Organosulfur Compounds from Garlic (Allium sativum) Oxidizing Canine Erythrocytes

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The sulfurous acid ester, trans-sulfurous acid allyl ester 3-allylsulfanyl-allyl ester 8, along with two known thiosulfimates was isolated from the aqueous ethanol extract of garlic (Allium sativum). The chemical structure of 8 was determined on the basis of spectroscopic data including high resolution mass and two-dimensional NMR techniques. All of these compounds induced methemoglobin formation in a canine erythrocyte suspension in vitro resulting in the oxidation of canine erythrocytes. This is the first report of sulfurous acid ester showing oxidant activity in canine erythrocytes.

Key words: Garlic, Oxidant, Sulfurous Acid Ester

Introduction

It is known that ingestion of garlic extracts induces hemolysis in sheep and dogs (Stevens, 1984 and Lee et al., 2000), while there is no report to evidence that consumption of garlic by man causes hemolytic anemia. Erythrocyte count in dogs given boiled garlic extract (5 g of whole garlic/kg of body weight, once a day for 7 days) decreased significantly (Lee et al., 2000). Increase of methemoglobin concentration, formation of eccentrocytes and Heinz body were observed in these dogs, suggesting the oxidation of blood. By this observation, it can be assumed that some constituents of garlic have the potential to oxidize erythrocyte membranes and hemoglobin, inducing hemolysis associated with the appearance of eccentrocytes in dogs. In addition, dogs with disease associated with oxidative stress and anemia may develop additive hemoglobin damage when fed food contains garlic. Thus, garlic has high toxicity potential to animals. The safety of the consumption of garlic by animals should be questioned. The objectives of our study are to isolate and identify compounds showing oxidant activity from garlic (Allium sativum) using an in vitro dog erythrocyte oxidation test.

In our previous study, a mixture of three sulfides, bis-2-propenyl trisulfide 1, bis-2-propenyl tetrarsulfide 2, and bis-2-propenyl pentasulfide 3, as well as two individual compounds: bis-2-propenyl thiosulfonate 4 and trans-sulfuric acid allyl ester 3-allylsulfanyl-allyl ester 5 were obtained from the aqueous ethanol extract of garlic (Hu et al., 2002). As a result of continuing research, bioassay-directed fractionation of the extract has now led to the isolation and characterization of one novel sulfurous acid ester and two known thiosulfimates as biologically active compounds. We report herein the isolation, structural elucidation and canine erythrocyte oxidizing activity of these compounds.

Materials and Methods

General

1H-NMR and 13C-NMR spectra were recorded with a JEOL JNM-270 spectrometer (1H: 270 MHz, 13C: 67.8 MHz). IR spectra were measured with
a Perkin Elmer System 2000 FT-IR spectrometer. Mass spectra, FIMS, EIMS and FIHRMS were recorded with a JEOL JMS-AX500 spectrometer and a JEOL JMS-SX102A spectrometer. HMOC and HMBC spectra were analyzed with a Bruker AM-500 FT-NMR spectrometer. Column chromatography was conducted with silica gel 60 (spherical, 70–140 mesh ASTM, Kanto Chemical). Silica gel 60 F$_{254}$ pre-coated plates (Merck) were used for analytical TLC and prep. TLC.

**Plant material**

Edible garlic was purchased in Japan, which was imported from China.

**Bioassay for the oxidation of canine erythrocytes**

The oxidation of canine erythrocytes was assayed by determining the methemoglobin formation in vitro. Whole blood from clinically normal dogs was drawn into a heparinized tube and centrifuged at 1250 $\times$ g for 7 min at 4°C. After removal of the layer of leukocytes and platelets, the erythrocytes were washed three times with 10 mm phosphate-buffered saline (PBS, pH 7.4) with 0.9% (w/v) sodium chloride, and resuspended in PBS with a packed cell volume of 25% (v/v). Five hundred $\mu$L of the erythrocyte suspension were incubated for 1 h at 37°C with each sample derived from garlic. The methemoglobin concentration was then measured as described by Hegesh et al. (1970), and expressed as percent of total hemoglobin. The same procedure without garlic extracts was used as a blank control.

**Bioassay-directed isolation of compounds**

Extraction and separation of the garlic-derived components was monitored with bioassay for oxidation of canine erythrocytes. Fresh garlic (8.7 kg) was cut into small pieces and soaked in four times its weight of 70% ethanol/distilled water (v/v). The immersion lasted for two weeks at room temperature. After filtration, the aqueous ethanol extract was concentrated, then partitioned between H$_2$O (1 L) and EtOAc (1 L). The aqueous layer was further extracted with EtOAc (1 L $\times$ 2), the combined EtOAc layer was concentrated then fractionated. The residue of EtOAc extract was subjected to column chromatography and successively eluted with CHCl$_3$ (500 ml), 3% MeOH/CHCl$_3$ (500 ml), 20% MeOH/CHCl$_3$ (500 ml) and MeOH (500 ml). Compounds 1–5 were obtained from the CHCl$_3$ eluate in our previous study (Hu et al., 2002).

The residue (2.95 g) from a 3% MeOH/CHCl$_3$ eluate was rechromatographed with 16% EtOAc/hexane, yielding three fractions: Fr. I (469.3 mg), Fr. II (431.2 mg) and Fr. III (269.8 mg). Fr. I was purified by a silica gel column using 1.5% MeOH/CHCl$_3$ as eluent to yield Fr. I-1 (282.8 mg). Fr. I-1 was subjected to prep. TLC, developed with 1% MeOH/CHCl$_3$ and gave a main band. Further purification of this band with prep. TLC using the solvent system 0.1% MeOH/CHCl$_3$ afforded 6 (9.3 mg).

Fr. II was applied to silica gel column chromatography eluted with 2% MeOH/CHCl$_3$ to give Fr. II-1 (210 mg) which was further purified by column chromatography using 1.5% MeOH/CHCl$_3$ as eluent to yield Fr. II-1-1 (29.8 mg). Finally, a single pure compound 7 (11.6 mg) was obtained from fractionation of Fr. II-1-1 by prep. TLC using the solvent system 45% EtOAc/hexane.

Fr. III was first subjected to silica gel column eluted with 2% MeOH/CHCl$_3$, yielding Fr. III-1 (82.6 mg). Fr. III-1 was purified by prep. TLC developed with 60% EtOAc/hexane to yield compound 8 (35.2 mg).

**Compound 6**

Compound 6 was obtained as a colorless oil; FIMS $m/z$ (rel. int.): 136 [M$^+$] (100). HRMS $m/z$ (rel. int.): 136.0005 (calcd. for C$_4$H$_8$OS$_2$: 136.0017).

$^1$H NMR (270 MHz, CDCl$_3$): $\delta$ 5.85 (1H, m, H-5), 5.40 (1H, dd, $J$ = 10, 1 Hz, H-6b), 5.30 (1H, dd, $J$ = 17, 1 Hz, H-6a), 3.75 (2H, m, H-4) and 2.60 (3H, s, H-1); $^{13}$C-NMR (67.8 MHz, CDCl$_3$): $\delta$ 125.8 (C-5), 124.0 (C-6), 60 (C-4) and 14.2 (C-1).

**Compound 7**

Compound 7 was obtained as a colorless oil; FIMS $m/z$ (rel. int.): 162 [M$^+$] (100). HRMS $m/z$ (rel. int.): 162.0183 (calcd. for C$_6$H$_{10}$OS$_2$: 162.0174).

$^1$H NMR (270 MHz, CDCl$_3$): $\delta$ 6.33 (2H, m, H-2 and H-3), 5.88 (1H, m, H-7), 5.44 (2H, m, H-8), 3.82 (2H, m, H-6) and 1.87 (3H, d, $J$ = 5.0 Hz, H-1); $^{13}$C-NMR (67.8 MHz, CDCl$_3$): $\delta$ 144.1 (C-3), 125.8 (C-7), 124.2 (C-8), 115.6 (C-2), 60 (C-6) and 19.5 (C-1).
 Compound 8 was obtained as a colorless oil; FDMS m/z (rel. int.): 234 [M+]+ (100). EIMS m/z (rel. int.): 41 (28.4), 67 (28.2), 73 (72.7), 103 (82.2), 111 (28.8), 129 (15.5), 145 (100), 157 (1.2), 171 (1.9), 177 (1.4), 218 (0.9), 234 (0.9). HRMS m/z (rel. int.): 234.0384 (calcd. for C$_9$H$_{14}$O$_3$S$_2$: 234.0385). IR $\nu_{KBr \max \text{cm}^{-1}}$: 2916, 1633, 1401, 1220, 1034, 990 and 927. $^1$H- and $^{13}$C-NMR data see Table I.

Results and Discussion

Using a bioassay-directed separation of the 3% MeOH/CHCl$_3$ eluate fraction, three oxidant active organosulfur compounds 6–8 were obtained. Compound 8 was obtained as volatile oil and gave a molecular weight and formula of 234 and C$_9$H$_{14}$O$_3$S$_2$ by FI and HRMS spectra, respectively. The IR spectrum displayed a sulfoxide stretch at 1034 cm$^{-1}$ in addition to a strong double bond absorption at 1636, 990 and 927 cm$^{-1}$. The $^1$H-NMR spectrum displayed signals for double bond methynes, one at $\delta$ 6.35 ($d$, $J$ = 14.8 Hz) and three at $\delta$ 5.83 ($m$). In addition, signals for two double bond methylenes ($\delta$ 5.38, $m$; 5.19, $d$, $J$ = 8.5 Hz) and three methynes adjacent to a double bond ($\delta$ 3.53, $ddd$, $J$ = 7.6, 6.9, 1.9 Hz; 3.49, $dd$, $J$ = 7.6, 5.6 Hz; 3.33, $d$, $J$ = 7.2 Hz) were observed. The $^{13}$C-NMR spectrum indicated nine nonequivalent carbons due to olefinic methylenes ($\delta$ 123.8 and 119.2), methines ($\delta$ 134.7, 132.5, 125.6 and 116.8) and aliphatic methylenes ($\delta$ 54.3, 53.0 and 41.3). Assignments of protons to the carbons were made by HMQC and HMBC experiments. These data suggested that compound 8 consisted three allyl fragments separated by a sul-
Fig. 2. Methemoglobin formation of canine erythrocytes by garlic-derived compounds. Five hundred µl of suspended canine erythrocyte (25% (v/v) packed cell volume) were incubated at 37°C with compounds derived from garlic. After one hour, the increase in methemoglobin concentration was measured (Hegesh et al., 1970). The methemoglobin concentration of blank control without garlic extracts was 1.1%. The dose of the test compound in each incubation was represented as the amount of garlic yielding the compound regardless of losses during purification. Corresponding to 10 g and 50 g garlic, the concentrations of the three compounds in the assay were calculated as the following: compound 6, 21.4 ppm and 107 ppm; compound 7, 26.6 ppm and 133 ppm; and compound 8, 81.0 ppm and 405 ppm. The results were based on the yields of compound 6–8 from garlic.

suggest the existence of other minor oxidants except the three compounds isolated in the present study.

The dose of the test compound in each incubation was shown as the amount of corresponding garlic weight. The values seem high, but are only theoretical ones, since the recovery ratios of the compounds from garlic are not taken into account. The concentrations of compounds 6, 7 and 8 in garlic were estimated to be 1.1, 1.3 and 4.1 mg/kg respectively by the yields of them. However, the actual concentrations of these compounds in garlic are thought to be much higher because of the inevitable loss during the purification and the volatility of these compounds. The three compounds identified in the present study may play an important role in oxidation of canine erythrocytes because they were found by the guidance of a bioassay of methemoglobin generating activity.

Hemolysis is associated with Heinz body formation within erythrocytes, which results from the precipitation and denaturation of hemoglobin molecules oxidatively damaged by n-propyl disulfide and three alkenyl thiosulfate compounds in onions (Gruhzit et al., 1931; Yamato et al., 1994, 1998, 1999). The groups of characteristic organosulfur compounds contained in garlic probably contributed to hemolytic activity and the oxidation mechanism of blood. Some constituents of garlic have the potential to oxidize erythrocyte hemoglobin and membranes. There is a possibility that garlic in pet food may cause hemolytic anemia due to some oxidants contained in garlic.

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