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Synthesis of 3,5-Di-*tert*-butyl-1,2-dihydroxybenzene Derivatives and Their Effect on Free-Radical Oxidation of Hexane and Oxygen Activation Ability of Neutrophils

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Abstract—C^6-Substituted derivatives of 3,5-di-*tert*-butyl-1,2-dihydroxybenzene have been synthesized, and their effect on radiation-induced free-radical oxidation of *n*-hexane and production of reactive oxygen and chlorine forms in neutrophils have been studied. It has been shown the introduction of the phenylhydrazone and phenylazomethine groups significantly increases the antioxidant activity of pyrocatechol derivatives. For six compounds, the ability to prevent the development of oxidative stress due to hyperproduction of active oxygen intermediates and $HOCl/OCl^-$ in neutrophils has been revealed.

Keywords: reactive oxygen and chlorine forms, antioxidant activity, myeloperoxidase, neutrophils, sterically hindered pyrocatechol, chemiluminescence

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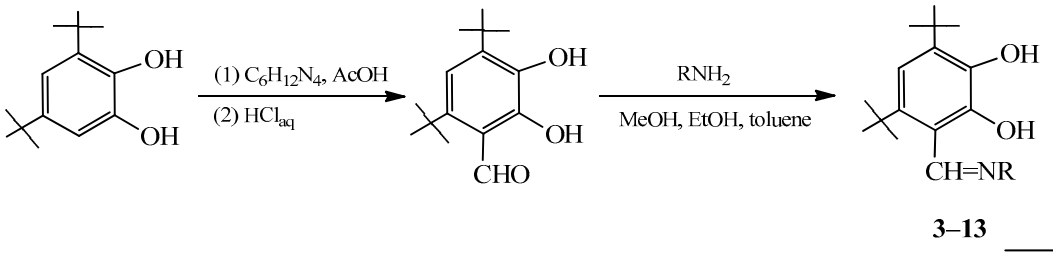
It is known that hyperproduction of active forms of oxygen and chlorine followed by intensification of free-radical reactions of damage of biomolecules in organism may be a reason of cardiovascular and oncological diseases, immunodeficiency, rheumatoid arthritis, and other pathologies [1]. Moreover, production of active forms of oxygen and chlorine and activation of peroxide oxidation of lipids accompany different virus infections [1]. Clinical and experimental studies have shown the efficiency of the use of antioxidant in chemotherapy of diseases caused by activation of free-radical processes in organism [1]. Thus, the development of rational methods of directed search for efficient regulators of free-radical reactions and potential drugs for the treatment of pathologies caused by oxidative stress in organism is promising.

Herein, the synthesis of a series of derivatives of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde [2] which strongly inhibits the processes involving peroxide and α-hydroxyalkyl radicals [3] was performed. The presence of an active formyl group in the structure of the parent compound allowed its condensation with different primary aliphatic amines and the formation of hybrid structures with potentially stronger antiradical and antioxidant properties.

In this study, we investigated antioxidant activity of 4,6-di-*tert*-butyl-3-phenyliminomethyl-1,2-dihydroxybenzene **1** and 2,4-di-*tert*-butyl-6-phenyliminomethylphenol **2** as the reference compounds and the prepared samples **3**–**13** in chemical (radiation-induced oxidation of hexane) and biological (generation of active forms of oxygen and chlorine in neutrophils of human blood and halogenating activity of myeloperoxidase) model systems. Cytotoxicity of the considered compounds against neutrophils was determined. The influence of the structure of the C^6-substituent of the prepared derivatives of 3,5-di-*tert*-butyl-1,2-dihidroxybenzene on the antioxidant activity and the formation of active forms of oxygen and chlorine in neutrophils.

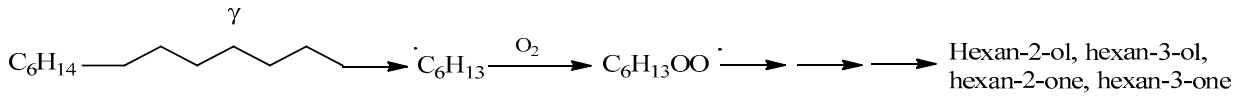
Synthesis of compounds **3**–**13** was carried out using condensation of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde with primary aliphatic and aromatic amines as well as phenyl hydrazine in equimolar ratio at heating in alcohol or toluene [Eq. (1)].



(1)

3–13

Antioxidant properties of the prepared compounds were investigated using the model of induced by γ-radiation oxidation of *n*-hexane in the presence of air. Peroxide ($ROO^{\bullet}$) radicals appeared in that process were then transformed into the corresponding alcohols and ketones. Hexanols and hexanones were the major products of *n*-hexane radiolysis in the presence of O_2 [Eq. (2)] [4].



(2)

The total yield of hexanols and hexanones in the presence of different additives was the indicator of their antioxidant activity and, therefore, the efficiency of their interaction with $ROO^{\bullet}$ radicals. Total yields of hexanols and hexanones formed in *n*-hexane radiolysis in the presence and in the absence of 3,5-di-*tert*-butyl-1,2-dihydroxybenzene derivatives are collected in Table 1.

It is known that the key reaction in phenol inhibition of oxidation of organic substances is the interaction of phenol with peroxide radicals forming the resonance-stabilized phenoxy radicals ($PhO^{\bullet}$). Then the formed radicals are combined with each other and $ROO^{\bullet}$ radicals [5].

Table 1. Influence of 3,5-di-*tert*-butyl-1,2-dihydroxybenzene derivatives **1**–**13** on total radiation-chemical yield of products of oxidative radiolysis of *n*-hexane

Comp. no.	Yield, $\times10^{-7}$ J/mol	Comp. no.	Yield, $\times10^{-7}$ J/mol
–	2.42±0.14	**8**	0.92±0.06
1	1.02±0.05	**9**	0.78±0.05
2	2.33±0.18	**10**	0.64±0.04
3	0.77±0.05	**11**	0.97±0.07
4	0.40±0.03	**12**	0.77±0.05
5	1.03±0.06	**13**	2.37±0.14
6	0.39±0.03	**14**	1.73±0.13
7	0.85±0.05	**15**	1.07±0.06

$$ROO^{\bullet} + PhOH \rightarrow ROOH + PhO^{\bullet}, \quad (3)$$

$$PhO^{\bullet} + ROO^{\bullet} \rightarrow \text{Molecular products}, \quad (4)$$

$$PhO^{\bullet} + PhO^{\bullet} \rightarrow \text{Molecular products}. \quad (5)$$

Incorporation of phenylhydrazone and phenylazomethine groups into 3,5-di-*tert*-butyl-1,2-dihydroxybenzene significantly enhanced the antioxidant activity. High reactivity in the reaction with peroxide radical was observed for compounds **1**, **3**–**12**: they reduced the radiation-chemical yield of the major products of *n*-hexane oxidation by 2.4–6.2 times. Antioxidant activity of those compounds was comparable with the well-known industrially used antioxidant ionol **15**, which has been investigated previously in a similar model system [6]. The studied compounds were also better antioxidants than 3,5-di-*tert*-butyl-1,2-dihydroxybenzene **14** [7]. It should be noticed that incorporation of phenylazomethine group into monophenol (compound **2**) did not affect the antioxidant activity. So, the presence of pyrocatechol (compounds **1** and **2**) or phenylazomethine (compounds **1** and **14**) units was necessary for the appearance of the antioxidant activity. Probably, the OH group of compound **2** was involved in intramolecular O–H···N type hydrogen bond, so, that compound did not exhibit activity in radical reactions.

The most active antioxidants were compounds **4** and **6**. Comparing compounds **4** and **6** with compound **1** (did not contain a hydroxy group in the benzene ring of phenylazomethine fragment) and **1**, **5**, **7** (the hydroxy group in benzene ring of phenylazomethine fragment was absent or methylated) revealed that the presence of the OH group in the *para* or *ortho* position

of phenylazomethine unit played a key role in the antioxidant activity of 3,5-di-*tert*-butyl-1,2-dihydroxybenzene derivatives. Such high reactivity of compounds **4** and **6** in the reaction with peroxide radical could be explained by possible formation of phenoxy radicals not only via the OH group of pyrocatechol but also via additional OH group of the phenylazomethine fragment. The presence of additional sulfhydryl group (compounds **9** and **10**), phenylhydrazone (**3**), and nitro (**12**) groups also provided the enhanced activity of the compounds in the reactions with peroxide radicals. Compound **13** with adamantylazomethine group was inactive in the radical reaction, probably due to the shielding of the hydroxy groups by bulky adamantyl substituent.

The compounds exhibiting the highest antioxidant activity in the chemical model system were chosen for further investigation. The study of their influence on the generation of active form of oxygen and chlorine by neutrophils of human blood, halogenating activity of myeloperoxidase, and ability to utilize $HOCl/OCl^-$ was carried out. Furthermore, vitality of those cells in the presence of the considered compounds was investigated.

Neutrophils are the main source of active forms of oxygen and chlorine in human and animal organisms. These cells recognize and destroy different pathogens, protecting the organism against infections [8]. They are predominantly present at the inflammation site on the early stages of the process. Phagocytosis of bacteria by neutrophils is conjugated with the activation of NADPH-oxidase, resulting in superoxide radical-anions formation [Eq. (6)] with further transformation into H_2O_2 under the action of superoxide dismutase [Eq. (7)] [9].

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{\bullet-} + H^+, \quad (6)$$

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2. \quad (7)$$

Hydrogen peroxide and chloride ions are the substrates for myeloperoxidase, the functioning of which is accompanied by the production of hypochloric acid [Eq. (8)] existing at physiological pH values as $HOCl/OCl^-$ [10].

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O. \quad (8)$$

$HOCl/OCl^-$ is the major agent destroying the foreign material in organism. Another important role of this oxidant is the regulation of the functional activity of cells by micromolar concentrations of $HOCl/OCl^-$ [11]. However, hypochloric acid and hypochlorite ions at high concentrations are the sources of free radicals which modify or destroy the vitally important biomolecules, leading to the development of many pathological states [1, 12].

The results of the investigation of influence of 3,5-di-*tert*-butyl-1,2-dihydroxybenzene derivatives on the processes of generation of active forms of oxygen and chlorine by neutrophils are given in Table 2. One can see that compounds **3**, **4**, **6**, **9**, **10**, and **12** significantly decreased the luminol-dependent chemiluminescence of neutrophils stimulated to phagocytosis. Compound **1** was an exception.

Since luminol is an indicator predominantly of hypochloric acid [13], the inhibition of luminol-dependent chemiluminescence of neutrophils could be related to the reduced production of $HOCl/OCl^-$ by those cells. That could be caused by several reasons, the major ones being the decrease in activity of myeloperoxidase, decrease in concentration of specific substrate of myeloperoxidase (hydrogen peroxide), and utilization of HOCl (product of the functioning of that enzyme).

The data on the influence of the analyzed compounds on lucigenin-dependent chemiluminescence of neutrophils are given in Table 2. According to obtained data, compound **9** did not affect that process. Other investigated compounds reduced the level of lucigenin-dependent chemiluminescence by 39.2–48.7%. The change in the intensity of lucigenin-dependent chemiluminescence correlated with the production of H_2O_2 by cells because lucigenin is used as indicator of superoxide radical-anions [14], which are formed during the activation of NADPH-oxidase. The yield of H_2O_2 might be decreased as a result of the decrease in the activity of NADPH-oxidase or interaction with H_2O_2. However, we obtained the data indicating no influence of the analyzed compounds on chemiluminescence yield in luminol–H_2O_2 system, which excluded their interaction with hydrogen peroxide.

The investigation of influence of the analyzed compounds on halogenating activity of myeloperoxidase (the major source of $HOCl/OCl^-$ in an organism) were carried out by measuring the total intensity of chemiluminescence in the myeloperoxidase–luminol–H_2O_2–Cl^- system. According to the data given in Table 2, the considered compounds effectively inhibited the luminol-dependent chemiluminescence in the system containing myeloper-

Table 2. Influence of 3,5-di-*tert*-butyl-1,2-dihydroxybenzene derivatives on free-radical processes in cellular and model systems and on vitality of neutrophils[a]

Comp. no.	R_{lum}, %	R_{luc}, %	R_{hal}, %	$R_{lum–NaOCl}$, %	V, %
1	–4.80±2.17	45.45±4.90	75.28±3.27	70.93±2.80	83.50±1.41
3	34.10±5.47	47.12±4.65	96.33±0.58	10.58±3.38	94.36±6.22
4	52.29±4.52	48.68±3.90	96.84±0.31	71.13±1.58	82.34±10.42
6	40.00±4.01	45.28±2.96	85.44±1.02	43.30±4.58	91.95±1.36
9	74.92±3.18	–7.13±5.74	87.30±1.23	–17.30±2.92	88.19±4.23
10	62.76±1.02	44.21±3.58	44.11±7.72	1.44±1.34	96.95±5.12
12	38.78±3.74	39.22±3.58	74.83±2.34	65.43±1.01	99.91±13.29
14	96.96±2.47	97.52±2.47	71.99±3.48	63.17±0.25	94.26±2.94

[a] (R_{lum}) the degree of inhibition of luminol-dependent chemiluminescence, (R_{luc}) the degree of inhibition of lucigenin-dependent chemiluminescence, (R_{hal}) the degree of inhibition of halogenating ability of myeloperoxidase, ($R_{lum–NaOCl}$) the degree of inhibition of chemiluminescence in luminol–NaOCl system, (V) vitality of neutrophils.

oxidase. The inhibiting effect of the analyzed compounds was decreased from 96.8 to 44.1% in following series: **3**, **4** > **6**, **9** > **1**, **12** > **10**.

The weakening of the luminol-dependent chemiluminescence in the system containing myeloperoxidase could be related to the inhibition of halogenating activity of the enzyme or the ability of the analyzed compounds to utilize hypochloric acid (the major product of the reaction catalyzed by myeloperoxidase) via a non-radical way. According to the data in Table 2, compounds **4**, **1**, and **12** inhibited the intensity of chemiluminescence in the luminol–NaOCl system by 70.9–65.4%, and the effect of compound **6** was as low as 43.3%. That fact indicated that those compounds could utilize hypochloric acid via a non-radical way**.** At the same time, compounds **3** and **10** did not affect the yield of chemiluminescence, and compound **9** insignificantly increased the yield, probably, by forming new radical products in the considered reaction. The comparison of the results obtained for the luminol–NaOCl and myeloperoxidase–luminol–H_2O_2–Cl^- systems revealed that compounds **3**, **4**, **6**, **9**, and **10** could effectively inhibit halogenating activity of myeloperoxidase. Analysis of the influence of the investigated compounds on the cell vitality (Table 2) indicated that the observed effects were not related to cytotoxic action of those compounds.

It should be noticed that we did not obtain quantitative correlation between the antioxidant activity of the analyzed compounds in the radiation-chemical and biochemical experiments (Tables 1 and 2), however, we observed a qualitative correlation between those effects. That could be caused by several reasons. Firstly, cells are complex multicomponent systems, in which the processes are interrelated and regulated by many intracellular and external factors. The investigated 3,5-di-*tert*-butyl-1,2-dihydroxybenzene derivatives differed in the hydrophobic properties. That fact could cause the different in their ability to penetrate through the cell membrane, localization in certain cell compartments and, therefore, interaction with certain biomolecules. The most important role in the regulation of cell functions is played by proteins. For example, a lot of proteins of cell membrane are receptors initing intracellular signaling process which controls important biochemical reactions in a cell, including generation of active forms of oxygen and chlorine in neutrophils. Protein enzymes act as catalysts. The change in the structure of protein molecules is a key factor of modification of their functions. In this study, we determined the integral response of the cells on the action of 3,5-di-*tert*-butyl-1,2-dihydroxybenzene derivatives (Table 2). The absence of quantitative correlation between the antioxidant activity of those compounds in chemical and biological systems was probably caused by a wide variety of molecular targets for them in neutrophils.

The most important regulators of protein functions by structural modification are compounds containing sulfhydryl groups. In our study, compounds **9** and **10** contained such fragments. One can see from the data in Table 2 that those compounds significantly decreased

the halogenating activity of myeloperoxidase and did not react with hypochlorite. We can therefore suggest that compounds **9** and **10** could modify the enzyme, decreasing the production of $HOCl/OCl^-$. The ability to efficiently inhibit the activity of myeloperoxidase practically without utilizing hypochlorite was also characteristic of compound **3**.

In summary, we showed that the synthesized 3,5-di-*tert*-butyl-1,2-dihydroxybenzene derivatives **3**–**12** (especially compounds **4** and **6**) exhibited high activity in the reaction with peroxide radicals. Compounds **3**, **4**, **6**, **9**, **10**, and **12** reduced the formation of active forms of oxygen and chlorine in neutrophils without cytotoxic action. Compounds **3**, **9**, and **10** efficiently inhibited the halogenating activity of myeloperoxidase without utilization of $HOCl/OCl^-$. For compounds **1**, **4**, **6**, and **12**, we figured out the ability to react with hypochlorite without formation of new radical products. The obtained data led to a conclusion that the prepared 3,5-di-*tert*-butyl-1,2-dihydroxybenzene derivatives **3**, **4**, **6**, **9**, **10**, and **12** could be considered as potential inhibitors of oxidative stress in human organism. That fact opens the possibilities for further application of these compounds in the development of new drugs for the treatment of pathologies caused by inflammation.

EXPERIMENTAL

2,6-Di-*tert*-butyl-4-methylphenol (ionol, **15**), *n*-hexane (97%), hexan-2-ol, hexan-2-one, hexan-3-ol, and hexan-3-one were purchased from Sigma-Aldrich.

^{1}H NMR spectra ($CDCl_3$ or DMSO-d_6 were used as solvents) were recorded using a Bruker ARX-400 spectrometer operating at 400 MHz. Mass spectra were registered with a Schimadzu QP-5000 spectrometer using direct injection of the specimens into an ion source with temperature of the source 200°C and ionization energy 70 eV. Melting points were determined using a Boëtius heated bench. Monitoring the reaction course and controlling purity of the obtained compounds were carried out using thin layer chromatography on Silufol UV-254 plates in hexane–chloroform–ethyl acetate system (5 : 2 : 1) (developing by iodine vapor).

Compounds **1**–**3**, **14** were synthesized as described elsewhere [2, 7, 15].

4,6-Di-*tert*-butyl-3-(2-hydroxyphenyliminomethyl)-1,2-dihydroxybenzene (4). A mixture of 0.25 g (1 mmol) of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde and 0.11 g (1 mmol) of *ortho*-aminophenol in 20 mL of anhydrous ethanol was refluxed for 3 h under stirring. The reaction mixture was kept overnight. The precipitate was filtered off and washed with cold anhydrous ethanol. Yield 0.29 g (85%), red powder, mp 195–198°C. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.46 s (9H, CH_3), 1.55 s (9H, CH_3), 6.89 s (1H, CH_{Ar}), 6.95–7.25 m (5H, $4CH_{Ar}$ + OH), 9.44 s (1H, CH=N). Mass spectrum, *m/z* (I_{rel}, %): 341 (87) $[M]^+$. $C_{21}H_{27}NO_3$.

4,6-Di-*tert*-butyl-3-(2-methoxyphenyliminomethyl)-1,2-dihydroxybenzene (5). 0.26 g (2 mmol) of *ortho*-anisidine was added to a solution of 0.5 g (2 mmol) of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde in 10 mL of anhydrous methanol. The obtained mixture was refluxed for 1 h and then cooled down. The precipitate was filtered off and washed with cold hexane. Yield 0.27 g (74%), red powder, mp 140–142°C. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.47 s (9H, CH_3), 1.53 s (9H, CH_3), 4.02 s (3H, CH_3O), 6.74 s (1H, CH_{Ar}), 7.04–7.32 m (4H, CH_{Ar}, OH), 9.43 s (1H, CH=N). Mass spectrum, *m/z* (I_{rel}, %): 355 (48) $[M]^+$. $C_{22}H_{29}NO_3$.

4,6-Di-*tert*-butyl-3-(4-hydroxyphenyliminomethyl)-1,2-dihydroxybenzene (6). A mixture of 0.25 g (1 mmol) of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde and 0.11 g (1 mmol) of *para*-aminophenol in 25 mL of anhydrous methanol was refluxed for 5 h under stirring. After removal of the solvent, the precipitate was recrystallized from heptane. Yield 0.26 g (76%), light-red powder, mp 148–150°C. ^{1}H NMR spectrum (DMSO-d_6), δ, ppm: 1.38 s (9H, CH_3), 1.48 s (9H, CH_3), 6.74 s (1H, CH_{Ar}), 6.94 d (2H, CH_{Ar}, $^3J_{HH}$ = 7.0 Hz), 7.15 d (2H, CH_{Ar}, $^3J_{HH}$ = 7.0 Hz), 8.17 s (1H, OH), 9.38 s (1H, CH_{Ar}), 9.80 s (1H, OH). Mass spectrum, *m/z* (I_{rel}, %): 341 (79) $[M]^+$. $C_{21}H_{27}NO_3$.

4,6-Di-*tert*-butyl-3-(4-methoxyphenyliminomethyl)-1,2-dihydroxybenzene (7). 0.25 g (2 mmol) of *para*-anisidine was added to a solution of 0.5 g (2 mmol) of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde in 10 mL of anhydrous methanol. After 30 min, the mixture was cooled down; the precipitate was filtered off and washed with cold hexane. Yield 2.84 g (80%), red crystals, mp 163–165°C. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.48 s (9H, CH_3), 1.54 s (9H, CH_3), 3.89 s (3H, OCH_3), 6.48 br. s (1H, OH), 6.84 s (1H, CH_{Ar}), 7.05 d (2H, CH_{Ar}, $^3J_{HH}$ = 7.0 Hz), 7.32 d (2H, CH_{Ar}, $^3J_{HH}$ = 7.0 Hz), 9.38 s (1H, CH=N). Mass spectrum, *m/z* (I_{rel}, %): 355 (24) $[M]^+$. $C_{22}H_{29}NO_3$.

4,6-Di-*tert*-butyl-3-(4-methylphenyliminomethyl)-1,2-dihydroxybenzene (8). A mixture of 2.5 g (10 mmol) of 3,5-di-*tert*-butyl-2-hydroxybenzaldehyde and 1.07 g (10 mmol) of *para*-toluidine in 30 mL of anhydrous methanol was refluxed for 30 min and kept overnight. The crystals were filtered off, washed with light ligroin, and recrystallized from ethanol. Yield 3.30 g (96%), light-red powder, mp 186–187°C. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.48 s (9H, CH_3), 1.55 s (9H, CH_3), 2.23 s (3H, CH_3), 6.84 s (1H, CH_{Ar}), 7.22–7.32 m (4H, CH_{Ar}), 9.39 s (1H, CH=N). Mass spectrum, *m/z* (I_{rel}, %): 339 (72) $[M]^+$. $C_{22}H_{29}NO_2$.

4,6-Di-*tert*-butyl-3-(2-sulfanylphenyliminomethyl)-1,2-dihydroxybenzene (9). A mixture of 0.25 g (1 mmol) of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde and 0.125 g (1 mmol) of aminothiophenol in 15 mL of anhydrous ethanol was refluxed for 5 h. After removal of the solvent, the residue was recrystallized from a heptane–ethanol mixture. Yield 0.28 g (78%), light-yellow powder, mp 112–114°C. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.43 s (9H, CH_3), 1.53 s (9H, CH_3), 3.67 br. s (1H, SH), 6.13 s (1H, OH), 6.85–7.48 m (5H, CH_{Ar}), 9.43 s (1H, CH=N). Mass spectrum, *m/z* (I_{rel}, %): 357 (77) $[M]^+$. $C_{21}H_{27}NO_2S$.

4,6-Di-*tert*-butyl-3-(2-sulfanylethyliminomethyl)-1,2-dihydroxybenzene (10). A solution containing 0.195 g (2 mmol) of cysteamine hydrochloride, 0.50 g (2 mmol) of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde, and 0.20 g (2 mmol) of triethylamine in 10 mL of anhydrous methanol was stirred at room temperature for 6 h. The precipitate was filtered off, methanol was evaporated under vacuum, the residue was washed with cold ethanol and recrystallized from hexane. Yield 0.44 g (72%), light-orange powder, mp 111–112°C. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.45 s (9H, CH_3), 1.49 s (9H, CH_3), 2.92 br. s (2H, CH_2), 3.83 br. s (2H, CH_2), 6.71 br. s (1H, OH), 7.30 s (1H, CH_{Ar}), 9.04 s (1H, CH=N). Mass spectrum, *m/z* (I_{rel}, %): 309 (100) $[M]^+$. $C_{17}H_{27}NO_2S$.

4,6-Di-*tert*-butyl-3-(4-bromophenyliminomethyl)-1,2-dihydroxybenzene (11). 0.17 g (1 mmol) of *para*-bromoaniline was added to a solution of 0.25 g (1 mmol) of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde in 10 mL of anhydrous methanol. After 0.5 h, the solution was cooled down, the precipitate was filtered off and washed with cold hexane. Yield 0.25 g (62%), red needle-shaped crystals, mp 195–196°C. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.45 s (9H, CH_3), 1.52 s (9H, CH_3), 6.87 s (1H, CH_{Ar}), 7.18 d (2H, CH_{Ar}, $^3J_{HH}$ = 7.0 Hz), 7.60 d (2H, CH_{Ar}, $^3J_{HH}$ = 7.0 Hz), 9.39 s (1H, CH=N). Mass spectrum, *m/z* (I_{rel}, %): 403 (34) $[M - H]^+$, 405 (34) $[M + H]^+$. $C_{21}H_{26}BrNO_2$.

4,6-Di-*tert*-butyl-3-(3-nitrophenyliminomethyl)-1,2-dihydroxybenzene (12). A mixture of 0.25 g (1 mmol) of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde and 0.14 g *meta*-nitroaniline in 20 mL of anhydrous ethanol was refluxed for 3 h and then cooled down. The precipitate was filtered off, washed with water, dried, and recrystallized from a heptane–*i*-PrOH mixture (10 : 1). Yield 0.25 g (68%), light-red needle-shaped crystals, mp 138–140°C. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.45 s (9H, CH_3), 1.55 s (9H, CH_3), 6.24 br. s (1H, OH), 6.92 s (1H, CH_{Ar}), 7.58–7.66 m (2H, CH_{Ar}), 8.10–8.19 m (2H, CH_{Ar}), 9.48 s (1H, CH=N). Mass spectrum, *m/z* (I_{rel}, %): 370 (100) $[M]^+$. $C_{21}H_{26}N_2O_4$.

4,6-Di-*tert*-butyl-3-(1-adamantyliminomethyl)-1,2-dihydroxybenzene (13). A mixture of 0.125 g (0.5 mmol) of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde, 0.075 g (0.5 mmol) of 1-adamantylamine, 15 mL of toluene, and catalytic amount of TsOH was refluxed for 15 h. After removal of toluene, the residue was dissolved in 30 mL of Et_2O. The obtained solution was washed with dilute H_2SO_4, $NaHCO_3$ solution, and water. The solvent was removed under vacuum, the residue was recrystallized from toluene. Yield 0.12 g (62%), orange crystalline powder, mp 263°C (decomp.). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.34 s (9H, CH_3), 1.42 s (9H, CH_3), 1.65–2.34 m (15H, $6CH_2$ + 3CH), 6.65 s (1H, OH), 7.29 s (1H, CH_{Ar}), 8.92 s (1H, CH=N). Mass spectrum, *m/z* (I_{rel}, %): 383 (39) $[M]^+$. $C_{25}H_{37}NO_2$.

10^{-3} M solutions of the investigated compounds in *n*-hexane were used for the radiation-chemical experiments. The solutions were irradiated in sealed glass ampoules using an MPX-γ-25M unit with ^{60}Co source. The absorbed dose rate was 0.40±0.02 Gy/s. The used dose range was 0.24–1.2 kGy.

Analysis of products of free-radical oxidation of *n*-hexane (hexan-2-ol, hexan-3-ol, hexan-2-one, and hexan-3-one) was performed by gas chromatography using a StabilWax-DA quartz capillary column (*l* = 30 m, 0.32 mm ID, 0.5 μm df) on a GC-17AAF/APC Shimadzu chromatograph equipped with flame ionization detector. Conditions: initial temperature 60°C, heating to 180°C at 8 deg/min, evaporator temperature 250°C, detector temperature 220°C, flow rate of the carrier gas (nitrogen) 30 cm/s.

The radiation-chemical yields (G, 10^{-7} J/mol) of the products of *n*-hexane radiolysis were calculated using linear parts of the dependences of their concentrations on the absorbed dose by least squares method. The error of the yield determination was below 10%.

The following items were used in the biochemical experiments: heparin (Belmedpreparaty), dextrane-500, histopack-1077, RPMI-1640 medium, luminol, lucigenin, chemotactic peptide fMLP (*N*-Formyl-Met-Leu-Phe), propidium iodide, tritone X-100, NaOCl, hydrogen peroxide (Sigma), ethanol, and homemade Earl's medium which contained: 0.12 mol/L of NaCl, 5.4×10^{-3} mol/L of KCl, 0.9×10^{-3} mol/L of $NaH_2PO_4\cdot2H_2O$, 0.8×10^{-3} mol/L of $MgSO_4\cdot7H_2O$, 1×10^{-3} of mol/L $CaCl_2$, 5.6×10^{-3} mol/L of glucose, and 26.2×10^{-3} mol/L of $NaHCO_3$ (Analiz-X). The analyzed compounds were dissolved in ethanol. The reference samples of cell suspensions and model systems contained the same amount of ethanol as the analyzed samples.

Neutrophils were isolated from fresh blood of health people using a standard procedure in gradient of histopack-1077 density [16]. The cells were resuspended in RPMI-1640 medium (pH = 7.4). The obtained suspension contained at least 96% of neutrophils; the analyzed sample contained 1×10^6 cells per 1 mL. For the isolation of myeloperoxidase, the neutrophils suspension was exposed to three cycles of freezing (at –20°C) and thawing (at 37°C), then the cells were centrifuged at 3000 rot/min for 10 min. The obtained supernatant containing myeloperoxidase was isolated from the precipitate and used for the analysis.

Generation of active forms of oxygen and chlorine by neutrophils and myeloperoxidase was investigated using chemiluminescence method with a BHL-1 biochemiluminometer (Belarus) containing a Unichrom system of the signal registration and processing (Belarus). Luminol was used as light emitter for determination of overall active oxygen and chlorine. Lucigenin was used for registration of superoxide radical-anions. In the experiments with neutrophils suspension, the measurements were performed in RPMI-1640 medium; in the case of myeloperoxidase, Earl's medium was used at 37°C. The volume of analyzed sample was 1 mL in both cases.

Before analysis, a neutrophil suspension with the investigated compound (concentration of compounds **1**, **3**, **4**, **6**, **9**, **10**, and **12** in the samples was 10^{-6} M) was incubated at 37°C for 20 min; then the activity of chemiluminescence caused by fMLP action was measured. The fMLP solution with concentration of 1×10^{-7} mol/L in 0.15 mol/L NaCl solution was added to the cells suspension 5 min after the start of adhesion.

Halogenating ability of myeloperoxidase was determined via the measurement of total intensity of chemiluminescence in a system containing luminol (5×10^{-5} mol/L), H_2O_2 (2×10^{-5} mol/L), and myeloperoxidase at pH = 5.2 in Earl's medium for 10 min. The volume of added supernatant containing myeloperoxidase was 100 μL, corresponding to myeloperoxidase content equal to 1×10^5 neutrophils per mL. Before measurement of halogenating ability of myeloperoxidase, the enzyme-containing supernatant was incubated with the analyzed compound for 10 min at 37°C.

Chemiluminescence in the 5×10^{-6} M of NaOCl–5×10^{-5} M of luminol and 1×10^{-4} M of H_2O_2–5×10^{-5} M of luminol systems was investigated for determination of the effect of pyrocatechol derivatives on the free-radical processes in the reaction of luminol oxidation by hypochlorite or H_2O_2. The integral intensity of chemiluminescence was assessed as the area under the kinetic curve. The degree of luminescence inhibition (R) was determined using Eq. (9).

$$R = (1 - \frac{I_i}{I_0}) \times 100\%. \quad (9)$$

Here R is the degree of luminescence inhibition, I_i and I_0 are total chemiluminescence intensities accounting for background irradiation in working and reference samples, respectively.

The cells vitality was assessed via fluorescence method using a CM 2203 Solar device (Belarus), with propidium iodide [17]. A phagocyte suspension was incubated with pyrocatechol derivatives for 1 h at 37°C. Then propidium iodide (4×10^{-6} mol/L) was added; after 5 min, the intensity of fluorescence F_{d1} (λ_{ex} = 530 nm, λ_{reg} = 640 nm) was measured for 2 min; the cells were destroyed by 0.2% Trilon X-100 (Tr X-100) and fluorescence F_{t1} was registered for 3 min. Cells vitality (V) in each sample was determined as a proportion of the number of alive cells and the total number of the cells in the sample by the Eq. (10).

$$V = \frac{(F_{t1} - F_{t2}) - (F_{d1} - F_{d2})}{(F_{t1} - F_{t2})} \times 100\%. \quad (10)$$

Here V is the cells vitality in the presence of the analyzed compound, F_{d1} and F_{t1} are parameters of

fluorescence intensity of propidium ion in the absence and in the presence of Tr X-100, respectively, for the sample, F_{d2} и F_{t2} are parameters of fluorescence intensity of propidium ion in the absence and in the presence of Tr X-100, respectively, in RPMI-1640 solution. For convenient comparison between the samples, V was normalized to the vitality in reference samples V_0 using Eq. (11).

$$\frac{V}{V_0} = \frac{(F_{t1} - F_{t2}) - (F_{d1} - F_{d2})}{(F_{t10} - F_{t2}) - (F_{d10} - F_{d2})} \times 100\%. \qquad (11)$$

Here V is cell vitality in the presence of the analyzed compound, V_0 is cell vitality in the reference sample, F_{d1} and F_{t1} are parameters of fluorescence intensity of propidium ion in the absence and in the presence of Tr X-100, respectively, for the sample, F_{d10} and F_{t10} are intensities of fluorescence F_{d1} and F_{t1} of the reference samples, F_{d2} and F_{t2} are parameters of fluorescence intensity of propidium ion in the absence and in the presence of Tr X-100, respectively, in a RPMI-1640 solution.

The results of intendent measurements (more than three ones) were averaged. The obtained data were reported as average value ± the product of standard deviation by the Student coefficient for the corresponding number of measurements (confidence level $p = 0.95$).

CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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