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Interaction of Phenolic Schiff Bases Bearing Sulfhydryl Moieties with 2,2-Diphenyl-1-picrylhydrazyl Radical: Structure–Activity Relationship Study †

Iveta Turomsha [1,2,*], Maxim Gvozdev [1,2], Natalia Loginova [1,2], Galina Ksendzova [1,2] and Nikolai Osipovich [2]

[1] Department of Chemistry, Belarusian State University, 14 Leningradskaya St., 220006 Minsk, Belarus; gvozdevmaximka@gmail.com (M.G.); loginonv@gmail.com (N.L.); ksja-bn@tut.by (G.K.)
[2] Research Institute for Physical Chemical Problems, Belarusian State University, 14 Leningradskaya St., 220030 Minsk, Belarus; osipovich@bsu.by
* Correspondence: ivetaturomsha@gmail.com
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Abstract: Current research on synthetic and naturally occurring phenolic compounds is centered around their prominent antioxidant properties. Since reactive oxygen (ROS) and nitrogen (RNS) species cause considerable damage to cellular components upon their overproduction, associated with the pathogenesis of degenerative, cardiovascular and oncological diseases, antioxidants may reduce the risk of developing such conditions. Because hydroxyl, amino and sulfhydryl groups present in their structure, antioxidants may function as hydrogen atom and electron donors, as well as metal-reducing and metal-chelating agents. We synthesized phenolic Schiff bases from 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde; *ortho*-, *meta*- and *para*-mercaptoanilines; and 2,2′- and 4,4′-disulfanediyldianilines. Their antioxidant properties were studied in a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay.

Keywords: Schiff bases; DPPH; antioxidant activity; thiols; phenolic compounds

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1. Introduction

Reactive oxygen (ROS) and nitrogen (RNS) species are essential molecules generated in cellular metabolism that play a significant role in the cell energy supply, chemical signaling, detoxification and immune function [1]. ROS and RNS include superoxide radical, hydroxyl–peroxyl, peroxyl, alkoxyl and hydroxyl free radicals, nitric oxide radicals and peroxynitrite anions [2]. However, in cases of disturbance of redox homeostasis, i.e., the disbalance between the processes of generation and neutralization of ROS and RNS, oxidative stress is observed in the cell. Oxidative damage may lead to the permeabilization of mitochondrial membranes, resulting in the disturbance of cellular energy homeostasis, the release of apoptosis-initiating factors and, eventually, in cell death [3]. Furthermore, oxidative stress involves the overproduction of free radical species that causes considerable damage to protein and lipid molecules, as well as to DNA [1,4]. Such harmful effects are associated with inflammation and hence with the pathogenesis of several diseases, namely neurodegenerative disorders, atherosclerosis, coronary heart disease, hypertension, muscular degeneration and cancer [5,6].

Redox homeostasis in the cellular environment is maintained by two major endogenous antioxidative defense mechanisms, enzymatic and non-enzymatic ones. Enzymatic defense systems comprise cascades of biochemical reactions where enzymes detoxify ROS and RNS by reacting directly with these species and/or by functioning as redox regulators [7]. Superoxide dismutases catalyze the neutralization of superoxide anion radicals, whereas glutathione peroxidase is involved in the removal of hydrogen peroxide from the

cell, as well as in the metabolism of lipid hydroperoxides formed as a result of oxidative stress [8]. The catalase enzyme participates in the detoxification of hydrogen peroxide, thereby modulating signal transduction and inducing tumor cell proliferation that stimulates the adaptation of the cells to oxidative stress [9,10].

Being less substrate-specific, non-enzymatic systems realize the primary mechanisms of antioxidant defense in the cellular response to oxidative stress. For instance, glutathione, ascorbic acid (vitamin C) as well as tocopherols and tocotrienols (vitamin E) mediate intracellular radical quenching and hence interfere with the chain reactions involved in ROS overproduction, including lipid peroxidation. Furthermore, vitamin E has been reported to participate in modulating the function of several enzymes, namely mitogen-activated protein kinase, protein kinase C, serine/threonine protein kinase Akt/PKB, protein tyrosine kinase, the pro-inflammatory enzymes phospholipase A2, 5-, 12- and 15-lipoxygenases, cyclooxygenase-2, nitric oxide synthase, etc. [11]. Therefore, it regulates cell survival, apoptosis, necrosis, adhesion and proliferation, as well as signal transduction.

Naturally occurring antioxidants comprise various classes of polyphenols, such as flavonoids, stilbenes, coumarins, curcuminoids, xanthones, secoiridoids, phenolic acids, acetophenones, benzophenones, high-molecular lignans and tannins, etc., that generally possess either a pyrogallol or a pyrocatechol fragment in their chemical structure [2,12]. Among them, flavonoids represent the most numerous group, being subdivided into flavones, flavonols, dihydroflavonols, flavanones, flavanonols, isoflavones, anthocyanidins, anthocyanins, proanthocyanidins and chalcones, all of them bearing two phenyl fragments and a heterocyclic fragment that form a prominent C6-C3-C6 structure [3,13]. Being mostly secondary metabolites of plants, naturally occurring polyphenols are essential for the development of plant pathogen resistance, as well as for their growth, reproduction and pigmentation [2]. Apart from being used as colorants, natural antioxidants find application in food preservation due to the presence of ortho-disubstituted phenolic fragments in their structure that allow for high toxicity, and hence, bactericidal properties [12].

In contrast to this, synthetic antioxidants mostly possess para-disubstituted phenolic structural fragments that render them less toxic [12]. The most widespread synthetic phenol derivatives are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertiary butylhydroquinone (TBHQ), commonly utilized as food additives in animal fats, cured meats and vegetable oils [14]. These compounds have been shown to interfere with the food deterioration process by quenching lipid hydroperoxide radicals.

The question remains, however, as to whether exogenous synthetic or naturally occurring antioxidants are able to serve as biological response modifiers, similarly to endogenous enzymes and small molecules, by interacting with a wide range of molecular targets essential to the cell functioning machinery. ROS and RNS scavenging by molecules that possess antioxidant properties is a prerequisite for the realization of a broad spectrum of biological activities, including anti-inflammatory, antibacterial, antiviral, antithrombotic, vasodilatory, hepatoprotective, antiallergic, anticarcinogenic, etc. [15].

A wide variety of assays applied for the estimation of antioxidant activity may be classified into three main categories: assays involving ROS-oxidizable substrate interactions (TBARS assay, diene conjugation measurements), assays involving a relatively stable single oxidizing agent (DPPH, ABTS, FRAP, CUPRAC methods), as well as assays relating antioxidant activity to electrochemical behavior (cyclic voltammetry) [16–18]. The second group of methods comprises free radical scavenging (DPPH and ABTS); metal ion-reducing, i.e., electron-scavenging, assays (FRAP and CUPRAC); and metal ion (Fe(II) and Cu(II))-chelating activity assays [19]. Recently, an antioxidant activity assay involving 4-nitroso-*N*,*N*-dimethylaniline bleaching by lipid hydroperoxides formed by the soybean lipoxygenase-1 enzyme has been proposed [20]. All of the methods significantly depend on the reaction conditions and are characterized by different mechanisms of antioxidant action, namely proton and/or electron transfer reactions [19].

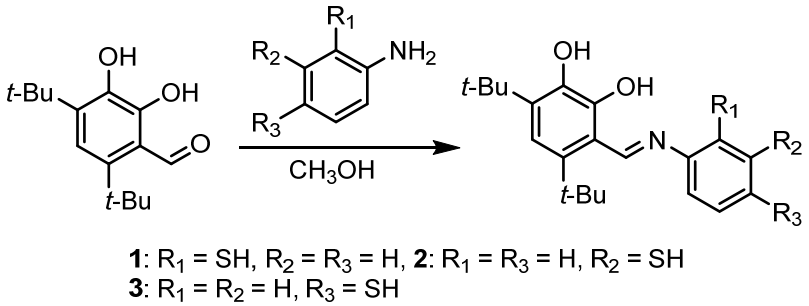
In this work, we utilized the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging method to investigate the antioxidant activity of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde-

derived phenolic Schiff bases bearing thiol groups from o-, m-, p-mercaptoanilines and 2,2′-, 4,4′-disulfanediyldianilines. Their DPPH radical-scavenging activity was further correlated with their chemical structure so as to determine essential structural characteristics that account for the potent antioxidant activity of such compounds.

2. Materials and Methods

2.1. Synthetic Procedure for Compounds **1**–**5**

A mixture of 0.50 g (2.0 mmol) of 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde and 2.0 mmol of *ortho*-, *metha*- and *para*-mercaptoanilines in 20 mL of anhydrous methanol was refluxed for 2 h (Figure 1). The obtained precipitate was filtered off and recrystallized from ethanol.



1: R_1 = SH, R_2 = R_3 = H, **2**: R_1 = R_3 = H, R_2 = SH
3: R_1 = R_2 = H, R_3 = SH

Figure 1. Synthetic scheme for compounds **1**–**3**.

Compounds **4** and **5** were obtained via a condensation reaction of the corresponding 2,2′- and 4,4′-disulfanediyldianilines with 4,6-di-*tert*-butyl-2,3-dihydroxybenzaldehyde, as shown above (Figure 2).

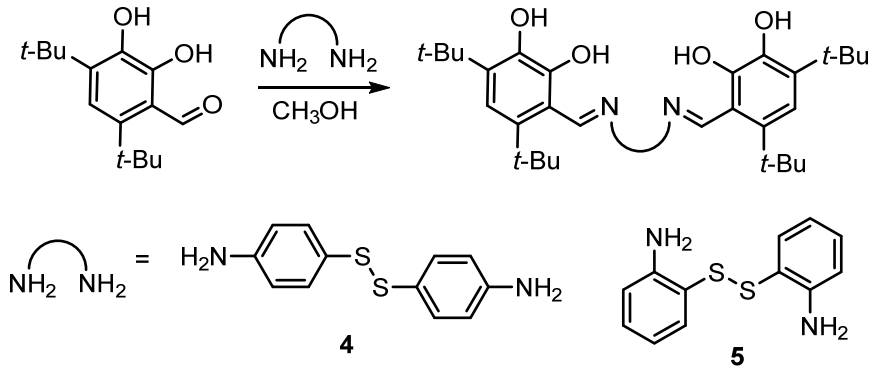


Figure 2. Synthetic scheme for compounds **4** and **5**.

4,6-di-*tert*-butyl-3-(2,3-dihydrobenzo[d]thiazol-2-yl)benzene-1,2-diol (**1**)

Pale-yellow solid, 78%. m.p. 112–114 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ, ppm: 1.35 s (18H, CH_3), 6.73 s (1H, CH_{arom}), 6.80–6.84 m (2H, CH_{arom}), 6.99–7.02 m (2H, CH_{arom}), 7.16 d (1H, CH, J = 7.8 Hz), 7.54–7.55 br m (1H, NH), 8.06 br. s (1H, OH), 10.28 s (1H, OH). FT-IR (ν, cm^{-1}): 3428 m, ν(O–H); 3332 m ν(N–H); 1153 m ν(C–O). UV-vis: λ, nm (logε, M^{-1} cm^{-1}): 226, 299 (3.78). EI, m/z (I_{rel}, %): 357 (77) $[M]^+$: $[C_{21}H_{27}NO_2S]^+$.

4,6-di-*tert*-butyl-3-(((3-mercaptophenyl)imino)methyl)benzene-1,2-diol (**2**)

Red solid, yield 68%. m.p. 138–140 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ, ppm: 1.38 s (9H, CH_3), 1.45 s (9H, CH_3), 5.75 br. s (1H, SH), 6.78 s (1H, CH_{arom}), 7.10 d (1H, CH_{arom}, J = 7.8 Hz), 7.24 d (1H, CH_{aroma}, J = 7.8 Hz), 7.29–7.31 m (1H, CH_{arom}), 7.37 t (1H, CH_{arom}, J = 7.8 Hz), 8.39 s (1H, OH), 9.35 s (1H, CH=N), 15.12 s (1H, OH). FT-IR (ν, cm^{-1}): 3346 s, ν(O–H); 1627 s ν(C=N); 1162 m ν(C–O), 1263 m ν(C_{arom}–N), 2577 w ν(S–H), 676 w, 646 w, 613 w ν(C–S). UV-vis: λ, nm (logε, M^{-1} cm^{-1}): 206 (4.43), 225 (4.36), 328 (4.27). EI, m/z (I_{rel}, %): 357 (92) $[M]^+$: $[C_{21}H_{27}NO_2S]^+$.

4,6-di-*tert*-butyl-3-(((4-mercaptophenyl)imino)methyl)benzene-1,2-diol (**3**)

Red solid, yield 64%. m.p. 189–191 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ, ppm: 1.37 s (9H, CH_3), 1.44 s (9H, CH_3), 5.65 br. s (1H, SH), 6.77 s (1H, CH_{arom}), 7.27–7.30 m (2H, CH_{arom}), 7.40– 7.42 m (2H, CH_{arom}), 8.25 s (1H, OH), 9.36 s (1H, CH=N), 15.26 s (1H, OH). FT-IR (ν, cm^{-1}): 3394 s, ν(O–H); 1610 s ν(C=N); 1162 m, 1097 m ν(C–O), 1247 m ν(C_{arom}–N), 2560 w ν(S–H), 705 w, 669 w ν(C–S). UV-vis: λ, nm (($\log\varepsilon$, M^{-1} cm^{-1}): 204 (4.57), 227 sh, 302 sh, 344 (4.45). EI, *m/z* (I_{rel}, %): 357 (92) $[M]^+$: $[C_{21}H_{27}NO_2S]^+$.

6,6′-((disulfanediylbis(4,1-phenylene))bis(azaneylylidene))bis(methaneylylidene))bis(3,5-di-*tert*-butylbenzene-1,2-diol) (**4**)

Orange solid, yield 79%; ^{1}H NMR (500 MHz, DMSO-d_6) δ, ppm: 1.37 s (18H, CH_3), 1.43 s (18H, CH_3), 6.77 s (2H, CH_{arom}), 7.41–7.44 m (4H, CH_{arom}), 7.64– 7.69 m (4H, CH_{arom}), 8.42 s (2H, OH), 9.38 s (2H, CH=N), 15.10 s (2H, OH). FT-IR (ν, cm^{-1}): 3430 m, ν(O–H); 1604 s, 1554 s ν(C=N); 1166 m ν(C–O). UV-vis: λ, nm ($\log\varepsilon$, M^{-1} cm^{-1}): 214, 300 sh, 347 (4.21). ESI-MS(+), *m/z* (I_{rel}, %): 735 (65) $[M+Na]^+$: $[C_{42}H_{51}N_2O_4S_2Na]^+$.

6,6′-((disulfanediylbis(2,1-phenylene))bis(azaneylylidene))bis(methaneylylidene))bis(3,5-di-*tert*-butylbenzene-1,2-diol) (**5**)

Orange solid, yield 55%; ^{1}H NMR (500 MHz, DMSO-d_6) δ, ppm: 1.39 s (18H, CH_3), 1.47 s (18H, CH_3), 6.84 s (2H, CH_{arom}), 7.32–7.44 m (2H, CH_{arom}), 7.39– 7.44 m (4H, CH_{arom}), 7.61–7.63 m (2H, CH_{arom}), 8.56 s (2H, OH), 9.45 s (2H, CH=N), 14.50 s (2H, OH). FT-IR (ν, cm^{-1}): 3519 m, 3495 m ν(O–H); 1596 s, 1553 s ν(C=N); 1167 m ν(C–O). UV-vis: λ, nm ($\log\varepsilon$, M^{-1} cm^{-1}): 223, 319 (4.47). ESI-MS(+), *m/z* (I_{rel}, %): 735 (100) $[M+Na]^+$: $[C_{42}H_{51}N_2O_4S_2Na]^+$.

2.2. General Methods

^{1}H NMR spectra (DMSO-d_6 and $CDCl_3$ were used as solvents) were recorded using a Bruker Avance-500 spectrometer operating at 500 MHz. ESI mass spectra of the ligands were registered with a Shimadzu LC-MS 2020 spectrometer using direct injection of the specimens into the ion source. The mass spectrometer was operated in the direct injection mode with electrospray ionization, with positive ion registration at a detector voltage of 1.2 kV, a heat block temperature of 400 °C and a desolvation line temperature of 250 °C. Nitrogen was used as a drying and nebulizer gas at flow rates of, respectively, 1.5 L/min and 15 L/min. UV-Vis absorption spectra of the compounds were recorded in acetonitrile (HPLC grade). Spectrophotometric experiments were performed with a Solar PB 2201 spectrophotometer using a quartz cuvette with a 1 cm optical path. The IR spectra were recorded using an ALPHA II FT-IR spectrometer in the wavelength range of 400–4000 cm^{-1} using a Smart Diffuse Reflectance accessory.

2.3. DPPH Radical-Scavenging Activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Sigma) was used. The compounds under study were prepared in ethanol and added to the ethanolic solution of DPPH (60 μM) up to the final concentrations of 60 μM. The decrease in absorbance was measured at 517 nm after 1 min. All the solutions were protected from light. The DPPH free radical-scavenging activity was calculated according to the following equation:

$$I, \% = \frac{A_0 - A_1}{A_0} \cdot 100\%, \quad (1)$$

A_1 and A_0 represent the absorbance of the samples, respectively with and without an antioxidant.

3. Results and Discussion

The molecular mechanisms involved in the realization of antioxidant activity by both endogenous and exogenous antioxidants may involve the following processes. First, the inhibition of free radical oxidation reactions may occur, which is realized through interference with the decomposition of lipid peroxides. Chain-breaking antioxidants are known to interrupt autooxidation chain reactions by the scavenging of ROS and RNS,

including singlet oxygen. There are antioxidants that inhibit the function of pro-oxidative enzymes, such as lipoxygenases, while certain molecules exert their antioxidant action by enhancing the activity of chain-breaking antioxidants, serving as synergists of the latter. In contrast to this, thiols and thioesters are known to be potent reducing agents that could scavenge hydroperoxides in a non-radical reaction pathway. Moreover, several antioxidant molecules bearing *N*-, *O*- and *S*-donor groups exhibit potent metal-chelating properties that allow for the removal of intracellular pro-oxidant species, namely iron(II) and copper(I) ions, by their coordination into metal complexes. The described mechanisms frequently coexist and may alter in response to the conditions of the cellular environment [20].

It should be noted that antioxidant properties of phenolic compounds are mainly associated with their relatively high reactivity in hydrogen abstraction reactions. This circumstance may be explained by the stabilization of the formed phenoxyl radical by delocalization of the unpaired electron in an extended conjugated aromatic system. Enhancement of the antioxidant activity of phenols and their derivatives is usually associated with the introduction of additional hydroxyl groups into their chemical structure, as well as with an decrease in sterical hindrance at the hydrogen abstraction site [21]. Pyrocatechol derivatives readily undergo hydrogen abstraction followed by the formation of a semiquinone radical that is additionally stabilized by an intramolecular hydrogen bond with the *ortho*-phenolic group. Moreover, the introduction of donor substituents into *ortho*- and *para*-positions to phenol hydroxyls is known to reduce the bond dissociation enthalpy (BDE) of the O–H bond, thereby stimulating the hydrogen abstraction process [22]. The antioxidant activity of pyrocatechol derivatives is further enhanced due to the possibility of subsequent quinone formation from the semiquinone radical in an oxidation process [23].

Phenolic Schiff bases bearing thiol groups in *para*-position to azomethine groups have been found to exhibit comparable DPPH radical-scavenging activity to their *meta*-thiosubstituted analogues (Table 1). Meanwhile, *ortho*-thiosubstituted derivatives display reasonably lower antioxidant activity, which is a consequence of the ring–chain tautomerism characteristic of such compounds that renders sulfhydryl groups incapable of hydrogen atom donation [24]. Another reason for the decreased antioxidant activity observed for the Schiff bases *ortho*-thiosubstituted with thiol groups is greater steric congestion caused by *ortho*- compared to *para*-substituents in a possible non-radical reaction pathway where thiols act as reducing agents. It should also be noted that diamine-derived Schiff bases possessing an increased number of phenolic groups display higher radical-scavenging activity than their monomeric counterparts, which is confirmed by their lower IC_{50} values (Table 1).

Table 1. DPPH radical-scavenging data.

Compound	IC_{50}, μM
1	24.93 ± 1.06
2	9.91 ± 0.53
3	10.43 ± 1.29
4	9.82 ± 0.53
5	10.70 ± 1.05
Trolox	17.11 ± 0.32

4. Conclusions

Phenolic compounds, particularly the synthesized phenolic Schiff bases, are known to effectively scavenge free radical species, namely ROS and RNS, in reactions that involve hydrogen atom transfer, electron transfer, metal ion reduction or metal ion chelation. The antioxidant properties of phenolic derivatives may provide a basis for their application in the treatment of certain diseases associated with the production of free radicals. The antioxidant activity of mono-thiosubstituted phenolic Schiff bases has been found to increase in the following order: *ortho*- < *meta*- ∼ *para*-mercaptoaniline derivatives. The reactivity of *ortho*-derivatives towards DPPH radicals is significantly influenced by their ring–chain

tautomerism. In contrast to the mono-thiosubstituted derivatives, their dimeric analogues exhibit higher antioxidant activity in the reactions of DPPH radical scavenging. The investigation of the structure–activity relationship of phenolic compounds remains relevant in terms of the determination of possible molecular mechanisms of antioxidant activity, as well as for the design of novel pharmacological agents.

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