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Antibacterial activity of naphthyl derived *bis*-(3-hydroxy-4-pyridinonate) copper(II) complexes against multidrug-resistant bacteria

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ABSTRACT

In this work, we report the synthesis and characterization of three novel copper(II) complexes of naphthyl derived 3-hydroxy-4-pyridinone chelators. Their antibacterial activity against several Gram-positive and Gram-negative reference strains and multidrug-resistant clinical isolates was assessed through determination of the minimum inhibitory concentration (MIC).

The complex **Cu(naph1pp)$_2$** shows the highest antibacterial activity, including against multidrug-resistant isolates, nonetheless, being more active against Gram-positive than Gram-negative bacteria. **Cu(naph1pp)$_2$** was further explored in combinatorial tests with ciprofloxacin against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE). The combination of **Cu(naph1pp)$_2$** and ciprofloxacin is considered additive, i.e., the effect of the two compounds combined is stronger than that of the individual compounds in the equivalent concentration.

1. Introduction

The development of new antimicrobial therapeutic agents is one of the main goals in medicinal chemistry. New compounds or strategies with antimicrobial activity are of extreme importance since we are running out of effective antibiotics towards the so-called superbugs harboring resistance to multiple antibiotics, which are now becoming globally spread and are jeopardizing the current treatment of many infections [1,2]. Carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum β-lactamases and carbapenemases, vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) are examples of multidrug-resistant bacteria towards which research, discovery, and development of new antibiotics must be focused, according to the World Health Organization [3].

To respond to these threats new antimicrobial platforms are being developed to prevent and treat infections caused by these resistant strains. In this context, metal-based antimicrobial macromolecules have emerged as an alternative to conventional platforms since they combine multiple mechanisms of action into one platform.

In the last years, many copper(II) complexes have been synthesized and their potential to act as antimicrobial and antifungal agents has been evaluated. In the synthesis of the new complexes, several ligands with antimicrobial activity have been used, and their complexation with copper seems to enhance such antimicrobial activity [4–8].

3-hydroxy-4-pyridinones (3,4-HPOs) are bidentate O,O-donor chelators with a 6-membered ring skeleton, that have been considered "privileged" structures for drug design. Their great interest in medicinal chemistry derives from the association of high/specific metal-chelating ability, easy derivatization and low toxicity [9–12]. Knowing that these ligands i) strongly bind copper(II) [13,14], ii) the [Cu(L)$_2$] species are dominant at physiological pH and iii) Cu^{2+} ions are kept complexed throughout the biological study, no toxic effects due to demetallation are expected.

Considering the versatile chemistry of the 3,4-HPOs, three bidentate ligands bearing a 3,4-HPO chelating moiety covalently bound to a monosubstituted naphthalene (**naph1pp** and **naph2pp**) or to a dansyl platform (**dansylpp**) were used as ligands in the synthesis of the copper (II) complexes (Fig. 1).

In **naph1pp** and **naph2pp** ligands, the naphthalene platform was attached to a 3,4-HPO ligand with the purpose of increasing the lipophilicity of the ligands and corresponding copper(II) complexes, and

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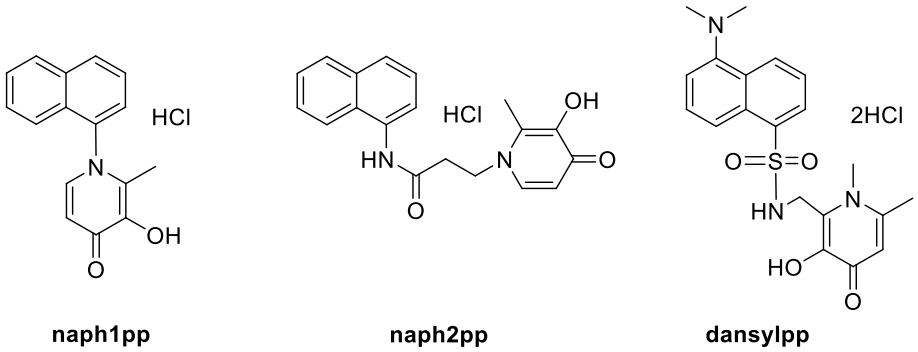

Fig. 1. Chemical structures of naphthyl derived 3,4-HPO ligands.

also because it has low molecular weight, biological compatibility and fluorescence properties. Considering these properties, the aim was to evaluate if the conjugation of the two molecules enhanced the antibacterial activity of each molecule alone. Naphthalene is part of the structure of a large number of molecules used in therapeutics, such as propranolol (beta-adrenergic blocking agent), naphazoline (a vasoconstrictor), naproxen and nabumetone (nonsteroidal anti-inflammatory agents) and methallenestril (a nonsteroidal estrogen) [15]. Moreover, naphthalene also presents other pharmacological properties, such as anti-protozoal and anti-tumor activity [16,17]. For the ligand **dansylpp**, a dansyl platform was used. This platform allowed not only to enhance water solubility but also to guarantee a maximum and constant fluorescence intensity in the pH range between 4 and 9, a property that can be further explored [18]. With this platform, the water solubility of the ligand was improved, while maintaining the same general structure of the previous ligands. The goal was to evaluate if the increase in water solubility of the ligand and copper(II) complex would influence positively the antibacterial activity.

The aim of this work was to infer the influence of particular features of the ligands and corresponding copper(II) complexes in their antimicrobial activity, which was assessed against several reference strains and multidrug-resistant isolates.

2. Experimental section

2.1. Chemicals and instrumentation

Reagents and solvents were purchased as reagent grade and used without further purification, unless otherwise stated.

Ligands 1,2-dimethyl-3-hydroxy-4-pyridinone (also known as, deferiprone®, L1, CP20 or Hdmpp), maltol, ethylmaltol and 1-aminonaphthalene were purchased from Sigma–Aldrich (grade puriss, p.a.) and were used as received. The remaining derivatives of 3-hydroxy-4-pyridinone ligands substituted with alkyl, naphthyl and dansyl groups were synthesized and purified as previously described [18–20].

High resolution MS analysis was carried out by electrospray ionization (ESI) using a LTQ Orbitrap TM XL hybrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) controlled by LTQ Tune Plus 2.5.5 and Xcalibur 2.1.0 with the following ESI source parameters: electrospray needle voltage 3.1 kV, sheath gas nitrogen 5, capillary temperature 275 °C, capillary voltage 9 V and tube lens voltage 85 V.

Elemental analyses were performed at the Unidad De Análisis Elemental of the Universidad de Santiago (Spain). EPR spectra were recorded using an X-band (9 GHz) Bruker ELEXSYS E 500 spectrometer equipped with a variable temperature unit (ER 4B1 VT), available at Laboratório de Ressonância Paramagnética Electrónica (Centro de Materiais da Universidade do Porto).

The stock solution of ciprofloxacin was prepared from ciprofloxacin hydrochloride, which was purchased from Sigma-Aldrich.

2.2. Synthesis

Copper(II) complexes, whose formulae and abbreviations are shown in Figs. 2 and S1, were synthesized following the method previously described [14,20], which is based on the reaction of the ligand with the metal ion salt in aqueous or aqueous/organic solution, depending on the lipophilicity of the ligand, using a 1:2.2 metal-to-ligand ratio, in order to avoid the concomitant formation of metal ion hydroxides. The

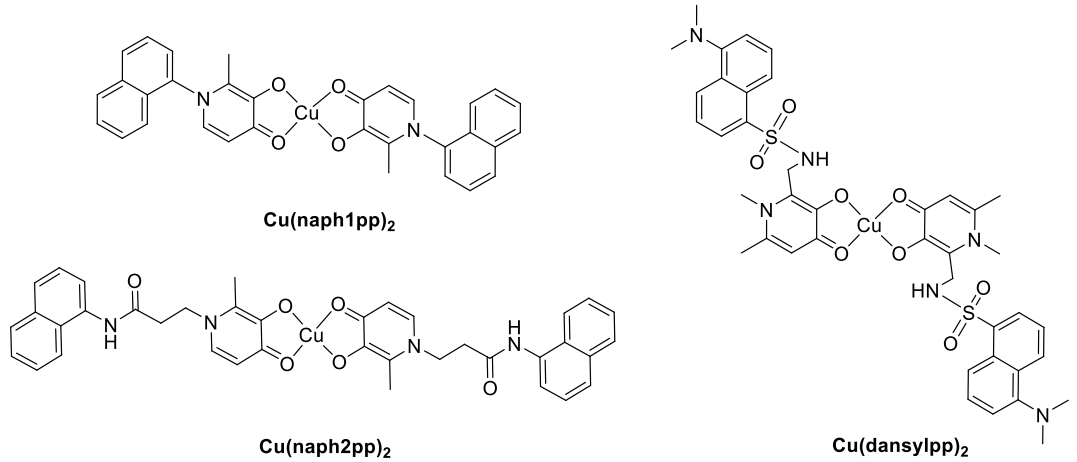

Fig. 2. Chemical structures of copper(II) complexes of naphthyl substituted 3,4-HPOs.

method also entails the ligand deprotonation prior to solution mixture by raising to 9 the pH of the solution containing the ligand. In this work, the ligand (22 mmol) was dissolved in water and a sodium hydroxide (NaOH) solution was added dropwise until pH = 9. The solution was stirred for 10 min followed by addition of 10 mmol of copper (II) nitrate dissolved in the minimum amount of water. For complexes **Cu(naph1pp)$_2$** and **Cu(naph2pp)$_2$**, the copper(II) acetate salt was used. The reaction was stirred at room temperature overnight to maximize the yield. The green solids obtained were filtered, washed with the minimum amount of cold water, transferred to a desiccator and dried under vacuum.

The copper(II) complexes were isolated in the solid state as green powders and characterized by elemental analysis. The elemental analysis of the alkyl copper(II) complexes are presented in SI.

Bis(3-hydroxy-2-methyl-1-naphthyl-4-pyridinonate)copper(II), ESI-HRMS: $[M + H]^+$ ($C_{32}H_{24}CuN_2O_4 + H^+$) m/z = 564.11011 (Δm = 0.7 ppm); **Cu(naph1pp)$_2$.2H$_2$O** $C_{32}H_{28}N_2O_6Cu$: found: C, 64.34; H, 4.75; N, 4.68, calcd: C, 64.04; H, 4.70; N, 4.67.

Bis(1-(N-naphthylcarbamoylpropyl)-3-hydroxy-2-methyl-4-pyridinonate)copper(II), ESI-HRMS: $[M + H]^+$ ($C_{38}H_{34}CuN_4O_6 + H^+$) m/z = 706.18471 (Δm = 1.6 ppm); **Cu(naph2pp)$_2$.3.5H$_2$O** $C_{38}H_{41}N_4O_{9.5}Cu$: found: C, 59.07; H, 4.13; N, 7.29, calcd: C, 59.33; H, 4.13; N, 7.29.

Bis(2-(*N*-dansylaminomethyl)-3-hydroxy-1,6-dimethyl-4-pyridinonate)copper(II), ESI-HRMS: $[M + H]^+$ ($C_{40}H_{44}CuN_6O_8S_2 + H^+$) m/z = 864.20308 (Δm = 2.4 ppm); **Cu(dansylpp)$_2$** $C_{40}H_{44}N_6O_8S_2Cu$: found: C, 55.70; H, 4.93; N, 9.22, calcd: C, 55.57; H, 5.13; N, 9.72.

2.3. Electron paramagnetic resonance

The samples were prepared by dissolution of the complexes in DMSO or MOPS and transferred to a capillary tube which was placed in the EPR quartz tube. EPR spectra were obtained in the following general experimental conditions: microwave frequency of 100 kHz, microwave power of 20 mW, modulation amplitude of 8 G, gain of 60 dB, acquisition time of 300 s and 10 scans, at 100 ± 1 K. The spectra were simulated with the computer suite program Bruker WinEPR/SimFonia.

2.4. Antimicrobial activity assays

2.4.1. Bacterial strains and growth conditions

All ligands and copper(II) complexes (Figs. 1 and 2) were tested against Gram-positive (*Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212) and Gram-negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) reference strains. Compounds shown in Fig. S1 were tested against the same strains as well as against multidrug-resistant isolates (Ec2-SA1, Pa4-SA2, Sa1-SA3, Ef1-SA4). Due to the stronger activity of **Cu(naph1pp)$_2$** against Gram-positive reference strains, this complex in particular was tested against several clinical multidrug-resistant isolates of methicillin-resistant *S. aureus* – MRSA (isolates Sa1-SA3, Sa3-SA3 and SA008) and of vancomycin-resistant *E. faecalis* – VRE (isolates Ef1-SA4, Ef2-SA4 and Ef3-SA4). All bacteria were grown in Mueller-Hinton II agar (MH agar – Liophilchem, Roseto degli Abruzzi (Te), Italy) from stored cultures at −80 °C. MH inoculated plates were incubated at 37 °C for 20–24 h before fresh colonies were picked to initiate each in vitro assay.

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

To evaluate the antibacterial activity of each ligand and copper(II) complex, MIC values were determined by the broth microdilution technique, following the recommendations of the Clinical and Laboratory Standards Institute [21]. Briefly, cation-adjusted Mueller-Hinton broth (MHB2 – Sigma-Aldrich) was used and dispensed into 96-well plates, where each ligand or complex was serially diluted (two-fold dilutions were performed). The highest in-test concentration was 256 or 512 μg/mL. All test-wells were inoculated with a bacterial concentration of approximately 5×10^5 CFU/mL. Positive and negative controls were used in each assay. The inoculated 96-well plates were incubated at 37 °C for 20–24 h. The MIC was the lowest concentration of the ligand or complex that completely inhibited the bacterial growth as detected by the unaided eye. An aliquot was taken from each well corresponding to the MIC and above the MIC and spread into MH agar plates with further incubation for 18–22 h at 37 °C. The lowest concentration at which no bacterial growth occurred was defined as the MBC.

2.4.3. Checkerboard method

Cu(naph1pp)$_2$ was combined, in two-drug combinations, with ciprofloxacin against MRSA and VRE isolates using the broth microdilution checkerboard method as previously described [22]. Through this method, the MICs of each single drug and of both drugs in combination were determined after 24 h of incubation at 37 °C. Those MIC values were then used to calculate the fractional inhibitory index (ΣFIC), interpreted as reported by Sopirala and collaborators [23]: ΣFIC ≤ 0.5, synergy; 0.5 < ΣFIC ≤ 1, additivity; 1 < ΣFIC ≤ 4, indifference; 4 < ΣFIC, antagonism.

2.4.4. Time-Kill method

The bactericidal effect of **Cu(naph1pp)$_2$** was also assessed by performing the time-kill method at its MIC value and 2× MIC, against *S. aureus* ATCC 25922 and *E. faecalis* ATCC 29212. Additionally, combinations of **Cu(naph1pp)$_2$** and ciprofloxacin that were found to be synergistic or additive by the checkerboard method were also explored by the time-kill curve method, according to the Clinical and Laboratory Standards Institute guidelines [24]. The time-kill assay was based in the broth macrodilution method. Briefly, MHB2 containing one or two drug combinations was inoculated with a mid-log-phase aliquot of the test bacterium, to yield a final concentration of approximately 10^6 CFU/mL, and incubated at 37 °C. Aliquots were removed at times 0, 2, 4, 8, 10 and 24 h, serially diluted in sterile 0.9% sodium chloride and plated in MH agar plates, which were incubated at 37 °C for 18–22 h, and then the number of CFU/mL were determined. By plotting the $\log_{10}$ CFU/mL versus time, time-kill curves were obtained. Bacteriostatic activity was defined as maintenance or reduction of < 99.9% (< 3 $\log_{10}$) of the total number of CFU/mL as in the original inoculum, while bactericidal activity was defined as a 99.9% reduction (≥ 3 $\log_{10}$) of the total number of CFU/mL in comparison to the original inoculum. Synergy was defined as a ≥ 2 $\log_{10}$ decrease in colony count of the two-drug combination after 24 h in comparison to the most active single agent [23,25].

3. Results and discussion

In this work, three novel copper(II) complexes were synthesized and their *in vitro* antibacterial activity was assessed. Such complexes included two 3,4-HPO: naphthalene conjugates (**naph1pp** and **naph2pp**) and a 3,4-HPO: dansyl conjugate (**dansylpp**). The results obtained by elemental analysis confirmed the synthesis of the complexes.

To compare the antibacterial activity of the new ligands and corresponding copper(II) complexes with previous reported 3,4-HPO ligands and copper(II) complexes, these were also tested herein in the same conditions.

3.1. Aqueous solution characterization

To characterize the coordination sphere of the novel copper(II) complexes EPR spectra were acquired in DMSO and MOPS at 100 K for **Cu(naph1pp)$_2$**, **Cu(naph2pp)$_2$** and **Cu(dansylpp)$_2$**.

The EPR spectra and the corresponding computer simulations (Figs. 3, S5, S6) obtained for the three copper(II) complexes indicate that the structure is similar for all of them. In Fig. 3, the spectra of complex **Cu(naph1pp)$_2$** in DMSO and MOPS and the corresponding computer simulations are depicted. The resolution of the spectrum in

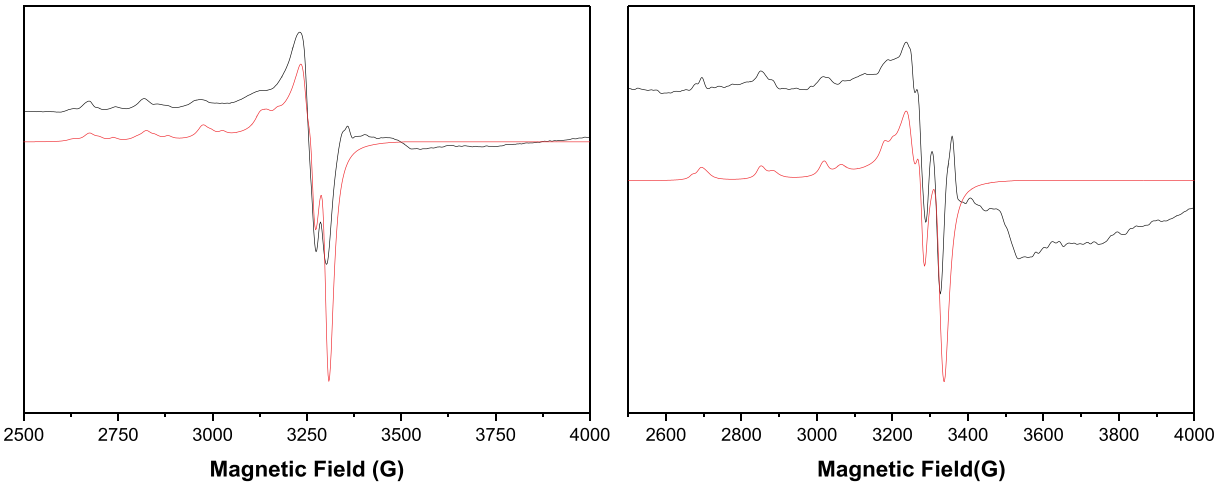

Fig. 3. EPR spectra (black) and computer simulations (red) of **Cu(naph1pp)$_2$** in DMSO (left) and MOPS(right) at 100 K.

Table 1
EPR Parameters for the complex **Cu(naph1pp)$_2$** in DMSO and MOPS.

Solvent		g-Value			A-value/10^{-4} cm^{-1}		
		g_{zz}	g_{xx}	g_{yy}	A_{zz}	A_{xx}	A_{yy}
DMSO	^{63}Cu	2.328	2.068	2.068	164.0	10.0	10.0
	^{65}Cu	2.34	2.070	2.070	186.0	10.0	10.0
	^{63}Cu	2.285	2.066	2.070	154.0	10.0	10.0
	^{65}Cu	2.312	2.070	2.070	160.0	10.0	10.0
MOPS	^{63}Cu	2.303	2.060	2.060	174.0	15.0	15.0
	^{65}Cu	2.300	2.060	2.060	190.0	15.0	15.0

MOPS is lower due to the lower solubility in the solvent. However, the computational simulation allowed the assignment of the Spin-Hamiltonian parameters for the copper(II) species and the values are presented in Table 1.

Both EPR spectra presented the typical features of 3,4-HPO copper (II) complexes with a square planar geometry with a tetragonal distortion and a $^2B_1$ ($d(x^2 - y^2)$) ground state [14,18,26–29]. The EPR parameters (Table 1) are characteristic of an interaction of an unpaired electron (S = 1/2) with the copper nuclei (^{65}Cu/^{63}Cu, I = 3/2) [14].

The spectra exhibit two well defined regions and in the lower field the characteristic four hyperfine lines of the two naturally occurring copper isotopes (^{65}Cu/^{63}Cu, I = 3/2) are discernible.

As other 3,4-HPO copper complexes, the EPR spectra showed differences when dissolved in different solvents, thus indicating that they are sensibly perturbed by interaction with neighboring solvent molecules. The results described are similar to those previously reported for copper(II) complexes with an O_4 coordination sphere [14,18,30].

In order to get insight on the speciation of the copper(II) complexes in solution we predicted the distribution diagrams for these complexes (Figs. 4, S7, S8) considering the values of the acidity constants of the ligands [20], metal ion and water protolysis constants, and the values of the stability constants obtained for the copper(II) complexes with 3,4-HPO ligands [13,14].

The predicted distribution diagram for the complex **Cu(naph1pp)$_2$** is depicted in Fig. 4. The diagram shows that the complex formation starts at very acidic pH values and indicate that at a physiological pH value the major complex species is **Cu(naph1pp)$_2$**.

Considering the aqueous solution characterization and analyzing the results from the EPR studies, it can be concluded that the structure of the copper complexes is not altered by the introduction of the substituents on positions 1 and 6 of the heterocyclic ligand and that an O_4 coordination sphere is present in all copper(II) complexes. The EPR spectra and predicted speciation diagram also indicate that the

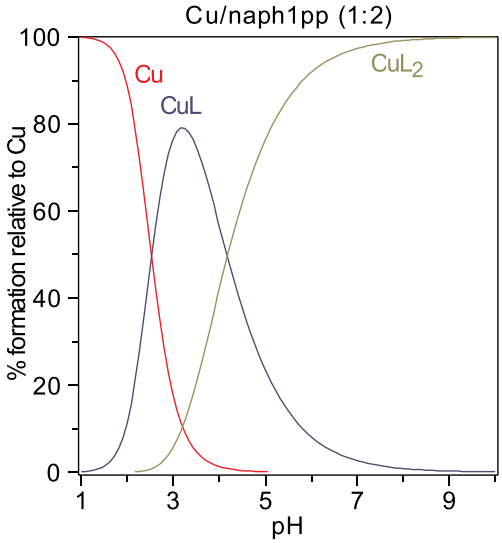

Fig. 4. Predicted speciation diagram for **Cu(naph1pp)$_2$** as a function of pH.

CuL_2 type complex is the sole copper(II) species present at pH values above 7.

3.2. Antimicrobial activity

3.2.1. MIC and MBC values

Since the new copper(II) complexes have distinctive structural characteristics, we sought to evaluate their influence on the antimicrobial activity.

The MIC values (and MBC values, when appropriate) of 1-aminonaphthalene, the three ligands and the corresponding copper(II) complexes against reference strains are presented in Table 2. To assure that the naphthalene unit did not affect the results per se, 1-aminonaphthalene was used as a control and no antimicrobial activity was observed.

Cu(naph1pp)$_2$ was the most active, particularly against Gram-positive bacteria, showing a bactericidal effect at a concentration of 2× MIC against *S. aureus* ATCC 25923 and at a concentration equal to the MIC against *E. faecalis* ATCC 29212 (Table 2 and Fig. 5). **Cu(naph1pp)$_2$** was also tested against several Gram-positive multidrug-resistant clinical isolates, including MRSA and VRE isolates (Table 3), and proved to be equally active against such isolates. We believe that these results are related not only to the lipophilicity of the complexes but also to

Table 2
MIC values in μg/mL (mM in parenthesis) of three naphthyl derived 3,4-HPO ligands and corresponding Cu(II) complexes, against Gram-positive and Gram-negative reference strains.

Compounds	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 25923	*E. faecalis* ATCC 29212
$Cu(naph1pp)_2$	256[1] (0.43)	512 (0.85)	64[2] (0.11)	128[3] (0.21)
$Cu(naph2pp)_2$	256 (0.33)	256 (0.33)	256 (0.33)	256 (0.33)
$Cu(dansyl)_2$	> 512 (> 0.59)	512 (0.59)	512 (0.59)	512 (0.59)
$Cu(dmpp)_2$	> 512 (> 1.51)	512 (1.51)	> 512 (> 1.51)	> 512 (> 1.51)
naph1pp	> 512 (> 1.78)	> 512 (> 1.78)	512 (1.78)	512 (1.78)
naph2pp	> 512 (> 1.43)	> 512 (> 1.43)	128 (0.36)	512 (1.43)
dansylpp	> 512 (> 0.95)	> 512 (> 0.95)	256 (0.48)	512 (0.95)
1-aminonaphthalene	512 (3.58)	512 (3.58)	512 (3.58)	512 (3.58)
Hdmpp	256 (1.84)	256 (1.84)	256 (1.84)	32/64 (0.23/0.46)

The MBC was [1] 512 μg/mL (0.85 mM); [2,3] 128 μg/mL (0.21 mM).

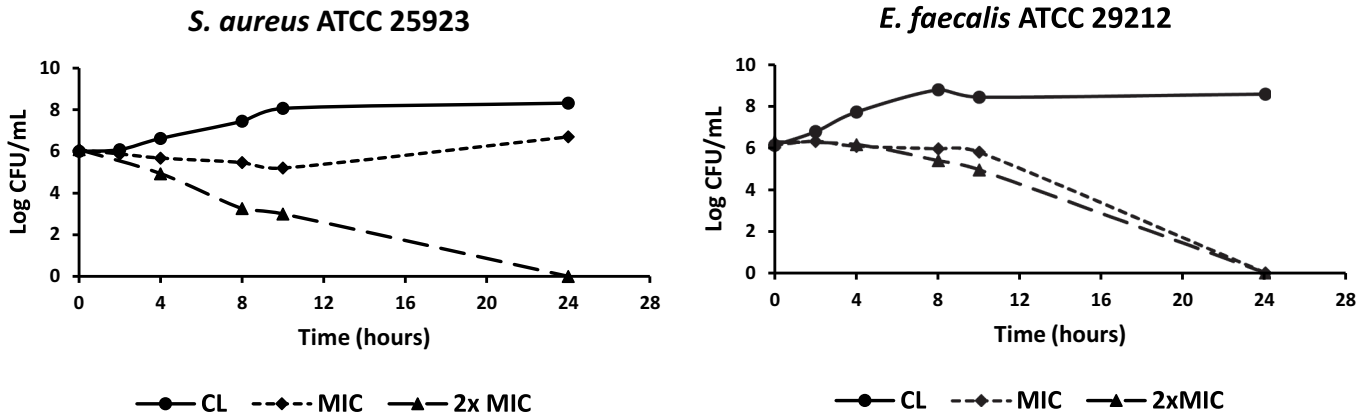

Fig. 5. Growth curves using viable counts of *S. aureus* ATCC 25923 and of *E. faecalis* ATCC 29212 after 24 h at 37 °C in LB medium, in absence (control) or presence of **$Cu(naph1pp)_2$** in a concentration corresponding to the MIC or 2× MIC.

Table 3
MIC values in μg/mL (mM in parenthesis) of **$Cu(naph1pp)_2$** against Gram-positive multidrug-resistant (MDR) isolates.

MDR isolates	$Cu(naph1pp)_2$
Sa1-SA3	64 (0.11)[a]
Sa3-SA3	64 (0.11)[a]
SA008	64 (0.11)[a]
Ef1-SA4	32 (0.05)[b]
Ef2-SA4	128 (0.21)[a]
Ef3-SA4	128 (0.21)[a]

Sa1-SA3, Sa3-SA3 and SA008 are MRSA isolates. Ef1-SA4, Ef2-SA4 and Ef3-SA4 are VRE isolates.
[a] The MBC was equal to the MIC.
[b] The MBC was 128 μg/mL (0.21 mM).

Table 4
MIC values of **$Cu(naph1pp)_2$** in combination with ciprofloxacin and respective FIC index values obtained from the checkerboard method.

MDR isolate	MIC (μg/mL)				ΣFIC
	Alone		In combination		
	$Cu(naph1pp)_2$	CIP	$Cu(naph1pp)_2$	CIP	
Ef1-SA4	32	128	4	32	0.38 (S)
Ef2-SA4	128	128	64	32	0.75 (A)
SA008	64	64	32	32	1.00 (A)
Sa1-SA3	64	256	8	128	0.63 (A)

CIP: ciprofloxacin; ΣFIC ≤ 0.5, synergy (S); 0.5 < ΣFIC ≤ 1, additivity (A); 1 < ΣFIC ≤ 4, indifference (I).

differences in the structure of the copper(II) complexes.

Regarding ligands **naph1pp** and **naph2pp**, some structural differences can be pointed out such as size and spatial disposition. As previously reported [19], the spatial disposition of dihydroxypyridinium and naphthalene entities differ in **naph1pp** and **naph2pp** cations due to different distances between the chelating and the fluorescent moieties. It is expected that for **$Cu(naph1pp)_2$** a smaller and less distorted complex is formed when compared with **$Cu(naph2pp)_2$** and that feature may explain the higher activity of **$Cu(naph1pp)_2$**.

Another feature that we wanted to assess with those two ligands was the influence of the amide group in the complex formed by the **naph2pp** ligand. This group is characteristic of several biologically active compounds, such as antimicrobials and antiprotozoals [31,32]. In the present study, the presence of this group did not enhance the antibacterial activity, neither of the ligand nor the copper(II) complex. Regarding the ligand **dansylpp** and the complex **$Cu(dansylpp)_2$**, the increase in water solubility of the ligand and copper(II) complex did not influence positively the antibacterial activity.

To the best of our knowledge, there are few studies regarding the antimicrobial activity of 3,4-HPOs complexes, namely copper(II) complexes. Zhou and collaborators studied the antimicrobial activity of several 3,4-HPO derivatives including the antimicrobial activity of the ligand **Hdmpp** (deferiprone) against *S. aureus, Bacillus subtilis, P. aeruginosa* and *E. coli* [33]. Another study concerning 3,4-HPOs copper(II) complexes referred the lack of antimicrobial activity of complex **$Cu(maltol)_2$** against susceptible Gram-positive (*E. faecalis* and *S. aureus*) and Gram-negative (*E. coli*, *Klebsiella pneumonia* and *P. aeruginosa*) strains [34].

To compare the MIC values of the naphthyl derived 3,4-HPOs and their copper(II) complexes with those consisting of 3-hydroxy-4-pyrones (3,4-HPs) and 3,4-HPOs ligands in the same conditions, a set of ligands and corresponding copper(II) complexes (Fig. S1, Table S1) was

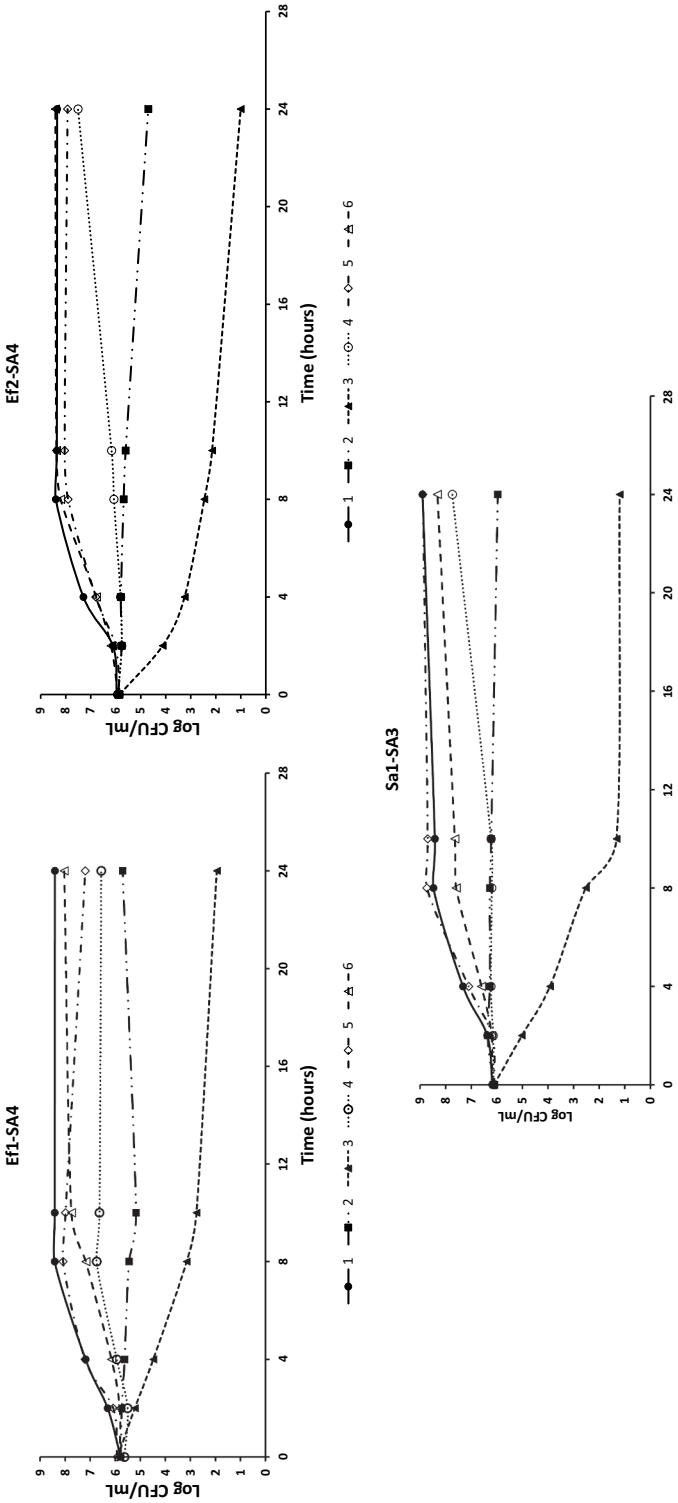

Fig. 6. Growth curves using viable counts of three multidrug-resistant Gram-positive isolates, Ef1-SA4, Ef2-SA4 and Sa1-SA3 after 24 h at 37 °C in LB medium. 1: control condition; 2: presence of **Cu(naph1pp)$_2$** at the MIC; 3: presence of CIP at the MIC; 4: presence of **Cu(naph1pp)$_2$** and CIP at the respective MICs obtained when in combination (Table 4); 5: presence of **Cu(naph1pp)$_2$** at a sub-MIC; 6: presence of CIP at a sub-MIC.

also tested. In accordance with a previous report [33], herein, the ligand **Hdmpp** was also more effective against Gram-positive species, particularly, *E. faecalis*. Equally, **Cu(maltol)$_2$** did not show antibacterial activity as reported previously [34]. In this set of copper(II) complexes, **Cu(ethylmaltol)$_2$** presented the best results, being more active against *E. faecalis* and equally effective against both reference and multidrug resistant isolates.

The increased activity of the naphthyl derived 3,4-HPOs copper complexes can be explained based on Overtone's concept and the Tweedy's Chelation theory [35,36]. According to the Overtone's concept of cell permeability, the lipid membrane of the cell favors the passage of lipid-soluble molecules which indicate that the antimicrobial activity depends on the lipophilicity of the compounds. Comparing the calculated values of logP for the ligands, obtained by MarvinView 19.8 software (htpp://www.chemaxon.com), **naph1pp** had the highest value clogP = 3.34, while **naph2pp** and **dansylpp** presented the values 2.75 and 2.19, respectively. The chelation of the copper(II) ion reduces its polarity due to the overlap of the ligand orbital and partial sharing of the positive charge of the copper(II) ion with the donor groups and possible π-electron delocalization over the whole chelate ring, which enhances the lipophilicity of the complexes and the penetration of the complexes into the lipid membranes. Considering the clogP of the **naph1pp** ligand, we expect that the complex **Cu(naph1pp)$_2$** also presents the highest lipophilicity. Besides that, this complex is the smallest and less distorted one and these characteristics can be paramount for cell permeation and antimicrobial activity.

So far, the antimicrobial activity of copper complexes has been attributed to the capacity to interact with DNA, namely through binding or cleavage [37–39]. Considering the molecular structure of the naphthyl derived 3,4-HPOs copper(II) complexes studied, we hypothesize that a similar mechanism may be responsible for the activity observed. Since DNA is an intracellular target, the efficiency shown by the different copper complexes in reaching it will vary according to their properties like lipophilicity and size. Nonetheless, the mechanism proposed is merely hypothetical, since our aim in the present study was not to assess the mechanism of action of these complexes. However, we intend to explore such goal in future studies.

3.2.2. Synergism or additivity between *Cu(naph1pp)$_2$* and ciprofloxacin

Based on the results obtained from the MIC experiments, complex **Cu(naph1pp)$_2$** was selected for synergy testing with a known antibiotic, ciprofloxacin. The combinatorial effects between **Cu(naph1pp)$_2$** and ciprofloxacin were tested against two MRSA and two VRE isolates using the checkerboard method, which allowed the calculation of the FIC index (ΣFIC). The results allowed to describe the interactions as synergistic or additive (Table 4), i.e., the MIC of **Cu(naph1pp)$_2$** and ciprofloxacin decreased, in more or less extent, when supplied simultaneously comparing to the MIC obtained when used solo.

Additionally, the time-kill assay was used as a complementary study to the checkerboard assay. Both **Cu(naph1pp)$_2$** and ciprofloxacin were tested alone and in combination against two VRE and one MRSA (Fig. 6). At a concentration equal to the MIC, **Cu(naph1pp)$_2$** showed to be bacteriostatic but not bactericidal against the three multidrug-resistant isolates assayed, and ciprofloxacin showed to be bactericidal in every case, as expected. **Cu(naph1pp)$_2$** and ciprofloxacin combined were more effective than each one used individually in the equivalent concentration (see conditions 5 and 6 in Fig. 6), nonetheless, that combination was not deemed synergistic but only additive against all three isolates assayed (Ef1-SA4, Ef2-SA4 and Sa1-SA3). Synergy could only be inferred if a ≥2 log10 decrease in CFU counts obtained in the two-drug combination after 24 h have happened.

A synergistic combination between a 3,4-HPO hexadentate-based dendrimeric chelator and norfloxacin against *S. aureus* and *E. coli* have been reported by others [33]. Lately, drug combinations have been considered as an alternative approach worth to be pursued in order to find effective solutions for the treatment of infections caused by multidrug-resistant pathogens, since the effort that has been applied in the development of new antibiotics has been relatively fruitless. Antibiotic-antibiotic combinations, and the pairing of an antibiotic with a non-antibiotic adjuvant are encouraging strategies to be studied and further explored [40].

4. Conclusions

In this work, we reported the synthesis and characterization of three novel copper(II) complexes and assessed their antibacterial activity against Gram-positive and Gram-negative reference strains and multidrug-resistant clinical isolates.

The complex **Cu(naph1pp)$_2$** showed the highest antibacterial activity against all strains and isolates, though presenting higher activity against Gram-positive bacteria. The antibacterial activity of **Cu(naph1pp)$_2$** in combination with ciprofloxacin against MRSA and VRE was further explored and the results showed that such combination enhanced the antibacterial activity and resulted in an additive effect.

Future work may take advantage of the fluorescent properties of **Cu(naph1pp)$_2$** that can allow to follow its entrance and distribution inside the bacteria. As previously reported [18], the complexation of the ligand with the copper(II) ion quenches its fluorescence. This feature can be used in further studies regarding the application of this complex since an “off-on” response can be indicative of the release of the copper (II) ion by the fluorescent ligand. In addition, future studies will be carried out to evaluate the mechanism of action of the synthesized complexes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2019.110704.

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