Supporting Information

Novel valproate half-sandwich rhodium and iridium conjugates to fight against multidrug-resistant Gram-positive bacteria

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1. General Materials and Methods

Materials. [Rh(η₅-C₅Me₅)Cl₂]₂ and IrCl₃ were used as received (Johnson Matthey). Deuterated solvents were purchased from Euriso-top. Other reagents were commercially available and were used as received.

Physical Measurements. ¹H and ¹³C(¹H) NMR spectra were recorded on a Bruker AC 300E, Bruker AV 400, or Bruker AV 600 NMR spectrometer and chemical shifts are reported in ppm and cited relative to SiMe₄ and using the residual proton impurities in the solvents for ¹H and ¹³C(¹H) NMR spectroscopy. Peak multiplicities are abbreviated as: s = singlet, m = multiplet, d = doublet, t = triplet, dd = doublet of doublet.

The C, H, and N analyses were performed with a Carlo Erba model EA 1108 microanalyzer, with an EAGER 200 software. The combustion of the samples was carried out in the presence of V₂O₅ and MgO as additives. The duration of the analysis was 15 minutes.

The UV/Vis spectra were registered in a Perkin-Elmer Lambda 750 S spectrometer. ESI mass (positive mode) analyses were carried out in a RP-HPLC/MS TOF 6220 equipped with a double binary pump (model G1312A), degasser, autosampler (model G1329A), diode array detector (model G1315D), and mass detector in series (Agilent Technologies 1200). Chromatographic analyses were performed with a Brisa C18 column (150 mm x 4.6 mm, 5 µm particle size); Teknokroma, Macclesfield, UK. The mobile phase was a mixture of (A) H₂O/HCOOH 0.1% and (B) acetonitrile/HCOOH 0.1%. The flow rate was 06 mL/min in a linear gradient. Chromatograms were recorded at λ=280 nm. The HPLC system was controlled by a ChemStation software (MASS HUNTER). The mass detector was an ion-trap spectrometer equipped with a dual-source electrospray APCI. Mass spectrometry data were acquired in the positive ionization mode. The ionization conditions were adjusted at 3508°C and 3 kV for capillary temperature and voltage, respectively.

Table S1. HPLC method

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<th>0.1 % formic acid in CH₃CN</th>
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2. Synthesis Procedures

Synthesis of H_{C}^{N} ligands

**Ligand HL1.** The synthesis of HL1 was carried out after slight modifications of bibliography. The diamine (c, methyl 3-amino-4-(butylamino)benzoate) was synthesized as previously reported by our group.1 The condensation of the aldehyde and the diamine (Scheme S1) was carried out following the procedure described in the literature.2 Briefly, after the solution of NaHSO₃ (24.2 mmol, 2.5 g) in 4 mL of water, 4-hydroxybenzaldehyde (2.42 mmol, 295 mg) was added and stirred 30 min at 100 ºC. Then, the diamine c (2.42 mmol, 540 mg) was solved in 4 mL of ethanol and added to the reaction mixture that was stirred for 18 h at 80 ºC. The precipitate was filtered and washed with water and hexane. A white solid of HL1 was obtained (628 mg, yield: 80%).

**Scheme S1.** Synthesis of H_{C}^{N} ligand HL1.

**HL1:** 80%. 80%. 1H NMR (400 MHz, CDCl₃) δH/ppm = 11.55 (s, 1H, Ar-OH), 8.54 (d, 4J = 1.6 Hz, 1H, Hₐ), 8.06 (dd, 34J = 8.6, 1.6 Hz, 1H, Hₐ), 7.45 (d, 3J = 8.6 Hz, 1H, Hₐ), 7.42 (d, 3J = 8.7 Hz, 2H, Hₐ), 6.85 (d, 3J = 8.7 Hz, 2H, Hₐ), 4.25 (t, 3J = 7.7 Hz, 2H, Hₐ), 3.95 (s, 3H, OCH₃), 1.88 – 1.73 (m, 2H, Hₐ), 0.89 (t, 3J = 7.3 Hz, 3H, Hₐ). 13C{1H} NMR (101 MHz, CDCl₃) δC/ppm = 167.5, 160.6, 156.1, 140.5, 138.1, 130.8 (Cₐ), 125.3, 124.8 (Cₐ), 120.9 (Cₐ), 118.4, 116.6 (Cₐ), 110.2 (Cₐ), 52.3 (OCH₃), 45.1 (Cₐ), 31.8 (Cₐ), 20.1 (Cₐ), 13.7 (Cₐ). HR-ESMS: m/z calc. for [M+H]⁺ at 325.1547, found at 325.1550. Anal. Calc. for C_{19}H_{20}N_{2}O_{3}: %C, 70.35; %H, 6.21; %N, 8.64. Found: %C, 70.42; %H, 6.28; %N, 8.56.

**Ligand HL2.** The synthesis of HL2 was carried out in different steps (see Scheme S2).3 In the first step, a solution of valproic acid (13.9 mmol, 1 g) in 6 mL of SOCl₂ was stirred at room temperature for 4 h under N₂ atmosphere. The mixture was evaporated and 2 mL of DMF were added. In the second step, 4-hydroxybenzaldehyde (12 mmol, 1.47 g) and triethylamine (72 mmol, 10 mL) were solved in 3 mL of dry DMF. Finally, the solution containing the chloride of valproate was added drop by drop into the solution containing
the aldehyde and stirred overnight at room temperature under inert atmosphere. After the reaction time, 50 mL of DCM were added and the mixture was extracted with 5x100 mL of water. The resulting solid was purified by silica gel column chromatography using DCM:MeOH (95:5) as eluent. A yellow oil of intermediate b was obtained (1.46 g, yield: 49%).

The last step consists on the condensation of the aldehyde and the diamine and was carried out following the procedure described in the literature. After the solution of NaHSO₃ (24.2 mmol, 2.5 g) in 4 mL of water, intermediate b (2.42 mmol, 600 mg) was added and stirred 30 min at 100 ºC. Then, the diamine c (2.42 mmol, 540 mg) was solved in 4 mL of ethanol and added to the reaction mixture that was stirred for 18 h at 80 ºC. The mixture was evaporated, solved in 50 mL of DCM and extracted with 50 mL of water. The oil obtained was purified by silica gel column chromatography with EtOAc:Hex (1:3) as eluent and a yellow oil of HL2 was obtained (818 mg, yield: 75%).

**Scheme S2. Synthesis of HC^N ligand HL2.**

**HL2:** 75%. ¹H NMR (300 MHz, CDCl₃) δH /ppm = 8.55 (d, ⁴J = 1.5 Hz, 1H, Hₐ), 8.06 (dd, ³⁴J = 8.5, 1.5 Hz, 1H, Hₖ), 7.77 (d, ³J = 8.6 Hz, 2H, H₂), 7.45 (d, ³J = 8.5 Hz, 1H, H₄), 7.28 (d, ³J = 8.6 Hz, 2H, H₅), 4.27 (t, ³J = 7.3 Hz, 2H, H₆), 3.96 (s, 3H, OCH₃), 2.72 – 2.59 (m, 1H, H₇), 1.89 – 1.69 (m, 4H, H₈–H₁₀), 1.66 – 1.53 (m, 2H, H₁₁–H₁₂), 1.52 – 1.38 (m, 4H, H₁₃–H₁₅), 1.37 – 1.22 (m, 2H, H₁₆), 0.98 (t, ³J = 7.2 Hz, 6H, H₁₇), 0.89 (t, ³J = 7.2 Hz, 3H, H₁₈). ¹³C{¹H} NMR (75 MHz, CDCl₃) δC /ppm = 174.8, 167.5, 154.4, 152.7, 138.6, 130.8 (CD), 126.8, 125.3, 124.9 (C₈), 122.5 (C₉), 122.1 (C₆), 110.1 (C₅), 52.3 (OCH₃).
Synthesis of [MCp*Cl(C^N)] (M = Ir, Rh) complexes

All complexes were synthetized under an N\textsubscript{2} atmosphere using standard Schlenk techniques as previously reported (Scheme S3).\textsuperscript{4} [Ir(n\textsuperscript{5}-C\textsubscript{5}Me\textsubscript{5})Cl\textsubscript{2}]\textsubscript{2} was synthesized by reaction of IrCl\textsubscript{3} (1 eq.) and pentamethylcyclopentadienyl (1.4 eq.) in methanol for 24 h at room temperature. The orange solid was isolated and dry under vacuum. For the synthesis of the complexes, a solution of the dimer (1 eq.), the corresponding ligand, HL\textsubscript{1} or HL\textsubscript{2} (2 eq.) and sodium acetate (2.5 eq.) in 10 mL of CH\textsubscript{2}Cl\textsubscript{2} was stirred for 24 h at room temperature. The precipitate was filtered and washed with diethyl ether. A pure yellow/orange solid was obtained for all the complexes.

![Scheme S3. General procedure for the synthesis of complexes IrL1-2 and RhL1-2.](image)

**IrL1**: 87%. \textsuperscript{1}H NMR (600 MHz, DMF-d\textsubscript{7}) \textit{\delta}_{H}/ppm = 9.97 (s, 1H, Ar-OH), 8.35 (d, \textsuperscript{4}J = 1.8 Hz, 1H, H\textsubscript{A}), 7.99 (dd, \textsuperscript{34}J = 8.6, 1.8 Hz, 1H, H\textsubscript{B}), 7.89 (d, \textsuperscript{5}J = 8.6 Hz, 1H, H\textsubscript{C}), 7.83 (d, \textsuperscript{3}J = 8.5 Hz, 1H, H\textsubscript{D}), 7.57 (d, \textsuperscript{4}J = 2.5 Hz, 1H, H\textsubscript{E}), 6.65 (dd, \textsuperscript{34}J = 8.5, 2.5 Hz, 1H, H\textsubscript{F}), 4.75 (t, \textsuperscript{3}J = 7.6 Hz, 2H, OCH\textsubscript{3}), 3.99 (s, 3H, OCH\textsubscript{3}), 1.95 – 1.88 (m, 2H, H\textsubscript{I}), 1.78 (s, 15H, H\textsubscript{K}), 1.52 – 1.44 (m, 2H, H\textsubscript{J}), 0.94 (t, \textsuperscript{3}J = 7.4 Hz, 3H, H\textsubscript{J}). \textsuperscript{13}C\textsuperscript{(1)}H NMR (151 MHz, DMF-d\textsubscript{7}) \textit{\delta}_{C}/ppm = 169.2, 167.0, 164.3, 160.2, 139.9, 139.7, 126.5 (C\textsubscript{D}), 125.4, 124.6, 123.9 (C\textsubscript{E}), 123.6 (C\textsubscript{F}), 117.9 (C\textsubscript{A}), 111.1 (C\textsubscript{C}), 110.16 (C\textsubscript{R}), 88.2, 52.1 (OCH\textsubscript{3}), 44.6 (C\textsubscript{G}), 31.9 (C\textsubscript{H}), 19.9 (C\textsubscript{I}), 13.6 (C\textsubscript{J}), 9.4 (C\textsubscript{K}). HR ESI-MS: \textit{m/z} calc. for [M-Cl]\textsuperscript{-} at 651.2199, found at 651.2191. Anal. Calc. for C\textsubscript{29}H\textsubscript{34}ClIrN\textsubscript{2}O\textsubscript{3}: %C, 50.76; %H, 4.99; %N, 4.08. Found: %C, 50.77; %H, 4.83; %N, 4.01.
**RhL1**: 67%. $^1$H NMR (600 MHz, DMF-$d_7$) δH /ppm = 9.99 (s, 1H, Ar-OH), 8.40 (d, $^4J = 1.5$ Hz, 1H, H$_A$), 7.98 (dd, $^{34}J = 8.6$, 1.5 Hz, 1H, H$_B$), 7.90 (d, $^3J = 8.6$ Hz, 1H, H$_C$), 7.78 (d, $^3J = 8.5$ Hz, 1H, H$_D$), 7.55 (d, $^4J = 2.4$ Hz, 1H, H$_E$), 6.66 (dd, $^{34}J = 8.5$, 2.4 Hz, 1H, H$_F$), 4.77 – 4.67 (m, 2H, H$_G$), 4.00 (s, 3H, OCH$_3$), 1.94 – 1.86 (m, 2H, H$_H$), 1.70 (s, 1H, H$_I$), 1.50 – 1.42 (m, 2H, H$_J$), 0.94 (t, $^3J = 7.4$ Hz, 3H, H$_K$). $^{13}$C($^1$H) NMR (151 MHz, DMF-$d_7$) δC /ppm = 167.1, 160.3, 158.9, 140.2, 140.1, 125.9 (C$_D$), 125.8, 124.8 (C$_E$), 124.5, 123.5 (C$_F$), 118.2 (C$_A$), 111.1 (C$_C$), 110.8 (C$_F$), 95.8, 95.8, 52.1 (OCH$_3$), 44.7 (C$_G$), 31.9 (C$_H$), 19.9 (C$_I$), 13.6 (C$_J$), 9.4 (C$_K$). HR ESI-MS: m/z calc. for [M-Cl]$^+$ at 561.1625, found at 561.1616. Anal. Calc. for C$_{29}$H$_{34}$ClN$_2$O$_3$Rh: %C, 58.35; %H, 5.74; %N, 4.69. Found: %C, 58.40; %H, 5.57; %N, 4.57.

**IrL2**: 46%. $^1$H NMR (600 MHz, CDCl$_3$) δH /ppm = 8.44 (d, $^4J = 1.5$ Hz, 1H, H$_A$), 8.03 (dd, $^{34}J = 8.6$, 1.5 Hz, 2H, H$_D$), 7.70 (dd, $^{34}J = 8.4$, 2.2 Hz, 2H, H$_D$); 6.82 (dd, $^{34}J = 8.4$, 2.2 Hz, 1H, H$_C$), 4.58 – 4.41 (m, 2H, H$_G$), 3.98 (s, 3H, OCH$_3$), 2.70 – 2.64 (m, 1H, H$_L$), 2.03 – 1.95 (m, 2H, H$_H$), 1.85 – 1.80 (m, 2H, H$_M$), 1.78 (s, 1H, H$_I$), 1.54 – 1.44 (m, 2H, H$_N$), 1.55 – 1.44 (m, 2H, H$_{I+O}$), 1.04 – 0.97 (m, 9H, H$_{J+P}$). $^{13}$C($^1$H) NMR (151 MHz, CDCl$_3$) δC /ppm = 175.5, 167.9, 167.2, 163.1, 152.3, 139.4, 139.3, 131.2, 130.1 (C$_{DE}$), 125.4, 125.0 (C$_{DE}$), 124.6 (C$_B$), 119.1 (C$_A$), 115.4 (C$_F$), 109.9 (C$_C$), 88.6, 52.4 (OCH$_3$), 45.8 (C$_L$), 45.1 (C$_G$), 35.0 (C$_{MN}$), 34.9 (C$_{MN}$), 20.9, 20.9 (C$_{IO}$), 20.4 (C$_{IO}$), 14.2 (C$_{JP}$), 13.9 (C$_{JP}$), 9.7 (C$_K$). HR ESI-MS: m/z calc. for [M-Cl]$^+$ at 777.3243, found at 777.3241. Anal. Calc. for C$_{37}$H$_{48}$ClIrN$_2$O$_4$: %C, 54.70; %H, 5.96; %N, 3.45. Found: %C, 54.68; %H, 5.87; %N, 3.42.
RhL2: 34%. ¹H NMR (401 MHz, CDCl₃) δH /ppm = 8.50 (d, ⁴J = 1.6 Hz, 1H, Hₐ), 8.02 (dd, ³⁴J = 8.6, 1.6 Hz, 1H, Hₖ), 7.71 (d, ⁴J = 2.3 Hz, 1H, Hₑ), 7.65 (d, ³J = 8.4 Hz, 1H, Hₙ), 7.40 (d, ³J = 8.6 Hz, 1H, Hₖ), 6.84 (dd, ⁴₃J = 8.4, 2.3 Hz, 1H, Hₖ), 4.53 – 4.38 (m, 2H, Hₖ), 3.98 (s, 3H, OCH₃), 2.72 – 2.63 (m, 1H, Hₖ), 2.02 – 1.91 (m, 2H, Hₖ), 1.87 – 1.76 (m, 2H, Hₖ), 1.60 – 1.56 (m, 2H, Hₖ), 1.54 – 1.44 (m, 6H, Hₖ), 1.04 – 0.97 (m, 9H, Hₖ). ¹³C(¹H) NMR (101 MHz, CDCl₃) δC /ppm = 175.5, 167.3, 159.0, 151.2, 139.8, 139.6, 131.7, 131.0 (Cₑ), 125.2, 124.6 (Cₙ), 124.5 (Cₖ), 119.5 (Cₖ), 116.3 (Cₖ), 109.9 (Cₙ), 96.2, 96.2, 52.4 (OCH₃), 45.8 (Cₖ), 45.2 (Cₖ), 34.9 (Cₖ), 34.9 (Cₖ), 31.9 (Cₖ), 20.9, 20.9 (Cₖ), 14.2 (Cₖ), 13.9 (Cₖ), 9.9 (Cₖ). HR ESI-MS: m/z calc. for [M-Cl]+ at 687.2665, found at 687.2681. Anal. Calc. for C₃7H₄₈Cl₆N₂O₄Rh: %C, 61.45; %H, 6.69; %N, 3.87. Found: %C, 61.41; %H, 6.65; %N, 3.72.
3. NMR spectra of new compounds

Figure S1. $^1$H NMR spectrum of HL1, 400 MHz, CDCl$_3$.

Figure S2. $^{13}$C NMR spectrum of HL1, 101 MHz, CDCl$_3$. 
**Figure S3.** HSQC 2D $^1$H-$^{13}$C NMR spectrum of HL1, 400 MHz, CDCl$_3$.

**Figure S4.** $^1$H NMR spectrum of HL2, 300 MHz, CDCl$_3$. 
Figure S5. $^{13}$C NMR spectrum of HL2, 75 MHz, CDCl$_3$.

Figure S6. COSY 2D $^1$H-$^1$H NMR spectrum of HL2, 300 MHz, CDCl$_3$. 
Figure S7. HSQC 2D $^1$H-$^{13}$C NMR spectrum of HL2, 300 MHz, CDCl$_3$.

Figure S8. $^1$H NMR spectrum of IrL1, 600 MHz, DMF-$d_7$. 
Figure S9. $^{13}$C NMR spectrum of IrL1, 151 MHz, DMF-$d_7$.

Figure S10. COSY 2D $^1$H-$^1$H NMR spectrum of IrL1, 600 MHz, DMF-$d_7$. 
Figure S11. HSQC 2D $^1$H-$^{13}$C NMR spectrum of IrL1, 600 MHz, DMF-$d_7$.

Figure S12. $^1$H NMR spectrum of RhL1, 600 MHz, DMF-$d_7$. 
Figure S13. $^{13}$C NMR spectrum of RhL1, 151 MHz, DMF-$d_7$.

Figure S14. COSY 2D $^1$H-$^1$H NMR spectrum of RhL1, 600 MHz, DMF-$d_7$. 
Figure S15. HSQC 2D $^1$H-$^1$C NMR spectrum of RhL1, 600 MHz, DMF-$d_7$.

Figure S16. $^1$H NMR spectrum of IrL2, 600 MHz, CDCl$_3$.
Figure S17. $^{13}$C NMR spectrum of IrL2, 151 MHz, CDCl$_3$.

Figure S18. COSY 2D $^{1}$H-$^{1}$H NMR spectrum of IrL2, 600 MHz, CDCl$_3$. 
Figure S19. NOESY 2D $^1$H-$^1$H NMR spectrum of IrL2, 600 MHz, CDCl₃.

Figure S20. HSQC 2D $^1$H-$^{13}$C NMR spectrum of IrL2, 600 MHz, CDCl₃.
Figure S21. $^1$H NMR spectrum of RhL2, 400 MHz, CDCl$_3$.

Figure S22. $^{13}$C NMR spectrum of RhL2, 101 MHz, CDCl$_3$. 
Figure S23. COSY 2D $^1$H-$^1$H NMR spectrum of RhL2, 400 MHz, CDCl$_3$.

Figure S24. NOESY 2D $^1$H-$^1$H NMR spectrum of RhL2, 400 MHz, CDCl$_3$. 
Figure S25. HSQC 2D $^1$H-$^{13}$C NMR spectrum of RhL2, 400 MHz, CDCl$_3$. 
4. Mass spectrometry

Figure S26. ESI-MS spectrum of HL1 (positive detection mode).
Figure S27. ESI-MS spectrum of HL2 (positive detection mode).
Figure S28. ESI-MS spectrum of IrL1 (positive detection mode).

Figure S29. ESI-MS spectrum of RhL1 (positive detection mode).
Figure S30. ESI-MS spectrum of IrL2 (positive detection mode).

Figure S31. ESI-MS spectrum of RhL2 (positive detection mode).
5. RP-HPLC of the complexes

![RP-HPLC of complexes in DMF](image)

**Figure S32.** HPLC of complexes in DMF.

6. UV/Vis spectra

![UV/Vis spectra](image)

**Figure S33.** Visible spectra of complexes in acetonitrile and water (1% DMF), 10 µM.
7. Stability study

*Stability in DMSO-d₆ by NMR*

![NMR spectra](image)

**Figure S34.** Stability of complex IrL1 in DMSO-d₆ by ¹H NMR at t= 0, 30 min and 1, 7 and 24 hours at r. t.
Figure S35. Stability of complex RhL1 in DMSO-$d_6$ by $^1$H NMR at t = 5 min and after 24 hours at r. t.

Stability in DMF-$d_7$ by NMR

Figure S36. Stability of complex IrL1 in DMF-$d_7$ by $^1$H NMR after 48 hours at r. t.
Figure S37. Stability of complex RhL1 in DMF-d7 by ^1H NMR after 48 hours at r. t.

Figure S38. Stability of complex IrL2 in DMF-d7 by ^1H NMR after 48 hours at r. t.

Figure S39. Stability of complex RhL2 in DMF-d7 by ^1H NMR after 48 hours at r. t.

Stability in RPMI (5% DMF) by UV/Vis

Figure S40. Stability of complexes in RPMI (5% DMSO) (10 µM) by UV/Vis after 24 hours of incubation at 37 °C.
8. Hydrolysis study of complex RhL2 by NMR

![Hydrolysis study of complex RhL2](image)

**Figure S41.** Hydrolysis study of complex RhL2 1 mM in MeOD:D$_2$O (2:1) by $^1$H NMR, 600 MHz at r.t. After 5 min (blue line), after 24 hours (red line), after 24 hours and the addition of Cl$^-$ (4 mM) (green line) and after 24 hours and the addition of Cl$^-$ (23 mM) (purple line).

9. X-Ray crystallographic analysis for RhL2

**X-Ray Structure Determinations.** Intensities were registered at low temperature on a Bruker D8QUEST diffractometer using monochromated Mo Kα radiation ($\lambda = 0.71073$Å). Absorption corrections were based on multi-scans (program SADABS).$^5$ Structures were refined anisotropically using SHELXL-2018.$^6$ Hydrogen atoms were included using rigid methyl groups or a riding model.

**Table S2.** Crystal data and structure refinement for AMC_23_0msp_a.

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<tr>
<td>Final R indices [I&gt;2sigma(I)]</td>
<td>R1 = 0.0260, wR2 = 0.0561</td>
</tr>
<tr>
<td>R indices (all data)</td>
<td>R1 = 0.0311, wR2 = 0.0612</td>
</tr>
<tr>
<td>Largest diff. peak and hole</td>
<td>1.298 and -0.638 e.Å⁻³</td>
</tr>
</tbody>
</table>
10. Antibacterial activity: MIC and MBC determination.

**Bacterial strains:** *S. aureus* CECT 5190 (methicillin resistant, Gram positive bacteria), *A. baumannii* ATCC 17978 (Gram negative bacteria) and *P. aeruginosa* PAO1 (Gram negative bacteria) were maintained in Mueller–Hinton (MH) broth or agar at 37 °C whereas *E. faecium* CECT 5253 (vancomycin resistant, Gram positive bacteria) was maintained in tryptic soy (TS) broth or agar.

Minimum Inhibitory concentration (MIC) determination was performed according to the broth microdilution plate method following CLSI criteria (Performance standards for antimicrobial susceptibility testing: 17th informational supplement M07-A9, Clinical and Laboratory Standards Institute., 2012) as previously described. The reported MIC values are the mean values of at least two independent experiments with four replicates.

Minimum bactericidal concentrations (MBC) were determined by plating 10 μL of the sample solutions from each well with no visible growth in MIC determination plates in MHA and then, by counting the number of colonies after 20 h of incubation at 37 °C. The concentration at which no colonies were grown corresponds to the MBC values.
11. Accumulation of metal complexes in bacteria

Bacterial inocula in the log phase were adjusted to 0.5 McFarland and then diluted 1:100 in conical tubes with 5 mL of fresh MHB and 1 μM of the metal complexes. After overnight incubation at 37 °C and 120 rpm, samples were centrifuged at 8000 rpm for 10 min. The pellet was washed twice with phosphate buffered saline (PBS) and finally, resuspended in 1 mL of PBS. 20 μL were used to record the OD600 with a microplate reader (Cytation 5 cell imaging multi-mode reader (Biotek Instruments, USA)). Then, samples were diluted in 3 mL of MilliQ water and digested with 65% HNO₃ for 24 h before the analysis with an 8900 ICP-MS (Agilent Technologies). Data are reported as the mean of two independent experiments with two replicates for each condition assayed.

12. References