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Stereochemical Course of the Generation of 3-Mercaptohexanal and 3-Mercaptohexanol by β-Lyase-Catalyzed Cleavage of Cysteine Conjugates

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The product resulting from the reaction between *E*-2-hexenal and L-cysteine was shown to be a diastereoisomeric mixture of 2-(2-*S*-L-cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid **1**. Treatment of the conjugate with two sources of cysteine-*S*-conjugate β-lyase (tryptophanase from *E. coli* and a crude enzyme extract prepared from *Eubacterium limosum*) resulted in the formation of 3-mercaptohexanal. The reaction proceeded with a slight preference for the (*S*)-configured product, however, with low conversion rate. The role of 3-*S*-L-cysteinylhexanal **2** as substrate for β-lyases was demonstrated by *in situ* generation of **2** from 3-*S*-(*N*-acetyl-L-cysteinyl)hexanal using acylase. Opposite enantioselectivity was observed for the liberation of 3-mercaptohexanol from 3-*S*-L-cysteinylhexanol **5** by the enzyme preparations from *Eubacterium limosum* and tryptophanase. Various yeasts produced 3-mercaptohexanol starting from **1** as well as from **5.** The reactions proceeded without preferential formation of one of the enantiomers.

KEYWORDS: Cysteine conjugate; thiol; β-lyase; enantioselectivity; 3-mercaptohexanal; 3-mercaptohexanol

INTRODUCTION

The investigation of cysteinylated nonvolatile precursors and the β-lyase-catalyzed liberation of sensorially active thiols has become an important area of flavor research (*1*−*7*). A typical example for a sulfur-containing flavor compound shown to be released from a cysteine conjugate is 3-mercaptohexanol (*3*−*5*), a powerful odorant first identified in passion fruits (*8*) and later reported in Sauvignon blanc wine (*9*). Its precursor 3-*S*-L-cysteinylhexanol has been detected in Sauvignon blanc must (*3*, *5*) and in passion fruit juice (*4*). The synthesis of this conjugate has been performed by Michael-type addition of L-cysteine to the α,β-unsaturated aldehyde *E*-2-hexenal and subsequent reduction using sodium borohydride (*3*−*5*). However, the structure of the assumed intermediate, named *S*-3-(hexan-1-al)-L-cysteine (*3*−*5*), has not been verified.

The potential to generate chiral thiols in optically enriched form using β-lyases has been demonstrated for the liberation of 8-mercapto-*p*-menthan-3-one from 8-*S*-L-cysteinyl-*p*-menthan-3-one (*6*). Lipase-catalyzed hydrolysis of 3-acetylthioesters obtained by addition of thioacetic acid to *E*-2-hexenal has been shown to proceed with preference of one of the enantiomers of 3-mercaptohexanal and 3-mercaptohexanol (*10*). The stereochemical course of the liberation of these sulfur-containing volatiles using β-lyases as catalysts has not been followed.

The objectives of this study were (i) to elucidate the structure of the adduct between *E*-2-hexenal and L-cysteine, (ii) to investigate its acceptance as substrate for β-lyases, and (iii) to investigate the stereochemical course of the liberation of chiral thiols from cysteinylated precursors using enzyme preparations and yeasts.

MATERIALS AND METHODS

Materials. *E*-2-Hexenal, L-cysteine (∼99%), *N*-acetyl-L-cysteine, Boc-L-cysteine and *N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA) were obtained from Fluka Chemie AG, Germany. [D_4]-methanol, D_2O, $CDCl_3$, and 4M HCl in 1,4-dioxane were from Sigma-Aldrich Chemie GmbH, Germany.

Tryptophanase (EC 4.1.99.1) (from *Escherichia coli*; 30 units/mg; 1 unit will release 1 μg of indole from L-tryptophan in 10 min at pH 8.3 at 37 °C) was purchased from Sigma-Aldrich Chemie GmbH, Germany. Acylase I (EC 3.5.1.14) (immobilized on Eupergit C from *Aspergillus*; 50 U/g moist material) was obtained from Fluka Chemie AG, Germany. *Eubacterium limosum* (ATCC 10825) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany. Fresh baker's yeast (Uniferm GmbH & Co. KG, Germany) was purchased in a local market. Freeze-dried wine yeast (SIHA Active Yeast 8, Burgundy yeast) was obtained from E. Begerow GmbH &

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Co., Germany. Two fresh beer yeasts (No. 34/70 and 184) were supplied by Hefebank Weihenstephan, Germany.

Syntheses. *2-(2-S-L-Cysteinylpentyl)-1,3-thiazolidine-4-carboxylic Acid* **1**. Method 1. *E*-2-Hexenal (1.2 mL, 10.0 mmol) was added to an aqueous solution (10 mL) of L-cysteine (1.21 g, 10.0 mmol) and potassium hydrogencarbonate (0.2 g, 2.0 mmol). The mixture was stirred at room temperature for 1 day under argon atmosphere. After addition of 20 mL of acetone, the formed brown precipitate was isolated by filtration and dried under vacuum after washing with 50 mL of acetone. A dark brown product was obtained. Yield, 0.92 g (2.9 mmol); mol yield from L-cysteine, 57%; purity determined by GC (system II) after trimethylsilylation, 32%. For further purification, the precipitate was redissolved in 10 mL of water, and the brown insoluble residue was filtered off. Addition of 20 mL of acetone to the solution resulted in the precipitation of a pale yellowish product, which was isolated by filtration and dried under vacuum. Yield, 0.27 g (0.85 mmol); mol yield from L-cysteine, 17%; purity determined by GC (system II) after trimethylsilylation, 76%.

Method 2. *E*-2-Hexenal (1.2 mL, 10.0 mmol) was added to a solution (50 mL) of L-cysteine (1.21 g, 10.0 mmol) in 40% aqueous ethanol. The reaction mixture was stirred for 5 h at room temperature. The pale yellowish precipitate appearing at the end of the reaction was isolated by filtration, washed with 20 mL of ethanol, and dried under vacuum. Yield, 1.0 g (3.1 mmol); mol yield from L-cysteine, 62%; purity determined by GC (system II) after trimethylsilylation, 98%.

GC retention index (SE−54) of TMS derivative, 2725. GC-MS of TMS derivative, *m*/*z* (relative intensity): 73 (55), 75 (11), 86 (11), 100 (15), 147 (12), 148 (14), 156 (10), 204 (10), 218 (100), 219 (22), 274 (23), 306 (14), 320 (3), 332 (2), 361 (2), 421 (1), 538 ($M^{+\cdot}$; 1). LC-MS, *m*/*z*: 323 (M + 1). FTIR, cm^{-1}, (%T): 1603 (63), 1430 (84), 1351 (79), 1301 (82), 850 (89).

3-S-(N-Acetyl-L-cysteinyl)hexanal ***3***. *E*-2-Hexenal (2.3 mL, 20 mmol) dissolved in 2 mL of methanol was added to an aqueous solution (20 mL) of *N*-acetyl-L-cysteine (3.3 g, 20 mmol) and potassium hydrogencarbonate (2.4 g, 24 mmol). The mixture was stirred at room temperature for 4 days. After adjusting the pH to 3 using 5% hydrochloric acid, the product was extracted with ethyl acetate (three times, 20 mL). The combined organic layers were washed with 20 mL of distilled water and dried over anhydrous sodium sulfate. Ethyl acetate was removed under reduced pressure; 1.1 g (4.1 mmol) of a pale yellowish, sticky liquid was obtained (mol yield 21%).

^{1}H NMR (500 MHz, $CDCl_3$), δ, ppm: 0.90 (3H, t, 7.3 Hz, H-6), 1.45 (2H, m, H-5), 1.60 (2H, m, H-4), 2.07, 2.12 (3H, H-10), 2.62 (2H, d, 7.1 Hz, H-2), 2.67 (2H, d, 6.8 Hz, H-2), 2.96, 3.11 (2H, m, H-7), 3.06 (2H, m, H-7), 3.20 (1H, m, H-3), 4.76 (1H, m, H-8), 4.84 (1H, m, H-8), 6.55 (1H, d, 6.9 Hz, NH), 7.04 (1H, d, 7.4 Hz, NH), 9.71 (1H, dd, 1.3, 2.2 Hz, H-1), 9.72 (1H, t, 1.3 Hz, H-1). ^{13}C NMR (125.6 MHz), δ, ppm: 201.5 (C-1), 173.3 (COOH), 172.0, 171.7 (C-9), 52.8, 52.6 (C-8), 49.0, 48.9 (C-2), 40.5 (C-3), 38.1 (C-4), 32.8 (C-7), 23.3 (C-10), 20.4 (C-5), 14.5 (C-6). FTIR, cm^{-1}, (%T): 1720 (59), 1624 (66), 1535 (68), 1375 (71), 1216 (66), 1183 (68), 1043 (76).

3-S-(N-Acetyl-L-cysteinyl)hexanol ***4***. 3-*S*-(*N*-Acetyl-L-cysteinyl)hexanal (105 mg, 0.4 mmol) was dissolved in 10 mL of distilled water, sodium borohydride (48 mg, 1.3 mmol) dissolved in 1 mL of water was added under ice-cooling, and the mixture was stirred for 1 h. After adjusting the pH to 3 using 5% hydrochloric acid, the product was extracted with ethyl acetate (three times, 10 mL). The combined organic layers were dried over anhydrous sodium sulfate. Ethyl acetate was removed under reduced pressure. Transparent sticky liquid (79 mg, 0.3 mmol) was obtained (mol yield 75%).

GC retention index (SE-54) of TMS derivative, 2174. GC-MS of TMS derivative, *m*/*z* (relative intensity): 73 (100), 75 (27), 83 (16), 103 (55), 116 (15), 117 (16), 129 (21), 157 (11), 214 (24), 246 (15), 258 (20), 348 (5), 392 (3), 407 ($M^{+\cdot}$; 0.4). ^{1}H NMR (500 MHz, $CDCl_3$), δ, ppm: 0.74, 0.75 (3H, t, 7.2 Hz, H-6), 1.27 (2H, m, H-5), 1.42 (2H, m, H-4), 1.58, 1.71 (2H, m, H-2), 1.91 (3H, s, H-10), 2.70 (1H, m, H-3), 2.78, 2.95 (2H, dd, 8.0, 13.9; 4.6, 13.9 Hz, H-7), 3.57 (2H, m, H-1), 4.40 (1H, dd, 4.7, 8.2 Hz, H-8), 4.42 (1H, dd, 4.6, 8.0 Hz, H-8). ^{13}C NMR (125.6 MHz), δ, ppm: 176.7 (C-9), 176.5 (COOH), 61.7 (C-1), 55.5, 55.4 (C-8), 44.8 (C-3), 38.8 (C-2), 38.7 (C-4), 33.1 (C-7), 24.0 (C-10), 21.7 (C-5), 15.5 (C-6).

3-S-L-Cysteinylhexanol ***5***. Boc-L-cysteine (1.6 g, 7 mmol) and triethylamine (1.2 g, 8.4 mmol) were dissolved in 10 mL of 1,4-dioxane under argon atmosphere. *E*-2-Hexenal (0.9 mL, 7.8 mmol) was added and the reaction mixture was stirred for 4 days at room temperature. 1,4-Dioxane was removed under reduced pressure. The residual yellow sticky liquid (ca. 2 mL) was washed by hexane (two times, 10 mL) and dried under reduced pressure, and 3.0 g of a yellowish sticky liquid was obtained and used for further synthesis. Synthesized 3-*S*-(Boc-L-cysteinyl)hexanal (1.0 g; 3.3 mmol) was dissolved in 20 mL of 0.5 M potassium phosphate buffer (pH 7.4). Sodium borohydride (250 mg, 6.6 mmol) in 5 mL of water was added under ice-cooling, and the reaction mixture was stirred for 1 h. The pH of solution was adjusted to 7 using 5% hydrochloric acid, and the product was extracted with ethyl acetate (4 times, 15 mL). The combined organic layers were washed with 10 mL of water and dried over anhydrous sodium sulfate. Ethyl acetate was removed under reduced pressure, 360 mg of transparent sticky liquid was obtained. To remove the Boc group, the obtained 3-*S*-(Boc-L-cysteinyl)hexanol was dissolved in 10 mL of 1,4-dioxane. A 4 M 1,4-dioxane solution of hydrochloric acid (10 mL) was added, and the reaction mixture was stirred for 1 h at room temperature (25 °C). 1,4-Dioxane was removed under reduced pressure. The residual sticky liquid was washed with 10 mL of diethyl ether and dried under reduced pressure. A transparent, sticky liquid (136 mg, 0.61 mmol) was obtained (mol yield from Boc-L-cysteine, 26%).

GC retention index (SE−54) of TMS derivative, 2022. GC-MS of TMS derivative, *m*/*z* (relative intensity): 73 (81), 100 (24), 147 (16), 218 (100), 219 (42), 220 (21), 232 (11), 233 (13), 320 (44), 321 (11), 394 (2), 422 (1), 437 ($M^{+\cdot}$; 0.1). ^{1}H NMR (500 MHz, $CDCl_3$), δ, ppm: 0.76 (3H, t, 7.2 Hz, H-6), 1.29 (2H, H-5), 1.45 (2H, H-4), 1.60, 1.75 (2H, H-2), 2.77 (1H, H-3), 3.01 (2H, H-7), 3.60 (2H, m, H-1), 4.07 (1H, dd, H-8). ^{13}C NMR (125.6 MHz), δ, ppm: 59.5 (C-1), 53.5(C-8), 43.2 (C-3), 36.8 (C-4), 36.5 (C-2), 30.1 (C-7), 19.6 (C-5), 13.5 (C-6).

3-Mercaptohexanal and 3-mercaptohexanol were synthesized according to the methods described previously (*10*).

Enzymatic Reactions. Substrates (25, 250, and 2500 nmol of **1**, **3**, and **5**, respectively) were dissolved in 250 μL of 50 mM potassium phosphate buffer (pH 7.4) containing 100 μM pyridoxal 5′-phosphate. Tryptophanase (0.4 mg) and the crude enzyme extract obtained from *E. limosum* (50 μL corresponding to 10 mg of wet cells) (*5*), respectively, were added, and the mixture was shaken (120 rpm) at 25 °C for 20 min. For the reaction with yeast, 2500 nmol of substrate was mixed with 250 mg of fresh baker's and beer yeast and 80 mg of dried wine yeast in 700 μL of 50 mM potassium phosphate buffer (pH 7.4) containing 100 μM pyridoxal 5′-phosphate and incubated in a sealed vial at 25/15 °C for 24 h/14 days. After the reaction, 2.5 μg of linalool was added as internal standard, and volatile compounds were extracted with dichloromethane (two times, 700 μL). The organic phase was dried over anhydrous sodium sulfate and subjected to GC analysis. For the experiments with the 3-*S*-(*N*-acetyl-L-cysteinyl)hexanal, 5 mg of acylase I and 10 μM of cobalt chloride were added to the reaction mixture (*11*).

Determination of Enantioselectivities. The enantioselectivities (*E*) of the reactions were calculated on the basis of the conversion rates (*c*), the enantiomeric excesses of the starting substrates (ee_0) and the enantiomeric excesses of the products (ee_p), determined via GC analysis on systems I−III, respectively. The following equation was used:

$$E = \frac{\ln\left[1 - c\left(\frac{1 + ee_p}{1 - ee_0}\right)\right]}{\ln\left[1 - c\left(\frac{1 - ee_p}{1 - ee_0}\right)\right]}$$

GC Analysis. Capillary GC was performed on the following three GC systems.

(I) A Carlo Erba MEGA2 gas chromatograph equipped with FID and FPD. Parallel detection was achieved by dividing the effluent of the column (DB-WAX, J&W; 60 m × 0.32 mm i.d.; film thickness 0.25 μm) via a chrom-fit connector and short pieces of deactivated fused silica capillaries to the two detectors. Split injection was performed at 215 °C, and column temperature was programmed from 40 °C (5 min hold) to 230 °C (25 min hold) at 4 °C/min. The detector

temperatures were 240 °C (FID) and 140 °C (FPD). Hydrogen was used as carrier gas at 105 kPa.

(II) A Carlo Erba GC 6000 gas chromatograph with FID; the column used was SE-54 (15 m × 0.25 mm i.d., film thickness 0.15 μm). Split injection was performed at 220 °C, and the column temperature was programmed from 50 °C (2 min hold) to 300 °C (5 min hold) at 4 °C/min. Hydrogen was used as carrier gas at 45 kPa.

(III) A Fisons GC 8000 gas chromatograph equipped with FID. The column used was a fused silica column (30 m × 0.25 mm i.d.) coated with an octakis(2,6-di-*O*-pentyl-3-*O*-butyryl)-γ-cyclodextrin (FS-LIPODEX E, Macherey-Nagel GmbH & Co., Germany). On-column injection was performed, and the column temperature was programmed from 40 °C (1 min hold) to 80 °C at 40 °C/min, from 80 to 90 °C at 0.5 °C/min and from 90 to 200 °C (10 min hold) at 5 °C/min for 3-mercaptohexanal, and from 40 °C (1 min hold) to 80 °C at 40 °C/min, from 80 to 110 °C at 1 °C/min and from 110 to 200 °C (10 min hold) at 5 °C/min for 3-mercaptohexanol. Hydrogen was used as carrier gas at 75 kPa.

Gas Chromatography-Mass Spectrometry. DB-WAXETR fused silica column (30 m × 0.25 mm i.d.; film thickness 0.5 μm; J&W) installed into a Finnigan GC8000 gas chromatograph equipped with a split/splitless injector and a Voyager mass spectrometer were used. The mass spectrometer was operated at scan mode at 20−600 amu, and the ionization voltage was 70 eV. The column temperature was programmed from 40 °C (5 min hold) to 240 °C (15 min hold) at 4 °C/min. The injector temperature was 215 °C, and the transfer line temperature was 230 °C. The injector was used as split mode, and the split flow was 27 mL/min. Helium was used as carrier gas at 75 kPa.

For the analysis of trimethylsilylated cysteine conjugates, an SE-54 fused silica column (15 m × 0.25 mm i.d., film thickness 0.15 μm) was used, and the temperature program was from 50 °C (2 min hold) to 250 °C (5 min hold) at 4 °C/min. Helium was used as carrier gas at 25 kPa.

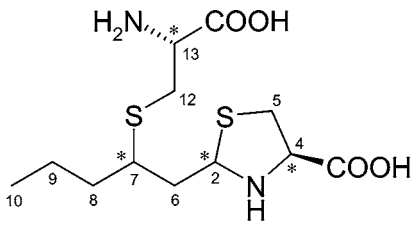
FTIR Spectroscopy. Infrared spectra were recorded with a Perkin-Elmer Spectrum One spectrometer with universal ATR sampling accessory.

NMR Spectroscopy. ^{1}H NMR and ^{13}C NMR spectra were recorded at 500.13 and 125.6 MHz, respectively, with an AVANCE 500 spectrometer (Bruker Instruments, Germany) equipped with a cryo platform and a triple resonance inverse probehead. Two-dimensional gradient-enhanced COSY, HMQC, HMBC, and NOESY experiments were performed according to standard Bruker software (XWINNMR 3.1). The mixing time was 1 s in the NOESY experiment. All measurements were done at 10 °C. The signal assignments are based on proton−proton (COSY, NOESY) and proton−carbon correlation experiments (HMQC, HMBC). Measurements were performed with the following solutions: **1** (2 mg in 0.5 mL of methanol-D_4), **3** (7 mg in 0.5 mL of $CDCl_3$), **4** (5 mg in 0.5 mL of D_2O), and **5** (5 mg in 0.5 mL of D_2O). ^{13}C chemical shifts were predicted using SPECINFO software (Chemical concepts 3.2.5).

RESULTS AND DISCUSSION

Reaction Between *E*-2-Hexenal and L-Cysteine. Structural elucidation by GC and GC-MS (trimethylsilyl derivative), LC-MS, and IR, as well as by ^{1}H and ^{13}C NMR revealed 2-(2-*S*-L-cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid **1** (**Figure 1**) as product of the reaction between *E*-2-hexenal and L-cysteine. The reaction does not stop at the level of the monoadduct formed by Michael addition to the double bond, but proceeds to the di-adduct, resulting in a thiazolidine moiety formed by reaction of cysteine with the aldehyde group. This route has been described for other α,β-unsaturated aldehydes, such as acroleine and crotonaldehyde (*12*). Alkaline conditions resulted in low yield of **1** and required an additional purification step, whereas the use of an aqueous ethanol solution as solvent yielded **1** in a purity of 98%. Neither NMR nor IR analysis indicated the presence of a free aldehyde group.

Adduct **1** possesses four asymmetric centers (**Figure 1**). Assuming that the configuration of the used L-cysteine is retained (i.e., (*R*)-configurations at C-4 and C-13), the formation of four isomers would have been expected (i.e., 2(*S*),4(*R*),7(*S*),13(*R*); 2(*S*),4(*R*),7(*R*),13(*R*); 2(*R*),4(*R*),7(*S*),13(*R*); and 2(*R*),4(*R*),7(*R*),13(*R*)). Their structures were investigated by means of ^{1}H and ^{13}C NMR. NMR analysis was hampered by the following facts: (i) low solubility of the compound in aprotic solvents, (ii) rapid degradation of the compound in protic solvents, and (iii) signal overlapping due to the presence of the diastereoisomeric forms. Using methanol-D_4 as solvent, the ^{1}H NMR signals of the mixture were well separated in the downfield region of the spectrum at 10 °C. For this reason, this experimental setting was used in all NMR experiments, despite of the low solubility of the compound in methanol (approximately 5 mg mL^{-1}).



1 A-D

diastereoisomer	molar ratio	ring configuration	absolute configuration at position			
			2	4	7	13
A	1.0	trans	(*S*)	(*R*)	(*R*)/(*S*)	(*R*)
B	1.0	trans	(*S*)	(*R*)	(*R*)/(*S*)	(*R*)
C	0.8	cis	(*R*)	(*R*)	(*R*)/(*S*)	(*R*)
D	0.5	cis	(*R*)	(*R*)	(*R*)/(*S*)	(*R*)

Figure 1. Diastereomeric products resulting from the reaction between *E*-2-hexenal and L-cysteine.

In the downfield region of the ^{1}H NMR spectrum, two sets of well-resolved signals were observed. The first set comprising four signals was detected at 5.14−4.81 ppm. A second group of four downfield-shifted signals was detected at 4.38−3.97 ppm. From the patterns of the coupling constants and the signal intensities it was concluded that the spectrum displays four isomers (A−D) of **1**. From the signal intensities, the molar ratios were estimated as 1.0:1.0:0.8:0.5 for diastereoisomers A, B, C, and D, respectively.

Owing to this complex mixture, the upfield-shifted region of the spectrum was highly crowded with severe signal overlap. Nevertheless, most of the signals could be assigned by two-dimensional proton−proton correlation experiments (COSY, NOESY) (**Table 1**). Comprehensive analysis of the coupling patterns in the COSY experiment showed that 2_A-2_D were involved in spin systems comprising protons connected to six aliphatic carbon atoms (H-2, H-6, H-7, H-8, H-9, H-10). These spin systems were terminated by methyl triplets at 0.9−1.0 ppm (H-10). The signals for H-7 (i.e., H-7_{A-D}) were detected at 2.8−3.0 ppm in line with an attachment of an *S*-R moiety (i.e., cysteinyl) at C-7. From the coupling pattern observed in the COSY experiment two additional spin systems were delineated for each diastereoisomer. Signals for H-4 were correlated with H-5 and H-5′ and signals for H-13 were correlated with H-12, respectively. On the basis of the coupling patterns and in conjunction with the chemical shifts, these spin systems were assigned to two cysteinyl moieties for each diastereoisomer.

Information about ^{13}C NMR chemical shifts could be gleaned from two-dimensional HMQC experiments. Moreover, chemical shifts predicted on the basis of a similarity search using the SPECINFO database were in almost perfect agreement with the observed data (**Table 1**). The proton signals for H-2 were correlated to carbon signals at 65−68 ppm which are typical chemical shifts for C-2 of similar thiazolidine ring systems (*13*).

Table 1. NMR Data of **1** (Diastereoisomeric forms A, B, C, and D)

	chemical shifts			coupling constant	correlation pattern	
		δ ^{13}C, ppm				
position	δ ^{1}H, ppm	observed	predicted[a]	J_{HH} Hz	COSY	NOESY
2_A	5.14 (dd)	65.3	63.6	9.6, 4.4	$6_A'$, 6_A	5_A, 7_A, 6_A
2_B	4.95 (t)	67.9		7.1	6_B	
2_C	4.89 (dd)	67.4		9.0, 4.6	$6_C'$, 6_C	4_C, 7_C, 6_C
2_D	4.81 (t)	68.1		6.6	6_D	4_D, 7_D, 6_D, 5_D
4_A	4.38(dd)	65.3	64.7	7.1, 4.1	5_A	5_A
4_B	4.32(dd)	66.0		7.1, 5.2	5_B	5_B
4_C	4.05(dd)	67.1		8.5, 7.2	5_C, $5_C'$	2_C, $5_C'$
4_D	3.97(dd)	67.1		9.0, 7.1	5_D, $5_D'$	2_D, $5_D'$
$6_A'$	2.13	40.5	30.5		2_A, 7_A	
6_A	1.85				2_A, 7_A	2_A
6_B	2.01	40.9			2_B, 7_B	
6_C	2.16	41.4			2_C, 7_C	2_C
$6_C'$	1.89				2_C, 7_C	
6_D	2.11	40.9			2_D, 7_D	2_D
7_A	3.01		39.5		$6_A'$, 6_A, 8_A	2_A
7_B	2.80				6_B, 8_B	
7_C	2.94				$6_C'$, 6_C, 8_C	2_C
7_D	2.86				6_D, 8_D	2_D
8_{A-D}	1.69–1.58	39.4–41.6	34.5		7_{A-D}, 9_{A-D}	
9_{A-D}	1.58–1.42	21.4	19.9		8_{A-D}, 10_{A-D}	
10_{A-D}	1.00–0.93	14.9	13.6		9_{A-D}	
13_{A-D}	3.80(dd)	56.3	63.9	7.6, 3.5	12_{A-D}	
	3.72	56.0				
	3.72					
	3.72					
5_A	3.37		32.9		4_A	2_A, 4_A
$5_D'$,$5_C'$	3.31				4_C, 4_D,5_D, 5_C	4_C, 4_D
5_B	3.23				4_B	4_B
5_C	2.96				4_C, $5_C'$	
5_D	3.03				5_D, $5_D'$	
12_{A-D}	3.05		33.5		13_{A-D}	
	3.14					
	3.02					
	2.97					

[a] ^{13}C NMR chemical shifts were predicted by the SPECINFO software package.

NOESY experiments allowed the determination of the configuration at C-2. The signals of H-2 of isomers C and D showed strong NOE interactions to the signals of H-4 (**Table 1**). Due to the proximity of H-2 and H-4 in *cis*-configured thiazolidine rings, the observed NOE correlations provide solid evidence for the assignments of isomers C and D to the *cis* forms of **1** (i.e., 2(*R*),4(*R*),7(*S*),13(*R*) or 2(*R*),4(*R*),7(*R*),13(*R*)). This assignment is in agreement with an earlier study, where the chemical shifts of H-2 and the coupling constant between H-4 and H-5 have been shown to follow a general rule for the *cis*/*trans* assignment of 2-substituted thiazolidine-4-carboxylic acid derivatives (*14*). Indeed, the chemical shifts of the *cis* forms of **1** (i.e., diastereomers C and D) are found at higher field than the corresponding signals of the *trans* forms (diastereomers A and B). Moreover, the sum of the coupling constants between H-4 and H-5 are less than 13 Hz for *trans* (i.e., 11.2 Hz for isomer A and 12.3 Hz for isomer B) and around 16 Hz for *cis* configuration (15.7 Hz for isomer C and 16.1 Hz for isomer D).

In conclusion, the assignments of the configurations at C-2 demonstrate the reaction between *E*-2-hexenal and L-cysteine to result in a mixture of *trans*- (A and B) and *cis*-isomers (C and D), at a ratio of 61:39%. An assignment of the absolute configurations at C-7 was not possible on the basis of the NMR data. However, an excess of 10% for (*R*)- or (*S*)-configuration at this position can be calculated from the ratios of the sums of A and C or B and C to those of A and D or B and D (**Figure 1**).

Enzyme-Catalyzed Transformations of 1. Adduct **1** was used as substrate for enzyme preparations from two sources: (i) a commercially available tryptophanase from *E. coli* and (ii) a cell free extract obtained from *E. limosum*. Both have been shown to possess β-lyase activity and to liberate thiols from a broad spectrum of precursors (*15*, *16*). As shown in **Table 2**, the application of both enzyme preparations resulted in the liberation of 3-mercaptohexanal. The identity of the generated thiol was confirmed by comparison of retention index and MS spectrum to those obtained from an authentic reference compound. Formation of the product by chemical cleavage of substrate could be ruled out by incubation under the same conditions without enzymes. The liberation of 3-mercaptohexanal from **1** by β-lyases indicates the presence of 3-*S*-L-cysteinylhexanal **2** as the actual substrate. Thiazolidine derivatives obtained from the reaction of cysteine with α,β-unsaturated aldehydes have been shown to be rather unstable in aqueous solutions and to be in equilibrium with cysteine, the monoadducts and eventually the parent aldehydes (*12*). Such an equilibrium has also been postulated for the formation of 2-furfuryl alcohol from the cysteine-furfural conjugate by baker's yeast (*7*). Although the presence of **2** in the reaction medium was not verified by chemical analysis, it is likely that the formation of 3-mercaptohexanal proceeds via routes **a** and **b** outlined in **Figure 2**.

Interestingly, the amount of 3-mercaptohexanal generated decreased with increasing amount of the thiazolidine offered as substrate to the enzymes. After administration of 2500 nmol

Table 2. β-Lyase-Catalyzed Formation of 3-Mercaptohexanal from 2-(2-*S*-L-Cysteinylpentyl)-1,3-thiazolidine-4-carboxylic Acid **1**[a]

		3-mercaptohexanal (n mol)			$E^{b,c}$	
enzyme	substrate (n mol)	(*R*)	(*S*)	conversion (%)	I	II
tryptophanase	25	2.4	2.6	20	1	1
	250	0.2	0.5	0.3	3	2
	2500	0.0	0.0	0		
E. limosum	25	0.1	0.2	2	2	1
	250	0.2	0.3	0.2	2	1
	2500	0.0	0.0	0		

[a] For conditions, see Materials and Methods. [b] Enantioselectivity calculated according to ref 17. [c] Based on an excess of 10% (*R*) (I) or 10% (*S*) (II) at position 7 of substrate **1**.

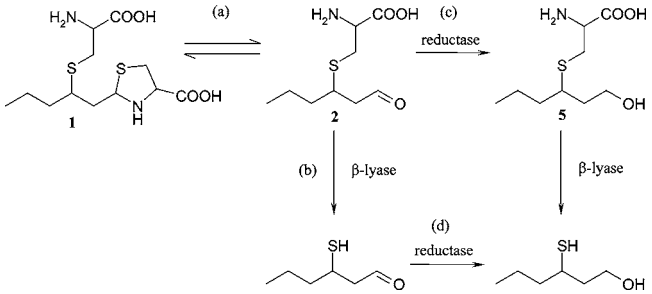


Figure 2. Hypothetical pathways involved in the transformation of **1** by β-lyase and yeast, respectively, in aqueous media.

of **1**, no product was formed at all. This indicates an inhibitory effect of the substrate.

The stereochemical course of the reaction was followed by enantiodifferentiation of 3-mercaptohexanal using octakis(2,6-di-*O*-pentyl-3-*O*-butyryl)-γ-cyclodextrin as chiral stationary phase. The data obtained were used to calculate conversion rates (*c*), enantiomeric excesses of product (ee_p) and enantioselectivities (*E*) applying equations previously described for kinetic resolutions (*17*). Both enzyme sources showed a preferred formation of the (*S*)-configured 3-mercaptohexanal. However, the enantioselectivities calculated demonstrate that the degree of stereoselectivity is only low.

Enzyme-Catalyzed Cleavage of 3-*S*-(*N*-Acetyl-L-cysteinyl)-hexanal 3. To avoid the formation of the thiazolidine derivative described above, *N*-acetyl-L-cysteine was used as reaction partner in the Michael addition to *E*-2-hexenal. NMR and FTIR analysis confirmed the presence of the aldehyde moiety in the resulting 3-*S*-(*N*-acetyl-L-cysteinyl)hexanal **3**. Because of the unstability of **3** under the conditions of trimethylsilylation, the synthesized aldehyde was converted to corresponding alcohol 3-*S*-(*N*-acetyl-L-cysteinyl)hexanol **4** for further confirmation of the structure. Compound **3** was not accepted as substrate by tryptophanase or the *E. limosum* extract. This is in accordance with the importance of the free amino group of the cysteinyl moiety demonstrated for other C−S β-lyases (*18*, *19*). However, after addition of an acylase to the reaction mixture, (i.e., an enzyme catalyzing a deacetylation) (*11*), a release of 3-mercaptohexanal was observed (**Table 3**). The amounts of product liberated were rather low. Nevertheless, the data confirm that 3-*S*-L-cysteinylhexanal **2** either present owing to the equilibrium state of **1** in aqueous solution or formed by acylase-catalyzed deacetylation of **3** acts as substrate for the C−S β-lyases applied. The preferred formation of the (*S*)-enantiomer starting from **3** is consistent with the stereochemical course observed for the enzyme-catalyzed reaction of **1**.

Enzyme-Catalyzed Cleavage of 3-*S*-L-Cysteinylhexanol 5. 3-*S*-L-Cysteinylhexanol **5** was synthesized by Michael-type addition of Boc-L-cysteine to *E*-2-hexenal, reduction with sodium borohydride and subsequent acidolysis. The compound was characterized by means of GC, GC-MS, LC-MS, ^{1}H, and ^{13}C NMR. The GC-MS data of the TMS-derivative were in

Table 3. Enzymatic Formation of 3-Mercaptohexanal from 3-*S*-(*N*-Acetyl-L-cysteinyl)hexanal **3** by Acylase I and β-Lyases[a]

		3-mercaptohexanal (n mol)				
enzyme	substrate (n mol)	(*R*)	(*S*)	conversion (%)	E^b	preferred product
tryptophanase	25	0.6	1.0	6	2	(*S*)
	250	3.7	10.6	6	3	(*S*)
	2500	1.9	12.2	1	7	(*S*)
E. limosum	25	0.1	0.2	1.0	3	(*S*)
	250	0.2	0.5	0.2	3	(*S*)
	2500	0.1	0.6	0.03	5	(*S*)

[a] For conditions, see Materials and Methods. [b] Enantioselectivity calculated according to ref 17.

Table 4. β-Lyase-Catalyzed Formation of 3-Mercaptohexanol from 3-Cysteinylhexanol **5**[a]

		3-mercaptohexanol (n mol)				
enzyme	substrate (n mol)	(*R*)	(*S*)	conversion (%)	E^b	preferred product
tryptophanase	25	7.6	6.5	57	2	(*S*)
	250	14.4	27.3	17	3	(*S*)
	2500	7.1	16.3	1	3	(*S*)
E. limosum	25	5.4	1.8	21	3	(*R*)
	250	18.8	5.7	10	3	(*R*)
	2500	29.6	8.5	2	3	(*R*)

[a] For conditions, see Materials and Methods. [b] Enantioselectivity calculated according to ref 17.

Table 5. Enzymatic Transformation of 2-(2-*S*-L-Cysteinylpentyl)-1,3-thiazolidine-4-carboxylic Acid **1** and 3-Cysteinylhexanol **5** by Yeasts

			3-mercaptohexanol (n mol)		
enzyme source	temp (deg C)	time (day)	(*R*)	(*S*)	conversion (%)
precursor **1**[a]					
baker's yeast	15	1	0.7	0.6	0.1
	15	14	16.9	17.9	1
	25	1	1.8	2.4	0.2
beer yeast (34/70)	15	1	0.3	0.2	0.02
	15	14	1.5	1.5	0.1
	25	1	1.4	1.4	0.1
beer yeast (184)	15	1	0.3	0.3	0.02
	15	14	2.4	2.4	0.1
	25	1	2.7	2.9	0.2
wine yeast (Siha 8)	15	1	0.3	0.4	0.03
	15	14	3.4	3.4	0.3
	25	1	0.5	0.7	0.05
precursor **5**[a]					
wine yeast (Siha 8)	25	1	1.2	1.1	0.1

[a] Amount of substrate, 2500 nmol.

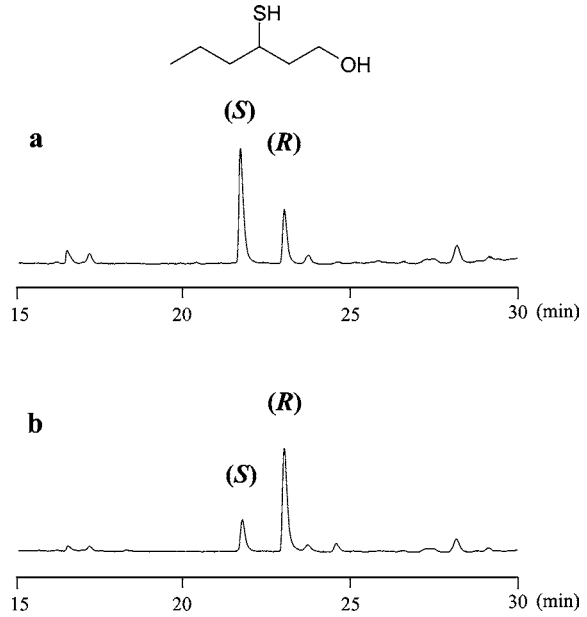


Figure 3. Enantiodifferentiation of 3-mercaptohexanol generated from 3-*S*-L-cysteinylhexanol **5** by tryptophanase (a) and a cell free extract from *E. limosum* (b). (GC system III; 2500 nmol of substrate; for other conditions, see Materials and Methods.)

agreement with those previously reported (*3*). ^{13}C NMR data revealed a mixture of two diasteroisomers; from the signal intensities molar ratios of about 52%:48% were estimated. As shown in **Table 4**, **5** was accepted as substrate by the two enzyme sources tested. Retention index and MS spectrum of the generated 3-mercaptohexanol were identical to those of an authentic reference compound. Resulting conversion rates by tryptophanase and the cell free extract from *E. limosum* are higher than those for **1**. However, they are still significantly lower than those observed under similar conditions for *S*-benzyl-L-cysteine and 8-*S*-L-cysteinyl-*p*-menthan-3-one (*6*).

Using octakis(2,6-di-*O*-pentyl-3-*O*-butyryl)-γ-cyclodextrin as chiral stationary phase, the enantiomers of 3-mercaptohexanol could be well separated. Enantiodifferentiation of the product liberated from **5** showed that the two enzyme preparations catalyze the cleavage of the C−S bond with preference for opposite enantiomers (**Figure 3**). Continuation of the reaction with tryptophanase to nearly complete cleavage (conversion > 95%) revealed a starting ratio of the substrate enantiomers of 55% (*R*):45% (*S*), thus confirming the NMR data.

Reaction with Yeast Cells. Compound **1** was accepted as substrate by different types of yeast (**Table 5**). Surprisingly, the reaction product was not 3-mercaptohexanal but 3-mercaptohexanol. Yeasts are well known to possess reductase activities (e.g., alcoholdehydrogenases) (*20*). Thus, it seems plausible that routes **c** or **d** outlined in **Figure 2** are involved in the formation of the alcohol. In accordance with the data observed for 8-*S*-L-cysteinyl-*p*-menthan-3-one, the β-lyase activities of the yeasts tested were rather low. However, the release rate of 3-mercaptohexanol is in the same order of magnitude as described for the inoculation of a model medium containing 3-*S*-L-cysteinylhexanol as precursor (*3*). The formation of 3-mercaptohexanol from **1** proceeded without preference of one of the enantiomers.

3-*S*-L-Cysteinylhexanol has been proposed as precursor of 3-mercaptohexanol in wine (*3*, *5*). The wine yeast tested in this study exhibited only low activity toward **5** and showed no significant differences in the rates of release for the two enantiomers (**Table 5**).

The systems investigated in this study suggest that β-lyases exhibit no or only moderate stereoselectivity when acting on 3-cysteinyl C_6-precursors. This is in line with the fact that 3-mercaptohexanol is present at rather low enantiomeric purity in passion fruits (*21*), in which 3-*S*-L-cysteinylhexanol **5** has been shown to act as precursor (*4*). On the other hand, the opposite stereoselectivities observed for the cleavage by tryptophanase and the extract from *E. limosum* indicate that further screening of the spectrum of β-lyases might result in biocatalysts suitable for generation of specific isomers of the important sulfur-containing volatiles 3-mercaptohexanal and 3-mercaptohexanol.

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NOTE ADDED AFTER ASAP

The original posting of December 16, 2003, has been corrected as of December 18, 2003, to show correct substrate values in Tables 3 and 4.

LITERATURE CITED

(1) Kerkenaar, A.; Schmedding, D. J. M.; Berg, J. Method for preparing thiol compounds. European Patent 0277688, 1988.

(2) Kerkenaar, A.; Schmedding, D. J. M.; Berg, J. Method for preparing thiol compounds with bacterial β-lyase. United States Patent 5578470, 1996.
(3) Tominaga, T.; Peyrot des Gachons, C.; Dubourdieu, D. A new type of flavor precursors in *Vitis vinifera* L. cv. Sauvignon blanc: *S*-cysteine conjugates. *J. Agric. Food Chem.* **1998**, *46*, 5215−5219.
(4) Tominaga, T.; Dubourdieu, D. Identification of cysteinylated aroma precursors of certain volatile thiols in passion fruit juice. *J. Agric. Food Chem.* **2000**, *48*, 2874−2876.
(5) 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.
(6) Wakabayashi, H.; Wakabayashi, M.; Eisenreich, W.; Engel, K.-H. Stereoselectivity of the β-lyase-catalyzed cleavage of *S*-cysteine conjugates of pulegone. *Eur. Food Res. Technol*. **2002**, *215*, 287−292.
(7) Huynh-Ba, T.; Matthey-Doret, W.; Fay, L. B.; Bel Rhlid, R. Generation of thiols by biotransformation of cysteine-aldehyde conjugates with baker's yeast. *J. Agric. Food Chem.* **2003**, *51*, 3629−3635.
(8) Engel, K.-H.; Tressl, R. Identification of new sulfur-containing volatiles in yellow passion fruits (*Passiflora edulis* f. *flavicarpa*). *J. Agric. Food Chem*. **1991**, *39*, 2249−2252.
(9) 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 Fragr. J.* **1998**, *13*, 159−162.
(10) Wakabayashi, H.; Wakabayashi, M.; Eisenreich, W.; Engel, K.-H. Stereoselectivity of the generation of 3-mercaptohexanal and 3-mercaptohexanol by lipase-catalyzed hydrolysis of 3-acetylthioesters. *J. Agric. Food Chem.* **2003**, *51*, 4349−4355.
(11) Giardina, T.; Biagini, A.; Dalle Ore, F.; Ferre, E.; Reynier, M.; Puigserver, A. The hog intestinal mucosa acylase I: Subcellular localization, isolation, kinetic studies, and biological function. *Biochimie* **1997**, *79*, 265−273.
(12) Esterbauer, H.; Ertl, A.; Scholz, N. The reaction of cysteine with α, β-unsaturated aldehydes. *Tetrahedron* **1976**, *32*, 285−289.
(13) Pesek, J. J. Quantitative determination of cis:trans isomeric ratios in substituted thiazolidines by carbon-13 magnetic resonance spectrometry. *Anal. Chem*. **1978**, *50*, 787−791.
(14) Restelli, A.; Annunziata, R.; Pellacini, F.; Ferrario, F. NMR determination of absolute configurations in 2-alkylthiazolidine-4-carboxylic acids. *J. Heterocyclic Chem*. **1990**, *27*, 1035−1039.
(15) Snell, E. E. Tryptophanase: structure, catalytic activities, and mechanism of action. *Adv. Enzymol. Relat. Areas Mol. Biol*. **1975**, *42*, 287−333.
(16) Larsen, G. L.; Stevens, J. L. Cysteine conjugate β-lyase in the gastrointestinal bacterium *Eubacterium limosum*. *Mol. Pharmacol*. **1985**, *29*, 97−103.
(17) Chen, C.-S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. Quantitative analyses of biochemical kinetic resolutions of enantiomers. *J. Am. Chem. Soc*. **1982**, *104*, 7294−7299.
(18) Tomisawa, H.; Suzuki, S.; Ichihara, S.; Fukazawa, H.; Tateishi, M. Purification and characterization of C−S lyase from *Fusobacterium varium*. *J. Biol. Chem*. **1984**, *259*, 2588−2593.
(19) Tateishi, M.; Suzuki, S.; Shimizu, H. Cysteine conjugate β-lyase in rat liver. *J. Biol. Chem*. **1978**, *253*, 8854−8859.
(20) Bränden, C.; Jörnvall, H.; Eklund, H.; Furugren, B. Alcohol dehydrogenases. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: London, United Kingdom, 1975; Vol. XI., pp103−190.
(21) Weber, B.; Dietrich, A.; Maas, B.; Marx, A.; Olk, J.; Mosandl, A. Stereoisomeric flavour compounds. LXVI. Enantiomeric distribution of the chiral sulphur-containing alcohols in yellow and purple passion fruits. *Z. Lebensm. Unters. Forsch*. **1994**, *199*, 48−50.

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