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Identification of (*S*,*S*)-γ-Glutamyl-(*cis*-*S*-1-propenyl)thioglycine, a Naturally Occurring Norcysteine Derivative, from the Chinese Vegetable *Toona sinensis*

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Supporting Information

ABSTRACT: Extracts of *Toona sinensis* shoots were studied to identify the precursors of volatile sulfur-containing flavor molecules. *T. sinensis* was found to contain new compounds (*S*,*S*)-γ-glutamyl-(*cis*-*S*-1-propenyl)thioglycine, **1**, (*S*,*S*)-γ-glutamyl-(*trans*-*S*-1-propenyl)thioglycine, **2**, and γ-glutamyl-(*cis*-*S*-1-propenyl)-cysteine, **3**. The structures of these compounds were determined by interpretation of multistage mass spectrometric (MS^n), 1D, and 2D NMR data. The absolute configuration of **1** was established by comparison of experimental with computed infrared and vibrational circular dichroism spectra. Because of the flexibility of the molecule and the novelty of the structure, the configuration was further confirmed by X-ray crystallography. Compounds **1** and **2** are the first examples of norcysteine-containing metabolites reported from nature. They may release thiols via cleavage of the amide bond by proteases, followed by spontaneous decomposition of the resulting unstable alk(en)yl norcysteine moiety.

KEYWORDS: *Toona sinensis, dipeptide, norcysteine, absolute configuration, thiol, cysteine S-conjugate*

INTRODUCTION

Chinese toon (*Toona sinensis* (A. Juss.) Roem, synonym *Cedrela sinensis*) is a rapidly growing deciduous tree that is widely distributed in northern and southeastern China and has a long history of consumption as a traditional vegetable, medicinal plant, and source of wood.[1] Every spring, the new growth, or tender shoots, of the *T. sinensis* tree are harvested and cooked in a variety of dishes. However, about 2 weeks after emerging, the leaves are no longer considered to be suitable for eating because they are said to become fibrous. The brief availability of this seasonal delicacy, as well as its compelling flavor, causes it to be much appreciated in large parts of northern China. In recent years, sprouts from *Toona* seeds have become an increasingly popular food. Both *T. sinensis* tender shoots and sprouts have a strong, fresh, and unique aroma that is characteristic of sulfur-containing volatile compounds.

The volatile sulfur compounds of onion, which are formed during cutting by a very fast enzymatic degradation of *S*-alk(en)yl-L-cysteine *S*-oxides, followed by a cascade of chemical reactions, contribute significantly to the characteristic aroma.[2] From the observation of fresh *Toona* flavor, we postulated that the aroma of *T. sinensis* is due to the formation of sulfur-containing volatile compounds that might be similar to those found in onion. However, only Mu et al.[3] have described the sulfur chemistry of *T. sinensis* shoots and identified a limited number of sulfur-containing volatile compounds. Considering the significant contribution that volatile sulfur compounds are believed to make to the complex alliaceous aroma of *T. sinensis*, we felt that the identification of nonvolatile precursors would be important for understanding the formation of sulfur-containing volatiles that occurs when the plant tissues are damaged by chopping or chewing. In this study, we investigated the sulfur-containing nonvolatile flavor precursors of *T. sinensis* that led to the discovery of two novel natural dipeptides containing thioglycine or norcysteine fragments, along with four cysteine derivatives, including one new cysteine dipeptide derivative and three known cysteine analogues.

MATERIALS AND METHODS

General Experimental Procedures. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded in D_2O and deuterated dimethyl sulfoxide (DMSO-d_6). In D_2O, sodium 3-(trimethylsilyl)-tetradeuteriopropionate was used as the internal standard. In DMSO-d_6, the residual solvent was used as the internal standard. Chemical shifts were recorded as δ values in parts per million (ppm). All relevant NMR signals for the diastereomeric mixtures were listed. Resonances were assigned by correlation spectroscopy (COSY), heteronuclear single-quantum correlation (HSQC), and heteronuclear multiple-bond correlation (HMBC) spectroscopy experiments. Gas chromatography (GC)−flame photometric detector (FPD)−mass spectrometry (MS) analysis was performed on a GC system (Agilent 6890) equipped with an electron impact−mass spectrometry (EI-MS) detector, an FPD, and a fused silica capillary column (DB1-MS, 30 m × 0.25 mm, film thickness = 0.25 μm). A standard GC method was used for the *T. sinensis* simultaneous distillation−extraction (SDE) extract: helium flow rate, 1 mL/min; oven temperature, 50 °C for 5 min, increased to 250 °C at 5 °C/min, and then held at 250 °C for 10 min. Reverse-phase (RP) high-performance liquid chromatography (HPLC) was

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Table 1. NMR Spectroscopic Data for Compound 1 (500 MHz, D_2O and DMSO-d_6)

	1 in D_2O		1 in DMSO-d_6		1 in DMSO-d_6 + D_2O[a]	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	175.9, C		170.5, C		170.4, C	
2	56.3, CH	3.90, t (6)	53.0, CH	3.45, br t	52.8, CH	3.38, m
3	28.8, CH_2	2.18, m	27.2, CH_2	1.89,m, 1.97, m	26.8,CH_2	1.88, m, 2.0, m
4	34.2, CH_2	2.54, t (8)	31.7, CH_2	2.34, t (8)	31.5,CH_2	2.32, m
5	176.5, C		171.1, C		171.1, C	
6	58.8, CH	5.44, s	55.7, CH	5.34, d (9)	55.4, CH	5.29, s
7	175.0, C		169.2, C		169.1, C	
8	121.5, CH	6.08, d (9)	122.9, CH	6.25, d (9)	122.9, CH	6.22, d (9)
9	134.2, CH	5.97, m	123.3, CH	5.62, m	123.3, CH	5.62, m
10	16.9, CH_3	1.70, dd (1, 7)	14.4, CH_3	1.58, d (7)	14.3, CH_3	1.56, d (7)
N−H				8.74, d (9)		

[a]Measured in about 0.3 mL of DMSO-d_6 with the addition of one drop of D_2O.

performed on an Agilent 1200 liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector (DAD), column oven (30 °C), and either an analytical (150 mm × 4.6 mm i.d., 3 μm) or semipreparative (150 mm × 10 mm i.d., 5 μm particle size) Supelco (Bellefonte, PA, USA) Discovery HS F5 column. Melting points were determined by using a capillary melting point apparatus and are reported uncorrected. LC-MS experiments were performed by using a Thermo Finnigan LXQ ion trap mass spectrometer equipped with an electrospray ionization (ESI) interface coupled to a Thermo Accela HPLC instrument with a PDA detector and an analytical Supelco Discovery HS F5 column (150 mm × 4.6 mm i.d., 3 μm). High-resolution electrospray ionization mass spectrometry (HRESIMS) was measured on a Bruker APEX III 7.0 T Fourier transform (FTMS) or IonSpec 4.7 T FTMS mass spectrometer. Thin-layer chromatography (TLC) was carried out on 0.25 mm silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). Silica gel 60 (0.040−0.063 mm, Merck) was used for column chromatography.

All commercially available reagents were used as received. All reactions were carried out under an inert atmosphere of nitrogen, unless stated otherwise. *S*-2-Propenyl-L-cysteine was synthesized by alkylation of L-cysteine hydrochloride with allyl bromide according to the procedure of Goudreau et al.[4] *cis*- and *trans*-*S*-1-propenyl-L-cysteine were prepared by isomerization of *S*-2-propenyl-L-cysteine using potassium *tert*-butoxide according to the procedure of Carson and Boggs.[5]

Plant Materials. The tender shoots of *T. sinensis* were purchased from a local market (Taian, Shandong province, China) in April 2009. The fresh sample was flash frozen in liquid nitrogen. Voucher specimens of both tender shoots and seeds (collection no. YGY2012-011) were collected from Xi'an Botanical Garden, Shanxi province of China (XBGH), and deposited at the herbarium of XBGH and Firmenich Aromatics (China) Co. (Firmenich). The specimen was identified by Dr. Bing Li (XBGH) and Dr. Yong-Ming Yuan (Firmenich). Red onion bulbs (*Allium cepa*) were purchased from a local supermarket in Shanghai, China.

Analyses of the Volatiles in *T. sinensis*. A sample of fresh *T. sinensis* shoots (100 g) was cooked in boiling water for 2 min, according to Chinese culinary tradition, and minced, and the aqueous slurry of cooked *T. sinensis* shoots was extracted with distilled dichloromethane (70 mL) using an SDE apparatus. The organic extract was dried over anhydrous Na_2SO_4, filtered, distilled using a Vigreux column, and concentrated to 0.15 mL for GC-FPD-MS analysis. Compounds were tentatively identified by comparison of MS data and retention indices with entries in the NIST05, Wiley 7, and Firmenich in-house MS databases.

Extraction and Analysis. Frozen shoots (80 g) were ground together with aqueous 5% trifluoroacetic acid (100 mL) and extracted with water (150 mL) at room temperature for 1 h. The resulting suspension was centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was filtered and analyzed by LC-MS eluted at 1.0 mL/min using a H_2O/MeCN gradient containing 0.1% formic acid (eluting with H_2O over 2 min, gradient 0−40% MeCN over 8 min, increasing to 95% MeCN over 0.1 min, and holding for 1 min). Crude extracts of *T. sinensis* sprouts, seeds, and onion were prepared and analyzed using the same procedure.

Isolation. A crude extract (1800 mL), prepared from 300 g of frozen *T. sinensis* shoots, was purified by ion exchange chromatography (100 g, Dow 50 WX8, 200−400 mesh, conditioned with 0.1 M HCl) and eluted with water (600 mL), followed by a gradient of aqueous NH_4OH applied in portions of 120 mL at concentrations of 0.3, 0.6, and 0.9 M (fraction 3), 1.2 M (fraction 4), and 1.5, 1.8, 2.1, and 2.4 M. Combining fractions 3 and 4, and then freeze-drying gave a brownish powder (lyophilisate, 6.1 g) enriched in **1**, **2**, **3**, and **5**, which was analyzed by LC-MS. The lyophilisate (1.0 g) was purified by solid-phase extraction (ENVI-18 packed SPE cartridge, Supelco), eluting with a gradient of 5, 10, 25, 50, and 100% MeCN (aq). Twenty fractions of 10 mL each were collected; fractions 1 and 2 were pooled and lyophilized to give a yellow solid (392 mg), fractions 3−6 to give a pale yellow solid (50 mg), and fractions 9 and 10 to give a brownish solid (5 mg). The lyophilized SPE solids were dissolved (0.1% formic acid (aq)), filtered, and purified by a semipreparative RP-HPLC, eluting at 3 mL/min with a gradient of MeCN containing 0.1% formic acid (B) in H_2O (A). Elution started with 0% B over 3 min, increased to 36% B (for pure **3**) over 18 min (5% B for pure **1** and **2**, 15% B for pure **5**), and then increased to 95% B in 0.1 min, which was held for 3 min. The SPE fractions from 300 g of frozen *T. sinensis* shoots were purified to give pure **1** (172 mg), **2** (30 mg), **3** (10 mg), and **5** (24 mg).

(S,S)-γ-Glutamyl-(cis-S-1-propenyl)thioglycine, ***1:*** amorphous white powder; $[\alpha]^{22}_D$ +258.6° (*c* 0.21, H_2O); IR (KBr pellet) ν_{max} 3034, 2933, 1723, 1647, 1514, 1378, 1229 cm^{-1}; ^1H NMR (500 MHz), ^{13}C NMR (125 MHz), see Table 1; HR-ESI-FTMS *m/z* 277.08575 $[M + H]^+$ (calcd for $C_{10}H_{17}N_2O_5S_1$, 277.08527).

(S,S)-γ-Glutamyl-(trans-S-1-propenyl)-thioglycine, ***2:*** amorphous white powder; $[\alpha]^{22}_D$ +233.5° (*c* 0.17, H_2O); ^1H NMR (500 MHz, DMSO-d_6) δ 6.14 (d, *J* = 15 Hz), 5.69 (m), 5.27 (br d), 3.41 (m), 2.33 (br t), 1.96 (m), 1.88 (m), 1.69 (d, *J* = 7 Hz); ^{13}C NMR (125 MHz, DMSO-d_6) δ 170.9 (s), 170.9 (s), 170.9 (s), 128.8 (d), 121.1 (d), 55.6 (d), 52.8 (d), 31.5 (t), 27.0 (t), 18.3 (q); HPLC/ESI + atmospheric pressure chemical ionization−time-of-flight-mass spectrometry (APCI-HR-TOF-MS) *m/z* 276.07869 [M] (calcd for $C_{10}H_{16}N_2O_5S_1$, 276.07799).

γ-Glutamyl-cis-S-1-propenyl cysteine, ***3:*** amorphous white powder; $[\alpha]^{23}_D$ +28.4° (*c* 0.40, H_2O); ^1H NMR (500 MHz, D_2O) δ 6.0 (d, *J* = 10 Hz), 5.77 (m), 4.48 (dd, *J* = 5, 8 Hz), 3.80 (m), 3.22 (dd, *J* = 5, 15 Hz), 3.05 (dd, *J* = 8, 15 Hz), 2.50 (t, *J* = 8 Hz), 2.16 (m), 1.66 (d, *J* = 7 Hz); ^{13}C NMR (125 MHz, D_2O) δ 178.4 (s), 177.3 (s), 176.7 (s), 129.8 (d), 126.8 (d), 57 (d), 57 (d), 37.8 (t), 34.6 (t), 29.3 (t) 16.9

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(q); HR-ESI-FTMS m/z 291.10147 $[M + H]^+$ (calcd for $C_{11}H_{19}N_2O_5S_1$, 291.10092).

Synthesis of γ-Glutamyl S-Propyl Thioglycine, 7. *N-Boc-L-glutamyl-D,L-2-hydroxyglycine Ethyl Ester, **8**.* To a solution of N-Boc glutamine (49.0 mg, 0.199 mmol) in acetone (2 mL) was added ethyl glyoxalate (50.4 mg, 0.247 mmol). After refluxing for 6 h, the mixture was concentrated in vacuo and then purified by HLB column to give **8** as a gum (63.6 mg, 91% yield, ~1:1 mixture of diastereomers by ^{1}H NMR). Only one peak, which contained both diastereomers, was detected by LC-MS without further optimization.

Colorless gum; ^{1}H NMR (500 MHz, CD_3OD) δ 5.55, 5.54 (1H, s), 4.21 (2H, q, J = 7.0 Hz), 4.10 (1H, br s), 2.34 (2H, t, J = 7 Hz), 2.14 (1H, m), 1.90 (1H, m), 1.43 (9H, s), 1.29 (3H, t, J = 7 Hz); ^{13}C NMR (125 MHz, CD_3OD) δ 175.9, 175.1, 171.6, 158.4, 80.9, 73.0, 63.1, 54.7, 33.4, 29.0, 28.9, 14.7; HPLC/ESI+APCI-HR-TOF-MS m/z 371.12820 [M] (calcd for $C_{14}H_{24}N_2O_8Na$, 371.14249).

*γ-Glutamyl-D,L-2-propylthioglycine Ethyl Ester, **9**.* To a solution of **8** (1.00 g, 2 mmol) and concentrated sulfuric acid (0.1 mL) in $CHCl_3$ (20 mL) and AcOH (2 mL) was added propyl mercaptan (0.36 mL, 3.99 mmol) dropwise at 0 °C. The mixture was stirred for 0.5 h at 0 °C and then for 6 h at room temperature. After **8** had been consumed, the mixture was evaporated to remove the chloroform and then stirred vigorously for 1 day at room temperature. The resulting mixture was concentrated in vacuo and then separated by HLB column to give **9** as a white solid (388 mg, 63% yield, 1:1 mixture of diastereomers). Two peaks attributed to diastereomers were detected by LC-MS.

Amorphous white powder; ^{1}H NMR (500 MHz, CD_3OD) δ 5.40, 5.39 (1H, s), 4.22 (2H, q, J = 7.0 Hz), 3.63 (1H, m), 2.67 (1H, m), 2.62 (1H, m), 2.50 (2H, m), 2.10 (2H, m), 1.63 (2H, m), 1.28 (3H, t, J = 7.0 Hz), 0.98 (3H, t, J = 7.0 Hz); ^{13}C NMR (125 MHz, CD_3OD) δ 174.8, 174.7, 173.9, 170.6, 63.2, 55.7, 55.1, 55.0, 34.0, 33.1, 32.9, 28.1, 28.0, 24.1, 14.7, 13.9; HPLC/ESI+APCI-HR-TOF-MS m/z 306.12386 [M] (calcd for $C_{12}H_{22}N_2O_5S_1$, 306.12494).

*L-Glutamyl-D,L-2-propylthioglycine, **7**.* To a solution of **9** (138 mg, 0.45 mmol) in tetrahydrofuran (THF) (5 mL) at 0 °C was added dropwise a precooled solution (0 °C) of lithium hydroxide hydrate (0.3 M in water) to adjust the pH to about 12. The mixture was stirred for 1.5 h at 0 °C and then was adjusted to pH 2 with 1 N HCl and separated by HLB column to give **7** as a white solid (97.6 mg, 78% yield, 1:1 mixture of diastereomers). Only one peak, which contained two diastereomers, was detected by LC-MS without further optimization.

Amorphous, white powder; $[\alpha]^{22}_D$ +159.6° (c 0.23, H_2O); ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.53 (1H, m, interchangeable H), 5.20 (1H, d, J = 8.5 Hz), 3.46 (1H, br s), 2.57 (1H, m), 2.52 (1H, m), 2.35 (2H, m), 1.98 (1H, m), 1.90 (1H, m), 1.52 (1H, m), 0.89 (3H, t, J = 7.5 Hz); ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.1, 170.9, 170.2, 54.1, 52.9, 31.7, 31.5, 27.0, 22.4, 13.3; HR-MALDI-FTMS m/z 279.1017 [M + H]$^+$ (calcd for $C_{10}H_{19}N_2O_5S_1$, 279.10092).

*Reduction of γ-Glutamyl-cis-S-1-propenyl-thioglycine, **1**, to **7**.* To a mixture of *cis*- and *trans*-γ-glutamyl-*S*-1-propenylthioglycine, **1** (50 mg, 0.18 mmol), in water (2 mL) was added Pd/C (5%, 25 mg), and the resulting slurry was stirred for 1 h. The mixture was filtered through Celite to remove Pd/C and washed with water (1 mL), and the combined aqueous solutions were transferred to a high-pressure reactor. The air in the system was purged three times with He. To the mixture was added additional Pd/C (5%, 25 mg), and the He in the system was replaced by purging with H_2 three times. The H_2 pressure was adjusted to 50 bar, and the mixture was stirred at room temperature for 12 h. The mixture was filtered through Celite to remove Pd/C and concentrated under vacuum to give pure γ-glutamyl *S*-propylthioglycine (**7**) (33 mg, 66% yield). The LC-MS retention times and MS, ^{1}H, and ^{13}C NMR data of **7** prepared by reduction were identical to those of **7** prepared synthetically.

*Derivatization of **1** To Yield **10**.* A crude sample of *T. sinensis* seed extract enriched in **1** (10.0 g, containing 17−20% **1**, 7.24 mmol) in a mixture of water (30 mL) and THF (30 mL) was treated with sodium bicarbonate (6.08 g, 72.4 mmol). The basic solution was treated with di-*tert*-butyl dicarbonate (7.90 g, 36.2 mmol) at room temperature for 24 h. The reaction mixture was concentrated under vacuum to remove THF. The residue was diluted with water (400 mL), acidified to pH 3 with 37% aqueous HCl, and extracted with EtOAc (3 × 100 mL). The combined organic phase was washed with water (100 mL) and extracted with 5% aqueous NaOH solution (4 × 50 mL). The alkaline aqueous product phase was acidified to pH 3 with 37% aqueous HCl and extracted with EtOAC (3 × 50 mL). The combined organic phase was washed with brine, dried, and filtered. After the removal of organic solvent, the crude product (1.96 g) was analyzed by TLC (1:1 MeOH/ethyl acetate, 5% acetic acid, $KMnO_4$ detection) and showed a single major product spot with an R_f of 0.5.

The crude product was esterified using diazomethane. A crude sample of the *tert*-butyl carbamate of **1** (1.96 g, 5.21 mmol) was treated with a freshly prepared ether solution of diazomethane until the yellow diazomethane color remained. The reaction was monitored by TLC to find a single new product spot at R_f 0.75 compared with the starting material at R_f 0.5. The reaction mixture was diluted with saturated $NaHCO_3$ solution (100 mL) and extracted with EtOAc (2 × 20 mL). The organic layer was washed with brine, dried, and filtered. After removal of the solvent, the crude product (1.17 g) was purified by silica chromatography using *n*-hexane/EtOAc (1:1) as the eluent to give a 7:3 mixture of **10** and its *trans* isomers (0.32 g, ~13% yield), which were analyzed by ^{1}H NMR.

The sample of **10** was successfully crystallized from *n*-hexane/EtOAc (1:1) to provide a pure sample of the *cis* isomer. X-ray quality crystals were grown from a THF solution of **10** by room temperature diffusion with *n*-hexane. This material was fully characterized by ^{1}H NMR, ^{13}C NMR, VCD, IR, and X-ray crystallography.

Optical rotations were obtained in 1 mL solutions with units of deg·cm^2·g^{-1}. IR and vibrational circular dichroism (VCD) spectra were recorded on a Chiral*IR*-2X FT-VCD spectrometer with single photoelastic modulator (PEM) (Bio Tools, Inc., Jupiter, FL, USA). A solution of **10** in $CDCl_3$ (6.3 mg/0.15 mL) in a 100 μm path length cell with BaF_2 windows was used for a 7 h measurement. The spectrum of $CDCl_3$ was measured for 3 h for VCD baseline correction and IR solvent subtraction. The PEM was optimized at 1400 cm^{-1} with a resolution of 4 cm^{-1}. ComputeVOA (BioTools, Inc.) was used to build the (*R*,*R*) and (*R*,*S*) configurations of **10** and to perform a conformation search at the molecular mechanics level. Geometry, frequency, IR, and VCD intensity calculations were performed at the density functional theory (DFT) level (B3LYP functional/6-31G(d) basis set) with Gaussian 09. Calculated frequencies were scaled by 0.97 and the IR and VCD frequencies converted to Lorentzian bands with 6 cm^{-1} half-width for comparison to experimental data. The experimental VCD and IR spectra were compared to the calculated Boltzmann averaged spectra for the three lowest energy conformers of each of the (*R*,*R*) and the (*R*,*S*) configurations. The CompareVOA program (BioTools) was used to evaluate the confidence levels of the assignments.

Single-crystal X-ray diffraction data were collected on a Bruker APEXII CCD area detector by using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å) at a temperature of 143(1) K.

*tert-Butylcarbamoyldimethyl-γ-glutamyl-(cis-S-1-propenyl)-thioglycine, **10**:* Fine, white needles; mp 78−80 °C; differential scanning calorimetry melting onset 86.7 °C, with decomposition observed at temperatures over 200 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.94 (d, J = 7 Hz, NH), 6.12 (d, J = 10 Hz, NH), 5.88 (m, 1H), 5.64 (d, J = 9 Hz, 1H), 5.24 (br d, J = 8 Hz), 4.32 (br s, 1H), 3.81 (s, 3H), 3.75 (s, 3H), 2.4 (m, 2H), 2.21 (m, 1H), 1.93 (m, 1H), 1.72 (dd, J = 1, 8 Hz, 3H), 1.45 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.6 (s), 171.0 (s), 169.0 (s), 155.7 (s), 130.0 (d), 119.3 (d), 80.2 (d), 60.3 (s), 53.8 (q), 53.1 (q), 52.5 (d), 32.3 (t), 28.9 (t), 28.3 (3q), 14.6 (q); HPLC/ESI+APCI-HR-TOF-MS m/z 404.16063 [M] (calcd for $C_{17}H_{28}N_2O_7S_1$, 404.16172).

Determination of γ-Glutamyl-(1-propenyl)thioglycine in Fresh *T. sinensis* Shoots. The water contents of the fresh *T. sinensis* shoots were determined by freezing using liquid nitrogen and then drying in a freeze-dryer. Weights were compared before and after freeze-drying. Typical water contents were around 85%. A crude extract of *T. sinensis* shoots (50 g) was prepared and analyzed by LC/UV/ESI-MS either directly (UV detection) or after 1:10 dilution (MS

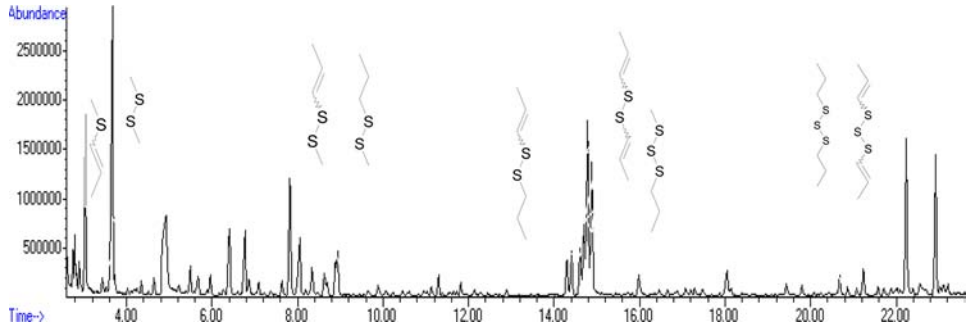

Figure 1. GC-MS-TIC chromatogram of *T. sinensis* shoot SDE extract.

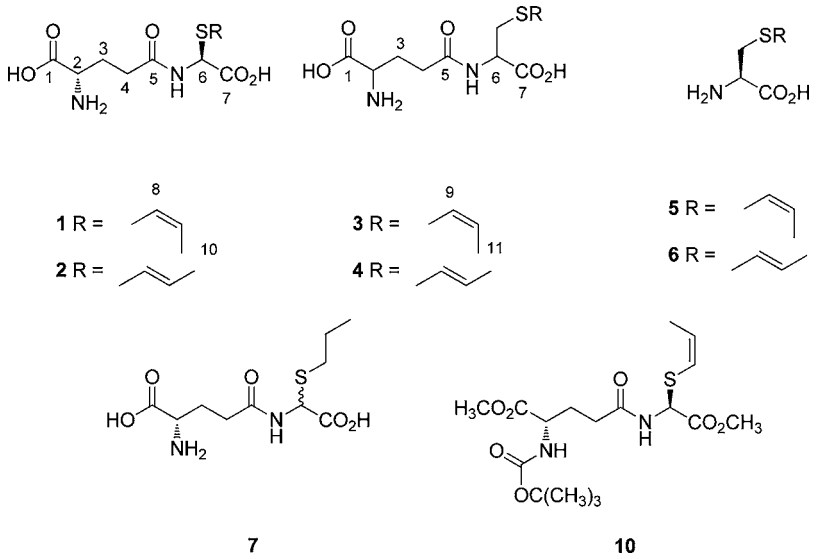

Figure 2. Sulfur-containing nonvolatile metabolites identified in *Toona sinensis* (**1**–**6**), a reduced analogue, **7**, and a derivative, **10**.

detection). An accelerated version of the gradient of the LC-MS method mentioned above was used. For these targeted quantitative analyses, the gradient started at 10% B over 1 min, increased to 50% B in 4 min, and then increased to 95% B in 0.1 min, which was held for 0.5 min, and then decreased to 10% B in 0.1 min and re-equilibrated for 5 min before the next injection. Volumes of 5 μL were injected. UV detection and quantification of γ-glutamyl-(1-propenyl)thioglycine were performed at 250 nm on the basis of calibration solutions containing 10, 30, 50, and 100 mg/L of the analyte. MS detection and quantification of γ-glutamyl-(1-propenyl)thioglycine were performed by monitoring the two transitions 277 → 203 and 277 → 130 in MS/MS mode (collision energy = 35%), on the basis of calibration solutions containing 5, 10, and 30 mg/L of the analyte.

RESULTS AND DISCUSSION

There are few reports about analyses of the volatiles in *T. sinensis*, especially descriptions of the volatile sulfur compounds.[3] An SDE of fresh *T. sinensis* shoots was performed and analyzed by GC-MS. By comparing the MS and retention indices of the volatile compounds in the extract with database entries, we identified more than 40 sulfur-containing compounds in the SDE extract, many of which proved to be mono-, di-, and trisulfides that contained a 1-propenyl group (Figure 1). The composition of sulfides in the *T. sinensis* extract was very similar to that reported from Egyptian onion oil and literature reports describing studies of onion volatiles.[6]

When cutting onions, the lachrymatory factor (propanethial *S*-oxide) is formed rapidly by reaction of enzymes with *S*-(1-(*E*)-propenyl)cysteine-*S*-oxide.[2] However, we found that when *T. sinensis* shoots or sprouts were crushed, no eye irritation occurred, indicating that *T. sinensis* did not contain or form propanethial *S*-oxide. From these observations, we assumed that either the nonvolatile flavor precursors or the enzymatic chemistries of *T. sinensis* and onion are different.

To test for the existence of sulfur-containing precursors, we prepared powder from intact freeze-dried *T. sinensis* sprouts and found it to be practically odorless, but upon addition of a small amount of water, the typical fresh, strong, and sulfur aroma of *T. sinensis* sprouts was perceived in a few seconds. We hypothesized that the complex alliaceous character of *T. sinensis* may be the result of enzyme activity upon nonvolatile precursors and that the enzyme was reactivated when the freeze-dried powder was brought into contact with water.

Uncrushed *T. sinensis* tender shoots were frozen using liquid nitrogen, ground with 5% aqueous trifluoroacetic acid to precipitate and deactivate the enzymes, centrifuged, and filtered to give a crude extract that was analyzed by LC-MS. Screening the major ion clusters and their isotopic ^{34}S-containing ions in the positive ion mode revealed two peaks with $[M + H]^+$ ions at m/z 277 containing sulfur atoms. A data acquisition experiment of m/z 277 with enhanced resolution of the scan range from 272 to 282 Da showed that the ion intensity of m/z 279 was about 5% of that of m/z 277, which is consistent, on the basis of the natural abundance of sulfur, with the presence of a single sulfur atom in the molecule. The detection of m/z 275 in LC-MS in negative ion mode at the same retention time confirmed that the molecular weights of these compounds, **1**

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and **2**, were 276 Da. Interestingly, these were the only two chemicals present in *T. sinensis* extracts with deactivated enzymes, that decomposed rapidly in the presence of active *T. sinensis* enzymes. Compounds **1** and **2** were purified by means of ion exchange chromatography, solid phase extraction, and semipreparative HPLC.

Compound **1** was obtained as an amorphous white powder, and its molecular formula was established as $C_{10}H_{16}N_2O_5S$ (see Figure 2 for structure) with four degrees of unsaturation on the basis of HRESIMS, NMR, and MS data. The ^{13}C NMR spectrum in D_2O showed a total of 10 carbon resonances, including three closely spaced carbonyls, two olefinic carbons, and five saturated aliphatic carbons, accounting for all four unsaturations. On the basis of the molecular formula and the carbonyl resonances at chemical shifts being consistent with acid or amide functionalities, compound **1** was suspected to be an *S*-alkyl dipeptide. The MS contained a characteristic glutamate ion at m/z 130 in the positive ion MS/MS experiment, which demonstrated a peptide bond linkage. Additionally, the cleavage of precursor ions at m/z 277 and 279 both formed product ion m/z 203, consistent with the loss of m/z 74 and 76, respectively, indicating the neutral loss of a propenethiol moiety. Structure elucidation was based on ^1H, ^{13}C, COSY, HSQC, and HMBC NMR experiments (Table 1).

The ^1H NMR spectrum (Table 1) in D_2O contained resonances assigned to one methyl (H-10, δ_H 1.70, J = 1, 7 Hz), two sp^3 methylenes (H-3, δ_H 2.18 and H-4, δ_H 2.54), two sp^3 methines (H-2, δ_H 3.90, J = 7 Hz and H-6, δ_H 5.44), and two olefinic protons (H-8, δ_H 6.08, d, J = 9 Hz, and H-9 δ_H 5.97). A COSY spectrum in D_2O contained three discrete spin systems, among which a doublet attributed to one proton of a double bond at δ 6.08 (CH-8) was coupled to a multiplet at 5.97 ppm (CH-9), and this was in turn coupled to a doublet that integrated for three protons at δ 1.70 (CH_3-10), indicating that **1** contained a propenyl moiety. The coupling constant of 9 Hz between H-8 and H-9 indicated the double bond had the *cis* configuration. Another spin system was identified from the coupling between a methine proton at δ 3.90 (CH-2) and a multiplet that integrated for two protons at δ 2.18 (CH_2-3), which was in turn coupled to a triplet that integrated for two protons at δ 2.54 (CH_2-4) and was consistent with the presence of a glutamyl moiety. An HMBC spectrum acquired in D_2O confirmed the assignment of the spin systems observed in the COSY; however, it was not possible to determine the connectivities of the spin systems because of insufficient resolution of the carbonyl carbons in D_2O. In DMSO-d_6, a doublet that resonated at δ 8.74 (N−H, J = 9 Hz) was coupled to a methine doublet at δ 5.34 (CH-6, J = 9 Hz) and constituted the third spin system. With the addition of one drop of D_2O in DMSO-d_6, the doublet at δ 8.74 in the ^1H NMR spectrum disappeared and the CH-6 doublet at δ 5.34 became a singlet, which suggested the presence of a norcysteine moiety consisting of a methine directly connected to an amide group, a sulfur atom, and an acid group. From the HMQC spectrum in DMSO-d_6 and a drop of D_2O, it was possible to assign the ^{13}C NMR spectrum as shown in Table 1. In the HMBC spectrum, correlations were observed from the proton resonances at 3.38 (CH-2), 1.88 (CH_2-3), and 2.0 (CH_2-3′) to the carbon resonance at 170.4 ppm (C-1). Additional HMBC correlations were observed from the proton resonances at 1.88 (CH_2-3), 2.0 (CH_2-3′), 2.32 (CH_2-4), and 5.29 (CH-6) to the carbon resonance at 171.1 ppm (C-5). These data demonstrated that a glutamate moiety was adjacent to the norcysteine group. The proton resonance at 5.29 ppm (CH-6) was also correlated to the carbon resonances at 169.2 (C-7) and 122.9 ppm (C-8), which confirmed that **1** contained a norcysteine group attached to a 1-propenyl moiety via a sulfide linkage. Thus, the structure of the compound with MW 276 was identified as **1**, γ-glutamyl-*cis*-*S*-1-propenylthioglycine.

Efforts to synthesize **1** were unsuccessful; however, both diastereomers of the reduced analogue, γ-glutamyl-*S*-propylthioglycine, **7**, were synthesized (Figure 5). Reduction of a sample of **1** isolated from *T. sinensis* gave a sample of **7**, which had LC retention times, mass spectra, and ^1H and ^{13}C NMR spectra that were identical to those of the synthetic sample of **7**. The synthetic diastereomers of **7** had LC retention times and NMR data that were indistinguishable; consequently, it was not possible to determine whether the natural product **1** existed as one diastereomer or as more than one. VCD was used to determine the configuration of **1**. To eliminate solvation by water and thus to simplify the theoretical calculations for **1**, a single derivative, **10**, of the natural compound was prepared by protection of the amino moiety as the *tert*-butyl carbamate and the two acid groups as methyl esters. Comparison of experimental and computed IR and VCD spectra allowed the assignment of the absolute configuration of **1** as (*S*,*S*) with a confidence level of 100%. By comparison, the confidence level of assignment as the (*S*,*R*) configuration was 13%. Serendipitously, derivative **10** was a crystalline solid, permitting confirmation of both the planar structure and stereochemistry of **1** by X-ray crystallography (Figure 3). X-ray quality crystals of **10** were grown from a tetrahydrofuran and hexane solution.

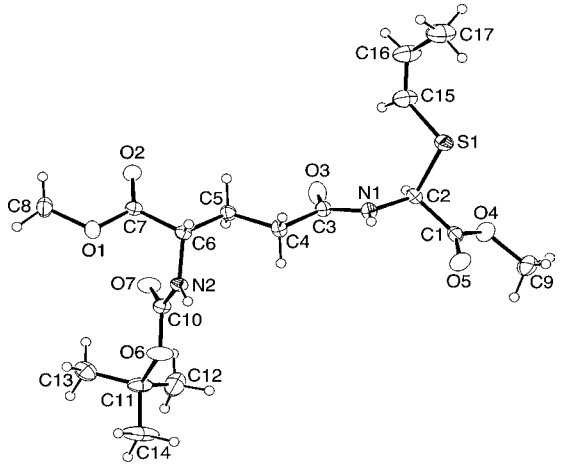

Figure 3. X-ray crystal structure of **10**.

Derivative **10**, $C_{17}H_{28}N_2SO_7$, crystallizes in the monoclinic space group P2 with a = 13.0700(3) Å, b = 5.0065(1) Å, c = 17.5507(3) Å, β = 93.4820(10)°, V = 1146.31(4) Å^3, Z = 2, and d_{calc} = 1.172 g/cm^3. X-ray intensity data were collected on a Bruker APEXII CCD area detector by using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å) at a temperature of 143(1) K. A total of 29600 reflections were measured over the ranges $1.89 \le \theta \le 25.05°$, $-15 \le h \le 15$, $-5 \le k \le 5$, and $-20 \le l \le 20$, yielding 4043 unique reflections (R_{int} = 0.0182). The structure was solved by direct methods. There was a region of disordered solvent for which a reliable disorder model could not be devised; the X-ray data were corrected for the presence of disordered solvent using SQUEEZE. Refinement was by full-matrix least-squares based

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on F^2. Refinement converged to $R_1 = 0.0284$ and $wR_2 = 0.0843$ for 3966 observed reflections for which $F > 4\sigma(F)$, $R_1 = 0.0289$, $R_2 = 0.0847$, and GOF = 1.087 for all 4043 unique, nonzero reflections and 251 variables. The maximum Δ/σ in the final cycle of least-squares was 0.001, and the two most prominent peaks in the final difference Fourier were +0.187 and −0.150 e/Å^3. The Flack absolute structure parameter refined to a value of 0.04(6), thus corroborating the assigned absolute structure. The X-ray data have been deposited in the Cambridge Database Centre (CCDC no. 906297). X-ray crystallography confirmed that **10** and, by inference, **1** exist in the (*S*,*S*) configuration.

Compound **2** was obtained as an amorphous white powder and exhibited the same molecular formula ($C_{10}H_{16}N_2O_5S$) as **1**, as determined by HRESIMS. A comparison of MS/MS data and NMR spectra indicated that **2** had a very similar structure to **1**. The large coupling constant (15 Hz) for $J_{8,9}$ revealed that **2** was the *trans* isomer of **1**. The similarities of the measured optical rotations of **1** and **2** ($[\alpha]^{22}_D$ +258.6° (*c* 0.21, H_2O) and $[\alpha]^{22}_D$ +233.5° (*c* 0.17, H_2O), respectively) were consistent with these metabolites possessing the same configurations.

The summed concentrations of metabolites **1** and **2** in extracts of *T. sinensis* shoots were determined to be approximately 10 g/kg dry weight (equal to approximately 1.5 g/kg wet weight).

From the identification of **1**, we postulated that the immediate precursor of *cis*-1-propenethiol, the major volatile compound released upon disruption of the tissues of *T. sinensis*, was the novel compound (*S*,*S*)-γ-glutamyl-(*cis*-*S*-1-propenyl)-thioglycine. Presumably 1-propenethiol reacted further to form the sulfides observed in organic extracts of *T. sinensis*, similar to the reactions of sulfur-containing volatile molecules that have been observed in chopped onions and garlic.[2] This is the first example of an α-thioglycine moiety occurring in a molecule found in nature. Interestingly, Kingsbury et al.[7] proposed the use of analogous synthetic dipeptides that contained α-thioglycine for portage of thiols into bacteria. Microorganisms, including *Escherichia coli*, have transport systems that can convey such dipeptides into the cell in which intracellular peptidase activity cleaves the peptide bond linking the two amino acids to release the unstable alk(en)ylthioglycine. This intermediate rapidly hydrolyzes to release ammonia, a thiol, and glyoxylic acid (Figure 4).[7] Synthetic compounds of this type have been studied as delivery systems for antimicrobial compounds and as probes for peptidase activity.[8−13]

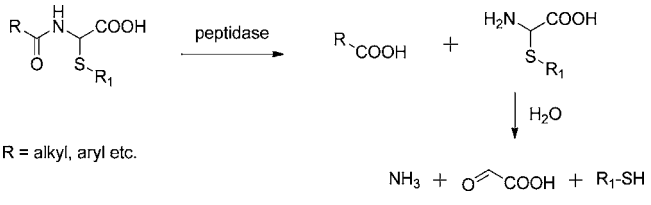

Figure 4. Mode of α-thioglycine-substituted peptide decomposition after peptidase cleavage.

In the crude extracts of *T. sinensis* shoots, with active or inactivated enzymes, two sulfur-containing compounds with protonated molecules $[M + H]^+$ *m*/*z* 162 in the positive-ion mode were detected in the LC-MS chromatogram at 7.72 and 7.92 min, with characteristic product ions *m*/*z* 145 (MS^2) and *m*/*z* 99, 73, and 55 (MS^3 of ion *m*/*z* 145). By means of elucidation by ^1H NMR and MS, the precursor and product ions of the peak that eluted at 7.93 min in the onion extract were found to be identical to those of *trans*-*S*-1-propenyl-L-cysteine, **6**, whereas another high-abundance peak that eluted at 7.72 min was separated, purified, and identified as *cis*-*S*-1-propenyl-L-cysteine, **5**; these findings were identical to those reported in the literature.[5,14] To the best of our knowledge, this is the first demonstration of the double bond of 1-propenyl-L-cysteine naturally occurring in a plant or food and having the *cis* configuration. This compound was first synthesized by Carson and co-workers[5,14] during the course of their analysis of onion sulfur chemistry in 1963.

Cysteine *S*-conjugates, such as *trans*-*S*-1-propenyl-L-cysteine, have been reported to be liberated from their corresponding γ-glutamyl dipeptides, which are widely distributed in the plant kingdom.[2,15] The crude onion extract was consequently screened for the precursor of *trans*-*S*-1-propenyl-L-cysteine, that is, γ-glutamyl-*trans*-*S*-1-propenyl-L-cysteine, **4**. In a positive-ion mode LC/ESI-MS experiment, the one peak with the expected protonated molecule $[M + H]^+$ 291, as well as three prominent secondary product ion peaks at *m*/*z* 170, 162, and 145, was detected at 9.73 min in the crude onion extract. The product ion spectra of the ^{34}S *m*/*z* 293 precursor ion isotopomer were recorded at *m*/*z* 170, 164, and 147, which indicated the presence of a sulfur atom in the two ions *m*/*z* 162 and 145. The characteristic ion recorded at *m*/*z* 162 suggested the loss of a γ-glutamyl moiety due to hydrogen rearrangement cleavage of the peptide bond. Fragmentation of the precursor ion *m*/*z* 162 in the MS^3 experiment, with a loss of NH_3 to produce ion *m*/*z* 145 as the base peak and the further product scan (MS^4) of ion *m*/*z* 145, exhibited characteristic ions *m*/*z* 99, 73, and 55, which were the same as the MS^2 and MS^3 fragmentation data for *trans*-*S*-1-propenyl-L-cysteine, **6**. These results allowed the tentative identification of this compound as γ-glutamyl-*trans*-*S*-1-propenyl-L-cysteine. The same method was applied to the analysis of the crude *T. sinensis* shoot extract, and two peaks with the ion $[M + H]^+$ 291 were detected at 9.62 and 9.73 min. Both of the ions had the same MS^2, MS^3, and MS^4 fragmentation spectra. Co-injection experiments of the *T. sinensis* and onion extracts were performed. A sample containing equal amounts of *T. sinensis* and onion extracts was prepared and analyzed by LC-MS. In the spiked extract, the peak at 9.73 min was obviously enhanced compared with the original crude *T. sinensis* extract. Consequently, the compound that eluted at 9.73 min in the crude *T. sinensis* extract was tentatively identified to be γ-glutamyl-*trans*-*S*-1-propenyl-L-cysteine, **4**.

The second peak with protonated molecule $[M + H]^+$ 291, which eluted at 9.62 min, was isolated using semipreparative HPLC and analyzed by LC/ESI-MS to show, in addition to the ion at *m*/*z* 291, two main product ions with *m*/*z* 162 and 145. The ^1H and ^{13}C NMR data were identical to those reported for γ-glutamyl-*trans*-*S*-1-propenyl-L-cysteine, **4**, except for the chemical shifts of the protons and carbons 9−11 and the coupling constants between H-9 and H-10.[16] The coupling constant of H-9 and H-10 was reported to be 15 Hz for compound **4**, whereas in **3** the coupling constant we observed for H-9 to H-10 was 9 Hz, allowing us to conclude that compound **3** was γ-glutamyl-*cis*-*S*-1-propenyl-L-cysteine.[5] Our conclusions regarding the identities of **3** and **4** were further supported by the fact that the HPLC retention order, wherein the *cis*- isomer eluted prior to the *trans* isomer, was consistent with the trend observed with metabolites **5** and **6** using the same HPLC column.

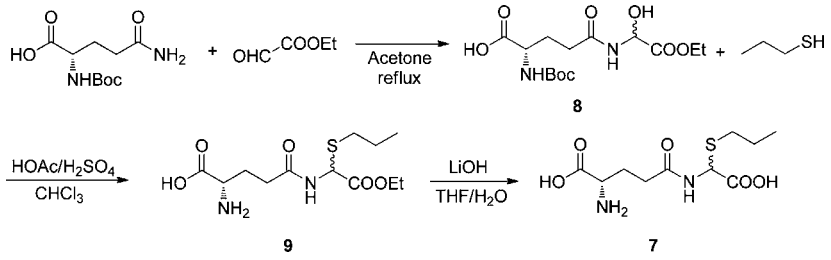

Figure 5. Synthesis of γ-glutamyl *S*-propylthioglycine **7**.

In this study it was determined that the nonvolatile precursors of 1-propenyl mono-, di-, and tri- sulfides in *T. sinensis* differed from those in onions, *A. cepa*. Whereas onions contain alkylcysteine oxides, which are cleaved by alliinases, *T. sinensis* contains metabolites that contain *S*-alkylnorcysteine moieties, which are presumably cleaved by proteases of *T. sinensis*. To preserve the unique, alliaceous flavor of *T. sinensis* shoots, care must be taken to prevent disruption of the plant cells resulting in premature release and subsequent loss of the unstable thiols that are critical to the flavor of authentic *Toona*.

■ ASSOCIATED CONTENT

Supporting Information

Comprehensive spectroscopic data for **1**−**10**. 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.

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■ ABBREVIATIONS USED

COSY, correlation spectroscopy; ESI, electrospray ionization; FPD, flame photometric detector; HRESIMS, high-resolution electrospray ionization mass spectrometry; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum correlation; RP-HPLC, reverse-phase high-performance liquid chromatography; SDE, simultaneous distillation extraction; SPE, solid phase extraction; VCD, vibrational circular dichroism

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