JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 9539–9546 9539
DOI:10.1021/jf902198e

Formation of Pigment Precursor (+)-1″-Methylene-6″-hydroxy-2*H*-furan-5″-one-catechin Isomers from (+)-Catechin and a Degradation Product of Ascorbic Acid in a Model Wine System

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The present study investigates the contribution of ascorbic acid to the formation of pigment precursors in model white wine systems containing (+)-catechin as the oxidizable phenolic substrate. The two main colorless products in these systems were structurally characterized as isomers of (+)-catechin substituted at either C6 or C8 on the A ring with a furan-type unit, namely, (+)-1″-methylene-6″-hydroxy-2*H*-furan-5″-one-6-catechin and (+)-1″-methylene-6″-hydroxy-2*H*-furan-5″-one-8-catechin. A known degradation product of ascorbic acid, L-xylosone, was separately prepared and, when reacted with (+)-catechin, generated the same (+)-furanone-catechin isomers as in model white wine systems. Incubation of these isomers in wine-like conditions yielded yellow xanthylium cation pigments. This study has shown that undesirable spoilage reactions (yellow coloration) can occur from a breakdown product of ascorbic acid−L-xylosone.

KEYWORDS: Ascorbic acid; (+)-catechin; L-xylosone; model white wine; oxidation; xanthylium cation; biomimetic synthesis

INTRODUCTION

Flavanols in wine, such as (+)-catechin (**Figure 1**), are well-known to react with aldehydes (*1*) formed or extracted during the winemaking process and aging. The most commonly occurring aldehyde in wine production is acetaldehyde, formed mainly by yeast during fermentation or by the oxidation of ethanol (*2*). Other compounds formed in wine that also contain an aldehyde group include glyceraldehyde, glyoxylic acid, and furfural. The first two may be generated under oxidative storage conditions of wine (*3*−*5*), whereas furfural can be extracted during the aging of wine in oak (*6*). Of these aldehydes, only glyoxylic acid (*7*) and furfural (*6*) have been shown to participate in xanthylium cation pigment formation after their reaction with (+)-catechin in model systems. However, pigments containing glyoxylic acid have been reported for red wine (*8*) but have not yet been observed in white wine.

Another wine constituent capable of producing electrophilic carbonyl compounds, despite its primarily antioxidant properties, is ascorbic acid (**Figure 1**). Ascorbic acid is naturally present in grapes but is usually rapidly consumed after crushing, typically due to ascorbic acid either scavenging oxygen or reducing *o*-quinone compounds formed from the enzymatic oxidation of phenolic compounds (*9*, *10*). The ascorbic acid present in white wine is mostly due to exogenous additions, often just before the bottling of white wine, although it may be used at various stages in the wine production process. The levels of ascorbic acid added may vary considerably, but it is typically added at rates ranging from 50 to 150 mg/L (*9*−*12*).

Ascorbic acid is added to white wine due to its ability to scavenge molecular oxygen efficiently, but in the process it is initially converted to dehydroascorbic acid and hydrogen peroxide. The dehydroascorbic acid then undergoes rapid degradation into a variety of species including numerous carboxylic acid, ketone, and aldehyde compounds (*13*−*16*) (**Figure 1**). Recent studies have shown the increased production of phenolic pigments from model wine systems of ascorbic acid and (+)-catechin and demonstrated that a degradation product emanating from ascorbic acid was able to react with (+)-catechin and form colored xanthylium cations (*17*, *18*). In general, phenolic pigments formed during the storage of white wine are undesirable as they are perceived as indicative of wine spoilage.

The behavior of (+)-catechin solutions in the presence of relatively high concentrations of ascorbic acid has been the focus of a recent study by Clark et al. (*19*). In this study, two colorless species were observed using HPLC-PDA in model wine samples containing iron(II), ascorbic acid, and (+)-catechin. The compounds accumulated while ascorbic acid was still present in solution and then underwent rapid degradation once the ascorbic acid was near depletion. Their degradation also coincided with the production of pigmented compounds in the model wine system. These colorless compounds thus appear to be key intermediates in the formation of pigments in model white wine systems. Despite intense effort, these compounds have until

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Published on Web 10/05/2009

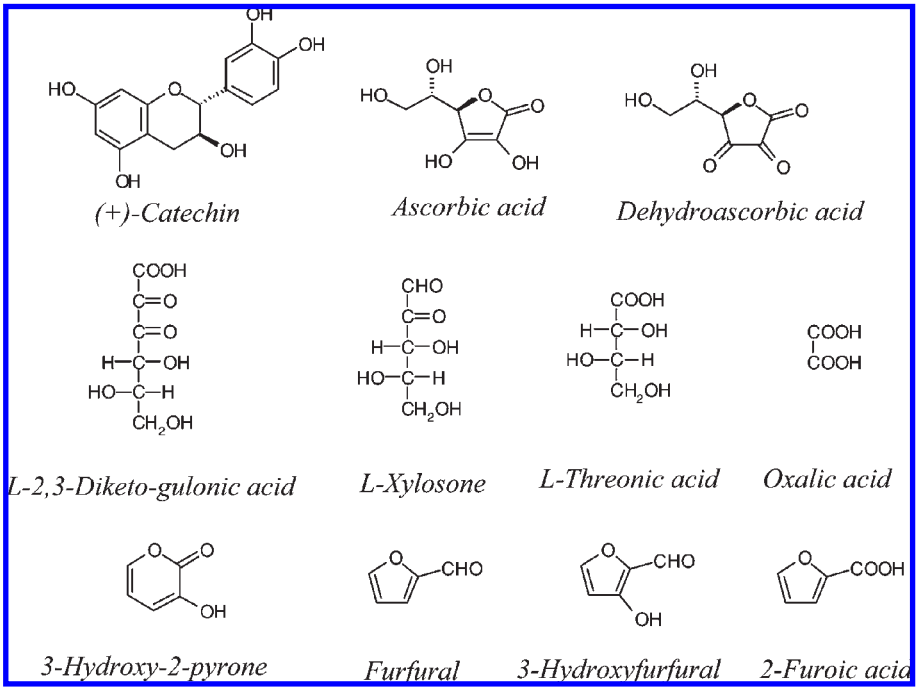

Figure 1. Molecular structures of (+)-catechin, ascorbic acid, and some reported degradation products of ascorbic acid.

now eluded identification. Furthermore, direct evidence that these compounds are indeed intermediates in pigment formation has not been available. This study addresses these two issues by identifying the colorless compounds as (+)-1″-methylene-6″-hydroxy-2*H*-furan-5″-one-6-catechin and (+)-1″-methylene-6″-hydroxy-2*H*-furan-5″-one-8-catechin and showing that these compounds react to give pigments. The study goes further to directly implicate a breakdown product of ascorbic acid, L-xylosone, as capable of reacting with (+)-catechin to generate the two (+)-furan-catechin isomers observed in model wine systems. Finally, the pigments formed by reaction of the (+)-furanone-catechin isomers were shown to be xanthylium cations, which have been reported in other model wine systems.

MATERIALS AND METHODS

General. Water purified through a Milli-Q (Millipore) water system (ISO 9001) was used for all solution preparations and dilutions. All glassware was soaked overnight in a 10% nitric acid (AnalR, BDH, Poole, U.K.) and then rinsed with copious amounts of water. L-Ascorbic acid (99%), L-(+)-tartaric acid (>99.5%), (+)-catechin hydrate (98%), potassium bitartrate (99%), d_6-DMSO (99.98%), L-(−)-xylose (99%), glyoxylic acid monohydrate (98%), and copper(II) acetate (98%) were purchased from Sigma-Aldrich (USA). Potassium hydroxide (AR grade, >85%) was obtained from BDH/Merck (Australia), and sulfuric acid (AR grade, >95%) and iron(II) sulfate heptahydrate (>98%) were purchased from Ajax Fine Chemicals (Australia). Ethanol (AR grade, >99.5%, Ajax Fine Chemicals), methanol (AR grade, >99.9%, Mallinckrodt Chemicals, USA), glacial acetic acid (AR grade, >99.7%, APS Ajax Fine Chemicals), hydrochloric acid (31.5%, AnalR, BDH, Australia), formic acid (98%, Fluka, Germany), and acetonitrile (HPLC grade, >99.9%, Ajax Fine Chemicals) were used without further purification.

Analytical LC analyses were conducted on an Ultra Performance Liquid Chromatography (UPLC) system consisting of a Waters Acquity binary solvent manager connected to a sample manager and a PDA detector all run by Empower^2 chromatography manager software. The column was a Waters Acquity BEH C18 (2.1 × 50 mm) with 1.7 μm particle diameter. Injection volume was 7.5 μL, column temperature 35 °C, and flow rate 0.45 mL/min; the elution gradient consisted of solvent A, 0.5% acetic acid in water, and solvent B, 0.5% acetic acid in methanol, as follows (expressed in solvent A followed by cumulative time): 100%, 0 min; 100%, 1 min; 95%, 1.31 min; 62%, 5.25 min; 56%, 6.27 min; 48%, 6.34 min; 45%, 7.22 min; 0%, 8.85 min; 0%, 9.40 min; 100%, 8.74 min; and 100%, 9.76 min.

LC-MS studies on the colorless compounds were conducted as described by Lutter et al. (*20*). LC-MS studies conducted on the xanthylium cations were conducted on an Agilent 1200 series triple-quadrapole (6410) HPLC-MS. The column and LC conditions were as described for the UPLC (above), except for an injection volume of 20 μL. The MS was operated with drying gas temperature at 350 °C, gas flow of 9 L/min, nebulizer pressure at 40 psi, and capillary voltage at 4 kV. MS analyses for the xanthylium cations were carried out in the positive ion mode with the fragmentor at both 50 and 150 V, the former providing parent ion signals and the latter inducing fragmentation.

Preparative HPLC separations were conducted on a Perkin-Elmer 250 binary LC pump connected to a Varian 320 Pro Star UV−visible detector (280 nm) controlled by a Varian Star (v 6.41) chromatography workstation. The column was a semipreparative 4 μm Phenomenex Synergy Hydro-RP C18 column (250 × 10 mm) coupled to a Phenomenex ODS Octadecyl C18 (10 × 10 mm) security guard column. Injection volume was 2 mL and flow rate 2 mL/min; the elution gradient consisted of solvent A, 2% formic acid in water, and solvent B, 2% formic acid in 80% acetonitrile, as follows (expressed in solvent A followed by cumulative time): 100%, 0 min; 85%, 5 min; 71%, 35 min; 0%, 40 min; 0%, 50 min; 100%, 55 min; 100%, 60 min. Lyophilization was performed on a Christ-Alpha 2-4D freeze-dryer (Biotech International, Germany).

High-resolution MS (HRMS) data were obtained on a 4.7 T FT-ICR Bruker Apex 3 mass spectrometer, running Bruker X-Mass software (v 7.0.2). The instrument was calibrated using sodium iodide in methanol. The samples were dissolved in methanol and infused at a flow rate of 150 μL/min in negative ionization mode (ESI^-). The mass range scanned was 45−1000 amu. Capillary exit voltage was −50 V, and drying gas temperature was 120 °C. Elemental analysis was run on a VG Opus 3.6 data station running VG elemental program.

NMR data were acquired on a Bruker DRX600 NMR spectrometer operating at a frequency of 600.18 MHz at 25 °C using TopSpin (version 1.3, Bruker GMBH). Samples were prepared in d_6-DMSO in Shigemi tubes (Sigma-Aldrich) and degassed (argon) before sealing. Spectral widths were set to allow at least 0.5 ppm either side of observed resonances. 1D NMR spectra were recorded (6009.62 Hz) with 32K data points zero filled to 64K and resolution enhanced using a Gaussian multiplication of the raw FID (Gaussian broadening = 0.2, line broadening = −2 Hz) before Fourier transformation. All 2D NMR experiments were run with quadrature

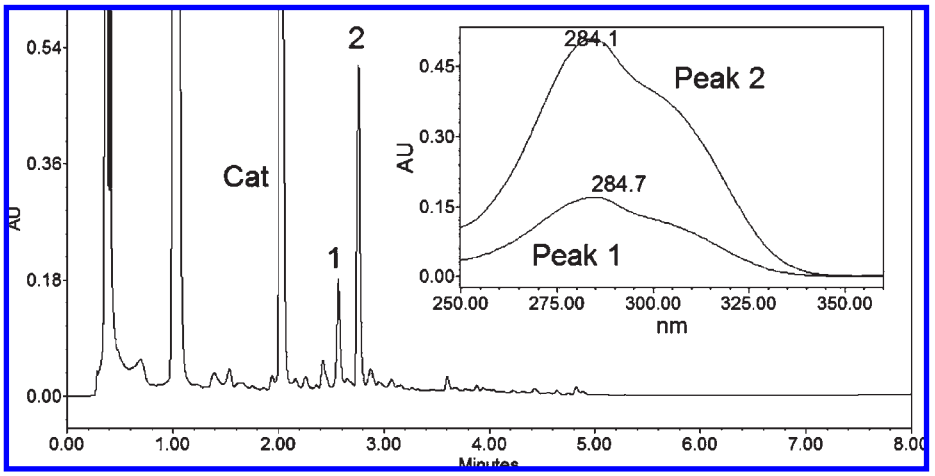

Figure 2. Liquid chromatogram at 280 nm of an ascorbic acid−(+)-catechin solution after 4 days at 45 °C with spectra of compounds **1** and **2** (inset). The model wine system consisted of a tartaric acid-buffered 12% ethanol solution (pH 3.2) with 1000 mg/L ascorbic acid, 500 mg/L catechin, and 1.5 mg/L iron(II). Peaks eluting before (+)-catechin are ascorbic acid and its degradation products.

detection with a ^{1}H spectral width of 6009 Hz and a recycle delay of 2 s. Chemical shifts were referenced to the residual d_5-DMSO (δ_H 2.49 ppm; δ_C 39.8 ppm). High-power ^{1}H $\pi/2$ pulses were determined to be 11.2 μs and low power (for MLEV spin lock), 30 ms. The ^{13}C high-power $\pi/2$ pulse was 11.0 μs, and a low-power pulse of 65 μs was used for GARP4 decoupling. Gradient pulses were delivered along the z-axis using a 100 step sine program.

Homonuclear proton−proton correlation was achieved with the eCOSY sequence (*21*) using gradient pulses for selection. The data were recorded in 4K data points in t_1 and in 512K data points in t_2. Points were predicted out to 8K data points in t_2 and 1K data points in t_1 (maximum entropy) and zero filled to 8K and 4K data points, respectively. The data were processed using a $\pi/2$ sine-bell shifted apodization in both dimensions and carefully phase corrected. TOCSY experiments were recorded over 2K data points in t_2 and 512 in t_1 using the X_M16 sequence (*22*) for the mixing time (30 ms; P29 = 50 μs, SP0 = 8.86 dB). The data were processed using a $\pi/2$ sine-bell shifted apodization in both dimensions. HSQC spectra were recorded over 2K data points in t_2 and 512 data points in t_1 (10−170 ppm) using the sensitivity-enhanced double INEPT transfer with no trim pulses and adiabatic pulses for ^{13}C decoupling in $f2$ (*23*). The spectra were made phase sensitive using echo−antiecho gradient selection (80:20:11:−5). Long-range ^{1}H−^{13}C correlation (HMBC) spectra were recorded with 2K data points in t_1 (10−210 ppm) and t_2 and processed with magnitude calculation in $f1$ to destroy all phase information. Spectra were obtained with a low-pass J-filter (145 Hz) to suppress one-bond correlations. No decoupling was used during the acquisition, and gradient pulses (50:30:40:1) were used for selection. Two experiments were run, optimized for a 20 and 2 Hz long-range coupling, respectively. Through-space connectivities were obtained using 2D homonuclear correlation via dipolar coupling (ROESY) using phase sensitive echo−antiecho gradient selection with a 250 ms mixing delay (2K × 512 data points).

Reactions under Various Model Wine Conditions. The composition of the model wine system was varied to establish the components essential for the formation of the colorless products. Ascorbic acid (1000 mg/L, 5.6 mM), (+)-catechin (500 mg/L, 1.6 mM), and iron(II) (1.5 mg/L, 0.027 mM) solutions were prepared in a tartaric acid-buffered model wine system (pH 3.2), with and without ethanol (12% v/v) present, as per Clark et al. (*19*). Tartaric acid- and formic acid-buffered model white wine (pH 3.2), ascorbic acid (500 mg/L, 2.8 mM), and (+)-catechin (250 mg/L, 0.8 mM) solutions, with and without ethanol (12% v/v) present, were prepared as per Barril et al. (*17*). The concentrations of the reactants are higher than those generally found in white wine but were required to generate sufficient amounts of oxidation products. It has been shown that the high concentration did not affect the species formed but only their rate of formation (*18*). All samples were prepared in triplicate, stored at 45 °C in a covered water bath (i.e., darkness), aerated daily (with rapid stirring for 2 min), and sampled every 2 or 4 days for chromatographic analysis. The uncertainties quoted are the 95% confidence limits (n = 3, Student's t test).

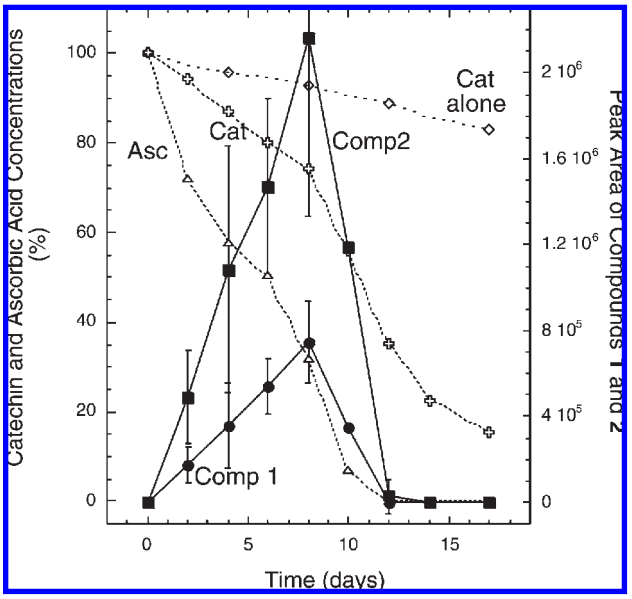

Figure 3. Formation of compounds **1** and **2** in a tartaric acid-buffered 12% ethanol solution with (+)-catechin (250 mg/L), ascorbic acid (500 mg/L), and 1.5 mg/L iron. Error bars indicate the 95% confidence limits (n = 3, Student's t test) and were omitted on day 10 for clarity. The peak areas and 95% confidence limits (× 10^6) for compounds **1** and **2** at day 10 were 0.3 ± 0.9 and 1 ± 9, respectively. The large uncertainty at day 10 was due to the rapid decrease in concentration of the compounds at this time.

Isolation of Reaction Products. A pH 3.2 solution was prepared by dissolving potassium bitartrate (2.4 g) and tartaric acid (1.2 g) in water (1 L). Ascorbic acid (1.0 g) and (+)-catechin (500 mg) were added to the solution, followed by 1.5 mg/L iron(II) (in the form of iron(II) sulfate heptahydrate), which was maintained in darkness at 45 °C, with daily aeration, until the colorless products formed reached a maximum concentration (8 days as determined by UPLC-PDA). At this time ascorbic acid (1.0 g) was added, to slow the otherwise rapid degradation of the accumulated colorless products, and the 1 L sample was stored at 4 °C.

The 1 L solution was then passed through a solid-phase extraction cartridge (Strata C18-E, 70 g/150 mL, Phenomenex, USA) to concentrate and provide a crude purification of the isomers. The cartridge was first conditioned with 80% aqueous methanol containing 2% formic acid (200 mL), followed by 2% formic acid (300 mL). The sample was absorbed and washed with 2% formic acid (300 mL) and eluted with 80% aqueous methanol with 2% formic acid (200 mL). The eluent was concentrated

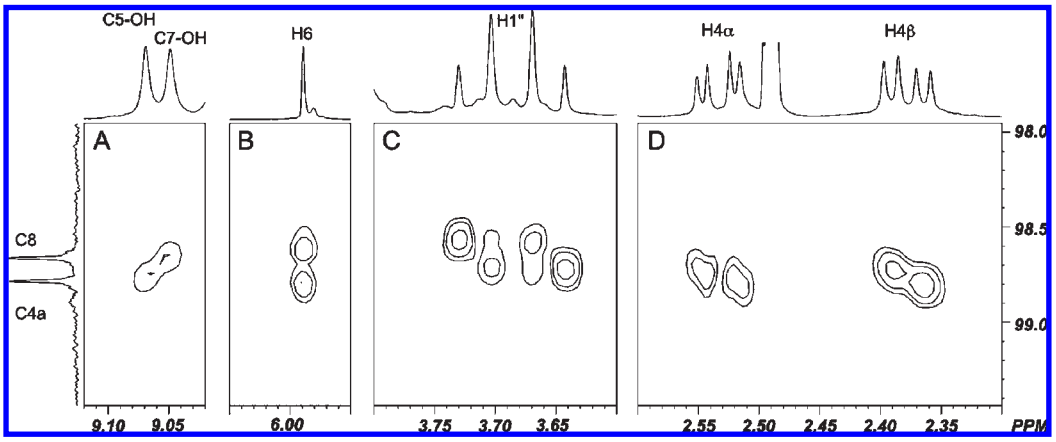

Figure 4. Heteronuclear multibond correlation spectrum of compound **2** showing correlations between C5OH and C4a and between C7OH and C8 (**A**); of H6 with both C8 and C4a (**B**); of $(H1'')_2$ and C8 (**C**); and $(H4)_2$ and C4a (**D**).

3-fold under a stream of nitrogen and injected directly onto preparative scale HPLC. Similar fractions were combined and blown down with a stream of nitrogen, and the aqueous residue was freeze-dried to yield the first eluting isomer as a pale yellow powder (15.0 mg; 2%): HRMS found, $[M - H]^-$, 401.088482, $C_{20}H_{17}O_9$ requires 401.087257; λ_{max} (0.5/24.8/74.7% (v/v) of acetic acid/methanol/water) 284 (300) nm; ^{1}H NMR (600 MHz, d_6-DMSO) δ 6.71 (d, J = 2.1 Hz, H2′), 6.68 (d, J = 8.1 Hz, H5′), 6.58 (dd, J = 8.2, 2.1 Hz, H6′), 5.87 (s, H8), 4.50 (d, J = 7.5 Hz, H2), 4.41 (s, $(H4'')_2$), 3.83 (m, H3), 3.78 (s, $(H1'')_2$), 2.64 (dd, J = 16.0, 5.5 Hz, H4α), 2.40 (dd, J = 16.0, 7.5 Hz, H4β); ^{13}C NMR (150.9 MHz, d_6-DMSO) δ 195.2 (C5″), 176.1 (C6″), 154.8 (C7), 154.2 (C5), 153.5 (C8a), 144.9 (C4′), 144.9 (C3′), 133.6 (C2″), 130.6 (C1′), 118.3 (C6′), 115.1 (C5′), 114.5 (C2′), 101.5 (C6), 99.9 (C4a), 94.5 (C8), 80.7 (C2), 72.3 (C4″), 66.3 (C3), 28.3 (C4), 21.6 (C1″).

The second (major) isomer to elute was similarly isolated as a pale yellow powder (25.0 mg; 4%): HRMS found, $[M - H]^-$, 401.086017, $C_{20}H_{17}O_9$ requires 401.087257; λ_{max} (0.5/24.8/74.7% (v/v) of acetic acid/methanol/water) 284 (300) nm; ^{1}H NMR (600 MHz, d_6-DMSO) δ 9.07 (bs, C5−OH), 9.05 (bs, C7−OH), 8.80 (bs, C4′−OH), 8.74 (bs, C3′−OH), 7.98 (bs, C6″−OH), 6.64 (d, J = 8.1 Hz, H5′), 6.64 (d, J = 2.1 Hz, H2′), 6.53 (dd, J = 8.2, 2.1 Hz, H6′), 5.99 (s, H6), 4.88 (bd, J = 5 Hz, C3−OH), 4.60 (d, J = 6.56 Hz, H2), 4.37 (AB, $W_{h/2}$ = 18.4 Hz, $(H4'')_2$), 3.82 (m, H3), 3.68 (AB, J = 16.05 Hz, $(H1'')_2$), 2.54 (dd, J = 16.05, 5.14 Hz, H4α), 2.38 (dd, J = 16.05, 7.10 Hz, H4β); ^{13}C NMR (150.9 MHz, d_6-DMSO) δ 195.1 (C5″), 176.0 (C6″), 154.5 (C5), 154.4 (C7), 153.3 (C8a), 144.7 (C4′), 144.6 (C3′), 133.7 (C2″), 130.9 (C1′), 117.6 (C6′), 115.1 (C5′), 114.1 (C2′), 98.8 (C4a), 98.7 (C8), 94.7 (C6), 80.7 (C2), 72.2 (C4″), 66.3 (C3), 27.0 (C4), 21.3 (C1″).

Synthesis of L-Xylosone and Subsequent Reaction with (+)-Catechin. The preparation of L-xylosone was conducted as described by Salomon et al. (*24*), following a method originating from Weidenhagen in 1937 using copper(II) acetate (*25*). Briefly, L-xylose (1 g, 6.7 mmol) was dissolved in aqueous methanol (95%; 60 mL), and the solution was boiled under reflux until the xylose was fully dissolved. Copper(II) acetate (4 g, 21.6 mmol) was then added in one portion, and the solution was boiled under reflux for a further 20 min. After cooling, the copper(I) oxide was removed by filtration, and the concentrated filtrate was percolated through a Chelex 100 ion-exchange resin (2.5 × 30 cm, Na^+ form; BioRad, USA) previously converted to the H^+ form. L-Xylosone was eluted with methanol to yield a pale yellow syrup (0.4 g, 40%).

L-Xylosone (1 g) was dissolved in a tartaric acid-buffered model wine system containing ethanol (50 mL) as prepared per Barril et al. (*17*). (+)-Catechin (500 mg) was added, and the solution was left to react in darkness at 45 °C. The formation of compounds **1** and **2** was monitored by UPLC-PDA every 12 h.

RESULTS AND DISCUSSION

Formation of Colorless Products in Various Model White Wine Systems. In model wine solutions containing ascorbic acid and (+)-catechin, colorless compounds giving rise to peaks 1 and 2 (**Figure 2**) were detected as previously reported (*19*). Initial studies were conducted to ensure that the colorless compounds emanated specifically from ascorbic acid and (+)-catechin and were not dependent on other components of the model wine system. For instance, an oxidative degradation product of tartaric acid, used to buffer wine-like media, is known to contribute to the formation of (+)-catechin oligomers through glyoxylic acid production (*5*, *7*).

The model wine systems were prepared at several concentrations of ascorbic acid, (+)-catechin, and iron(II), with the presence or absence of ethanol and with different supporting buffer systems of either tartaric acid or formic acid. **Figure 3** provides an example of the production and decay of compounds **1** and **2** in a model wine system containing (+)-catechin and ascorbic acid. In this case, ascorbic acid was almost depleted by day 10. As reported previously (*19*), compounds **1** and **2** in the model wine samples increased to a maximum concentration and then began to decrease in concentration once ascorbic acid was near depletion. The formation of compounds **1** and **2** is consistent with the initial degradation of (+)-catechin, as the polyphenol decay was immediate and faster than in the absence of ascorbic acid ((+)-catechin alone in **Figure 3**).

In all combinations of the model wine system, the formation of compounds **1** and **2** was reliant upon only the presence of both (+)-catechin and ascorbic acid. Other components of the model wine system, particularly iron (0−1.5 mg/L) and/or ethanol (0−12% (v/v)), had some impact on the rate of formation of compounds **1** and **2** but were not essential for the formation of these colorless compounds.

Identification of Compounds 1 and 2. Compounds **1** and **2** yielded identical ESI-MS spectra with peaks at m/z 401 and 403 in negative and positive ionization mode, respectively. High-resolution MS data established a molecular formula of $C_{20}H_{18}O_9$ (±3 ppm) for both compounds, indicating 12 degrees of unsaturation. These facts coupled with identical UV−visible spectra (**Figure 2**, inset) suggested that the compounds were isomers and contained the (+)-catechin chromophore (λ_{max} = 278 nm), albeit slightly bathochromically shifted to 284 nm. Loss of 113 amu from the compounds gave ions with the same mass as catechin-H^+ (m/z 289 (negative ionization mode); $C_{15}H_{13}O_6$), suggesting that the two compounds were both substituted with a $C_5H_5O_3$ fragment, composed, as determined by NMR, of a ketone (δ_C 195.1) and an enol (δ_C 176.0, 133.7), a methylene next to oxygen (δ_C 72.2), and an aliphatic methylene (δ_C 21.3). With 3 degrees of unsaturation for the substitution unit, this suggested a 5-methylene-4-hydroxy-2*H*-furan-3-one structure. Similar structures have been reported as products of acid treatment of xylitol (*26*). Two-dimensional

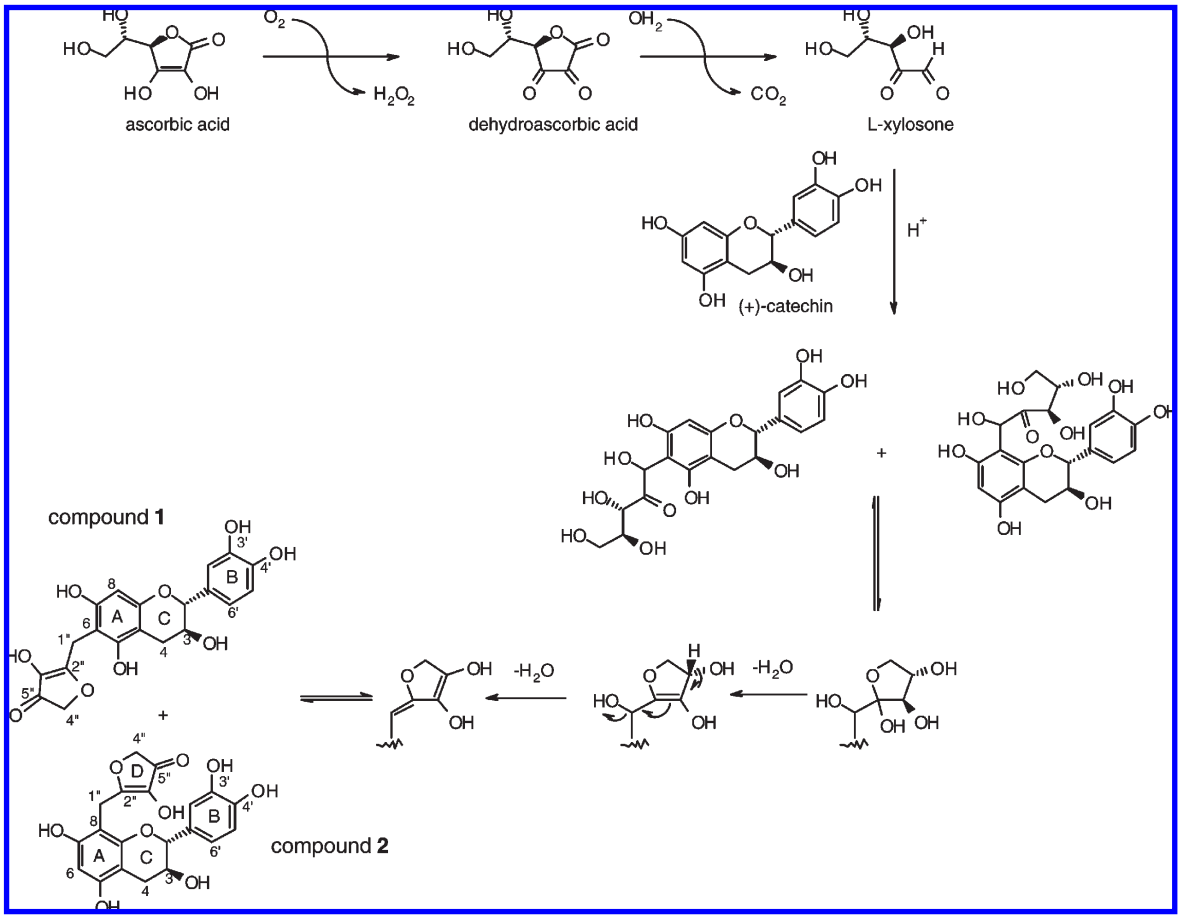

Figure 5. Scheme for the hypothetical formation of compounds **1** and **2**.

NMR evidence supported this structure for the substitution fragment. In particular, the two methylenes both showed $^3J_{CH}$ couplings to the enol carbon at 176.0 ppm but no couplings to each other. The methylene bonded to an oxygen (δ_C 72.2) was coupled to the ketone (δ_C 195.1) in the HMBC spectrum, whereas the aliphatic methylene (δ_C 21.3) was coupled to the enol (δ_C 133.7), indicating that this was the attachment point to the (+)-catechin. In the ^{1}H NMR spectrum of compound **2**, all of the (+)-catechin signals could be identified except one singlet for one of the A ring protons, suggesting that the fragment was linked to C6 or C8. This type of substitution is well-known for flavanols (*5*, *27*).

For compound **2**, the HSQC spectrum showed three methylenes, one was clearly that of the (+)-catechin C ring ((H4)$_2$ 2.54/2.38 ppm) as it showed long-range J_{CH} couplings to C5 (δ_C 154.5), C8a (δ_C 153.3), C4a (δ_C 98.8), C3 (δ_C 66.3), and C2 (δ_C 80.7) in the HMBC spectrum. C5 was differentiated from C8a on the basis of the former's $^2J_{CH}$ coupling to an exchangeable proton at 9.07 ppm. This exchangeable proton (9.07 ppm) was also coupled to C4a and C6 ($^3J_{CH}$), indicating that the attachment point of the −OH was C5. The other exchangeable proton on ring A (C7OH) was characteristically coupled to C8 (**Figure 4**). H6 was coupled to C4a and C8, with the latter also coupled to (H1″)$_2$ (**Figure 4**). (H1″)$_2$ was also coupled to C8a and C7 in the HMBC spectrum, unequivocally indicating that the furanone was attached at C8. As expected, H6 showed equally intense ROE correlation to C5−OH and C7−OH, the latter also showing a correlation to (H1″)$_2$. All other spectral data were consistent with structure **2** for the later eluting isomer (**Figure 5**).

Compound **1** was postulated to be the C6-attached isomer as has been reported for other aldehydes (*28*). In this case the point of attachment was confirmed by the fact that in compound **2**, both (H4)$_2$ and (H1″)$_2$ were coupled to C8a, whereas in compound **1** both were coupled to C5 (δ_C 154.2) in the HMBC spectrum. As in compound **2**, (H4)$_2$ was also coupled to C8a and (H1″)$_2$ was coupled to C7. Finally, the attachment point at C6 was confirmed by HMBC correlations between H8 (5.87 ppm) and C6 (101.5 ppm) and C4a (99.9 ppm) as expected but also C7 (154.8 ppm) and C8a (153.5 ppm).

Table 1. 440 nm Absorbance of Samples during Storage at 45 °C in Darkness[a]

sample	day 0	day 14
(+)-catechin	0.001 ± 0.002	0.031 ± 0.002
compound **1**	0.005 ± 0.003	0.09 ± 0.02
(+)-catechin + compound **1**	0.010 ± 0.004	0.154 ± 0.006
compound **2**	0.009 ± 0.001	0.13 ± 0.02
(+)-catechin + compound **2**	0.011 ± 0.001	0.29 ± 0.01

[a] All samples were in a formic acid-buffered 12% aqueous ethanol solution. (+)-Catechin, 0.25 mM; compound **1** 0.02 mM; and compound **2**, 0.1 mM. Uncertainties are expressed as the 95% confidence limits (n = 3, Student's t-test).

Compounds **1** and **2** (**Figure 5**) are the first reported products to form between a carbon-based degradation product of ascorbic acid and (+)-catechin. Their formation explains the accelerated degradation of (+)-catechin that has been reported (*19*) in model wine solutions containing ascorbic acid. The formation of compounds **1** and **2** also confirms that (+)-catechin is not being directly oxidized in the presence of ascorbic acid but is rather forming an adduct with a degradation compound of ascorbic acid. These results highlight the multifaceted role that ascorbic acid may have in wine or other food systems. On the one hand, ascorbic acid undoubtedly acts as an antioxidant; on the other,

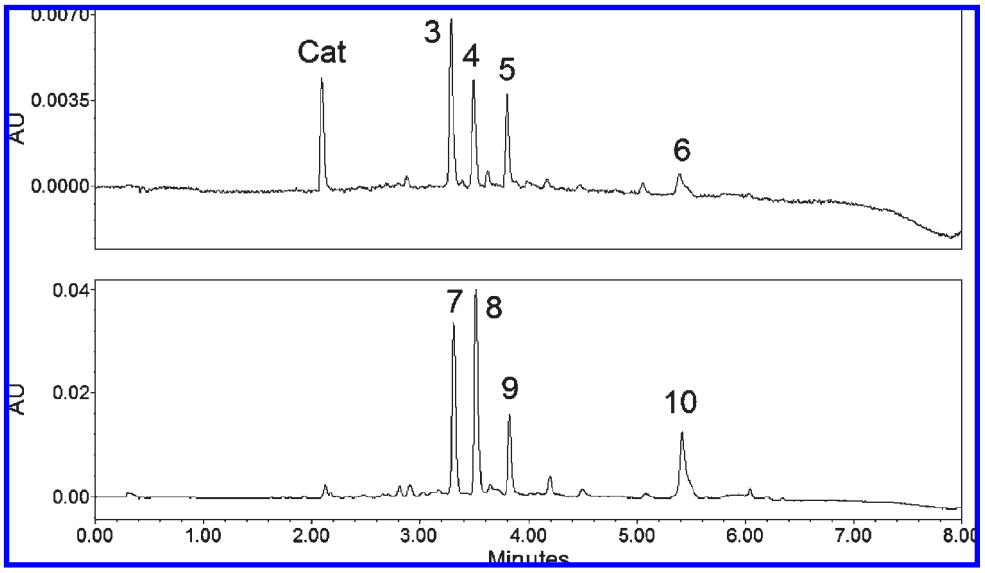

Figure 6. Liquid chromatograms at 440 nm of a solution of (+)-catechin and compound **1** (upper trace) and of (+)-catechin and glyoxylic acid (lower trace) after 14 days at 45 °C. The solutions consisted of a formic acid-buffered 12% ethanol solution.

breakdown products of ascorbic acid are capable of further reactions. In wines, those reactions may be with flavanols (e.g., (+)-catechin), which are the compounds most correlated to the browning of white wine.

Compounds **1** and **2** are stable substituted (+)-catechin monomers, provided some ascorbic acid remains in the model wine system. Other examples of stable (+)-catechin−adduct monomers are formaldehyde-substituted (+)-catechins generated as minor products in the reaction between glyoxylic acid and (+)-catechin (*29*) and certain oak-derived aldehydes that also form pyrylium-chromophores on addition to single (+)-catechin units (*30*). This is in contrast to many other (+)-catechin addition products that readily undergo further addition and form bridged-(+)-catechin dimers. The reliance on ascorbic acid for the stability of compounds **1** and **2** suggests that an oxidation step is required for their conversion to some other species (see below).

Formation and Fate of the Isomers as Model Wine System Components. Of the many degradation products of ascorbic acid that are reported in the literature (*13*−*16*)), L-xylosone (**Figure 1** appeared to be a reasonable candidate as a precursor to compounds **1** and **2**. Indeed, a retro-synthetic analysis (**Figure 5**) suggested that xylosone could be involved in the formation of **1** and **2**. Indeed, no other known degradation product of ascorbic acid allowed such feasible formation.

L-Xylosone, an aldehyde, is formed from the hydrolysis and subsequent decarboxylation of dehydroascorbic acid (*14*), but rapidly degrades to other aldehydes and ketones (*13*, *15*). Its lack an appropriate chromophore to allow detection above 250 nm, and its relatively polar character and expected elution near the injection front under the chromatographic conditions used here means that its detection by the system utilized in this experiment was not possible. Consequently, L-xylosone was synthesized as per the method described by Salomon et al. (*24*) and reacted with (+)-catechin in a model wine system consisting of a tartaric acid-buffered 12% ethanol solution. Colorless compounds were subsequently generated that had identical retention times, UV−visible spectra, and MS data as compounds **1** and **2** (as reported above). This confirmed that L-xylosone was the precursor to compounds **1** and **2** in model wine solutions of (+)-catechin and ascorbic acid.

Given that past work showed a link between the degradation of compounds **1** and **2** with the accelerated production of color in wine-like solutions (*19*), experiments were conducted to confirm the ability of compounds **1** and **2** to form colored compounds. Indeed, when left at room temperature after NMR analyses, the solution of both isolated isomers developed deep golden-brown coloration. When 0.02 mM compound **1** and 0.1 mM compound **2** were prepared ($n = 3$) at (+)-catechin concentrations of 0 and 0.25 mM in the model wine solution (formic buffer), they initially afforded 440 nm absorbances (a measure of yellow color) of < 0.012 absorbance unit. However, after incubation at 45 °C in darkness, the samples all increased in 440 nm absorbance to reach the values indicated in **Table 1**. It is evident that both compounds **1** and **2** were precursors to colored compounds and that the presence of 0.25 mM (+)-catechin enhanced the formation of colored compounds by compounds **1** and **2**.

Analysis by UPLC at day 14 revealed that all samples with added compound **1** or **2**, regardless of catechin addition, had three major peaks (3−5, **Figure 6**) in the 440 nm chromatograms. The UV−visible spectra extracted from the UPLC-PDA demonstrated that these major peaks (3−5, **Figure 6**), in all of the samples with compound **1** or **2** present, showed identical absorbance maxima at 280 and 440 nm with a shoulder at 310 nm. LC-MS data associated with these peaks all showed m/z signals at 617 in the positive ionization mode and the major fragmentation ion at m/z 465.

Such MS and UV−visible data were consistent with the xanthylium cation pigments derived from (+)-catechin and glyoxylic acid (**Figure 7**). The reaction of glyoxylic acid with (+)-catechin can generate six isomeric forms of the yellow xanthylium cations (*8*), albeit at different rates of formation, one of which is shown in **Figure 7**. These yellow pigments have UV−visible maxima at 280 and 440 nm with a shoulder at 310 nm (*7*) and a molecular mass of 617 amu (*7*) as well as the characteristic retro-Diels−Alder degradation of the dihydropyran moiety to form the m/z 465 fragment (positive ionization mode) (*31*).

To confirm that compounds **1** and **2** indeed form xanthylium cations, it was necessary to compare retention times of the purported xanthylium cations (as reported above) with those of genuine xanthylium cations synthesized from (+)-catechin and glyoxylic acid (*7*). Thus, a solution of xanthylium cations was prepared from 0.50 mM (+)-catechin and 0.25 mM glyoxylic acid by incubation at 45 °C in a tartaric acid-buffered model wine solution. The solution was analyzed by UPLC-PDA, and the

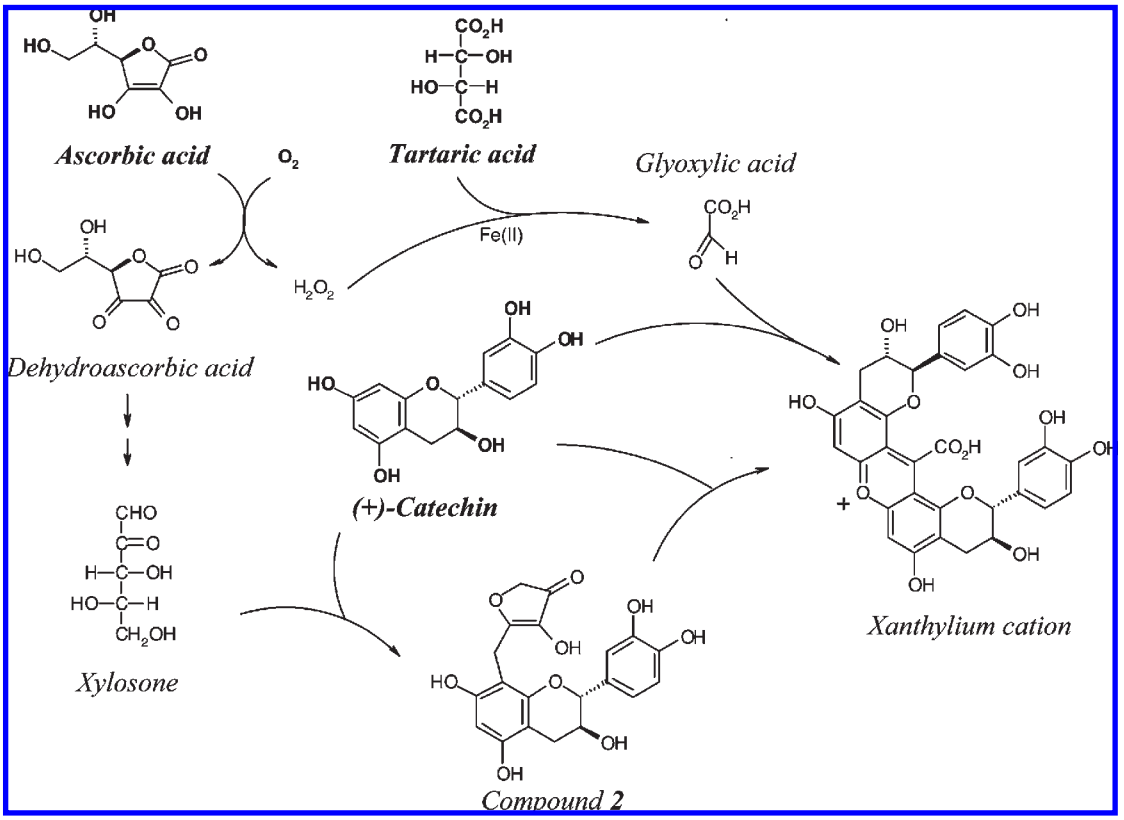

Figure 7. Formation of xanthylium cations from (+)-catechin and ascorbic acid in a tartaric acid-buffered model wine system.

peaks (7−9, **Figure 6**) corresponding to the xanthylium cations were confirmed by their UV−visible spectra and HPLC-MS analysis (data as described earlier). The remaining major peak in this solution (peak 10, **Figure 6**) corresponded to the ethyl ester of the xanthylium cation, which has also been described previously (*29*). The retention times of the xanthylium cations generated from glyoxylic acid with (+)-catechin (peaks 7−9, **Figure 6**) were at retention times identical to those of the major pigments in all of the samples containing compound **1** or **2** (peaks 3−5, **Figure 6**). The matching retention times were confirmed with the use of three different HPLC gradient systems and two different chromatography columns (data not shown). Consequently, the results demonstrate that compounds **1** and **2** form the same xanthylium cations as those generated by (+)-catechin and glyoxylic acid. Intriguingly, this was the case whether compound **1** or **2** was incubated with (+)-catechin or not, but more xanthylium cations were generated if (+)-catechin was present (**Table 1**).

The actual mechanism for the conversion of compounds **1** and **2** to xanthylium cations is not certain and is the subject of ongoing investigations. However, the results do establish a mechanistic link between (+)-catechin, a degradation product of ascorbic acid, and the production of xanthylium cations (**Figure 7**) that has previously been postulated (*17*, *18*). The results also add xylosone to the list of aldehyde compounds (i.e., glyoxylic acid (*7*) and glyoxal (*32*)) that can react with (+)-catechin to generate the xanthylium cation pigments such as that shown in **Figure 7**. Such a mechanism will be relevant to all beverages that contain flavanols and ascorbic acid, such as fruit juices. Furthermore, it will also be particularly relevant to wines with relatively high xylosone concentration, such as those produced from grapes infected with *Botrytis cinerea* (*33*).

In conclusion, the results of this study show a unique outcome from the reactions of two "antioxidants" in wine-like conditions, ascorbic acid and (+)-catechin. The process is far more complex than the anticipated and simple transfer of two electrons and two protons and demonstrates that ascorbic acid is a highly unpredictable molecule capable of inducing a variety of reaction processes. From a model white wine system, two new "natural" products derived from (+)-catechin and ascorbic acid were isolated. The two compounds were unequivocally identified on the basis of spectral data, primarily NMR spectroscopy. A retrosynthetic study indicated that the compounds could be formed from L-xylosone, a known degradation product of ascorbic acid, and (+)-catechin. Both isomers were independently synthesized from L-xylosone to establish this compound as a key precursor to compounds **1** and **2**. The accumulation of the isomers (**1** and **2**) depends on the presence of ascorbic acid, and they eventually react further to give colored xanthylium cation pigments.

ABBREVIATIONS USED

HPLC-DAD, high-performance liquid chromatography with diode array detection; DMSO, dimethyl sulfoxide; UV, ultraviolet; HRMS, high-resolution mass spectrometry; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; ESI, electrospray ionization; NMR, nuclear magnetic resonance; FID, free induction decay; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence; INEPT, insensitive nuclei enhanced by polarization transfer; HMBC, heteronuclear multibond correlation; ROESY, rotating frame Overhauser effect spectroscopy.

ACKNOWLEDGMENT

We are indebted to John Allen from the (Research School of Chemistry) Australian National University for the high-resolution mass spectra.

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Received June 25, 2009. Revised manuscript received August 27, 2009. Accepted August 31, 2009. This project was supported by the Australian Grape and Wine Research and Development Corporation (CSU03/02). We acknowledge the Australian Research Council and Macquarie University for funding of the Macquarie University NMR Facility.