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New Cysteine-*S*-Conjugate Precursors of Volatile Sulfur Compounds in Bell Peppers (*Capsicum annuum* L. Cultivar)

Christian Starkenmann* and Yvan Niclass

Corporate R&D Division, Firmenich SA, P.O. Box 239, CH-1211 Geneva 8, Switzerland

Supporting Information

ABSTRACT: The objective of this study was to verify whether the volatile organic sulfur compounds recently discovered in bell pepper (*Capsicum annuum*, L. cultivars), such as the mercapto-ketones: 4-sulfanyl-2-heptanone and 2-sulfanyl-4-heptanone, the mercapto-alcohols: 4-sulfanyl-2-heptanol and 2-sulfanyl-4-heptanol, and heptane-2,4-dithiol, originate from their corresponding cysteine-*S*-conjugates. Analysis of aqueous extracts of red and green bell pepper by ultraperformance liquid chromatography−mass spectrometry with electrospray ionization in the positive mode (UPLC−MS ESI$^+$) displayed masses corresponding to the expected cysteine-*S*-conjugates. To confirm this observation, four cysteine-*S*-conjugates were prepared as authentic samples: *S*-(3-hydroxy-1-methylhexyl)-L-cysteine, *S*-(3-hydroxy-1-propylbutyl)-L-cysteine, *S*-(3-oxo-1-propylbutyl)-L-cysteine, and (2*R*,2′*R*)-3,3′-(4-hydroxyheptane-2,6-diyl)bis(sulfanediyl) bis(2-aminopropanoic acid). By comparison with the fragmentation patterns and retention times of synthetic mixtures of cysteine-*S*-conjugate diastereoisomers, the natural occurrence of cysteine conjugates was confirmed in bell peppers. In addition, the cysteine-*S*-conjugates from red and green bell pepper extracts were concentrated by ion exchange chromatography and the fractions incubated with a β-lyase (apotryptophanase). The liberated thiols were concentrated by affinity chromatography, and their occurrence, detected by gas chromatography−mass spectrometry, confirmed our predictions. Moreover, 3-sulfanyl-1-hexanol was also detected and the occurrence of *S*-(1(2-hydroxyethyl)butyl)-L-cysteine confirmed. A quantitative estimation based on external calibration curves, established by UPLC−MS ESI$^+$ in selected reaction monitoring mode, showed that cysteine-*S*-conjugates were present at concentrations in the range of 1 to 100 μg/kg (±20%).

KEYWORDS: cysteine-*S*-conjugate, bell peppers, *Capsicum annuum*, sulfur compounds, thiols, *S*-(3-hydroxy-1-methylhexyl)-L-cysteine, *S*-(3-hydroxy-1-propylbutyl)-L-cysteine, *S*-(3-oxo-1-propylbutyl)-L-cysteine, (2*R*,2′*R*)-3,3′-(4-hydroxyheptane-2,6-diyl)-bis(sulfanediyl)bis(2-aminopropanoic acid)

INTRODUCTION

The glutathione cycle is a biochemical pathway with various benefits for living organisms, mainly as a detoxification agent. Glutathione (γ-glutamine-cysteine(SH)-glycine; GSH) reacts with electrophiles, the conjugation being catalyzed by a large group of enzymes classified as glutathione transferases. The GSH-conjugates are metabolized to give cysteine-*S*-conjugates.[1−3] The cysteine conjugates can then be further degraded with β-lyases from plants to form volatile organic thiols, pyruvate, and ammonia.[4] An interesting feature of these cysteine-*S*-conjugates is that they are hydrolyzed not only by plant enzymes but also by salivary enzymes from bacteria in the mouth or even farther down the digestive track.[5] This feature explains the persistence of the perception of sulfur odor in the mouth for fruits or vegetables that include sulfur compounds.

Numerous publications describe the volatile constituents of bell peppers, *Capsicum annuum* L. (Solanaceae),[6−10] which include a large number of cultivars. Methoxypyrazine derivatives such as 2-methoxy-3-isobutylpyrazine,[6] as well as sulfur compounds such as 2-heptanethiol **1**,[11] are recognized as important contributors to the aroma of bell peppers. Buttery et al.[6] first analyzed a steam-distillate of bell peppers, identifying unsaturated C7- and C9-ketones. Recently, Naef et al. discovered 21 new sulfur compounds in red bell peppers, some of which had structures that could be explained by the Michael addition of cysteine to α,β-unsaturated ketones discovered in bell pepper.[12]

In a previous study to search for the precursor of **1**, we prepared an extract from 1.5 kg of green bell peppers and proved the occurrence of *S*-(2-heptyl)-L-cysteine **2**[5] in a fraction obtained by ion exchange chromatography. Out of curiosity, we analyzed this fraction using ultraperformance liquid chromatography−mass spectrometry electrospray ionization (UPLC−MS ESI$^+$) in single ion monitoring (SIM) mode to find out whether any molecular weights would correspond to the adduct of cysteine α,β-unsaturated C7 ketones. We found compounds corresponding to $C_{10}H_{19}NO_3S$ M + H = 234, $C_{10}H_{21}NO_3S$ M + H = 236, $C_{13}H_{26}N_2O_4S_2$ M + H = 339, possibly a double adduct of cysteine and $C_9H_{19}NO_3S$ M + H = 222.

This paper describes the synthesis of the cysteine conjugates prepared with (3*E*)-hepten-2-one **3** and (2*E*)-hepten-4-one **4**, as well as the analytical studies of two types of bell pepper to verify their occurrence.

MATERIALS AND METHODS

Materials. Green and red bell peppers were purchased from a local market farm in August 2010 (www.mattines.ch). Chemical reagents and solvents were of analytical quality and purchased from Fluka-Sigma-Aldrich (Buchs, Switzerland), Novabiochem (Darmstadt, Germany),

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SDS Carlo Erba Reactifs (Val-de-Reuil, France), and Acros (Morris Plains, NJ).

UPLC−MS. Analyses were performed on a Waters (Baden-Dättwil, Switzerland) Acquity system coupled to a mass spectrometer. The separations and quantifications were performed on an Acquity BEH-C18 column (2.1 mm i.d. × 100 mm, 1.7 μm). The elution solvents were CH_3CN containing 0.1% of formic acid (solvent B) and water containing 0.1% of formic acid (solvent A). The gradient profile started at 10% of B, which was held for 0.5 min, increased to 30% of B in 8.4 min, and then to 90% of B in 2.1 min. The flow rate was 0.3 mL/min. The mass spectrometer was a Thermo Finnigan LXQ with an ESI source (HESI-II) operated in positive mode. The spray voltage was 4.0 kV. The capillary temperature was 349 °C. The sheath gas was nitrogen at a flow rate of 60 (Finnigan arbitrary units). The auxiliary gas was also nitrogen at a flow rate of 5 (Finnigan arbitrary units). Authentic samples were injected first in full MS mode and then in MS−MS mode, with wide band activation at 35 eV. Analyses of bell pepper extracts were performed in selected reaction monitoring (SRM) mode at 35 eV, according to the fragmentation pattern of the target molecule.

Gas Chromatography (GC)−MS. Compound identifications were performed on a GC 6890 (Agilent, Palo Alto, CA) equipped with a fused silica SPwax-10 (30 m × 0.25 mm i.d., 0.25 μm film thickness) capillary column (Supelco, Bellefonte, PA). The initial oven temperature was held at 50 °C for 5 min and then increased at 5 °C/min to 250 °C. The injection port was operated in split mode 1/10, the injection volume was 1 μL, and the carrier gas was helium at a constant flow rate of 0.7 mL/min. The column was coupled to a 5973N Inert MS (Agilent). The mass spectra in electron impact mode were measured at 70 eV in a scan range from 30 to 300 m/z. The linear retention indices (LRIs) were calculated by linear extrapolation from the retention times of the analytes and the two closest alkanes eluting just before and just after the analyte.

Nuclear Magnetic Resonance (NMR) Spectra. ^{1}H- and ^{13}C NMR spectra were recorded in D_2O on a Bruker Avance-500 (Zurich, Switzerland) at 500.13 and 125.76 MHz, respectively. Sodium 3-(trimethylsilyl)tetradeuteriopropionate was used as an internal standard (IS) to calibrate the chemical shifts.

Centrifugal Partition Chromatography (CPC). A CPC 1000 from Armen Instrument (Saint Avé, France) was used for preparative liquid−liquid chromatography. The solvent mixture was butanol, acetic acid, and water (3.8:1.2:5), prepared the day before the separation. The rotation was 2600 rpm, the flow rate was 5 mL/min, and the volume of the fractions collected was 20 mL.

***S*-(3-Oxo-1-propylbutyl)-L-cysteine 5.** *N*-Boc-cysteine (2.0 g, 9 mmol), Cs_2CO_3 (1.5 g, 4.6 mmol), (3*E*)-hepten-2-one **3** (1.57 g, 14 mmol), and acetonitrile (3 mL) were stirred overnight at room temperature (∼22 °C), and then concentrated HCl (12 mL) was added dropwise at 0 °C. After 1 h, the solution was loaded on a Dowex 50WX8, H^+, 400 mesh (70 g) column preconditioned with distilled water. The column was eluted with water to neutrality (∼200 mL) and then stepwise eluted with 100 mL portions of 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, and 2.1 molar solutions of aqueous ammonia. Compound **5** eluted with 0.9 M ammonia. We obtained 950 mg (yield 50%) after lyophilization. UPLC−MS ESI$^+$ (rt 3.35 min). MS−MS: M + H = 234 (fragments selected for SRM: 122 and 87). Mixture of two diastereoisomers: ^{1}H NMR (500 MHz, D_2O) δ 4.11 (dd, J = 7.1, 3.8, 1H); 4.04 (dd, J = 7.2, 3.9, 1H); 3.23−3.02 (m, 6H); 2.96−2.77 (m, 4H); 2.23 (s, 3H); 2.22 (s, 3H); 1.63−1.53 (m, 4H); 1.46−1.33 (m, 4H); 0.89 (t, J = 7.3, 6H). Major diastereoisomer (55%/45%,): ^{13}C NMR (125 MHz, D_2O) δ 216.7 (s), 174.7 (s), 56.4 (d), 50.9 (t), 43.4 (d), 39.8 (t), 33.7 (t), 32.8 (q), 22.3 (t), 16.0 (q). Minor diastereoisomer: ^{13}C NMR (125 MHz, D_2O) δ 216.7 (s), 174.6 (s), 56.6 (d), 51.3 (t), 43.7 (d), 39.5 (t), 33.5 (t), 32.7 (q), 22.3 (t), 15.9 (q).

Preparation of *S*-(3-Hydroxy-1-propylbutyl)-L-cysteine 7. *N*-Boc-cysteine (2.0 g, 9 mmol), Cs_2CO_3 (1.5 g, 4.6 mmol), (3*E*)-hepten-2-one **3** (1.57 g, 14 mmol), and acetonitrile (3 mL) were stirred overnight at room temperature (∼22 °C). $NaBH_4$ (304 mg, 8 mmol) was added portionwise to the reaction mixture at 0 °C. The reaction was stirred for 1 h at room temperature, and then concentrated HCl (12 mL) was added dropwise at 0 °C. After 1 h, the solution was loaded on a Dowex 50WX8, H^+, 400 mesh (70 g) column preconditioned with distilled water. The column was eluted with water to neutrality (∼200 mL) and then stepwise eluted with 100 mL portions of 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, and 2.1 molar solutions of aqueous ammonia. Compound **7** was eluted in two fractions, 1.2 and 1.5 M NH_4OH; after lyophilization, we obtained 1.94 g (yield 91%). UPLC−MS ESI$^+$ (rt 2.59−2.70 min). MS−MS: M + H = 236 (fragments selected for SRM: 147, 115, 97). Mixture of four diastereoisomers: ^{1}H NMR (500 MHz, D_2O) δ 4.15−4.06 (m, 2H); 4.05−3.97 (m, 2H); 3.95−3.89 (m, 4H); 3.17−2.98 (m, 8H); 2.95−2.85 (m, 4H); 1.79−1.53 (m, 16H); 1.50−1.37 (m, 8H); 1.21 (d, J = 6.2, 9H); 1.20 (d, J = 6.2, 3H); 0.91 (t, J = 7.3, 12H). Mixture of four diastereoisomers: ^{13}C NMR (125 MHz, D_2O) δ 175.64 (s), 175.56 (s), 175.54 (s), 175.5 (s), 68.6 (d), 68.5 (d), 68.1 (2d), 57.02 (d), 56.96 (d), 56.95 (d), 56.87 (d), 46.0 (2t), 45.8 (2t), 45.7 (d), 45.4 (t), 45.3 (2d), 45.2 (d), 40.1 (t), 40.0 (t), 38.78 (t), 38.77 (t), 33.3 (t), 33.2 (t), 33.1 (t), 33.08 (t), 25.3 (q), 25.2 (q), 25.0 (2q), 22.02 (t), 22.0 (t), 21.9 (t), 21.8 (t),16.04 (2q), 16.01 (q), 15.99 (q).

Preparation of *S*-(3-Hydroxy-1-methylhexyl)-L-cysteine 8. *N*-Boc-cysteine (2.0 g, 9 mmol), Cs_2CO_3 (1.5 g, 4.6 mmol), (2*E*)-hepten-4-one **4** (1.57 g, 14 mmol), and acetonitrile (3 mL) were stirred overnight at room temperature (∼22 °C). $NaBH_4$ (304 mg, 8 mmol) was added portionwise to the reaction mixture at 0 °C. The reaction was stirred for 1 h at room temperature, and then concentrated HCl (12 mL) was added dropwise at 0 °C. After 1 h, the solution was loaded on a Dowex 50WX8, H^+, 400 mesh (70 g) column that had been conditioned with distilled water. The column was eluted with water to neutrality (∼200 mL) and then stepwise eluted with 100 mL portions of 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, and 2.1 molar solutions of aqueous ammonia. The fractions were analyzed by liquid chromatography−MS (LC−MS), and compound **8** was found in the fraction eluted with 1.5 M ammonia. The fraction containing a mixture of diastereoisomers of compound **8** was lyophilized to give 1.88 g (yield 88%). UPLC−MS ESI$^+$ (rt 2.69−2.77 min). MS−MS: M + H = 236 (major fragments for SRM: 97, 115, 147). Mixture of four diastereoisomers: ^{1}H NMR (500 MHz, D_2O) δ 3.96−3.86 (m, 6H); 3.84−3.76 (m, 2H); 3.23−2.99 (m, 12H); 1.79−1.70 (m, 2H); 1.69−1.57 (m, 6H); 1.51−1.29 (m, 28H); 0.91 (t, J = 7.2, 12H); ^{13}C NMR (125 MHz, D_2O) δ 175.63 (s), 175.6 (2s), 175.58 (s), 71.6 (2d), 71.55 (d), 71.5 (d), 56.9 (d), 56.8 (d), 56.76 (d), 56.7 (d), 46.4 (t), 46.3 (2t), 46.0 (t), 41.7 (d), 41.68 (t), 41.6(t), 41.4 (t), 40.6 (d), 40.0 (d), 39.5 (d), 39.4 (d), 33.3 (t), 33.2 (t), 33.1 (2t), 24.6 (q), 24.4 (q), 22.7 (q), 22.6 (q), 20.9 (4t), 16.2 (2q), 16.1 (2q).

***Syn*/*anti* 2,4-Heptanediol 9.** (3*E*)-Hepten-2-one (2.2 g, 20 mmol) **2**, benzyl alcohol (19 g 180 mmol), and tetramethyl guanidine (0.46 g, 4 mmol) were stirred at room temperature for 48 h. EtOH (70 mL) and $NaBH_4$ (760 mg) were added. After 8 h, the solvent was removed under vacuum and the residue extracted with Et_2O and washed with 0.1 M HCl. The organic phase was dried over $MgSO_4$ and concentrated under vacuum. The crude oil was distilled on a 1 cm Vigreux column under reduced pressure (1 mbar, oil bath 80 °C) to remove benzyl alcohol. We obtained 2.5 g (yield 56%) of both *syn* and *anti* diastereoisomers. The residue was then rediluted in EtOH (25 mL) and deprotected by catalytic hydrogenation (Pd/C 5%, 250 mg) overnight. The reaction mixture was filtered and the EtOH removed under reduced pressure to obtain 1.2 g (yield 80%) of the *syn* and *anti* diols **9**. The structure was confirmed by GC−MS (same MS for both stereoisomers and same retention time on GC_{SBP-1} column) and by ^{1}H and ^{13}C NMR. MS: m/z 132 (0), 114 (5), 89 (45), 73 (50), 71 (62), 55 (76), 45 (90), 43 (100). ^{13}C NMR (125 MHz, $CDCl_3$) (*syn* or *anti*): δ 72.7 (d), 69.1 (d), 44.7 (t), 40.4 (t), 24.1 (q), 18.5 (t), 14.1 (q). ^{13}C NMR

(125 MHz, $CDCl_3$) (*syn* or *anti*): δ 68.9 (d), 65.3 (d), 44.2 (t), 39.6 (t), 23.5 (q), 19.0 (t), 14.1 (q).

(2*R*,2′*R*)-3,3′-(4-Hydroxyheptane-2,6-diyl)bis(sulfanediyl)-bis(2-aminopropanoic acid) 10. To a solution of the diol **9** (660 mg, 5 mmol) in toluene (5 mL) containing triethylamine (3.35 mL, 30 mmol), methanesulfonyl chloride (1 mL, 20 mmol) was added dropwise at −30 °C. The reaction mixture was poured on a mixture of ice and 2 M HCl and extracted with Et_2O, and the organic phase was dried on $MgSO_4$. The solvent was removed under vacuum to give 1.2 g (yield 84%) of the bis mesilate. MS: *m*/*z* 288 (1), 175 (55), 149 (100), 123 (30), 97 (95), 79 (56), 71 (43), 55 (75). ^{13}C NMR (125 MHz, $CDCl_3$): δ 79.1 (d), 75.7 (d), 41.8 (t), 38.8 (q), 38.7 (q), 36.5 (t), 21.2 (q), 18.2 (t), 13.7 (q).

The doubly protected *N*,*O*(BOC)$_2$-cysteine (1.2 g, 4.5 mmol), prepared according to Chevallet et al.,[13] was dissolved in *N*-methylpyrrolidone (2.5 mL) in the presence of Cs_2CO_3 (1.5 g, 4.6 mmol) and stirred for 2 h at 22 °C before addition of the double-mesylated alcohol prepared earlier (430 mg, 1.5 mmol). The mixture was heated at 80 °C for 16 h. The reaction mixture was extracted with EtOAc, and salts were removed by extraction with H_2O. The organic phases were dried on $MgSO_4$ and concentrated to obtain crude brownish oil (1.97 g). By thin layer chromatography (HPTLC silica gel 60 F254, Merck 1.05628.0001) with the elution system hexane/ethyl acetate (4:1), the starting material had an R_f of 0.5, and two new spots with an R_f of 0.69 and 0.84 were observed. This crude mixture was diluted in a solution of CH_2Cl_2 (5 mL) and trifluoroacetic acid (TFA; 10 mL) and stirred for 2 h at 22 °C. The TFA was removed by azeotropic distillation in the presence of toluene (2 × 25 mL). The crude oil was then diluted in water, loaded on a Dowex 50 × 8 column, and eluted as described earlier. By LC−MS, a peak with molecular mass corresponding to the desired compound was found in three fractions (0.4 g), which eluted with 0.9, 1.2, and 1.5 M ammonia. The product was further purified by CPC (Armen Instrument). The solvent system was butanol (3.8 parts), acetic acid (1.2 parts), and water (5.0 parts), and the partition coefficient was K = 0.53. The flow rate was 6 mL/min, the mobile phase volume 100 mL, the stationary phase 400 mL, the rotation 2500 rpm, and the pressure 37 bar. By our calculations, the product should have been in fraction 26, and it was effectively recovered in fractions 20 to 26. The solvent was removed under reduced pressure, water was added, and the solution was finally lyophilized to give 75 mg (yield 14.8%) of a white powder corresponding to the desired compound **10**. UPLC−MS ESI^+ (rt 2.35 −2.46 min). MS−MS: M + H = 339 (major fragments for SRM: 129, 154, 218). ^{13}C NMR (125 MHz, D_2O) tentative description of a mixture of diastereoisomers: δ 173.6−173.7 (2s), 55.8 (2d), 46.7 (d), 44.2−44.4 (t), 41.0 (d), (39.0, 39.1, 39.4) (t), (32.0, 32.1, 32.3, 32.4, 32.5, 33.7) (t), (23.2, 23.3, 26.1) (q), (20.0, 22.0, 22.2) (t), 16.0 (q).

Analysis of Cysteine-*S*-Conjugates in Bell Peppers. One kilogram of green bell peppers and 1 kg of red bell peppers were mixed with water (750 mL), ethanol (200 mL), and formic acid (5 mL) and processed in a food processor to obtain a slurry (2−3 min). An IS, *S*-[1-(2-hydroxyethyl)-1-methylbutyl]-L-cysteinyl glycine (1.0 mg), was added (M + H = 293).[14] The emulsion was centrifuged at 8250*g* for 45 min at 5 °C. The clear supernatant (1550 mL) was concentrated under partial vacuum at 30 °C to give 150 mL. This was used for quantitative analysis (1 mL). The remainder was loaded on a Dowex column. The column was eluted with water to neutrality (∼250 mL) and then stepwise eluted with 100 mL portions of 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, and 2.8 molar solutions of aqueous ammonia. Fractions that eluted with 0.9 M ammonia to 2.1 M ammonia solutions were pooled together, the ammonia was removed under vacuum, and the extracts were lyophilized to give 3.89 g for the green bell pepper and 6.11 g for the red bell pepper powder. These powders were used for enzymatic treatment.

Calibration curves were established in SRM mode with the authentic samples at different concentrations in 0.1% water/formic acid of 0.01, 0.10, 1.00, 10.00 μg/mL. Calibration curves had excellent values for the square of the Pearson correlation coefficient, from 0.9994 to 1.0000.

Release of Free Thiols from Cysteine-*S*-Conjugates. A buffered solution of potassium phosphate (100 mM, pH 8) containing ethylenediaminetetraacetic acid (1 mM), pyridoxal 5 phosphate (0.1 mM), and L-glutathione in reduced form (1 mM) was prepared. Apotryptophanase (from *Escherichia coli*, activity 75−150 units/mg, Sigma-Aldrich) was freshly prepared (1 mg in 0.5 mL of buffer). Two solutions of standard containing **5**, **7**, and **12** (0.01 mL, containing 10 μg of each precursor) were added to the buffer (1.5 mL). Two solutions containing the green and red bell pepper Dowex fraction (1 g) in the buffer (5 mL) were prepared. The enzyme (0.5 mL) was added to one of the standard solutions and one of the green or red bell pepper solutions. The six mixtures were incubated for 30 min at 35 °C in sealed vials. The incubated mixtures were cooled in ice and dichloromethane (0.5 mL) added. The solution was vigorously stirred and then centrifuged. The organic phases were dried over Na_2SO_4 and loaded on a Pasteur pipet equipped with a small cotton plug and loaded with 1 mL of Affigel, and then the free thiols were eluted as described previously.[12,15] The concentrated thiol fraction (about 20−50 μL) was injected on the GC−MS, and the occurrence of the thiols of interest was searched with the MS in SIM mode. The structure was confirmed by MS fragmentation pattern and by linear retention index (on polar columns) in accordance with the authentic samples.[12] The water phase was also injected on the LC−MS to check whether the precursors were transformed.

RESULTS AND DISCUSSION

Preparation of Authentic Samples. Authentic samples **5**−**8** were synthesized from *N*-Boc-cysteine by Michael-type addition to the corresponding α,β-unsaturated ketones (*E*)-3-hepten-2-one **3** and (*E*)-2-hepten-4-one **4** (Figure 1). The synthesis was performed with cesium carbonate (Cs_2CO_3) as a base. Various bases were used to perform the Michael addition of cysteine or glutathione to α,β-unsaturated aldehydes, including NaOH,[16] Na_2CO_3,[17] and Et_3N,[18] but Cs_2CO_3 is thought to be more efficient because cesium thiolate is well soluble in organic solvent. Cesium thiolate also displayed very good nucleophilicity[19] and was previously used for the addition of *N*-Boc-cysteine to (*E*)-2-hexenal.[20]

The nucleophilic sulfur atom can approach the sp^2 carbon of the α,β-unsaturated aldehydes from the *si*-face or the *re*-face. In basic conditions, we do not expect diastereoselectivity and therefore we expect to obtain two diastereoisomers of **5** and **6**. In nature this reaction is catalyzed by glutathione transferase, but the stereoselectivity of this class of enzyme has not been reported. Compound **5** was carefully purified and analyzed by NMR. It was obtained with a moderate yield of 50%, which could be explained by the retro-Michael occurring during the Dowex purification. The ^1H NMR of **5** confirms the presence of the α proton of α-amino acid at 4.11 ppm and at 4.04 of the two diastereoisomers. The proton signal integration displayed a 3:2 diastereoisomeric ratio. All other signals, such as a singlet at 2.23 and at 2.22 ppm, which are typical for a methyl linked to a carbonyl, are in agreement with the expected compound **5**. The ^{13}C NMR also confirmed the structure with, for example, the presence of a singlet at 216.74 and at 216.71 ppm for the ketone function, or the typical doublet at 56.61 and at 56.40 for the α carbon of α-amino acids. Compound **6** was lost during purification; a crude sample was analyzed by UPLC−MS and MS−MS to record its spectral data. The two diastereoisomers of **5** or **6** are

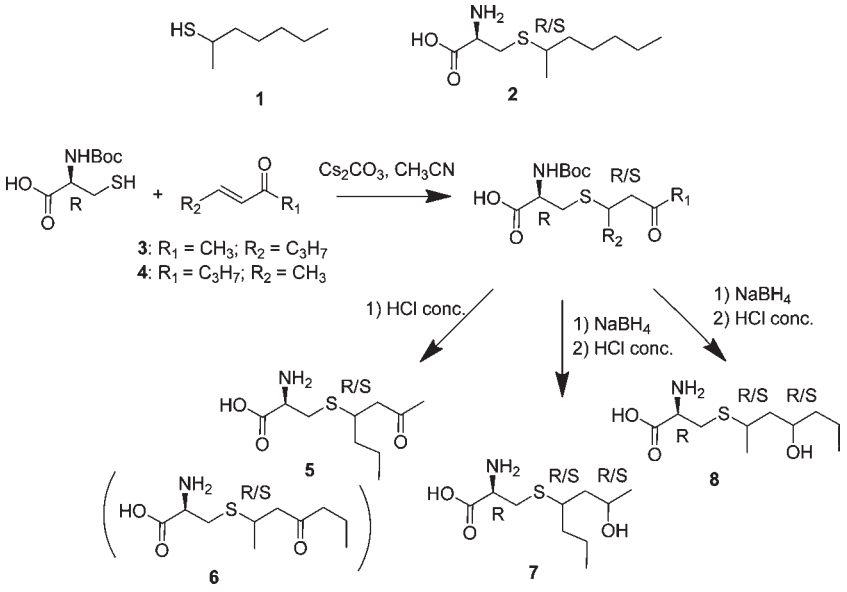

Figure 1. Structures of compounds **1** and **2** reported previously to occur naturally in bell peppers.[5] Synthesis of cysteine-*S*-conjugates **5**−**8** used as authentic samples.

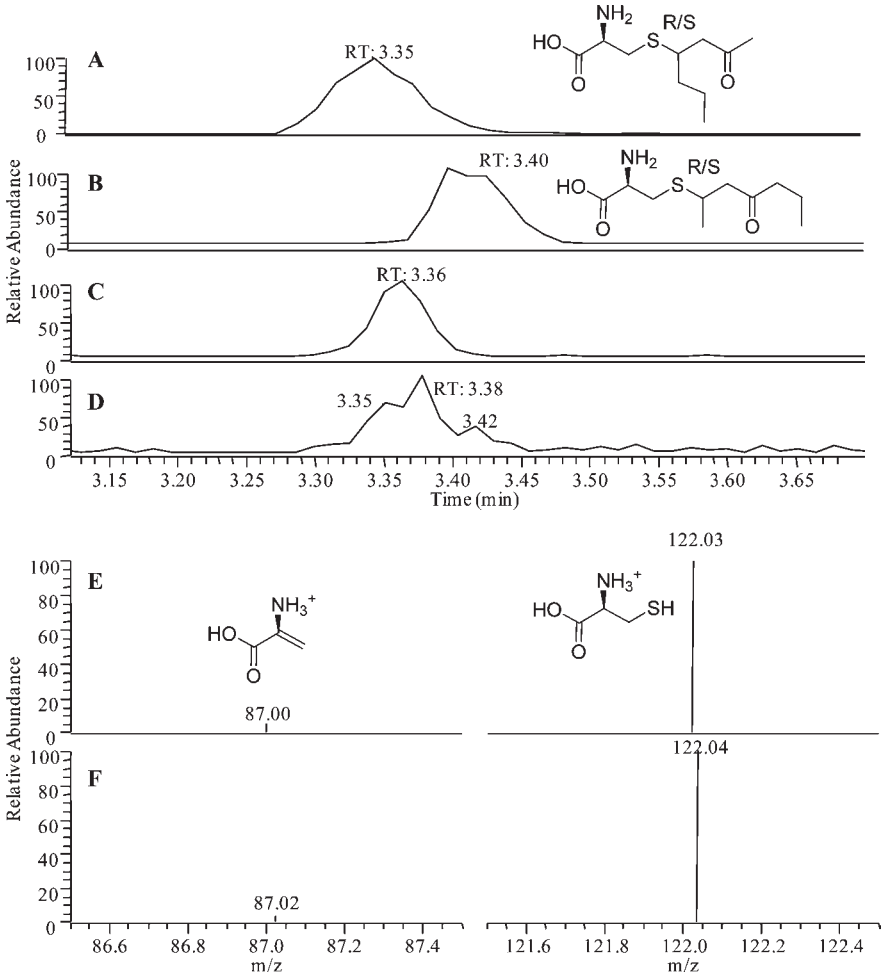

Figure 2. (A) UPLC−MS trace of compound **5** monitored in SRM mode (ESI^+). SRM MS−MS of M + H = 234, collision energy: 35 eV [86.50−87.50, 21.50−122.50]. (B) UPLC−MS trace of compound **6**, same conditions. (C) UPLC−MS trace of green bell pepper in same conditions. (D) UPLC−MS trace of red bell pepper extract in same conditions. (E) MS−MS of red bell pepper (D) from 3.32 to 3.40 min. (F) MS−MS of red bell pepper (D) from 3.40 to 3.42 min.

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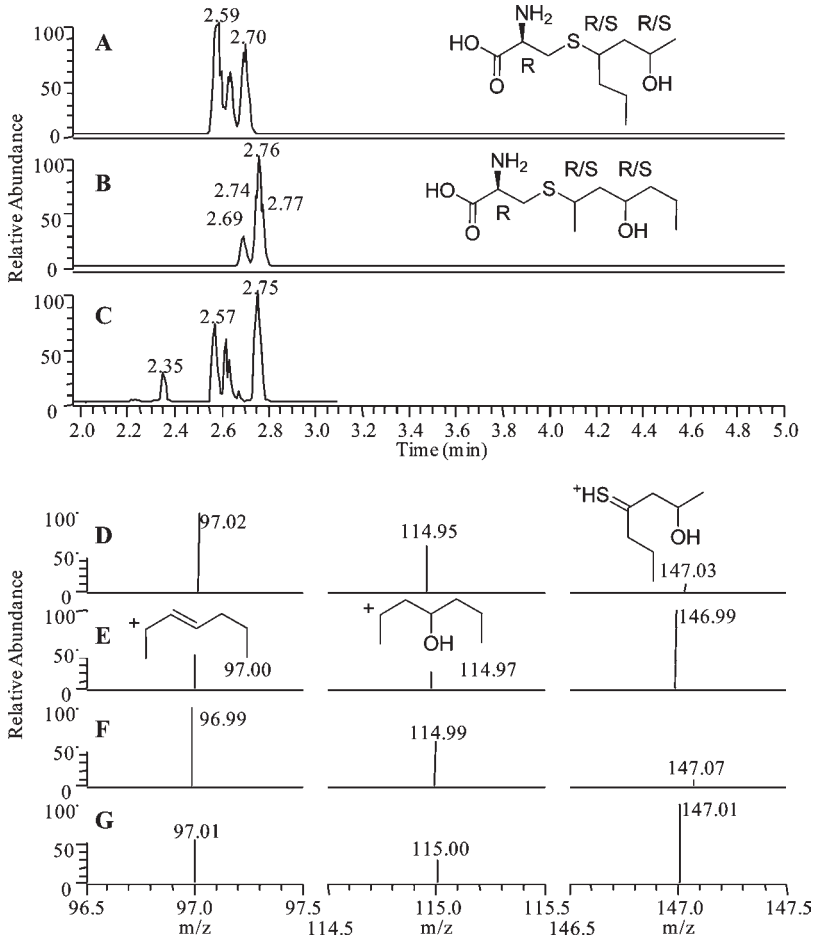

Figure 3. (A) UPLC−MS trace of diastereoisomers corresponding to **7** monitored in SRM MS−MS of M + H = 236, collision energy: 35 eV [96.50−97.50; 114.5−155.50; 146.50−147.50]. (B) UPLC−MS trace of compound **8**, same SRM mode. (C) UPLC−MS trace of red bell pepper, same SRM mode. (D) MS−MS fragment intensities of **7**. (E) MS−MS fragment intensities of **8**. (F) MS−MS of red bell pepper extract (C) from 2.55 to 2.63 min. (G) MS−MS of red bell pepper extract (C) from 2.73 to 2.77 min.

not separated, but compounds **5** and **6** have different retention times. The fragmentation in MS−MS of (M + H = 234) displayed two major fragments, *m/z* 122 corresponding to cysteine as a result of the retro-Michael, and *m/z* 87 as a result of the elimination of H_2S from cysteine (Figure 2A, 2B).

Compounds **7** and **8** were prepared via the reduction of the *N*-Boc protected adduct. The reduction is not stereoselective; therefore, we obtained a mixture of the four possible diastereoisomers. The proton NMR analysis for compound **7** clearly showed four embedded signals for the α proton of the α-amino acid moiety at 4.15−4.06 and 4.05−3.97 ppm, as well as for the corresponding carbon in ^{13}C NMR: 57.02, 56.96, 56.95, 56.87 ppm. The NMR analysis of compound **8** displayed the same pattern, confirming the expected structure of **8**; however, it contained 5% of cystine. These references were injected on the UPLC−MS−MS. For compound **7**, a cluster of peaks representing all of the possible diastereoisomers was observed at M + H = 236 (Figure 3A). The peak shape of compound **8** was even less instructive about diastereoisomer distribution, but it eluted slightly later compared with **7**, which allowed us to selectively look for it in bell pepper. The resolution of peaks between **7** and **8** was not good enough to quantify **7** and **8** independently (Figure 3B). To prove the occurrence of **7** in bell pepper, the mass of 236 was fragmented and monitored in SRM mode *m/z* = 147 ($C_7H_{15}OS$), corresponding to the β-elimination of cysteine, and then to the loss of the sulfur atom *m/z* 115 ($C_7H_{15}O$), and finally to the loss of water *m/z* 97 (C_7H_{13}) (Figure 3D). Compound **8** displayed the same fragmentation pattern (Figure 3D, 3E). These fragmentations were proposed by Mass Frontier software (Thermo Scientific).

The preparation of **10** was more difficult. Benzyl alcohol was added to (*E*)-3-hepten-2-one **4**. After reduction, heptane-2,4-diol **9** was obtained and treated with mesyl chloride to transform the two hydroxyl functions into good leaving groups, which were then substituted by the nucleophilic addition of doubly protected cysteine (Figure 4). Ion exchange chromatography did not afford product **10** in sufficient purity. For this reason, we further purified the fraction by CPC.[21] The advantage of this technology, compared with preparative high-performance liquid chromatography, is that it is possible to load more than 1 g per injection. Once the solvent system is established, according to the *K* (partition coefficient) and the operating parameters, it is possible to determine the fraction in which the product should be recovered.[22] Therefore, when the natural extract is processed under the same conditions, it is possible to locate quite precisely the fraction in which the targeted compound should be present (these results are not documented). The major drawback of the CPC is the use of a large amount of solvent; in our case, the

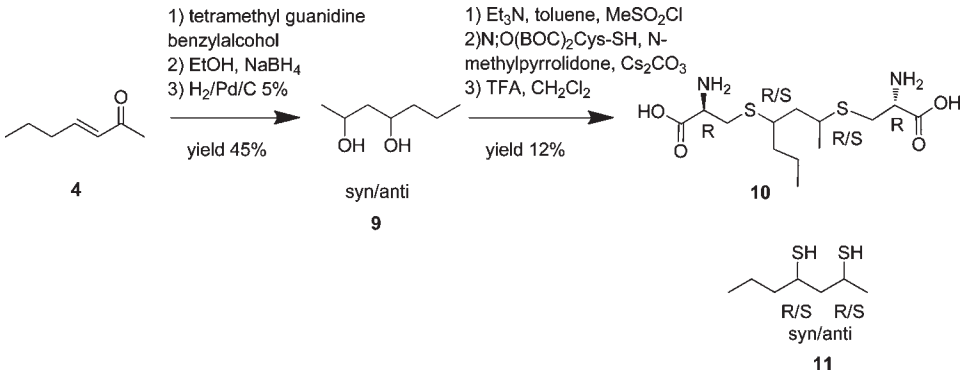

Figure 4. Preparation of bis-cysteine-*S*-conjugate **10**.

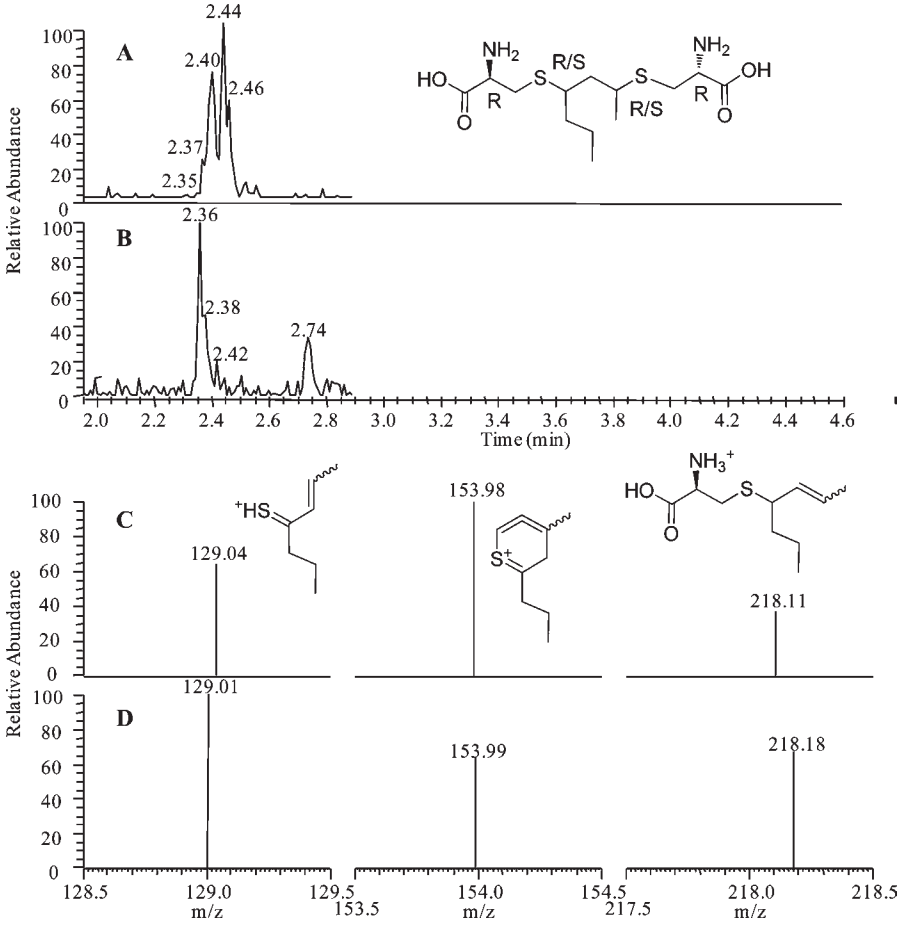

Figure 5. (A) UPLC−MS trace of diastereoisomers of **10** monitored in SRM: MS−MS M + H = 339, collision energy 35 eV [128.50−129.50; 153.5−154.50; 217.50−218.50]. (B) Red bell pepper extract. (C) Intensity of 10 fragments at 2.35−2.46 min. (D) Fragments observed in red bell pepper extract at 2.36−2.39 min (from B).

solvent system was butanol, acetic acid, and water, which was difficult to remove. We also observed background noise from contaminants originating from butanol and acetic acid. We obtained a white powder that was soluble in 0.1 M DCl to measure NMR. The spectra are complicated because of the presence of all diastereoisomers, but in ^{1}H NMR at 4.35−4.25 there is a typical signal for the α-proton of cysteine, as well as a broad signal at 3.28−3.10 corresponding to the $-CH_2-$ of cysteine. The doublet accounting for the methyl CH_3-CH−(SR)−R was attributed to complex signals of embedded doublets at 1.3 ppm. The methyl at the end of the chain gave a triplet at 0.9 ppm. The ^{13}C NMR is also very complex and tentatively described in the Materials and Methods and added as Supporting Information. The UPLC-ESI$^+$ displayed M + H = 339, corresponding to the chemical formula $C_{13}H_{26}N_2O_4S_2$ in ESI$^+$ MS−MS. The fragments used in SRM mode were (m/z = 218) resulting from the loss of one cysteine, and then a fragment resulting from the beta elimination of a second cysteine (m/z = 154) (although no fragments were suggested by Mass Frontier), and finally m/z = 129, which seems to correspond to the chemical

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Table 1. Summary of the Results Obtained by Quantification in SRM Mode for Red and Green Bell Peppers

	concentration[a] (μg/kg)				
bell pepper type	**2**	**5**	**7 + 8**	**12**	IS
green	4.7 ± 0.2	3.3 ± 0.5	67.7 ± 13.5	3.2 ± 0.6	587 ± 32.9
red	0.2 ± 0.1	14.5 ± 0.2	107.7 ± 21.4	0.8 ± 0.2	549 ± 86.2

[a] Values represent the mean ± SD. IS, internal standard.

formula $C_7H_{13}S^+$ (Figure 4A, 4B). We are confident that we detected the authentic sample of the precursor of 2,4-heptanedithiol **11** or the corresponding dithiolane,[12] in the volatile of the bell pepper.

Analysis of Bell Peppers. Fresh extracts were prepared from red and green bell peppers. Compound **5** was searched at 3.35 min in all bell peppers, in SRM mode *m/z* 122 and 87 coming from M + H = 234. Its occurrence was obvious in green bell pepper (Figure 2C, 2E). We also observed the other expected compound **6** at 3.42 min (Figure 2D, 2F), but only in red bell pepper and with less abundance. The alcohol derivatives **7** and **8** were detected in all bell peppers analyzed; Figure 3C, 3F, and 3G illustrate their occurrence in red bell pepper. The presence of the two diastereoisomers of 2,4-heptane dithiol **11** in the volatile portion of green bell pepper[12] led us to postulate the occurrence of **10**. This postulate was positively confirmed by the detection at 2.36 and 2.42 min of a mass of M + H = 339 having the same fragmentation pattern as the authentic sample (Figure 5B, 5D), even though the peak shape of the four possible diastereoisomers **10** is quite different from that of the diastereoisomer distribution in the natural product. The occurrence of (*E*)-2-hexenal was documented in green bell pepper;[9,10] for this reason, we expected to find *S*-(1-(2-hydroxyethyl)butyl)-L-cysteine **12**, which was prepared previously.[20] In all extracts we detected it at 2.04 min, which was the same retention time of reference **12**, fragments *m/z* 187, 101, and 83 coming from M + H = 222. This finding also confirms the occurrence of **12** in bell peppers.

Quantitative Analysis of the Precursors 2, 5, 7, 8, and 12. The calibration curves were made with the authentic samples. Compound **6** decomposed during its purification, and thus we omitted this compound from the analysis. The ^{1}H NMR of compound **8** displayed the presence of cystine (11%); because of this contamination, no calibration curves were made for **8**. The ^{1}H NMR of compounds **2**, **5**, **7**, and **12** displayed purity of more than 95%, and no other compounds were detected by UPLC−MS. These compounds are considered to be obtained in their neutral form (R−COO^-, R−NH_3^+) according to the conditions used for the elution of the Dowex column. Although we have no information regarding the possible presence of water, the powders were free-flowing; therefore, we ignored the possible presence of water, in agreement with the observations of other groups that have also performed several quantifications with cysteine-*S*-conjugates.[16,18] For compounds **7** and **8**, we considered a peak area from 2.57 to 2.75 min and we used the calibration curve for **7**. This gave a reasonable idea of the content of these conjugates, as the objective was not to obtain precise quantification. Previous results obtained for **2**[5] demonstrated variations in the content of these precursors by a factor of close to 20 between different lots. The quantification was performed on the crude extract of one red bell pepper lot and one green bell pepper lot, repeated twice. The results are summarized in Table 1, the values representing the average of two measures in SRM mode and the variation (±) reflecting the standard deviation (SD). The more abundant conjugates were **7** and **8**. Compound **5** was present in red and green bell pepper. Compounds **2** and **12** were more abundant in green bell pepper, but to confirm these trends, more data should be obtained from many different lots of green and red bell pepper (Table 1). The IS quantified at 587 (±33) μg/kg in the green bell pepper and at 549 (±86) μg/kg in the red bell pepper indicated a recovery factor of 59% and 55%, but the recovery factor was not used to adjust the concentration values of **2**, **5**, **7** + **8**, and **12**.

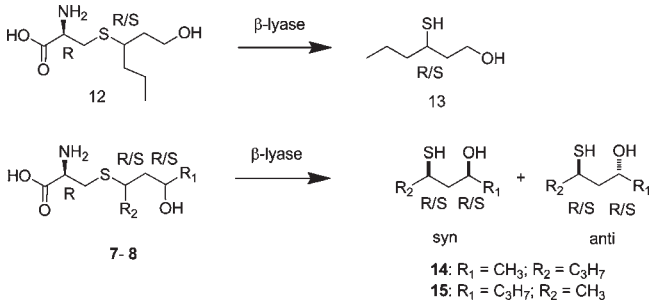

Figure 6. Chemical scheme of volatile thiols obtained after enzymatic treatment.

Release of Volatile Thiols. Thiols can be released from cysteine-*S*-conjugates by enzymes called β-lyases.[4] Confirming proof of the occurrence of cysteine conjugates as precursors of volatile thiols would be to release the corresponding free thiol from the fraction containing them when treated with a β-lyase. Apotryptophanase (EC 4.1.99.1) is a commercially available β-lyase. It was used according to a protocol published by Peyrot des Gachons et al. in 2000.[23] The incubation was performed on a model system containing 10 μg each of **5**, **7**, and **12** in 1.5 mL of buffer (Figure 6). After incubation, no precursors were detected in the water phase. The volatile organic sulfur, extracted with dichloromethane and injected on GC−MS, confirmed the formation of compounds **13** and **14**, as well as 4-sulfanyl-2-heptanone from **5**. Conjugate **7** was found to release the two expected *syn* and *anti* diastereoisomers **14**, LRIs 1742 (reference 1746) and 1760 (reference 1763).[12]

The estimated concentration of conjugates in the Dowex fraction was 107.7 (±21.4) μg/kg for **7** and **8** together in red bell pepper. From 1 g of the Dowex fraction, if the enzymatic transformation converts all of the precursors in the volatile thiols, after concentration of the CH_2Cl_2 extract, we expect to obtain a concentration of free thiols of about 1 mg/kg, which is close to the detection limit in GC−MS-SIM (signal/noise <5). The problem was the presence of many other compounds extracted with dichloromethane. Therefore, we had two options: to further purify the fraction containing the cysteine-*S*-conjugates or to purify the organic extract containing free thiols by affinity chromatography (Affigel).[10,12] The concentration of the volatile organic thiols was the option chosen, after which we were able to confirm by GC−MS the formation of the stereoisomers of **14** and **15** with LRIs, consistent with those published (1720, reference 1722 and 1740, reference 1745)[12] in red bell pepper and of **5**, **7**, **8**, and **12** in green bell pepper.

This work confirms that cysteine-*S*-conjugates previously detected in *Vitis vinifera*,[23,24] *Allium* sp.,[25] *Asparagus*,[26] and *Citrus*[20] are ubiquitous in natural products and represent one major precursor pathway to volatile organic sulfur compounds. Cysteine-*S*-conjugates are important flavor precursors,[27] and

agronomists should take into account the importance of this metabolic pathway to develop tasty new bell peppers.

■ ASSOCIATED CONTENT

Supporting Information. Additional information as discussed in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*Tel: +41 22 780 34 77. Fax: +41 22 780 33 34. E-mail: Christian.starkenmann@firmenich.com.

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■ ABBREVIATIONS USED

CPC, centrifugal partition chromatography; ESI^+, electrospray ionization source operated in positive mode; GC, gas chromatography; GSH, reduced glutation; IS, internal standard; LC−MS, liquid chromatography−mass spectrometry; LRIs, linear retention indices; MS, mass spectrometry; NMR, nuclear magnetic resonance; SIM, single ion monitoring; SRM, selected reaction monitoring; TFA, trifluoroacetic acid; UPLC−MS, ultraperformance liquid chromatography−mass spectrometry

■ REFERENCES

(1) Lamoureux, G. L.; Rusness, D. G. The role of glutathione and glutathione-S-transferases in pesticide metabolism, selectivity, and mode of action in plants and insects. In *Glutathione: Chemical, Biochemical and Medical Aspects*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley and Sons: New York, 1989; Vol. 3B, pp 153−196.

(2) Marrs, K. A. The functions and regulation of glutathione-S-transferases in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1996**, *47*, 127–158.

(3) Dixon, D. P.; Skipsey, M.; Edwards, R. Roles for glutathione transferases in plant secondary metabolism. *Phytochemistry* **2010**, *71*, 338–350.

(4) Cooper, A. Mechanisms of cysteine-*S*-conjugate β-lyase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1998**, *72*, 199–238.

(5) Starkenmann, C.; Le Calvé, B.; Niclass, Y.; Cayeux, I.; Beccucci, S.; Troccaz, M. Olfactory perception of cysteine-*S*-conjugates from fruit and vegetables. *J. Agric. Food Chem.* **2008**, *56*, 9575–9580.

(6) Buttery, R. G.; Seifert, R. M.; Guadagni, D. G.; Ling, L. C. Characterization of some volatile constituents of bell peppers. *J. Agric. Food Chem.* **1969**, *17*, 1322–1327.

(7) Keller, U.; Flath, R. A.; Thomas, R.; Teranishi, R. Volatiles from red peppers (*Capsicum* spp.). In *Quality of Selected Fruits and Vegetables of North America*; Teranishi, R., Barrera-Benitez, H., Eds.; ACS Symposium Series 170; American Chemical Society: Washington, DC, 1981; pp 137−146.

(8) Luning, P. A.; de Rijk, T.; Wichers, H. J.; Roozen, H. P. Gas chromatography, mass spectrometry, and sniffing port analyses of volatile compounds of fresh bell peppers (Capsicum annuum) at different ripening stages. *J. Agric. Food Chem.* **1994**, *42*, 977–983.

(9) Zimmermann, M.; Schieberle, P. Important odorants of sweet bell pepper powder (*Capsicum annuum* cv. annuum): Differences between samples of Hungarian and Moroccan origin. *Eur. Food Res. Technol.* **2000**, *211*, 175–180.

(10) Mazida, M. M.; Salleh, M. M.; Osman, H. Analysis of volatile aroma compounds of fresh chilli (*Capsicum annuum*) during stages of maturity using solid phase microextraction (SPME). *J. Food Compos. Anal.* **2005**, *18*, 427–437.

(11) Simian, H.; Robert, F.; Blank, I. Identification and synthesis of 2-heptanethiol, a new flavor compound found in bell peppers. *J. Agric. Food Chem.* **2004**, *52*, 306–310.

(12) Naef, R.; Velluz, A.; Jaquier, A. New volatile sulfur-containing constituents in simultaneous distillation-extraction extract of red bell peppers (*Capsicum annuum*). *J. Agric. Food Chem.* **2008**, *56*, 517–527.

(13) Chevallet, P.; Garrouste, P.; Malawska, B.; Martinez, J. Facile synthesis of tert-butyl ester of N-protected amino acids with tert-butyl bromide. *Tetrahedron Lett.* **1993**, *34*, 7409–7412.

(14) Starkenmann, C.; Niclass, Y.; Troccaz, M.; Clark, A. J. Identification of the precursor of (*S*)-3-methyl-3-sulfanylhexan-1-ol, the sulfury malodour of human axilla sweat. *Chem. Biodivers.* **2005**, *2*, 705–716.

(15) Full, G.; Schreier, P. Covalent chromatography. A valuable method for the aroma analysis of thiols at trace levels. *Lebensmittelchemie* **1994**, *48*, 1–4.

(16) Luisier, J. L.; Buettner, H.; Volker, S.; Rausis, T.; Frey, U. Quantification of cysteine S-conjugate of 3-sulfanylhexan-1-ol in must and wine of petite arvine vine by stable isotope dilution analysis. *J. Agric. Food Chem.* **2008**, *56*, 2883–2887.

(17) Starkenmann, C. Analysis of a model reaction system containing cysteine and (E)-2-methyl-2-butenal, (E)-2-hexenal, or mesityl oxide. *J. Agric. Food Chem.* **2003**, *51*, 7146–7155.

(18) Grant-Preece, P. A.; Pardon, K. H.; Capone, D. L.; Cordente, A. G.; Sefton, M. A.; Jeffery, D. W.; Elsey, G. M. Synthesis of wine thiol conjugates and labeled analogues: Fermentation of the glutathione conjugate of 3-mercaptohexan-1-ol yields the corresponding cysteine conjugate and free thiol. *J. Agric. Food Chem.* **2010**, *58*, 1383–1389.

(19) Keinan, E.; Eren, D. An improved method for S_N2-type demethoxycarbonylation of activated esters with 4-aminothiophenol and a cesium catalyst. *J. Org. Chem.* **1986**, *51*, 3165–3169.

(20) Starkenmann, C.; Niclass, Y.; Escher, S. Volatile organic sulfur-containing constituents in *Poncirus trifoliata* (L.) Raf. (Rutaceae). *J. Agric. Food Chem.* **2007**, *55*, 4511–4517.

(21) Rolet-Menet, M.-C. Centrifugal partition chromatography. In *Encyclopedia of Chromatography*, 2nd ed.; Cazes, J., Ed.; Taylor & Francis: London, 2008.

(22) Berthod, A.; Friesen, J. B.; Inui, T.; Pauli, G. F. Elution-extrusion countercurrent chromatography: Theory and concepts in metabolic analysis. *Anal. Chem.* **2007**, *79*, 3371–3382.

(23) Peyrot des Gachons, C.; Tominaga, T.; Dubourdieu, D. Measuring the aromatic potential of *Vitis vinifera* L. Cv. Sauvignon Blanc grapes by assaying S-cysteine conjugates, precursors of the volatile thiols responsible for their varietal aroma. *J. Agric. Food Chem.* **2000**, *48*, 3387–3391.

(24) Tominaga, T.; Peyrot des Gachons, C.; Dubourdieu, D. A new type of flavor precursors in *Vitis vinifera* L. cv. Sauvignon Blanc: S-cysteine conjugates. *J. Agric. Food Chem.* **1998**, *46*, 5215–5219.

(25) Brewsler, J. L.; Rabinowitch, H. D. Onions and allied crops. In *Biochemistry Food Science*; Brewster, J. L., Rabinowitch, H. D., Eds.; CRC Press Inc.: Boca Raton, FL; 1989, Vol. 3, pp 1−288.

(26) Parry, R. J.; Mizusawa, A. E.; Chiu, I. C.; Naidu, M. V.; Ricciardone, M. Biosynthesis of sulfur compounds. Investigations of the biosynthesis of asparagusic acid. *J. Am. Chem. Soc.* **1985**, *107*, 2512–2521.

(27) Starkenmann, C.; Troccaz, M.; Howell, K. The role of cysteine and cysteine-*S*-conjugates as odour precursors in the flavour and fragrance industry. *Flav. Fragr. J.* **2008**, *23*, 369–381.