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Copper(II) complexes with terpene derivatives of ethylenediamine: synthesis, and antibacterial, antifungal and antioxidant activity†

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The synthesis of new chiral copper(II) complexes with terpene derivatives of ethylenediamine and the results of studying their antibacterial, antifungal and antioxidant activity *in vitro* are discussed. All studied copper complexes (**1–4**) showed significantly higher antifungal activity against the strains of *C. albicans*, *S. salmonicolor* and *P. notatum* compared to the activity of the clinical antifungal drug amphotericin. High antibacterial activity of copper complexes with terpene derivatives of ethylenediamine was revealed against the *S. aureus* (MRSA) strain, which is resistant to the reference antibiotic ciprofloxacin. Using various test systems, a comparative assessment of the antioxidant activity (AOA) of the synthesized copper complexes and the ligands was carried out. The salen-type complex **4**, which has the highest AOA in the model of initiated oxidation of a substrate containing animal lipids, was superior to other copper complexes in the ability to protect erythrocytes under conditions of H_2O_2-induced hemolysis.

Introduction

Metal complexes with organic ligands are being actively investigated as pharmacological agents, some of which have already found application in medical practice.[1–5] The idea of using a three-dimensional metal complex matrix to create a new drug is very promising, since a high degree of variability is possible.[5] Using metals with variable valence, for example Cu(I/II), it is possible to ensure their active participation in redox biochemical processes. Moreover, ligands can stabilize one or another oxidation state of the metal. In general, by varying the nature of the metal center, it is possible to control the kinetic parameters (rate of ligand exchange) and thermodynamics (strength of metal–ligand binding, level of redox potential). The variation in the nature of the ligands is almost limitless. This also applies to the internal coordination sphere and the external one, which provides anion exchange. Organic compounds of various classes were studied as starting ligands.[6] First of all, these are N-donor imines, amines, heterocyclic compounds, as well as ligands of the salen type and sulfur-containing ligands. In most works, the fact of a higher biological activity of the metal complex in comparison with the corresponding ligand was established. Metal complexes with a certain type of ligand can provide targeted pharmacological properties.

Screening for antimicrobial activity of a wide range of metal-containing compounds was carried out within the framework of an international project Community for Open Antimicrobial Drug Discovery.[1] It was found that in the group of metal complexes the proportion of compounds with high efficiency is 9.9% in comparison with organic molecules (0.9%). The great interest of researchers in copper complexes is confirmed by the fact that in 2020 alone, more than 60 works were published devoted to the synthesis and study of the antimicrobial properties of these compounds. Our review is devoted to the analysis of these results.[6]

Copper complexes are characterized by great potential for biomedical applications, which is confirmed by numerous studies. A number of works report on the AOA of copper complexes.[7–17] Recently, much attention has been paid to the development and use of copper complexes with the superoxide dismutase (SOD) mimetic activity.[18–24] To test free-radical-scavenging properties of drugs is a promising protocol for enlarging the applicability of the clinic drugs.[25] Copper stands out among transition metals for its remarkable affinity for biological ligands and redox properties that make it an ideal metal to drive biochemical reactions involving redox and oxygen chemistry. But the same chemical properties that make Cu

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a superb enzymatic co-factor render the metal potentially toxic, particularly for unicellular microbes.[26]

Analysis of the literature data allows us to conclude that the synthesis and study of the biological activity of copper complexes is very promising from the point of view of obtaining new pharmacological preparations. This work discusses the synthesis of chiral copper(II) complexes **1–4** with terpene derivatives of ethylenediamine and the results of studying their biological activity. Antibacterial and antifungal activity was determined by inhibiting cell growth of five bacteria types (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Mycobacterium vaccae*, *Bacillus subtilis*, *Escherichia coli*) and three types of fungi (*Candida albicans*, *Sporobolomyces salmonicolor*, *Penicillium notatum*). These types of widespread bacteria and fungi possess multiple mechanisms of resistance to a number of clinical drugs, which makes actual the search for new bactericidal and fungicidal drugs. A comparative assessment of the AOA of the synthesized copper complexes was carried out *in vitro* using test systems of varying degrees of complexity (including mammalian erythrocytes).

Results and discussion

Synthesis of copper(II) complexes

Terpene ligands are successfully used in coordination chemistry for the synthesis of chiral metal complexes.[27] In our work, we used terpene derivatives of ethylenediamine **L1–L4** as polydentate ligands for the preparation of copper chelate complexes (Fig. 1). We have previously described the synthesis and characteristics of these compounds: **L1**,[28] **L2**,[29] **L3**,[30] **L4**.[31]

The synthesis of copper complexes **1–4** (Fig. 2) was carried out by direct interaction of equimolar amounts of the starting ligand and copper chloride in methanol at room temperature. After removal of the solvent and recrystallization of the solid residue, substances were obtained in the form of powders. The structure of the obtained metal complexes was confirmed by the data of elemental analysis, mass spectrometry, and IR spectroscopy. The IR spectra show a shift in the band of stretching vibrations of the C=N bond upon passing from the free ligand to the corresponding complex, which indicates the participation of imine nitrogen in coordination. For complexes **1–4** in the region 410–490 cm^{-1} absorption bands are observed corresponding to stretching vibrations of Cu–N bonds. For complex **4**, the participation of the phenol hydroxyl in coordination can be confirmed by the fact that the band due to O–H shifted from 3254 cm^{-1} (**L4**) to 3439 cm^{-1} (**4**). In the case of compounds **1**

and **2**, no such regularities were noted. The mass spectra of compounds **1–4** show the presence of peaks of molecular ions, the low intensity of which indicates the instability of the initial molecule under conditions of electron impact. The data obtained provide good agreement for the molecular formula of these complexes. To determine the metal complexes charge, the molar conductivity of complexes **1–4** measured in DMSO (10^{-3} M) at 25 °C. Low molar conductance values (18–35 S cm^2 mol^{-1}) suggests complexes are non-electrolytes in nature.[32]

According to X-ray diffraction (XRD) analysis, compound **3** (Fig. 3) crystallizes in a non-centrosymmetric space group, which confirms the chirality of the compound. The central copper ion coordinates two N atoms, the O atom of the OH group of the tridentate ligand **L3**, and two Cl atoms (coordination number 5). As a result of the coordination interaction of the Cu^{2+} ion with the ligand **L3**, two five-membered chelate rings CuC$_2$N$_2$ and CuC$_2$NO are closed. In this case, the *S*-configuration of the chiral nitrogen N1 is fixed. The Cl$_2$N$_2$O coordination polyhedron has the shape of a trigonal bipyramid. This geometry for copper(II) complexes is not unique and has been noted in a number of studies.[33] Angular structural parameter τ_5, as general descriptor five-coordinate centric molecules,[34] determined for **3**. Its value is 0.6133. According to this criterion, the Cl$_2$N$_2$O coordination polyhedron in the solid state has a trigonal–bipyramidal like geometry.

Antibacterial and antifungal activity

Studies have shown that, in general, copper complexes **1–4** showed high antibacterial and antifungal activity (Table S1†). In particular, a high antibacterial activity of **1–4** was revealed against the methicillin-resistant *S. aureus* (MRSA) strain, which was also resistant to the reference antibiotic ciprofloxacin (Fig. 4). In relation to the *S. aureus* (511 B3) strain, the activity of complexes **1–4** is comparable to that of ciprofloxacin. All studied copper complexes (**1–4**) showed significantly higher antifungal activity against strains of *Candida albicans*, *Sporobolomyces salmonicolor* and *Penicillium notatum* compared to the activity of the clinical antifungal drug amphotericin (Fig. 5).

For comparison, the antimicrobial activity of free ligands **L1–L4** was evaluated and it was found that they are inactive against the line of investigated strains of pathogenic microorganisms. The data obtained confirm the regularity established in many works – a higher antimicrobial activity of metal complexes compared to free ligands.[11,19,35,36] Scientists explain this result by an increase in the lipophilicity of the Cu^{2+} ion due to coordination with the organic ligand. Subsequently, the penetration of
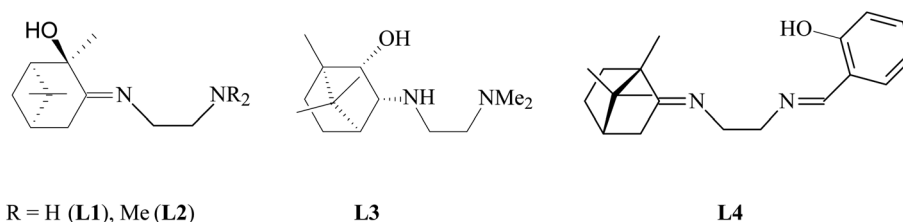

R = H (**L1**), Me (**L2**)   **L3**   **L4**

Fig. 1 Ligands **L1–L4** for the copper complexes synthesis.

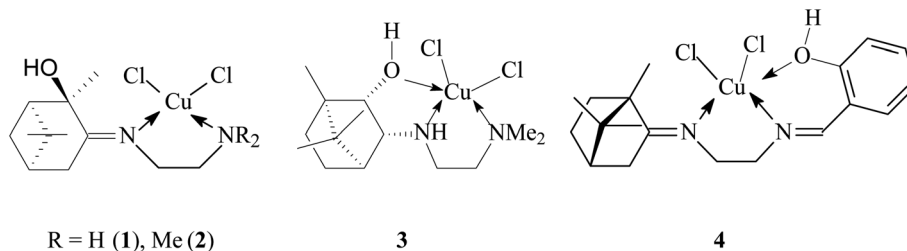R = H (**1**), Me (**2**) **3** **4**

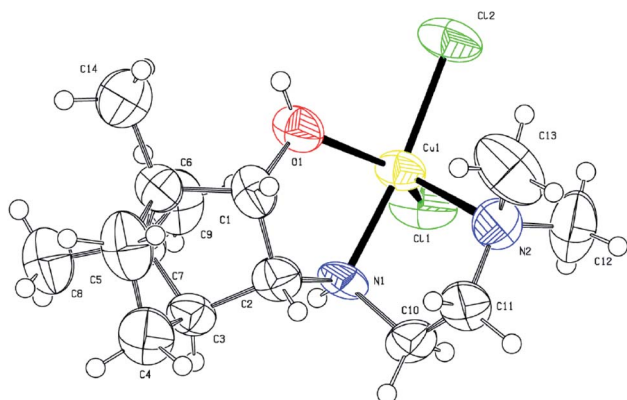Fig. 2 New copper complexes **1–4** with terpene derivatives of ethylenediamine.

Fig. 3 ORTEP drawing of the molecular structure **3**. Thermal ellipsoids are shown at the 50% probability level.
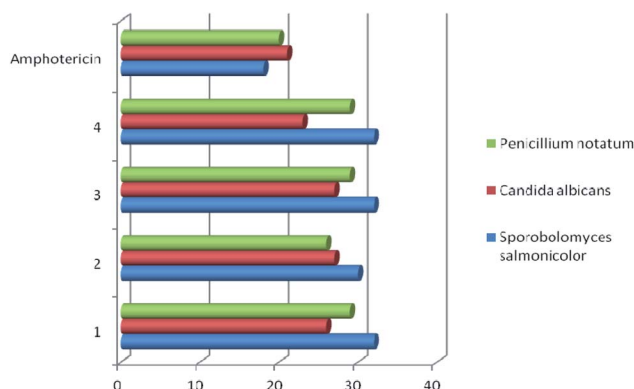

Fig. 5 Antifungal activity of copper complexes **1–4** *vs.* clinical fungicide amphotericin. Agar diffusion assay, inhibition zone diameter, mm.
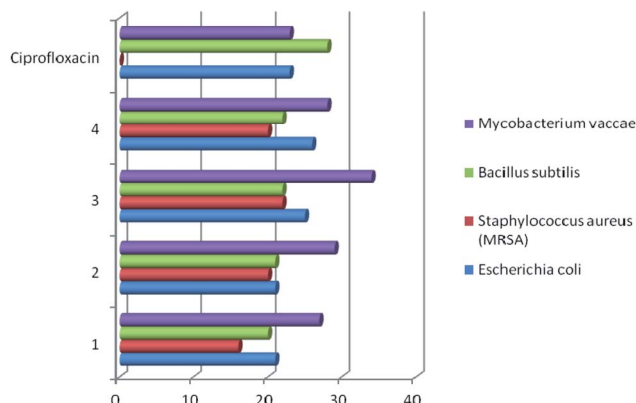

Fig. 4 Antibacterial activity of copper complexes **1–4** *vs.* clinical antibiotic ciprofloxacin. Agar diffusion assay, inhibition zone diameter, mm.

the complexes into lipid membranes will be enhanced and then blocking the Cu(II) binding sites in the enzymes of microorganisms. The authors[36] add that "the enhancement of the activity can occur not only due the increase of lipophilicity but alternatively it may be due to the formation of reactive oxygen species through the reduction of Cu(II) to Cu(I) in intracellular environment, which can cause the death of the microorganism".
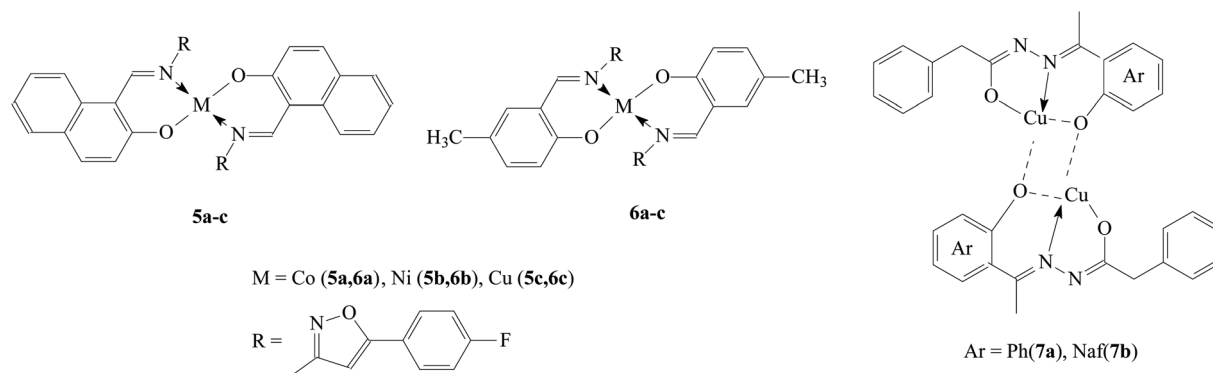
It is difficult to make a correct comparison of the literature data on the antimicrobial activity of copper complexes and our

results. There are a lot of examples,[6] however, the difficulty lies in the fact that the assessment of activity was carried out by various methods, the data are presented in various quantitative units. We have tried to make a small selection of comparable data.[11,12] The results are included in the Table S1.†

A series of bivalent metal complexes **5a–c** and **6a–c** (Fig. S1†) with Schiff base ligands have been synthesized.[12] The antimicrobial screening results displayed that the Schiff base ligands were moderately active against bacterial (*P. aeruginosa*, *E. coli*, *S. aureus*, *B. amyloliquefaciens*) and fungal (*M. phaseolina*, *S. rolfsii*) strains whereas the corresponding metal complexes **5a–c** and **6a–c** show higher activity than free Schiff base ligands. From the above results it is concluded that, among all, copper complexes **5c** and **6c** show highest potential activity against mentioned bacterial and fungal species.

Thermal reactions of Cu²⁺ ions with the two Schiff base ligands [*N*′-(1-(2-hydroxyphenyl)ethylidene)-2-phenylacetohydrazide] and [*N*′-((1-hydroxynaphthalen-2-yl)methylene)-2-phenylacetohydrazide] resulted in formation of the binuclear complexes **7a** and **7b**.[11] The two ligands showed no activity towards either the bacteria (*E. coli*, *S. aureus*) or the fungi (*A. flavus*, *C. albicans*). On the other hand, the results indicated high antimicrobial activities for the complexes **7a**, **7b** and were found to be comparable with that of standards (ampicillin or amphotericin B). Accordingly, it could be concluded that the Cu²⁺ ions improved the antimicrobial activity of the compounds.

These examples indicate comparable antibacterial activity of the copper complexes **1–4** obtained by us and compounds **5c**,

5a–c

6a–c

M = Co (**5a,6a**), Ni (**5b,6b**), Cu (**5c,6c**)

R =

Ar = Ph(**7a**), Naf(**7b**)

6c, **7a**, **7b** described in the literature[11,12] against *E. coli*, *P. aeruginosa*, and *S. aureus* strains. The antifungal activity of complexes **1–4** against *Candida albicans* is twice as high than that established for compounds **7a** and **7b**.[11]

Antioxidant activity

For the obtained complex compounds of copper **1–4** (Fig. 2), as well as free ligands **L1**, **L2** (Fig. 1), a comparative assessment of AOA was carried out using various test systems. We employ a common set of methods, which were successfully used by us in previous studies, to investigate the antioxidant properties of the prepared compounds.[37–40]

The AOA of compounds was assessed (*in vitro*) by the ability to inhibit lipid peroxidation (LPO) processes in a substrate containing lipids of the laboratory mice brain. In this case, two methods of LPO initiation were used – Fe^{2+}/ascorbate or H_2O_2. The content of LPO secondary products reacting with 2-thiobarbituric acid (TBA-reactive substances, TBA-RS) was determined spectrophotometrically (Fig. 6 and Table S2†). From the analysis of the data obtained at a concentration of compounds of 500 μM, it follows that regardless of the method of LPO initiation in both pairs of ligand-complex, the highest inhibitory activity was observed for copper complexes. In particular, complex **1** is more active than ligand **L1**, complex **2** is more active than ligand **L2**. Copper complexes **3** and **4** are also characterized by a high AOA at the indicated concentration. Note that the results obtained upon initiation of LPO by various methods are closely correlated with each other (Spearman's correlation coefficient $R_s = 0.75$; significance level $p = 0.05$; sample size $n = 7$).

With a decrease in concentration to 100 μM, complex **4** practically does not lose its ability to inhibit LPO and retains leading position (Fig. 6 and Table S2†). This compound is comparable in activity to the standard antioxidant 2,6-di-*tert*-butyl-4-methylphenol (BHT) for both methods of LPO initiation (Fe^{2+}/ascorbate or H_2O_2). Structurally, the copper complex **4** has significant differences from compounds **1–3**, since it contains N,N,O-donor tridentate ligand of salen type. It can be assumed that the high AOA **4** is provided by the presence of

a phenolic fragment. For further research, we define this type of complexes as the most promising.

The absence of an increase (and even a slight decrease) in the antioxidant activity with an increase in the drug concentration, as is observed for complex **4**, was repeatedly noted earlier for structures of another type.[38,39,49] The authors attribute this to the possible pro-oxidant effect of these highly active compounds at high concentrations.

Before carrying out a comparative study of the membrane-protective activity (MPA) of compounds **1–4**, **L1**, **L2** on the model of oxidative hemolysis of erythrocytes, we assessed their toxicity by the degree of hemolysis of erythrocytes. It was established (Fig. 7 and Table S3†) that almost all tested compounds at 10 μM are characterized by low hemolytic activity – the death of erythrocytes in their presence slightly exceeds spontaneous. Only in the presence of complex **2** erythrocyte hemolysis exceeds the control values by about two times. Further studies of the biological activity of the compounds using blood cells were carried out at 1 μM.

MPA of the tested compounds (copper complexes **1–4**, ligands **L1** and **L2**) was determined by the degree of inhibition of oxidative hemolysis in red blood cells. 2,2′-Azobis-(2-amidinopropane)hydrochloride (AAPH) or H_2O_2 was used to initiate oxidative hemolysis. In an aqueous medium at physiological temperatures AAPH generates the formation of peroxyl radicals that are unable to penetrate into the cell and act on the membrane from the outside.[41,42] Oxidative hemolysis initiated by AAPH is widely used for the detection of AOA and MPA of various compounds and plant extracts.[41–45] It was found that all studied compounds (copper complexes **1–4**, ligands **L1** and **L2**) have statistically significant MPA, which follows from the decrease in the rate of cell death in their presence under the influence of AAPH radicals (Table 1). Two hours after the induction of oxidative stress, the level of hemolysis in the control was 23.2%, in the presence of copper complexes **1–4** – only 6.2–10.9%, ligands **L1** and **L2** – 12.1–15.0%. The highest MPA among all studied compounds was found for complex **2**, which was superior in activity not only to the corresponding ligand **L2**, but also to BHT, slowing down the rate of oxidative hemolysis during the experiment.
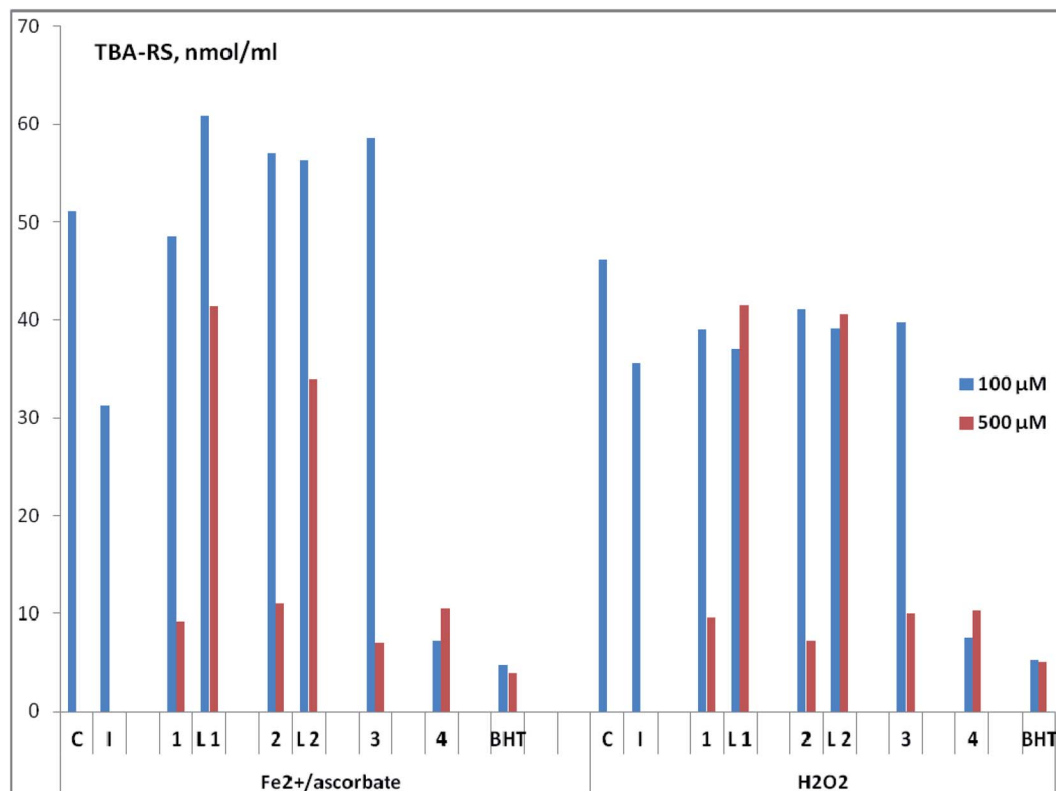
Fig. 6   AOA of the test compounds (copper complexes 1–4, ligands L1 and L2) at concentrations of 100 and 500 μM. C – control without test compounds. I – intact samples (without initiated oxidation). BHT – standard antioxidant 2,6-di-tert-butyl-4-methylphenol.
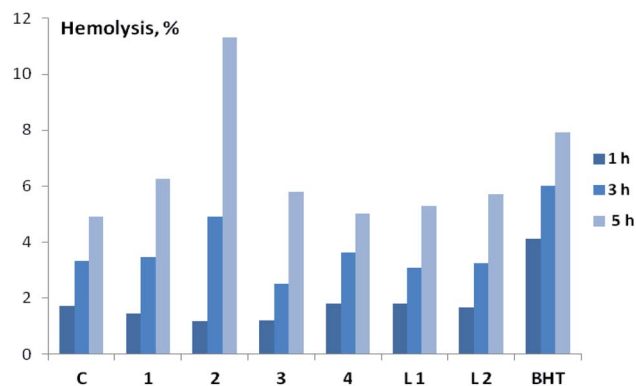

Fig. 7   Hemolytic activity of the test compounds (copper complexes 1–4, ligands L1 and L2) at a concentration of 10 μM after 1, 3 and 5 h of incubation. C – control without test compounds. BHT – standard antioxidant 2,6-di-tert-butyl-4-methylphenol.

Table 1   MPA of the test compounds (copper complexes 1–4, ligands L1 and L2) at a concentration of 1 μM under the conditions of AAPH- and H_2O_2-induced hemolysis

	Hemolysis, %				
Variant	1 h	2 h	3 h	4 h	5 h
AAPH-induced hemolysis					
C^a	2.7 ± 0.0	23.2 ± 0.8	55.9 ± 0.7	75.6 ± 0.8	81.8 ± 0.4
1	2.8 ± 0.0	9.3 ± 0.2	42.4 ± 0.6	69.1 ± 0.8	81.3 ± 0.6
2	2.4 ± 0.1	6.2 ± 0.7	31.6 ± 0.1	62.0 ± 0.7	75.5 ± 0.7
3	2.8 ± 0.0	10.4 ± 0.6	47.1 ± 1.4	69.4 ± 1.1	81.2 ± 0.8
4	2.5 ± 0.0	10.9 ± 0.4	45.5 ± 0.6	70.8 ± 0.4	82.9 ± 0.4
L1	2.7 ± 0.0	12.1 ± 1.0	42.7 ± 0.6	66.7 ± 0.8	79.0 ± 0.7
L2	2.9 ± 0.2	15.0 ± 1.3	47.5 ± 1.2	68.7 ± 0.6	80.7 ± 1.7
BHT^b	3.0 ± 0.1	16.0 ± 0.8	46.2 ± 0.8	70.7 ± 0.8	81.0 ± 0.3
H_2O_2-induced hemolysis					
C	11.5 ± 0.7	23.1 ± 0.5	30.7 ± 0.7	36.7 ± 0.6	39.9 ± 0.4
1	7.1 ± 0.4	17.9 ± 0.6	24.8 ± 0.9	30.6 ± 0.8	34.5 ± 0.8
2	6.9 ± 0.5	18.5 ± 0.5	25.5 ± 0.3	29.4 ± 0.2	33.5 ± 0.4
3	11.0 ± 0.5	21.4 ± 0.6	29.1 ± 0.8	36.8 ± 0.4	40.5 ± 0.8
4	10.9 ± 0.6	16.4 ± 0.4	20.9 ± 0.5	25.0 ± 0.4	28.0 ± 0.7
L1	8.0 ± 0.5	22.6 ± 0.8	29.8 ± 1.5	34.1 ± 1.0	36.3 ± 1.0
L2	10.3 ± 0.6	21.8 ± 0.7	26.7 ± 0.5	29.7 ± 0.3	31.2 ± 0.3
BHT	5.6 ± 0.4	9.2 ± 0.9	11.9 ± 0.5	18.4 ± 0.6	22.3 ± 1.1

^{a} C – control without test compounds. ^{b} BHT – standard antioxidant 2,6-di-tert-butyl-4-methylphenol.

Hydroperoxide (H_2O_2), along with AAPH, is also widely used to initiate oxidative hemolysis in the study of the antioxidant and membrane-protective properties of various compounds.[44,46,47] Unlike AAPH, this compound easily penetrates into the erythrocyte, and its primary target is hemoglobin.[42] The results of a comparative MPA assessment of the studied compounds (copper complexes 1–4, ligands L1 and L2) under H_2O_2-induced hemolysis are presented in Table 1. In this experiment, the highest activity among complexes 1–4 was found for the salen type compound 4, which contains
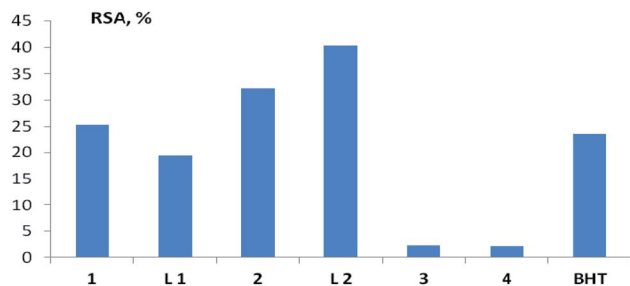
Fig. 8 RSA of the test compounds (copper complexes **1–4**, ligands **L1** and **L2**) in the test with DPPH at a concentration of 100 μM. BHT – standard antioxidant 2,6-di-*tert*-butyl-4-methylphenol.

a phenolic fragment and is distinguished by the highest AOA in the model of initiated oxidation of a substrate containing animal lipids.

In the study of the AOA of various compounds and extracts, the assessment of radical scavenging activity (RSA) by their ability to interact with the stable chromogen radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) is widely used.[48–51] In a number of works, this method was also used to characterize the AOA of copper complexes with ligands of various structures.[7–12,16] In our studies, the largest RSA, comparable to that of BHT, was found for copper complexes **1** and **2**, as well as their ligands **L1** and **L1** (Fig. 8 and Table S4†). For complex **2**, this result is consistent with the data obtained in the test for inhibition of oxidative hemolysis initiated by AAPH radicals. It is possible that the high RSA of compounds **1** and **2** is due to the fact that they belong to the derivatives of the pinane series, which are more prone to oxidation reactions, in contrast to the bornane rings (**3**, **4**).

It should be noted that for the studied compounds (copper complexes **1–4**, ligands **L1** and **L2**), the RSA, assessed in the test with DPPH, does not correlate with the results obtained in biologically relevant test systems, and does not allow predicting the activity of the considered terpene ligands and their copper complexes in contact with living cells. Spearman's rank correlation coefficient (R_s) between RSA and AOA, assessed by the ability to inhibit LPO with both initiation methods, ranged from -0.18 to 0.43 ($n = 7$; $p > 0.05$), between RSA and erythrocyte survival under conditions of AAPH-induced hemolysis from -0.64 to 0.18 ($n = 7$; $p > 0.05$).

Significant differences in the results depending on the method used were noted in the study of AOA Schiff-base ligands containing carboxylate groups and their copper and nickel complexes.[14] A similar result was noted for the complexes Pd(II), Fe(II), Zn(II), Mn(II), Cu(II) and VO(IV) *c* multifunctional Schiff base.[16] Our data confirm the conclusions of other researchers[41,52,53] that for a correct assessment of AOA one should not be limited to studies of RSA alone, it is desirable to use more relevant biological test systems.

Conclusion

New chelate complexes of copper(II) (**1–4**) with terpene derivatives of ethylenediamine have been synthesized and characterized. The structure of compound **3** was confirmed by XRD. A high antibacterial activity of copper complexes **1–4** was revealed against a methicillin-resistant strain of *Staphylococcus aureus* (MRSA), resistant, among other things, to the reference antibiotic ciprofloxacin. All studied copper complexes (**1–4**) showed significantly higher antifungal activity against the strains of *Candida albicans*, *Sporobolomyces salmonicolor* and *Penicillium notatum* compared to the activity of the clinical antifungal drug amphotericin. At the same time, no dependence on the structure of the initial ligand was revealed.

Using test systems of varying degrees of complexity, the AOA of copper complexes **1–4** and terpene ligands **L1** and **L2** were assessed. Regardless of the method of lipid peroxidation initiation (Fe^{2+}/ascorbate or H_2O_2), in both ligand–complex pairs (**L1-1** and **L2-2**), the highest inhibitory activity was observed for complexes **1** and **2**. Salen type complex **4**, characterized by the highest AOA in the model of initiated oxidation of a substrate containing animal lipids and showing no hemolytic activity even at a concentration of 10 μM, was superior to other copper complexes in their ability to protect erythrocytes under H_2O_2-induced hemolysis. It was found that all tested compounds at a concentration of 10 μM are characterized by low hemolytic activity – the death of erythrocytes in their presence slightly exceeds spontaneous, which allows them to be classified as low-toxic.

Experimental section

Synthesis of copper complexes

Materials and methods. All reagents and solvents were purchased from Sigma-Aldrich, Merck and Fluka. All solvents (ethanol, methanol, chloroform, dimethylsulfoxide, acetone, diethyl ether, hexane) were of reagent grade and used without further purification.

The FT-IR spectra of the synthesized novel complexes were recorded in the 200–4000 cm^{-1} region on FT-IR spectrometer Shimadzu IR Prestige 21 on thin films or KBr tablets. Optical rotations were obtained with automatized digital polarimeter Optical Activity PolAAr 3001 England. Melting points were determined with Gallencamp-Sanyo apparatus. Elemental analyses were performed by using vario Micro cube apparatus. Mass spectra were recorded on a gas chromatography-mass spectrometer Shimadzu GCMS-QP2010 Plus. Molar conductance of the complexes in DMSO (10^{-3} M) measured by Elins Z-1000P Impedance Meter.

The following terpene derivatives of ethylenediamine were used as ligands, the synthesis of which was described by us earlier: (*E*)-N^1-[(1*R*,2*R*,5*R*)-2-oxy-2,6,6-trimethylbicyclo[3.1.1]heptane-3-ylidene]ethane-1,2-diamine (**L1**);[28] (*E*)-N^1,N^1-dimethyl-N^2-[(1*R*,2*R*,5*R*)-2-oxy-2,6,6-trimethylbicyclo[3.1.1]heptane-3-ylidene]ethane-1,2-diamine (**L2**);[29] N^1,N^1-dimethyl-N^2-[(1*R*,2*S*,3*R*,4*S*)-2-oxy-1,7,7-trimethylbicyclo[2.2.1]heptane-3-yl]ethane-1,2-diamine (**L3**);[30] (*E*,*E*)-N^1-[(1*S*,4*S*)-1,7,7-trimethylbicyclo[2.2.1]heptane-2-ylidene]-N^2-(2-hydroxybenzylidene)ethane-1,2-diamine (**L4**).[31]

General procedure for the synthesis of copper complexes 1–4. A solution of CuCl$_2$·2H$_2$O (0.5 mmol) and corresponding

ligand **L1–L4** (0.5 mmol) in 10 mL of methanol was stirred for 8 h at room temperature. Copper(II) complexes were obtained in the form of powders after removal of the solvent and recrystallization from a mixture of acetone – diethyl ether.

Physico-chemical and spectral characteristics of copper complexes 1–4. Dichloro{(*E*)-N^1-[(1*R*,2*R*,5*R*)-2-oxy-2,6,6-trimethylbicyclo[3.1.1]heptane-3-ylidene]ethane-1,2-diamine-N^1,N^2} copper(II) (**1**): green powder; yield 84%; m.p.: 107 °C (decomp.); [*α*]$_D$ + 416.0 (*c* 0.05, EtOH); selected FT-IR data, *ν*, cm^{-1}: 3229 broad (OH), 3132 broad (NH$_2$), 1674 (C=N), 467, 457, 406 (Cu–N). Complex **1** dissolves in EtOH, DMSO. Elemental analysis (%) calcd for C$_{12}$H$_{22}$N$_2$OCl$_2$Cu·H$_2$O: C 39.73; H 6.67; N 7.72; found: C 39.53; H 5.93; N 7.59. Molar conductance (DMSO, *Λ*$_m$, S cm^2 mol^{-1}): 32.4. MS (*m/z*): 343.2 [M]$^+$ for C$_{12}$H$_{22}$N$_2$OCl$_2$Cu.

Dichloro{(*E*)-N^1,N^1-dimethyl-N^2-[(1*R*,2*R*,5*R*)-2-oxy-2,6,6-trimethylbicyclo[3.1.1]heptane-3-ylidene]ethane-1,2-diamine-N^1,N^2}copper(II) (**2**): green powder; yield 82%; m.p.: 86 °C (decomp.); [*α*]$_D$ + 487.1 (*c* 0.1, EtOH); selected FT-IR data, *ν*, cm^{-1}: 3163 broad (OH), 1672 (C=N), 460, 432 (Cu–N). Complex **2** dissolves in EtOH, DMSO, acetone. Elemental analysis (%) calcd for C$_{14}$H$_{26}$N$_2$OCl$_2$Cu·H$_2$O: C 43.02; H 7.22; N 7.17; found: C 43.61; H 7.10; N 7.08. Molar conductance (DMSO, *Λ*$_m$, S cm^2 mol^{-1}): 28.5. MS (*m/z*): 372.2 [M + H]$^+$ for C$_{14}$H$_{26}$N$_2$OCl$_2$Cu.

Dichloro{N^1,N^1-dimethyl-N^2-[(1*R*,2*S*,3*R*,4*S*)-2-oxy-1,7,7-trimethylbicyclo[2.2.1]heptane-3-yl]ethane-1,2-diamine-N^1,N^2,O}copper(II) (**3**): green powder; yield 66%; m.p.: 124 °C (decomp.); [*α*]$_D$ − 207.1 (*c* 0.04, EtOH). FT-IR, *ν*, cm^{-1}: 3228 broad (OH), 3184 (NH), 443, 476 (Cu–N). Complex **3** dissolves in EtOH, DMSO. Elemental analysis calcd (%) for C$_{14}$H$_{28}$N$_2$OCl$_2$Cu: %: C 44.86; H 7.53; N 7.47; found: C 45.02; H 7.12; N 7.41. MS (*m/z*): 374.1 [M + H]$^+$ for C$_{14}$H$_{28}$N$_2$OCl$_2$Cu.

Dichloro{(*E*,*E*)-N^1-[(1*S*,4*S*)-1,7,7-trimethylbicyclo[2.2.1] heptane-2-ylidene]-N^2-(2-hydroxybenzylidene)ethane-1,2-diamine-N^1,N^2,O}copper(II) (**4**): purple powder; yield 83%; m.p.: 182 °C (decomp.); [*α*]$_D$ + 68.0 (*c* 0.05, H$_2$O); selected FT-IR data, *ν*, cm^{-1}: 3439 broad (OH), 1695 (C=N), 1624 (C=N), 467, 422 (Cu–N). Complex **4** dissolves in H$_2$O, DMSO. Elemental analysis (%) calcd for C$_{19}$H$_{26}$N$_2$OCl$_2$Cu: %: C 52.72; H 6.05; N 6.47; found: C 52.32; H 6.18; N 6.38. Molar conductance (DMSO, *Λ*$_m$, S cm^2 mol^{-1}): 34.5. MS (*m/z*): 432.1 [M]$^+$ for C$_{19}$H$_{26}$N$_2$OCl$_2$Cu.

Crystal structure determination of complex 3. XRD experiment was accomplished on the base of centre collective using "SAOC" IOS UB RAS. XRD data were collected on the automated four-circle diffractometer with CCD "Xcalibur S" according to the standard procedure (MoK$_α$-irradiation, graphite monochromator, ω-scans with 1° step at *T* = 295(2) K). Empirical absorption correction was applied. Using Olex2,[54] the structure was solved with the SHELXS[55] structure solution program using Direct Methods and refined with the SHELXL[55] refinement package using Least Squares minimization in anisotropic approximation for non-hydrogen atoms. H-atoms were placed in the calculated positions and refined isotropically in the "rider" model. Crystal data for compound: crystal monoclinic, space group *P*2$_1$, *a* = 7.1957(10) Å, *b* = 12.0448(13) Å, *c* = 10.685(2) Å, *β* = 101.652(15)°, *V* = 907.0(2) Å^3, *μ* = 1.496 mm^{-1}. On the angles of the scattering 7.138° ≤ 2*θ* ≤ 61.772° 6643

reflections were collected, 4237 independent reflections (*R*$_{int}$ = 0.0747). Final *R*-indexes: *R*$_1$ = 0.1501, w*R*$_2$ = 0.3056 (all data), *R*$_1$ = 0.0985, w*R*$_2$ = 0.2429 (*c I* > 2*σ*(*I*)), GooF = 1.028. Largest diff. peak/hole 1.77/−0.91 eÅ^{-3}. XRD data were registered in the Cambridge Structural Database (CCDC 2113093).

$$\tau_5 = (\beta - \alpha)/60° = (172.2 - 135.4)/60 = 0.6133$$

Evaluation of antimicrobial activity

The microorganisms used in this study are *Pseudomonas aeruginosa* (*P. aeruginosa*) [SG137 B7], *P. aeruginosa* [K799/61 B9], *Escherichia coli* (*E. coli*) [458 B4], *Staphylococcus aureus* (*S. aureus*) [511 B3], *S. aureus* (MRSA) [134/93 R9], *Mycobacterium vaccae* (*M. vaccae*) [10670 M4], *Bacillus subtilis* (*B. subtilis*) [6633 B1] as pathogenic bacterial strains and *Sporobolomyces salmonicolor* (*S. salmonicolor*) [549 H4], *Candida albicans* (*C. albicans*) [H8], *Penicillium notatum* (*P. notatum*) [JP36 P1] as pathogenic fungal strains.

The antimicrobial activity of Cu(II) complexes were screened by agar well diffusion method.[56,57] Experimental compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration 1 mg mL^{-1}. The bacteria were cultivated on standard I nutrient agar in Petri dishes at 37 °C. After inoculation, a disc (9 mm in diameter) was removed from the center of the Petri dish and 50 μL (corresponds to 50 μg of pure compound) of the test solution was added to the cavity. After 18 hours of incubation at the respective temperatures, growth inhibition or promotion occurred as a halo around the inoculation hole. Ciprofloxacin (5 μg mL^{-1} in deionized water) was used as the antibacterial reference compound. When studying the antifungal activity, the amphotericin (Amph) was used as a comparison drug. The diameter of the inhibition zones was measured (in mm) and documented as given in Table S1.† All biological assays done in triplicate.

Evaluation of the antioxidant activity

Materials and methods. In this work we used FeSO$_4$ (Reachim Ltd, Moscow, Russian Federation), phosphate buffered saline (pH 7.4) (Sigma-Aldrich, USA); 2,2-diphenyl-1-picrylhydrazyl (CAS Number 1898-66-4, Alfa Aesar), 2,2′-azobis-(2-amidinopropane)hydrochloride (CAS Number 2997-92-4, Alfa Aesar), trichloroacetic acid (CAS Number 76-03-9, Alfa Aesar), thiobarbituric acid (CAS Number 504-17-6, Alfa Aesar), and ascorbic acid (CAS Number 50-81-7, ICN Biomedicals, USA).

When studying the AOA of the compounds, we performed no experiments on animals: the analyzes were carried out exclusively *in vitro*. For these purposes, we used the erythrocytes and brain tissue of intact laboratory mice obtained from the scientific collection of experimental animals at the Institute of Biology, Komi SC, UB RAS (https://www.ckp-rf.ru/usu/471933/). The animals were kept in accordance with bioethical conditions (Protocol No. 1, dated 24.01.2017, approved by Academic Council of the Institute of Biology of the Komi Scientific Center of the Ural Branch of the RAS).

To study AOA, stock solutions of the studied compounds (copper complexes **1–4**, ligands **L1** and **L2**) in ethanol with a concentration of 5 mM were prepared.

Radical scavenging activity. The RSA of the compounds was evaluated by their ability to interact with the stable radical of DPPH.[51] Test compound stock solution was added to 0.0015% DPPH solution in MeOH (final concentration of compounds 100 μM). Then, each mixture was shaken vigorously and kept in the dark at room temperature for 30 min. The absorption decrease was measured at λ 517 nm using a Thermo Spectromic Genesys 20 spectrophotometer. RSA was calculated as a percentage of DPPH discoloration using the following equation:

$$RSA, \% = 100 \times (1 - At/Ac),$$

where At is the absorbance of the solution containing the test compound and Ac is the absorbance of the control sample with all reagents except for the compound.

The ability to inhibit lipid peroxidation. The AOA was studied (*in vitro*) by the ability to inhibit LPO processes in a substrate containing lipids of laboratory mice brain.[58,59] Briefly, extracted brain was homogenized in physiological saline (pH = 7.4) and centrifuged. Then test compound stock solution was added to the supernatant (final concentration 100 μM or 500 μM) and LPO was initiated by $FeSO_4$/ascorbic acid or H_2O_2. Samples were stirred gently for 1 h at 37 °C in a thermostatic shaker Biosan ES-20. Content of LPO secondary products reacting with TBA (TBA-RS, nmol mL^{-1}) was determined spectrophotometrically at $\lambda = 532$ nm using the extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.[60]

Hemolytic activity. Toxicity, AOA and MPA of the tested compounds (copper complexes **1–4**, ligands **L1** and **L2**), were investigated using a 0.5% (v/v) suspension of laboratory mice red blood cells (RBCs) in phosphate buffered saline (pH 7.4). The compounds ability to induce hemolysis defined their toxicity (*in vitro*). The solution of tested compound was added to a suspension of erythrocytes (final concentration 10 μM) and incubated at 37 °C for 5 h in a shaker Biosan ES-20. After 1, 3 and 5 h of incubation an aliquot was taken from the suspension, centrifuged for 5 min (1600 g); hemolysis was determined by the hemoglobin content in the supernatant on Thermo Spectromic Genesys 20 spectrophotometer at $\lambda = 541$ nm. The percentage of hemolysis was calculated relative to complete hemolysis of the sample, where it was triggered by addition of distilled water.

MPA was determined by the degree of inhibition of oxidative hemolysis in RBCs. For this purpose, 30 min after the test compounds solution was added to the of the erythrocyte suspension hemolysis was initiated with H_2O_2 (0.006%) or AAPH (3 mM). Then the reaction mixture was incubated in a thermostatic shaker Biosan ES-20 with slow stirring at 37 °C temperature for 5 hours. Each hour an aliquot was taken from the incubated samples, centrifuged, hemolysis was determined by the hemoglobin content in the supernatant on Thermo Spectromic Genesys 20 spectrophotometer at $\lambda = 524$ nm.[41]

Statistical analysis. Each experiment was carried out in 4–11 replicates. Statistical analysis was done using Microsoft Office Excel 2007 and Statistica 6.0 software package. Experimental results are presented as arithmetic means with standard errors. Regression analyses were performed, and Spearman's rank correlation coefficient was calculated in order to evaluate the interrelation between certain parameters.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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