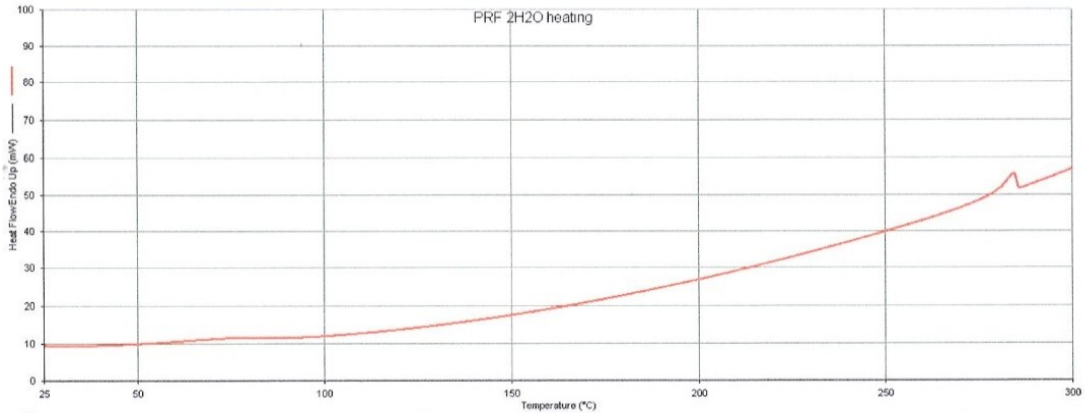


Fig. ESI-7. DSC trace for PF·$2H_2O$.

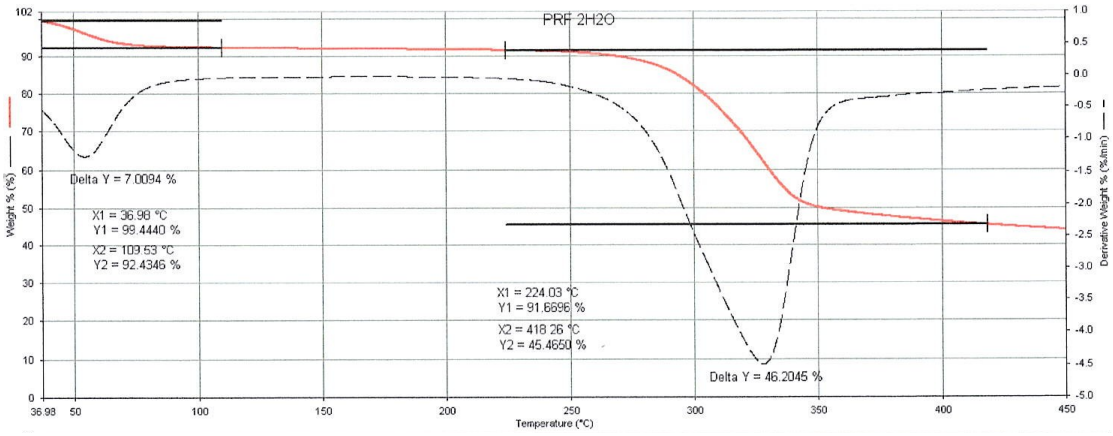


Fig. ESI-8. TGA trace for PF·$2H_2O$.

Fig. ESI-9. DSC trace for PF·CuCl.

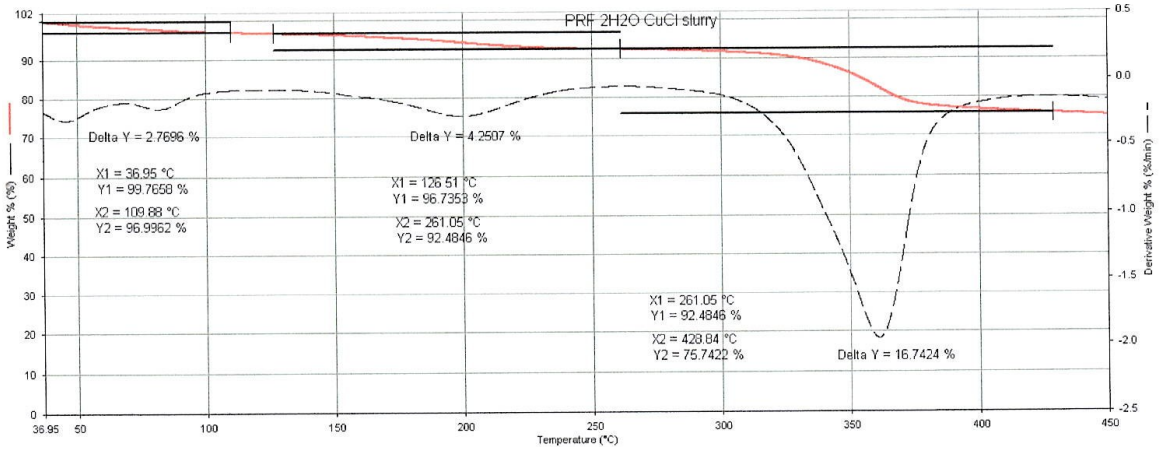


Fig. ESI-10. TGA trace for PF·CuCl.

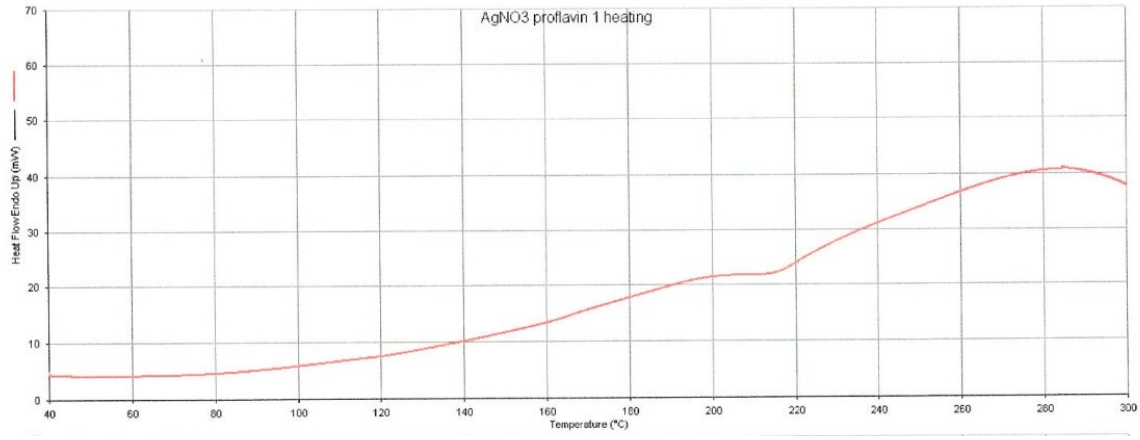


Fig. ESI-11. DSC trace for PF·$AgNO_3$.

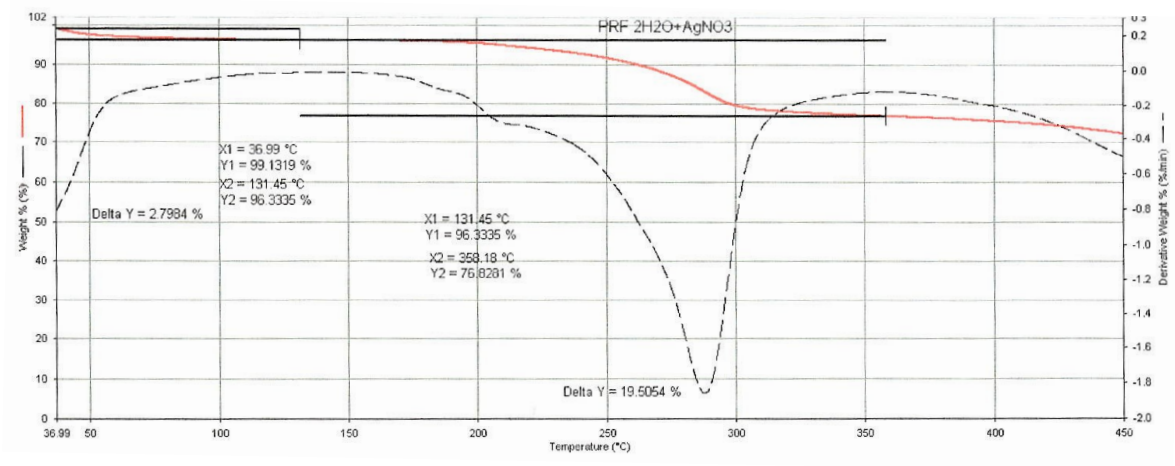


Fig. ESI-12. TGA trace for PF·$AgNO_3$ (low temperature mass loss is due to adsorbed water).

2. Antimicrobial Assays

Working stocks of the various compounds were made fresh and used within 24 h at a concentration of 25 mg/mL in double distilled water producing solutions or slurries. Samples evaluated included: proflavine (PF) and the copper derivative **1a** and silver derivative **1b**; methyl viologen dichloride ($MVCl_2$) and the Cu derivatives **2a** and **2b**. In addition to these the metal salts of $CuSO_4$, CuCl, $CuCl_2$, $AgNO_3$, are included as comparators as well as silver oxide (Ag_2O) nanoparticles. Antimicrobial testing was performed using the pathogen indicator quality control strains obtained from the ATCC strain bank (https://www.atcc.org): *Pseudomonas aeruginosa* ATCC27853, *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC 25922. All cultures were grown on Lysogeny broth media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 10 g/L bacto agar).

Three different methods for evaluating the antimicrobial activity were performed. Each assay was performed by at least 3 independent biological trials with at least 2 technical replicates per trial. All data within a given technical trial was normalized to a zone of inhibition of 10 units. The normalized data was then used to produce an average of trials and the standard deviation calculated and plotted. Statistical evaluation performed by a paired T-test.

2.1 Method 1. From overnight cultures (37°C with shaking at 200 rpm), 100 µL were spread on LB agar plates and allowed to dry for 30 min. A 1.5 mm diameter hole was produced using a sterile wood inoculant swab stick. From each of the compound stock solutions 3 µL were delivered to each of the wells. The plate was then transferred to a 37°C incubator and the bacterial lawns were allowed to grow for 24 hours before their zone of inhibition diameters were measured.

2.2 Method 2: Blank 5 mm diameter Antimicrobial susceptibility disks (Oxoid) were add to the compound stocks and allowed to soak for 1 hour to adsorb the compounds (vials containing the stock slurries were mixed by inversions several times during the hour). As in method 1 a bacterial lawn of each strain was produced by spreading 100 µL on LB agar plates and allowed to dry for 30 min. The soaked disks were then transferred to the plates which were incubated for 24 h before their zone of inhibition diameters were measured.

2.3. Method 3: As in method 2 susceptibility disks were prepared with each compound. However, the disks were dried at 37°C after removal from the stock solution. In this method a robust bacteria lawn was produced inoculated with 200 µL of overnight culture.

However, the bacteria were allowed to grow for 24 h prior to having the disk placed on the lawn in order to evaluate the ability of the compounds impregnated into the disk could kill and lyse pre-grown bacteria.

References

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