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Stereoselective Formation of the Varietal Aroma Compound Rose Oxide during Alcoholic Fermentation

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The potent aroma compound rose oxide was quantified in several white wines by a headspace solid-phase microextration stable isotope dilution assay (HS-SPME-SIDA) and the enantiomeric ratios of the *cis* diastereomers were determined by enantioselective capillary GC. The most odor-active stereoisomer (–)-*cis*-rose oxide was detectable in all investigated white wines ranging from 0.2 to 12 μg/L. However, its contribution to the overall aroma in some white wine varieties can be neglected as indicated by a low odor activity value (OAV). The highest concentrations were found in Gewürztraminer wines, confirming the importance of rose oxide as a varietal aroma compound in this variety. Surprisingly, the enantiomeric ratio of *cis*-rose oxide in all investigated wines was substantially lower than in nonfermented musts and in some wines almost racemic *cis*-rose oxide was detected. Fermentation studies with a model must that contained deuterated water revealed that yeast is capable of reducing the precursor 3,7-dimethyl octa-2,5-dien-1,7-diol (geranyl diol I) yielding 3,7-dimethyl-5-octen-1,7-diol (citronellyl diol I) that gives rise to *cis*- and *trans*-rose oxide after acid catalyzed cylization. The deuterium labeling pattern of the resulting rose oxide stereoisomers and a clearly detectable kinetic isotope effect indicate that at least two different reductive pathways in yeast exist that yield *cis*-rose oxide with different enantiomeric ratios altering the genuine enantiomeric ratio in grape musts. The presence of (+)-*cis*-rose oxides in wines can therefore be attributed to the reductive yeast metabolism during fermentation. This observation corroborates recent findings that the modification of terpene derived varietal aroma is an integral part of yeast metabolism and not only a simple hydrolytical process.

KEYWORDS: ***Vitis vinifera*; *Saccharomyces cerevisiae*; wine aroma; terpenes; biotransformation; deuterium labeling**

INTRODUCTION

Rose oxide is a chiral monoterpenoid ether that can be detected in various essential oils (*1*). It has also been shown to contribute significantly to the varietal aroma of Gewürztraminer wines (*2, 3*). Its biosynthesis in grape berries has been recently investigated by deuterium labeling studies (*4*), and the enantiomeric ratios of the *cis* diastereomer were determined in grape musts of different varieties by stir-bar sorptive extraction (*5*). It could be shown that (–)-*cis*- and *trans*-rose oxide is generated by stereoselective reduction of geraniol followed by allylic hydroxylation and acid-catalyzed cyclization. Consequently, *cis*- and *trans*-rose oxide are present at high enantiomeric purities in grape musts ranging from 97 to 88% in favor of the (–)-enantiomer (*5*). However, the only enantiomeric ratio of *cis*-rose oxide that has been reported up to now in the literature for a Gewürztraminer wine is 70:30 in favor of the (–)-enantiomer (*2*) and is hence considerably lower than the enantiomeric ratio found for grape musts. We have therefore developed an enantioselective headspace solid-phase microextration stable isotope dilution assay (HS-SPME-SIDA) to quantify the rose oxide enantiomers in different white wines and to assess the contribution to the overall aroma by calculating the odor activity values for the most odor-active (–)-*cis*-enantiomer (*6*). Additionally, fermentation studies using a model must that contained deuterium oxide were conducted giving evidence for yeast mediated (+)-*cis*-rose oxide formation, thus explaining the rather low enantiomeric purity of *cis*-rose oxide in wines when compared to the corresponding musts.

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MATERIALS AND METHODS

Reagents and Standards. (+/−)-*cis*/*trans*-Rose oxide was supplied by Roth (Karlsruhe, Germany). All other reagents and solvents were purchased from Fluka or Aldrich (both Buchs, Switzerland). *cis*- and *trans*-[6,6-$^2H_2$]rose oxide were prepared as previously described (*7*). Pure *cis*-[6,6-$^2H_2$]rose oxide was obtained by flash chromatography and pentane/ether (9:1, v/v) as eluent. (*E*)-3,7-Dimethyl octa-2,5-dien-1,7-diol and (*E*)-3,7-dimethyl octa-2,7-dien-1,6-diol (geranyldiol I and geranyldiol II) were prepared from geraniol by peroxidation and reduction as previously described for the corresponding citronellol derived diols (*8*). MS data of the synthesized diols were in good agreement with previously published data (*9*).

Samples. Samples were obtained from the swiss wine market within the scope of the official food control program conducted by the food control authority of the canton Geneva.

Gas Chromatography−Mass Spectrometry (GC−MS) Conditions. GC−MS analysis was carried out using a Varian Chrompack CP-3000 GC coupled to a Saturn 2000 ion-trap MS (both Varian, Palo Alto, CA). The column was a self-prepared 30 m × 0.25 mm i.d., 0.25 μm film thickness DiMe-β (heptakis-(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin) in SE 52 (*10*). Helium was the carrier gas at a flow rate of 0.8 mL/min. The oven program was 5 min at 40 °C, 5 °C/min up to 75 °C, 1 °C/min up to 85 °C held for 10 min, and finally, 5 °C/min up to 180 °C held for 5 min. The injector was kept at 250 °C. Splitless injection was used, and the split valve was opened after 2 min with a split/column flow ratio of 100. The mass spectra were recorded in full-scan mode (*m*/*z* 40–250 mass range). The electron impact energy for ionization was 70 eV. For the quantification of rose oxide mass lane *m*/*z* 139 was monitored. The internal standard *cis*-[6,6-$^2H_2$]rose oxide was monitored at mass lane *m*/*z* 141.

Headspace Solid-Phase Microextraction (HS-SPME). In a 25 mL vial, 5 mL of each wine sample was spiked with 10 μL of a solution of the internal standard *cis*-[6,6-$^2H_2$]rose oxide in 10% ethanol to give a final concentration of 4 μg/L. Sodium chloride was added to give a final concentration of 0.1 g/mL. The vial was closed and equilibrated for 1 h at room temperature. For HS-SPME sampling (*11*), a 1 cm fused silica fiber coated with 100 μm PDMS was used (Supelco, Bellefonte, PA). After 20 min of extraction time, the fiber was inserted into the injection port of the GC−MS system and thermally desorbed for 3 min.

Calibration of the Stable Isotope Dilution Assay. A standard solution of rose oxide in pure ethanol was serially diluted with 10% ethanol followed by addition of the internal standard (IST) *cis*-[6,6-$^2H_2$]rose oxide in 10% ethanol to give a final concentration of 4 μg/L IST. The obtained standard solutions were analyzed by HS-SPME as described above. Integrated peak area ratios (peak area IST at *m*/*z* 141/peak area of rose oxide at *m*/*z* 139) were calculated and plotted against the concentration ratios (4 μg/L IST/μg/L rose oxide). The resultant curve was linear (response ratio = 0.9429 × concentration ratio − 0.0333) with $R^2 = 0.9997$ for a range between 0.1 and 12 μg/L. A typical wine sample that contained *cis*-rose oxide at a concentration level of 5 μg/L was analyzed ($n = 3$) to check the coefficient of variance (CV) of the analytical method. The CV was determined to be 4.7%, which compares well to similar SIDA methods for quantification of wine aroma impact compounds (*12*).

Model Fermentations. A total of 25 mL of a model must containing 50 mg/mL glucose was adjusted to pH 3.5 with tartaric acid. The model must was spiked with different possible precursors of rose oxide (geraniol, citronellol, (*E*)-3,7-dimethyl octa-2,5-dien-1,7-diol, and (*E*)-3,7-dimethyl octa-2,7-dien-1,6-diol) to give a final concentration of 500 μg/L of each precursor. After the addition of 100 μL yeast suspension (0.1 g/mL of *Saccharomyces cerevisiae*, strain CEPPO 20, Littorale Oenologie, Servian, France), the model must was fermented at 30 °C for 24 h followed by the analysis of the generated metabolites using HS-SPME as described above. Model fermentations were also conducted using deuterium oxide as the solvent. Unlabeled rose oxide that was generated in model fermentations with water as the solvent was quantified by the stable isotope dilution assay as described above. All fermentation studies (solvent H_2O and D_2O) were carried out at least 3 times.

RESULTS

The total concentration and the enantiomeric ratio of *cis*-rose oxide in different white wine varieties were determined by a headspace solid-phase microextration stable isotope dilution assay (HS-SPME-SIDA). *cis*-[6,6-$^2H_2$]Rose oxide was used as internal standard and detected selectively on mass lane *m*/*z* 141, whereas genuine rose oxide was detected on mass lane *m*/*z* 139 as illustrated by parts **A**−**D** of **Figure 1**. The highest concentrations were found in Gewürztraminer wines confirming the importance of rose oxide as a varietal aroma compound in this variety (*2, 3*) (**Table 1**). In all other analyzed wines, rose oxide was detectable as well, albeit at lower concentrations.

The highest enantiomeric purity for *cis*-rose oxide was 76% in favor of the (–)-enantiomer, and in some wines, *cis*-rose oxide was present as an almost racemic mixture (**Table 1**). Because in a previous study, rose oxide was detected at high enantiomeric purities in grape musts ranging from 97 to 88% in favor of the (–)-enantiomer (*5*), this finding is rather surprising and raises the question of the origin of the elevated (+)-*cis*-rose oxide concentrations. Preliminary studies with deuterium oxide enriched must showed the formation of deuterium labeled rose oxide during alcoholic fermentation (*13*). Therefore, fermentation studies with model musts were carried out that were spiked with different potential precursors for rose oxide. Geraniol, citronellol, and a mixture of the geraniol derived diols I and II ((*E*)-3,7-dimethyl octa-2,5-dien-1,7-diol and (*E*)-3,7-dimethyl octa-2,7-dien-1,6-diol) were tested as precursors at a concentration of 500 μg/L each. All tested precursor are genuine monoterpenes in grape musts and occur in free or glycosidically bound form (*14*). Neither geraniol nor citronellol gave any detectable rose oxide after fermentation. However, when a 1:1 mixture of (*E*)-3,7-dimethyl octa-2,5-dien-1,7-diol and (*E*)-3,7-dimethyl octa-2,7-dien-1,6-diol was applied, *cis*- and *trans* rose oxide was detectable. The biotransformation is clearly stereoselective as indicated by an enantiomeric ratio of 85:25 in favor of the (+)-*cis*-enantiomer (**Figure 2A**). The total *cis*-rose oxide concentration was up to 15 ± 3 μg/L and compares well to the concentrations of (+)-*cis*-rose oxide that can be found in the investigated wines.

To gain more insight into the formation mechanism of rose oxide in yeast fermentations were also carried out with deuterium oxide as the solvent. In the presence of deuterium oxide, the enantiomeric ratio of the generated *cis*-rose oxide is altered indicating a kinetic isotope effect (**Figure 2B**). When mass lanes *m*/*z* 139 and 141 are extracted selectively, it becomes evident that at least two different isotopologues are generated that can be distinguished by their enantiomeric ratios (parts **C** and **D** of **Figure 2**). Unlabeled *cis*-rose oxide is visible on mass lane *m*/*z* 139 and is almost racemic, whereas labeled [4,5-$^2H_2$](+)-*cis*-rose oxide is visible on mass lane *m*/*z* 141. The mass spectrum of the generated labeled [4,5-$^2H_2$](+)-*cis*-rose oxide is in agreement with an incorporation of the deuterium atoms in positions 4 and 5 as can be deduced from the fragmentation mechanism of rose oxide in electron impact mass spectrometry (*15*). Minor amounts of [6-^2H](+)-*cis*- and [4,5,6-$^2H_3$](+)-*cis*-rose oxide were detectable as well on mass lanes *m*/*z* 140 and 142, respectively, which probably arise from the reduction of the corresponding unsaturated aldehyde that is formed as an intermediate during double bond reduction by yeast enoate reductase as described below (*16*). These findings demonstrate that at least two different reductive pathways in yeast exist that yield *cis*-rose oxide with different enantiomeric ratios, altering the genuine enantiomeric ratio in grape musts. For the majority of redox enzymes, NAD and its respective

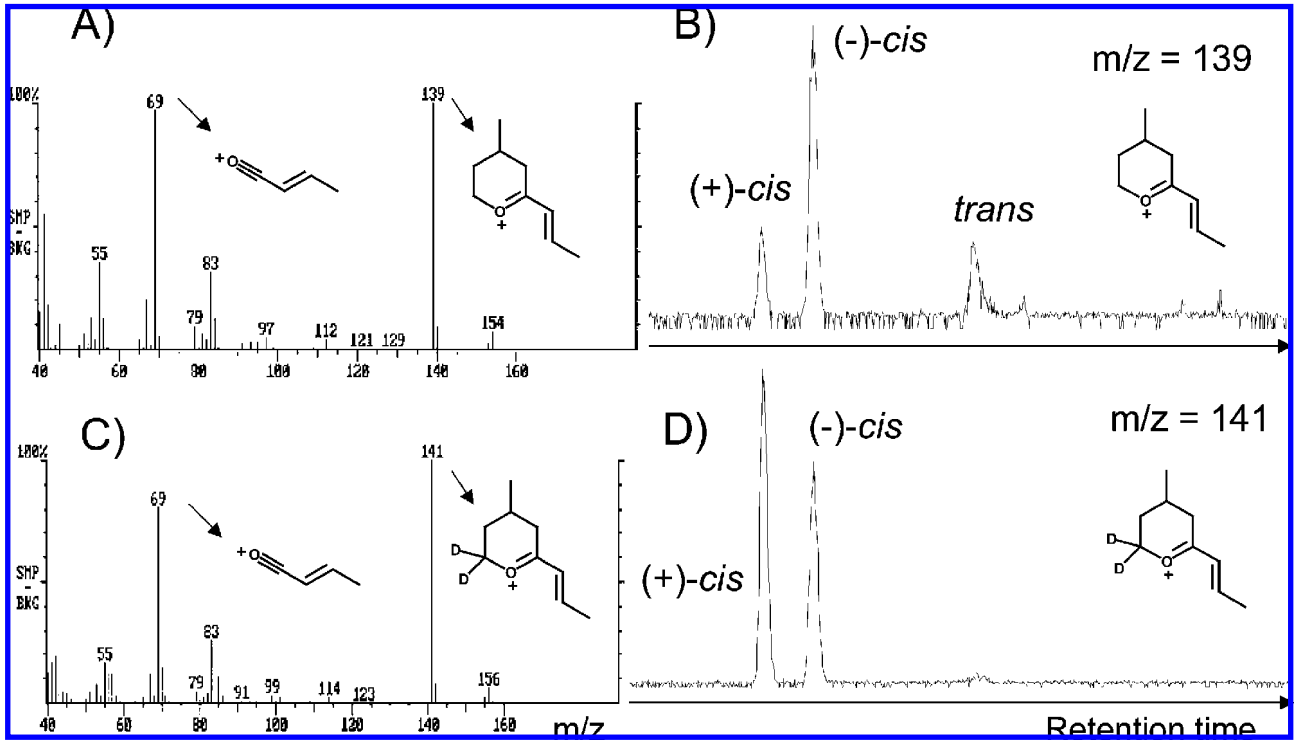

Figure 1. Mass spectra of genuine (**A**) and *cis*-[6,6-$^2H_2$]rose oxide (**C**). Fragments are assigned to the corresponding mass peaks according to Wüst et al. (*15*). The chromatograms resulted from a stable isotope dilution assay of a typical Gewürztraminer wine that was spiked with 4 μg/L of the labeled internal standard (IST). On mass lane *m*/*z* 139, the genuine rose oxides are detected (**B**), and on mass lane *m*/*z* 141, the IST is detected (**D**).

Table 1. Enantiomeric Ratios and Total Concentrations of *cis*-Rose Oxide in Different White Wines as Determined by HS-SPME-SIDA

wine	percentage of (+)-*cis*	percentage of (–)-*cis*	total concentration (+/−)-*cis*-rose oxide (μg/L)[a]	odor activity value (OAV)[b]
Gewürztraminer	41	59	3	9
	39	61	6	19
	44	56	8	24
	24	76	12	49
	25	75	4	16
	42	58	7	21
Muscat	38	62	2	7
	54	46	4	9
	46	54	10	28
	34	66	3	9
	57	43	2	4
	40	60	3	9
	38	62	0.7	2
	30	70	2	8
Silvaner	60	40	4	8
	62	38	2	5
	59	41	2	4
Traminer	25	75	2	6
Païen	47	53	0.6	2
Himbertscha	41	59	0.7	2
Petite Arvine	42	58	0.4	1
Humagne Blanc	41	59	0.3	<1
Lafnetscha	52	48	0.3	<1
Plantscher	33	67	0.2	<1
Gwäss	48	52	0.2	<1

[a] The results are given as the mean value of two independent analyses. [b] For calculating the OAVs, an odor threshold value of 0.2 μg/L for (–)-*cis*-rose oxide was used (*2, 3*).

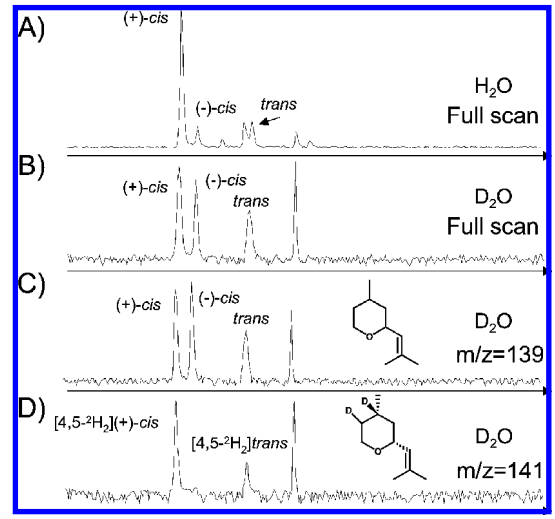

Figure 2. HS-SPME GC−MS analysis of the generated rose oxide stereoisomers of a model must that was spiked with 500 μg/L (*E*)-3,7-dimethyl octa-2,5-dien-1,7-diol and (*E*)-3,7-dimethyl octa-2,7-dien-1,6-diol and fermented with *S. cerevisiae* (**A**). When water as solvent for the model must is replaced by deuterium oxide, the enantiomeric ratio of the resulting *cis*-rose oxide is altered (**B**). Selective ion extraction of the full-scan chromatogram in B reveals the presence of almost racemic nonlabeled *cis*-rose oxide on mass lane *m*/*z* 139 (**C**) and the presence of (+)-*cis*-[4,5-$^2H_2$]rose oxide on mass lane *m*/*z* 141 (**D**).

phosphate NADP are required by about 80 and 10% of redox enzymes, respectively. In the asymmetric reduction with yeast, the cofactor NAD(P)H plays an important role in acting as porter to capture a hydride from a reductant and deliver it later to a substrate. To be effectively reduced by a yeast enoate reductase, the double bond has to be activated by an electron-withdrawing substituent. Allylic alcohols, such as geranyl diol I, in our study are used as substrates, which are transformed via the corresponding unsaturated aldehydes (*16*). During asymmetric reduction of carbonyl compounds by yeast it has been found that the

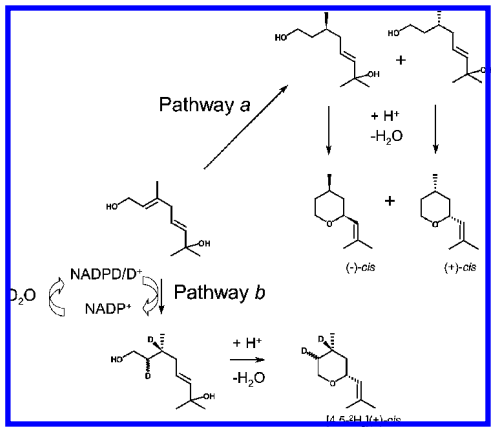

Figure 3. Proposed biosynthetic pathways a and b for the formation of the labeled and nonlabeled rose oxide stereoisomers by yeast fermentation of the model must that contained (*E*)-3,7-dimethyl octa-2,5-dien-1,7-diol as precursor and deuterium oxide as the solvent. $NADP^+$ is the coenzyme of a hypothetical reductase.

hydrogen atoms were transferred from the cofactor NADPH and the hydrogen was provided by the added glucose or certain unidentified substances stored in the yeast cells (*17*). Thus, there are at least two different NADPH pools in yeast, which are differentially labeled with deuterium, when fermentation is carried out in deuterium oxide. Moreover, Fronza and co-workers have already shown by fermentation studies using deuterium oxide as the solvent that the saturation of a double bond of a substrate by yeast that gives rise to the formation of raspberry ketone occurs through the intervention of at least two enzymic systems acting with different stereochemistry (*18*). This dual pathway of reduction is hence well-documented in the literature.

Figure 3 illustrates both conceivable biosynthetic pathways of rose oxide formation in yeast. The stereoselective reduction of the C2 double bond introduces two deuterium atoms at C2 and C3. The coenzym NAD(P) of the hypothetical reductase is regenerated by incorporating deuterium from the solvent deuterium oxide (pathway b). The resulting labeled citronellyl diol yields rose oxide by acid-catalyzed cyclization. Contrarily, pathway a is characterized by the action of a nonselective reductase that transfers two nonsolvent exchangeable hydrogen atoms and yields almost racemic *cis*-rose oxide. It should be noted that the isomeric (*E*)-3,7-dimethyl octa-2,7-dien-1,6-diol may be reduced as well by yeast. However, the resulting diol can not cyclize to yield rose oxide under the given conditions (*19*) and can be ruled out as a precursor.

DISCUSSION

The results of this study corroborate recent findings that the modification of terpene-derived varietal aroma is an integral part of yeast metabolism and not only a simple hydrolytical process (*20*). Although the main stereoisomer of rose oxide that was generated by the used yeast strain is the (+)-*cis*-enantiomer, which has a relatively high odor threshold value of 50 μg/L (*6*), the amounts of generated (–)-*cis*-rose oxide are in some cases sufficiently high enough to modify the aroma of the corresponding wine. The findings are also of interest for the evaluation of authentic food aroma compounds by enantioselective GC because the enantiomeric ratio of a chiral aroma compound obviously can be altered by the fermentation.

Whereas the presented fermentation experiments perfectly explain the presence of (+)-*cis*-rose oxide in wine, it is also possible that beside the identified precursor (*E*)-3,7-dimethyl octa-2,5-dien-1,7-diol other precursors exist that can yield rose oxide after yeast-mediated biotransformation. Nerol-derived diols may be direct substrates as well that yield rose oxide with different enantiomeric ratios. The screening for other precursors and the optimization of the biotransformation conditions, such as temperature, pH value, and yeast strain, are currently in hand.

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NOTE ADDED AFTER PRINT PUBLICATION

The print publication of February 27, 2008 (Vol. 56, Issue 4), contained erroneous presentations of minus signs. These have been corrected April 2, 2008. An addition/correction also appears in the April 9, 2008 (Vol. 56, Issue 7), print issue.

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