Copper complexes with nitronylnitroxyl radicals as lipoxygenase inhibitors with antioxidant activity*

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The effects of the complexes $Cu(L^RH)_2(NO_3)_2$ (R = H, Me) with the nitronylnitroxyl radicals L^RH on the lipoxygenase (LOX-1) activity upon peroxidation of linoleic acid were studied. The complexes under study are reversible enzyme inhibitors, competitive and non-competitive inhibition type being observed for the complex with the methyl-substituted and unsubstituted ligands, respectively. The results of molecular docking showed that the complexes under study are incorporated into the hydrophobic pocket of the LOX-1 active site and the change in the type of inhibition can be explained by introduction of additional methyl group into the imidazole fragment of the ligand, which provides a slightly more favorable interaction of the inhibitor with the enzyme and results in the non-competitive mechanism of inhibition. The starting diamagnetic compounds (L^RH_2), nitronylnitroxyl radicals (L^RH), and Cu^{II} complexes exhibit high activity in the nonenzymatic peroxidation of linoleic acid, as well as in generation of the superoxide radical anion $O_2^{\cdot -}$ in the xanthine—xanthine oxidase system.

Key words: nitronylnitroxyl radicals, copper complexes, lipoxygenase, linoleic acid, xanthine oxidase, antioxidants, inhibitors, molecular docking.

Lipoxygenases form a family of non-heme iron-containing dioxygenases, which catalyze regio- and stereoselective oxidation of polyunsaturated fatty (arachidonic, linoleic, linolenic) acids containing one or more (1*Z*,4*Z*)-pentadiene fragments to the corresponding (1*S*,2*E*,4*Z*)-hydroperoxides.[1,2] This reaction relates to the radical peroxidation processes and is the first step in the biosynthesis of leukotrienes, which are mediators of various inflammatory processes and allergic reactions involved in the pathogenesis of neoplastic diseases, asthma, and atherosclerosis.[3—7] In addition, the side products of the lipoxygenase reaction are reactive oxygen species (ROS), *viz.*, superoxide radical anion and hydrogen peroxide, which determines also the role of lipoxygenase in the pathogenesis of diseases induced by oxidative stress.[8]

Thus, lipoxygenase is an important pharmaceutical target and, therefore, many attempts have been made in recent years to find a selective lipoxygenase inhibitor.[9—12] However, only one medical product for treatment of asthma, *viz.*, Zileuton® (see Ref. 13), has entered the market, but this drug was ineffective in the treatment of other diseases whose pathogenesis is associated with leukotrienes. Therefore, the search for novel compounds capable of inhibiting lipoxygenase effectively and reversibly is of current concern.

The search for inhibitors is carried out among organic compounds; however, the data on the ability of metal complexes, for example, the copper complexes with diene and heterocyclic ligands,[14] to exhibit inhibiting activity towards lipoxygenase have been obtained recently.[15]

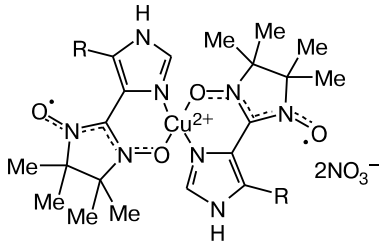
The stable nitroxyl radicals, which are inhibitors of the ROS formation, antioxidants, radioprotectors, contrast agents, and antiproliferative agents, attract attention of researchers in recent years.[16] Great advances have been achieved in the development of the methods for the preparation of metal complexes with ligands, *viz.*, nitroxyl radicals, and investigation of wide range of their properties.[17—19]

The aim of the present work is to study the effects of the Cu^{II} complexes with the nitronylnitroxyl radicals $Cu(L^RH)_2(NO_3)_2$ (**1a**,**b**) on the enzymatic activity of lipoxygenase upon peroxidation of linoleic acid, as well as upon generation of the superoxide radical anion $O_2^{\cdot -}$ in

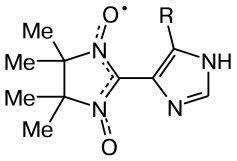
* Dedicated to the Academician of the Russian Academy of Sciences R. Z. Sagdeev on the occasion of his 70th birthday.

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 12, pp. 2514—2521, December, 2011.
1066-5285/11/6012-2564

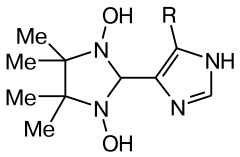
the xanthine—xanthine oxidase system. As a comparison, the activity of the nitronylnitroxyl radicals L^RH (**2a**,**b**) in the same processes and the diamagnetic precursors L^RH_2 (**3a**,**b**) in the hydrogen-atom transfer was studied using 1,1-diphenyl-2-picrylhydrazyl (DPPH).



1a,b



2a,b



3a,b

R = H (**a**), Me (**b**)

Experimental

Compounds **1a**,**b**, **2a**,**b**, and **3a**,**b** were prepared according to known procedures.[20—22] DPPH (Aldrich), ethanol (96%), copper(II) chloride (reagent grade), neocuproine (2,9-dimethyl-1,10-phenanthroline, Aldrich), lipoxidase from glycine max (soybean, Type I-B, Sigma), linoleic acid (Sigma, ≥99%), xanthine (Sigma), xanthine oxidase (Sigma), tetrazolium blue chloride (Merck), bovine serum albumine, thiobarbituric acid (TBA, Sigma—Aldrich), ionol (2,6-di-*tert*-butyl-4-methylphenol, Aldrich), Trolox (Acros Organics), EDTA (reagent grade), trichloroacetic acid (reagent grade), dimethylsulfoxide (DMSO, reagent grade) were used. Solvents were purified according to the standard procedures.[23] Distilled water, ammonium acetate, boric acid, sodium bicarbonate, sodium hydrophosphate, and sodium dihydrophosphate (reagent grade) were used for the preparation of buffer solutions.

A Thermo Evolution 300 BB spectrophotometer (UK), Zenyth200rt 96-well plate spectrophotometer (Austria), a Termit thermostat (Russia), and a Hettich Universal 320R centrifuge (Germany) were used.

Determination of the activity of lipoxygenase was performed by spectrophotometry.[24] The content of hydroperoxide (the oxidation product of linoleic acid) was measured at λ_{max} = = 234 nm. The sample contained a solution of linoleic acid (2 mL, $0.3 \cdot 10^{-3}$ mol L^{-1}), borate buffer (0.89 mL, pH 9.0), and a solution of the compound under study (0.01 mL) in DMSO. The reaction was initiated by addition of a solution of the enzyme (0.1 mL, 500 units) and the measurements were carried out for 10 min at 25 °C. The measurments were performed using six concentrations for each compound in the range of (10—100) 10^{-6} mol L^{-1}. All experiments were repeated three times.

The inhibition rate of lipoxygenase (A(%)) was determined by the formula

$$A(\%) = (v_0/v_0') \cdot 100\%,$$

where v_0 and v_0' are initial rates of the enzymatic reaction in the presence and absence (control) of the compounds under study, respectively.

The initial rate (v_0 and v_0') was calculated by the formula

$$v_0 = \Delta C/\Delta t = \Delta A/(\Delta t \cdot \varepsilon) = \mathrm{tg}\alpha/(\Delta t \cdot \varepsilon),$$

where C is the product concentration, t is the reaction time, ε is the molar extinction coefficient, tgα is the slope of the kinetic curve (time dependence of the product concentration).

The inhibition concentration IC_{50} was estimated by approximation of the obtained data by the general logistic curve

$$A(\%) = (1/(1 + [I]/IC_{50})) \cdot 100\%,$$

where $[I]$ is the inhibitor concentration.

In a series of the experiments for determination of the inhibition reversibility, the reaction mixture contained borate buffer (0.89 mL, pH 9.0), a solution of inhibitor in DMSO (0.01 mL), and a solution of the enzyme (0.1 mL, 500 units). The solution was kept for different time intervals (1, 5, and 10 min) and the reaction was initiated by addition of a solution of linoleic acid (2 mL, 0.45 mmol L^{-1}).

Determination of the inhibition type of lipoxygenase. The kinetic parameters and inhibition type of lipoxygenase were evaluated according to Ref. 25 by changing the concentration of linoleic acid in the range of (10—1000) 10^{-6} mol L^{-1} at constant concentration of inhibitor, which was close to the IC_{50} value for this compound.[25] The kinetic parameters K_m (Michaelis constant) and V_{max} (the reaction rate upon saturation of the enzyme with a substrate) were determined graphically in the Lineweaver—Berk coordinates (the double inverse coordinates method).[25]

Enzymatic generation of the superoxide radical anion $O_2^{\cdot -}$ in the xanthine—xanthine oxidase system. The effects of compounds **1a**,**b** on enzymatic generation of the superoxide radical anion $O_2^{\cdot -}$ in the xanthine—xanthine oxidase system were estimated by the reduction rate of tetrazolium blue into formazan.[26] The composition of the reaction mixture was as follows: carbonate buffer (2.7 mL, 40 mmol L^{-1}, pH 10.0) containing EDTA (0.1 mmol L^{-1}), a solution of xanthine (0.06 mL, 10 mmol L^{-1}), a 0.5% bovine serum albumin (0.33 mL), tetrazolium blue (0.03 mL, 2.5 mmol L^{-1}), and a solution of the compound under study in DMSO (0.06 mL). Xanthine oxidase (0.02 mL, 0.04 unit) was added to the mixture at 25 °C and the absorption at λ_{max} = = 560 nm was measured for 300 sec. The control experiment was performed in the presence of DMSO (0.06 mL). The measurments were performed using six concentrations for each compound. All experiments were performed three times.

A series of experiments were performed to estimate the activity of xanthine oxidase in the presence of the compounds under study by determination of the formation rate of uric acid. The composition of the reactoin mixture was as follows: carbonate buffer (2.76 mL, 40 mmol L^{-1}, pH 10.0) containing EDTA (0.1 mmol L^{-1}), a solution of xanthine (0.06 mL, 10 mmol L^{-1}), and a solution of the compound under study in DMSO (0.06 mL). The reaction was initiated by addition of xanthine oxidase (0.12 mL, 0.04 unit) and the absorption at λ_{max} = 293 nm was measured for 300 sec.

Nonenzymatic peroxidation of linoleic acid. The effects of compounds **2a**,**b** and **3a**,**b** on the nonenzymatic peroxidation of linoleic acid were estimated according to a known procedure.[27] Phosphate buffer (2 mL, pH 7.4) was added to a solution of linoleic acid (0.034 mL) in ethanol (1.97 mL). The reaction mixture contained a solution of linoleic acid (0.33 mL), a solution of $FeCl_2$ (0.066 mL, 4 mmol L^{-1}), phosphate buffer (0.164 mL), and a solution of the compound under study in ethanol (1 mL, 30 mmol L^{-1}).[28] The resulted solution was incubated for 30 min at 37 °C, then a solution (0.84 mL) containing 0.25 *M* HCl, 10% trichloroacetic acid, and 0.6% TBA was added. The mixture was heated at 95 °C for 15 min, centrifuged and the absorption at λ_{max} = 532 nm was measured on a 96-well plate spectrophotometer.

DPPH test. The reducing ability of compounds **2a**,**b** and **3a**,**b** was estimated by spectrophotometry at 517 nm using DPPH (see Ref. 29). The reaction was performed in the wells of a 96-well plate. The reaction mixture contained DPPH (0.1 mL, 0.2 mmol L^{-1}), a solution of the compounds under study with different concentration (0.1 mL, 0.01—0.2 mmol L^{-1}). The reaction was performed at 25 °C for 30 min. The percentage antioxidant activity ($I(\%)$) was calculated from the formula

$$I(\%) = (A_0 - A_1)/A_0 \cdot 100\%,$$

where A_0 is the absorption of a control solution of DPPH and A_1 is the absorption of a reagent solution.

The effective concentration EC_{50} was estimated by approximation of the resulted data by the general logistic curve:

$$I(\%) = 100 \cdot (1/(1 + [C]/EC_{50})),$$

where [*C*] is a concentration of the compound under study.

CUPRAC test. The redox activity of compounds **2a**,**b** and **3a**,**b** was estimated by the CUPRAC method.[30] The experimental data were recorded by an increase in the absorption of a solution at 450 nm, which corresponds to the maximum absorption of the copper(I) neocuproin complex. The measurements were performed at 25 °C using a 96-well plate spectrophotometer. The reaction mixture contained acetate buffer (0.05 mL, pH 7.0), a solution of $CuCl_2$ (0.05 mL, 0.01 mol L^{-1}), a solution of neocuproin (0.05 mL, 7.5 mmol L^{-1}), and a solution of the compound under study in ethanol (0.05 mL, 2 mmol L^{-1}).

The results were represented in trolox equivalents (TEAC). The value of trolox equivalent was determined from the absorption value using the calibration curve, *viz.*, concentration dependence of the reduced copper complex on the trolox content.

Molecular docking. Docking of the inhibitor molecules into the lipoxygenase active site was carried out using the AutoDock 4.2 program, which includes a genetic algorithm.[31,32]

The protein structure (the protein data bank PDB[33], the access code 1IK3[34]) was prepared by removal of the crystallization water molecules and ligand structures, addition of polar hydrogen atoms, and energy minimization (100 iterations by the Powell method in the Tripos force field[35]) using the Sybyl 8.0 software package.[36]

The ligand atomic coordinates were extracted from the X-ray diffraction data files[20,21] (CCDC 199137) using the PLATON program[37] and then optimized using the Sybyl software. As it is impossible to consider radical particles by the molecular mechanics methods due to the absence of the parameters for such particles in force fields, the corresponding molecular fragments were represented as N-oxides. The nitrate ions were eliminated from the complex structures before docking.

The systems were prepared for docking using the MGLTools 1.5.4 software package.[38] The atomic charges upon docking were calculated according to the Gasteiger scheme,[39] the charge +3 was assigned to the iron atom and the charge +2 was assigned to the copper atom. The van der Waals radius for the copper atom was set equal to 1.4 Å. Mesh fields were built for the spatial region including only the catalytic site of the enzyme. Upon docking, 100 startups of the genetic algorithm were performed for each complex. The results were clustered using the threshold root-mean-square deviation of 2.0 Å.

Results and Discussion

Soybean lipoxygenase-1 (LOX-1) is frequently used as a model enzyme during the study of homological lipoxygenase family (EC 1.13.11.12), in particular, 5-lipoxygenases acting in the human body. 5-Lipoxygenase and LOX-1 catalyze oxidation of linoleic acid to 9-(*S*)-hydroperoxy-10*E*,12*Z*-octadiene acid (HPOD). In the present work, the study of the activity of the copper complexes with the nitronylnitroxyl radicals **1a**,**b** and ligands **2a**,**b** were performed by the example of LOX-1. Linoleic acid was used as a substrate. The reaction was monitored by spectrophotometry at λ_{max} = 234 nm by the formation of 9-(*S*)-hydroperoxy-10*E*,12*Z*-octadiene acid ($\varepsilon = 2.5 \cdot 10^4$ L mol^{-1} $см^{-1}$) (see Refs 2 and 20).

Inhibition of LOX-1. The effects of complexes **1a**,**b** on the activity of LOX-1 are illustrated by Figs 1—5. It is important to note that the experimental data obtained in the present work evidence that the starting organic ligands, *viz.*, the stable nitronylnitroxyl radicals **2a**,**b**, do not affect the oxidation of linoleic acid catalyzed by lipoxygenase. The enzymatic reaction rates in the presence of ligands **2a**,**b** differ by 1±0.3% from those of the control reaction performed without additives.

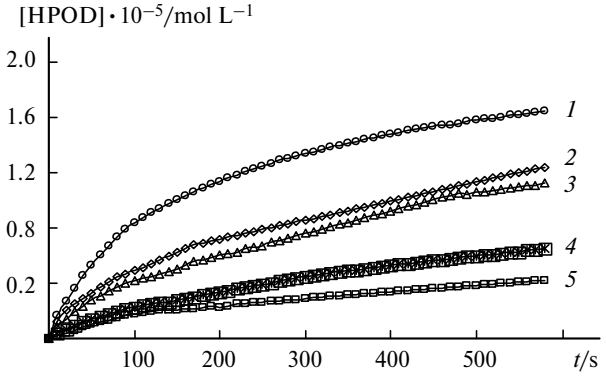


Fig. 1. The kinetic accumulation curves of HPOD in the absence of inhibitor (*1*) and in the presence of complex **1a** at concentration of $1.1 \cdot 10^{-5}$ (*2*), $1.4 \cdot 10^{-5}$ (*3*), $2.7 \cdot 10^{-5}$ (*4*), and $3.3 \cdot 10^{-5}$ mol L^{-1} (*5*).

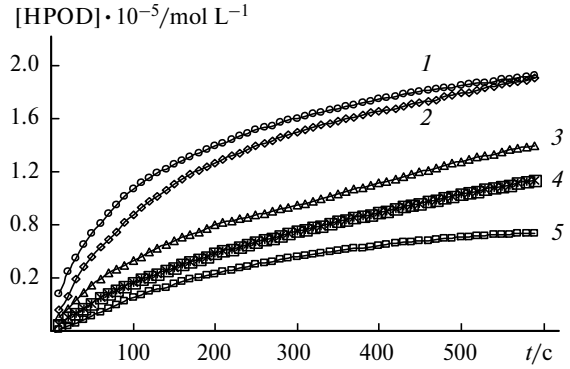


Fig. 2. The kinetic accumulation curves of HPOD in the absence of inhibitor (*1*) and in the presence of complex **1b** at concentration of $1.1 \cdot 10^{-5}$ (*2*), $1.4 \cdot 10^{-5}$ (*3*), $2.7 \cdot 10^{-5}$ (*4*), and $3.3 \cdot 10^{-5}$ mol L^{-1} (*5*).

The experimental kinetic accumulation curves of HPOD at the constant concentration of the substrate and different concentrations of complexes **1a**,**b** are shown in Figs 1 and 2.

The IC_{50} values were determined by approximation of the concentration dependence by the logistic curve. The resulted curves are shown in Fig. 3 and the kinetic data are given in Table 1. The IC_{50} values for complexes **1a**,**b** are $(29.2 \pm 4.7) \cdot 10^{-6}$ and $(20.1 \pm 3.9) \cdot 10^{-6}$ mol L^{-1}, respectively.

To establish the reversibility of inhibition of LOX-1 by the copper complexes **1a**,**b**, the enzyme was incubated in a buffer solution in the presence of complex and the kinetic curves were recorded after different time intervals (1, 5, and 10 min) by adding a solution of linoleic acid in buffer to the reaction mixture. Preincubation of LOX-1 with complexes **1a**,**b** does not change the initial rate of the enzymatic reaction, which evidences the reversibility of the enzyme inhibition.

The experimental kinetic curves obtained for different concentrations of the substrate in the Lineweaver—Berk coordinates are shown in Figs 4 and 5 and demonstrate the type of inhibition of lipoxygenase by complexes **1a**,**b** depending on the substrate concentration. The concurrence of straight lines in one y-coordinate (see Fig. 4) suggests that complex **1a** inhibites the enzyme being in competition with the substrate for the enzyme-binding site.

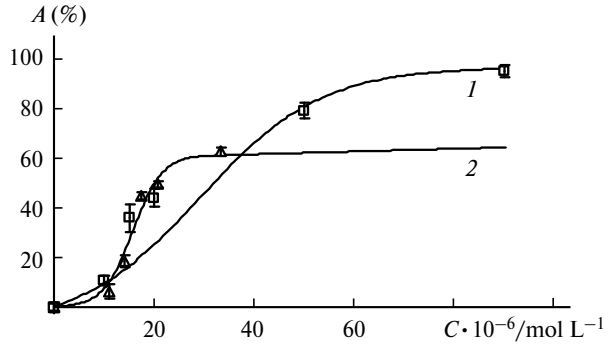


Fig. 3. Dependence of the change in the inhibition rate of LOX-1 on the concentration of complexes **1a** (*1*) and **1b** (*2*).

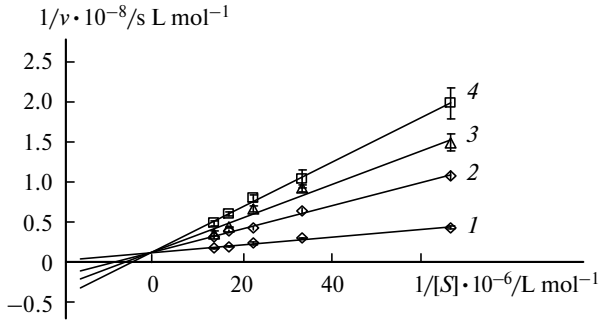


Fig. 4. The kinetic curves of the lipoxygenase (LOX-1) inhibition in the Lineweaver—Berk coordinates in the absence of inhibitor (*1*) and in the presence of complex **1a** at concentration of $1 \cdot 10^{-5}$ (*2*), $2 \cdot 10^{-5}$ (*3*), and $3 \cdot 10^{-5}$ mol L^{-1} (*4*).

Whereas it follows from Fig. 5 that complex **1b** does not compete with linoleic acid (as a substrate) for the enzyme-binding site and, consequently, exhibits the noncompetitive type of inhibition.

Thus, the data obtained suggest reversible inhibition of LOX-1 for both compound **1a** and compound **1b**, the competitive mechanism of inhibition in the case of complex **1a**, and the noncompetitive mechanism of inhibition for complex **1b**.

Molecular docking. To interpret the obtained experimental data on the inhibition type typical of complexes **1a,b**, we performed molecular docking. The results from docking of compounds **1a,b** are shown in Fig. 6. Both compounds are characterized by incorporation into the hydrophobic packet of the active site analogously to that identified earlier.[40]

Nevertheless, the orientations of two compounds under study differ noticeably. Compound **1b** is characterized

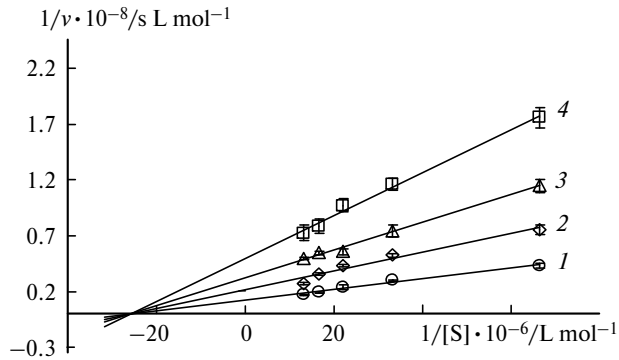


Fig. 5. The kinetic curves of the lipoxygenase (LOX-1) inhibition in the Lineweaver—Berk coordinates in the absence of inhibitor (*1*) and in the presence of complex **1b** at concentration of $1 \cdot 10^{-5}$ (*2*), $2 \cdot 10^{-5}$ (*3*), and $3 \cdot 10^{-5}$ mol L^{-1} (4).

Table 1. Kinetic parameters of inhibition of LOX-1 by complexes **1a**,**b***

Complex	$C \cdot 10^{-6}$	$K_m \cdot 10^3$ /mol L^{-1}	$V_{max} \cdot 10^{-5}$ /mol L^{-1} s^{-1}	Type of inhibition
1a	12.5	0.113	7.8	Competitive
	20.0	0.156	7.5	
	30.0	0.200	7.2	
1b	12.5	0.039	4.7	Non-competitive
	20.0	0.039	3.1	
	30.0	0.039	2.0	

* In the absence of complexes, $K_m = 0.040 \cdot 10^{-3}$ mol L^{-1}, $V_{max} = 8.4 \cdot 10^{-5}$ mol L^{-1} s^{-1}.

by a larger number of hydrophobic interactions than that for **1a** due to the presence of two methyl group contacting with the amino-acid residues Val372 (the minimum distance $r_{C...C}$(min) = 3.27 Å) and Val571 ($r_{C...C}$(min) = 2.10 Å). There are also hydrophobic interactions with the residues Thr728 ($r_{C...C}$(min) = 2.1 Å), Leu773 ($r_{C...C}$(min) = 2.26 Å), and Leu565 ($r_{C...C}$(min) = 2.43 Å), only the latter occurs in the orientation proposed for the complex of LOX-1 with **1a** ($r_{C...C}$(min) = 2.67 Å). In addition, there is a hydrophobic interaction between the ligand and the amino-acid residue Ile770 ($r_{C...C}$(min) = 2.62 Å) in the complex of LOX-1 with **1a**, which is absent in the complex of LOX-1 with **1b**. The hydrogen bond formation with the residues Arg726 (the distance $r_{O...N}$ = 4.29 Å), Ser510 ($r_{O...O}$ = 3.26 Å), and Thr575 ($r_{N...O}$ = 3.69 Å) is possible for the nitroxyl group of complex **1b**. The amide groups of the glutamine residues Gln716 and Gln514 can reorientate upon binding to complex **1b** in such a way that provides the interaction with the copper atom; however, such bonds were not identified upon docking due to the fact that the mobility of side chains of the protein was not studied. Complex **1a** is located in the binding cavity so that the formation of side interactions is unlikely. In addition, this complex has a nonfavorable close contact with the amino-acid residue His518 involved in complexation with the iron atom; however, the orientation analogous to that of **1b** was not found upon docking. Upon interaction with the inhibitor, the interacion of this hystidine residue is possible in principle not only with the iron atom of the active site, but also with the copper atom of the inhibitor. The hydrogen bond formation between the copper complex and the binding site in the protein is unlikely due to a high hydrophobicity of the binding region and structural rigidity of the copper complex, which, in turn, dismisses a possibility of the hydrogen bond formation. However, it shoud be noted that there exists a hydrogen bond between the NH proton of the imidazole ring of complex **1a** and the amide oxygen atom of the Gln514 main chain (not shown in Fig. 6, *a*). None of the proposed structures of the complex "enzyme—inhibitor" suggest direct interaction of inhibitors with the iron atom or the hydroxide ion positioned in its coordination sphere.

The distances between the copper atom and the iron atom of the enzyme active site (Fig. 7) for complexes **1a** and **1b** are 7.37 and 9.15 Å, respectively. These data allows considering complexes **1a,b** as redox inhibitors[12] whose activity is based on the electron transfer from the Cu^{2+} ion

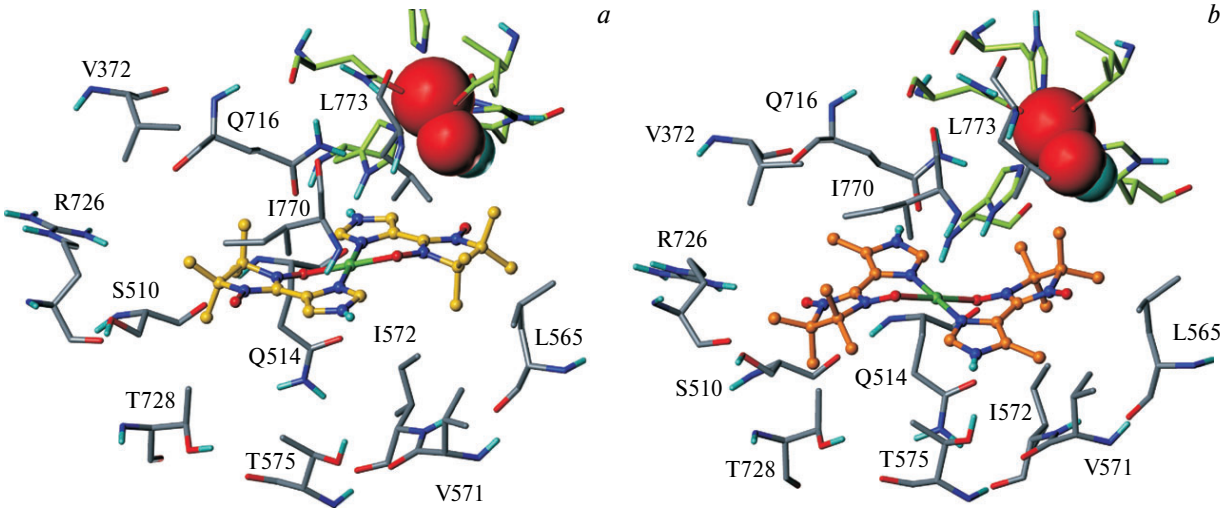


Fig. 6. The results from docking of compounds **1a** (*a*) (the carbon atoms are shown in yellow) and **1b** (*b*) (the carbon atoms are shown in orange). The copper complexes are shown using the ball-and-stick model. The [FeOH] fragment in the active site is shown with preservation of the van der Waals atom radii. The carbon atoms of the amino-acid residues coordinated to the iron atom are shown in green and the carbon atoms of the amino-acid residues contacting with the inhibitor are shown in blue, the nitrogen atoms are shown in dark-blue, the hydrogen atoms are shown in blue, the oxygen atoms are shown in red, and the copper atoms are shown in bright-green. *Note*. Figure 6 is available in full-color in the online version of the journal (http://www.springerlink.com).

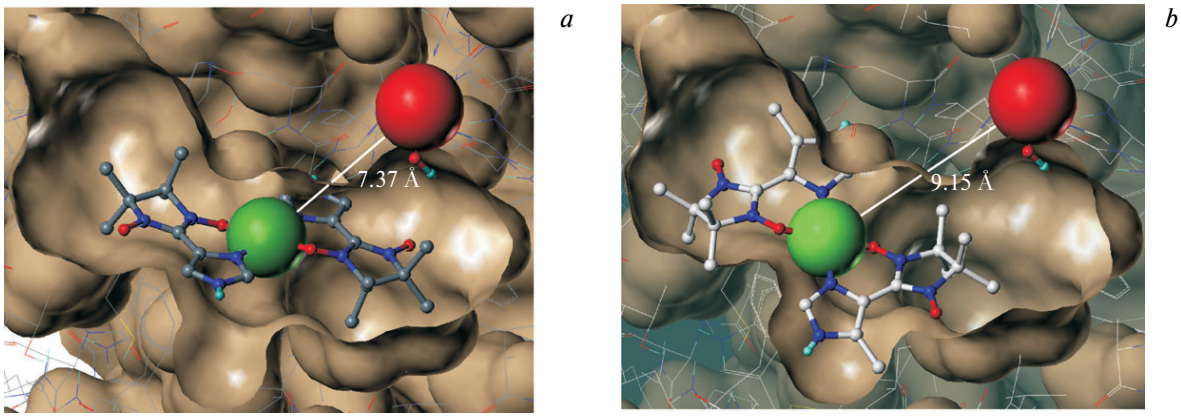


Fig. 7. The results from docking of compounds **1a** (*a*) and **1b** (*b*). The distances between the copper atoms (green) in complexes **1a,b** and the iron atom (red) in the active site are indicated. *Note*. Figure 7 is available in full-color in the online version of the journal (http://www.springerlink.com).

to the Fe^{3+} ion in the binding site of the inhibitor. However, a considerable part of the cavity, which is usually occupied by the LOX-1 native substrates, in this case is occupied by the copper complexes, which seems to confirm their inhibitory activity.

Unusual change in the type of inhibition mechanism on going from **1a** to **1b** cannot be explained reliably without invoking X-ray diffraction. Nevertheless, one can assume based on the results from docking that upon introduction of the additional methyl group into the imidazole fragment (complex **1b**), the orientation of the copper complex can occur in the binding cavity of LOX-1, which is characterized by a larger number of hydrophobic contacts compared to the orientation typical of **1a**. As a consequence, the interaction of inhibitor with lipoxygenase becomes more favorable and results in the noncompetitive mechanism at the concentration range of the substrate under study.

Nonenzymatic peroxidation of linoleic acid. To study the effects of complexes **1a**,**b** and the corresponding ligands, *viz.*, stable nitronylnitroxyl radicals **2a**,**b**, and their reduced forms **3a**,**b**, on the nonenzymatic peroxidation of the substrate, oxidation of linoleic acid induced by the Fe^{2+} ions was used. We determined by spectrophotometry the content of the TBA-reactive substances, *viz.*, the carbonyl compounds that are produced upon decomposition of hydroperoxyoctadiene acid in the presence of air. The results are shown in Fig. 8 as a percentage content of the TBA-reactive substances (TBARS) relatively to the control. The known antioxidant ionol (2,6-di-*tert*-butyl-4-methylphenol) was used as a reference.

The presence of compound **2a** had no effect on peroxidation of linoleic acid. The antioxidant activities of radical **2b** and copper complexes **1a**,**b** are comparable with that of ionol (~61, 70, 82, and 81%, respectively). As seen from Fig. 8, compounds **3a**,**b** have the highest activities exceeding considerably that of ionol (48 and 17%, respectively), which allows assumption of their high efficiency as promising antioxidants.

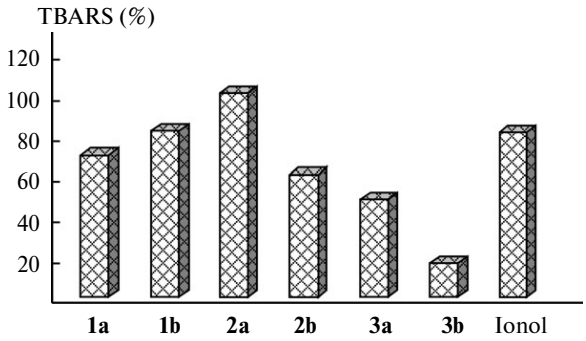


Fig. 8. The content of the TBA-reactive peroxidation products of linoleic acid in the presence of complexes **1a**,**b**, **2a**,**b**, and **3a**,**b** (the concentration of the compounds under study is 1.6 mmol L^{-1} (mg of linoleic acid)$^{-1}$, 37 °C, $FeSO_4 \cdot 4H_2O$, 30 min).

DPPH test. To evaluate the nonspecific antioxidant activity of compounds **3a**,**b** showing the maximum effect on inhibition of the chain radical process, a test was performed, which uses the model hydrogen abstraction from a potential antioxidant molecule by the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). This process was monitored by the change in the characteristic absorption band of DPPH at $\lambda_{max} = 517$ in the electronic absorption spectra. Figure 9 shows the dependence of reduction of the DPPH radical on the concentrations of compounds **3a**,**b**.

The EC_{50} values for compounds **3a,b** are 41.7 and $33.7 \cdot 10^{-6}$ mol L^{-1}, respectively. The EC_{50} values for trolox and ionol are 46.4 and $101.7 \cdot 10^{-6}$ mol L^{-1}, respectively, which confirms also high antioxidant activities of ligands **3a**,**b**.

CUPRAC test. To study the ability of compounds **1a**,**b**, **2a**,**b**, and **3a**,**b** to show antioxidant activity as reagents in

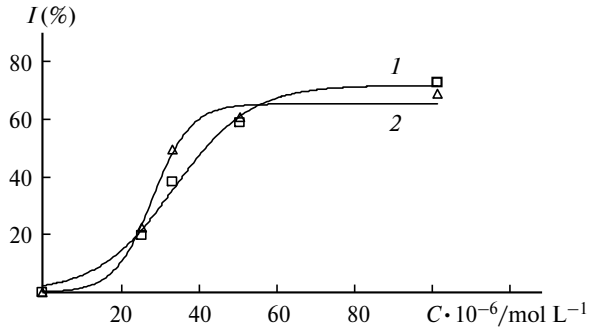


Fig. 9. The concentration dependence of the reduction of the DPPH radical in the presence of compounds **3a** (*1*) and **3b** (*2*) (25 °C, EtOH, 30 min).

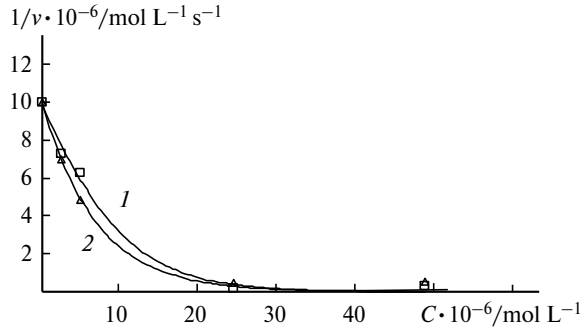


Fig. 11. Change in the accumulation rate of formazan in the xanthine—xanthine oxidase system depending on the concentration of complexes **1a** (*1*) and **1b** (*2*).

the electron-transfer process, the CUPRAC method was used. The experimental data expressed in the equivalents of trolox (TEAC)[30] are shown in Fig. 10. Compounds **3a**,**b** exhibited a moderate activity during reduction of Cu^{2+} under the experimental conditions, the TEAC values were 65±3 and 66±3% of the activity of trolox, respectively. The copper complexes **1a**,**b** and the corresponding ligands, *viz.*, stable radicals **2a**,**b**, exhibited an extremely low activity. The TEAC values were 10±1, 38±1, 1±1, and 28±2% for **2a**, **2b**, **1a**, and **1b**, respectively.

Thus, the data obtained do not allow us to assume that the redox mechanism accounts for the activity of complexes **1a**,**b**.

Enzymatic generation of the superoxide radical anion $O_2^{\cdot -}$ in the xanthine—xanthine oxidase system. It is known that the lipoxygenase-catalyzed oxidation is accompanied by the formation of the superoxide radical anion.[12,40,41] The ability to reduce the superoxide radical anion was defined[26] for the compounds under study using the xanthine—xanthine oxidase system where $O_2^{\cdot -}$ is generated. In addition, the effects of complexes **1a**,**b** on the xanthine oxidase activity were estimated by the rate of uric acid formation.[26] It was shown that compounds **1a**,**b** have no effect on the activity of the enzyme. Radicals **2a**,**b** do not affect the rate of the formazan formation in the reaction mixture. At the same time, their copper complexes **1a**,**b** possess a pronounced ability to bind to the superoxide radical anion. The dependence of the accumulation rate of formazan in the xanthine—xanthine oxidase system on the concentrations of complexes **1a**,**b** are shown in Fig. 11.

The IC_{50} values for complexes **1a**,**b** are $(8.7\pm0.8)\cdot10^{-6}$ and $(4.4\pm0.5)\cdot10^{-6}$ mol L^{-1}, respectively. The values obtained characterize these complexes as active reducing agents of the superoxide radical anion, which suggests their antioxidant efficiency upon generation of active oxygen metabolites, particularly, $O_2^{\cdot -}$.

Thus, the copper complexes with the stable nitronylnitroxyl radicals are reversible inhibitors of LOX-1 and possess a high antioxidant activity upon the radical peroxidation of substrates and formation of the superoxide radical anion, as well.

This work was financially supported by the Russian Foundation for Basic Research (Projects Nos 09-03-00090, 11-03-12088-ofi-m, and 11-03-01165), Department of Chemistry and Material Sciences of the Russian Academy of Sciences (Project according to Program No. 9 "Medicinal Chemistry"), Interdisciplinary Integration Project of the Siberian Branch of the Russian Academy of Sciences (Project No. 93).

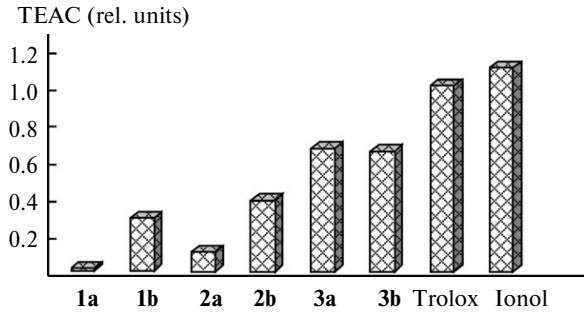


Fig. 10. The values of the antioxidant activities of the compounds under study obtained by the CUPRAC method.

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Received June 23, 2011;
in revised form November 18, 2011