JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Identification and Quantitation of 3-*S*-Cysteinylglycinehexan-1-ol (Cysgly-3-MH) in Sauvignon blanc Grape Juice by HPLC-MS/MS

Dimitra L. Capone,[†,‡] Kevin H. Pardon,[†] Antonio G. Cordente,[†] and David W. Jeffery*[,‡]

[†]The Australian Wine Research Institute, P.O. Box 197, Glen Osmond, South Australia 5064, Australia

[‡]School of Agriculture, Food and Wine, Waite Research Institute, The University of Adelaide, P.M.B. 1, Glen Osmond, South Australia 5064, Australia

ABSTRACT: Precursors to varietal wine thiols are a key area of grape and wine research. Several such precursors, in the form of odorless conjugates, have been closely studied in recent years. A new conjugate has now been identified as 3-*S*-cysteinylglycinehexan-1-ol (Cysgly-3-MH), being the dipeptide intermediate between cysteine and glutathione precursors of tropical thiol 3-mercaptohexan-1-ol (3-MH). Authentic Cysgly-3-MH was produced via enzymatic transformation of the glutathione conjugate and used to verify the presence of both diastereomers of Cysgly-3-MH in Sauvignon blanc juice extracts. Cysgly-3-MH was added into our HPLC-MS/MS precursor method, and the validated method was used to quantify this new analyte in a selection of Sauvignon blanc juice extracts. Cysgly-3-MH was found in the highest concentrations (10−28.5 μg/L combined diastereomer total) in extracts from berries that had been machine-harvested and transported for 800 km in 12 h. This dipeptide conjugate was much less abundant than the glutathione and cysteine conjugates in the samples studied. On the basis of the results, the new cysteinylglycine conjugate of 3-MH seemingly has a short existence as an intermediate precursor, which may explain why it has not been identified as a natural juice component until now.

KEYWORDS: wine aroma, 3-mercaptohexan-1-ol, wine thiol precursors, HPLC-MS/MS, identification, dipeptide intermediate

INTRODUCTION

Varietal thiols such as 3-mercaptohexan-1-ol (3-MH) and 4-mercapto-4-methylpentan-2-one are undoubtedly important to the characteristic aromas of wines such as Sauvignon blanc. These compounds are found as odorless precursors (Figure 1) in grape juices and musts, bound to cysteine or glutathione (GSH), with release of the volatile thiols requiring enzymatic reactions such as those which occur during fermentation. Because of the biological role of GSH in plants,[1−4] it is understandable that incorporation of volatile thiol structures in the form of *S*-conjugates is a phenomenon that is not limited to grape products. Cysteine conjugates that lead to volatile thiols have been reported in passion fruit juice,[5] bell pepper,[6,7] and native fruit related to citrus,[8] and new conjugates have continued to be identified in grape musts such as those derived from botrytized fruit.[9]

Naturally, the presence of the precursors should relate in some way to wine thiol concentrations,[10,11] but there appears to be a large disparity between the amount of 3-MH determined in wine and the disappearance of precursors present in the juice.[12] Ideally we would like to account for the evolution of these sulfur-containing molecules between juice and wine to reconcile precursor and volatile thiol concentrations, so viticultural and winemaking effects can be fully examined and exploited. To do this, however, there needs to be the continued identification of new compounds in grape juices and wines. For instance, the presence of dipeptide intermediates was proffered in Grant-Preece et al. to account for the loss of material during fermentation of 3-*S*-glutathionylhexan-1-ol (Glut-3-MH **3**) to release 3-*S*-cysteinylhexan-1-ol (Cys-3-MH **1**) and 3-MH, but their natural existence was not verified.[13]

The breakdown of GSH into its cysteine counterpart in biological systems is well-known,[14] and a number of authors have concluded, quite rightly it seems, that the 3-*S*-cysteinylglycinehexan-1-ol (Cysgly-3-MH **2**, Figure 1) intermediate should also feature in this pathway in grapes.[4,11,15] Research by Peyrot des Gachons et al. indicated the presence of Cysgly-3-MH **2** only when a must was passed down a column containing γ-glutamyltranspeptidase (GGT), an enzyme responsible for cleaving the glutamate from GSH to yield cysteinylglycine, yet they could not determine the presence of **2** in the must without enzyme treatment.[16] Kobayashi et al. also examined aspects related to thiol conjugates as well as grapevine enzymes including GGT, but were unable to verify the natural presence of **2** on two occasions.[4,11]

We undertook experiments to address the proposition that Cysgly-3-MH **2** was indeed present in Sauvignon blanc juice and extended our precursor method[17] to include this analyte. Results from the evaluation of an array of Sauvignon blanc juice samples by HPLC-MS/MS are also presented, highlighting the fleeting nature of this newly identified conjugate.

MATERIALS AND METHODS

Materials. Isotopically labeled and unlabeled compounds were previously synthesized according to the procedures of Grant-Preece et al.[13]

Received: June 27, 2011
Revised: September 8, 2011
Accepted: September 10, 2011
Published: September 26, 2011

dx.doi.org/10.1021/jf202543z | *J. Agric. Food Chem.* 2011, 59, 11204–11210

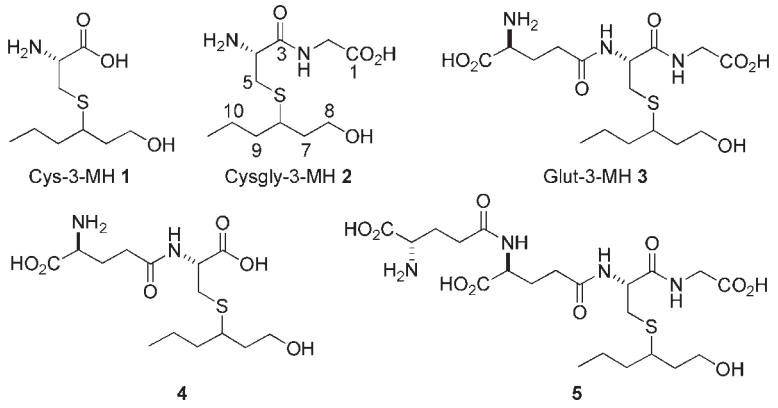

Figure 1. Structures of 3-mercaptohexan-1-ol-related compounds 3-*S*-cysteinylhexan-1-ol (**1**), 3-*S*-cysteinylglycinehexan-1-ol (**2**), and 3-*S*-glutathionylhexan-1-ol (**3**) along with putative glutamate derivatives **4** and **5**. Atom numbering shown for 3-*S*-cysteinylglycinehexan-1-ol (Cysgly-3-MH **2**) relates to the NMR peak assignments.

and Pardon et al.[18] The compounds used were *S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl]-L-cysteine (Cys-3-MH **1**); *S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl-1,2,2,3,3,4,4,4-d_8]-L-cysteine (d_8-Cys-3-MH); γ-L-glutamyl-*S*-[(1*R*/*S*)-1-(2-hydroxyethyl)butyl]-L-cysteinylglycine (Glut-3-MH **3**); and γ-L-glutamyl-*S*-[(1*R*/*S*)-1-(2-hydroxyethyl-2-d_1)butyl-1,2,2,3,3,4,4,4-d_8]-L-cysteinylglycine (d_9-Glut-3-MH). Stock solutions of standards were prepared volumetrically in Milli-Q water, and working solutions were stored at 4 °C until required. Solvents were of Merck HPLC grade for HPLC-MS analysis (Rowe Scientific, Lonsdale, SA, Australia). All chemicals were of analytical reagent grade unless otherwise stated, and water was obtained from a Milli-Q purification system (Millipore, North Ryde, NSW, Australia). Merck solvents and Merck or BDH reagents were purchased from Rowe Scientific, whereas other chemicals and octadecyl-functionalized silica gel (200−400 mesh, 16−18% carbon loading) used for low-pressure C18 chromatography were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia).

Nuclear Magnetic Resonance (NMR) Analysis. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded with a Bruker spectrometer operating at 400 MHz. Chemical shifts were recorded as δ values in parts per million (ppm), and resonances were assigned by routine 2D correlation experiments. Spectra were acquired in deuterium oxide (D_2O) at ambient temperature with a small quantity of acetonitrile added as an internal reference (2.06 and 1.47 ppm for ^{1}H and ^{13}C spectra, respectively).

High-Resolution Mass Spectrometry (HRMS). Spectra were obtained on a Bruker microTOF-Q II with atmospheric pressure chemical ionization (APCI) in positive mode. Samples dissolved in water at concentrations of approximately 1−2 mg/L were analyzed by flow injection.

Melting Points. A Buchi Melting Point B-540 unit was used, and melting points were uncorrected.

Enzymatic Treatment of Glut-3-MH 3. Reactions were conducted at 28 or 37 °C for 90 min or 24 h in a solution containing 3.3 mg of **3** (8 mM), 50 mM Tris-HCl buffer (pH 8.5), 5 units of GGT from equine kidney (type VI, Sigma-Aldrich), and histidine in a total volume of 1 mL. Histidine was used as the amino acid acceptor at different concentrations relative to **3** (0, 8, 16, 40, and 80 mM). Histidine stock was adjusted to pH 8.5 before use with 5 M KOH. At the end of the enzymatic reaction, samples were filtered using an Ultrafree-MC (10 kDa) centrifugal filter unit (Millipore, North Ryde, NSW, Australia) to remove the enzyme prior to HPLC-MS analysis.

Preparation of *S*-[(1*R*/*S*)-1-(2-Hydroxyethyl)-1-butyl]-L-cysteinylglycine (Cysgly-3-MH 2). A solution containing **3** (64.0 mg, 0.157 mmol, 8 mM) and Tris-HCl buffer (pH 8.5) was treated as above with GGT and 80 mM histidine for 24 h at 28 °C and centrifuge filtered. The solution was adjusted to pH 3.2 using 2 M HCl, and the solvent was removed under reduced pressure. The crude, white residue was purified by low-pressure C18 reversed-phase chromatography (column diameter, 15 mm; bed height, 300 mm). Elution was performed with a gradient from water to 20% ethanol/water, and the fractions that gave a positive reaction to ninhydrin when spotted on a silica TLC plate were assessed by HPLC-MS. Combination of appropriate fractions and concentration under reduced pressure afforded a diastereomeric mixture of Cysgly-3-MH **2** as a white solid (30.5 mg, 70%), which was >95% pure (HPLC-MS), mp >200 °C (decomposed above 134.5 °C).

^{1}H NMR δ 4.09 (1H, bs*, H_5); 3.92 (1H, d‡, J = 17.2 Hz, H_{2a}); 3.78−3.71 (2H, m*, H_{10}); 3.70 (1H, d‡, J = 17.2 Hz, H_{2b}); 3.11−2.97 (2H, m*, H_6); 2.92−2.83 (1H, m*, H_8); 1.92−1.83 (1 H, m*, H_{9a}); 1.78−1.69 (1H, m*, H_{9b}); 1.65−1.51 (2H, m*, H_{11}); 1.48−1.35 (2H, m*, H_{12}); 0.90 (3H, t‡, J = 7.2, Hz, H_{13}). ^{13}C NMR δ 176.6‡ (C_1); 170.1‡ (C_4); 59.71, 59.73 (C_{10}); 53.8‡ (C_5); 44.0‡ (C_2); 43.25, 43.26 (C_8); 37.0‡ (C_{11}); 36.7, 36.8 (C_9); 31.5, 31.6 (C_6); 19.85, 19.91 (C_{12}); 13.8‡ (C_{13}). APCI-HRMS (m/z) calcd for $C_{11}H_{23}N_2O_4S^+$ ([M + H]$^+$), 279.1373; found, 279.1386. For the NMR data, * indicates overlapping signals from the two diastereomers, and ‡ indicates that the signals from the two diastereomers coincided. Atom numbering for the NMR assignments of **2** is shown in Figure 1. NMR data for this compound have been reported previously and are generally in good accord with our findings, although the signal for H_6 was not reported in that publication.[19]

Grape Samples. Sauvignon blanc grapes used in this the study were commercially transported samples with antioxidant additions[20] and samples from five Adelaide Hills clones[12] described previously. For the transport study, replicated treatments (in 2.5 tonne picking bins) involved machine-harvested grapes dosed with various levels of SO_2 (added as potassium metabisulfite) and/or ascorbic acid in the vineyard after harvest. The bins were sampled immediately (no transport) and again at a winery after being transported approximately 800 km in around 12 h (transport). For the Adelaide Hills clone study, samples were hand-harvested approximately every 2 weeks from veraison to harvest from five clones (Q9720, H5V10, F7V7, 5385, F4V6) located in consecutive rows in a single vineyard. Full details of the samples and preparation methods for these trials are described elsewhere.[12,20]

HPLC-MS Analysis of 3-MH Conjugates. *Sample Preparation.* Extracts of homogenates were prepared for HPLC-MS/MS analysis according to the procedures outlined in Capone et al.[12] The existing labeled internal standards (d_8-Cys-3-MH and d_9-Glut-3-MH) were assessed for calibration of (*R*/*S*)-Cysgly-3-MH **2**. Reconstituted sample extracts were stored at −20 °C after initial analysis and could be reanalyzed without detrimental effects for at least 12 months.

HPLC-MS Instrumentation. All HPLC-MS and MS/MS analyses were carried with an Agilent 1200 instrument (Agilent, Forest Hill, VIC, Australia) equipped with a binary pump and connected in series to a 4000 Q Trap hybrid tandem mass spectrometer with TurboV source and TurboIonSpray probe (Applied Biosystems/MDS Sciex, Concord, ON, Canada) as previously described.[17]

Mass Spectrometer Conditions. Multiple reaction monitoring (MRM) was performed under the conditions described previously.[17] The mass spectrometer parameters were modified to incorporate Cysgly-3-MH **2** mass transitions (m/z 279.4 → 262.2, 279.4 → 162.1, and 279.4 → 149.0) based on infusion of the synthetic reference material. For enhanced product ion (EPI) spectra, Q1 had unit resolution, the scan rate was 1000 Da/s, dynamic fill time was selected for the ion trap, and spectra were recorded between m/z 50 and 285 for a parent ion of m/z 279.2. The collision energy spread (CES) was set at 10, and the remaining parameters were the same as for MRM experiments. Selected ion monitoring (SIM) was undertaken with the same mass spectrometer source parameters used for MRM (except declustering potential, which was increased to 60 V) while monitoring the following ions: m/z 537.3 for **5**, m/z 408.2 for Glut-3-MH **3**, m/z 351.2 for **4**, m/z 279.1 for Cysgly-3-MH **2**, and m/z 222.1 for Cys-3-MH **1**.

Co-injection Experiments. An extract obtained from a Sauvignon blanc grape sample that was dosed with 50 mg/L of SO_2 and 100 mg/L of ascorbic acid for a transport study[20] was analyzed by HPLC-MS using MRM. A 20 μL aliquot of this extract was reanalyzed after being spiked with 20 μL of the extract from a 50 μg/L solution of synthetic (*R*/*S*)-Cysgly-3-MH **2** standard. This spiking and analysis was repeated with a 10 μL aliquot of sample extract and 30 μL of synthetic standard extract.

Analytical Method Validation. The analytical method was validated by a series of duplicate standard additions of unlabeled (*R*/*S*)-Cysgly-3-MH **2** (0, 0.5, 5, 10, 25, 50, 100, and 250 μg/L) to a Chardonnay juice (20.0 °Brix, pH 3.32, titratable acidity 4.7 g/L) and to Milli-Q water. To determine the precision and recovery of the analysis in these matrices, seven replicate samples were spiked at 10 μg/L of (*R*/*S*)-Cysgly-3-MH **2**. The limit of detection (LOD) and limit of quantitation (LOQ) were determined using the visual evaluation method. LOD was determined by establishing the minimum level at which the analyte could be reliably detected from the analysis of samples with known concentrations of analyte (signal-to-noise ratio of about 3). LOQ was determined by establishing the minimum level at which the analyte could be quantified with acceptable accuracy and precision (<2% relative standard deviation) from the analysis of samples with known analyte concentrations. For quantifying the analytes in batches of unknown samples, duplicate standards in water were prepared at the same time as the juice samples with (*R*/*S*)-Cysgly-3-MH **2** concentrations of 0, 10, 50, and 250 μg/L. All validation and calibration samples were extracted and analyzed according to the method.

Statistical Analysis. The results reported for the calibration of the method were derived from the average of two replicate measurements for each concentration of analyte (and seven replicates for repeatability samples). Trial effects were analyzed using one-way or two-way analysis of variance (ANOVA) (GenStat 11.0, VSN International Ltd., Hemel Hempstead, U.K.). Other statistical data were obtained using Microsoft Excel 2007.

RESULTS AND DISCUSSION

Preparation of Cysgly-3-MH 2. With (*R*/*S*)-Glut-3-MH **3** on hand, it was deemed quicker to optimize glutamate cleavage with GGT to prepare (*R*/*S*)-Cysgly-3-MH **2**, rather than use protecting group strategies and conjugate addition of protected L-cysteine or L-cysteinylglycine to (*E*)-2-hexenal, followed by reduction of the aldehyde. GGT catalyzes the transfer of the γ-glutamyl group of GSH (or its conjugates) to an amino acid or peptide acceptor to produce L-cysteinylglycine. We therefore tested the optimal conditions for a crude enzyme preparation of equine kidney GGT to cleave the glutathione conjugate (Glut-3-MH **3**) to yield practical quantities of the corresponding Cysgly-3-MH **2** for use as an analytical standard.

Table 1. Decomposition of 3-*S*-Glutathionylhexan-l-ol (3) (8 mM) by Equine Kidney GGT in Tris-HCl Buffer (pH 8.5) with the Addition of Different Equivalents of Histidine (Relative to the Initial Concentration of 3) Determined Using HPLC-MS in SIM Mode after 90 min (and 24 h)

	relative conjugate concentration[a] (%)				
histidine (mM)	m/z 222 **1**	m/z **279** **2**	m/z 408 **3**	m/z 351 **4**	m/z 537 **5**
0	3.0	23.1	66.7	0.9	6.3
8	2.5	36.5	56.8	0.5	3.7
16	2.1	45.1	49.7	0.3	2.8
40	1.4	60.9	35.9	0.1	1.7
80	0.7 (6.5)	70.9 (**86.3**)	27.3 (6.9)	<0.1 (0.1)	1.0 (0.2)

[a] Relative concentrations are based on peak areas of the reaction mixture.

The results in Table 1 confirm that equine kidney GGT can use Glut-3-MH **3** as a substrate under the conditions employed. When no histidine was added, 23.1% of **3** (based on relative peak areas for the conjugates) was converted into Cysgly-3-MH **2** after 90 min at 28 °C. The addition of increased concentrations of histidine to the reaction mixture boosted the formation of **2**, and with 10 equivalents of histidine (80 mM), the formation of **2** reached 70.9%. In these conditions, extending incubation to 24 h increased the yield to 86.3% (Table 1), whereas incubation at 37 °C for 90 min had a minor effect, yielding 75% of **2** (data not shown). Moreover, the addition of increased concentrations of histidine reduced the formation of byproducts of the reaction (Table 1) such as the putative dipetide **4** and tetrapeptide **5** (Figure 1) and Cys-3MH **1**, making the purification of Cysgly-3-MH **2** easier. Conjugate **5** can be formed in an autotranspeptidation reaction, when glutathione acts both as donor and acceptor of the γ-glutamyl group.[21] The formation of Cys-3MH **1** may be a consequence of enzymatic hydrolysis of glutamyl and glycine residues or hydrolysis and transfer of them to acceptor amino acids,[14] and with commercial, crude GGT preparations, peptidase impurities likely give rise to the cysteine conjugate.[16]

The optimized conditions were applied to around 60 mg of (*R*/*S*)-Glut-3-MH **3** (80 mM histidine, 28 °C, 24 h) to give (*R*/*S*)-Cysgly-3-MH **2** in 70% yield after low-pressure C18 purification, with a purity of >95% as determined by HPLC-MS analysis. Fractions were collected using a water/ethanol gradient and assessed initially by spotting on silica TLC plates and visualizing with ninhydrin spray. Fractions testing positive for amino acid were assessed by HPLC-MS, and those fractions containing **2** were combined. The synthetic material was fully characterized by NMR, HPLC-MS, and MS/MS experiments. Cysgly-3-MH **2** has been identified previously in human axilla sweat, and our data are in good agreement with those ascribed to the synthesized material in that paper.[19]

Identification of Cysgly-3-MH 2 in Sauvignon blanc Juice. Authentic (*R*/*S*)-Cysgly-3-MH **2** was analyzed by HPLC-MS/MS in EPI mode. The mass spectrum obtained for the earlier eluting peak in the chromatogram, assigned as (*S*)-Cysgly-3-MH by analogy with the known precursor elution orders,[17] appears in

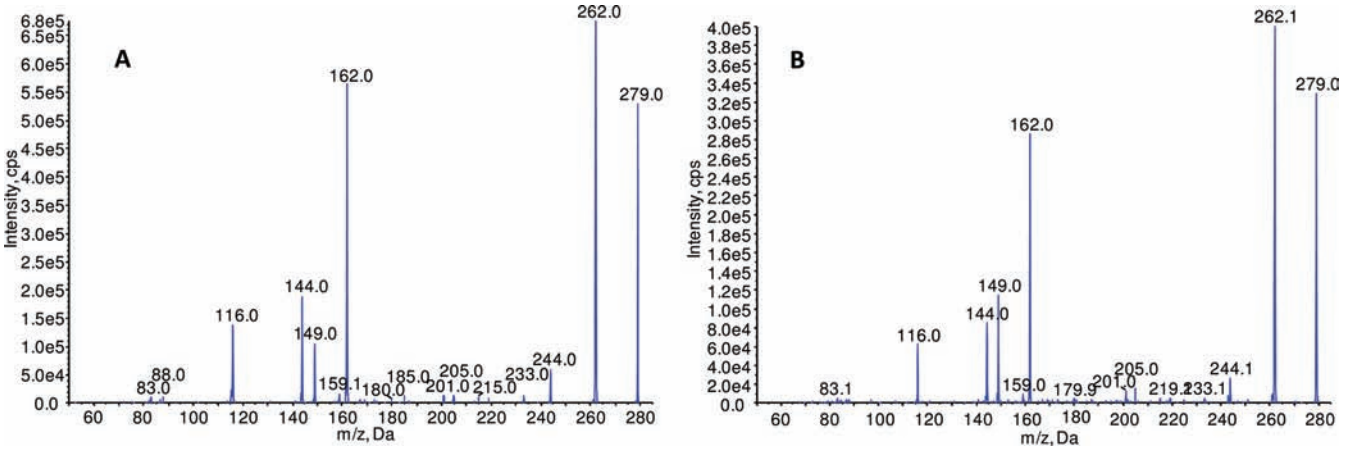

Figure 2. EPI mass spectra for extracts of (A) authentic (*S*)-3-*S*-cysteinylglycinehexan-1-ol ((*S*)-**2**) 50 μg/L standard and (B) naturally present 3-*S*-cysteinylglycinehexan-1-ol (**2**) in a transported Sauvignon blanc sample dosed with 50 mg/L SO_2 and 100 mg/L ascorbic acid at the time of harvest. The (*S*)-designation relates to the alkyl chain stereocenter, and the peak was assigned on the basis of the elution order of related conjugates.[17]

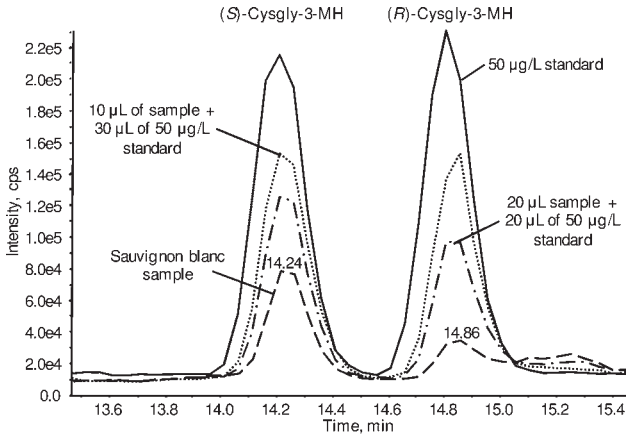

Figure 3. Overlaid and expanded MRM chromatograms showing co-injections for an extract of transported Sauvignon blanc fruit spiked with increasing amounts of the extract from a 50 μg/L solution of authentic (*R*/*S*)-3-*S*-cysteinylglycinehexan-1-ol (**2**). The diastereomers were assigned to the peaks on the basis of the elution order of related conjugates.[17] The (*S*)- and (*R*)-designations relate to the alkyl chain stereocenter.

Figure 2A, and an identical mass spectrum was observed for the (*R*)-Cysgly-3-MH diastereomer eluting as a similarly sized peak 0.5 min later (data not shown). Several juice sample extracts arising from machine-harvested Sauvignon blanc fruit that was transported 800 km to a winery[20] were analyzed using the EPI method. One of these samples contained peaks at the appropriate retention times, and the mass spectrum shown in Figure 2B of the first eluting peak was identical to that of the authentic compound (Figure 2A). There was also a smaller peak with an identical mass spectrum for the naturally present (*R*)-Cysgly-3-MH diastereomer eluting 0.5 min later (data not shown). The fragmentation patterns of authentic and natural compounds (protonated molecular ion *m*/*z* 279) were entirely consistent with the reported fragmentation[17] of the daughter ions observed at *m*/*z* 279 and 288 for Glut-3-MH **3** and d_9-Glut-3-MH, respectively, which resulted from the neutral loss of glutamate from the parent compounds to yield the cysteinylglycine daughter ions.

Co-injection experiments were undertaken to verify the identification of **2** in a Sauvignon blanc juice extract. Additions of a standard solution of synthetic (*R*/*S*)-Cysgly-3-MH **2** to the juice extract used for initial identification yielded symmetrical peak enhancements for the two peaks present in the chromatograms (Figure 3). These two peaks were therefore confirmed as belonging to the two diastereomers of **2** present naturally in the Sauvignon blanc grape juice extract. Along with the mass spectra obtained from EPI experiments, these co-injections provided positive evidence for the detection of both diastereomers of Cysgly-3-MH **2** in a Sauvignon blanc juice for the first time.

Although it should be noted that this work was conducted on methanolic extracts that were frozen, the treatment the original juices underwent to give the extracts meant the likelihood of **2** forming enzymatically during storage was extremely remote. Indeed, quantitative analysis of fresh extracts as discussed later also revealed the presence of Cysgly-3-MH **2**, so we were confident in the results, especially considering what we have formerly revealed about precursor stability during storage.[12] The existence of Cysgly-3-MH **2** had been hypothesized previously[4,11,15] and is no doubt an intermediate in the metabolic breakdown of Glut-3-MH **3** to Cys-3-MH **1** such as during fermentation. With its presence verified, we turned our attention to adding **2** into our precursor method[17] and applying it to the quantitative analysis of various Sauvignon blanc juice extracts.

Method Validation. Synthetic (*R*/*S*)-Cysgly-3-MH **2** was infused into the MS to determine the MRM transitions (listed under Materials and Methods) to add to the existing precursor method.[17] Stable isotope dilution analysis (SIDA) was used for quantitative determination of **2** in juice samples. Calibrations were developed using either d_8-Cys-3-MH or d_9-Glut-3-MH as internal standard (both were present in the labeled standard mix added prior to SPE) with synthetic Cysgly-3-MH **2**. The standard addition functions obtained for **2** using either internal standard were linear throughout the concentration range (0−250 μg/L), with minimum coefficients of determination (R^2) of 0.99 in juice or water. Chromatographically, Cysgly-3-MH **2** was of intermediate polarity compared to Cys-3-MH **1** and Glut-3-MH **3**, with the HPLC retention times for the dipeptide diastereomers appropriately falling between the single amino acid and tripeptide conjugates (Figure 4). On the basis of average recoveries using 10 μg/L of spiked Cysgly-3-MH **2** in juice

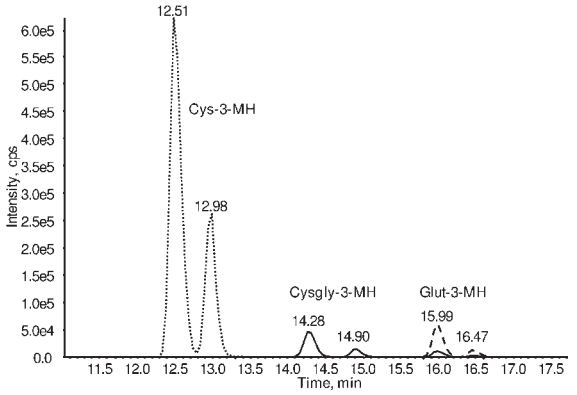

Figure 4. Expanded MRM chromatogram overlays showing resolved diastereomers and elution order of 3-*S*-cysteinylhexan-1-ol (**1**) (m/z 222.1 → 83.0), 3-*S*-cysteinylglycinehexan-1-ol (**2**) (m/z 279.4 → 162.1), and 3-*S*-glutathionylhexan-1-ol (**3**) (m/z 408.6 → 262.2) in the extract from a transported Sauvignon blanc sample dosed with 50 mg/L SO_2 and 100 mg/L ascorbic acid at the time of harvest. Note that the relative peak sizes are not indicative of the concentrations for different precursor types.

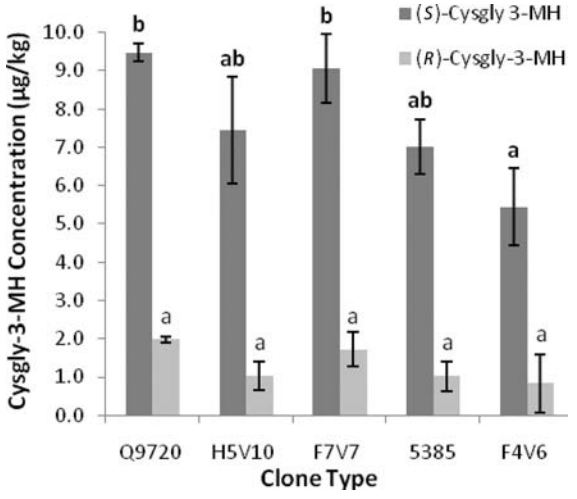

Figure 5. Concentrations of (*S*)- and (*R*)-3-*S*-cysteinylglycinehexan-1-ol (**2**) (μg/kg) determined for grapes obtained at harvest for five Sauvignon blanc clones colocated in an Adelaide Hills vineyard. Details of the clone samples were reported previously.[12] Error bars represent the standard deviation of three replicates. The (*S*)- and (*R*)-designations relate to the alkyl chain stereocenter. There were statistically significant differences between the clone types ($p = 0.002$ for (*S*)-**2** and $p = 0.049$ for (*R*)-**2**). Different letters for the same precursor diastereomer indicate significant differences ($p < 0.05$) between the means ((*S*)-Cysgly-3-MH **2** is differentiated with bold letters).

(81.4 ± 10.4% using d_9-Glut-3-MH as internal standard, 98.7 ± 5.7% using d_8-Cys-3-MH), we decided to use labeled Cys-3-MH when calibrating and quantifying **2**. The relative standard deviation for repeatability at 10 μg/L of added **2** was 5.8%, providing acceptable method precision. The LOQ and LOD, determined to be 0.5 and 0.2 μg/L, respectively, were perfectly adequate for our purposes. White grape juice and water gave almost identical calibration function slopes when using d_8-Cys-3-MH, and the quantitative analysis was independent of the matrix (data not shown).

Evaluation of Cysgly-3-MH 2 in Sauvignon blanc Juice Samples. *Adelaide Hills Clones.* Cysgly-3-MH **2** was assessed in extracts obtained during the maturation of grapes from five Sauvignon blanc clones planted in a single Adelaide Hills location. The concentrations of Cys-3-MH **1** and Glut-3-MH **3** and basic chemical parameters of the clones at each sampling time point were reported previously.[12] The levels of **2** were proportionally very low, being barely detectable during the early sampling time points (data not shown), rising to a maximum of only around 11 μg/L (combined diastereomer total) at harvest (Figure 5). There were some differences between the five clones at harvest, particularly for (*S*)-**2**, but the general trend for Cysgly-3-MH **2** concentrations was consistent among the clones during ripening and in agreement with the profiles of **1** and **3** reported for these samples.[12] The (*S*)-Cysgly-3-MH diastereomer was around 4.5−7.5 times more abundant than the (*R*)-diastereomer, which is in accord with the diastereomer distributions observed previously for cysteine and glutathione conjugates.[12,17,20] The quantities of **2** determined for these samples were much lower than the amounts of **1** and **3** already reported, indicating this intermediate between cysteine and glutathione conjugates was relatively short-lived in these grape samples. Nonetheless, a level of 10 μg/L of Cysgly-3-MH **2**, if completely transformed into Cys-3-MH **1** with subsequent release of 3-MH during fermentation, could theoretically yield 480 ng/L of 3-MH based on 10% conversion of the cysteine conjugate. This is a significant amount of potential 3-MH that has been masked and unaccounted for in previous studies evaluating precursor transformations during fermentation. Clearly, within grape berries Cysgly-3-MH **2** is readily converted to Cys-3-MH **1**; whether yeast can utilize **2** itself or first have to metabolize it to **1** needs to be determined.[22] It is conceivable that transformation to Cys-3-MH **1** by yeast would occur first, because the more readily metabolized precursor of 3-MH seems to be the cysteine conjugate.[12,20,22]

Transport and Antioxidant Effects. Sample extracts from a transport trial were analyzed for Cysgly-3-MH **2** to assess the effects of machine-harvesting and application of antioxidants in the vineyard. The stark effects of grape transportation and high SO_2 levels were highlighted previously for Cys-3-MH **1** and Glut-3-MH **3**.[20] The apparent formation and breakdown of **3** during transportation provided much higher amounts of **1** (up to 270 μg/L)[20] than ordinarily observed in our other studies (up to 55 μg/L).[12,17] This indicated that Cysgly-3-MH **2**, the obvious intermediate in the breakdown of **3**, should also be formed in determinable quantities. In accord with these expectations, total Cysgly-3-MH **2** was quantified in the sample extracts at up to 28.5 μg/L (Figure 6), with the concentrations generally mirroring the trends asssociated with transport and antioxidant effect detailed previously.[20] As such, transportation significantly increased the abundance of **2** relative to nontransported samples by up to 20-fold, and the application of 500 mg/L of SO_2 to the picking bins in the vineyard yielded considerably lower levels. Furthermore, Cysgly-3-MH **2** concentrations in the transported samples were highest without antioxidant addition, whereas the use of 50 mg/L of SO_2, with or without ascorbic acid, yielded the second highest amounts of **2**. These results generally compare well with what we found for Cys-3-MH **1** and Glut-3-MH **3** and can be explained in the same way on the basis of the potential effects of SO_2.[20] In particular, high SO_2 additions could affect enzymatic processes (i.e., oxidation or conjugation reactions),

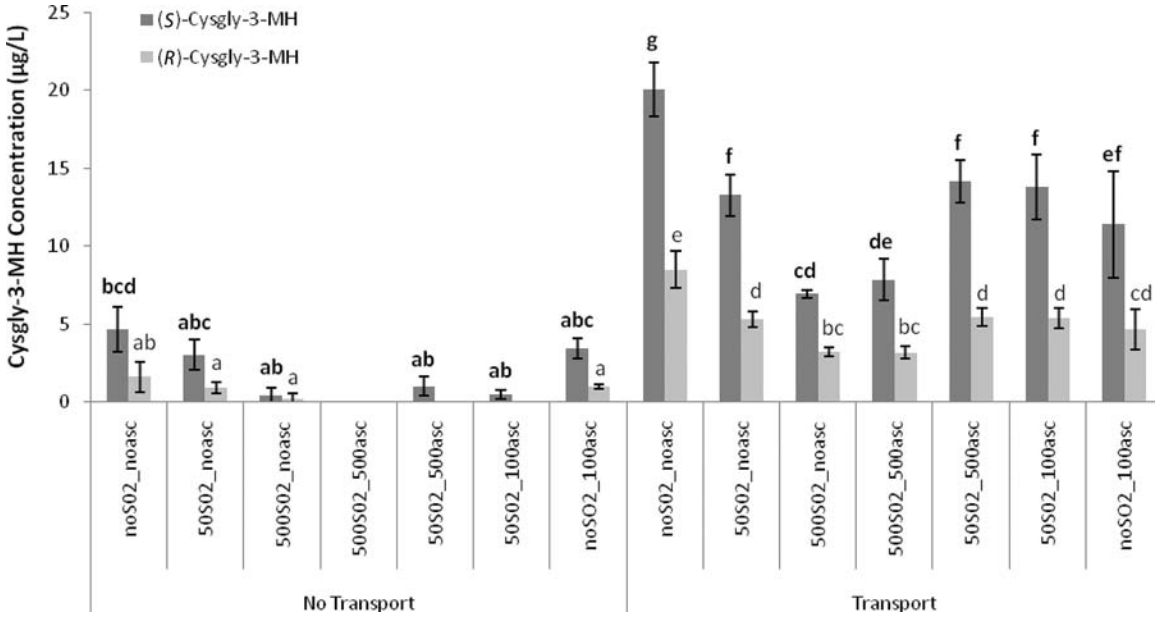

Figure 6. Concentrations of (*S*)- and (*R*)-3-*S*-cysteinylglycinehexan-1-ol (**2**) (µg/L) determined for Sauvignon blanc grapes that underwent antioxidant addition in the vineyard and transportation. Details of the treatments were reported previously.[20] The *x*-axis labels refer to the additions of antioxidants (SO_2 and ascorbic acid) (mg/L). Error bars represent the standard deviation of three replicates. The (*S*)- and (*R*)-designations relate to the alkyl chain stereocenter. There were statistically significant differences ($p < 0.001$) between the treatments and due to the effects of transport. Different letters for the same precursor diastereomer indicate significant differences ($p < 0.05$) between the means ((*S*)-Cysgly-3-MH **2** is differentiated with bold letters).

thereby limiting formation of **3** or preventing its degradation into the related conjugates **1** and **2**.

In accord with the results for the Adelaide Hills clones, the levels of **2** in the transport samples were much lower than those for the cysteine or glutathione conjugates, again indicating the fleeting nature of the cysteinylglycine conjugate. This might clarify why others have failed to identify this compound, as it seems that significant amounts are accumulated only during particular types of grape processing.

The presence of Cysgly-3-MH **2** has been verified for the first time in this study. It has been detected at low micrograms per liter levels in fruit from Sauvignon blanc clones at harvest and was found at meaningful concentrations only in Sauvignon blanc fruit that had been machine-harvested and transported for 12 h to a winery. The indications are that **2** is a short-lived intermediate between the cysteine and glutathione conjugates. This helps explain why it has eluded identification until now, even though its role has been hypothesized several times. The contribution of **2** to wine 3-MH concentrations needs to be evaluated, but its identification has improved our ability to account for thiol precursors and varietal thiol 3-MH in wine.

AUTHOR INFORMATION

Corresponding Author

*Phone: +61 8 8303 6649. Fax: +61 8 8303 7116. E-mail: david.jeffery@adelaide.edu.au.

Funding Sources

The Australian Wine Research Institute (AWRI) and The University of Adelaide are members of the Wine Innovation Cluster in Adelaide. The work was performed at AWRI and 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.

ACKNOWLEDGMENT

We thank AWRI researchers Dr. Cory Black for constructive feedback on synthetic aspects of the work and Dr. Yoji Hayasaka for mass spectrometry expertise. We are grateful to members of the Australian wine industry for their continued support, provision of numerous fruit and juice samples, and for access to vineyards.

ABBREVIATIONS USED

3-MH, 3-mercaptohexan-1-ol; GSH, glutathione; Glut-3-MH, 3-*S*-glutathionylhexan-1-ol; Cys-3-MH, 3-*S*-cysteinylhexan-1-ol; Cysgly-3-MH, 3-*S*-cysteinlyglycinehexan-1-ol; GGT, γ-glutamyltranspeptidase; HPLC-MS/MS, high-performance liquid chromatography−tandem mass spectrometry; TLC, thin layer chromatography; MRM, multiple reaction monitoring; EPI, enhanced product ion; CES, collision energy spread; SIM, selected ion monitoring; LOD, limit of detection; LOQ, limit of quantitation; SIDA, stable isotope dilution analysis.

REFERENCES

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

(2) 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.

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

(4) Kobayashi, H.; Takase, H.; Suzuki, Y.; Tanzawa, F.; Takata, R.; Fujita, K.; Kohno, M.; Mochizuki, M.; Suzuki, S.; Konno, T. Environmental stress enhances biosynthesis of flavor precursors, *S*-3-(hexan-1-ol)-glutathione and *S*-3-(hexan-1-ol)-L-cysteine, in grapevine through glutathione *S*-transferase activation. *J. Exp. Bot.* **2011**, *62*, 1325–1336.

(5) Tominaga, T.; Dubourdieu, D. Identification of cysteinylated aroma precursors of certain volatile thiols in passion fruit juice. *J. Agric. Food Chem.* **2000**, *48*, 2874–2876.

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

(7) Starkenmann, C.; Niclass, Y. New cysteine-*S*-conjugate precursors of volatile sulfur compounds in bell peppers (*Capsicum annuum* L. cultivar). *J. Agric. Food Chem.* **2011**, *59*, 3358–3365.

(8) 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.

(9) Thibon, C.; Shinkaruk, S.; Jourdes, M.; Bennetau, B.; Dubourdieu, D.; Tominaga, T. Aromatic potential of botrytized white wine grapes: identification and quantification of new cysteine-*S*-conjugate flavor precursors. *Anal. Chim. Acta* **2010**, *660*, 190–196.

(10) 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.

(11) Kobayashi, H.; Takase, H.; Kaneko, K.; Tanzawa, F.; Takata, R.; Suzuki, S.; Konno, T. Analysis of *S*-3-(hexan-1-ol)-glutathione and *S*-3-(hexan-1-ol)-L-cysteine in *Vitis vinifera* L. cv. Koshu for aromatic wines. *Am. J. Enol. Vitic.* **2010**, *61*, 176–185.

(12) Capone, D. L.; Sefton, M. A.; Jeffery, D. W. Application of a modified method for 3-mercaptohexan-1-ol determination to investigate the relationship between free thiol and related conjugates in grape juice and wine. *J. Agric. Food Chem.* **2011**, *59*, 4649–4658.

(13) 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.

(14) McIntyre, T. M.; Curthoys, N. P. The interorgan metabolism of glutathione. *Int. J. Biochem.* **1980**, *12*, 545–551.

(15) Ribéreau-Gayon, P.; Glories, Y.; Maujean, A.; Dubourdieu, D. Varietal aroma. In *Handbook of Enology*; Wiley: West Sussex, U.K., 2006; Vol. 2, pp 205−230.

(16) 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.

(17) 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*, 1390–1395.

(18) 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.

(19) 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. Biodiversity* **2005**, *2*, 705–716.

(20) Capone, D. L.; Jeffery, D. W. Effects of transporting and processing Sauvignon blanc grapes on 3-mercaptohexan-1-ol precursor concentrations. *J. Agric. Food Chem.* **2011**, *59*, 4659–4667.

(21) Karkowsky, A. M.; Bergamini, M. V.; Orlowski, M. Kinetic studies of sheep kidney γ-glutamyl transpeptidase. *J. Biol. Chem.* **1976**, *251*, 4736–4743.

(22) Winter, G.; Van Der Westhuizen, T.; Higgins, V. J.; Curtin, C.; Ugliano, M. Contribution of cysteine and glutathione conjugates to the formation of the volatile thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) during fermentation by *Saccharomyces cerevisiae*. *Aust. J. Grape Wine Res.* **2011**, *17*, 285–290.