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New Insights on 3-Mercaptohexanol (3MH) Biogenesis in Sauvignon Blanc Wines: Cys-3MH and (*E*)-Hexen-2-al Are Not the Major Precursors

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The molar conversion yield of Cys-3MH into 3MH, during alcoholic fermentation, was traced using a deuterated isotope of the precursor added to different Sauvignon Blanc musts. This yield is close to that found in synthetic media supplemented with synthetic Cys-3MH, that is, below 1%. Yet, this represents only 3−7% of the total 3MH production in wine. This clearly shows that Cys-3MH is a precursor of 3MH, but not the main one in the different musts tested. The contribution of (*E*)-hex-2-enal, which has been suggested as another potential precursor of 3MH, was discarded as well, as shown using also a deuterated analogue. The third suggested precursor of 3MH is a glutathionyl-3MH (G-3MH), which upon proteolytic degradation could release Cys-3MH. The knockout of the *OPT1* gene, which encodes the major glutathione transporter, reduces 3MH accumulation by a 2-fold factor in grape must as compared to the wild type strain. Consequently, it is deduced that major 3MH precursor(s) are transported into yeast via Opt1p, which is in favor of G-3MH being a 3MH precursor. This work opens the search for the major natural precursor(s) of 3MH in Sauvignon Blanc must.

KEYWORDS: Thiol; precursor; Sauvignon blanc

INTRODUCTION

Some thiols contribute to the pleasant aroma of some fruits such as grapefruit (*1*), passion fruit (*2*), and guava (*3*). Volatile thiols have also been identified in wines, and especially in Sauvignon Blanc wines (*4, 5*).

3-Mercaptohexanol (3MH), 3-mercaptohexyl acetate (3MHA), 4-mercapto-4-methylpentan-2-one (4MMP), 4-mercapto-4-methylpentan-2-ol (4MMPOH), and 3-mercapto-3-methylbutan-1-ol (3MMB) are volatile thiols that strongly contribute to the flavor of Sauvignon Blanc wines (*5*), especially 3MH and its acetate (*6*). Fruity aromas and very low perception thresholds characterize these two thiols. The aromas of the *R* and *S* forms of 3MH in hydroalcoholic model solution were shown to be slightly different, with aromas reminiscent of grapefruit and passion fruit, respectively, but with similar perception thresholds (50 and 60 ng L^{-1}, respectively). The two enantiomers of 3MHA, produced from 3MH during fermentation, by the action of the yeast ester-forming alcohol acetyltransferase (*7, 8*), exhibit different aromas and perception thresholds: the *R* form is reminiscent of passion fruit and is less odoriferous (perception threshold of 9 ng L^{-1}) than the *S* form, which has a more herbaceous odor of boxwood with a perception threshold of 2.5 ng L^{-1} (*9*). 3MH and 3MHA are key compounds of the aroma typicity of young Sauvignon Blanc wines, but their general impact in wines made from other *Vitis vinifera* L. varieties is taken more and more into account. For example, these compounds have been already identified not only in white wines made from Petit Manseng and Gros Manseng (*10*), Maccabeo (*11*), Muscadet and Bacchus (*12, 13*) but also in red varieties such as Grenache (*14*), Merlot, and Cabernet Sauvignon grapes (*15*), in which they can exert a significant aroma impact, depending on their concentration and the general aroma complexity of the wine.

3MH is generated during the fermentation process, by yeast, from precursors initially present in the must.

The first of these precursors was described as an *S*-cysteine conjugate: *S*-3-(hexan-1-ol)-L-cysteine (Cys-3MH) (*16*). First, nonvolatile crude extracts obtained from Sauvignon Blanc must were shown to contain Cys-3MH (*17*). In addition, the in vitro action of a cell-free enzyme extract of *Eubacterium limosum* (containing β-carbon−sulfur lyase enzymatic activity) on this Sauvignon Blanc extract allowed the release of 3MH, this release being correlated with Cys-3MH decrease (*17*). Consequently, it was deduced that the in vivo volatile thiol release by yeast from its corresponding precursor may involve similar yeast carbon−sulfur lyase enzymatic activities. Studies on putative yeast carbon−sulfur lyases have been performed (*18*), but no single key enzyme could be identified. Several other studies

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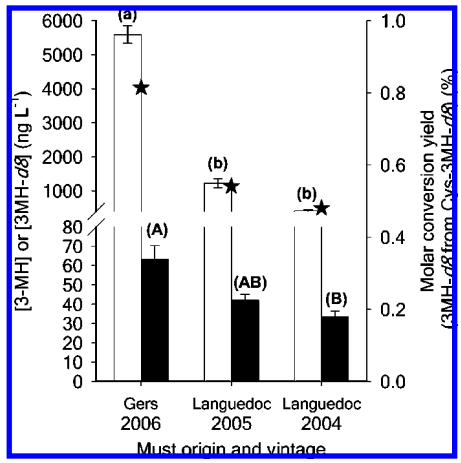

Figure 1. Production of 3MH (white bars), production of 3MH-d_8 (black bars) from 12.5 μg L^{-1} of Cys-3MH-d_8 added to three Sauvignon Blanc grape musts, and molar conversion yield of 3MH-d_8 from Cys-3MH-d_8 added (black stars) by strain ES1 (mean and standard deviation of duplicates). The same letters in parentheses indicate homogeneous groups at the 95% confidence level, as tested by Tukey statistical test.

confirmed the hypothesis that Cys-3MH was a precursor of 3MH. In Cabernet Sauvignon and Merlot rosé wines, a correlation between Cys-3MH content of the must and 3MH concentration in the final wine was demonstrated (*15*). It has also been shown that as 3MH concentration increases in the medium during fermentation, Cys-3MH concentration decreases (*19*). In addition, it was shown that the increase of Cys-3MH concentration by prolonged skin contact allowed an increase in the final amount of 3MH in wine, because Cys-3MH concentration was almost 8 times higher in the skin than in the pulp (*15, 20*).

Calculation of the molar transformation yields of the cysteinylated precursor into its corresponding thiol from literature data gives variable but always low results: 0.1−12% conversion yield of Cys-3MH into 3MH (and 3MHA) (*15, 19, 21*). Conversion yields from Cys-3MH are particularly low on synthetic media (below 1%) compared to those calculated on grape musts (sometimes above 10%) (*19, 21, 22*).

An alternative biogenetic pathway from (*E*)-hex-2-enal leading to 3MH was also demonstrated by the addition of the deuterated analogue of (*E*)-hex-2-enal to Melon B. must (*12, 23*). This pathway needs sulfur addition on the carbonyl precursor, but the nature of the implied sulfur compound was not elucidated. (*E*)-Hex-2-enal is a well-known prefermentary compound. It is generated in must from unsaturated lipids, in concentrations ranging from a few to hundreds of micrograms per liter, depending on grape variety and prefermentary treatment (*24*). At these concentrations, the production of 3MH from (*E*)-hex-2-enal could be significant. Anyway, in the case studied by Schneider et al. (*23*), an accurate quantification of the proportion of deuterated 3MH produced from deuterated (*E*)-hex-2-enal and natural 3MH showed that this pathway afforded about 10% of the total 3MH in these fermentation conditions. Nevertheless, other related compounds such as (*E*)-hex-2-enol and (*Z*)-hex-2-enol or unsaturated intermediates could also lead to 3MH by a similar pathway.

Finally, another 3MH precursor was identified in Sauvignon Blanc and Gros Manseng musts: *S*-3-(hexan-1-ol)-glutathione (G-3MH) (*25*). In Gros Manseng must, G-3MH is 5 times more abundant than Cys-3MH, whereas it is only 1.5 times more abundant in Sauvignon Blanc must, meaning that the relative concentrations of Cys-3MH and G-3MH could depend on the vine variety. By the action of γ-glutamyltranspeptidase and carboxypeptidase enzymatic activities, G-3MH generated Cys-3MH (*25*), although these enzymes have never been identified in grapes.

After several experiments on synthetic media complemented with Cys-3MH and on actual grape musts, we reached the conclusion that on Sauvignon Blanc grape musts the conversion yields of Cys-3MH into 3MH and 3MHA were 10−100 times higher than on synthetic culture media enriched with Cys-3MH (*26, 27*). These yields on grape musts varied significantly from one must to another, even with the same yeast strain (*27*). In addition, there was no correlation between the initial amount of Cys-3MH available in the must and the production of 3MH and 3MHA.

These contradictory results, not only between synthetic culture media enriched with Cys-3MH and grape musts but also within the different grape musts tested, led us to investigate more precisely the Cys-3MH content of Sauvignon Blanc musts and the corresponding 3MH production and to then reconsider the origin of 3MH in wine.

MATERIALS AND METHODS

Chemicals. Analytical reagents have been purchased from Sigma-Aldrich.

3MH, 3MH-d_2, 3MH-d_8, 3MHA, and 3MHA-d_5. 3MH and 3MHA were purchased from Interchim, Montlucon (France). 3MH-d_2 and 3MHA-d_5 were synthesized as reported in a previous paper (*28*). 3MH-d_8 was synthesized from hexenal-d_8 as reported in a previous paper (*23*).

Synthesis of Cys-3MH, Cys-3MH-d_2 and Cys-3MH-d_8. Cys-3MH (*S*-3-(hexan-1-ol)-L-cysteine was synthesized according to the method previously reported (*10*) by the addition of N-Boc-cysteine to (*E*)-hex-2-enal. Cys-3MH-d_8 ([$^2H_8$]-*S*-3-(hexan-1-ol)-L-cysteine) was also synthesized according to this method, by the addition of N-Boc-cysteine to [$^2H_8$]-(*E*)-hex-2-enal. Cys-3MH-d_2 ([$^2H_2$]-*S*-3-(hexan-1-ol)-L-cysteine) was synthesized according to the method previously reported (*10*) by the addition of Boc-lactone on 3MHA-d_2. 3MHA-d_2 was also synthesized in our laboratory, according to the method previously described by Kosteridis et al. (*28*).

Synthesis of [$^2H_8$]-(E)-Hex-2-enal. [$^2H_8$]-(*E*)-Hex-2-enal (hexenal-d_8) was synthesized from [$^2H_{10}$]-butanol in two steps: the first step led to [$^2H_8$]-butanal production and was based on Dess−Martin periodinane oxidation (*29*), and the second led to hexenal-d_8 and was based on Wittig reactivity according to the method previously described by Schneider (*12*).

Yeast Strains. Two commercial wine yeast strains of *Saccharomyces cerevisiae*, ES1 and ES2, were used in this study. Two laboratory yeast strains of *S. cerevisiae* from EUROSCARF were also used: BY4743 and its deletion mutant BY4743 *opt1*Δ.

Culture Media and Fermentation Conditions. Three French Sauvignon Blanc grape musts were used: two from Languedoc (vintages 2004 and 2005) containing 11.5 and 12.5 μg L^{-1} of Cys-3MH, respectively, and one from Gers (vintage 2006) containing 35 μg L^{-1} of Cys-3MH.

Experiments were conducted at laboratory scale in handmade glass fermenters with a working volume of 1.1 L. Fermenters were fitted with fermentation locks (CO_2 bubbling outlets filled with water), and fermentations were conducted under anaerobic conditions with constant stirring under isothermal conditions (22 °C for industrial strains and 24 °C for laboratory strains). For inoculation of enological strains, active dry yeasts (ADY) were simply rehydrated as recommended by the manufacturer. Briefly, 2 g of dry yeast was suspended in 20 mL of warm water (37 °C) containing glucose (50 g L^{-1}). This suspension was incubated for 30 min at 37 °C with strong agitation every 15 min. Two milliliters of this suspension was used to inoculate 1 L of the fermentation medium, giving a cell concentration of about 2 × 10^6 cells mL^{-1}.

BY4743 laboratory strains were used to ferment the must from Gers (2006), complemented with amino acids to correct yeast auxotrophies. Fermenters were inoculated at 2 × 10^6 cells mL^{-1}, from precultures. Precultures were conducted on the same must at 28 °C under stirring

Table 1. Calculation of the 3MH Amount Generated from Cys-3MH Naturally Present in Grape Musts from the Conversion Yields Obtained from Cys-3MH-d_8 into 3MH-d_8

must origin	vintage	total 3MH (ng L^{-1})	molar yield of 3MH-d_8 from Cys-3MH-d_8 (%)	natural Cys-3MH (μg L^{-1})	3MH calcd from Cys-3MH (ng L^{-1})	3MH originated from Cys-3MH on the total 3MH (%)
Gers	2006	5590	0.81	35.0	170	3
Languedoc	2005	1226	0.54	12.5	40	3
Languedoc	2004	418	0.48	11.5	33	7

for 24 h in 50 mL sterile Erlenmeyer flasks (working volume of 20 mL, heat-sterilized at 120 °C, 20 min). Cells were counted before centrifugation of the volume of preculture (1500*g*, 5 min) necessary for a 2×10^6 cells mL^{-1} inoculation. Supernatants were discarded, and cell pellets were used for inoculation. All manipulations were performed in sterile conditions.

Fermentation progress was followed by fermenter weighing: the amount of CO_2 released was determined by automatic measurement of fermenter weight loss every 20 min (*30*).

Completed fermentations were clarified by centrifugation (1800*g* for 10 min), and 500 mL of supernatant was treated for thiol extraction, immediately after fermentation. Experiments were conducted in duplicates.

Cell Counting. Yeast culture samples were diluted with Isoton II (Beckman-Coulter, Margency, France) for cell enumeration (1000−2500 times). After sonication (35 s, 10 W), cells were counted with an electronic Coulter counter (model Z2, Beckman-Coulter, Margency, France) fitted with a 100 μm aperture probe.

Analysis of 3MH, 3MH-d_8, 3MHA, and 3MHA-d_8. Extraction of the volatile thiols and purification of the extracts were performed according to the method previously reported (*12, 13*). 3MH-d_2 and 3MHA-d_5 were used as internal standards for stable isotope dilution assay. Typically, 500 ng L^{-1} of 3MH-d_2 and 50 ng L^{-1} of 3MHA-d_5 were added to 500 mL of fermented synthetic medium. The final purified extracts were analyzed by gas chromatography coupled with ion trap tandem mass spectrometry (GC-ITMS-MS) (*10*). In our experiments, 3MHA-d_8 was always below the detection limit (10 ng L^{-1}). New calibration solutions were prepared for each series of experiments. The peak area ratios for the selected quantifiers were plotted against the concentration ratios. Calibration concentration ratio and absolute area value were adapted to results obtained on the extract analysis. The standard errors for quantification of 3MH, 3MH-d_8, and 3MHA in these conditions are 10 and 5%, respectively.

Analysis of Cys-3MH. Cys-3MH-d_2 was added to defrosted supernatant as internal standard (typically, 50 μg L^{-1} of Cys-3MH-d_2 was added to 25 mL of supernatant). Initial or fermented musts were extracted on cation exchange Dowex resin (50wX4-100; Sigma Aldrich). Derivatization of the extract with ethyl chloroformate and analysis of the final derivatized extract by gas chromatography−electronic impact mass spectrometry (GC-ECMS) was done as previously described (*10*). Calibration curves were plotted for the target compound, that is, natural Cys-3MH. After concentration to dryness under nitrogen flow at 45 °C, calibration solutions containing the target compounds at serial dilutions and the fixed amount of Cys-3MH-d_2 used in the samples were derivatized with ethyl chloroformate (*10*). The peak area ratios for the selected quantifiers were plotted against the concentration ratios. Calibration concentrations ratio and absolute area value were adapted to results obtained on the extract analysis.

Analysis of (E)-Hex-2-enal and Related Compounds in Grape Must. One hundred milliliters of grape must was placed in a separator funnel cooled at 1 °C in an ice bath under nitrogen, and 65 μg of 4-nonanol was added as an internal standard. After homogenization, liquid−liquid extraction of the volatiles was performed with 30 mL of dichloromethane (Pestanal 99.8%, Riedel de Haën). The mixture was then stirred during 20 min at 500 rpm before a second addition of 30 mL of dichloromethane. The mixture was then stirred again during 20 min at 500 rpm. After that, the mixture was centrifuged for 25 min at 10000*g* (4 °C), and the organic phase was collected and dried over sodium sulfate. Finally, the extract was concentrated by partial rectification on a Vigreux column at 45 °C, to a final volume of about 4 mL. Analysis of the final extract was performed by gas chromatography−electronic impact mass spectrometry (GC-EIMS). Analysis was performed in the full-scan mode in the mass range from 29 to 350 amu. Data acquisition and treatment were performed with Hewlett-Packard 5989 B.05.02 MS Chemstation software.

Statistical Analysis. The KyPlot (version 2.15) free software (http://www.qualest.co.jp/Download/KyPlot) was used to perform ANOVA and Tukey tests (pairwise comparisons for one-way layout design) to classify the data into homogeneous groups.

RESULTS

Is 3MH Precursor in Sauvignon Blanc a Cysteinylated Precursor? To check if 3MH was indeed generated from Cys-3MH, we complemented several Sauvignon Blanc grape musts with the deuterated analogue Cys-3MH-d_8. After fermentation, the amount of 3MH-d_8 was quantified. Calculation of the molar conversion yield of 3MH-d_8 from Cys-3MH-d_8 allowed us to estimate the production of 3MH from the natural form of Cys-3MH by reporting the conversion yield of the deuterated to the natural one.

Sauvignon Blanc musts from Languedoc (2004 and 2005 vintages) and from Gers (2006 vintage) were complemented with 12.5 μg L^{-1} of Cys-3MH-d_8 and fermented with strain ES1 (1.1 L fermentors, 22 °C, ADY). Results are given in **Figure 1**.

The amount of 3MH-d_8 produced and the conversion yields calculated from Cys-3MH-d_8 were all below 1% (**Table 1**). Considering that the transformation yields are similar for Cys-3MH and Cys-3MH-d_8, we calculated the amount of 3MH that originated from natural Cys-3MH. Results are given in **Table 1**.

Table 1 demonstrates that only a small part of the total 3MH amount came from Cys-3MH (3−7%). Thus, in none of the three Sauvignon Blanc musts tested was the main 3MH precursor Cys-3MH. We cannot exclude that it could be the case in other Sauvignon Blanc wines. Nevertheless, in our experiments we obtained three wines (concentration of 3MH ranging from 400 to 5500 ng L^{-1}) that gave an impression of low to very strong aromatic typicity (data not shown) and in none of them did Cys-3MH play a central role.

To check that this limited conversion from Cys-3MH was not due to the yeast strain, we also tested strain ES2 in the same conditions [Sauvignon Blanc must from Gers (2006 vintage) complemented with 12.5 μg L^{-1} of Cys-3MH-d_8] and quantified the corresponding thiol productions. Results are given in **Figure 2**.

Similar results were obtained with strain ES2: a final conversion yield from Cys-3MH-d_8 of 0.6% was obtained, that is to say, 130 ng L^{-1} of 3MH obtained from the 35 μg L^{-1} of Cys-3MH initially present in the must. This represents <5% of the final amount of 3MH.

With the two strains considered, Cys-3MH was clearly not the most important origin of 3MH in the Sauvignon Blanc must

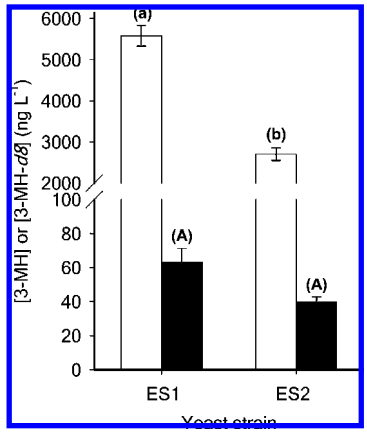

Figure 2. Production of 3MH (white bars) and production of 3MH-d_8 from 12.5 μg L^{-1} of Cys-3MH-d_8 added to Sauvignon Blanc grape must from Gers (2006 vintage) and fermented by strains ES1 and ES2 (black bars) (mean and standard deviation of duplicates). The same letters in parentheses indicate homogeneous groups at the 95% confidence level, as tested by Tukey statistical test.

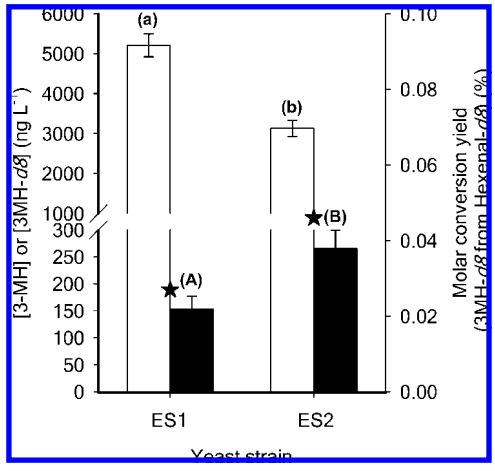

Figure 3. Production of 3MH (white bars), production of 3MH-d_8 from 1200 μg L^{-1} of hexenal-d_8 added to Sauvignon blanc grape must from Gers (2006 vintage) (black bars), and molar conversion yield of 3MH-d_8 from hexenal-d_8 added (black stars) to strains ES1 and ES2 at 22 °C (mean and standard deviation of duplicates). The same letters in parentheses indicate homogeneous groups at the 95% confidence level, as tested by Tukey statistical test.

tested. In our conditions, this production pathway always led to <10% of the total amount of 3MH. We therefore investigated other production pathways for 3MH.

Is (E)-Hex-2-enal in Sauvignon Blanc a Carbonyl Precursor of 3MH? The alternative biogenetic pathway leading to 3MH from (*E*)-hex-2-enal was previously demonstrated (*12, 23*). Following the same procedure, we complemented the Sauvignon Blanc grape must from Gers (2006 vintage) with 1200 μg L^{-1} of hexenal-d_8. As the sulfur donor is not elucidated, but could be H_2S, two different yeast strains (ES1 and ES2) that potentially have different H_2S productions were again tested. Results are given in **Figure 3**.

In this grape must, 16 μg L^{-1} of (*E*)-hex-2-enal was quantified. Reporting the conversion yield obtained with the hexenal-d_8 added (below 0.05%), we can estimate that, whatever the strain considered, <5 ng L^{-1} of 3MH was produced through this pathway. This represents <1‰ of the total amount of 3MH detected. This result was also obtained with a third industrial yeast strain (data not shown).

On the must studied (from Gers, 2006 vintage) the 3MH production through the hexenal pathway was thus negligible. We also quantified other related volatile compounds such as (*E*)-hex-2-enol and (*Z*)-hex-2-enol, which could lead to 3MH by similar pathways, but none of them occurred in significant quantities (data not shown).

In the Sauvignon Blanc musts from Languedoc, (*E*)-hex-2-enal was below the detection threshold and no related volatile compounds occurred in significant quantities. Therefore, we did not perform the test with hexenal-d_8 on these musts.

Finally, it can be concluded that, in our conditions, none of the pathways that have been clearly shown to be related to 3MH production [from Cys-3MH (*16*) and (*E*)-hex-2-enal (*23*)] played a key role in the final 3MH amount generated in the corresponding wines. The last suggested production pathway was from *S*-3-(hexan-1-ol)-glutathione (G-3MH) (*25*). This last pathway was therefore investigated.

Is 3MH Precursor in Sauvignon Blanc a Glutathionylated Precursor? The best way to investigate the role of G-3MH in 3MH production would be, as previously done for Cys-3MH and (*E*)-hex-2-enal, to complement a grape must with the labeled analogue of G-3MH and quantify the corresponding labeled 3MH produced at the end of fermentation.

Unfortunately, we did not have a labeled analogue of G-3MH at our disposal. Therefore, an indirect way of investigation was chosen to get a better idea of the potential role of G-3MH in 3MH formation. We used a laboratory *S. cerevisiae* yeast strain deleted for the gene encoding the main glutathione transporter (*OPT1*) (*31*). *OPT1*, also known as *HGT1* (open reading frame *YJL212*c), was shown to be a high-affinity glutathione transporter (K_m = 54 μM) from the yeast *S. cerevisiae* (*31*). It belongs to an oligopeptide permease transporter family (*32*) and also transports oligopeptides consisting of four to five amino acids (*33*). The function of Opt1p, as a peptide transporter, may represent a way for the cell to incorporate sulfur (*34*). It was shown that yeast strains deleted for *OPT1* did not show any detectable plasma membrane glutathione transport. Such *opt1*Δ deletion is lethal in a mutant defective in glutathione biosynthesis (*gsh1*Δ). The specific repression of the transport activity by glutathione argues strongly for Opt1p being primarily a glutathione transporter. Even if a second glutathione transporter exists, its contribution to glutathione uptake is considered to be very minimal.

Our hypothesis is that G-3MH would be transported into the yeast through Opt1p.

The BY4743 strain and its mutant BY4743*opt1*Δ were tested on the Sauvignon Blanc grape must from Gers (2006 vintage), on which the natural production of 3MH was the most important. Results are shown in **Figure 4**. In this case, fermentations were stopped at 8% of residual sugars, to limit uncontrolled variations possibly induced by sluggish fermentation with laboratory strain on actual grape must.

This experiment demonstrated that in the absence of Opt1p, the production of 3MH and 3MHA was divided by a factor 2. Thus, at least half of the main precursor(s) of 3MH enter(s) the cell through Opt1p. In the absence of this transporter, the precursor uptake limited significantly the production of 3MH and 3MHA by yeast.

DISCUSSION

This work raised new questions on the nature of 3MH precursor in grape must.

On three French Sauvignon Blanc musts, addition of a deuterated analogue of Cys-3MH to the must allowed us to

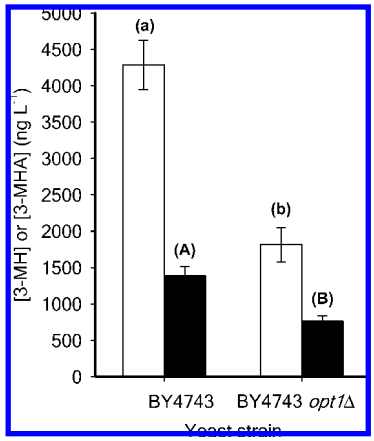

Figure 4. 3MH (white bars) and 3MHA (black bars) production by strain BY4743 and its deletion mutant BY4743 *opt1*Δ on Sauvignon Blanc grape must from Gers (2006 vintage) at 24 °C (mean and standard deviation of duplicates). The same letters in parentheses indicate homogeneous groups at the 95% confidence level, as tested by Tukey statistical test.

quantify that only 3−7% of the total 3MH production in fact originated from Cys-3MH. Moreover, also by addition of a deuterated analogue, it was demonstrated that (*E*)-hex-2-enal was not a major precursor of 3MH.

In our cases, the absence of correlation between results obtained on synthetic media compared to those obtained on grape musts could be explained by the fact that the precursor used in synthetic media, Cys-3MH, was not the main precursor in the grape musts tested. If the conclusion that Cys-3MH is not a major precursor of 3MH was extended to grape musts in general, it could explain the difficulties encountered, not only by us but also by the scientific community, to mimic thiol production in synthetic culture media (differences in the molar production yields) (*8, 18, 22*) and the differences observed between results obtained on synthetic media and grape musts (regarding the influence of the fermentation temperature for example) (*21, 35*). For this, further experiments should be conducted on additional grape musts to validate that neither Cys-3MH nor (*E*)-hex-2-enal is ever a major precursor of 3MH.

However, Cys-3MH was shown to be present in must at a concentration of about 10 μg L^{-1}. If the conversion yield of this precursor into 3MH was close to 100%, enough 3MH would be produced for the wine to exhibit a strong aromatic typicity. Such a complete conversion could have happened with the mutant of a commercial wine yeast strain, constructed by Swiegers et al. (*8*), in which the *Escherichia coli tna*A gene, encoding a tryptophanase with strong cysteine-β-lyase activity, was cloned and overexpressed.

In addition, the major 3MH precursor(s) present in the must could be cleaved by cysteine-β-lyase activity to liberate 3MH. In this case the indirect quantification of the must precursors using the tryptophanase activity, as described by Peyrot Des Gachons et al. (*36*), would in fact give an estimation of the whole thiol potential of the must and not of the cysteinylated precursors content. The must aromatic potential quantified with this method would therefore be correlated to the final thiol amount (*20, 36*). However, this hypothesis would need further investigations.

Regarding G-3MH being a major precursor of 3MH, the limitation in 3MH production induced by *OPT1* deletion cannot be taken as a demonstration that G-3MH is indeed a 3MH precursor. From this result, two hypotheses could be made:

(1) Half of the 3MH precursor contained in this must entered the cell through Opt1p and therefore exhibited a "glutathionylated-like structure", most probably an *S*-glutathione conjugate like G-3MH but also maybe a tetra- or penta-peptide structure.

(2) Glutathione could be an activator of 3MH release from its actual precursor. Glutathione uptake was strongly limited in the absence of Opt1p, and 3MH liberation would thus have been down-regulated by the limited glutathione intracellular content. This hypothesis could be tested by glutathione quantification and addition in musts. Nevertheless, the antioxidant effect of glutathione on free thiols should also be taken into consideration within this experiment.

Peyrot Des Gachons et al. (*25*) also suggested that G-3MH could itself be a Cys-3MH precursor. Nevertheless, because of the very low conversion yields obtained from Cys-3MH, if generation of 3MH from G-3MH was going through a first cleavage step to Cys-3MH, the yields would remain very low and significant quantities of G-3MH would be needed. Therefore, a direct liberation of 3MH from G-3MH would be more profitable.

Besides, even if the 3MH production decreased significantly with the deletion of *OPT1*, the deletion mutant strain still produced half of the 3MH final amount of the corresponding wild type strain. This remaining production could have had different origins: (1) G-3MH that would enter the cell htrough other transporter(s) or (2) other precursor(s) that would also enter the cell through other transporter(s). Depending on the results, double (or more) deletion mutants should be tested.

Deuterated G-3MH is now being synthesized in our laboratory. This labeled analogue should then be used to quantify the importance of G-3MH in the production of 3MH. This information shall orientate further projects on the Sauvignon Blanc aroma topic.

It is now of great importance to identify actual thiol precursor(s). It should indeed be noted that as long as the precursor is not identified (and synthesized), it is not possible to conduct experiments on model synthetic media. Furthermore, its structure would give leads for the understanding of the transport and conversion pathways.

LITERATURE CITED

(1) Buettner, A.; Schieberle, P. Characterization of the most odor-active volatiles in fresh, hand-squeezed juice of grapefruit (*Citrus paradisi* Macfayden). *J. Agric. Food Chem.* **1999**, *47*, 5189–5193.

(2) Engel, K. H.; Tressl, R. Identification of a new sulfur containing volatiles in yellow passion fruits (*Passiflora edulis* f. *flavicarpa*). *J. Agric. Food Chem.* **1991**, *39*, 2249–2252.

(3) Du Plessis, C. S.; Augustyn, O. P. H. Initial study on the guava aroma of Chenin blanc and Colombard wines. *S. Afr. J. Enol. Vitic.* **1981**, *2*, 101–103.

(4) Darriet, P.; Tominaga, T.; Lavigne, V.; Boidron, J.-N.; Dubourdieu, D. Identification of a powerful aromatic component of *Vitis vinifera* L. var. Sauvignon wines: 4-mercapto-4-methylpentan-2-one. *Flavour Fragrance J.* **1995**, *10*, 385–392.

(5) Tominaga, T.; Furrer, A.; Henry, R.; Dubourdieu, D. Identification of new volatile thiols in the aroma of Vitis vinifera L. var. Sauvignon blanc wines. *Flavour Fragrance J.* **1998**, *13*, 159–162.

(6) Tominaga, T.; Darriet, P.; Dubourdieu, D. Identification of 3-mercaptohexanol acetate, compound having a powerful odor reminiscent of box-tree, involved in the aroma of Sauvignon wines. *Vitis* **1996**, *35*, 207–210.

(7) Swiegers, J. H.; Willmott, R.; Hill-Ling, A.; Capone, D. L.; Pardon, K. H.; Elsey, G. M.; Howell, K. S.; De Barros Lopes, M. A.; Sefton, M. A.; Lilly, M.; Pretorius, I. S. Modulation of volatile thiol and ester aromas in wine by modified wine yeast. *Proceedings of the Weurman Flavour Research Symposium, Developments in Food Science*, Roskilde, Denmark, June 21−24, 2005; Elsevier: Amsterdam, The Netherlands, 2005.

(8) Swiegers, J. H.; Pretorius, I. S. Modulation of volatile sulfur compounds by wine yeast. *Appl. Microbiol. Biotechnol.* **2007**, *74*, 954–960.
(9) Tominaga, T.; Niclass, Y.; Frerot, E.; Dubourdieu, D. Stereoisomeric distribution of 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate in dry and sweet white wines made from *Vitis vinifera* (Var. Sauvignon Blanc and Semillon). *J. Agric. Food Chem.* **2006**, *54*, 7251–7255.
(10) Dagan, L. Potentiel aromatique des raisins de *Vitis vinifera* L. cv. Petit Manseng et Gros Manseng. Contribution à l'arôme des vins de pays des Côtes de Gascogne., Agro M, 2006.
(11) Escudero, A.; Gogorza, B.; Melus, M. A.; Ortin, N.; Cacho, J.; Ferreira, V. Characterization of the aroma of a wine from maccabeo. Key role played by compounds with low odor activity values. *J. Agric. Food Chem.* **2004**, *52*, 3516–3524.
(12) Schneider, R. Contribution à la conaissance de l'arôme et du potentiel aromatique du Melon B (*Vitis vinifera* L.) et des vins de Muscadet, Montpellier II, 2001.
(13) Schneider, R.; Kotseridis, Y.; Ray, J. L.; Augier, C.; Baumes, R. Quantitative determination of sulfur-containing wine odorants at sub parts per billion levels. 2. Development and application of a stable isotope dilution assay. *J. Agric. Food Chem.* **2003**, *51*, 3243–3248.
(14) Ferreira, V.; Ortin, N.; Escudero, A.; Lopez, R.; Cacho, J. Chemical characterization of the aroma of Grenache rose wines: aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. *J. Agric. Food Chem.* **2002**, *50*, 4048–4054.
(15) Murat, M. L.; Tominaga, T.; Dubourdieu, D. Assessing the aromatic potential of Cabernet Sauvignon and Merlot musts used to produce rose wine by assaying the cysteinylated precursor of 3-mercaptohexan-1-ol. *J. Agric. Food Chem.* **2001**, *49*, 5412–5417.
(16) Tominaga, T.; Masneuf, I.; Dubourdieu, D. A S-cysteine conjugate, precursor of aroma of white Sauvignon. *J. Int. Sci. Vigne Vin* **1995**, *29* (4), 227–232.
(17) Tominaga, T.; Peyrot des Gachons, C.; Dubourdieu, D. A new type of flavor precursor in *Vitis vinifera* L. cv. Sauvignon Blanc: *S*-cysteine conjugates. *J. Agric. Food Chem.* **1998**, *46*, 5215–5219.
(18) Howell, K. S.; Klein, M.; Swiegers, J. H.; Hayasaka, Y.; Elsey, G. M.; Fleet, G. H.; Hoj, P. B.; Pretorius, I. S.; de Barros Lopes, M. A. Genetic determinants of volatile-thiol release by *Saccharomyces cerevisiae* during wine fermentation. *Appl. Environ. Microbiol.* **2005**, *71*, 5420–5426.
(19) Dubourdieu, D.; Tominaga, T.; Masneuf, I.; Peyrot des Gachons, C.; Murat, M. L. The role of yeasts in grape flavor development during fermentation: the example of Sauvignon blanc. *Am. J. Enol. Vitic.* **2006**, *57*, 81–88.
(20) Peyrot des Gachons, C.; Tominaga, T.; Dubourdieu, D. Localization of *S*-cysteine conjugates in the berry: effect of skin contact on aromatic potential of *Vitis vinifera* L. cv. Sauvignon Blanc must. *Am. J. Enol. Vitic.* **2002**, *53*, 144–146.
(21) Masneuf-Pomarede, I.; Mansour, C.; Murat, M. L.; Tominaga, T.; Dubourdieu, D. Influence of fermentation temperature on volatile thiols concentrations in Sauvignon blanc wines. *Int. J. Food Microbiol.* **2006**, *108*, 385–390.
(22) Howell, K. S.; Swiegers, J. H.; Elsey, G. M.; Siebert, T. E.; Bartowsky, E. J.; Fleet, G. H.; Pretorius, I. S.; de Barros Lopes, M. A. Variation in 4-mercapto-4-methyl-pentan-2-one release by *Saccharomyces cerevisiae* commercial wine strains. *FEMS Microbiol Lett.* **2004**, *240*, 125–129.
(23) Schneider, R.; Charrier, F.; Razungles, A.; Baumes, R. Evidence for an alternative biogenetic pathway leading to 3-mercaptohexanol and 4-mercapto-4-methylpentan-2-one in wines. *Anal. Chim. Acta* **2006**, *536*, 58–64.
(24) Cordonnier, R.; Bayonove, C. Etude de la phase perfermentaire de la vinification: extraction et formation de certains composés de l'arôme; cas des terpenols, des aldehydes et des alcools en C6. *Connaiss. Vigne Vin* **1981**, *15*, 269–286.
(25) Peyrot Des Gachons, C.; Tominaga, T.; Dubourdieu, D. Sulfur aroma precursor present in S-glutathione conjugate form: identification of *S*-3-(hexan-1-ol)-glutathione in must from *Vitis vinifera* L. cv. Sauvignon blanc. *J. Agric. Food Chem.* **2002**, *50*, 4076–4079.
(26) Subileau, M.; Salmon, J. M.; Schneider, R.; Degryse, E. Precursor uptake modulates the production of aromatic thiols during fermentation. *FEMS Yeast Res.* **2008**, *8*, 771–780.
(27) Subileau, M. Parameters influencing varietal thiol release by strains of Saccharomyces cerevisiae: from a controlled synthetic medium to the complexity of Sauvignon blanc must, SupAgro, 2008.
(28) Kotseridis, Y.; Ray, J. L.; Augier, C.; Baumes, R. Quantitative determination of sulfur containing wine odorants at sub-ppb levels. 1. Synthesis of the deuterated analogues. *J. Agric. Food Chem.* **2000**, *48*, 5819–5823.
(29) Lévêque, L.; Le Blanc, M.; Pastor, R. Synthesis of per(poly)fluoroalkyl aldehydes RF(CH2nCHO). *Tetrahedron Lett.* **1998**, *39*, 8857–8860.
(30) Sablayrolles, J. M.; Barre, P.; Grenier, P. Design of a laboratory automatic system for studying alcoholic fermentations in anisothermal enological conditions. *Biotechnol. Tech.* **1987**, *1*, 181–184.
(31) Bourbouloux, A.; Shahi, P.; Chakladar, A.; Delrot, S.; Bachhawat, A. K. Hgt1p, a high affinity glutathione transporter from the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **2000**, *275*, 13259–13265.
(32) Lubkowitz, M. A.; Barnes, D.; Breslav, M.; Burchfield, A.; Naider, F.; Becker, J. M. Schizosaccharomyces pombe isp4 encodes a transporter representing a novel family of oligopeptide transporters. *Mol. Microbiol.* **1998**, *28*, 729–741.
(33) Hauser, M.; Narita, V.; Donhardt, A. M.; Naider, F.; Becker, J. M. Multiplicity and regulation of genes encoding peptide transporters in *Saccharomyces cerevisiae*. *Mol. Membr. Biol.* **2001**, *18*, 105–112.
(34) Wiles, A. M.; Cai, H.; Naider, F.; Becker, J. M. Nutrient regulation of oligopeptide transport in *Saccharomyces cerevisiae*. *Microbiology* **2006**, *152*, 3133–3145.
(35) Swiegers, J. H.; Francis, I. L.; Herderich, M. J.; Pretorius, I. S. Meeting consumer expectations through management in vineyard and winery: the choice of yeast for fermentation offers great potential to adjust the aroma of Sauvignon Blanc wine. *Aust. N.Z. Wine Ind.* **2006**, *21*, 34–42.
(36) Peyrot Des Gachons, C.; Tominaga, T.; Dubourdieu, D. Measuring the aromatic potential of *Vitis vinifera* L. cv. Sauvignon blanc grapes by assaying *S*-cysteine conjugates, precursors of the volatile thiols responsible for their varietal aroma. *J. Agric. Food Chem.* **2000**, *48*, 3387–3391.

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