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4-Mercaptoimidazoles Derived from the Naturally Occurring Antioxidant Ovothiols 1. Antioxidant Properties

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4-Mercaptoimidazoles derived from the naturally occurring family of antioxidants, the ovothiols, were assayed for their antioxidant properties. These compounds are powerful HOCl scavengers, more potent than the aliphatic thiol N-acetylcysteine. They react slowly with hydrogen peroxide with second order rate constants of $0.13–0.89\,M^{-1}\,s^{-1}$. Scavenging of hydroxyl radical occurs at a diffusion-controlled rate ($k = 2.0–5.0 \times 10^{10}\,M^{-1}\,s^{-1}$) for the most active compounds, which are also able to inhibit copper-induced LDL peroxidation. The combination of radical scavenging and copper chelating properties may explain the inhibitory effects on LDL peroxidation. Two molecules of mercaptoimidazole can chelate a copper ion and form a square planar complex detected by EPR. Compounds bearing an electron-withdrawing group on position 2 of the imidazole ring are the most potent antioxidant molecules in this series.

Keywords: 4-Mercaptoimidazoles, hypochlorous acid, hydrogen peroxide, hydroxyl radical, lipid peroxidation

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; DETAPAC, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethylpyrroline-N-oxide; DPPH, 2,2′-diphenyl-1-picrylhydrazine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; LDL, low density lipoprotein; NAC, N-acetylcysteine; PBS, phosphate-buffered saline

INTRODUCTION

Ovothiols, a redox-active family of 1-methyl-4-mercaptohistidines, were discovered during the study of the fertilisation of eggs from various invertebrates.[1–4] The remarkable abundance of the ovothiols in eggs (5 mM) suggested that they play essential biological functions. They

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were shown to act as cofactors for the cyanide-resistant [NAD(P)H] oxidase activity of ovoperoxidase,[3,5,6] although this does not seem to be their physiological role. The main function of these compounds is the protection of eggs from oxidative damage during the respiratory burst which takes place immediately after fertilisation. Indeed, invertebrate eggs construct a protective fertilisation envelope after sperm entry. This results from the action of an egg enzyme, ovoperoxidase, which extensively catalyses the cross-linking of tyrosyl residues using H_2O_2 produced in a burst as an extracellular oxidant.[7] The persistence of high concentrations of ovothiols for several hours after fertilisation suggested their intervention to limit the deleterious effects resulting from this oxidative stress. The antioxidant character of ovothiols has been investigated *in vitro*. They act as glutathione peroxidase mimics: they are rapidly oxidised by hydrogen peroxide at five times the rate measured for glutathione and are regenerated by reducing glutathione, their redox potentials being considerably more positive than that of glutathione.[8,9] In addition ovothiols were found to scavenge Fremy's salt and Banfield's radical much more rapidly than glutathione,[6] tyrosyl,[6] superoxide and linoleate peroxyl radicals with efficiency comparable to that of ascorbic acid and the tocopherol analogue Trolox.[10] Ovothiols are more reactive than glutathione as one-electron donors. This facile one-electron donation makes ovothiols together with ascorbate, amongst the most efficient antioxidants from natural origin. Because the reduction potential of the ovothiyl radical is lower than that of vitamin E, these heterothiols are able to repair the vitamin E radical, involved in membrane protection.[10] The acidity of the thiol function also differentiates ovothiols from aliphatic thiols such as glutathione. The thiol pK_a of a model 4-mercaptoimidazole, 2.3[11] is considerably lower than that of glutathione (thiol pK_a 8.65). The mercaptoimidazole moiety of ovothiols thus exists predominantly as the imidazolium thiolate form at physiological pH, so that ovothiols are moderately good H-atom donors.[10]

These observations prompted us to design a series of ovothiol derivatives having various substituents on the mercaptoimidazole ring.[12] The radical scavenging properties of the newly synthesised compounds were evaluated using 2,2-diphenyl-1-picrylhydrazyl and Fremy's salt radicals. In both tests, compounds bearing a strong electron-withdrawing group at C-2 appear as the most active imidazole derivatives.

In view of the importance of thiols in antioxidant therapy,[13–16] we report here a detailed study of the rate of reaction of 4-mercaptoimidazoles with various oxidants: H_2O_2, $HO^\bullet$ and HOCl. Their inhibitory effects on copper-induced LDL peroxidation and their interaction with Cu^{2+} were also investigated.

MATERIALS AND METHODS

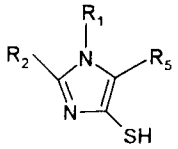
Chemicals and reagents were of the highest quality available and were purchased from Sigma Aldrich Company. All 4-mercaptoimidazoles (Table I) were obtained by chemical syntheses according to a previously reported method.[12] Thiol concentrations were determined using 5,5′-dithiobis(2-nitrobenzoic acid), DTNB.[17] Stock solutions ($0.01\,mol\,l^{-1}$) were freshly prepared with ethanol and buffer solutions with Milli-Q water (conductivity $< 10^{-18}$ $\Omega^{-1}\,cm^{-1}$).

^1H NMR spectra were recorded on a Brüker AM 300 WB (300 MHz). Mass spectra analyses were performed on a Finnigan MAT Vision 2000 mass spectrometer (MALDI-TOF). UV measurements were made on a UVIKON 932 spectrophotometer.

Reaction with Hypochlorous Acid

HOCl was produced immediately before use by adjusting sodium hypochlorite ($Na^+\,OCl^-$) to pH 6.2 with dilute H_2SO_4. Oxidation of 4-mercaptoimidazoles was followed spectrophotometrically

TABLE I Structure and reactivity towards H_2O_2 of test compounds



Entry	R_1	R_2	R_5	k [a] ($M^{-1}s^{-1}$)	k/k_{NAC} [b]
1	$-CH_3$	$-H$	$-CH_3$	0.33 ± 0.05 (263)	2.5
2	3,4–diMeO–C_6H_3	$-H$	$-H$	0.31 ± 0.03 (244)	2.4
3	4–MeO–C_6H_4	$-H$	$-H$	0.37 ± 0.05 (242)	2.8
4	2–MeO–C_6H_4	$-H$	$-H$	0.45 ± 0.03 (235)	3.5
5	$-CH_3$	4–Cl–C_6H_4	$-H$	0.31 ± 0.04 (313)	2.4
6	$-CH_3$	3–Cl–C_6H_4	$-H$	0.68 ± 0.05 (313)	5.2
7	$-CH_3$	2–Cl–C_6H_4	$-H$	0.54 ± 0.04 (300)	4.2
8	$-CH_3$	3–CF_3–C_6H_4	$-H$	0.89 ± 0.05 (319)	6.8
9	$-CH_3$	$-H$	2–CF_3–C_6H_4	—	—
10	$-CH_3$	$-H$	2–Cl–C_6H_4	0.19 ± 0.02 (275)	1.5
11	$-CH_3$	$-H$	4–Cl–C_6H_4	0.13 ± 0.02 (295)	1.0
12	$-CH_3$	$-H$	4–MeO–C_6H_4	0.38 ± 0.03 (271)	2.9
13	$-CH_3$	$-CF_3$	2–MeO–C_6H_4	0.69 ± 0.06 (275)	4.6

[a]Second order rate constants of reaction of 4-mercaptoimidazoles (0.1 mM) with H_2O_2 (1 mM) at 20°C in a 20 mM KH_2PO_4–KOH buffer pH 7.2 containing 2 mM EDTA. The wavelengths (nm) of the titrations are bracketed. The oxidation of compound **9** could not be followed since an insolute appeared in the cuvette.
[b]k_{NAC}: second order rate constant of reaction of N-acetylcysteine with H_2O_2.

and the disappearance of the –SH group was measured using DTNB. A solution of 100 μM test compound in a 50 mM KH_2PO_4–KOH buffer pH 6.6 was incubated with increasing quantities of HOCl until no further spectral modification was observed.

Scavenging of Hydrogen Peroxide

Experiments were conducted in a 20 mM KH_2PO_4–KOH buffer pH 7.2 containing 2 mM EDTA. The rate of reaction of compounds with H_2O_2 was followed spectrophotometrically by measuring the absorbance at 240–320 nm, which corresponds to a maximum in absorbance of the –SH group of the 4-mercaptoimidazoles. The compound (final concentration 0.1 mM) was incubated in the presence of a ten fold excess of hydrogen peroxide at 20°C. Given the stoichiometry of the following equation:[8]

$$2RSH + H_2O_2 \rightarrow RSSR + 2H_2O$$

the rate, ν, of the reaction can be written as

$$\nu = -\frac{1}{2}\frac{d[RSH]}{dt} = -\frac{d[H_2O_2]}{dt} = \frac{d[RSSR]}{dt} = \frac{1}{2}\frac{d[H_2O]}{dt}$$

The rate of the process has been reported to be first order in H_2O_2 and thiol.[8] In our case, in presence of an excess of H_2O_2, the rate is only first order in thiol, and the following straight line equation can be established:

$$\ln\left[\frac{A_{\lambda,\infty} - A_{\lambda,0}}{A_{\lambda,\infty} - A_{\lambda}}\right] = 2 \cdot k \cdot c_0 \cdot t$$

where $A_{\lambda,0}$, $A_{\lambda,\infty}$ and A_{λ} are the absorbance at the wavelength λ at the beginning, the end, and the time t of the process, respectively; c_0 is the constant concentration of H_2O_2 and k the rate constant of the reaction.

This latter equation provides a convenient way to calculate the rate constant k of the oxidation

reaction of thiol compound by H_2O_2 (Table I). The loss of the –SH group of NAC was determined using the DTNB reagent.

Scavenging of Hydroxyl Radicals

EPR measurements were recorded with a Varian E-109 spectrometer operating at 9.5 GHz with a 100 kHz high-frequency modulation. The sample solutions were examined in a flat quartz cell inserted in an E-238 cavity operating in the TM_{110} mode. Hydroxyl radicals were generated by the Fe(II)/H_2O_2 system (0.1 mM H_2O_2, 0.1 mM $FeSO_4$). The ability of the thiols to scavenge $HO^\bullet$ radicals was determined by measuring the inhibition of formation of 5,5-dimethylpyrroline N-oxide (DMPO)/$HO^\bullet$ adducts. DMPO was purified by vacuum distillation prior to use. The rate constants of reaction of thiols with $HO^\bullet$ radicals were calculated from kinetic competition studies with DMPO,[18] based on the rate constant of the reaction of DMPO with $HO^\bullet$, $k_{DMPO} = 2.0 \times 10^9\ M^{-1}\ s^{-1}$.[19]

The linear relationship $i_0/i = 1 + k_{RSH}/k_{DMPO}$ ([RSH]/[DMPO]) was used, where i_0 and i are the rates of hydroxyl radical trapping in the absence and presence of the competitor, respectively; k_{RSH} and k_{DMPO} are second-order rate constants for the reaction of $HO^\bullet$ with the competitor and DMPO, respectively.

Ethanol was used to obtain the complete dissolution of the lipophilic test compound. The mercaptoimidazole (1.0 mM), ethanol (1.7 M), $FeSO_4$ (0.1 mM), DMPO (8.0 mM) and DETAPAC (50 mM) were incubated in buffer solution and the reaction was initialised by addition of H_2O_2 (0.1 mM).

LDL Peroxidation

LDL was isolated from plasma of normolipidemic donors by low speed ultracentrifugation in the density range of 1.019–1.063 g/ml as previously described.[20] The preparation was dialysed extensively against 10 mM phosphate-buffered saline (PBS, pH 7.2) at 4°C for 4 h to remove EDTA and stored at 4°C under nitrogen. The isolated LDL was diluted with 10 mM PBS, pH 7.2, to a final protein concentration of 100 μg/ml (determined by the Peterson's method[21]).

To initiate peroxidation, an aliquot of a freshly prepared stock aqueous solution of $CuSO_4$ was added to LDL (100 μg/ml) in PBS to yield 1.66 μM final copper concentration. Where indicated, prior to copper addition, aliquots of freshly prepared stock solutions of test compounds in ethanol were immediately added and mixed to yield final concentrations of 0–100 μM. The final ethanol concentration never exceeded 1% (v/v) and the same amount of ethanol was added in the corresponding blanks. LDL was incubated at 30°C for 8 h. Conjugated dienes (O.D. 234 nm) were measured every 10 min to assess the degree of LDL oxidation.[22] We defined the 50% efficacious dose (ED_{50}) of a test compound as the concentration that increased the control lag time of oxidised LDL by 1.5 times. They were calculated from semilogarithmic plots (log of drug dose versus lag time expressed as percent of control).

Copper Chelation

Cu^{2+} binding studies were performed using absorption spectrophotometry. Mercaptoimidazoles (100 μM) were incubated with $CuSO_4$ (0–100 μM) in ethanol at ambient temperature and the absorption spectra (200–400 nm) of the solutions were recorded against a buffer blank containing the same copper amounts. Ethanol was used as solvent to avoid precipitation of the copper complexes. Reactions were simultaneously monitored by thin layer chromatography. EPR spectra of the copper : thiol complexes were obtained at liquid nitrogen temperature (77 K). The g factor measurements were related to the "strong pitch", $g = 2.0028$. The geometry of the complexes was also determined by molecular modelling. Calculations were performed with Spartan® Plus 5.0, using the PM3 (TM) semi-empirical quantum method in the UHF formalism

and with a multiplicity of 2. All the geometries were optimised by minimising their total energy.

RESULTS

Reaction with Hypochlorous Acid

Incubation of 4-mercaptoimidazoles with HOCl caused important changes in their absorption spectrum, consistent with the oxidation of the thiols into disulphides (Figure 1). This was confirmed by ^{1}H NMR spectroscopy (disappearance of the SH proton and shift of the 5-H signal) and mass spectrometry (apparition of the disulphide peak). Reactions were very fast and complete oxidation was obtained within a few seconds; 0.6 equivalent of HOCl (60 μM) afforded total oxidation of the thiol compounds except for compound **1** and N-acetylcysteine. This is in good agreement with the stoichiometry (0.5) of the following reaction equation:

$$2RSH + ClO^- \rightarrow RSSR + Cl^- + H_2O;$$

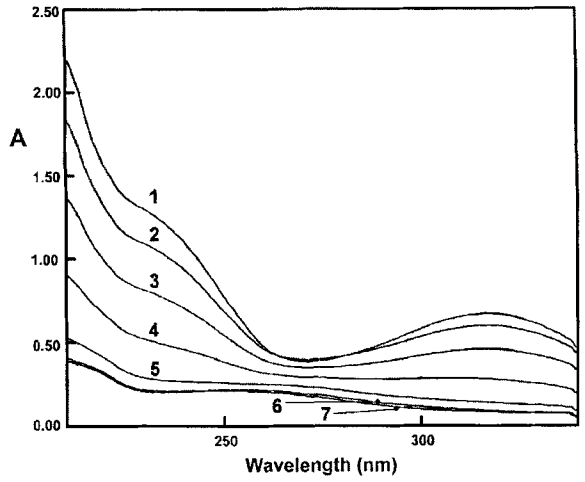


FIGURE 1 Spectral changes observed upon reaction of HOCl with compound **6**. Spectrum 1, 100 μM compound **6** alone; Spectrum 2, 100 μM compound **6** + 10 μM HOCl; Spectrum 3, 100 μM compound **6** + 20 μM HOCl; Spectrum 4, 100 μM compound **6** + 30 μM HOCl; Spectrum 5, 100 μM compound **6** + 40 μM HOCl; Spectrum 6, 100 μM compound **6** + 50 μM HOCl; Spectrum 7, 100 μM compound **6** + 60 μM HOCl.

30% and 65% of the initial SH concentrations were still measured after incubation of compound **1** and NAC with 60 μM HOCl, indicating lower reactivities of both compounds towards HOCl.

Scavenging of Hydrogen Peroxide

Oxidation of thiols by H_2O_2 afforded the disulphides as revealed by ^{1}H NMR spectroscopy and mass spectrometry. Initial experiments showed that when 4-mercaptoimidazoles (final concentration 0.1–0.5 mM) were incubated with H_2O_2 (1.0 mM final concentration), the rate of thiol consumption was proportional to the concentrations of thiol compound and hydrogen peroxide. Second-order rate constants were calculated from combined data (Table I) and referenced to that obtained for N-acetylcysteine ($k = 0.13\,M^{-1}\,s^{-1}$). The ovothiol derivatives reacted slowly with H_2O_2 with rate constants between 0.13 and $0.89\,M^{-1}\,s^{-1}$ at 20°C. Nevertheless they were all more effective than NAC at consuming H_2O_2.

Scavenging of Hydroxyl Radicals

In the presence of DMPO, ethanol (1.7 M) and a Fe(II)/H_2O_2–HO$^\bullet$ generating system, the α-hydroxyethyl radicals reacted with DMPO to yield a spectrum with hyperfine splitting constants $a_N = 15.7$ G and $a_H = 23.0$ G, which was completely distinct from the DMPO/HO$^\bullet$ spectrum ($a_N = a_H = 14.5$ G; Figure 2).[19] Addition of a competitor such as compound **8** caused a marked decrease in the intensities of both DMPO adducts. This indicates that compound **8** is a potent scavenger of hydroxyl radicals. The production of α-hydroxyethyl radicals formed upon the reaction of ethanol with HO$^\bullet$ is lowered. Similar behaviours were observed for compounds **6**, **7** and **10**. Assuming a rate constant of $2.1 \times 10^9\,M^{-1}\,s^{-1}$ for reaction of DMPO with HO$^\bullet$,[19] these thiols gave rate constants between $1.0 \times 10^9\,M^{-1}\,s^{-1}$ and $5.0 \times 10^{10}\,M^{-1}\,s^{-1}$ (Table II). For the other compounds, there was no significant variation of the DMPO/HO$^\bullet$ adduct.

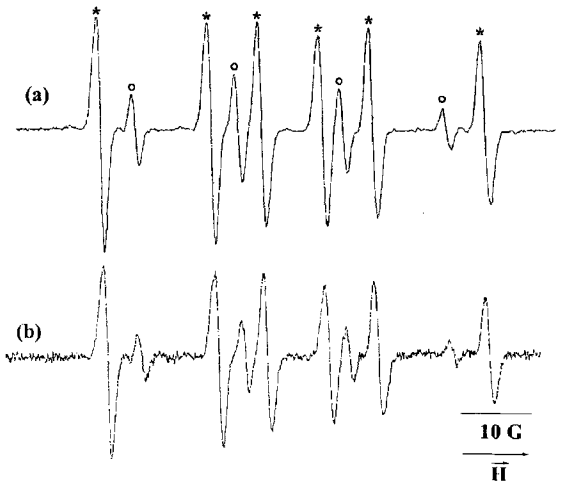


FIGURE 2 (a) DMPO radical adducts formation in the Fe(II)/H_2O_2 $HO^\bullet$ radical generating system (0.1 mM H_2O_2, 0.1 mM $FeSO_4$). All samples contained 8.0 mM DMPO, 1.7 M ethanol and 50 mM DETAPAC. The spectrum is a combination of DMPO/$HO^\bullet$ (○) and DMPO/α-hydroxyethyl (*) adducts. Gain: 6.3×10^3. (b) 1.0 mM compound **8**. Gain: 1.6×10^4.

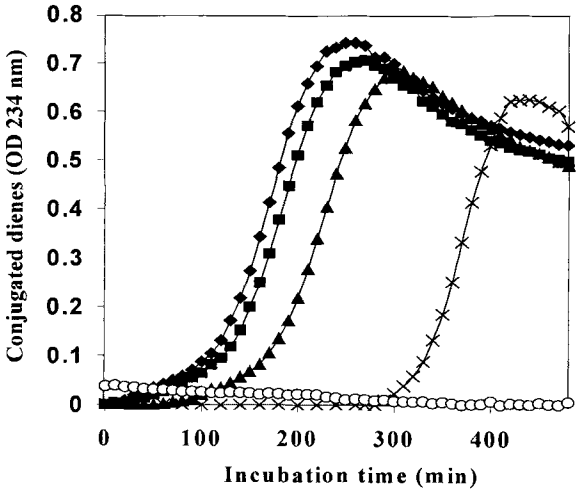


FIGURE 3 Compound **7** prevents LDL peroxidation. The effects of compound **7** on Cu^{2+}-mediated peroxidation of LDL measured by the formation of conjugated dienes at 30°C (O.D. 234 nm). LDL (0.1 mg/ml) incubated with 1.66 μM Cu^{2+} in phosphate buffer (pH 7.2) with varying concentrations of compound **7**: 0 (◆), 1.0 (■), 2.5 (▲), 5.0 (×) and 7.5 μM (○).

TABLE II Activities of mercaptoimidazoles as hydroxyl radical scavengers and as inhibitors of copper-induced LDL peroxidation

	k[a] ($10^9 M^{-1} s^{-1}$)	ED_{50}[b] (μM)
2	—	—
5	5.0	3.8
6	20.0	5.8
7	6.0	2.7
8	50.0	6.2
9	1.0	50.0
10	40.0	5.5
11	—	—
13	—	60.0

[a]Rates of reaction of 4-mercaptoimidazoles with hydroxyl radical at 25°C.
[b]Efficacious drug doses necessary to increase by 50% the lag time of LDL (100 μg/ml) oxidised in presence of copper (1.66 μM) for 8 h at 30°C. ED_{50} values were calculated as described in Materials and Methods. Shown are the mean values from three independent experiments.

Effects of 4-Mercaptoimidazoles on Copper-induced LDL Oxidation

Copper-induced LDL oxidation is a common model for the study of LDL oxidative modification. The lag time prior to the exponential increase in conjugated dienes in LDL incubated with 1.66 μM copper was considerably increased in presence of mercaptoimidazoles such as compound **7** (Figure 3). The results listed in Table II show that imidazole derivatives bearing an electron-withdrawing group at C-2 (compounds **5–8**) are the most effective inhibitors of LDL oxidation ($ED_{50} = 2.7–6.2$ μM). Their activity is superior to that of vitamin E ($ED_{50} = 10$ μM) and melatonin ($ED_{50} = 79$ μM).[23] Compounds with a N-1 or a C-5 substitution (**2**, **9**, **11**, **13**) are far less active except compound **10**. The thiol function is undoubtedly responsible for the antioxidant properties because the corresponding disulphides have no effect. Interestingly, several imidazoles exhibited both pro- and antioxidant properties depending on their concentration. For example compounds **5** and **10** promoted LDL peroxidation at low concentrations (< 3 μM) but inhibited the oxidative reaction at higher concentrations.

Copper Chelating Properties

Upon addition of Cu^{2+}, the UV absorption spectrum of a 100 μM mercaptoimidazole solution underwent strong modifications (Figure 4).

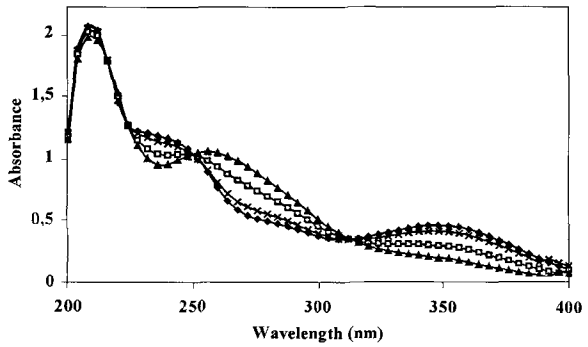


FIGURE 4 Spectral changes upon reaction of compound **6** (100 μM in ethanol) with increasing concentrations of copper ion: (◆) 0, (×) 10, (□) 35, (▲) 50 μM.

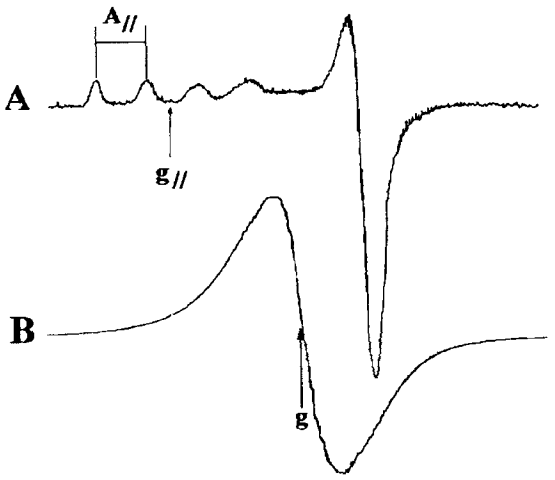


FIGURE 5 (A) EPR spectrum of 5 mM compound **8** incubated with 1 mM $CuSO_4$ in ethanol. (B) EPR spectrum of a 1 mM $CuSO_4$ solution in ethanol. Experimental settings: 100 kHz modulation frequency, 10 G modulation amplitude, 10 mW microwave power and 3.2×10^2 receiver gain.

The effect saturated at copper concentrations over 50 μM. Thin layer chromatography was used to monitor the reactions. The apparition of a slowly migrating product was detected concomitantly with loss of the thiol compound. The thiol band was totally extinguished in presence of 50 μM copper. The experiments suggested that two molecules of mercaptoimidazole can complex one copper ion. The complex was characterised by EPR and mass spectrometry.

Figure 5B displays the EPR spectrum of $CuSO_4$ (1 mM) obtained in liquid nitrogen (77 K). This spectrum exhibited an isotropic g value of

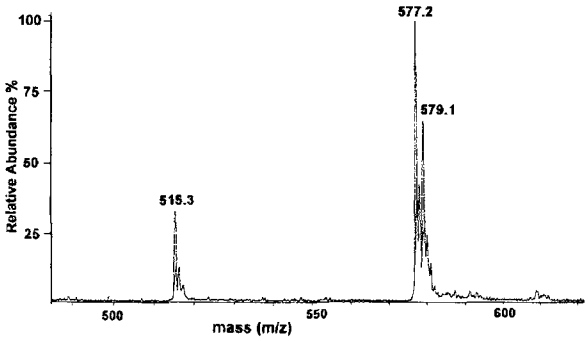


FIGURE 6 MALDI-TOF mass spectrometric analysis (positive ions) of the reaction of 0.01 M compound **8** with 0.01 M Cu^{2+} in ethanol.

2.036 for free copper in ethanol. Upon addition of compound **8** (5 mM), the EPR spectrum exhibited parallel components with a $g_{||}$ value of 2.14 and an hyperfine splitting constant, $A_{||}$ of 115 G and a perpendicular component at higher field (Figure 5A). The relative position of these components is commonly observed for normal tetragonal Cu(II) complexes. The double integral of the signal from the Cu(II) complex was compared with uncomplexed Cu(II). This showed that slight Cu(I) formation (about 10%) occurs in the complex.

Mass spectrometry analysis of a crude product containing compound **8** incubated with 1 mol Cu^{2+} equivalent (Figure 6) revealed the presence of a major ion at $m/e = 577$. This ion was attributed to $(2M + {}^{63}Cu - 2H)$ [M corresponding to the molecular weight of the thiol compound]. The next ion at $m/e = 579$ would correspond to the same complex with the other isotope of copper (^{65}Cu) and the species at $m/e = 515$ would correspond to the disulphide compound.

The geometry of the complexes was investigated by molecular mechanics calculations. Different starting structures were used to initialise the geometry optimisation process, i.e. tetrahedral, square planar, and any other geometry. A square planar geometry is by far the most stable conformation, as expected from the EPR experimental data. Each of the two 4-mercaptoimidazoles is interacting with copper via the sulphur atom and the nitrogen atom on position 3 of the

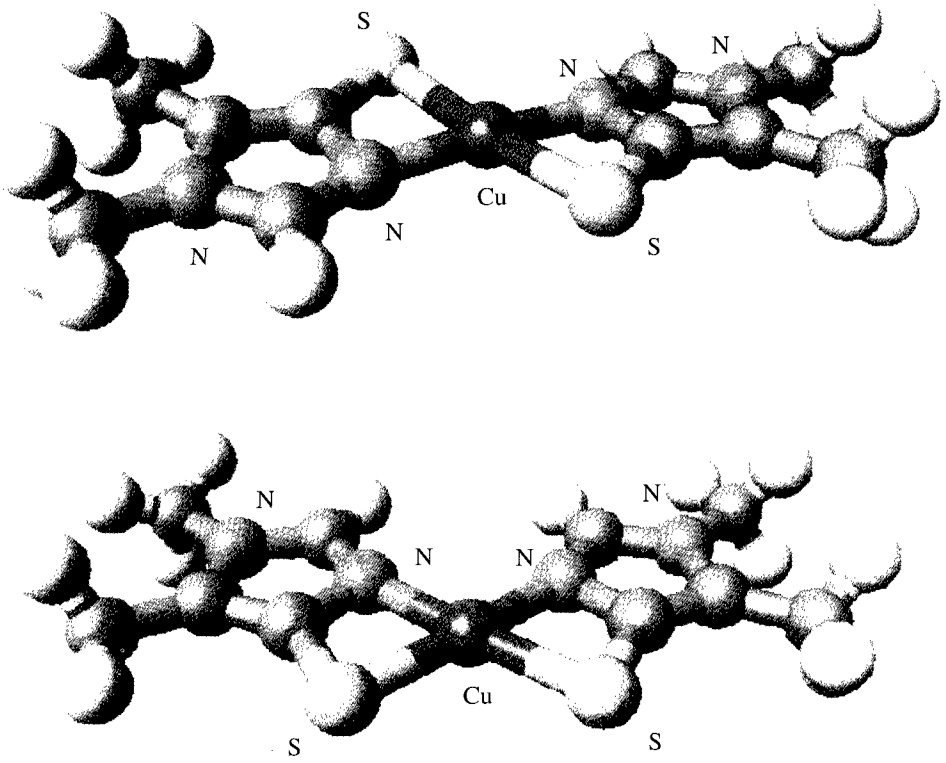


FIGURE 7 Geometry of the copper complexes calculated for compound **1** by molecular modelling using Spartan® Plus 5.0 and the PM3 (TM) semi-empirical quantum method. Upper representation: trans complex; lower representation: cis complex. (See Color plate I at the end of this issue.)

imidazole ring. The trans complex is 2.9 kcal/mol lower in energy than the cis complex (Figure 7).

DISCUSSION

Results obtained from the different assays corroborate the fact that compounds bearing electron-withdrawing group on C-2 position of the imidazole ring are the most antioxidant ovothiol derivatives.

In presence of the myeloperoxidase-derived strong oxidant HOCl,[24] complete oxidation of the thiol compounds into disulphides was very fast whereas NAC was partially oxidised under the same conditions. Thus mercaptoimidazoles represent excellent HOCl scavengers since NAC is reported as a potent scavenger.[25]

Low second-order kinetic constants (Table I) were determined at 20°C for the reaction of 4-mercaptoimidazoles and NAC with H_2O_2 ($0.13–0.89\,M^{-1}\,s^{-1}$). These values are in good agreement with the data reported by Turner *et al.* with regard to the temperature difference between the two experiments.[8] Compounds bearing electron-withdrawing groups on position 2 (**6**, **7**, **8**, **13**) are about 4–7 times more reactive than NAC and emerge as the most potent scavengers (except for compound **5**).

EPR experiments revealed that compounds **5–10** are powerful scavengers of hydroxyl radical, yielding high rate constants (Table II). Compounds **6**, **8** and **10** react with $HO^{\bullet}$ radicals at a virtually diffusion-controlled rate ($k = 2.0–5.0 \times 10^{10}\,M^{-1}\,s^{-1}$), like glutathione,[26] captopril[14] and ergothioneine.[27] Surprisingly, we

did not detect thiyl radical adducts in contrast to experiments with aliphatic thiols.[14,28] Several factors may disfavour the formation of DMPO thiyl adducts: the presence of competing $HO^{\bullet}$ and $HOEt^{\bullet}$ radicals which are more reactive towards DMPO than thiyl radicals ($k = 10^7$–$10^8\,M^{-1}\,s^{-1}$)[29,30] and the simultaneous rapid decay of thiyl radicals ($k = 6.0 \times 10^8\,M^{-1}\,s^{-1}$).[10] Dimerisation to form the disulphides and reaction with the parent thiolates to form highly reducing disulphide radical anions $RSSR^{\bullet-}$ may represent the main processes accounting for this decay. The last route is expected to be favoured by the typically low pK_a values of the thiol groups.

Mercaptoimidazoles, like other aliphatic thiols[31–34] delayed significantly the appearance of conjugated dienes during copper-induced LDL oxidation. Here again the most active compounds **5–8** are C-2 substituted (ED_{50} 2.7–6.2 μM) and compound **10** is the only C-5 substituted molecule with a similar ED_{50} value (Table II). Their inhibitory effects depend on several factors.

The sensitivity of the thiol compounds to autoxidation may play a role since the inactive disulphides can form at 30°C and even at lower temperatures.[12] The action of heterothiols against LDL peroxidation can also be related to their ability to scavenge initiating and propagating radicals. But a strict correlation cannot be established. Thus compounds **11** and **13**, which are powerful scavengers of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical,[12] are far less effective at inhibiting LDL peroxidation. The ability of two molecules of mercaptoimidazole to chelate a copper ion and form a square planar complex was clearly evidenced. EPR experiments showed that there is a small proportion of copper which is reductively inactivated in the complex. This was confirmed using the specific bathocuproine/Cu^+ complex (data not shown). Copper chelation promotes another mechanism for the inhibition of LDL peroxidation, which was initially proposed for captopril[14,35] and dihydrolipoic acid.[31] The most active compounds **6**, **7**, **8** and **10** were also assayed for their ability to inhibit azo compound-initiated oxidative modification of LDL, using 2,2′-azo-bis(2-amidinopropane)dihydrochloride (AAPH). ED_{50} values of 67, 11.4, 27 and 100 μM were obtained for these four compounds respectively, showing that their antioxidant activity (lower in the metal-independent system) is based, at least partially, on copper chelation. Thus we can reasonably propose that the inhibitory effects observed for compounds **6**, **7**, **8** and **10** result mainly from the combination of their radical scavenging and copper chelating properties.

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