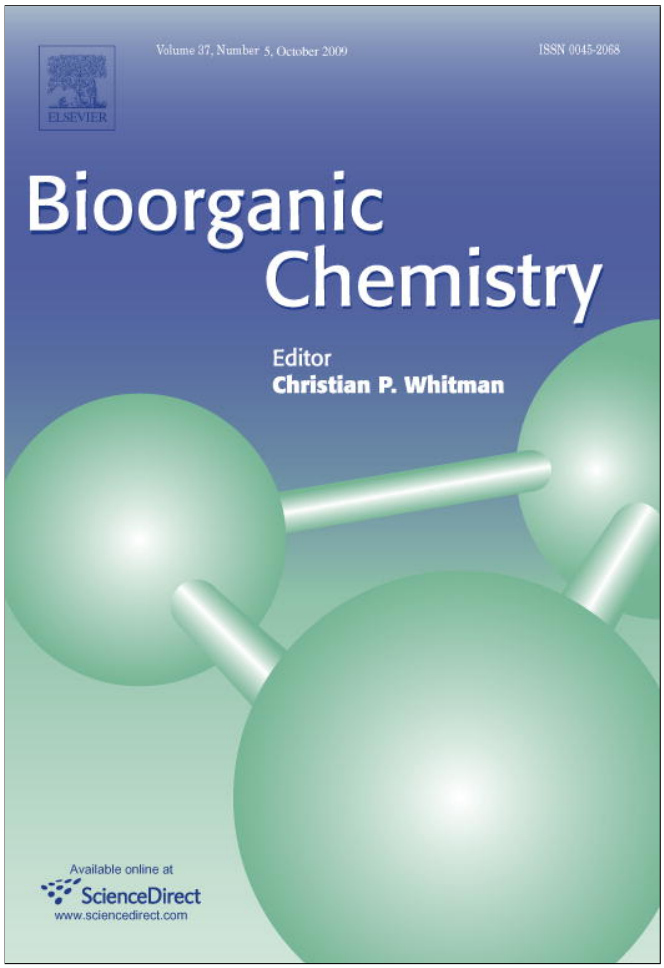

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Diethanolamine Pd(II) complexes in bioorganic modeling as model systems of metallopeptidases and soybean lipoxygenase inhibitors

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ABSTRACT

The reaction of $PdCl_2$ with diethanolammonium chloride (DEAxHCl), in the molar ratio 1:2, affords the $[HDEA]_2[PdCl_4]$ complex (**1**). The hydrolytic activity of the novel Pd(II) complex **1** was tested in reaction with *N*-acetylated *L*-histidylglycine dipeptide (AcHis-Gly). Complex **1**, as well as earlier prepared *trans*-$[PdCl_2(DEA)_2]$ complex (**2**), and DEA, as their precursor, were tested for their *in vitro* free radical scavenging activity. UV absorbance-based enzyme assays were done in order to evaluate their inhibitory activity of soybean lipoxygenase (LOX). Also, assays with superoxide anion radical were done. The scavenging activities of the complexes were measured and compared with those of their precursors and caffeic acid. Complex **2** exhibits the highest antioxidant activity and the highest inhibitory effect against the soybean LOX.

1. Introduction

It was shown that some of palladium(II) complexes, as model systems in bioorganic chemistry, can be interesting as artificial metallopeptidases, inhibitors of enzymes, and free radical scavengers.

Interest in the study of interactions of platinum(II) and palladium(II) complexes with sulfur- and histidine-containing peptides and proteins became of capital importance after the discovery that their aqua complexes can be promising reagents for the selective hydrolytic cleavage of the above mentioned peptides [1–12]. Furthermore, it was found that some of palladium complexes can be enzyme inhibitors. These compounds inhibit various enzymes such are RNA polymerase [13], topoisomerase III, reverse transcriptase (by free radical generation) [14], leukemia virus reverse transcriptase [15], cellobiohydrolase [16], and soybean LOX [11]. Some palladium complexes have been evaluated for their anti-inflammatory and antioxidant activities *in vitro,* whereby palladium complexes exhibited higher activity compared to the activity of the ligand [12,17,18].

The lipoxygenases are non-heme iron containing enzymes which catalyze the oxidative metabolism of fatty acids, and are useful target for the design and development of new drugs that substantially inhibit the generation of the final inflammatory products and the propagation of inflammation [19,20]. Linoleic acid is the primary substrate in the reaction of dioxigenation of polyunsaturated fatty acids catalyzed by plant LOX, while the mammalian isozymes mainly catalyze the metabolism of arachidonic acid [21]. In mammalian cells, LOXs play an essential role in the biosynthesis of many bioregulatory molecules such as leukotrienes, lipoxins and hepoxylines. These compounds are mediators in the pathophysiology of variety of diseases, for example bronchial asthma, psoriasis and inflammation [22]. The fatty acid hydroperoxides, generated in the reaction of dioxigenation of polyunsaturated fatty acids catalyzed by LOX, significantly contribute to the creation of atherosclerotic lesions [23]. These compounds have critical influence on the development of several human cancers [24]. For these reasons, extensive research was done to find efficient inhibitors of LOX activity [21,25–33]. Most of these studies have used readily obtainable soybean lipoxygenase, which is a homologue of mammalian lipoxygenase and well examined [34,35]. Most of the lipoxygenases inhibitors are antioxidants or free radical scavengers, since lipoxygenation occurs via a carbon-centered radical.

In the present study, a new diethanolammonium–tetrachloridopalladate(II) complex (**1**) ($[HDEA]_2[PdCl_4]$, where DEA is diethanolamine), was synthesized and employed to study its hydrolytic activity in the reaction with *N*-acetylated *L*-histidylglycine dipeptide (AcHis-Gly). New complex **1** and earlier prepared diethanolamine complex *trans*-$[PdCl_2(DEA)_2]$ complex (**2**), as well as diethanolamine, precursor of these compounds, were tested for their LOX inhibitory activity and free radical scavenging activity.

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2. Materials and methods

The compounds D_2O, DNO_3, and $PdCl_2$ were obtained from Aldrich Chemical Co. All common chemicals were of reagent grade. Dipeptide *L*-histidylglycine (His-Gly), soybean lipoxygenase, linoleic acid sodium salt, NADH, and nitrotetrazolium blue (NBT) were obtained from Sigma Chemical Co. *N*-methylphenazonium-methyl sulfate and diethanolamine (DEA; $NH(CH_2CH_2OH)_2$) were purchased from Fluka. The terminal amino group in His-Gly was acetylated by standard method to obtain AcHis-Gly [1].

All pH measurements were made at 25 °C. The pH meter (Iskra MA 5704) was calibrated with Fischer certified buffer solutions of pH 4.00 and 7.00. The results were not corrected for the deuterium isotope effect. Reactions of AcHis-Gly with palladium(II) complex in D_2O solutions were followed by ^1H NMR spectroscopy using a Varian 200 MHz spectrometer. The palladium(II) complex **1** and peptide were mixed in an NMR tube in molar ratio 1:2. The pH was 2.0. The internal reference was TSP (sodium trimethylsilylpropane-3-sulfonate). The IR spectra were recorded on a Perkin-Elmer Spectrum One FT-IR spectrometer using the KBr pellet technique. Elemental microanalyses for carbon, hydrogen, and nitrogen were performed at the Faculty of Chemistry, Belgrade University.

2.1. Computational method

All calculations were conducted using Gaussian03 [36] with the B3LYP hybrid functional [37–39]. The triple split valence basis set 6-311G(d,p) was used for C, H, O, N, and Cl [40], whereas LANL2DZ + ECP [41] was employed for the Pd center. Geometrical parameters of all investigated species were optimized in vacuum. Vibrational analysis was performed for all structures. All calculated structures were verified to be local minima (all positive eigenvalues) for ground state structures by frequency calculations. The natural bond orbital analysis [42,43] (Gaussian NBO version) was performed for all structures.

3. Synthesis of the diethanolammonium–tetrachloridopalladate (II) complex, $[HDEA]_2[PdCl_4]$

3.1. Synthesis of the diethanolammonium chloride, [HDEA]Cl

Diethanolamine hydrochloride was prepared by slowly dropping hydrochloric acid to diethanolamine dichlormetane solution. Hydrochloric acid was added excessively to 5% of the stoichiometric amount. Resulting solution was mixed during 2 h at room temperature. Finally, volatile component was evaporated in vacuo. Prepared ionic liquid is colourless viscous liquid whose boiling point is 112 °C.

Spectral characterization of the ionic liquid [HDEA]Cl: ^1H NMR spectrum (200 MHz, D_2O): $\delta = 3.266$ (4H, $-CH_2-NH_2$, *t*, $J = 5.0$ Hz) ppm; 3.891(4H, $-CH_2-OH$, *t*, $J = 5.0$ Hz) ppm. IR (film): $\upsilon = 325$, 631, 949, 1039, 1064, 1408, 2852, 3336, 3384 cm^{-1}.

3.2. Synthesis of the $[HDEA]_2[PdCl_4]$ complex

The $[HDEA]_2[PdCl_4]$ complex was synthesized starting from $PdCl_2$ and two equivalents of diethanolamine hydrochloride, according to the procedure published earlier [11]. In the course of 3 h, the reaction of 0.1774 g (0.001 mol) of $PdCl_2$ dissolved in 15 mL of water with 0.283 g (0.002 mol) of diethanolamine hydrochloride, at 50–60 °C, afforded an orange–brown solution which was left at room temperature for 2 days. The precipitated brown crystals were filtered off, washed with ethanol, air-dried and showed a melting point of 115–116 °C. Yield 0.446 g (97%). Calculated for $[HDEA]_2[PdCl_4] = C_8H_{22}O_4N_2Cl_4Pd$ ($FW = 460.42$): C, 20.95; N, 6.09; H, 5.21%; found: C, 21.01; N, 6.13; H, 5.18%.

Spectral characterization of the complex $[HDEA]_2[PdCl_4]$: ^1H NMR spectrum (200 MHz, D_2O): $\delta =$, *t*, 3.24 (4H, $-CH_2-NH$ $J = 5.2$ Hz), 3.86 (4H, $-CH_2-OH$, *t*, $J = 5.2$ Hz) ppm; IR (KBr): $\upsilon = 323$, 698, 958, 1065, 1088, 1405, 1574, 2874, 3299, 3343 cm^{-1}.

3.3. Soybean lipoxygenase inhibition study in vitro

In vitro study was evaluated as reported previously [44]. The tested compounds dissolved in ethanol were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 cm^3 of enzyme solution ($1/9 \times 10^{-4}$ w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor.

3.4. Assay of superoxide anion radical scavenging activity [44,45]

The superoxide-producing system was set up by mixing phenazine methylsulfate (PMS), NADH and air-oxygen. The production of superoxide was estimated by the nitroblue tetrazolium method. The reaction mixture containing investigated compound, 3 μM PMS, 78 μM NADH, and 25 μM NBT in 19 μM phosphate buffer pH 7.4 was incubated for 2 min at room temperature and the absorption measured at 560 nm against a blank containing PMS. The tested compounds were preincubated for a blank containing PMS. The tested compounds were preincubated for 2 min before adding NADH.

4. Results and discussion

4.1. Structure examination of $[HDEA]_2[PdCl_4]$ complex (**1**)

The structure of complex $[HDEA]_2[PdCl_4]$ was examined using DFT method. The optimized structure of complex is presented in Fig. 1. $[PdCl_4]^{2-}$ anion exhibits square planar coordination, where the four chlorine anions lie in the equatorial plane, whereas the two protonated diethanolamine cations form hydrogen bonding with chlorido ligands. *Cis*- and *trans*-chlorido ligands form with palladium bond angles of 90° and 180°, respectively. The Pd–Cl bond length is equal to 2.40 Å, whereas the distances between chlorido ligands and hydrogens bonded to nitrogen lie in the range of 2.10–2.15 Å. The NBO analysis of the complex reveals covalent Pd–Cl bonds, with hybrid compositions of $0.41(sp^2d)_{Pd} + 0.91(sp^{5.80})_{Cl}$.

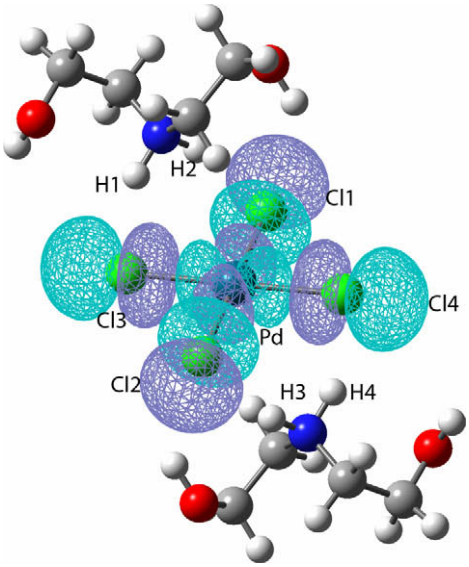


Fig. 1. Optimized geometry of complex **1** with delineated LUMO map.

Lower occupancy in all σ Pd–Cl orbitals (1.90) is due to donation of density from each σ bonding orbital to the *trans*- σ^* Pd–Cl antibonding orbital, in accord with the usual chemical picture of delocalized chemical systems.

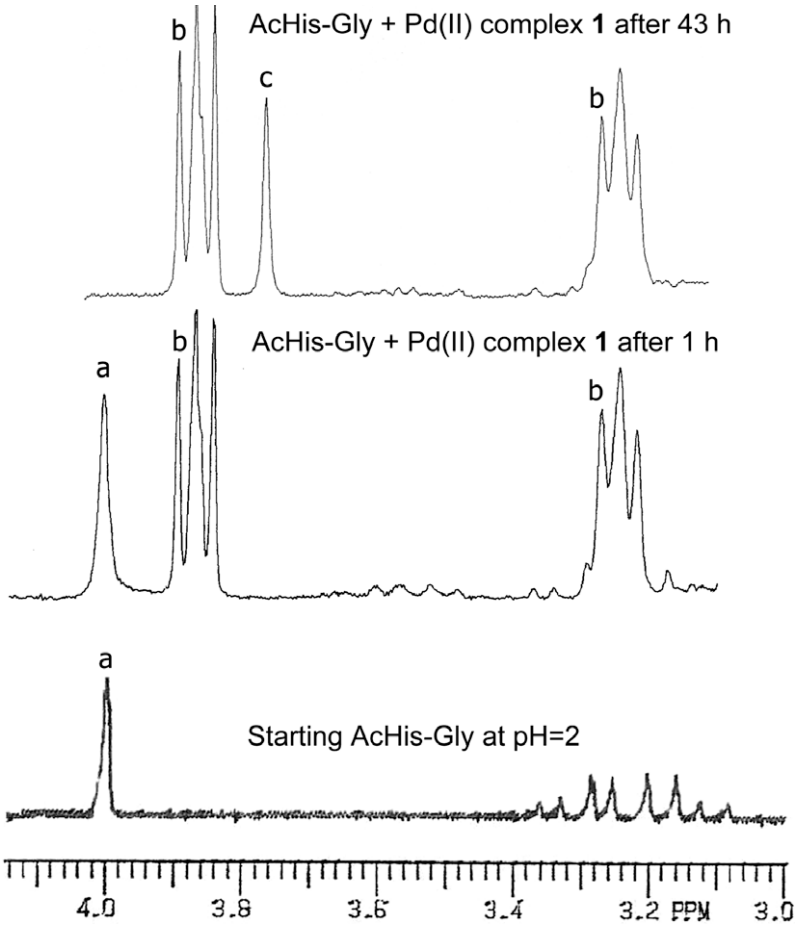
The vibrational analysis of complex **1** was performed. There are characteristic vibrations at 3740 cm^{-1} (stretching vibrations of OH groups of protonated diethanolamine), 3128 and 3042 cm^{-1} (stretching vibrations of NH_2^+ ions), 1623 cm^{-1} (deformational vibrations of NH_2^+ ions), 3011 cm^{-1} (stretching vibrations of CH_2 groups), and 1505 (deformational vibrations of CH_2 groups). These values are significantly different from the experimental values of 3343, 3051, 2874, 1574, 2912, and 1455 cm^{-1}. It is well known that the computed values of vibrational frequencies contain systematic error due to neglect of electron correlation. There is also a contribution to the error due to the fact that the calculated frequencies come from treating the potential energy surface near the stationary point as a harmonic oscillator. In reality, the potential energy surface near the stationary point is generally anharmonic. This results in overestimating the vibrational frequencies values, on average, by anywhere between 0% and 20%, implying that they need to be scaled to provide satisfactory approximation of experimental vibrational frequencies. By applying a scaling factor for B3LYP/6-311G(d,p) of 0.967 ± 0.021 [46] to the computed values of vibrational frequencies for complex **1**, the agreement with experimental data is improved. The only exception is the stretching vibration of OH groups of protonated diethanolamine, which can be attributed to association of complex via hydrogen bonds crosswise OH groups. Such agreement between experimental and computational results confirms the predicted structure of complex **1**. These results were used in order to describe the structure–activity relationship (SAR) of complex compound **1**.

4.2. Hydrolytic reaction of $[HDEA]_2[PdCl_4]$ complex with AcHis-Gly

The Pd(II)-diethanolamine complex *trans*-$[PdCl_2(DEA)_2]$ [11] showed selective hydrolytic activity in the reaction with *N*-acetylated *L*-histidylglycine dipeptide (AcHis-Gly). We supposed that novel Pd(II)-diethanolamine complex **1** can be artificial metallopeptidase, also. To confirm our presumption, we performed the reaction of this complex and dipeptide AcHis-Gly (molar ratio 1:2) at pH 2.0 and 60 °C in D_2O, and studied by ^1H NMR spectroscopy. In our experiment, under the above mentioned conditions, only one complex and selective cleavage of peptide bond were observed after 15 h. The NMR spectra showed decreasing of the singlet at 4.00 (due to mehylene glycine protons of the non-hydrolyzed substrate) and increasing of the singlet at 3.77 ppm for these protons in free glycine. After 43 h of heating the reaction mixture at 60 °C, the intensity of singlet at 3.77 ppm was not changed, Graphic 1. Addition of amino-acid glycine to the reaction mixture caused an increase of the signal at 3.77 ppm. Under these reaction conditions free acetic acid was not detected by NMR spectroscopy during investigated reaction time.

4.3. Soybean lipoxygenase inhibition study in vitro [44]

In continuation of our study on biological significant derivatives of histidine, this part of our work is devoted to the study of *in vitro*



Graphic 1. Parts of ^1H NMR spectra for the hydrolytic reaction of AcHis-Gly with $[HDEA]_2[PdCl_4]$ complex as function of time at pH = 2 and 60 °C in D_2O as solvent. The chemical shifts are in ppm relative to TSP. Resonance are indicated as follows: (a) methylene glycine protons of the starting dipeptide; (b) protons of the protonated diethanolamine units of the $[HDEA]_2[PdCl_4]$ complex (**1**); and (c) methylene protons of the free glycine.

inhibition of soybean lipooxygenase (LOX), as a histidine-containing enzyme. Availability and stability of mammalian lipoxygenases is limited, and therefore research on lipoxygenases was done with readily obtainable enzyme from soybean seeds. The active site in soybean LOX is non-heme Fe(III) atom coordinated by three histidines, isoleucine, asparagine and a hydroxide group [47], Fig. 2. Inhibition of this enzyme was investigated by using complexes **1** and **2**, and DEA.

Considering the radical mechanism of inhibition of LOX and the fact that palladium(II) ion is "soft" Lewis acid, we assumed that complexes **1** and **2** can act as free radical scavengers in LOX-catalyzed reaction of dioxigenation of fatty acids.

The generally accepted mechanism of action of LOX is radical mechanism which involves hydrogen abstraction from the fatty acid by the iron(III)-hydroxide group, accompanied with the reduction of LOX to Fe(II) form (Scheme 1) The obtained pentadienyl radical is then trapped by dioxigen to yield a peroxyl radical of fatty acid as a catalytic intermediate. Reduction of the peroxyl radical by the Fe(II) ion yields the hydroperoxide product and regenerates the Fe(III) ion (active LOX form) [48].

Studies on inhibitors of soybean LOX show several possible mechanisms, e.g., by binding inhibitor to sites around the active site of the enzyme molecule [49], by preventing the formation of the activated Fe(III) form of LOX [50] or by trapping the free radicals formed during the lipoxygenase-catalyzed oxygenation of polyunsaturated fatty acids [51]. In our cases it is reasonable to expect that palladium(II)–chlorido complexes, as electrophiles, react as free radical scavengers by trapping radical intermediate(s) and blocking the catalytic cycle.

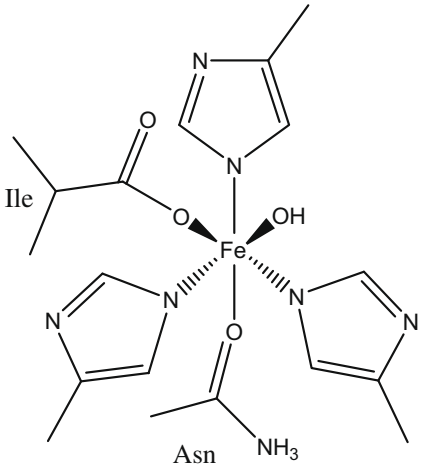


Fig. 2. The active site in soybean LOX.

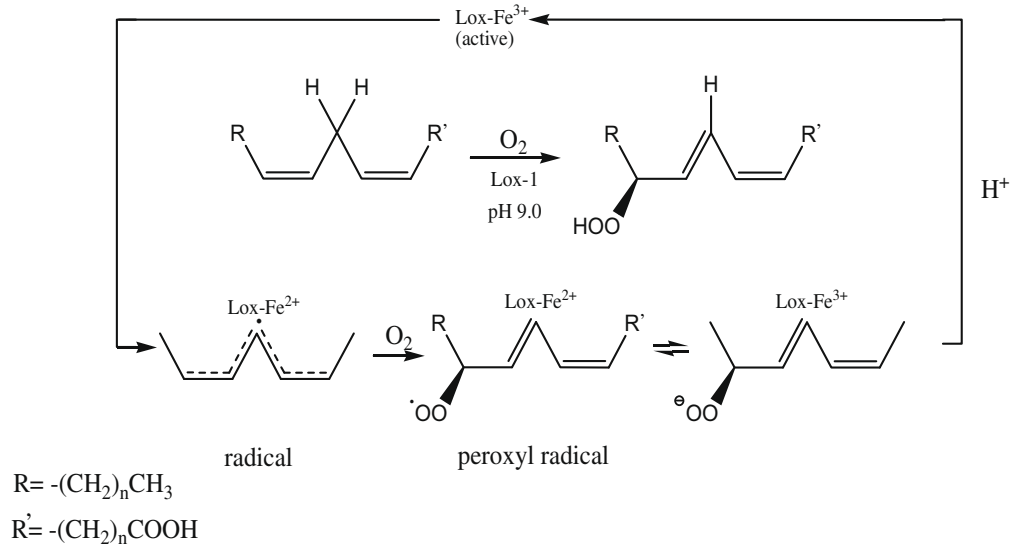
UV absorbance-based enzyme assays with diethanolamine palladium(II) complexes **1** and **2**, and DEA were done in order to evaluate their inhibitory activity of soybean LOX. Perusal of% inhibition values, or IC_{50} values, shows that DEA as a complex precursor has lower IC_{50} than complexes. Significant higher inhibitor activity of complex **1**, and especially **2**, relative to DEA (Table 1) clearly shows that palladium(II)-chlorido moiety of these complexes is meritorious for higher inhibitor activity. Complex **2** is very active and more potent than the reference compound caffeic acid. These results are supported by our DFT calculations and structure–activity relationship (SAR) investigation. In Fig. 1 the LUMO map of complex **1** is depicted. It is shown that the most electron-deficient area of complex **1** is delocalizated over palladium (II) ion and four chlorido ligands, namely over the $[PdCl_4]^{2-}$ ion. Therefore, the $[PdCl_4]^{2-}$ moiety of the complex acts as weak Lewis acid capable for accepting electrons from other species, such as radicals. In case of complex **2**, LUMO orbital is delocalizated over palladium (II) ion, two chlorido ligands and two nitrogen atoms of diethanolamine ligands, Fig. 3. For this reason, one can suppose that complexes **1** and **2** can be used as successful radical scavengers.

In order to prove free radical scavenger properties of complexes **1** and **2**, and DEA, assays *in vitro* with superoxide anion radical were done. The superoxide-producing system was set up by mixing phenazine methosulfate (PMS), nicotinamide-adenine-dinukle-

Table 1
% Superoxide radical scavenging activity ($O_2^{\cdot-}$); *in vitro* inhibition of soybean lipoxygenase (LOX) (IC_{50}).

Compound	($O_2^{\cdot-}$) (%) 0.01 mM	($O_2^{\cdot-}$) (%) 0.1 Mm	LOX IC_{50} (μM)
Complex **1**	78	100	210
Complex **2**	64	100	76
DEA	83	92	500
CA		45	600

CA caffeic acid; DEA diethanolamine.
Each value represents the mean of two independent experiments.



Scheme 1. Radical mechanism for lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids under aerobic conditions.

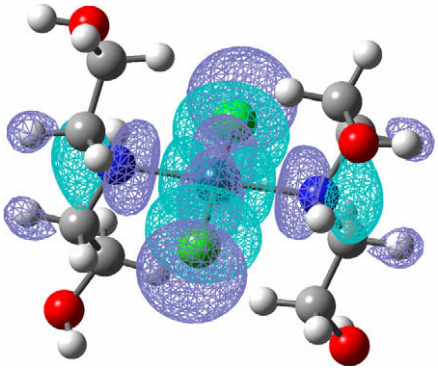


Fig. 3. Optimized geometry of complex **2** with delineated LUMO map [52,53].

otide (NADH) and air-oxygen. Generation of the superoxide radical anion is a first step of the spectrophotometric method that has been applied to the measurement of the antioxidant activity of investigated complexes. The complexes showed very high scavenging activity which was concentration dependent. Diethanolamine, as precursor of the investigated complexes, exhibited lower activity, Table 1. The complexes **1** and **2**, as well as DEA, are very active and more potent than the reference compound caffeic acid.

5. Concluding remarks

The reaction between $PdCl_2$ and diethanolammonium chloride in a molar ratio of 1:2, leads to the easy formation of the $[HDEA]_2[PdCl_4]$ complex (**1**), whose structure was optimized by DFT methods. In order to prove hydrolytic activity, the reaction between the $[HDEA]_2[PdCl_4]$ complex with MeCOHis-Gly dipeptide at pH = 2.0 and 60 °C was done. Selective cleavage of peptide bond was observed under these experimental conditions during the course of 43 h. Also, complexes **1** and **2**, and DEA, as their precursor, were tested for their *in vitro* soybean LOX inhibitory and free radical scavenging activities. UV absorbance-based enzyme assay and assay with superoxide anion radical were done. The scavenging activities of the complexes were measured and compared with those of their precursor and vitamin C. Complex **2** with high antioxidant ability and low IC_{50} value is considered as agent with potential antioxidant activity, and can therefore be candidate for further stages of screening *in vitro* and/or *in vivo*.

Acknowledgments

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