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Antioxidant activity of thioureidic derivatives I

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Abstract

Recent developments in biomedical science have shown that free radicals are involved in many diseases. They attack the unsaturated fatty acids in the biomembrane resulting in membrane lipid peroxidation, which is strongly connected to aging, carcinogenesis and atherosclerosis. Free radicals also attack DNA and cause mutation leading to cancer. In addition lipid peroxidation is an important factor of deterioration in the processing and storage of food. Therefore, it is important to search for new effective radical scavengers (Sci. Rev. 2 (1997) 152; J. Nat. Prod. Rev. 63 (2000) 1035). In this manuscript we describe the antioxidant activity of new thioureidic compounds.

Keywords: Thioureidic compounds; Antioxidation activities

1. Introduction

Several studies have investigated the in vitro biological activity of synthetic thioureidic derivatives and have established important structure–activity relationships. Thus, we have investigated the free-radical scavenging and antioxidant effectiveness of synthetic thioureidic derivatives, which demonstrated antiamebic activity, by using different in vitro methods:

1) in aqueous phase, detection of the relative antioxidant capability to scavenge the chromogenic radical cation 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonate ($ABTS^{+\bullet}$) in comparison with Trolox and expressed as TEAC [1,2]. $ABTS^{+\bullet}$, cation radical, is a blue–green cromogen with a characteristic absorption at 734 nm. The antioxidant activities of compounds are expressed as TEAC, trolox equivalent antioxidant capacity;
2) heat-induced oxidation of an acqueous emulsion system of β-carotene and linoleic acid was measured by the method described by Pratt (1992) [3];
3) superoxide anion enzymatic generation assay. Superoxide anion was generated in an enzymatic system by preparing a mixture of xanthine and xanthine oxidase [4];
4) xanthine oxidation inhibition assay; in this assay xanthine oxidase activity was evaluated by the spectrophotometric measurement of the formation of uric acid by xanthine [4].

2. Materials and methods

2.1. Chemicals

ABTS (2,2′-azinobis-3-ethilbenzothiazoline-6 sulfonic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, linoleic acid, Tween 20, butylhydroxytoluene, β-carotene, EDTA, bovine serum albumin (BSA), nitroblue–tetrazolium (NBT), xanthine, xanthine oxidase (XOD), sodium carbonate, sodium phosphate monobasic and sodium phosphate dibasic were obtained by Sigma Aldrich (Gillingham, Dorset, UK). The solvents were obtained by Carlo Erba reagent. Nanopure water was prepared by Milli-Q apparatus.

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Melting points were determined by a Kofler apparatus and are reported in Table 5, ^{1}H NMR spectra were recorder on a Bruker DRX 600 spectrometer in DMSO-d_6 solvent. Chemical shift, expressed as d (ppm), are shown in Table 6. The purity of the compounds was checked by thin layer chromatography (TLC), using ethyl acetate as eluente: each compound was considered pure when a chromatographic run of the recrystallised sample gave a single spot in the TLC plate (Table 5).

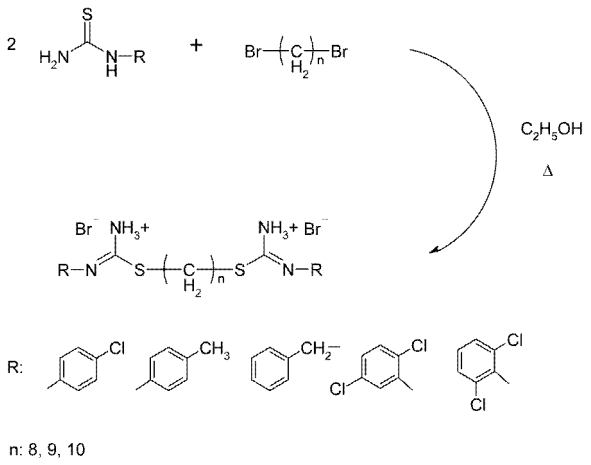
2.3. Preparetion of 1–6 compounds

The compounds 1–6 (see Fig. 1) have benn synthesized as follows. Two grams of the suitable thiourea and dibromoalkane (1/0.5) was refluxed in anhydrous ethanol for 4–5 h. The reaction mixture was evaporated under vacuum. The residue was crystallised from acetonitrile solvent, to give the bis-thioureidic salts (Scheme 1).

2.4. Antioxidant activity

2.4.1. Free radical scavenging assay of pure compound

In the way of investigate the free radical scavenging activity of synthetic thioureidic derivatives, we used the TEAC assay, based on the ability of the antioxidant to scavenge the $ABTS^{+\bullet}$ (Table 1), according to Re et al. [5–7]. $ABTS^{+\bullet}$ cation radical was produced by the reaction between ABTS 7 mM in water and potassium persulfate 2.45 mM, stocked in the dark at room temperature for 12 h. $ABTS^{+\bullet}$ is a blue–green cromogen with a characteristic absorption at 734 nm. The $ABTS^{+\bullet}$ solution was diluted with PBS, pH 7.4, to an absorbance of 0.70 at 734 nm and equilibrated at 30 8C. Samples were diluted with methanol to have 0.3, 0.5, 1, 1.5, 2 solutions. The reaction was enhanced by the addition of 1 ml of diluted ABTS to 10 μl of each solution of sample or Trolox (standard), or 10 ml of methanol (blank). The determination was repeated three times for each sample solution. The percentage inhibition of absorbance at 734 nm was calculated for each concentration in function of the blank's absorbance. The percentage inhibition was plotted as a function of concentration compound or standard. The antioxidant activities of compounds expressed as TEAC. The TEAC value is defined as the concentration of standard Trolox solution with equivalent percentage inhibition to a 1 mM concentration solution of the compound after investigation.



Scheme 1.

Table 1
Trolox equivalent antioxidant capacity (mM) of synthetic thioureidic derivatives [a,b]

	TEAC
Compound 1	0.3740 ± 0.0010
Compound 2	0.0857 ± 0.0051
Compound 3	0.2150 ± 0.0020
Compound 4	0.3473 ± 0.0045
Compound 5	0.0683 ± 0.0064
Compound 6	0.0747 ± 0.0032

[a] Mean ± SD of three determinations.
[b] Trolox equivalents.

2.4.2. Autoxidation of b-carotene [3,4]

2.4.2.1. Autoxidation of b-carotene. Heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid was measured by the method described by Pratt (1992) [7–9]. Quantity of linoleic acid (20 mg) and Tween 20 (200 mg) were placed in a flask, and a solution of 2 mg of β-carotene in 10 ml of $CHCl_3$ was added. After removal of $CHCl_3$, 50 ml of oxygenated distilled water were added. Aliquots of each compound (200 μl), dissolved in ethanol to a 15 μg/ml solution, were added to each flask with shaking. Samples without test compounds were used as blanks and sample with 2,6 di-tert-butyl-4-methylphenol. BHT was used as a control substance. Samples were subjected to the oxidation, by placing in an oven at 50 8C for 3 h. The absorbance was read at 470 nm at regular intervals to monitor the rate of bleaching of β-carotene. The antioxidant activities was expressed as AA (Table 2), calculated with the equation:

$$\text{Inhibitory ratio (AA)} = 100[1 - A_0 - A_t]/A_{00} - A_{0t}$$

where: A_0 is the absorbance at the beginning of the incubation, with compound; A_t is the absorbance at the time t, with compound; A_{00} is the absorbance at beginning of the incubation, without compound; A_{0t} is the absorbance at the time t, without compound.

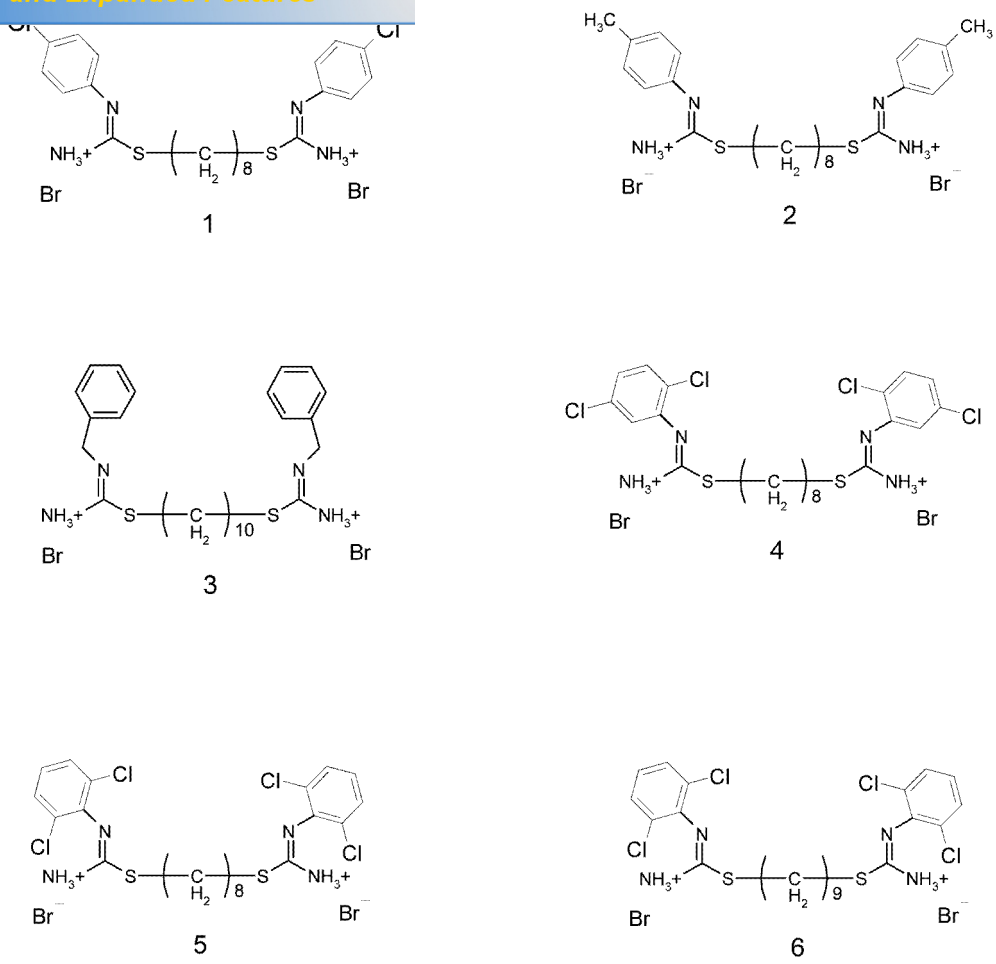


Fig. 1.

2.4.3. Superoxide anion enzymatic generation assay [3,4]

Superoxide anion was generated in an enzymatic system by preparing a mixture of xantine and xantine oxidase, as indicated in literature [8–12]. The reaction mixture included 0.1 mM EDTA, 50 μg/ml bovine serum albumine (BSA), 25 μM nitroblue tetrazolium, 0.1 mM xanthine and 3.3×10^{-3} U xanthine oxidase (XOD) in 40 mM sodium carbonate buffer (pH 10.2) in a final volume of 3 ml. After incubation at 25 8C with increasing concentrations of samples, the absorbance of formazan produced was determined at 560 nm.

The inhibitory effects of samples on the generation of superoxide anion were estimated by the equation:

$$\text{Inhibitory ratio} = (A_0 - A_1) \times 100 / A_0$$

where: A_0 is the absorbance with no addition of sample; A_1 is the absorbance with addition of sample.

The inhibitory ratio for each sample was plotted as a function of the concentration, then was calculated the IC_{50} value, with the statistical method of linear regression.

Table 2
Inhibition of autoxidation of β-carotene, AA%

	1 [a] h	2 [a] h
Compound 1	53.08	31.161
Compound 2	45.64	27.479
Compound 3	42.98	23.229
Compound 4	41.47	25.439
Compound 5	0	
Compound 6	0	
BHT	71.85	64.451

[a] Positive control.

2.4.4. Xanthine oxidation inhibition assay[4]

Xanthine oxidase inhibition activity was evaluated by the spectrophotometric measurement of the formation of uric acid by xanthine, as described in literature [9–11]. A 100 μM solution of xanthine in 0.1 M phosphate buffer pH 7.8 with 0.04 U/ml of xanthine oxidase was incubated for 10 min at room temperature and read at 295 nm against a blank sample which did not contain the enzyme. Various concentrations of testing compounds were added to samples before the enzyme has

Superoxide anion scavenging activity [a,b]

	IC_{50} (μM/l)
Compound 1	75.3±2.1
Compound 2	124.7±2.0
Compound 3	38.0±0.4
Compound 4	17.3±0.4
Compound 5	26.2±2.8
Compound 6	17.9±0.5

[a] Mean±SD of three determinations.
[b] SOD equivalents.

been instilled and their effect on the generation of uric acid was used to calculate regression lines and IC_{50} value.

3. Results

3.1. Free Radical scavenging assay

Trolox (a water soluble vitamin E analogue) equivalent antioxidant capacity (TEAC) has been used to determine the radical scavenging abilities of synthetic thioureidic derivatives as electron or $H^{\bullet}$ donating agents throughout their ability to scavenge $ABTS^{+\bullet}$ The TEAC value resulting for compounds 1–6 are summarized in Table 1.

3.2. Linoleic acid autoxidation assay

The antioxidant effect of synthetic thioureidic derivatives was also measured by autoxidation of β-carotene assay. The value of antioxidant activity (AA) measured a $t = 60$ and 120 min for compounds, employing bleaching of β-carotene as a model system, are showed in Table 2.

3.3. Superoxide anion enzymatic generation assay

Superoxide anion is one of the more aggressive reactive oxigen species (ROS), product in human organism. Superoxide anion destroy endothelium derived relaxing factor (EDRF) while its products, hydroxyl radicals and lipid peroxides inhibit prostacyclin generation. Phenolic compounds like flavonoids have been shown to scavenge free radicals and their vasoprotective action has been associated with this particular property.

Using an enzymatic biological generator of superoxide anion we have compared the free radical scavenging activity of synthetic thioureidic derivatives with the data reported in literature for flavonoids and other antioxidant compounds. The xanthine oxidase system generated superoxide anions as measured by the reduction of NBT, this reaction was inhibited from SOD in a concentration-dependent mode. The compounds investigated have inhibited the development of the colour produced during the reaction of superoxide anion with NBT, in a moderate range of activity. The IC_{50} value is inferior to 50 μg/ml (Table 3).

3.4. Xanthine oxidase activity assay

In the way of exclude the hypothesis that the superoxide anion scavenging activity was a result of an inhibition of xanthine oxidase enzymatic system, we have investigated the activity of the six compounds as inhibitors against the product of uric acid from xanthine in the oxidation reaction catalyzed from xanthine oxidase.

We used a simple spectrophotometric assay, that permitted to measure the production of uric acid from xanthine. Finally synthetic thioureidic derivatives showed a good activity as inhibitors against the product of uric acid from xantine in the oxidation reaction catalysed from xantine oxidase, that partially explain the reduced production of superoxide anion. Data are shown in Table 4.

4. Discussion

A fascinating hypothesis raised in the past few years is that the health-promoting action of some foods could be due to the presence of nonessential components, such as polyphenols (many with antioxidant potential) that could contribute to the modulation of the oxidative balance in vivo

On the other hand reactive oxygen species (ROS) are considered related to many diseases, including atherosclerosis, liver injury, aging, inflammation, neurovegetative diseases, and cancer.

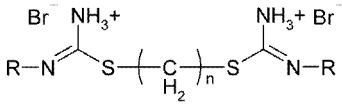
The present study deals with the activity of synthetic thioureidic derivatives in several in vitro systems for evaluate the mechanism of their antioxidative effects.

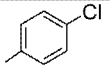
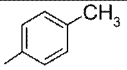
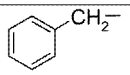
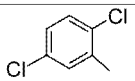
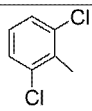
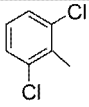
Table 4
Xanthine oxidase activity inhibition [a]

	IC_{50} (μM/l)
Compound 1	31.2±0.2
Compound 2	10.6±1.1
Compound 3	17.3±1.3
Compound 4	13.5±2.0
Compound 5	10.2±0.3
Compound 6	16.6±1.8

[a] Mean±SD of three determinations; superoxide dismutase inactive at 1–100 units/ml.

Physical and analytical data of compounds 1–6



N° Composto	n	R	Melting point °C	Yield %
1[12]	8		oil	81
2	8		215	70
3	10		oil	55
4	8		156	75
5	8		208	83
6	9		132	60

Crystallisation solvent: acetonitrile

Crystallisation solvent: acetonitrile.

We have used different models for studying antioxidant activity of synthetic thioureidic derivatives.

Synthetic thioureidic derivatives showed a low activity as radical scavengers. The presence of methyl substituent on aromatic rings give an increment of the activity in this experimental model, as a matter of their capacity of working as terminator in the radical chain.

In the model of autoxidation of linoleic acid, the data show that these compounds have a interesting activity at concentration of 12 μg/ml, in comparison with synthetic

Table 6
^{1}H NMR date (d ppm) of compounds 1–6 (DMSO-d_6) and signal attribution.

Compound 1	1.62–1.27 (m, 12H, 6CH_2); 3.34–3.32 (m, 4 H, 2S–CH_2); 7.44–7.38 (8H, H–Ar)
Compound 2	1.25–1.20 (s, 6H, 2CH_3); 1.68–1.30 (m, 12H, 6CH_2); 3.32–3.25 (m, 4H, 2S–CH_2); 7.45–7.35 (8H, H–Ar)
Compound 3	1.30–1.25 (m, 16H, 8CH_2); 3.32–3.25 (m, 4H, 2S–CH_2); 3.40–3.48 (M, 4H, 2CH_2); 7.40–7.30 (10H, H–Ar)
Compound 4	1.70–1.28 (m, 12H, 6CH_2); 3.30–3.20 (m, 4H, 2S–CH_2); 7.45–7.50 (2H, H–Ar); 7.80–7.84 (1H, H–Ar)
Compound 5	1.62–1.30 (m, 12H, 6CH_2); 3.20–3.45 (m, 4H, 2S–CH_2); 7.45–7.47 (3H, H–Ar)
Compound 6	1.65–1.33 (m, 14H, 7CH_2); 3.33–3.27 (m, 4H, 2S–CH_2); 7.40–7.65 (3H, H–Ar)

this test.

In the superoxide anion generation assay, synthetic thioureidic derivatives have a low activity. On the other hand it is important to observe synthetic thioureidic derivatives were active in direct inhibition of xanthine oxidase.

The antioxidant effectiveness of the studied compounds seems to be related to the presence of aliphatic substituents in the aromatic rings. In the future, further studies with different substituents will be performed.

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