RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2016, **6**, 86695

Copper(II) complexes with aromatic nitrogen-containing heterocycles as effective inhibitors of quorum sensing activity in *Pseudomonas aeruginosa*†

Biljana Đ. Glišić,*[a] Ivana Aleksic,[b] Peter Comba,*[c] Hubert Wadepohl,[c] Tatjana Ilic-Tomic,[b] Jasmina Nikodinovic-Runic*[b] and Miloš I. Djuran[a]

Five copper(II) complexes **1–5** with aromatic nitrogen-containing heterocycles, pyrimidine (pm, **1**), pyrazine (pz, **2**), quinazoline (qz, **3** and **4**) and phthalazine (phtz, **5**) have been synthesized and structurally characterized by spectroscopic and single-crystal X-ray diffraction techniques. The crystallographic results show that, dependent on the ligand structure, complexes **1–5** are of different nuclearity. The antimicrobial efficiency of complexes **1–5** has been evaluated against three clinically relevant microorganisms and none of the complexes showed significant growth inhibiting activity, with values of minimum inhibitory concentrations (MIC) in the mM range. Since in many bacteria, pathogenicity and virulence are regulated by intercellular communication processes, quorum sensing (QS), the effect of the copper(II) complexes on bacterial QS has also been examined. The results indicate that the investigated complexes inhibit violacein production in *Chromobacterium violaceum* CV026, suggesting an anti-QS activity. In order to differentiate, which of the QS pathways was affected by the copper(II) complexes, three biosensor strains were used: the PAO1 ΔrhlIpKD-rhlA and the PA14-R3ΔlasIPrsaI lux strain to directly measure the levels of C4-HSL (*N*-butanoyl-homoserine lactone) and 3OC12-HSL (*N*-3-oxo-dodecanoyl-homoserine lactone), respectively, and PAO1 ΔpqsA mini-CTX luxPpqsA for the detection of AHQs (2-alkyl-4-quinolones). Complexes **1–5** were shown to be efficient inhibitors of biofilm formation of the human opportunistic pathogen *Pseudomonas aeruginosa* PAO1, with the qz-containing complex **3** being the most active. Finally, the most anti-QS-active complexes **1** and **3** showed synergistic activity against a multi-drug resistant clinical isolate of *P. aeruginosa*, when supplied in combination with the known antibiotics piperacillin and ceftazidime.

Received 6th August 2016
Accepted 5th September 2016

DOI: 10.1039/c6ra19902j

www.rsc.org/advances

Introduction

Six first row d-block metals, manganese, iron, cobalt, nickel, copper and zinc, are essential micronutrients in all living organisms and the concept of trace metal homeostasis, in which various cellular actions maintain the fine balance between nutrition, immunity and toxicity, is well developed.[1,2] Copper is an essential trace element for virtually all organisms, yet its accumulation beyond homeostatic levels is highly toxic in bacterial cells, fungi and mammals.[3] Based on the ability of copper to cycle between reduced (Cu^{I}) and oxidized (Cu^{II}) states, this metal serves as a cofactor for enzymes that generate ATP and mature hormones, function in neurotransmitter biogenesis and disproportionation of superoxide anions, and pump iron across membranes.[4] In one form or another, copper has been used through the ages as a potent antimicrobial agent to sterilize water and treat wounds, and currently, it is used as an antimicrobial surface in veterinary and healthcare settings, where studies have shown a reduction in nosocomial infection in hospitals that have implemented the use of copper surfaces on doorknobs, handrails and other surfaces.[5,6]

Simple inorganic salts of copper are usually toxic, but as a transition metal ion it forms a large number of complexes. Administration of copper ions in a form of a complex could have the advantage in selective delivery of copper ions or radionuclides to diseased tissues.[7] Considering this, a large number of copper(II) complexes have been synthesized and explored for their biological activities. Various copper(II) complexes with

[a]*Department of Chemistry, Faculty of Science, University of Kragujevac, R. Domanovića 12, PO Box 60, 34000 Kragujevac, Serbia. E-mail: bglisic@kg.ac.rs*

[b]*Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, 11000 Belgrade, Serbia. E-mail: jasmina.nikodinovic@gmail.com*

[c]*Anorganisch-Chemisches Institüt and Interdisciplinary Center for Scientific Computing, Heidelberg University, Im Neuenheimer Feld 270, 69120 Heidelberg, Germany. E-mail: peter.comba@aci.uni-heidelberg.de*

† Electronic supplementary information (ESI) available: Fig. S1, Tables S1 and S2. CCDC 1494330–1494334. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c6ra19902j

different ligands were reported to possess antibacterial and antifungal activity and the approach to use ligands which already have antimicrobial activity that is further enhanced by complexation with copper has been widely utilized.[8–11] In addition to its long history of medical applications as antimicrobial as well as anti-inflammatory or antiarthritic agents, copper(I) and copper(II) complexes have not been examined extensively as potential anticancer drugs until recently.[12,13]

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacteria, still considered as one of the most problematic microorganisms due to its chromosomal plasticity and ability to develop mutational resistance against a variety of antibiotics.[14] The antibiotic treatment choices for already existing or emerging hard-to-treat multidrug-resistant bacterial infections are very limited, due to the fact that the vast majority of current antibiotics target the same bacterial cellular processes (*i.e.* translational machinery, cell wall biosynthesis, DNA/RNA metabolism), therefore the identification of new targets as well as new antimicrobials is urgently required.[15] A relatively innovative strategy to combat bacterial infections relies on specific inhibition of bacterial virulence, hence the ability to cause disease rather than bacterial growth.[16] The use of antivirulence drugs could have the advantage of reducing bacterial adaptability to the host environment, facilitating the host immune system to combat the infection and reducing the strong selective pressure exerted by conventional antibiotics.[17] In many pathogenic bacteria, virulence is coordinated and controlled by quorum sensing (QS) circuits, which make them particularly attractive targets for antivirulence compounds.[18,19] Several recent reports suggest that anti-QS agents hold significant promise as resistance-robust antimicrobial drugs.[20–22] QS refers to cell to cell communication between microorganisms that occurs *via* production and reception of signal molecules and controls bacterial population-dependant gene expression.[23,24] These genes include those involved in improving bacterial survival under various threats such as virulence and pathogenicity, in the secondary metabolite production, plasmid transfer, motility and biofilm formation.[23,25]

The pathogenic potential of *P. aeruginosa*, like many other Gram-negative bacteria, relies on the coordinated expression of a large array of virulence factors, the majority of which are positively controlled by QS.[26,27] The *P. aeruginosa* QS network is elaborate and consists of three different QS systems, based on the production of specific signal molecules: *N*-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL), *N*-butanoyl-homoserine lactone (C4-HSL) and 2-heptyl-3-hydroxy-4-quinolone (PQS).[27,28] The importance of QS for *P. aeruginosa* pathogenicity and its suitability as a target for the development of anti-*Pseudomonas* drugs is undisputed,[29,30] while quorum-sensing regulation of a copper ion toxicity in this strain has also been described.[31] The majority of quorum disrupting compounds identified to date include furanones and other organic molecules that competitively bind to receptor molecules, some antibiotics, and quorum signal degrading enzymes,[32–34] while, to the best of our knowledge, there is no literature data concerning the effect of metal complexes on QS.

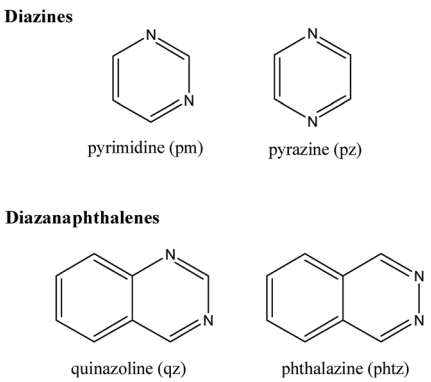
In the present study, copper(II) complexes with aromatic nitrogen-containing heterocycles (N-heterocycles), two diazines (pyrimidine, pm and pyrazine, pz), and two diazanaphthalenes (quinazoline, qz and phthalazine, phtz) were synthesized and structurally characterized by spectroscopic (IR and UV-vis) and crystallographic methods. The choice of the ligands for the synthesis of the corresponding copper(II) complexes was based on their great importance in coordination, bioinorganic and supramolecular chemistry.[35] For instance, the investigated N-heterocycles and their derivatives are structural moieties of many natural products and biologically active compounds, that have shown antimicrobial, antitumor, anticonvulsant and anti-inflammatory activity.[36,37] All synthesized copper(II) complexes were assessed for their *in vitro* antimicrobial and antiproliferative activities against a normal human cell line, as well as for the antivirulence effects targeting bacterial QS, using *Pseudomonas aeruginosa* as the model organism.

Results and discussion

Synthesis and structural characterization of the copper(II) complexes with N-heterocycles

Two diazines (pyrimidine, pm and pyrazine, pz), and two diazanaphthalenes (quinazoline, qz and phthalazine, phtz) were used as ligands for the synthesis of copper(II) complexes (Scheme 1). All these N-heterocycles, both monocyclic and bicyclic, contain two nitrogen atoms within one ring, but at different positions, *i.e.* 1,3 for pm and qz, 1,4 for pz and 2,3 for phtz, and upon coordination to the metal ion they can form complexes of different nuclearity. Accordingly, our results show that the pm-containing Cu(II) complex **1** is polynuclear, while complexes with pz (**2**) and qz (**3** and **4**) are mononuclear species, and the reaction between the Cu(II) ion and phtz resulted in the formation of the trinuclear complex **5**. The stoichiometries of all these complexes were confirmed by elemental microanalyses, and their structures were elucidated from UV-vis and IR spectroscopy and single-crystal X-ray diffraction analysis.

Diazine-containing copper(II) complexes 1 and 2. The molecular structures of the diazine-containing copper(II) complexes **1** and **2** along with the crystal packing of **1** are shown



Scheme 1 Schematic drawing of N-heterocyclic ligands used in this study.

in Fig. 1. Selected bond distances (Å) and valence angles (°) in these two complexes are listed in Table 1.

In pyrimidine-containing complex **1**, the Cu(II) ion is located at a center of inversion and coordinated by two pyrimidines and two water molecules equatorially, and two axial nitrates (Fig. 1a). As a consequence of pseudo-Jahn–Teller distortion, the axial Cu–O1(nitrate) bonds (2.3384(9) Å) are significantly longer than the equatorial Cu–O4(water) and Cu–N1(pm) bonds at 2.0063(8) and 2.0235(9) Å, respectively (Table 1), indicating that complex **1** has a distorted octahedral geometry. Moreover, a large deviation of the angle O4–Cu–O1 (81.83(4)°) from the ideal angle of 90° confirms the distortion from a regular octahedron. The Cu–N1(pm) bond distance is in accordance with that in the previously characterized pyrimidine-containing copper(II) complexes,[38] of which one has identical structure to the presently investigated complex **1**. However, the previously reported copper(II) complex was obtained as a mixture with mononuclear $[Cu(NO_3)_2(H_2O)_2(pm)_2]$ from the reaction of $Cu(NO_3)_2$ and pm in water.[38] As previously found for some pyrimidine metal complexes,[39,40] in **1**, the N-heterocycle acts as a bridging ligand between two Cu(II) ions, forming a one-dimensional coordination chain (Fig. 1b). The Cu···Cu distance within one chain is 5.717(2) Å and the interchain Cu···Cu distance is 6.843(2) Å. The coordination chains are connected by a hydrogen bonding network (see Table S1†).

The pyrazine-containing complex **2** is a mononuclear species (Fig. 1a). It consists of a square planar array of nitrogen atoms of four monodentatedly coordinated pz ligands with axial

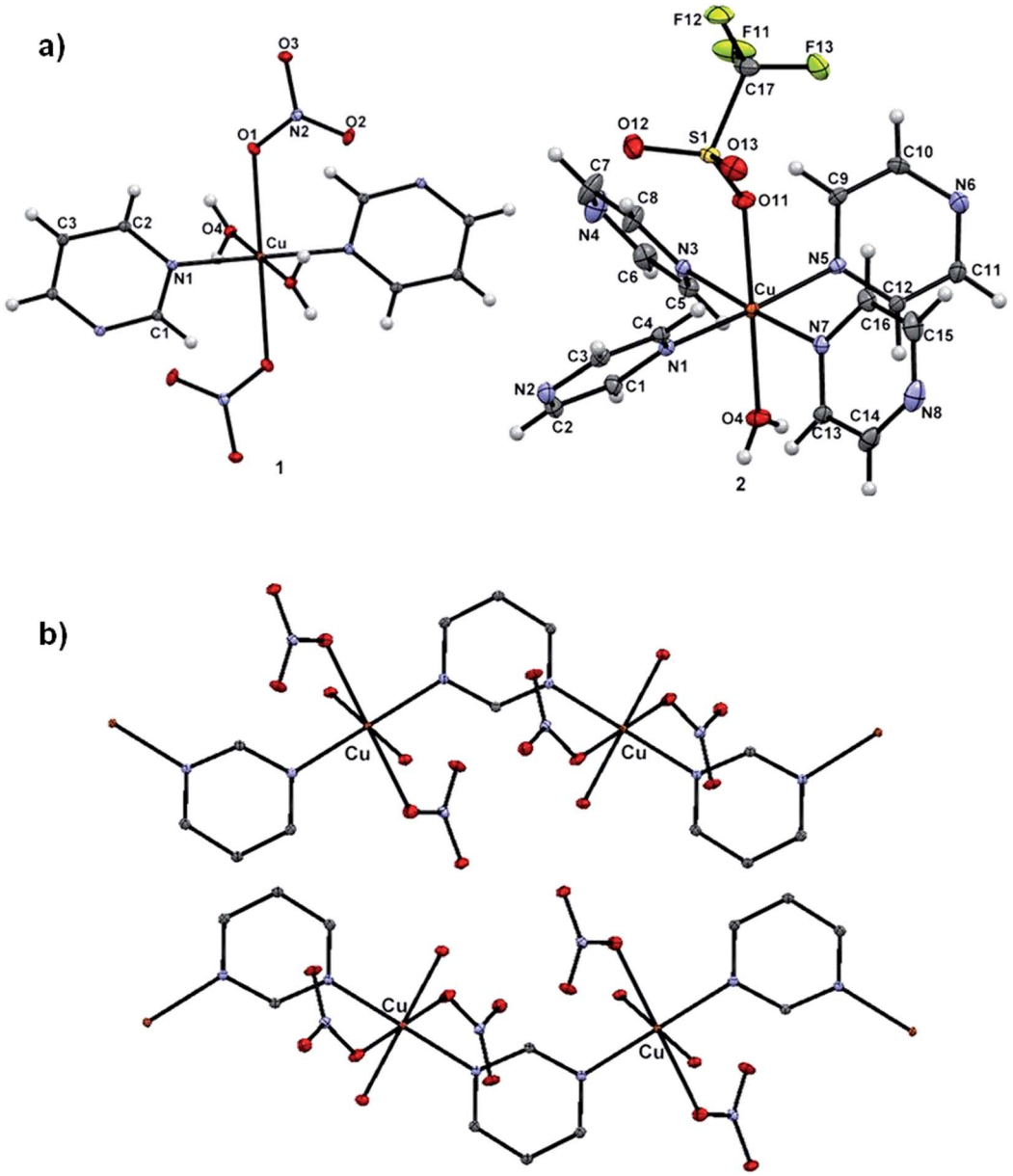


Fig. 1 (a) Molecular structures of diazine-containing copper(II) complexes **1** and **2**, and (b) the crystal packing of **1**. Non-coordinating triflate anion in **2** is omitted for clarity. Displacement ellipsoids for both of complexes are drawn at 50% probability level and H atoms are represented by spheres of arbitrary size.

Table 1 Selected bond distances (Å) and valence angles (°) in diazine-containing copper(II) complexes **1** and **2**[a]

1		**2**	
Cu–O4	2.0063(8)	Cu–O4	2.3041(17)
Cu–O4^{i}	2.0064(8)	Cu–O11	2.3816(16)
Cu–O1^{i}	2.3384(9)	Cu–N1	2.0374(15)
Cu–O1	2.3384(9)	Cu–N3	2.0385(16)
Cu–N1	2.0235(9)	Cu–N5	2.0345(16)
Cu–N1^{i}	2.0235(9)	Cu–N7	2.0360(16)
O4–Cu–O4^{i}	180.0	O4–Cu–O11	178.45(5)
O4–Cu–O1^{i}	98.17(4)	N1–Cu–O4	90.21(6)
O4^{i}–Cu–O1^{i}	81.83(4)	N1–Cu–O11	88.41(6)
O4–Cu–O1	81.83(4)	N1–Cu–N3	90.05(6)
O4^{i}–Cu–O1	98.17(4)	N3–Cu–O4	91.40(5)
O4–Cu–N1	87.73(4)	N3–Cu–O11	89.31(5)
O4–Cu–N1^{i}	92.27(4)	N5–Cu–O4	93.97(6)
O4^{i}–Cu–N1^{i}	87.73(4)	N5–Cu–O11	87.40(6)
O4^{i}–Cu–N1	92.27(4)	N5–Cu–N1	175.76(5)
O1–Cu–O1^{i}	180.0	N5–Cu–N3	90.51(6)
N1^{i}–Cu–O1^{i}	91.67(3)	N5–Cu–N7	88.53(6)
N1^{i}–Cu–O1	88.32(3)	N7–Cu–O4	92.21(5)
N1–Cu–O1	91.68(3)	N7–Cu–O11	87.09(5)
N1–Cu–O1^{i}	88.33(3)	N7–Cu–N1	90.64(6)
N1–Cu–N1^{i}	180.0	N7–Cu–N3	176.32(6)
N2–O1–Cu	125.44(6)	S1–O11–Cu	151.35(8)
C1–N1–Cu	118.10(6)	C1–N1–Cu	121.40(12)
C2–N1–Cu	123.90(6)	C4–N1–Cu	121.52(11)
		C5–N3–Cu	120.57(11)
		C8–N3–Cu	122.42(12)
		C9–N5–Cu	121.77(11)
		C12–N5–Cu	121.02(11)
		C13–N7–Cu	120.22(11)
		C16–N7–Cu	122.22(12)
		O11–S1–C17	101.52(8)
		O12–S1–O11	115.02(8)
		O12–S1–O13	115.06(9)
		O12–S1–C17	104.93(9)
		O13–S1–O11	114.02(8)
		O13–S1–C17	104.12(9)

[a] Symmetry code: (i) $-x + 3/2$, $-y + 3/2$, $-z + 1/2$.

coordination sites occupied by oxygen atoms belonging to trifluoromethanesulfonate (triflate) and water. The coordination sphere in **2** is strongly distorted from regular octahedral geometry: the Cu–O4(water) and Cu–O11(triflate) bond lengths of 2.304(2) and 2.382(2) Å, respectively, are significantly longer than the mean Cu–N(pz) bonds of 2.037 Å (Table 1), reflecting a large tetragonal distortion. The mean Cu–N(pz) bond distance in **2** compares well with that found in other mononuclear copper(II) complexes with equatorial nitrogen-containing ligands.[38,41] The coordinated triflate anion adopts a staggered ethane-like conformation around S–C bond, with O–S–O angles (mean 114.7°) greater than the C–S–O angles (mean 103.5°) (Table 1). The O13 atom of the coordinated triflate anion acts as acceptor in intermolecular hydrogen bonding with the coordinated water molecules (Table S1†).

Diazanaphthalene-containing copper(II) complexes 3–5. The molecular structures of the copper(II) complexes with quinazoline (**3** and **4**) and phthalazine (**5**) are shown in Fig. 2, while their selected bond distances (Å) and valence angles (°) are listed in Table 2.

Complexes **3** and **4** have monodentatedly coordinated quinazoline ligands and are mononuclear. In complex **3**, the Cu(II) ion is equatorially coordinated to two nitrogen atoms of quinazolines and two oxygen atoms of water molecules, and two axial oxygen atoms from nitrate anions complete the coordination sphere (Fig. 2). Complex **3** has a distorted octahedral geometry with the axial Cu–O1(nitrate) bonds (2.395(1) Å) significantly longer than the equatorial Cu–O4(water) and Cu–N1(qz) bonds at 1.982(1) and 2.012(1) Å, respectively (Table 2). Like in the pyrimidine-containing complex **1**, the angle O4–Cu–O1 (98.21(5)°) deviates from the ideal angle of 90°.

Complex **4** crystallizes as an acetonitrile solvate. The coordination geometry around Cu(II) in **4** (Fig. 2), is intermediate between trigonal bipyramidal and square pyramidal (TBDSBP).[42] This emerges from the trigonality index[43] τ of 0.35, $\tau = (\beta - \alpha)/60°$ where β = N1–Cu–N3 = 177.20(4)° and α = O1–Cu–O4 = 156.48(4)° (square pyramidal and trigonal bipyramidal geometries result in τ values of 0 and 1, respectively).[43] The corners of the plane in the coordination geometry of **4** are occupied by two nitrogen atoms of qz ligands (N1 and N3) and two oxygen atoms of the monodentatedly coordinated nitrate anions (O1 and O4) (Fig. 2). The remaining coordination site at the axial position in **4** is occupied by a water molecule. The lengths of Cu–N1(qz) and Cu–N3(qz) of 2.023(1) and 2.028(1) Å, respectively, compare well to those in the qz-containing complex **3** (2.012(1) Å) (Table 2). The O2 nitrate oxygen atom, as well as the uncoordinated N4 nitrogen atom of the qz ring, are engaged in intermolecular hydrogen bonding with the coordinated water molecule (Table S1†).

The phthalazine-containing copper(II) complex **5** has a trinuclear structure, in which the Cu(II) ions are linked by bridging phtz, nitrate and hydroxide ligands (Fig. 2). The two further nitrate anions are not coordinating. This results in six-coordination of the central Cu(II) ion, which is surrounded equatorially by two N3 nitrogen atoms of phthalazines and two O2 oxygen atoms of hydroxides, while two O4 oxygen atoms of nitrates are at the axial positions. This Cu(II) ion (designated as Cu2 in the ORTEP drawing of **5**; Fig. 2) has a pseudo-Jahn–Teller distorted octahedral geometry, emerging from the fact that the axial Cu2–O4(nitrate) bond (2.396(2) Å) is significantly longer than those in the equatorial plane, *i.e.* Cu2–N3(phtz), 2.052(2) Å, and Cu2–O2(hydroxide), 1.904(2) Å (Table 2). Contrary to the central Cu2, both of the terminal Cu(II) ions (Cu1) are pentacoordinated (Fig. 2). Along with the bridging phtz, nitrate and hydroxide ligands, the coordination around Cu1 ions is completed by monodentatedly coordinated phtz and water ligands. The Addison parameter τ[43] of the pentacoordinated Cu1 ions is 0.012, (β = O2–Cu–O1 = 178.24(7)° and α = N1–Cu–N11 = 177.54(7)°), indicating that the geometry around the metal is very close to the perfect square pyramidal. The apical Cu1–O3(nitrate) bond (2.322(2) Å) is longer than the bonds in the square plane (Table 2). The Cu1–N1(phtz) bond involving the nitrogen atom of the monodentatedly coordinated phtz (2.020(2) Å) is slightly shorter than that involving the nitrogen atom of the bridging one (Cu1–N11(phtz) at 2.050(2) Å). This structural characteristic was

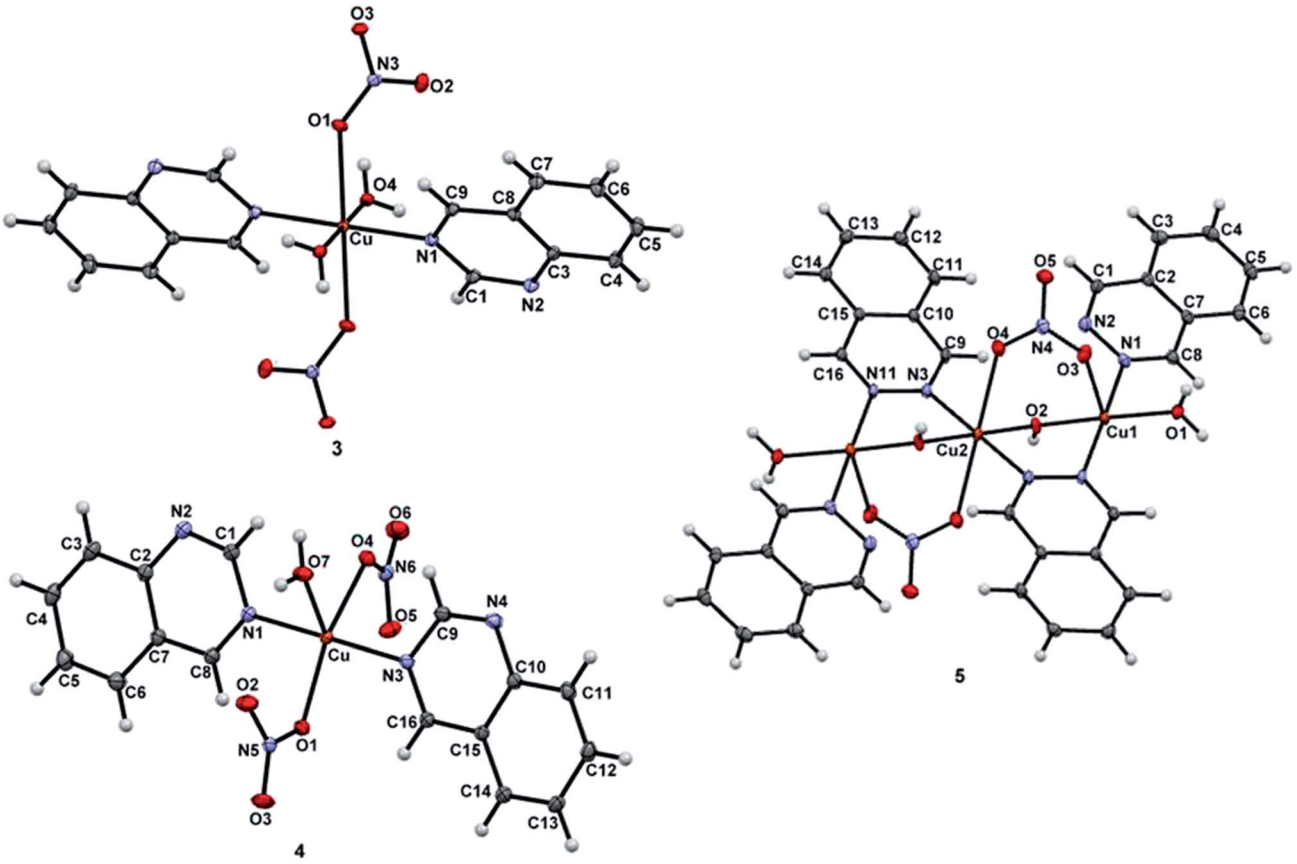


Fig. 2 Molecular structures of diazanaphthalene-containing copper(II) complexes **3–5**. Solvent of crystallization (acetonitrile) and non-coordinating nitrate anion in **4** and **5**, respectively, are omitted for clarity. Displacement ellipsoids are drawn at 50% probability level and H atoms are represented by spheres of arbitrary size.

also previously noted for the trimetallic copper(II) complex with pyridazine (pydz), in which both coordination modes of pydz (monodentate and bidentate bridging) were observed.[44]

Spectroscopic characterization

The IR spectroscopic data for copper(II) complexes **1–5** are listed in the Experimental section (*vide infra*). Except for the peaks that are attributed to the coordinated N-heterocycles, the IR spectra of the complexes show the typical peaks of the corresponding anion and water molecules. Thus, a broad absorption at ~3400 cm^{-1} attributed to the stretching vibration of OH confirms the presence of a coordinated water molecule and hydroxide anion in **1–4** and **5**, respectively.[45] In the IR spectra of **1**, **3** and **4**, the band corresponding to the asymmetric stretching modes of nitrate is split into two bands with a relatively small separation ($\Delta\nu$ = 31 cm^{-1} for **1** and **3** and 27 cm^{-1} for **4**). This is in accordance with nitrate coordination to the Cu(II) ion in these complexes.[46] Moreover, two very weak bands in the overtone region at ~1767 and ~1751 cm^{-1} for complexes **1**, **3** and **4** can be ascribed to the combination of symmetric stretching and in-plane bending of nitrate, indicating that this anion is monodentatedly coordinated to Cu(II).[47] On the other hand, complex **5** exhibits three discernible nitrate combination band absorptions, indicating the presence of coordinated and uncoordinated nitrate groups in this complex.[47] The additional band in the region of 1300–1320 cm^{-1} in the IR spectrum of **5** is also in accordance with the presence of uncoordinated nitrate in its structure.[46]

The IR spectrum of **2** with a monodentate triflate and triflate incorporated in the crystal lattice, shows a number of strong absorptions in the 1000–1300 cm^{-1} region. In order to find a correlation between vibrational frequencies and the mode of coordination, much attention has been devoted to the 1200–1300 cm^{-1} region, due to the fact that splitting of the ν_{as}(S–O) band is due to the coordination of triflate in monodentate or bidentate fashion.[48] By comparison of the IR spectrum of **2** with that of $Cu(CF_3SO_3)_2$, the band at 1286 cm^{-1} was assigned to the asymmetric stretching mode of S–O of the uncoordinated $CF_3SO_3^-$ anion. The frequencies at 1252 and 1230 cm^{-1} are due to the ν_{as}(S–O) of the monodentate triflate. The presence of these two bands is in accordance with that previously observed for the monodentate triflate in $[Cu(bpt)(CF_3SO_3)(H_2O)]_2$ (Hbpt = 3,5-bis(pyridin-2-yl)-1*H*-triazole)[49] and $[Cu(CF_3SO_3)_2(py)_4]$ (py = pyridine) complexes.[41]

The wavelengths of maximum absorption for the copper(II) complexes **1–5** (λ_{max}, nm) and molar extinction coefficients (ε, M^{-1} cm^{-1}) determined after dissolution of the complexes, are listed in the Experimental section. The investigated copper(II) complexes exhibit a single band in the expected region,[50] assigned to the d_{z^2}, d_{xy}, d_{xz}, $d_{yz} \rightarrow d_{x^2-y^2}$ transitions with a $d_{x^2-y^2}$ ground state.[51] The energy of absorption maxima for complexes **1–5** increases in the following order: **3** < **1** ≈ **2** < **4** < **5**.

Table 2 Selected bond distances (Å) and valence angles (°) in diazanaphthalene-containing copper(II) complexes **3–5**[a]

3		**4**		**5**	
Cu–O4	1.9816(11)	Cu–O1	1.9925(12)	Cu1–O1	2.0335(16)
Cu–O1	2.3951(12)	Cu–O4	2.0282(13)	Cu1–O2	1.8937(16)
Cu–N1	2.0123(13)	Cu–O7	2.2153(13)	Cu1–O3	2.3216(17)
Cu–N1^{i}	2.0123(13)	Cu–N1	2.0229(13)	Cu1–N1	2.0202(19)
		Cu–N3	2.0281(13)	Cu1–N11	2.0497(19)
				Cu2–O2	1.9038(16)
				Cu2–O4	2.3955(18)
				Cu2–N3	2.0522(19)
O4–Cu–O4^{i}	180.0	O1–Cu–O4	156.48(4)	O1–Cu1–O3	81.05(6)
O4–Cu–O1^{i}	81.79(5)	O1–Cu–O7	121.23(4)	O1–Cu1–N11	92.05(7)
O4–Cu–O1	98.21(5)	O1–Cu–N1	91.28(4)	O2–Cu1–O1	178.24(7)
O4–Cu–N1	93.28(5)	O1–Cu–N3	88.50(4)	O2–Cu1–O3	99.89(7)
O4–Cu–N1^{i}	86.73(5)	O4–Cu–O7	82.28(4)	O2–Cu1–N1	91.41(7)
O1–Cu–O1^{i}	180.0	O4–Cu–N3	91.36(4)	O2–Cu1–N11	86.47(7)
N1–Cu–O1	92.61(4)	N1–Cu–O4	87.73(4)	N1–Cu1–O1	90.06(7)
N1–Cu–O1^{i}	87.39(4)	N1–Cu–O7	93.37(5)	N1–Cu1–O3	91.46(7)
N1–Cu–N1^{i}	180.0	N1–Cu–N3	177.20(4)	N1–Cu1–N11	177.54(7)
N3–O1–Cu	129.16(8)	N3–Cu–O7	89.13(4)	N11–Cu1–O3	90.11(7)
C1–N1–Cu	124.66(8)	N5–O1–Cu	115.04(8)	O2–Cu2–O2^{ii}	180.00(9)
C9–N1–Cu	118.40(9)	N6–O4–Cu	105.65(7)	O2–Cu2–O4^{ii}	91.59(7)
		C1–N1–Cu	119.27(8)	O2–Cu2–O4	88.35(7)
		C8–N1–Cu	123.95(8)	O2–Cu2–N3^{ii}	86.92(7)
		C9–N3–Cu	117.84(8)	O2–Cu2–N3	93.08(7)
		C16–N3–Cu	125.25(8)	O4^{ii}–Cu2–O4	180.0
				N3–Cu2–O4	83.10(7)
				N3–Cu2–O4^{ii}	96.90(7)
				N3–Cu2–N3^{ii}	180.0
				Cu1–O2–Cu2	116.27(9)
				N4–O3–Cu1	120.82(14)
				N4–O4–Cu2	130.68(15)
				N2–N1–Cu1	116.19(14)
				C8–N1–Cu1	122.51(16)
				N11^{ii}–N3–Cu2	118.16(13)
				C9–N3–Cu2	120.49(15)
				N3^{ii}–N11–Cu1	114.38(14)
				C16^{ii}–N11–Cu1	125.63(15)

[a] Symmetry codes: (i) $-x$, $-y$, $-z$; (ii) $-x+2$, $-y$, $-z$.

Antimicrobial and antiproliferative activity of copper(II) complexes

The MIC values of copper(II) complexes **1–5**, as well as $Cu(NO_3)_2\cdot 3H_2O$ and $Cu(CF_3SO_3)_2$ salts used for their synthesis, were determined against pathogenically relevant Gram-negative *Pseudomonas aeruginosa* (PAO1 strain, as well as clinical isolate DM-18 that showed higher resistance to a wide panel of antibiotics), Gram-positive *Staphylococcus aureus* and the fungal yeast representative *Candida albicans* (Table 3). We have previously established that the used N-heterocyclic ligands (Scheme

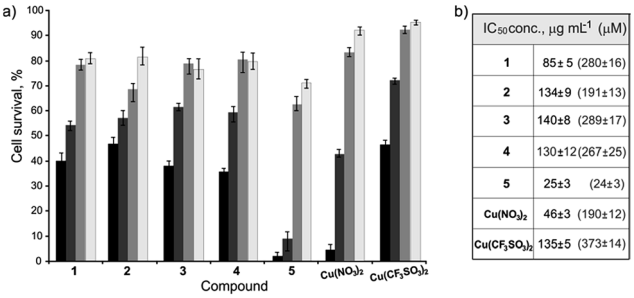
Table 3 Antimicrobial activity of the copper(II) complexes **1–5** and the corresponding salts used for their synthesis (MIC) against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*

	P. aeruginosa PAO1		*P. aeruginosa* DM-18		*S. aureus*		*C. albicans*	
Copper(II) compound	μg mL^{-1}	mM	μg mL^{-1}	mM	μg mL^{-1}	mM	μg mL^{-1}	mM
1	1000	3.29	>1000	>3.29	1000	3.29	1000	3.29
2	500	0.71	>1000	>1.43	500	0.71	500	0.71
3	>1000	>2.07	>1000	>2.07	>1000	>2.07	>1000	>2.07
4	1000	2.06	>1000	>2.06	1000	2.06	1000	2.06
5	1000	0.97	>1000	>0.97	1000	0.97	1000	0.97
$Cu(NO_3)_2\cdot 3H_2O$	500	2.07	1000	4.14	500	2.07	250	1.03
$Cu(CF_3SO_3)_2$	1000	2.76	1000	2.76	1000	2.76	500	1.38

1) did not show significant antimicrobial activity against these strains nor antiproliferative effect on normal human fibroblast cell line.[52,53] Overall, none of the tested copper(II) compounds showed significant antimicrobial activity under the tested conditions with MIC values mostly $\geq$1 mg mL^{-1} (mM range). Given the numerous literature data on antimicrobial properties of Cu(II) ions, these results were surprising and confusing at the beginning. Nonetheless, they are in line with MIC values previously determined for copper(II) complexes with 2-acetylpyridine- and 2-benzoylpyridine-derived hydrazones, as well as for $CuCl_2 \cdot 2H_2O$.[54] While we have not observed any difference in the activities against bacterial and fungal strain, Despaigne *et al.*[54] reported slightly better activity against *C. albicans*, which was still quite high (220–1600 μM). Similarly, but in the different experimental setup, when disc diffusion was used to determine the antimicrobial potency, neither discs loaded with $CuCl_2 \cdot 6H_2O$ and [Cu(ma)$_2$] (Hma = maltol) showed any growth inhibition against five different bacterial pathogens, including *P. aeruginosa* and *S. aureus*.[55] Even with copper(I) complexes containing phenanthroline-type ligands, poor activity against *P. aeruginosa* has been reported.[56] On the contrary, complexation with Cu(II) usually improves the antimicrobial potential of the already active ligands, *e.g.* with 3,4,7,8-tetramethyl-1,10-phenanthroline and 4,7-diphenyl-1,10-phenanthroline with MIC values in the 1–25 μM range.[11] It appears that the activity and nature of the ligand has a marked effect on the bacterial uptake and defines the activity and lipophilicity of the complex.

The ability of bacteria to survive in the presence of soluble copper salts or on solid copper surfaces is dependent on the expression of copper tolerance genes for example copper exporting ATPase-type pumps.[57] For instance, it was shown that multiple genes exist in *P. aeruginosa* that are responsible for copper resistance and that mutations in the copper homeostasis *cueA* gene encoding P-type ATPase reduce its pathogenicity in the murine model.[58,59] Several recent studies suggest that the same pathways of copper tolerance within certain pathogenic bacteria are required to survive the innate immune response during infection. This relatively new field of biometal research underscores a novel role for copper in host immunity and emphasizes the medical importance of understanding copper homeostasis at the host–pathogen interface.[2,60]

The studied copper(II) compounds were not efficient antiproliferative agents, even though IC_{50} values determined for 48 h exposure of the MRC5 cell line were from 3.7- to 40.0-fold lower in comparison to the MIC values for **2** and **5**, respectively (Fig. 3 and Table 3). Most cytotoxic proved to be trinuclear complex **5** containing phthalazine ligand. Again, all N-heterocyclic ligands exhibited low cytotoxic activity against MRC5 cells (results not shown). Recently, IC_{50} values between 4.8–53.0 μM were reported for copper(II) complexes with heterocyclic bases (1,10-phenanthroline and 2,2′-bipyridine) and 3-hydroxypicolinic acid against MRC5 cells after 24 h exposure.[13] Although numerous copper(II) complexes have been investigated for antitumoral purposes with general [Cu(L_1-*N*,*N*)L_2]X structure (Casiopeinas) and described to interact with mitochondria, DNA, inhibiting oxidative phosphorylation and causing cellular ATP depletion,[61] the compounds described in this study were not further persuaded in this context due to the relatively low cytotoxicity (Fig. 3).



IC_{50} conc., μg mL^{-1} (μM)	
1	85 ± 5 (280±16)
2	134±9 (191±13)
3	140±8 (289±17)
4	130±12 (267±25)
5	25±3 (24±3)
$Cu(NO_3)_2$	46±3 (190±12)
$Cu(CF_3SO_3)_2$	135±5 (373±14)

Fig. 3 *In vitro* antiproliferative effect of copper(II) compounds on human fibroblasts (MRC5) following 48 h exposure (a) at a range of concentrations (■ – 250; ■ – 50; ■ – 10; □ – 5 μg mL^{-1}) and (b) calculated IC_{50} values (IC_{50} = concentration required to inhibit 50% cell growth).

Effect of copper(II) complexes on bacterial quorum sensing (QS)

In many bacteria, including *P. aeruginosa*, pathogenicity is regulated by an intercellular communication process called quorum sensing (QS). So far, QS is considered one of the most promising targets for antivirulence therapies.[20,33] Prompted by the finding that $CuSO_4$ showed a negative effect on the virulence of a pathogenic *Edwardsiella tarda* and pathogenic *Vibrio harveyi* strains,[62,63] we have decided to examine whether the copper(II) compounds utilized within this study exerted an effect on bacterial QS.

Effect on violacein and pyocyanin production. Firstly, all copper(II) compounds were tested for the ability to inhibit violacein production in *Chromobacterium violaceum* CV026 in concentration of 50 μg per disc (Fig. 4). Wild-type *C. violaceum* is

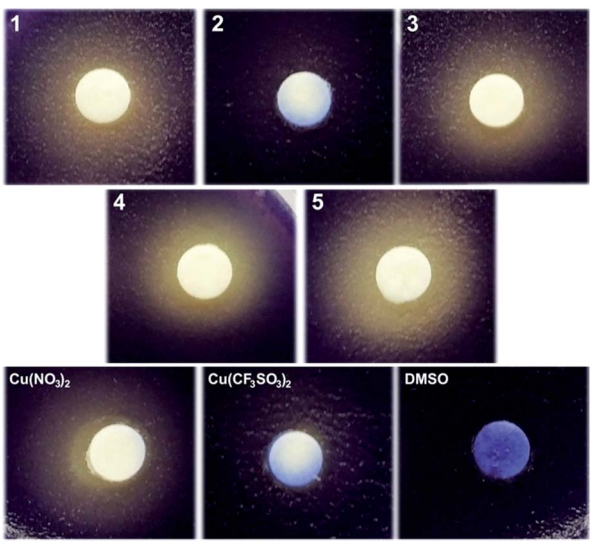


Fig. 4 Inhibition of violacein synthesis in *Chromobacterium violaceum* CV026 by the investigated copper(II) compounds (50 μg per disc).

a Gram-negative bacterium that produces the characteristic purple pigment, violacein, when AHL (*N*-acyl homoserine lactone) signaling molecules reach a threshold level, while CV026 is a derivative strain harboring a LuxR receptor homologue and a corresponding QS-controlled promoter fused to a violacein production gene cluster and it is unable to produce violacein unless exogenous AHLs are supplied.[64,65] Based on this readily observed pigmentation phenotype, *C. violaceum* strains can be used to detect various aspects of AHL-mediated QS activity.[66] Colorless, but not clear zones around the compound-loaded discs in our assay, indicated anti-QS activity, observed with the copper(II) compounds in the following order **5** > **1** > **4** ≥ **3** > $Cu(NO_3)_2 \cdot 3H_2O$, while **2** and $Cu(CF_3SO_3)_2$ did not inhibit violacein production under these conditions (Fig. 4).

Notably, $Cu(NO_3)_2 \cdot 3H_2O$ caused a zone of clearance immediately around the disc, confirming the activity on the bacterial growth. This initial screen, lead us to conclude that the copper(II) compounds have the potential to interfere with the AHL-dependant QS pathway. Surprisingly, in the same concentration, none of the compounds had the ability to affect the pyocyanin production in *P. aeruginosa* PA14 strain (Fig. S1†). Pyocyanin is a blue-green phenazine pigment also associated with AHL concentration,[67] suggesting either strain dependence or that much higher concentrations are required to affect pyocyanin production.

Effect on specific QS pathways in *P. aeruginosa* PAO1. The opportunistic pathogen *P. aeruginosa* PAO1 uses elaborate interconnected QS pathways to regulate biofilm formation, group motility and an arsenal of virulence factors in order to overwhelm host defenses and establish chronic infections. Therefore, it was important to differentiate which of the QS pathways was affected by the copper(II) compounds used in this study. Hence, three biosensor strains developed for this purpose were included: the PAO1 ΔrhlIpKD-rhlA biosensor was used to directly measure the levels of C4-HSL (*N*-butanoyl-homoserine lactone), and the PA14-R3ΔlasIPrsaI lux strain was used to measure the levels of 3OC12-HSL (*N*-3-oxo-dodecanoyl-homoserine lactone) in the culture supernatants of *P. aeruginosa* PAO1, while the detection of AHQs (2-alkyl-4-quinolones), such as PQS (2-heptyl-3-hydroxy-4-quinolone) and HHQ (2-heptyl-4-quinolone), was performed by the biosensor PAO1 ΔpqsA mini-CTX luxPpqsA (Fig. 5).

The production of short chain acyl homoserine lactones such as C4-HSL by *P. aeruginosa* PAO1 was the least affected by the copper(II) compounds. Only pm-containing **1**, pz-containing **2** and $Cu(NO_3)_2 \cdot 3H_2O$ significantly affected the production of C4-HSL negatively for 30% and positively for 25% in comparison to DMSO control, respectively (Fig. 5). The polynuclear complex **1** significantly reduced the production of 3OC12-HSL for 40% and had no effect on AHQs production, indicating that it specifically affected HSL mediated QS in *P. aeruginosa* PAO1.

The production of longer chain acyl homoserine lactones, such as 3OC12-HSL was affected by all copper(II) complexes and not by inorganic salts to a various extent (Fig. 5). Apart from complex **1**, all other compounds caused higher production of this signaling molecule between 25–50% in comparison to the DMSO control, with the highest effect caused by the qz-containing complex **4**.

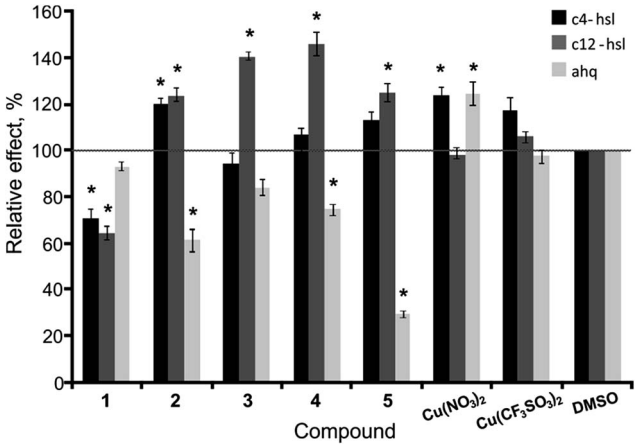


Fig. 5 Effect of copper(II) compounds (50 μg mL^{-1}) on acyl homoserine lactone (AHL) production in *P. aeruginosa* PAO1 (■ – short-chain (C4-HSL); ▣ – long chain (3OC12-HSL) and □ – AHQs) (* = statistically significant differences between compounds and DMSO control).

The production of diverse 2-alkyl-4-quinolones (AHQs), which act as QS signal molecules and are also involved in the regulation of many virulence factors in *P. aeruginosa* PAO1 (*i.e.*

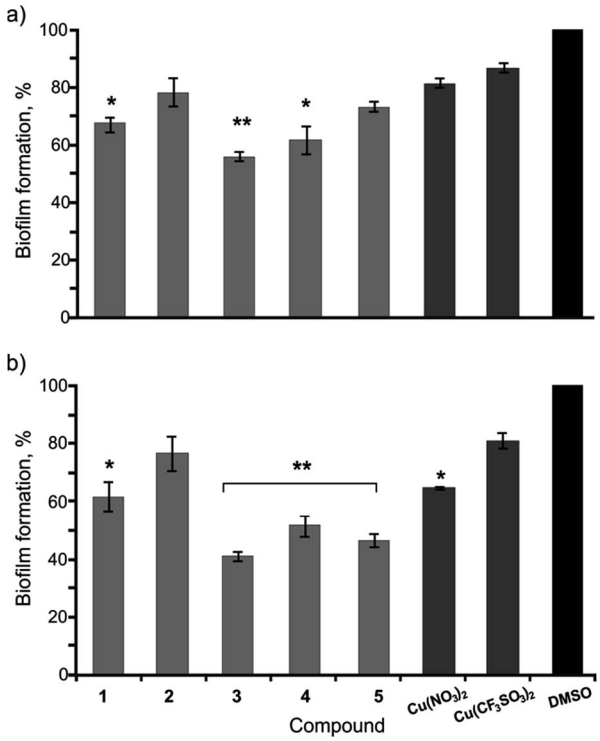


Fig. 6 *P. aeruginosa* PAO1 biofilm formation in the presence of concentrations of (a) 50 and (b) 250 μg mL^{-1} of copper(II) compounds. The biofilm formation in the presence of DMSO was considered to represent 100% biofilm formation (results were considered significant when compared to DMSO control; *$p < 0.05$, **$p < 0.01$).

Table 4 Minimal inhibitory concentration (MIC, μg mL^{-1}) of individual antibiotics and in the presence of copper(II) compounds against *P. aeruginosa* clinical isolate DM-18

	Cu(II) Compound								
	1			**3**			$Cu(NO_3)_2 \cdot 3H_2O$		
Antibiotic	—	500 μg mL^{-1}	1000 μg mL^{-1}	—	500 μg mL^{-1}	1000 μg mL^{-1}	—	500 μg mL^{-1}	1000 μg mL^{-1}
Piperacillin	250	125	62.5	250	125	62.5	250	125	62.5
Ceftazidime	1000	500	250	Indifferent			1000	500	250

elastase, pyocyanin, rhamnolipids, biofilm development), was negatively affected by Cu(II) complexes **5** > **2** > **4** and has been stimulated by $Cu(NO_3)_2 \cdot 3H_2O$ salt for 25% (Fig. 5). The corresponding N-heterocyclic ligands, supplied in 50 μg mL^{-1}, exerted no significant effect on any of the biosensors (results not shown).

These results indicated that the tested copper(II) compounds, that showed no significant effect on the growth of *P. aeruginosa* strains (Table 3), were able to successfully modulate the production of signaling molecules that are part of the QS system (Fig. 4 and 5). It appears that the acyl homoserine lactone and the quinolone-mediated QS system in *P. aeruginosa* PAO1 were affected, with the quinoline system more reduced and the longer chain AHL more stimulated. The quinoline system is usually transferred *via* extremely hydrophobic molecules, usually in membrane vesicles,[68] so structural differences of the selected copper(II) complexes could not account for the observed differences in the QS modulating activity.

Effect on *P. aeruginosa* PAO1 biofilm formation. The involvement of cell-to-cell signals in the development of a bacterial biofilm has been recognized for more than 20 years,[69] and the recent report of Ni(II) and Cd(II) ions successfully preventing biofilm formation of *Burkholderia multivorans* without affecting its viability[70] provided further reason to examine the effect of Cu(II) complexes on the ability of PAO1 to form biofilms. Indeed, copper(II) complexes showing QS antagonistic activity were also able to reduce biofilm formation, when supplied in two different concentrations of 50 and 250 μg mL^{-1} (Fig. 6). Inorganic salts were less successful in biofilm prevention and only $Cu(NO_3)_2 \cdot 3H_2O$ supplied in 250 μg mL^{-1} reduced the biofilm formation by 30%.

At concentration of 50 μg mL^{-1}, complexes **1**, **3** and **4** showed the best antibiofilm activity, reducing the biofilm formation between 23–38% in comparison to the DMSO treated control (Fig. 6a). When the compounds were supplied in 250 μg mL^{-1}, the negative effect was more pronounced (**3** > **5** > **4** > **1** > $Cu(NO_3)_2 \cdot 3H_2O$), causing biofilm inhibition between 30–60% (Fig. 6b). Both of these concentrations had no effect on bacterial growth (Table 3), while most of the compounds were cytotoxic at 250 μg mL^{-1} (Fig. 3). Overall, qz-containing complex **3** have the most pronounced ability to inhibit biofilm formation of *P. aeruginosa* PAO1. To the best of our knowledge, this is the first report of a copper(II) complex as efficient inhibitor of biofilm formation of extremely hard to eradicate human opportunistic pathogen *P. aeruginosa* PAO1.

Synergy with known antibiotics

Based on the results of the anti-QS activity, we have selected complexes **1** and **3**, and $Cu(NO_3)_2 \cdot 3H_2O$ to examine whether they show any synergistic activity with known antibiotics, when supplied in the combination. For this purpose, the clinical isolate *P. aeruginosa* DM-18, that showed increased resistance to a wide panel of antibiotics, including newer generation piperacillin and ceftazidime, was selected (Table 4). With piperacillin, addition of 500 μg mL^{-1} of the copper(II) compounds that had no effect on planktonic growth of *P. aeruginosa* DM-18 (Table 3), reduced the MIC two fold in the case of all three tested copper(II) compounds, and four-fold when 1000 μg mL^{-1} were used (Table 4). In the case of ceftazidime, the effect of **3** was indifferent, while the polynuclear complex **1** and $Cu(NO_3)_2 \cdot 3H_2O$ enhanced the effect of the antibiotic (Table 4). Similarly, organic quorum sensing inhibitors, such as cinnamaldehyde, were found to increase the success of the antibiotic treatment by increasing the susceptibility of bacterial biofilms and/or by increasing the host survival, following an infection with *P. aeruginosa*.[71] This effect was not previously reported for copper(II) complexes.

One of the strategies to overcome the problem of resistance is the application of commonly used antibiotics in combination with different agents.[72,73] Metal ions and especially metallic nanoparticles have shown great promise when used in combination with a number of antibiotics such as ampicillin, kanamycin, erythromycin and chloramphenicol against different Gram positive or Gram negative strains.[74] For instance, Au(III) ions enhance the efficacy of a number of commonly used antibiotics against *P. aeruginosa* up to 146%.[75]

Conclusions

In summary, five copper(II) complexes with the diazines pyrimidine (pm, **1**) and pyrazine (pz, **2**), and the diazanaphthalenes quinazoline (qz, **3** and **4**) and phthalazine (phtz, **5**) were synthesized and structurally characterized by spectroscopy and crystallography. The results of the crystallographic analysis show that the nuclearity of the N-heterocycle-copper(II) complexes can be tuned by modification of the ligand structure. The investigation of the antimicrobial properties of these copper(II) complexes shows that, although they are not efficient growth inhibitors of *Pseudomonas aeruginosa* strains, they are able to inhibit quorum sensing (QS) by successful modulation

of production of signaling molecules that are part of the QS system. Beside the action of copper(II) complexes **1–5** on the acyl homoserine and AHQs level, they are also potent inhibitors of biofilm formation in *P. aeruginosa* PAO1. These findings could serve as a basis for further complexation of Cu(II) ions with small organic molecules that have already shown strong anti-QS activity,[68] which can be of importance for the development of novel antivirulence therapeutic agents that attenuate virulence without killing or a pronounced effect on the growth, thus offering a lower risk for resistance development. Finally, using a combination approach, we confirmed the synergies of the most anti-QS active complexes **1** and **3** and two currently used organic-based antibiotics, piperacillin and ceftazidime against multi-drug resistant clinical isolate of *P. aeruginosa*, indicating that this combination therapy could be considered as a new approach, and common antibiotics may have an even broader range of medical applications in the future.

Experimental

Materials

Distilled water was demineralized and purified to a resistance of greater than 10 MΩ cm^{-1}. Copper(II) salts ($Cu(NO_3)_2 \cdot 3H_2O$ and $Cu(CF_3SO_3)_2$), ethanol, methanol, acetonitrile and dimethyl sulfoxide were purchased from Sigma-Aldrich. Pyrimidine (pm), pyrazine (pz), quinazoline (qz) and phthalazine (phtz) were obtained from ABCR. All reactants were of analytical reagent grade and used without further purification.

Synthesis of copper(II) complexes 1 and 3–5

Copper(II) complexes with pyrimidine, quinazoline and phthalazine, $[Cu(NO_3)_2(H_2O)_2(pm)]_n$ (**1**), $[Cu(NO_3)_2(H_2O)_2(qz)_2]$ (**3**), $[Cu(NO_3)_2(H_2O)(qz)_2] \cdot 0.5CH_3CN$ (**4**) and $[Cu_3(NO_3)_2(OH)_2(H_2O)_2(phtz)_4](NO_3)_2$ (**5**), were synthesized according to the modified procedure for the preparation of copper(II) complexes with pyridazine[44] and quinoxaline.[76]

The solution of 1.0 mmol (241.6 mg for **1**, **3** and **5**) or 0.2 mmol (48.3 mg for **4**) of $Cu(NO_3)_2 \cdot 3H_2O$ in 5.0 mL of ethanol was added slowly under stirring to the solution containing 1.0 mmol of the corresponding N-heterocyclic ligand (80.4 μL of 98% pyrimidine (**1**), 130.2 mg of quinazoline (**3** and **4**) and phthalazine (**5**)) in 5.0 mL of ethanol. The reaction mixture was stirred at room temperature for 3 h. The solid product of complexes **1**, **3** and **4** precipitated from the reaction mixtures was filtered off and dissolved in 20.0 mL of acetonitrile. These complexes were crystallized after acetonitrile solutions were left to stand in the refrigerator at +4 °C for five days. The light blue crystals of **1** and green crystals of **3** and **4** suitable for single-crystal X-ray crystallography were filtered off and dried at ambient temperature. On the other hand, complex **5** was crystallized from the mother ethanol solution after its cooling in the refrigerator for several days. Yield (calculated on the basis of $Cu(NO_3)_2 \cdot 3H_2O$): 239.9 mg (79%) for **1**, 150.0 mg (62%) for **3**, 65.2 mg (67%) for **4** and 105.5 mg (41%) for **5**.

Anal. calcd for **1** = $C_4H_8CuN_4O_8$ (M_r = 303.68): C, 15.82; H, 2.66; N, 18.45. Found: C, 15.89; H, 2.73; N, 18.81%. IR (KBr, ν, cm^{-1}): 3446(br), 3115(w), 3093(w), 3032(w), 1767(w), 1751(w), 1639(w), 1599(m), 1464(w), 1403(m), 1384(vs), 1353(s), 1218(w), 1182(w), 1143(w), 1084(w), 1036(w), 833(w), 805(w), 684(w). UV-vis (DMSO, λ_{max}, nm): 838 (ε = 23.0 M^{-1} cm^{-1}).

Anal. calcd for **3** = $C_{16}H_{16}CuN_6O_8$ (M_r = 483.89): C, 39.71; H, 3.33; N, 17.37. Found: C, 39.69; H, 3.38; N, 17.68%. IR (KBr, ν, cm^{-1}): 3411(br), 3114(w), 3090(w), 3020(w), 1763(w), 1751(w), 1670(w), 1621(s), 1589(m), 1574(m), 1491(m), 1384(vs), 1353(s), 1240(m), 1212(m), 1155(m), 1136(m), 1059(w), 1013(w), 969(w), 944(w), 925(m), 871(w), 825(w), 790(m), 750(s), 636(m), 550(w), 484(m). UV-vis (DMSO, λ_{max}, nm): 842 (ε = 23.6 M^{-1} cm^{-1}).

Anal. calcd for **4** = $C_{17}H_{15.50}CuN_{6.50}O_7$ (M_r = 486.40): C, 41.98; H, 3.21; N, 18.72. Found: C, 42.00; H, 3.14; N, 18.72%. IR (KBr, ν, cm^{-1}): 3453(br), 3115(w), 3066(w), 1767(w), 1751(w), 1621(s), 1575(m), 1490(m), 1384(vs), 1357(m), 1290(m), 1213(m), 1155(m), 1136(m), 1059(w), 1014(w), 969(w), 942(w), 925(m), 812(w), 791(m), 751(m), 636(m), 551(w), 484(m). UV-vis (DMSO, λ_{max}, nm): 836 (ε = 26.1 M^{-1} cm^{-1}).

Anal. calcd for **5** = $C_{32}H_{30}Cu_3N_{12}O_{16}$ (M_r = 1029.30): C, 37.34; H, 2.94; N, 16.33. Found: C, 37.98; H, 3.09; N, 16.57%. IR (KBr, ν, cm^{-1}): 3455(br), 3066(w), 3047(w), 3000(w), 1772(w), 1760(w), 1752(w), 1621(m), 1384(vs), 1354(s), 1335(sh), 1320(m), 1280(m), 1249(m), 1224(m), 1161(m), 1043(w), 1013(w), 987(m), 933(w), 824(w), 765(m), 652(w), 571(w), 471(m). UV-vis (DMSO, λ_{max}, nm): 760 (ε = 65.1 M^{-1} cm^{-1}).

Synthesis of copper(II) complex 2

Copper(II) complex with pyrazine, $[Cu(CF_3SO_3)(H_2O)(pz)_4]CF_3SO_3$ (**2**), was synthesized according to the modified procedure for the preparation of $[Cu(CF_3SO_3)_2(pz)_4] \cdot H_2O$ complex.[41] The solution of 0.3 mmol of $Cu(CF_3SO_3)_2$ (108.5 mg) in 2.0 mL of hot methanol was added under stirring to the hot solution of pyrazine (4.29 mmol, 343.6 mg) dissolved in 5.0 mL of methanol. After standing of the mother methanol solution in the refrigerator for 2 days, the blue crystals of complex **2** were formed. These were filtered off and dried at ambient temperature. Yield (calculated on the basis of $Cu(CF_3SO_3)_2$): 155.4 mg (74%). Anal. calcd for **2** = $C_{18}H_{18}CuF_6N_8O_7S_2$ (M_r = 700.06): C, 30.88; H, 2.59; N, 16.01. Found: C, 30.29; H, 2.63; N, 16.05%. IR (KBr, ν, cm^{-1}): 3443(br), 3138(w), 3102(w), 3051(w), 1648(w), 1419(s), 1286(vs), 1252(vs), 1230(s), 1177(s), 1151(s), 1125(m), 1084(w), 1056(m), 1034(s), 974(w), 805(m), 639(s), 575(w), 516(m), 493(w), 457(w). UV-vis (DMSO, λ_{max}, nm): 838 (ε = 27.4 M^{-1} cm^{-1}).

Measurements

Elemental analyses for carbon, hydrogen and nitrogen were performed on a Vario EL instrument by the Microanalytical Laboratory, Department of Organic Chemistry, Heidelberg University. Infrared spectra were recorded as KBr pallets on a Perkin Elmer Spectrum 100 spectrometer over the range of 450–4000 cm^{-1}. The UV-vis spectra were recorded on a Cary 100 spectrophotometer (Varian, USA), after dissolving of the corresponding copper(II) complex in dimethyl sulfoxide (DMSO), over the wavelength range of 200–1100 nm. The concentration of the copper(II) complex was 2.5 mg mL^{-1}.

X-ray crystal structure determinations

Crystal data and details of the structure determinations are listed in the ESI (Table S2†). Full shells of intensity data were collected at low temperature with an Agilent Technologies Supernova-E CCD diffractometer (Mo-K_α radiation, microfocus X-ray tube, multilayer mirror optics; complex **5**) or a Bruker AXS Smart 1000 CCD diffractometer (Mo-K_α radiation, sealed X-ray tube, graphite monochromator; all other compounds). Detector frames (typically w-, occasionally j-scans, scan width 0.4–1°) were integrated by profile fitting.[77–79] Data were corrected for air and detector absorption, Lorentz and polarization effects[78,79] and scaled essentially by application of appropriate spherical harmonic functions.[80–82] Absorption by the crystal was treated numerically (Gaussian grid, complex **5**)[83] or with a semiempirical multiscan method augmented by a spherical correction (as part of the scaling process; all other complexes).[81,82] For the dataset collected with the microfocus tube(s) an illumination correction was performed as part of the numerical absorption correction.[78,81] The structures were solved by the heavy atom method combined with structure expansion by direct methods applied to difference structure factors[84] (complex **3**) or by the charge flip procedure[85] (all other complexes) and refined by full-matrix least squares methods based on F^2 against all unique reflections.[86] All non-hydrogen atoms were given anisotropic displacement parameters. An empirical extinction correction[87] was applied to the data of complex **1**.[86]

Hydrogen atoms were generally input at calculated positions and refined with a riding model. When justified by the quality of the data the positions of some or all hydrogen atoms were taken from difference Fourier syntheses and refined. Except for complex **5**, hydrogens on oxygen were always fully refined. For **5**, difference Fourier syntheses showed two shallow maxima close to the terminal oxygen atom O1 on Cu1, which were attributed to the two hydrogen atoms of a water ligand (d_{O-H} = 0.86 and 0.81 Å, angle(HOH) = 107°). Inspection of the hydrogen bond network indicated possible disorder, with the two water hydrogen donors to choose from three nitrate oxygen acceptors. Independent refinement of these hydrogens did not result in a reasonable geometry nor was it possible to identify a third Fourier peak resulting from disorder. Constrained refinement was therefore carried out with the water molecule treated as a rigid group pivoting on the oxygen atom and the initial positions of the hydrogen atoms taken from the Fourier peaks. Drawings were prepared with MERCURY.[88]

Bacterial strains, growth media and culture conditions

Biosensor strain *Chromobacterium violaceum* CV026 [89] was provided by Prof. Vittorio Venturi (ICGB, Trieste, Italy). *Pseudomonas aeruginosa* PAO1, *P. aeruginosa* PA14, *P. aeruginosa* PAOJP2, *P. aeruginosa* PA14-R3 and PAO1 ΔpqsAminiCTXluxPpqsA were kindly provided by Dr Livia Leoni, Department of Biology, University Roma Tre, Italy. Bacteria were routinely grown in Luria–Bertani (LB) medium (1%, (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract) with or without agar (1.5%, w/v) with shaking (180 rpm) at 37 °C. TSB (tryptone soy broth) (Oxoid) was used for growth of *P. aeruginosa* PAO1 in the assay of biofilm formation. When required, antibiotics kanamycin, streptomycin and tetracycline (BioReagent, Sigma-Aldrich, Germany) were incorporated into growth medium at the concentration of 30 or 200 μg mL^{-1} in the case of kanamycin, 20 μg mL^{-1} for streptomycin and 100 μg mL^{-1} for tetracycline.

P. aeruginosa strain DM-18, clinical isolate, showing reduced susceptibility towards commonly used antibiotics including ceftazidime, amikacin, enrofloxacin, piperacillin, colistin, imipenem and meropenem, was kindly provided by Prof. Dusan Misic (Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Belgrade) and used in experiments to determine the synergy of copper(II) compounds and antibiotics, namely piperacillin and ceftazidime. *P. aeruginosa* strain DM-18 was routinely grown in LB medium. Piperacillin and ceftazidime were purchased from Sigma-Aldrich, Germany.

Minimal inhibitory concentration (MIC) determination and antiproliferative activity

To determine minimal inhibitory concentrations (MIC) of each copper(II) complex and salt, N-heterocyclic ligand and antibiotic against bacterial (*P. aeruginosa* and *S. aureus*) and fungal (*C. albicans*) strains, standard broth microdilution method has been used in LB broth.[89] Tested compounds were included in concentrations from 0.8 to 1000 μg mL^{-1}. The carrier solvent DMSO was used as a control. Cell growth was measured *via* optical density at 600 nm (OD_{600}) using Tecan Infinite 200 Pro multiplate reader (Tecan Group, Männedorf, Switzerland) after 24 h incubation at 37 °C.

Synergy tests. To determine the synergy between the selected copper(II) compounds (**1**, **3** and $Cu(NO_3)_2 \cdot 3H_2O$) and antibiotics, piperacillin and ceftazidime, against *P. aeruginosa* DM-18, previously described checkerboard microdilution method has been used. Bacterial suspension with turbidity equivalent to 0.5 McFarland standards was adjusted to give a final inoculum 3×10^5 to 5×10^5 colony forming units (CFU) mL^{-1}, in 100 μL LB broth and incubated for 24 h at 37 °C. Plates were read for visual turbidity and spectrophotometrically at 600 nm, and results were recorded after 24 h of incubation at 37 °C as turbidity in wells indicated growth of the microorganism. The MIC was determined in the microtiter plate with the lowest drug concentration at which there was no visible growth. The MICs of single antibiotics (MIC_A) and in combination with Cu(II) compound (MIC_{AB}) were determined after 24 h of incubation at 37 °C. MIC_{AB} was defined as the MIC of drug A in the presence of compound B.[90]

Antiproliferative assay. Antiproliferative activities of copper(II) complexes and control compounds were measured using the methods described previously.[91] MRC5 cells (human lung fibroblast, obtained from ATCC) were plated in a 96-well flat-bottom plate at a concentration of 1×10^4 cells per well, grown in humidified atmosphere of 95% air and 5% CO_2 at 37 °C, and maintained as monolayer cultures in RPMI-1640 medium supplemented with 100 μg mL^{-1} streptomycin, 100 U

mL^{-1} penicillin, and 10% (v/v) fetal bovine serum (FBS). After 24 h of MRC5 cells incubation, the media containing increasing concentrations of each tested compound (5, 10, 50 and 250 μg mL^{-1}) were added to the cells. Control cultures received the solvent DMSO and blank wells contained 200 μL of growth medium. After 48 h of incubation, cells proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Cell proliferation was determined from the absorbance at 540 nm on Tecan Infinite 200 Pro multiplate reader (Tecan Group, Männedorf, Switzerland). The MTT assay was performed two times in four replicates and the results were presented as percentage of the control (untreated cells) that was arbitrarily set to 100%.

Effect of copper(II) compounds on violacein synthesis in *Chromobacterium violaceum* CV026 and pyocyanin synthesis in *Pseudomonas aeruginosa* PA14

C. violaceum CV026, mini-Tn5 mutant, dependent on exogenous AHLs for violacein production, was used as an indicator organism to monitor QS inhibition in disc diffusion assay.[64] Briefly, semi-solid LB agar (0.3%, w/v; 5 mL) was seeded with 50 μL of an overnight culture of *C. violaceum* CV026, supplemented by *N*-hexanoyl-L-homoserine lactone (Sigma, Germany) to a final concentration of 5 μM and poured over the surface of LB agar plates. When the overlaid agar had solidified, sterilized discs containing 50 μg of each compound were placed on the plates. Petri dishes were incubated in the upright position overnight (30 °C) and examined for the violacein synthesis. Inhibition of violacein synthesis was defined by the presence of white haloes in a purple background.

Overnight culture of *P. aeruginosa* PA14 grown in Kings Medium A (Himedia, Mumbai, India) was diluted to OD_{600} of 0.2 (5 mL). Different copper(II) compounds at a concentration of 50 μg mL^{-1} were added to the flasks with *P. aeruginosa* PAO1 and incubated at 37 °C for 24 h. Cultures were then extracted with chloroform (3 mL), chloroform layer was transferred to fresh tube and mixed with 0.2 M HCl (1 mL) giving it a pink to deep red color, indicating the presence of pyocyanin. To determine the amount of pyocyanin the absorbance was measured at 520 nm.[92] The experiment was done in triplicate and repeated two times. The values were expressed as ratio $(OD_{520}/OD_{600}) \times 100$.

Assays for acyl homoserine lactones (AHL) production in *P. aeruginosa*

For the extraction of AHLs, *P. aeruginosa* PAO1 was grown in LB broth at 37 °C for 24 h, supplemented with appropriate test compound to a final concentration of 50 μg mL^{-1} from DMSO stock solution. Control containing the equivalent amount of DMSO was also included. After 24 h of growth, optical density of the culture at 540 nm (OD_{540}) was determined and the cells were centrifuged. Supernatants (20 mL) from these cultures were extracted with the same volume of acidified ethyl acetate (acetic acid, 0.1% (v/v)) for 10 min with shaking and centrifuged to separate aqueous and ethyl acetate phase. Ethyl acetate phase was transferred to a clean bottle and solvent was removed under reduced pressure and the remaining residue weighted and dissolved in the same solvent in an appropriate amount which corresponded to following ratio: 1 μL of final extract corresponding to 1×10^9 cells of the original culture.[93] Experiments were performed in triplicate. Concentrated extracts were used in AHL assays. Semi-solid LB agar (0.3%, w/v; 5 mL) was seeded with 50 μL of an overnight culture of *C. violaceum* CV026 and poured over the surface of LB agar plates. When the overlaid agar had solidified, sterilized discs containing 10 μL of each AHL extract were placed on the surface of plates. These Petri dishes were incubated in the upright position overnight (30 °C) and examined for the stimulation of violacein synthesis. QS induction was detected as purple pigmentation of bioreporter strain grown around the discs. Measurements were made from the outer edge of the discs to the edge of the zones of QS-stimulation or QS-inhibition. Assays were performed in triplicate.

For the micro-volumetric determination of long-chain (3OC12-HSL) and short-chain AHLs (C4-HSL) levels in *P. aeruginosa* PAO1 culture supernatants, cultures were grown overnight at 37 °C in LB. Cultures were diluted 1 : 1000 in 10 mL LB in the presence of copper(II) compounds or DMSO and grown at 37 °C. After 6 h of growth the OD_{600} was measured, and 2 mL of culture was centrifuged and the supernatant was filtered and stored at −20 °C until used. Aliquot of culture supernatants (20 μL) was added to 180 μL of LB inoculated with *P. aeruginosa* PA14-R3 (measurements of 3OC12-HSL) or *P. aeruginosa* PAOJP2 (measurements of C4-HSL) (final $OD_{600} = 0.045$).[94] Plates were incubated at 37 °C with shaking, and cell density (OD_{600}) and bioluminescence (light counts per second, LCPS) were simultaneously measured after 4 h of incubation using Tecan Infinite 200 Pro multiplate reader (Tecan Group Ltd., Männedorf, Switzerland). Luminescence values were normalized per cell density.

2-Alkyl-4-quinolones (AHQs) production assay. For the detection and quantification of quorum sensing molecules 2-alkyl-4-quinolones (AHQs), such as 2-heptyl-3-hydroxy-4 quinolone (PQS) and 2-heptyl-4-quinolone (HHQ), which represent the major *P. aeruginosa* AHQs signal molecules present in bacterial culture supernatants, a lux-based *P. aeruginosa* AHQ sensor was employed in liquid microtiter plate assay. PAO1 ΔpqsA mini-CTX luxPpqsA biosensor was used for detection and quantification of AHQs molecules extracted from *P. aeruginosa* PAO1 cultures as described previously.[95]

Activity of copper(II) compounds on *P. aeruginosa* biofilm formation

P. aeruginosa biofilms were grown in TSB medium without shaking conditions. The effect of copper(II) compounds on biofilm forming ability was tested at concentrations of 50 and 250 μg mL^{-1}, using polystyrene flat-bottomed microtitre 96 well plates as previously described with some modifications.[96] Briefly, overnight culture of *P. aeruginosa* was diluted in a fresh LB medium to OD_{600} of 0.2 and 100 μL of the diluted culture with appropriate copper(II) compound. After incubation for 24 h

at 37 °C, the plate was washed twice with sterile water, dried, stained for 30 min with 0.4% crystal violet in order to determine biofilm formed. Stained cells were washed with sterile water to remove unbound crystal violet. After drying, 150 μL of 30% (v/v) acetic acid was added to solubilize the dye. After 10 min, the content of the wells was homogenized and the OD of samples was measured at 550 nm using Tecan Infinite 200 Pro multiplate reader (Tecan Group, Männedorf, Switzerland). The experiment was done in quintuplicate and repeated two times.

Statistical analysis

For each species, three samples were used and all the assays were carried out in triplicate. The results were expressed as mean values and standard errors, and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This analysis was carried out using SPSS v. 18.0 program.

Acknowledgements

This work has been financially supported by the Ministry of Education, Science and Technological Development, Republic of Serbia, under Grants No. 172036 and 173048. B. Đ. G. gratefully acknowledges financial support from the German Academic Exchange Service (DAAD) during postdoctoral stay at Heidelberg University, Germany. Dr Dusan Misic (Faculty of Veterinary Medicine, University of Belgrade) is acknowledged for supplying clinical isolate.

Notes and references

1 M. I. Hood and E. P. Skaar, *Nat. Rev. Microbiol.*, 2012, **10**, 525–537.
2 K. Y. Djoko, C.-L. Y. Ong, M. J. Walker and A. G. McEwan, *J. Biol. Chem.*, 2015, **290**, 18954–18961.
3 S. Lutsenko, *Curr. Opin. Chem. Biol.*, 2010, **14**, 211–217.
4 B.-E. Kim, T. Nevitt and D. J. Thiele, *Nat. Chem. Biol.*, 2008, **4**, 176–185.
5 E. Ladomersky and M. J. Petris, *Metallomics*, 2015, **7**, 957–964.
6 A. L. Casey, D. Adams, T. J. Karpanen, P. A. Lambert, B. D. Cookson, P. Nightingale, L. Miruszenko, R. Shillam, P. Christian and T. S. J. Elliott, *J. Hosp. Infect.*, 2010, **74**, 72–77.
7 P. Szymański, T. Frączek, M. Markowicz and E. Mikiciuk-Olasik, *Biometals*, 2012, **25**, 1089–1112.
8 B. K. Singh, N. Bhojak, P. Mishra and B. S. Garg, *Spectrochim. Acta, Part A*, 2008, **70**, 758–765.
9 A. P. Singh, N. K. Kaushik, A. K. Verma, G. Hundal and R. Gupta, *Eur. J. Med. Chem.*, 2009, **44**, 1607–1614.
10 M. N. Patel, P. A. Parmar and D. S. Gandhi, *Bioorg. Med. Chem.*, 2010, **18**, 1227–1235.
11 N. S. Ng, M. J. Wu, C. E. Jones and J. R. Aldrich-Wright, *J. Inorg. Biochem.*, 2016, DOI: 10.1016/j.jinorgbio.2016.06.006.
12 C. Santini, M. Pellei, V. Gandin, M. Porchia, F. Tisato and C. Marzano, *Chem. Rev.*, 2014, **114**, 815–862.
13 J. do Couto Almeida, I. M. Marzano, M. Pivatto, N. P. Lopes, A. M. Da Costa Ferreira, F. R. Pavan, I. C. Silva, E. C. Pereira-Maia, G. Von Poelhsitz and W. Guerra, *Inorg. Chim. Acta*, 2016, **446**, 87–92.
14 M. C. Wolfgang, B. R. Kulasekara, X. Liang, D. Boyd, K. Wu, Q. Yang, C. G. Miyada and S. Lory, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 8484–8489.
15 R. I. Aminov, *Front. Microbiol.*, 2010, **1**, 134.
16 L. Cegelski, G. R. Marshall, G. R. Eldridge and S. J. Hultgren, *Nat. Rev. Microbiol.*, 2008, **6**, 17–27.
17 D. A. Rasko and V. Sperandio, *Nat. Rev. Drug Discovery*, 2010, **9**, 117–128.
18 J. Njoroge and V. Sperandio, *EMBO Mol. Med.*, 2009, **1**, 201–210.
19 W. R. J. D. Galloway, J. T. Hodgkinson, S. Bowden, M. Welch and D. R. Spring, *Trends Microbiol.*, 2012, **20**, 449–458.
20 F. Imperi, F. Massai, C. R. Pillai, F. Longo, E. Zennaro, G. Rampioni, P. Visca and L. Leoni, *Antimicrob. Agents Chemother.*, 2013, **57**, 996–1005.
21 J. P. Gerdt and H. E. Blackwell, *ACS Chem. Biol.*, 2014, **9**, 2291–2299.
22 M. A. Welsh, N. R. Eibergen, J. D. Moore and H. E. Blackwell, *J. Am. Chem. Soc.*, 2015, **137**, 1510–1519.
23 S. Atkinson and P. Williams, *J. R. Soc., Interface*, 2009, **6**, 959–978.
24 S. Uroz, Y. Dessaux and P. Oger, *ChemBioChem*, 2009, **10**, 205–216.
25 M. Juhas, L. Eberl and B. Tümmler, *Environ. Microbiol.*, 2005, **7**, 459–471.
26 J. L. Veesenmeyer, A. R. Hauser, T. Lisboa and J. Rello, *Crit. Care Med.*, 2009, **37**, 1777–1786.
27 P. Williams and M. Cámara, *Curr. Opin. Microbiol.*, 2009, **12**, 182–191.
28 M. Schuster, D. J. Sexton, S. P. Diggle and E. P. Greenberg, *Annu. Rev. Microbiol.*, 2013, **67**, 43–63.
29 C. Winstanley and J. L. Fothergill, *FEMS Microbiol. Lett.*, 2009, **290**, 1–9.
30 T. Bjarnsholt, T. Tolker-Nielsen, N. Høiby and M. Givskov, *Expert Rev. Mol. Med.*, 2010, **12**, e11.
31 J. T. Thaden, S. Lory and T. S. Gardner, *J. Bacteriol.*, 2010, **192**, 2557–2568.
32 N. Amara, B. P. Krom, G. F. Kaufmann and M. M. Meijler, *Chem. Rev.*, 2011, **111**, 195–208.
33 M. G. P. Page and J. Heim, *Curr. Opin. Pharmacol.*, 2009, **9**, 558–565.
34 W. R. J. D. Galloway, J. T. Hodgkinson, S. D. Bowden, M. Welch and D. R. Spring, *Chem. Rev.*, 2011, **111**, 28–67.
35 C. J. Sumby, *Coord. Chem. Rev.*, 2011, **255**, 1937–1967.
36 D. J. Connolly, D. Cusack, T. P. O'Sullivan and P. J. Guiry, *Tetrahedron*, 2005, **61**, 10153–10202.
37 M. A. J. Duncton, E. L. Piatnitski, R. Katoch-Rouse, L. M. Smith II, A. S. Kiselyov, D. L. Milligan, C. Balagtas, W. C. Wong, J. Kawakami and J. F. Doody, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 1579–1581.
38 M. Yasui, Y. Ishikawa, N. Akiyama, T. Ishida, T. Nogami and F. Iwasaki, *Acta Crystallogr., Sect. B: Struct. Sci.*, 2001, **57**, 288–295.

39 C. V. K. Sharma and R. D. Rogers, *Cryst. Eng.*, 1998, **1**, 19–38.
40 N. D. Savić, B. Đ. Glišić, H. Wadepohl, A. Pavic, L. Senerovic, J. Nikodinovic-Runic and M. I. Djuran, *MedChemComm*, 2016, **7**, 282–291.
41 J. S. Haynes, S. J. Rettig, J. R. Sams, J. Trotter and R. C. Thompson, *Inorg. Chem.*, 1988, **27**, 1237–1241.
42 R. Balamurugan, M. Palaniandavar, H. Stoeckli-Evans and M. Neuburger, *Inorg. Chim. Acta*, 2006, **359**, 1103–1113.
43 A. W. Addison, T. N. Rao, J. Reedijk, J. van Rijn and G. C. Verschoor, *J. Chem. Soc., Dalton Trans.*, 1984, 1349–1356.
44 T. Otieno, S. J. Rettig, R. C. Thompson and J. Trotter, *Inorg. Chem.*, 1995, **34**, 1718–1725.
45 L. K. Thompson, T. C. Woon, D. B. Murphy, E. J. Gabe, F. L. Lee and Y. Le Page, *Inorg. Chem.*, 1985, **24**, 4719–4725.
46 A. S. Potapov, E. A. Nudnova, A. I. Khlebnikov, V. D. Ogorodnikov and T. V. Petrenko, *Inorg. Chem. Commun.*, 2015, **53**, 72–75.
47 A. B. P. Lever, E. Mantovani and B. S. Ramaswamy, *Can. J. Chem.*, 1971, **49**, 1957–1964.
48 G. A. van Albada, W. J. J. Smeets, A. L. Spek and J. Reedijk, *Inorg. Chim. Acta*, 1997, **260**, 151–161.
49 R. Prins, P. J. M. W. L. Birker, J. G. Haasnoot, G. C. Verschoor and J. Reedijk, *Inorg. Chem.*, 1985, **24**, 4128–4133.
50 B. J. Hathaway, in *Comprehensive Coordination Chemistry*, ed. G. Wilkinson, R. D. Gillard and J. A. McCleverty, Pergamon, Oxford, vol. 5, 1987.
51 N. S. Drašković, D. D. Radanović, U. Rychlewska, B. Warżajtis, I. M. Stanojević and M. I. Djuran, *Polyhedron*, 2012, **43**, 185–193.
52 B. Đ. Glišić, L. Senerovic, P. Comba, H. Wadepohl, A. Veselinovic, D. R. Milivojevic, M. I. Djuran and J. Nikodinovic-Runic, *J. Inorg. Biochem.*, 2016, **155**, 115–128.
53 N. D. Savić, D. R. Milivojevic, B. Đ. Glišić, T. Ilic-Tomic, J. Veselinovic, A. Pavic, B. Vasiljevic, J. Nikodinovic-Runic and M. I. Djuran, *RSC Adv.*, 2016, **6**, 13193–13206.
54 A. A. R. Despaigne, F. B. Da Costa, O. E. Piro, E. E. Castellano, S. R. W. Louro and H. Beraldo, *Polyhedron*, 2012, **38**, 285–290.
55 K. D. Mjos, E. Polishchuk, M. J. Abrams and C. Orvig, *J. Inorg. Biochem.*, 2016, DOI: 10.1016/j.jinorgbio.2016.02.026.
56 R. Starosta, K. Stokowa, M. Florek, J. Król, A. Chwilkowska, J. Kulbacka, J. Saczko, J. Skała and M. Jeżowska-Bojczuk, *J. Inorg. Biochem.*, 2011, **105**, 1102–1108.
57 C. L. Dupont, G. Grass and C. Rensing, *Metallomics*, 2011, **3**, 1109–1118.
58 J. Elguindi, J. Wagner and C. Rensing, *J. Appl. Microbiol.*, 2009, **106**, 1448–1455.
59 W. R. Schwan, P. Warrener, E. Keunz, C. K. Stover and K. R. Folger, *Int. J. Med. Microbiol.*, 2005, **295**, 237–242.
60 V. Hodgkinson and M. J. Petris, *J. Biol. Chem.*, 2012, **287**, 13549–13555.
61 A. Marín-Hernández, I. Gracia-Mora, L. Ruiz-Ramírez and R. Moreno-Sánchez, *Biochem. Pharmacol.*, 2003, **65**, 1979–1989.
62 Y.-H. Hu, W. Dang, C.-S. Liu and L. Sun, *Lett. Appl. Microbiol.*, 2010, **50**, 97–103.
63 T. Nakayama, N. Nomura and M. Matsumura, *J. Appl. Microbiol.*, 2007, **102**, 1300–1306.
64 K. H. McClean, M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. A. B. Stewart and P. Williams, *Microbiology*, 1997, **143**, 3703–3711.
65 T. Morohoshi, M. Kato, K. Fukamachi, N. Kato and T. Ikeda, *FEMS Microbiol. Lett.*, 2008, **279**, 124–130.
66 W. Chu, D. A. Vattem, V. Maitin, M. B. Barnes and R. J. C. McLean, *Methods Mol. Biol.*, 2011, **692**, 3–19.
67 T. Das and M. Manefield, *PLoS One*, 2012, **7**, e46718.
68 J. Hodgkinson, S. D. Bowden, W. R. J. D. Galloway, D. R. Spring and M. Welch, *J. Bacteriol.*, 2010, **192**, 3833–3837.
69 D. G. Davies, M. R. Parsek, J. P. Pearson, B. Iglewski, J. W. Costerton and E. P. Greenberg, *Science*, 1998, **280**, 295–298.
70 L. M. Vega, J. Mathieu, Y. Yang, B. H. Pyle, R. J. C. McLean and P. J. J. Alvarez, *Int. Biodeterior. Biodegrad.*, 2014, **91**, 82–87.
71 G. Brackman, P. Cos, L. Maes, H. J. Nelis and T. Coenye, *Antimicrob. Agents Chemother.*, 2011, **55**, 2655–2661.
72 Y. T. Tan, D. J. Tillett and I. A. Mackay, *Mol. Med. Today*, 2000, **6**, 309–314.
73 G. D. Wright, *Chem. Biol.*, 2000, **7**, R127–R132.
74 A. M. Fayaz, K. Balaji, M. Girilal, R. Yadav, P. T. Kalaichelvan and R. Venketesan, *J. Nanomed. Nanotechnol.*, 2010, **6**, 103–109.
75 Z. E. Nazari, M. Banoee, A. A. Sepahi, F. Rafii and A. R. Shahverdi, *Gold Bull.*, 2012, **45**, 53–59.
76 H. W. Richardson, W. E. Hatfield, H. J. Stoklosa and J. R. Wasson, *Inorg. Chem.*, 1973, **12**, 2051–2053.
77 K. Kabsch, in *International Tables for Crystallography*, ed. M. G. Rossmann and E. Arnold, Kluwer Academic Publishers, Dordrecht, vol. F, ch. 11.3, 2001.
78 CrysAlisPro, Agilent Technologies UK Ltd., Oxford, UK, 2011–2014, and Rigaku Oxford Diffraction, Rigaku Polska Sp.z o.o., Wrocław, Poland, 2015.
79 SAINT, Bruker AXS GmbH, Karlsruhe, Germany, 1997–2013.
80 R. H. Blessing, *Acta Crystallogr., Sect. A: Found. Crystallogr.*, 1995, **51**, 33–38.
81 SCALE3 ABSPACK, CrysAlisPro, Agilent Technologies UK Ltd., Oxford, UK, 2011–2014, and Rigaku Oxford Diffraction, Rigaku Polska Sp.z o.o., Wrocław, Poland, 2015.
82 (*a*) G. M. Sheldrick, *SADABS*, Bruker AXS GmbH, Karlsruhe, Germany, 2004–2014; (*b*) L. Krause, R. Herbst-Irmer, G. M. Sheldrick and D. Stalke, *J. Appl. Crystallogr.*, 2015, **48**, 3–10.
83 W. R. Busing and H. A. Levy, *Acta Cryst.*, 1957, **10**, 180–182.
84 (*a*) P. T. Beurskens, in *Crystallographic Computing 3*, ed. G. M. Sheldrick, C. Krüger and R. Goddard, Clarendon Press, Oxford, UK, 1985, p. 216; (*b*) P. T. Beurskens, G. Beurskens, R. de Gelder, J. M. M. Smits, S. Garcia-Granda and R. O. Gould, *DIRDIF-2008*, Radboud University Nijmegen, The Netherlands, 2008.
85 (*a*) L. Palatinus, SUPERFLIP, EPF Lausanne, Switzerland and Fyzikální ústav AV ČR, v. v. i., Prague, Czech Republic, 2007–

2014; (*b*) L. Palatinus and G. Chapuis, *J. Appl. Crystallogr.*, 2007, **40**, 786–790.

86 (*a*) G. M. Sheldrick, *SHELXL–20xx*, University of Göttingen and Bruker AXS GmbH, Karlsruhe, Germany, 2012–2014; (*b*) G. M. Sheldrick, *Acta Crystallogr., Sect. A: Found. Crystallogr.*, 2008, **64**, 112–122; (*c*) G. M. Sheldrick, *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.*, 2015, **71**, 3–8.

87 A. C. Larson, in *Crystallographic Computing*, ed. F. R. Ahmed, S. R. Hall and C. P. Huber, Munksgaard, Copenhagen, 1970, p. 291.

88 I. J. Bruno, J. C. Cole, P. R. Edgington, M. Kessler, C. F. Macrae, P. McCabe, J. Pearson and R. Taylor, *Acta Crystallogr., Sect. B: Struct. Sci.*, 2002, **58**, 389–397.

89 J. T. Casey, C. O'Cleirigh, P. K. Walsh and D. G. O'Shea, *J. Microbiol. Methods*, 2004, **58**, 327–334.

90 M. M. Sopirala, J. E. Mangino, W. A. Gebreyes, B. Biller, T. Bannerman, J.-M. Balada-Llasat and P. Pancholi, *Antimicrob. Agents Chemother.*, 2010, **54**, 4678–4683.

91 M. B. Hansen, S. E. Nielsen and K. Berg, *J. Immunol. Methods*, 1989, **119**, 203–210.

92 D. W. Essar, L. Eberly, A. Hadero and I. P. Crawford, *J. Bacteriol.*, 1990, **172**, 884–900.

93 I. Bertani, G. Rampioni, L. Leoni and V. Venturi, *BMC Microbiol.*, 2007, **7**, 71.

94 (*a*) K. Duan and M. G. Surette, *J. Bacteriol.*, 2007, **189**, 4827–4836; (*b*) F. Massai, F. Imperi, S. Quattrucci, E. Zennaro, P. Visca and L. Leoni, *Biosens. Bioelectron.*, 2011, **226**, 3444–3449.

95 M. P. Fletcher, S. P. Diggle, S. A. Crusz, S. R. Chhabra, M. Cámara and P. Williams, *Environ. Microbiol.*, 2007, **9**, 2683–2693.

96 B. M. Coffey and G. G. Anderson, in *Pseudomonas Methods and Protocols*, ed. A. Filloux and J.-L. Ramos, Humana Press, New York, 2014, pp. 631–643.