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Characterization of Phenolic Constituents Inhibiting the Formation of Sulfur-Containing Volatiles Produced during Garlic Processing

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Supporting Information

ABSTRACT: Garlic (*Allium sativum* L.), which is a widely distributed plant, is globally used as both spice and food. This study identified five novel phenolic compounds, namely, 8-(3-methyl-(*E*)-1-butenyl)diosmetin, 8-(3-methyl-(*E*)-1-butenyl)chrysin, 6-(3-methyl-(*E*)-1-butenyl)chrysin, and Alliumones A and B, along with nine known compounds **6**−**14** from the ethanol extract of garlic. The structures of these five novel phenolic compounds were established via extensive 1D- and 2D-nuclear magnetic resonance spectroscopy experiments. The effects of the phenolic compounds isolated from garlic on the enzymatical or nonenzymatical formation of sulfur-containing compounds produced during garlic processing were examined. Compound **12** significantly reduced the thermal decomposition of alliin, whereas compound **4** exhibited the highest percentage of alliinase inhibition activity (36.6%).

KEYWORDS: *Allium sativum, phenolic compounds, alliin, alliinase*

INTRODUCTION

Garlic (*Allium sativum* L.), which is a widely distributed plant, has been used as spice, food, and folk medicine worldwide for hundreds of years.[1,2] Garlic is a leading herbal remedy used by alternative medical practitioners. Garlic exhibits a wide range of therapeutic effects, such as hypolipidaemic,[3] antiatherosclerotic,[4] antidiabetic,[5] antimicrobial,[6] anticarcinogen,[7] and immunomodulatory[8] activities.

The characteristic smell and health activities of garlic result from sulfur-containing volatiles;[9] these substances are formed when fresh, raw garlic is chopped or crushed, thereby rupturing the intracellular compartments that contain the nonprotein amino acid alliin (*S*-allyl-L-cysteine sulfoxide), pyridoxal-5-phosphate-dependent enzyme alliinase (L-(+)-*S*-alk(en)-ylcysteine sulfoxide lyase, EC 4.4.1.4), and α, β-eliminating endogenous lyase from *Allium* spp.[10,11] or from alliin, which is also a product nonenzymatically formed when garlic or alliin is thermally processed at temperatures >100 °C.[9,12−14]

Aside from S-containing volatiles, garlic also contains phenolic compounds, including the flavonoids[15−17] myricetin, quercetin, and apigenin, as well as phenolic acids,[18] such as gallic, chlorogenic, ferulic, *o*-coumaric, and cinnamic acids, the contents of which were affected by the genotype and location of garlic and the processing condition of "seed" cloves. For instance, the white and Chinese garlic cultivars contained higher contents of total phenolics and ferulic acid than those of the purple garlic cultivars;[18] phenolic compounds were more abundant in the leaves than in the bulbs;[19] and low-temperature conditioning of garlic seed cloves increased the synthesis of phenolic compounds, resulting in a threefold content increase compared with those conditioned at room temperature.[20] Most phenolic compounds exhibit interesting biochemical properties, such as anti-HIV property,[21] capability to protect low-density lipoprotein cholesterol from oxidation,[22] antioxidative characteristics,[23−26] inhibition of ACE activity.[27] and binding affinity toward P-glycoprotein.[28]

Besides having various biological activities, phenolic compounds usually play important roles in food processing.[29] They easily form complexes with other food component such as protein and lipid via hydrogen bonding interactions, leading to changes in physicochemical properties of the latter such as solubility, thermal stability, and digestibility.[30−34] Therefore, phenolic compounds, as the major metabolites of garlic, could interact with alliin or alliinase during garlic processing. These phenolic compounds affect the formation of S-containing compounds, thereby altering the flavor and health benefits of garlic. However, little information is available on the systematic isolation of the phenolic compounds from garlic and the effect of such compounds on alliin or alliinase.

This research aimed to characterize phenolic compounds from garlic and study the effects of them on the enzymatical or nonenzymatical formation of S-containing compounds. Fourteen phenolic compounds that were isolated from garlic, including five novel flavones, were investigated to determine the inhibitory activities of the compounds on the enzymatical or nonenzymatical formation of S-containing compounds. To our knowledge, this study provided the first evidence of the functions of phenolic compounds in garlic during alliinase catalyst reaction and thermal decomposition of alliin.

MATERIALS AND METHODS

General Experimental Procedures. All chemicals, including alliin (98.5%) and alliinase (EC 4.4.1.4, 1000 U/mg), were purchased

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from Aladdin Industrial Corporation (China). Distilled water was used throughout the experiments. All the experiments were performed at room temperature unless stated otherwise. UV/vis spectra were obtained using a Spectrumlab 53 UV−visible spectrophotometer (Shanghai Lengguang Technology Co., Ltd., Shanghai, China). Nuclear magnetic resonance (NMR) spectra were acquired using a 400 MHz NMR machine (Bruker), and IR spectra were obtained using a Fourier transform-infrared spectrophotometer (Nicolet Avatar). All the experiments were performed in triplicate.

Plant Material. In January 2014, garlic (*Allium sativum* L.) was collected from Guangzhou, which is located in the Guangdong province of China. Voucher specimens (Accession No. 201410005) were deposited at the herbarium of the Jinan University.

Extraction and Isolation. Around 25 kg of air-dried garlic was peeled and extracted thrice with 95% ethanol (3 × 30 L) at 80 °C. The extract was evaporated to dryness in a vacuum at 60 °C, and 1500.5 g of the crude extract was obtained. The crude extract was purified using an Amberlite A21 column (dimension of column: 10 cm, flow rate: 20 mL/min, eluted with 0%, 25%, 50%, 75%, 90%, and 100% ethanol, volume of solvent: 6 × 40 L) to afford six fractions, namely, fractions 1−6. Fraction 1 contained high amounts of sugar and amino acids, which were not further separated. Fraction 2 (10.1 g, 25% ethanol fraction) was further separated by passing the compounds to a Sephadex LH-20 column (dimension of column: 4 cm, flow rate: 2 mL/min, solvent: MeOH, volume of solvent: 2000 mL) to afford two fractions, namely, fractions 2A and 2B. After evaporation of the solvent at a reduced pressure, fraction 2A (4.2 g) was passed through a 100 g silica gel column by using MeOH/CH_2Cl_2 (2:10) as the eluting solvent to afford compound **12** (300 mg). Fraction 2B (2.4 g) was also passed through a 50 g silica gel column, developed with PE/EtOAc (5:0.5, 5:1, and 5:2), and monitored via thin-layer chromatography by using PE/EtOAc (5:1) to afford compounds **8** (20 mg, R_f = 0.35) and **9** (10 mg, R_f = 0.40). Fraction 3 (14.0 g, 50% ethanol fraction) was separated using a silica gel column (200 g) and eluted by PE/EtOAc (5:1). Subsequently, the fraction was separated by a Sephadex LH-20 column (dimension of columns: 1 cm, flow rate: 0.2 mL/min, solvent: MeOH, volume of solvent: 200 mL) to afford compound **10** (8.8 mg) and another fraction (subfraction A). Subfraction A was further purified by HPLC [250 mm × 10 mm YMC-Pack-C_{18} column, mobile phase: MeOH/H_2O (30:70), detection wavelength: 254 nm, flow rate: 1.0 mL/min] to obtain compound **7** (8.3 mg, Rt = 10.5 min). Fraction 4 (12.1 g, 75% ethanol fraction) was subjected to RP_{18} gel (200 g) column by using MeOH−H_2O (from 8:2 to 9:1) as the eluting solvent to obtain two fractions, namely, 4A and 4B. Fraction 4A was separated using a silica gel column and EtOAc/PE (1:5) as the eluting solvent. Fraction 4A was then purified via preparative TLC CH_2Cl_2/PE(1:1) to afford compound **1** (11 mg, R_f = 0.5). Fraction 4B was subjected to a Sephadex LH-20 column (dimension of column: 1 cm, flow rate: 0.1 mL/min, solvent: MeOH, volume of solvent: 180 mL) and then purified via preparative HPLC [250 mm × 10 mm YMC-Pack-C_{18} column, mobile phase: MeOH/H_2O (20:80), detection wavelength: 254 nm, flow rate: 1.0 mL/min] to obtain compounds **4** (10 mg, Rt = 13.5 min) and **5** (11 mg, Rt = 14.8 min). Fraction 5 (25.8 g, 90% ethanol fraction) was separated using a silica gel column and EtOAc/PE (1:5) to afford three fractions, including 5A−5C. Fraction 5A was separated by a Sephadex LH-20 column (dimension of column: 1 cm, flow rate: 0.2 mL/min, solvent: CH_3COCH_3, volume of solvent: 170 mL) to obtain compound **14** (14.6 mg), and fraction 5B was chromatographed using a silica gel column (400 g) with CH_2Cl_2/MeOH mixtures of increasing polarity to afford compound **11** (10.4 mg). Fraction 5C was separated using a Sephadex LH-20 column (dimension of column: 1 cm, flow rate: 0.2 mL/min, solvent: MeOH/H_2O (1:1), volume of solvent: 240 mL) to yield compound **6** (18.8 mg). Meanwhile, fraction 6 (10.2 g, 100% ethanol fraction) was separated by silica and developed with CH_2Cl_2/MeOH (100:1, 100:5, 10:1) to obtain two fractions, namely, fractions 6A and 6B. Fraction 6A was purified using a silica gel column [30g, CH_2Cl_2/CH_3COCH_3 (100:1)] and further prepared via preparative TLC (EtOAc/PE (5:1) to obtain compounds **2** (8.1 mg, R_f = 0.4) and **3** (8.4 mg, R_f = 0.34). Fraction 6B was separated using a silica gel column (30 g, CH_2Cl_2/MeOH [50:1]) to obtain compound **13** (10.9 mg).

*8-(3-Methyl-(E)-1-butenyl)diosmetin (**1**).* Yellow powder; $C_{21}H_{20}O_6$; m.p.194−196 °C; UV (MeOH) λ_{max} (log ϵ) 220 (2.24), 267 (4.12) nm; IR (KBr) υ_{max}: 3377, 2949, 1643, 1580, 1399, and 851 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 6.58 (1H, s, H-3), 6.24 (1H, s, H-6), 7.39 (1H, d, J = 2.0 Hz, H-2′), 7.04 (1H, d, J = 8.4 Hz, H-5′), 7.48 (1H, dd, J = 8.4 and 2.0 Hz, H-6′), 3.94 (3H, s, OCH_3), 6.54 (1H, d, J = 16.4 Hz, H-1″), 6.48 (1H, dd, J = 16.4 and 6.8 Hz, H-2″), 2.53 (1H, m, H-3″), and 1.18 (6H, d, J = 6.8 Hz, H-4″ and H-5″); HR−ESI−MS, m/z 369.1331 $[M + H]^+$ (calcd for $C_{21}H_{21}O_6$, 369.1333).

*8-(3-Methyl-(E)-1-butenyl)chrysin (**2**).* Yellow powder; $C_{20}H_{18}O_4$; m.p.184−186 °C; UV (MeOH) λ_{max} (log ϵ) 223 (2.38), 276 (4.49) nm; IR (KBr) υ_{max}: 3401, 2926, 1607, 1578, 1422, and 872 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 6.61 (1H, s, H-3), 6.17 (1H, s, H-6), 7.88 (2H, d, J = 7.6 Hz, H-2′ and H-6′), 7.52 (3H, m, H-3′, H-4′, and H-5′), 6.46 (1H, d, J = 16.4 Hz, H-1″), 6.43 (1H, dd, J = 16.4 and 6.4 Hz, H-2″), 2.46 (1H, m, H-3″), and 0.89 (6H, d, J = 6.8 Hz, H-4″ and H-5″); HR−ESI−MS, m/z 323.1279 $[M + H]^+$ (calcd for $C_{20}H_{19}O_4$, 323.1278).

*6-(3-Methyl-(E)-1-butenyl)chrysin (**3**).* Yellow powder; $C_{20}H_{18}O_4$; m.p.188−189 °C; UV (MeOH) λ_{max} (log ϵ) 218 (2.36), 275 (3.11) nm; IR (KBr) υ_{max}: 3363, 2924, 1644, 1580, 1379, and 850 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 6.67 (1H, s, H-3), 6.43 (1H, s, H-8), 7.54 (2H, d, J = 6.8 Hz, H-2′ and H-6′), 7.95 (2H, dd, J = 6.8 and 2.0 Hz, H-3′ and H-5′), 7.56 (1H, d, J = 6.8 Hz, H-4′), 6.63 (1H, d, J = 16.4 Hz, H-1″), 6.68 (1H, dd, J = 16.4 and 6.4 Hz, H-2″), 2.44 (1H, m, H-3″), 1.07 (6H, d, J = 6.8 Hz, H-4″ and H-5″); HR−ESI−MS, m/z 323.1271 $[M + H]^+$ (calcd for $C_{20}H_{19}O_4$, 323.1278).

*Alliumone A (**4**).* Yellow powder; $C_{35}H_{28}O_8$; m.p.312−314 °C; UV (MeOH) λ_{max} (log ϵ) 216 (2.44), 277 (5.19), nm; IR (KBr) υ_{max}: 3469, 2924, 1647, 1580, 1397, and 845 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 6.61 (2H, s, H-3), 6.16 (2H, s, H-6), 7.14 (4H, dd, J = 8.0 and 7.8 Hz, H-2′ and H-6′), 7.62 (4H, d, J = 7.8 Hz, H-3′ and H-5′), 7.22 (2H, dd, J = 8.0 and 7.8 Hz, H-4′), 5.54 (1H, t, J = 8.0 Hz, H-1″), 2.12 (2H, t, J = 8.0 Hz, H-2″), 1.44 (1H, m, H-3″), 0.84 (6H, d, J = 6.4 Hz, H-4″ and H-5″); HR−ESI−MS, m/z 577.1840 $[M + H]^+$ (calcd for $C_{35}H_{29}O_8$, 577.1857).

*Alliumone B (**5**).* Yellow powder; $C_{35}H_{28}O_8$; m.p.331−333 °C; $[\alpha]_{20}^{D}$ +10.04 (c 0.001, methanol); UV (MeOH) λ_{max} (log ϵ) 216 (2.78), 277 (5.79), and 345 (2.45) nm; IR (KBr) υ_{max}: 3433, 2953, 1656, 1590, 1416, and 878 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) unit I: δ 6.72 (1H, s, H-3), 6.34 (1H, s, H-8), 7.59 (2H, m, H-2′ and H-6′), 8.34 (2H, dd, J = 7.8 and 1.2 Hz, H-3′ and H-5′), 7.51 (1H, m, H-4′), 5.74 (1H, t, J = 8.4 Hz, H-1″), 2.28 (1H, m, H-2″α), 2.21 (1H, m, H-2″β), 1.44 (1H, m, H-3″), 0.92 (3H, m, H-4″), 0.93 (3H, m, H-5″); unit II: δ 6.60 (1H, s, H-3), 6.12 (1H, s, H-6), 7.50 (2H, m, H-2′ and H-6′), 7.94 (2H, d, J = 7.0 Hz, H-3′ and H-5′), 7.57 (1H, m, H-4′); HR−ESI−MS, m/z 577.1857 $[M + H]^+$ (calcd for $C_{35}H_{29}O_8$, 577.1857).

Alliin and Allicin Analysis. Quantitative analyses of alliin and allicin were performed using an Agilent 1260 infinity quaternary liquid chromatography (Hewlett-Packard, Wilmington, NC) equipped with a 250 cm × 4.6 mm ODS-A-C_{18} (5 nm particle size) column. The HPLC conditions were as follows: alliin and allicin, as mobile phase of MeOH/H_2O (20:80) and (15:85), detection wavelength of 220 and 254 nm, and flow rate of 0.8 and 1.0 mL/min, respectively.

Allicin Synthesis. Alliin was synthesized as previously described with slight modifications.[35] Diallyl disulfide was placed under vacuum at 0 °C to remove traces of allyl disulfide. A solution of diallyl disulfide (1.00 g, 7.0 mmol) in chloroform (30 mL) and *m*-chloroperbenzoic acid (77%, 1.79 g, 8.0 mmol) in chloroform (5 mL) was added dropwise at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. Anhydrous sodium carbonate (8 g) was added in small portions with vigorous stirring. The reaction mixture was stirred for an additional 0.5 h at 0 °C and then filtered through a pad of Celite and magnesium sulfate. The filtrate was concentrated under reduced pressure to yield 0.90 g (85% yields) of crude allicin, which was purified by column chromatography (PE: EtOAc, 80:20). ^{1}HNMR (400 MHz,

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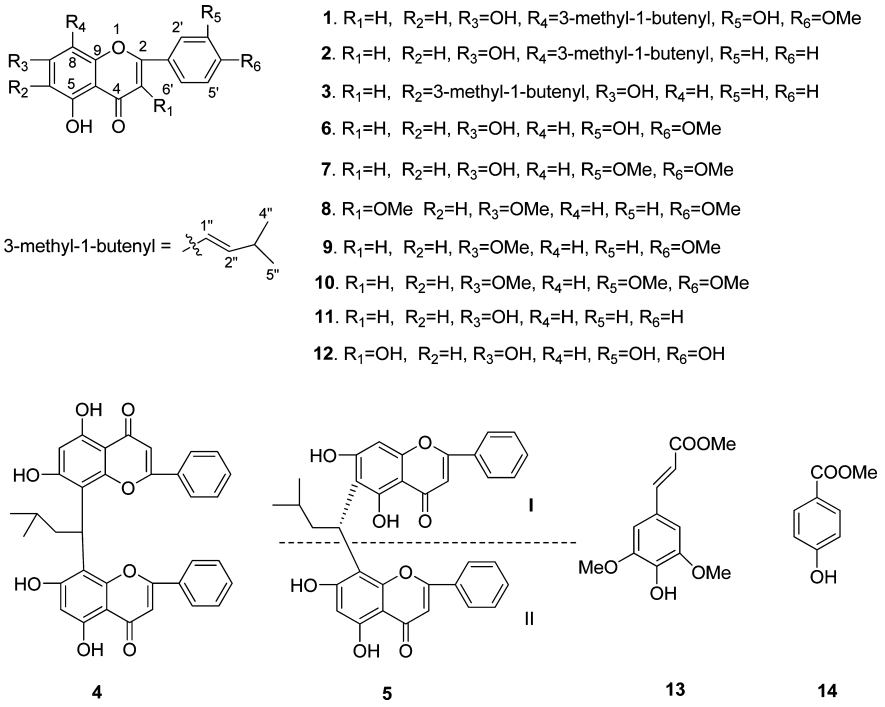

Figure 1. Structures of compounds **1**−**14**.

CD_3CO_2D): δ 3.72−3.92 (m, 4H), 5.20−5.56 (m, 4H), 5.88−5.99 (m, 2H); ^{13}CNMR (100 MHz, CD_3CO_2D): δ 35.0, 59.9, 119.1, 124.0, 125.8, 132.9 ppm.

Thermal Decomposition of Alliin. The thermal decomposition of alliin was estimated by determining its content in the samples according to a previously described method.[12,36] Exactly 5 mg of alliin, 1.0 mg of phenolic compounds isolated from garlic (control: no phenolic compound added), and water were placed in a 5 mL glass tube. The tube was then sealed and equilibrated for 24 h. After equilibration, the tube was heated in an oven at 120 °C for 2 h, cooled in a freezer to −18 °C, and crushed into water to a total volume of 5 mL. The resulting solution was immediately analyzed for alliin content.[10,21] The inhibition percentage was calculated as follows: inhibition percent = [(content of alliin − content of control)/content of control] × 100%. Triplicate measurements were performed.

Assay of Alliinase Activity. Alliinase activity was assayed by determining the production of pyruvate from alliin according to a previously described method with slight modifications.[10] Briefly, the alliinase activity was assayed by determining the production of allicin from alliin. Around 0.5 mL of a 890 U mL^{-1} alliinase sample was added to 1 mL of the standard reaction mixture containing 60 mmol L^{-1} sodium phosphate buffer (pH = 6.5), 25 μmol L^{-1} pyridoxal phosphate, 1 mmol L^{-1} phenolic compounds isolated from garlic (control: no addition of phenolic compound), and 20 mmol L^{-1} pure alliin as substrate. The enzymatic reaction was incubated at 25 °C for 5 min, and the reaction was terminated by adding 2 mL of 0.1 g L^{-1} trichloroacetic acid. The solution was centrifuged to remove the precipitated protein, and the supernatant was assayed for pyruvate concentration via colorimetric analysis. Around 0.5 mL of 1.0 g kg^{-1} 2,4-dinitrophenylhydrazine was added to the supernatant and incubated at 25 °C for 5 min. Exactly 5 mL of 0.5 mol L^{-1} NaOH was then added to the solution and incubated at 25 °C for 10 min. The absorbance at 520 nm was determined using a Spectrumlab 53 UV−visible spectrophotometer (Shanghai Lengguang Technology Co., Ltd., Shanghai, China). The pyruvate concentration was calculated according to the pyruvate standard curve, and the inhibition percentage was calculated as follows: alliinase inhibition (%) = [(content of control − content of pyruvate)/content of control] × 100%. Triplicate measurements were performed.

Procedure for Allicin Decomposition. Allicin decomposition was estimated by determining the content of allicin in the samples according to a previously described method.[35] Exactly 0.5 mg of phenolic compound (control: no phenolic compound added) was added into 0.5 mL of 10 mmol L^{-1} allicin aqueous solution at room temperature. The aliquots were withdrawn from the reaction mixture at a regular time interval (24 h). The resulting solution was immediately analyzed for allicin content, after which the decomposition percentage of allicin was calculated. Each value is the average obtained from three runs of chromatography performed on the sample. Allicin decomposition (%) = [(initial content of allicin − content of allicin)/initial content of allicin] × 100%.

RESULTS AND DISCUSSION

Identification of Compounds 1−14. Exactly 95% aqueous ethanol extract of garlic was separated successively by partitioning the extract using Amberlite A21 column (eluted with 0%, 25%, 50%, 75%, 90%, and 100% ethanol) and repeated Sephadex LH-20 columns. Gel column chromatography afforded five new phenolic compounds **1**−**5** (Figure 1). Nine known compounds, namely, diosmetin (**6**),[37] 2-(3,4-dimethoxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one (**7**),[38] 5-hydroxy-3,7-dimethoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one (**8**),[39] 5-hydroxy-7-methoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one (**9**),[40] 2-(3,4-dimethoxyphenyl)-5-hydroxy-7-methoxy-4*H*-chromen-4-one (**10**),[41] chrysin (**11**),[42] quercetin (**12**),[4,43] (*E*)-methyl 3-(4-hydroxy-3,5-dimethoxyphenyl)-acrylate (**13**),[44] and methyl-4-hydroxybenzoate (**14**),[45] were also obtained and identified based on their spectral data and comparison with literature reports.

Compound **1** exhibited the molecular structure $C_{21}H_{20}O_6$. This result was inferred from the HR-EIMS, ^{13}C NMR, and DEPT data, which indicated 12 degrees of unsaturation. The IR spectrum indicated the presence of hydroxyl groups (3377 cm^{-1}) and a carbonyl group (1643 cm^{-1}), which was conjugated with an aromatic ring. The ^{1}H NMR spectrum provided signals of most functional groups, including five

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aromatic protons [δH 6.58 (1H, s), 6.24 (1H, s), 7.39 (1H, d, *J* = 2.0) 7.04 (1H, d, *J* = 8.4 Hz), and 7.48 (1H, dd, *J* = 8.4 and 2.0 Hz)] and a 3-methyl-1-butenyl group [6.54 (1H, d, *J* = 16.4 Hz, H-1″), 6.48 (1H, dd, *J* = 16.4 and 6.8 Hz, H-2″), 2.53 (1H, m, H-3″), and 1.18 (6H, d, *J* = 6.8 Hz, H-4″ and H-5″)]. The ^{13}C NMR spectrum (Table 1) also exhibited signals for one

Table 1. ^{13}C NMR Chemical Shift Assignments (δ) of 1−5 (in CD_3OD, 100 MHz)

				4	5	
positions	1	2	3	unit I, II	unit I	unit II
2	166.1	165.3	164.7	165.5	165.3	164.5
3	104.3	105.7	105.6	106.2	105.3	105.0
4	184.2	184.0	183.4	183.6	183.1	184.0
5	161.1	161.1	160.3	161.2	161.0	160.8
6	100.0	100.5	111.5	103.5	116.7	103.3
7	164.1	165.5	168.6	172.1	171.4	172.8
8	106.6	106.8	95.7	110.4	97.7	112.0
9	155.9	155.9	157.7	157.3	158.3	157.2
10	105.3	105.1	104.3	104.0	104.1	103.4
1′	125.3	132.7	132.6	132.2	133.1	132.9
2′	114.2	130.1	130.2	129.8	130.1	129.9
3′	148.3	127.5	127.3	127.3	128.0	127.2
4′	152.6	132.9	132.6	132.2	132.7	132.6
5′	112.4	127.5	127.3	127.3	128.0	127.2
6′	120.1	130.1	130.2	129.8	130.1	129.9
1″	116.9	117.3	118.2	30.1	28.0	
2″	142.7	142.3	141.9	42.0	41.0	
3″	34.2	34.1	34.5	26.9	27.7	
4″	23.2	23.0	23.3	23.0	23.1	
5″	23.2	23.0	23.3	23.0	23.3	
MeO	56.4					

carbonyl group, seven aromatic methane groups, and seven aromatic quaternary C, including at least four oxygenated C signals at δC 155.9, 161.1, 164.1, and 166.1. This result revealed that compound **1** contained a flavone skeleton. Compound **1** was deduced as 3-methyl-1-butenyl group-substituted diosmetin[22] based on 2D NMR spectrum. In the HMBC spectrum, the following long-range cross peaks (^2J and ^3J) were observed in a ring: singlet at δH 6.24 (H-6) with C-5 (δC 161.1), C-7 (δC 164.1), C-8 (δC 106.6), and C-10 (δC 105.3); as well as methane doublet at δH 6.54 (H-1″) correlated with C-7 (δC 164.1), C-8 (δC 106.6), and C-9 (δC 155.9), which indicated that the 3-methyl-1-butenyl group was attached at C-8. An aromatic methoxy group at δH 3.94 correlated with C-4′ (δC 152.6), and another proton at δH 6.48 (H-2′) showed cross peaks with C-6′ (δC 120.1), C-3′ (δC 148.3), and C-4′ (δC 152.6). These results indicated the presence of a 3′-hydroxyl-4′-methoxybenzoyl moiety. The stereochemistry at C-2″ was *E* because the coupling constant between H-1″ and H-2″ was 16.4 Hz.[46] Thus, the structure of compound **1** was (*E*)-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-8-(3-methylbut-1-enyl)-4*H*-chromen-4-one, which was named as 8-(3-methyl-(*E*)-1-butenyl) diosmetin.

Compound **2** was obtained as a yellow amorphous powder. The EIMS exhibited a molecular ion peak at *m*/*z* 323 [M + 1]. The elemental formula of the compound was $C_{20}H_{18}O_4$, as confirmed by the results of HR−ESI−MS ([M + H] 323.1279; calcd for $C_{20}H_{19}O_4$, 323.1278). The IR and UV data of compound **2** were compared with those of compound **1**, and the same basic skeleton as compound **1** was observed in compound **2**. The ^{13}C NMR spectra of **2** displayed signals for one carbonyl group, seven aromatic methane groups, including two symmetrical aromatic methane groups, as well as seven aromatic quaternary C, including three oxygenated C signals at δC 165.3, 161.1, and 165.5. Analysis of the ^{13}C NMR of compound **2** showed that an aromatic methoxy and two oxygenated aromatic quaternary C signals were eliminated, and two aromatic methane signals were added compared with those of compound **1**, which indicated that the C-3′ and C-4′ positions cannot be substituted by the methoxy and hydroxyl groups. The results revealed that compound **2** contained a chrysin skeleton.[42] Compound **2** was deduced as an isomer of 8-prenychrysin.[47] By contrast, the ^{13}C NMR and DEPT data of **2** are similar to those of 8-prenylchrysin, except that a downfield quaternary carbon signal and a methylene signal in 8-prenylchrysin were replaced by two methane signals in **2**, which indicated that **2** contained 3-methyl-1-butenyl group instead of prenyl group in 8-prenylchrysin.[44] In the HMBC NMR experiments, proton δH 6.46 (H-1″) showed correlations with C-7 (δC 165.5), C-8 (δC 106.8), and C-9 (δC 155.9), thereby confirming that the 3-methyl-1-butenyl group was attached at C-8. Accordingly, compound **2** was identified as (*E*)-5,7-dihydroxy-8-(3-methylbut-1-enyl)-2-phenyl-4*H*-chromen-4-one, which was named as 8-(3-methyl-(*E*)-1-butenyl)-chrysin.

Compound **3** exhibited the same molecular formula ($C_{20}H_{18}O_4$) according to the HR−ESI−MS ([M + H] 323.1271; calcd for $C_{20}H_{19}O_4$, 323.1278). The IR bands showed the presence of OH groups (3363 cm^{-1}). A comparison of the NMR spectra of compounds **2** and **3** (Table 1) showed the similarity of the two compounds. The major difference indicated that one aromatic methane signal at δC 100.5 and an aromatic quaternary C signal at δC 106.8 in compound **2** were replaced by that at δC 95.7 and δC 111.5 in compound **3**. This finding implied that the 3-methyl-1-butenyl group, which substituted the position in compound **3**, was different from that in compound **2**. The correlations between a methane doublet at δH 6.63 (H-1″) and C-7 (δC 168.6), C-8 (δC 95.7), and C-9 (δC 157.7) in the HMBC spectrum suggested that the 3-methyl-1-butenyl group was linked to a C-6 carbon. Thus, the structure was characterized as (*E*)-5,7-dihydroxy-6-(3-methylbut-1-enyl)-2-phenyl-4*H*-chromen-4-one, which was named as 6-(3-methyl-(*E*)-1-butenyl)chrysin.

Compound **4** was isolated as a yellow powder. The molecular formula of compound **4** was $C_{35}H_{28}O_8$ according to the results of ^{13}C (DEPT), NMR spectra, and HR−ESI−MS ([M + H] 577.1840; calcd for $C_{35}H_{29}O_8$, 577.1857). The ^{1}H and ^{13}C NMR spectral data of compound **4** were analogous to those of compound **2**. The ^{13}C NMR spectrum showed that two signals, which were ascribed to methane C atoms (δC = 30.1 ppm) at C-1″ and C-2″ (δC = 42.0 ppm) in compound **4**, were observed in place of the two olefinic C atoms (δC = 117.3 and 142.3 ppm, respectively) in compound **2**. The characteristic proton signal at δH = 5.54 ppm, which was attributed to C-1″ (δC = 30.1 ppm), suggested that C-1″ was linked with two symmetric flavone moieties that combined the molecular formula with ^{13}C NMR spectral data and was confirmed by the significant HMBC cross peaks from the methane proton at δH 5.54 to C-8 (δC = 110.4 ppm) and C-7 (δC = 172.1 ppm). Thus, compound **4** was determined as 8,8′-(3-methylbutane-1,1-diyl)bis(5,7-dihydroxy-2-phenyl-4*H*-chromen-4-one), which is a novel natural compound named *Alliumone A*.

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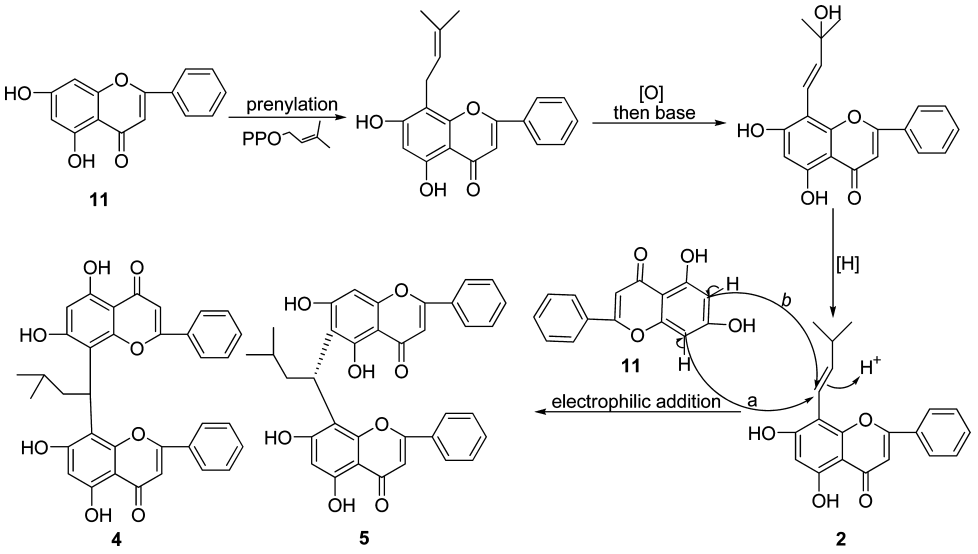

Figure 2. Proposed biosynthesis of **4** and **5**.

Compound **5** was obtained as a yellow amorphous powder. The molecular formula of compound **5** was $C_{35}H_{28}O_8$ according to the ^{13}C (DEPT), NMR spectra, and HR−ESI−MS ([M + H] 577.1857; calcd for $C_{35}H_{28}O_8$, 577.1857). These results were the same as those for compound **4**. The NMR signals of compound **5** appeared in pairs at the same region as those of compound **4**, which suggested that compound **5** was the nonsymmetrical isomer of compound **4**. The presence of a correlation peak from the characteristic proton signal at δH = 5.74 ppm, which was ascribed to C-1″, C-8, and C-6, in the HMBC experiment (δC = 28.0 ppm), demonstrated that C-1″ was attached to the C-8 of unit I and C-6 of unit II. A detailed analysis of the HMBC, H-2″(I) to C-4″(I) and C-1″(I), H-8(I) to C-10(I) and C-7(I), H-6(II) to C-10(II) and C-8(II), H-3(I II) to C-4(I II), C-10(I II), and C-1′(I II), as well as H-4′(I II) to C-3′/5′(I II) and C-2′/6′(I II), facilitated the assignment of all the protons and Cs. An *S* configuration of a chiral center was tentatively assigned according to the optical rotation [$(\alpha)_{20}^{D}$ +10.04 (c 0.001, methanol)] of compound **5** with that of (*S*)-1-ferrocenyl-4-(1-cyclopentylethyl)benzene.[48] In conclusion, the structure of compound **5** was determined as (*S*)-8-(1-(5,7-dihydroxy-4-oxo-2-phenyl-4*H*- chromen-6-yl)-3-methylbutyl)-5,7-dihydroxy-2-phenyl-4*H*-chromen-4-one, which is a novel natural compound named *Alliumone B*.

A plausible biogenetic pathway to **4** and **5** was proposed (Figure 2). Compound **11** generated 8-prenylchrysin though prenylation reaction. The prenyl group of the latter was oxidized and then basified, leading to form 8-(3-hydroxy-3-methyl-but-1-enyl)chrysin, which was similar to the last step of the formation of hyperualones B.[49] The resulting compound was reduced to generate **2**, which further produced the electrophilic addition reaction at the C(1″)= C(2″) bond with C-8 of **11** via a or C-6 of **11** via b (Figure 2), thereby forming **4** and **5**, respectively.

Effect of Phenolic Compounds on Thermal Degradation of Alliin. Previous research reported that alliin is self-degraded or thermally decomposed to generate volatile S-containing compounds.[12−14] These compounds are generated from alliin via hydrolysis or free-radical rearrangement.[14] Most phenolic compounds are strong radical scavengers; thus, we investigated the effects of phenolic compounds isolated from garlic on the thermal decomposition of alliin (Figure 3). The phenolic

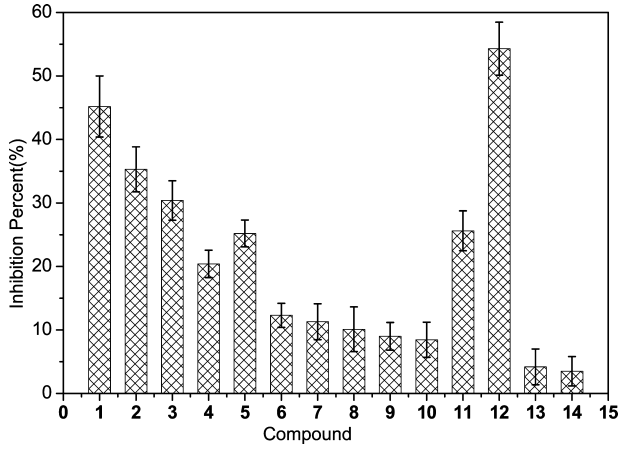

Figure 3. Effect of phenolic compounds from garlic on thermal degradation of alliin.

compounds positively affected the inhibition of the thermal degradation of alliin, in which addition of flavones, such as compounds **11** and **12,** led to stronger inhibition than that of other phenolic compounds, including **13** and **14**. Compound **12** exhibited a maximum inhibition of about 51% compared with compound **14,** which exhibited 5.2% inhibition.

The hydroxyl group in the flavones affected the inhibition ability of the compounds. Compound **11** exhibited more potential inhibition ability than those of compounds **9** and **10** because the latter compounds lacked a hydroxyl substituent at the 7-position of the A ring. This result suggested that the hydroxyl group substituent at the 7-position of the A ring can increase the inhibition ability of the compound. The 3-methyl-1-butenyl group substituent on the flavone skeleton also increased the inhibition capability of the flavone, particularly when the substituent was located at the 8-position of the A ring. This result was exhibited by compounds **2**, **3**, and **11**. Compounds **2** and **3** exhibited one 3-methyl-1-butenyl group substituent at the 8- and 6-positions, respectively, whereas compound **11** lacked a 3-methyl-1-butenyl group substituent in the skeleton. Thermal degradation assay results showed that compounds **2** and **3** exhibited stronger inhibition ability than that of compound **11**. Compound **2** also exhibited better

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inhibition activity than compound **3**, which suggested that the presence of a 3-methyl-1-butenyl group at the 8-position in the skeleton caused stronger inhibition ability relative to that with a 3-methyl-1-butenyl group at the 6-position.

The above results indicated that most flavones clearly exhibited strong inhibition during the alliin degradation. This finding was expected because the flavones behaved as strong radical scavengers, which prevented free radical reaction during the thermal process. The 3-methyl-1-butenyl group substituent, which behaved as an electron-donating substituent at the ring, also decreased the vertical ionization potentials, thereby resulting in better antioxidant activity.[50] This behavior can strongly inhibit the thermal degradation of alliin.

Effect of Phenolic Compounds on Alliinase Activity. The alliinase catalyst reaction was affected by the temperature, pH, concentration, solvent, and presence of additives.[8,9,51,52] However, the functions of the phenolic compounds, which are major secondary metabolites of garlic in the alliinase activity, remain unknown. Figure 4 shows the effects of 14

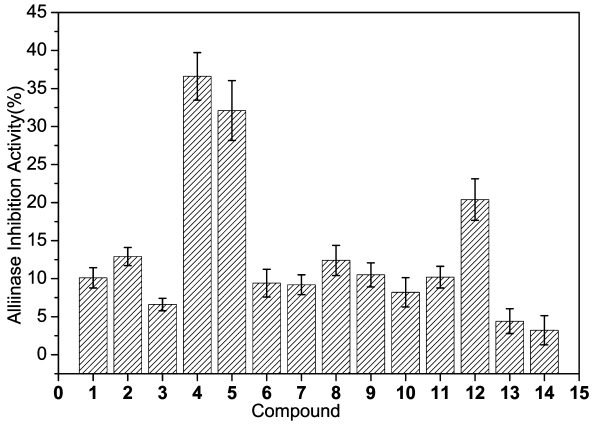

Figure 4. Alliinase inhibition activity of phenolic compounds isolated from garlic.

phenolic compounds isolated from garlic on the alliinase activity. The results indicated that flavones with more hydroxyls (e.g., compound **12**) exhibited higher inhibitory activity than that with more methoxyls (e.g., compounds **8** and **9**). Biflavone **4** also showed the highest percentage of alliinase inhibition activity at 36.6%. Moreover, compound **5** exhibited 32.7% alliinase inhibition activity. The alliinase inhibition activities of compounds **4** and **5** were correspondingly 3.6 and 3.2 times more potent than that of compound **11**. These results implied that the efficiency of flavones as inhibitors of alliinase depended largely on their chemical structures, relative orientation, and number of hydroxyl groups attached to the aromatic ring.

In addition to interactions with alliinase and alliin, phenolics may also interact with allicin as the reactive species formed upon the alliinase cleavage of alliin. Allicin is very unstable and thus easily decomposes into S-containing volatiles. Phenolics could affect allicin stability. Therefore, two phenolics **4** and **12**, which respectively showed strong interaction with alliinase and alliin, were selected to evaluate their effects on allicin stability (Figure 5). After 144 h of the test, the addition of phenolics **4** and **12** caused the decomposition percentage of allicin to reach 47% and 48%, respectively, compared with the control at 52%. This finding is consistent with that obtained by Fujisawa.[53] This result implies that the addition of phenolics slightly promoted allicin stability at room temperature.

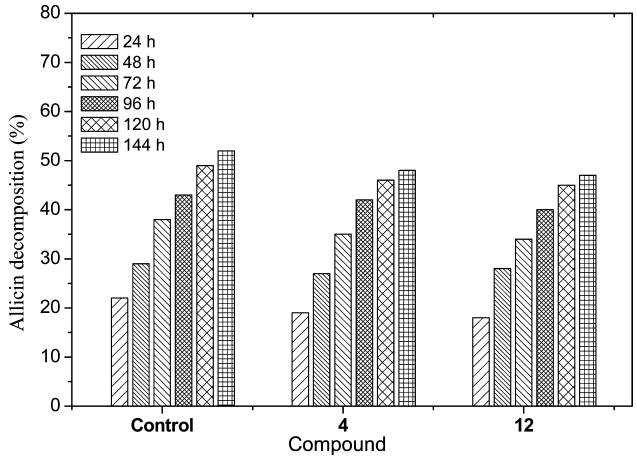

Figure 5. Decomposition percentage of allicin with phenolic compounds **4** and **12**. Each value is the average obtained from three runs of chromatography performed on the sample.

In the present study, five novel flavones, namely, 8-(3-methyl-(*E*)-1-butenyl)diosmetin, 8-(3-methyl-(*E*)-1-butenyl)-chrysin, 6-(3-methyl-(*E*)-1-butenyl)chrysin, and Alliumones A and B, together with nine known compounds **6**−**14**, were identified from the ethanol extract of garlic. To our knowledge, this research is the first systematic report on phenolic compounds from garlic and thus provides the first evidence of the effects of phenolic compounds in garlic on the enzymatical or nonenzymatical formation of S-containing compounds. This study demonstrated that compounds **12** exhibited the strongest inhibition ability for the thermal degradation of alliin (51%), and biflavone **4** showed the highest percentage of alliinase inhibition activity (36.6%). The results may serve as important references for garlic processing.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR, ^{13}C (DEPT) NMR, HSQC, HMBC, and ^{1}H−^{1}H COSY spectra and HR−ESI−MS data of compounds **1**−**5** and ^{1}H and ^{13}C NMR data of compounds **6**−**14**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

PE, petroleum ether; EtOAc, ethyl acetate; R_f, retention factor; Rt, retention time; HIV, human immunodeficiency virus; ACE, angiotensin 1 converting enzyme; TLC, thin-layer chromatography

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