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Rates of Formation of *cis*- and *trans*-Oak Lactone from 3-Methyl-4-hydroxyoctanoic Acid

KERRY L. WILKINSON,[†,‡] GORDON M. ELSEY,*[,†,‡] ROLF H. PRAGER,[†] ALAN P. POLLNITZ,[‡] AND MARK A. SEFTON[‡]

School of Chemistry, Physics and Earth Sciences, Flinders University of South Australia, P.O. Box 2100, Adelaide, South Australia 5001, Australia, and The Australian Wine Research Institute, P.O. Box 197, Glen Osmond, South Australia 5064, Australia

The rates of formation of both *cis*- and *trans*-oak lactone from the corresponding isomers of 3-methyl-4-hydroxyoctanoic acid have been measured in model wine at room temperature for a range of pH values. The half-life for formation of the *trans*-isomer at pH 2.9 was calculated to be 3.1 h, whereas that for the *cis*-isomer, at the same pH, was calculated to be 40.5 h. The k_{trans}/k_{cis} ratio in model wine was found to 12.86 ± 1.34 over the range of pH values employed. A reason for the more facile formation of the *trans*-isomer, based on conformational reasons, has been proposed. In acidic aqueous media the equilibrium between the oak lactones and their corresponding ring-opened analogues was found to favor the former entirely, with no evidence for the latter being found. Implications of the present study for the future analysis of oak samples, as well as for the interpretation of existing data, are discussed.

KEYWORDS: Oak; wine; oak lactone; whiskey lactone; kinetics; lactonization

INTRODUCTION

The *cis*-isomer of the so-called oak lactones, (4*S*,5*S*)-5-butyl-4-methyl-4,5-dihydro-2(3*H*)-furanone (**1**) (**Figure 1**), is regarded as among the most important of the volatile components of oakwood that are extracted into wine or spirits during barrel-aging. Recent experiments (*1*) have shown that the nature-identical enantiomer of *cis*-oak lactone had aroma detection thresholds of 23 μg/L in a dry white wine and 46 μg/L in a red wine, although there appeared to be a substantial variation in the sensitivity of individual panelists to this compound.

There are many factors that influence the amount of *cis*- and *trans*-oak lactone that can be extracted from oak. They include the composition of the unseasoned wood, the seasoning and toasting processes, and the length of time alcoholic beverages spend in oak cooperage [the literature on *cis*- and *trans*-oak lactone has been comprehensively reviewed (*2*)]. Such studies indicate that one or more oak lactone precursors, present in oakwood, are able to generate oak lactone during these processes. Otsuka et al. (*3*) reported the isolation of a 3-methyl-4-hydroxyoctanoic acid derivative, which they termed an oak lactone precursor, and proposed the methylated gallic acid derivative **2** (**Figure 1**) as the structure for this compound. However, an authentic sample of this structure synthesized in our laboratory had completely different properties from the isolated compound (*4*). More recently, Masson et al. (*5*) identified a galloylated glucoside of (3*S*,4*S*)-3-methyl-4-hydroxyoctanoic acid as a component of oakwood. Both this compound **4** and the simple glucoside **3** are able to generate *cis*-oak lactone during barrel-toasting, and perhaps also during seasoning, but not during wine conservation in the absence of enzymic activity (*1*).

Singleton (*6*) has described cases in which the oak aroma in wine continued to intensify for some time after the removal of oak chips. Were such intensification of aroma due to increases in *cis*-oak lactone concentration, then this would also indicate the presence in the wines of a precursor form other than the glucoside **3** or its derivative **4**, which are essentially unreactive under wine conservation conditions. Pollnitz (*7*) has also observed small increases in *cis*- (but not *trans*-) oak lactone concentration in model wine extracts of oak chips following the removal of the chips from the wine. A potential candidate for the continued formation of *cis*-oak lactone in wine following the removal of oak contact is the aglycon component of **3**, 3-methyl-4-hydroxyoctanoic acid (**6**). This compound could quite conceivably form in oakwood as a result of cleavage of the glucose unit (from **3**) or the galloylglucose unit (from **4**), during seasoning or toasting, via chemical or biological processes.

In a study of wines fermented and then stored in oak cooperage, Waterhouse and Towey (*8*) reported significant differences in the ratio of *cis/trans*-oak lactone isomers between wines stored in American compared with French barrels. These authors discussed the factors that might be responsible for the changes, observed by other authors, in the *cis/trans* ratio or in

* Corresponding author (telephone +61 8 8201 3071; fax +61 8 8201 2905; e-mail Gordon.Elsey@flinders.edu.au).
† Flinders University.
‡ The Australian Wine Research Institute.

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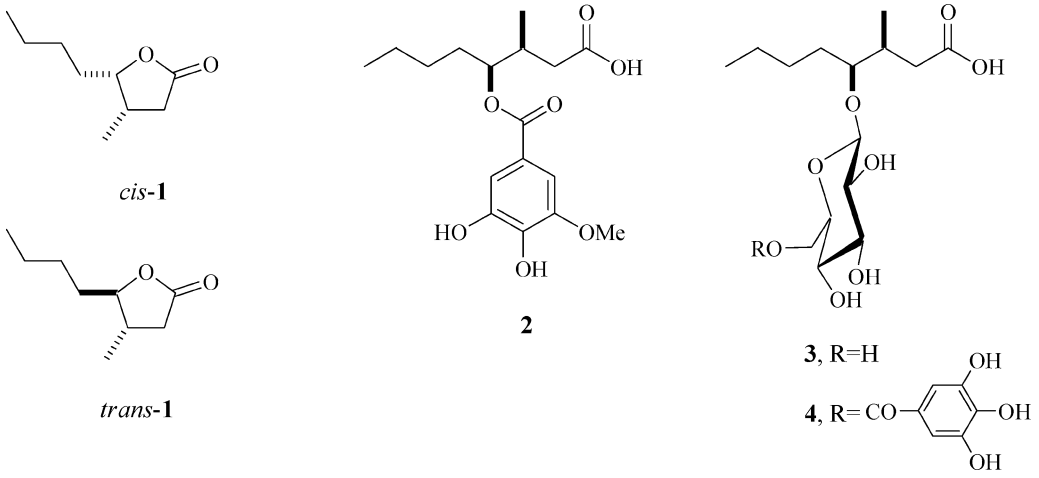

Figure 1. Structures of the oak lactones (**1**), proposed precursor **2**, and isolated precursors **3** and **4**.

the amounts of extractable oak lactone which result from toasting or seasoning. Waterhouse and Towey speculated that such observations are a result of chemical equilibria between the oak lactones (**1**), their open-chain hydroxy acid equivalents **6**, and the ethyl esters of such hydroxy acids. Maga (*2*) has stated that acceptance of this hypothesis would require the disregarding of all previous data in relation to the roles played by *cis*- and *trans*-oak lactone in alcoholic beverage flavor, because such data "were subject to artifacts inherent in the analytical process".

This study was undertaken to determine (i) the rate of lactonization of 4-hydroxy-3-methyloctanoic acid; (ii) the equilibria among oak lactone, the corresponding hydroxy acid, and the ethyl ester of the hydroxy acid for each of the *cis*- and *trans*-isomers; (iii) the effects of pH and ethanol concentration in these studies; and (iv) the implications of the results for carrying out analysis of oak extracts and interpreting the results of previously published analyses.

MATERIALS AND METHODS

Chemicals were purchased from Sigma-Aldrich. All solvents used were of HPLC grade from OmniSolv (Darmstadt, Germany). All organic solvent solutions were dried over anhydrous sodium sulfate before being filtered. Oak lactone was quantified by the SIDA method reported previously (*9*). pH measurements were made with an EcoScan pH 5/6 meter (Eutech Instruments, Singapore), which was calibrated before use.

Preparation of Potassium 3-Methyl-4-hydroxyoctanoate Stock Solution for Lactonization Study. To a 50:50 mixture of racemic *cis*-**1** and racemic *trans*-**1** (1.0061 g, 6.45 mmol) in ethanol (10 mL) was added a solution of potassium hydroxide (347 mg, 6.19 mmol) in water (20 mL). After 16 h at room temperature, water (50 mL) was added, followed by extraction with diethyl ether (3 × 25 mL) to remove residual oak lactone. The combined ether extracts were quantified for oak lactone and revealed the presence of *cis*-oak lactone (108 mg) and *trans*-oak lactone (74 mg). The aqueous solution [containing *trans*-**5** (584 mg) and *cis*-**5** (537 mg)] was diluted with water to 100 mL. This stock solution therefore comprised 5.84 g/L *trans*- and 5.37 g/L *cis*-potassium 3-methyl-4-hydroxyoctanoate. This corresponds to a total concentration of **5** of 11.21 g/L.

Preparation of Potassium 3-Methyl-4-hydroxyoctanoate Stock Solution for pK_a Determinations. To a 50:50 mixture of racemic *cis*-**1** and racemic *trans*-**1** (1.0134 g, 6.49 mmol) in ethanol (4 mL) was added a solution of potassium hydroxide (303 mg, 5.40 mmol) in water (20 mL). After 72 h at room temperature, water (50 mL) was added, followed by extraction with diethyl ether (3 × 25 mL) to remove residual oak lactone. The combined ether extracts were quantified for oak lactone and revealed the presence of *cis*-oak lactone (118 mg) and *trans*-oak lactone (78 mg). The aqueous solution [containing *trans*-**5** (582 mg) and *cis*-**5** (528 mg)] was diluted with water to 100 mL. This stock solution therefore comprised 5.82 g/L *trans*- and 5.28 g/L *cis*-potassium 3-methyl-4-hydroxyoctanoate. This corresponds to a total concentration of **5** of 11.10 g/L.

Preparation of Oak Lactone Solution. A stock solution of oak lactone was prepared by dissolving a 50:50 mixture of racemic *cis*-**1** and racemic *trans*-**1** (~1.5 mg) in ethanol (250 mL). The total oak lactone concentration was accurately measured as 5.32 mg/L, by gas chromatography−mass spectrometry (GC-MS) quantification (*9*).

Determination of pK_a of 3-Methyl-4-hydroxyoctanoic Acid (6). An aqueous solution of potassium 3-methyl-4-hydroxyoctanoate (111.0 mg, 0.523 mmol) was prepared by diluting the stock solution of **5** (10 mL, 11.10 g/L) to a total volume of 100 mL with water. To this was added aqueous hydrochloric acid (0.1M) with stirring, at 25 °C. The pH of the solution was measured after the addition of 2.11 mL (0.4 equiv), 2.61 mL (0.5 equiv), and 3.11 mL (0.6 equiv). The pK_a of **6** was measured in both model wine (12% ethanol) and model spirit (40% ethanol) in an identical manner.

Effect of Sodium Bicarbonate Addition on Oak Lactone Concentration. Model wine (10% ethanol) solutions of **1** (532 μg/L) were prepared by diluting the stock solution of **1** (1 mL, 5.32 mg/L) to a total volume of 10 mL with water. To this solution was added saturated sodium bicarbonate (5 mL). The solution was then sealed in glass ampules (2 mL). Samples were analyzed (in duplicate) for oak lactone content by GC-MS using SPME, after 1, 2, and 7 days at room temperature.

Effect of Sodium Bicarbonate Addition on 3-Methyl-4-hydroxyoctanoic Acid Concentration. Model wine (10% ethanol) solutions of **6** (10 mL, 412 μg/L) were prepared by diluting the stock solution of **5** (50 μL, 11.21 mg/mL) to a total volume of 10 mL with ethanol (1 mL) and water. To this solution was added saturated sodium bicarbonate (5 mL). The solution was then sealed in glass ampules (2 mL). Samples were analyzed (in duplicate) for oak lactone content by GC-MS using SPME, after 1, 2, and 7 days at room temperature.

Determination of Lactonization Rates for the Conversion of 6 into 1. Model wine (12% ethanol) solutions of **6** (*cis*, 441 μg/L; *trans*, 479 μg/L) were prepared by diluting the stock solution of **5** (1 mL; *cis*, 5.37 g/L; *trans*, 5.84 g/L) with ethanol (12 mL) and a buffer solution of the appropriate pH to a total volume of 100 mL. For a desired pH of 2.9, it was found that a buffer at pH 2.8 was required, with the final pH being obtained after addition of the ethanol solution of **5**. This relationship between added buffer pH and the final obtained pH was consistent over the pH range utilized in this study. Aliquots (5 mL) were taken (in duplicate) over time, quenched with a saturated solution of sodium bicarbonate (5 mL), and analyzed using SPME by GC-MS for oak lactone content as described by Pollnitz et al. (*9*). Additionally, the extent of lactonization of **6** was investigated in model wine at pH 1.0 (accurate rates were not determined) and showed that both isomers reached completion within 2 h. The rates of lactonization in model spirit (40% ethanol) were measured in an analogous manner. For this medium, a buffer solution of pH 5.0 was employed, which corresponded to a final pH of 5.5 after dilution.

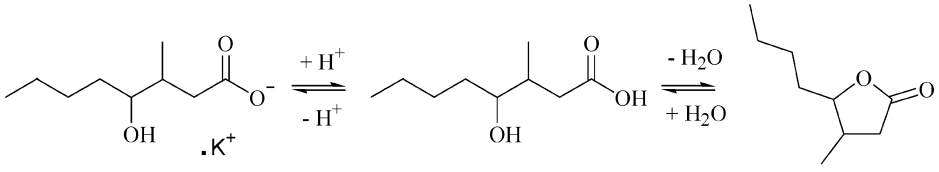

Figure 2. Formation of oak lactones (**1**) from the open-chain carboxylate species **5**.

Oak Lactone Stability in Model Wine. Model wine (10% ethanol) solutions of **1** (532 μg/L) were prepared by diluting the stock solution of **1** (1 mL, 5.32 mg/L) to a total volume of 10 mL with either pH 1 or pH 2.8 buffer solutions. Samples were analyzed (in duplicate) for oak lactone content by GC-MS using SPME, after 0, 2, 6, and 336 h at room temperature.

Studies on the Formation of Ethyl 3-Methyl-4-hydroxyoctanoate in Model Wine. Model wine (10% ethanol) solutions of **1** (532 μg/L), prepared by diluting the stock solution of **1** (10 mL, 5.32 mg/L) to a total volume of 100 mL with pH 2.8 buffer solution, and **6** (*cis*, 441 μg/L; *trans*, 479 μg/L), prepared by diluting the stock solution of **5** (1 mL; *cis*, 5.37 g/L; *trans*, 5.84 g/L) to a total volume of 100 mL with ethanol (10 mL) and pH 2.8 buffer solution, were analyzed by GC-MS using SPME, in both SCAN and selected ion monitoring (SIM) modes, after 28 days at room temperature.

RESULTS AND DISCUSSION

Methodology Employed. The approach taken to determine the equilibrium constants and reaction rates for the processes shown in **Figure 2** was based on two assumptions. First, under acidic conditions, the first step (protonation of the carboxylate species **5**) occurs rapidly. As the pK_a of the resulting acid **6** is expected to be similar to that of other carboxylic acids, it is unlikely to undergo significant reionization. The pK_a of **6** can then be determined according to the Henderson−Hasselbach equation by direct measurement of the pH at 50% neutralization, that is, when the concentrations of acid and base are exactly equal:

$$\mathrm{pH} = \mathrm{p}K_a - \log_{10}([\mathrm{HA}]/[\mathrm{A}^-])$$

The second assumption was that the second step from **Figure 2**, cyclization to produce oak lactone, would be relatively slow. This would allow the rate of lactonization to be determined by "freezing" the reaction at various intervals and measuring the amount of oak lactone formed. We proposed to quench the lactonization reaction by neutralizing the mixture, through the addition of saturated sodium bicarbonate solution. That the quenching procedure itself would not influence the concentration of oak lactone was established by adding an excess of saturated sodium bicarbonate to model wine solutions of either **1** or the open-chain derivative **6**. The oak lactone content of these solutions was then monitored by GC-MS after 1, 2, and 7 days. The solution of **1** was unaffected by added sodium bicarbonate after 2 days, although a small decrease ($\leq$10%) was observed after 7 days. Furthermore, only trace amounts of oak lactone could be detected in the solutions of **6**, even 7 days postquenching. Therefore, samples taken during the lactonization rate study were quenched with saturated sodium bicarbonate solution and subsequently analyzed by GC-MS within 24 h, without exception. Because the quantification method utilized (*9*) allows the concentrations of both *cis*- and *trans*-oak lactone to be independently measured during a single analysis, the rate study was performed on an oak lactone sample comprising a 50:50 mixture of racemic *cis*- and *trans*-oak lactone. Additionally, to avoid artifactual formation of oak lactone in the GC-MS injector block, as observed by Pollnitz et al. (*9*), samples were analyzed by solid-phase microextraction (SPME) of the headspace, rather than by liquid−liquid extraction.

Table 1. Measured pH and pK_a Values for **6** in a Variety of Media

	medium[a]			
	HCl added[b]	water	model wine	model spirit
	0.4	4.45	4.77	5.51
pH[c]	**0.5**[d]	**4.26**	**4.57**	**5.29**
	0.6	4.06	4.34	5.04

[a] Model wine, 12% (v/v) EtOH in H_2O; model spirit, 40% (v/v) EtOH in H_2O. [b] Molar equivalents of HCl added relative to **5**. [c] Mean values from two replicates. [d] pH = pK_a at this point, according to Henderson–Hasselbach equation.

Table 2. Calculated Rate Constants, Half-Lives, and Rate Ratios for the Conversion of **6** into **1** at 25 °C

		cis-**1**		*trans*-**1**		
medium[a]	pH[b]	10^3k[c,d]	$t_{1/2}$[e]	10^3k	$t_{1/2}$	k_{trans}/k_{cis}
model wine	2.9	17.1	40.5	222.5	3.1	13.0
	3.1	10.3	67.3	139.0	5.0	13.5
	3.3	8.0	86.6	104.8	6.6	13.1
	3.5	4.2	165.0	44.4	15.6	10.6
	3.7	1.8	385.1	25.3	27.4	14.0
						12.86 ± 1.34[f]
model spirit	5.5	0.1	6931.0	2.1	330.1	21.0

[a] Model wine, 12% (v/v) EtOH in H_2O; model spirit, 40% (v/v) EtOH in H_2O. [b] pH error, ±0.05. [c] Units of k, h^{-1}. [d] Correlation coefficients were >0.99 in all cases, except for *cis*-oak lactone formation in model spirit, for which the value was 0.978. [e] Units of $t_{1/2}$, h. [f] Error expressed as standard deviation.

Measurement of pK_a Values of 3-Methyl-4-hydroxyoctanoic Acid (6). The pK_a of a 50:50 mixture of both *threo*- and *erythro*-3-methyl-4-hydroxyoctanoic acid (**6**) was determined by measuring the pH of an aqueous solution of the potassium salt **5**, upon the addition of hydrochloric acid. The pH and pK_a values are collected in **Table 1**. The value for **6** in aqueous solution, 4.26, is similar to that for acetic acid, 4.76 (*10*), supporting the first assumption outlined above. As can be observed in **Table 1**, as the ethanol content of the medium increases, there is a concomitant increase in the value of pK_a. This has been observed before, in connection with the pK_a of substituted aryl carboxylic acids (*11*), and is almost certainly a reflection of the lower ionizing power of the solution as the aqueous content is reduced.

Measurements of Rate Constants for Conversion of 6 into 1. A model wine solution of **6** was prepared from a solution of **5** and a potassium hydrogen tartrate buffer solution adjusted to the required pH. Aliquots were taken at various intervals, quenched by the addition of saturated sodium bicarbonate, and analyzed by GC-MS to determine the concentrations of both oak lactones. The pH values, rate constants, half-lives, and *trans*/*cis* ratios are collected in **Table 2**. The solution showed good first-order kinetics for the formation of both *cis*-oak lactone and *trans*-oak lactone, as evidenced by the excellent r^2 values

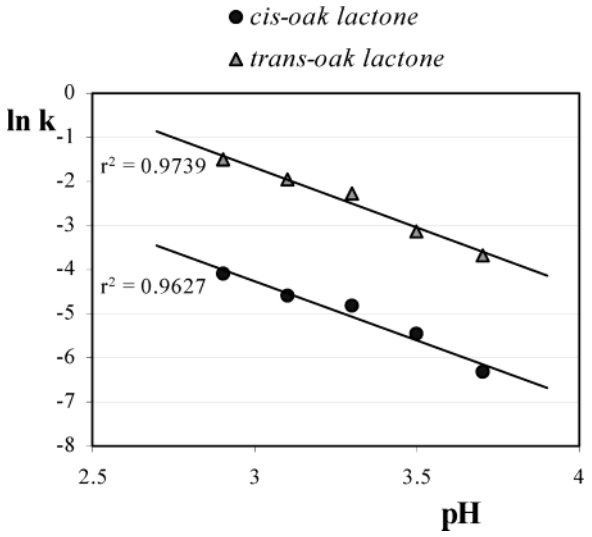

Figure 3. Plot of ln *k* versus pH for formation of both *cis*- and *trans*-oak lactone.

obtained. As expected for this acid-mediated reaction, the rate is strongly influenced by the pH of the medium (**Figure 3**).

A clear difference in rates of lactonization is evident between the isomers. The ratio of the rates changes little, irrespective of the medium or the pH. In the case of the rates measured in the model spirit, the value for *cis*-oak lactone is based on data collected over substantially less than 1 half-life (24 days of monitoring, compared with a calculated half-life of ≈300 days). The correlation coefficient for this particular measurement is slightly lower than the others, allowing one to suggest that the slightly higher ratio for this medium (k_{trans}/k_{cis} = 21.0) is a manifestation of the less accurate measurement of the rate constant for *cis*-oak lactone in this solution. This difference in reactivity between the isomers was also observed during the synthesis of the glycoconjugates of **6** (*1*). One possible reason for the more facile ring closure to the *trans*-isomer can be seen by considering the Newman projections for each isomer of **6** (**Figure 4**). For this isomer, lactonization must proceed from a conformer such as **A** or **C**, that is, where the C_1 carbonyl and C_4 hydroxyl groups are in close proximity. Conformer **A** is favored, having only two gauche interactions, whereas conformers **B** and **C** each have three such interactions. Ring closure, therefore, occurs from the most populated conformer. However, for the *cis*-isomer, lactonization must proceed from a conformer such as **D** or **E**, but the preferred conformer is actually **F**. Ring closure is now occurring from the least populated conformer and would therefore be expected to be slower, relative to the *trans*-isomer. An alternative explanation is that there is an unfavorable steric interaction between the methyl and butyl groups in both the transition state and product for formation of the *cis*- but not the *trans*-isomer.

Implications for Previous Studies. Finally, we wished to address the points raised by Waterhouse and Towey (*8*). To investigate the equilibria between oak lactone (**1**), the corresponding hydroxy acid **6**, and the ethyl ester of **6** for each of the *cis*- and *trans*-isomers, aqueous ethanol solutions of oak lactone at pH 1.0 and 2.9 were prepared, and the concentration of oak lactone was monitored over time. The oak lactone content of these solutions was found to remain constant over 28 days, suggesting that ring opening of the lactone is unlikely under such conditions. Additionally, solutions of **6** at pH 1.0 reached the theoretical maximum values (of both *cis*- and *trans*-oak lactone) within 2 h. The potential formation of ethyl 3-methyl-4-hydroxyoctanoate, as a result of ethanolysis reactions, as proposed by Waterhouse and Towey (*8*), was also investigated. Model wine solutions of both **1** and **6** were analyzed by GC-MS in both scan and SIM modes. However, despite comparison with mass spectrometric data from a sample synthesized in an analogous manner to the corresponding isopropyl ester (*12*), we found no evidence to support the formation of the ethyl esters, even though these esters could be seen easily by GC-MS. Therefore, even in an aqueous environment, the oak lactones will be found almost entirely in the ring-closed form at equilibrium.

Waterhouse and Towey (*8*) discussed data from Chatonnet (*13*) which showed that the ratio of *cis*/*trans*-oak lactone in extracts of toasted wood increased with increasing toasting levels. They hypothesised that the open-chain form of oak lactone closes during toasting and "the *cis*-lactone, for kinetic or thermodynamic reasons, dehydrates to a greater extent under these conditions, and the ratio of *cis* to *trans* isomers increases as well". However, as shown in **Table 2**, the *trans*-lactone is formed in aqueous solution more rapidly than the *cis*-isomer from their respective hydroxy acids at all pH values. Furthermore, hydrolytic studies indicate that the *trans*-isomer, as expected from steric considerations, is also the thermodynamically more favorable product (*1*). Another explanation for the

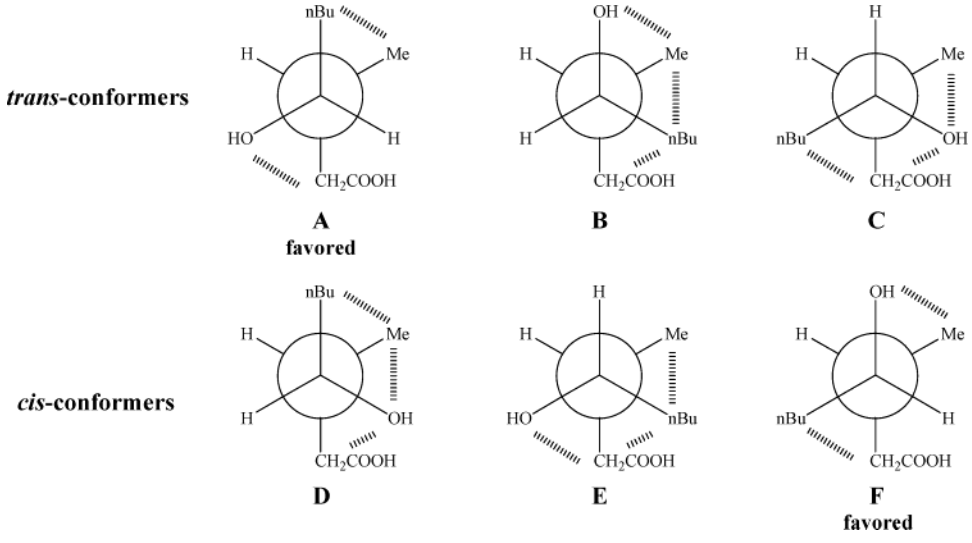

Figure 4. Conformers available to the different isomers of **6**.

findings of Chatonnet (*13*), supported by the data in **Table 2**, is that the conditions of extraction he employed (4 weeks of maceration at 18 °C, pH 3.5) were sufficient for any (3*S*,4*R*)-hydroxy acid that might have been present to ring-close to the *trans*-isomer but were likely to be insufficient for the corresponding (3*S*,4*S*)-hydroxy acid (measured half-life of ≈7 days at 25 °C) to completely lactonize to the *cis*-form. If most of the oak lactone were in the open-chain form in unheated wood but ring-closed in heated samples, then this could explain the observed increases of *cis*-oak lactone in the latter. An alternative explanation for Chatonnet's data is that the results are a real reflection of total oak lactone in the wood and that the increased concentration in *cis*-oak lactone is a result of decomposition of the recently identified galloyl glucoside **4** of the hydroxy acid **6**.

The assertion of Waterhouse and Towey (*8*) that when the ratio of *cis*- to *trans*-oak lactone "is determined from wine stored in barrels for 8 months, the ratio is always near the range 1−2 regardless of toasting levels..." becomes questionable when one examines the original source of these data (*13*). The ratios quoted by Waterhouse and Towey are actually composites for wines stored in barrels made from two different species of oak from two different regions (Allier and Limousin). There was a trend for the *cis*/*trans* ratio in wines in the Limousin oak to increase with increasing toasting level (from 1.4 to 4.9 for light to very heavy toast). For the wine in Allier barrels, the ratios varied in a nonsystematic way from 0.8 to 3.1, albeit with the highest ratio at the highest toast level. Although the ratios for the higher toast levels were not as high as those observed for the laboratory experiments, this could simply be because the latter were obtained from shavings of the outer 3 mm of wood, rather than from intact staves.

As further support for their hypothesis, Waterhouse and Towey (*8*) quoted the results of a seasoning trial by Sefton and colleagues (*14*), in which the levels of oak lactone were observed to increase under Australian climatic conditions. However, these seasoning results cannot be a manifestation of the equilibrium between oak lactone and its open-chain forms. As the present study shows, the extraction conditions employed by Sefton et al. (*14*) (model wine, pH 3.2, 50 °C, 7 days) are more than sufficient to ensure the near-complete conversion of any open-chain form into oak lactone.

Any free hydroxyacid present in oak or oak extracts is going to be subject to lactonization to produce oak lactone. Our results show that this process is essentially irreversible at wine pH. Thus, any aglycon extracted into wine will be completely converted to the lactone form over time. The extraction and analysis of wine/oak samples could potentially lead to misleading results if adequate time for lactonization is not taken into consideration. Oak extracts are often prepared by extraction with ethanol/water or acetone/water at room temperature, overnight (i.e., 16 h) (*15*, *16*). Under such conditions lactonization of any hydroxy acid **6** that might be present is likely to be incomplete and could result in the underestimation of *cis*-oak lactone concentration. For analyses specific to oak lactone, we recommend that the extracting medium be kept acidic (pH ≈3.0), and subsequently heated (≈50 °C) for several days, to ensure complete lactonization of any 3-methyl-4-hydroxyoctanoic acid which might be present.

The results from the present study indicate that lactonization of the *trans*-isomer of **6** would reach completion after several days at room temperature, whereas lactonization of the *cis*-isomer could take up to several weeks, depending on the pH of the wine. Therefore, it is anticipated that both isomers would undergo complete lactonization during barrel maturation. Likewise, although the half-life for formation of *cis*-oak lactone was determined to be almost 300 days in model spirit at pH 5.5, given that most spirits are barrel-aged for several years, near-complete lactonization is likely to be achieved.

The addition of oak staves, chips, shavings, or powder to wine, as a more rapid and economical method of oak treatment (*17*), allows the incorporation of oak flavors and interaction of wood and wine constituents without traditional barrel maturation. The increased surface area of chips or shavings (as compared with barrels) results in greater rates of extraction, and so wines typically receive only several days of contact with oak, depending on the preference of the winemaker. Given that the half-life for formation of *cis*-oak lactone was determined to be ≈14 days at pH 3.7, the formation of *cis*-oak lactone after the oak has been removed is possible. This provides a plausible explanation for the intensification of oak aroma even after the removal of oak, as reported by Singleton (*6*).

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