JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 1383–1389 1383
DOI:10.1021/jf9037198

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

Paris A. Grant-Preece,[†] Kevin H. Pardon,[‡] Dimitra L. Capone,[‡] Antonio G. Cordente,[‡] Mark A. Sefton,[‡,§] David W. Jeffery,*[,‡] and Gordon M. Elsey[†,‡,§]

[†]School of Chemistry, Physics and Earth Sciences, Flinders University, P.O. Box 2100, Adelaide, South Australia 5001, Australia and [‡]The Australian Wine Research Institute, P.O. Box 197, Glen Osmond, South Australia 5064, Australia. [§]Present address: School of Agriculture, Food and Wine, The University of Adelaide, Waite Campus, PMB 1, Glen Osmond, South Australia 5064, Australia.

Synthesis of the putative wine thiol precursor 3-*S*-glutathionylhexan-1-ol (Glut-3-MH) has been undertaken to provide pure reference materials for the development of HPLC-MS/MS methods for precursor quantitation in grape juice and wine, and for use in fermentation experiments. Labeled thiol conjugates were also prepared for use as internal standards. Purification and fermentation of a single diastereomer of Glut-3-MH with VIN13 (*CSL1*) yielded not only the (*R*)-enantiomer of the wine impact odorant 3-mercaptohexan-1-ol (3-MH) but also the cysteine conjugate intermediate as a single (*R*)-diastereomer, as determined by HPLC-MS/MS. Chiral GC-MS was used to quantify the total amount of (*R*)-3-MH released from the ferments, resulting in a molar conversion yield of the glutathione conjugate of about 3%. Enzymatic degradation of the single (*R*)-Glut-3-MH diastereomer with a γ-glutamyltranspeptidase confirmed the stereochemical relationship to the related cysteine conjugate. This is the first demonstration that Glut-3-MH can liberate 3-MH under model fermentation conditions, where the cysteine conjugate is also formed in the process. This furthers our understanding of the nature of wine thiol precursors and opens avenues for additional studies into formation and interchange of wine thiols and their precursors.

KEYWORDS: Synthesis; fermentation; diastereoisomers; wine thiol precursors; varietal thiols; elution order; HPLC-MS/MS; chiral analysis; GC-MS

INTRODUCTION

Volatile, polyfunctional thiols are compounds which can impart pleasant, varietal aromas to wine. The compounds responsible for these varietal aromas were first identified in various fruits, such as black currant, grapefruit, passion fruit, or guava, and described in Sauvignon Blanc wine for the first time in 1995 (*1*, *2*). **Figure 1** shows thiols which exhibit a typical Sauvignon Blanc aroma in wine, namely: 4-mercapto-4-methylpentan-2-one (4-MMP, **1**), with a characteristic aroma described as "black currant", "boxwood", and "broom" (*2*); 3-mercaptohexyl acetate (3-MHA, **2**), with an aroma described as "grapefruit", "passion fruit", and "box tree" (*3*); and 3-mercaptohexan-1-ol (3-MH, **3**), described similarly to **2** with an aroma of "passion fruit" or "grapefruit" (*4*). These important wine thiols are extremely potent and have some of the lowest odor detection thresholds of any compound found in foods or beverages. The odor detection thresholds of **1**, racemic **2**, and racemic **3** were determined to be 3 ng/L in white and red wine (*2*), 4 ng/L in 12% aqueous ethanol (*3*), and 60 ng/L in 12% aqueous ethanol (*4*), respectively.

Stereochemistry can be important to the sensory characteristics of wine thiols. Both enantiomers of 3-MHA (**2**) and 3-MH (**3**) (**Figure 1**) are present in Sauvignon Blanc and Semillon wines (*5*). The odor detection thresholds for the (*R*)- and (*S*)-enantiomers of **3** were reported to be 50 and 60 ng/L, respectively, in model wine, while the reported detection thresholds for (*R*)-**2** and (*S*)-**2** were 9 and 2.5 ng/L, respectively (*5*). Furthermore, the odor qualities of the 3-MH enantiomers differed, with descriptions of "grapefruit" and "citrus peel" for (*R*)-**3** and "passion fruit" for (*S*)-**3** (*5*).

The thiols **1**−**3** are not only varietal impact aroma compounds of Sauvignon Blanc; they have also been identified in wines made from many different cultivars of *Vitis vinifera*, such as Scheurebe, Gewürztraminer, Riesling, Colombard, Petit Manseng, Semillon, Cabernet Sauvignon, and Merlot (*4*, *6*−*10*).

The varietal thiols **3** and **1** are not present in grape juice to any appreciable extent but are conjugated as odorless precursors (**Figure 2**). Initially, *S*-cysteinyl conjugates of **3** (i.e., (*R*/*S*)-**4**) and **1** (i.e., **6**) were identified as putative precursors to the

*Corresponding author. Telephone: +61 8 8313 6600. Fax: +61 8 8313 6601. E-mail: david.jeffery@awri.com.au.

Published on Web 01/15/2010

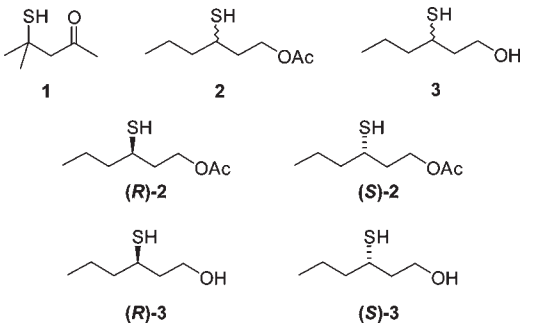

Figure 1. Structures of thiols important to the varietal aroma of some wine styles.

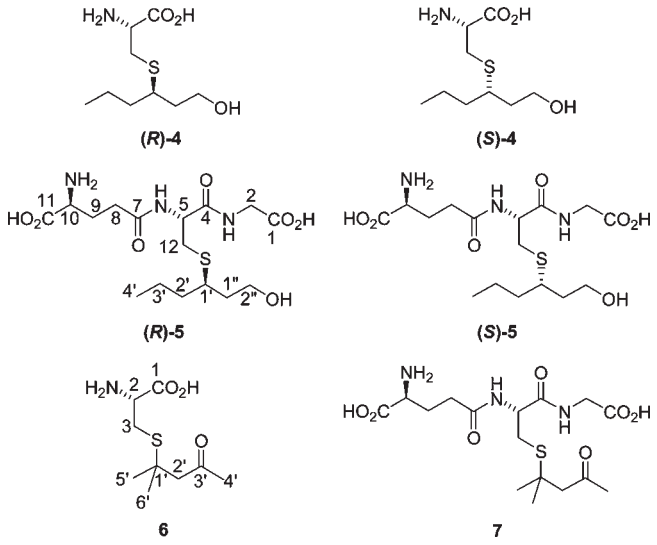

Figure 2. Structures of potential precursors to important wine thiols, including the numbering scheme employed for **5** and **6**.

corresponding thiols in a Sauvignon Blanc juice (*11*). Subsequently, the *S*-glutathione conjugate of **3** (i.e., (*R*/*S*)-**5**) was discovered in Sauvignon Blanc juice (*12*), and more recently, the glutathione conjugate of **1** (i.e., **7**) was described (*13*).

Recently, the individual diastereomers of **4** were prepared for the first time and were shown to undergo cleavage to their respective enantiomers of **3** by the action of apotryptophanase enzyme (*14*). When (*R*)-**4** and (*S*)-**4** were added to ferments conducted with the wine yeast *Saccharomyces cerevisiae*, the same conversions were observed. Subsequently, however, Subileau et al. (*15*) have shown that these *S*-cysteinyl conjugates are only minor precursors of **3** in wine. They inferred that glutathione derivatives might be more significant precursors of the volatile thiols important to wine aroma. Therefore, biochemical glutathione conjugation requires careful consideration. It is known that glutathione present in grapes rapidly decreases in concentration after crushing because of the onset of redox and enzymatic processes (*16*). Also, plants utilize glutathione conjugation as a protective mechanism to detoxify cells and as an antioxidant (*17*−*20*), and cysteine conjugates arise from the degradation of the corresponding glutathione conjugates. This might help to explain the presence of glutathione and cysteine conjugates of varietal thiols in grape juices/musts.

In order to understand further the relationship between thiol precursors in juice and varietal thiols in wine, studies requiring compound synthesis, fermentation, and analytical method development were carried out. This work was undertaken to provide the diastereomers of *S*-glutathionyl conjugate **5**, the pure diastereomer (*R*)-**5**, and the various labeled conjugates for use as internal standards in the development of analytical methods. The fate of the synthetic glutathione conjugate of 3-MH during fermentation was also investigated. The following paper in this issue (*21*) describes the development of methods for the quantitation of 3-MH conjugates and their application to grape and wine samples.

MATERIALS AND METHODS

Materials. All chromatographic solvents were high performance liquid chromatography (HPLC) grade and reaction solvents were of the highest commercial grade available (Rowe Scientific, Lonsdale, South Australia, Australia). All chemicals were analytical reagent grade unless otherwise stated, and octadecyl-functionalized silica gel (200−400 mesh, 16−18% carbon loading) was used for low-pressure C18 chromatography (Sigma-Aldrich, Castle Hill, New South Wales, Australia). Water was obtained from a Milli-Q purification system (Millipore, North Ryde, New South Wales, Australia). Diethyl ether and THF were distilled from sodium/benzophenone immediately prior to use. Stock solutions of standards were prepared volumetrically in Milli-Q water and stored at −20 °C until required. All prepared solutions were % v/v with the balance made up with Milli-Q water, unless otherwise specified. The d_8-ethyl hexenoate previously reported (*14*) was reduced to either d_8-hexenol or d_{10}-hexenol by use of lithium aluminum hydride and lithium aluminum deuteride, respectively. The corresponding d_8-hexenal and d_9-hexenal were obtained by Swern oxidation (*22*). d_{10}-Mesityl oxide was prepared as described previously (*23*).

NMR Analysis. Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded with a Varian Gemini 300 spectrometer operating at 300 MHz for proton and 75.5 MHz for carbon nuclei. Chemical shifts were recorded as δ values in parts per million (ppm). Spectra were acquired in chloroform-*d* or deuterium oxide (D_2O) at ambient temperature, and resonances were assigned by routine 2D correlation experiments.

High Resolution Mass Spectrometry (HRMS). Spectra were obtained on a Bruker micrOTOF-Q II with electrospray ionization (ESI) in positive mode. Samples dissolved in water at concentrations of approximately 2−10 mg/L were analyzed by flow injection.

Optical Rotations. Specific rotations were recorded with a PolAAr 21 polarimeter, referenced to the sodium D line (589 nm) at 20 °C, using the spectroscopic grade solvents specified and at the concentrations (*c*, g/100 mL) indicated. The measurements were carried out in a cell with a 1 dm path length.

γ-L-Glutamyl-*S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl]-L-cysteinylglycine (3-*S*-Glutathionylhexan-1-ol or Glut-3-MH) (5). Glutathione (2.0 g, 6.5 mmol) in 70% aqueous acetonitrile (19.2 mL) was treated with pyridine (1.03 g, 13 mmol) and (*E*)-2-hexenal (0.64 g, 6.5 mmol). After stirring for 45 h at room temperature, the reaction was diluted with water (40 mL) and the mixture was washed with dichloromethane. The aqueous layer was concentrated under reduced pressure at 50 °C to give a colorless solid (2.49 g, 85%). A portion of this product (1.42 g, 3.5 mmol) in ice water (16 mL) was treated with $NaBH_4$ (0.26 g, 7.0 mmol) and stirred at 0 °C for 3 h before being acidified with 1 M HCl to pH 3.1. The mixture was loaded onto a C18 reversed-phase low-pressure column (45 g, 15 cm × 2 cm bed) and eluted successively with 1% aqueous ethanol, 5% aqueous ethanol, 15% aqueous ethanol, and 40% aqueous ethanol. Fractions from the first two eluents were concentrated under reduced pressure to provide the title compound **5** as a white powder (0.488 g, 34%; 58% based on recovered aldehyde); mp 106−108 °C.

^{1}H NMR (δ *ppm, D_2O):* 4.52 (1H, m*, H_5); 3.93 (2H, s‡, H_2); 3.78 (1H, t‡, J = 6.5 Hz, H_{10}); 3.75−3.60 (2H, m*, $H_{2''}$); 2.93 (1H, dd‡, J = 13.8, 5.0 Hz, H_{12a}); 2.91−2.71 (2H, m*, $H_{1',12b}$); 2.51 (2H, app t‡, $J \sim 6.7$ Hz, H_8); 2.12 (2H, app q‡, $J \sim 6.7$ Hz, H_9); 1.90−1.31 (6H, m*, $H_{2',3',1''}$); 0.85, 0.84 (3H, 2 × t*, J = 7.1 Hz, $H_{4'}$).

^{13}C NMR (δ *ppm, D_2O):* 174.8‡, 173.7‡, 172.8‡, 172.7‡ ($C_{1,4,7,11}$); 59.4, 59.3 ($C_{2''}$); 53.9‡ (C_{10}); 53.7, 53.6 (C_5); 42.5, 42.4 ($C_{1'}$); 41.7‡ (C_2); 36.7, 36.6, 36.5, 36.4 ($C_{2',1''}$); 31.4‡ (C_8); 31.16, 31.15 (C_{12}); 26.2‡ (C_9); 19.5, 19.4 ($C_{3'}$); 13.32, 13.29 ($C_{4'}$).

ESI-HRMS (*m*/*z*). Calcd for $C_{16}H_{29}N_3NaO_7S^+$ $[M + Na]^+$, 430.1618; found, 430.1585.

ESI-MS (*m*/*z*). 408.0 $[M + H]^+$; 430.0 $[M + Na]^+$; 446.0 $[M + K]^+$.

In the NMR data, * indicates overlapping signals from the two isomers, and ‡ indicates that the signals from the two isomers coincided.

γ-L-Glutamyl-*S*-[(1*R*/*S*)-1-(2-hydroxyethyl-d_1)butyl-1,2,2,3,3,4,4,4-d_8]-L-cysteinylglycine (d_9-5). Glutathione (0.284 g, 0.92 mmol) was treated with d_9-(*E*)-2-hexenal (93.3 mg, 0.88 mmol) exactly as described above. Reduction of the carbonyl was effected with $NaBH_4$ (0.10 g, 2.68 mmol), and C18 chromatography as before provided the d_9-analogue of **5** as a white solid (160 mg, 44%). The spectral data of **d_9-5** were entirely consistent with those of the unlabeled compound, with the difference being the absence of signals corresponding to the labeled positions in the ^1H NMR spectrum.

ESI-HRMS (*m*/*z*). Calcd for $C_{16}H_{20}D_9N_3NaO_7S^+$ $[M + Na]^+$, 439.2180; found, 439.2176.

ESI-MS (*m*/*z*). 417.2 $[M + H]^+$; 439.0 $[M + Na]^+$; 455.0 $[M + K]^+$.

N-Boc-L-Glutathione Dimethyl Ester (8). L-Glutathione monohydrochloride was converted into the dimethyl ester as described by Falck et al. (*24*) to afford a white solid with spectroscopic parameters in close agreement with those reported previously (*24*).

$[\alpha]_D$ −10.0 (*c* 0.7, $CHCl_3$), lit. (*24*) −6.8 (*c* 1.3, $CHCl_3$).

N-Boc-γ-L-Glutamyl-*S*-[(1*R*/*S*)-1-(2-oxoethyl)butyl]-L-cysteinylglycine Dimethyl Ester (9). (*E*)-2-Hexenal (42 μL, 0.36 mmol) was added to a solution of the Boc-protected diester **8** (0.16 g, 0.36 mmol) and triethylamine (0.57 mL, 4.1 mmol) in dichloromethane (5 mL). After stirring at room temperature for 23 h, the mixture was diluted with dichloromethane (20 mL) and washed successively with 0.2 M HCl (10 mL), water (25 mL), and brine (25 mL). The organic phase was dried (Na_2SO_4), concentrated under reduced pressure, and purified on silica gel with dichloromethane/ethyl acetate (2:3 v/v) to give **9** as a white solid (30.5 mg, 16%) after solvent removal.

^1H NMR (δ *ppm, $CDCl_3$*): 9.73, 9.71 (1H, 2 × t, $J \sim 1.5$ Hz, CHO); 4.66 (1H, m*, H_5); 4.26 (1H, m*, H_{10}); 4.01, 3.99 (2H, 2s, H_2); 3.71 (6H, s‡, COOMe); 3.32−2.64 (5H, m*, $H_{1'',1',12}$); 2.38 (2H, m*, H_8); 2.20−1.84 (2H, m*, H_9); 1.60−1.30 (4H, m*, $H_{2',3'}$); 1.39 (9H, s‡, *t*-Bu); 0.89 (3H, 2 × t‡, $J \sim 7.0$ Hz, $H_{4'}$).

In the NMR data, * indicates overlapping signals from the two isomers, and ‡ indicates that the signals from the two isomers coincided.

N-Boc-γ-L-Glutamyl-*S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl]-L-cysteinylglycine Dimethyl Ester (10). Sodium borohydride (1.0 mg, 24 μmol) in methanol (1.2 mL) was added dropwise to a solution of the protected aldehyde **9** (25.6 mg, 48.9 μmol) in methanol (2 mL) at 0 °C. After 5 min, a second portion of sodium borohydride (1 mg) in methanol was added. After stirring for 3 h at room temperature, the solvent was removed under reduced pressure to afford the hexanol adduct **10** as a pale yellow oil (23.5 mg, 92%).

^1H NMR (δ *ppm, $CDCl_3$*): 4.66 (1H, m*, H_5); 4.30 (1H, m*, H_{10}); 4.10−3.92 (2H, m*, H_2); 3.88−3.62 (8H, m*, COOMe, $H_{2''}$); 3.00−2.82 (3H, m*, $H_{1',12}$); 2.36−2.31 (2H, m*, H_8); 2.20−1.30 (8H, m*, $H_{1'',2',3',9}$); 1.39 (9H, s‡, *t*-Bu); 0.88, 0.87 (3H, 2 × t*, $J \sim 7.0$ Hz, $H_{4'}$).

In the NMR data, * indicates overlapping signals from the two isomers, and ‡ indicates that the signals from the two isomers coincided.

The approximately 1:1 mixture of diastereomers was subjected to chromatography on silica with 100% ethyl acetate. This provided a pure fraction of one diastereomer, designated "front" because it eluted first, as well as mixtures of the "front" and "back" isomers.

"Front" diastereomer of 10.

$[\alpha]_D$ −25.0 (*c* 0.2, $CHCl_3$).

^1H NMR (δ *ppm, $CDCl_3$*): 4.66 (1H, app q, $J \sim 7.2$ Hz, H_5); 4.36 (1H, br s, H_{10}); 4.07 (1H, dd, $J = 18.3$, 5.7 Hz, H_{2a}); 3.99 (1H, dd, $J = 18.3$, 5.4 Hz, H_{2b}); 3.84−3.66 (2H, m, $H_{2''}$); 3.75, 3.74 (6H, 2 × s, COOMe); 3.05−2.92 (2H, m, $H_{1',12a}$); 2.85 (1H, dd, $J = 14.3$, 7.0 Hz, H_{12b}); 2.37 (2H, t, $J = 6.9$ Hz, H_8); 2.19 (1H, m, H_{9a}); 1.90 (1H, m, H_{9b}); 1.63−1.44 (6H, m, $H_{1'',2',3'}$); 1.43 (9H, s, *t*-Bu); 0.91 (3H, t, $J = 7.2$ Hz, $H_{4'}$).

"Front" Isomer of γ-L-Glutamyl-*S*-[1-(2-hydroxyethyl)butyl]-L-cysteinylglycine Monohydrochloride (5). Sodium hydroxide solution (0.68 M, 93 μL, 63 μmol) was added to a solution of the "front" isomer of **10** (34.0 mg, 63 μmol) in 90% aqueous methanol (3.2 mL), and the mixture was stirred at room temperature. After 4 h, a second equal portion of hydroxide solution was added, and stirring continued for a further 8 h. The mixture was diluted with water (50 mL) and extracted with dichloromethane (2 × 40 mL). The aqueous layer was then acidified with 1 M HCl to pH 2 and extracted with ethyl acetate (4 × 30 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure (26.1 mg, 90%), and a solution of trifluoroacetic acid (108 μL, 1.40 mmol) in dichloromethane (2.8 mL) was added. After stirring at room temperature for 10 h, the solvent was removed and the residue passed through a small column of Amberlite IRA400 chloride form ion-exchange resin, with water as the eluent. The water was removed under reduced pressure to yield pure "front" diastereomer of **5** as a white solid. Subsequent experiments showed this diastereomer to be the (*R*)-isomer of **5** (**Figure 2**).

$[\alpha]_D$ −1.5 (*c* 0.7, H_2O).

^1H NMR (δ *ppm, D_2O*): 4.52 (1H, m, H_5); 4.01−3.93 (3H, m, $H_{2,10}$); 3.69 (2H, t, $J = 6.6$ Hz, $H_{2''}$); 3.03 (1H, dd, $J = 13.8$, 5.1 Hz, H_{12a}); 2.91−2.78 (2H, m, $H_{1',12b}$); 2.55 (2H, m, H_8); 2.19 (2H, app q, $J \sim 7.0$ Hz, H_9); 1.90−1.34 (6H, m, $H_{1'',2',3'}$); 0.87 (3H, t, $J = 7.2$ Hz, $H_{4'}$).

^{13}C NMR (δ *ppm, D_2O*): 174.8, 173.7, 172.8, 172.7 ($C_{1,4,7,11}$); 59.5 ($C_{2''}$); 53.7 (C_{10}); 53.0 (C_5); 42.6 ($C_{1'}$); 41.3 (C_2); 36.7, 36.4 ($C_{2',1''}$); 31.2 (C_8); 31.1 (C_{12}); 25.9 (C_9); 19.5 ($C_{3'}$); 13.3 ($C_{4'}$).

ESI-HRMS (*m*/*z*). Calcd for $C_{16}H_{29}N_3NaO_7S^+$ $[M + Na]^+$, 430.1618; found, 430.1592.

***S*-[(1*R*/*S*)-1-(2-Hydroxyethyl)butyl-1,2,2,3,3,4,4,4-d_8]-L-cysteine (d_8-4).** This compound was prepared exactly as described in ref *14*, except that d_8-(*E*)-2-hexenal was used. The final product was obtained in 51% yield (based on d_8-(*E*)-2-hexenal) as a pair of diastereomers whose spectral data were entirely consistent with those of the unlabeled compound (*14*), with the only difference being the absence of signals corresponding to the labeled positions in the ^1H NMR spectrum.

ESI-HRMS (*m*/*z*). Calcd for $C_{19}H_{11}D_8NNaO_3S^+$ $[M + Na]^+$, 252.1477; found, 252.1461.

ESI-MS (*m*/*z*). 230.2 $[M + H]^+$.

***S*-(1,1-Di(methyl-d_3)-3-oxobutyl)-L-cysteine (d_6-6).** d_{10}-Mesityl oxide (2.00 g, 18.6 mmol) was added to L-cysteine (2.32 g, 18.6 mmol) and pyridine (2.79 g, 35.3 mmol) in water (41 mL), and the solution was stirred at room temperature. After 72 h, the mixture was acidified with 1 M HCl to approximately pH 3.5 and extracted with dichloromethane (2 × 25 mL). The aqueous residue was loaded onto a C18 column (200 g, 28 cm × 6 cm bed) and eluted successively with pH 3.5 HCl (150 mL) followed by 1% ethanol in pH 3.5 HCl (300 mL). The latter fraction was concentrated under reduced pressure to yield the d_6-labeled product as a white solid (3.52 g, 83%).

^1H NMR (δ *ppm, D_2O*): 3.92 (1H, dd, $J = 7.4$, 4.2 Hz, H_2); 3.18 (1H, dd, $J = 13.9$, 4.2 Hz, H_{3a}); 3.04 (1H, dd, $J = 13.9$, 7.4 Hz, H_{3b}); 2.70 (2H, br s, $H_{2'}$); 2.09 (3H, s, H4′).

ESI-HRMS (*m*/*z*). Calcd for $C_9H_{11}D_6NNaO_3S^+$ $[M + Na]^+$, 248.1197; found, 248.1188.

ESI-MS (*m*/*z*). 226.3 $[M + H]^+$; 248.3 $[M + Na]^+$; 264.1 $[M + K]^+$.

γ-L-Glutamyl-*S*-(1,1-di(methyl-d_3)-3-oxobutyl-2,4,4,4-d_4)-L-cysteinylglycine (d_{10}-7). This compound was prepared as described in ref *13*, except d_{10}-mesityl oxide was used. The final product was obtained in 81% yield (based on d_{10}-mesityl oxide) as a single isomer which was recrystallized from a solution of 90% aqueous ethanol. The spectral data were entirely consistent with those of the unlabeled compound (*13*), with the only difference being the absence of signals corresponding to the labeled positions in the ^1H NMR spectrum.

ESI-HRMS (*m*/*z*). Calcd for $C_{16}H_{17}D_{10}N_3NaO_7S^+$ $[M + Na]^+$, 438.2085; found, 438.2066.

ESI-MS (*m*/*z*). 415.8 $[M + H]^+$; 438.4 $[M + Na]^+$; 454.4 $[M + K]^+$.

Assessment of d_{10}-7 Stability under Acidic Conditions. **d_{10}-7** (approximately 30 mg) was dissolved in water (1 mL), yielding a solution of approximately pH 3.2, which was stirred at room temperature. After 1, 2, and 24 h, a small portion of this solution was removed, diluted to approximately 1 mg/L with water, and infused into the mass spectrometer. After 4 days of stirring at room temperature, another small aliquot was diluted with 40% aqueous acetonitrile containing 0.5% formic acid to approximately 1 mg/L, infused into the mass spectrometer, and reinfused after standing for 1 h and then 1 day.

Microbial Strains, Media, and Culture Conditions. The *Saccharomyces cerevisiae* strains used in this study were commercial wine yeast VIN13 (Anchor Yeast, Cape Town, South Africa) and VIN13 (*CSL1*) (AWRI 1655), a modified strain that expresses the *Escherichia coli tna*

A gene, which encodes a tryptophanase with strong cysteine-β-lyase activity (*25*). Yeast cultures were routinely grown at 28 °C in YPD (1% yeast extract, 2% peptone, 2% glucose). For the fermentation experiments, the following model fermentation media was used: 0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate (Jomar Biosciences, Kensington, South Australia, Australia), 4% glucose, and 0.1% ammonium sulfate.

Fermentation Conditions. Starter cultures were produced for the VIN13 and VIN13 (*CSL1*) yeast strains by growing cells in YPD medium for 24 h to stationary phase at 28 °C at 200 rpm. Cells were washed with water and resuspended in water, and the optical density at 600 nm was measured to estimate the cell concentration. The starter cultures were inoculated in equal cell numbers at 2×10^6 cells/mL into 250 mL of model fermentation media spiked with either the (*R*)-**5** diastereomer or a diastereomeric mixture of **4**, at final concentrations of 3 mg/L. Negative controls where no yeast cells were added were also included. Fermentations were conducted at 22 °C, without perturbation, in 250 mL conical flasks fitted with an air lock filled with sterile water and side arm septa for sampling. Samples for GC-MS and HPLC-MS analysis were taken after 5 days of fermentation and clarified by centrifugation (2500*g* for 5 min) to remove yeast cells, and supernatants were stored at −20 °C until further analysis. Residual sugar concentrations were determined using Bayer Clinitest Reagent Tablets (Winemaking Supplies and Services, Hallam, Victoria, Australia). All fermentations involving (*R*)-**5** were performed in triplicate.

Enzymatic Treatment of Fermentation Samples. Reactions were conducted at 28 °C for 16 h in a solution containing 600 μL of the clarified fermentation sample, 50 mM Tris-HCl buffer (pH 8.5), 3 units of γ-glutamyltranspeptidase (GGT) from equine kidney, type VI (Sigma-Aldrich), and 2 mM histidine in a total volume of 1 mL. Histidine was used in excess as the amino acid acceptor. At the end of the enzymatic reaction, samples were filtered using an Ultrafree-MC (10 kDa) centrifugal filter unit (Millipore, North Ryde, New South Wales, Australia) to remove the enzyme prior to HPLC-MS analysis.

Chiral GC-MS Analysis. Samples were analyzed with a Hewlett-Packard (HP) 6890N gas chromatograph fitted with a Gerstel MPS2 autosampler and coupled to a HP 5973N mass spectrometer. The details for sample preparation and analysis are identical to those described in ref *14*.

HPLC-MS Instrumentation. All HPLC-MS analyses were carried out with an Agilent 1200 instrument (Agilent, Forest Hill, Victoria, Australia) equipped with a binary pump and connected in series to a 4000 Q Trap hybrid tandem mass spectrometer with a TurboV source and TurboIonSpray probe (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Data acquisition and processing were performed using Applied Biosystems/MDS Sciex Analyst software (version 1.5). The full details for the HPLC and mass spectrometer parameters appear in the following paper in this issue (*21*).

Co-injection Experiments. Unspiked reference, ferment, and enzyme-treated samples were analyzed by HPLC-MS/MS using multiple reaction monitoring (MRM), and they were reanalyzed after addition of varying quantities of 1:1 diastereomeric mixtures of the pure reference compounds.

Semiquantitation Using Labeled Standards. Samples to be quantified were spiked with 1:1 diastereomeric mixtures of the relevant deuterated reference compounds and analyzed by HPLC-MS/MS using MRM. The response factors for the appropriate labeled standard diastereomers were considered to be equal to the response factors of the analytes, and the following simple calculation was used: (area analyte/area labeled standard) × concentration of labeled standard. The amount of labeled standard used was adjusted to give a similar peak height to the analyte of interest.

RESULTS AND DISCUSSION

Synthesis of Compounds. In order to further our research into the formation and reactivity of precursors to important wine thiols, we required samples of the 3-MH glutathione conjugate **5** in a stereochemically defined form, as well as the deuterium labeled analogue, and labeled analogues of **4**, **6**, and **7**. We had earlier reported the synthesis of the individual diastereomers (*R*)-**4** and (*S*)-**4** of the cysteine conjugate of 3-MH (*14*) and the unlabeled

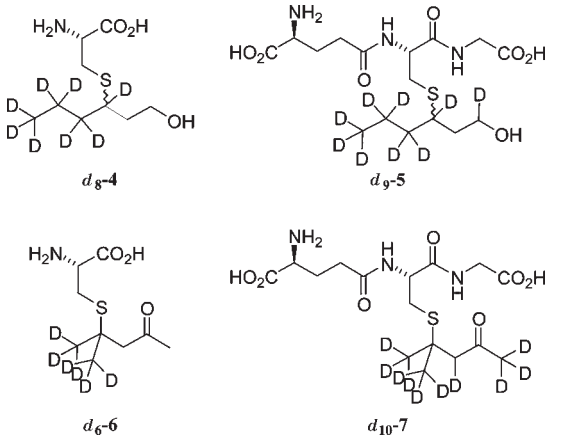

Figure 3. Position of labeling of thiol precursors prepared for use as internal standards.

glutathione conjugate **7** of 4-MMP (*13*). The labeled analogues of cysteine conjugates **4** and **6** were prepared by adapting published methods. Labeled Cys-3-MH (***d*$_8$-4**) (**Figure 3**) was prepared by addition of Boc-protected cysteine to d_8-(*E*)-2-hexenal followed by carbonyl reduction, as reported for the unlabeled compound by Pardon et al. (*14*). Labeled Cys-4-MMP (***d*$_6$-6**) (**Figure 3**) was formed by addition of cysteine to d_{10}-mesityl oxide as reported by Howell et al. (*26*) for the unlabeled analogue. It was observed that the conditions for product isolation (acidification with HCl, chromatography, and prolonged heating to remove solvent) for labeled Cys-4-MMP effected back-exchange of four of the deuterium labels (those alpha to the carbonyl group) to give the d_6-labeled compound ***d*$_6$-6**, as evidenced by the proton NMR and mass spectra. The remaining six labels are not activated toward substitution. This back-exchange was observed previously by Hebditch et al. (*27*), who also obtained the d_6-product from d_{10}-mesityl oxide.

The labeled analogue of **7** was prepared by adapting the method of Fedrizzi et al. (*13*), who prepared the unlabeled compound. In contrast to the isolation of labeled Cys-4-MMP (***d*$_6$-6**), however, labeled Glut-4-MMP (***d*$_{10}$-7**) (**Figure 3**) was obtained after recrystallization from aqueous ethanol. Under these neutral conditions, product isolation did not cause back-exchange of the labels and the product was isolated as the d_{10}-analogue of **7**. Nevertheless, the loss of some labels from the acidic workup and isolation conditions of the cysteine analogue raised concerns that partial or complete exchange could occur under the conditions of the HPLC-MS stable isotope dilution analysis (SIDA) method intended for quantitation of the conjugate. This concern was allayed, however, by stirring a solution of ***d*$_{10}$-7** in pH 3.2 solution for up to four days with no loss of label evident by infusion MS analysis. Additionally, after 4 days, an aliquot was diluted with 40% acetonitrile containing 0.5% formic acid (to emulate the mobile phase composition at the time this compound elutes by HPLC) and analyzed by infusion several times over 24 h, again with no loss of label.

Previously, we employed a strategy involving doubly protected cysteine to provide diastereomerically pure samples of each of the cysteine conjugates of 3-MH (*14*). We employed the same strategy (**Figure 4**) to prepare a pure sample of one of the individual glutathione conjugates, which was subsequently shown to possess the (*R*)-configuration at the alkyl chain stereocenter. This was termed the "front" diastereomer, based on the elution order of the protected form of the isomers of **5** from a silica column. Additionally, a diastereomeric mixture of Glut-3-MH (**5**) was prepared by the addition of glutathione to (*E*)-2-hexenal, followed

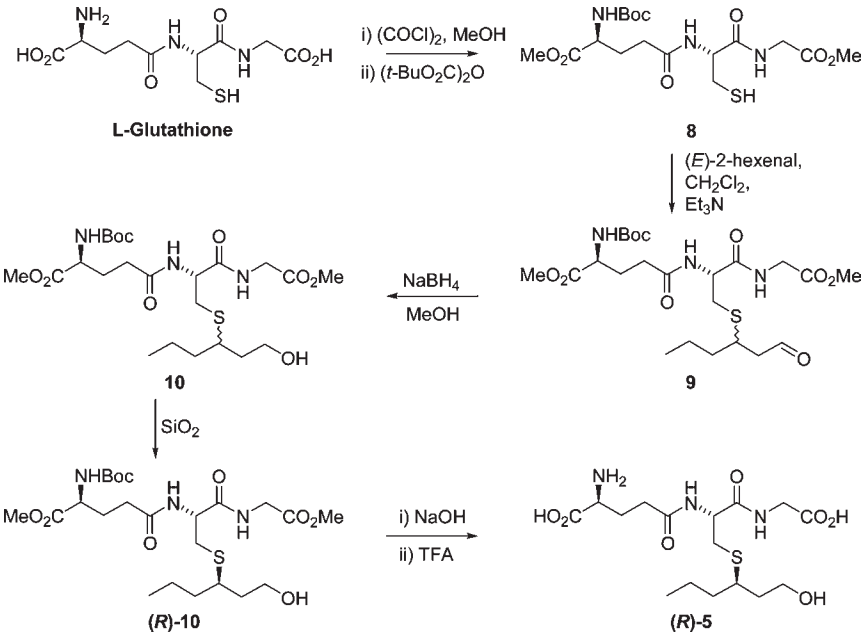

Figure 4. Synthetic route to the (*R*)-diastereomer of **5**.

by reduction with sodium borohydride and C18 purification. Using the same procedure, labeled Glut-3-MH (**d_9-5**) (**Figure 3**) was synthesized from glutathione and d_9-(*E*)-2-hexenal.

Fermentation of 4 and Purified "Front" Diastereomer of 5. Fermentations were undertaken using a commercial wine yeast strain (VIN13) and a modified VIN13 (*CSL1*) strain with overexpression of an *E. coli* gene (*tna* A) encoding for an enzyme with cysteine-β-lyase activity. The latter strain was chosen for its ability to release larger quantities of the volatile thiol **3** from its nonvolatile cysteinylated precursor **4**, during fermentation (*25*). Both yeasts were utilized to compare the effectiveness of the modified yeast strain with its parent in the release of **3** from the glutathione precursor **5**. Fermentation of the cysteine conjugates was undertaken as a means of checking that the ferments had carbon−sulfur lyase activity as expected; hence, these were not replicated. Samples were taken for analysis after 5 days of fermentation, when all the ferments were dry (residual sugar less than 2 g/L).

Chiral analysis and quantitation of the enantiomers of **3** in fermentation samples were performed according to the method described by Pardon et al. (*14*), which has a limit of detection of 5 μg/L. When the "front" diastereomer of **5** was fermented with the parent VIN13 yeast, neither enantiomer of the thiol **3** was detected (**Table 1**). Similarly, **3** was not detected in the control (unfermented) samples. However, when the "front" diastereomer was subjected to the VIN13 (*CSL1*) yeast, an average of 31 μg/L of (*R*)-**3** was detected for three replicate fermentations. There was no evidence for formation of the opposite (*S*)-enantiomer from fermentation of the single "front" diastereomer. From this result it can be inferred that glutathione conjugate **5** present in grape juice is a precursor to 3-MH in wine, although the conversions, in this case, were only in the order of 3%. Also, the chiral analysis allowed the alkyl chain stereocenter for the "front" diastereomer of **5** to be assigned as the (*R*)-configuration.

The concentrations of (*R*)-**3** and (*S*)-**3** present after fermentation of a 1:1 diastereomeric mixture of **4** fermented with the modified VIN13 (*CSL1*) yeast were 53 and 141 μg/L, respectively (**Table 1**). This result demonstrated that the VIN13 (*CSL1*) strain was more efficacious in effecting the release of volatile thiols from their conjugate precursors than the parent strain. Interestingly,

Table 1. Formation of (*R*)-**3** and (*S*)-**3** after Fermentation with Either the Parent VIN13 or the Modified VIN13 (*CSL1*) Yeast

	conjugate	thiol quantified (μg/L)	
yeast	fermented	(*R*)-**3**	(*S*)-**3**
control	**4** (1:1)[a]	nd[c]	nd
VIN13	**4** (1:1)[a]	nd	nd
VIN13 (*CSL1*)	**4** (1:1)[a]	53	141
control	(*R*)-**5**[b]	nd	nd
VIN13	(*R*)-**5**[b]	nd	nd
VIN13 (*CSL1*)	(*R*)-**5**[b]	31.0 ± 1.0	nd

[a] Single fermentation. [b] Mean of triplicate fermentations. [c] nd, not detected (limit of detection 5 μg/L).

the modified yeast preferentially consumed the (*S*)-**4** diastereomer, resulting in an (*R*)-**3**/(*S*)-**3** ratio of about 1:3. This diastereoselectivity was probably due to overexpression of tryptophanase by the yeast, which has been reported by Wakabayashi et al. (*28*) in the formation of an approximate 1:2 mixture of (*R*)-**3**/(*S*)-**3** from a diastereomeric mixture of **4**. The conversion of precursor **4** into thiol **3** was approximately 14% with the modified yeast. Again, neither enantiomer of **3** was detected with the parent yeast.

HPLC-MS/MS Analysis of Precursors. We had previously described the synthesis and stereochemical assignment of the individual diastereomers of the cysteine conjugate of 3-MH (**4**) (*14*). Using the HPLC conditions described in the following paper in this issue (*21*), we have now been able to resolve a 1:1 diastereomeric mixture of **4** and show that the first and second eluting isomers were the (*S*)- and (*R*)-forms, respectively (e.g., **Figure 5A**). Similarly, the two glutathione diastereomers **5** were also separated under the same conditions, and again, the "front" diastereomer (i.e., (*R*)-isomer of **5**) was the second eluting diastereomer (e.g., **Figure 5B**). HPLC-MS/MS analysis (*21*) of the "front" isomer of **5** obtained from purification on silica showed an isomeric purity of $>97\%$, with $<3\%$ of the corresponding (*S*)-isomer present (**Figure 5C**).

Fermenting the purified (*R*)-**5** diastereomer with VIN13 (*CSL1*) led to a reduction in the amount of (*R*)-**5** remaining in the media and formation of the corresponding (*R*)-**4** diastereomer

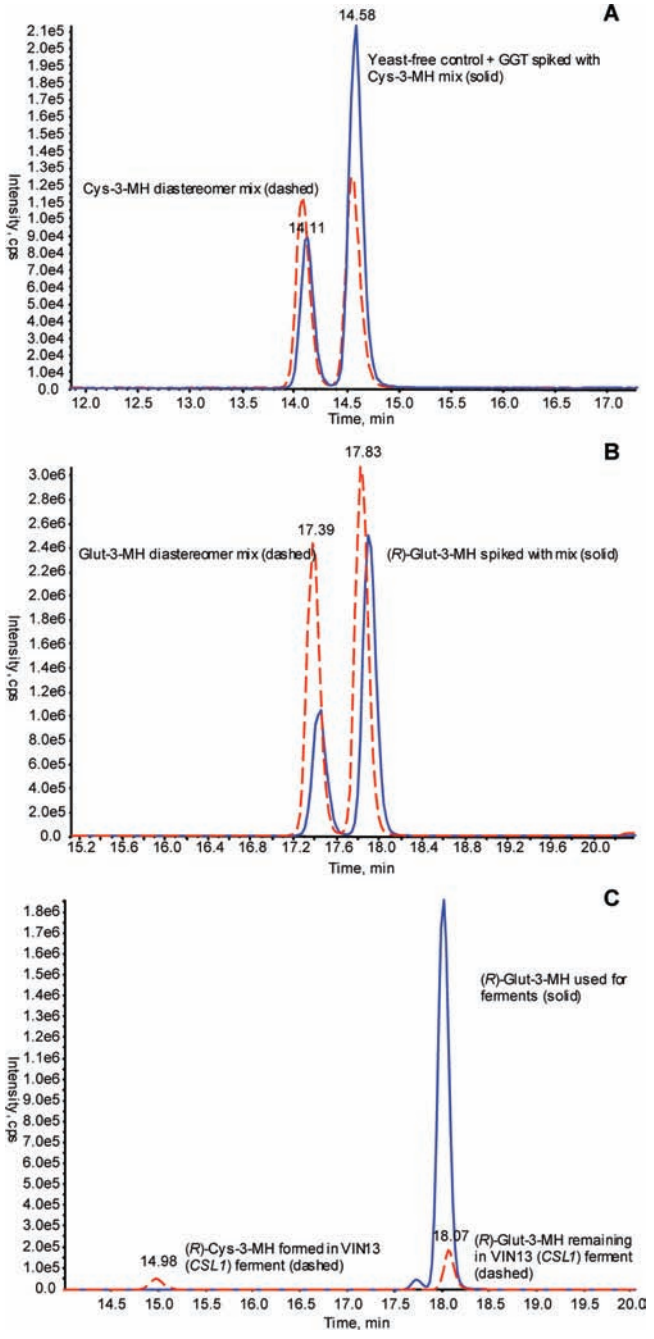

Figure 5. Overlaid MRM total ion chromatograms of (**A**) a diastereomeric mixture of **4** spiking solution (dashed line) and yeast-free control fermentation sample treated with GGT and spiked with the diastereomeric mixture of **4** (solid), showing enhancement of the right-hand peak at 14.6 min in the solid trace, (**B**) a diastereomeric mixture of **5** spiking solution (dashed line) and pure (*R*)-**5** diastereomer spiked with the diastereomeric mixture of **5** (solid line), showing enhancement of the right-hand peak at 17.8 min in the solid trace, and (**C**) synthesized (*R*)-**5** diastereomer (>97% diastereomeric excess by HPLC) used for fermentations (solid), and VIN13 (*CSL1*) fermentation sample (dashed) showing (*R*)-**4** at 15 min formed during fermentation and residual (*R*)-**5** at 18 min.

(**Figure 5C**). No diastereomers of **4** were detected in the control experiments where no yeast was added, indicating there was no chemical hydrolysis of the glutamate and glycine residues of the glutathione conjugate (data not shown). Considering that the modified yeast VIN13 (*CSL1*) was constructed to release thiols from cysteine conjugates (*26*), it is possible that the yeast first converted the glutathione conjugate to the corresponding cysteine conjugate, which was then cleaved to yield the free thiol (*R*)-**3**. It is also possible that dipeptide conjugates of 3-MH were formed during fermentation, but as the MRM conditions used to measure thiol conjugates (*21*) were only developed for compounds for which we had authentic samples (and were therefore able to record their mass spectra), any dipeptide intermediates that might have been present would not have been detected.

Table 2. Amount of Precursor in Selected Samples Quantified Using Labeled Internal Standards

	precursor quantified (μg/L)	
sample	(*R*)-Cys-3-MH (%)[d]	(*R*)-Glut-3-MH
VIN13 (*CSL1*)[a]	55 (3.7%)	480
VIN13 (*CSL1*) + GGT[b]	350 (23.5%)	10
yeast-free control + GGT[c]	599 (40.2%)	10

[a] 3 mg/L (*R*)-Glut-3-MH·HCl fermented with VIN13 (*CSL1*) yeast. [b] 3 mg/L (*R*)-Glut-3-MH·HCl fermented with VIN13 (*CSL1*) yeast then treated with GGT. [c] 3 mg/L (*R*)-Glut-3-MH·HCl control treated with GGT. [d] As a molar % of (*R*)-Glut-3-MH added.

γ-Glutamyltranspeptidase (GGT) is an enzyme that catalyzes the transfer of the γ-glutamyl group of glutathione (GSH) or GSH conjugates to an acceptor amino acid. Cysteine conjugates may also result after enzymatic hydrolysis of glutamyl and glycine residues or hydrolysis and transfer of residues to acceptor amino acids (*29*). With commercially available crude enzyme preparations of GGT, cysteine conjugate formation is likely due to other peptidase impurities (*12*). In agreement with this observation, we found that when samples containing the "front" diastereomer of **5** were treated with GGT, the (*R*)-isomer of **4** was detected but the (*S*)-isomer was not. These fermentation and enzyme conversions of "front" **5** to (*R*)-**4** confirmed the assignment of the "front" isomer of **5** as the (*R*)-diastereomer.

Quantitation of (*R*)-4 and (*R*)-5 Remaining after Treatments. Diastereomeric mixtures of labeled standards were used to quantify the (*R*)-**5** and (*R*)-**4** remaining after fermentation and/or GGT treatment (**Table 2**). For the VIN13 (*CSL1*) ferment, 480 ppb of (*R*)-**5** and 55 μg/L of (*R*)-**4** remained after fermentation. These values obviously do not account for the initial amount of (*R*)-**5** added to the ferments (3 mg/L as the monohydrochloride salt), as some was converted to the free thiol, and an indeterminate amount might have been converted to dipeptide conjugates of 3-MH and/or other breakdown products of the glutathione precursor.

Quantitation of the precursors after treatment of the VIN13 (*CSL1*) ferment and yeast-free control samples with GGT revealed that only a small amount (10 μg/L) of (*R*)-**5** remained and a substantial amount of (*R*)-**4** was formed (350 and 599 μg/L for enzyme treated VIN13 (*CSL1*) and control samples, respectively). The molar yields of (*R*)-Cys-3-MH (**Table 2**) show that this was not formed from stereospecific conversion of trace impurities in the synthetic (*R*)-glutathione conjugate **5**.

Synthesis of a range of compounds related to important wine thiols has enabled the development of an HPLC-MS/MS method for the quantitation of varietal thiol precursors, described in the following paper in this issue (*21*). It has also provided material for fermentation experiments to evaluate the effect of yeasts on the release of thiols such as 3-MH and to relate precursor and free thiol stereochemistry. Fermentation of a pure Glut-3-MH diastereomer yielded the corresponding enantiomer of 3-MH,

as determined by chiral GC-MS, along with the related Cys-3-MH intermediate. In the model fermentation media used in this study, we have shown that Glut-3-MH can liberate 3-MH during fermentation and that the cysteine conjugate is formed in the process, which suggests this might also occur during fermentation of a grape juice/must. This furthers our understanding of the nature of wine thiol precursors and allows additional studies to be undertaken.

ABBREVIATIONS USED

4-MMP, 4-mercapto-4-methylpentan-2-one; 3-MHA, 3-mercaptohexyl acetate; 3-MH, 3-mercaptohexan-1-ol; Glut-3-MH, 3-*S*-glutathionylhexan-1-ol; GGT, γ-glutamyltranspeptidase; MRM, multiple reaction monitoring; Cys-3-MH, 3-*S*-cysteinylhexan-1-ol; Cys-4-MMP, 4-*S*-cysteinyl-4-methylpentan-2-one; SIDA, stable isotope dilution analysis.

ACKNOWLEDGMENT

We thank Markus Herderich for manuscript evaluation and helpful suggestions.

LITERATURE CITED

(1) Fischer, U. Wine Aroma. In *Flavours and Fragrances*; Springer: Berlin Heidelberg, 2007; pp 241−267.
(2) Darriet, P.; Tominaga, T.; Lavigne, V.; Boidron, J.-N.; Dubourdieu, D. Identification of a powerful aromatic component of *Vitis vinifera* L. var. Sauvignon wines: 4-mercapto-4-methylpentan-2-one. *Flavour Fragr. J.* **1995**, *10*, 385–392.
(3) Tominaga, T.; Darriet, P.; Dubourdieu, D. Identification of 3-mercaptohexyl acetate in Sauvignon wine, a powerful aromatic compound exhibiting box-tree odor. *Vitis* **1996**, *35*, 207–210.
(4) Tominaga, T.; Furrer, A.; Henry, R.; Dubourdieu, D. Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. *Flavour Fragr. J.* **1998**, *13*, 159–162.
(5) Tominaga, T.; Niclass, Y.; Frérot, E.; Dubourdieu, D. Stereoisomeric distribution of 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate in dry and sweet white wines made from *Vitis vinifera* (Var. Sauvignon Blanc and Semillon). *J. Agric. Food Chem.* **2006**, *54*, 7251–7255.
(6) Aznar, M.; López, R.; Cacho, J. F.; Ferreira, V. Identification and quantification of impact odorants of aged red wines from Rioja. GC-Olfactometry, quantitative GC-MS, and odor evaluation of HPLC fractions. *J. Agric. Food Chem.* **2001**, *49*, 2924–2929.
(7) Murat, M.-L.; Masneuf, I.; Darriet, P.; Lavigne, V.; Tominaga, T.; Dubourdieu, D. Effect of *Saccharomyces cerevisiae* yeast strains on the liberation of volatile thiols in Sauvignon blanc wine. *Am. J. Enol. Vitic.* **2001**, *52*, 136–139.
(8) Tominaga, T.; Baltenweck-Guyot, R.; Peyrot des Gachons, C.; Dubourdieu, D. Contribution of volatile thiols to the aromas of white wines made from several *Vitis vinifera* grape varieties. *Am. J. Enol. Vitic.* **2000**, *51*, 178–181.
(9) Tominaga, T.; Murat, M.-L.; Dubourdieu, D. Development of a method for analyzing the volatile thiols involved in the characteristic aroma of wines made from *Vitis vinifera* L. Cv. Sauvignon Blanc. *J. Agric. Food Chem.* **1998**, *46*, 1044–1048.
(10) Guth, H. Quantitation and sensory studies of character impact odorants of different white wine varieties. *J. Agric. Food Chem.* **1997**, *45*, 3027–3032.
(11) 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.
(12) Peyrot des Gachons, C.; Tominaga, T.; Dubourdieu, D. Sulfur aroma precursor present in *S*-glutathione conjugate form: identification of *S*-3-(hexan-1-ol)-glutathione in must from *Vitis vinifera* L. cv. Sauvignon Blanc. *J. Agric. Food Chem.* **2002**, *50*, 4076–4079.
(13) Fedrizzi, B.; Pardon, K. H.; Sefton, M. A.; Elsey, G. M.; Jeffery, D. W. First identification of 4-*S*-glutathionyl-4-methylpentan-2-one, a potential precursor of 4-mercapto-4-methylpentan-2-one, in Sauvignon Blanc juice. *J. Agric. Food Chem.* **2009**, *57*, 991–995.
(14) Pardon, K. H.; Graney, S. D.; Capone, D. L.; Swiegers, J. H.; Sefton, M. A.; Elsey, G. M. Synthesis of the individual diastereomers of the cysteine conjugate of 3-mercaptohexanol (3-MH). *J. Agric. Food Chem.* **2008**, *56*, 3758–3763.
(15) Subileau, M.; Schneider, R.; Salmon, J.-M.; Degryse, E. New insights on 3-mercaptohexanol (3MH) biogenesis in Sauvignon Blanc wines: Cys-3MH and (*E*)-hexen-2-al are not the major precursors. *J. Agric. Food Chem.* **2008**, *56*, 9230–9235.
(16) Adams, D. O.; Liyanage, C. Glutathione increases in grape berries at the onset of ripening. *Am. J. Enol. Vitic.* **1993**, *44*, 333–338.
(17) Marrs, K. A. The functions and regulation of glutathione *S*-transferases in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1996**, *47*, 127–158.
(18) Edwards, R.; Dixon, D. P.; Walbot, V. Plant glutathione *S*-transferases: enzymes with multiple functions in sickness and in health. *Trends Plant Sci.* **2000**, *5*, 193–198.
(19) Alscher, R. G. Biosynthesis and antioxidant function of glutathione in plants. *Physiologia Plantarum* **1989**, *77*, 457–464.
(20) Noctor, G.; Foyer, C. H. Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 249–279.
(21) Capone, D. L.; Sefton, M. A.; Hayasaka, Y.; Jeffery, D. W. Analysis of precursors to wine odorant 3-mercaptohexan-1-ol using HPLC-MS/MS: resolution and quantitation of diastereomers of 3-*S*-cysteinylhexan-1-ol and 3-*S*-glutathionylhexan-1-ol. *J. Agric. Food Chem.* **2010**, *58*, http://dx.doi.org/10.1021/jf903720w.
(22) Mancuso, A. J.; Swern, D. Activated dimethyl sulfoxide—useful reagents for synthesis. *Synthesis* **1981**, 165–185.
(23) Kotseridis, Y.; Ray, J.-L.; Augier, C.; Baumes, R. Quantitative determination of sulfur containing wine odorants at sub-ppb levels. 1. Synthesis of the deuterated analogues. *J. Agric. Food Chem.* **2000**, *48*, 5819–5823.
(24) Falck, J. R.; Sangras, B.; Capdevila, J. H. Preparation of *N*-^{t}Boc L-glutathione dimethyl and di-tert-butyl esters: versatile synthetic building blocks. *Bioorg. Med. Chem.* **2007**, *15*, 1062–1066.
(25) Swiegers, J. H.; Capone, D. L.; Pardon, K. H.; Elsey, G. M.; Sefton, M. A.; Francis, I. L.; Pretorius, I. S. Engineering volatile thiol release in *Saccharomyces cerevisiae* for improved wine aroma. *Yeast* **2007**, *24*, 561–574.
(26) Howell, K. S.; Swiegers, J. H.; Elsey, G. M.; Siebert, T. E.; Bartowsky, E. J.; Fleet, G. H.; Pretorius, I. S.; de Barros Lopes, M. A. Variation in 4-mercapto-4-methyl-pentan-2-one release by *Saccharomyces cerevisiae* commercial wine strains. *FEMS Microbiol. Lett.* **2004**, *240*, 125–129.
(27) Hebditch, K. R.; Nicolau, L.; Brimble, M. A. Synthesis of isotopically labelled thiol volatiles and cysteine conjugates for quantification of Sauvignon Blanc wine. *J. Label Compd. Radiopharm.* **2007**, *50*, 237–243.
(28) Wakabayashi, H.; Wakabayashi, M.; Eisenreich, W.; Engel, K.-H. Stereochemical course of the generation of 3-mercaptohexanal and 3-mercaptohexanol by β-lyase-catalyzed cleavage of cysteine conjugates. *J. Agric. Food Chem.* **2004**, *52*, 110–116.
(29) McIntyre, T. M.; Curthoys, N. P. The interorgan metabolism of glutathione. *Int. J. Biochem.* **1980**, *12*, 545–551.

Received for review October 23, 2009. Revised manuscript received December 23, 2009. Accepted December 26, 2009. The Australian Wine Research Institute, a member of the Wine Innovation Cluster in Adelaide, is supported by Australia's grapegrowers and winemakers through their investment body, the Grape and Wine Research Development Corporation, with matching funds from the Australian government.