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Identification of a Sotolon Pathway in Dry White Wines

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Sotolon (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone) is a chiral furanone, an aroma compound known to be responsible for premature-aging flavor in dry white wines. Sotolon generally results from mild oxygenation during bottle aging, and until now, its formation pathways had not been elucidated. The ability of the main precursors described in the literature under very different experimental conditions to produce sotolon was tested. In model wine solution maintained at 40 °C for 6 months, sotolon was produced by the oxidative degradation of ascorbic acid. By use of GC−MS, 2-ketobutyric acid, produced by the oxidative degradation of the ascorbic acid in the model wine solution, was identified as a potent precursor of sotolon in this pathway. Ascorbic acid is an exogenous compound, added before bottling, but 2-ketobutyric acid was found even in white wines that had not been supplemented. Consequently, this sotolon formation pathway is also valid in white wines with no added ascorbic acid. In addition, we showed that *Saccharomyces cerevisiae* strains were capable of producing variable concentrations of this ketone during alcoholic fermentation. In model wine solution, certain yeast strains released large quantities of 2-ketobutyric acid, similar to those found in oxidized dry white wines. In view of these results, the role of yeast strains in this premature-aging phenomenon of dry white wines is discussed. Finally, these investigations revealed that one chemical mechanism responsible for the low concentrations of sotolon found in prematurely aged white wines made from various grape varieties was an aldol condensation between 2-ketobutyric acid and acetaldehyde.

KEYWORDS: Wine; sotolon; oxidation; premature aging; ascorbic acid; pathway; sauvignon blanc; yeast

INTRODUCTION

In 1967, Sulser (*1*) identified sotolon (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone) for the first time in vegetable protein hydrolyzates with an aroma reminiscent of walnuts. Later, this compound was identified as a key flavor in coffee (*2*), rye bread crust (*3*), raw cane sugar (*4*), aged sake (*5*), dessert wine (*6*), and fenugreek seeds (*7*). The positive contribution of sotolon to specific nuances of "vins jaunes" (*8*), port wines (*9*), fortified Madeira wines (*10*), and French fortified wines (VDN) (*11*) is well-known. However, in the case of dry white wines, the presence of this very intense aroma compound is associated with a deterioration of the bouquet during aging (*12*−*14*).

The significant contribution of this furanone to such various foods probably explains why a great deal of research has focused on identifying its precursors and formation pathways in various media. According to several studies, amino acids constitute a potential source of this furanone in wines. Historically, Sulser was the first to describe a sotolon formation pathway, via a degradation product of threonine. Its enzymatic or chemical deamination followed by an aldol condensation with acetaldehyde led to the formation of sotolon in French "vin jaunes" (*15*, *16*) and fortified wines (*11*). Takahashi (*5*) and later Kobayashi (*17*) reported elsewhere on the formation of sotolon in sake by a similar chemical route. Inspired by Kobayashi's hypothesis, Cutzach (*18*) suggested that sotolon was also produced by an aldol condensation between glutamic and pyruvic acids in fortified wines. Dubois (*19*) detected sotolon after heating (100 °C, 24 h) a dilute acid solution (HCL, 6 N) containing pyruvic acid and 2-ketobutyric acid.

A strict oxidative mechanism, based on the peroxidation of acetaldehyde, also yielded sotolon and, according to Pisarnitzky (*20*), may be responsible for the high levels of sotolon found in oxidative wines, such as Sherry and Madeira.

Maillard reactions in several combinations of binary mixtures of cysteine and three sugars, ribose (*21*), glucose, and rhamnose (*22*), resulted in the formation of sotolon. According to Hofmann (*23*), heating an aqueous solution (145 °C, 20 min, pH 5.0) containing hydroxyacetaldehyde and butane-2,3-dione (diacetyl) generated a significant amount of sotolon. However, the yield of this pathway was almost 60 times lower at pH 3.0. These mechanisms seemed less likely in our case, as these reactions occur under much harsher conditions than those found in wine during aging.

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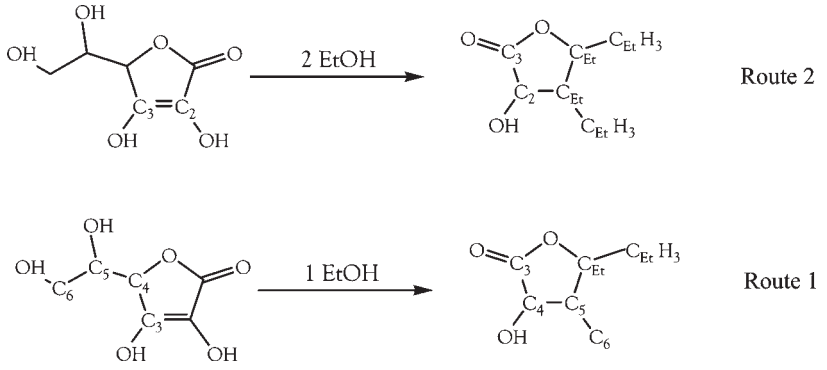

Figure 1. Sotolon formation pathways from ascorbic acid and ethanol postulated by König (*24*).

More recently, another sotolon formation pathway in food was demonstrated in a citrus soft drink by König (*24*): oxidative degradation of ascorbic acid in an acidic medium containing ethanol (**Figure 1**).

A more specific route involved thermo-induced oxidative deamination of 4-hydroxyisoleucine, an amino acid found in high concentrations in fenugreek seeds (*25*). Therefore, sotolon formation occurs under a wide range of conditions, from mild oxidation and conservation to more intensive roasting.

In the first part of our investigations, the presence of non-racemic forms of sotolon in dry white wines was demonstrated and their possible influence on the perception threshold of sotolon in wines explored (*26*). Moreover, we recently confirmed the role of molecular oxygen in sotolon formation in bottled dry white wines (*13*). While oxidation phenomena are generally considered to be favorable, or even essential, for the proper development of sweet fortified wine aromas, for example, the same is not true of dry white wines, where oxygen transfer is deliberately restricted.

Our research into the oxidative formation mechanisms of sotolon in dry white wines drew inspiration from the work of Takahashi (*5*), later used by Cutzach (*11*). These authors studied the role of the aldol condensation reaction between 2-ketobutyric acid and acetaldehyde in the sotolon formation in a specific model dilute alcohol solution (high ethanol and reducing sugar levels) during oxidative aging.

The aim of this study was to investigate a potentially stereospecific formation pathway capable of producing the low sotolon concentrations found in oxidized dry white wines. Following a large-scale review of sotolon pathways in nature, isotopically labeled precursors were used to reveal the sotolon formation pathway in dry white wine. The formation of sotolon from these precursors was also studied in laboratory experiments using both model wine solution and dry white wines. The main aim of future research will be to determine the optimum conditions for the formation of this undesirable compound in order to control this phenomenon and prevent sotolon formation in dry white wines during aging.

MATERIALS AND METHODS

Reagents. 3-Octanol (>99%), 4,5-dimethyl-3-hydroxy-2(5)*H*-furanone (>99%), 2-ketobutyric acid (97%), diethyl oxalpropionate (>99%), boric acid (99.5%), formaldehyde, acetaldehyde (99.5%), propanaldehyde (99.5%), sodium ethylendiaminetetraacetic acid salt (EDTA, 100%), D-(+)-glucose (99.5%), D-(−)-fructose (99.5%), L-ascorbic acid (99%), D-isoascorbic acid (>99%), NaOH, and [1,1,2,2,2-D_5]ethanol were all from Sigma-Aldrich (St. Quentin Fallavier, France). Anhydrous sodium sulfate (99%), Fe(SO_4), dichloromethane (Rectapur grade), and acetonitrile (HPLC grade) were supplied by Prolabo (France).

Wine Samples. The white wine was a wood-aged Pessac Leognan sauvignon blanc from the 1997 vintage. Sulfur dioxide and ascorbic acid were added to the wine before bottling in 750 mL bottles in 1998. Final concentrations of free sulfur dioxide and ascorbic acid at bottling were 30 and 50 mg/L, respectively. Bottles were sealed with top-grade natural corks and aged in a temperature-controlled cellar. In 2005, bottles were sampled randomly for analysis. Commercial wine untreated with ascorbic acid at bottling was used for accelerated aging experiments.

Model Wine Solutions. The composition of model wine solutions for determining sotolon pathways was as follows: 12% vol EtOH, 5 g/L tartaric acid, 750 mg/L glucose, 750 mg/L fructose, 1 mg/L Fe(II), pH 3.5 (5 M NaOH). The wine model solution used to study the reaction between acetaldehyde and 2-ketobutyric acid consisted of 12% vol ethanol and 5 g/L tartaric acid, pH 3.5 (5 M NaOH). For all experiments, the oxygen content was adjusted to 8 mg/L by air bubbling before the bottles were sealed. Dissolved oxygen was measured using an oxygen electrode (Orbisphere, model 31120).

Extraction Procedure. Wine samples and model wine solutions (100 mL) were spiked with 100 μL of 3-octanol in 100 mg/L ethanol solution as an internal standard and 15 g of anhydrous sodium sulfate (its higher ionic strength enhances extractability). Wines were extracted three times with 10, 5, and 5 mL of CH_2Cl_2 (magnetic stirring: 10, 5, 5 min; 750 rpm). The three organic phases obtained were blended, dried over anhydrous sodium sulfate, and concentrated to 0.5 mL under a nitrogen stream. An amount of 2 μL of the extract was injected into the GC with an MS detector.

Gas Chromatography−Mass spectrometry (GC−MS) Conditions. A Star 3400CX gas chromatograph fitted to a Saturn 2000 electronic ion trap mass spectrometer from Varian was used to analyze the organic extract.

Sotolon and 2-Ketobutyric Acid Quantification. Chromatographic conditions were the same as in ref *13*. Briefly, the fused silica column was coated with SPB1 (apolar) from Supelco (Saint Quentin Fallavier, France), 60 m × 0.25 mm; film thickness 1 μm. The carrier gas was He (Linde gas, Bordeaux, France), 5.3 grade, with a flow rate of 0.9 mL/min. A Varian 1078 programmable-temperature injector was used to inject the 2 μL sample. The injector was initially set to 180 °C for 0.3 min. Then the temperature was raised to 230 at 180 °C/min and maintained for 30 min. Oven temperature was initially set to 45 °C for 1 min and then raised to 200 °C at 3 °C/min and to 270 °C at 15 °C/min and held at that temperature for a further 10 min. The programmable-temperature injector was as previously described. The transfer line and manifold were maintained at 210 and 70 °C, respectively. Trap temperature was maintained at 170 °C. Axial modulation was 3.5 V. Injection (2 μL) was in splitless mode (closure time: 0.75 min); split flow 50 mL/min. Ions m/z 57, 59, and 83 were used to quantify 2-ketobutyric acid, octanol-3, and sotolon, respectively. Ion 128 confirmed the presence of sotolon. Linear retention indices (LRI) were obtained by simultaneous injection of samples and a series of alkanes (C7−C23) (*27*).

Stereoisomer Distribution Analysis of Sotolon. Materials and chromatographic conditions were previously described in ref *26*. Briefly, the precolumn was a fused silica column coated with polar phase (BP20; 2 m × 0.25 mm i.d.; 0.25 μm film thickness) (SGE, France) followed by a 30 m × 0.25 mm i.d. fused silica column coated with a 0.25 μm film of a 20% β-cyclodextrin solution in 35% phenylmethylpolysiloxane (HPchiral, from J&W, France). The carrier gas was He (Linde gas, Bordeaux),

Table 1. Main Sotolon Precursors Identified in Foods and Tested in Model Wine Solution from Harsh (100 °C, 5 days) to Mild Conditions (45 °C, 6 Months) ($n = 2$)

					sotolon (μg/L)		
code	ref	model system	usual concentrations in white wines[b] (mg/L)	concentrations (mg/L)	100 °C 5 days	70 °C 30 days	45 °C 6 months
1	*24*	ascorbic acid	0−150	100	55.3 (5.5)	15.4 (0.9)	35.2[h] (4.2)
		ethanol	10−14[f]	12[f]			
2	*18*	pyruvic acid	11−460	100	30.0 (4.6)	3.1 (0.3)	Nd[a]
		glutamic acid	8−35	50			
3	*19*	pyruvic acid	11−460	100	Nd[a]		
		2-ketobutyric acid	0.5−11[c]	10			
4	*20*	acetaldehyde	20−200[g]	5	Nd[a]		
5	*5*, *11*	threonine	10−100	10	42.9 (5.3)	5.6 (0.3)	Nd[a]
		ethanol	10−14[f]	12[f]			
6	*34*	diethyl oxalpropionate	0.1−3.6[c]	0.05	1.0	Tr	
7	*21*	cysteine	0.1− 5[d]	10	Nd[a]		
		ribose	100[e]	100			
8		model solution			1.5 (0.3)	Nd[a]	Nd[a]

[a] Nd: not detected. Tr: trace (μg/L). [b] From refs *33* and *35* unless otherwise indicated. [c] Our results. [d] From ref *36*. [e] From ref *37*. [f] % vol. [g] total concentration (free and bound forms). [h] $n = 3$.

5.3 grade, with a flow rate of 1 mL/min. A Varian 1078 programmable-temperature injector was used to inject the 2 μL sample. Injector temperature was programed as in the previous section. Oven temperature was initially set to 50 °C for 0.1 min, raised to 110 at 1 °C/min, held at that temperature for 20 min, and then raised to 210 at 3 °C/min and held at that temperature for a further 20 min.

Diethyl Oxalpropionate Quantification. The extraction procedure was as previously described for sotolon and 2-ketobutyric acid. Chromatographic conditions were the following: column SPB1 (30 m, 0.25, 1 μm), flow rate 0.9 mL/min; oven temperature, 45 °C for 1 min, programed at a rate of 3 °C/min to 230 °C, held at that temperature for 20 min. Impact electronic mode was chosen for quantification of the internal standard ($m/z = 59$). By use of isobutane as reagent gas (Linde gas), the fragmentation pattern of the ester showed a specific, medium sized peak at $m/z = 203$, corresponding to $[M + H]^+$. Chemical ionization mode was used to quantify this ester in wines.

Ascorbic Acid Quantification. The assay was based on the method described by Cancalon (*28*) and Liao (*29*), modified as follows. Capillary electrophoresis (HP^{3D} Hewlett-Packard) was coupled with a diode array detector ($\lambda = 270$ nm). The pH of the borate buffer (100 mM) was adjusted to 9.3 by adding NaOH (1 M) and supplementing with 5% acetonitrile. The total length of the fused silica capillary (Agilent, France) was 64 cm, and the internal diameter was 50 μm. Hydrostatic injection of the sample was 15 s, 50 mb. The +30 kV voltage applied throughout separation generated a 44 μA current. Separation was carried out at a constant temperature of 23 °C. After each analysis, the capillary was rinsed with NaOH (0.1 M, 3 min), H_2O (1 min), and borate buffer (3 min) in turn. A 400 μL wine sample was used for the ascorbic acid assay, supplemented with 100 μL HCL/EDTA solution (100/10 mM) and 10 μL D-isoascorbic acid in 1 g/L HCL/EDTA solution as internal standard.

Validation of Analytical Conditions, Calibration Graphs, Repeatability. 2-Ketobutyric acid containing traces of sotolon was purified as described in ref *16*. The standard range was prepared by adding increasing concentrations of sotolon, 2-ketobutyric acid, diethyl oxalpropionate, and ascorbic acid to a dry white wine, from 1 to 20 μg/L, from 0.5 to 20 mg/L, from 0.1 to 5 μg/L, and from 20 to 150 mg/L, respectively. Linear regressions and correlation coefficients were calculated according to the least-squares method. Results were as follows: $43.59(h/h_{ei}) - 0.57$ ($R^2 = 0.998$); $6.00(h/h_{ei}) + 0.11$ ($R^2 = 0.998$); $218.1(h/h_{ei}) + 0.21$; $26.52(S/S_{ei}) + 1.83$ ($R^2 = 0.998$). The within-day reproducibility of the methods was calculated from the analysis of five replicates of the same wine sample on the same day. The coefficients of variation were 5.2% (6 μg/L), 6.8% (3 mg/L), 5.5% (3 μg/l), and 3.5% (20 mg/L), respectively.

Determination of the Equilibrium Constant for the Carbonyl Bisulfite Combination. The dissociation constant of the bisulfite combination characterizes the affinity of a compound for SO_2. K_d was calculated according to ref *30*. Determinations were carried out in triplicate in aqueous buffer solution (4 g/L tartaric acid, pH 3.5 using 5 M NaOH). A solution initially containing 1 mM 2-ketobutyric acid was divided into seven fractions and supplemented with SO_2 (5, 10, 20, 40, 60, 120, and 200 mg/L). The bottles, filled and hermetically sealed, were kept at 25 °C. After 7 days, once equilibrium had been established, free and total SO_2 were assayed by iodometry (*31*).

Fermentations. The model solution was prepared according to Marullo (*32*), with slight modifications: 110 g/L fructose and 110 g/L glucose. Commercial starter IOC was from Lallemand, and RMS2 and BO213 were from Laffort Enologie (Bordeaux, France). The other *S. cerevisiae* yeast strains (K, EC, CV, DV, M, CDV, BV) were from a private collection (Faculté d'Oenologie de Bordeaux). 2-Ketobutyric acid release was measured at the end of the fermentation process (reducing sugar of < 2 g/L), which took place in 1 L bioreactors at 24 °C. An amount of 150 mL was centrifuged before extraction to pellet the cells.

RESULTS AND DISCUSSION

Identification of Sotolon Precursors in Model Wine Solution during Aging. The first step in identifying a sotolon formation pathway in dry white wine during aging was to evaluate the ability of most of the reactions described in the literature in numerous foods (**Table 1**) to generate sotolon in model wine solution. All the precursors tested were added to the model solution in the range of concentrations found in white wines. Experimental parameters ranged from extreme values to the usual mild reaction conditions in wine during aging.

Most of the precursors tested, except 2-ketobutyric acid and diethyl oxalpropionate, had already been identified in dry white wines. By use of conventional GC−MS in EI or CI (C_4H_{10}) mode, the concentrations of these two compounds were assayed in a small series of commercial white wines. Average concentrations of 2-ketobutyric acid and diethyl oxalpropionate ranged from 0.3 to 16 mg/L and from 0.1 to 3.6 μg/L, respectively. Concentration of this ester was reported for the first time in wine.

After 5 days at 100 °C, no sotolon production was detected in samples for pathways described in the literature as strongly temperature dependent (**Table 1**, codes 6 and 7). This indicated that no significant chemical reactions producing sotolon between these compounds took place during wine aging, so these reactions were not tested any further. After 6 months at 40 °C, none of the precursors already identified in wines or pathways already tested in model wine solution (**Table 1**, codes 2−5) were able to produce sotolon. Only the oxidative degradation of ascorbic acid was capable of generating sotolon in model wine solution containing low concentrations of reducing sugar under such mild reaction conditions. After 6 months at 40 °C, ascorbic acid had completely disappeared from the model solution (result not shown), resulting in a high concentration of sotolon (35.2 μg/L), compared to its perception threshold in model solution (2 μg/L).

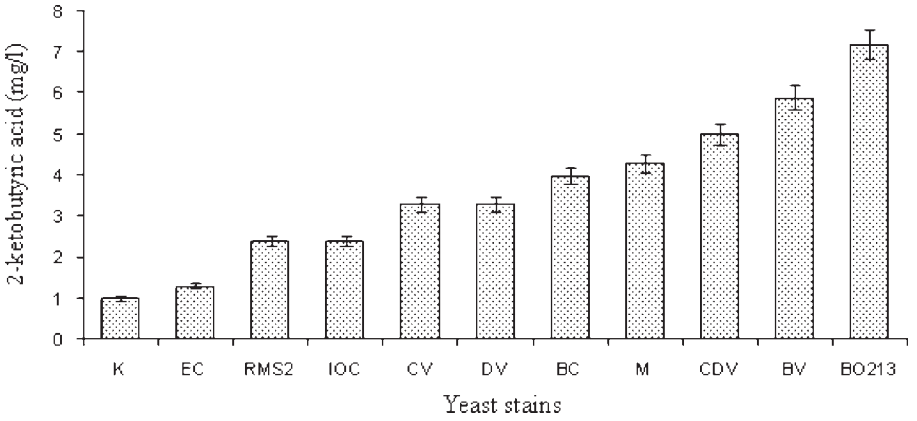

Figure 2. Effect of *S. cerevisiae* strains on the production of 2-ketobutyric acid in the synthetic medium ($n = 2$).

Table 2. Impact of Ethanol (12% vol) on 2-Ketobutyric Acid and Sotolon Formation from Ascorbic Acid (AA) Degradation (3 g/L) in Model Solution Heated for 2 Days at 70 °C (Dissolved Oxygen, 8 mg/L) ($n = 3$)

	control	control + AA	control + AA + EtOH
2-ketobutyric acid (mg/L)	Nd[a]	Nd[a]	0.8 (0.08)
sotolon (μg/L)	Nd[a]	Nd[a]	30 (1.6)

[a] Nd: not detected.

Table 3. Sotolon Formation According to Acetaldehyde Concentration in Model Wine Solution Containing 2-Ketobutyric Acid (10 mg/L)[a]

	acetaldehyde (μg/L)				
	0	100	200	500	1000
sotolon (μg/L)	Nd	Nd	Nd	trace	1.1 (0.2)

[a] Determined at 40°C, 30 days. $n = 3$. Nd: not detected.

Ascorbic acid is a natural antioxidant, present in small quantities in grapes, that rapidly disappears during initial aeration of the must. It is used during white wine vinification and generally added just before bottling (*33*). A great deal of research has already investigated the advantages and disadvantages of ascorbic acid for preventing browning and aroma degradation in white wines. Consequently, ascorbic acid addition to white wines during bottling is controversial, and some winemakers do not use it. Hence, even if ascorbic acid is a potential sotolon precursor in white wines, it cannot be responsible for the presence of sotolon in white wines untreated with this antioxidant. One logical hypothesis was that a byproduct of ascorbic acid oxidation was a key intermediate in the sotolon formation pathway.

Oxidative Decomposition of Ascorbic Acid. Thermal or oxidative degradation of ascorbic acid generates several families of chemicals, depending on the conditions: aliphatic compounds, many ketones, and aldehydes, as well as keto acids, lactones, and some pyrones (*38*, *39*). By studying the oxidative degradation of deuterated and ^{13}C-labeled ascorbic acid with ethanol, König postulated two formation pathways from these precursors (**Figure 2**). Because of this approach, this author identified carbons from ascorbic acid and ethanol in the carbon skeleton of sotolon. This work was extended to study labeled sotolon fragments. Sotolon formed via route 1 was generated from carbons 3−6 of ascorbic acid. Sotolon was assumed to originate from an aldol reaction between compounds with two and four carbon atoms in their skeleton. This prompted us to search for 2-ketobutyric acid among the oxidation degradation byproduct of ascorbic acid.

By use of GC−MS in full scan mode, 2-ketobutyric acid was identified as a minor degradation product of ascorbic acid in model wine solutions (12% vol, pH 3.5) under our temperature conditions. In view of the low mass and poor specificity of the only fragment ($m/z = 57$ (100), $[C_2H_5CO]^+$) found on the fragmentation spectrum of 2-ketobutyric acid, the chromatographic conditions were optimized to obtain a well-resolved peak. Identification was confirmed by co-injecting and comparing its mass spectrum with that of the pure compound ($IRL_{SPB1} = 773$). After 2 days at 70 °C, significant amounts of sotolon and 2-ketobutyric acid were formed in dilute alcohol solution containing ascorbic acid (**Table 2**), while none of these compounds were obtained from the degradation of ascorbic acid in aqueous acid solution.

In order to clarify the mechanistic description of the degradation process, the presence of ethanol on the skeleton of 2-ketobutyric acid was evaluated using deuterated ethanol. Investigation of a sample containing [1,1,2,2,2-D_5]ethanol and ascorbic acid by GC−MS indicated that 2-ketobutyric acid did not contain the labeled isotope in its skeleton and was therefore formed via oxidative degradation of ascorbic acid. This finding was in agreement with the route 1 proposed by König.

Sotolon Formation from 2-Ketobutyric Acid and Acetaldehyde. The formation of sotolon from these two precursors had already been confirmed by Pham in Vin Jaune model solution (*16*), where high concentrations of acetaldehyde and 2-ketobutyric acid resulted in a high sotolon content. As wine contains free sulfur dioxide (in HSO_3^- and H_2SO_3 form), some of the compounds bearing carbonyl groups are present in bound forms, e.g. α-hydroxysulfonic acids (*33*). Acetaldehyde is known to combine very effectively with sulfur dioxide, so only limited amounts are present in the free form. Blouin (*40*) estimated that with 30 mg/L free sulfur dioxide, only 0.04% of the acetaldehyde in wine remained free. During wine aging, when free sulfur dioxide is no longer present, weak dissociation of sulfurous aldehydic acid may release trace of ethanal. Concentrations of 2-ketobutyric in wine were about 0.3−16 mg/L. For this reason, we hypothesized that the acetaldehyde concentration influenced sotolon formation during wine aging.

Experiments on the reaction of low concentrations of acetaldehyde in the presence of 2-ketobutyric acid (10 mg/L) under mild conditions (40 °C, 30 days) revealed that sotolon was formed when acetaldehyde content exceeded 500 μg/L. Low concentrations of sotolon, up to 1 μg/L (**Table 3**), were detectable after a short time. As already observed, the aldol reaction between these carbonyl compounds in dilute alcohol solution is slow, probably because of the presence of high concentrations of acetaldehyde in hydrate form. Chiral analysis of samples also showed that sotolon was produced in racemic form. However, although aldol

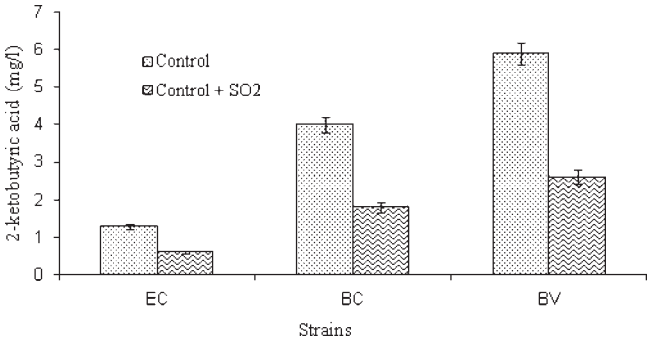

Figure 3. Effect of SO_2 addition (50 mg/L) on the 2-ketobutyric acid concentrations of model solutions fermented by three *S. cerevisiae* strains ($n = 3$).

condensation between these two compounds constitutes a viable pathway for generating sotolon in wine, its racemic nature indicated that it may have been formed by an enantioselective reaction via the mediation of the chiral inducers present in wine. Sotolon has previously been shown to racemize slowly in an acidic medium (*41*). Therefore, we cannot rule out the possibility that chiral vectors, such as amino acids and, especially, proline, catalyze aldol reactions in an enantioselective fashion and that the enantioenriched sotolon thus formed then racemizes during aging (*41*). The type of solvent is also known to have a significant impact on such reactions. The best results were obtained by several teams (*41*, *42*) using polar aprotic solvent, so the relevance of these reactions in dilute alcohol solution must be investigated carefully.

Biochemical Origin of 2-Ketobutyric Acid in Dry White Wines. Previous evidence that sotolon is systematically present in prematurely aged white wines (*13*) was confirmed by our research. Indeed, the sotolon formation pathway described above involves two carbonyl compounds found in all white wines. Acetaldehyde is known to be a byproduct of alcoholic fermentation in wine. Because of wine's high ethanol concentration, it is also generally accepted that acetaldehyde is one of the main volatile compounds generated during oxidation (*33*). Concentrations vary significantly from one wine to another mainly because of variations in winemaking and storage conditions. As previously indicated, the presence of free acetaldehyde in wines is mainly dependent on the free sulfur dioxide content. Moreover, a recent study demonstrated its limited organoleptic impact on oxidized dry white wines (*12*). Factors affecting the 2-ketobutyric acid concentrations in wines are more debatable.

In S. *cerevisiae*, the biosynthesis of several higher alcohols is closely related to the aspartate-derived amino acid metabolism, whereby aspartate is converted via homoserine into threonine or methionine (*43*). The threonine is initially deaminated by threonine deaminase, forming 2-keto acid, the precursor of the higher alcohol *n*-propanol (Ehrlich's catabolism mechanism). 2-Ketobutyric acid is a key intermediate in this pathway. Moreover, a general variability in yeast strains was observed to affect the release of higher alcohols in wine. Furthermore, the choice of yeast strain has a decisive impact on wine flavor, especially the varietal aroma *V. Vinifera* L. var. sauvignon blanc wines (*44*).

For these reasons, we tested the capacity of 11 yeast strains to produce 2-ketobutyric during alcoholic fermentation of synthetic grape juice. All the fermentation processes were completed in comparable times.

As shown in **Figure 2**, the yeast strain had a major impact on 2-ketobutyric acid levels at the end of the fermentation process. The highest concentration exceeded 7.1 mg/L, while the lowest was 0.9 mg/L. Commercial yeast strains (RMS2 and IOC) produced lower concentrations than most of the *S. cerevisiae* strains from the private collection. This effect may be due to a modification in threonine deaminase activity.

Table 4. Impact of Ascorbic Acid Addition (50 mg/L) on 2-Ketobutyric Acid and Sotolon Concentrations in Dry White Wine Saturated with Oxygen after 6 Months of Accelerated Aging (40 °C) ($n = 3$)

	control	control (6 months)	AA (6 months)
2-ketobutyric acid (mg/L)	0.9 (0.3)	3.3 (0.5)**	7.5 (0.4)**
sotolon (μg/L)	Nd[a]	7.5 (0.9)**	12.1 (0.7)**

[a] nd: not detected. **: significant value (ANOVA), $p < 0.01$.

It was previously demonstrated that the free acetaldehyde concentration was probably the limiting factor for sotolon production in wine rather than the 2-ketobutyric acid content. However, according to our previous result, we cannot exclude the possibility that a high 2-ketobutyric acid content at the end of the fermentation process, induced by specific yeast strains, leads to the development of higher sotolon concentrations during aging.

Impact of Sulfur Dioxide. To validate the quantification results, the proportion of bound forms of 2-ketobutyric acid in the form of α-hydroxysulfonic acids was assayed. Three compounds were tested: formaldehyde, acetaldehyde, and propanaldehyde. High concentrations of these compounds were added to several dry white wines selected for their different degrees of oxidation and free sulfur dioxide levels (*45*). The goal was to displace the equilibrium between the free and potential bound forms of 2-ketobutyric acid. After 24 h, free SO_2 levels were very low, indicating that it had combined with aldehydes, whereas 2-ketobutyric acid was not detected in any sample (results not shown). Considering the reactivity of 2-ketobutyric acid with excess acetaldehyde, this common approach was not appropriate in this case.

We determined the affinity to SO_2 of a solution containing 1 mM 2-ketobutyric acid. The dissociation constant of the carbonyl bisulfite, $K_d = 0.6$ mM, was very close to that determined for other 2-ketoacids found in wine (*40*). Thus, a 25 mg/L free SO_2 concentration was capable of binding approximately 50% of the 2-ketobutyric acid in a model wine solution. We then validated this result in a more complex matrix. Three of the model solutions fermented in the previous experiment were selected for their 2-ketobutyric acid concentrations. 2-Ketobutyric acid was assayed 1 week after the addition of 50 mg/L SO_2 (**Figure 3**). The experiment was repeated three times with each sample. Adding SO_2 to fermented model solutions resulted in a 50% decrease in keto acid, irrespective of the yeast strain. Free SO_2 content was 30 mg/L. These results were consistent with the K_d calculated for 2-ketobutyric acid.

Validation of this Pathway in Dry White Wines. The model wine solution study was extended to wine containing ascorbic acid following accelerated aging in the laboratory or slow aging in bottle. The aim was to evaluate the role of ascorbic acid as a 2-ketobutyric acid precursor and to confirm the validity of the chemical mechanism proposed above.

The ascorbic acid concentration in the laboratory experiments was 50 mg/L, corresponding to the average amount added to wine before bottling. As shown in **Table 4**, following heat treatment of the wine (40 °C, 6 months) saturated with oxygen, significant amounts of 2-ketobutyric acid and sotolon were produced. The increase was more marked in wines supplemented with ascorbic acid (+ 56% and +38%, respectively). According to the sotolon formation pathway described previously, these results clearly suggested a direct link between the presence of ascorbic acid in the samples and the final sotolon content. In addition, it was obvious that mild accelerated-aging conditions induced other

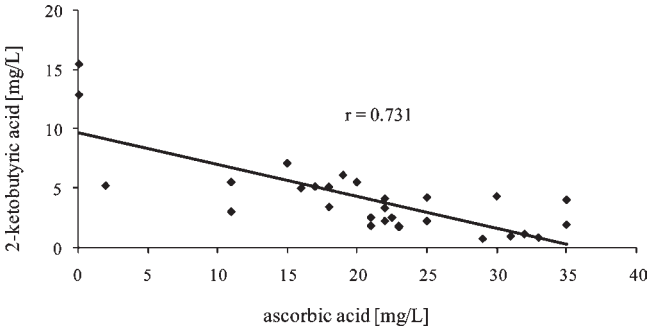

Figure 4. Correlation between ascorbic acid and 2-ketobutyric acid concentrations in dry white wines.

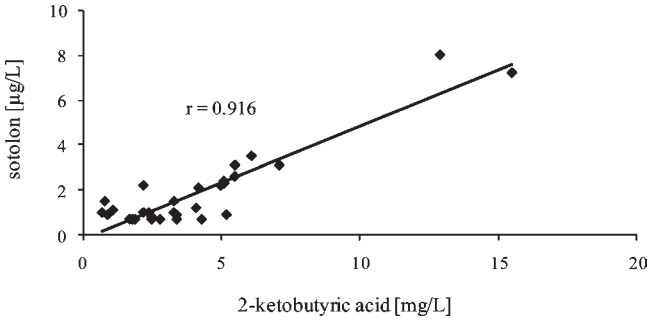

Figure 5. Correlation between sotolon and 2-ketobutyric acid concentrations in dry white wines.

mechanisms capable of generating 2-ketobutyric acid and sotolon. Other compounds and pathways may act as important precursors of 2-ketobutyric acid and sotolon in dry white wines.

Aging in Bottle. The following experiment was conducted to validate the suggested pathway for sotolon formation in dry white wines. The sotolon and 2-ketobutyric acid concentrations were assayed in 40 bottles of dry white Pessac Leognan wine, from the same vintage (1997) and bottling batch. The wine was supplemented with 50 mg/L ascorbic acid at bottling, and bottles were sealed with top grade natural corks. After 7 years' aging, the ascorbic acid levels in these wines ranged from under 1 mg/L (the method quantification limit) to 35 mg/L, corresponding to a decrease of 30−100%. This huge range of ascorbic acid concentrations was probably associated with uncontrolled cork permeability to oxygen (*46*, *47*).

These two parameters were correlated fairly well ($r = 0.731$, $p < 0{,}001$; **Figure 4**). The level of this keto acid in dry white wines during mild oxidative aging was not exclusively linked with the presence of ascorbic acid. Consequently, ascorbic acid may be considered one among several 2-ketobutyric acid precursors in wine.

In contrast, 2-ketobutyric acid and sotolon concentrations were very highly correlated ($r = 0.916$, $p < 0{,}001$) (**Figure 5**). Sotolon was quantified in all samples at levels ranging from 1.1 to 7.9 μg/L. Maximum values were found in the samples with the lowest ascorbic acid content, reaching levels well above the perception threshold in model wine solution (2 μg/L). A similar range was found for 2-ketobutyric acid, also reaching maximum values (15.4 mg/L) in oxidized white wines. These results demonstrate that, in wine, 2-ketobutyric acid, via aldol condensation with acetaldehyde, is responsible for the small amounts of sotolon found in prematurely aged dry white wines.

2-Ketobutyric acid was demonstrated to be one of the precursors of sotolon, and ascorbic acid addition at bottling contributed, to a lesser extent, to 2-ketobutyric acid formation in dry white wines. These results support a new "working hypothesis" to explain sotolon formation during white wine aging in barrel or in bottle.

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