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Identification and Characterization of Sulfur Heterocyclic Compounds That Contribute to the Acidic Odor of Aged Garlic Extract

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ABSTRACT: The aroma of aged garlic extract (AGE) has been recently characterized as a complexity of seasoning-like, metallic, fatty, and acidic notes; most of the important aroma compounds were identified in a previous study. Besides the 25 previously identified aromas of AGE, several of the odor compounds that contribute to the acidic notes were isolated and identified using various analytical techniques, including gas chromatography coupled with an olfactometry monitoring system (GC−O), accurate and high-performance preparative GC system, GC−MS analysis, and sensory evaluation. The identified aromas include: 2,4-dimethyl-1,3-dithiolane, 2,5-dimethyl-1,4-dithiane, and 2,6-dimethyl-1,4-dithiane. Interestingly, AGE contains all stereoscopic isomers of each of these components. An aroma recombinant composed of the newly identified acidic odors with other key odorants showed good agreement with the aroma of AGE.

KEYWORDS: *aged garlic extract, acidic odor, organosulfur compounds, 2,4-dimethyl-1,3-dithiolane, 2,5-dimethyl-1,4-dithiane, 2,6-dimethyl-1,4-dithiane*

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INTRODUCTION

Garlic (*Allium sativum* L.) is a vegetable that is used worldwide as a seasoning and has been used traditionally as a medicinal plant.[1] Various studies have been conducted to elucidate the pharmacological effects of garlic; a number of active principle compounds of garlic have been identified.[2,3] Among the known active compounds, the sulfur-containing volatile compound diallylthiosulfinate, known as allicin, is an important contributor to the biological activities of garlic.[4,5]

Allicin is produced from alliin (*S*-allylcysteine sulfoxide) by alliinase when fresh garlic is mechanically damaged, for example, by slicing or grating.[6] Allicin is gradually degraded to ajoenes, allyl sulfides, and vinyl dithiins.[7] These degradation products have also been reported to exhibit pharmacological effects, such as anticancer, antimicrobial, antioxidant effects, and protect against cardiovascular disease.[2,4,5] Although allicin and some of its degradants have beneficial effects in humans, allicin is also the principal compound associated with the pungent odor of fresh garlic. Allicin is degraded to malodorous volatiles (e.g., allyl methyl sulfide and allyl mercaptan) in the mouth and gut, which results in an unpleasant breath odor.[8−10] Because of these undesirable effects some people avoid fresh garlic, despite its significant health benefits.

In an effort to reduce the pungent smell of fresh garlic, various treatments and processes have been investigated; however, most of these efforts also reduce the pharmacological activities to some extent.[11,12] Aged garlic extract (AGE) is prepared by extracting sliced garlic in aqueous ethanol and naturally aging for >10 months.[13] During this process, allicin is decomposed completely, and thus, the pungent odor of garlic is substantially reduced.[14] In addition, it was found that AGE contains characteristic sulfur-containing amino acids, such as *S*-1-propenylcysteine and *S*-allylcysteine,[15] which exhibits beneficial pharmacological effects, such as antioxidant, antihypertensive, and immunomodulatory effects.[16−18]

Because few studies have focused on the aroma of AGE, we recently characterized the aroma components in AGE using sensomics to elucidate the effect of the aging process on the aroma of AGE.[14] Twenty-five compounds, including sulfur-containing compounds, phenols, and esters, were identified as key odorants, and these key aromas were mostly absent in fresh garlic.[19,20] The aroma components of AGE were also found to be different from those of other garlic products, such as heated garlic and black garlic.[19−21] However, the key aroma compounds contributing to the acidic note of AGE remain unclear. Further investigation of the aroma of AGE using various analytical techniques led to the isolation and identification of additional important aroma compounds that contribute to the acidic note; these results are reported herein.

MATERIALS AND METHODS

Materials. AGE was provided by Wakunaga Pharmaceutical Co., Ltd. AGE was prepared by slicing garlic cloves and soaking them in an aqueous ethanol solution, followed by extraction and aging for >10 months at room temperature in an air tight container, and subsequent filtering of the solution.[13]

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Chemicals. All chemicals used in this study were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Kishida Chemical (Osaka, Japan), or Kanto Chemical Co., Ltd. (Tokyo, Japan). The purities of the chemicals used for sensory analysis were >95% (GC-FID).

Synthesis of (2*R*,4*S*)- and (2*S*,4*S*)-2,4-Dimethyl-1,3-dithiolane (1). *(R)-1,2-Dibromopropane (**5**).* Powdery triphenylphosphine (5.77 g, 22.0 mmol) was added to (*S*)-1,2-propanediol (**4**) (761 mg, 10.0 mmol) and tetrabromomethane (7.30 g, 22.0 mmol) in dichloromethane (50 mL) at 0 °C. The mixture was stirred for 6 h at room temperature. The reaction mixture was concentrated *in vacuo* and extracted with *n*-pentane. The extracts were dried (Na_2SO_4) and the solvent was removed *in vacuo*. The residue was purified on a short silica gel column using *n*-pentane as an eluent to yield a mixture of (*R*)-**5**, tetrabromomethane, and tribromomethane (6.60 g). The mixture was used for the next step without further purification. (*S*)-**5** was obtained from (*R*)-**4** by the same manner as described above.

*(S)-1,2-Propanedithiol, Diacetate (**6**).* K_2CO_3 (1.52 g, 11.0 mmol) was added to a solution of (*R*)-**5** and thioacetic acid (1.67 g, 22.0 mmol) in dimethylformamide (DMF) (10 mL) at 0 °C. The mixture was stirred for 2 h at room temperature. The reaction mixture was then extracted with diethyl ether. The extracts were washed with water and dried (Na_2SO_4), and then the solvent was removed *in vacuo*. The residue was purified on a silica gel column using *n*-hexane/AcOEt (9/1, v/v) to yield (*S*)-**6** (810 mg, 4.21 mmol). (*R*)-**6** was obtained from (*S*)-**5** by the same manner as described above. ^{1}H NMR of (*S*)-**6** and (*R*)-**6** were identical to that of the racemate. ^{1}H NMR for **6** (400 MHz, $CDCl_3$): δ 1.32 (d, 3H, *J* = 7.2 Hz), 2.30 (s, 3H), 2.34 (s, 3H), 3.10−3.20 (m, 2H), 3.65−3.75 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 19.6, 30.5, 30.6, 35.0, 39.2, 194.9, 195.1.

*(S)-1,2-Propanedithiol (**7**).* (*S*)-**6** (192 mg, 1.00 mmol) was dissolved in anhydrous diethyl ether (5 mL) and added dropwise to a suspension of lithium aluminum hydride (75.9 mg, 2.00 mmol) in anhydrous diethyl ether (1 mL) at 0 °C. The mixture was stirred for 3 h at room temperature. Lithium aluminum hydride was quenched by the slow addition of water and 15% NaOH aqueous solution at 0 °C. The reaction mixture was filtered and neutralized with hydrochloric acid. The solution was extracted with *n*-pentane and washed with water. The extracts were dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue was purified on a silica gel column using *n*-pentane/Et_2O (49/1, v/v) to yield (*S*)-**7** (91.1 mg, 0.842 mmol). (*R*)-**7** was obtained from (*R*)-**6** by the same manner as described above. ^{1}H NMR and MS of (*S*)-**7** and (*R*)-**7** were identical to those of the racemate. ^{1}H NMR for **7** (400 MHz, $CDCl_3$): δ 1.41 (d, 3H, *J* = 7.2 Hz), 1.63 (dd, 1H, *J* = 8.6 and 8.6 Hz), 1.82 (d, 1H, *J* = 6.8 Hz), 2.69−2.74 (m, 2H), 2.99−3.10 (m, 1H); MS-EI *m*/*z* (intensity in relative %): 39 (27), 41 (62), 45 (28), 47 (40), 59 (23), 60 (23), 61 (100), 74 (47), 75 (20), 108 (52; M^+). ^{13}C NMR (100 MHz, $CDCl_3$): δ 23.3, 35.5, 38.4.

*(2R,4S)- and (2S,4S)-2,4-Dimethyl-1,3-dithiolane (**1**).* Acetaldehyde (22 mg, 0.50 mmol) was added to a solution of (*S*)-**7** (55 mg, 0.50 mmol) and boron trifluoride diethyl ether complex (50 mg) in dichloromethane (2 mL). The mixture was stirred for 0.5 h at room temperature and then heated to 40 °C for 1 h. The reaction mixture was cooled and subsequently dissolved in dichloromethane. The extracts were washed with water and then dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue was purified on a silica gel column using *n*-pentane/Et_2O (49/1, v/v) to yield compound **1** (35 mg, 0.26 mmol). (2*R*,4*S*)-**1** and (2*S*,4*S*)-**1** were purified and isolated by preparative GC on an InertCap Pure WAX column (30 m × 0.53 mm i.d., 1 μm film thickness, GL Sciences, Tokyo, Japan). The enantiomeric excesses of isolated (2*R*,4*S*)-**1** and (2*S*,4*S*)-**1** were determined to be 75 and 74%, respectively, by GC analysis using a β-DEX 225 column [30 m × 0.25 mm i.d., 0.25 μm film thickness, (Merck, Darmstadt, Germany)]. ^{1}H NMR, ^{13}C NMR and MS of (2*R*,4*S*)-**1** and (2*S*,4*R*)-**1** were identical to those of the racemate. ^{1}H NMR for (2*R*,4*S*)-**1** and (2*S*,4*R*)-**1** (400 MHz, $CDCl_3$): δ 1.45 (d, 3H, *J* = 6.4 Hz), 1.64 (d, 3H, *J* = 6.6 Hz), 2.97 (dd, 1H, *J* = 7.8 and 11.5 Hz), 3.24 (dd, 1H, *J* = 4.8 and 11.5 Hz), 3.74−3.84 (m, 1H), 4.64 (q, 1H *J* = 6.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.2, 24.1, 45.4, 48.8, 51.5; MS-EI *m*/*z* (intensity in relative %): 41 (28), 45 (33), 59 (53), 60 (19), 64 (20), 74 (25), 92 (41), 119 (81), 134 (100; M^+). ^{1}H NMR for (2*S*,4*S*)-**1** and (2*R*,4*R*)-**1** (400 MHz, $CDCl_3$): δ 1.41 (d, 3H, *J* = 6.8 Hz), 1.61 (d, 3H, *J* = 6.6 Hz), 2.92 (dd, 1H, *J* = 7.2 and 11.5 Hz), 3.35 (dd, 1H, *J* = 5.0 and 11.5 Hz), 3.86−3.95 (m, 1H), 4.67 (q, 1H, *J* = 6.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.1, 25.5, 46.1, 47.9, 50.0; MS-EI *m*/*z* (intensity in relative %): 41 (27), 45 (32), 59 (51), 60 (19), 64 (19), 74 (24), 92 (39), 119 (81), 134 (100; M^+).

*Synthesis of 2,5-Dimethyl-1,4-dithiane (**2**).* Compound **2** was synthesized according to the method of Iranpoor and Owji.[22] ^{1}H NMR for (2*S*,5*S*)-**2** and (2*R*,5*R*)-**2** (400 MHz, $CDCl_3$): δ 1.41 (d, 6H, *J* = 6.8 Hz), 2.74−2.82 (m, 2H), 2.94−3.03 (m, 4H); MS-EI *m*/*z* (intensity in relative %): 41 (19), 45 (33), 59 (24), 60 (88), 74 (26), 75 (50), 88 (8), 106 (42), 133 (5), 148 (100; M^+). ^{1}H NMR for *meso-trans*-**2** (400 MHz, $CDCl_3$): δ 1.21 (d, 6H, *J* = 6.8 Hz), 2.54−2.70 (m, 4H), 3.12−3.22 (m, 2H); MS-EI *m*/*z* (intensity in relative %): 41 (17), 45 (18), 59 (20), 60 (68), 74 (22), 75 (35), 106 (38), 133 (6), 148 (100; M^+). ^{13}C NMR analysis was not conducted because of the limited amount of sample.

*Synthesis of 2,6-Dimethyl-1,4-dithiane (**3**).* A solution of compound **7** (11 mg, 0.10 mmol) in DMF (20 mL) was added dropwise for 30 min with stirring to a solution of compound **5** (20 mg, 0.10 mmol) and K_2CO_3 (14 mg, 0.20 mmol) in DMF (30 mL). The mixture was stirred for 30 min in a N_2 atmosphere, and then dissolved in dichloromethane and washed with water. The extracts were dried (Na_2SO_4), and the solvent was removed *in vacuo*. The residue was purified on a silica gel column using *n*-pentane/Et_2O (49/1, v/v) followed by preparative GC. *meso-trans*-**2** and *meso-cis*-**3** were obtained by the reaction of (*S*)-**5** and (*S*)-**7** under the same reaction conditions used for the synthesis of racemic compound **3**. *meso-trans*-**2** and *meso-cis*-**3** were separated by preparative GC on a DB-5 column (30 m × 0.53 mm i.d., 1 μm film thickness, J&W Scientific). (2*R*,5*R*)-**2** and (2*R*,6*R*)-**3** were obtained by the reaction of (*S*)-**5** and (*R*)-**7** under the same reaction conditions. (2*S*,5*S*)-**2** and (2*S*,6*S*)-**3** were obtained by the reaction of (*R*)-**5** and (*S*)-**7** by the same manner. *cis*-**2** ((2*S*,5*S*)-**2** and (2*S*,5*S*)-**2**) and *trans*-**3** ((2*S*,6*S*)-**3** and (2*R*,5*R*)-**3**) were purified by preparative GC on a DB-5 column (60 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific). The enantiomeric excesses of (2*R*,5*R*)-**2**, (2*S*,5*S*)-**2**, (2*S*,6*S*)-**3**, and (2*R*,6*R*)-**3** were determined to be >95% by GC analysis using a β-DEX 225 column (30 m × 0.25 mm i.d., 0.25 μm film thickness). ^{1}H NMR and MS of (2*S*,6*S*)-**3** and (2*R*,5*R*)-**3** were identical to those of the racemate. ^{1}H NMR for *meso-cis*-**3** (400 MHz, $CDCl_3$): δ 1.21 (d, 6H, *J* = 6.8 Hz), 2.70−2.91 (m, 4H), 2.95−3.07 (m, 2H); MS-EI *m*/*z* (intensity in relative %): 41 (15), 45 (17), 59 (18), 60 (55), 74 (17), 75 (18), 88 (4), 106 (56), 133 (3), 148 (100; M^+).^{1}H NMR for (2*S*,6*S*)-**3** and (2*R*,5*R*)-**3** (400 MHz, $CDCl_3$): δ 1.40 (d, 6H, *J* = 6.8 Hz), 2.60 (dd, 2H, *J* = 7.2 and 13.6 Hz), 2.96 (dd, 2H, *J* = 2.4 and 13.6 Hz), 3.13−3.22 (m, 2H); MS-EI *m*/*z* (intensity in relative %): 41 (18), 45 (19), 59 (24), 60 (63), 74 (18), 75 (23), 106 (54), 133 (3), 148 (100; M^+). ^{13}C NMR analysis was not conducted because of the limited amount of sample.

Purification and Isolation of the Acidic Odorants of AGE. AGE (500 mL) was diluted with 2.5 L of purified water, and the solution was passed through a column packed with 12 g of Porapak Q (GL Sciences, Tokyo, Japan), a ethylvinylbenzene−divinylbenzene copolymer, to adsorb the organic compounds. After the column was washed with water, the organic compounds adsorbed on the polymer were eluted with dichloromethane (200 mL). The organic compounds of AGE were collected from a total of 2000 mL of AGE. The extracts in 800 mL of dichloromethane were fractionated by solvent-assisted flavor evaporation (SAFE) without concentration to collect relatively high volatile compounds from the extract matrix.[23] The collected volatile compounds were dried (Na_2SO_4) and concentrated to 1 mL using a Vigreux column (150 mm × 6 mm i.d., Aldrich, Germany). The volatile compounds were then dissolved in *n*-hexane and applied onto a silica gel column (8 g). The *n*-pentane/Et_2O solvent system, which exhibits a very low boiling point,

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was used as the eluent to prevent the loss of highly volatile compounds. The column was eluted with 30 mL of *n*-pentane/Et_2O (97/3, v/v, Fr. 1), followed by 30 mL of *n*-pentane/Et_2O (97/3, v/v, Fr. 2), 30 mL of *n*-pentane/Et_2O (97/3, v/v, Fr. 3), 50 mL of *n*-pentane/Et_2O (90/10, v/v, Fr. 4), 50 mL of *n*-pentane/Et_2O (70/30, v/v, Fr. 5), 50 mL of *n*-pentane/Et_2O (50/50, v/v, Fr. 6), and 50 mL of Et_2O (Fr. 7). The fractions were subjected to GC−olfactometry (GC−O) analysis. The acidity aromas were isolated by preparative GC on an InertCap Pure WAX column (30 m × 0.53 mm i.d., 1 μm film thickness).

Preparative GC. Preparative GC was performed using an Agilent 7890 A gas chromatograph (Agilent Technology, CA, USA) equipped with collection traps (VPS 2800, GL Sciences). The column effluent was split 5:95 to the flame ionization detector (FID, 230 °C) and the collection trap (−15 °C).

For a fine separation and purification to prepare authentic specimens at high purity for sensory analysis, another preparative GC system equipped with a different collection system was used, as described by Nojima et al.[24] Short megabore capillary columns (DB-5, 20 cm, 1 μm film thickness) were used as traps for the collection system. As it is not necessary to cool the traps during the collection process, the same trap can be used for successive collections of the same GC fraction during sequential injections.[24] The traps were extracted with 100 μL of dichloromethane to recover the volatile compounds.

Preparative GC Condition for 2,4-Dimethyl-1,3-dithiolane. An Inert cap Pure WAX column (30 m × 0.53 mm i.d., 1 μm film thickness) was used. The injector temperature was 250 °C and the samples (3.0 μL) were injected by the splitless mode. Helium was used as the carrier gas at a flow rate of 6.0 mL/min. The oven temperature was held at 40 °C for 2 min for the preparation of AGE volatiles, then increased at a rate of 6°C/min to 230 °C, and further held at 230 °C for 5 min. The oven temperature was held at 60 °C for 2 min for the preparation of compound **1**, then increased at a rate of 1.5°C/min to 100 °C, further increased at a rate of 12°C/min to 230 °C, and then held at 230 °C for 5 min.

Preparative GC Condition for *trans*-2,5-Dimethyl-1,4-dithiane and *cis*-2,6-Dimethyl-1,4-dithiane. A DB-5 column (30 m × 0.53 mm i.d., 1 μm film thickness) was used. The injector temperature was 250 °C, and the samples (3.0 μL) were injected by the splitless mode. Helium was used as the carrier gas at a flow rate of 6.0 mL/min. The oven temperature was held at 60 °C for 2 min, increased at a rate of 6°C/min to 150 °C, then further increased at a rate of 12°C/min to 230 °C, and held at 230 °C for 5 min.

Preparative GC Condition for *cis*-2,5-Dimethyl-1,4-dithiane and *trans*-2,6-Dimethyl-1,4-dithiane. A DB-5 column (60 m × 0.25 mm i.d., 0.25 μm film thickness) was used. The injector temperature was 250 °C, and the samples (1.0 μL) were injected by the split mode (10:1). The oven temperature was held at 65 °C for 60 min, then increased at a rate of 12°C/min to 230 °C, and held at 230 °C for 5 min. Helium was used as the carrier gas at a flow rate of 1.6 mL/min.

GC−O Analysis. The odor qualities of each odorant were evaluated using GC−O by at least 5 trained panelists at concentrations near each threshold level. GC−O analysis was performed using an Agilent 7890 A gas chromatograph (Agilent Technology, CA, USA) equipped with an olfactory port (OP275, GL Science). A β-DEX 225 column (30 m × 0.25 mm i.d.), 0.25 μm film thickness, (Merck, Darmstadt, Germany) was used. The samples (1.0 μL) were injected by the split mode (10:1). The column temperature was held at 85 °C for 27 min [except for (2*R*,4*S*)-**1** and (2*S*,4*R*)-**1**], then increased at a rate of 8 °C/min to 220 °C, and held at 220 °C for 5 min. For (2*R*,4*S*)-**1** and (2*S*,4*R*)-**1**, the column temperature was held at 75 °C for 35 min, then increased at a rate of 8 °C/min to 220 °C, and held at 220 °C for 5 min. Helium was used as the carrier gas at a flow rate of 1.6 mL/min. The flow split ratio between the FID and the olfactory port was 1:1. The FID and the sniffing port were held at 250 °C.

GC−MS Analysis. An Agilent 7890 A gas chromatograph equipped with a 5975 C mass spectrometer was used. The ion energy for electron impact was 70 eV. The mass scan range was *m*/*z* 33−450. The oven temperature was held at 40 °C for 2 min, increased at a rate of 6 °C/min to 230 °C, and then held at 230 °C for 5 min. Selected ion monitoring (SIM) mode was used for quantitative analysis of odorants. 1-Octanol was used an internal standard (IS). The IS was spiked into 100 mL of AGE samples at a concentration of 0.106 mg/L and then subjected to purification steps. The concentrations of the odorants were determined by calibration curves of IS and each odorant. Preliminary purification of odorants was performed using a Porapak Q column and SAFE. We monitored the following ions by SIM (*m*/*z*): **1** (134, 119); **2** (148, 106); **3** (148, 106); and 1-octanol (IS, 84, 70). Linear retention indices (RIs) of each odorant were calculated by GC−MS analysis by using *n*-alkanes C6−C26 on a DB-WAX column (60 m × 0.25 mm i.d., 0.25 μm film thickness) and C6−C18 on a DB-5 column (60 m × 0.25 mm i.d., 0.25 μm film thickness).

Nuclear Magnetic Resonance (NMR) Spectroscopy. ^1H, ^{13}C NMR and $^1H-^1H$ correlation spectroscopy ($^1H-^1H$ COSY) experiments were performed using an Agilent 400-MR (Agilent Technologies, Tokyo, Japan). $CDCl_3$ was used as the solvent. The chemical shifts were referenced to tetramethylsilane.

Sensory Analysis. Sensory analysis was performed by 20 trained panelists. The sensory attributes and aroma references were used to define the following aromas: acid (acetic acid), cabbage (dimethyl sulfide), cooked potato (methional), pungent (sliced fresh garlic), seasoning (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone), caramel (maltol), metallic (uncured ham), and fatty (bacon). Panelists were trained to share common sensory attributes and intensities using aroma reference solutions before sensory analysis. The intensities were scored from 0 (no odor) to 2.0 (strong odor) in increments of 0.5 (0, 0.5, 1.0, 1.5, and 2.0).

Odor thresholds in water were determined by the triangular test according to Czerny et al.[25] Two 45 mL glass containers containing purified water (20 mL) were used as blank samples. One vessel contained aqueous solution of odorants. The solution was diluted stepwise 1:2 with water. The samples were tested by 20 trained panelists. The detection odor threshold of each odorant was calculated according to Czerny et al.[25] Odor activity values (OAVs) were calculated by dividing the concentrations by the odor thresholds of the compounds in water.

Statistical Analysis. Sensory test results were analyzed by analysis of variance (ANOVA) followed by Tukey−Kramer multiple comparisons test using JMP 11 software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Isolation and Specification of the Acidic Note of AGE. Volatile compounds of AGE were collected and partially purified by SAFE, and then separated into 7 fractions (Fr. 1−7) by silica gel column chromatography (Figure 1). Significant acidic and sulfury odors were found in Fr. 3 by the sensory test; this fraction was then subjected to GC−O analysis. Three acidic aromas and one strong sulfury aroma were found in the fraction at RIs of 1422, 1542, 1599, and 1430 on a DB-WAX column, respectively. One of these (RI = 1542) was specified in our previous study as an important key aroma in AGE.[14] The RIs of the odors are reported in Table 1. The mass spectra of all 4 odorants clearly show sulfur isotopic patterns, but their fragmentation patterns were not found in the databases (NIST 08 and Wiley W9N08). Their molecular ions were tentatively determined as follows: RI 1422, *m*/*z* 134; RI 1430, *m*/*z* 134; RI 1542, *m*/*z* 148; and RI 1599, *m*/*z* 148, respectively. The compounds were further purified and isolated using preparative GC for NMR analysis.

Identification of 2,4-Dimethyl-1,3-dithiolane. The GC peaks at RIs of 1422 and 1430 on a DB-WAX column were collected in the same fraction by preparative GC (Fr. 3−1) and

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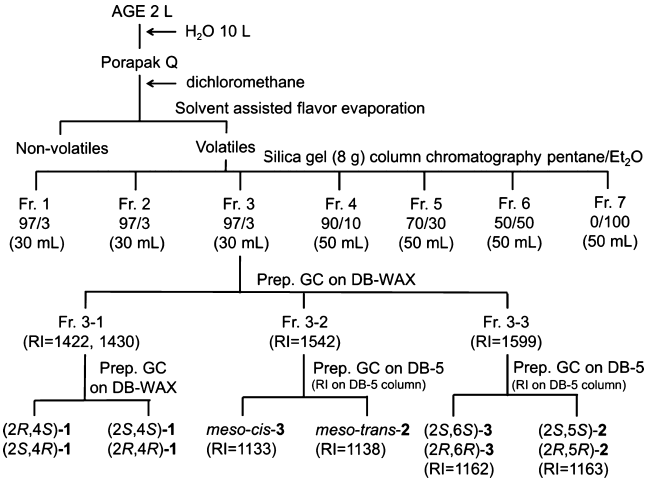

Figure 1. Isolation scheme for acidic odor components from aged garlic extract (AGE). **1**: 2,4-dimethyl-1,3-dithiolane, **2**: 2,5-dimethyl-1,4-dithiane, and **3**: 2,6-dimethyl-1,4-dithiane.

subjected to further analysis by GC−O, GC−MS (using a different polarity column), and NMR. Both peaks also exhibited strong odors as confirmed by GC−O analysis using a DB-5 column. The mass spectra showed similar patterns (Figures S1 and S2), suggesting that the compounds were related to each other. Fr. 3−1 was subjected to NMR analysis without further purification (Figures S3 and S4). The ^{1}H NMR spectrum of Fr. 3−1, that is, a mixture of two compounds, was easily assigned to each of the two individual compounds based on the ^{1}H−^{1}H COSY results and integration results. Based on the NMR and MS results of the mixture, these odorants were tentatively identified as a mixture of diastereomers of compound **1**. Compound **1** was synthesized, and the structures of the odorants eluted at RI 1422 and 1430 on a WAX column were found to be *cis*- and *trans*-2,4-dimethyl-1,3-dithiolane, respectively. The identities were subsequently confirmed by matching their mass spectra, RIs, ^{1}H NMR spectra, and odor qualities with those of the synthesized authentic compounds. The position of the *cis*−*trans* isomer was determined by comparing the NMR data to that in previous report.[26]

Compound **1** exhibits two asymmetric centers, and thus, has four stereoisomers. All four isomers were synthesized to determine the absolute configuration of **1** (Figure 2). Each of these isomers were completely separated on a β-DEX 225 column. The GC peaks with retention times of 28.3 and 29.0 min for *cis*-**1** were determined to be (2*S*,4*R*)-**1** and (2*R*,4*S*)-**1**, respectively. For *trans*-**1**, retention times of 16.7 and 17.2 min were determined to be (2*S*,4*S*)-**1** and (2*R*,4*R*)-**1**, respectively. AGE contains enantiomers of both diastereomers at a ratio of about 50:50. To the best of our knowledge, this is the first time these compounds were identified in food products.

The concentrations of *cis*-**1** and *trans*-**1** were found to be 1.27 and 0.78 mg/L in AGE, respectively (Table 1). These odorants were not detected in fresh garlic (data not shown). In our preliminary experiment, compound **1** was shown to be generated by simply mixing and heating acetaldehyde, hydrogen sulfide and allyl mercaptan in aqueous ethanol; these starting materials are easily generated from ethanol and allicin.[27,28] Thus, it appears that these compounds are generated by a similar process during aging.

Characterization of the Aroma of 2,4-Dimethyl-1,3-dithiolane. The odor qualities of each stereoisomer were evaluated by GC−O (Table 2). For *cis*-**1**, the odor quality of (2*R*,4*S*)-**1** was described as acid and malty, whereas that of (2*S*,4*R*)-**1** was described as garlic and gasoline. For *trans*-**1**, (2*S*,4*S*)-**1** was described as sulfur and sweet, whereas (2*R*,4*R*)-**1** was described as garlic and green. These results suggest that the odor qualities depend on the stereochemistry of compound **1**.

Odor thresholds of compound **1** were determined using synthetic racemic mixtures because the threshold test needs a relatively large amount and high purity of compound, which is not easy to prepare. The thresholds of *cis*-**1** and *trans*-**1** were determined as 14.7 and 5.7 μg/L, respectively (Table 1). OAVs of *cis*-**1** and *trans*-**1** in AGE were 86 and 131, respectively (Table 1). The OAV of *trans*-**1** was the 9th highest in AGE.

Identification of 2,5- and 2,6-Dimethyl-1,4-dithiane. The GC peaks at RIs of 1542 and 1599 on a DB-WAX column were collected by preparative GC (Fr. 3−2 and Fr. 3−3, respectively; Figure 1) and subjected to further analysis by GC−O and GC−MS using a different polarity column. Two peaks were detected for each fraction (Fr. 3−2 and Fr. 3−3) by GC analysis using a DB-5 column, indicating these were coeluted on a DB-WAX column. All 4 peaks from the two fractions exhibited strong odors by GC−O analysis using a

Table 1. RIs, Concentrations, Odor Thresholds, and OAVs of 2,4-Dimethyl-1,3-dithiolane, 2,5-Dimethyl-1,4-dithiane, and 2,6-Dimethyl-1,4-dithiane and Their Respective *cis*−*trans* Isomers in AGE

odorant	RI DB-5	RI DB-WAX	concentration in AGE[a] (mg/L)	odor threshold in water (μg/L)	OAV	quantified ion[d] (m/z)	correlation coefficient	calibration range[e] (mg/L)	LOD[f] (μg/L)	LOQ[f] (μg/L)
cis-2,4-dimethyl-1,3-dithiolane[b]	1034	1422	1.27 ± 0.036	14.7	86	134	0.9998	0.28−2.27	2.1	6.4
trans-2,4-dimethyl-1,3-dithiolane[b]	1036	1430	0.78 ± 0.036	5.7	139	134	0.9999	0.32−2.57	2.5	7.5
cis-2,5-dimethyl-1,4-dithiane[b]	1163	1599	0.14 ± 0.021	not determined[c]	not determined[c]	106	0.9970	0.024−0.24	4.6	13.7
trans-2,5-dimethyl-1,4-dithiane	1138	1542	0.11 ± 0.008	0.17	647	106	1.0000	0.027−0.27	6.0	18.0
cis-2,6-dimethyl-1,4-dithiane	1133	1542	0.17 ± 0.000	9.9	17	106	0.9998	0.063−0.63	4.7	14.0
trans-2,6-dimethyl-1,4-dithiane[b]	1162	1599	0.36 ± 0.031	not determined[c]	not determined[c]	106	0.9996	0.060−0.60	4.4	13.3

[a]Mean values of triplicate measurements with standard deviations. [b]Concentration, odor threshold, and OAV were determined as a racemic mixture. [c]Preparation of high purity specimens for the odor threshold test was difficult. [d]Ions selected for quantitative analysis. [e]Calibration range of the corresponding concentration in aged garlic extract. [f]The limits of detection were estimated as the concentrations of a standard that produced a signal-to-noise ratio of 3. The limits of quantitation were estimated as the concentrations of a standard that produced a signal-to-noise ratio of 10.[33]

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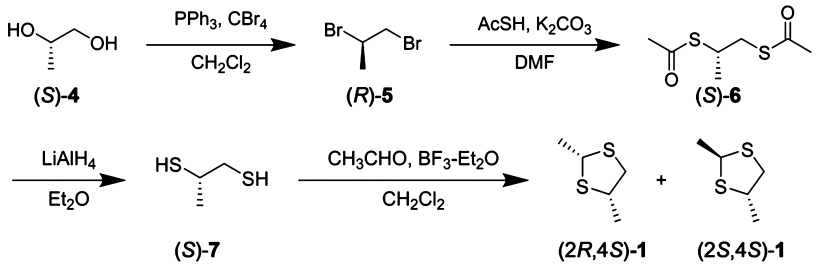

Figure 2. Synthesis of stereoisomers of 2,4-dimethyl-1,3-dithiolane.

Table 2. Odor Qualities of 2,4-Dimethyl-1,3-dithiolane, 2,5-Dimethyl-1,4-dithiane, and 2,6-Dimethyl-1,4-dithiane and Their Respective Stereoisomers by GC-O Analysis

odorant	odor quality
(2*S*,4*R*)-2,4-dimethyl-1,3-dithiolane	garlic, gasoline
(2*R*,4*S*)-2,4-dimethyl-1,3-dithiolane	acid, malty
(2*S*,4*S*)-2,4-dimethyl-1,3-dithiolane	garlic, sulfury, sweet
(2*R*,4*R*)-2,4-dimethyl-1,3-dithiolane	garlic, green
(2*S*,5*S*)-2,5-dimethyl-1,4-dithiane	sulfury, leather, gassy
(2*R*,5*R*)-2,5-dimethyl-1,4-dithiane	peppery, citrus
meso-trans-2,5-dimethyl-1,4-dithiane	green, metallic, acid, mushroom
meso-cis-2,6-dimethyl-1,4-dithiane	rubber, fruity, green, woody, acid
(2*S*,6*S*)-2,6-dimethyl-1,4-dithiane	sulfury, rubber, garlic
(2*R*,6*R*)-2,6-dimethyl-1,4-dithiane	woody, seasoning, sulfury, rubber

DB-5 column. The mass spectra of these odorants showed similar patterns (Figures S5−S8), suggesting that they are related compounds that exhibit similar chromatographic behaviors on a silica gel column, as well as both polar and nonpolar GC columns. Fr. 3−2 and Fr. 3−3 were then subjected to NMR analysis without further purification (Figures S9−S12). The ^{1}H NMR spectrum of Fr. 3−2 was easily assigned to each of the individual compounds based on the ^{1}H−^{1}H COSY results and integration results. Based on the NMR and MS results of the mixture, the odorants in Fr. 3−2 were tentatively identified as stereoisomers of compound **2** or **3**. The ^{1}H NMR spectrum of Fr. 3−3 was also easily assigned to each of the individual compounds, and the odorants in Fr. 3−3 were also tentatively identified as stereoisomers of compound **2** or **3**.

All stereoisomers of compounds **2** and **3** were synthesized and the structures of the odorants in Fr. 3−2 were identified as (2*R*,6*S*)-2,6-dimethyl-1,4-dithiane (*meso-cis*-**3**) and (2*R*,5*S*)-2,5-dimethyl-1,4-dithiane (*meso-trans*-**2**) by matching their mass spectra, RIs, ^{1}H NMR spectra, and odor qualities with those of synthesized authentic compounds. The odorants in Fr. 3−3 were identified as *cis*-**2** ((2*S*,5*S*)-**2** or (2*R*,5*R*)-**2**), and *trans*-**3** ((2*R*,6*R*)-**3** or (2*S*,6*S*)-**3**), without absolute configuration.

All isomers of *cis*-**2** and *trans*-**3** were synthesized to determine their absolute configurations (Figure 3). The two enantiomers of each of *cis*-**2** and *trans*-**3** were completely separated on a *β*-DEX 225 column. For *cis*-**2**, the GC peaks with retention times of 22.4 and 23.2 min were determined to be (2*S*,5*S*)-**2** and (2*R*,5*R*)-**2**, respectively. For *trans*-**3**, the GC peaks with retention times of 21.8 and 22.3 min were determined to be (2*R*,6*R*)-**3** and (2*S*,6*S*)-**3**, respectively. AGE contains enantiomers of both **2** and **3** at a ratio of about 50:50. Yu et al. reported that compound **2** was tentatively identified in baked blanched garlic, fried blanched garlic, and thermally degraded alliin.[28,29] Compound **3** was identified in simulated

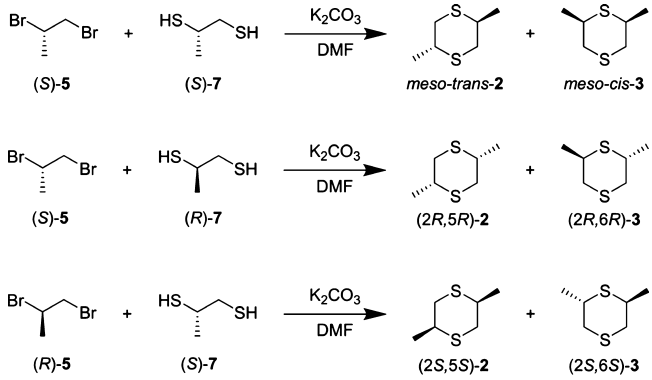

Figure 3. Synthesis of stereoisomers of 2,5-dimethyl-1,4-dithiane, and 2,6-dimethyl-1,4-dithiane.

meat flavors[30] and thermal treatment of the meat broths in the presence of alkyl-mercaptopropanol.[31] To date, the aroma properties of compounds **2** and **3** have not been well studied.

The concentrations of *cis*-**2**, *trans*-**2**, *cis*-**3** and *trans*-**3** in AGE were determined to be 0.14, 0.11, 0.17, and 0.36 mg/L, respectively (Table 1). These odorants were not detected in fresh garlic (data not shown). Yu et al. proposed that compound **2** is the cyclization product of two allyl mercaptan molecules.[28] Although it seems likely that a similar reaction occurred during the aging process, this remains to be clarified. To better understand the generation pathway of the various component compounds and the corresponding change in the aroma profile of AGE, a systematic investigation of the aroma profiles of AGE during the aging process is necessary and should be the subject of future research.

Characterization of the Aroma of 2,5- and 2,6-Dimethyl-1,4-dithiane. Each stereoisomer of compounds **2** and **3** yielded different odor qualities (Table 2). In particular, *trans*-**2** exhibits a unique and strong odor (green, metallic, garlic, and mushroom) compared with the other isomers. The odorant reported as the key aroma compound was determined to be *trans*-**2** upon comparison of retention times and odor quality. The odor detection thresholds of *trans*-**2** and *cis*-**3** in water were determined to be 0.17 and 9.9 *μ*g/L, respectively (Table 2). The odor of *trans*-**2** was just as strong as the well-known flavoring methional (odor threshold: 0.43 *μ*g/L) and dimethyl sulfide (odor threshold: 0.84 *μ*g/L).[25] The quantities of pure *cis*-**2** and *trans*-**3** were too small to determine odor thresholds. The OAV of *trans*-**2** was determined to be 647, the fifth highest in AGE. Kubec et al. reported the aroma characters of an isomeric pair of compound **2**, noting that one isomer had an unpleasant odor, while the other had a mushroom-like odor with a considerably lower threshold value.[32] The aroma character reported in the literature for the later isomer is very similar to our evaluation results for *trans*-**2**.

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While the configurations of compound **2** were not determined in the previous report, we herein succeeded in determining the absolute configurations.

Sensory Analysis of Aroma Recombinant. The aroma profiles of recombinant samples were evaluated by sensory analysis to determine the contributions of compounds **1**, **2** and **3** to the acidic note of AGE. The following two aroma recombinates were evaluated: (i) 25 compounds in aqueous ethanol solution at their actual AGE concentrations reported in a previous report[14] and (ii) a blend of the 25 compounds and compounds **1**, **2**, and **3** at their actual AGE concentrations. The average scores of 20 panelists are shown in Figure 4.

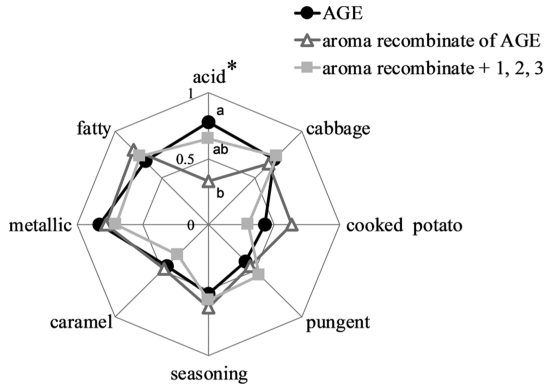

Figure 4. Aroma profiles of AGE and aroma recombinate of AGE. **1**: 2,4-dimethyl-1,3-dithiolane, **2**: 2,5-dimethyl-1,4-dithiane, and **3**: 2,6-dimethyl-1,4-dithiane. Each of the attributes denoted with different letters indicate significant differences (ANOVA, Tukey's post-hoc test, $p < 0.05$).

ANOVA showed significant differences for the scores for "acid" ($p < 0.05$) and no significant differences for that of other sensory attributes. The score for "acid" of the aroma recombinant without compounds **1**, **2**, and **3** (0.33 points) was less intense ($p < 0.05$) compared with that of original AGE (0.78 points). Addition of compounds **1**, **2**, and **3** resulted in good agreement in the intensity of "acid" (0.65 points) with that of original AGE. A blend of the 25 compounds and compounds **1**, **2**, and **3** in aqueous ethanol was able to simulate the overall aroma of AGE, including the other 7 descriptors.

In conclusion, we further investigated the aroma of AGE using various analytical techniques, such as GC−O, accurate and high-performance preparative GC system, GC−MS analysis, and sensory evaluation, in an effort to better understand the aroma of AGE. 2,4-Dimethyl-1,3-dithiolane (**1**), 2,5-dimethyl-1,4-dithiane (**2**), and 2,6-dimethyl-1,4-dithiane (**3**) and their respective stereoisomers were identified as key aroma components of the acidic note of AGE. Our studies revealed the chemical composition of the aroma of AGE, and we hope this finding leads researchers to develop a method and process to further reduce the pungent smell of fresh garlic. In addition, we also hope these newly identified sulfur heterocyclic odorants, which exhibit characteristic flavors, lead to the development of new flavorings and pharmacological compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c06634.

^{1}H NMR, ^{1}H−^{1}H COSY and mass spectra of compounds **1**, **2**, and **3** in AGE; mass spectrum of *cis*-2,4-dimethyl-1,3-dithiolane in AGE; mass spectrum of *trans*-2,4-dimethyl-1,3-dithiolane in AGE; ^{1}H NMR spectrum of the mixture of *cis*-2,4-dimethyl-1,3-dithiolane and *trans*-2,4-dimethyl-1,3-dithiolane in AGE; ^{1}H−^{1}H COSY spectrum of the isolated mixture of *cis*-2,4-dimethyl-1,3-dithiolane and *trans*-2,4-dimethyl-1,3-dithiolane in AGE; mass spectrum of *cis*-2,5-dimethyl-1,4-dithiane in AGE; mass spectrum of *trans*-2,5-dimethyl-1,4-dithiane in AGE; mass spectrum of *cis*-2,6-dimethyl-1,4-dithiane in AGE; mass spectrum of *trans*-2,6-dimethyl-1,4-dithiane in AGE; ^{1}H NMR spectrum of the mixture of *trans*-2,5-dimethyl-1,4-dithiane and *cis*-2,6-dimethyl-1,4-dithiane in AGE; ^{1}H−^{1}H COSY spectrum of the isolated mixture of *trans*-2,5-dimethyl-1,4-dithiane and *cis*-2,6-dimethyl-1,4-dithiane in AGE; ^{1}H NMR spectrum of the mixture of *cis*-2,5-dimethyl-1,4-dithiane and *trans*-2,6-dimethyl-1,4-dithiane in AGE; and ^{1}H−^{1}H COSY spectrum of the isolated mixture of *cis*-2,5-dimethyl-1,4-dithiane and *trans*-2,6-dimethyl-1,4-dithiane in AGE (PDF)

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Notes

The authors declare no competing financial interest.

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