# A New Type of Flavor Precursors in *Vitis vinifera* L. cv. Sauvignon Blanc: *S*-Cysteine Conjugates

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Three flavor-active volatile thiols (4-mercapto-4-methylpentan-2-one, 4-mercapto-4-methylpentan-2-ol and 3-mercaptohexan-1-ol) involved in *Vitis vinifera* L. var. Sauvignon blanc wine aroma can be generated in vitro from nonvolatile must extracts by the enzyme action of a cell-free extract obtained from the gastrointestinal bacterium *Eubacterium limosum*. The specificity of a cysteine conjugate $\beta$-lyase (EC 4.4.1.13) which is contained in the cell-free extract, strongly suggested the existence of precursor for these volatile thiols having a structure of *S*-cysteine conjugate. Gas-phase chromatography analysis coupled with mass spectrometry of must extract in the form of trimethylsilylated derivatives, verified this hypothesis. The release of flavor-active volatile thiols during alcoholic fermentation of the must is shown to be due to the degradation, by yeast, of the corresponding *S*-cysteine conjugates. This mechanism explains the amplification of the typical Sauvignon blanc grape aroma during alcoholic fermentation.

**Keywords:** *Cysteine conjugate β-lyase; Eubacterium limosum; flavor precursor; metal ion chelated column; Sauvignon blanc; S-cysteine conjugate; varietal aroma*

## INTRODUCTION

Most of the flavor precursors identified up until now in fruits (Ho et al., 1990; Stahl-Biskup et al., 1993), particularly in grapes (Williams et al., 1982; Strauss et al., 1988; Winterhalter et al., 1990), are glycosides of monoterpenes or $C_{13}$ norisoprenoids. Several of the corresponding aglycones (free forms) are involved in the aromas of floral grape varieties such as Muscats (Ribéreau-Gayon et al., 1975). With "floral" varieties, musts and wines have similar terpene aroma because there is little transformation of monoterpenes glycosides to volatile aglycones by yeasts metabolism during alcoholic fermentation. In contrast, "simple" or "non-floral" grape varieties, like Sauvignon blanc, may have characteristic varietal aromas that are much more intense in wine after fermentation than in must (Peynaud, 1980), but the mechanism of the development of such aromas has never been elucidated.

Three highly aromatic volatile thiols (4-mercapto-4-methypentan-2-one, 4-mercapto-4-methylpentan-2-ol, and 3-mercaptohexan-1-ol) were recently identified in Sauvignon blanc wines (Darriet et al., 1995; Tominaga et al., 1998a) where they were responsible for the overtones of box tree, broom, and passion fruit considered typical of this grape variety (Tominaga et al., 1998b). However, only trace levels of the compounds occur in the must of this cultivar. The presence of a nonvolatile, non-glycosylated precursor of 4-mercapto-4-methypentan-2-one in Sauvignon blanc must has been shown (Darriet et al., 1993), but the structure of this compound had not been elucidated.

This paper describes the identification of flavor precursors for the three volatile thiols involved in the aroma of Sauvignon wines. The amplification of these aromas, from must to wine, by yeast during alcoholic fermentation is also discussed.

## MATERIALS AND METHODS

**Preparation of Crude Extract Containing Sulfur Flavor Precursors (CESFPs).** Sauvignon blanc must, prior to fermentation, was obtained from a winery of the Bordeaux area. A crude extract likely to contain sulfur flavor precursors was obtained by processing 45 L of must by adsorption chromatography on $C_{18}$ silica (Alltech; Econosil C18) eluted by 1% ethanol (Darriet et al., 1993). The eluates were evaporated to dryness in a vacuum and then reconstituted in 4.5 mL of distilled water.

**Enzyme Release of Volatile Thiols from CESFPs.** Alliin lyase (EC 4.4.1.4) was partially purified from garlic (Mazelis and Crews, 1968). Crude preparation of cysteine conjugate $\beta$-lyase (EC 4.4.1.13) was obtained from *Eubacterium limosum* (strain ATCC 10829, Biovalley, F-94381, Bonneuil/Marne) cultivated under the conditions described by Kerkenaar et al. (1988) and isolated by the method of Tomisawa et al. (1984). A total of 50 $\mu$L of CESFPs was incubated with 25 $\mu$L of cell-free extract of this bacterium or garlic extract in 250 $\mu$L of reagent medium consisting of a potassium phosphate buffer (100 mM, pH 8.0) containing 1 mM EDTA, 0.1 mM pyridoxal phosphate, and 1 mM glutathione. After 15 min, the volatile products formed were extracted with 200 $\mu$L of dichloromethane containing 75 ng of methyl isothiocyanate as an internal standard. The organic phase was collected by centrifugation and concentrated to 50 $\mu$L under a nitrogen flow.

**Purification of the CESFPs.** A total of 3 mL of CESFPs was percolated on a strongly basic anion column (0.5 × 2 cm) (Dowex A2, Sigma; code I-7396). The fraction that was not retained was adjusted to pH 8 by adding a 1 M potassium phosphate buffer and then applied to a chelating Sepharose 4B (Pharmacia; code 17-0575-01) column (0.5 × 2 cm) containing $Cu^{2+}$ immobilized two-thirds of the way up. After being rinsed with the potassium phosphate buffer, 0.05 M at pH 8, the column was eluted by 2 mL of hydrochloric acid at 50 mM. The eluate was evaporated to dryness in a vacuum, and the

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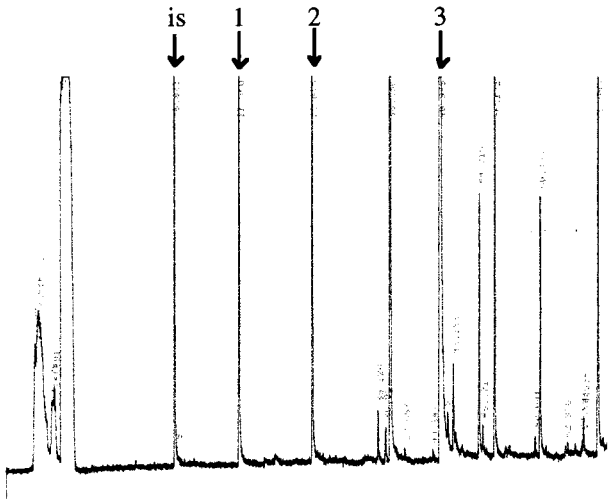


**Figure 1.** Release of volatile thiols from Sauvignon blanc must extract due to the enzyme action of cell-free extract from *Eubacterium limosum* possessing a high activity of cysteine conjugate $\beta$-lyase (EC 4.4.1.13) (is, internal standard; 1, 4-mercapto-4-methylpentan-2-one; 2, 4-mercapto-4-methylpentan-2-ol; 3, 3-mercaptohexan-1-ol).

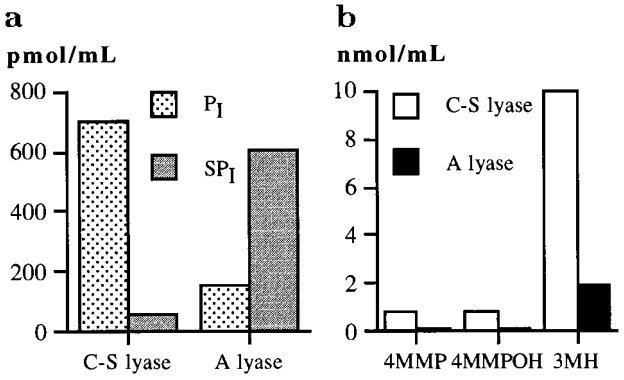


**Figure 2.** Enzyme release of volatile thiols by a cell-free extract from *Eubacterium limosum* (C−S lyase) or garlic extract (A lyase) acting on various flavor precursor substrates. (a) Synthesized flavor precursors: *S*-4-(4-methylpentan-2-one)-L-cysteine ($P_I$), and *S*-4-(4-methylpentan-2-one)-L-cysteine sulfoxide ($SP_I$). (b) Natural flavor precursors in Sauvignon blanc must extract. 4MMP, 4-mercapto-4-methylpentan-2-one; 4MMPOH, 4-mercapto-4-methylpentan-2-ol; 3MH, 3-mercaptohexan-1-ol.

residue was suspended in 500 $\mu$L of absolute ethanol. Finally, the soluble fraction was evaporated to dryness.

**Synthesis of *S*-Cysteine Conjugates.** *S*-4-(4-Methylpentan-2-one)-L-cysteine (PI) was obtained by an addition reaction of L-cysteine hydrochloride (Sigma; code C-4820) (200 mg in 20 mL of water) with 1 mL of mesityl oxide (Aldrich; code M785-5). After one night at room temperature with magnetic stirring, the product was purified by adsorption on a silica column $C_{18}$ (2.2 × 2 cm) and eluted by 25 mL of 1% aqueous ethanol. The eluate was evaporated to dryness in a vacuum at 35 °C and diluted in 2 mL of water. *S*-4-(4-Methylpentan-2-ol)-L-cysteine (PII) was obtained by reducing of PI with sodium borohydride (Tominaga et al., 1998a). *S*-3-(Hexan-1-ol)-L-cysteine (PIII) was obtained by reducing *S*-3-(hexan-1-al)-L-cysteine prepared from 1 mL of *trans*- 2-hexenal (Aldrich; code 13,265-9) and L-cysteine in the same way as PI. *S*-4-(4-Methylpentan-2-one)-L-cysteine sulfoxide (SPI) was obtained by oxidation of PI with $H_2O_2$ (Synge and Wood, 1956). The quantity of *S*-cysteine conjugates was determined by the ninhydrin method (Rosen, 1957) using *S*-ethylcysteine as standard.

**Microvinification.** Fermentation medium used was: glucose 85 g/L, fructose 85 g/L, L-tartaric acid 3 g/L, citric acid 0.3 g/L, L-asparagine 2 g/L, potassium phosphate monobasic 2 g/L, ammonium sulfate 2 g/L, magnesium sulfate 0.2 g/L, manganese(II) sulfate 0.01 g/L, mesoinositol 0.3 g/L, all in 1 L of distilled water adjusted to pH 3.5 with solid potassium hydroxide. After autoclaving, 10 mL/L of vitamin solution sterilized by filtration (D-biotin 4 mg/L, thiamine hydrochloride 100 mg/L, pyridoxine hydrochloride 100 mg/L, nicotinic acid 100 mg/L, d-panthothenic acid hemicalcium salt 100 mg/L, *p*-aminobenzoic acid 100 mg/L) and cellulose (0.4 g/L) was added. The medium was inoculated with *Saccharomyces cerevisiae* (0.1 g/L) [strain VL3c, CLIB (Collection de Levures d'Interêt Biotechnologique, Paris, France) 2016]. After fermentation, $SO_2$ (0.05 g/L) was added.

**GC Analyses.** The volatile thiols released in vitro were analyzed by GC coupled with flame photometric detector (FPD) under the conditions described by Tominaga and Dubourdieu (1997). The volatile thiols released from Sauvignon blanc must or model medium with or without pimaricin (50 mg/L) during fermentation were purified and measured by GC/MS (Tominaga et al., 1998b).

Purified samples of CESFPs were trimethylsilylated with 50 $\mu$L of a mixture of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce; code 38827), trimethylchlorosilane (TMCS) (Pierce; code 88530), and pyridine (3:1:3) at 70 °C for 15 min. Trimethylsilylated material (2 $\mu$L) was analyzed by GC/FPD or GC/MS on a BPX-5 column (SGE, 50 m × 0.22 mm, 0.25 $\mu$m) under the conditions previously described (Tominaga and Dubourdieu, 1997) with a starting temperature of 100 °C.

The concentration of *S*-3-(hexan-1-ol)-L-cysteine in a model fermentation medium with or without pimaricin (50 mg/L) was measured by GC/MS, as follows. The medium was centrifuged at 3800*g* for 5 min and filtered on 1.2 $\mu$m membrane. To 1 mL of the filtered supernatant was added 100 $\mu$L of hydrochloric acid (50 mM) containing 780 pmol of *S*-ethylcysteine as an internal standard. It was then purified on a strongly acidic cation exchanger column (0.7 × 1.5 cm) (Dowex 50, Sigma; code 50WX4-200R). The column was rinsed with 5 mL of hydrochloric acid (5 mM). The resin was then placed in a small centrifuging tube with 500 $\mu$L of ammonia solution (1.3 M). A 100 $\mu$L sample of the supernatant, collected by centrifugation at 5000*g* for 1 min, was evaporated and trimethylsilylated using the method previously described. MS detection was carried out in SIM mode (ion selected; $m/z$ = 250 for internal standard, $m/z$ = 320 for the precursor).

## RESULTS

Nonvolatile crude extracts containing sulfur flavor precursors (CESFPs) were obtained from Sauvignon blanc must by adsorption on a $C_{18}$ silica column eluted by ethanol (Darriet et al., 1993). CESFPs were then subjected to the action of a cell-free extract from *Eubacterium limosum*, which has a cysteine conjugate $\beta$-lyase (EC 4.4.1.13) (Larsen and Stevens, 1986). Following incubation for 15 min at 30 °C, the volatile compounds released were analyzed by GC/FPD (Figure 1). Peaks 1−3 were identified by mass spectrometry analysis. 4-Mercapto-4-methylpentan-2-one (peak 1), 4-mercapto-4-methylpentan-2-ol (peak 2), and 3-mercaptohexan-1-ol (peak 3) were formed by the action of the bacterial extract on CESFPs. No thiols were released if bacterial extract was treated by heating. In view of the substrate specificity of the $\beta$-lyase in the bacterial preparation (Larsen and Stevens, 1986), it was envisaged that the precursors of these various thiols in grape could be *S*-cysteine conjugates or their sulfoxides. The validity of this hypothesis was demonstrated by the following experiment.

The three *S*-cysteine conjugates able to release the volatile thiols [*S*-4-(4-methylpentan-2-one)-L-cysteine, *S*-4-(4-methylpentan-2-ol)-L-cysteine, and *S*-3-(hexan-1-ol)-L-cysteine], labeled PI, PII, and PIII, respectively,

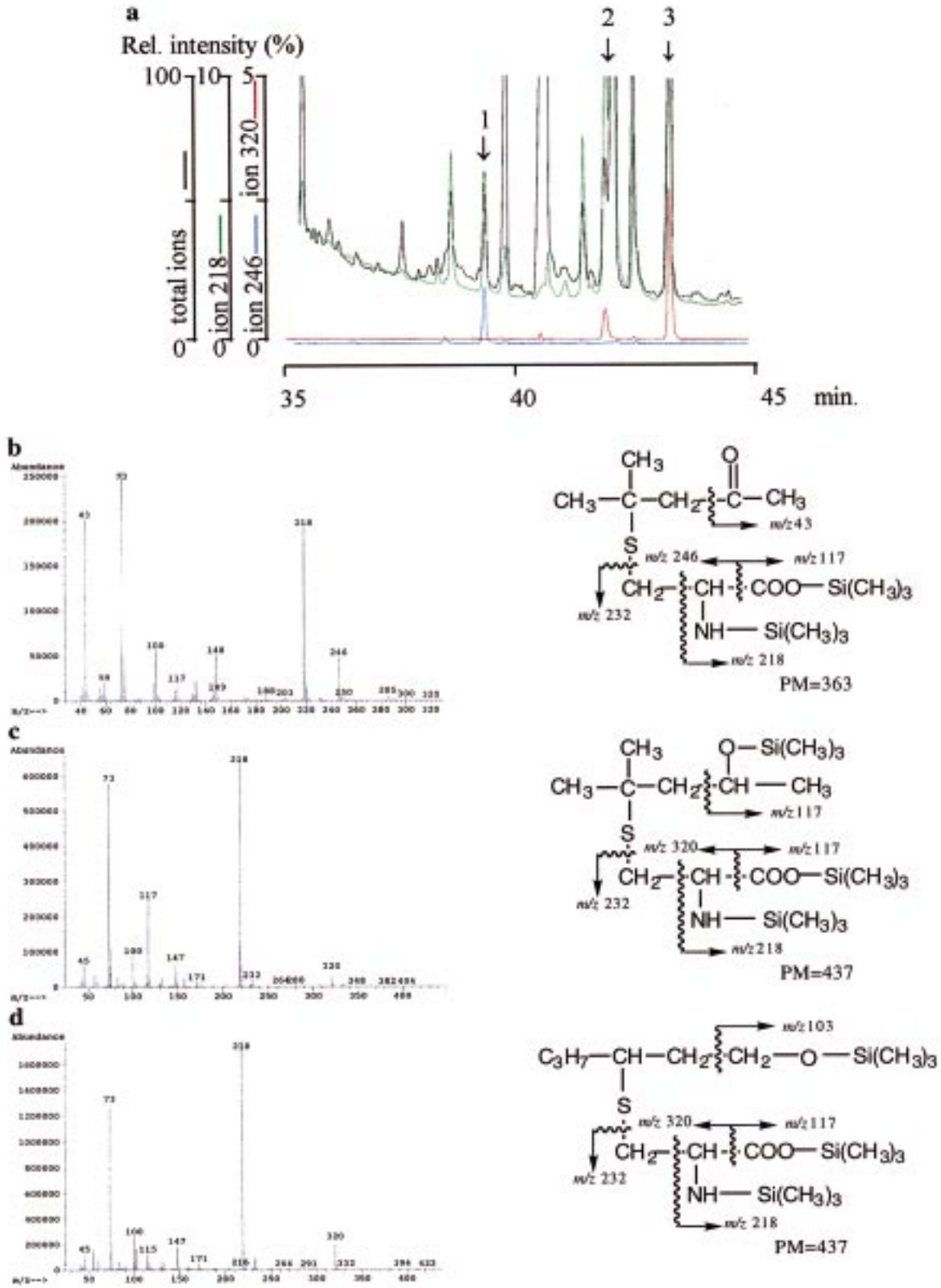


**Figure 3.** Identification by GC/MS of flavor precursors in Sauvignon blanc must. (a) Chromatogram obtained by trimethylsilylation of the purified must extract with detection of total ions (black), ion 218 (green), ion 246 (blue), and ion 320 (red). (b−d) Mass spectra and interpretation of mass fragments obtained from peaks 1−3 corresponding to *S*-4-(4-methylpentan-2-one)-L-cysteine (PI), *S*-4-(4-methylpentan-2-ol)-L-cysteine (PII), and *S*-3-(hexan-1-ol)-L-cysteine (PIII).

were synthesized in the laboratory, as was *S*-4-(4-methylpentan-2-one)-L-cysteine sulfoxide (SPI). The quantity of 4-mercapto-4-methylpentan-2-one released, due to the action of the $\beta$-lyase of *Eubacterium limosum* (EC 4.4.1.13) on PI and SPI (4 nmol of each) under the same conditions, was compared (Figure 2a). It was much higher in the case of PI. On the other hand, alliin lyase (EC 4.4.1.4), purified from garlic, released much more of this thiol from SPI than from PI. The mechanism by which volatile thiols are released from *S*-

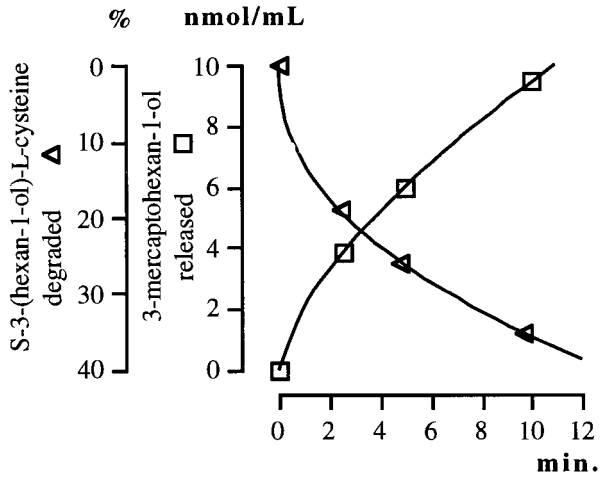


**Figure 4.** Release in vitro of 3-mercaptohexan-1-ol by the enzyme action of a cell-free extract from *Eubacterium limosum* on *S*-3-(hexan-1-ol)-L-cysteine in Sauvignon blanc must extract: release of 3-mercaptohexan-1-ol (□), degradation of *S*-3-(hexan-1-ol)-L-cysteine (△).

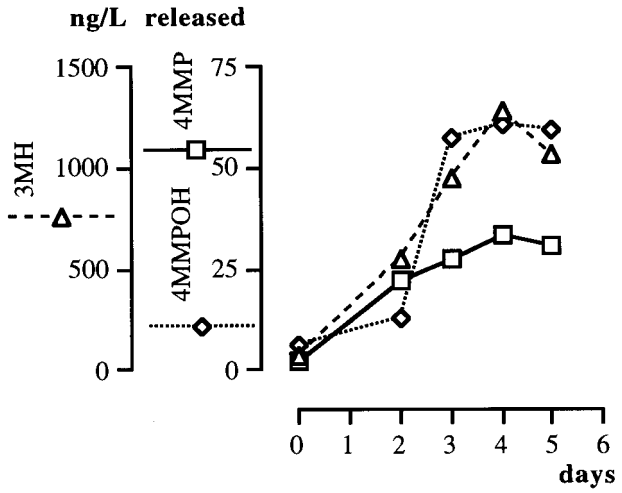


**Figure 5.** Release of volatile thiols during alcoholic fermentation from Sauvignon blanc must. Day 0 corresponds to inoculation with the VL3c yeast strain (4MMP, 4-mercapto-4-methylpentan-2-one; 4MMPOH, 4-mercapto-4-methylpentan-2-ol; 3MH, 3-mercaptohexan-1-ol).

cysteine conjugate sulfoxides probably involves intermediate components (derivatives of sulfenic acid or thiosulfinate) (Chin and Lindsay, 1994). The fact that the $\beta$-lyase of *Eubacterium limosum* released much higher amounts of volatile thiols from CESFPs than alliin lyase (Figure 2b) seems to indicate that the precursor components of the aromatic thiols in Sauvignon blanc are *S*-cysteine conjugates rather than *S*-cysteine conjugate sulfoxides.

Direct identification of the *S*-cysteine conjugates was carried out as follows. CESFPs containing the sulfur flavor precursors to be identified were purified by percolation on a chelating Sepharose 4B column, which has the property of fixing certain amino acids via the intermediary of chelated metal ion (Belew and Porath, 1990). The retained fraction was eluted by hydrochloric acid (50 mM). Once the eluted fraction had evaporated to dryness, the residue was extracted with ethanol and then dried, and the purified flavor precursor extracts were analyzed by GC/MS in the form of trimethylsilylated derivatives. The same chromatography analyses were carried out on PI, PII, and PIII. Figure 3a shows

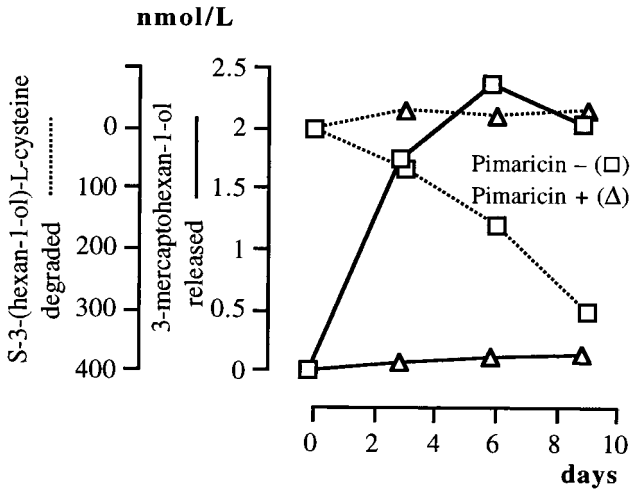


**Figure 6.** Release of 3-mercaptohexan-1-ol during alcoholic fermentation from a model medium to which synthesized *S*-3-(hexan-1-ol)-L-cysteine was added in the absence (□) and presence (△) of primaricin. 3-Mercaptohexan-1-ol released and *S*-3-(hexan-1-ol)-L-cysteine degraded were expressed by — and ---, respectively. Day 0 corresponds to inoculation with the VL3c yeast strain.

the chromatograms obtained by trimethylsilylation of the purified must extract. The retention times and mass spectra of peaks 1−3 correspond to those of silylated PI, PII, and PIII (Figure 3b−d).

When the cell-free extract from *Eubacterium limosum* was allowed to act on the CESFPs, the release of volatile thiols in vitro resulted from the enzyme degradation of *S*-cysteine conjugates as shown in Figure 4, as an example, where 3-mercaptohexan-1-ol and *S*-3-(hexan-1-ol)-L-cysteine were measured by GC/FPD. During alcoholic fermentation of Sauvignon blanc must, a clear increase in the concentration of aromatic volatile thiols is observed (Figure 5). This increase in these important volatile thiols by yeast (*Saccharomyces cerevisiae*) metabolism is due to a degradation of the cysteinylated flavor precursors of the grapes, leading to the formation of the corresponding volatile thiols as shown in the following experiment. A fermentation model medium, added with laboratory-synthesized *S*-3-(hexan-1-ol)-L-cysteine (1 nmol/mL) was inoculated with *Saccharomyces cerevisiae* (strain VL3c). Figure 6 shows the release of 3-mercaptohexan-1-ol during alcoholic fermentation. A small fraction of the flavor precursor degraded was transformed into free aroma. If fermentation was inhibited by pimaricin, aroma release and precursor degradation were very limited. The release of 3-mercaptohexan-1-ol was proportional to the concentration of *S*-3-(hexan-1-ol)-L-cysteine in the fermentation medium (data not shown).

## DISCUSSION

A little information is available on the *S*-cysteine conjugates in plants. However, the *S*-cysteine conjugate sulfoxides known up until now in plants were found in Amaryllidaceae, Cruciferae, and Liliaceae (Kjær, 1963; Fowden, 1964; Virtanen, 1965), in particular the species *Allium* (Stoll and Seebeck, 1951). The development of aromas in these plants, which involves an endogenous enzyme activity (Mazelis, 1963; Mazelis and Crews, 1968; Tobkin and Mazelis, 1979) was only triggered by mechanical damage to their tissues (Virtanen, 1965; Lancaster and Collin, 1981). *S*-cysteine conjugate has

now been identified for the first time as flavor precursors in grape. In Sauvignon blanc, the volatile thiols characteristic of this grape variety are only present in trace amounts; however their odor activity values of these thiols are significant (Darriet et al., 1995; Guth, 1997; Tominaga et al., 1998a, 1998b). They are released during alcoholic fermentation due to the degradation, by yeast, of the corresponding *S*-cysteine conjugates. This previously unexplained amplification of grape aromas by fermentation has now been understood; however, the secondary yeast metabolism involved in this transformation must now be elucidated.

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