Rationalizing the Origin of Solerone (5-Oxo-4-hexanolide): Biomimetic Synthesis and Identification of Key Metabolites in Sherry Wine

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A biomimetic synthesis of solerone (5-oxo-4-hexanolide, **1**) using both enzymatic and acid-catalyzed reactions was performed. Starting from L-glutamic acid 5-ethyl ester (**2**) enzymatic oxidative deamination followed by subsequent decarboxylation of the corresponding 2-oxoglutaric acid 5-ethyl ester (**3**) led to ethyl 4-oxobutanoate (**4**). In the presence of pyruvate, **4** served as key substrate for a novel acyloin condensation catalyzed by pyruvate decarboxylase (EC 4.1.1.1) from *Saccharomyces cerevisiae*. Finally, the resulting ethyl 4-hydroxy-5-oxo-hexanoate (**5**) was easily converted into solerone (**1**) in the presence of acid. The acyloin condensation of **3** with acetaldehyde to ethyl 5-hydroxy-4-oxohexanoate (**6**) revealed an alternative route to solerone (**1**). Acid-catalyzed lactonization of **6** produced 4-oxo-5-hexanolide (**7**) as well as **5** and **1** via keto−enol tautomerization. Confirming the relevance of the proposed biogenetic pathway, the solerone precursors **2**−**6** as well as δ-lactone **7** were identified in sherry by GC/MS analysis for the first time.

Keywords: *Sherry; solerone; biogenesis; pyruvate decarboxylase*

INTRODUCTION

Solerone (5-oxo-4-hexanolide, **1**) is a known constituent of wines (Schreier, 1979), in particular, flor sherry (Augustyn et al., 1971). Recently, it has also been identified in dried figs (Näf et al., 1995). While the contribution of solerone (**1**) to the aroma of sherry is controversially discussed (Martin et al., 1991), it is generally accepted that **1** is enzymatically formed during the course of sherry fermentation under oxidative conditions. However, experimental information on both enzymes and key metabolites involved in the biosynthesis of **1** is rather scarce to date. ^{14}C-labeling experiments indicated the involvement of glutamic acid and ethyl 4-oxobutanoate (**4**) (Fagan et al., 1981; Wurz et al., 1988), but **4** has not been detected in sherry as yet. Results of the enantiodifferentiation of naturally occurring **1** and the structurally related solerol (5-hydroxy-4-hexanolide) allowed no conclusions with respect to the origin of **1** (Hollnagel et al., 1991; Guichard et al., 1992; Krajewski et al., 1995).

To rationalize the origin of solerone **1** through biomimetic synthesis and further identification of the metabolites involved, both enzymatic and acid-catalyzed reactions were performed. The results are described in this paper.

EXPERIMENTAL PROCEDURES

General. L-Glutamic acid 5-ethyl ester, thiamin diphosphate hydrochloride, L-amino acid oxidase (EC 1.4.3.2) from *Crotalus adamanteus* venom (0.52 unit/mg), catalase (EC 1.11.1.6) from bovine liver (38 000 units/mg of protein, 21 mg of protein/mL of enzyme preparation), pyruvate decarboxylase (EC 4.1.1.1) from *Saccharomyces carlsbergensis* (5.3 units/mg of protein, 15 mg of protein/mL of enzyme preparation), and baker's yeast (*Saccharomyces cerevisiae*, fast dried, type II) were purchased from Sigma Chemical Co. All commercial chemicals (Aldrich) used were of analytical grade quality, and the solvents were redistilled before use. NMR spectra were recorded on a Bruker WM 400 spectrometer.

Sherry Samples. Samples of two Spanish sherries were purchased in a grocery: Oloroso "Torre Breva, Orleans-Borbon" from Sanlucar de Barrameda, Spain, and Manzanilla "Domecq, Rio Viejo" from Jerez de la Frontera, Spain.

Isolation Procedures. *Method A.* Sherry (300 mL) was adjusted to pH 4.5 with 1.5 M NaOH and passed slowly down a cation-exchange column (Dowex 50-WX4, Serva, Na^+-form). The retained interfering matrix was removed by washing with 200 mL of distilled water. The amino acids were recovered with saturated NaCl solution and subsequently with a saturated NaCl solution adjusted to pH 10 with Na_2CO_3. The combined salt solutions were adjusted to pH 7, and water was removed at 40 °C *in vacuo*. The residue was directly used for the formation of trifluoroacetyl derivates of amino acids.

Method B. Sherry (300 mL) was saturated with NaCl and extracted with three batches of CH_2Cl_2 (300 mL; 16 h each). The extract was dried over anhydrous Na_2SO_4 and concentrated to 1.5 mL at 40 °C under reduced pressure.

Formation of Trifluoroacetyl Derivates. An aliquot (5.0 g) of the water-free residue (isolation method A) was suspended in 7 mL of dichloromethane. After addition of 0.5 mL of trifluoroacetic anhydride (TFAA), the sample was heated in a screw-capped vial (Teflon septum) at 120 °C for 5 min in an oil bath behind a safety shield. The solution was filtered off, concentrated *in vacuo*, and analyzed by GC/MS.

*Ethyl 3-[2′-(Trifluoromethyl)-5′(4′H)-oxazolon-4′-yl]propanoate (**8**).* L-Glutamic acid 5-ethyl ester (10 mg) was treated with TFAA as described above: MS, *m*/*z* (%) 253 (0.1), 225 (6), 208 (100), 181 (18), 180 (10), 167 (10), 152 (16), 136 (23), 122 (31), 116 (19), 100 (38), 86 (20), 84 (20), 82 (38), 72 (20), 69 (27), 55 (83), 54 (94), 44 (33), 41 (52).

Gas Chromatography/Mass Spectrometry (GC/MS). A Varian 3300 gas chromatograph equipped with a split injector (220 °C; 1:10) was directly coupled with a Finnigan MAT 44 mass spectrometer. Separation of volatiles was achieved on a J&W fused silica DB-Wax capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm, using helium as carrier gas). The temperature program was as follows: 3 min isothermal at 50 °C, raised to 240 °C at 4 °C/min. The temperature of the ion source and all connection parts was 220 °C, the electron energy for all EI mass spectra was 70 eV, and the cathodic current was 0.7 mA.

Biomimetic Synthesis and Preparation of Reference Compounds. *2-Oxoglutaric acid 5-ethyl ester* (**3**) was synthesized by treatment of 13 mM L-glutamic acid 5-ethyl ester (**2**) in 70 mL of NaH_2PO_4/Na_2HPO_4 buffer (pH 7.2) with 6 units of L-amino acid oxidase (safety information as provided by the

S0021-8561(96)00316-0 CCC: $14.00

supplier should be taken into consideration) in the presence of 800 units of catalase according to the procedure of Meister (1952), yielding 68% **3**: MS, *m/z* (%) 128 (18), 119 (5), 102 (6), 101 (100), 100 (8), 74 (19), 73 (46), 56 (24), 55 (55), 45 (48), 43 (15); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26 (t, $J_{1',2'} = 7.1$ Hz, 2′-H), 2.72 (t, $J_{3,4} = 6.4$ Hz, 4-H), 3.21 (t, 3-H), 4.15 (q, 1′-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1 (C-2′), 28.0 (C-4), 32.7 (C-3), 61.2 (C-1′), 159.8 (C-5), 172.1 (C-1), 194.2 (C-2).

*Ethyl 4-Oxobutanoate (**4**).* The reaction mixture contained 2-oxoglutaric acid 5-ethyl ester (30 mM), pyruvate decarboxylase (0.17 unit/mL), thiamin diphosphate (5 mM), and $MgSO_4$ (5 mM) in 1 mL of 0.2 M citrate buffer (pH 6.0). After 24 h at room temperature, the reaction was terminated and the solution was extracted with dichloromethane, dried over anhydrous Na_2SO_4, and concentrated in a stream of dry nitrogen for GC/MS analysis. A conversion rate of 70 % was measured for the decarboxylation product **4**. When the same procedure was repeated with 2-oxoglutaric acid instead of ester **3**, no decarboxylation product was detected.

Accordingly to Suomalainen (1968) we decarboxylated **3** by baker's yeast, yielding 62% **4**. Additionally, **4** was synthesized from γ-butyrolactone (Banerji et al., 1987); its spectral properties were in good agreement with data previously published (Smith et al., 1991; Kuehne and Pitner, 1989).

*Ethyl 4-Hydroxy-5-oxohexanoate (**5**). Method A (Biotransformation by Intact Cells, cf. Ohta et al., 1992).* The fermenting medium consisted of 2.0 g of dried baker's yeast (prior to incubation suspended in 10 mL of distilled water for 15 min), ethyl 4-oxobutanoate (77 mM), sodium pyruvate (1.5 M), and acetaldehyde (180 mM). After 24 h of stirring at room temperature, the yeast was filtered off with Celite. The filtrate was saturated with NaCl, extracted with dichloromethane, dried over anhydrous Na_2SO_4, and concentrated to 1 mL at 40 °C *in vacuo*. Besides acetoin and γ-butyrolactone, minor amounts of acyloin condensation product **5** (2%) were formed.

Method B (Enzymatic Synthesis). Pyruvate decarboxylase (0.1 unit/mL) was presaturated with thiamin diphosphate (0.05 mM) and $MgSO_4$ (1.0 mM) in 1.0 mL of 0.2 M citrate buffer (pH 6.0). After 30 min of stirring, ethyl 4-oxobutanoate (10 mM) and sodium pyruvate (30 mM) were added and stirring was continued for 3 days at room temperature. Saturation with NaCl was followed by extraction with dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in a stream of dry nitrogen. Subsequent GC/MS analysis revealed a conversion rate of 4% to ketol **5**. When the same procedure was repeated with 4-oxobutyric acid instead of ester **4**, no acyloin condensation product was detected.

Method C (Chemical Synthesis). To a solution of 3.9 mmol (500 mg) of solerone in 1.7 mL of ethanol was added 0.017 mmol of concentrated H_2SO_4. After 19 h of stirring at room temperature, the pH was adjusted to 7.0 by 0.034 mmol of $CaCO_3$. Stirring was continued for another hour, the suspension was filtered off, and the solvent was evaporated. The yellowish oil (204 mg) still contained about 10% solerone and other byproducts. Samples for spectroscopic purposes were purified by HPLC on a silica column (Eurosphere Si 100, Knauer, Berlin, Germany, 250 × 4 mm, 5 μm, methyl *tert*-butyl ether/pentane 7/3 to 10/0 in 30 min; detection at 275 nm): MS, *m/z* (%) 131 (13), 129 (10), 111 (4), 88 (5), 85 (100), 60 (5), 57 (25), 55 (14), 45 (14), 44 (10), 43 (76), 40 (24); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (t, $J_{1',2'} = 7.1$ Hz, 2′-H), 1.71−1.85 (m, 3-H), 2.26 (s, 6-H), 2.44−2.58 (m, 2-H), 4.15 (q, 1′-H), 4.21−4.27 (m, 4-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1 (C-2′), 25.2 (C-6), 28.5/29.5 (C-2/C-3), 60.6 (C-1′), 75.7 (C-4), 173.2 (C-1), 209.4 (C-5).

*Solerone (**1**). Method A.* (*S*)-Solerone (**1**) was prepared from L-glutamic acid as published previously (Berti et al., 1983). The spectroscopic data of **1** did correspond to those published by Mosandl and Hollnagel (1989).

Method B. An aqueous solution of 10 mg of hydroxy ester **5** was mixed with catalytic amounts of concentrated H_2SO_4 and stirred for 15 h at room temperature. The mixture was extracted with dichloromethane, and the combined organic layers were dried (anhydrous Na_2SO_4) and concentrated in a stream of nitrogen. GC/MS analysis showed equimolar amounts of **5** and **1**.

Ethyl 5-Hydroxy-4-oxohexanoate (**6**). *Method A (Enzymatic Synthesis).* Pyruvate decarboxylase (0.5 unit/mL) was presaturated with thiamin diphosphate (0.05 mM) and $MgSO_4$ (1.0 mM) in 1 mL of 0.2 M citrate buffer (pH 6.0). After 30 min of stirring, 2-oxoglutaric acid 5-ethyl ester (**3**) (10 mM) and acetaldehyde (50 mM) were added and stirring was continued for 3 days at room temperature. Saturation with NaCl was followed by extraction with dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in a stream of dry nitrogen prior to GC/MS analysis. Besides ketol **6** (4% yield) the decarboxylation product **4** was found.

Method B (Chemical Synthesis). The ethanolysis of 0.2 mmol of 4-oxo-5-hexanolide (**7**) in 3 mL of ethanol was accomplished after 30 min of refluxing with catalytic amounts of concentrated HCl. The solution was neutralized with $CaCO_3$ and dried (anhydrous Na_2SO_4). Compound **6** was purified by flash chromatography on silica gel (diethyl ether/pentane 5/5 to 9/1), and a colorless oil was received (34% yield): MS, *m/z* (%) 146 (1), 131 (2), 130 (2), 129 (30), 111 (6), 102 (32), 101 (50), 85 (13), 75 (5), 74 (24), 73 (20), 57 (14), 56 (17), 55 (34), 45 (100), 43 (59); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26 (t, $J_{1',2'} = 7.1$ Hz, 2′-H), 1.42 (d, $J_{5,6} = 7.0$ Hz, 6-H), 2.61−2.77 (m, 2-H, 3-H), 4.13 (q, 1′-H), 4.31 (q, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1 (C-2′), 19.8 (C-6), 28.0 (C-2), 32.2 (C-3), 60.8 (C-1′), 72.8 (C-5), 172.3 (C-1), 210.8 (C-4). GC/MS analysis of the crude oil revealed about 40% **5** and 5% **1** as byproducts.

*4-Oxo-5-hexanolide (**7**).* An aqueous solution of 1 mg of hydroxy ester **6** was mixed with catalytic amounts of concentrated H_2SO_4 and stirred for 16 h at room temperature. The mixture was extracted with CH_2Cl_2, and the combined organic layers were dried over Na_2SO_4 and concentrated in a stream of dry nitrogen. The product distribution was monitored by GC/MS showing 60% **7**, 30% **1**, and 10% **5**. Following a simplified procedure of Georgiadis et al. (1991), the δ-lactone **7** was additionally synthesized starting from 2-acetylfuran: MS, *m/z* (%) 128 (10), 100 (6), 85 (2), 84 (3), 60 (2), 57 (6), 56 (100), 55 (7), 45 (17), 44 (9), 43 (50), 42 (25); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.9 (C-6), 28.3 (C-2), 33.2 (C-3), 79.5 (C-5), 169.8 (C-1), 205.2 (C-4). ^{1}H NMR data were identical with those from Georgiadis et al. (1991).

RESULTS AND DISCUSSION

Biomimetic Synthesis of Solerone. We applied pyruvate decarboxylase (EC 4.1.1.1) (PDC) as key enzyme for the biomimetic synthesis elucidating the formation of solerone **1** (Figure 1). The thiamin diphosphate dependent enzyme from *S. cerevisiae* is responsible for the decarboxylation of pyruvate during the course of alcoholic fermentation. After loss of carbon dioxide from 2-oxoacids, the resulting aldehyde is released. Alternatively, the cofactor-bound decarboxylation product can react further with an another aldehyde. By the latter acyloin condensation a new carbon−carbon bond will be formed, thus opening a biosynthetic way to α-hydroxy carbonyl compounds (Koga, 1995; Kren et al., 1993).

While in the presence of 2-oxoglutaric acid neither decarboxylation nor acyloin condensation has been observed, as one could expect from results published previously (Suomalainen et al., 1969), we succeeded in the enzymatic conversion of the mono ethyl ester **3** to ethyl 4-oxobutanoate (**4**), using both whole yeast cells (*S. cerevisiae*) and purified PDC. The oxo ester **4** served as substrate for a second reaction catalyzed by PDC. Formation of a new carbon−carbon bond was accomplished in the presence of pyruvic acid, which acted as donor of a C_2 unit. Thus, ethyl 4-hydroxy-5-oxohexanoate (**5**) was obtained for the first time as the

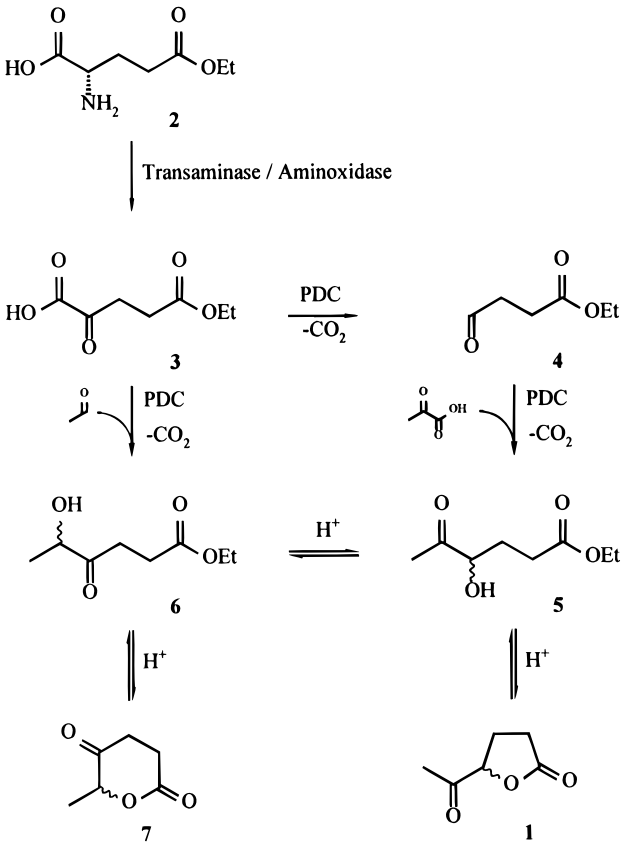

Figure 1. Proposed biogenesis of solerone (**1**) in sherry.

result of an enzymatic acyloin condensation. Finally, traces of acid induced the lactonization of hydroxy ester **5**, indicating it as direct precursor of solerone (**1**) (Figure 1).

These results demonstrated the importance of the ethyl ester function, which has not been given attention in previous discussions on the biogenesis of **1** (Muller et al., 1972, 1973). Consequently, we focused our interest on glutamic acid 5-ethyl ester (**2**) as potential precursor of the corresponding 2-oxoglutaric acid 5-ethyl ester (**3**). Our view was supported by earlier ^{14}C-labeling experiments, in which the involvement of L-glutamic acid in the biosynthesis of **1** has been indicated (Fagan et al., 1981; Wurz et al., 1988). In addition, diethyl glutamate has already been identified in sherry (Heresztyn, 1984; Herraiz and Ough, 1992). While amino acids can be transformed to 2-oxoacids by pyridoxal-dependent transaminases as well, we applied the oxidative deamination of **2** catalyzed by L-amino acid oxidase (EC 1.4.3.2) (Meister, 1952). The use of an oxygen-electrode enabled direct monitoring of the reaction progress. Hydrogen peroxide had to be destroyed with catalase (EC 1.11.1.6) to avoid oxidative decarboxylation and degradation of **3**. By this enzymatic oxidation reaction 5-ethyl 2-oxoglutarate (**3**) was obtained with excellent yield.

The proposed biosynthetic pathway describing the transformation of ethyl glutamate (**2**) to solerone (**1**) via ethyl 4-oxobutanoate (**4**) is in good agreement with previously reported radiotracer experiments (Freeman et al., 1977; Fagan et al., 1981; Wurz et al., 1988). In addition, we evaluated another yet unknown route to solerone (**1**). Starting from 2-oxoglutaric acid 5-ethyl ester (**3**), the PDC-catalyzed acyloin condensation with acetaldehyde yielded in one step ethyl 5-hydroxy-4-oxohexanoate (**6**). Acid-catalyzed lactonization of **6** led

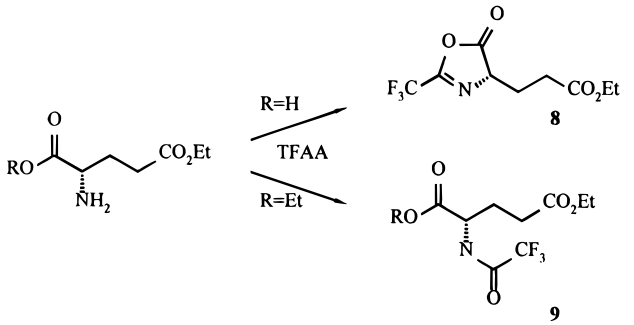

Figure 2. Trifluoroacetylation of glutamic acid derivates with TFAA prior to GC/MS analysis.

Table 1. Solerone and Key Progenitors As Identified in Sherry Samples

compound	Ri[a]	concn[b]
L-glutamic acid 5-ethyl ester (**2**)	1983[c]	+
solerone (**1**)	2113	+
ethyl 4-hydroxy-5-oxohexanoate (**5**)	2135	+
ethyl 5-hydroxy-4-oxohexanoate (**6**)	2189	+
4-oxo-5-hexanolide (**7**)	2194	+
ethyl 4-oxobutanoate (**4**)	2280	+
2-oxoglutaric acid 5-ethyl ester (**3**)	2374	+++

[a] Linear retention indices based on a series of hydrocarbons. For details, see Experimental Procedures. [b] Relative data evaluated by addition of external standard (2.0 mg/L 2-undecanol; Ri = 1704): +, <0.5 mg/L; ++, 0.5−10 mg/L; +++, 10−50 mg/L. The concentrations were comparable in both sherry wines. [c] Ri of the trifluoroacetyl derivate **8**.

to 4-oxo-5-hexanolide (**7**). As byproducts, substantial amounts (up to 40%) of ethyl 4-hydroxy-5-oxohexanoate (**5**) and solerone (**1**) were formed. A similar rearrangement yielding solerol (5-hydroxy-4-hexanolide) has been observed on storage of 4-hydroxy-5-hexanolide (Hollenbeak and Kuehne, 1974).

Identification of the Proposed Key Metabolites 2−7 in Sherry Wine. To identify the postulated metabolites in sherry wine, a Manzanilla sherry and an Oloroso sherry were analyzed. Neutral and acidic sherry constituents were analyzed by GC/MS after solvent extraction. Selective extraction of amino acid ethyl ester **2** was achieved using a strongly acidic cation exchange resin. To avoid the artifactual formation of ethyl esters, the method described by Herraiz and Ough (1992) was chosen. Control experiments revealed no amino acid ethyl ester formation during extraction. Prior to GC/MS analysis mono ethyl ester **2** had to be derivatized with TFAA, yielding ethyl 3-[2′-(trifluoromethyl)-5′(4′*H*)-oxazolon-4′-yl]propanoate (**8**) (Bergman and Lidgren, 1989). Diethyl glutamate was simultaneously detected as *N*-trifluoroacetyl derivate **9** (Figure 2). For the first time, L-glutamic acid 5-ethyl ester (**2**), 2-oxoglutaric acid 5-ethyl ester (**3**), ethyl 4-oxobutanoate (**4**), ethyl 4-hydroxy-5-oxohexanoate (**5**), ethyl 5-hydroxy-4-oxohexanoate (**6**), and 4-oxo-5-hexanolide (**7**) were identified in sherry by comparison of chromatographic and spectroscopic data with those of authentic reference substances. The newly identified compounds **2**−**7** are listed in Table 1.

Conclusion. The biogenesis of solerone **1** and related compounds was successfully rationalized by biomimetic model reactions. As key step we established the pyruvate decarboxylase catalyzed acyloin condensation of pyruvic acid with ethyl 4-oxobutanoate (**4**) or ethyl 2-oxoglutarate (**3**) with acetaldehyde. The importance of the ethyl ester function in **3** and **4** serving as substrates for the enzymatic formation of α-hydroxy

ketones **5** and **6** was demonstrated. The distinct equilibrium between isomeric acyloins **5** and **6** and the corresponding lactones was disclosed. Finally, the identification of six yet unknown sherry compounds including acyloins **5** and **6**, which have been synthesized for the first time, confirmed the relevance of the biosynthetic pathway.

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Received for review May 1, 1996. Accepted November 8, 1996.[⊗] M.H. acknowledges generous funding provided by the Deutsche Forschungsgemeinschaft DFG, Bonn (He 2599/1-1), and the Deutscher Akademischer Austauschdienst DAAD. Furthermore, the Australian Wine Research Institute (AWRI, Adelaide) and Grape and Wine Research and Development Council are thanked for support.

JF960316J

[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1997.