Synthesis and characterization of a 13-member macrocycle functionalized by tyramine arms: Complexation with Cu\(^{2+}\) and antioxidant capacity

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A new macrocycle bearing arms through amide linkages, 2,2’-(2,9-dioxo-1,4,7,10-tetraazacyclododecane-4,7-diyl)bis[N-(4-hydroxyphenethyl)acetamide] abbreviated as L13Tyra, has been synthesized with amide-coupling agents in a microwave reactor. This macrocycle involves two potential metal-coordinating and bioactive sites, i.e., the tetraaza-macro cyclic ring (with a low basicity of the amino nitrogen) and the pendant phenol arms (with a small pK\(_a\), 8.6). The Cu\(^{2+}\) complex has different compositions in solid and in solution. An X-ray crystal study shows that a mononuclear complex [Cu(L13Tyra – 2H)]\(^0\) is formed with a square coordination of two deprotonated amide nitrogen and two amino nitrogen atoms of the macrocyclic ring; a carbonyl oxygen atom from a pendant arm occupies an axial site to construct a square pyramid. UV–Vis spectrometric titrations in aqueous solutions indicate the formation of a binuclear complex [Cu\(_2\)(L13Tyra – 4H)(H\(_2\)O)]\(^x\)\(^+\) in which phenolate oxygen atoms of the tyramine arms coordinate a Cu\(^{2+}\) ion in addition to the coordination of the macrocyclic chelate; such a binuclear structure is maintained only in solution. The uncoordinated ligand has a high antioxidant capacity with a TEAC (Trolox equivalent antioxidant capacity) assay comparable to that of ascorbic acid, thanks to the phenolic OH of the tyramine arms. Copper(II) ion works as an inhibitor against the activity; the TEAC assay of the binuclear complex is as small as one-twentieth that of the uncoordinated ligand. Antiproliferative and cytotoxic assays with normal and cancer cell lines show no toxicity for both the ligand and its Cu\(^{2+}\) complex.

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1. Introduction

The design and synthesis of polyazamacrocycles and their metal complexes have received considerable attention because of their relationship to biomimetic and catalytic systems [1,2] and their widespread applications in biology, medicine [3–5], pharmaceutics [6,7], supramolecular chemistry [8], analytical chemistry [6] and new functional materials [8]. Macro cyclic ligands have a great advantage over acyclic ligands as they offer the benefit of highly stable complex formation and, through functionalization, the opportunity to fine tune in the coordination environment (or ‘macro cyclic effect’) [4,6]. Macrocycles with functional pendant arms are important and powerful chelating agents, which have attracted interest because of their unique coordination and structural properties [2]. The complexation properties of polyazamacrocycles are governed mainly by the macrocyclic ring size [1,8]. Pendant arms allow to introduce new properties into macrocyclic ligands depending on the nature of their functional groups [3,9]. For example, N-functionalization of polyazamacrocycles can enhance their metal ion selectivity and cause their metal complexes to be more thermodynamically and kinetically stable [1,3,9,10].

As macrocycles with a new type of pendant arms, we have synthesized tetraaza-macrocycles bound with phenyl arms through...
amid linkage (L13Fea in Scheme 1), and found that the pendant arms have a great influence on the complexation properties [11]. The advantages of amid linkages include the high stability and the functionality of carbonyl oxygen and acidic NH hydrogen. If additional metal-coordinating and/or bioactive sites are introduced into pendant arms, the resulting macrocycles are expected to retain useful properties and functions [2,3]. In line with this viewpoint, the present work reports that functional tyramine arms can be bound to a 13-membered macrocycle through amid linkages, as shown in Scheme 2: the new ligand, abbreviated as L13Tyra, is \(2,2\)\(-(2,9-dioxo-1,4,7,10-tetraazacyclotridecane-4,7,10-triyl)bis(N-(4-hydroxyphenethyl)acetamide). The phenol OH of tyramine arms is expected to work as an additional metal-coordinating and bioactive site. The complexes of L13Tyra with copper(II) and/or bioactive site. The complexes of L13Tyra with copper(II) have been studied by crystal X-ray diffraction, UV–Vis spectrometry, EPR and IR spectroscopies. The antioxidant capacity of the uncoordinated macrocycle has been evaluated by complexometry with EDTA. The advantages of amid linkages include the high stability and the functionality of carbonyl oxygen and acidic NH hydrogen. If additional metal-coordinating and/or bioactive sites are introduced into pendant arms, the resulting macrocycles are expected to retain useful properties and functions [2,3].

2. Experimental

2.1. Chemicals and materials

\(N,N\)-Dimethylformamide provided from Fisher Scientific was dried with molecular sieves for 24 h before use. All other reagents and solvents were Aldrich products, and they were used without further purification. Copper(II) chloride solutions were standardized by complexometry with EDTA.

Macrocyle L13 (Scheme 2) was obtained by the method reported previously, and its purity was checked by melting point, IR and \(^1\)H NMR as compared with those reported previously [12,13].

2.2. Physical and spectroscopic measurements

Melting points were determined with a Mel-Temp II apparatus. IR spectra were recorded on KBr pellets by a Perkin-Elmer FT-IR Spectrometer Model Frontier. UV–Vis spectra were measured on a diode array spectrophotometer Agilent model 8453. EPR measurements were made at room temperature or 77 K in quartz tubes with a Jeol JES-TE300 spectrometer equipped with a cylindrical cavity (TE011 mode) operating at X band frequency (9.4 GHz) at 100 kHz field modulation. The mass spectra were obtained for methanol solutions at the University of Arizona Mass Spectroscopy Facility (Tucson, AZ, USA). The elemental analyses were performed by ALS Environmental Service (Tucson, AZ, USA).

The NMR spectra (400 MHz for \(^1\)H, 100 MHz for \(^13\)C) were recorded on a Bruker Avance 400 spectrometer in the indicated solvents. For the studies of protonation, the \(^1\)H NMR spectra were obtained in \(D_2O\) solutions at a probe temperature of approximately 23 °C. The internal reference was sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). A minimum quantity of a dilute KOH-d or HCl-d solution was used for adjusting the \(pD\) of the sample solutions. The pH value of each sample solution was measured with a long-stem combination electrode inserted into the NMR tube after NMR experiments. The electrode was calibrated with the standard aqueous buffers, and the measured pH values were converted to the \(pD\) values by the relation \(pD = pH_{meas} + 0.45\) [14].

2.3. Synthesis of macrocycle L13Tyra

The amidation of the carboxylic pendant arms of L13 was carried out by means of a CEM microwave reactor model Discover. A 10 mL reactor vessel was loaded with 0.3 mmol (0.10 g) of L13, 0.66 mmol (0.09 g) of 1-hydroxy-7,10-tetraazacyclotridecane-4,7,10-triyl)bis(N-(4-hydroxyphenethyl)acetamide). The phenol OH of tyramine arms is expected to work as an additional metal-coordinating and bioactive site. The complexes of L13Tyra with copper(II) have been studied by crystal X-ray diffraction, UV–Vis spectrometry, EPR and IR spectroscopies. The antioxidant capacity of the uncoordinated macrocycle has been evaluated to be comparable to that of ascorbic acid; Cu\(^{2+}\) ion is a strong inhibitor against the antioxidant function.

\[ \text{L13} + \text{HOBt} + \text{DIC} \xrightarrow{\text{DMF, MW}} \text{L13Fea} \]

The Cu²⁺ complex was prepared by a reaction between L13Tyra ligand and copper(II) carbonate as follows [15]. Solid copper(II) carbonate in excess (0.38 mmol, 0.048 g) was added to the ligand (0.17 mmol, 0.01 g) in water (10 mL). After the mixture was stirred overnight at a temperature of 40°C, excess carbonate was removed by filtration using Acrodisc/C210 membrane (Sigma–Aldrich). When ethanol was added to the filtrate, the metal complex was obtained as a purple solid. The product was recrystallized as a microcrystalline solid from an aqueous solution by adding acetone. Recrystallization from hot water yielded rhombohedral crystals. Yield: 90%. IR (KBr): ν = 3251 cm⁻¹, ν–OH = 3098 cm⁻¹, ν–C=H 2921 cm⁻¹, ν–C=O 1661 cm⁻¹, ν–C=O 1615 cm⁻¹, ν–N–H 1442 cm⁻¹, νp–subt 825 cm⁻¹ (see A1, Supplementary material).

2.5. UV–Vis studies of Cu²⁺ complex formation

For a study of complexation at a constant pH, the UV–Vis spectrum of the ligand was monitored by adding a Cu²⁺ solution into an L13Tyra solution. A stock solution of the ligand at a concentration 10⁻⁴ M (M = mol dm⁻³) was prepared by dissolving a suitable amount of the ligand in 0.1 M tris(hydroxymethyl)aminomethane (known as Trizma) buffer at pH 7.2. A stock solution of CuCl₂ was prepared so that the molar concentration was about 30 times that of the stock solution of the ligand. A quartz cuvette of the spectrometer was filled with 3 mL of the ligand stock solution, and then 10 μL aliquots of the metal stock solution were added successively with a calibrated micropipette so that the ratios of the total concentrations [Cu²⁺][L]t were in a desired range. The spectra were recorded. At each measurement, the absorption spectrum was confirmed to be unchanged with time.

For a study by complexation by a pH-variation method, a stock solution of the ligand at a concentration 10⁻⁴ M (M = mol dm⁻³) was prepared in 5 mL of HPLC-grade water) with potassium persulfate (58.47; H, 7.28; N, 14.11. Found: C, 58.53; H, 7.38; N, 14.14%.

2.6. X-ray crystal analysis of CuL13Tyra complex

Crystals of CuL13Tyra complex suitable for single crystal X-ray diffraction (SXD) were obtained by the method described in Section 2.4. Purple-blue crystals with appropriate dimensions 0.121 mm × 0.143 mm × 0.174 mm were selected for the X-ray diffraction experiment at T = 120(2) K. Diffraction data were collected on a Bruker SMART DUO diffractometer system equipped with a curved graphite monochromator and a Mo Kα sealed tube (λ = 0.71073 Å). A total of 2060 frames were collected. For data collection, Bruker APEX3 Software was used [16]. The frames were integrated with the Bruker SAINT Software package [17] using a narrow-frame algorithm. The integration of the data using a monoclinic unit cell yielded a total of 54,784 reflections to a maximum θ angle of 25.71° (0.82 Å resolution); 6519 reflections were independent (average redundancy 8.404, completeness = 99.9%, Rint = 5.72%, Rfree = 3.29%), and 5233 (80.27%) were greater than 2σ(I). The final cell constants of a = 15.7906(14) Å, b = 12.7038(10) Å, c = 17.6394(14) Å, β = 104.8070(10)°, volume = 3421.0(5) Å³, are based upon the refinement of the XYZ-centroids of 9952 reflections above 2σ(I) with 4.454° < 2θ < 49.66°. Data were corrected for absorption effects by the Multi-Scan method (SADABS) [18]. The ratio of minimum to maximum apparent transmission was 0.937. The calculated minimum and maximum transmission coefficients (based on crystal size) are 0.8870 and 0.9200.

The structure was solved and refined with the Bruker SHELXTL Software Package [19] on the space group P2₁/c with Z = 4 for the formula unit, CₓHᵧCuₓNₓO₁₁. Structure refinement was carried out using SHELXL2014 [20]. The final anisotropic full-matrix least-squares refinement on F² with 466 variables converged at Rs = 3.46%, for the observed data and wR₂ = 7.79% for all data. The goodness-of-fit was 1.056. The largest peak in the final difference electron density synthesis was 0.376 e Å⁻³ and the largest hole was −0.496 e Å⁻³ with an RMS deviation of 0.056 e Å⁻³. On the basis of the final model, the calculated density was 1.398 g cm⁻³ and F(000) = 1524 e. OLEX2 [21], SHELXE [22], ORTEP-3 for Windows [23] and Mercury 3.6 [24] were used for molecular graphics. Material for publication was prepared using apo [16].

Table 1 displays relevant crystal data, data collection and final structure refinement parameters for CuL13Tyra complex. CH₂ and Cromatic–H hydrogen atoms are placed at calculated positions and refined with a riding model, N–H and Hwater hydrogen atoms have been located from interactive examination of difference Fourier maps following least squares refinement with Uiso(Hwater) = 1.2, Uiso(HOH) = 1.5 and Uiso(HH2O) = 1.5, respectively.

2.7. Evaluation of antioxidant capacity

Antioxidant capacity was evaluated by Trolox equivalent antioxidant capacity (known as TEAC) assay, which is based on the capacity of a sample to inhibit the radical ABTS⁺ of 2,2′-azino-bis(3-ethylbenzotriazoline-6-sulfonic) acid (ABTS) as compared with the antioxidant reference standard Trolox® [25]. ABTS⁺ radical was generated by chemical reaction of ABTS (19.2 mg dissolved in 5 mL of HPLC-grade water) with potassium persulfate (g cm⁻³) 4.0

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<th>Crystallographic data for CuL13Tyra complex.</th>
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(88 μL of a 0.0378 g mL⁻¹ solution); the reaction diagram is shown in S1 in Supplementary materials [26]. The reaction mixture was incubated in the dark at room temperature for 12–16 h. To 1 mL of the resulting solution was added 88 mL of pure ethanol. The radical concentration was adjusted so that the absorbance was 0.7 ± 0.02 at 734 nm.

The reaction was initiated by mixing 245 μL of the ABTS⁺ solution and 5 μL of a 1 × 10⁻³ M sample solution under study and ascorbic acid solution as control. Absorbance was monitored at 734 nm at reaction times of 1 and 6 min. The percentage of inhibition was calculated and the results were expressed as μmol of TE/100 g (TE: Trolox Equivalents). The absorbance was read with a microplate reader device (BMG Labtech Inc., Model Omega) at a wavelength of 734 nm. Each sample was analyzed in triplicate.

Another evaluation of antioxidant capacity was performed as DPPH assay; the 1H NMR shifts of L13Tyra were determined before and after a treatment with the stable free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) in DMSO-d₆. One-half mL of a 0.005 M L13Tyra solution was placed in an NMR tube. After the 1H NMR spectrum of the ligand was recorded, an equimolar amount of DPPH was added into the NMR tube. The mixture was allowed to stand for 3 min at room temperature until the purple color of DPPH faded, and it was subjected to 1H NMR analysis. The spectra were analyzed with MESTREC software (MestReNova v9.0.1-13254, Mestrelab Research S.L., Spain).

2.8. Antiproliferative activity assay

ARPE-19 (human retinal pigment epithelium, normal cell line) and HeLa (human cervix carcinoma) cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS). The cells were incubated in an atmosphere of 5% CO₂ at 37 °C. The cytotoxic effect of L13Tyra and L13tyra-Cu against ARPE-19 and HeLa cell lines was evaluated by a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay [27]. MTT is a colorimetric assay based on the fact that mitochondrial oxidoreductase enzymes are capable of reducing the blue tetrazolium dye MTT (yellow color) to its purple insoluble formazan. The cellular oxidoreductase enzymes may, under defined conditions, reflect the normal function of mitochondria and the cell viability. ARPE-19 and HeLa cells were seeded (1 × 10⁴ cells/well) in a 96-well plate with DMEM medium. Then, the cells were treated with various concentration of L13Tyra or L13tyra-Cu dissolved in deionized water (3.125, 6.25, 12.5 and 25 μg/mL), and incubated for 48 h. After incubation 10 μL of MTT solution (5 mg/mL; Sigma, USA) was added to each well, followed by 4 h incubation at 37 °C. Formazan crystals were dissolved with acidic isopropanol, and the plates were read on an ELISA plate reader using a test wavelength of 570 nm. Plates were normally read within 10 min after adding isopropanol. The antiproliferative activity of the test compound was determined as percent cell viability. The experiment was performed in triplicate and the medium without ligand served as control.

3. Results and discussion

3.1. Synthesis of L13Tyra

The reaction shown in Scheme 2, i.e., the conversion of carboxylate into amide group, was performed through active ester by an in situ method using coupling agents, N,N-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole hydrate (HOBt). The reaction was standardized by microwave heating. This methodology allows the formation of the amide group in the pendant arms, while the OH group of tyramine is protected and the macrocyclic ring remains unchanged, affording the new amide derivative L13Tyra in Scheme 2. The amide linkages were stable during any experiment in the present work.

The formation of L13Tyra has been confirmed by IR, ¹H NMR and mass spectroscopies, as described in experimental section. The IR spectra, for example, give evidence for the presence of phenol group: a strong 3315 cm⁻¹ band assigned to O–H stretching, a C–O vibration band at 1107 cm⁻¹, and an out-of-plane O–H bending vibration (823 cm⁻¹) of p-substituted phenyl ring. The disappearance of carboxylic acid bands also supports the occurrence of the amidation (see A2 in Supplementary materials).

3.2. Species formed in aqueous medium

The pD dependences of the ¹H NMR chemical shifts δ observed for L13Tyra are shown in Fig. 1. Aromatic protons labeled i and b as shown in Scheme 2 undergo upfield shift, or the δ values decrease, with increasing pD in the range of 9.5–12.5, indicating that acid dissociation occurs from the phenol O–H to yield (L13Tyra – H⁺)⁺ (hereafter, this species is denoted by the short formula L13Tyra⁺). The signals of aliphatic protons a, b and c shift downfield with a decrease in pD from 5.5 to 2, concluding that the amino nitrogen in the macrocyclic ring is protonated to form L13TryaH⁺ species at the low pD values. The added proton is populated equally on the two nitrogen atoms in the >NCH₂CH₂N< unit because every CH₂ proton adjacent to the nitrogen atoms exhibits a single peak. These protonation species are shown in Fig. 1. The shift δ of proton j upon protonation is given by the following function of pD:

δj (pD) = δj0 + δj1 · 10⁻pD + δj2 · 10⁻2pD/1 + β1 · 10⁻pD + β2 · 10⁻2pD

Here, δj0 is the δ of proton j in the species L13Tyra⁺ formed at the first protonation step that occurs simultaneously at two chemically equivalent phenolate oxygen atoms, δj1 is the δ of L13TyraH⁺ yielded at the second protonation step on amino nitrogen, and β1 and β2 are the first and second overall protonation constants in a D₂O medium, respectively. These constants are given by stepwise constants as β1 = KDO and β2 = KDP · KON, where KDO is the protonation constant of the phenolate oxygen (i.e., the inverse of the acid dissociation constant of O–H) and KON is the protonation constant of the amino nitrogen. The shifts of all proton signals are well reproduced by Eq. (1) with log KDO = 10.99 and log KON = 3.71. This log KON value is close to 3.49 of L13Fea whose molecular structure is shown in Scheme 1 [11]. Both values are much smaller than 6.41 reported for the parent macrocycle L13 [28]. The extremely low basicity of the amino nitrogen in L13Tyra is a result of the replacement of CH₂CO₂⁻ arms with electron-withdrawing CH₂CONH⁻. More effectively, the amide hydrogen of the arm constructs a hydrogen-rich environment, together with the amide hydrogen of the macrocyclic ring, around the amino nitrogen so that it works as a barrier against protonation to decrease log KON.

The effect of pH on L13Tyra was also monitored by UV–Vis spectroscopy (Fig. 2). At pH below 6.5, two absorption bands of tyramine ring are observed at 225 and 276 nm, and sharply intensify with increasing pH. This bathochromic shift is consistent with deprotonation of the tyramine moieties to form the corresponding phenolate. Observation of isosbestic points at 265 and 2S5 nm indicates the presence of two species in equilibrium. The inset of
the dissociation of NH proton has a small effect on the electronic state of the aromatic system, resulting in the small changes in absorbance. This acid dissociation process was not detected by \textsuperscript{1}H NMR; probably it occurs out of pD range studied in D\textsubscript{2}O.

3.3. Crystal structure of CuL\textsubscript{13}Tyra complex

Copper(II) complex of L\textsubscript{13}Tyra crystallizes in the monoclinic system with the P\textsubscript{2}\textsubscript{1}/c space group. Relevant crystallographic information and final refinement parameters are displayed in Table 1. Selected bond distances, angles and torsion angles are given in Tables S.1–S.3 (see ESI). The asymmetric unit is comprised by one molecule of the macrocycle L\textsubscript{13}Tyra with one copper atom and five water molecules. Fig. 3 displays the asymmetric unit.

The compound is formulated by [Cu(L\textsubscript{13}Tyra – 2\text{H})]\textsubscript{5}H\textsubscript{2}O in which two hydrogens are dissociated from the amide groups of the macrocyclic ring. The main structural feature is that the tetraaza-macrocycle coordinates through its nitrogen atoms with Cu(II) forming an equatorial distorted-square base. The metal atom is located at a height of 0.34(1) Å above the main plane formed by the nitrogen atoms. A carbonyl oxygen atom from one of the tyramine arms is also ligated to Cu(II) in an axial coordination site, leading to a distorted square-pyramidal geometry. The Cu–N distances range between 1.9170 (17) and 2.0522 (17) Å, whereas the Cu1–O5 distance is 2.3778 (15) Å.

The bond lengths of Cu1–N1 and Cu1–N2 are slightly shorter than Cu1–N3 and Cu1–N4 (see Table S3 ESI). This asymmetric bonding indicates an elongated square ellipsoid coordination. The degree of tetragonal distortion is 0.93 in the CuL\textsubscript{13}Tyra complex, suggesting a limited tetragonal-octahedral geometry [35]. The same type of square planar coordination involving deprotonated amide nitrogen has been reported for the X-ray structures of Cu\textsuperscript{2+} complexes with amide-based macrocycles [36,37]. Deprotonated amide nitrogen can form a strong coordinate bond because the bond length, 1.13 Å, is much smaller than Cu\textsuperscript{2+}–N\textsubscript{0} distance, causes the distortion of the tetraaza-coordination geometry.

Fig. 2. Studies of pD effect on the UV–Vis spectrum of \textsuperscript{1}H NMR. The inset presents the changes in absorbance at 240, 276 and 295 nm.
dimethyl-2,6,13,17-tetra-azatricyclo-(16.4.0.7,12) docosane-2-acetic acid)-copper(II) diperchlorate hemihydrate (CSD-SUSVIL) [38] and 2.414 Å in (4-(t-butoxycarbonylmethyl)-1,4,8,11-tetraazabicyclo(10.2.2) hexadecane-\(N,N^0,N^00,N^000,O\))-copper(II) trichloro-copper(I) (CSD-KEYHOM) [39]. The bond length 2.3778(15) Å in CuL13Tyra is intermediate between these values, suggesting that the coordinate bond is weak.

Two tyramine arms are crystallographically inequivalent: one arm is anchored on the metal ion through the weak coordinate bond of carbonyl oxygen O5, and the other is linked to a neighboring molecule with the hydrogen bond N6–H6N/C1/C1/C1O6#2 (see Table S7, ESI). Still they are oriented to the same direction with respect to the macrocyclic chelate, and their phenol oxygen atoms O4 and O6 are connected by hydrogen bonding with two ordered water molecules.

3.4. Cu\(^{2+}\) complexation in aqueous solution

Coordination of L13Tyra with Cu\(^{2+}\) in solution was studied at pH 7.2 by means of UV–Vis spectrometric titrations. Fig. 4 shows the near-UV and visible spectra in the presence of Cu\(^{2+}\) at different concentrations. In addition to the 225 and 276 nm bands from the tyramine unit, a new charge-transfer (CT) absorption appears as a shoulder at 312 nm, and a d–d transition band at 550 nm. The absorbance of every new band increases linearly with the total Cu concentration [Cu]\(_t\) up to the ratio [Cu]\(_t\)/[L]\(_t\) = 2, above which the absorbance remains constant with the molar absorptivity \(\varepsilon_{\text{max}}\) = 1.31 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\) for the CT band and 300 for the d–d band (see A6 in Supplementary materials). The absorbance versus [Cu]\(_t\) plots suggest the formation of a binuclear complex, in contrast to the mononuclear complex in crystal. The binuclear stoichiometry in solution was confirmed by Job’s method as well (see A7 in Supplementary materials).

An aqueous solution of a metal–ligand 2:1 mixture is colored purple at pH above 2.5, and the color is deepened with increasing pH as the CT and d–d bands strengthen. The absorption-spectral pattern of the solution is changed with pH; some typical spectra are represented in Fig. 5, and the absorbance is plotted against pH for the 276, 295, and 550 nm bands in Fig. 6. The d–d band at 550 nm with the high absorptivity resembles that reported for the Cu\(^{2+}\) complexes of L13 and its analogues; the first coordination sphere in these complexes is a square plane consisting of two amino nitrogen and two deprotonated amide nitrogen atoms within a macrocyclic ring [11,40]. This mode of coordination is essentially identical with that found in the X-ray structure shown in Fig. 4. This fact suggests that one of the Cu\(^{2+}\) ions in the binuclear L13Tyra complex forms a tetraaza-macrocyclic metal chelate.

In the pH range where the complexation is confirmed by the CT and d–d bands, the 295 nm band originating from the tyramine unit is intensified as well. This spectral change suggests that the phenol is deprotonated to coordinate a Cu\(^{2+}\) ion [41–44]. Therefore, the second metal ion in the binuclear complex is coordinated with the oxygen atoms of the tyramine groups, and the remaining coordination sites are occupied by water molecules and/or hydroxyl ions to form a tetrahedral or distorted square pyramidal geometry.
The square planar Cu$^{2+}$ unit. Since the 550 nm band strengthens around the second Cu$^{2+}$ ion by oxygen atoms is so weak that the so weak as to be broken by hydration of Cu$^{2+}$ in water. In the solu-
mine-originating bands and the metal d–d band are changed in 550 nm bands in Fig. 6. Since all the bands including the tyra-
Fig. 6. Changes in absorbance of Cu$_2$-L13Tyra at 276 nm, 295 nm and 550 nm with pH, and species distribution obtained from the spectral change.

The coordinate bond of carbonyl oxygen in crystal is expected to be so weak as to be broken by hydration of Cu$^{2+}$ in water. In the solu-
tions studied, therefore, two phenol rings are extended from the macrocyclic chelate in an equivalent manner, and they can approach closer to each other due to a hydrophobic and π–π interaction; such a conformation allows the coordination of two pheno-
late oxygen atoms with a Cu$^{2+}$ ion to construct a huge metal chelate ring, and hence a binuclear complex can be formed. Coordi-
nation of the pendant amide group is ruled out because such a coordination is not found for L13Fea, which bears phenyl group in place of phenol group (Scheme 1) [12]. The ligand field formed around the second Cu$^{2+}$ ion by oxygen atoms is so weak that the d–d band probably appears around 720 nm with ε \approx 20–50 M$^{-1}$ cm$^{-1}$ [41,45–47], and it is masked with the strong 550 nm band from the square planar Cu$^{2+}$ unit. Since the 550 nm band strengthens synchronously with the CT band in a linear manner with the total Cu concentration [Cu]$_t$ up to [Cu]$_t$/[L]$_t$ = 2, the two coordination sites are bound to Cu$^{2+}$ ions at the same time. This simultaneous coordination process is supported by the pH variation of the tyra-
mine-originating bands as described below.

The spectral pattern of a metal–ligand 2:1 mixture in solution is strongly dependent on pH, as represented for the 276, 295, and 550 nm bands in Fig. 6. Since all the bands including the tyra-
mine-originating bands and the metal d–d band are changed in the pH range 5–7 in a synchronized manner, the complexations occur simultaneously at the two coordination sites, i.e., the macro-
cyclic ring and the phenolate arms. This coordination scheme is consistent with the stoichiometry determined from the spectral change with [Cu]$_t$/[L]$_t$ ratios at pH 7.2 [Fig. 4; A6 and A7 in Supple-
mentary materials]. At pH above 9, the 295 nm band is intensified while the 276 band is weakened, suggesting a change in coordi-
nation around a Cu atom bonded to phenolate oxygen; probably, two of coordinated water molecules are converted to hydroxyl ions at the high pH. The overall complexation is expressed by the follow-
ing equilibria:

\[
2\text{Cu} + \text{L13Tyra} \rightarrow [\text{Cu}_2\text{L13Tyra}]^{4+} + 4\text{H}^+ \tag{2}
\]

\[
[C\text{u}_2\text{L13Tyra}]^{4+} \leftrightarrow [\text{Cu}_2\text{L13Tyra}^{2+}(\text{OH})_2]^{2+} + 2\text{H}^+ \tag{3}
\]

Here, L13Tyra$^{4+}$ is the abbreviation of [L13Tyra – 4H]$^{4+}$ species in which two amide protons are released from the macrocyclic ring and two phenol protons are dissociated from the tyramine arms; water molecules are discarded from the chemical formula and the equilibrium equations. Every absorption band exhibits a two-
step pH variation corresponding to the two equilibria (Fig. 6). The most pronounced change is observed for the 295 nm band. Its absorptivity is ca. 5 times as large as that of the free ligand, and the absorbance change of the ligand is very small compared with that of the metal–ligand mixture at the wavelength. When the absorbance from the uncoordinated ligand is neglected for the above reason, the equilibrium constants can be determined from the inflection points of the absorbance versus pH curve of the 295 nm band as log K$_1$ = 5.65(7) for the first equilibrium expressed by Eq. (2) and log K$_2$ = 10.37(3) for the equilibrium shown by Eq. (3). These equilibrium constants give species distribution included in Fig. 6.

Evidently, the composition of the Cu complex in solution is differ-
ent from that in crystal. The coordination of the second Cu$^{2+}$ ion with phenolate oxygen is readily breakable under some circum-
stances because the so-called chelate effect is inoperative for the huge chelate ring and the large steric effect is unfavorable for the stabilization. In fact, the electrospray (ESI) mass spectrum of a methanol solution shows only a mononuclear species [Cu(L13Tyra – 2H)]$^{+}$ (m/z = 652.2) and its dimeric species [(Cu(L13Tyra – 2H))$_2$ + Na]$^{+}$ (m/z = 1283.4) even for Cu$_2$L13Tyra (see A8 in Supplementary materials); the ionization in the gas phase proceeds by the capture of a Na$^+$ ion from a glassware employed [48–51] and the dissociation of a weakly bound Cu$^{2+}$ ion from the molecule [52]. The formation of the mononuclear complex in methanol has been confirmed, as a methanol solution quenched to a glass at 77 K exhibits only a single Cu$^{2+}$ signal characteristic of a distorted square planar or square pyramidal coordination (see A9 in Supplementary materials) [53]. In the process of crystallization from a hot solution,
the binuclear complex releases a Cu$^{2+}$ ion from the phenolate coordination site, yielding the mononuclear complex as found in the X-ray study. The phenolate coordination may be short-lived in the complexation equilibrium in solution, but it is detectable by UV–Vis spectroscopy.

3.5. Evaluation of the antioxidant capacity by TEAC assay

The ligand L13Tyra in its free form has a very high antioxidant capacity (0.1384 g TEa/mol), which falls into the range of the antioxidant capacity presented by ascorbic acid (0.1678 g TEa/mol) at the same molar concentration, and it is higher than the TEAC assays of other compounds having similar structures (Fig. 7). Comparison with L13Fea concludes that the introduction of OH groups to the para-position in L13Tyra dramatically improves the antioxidant capacity. The presence of phenolic groups in many natural and synthetic compounds results in their high antioxidant capacity when compared with other compounds lacking such groups; phenolic acids and flavonoids containing phenolic groups are often reported to neutralize reactive oxygen species (ROS) [54,55]. The antioxidant capacity of L13Tyra is comparable to phenolic acids although it is lower than flavonoids as well as vitamins C and E.

Coordination with copper inhibits antioxidant capacity of L13Tyra; the capacity of CuL13Tyra (0.014 g TEa/mol) is only one-tenth that of the free ligand molecule, and even lower activity (0.0063 g TEa/mol) is found for Cu$_2$L13Tyra that is formed in solutions (Fig. 7). On the other hand, high antioxidant capacities are reported for binuclear Cu$^{2+}$ and Fe$^{3+}$ complexes with EDTA-based cyclophanes having a large molecular size [56,57]. These cyclophane chelates of copper(II) bear a square coordination geometry, and their antioxidant capacities are due to their conformational flexibility which promotes interaction with ABTS$^+$ radical [57]. A resembling square geometry is formed in the poor oxidant CuL13-Trya as well, but its chelate ring is small and consequently less flexible. This high rigidity may be unfavorable for interaction with the ABTS$^+$ radical. Furthermore, the coordination of Cu$^{2+}$ to the phenolate oxygen is effective enough to hinder the antioxidant capacity of the phenol group despite the weak coordinate bond. As a whole, Cu$^{2+}$ ion works as an inhibitor against the antioxidant L13Tyra through coordination.

3.6. Evaluation of the antioxidant capacity by $^1$H NMR

The mechanisms involved in radical quenching can be elucidated by NMR studies of a reaction with DPPH (1,1-diphenyl-2-picrylhydrazyl) used as a model radical [58]. Fig. 8 shows the $^1$H NMR spectra of L13Tyra in DMSO-$d_6$ at a concentration of 0.005 M before and after a reaction with DPPH. Before the reaction, L13Tyra exhibits a hydroxyl proton signal at 9.23 ppm with broadening (fwhm 9.4 Hz) due to a proton exchange probably through intramolecular hydrogen bonds kept in the non-aqueous solvent. When an equimolar amount of DPPH was added to the solution of L13Tyra, the deep purple color of the radical faded to brown in a reaction time of ca. 3 min; the macrocycle can quickly neutralize the free radical, in consistency with the high antioxidant capacity demonstrated by the TEAC assay. After this reaction, the NMR spectrum exhibited some new signals (marked with an asterisk in Fig. 8) attributable to neutralized DPPH or decomposition products. Regarding the spectrum of L13Tyra, the OH signal shifted upfield by 0.02 ppm with narrowing to an fwhm of 2.0 Hz, and the

![Fig. 8. $^1$H NMR spectrum of L13Tyra (top) and the spectrum after treatment with DPPH radical (bottom) in DMSO-$d_6$. The asterisked signals originate from neutralized DPPH or decomposition products. The amide NH signals consist of two triplets. The aliphatic proton signals are unchanged before and after the treatment.](http://example.com/image1.png)

![Fig. 9. Antiproliferative activity (% cell viability) of L13Tyra ligand on two cell lines; (A) normal cell line (ARPE-19, human retinal pigmented epithelium, normal cell line) and (B) cancer cell line (HeLa, human cervix carcinoma). The concentrations used of the compound from 3.125 to 25 µg/mL. The data are presented as mean ± SD of three independent experiments.](http://example.com/image2.png)

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Fig. 10. Observation at 48 h in inverted microscope. Antiproliferative activity of L13Tyra ligand on cancer cell line HeLa, at concentrations of (A) 3.125 μg/mL, (B) 6.25 μg/mL, (C) 12.5 μg/mL and (D) 25 μg/mL. All images are magnified at 10×.

Fig. 11. Observation at 48 h in inverted microscope. Antiproliferative activity of L13Tyra ligand on normal cell line ARPE-19, at concentrations of (A) 3.125 μg/mL, (B) 6.25 μg/mL, (C) 12.5 μg/mL and (D) 25 μg/mL. All images are magnified at 10×.
3.7. Antiproliferative activity

The antiproliferative activity of L13Tyra ligand was evaluated with the MTT colorimetric assay on a cancer cell line (HeLa, human cervix carcinoma) and a normal cell line (ARPE-19, human retinal pigmented epithelium, normal cell line).

The antiproliferative activity assays in vitro (Fig. 9) are expressed as percentage values of cellular viability after 48 h of continuous exposure to different concentrations of a test compound. The results were compared with the criteria established by the United States National Cancer Institute; in the case of pure compounds, they are considered active if their IC50 values are lower than 4 μg/mL [59].

The compounds studied have no antiproliferative activity on the two cell lines employed, because of values above 58% cell viability at the highest concentration 25 μg/mL and viability close to 100% at a concentration of 3.125 μg/mL after 48 h of exposure under the working conditions. The obtained MTT assay is consistent with observation with an inverted microscope (Figs. 10 and 11), which shows the integrity of the cell morphology and growth when exposed to each of the compounds under study. This was observed in both cell lines without significant differences.

4. Conclusions

Tyramine incorporated as pending arms in L13Tyra functions as a metal-chelating and radical-scavenging site. Furthermore, the side arms control the chemical and metal-coordinating properties of the amide-based tetraaza-macrocyclic ring. The basicity of the amino nitrogen is markedly lowered. The amide hydrogen is readily replaced by a Cu2+ ion even in acidic solution so that a tetraaza-macrocyclic metal chelate is formed. The phenol oxygen of the tyramine arms can work as the second coordination site toward a Cu2+ ion to yield a binuclear complex in solution. The binuclear complex is maintained only in solution; since the Cu–O coordinate bond is weakened by a steric effect, the dissociation of the Cu2+ ion from the phenol group occurs during crystal growth to result in the formation of a mononuclear complex (with the tetraaza-chelation) in solid as confirmed by X-ray crystallography.

L13Tyra is a powerful antioxidant comparable to ascorbic acid, as a result of the availability of phenolic group which can neutralize free radicals through hydrogen atom transfer. The antioxidant capacity is inhibited effectively by the Cu2+ coordination. Antiproliferative and cytotoxic assays show no toxicity of the L13Tyra ligand as well as its Cu2+ complex.

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Appendix A. Supplementary data

CCDC 1487648 contains the supplementary crystallographic data for CuL13Tyra complex. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or e-mail: deposit@ccdc.cam.ac.uk. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.poly.2016.10.028.

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