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Isolation and Identification of Organosulfur Compounds Oxidizing Canine Erythrocytes from Garlic (*Allium Sativum*)

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Five compounds oxidizing canine erythrocytes were isolated from an aqueous ethanol garlic extract by silica gel column chromatography and preparative thin-layer chromatography. On the basis of nuclear magnetic resonance, infrared spectroscopy, and mass spectrometry, they were identified as three known compounds: bis-2-propenyl trisulfide (**1**), bis-2-propenyl tetrasulfide (**2**), and bis-2-propenyl pentasulfide (**3**) as well as two novel compounds, bis-2-propenyl thiosulfonate (**4**) and *trans*-sulfuric acid allyl ester 3-allylsulfanyl-allyl ester (**5**). A mixture of compounds **1**−**3** and compounds **4** and **5** induced methemoglobin formation in canine erythrocyte suspension in vitro resulting in the oxidation of canine erythrocytes. These groups of characteristic organosulfur compounds contained in garlic probably contribute to oxidations in blood. The constituents of garlic have the potential to oxidize erythrocytes and hemoglobin, suggesting that foods containing quantities of garlic should be avoided for feeding dogs.

KEYWORDS: Garlic; oxidant; methemoglobin; sulfide; thiosulfonate; sulfuric acid ester

INTRODUCTION

In veterinary science, it is known that onions are oxidatively toxic to erythrocytes resulting in hemolytic anemia in domestic animals, such as dogs, cats, horses, sheep, and cattle (*1*, *2*). The causative toxic compounds have been isolated from onion (*3*). Onions contain characteristic organosulfur compounds that contribute to its hemolytic activity (*4*). Garlic (*Allium sativum*), a member of the family liliaceae, has been used since ancient times as a pharmaceutical. The lowering of cholesterol, inhibition of platelet aggregation, immune enhancement, and antioxidant activity are some of its many reported functions (*5*, *6*). Modern scientific research is confirming many of these properties, defining mechanisms of action, and exploring the potential of garlic to prevent and treat disease. However, when a large amount of garlic is consumed, undesirable effects, such as burning sensations and diarrhea, have been reported to occur in humans (*7*). Toxicity tests of garlic have shown that raw garlic powder can cause severe inflammation and damage to canine gastric mucosa, and enteric-coated garlic tablets caused epithelial cell loss at the top of the ileum crypts when administered orally (*8*). Ingestion of garlic extracts has also been shown to induce hemolysis in dogs and sheep (*9*, *10*). Garlic-induced hemolysis occurs due to oxidative damage including formation of eccentrocytes and Heinz bodies (*9*). Eccentrocytes are erythrocytes with hemoglobin located to one side of the cell, leaving an open area of cytoplasm containing little hemoglobin. They result from oxidant injury to the membrane. These results showed that garlic has a high potential toxicity to dogs. The safety of the consumption of garlic by animals should be questioned. The objectives of this study were to isolate and identify compounds showing oxidant activity from garlic using an in vitro dog erythrocyte oxidation test.

MATERIALS AND METHODS

Garlic. Garlic was purchased from Hokkaido University Cooperation Shop.

Extraction and Isolation. 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 2 weeks at room temperature. After the mixture was filtered, the aqueous ethanol extract was concentrated and then partitioned between water (1 L) and EtOAc (1 L). The aqueous layer was further extracted with EtOAc (1 L × 2), and the combined EtOAc layer was concentrated and then fractionated as shown in **Figure 1**. The EtOAc fraction was subjected to column chromatography and eluted with $CHCl_3$ (500 mL), 3% MeOH/$CHCl_3$ (500 mL), 20% MeOH/$CHCl_3$ (500 mL), and MeOH (500 mL) successively. The residue from the $CHCl_3$ eluate was rechromatographed successively with 3% EtOAc/hexane (150 mL), 50% EtOAc/hexane (150 mL), and EtOAc (150 mL). The separation was monitored by an oxidant activity bioassay. Finally, two single pure compounds (**4** and **5**) and an active mixture (compounds **1**−**3**) were obtained from

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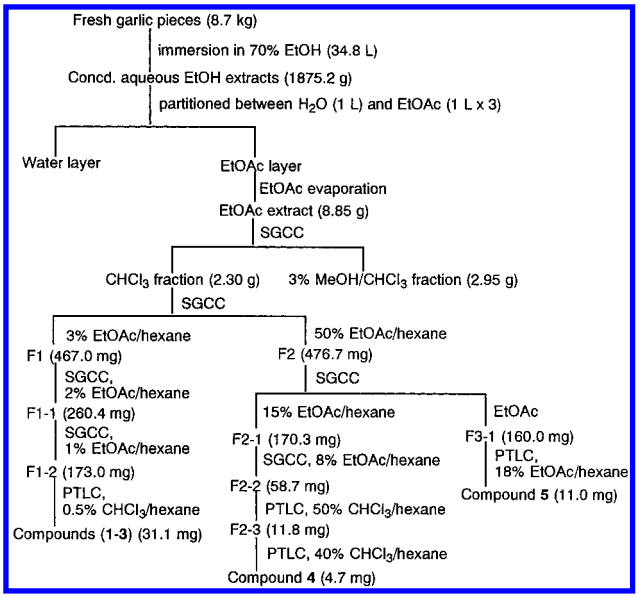

Figure 1. Scheme for separation procedure of oxidant active compounds in garlic. Isolated fractions without oxidant activity are not shown.

Table 1. ^{1}H and ^{13}C NMR Spectral Data of Compounds **4** and **5**[a]

	compd **4**		compd **5**	
position	^{1}H	^{13}C	^{1}H	^{13}C
1	3.72 (d, J = 7.2 Hz)	39.5	3.75 (d, J = 7.5 Hz)	54.5
2	5.85 (m)	131.0	5.85 (m)	114.5
3	5.26 (m)	120.0	6.39 (dt, J = 15.8, 1.0 Hz)	136.5
1′	3.92 (d, J = 7.2 Hz)	67.0	3.65 (d, J = 7.5 Hz)	56.5
2′	5.85 (m)	31.0	5.88 (m)	124.5
3′	5.48 (m)	126.0	5.48 (m)	125.0
1″			3.35 (d, J = 7.3 Hz)	42.0
2″			5.88 (m)	132.0
3″			5.19 (d, J = 7.9 Hz)	119.5

[a] Assignments were based on DEPT, ^{1}H–^{1}H COSY, HMQC, and HMBC experiments.

fractionations of 50% EtOAc/hexane (F2) and 3% EtOAc/hexane eluate (F1), respectively. Further separation of the active mixture was not possible due to its instability and volatility. However, spectroscopic analysis showed that this mixture contained three known sulfides (compounds **1**−**3**).

Compounds 1−3. Colorless oil. Field ionization mass spectroscopy (FIMS) *m*/*z* (relative intensity): 178 [M^+, compound **1**] (30.1), 210 [M^+, compound **2**] (86.6), 242 [M^+, compound **3**] (100). Electron impact mass spectroscopy (EIMS) *m*/*z* (relative intensity): 54 (15.3), 59 (100), 78 (11.4), 104 (34.2), 113 (38.3), 130 (95.6), 146 (14.2), 172 (11.4), 178 (8.3), 181 (45.4), 210 (8.3), 213 (58.3), 215 (27.2), 242 (4.0). High-resolution mass spectroscopy (HRMS) *m*/*z* (relative intensity): 177.9987 (calcd for $C_6H_{10}S_3$, 177.9946), 209.9627 (calcd for $C_6H_{10}S_4$, 209.9667) and 241.9312 (calcd for $C_6H_{10}S_5$, 241.9388). IR γ_{max}^{KBr} (cm^{-1}): 3082, 2921, 1634, 1422, 1397, 1217, 985, 919, 720.

Compound 4. Colorless oil. FIMS *m*/*z* (relative intensity): 178 [M^+] (100). EIMS *m*/*z* (relative intensity): 41 (52.9), 73 (100), 81 (10.4), 99 (14.9), 114 (12.8), 149 (2.0), 178 (5.3). HRMS *m*/*z* (relative intensity): 178.0114 (calcd for $C_6H_{10}O_2S_2$, 178.0123). IR γ_{max}^{KBr} (cm^{-1}): 1636, 1559, 1507, 1324, 1126, 941. ^{1}H- and ^{13}C nuclear magnetic resonance (NMR) data, see **Table 1**.

Compound 5. Colorless oil. FIMS *m*/*z* (relative intensity): 250 [M^+] (100). EIMS *m*/*z* (relative intensity): 41 (42.3), 67 (36.7), 73 (76.8), 103 (62.8), 111 (33.7), 145 (100), 250 (21.1). HRMS *m*/*z* (relative intensity): 250.3398 (calcd for $C_9H_{14}O_4S_2$, 250.0334). IR γ_{max}^{KBr} (cm^{-1}): 2918, 1636, 1422, 1398, 1316, 1124, 990, 937. ^{1}H- and ^{13}C NMR data, see **Table 1**.

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*g* for 7 min at 4 °C. After the layer of leukocytes and platelets was removed, 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 microliters of the erythrocyte suspension was incubated for 1 h at 37 °C with each sample derived from garlic. The methemoglobin concentration was then measured as described by Hegesh (*11*) and expressed as percent of total hemoglobin. The same procedure without garlic extracts was used as a blank control.

Structure Determination. ^{1}H NMR and ^{13}C NMR spectra were recorded with a JEOL JNM-270 spectrometer (^{1}H: 270 MHz, ^{13}C: 67.8 MHz). IR spectra were measured with a Perkin-Elmer System 2000 Fourier transform infrared (FTIR) spectrometer. Mass spectra, FIMS, EIMS, and field ionization high-resolution mass spectroscopy (FI-HRMS) were recorded with a JEOL JMS-AX500 spectrometer and a JEOL JMS-SX102A spectrometer. Distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (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} precoated plates were used for analytical thin-layer chromatography (TLC) and preparative TLC (Merck).

RESULTS AND DISCUSSION

Isolation and Identification of Oxidizing Compounds. The $CHCl_3$ eluate fraction yielded an active mixture containing three sulfides (compounds **1**−**3**) and two individual active compounds (**4** and **5**). The active mixture was obtained as a volatile oil. The FIMS spectrum showed three molecular ion peaks indicating that the mixture consisted of three compounds. The molecular formulas of peaks at *m*/*z* 242, 210, and 178 were determined to be $C_6H_{10}S_5$, $C_6H_{10}S_4$, and $C_6H_{10}S_3$, respectively, by HRMS. The IR spectrum showed the absorption band for the vinyl group (1634, 985, and 919 cm^{-1}). In the ^{1}H NMR spectrum, there were signals of three kinds of protons: double-bond methylenes (δ 5.23, 6H, m), double-bond methines (δ 5.86, 3H, m), and methylenes linked to double bonds (δ 3.58, 2H, d, J = 7.4 Hz; 3.49, 4H, d, J = 7.3 Hz). The ^{13}C NMR spectrum also showed the signals for the above-mentioned carbons. These data were in good accordance with those for symmetric bis-2-propenyl polysulfides. Thus, the components of the mixture were determined to be bis-2-propenyl trisulfide (**1**), bis-2-propenyl tetrasulfide (**2**), and bis-2-propenyl pentasulfide (**3**).

Compound **4** was obtained as a volatile oil and gave a molecular weight and formula of 178 and $C_6H_{10}O_2S_2$ by FI and HRMS spectra, respectively. The IR spectrum indicated the presence of thiosulfonate (1324 and 1126 cm^{-1}) and double bonds (1636 and 941 cm^{-1}). A diallyl thiosulfonate skeleton was deduced from the ^{1}H and ^{13}C NMR spectra. Thus, in the ^{1}H NMR, there were signals due to two allyl groups: two double-bond methines (δ 5.85, m), two double-bond methylenes (δ 5.48, 2H, m; 5.26, 2H, m), and two methylenes (δ 3.72, 2H, d, J = 7.2 Hz; δ 3.92, 2H, d, J = 7.2 Hz). The ^{13}C NMR spectrum also showed the signals for the above-mentioned carbons (see **Table 1**). Assignments of protons to the carbons were made by HMQC. The structure of each allyl fragment was resolved by HMBC and correlation spectroscopy (COSY) experiments. Hence, the structure of compound **4** was determined to be bis-2-propenyl thiosulfonate (**Figure 2**).

Compound **5** was obtained as a volatile oil and gave a molecular weight and formula of 250 and $C_9H_{14}O_4S_2$ by FI and HRMS spectra, respectively. The IR spectrum suggested the presence of sulfonyl (1123 and 1316 cm^{-1}) and double bonds

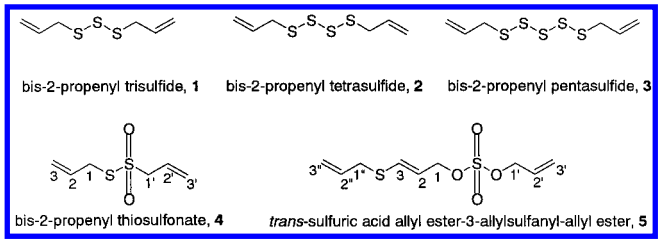

Figure 2. Structures of oxidant active compounds derived from garlic.

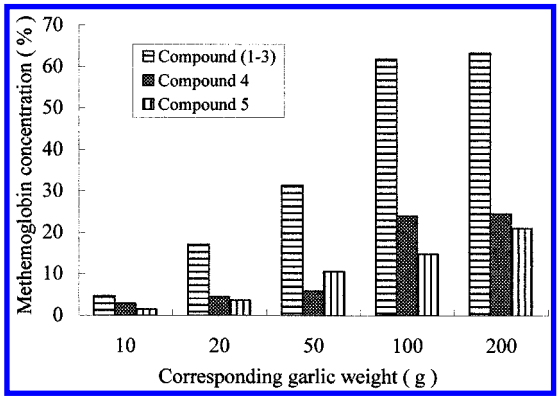

Figure 3. Methemoglobin formation of canine erythrocytes by garlic-derived compounds. Five hundred microliters of suspended canine erythrocyte (25% (v/v) packed cell volume) was incubated at 37 °C with compounds derived from garlic. After 1 h, the increase in methemoglobin concentration was measured (*11*). 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.

(1636, 990, and 937 cm^{-1}). The ^1H NMR spectrum displayed signals for double-bond methines, one at δ 6.39 (dt, $J = 15.8$, 1.0 Hz) and three at δ 5.88 (m). In addition, signals for two double-bond methylenes (δ 5.48, m; 5.19, d, $J = 7.9$ Hz) and three methylenes adjacent to a double bond (δ 3.75, d, $J = 7.5$ Hz; 3.65, d, $J = 7.5$ Hz; 3.35, d, $J = 7.3$ Hz) were observed. The ^{13}C NMR spectrum had signals due to methylenes (δ 125 and 119.5), methines (δ 136.2, 132, 124.5, and 114.2), and methylenes (δ 56.5, 54.5, and 42.0). Assignments of protons to the carbons were made by HMQC (**Table 1**). These data suggested that compound **5** consisted of three allyl fragments separated by a sulfur and a sulfonyl. COSY and HMBC experiments showed $^1H-^1H$ and $^1H-^{13}C$ correlations in each allyl fragment. The $-CH{=}CHCH_2-$ moiety was determined to be the trans isomer based on the J value (15.8 Hz) of the methine. Fragment peaks at m/z 41 and 145 in EIMS were assigned to $[CH_2{=}CHCH_2]^+$ and $[CH{=}CHCH_2SO_2CH{=}CHCH_2]^+$. Therefore, compound **5** was determined to be *trans*-sulfuric acid allyl ester 3-allylsulfanyl-allyl ester (**Figure 2**).

Oxidant Activity of Compounds Derived from Garlic. The relative oxidant activity of the garlic-derived compounds was examined by the increase in methemoglobin concentration. Results of the oxidant activity (**Figure 3**) showed that the mixture of compounds **1**−**3** possessed much stronger oxidant activity than compounds **4** and **5**. With an increase in the amount of garlic, the oxidant activity increased dose dependently. The ethyl acetate extract (**Figure 1**) showed much stronger oxidant activity with 47.45% of methemoglobin concentration at 5 g of corresponding garlic weight as compared with single pure compounds isolated from the extract. Furthermore, the water layer (**Figure 1**) also showed oxidant activity with 46.72% of methemoglobin concentration at 5 g of corresponding garlic weight. All of these suggest the existence of other minor oxidants except the five 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 values, since the recovery ratios of the compounds from garlic are not taken into account. The concentrations of the mixture of compounds **1**−**3** and compounds **4** and **5** in garlic were estimated to be 3.58, 0.55, and 1.26 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 five compounds identified in the present study may play an important role in the oxidation of canine erythrocytes because they were found with 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 alk(en)yl thiosulfate compounds in onions (*3*, *12*, *13*). The groups of characteristic organosulfur compounds contained in garlic probably contributed to hemolytic activity and the oxidation mechanism of blood. The 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.

ABBREVIATIONS USED

PBS, phosphate-buffered saline; FIMS, field ionization mass spectrometry.

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