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RADICAL SCAVENGING CAPACITY OF *N*-(2-MERCAPTO-2-METHYLPROPIONYL)-L-CYSTEINE: DESIGN AND SYNTHESIS OF ITS DERIVATIVES WITH ENHANCED POTENTIAL OF SCAVENGE HYPOCHLORITE

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ABSTRACT

High-molar-mass hyaluronan (HA) – glycosaminoglycan composed of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid linked by β-(1→4) and β-(1→3) glycoside bonds – is an essential component of the extracellular matrix in many tissues. It is rather susceptible to free-radical mediated degradation resulting in various physiological and pathological consequences. In the present study, we investigated the effect of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine against free-radical degradation of high-molar-mass HA. We applied rotational viscometry to record time-dependent changes of the dynamic viscosity of HA solutions, which reflected a decrease of HA molar-mass and thereby its fragmentation. The oxidative degradation of high-molar-mass hyaluronan was evoked by the Weissberger biogenic oxidative system comprising Cu(II) and ascorbate. Previously, we proved that stage I of oxidative HA degradation was mediated predominantly by hydroxyl radicals while stage II by peroxyl-type radicals. To determine the scavenging activity of the compound tested, ABTS and DPPH assays were used. Oximetry was used to monitor the consumption of oxygen by the treated HA solutions.

In this study, rotational viscometry demonstrated a significant ability of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine to protect high-molar-mass HA from oxidative degradation in both stage I and II. This implies that this substance may possess a certain scavenging activity towards hydroxyl- and peroxyl-type radicals. Furthermore, the IC_{50}

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values obtained by the ABTS and DPPH assays revealed a high free-radical scavenging activity of this substance. Oxygen consumption in the reaction mixture containing the Weissberger biogenic oxidative system was found to occur along the very similar trajectory as the curve recorded by rotational viscometry. After addition of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine into the reaction mixture, consumption of oxygen was significantly inhibited. These results may indicate that the possible mechanism of action of this compound, revealed as an effective remedy in treating arthritic joints, could be its free-radical scavenging activity and hence the ability to protect high-molar-mass HA of synovial fluid from its oxidative degradation. In the last part, based on existing literature, we discuss the design and synthetic routes towards the preparation of a new derivative of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine. This novel compound should react with another reactive species formed during oxidative stress, the hypochlorite, plausibly damaging HA, while the beneficial activity of the parent molecule, *i.e.* the ability to scavenge hydroxyl- and peroxyl-type radicals, would be maintained. Preliminary synthetic results are also described.

Keywords: ABTS, DPPH, extracellular matrix, glycosaminoglycans, oximetry, rotational viscometry, thiols

1. INTRODUCTION

Many human diseases are associated with harmful actions of reactive oxygen species (Halliwell and Gutteridge, 1989). These species are involved in oxidative modification and damage of essential biomacromolecules *in vivo*. Of them, high-molar-mass hyaluronan (HA) is of particular interest (Jiang et al., 2011). The reported reduction of HA molar-mass in the synovial fluid of patients suffering from arthritic diseases led to *in vitro* studies on HA degradation by reactive oxygen species (Parsons et al., 2002). One of the earliest investigations was carried out by Pigman et al. (1961) and since then numerous studies have investigated the action of various reactive oxygen species (ROS) on HA.

Hyaluronan (Figure 1) is a glycosaminoglycan composed of repeating disaccharide units formed by *N*-acetyl-D-glucosamine and D-glucuronic acid. These disaccharide units are linked together by β-(1–3) and β-(1–4) glycosidic bonds (Meyer, 1934, Laurent et al., 1995, Noble, 2002, Jiang et al., 2011, Erickson, 2012). HA is a main component of extracellular matrix in many tissues and organs, e.g. the skin, vitreous humor, trachea, heart valves, and synovial joints. The extracellular matrix represents a foundation of tissue microenvironments, which provide biophysical and biochemical cues that maintain tissue architecture integrity (Veiseh and Turley, 2011).

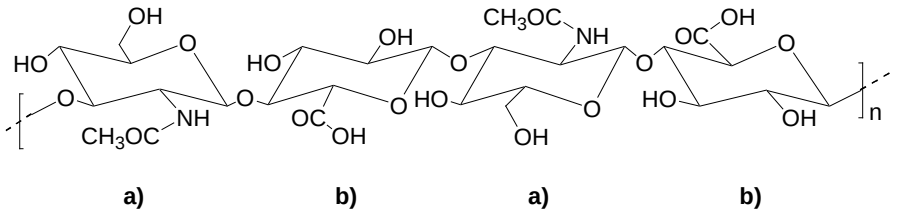


Figure 1. Chemical structure of a disaccharide unit of hyaluronan: a) *N*-acetyl-D-glucosamine, b) D-glucuronic acid.

Components of the extracellular matrix include glycosaminoglycans, collagen and other fibrous proteins, glycoproteins, and specialized polysaccharides that form a jellylike or watery "ground substance". Glycosaminoglycans, for example chondroitin sulfate, heparan sulfate, dermatan sulfate, and also hyaluronan, are major components of the extracellular matrix molecules (Table 1) (DeAngelis, 2002, Dreyfuss et al., 2009, Hull et al., 2012).

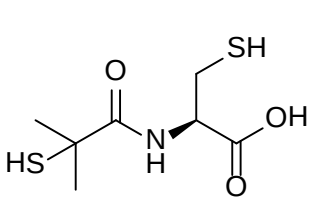
Table 1. Types of glycosaminoglycans

NAME	Approx. M_w (Da)	COMPONENTS		
		SACCHARIDE	SULFATE	PROTEOGLYCANS
Chondroitin sulfate	$(10–50) \times 10^3$	Glucuronic acid Galactosamine	✓	✓
Heparan sulfate	$(10–50) \times 10^3$	Glucuronic acid Iduronic acid Glucosamine	✓	✓
Dermatan sulfate	$(10–50) \times 10^3$	Iduronic acid Galactosamine	✓	✓
Keratan sulfate	$(5–15) \times 10^3$	Galactose Glucosamine	✓	✓
Hyaluronan	$10^5–10^7$	Glucuronic acid Glucosamine	—	—

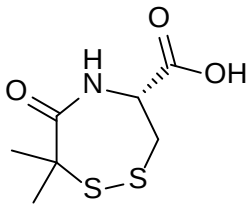
✓ present
— absent

Glycosaminoglycans are complex polysaccharides with important roles in various physiological events, such as cell growth, morphogenesis, differentiation, cell migration and bacterial/viral infections (Lee and Spicer, 2000, Yamada, 2011).

N-(2-Mercapto-2-methylpropionyl)-L-cysteine (1), illustrated in Figure 2, is clinically used for treatment of rheumatoid arthritis and inflammation diseases (Horwitz, 2003). It has been confirmed that this drug is efficacious in reducing acute phase of inflammation and oxidative stress. A plausible mechanism of its action is the formation of a 7-membered cyclic disulfide – (*R*)-7,7-dimethyl-6-oxo-1,2,5-dithiazepane-4-carboxylic acid (2) in response to reaction with oxidants such as hydroxyl and other radicals (Figure 2).



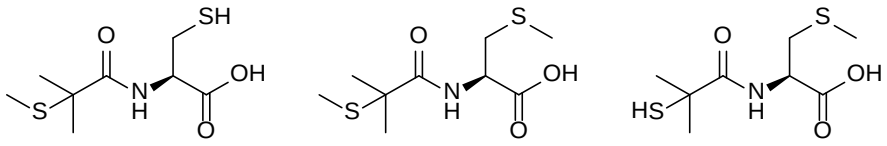
1 (M = 223.31)



2 (M = 221.31)

Figure 2. Chemical structure of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine (1) and corresponding 7-membered cyclic disulfide (2).

N-(2-mercapto-2-methylpropionyl)-L-cysteine is metabolized *in vivo* to cyclic disulfide **2** (Figure 2) and *S*-methyl- and *S,S'*-dimethyl derivatives **3** and **4**, respectively (Figure 3).



3 (M = 237.31) **4** (M = 251.31) **5** (M = 237.31)

Figure 3. Chemical structures of *S*-methylated *N*-(2-mercapto-2-methylpropionyl)-L-cysteine derivatives.

Together with the parent structure, these compounds have been investigated in connection with their immunomodulating effects (Matsuno et al., 1998) as well as in respect to their potential to improve impaired angiogenesis *via* inducing synthesis of vascular endothelial growth factor (VEGF) (Distler et al., 2004).

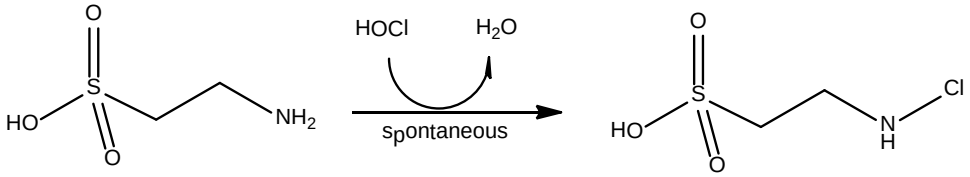
Interaction of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine (**1**) and its derivatives **3**, **4**, **5** (Figure 3) with human serum albumin (HSA) has been examined in a study by Narazaki et al. (1996), aimed to elucidate the reaction mechanism of covalent binding between HSA and thiol containing compounds.

The ability of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine and its oxidized derivative **2** to induce glutathione biosynthesis *via* activation of the antioxidant-response element (ARE) has also been studied and possible chemopreventive properties of these compounds against cancer have been suggested (Wielandt et al., 2006).

Together with reactive oxygen species produced by neutrophilic polymorphonuclear leukocytes, HOCl and taurine-*N*-monochloramine (TauCl, **7**, Scheme 1) are also end-products of the neutrophilic polymorphonuclear leukocyte respiratory burst (Mainnemare et al., 2004).

In human neutrophils, HOCl is generated from hydrogen peroxide by the action of myeloperoxidase in the presence of chloride anion (Weiss et al., 1982). Hypochlorous acid activates tyrosine kinase signaling cascades, leading to increased production of extracellular matrix components, growth factors, and inflammatory mediators.

HOCl also increases the capacity of α_2-macroglobulin to bind TNF-alpha, IL-2, and IL-6. As the oxygen-chlorine bond is highly polarized, HOCl is the source of electrophilic Cl atom. The reaction of this bond with endogenic substrates can result in generation of toxic intermediates, which may eventually lead to cell death (Thomas et al., 1983, Grisham et al., 1984).



6 (M = 125.15) **7** (M = 159.6)

Scheme 1. Formation of taurine-*N*-monochloramine.

Relatively stable TauCl (**7**) is formed in a reaction of HOCl with taurine (**6**), which is also generated by neutrophils. Compared to HOCl, TauCl is less toxic and inhibits the production of inflammatory mediators, prostaglandins, and nitric oxide (Mainnemare et al., 2004).

For targeting oxygen radicals and hypochlorous acid at the same time, the two compounds – *N*-(2-mercapto-2-methylpropionyl)-L-cysteine and taurine might be administered simultaneously. However, such application has certain limitations, mainly due to different distribution and hence bioavailability of these distinct compounds within the body compartments. It is desirable to have both functions in one single molecule which would be then able to deliver both effects to the target site at once. Therefore, we turned our focus to extend the activity of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine by the ability of taurine to scavenge HOCl.

Accordingly, the aims of the present study were (i) to establish the scavenging activity of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine by the ABTS and DPPH decolorization assays; (ii) to study the mode of action of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine *in vitro* by rotational viscometry; (iii) to assess the effect of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine on the degradation of HA by means of oximetry, *i.e.* to compare the consumption of oxygen in the HA solutions containing the Weissberger´s biogenic oxidative system in the presence and absence of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine; and (iv) to design and synthesize a *N*-(2-mercapto-2-methylpropionyl)-L-cysteine derivative capable of scavenging HOCl by combining the key structural features of the parent molecule and taurine. The original activity of the parent drug has to be retained and molar-mass of the resulting derivative should not exceed 500 Da.

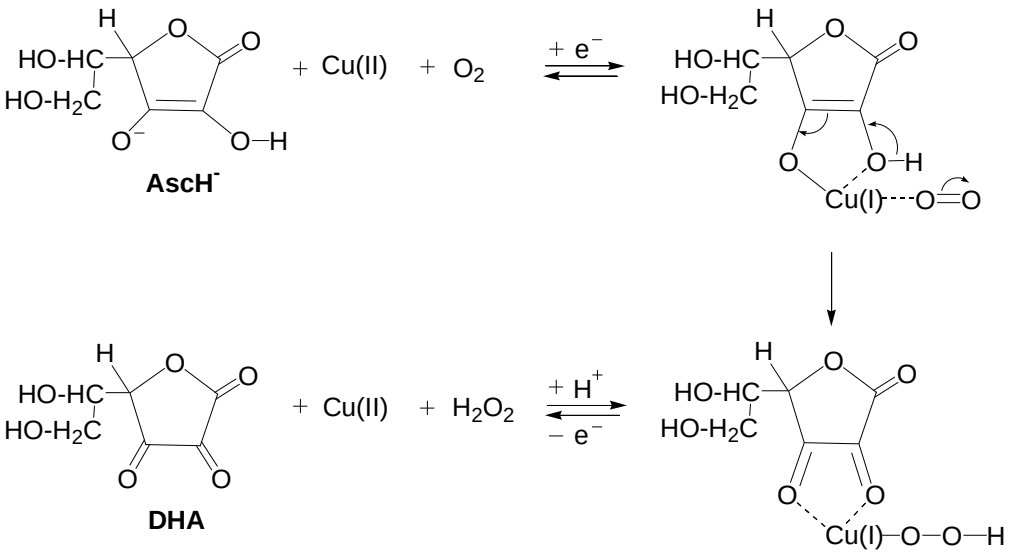
2. RESULTS AND DISCUSSION

Oxygen is an indispensable element for aerobic organisms. It is required especially for energy metabolism. Sufficient tissue oxygenation is therefore a prerequisite for adequate energy production that is essential for the maintenance of key cell functions (Schreml *et. al.*, 2010). On the other hand, utilization of the electronegative potential of molecular oxygen results also in a certain production of reactive oxygen species and nitrogen species (RNS). Overproduction of ROS, RNS and reactive chlorous species may lead to oxidative stress. In the inflammatory reaction, ROS are among the products released by activated neutrophils, monocytes, macrophages, endothelial cells and fibroblasts into the affected tissue (Schreml *et. al.*, 2010). In the acute phase of inflammation, the targeted treatment with antioxidants may be expected to modulate the increase of ROS and/or RNS towards their baseline levels. Our present study suggests that compound 1 may act in this manner.

2.1. Weissberger Biogenic Oxidative System

As an established method for the initiation of radical degradation, we decided to employ the Weissberger oxidative system under biogenic conditions (Scheme 2). Reaction between $AscH^-$ and Cu(II) in the presence of oxygen gives hydrogen peroxide and since ascorbate

reduces Cu(II) to cuprous ions it is feasible to assume that Cu(I) and hydrogen peroxide generates hydroxyl radicals (Valachová et al., 2010a).



2 Cu(I) + H_2O_2 → 2 Cu(II) + $^{\bullet}OH$ + OH^- (Fenton-like reaction)

Scheme 2. The Weissberger biogenic oxidative system. $AscH^-$ and DHA denote ascorbate anion and dehydroascorbate, respectively.

The $^{\bullet}OH$ radicals abstract proton from the HA macromolecule. The resulting HA (macro)radical ($A^{\bullet}$) reacts under aerobic conditions with molecular oxygen yielding a secondary peroxyl (macro)radical (A-O-$O^{\bullet}$).

The latter participates in the phase of propagation of free-radical-mediated oxidative degradation of HA (Kvam et al., 1993; Greenwald et al., 1980; Wong et al., 1981; Saari et al., 1993; Hawkins et al., 1996).

2.2. Rotational Viscometry

Figure 4 illustrates changes of the dynamic viscosity of the HA solution due to a pro-oxidative action of the two reactants, namely Cu(II) and ascorbic acid. After a few-minute induction period the initial value of the HA solution dynamic viscosity equaling 11.48 mPa·s started to gradually decrease.

After a 5-h measurement, dynamic viscosity of the HA solution reached the value 7.35 mPa·s. As already proved, the decrease of the HA solution viscosity is a result of the degradation of high-molar-mass HA, whose initial M_w value equals 970 kDa. The rate of decrease of the dynamic viscosity value in the basic experimental set is sufficiently high to be used as the probe reference.

This reference curve (Figure 4, curve 0) is depicted also in the next figures (Figure 5 and 6, curve 0).

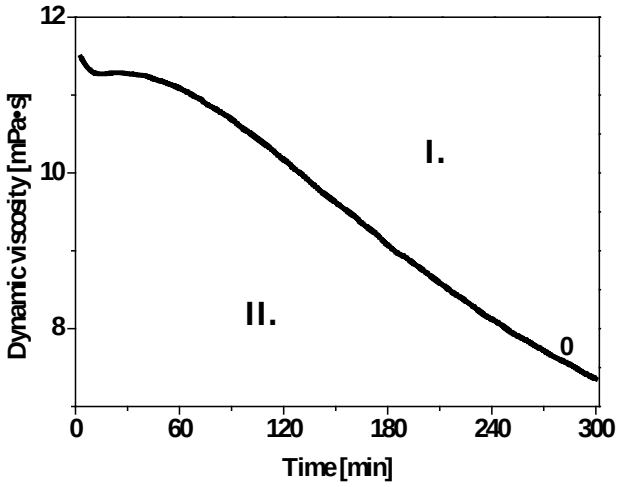


Figure 4. Free-radical degradation of high-molar-mass HA by the action of the pro-oxidative system containing 1 μM Cu(II) *plus* 100 μM ascorbate (curve 0).

It can be expected that lowering of the concentration ratio of cupric chloride/ascorbate, e.g. by applying higher concentrations of Cu(II) solutions, would lead to increasing the rate of HA degradation, reflected by the probe reference. Indeed, any pro-oxidative action of a substance added into the reference oxidative system would results in a greater decline of the curves situated within area II. (Figure 4). On the contrary, any anti-oxidative effect of a compound added into the same system would cause a retardation of the dynamic viscosity drop.

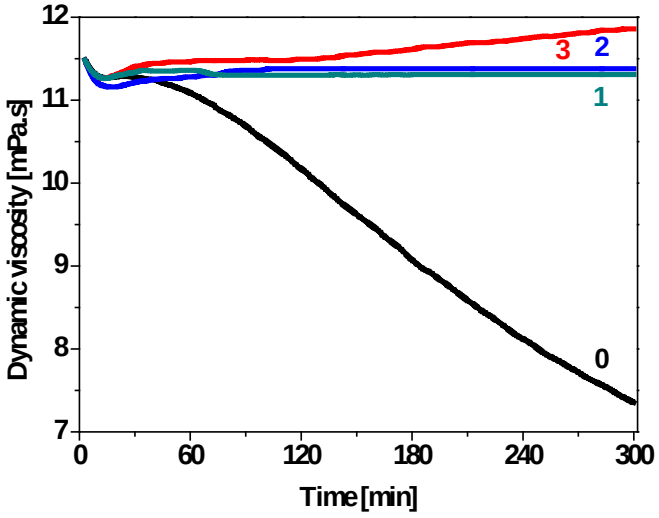


Figure 5. Effect of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine on HA degradation induced by the Weissberger biogenic oxidative system – 1.0 μM Cu(II) and 100 μM ascorbate (curve 0). Concentrations of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine in the reaction mixture were 1, 10 and 100 μM, curves 1, 2 and 3, respectively. The drug was added before the onset of the HA oxidative degradation.

In a limiting situation providing that the high-molar-mass HA is completely protected against any degradation, we can speak about a total inhibitory action of the admixed anti-oxidative compound with resulting curves situated in area I (Figure 4). Hence, area II. represents the action of the compounds functioning in a pro-oxidative mode, while area I. is represented by the action of the compounds acting in an anti-oxidative mode.

Our results suggest that addition of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine (1, 10 and 100 μM) at the beginning of free-radical degradation of high-molar-mass HA led to a complete inhibition of HA degradation (Figure 5).

Similar effects of this substance were observed when it was added to the reaction mixture 1 hour after the onset of HA oxidative degradation (Figure 6). The compound tested acted in an anti-oxidative mode. These results demonstrated the ability of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine to act as an effective scavenger of reactive oxygen species, such as $^{\bullet}$OH and peroxyl-type radicals (Figure 5 and 6, respectively).

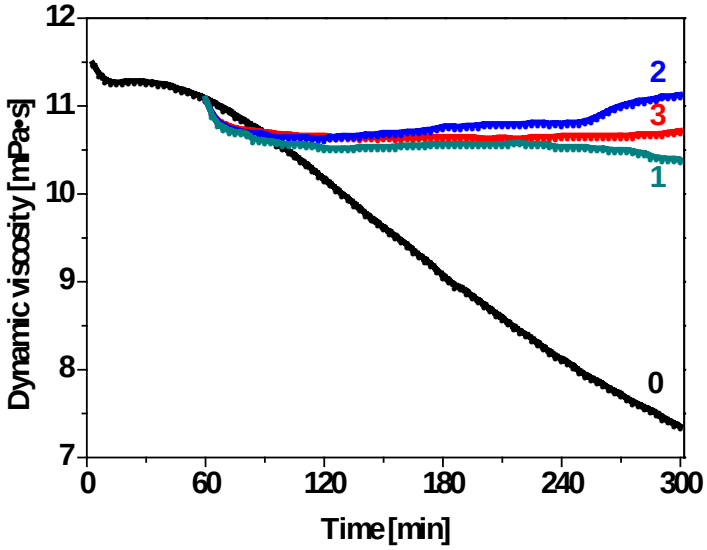


Figure 6. Effect of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine on HA degradation induced by the Weissberger biogenic oxidative system – 1.0 μM Cu(II) and 100 μM ascorbate (curve 0). Concentrations of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine in the reaction mixture were 1, 10 and 100 μM, curves 1, 2 and 3, respectively. The drug was added 1 h after the onset of the HA oxidative degradation.

2.3. ABTS and DPPH Assays

Compound 1, a low-molar-mass cysteine derivative containing two thiol groups (Figure 2), was classified as a more potent thiol donor than any other cysteine derivative (Horwitz, 2003). We proposed that *N*-(2-mercapto-2-methylpropionyl)-L-cysteine potentially acted as an H donor. The chemical structure of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine predetermines this drug to be an effective reducing agent as it contains two thiol groups, potential donors of two electrons. Indeed, under our experimental conditions, it was found to be a very potent antioxidant (see Table 2).

Table 2 depicts the IC_{50} values of the ABTS and DPPH assays for *N*-(2-mercapto-2-methylpropionyl)-L-cysteine and quercetin. The IC_{50} values obtained for quercetin by these assays were 2.9 and 4.4 µM, respectively (Valachová et al., 2010b). Based on its IC_{50} values (4.0 and 9.0 µM, respectively), *N*-(2-mercapto-2-methylpropionyl)-L-cysteine may be classified as an effective scavenger of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. Quercetin is a substance classified as the standard natural antioxidant. The IC_{50} values were calculated from the respective dose-response inhibition curves.

Table 2. IC_{50} values of the compounds tested determined by ABTS and DPPH decolorization assays (n=4; data expressed as mean ± s.e.m.; significance of differences was determined by the One-Way ANOVA test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the quercetin value)

SUBSTANCE	ABTS IC_{50}[µM]	DPPH IC_{50}[µM]
N-(2-mercapto-2-methylpropionyl)-L-cysteine	4.0 ± 0.4 n.s.	9.0 ± 0.4 ***
Quercetin	2.9 ± 0.2	4.4 ± 0.2

n.s. – not significant.

The decolorization reaction of the $ABTS^{\bullet+}$ radical cation solution or that of the $DPPH^{\bullet}$ radical can be simply described by the chemical reaction during which a proper reductant, in our case *N*-(2-mercapto-2-methylpropionyl)-L-cysteine, provides an electron to the acceptor – the $ABTS^{\bullet+}$ radical cation or $DPPH^{\bullet}$ radical. The reaction of a thiol-derived compound (R-SH), which itself undergoes oxidation, can be described as $R–SH - e^{-} \rightarrow R–S^{\bullet} + H^{+}$.

2.4. Oximetry

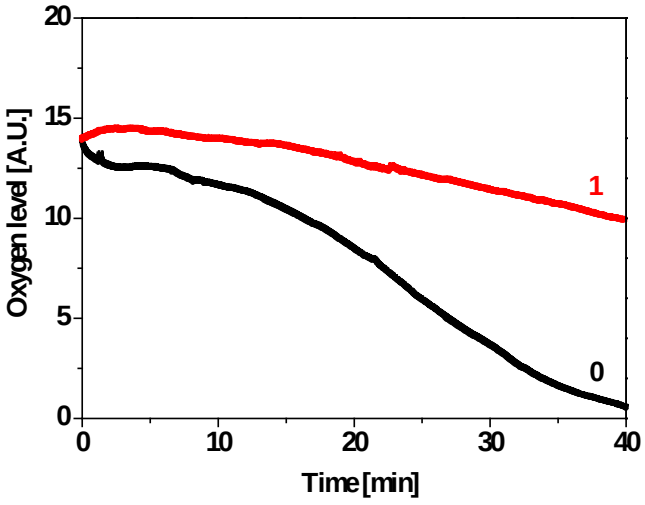


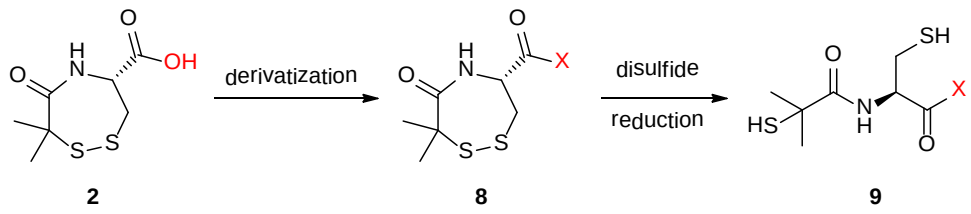
Figure 7. Oxygen concentration decrease in the reaction mixture with the Weissberger biogenic oxidative system – 1.0 µM Cu(II) and 100 µM ascorbate (curve 0). Addition of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine (100 µM) significantly inhibited the oxygen consumption by the Weissberger biogenic oxidative system (curve 1).

Oxygen consumption in the reaction mixture containing the Weissberger biogenic oxidative system, i.e. 1.0 µM Cu(II) and 100 µM ascorbate (curve 0, Figure 7), was found to occur along the very similar trajectory as the curve recorded by rotational viscometry (compare with the curve 0 in Figure 5). After addition of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine in 100 µM concentration into the reaction mixture (curve 1, Figure 7), consumption of oxygen was significantly inhibited. This finding indicated that the substance analyzed effectively reduced consumption of oxygen by the Weissberger biogenic oxidative system.

We speculate that this effect of substance **1** may occur due to the effective inhibition of the Weissberger biogenic oxidative system mediated free-radical generation resulting in the reduction of its cycling rate and thus decreasing oxygen consumption (Scheme 2).

2.5. Synthesis of *N*-(2-Mercapto-2-Methylpropionyl)-L-Cysteine Derivative

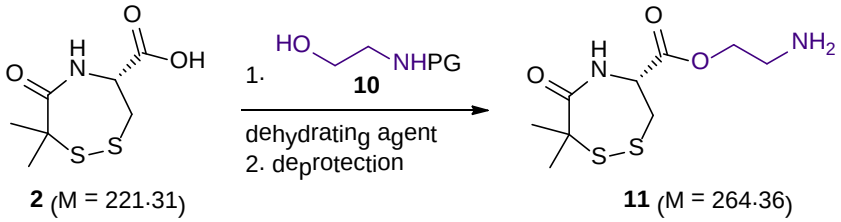
When a molecular structure is to be modified, a detailed investigation of its features is required. Taking into account the mode of action of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine (1) and the resulting disulfide metabolite (*R*)-7,7-dimethyl-6-oxo-1,2,5-dithiazepane-4-carboxylic acid (2), it is evident that the two thiol functional groups are inevitable for the original activity of the compound. These have to be retained. Of the remaining groups, only carboxylate is amenable to simple modification. From the synthetic point of view, however, free thiol groups complicate synthetic strategy, as once deprotonated, they are fairly nucleophilic and could readily compete with -COO^- or form cyclic thioester under dehydrating conditions. We therefore thought it would be elegant to choose the available compound 2 as the starting material for the transformation followed by the reduction of the disulfide bond. In this way, the –S-S– bond acts as a protecting group for the thiols (Scheme 3).



Scheme 3. The synthetic strategy for derivatization of 1. Cyclic disulfide 2 is first transformed to an intermediate compound 8. Subsequently, the free thiols are regenerated by –S-S– reduction to give the target structure 9.

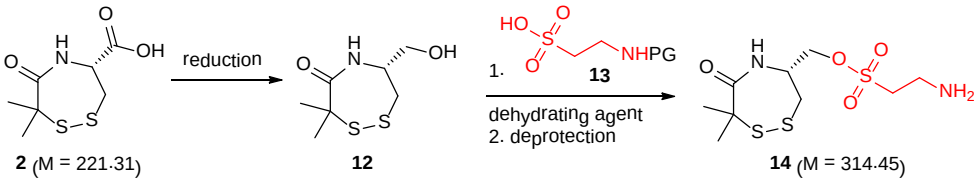
HOCl scavenging activity of taurine is due to the primary amino group in its structure. Direct reaction of 2 with taurine is not feasible because the NH_2 group would preferably form an amide with the carboxylic function. It is conceivable to first protect the amino group of taurine, but still the resulting mixed carboxylic-sulfonic anhydride would be prone to hydrolysis and at least two more reaction steps would be necessary. Introduction of -CH_2-CH_2-NH_2 moiety to (*R*)-7,7-dimethyl-6-oxo-1,2,5-dithiazepane-4-carboxylic acid (2) could also be carried out by means of:

- esterification with commercially available *N*-protected aminoethanol 10 in the presence of a dehydrating agent (Scheme 4). However, conditions for the removal of the protecting group (PG) would have to be compatible with the rest of the molecule (ester and amide functions).



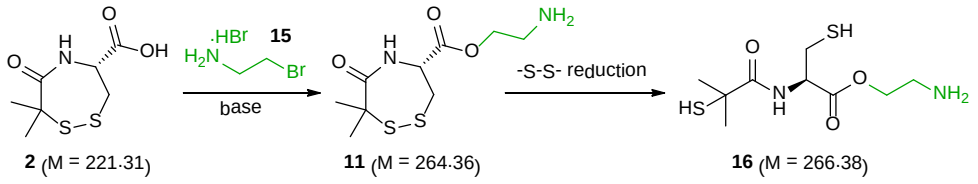
Scheme 4. Esterification approach with *N*-protected aminoethanol 10.

- reduction of carboxylate to carbinol 12 and subsequent reaction with *N*-protected taurine 13 (Scheme 5). Yet, this approach would present a significant alteration to the parent structure 1. Moreover and again, the reducing conditions in the first step would have to be compatible with the amide and disulfide functions.



Scheme 5. Carboxylate reduction in 2 followed by esterification with N-protected taurine 13.

- direct alkylation of carboxylate in 2 by 2-bromoethylamine salt 15 in the presence of a base. The reaction should proceed under mild conditions and besides 2-bromoethylamine, a number of analogous reagents is commercially available as hydrochlorides or -bromides for further investigation. Moreover, to increase the solubility of target compound 16 in water, its primary amino group could be eventually transformed into hydrochloride. We decided to favor this approach.



Scheme 6. Direct alkylation of carboxylate in 2 with 2-bromoethylamine salt 15.

The alkylation reaction of (*R*)-7,7-dimethyl-6-oxo-1,2,5-dithiazepane-4-carboxylic acid (**2**) was performed as described in *Experimental procedures*. After time-consuming column chromatography, 10 mg of the product was obtained in the form of yellow oil. Its ^{1}H NMR spectrum is depicted in Figure 8. Under identical reaction conditions, stoichiometric addition of silver(I) salt ($AgNO_3$), contrary to the previously described procedure in the literature, did not lead to any improvement (Aron et al., 2005).

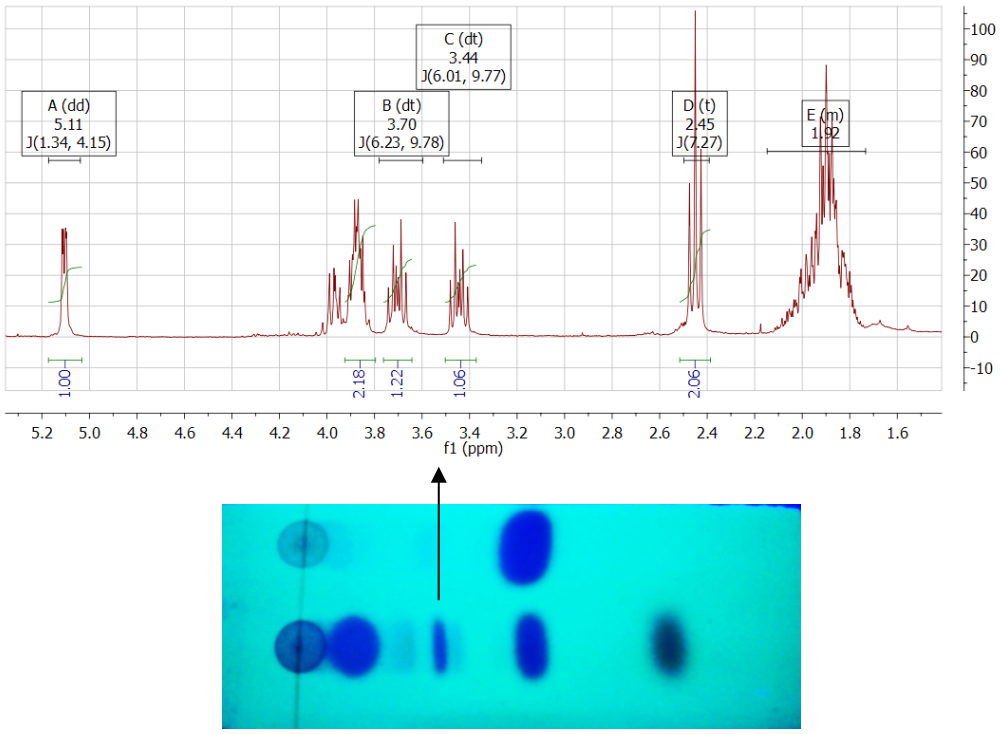


Figure 8. ^{1}H NMR spectrum of a (*R*)-7,7-dimethyl-6-oxo-1,2,5-dithiazepane-4-carboxylic acid alkylation product and reaction TLC plate. The arrow indicates which fraction was used to obtain the spectrum.

Based on these preliminary results, the first reaction step did indeed afford a new compound. Nevertheless, its structure has not been fully elucidated. In its ^{1}H NMR spectrum (Figure 8), the triplet at 2.45 ppm with doubled integrated intensity (and therefore corresponding to 2 hydrogen atoms) clearly suggests incorporation of -CH_2-CH_2- moiety into the structure. The triplet arising from the nearby -CH_2- is presumably hidden in the multiplet at 1.76-2.11 ppm. Detailed analysis of the spectrum would be possible after additional purification. Further investigations are underway.

CONCLUSION

In conclusion, our experimental findings suggest that *in vivo* the beneficial effect of the disease-modifying anti-rheumatic drug *N*-(2-mercapto-2-methylpropionyl)-L-cysteine may be partly due to its ability to protect the synovial joint high-molar-mass HA from oxidative

degradation. Particularly the free-radical scavenging ability of this drug is likely to be involved.

By combining the beneficial features of the compound studied and those reported for taurine, we have designed a synthetic route towards a new *N*-(2-mercapto-2-methylpropionyl)-L-cysteine derivative with an increased potential to act as a scavenger of hypochlorite. However, further elaboration of the synthetic procedure employed (replacement of base, solvent) and evaluation of the structure of the compound obtained is still required.

4. EXPERIMENTAL PROCEDURES

4.1. Biopolymer and Chemicals

High-molar-mass hyaluronan sample P0207-1A (M_w = 970 kDa) was purchased from Lifecore Biomedical Inc., Chaska, MN, U.S.A. Analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ were purchased from Slavus Ltd., Bratislava, Slovakia. 2,2´-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS; *purum*, >99%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-bromoethylamine hydrobromide (99%) and *N*,*N*-dimethylformamide (DMF, puriss., absolute, over molecular sieve) were the products of Sigma-Aldrich GmbH, Steinheim, Germany. *N*-(2-mercapto-2-methylpropionyl)-L-cysteine and (*R*)-7,7-dimethyl-6-oxo-1,2,5-dithiazepane-4-carboxylic acid were gifts from Santen Co., Osaka, Japan. Potassium persulfate (*p.a.* purity; max 0.001% nitrogen), silver(I) nitrate (*p.a.*) and L-ascorbic acid used were the products of Merck KGaA, Darmstadt, Germany. Ethanol (96%) and distilled methanol, both *p.a.* purity grade, were purchased from Mikrochem, Pezinok, Slovakia. Redistilled deionized high quality water, with conductivity of <0.055 μS/cm, was produced by using the TKA water purification system from Water Purification Systems GmbH, Niederelbert, Germany.

4.2. Solutions

Solutions for rotational viscometry were prepared as follows: the high-molar-mass hyaluronan sample solutions (2.5 mg/ml) were prepared in the dark at room temperature in 0.15 M aqueous NaCl in two steps: first, 4.0 ml and after 6 h the same solvent in the volume of 3.90 or 3.85 ml were added, when working in the absence or presence of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine, respectively. The stock solutions of ascorbate, *N*-(2-mercapto-2-methylpropionyl)-L-cysteine (16, 1.6, 0.16 mM), and cupric chloride (16 mM diluted to a 160 μM solution) were also prepared in 0.15 M aqueous NaCl.

Solutions for oximetry were prepared as follows: the HA sample solutions (2.5 mg/ml) were prepared in the dark at room temperature in 0.15 M aqueous NaCl in two steps: first, 1.0 ml of the solvent was added to 5 mg of HA and after 6 h another 1.0 ml of the solvent was added. The stock solution of ascorbate (16 mM) was prepared in deionized water, while solutions of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine (16 mM), and cupric chloride (16 mM diluted to a 160 μM solution) were prepared in 0.15 M aqueous NaCl.

4.3. Rotational Viscometry

The dynamic viscosity of the reaction mixture (8 ml; 0.15 M aqueous NaCl) containing high-molar-mass HA,) Cu(II) ions (1 μM) and ascorbate (100 μM in the absence and presence of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine (1, 10 and 100 μM) was monitored by a Brookfield LVDV-II+PRO digital rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.) at 25.0 ± 0.1°C at a shear rate of 237.6 s^{-1} for 5 h in a reservoir-spindle couple made of Teflon® (Hrabárová et al., 2009, 2012, Stankovská et al., 2010, Surovčíková et al., 2012). The drug was introduced into the reservoir vessel before initiating HA oxidative degradation or 1 h after the reaction onset (Baňasová et al., 2012, 2013, Valachová et al., 2011, 2012, 2013).

4.4. ABTS and DPPH assays

The standard ABTS decolorization assay was utilized as reported previously (Re *et. al.*, 1999, Cheng *et. al.*, 2006). Briefly, the aqueous solution of $ABTS^{\bullet+}$ cation radical was prepared 24 h before the measurements at room temperature as follows: ABTS aqueous stock solution (7 mmol/l) was mixed with $K_2S_2O_8$ aqueous solution (2.45 mmol/l) in equivolume ratio. The next day, 1.1 ml of the resulting solution was diluted with 96% ethanol to the final volume of 50 ml. The ethanol-aqueous reagent in the volume of 250 μl was added to 2.5 μl of the ethanolic solutions of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine. The concentration of stock solutions of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine was 101−0.808 mmol/l. The light absorbance of the sample mixtures was recorded at 734 nm 6^{th} minutes after mixing the reactants.

For the DPPH decolorization assay, 2,2-diphenyl-1-picrylhydrazyl (1.1 mg) was dissolved in 50 ml of distilled methanol to generate $DPPH^{\bullet}$. The $DPPH^{\bullet}$ radical solution in the volume of 225 μl was added to 25 μl of the methanolic solutions of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine (in the concentration range of 10–0.078 mmol/l) and in the 30^{th} min the absorbance of the samples was measured at 517 nm.

All measurements of both assays were performed quadruplicately in 96-well Greiner UV-Star microplates (Greiner-Bio-One GmbH, Germany) by using the Tecan Infinite M 200 reader (Tecan AG, Austria).

4.5. Oximetry

The oxygen consumption of the reaction mixture (1600 μl; 0.15 M aqueous NaCl) containing high-molar-mass HA, Cu(II) ions (1 μM) and ascorbate (100 μM) in the absence and presence of *N*-(2-mercapto-2-methylpropionyl)-L-cysteine (100 μM) was monitored by a Strathkelvin 782 2-Chanel Oxygen System version 1.0 (Strathkelvin Instruments, Ltd., UK). Oximetric experiments were carried out at 37°C and oxygen consumption was recorded for 40 min.

The oxygen electrodes used had the advantage of small size, high precision, and the ability to operate in either stirred or unstirred media.

4.6. Synthesis of *N*-(2-Mercapto-2-Methylpropionyl)-L-Cysteine Derivative

The alkylation of (*R*)-7,7-dimethyl-6-oxo-1,2,5-dithiazepane-4-carboxylic acid (**2**) was carried out as follows: **2** (50 mg, 0.23 mmol) was dissolved in 1 ml of DMF, and triethylamine (0.11 ml, 0.79 mmol) was added drop-wise *via* syringe followed by 2-bromoethylamine hydrobromide (70 mg, 0.34 mmol). The mixture was heated to 50°C for 1 hour and then left to reach room temperature. Reaction progress was monitored by thin-layer chromatography (TLC) on Merck silica gel F-254 plates and visualization was performed using UV light (254 nm). After 24 hours, the reaction mixture was diluted with distilled water, extracted with ethyl acetate (3×), dried over Na_2SO_4 and concentrated under reduced pressure. The crude mixture was separated by column chromatography ($CHCl_3$:CH_3OH = 30:1) on Merck silica gel 60. Structure of the compounds was analyzed by ^1H nuclear magnetic resonance (NMR) spectroscopy on a Varian Mercury Plus Instrument (300 MHz) at 20°C in $CDCl_3$ with $(CH_3)_4Si$ (0.00 ppm) as an internal standard.

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