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In vitro assessment of the antioxidant activity of new benzimidazole-2-thione hydrazone derivatives and DFT study of their mechanism of action

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Benzimidazole-2-thione derivatives containing hydrazone moieties designed as melatonin analogues were effective in inhibiting induced oxidative stress and acted as potential hepatoprotectors. As a continuation from our previous work, we have synthesized new derivatives of the benzimidazole-2-thione containing residues of vanillin, syringaldehyde and veratraldehyde. We estimated their radical scavenging potential in systems containing stable free radicals (ABTS and DPPH) and evaluated their protection effect against ferrous iron induced oxidative damage of lecithin.

The studied compounds demonstrated a different extent of scavenging effect against both stable free radicals, which could be attributed to their structural dissimilarity and to the different mechanism of radical neutralization. All compounds demonstrated capability to diminish the concentration of ABTS. In the DPPH system no statistically significant decrease of the absorbance of the samples containing veratraldehyde residue was observed. Comparison of the C-50 values for the vanillin and syringaldehyde containing compounds estimated using linear regression analysis, denoted necessity of lowering the concentration in order to observe 50 % radical scavenging activity than the reference Trolox in both used systems.

All the tested compounds decreased the ferrous iron induced oxidative molecular damage. The observed protection effect was in the same concentration range as the one of strong reference antioxidants, such as Trolox and Quercetin.

Different possible mechanisms, such as hydrogen atom transfer (HAT), single-electron transfer (SET-PT), sequential proton loss electron transfer (SPLET) were studied by DFT computations of the respective reaction enthalpies in polar and nonpolar solvents.

Keywords: benzimidazoles, antioxidants, ABTS, DPPH, DFT

INTRODUCTION

The administration of antioxidants is a leading strategy in the prevention and treatment of health disorders resulting from decreased antioxidant capacity [1, 2]. The development of new antioxidants based on the structural resemblance between N-substituted benzimidazole derivatives and melatonin has demonstrated promising results [3-5]. In a previous study we synthesized series of N,N'-disubstituted benzimidazole-2-thiones with extended side chains coupled with benzaldehydes, containing methoxy- and fluoro- substituents. It was shown that the compounds affect the cell viability and the levels of lactate dehydrogenase, glutathione and malonaldehyde in isolated rat hepatocytes [6]. Among all the tested compounds, the unsubstituted benzimidazole-2-thione hydrazone containing methoxyphenyl moieties preserved to the highest degree the functional-metabolic status of the hepatocytes and exhibited cytoprotective and antioxidant effects similar to those of Quercetin. This compound was selected as a promising structure for further research. It was proved that benzimidazole-2-thione derivatives containing hydrazone moieties designed as melatonin analogues could inhibit induced oxidative stress and act as potent hepatoprotectors [6]. As a continuation of our previous work we are currently reporting the synthesis of new derivatives of the benzimidazole-2-thione containing residues of vanillin, syringaldehyde and veratraldehyde that will be further investigated for neuroprotective action. The new compounds were assessed for *in vitro* antioxidant activity by ABTS, DDPH and ferrous iron induced peroxidation in a lipid containing model system.

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EXPERIMENTAL PART

General procedure for preparation of compounds 1-3

To a solution of 0.001 mol 3,3'-(2-thioxo-1H-benzo[d]imidazole-1,3(2H)-diyl)dipropanehydrazide in 50 ml absolute ethanol 0.0025 mol of the corresponding benzaldehydes were added. The solution was refluxed for 1 h and the solid product was filtered and purified with ethanol.

3,3'-(2-thioxo-1H-benzo[d]imidazole-1,3(2H)-diyl)bis(N'-(3,4 dimethoxybenzylidene)propanehydrazide) (**1**)

Yield 73%, Mp 239-241 °C, IR (ν_{max}/cm^{-1}) 3188 (νN-H); 2957 $\nu_{as}CH_3$); 2832 (ν_sCH_3); 1663 (νC=O)

amide I; 1642 (νC=N); 1575 (δN-H); 1270 (νC-O-C);

^{1}H NMR (250 MHz, DMSO-d6) δ, ppm: 11.23 – 11.33 (m, 2H, NH); 8.01-8.03 (d, *J* = 4.4 Hz, 1H, CH), 7.78 – 7.81 (d, *J* = 6.7 Hz, 1H, CH); 7.40 – 7.51 (m, 2H, Ar-H); 7.19 – 7.26 (m, 4H, Ar-H); 6.92 – 7.26 (m, 4H, Ar-H); 4.47 – 6.60 (m, 4H, CH_2); 3.76 – 3.80 (m, 12H, CH_3); 3.04 – 3.13 (m, 2H, CH_2); 2.72 – 2.79 (m, 2H, CH_2).

^{13}C NMR (151 MHz, DMSO-d6) δ, ppm: 172.59, 168.28, 166.73, 150.80, 149.33, 147.42, 144.31, 131.72, 127.04, 123.22, 122.54, 121.49, 111.67, 108.90, 108.53, 55.92, 55.84, 32.63, 30.97.

3,3'-(2-thioxo-1H-benzo[d]imidazole-1,3(2H)-diyl)bis(N'-(4-hydroxy-3,5-dimethoxybenzylidene)propanehydrazide) (**2**)

Yield 75%, Mp 284-286 °C, IR (ν_{max}/cm^{-1}) 3324 (νO-H); 3210 (νN-H); 2940 ($\nu_{as}CH_3$); 2844 (ν_sCH_3); 1670 (νC=O) amide I; 1656 (ν C=N); 1556 (δ N-H); 1315 (ν C-O-C);

^{1}H NMR (250 MHz, DMSO-d6) δ, ppm: 11.23 – 11.31 (m, 2H, NH); 8.86 (s, 1H, OH), 8.81 – 8.83 (d, *J* = 5.4 Hz, 1H, OH); 7.96 – 7.98 (d, *J* = 5.2 Hz, 1H, CH); 7.75 – 7.76 (d, *J* = 3.0 Hz, 1H, CH); 7.40 – 7.57 (m, 2H, Ar-H); 7.23 – 7.26 (m, 2H, Ar-H); 6.86 – 6.92 (m, 4H, Ar-H); 6.92 – 7.26 (m, 4H, Ar-H); 4.47 – 6.59 (m, 4H, CH_2); 3.76 – 3.80 (m, 12H, CH_3); 3.05 – 3.13 (m, 2H, CH_2); 2.72 – 2.78 (m, 2H, CH_2).

^{13}C NMR (151 MHz, DMSO-d6) δ, ppm: 172.19, 168.50, 166.26, 148.52, 147.51, 144.62, 138.06, 131.88, 124.81, 124.70, 123.22, 110.06, 104.98, 56.52, 56.45, 33.02, 31.14, 19.11.

3,3'-(2-thioxo-1H-benzo[d]imidazole-1,3(2H)-diyl)bis(N'-(4-hydroxy-3-methoxybenzylidene)propanehydrazide) (**3**)

Yield 82%, Mp 229-231 °C, IR (ν_{max}/cm^{-1}) 3325 (νO-H); 3178 (νN-H); 2952 ($\nu_{as}CH_3$); 2840 (ν_sCH_3); 1655 (νC=O) amide I; 1654 (ν C=N); 1559 (δ N-H); 1280 (ν C-O-C);

^{1}H NMR (250 MHz, DMSO-d6) δ, ppm: 11.15 – 11.32 (m 2H, NH); 9.53 – 9.60 (m, 2H, OH); 7.93 – 7.95 (d, *J* = 10.7 Hz, 1H, CH), 7.72 – 7.74 (d, *J* = 10.8 Hz, 1H, CH); 7.39 – 7.45 (m, 2H, Ar-H); 7.20 – 7.25 (m, 3H, Ar-H); 7.13 – 7.16 (dd, J = 9.9 , 1.9 Hz 1H, Ar-H); 6.99-7.02 (m, 1H, Ar-H); 6.91 – 6.97 (m, 1H, Ar-H); 6.74 - 6.80 (m, 2H, Ar-H); 4.44 – 4.56 (m, 4H, CH_2); 3.75 – 3.78 (m, 6H, CH_3); 3.02 – 3.09 (m, 2H, CH_2); 2.69 – 2.74 (m, 2H, CH_2).

^{13}C NMR (151 MHz, DMSO-d6) δ, ppm: 172.34, 168.56, 149.02, 148.24, 148.28, 144.33, 131.79, 125.87, 123.30, 123.23, +122.33, 121.43, 115.78, 109.99, 56.05, 55.97, 31.03, 18.77.

Antioxidant anti-radical potential estimation

Two sets of samples were prepared – a control sample, where the tested substance has been omitted, and samples containing different concentrations of the studied compound. Prior to each experiment fresh radical solutions were prepared. Trolox was used as a referent compound. Based on the obtained results for the controls and the absorbance at different concentration of the tested compounds, the % radical scavenging activity was calculated (% RAS).

ABTS assay – the procedure was performed according to Re et al. [7]. The radical cation was produced in buffered water by the mixing of 14 mM ABTS stock solutions with a strong oxidizing agent (potassium persulfate 2.45 mM - final concentration). The obtained mixture was allowed to stand in the dark for 12–16 h (until the reaction was complete and the absorbance values were stable). The suspension was then diluted by mixing 1 ml $ABTS^{\bullet+}$ with PBS to obtain a final working solution with absorbance 0.70 ± 0.01 units at 734 nm. The photometric assay was conducted by measuring the reduction in absorbance of 2 ml of $ABTS^{\bullet+}$ after adding the derivatives at 734 nm exactly 60 min after the mixing.

DPPH assay – the experimental procedure was performed as described by Groupy et al. [8]. A purple colored working solution of the DPPH radical was prepared in ethanol with absorbance of 1 at 517 nm. Two milliliters of the working solution of the radical were allowed to react with different concentrations of the tested compounds for 1 hour in the dark at room temperature. After the incubation the decrease in absorbance at 517 nm was registered and the radical scavenging activity was calculated.

Protection effect of the studied compounds during ferrous iron induced peroxidation in a lipid containing model system – thiobarbituric acid reactive species assay (TBARS) was used to assess lipid peroxidation. The experiments were performed according to Asakawa and Matsushita [9]. The peroxidation was initiated in a system containing lecithin as an oxidisable substrate - 1 mg.ml^{-1} using $FeCl_2$ with final concentration of 0.1 mmol.l^{-1}. Two groups of samples were prepared: controls and benzimidazole-2-thione derivative containing ones. All the samples were incubated in a water bath at 37°C for 30 min. Then under acidic conditions and at high temperatures (addition of 0.5 ml of 2.8% trichloroacetic and 0.5 ml of thiobarbituric acid and incubation at 100°C water bath for 20 min) the generation of a pink colored product resulting from the reaction between TBA and MDA was observed. All samples were centrifuged at 3000 rpm for 20 min and the absorbance of the probes was determined at 532 nm. The degree of damage of the lipid molecules

was presented as a percentage from the controls where the maximal molecular damage was observed.

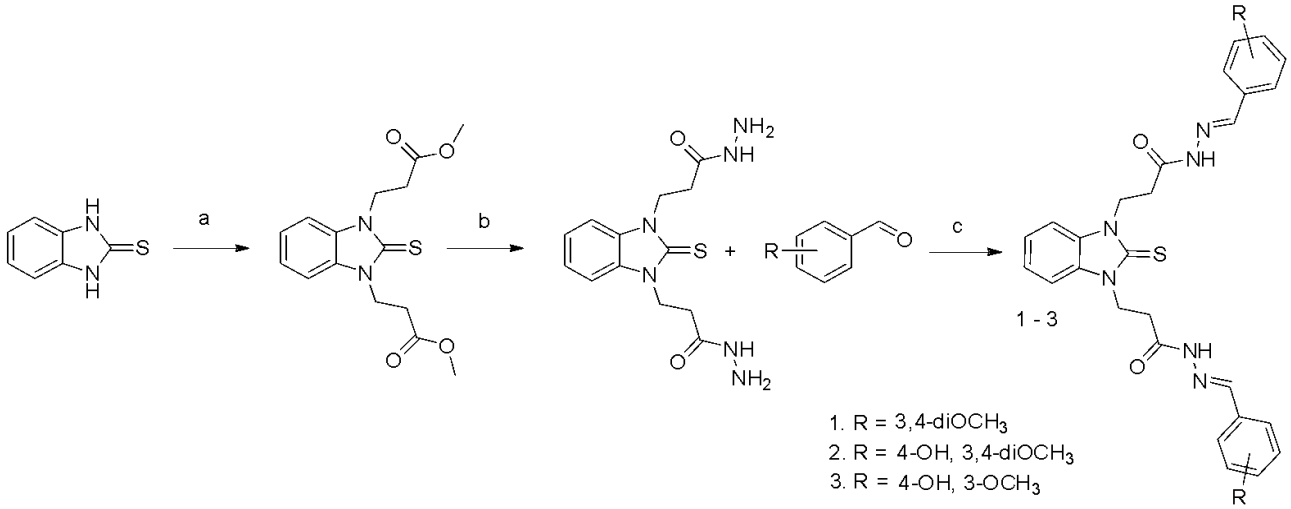
Computational details

Geometry optimizations and frequency calculations were carried out using the B3LYP functional and 6-31+G(d) basis set included in the Gaussian 09 suite program [10-12]. Geometry optimization was performed by an analytical gradient technique without symmetry restrictions and the stationary points found on the potential energy hypersurface were confirmed as minima by the absence of imaginary frequencies. Dissociation enthalpies (BDE), ionization potentials (IP) and proton affinities (PA) were calculated at 298 K based on the equations provided in literature [13]. The corresponding reaction enthalpies in water were obtained with the Integral Equation Formalism Polarizable Continuum Model (IEF-PCM) [14] on the same level of theory. Enthalpies of hydrated electron and proton were applied in accordance with the values reported by Klein et al. [13].

RESULTS

Chemistry

The initial ester and hydrazide precursors were synthesized as previously reported [15]. The new hydrazone derivatives **1-3** were synthesized by refluxing the hydrazide compound in absolute ethanol with the veratraldehyde, syringaldehyde or vanillin (Scheme 1).



Scheme 1. Reagents and conditions: a) methyl acrylate, DMF, refluxing; b) hydrazine hydrate, ethanol solution, refluxing; c) veratraldehyde, syringaledhyde or vanillin, ethanol, refluxing

The molecular geometry of the studied compounds is determined mainly by the orientation of the flexible hydrazone chains linking the flat benzimidazole-2-thione and aryl rings, and the possibility for rotation around single bonds within it. As it is shown in Figure 1, it could lead to several possible conformations. It is known from previous X-ray studies on N,N'-disubstituted benzimidazole-2-thione esters that C-H…S and C-H…O interactions stabilize the structure in the crystal state [15]. It was also found for the studied compounds in isolated state by the DFT optimization (conformer **2_C1**, Figure 1). The lack of such interactions in one of the hydrazone chains (conformer **2_C2**) or in both of them (conformer **2_C3**) results in a higher total energy of the respective conformers. Another factor enhancing the molecular structure stability is the formation of an intramolecular hydrogen bond between the H-atom from the azomethine and the carbonyl group due to rotation around the N-N bond (conformer **2_C1** has lower energy compared to conformer **2_C4**). On the other hand, the energy differences between all presented conformers are small and they might be coexisting in solution. In accordance with this, multiple sets of resonances were registered in the ^{1}H NMR spectra of the compounds.

In vitro assessment of the antioxidant activity

For the estimation of the anti-radical potential ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging methods were chosen. They are *in vitro* methods based on the reduction of stable radicals by antioxidants, which is related to a change in the color of the sample solution.

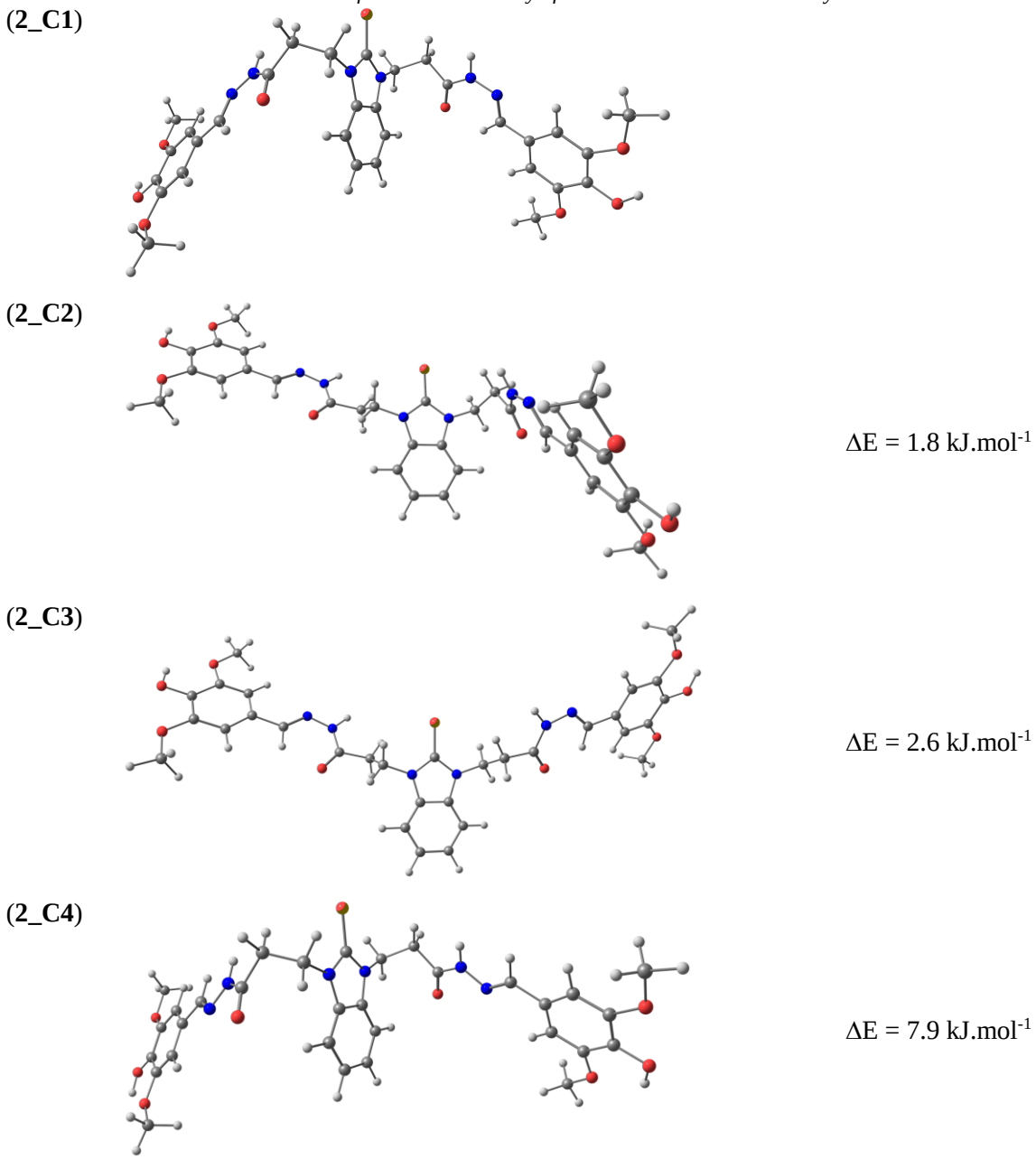


Figure 1. Conformers resulting from different orientations of the hydrazone chains and rotation around the single N-N bond, presented by compound **2;** $\Delta E = E_n - E_1$.

The extent of the observed color alterations (decolorization) is evaluated using spectrophotometric methods and the obtained decrease in the absorbance is used as a measure of the anti-radical properties of the tested substances. Despite the fact, that both radicals are not biologically relevant, since they are chemically dissimilar to any of the radicals responsible for the autoxidation processes in the real living systems, these methods are commonly used both for the preliminary screening of a broad spectrum of tested samples, including newly synthesized compounds, biological probes, extracts etc. The reason for this is the fact that they are commercially available assays that are easy to perform, relatively cheap and reproducible. Both radicals exhibit different mechanisms of action in regard to the potential scavenging agents, which gives possibilities to use both systems for the determination of different aspects of their potential radical trapping power.

The capability of the tested benzimidazole-2-thione derivatives to decrease the concentration of stable free radicals in ABTS and DPPH assays was evaluated. In all the samples containing the tested compounds we observed a decrease of the absorbance value in the presence of the ABTS radical compared to the control ones. For the **3** and **2** containing compounds the experiments were performed in the concentration range from 0 to 9 μmol/L, due to the fact that at higher concentration

full decolorization of the sample solution was observed. In the presence of **1** a decrease of the absorbance was also observed, but this effect was witnessed at higher concentrations *i.e.* the experimental concentration range was from 0 to 90 μmol.l^{-1}. In all the experiments we observed a linear concentration/RSA dependence (R^2 values varying between 0.966 and 0.989) and with the increase of the compounds concentration the RSA also increased. At the maximal tested concentration of 9 μmol.l^{-1}, the **3** and **2** had RSA respectively 80% and 98% higher than the one of the used reference Trolox. Compound **1** exhibited RSA of 20% at 90 μmol.l^{-1}. Comparison of the C-50 values of **2** and **3** ($C\text{-}50_2$ = 3.28 μmol.l^{-1}and $C\text{-}50_3$ = 5.32 μmol.l^{-1}) with the referent Trolox (C-50 Trolox = 11.97 μmol.l^{-1}) demonstrated that **2** and **3** required a lower concentration than Trolox in order to reach 50% RSA at the same experimental conditions (Figure 2).

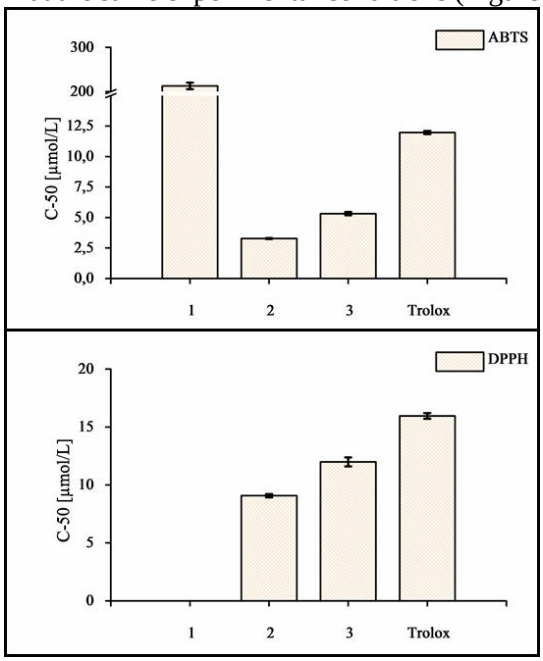


Figure 2: Comparison of the C-50 values of the tested hydrazones calculated on the basis of the concentration/RSA% relationship estimated using the stable free radical containing model systems. No data for **1** in the DPPH systems has been presented due to lack of activity.

In the DPPH model system no statistically significant decrease of the absorbance of the samples containing **1** compared to the control ones was observed at concentration 90 μmol.l^{-1} – suggesting lack of RSA of this compound. **3** and **2** again demonstrated capability to decolorize the sample solution in a concentration dependent manner. The same concentration range as in the ABTS containing systems was used but the estimated values were significantly lower compared with the ones from the first radical scavenging method. Again we witnessed lower C-50 values of the hydrazones **3** and **2** ($C\text{-}50_3$ = 11.99 μmol.l^{-1} and $C\text{-}50_2$= 9.07 μmol.l^{-1}) compared to the reference ($C\text{-}50_{Trolox}$ = 15.93 μmol.l^{-1}).

The capability of the studied compounds to decrease the TBARS generation in lecithin containing model systems has been estimated. The evaluation of the potency of newly designed compounds to influence peroxidation processes in lipid containing systems is important in view of estimation of their potential to decrease or initiate and accelerate the oxidative molecular changes of the components of the biological membranes. This is due to the fact that it subsequently could lead to alterations in the membrane transport, structural changes in membrane potential and generation of toxic products.

The performed experiments denoted decrease of the absorbance values measured at 532 nm of all samples containing hydrazones compared to the control ones. This suggests diminishment of the molecular damage presented as % from the control and decrease the extent of the peroxidation process of the lecithin molecules in the presence of the benzimidazole-2-thione derivatives (Figure 3).

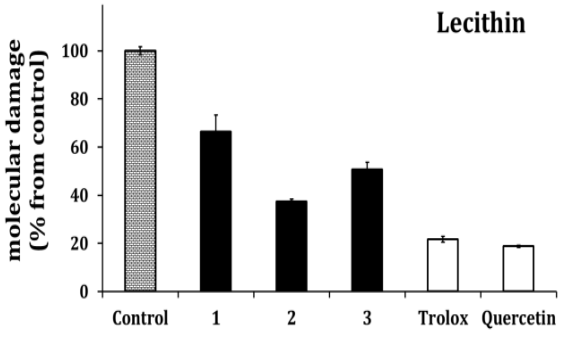


Figure 3. Effect of the hydrazone derivatives and the referent Trolox and Quercetin on *in vitro* Fe (II) induced oxidative damage in the lecithin system at 90 μmol.l^{-1} concentration.

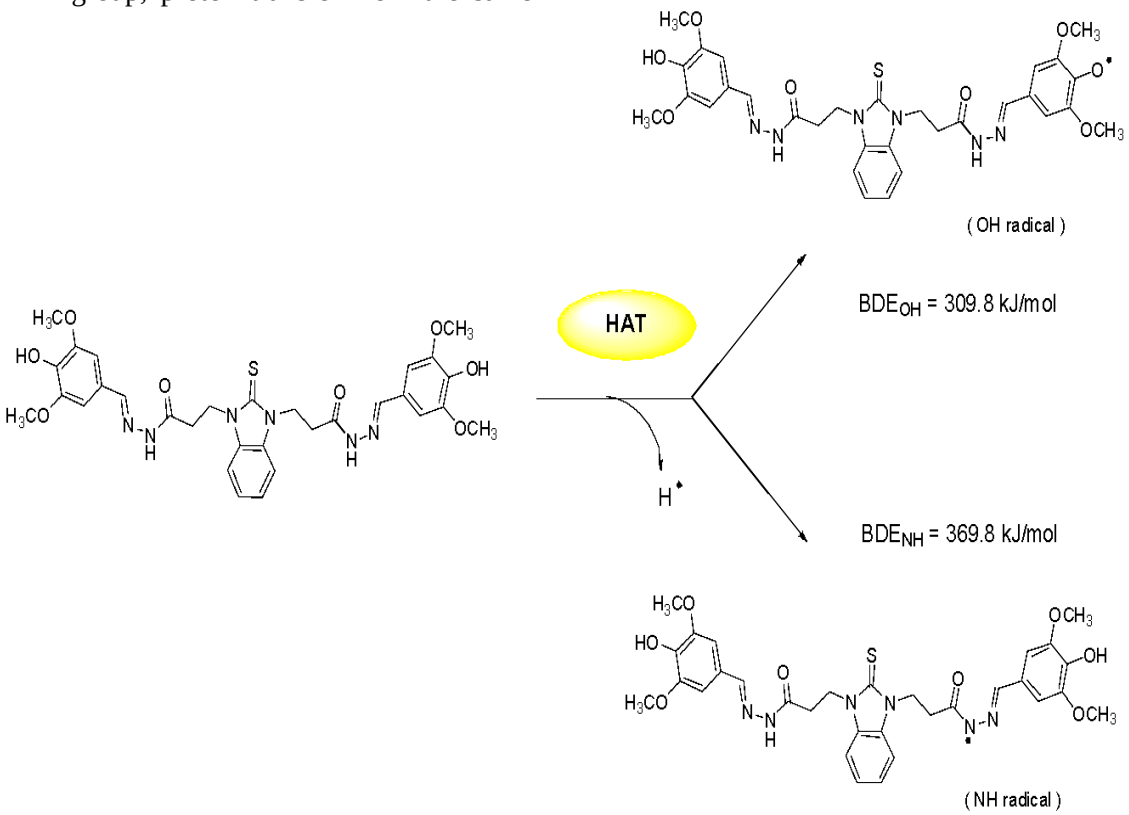
The observed decrease in absorbance was different and depended on the type of the structural changes in the tested molecules. For all of them the effect was in the same concentration range as the one in Trolox and Quercethin. Again, as in the stable free radical containing systems, the potency of the benzimidazole-2-thione derivatives decreased in the following order: **2** > **3** > **1**. Despite the fact that the most potent of the tested hydrazones - **2** decreased the calculated percentage of molecular damage with nearly 60 % compared to the controls, none of the tested compounds was able to reduce the molecular damage in greater extent than the referent compounds. In the samples containing Quercetin and

Trolox this parameter stayed twice lower, around 20%.

Computational study on possible radical scavenging mechanisms

In order to complement the obtained experimental data and suggest the most probable mechanism of antioxidant action, we have carried out a DFT study on the reaction enthalpies of compound **2** which have shown the most potent antioxidant effects among the studied hydrazones. The antioxidant activity of **2** can take place through several mechanisms: hydrogen atom transfer to the free radicals by cleavage of the hydroxyl O-H or amide N-H group, proton transfer from the same bonds or alternatively single electron transfer from the aromatic system. Moreover, the mechanism could change depending on the medium polarity. Therefore, we have estimated the bond dissociation enthalpy, ionization potential and proton affinity of **2** in gas phase and in water.

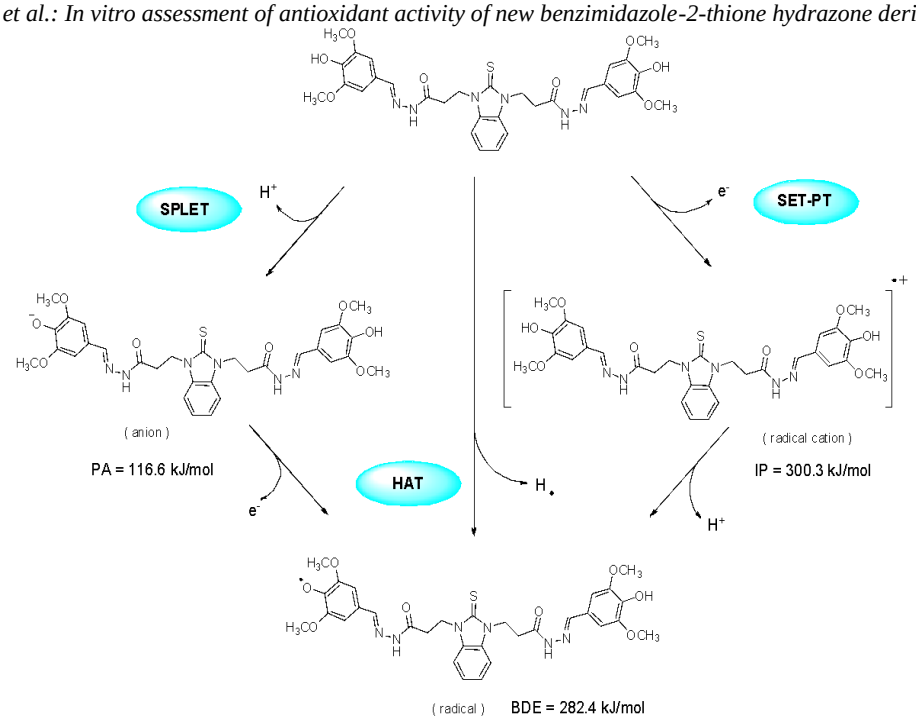
In non-polar medium H-transfer from the hydroxyl group is connected with a BDE value of 309 kJ.mol^{-1} (Scheme 2). The BDE value for cleavage of the N-H group is 369 kJ.mol^{-1}, which is close to that for the earlier studied hydrazone containing o-methoxy group in the phenyl ring [6].



Scheme 2. Hydrogen atom abstraction from the hydroxyl and amide group of **2** in nonpolar medium

The BDE values for the formation of (Z)-4-hydroxyhex-2-enyl and (Z)-4-hydroperoxyhex-2-enyl radical as model lipid radicals were previously computed at the same level of theory [6] and were found to be 406 and 321 kJ.mol^{-1}, respectively. The predicted BDE values for **2** are lower than both values, therefore it can be concluded that the hydroxyl-containing hydrazones could trap not only the OH$^{\cdot}$ / OR$^{\cdot}$, but also OOH$^{\cdot}$ / OOR$^{\cdot}$ radicals. It is in good agreement with the improved antioxidant potency of compounds **2** and **3** in comparison to the earlier studied methoxy and fluoro-containing hydrazones [6]. The methoxy-substituted derivative **1** where only the N-H bonds could be cleaved is expected to be effective only against the OH$^{\cdot}$ / OR$^{\cdot}$ radicals.

In water the BDE$_{O-H}$ value of **2** is lowered by almost 20 kJ.mol^{-1} (Scheme 3), but nevertheless it is twice larger than its proton affinity PA. Deprotonation becomes much more favorable than direct hydrogen atom transfer since the formed ionic species are stabilized by the water. Therefore, the SPLET mechanism is expected to be the main mechanism of free radical scavenging in water and in polar organic solvents.



Scheme 3. Preferred mechanism of antioxidant action of **2** in water.

CONCLUSIONS

New series of N,N'-disubstituted benzimidazoles containing methoxy and hydroxyl substituents were synthesized as promising radical scavengers for neuroprotective application. The structural modification led to an improved *in vitro* antioxidant activity. All of the compounds showed ABTS scavenging activity, while in the DPPH assay only the hydroxyl compounds were effective. All the tested compounds decreased the ferrous iron induced molecular damage in the same concentration range Trolox and Quercetin. From the computed reaction enthalpies for HAT, SET and SPLET mechanisms it can be concluded that in nonpolar medium the hydroxyl-containing hydrazone derivatives might efficiently donate hydrogen atoms from the hydroxyl groups via the HAT mechanism, whereas in polar medium they can deactivate the free radicals through proton transfer, i.e. the SPLET mechanism.

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