

## Research Article

# Antioxidative Activity of Ferrocenes Bearing 2,6-Di-*Tert*-Butylphenol Moieties

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The antioxidative activity of ferrocenes bearing either 2,6-di-*tert*-butylphenol or phenyl groups has been compared using DPPH (1,1-diphenyl-2-picrylhydrazyl) test and in the study of the *in vitro* impact on lipid peroxidation in rat brain homogenate and on some characteristics of rat liver mitochondria. The results of DPPH test at 20°C show that the activity depends strongly upon the presence of phenolic group but is improved by the influence of ferrocenyl fragment. The activity of N-(3,5-di-*tert*-butyl-4-hydroxyphenyl)iminomethylferrocene (**1**), for instance, was 88.4%, which was higher than the activity of a known antioxidant 2,6-di-*tert*-butyl-4-methylphenol (BHT) (48.5%), whereas the activity of N-phenyl-iminomethylferrocene **2** was almost negligible –2.9%. The data obtained demonstrate that the compounds with 2,6-di-*tert*-butylphenol moiety are significantly more active than the corresponding phenyl analogues in the *in vitro* study of lipid peroxidation in rat brain homogenate. Ferrocene **1** performs a promising behavior as an antioxidant and inhibits the calcium-dependent swelling of mitochondria. These results allow us to propose the potential cytoprotective (neuroprotective) effect of ditopic compounds containing antioxidant 2,6-di-*tert*-butylphenol group and redox active ferrocene fragment.

## 1. Introduction

Oxidative stress has been found to play a critical role in numerous disease conditions including neurodegeneration [1–3].

The antioxidative defense system in living organism regulates a disturbance in the prooxidant-antioxidant balance and protects the cell damage induced by high level of oxidative stress. Among the classes of well-known natural antioxidants-vitamins E group, ascorbic acid, glutathione, and so forth,  $\alpha$ -tocopherol and its synthetic analogues, sterically hindered phenols, are of particular importance [4]. The substituted 2,6-dialkylphenols are widely used as inhibitors of free radicals formation in the oxidative destruction of natural and synthetic substrates. The mechanism of their physiological action is associated with the stable phenoxyl radicals' formation in the process of hydrogen atom abstraction by highly reactive peroxy radicals of lipids [5].

The goal of this study was to optimize the effect of 2,6-di-*tert*-butylphenol and to increase the stability of

the corresponding phenoxyl radicals responsible for their antioxidative activity. The approach based on modification of phenolic antioxidants *via* incorporation of ferrocenyl moiety in their molecules seems to be a promising one. Previously we have reported the synthesis, electrochemical characteristics, and ESR study of novel ferrocenes with redox active 2,6-di-*tert*-butylphenol fragments (compounds **1,3**) [6]. These compounds exhibit the properties of multistep redox systems, and the intramolecular electron transfer between two redox active sites of the molecule (the phenol and ferrocene groups) was observed. The high stability of phenoxyl radical species formed in the oxidation is in agreement with a certain degree of electronic delocalization over the molecule.

On the other hand, the ferrocene derivatives show a wide spectrum of physiological activity [7–9].

The incorporation of ferrocene into an anticancer drug tamoxifen, a selective estrogen receptor modulator, containing phenol was reported. The activity of these novel ferrocene derivatives (ferrocifens) was found to be associated with the

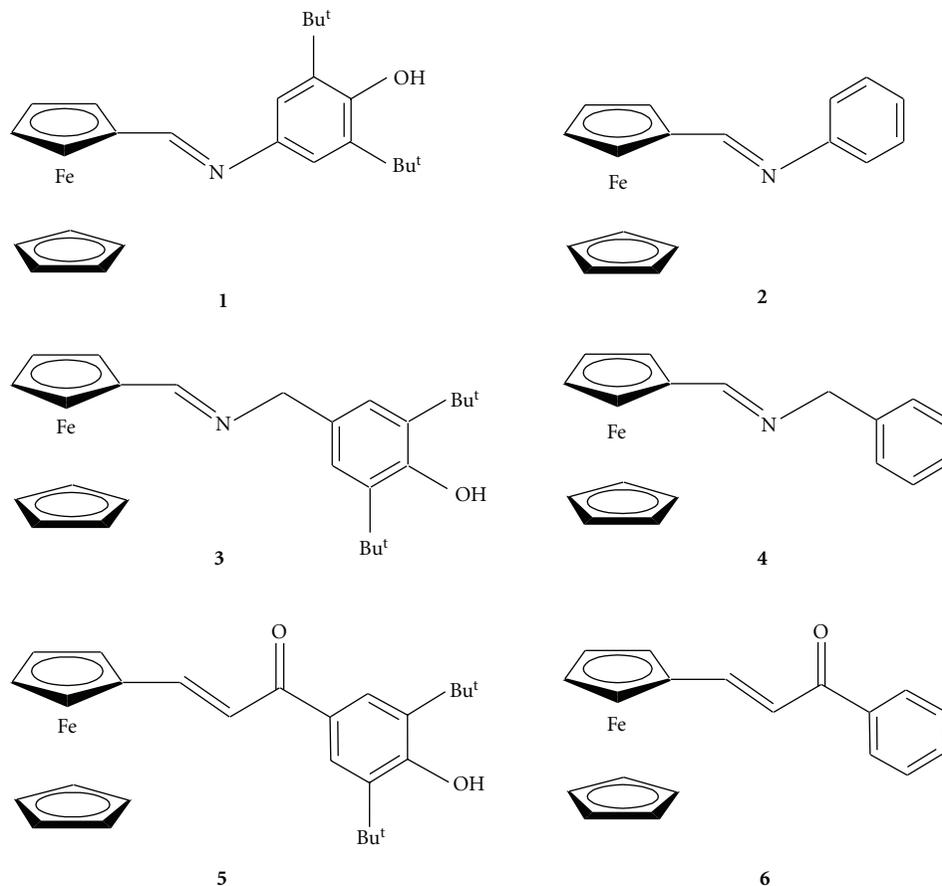


FIGURE 1: Structures of compounds 1–6.

proton-coupled electron transfer between ferrocenium ion and phenol group that occurs in their oxidized species [10–12].

The antioxidative activity in scavenging of superoxide radical-anion  $O_2^{\cdot-}$  and  $HO^{\cdot}$  radical was observed for recently synthesized ferrocenes containing nitroxides radicals as substituents [13].

As it has been reported earlier, diselenides having redox-active ferrocenyl units show peroxidase-like antioxidant activity mimicking selenoenzyme glutathione peroxidase that protects the cell membranes from oxidative damage [14].

In our previous study, we have observed the modulation of the antioxidative effect of metalloporphyrins bearing 2,6-di-*tert*-butylphenol pendants by the metal nature [15].

In this study we compared the antioxidative activity of 1–6 presenting the pairs of compounds bearing either 3,5-di-*tert*-butyl-4-hydroxyphenyl or phenyl substituents linked to the ferrocene by various spacers (Figure 1).

## 2. Materials and Methods

**2.1. Ferrocenes.** N-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-iminomethylferrocene (1), N-phenyliminomethylferrocene (2), N-(3,5-di-*tert*-butyl-4-hydroxybenzyl)-iminomethylferrocene (3), N-benzyliminomethylferrocene (4), (3,5-di-*tert*-

butyl-4-hydroxyphenyl)-3-ferrocenylpropen-2-on (5), and phenyl-3-ferrocenylpropen-2-on (6) were synthesized as described previously [6, 16].

**2.2. DPPH Radical Scavenging Activity.** The free radical-scavenging activity was evaluated using the stable radical DPPH, according to the method described by Brand-Williams et al. [17] with a slight modification.

Each compound was tested for antioxidant activity against DPPH radical at a molar 1:1 ratio. One mL of antioxidant solution in methanol was added to 1 mL of DPPH solution in methanol so that the final DPPH and antioxidant concentration can be 0.1 mM. The samples were incubated for 30 minutes at 20°C in methanol and the decrease in the absorbance of DPPH solution was measured at 517 nm, using a Thermo Evolution 300 BB spectrophotometer. The results were expressed as scavenging activity, calculated as follows:

$$\text{Scavenging activity, \%} = \left[ \frac{(A_0 - A_1)}{A_0} \right] \times 100. \quad (1)$$

The concentration of antioxidant needed to decrease 50% of the initial substrate concentration ( $EC_{50}$ ) is a parameter widely used to measure the antioxidant effect [18]. For determination of  $EC_{50}$ , the values of DPPH solution

absorbance which decrease after 30 minutes were used. The  $EC_{50}$  values were calculated graphically by plotting scavenging activity against compound concentration. Different sample concentrations (0.01, 0.02, 0.05, and 0.1 mM) were used in order to obtain kinetic curves and to calculate the  $EC_{50}$  values. The lower  $EC_{50}$  means the higher antioxidant activity.

**2.3. Rat Brain Homogenates (RBH) and Rat Liver Mitochondria (RLM) Preparation.** On the day of the experiment, adult Wistar male rats fasted overnight were euthanized in a  $CO_2$ -chamber followed by decapitation. The procedure was in compliance with the Guidelines for Animal Experiments at Institute of Physiologically Active Compounds of Russian Academy of Sciences.

The brains were rapidly removed and homogenized in 0.12 M HEPES/0.15 M NaCl, pH 7.4 buffer (HBS) (10 mg/gr wet weight) and used immediately for assay.

Mitochondria were isolated from homogenates of livers of adult Wistar strain rats, fasted overnight, in a 5 mM HEPES buffer, pH 7.4, containing 210 mM mannitol, 70 mM sucrose, and 1 mM EDTA, by conventional differential centrifugation [19].

Protein concentrations in RBH and RLM were determined by the biuret assay using bovine serum albumin as a standard [20].

**2.4.  $Fe^{3+}$ -Induced Lipid Peroxidation Assay.** The extent of lipid peroxidation (LP) was estimated by the levels of malondialdehyde measured using the thiobarbituric acid reactive substances (TBARS) assay. Isolated mitochondria are metabolically active and tightly coupled as shown by respiratory control ratio values, which were about 4 with glutamate-malate as substrate as measured by mitochondrial oxygen consumption at Oroboros oxygraph (Anton Paar, Austria) in a medium containing 10 mM  $KH_2PO_4$  (or  $NaH_2PO_4$ ), 60 mM KCl, 60 mM Tris, 5 mM  $MgCl_2$ , 110 mM mannitol, and 0.5 mM  $EDTA-Na_2$ , pH 7.4.

Study of compounds influence on LP of the RBH was carried out at 30°C for 40 minutes in 0.25 mL of the RBH in HBS (2 mg of protein · mL<sup>-1</sup>) in the presence or absence of compounds or vehicle (DMSO). LP was induced by using  $Fe^{3+}$  (0.5 mM  $Fe(NH_4)(SO_4)_2$ ) as an oxidizing agent [21]. Then 0.25 mL aliquots were mixed with 0.5 mL thiobarbituric acid (TBA) medium containing 250 mM HCl, 15% trichloroacetic acid, and 3 mM TBA, heated at 95°C for 15 minutes, cooling at 4°C then probes centrifuged (10 minutes at 10 000 g) and the supernatants transferred into 96-plate and absorbance was measured at 530–620 nm at the Wallac Victor 1420 Multilabel Counter (PerkinElmer Wallac).

All the experiments were performed using four independent experiments with different brain homogenate preparations. Data are normalized to control probe with oxidant as 100% and blank probe with diluent but without oxidizing agent. Preliminary experiments were done in the absence of compounds interaction with thiobarbituric acid. The values are expressed as mean% ± SD. The concentrations of ferrocenes giving half-maximal inhibition ( $IC_{50}$ ) of LP were determined by dose-effect analysis.

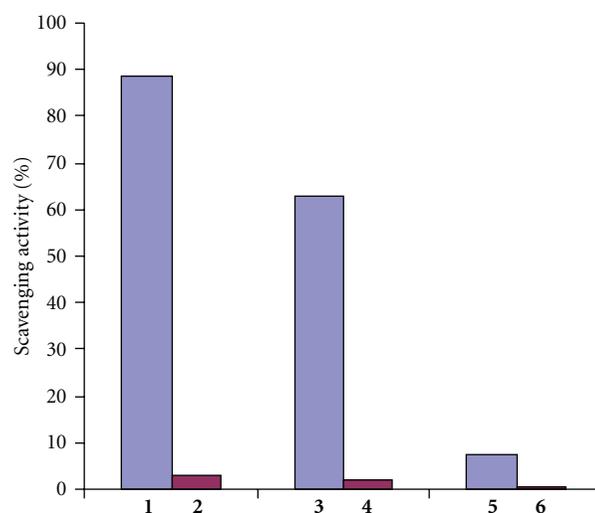


FIGURE 2: Scavenging activity for compounds 1–6 in DPPH test (MeOH, 20°C, 100  $\mu$ M).

**2.5. Mitochondrial Swelling Assay.** Mitochondrial swelling caused by influx of solutes through open mitochondrial permeability transition (MPT) pores results in an increase in light transmission (i.e., a reduced turbidity). This turbidity change offers a convenient and frequently used assay of the MPT by measurement of absorbance in mitochondrial suspensions. The MPT induced by  $Ca^{2+}$  was monitored by absorbance changes at 540 nm in a Beckman DU 640 spectrophotometer in 1 mL of buffer A plus 0.8  $\mu$ M rotenone, 5 mM succinate, 1 mM  $KH_2PO_4$ , and 0.5 mg protein of isolated liver mitochondria at 30°C and continuous stirring [19]. Swelling rate is quantified as  $\Delta A_{540}/\text{min}/\text{mg}$ , calculated, in all cases, from a tangent to the steepest portion of the plot of  $A_{540}$  versus time.

**2.6. Measurement of Mitochondrial Membrane Potential.** The same experimental conditions were used for the assessment of alterations of the mitochondrial membrane potential, except that safranin was included in incubation medium at a final concentration of 10  $\mu$ M and succinate was added after the compound. This concentration of safranin was determined before hand as the optimal compromise between signal/baseline ratio and interference of safranin itself with swelling induced by Ca/Pi (safranin tended to enhance Ca/Pi-induced swelling at concentrations above 20  $\mu$ M) [19]. Changes in the status of the MPT pore are assessed spectrophotometrically at 524 versus 554 nm in a Beckman DU 640 spectrophotometer at 30°C and continuous stirring.

### 3. Results and Discussion

We have compared the antioxidative activity of 1–6 presenting the pairs of compounds bearing either 3,5-di-*tert*-butyl-4-hydroxyphenyl or phenyl substituents linked to the ferrocene by various spacers (Figure 1).

The scavenging activity has been studied in the process of hydrogen atom transfer to the stable free radical DPPH

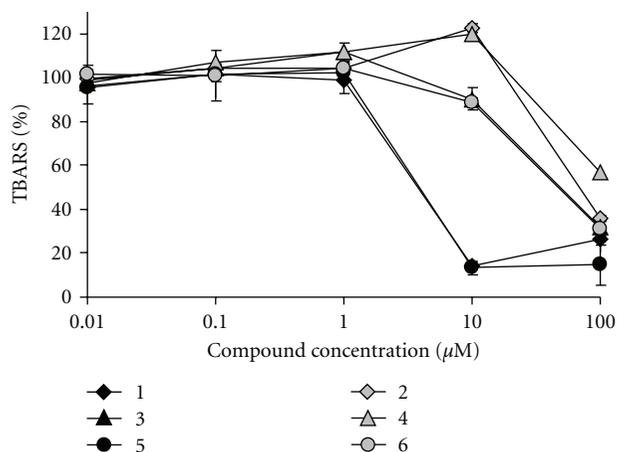


FIGURE 3: The relative content of TBARS in the lipid peroxidation of rat brain homogenates as nonenzymatic process in the presence of 10  $\mu\text{M}$  1–6 (0.5 mM  $\text{Fe}(\text{NH}_4)(\text{SO}_4)_2$ ).

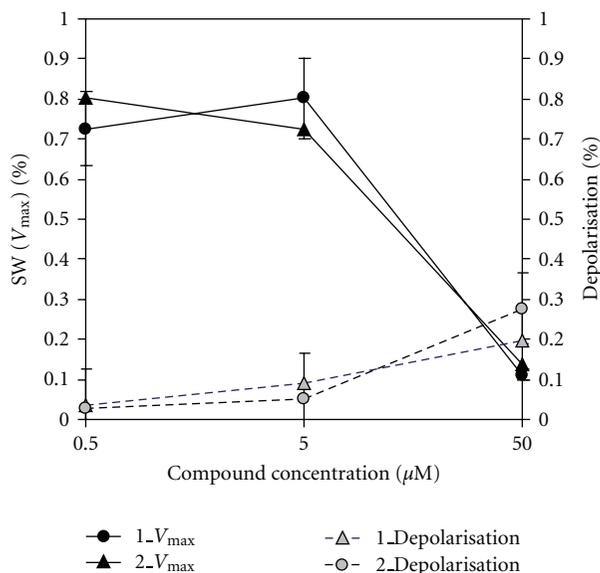


FIGURE 4: Influence of 0.1 mM ferrocenes 1 and 2 on mitochondrial swelling and transmembrane potential (the values were determined and expressed as % of control).

[22]. The results of DPPH test at 20°C show that the activity depends strongly upon the presence of phenolic group in the presented pairs of compounds. The activity of 1, for instance, was 88.4% that is higher than that of a known antioxidant 2,6-di-*tert*-butyl-4-methylphenol (BHT) (48.5%) whereas the activity of 2 bearing phenyl substituent was almost negligible –2.9% (Figure 2). The values of scavenging activity of compounds 3, 5 were lower, and in the case of 3 the decrease in activity was more pronounced. Evidently the activity extent of the compounds tested depends on their molecular structures. The HO-group of 2,6-di-*tert*-butylphenol is the key site in the molecule that is involved in hydrogen transfer to DPPH. However, despite the presence of ferrocene moiety in all the compounds

TABLE 1: The values of  $\text{IC}_{50}$  in the antioxidative activity assay in rat brain homogenates for compounds 1–6.

Compound	$\text{IC}_{50}$ $\mu\text{M}$ (0.5 mM $\text{Fe}(\text{NH}_4)(\text{SO}_4)_2$ )
1	$3.7 \pm 1.0$
2	$70.4 \pm 11.1$
3	$47.3 \pm 2.4$
4	$100 \pm 15.0$
5	$3.9 \pm 1.8$
6	$47.8 \pm 1.6$

they differ significantly containing linkers of various length and conjugation ability ( $-\text{CH}=\text{N}-$ , 1;  $-\text{CH}=\text{N}-\text{CH}_2-$ , 2;  $-\text{CH}=\text{CH}-\text{C}(\text{O})-$ , 3). The decrease of conjugation in their molecules containing either  $\text{CH}_2$  or  $\text{CO}$  groups in linkers leads to the decrease of metal influence on the stability of radicals formed as it has been observed previously [6]. However, it should be mentioned that the activity of 3 is much higher than that of 5 with N atom possessing a lone electron pair in linker that improves the influence of ferrocene moiety.

To compare the activity of compounds under investigation with that of widely known antioxidant parameter,  $\text{EC}_{50}$  was determined for the more efficient ferrocene 1 and 2,6-di-*tert*-butyl-4-methylphenol (BHT).  $\text{EC}_{50}$  values after 30 minutes of experiment at 20°C for 1 and BHT are 34.6 and 105.4  $\mu\text{M}$ , respectively. Therefore, the result obtained shows a more pronounced effect of ferrocenyl derivative of 2,6-di-*tert*-butylphenol.

In order to study the antioxidant effect of ferrocenes 1–6 in biologically significant *in vitro* test system, we have investigated the compounds influence on  $\text{Fe}^{3+}$ -induced peroxidation of brain homogenate lipids (LP) as a nonenzymatic process by addition of  $(\text{NH}_4)\text{Fe}(\text{SO}_4)_2$ . The level of LP was followed by the accumulation of products that reacted with thiobarbituric acid—TBARS. The samples of Wistar strain rats homogenates were divided as following: one control homogenate and samples of homogenate with addition of compounds under investigation. TBARS concentrations were determined in homogenates by measuring the intensity of the solution color at 530 nm using UV-VIS spectroscopy [23].

The data of antioxidative activity assay of 1–6 presenting the pairs of compounds bearing either 3,5-di-*tert*-butyl-4-hydroxyphenyl or phenyl substituents linked to the ferrocene by various spacers are shown in Figure 3. The  $\text{IC}_{50}$  values are summarized in Table 1.

The data of antioxidative activity assay of ferrocenes 1–6 indicate the influence of 2,6-di-*tert*-butylphenol group as it was observed in DPPH test. Ferrocene 1 performs an effective inhibitory action in concentrations range at 10–100  $\mu\text{M}$  (Figure 3, curve 1). The decrease in peroxidation level is more than 10%.

In contrast to DPPH test, the data of this assay reveal the antioxidant activity of all studied compounds. These results allow us to suggest that the ferrocene moiety participates in antioxidative potential of these compounds. However,

the compounds **1**, **3**, **5** bearing 2,6-di-*tert*-butylphenol are significantly more active than the corresponding phenyl analogues. This effect is most obvious at concentration 10  $\mu\text{M}$  of compounds (Figure 3). Moreover, at this concentration some pro-oxidant effect of compounds **2** and **4** could be observed. This fact might be associated with the influence of iron center in the molecules of ferrocenes that participates in redox processes and therefore promotes the peroxidation.

The involvement of ferrocene group in the peroxidation process might be associated with the oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  in the oxidative medium that leads to the formation of ferrocenium cation. As it was proved earlier [24] ferrocenium cations react easily with molecular oxygen and produce reactive peroxy radical cations. On the other hand, in the presence of antioxidant, namely BHT, ferrocenium cation can be stabilized due to the reduction that takes place between the cation and antioxidant. The principal consequence of this electron/proton coupled reaction is the reversibility of ferrocene/ferrocenium redox system. This fact might support the proposition of the intramolecular redox process in ferrocene species containing 2,6-di-*tert*-butylphenol fragment (compounds **1**, **3**, **5**).

To study proapoptotic/antiapoptotic effect of ferrocene **1** with 2,6-d-*tert*-butylphenol group which shows the more promising activity in both tests and to compare it with the effect of its analog **2** bearing phenyl substituent, we have investigated the influence of these compounds on two main characteristics of mitochondria: calcium-induced mitochondrial swelling (SW) that represents the mitochondrial permeability pores opening (which causes cell death), and mitochondrial membrane potential.

It was shown that at concentration 0.1 mM ferrocenes **1** and **2** slightly depolarize the mitochondria (up to 25%) (Figure 4). On the other hand, these compounds inhibit the calcium-dependent swelling of mitochondria and this effect could not be the consequence of the depolarisation only. In both cases the effects on mitochondrial swelling and mitochondrial membrane potential obtained for ferrocene **1** are less pronounced than for **2**. These data allow us to propose the potential cytoprotective (neuroprotective) effect of compounds studied.

#### 4. Conclusion

The antioxidative activity of ferrocenes bearing either 2,6-di-*tert*-butylphenol or phenyl groups, studied using DPPH test, depends strongly upon the presence of phenol group and the conjugation between peroxy radical formed and ferrocene unit. The compounds **1**, **3**, **5** bearing 2,6-di-*tert*-butylphenol are significantly more active than the corresponding phenyl analogues in the in vitro lipid peroxidation in rat brain homogenate. N-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-iminomethylferrocene (**1**) performs a promising behavior as an antioxidant and inhibits the calcium-dependent swelling of mitochondria. The results allow us to propose the potential cytoprotective (neuroprotective) effect of ditopic compounds containing antioxidant 2,6-di-*tert*-butylphenol group and redox active ferrocene fragment.

#### Abbreviations

BHT:	butylated hydroxytoluene (2,6-di- <i>tert</i> -butyl-4-methylphenol)
DPPH:	1,1-diphenyl-2-picrylhydrazyl ( $\alpha,\alpha$ -diphenyl- $\beta$ -picrylhydrazyl)
HBS:	HEPES buffered saline
LP:	lipid peroxidation
MPT:	mitochondrial permeability transition
RBH:	rat brain homogenate
RLM:	rat liver mitochondria
TBA:	thiobarbituric acid
TBARS:	thiobarbituric acid reactive substances.

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