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Low-Toxicity Complexes with Nitrogen-Containing Ligands as Promising New Agents for Treatment and Prevention of Free Radical States

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Abstract—A method for synthesis of complexes with 1,5-pentamethylenetetrazole ligand was proposed. These compounds hold promise for antioxidant treatment of so-called "free radical" diseases of different geneses, including those caused by unfavorable environmental factors. The antioxidant activity of the complexes synthesized was tested by two independent methods: by assessing the ability to inhibit lipid peroxidation and by measuring the SOD-like activity.

Keywords: Unfavorable factors, free radical state, antioxidants, SOD-like activity, complexes of 1,5-pentamethylenetetrazole.

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All organs and systems in human body require oxygen to actively function. The main metabolic processes in humans are underlain by redox reactions. In the mitochondria, up to 5% molecular oxygen forms highly reactive free radicals, rather than is converted to water [1]. In this context, a special role belongs to free-radical processes producing oxygen-containing radicals and peroxides. These reactive oxygen species, commonly referred to as ROSs, exhibit strong toxic effects [2]. With their high reactivity, ROSs react with a broad range of biomolecules [3–6]. Maintaining the proper level of the common pool of free radicals under adverse environmental conditions is a major challenge of our time [7–10].

The formation of free radicals is an important protective mechanism underlying the nonspecific immunity [11, 12]: Phagocytosis leads to a multiple increase in the content of free radicals in the phagocytic cells with a simultaneous increase in oxygen consumption by a factor of 20 or greater [13].

At the same time, excessive activation of free-radical oxidation reactions is a typical pathological process occurring as a consequence of various diseases [14] and injurious exposures of the body. It was demonstrated that free radicals are involved in the pathogenesis of many diseases (shocks of various geneses, atherosclerosis, disorders of cerebral, peripheral, and coronary circulation [15], diabetes mellitus and diabetic angiopathy [16], rheumatoid, inflammatory, and degenerative diseases of the musculoskeletal system, eye lesions, lung diseases, thermal damages, various toxications, cancer [17, 18], and reperfusion injuries [19–22]). Excess production of free radicals and exposure to factors intensifying their generation constitute the major cause of premature aging [23, 24].

Human body has its own natural mechanisms to cope with excess free radicals: A complex multilevel antioxidant system includes enzymes, vitamins, and low-molecular-weight compounds [25–27].

Today, however, under conditions of technogenic civilization, humans are exposed to a multitude of xenobiotics, and human body is incapable of neutralizing all the excess free radicals using its own resources. This results in an imbalance between the substances promoting production of free radicals (pro-oxidants) and those reducing the level of radical metabolites, antioxidants (AOs), thereby leading to an oxidative stress [28–30]. Thus, proper functioning of the natural antioxidant system of humans requires constant supply of specific agents [31].

There exist a fairly broad spectrum of such agents having different structures and origins. Based on the currently adopted principles, antioxidants are traditionally classified as follows [32, 33]:

(1) Antiradical agents:

(1.1) Endogenous compounds: vitamins E, C, and A, carotene (pro-vitamin A), ubiquinone, and lycopene and

(1.2) Synthetic agents: ionol (dibunol), emoxipine, probucol (phenbutol), dimethyl sulfoxide (dimexide), and oiliphenum (hypoxene);

(2) Antioxidant enzymes and their activators: superoxide dismutase (Erisod, Ergotein), and sodium selenite; and

(3) Substances that block free radical formation: allopurinol (milurite) and antihypoxants.

Although a wide range of agents controlling the free-radical processes in human body are already in use, intensive search for new agents able to interfere with the processes involving reactive oxygen species is, however, under way now. Relatively recently, testing complexes as antioxidant agents has been initiated [34–37]. Among them, complexes with nitrogen-containing ligands are considered today to constitute a promising class of antioxidants [38–40]. It was found that antioxidant compounds with metals significantly extend the lifespan in animals under acutely emerging hypoxic conditions. In particular, distinct antihypoxic action was revealed for divalent copper-ion complexes [41–43].

Here, we synthesized new copper complexes with a polynitrogen ligand, 1,5-pentamethylenetetrazole (PMT) (corazole): copper(II) perchlorate bis(1,5-pentamethylenetetrazole) $[Cu(PMT)_2]\cdot(ClO_4)_2$, copper(II) perchlorate tetrakis(1,5-pentamethylenetetrazole) $[Cu(PMT)_4](ClO_4)_2$, copper(II) chloride (1,5-pentamethylenetetrazole) $[Cu(PMT)]Cl_2$, and copper(II) ascorbate (1,5-pentamethylenetetrazole) $[Cu(PMT)_{1/2}]\cdot(HAsc)_2$ and examined their antioxidant properties.

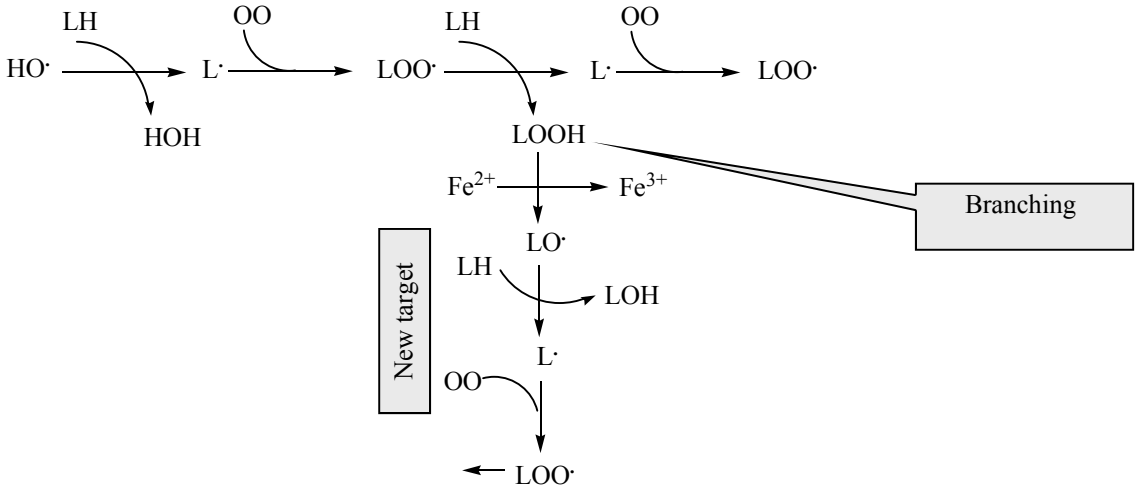
The antioxidant action of agents is underlain by their ability to interact with ROSs: H_2O_2, $OH^{\cdot}$, and $O_2^{-\cdot}$. Classic antioxidants react with all the above-listed reactive oxygen species, but the rate of reactions with various ROSs depends primarily on the structure of a particular AO [44]. Each antioxidant reacts preferentially with one specific oxygen radical. For the above-mentioned complexes, the most likely may be the reaction with $O_2^{-\cdot}$ via accepting one of the electrons thereof, whereby the radical-chain reactions of oxidation of biomolecules will be terminated.

Since corazole (1,5-pentamethylenetetrazole) proper may exhibit pronounced biological properties and act as a free radical scavenger, we had to evaluate its potential antioxidant activity.

Considering simultaneous generation of multiple ROSs under standard conditions, as well as their interconversions, we employed several methods for evaluating the antioxidant activity in order to arrive at a comprehensive picture.

1. Effect of the Agents Synthesized on the Fe^{+2}-Initiated Chemiluminescence in a Liposome Suspension

The antioxidant properties of an agent are traditionally evaluated basing on its ability to inhibit lipid peroxidation (LPO) [45].



Schematic diagram of the lipid peroxidation process.

Table 1. Comparison of the abilities of the complexes synthesized to inhibit chemiluminescence when in the concentration of 5×10^{-6} M in a liposome suspension at 20°C, pH = 7.4, c($FeSO_4$) = 2.4×10^{-3} M

Comp. no.	Compound	Quenching			
		light sum, rel. units	decrease in the light sum, %	peak intensity, rel. units	decrease in the peak intensity, %
	Control	116±3	0	109±3	0
I	$[Cu(PMT)_2](ClO_4)_2$	67±3	42	106±3	3
II	$[Cu(PMT)_4](ClO_4)_2$	108±3	7	144±3	Growth 32
III	$[Cu(PMT)]Cl_2$	51±3	56	107±2	2
IV	$[Cu(PMT)_{1/2}](HAsc)_2$	84±2	27	123±2	Growth 13
V	PMT	84±2	27	130±2	Growth 19
VI	KHAsc	96±2	17	138±2	Growth 27
VII	$CuSO_4\cdot5H_2O$	85±2	27	48±2	56

Among the main substrates for free-radical reactions are lipids, above all polyunsaturated fatty acid molecules and the lipid components of very-low- and low-density lipoproteins. These substrates are involved in a complex free-radical process, LPO [45–47].

To date, lipid peroxidation is fairly well understood due to the availability of chemical kinetic data and is regarded as a radical chain process [45]. Figure presents a schematic diagram of this process.

Lipid peroxidation is accompanied by weak chemiluminescence [48]; an agent reducing the chemiluminescence intensity in a system is considered as exhibiting antioxidant properties.

The stronger the chemiluminescence quenching effect produced by an agent, the higher its antioxidant properties. In our studies we used standard systems. One such system is a liposome suspension of egg yolk in a buffer or saline [49]. For our experiments we selected a liposome suspension in phosphate buffer with pH = 7.4. Cysteamine ($NH_2CH_2CH_2SH$), which is well known for its ability to capture oxygen radicals and quench chemiluminescence, served as the reference [50]. It should be noted that thiol-containing compounds usually exhibit high antioxidant activities [51, 52].

A decrease in the chemiluminescence, caused by the ligand (1,5-pentamethylenetetrazole) and its complexes, is suggestive of their antioxidant properties. The tested compounds producing chemiluminescence quenching effects close to that achieved with cysteamine at identical concentrations may be considered as promising antioxidants. The chemiluminescence quenching abilities of the agents are presented in Table 1.

The chemiluminescence quenching was assessed from decreases in two parameters:

– light sum and

– peak intensity.

It should be noted that changes in the light sum and peak intensity do not exhibit similar trends. However, the light sum is most often used to describe the antioxidant activity. This choice is justified since, in the development of a chain process in a biosystem, formation of free radicals may proceed in a nonuniform manner, with possible short-term acceleration of radical reactions, but the total number of the radicals produced in a certain period may be smaller. With the light sum used for characterizing the peroxidation intensity, all the compounds tested by us (**I–VII**) may be considered as antioxidants (see Table 1).

Introduction of corazole (PMT) into the inner sphere causes the antioxidant activity to increase. In the case of compounds **I** and **II**, the chemiluminescence quenching effect depends on the number of the corazole molecules coordinated. With two corazole molecules in the inner sphere of the complex the chemiluminescence level decreases by 42%, thereby indicating a pronounced antioxidant activity. Introduction of another two corazole molecules leads to nearly complete loss of antioxidant properties, as evidenced by the recovery of the chemiluminescence level to that of the control.

The antioxidant activity of the complexes is weakly affected by the nature of the anion (compounds **III** and **I**). However, introduction into the outer sphere of a reducing agent, ascorbic acid (HAsc), significantly deteriorates the antioxidant activity. It is impossible to clearly identify how the antioxidant activity depends on the nature of the anion, since the number of the corazole molecules coordinated in the inner sphere of

the complex varies with the anion. In this connection, it was of interest to examine the SOD-like activity of the agents tested.

The choice of this approach was dictated by the fact that the processes of the type can be fairly effectively implemented in vivo [53, 54].

$$Me^{n+} + O_2 \rightarrow Me^{(n+1)+} + O_2^{\bullet -}, \quad (1)$$

$$Me^{n+} + O_2^{\bullet -} \rightarrow Me^{(n-1)+} + O_2. \quad (2)$$

2. Determination of the SOD-Like Activity

The ability of agents to catalyze the reaction

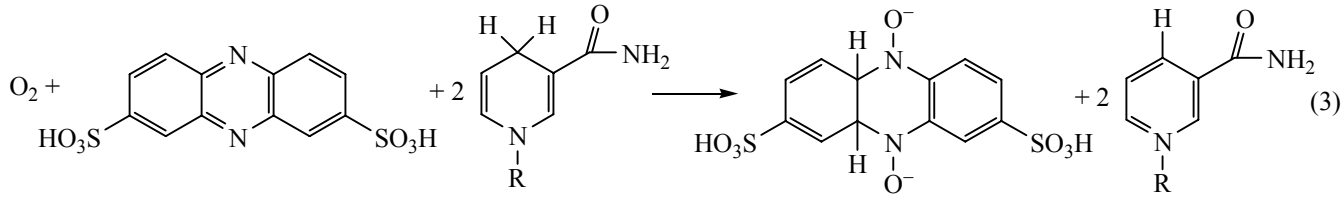
$$2O_2^{\bullet -} + 2H^+ = H_2O_2 + O_2$$

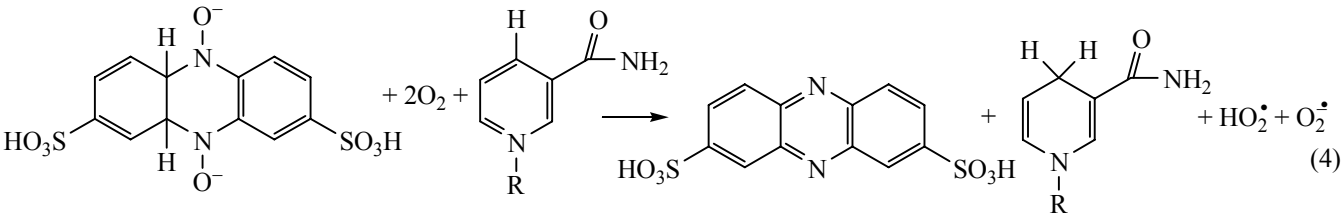
or in another way to remove the superoxide anion radical from the system is called the SOD-like activity [55], since the removal of superoxide anion radical in vivo is catalyzed by a particular enzyme, superoxide dismutase (SOD) [56, 57].

Complexes can affect the superoxide anion radical levels in biological systems [55].

We assessed the SOD-like activity from the ability of the agents synthesized to inhibit the reduction of tetrazolium to formazan under standard conditions.

The reactions in the test system comprised of nitrotetrazolium blue, reduced nicotinamide adenine dinucleotide ($NADH_2$), and phenazine methosulfate (PMS), are represented by Eqs. (3)–(6).





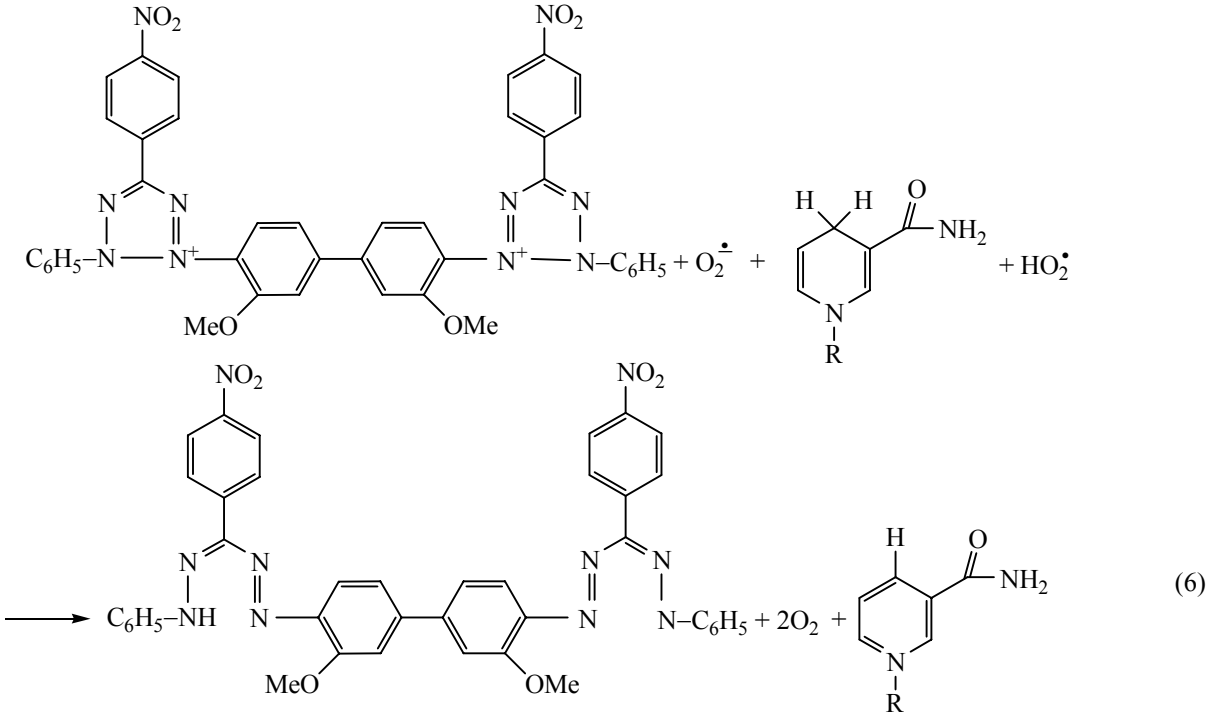


Table 2. The SOD-like activity of the complexes in the presence of EDTA[a]

Comp. no.	Compound	Optical density *D*	Quenching, %
	Control	0.268±0.02	0
I	$[Cu(PMT)_2](ClO_4)_2$	0.229±0.02	16±3
II	$[Cu(PMT)_4](ClO_4)_2$	0.223±0.02	17±3
III	$[Cu(PMT)]Cl_2$	0.183±0.02	32±3
IV	$[Cu(PMT)_{1/2}](HAsc)_2$	0.199±0.02	26±4
V	PMT	0.242±0.01	10±5
VI	KHAsc	0.241±0.01	10±1
VII	$CuSO_4 \cdot 5H_2O$	0.167±0.02	38±4

[a] EDTA is ethylenediaminetetraacetate

Table 3. The SOD-like activity of the complexes in the absence of EDTA[a]

Comp. no.	Compound	Optical density *D*	Quenching, %
	Control	0.180±0.02	0
I	$[Cu(PMT)_2](ClO_4)_2$	0.109±0.02	39±3
II	$[Cu(PMT)_4](ClO_4)_2$	0.106±0.02	41±3
III	$[Cu(PMT)]Cl_2$	0.090±0.02	50±3
IV	$[Cu(PMT)_{1/2}](HAsc)_2$	0.094±0.02	48±5
V	PMT	0.167±0.01	7±1
VI	KHAsc	0.179±0.01	1±4
VII	$CuSO_4 \cdot 5H_2O$	0.110±0.02	39±2

[a] EDTA is ethylenediaminetetraacetate

The data on inhibition of the reduction of nitrotetrazolium blue to formazan by the corazole complexes synthesized are presented in Table 2.

The standard method for measuring the superoxide dismutase activity involves introduction of EDTA into the reaction medium [58].

As known [59], EDTA is a good complexing agent able of substituting polynitrogen ligands in the inner sphere of complexes. Hence, we had to elucidate how it could affect the results that we obtained [60]. To this end, similar experiments were conducted in the system free from EDTA (Table 3).

From Tables 2 and 3 we can conclude the following:

(1) Studies of the SOD-like activity revealed a pronounced ability of copper sulfate **VII** to inhibit the reduction of nitrotetrazolium blue to formazan. Close activity levels are exhibited by coordination compounds **I–IV** tested by us, whereas the free ligand and the salts in the absence of the copper cation lack an antioxidant activity. Presumably, removal of $O_2^{\bullet -}$ depends on the presence of copper which is reduced as follows:

$$Cu^{2+} + O_2^{\bullet -} \rightarrow Cu^{+} + O_2.$$

(2) When introduced into the system, EDTA does not basically affect the ability of copper-containing agents to react with superoxide radical anion ($O_2^{\bullet -}$) but is likely to be coordinated by the copper ion. As a result, corazole is partially or completely displaced from complexes **I** and **II**, or enters the inner sphere of complexes **III** and **IV**, thereby increasing the coordination number of copper to four. Most probably, this is specifically responsible for a decrease in the SOD-like activity of **I, II** and **III, IV** in the EDTA-containing medium compared to that exhibited by these compounds in the samples free from EDTA.

(3) In the EDTA-containing system, the ability to display a SOD-like activity is affected by the outer-sphere anion. The reactivity decreases most significantly in the case of the agents containing perchlorate anion (complexes **I** and **II**).

Thus, in two independent tests the antioxidant activity was exhibited by the same agent, $[Cu(PMT)]Cl_2$.

Therefore, we can presume that the chemiluminescence quenching effect is due to termination of the sequential oxygen reduction chain:

$$O_2 + e^- \rightleftarrows O_2^{\bullet -} + e^- \rightleftarrows O_2^{2-} + e^- \rightleftarrows [O^- + O^{2-}] + e^- \rightleftarrows 2O^{2-}$$

$$+H^+ \; - \; H^+ \qquad 2H^+ \; - \; 2H^+ \qquad +3H^+ \; - \; 3H^+ \qquad +4H^+ \; - \; 4H^+$$

$$HO_2^{\bullet} \qquad H_2O_2 \qquad HO^{\bullet} + H_2O \qquad 2H_2O$$

(ROO$^{\bullet}$ analog) (ROOH analog)

via elimination of O_2^- from the system. In this case, formation of a hydroxyl radical, an active LPO initiator, does not take place, which fact is specifically responsible for a decrease in the chemiluminescence intensity.

Thus, the chemiluminescence quenching effect and the SOD-like activity make $[Cu(PMT)]Cl_2$ a potential and promising antioxidant.

EXPERIMENTAL

Preparation of the complexes *(general procedure)*. The complexes were synthesized according to the reaction below:

$$M(An)x + nPMT \rightleftarrows [M(PMT)_n](An)_x,$$
$$M = Cu(II);\ An = Cl^-, ClO_4^-;\ n = 1, 2, 4.$$

The calculated amount of PMT is added to an aqueous salt solution under stirring at room temperature, after which the reaction mixture is heated to 60°C and kept at this temperature for 3 h. After cooling to 17°C the resulting precipitate is filtered off, washed with ethyl acetate (20 mL), and recrystallized from isopropyl alcohol.

Synthesis of copper(II) perchlorate bis(1,5-pentamethylenetetrazole) $[Cu(PMT)_2](ClO_4)_2$. Yield 60%. IR spectrum, ν, cm^{-1}: 2860 [$\nu_s(CH_2)$]; 1925 [$\nu_{as}(CH_2)$]; 1470 [$\delta(CH_2)$]; 1454 (N=N); 1352, 1337 (C=N); 1282, 1268 (N–N); 1100–800 (Tz). ^{1}H NMR (DMS-d_6) spectrum, δ, ppm: 4.41 [2H, CH_2–(N^1)]; 2.92 s[2H, $CH_{2'}$ (C^5)]; 1.81 s (2H, C^7), 1.70 s (2H, C^9), 1.57 s (2H, C^8). Found, %: C 34.99; H 5.1; N 27.49. $C_{12}H_{20}Cl_2{\cdot}CuN_8O_8$. Calculated, %: C 35.4; H 5.95; N 27.5.

Synthesis of copper(II) perchlorate tetrakis(1,5-pentamethylenetetrazole) $[Cu(PMT)_4](ClO_4)_2$. Yield 0.61 g of a light blue product (60% of theory). IR spectrum, ν, cm^{-1}: 2860 [$\nu_s(CH_2)$]; 1925 [$\nu_{as}(CH_2)$]; 1470 ($\delta(CH_2)$]; 1454 (N=N); 1352, 1337 (C=N); 1282, 1268 (N–N); 1100-800 (Tz). ^{1}H NMR (DMS-d_6) spectrum, δ, ppm: 4.41 s [CH_2–(N^1)]; 2.92 S (2H, C^7); 1.81 s (2H, C^7), 1.70 s (2H, C^9), 1.57 s (2H, C^8). $C_{24}H_{40}Cl_2Cu{\cdot}N_{16}O_8$. Calculated, %: C 35.4; H 4.95; N 27.5.

With isopropyl alcohol as the solvent, the end product was obtained in a higher yield. Yield 0.81 g of a light blue product (80% of theory). IR spectrum, ν, cm^{-1}: 2860 [$\nu_s(CH_2)$]; 1925 [$\nu_{as}(CH_2)$]; 1470 [$\delta(CH_2)$]; 1454 (N=N); 1352, 1337 (C=N); 1282, 1268 (N–N); 1100–800 (Tz). ^{1}H NMR (DMS-d_6) spectrum, δ, ppm: 4.41 [2H, CH_2–(N^1)]; 2.92 s[2H, $CH_{2'}$ (C^5)]; 1.81 s (2H, C^7), 1.70 s (2H, C^9), 1.57 s (2H, C^8). Found, %: C 35.2; H 5.2; N 27.5.

Synthesis of copper(II) chloride (1,5-pentamethylenetetrazole) $[Cu(PMT)]Cl_2{\cdot}4H_2O$. Yield 0.83 g (83% of theory). Calculated, %: C 21.21; H 5.31; N 16.49. $C_6H_{18}Cl_2CuN_4O_4$. Found, %: C 21.17; H 5.37; N 16.13. IR spectrum, cm^{-1}: 3163 [$\nu_{as}(H_2O)$]; 1629 [$\delta(H_2O)$]; 2943 [$\nu_s(CH_2)$]; 1477 [$\delta(CH_2)$]; 2943, 1535, 1421, 968 (Tetr).

We used PMT available from ACROS ORGANICS as finished product (98% pure, mp 58.5°C).

Determination of the SOD-like activity. The SOD-like activity of the complexes synthesized was determined by the spectrophotometric method.

We used the following chemicals:

(1) A solution of nitrotetrazolium blue: 100 mg of anhydrous compound was dissolved in 50 mL of distilled water using a hot water bath, filtered, and stored in a dark-colored container (because of photosensitivity).

(2) A solution of phenazine methosulfate (PMS): 1 mg was dissolved in 100 mL of distilled water and stored in a dark-colored container (because of photosensitivity).

(3) A solution of nicotinamide dinucleotide phosphate ($NADH_2$): 6 mg of anhydrous product was dissolved in 3 mL of tris-EDTA buffer (pH 8.0) and 1.8 mL of distilled water and stored in a dark-colored container (because of photosensitivity).

(4) Phosphate buffer, pH = 7.8.

The reaction mixture had the following composition:

(a) 0.2 mL of EDTA (ethylenediaminetetraacetate) solution;

(b) 0.1 mL of a gelatin solution;

(c) 0.5 mL of nitrotetrazolium blue;

(d) 0.1 mL of PMS (phenazine methosulfate); and

(e) 2.0 mL of phosphate buffer.

The experiments aimed to elucidate how the complexes affect the reduction of nitrotetrazolium blue to formazan were performed as follows. A control sample and a test sample were prepared in parallel. Into the control sample, 2.0 mL of the buffer, 0.5 mL of the tetrazolium solution, 0.1 mL of the PMS

solution, 0.2 mL of distilled water, and 0.2 mL of the $NADH_2$ solution were added sequentially. After $NADH_2$ was introduced into the reaction medium, a stopwatch was started, and the samples were placed into a thermostat under the dark conditions. After 10 min the optical density of the solution was measured at $\lambda = 540$ nm with reference to the buffer solution. The test sample was treated in a similar manner, except that 0.05–0.2 mL of the solution of the complex was added instead of distilled water, with the missing quantity made up by distilled water. In both the control and test samples, nitrotetrazolium blue was reduced to formazan. Comparison of the optical densities of the control and test samples allowed evaluating the effect of the agent on reduction of nitrotetrazolium blue to formazan. Each measurement was made in triplicate–quintuplicate.

Examination of the antioxidant activity based on the chemiluminescence quenching effect produced by the complexes synthesized was performed under standard conditions in a liposome suspension of egg yolk in phosphate buffer, pH 7.4, with cysteamine as the reference.

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