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In silico and in vitro evaluation of the biological activity of some organic sulfur-containing compounds

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Abstract: It was predicted, using the Prediction of Activity Spectra for Substances online software, that organic sulfur-containing compounds 2,6-di-*tert*-butyl-4-mercaptophenol (**1**), 2,6-di-*tert*-butyl-4-(3-mercaptopropyl)phenol (**2**), bis-3-[3,5-di-*tert*-butyl-4-hydroxyphenyl)propyl] sulfide (**3**), bis(3,5-di-*tert*-butyl-4-hydroxyphenyl) disulfide (**4**), diphenyl disulfide (**5**), dibenzyl disulfide (**6**), dibutyl disulfide (**7**), di-*tert*-butyl disulfide (**8**), diallyl disulfide (**9**), and methyl propyl trisulfide (**10**) could possess antioxidant activity and act as active scavengers of oxygen metabolites. The antiradical activity of these species was analyzed and it was shown that they were able to react with electrochemically generated the superoxide anion radical. The rate constants for reactions of **1**–**10** with the superoxide anion radical were calculated. The effect of the organic sulfur compounds on the rate of generation $O_2^{-\bullet}$ on adrenaline oxidation in an alkaline medium and on the ability of the biopreparation, based on the liver of Russian sturgeon, to deactivate $O_2^{-\bullet}$ was studied. The effect of the organic sulfur compounds on the rate of $O_2^{-\bullet}$ generation in the model system of the quinoid oxidation of adrenaline in an alkaline medium and on the ability of the biopreparation to deactivate $O_2^{-\bullet}$ was studied. It was found that $O_2^{-\bullet}$ generation rate decreased in the presence of the studied compounds, evidencing for their antiradical activity. Increase of superoxide dismutation activity of the biopreparation in the presence of compounds **1**–**10** was also shown, and this fact could indicate their ability to increase the antioxidant status and decrease the probability of oxidative stress development. It was found that compounds **1**–**10** did not inhibit enzyme lipoxygenase, which indicated the absence of antiinflammatory activity. The results of the forecast were consistent with the data of the experimental studies.

Key words: Organic sulfur-containing compounds, superoxide anion radical, superoxide dismutase, in silico, cyclic voltammetry, in vitro

1. Introduction

The latest data have shown that oxidative stress is involved in the pathogenesis of various intractable diseases [1,2]. In living organisms, oxygen can exhibit toxic properties due to the formation of reactive oxygen species (ROS), which damage membrane lipids, proteins, and DNA; therefore, antioxidants should be used to neutralize ROS actions [3]. Many antioxidants are used in medicine, but some of them are not efficient enough or have unwanted side effects; thus, the new representatives of this class should be developed [4,5].

Sulfur-containing compounds can play an important role in the formation of the protective systems of an

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organism and act as agents that cause the decomposition of hydroperoxides [6]. Some natural sulfur-containing compounds have outstanding biological activity, for example, diallyltrisulfide, which is found in garlic, was intensively studied as an agent for the treatment of cancer and cardiovascular disease [7]. Diallylpolysulfides increase the glutathione level in cells and act as powerful inductors of detox enzymes [8]. For example, the organosulfur compound allicin, which is also found in garlic, demonstrates great antioxidant properties, since it decreases the formation of ROS in cells and prevents oxidative stress [9–11]. Allicin is very unstable and can decompose with the formation of other sulfur-containing compounds, such as diallyl disulfide [12]. The health benefits of organosulfur-containing foods are well known and have been attributed to prooxidant or/and antioxidant activities. The effects of garlic are similar to those of H_2S, which can be released from this plant under certain conditions, but recent studies have suggested that polysulfides could actually be the mediators of physiological signaling [13–15]. It is also well-known that phenolic antioxidants can act as inhibitors for radical chain reactions in organic substrate autoxidation; hence, the derivatives of hindered 2,6-dialkylphenols are used as models of vitamin E in industry and medicine. The use of polyfunctional compounds combining both the antioxidant 2,6-di-*tert*-butylphenol and sulfur-containing groups has been proposed as a way to enhance their antioxidant efficiency. Sulfur-containing phenolic compounds can exhibit their antioxidant activity in several ways: 1) radical scavenging activity toward either reactive species, such as $HO^{\bullet}$ and $O_2^{-\bullet}$, or toward lipid peroxidizing radicals such as $R^{\bullet}$, $RO^{\bullet}$, and $ROO^{\bullet}$; 2) the prevention of the transition metal-catalyzed production of reactive species through metal chelation; and 3) decomposition of hydroperoxides without ROS formation.

Aside from organic sulfur species, which are essential and indispensable constituents of living organisms, there is a plethora of less widespread organic sulfur-containing compounds that possess much less common and often unexpected properties and biological activity. In this view, the aim of this study was to predict the antioxidant activity of a row of synthetic organic sulfur-containing compounds, as shown in Figure 1: 2,6-di-*tert*-butyl-4-mercaptophenol (**1**), 2,6-di-*tert*-butyl-4-(3-mercaptopropyl)phenol (**2**), bis-3-[3,5-di-*tert*-butyl-4-hydroxyphenyl)propyl] sulfide (**3**), bis(3,5-di-*tert*-butyl-4-hydroxyphenyl) disulfide (**4**), diphenyl disulfide (**5**), dibenzyl disulfide (**6**), dibutyl disulfide (**7**), di-*tert*-butyl disulfide (**8**), diallyl disulfide (**9**), and methyl propyl trisulfide (**10**) using in silico methods, and to compare the results with experimental data in vitro. Synthetic analogues of the natural compounds were studied and compared with biologically-active disulfides, which can be found in plant products (di-*tert*-butyl disulfide, dibutyl disulfide, methylpropyl trisulfide, and diallyl disulfide). These compounds contain sulfur atoms and sterically-hindered phenolic fragments, which should favor an increase in their antioxidant activity. The combination of methods in silico and in vitro allowed for the effective primary screening of physiologically active compounds for further selection of the potential drug candidates.

2. Results and discussion

2.1. In silico studies

Antioxidants protect organisms from highly reactive free radicals, especially ROS, which are capable of oxidizing biomolecules, causing different human diseases [16]. The search for novel antioxidant agents that prevent or reduce the impact of oxidative stress on cells is a topical scope. Forecasting of biological activity of organic sulfur-containing compounds is important for the development of new drugs. In this study, the forecast of biological activity of compounds **1**–**10** was performed using the Prediction of Activity Spectra for Substances (PASS) software (PharmaExpert.ru ©2011, 2017, Version 2.0) [17]. As shown in Table 1, according to the

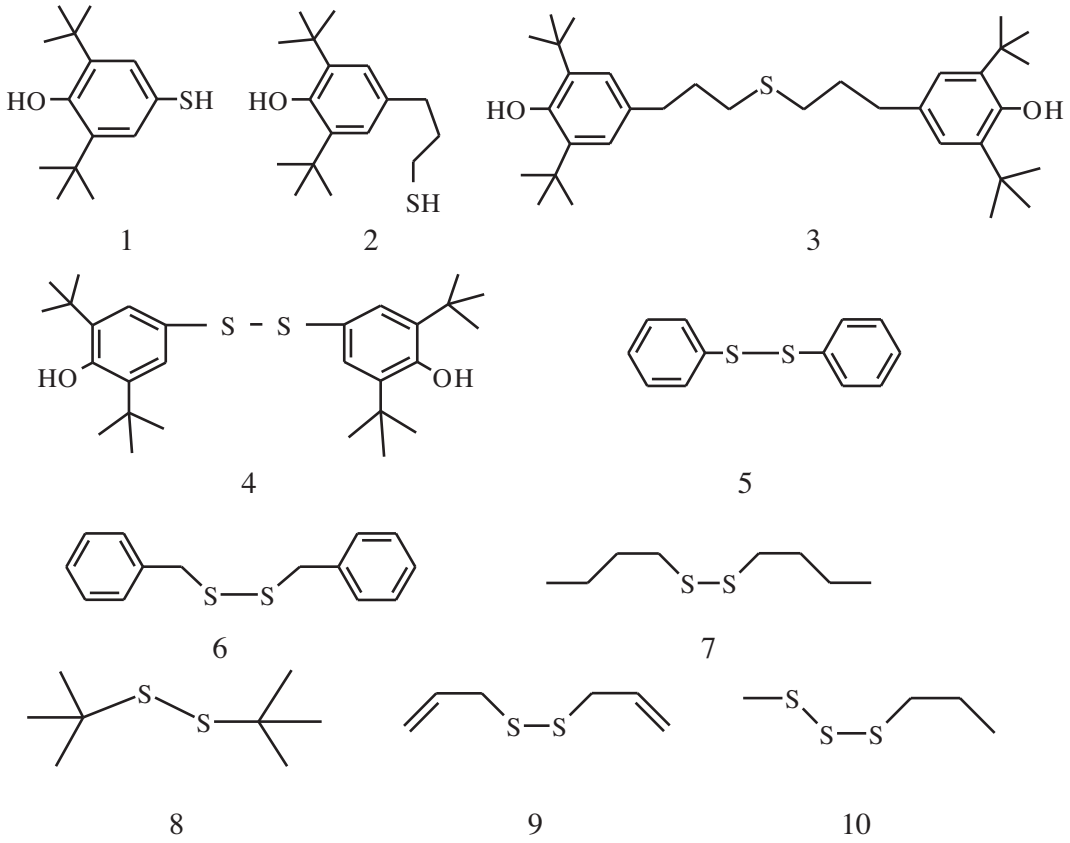

Figure 1. Structural formula of the studied compounds: 2,6-di-*tert*-butyl-4-mercaptophenol (**1**), 2,6-di-*tert*-butyl-4-(3-mercaptopropyl)phenol (**2**), bis-3-[3,5-di-*tert*-butyl-4-hydroxyphenyl)propyl] sulfide (**3**), bis(3,5-di-*tert*-butyl-4-hydroxyphenyl) disulfide (**4**), diphenyl disulfide (**5**), dibenzyl disulfide (**6**), dibutyl disulfide (**7**), di-*tert*-butyl disulfide (**8**), diallyl disulfide (**9**), and methyl propyl trisulfide (**10**).

computer forecast for compounds **1**–**10** the probability of antioxidant activity was revealed. The spectrum of biological activity for compounds **1**–**10** is presented in the form of a list of activity types, for which the probability of presence (P_a) and the probability of lack of activity (P_i) were calculated, the P_a and P_i values were independent and $P_a > P_i$.

A high P_a value for certain bioactivity types could be caused by similarity of the structures of the molecules of the tested compounds to the structures of known pharmacologically active substances. Among the large number of predicted types of bioactivity of compounds **1**–**10,** those that were closely related to antioxidant properties, namely, those with the ability to act as antioxidants and traps of ROS initiating oxidative processes, and antidotes, including antidotes that bind heavy metals, were selected. For all of the compounds, the probability to act as a ROS scavenger was shown; this can be a measure of antioxidant activity. In order to check the forecast, the antioxidant activity of the compounds **1**–**10**, investigations using different model systems in vitro were carried out.

2.2. Evaluation of the antioxidant properties of compounds by cyclic voltammetry

Oxygen metabolism in living organisms generally proceeds as a 2-electron reduction by the respiratory chain, resulting in water molecule formation, similar to the mechanism of oxygen electrochemical reduction at the electrode in an electrochemical cell [18]. The highly-reactive $O_2^{-\bullet}$ is formed by 1-electron reduction as a by-

Table 1. Antioxidant activity prediction for compounds **1–10.**

Compound	Oxygen scavenger		Nitric oxide scavenger		Free radical scavenger		Antidote	
	P_a	P_i	P_a	P_i	P_a	P_i	P_a	P_i
1	0.581	0.027	0.250	0.020	0.589	0.006	0.367	0.014
2	0.707	0.005	0.205	0.046	0.618	0.005	0.526	0.004
3	0.546	0.036	0.230	0.030	0.557	0.007	0.333	0.022
4	0.549	0.005	0.519	0.044	0.473	0.012	0.317	0.027
5	0.621	0.018	0.274	0.013	0.230	0.056	0.228	0.078
6	0.643	0.014	0.242	0.024	0.205	0.072	0.216	0.090
7	0.738	0.004	0.254	0.019	0.275	0.037	0.278	0.043
8	0.652	0.013	0.291	0.009	0.210	0.068	0.310	0.029
9	0.592	0.024	0.295	0.008	0.325	0.026	0.304	0.031
10	0.570	0.030	0.192	0.056	-	-	0.160	0.156

P_a: probability of presence of biological activity, P_i: probability of lack of biological activity.

product in the oxidation metabolism process, where it can be further oxidized to O_2 or reduced to H_2O_2.

During normal biological processes, $O_2^{-\bullet}$ and H_2O_2 are formed in small quantities and there is a natural defense antioxidant system, such as superoxide dismutase (SOD), which efficiently removes superoxides and other ROS. Under certain conditions, for example, the intake of drugs, UV-radiation, or metabolic dysfunction, the level of ROS ($O_2^{-\bullet}$, $HO^{\bullet}$, and $NO^{\bullet}$) generation exceeds the normal defense capacity of the body, which can result in harmful effects on tissues [19].

Thus, the $O_2^{-\bullet}$ is highly reactive and toxic, and it can cause oxidation of biomolecules as well as initiate radical-chain oxidation in tissues [20]. Numerous mechanisms of antioxidant defense are considered as the natural means of cell defense against the consequences of oxidation stress. The compounds, which can rapidly react with $O_2^{-\bullet}$, are promising antioxidants, because their reactions can result in a decrease in the toxic action of ROS and can stop the radical-chain processes.

To check (to confirm or disprove) the predicted activity of ROS acting as traps, the possibility of organic sulfur-containing compound interaction with electrochemically generated $O_2^{-\bullet}$ was investigated by cyclic voltammetry in a 3-electrode cell in CH_3CN on a platinum electrode using an IPC-Pro potentiostat.

It is known that the electrochemical reduction of oxygen to $O_2^{-\bullet}$ (cathode wave) and reverse oxidation (anode wave) can be detected by cyclic voltammetry in the aprotic media [21,22]. If the potential antioxidant is added to the aprotic solution, a decrease in the height of the anodic peak, corresponding to a decrease of the dissolved oxygen reduction to $O_2^{-\bullet}$, is detected. This criterion was convenient because electrochemically generated $O_2^{-\bullet}$ is a long-living species and no byproduct was generated [23].

The interaction of sulfur-containing organic compounds **1-10** with an electrochemically generated $O_2^{-\bullet}$ in CH_3CN on a platinum electrode was studied. The use of an aprotic medium, such as CH_3CN, allowed the avoidance of electrochemically generated $O_2^{-\bullet}$ disproportiation; hence, this radical remained stable, even in voltammetric sweeps at a low scanning rate [24,25]. The stability of electrochemically generated $O_2^{-\bullet}$ in CH_3CN was tested by analysis of the anodic oxidation current observed on the reverse scan (–2.0 to 0.5 V).

As shown in Figure 2, the form of cyclic voltammogram of the electroreduction of oxygen changed in the presence of compound **3.** CV curves for the other compounds were very similar. An increase in the oxygen reduction cathodic peak, decrease in the oxygen oxidation anodic peaks, and new peak in the anode region (E_{pa} = –0.2 ÷ 0.1 V) were found.

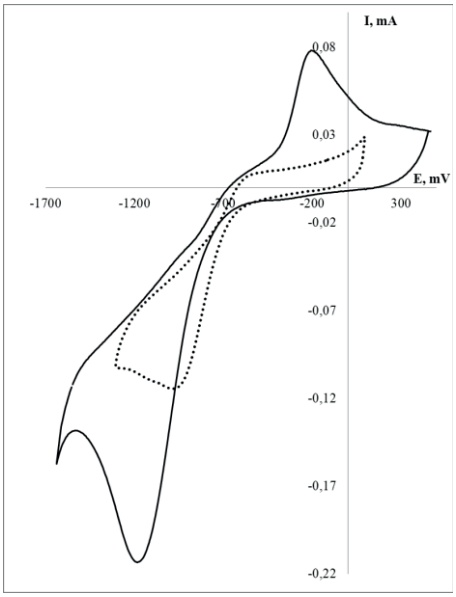

Figure 2. Cyclic voltammograms of $O_2^{-\bullet}$ in the presence of compound **3** (--) and without any additives (···) in CH_3CN (0.1 M *tert*-Bu_4NClO_4, 25 °C ± 1, and scan rate of 0.2 V s^{-1}).

A decline in the anodic peak of $O_2^{-\bullet}$ oxidation indicated that all of the compounds reacted irreversibly with the superoxide anion-radical. As shown in Table 2, based on the obtained voltammetric data, the rate constants (k) of the interaction of compounds **1**–**10** with $O_2^{-\bullet}$ were calculated. The calculations were based on the values of the relative increase in the oxygen reduction currents in the presence of the studied compounds, as described previously.

The values of these constants varied in the range of $0.003 \times 10^3 \div 1.68 \times 10^3$ L $mol^{-1} s^{-1}$. Such a wide range could be caused by the influence of the structure of organic sulfur-containing compounds, in particular the amount sulfur atoms, the nature of the functional groups, as well as the steric effects of the substituents.

Notably, the k value for **5** was significantly higher than for the other studied compounds, while in the case of **1**, **8**, and **9**, the k values were the lowest. Thus, the size of the molecule (and diffusion rate in the solutions, which depends on the size) did not play a critical role in the determination of the k value, because molecules the size of **5** were apparently not the lowest, and the molecular sizes of **1**, **8**, and **9** were apparently not the largest in the row of the studied compounds. Moreover, it can be noted that the presence of the 2,6-di-*tert*-butyl-4-mercaptophenolic or 2,6-di-*tert*-butyl-4-phenolic group was not the sole factor that governed the rate of compound interaction with $O_2^{-\bullet}$, as can be concluded from a comparison of the k values for **1**–**4**.

In this study, the oxygen reduction stage on the CV curves was transformed into an irreversible one in the presence of phenolic sulfur-containing compounds, which can be evidenced by their interaction with $O_2^{-\bullet}$. Furthermore, a new anodic peak at a potential of + 0.2 V occurred, which was presumably associated with the formation of the phenolate anion or thiolate anion in the reaction:

Table 2. Rate constants (k) of the interaction of compounds **1**–**10** with $O_2^{-\bullet}$ and antiradical capacity indicators.

Compound	k × 10^{-3}, L $mol^{-1}s^{-1}$	Antiradical activity, % of control	SOD activity of the sturgeon liver, % of control
1	0.009 ± 0.002	57 ± 4.5	32 ± 4.3
2	0.750 ± 0.015	58 ± 3.8	4 ± 0.5
3	0.016 ± 0.002	95 ± 4.1	4 ± 0.9
4	0.401 ± 0.008	63 ± 4.8	15 ± 3.0
5	1.683 ± 0.013	44 ± 2.9	39 ± 4.8
6	0.059 ± 0.002	80 ± 3.5	56 ± 4.4
7	0.018 ± 0.002	137 ± 3.6	72 ± 4.3
8	0.010 ± 0.004	106 ± 2.8	81 ± 3.6
9	0.003 ± 0.001	20 ± 3	133 ± 1.4
10	0.340 ± 0.009	11 ± 1.8	71 ± 2.8

The level of adrenaline autooxidation products accumulation in the bicarbonate buffer in the presence of the biopreparation was taken as 100% (control). The average values for a series of experiments are given; all differences from the control experimental group were $P < 0.05$. The values are expressed as mean ± standard deviation.

$$ROH + O_2^{-\bullet} \rightarrow RO^- + HOO^\bullet,$$

$$RSH + O_2^{-\bullet} \rightarrow RS^- + HOO^\bullet.$$

Such changes suggested that phenolic sulfur-containing compounds, in reaction to the superoxide anion radical, acted as proton donors (that is, Brønsted acids), which was in good agreement with the literature data [26,27].

The mechanism of RSH interaction with $O_2^{-\bullet}$ was discussed and it was proposed that the reaction of thioles occurred by the scheme [28]:

$$RSH + O_2^{-\bullet} \rightarrow RS^\bullet + HOO^-.$$

Thus, it can be concluded that compounds **1** and **2** could interact with $O_2^{-\bullet}$, both through the OH-group and the SH-group. In the case of compound **1**, interaction with $O_2^{-\bullet}$ could lead to the formation of stable phenolic or thiyl radicals, in contrast to compound **2**, where only phenoxyl could form, because the SH-group was separated from the aromatic fragment.

As a consequence, it was expected that compound **1** should possess higher antioxidant activity; however, the experimental data on the interaction of RSH with electrochemically generated $O_2^{-\bullet}$ did not confirm this supposition. The higher constant interaction of compound **2** with the electrochemically generated superoxide-radical compared to compound **1** may be explained by the supposition that the reaction involves the SH-group in both compounds, and in compound **2,** this group was sterically more accessible when compared to compound **1**.

According to the data of the same study, the reaction of disulfides with $O_2^{-\bullet}$ occurs by the scheme [28]:

$$R_2S_2 + O_2^{-\bullet} \rightarrow RSOO^\bullet + RS^-.$$

In the case of compounds **5** and **6**, the high constant could be explained by higher reactivity of the aromatic disulfides, which underwent the nucleophilic attack of $O_2^{-\bullet}$ on the S-S bond faster when compared to the aliphatic disulfides (the first step of the overall oxidation pathway). Presumably, the absence of sterically hindered substituents in compound **5** also facilitated the attack of the superoxide radical anion on the S-S bond.

2.3. SOD-protective activity and pro-/antioxidant activity

The reaction of adrenaline autooxidation in the alkaline carbonate buffer was used for the determination of the SOD enzymatic activity, as a good model of the quinoid oxidation of adrenaline [29,30]. Scavenging superoxide anion-radical enzyme SOD inhibits the accumulation of adrenochrome. The sequence of reactions of adrenaline autooxidation, resulting in adrenochrome formation, has been described previously [31–33]. SOD is present in almost all aerobic cells and extracellular fluids, and breaks down $O_2^{-\bullet}$ into O_2 and H_2O_2 (the process is facilitated by Cu, Zn, Mn, and Fe), which plays a major role in lipid peroxidation. Carbonate/bicarbonate ions are present in the living nature and involved involvement of free radicals processes proceeding in the organism. The role of carbonate/bicarbonate ions has been recognized in the reaction of the superoxide anion radical generation with adrenaline autooxidation in an alkaline buffer (a model of quinoid adrenaline oxidation in the body). It was shown that these ions were directly involved, not only in the formation of the superoxide anion radical, but also other radicals derived from the carbonate/bicarbonate buffer.

The influence of sulfur-containing compounds on the rate of $O_2^{-\bullet}$ generation in the model system of adrenaline (epinephrine) quinoid oxidation in the alkaline bicarbonate buffer and on the SOD-protective activity of a biopreparation, based on the liver of Russian sturgeon, was studied. The antiradical activity of compounds **1**–**10** towards $O_2^{-\bullet}$ was studied using the superoxide producing reaction of adrenaline autooxidation in an alkaline medium (pH 10.65), and the formation of adrenochrome was detected at a wavelength of 347 nm (Table 2). The effect of compounds **1**–**10** on the SOD activity of the biopreparation, i.e. the ability to inhibit $O_2^{-\bullet}$ production, was also investigated on this model system (Table 2).

It was found that the studied compounds **1**–**10** slightly increased the SOD-protective activity of the liver of Russian sturgeon, slowing down the rate of adrenaline oxidation. The value of adrenaline autooxidation in the alkaline medium without the addition of the compounds **1**–**10** was taken as 100%. The increase in the $O_2^{-\bullet}$ generation rate (activity $>100\%$) was evidenced by the prooxidant activity of the compounds. It was found that compounds **7**–**8** exhibited prooxidant activity, increasing the rate of $O_2^{-\bullet}$ generation by 37% and 6%, which corresponded to 137% and 106%, respectively, shown in Table 2. Similarly, the decrease in the $O_2^{-\bullet}$ generation rate (activity $<100\%$) was evidenced by the antioxidant activity of the compounds. For example, the antiradical activity of compounds **9** and **10** was 20% and 11%, respectively, corresponding to a decrease of 80% and 89%.

Notably, these compounds are present in onions and garlic, which confirms the healing properties of these products. Compounds **2**–**4** did not demonstrate pronounced antiradical activity, despite the presence of a sterically hindered phenolic fragment. All of the studied polysulfides increased the SOD-protective activity of the biopreparation (cytosolic fraction of Russian sturgeon liver homogenate), except for compound **9**, where the addition of the latter increased the $O_2^{-\bullet}$ generation rate by 33% in the presence of the biopreparation. It can be concluded that diallyl disulfide exhibited prooxidant activity.

Thus, the antiradical and SOD-protector activity of the organosulfuric compounds was found in the

model system of the adrenaline autooxidation, in an alkaline medium, for all of the compounds, except for **7**–**9**. However, in the group of studied compounds, the rules governing the change in the rate of $O_2^{-\bullet}$ generation, depending on the number of sulfur atoms and the nature of the organic radical, were not found in this model system.

2.4. Superoxide anion radical scavenging activity (NBT assay)

It is known that extracellular free radicals are produced in vivo by several oxidative enzymes in the human body, including xanthine oxidase and lipooxygenase [34,35]. Xanthine oxidase, a molybdenum-containing enzyme, produces the superoxide anion radical as a normal product [36].

Herein, the absorption scavenging activity of the superoxide radical generated in the enzymatic system in the presence of compounds **1**–**10** was investigated by the reduction of nitroblue tetrazolium (NBT). Inhibition of blue formazan production can be expressed as a decrease in absorption at 560 nm, due to $O_2^{-\bullet}$ scavenging relative to the control experiment. Only compounds **1**–**2** showed 40%–55% of the activity on the absorption of the superoxide anion-radical ($P < 0.05$) relative to the control, and such activity can be associated with the presence of the SH-groups in these compounds. The antiradical and SOD-protector activity of the compounds **1**–**6** and **10** was found in the model system of adrenaline autooxidation in an alkaline medium (Table 2), but in contrast, only phenolic compounds **1**–**2** showed 40%–55% of activity on the superoxide anion-radical absorption relative to the control ($P < 0.05$) in the enzymatic system due to NBT reduction.

Consequently, the interaction of $O_2^{-\bullet}$ with the sulfur-containing phenolic compounds proceeds more easily through the SH-group, but not through the phenolic OH-group in this model system. Thus, the reactivity of compounds **1**–**10** with $O_2^{-\bullet}$ in reaction to the NBT was assessed and it was found that compounds bearing the SH-group were more active than compounds with a phenolic group.

2.5. Lipoxygenase (LOX) inhibitory assessment

The roles of the cyclooxygenase enzyme and LOX in the inflammatory process were extensively explored [37–39]. It was shown that the metabolism of arachidonic acid generated a host of proinflammatory metabolites. Leucotrienes were synthesized due to the LOX activity and had a major role in the inflammatory process. During oxidative stress, the LOX could exhibit uncontrolled activity and was able to cause cell membrane destruction by phospholipids oxidation. Compounds of the di-*tert*-butyl phenol class were found to have inhibited the cyclooxygenase and LOX enzymes with proven efficiency in tumor growth arresting [40].

In this study, the antiinflammatory activity of organic sulfur-containing compounds **1**–**10** was evaluated according to their ability to inhibit the LOX. It was found that compounds **1**–**10** did not inhibit the LOX enzyme, which indicated the absence of their antiinflammatory activity.

3. Conclusion

The antioxidant activity of organic sulfur-containing compounds was studied in silico and in vitro. It was shown that the compounds had protective action against oxidative damage. Among the investigated organic sulfur-containing compounds, 2,6-di-*tert*-butyl-4-mercaptophenol (**1**), 2,6-di-*tert*-butyl-4-(3-mercaptopropyl)phenol (**2**), and diphenyl disulfide (**5**) showed a slightly better antioxidant effect; however, the influence of the hydroxyl group and the quantity of sulfur atoms on their antioxidant effect was unclear.

Considering that the same compounds could have both anti- or prooxidant effects under different conditions, it was important to conduct a comprehensive study of the antioxidant activity of the compounds on various model systems. This allowed a complete evaluation of the reactivity of the substances and determination of the nature of its antioxidant action.

The results of the computer screening performed in this study were confirmed by the data of the experimental in vitro studies. It was found that sulfur-containing compounds could act as active scavengers of oxygen metabolites, which were predicted in silico. The results of the prediction can be used in planning further experimental studies of in vitro and in vivo systems.

On the basis of the results, it was concluded that sulfur-containing compounds can be useful for inhibiting oxidative stress as well as diminishing cell damage. However, in terms of oxidative stability, further investigation is needed to determine the antioxidant effects of food with sulfur-containing compounds as additives.

4. Methods

4.1. General procedures

Compounds **1**–**3**, **5**–**10**, and all of the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise. Compound **4** was synthesized as described previously [41].

4.2. Forecast of biological activity in silico

The spectrum of the biological activity of compounds **1**–**10** was predicted in silico using PASS and general unrestricted structure-activity relationships (Way2Drug.com, 2011–2016) software (Lagunin et al., 2011). The structures were drawn using CHEM Sketch package 11.0 (ACD/Labs, Toronto, Canada). The spectrum of biological activity for the substances was presented in the form of a list of types of biological activity, for which the probability of presence (P_a) and probability of lack of activity (P_i) were calculated. The P_a and P_i values are independent and their values varied from 0 to 1. In this paper, the types of biological activity for which P_a was higher than P_i were evaluated.

4.3. Electrochemical measurements

Electrochemical studies were performed in a CH_3CN solution of 0.1 M Bu_4NClO_4 using the 3-electrode cell method with an IPC-pro potentiostat. The working electrode was a stationary platinum electrode with a diameter of 3 mm, while the auxiliary electrode was a platinum plate ($S = 18\ mm^2$). The reference electrode was (Ag/AgCl/KCl) with a waterproof diaphragm. The potential sweep rate was 0.2 V s^{-1}. Bu_4NClO_4 (99%, Acros) for the supporting electrolyte, and it was twice recrystallized from the aqueous reference standard and dried for 48 h in vacuum at 50 °C. The concentration of the studied compounds was 5 mM. A method for evaluation of the superoxide scavenging capacity based on the relative increase in oxygen reduction current in the presence of antioxidants was used [23]. The calculation was performed on the basis of the relative increase in the oxygen reduction current in the presence of compounds **1**–**10**.

4.4. Determination of the SOD-protective activity and pro-/antioxidant activity

SOD-protective activity of the biopreparation was the ability to utilize the superoxide anion radical $O_2^{-\bullet}$, as determined by the method of Sirota [42]. A cytosolic fraction of the Russian sturgeon liver homogenate was used as the source of SOD.

a) Preparation of a cytosolic fraction of the Russian sturgeon liver homogenate

First, the sturgeon liver was washed with cold 0.2 M Tris buffer (pH 7.8) to remove any traces of blood. All of the procedures were performed at a temperature of 0–4 °C. Next, a homogenate was obtained using a Potter homogenizer (Thomas Scientific, NJ, USA) in 0.2 M Tris buffer at a ratio of 1:10. The homogenate was then centrifuged for 10 min at 1000 g to remove partially destroyed cells and nuclei. After centrifugation, the precipitate had to be in the form of a dense layer at the bottom of the tube and the centrifugate had to be transparent. To extract the liquid, the end of a capillary pipette was immersed in the centrifugate without touching the sediment and the walls of the tube. The resulting supernatant contained the enzymes of the cytosolic fraction of the liver homogenate, including the SOD.

b) Determination of the SOD-protective activity of the biopreparation

Here, 0.1 mL of the biopreparation was added to a cuvette with 2 mL of bicarbonate buffer (pH 10.65) and 0.1 mL of 0.1% adrenaline solution, and was thoroughly and quickly mixed. The increase in optical density against a control solution containing the same components, in addition to adrenaline, was measured. The rate of adrenaline oxidation without the biopreparation and in the presence of the biopreparation was evaluated by the change in optical density, measured at 347 nm for 3 min. To determine the order of this reaction, a graphical method was used, which consisted in determining a linearly-dependent function on time. The kinetic curve of the autooxidation of the adrenaline in the coordinates LnD/Do = f (Time) was plotted. The decrease in the rate of the process in the presence of the biopreparation was used to characterize the SOD-protective activity.

c) Determination of the pro-/antioxidant activity

Here, 2 mL of 0.2 M bicarbonate buffer (pH 10.65), 0.1 mL of the tested compound (initial concentrations of these compounds 25 μM), and 0.1 mL of a 0.1% solution of adrenaline were added to the cuvette. The operations described above were then repeated.

4.5. Inhibition of the superoxide radical anion formation by xanthine oxidase (NBT assay)

Ethylenediaminetetraacetic acid (EDTA), xanthine, bovine serum albumin, NBT, and xanthine oxidase (25 MU) were purchased from Sigma-Aldrich. The superoxide anions were generated enzymatically by the xanthine oxidase system. The reaction mixture consisted of 2.70 mL of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH, 10.0), 0.06 mL of 10 mM xanthine, 0.03 mL of 0.5% bovine serum albumin, 0.03 mL of 2.5 mM NBT, and 0.06 mL of the sample solution in dimethyl sulfoxide (DMSO). Next, 0.12 mL of xanthine oxidase (0.04 units) was added to the mixture at 25 °C, and the absorbance at 560 nm (by formation of blue formazan) was measured using an Anthos Zenyth 200rt microplate spectrophotometer (Biochrom Ltd., Cambridge, UK) for 60 s. A control experiment was also carried out by replacing the sample solution with the same amount of DMSO.

Inhibition I (%) = $[(1 - A_i/A_0) \times 100\%]$, where A_i is the absorbance in the presence of the testing compound and A_0 is the absorbance of the blank solution [43]. All of the experiments were performed 3 times.

4.6. LOX activity

LOX type 1-B from glycine max (soybean), boric acid, linoleic acid, ammonium acetate, copper(II) chloride, and ethanol (96%) were purchased from Sigma-Aldrich and were used with no further purification. The LOX inhibition activity was determined spectrophotometrically by measuring the increase in absorbance at 234 nm for the oxidation of linoleic acid [44]. The reaction mixture contained the test compounds dissolved in DMSO at

initial concentrations of 0.05 ÷ 2 mM or 0.03 mL DMSO (blank) and 1 mL of 0.3 mM linoleic acid in a borate buffer (pH 9.0), and 0.3 mL of borate buffer. The total sample volume was 1.5 mL, while the final concentration in DMSO was 0.33% v/v. The reaction was started by adding 0.17 mL of the LOX solution (500 units) to the borate buffer. The increase in absorbance was measured every 10 s for 10 min under a controlled temperature of 25 °C. The degree of LOX activity (A, %) in the presence of the compounds was calculated according to the method reported in the literature [45].

A, % = (ν_0 in the presence of inhibitor/ν_0 in the absence of inhibitor) × 100%, where ν_0 is the initial rate.

The value of the initial rate (ν_0, μM min^{-1}) was calculated according to formula:

$$\nu_0 = \Delta C/\Delta t = \Delta A/\Delta t\varepsilon = \mathrm{tg}\alpha/\Delta t\varepsilon,$$

where C is the product concentration (hydroperoxy-linoleic acid), t is the reaction time, ε is the molar absorbance coefficient of hydroperoxy-linoleic acid, and tgα is the slope of the kinetic curve plotted as absorbance *vs.* time.

IC_{50} values were obtained graphically by plotting a logistic curve on the inhibition activity (A, %) *vs.* inhibitor concentration ([I], μM) coordinates. The general curve equation was as follows:

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

4.7. Statistical analysis

The average values obtained in the independent experiments for 3–5 parallel measurements in each experiment were given. The received data were processed on a PC using MS Excel. The results were presented in the form of mean ± standard deviation. Statistical significance was assessed using the Student t-test.

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