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## Synthesis, characterization and *in vitro* antiproliferative and antibacterial studies of tetraazamacrocyclic complexes of Co(II) and Cu(II) with pyromellitic acid

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New cationic tetranuclear Co(II) and neutral binuclear Cu(II) complexes with tpmc (*N,N,N'',N'''*-tetrakis-(2-pyridylmethyl)-1,4,8,11-tetraazacyclotetradecane) and bridging pyromellitate ligand pma (tetraanion 1,2,4,5-benzenetetracarboxylic acid) were isolated. The composition of the compounds is proposed based on elemental analyses (C, H, N, M = Cu, Co), molar conductivity determination, UV-vis, FTIR, EPR, LC-MS and reflectance spectroscopy, magnetic measurements, cyclic voltammetry, as well as TG and DTA. It is proposed that in  $[\text{Co}_4(\text{pma})(\text{tpmc})_2](\text{ClO}_4)_4 \cdot 6\text{H}_2\text{O}$  (**1**), cobalt(II) is six-coordinate out of cyclam rings and one  $\text{OCO}^-$  from pma participates in coordination with one Co(II). In the case of  $[\text{Cu}_2(\text{pma})\text{tpmc}] \cdot 8\text{H}_2\text{O}$  (**2**), one  $\text{OCO}^-$  from pma bridges two Cu(II). The cytotoxic activity of **1** and **2** was tested against tumor cell lines human cervix adenocarcinoma (HeLa), estrogen-receptor-positive human breast cancer (MCF-7), human myelogenous leukemia (K562) and the human Caucasian Burkitt's lymphoma (Ramos). The  $\text{IC}_{50}$  values for **1** and **2** were within the range  $44.66 \pm 2.39$  to  $152.40 \pm 2.28$   $\mu\text{M}$ , and from  $140.88 \pm 3.51$  to  $192.05 \pm 2.09$   $\mu\text{M}$ , respectively. Both **1** and **2** were tested for antimicrobial activity. We determined that minimal inhibitory concentration for **1** against *Staphylococcus aureus*, *Bacillus subtilis* and *Klebsiella pneumoniae* was 25 mM. Complex **2** did not express activity against tested microbial strains.

**Keywords:** Co(II) and Cu(II) Complexes; tpmc; Pyromellitate ligand; Antimicrobial activity; Cytotoxic activity

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## 1. Introduction

Tetraazamacrocyclic ligands and their metal complexes are very attractive compounds and have been intensively studied over the last decades because of their interesting structure, redox properties, possible applications as catalysts, new magnetic materials, ion selective potentiometric sensors, agents for nucleic acid cleavage, *etc.* [1-4]. Their potential application as antitumor [5], antiviral (including HIV activity) [6-7], antibacterial, antifungal or antimalarial agents have been examined [8-12].

Tetraazamacrocyclic ligand with four pendant arms, *N,N',N'',N'''*-tetrakis(2-pyridylmethyl)-1,4,8,11-tetraazacyclotetradecane (tpmc), forms mono-, bi- or tetranuclear complexes having various structures depending on the metal center and the number of co-ligands. Metal centers are coordinated with tpmc in *exo* or *endo* mode. In bi- and tetranuclear complexes, depending on the number, type and size of donor atoms of additional ligands, metal centers, as well as the reaction conditions, different modes of macrocyclic conformations were found. Macrocyclic ligand adopts *boat* conformation when the additional ligand is bonded in a bridged or a combined chelate-bridged manner and a *chair* conformation when two other ligands are independently bonded in *trans* position. Metal centers are also bridged with N-(CH<sub>2</sub>)<sub>3</sub>-N fragments of the macrocyclic ring. Many macrocyclic mixed ligand Cu(II) and Co(II) complexes were previously described [13-19].

Polycarboxylates are used for the synthesis of hybrid materials, as multidentate, forming several possible coordination modes (monodentate, bis-monodentate, bidentate, tridentate) of the independent carboxylate groups [20-24]. Aromatic tetracarboxylic derivatives such as 1,2,4,5-benzenetetracarboxylic acid (H<sub>4</sub>pma, commonly known as pyromellitic acid) belong to important O-donor ligands which have been used to prepare metal-organic hybrid compounds [25]. It consists of a rigid planar C<sub>10</sub> backbone due to the  $\delta$ -bonding of the benzene ring and four freely rotating and flexible carboxylate groups. It can be deprotonated to give four available charges. Compounds with anion of a single negative charge are less common. The known compounds have dimeric units bridged by either H<sub>2</sub>pm<sup>2-</sup> or pm<sup>4-</sup>, a one-dimensional chain structure with bridging pm<sup>4-</sup>, a square grid of two-dimensional layers with bridging pm<sup>4-</sup>, and three-dimensional architectures with bridging pm<sup>4-</sup> [25b-f]. In some cases the structures have cavities and channels which are filled with water. Pyromellitic acid is an important raw material for the plastics industry due to its excellent thermal and mechanical properties.

Copper, as a biologically important metal, plays an important role in endogenous processes like oxidative DNA-damage associated with aging and cancer. Many studies have focused on binuclear copper complexes due to their presence in metalloproteinase as well as their affinity for DNA [26-28].

In order to get better insight into the coordination chemistry of pyromellitate-containing cobalt(II) and copper(II) complexes, we have prepared two new analogous macrocyclic complexes of Co(II) and Cu(II) with tetracarboxylate ligand (pyromellitic acid). Their synthesis, physical and chemical properties, antimicrobial and cytotoxic potentials are reported in this paper.

## 2. Experimental

### 2.1. Preparation

**Caution!** Metal-organic perchlorate salts must be handled with caution! Use only the absolute minimum of material required!

Ligand tpmc was prepared and purified according to a procedure reported [29]. All other chemicals of p.a. grade are used as supplied, with the exception of CH<sub>3</sub>CN used for electrical conductivity measurements, recording electronic and LC/MS spectra, which was of HPLC grade purity.

**2.2.1. [Co<sub>4</sub>(pma)(tpmc)<sub>2</sub>](ClO<sub>4</sub>)<sub>4</sub>·6H<sub>2</sub>O (1).** The complex was prepared by adding aqueous solution of pyromellitic acid to a suspension of 188.3 mg (0.515 mmol) Co(ClO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O and 145.5 mg (0.258 mmol) tpmc in 20 mL CH<sub>3</sub>CN. The amounts correspond to the mole ratio of the components of 4:2:1.5, respectively. The pH of the aqueous solution (15 mL) of pyromellitic acid (50.5 mg, 0.197 mmol) was adjusted to 5.5 by the dropwise addition of aqueous solution of 0.1 M NaOH. The solution of pyromellitic acid was added carefully to a suspension of Co(ClO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O and tpmc with stirring. The mixture was stirred and refluxed at 80 °C on a water bath for 3 h. The purple solution was concentrated to ½ of the starting volume and cooled in a refrigerator. After a few days the purple microcrystals were filtered and recrystallized from CH<sub>3</sub>CN-H<sub>2</sub>O (10:1, v/v). The compound was dried at room temperature. Yield 81%, FW = 2121.39. Anal. Calcd. for Co<sub>4</sub>C<sub>78</sub>H<sub>102</sub>N<sub>16</sub>O<sub>30</sub>Cl<sub>4</sub> (%): C, 44.16; H, 4.85; N, 10.56; Co, 11.10. Found: C, 44.54; H, 4.93; N, 10.62; Co, 10.73. It was (T = 20±2 °C) well soluble in CH<sub>3</sub>CN,

DMSO and DMF, insoluble in CH<sub>3</sub>OH, EtOH, (CH<sub>3</sub>)<sub>2</sub>CO and H<sub>2</sub>O;  $\mu_{\text{eff}}$  (room T) = 4.96 BM/Co(II);  $\Lambda_{\text{M}} = 636 \text{ S cm}^2 \text{ mol}^{-1}$ . FTIR (KBr):  $\nu = 3441, 1610, 1104, 625, 1546, 1400, 481, 419 \text{ cm}^{-1}$ ; UV-vis (CH<sub>3</sub>CN):  $\lambda_{\text{max}} = 527 \text{ nm}$  ( $\epsilon = 264 \text{ M}^{-1}\text{cm}^{-1}$ ); MS (LC-MS, 4000 V), (positive):  $m/z$  (%) 667.9 (7.83), 565.7 (3.01), 565.4 (7.83), 333.2 (16.27), 284.2 (39.16), 227.0 (7.23), 189.1 (3.01).

**2.2.2. [Cu<sub>2</sub>(pma)tpmc]·8H<sub>2</sub>O (2).** **2** was obtained by the reaction of Cu(NO<sub>3</sub>)<sub>2</sub>·3H<sub>2</sub>O (100 mg, 0.414 mmol), tpmc (116.9 mg, 0.206 mmol) and pyromellitic acid (78.9 mg, 0.310 mmol) (neutralized previously to pH 6.0 with NaOH) (molar ratio 2:1:1.5) in CH<sub>3</sub>OH-H<sub>2</sub>O (1:1; v/v). Mixture was stirred and refluxed (~80 °C) 3 h, concentrated to ½ of the initial volume and left in the refrigerator overnight. Microcrystalline blue product was separated by suction, dried, powdered, washed with ethanol, cold water and dried at room temperature. Yield 79%, FW = 1086.04. Anal. Calcd. for C<sub>44</sub>H<sub>74</sub>O<sub>22</sub>N<sub>8</sub>Cu<sub>2</sub> (%): C, 48.66; H, 5.75; N, 10.32; Cu, 11.69. Found: C, 48.30; H, 5.43; N, 9.99; Cu, 11.39. Product was insoluble in common solvents (H<sub>2</sub>O, CH<sub>3</sub>OH, EtOH, CH<sub>3</sub>CN, DMF, (CH<sub>3</sub>)<sub>2</sub>CO) except limited in DMSO.  $\mu_{\text{eff}}$  (room T) = 2.07 BM/Cu (II); FTIR (KBr):  $\nu = 3452, 1609, 1572, 1381, 469, 429 \text{ cm}^{-1}$ ; Reflectance spectrum  $\lambda_{\text{max}} = 654 \text{ nm}$ . By heating microcrystals, its color changes at ~110 °C turquoise, above ~190 °C deep green, and from 210-250 °C became brown, suggesting liberation of lattice water and some decomposition of the ligand(s) and/or complex.

## 2.2. Measurements

Elemental analyses were performed by standard methods in the Center for Instrumental Analyses ICTM in Belgrade. The Cu and Co contents were determined by using an atomic absorption spectrophotometer PERKIN-ELMER AAS-5100/PC. Electronic absorption spectrum of **1** in CH<sub>3</sub>CN (1×10<sup>-3</sup> M) was recorded on a GBC UV-VIS Cintra 20 spectrophotometer. Reflectance spectrum of **2** was recorded on a CARY 17D spectrophotometer using MgO as standard. The correction for the position of broad maxima were performed by a computer using Kubelka-Munks' function. Magnetic susceptibility measurements were made at room temperature (20±2 °C) using an MSB-MKI balance (Sherwood Scientific Ltd., England). Data were corrected for diamagnetic susceptibilities using Pascal's constants [30]. FTIR spectra (KBr disc technique) were recorded using a Perkin-Elmer FTIR 31725X spectrophotometer from 400-4000 cm<sup>-1</sup>.

Molar electrical conductivity in CH<sub>3</sub>CN for **1** ( $1 \times 10^{-3}$  M) was measured at room temperature on a Hanna instruments HI 8820N conductometer. LC/MS (4000 V) spectrum for **1** was carried out on MS system consisting of 6210 (Time-of-Flight) (G1969 A, Agilent Technologies) in CH<sub>3</sub>CN (~1 mg of the sample/mL) in positive ion mode in the range 100-2000 m/z. Mobile phase was 0.2% solution of HCOOH in the mixture of CH<sub>3</sub>CN-H<sub>2</sub>O (50:50; v/v). For the collection and processing of the results Mass-Hunter Workstation software was used. The most intense peak at ~282 m/z originated from solvent system. MS peaks were measured up to 700 m/z. The simultaneous TG-DTA experiments were carried out using a SDT 2960 thermal analyzer in air (flow rate = 90 mL/min) from 20 to 580 °C (heating rate = 15 °C/min) using platinum cups. EPR spectrum of powdered sample was recorded at room temperature and 77 K on a Bruker ELEXSYS E 500 CW-EPR spectrometer operating at X-band frequency and equipped with an ER 036TM NMR Teslameter and E 41 FC frequency counter.

Electrochemical measurements for **2** were performed by METHROME 797 VC Computrace in a standard three-electrode cell: Pt disc as working, standard AgCl/Ag as reference and Pt as auxiliary electrodes. Measurements were carried out in 10 mL of DMSO. Concentration of the complex was about  $1 \times 10^{-4}$  M. CV was performed with the sweep rate of 50, 100 and 200 mV/s, respectively, with the potential ranged from -1 V to 1 V *versus* AgCl/Ag, at room temperature (20±2 °C). Oxygen was removed from the system by continuous bubbling with oxygen free N<sub>2</sub>.

### **2.3. *In vitro* evaluation of antiproliferative and antimicrobial activity**

**2.3.1. Antiproliferative activity.** Stock solutions (10 µM) of **1** and **2** were made in DMSO, filtered through Millipore filters (0.22 µm), and diluted to the relevant working concentrations for use in the nutrient medium. The nutrient medium was RPMI 1640 for all cells without phenol red, supplemented with L-glutamine (3 mM), streptomycin (100 µg/mL), and penicillin (100 IU/mL), fetal bovine serum (10%; FBS; 56 °C heat-inactivated) and HEPES (25 mM), adjusted to pH 7.2 (bicarbonate solution). RPMI-1640, FBS, Hepes and L-glutamine were products of Sigma Chemical Co., St. Louis, MO, USA.

The human cervix adenocarcinoma (HeLa) and estrogen-receptor-positive human breast cancer (MCF-7) cells were cultured as monolayers in the nutrient medium (see above), while the human myelogenous leukemia (K562) and the human Caucasian Burkitt's lymphoma (Ramos)

cells were maintained as suspension cultures in the same nutrient medium. All these cells were grown at 37 °C in 5% CO<sub>2</sub> and a humidified air atmosphere.

The metabolic activity was assessed by using the CellTiter 96H Aqueous One Solution Cell Proliferation Assay (Promega, Madison/WI, USA), in accord with the manufacturer's instructions.

The HeLa and MCF-7 cells were seeded ( $5 \times 10^4$ /mL) into 96-well microtiter plates and 20 h later, after cell adherence, five different concentrations of the test compounds were added to the wells. The final test compound concentrations were in the range of 12.5 μM to 200 μM. In addition, we tested the activity of the starting compounds (tpmc and pma) in the same concentration range. Only the nutrient medium was added to the cells in the control wells. Cisplatin was used as a positive control. For the K562 and Ramos cells the test compounds were added to cell suspensions ( $5 \times 10^4$ /mL) 2 h after cell seeding, to the same final concentrations applied to the HeLa and MCF-7 cells. All experiments were carried out in triplicate. Nutrient medium with the corresponding concentrations of the test compounds, but without cells, was used as the blank probe [31].

After 48 h, 100 μL of cell suspension was incubated with 10 μL of the supplied MTS tetrazolium compound. After 3 h of incubation under standard conditions, the absorbance was measured at 492 nm by using an automated microplate reader Tecan Safire (Tecan, Mannedorf/Zürich, Switzerland).

To achieve cell survival (%), absorbance at 492 nm of a sample with cells grown in the presence of various concentrations of compounds tested was divided with absorbance of control sample (the absorbance of cells grown in nutrient medium only). The absorbance of blank probe was always subtracted from absorbance of a corresponding sample with cells. IC<sub>50</sub> was defined as the concentration of the compound inhibiting the cell survival by 50%, compared to a vehicle-treated control cells. All experimentally obtained IC<sub>50</sub> data were means of three measurements.

**2.3.2. Three-dimensional invasion assay.** The effect of **1** to impair tumor cell invasion in a 3D model was evaluated using multi-cellular tumor spheroids (MCTS). MCTS were prepared according to the hanging drop method [32]. Twenty μL of HeLa cells suspension (150 cells per drop) were placed onto the lids of 90 mm tissue-culture dishes which were inverted over 10 mL of water. After 5 days, the formed aggregates were transferred to wells of Lab-Tek™ Chambered



Coverglass (Nalge Nunc International, Penfield, NY, USA) which had been coated with 70  $\mu\text{L}$  of Matrigel (5 mg/mL) in SFM. Then, spheroids were covered with an additional 70  $\mu\text{L}$  of Matrigel and after 20 min at 37  $^{\circ}\text{C}$  covered with 400  $\mu\text{L}$  of the medium. Complex **1** (in concentration equal to  $\text{IC}_{50}$ ) was added to the Matrigel and the growth of spheroids was monitored for three days. As a control, only the medium was added to Matrigel. Images of tumor spheroids were obtained by using inverted microscope Olympus, Japan.

**2.3.3. Antimicrobial activity.** The antimicrobial activity of **1** and **2** was tested against Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212) and *Bacillus subtilis* (ATCC 6633), Gram-negative bacteria *Escherichia coli* (ATCC 10536) and *Klebsiella pneumoniae* (ATCC 13883), and against *Candida albicans* (ATCC 10231) as yeast. Microorganisms were provided by the Institute of Immunology and Virology, Torlak, Belgrade. Each species was maintained on Mueller-Hinton agar (MHA), which was also used to confirm the absence of contamination and validity of the inocula. Before testing, each species was recovered by subculturing in Mueller-Hinton broth (MHB) aerobically for 24 h at 37  $^{\circ}\text{C}$ . For *Candida albicans* Sabourad agar (SA) and Sabourad dextrose broth (SB) were used. Working concentrations (approximately  $10^6$  CFU/mL for bacteria,  $10^5$  CFU/mL for *Candida albicans*) used for antimicrobial activity assays were prepared by dilution of the culture in microbiological medium (for bacteria MHB, for *Candida albicans* SB). Compounds were dissolved in DMSO (2%) to prepare stock solutions sterilized by filtration through a 0.22-mm membrane filter (Sartorius AG, Germany) and further diluted with MHB to working solutions. DMSO was chosen as a non-toxic solvent [33, 34].

Broth microdilution method was employed to determine minimum inhibitory concentrations (MICs). The concentration of compounds ranged from 100 to 3.125 mM. Test bacterial culture (100  $\mu\text{L}$ ) in a MHB was added to the wells of a sterile 96-well microtiter plate (Sarstedt, Numbrecht, Germany) already containing 100  $\mu\text{L}$  of twofold serially diluted compound in MHB. Also, 100  $\mu\text{L}$  *Candida albicans* in SB was added to the wells 96-well microtiter plate already containing 100  $\mu\text{L}$  of twofold serially diluted compounds in SB. The final volume in each well was 200  $\mu\text{L}$ . The microplates were prepared in triplicate and incubated aerobically for 24 h at 37  $^{\circ}\text{C}$ . Wells with MHB were used as a sterility control, while negative controls were wells with tested compound in 100  $\mu\text{L}$  of MHB, but without bacteria. Positive



controls were wells with 100 mL suspension of microorganisms in 100  $\mu$ L of MHB (or SB for *Candida albicans*) and wells with bacterial suspension in a MHB with DMSO (or SB for *Candida albicans*), (to prove that DMSO had no inhibition effect on the microorganism's growth).

To indicate cellular respiration, 2,3,5-triphenyltetrazolium chloride (TTC) (Aldrich Chemical Company Inc., Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium. The final concentration of TTC after inoculation was 0.05%. Viable microorganisms enzymatically reduced the white TTC to a pink TPF (1,3,5-triphenylformazan). The MIC was defined as the lowest concentration of the investigate compounds at which the microorganism does not demonstrate visible growth.

### 3. Results and discussion

#### 3.1. Preparation

Complexes  $[\text{Co}_4(\text{pma})(\text{tpmc})_2](\text{ClO}_4)_4 \cdot 6\text{H}_2\text{O}$  (**1**) and  $[\text{Cu}_2(\text{pma})\text{tpmc}] \cdot 8\text{H}_2\text{O}$  (**2**) were prepared *via* the reaction of  $\text{Co}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$  and  $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$  with the ligand tpmc and previously neutralized pyromellitic acid in molar ratio 4:2:1.5 for **1** and 2:1:1.5 for **2**. The complexes were obtained in good yield (~80%). The compounds were characterized by elemental analysis and spectroscopic techniques. Both complexes are microcrystalline solids, which are stable in air. Unfortunately, all attempts to grow single-crystals suitable for X-ray analyses of these complexes failed. For this reason, the structure of the complex is proposed based on spectroscopic and TG/DTA measurements and by comparison of the obtained data with some of the aromatic carboxylate complexes previously described. For some of them the structure was confirmed by X-ray analysis [18, 35].

#### 3.2. LC-MS, UV-vis, reflectance, FTIR, EPR spectra and magnetic measurements

Electronic spectral data of **1** were recorded in  $\text{CH}_3\text{CN}$ . In the visible region, there is a band at 527 nm ( $\epsilon = 264 \text{ M}^{-1}\text{cm}^{-1}$ ) and shoulder at 475 nm ( $\epsilon = 219 \text{ M}^{-1}\text{cm}^{-1}$ ) and are due to  ${}^4\text{T}_1(\text{F}) \rightarrow {}^4\text{T}_{1g}(\text{P})$  and  ${}^4\text{T}_1(\text{F}) \rightarrow {}^4\text{A}_{2g}(\text{F})$  transitions, respectively, typical for six-coordinate Co(II) complexes [36, 37], while the shape and position of the bands agree with those for high-spin Co(II) complexes with three unpaired electrons. Position of the band at 527 nm of **1** is very close to those of complexes Co(II)-tpmc with aromatic and  $\alpha,\omega$ -dicarboxylates, in the range 508-

542 nm [17, 19]. This indicates the presence of the same chromophore ( $\text{CoN}_4\text{O}_2$ ). Much higher  $\epsilon$  value of  $264 \text{ M}^{-1}\text{cm}^{-1}$  (**1**) than related dinuclear complexes ( $\epsilon = 75\text{-}94 \text{ M}^{-1}\text{cm}^{-1}$  for aromatic and  $\epsilon = 95\text{-}109 \text{ M}^{-1}\text{cm}^{-1}$  for aliphatic dicarboxylate macrocycle complexes) may be a consequence of the asymmetry and is consistent with the proposed formula for tetranuclear complex. In the UV region of the electronic spectrum of **1**, several sharp unresolved bands are present in the range of 252-310 nm ( $\epsilon = 5439\text{-}5766 \text{ M}^{-1}\text{cm}^{-1}$ ) belonging to CT transitions. The Vis spectral data for **1** and related complexes are shown in table 1.

For **2**, the geometry is not easy to predict due to its solubility and scarce information. In reflectance spectrum, a band at 654 nm corresponds to d-d transitions in Cu(II) complexes. The absorption bands in the reflectance spectra of similar Cu(II) complexes are also in the range 621-654 nm [35, 38, 39]. According to this result it can be assumed that **2** has the same chromophore ( $\text{CuN}_4\text{O}$ ) and five-coordinate Cu(II) as in the similar compounds.

In LC-MS spectrum of **1**, there is no peak for molecular ion. The peaks and corresponding intensities from the fragmentation of the complex are given in table 2. The peak characteristic for the tpmc ligand also appears in the MS spectrum of **1** at 565,4 (7.83%)  $m/z$ , ( $\text{C}_{34}\text{H}_{44}\text{N}_8^+$ ) [39, 40].

**3.2.1. FTIR spectra.** In the FTIR spectra of the complexes, some common characteristic bands are observed [41, 42]: a broad strong band at  $\sim 3400 \text{ cm}^{-1}$  belongs to the stretching vibrations of crystal water,  $\nu(\text{H}_2\text{O})$ ; a strong band at  $\sim 1610 \text{ cm}^{-1}$  is typical for the skeletal vibration of pyridine from tpmc; the strongest band in the spectrum **1** at  $1100 \text{ cm}^{-1}$  belongs to the  $\nu(\text{ClO}_4^-)$  and the sharp, medium intensity band at  $\sim 620 \text{ cm}^{-1}$  to  $\delta(\text{ClO}_4^-)$ . FTIR spectra of the complexes and free ligands were used for the prediction of the composition as well as the proposition of the more favorable coordination modes. From shifting of the  $\nu_{\text{asym}}(\text{OCO})$  and  $\nu_{\text{sym}}(\text{OCO})$  bands in FTIR spectra of the complexes (table 1) in relation with those found in the spectrum of the pma ligand recorded for comparison ( $\nu_{\text{asym}}(\text{OCO}^-) = 1580 \text{ cm}^{-1}$  and  $\nu_{\text{sym}}(\text{OCO}^-) = 1385 \text{ cm}^{-1}$ ,  $\Delta\nu = 195 \text{ cm}^{-1}$ ), it is obvious that  $\text{OCO}^-$  groups are coordinated. Additional evidence for their coordination is the occurrence of the weak bands of  $\nu(\text{M-O})$  at  $\sim 420 \text{ cm}^{-1}$ , while bands at  $480\text{-}470 \text{ cm}^{-1}$  could be ascribed to  $\nu(\text{M-N})$  confirming the binding of macrocycle.

It is also known that the six-coordination is favorable for Co(II) ion [37, 42]. Significantly lower  $\Delta\nu$  value in **1** related to a pyromellitate ligand and  $\nu_{\text{asym}}$  displacement

(table 1) towards lower values compared to the polycarboxylate indicates the combined chelate-bridging manner of coordination [42]. Probably, oxygen atoms from one of the  $\text{OCO}^-$  groups are bound to the same  $\text{Co(II)}$  ion, thus forming 4-membered rings (figure 1a) rather than enlarged (7-9 membered) rings (figure 1b and 1c). Similar coordination was proposed for aliphatic dicarboxylic anion in  $\text{Co(II)-tpmc}$  complexes [19].

In **2**, bathochromic shift of  $\nu_{\text{asym}}$  and  $\nu_{\text{sym}}$  and the approximate  $\Delta\nu$  values of free ligand and complexes point to the bridging binding of the pyromellitate anions. The presence of four  $\text{OCO}^-$  groups in the pyromellitate ligand provides opportunities for a variety of coordination modes [42, 43], but it is likely that only one carboxylate group participates in the coordination of  $\text{Cu(II)}$  ions, while the remaining groups either participate in hydrogen bonding with the water molecules in the crystal or even participate in the creation of the polymer structure. Monodentate coordination mode of two  $\text{OCO}^-$  groups in bridging coordination can also be excluded because, in that case, unstable formed chelate rings would have 11 or more members (figure 1d). In that case,  $\Delta\nu$  is significantly higher than  $\Delta\nu$  of uncoordinated carboxylate anion [42, 43]. The engagement of more than two oxygen atoms from carboxylate ligands (each O is coordinated to one metal) in the coordination cannot be considered because it would be in contradiction with the diffusion-reflectance spectra and copper(II) coordination number of 5, as well as the particular chromophore and other methods applied. It is possible that one  $\text{OCO}^-$  from  $\text{pma}^{4-}$  ligand bridges  $\text{Cu(II)}$  atoms in **2** (figure 1e) and the rest of the O atoms are involved in the formation of H-bonds with water molecules. Other bridged coordination mode as well as the possible polymer structure of the complex cannot be excluded.

In table 1 the electronic and FTIR spectral data, the molar electrical conductivity ( $\Lambda_M$ ) and the magnetic moments ( $\mu_{\text{eff}}$ ) of **1** and **2** together with the corresponding data for some similar complexes from the literature are presented. It is evident that five-coordinate  $\text{Cu(II)}$  forms binuclear (benzoate, Hpht) or tetranuclear complexes (ipht) with the tpmc in a *boat* conformation (figure 1f) [18, 35]. Carboxylic group bridges two metallic centers within the same  $[\text{Cu}_2\text{tpmc}]^{4+}$  unit. This type of coordination mode for one carboxylic group ( $\mu\text{-O,O'}$ ) was confirmed previously by X-ray structure analyses of the complexes with bridging benzoate, isophthalate or succinate dianions [18, 35]. Complexes with dicarboxylate anions were tetranuclear. By comparing the spectroscopic properties of **2** with mono- and dicarboxylate complexes (table 1), it can be assumed that the same coordination pattern of carboxylate group,  $\mu\text{-O,O'}$  is present. To

our knowledge, all complexes of Co(II) with dicarboxylate anions were binuclear with a coordination number of 6 for Co(II) [17, 19]. For the Co(II)-tpmc complexes, a combined chelate-bridging coordination of dicarboxylate was proposed. Since there were no single-crystal X-ray structural data for similar Co(II) complexes, on the basis of spectral characteristics, for **1** a coordination number of 6 is proposed.

Macrocyclic ligand tpmc is very flexible, adapting itself to different radii of the metal ions, as well as to the bridging ligand, thus causing diverse geometries and properties of the complexes. The greater tendency of Co(II), than Cu(II) under similar conditions, to form six-coordinate species could explain some of these differences. Complex **1** is cationic tetranuclear with formula  $[\text{Co}_4(\text{pma})(\text{tpmc})_2](\text{ClO}_4)_4 \cdot 6\text{H}_2\text{O}$  in which the six coordination sites around the Co(II) are occupied by four N atoms from the macrocyclic ligand tpmc (two cyclam and two pyridyl N, figure 1f) and two oxygen atoms from pyromellitate anion. Complex **2** is neutral binuclear with formula  $[\text{Cu}_2(\text{pma})\text{tpmc}] \cdot 8\text{H}_2\text{O}$ . Each Cu(II) ion is five-coordinate with four N atoms (two from the cyclam and two from adjacent pyridyl groups, figure 1f) and O atom from the pyromellitate anion.

**3.2.2. Magnetic properties.** The magnetic moments of Co and Cu complexes are 4.96 BM/Co and 2.07 BM/Cu, respectively. Magnetic moment value at room temperature for **1** of 4.96 BM/Co is higher than spin-only value of 3.87 BM/Co and in accord with the values experimentally found for high-spin Co(II) complexes with three unpaired electrons 4.7-5.2 BM/Co [18, 19, 44]. Magnetic moment of 2.07 BM per Cu(II) at room temperature was in the range 1.75-2.20 BM/Cu found previously for similar five-coordinate Cu(II) complexes with one unpaired electron [18, 35, 39, 44].

EPR spectrum of powdered complex **2** is shown in figure 2. The EPR spectrum of **2** is typical for copper(II) in axially elongated tetragonal surrounding. This refers to the presence of a structure with a value of the effective spin  $S = 1/2$  and  $d_{x^2-y^2}$  magnetic orbital occupied by one unpaired electron [45, 46]. For neutral complex  $[\text{Cu}_2(\text{pma})\text{tpmc}] \cdot 8\text{H}_2\text{O}$  (**2**), square planar arrangement four N atoms from tpmc in  $[\text{Cu}_2\text{tpmc}]^{4+}$  with the O atoms from  $\text{OCO}^-$  occupying definite positions along the tetragonal axis can be proposed [47]. The weak coordination of the O atoms imposes an elongated structure. It is intermediate between the free and the fully coordinated species [47]. The shape of spectrum is temperature independent.

Similar EPR spectrum to **2** has been observed for related complex  $[\text{Cu}_2(\text{Hpht})\text{tpmc}](\text{ClO}_4)_3 \cdot 3\text{H}_2\text{O}$  [38]. It suggests a possible square pyramidal geometry around the Cu(II) ion in **2**, which would be in accord with the proposed  $\text{CuN}_4\text{O}$  chromophore. EPR spectrum of **2** is similar to the EPR spectra of some Cu(II) complexes with  $\mu$ -coordinated carboxylate anions, in which the X-ray analyses confirmed longer axial Cu(II)-O bond in relation to the others in the square-pyramidal plane [48].

### **3.3. Thermal analysis**

Complex **2** undergoes a three-step decomposition, where the first endothermic step is the dehydration (figure 3). It starts at room temperature and ends at 130 °C. The weight loss corresponds to the evaporation of 15 molecules  $\text{H}_2\text{O}$  (calculated: 22.3%; found: 23.1%). In the range 130-160 °C, the dehydrated compound is stable. The second stage in the range 160-200 °C is due to loss of the carboxylate groups from pyromellitate ligand (calculated: 14.5%; found: 14.4%) and is accompanied by an exothermic heat effect. The shape of the TG curve and the high exothermic character of the decomposition refer to the burning of the sample with a final product at 400 °C which most probably belongs to CuO (calculated: 13.1%; found: 12.5%).

The previously discussed methods for the characterization of both complexes indicate that the analytical data of both complexes are compatible with the proposed composition.

### **3.4. Electrochemical properties**

CV curves for **2** were recorded in DMSO in the range of potential -1.0 to 1.0 V with scan speed 50, 100 and 200 mV/s (figure 4). No peak current was observed. The absence of peaks at about -0.34 V in the cathodic part from the reduction and around 0.00 V in the anodic part from the oxidation of copper on the electrodes confirmed the electrochemical stability of the studied complex under the given experimental conditions independent on the scan rate.

The electrochemical stability of **2** indicates that the dissolution of the complex in DMSO is not causing the dissociation of the complex. A similar electrochemical behavior was observed in most of the Cu(II)-tpmc complexes with bridged  $\mu$ -coordinated aromatic/aliphatic mono/dicarboxylates [35, 38, 39]. Comparison of the UV-vis and diffusion reflectance spectra of those complexes shows that the dissolution causes a change in conformation of the macrocycle

rather than dissociation of the complex cation. Stability of **2** during the dissolution indicates that the antiproliferative activity is derived from the complex.

### **3.5. Antiproliferative and antibacterial activity**

The cytotoxicity of **1** and **2** was tested with selected human cancer cell lines: cervix adenocarcinoma (HeLa), estrogen-receptor-positive human breast cancer (MCF-7), human myelogenous leukemia (K562) and the human Caucasian Burkitt's lymphoma (Ramos) cells. The cytotoxic properties of target compounds are presented in table 3 and figures 5 and 6. Cell survival was determined by MTS test, after 48-h exposure to compounds (figures 5 and 6). Both compounds have promoted significant decreases in the metabolic activity of the HeLa, MCF-7, K562 and Ramos cell, which occurred in a dose-dependent fashion.

The use of metal complexes in the pharmaceutical industry has recently increased and as a result, novel metal-based complexes have started an interest as potential anticancer agents. Copper, which is an essential trace element in all living organisms, is important in maintaining the function of numerous proteins and enzymes. It has recently been demonstrated that Cu complexes may be used as tumor-specific proteasome inhibitors and apoptosis inducers by targeting the ubiquitin-proteasome pathway (UPP) [49]. The UPP plays an important role in cell growth and apoptosis and has been shown as a novel target for cancer therapy [50]. Also, Cu complexes have shown the potential to lead to apoptosis in PC-3 (human prostate cancer cells) and in MDA-MB-231 (human breast cancer cells) but not in nontumorigenic MCF-10A cells [51]. The cobalt complexes are of more limited medical use compared to copper complexes. Furthermore, cobalt complexes possess *in vivo* insulin-like properties [52], antifungal [53], anti-oxidant activity [54] and antimicrobial activities [55, 56].

There are data indicating that cobalt-containing complexes are very interesting because they can be potent antitumor agents [57-59]. In addition, the antitumor active cobalt(II) complexes depend on the type and position of substituent in the aromatic rings [60].

The hanging drop method represents an attractive alternative for MCTS production because it is mild, can be applied to a wide variety of cell lines, and can produce spheroids of a homogeneous size without the need for sieving or manual selection. The method has applications for basic studies of physiology and metabolism, tumor biology, toxicology, cellular organization,

and development of bioartificial tissue. In figure 7, it can be noticed that complex  $[\text{Co}_4(\text{pma})(\text{tpmc})_2](\text{ClO}_4)_4 \cdot 6\text{H}_2\text{O}$  diminished migration of HeLa cells [32].

Compounds **1** and **2** were tested for antimicrobial activity against three Gram-positive and two Gram-negative bacterial strains, as well as against one strain of yeast *Candida albicans*.

The activity was observed for **1** against *Staphylococcus aureus* (MIC = 25 mM), *Bacillus subtilis* (MIC = 25 mM) and *Klebsiella pneumoniae* (MIC = 25 mM), whereas the strains of *Enterococcus faecalis*, *Escherichia coli* and *Candida albicans* were not susceptible.

Experimental results obtained from the study of antimicrobial activity demonstrate that **1** displays bacteriostatic activity in the concentration of 25 mM (53  $\mu\text{g}/\text{mL}$ ).

Complex **2** did not express activity against tested microbial strains (table 4). The data from the literature suggest that MIC is influenced by the nature of the metal ion as well as by the presence of an anion in the coordination sphere [61]. The data so far show that the antibacterial activity of the complex of tetraazamacrocyclic metal complexes can be interpreted in terms of the theory of chelation. Chelation/coordination reduces the polarity of the metal. The chelation process increases the lipophilic nature of the central metal, favoring the path through the lipid layer of the membrane, which transmits the metal complex to the bacterial membrane more effectively, by blocking the locations of metal binding to microorganisms. This prevents protein synthesis and growth of microorganisms [62-65].

#### 4. Conclusion

In this article we reported the synthesis, spectroscopic, electrochemical and thermal properties of new Co(II) and Cu(II) mixed-ligand complexes with octaazamacrocyclic ligand tpmc and polycarboxylate-pyromellitic acid. The Co(II) complex is tetranuclear in which the six coordination sites around the Co(II) are occupied by four nitrogen atoms from the macrocyclic ligand tpmc (two cyclam and two pyridyl N) and two oxygen atoms from piromellitate anion. All oxygen atoms are engaged in coordination and probably one carboxylate group connecting the Co(II) ions as bidentate. The complex Cu(II) is neutral binuclear with five-coordinate Cu(II) ions, four N atoms (two cyclam and two pyridyl N) and O atom from the pyromellitate anion. One carboxylate group is bridging two Cu(II) ions inside tpmc unit. Cobalt(II) complex showed antibacterial activity against bacteria and cytotoxic activity against four human cancer cell lines.



These results indicate that the type of metal ion in the complex can affect their composition, properties, anti-proliferative and antimicrobial activity.

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## Figure captions

Figure 1. Selected coordination modes of pma ligand (a-e) and *exo* coordination of M=Co(II), Cu(II) in binuclear tpmc unit where tpmc adopted *boat* conformation (f).

Figure 2. EPR spectrum at room temperature for **2** (similar spectrum is observed at LN<sub>2</sub> temperature).

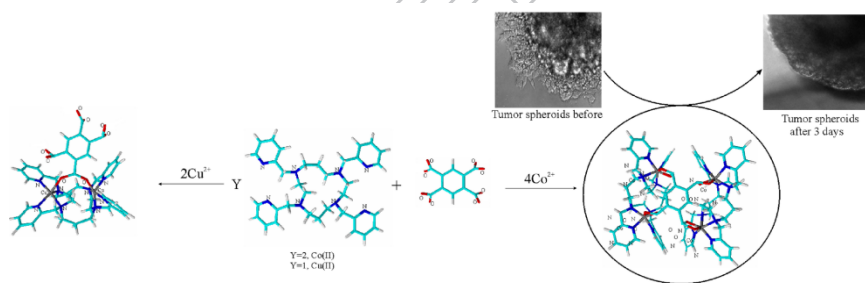
Figure 3. Simultaneous TG-DTA of **2**.

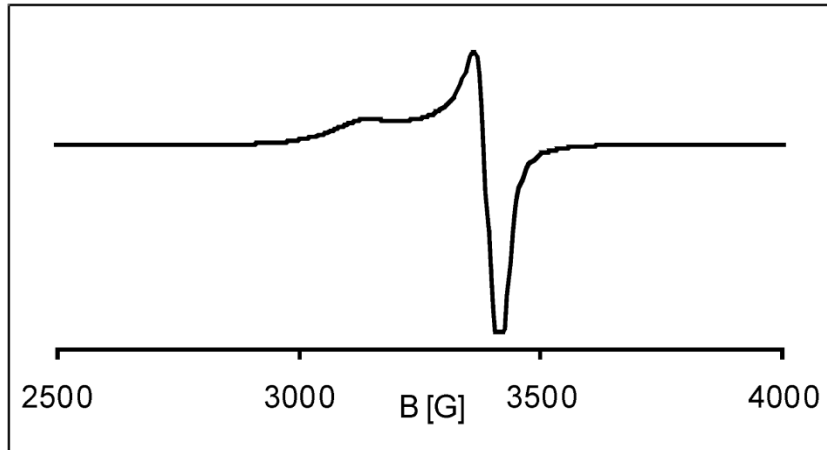
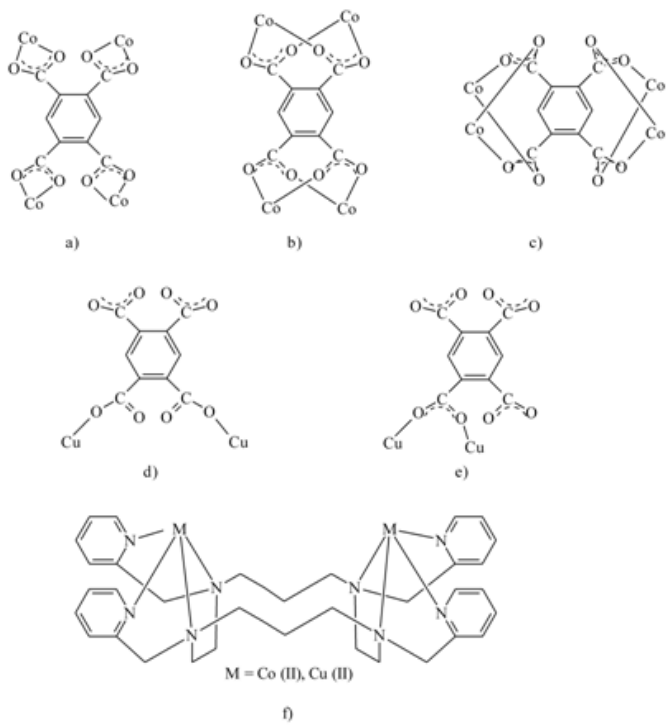
Figure 4. Cyclic voltammogram of **2** in DMSO (with scan speed of 100 mV/s).

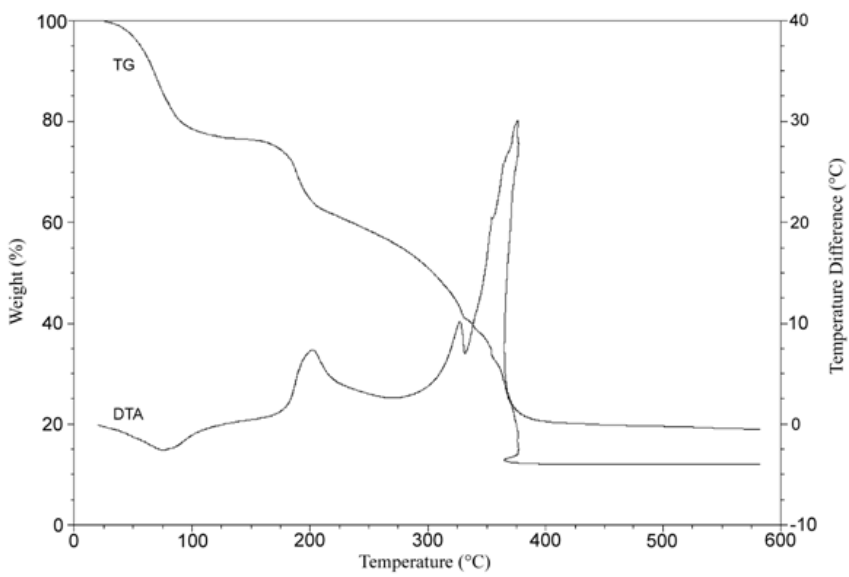
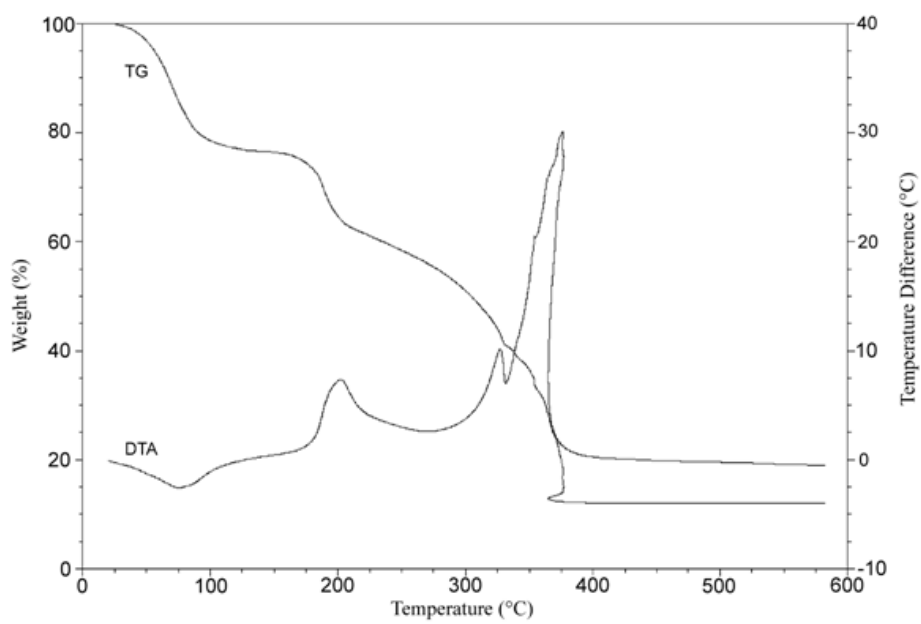
Figure 5. Cells were incubated with various concentrations of **1** and **2**. After 48 h of incubation, MTS reagent was added. Obtained data show the optical density of cell cultures. Each experiment was repeated three times and the results were presented as the mean value of  $\pm$  SD.

Figure 6. Dose-response curves for the cytotoxicity of **1** and **2** toward HeLa, MCF-7, K562 and Ramos cells. Percentage of viable cells was plotted against various concentrations of investigated compounds.

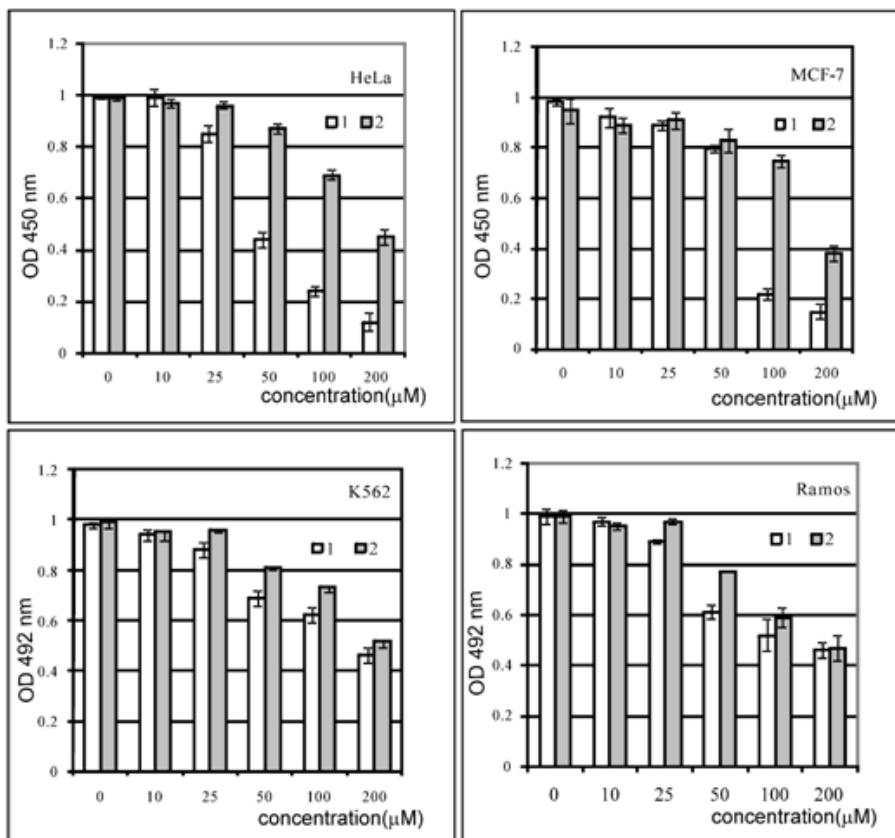
Figure 7. [Co<sub>4</sub>(pma)(tpmc)<sub>2</sub>](ClO<sub>4</sub>)<sub>4</sub>·6H<sub>2</sub>O (**1**) to impair invasion of HeLa cells in a 3D *in vitro* model of tumor invasion. (A) MCTS were implanted in Matrigel and covered with growth medium. (B) MCTS were implanted in Matrigel and covered with growth medium with **1**. Representative images of HeLa MCTS were obtained at day three after treatment with **1**.





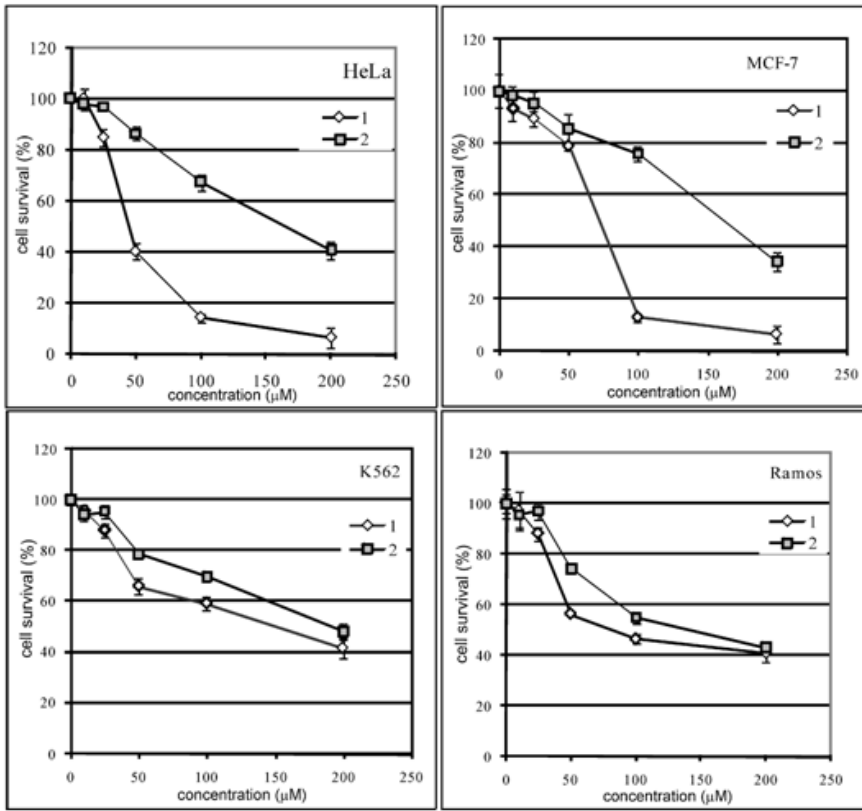






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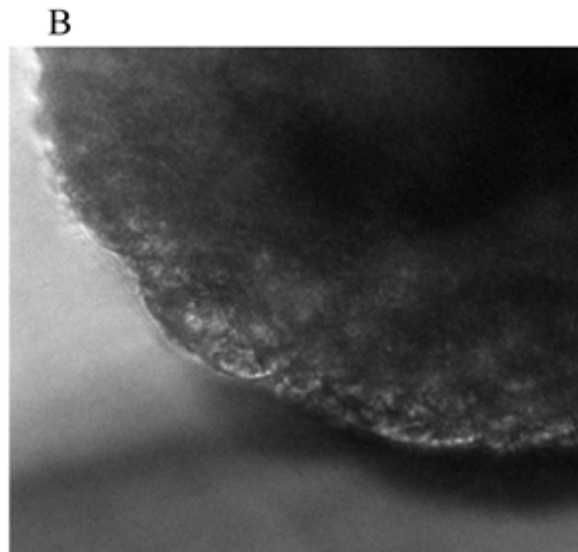
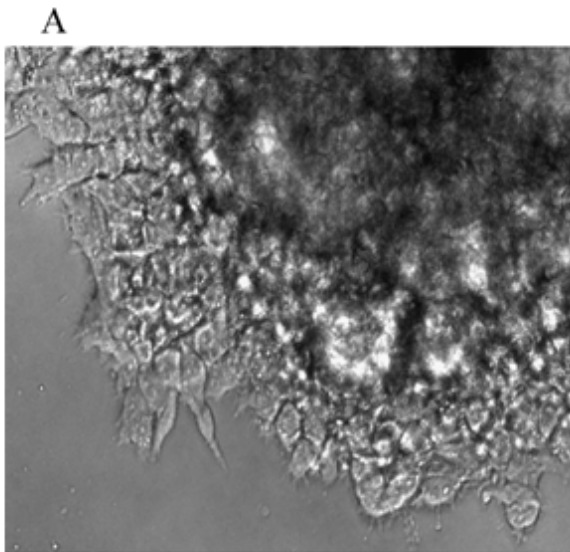


Table 1. Electronic and FTIR spectral data, molar electrical conductivity ( $\Lambda_M$ ) and magnetic moments ( $\mu_{\text{eff}}$ ) of **1** and **2** along with some similar compound from the literature [17, 18, 35, 38].

Complexes	$\lambda_{\text{max}}$ ( $\epsilon$ ) [nm ( $M^{-1}$ $\text{cm}^{-1}$ )]	$\Lambda_M$ [ $S$ $\text{cm}^2 \text{mol}^{-1}$ ] <sup>c</sup> (electrolyt e type)	$\mu_{\text{eff}}$ [BM/M(II )]	FTIR		
				$\nu_{\text{asym}}$ (OC O)	$\nu_{\text{sym}}$ (OC O)	$\Delta\nu$
	516 (75),					
[Co <sub>2</sub> (C <sub>6</sub> H <sub>5</sub> COO) <sub>2</sub> tpmc](ClO <sub>4</sub> ) <sub>2</sub> ·3H <sub>2</sub> O *	sh 479 (66) <sup>a</sup> - b	143 (1:2)	4.81	1580	1413	16 7
	523 (86),					
[Co <sub>2</sub> (pht)tpmc](ClO <sub>4</sub> ) <sub>2</sub> ·2H <sub>2</sub> O <sup>*</sup>	sh 487 (74) <sup>a</sup> - b	172 (1:2)	4.74	1544	1423	12 1
	508 (94),					
[Co <sub>2</sub> (ipht)tpmc](ClO <sub>4</sub> ) <sub>2</sub> ·4H <sub>2</sub> O <sup>*</sup>	sh 485 (89) <sup>a</sup> - b	116 (1:2)	5.01	1538	1425	11 3
	527 (264),					
[Co <sub>4</sub> (pma)(tpmc) <sub>2</sub> ](ClO <sub>4</sub> ) <sub>4</sub> ·6H <sub>2</sub> O ( <b>1</b> )	sh 475 (219) <sup>a</sup> - b	636 (1:4)	4.96	1546	1400	14 6
	527 (264),					
[Cu <sub>2</sub> (C <sub>6</sub> H <sub>5</sub> COO)tpmc](ClO <sub>4</sub> ) <sub>3</sub> ·CH <sub>3</sub> O H <sup>*</sup>	671 (365) <sup>a</sup>	310 (between	1.88	1555	1382	17 3

	, 621 <sup>b</sup>	1:2 i 1:3)				
[Cu <sub>2</sub> (Hpht)tpmc](ClO <sub>4</sub> ) <sub>3</sub> ·3H <sub>2</sub> O*	658 (335) <sup>a</sup> , 643 <sup>b</sup>	435 (≈1:3)	1.74	1713, 1576, 1565	1391, 1357, 1298	19 6 6
[Cu <sub>4</sub> (ipht)(tpmc) <sub>2</sub> ](ClO <sub>4</sub> ) <sub>6</sub> ·NaClO <sub>4</sub> ·2H <sub>2</sub> O*	676 (652) <sup>a</sup> , 654 <sup>b</sup>	800 (1:6)	1.76	1573, 1560	1387	18 0
[Cu <sub>2</sub> (pma)tpmc]·8H <sub>2</sub> O (2)	<sup>-a</sup> , 654 <sup>b</sup>	-	2.07	1572	1381	19 1

<sup>a, c</sup> in CH<sub>3</sub>CN solution, <sup>b</sup> reflectance spectra; \* data from literature [17, 18, 35, 38], H<sub>2</sub>pht = phthalic acid, H<sub>2</sub>ipht = isophthalic acid, H<sub>4</sub>pma = piromellitic acid

Table 2. The fragmentation ions of **1** in LC-MS spectrum (positive ion mode).

Fragmentation ions	<i>m/z</i> <sup>a</sup>	Relative intensity (%)
[Co <sub>2</sub> (C <sub>6</sub> H <sub>2</sub> (COOH) <sub>4</sub> )(C <sub>34</sub> H <sub>44</sub> N <sub>8</sub> )](ClO <sub>4</sub> ) <sub>4</sub> <sup>4+</sup>	333.2 (333.5)	16.27
[Co <sub>2</sub> (C <sub>6</sub> H <sub>2</sub> (COO) <sub>2</sub> (COOH) <sub>2</sub> )(C <sub>34</sub> H <sub>44</sub> N <sub>8</sub> )](ClO <sub>4</sub> ) <sub>4</sub> <sup>2+</sup>	667.9 (667.3)	7.83
[Co <sub>2</sub> (C <sub>6</sub> H <sub>2</sub> (COOH) <sub>4</sub> )(C <sub>34</sub> H <sub>44</sub> N <sub>8</sub> )](ClO <sub>4</sub> ) <sub>2</sub> <sup>6+</sup>	189.1 (189.0)	3.01
[Co <sub>2</sub> (C <sub>6</sub> H <sub>2</sub> (COO)(COOH) <sub>3</sub> )(C <sub>34</sub> H <sub>44</sub> N <sub>8</sub> )](ClO <sub>4</sub> ) <sub>2</sub> <sup>5+</sup>	227.0 (226.8)	7.23
[Co <sub>2</sub> (C <sub>6</sub> H <sub>2</sub> (COO) <sub>2</sub> (COOH) <sub>2</sub> )(C <sub>34</sub> H <sub>44</sub> N <sub>8</sub> )](ClO <sub>4</sub> ) <sub>2</sub> <sup>4+</sup>	284.2 (283.3)	39.16
[Co <sub>2</sub> (C <sub>6</sub> H <sub>2</sub> (COO) <sub>4</sub> )(C <sub>34</sub> H <sub>44</sub> N <sub>8</sub> )](ClO <sub>4</sub> ) <sub>2</sub> <sup>2+</sup>	565.7 (565.5)	3.01
C <sub>34</sub> H <sub>44</sub> N <sub>8</sub> <sup>+</sup>	565.4 (564.8)	7.83

<sup>a</sup> Calculated values are given in parentheses

Table 3. Concentrations of **1**, **2** and ligands that induced 50% decrease in cell survival (IC<sub>50</sub>).

Compound	IC <sub>50</sub> ±SD (μM)			
	HeLa	MCF-7	K 562	Ramos
[Co <sub>4</sub> (pma)(tpmc) <sub>2</sub> ](ClO <sub>4</sub> ) <sub>4</sub> ·6H <sub>2</sub> O	44.66±2.39	71.79±2.94	152.40±2.28	81.66±0.79
[Cu <sub>2</sub> (pma)tpmc]·8H <sub>2</sub> O	165.19±1.37	163.20±6.90	192.05±2.09	140.88±3.51
tpmc	>200	>200	>200	>200
pma	>200	>200	>200	>200
cisplatin	10.9±3.5	4.9±1.7	7.9±2.5	5.9±3.8

All data are results from three independent experiments, each carried out in triplicate. The results are presented as mean IC<sub>50</sub> ± standard deviation, μM.

Table 4. Antimicrobial activity of **1** and **2** and expressed as MIC (mM and μg/mL), determined by the broth microdilution methods.

Microbial strain	Co <sub>4</sub> (pma)(tpmc) <sub>2</sub> ](ClO <sub>4</sub> ) <sub>4</sub> ·6H <sub>2</sub> O mM (μg/mL)	[Cu <sub>2</sub> (pma)tpmc]·8H <sub>2</sub> O mM (μg/mL)	Ampicillin μg/mL	Nystatin μg/mL
<i>S. aureus</i>	25 (53)	> 100 (>119)	1	nt
<i>B. subtilis</i>	25 (53)	> 100 (>119)	8	nt
<i>E. faecalis</i>	> 100 (>212)	> 100 (>119)	8	nt
<i>E. coli</i>	> 100 (>212)	> 100 (>119)	2	nt
<i>K. pneumoniae</i>	25 (53)	> 100 (>119)	2	nt
<i>C. albicans</i>	> 100 (>212)	> 100 (>119)	nt	4

nt = not tested