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Free radical scavenging activity of novel thiazolidine-2,4-dione derivatives†

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ABSTRACT: Free radical activity towards superoxide anion radical ($O_2^{\bullet}$), hydroxyl radical (*HO*·) and 2,2-diphenyl-1-picrylhydrazyl (*DPPH*·) of a series of novel thiazolidine-2,4-dione derivatives (TSs) was examined using chemiluminescence, electron paramagnetic resonance (EPR) and EPR spin trapping techniques. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was applied as the spin trap. Superoxide radical was produced in the potassium superoxide/18-crown-6 ether dissolved in dimethyl sulfoxide. Hydroxyl radical was generated in the Fenton reaction (Fe(II) + H_2O_2. It was found that TSs showed a slight scavenging effect (15–38% reduction at 2.5 mmol/L concentration) of the DPPH radical and a high scavenging effect of $O_2^{\bullet}$ (41–88%). The tested compounds showed inhibition of *HO*· -dependent DMPO-OH spin adduct formation (the amplitude of EPR signal decrease ranged from 20 to 76% at 2.5 mmol/L concentration. Our findings present new group compounds of relatively high reactivity towards free radicals. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: 1,1-diphenyl-2-picrylhydoxyl radical; chemiluminescence; EPR; hydroxyl radical; spin trapping; superoxide radical; thiazolidine 2,4-dione derivatives

Introduction

A considerable amount of evidence indicates that reactive oxygen species (ROS) such as hydroxyl radical (*HO*·) and superoxide anion radical ($O_2^{\bullet}$) may play a dual function depending on their concentration. ROS are produced in cells under normal physiological conditions during redox reactions and have useful physiological functions, e.g. as in cellular signaling systems (1,2), but generated in excess participate in several physiopathologies. They can damage biomolecules and are involved in the etiology of several human chronic diseases and aging (3,4). Growing experimental data suggest that cancer cells are under increased oxidative stress and contain increased concentrations of ROS compared to normal cells (5). In addition, studies provide evidence that during inflammatory processes, endogenous overgeneration of ROS occurs, and that tumorigenesis is associated with inflammation (6).

The investigation of biological and antioxidant activities of natural substances and natural derivatives having different substituents or even synthetic analogues capable of quenching toxic free radicals, generated under oxidative stress conditions, are the topic of much research (7–10).

Based on our promising studies on thiazolyl thiazolidinedione derivatives, concerning their antioxidant capacities, we have undertaken evaluation of the scavenging property of some novel thiazolidine-2,4-dione derivatives (TSs) (Fig. 1) for $O_2^{\bullet}$, *HO*· and 2,2-diphenyl-1-picrylhydrazyl radical (*DPPH*·).

Materials and methods

The compounds tested were synthesized by the Knoevenagel reaction of 3-substituted benzyl-2,4-thiazolidinediones and phenacyl-2,4-thiazolidinediones with 4-chloro-2-(5-methyl[1,2,4]triazol-3-ylsulfanyl)-thiazole-5-carbaldehyde using an acetic acid/sodium acetate mixture (11). All reagents for synthesis were purchased from E. Merck (Darmstadt, Germany) and Aldrich (Milwaukee, MI, USA).

4,5-Dihydroxy-1,3-benzene-disulfonic acid (Tiron), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), 6-hydroxy-2,5,7,8-tetramethyl-2 carboxylic acid (Trolox), 18-crown-6 (1,4,7,10,13,16) hexaoxacy clooctadecane and ascorbic acid were from Merck (Darmstadt, Germany); potassium superoxide (KO_2) and ammonium ferrous sulfate hexahydrate were from Fluka (Buchs, Switzerland); and p-nitroblue-tetrazolium chloride was from Sigma (St Louis, MO, USA). Anhydrous dimethyl sulfoxide (DMSO) was purchased from Aldrich (Milwaukee, WI, USA).

Superoxide radical was prepared according to the procedure given by Valentine *et al*. (12). A 60-mg aliquot of 18-crown-6

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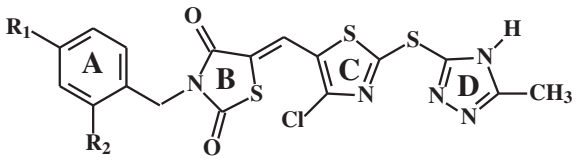
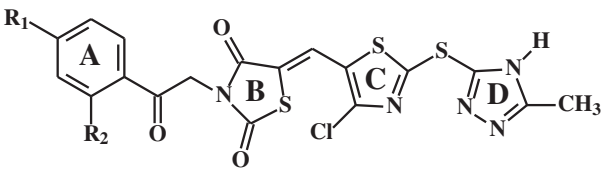


Compound 1a - 1f	Substituents	
	R_1	R_2
a	H	H
b	F	H
c	Cl	H
d	Br	H
e	Cl	Cl
f	NO_2	H



Compound 2a - 2f	Substituents	
	R_1	R_2
a	H	H
b	F	H
c	Cl	H
d	Br	H
e	Cl	Cl
f	NO_2	H

Figure 1. Chemical structure of thiazolidine-2,4-dione derivatives.

was dissolved in 10 mL of dry DMSO and then KO_2 (7 mg) was quickly introduced into the flask using a syringe to avoid contact with air humidity. The mixture was stirred for 1 h to give a pale yellow solution of 10 mmol/L $O_2^{\bullet-}$, which was stable at room temperature for at least 1 h. The $O_2^{\bullet}$ concentration was determined using ultraviolet spectra ($\lambda_{max} = 251$ nm, $\varepsilon = 2686 \pm 29$ mol/cm).

Chemiluminescence (CL) intensity was recorded using an EMI9553Q photomultiplier with a S20 cathode sensitive in the range 200–800 nm, connected to a Zeiss K-200 recorder. Reagents were introduced to a thermostated glass cuvette placed in a light tight chamber using polyethylene pipes with aid of semiautomatic syringes. A personal computer equipped with a home-made software program was used to monitor and analyze the output signal. The effects of inhibitors and scavengers were expressed as the percentage inhibition of original measurements of the CL intensity:

$$R = [(I_o - I)/I_o] \times 100\%$$

where I_o and I represent the CL intensities measured in the absence of an inhibitor (TS) (the control reaction) and I is that in the presence of a tested compound.

The hydroxyl radical scavenging activity of TDs was measured using the Fenton reaction in a sodium trifluoroacetate solution and ferrous ions as a catalyst (13,14)

$$Fe(II) + H_2O_2 \rightarrow HO^{\bullet} + Fe(II) + HO^{-} \quad (1)$$

and DMPO as a spin trap. The final concentrations of reagents were: 10 mmol/L sodium trifluoroacetate, 0.625 mmol/L ammonium ferrous sulfate, 0.5 mmol/L H_2O_2, sodium trifluoroacetate buffer (0.02 mol/L, pH 6.15) and 25 mmol/L DMPO dissolved in water. Hydroxyl radical reacts with the DMPO trap giving a stable, easy measurable with electron paramagnetic resonance (EPR) the $DMPO - OH^{\bullet}$ spin adduct (15):

$$HO^{\bullet} + DMPO \rightarrow DMPO - \dot{O}H \quad (2)$$

The reaction mixture was placed in the EPR cavity using a quartz flat cell within an optical path length of 0.25 mm. The EPR signal was analyzed approximately 1 min after the introduction of ammonium ferrous sulfate.

The testing of the dependence of the relative height of the second line in the four-line EPR spectrum of the $DMPO - OH^{\bullet}$ spin adduct, formed in the absence (H_o) and the presence of the $HO^{\cdot}$ scavenger (H), allows calculation of the EPR ratios (Q):

$$Q(\%) = [(H_o - H)/H_o] \times 100\%$$

To evaluate the antioxidative property (i.e. the interaction of a potential antioxidant; a TS) we applied the DPPH radical reduction from a method given by Nanjo *et al.* (16). The DMSO solution of each tested compound or DMSO itself as a control was added to $DPPH^{\cdot}$ dissolved in ethanol. After mixing for 10 s the solution was introduced into a flat quartz cell with an optical path length of 0.25 mm, and the EPR signal was detected as a function of time. The reduction of $DPPH^{\cdot}$ by a tested compound was followed by the decrease in its EPR signal. Scavenging activity of TSs was calculated after 5 min according to the following equation:

$$R = [(H_o - H)/H_o] \times 100\%$$

where H_o is the relative height of the third peak in the EPR spectrum in the absence of an inhibitor (considered as a control reaction) and H- is the relative peak hight that is measured in the presence of an inhibitor. EPR spectra were recorded at room temperature with an X-band standard spectrometer operating at 9.3 GH, using a modulation frequency of 100 kHz.

Results and discussion

Within the framework of this study, the primary characterization of the free radical scavenging activity of 12 novel synthesized compounds was performed employing the DPPH radical. The radical has a proton, for which an EPR spectrum is shown in Fig. 2. The spectrum exhibits five splitting lines and decreases significantly on exposure to proton radical scavengers. The capacity of antioxidants to scavenge the DPPH radical has been exemplified by their action as hydrogen atom donors (17). The method was successfully applied for the primary characterization of free radical scavenging and antioxidant activities of several natural and synthetic substances (18–20). All tested TSs

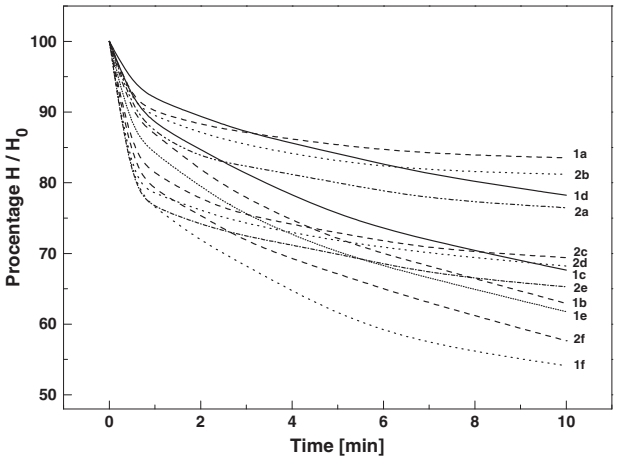


Figure 2. EPR time course of the reduction of the DPPH free radical by 2.5 mmol/L TSs (curves 1–12) in DMSO (25% v/v)/C2H5OH (75% v/v). Inset graph shows the EPR spectrum of the DPPH radical detected in the absence of a scavenger. Denotation of the TSs are given in Fig. 1. The remaining conditions are reported under the Materials and Methods section. [Correction made here after initial online publication.]

were able to scavenge the DPPH radical. The reactions between *DPPH·* and TSs were biphasic, similar to the reference antioxidants the vitamin E analog Trolox and ascorbic acid (data not shown). The kinetic curves exhibit two features, first the rapid decrease lasting about 1 min, and the second slower with hyperbolic curves, reaching a steady state in $t > 10$ min. Figure 3 summarises the *DPPH·* scavenging ratio of the examined TSs, ascorbic acid and Trolox.

Generally, the compounds presented only a slight effect in the range 15–39% at the maximum tested concentration (2.5 mmol/L). Our observations demonstrate that the TSs studied show a *DPPH·* reduction much lower than ascorbic acid and Trolox at lower concentration (50%). However, the detected positive *DPPH·* test suggests that all examined compounds can react with free radicals. To confirm the free radical scavenging ability of TSs compounds we examined their activity towards $O_2^{\bullet-}$ and HO radicals. Superoxide anion radical produced from KO_2 in $(CH_3)_2SO$ in the presence of 18-crown-6 ether as a solubilizing agent (21) exhibits a strong light emission. The kinetic curve of the CL shows an initial rapid approximately first-order decay followed by a very small decrease lasting at least 3 min (Fig. 4). This reaction has become very useful for detecting antioxidative effects of biologically important substances and selection of natural compounds inhibiting the $O_2^{\bullet-}$ radical (9,10,22,23). Reactions responsible for generation of the CL emitter (pairs of singlet oxygen) from the $O_2^{\bullet-}$/DMSO system have been reported in previous papers (21,24–26), and the main reaction responsible for the $^1O_2$ production is removal of a proton from DMSO (21).

$$2O_2^{\bullet-} + 2(CH_3)_2SO \rightarrow {}^1O_2 + (CH_3)_2SO_2 + CH_3SOCH_2 + OH^- \quad (4)$$

All examined TSs (dissolved in DMSO) added to the $O_2^{\bullet-}$/ DMSO system exerted a short-lasting '"flash' followed by a decrease in light emission. Figure 4 (curves 3 and 4) shows the representative kinetic curves for compounds **1f** and **2f**, respectively. Curve 2 presents an effect of DMSO at the same concentration as in the TS compound/DMSO sample; this reaction was considered as a control. The examined compounds showed a high quenching ratio ranging from 49% to 88% at a concentration of 1 mmol/L (Fig. 5). Only one compound, **1e** showed a scavenging effect lower than that of an inhibitor specific to $O_2^{\bullet-}$, such as p-nitrobluetetrazolium chloride at the same concentration.

The results from the $O_2^{\bullet-}$ scavenging assay show, once again, the important property of TSs, i.e. the antioxidant ability. For $O_2^{\bullet-}$ activity, three types of reactions are proposed: action as an oxidant; reducing agent; and powerful nucleophile in aprotic solvents (27,28). The strongly decreased CL from the $O_2^{\bullet-}$/DMSO system in the presence of TSs shows that the compounds were able to scavenge $O_2^{\bullet-}$ species directly by hydrogen transfer:

$$O_2^{\bullet-} + H^{\bullet} \rightarrow HOO^- \quad (5)$$

$$HOO^- + (CH_3)_2SO \rightarrow (CH_3)_2SO_2 + OH^- \quad (6)$$

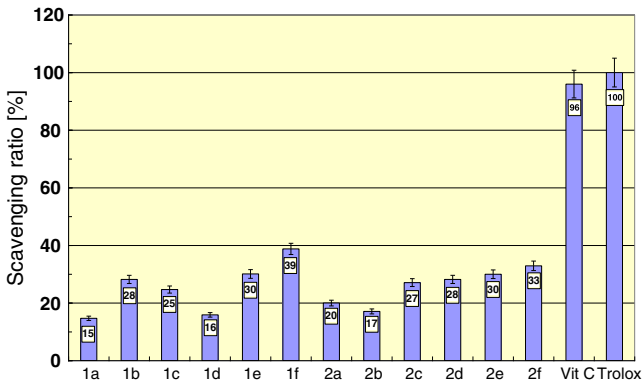


Figure 3. Scavenging effects of thiazolidine-2,4-dione derivatives (2.5 mmol/L), ascorbic acid and Trolox (1.25 mmol/L) on DPPH radical. Analysis was performed 5 min after addition of scavenging compound. The electron paramagnetic resonance settings were microwave power 20 mW; modulation amplitude 0.2 G; time constant 0.2 s; receiver gain 3.2×10^4. Temperature 295 K. Reagent concentrations are the same as in Fig. 2.

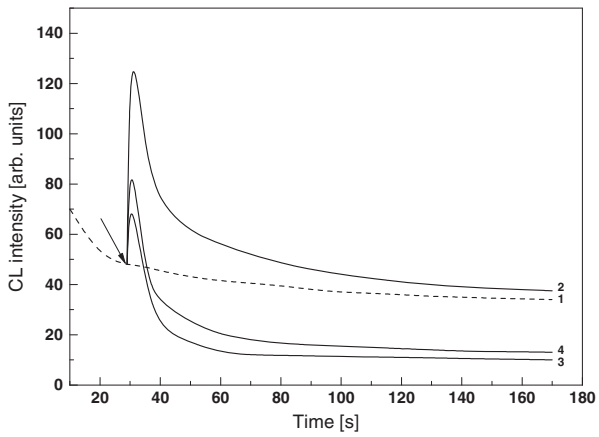


Figure 4. The effect of the TSs (2.5 mmol/L) on the chemiluminescence intensity accompanying 1 mmol/L $O_2^{\bullet-}$ generated in DMSO. Curve 1 — the chemiluminescence kinetic from $O_2^{\bullet-}$ alone; curve 2, CL recorded under the same condition as curve 1 but after an addition of 0.5 mL DMSO (control); curve 3 and 4 are the same as the control but in the presence of compounds **1f**, **2f** dissolved in 0.5 mL DMSO (1.76 mol/L), respectively. An arrow indicates the moment of the compound or DMSO addition. Temperature 296 K. [Correction made here after initial online publication.]

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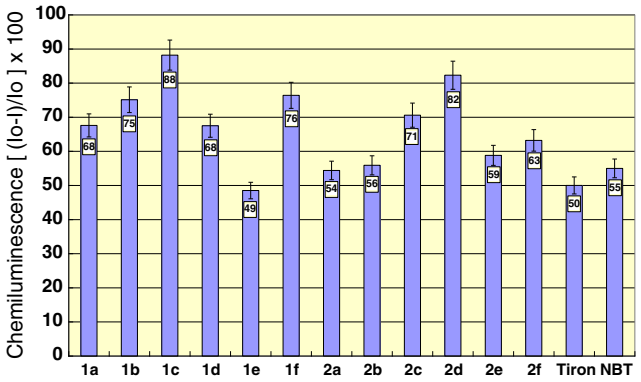

Figure 5. Scavenging effect of thiazolidine-2,4-dione derivatives, Tiron and p-nitroblue-tetrazolium chloride (NBT; 1 mmol/L) on the chemiluminescence from 1 mmol/L $O_2^{\bullet}$ radicals generated in dimethyl sulfoxide. The remaining conditions are the same as in Fig. 4.

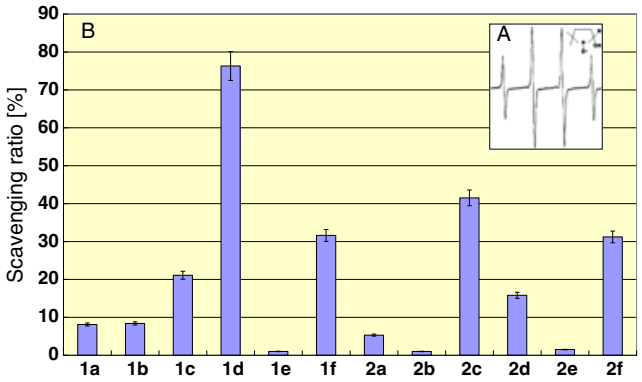

Figure 6. (A) The electron paramagnetic resonance spectrum of the DMPO–OH spin adduct arising from the Fenton reaction. (B) The inhibitory effect of thiazolidine-2,4-dione derivatives compounds (2.5 mmol/L) on the DMPO–OH radical formation. The electron paramagnetic resonance settings were microwave power 20 mW; modulation amplitude 0.5 G; time constant 0.3 s; receiver gain 4×10^4. Temperature 295 K.

The protective effect of TSs against $O_2^{\bullet}$, although observed in a non-cellular system, is interesting from the antioxidant point of view in cellular systems. The overproduction of ROS in a cell is the main reason for changes caused by oxidative stress. Superoxide radical is the first product of phagocytosis occurring during inflammation as a result of respiratory burst (i.e. strongly increased consumption of oxygen) (29,30).

Although $O_2^{\bullet}$ is a short-lived species undergoing conversion to a rather unreactive molecule hydrogen peroxide (H_2O_2) (31,32):

$$2O_2^{\bullet} + 2H^+ \rightarrow H_2O_2 + {}^1O_2 \qquad (7)$$

the species is considered as a precursor of the strongest reactive oxidizing agent HO radical, generated in a cell through the Haber–Weiss/Fenton type reactions (equation 1).

Equation 7 also generates the excited states of molecular oxygen, i.e. singlet oxygen ($^1O_2$), which is a strong oxidant in a cell. To confirm further the free radical scavenging activity of the TSs tested, we examined their reactivity towards $HO^{\cdot}$, using the EPR spectroscopy and spin-trapping technique with DMPO as a trap. Hydroxyl radical reacts with DMPO at a very high rate constant ($k = 4.3 \times 10^9$ mol/s), and arising spin adduct $DMPO-OH^{\bullet}$ exhibits a four-line spectrum with 1:2:2:1 signal intensity and nitrogen (a_N) and β proton (a_H) hyperfine coupling constants $a_N = a_H = 14.9$ G [the spectrum was presented in a previous paper (11)].

To verify whether $HO^{\cdot}$ has been trapped by the DMPO spin trap we repeated the experiments in the presence of ethanol, which is considered as a strong inhibitor of $HO^{\cdot}$. Consistent with findings of Finkelstein *et al.* (15) we observed quenching of the $DMPO-OH^{\bullet}$ spin adduct spectrum by approximately 30% in the presence of ethanol (10% v/v) and the appearance of a new six-line spectrum with $a_N = 15.8$ G and $a_H = 2.8$ G (data not shown). The spectrum is due to spin trapping of the α-hydroxyethyl radical (33):

$$C_2H_5OH + HO^{\bullet} \rightarrow C_2H_5\dot{C}HOH \qquad (8)$$

The $C_2H_5\dot{C}HOH$ radical as a strong antioxidant (34) can oxidize Fe(II) to Fe(III) inhibiting the $HO^{\cdot}$ generation in the Fenton reaction, thereby inhibits the EPR signal from the $DMPO-OH^{\bullet}$ spin adduct.

As shown in Fig. 6 from group 1 only compounds **1c**, **1d** and **1f** were effective in scavenging $HO^{\cdot}$ ranging from 20% to 76%. In contrast, compounds **1a**, **1b** and **1e** were practically without effect. From group 2 only three compounds of six (i.e. **2c**, **2d**, **2f**) showed the scavenging activity ranging from 16% to 41%. This finding implies that compounds from group 2 were less efficient in the $HO^{\cdot}$ scavenging than from group 1.

The benzene ring, present in both groups of TSs plays an essential role in the $HO^{\cdot}$ scavenging process. Findings for compounds from group **1** effective in scavenging $HO^{\cdot}$ above 20% indicated that 4-Cl, 4-Br and 4-NO_2 substitution in the benzene ring plays an important role in radical scavenging activity. Compounds from group **2**, which differ from those in group **1** by the presence of a carbonyl group and having 4-Cl, 4-Br or 4-NO_2 substitution on the benzene ring were able to scavenge HO radicals. This fact points to a likely contribution of the Cl, Br and NO_2 substituents to the molecular stabilization, increasing TSs reactivity towards $HO^{\cdot}$. The findings also suggest that presence of the second Cl atom on the benzene ring suppresses reactivity of the tested compound with this radical. The TS compounds from both groups with no substituents in the benzene ring showed no activity.

In conclusion, the scavenging properties for ROS and the DPPH radical of TSs were examined for the first time. The results showed that TSs examined were effective and efficient in scavenging $O_2^{\bullet}$, and some of them were effective as $HO^{\cdot}$ scavengers. Considering the structural similarity between the groups of compounds examined, it is very likely that the $O_2^{\bullet}$ scavenging activity depends on the hydrogen donating ability of the D ring, while the $HO^{\cdot}$ scavenging activity depends on the type of substituents on the benzene ring.

The examined compounds, therefore, are promising molecules to prevent or control oxidative stress.

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