Harnessing the Dual Antimicrobial Mode of Action Using the Principle of the Irving-Williams Series to Completely Eradicate *Staphylococcus aureus*

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Experimental procedures

Synthesis and characterization of BQ: In an oven-dried 50-mL round bottom flask, 2-methyl-8-quinolinol (0.5 g, 3.14 mmol, 1 equiv) and N-bromosuccinimide (1.23g, 6.91 mmol, 2.2 equiv) were dissolved in dry toluene (20 mL) under argon atmosphere. The reaction mixture was stirred for 2 h at room temperature. The solvents were evaporated in vacuo, and the residue was dissolved in dichloromethane and adsorbed in silica. The residue was purified with column chromatography using dichloromethane as an eluent to obtain the product (0.99 g, 100% yield). (**Scheme S1**). Spectroscopic characterization, including UV-Vis (**Figure S1**), FT-IR (**Figure S2**), ^{1}H NMR (**Figure S3),** ^{13}C NMR (**Figure S4**), and ESI-HRMS (**Figure S5**). Melting point: 123-125 ºC.

Synthesis and characterization of 1: To a solution of BQ (2 mmol, 0.1 g) in ethanol (20 mL) was added Na_2CO_3 (1.5 mmol, 0.03g) in deionized water followed by manganese chloride tetrahydrate

(1 mmol, 0.026 g) in ethanol/ deionized water (10 mL). The resulting mixture was stirred for 3 h at room temperature. The yellow-brown precipitate was collected by filtration, followed by washing with ethanol/ deionized water three times and dried in a vacuum oven. Elemental analysis on C, H and N showed the crude product contains two coordinated and four crystallization H_2O molecules (see **Table 1**). The product was purified by recrystallization in hot DMSO to give dark red plate-shaped single crystals (0.084 g, 61% yield). Spectroscopic characterization, including elemental analysis (**Table S1**), UV-Vis (**Figure S1**), FT-IR (**Figure S2**), and single crystal X-ray diffraction (SCXRD) (**Figure 1**, **Tables S2 and S3**), indisputably established the identity of the product as Mn with purity ≥98%. Melting point: decomposed at 280 ºC.

Metal-Exchange Studies: We describe a methodology for studying the metal-exchange of Mn with Fe(II), Cu(II), and Zn(II). **1** was dissolved in an ethanol/DMSO mixture (i.e., 50% DMSO). Stock solutions of $FeCl_2$, $CuCl_2$, and $ZnCl_2$ were prepared at a concentration of 30 μg/mL. The solution of **1** was added to a UV-VIS cuvette at a concentration of 3.125μg/mL, followed by the addition of each metal solution at a 10X higher concentration than of **1**. The mixture was allowed to equilibrate before being measured using a UV-VIS spectrophotometer from 235-450 nm. The measurement was conducted multiple times. The spectra were analyzed to determine the extent of metal-ligand exchange of **1** with Fe(II) (**Figure 2**), Cu(II) **(Figure S6**), or Zn(II) (**Figure S7**).

Minimum inhibitory concentration (MIC) assays. In this study, the minimum inhibitory concentrations (MICs) of BQ, ciprofloxacin, clioquinol, and **1** against four strains of bacteria, including MSSA; ATCC 6538, $MRSA^{\alpha}$; ATCC BAA-44, $MRSA^{\beta}$; ATCC BAA-1717, and VISA; ATCC 700699, were determined using the Clinical and Laboratory Standards Institute (CLSI) recommended broth microdilution method and guidelines.[1] The bacterial samples, with a

concentration of 1×10^6 CFU/mL, were exposed to various concentrations of BQ, ciprofloxacin, clioquinol, and **1** in a 96-well plate. After 24 h of incubation, the MICs were determined as the lowest concentrations at which no visible bacterial growth was observed. This widely accepted and standardized method provides a reliable assessment of the antimicrobial activity of these compounds against a range of bacterial strains.

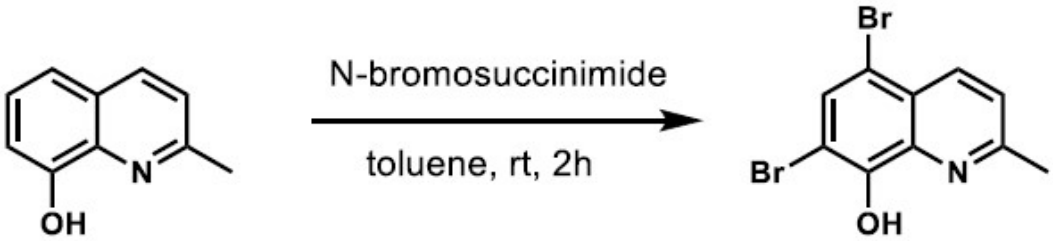
Investigation of antibacterial activity of 1 and BQ: A single colony of *S. aureus* bacteria was cultured in 5 mL of TSB bacteria medium at 37 °C and 180 rpm for 24 h. The bacterial suspension was diluted to 1:100 in a new medium and incubated at 180 rpm for 4 h at 37 °C to establish the bacteria density of 1×10^9 CFU/mL. Then, 10 μL of a diluted bacterial solution (1×10^6 CFU/mL) was added to 990 μL of the medium followed by treatment with a 10 μL DMSO solution containing a specific amount of BQ or **1,** and the culture tubes were incubated in an Incu-Shaker for 24 h. The number of colonies on the agar plates was counted to determine the CFU. All measurements were carried out in triplicate.[2]

Bacterial reactive oxygen species (ROS) measurements: A previously described procedure was followed to determine the intracellular levels of bacterial reactive oxygen species (ROS) in MSSA. MSSA bacteria were exposed to various concentrations of BQ, **1**, and a control. The samples were incubated at 37°C with agitation for 2 h. Bacterial pellets were collected by centrifugation and washed with Hank's balanced salt solution (HBSS 1X). The bacterial suspensions were then incubated in the dark at 37°C with 20 μM of 2′,7′- dichlorofluorescein diacetate (DCFH-DA) for 30 min. The fluorescence intensity of the bacterial suspension was measured at 497/529 nm (excitation/emission) wavelength using a SpectraMax M4 microplate reader. This method allows for the measurement of ROS levels in MSSA and the comparison of the effects of BQ and **1** on

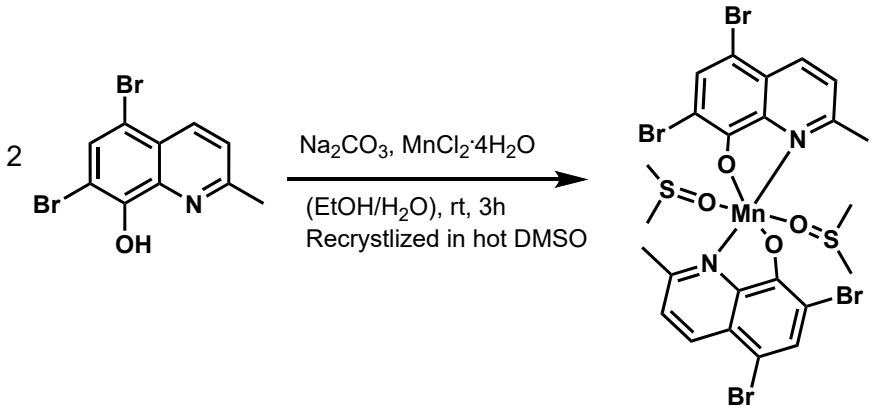
ROS production with a control sample. The previously described procedure was used as a reference for these measurements.[2]

***In vitro* assays of resistance development:** We carried out the resistance development assays by successive passaging of bacteria using a procedure described previously.[3,4] By serially exposing ciprofloxacin or **1** to the MSSA (ATCC 6538) for 24 days or 7 days, respectively, the potential for the development of resistance was determined. After 24-h incubation at 37 °C, bacteria grew at the highest concentration of ciprofloxacin or **1.** They were collected again, and MIC values were determined. This procedure was repeated for 30 passages for MSSA bacteria. The results were represented as the fold change in MIC with respect to the MIC value on day 1.

Cell viability (MTT) assays: The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method was used for the assessment of cell viability. The cells were seeded into 96-well plates with a volume of 100 μL cell suspensions (2×10^5 cells/mL), and incubation for a day was allowed at a controlled temperature of 37 °C and CO2 level of 5%. Following this, each well was treated with 100 μL of fresh medium containing different concentrations of **1**, and incubation was allowed for a further 24 hours. Post this incubation period, treatment with 10 μL of MTT reagent was given for 2 hours at 37 °C, followed by the addition of 100 μL of a DMSO solution to every well. The plate was then covered and left in darkness for another 2 hours at the same temperature. Absorbance at 570 nm was measured using a SpectraMax M4 microplate reader. The entire procedure was conducted in three independent instances. Lastly, the data gathered was processed with Origin and GraphPad Prism software to ascertain IC_{50} values. The results were shown as a percentage of the viable cells compared to the cells that received no treatment (control group).[5]



Scheme S1. Synthetic procedure for the preparation of BQ



Scheme S2. Synthetic procedure for the preparation of **1**

Table S1 Results of the elemental analysis of $[Mn(BQ)_2(DMSO)_2]$

Elements	$C_{24}H_{24}N_2O_4S_2Br_4Mn$	
	Calculated %	Experimental %
Carbon (C)	34.19	33.88
Hydrogen (H)	2.87	2.34
Nitrogen (N)	3.32	3.89

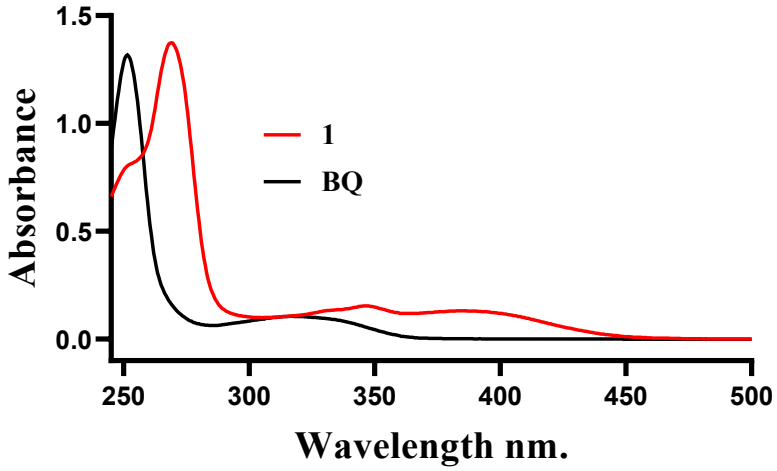


Figure S1. UV-vis spectra of **1** compared to BQ in DMSO (both spectra were recorded with a SHIMADZU UV-2401PC spectrophotometer in DMSO)

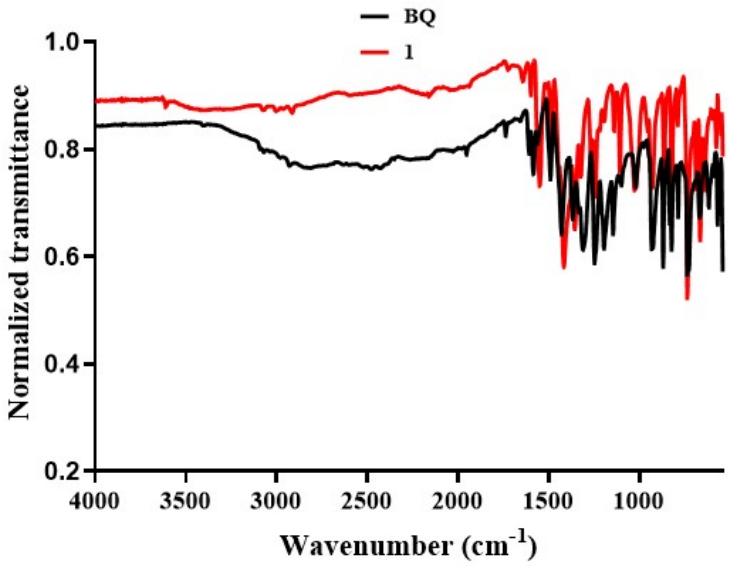


Figure S2. FT-IR spectra of **1** compared to BQ (both spectra were recorded with a Bruker Vector 33 Fourier Transform Infrared Spectrophotometer)

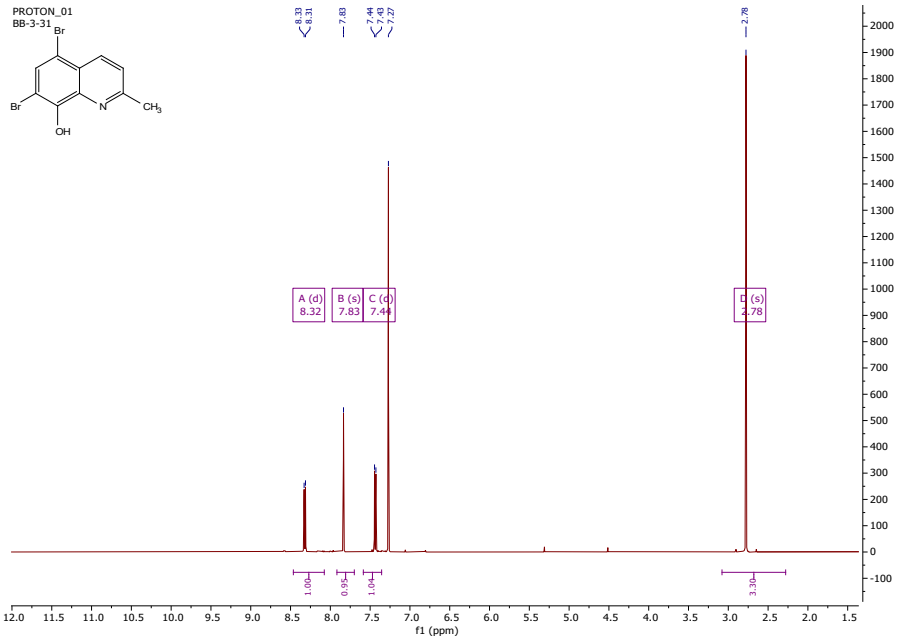


Figure S3. ^{1}H NMR spectrum of BQ in chloroform-d[1](500 MHz, $CDCl_3$): δ 8.32 (d, J = 8.6 Hz, 1H), 7.83 (s, 1H), 7.44 (d, J = 8.6 Hz, 1H), 2.78 (s, 3H) (Please note that the peak at 7.27 is the solvent peak)

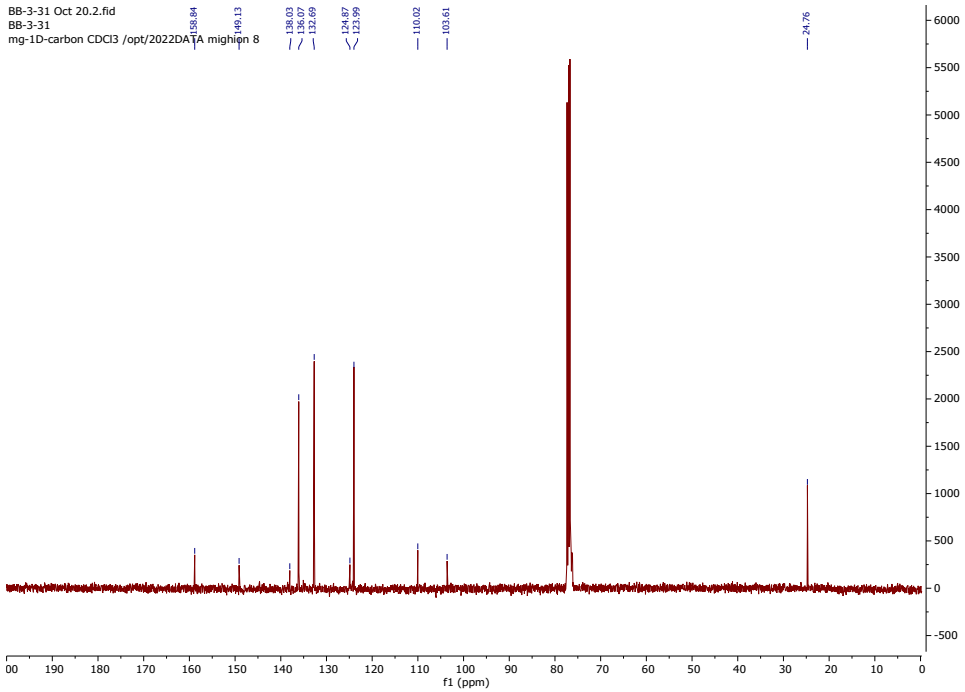


Figure S4. ^{13}C NMR spectrum of BQ in chloroform-d[1](500 MHz, $CDCl_3$): δ 158.84, 149.13, 138.03, 136.07, 132.69, 124.87, 123.99, 110.02, 103.61, 24.76 (Please note that the peak at 77.16 is the solvent peak)

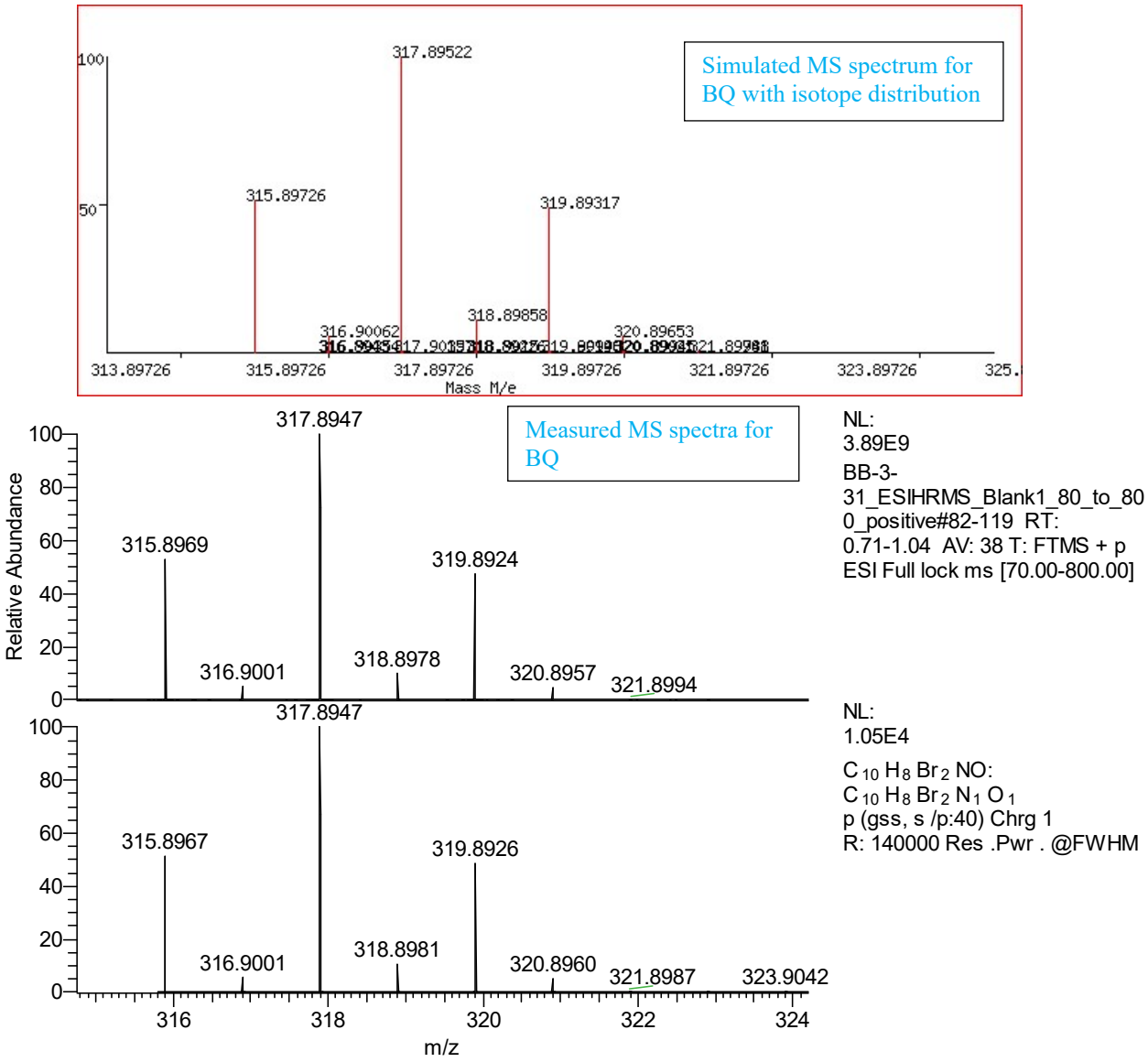


Figure S5. ESI-HRMS for BQ; $[M+H]^+ = C_{10}H_8Br_2NO^+$ (Bruker Esquire ESI-HRM was used to measure these MS spectra of BQ)

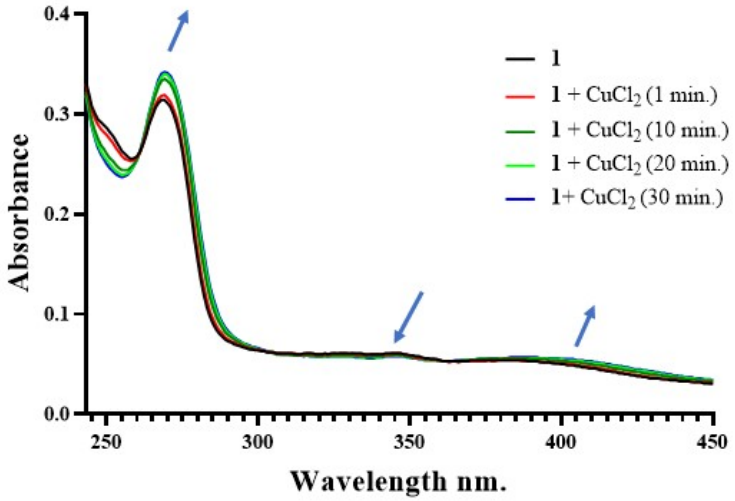


Figure S6. Metal-exchange reaction between **1** and $CuCl_2$ as followed by UV-VIS spectroscopy, showing the release of Mn(II) and concomitant conversion of **1** into $Cu(BQ)_2$ in solution (The concentration of **1** was 3.125µg/mL and the concentration of $CuCl_2$ was 30 µg/mL)

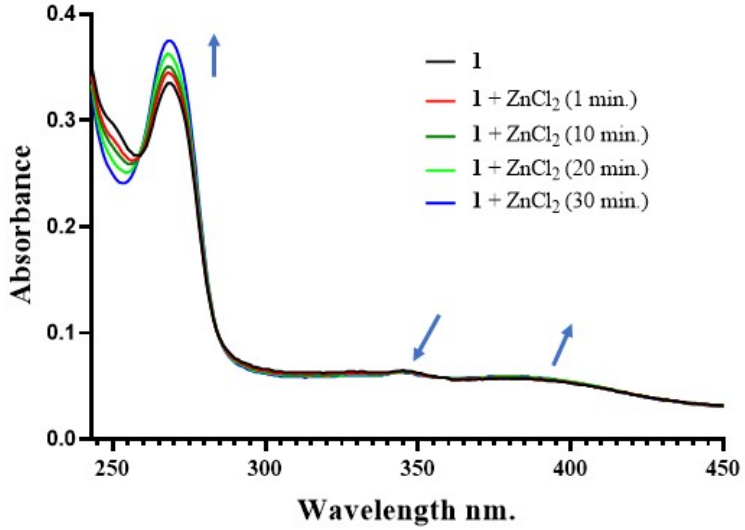


Figure S7. Metal-exchange reaction between **1** and $ZnCl_2$ as followed by UV-VIS spectroscopy, showing the release of Mn(II) and concomitant conversion of **1** into $Zn(BQ)_2$ in solution (The concentration of **1** was 3.125µg/mL and the concentration of $ZnCl_2$ was 30 µg/mL)

Table S2. Crystal data and refinement results of **1**.

CCDC deposition no.	**CCDC-2267020**	
Formula	$C_{24}H_{24}N_2O_4S_2Br_4Mn.C_2H_6OS$	
Formula weight	921.28	
Temperature	150(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	Cc	
Unit cell dimensions	a = 21.7849(4) Å	α = 90°
	b = 15.7021(4) Å	β = 112.1318(11)°
	c = 10.5287(3) Å	γ = 90°
Volume	3336.17(15) $Å^3$	
Z	4	
Density (calculated)	1.834 Mg/m^3	
Absorption coefficient	5.412 mm^{-1}	
F(000)	1812	
Crystal size	0.152 x 0.082 x 0.061 mm^3	
θ range for data collection	1.643 to 28.306°	
Index ranges	$-29 \le h \le 29, -20 \le k \le 20, -14 \le l \le 14$	

Reflections collected	41048
Independent reflections	8253 [R_{int} = 0.0300]
Completeness to θ = 25.242°	100.0 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.862 and 0.758
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	8253 / 2 / 379
Goodness-of-fit on F^2	0.982
Final R indices [I>2σ(I)]	R1 = 0.0237, wR2 = 0.0488
R indices (all data)	R1 = 0.0294, wR2 = 0.0500
Absolute structure parameter	0.014(6)
Largest diff. peak and hole	1.307 and -0.433 eÅ^{-3}

Table S3 Selected bond lengths [Å] and angles [°] in **1**.

Mn-O(1)	2.098(3)	Mn-O(2)	2.114(3)
Mn-O(4)	2.175(3)	Mn-O(3)	2.187(3)
Mn-N(2)	2.352(3)	Mn-N(1)	2.374(3)
Br(1)-C(2)	1.896(4)	Br(2)-C(4)	1.908(4)
Br(3)-C(12)	1.898(4)	Br(4)-C(14)	1.894(4)
S(1)-O(3)	1.501(3)	S(1)-C(21)	1.777(6)
S(1)-C(22)	1.786(6)	S(2)-O(4)	1.517(3)
S(2)-C(23)	1.771(4)	S(2)-C(24)	1.784(5)
O(1)-C(1)	1.285(4)	O(2)-C(11)	1.290(5)
N(1)-C(9)	1.334(5)	N(1)-C(6)	1.370(5)
N(2)-C(19)	1.324(5)	N(2)-C(16)	1.375(4)
C(1)-C(2)	1.394(5)	C(1)-C(6)	1.449(5)
C(2)-C(3)	1.403(5)	C(3)-C(4)	1.357(6)
C(4)-C(5)	1.416(5)	C(5)-C(7)	1.410(6)
C(5)-C(6)	1.422(5)	C(7)-C(8)	1.359(6)
C(8)-C(9)	1.408(6)	C(9)-C(10)	1.488(6)
C(11)-C(12)	1.399(5)	C(11)-C(16)	1.449(5)
C(12)-C(13)	1.391(5)	C(13)-C(14)	1.367(6)
C(14)-C(15)	1.421(5)	C(15)-C(16)	1.407(5)
C(15)-C(17)	1.419(6)	C(17)-C(18)	1.366(6)
C(18)-C(19)	1.423(5)	C(19)-C(20)	1.500(5)
S(3)-O(5)	1.500(3)	S(3)-C(26)	1.769(4)
S(3)-C(25)	1.787(5)		

O(1)-Mn-O(2)	177.50(11)	O(1)-Mn-O(4)	85.89(10)
O(2)-Mn-O(4)	93.39(10)	O(1)-Mn-O(3)	91.22(11)
O(2)-Mn-O(3)	86.42(11)	O(4)-Mn-O(3)	92.89(11)
O(1)-Mn-N(2)	108.43(11)	O(2)-Mn-N(2)	73.93(10)

O(4)-Mn-N(2)	88.94(10)	O(3)-Mn-N(2)	160.34(11)
O(1)-Mn-N(1)	74.05(10)	O(2)-Mn-N(1)	107.00(11)
O(4)-Mn-N(1)	158.18(11)	O(3)-Mn-N(1)	95.99(12)
N(2)-Mn-N(1)	89.34(11)	O(3)-S(1)-C(21)	106.6(3)
O(3)-S(1)-C(22)	105.6(2)	C(21)-S(1)-C(22)	98.1(3)
O(4)-S(2)-C(23)	104.14(18)	O(4)-S(2)-C(24)	105.17(19)
C(23)-S(2)-C(24)	98.2(2)	C(1)-O(1)-Mn	119.5(2)
C(11)-O(2)-Mn	118.9(2)	S(1)-O(3)-Mn	141.02(19)
S(2)-O(4)-Mn	126.87(16)	C(9)-N(1)-C(6)	118.8(3)
C(9)-N(1)-Mn	131.2(3)	C(6)-N(1)-Mn	110.0(2)
C(19)-N(2)-C(16)	119.0(3)	C(19)-N(2)-Mn	130.3(2)
C(16)-N(2)-Mn	110.5(2)	O(1)-C(1)-C(2)	124.1(3)
O(1)-C(1)-C(6)	121.0(3)	C(2)-C(1)-C(6)	114.8(3)
C(1)-C(2)-C(3)	123.9(4)	C(1)-C(2)-Br(1)	117.6(3)
C(3)-C(2)-Br(1)	118.5(3)	C(4)-C(3)-C(2)	119.9(4)
C(3)-C(4)-C(5)	121.3(4)	C(3)-C(4)-Br(2)	118.8(3)
C(5)-C(4)-Br(2)	119.9(3)	C(7)-C(5)-C(4)	125.2(4)
C(7)-C(5)-C(6)	117.0(4)	C(4)-C(5)-C(6)	117.9(3)
N(1)-C(6)-C(5)	122.5(3)	N(1)-C(6)-C(1)	115.4(3)
C(5)-C(6)-C(1)	122.2(3)	C(8)-C(7)-C(5)	119.6(4)
C(7)-C(8)-C(9)	120.8(4)	N(1)-C(9)-C(8)	121.4(4)
N(1)-C(9)-C(10)	118.0(4)	C(8)-C(9)-C(10)	120.6(4)
O(2)-C(11)-C(12)	124.6(3)	O(2)-C(11)-C(16)	120.7(3)
C(12)-C(11)-C(16)	114.7(3)	C(13)-C(12)-C(11)	123.3(4)
C(13)-C(12)-Br(3)	119.6(3)	C(11)-C(12)-Br(3)	117.0(3)
C(14)-C(13)-C(12)	120.7(3)	C(13)-C(14)-C(15)	120.1(3)
C(13)-C(14)-Br(4)	119.2(3)	C(15)-C(14)-Br(4)	120.7(3)
C(16)-C(15)-C(17)	117.0(3)	C(16)-C(15)-C(14)	118.3(3)
C(17)-C(15)-C(14)	124.7(4)	N(2)-C(16)-C(15)	122.7(3)
N(2)-C(16)-C(11)	114.9(3)	C(15)-C(16)-C(11)	122.4(3)
C(18)-C(17)-C(15)	119.9(4)	C(17)-C(18)-C(19)	119.6(4)
N(2)-C(19)-C(18)	121.7(3)	N(2)-C(19)-C(20)	117.7(3)
C(18)-C(19)-C(20)	120.5(4)	O(5)-S(3)-C(26)	105.8(2)
O(5)-S(3)-C(25)	107.4(2)	C(26)-S(3)-C(25)	96.6(2)

References

1. Abeydeera, N., Yu, B., Pant, B. D., Kim, M.-H., Huang, S. D., Biomater. Sci. 2022, 10, 474.
2. Abeydeera, N., Benin, B.M., Mudarmah, K., Pant, B.D., Chen, G., Shin, W.S., Kim, M.H. and Huang, S.D., Antibiotics 2023, 12, 886.
3. Baylay, A. J., Piddock, L. J., Webber, M. A., 2019, 1–26.
4. Dassanayake, T. M., Dassanayake, A. C., Abeydeera, N., Pant, B. D., Jaroniec, M., Kim, M.-H., Huang, S. D., Sci. 2021, 9 (23), 7996–8006.
5. Abeydeera, N.; Stilgenbauer, M.; Pant, B.D.; Mudarmah, K.; Dassanayake, T.M.; Zheng,Y.-R.; Huang, S.D. *Molecules* **2023**, *28*, 4917.