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Synthesis, structure, urease inhibitory, and cytotoxic activities of two complexes with protocatechuic acid derivative and phenanthroline

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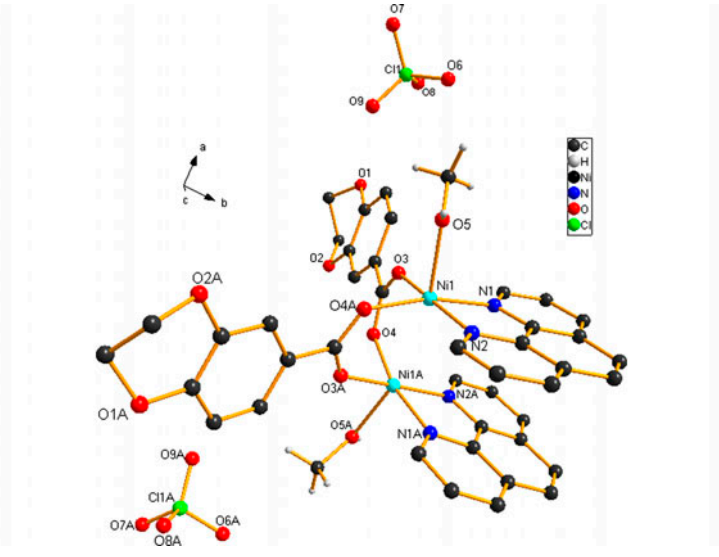

Two complexes, $[Cu^{II}(L^1)(phen)_2](ClO_4)$ (**1**) and $[Ni^{II}_2(L^1)_2(phen)_2(MeOH)_2](ClO_4)_2$ (**2**), with HL^1, a ligand derived from protocatechuic acid (=2,3-dihydrobenzo[*b*][1,4]dioxine-6-carboxylic acid) and phen (=1,10-phenanthroline) were synthesized and characterized by C, H, and N elemental analysis, UV–vis, FT-IR, and single-crystal X-ray diffraction, which revealed that **1** is mononuclear and **2** is dinuclear. Both complexes crystallized in monoclinic space group *C2/c*. The urease inhibitory

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activity and *in vitro* cytotoxic activity of **1** and **2** were tested. The complexes showed strong inhibitory activity against *jack bean* urease and significantly suppressed the growth of A549, L929, and SW620 cell lines.

Keywords: Urease inhibitors; Cytotoxicity; Complex; Protocatechuic acid

1. Introduction

Urease (urea amidohydrolase; E.C.3.5.1.5) is a nickel-containing metalloenzyme that catalyzes rapidly the hydrolysis of urea to form ammonia and carbamate [1, 2]. Urease is widely distributed in a variety of algae, bacteria, fungi, and plants. The reaction catalyzed by urease may cause an accumulation of ammonia and accompanying pH elevation, which has important negative implications in medicine and agriculture [3–6]. The Gram-negative bacterium *Helicobacter pylori* is associated with severe gastric pathologies, including peptic ulcer, chronic active gastritis, and gastric cancer. This micro-organism is able to invade and colonize the human stomach, directly interacting with gastric epithelial cells, mainly because of its high urease activity. In 1994, *H. pylori* was classified as a type I carcinogen for humans by the IARC/WHO [7]. Compounds having urease inhibitory and anticancer activities are very important in the treatment of the infections caused by urease-producing bacteria. Some metal complexes have been reported to show anticancer [8–12] and urease inhibitory activities [13–17].

Protocatechuic acid (3,4-dihydroxybenzoic acid) is a simple phenolic compound widely distributed in nature. It is detected in almost all plants and is one of the biologically active components of some medicinal plants, including those used in natural medicines. Protocatechuic acid has antioxidant and antiradical activity and chemopreventive ability in chemically induced carcinogenesis [18–20]. Some complexes with protocatechuic acid and its derivatives as ligands have been reported [21–23]. Herein, we report the synthesis of two new complexes with a protocatechuic acid derivative as ligand. The structure, the urease inhibitory activities, and the antiproliferative activities of the two complexes were evaluated.

2. Experimental

2.1. *General methods and material*

Unless otherwise stated all solvents were of reagent grade quality and purchased commercially. All chemicals were also commercially available and used without purification. Elemental analyses were performed on a Perkin-Elmer 240C elemental analyzer.

2.2. *Synthesis of 2,3-dihydrobenzo[b][1,4]dioxine-6-carboxylic acid*

The protocatechuic acid derivative ligand 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carboxylic acid ($C_9H_8O_4$, HL_1) was synthesized with protocatechuic acid (3,4-dihydroxybenzoic acid) and 1,2-dibromoethane (scheme 1) according to our previously reported method [23]. Yield 81%. M.p. 133–136 °C. Anal. Calcd for $C_9H_8O_4$ (%): C, 60.00; H, 4.48. Found (%): C, 60.03; H, 4.45. Complexes **1** and **2** were synthesized by the reaction of HL_1 with 1,10-phenanthroline (=phen) and the corresponding metal salts (scheme 2).

2.3. *Synthesis of $[Cu^{II}(L^1)(phen)_2](ClO_4)$ (1)*

HL^1 (0.2 mM, 0.036 g) and KOH (0.2 mM, 0.0112 g) were added to 5 mL methanol, the mixture was stirred for 30 min at room temperature to give clear solution, then $Cu(ClO_4)_2{\cdot}6H_2O$ (0.1 mM, 0.036 g) and phen (0.1 mM, 0.018 g) and 5 mL solvent (ethanol : acetone = 1 : 1) were added to the solution and the mixture was stirred for 30 min to give a green clear solution. After keeping the solution in air for 15 days, green block-shaped single crystals of **1** suitable for structure determination were obtained on slow evaporation of the solvent. Crystals were isolated by filtration, washed with cold ethanol, and then dried in air. Yield 81%. Anal. Calcd for $C_{50}H_{46}Cu_2N_2O_{16}$ (%): C, 56.42; H, 3.30; N, 7.97. Found (%): C, 55.88; H, 3.28; N, 7.93.

2.4. *Synthesis of $[Ni^{II}_2(L^1)_2(phen)_2(MeOH)_2](ClO_4)_2$ (2)*

HL^1 (0.2 mM, 0.036 g) and KOH (0.2 mM, 0.0112 g) were added to 3 mL dichloromethane, the mixture was stirred for 10 min at room temperature to give clear solution. The solution was transferred to a glass tube, then 2 mL mixed solution (methanol : dichloromethane = 1 : 1) was added to the glass tube and afterwards 3 mL of a methanolic solution of $Cu(ClO_4)_2{\cdot}6H_2O$ (0.1 mM, 0.036 g) was added to the glass tube. After keeping the glass tube in air for 15 days, green block-shaped single crystals of **2** suitable for structure determination were obtained at the middle of the tube on slow diffusion of the ligand and metal salt layer. Crystals were isolated by filtration and washed with cold methanol and then dried in air. Yield 75%. Anal. Calcd for $C_{44}H_{36}Ni_2N_4O_{18}Cl_2$ (%): C, 48.17; H, 3.31; N, 5.11. Found (%): C, 48.28; H, 3.31; N, 5.16.

Table 1. Crystal and experimental data for **1** and **2**.

	1	**2**
Molecular formula	$C_{33}H_{23}CuN_4O_8Cl$	$C_{44}H_{38}Ni_2N_4O_{18}Cl_2$
Molecular weight	702.55	1099.10
Temperature (K)	293	293
Radiation λ	Mo Kα (0.7107 Å)	Mo Kα (0.7107 Å)
Crystal system	Monoclinic	Monoclinic
Space group	*C2/c*	*C2/c*
a (Å)	20.5362(9)	23.4889(13)
b (Å)	22.7211(10)	9.9273(6)
c (Å)	15.3901(7)	21.8489(13)
α (°)	90	90
β (°)	123.863(1)	117.541(2)
γ (°)	90	90
V (Å^3)	5963.0(5)	4517.4(5)
Z	8	4
D_c (g cm^{-3})	1.565	1.616
Crystal size (mm^3)	0.25 × 0.23 × 0.17	0.30 × 0.28 × 0.26
F (0 0 0)	2872	2256
θ range (°)	2.4–26.6	2.0–25.1
Reflections collected/unique	30,468/6241 [R_{int} = 0.056]	21,011/4004 [R_{int} = 0.058]
Reflns. obs. [$I > 2\sigma(I)$]	4057	2744
Goodness of fit on F^2	1.042	1.023
Data/parameters/restraints	6241/424/0	4004/373/48
Largest diff. peak and hole (e Å^{-3})	0.689 and −0.525	0.49 and −0.451
R_1, wR_2 [$I > 2\sigma(I)$][a]	0.0636, 0.1398	0.0457, 0.0981
R_1, wR_2 (all data)[a]	0.1134, 0.1638	0.0839, 0.1132

[a]$R_1 = F_o - F_c / F_o$, $wR_2 = [\sum w(F_o^2 - Fc^2) / \sum w(F_o^2)^2]^{1/2}$ $\sum$.

Table 2. Selected bond distances (Å) and angles (°) for **1** and **2**.

1	
Cu(1)–N(1) 1.992(3)	Cu(1)–N(2) 2.046(3)
Cu(1)–N(3) 2.002(3)	Cu(1)–O(1) 1.994(3)
Cu(1)–N(4) 2.204(3)	
N(1)–Cu(1)–O(1) 89.71(12)	N(1)–Cu(1)–N(3) 175.94(13)
O(1)–Cu(1)–N(3) 0.99(12)	N(1)–Cu(1)–N(2) 81.35(13)
O(1)–Cu(1)–N(2) 155.46(13)	N(3)–Cu(1)–N(2) 96.43(13)
N(1)–Cu(1)–N(4) 104.25(13)	O(1)–Cu(1)–N(4) 89.84(12)
N(3)–Cu(1)–N(4) 79.75(13)	N(2)–Cu(1)–N(4) 114.47(13)
2 (#1: $-x, y, -z+1/2$)	
Ni(1)–O(3) 1.928(3)	Ni(1)–O(4)#1 1.954(3)
Ni(1)–N(2) 2.003(4)	Ni(1)–N(1) 2.031(4)
Ni(1)–O(5) 2.192(4)	O(4)–Ni(1)#1 1.954(3)
O(3)–Ni(1)–O(4)#1 94.73(15)	O(3)–Ni(1)–N(2) 172.48(16)
O(4)#1–Ni(1)–N(2) 91.02(15)	O(3)–Ni(1)–N(1) 91.39(15)
O(4)#1–Ni(1)–N(1) 166.51(16)	N(2)–Ni(1)–N(1) 81.99(16)
O(3)–Ni(1)–O(5) 92.15(15)	O(4)#1–Ni(1)–O(5) 93.38(16)
N(2)–Ni(1)–O(5) 92.34(15)	N(1)–Ni(1)–O(5) 98.39(16)

2.5. *Crystal structure determination*

X-ray diffraction intensities were collected using a Bruker SMART APEX-II CCD area detector equipped with graphite-monochromated Mo-Kα radiation ($\lambda = 0.71073$ Å). Absorption correction was applied by SADABS [24]. The structure was solved by direct methods and refined on F^2 by full-matrix least squares using Bruker's SHELXTL-97 program [25]. All nonhydrogen atoms were refined anisotropically. The hydrogens were placed in calculated positions and constrained to ride on their parent atoms. There are 48 restraints used to deal with the structure disorder during the refinement of **2**. The details of the crystallographic data are summarized in table 1, selected bond lengths and angles are listed in table 2 and geometrical parameters for hydrogen bonds are shown in table 3.

2.6. *Measurement of the inhibitory activity against jack bean urease*

Jack bean urease was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). The measurement of urease was carried out according to the literature [26, 27]. Generally, the

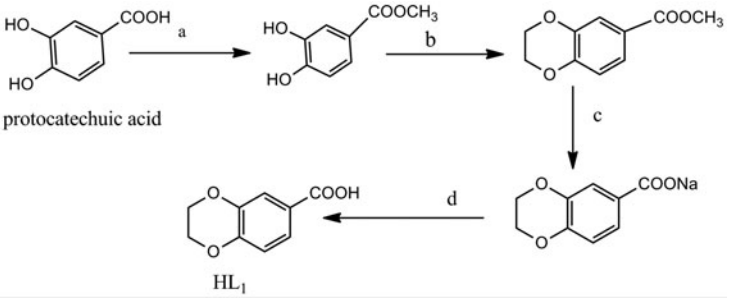
Table 3. Geometrical parameters for hydrogen bonds.

Hydrogen bonds	Symmetry code	D–H (Å)	H···A (Å)	D···A (Å)	D–H···A (°)
1					
C2–H2…O7	$[1/2-x, 1/2-y, 1-z]$	0.9300	2.4100	3.124(8)	134.00
C4–H4…O6	$[-x, y, 1/2-z]$	0.9300	2.5200	3.424(8)	164.00
C6–H6…O5	$[-x, y, 1/2-z]$	0.9300	2.5900	3.427(10)	151.00
C7–H7…O2	$[-x, -y, -z]$	0.9300	2.3100	3.167(7)	153.00
C10–H10…O8	$[x, -y, -1/2+z]$	0.9300	2.5900	3.278(10)	131.00
2 (1#: $[-x, y, 1/2-z]$)					
O5–H5A…O7	$[1/2-x, 1/2+y, 1/2-z]$	0.9000	1.8500	2.682(7)	152.00
C9–H9…O2	$[-x, 1+y, 1/2-z]$	0.9300	2.5800	3.392(5)	146.00
C10–H10…O7	$[-1/2+x, 1/2-y, -1/2+z]$	0.9300	2.5400	3.377(8)	150.00
C11–H11…O4	$[-x, y, 1/2-z]$	0.9300	2.5600	3.040(6)	112.00

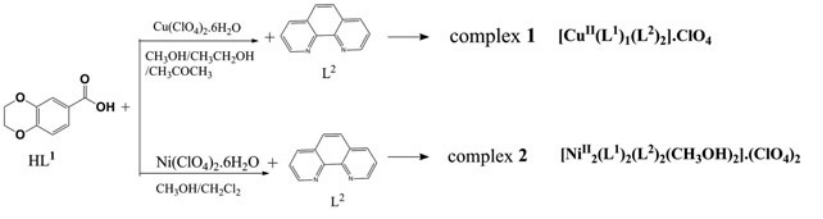
assay mixture, containing 25 µL of *jack bean* urease (10 kU/L) and 25 µL of the tested samples (complexes, also their parent ligands and metal salts) of various concentrations [dissolved in DMSO : H_2O = 1 : 1 (v : v)], was pre-incubated for 1 h at 37 °C in a 96-well assay plate. After pre-incubation, 0.2 mL of 100 mM phosphate buffer at pH 6.8 containing 500 mM urea and 0.002% phenol red was added and incubated at 37 °C. The reaction time was measured by microplate reader (570 nm), which was required to produce enough ammonium carbonate to raise the pH of a phosphate buffer from 6.8 to 7.7, the end-point being determined by the color of phenol red indicator.

2.7. *Measurement of the* in vitro *cytotoxic activity*

The *in vitro* cytotoxic activities of the complexes to human lung cancer cell line A549, the mouse fibrosarcoma cell line L929 and human colorectal cancer cell line SW620 were evaluated as described in the literature [28] with some modifications. Target tumor cells were grown to log phase in *RPMI* 1640 medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 µg/mL streptomycin. After reaching a dilution of 3×10^4 cells mL^{-1} with the medium, 90 µL of the obtained cell suspension was added to each well of the 96-well culture plates. Subsequently, incubation was performed at 37 °C in 5% CO_2 atmosphere for 24 h before the cytotoxicity assessment. Ten microliters of tested samples at pre-set concentrations were added to wells with cisplatin as a positive reference, the solvent control (0.5%DMSO) in complete medium. After 48 h exposure, 20 µL of PBS containing 2.5 mg mL^{-1} of MTT was added to each well. After 4 h, the medium was



Scheme 1. Synthesis of HL_1. Reagents and conditions: (a) MeOH, H_2SO_4, reflux, 8 h; (b) $Br(CH_2)_2Br$, acetone, reflux, 24 h; (c) NaOH, H_2O, reflux, 5 h; (d) HCl.



Scheme 2. Synthesis of **1** and **2**.

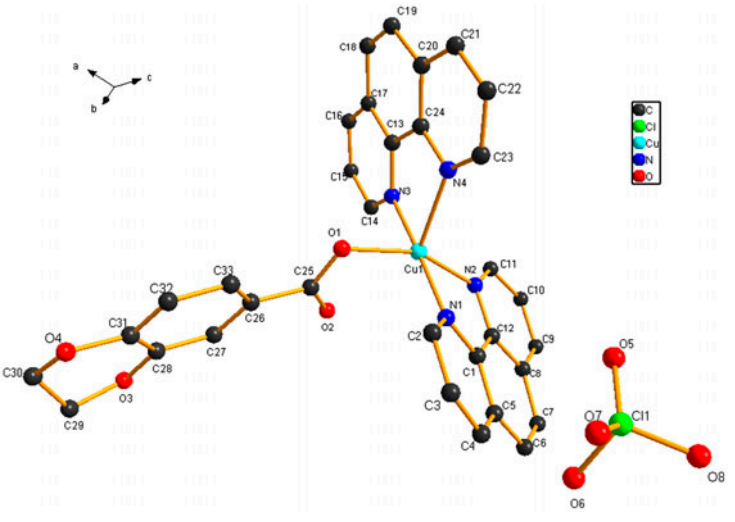


Figure 1. A perspective view of **1** (hydrogens are omitted for clarity).

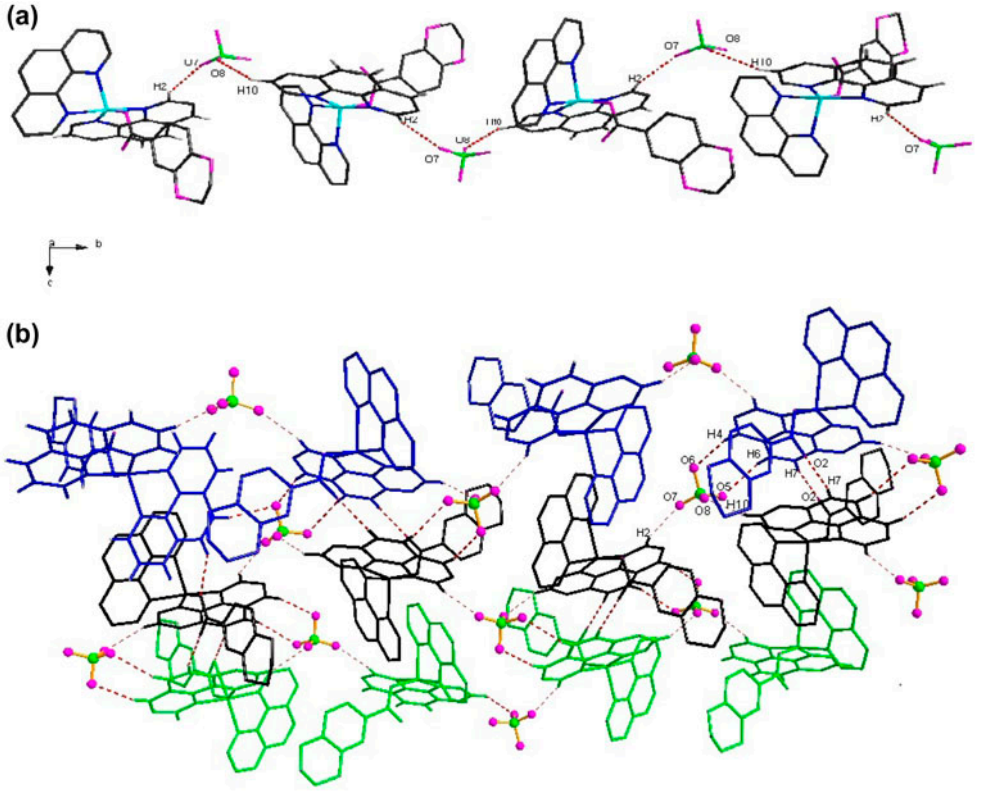


Figure 2. View of the hydrogen bond-driven 1-D and 2-D network of **1** (2a: 1-D chain running along the *b* axis; 2b: 2-D network in the *bc* plane). Hydrogen bonds are shown as dashed lines.

replaced by 150 µL DMSO to dissolve the purple formazan crystals produced. The absorbance at 570 nm of each well was measured with an ELISA plate reader. The data represented the mean of three independent experiments in triplicate and were expressed as means ± SD. The IC_{50} value was defined as the concentration at which 50% of the cells could survive.

3. Results and discussion

3.1. *Crystal structure description of the complexes*

3.1.1. Structure of 1. $[Cu^{II}(L^1)(phen)_2](ClO_4)$ (**1**) crystallizes in the monoclinic space group *C2/c*. The perspective views of the crystal structures of **1** are shown in figure 1. Each molecule consists of one copper ion, one 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carboxylato (L_1) ligand, two phen ligands, and one perchlorate. The distance between Cu and O2 (2.677 Å) is out of the range of the typical Cu–O coordination bond distance (1.99–2.34 Å) and can be defined as weak interaction. Copper ion is five-coordinate with one O of L^1 and four N of two phen ligands in a slightly distorted square pyramidal arrangement with trigonality index, $\tau = 0.1$ $\{\tau = [(\beta - \alpha)]/60$, where α and β are the two largest angles $(\beta > \alpha)\}$ [29], thus forming a $[CuON_4]$ chromophore, where the L^1 and

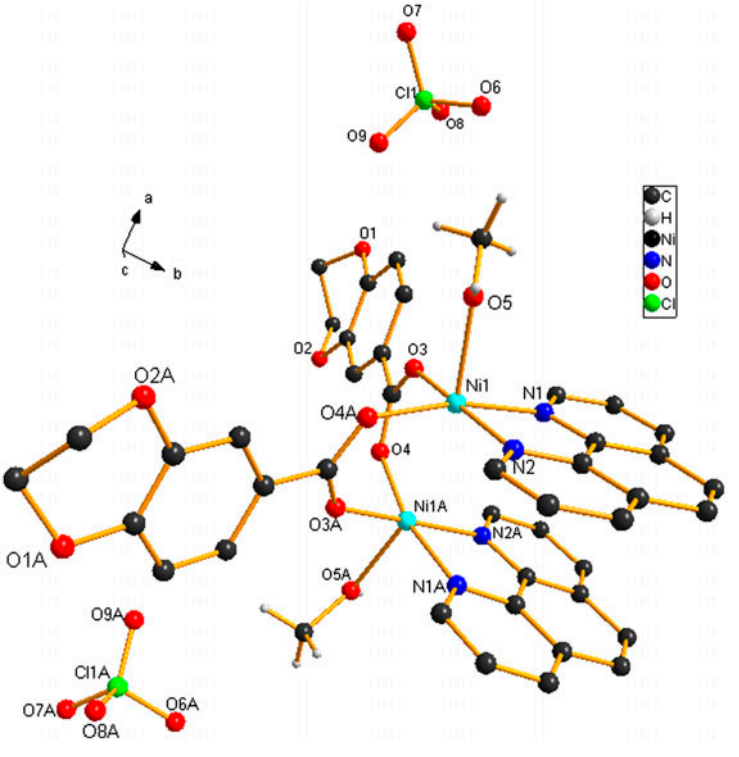


Figure 3. A perspective view of **2** (hydrogens are omitted for clarity).

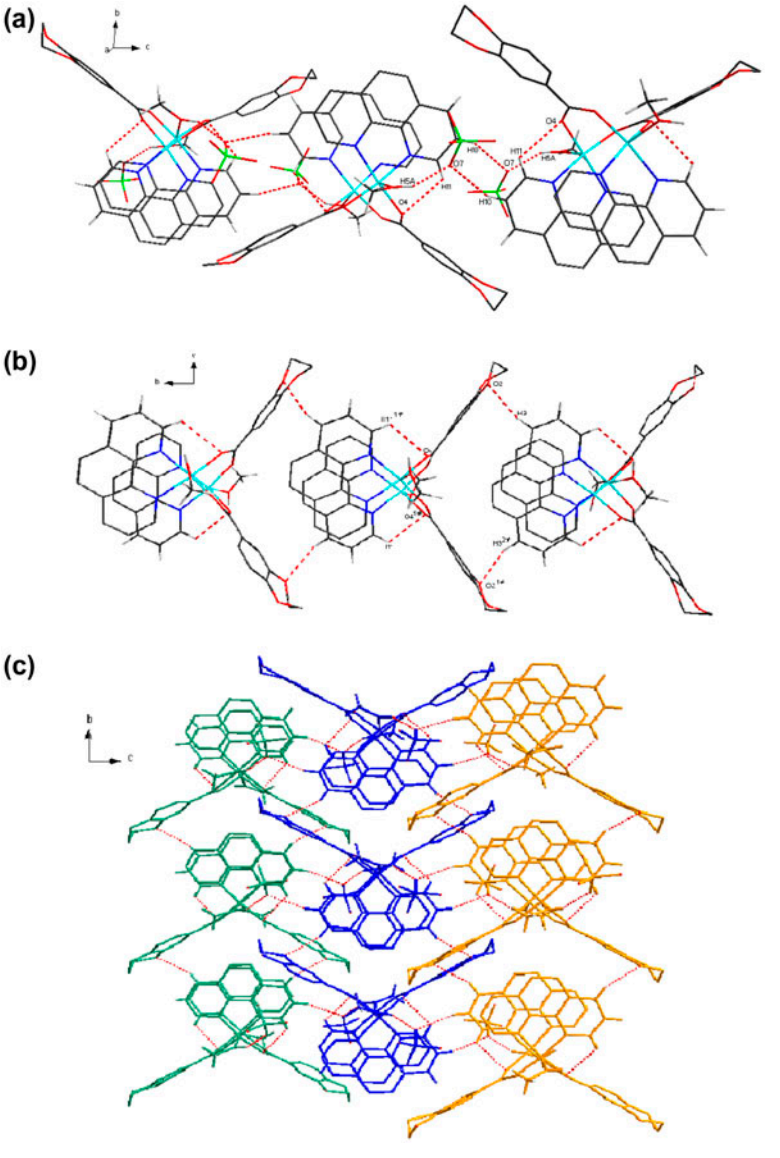


Figure 4. View of the hydrogen bond-driven 1-D and 2-D network of **2** (4a: 1-D chain running along the *c* axis; 4b: 1-D chain running along the *b* axis; 4c: 2-D network in the *bc* plane). Hydrogen bonds are shown as dashed lines.

phen are monodentate and chelating bidentate, respectively. The bond distances are shown in table 2. In the crystal, the ClO_4^- link adjacent molecules into 1-D chains along the *b* axis through intermolecular H bonds H2 $(0.5 - x, -0.5 + y, 0.5 - z]$…O7–Cl2–O8 $(x, -y, -0.5 + z)$…H10 $[x, y, z]$ (figure 2 and table 3). The free ClO_4^- anions also act as bridges to link the chains (running along the *b* axis, highlighted by different color) into a 2-D network (figure 2 and table 3) via six intermolecular H bonds: Cl2–O5 $(x, -y, -0.5 + z)$

…H6 (−*x*, −*y*, −*z*), Cl2–O6 (*x*, −*y*, −0.5 + *z*)…H4 (−*x*, −*y*, −*z*), Cl2–O7 (*x*, −*y*, −0.5 + *z*)…H2 (0.5 − *x*, −0.5 + *y*, 0.5 − *z*), Cl2–O8 (*x*, −*y*, −0.5 + *z*)…H10 (*x*, *y*, *z*), O2 (*x*, *y*, *z*)…H7 (−*x*, −*y*, −*z*)–C7 (−*x*, −*y*, −*z*) and O2 (−*x*, −*y*, −*z*)…H7 (*x*, *y*, *z*)–C7 (*x*, *y*, *z*). In free ClO_4^-, O5 and O6 act as H-acceptors to H6 and H4 of one 1-D chain extended along the *b* axis, O7 and O8 act as H-acceptors to H2 and H10 of another 1-D chain extended along the *b* axis, thus linking adjacent 1-D chains into the 2-D network in the *bc* plane. The intermolecular H bonds O2(*x*, *y*, *z*)…H7(−*x*, −*y*, −*z*)–C7(−*x*, −*y*, −*z*) and O2(−*x*, −*y*, −*z*)…H7 (*x*, *y*, *z*)–C7 (*x*, *y*, *z*) are also involved in the formation of the 2-D network.

3.1.2. Structure of 2. $[Ni_2(L^1)_2(phen)_2(CH_3OH)_2](ClO_4)_2$ (**2**) is in the monoclinic space group *C2/c*. The perspective views of the crystal structures of **2** are shown in figure 3. In each molecule, there are two nickel ions, two L^1 anionic ligands, two phen, and two methanol ligands. Each nickel is five-coordinate with two O from two L^1, two N from one phen, and one O from methanol in the distorted square pyramidal arrangement ($\tau = 0.34$), thus forming a $[NiO_3N_2]$ chromophore. Two nickel ions are linked by two bridging bidentate carboxylates of L^1. Oxygens of two L^1 anions and two N from one phen form the basal plane with the O of methanol in the axial position. The main bond lengths and angles are shown in table 2. In a free ClO_4^- anion, O7 is a H-acceptor to H10 and H5A from two adjacent molecules forming a 1-D chain extended along the crystallographic *c* axis [figure 4(a) and table 3]; intermolecular H bond C9–H9…O2 (−*x*, 1 + *y*, 1/2 − *z*) links the adjacent molecules into a 1-D chain along the *b* axis [figure 4(b) and table 3], thus, the H bonds link the chains into the 2-D network in the *bc* plane [figure 4(c) and table 3].

3.2. *Infrared and electronic spectra*

Infrared spectra of HL^1 and the two complexes provide information about the metal–ligand bonding. For HL^1, the strong absorption band at 1686 cm^{-1} is assigned to the C=O(Ar–COOH) stretching vibration and the broad absorption bands at 2500–2700 cm^{-1} are assigned to the O–H(Ar–COOH) stretching vibrations. In **1** and **2**, the two bands disappear indicating complete deprotonation of the carboxylic acid. The characteristic bands of the carboxylate ligands have typical $\nu(OCO)_{asym}$ {1561 cm^{-1} in **1**, 1563 cm^{-1} in **2**} and $\nu(OCO)_{sym}$ {1353 cm^{-1} in **1**, 1431 cm^{-1} in **2**} values. In **1**, the calculated value of Δ(OCO) {$\nu(OCO)_{asym}$–$\nu(OCO)_{sym}$} is 208 cm^{-1} indicating HL^1 is monodentate since the Δ(OCO) value (>200 cm^{-1}) is correct for a monodentate carboxylate. In **2**, the Δ(OCO) value is 132 cm^{-1} with HL_1 a bidentate ligand (bidentate carboxylate ligand <200 cm^{-1}) [30]. The sharp absorption at 1109 cm^{-1} in **1** and 1099 cm^{-1} in **2** is bands of ClO_4^-. In **2**, the broad and medium weak bands around 3449 cm^{-1} are due to the O–H stretch of coordinated MeOH. The electronic spectra of HL^1 and the complexes are recorded in DMSO : H_2O (V : V = 1 : 1) solutions. The weak broad bands observed between 600 and 800 nm in **1** and **2** were assigned to d–d transitions of the complexes, presenting evidence that the complexes keep their structures in solution.

3.3. *Inhibitory activity against jack bean urease*

The abilities of the ligands, Cu^{2+}, Ni^{2+}, and complexes inhibiting urease were studied by the IC_{50} values of the material tested against *jack bean* urease according to the literature

Table 4. Inhibition of urease by the tested materials.

Tested materials	IC_{50} (μM)
HL^1	>100
phen	>100
$Ni(ClO_4)_2·6H_2O$	6.69
$Cu(ClO_4)_2·6H_2O$	1.69
$[Cu(L^1)(phen)_2](ClO_4)$, **1**	0.14
$[Ni_2(L^1)_2(phen)_2(MeOH)_2](ClO_4)_2$, **2**	0.065
Acetohydroxamic acid	12.5

HL^1 = 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carboxylic acid; phen = 1, 10-phenanthroline.

Table 5. The antiproliferative activity of the tested materials.

	IC_{50} (μM)		
Test material	A549	L929	SW620
1	14 ± 3	13 ± 2	24 ± 3
2	18 ± 3	13 ± 3	9 ± 2
cisplatin	40 ± 6	12 ± 2	21 ± 3
HL^1	>100	>100	>100
$Cu(CH_3COO)_2$	>100	>100	>100
$Ni(CH_3COO)_2$	>100	>100	>100

phenol-red method. The results are summarized in table 4. The IC_{50} value of HL^1 and phenanthroline are >100 μM. Under the same conditions, **1** and **2** show much higher inhibitory activity against *jack bean* urease with an IC_{50} value of 0.14 and 0.065 μM, respectively, with the acetohydroxamic acid (IC_{50} = 12.5 μM) as a standard reference against urease.

3.4. *In vitro anticancer activities*

The cytotoxic properties of the complexes and their free ligands along with the clinically used drug cisplatin were investigated using a MTT assay against three cell lines. The free ligands, Cu^{2+} and Ni^{2+} ions (as metal salt) were noncytotoxic at concentrations up to 100 μM. The two complexes exhibited excellent antitumor activities to the human lung cancer cell line A549, mouse fibrosarcoma cell line L929, and human colorectal cancer cell line SW620 (table 5).

4. Conclusion

The present study reports the synthesis, structures, urease inhibitory, and *in vitro* cytotoxic activities of two complexes with a protocatechuic acid derivative as ligand. The complexes exhibit stronger urease inhibitory and cytotoxic activities than their parent ligands and metal ion. The activity probably is due to the strong Lewis acid properties of metal ions and the ligands strengthening the inhibitory activity of the complexes. The results are in accord with those reported previously for some metal complexes having stronger urease inhibitory and

in vitro cytotoxic activities [15, 23, 31–35]. Compared with the data reported, the complexes exhibit fairly strong urease inhibitory and cytotoxic activities, and may be used as urease inhibitors and anticancer agents. Detailed investigation is continuing to study the inhibitory mechanism.

Supplementary material

Crystallographic data for the structural analysis have been deposited with the Cambridge Crystallographic Data Center (CCDC 1026931 for **1** and 1026932 for **2**). These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Center, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: (+44) 1223 336033; E-mail: deposit@ccdc.cam.ac.uk or www: http://www.ccdc.cam.ac.uk).

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental data

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