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Redox-active metal(II) complexes of sterically hindered phenolic ligands: Antibacterial activity and reduction of cytochrome *c*

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ABSTRACT

The synthesis and physico-chemical characterization of Fe(II) and Mn(II) complexes of 4,6-di-*tert*-butyl-3[(2-hydroxyethyl)sulphanyl]-1,2-dihydroxybenzene (HL^I) and 2-amino-4,6-di-*tert*-butylphenol (HL^{II}) were carried out. Antibacterial activity of the Co(II), Fe(II) and Mn(II) complexes was evaluated in comparison with Cu(II) complexes and three common antibiotics; it was found to follow the order: (1) $Cu(L^I)_2 > Co(L^I)_2 > Fe(L^I)_2 \geqslant Mn(L^I)_2 > HL^I$; (2) $Cu(L^{II})_2 > Co(L^{II})_2 > HL^{II} > Fe(L^{II})_2 \geqslant Mn(L^{II})_2$; and their reducing ability (determined electrochemically) followed the same order. Spectrophotometric investigation was carried out in order to estimate the rate of the reduction of bovine heart cytochrome *c* with the ligands and their metal(II) complexes. NADPH:cytochrome P450-reductase was found to increase the rate of cytochrome *c* reduction with HL^I and HL^{II} ligands, while adrenodoxin in couple with NAD(P)H: adrenodoxin reductase had no substantial effect thereon. It was shown that the reduction of cytochrome *c* with these compounds cannot be related solely to the facility of their oxidation or ionization.

1. Introduction

Biomedical investigations have a significant place among various aspects of bio-coordination chemistry. They comprise both development of new bioactive coordination compounds and investigation of plausible mechanisms of their interaction with cell components. Electron transport is known to be one of the key forms of the cell metabolism. In particular, it provides the processes of biological oxidation (respiration) localized in mitochondrions [1]. Research on the effect of some transition metal complexes upon fungal and mammalian cell organelles has shown that they have the potential to damage mitochondrial function and uncouple respiration [2–6]. In this connection it may be expected that metal complexes able to participate in redox processes and affect the electron-transport cell systems will be promising in the search for potential chemotherapeutical agents.

Previously we have found that some mono- and di-substituted derivatives of sterically hindered phenolic ligands as well as their complexes with Cu(II), Co(II), Ni(II) ions exhibit antiviral, antibacterial and antifungal activities, the complexes mostly demonstrating a higher level of activity than the parent ligands [7–12]. Furthermore, using the method of cyclic voltammetry, we have shown these compounds to be also of a pronounced reducing ability correlating with antifungal activity in a limited series of these compounds [11,12]. This allowed us to suggest that redox processes could play an important part in realizing the mechanism of biological activity of the said compounds. Thus, one of the possible types of their biological macromolecular targets may be comprised by oxidoreductases which are components of mammalian electron transport chains, namely: cytochrome *c* (Cyt *c*), NADPH: cytochrome P450-reductase (P450R), NAD(P)H: adrenodoxin (Adx) and adrenodoxin reductase (AdR) [13]. There are a number of reasons motivating interest in these particular electron-transfer proteins. Cyt *c* transfers electrons from the complex III (coenzyme Q-cytochrome *c* reductase) to complex IV (cytochrome *c* oxidase) in the mitochondrial respiratory chain [14,15]. It is noteworthy that mammalian Cyt *c* is able to oxidize different catechols, quinols and *o*-aminophenols into respective quinons [16–19]. P450R is an electron-donating partner of cytochromes P450 of endoplasmic reticulum [20,21]. Adx in couple with AdR maintain the activity of cytochromes P450 in mitochondrions of adrenal cortex [22,23]. P450R and AdR accept two electrons from NADPH and transfer them one after another to their oxidizer partners. Catalysis by cytochromes P450 of a number of key reactions of metabolism of several classes of endogenic and exogenic compounds including pollutants and the vast majority of medicines makes the

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examination of the interactions of potential medicines with these enzyme systems fundamentally important [24]. Furthermore, P450R and AdR are able to catalyze single-electron reduction of quinons [25–27]. It is quite important that microorganisms possess cytochromes *c* of their own, as well as oxidoreductases with structure–functional properties similar to those of mammalian P450R, AdR or Adx [28]. Some microbial "analogs" of P450R, AdR and Adx are capable of transferring electrons to mammalian Cyt *c* and P450, forming catalytically active artificial systems [22,29–31]. Account should also be taken of the known relation of the above-mentioned enzyme systems to generation of reactive oxygen species and to apoptosis [32–36].

In the present work the reduction of bovine heart Cyt *c* with two derivatives of sterically hindered phenolic ligands – 4,6-di-*tert*-butyl-3[(2-hydroxyethyl)sulphanyl]-1,2-dihydroxybenzene (HL^I) and 2-amino-4,6-di-*tert*-butylphenol (HL^{II}) – as well as with their redox-active transition metal (copper, cobalt, manganese, iron) complexes, and the effect of electron transfer proteins of mammalian P450-dependent monooxygenase systems on this process were investigated spectrophotometrically. We also report here the synthesis and characterization of Fe(II) and Mn(II) complexes with aforesaid ligands in order to compare their coordinative behavior in relation to Fe(II), Mn(II) ions with the results obtained before for their Cu(II) and Co(II) complexes [11,12], and to assess the influence of complexation on their biological activity and redox properties.

The results obtained are discussed in the context of presumed interconnection of the capacity of the compounds under study for reducing Cyt *c*, their antibacterial activity, redox properties determined electrochemically, and other physico-chemical characteristics.

2. Experimental

2.1. Materials and methods

Chemicals were purchased from commercial sources and were used without further purification. 4,6-di-*tert*-butyl-3[(2-hydroxyethyl)sulphanyl]-1,2-dihydroxybenzene (HL^I) and 2-amino-4,6-di-*tert*-butylphenol (HL^{II}) were prepared according to methods reported previously [11,12]. The purity of compounds was checked by TLC. Elemental analyses were carried out with the instrument Vario EL (CHNS mode). Metals and sulfur were determined using an atomic emission spectrometer with an inductively coupled plasma excitation source (Spectroflame Modula). Infrared spectra of solids were recorded with a Nicolet 380 spectrometer in the wavelength range 4000–400 cm^{-1} at room temperature, using «Smart Performer». Thermal analysis was performed with a Simultaneous Thermal Analyzer STA 449 C. XRD analysis was carried out with an HZG 4A diffractometer (Co Kα or Cu Kα radiation, MnO_2-filter). ESR spectra of polycrystalline samples were measured with ERS-220 X-band spectrometer (9.45 GHz) at room temperature and at 77 K, using 100 kHz field modulation; *g* factors were quoted relative to the standard marker DPPH. Magnetic susceptibility was determined with the use of a Gouy balance at room temperature using $Hg[Co(SCN)_4]$ as a calibrant. UV–Vis spectra were recorded with a SPECORD M500 spectrophotometer. The molar conductance of 10^{-3} mol·l^{-1} solutions of the metal(II) complexes in acetonitrile was measured at 20 °C using a TESLA BMS91 conductometer (cell constant 1.0). Electrochemical measurements were performed under dry nitrogen in a three-electrode two-compartment electrochemical cell using a glassy-carbon (GC) working electrode, Pt auxiliary electrode and Ag|AgCl|0.1 mol l^{-1} $(C_2H_5)_4NCl$ reference electrode. The supporting electrolyte was 0.1 mol l^{-1} $(C_2H_5)_4NClO_4$. The Ag|AgCl|0.1 mol l^{-1} $(C_2H_5)_4NCl$ reference electrode was calibrated with the ferrocenium|ferrocene redox couple located at $E_{1/2}$ = +0.52 V. Acetonitrile was used as a solvent.

2.2. Synthesis of the metal(II) complexes with 4,6-di-tert-butyl-3[(2-hydroxyethyl)sulphanyl]-1,2-dihydroxybenzene (HL^I) and 2-amino-4,6-di-tert-butylphenol (HL^{II})

Based on our previous findings [9–12], the preparation of metal(II) complexes followed a common procedure. A solution of 0.050 mmol $M(CH_3COO)_2$ (M = Fe(II) and Mn(II)) in 10 ml of water was added dropwise to a colorless solution of 0.100 mmol of a ligand dissolved in 10 ml of ethanol (molar ratio M(II):L = 1:2). As these ligands can be oxidized by oxygen, argon was bubbled through the solutions (pH ⩽ 6) during the synthesis to ensure the absence of oxygen. Colored precipitates of Fe(II) and Mn(II) complexes formed instantaneously. After 1.5 h stirring, they were collected on membrane filters (JG 0.2 μm), washed with ethanol and water, and dried in vacuo (yield>70 %).

2.2.1. $Cu(L^I)_2$ complex

For elemental analyses data of $Cu(L^I)_2$ see Refs. [9,12].

2.2.2. $Co(L^I)_2$ complex

For elemental analyses data of $Co(L^I)_2$ see Ref. [12].

2.2.3. $Fe(L^I)_2$ complex

Blue. Yield: 70–75%. *Anal* Calc. for $C_{32}H_{50}S_2O_6Fe$: C, 59.09; H, 7.76; S, 9.87; Fe, 9.59. Found: C, 58.95; H, 7.40; S, 9.80; Fe, 9.49%.

2.2.4. $Mn(L^I)_2$ complex

Yellow-green. Yield: 55–60%. *Anal.* Calc. for $C_{32}H_{50}S_2O_6Mn$: C, 59.17; H, 7.77; S, 9.88; Mn, 8.47. Found: C, 59.10; H, 7.68; S, 9.79; Mn, 8.41%.

2.2.5. $Cu(L^{II})_2$ complex

For elemental analyses data of $Cu(L^{II})_2$ see Refs. [10,11].

2.2.6. $Co(L^{II})_2$ complex

For elemental analyses data of $Co(L^{II})_2$ see Ref. [11].

2.2.7. $Fe(L^{II})_2$ complex

Green. Yield: 77–80%. *Anal* Calc. for $C_{28}H_{44}N_2O_2Fe$: C, 67.72; H, 8.94; N, 5.65; Fe, 11.25. Found: C, 67.61; H, 8.82; N, 5.58; Fe, 11.14%.

2.2.8. $Mn(L^{II})_2$ complex

Green. Yield: 80–82%. *Anal.* Calc. for $C_{28}H_{44}N_2O_2Mn$: C, 67.84; H, 8.96; N, 5.65; Mn, 11.09. Found: C, 67.79; H, 8.88; N, 5.57; Mn, 11.01%.

2.3. Physico-chemical characterization

2.3.1. $Cu(L^I)_2$ complex

For physical and spectral characteristics of $Cu(L^I)_2$ see Refs. [9,12].

2.3.2. $Co(L^I)_2$ complex

For physical and spectral characteristics of $Co(L^I)_2$ see Ref. [12].

2.3.3. $Fe(L^I)_2$ complex

Molar conductivity (in acetonitrile): Λ_{mol} = 6.9 Ω^{-1} cm^2 mol^{-1}. TG/DTA data: no weight loss was observed until decomposition which began about 190 °C, with an endothermic peak at 240 °C and an exothermic one at 350 °C, ultimately leaving FeO as the residue. The maximal weight loss of 87.8% corresponds to the loss of two ligand molecules in the $Fe(L^I)_2$ complex (Calc. 88.9%). Promi-

nent IR absorption bands (ν, cm^{-1}): 3360w (O–H), 1543w and 1481w (C=C arom), 1180w and 1058m (C–O), 812w and 619m (C–S), 592m and 553m (Fe–O). UV–Vis data (acetonitrile) (λ_{max}, nm (lg ε)): 590 (3.34), 415 (3.29), 305 (3.94), 255 (3.99), 225 (4.45). μ_{eff} (RT, BM) = 0.

2.3.4. $Mn(L^I)_2$ complex

Molar conductivity (in acetonitrile): Λ_{mol} = 3.5 Ω^{-1} cm^2 mol^{-1}. TG/DTA data: no weight loss was observed until decomposition which began about 220 °C, with an endothermic peak at 285 °C and an exothermic one at 360 °C, ultimately leaving MnO as the residue. The maximal weight loss of 86.6% corresponds to the loss of two ligand molecules in the $Mn(L^I)_2$ complex (Calc. 87.5%). Prominent IR absorption bands (ν, cm^{-1}): 3425w (O–H), 1586w and 1511w (C=C arom), 1166m, 1069m and 1019m (C–O), 818w, 647m and 628m (C–S), 560w and 510w (Mn–O), 345w (Mn–S). UV–Vis data (acetonitrile) (λ_{max}, nm (lg ε)): 570 (3.39), 410 (3.55), 370 (3.56), 310 (3.65), 255 (3.74), 225 (3.96). ESR data: g_{iso}=2.012. μ_{eff} (RT, BM) = 1.75.

2.3.5. $Cu(L^{II})_2$ complex

For physical and spectral characteristics of $Cu(L^{II})_2$ see Refs. [10,11].

2.3.6. $Co(L^{II})_2$ complex

For physical and spectral characteristics of $Co(L^{II})_2$ see Ref. [11].

2.3.7. $Fe(L^{II})_2$ complex

Molar conductivity (in acetonitrile): Λ_{mol} = 5.3 Ω^{-1} cm^2 mol^{-1}. TG/DTA data: no weight loss was observed until decomposition which began about 170 °C, with an endothermic peak at 225 °C and an exothermic one at 330 °C, ultimately leaving FeO as the residue. The maximal weight loss of 84.4% corresponds to the loss of two ligand molecules in the $Fe(L^{II})_2$ complex (Calc. 85.5%). Prominent IR absorption bands (ν, cm^{-1}): 3367m (N–H), 1593w and 1484m (C=C arom), 1244m (C–N), 1097w, 1038w and 1022m (C–O), 596m and 563m (Fe–O), 485w (Fe–N). UV–Vis data (acetonitrile) (λ_{max}, nm (lg ε)): 600 (3.17), 440 (3.35), 400 (3.39), 350 (3.64), 285 (3.80), 240 (4.13). μ_{eff} (RT, BM) = 0.

2.3.8. $Mn(L^{II})_2$ complex

Molar conductivity (in acetonitrile): Λ_{mol} = 3.9 Ω^{-1} cm^2 mol^{-1}. TG/DTA data: no weight loss was observed until decomposition which began about 180 °C, with two endothermic peaks, at 185 °C (without any noticeable weight loss) and at 282 °C, and an exothermic one at 410 °C, ultimately leaving MnO as the residue. The maximal weight loss of 84.6% corresponds to the loss of two ligand molecules in the $Mn(L^{II})_2$ complex (Calc. 85.7%). Prominent IR absorption bands (ν, cm^{-1}): 3351m (N–H), 1598m and 1495m (C=C arom), 1283m (C–N), 1159s, 1100s, 1047m and 1008m (C–O), 543m and 526w (Mn–O), 427w (Mn–N). UV–Vis data (acetonitrile) (λ_{max}, nm (lg ε)): 590 (3.24), 480 (3.27), 420 (3.27), 350 (3.42), 290 (3.42), 235 (3.50). ESR data: g_{iso} = 2.04. μ_{eff} (RT, BM) = 1.68.

2.4. Antibacterial assays

The following test microorganisms (collection of Department of Microbiology, Belarusian State University) were used: *Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens, Salmonella typhimurium*, *Bacillus subtilis, Sarcina lutea, Staphylococcus saprophyticus, Staphylococcus aureus, Mycobacterium smegmatis.* Antibacterial activity of the compounds was estimated by a minimum inhibitory concentration (MIC, μg ml^{-1}) as described elsewhere [7,37]. The compounds being tested were dissolved in dimethyl sulfoxide (DMSO). A twofold serial dilution from 200 to 3.1 μg ml^{-1} was used. The absence of microbial growth after an incubation period of 48 h at 37 °C for bacteria was taken to be a criterion of effectiveness. In every case MIC was determined as the lowest concentration of the compound under study which inhibits the visible microbial growth, compared with the control system in which the microorganisms were grown in the absence of any test compound. The amount of DMSO in the medium was 1% and did not affect the growth of the tested microorganisms. There were three replicates for each dilution. Results were always verified in three separate experiments.

2.5. Reduction of cytochrome c

Bovine heart cytochrome *c* (Sigma) was used. Cytochrome P450 cholesterol side chain cleavage (CYP11A1, P450scc), Adx and AdR were isolated from bovine adrenal cortex according to [38]. Human P450R was purified according to [39]. Spectrophotometric experiments were performed with a Shimadzu UV-1202 spectrophotometer using quartz cuvette with 1 cm optical path. Cyt *c* concentration was determined on its interaction with excess sodium dithionite, using the absorption coefficient ε_{550} = 21 $mmol^{-1}$ l cm^{-1} [40]. Ar-saturated acetonitrile solutions of the ligands and complexes under study and Cyt *c* (7 μmol l^{-1}) were used. Experiments were performed in 10 mmol·l^{-1} sodium phosphate buffer (pH 7.4) at 20 °C. Aliquots of the compounds under study were added to Cyt *c* solution up to the final concentrations 35.0 or 17.4 μmol l^{-1}. If necessary, P450R, Adx and AdR were first added to Cyt *c*. The initial rate of Cyt *c* reduction (ν) was evaluated by the slope of the kinetic curve A_{550} versus time according to [41,42]. The results were confirmed in three independent experiments.

3. Results and discussion

3.1. Physico-chemical characterization

The solid products resulting from the interaction of Fe(II) and Mn(II) ions with HL^I and HL^{II} ligands were well characterized by means of elemental analysis, TG/DTA, FT-IR, UV–Vis, ESR, magnetic susceptibility and conductance measurements. The elemental analyses data for the Fe(II) and Mn(II) complexes are in agreement with the general formula ML_2 (Sections 2.2.3, 2.2.4, 2.2.7 and 2.2.8). Thermal analysis in air flow with identification of the final products by X-ray powder diffraction has shown all the complexes to be anhydrous and unsolvated, because their DTA curves lack any endothermic peaks over a wide range from 70 to 160 °C. The agreement between the experimental and theoretical weight losses for the above processes confirms the above-mentioned general formula of the metal complexes (Sections 2.3.3, 2.3.4, 2.3.7 and 2.3.8).

These complexes were insoluble in water, ethanol, methanol, diethyl ether, but they were soluble in acetonitrile and dimethyl sulfoxide. The conductivity data indicate their being essentially non-electrolytes in acetonitrile [43], and suggest that the two bidentate ligands may be coordinated to Fe(II) and Mn(II) ions as monoanionic species.

All the complexes were characterized by X-ray patterns of their own, differing significantly from that of the ligand. However, a full structural analysis could not be performed because no single crystals suitable for X-ray diffraction studies were obtained. Because of the well-known difficulties in direct X-ray investigations of polycrystalline metal complexes with sterically hindered ligands [44,45], the geometrical arrangement of the ligating atoms in the metal complexes was investigated by several spectroscopic and magnetochemical methods.

To specify the coordination cores in the Fe(II) and Mn(II) complexes, we used IR spectroscopy (Sections 2.3.3, 2.3.4, 2.3.7 and 2.3.8). In the spectra of the ligands HL^I and HL^{II} there are some bands in the range from 3536 to 3290 cm^{-1}, which may be assigned to the stretching vibrations of hydroxyls [46]. In the spectra of $Fe(L^I)_2$ and $Mn(L^I)_2$ complexes only one band at 3360 cm^{-1} is observed in this region, and it is absent in the spectra of $Fe(L^{II})_2$ and $Mn(L^{II})_2$ complexes, which suggests the ligand being coordinated in the form of monoanion. The bands at 1200–1060 cm^{-1} in the spectra of the ligands, assigned to the vibrations of C–O bond, are shifted towards lower frequencies in the spectra of metal(II) complexes, indicating metal(II) being coordinated with hydroxyl of the ligand [46]. The changes in the frequencies of (C=C) stretching vibrations of aromatic ring in the spectra of metal complexes compared to those of the ligand HL^I (1604, 1561 cm^{-1}) or the ligand HL^{II} (1593 cm^{-1}) also are evidence in favor of the coordination bond formation. The shift of the bands at 850 and 680 cm^{-1} (assigned to C–S bond vibrations) in the spectra of $Fe(L^I)_2$, $Mn(L^I)_2$ complexes as well as of the band at 1292 cm^{-1} (assigned to C–N bond vibrations) in the spectra of $Fe(L^{II})_2$, $Mn(L^{II})_2$ complexes to the low-frequency region suggests that sulfur and nitrogen are involved in the complexation. It should be noted that in the spectra of all the complexes under study there are new bands in the region from 596 to 480 cm^{-1}, which may be assigned to the stretching vibrations of M–O and M–N bonds. M–S stretching vibration frequencies are registered in the region 350–320 cm^{-1}. The above-listed facts suggest that sulphanyl and amino groups as well as deprotonated hydroxyls take part in forming the MS_2O_2 or MN_2O_2 coordination cores in the metal(II) complexes.

The ESR spectra of the $Mn(L^I)_2$ and $Mn(L^{II})_2$ complexes show a broad isotropic signal (200–280 G) at liquid nitrogen temperature with g_{iso} values of 2.012 and 2.04, respectively. According to [47,48], such parameters are characteristic of Mn(II) complexes with a distorted form of coordination polyhedrons and with virtually no interaction between manganese atoms. No signal of stabilized radicals present in ESR spectra as well as the ν(C=O) stretching vibrations lacking in IR spectra of metal(II) complexes, respectively in the ranges of 1400–1500 cm^{-1} confirm the phenolate character of the ligands.

In the electronic absorption spectra of the $Fe(L^I)_2$, $Mn(L^I)_2$, $Fe(L^{II})_2$ and $Mn(L^{II})_2$ complexes in acetonitrile solution the absorption maxima in the high-energy region 225–350 nm belong to intraligand transitions (Sections 2.3.3, 2.3.4, 2.3.7 and 2.3.8). The absorption maxima appearing in the spectra of the $Fe(L^I)_2$ and $Mn(L^I)_2$ at 305–310 nm and 370–415 nm are indicative of the ligand-to-metal(II) charge transfer transitions, respectively $S \rightarrow M^{II}$ and $O_{phenolate} \rightarrow M^{II}$ [49]. The spectra of the $Fe(L^{II})_2$ and $Mn(L^{II})_2$ complexes exhibit absorption bands at 405–420 nm and 440–480 nm, which can be attributed, respectively to $N(\sigma) \rightarrow M^{II}$ and $O_{phenolate} \rightarrow M^{II}$ charge transfer transitions [49,50].

According to the literature [49,51–53], the absorption maxima observed in the spectra of $Fe(L^I)_2$ and $Fe(L^{II})_2$ complexes about 590–600 nm are due to *d–d* transitions characteristic of low-spin Fe(II) complexes with the square planar (or near to it) form of coordination polyhedron [52,54]. This conclusion is in agreement with ESR-spectroscopic data and values of effective magnetic moments of $Fe(L^I)_2$ and $Fe(L^{II})_2$ complexes ($\mu_{eff} = 0$), indicative of their diamagnetism. Hence, the ligands HL^I and HL^{II} exert the crystal field strong enough for spin coupling in Fe(II) ion and form low-spin complexes with this ion. Spin coupling is also characteristic of Mn(II) ion in $Mn(L^I)_2$ and $Mn(L^{II})_2$ complexes, which is supported by the values of effective magnetic moment μ_{eff} of these complexes (1.75 and 1.68 BM). Note that most of the scarce low-spin Mn(II) complexes known contain redox-active ligands, and their absorption spectra have *d–d* bands at 380–400 nm and 520–590 nm, which are characteristic of the square planar chromophore [47,49,55–57]. These bands were found in the spectra of $Mn(L^I)_2$ and $Mn(L^{II})_2$ complexes, which may be indicative of the square planar form of their MnS_2O_2 и MnN_2O_2 chromophores.

This view of coordination cores agrees with the data obtained by physico-chemical methods for the Cu(II) and Co(II) complexes of the ligands HL^I and HL^{II} investigated previously [9,10]. In the light of the spectral data, magnetic moment, and analytical results the mode of bonding in the metal(II) complexes can be represented as shown below (Scheme 1):

The electrochemical redox properties of the ligands and their metal(II) complexes were investigated in de-aerated acetonitrile solution of these compounds by cyclic voltammetry in the potential range from −2.2 to +2.2 V (for full experimental details see the Section 2.1). Selected data are presented in Table 1. The data obtained allow one to make conclusions about the reducing ability of the compounds under study. In this connection it was taken into consideration that *o*-diphenol derivatives readily undergo electrochemical oxidation to give respective semiquinones and benzoquinones [58–60]; besides, oxidation of sulfur atom in the side chain of the ligand HL^I is possible. The investigation carried out showed that the ligand HL^I and its metal complexes can be arranged into a sequence according to their reducing ability, the formal potential of the redox system $E^1_{1/2}$ being used as its criterion (according to [61]):

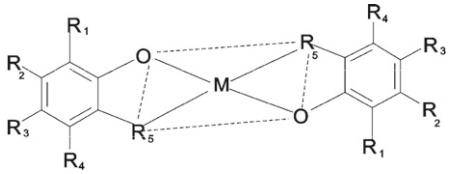
$Cu(L^I)_2 > Co(L^I)_2 > Fe(L^I)_2 \geqslant Mn(L^I)_2 > HL^I$ (Table 1). The formal potential is calculated as the average potential of the peaks found by the cyclic voltammetry method: $E^1_{1/2} = (E^1_{pa} + E^1_{pc})/2$. These findings show that the processes of oxidation for these metal complexes begin at potentials much more cathodic than those for the ligand HL^I (Table 1). Comparison of the redox properties of the ligand HL^{II} and its metal(II) complexes shows that their reducing ability follows the order: $Cu(L^{II})_2 > Co(L^{II})_2 > HL^{II} \approx Fe(L^{II})_2 > Mn(L^{II})_2$. It is $Cu(L^{II})_2$ and $Co(L^{II})_2$ complexes that are characterized by the most cathodic potentials in these processes, with $Fe(L^{II})_2$ complex and the ligand HL^{II} ranking next, their redox properties being virtually the same, while redox processes for $Mn(L^{II})_2$ begin at much more anodic potentials (Table 1). Thus, in the series of the compounds studied it is Cu(II) complexes that are the most readily oxidized.

3.2. Biological evaluation

3.2.1. Antibacterial activity

MIC values of the ligands HL^I and HL^{II} and their Mn(II), Fe(II), Co(II), Cu(II) complexes are listed in Table 2. For the ligands and their Cu(II) complexes, continuing our previous study [7,10], we have expanded the spectrum of Gram-positive bacteria, and it was for the first time that the antibacterial activity of Mn(II), Fe(II), Co(II) complexes of these ligands was investigated. Commonly used antibiotics streptomycin, tetracycline and chloramphenicol were tested as positive controls.

Evaluating the antibacterial activity of the ligands and their metal(II) complexes, we can note that they demonstrated a low inhib-



$M(L^I)_2$: R_1=OH, R_2= R_4=$C(CH_3)_3$, R_3=H, R_5=$S(CH_2)_2OH$, M=Fe(II), Mn(II)

$M(L^{II})_2$: R_1= R_3= $C(CH_3)_3$, R_2= R_4=H, R_5=NH_2, M=Fe(II), Mn(II)

Scheme 1.

Table 1
Cyclic voltammetry data (anodic scan) for the ligands HL^{I}, HL^{II} and their metal(II) complexes.

Compound	E^1_{pa} (V)	E^1_{pc} (V)	$E^1_{1/2}$ (V)	E^2_{pa} (V)	E^2_{pc} (V)	E^3_{pa} (V)	E^3_{pc} (V)	E^4_{pa} (V)	E^4_{pc} (V)	E^5_{pa} (V)
HL^{I}[a]	1.20	0.45	0.83	1.40[c]		1.77[c]		2.10[c]		
$Cu(L^{I})_2$[a]	0.23	−0.02	0.11	1.20[c]		1.40[c]		1.75[c]		2.10[c]
$Co(L^{I})_2$[a]	0.87	0.34	0.61	1.20[c]		1.40[c]		1.75[c]		2.10[c]
$Mn(L^{I})_2$	1.20	0.26	0.73	1.40[c]		1.70[c]		1.93[c]		
$Fe(L^{I})_2$	1.05	0.39	0.72	1.40[c]		1.70[c]				
HL^{II}[b]	0.58	0.49	0.53	1.72[c]						
$Cu(L^{II})_2$[b]	0.27	0.14	0.21	0.61	0.23	0.89	0.51	1.27	1.07	
$Co(L^{II})_2$[b]	0.65	0.22	0.44	1.20	0.53					
$Mn(L^{II})_2$	0.95	0.59	0.77							
$Fe(L^{II})_2$	0.57	0.48	0.53	1.70[c]						

[a] Ref. [12].
[b] Ref. [11].
[c] Irreversible process, it is just the anodic peak that is observed.

Table 2
Antibacterial activity of the free ligands and their metal(II) complexes evaluated by minimum inhibitory concentration (MIC, μg ml^{-1}).

Compound	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Salmonella typhimurium*	*Escherichia coli*	*Bacillus subtilis*	*Sarcina lutea*	*Staphylococcus saprophyticus*	*Staphylococcus aureus*	*Mycobacterium smegmatis*
HL^{I}	>100[a]	>100[a]	>100	>100[a]	>100	>100	25[a]	>100	50
$Cu(L^{I})_2$	>100[a]	>100[a]	>100	>100[a]	12.5[a]	6.2[a]	12.5[a]	6.2	12.5
$Co(L^{I})_2$	>100	>100	>100	>100	12.5	12.5	25	25	12.5
$Fe(L^{I})_2$	>100	>100	>100	>100	100	25	100	100	25
$Mn(L^{I})_2$	>100	>100	>100	>100	100	50	100	100	25
HL^{II}	>100[b]	>100[b]	>100	>100[b]	25[b]	12.5[b]	12.5[b]	12.5	12.5
$Cu(L^{II})_2$	>100[b]	>100[b]	>100	>100[b]	6.2[b]	3.1[b]	6.2[b]	6.2	6.2
$Co(L^{II})_2$	>100	>100	>100	>100	12.5	6.2	6.2	12.5	12.5
$Fe(L^{II})_2$	>100	>100	>100	>100	50	12.5	50	50	12.5
$Mn(L^{II})_2$	>100	>100	>100	>100	>100	>100	50	50	>100
Streptomycin	>100	6.2	12.5	<3.1	6.2	12.5	6.2	3.1	3.1
Tetracycline			6.2	3.1	6.2	6.2	6.2	<3.1	
Chloramphenicol	12.5		6.2	6.2	<3.1		6.2	6.2	12.5

[a] Ref. [7].
[b] Ref. [10].

iting ability toward Gram-negative bacteria: MIC > 100 μg ml^{-1} (Table 2). The results of estimating their antibacterial activity against Gram-positive bacteria are informative enough. The activity of the Cu(II) and Co(II) complexes against most of Gram-positive bacteria is higher than that of the ligands HL^{I} and HL^{II} (Table 2). Gram-positive bacteria are more sensitive to these complexes and give no growth if concentrations of the complexes are above 6.2 μg ml^{-1}. The high activity of the metal(II) complexes under study against *Mycobacterium smegmatis* is worthy of particular notice. $Cu(L^{II})_2$ complex is more active against *Bacillus subtilis*, *Sarcina lutea* and *Mycobacterium smegmatis* than the ligands and other metal(II) complexes. In general, we have revealed a significant difference of MIC for $Cu(L^{I})_2$, $Co(L^{I})_2$, $Cu(L^{II})_2$, $Co(L^{II})_2$ and $Fe(L^{I})_2$, $Mn(L^{I})_2$, $Fe(L^{II})_2$, $Mn(L^{II})_2$ complexes against *Bacillus subtilis*, *Sarcina lutea*, *Staphylococcus saprophyticus, Staphylococcus aureus* and *Mycobacterium smegmatis* (Table 2).

None of the starting metal salts acts against bacteria up to the dose of 200 μg ml^{-1}. Data reported in Table 2 clearly point out that in the cases mentioned above the antibacterial activity of the metal(II) complexes synthesized does not correlate with the toxicity of the metal(II) ions to the bacteria tested.

It is noteworthy that by and large the antibacterial activity of the compounds examined follows the order: (1) $Cu(L^{I})_2 > Co(L^{I})_2 > Fe(L^{I})_2 \geqslant Mn(L^{I})_2 > HL^{I}$; (2) $Cu(L^{II})_2 > Co(L^{II})_2 > HL^{II} > Fe(L^{II})_2 \geqslant Mn(L^{II})_2$; and their reducing ability (determined electrochemically) follows the same order, as it was shown above (Table 1).

When evaluating the bioactivity of the metal complexes against several bacteria it can be noted that the Cu(II) and Co(II) complexes may be considered as potential antibacterial agents, particularly when their activity is comparable with the inhibiting effect of such antibiotics as streptomycin, tetracycline and chloramphenicol (Table 2).

3.2.2. Reduction of cytochrome c

The results of spectrophotometric investigation of redox interaction of oxidized form of Cyt *c* with the HL^{I} and HL^{II} ligands and their Mn(II), Fe(II), Co(II) and Cu(II) complexes are given in Tables 3 and 4, and Fig. 1. The characteristic absorption bands at 550 and 520 nm appearing when the ligands or their metal complexes are added to the solution of the oxidized Cyt *c* bear witness to the ability of these compounds to reduce Cyt *c* in vitro, thus substantiating the previous findings [16–19], according to which different catechols, quinols and *o*-aminophenols transfer electrons in an outer-sphere process involving the exposed heme edge surrounded by positively charged amino acid residues; aromatic redox active amino acid residues also can be potential participants of the electron

Table 3
Rates of reduction of Cyt *c* (v) with the ligands HL^{I}, HL^{II}, and their metal(II) complexes.[a]

Compound	v (nmol min^{-1})	Compound	v (nmol min^{-1})
HL^{I}	3.1	HL^{II}	10.0
$Cu(L^{I})_2$	2.4	$Cu(L^{II})_2$	4.0
$Co(L^{I})_2$	1.9	$Co(L^{II})_2$	5.8
$Fe(L^{I})_2$	0.9	$Fe(L^{II})_2$	7.0
$Mn(L^{I})_2$	2.2	$Mn(L^{II})_2$	5.7

[a] The final concentrations of Cyt *c* and the complex (or the ligand) were respectively, 7 and 35 μmol l^{-1}.

Table 4
Effect of electron-transfer proteins of P450-dependent monooxygenase systems on the rate of Cyt *c* (7 μmol l^{-1}) reduction (*v*) with HL^{I} and HL^{II} ligands (17.4 μmol l^{-1}).

Ligand	Enzyme/concentration (nmol l^{-1})	*v* (nmol min^{-1})	Ligand	Enzyme/concentration (nmol l^{-1})	*v* (nmol min^{-1})
HL^{I}		1.0	HL^{II}		6.1
HL^{I}	P450R/170	1.3	HL^{II}	P450R/170	17.2
HL^{I}	P450R/330	1.9	HL^{II}	P450R/330	28.0
HL^{I}	Adx + AdR/200 + 200	1.0	HL^{II}	Adx + AdR/200 + 200	6.2

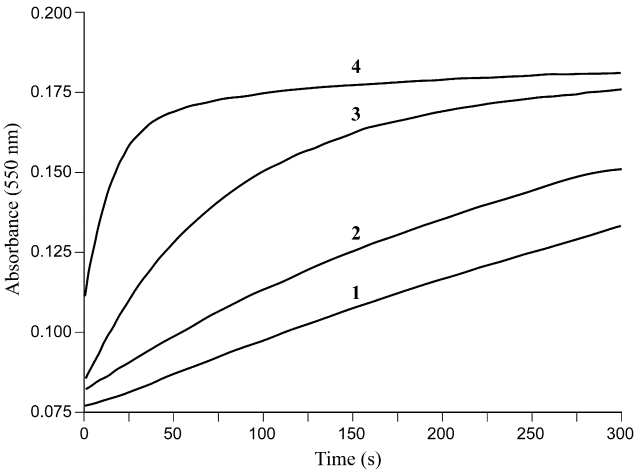


Fig. 1. Kinetic data for a change in absorbance of the Cyt *c* solution (at 550 nm) after adding the ligand HL^{I} (without P450R (1) and with P450R (2)) and the ligand HL^{II} (without P450R (3) and with P450R (4)). Concentration of the ligands HL^{I} and HL^{II} is 17 μmol l^{-1}.

transfer [17]. On the other hand, these ligands were unable to reduce P450scc under the same experimental conditions (save that the buffer was supplemented with 0.1% of sodium chlorate to solubilize P450).

We have found that the rate of Cyt *c* reduction with HL^{II} ligand is higher than that with HL^{I} ligand (Table 3). Taking into account the findings presented in [16–19], it can be suggested that the most probable route of oxidation of the ligands HL^{I} and HL^{II} in the system of interest in vitro under anaerobic conditions involves single-electron stages of their monoanionic forms being oxidized to *o*-benzoquinones upon interacting with Cyt *c* via intermediate formation of respective *o*-benzosemiquinone or *o*-iminobenzosemiquinone. The higher rate of reduction with the ligand HL^{II} compared to that with the ligand HL^{I} can be due first of all to a higher reducing ability of the former (Section 3.1, Table 1), as their ionization constants, characterizing their ability to form monoanions by hydroxyls being deprotonated, are virtually comparable (pH 9.5–9.9 [8]).

Metal complexes of the ligands HL^{I} and HL^{II} also reduce Cyt *c*. It should be taken into account that metal complexes can interact with Cyt *c* both in their molecular form and via ligand monoanions and metal ions formed upon their dissociation. In this connection the fact that the rates of Cyt *c* reduction with metal complexes and with respective ligands differ can be due to steric and diffusion factors. Furthermore, attention should be paid to the values of stability constants of these metal complexes as well as to the ability of the respective metal ions to reduce Cyt *c* (for example, Fe(II) [62], Co(II) [63]) or to oxidize its reduced form (for example, Cu(II) [64,65]). The values of stability constants for the complexes under investigation were previously determined in our studies [8,66] and vary in the range of lg β = 5.5–7.2. $Cu(L^{II})_2$ is the most stable complex (lg β = 7.2). The complexes $Cu(L^{I})_2$, $Co(L^{I})_2$ and $Co(L^{II})_2$ have intermediate stability constant values (lg β = 6.6–6.7), while $Fe(L^{I})_2$, $Mn(L^{I})_2$, $Fe(L^{II})_2$ and $Mn(L^{II})_2$ are the less stable complexes (lg β = 5.5–5.7).

The complex $Cu(L^{I})_2$ in the series of metal complexes with HL^{I} ligand had the highest *v* value (Table 3); this complex also exhibits the highest reducing ability (determined electrochemically) in this series (Table 1). The rates of Cyt *c* reduction with $Co(L^{I})_2$ and $Mn(L^{I})_2$ complexes are comparable in values, but these complexes are noticeably different in their reducing ability (Tables 1 and 3). The complex $Fe(L^{I})_2$, while having virtually the same reducing ability (determined electrochemically) as that of $Mn(L^{I})_2$ (Table 1), ranks substantially below the latter and the other complexes of HL^{I} in the rate of Cyt *c* reduction (Table 3).

In the series of complexes with the ligand HL^{II} the complex $Fe(L^{II})_2$ shows the highest rate of Cyt *c* reduction, approaching the rate of redox process with HL^{II} ligand participating (Table 3). It should be emphasized that their reducing ability (determined electrochemically) is virtually the same (Table 1), even though they are not the most active reductants in this series of compounds and are characterized by less cathodic $(E^{a}_{p1} + E^{c}_{p1})/2$ values than those for $Cu(L^{II})_2$ and $Co(L^{II})_2$. Nevertheless, the latter two didn't show a high rate on reducing Cyt *c* (Table 3), apparently because their stability constants are 1–1.5 orders of magnitude higher than that of the complex $Fe(L^{II})_2$. Hence, on ionization these complexes less readily form ligand monoanions which are the most probable reducing agents for Cyt *c*. The results obtained suggest that ionization of metal complexes can play a significant part in Cyt *c* reduction. The rates of Cyt *c* reduction with the complexes $Co(L^{II})_2$ and $Mn(L^{II})_2$ turned out to be comparable (Table 3), even though the complexes differ significantly in their reducing ability (determined electrochemically) (Table 1).

P450R was found to catalyze Cyt *c* reduction with HL^{I} and HL^{II} ligands in concentration-dependent manner (Table 4 and Fig. 1). Thus, when P450R is added to Cyt *c* solution in molar ratio of about 1:20, the rate of its reduction with HL^{I} and HL^{II} ligands is increased, respectively 2 and 4.5 times, which is in line with their particular reducing abilities. P450R is known to be capable of catalyzing one-electron reduction of Cyt *c* and various quinones [25,26, 34,35]. On this basis we suggested that P450R can accept electrons from HL^{I} and HL^{II} ligands (or their semiquinone forms) and then transfer them to Cyt *c*, providing an additional route for its reduction.

Note that using mitochondrial P450-dependent electron-transfer system (Adx in couple with AdR) did not effect an increase in the rate of Cyt *c* reduction with HL^{I} and HL^{II} ligands under the experimental conditions (Table 4), although AdR is known to be capable of catalyzing the reduction of various quinones [27], and the system Adx–AdR – of catalyzing Cyt *c* reduction [23].

In spite of the fact that in some cases we have found a correlation between the rates of Cyt *c* reduction and physico-chemical characteristics of the compounds under study, the reduction of Cyt *c* with these compounds may not be related solely to the facility of their oxidation or ionization [67,68].

4. Conclusion

The ability of two derivatives of sterically hindered phenolic ligands HL^{I} and HL^{II} and their complexes with redox active Cu(II), Co(II), Mn(II), Fe(II) ions to reduce mammalian Cyt *c* was

demonstrated by means of absorption spectrophotometry. $Cu(L^I)_2$, HL^I, $Fe(L^{II})_2$, and HL^{II} were found to be characterized by the highest rates of Cyt *c* reduction. It points to the fact that there is no direct correlation between redox or some other physico-chemical properties of the compounds under study and their ability to enter into redox interaction with biological subjects. Hence, the reduction of Cyt *c* by the ligands and their Cu(II), Co(II), Fe(II) and Mn(II) complexes may not be related solely to the facility of their oxidation or ionization. P450R was also shown to increase the rate of Cyt *c* reduction with HL^I and HL^{II} ligands, probably due to providing an additional route of transferring electrons to Cyt *c*.

The antibacterial activity of these compounds was found to follow the order: (1) $Cu(L^I)_2 > Co(L^I)_2 > Fe(L^I)_2 \geqslant Mn(L^I)_2 > HL^I$; (2) $Cu(L^{II})_2 > Co(L^{II})_2 > HL^{II} > Fe(L^{II})_2 \geqslant Mn(L^{II})_2$, and their reducing ability (determined electrochemically) followed the same order. These sequences are not entirely the same as those characterizing the decrease of the rates of Cyt *c* reduction with the ligands and their metal complexes: (1) $HL^I > Cu(L^I)_2 \geqslant Mn(L^I)_2 \geqslant Co(L^I)_2 > Fe(L^I)_2$; (2) $HL^{II} > Fe(L^{II})_2 > Mn(L^{II})_2 \sim Co(L^{II})_2 > Cu(L^{II})_2$. Nevertheless, the results obtained bring out clearly that redox interaction with oxidoreductases as macromolecular targets can be essential for realizing their biological effects. It should be noted that when evaluating the above-mentioned compounds as potential antibacterial and antioxidant agents, their ability to reduce Cyt *c* in mammalian mitochondrions should be regarded as either a side or a pharmacological effect. It is of particular importance in the case of metal complexes more lipophilic in comparison with parent ligands.

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