CRYSTAL STRUCTURE, CHARACTERISATION AND BIOLOGICAL ACTIVITY OF COPPER(II)-CIPROFLOXACIN IONIC COMPOUND

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Abstract

The title compound, ciprofloxacinium(1+) ciprofloxacinium(2+) tetrachlorocuprate(II) chloride hydrate, $(cfH_2)(cfH_3)[CuCl_4]Cl\cdot H_2O$, **(1)**, was isolated and its structure was determined by X-ray crystallography. This is a typical ionic compound with no direct bonds between the quinolone and metal. Both ciprofloxacin (cfH = 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid) molecules are nonequivalent and are protonated thus being unable to coordinate to the copper. The new complex has been characterised by intrinsic fluorescence emission and UV spectroscopy. The title compound as well as two previously isolated copper complexes, $[Cu(cfH)(H_2O)_3]SO_4\cdot 2H_2O$, **(2)** and $[Cu(cfH)_2]Cl_2\cdot 6H_2O$, **(3)**, were tested against the growth of various Gram positive and Gram negative microorganisms. Antimicrobial activities were evaluated using the agar diffusion test. Since ciprofloxacin alone has the ability to bind to DNA, the binding of a new compound has also been tested by UV spectroscopy. Our results reveal that $(cfH_2)(cfH_3)[CuCl_4]Cl\cdot H_2O$ slightly thermally destabilise the linear double stranded DNA at pH 7.0.

Introduction

Quinolones are a large group of synthetic antibacterial agents used in a clinical practice for the treatment of variety of bacterial infections.[1] Ciprofloxacin (Figure 1) is the quinolone family member and is amongst other also the drug of choice for treating victims infected by anthrax.[2] The U.S. Food and Drug Administration (FDA) approved cfH for post-exposure inhalational anthrax and after first confirmed cases of anthrax found in USA in 2001 the demand for cfH has enormously increased.

In our previous studies we have already shown that from acidic solutions of quinolone and various metals, ionic type compounds could be isolated.[3-10]

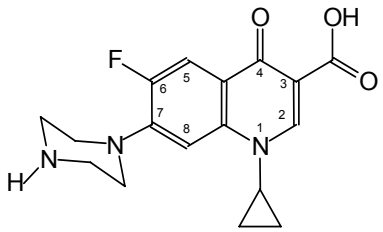

Figure 1: Chemical structure of ciprofloxacin (cfH)

It was already reported that some quinolone-metal complexes exert biological activity against various microorganisms or have some other positive effects in the treatment of certain diseases.[11-18] Certain bacterial infections now defy all known antibiotics and antibiotic resistance is a growing problem.[19] There is a great need for new antibacterial agents and metal complexes could play an important role in this field. Our aim was to determine the biological activity of the title compound and to recognise its interaction with DNA. Prior to this it was essential to determine the exact formula, crystal structure and other properties of the title compound.

Experimental

*Synthesis of $(cfH_2)(cfH_3)[CuCl_4]Cl\cdot H_2O$ (**1**)*

Ciprofloxacin hydrochloride hydrate (0.2594 mmol) was dissolved in concentrated hydrochloric acid and copper(II) chloride dihydrate (0.1297 mmol) was added during the stirring. Orange crystals suitable for X-ray diffraction analysis were obtained from yellow solution by slow evaporation of the solvent. Found: C, 43.92; H, 4.23; N, 9.04. $C_{34}H_{41}Cl_5CuF_2N_6O_7$ requires C, 44.16; H, 4.44; N, 9.09%.

Compounds $[Cu(cfH)(H_2O)_3]SO_4\cdot 2H_2O$, **(2)** and $[Cu(cfH)_2]Cl_2\cdot 6H_2O$, **(3)** were prepared as reported elsewhere.[4, 5]

Analyses and physical measurements

CHN analyses: The analyses of carbon, hydrogen and nitrogen were carried out on a Perkin-Elmer 204C microanalyzer.

X-ray structure analysis: Diffraction data were collected on a Nonius Kappa CCD diffractometer with graphite monochromated Mo$K\alpha$ radiation. They were processed using DENZO[20] program. Structure was solved by direct methods using SIR97.[21] We

employed block-diagonal least-squares refinement on F magnitudes with anisotropic displacement factors for all non-hydrogen atoms using Xtal3.4[22] program. The positions of hydrogen atoms were obtained from the difference Fourier map. The resulting crystal data and details concerning data collection and refinement are quoted in Table 1. Selected bond distances are given in Table 2. All geometrical details and other crystallographic data for compound **(1)** have also been deposited with the Cambridge Crystallographic Data Center as supplementary material with the deposition number CCDC 178211. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK.

UV-vis spectroscopy: UV-absorbance measurements were conducted in a Cary 1 UV-visible spectrophotometer (Varian, Australia) using matched 1 cm path length quartz cuvettes. The spectrophotometer was equipped with a thermoelectrically controlled cell holder.

The molar extinction coefficient of compound **(1)** was determined spectrophotometrically at 275 nm. The drug was dried overnight at 130 °C and a precisely weighted amount using a high precision balance (Sartorius Analytic A 210P, Sartorius GmbH, Germany) was dissolved in triply distilled water. From the slope of the line, A_{275} vs. concentration (Beer's law), the molar extinction coefficient of compound **(1)**, ε_{275}, was determined to be 83,700 ± 1,600 $M^{-1}cm^{-1}$ at 25 °C. For UV-absorbance measurements the concentration of compound **(1)** was 10 μM.

Natural genomic DNA (Calf Thymus DNA) was purchased from Pharmacia Biotech (Uppsala, Sweden). This DNA was of the highest grade commercially available. Before use it was thoroughly dialysed against corresponding buffer solution. The concentration of DNA was determined spectrophotometrically at 25 °C using the molar extinction coefficient, ε_{259} = 12,800 $M^{-1}cm^{-1}$, expressed in molar concentration of base pairs.[23] Unless otherwise stated, the buffer solution (pH 7.0) used in our experiments with Calf Thymus DNA consisted of 10 mM cacodylate containing 108.6 mM Na^+.

Absorbance *versus* temperature profiles were measured at 260 nm. The heating rate was 1.0 °C/min. For each optically detected transition, the temperatures of half-transition (T_m) were determined. Melting experiments of DNA at different ratios of drug to DNA (R from 0 to 1) were performed at pH 7.0 (10 mM Cacodylate buffer and 108.6

mM Na^+). The concentration of DNA was 20 μM (per base pair). To avoid complications from the contribution of cfH or compound **(1)** to the absorbance spectrum of DNA, the reference cuvette was filled with the solution of cfH or compound **(1)** of the same concentration as the sample cuvette.

Fluorescence emission spectroscopy: Intrinsic fluorescence emission spectra of compound **(1)** (titrated by HCl or NaOH) were performed at 25 °C in a Perkin-Elmer Model LS-50 Luminescence spectrometer equipped with a water thermostated cell holder using 1 cm path length quartz cuvette. The emission spectra were recorded in the range from 350 to 625 nm. Fluorescence titrations profiles were measured by incrementally adding aliquots of reagent (HCl or NaOH) in a cuvette containing a known and always constant concentration of compound **(1)** (1 μM). The emission spectra of compound **(1)**, from which the corresponding emission spectra of pure solvent (background intensity) were subtracted, were further multiplied for dilution factor and corrected for PM-tube response using a fluorescence spectrum of Quinine sulphate ($c = 2.5 \cdot 10^{-7}$ M) in 0.1 M perchloric acid as a standard.

Antimicrobial activity: Antimicrobial activity of the compounds was determined against the growth of the following bacterial strains: *Staphylococcus aureus, Streptococcus salivarius, Micrococcus luteus, Bacillus cereus, Bacillus subtilis, Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhimurium*. All the used bacterial strains were obtained from the local collection at the Department of Biology, University of Ljubljana. Antimicrobial activities were evaluated using the agar diffusion test. The tested bacteria were allowed to grow overnight and their concentration was then determined. Bacterial culture was incorporated to Lauria Broth nutrient agar, which was previously cooled to 42 °C. The final concentration of bacteria, was approximately $5 \cdot 10^5$ CFU/mL (CFU – colony forming unit). Twenty milliliters of inoculated medium was poured into petri dishes and kept at 4 °C until use. Circles of agar ($\Phi = 1$ cm) were cut out from the cooled medium. The MIC (minimal inhibitory concentration) values of compounds **(1), (2)** and **(3)** were determined using ciprofloxacin as a reference substance. MIC represents the lowest concentration of an antibiotic that will inhibit the growth of a tested organism. For estimating MIC, the antibacterial substances were diluted gradually in 10 mM potassium

phosphate buffer (pH = 7.4), containing 2% DMSO. Hundred milliliters of each dilution were poured into the holes cut in the inoculated medium, after that the system was kept at 37 °C for 24 h. Finally, the diameters of inhibition zones were measured.

Results and discussion

X-Ray structure analyses

The asymmetric unit of compound **(1)** is shown in Figure 2. Figure was drawn with the aid of ORTEP[24] program. The asymmetric unit contains two complex $[CuCl_4]^{2-}$ anions, two chloride ions, two water molecules and four protonated ciprofloxacin molecules **a**, **b**, **c** and **d**. In all four cases terminal nitrogen atom of the piperazine ring - N(74) is protonated. In ciprofloxacin cations **b** and **d** also oxygen O(4) is protonated, so their charge is +2.

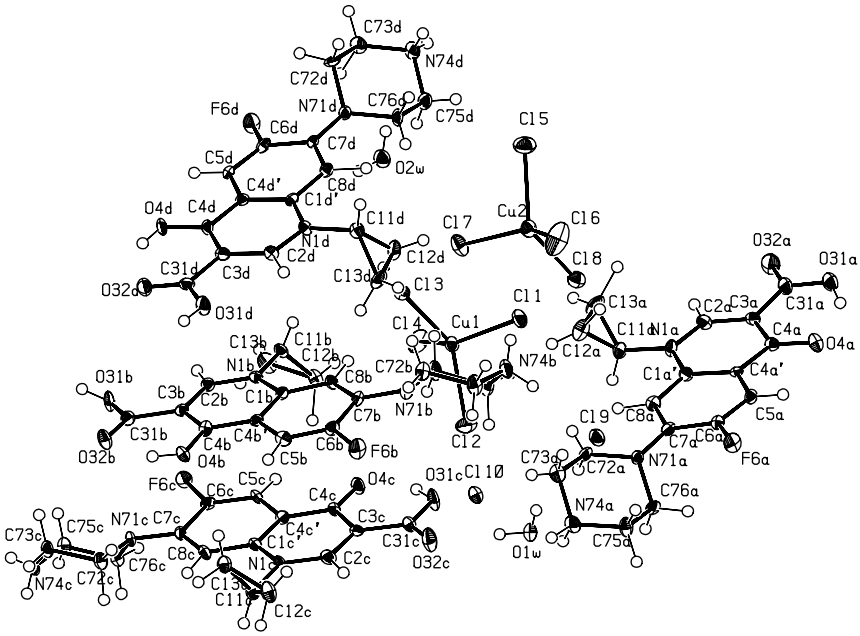

Figure 2: Ortep view of the asymmetric unit of compound **(1)**

The unit cell parameters of compound **(1)** are very similar to two norfloxacin (nfH) analogues[3] with formulas: $(nfH_2)(nfH_3)[CuCl_4]Cl{\cdot}H_2O$ and $(nfH_2)(nfH_3)[ZnCl_4]Cl{\cdot}H_2O$, which are isostructural and both crystallise in the centric $P2_1/c$ space group. On the basis

of this similarity and the similarity of cfH with nfH we had expected that also compound **(1)** is isostructural with the above mentioned structures. But analysis of the diffraction data (systematic absences and the statistics of reflections) suggested lower symmetry - acentric space group $P2_1$, so there are two units of (cfH2)(cfH3)[CuCl4]Cl·H2O in the asymmetric unit.

Table 1: Crystal data, data collection and structure refinement of compound **(1)**

$C_{68}H_{82}Cl_{10}Cu_2F_4N_{12}O_{14}$	D_x = 1.621 Mg m^{-3}
M_r = 1849.09	Mo $K\alpha$ radiation
Monoclinic, $P2_1$	Cell parameters from 8820 reflections
a = 14.7409(1) Å	θ = 1.0 - 27.48^0
b = 20.3550(2) Å	μ = 0.996 mm^{-1}
c = 13.7359(1) Å	T = 150(1) K
β = 113.1784(4)^0	Prism
V = 3788.80(5) Å^3	Orange
$F(000)$ = 1900	0.32 x 0.08 x 0.08 mm
Z = 2	
Nonius Kappa CCD diffractometer	8931 unique reflections
ω scans	7542 reflections with $F^2>2.0\sigma(F^2)$
Multiscan absorption correction	R_{int} = 0.063
79632 integrated reflections	θ range = 1.5 – 27.5^0
Refinement on F	Empirical weighting scheme
R (on F_{obs}) = 0.052	$(\Delta/\sigma)_{max}$ = 0.0055
wR (on F_{obs}) = 0.057	$(\Delta/\sigma)_{ave}$ = 0.00016
8474 contributing reflections	$\Delta\rho_{max}$ = 1.93 eÅ^{-3}
991 parameters	$\Delta\rho_{min}$ = -1.32 eÅ^{-3}
H-atom parameters not refined	

Rejecting $h,0,l$ (for l odd) reflections we were able to solve the structure also in the centric $P2_1/c$ space group. The asymmetric unit was two times smaller and similar to those of norfloxacin analogues. But R_{iso} of that model was very high (above 30%) and further refinement was unsuccessful.

Table 2: Selected bond distances (Å) in the structure of compound **(1)**

Cu(1)-Cl(1)	2.230(3)	Cu(2)-Cl(5)	2.293(4)
Cu(1)-Cl(2)	2.266(4)	Cu(2)-Cl(6)	2.206(5)
Cu(1)-Cl(3)	2.262(4)	Cu(2)-Cl(7)	2.229(3)
Cu(1)-Cl(4)	2.246(4)	Cu(2)-Cl(8)	2.264(4)
O(4a)-C(4a)	1.247(14)	O(4c)-C(4c)	1.259(14)
O(31a)-C(31a)	1.310(15)	O(31c)-C(31c)	1.325(15)
O(32a)-C(31a)	1.216(17)	O(32c)-C(31c)	1.216(17)
C(3a)-C(4a)	1.459(17)	C(3c)-C(4c)	1.435(17)
C(3a)-C(31a)	1.498(16)	C(3c)-C(31c)	1.481(16)
O(4b)-C(4b)	1.329(14)	O(4d)-C(4d)	1.322(14)
O(31b)-C(31b)	1.303(16)	O(31d)-C(31d)	1.300(16)
O(32b)-C(31b)	1.224(14)	O(32d)-C(31d)	1.235(14)
C(3b)-C(4b)	1.395(17)	C(3d)-C(4d)	1.413(18)
C(3b)-C(31b)	1.500(15)	C(3d)-C(31d)	1.475(16)

It has to be mentioned that we had collected X-ray data for compound **(1)** already before; firstly at room temperature on an Enraf-Nonius CAD-4 and secondly also at 150 K on Nonius Kappa CCD diffractometer. It is interesting that analysis of those diffraction data (systematic absences and the statistics of reflections) suggested $P2_1/c$ space group. In both cases the solution of the phase problem resulted in the structure model very similar to those of analogous norfloxacin compounds. But further refinement resulted in unacceptable large R values and disorder in some parts of the structure. The result was not better using two times larger model and $P2_1$ space group. Since both data collections were undertaken using crystals from the same batch and since low temperature data excluded dynamical type of disorder, we had suspected that the reason might have been the static disorder. So, new crystals were carefully prepared and new low temperature X-ray diffraction data were collected which finally resulted in the successful structure analysis, described in this paper. As in the above mentioned analogous nfH compounds[3], ciprofloxacin molecules are not coordinated to copper(II) ions. Each of central atoms Cu(1) and Cu(2) are surrounded by four chloride ions in the form of distorted tetrahedron. The angles Cl-Cu-Cl are between 94.8(1) to 138.5(1)°, and distances Cu-Cl between 2.206(5) and 2.293(4) Å. These and the bond distances of protonated cfH molecules are within expected ranges and in an agreement with the values reported for corresponding type of bonds.[25,26]

In all cfH molecules O(31) atom is bonded to C(31) and to a hydrogen atom. This is

in agreement also with C(31)-O(31) distances (from 1.300(16) to 1.325(15) Å) which indicate single bond. Bonds C(31)=O(32) have in all four cases double bond character (distances are from 1.216(17) to 1.235(14) Å). In **a** and **c** molecules the bond C(4)=O(4) has double bond character (bond lengths are 1.247(14) and 1.259(14) Å) while in **b** and **d** molecules O(4) atom is protonated and bonded to C(4) atom by single bond (distances are 1.329(14) and 1.322(14) Å). In the later case, atom O(4) is a donor of intramolecular hydrogen bond to O(32) atom from carboxyl group of the same molecule. Also in **a** and **c** molecules O(4) atom and carboxyl group are connected by intramolecular hydrogen bond, but O(4) atom in this case acts as an acceptor. The donor of this intramolecular hydrogen bond is O(31). This is possible since the orientation of carboxyl group in **a** and **c** molecules differ from those in **b** and **d**. Torsion angles C(4)-C(3)-C(31)-O(31) in **a** to **d** are -1(2), -178(1), -3(2) and -177(1)°, respectively. There are differences also in the role of carboxyl group in the intermolecular hydrogen bonding. In **a** and **c** molecules O(32) atom is an acceptor of intermolecular N-H...O hydrogen bond while in **b** and **d** molecules (O31) atom is a donor of intermolecular O-H...Cl hydrogen bond. Hydrogen bonding contact distances are given in Table 3. Similarly as was observed in both analogous nfH compounds[3], there are layers in the structure. The distances between aromatic rings from the neighbouring layers are around 3.5 Å, so the interactions between π-electronic systems are possible.[27]

Table 3: Hydrogen bonding contact distances in the structure of compound **(1)**

donor...acceptor	contact distance (Å)	donor...acceptor	contact
	intermolecular hydrogen bonds		
O(1w)...Cl(9)	3.247(8)	O(2w)...Cl(10)	3.265(9)
O(1w)...O(32c)	2.770(13)	O(2w)...O(32a)	2.862(13)
N(74a)...O(1w)	2.785(16)	N(74c)...O(2w)	2.846(16)
N(74a)...Cl(5)	3.195(13)	N(74c)...Cl(3)	3.176(9)
N(74b)...Cl(8)	3.120(10)	N(74d)...Cl(2)	3.179(13)
N(74b)...Cl(9)	3.148(14)	N(74d)...Cl(10)	3.108(13)
O(4b)...Cl(5)	3.464(9)	O(4d)...Cl(1)	3.134(8)
O(31b)...Cl(10)	2.947(8)	O(31d)..Cl(9)	3.000(8)
	intramolecular hydrogen bonds		
O(31a)...O(4a)	2.548(13)	O(31c)...O(4c)	2.515(13)
O(4b)...O(32b)	2.571(12)	O(4d)..O(32d)	2.557(13)

Characterisation of $(cfH_2)(cfH_3)[CuCl_4]Cl\cdot H_2O$ by spectroscopic techniques

Figures 3A and B show the UV absorption and intrinsic fluorescence emission spectra of compound **(1)** at pH 7.0. Figure 3C shows the changes in the fluorescence emission intensity at 450 nm with pH, respectively.

The position of wavelengths of maximum intensity, λ_{max}, in the UV-absorbance spectrum of compound **(1)** observed at 272 ± 1, 322 ± 1 and 332 ± 1 nm (Figure 3A) are not different from those observed for free cfH at similar solution conditions.[28] Similarly, the position of wavelength of maximum intensity, λ_{max}, in fluorescence emission spectrum of compound **(1)** (Figure 3B) occurs at the same wavelength as for cfH.[28]

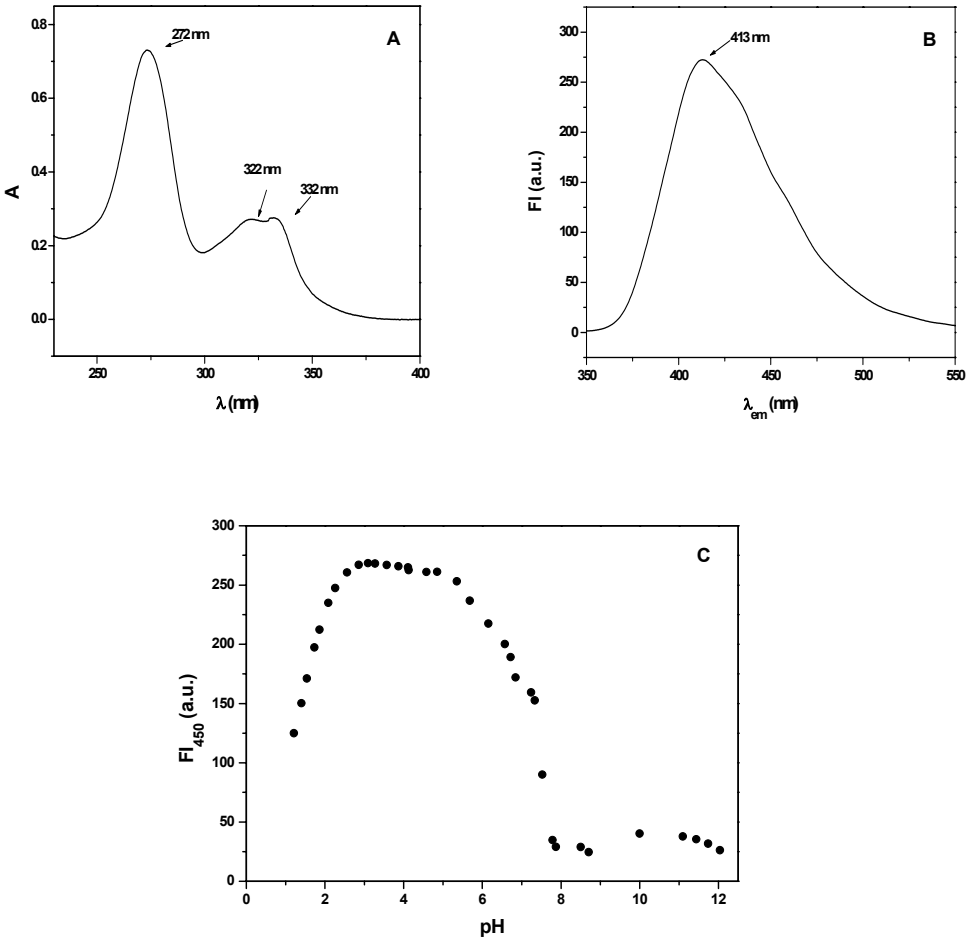

Figure 3: The absorbance (A) and intrinsic fluorescence emission spectra (B) of compound **(1)** at pH 7.0. (C) The pH dependence of compound **(1)** single wavelength fluorescence intensity, FI, at 450 nm. λ_{ex} was 330 nm.

Noticeable changes in the fluorescence intensity at 450 nm upon changes in pH appear in the pH range below 3 and between 5 and 8, indicating the presence of various species in the solution of compound **(1)**, similarly as it was observed for cfH before.[28] It has been reported that cfH exists in a cationic form below pH 5, as a mixture of anions, cations, and zwitterions in the pH range from 5 to 10, and as the anion at pH higher than 10.[29, 30]

Taken together, these data indicate that the spectroscopic properties of the title compound do not distinguish significantly from cfH. It seems that at the conditions used (low concentration of the compound **(1)** and indirectly the concentration of Cu^{2+} ions) there are no other interactions except ionic between the metal and ligand which influence only the fluorescence intensity quenching.

Antimicrobial activity of the compounds

The susceptibility of the bacterial strains to the test agents is presented in Table 4. Graphical representation of minimal inhibitory concentration of an antibiotic, expressed in terms of molarity, is shown in Figure 4.

Table 4: Antibacterial activity of **cfH**, compound **(1)**, **(2)**, and **(3)**, expressed as the MIC

		MIC (μg/ml)		
Microorganism	**cfH**	**1**	**2**	**3**
Staphylococcus aures	1.5	2.5	4	2.5
Streptococcus salivarius	0.075	0.075	0.1	0.075
Micrococcus luteus	15	20	30	15
Bacillus cereus	0.5	0.75	2.5	0.75
Bacillus subtilis	0.3	0.3	0.5	0.3
Escherichia coli	0.08	0.2	0.2	0.1
Proteus vulgaris	0.075	0.2	0.2	0.2
Klebsiella pneumoniae	0.25	0.4	0.6	0.3
Pseudomonas aeruginosa	0.8	0.8	0.8	0.8
Salmonella typhimurium	0.2	0.2	0.5	0.2

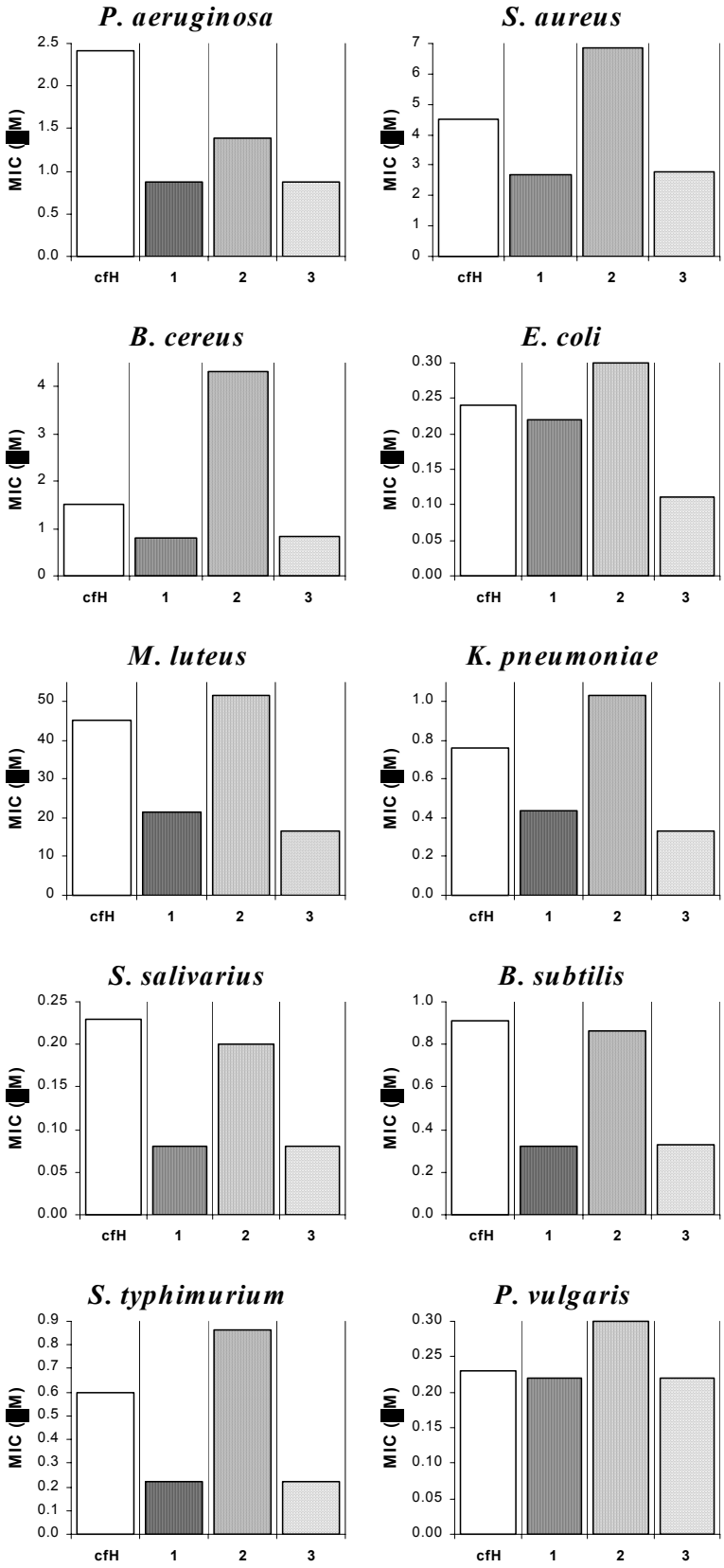

Figure 4: Graphical representation of MIC for **cfH**, compound **(1)**, **(2)**, and **(3)**

The results of bioactivity testing (MIC values), expressed in mass concentration (Table 4) (which is normally used in the presentation of bioactivity data) could be a bit misleading. First impression is that the complexes **(1)-(3)** are of comparable or even lower activity than free cfH. But if we convert these values to the molar concentrations (Figure 4), the following two conclusions could be drawn:

- formula weights of **(1)** and **(3)** are comparable (904.5 and 924.0 g/mol) and both complexes contain two molecules of cfH per formula. As shown in Figure 4 the activity of both compounds is comparable for most microorganisms. It is possible that both compounds are converted to similar species in solution,
- it seems that the activity mostly depends on the amount of cfH in the sample. So it is reasonable that MIC values expressed in molar concentrations of **(1)** and **(3)** are roughly two times lower than the values for cfH (Figure 4).

*Compound **(1)** binding to DNA*

The melting temperatures of the investigated systems are presented in Table 5.

Table 5: The melting temperatures, T_m (°C), of the genomic Calf Thymus DNA (CT-DNA) at pH 7.0 (10 mM Cacodylate buffer and 108.6 mM Na^+) in the presence of cfH and compound **(1)**. Molar ratio of drug to DNA is 1:1. Melting temperature of Calf Thymus DNA, $T°_m$, is 86.4 ± 0.5 °C *; $\Delta T = T_m - T°_m$

	T_m(°C)	ΔT (°C)
CT-DNA + cfH*	82.9 ± 0.5	-3.5 ± 1.0
CT-DNA + compound (1)	84.4 ± 0.5	-2.0 ± 1.0

*data out from Vilfan et. al[28]

Ciprofloxacin and compound **(1)** have similar effect on the thermal stability of natural linear genomic CT-DNA. Both compounds slightly thermally destabilise the linear double stranded DNA at employed solution conditions. The decrease in thermal stability of genomic CT-DNA upon addition of cfH or compound **(1)** suggests that the ligand preferentially interacts with single stranded DNA rather than double stranded

DNA as was already reported in the literature for similar quinolone systems.[31] It could also be concluded that at the experimental conditions used, copper ions do not considerably affect the melting temperatures of DNA.

Acknowledgements

The diffraction data for the title compound were collected on the Kappa CCD Nonius diffractometer at the Laboratory of Inorganic Chemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia. We acknowledge with thanks the financial contribution of the Ministry of Science and Technology, Republic of Slovenia through grants X-2000 and PS-511-103, which thus made the purchase of the apparatus possible.

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Supplementary material

Further details of the refinement and view of the layers in the structure could be obtained on request from the authors.

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Povzetek

Sintetiziran je bil nov kompleks med bakrom in ciprofloksacinom s formulo $(cfH_2)(cfH_3)[CuCl_4]Cl{\cdot}H_2O$ (**1**). Rentgenska strukturna analiza je pokazala, da je to tipična ionska spojina brez usmerjenih vezi med kinolonom in kovino, kjer molekuli ciprofloksacina (cfH=1-ciklopropil-6-fluoro-1,4-dihidro-4-okso-7-(1-piperazinil)-3-kinolon karboksilna kislina) nista ekvivalentni. Obe sta protonirani, kar jima onemogoča koordinacijo na baker. Sintetizirani kompleks je bil okarakteriziran z dvema spektroskopskima tehnikama, fluorescenčno emisijsko in UV spektroskopijo. Skupaj z dvema predhodno izoliranima bakrovima kompleksoma, $[Cu(cfH)(H_2O)_3]SO_4{\cdot}2H_2O$ (**2**) in $[Cu(cfH)_2]Cl_2{\cdot}6H_2O$ (**3**), je bil testiran proti rasti raznih Gram pozitivnih in Gram negativnih bakterij. S pomočjo UV spektroskopije je bila določena sposobnost vezave novega kompleksa na DNA. Ugotovljeno je bilo, da tako kot ciprofloksacin, tudi izolirani kompleks pri pH 7.0 nekoliko termično destabilizira dvojno vijačnico.