Free Rad. Res., Vol. 32, pp. 525–533
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4-Mercaptoimidazoles Derived from the Naturally Occurring Antioxidant Ovothiols 2. Computational and Experimental Approach of the Radical Scavenging Mechanism

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Accepted by Prof. H.Sies

(Received 5 July 1999; In revised form 2 November 1999)

The radical-scavenging mechanism of fourteen 4-mercaptoimidazoles, derived from the natural family of ovothiols, was studied via a QSAR approach, cyclic voltammetry, ESR and NMR spectroscopy. A significant correlation was found between the DPPH scavenging abilities of test compounds and thermodynamic parameters like overall ease of disulphide formation. The production of a disulphide compound via thiyl radical formation is proposed. Upon DPPH scavenging, hydrogen abstraction from thiols yields transient short-lived thiyl radicals, which were characterised by ESR and rapidly dimerise to form a disulphide compound. Cyclic voltammetry showed that the best DPPH scavengers exhibit low oxidation potentials for their oxidation to disulphides.

Keywords: 4-Mercaptoimidazoles, quantitative structure–activity relationship (QSAR), ESR spectroscopy, redox potentials, radical scavenging, thiyl radical

INTRODUCTION

We have previously reported the synthesis of several 4-mercaptoimidazoles derivatives and presented preliminary data on their radical scavenging activity.[1] Their antioxidant properties towards several oxygen-derived toxic species is presented in the preceding paper. To investigate further their radical scavenging mechanism, a QSAR study on the ability of the compounds to scavenge the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was performed. Redox potentials were measured by cyclic voltammetry. In addition ESR spectroscopy was used to characterise the thiyl radical formed upon DPPH and $HO^{\bullet}$ scavenging or under an imposed positive oxidative potential.

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MATERIALS AND METHODS

Computer Calculations

All the calculations were performed using MOPAC 6 or Spartan Plus 5.0 with the AM1 semi-empirical mechanical quantum algorithm. Standard bond lengths and angles were used as a starting point for the geometry. Compounds geometries were optimised by minimising the total energy using the key word AM1, PRECISE and GNORM = 0.01. Transition states were localised using the TS algorithm of MOPAC.

Cyclic Voltammetry

Cyclic voltammetry measurements were made with a SOLEA TACUSSEL PJT 120 potentiostat equipped with a programmable interface SOLEA TACUSSEL IMT1. Working and auxiliary electrodes were a platinum CTV 101T and a platinum wire, respectively. The standard Calomel reference electrode was separated from the bulk of the solution by KCl saturated solution with a glass frit. Scan rate of 50 mV s^{-1} gave the best results. The electrolyte support salt was tetraethylammonium perchlorate ($N^+Et_4ClO_4^-$) (0.1 N) and all solutions were made in analytical grade MeCN to obtain complete dissolution of the lipophilic test compounds. Solutions (1.0 mM) were deoxygenated with a stream of dry nitrogen. A nitrogen blanket was maintained above the solutions during the electrochemical measurements.

ESR Spin Trapping and NMR Experiments

X-band ESR spectra were obtained with a Varian E-9 operating at 100 kHz of modulation frequency at room temperature (20°C). The ESR measurements coupled to electrochemistry were performed in a TM cavity mode with 0.1 cm flat quartz cell. The anodic and cathodic platinum electrodes were connected to a SOLEA TACUSSEL PJT 120 potentiostat to monitor the imposed voltage. Calomel electrode was used as reference.

For spin trapping ESR experiments in combination with electrochemistry, a 1 mM mercaptoimidazole solution in DMSO was incubated with 32 mM DMPO. Use of DMSO as a solvent gave the best analysable DMPO adducts.

For experiments in presence of DPPH, thiol compound (15 mM), DPPH (5 mM) and DMPO (400 mM) were incubated in benzene and the spectra were recorded immediately after initiation of the reaction. Solvent was benzene to allow a strict comparison of the DMPO adducts with previously reported similar studies.

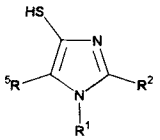
^{1}H NMR spectra were recorded on a Bruker AC300 and performed in $CDCl_3$. Chemical shifts (δ) are referenced to internal solvent and reported relative to $SiMe_4$.

RESULTS

Structure–Activity Relationships

The scavenging activity of the compounds towards the DPPH radical has been published elsewhere[1] and the log(1/IC_{50}) values are given in Table I. The proposed scavenging mechanism

TABLE I Structures and activities towards DPPH radical of the studied 4-mercaptoimidazoles



Entry	R^1	R^2	R^5	log(1/IC_{50})
1	CH_3	H	CH_3	2.538
2	3,4-diMeO-Ph	H	H	3.111
3	4-MeO-Ph	H	H	2.871
4	2-MeO-Ph	H	H	2.680
5	CH_3	4-Cl-Ph	H	3.297
6	CH_3	3-Cl-Ph	H	4.482
7	CH_3	2-Cl-Ph	H	4.721
8	CH_3	2-CF_3-Ph	H	4.409
9	CH_3	3-CF_3-Ph	H	4.721
10	CH_3	H	2-CF_3-Ph	4.638
11	CH_3	H	2-Cl-Ph	4.721
12	CH_3	H	4-Cl-Ph	4.699
13	CH_3	H	4-MeO-Ph	2.883
14	CH_3	CF_3	2-MeO-Ph	4.638

involves hydrogen abstraction from the thiol upon reaction with DPPH and formation of a transient thiyl radical species. The combination of two thiyl radicals leads to the formation of disulphide compounds (Figure 1). Disulphides can also be obtained by the formation of transient disulphide radical anions $RSSR^{\bullet -}$ resulting from the reaction of thiyl radicals with parent thiolates. These highly reducing species are easily oxidised to disulphides. This alternative route gives the same reaction enthalpy for disulphide formation. The reaction coordinate method was used to follow these two reactions.

For hydrogen abstraction, the reaction coordinate corresponds to the distance between the hydrogen and the sulphur atom, and for disulphide formation, it corresponds to the distance between the two sulphur atoms. As described in a previous study on hydrogen abstraction from phenolic compounds,[2] all calculations were performed using AM1 method at the Unrestricted Hartree Fock level.[3] Activation energy (ΔH_{a_1}), reaction enthalpy (ΔH_{r_1}) for hydrogen abstraction and reaction enthalpy (ΔH_{r_2}) for disulphide formation are collated in Table II. Hydrogen abstraction is energetically unfavoured, with a ΔH_{r_1} of about

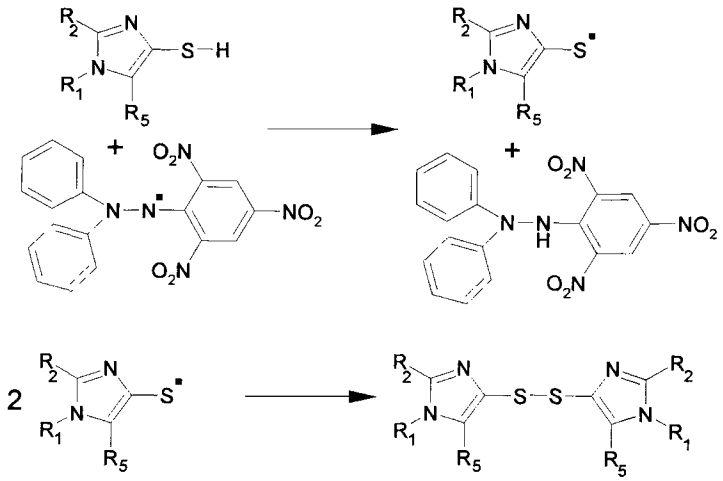


FIGURE 1 Potential DPPH scavenging mechanism by 4-mercaptoimidazoles.

TABLE II Thermodynamic parameters and spin density of the compounds

Entry	ΔH_{a_1} [a]	ΔH_{r_1} [a]	ΔH_{r_2} [a]	ds1	ds2	ds3	ds4	ds5	dsS
1	74.471	23.907	−40.789	0.0044	−0.1108	0.1455	−0.2945	0.5180	0.7356
2	76.557	24.029	−39.550	0.0379	−0.1454	0.1540	−0.3230	0.5170	0.7522
3	76.625	24.498	−40.169	0.0374	−0.1422	0.1531	−0.3202	0.5171	0.7508
4	72.488	23.255	−39.876	0.0252	−0.1236	0.1395	−0.3007	0.5049	0.7486
5	71.674	22.560	−40.657	0.0341	−0.1998	0.2312	−0.3186	0.5205	0.7660
6	74.387	24.584	−42.378	0.0328	−0.1975	0.2299	−0.3171	0.5183	0.7664
7	73.791	24.592	−42.461	0.0292	−0.1651	0.1946	−0.3028	0.5047	0.7626
8	76.284	24.875	−42.904	0.0194	−0.1567	0.1932	−0.3022	0.4975	0.7698
9	74.168	24.939	−42.523	0.0262	−0.1866	0.2241	−0.3164	0.5143	0.7705
10	76.811	24.974	−42.331	0.0219	−0.1500	0.1723	−0.3442	0.5566	0.7413
11	76.409	24.648	−41.726	0.0090	−0.1380	0.1657	−0.3350	0.5536	0.7400
12	76.210	24.097	−41.381	−0.0060	−0.1404	0.1637	−0.3424	0.5597	0.7090
13	74.748	22.642	−40.393	−0.0153	−0.1237	0.1526	−0.3250	0.5513	0.7041
14	76.844	24.738	−46.998	−0.0553	−0.1286	0.2088	−0.3084	0.4924	0.7595

[a] Energies: kcal/mol.

24 kcal/mol whereas the disulphide formation from thiyl radicals is facilitated with an average (ΔH_{r_2}) of −41 kcal/mol. It was not possible to compute the activation energy for the disulphide formation reaction because of the ghost-orbital effect.

A correlation can be found between (ΔH_{r_2}) and $\log(1/IC_{50})$:

$$\log(1/IC50) = -24.83 - 0.693\Delta H_{r_2} \quad (1)$$
$$n = 13 \quad R = 0.86 \quad F = 31.7 \ (F_{0.99} = 9.65)$$
$$Q = 0.81$$

R is the correlation coefficient, F the Fischer test and Q represents the cross-validated correlation coefficient obtained by the Leave-One-Out method. The more the disulphide formation reaction is energetically favourable, the more the reactivity of a 4-mercaptoimidazole compound towards DPPH is enhanced. The most active compound is compound **14**, which has the higher absolute ΔH_{r_2}. In fact, the IC_{50} values measured with the DPPH scavenging test cannot be lower than 25 μM, which is the limit value for all the most active compounds. To take into account the experimental limitation, Equation (1) can be modified as follows:

$$\log(1/IC_{50}) = \alpha \cdot \left(1 + (CIC_{50}/\alpha)^2\right)^{1/2} \quad \text{or}$$
$$\log(1/IC_{50}) = \alpha \cdot \ln\left(1 + \exp(CIC_{50}/\alpha)\right)$$

where CIC_{50} is a IC_{50} value calculated using an equation similar to Equation (1), and α is the limit value for the IC_{50} values. The equation is applicable to all 14 compounds. Optimisations of the different parameters yield a value of 19 μM for α with the logarithmic function and 26 μM with the square root function. This is in good agreement with the experimental data. A graphical representation of these equations is shown in Figure 2.

No satisfactory correlation could be found with ΔH_{a_1} or ΔH_{r_1}. The disulphide formation from the thiyl radicals seems to be the limiting step of the DPPH scavenging by 4-mercaptoimidazoles. Spin

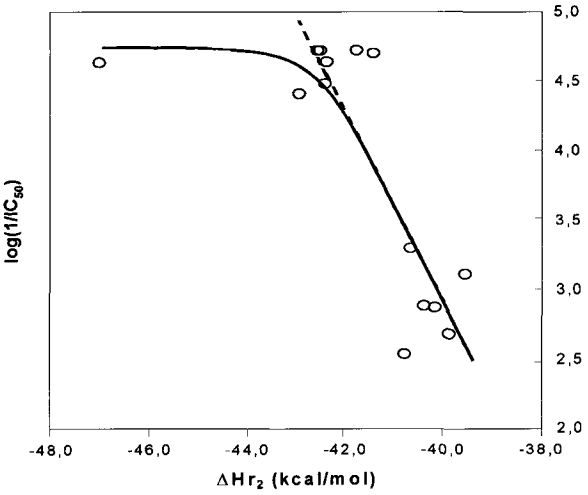


FIGURE 2 Plots of DPPH activity versus ΔH_{r_2}. Experimental data points (○), data calculated from Equation (1) (dashed line) and data calculated with experimental limit (solid line).

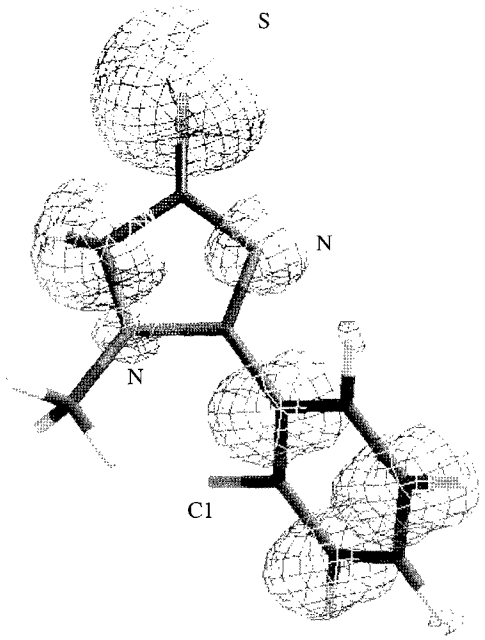


FIGURE 3 Spin density localisation for the thiyl form of compound 7 obtained with Spartan Plus 5.0. (See Color plate II at the end of this issue.)

density properties of the thiyl radicals were calculated for each atom. The values are reported in Table II and graphically displayed in Figure 3. The unpaired electron of the thiyl radical is

delocalised over the whole molecule. Nevertheless the highest density values were found on the sulphur atom and on carbons 4 and 5 of the imidazole ring. Holler and Hopkins proposed that the unusual stability of these thiyl radicals may result from delocalisation of the electron deficiency over the adjoining mercaptoimidazole nucleus, essentially on the N-1 nitrogen atom.[4] Our computational method supports this hypothesis only partially. We found that the delocalisation of the thiyl radical reaches the C-5 atom, but not the adjacent N-1 atom.

Moreover a strong correlation ($R = 0.85$) was found between the spin density on carbon 4 (ds4) of the imidazole ring with the spin density on carbon 5 (ds5). For this reason we used the spin density on the sulphur atom (dsS) and on carbon 5 to investigate structure activity relationships. Because a relationship between $\log(1/IC_{50})$ and spin density may not be linear, we also incorporated the square parameters $ds5^2$ and dsS^2 as well as the cross parameter $ds5 \cdot dsS$ was added. A systematic analysis of all possible correlations was carried out, and the three best equations were:

$$\log(1/IC_{50}) = -283.90 + 692.2\,dsS + 887.5\,ds5^2 - 1210\,ds5 \cdot dsS$$
$$n = 14 \quad R = 0.88 \quad F = 12.1\ (F_{0.99} = 6.55) \quad Q = 0.77$$

$$\log(1/IC_{50}) = 415.0 + 30.83\,dsS^2 - 1658.6\,ds5 + 1602.5\,ds5^2$$
$$n = 14 \quad R = 0.86 \quad F = 9.34\ (F_{0.99} = 6.55) \quad Q = 0.74$$

$$\log(1/IC_{50}) = 398.5 + 45.20\,dsS - 1658.8\,ds5 + 1602.4\,ds5^2$$
$$n = 14 \quad R = 0.85 \quad F = 9.04\ (F_{0.99} = 6.55) \quad Q = 0.74$$

To use all the information of the above correlations without overfitting, the three equations were averaged. Figure 4 displays the variation

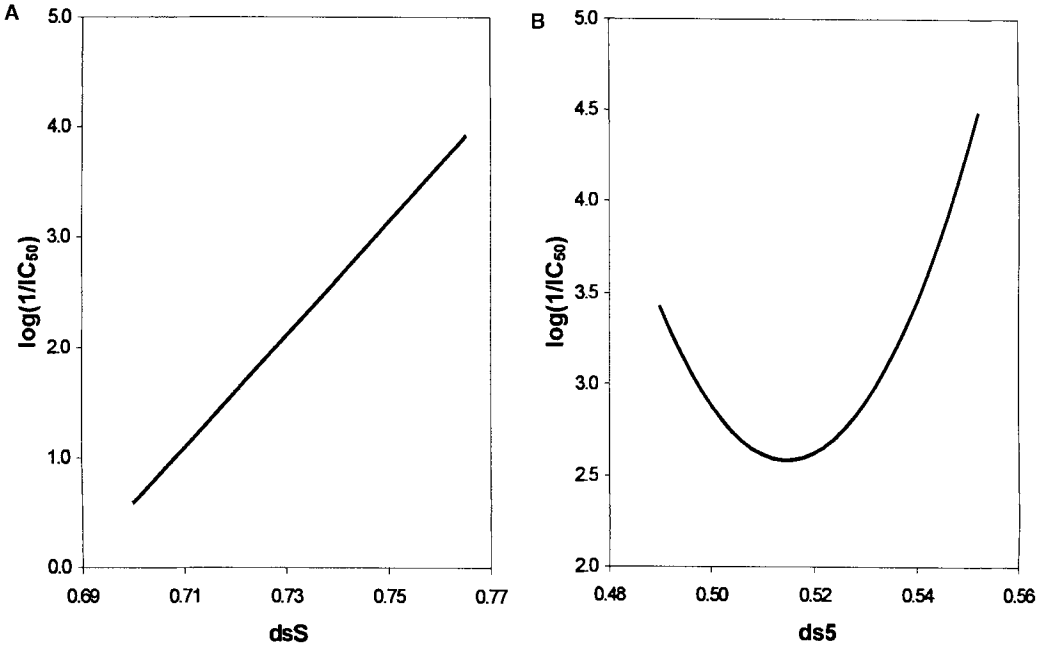


FIGURE 4 Plots of calculated DPPH activity vs. dsS with ds5 fixed to 0.52 (A) and vs. ds5 with dsS fixed to 0.74 (B).

of $\log(1/IC_{50})$ as a function of ds5 or dsS when dsS or ds5 are set as constant, respectively. The relation between ds5 and $\log(IC_{50})$ shows a minimum whereas the relation between $\log(1/IC_{50})$ and dsS is essentially linear. A 4-mercaptoimidazole compound is most active towards DPPH when the spin density on the sulphur atom is high. This is in agreement with the correlation between the activity and ΔH_{r_2}, since a strong localisation of the unpaired electron on the sulphur atom should facilitate the disulphide formation.

Since ΔH_{r_2}, dsS and ds5 are not interdependent, it is possible to use them all in a single equation. The following correlation can be drawn:

$$\log(1/\mathrm{IC50}) = -152.11 - 0.406\Delta H_{r_2} + 324.4\,\mathrm{dsS} + 419.6\,\mathrm{ds5}^2 - 559.0\,\mathrm{ds5}\cdot\mathrm{dsS} \quad (2)$$

$$n = 13 \quad R = 0.94 \quad F = 15.9\ (F_{0.99} = 7.01)$$

$$Q = 0.80$$

A graphical representation of this correlation is given in Figure 5 and the randomisation test confirms the validity of this equation.[5]

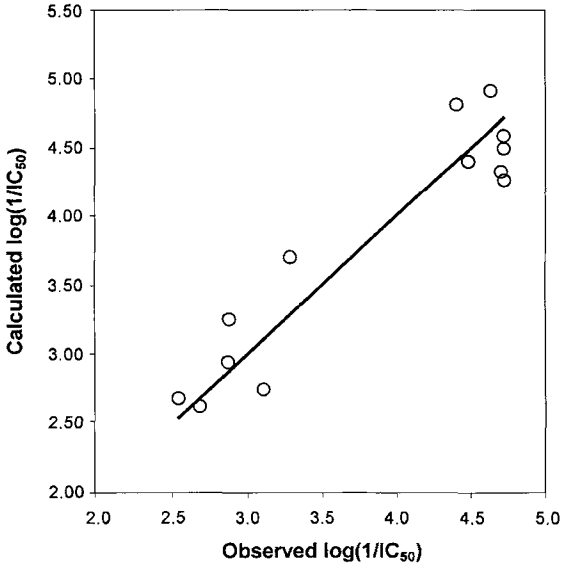


FIGURE 5 Plots of DPPH activity calculated by Equation (2) vs. observed activity.

Cyclic Voltammetry

The observable redox processes were previously reported by Bradbury *et al.*:[6]

1 $\quad 2\,RSH + 2e^- \rightarrow 2\,RS^- + H_2$

1′ $\quad 2\,RS^- + H_2 \rightarrow 2\,RSH + 2e^-$

2 $\quad RSSR + 2e^- \rightarrow 2\,RS^-$

2′ $\quad 2\,RS^- \rightarrow RSSR + 2e^-$

3′ $\quad 2\,RSH \rightarrow RSSR + 2H^+ + 2e^-$

4′ $\quad RSSR \rightarrow [RSSR]^{+\bullet} + e^-$

Table III gives the redox potentials adjusted to the normal hydrogen electrode and a typical voltammogram is displayed in Figure 6. The redox processes are irreversible and the oxidation potential 3′ of thiol in disulphide exhibits a strong dependence upon the activity of the compounds as DPPH scavengers. Typically, the oxidation potential of reaction 3′ for an inactive DPPH scavenger is about 1.20 V, whereas it is 0.50 V for an active one. This is in agreement with the postulated mechanism of oxidation of the thiol into the disulphide compound while scavenging DPPH. Moreover, the 0.50 V oxidation potential value is lower than those observed for

TABLE III Redox potentials for 1.0 mM solutions of 4-mercaptoimidazoles in MeCN at 25°C

Entry	Potentials in volt vs normal hydrogen electrode					
	1	2	1′	2′	3′	4′
1	−0.64	−1.51	−0.24	0.19	1.26	1.70
2	−0.70	−1.46	−0.58	−0.01	1.15	1.60
3	−0.56	−1.45	−0.61	−0.09	1.16	1.54
4		−1.55	−0.57	−0.11	1.18	1.46
5	−0.90	−1.57	−0.40	−0.12		1.33
6	−0.75	−1.39		−0.20	0.51	1.26
7		−1.37	−0.31	−0.08	0.60	1.36
9	−0.87	−1.53	−0.37	−0.07		1.41
10	−0.68	−1.44			0.49	1.38
11	−0.57	−1.42		−0.19	0.43	1.31
12	−0.62	−1.42			0.41	1.23
13	−0.74				1.16	1.42
14	−0.70	−1.35		−0.06		
NAC		−1.09			0.97	
Thiophenol		−1.23		0.08	1.22	1.50

Potentials were obtained from cyclic voltammograms at a scan rate of 50 mV s^{-1}. 1, 2, 1′, 2′, 3′, 4′ are half redox reactions explicited in the text.

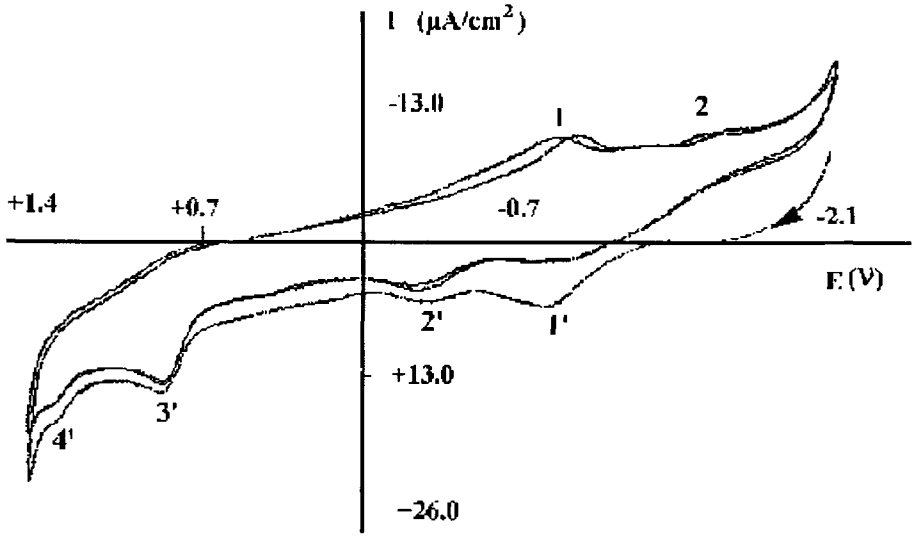


FIGURE 6 Cyclic voltammogram for a solution of 1.0 mM compound **2** in MeCN containing 0.1 N tetraethylammonium perchlorate at a scan rate of 50 mV s^{-1}.

thiophenol (1.22 V) and N-acetylcysteine (0.97 V) under identical conditions.

ESR Experiments

Coupling to Electrochemistry

In order to identify the thiyl radical intermediate, we performed experiments by coupling electrochemistry technique with ESR measurements. For this study we have measured the ESR signal under potential at the characteristic values found with the cyclic voltammetry experiments for disulphide formation from thiols. Figure 7(a) displays the experimental spectrum obtained with compound **9** using DMSO as a solvent and DMPO as a spin trap. The potential value was monitored above the oxidation potential of 0.36 V. Under these conditions, hyperfine splitting constants of $a_N = 13.9$ G and $a_H = 11.8$ G are assigned to the thiyl radical spin adduct. These values are consistent with those reported by Josephy *et al.*[7] with several thionitrite compounds. Similar signals with identical hyperfine splitting constants values were obtained for all DPPH scavenging

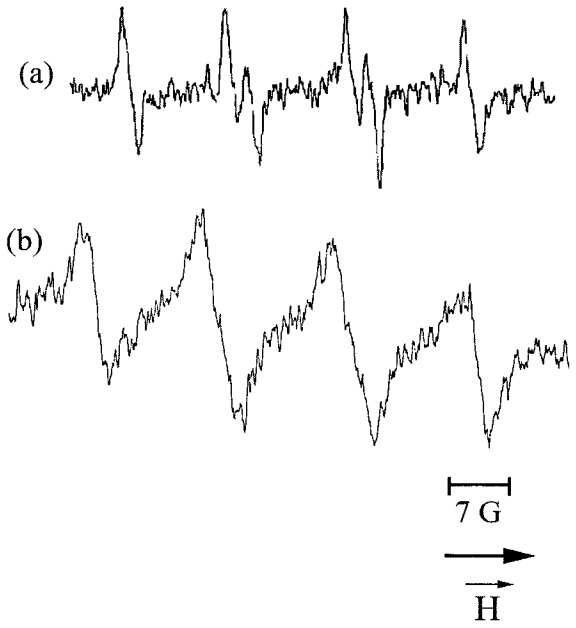


FIGURE 7 (a) Experimental spin trapping ESR spectrum of 1 mM compound **9** at +0.5 V in combination with electrochemistry in DMSO at room temperature. Experimental settings: microwave power 10 mW, amplitude modulation of 1.0 G and receiver level gain was 2.0×10^4. (b) ESR spectrum obtained after mixing 5 mM DPPH, 15 mM compound **9** and 400 mM DMPO in benzene. Experimental settings: microwave power 20 mW, amplitude modulation of 0.8 G and receiver level gain was 1.0×10^5.

compounds. In contrast, no thiyl radical spin adduct was detected for inactive compounds.

Spin Trapping in Presence of DPPH

Attempts to detect DMPO/thiyl adducts using Fenton reagent failed (see preceding paper). So we turned to the DPPH radical. Figure 7(b) shows the experimental spectrum obtained with compound **9** using benzene as a solvent, DMPO as a spin trap and DPPH as the oxidising agent. Short-lived DMPO adduct with hyperfine splitting constants $a_N = 13.6$ G and $a_H = 14.5$ G (line width 1.5 G) was observed. This is in good agreement with hyperfine parameters obtained for phenyl thiyl radical spin adducts in the same solvent.[8]

Hydroxyl Radical Scavenging Activity

^{1}H NMR experiments in $CDCl_3$ were performed with the most active compound **9** to validate the radical scavenging mechanism. Figure 8(a) shows the region of SH resonance frequency with a broad line resonance at 2.1 ppm.[1] After incubating 1.0 mM compound **9** and Fenton reagent (0.1 mM H_2O_2, 0.1 mM $FeSO_4$), the solution was evaporated and the residual material dissolved in $CDCl_3$ solvent for NMR experiments. The thiol resonance line at 2.1 ppm almost entirely disappeared (Figure 8(b)) and there was a shift of the 5-H signal from 7.32 to 7.50 ppm suggesting that the molecule has reacted with $HO^\bullet$ to yield the disulphide compound.[1]

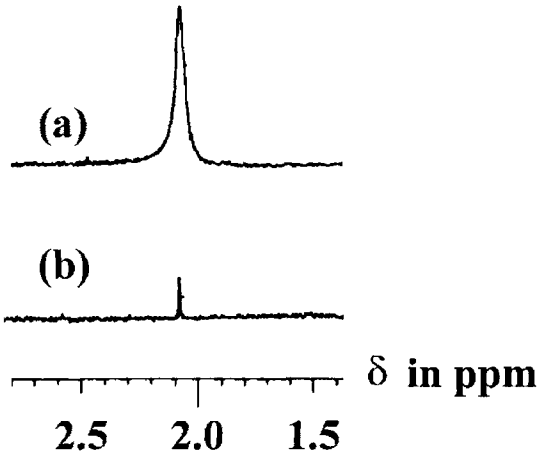


FIGURE 8 ^{1}H NMR spectrum of thiol region of native compound **9** in $CDCl_3$ at 300 MHz (a), after Fenton reaction system (0.1 mM H_2O_2, 0.1 mM $FeSO_4$ (b).

DISCUSSION

The experimental data provide strong evidences for the formation of disulphide compounds via thiyl radical formation upon DPPH scavenging by 4-mercaptoimidazole compounds. This mechanism is supported by both the QSAR and physicochemical studies.

Ovothiols [9,10] and 4-mercaptoimidazoles[1] were shown to efficiently scavenge free radicals and the pathway for free radical scavenging was assumed to involve transient thiyl radicals, as observed for many other aliphatic thiols. Here we demonstrated by ESR experiments the existence of such thiyl radical intermediates. First they were identified by coupling electrochemistry with ESR. Then thiyl radicals were trapped by DMPO during DPPH scavenging by 4-mercaptoimidazoles. In this way, we can directly implicate thiyl radical formation in the radical scavenging mechanism.

Their formation occur by hydrogen abstraction from the thiol function, as evidenced by ^{1}H NMR experiments. The ΔH_{r_1} values predicted by the AM1 method for thiol conversion to thiyl radical suggested that this first step is slightly endothermic and that the overall proposed mechanism may not be feasible. However, the critical step in scavenging DPPH by 4-mercaptoimidazoles turned out to be the second one involving the formation of disulphide compounds from thiyl radicals. This process was predicted to be exergonic. A 4-mercaptoimidazole is active towards DPPH as its disulphide formation is energetically favourable. This is the case for compounds **6–10** and **14** substituted with electron-withdrawing groups. This hypothesis is consistent with the fact that active and inactive compounds towards DPPH can be discriminated by the potential of

the cyclic voltammogram peak for the oxidation of thiol to disulphide. The best DPPH scavengers exhibit low oxidation potentials for this redox reaction.

In this report, a model was derived to correlate the radical scavenging abilities of 4-mercaptoimidazoles to calculated parameters. A highly significant correlation ($R = 0.94$) was obtained using the reaction enthalpy for disulphide formation from thiyl radicals (ΔH_{r_2}), the spin density on carbon 5 (ds5) and the spin density on the sulphur atom (dsS). This model may be useful in predicting the antioxidant activities of new ovothiol derivatives. The thermodynamical investigation performed in this work does not take into account the kinetical aspects of the considered pathway. However, since all the reactions investigated involve free radicals, which are expected to react at near-diffusion-controlled rates, it seems likely that the efficiency as a free-radical trapper of a compound relies mainly on the thermodynamics of the reactions and on the activities of the relevant species. In this sense, it has been demonstrated that the rate constants for the reaction of 4-mercaptoimidazoles with hydroxyl radicals (see preceding paper) is very high (of the order of $10^{10}\,M^{-1}\,s^{-1}$).

The ability of 4-mercaptoimidazoles to scavenge free radicals may be physiologically used. These compounds may protect cells *in vivo* through $HO^{\bullet}$ scavenging. They react with $HO^{\bullet}$ radicals with high rate constants to yield disulphide compounds. In addition, physiological thiols like glutathione may regenerate 4-mercaptoimidazoles from the oxidised disulphide forms.[11]

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