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ANTIOXIDANT PROPERTIES OF 2-IMIDAZOLONES AND 2-IMIDAZOLTHIONES

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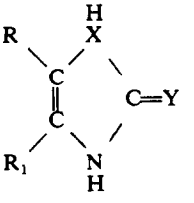
Abstract—Uric acid has been postulated to be an important antioxidant and free radical scavenger in humans. Other purines, such as xanthine, that lack an 8-oxo group on the imidazole ring do not show antioxidant properties. For this reason, the antioxidative activities of 2-imidazolones and 2-imidazolthiones were compared to that of uric acid. 2-Imidazolthiones reacted with the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) at rates comparable to those of uric acid and other antioxidants. 2-Imidazolones also reacted with DPPH, although at a much slower rate than the 2-imidazolthiones. The 2-imidazolthiones protected oxyhemoglobin from oxidation to methemoglobin by sodium nitrite; the 2-imidazolones had little effect on the oxidation of oxyhemoglobin by nitrate. Most of the 2-imidazolthiones and 2-imidazolones protected both porcine and bovine erythrocytes from hemolysis by *t*-butyl hydroperoxide. Although 2-imidazolthiones were more reactive than 2-imidazolones in the assays using DPPH and the oxidation of oxyhemoglobin, both types of compounds may be useful as antioxidants *in vivo*.

Uric acid has been postulated to be an important antioxidant and free radical scavenger in humans. Its presence in higher concentrations in plasma of humans, compared to other primates, has been suggested to be a major factor in lengthening life span and decreasing age-specific cancer rates in humans [1]. 3-*N*-Ribosyluric acid has been proposed as an antioxidant and free radical scavenger in the bovine erythrocyte [2]. Since compounds such as xanthine, hypoxanthine, adenine, and guanine, which are also purines, but lack an 8-oxo group on the imidazole ring of the purine, do not show antioxidant properties [1, 3], the 2-imidazolone portion of the purine molecule may be important for the antioxidant properties of urates. It was shown recently that 2-imidazolone reacted with the stable free radicals 1,1-diphenyl-2-picrylhydrazyl (DPPH) and galvinoxyl, although at a much slower rate than uric acid [4], suggesting that 2-imidazolones have potential as antioxidants. In addition, some 2-mercapto-4-substituted imidazole derivatives have been reported to exhibit anti-inflammatory activity against carrageenan-induced rat paw edema [5]. The research reported here was conducted to determine whether some 2-imidazolones and 2-imidazolthiones that were substituted at the 4 and 5 positions (Table 1) have increased activity as antioxidants compared to 2-imidazolone. Some of the 2-imidazolones used in this study have been reported to have cardiotonic activity [6], and two of these are undergoing clinical trials for the treatment of congestive heart failure [7, 8]. Since ischemic heart disease is a major cause of congestive heart failure [9], and since oxygen-derived free radicals have been implicated in the pathological processes associated with myocardial ischemia [10], the antioxidant activities of these compounds could have additional benefit.

Table 1. Structures of the 2-imidazolones and the 2-imidazolthiones used in this study



	R	R_1	X	Y
1.	H	H	N	O
2.	H	CH_3	N	O
3.	CH_3	$COCH_3$	N	S
4.	CH_3	$CONH_2$	N	O
5.	CH_3	$CONH_2$	N	S
6.	CH_3	$CON(CH_3)_2$	N	O
7.	CH_3	$CON(CH_3)_2$	N	S
8.	CH_3	$COCF_3$	N	O
9.	CF_3	$COOC_2H_5$	N	O
10.	CH_3	$COOC_2H_5$	N	S
11.	H	CO *n*-C_3H_7	N	O
12.	CH_3	COC_6H_5	N	S
13.	C_2H_5	COC_5H_4N	N	O
14.	C_2H_5	COC_5H_4NO	N	O
15.	CH_3	COC_5H_4N	S	O
16.	CH_3	$COC_6H_4OCH_3$	N	O
17.	CH_3	$COC_6H_4OCH_3$	N	S
18.	CH_3	$COC_6H_4SCH_3$	N	O
19.	CH_3	$COC_6H_4SOCH_3$	N	O
20.	CH_3	$COC_6H_4SO_2CH_3$	N	O

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MATERIALS AND METHODS

Reactivity with DPPH. The reactivity of the 2-imidazolones and the 2-imidazolthiones with DPPH was carried out by a method similar to that previously used [3]. The 2-imidazolones and 2-imidazolthiones were dissolved in 95% ethanol at a concentration of 0.11 mM. The solution (2.7 ml) was placed in a cuvette, 0.3 ml of 1.0 mM DPPH in 95% ethanol was added, the sample was mixed, and the cuvette was placed in a Beckman model 25 spectrophotometer equipped with an automatic sample changer. The samples were read every minute at 517 nm. The rate of the reaction over the first 5 min was used to calculate an initial rate. Uric acid and glutathione were dissolved in deionized water at a concentration of 1.0 mM and 0.3 ml added to 2.7 ml of 0.11 mM DPPH.

Effect on methemoglobin formation. The measurement of the abilities of the 2-imidazolones and the 2-imidazolthiones to prevent the oxidation of oxyhemoglobin to methemoglobin by nitrite was carried out as described previously [11]. Blood samples from pigs and cattle were collected into heparin immediately after the animals were slaughtered. The blood was centrifuged at 2000 *g* for 20 min at 0°. The plasma and buffy coat of white cells were removed by aspiration, and the erythrocytes were washed three times with 3 vol. of phosphate-buffered saline. The washed cells were resuspended in 20 vol. of 20 mM phosphate buffer to lyse the cells. The hemolysate was centrifuged at 25,000 *g* for 60 min, and the lysate was carefully removed from the membrane pellet with a pipet. Fifty milliliters of the lysate was dialyzed against 2 liters of 20 mM phosphate buffer, pH 7.4, for 24 hr with one change of buffer. The preparations of dialyzed oxyhemoglobin from both cattle and pigs were assayed for glutathione [12], and the preparation from cattle was also assayed for 3-*N*-ribosyluric acid [13]; neither antioxidant was detected in the oxyhemoglobin after dialysis. The oxyhemoglobin (85 μM), with or without the 2-imidazolone or the 2-imidazolthione, was mixed with sodium nitrite (final concentration 0.6 mM), and the formation of methemoglobin was followed by reading the absorbance at 631 nm at 1-min intervals [14].

Protection of erythrocytes from hemolysis. The measurement of the abilities of the 2-imidazolones and the 2-imidazolthiones to protect erythrocytes from hemolysis by *t*-butyl hydroperoxide was carried out as described by Ames *et al.* [1]. Washed red blood cells from cattle or pigs and 0.2 mM *t*-butyl hydroperoxide with and without 50 μM 2-imidazolones or 2-imidazolthiones were incubated in isotonic buffer (total volume of cells, 5 ml; 25-ml Erlenmeyer flasks) for 16 hr at 37° in a New Brunswick Scientific gyrotory water bath shaker at 100 rpm. A 0.5-ml sample of the erythrocyte suspension was added to 4.5 ml of isotonic buffer containing 100 mg of NaCN and 300 mg of $K_3Fe(CN)_6$ per liter. The samples were centrifuged at 2500 *g* for 10 min to remove the intact cells, and the supernatant solution was read at 540 nm. A second 0.5-ml sample was added to the aqueous cyanide solution to give a 100% hemolysis value.

Chemicals. Uric acid and imidazole were obtained from the Sigma Chemical Co., St. Louis, MO. Reduced glutathione was from the United States Biochemical Corp., Cleveland, OH. Sodium nitrite was from the Fischer Chemical Co., Fair Lawn, NJ. The 2-imidazolones and 2-imidazolthiones used in these experiments were obtained from Merrell Dow Pharmaceuticals Inc., Cincinnati, OH.

Table 2. Reaction of 2-imidazolones and 2-imidazolthiones with 1,1-diphenyl-2-picrylhydrazyl

Compound added		ΔA_{517}/min
No addition		0.000
+100 μM	1	0.002 ± 0.001*
+	2	0.011 ± 0.001
+	3	0.658 ± 0.018
+	4	0.009 ± 0.001
+	5	1.133 ± 0.055
+	6	0.015 ± 0.002
+	7	0.923 ± 0.032
+	8	0.007 ± 0.001
+	9	0.004 ± 0.001
+	10	0.620 ± 0.053
+	11	0.002 ± 0.001
+	12	0.535 ± 0.034
+	13	0.022 ± 0.002
+	14	0.049 ± 0.001
+	15	0.002 ± 0.001
+	16	0.012 ± 0.001
+	17	0.121 ± 0.022
+	18	0.018 ± 0.003
+	19	0.022 ± 0.003
+	20	0.019 ± 0.003
+	Glutathione	0.006 ± 0.001
+	Imidazole	0.000
+	Uric acid	0.761 ± 0.034

* Values are the mean ± SD for four separate experiments.

RESULTS

Reactivity with DPPH. Antioxidants react with the stable free radical DPPH and convert it to 1,1-diphenyl-2-picrylhydrazine [15]. The change in absorbance produced in this reaction has been used to assay for antioxidants in animal tissues and to test the ability of barbiturates to act as free radical scavengers [16, 17]. Although imidazole did not react with DPPH, all of the 2-imidazolones used in this study reacted with DPPH. These results support the idea that the 2-oxo substitution on the imidazole ring is important in the antioxidant activity of these compounds. Most of the 4 and 5 substituted compounds reacted more rapidly than the parent compound 2-imidazolone, although they reacted at a much slower rate than did uric acid (Table 2). The 2-imidazolthiones (compounds 3, 5, 7, 10, 12 and 17) were much more reactive than the 2-imidazolones or glutathione, another sulfhydryl-containing compound. When a sulfur atom replaced one of the nitrogens in the imidazole ring, the thiazolone (compound 15) reacted at a slower rate than a structurally similar 2-imidazolthione (compound 13) and at the same rate as 2-imidazolone (compound 1).

Methemoglobin formation. The oxidation of

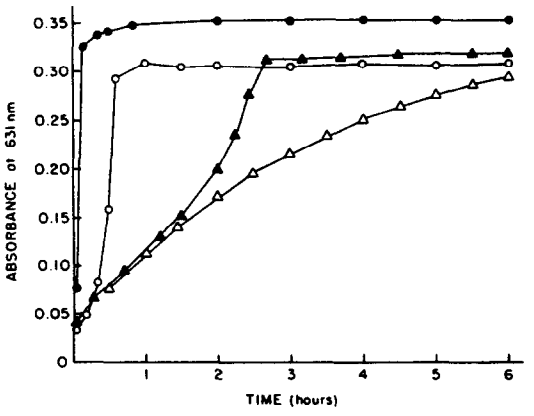

Fig. 1. Time course of methemoglobin formation (ΔA_{631}) induced by 0.875 mM sodium nitrite alone (●) and in the presence of a 6.25 μM (○), 12.5 μM (▲), or 25 μM concentration of compound 12 (△).

Table 3. Effects of 2-imidazolones and 2-imidazolthiones on the formation of methemoglobin

Compound added		Time to form 50% metHb (min)
0.6 mM Sodium nitrite		6.8 ± 1.2*
+50 μM	1	7.2 ± 2.4
+	2	20.2 ± 6.3†
+	3	120.0 ± 14.9†
+	4	5.7 ± 1.7
+	5	144.0 ± 16.0†
+	6	6.1 ± 1.4
+	7	142.0 ± 14.4†
+	8	8.7 ± 5.6
+	9	10.5 ± 4.6
+	10	156.0 ± 13.7†
+	11	4.6 ± 3.5
+	12	151.0 ± 14.8†
+	13	5.7 ± 1.6
+	15	3.7 ± 1.0†
+	16	5.2 ± 1.3
+	17	149.8 ± 18.1†
+	18	3.3 ± 1.0†
+	19	3.4 ± 1.0†
+	20	7.8 ± 3.5
+	Uric acid	137.0 ± 6.8†
+	Imidazole	7.8 ± 1.8

* Values are the mean ± SD from four experiments.
† Statistically significant difference ($P < 0.05$) from oxyhemoglobin incubated with nitrite alone (two-tailed Student's *t*-test).

oxyhemoglobin to methemoglobin by sodium nitrite occurs in two stages. There is an initial, slow phase followed by a rapid, autocatalytic phase which carries the reaction to completion [11, 18]. The autocatalytic phase is depicted in Fig. 1. Most of the 2-imidazolones had either no effect or they accelerated (compounds 15, 18 and 19) the rate of formation of methemoglobin by sodium nitrite (Table 3). The 2-imidazolthiones (compounds 3, 5, 7, 10, 12 and 17) inhibited the formation of methemoglobin. Less than 50% of the oxyhemoglobin was converted to methemoglobin in 50 min in the presence of the 2-imidazolthiones, whereas the control was oxidized completely in 10 min.

In studies carried out to determine the effect of the 2-imidazolthiones on the rate of methemoglobin formation in the presence of 0.6 mM nitrite, it was found that concentrations as low as 6.25 μM decreased this rate (Fig. 1). At this concentration of any of the 2-imidazolthiones tested, the oxidation of oxyhemoglobin by nitrite showed a delay in the formation of methemoglobin, but the two stages were still distinct. At a concentration of 25 μM of any of the 2-imidazolthiones, the oxidation was curvilinear over 6 hr of incubation, and two stages were not seen.

Erythrocyte lysis. Urates have been shown to protect erythrocytes from hemolysis produced by *t*-butyl hydroperoxide [2, 19]. Except for imidazole, compound 11, and compound 15, all of the 2-imidazolones and 2-imidazolthiones used in this study protected both cattle and pig erythrocytes from hemolysis by 0.2 mM *t*-butyl hydroperoxide (Table 4). The data shown in Table 4 are for pig erythrocytes.

Table 4. Effects of 2-imidazolones and 2-imidazolthiones on hemolysis of pig erythrocytes

Compound added		% Hemolysis
No addition		6 ± 1*
+200 μM *t*-Butyl hydroperoxide		76 ± 15
+50 μM	1	25 ± 10†
+	2	15 ± 5†
+	3	22 ± 6†
+	4	23 ± 4†
+	5	29 ± 5†
+	6	27 ± 7†
+	7	31 ± 19†
+	8	14 ± 2†
+	9	15 ± 6†
+	10	19 ± 5†
+	11	75 ± 16
+	12	24 ± 7†
+	13	36 ± 6†
+	15	60 ± 13
+	16	47 ± 16†
+	17	24 ± 7†
+	18	44 ± 7†
+	19	26 ± 10†
+	20	26 ± 13†
+	Imidazole	67 ± 16
+	Uric acid	18 ± 6†

* Values are the mean ± SD from four experiments.
† Statistically significant difference ($P < 0.05$) from the erythrocytes treated with 200 μm *t*-butyl hydroperoxide (two-tailed Student's *t*-test).

DISCUSSION

This study was undertaken to ascertain whether some 2-imidazolones and some of their sulfur-containing analogues have antioxidant properties, and how they compared to urates in this regard. Three systems were used to test these compounds. The first of these was their reaction with DPPH. The 2-imidazolones, the imidazothiones, and a thiazolone

all reacted with DPPH. However, the imidazolones and the thiazolone reacted at a rate similar to glutathione and much slower than that of uric acid. The 2-imidazolthiones, however, reacted at rates greater than that of the 2-imidazolones or of glutathione. The high reactivity seems dependent on the presence of a free sulfhydryl group since the thiazolone used in these studies had very low activity with DPPH. At physiological pH, the sulfur of the imidazolthiones would be in the sulfhydryl form. 1-Methyl-2-mercaptoimidazole (methimazole), an antithyroid agent with several structural features related to the imidazolthiones used in this study, was reported recently to protect sensitive enzymes from inactivation by $OH\cdot$ and I_2^- [20]. The authors suggested that methimazole may react directly with these free radicals. It also reacted rapidly with DPPH under the conditions used here.

The imidazolones did not protect oxyhemoglobin from oxidation to methemoglobin by nitrite. In some cases the 2-imidazolones stimulated this oxidation. The reason for this stimulation is unknown. Uric acid has been shown to stimulate the inactivation of alcohol dehydrogenase by the hydroxyl radical and the rate of oxidation of oxyhemoglobin produced by *t*-butyl hydroperoxide [19, 21]. It was suggested that the radical formed in the reaction of these compounds with uric acid was responsible for the stimulation. The protection afforded by the 2-imidazolthiones was at concentrations which were much less than those of the nitrite present. The ability of these compounds to protect at this low molar ratio may be a result of their reacting with the hydrogen peroxide or superoxide formed by the oxidation of oxyhemoglobin rather than a direct reaction with the nitrite, as has been suggested for the protection by uric acid [11]. This reaction would prevent the onset of the autocatalytic phase of the reaction, but the first stage reaction might not be inhibited by these agents.

Most of the 2-imidazolones protected both porcine and bovine erythrocytes from hydrolysis by *t*-butyl hydroperoxide, which supports the view that these compounds have antioxidant properties. However, the antioxidant properties of the cardiotonically active 2-imidazolones (piroximone and enoximone, compounds 13 and 18) may be too weak to be of any biological significance [6, 7]. Nevertheless, the antioxidant activity of some of the other 2-imidazolones as well as that of the more potent 2-imidazolthiones may have biological usefulness. Further studies will be required to ascertain whether the 2-imidazolthiones and 2-imidazolones have important *in vivo* activity as antioxidants.

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