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Enzyme-catalyzed transformations of sulfur-containing flavor precursors

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1. **Introduction** 1

2. **Background** 5
2.1. Sulfur-containing flavor compounds in foods 5
2.2. Chirality of flavor compounds 8
2.3. Enzymatic generation of sulfur-containing flavor compounds 14
2.3.1. C-S β-lyase-catalyzed transformations 17
2.3.2. Lipase-catalyzed transformations 24

3. **Materials and Methods** 30
3.1. **Materials** 30
3.1.1. Chemicals 30
3.1.2. Enzymes and enzyme preparations 31
3.1.3. Microorganisms 32
3.1.4. Plants 32
3.2. **Syntheses** 32
3.2.1. Cysteine, homocysteine and glutathione conjugates 32
3.2.2. Thioesters 47
3.2.3. Thiols 49
3.3. **Culturing and preparation of the crude enzyme extact** 50
3.3.1. Extract from *Eubacterium limosum* 50
3.3.2. Preparation of acetone powders 50
3.4. **Enzymatic reactions** 51
3.4.1. C-S lyases 51
3.4.2. Lipases 53
3.4.3. Acylase 54
3.5. **Analyses** 54
3.5.1. Work-up of enzymatic reaction product 54
3.5.2. GC, GC-MS 55
3.5.3. NMR 58
3.5.4. LC-MS 59
3.5.5. FT-IR 59
3.5.6. Protein content 59

4. **Results and Discussion** 60
4.1. **C-S β-Lyase-catalyzed transformations** 60
4.1.1. Cysteine, homocysteine and glutathione conjugates of pulegone 60
4.1.1.1. Syntheses and structural elucidations 60
4.1.1.2. Enzymatic cleavage 69
4.1.1.3. Screening for β-lyases from other sources 77
4.1.1.4. Discussion 81
4.1.2. Cysteine conjugates of C_6-compounds 85
4.1.2.1. Syntheses and structural elucidations 85
4.1.2.2. Enzymatic cleavage 91
4.1.2.3. Discussion 98
4.1.3. β-Lyase-catalyzed transformations of other substrates 101
4.1.3.1. Syntheses and structural elucidations 101
4.1.3.2. Enzymatic transformations 104
4.1.3.3. Discussion 106
4.2. **Lipase-catalyzed transformations of thioesters** 108
4.2.1. Syntheses of thioesters 108
4.2.2. Lipase-catalyzed kinetic resolutions of thioesters 108
4.2.2.1. Activities and enantioselectivities of lipase preparations 108
4.2.2.2. Determination of absolute configurations 113
4.2.2.3. Influence of immobilization 115
4.2.2.4. Influence of co-solvent 115
4.2.2.5. Influence of structural modifications 117
4.2.3. Sensory properties of thioesters and thiols 118
4.2.4. Discussion 120
5. **Summary** 123
6. **Zusammenfassung** 125
7. **References** 127

Note: Some of the compounds have been numbered in the text. The numbering has not been applied consecutively, but has been restarted in each of the major chapters.

1. Introduction

The use of enzymes as biocatalysts is a well-established approach in flavor chemistry (*Berger, 1995*). Hydrolases play outstanding roles and their use for the liberation of flavor compounds from non-volatile precursors or for kinetic resolutions of chiral substrates has been studied extensively (*Teranishi et al., 1992*).

Sulfur-containing volatiles especially thiols belong to the most important flavor compounds occurring in foods (*Engel, 1999; Blank, 2002*). Sulfur-containing volatiles are not only generated in the course of the thermal treatment of foods (*Mussinan and Keelan, 1994; Mottram and Mottram, 2002*) but are also biosynthesized in various plants, especially tropical fruits (*Engel, 1999; Goeke, 2002*). Passion fruits are a typical example of a fruit, the flavor of which is determined by sulfur-containing compounds (*Werkhoff et al., 1998*). 3-Mercaptohexanol, firstly identified in yellow passion fruits (*Engel and Tressl, 1991*) and later also described as volatile constituent of Sauvignon blanc wine (*Tominaga et al., 1998a*) plays an important role in this spectrum. The corresponding aldehyde 3-mercaptohexanal had been described as synthetic intermediate (*Winter et al., 1976*). Later it has been reported as flavor compound in cooked liver and was described as imparting “tropical fruit”-type aroma notes (*Werkhoff et al., 1996*). Synthesis via combinatorial approach and sensory evaluation by gas chromatography/olfactometry revealed this mercaptoaldehyde to have a citrus peel note (*Vermeulen and Colin, 2002*).

With the interest in the biogenesis of volatile sulfur-containing compounds, the investigation of cysteinylated non-volatile precursors and the β-lyase-catalyzed liberation of sensorially active thiols has become an important area of flavor research (*Kerkenaar et al., 1988; Kerkenaar et al., 1996; Huynh-Ba et al., 1998; Tominaga et al., 1998b; Tominaga and Dubourdieu, 2000; Peyrot des Gachons et al., 2000; Huynh-Ba et al., 2003*).

Cysteine-*S*-conjugate β-lyases (EC 4.4.1.13) isolated from gastrointestinal microorganisms have been shown to catalyze the cleavage of the carbon-sulfur bond in various *S*-aryl, *S*-aralkyl, and *S*-alkyl cysteines (*Tomisawa et al., 1984; Larsen and Stevens, 1986*). These enzymes have been proposed as catalysts for the formation of sulfur-containing volatiles from cysteine conjugates of α,β-unsaturated aldehydes and ketones (*Kerkenaar et al., 1988*).

Recently, this class of enzymes has attracted new attention, because *S*-cysteine conjugates have been described as a new type of non-volatile flavor precursors in *Vitis vinifera* and passion fruits, and cysteine β-lyases proved to be suitable to release volatile thiols from these conjugates (*Tominaga et al., 1998b; Tominaga and Dubourdieu, 2000; Peyrot des Gachons et al., 2000*).

A typical example for a sulfur-containing flavor compound shown to be released from a cysteine conjugate is 3-mercaptohexanol (*Tominaga et al., 1998b; Tominaga and Dubourdieu, 2000; Peyrot des Gachons et al., 2000*). Its precursor 3-*S*-L-cysteinylhexanol has been detected in Sauvignon blanc must (*Tominaga et al., 1998b; Peyrot des Gachons et al., 2000*) and in passion fruit juice (*Tominaga and Dubourdieu, 2000*). 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 (*Tominaga et al., 1998b; Tominaga and Dubourdieu, 2000; Peyrot des Gachons et al., 2000*). However, the structure of the assumed intermediate, named *S*-3-(hexan-1-al)-L-cysteine (*Tominaga et al., 1998b; Tominaga and Dubourdieu, 2000; Peyrot des Gachons et al., 2000*), has not been verified.

Another typical example is 8-mercapto-*p*-menthan-3-one, a powerful odorant occurring in buchu leef oil (*Sundt et al., 1971; Lamparsky and Schudel, 1971*) and imparting the typical “cassis”-type aroma. The four stereoisomers have been shown to differ significantly in their sensory properties (*Köpke and Mosandl, 1992*) and their naturally occurring distribution has been determined (*Köpke et al., 1994*). The generation of 8-mercapto-*p*-menthan-3-one from

8-*S*-L-cysteinyl-*p*-menthan-3-one by an extract from *E. limosum* having β-lyase activity has been described (*Kerkenaar et al., 1988*). However, the stereochemical course of this enzyme-catalyzed reaction had not been considered.

Lipases constitute another class of enzymes which are well-established biocatalysts widely used for regioselective and enantioselective biotransformations (*Koskinen and Klibanov, 1996; Faber, 2000*). For esters, alcohols and acids many examples of kinetic resolutions of enantiomers via hydrolysis, transesterification and esterification have been described (*Theil, 1995; Reetz, 2002*). Analogous reactions have been reported for thioacids and esters (*Zaks and Klibanov, 1985; Sproull et al., 1997; Caussette et al., 1997; Weber et al., 1999*). Apart from a first communication on the lipase-catalyzed hydrolysis of 3-acetylthiocycloheptene (*Iriuchijima and Kojima, 1981*), the exploitation of the stereoselectivity of enzyme-catalyzed reactions of sulfur-containing esters started rather late (*Bianchi and Cesti, 1990; Baba et al., 1990*). In the meantime various approaches have been described (*Frykman et al., 1993; Öhrner et al., 1996; Izawa et al., 1997*), many of them focusing on the enzymatic resolution of 2-arylpropionates, an important class of non-steroidal anti-inflammatory drugs (*Um and Drueckhammer, 1998; Chang et al., 1998; Chen et al., 2002*).
Considering the importance of sulfur-containing compounds in flavor chemistry, it is not surprising that enzyme-catalyzed reactions have also been proposed as strategies to obtain flavoring compounds. Lipase-catalyzed syntheses (*Cavaille-Lefebvre and Combes, 1997; Cavaille-Lefebvre et al., 1998*) as well as hydrolyses of thioesters (*Bel Rhlid et al., 2001; Bel Rhlid et al., 2002*) have been described as useful approaches. Recently, the potential to use porcine liver esterase for the generation of 3-mercaptohexanal by hydrolysis of 3-acetylthiohexanal has been indicated (*Bel Rhlid et al., 2003*). However, the stereochemical course of the reaction had not been followed.

The objectives of this study were (i) to investigate the potential of β-lyases from

different sources to release thiol compounds from corresponding cysteine conjugates, (ii) to screen lipases from different sources for their potential to generate 3-mercaptohexanal and 3-mercaptohexanol by hydrolysis of the corresponding thioesters, and (iii) to focus on the capability of these biocatalysts to discriminate between substrate enantiomers and diastereoisomers, respectively.

2. Background

2.1. Sulfur-containing flavor compounds in foods

Sulfur-containing volatiles constitute one of the most potent classes of flavor compounds occurring in foods. From the dawn of flavor chemistry, sulfur-containing compounds have been attracting special attention. Already in *1976*, *Maga* reviewed volatile thiol compounds in food. He described over 60 foods in which thiols have been identified and described sensory properties of more than 70 thiols. As reviewed by *Blank* (*2002*), about 700 sulfur-containing substances have been reported as volatile compounds in food, corresponding to approximately 10 % of the total number of volatiles listed (*Nijssen et al., 1996*).

Table 2.1.1 Sulfur-containing character impact compounds in foods

Compound	Occurrence
methanethiol	cabbage
ethanethiol	broccoli
propanethiol	leek
allylthiol	garlic
propenylthiol	onion
3-mercaptohexanol	passion fruits, Sauvignon blanc
4-mercapto-4-methylpentan-2-ol	passion fruits, Sauvignon blanc
4-mercapto-4-methylpentan-2-one	Sauvignon blanc, grapefruit
3-mercapto-3-methylbutanol	yellow passion fruits
4-methoxy-2-methyl-2-butanethiol	blackcurrant, olive oil
2-methyl-3-furanthiol	beef meat (cooked)
(*R*)-1-*p*-menthen-8-thiol	grapefruit
methional	potato chips
2-furfurylthiol	coffee (roasted)
(2*R*,3*S*)-3-mercapto-2-methyl-1-pentanol	onion (fresh)
2-methyl-4-propyl-1,3-oxathiane	passion fruits
ethyl 3-mercaptopropanoate	grape (concorde)
ethyl 3-(methylthio)-propanoate	pineapple
8-mercapto-*p*-menthan-3-one	buchu leaf oil

Adapted from *Blank, 2002*

They comprise more than 440 sulfides, ~100 thiazoles, ~100 thiophenes, and ~60 thiols. Many S-compounds have been reported to act as so-called character-impact compounds, i.e. to impart typical flavor notes to foods. Examples are listed in Table 2.1.1.

The essential roles of these constituents are due to their low odor thresholds. In Table 2.1.2, the odor thresholds of some sulfur-containing volatiles are compared to those of other well-known flavor compounds.

Table 2.1.2 Comparison of the thresholds of sulfur-containing volatiles and other aroma compounds

Compound	Source	Odor threshold (ppb in water)
methanethiol	*brassica*, *allium* plants	0.02[a]
furfurylthiol	coffee	0.005[a]
4-mercapto-4-methylpentan-2-one	passion fruits, grapefruit, wine	0.0001[b]
1-*p*-menthen-8-thiol	grapefruit	0.00002[a]
vanillin	vanilla bean	680[a]
geraniol	rose flower, plants	40[a]
(*E*)-2-hexenal	leaves, vegetables	17[a]
4-decanolide	peach	7[a]
isoamyl acetate	banana	2[a]
sotolone	crude sugar, soy sauce	0.001[a]

[a] *Rychlik et al., 1998*; [b] *Buettner and Schieberle, 2001a*

The uniqueness of volatile sulfur compounds also becomes obvious when comparing the flavor properties of oxygen-containing compounds to those in which oxygen has been replaced by sulfur (Table 2.1.3).

Table 2.1.3 Comparison of the odor properties of sulfur-containing volatiles to those of the corresponding oxygen-analogues[a]

OH	pungent [b] (9000 ppb)	SH	onion, cabbage-like [c] (3 ppb)
OH	fruity [b] (500 ppb)	SH	putrefaction of onion [e] (6 ppb) [c]
OH	rose-like [b] (40 ppb)	SH	grapefruit-like [d]
O OH	nearly odorless, weak caramel-like [c]	O SH	coffee (roasted) [b] (0.005 ppb)

[a] Odor threshold values in water are shown in parenthesis
[b] *Rychlik et al., 1998*; [c] *Leffingwell, 2004*; [d] *Helmlinger et al., 1974*;
[e] Meilgaard, *1975*

Odor thresholds of thiol compounds also depend strongly on their structures. As shown in Table 2.1.4, tertiary thiols have much lower threshold values than the corresponding primary or secondary thiols (*Meilgaard, 1975*).

Table 2.1.4 Correlation between structures and odor thresholds of sulfur-containing compounds

Compound	Structural feature	Odor threshold [µg/L beer]
1-butanethiol	primary SH	0.7
2-butanethiol	secondary SH	0.6
2-methyl-1-propanethiol	primary SH	2.5
2-methyl-2-propanethiol	tertiary SH	0.08
3-methyl-2-butanethiol	secondary SH	0.2
2-methyl-2-butanethiol	tertiary SH	0.00007

2.2. Chirality of flavor compounds

Chirality in flavor perception

The first data regarding the enantioselective perception of chiral odorants were published by *Rienäcker and Ohloff* (*1961*). They described (+)-β-citronellol to have typical citronella odor, while (-)-β-citronellol was found to exhibit a geranium-type note. Another classical example demonstrating the importance of chirality in flavor perception is carvone (*Leitereg et al., 1971*). Both enantiomers

Table 2.2.1 Enantiomers showing different odor properties

Compound	Odor description
7-hydroxy-6,7-dihydro-citronellal	(+): lily of the valley with green minty notes (–): sweet lily of the valley note
linalool	(+): sweet, petitgrain (–): woody, lavender
nootkatone	(+): grapefruit (–): woody, spicy
nerol oxide	(+): green, floral (–): green, spicy, geranium
androstenone	(+): odorless (–): sweaty, urine, strong, musky
menthol	(–): sweet, fresh, minty, strong, cooling effect (+): dusty, vegetable, less minty, less cooling
limonene	(+): orange (–): turpentine

Adapted from *Brenna et al., 2003*

occur in nature and exhibit very different flavor characteristics. (*R*)(-)-Carvone possesses the odor of spearmint (threshold in water: 2 ppb), whereas (*S*)(+)-carvone has caraway odor (threshold in water: 85 – 130 ppb). To date, more than 320 enantiomeric pairs have been reported to exhibit either different odor properties or differences in odor intensities (*Leffingwell, 2004; Brenna et al., 2003*). Examples are shown in Table 2.2.1.

An impressive example of the influence of chirality on the sensory properties of sulfur-containing compounds is 1-*p*-menthene-8-thiol. It has been reported as extremely potent constituent of grapefruit juice (*Citrus paradisi* Macfayden) (*Demole and Enggist, 1982; Demole et al., 1982*). Later it was also identified in orange (*Buettner and Schieberle, 2001b*), yuzu (*Yukawa et al., 1994*) and grape must (*Serot et al., 2001*).
Demole et al. (1982) reported both enantiomers to exhibit grapefruit-like odor with thresholds in water of 0.00002 ppb for the (*R*)- and 0.00008 ppb for the (*S*)-enantiomer. Several years later, *Lehmann et al.* (*1995*) separated the enantiomers of 1-*p*-menthene-8-thiol by means of capillary GC using a chiral stationary phase. GC/Olfactometry of the two antipodes revealed that only the (*R*)-enantiomer has a strong grapefruit-like odor whereas the (*S*)-enantiomer was described as weak, non-specific and nearly odorless. The comparison of the odor properties of α-terpineol and 1-*p*-menthene-8-thiol demonstrates the tremendous impact of the replacement of a tertiary hydroxy group by a thiol moiety (Table 2.2.2).

Table 2.2.2 Odor properties of 1-*p*-menthen-8-thiol (**1**) and α-terpineol (**2**)

Odor evaluation (GC/O)			
(*R*)-**1**	(*S*)-**1**	(*R*)-**2**	(*S*)-**2**
SH	SH	OH	OH
grapefruit-like, strong impact	weak, nonspecific, neary odorless	flowery, sweet, lilac	tarry, reminiscent of cold pipe

Adapted from *Lehmann, 1995*

The effect of chirality on odor properties of sulfur containing compounds is also obvious for 2-methyl-4-propyl-1,3-oxathiane, occurring in passion fruits, and its homologues (Table 2.2.3). Each pair of enantiomers and diastereoisomers of the sulfoxide, the methylated and the de-methylated derivative showed different odor properties.

Lactones are also well-known naturally occurring chiral flavor compounds. 4-Alkyl substituted γ-lactone enantiomers exhibit differences in odor qualities as well as in odor intensities (*Brenna et al., 2003*). The sensory properties of a homologous series of γ- and δ-thiolactones resulting from the replacement of the ring-oxgen in γ- and δ-lactones by sulfur have been assessed (*Schellenberg, 2002*). The basic coconut-note of the oxygen-containing lactones was complemented by attractive fruity, tropical notes. Significant differences between enantiomers were observed for δ-thiooctalactone and δ-thiodecalactone (*Engel et al., 2001*).

Table 2.2.3 Odor descriptions of stereoisomeric oxathianes

structure	configuration	odor description	reference
	cis-(2*R*,4*S*)	sulfury, herbaceous-green, roasty, blackcurrant, fruity, raspberry	a, b
	trans-(2*S*,4*S*)	sulfury, bloomy-sweet, less strong than (2*R*,4*S*)	a, b
	cis-(2*S*,4*R*)	fatty, green fruity, tropical fruits, grapefruit, fruity, exotic fruit, passion fruits, green	a, b
	trans-(2*R*,4*R*)	green-grass root, earthy, raddish	a, b
	(2*R*,3*R*,4*S*)	intensive, pungent, green, rotten	c
	(2*R*,3*S*,4*S*)	green-grass, longlasting sulfur note	c
	(2*S*,3*S*,4*R*)	intensive, exotic fruits, volatile	c
	(2*S*,3*R*,4*R*)	intensive, green, reminiscent of rhubarb	c
	(4*S*)	artificial, sulfury, burnt rubber-like	a
	(4*R*)	artificial fruity, fatty, slight grapefruit note	a
	(4*S*)	typical carrot note, sweet	a
	(4*R*)	slight fruity, soft lemon note	a

a: *Mosandl and Heusinger, 1985*; b: *Singer et al., 1986*; c: *Singer et al., 1987*

Chiral analysis of flavor compounds

The most popular approach to the separation of enantiomeric pairs involves diastereomorphous interaction with a chiral environment. This task can be accomplished by derivatization of the analyte with a chiral auxiliary, followed by separation of the diastereoisomers in an achiral environment, e.g., by chromatographic or electrophoretic methods. For a long time, derivatization of enantiomers to diastereomers, originally developed by *Bailey and Hass* (*1941*), was the only method available. According to those approaches, alcohols were derivatized with 2-acetyllactic acid and acids with (-)-menthol, for example. It

turned out that this methodology suffered from several drawbacks, the most serious being the lack of complete enantiomeric purity of the chiral derivatization agent.

More recently, several methods involving reversible weak interactions with a chiral environment were introduced. Here, a lack of complete enantiomeric purity does not result in a signal that may be erroneously attributed to the minor enantiomer of the analyte; instead, such a deviation will typically result in a decrease in the degree of discrimination.

These methods comprise chiral stationary phases, mobile phase additives, buffer additives, solvating agents and lanthanide shift reagents. The most important chiral stationary phases used in gas chromatography can be divided into three main classes: amide phases (hydrogen bonds) (*König et al., 1981*), metal complex phases (complexation) (*Schurig and Bürkle 1982*) and cyclodextrin phases (inclusion) (*König et al., 1988*).

In *1983*, the first application of a cyclodextrin phase to the separation of enantiomers by GC was reported by *Koscielski et al.* (*1983*). These authors separated the enantiomers of α- and β-pinene, respectively, on celite coated with an aqueous formamide solution of α- and β-cyclodextrins in packed column gas solid chromatography.

Cyclodextrins are cyclic oligomers (named α- (n=6), β- (n=7) and γ- (n=8)) of glucopyranose connected by α-1,4-glucoside bonds which are able to form inclusion complexes in their cavities (Figure 2.2.1). They have three free hydroxy groups on 2- (secondary), 3- (secondary) and 6- (primary) position that can be modified by various substituents.

Since *1988* selectively alkylated and/or acylated α-, β- and γ-cyclodextrins have been synthesized, serving as chiral stationary phases in enantioselective gas chromatography. Starting from peralkylated material, e.g. permethylated cyclodextrin (*König et al., 1988*), many types of cyclodextrin derivatives, some of them very efficient for flavor analysis, were proposed during the following years by many researchers (*Armstrong et al., 1990; Schmarr et al., 1991; Bicchi et al., 1992*). 6-*O*-*tert*-butyldimethylsilyl derivatives such as heptakis(2,3-di-*O*-methyl -6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin were reported to show great potential

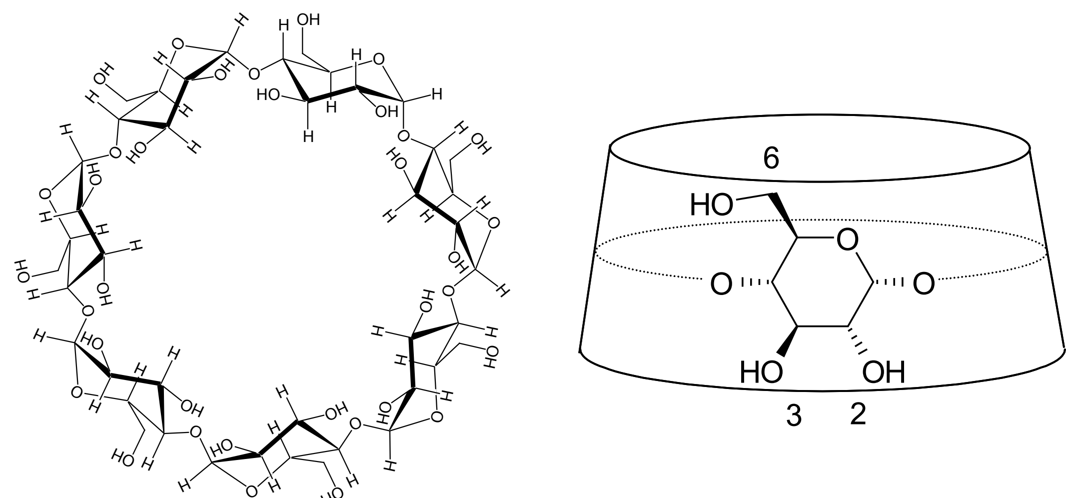


Figure 2.2.1 Molecular structure (left) and cavity model (right) of β-cyclodextrin

for the enantiomeric separation for various substance classes (*Dietrich et al., 1992b*). Moreover, it was demonstrated that 2,3-di-*O*-acetyl modification like in heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin results in high specificity for the enantiomeric separation especially of oxygen-containing compounds (*Dietrich et al., 1992a*).

Meanwhile, nearly every chiral flavor compound has been made amenable to enantiomer separation by GC, although a researcher in this field will find it difficult to search the most suitable chiral stationary phase for a given separation problem.

As compared to routine analysis of achiral compounds, the separation of enantiomers requires a more sophisticated instrumentation. Problems may arise from overlapping of peaks, leading to wrong peak assignments and misinterpretation of enantiomer ratios. Therefore, a so-called multidimensional gas chromatographic system (MDGC) composed of an achiral pre-column and a chiral main column should be used (*Bernreuther et al., 1989; Krammer et al., 1990; Palm et al., 1991; Werkhoff et al., 1991*). Such a combination also makes the sample preparation easier and less time-consuming.

The combination of enantioselective GC with GC-sniffing proved especially useful for the characterization of the odors of single enantiomers (*Lehmann et al., 1995*).

2.3. Enzymatic generation of sulfur-containing flavor compounds

On one hand, sulfur-containing volatiles are formed in the course of thermal processing of foods resulting from reactions of sulfur-containing amino acids in the course of the Maillard reaction (*Mottram and Mottram, 2002*). On the other hand, they also constitute essential components of the biogenetically derived aroma patterns of biological systems such as fruits (*Engel, 1999; Goeke, 2002*).

Sulfur is essential for plant growth and found in two sulfur-containing amino acids, cysteine and methionine (*Saito, 2000*). Sulfur is taken up by plants in its inorganic sulfate form (SO_4^{2-}) from external environments (i.e. the soil) into the symplastic system. The vacuole is presumed to be the major compartment for sulfate storage within cells. Sulfate is then converted to sulfite (SO_3^{2-}) via adenosine 5'-phosphosulfate (APS) by the action of ATP sulfurylase and APS reductase. Sulfite is then reduced by sulfite reductase into S^{2-}. The final step in cysteine synthesis is the incorporation of the sulfide moiety at the β-position of alanine. The carbon skeleton is derived from serine via *O*-acetylserine (Figure 2.3.1).

Methionine is further synthesized in three steps from cysteine and *O*-phosphohomoserine. Firstly, cystathionine is synthesized by the action of cystathionine γ-synthase, then the β-C-S bond is cleaved by cystathionine β-lyase to produce homocysteine, and finally methionine synthase transfers a methyl group to homocysteine to produce methionine.

After cysteine and methionine are synthesized, sulfur can be incorporated into proteins and a variety of other compounds. For example, sulfur's proposed entry into the flavor pathway of onion has been reported to start from cysteine via glutathione (*Block, 1992*).

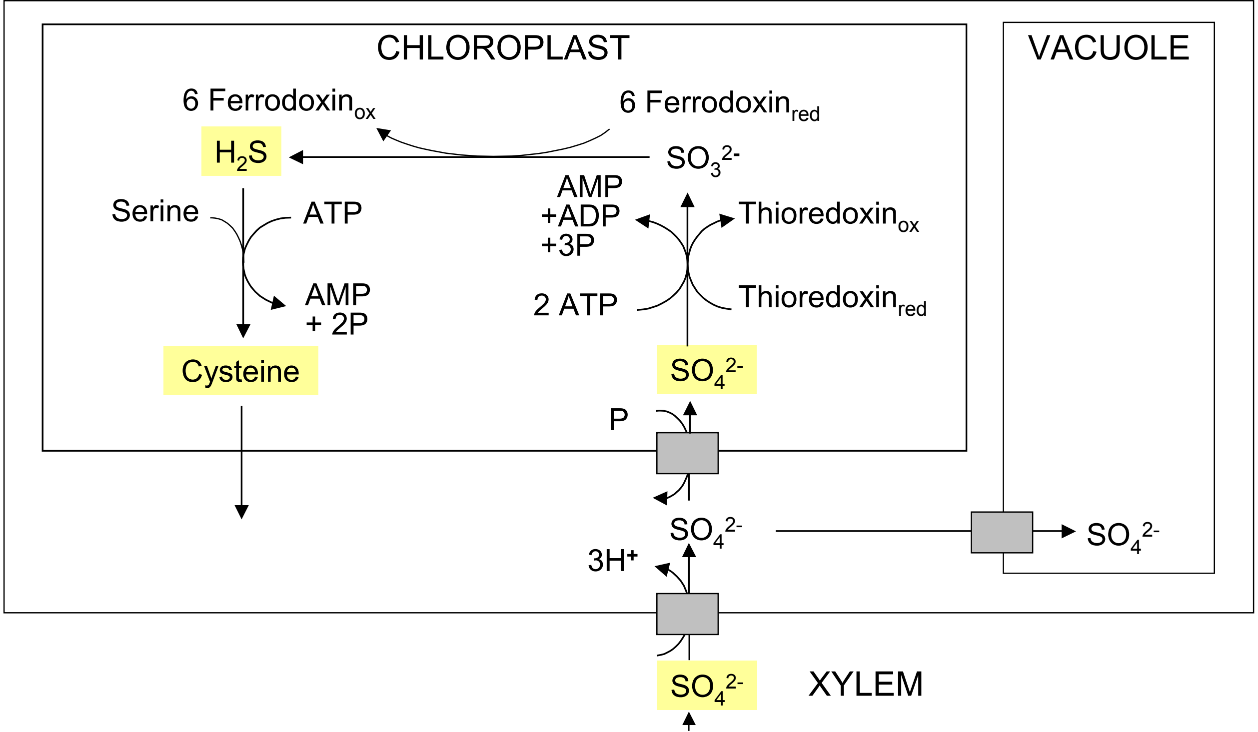


Figure 2.3.1 Uptake of sulfur by plants

The flavor of many vegetables is due to volatile sulfur-containing compounds formed by a variety of enzymatic reactions starting from non-volatile precursors. Classical examples are glucosinolates and *S*-alkyl or *S*-alkenyl-L-cysteine *S*-oxides.

Glucosinolates are non-volatile precursors which are hydrolyzed to volatiles when plant tissues are disrupted or damaged (*Chen and Andreasson, 2001*). Almost all vegetables containing glucosinolates belong to the family Cruciferae which includes *Brassica* plants such as mustard (*Kojima et al., 1973*), horseradish (*Gilbert and Nursten, 1972*), watercress (*MacLeod and Islam, 1975*), cabbage, cauliflower and broccoli (*Buttery et al., 1976*). Plants containing glucosinolates also contain enzymes degrading these compounds. These enzymes, called myrosinases (EC 3.2.3.1), catalyze the hydrolysis of the thioglucosidic linkage in glucosinolates to produce thiohydroxamate-*O*-sulfonate. This is normally followed by a Lossen-type rearrangement to yield isothiocyanates. This represents the major degradation pathway under

conditions normally prevailing in crushed or injured plant tissues. The isothiocyanates are pungent compounds and key contributors to the characteristic flavor of Cruciferae crops. There are several possibilities for the degradation of glucosinolates resulting in a variety of products as shown in Figure 2.3.2.

Figure 2.3.2 Products resulting from degradation of glucosinolates
Adapted from *Chen and Andreasson, 2001*

The *S*-alkyl and *S*-alkenyl-L-cysteine *S*-oxides are precursors of the characteristic aroma of *Allium* genus plants which include garlic, leek, onion and shallot. When *Allium* species tissues are disrupted, *S*-alk(en)yl cysteine *S*-oxides (R=methyl, 1-propenyl, 2-propenyl or *n*-propyl) are cleaved by alliinase (EC 4.4.1.4), a kind of C-S lyase, to reactive sulfenic acids [RSOH] which condense to yield thiosulfinate esters [RS(O)SR] as shown in Figure 2.3.3.

Figure 2.3.3 Cleavage of *S*-alkyl cysteine *S*-oxide by alliinase

The saturated and unsaturated thiosulfinates are the primary constituents responsible for the odor of freshly cut *Allium* species (*Block et al., 1992*). These very labile and reactive thiosulfinates are converted to di- and poly-sulfides. Allicin (2-propene-1-sulfinothioic acid *S*-2-propenyl ester) is the predominant aroma principle of freshly cut garlic. The organosulfur chemistry of *Allium* genus plants has been extensively reviewed by *Block* (*1992*).

2.3.1. C-S β-lyase-catalyzed transformations

A number of enzymes are known to catalyze β-elimination reactions of *S*-substituted cysteines to yield pyruvate, ammonia and the corresponding thiols (Fig. 2.3.4).

Figure 2.3.4 Reaction catalyzed by C-S β-lyases

Alliin lyase (EC 4.4.1.4), cystathionine β-lyase (cystine lyase) (EC 4.4.1.8), alkylcysteine β-lyase (EC 4.4.1.6) and cysteine conjugate β-lyase (EC 4.4.1.13) are examples of enzymes exhibiting this type of activities for β-elimination reactions. They require pyridoxal 5'-phosphate (PLP) as co-factor. Properties of these enzymes are summarized in Table 2.3.1.

Table 2.3.1. Enzymes exhibiting C-S β-lyase activities.

enzyme	source	substrate	pH optimum
alliin lyase (EC 4.4.1.4)	onion[a], garlic[a], leek[b], *Penicillium corymbiferum*[c], etc.	*S*-(1-propenyl)-L-cysteine sulfoxide; *S*-allyl-L-cysteine sulfoxide	7.8 - 9.5
cystathionine β-lyase (EC 4.4.1.8) (cystine lyase)	turnip[d], cabbage[d], broccoli[d], spinach[d], *E. coli*[e], *Lactococcus lactis*[f], *Synechocystis* sp.[g], etc.	L-cystine; L-cysteine; L-cysteine-S-SO_3; *S*-methyl-L-cysteine sulfoxide; cystathionine	8.5 - 9.0
alkylcysteine β-lyase (EC 4.4.1.6)	*Acacia farnesiana*[h] *Pseudomonas* sp.[i], *Bacillus* sp.[j], etc.	L-djenkolate; *S*-methyl-L-cysteine; *S*-benzyl-L-cysteine	7.8 - 9.5
cysteine conjugate β-lyase (EC 4.4.1.13)	rat[k], mouse[l], hamster[l], guinea pig[l], bovine[m], human[n], tapeworm[o], *Mucor javanicus*[p], *Fusobacterium varium*[q], *Eubacterium limosum*[r], etc.	*S*-(2-benzothiazolyl)-L-cysteine; *S*-2,4-dinitrophenyl-L-cysteine; *S*-(2-benzothiazolyl)-L-cysteine	7.4 - 8.8

[a]*Nock and Mazelis, 1987*; [b]*Won and Mazelis, 1989*; [c]*Durbin and Uchytil, 1971*; [d]*Ramirez and Whitaker, 1998*; [e]*Dwivedi et al., 1982*; [f]*Alting et al., 1995*; [g]*Clausen et al., 2000*; [h]*Mazelis and Creveling, 1975*; [i]*Kamitani et al., 1991*; [j]*Kamitani et al., 1990*; [k]*Stevens et al., 1986*; [l]*Elfarra and Hwang, 1990*; [m]*Kishida et al., 2001*; [n]*Lash et al., 1990*; [o]*Adcock et al., 1999, 2000*; [p]*Shimomura et al., 1992*; [q]*Tomisawa et al., 1984*; [r]*Larsen and Stevens, 1986*

Alliin lyase and cystathionine β-lyase occur in *Allium* and *Brassica* species and are well-known to contribute to the formation of important sulfur-containing volatiles in these plants.

Alliin lyase catalyzes the cleavage of *S*-alkyl or *S*-alkenyl-L-cysteine sulfoxide to yield *S*-alkyl-sulfenic acid. The predominant natural substrates are *S*-(1-propenyl)-L-cysteine sulfoxide in onion and *S*-allyl-L-cysteine sulfoxide in garlic. In garlic, the enzymatically formed allyl sulfenic acid is converted to allicin (diallyl thiosulfinate) and further converted to several disulfide and trisulfide

compounds.

Cystathionine β-lyases cleave L-cystine or L-cystathionine through an α,β-elimination reaction yielding thiocysteine or homocysteine, pyruvate and ammonia. This enzyme has been purified and characterized from several *Brassica* vegetables such as turnip, cabbage, spinach and broccoli (*Ramirez and Whitaker, 1998*). The enzyme has been shown to be responsible for the off-flavor deterioration of unblanched broccoli. A mechanism for the formation of volatile sulfur-containing compounds such as dimethyl disulfide and dimethyl trisulfide from *S*-methyl-cysteine sulfoxide by cystathionine β-lyase has been proposed (*Marks et al., 1992*). Cystathionine β-lyase has also been reported to be involved in the biosynthesis of methionine in Gouda cheese. Methionine is further transformed by the same enzyme to methanethiol, a putative precursor of important flavor compounds (*Alting et al., 1995*).

Alkylcysteine β-lyase and cysteine conjugate β-lyase accept *S*-alkyl-, *S*-aralkyl- or *S*-aryl-L-cysteine, rather than the sulfoxides as substrates.

Alkylcysteine β-lyase has been found in *Acacia* spieces (*Mazelis and Creveling, 1975*) and *Bacillus* sp. (*Kamitani et al., 1991*). The natural substrate of the enzyme is reported to be L-djenkolate, which is cleaved to *S*-(mercaptomethyl)cysteine. The enzyme from *Acacia* plant catalyzes the β-elimination of both the thioether and sulfoxide form of the substrate.

Cysteine conjugate β-lyase has been firstly isolated from rat liver (*Tateishi et al., 1978*). Later it has been characterized and partially purified from mammals (*Stevens et al., 1986; Elfarra and Hwang, 1990; Kishida et al., 2001; Lash et al., 1990*), parasitic helminths (*Adcock et al., 1999; 2000*) and microorganisms (*Shimomura et al., 1992; Tomisawa et al., 1984; Larsen and Stevens, 1986*).

Generally, cysteine conjugate of aromatic compounds mainly serve as substrates for this enzyme. The enzymes extracted from bacteria have rather broad substrate specificities. The cleavage of cysteine conjugates containing simple *S*-alkyl groups (e.g. *S*-methyl- or *S*-ethyl-), halogenated groups (e.g. *S*-1,2-dichlorovinyl-) and an amino acid moiety (e.g. cystathionine) could be catalyzed by cysteine conjugate β-lyase from *Fusobacterium varium* or

Eubacterium limosum. However, for the mammalian enzymes, simple *S*-alkyl cysteine conjugates are no substrates. The activity is generally inhibited by hydroxylamine, in most cases also by pottasium cyanide.

Purified cysteine conjugate β-lyase from rat liver has been shown to have kynureninase (EC 3.7.1.3) activity (*Stevens, 1985*). The enzyme obtained from rat kidney has been reported to be identical to glutamine transaminase K (EC 2.6.1.64) (*Stevens et al., 1986*). Some of the enzymes extracted from parasitic helminths have aspartate and alanine aminotransferase and γ-glutamyl transpeptidase activities.

There are other mammalian PLP-containing enzymes which have been shown to catalyze a cysteine *S*-conjugate β-lyase reaction. Examples are pig heart alanine aminotransferase, pig heart aspartate aminotransferase, human branched-chain amino acid aminotransferase, rat kidney alanine-glyoxylate aminotransferase isozyme II and rat kidney high M_r protein (M_r >200,000) (*Cooper et al., 2002a,b; 2003*).

Cysteine *S*-conjugates are intermediates in the mercapturate pathway [xenobiotic → glutathione *S*-conjugate → cysteinylglycine *S*-conjugate → cysteine *S*-conjugate ⇌ *N*-acetyl-L-cysteine *S*-conjugate → excretion] (Figure 2.3.5). This pathway is important for the detoxification of exogenous electrophiles and in the elimination of some endogenous electrophiles because most mercapturates (*N*-acetyl-L-cysteinyl *S*-conjugates) are less toxic and more water-soluble than the parent compounds and are readily excreted (*Cooper et al., 2002b*).

Halogenated alkenes such as trichloroethylene, tetrafluoroethylene and chlorotrifluoroethylene are metabolized at least in part to the corresponding cysteine *S*-conjugate. These cysteine conjugates are suggested to be nephrotoxic partly due to the high reactivity of the sulfur-containing fragment eliminated by the action of cysteine *S*-conjugate β-lyases. On the other hand, considering the prominent location of cysteine conjugate β-lyase in the human (or mammalian) kidney, this enzyme could be a target for prodrugs to kidney cancer. After administration of *S*-(6-purinyl)-L-cysteine to rats, the concentrations

Figure 2.3.5 Mercapturic acid pathway
(a) glutathione *S*-transferase (b) γ-glutamyltransferase
(c) cysteinylglycine dipeptidase (d) L-cysteine *S*-conjugate β-lyase
(e) L-cysteine *S*-conjugate *N*-acetyltransferase (f) acylase

of 6-mercaptopurine (anti cancer compound) and its metabolite were 90- and 2.5- fold higher in kidney than in plasma and liver, respectively (*Hwang and Elfarra, 1991*). The use of the selenocysteine Se-conjugates as potential prodrugs has also been proposed because of the higher reactivity of the enzyme for these compounds (*Commandeur et al., 2000; Rooseboom et al., 2000*).

The wide distribution of cysteine conjugate β-lyase in gastrointestinal bacteria such as *Bacteroides* sp., *Eubacterium* sp. and *Fusobacterium* sp. (*Larsen, 1985*) or in parasitic helminths (*Adcock et al., 1999*) suggests an important role of the

intestinal microflora in the *in vivo* formation of thio- or methylthio-containing metabolites of various xenobiotics. Fungal enzymes may contribute to the degradation of pesticides in soil which contains many kinds of halogenated compounds (*Shimomura et al., 1992*).

The mechanisms of PLP-dependent enzymes are well studied. PLP binds the enzyme as an aldimine with the ε-amino group of a lysine residue. Entry of a substrate amino acid into the active site results in transimination to form a new substrate-PLP complex (Schiff base) (*John, 1995*). These mechanism is outlined for the *S*-cysteine conjugate as a substrate in Figure 2.3.6.

Enzyme-PLP internal Schiff base

Substrate-PLP external Schiff base

Figure 2.3.6 Mechanism of the reaction of the active site of the enzyme with PLP and substrate.

The resulting free lysine amino function of the enzyme can remove a proton from the α-position of the amino acid moiety of the substrate-PLP Schiff base to produce a resonance-stabilized enzyme-bound anion. This may reprotonate at the α-carbon resulting in the elimination of an electronegative β-substituent. Because all of these processes depend on initial stabilization of negative charge through the extended π-system, optimal orbital overlap requires that the

π-system be coplanar during reaction and that the σ-bonds which are broken be perpendicular to this plane (*Dunathan and Voet, 1974*), as shown in Figure 2.3.7.

Figure 2.3.7 Mechanism for the β-elimination of the substrate-PLP Schiff base

Such stereoelectronic requirements enable PLP-dependent enzymes to enhance reaction rates and control specificity of bond cleavage by proper conformational orientation (*Vederas and Floss, 1980*). It is also proposed that the reaction catalyzed by alkylcysteine β-lyase from *Acacia* sp. takes place on only one side of a planar coenzyme-substrate complex (*Tsai et al., 1978*). The PLP binding site of the onion alliin lyase which is shown to have high catalytic activity of cysteine conjugate β-lyase was identified as Lys 285 in the amino acid sequence (*Kitamura et al., 1997*).

Recently, cysteine *S*-conjugates have been reported as other type of naturally occurring non-volatile sulfur-containing flavor precursors in passion fruits and grape must (*Tominaga et al., 1998b; Tominaga and Dubourdieu, 2000*). Thiol compounds are generated by the action of C-S β-lyase from these precursors (Figure 2.3.8).

Figure 2.3.8 Sulfur-containing volatiles formed by β-lyase-catalyzed cleavage of non-volatile precursors in passion fruits (*Tominaga and Dubourdieu, 2000*)

2.3.2. Lipase-catalyzed transformations

Lipases (EC 3.1.1.3), a class of enzymes belonging to the serine hydrolases, are enzymes occurring ubiquitously in microorganisms, plants and amimals. Their biological function in plants and animals is to catalyze the hydrolysis of triacyl glycerols to yield free fatty acids. A number of fungal and bacterial species were found to be efficient lipase producers, and the lipases have been studied from academic and industrial viewpoints. Their industrial importance is based on their broad substrate specificity promoting a wide range of biocatalytic reactions. Lipases are active both at oil/water interfaces and in organic solvents. Many lipases are heat-stable (up to 100 °C) and they do not need co-factors.

Microbial lipases have found application in several fields of industry, e.g. in the manufacturing of foods, leather, pharmaceuticals, cosmetics. They are extensively used in the dairy industry for the hydrolysis of milk fat and in the cheese manufacturing industry. Lipases are also being intensively investigated with regard to the modification of oils rich in high-value polyunsaturated fatty acids such as arachidonic, eicosapentaenoic or docosahexaenoic. Probably, the most elegant application of lipases is their use in enantioselective syntheses of optically active compounds from *meso* reactants. The resulting optically pure compounds are often difficult to obtain by alternative routes and can be of great synthetic and applicational value.

Lipases catalyze the hydrolytic cleavage of the C-O bond in esters, resulting in the liberation of alcohols. In analogy, thiol compounds will be generated from thioesters by the action of hydrolytic enzymes (Figure 2.3.9).

$$R\text{-}O\text{-}C(=O)\text{-}R' + H_2O \underset{}{\overset{\text{lipase}}{\rightleftharpoons}} R\text{-}OH + HO\text{-}C(=O)\text{-}R'$$

$$R\text{-}S\text{-}C(=O)\text{-}R' + H_2O \underset{}{\overset{\text{lipase}}{\rightleftharpoons}} R\text{-}SH + HO\text{-}C(=O)\text{-}R'$$

Figure 2.3.9 Generation of alcohols and thiols by lipase-catalyzed hydrolysis of esters and thioesters, respectively

Lipases can hydrolyze and form carboxylic ester bonds like proteases and esterases, but their molecular mechanism is different. The most important difference between lipases and esterases is the physicochemical interaction with their substrates. Esterases show a ‘normal’ Michaelis-Menten activity depending on the substrate concentration, but lipases display almost no activity as long as the substrate is in a dissolved monomeric state. However, when the substrate

concentration is gradually enhanced beyond its solubility limit by forming a second (lipophylic) phase, a sharp increase in lipase-activity takes place. This phenomenon has been called the ‘interfacial activation’ (*Verger, 1997*). The molecular rationale for this ‘interfacial activation’ has been explained as a rearrangement process within the enzyme. A freely dissolved lipase in the absence of an aqueous / lipid interface resides in its inactive state, because a part of the enzyme molecule covers the active site. When the enzyme contacts the interface of a biphasic water-lipid system, a short α-helix, the so-called ‘lid’, is folded back. Thus, by opening its active site, the lipase is rearranged into its active state.

Microbial and mammalian lipases show no obvious similarities in their primary structures. A large sequence variety is found within the group of microbial lipases. But the sequence alignment of lipases has shown a consensus sequence Gly – X – Ser – X – Gly that exists in most mammalian and microbial lipases around the active serine. The mutations in the consensus sequence of *Candida antarctica* lipase B have been shown to change its activities, specificities and thermostabilities (*Patkar et al., 1997; 1998*).

Due to the unique environment around their active site, lipases are capable to calalyze a wide range of racemic resolutions. Lipase-catalyzed reactions belong to three categories: asymmetric hydrolysis, asymmetric esterification and asymmetric transesterification.

The stereoselectivity is said to be affected by the structure of enzyme, the structure of substrate, the reaction conditions such as the presence of a co-solvent and an immobilization of the enzyme.
The use of organic media in biocatalytic transformations has many advantages such as easy work-up due to low boiling point, increased solubility of substrate and prevention of microbial contaminations. However, the most important advantage of using organic media is the possibility to change the properties of enzymes as regards chemo-, regio- and enantioselectivity due to the change of the rigidity of the enzyme conformation by influencing the formation of hydrogen

bonds in organic media or shifting thermodynamic equilibria. As a consequence, the outcome of an enzyme-catalyzed reaction may be controlled by choosing the appropriate organic solvent. This technique has been commonly denoted as “medium engineering”. The influence of organic solvents on enzyme enantioselectivity has also been observed with lipases as a general phenomenon (*Faber et al., 1993*). In those cases, the stereochemical preference of an enzyme for one specific enantiomer usually remains the same, although its selectivity may vary significantly depending on the solvent.

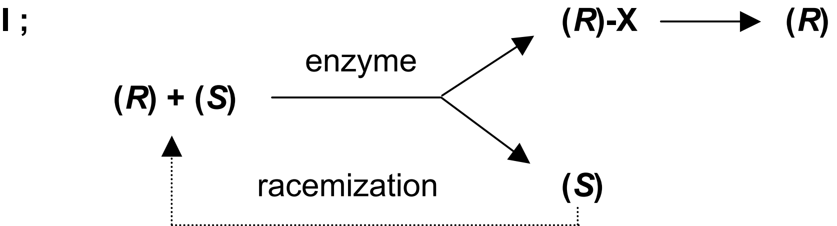
Experimentally determined stereoselectivities of microbial lipases for secondary alcohols were analyzed and simplified as empirical rules. The simplest model to predict stereoselectivity distinguishes between a fast and slow reacting enantiomer of a secondary alcohol substrate by simply comparing the relative sizes of the substituents at the stereocenter (*Kazlauskas et al., 1991; Cygler et al., 1994*).

The production of optically pure (enriched) compounds by kinetic resolution of a racemic mixture is one of the most exciting applications of lipases. These processes can be represented in a simplified diagram as shown in Figure 2.3.10. The residual enantiomer can be racemized chemically and recycled for industrial use.

A variety of applications of such lipase-catalyzed kinetic resolutions has been reported (*Koskinen and Klibanov, 1996; Faber, 2000*).

Lipase-catalyzed thiotransesterifications resulting in thioesters have been reported (*Zaks and Klibanov, 1985; Sproull et al., 1997; Caussette et al., 1997; Weber et al., 1999*) but no kinetic resolutions are described. Apart from a first communication on the lipase-catalyzed hydrolysis of 3-acetylthiocycloheptene (*Iriuchijima and Kojima, 1981*), the exploitation of the stereoselectivity of enzyme-catalyzed reactions of sulfur-containing esters started rather late (*Bianchi and Cesti, 1990; Baba et al., 1990*). In the meantime various approaches have been described showing the transesterification between

I ;



II ;

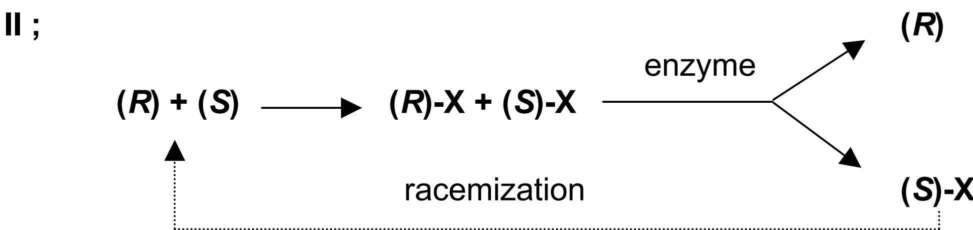


Figure 2.3.10 Enzyme-assisted enantiomeric discrimination (exemplarily, the course of the reactions based on a preference of the (*R*)-enantiomer is shown)

S-ethyl thiooctanoate and secondary alcohols (*Frykman et al., 1993*), the transesterification between several thiols and secondary alcohols (*Öhrner et al., 1996*) and hydrolysis of thioesters (*Izawa et al., 1997*). Other reports focus on the enzymatic resolution of 2-arylpropionates, an important class of non-steroidal anti-inflammatory drugs (*Um and Drueckhammer, 1998; Chang et al., 1998; Chen et al., 2002*).

Considering the importance of sulfur-containing compounds in flavor chemistry, it is not surprising that enzyme-catalyzed reactions have also been proposed as strategies to obtain flavoring compounds. Lipase-catalyzed syntheses of short chain thioesters (*Cavaille-Lefebvre and Combes, 1997; Cavaille-Lefebvre et al., 1998*) as well as hydrolyses of thioesters (*Bel Rhlid et al., 2001; Bel Rhlid et al., 2002*) have been described as useful approaches. Just recently, the potential to use lipases and an esterase for the generation of thiol flavor compounds, such as furfurylthiol, 2-methyl-3-mercaptofuran and 3-mercaptohexanal by hydrolysis of the corresponding thioesters has been reported (*Bel Rhlid et al., 2003*) (Figure

2.3.11). However, the stereochemical course of the reactions had not been followed.

lipase (from *Candida rugosa*, porcine pancreas)
esterase (from porcine liver)

furfurylthiol

2-methyl-3-mercaptofuran

3-mercaptohexanal

Figure 2.3.11 Enzymatic generation of thiols from thioesters

3. Materials and Methods

3.1. Materials

3.1.1. Chemicals

Acetone	Riedel de Haën 24201
N-Acetyl-L-cysteine	Fluka 01039
Benzaldehyde	Fluka 12010
S-Benzylcysteine (97 %)	Aldrich B1980-0
Benzylmercaptane	Fluka 13540
Boc-L-cysteine	Fluka 15411
1-Bromoheptane	Aldrich B6757-0
$CDCl_3$	Merck 102450
2-Chlorocyclohexanol	Aldrich C3240-2
p-Chloromercuribenzoic acid	Fluka 25010
2-Cyclohexenone	Fluka 29255
Cyclohexylaldehyde	Aldrich 10846-4
Cyclohexylmethylbromide	Fluka 17612
D-Cysteine (~99 %)	Fluka 30095
L-Cysteine (~99 %)	Fluka 30090
D_2O	Merck 113366
Dichloromethane	Riedel de Haën 24233
Diethylether (distilled prior to use)	Condea Chemie 33/1155
Furfural	Fluka 48070
Glutathione	Fluka 49750
HCl 4 M in 1,4-dioxane	Aldrich 34554-7
Heptanal	Fluka 75170
3-Hepten-2-one	Avocado Res. Chem. 19086
E-2-Hexenal	Fluka 53000
DL-Homocysteine	Fluka 53510
(-)-Linalool	Fluka 62139
8-Mercapto-*p*-menthan-3-one (*cis* and *trans*)	Aldrich W311770-5
Mesityl oxide	Aldrich 28183-2
$[D_4]$-Methanol	Merck 106028
N-Methyl-*N*-trimethylsilyltrifluoracetamide	Fluka 69482
3-Nonen-2-one	Aldrich 26253-6
3-Octen-2-one	ABCR AV 14652

3-Penten-2-one	Fluka 77042
Potassium hydrogencarbonate	Fluka 60339
(*R*)-(+)-Pulegone (>95 %)	Fluka 82570
(*S*)-(-)-Pulegone (~99 %)	Fluka 82579
Pyridoxal-5'-phosphate	Fluka 82870
Sodium borohydride	Merck 806372
Sodium hydrogensulfite monohydrate	Riedel de Haën 13590
Sodium sulfate	Merck 106649
Thioacetic acid	Fluka 88620
Thiobenzoic acid	Fluka 88500

3.1.2. Enzymes and enzyme preparations

Acylase I	Fluka 01824
L-Methionine γ-lyase	Sigma M4545
Tryptophanase	Sigma T0754
Esterase from porcine liver (PLE)	Sigma E3019
Lipases from	
Aspergillus niger (abbreviation: ANL)	Fluka 62301
Aspergillus oryzae (AOL)	Fluka 95184
Candida antarctica (CAL)	Fluka 62299
Candida antarctica lipase A (CAL-A)	Fluka 62287
Candida antarctica lipase B (CAL-B)	Fluka 62288
Candida antarctica lipase B (CAL-B resin)	Sigma L4777
Candida rugosa (CRL)	Sigma L1754
Mucor javanicus (MJL)	Sigma L8906
Mucor miehei (MML)	Sigma L9031
Penicillium roqueforti (PRL)	Fluka 62308
porcine pancreas (PPL)	Sigma L3126
Pseudomonas cepacia (PCL)	Sigma L9156
Rhizopus oryzae (ROL)	Fluka 80612
Thermomyces lanuginosus (TLL)	Sigma L0902
wheat germ (WGL)	Sigma L3001
E. coli liophilised cell	Sigma EC-9637
Pig kidney acetone powder	Sigma K7250
Rat liver acetone powder	Sigma L1380
Yeast enzyme concentrate	Sigma Y2875

3.1.3. Microorganisms

Eubacterium limosum (ATCC 10825) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany. Fresh baker's yeast (F.X. Wieninger Hefefabrik, Germany and 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 & Co., Germany. Two fresh beer yeasts (No. 34/70 and 184) were supplied by Hefebank Weihenstephan, Germany.

3.1.4. Plants

Blackcurrant (*Ribes Nigrum* L. cultivar Ben Sarek) and box tree (*Buxus sempervirens* L. var. arborescens) were purchased in a local market and their young intact leaves were used. Yellow passion fruits (*Passiflora edulis* f. *flavicarpa*) of Colombia origin were purchased in a local market. Some of the unripe fruits were used immediately, others were kept at room temperature for 3 weeks for ripening before they were used as enzyme source. In addition, yellow passion fruits plants were grown from the seeds taken from ripened fruits and their young intact leaves were used.

3.2. Syntheses

3.2.1. Cysteine, homocysteine and glutathione conjugates

Cysteine conjugates of pulegone

The synthesis was performed according to a previously reported method (*Kerkenaar et al., 1988*). L-Cysteine (1.21 g, 10.0 mmol) and potassium hydrogencarbonate (0.2 g, 2.0 mmol) were dissolved in 10 mL of distilled water. (*S*)-Pulegone (1.64 mL, 10.0 mmol) was added at room temperature (25 °C). The reaction mixture was continuously stirred for 4 days. The generated white precipitate was isolated by filtration, washed with 20 mL of water and 20 mL of acetone, and dried under vacuum. The purities were determined by GC analysis after trimethylsilylation, using *S*-benzylcysteine as internal standard. 1.83 g (6.70 mmol) of a white powder was obtained (mol yield from (*S*)-pulegone: 67 %; purity: 91 %). The L-cysteine conjugate of (*R*)-pulegone was synthesized

according to the same procedure; 1.61 g (5.90 mmol) of conjugate was obtained (mol yield from (*R*)-pulegone: 59 %; purity: 68 %).

For further purification of the diastereomeric mixtures, the precipitates (0.2 g) were dissolved in 3 mL of distilled water. Acetone (6 mL) was added subsequently to the solutions. After refrigeration, white precipitates (ca 0.1 g) were obtained which were filtrated and dried under vacuum. Final purities were as follows; (*S*)-pulegone conjugate: purity determined by GC (system II) after trimethylsilylation: 99.5 %; purity based on comparison to *S*-benzylcysteine: 93.8 %; (*R*)-pulegone conjugate: purity determined by GC (system II) after trimethylsilylation: 95.3 %; purity based on comparison to *S*-benzylcysteine: 82.6 %.

D-Cysteine conjugates were synthesized in the same way. Yields and purities were comparable to those obtained for the L-cysteine conjugates. Mol yield from (*S*)-pulegone: 55 %; purity determined by GC (system II) after trimethylsilylation: 67.5 %; purity based on comparison to *S*-benzylcysteine: 58.6 %; mol yield from (*R*)-pulegone: 70 %; purity determined by GC (system II) after trimethylsilylation: 90.7 %; purity based on comparison to *S*-benzylcysteine: 89.0 %.

Homocysteine conjugate of pulegone

DL-Homocysteine (0.99 g, 7.3 mmol) and potassium hydrogencarbonate (0.2 g, 2.0 mmol) were dissolved in 10 mL of distilled water. (*R*)-Pulegone (1.2 mL, 7.4 mmol, dissolved in 1 mL methanol) was added at room temperature (25 °C). The reaction mixture was continuously stirred under argon atmosphere for 3 days. The generated white precipitate was isolated by filtration, washed with 10 mL of water and 20 mL of acetone, and dried under vacuum. The purity was determined by GC analysis after trimethylsilylation using *S*-benzylcysteine as internal standard.

Yield: 1.1 g (3.9 mmol); mol yield from cysteine: 53 %; purity determined by GC (system II) after trimethylsilylation: 85.2 %; purity based on comparison to *S*-benzylcysteine: 67.0 %.

GC retention indices (SE-54) of TMS derivatives (relative amounts are given in parentheses): 2354 (31 %), 2360 (25 %), 2373 (24 %), 2379 (20 %). GC-MS of TMS derivative, *m/z* (relative intensity): 73 (51), 218 (100), 219 (19), 278 (33),

314 (6), 431 ($M^{+\cdot}$; 6). The mass fragment patterns were virtually identical for the four peaks.

Glutathione conjugate of pulegone

Glutathione (1.54 g, 5.0 mmol) and potassium hydrogencarbonate (0.7 g, 7.0 mmol) were dissolved in 5 mL of distilled water. (*R*)-Pulegone (0.81 mL, 5.0 mmol, dissolved in 0.8 mL methanol) was added at room temperature (25 °C). The reaction mixture was continuously stirred under argon atmosphere for 4 days. After adjusting the pH to 5 using 5 % hydrochloric acid, the reaction mixture was loaded on a Dowex 50 cation exchange resin column (ϕ 10mm× 250mm; H^+-form; 100/200 mesh). The column was washed by 100 mL of water and eluted by 0.1 N aqueous ammonia. The fractions exhibiting ninhydrin activity were collected and concentrated under reduced pressure (40 °C, aspirator). Acetone was added gently as upper layer on the residual stiky syrup (ca. 0.3 mL) and stored in the refrigerator. After 7 days, the product was dehydrated and changed to white solid. Then the product was dried under vacuum after removing acetone by decantation.

Yield: 0.22 g (0.48 mmol); mol yield from glutathione: 9.6 %; purity from LC-UV peak area: 53 % (total of two peaks).

2-(2-S-L-Cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid

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
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

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. 79 mg (0.3 mmol) of transparent sticky liquid was obtained (mol yield 75 %; purity determined by GC (system II) after trimethylsilylation: 52.0 %).

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). ^1H 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-Cysteinyl-1-hexanol

N-tert-Butyloxycarbonyl-L-cysteine (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 with hexane (two times, 10 mL) and dried under reduced pressure. 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. 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 diethylether and dried under reduced pressure. 136 mg (0.61 mmol) of a transparent, sticky liquid was obtained (mol yield through from Boc-L-cysteine: 26 %; purity determined by GC (system II) after trimethylsilylation: 85.8 %; purity based on comparison to *S*-benzylcysteine: 53.3 %).

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).

4-S-L-Cysteinyl-2-pentanone

L-Cysteine (4.9 g, 40 mmol) and potassium hydrogencarbonate (0.6 g, 6 mmol) were dissolved in 30 mL of distilled water. 3-Penten-2-one (4.0 mL, 40 mmol) dissolved in 4 mL methanol was added under ice-cooling. The reaction mixture was continuously stirred under argon atmosphere for 4 days at room temperature (25 °C). The reaction mixture was concentrated to 10 mL under reduced pressure (40 °C, aspirator). 20 mL of acetone were added to reaction mixture and after 1 day of refrigeration the generated pale yellowish gel was isolated by filtration, washed with 40 mL of acetone, and dried under vacuum. For further purification, the precipitate was redissolved in 7 mL of water and the insoluble residue was filtered off. Addition of 50 mL of acetone to the solution resulted in the precipitation of a pale yellowish product after 2 days refrigeration which was isolated by filtration, washed by 30 mL of acetone and dried under vacuum.

4-S-L-Cysteinyl-2-heptanone, 4-S-L-cysteinyl-2-octanone and 4-S-L-cysteinyl-2-nonanone

L-Cysteine (1.21 g, 10.0 mmol) and potassium hydrogencarbonate (0.2 g, 2.0 mmol) were dissolved in 10 mL of distilled water, 3-hepten-2-one (1.56 mL, 12.0 mmol), 3-octen-2-one (1.78 mL, 12.0 mmol) and 3-nonen-2-one (1.98 mL, 12.0 mmol), respectively, were added at room temperature (25 °C). The reaction mixture was continuously stirred under argon atmosphere for 1 day (3-octen-2-one and 3-nonen-2-one) or 2 days (3-hepten-2-one).

In the case of 3-octen-2-one and 3-nonen-2-one, 10 mL of water and 20 mL of acetone were added to reaction mixture and after 1 h the generated white precipitate was isolated by filtration, washed with 40 mL of acetone, and dried under vacuum.

In the case of 3-hepten-2-one, after the pH of reaction mixture was adjusted to 6 by 5 % HCl aq,10 mL of ether and 50 mL of acetone were added to the reaction mixture and after 1 day refrigeration, the generated white gel was isolated by filtration, washed with 40 mL of acetone, and dried under vacuum.

The purities were determined by GC analysis after trimethylsilylation using *S*-benzylcysteine as internal standard.

Yields and analytical data:

4-S-L-Cysteinyl-2-pentanone

2.8 g (13.5 mmol); mol yield from cysteine: 33.5 %; purity determined by GC (system II) after trimethylsilylation: 19.8 %; purity based on comparison to *S*-benzylcysteine: 15.6 %. GC retention index (SE-54) of TMS derivative: 1819. GC-MS of TMS derivative, *m/z* (relative intensity): 43 (27), 73 (89), 100 (33), 147 (20), 148 (23), 157 (23), 218 (100), 219 (31), 232 (64), 306 (2), 334 (0.6), 349 ($M^{+\cdot}$; 0.3).

4-S-L-Cysteinyl-2-heptanone

0.90 g (3.87 mmol); mol yield from cysteine: 39 %; purity determined by GC (system II) after trimethylsilylation: 41.4 %; purity based on comparison to *S*-benzylcysteine: 20.1 %. GC retention index (SE-54) of TMS derivative: 1947. GC-MS of TMS derivative, *m/z* (relative intensity): 43 (19), 73 (55), 100 (17), 148

(14), 185 (11), 218 (100), 219 (20), 260 (34), 304 (0.2), 334 (0.6), 362 (0.4), 377 ($M^{+\cdot}$; 0.1).

4-S-L-Cysteinyl-2-octanone

0.44 g (1.79 mmol); mol yield from cysteine: 18 %; purity determined by GC (system II) after trimethylsilylation: 59.1 %; purity based on comparison to *S*-benzylcysteine: 44.0 %. GC retention index (SE-54) of TMS derivative: 2028. GC-MS of TMS derivative, *m/z* (relative intensity): 43 (23), 73 (64), 100 (19), 148 (16), 199 (11), 218 (100), 219 (25), 274 (40), 318 (0.3), 348 (0.7), 376 (0.5), 391 ($M^{+\cdot}$; 0.2).

4-S-L-Cysteinyl-2-nonanone

2.36 g (9.03 mmol); mol yield from cysteine: 90 %; purity determined by GC (system II) after trimethylsilylation: 43.5 %; purity based on comparison to *S*-benzylcysteine: 18.2 %. GC retention index (SE-54) of TMS derivative: 2110. GC-MS of TMS derivative, *m/z* (relative intensity): 43 (18), 73 (55), 100 (15), 148 (13), 213 (8), 218 (100), 219 (21), 288 (32), 332 (0.3), 362 (0.5), 387 (1), 390 (0.5), 405 ($M^{+\cdot}$; 0.1).

3-S-L-Cysteinylcyclohexanone

L-Cysteine (6.3 g, 52 mmol) and potassium hydrogencarbonate (1.0 g, 10 mmol) were dissolved in 50 mL of distilled water. 2-Cyclohexenone (5 mL, 52 mmol) dissolved in 5 mL methanol was added under ice-cooling. The reaction mixture was continuously stirred under argon atmosphere for 3 days at room temperature (25 °C). The reaction mixture was concentrated to 30 mL under reduced pressure (40 °C, aspirator). 20 mL of acetone were added to reaction mixture and after 1 h the generated white precipitate was isolated by filtration, washed with 40 mL of acetone, and dried under vacuum.

Yield: 2.80 g (12.9 mmol); mol yield from cysteine: 24.9 %; purity determined by GC (system II) after trimethylsilylation: 81.7 %; purity based on comparison to *S*-benzylcysteine: 17.5 %.

GC retention index (SE-54) of TMS derivative: 2135 (83 %), 2171 (17 %). GC-MS of TMS derivative, *m/z* (relative intensity): 45 (11), 73 (84), 100 (9), 147

(10), 169 (100), 170 (42), 201 (8), 218 (42), 233 (26), 328 (1).

3-S-L-Cysteinylcyclohexanol

Synthesized 3-*S*-L-cysteinylcyclohexanone (1.4 g, 6.3 mmol) was dissolved in 10 mL of distilled water. Sodium borohydride (1.3 g, 33 mmol) dissolved in 1 mL of water was added under ice-cooling and the mixture was stirred for 2 h. After adjusting the pH to 3 using 5 % hydrochloric acid, the reaction mixture was loaded on Dowex 50 cation exchange resin column (ϕ 10mm × 250mm; H^+-form; 100/200 mesh). The column was washed with 100 mL of water and eluted by 0.1 N aqueous ammonia. The eluted fraction exhibiting ninhydrin activity was collected and concentrated to 10 mL under reduced pressure (40 °C, aspirator). 40 mL of acetone was added and the generated white precipitate was isolated by filtration, washed with 10 mL of acetone and dried under vacuum. The purity was determined by GC analysis after trimethylsilylation using *S*-benzylcysteine as internal standard.

Yield: 0.40 g (1.8 mmol); mol yield from 3-*S*-L-cysteinylcyclohexanone: 29 %; purity determined by GC (system II) after trimethylsilylation: 50.9 %; purity based on comparison to *S*-benzylcysteine: 15.3 %.

GC retention index (SE-54) of TMS derivative: 2133. GC-MS of TMS derivative, *m/z* (relative intensity): 73 (43), 81 (8), 100 (9), 147 (6), 218 (100), 219 (18), 318 (16), 392 (0.8), 420 (0.6), 435 ($M^{+\cdot}$; 0.1).

2-S-L-Cysteinylcyclohexanol

L-Cysteine (3.69 g, 30.5 mmol) and sodium hydroxide (1.35 g, 33.8 mmol) were dissolved in 30 mL of 60 % aqueous methanol. 2-Chlorocyclohexanol (4.0 mL, 33.6 mmol) dissolved in 4 mL methanol was added under ice-cooling. The reaction mixture was continuously stirred for 2 days at room temperature (25 °C) under argon atmosphere. At the end of reaction the pH of solution was adjusted to 7 by 5 % hydrochloric acid and the aqueous layer was washed by 10 mL of dichloromethane. The aqueous layer was concentrated to 10 mL under reduced pressure (40 °C, aspirator). After filtering off precipitate generated, 40 mL of acetone was added to the reaction mixture. The generated white gel was isolated by filtration, washed with 40 mL of acetone and dried under vacuum.

The purity was determined by GC analysis after trimethylsilylation using *S*-benzylcysteine as internal standard.
Yield: 0.61 g (2.8 mmol); mol yield from cysteine: 9.1 %; purity determined by GC (system II) after trimethylsilylation: 80.8 %; purity based on comparison to *S*-benzylcysteine: 26.6 %.
GC retention index (SE-54) of TMS derivative: 2065. GC-MS of TMS derivative, *m/z* (relative intensity): 73 (79), 100 (16), 146 (22), 147 (12), 201 (13), 218 (100), 219 (28), 318 (28), 420 (1), 435 ($M^{+\cdot}$; 0.1).

4-S-L-Cysteinyl-4-methyl-2-pentanone

L-Cysteine (5.0 g, 41 mmol) and potassium hydrogencarbonate (0.6 g, 6 mmol) were dissolved in 30 mL of distilled water, mesityl oxide (4.7 mL, 41 mmol) dissolved in 4 mL methanol were added under ice-cooling. The reaction mixture was continuously stirred under argon atmosphere for 3 days at room temperature (25 °C). The reaction mixture was concentrated to 10 mL under reduced pressure (40 °C, aspirator). 30 mL of acetone were added to the reaction mixture and after 1 day of refrigeration the generated white gel was isolated by filtration, washed with 40 mL of acetone, and dried under vacuum. For further purification, the precipitate was redissolved in 15 mL of water and the insoluble residue was filtered off. Addition of 200 mL of acetone to the solution resulted in the precipitation of a pale yellowish product after 1 day refrigeration which was isolated by filtration, washed by 50 mL of acetone and dried under vacuum. The purity was determined by GC analysis after trimethylsilylation using *S*-benzylcysteine as internal standard.
Yield: 0.93 g (4.2 mmol); mol yield from cysteine: 10.5 %; purity determined by GC (system II) after trimethylsilylation: 40.3 %; purity based on comparison to *S*-benzylcysteine: 38.1 %.
GC retention index (SE-54) of TMS derivative: 1864. GC-MS of TMS derivative, *m/z* (relative intensity): 43 (27), 73 (54), 100 (17), 148 (23), 218 (100), 219 (18), 246 (26), 290 (0.4), 320 (0.2), 348 (0.2), 363 ($M^{+\cdot}$; 0.1).

6-S-L-Cysteinyl-1-hexanol

L-Cysteine (0.60 g, 5.0 mmol) and sodium hydroxide (0.20 g, 5.0 mmol) were

dissolved in 5 mL of distilled water, 1-bromo-6-hexanol (0.80 mL, 6.1 mmol) dissolved in 5 mL methanol was added under ice-cooling. The reaction mixture was continuously stirred for 1 day at room temperature (25 °C) under argon atmosphere. 10 mL of acetone was added to the reaction mixture and after 4 h the generated white precipitate was isolated by filtration, washed with 10 mL of acetone, and dried under vacuum. The purity was determined by GC analysis after trimethylsilylation using *S*-benzylcysteine as internal standard.

Yield; 0.96 g (4.3 mmol); mol yield from cysteine: 87 %; purity determined by GC (system II) after trimethylsilylation: 94.6 %; purity based on comparison to *S*-benzylcysteine: 97.7 %.

GC retention index (SE-54) of TMS derivative: 2222. GC-MS of TMS derivative, *m/z* (relative intensity): 73 (72), 100 (29), 115 (13), 147 (20), 218 (100), 219 (40), 320 (48), 394 (6), 422 (2), 437 ($M^{+\cdot}$; 0.1).

2-Alkyl (Aryl)-1,3-thiazolidine-4-carboxylic acid

The syntheses were performed according to a previously described procedure (*Huynh-Ba et al., 1998; 2003*). L-Cysteine (2.0 g, 16.5 mmol) was dissolved in 50 mL of 40 % aqueous ethanol. Furfural (1.6 mL, 19.3 mmol), benzaldehyde (2.0 mL, 19.8 mmol), cyclohexylaldehyde (2.4 mL, 19.9 mmol) and heptanal (2.7 mL, 19.3 mmol), respectively, were added. The reaction mixture was stirred for 1 h at room temperature and subsequently for 1 h under ice-cooling. The white precipitates appearing at the end of the reaction were isolated by filtration, washed with 40 mL of ethanol and 10 mL acetone and dried under vacuum. The purity was determined by GC peak area analyzed after trimethylsilylation.

Yields and analytical data:

2-Furyl-1,3-thiazolidine-4-carboxylic acid

2.47 g (12.4 mmol); mol yield from cysteine: 75 %; purity determined by GC (system II) after trimethylsilylation: 94.8 %. GC retention index (SE-54) of TMS derivative: 1943. GC-MS of TMS derivative, *m/z* (relative intensity): 73 (100), 75 (17), 81 (17), 94 (15), 147 (24), 224 (37), 226 (25), 297 (6), 328 (25), 343 ($M^{+\cdot}$; 2). NMR data are shown in Table 3.2.1.

Table 3.2.1 NMR data of 2-furyl-1,3-thiazolidine-4-carboxylic acid

Position	Chemical shift		Coupling constant	Correlation pattern		
	δ ^{1}H, ppm	δ ^{13}C, ppm	J_{HH}, Hz	COSY	HMQC	HMBC
2a	5.82	62.88			2a	4a, 5a', 5a, 9a(W)
4a	4.33	64.67	6.3, 12.7	5a’, 5a	4a	2a, 5a', 5a
5a	3.23	36.94	5.8, 10.5	4a, 5a'	5a	2a, 4a
5a’	3.47	36.94		4a, 5a	5a'	5a, 2a
6a		153.27				2a, 7a, 8a, 9a
7a	6.41	107.62			7a	7a, 9a
8a	6.37	110.36	1.8, 3.1	8a	8a	7a
9a	7.49	143.01	0.8, 1.7	7a	9a	4a, 5a, 5a’
COOH		172.87				
2b	5.65	63.69			2b	4(W), 5b, 5b’
4b	4.01	65.49	7.1, 8.8	5b’, 5b	4b	2b(W), 5b, 5b’
5b	3.19	37.61	8.8, 10.2	4b, 5b’	5b	2b, 4b
5b’	3.18	37.61		4b, 5b	5b’	2b, 4b, 5b
6b	3.47	151.03				2b, 9b
7b	6.46	108.09	3.3		7b	7b, 9b
8b	6.41	110.55		8b	8b	7b, 9b
9b	7.53	143.22	0.8, 1.7	7b	9b	7b
COOH		172.63				4b, 5b, 5b’

2-Phenyl-1,3-thiazolidine-4-carboxylic acid

3.07 g (14.7 mmol); mol yield from cysteine: 89 %; purity determined by GC (system II) after trimethylsilylation: 75.3 %. GC retention index (SE-54) of TMS derivative: 2080. GC-MS of TMS derivative, *m/z* (relative intensity): 45 (13), 73 (100), 91 (18), 104 (11), 147 (23), 178 (12), 190 (10), 234 (20), 236 (25), 280 (9), 338 (11), 353 ($M^{+\cdot}$; 0.3). NMR data are shown in Table 3.2.2.

2-Cyclohexyl-1,3-thiazolidine-4-carboxylic acid

3.40 g (15.8 mmol); mol yield from cysteine: 96 %; purity determined by GC (system II) after trimethylsilylation: 95.1 %. GC retention index (SE-54) of TMS derivative: 2019. GC-MS of TMS derivative, *m/z* (relative intensity): 73 (100), 95 (29), 116 (30), 122 (22), 147 (21), 174 (28), 240 (47), 242 (38), 248 (19), 291 (12), 344 (8), 359 ($M^{+\cdot}$; 0.4). NMR data are shown in Table 3.2.3.

Table 3.2.2 NMR data of 2-phenyl-1,3-thiazolidine-4-carboxylic acid

Position	Chemical shift		Coupling constant	Correlation pattern		
	δ ^{1}H, ppm	δ ^{13}C, ppm	J_{HH}, Hz	COSY	HMQC	HMBC
2a	5.78	69.87			2a	4a, 5a, 7-10a
4a	4.44	67.76	4.7, 7.2	5a’, 5a	4a	2a, 5a, 5a', 7-10a
5a	3.43	32.82	4.7, 10.8	4a, 5a'	5a	2a, 4a, 7-10a
5a’	3.54	32.82		4a, 5a	5a'	2a, 4a, 7-10a
6a	7.59	138.2			6a	2a, 4a, 5a, 5a'
7-10a	7.59	127.7-128.7			7-10a	2a, 4a, 5a, 5a'
COOH		172.65				
2b	5.61	70.97			2b	4b, 5b, 5b’, 7-10b
4b	4.12	65.40	7.5, 14.9	5b’, 5b	4b	2b, 5b, 5b’, 7-10b
5b	3.43	37.50	4.7, 10.8	4b, 5b’	5b	2b, 4b, 7-10b
5b’	3.54	37.50		4b, 5b	5b’	2b, 4b, 7-10b
6b	7.59	138.2			6b	2b, 4b, 5b, 5b’
7-10b	7.40	127.7-129.8			7-10b	2b, 4b, 5b, 5b’
COOH		172.72				

Table 3.2.3 NMR data of 2-cyclohexyl-1,3-thiazolidine-4-carboxylic acid

Position	Chemical shift		Coupling constant	Correlation pattern		
	δ ^{1}H, ppm	δ ^{13}C, ppm	J_{HH}, Hz	COSY	HMQC	HMBC
2a	4.61	71.91	8.3	6a	2a	4a, 5a, 10-11a
4a	4.32	64.90	5.9	5a	4a	2a, 5a
5a	3.33	33.37	1.7, 3.3	4a	5a	2a, 4a
5a’	3.33	33.37	1.7, 3.3	4a		2a, 4a
6a	1.82	25.50		2a, 7-8a	6a	7-8a, 10-11a
7-8a	1.33	25.70		6a	7-8a	6a
9a	2.00	30.81		10-11a	9a	2a
10-11a	1.18	30.95		6a, 9a	10-11a	2a, 6a, 7-8a
COOH		171.40				
2b	4.42	72.38	8.5	6b	2b	4b, 5b, 5b’, 10-11b
4b	4.09	65.56	7.5, 15.3	5b’, 5b	4b	5b, 5b’, 10-11b
5b	3.14	33.78	7.7, 11.0	4b, 5b’	5b	2b, 6b, 7-8b
5b’	3.38	33.87	7.1, 11.0	4b, 5b	5b’	2b, 6b, 10-11b
6b	1.74	31.00		2b, 10-11b	6b	2b, 7-8b, 10-11b
7-8b	1.33	25.70			7-8b	6b
9b	1.74	31.00		2b, 10-11b	9b	2b
10-11b	1.18	30.95		6b, 9b	10-11b	2b, 6b, 7-8b
COOH		171.55				

2-n-Hexyl-1,3-thiazolidine-4-carboxylic acid

2.99 g (13.8 mmol); mol yield from cysteine: 83 %; purity determined by GC (system II) after trimethylsilylation: 74.3 %. GC retention index (SE-54) of TMS derivative: 1932 (67 %), 1962 (33 %). GC-MS of TMS derivative, *m/z* (relative intensity): 73 (78), 86 (20), 147 (11), 184 (5), 244 (82), 276 (100), 300 (2), 318 (2), 346 (1), 361 ($M^{+\cdot}$; 0.2). NMR data are shown in Table 3.2.4.

Table 3.2.4 NMR data of 2-*n*-hexyl-1,3-thiazolidine-4-carboxylic acid

Position	Chemical shift		Coupling constant	Correlation pattern		
	δ ^{1}H, ppm	δ ^{13}C, ppm	J_{HH}, Hz	COSY	HMQC	HMBC
2a	4.84	65.69		6a, 6a'	2a	4a, 5a, 5a'
4a	4.43	64.19	5.9, 14.0	5a, 5a'	4a	5a, 5a'
5a	3.50	32.20		4a	5a	4a, 7-9a, 11a
5a’	3.35	32.20		4a	5a'	4a, 7-9a, 11a
6a	2.14	33.50		6a'		4a, 11a
6a'	1.91	33.50		6a		
7-9a	1.30	22.05-31.82		11	7-9a	11a
11a	0.89	13.62	6.9, 14.0	7-9a	11	7-9a
COOH		169.60				
2b	4.74	66.27	5.5, 8.6	6b’	2b	
4b	4.33	64.68	6.9, 7.2, 14.1	5b’, 5b	4b	5b, 5b’
5b	3.49	33.18		4b	5b	4b, 7-9b, 11b
5b’	3.37	33.18		4b	5b’	4b, 7-9b, 11b
6b	2.14	33.50		6b’	6b	4b, 7-9b
6b’	1.83	33.50		6b	6b’	7-9b
7-9b	1.30	22.05-31.82		11	7-9b	11b
11b	0.89	13.40		7-9b		7-9b
COOH		171.56				

S-Alkyl-L-cysteine derivatives

S-Alkyl-L-cysteine derivatives were synthesized according to the general method reported previously (*Vince and Wadd, 1969*). Cysteine was used as nucleophile instead of glutathione and alkylbromide or alkylchloride were used as alkylhalide instead of alkyliodide.

S-n-Heptyl-L-cysteine

L-Cysteine (2.0 g, 16.5 mmol) and sodium hydroxide (0.66 g, 16.5 mmol) were dissolved in 30 mL of 40 % aquaous methanol. Heptylbromide (3.0 mL, 19.1 mmol) dissolved in 5 mL methanol was added under ice-cooling. The reaction mixture was continuously stirred for 2 days under argon atmosphere. The generated white precipitate was isolated by filtration, washed with 20 mL of water and 40 mL of acetone, and dried under vacuum. The purity was determined by GC analysis after trimethylsilylation.

Yield: 2.75 g (12.5 mmol); mol yield from cysteine: 76 %; purity determined by GC (system II) after trimethylsilylation: 92.0 %. GC retention index (SE-54) of TMS derivative: 1948. GC-MS of TMS derivative, *m/z* (relative intensity): 73 (87), 100 (35), 147 (22), 218 (100), 219 (49), 246 (82), 320 (11), 348 (2), 363 ($M^{+\cdot}$; 0.1), 364 ($MH^{+\cdot}$; 0.2). NMR data are shown in Table 3.2.5.

Table 3.2.5 NMR data of *S-n*-heptyl-L-cysteine

Position	Chemical shift		Coupling constant	Correlation pattern		
	δ ^{1}H, ppm	δ ^{13}C, ppm	J_{HH}, Hz	COSY	HMQC	HMBC
2	3.60	54.41	3.9, 8.8	3, 3’	2	3, 8
3’	3.12	34.39	3.8, 14.4	2, 3	3’	
3	2.85	34.13	8.8, 14.3	3'		6, 9, 10
4	3.46	33.37	6.8, 13.5	5	4	5
5	1.86	33.03	6.7, 21.7	4, 6	5	4
6	1.44	22.65	7.5, 25.1	5	6	
7	1.63	29.58		8	7	
8	2.85	31.68	7.4, 14.8	7	8	9
9	1.34	30.93		10	9	3, 10
10	0.936	13.80		9	10	3, 9
COOH		173.60				

S-Cyclohexylmethyl-L-cysteine

L-Cysteine (2.0 g, 16.5 mmol) and sodium hydroxide (0.66 g, 16.5 mmol) were dissolved in 30 mL of 40 % aquaous methanol. Cyclohexylmethylbromide (2.7 mL, 19.5 mmol) dissolved in 5 mL methanol was added under ice-cooling. The reaction mixture was continuously stirred for 4 days under argon

atmosphere. At the end of reaction a white creamy layer appeared. The upper aqueous layer was removed by decantation, the creamy layer was washed with 10 mL of water, and 30 mL of acetone were added. The generated white precipitate was isolated by filtration, washed with 40 mL of acetone and dried under vacuum. The purity was determined by GC analysis after trimethylsilylation.

Yield: 0.74 g (3.42 mmol); mol yield from cysteine: 21 %; purity determined by GC (system II) after trimethylsilylation: 42.8 %. GC retention index (SE-54) of TMS derivative: 1987. GC-MS of TMS derivative, *m/z* (relative intensity): 73 (70), 100 (23), 147 (13), 218 (100), 219 (33), 244 (59), 318 (6), 346 (1), 361 ($M^{+\cdot}$; 0.1). NMR data are shown in Table 3.2.6.

Table 3.2.6 NMR data of *S*-cyclohexylmethyl-L-cysteine

Position	Chemical shift		Coupling constant	Correlation pattern		
	δ ^{1}H, ppm	δ ^{13}C, ppm	J_{HH}, Hz	COSY	HMQC	HMBC
2	3.57	54.59	3.78, 8.83	3, 3'	2	3’, 4
3	2.83	35.20	8.83, 14.34	2, 3'	3	3', 6
3’	3.09	35.14	3.94, 14.19	2, 3	3’	4
4	2.50	39.21	1.39, 6.94	5	4	3, 3’
5	1.51	31.81		4	5	9
6	1.75	26.29		9	6	4, 5
7	1.68	29.50		9	7	4, 2
8	1.90	32.88		10	8	5
9	1.29	29.74		6, 7	9	5
10	0.99	32.92		8	10	2, 4, 7
COOH		175.48				

3.2.2. Thioesters

Thioesters were synthesized by Michael-type addition of thiocarboxylic acid to α,β-unsaturated carbonyls (*Stoffelsma and Pypker, 1977; Annunziata et al., 1992*).

3-Acetylthiohexanal

A mixture of *E*-2-hexenal (2.3 mL, 20 mmol) and thioacetic acid (2.1 mL, 30 mmol) was stirred for 2 h under ice-cooling and for another 24 h at room temperature (25 °C). After removing the excess of thioacetic acid under reduced pressure at 40 °C, 3.48 g (20 mmol) of a pale yellow, sticky liquid was obtained (mol yield from *E*-2-hexenal: 100 %; purity: 95 % by GC). GC retention indices: DB-1: 1231, SE-54: 1266, DB-WAX: 1845. GC-MS (*m/z* (relative intensity)): 43 (100), 131 (18), 41 (18), 55 (15), 99 (10), 103 (9), 89 (7), 70 (7), 69 (7), 114 (4), 174 (M+; 2). ^{1}H NMR (500 MHz, $CDCl_3$): δ, ppm; 0.89 (3H, t, 7.2 Hz, H-6), 1.37 (4H, m, H-4, H-5), 2.30 (3H, s, CH_3-CO), 2.69 (2H, m, H-2), 3.91 (1H, qui, 6.8 Hz, H-3), 9.67 (1H, t, 1.8 Hz, H-1). ^{13}C NMR (125.6 MHz): δ, ppm; 200.5 (*C*O-CH_3), 195.6 (C-1), 49.2 (C-2), 38.7 (C-3), 36.9 (C-4), 32.9 (CO-*C*H_3), 20.5 (C-5), 14.9 (C-6).

3-Benzoylthiohexanal

A mixture of *E*-2-hexenal (2.3 mL, 20 mmol) and thiobenzoic acid (3.5 mL, 30 mmol) was stirred for 2 h under ice-cooling and for another 24 h at room temperature (25 °C). After dissolving the reaction mixture in 20 mL of dichloromethane, the solution was washed with 10 mL of 0.1 M sodium phosphate buffer (pH 8.5) and two times with 10 mL of distilled water. After drying over anhydrous sodium sulfate, dichloromethane was removed under reduced pressure at 40 °C. 4.96 g (21 mmol) of a yellowish, sticky liquid was obtained (mol yield from *E*-2-hexenal: 105 %; purity: 79 % by GC). GC retention indices: DB-1: 1835, SE-54: 1860. GC-MS (*m/z* (relative intensity)): 105 (100), 77 (30), 51 (10), 106 (8) 139 (5), 41 (4) 208 (1), 131 (1), 114 (1), 236 (M+; 1). ^{1}H NMR (500 MHz, $CDCl_3$): δ, ppm; 0.93 (3H, t, 7.2 Hz, H-6), 1.45 (2H, m, H-5), 1.72 (2H, qua, 7.5 Hz, H-4), 2.81 (2H, m, H-2), 4.16 (1H, qui, 6.8 Hz, H-3), 7.42 (2H, m, *m*-Ph), 7.55 (1H, m, *p*-Ph), 7.92 (2H, m, *o*-Ph), 9.74 (1H, t, 1.8 Hz, H-1). ^{13}C NMR (125.6 MHz): δ, ppm; 200.2 (*C*O-Ph), 191.3 (C-1), 136.8, 133.5, 128.6, 127.2 (Ph), 49.0 (C-2), 38.3 (C-3), 36.6 (C-4), 20.3 (C-5), 13.7 (C-6).

3-Acetylthiohexanol

A solution of 3-acetylthiohexanal (112 mg, 0.64 mmol) in 5 mL of methanol was

added to 20 mL of 0.5 M potassium phosphate buffer (pH 7.4). After dropwise addition of sodium borohydride (50 mg, 1.3 mmol dissolved in 2 mL of water) to the stirred solution under ice-cooling, the solution was stirred for another 30 min. The pH was adjusted to 5 using 2 N sulfonic acid, and the solution was extracted with dichloromethane (two times 10 mL). After washing (10 mL of distilled water) and drying (anhydrous sodium sulfate) of the combined extracts, dichloromethane was removed under reduced pressure at 40 °C. 97.3 mg (0.55 mmol) of a transparent liquid was obtained (mol yield from 3-acetylthiohexanal: 86 %; purity: 95 % by GC). GC retention indices: DB-1: 1293, DB-WAX: 2090. GC-MS (*m/z* (relative intensity)): 43 (100), 55 (52), 88 (27), 41 (25), 83 (19), 82 (18), 116 (15), 133 (7), 101 (7), 176 (M+; 1). ^{1}H NMR (500 MHz, $CDCl_3$): δ, ppm; 0.89 (3H, t, 7.3 Hz, H-6), 1.5-1.4 (4H, m, H-4, H-5), 2.34 (3H, s, CH_3-CO), 1.98 (2H, m, H-2), 3.62 (1H, nd, H-3), 3.62 (1H, nd, H-1). ^{13}C NMR (125.6 MHz): δ, ppm; 198.6 (*C*O-CH_3), 60.2 (C-1), 41.6 (C-2), 39.2 (C-3), 37.6 (C-4), 31.1 (CO-*C*H_3), 20.6 (C-5), 14.1 (C-6).

3.2.3. Thiols

8-Mercapto-p-menthan-3-one

Sodium hydrogen sulfide monohydrate (1.43 g, 19.3 mmol) was dissolved in 10 mL of distilled water. 0.1 mL (0.616 mmol) of (*S*)-pulegone and (*R*)-pulegone, respectively, were added. After stirring the solution at room temperature for 2 days, the pH was adjusted to 6 using 5 % hydrochloric acid. Excess hydrogen sulfide was purged off and the product was extracted with 10 mL of dichloromethane. The separated dichloromethane layer was dried over anhydrous sodium sulfate and used for GC analysis.

3-Mercaptohexanal

3-Acetylthiohexanal (113 mg, 0.65 mmol) dissolved in 1 mL of methanol was added to 10 mL of 0.5 N sodium hydroxide aqueous solution and the mixture was stirred for 30 min under ice-cooling. The pH was adjusted to 2 using 2 N sulfonic acid, and the solution was extracted with dichloromethane (two times 10 mL). After washing (two times 10 mL of distilled water) and drying (anhydrous

sodium sulfate), the dichloromethane solution was subjected to GC and GC/MS analysis. GC retention indices: DB-1: 1002, SE-54: 1032, DB-WAX: 1359. GC-MS (*m/z* (relative intensity)); 55(100), 41(70), 42(62), 70(40), 61(34), 81(25), 57(24), 43(23), 80(22), 114(16), 99(16), 132(M+; 17).

3.3. Culturing and preparation of the crude enzyme extract

3.3.1. Extract from *Eubacterium limosum*

E. limosum (ATCC 10825) was cultured at 37 °C under anaerobic conditions in 1 L of a medium prepared as previously described (*Kerkenaar et al., 1988*). After 48 h, cells were harvested by centrifugation and washed twice with 600 mL of 50 mM potassium phosphate buffer (pH 7.4) containing 100 μM pyridoxal 5'-phosphate. The wet weight yield was about 2 g of cells per liter of culture medium. Extraction of the crude enzyme was carried out according to a previously described procedure (*Tomisawa et al., 1984*). Cells were suspended in 10 mL of 50 mM potassium phosphate buffer (pH 7.4) containing 100 μM pyridoxal 5'-phosphate. The suspension was sonicated for 2 min and centrifuged at 10,000 x g for 30 min. The supernatant was separated, freeze-dried and used as crude enzyme extract.

3.3.2. Preparation of acetone powders

Fresh fruits or leaves were crushed in liquid nitrogen and homogenized with cold acetone (4 to 6 vol., v/w at –20 °C) using a Warring blendor for 1 min in the ice bath. The slurry was filtered and the residue was further homogenized with the same volume of cold acetone four times. The powder obtained after removal of acetone from the final powder in a vacuum dessicator was stored at –20 °C and used as enzyme source (*Mazelis and Creveling, 1975; de los Angels Serradell et al., 2000*). The yields obtained from the various plant materials are listed in Table 3.3.1.

Table 3.3.1 Yields of acetone powders obtained from various plant materials

	starting material (g)	acetone powder (mg)
onion	32	880
blackcurrant (leaf)	23	2920
box tree (leaf)	12	3630
passion fruits (ripened)	19	660
passion fruits (unripened)	7	230
passion fruits (internal rind layer)	15	620
passion fruits (leaf)	7	730

3.4. Enzymatic reactions

3.4.1. C-S lyases

Stability of cysteine conjugates

A mixture (2500 nmol) consisting of equal amounts of purified cysteine conjugates of (*R*)- and (*S*)-pulegone was suspended or dissolved in 250 µL of acetonitrile, methanol, distilled water and 50 mM potassium phosphate buffer (pH 7.4), respectively. The solutions were shaken at 25 °C for 20 min and 2.5 µg of benzylmercaptane were added as internal standard. The acetonitrile solution was directly used for GC analysis. The methanol sample was mixed with 400 µL of water and extracted twice with 800 µL of pentane-ether (1:1). The organic phase was dried, concentrated and subjected to GC analysis. The aqueous solutions were extracted using dichloromethane as described for the enzyme-catalyzed reactions. Initial contents of pulegone in the conjugates were determined by extraction with dichloromethane and subsequent GC analysis.

Determination of C-S β-lyase activity

C-S β-Lyase activities were determined using *S*-benzyl-L-cysteine as a substrate. 2500 nmol of *S*-benzyl-L-cysteine was dissolved in 250 µL of 50 mM potassium phosphate buffer (pH 7.4) containing 100 µM pyridoxal 5'-phosphate. Tryptophanase (0.1 mg) and the crude enzyme extract obtained from *E. limosum* (50 µL corresponding to 10 mg of wet cells), respectively, were added and the mixture was shaken (140 rpm) at 37 °C for 5 min. After adding 2.5 µg of

benzylalcohol as internal standard, the generated benzylmercaptane was extracted with dichloromethane (2 x 700 µL). The separated organic phase was dried over anhydrous sodium sulfate and concentrated to about 100 µL under nitrogen flow. Enzyme activities were calculated from the ratios of the GC peak areas of benzylmercaptane to that obtained for the internal standard.

Enzymatic reaction-1

A mixture of equal amounts of the purified cysteine conjugate diastereoisomers derived from (*R*)-pulegone (98.2 % (1*R*,4*R*)) and (*S*)-pulegone (97.4 % (1*S*,4*S*)) was used as substrate. Three different amounts (22, 220, 2200 nmol) of this mixture were dissolved in 250 µL of 50 mM potassium phosphate buffer (pH 7.4) containing 100 µM pyridoxal 5'-phosphate. Tryptophanase (0.1 mg) (EC 4.1.99.1, from *E. 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) and the crude enzyme extract obtained from *E. limosum* (50 µL corresponding to 10 mg of wet cells), respectively, were added and the mixture was shaken (140 rpm) at 25 °C for 20 min. For the reaction with yeast, the same amounts of substrate were mixed with 250 mg of yeast in 700 µL of buffer (pH 7.4) containing 100 µM pyridoxal 5'-phosphate and incubated at 25 °C for 24 h. After adding 2.5 µg of benzylmercaptane as internal standard, volatile compounds were extracted from the reaction mixture with dichloromethane (700 µL x 2). The separated organic phase was dried over anhydrous sodium sulfate, concentrated to about 100 µL under nitrogen flow and subjected to GC analysis.

For the measurement of diastereoselectivity, the synthesized non-purified cysteine conjugates were used as substrate (2500 nmol). Other conditions were as described above.

For the reactions using other enzyme sources, 2500 nmol of substrates and the amounts of enzyme preparations given in chapter 4.1.1.3 were used. Other conditions were the same as those for yeast-catalyzed reactions. The following enzyme sources were used: *E. coli* lyophilized cells: from strain W (ATCC 9637). Yeast enzyme concentrate: hexokinase 0.8 units/mg solid (1 unit will phosphorylate 1 µmol of D-glucose/min) and alcohol dehydrogenase 24 units/mg solid (1 unit will convert 1 µmol of ethanol to acetaldehyde/min). Rat

liver acetone powder: alcohol dehydrogenase 1 unit/g. Pig kidney acetone powder: alkaline phosphatase 133 units/g solid (1 unit will hydrolyze 1 μmol of *p*-nitrophenyl phosphate/min). Acetone powders from plant materials were prepared as described in chapter 3.3.2.

For the reaction using other substrates except 2-(2-*S*-L-cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid, 3-*S*-(*N*-acetyl-L-cysteinyl) hexanal and 3-*S*-L-cysteinyl-1-hexanol, 2500 nmol of substrates were used. Other conditions were same with those for tryptophanase reactions.

Enzymatic reaction-2

25, 250 and 2500 nmol of 2-(2-*S*-L-cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid, 3-*S*-(*N*-acetyl-L-cysteinyl)hexanal and 3-*S*-L-cysteinyl-1-hexanol, 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), 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, respectively, in 700 μL of 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.

3.4.2. Lipases

50 μmol of the thioester substrate (8.7 mg of 3-acetylthiohexanal, 11.8 mg of 3-benzoylthiohexanal and 8.8 mg of 3-acetylthiohexanol, respectively) was added to 500 μL of 50 mM potassium phosphate buffer (pH 7.4). The enzyme preparation (ROL 10 mg; ANL 50 mg; WGL 25 mg; MJL 25 mg; PRL 50 mg; MML 5 mg; PCL 25 mg; PPL 2 mg; CRL 25 mg; PLE 0.4 mg; AOL 20 mg; TTL 100 mg; CAL 10 mg; CAL-A 10 mg; CAL-B 10 mg; CAL-B resin 10 mg) was added and the mixture was stirred magnetically (300rpm) with a teflon bar at 25 °C. After 2 h,

an aliquot of 20 µL of the reaction mixture was extracted with 500 µL of dichloromethane. The organic phase was dried over anhydrous sodium sulfate and subjected to GC analysis.

For the experiments on the co-solvent effects, 10 to 40 vol.% of acetone and *tert*-butanol, respectively, was added to the buffer solution.

3.4.3. Acylase

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 (*Giardina et al., 1997*). Other conditions were same as described above for the enzymatic reaction-2.

3.5. Analyses

3.5.1. Work-up of enzymatic reaction product

Kinetic resolution of 3-acetylthiohexanal (350 µmol substrate; reaction time 8 h) was performed as described above using CAL-B as catalyst. The organic extract obtained was concentrated to 100 µL by using a nitrogen stream and added to 10 mL of ice-cooled 0.1 M potassium phosphate buffer (pH 7.4). After addition of sodium borohydride (3.7 mg, 98 µmol in 1 mL distilled water), the solution was stirred continuously for 1 h under ice-cooling. The pH was adjusted to 3 using 2 N sulfonic acid, and the solution was extracted two times with 10 mL of diethyl ether. The combined extracts were washed (two times with 10 mL of distilled water) and dried over anhydrous sodium sulfate.

Separation of 3-acetylthiohexanol and 3-mercaptohexanol was achieved by using *p*-hydroxymercuribenzoate (*Darriet et al., 1995*). The organic solution was extracted three times (each for 5 min) with 10 mL of an aqueous solution (pH 8.5) of *p*-hydroxymercuribenzoate (2.5 mM) prepared from *p*-chloromercuribenzoate (*Boyer, 1954*). The organic phase was washed with 10 mL of distilled water and dried over anhydrous sodium sulfate. 3-Acetylthiohexanol contained in this solution was converted to 3-mercaptohexanol by alkaline hydrolysis according to the procedure described above and subjected to GC on the chiral stationary phase. The aqueous phase

was washed with 10 mL of dichloromethane followed by addition of L-cysteine (0.91 g; 7.5 mmol) dissolved in 10 mL distilled water. After 10 min equilibrium at room temperature, the pH was adjusted to 6 by addition of 5 % hydrochloric acid. The released 3-mercaptohexanol was isolated by extraction with dichloromethane (two times 20 mL; each for 5 min). The combined extracts were washed with 10 mL of distilled water, dried over anhydrous sodium sulfate and also subjected to GC on the chiral stationary phase.

3.5.2. GC, GC-MS

The cysteine conjugates were analyzed after trimethylsilylation. About 1 mg of sample was suspended in 100 µL of acetonitrile (dried over molecular sieve 4 Å). After addition of 50 µL of MSTFA, the sample was kept at 70 °C for 15 min. 0.2-1.0 µL of this solution was subjected to GC analysis.

Capillary GC was performed on the following 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 x 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 (split flow: 25 mL/min), and column temperature was programmed from 40 °C (5 min hold) to 230 °C (25 min hold) at 4 °C/min. The detector temperature was 240 °C for FID and 140 °C for FPD. Hydrogen was used as carrier gas at 105 kPa.

(II): A Carlo Erba GC 6000 gas chromatograph with FID; the columns used were DB-1 (30 m x 0.25 mm i.d., film thickness 1 µm) and SE-54 (15 m x 0.25 mm i.d., film thickness 0.15 µm), respectively. Split injection was performed at 220 °C (split flow: 29 mL/min), 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 80 kPa for DB-1 and 45 kPa for SE-54.

(III): Two Fisons GC 8000 gas chromatographs equipped with FID detectors and coupled via a moving capillary stream switching (MCSS) system (*Weber et al.,*

1995) were used. A DB-5 fused silica column (28 m x 0.25 mm i.d., film thickness 0.25 µm; J&W) was used as the precolumn and a fused silica column (30 m x 0.25 mm i.d.) coated with 33 % heptakis(per-*O*-ethyl)-β-cyclodextrin in OV1701-vi (PerEt-β-CD) as the main column. Split injection was performed at 205 °C, and the precolumn temperature was programmed from 40 °C (5 min hold) to 115 °C at 3 °C/min and from 115 to 240 °C (15 min hold) at 15 °C/min. For the main column it was from 60 °C (20 min hold) to 110 °C at 1 °C/min and 110 to 150 °C (5 min hold) at 15 °C/min. Hydrogen was used as carrier gas at 150 kPa. Transfers onto the main column using cut time intervals were 15.4-16.1 min for 3-mercaptohexanal and 27.2-27.7 min for 3-acetylthiohexanal. The data recording as well as the control of the MCSS system was managed with Chrom-Card® for Windows software (Fisons Instruments) on work station.

(IV): A Carlo Erba GC 6000 gas chromatograph equipped with FID; the column used was a fused silica column (30 m x 0.25 mm i.d.) coated with a 0.25 µm film of 50 % heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin in OV1701-vi (DiMe-β-CD). Split injection was performed at 200 °C, and the column temperature was programmed from 50 °C (2 min hold) to 200 °C (5 min hold) at 3 °C/min. Hydrogen was used as carrier gas at 80 kPa. For the analysis of 3-benzoylthiohexanal, the column temperature was programmed from 120 to 150 °C at 2 °C/min, 150 to 180 °C (5 min hold) at 0.2 °C/min.

(V): 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 a 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.

(VI): The analysis of stereoisomers of 8-mercapto-*p*-menthan-3-one was

performed according to literature (*Köpke et al., 1992*). A Fisons GC 8000 equipped with a split/splitless injector and FID detector was used. Column: 30 m x 0.25 mm i.d. chiral column with a 0.25 µm film of 50 % octakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-γ-cyclodextrin in OV1701-vi was coupled via press-fit connection behind a 27 m x 0.32 mm i.d. Superox 20M (0.25 µm film thickness). The column temperature was programed from 80 °C (0 min hold) to 110 °C at 1 °C/min, from 110 to 130 °C at 0.5 °C/min and from 130 to 205 °C at 5 °C/min (10 min hold). Hydrogen was used as carrier gas at 150 kPa. Injector temperature was 205 °C and injection mode was split with split flow of 30 mL/min.

GC/O was performed on the following GC systems.
(I): A DB-WAX column (55 m x 0.32 mm i.d.; film thickness 0.25 µm; J&W) installed into Carlo Erba GC4200 gas chromatograph equipped with a split/splitless injector and FID. At the end of column the effluent was splitted 1:1 for FID and sniffing port with chrom-fit® connector and deactivated fused silica tube. The column temperature was programmed from 60 °C (5 min hold) to 230 °C at 4 °C/min. The injector temperature was 215 °C, and the detector temperature was 240 °C for FID and 200 °C for sniffing port. Makeup flow of 20 mL/min nitrogen was used for sniffing port. Hydrogen was used as carrier gas at 100 kPa.

(II): A DiMe-β-CD column installed into HP5890II gas chromatograph equipped with a cooled-on-column injector and FID. The effluent was splitted to FID and sniffing port as described above. The typical temperature program was from 40 °C (2 min hold) to 80 °C at 40 °C/min, 80 to 140 °C at 2 °C/min, 140 to 200 °C (5 min hold) at 20 °C/min. The injector temperature was controlled as oven tracking mode, and the detector temperature was 220 °C for FID and 200 °C for sniffing port. Hydrogen was used as carrier gas at 70 kPa.

Gas chromatography - Mass Spectrometry conditions were as follows.
DB-WAXETR fused silica column (30 m x 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. 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.

Conditions for analysis of trimethylsilylated cysteine conjugates were as follows: SE-54 fused silica column (15 m x 0.25 mm i.d., film thickness 0.25 µm) was used. The column temperature was programed from 50 °C (2 min hold) to 300 °C (5 min hold) at 4 °C/min. Helium was used as carrier gas at 25 kPa. Injector temperature was 210 °C, injector mode was split with split flow of 32 mL/min. The ionization voltage was 70 eV.

For the analysis of 3-benzoylthiohexanal, SE-54 fused silica column (15 m x 0.25 mm i.d., film thickness 0.15 µm) was used and the temperature programm 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.

3.5.3. NMR

^{1}H NMR and ^{13}C NMR spectra were recorded at 500.13 MHz and 125.6 MHz, respectively, with an AVANCE 500 spectrometer (Bruker Instruments, Germany) equipped with a cryo platform and a triple resonance inverse probe head. Two-dimensional gradient-enhanced COSY, HMQC, HMBC and NOESY experiments were performed according to standard Bruker software (XWINNMR 3.1). Composite pulse decoupling was used in the ^{13}C NMR experiments. In the NOESY experiment, the relaxation delay and the mixing delay were 5 sec and 1 sec, respectively. The signal assignments are based on proton-proton (COSY, NOESY) and proton-carbon correlation experiments (HMQC, HMBC). (1*R*, 4*R*)- and (1*S*,4*S*)-8-*S*-L-cysteinyl-*p*-menthan-3-one and other cysteine conjugate except cysteine conjugates of C_6-compounds were measured in [D_4]-methanol. The sample temperature was 25 °C. For cysteine conjugates of C_6 compounds measurements were performed with the following conditions: 2-(2-*S*-L-Cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid (2 mg in 0.5 mL of

methanol-D_4; 10 °C), 3-*S*-(*N*-Acetyl-L-cysteinyl)hexanal (7 mg in 0.5 mL of $CDCl_3$; 10 °C), 3-*S*-(*N*-Acetyl-L-cysteinyl)hexanol (5 mg in 0.5 mL of D_2O; 10 °C) and 3-*S*-L-Cysteinyl-1-hexanol (5 mg in 0.5 mL of D_2O; 10 °C). ^{13}C chemical shifts were predicted using SPECINFO software (Chemical concepts 3.2.5). Thioesters were measured in 0.5 mL of [D]-chloroform.

3.5.4. LC-MS

ESI-MS^n spectra were recorded using a Thermo Finnigan LCQ Deca XP LC/MS^n system (Thermo Electron Corp.). Conditions were as follows: interface temp.: 300 °C; interface voltage: 45 kV; tube offset: 25 V; mass range: 50~600 *m/z*; polarity: positive and/or negative.

(I): Column: Hypersil BDS C18 5 µ 2.1 mm i.d. x 150 mm; eluent: 0.1 % HCOOH in MeOH aq. linear gradient 10 % to 100 % of MeOH from 2 to 12 min; flow rate: 300 µL / min, main peak appeared around 9 min.

(II): Column: Capcel pak C18 2 mm i.d. x 150 mm; eluent: 0.1 % HCOOH in acetonitrile aq. linear gradient 5 % to 40 % of acetonitrile in 20 min; flow rate: 0.2 mL / min.

3.5.5. FT-IR

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

3.5.6. Protein content

Protein contents of the enzyme preparations were determined according to the Bradford method (*Kruger, 1994*).

4. Results and Discussion

4.1. C-S β-Lyase-catalyzed transformations

4.1.1. Cysteine, homocysteine and glutathione conjugates of pulegone

4.1.1.1. Syntheses and structural elucidations

Cysteine conjugates of pulegone

Cysteine conjugates of pulegone were synthesized by Michael-type addition of L-cysteine to the α,β-unsaturated carbonyls (*R*)- and (*S*)-pulegone (**1, 2**), according to the scheme outlined in Figure 4.1.1.

+ cysteine

1 (*R*) **2** (*S*)

3a (1*R*, 4*R*) **4a** (1*S*, 4*S*)
3b (1*R*, 4*S*) **4b** (1*S*, 4*R*)

Figure 4.1.1 Synthesis of cysteine conjugates of pulegone

Michael-type addition of thiolate anions to α,β-unsaturated carbonyls is a well-known approach for the synthesis of thioethers (*Stoffelsma and Pypker, 1977; Annunziata et al., 1992*). The method has been applied to obtain cysteine conjugates of a spectrum of α,β-unsaturated aldehydes and ketones, including pulegone (*Kerkenaar et al., 1988; 1996*). However, the stereochemical course of these additions had not been investigated.

Under the conditions applied in this study, for each of the reactions starting from (*R*)- and (*S*)-pulegone, respectively, GC analysis of the trimethylsilylated products revealed the presence of a pair of compounds, one of them being present in high excess. The purities of the major products could be further

improved by reprecipitation from water/acetone. As shown examplarily for the conjugates obtained from (*R*)-pulegone in Figure 4.1.2, the purity of the first eluting main compound **(I)** could be increased from 94.6 % to 98.2 % by means of recrystallization. The same degree of purification could be achieved for the major product resulting from the reaction of (*S*)-pulegone (Table 4.1.1).

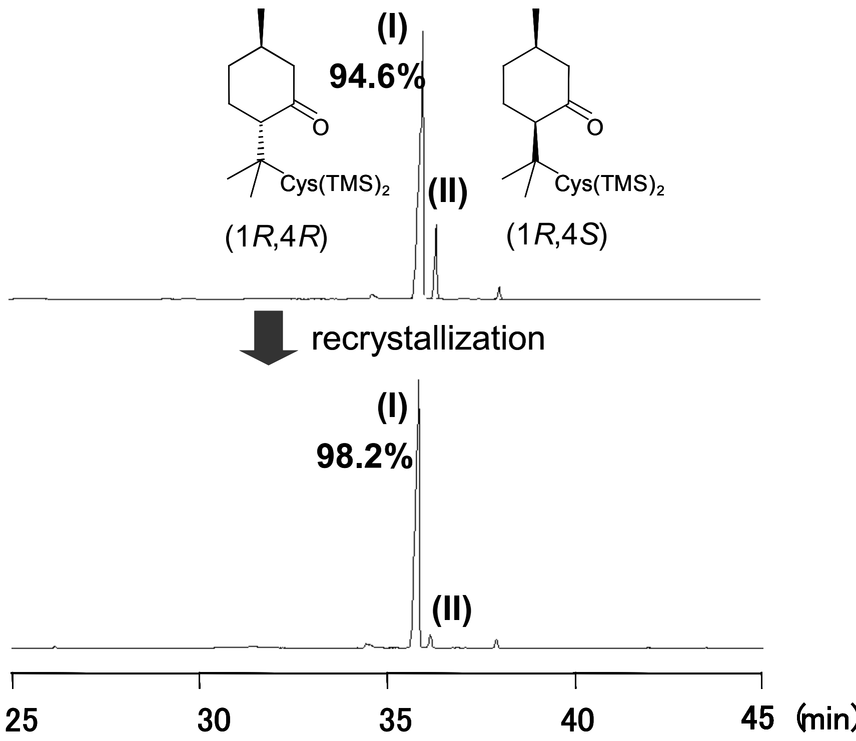


Figure 4.1.2 GC analysis of trimethylsilylated crude and purified cysteine conjugates of (*R*)-pulegone
(for conditions, see Materials and Methods, GC system II)

Capillary GC retention indices and mass spectra of the trimethylsilylated reaction products are given in Table 4.1.1. The molecular weights and the fragmentation patterns obtained by LC-MS of the purified cysteine conjugate of (*R*)-pulegone are shown in Figure 4.1.3. Virtually the same data were obtained from that of (*S*)-pulegone.

This set of analytical information indicated the compounds to be the four diastereomeric products (**3a**, **3b**, **4a** and **4b**) expected from the addition of cysteine to the double bond of pulegone (Figure 4.1.1).

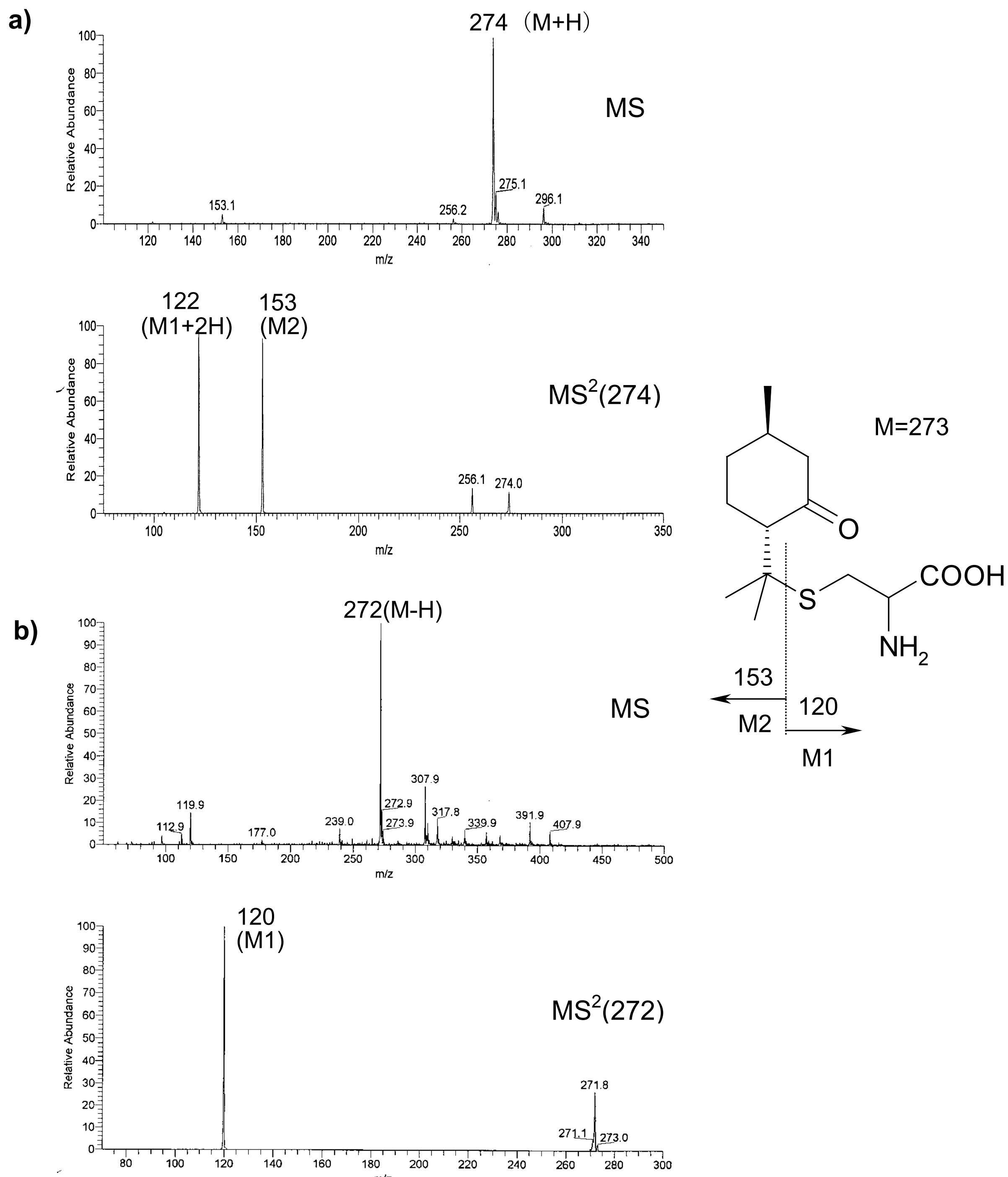


Figure 4.1.3 ESI-MS^n analysis of 8-*S*-cysteinyl-*p*-menthan-3-one

a) Detection polarity : positive

b) Detection polarity : negative

Table 4.1.1 Analytical data of 8-*S*-L-cysteinyl-*p*-menthan-3-one stereoisomers obtained by addition of L-cysteine to (*R*)-pulegone (**3a**, **3b**) and (*S*)-pulegone (**4a**, **4b**)

		L-cysteine conjugates of			
		(*R*)-pulegone		(*S*)-pulegone	
		(I)[a]	**(II)**	**(I)**	**(II)**
		3a (1*R*,4*R*)	**3b** (1*R*,4*S*)	**4a** (1*S*,4*S*)	**4b** (1*S*,4*R*)
Distribution (%)	raw	94.6	5.4	95.1	4.9
	purified	98.2	1.8	97.4	2.6
KI (SE-54) [b,c]		2224	2243	2224	2243
GC-MS [c] *m/z* (relative intensity, %)		300 (62), 219 (33), 218 (100), 153 (35), 148 (66), 147 (36), 109 (30), 100 (60), 73 (95), 69 (64)	300 (19), 219 (18), 218 (100), 153 (9), 148 (27), 147 (17), 109 (13), 100 (23), 73 (78), 69 (28)	300 (33), 219 (20), 218 (100), 153 (18), 148 (40), 147 (20), 109 (18), 100 (33), 73 (93), 69 (39)	300 (13), 219 (15), 218 (77), 153 (10), 148 (29), 147 (20), 109 (18), 100 (27), 73 (100), 69 (38)

[a] Roman numbers correspond to the GC order of elution of the trimethylsilylated derivatives (cf. Figure 4.1.2)

[b] Kovats retention indices

[c] TMS derivatives

Verification of the structures was achieved by NMR investigation of the purified major products **3a** and **4a**. The assignments of the (1*R*,4*R*) configuration for **3a** and the (1*S*,4*S*) configuration for **4a** (Fig. 4.1.4) were based on the following results:

(3a) **(4a)**

Figure 4.1.4 Structures of the major cysteine conjugates obtained from (*R*)-pulegone (product **3a**) and (*S*)-pulegone (product **4a**)
3a: (1*R*,4*R*)-8-*S*-cysteinyl-*p*-menthan-3-one, *trans*
4a: (1*S*,4*S*)-8-*S*-cysteinyl-*p*-menthan-3-one, *trans*
*protons on cysteine residue are omitted

Using deuterated methanol as solvent, the ^{1}H NMR signals were well separated and resolved at a transmitter frequency of 500 MHz. The NMR data of **3a** and **4a** were virtually identical. ^{1}H NMR chemical shifts, signal multiplicities, coupling constants, and correlation patterns in two-dimensional NOESY and COSY experiments determined for **3a** are summarized in Table 4.1.2. The signal assignments were based on two-dimensional COSY experiments in conjunction with chemical shift considerations. Coupling constants extracted from the one-dimensional ^{1}H spectrum were also a useful source for structural information. For rigid hexane ring systems, geminal constants ($2\ J_{HH}$) typically range from 12 to 14 Hz, whereas vicinal coupling constants ($3\ J_{HH}$) range from 11 to 13.5 Hz for axial-axial couplings, from 3.5 to 4.5 Hz for axial-equatorial couplings, and from 2.5 to 3 Hz for equatorial-equatorial couplings (*Croasmun and Carlson, 1994*). On this basis, the coupling constants for the H-4 signal (2.66 ppm) establish the axial position of H-4 with an axial-axial coupling of

12.5 Hz to the axial H-5 proton, and an axial-equatorial coupling of 4.6 Hz to the equatorial H-5 proton. As expected, the bulky residue at position 4 is in the thermodynamically favored equatorial position (Fig. 4.1.4).
The coupling constants detected for the position 2 and 6 signals indicated axial orientation for H-1. More specifically, for one of the H-2 signals (H-2ax, 2.17 ppm) two large coupling constants (around 12 Hz each) were observed, one of which was caused by a geminal coupling and the other was caused by an axial-axial coupling (between H-2ax and H-1ax). Similarly, one of the H-6 signals (1.47 ppm) was characterized by three large coupling constants (around 13 Hz each) indicating one geminal coupling (between H-6ax and H-6eq) and two axial-axial couplings (between H-6ax and H-1ax and between H-6ax and H-5ax, respectively).
Independently, the structures **3a** and **4a** shown in Figure 4.1.4 were confirmed by two-dimension NOESY experiments where through-space correlations were observed between the axial protons at C-1 and C-5, and between the axial protons at C-2 and C-4, respectively.

In conclusion, the addition of cysteine to the double bond of pulegone via Michael-addition mechanism results in the preferred formation of the *trans*-configured diastereoisomeric products, irrespective of the configuration at position C1 of the starting material.

Table 4.1.2 NMR data of (1*R*,4*R*)-8-*S*-L-cysteinyl-*p*-menthan-3-one (**3a**)

Position	Chemical Shifts δ^1H^a (ppm)	Coupling Constants J_{HH}^b (Hz)	Correlation Patterns	
			NOESY	COSY
12	3.64 (dd)	9.1 (11), 3.6 (11’)	10, 5_{ax}, 9	11, 11’
11’	3.20 (dd)	13.7 (11), 3.6 (12)	11, 10, 5_{ax}, 9	12, 11
11	2.87 (dd)	13.7 (11’), 9.1 (11)	11’, 10, 5_{ax}, 9	12, 11’
4_{ax}	2.66 (dd)	12.5 (5_{ax}), 4.6 (5_{eq})	10, 9, 6_{ax}, 2_{ax}	5_{ax}, 5_{eq}
5_{eq}	2.56 (m)		10, 5_{ax}	4_{ax}, 5_{ax}, 6_{ax}
2_{eq}	2.21 (dd)	12.1 (2_{ax}), 5.0 (1_{ax})	1_{ax}	1_{ax}, 2_{ax}
2_{ax}	2.17 (t)	12.1 (2_{eq}), 12.1 (1_{ax})	4_{ax}, 7, 6_{ax}	1_{ax}, 2_{eq}
6_{eq}	1.91 (dm)	12.9 (6_{ax})	5_{eq}, 7, 6_{ax}	6_{ax}, 5_{ax}
1_{ax}	1.84 (m)		2_{eq}, 7, 5_{ax}	2_{ax}, 2_{eq}, 7, 6_{ax}
5_{ax}	1.54 (dqua)	13.0 (5_{eq}), 13.0 (4_{ax}), 13.0 (6_{ax}), 3.0 (6_{eq})	1_{ax}, 5_{eq}	4_{ax}, 5_{eq}, 6_{eq}, 6_{ax}
9	1.54 (s)		4_{ax}, 11, 11’, 12	
6_{ax}	1.47 (dqua)	12.9 (6_{eq}), 12.9 (5_{ax}), 12.9 (1_{ax}), 3.5 (5_{eq})	6_{eq}, 2_{ax}, 7, 4_{ax}	6_{eq}, 5_{ax}, 1_{ax}, 5_{eq}
10	1.37 (s)		5_{eq}, 4_{ax}, 11, 11’, 12	
7	1.03 (d)	6.5 (1_{ax})	6_{ax}, 1_{ax}, 6_{eq}, 2_{ax}	1_{ax}

[a] Referenced to the methyl signal of MeOD (δ^1H, 3.30 ppm). Signal multiplicities are indicated in parentheses (d, doublet; dd, double-doublet; t, triplet; s, singlet; m, multiplet; dm, doublet of multiplets; dqua, quartet of doublets)

[b] Coupling partners are indicated in parentheses.

Homocysteine conjugate of pulegone

Comparable to cysteine, homocysteine can act as nucleophile, resulting in the generation of the corresponding conjugates of α,β-unsaturated compounds. The gas chromatgraphic separation of the trimethylsilylated products obtained by reaction of (*R*)-pulegone and DL-homocysteine is shown in Figure 4.1.5. The virtually identical mass fragment patterns determined by GC/MS (see chapter 3.2.1) indicated the presence of four diastereoisomers resulting from the addition of DL-homocysteine. Pending a final structural assignment by NMR, the ratios of the GC peaks indicate that almost equal amounts of *cis*- and *trans*- isomers were formed. This is in sharp contrast to the results obtained for the addition of cysteine (95 % *trans*- : 5 % *cis*-isomer). It is also interesting to note, that apparently the conjugates of D- and L-homocysteine exhibit slightly different retention times. On the contrary, the conjugates of D- and L-cysteine showed identical retention behavior under the same conditions.

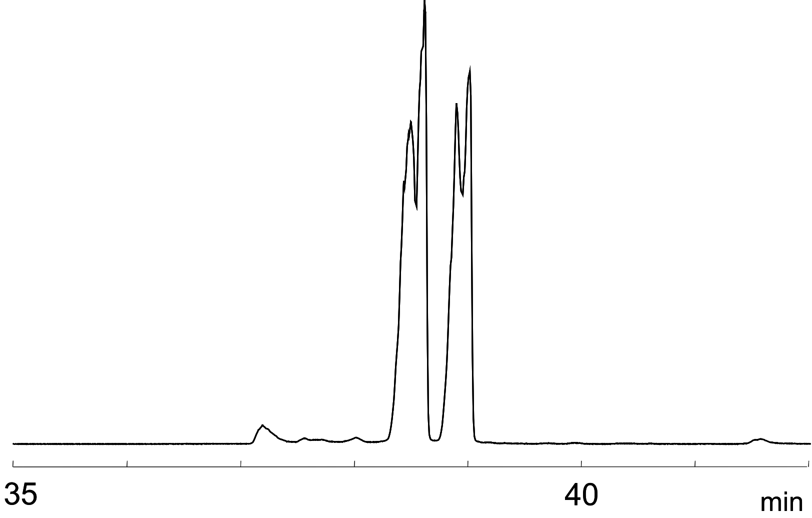


Figure 4.1.5 GC analysis of the trimethylsilylated products obtained after addition of DL-homocysteine to (*R*)-pulegone (for conditions, see Materials and Methods, GC system II)

Glutathione conjugate of pulegone

In analogy to cysteine and homocysteine, glutathione can be used as nucleophile in the addition to α,β-unsaturated carbonyl compounds. LC-MS analysis of the product obtained by reaction of glutathione and (*R*)-pulegone revealed the presence of two compounds (Figure 4.1.6). The MS spectra indicated them to be the two diastereoisomers expected due to *cis*- and *trans*-addition (MH^+: 460), respectively. Again, the ratio of peak areas (39 % : 61 %) was quite different from that observed for the addition of cysteine.

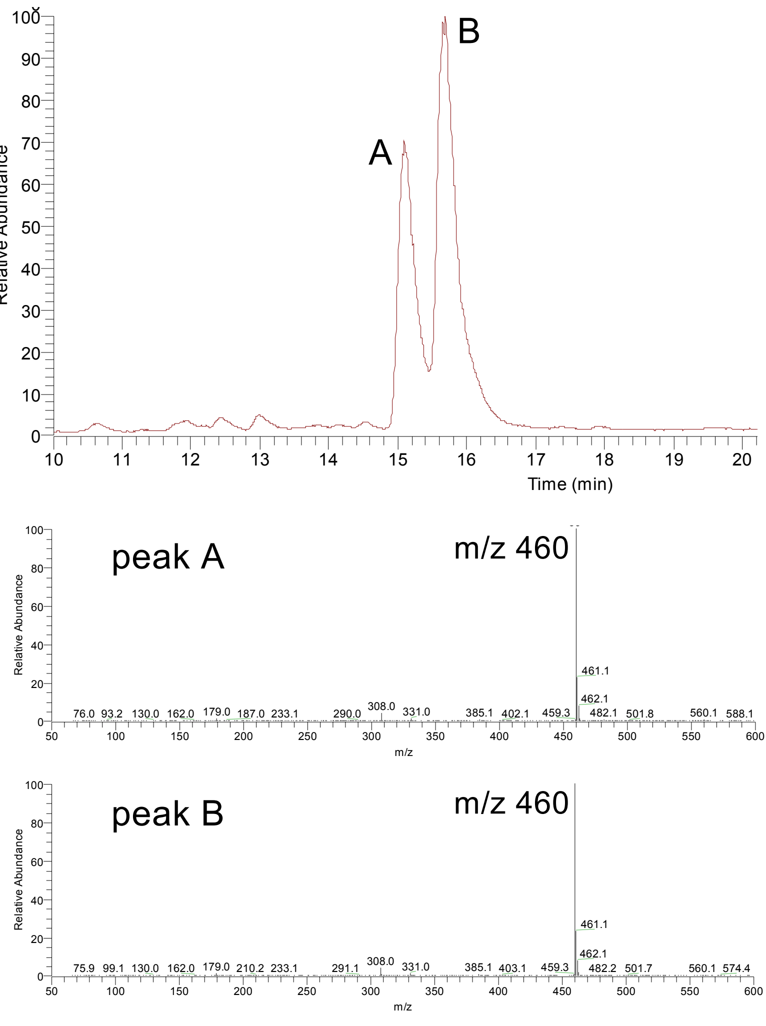


Figure 4.1.6 LC-MS analysis of 8-*S*-glutathionyl-(1*R*)-*p*-menthan-3-one
TIC (*m/z*=50-600; upper) and MS spectrum of peak A and B (lower)
(for conditions, see Materials and Methods, LC-MS system II)

4.1.1.2. Enzymatic cleavage

Cysteine conjugates

By the action of β-lyase, the synthesized cysteine conjugates of pulegone were cleaved to generate the corresponding thiol 8-mercapto-*p*-menthane-3-one as shown in Figure 4.1.7.

+ C-S β-lyase

3a (1*R*,4*R*) **4a** (1*S*,4*S*)
3b (1*R*,4*S*) **4b** (1*S*,4*R*)

5a (1*R*,4*R*) **6a** (1*S*,4*S*)
5b (1*R*,4*S*) **6b** (1*S*,4*R*)

Figure 4.1.7 Enzymatic cleavage of cysteine conjugates of pulegone

Stability of cysteine conjugates

Former studies on the enzyme-catalyzed transformation of cysteine conjugates of pulegone had indicated the formation of pulegone due to chemical cleavage of the precursor (*Kerkenaar et al., 1988*). In order to clarify this phenomenon, the stabilities of the synthesized conjugates were investigated in different solvents. As shown in Table 4.1.3, the conjugates are rather stable in non-aqueous solvent. In buffer solution, however, up to 54 % of the conjugate is cleaved to yield pulegone after 24 h. The data suggest that the liberation of pulegone proceeds via a reversed Michael-type addition and the formation of the intermediate carbonium ion at C-8 is favored in protic solvents. The minor amounts of chemically formed pulegone (3 and 4.5 %) observed in buffer after 20 min., i.e. the time used for the enzyme-catalyzed transformations, were taken into account when calculating the conversion rates in the course of the enzyme-catalyzed reactions.

Table 4.1.3 Liberation of (*R*)- and (*S*)-pulegone, respectively, by chemical cleavage of the 8-*S*-L-cysteinyl conjugates in different solvents[a]

time (h)	0.3		2		24	
	pulegone (mol %)[b]					
	(*R*)	(*S*)	(*R*)	(*S*)	(*R*)	(*S*)
acetonitrile	2.0	0.2	1.8	0.2	2.9	0.5
methanol	0.6	0.1	1.7	0.2	3.0	0.9
water	1.4	0.8	2.6	3.8	19.9	36.1
buffer[c]	3.0	4.5	8.7	15.6	39.2	54.2

[a] Initial contents of pulegone in conjugates were 3.4 % in (*R*)-pulegone conjugate and 0.2 % in (*S*)-pulegone conjugate
[b] mol % to original corresponding cysteine conjugate (2500 nmol / 250 µl of each solvent)
[c] 50 mM potassium phosphate buffer (pH 7.4)

Enzymatic cleavage of cysteine conjugates

β-Lyase from three sources was used for the enzyme-catalyzed transformations of the cysteine conjugates of pulegone: (i) a cell-free extract obtained from *E. limosum*, (ii) a commercially available tryptophanase preparation from *E. coli*, and (iii) baker's yeast (*Saccharomyces cerevisiae*). This selection was based on the following considerations: β-Lyase from *E. limosum* had been shown to possess activity towards several cysteine conjugates (*Larsen and Stevens, 1986*), including those present in must from Sauvignon blanc (*Tominaga et al., 1998b*) and in passion fruits (*Tominaga and Dubourdieu, 2000*). Apotryptophanase had been suggested as diagnostic tool to assess the aromatic potential of wine grapes (*Peyrot des Gachons et al., 2000*). Tryptophanase had been also shown to possess β-lyase activity to liberate thiols from a broad spectrum of precursors (*Snell, 1975*). The involvement of *Saccharomyces cerevisiae* in the degradation of cysteinylated flavor precursors in grapes had been demonstrated in model fermentations (*Tominaga et al., 1998b*).

As shown in Table 4.1.4, 8-*S*-L-cysteinyl-*p*-menthan-3-one was accepted as substrate by the three enzyme sources tested. Retention time and MS spectrum

Table 4.1.4 Enzyme-catalyzed generation of 8-mercapto-*p*-menthan-3-one from 8-*S*-L-cysteinyl-*p*-menthan-3-one

Initial amout of substrate (nmol)	Tryptophanase		*E. limosum*		Yeast	
	product (nmol)	conv. [a] (%)	product (nmol)	conv. (%)	product (nmol)	conv. (%)
22	18.0	86.9	8.6	48.8	- [b]	-
220	83.5	42.1	57.3	30.4	0.4	0.2
2200	121.9	6.4	106.0	5.8	4.2	0.2

[a] conversion rate = generated 8-mercapto-p-menthan-3-one / (initial substrate – chemically cleaved pulegone)

[b] not detected

of the generated 8-mercapto-*p*-menthan-3-one were identical to those of an authentic reference compound. Quantification was performed using benzylmercaptane as internal standard. Chemical formation of this product could be ruled out by incubation under the same conditions without the enzyme preparations.

In order to put the activities observed for the pulegone conjugates into perspective, they were compared to those towards *S*-benzylcysteine, a substrate known to be accepted by β-lyases (*Tomisawa et al., 1984; Larsen and Stevens, 1986*). The tryptophanase preparation used (protein content 31 µg / 0.1 mg) released 12 µg of benzylmercaptane / 10 min / 0.1 mg. The extract obtained from *E. limosum* (protein content 76 µg / 50µL) exhibited an activity of 7 µg of benzylmercaptane / 10 min / 50 µL. The C-S β-lyase activities towards the pulegone conjugates (10 µg of 8-mercapto-*p*-menthan-3-one / 10 min) were in the same order of magnitude. Yeast showed considerably lower conversion rates which might explained by hindered diffusion of the substrate due to the use of whole cells.

D-cysteine conjugates of (*R*)- and (*S*)-pulegone were not accepted as substrates by tryptophanase and the extract of *E. limosum*. This is in accordance with the specificities reported for cysteine conjugate β-lyase from *E. limosum*: *S*-Benzyl-D-cysteine and *S*-ethyl-D-cysteine were not accepted as substrate although the corresponding conjugates of L-cysteine were cleaved (*Larsen and Stevens, 1986*).

Enantioselectivity of the enzyme-catalyzed reactions

The stereoselectivity of the enzyme-catalyzed transformation of the cysteine conjugates of pulegone was followed by determining the configuration of the formed 8-mercapto-*p*-menthan-3-one. The stereoisomers of 8-mercapto-*p*-menthan-3-one were separated by capillary gas chromatography according to a previously described procedure: octakis (2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-γ-cyclodextrin was used as chiral stationary phase and the selectivity was adjusted by coupling the chiral column to a polar, non-chiral column (*Köpke et al., 1992*). The separation of 8-mercapto-*p*-menthan-3-one stereoisomers obtained by reaction of (*S*)- and (*R*)-pulegone with sodium hydrogen sulfide monohydrate is shown in Figure 4.1.8. The pattern of diastereoisomers reflects the preferred formation of the *trans*-configured products as mentioned in Chapter 4.1.1.1.

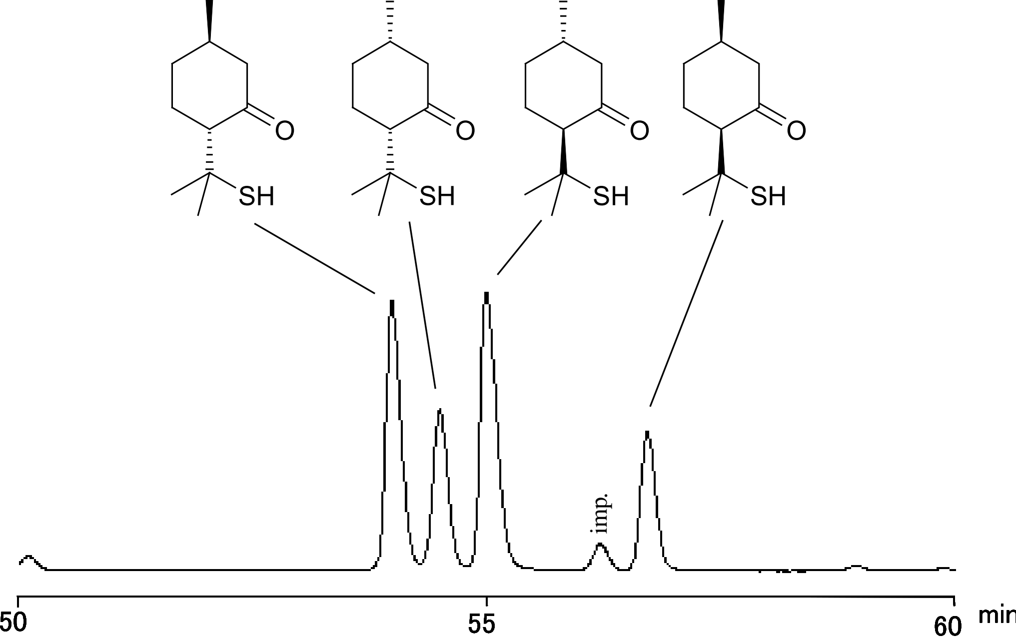


Figure 4.1.8 GC analysis of 8-mercapto-*p*-menthan-3-one using octakis(2,3-di-*O*-acetyl-6-O-*tert*-butyldimethylsilyl)-γ-cyclodextrin as chiral stationaly phase.
(for conditions, see Materials and Methods, GC system VI)

The enantioselectivities of the enzyme-catalyzed reactions were calculated on the basis of equations developed for the quantitative treatment of biochemical kinetic resolutions (*Chen et al., 1982*). If an enzyme reacts with a racemic mixture and the enzyme shows enantioselectivity, each of the enantiomers can be considered as separate substrate. Enantiodiscrimination is determined by the differences in binding of the enantiomers, expressed as K_m, and in the conversion of the enzyme-substrate complexes into the products, expressed as k_{cat}. The enantioselectivity of the reaction is described by the ratio of the resulting specificity constants, i.e. the ratios of k_{cat} and K_m for each enantiomer. Equation 1 shows the ratio for the case of preference of the (*R*)-enantiomer in the course of the kinetic resolution.

$$\text{Enzyme + Substrate } (R, S) \underset{(K_m)_S}{\overset{(K_m)_R}{\rightleftarrows}} \begin{cases} [ER] \underset{}{\overset{(k_{cat})_R}{\rightleftarrows}} \text{Product } (R) \\ [ES] \underset{(k_{cat})_S}{\rightleftarrows} \text{Product } (S) \end{cases}$$

$$E = \frac{k_R}{k_S} = \frac{(k_{cat}/K_m)_R}{(k_{cat}/K_m)_S} \qquad \text{(eq. 1)}$$

According to previously developed equations (*Chen et al., 1982*), the enantiomeric ratio (*E*) can be determined experimentally not only by following these biochemical constants, but also by measuring conversion rates (*c*) and enantiomeric excesses (*ee*) of substrates or products in the course of the reaction.

$$E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]} \qquad \text{(eq. 2)}$$

where

$$c = \frac{P_a + P_b}{A_0 + B_0} \qquad (c = \text{conversion rate})$$

$$ee_p = \frac{P_a - P_b}{P_a + P_b} \qquad (ee_p = \text{enantiomeric excess of product})$$

A_0, B_0 ; initial amount of each enantiomeric substrate A and B
P_a, P_b; amount of product after enzymatic reaction from A and B, respectively

Using this concept, the enantioselectivity of the enzyme-catalyzed cleavage of 8-*S*-L-cysteinyl-*p*-menthan-3-one was investigated using a mixture of equal amounts of the major products obtained after addition of cysteine to (*R*)- and (*S*)-pulegone, i.e. the (1*R*,4*R*)- and (1*S*,4*S*)-stereoisomers.
The comparison of the resulting initial distribution of the enantiomeric substrates **3a** (1*S*,4*S*) and **4a** (1*R*,4*R*) to that of the formed products **5a** and **6a** revealed a preference of the (1*S*,4*S*)-stereoisomer by tryptophanase (Table 4.1.5). The resulting value of 2 demonstrates that the degree of enantiodiscrimination is only low. The preference for one of the enantiomers was even less pronounced for *E. limosum*. However, it is noteworthy that the opposite substrate stereoisomer was cleaved faster by this enzyme preparation.

Table 4.1.5 Enantioselectivity of the enzyme-catalyzed generation of 8-mercapto-*p*-menthan-3-one from 8-*S*-L-cysteinyl-*p*-menthan-3-one

	8-*S*-L-cysteinyl-*p*-menthan-3-one		8-mercapto-*p*-menthan-3-one			
			tryptophanase		*E. limosum*	
	nmol	%	nmol	%	nmol	%
(1*S*,4*S*)	1124	52.5	71.6	68.0	41.9	43.3
(1*R*,4*R*)	1015	47.5	33.7	32.0	55.0	56.7
E			2[a]		1.5[b]	

[a] enantioselectivity for (1*S*,4*S*)
[b] enantioselectivity for (1*R*,4*R*)

Diastereoselectivity of the enzyme-catalyzed reactions

Diastereoselectivity was investigated by using the non-purified 8-*S*-cysteinyl-*p*-menthan-3-one stereoisomers **3a**, **3b** and **4a**, **4b**, respectively, as substrates. The effect was quantified by calculating a diastereomeric ratio *D*, analogous to the *E*-value described above. The calculations had to take into consideration that the diastereoisomeric excess of the starting mixture of isomers was greater than zero. According to *Chen et al.* (*1982*), in such a case the kinetic resolution can be described by equation 3.

$$1-c\left(\frac{1+de_p}{1+de_0}\right)=\left[1-c\left(\frac{1-de_p}{1-de_0}\right)\right]^D \qquad \text{(eq. 3)}$$

where

$$de_o=\frac{A_0-B_0}{A_0+B_0} \qquad \text{(diastereomeric excess of substrate, A > B)}$$

$$de_p=\frac{P_a-P_b}{P_a+P_b} \qquad \text{(diastereomeric excess of product, } P_a > P_b\text{)}$$

Transformation of eq. 3 resulted in a correlation analogous to eq. 2, which allows the calculation of the *D* value on the basis of the diastereomeric exesses of the product (de_p) and the starting substrate (de_0).

$$D=\frac{\ln\left[1-c\left(\frac{1+de_p}{1+de_0}\right)\right]}{\ln\left[1-c\left(\frac{1-de_p}{1-de_0}\right)\right]} \qquad \text{(eq. 4)}$$

If an enzyme prefers the minor substrate B, the following equation has to be used.

$$D=\frac{\ln\left[1-c\left(\frac{1-de_p}{1-de_0}\right)\right]}{\ln\left[1-c\left(\frac{1+de_p}{1+de_0}\right)\right]} \qquad \text{(eq. 5)}$$

As shown in Table 4.1.6, both enzyme preparations showed a preference for the *cis*-configured (1*R*,4*S*)- and (1*S*,4*R*)-diastereoisomers that means the minor diastereoisomer was preferred by the enzyme. The most pronounced discrimination (*D* = 11) was observed for the (1*R*,4*S*)-isomer by tryptophanase.

Table 4.1.6 Diastereoselectivity of the enzyme-catalyzed generation of 8-mercapto-*p*-menthan-3-one from 8-*S*-L-cysteinyl-*p*-menthan-3-one

	8-*S*-L-cysteinyl-*p*-menthan-3-one		8-mercapto-*p*-menthan-3-one			
			tryptophanase		*E. limosum*	
	nmol	%	nmol	%	nmol	%
(1*R*,4*R*)	2365	94.6	54.9	63.7	40.3	93.4
(1*R*,4*S*)	135	5.4	31.3	36.3	2.8	6.6
D[a]			11		1.2	
(1*S*,4*S*)	2378	95.1	117.5	88.0	45.2	81.2
(1*S*,4*R*)	122	4.9	16.0	12.0	10.5	18.8
D[b]			3		5	

[a] diastereoselectivity for (1*R*,4*S*)
[b] diastereoselectivity for (1*S*,4*R*)

Enzymatic cleavage of homocysteine and glutathione conjugates

Tryptophanase and a commercially available L-methionine γ-lyase preparation from *Pseudomonas putida* were used for the enzyme-catalyzed transformations of the glutathione and homocysteine conjugates of (*R*)-pulegone. As shown in Table 4.1.7, tryptophanase exhibited activity towards the glutathione conjugates (0.1 % conversion). As expected, the DL-homocysteine conjugates were not accepted by this β-lyase. These substrates were only cleaved by action of the L-methionine γ-lyase (0.4 % conversion). In both cases, chemical formation of 8-mercapto-*p*-menthan-3-one could be ruled out by incubation under the same conditions without the enzyme preparations. 8-Mercapto-*p*-menthan-3-one generated from glutathione conjugates by tryptophanase was nearly racemic.

Table 4.1.7 Enzyme-catalyzed generation of 8-Mercapto-*p*-menthan-3-one from corresponding glutathione and homocysteine conjugates of (*R*)-pulegone

substrate	amount of substrate (nmol)	enzyme	8-mercapto-*p*-menthan-3-one (nmol)	de_p[a]	*D*
glutathione conjugates	1300	tryptophanase[b]	1.0	0.03	1.5[d]
DL-homocysteine conjugates	1700	tryptophanase[b]	0	-	-
DL-homocysteine conjugates	1700	L-methionine γ-lyase[c]	6.1	0.38	1.8[e]

[a] diastereomeric excess for (1*R*,4*R*) in the product
[b] amount of enzyme: 0.1 mg
[c] amount of enzyme: 5.0 mg
[d] diastereoselectivity for (1*R*,4*S*), as de_0 was 0.22 for (1*R*,4*R*)
[e] diastereoselectivity for (1*R*,4*R*), as de_0 was 0.12 for (1*R*,4*R*)

4.1.1.3. Screening for β-lyases from other sources

Cysteine conjugate β-lyases occur widely in nature and have been described in bacteria as well as in mammalian tissues (chapter 2.3.1). In higher plants, however, only alliin lyase extracted from onion has been reported to have cysteine conjugate β-lyase activity (*Kitamura et al., 1997*). Moreover, *Acacia* sp. have been shown to possess alkylcysteine β-lyase (*Mazelis and Crevling, 1975*; *Sweet and Mazelis, 1987*) which catalyzes the cleavage of C-S bond of alkyl- or aryl-cysteine instead of sulfoxide.

Plants in which thiol compounds have been reported as important flavor components and/or cysteinylated precursors have been identified seemed to be promising candidates to screen for β-lyase activity. The structures of the compounds guiding the selection are shown in Figure 4.1.9.

8-mercapto-*p*-menthan-3-one | 2-mercapto-2-methyl-4-methoxybutane | 4-mercapto-4-methyl-2-pentanone | 3-mercaptohexanol

Figure 4.1.9 Character impact thiol compounds occurring in plants

8-Mercapto-*p*-mentan-3-one, the compond formed upon β-lyase-catalyzed cleavage of cysteine conjugates of pulegone as described in chapter 4.1.1.2, is a powerful flavor compound occurring in buchu leef oil (*Sundt et al., 1971*; *Lamparsky and Schudel, 1971*) and imparting the typical “cassis”-type aroma. It is used as flavoring material in many food products, although the compound itself has not been identified in blackcurrant. On the other hand, the sulfur-containing compound 2-mercapto-2-methyl-4-methoxybutane has been found in blackcurrant buds and is reported to have a characteristic catty note (*Rigaud et al., 1986*). Box tree is also well known to have a strong catty odor. The compound responsible for this odor has been shown to be 4-mercapto-4-methyl-2-pentanone (*Tominaga and Dubourdieu, 1997*), a substance having the same skeleton as 8-mercapto-*p*-mentan-3-one. Therefore, the young leaves of blackcurrant and box tree were screened as potential enzyme sources.

Passion fruits has been reported to contain 3-mercaptohexanol as aroma-active thiol (*Engel and Tressl, 1991*) and the presence of cysteinylated precursors of 3-mercaptohexanol and 3-mercapto-3-methylbutanol has been demonstrated (*Tominaga and Dubourdieu, 2000*). However, so far no activities of cysteine conjugate β-lyases have been reported in passion fruits. Therefore, leaves as well as fruits of this plant were screened for cysteine conjugate β-lyase activity.

As comparator, onions known to possess cysteine conjugate β-lyase activity (*Kitamura et al., 1997*) were also investigated as enzyme source.

For enzyme isolation, acetone powders were prepared. The yields obtained from the various plant materials are given in chapter 3.3.2.
These plant-derived acetone powders were compared to acetone powders from animal sources. Cysteine conjugate β-lyase activities have been reported in rat liver (*Stevens, 1985*), and in the kidneys of rat (*Stevens et al., 1986*), bovine (*Kishida et al., 2001*) and humans (*Perry et al., 1995*). Thus, commercially available acetone powders from rat liver and pig kidney were tested.
The preparations were screened for activities of β-lyases using *S*-benzylcysteine and 8-*S*-cysteinyl-*p*-menthan-3-one as substrates. The results are summarized in Table 4.1.8. For comparison, the previously described results obtained by using tryptophanase, the extract of *E. limosum* and yeast cells, respectively, (chapter 4.1.1.2) are also included in the Table. They were complemented by data on lyophilised *E. coli* cells and a yeast enzyme concentrate.

The strain W of *E. coli* has been reported to possess cystathionine β-lyase activity (*Delavier-Klutchko and Flavin, 1965*). The rate of conversion of 8-*S*-cysteinyl-*p*-menthan-3-one observed for lyophylised cells of this strain was in the same order of magnitude as that for the tryptophanase preparation isolated from *E. coli*. However, a 500 times higher amount of enzyme preparation and prolonged reaction time were required. The quantitative comparison of the data is hampered by the fact that no units had been provided for the lyophylised cell preparation by the supplier.
The hindered diffusion of substrates due to the use of whole cells was considered as one of the reasons for the only low C-S β-lyase activities observed in reactions catalyzed by fresh baker's yeast. However, the conversion rates could not be improved using a commercially obtained yeast enzyme concentrate.
Both acetone powders from animal sources showed low activities towards *S*-benzylcysteine and 8-*S*-cysteinyl-*p*-menthan-3-one under the experimental conditions applied. It has been demonstated that the addition of pyridoxal 5'-phosphate during purification is required in order to stabilize cysteine

Table 4.1.8 Activities of β-lyase preparations from various sources

enzyme source	amount of enzyme preparation (mg)[a]	reaction time (h)	conversion rate (%)	
			S-benzyl cysteine[b]	8-*S*-cysteinyl-*p*-menthan-3-one[c]
tryptophanase (*E. coli*)	0.1	0.3	6.4	6.4
E. limosum (extract)	50[d]	0.3	1.1	5.8
yeast (cells)	250	24	-[e]	0.2
E. coli (lyophilised cell)	50	4	-	5.3
yeast (enzyme concentrate)	25	4	-	0.03
acetone powders (animals)				
rat liver	20	6	0.05	0.08
pig kidney	20	6	0.08	0.01
acetone powders (plants)				
onion (bulb)	15	6	0.15	-
blackcurrant (leaf)	20	6	0.07	0.44
box tree (leaf)	20	6	0.08	0.12
passion fruits (ripened fruit)	15	6	0.1	-
passion fruits (unripened fruit)	15	6	nd[f]	-
passion fruits (internal rind layer)	15	6	nd	-
passion fruits (leaf)	15	6	tr[g]	-

[a] amount per reaction; [b] amount of substrate: 2500 nmol; [c] amount of substrate: 2200 nmol; [d] µl; [e] not determined; [f] not detected (c < 0.0005 %); [g] trace amount detected (c < 0.01 %)

conjugate β-lyases in bacterial and mammalian tissues (*Larsen and Stevens, 1986*). No information has been available on the procedure applied to obtain the commercially purchased acetone powders from rat liver and pig kidney. The lacking use of pyridoxal 5'-phosphate may be one of the reasons for the rather low C-S β-lyase activities observed in these enzyme preparations.

By investigation of the enzyme preparations from blackcurrant and box tree leaves and ripened passion fruits, cysteine conjugate β-lyase activities could be demonstrated for the first time in these plants. The activities toward

S-benzylcysteine were in the same order of magnitude as those determined in onions. Blackcurrant and box tree leaves were active towards both substrates, and interestingly, blackcurrant leaves showed about 6 times higher activities for 8-*S*-cysteinyl-*p*-menthan-3-one than for *S*-benzylcysteine. In passion fruits, β-lyase activity was measurable only in the pulp of ripened fruit.

4.1.1.4. Discussion

The addition of cysteine to the double bond of pulegone results in the preferred formation of the *trans*-configured diastereoisomeric products, irrespective of the configuration at position C1 of the starting material. In combination with a subsequent purification step, the procedure is a suitable approach to obtain (1*R*,4*R*)- and (1*S*,4*S*)-8-*S*-L-cysteinyl-*p*-menthan-3-one in sufficient amounts in high purities. The corresponding *cis*-stereoisomers [(1*R*,4*S*)- and (1*S*,4*R*)-] are not accessible via this reaction pathway in pure form.
The ratio of *trans*- and *cis*-diastereoisomers of 8-mercapto-*p*-menthan-3-one obtained by Michael addition of hydrogen sulfide to pulegone had been reported as 2 : 1 (*Sundt et al., 1971*; *Kerkenaar et al., 1988*; *Köpke and Mosandl, 1992*). This indicates that the increased bulkiness of the cysteine residue compared to H_2S might be the reason for the more pronounced formation of *trans*-isomers resulting from the addition of cysteine.
However, this explanation is not in agreement with the high proportions of *cis*-stereoisomers resulting from the addition of homocysteine and glutathione, respectively, to pulegone. The *cis*-isomers of these conjugates may be stabilized by specific intramolecular interactions. More detailed NMR studies (e.g. NOE measurements) could be helpful to elucidate these phenomena.

8-Mercapto-*p*-menthan-3-one generated from 8-*S*-cysteinyl-*p*-menthan-3-one by β-lyase-catalyzed cleavage is a powerful flavor compound occurring in buchu leef oil (*Sundt et al., 1971; Lamparsky and Schudel, 1971*) and imparting the typical “cassis”-type aroma. The four stereoisomers have been shown to differ significantly in their sensory properties as shown in Figure 4.1.10 (*Köpke and*

Mosandl, 1992). From a flavoring point of view, the (1*S*,4*R*)- and the (1*R*,4*R*)-diastereoisomer seem to be the most desirable. Only the (1*S*)-configured diastereoisomers have been reported to occur naturally (*Köpke et al., 1994*). The *cis*-(1*S*,4*R*) isomer is predominant in genuine buchu leaf oil (*Kaiser et al., 1975; Köpke et al., 1994*). However, the ratio of *cis*- / *trans*-diastereoisomers is strongly influenced by the duration of distillation applied to obtain the essential oil. In a sample prepared by steam distillation for 8 h, a ratio of *cis*-(1*S*,4*R*) / *trans*-(1*S*,4*S*) of approximately 60 % / 40 % has been observed (*Köpke et al., 1994*).

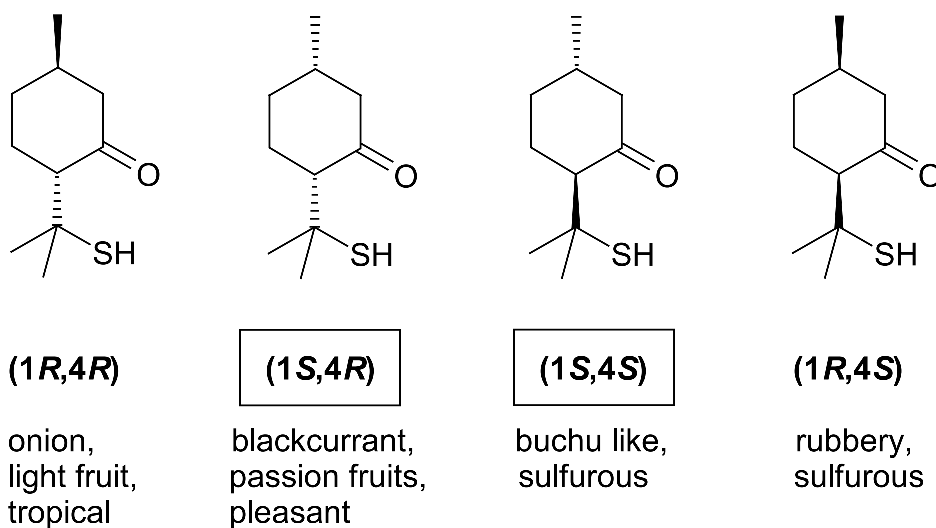


Figure 4.1.10 Flavor properties of 8-mercapto-*p*-menthan-3-one isomers (The diastereoisomers marked by boxes are those occurring in buchu leaf oil.)

The degree of enantioselectivity observed for the enzyme-catalyzed cleavages of cysteine conjugates by tryptophanase and the extract from *E. limosum* was low. As regards applications for preparative purposes, the discrimination of diastereoisomers of the pulegone conjugates is also moderate compared to other kinetic resolutions described (*Koskinen and Klibanov, 1996*).

E-values above 30 are regarded as exellent for kinetic resolutions. Those in a range between 15 and 30 are considered as moderate to good for practical

purposes (*Faber, 2000*). However, even kinetic resolutions exhibiting only low enantiomeric ratios can be exploited to obtain optically enriched compounds, if the target-enantiomer is the one being accumulated in the remaining substrate (*Straathof and Jongejan, 1997*). Therefore, the low enantioselectivity of tryptophanase observed towards a racemic mixture of the *trans*-configured stereoisomers with (1*S*)- and (1*R*)-configuration, respectively, can be used to obtain the naturally occurring (1*S*,4*S*)-diastereomer as product; the more attractive (1*R*,4*R*)-diastereoisomer could then released from the enantiomerically enriched remaining substrate via a non-specific way.
Regarding their diastereoselectivity, both enzyme preparations tested showed preference for the *cis*-configured stereoisomers. For tryptophanase this preference (*D* = 11) was more pronounced for the (1*R*)-configured substrates; the extract of *E. limosum* exhibited diastereoselectivity (*D* = 5) only for the naturally occurring (1*S*,4*R*)-stereoisomer. An exploitation of this diastereoselectivity for preparative purposes is hampered by the fact that the chemical synthesis of the cysteine conjugates of pulegone via Michael addition resulted in starting substrates in which the *cis*-configured diastereoisomers occur only at a proportion of approximately 5 %.
On the other hand, the addition of glutathione to pulegone was shown to result in much higher proportions of the *cis*-stereoisomers. Enzymatic cleavage of these conjugates using tryptophanase resulted in only low conversion into 8-mercapto-*p*-menthan-3-one. However, *S*-3-(hexan-1-ol)-glutathione has recently been detected in musts of Gros Manseng and Sauvignon blanc by liquid secondary ion mass spectrometry (*Peyrot des Gachons et al., 2002*) and it has been shown that *S*-3-(hexan-1-ol)-L-cysteine is generated by treatment of these musts by immobilized γ-glutamyltranspeptidase.

In vivo, glutathione conjugates are generated for detoxification and are metabolized to cysteine conjugates in the mercapturic acid pathway (*Cooper, 1998*). For haloalkenes it has been demonstrated that the enzyme-catalyzed addition of glutathione in mammals may proceed regio- and stereospecifically. In rat liver microsomal glutathione *S*-transferase catalyses the stereoselective addition of glutathione to chlorotrifluoroethene, whereas the cytosolic enzyme

exhibited no enantioselectivity (*Dekant, 2003*; *Hargus et al., 1991*).
The conformations of glutathione and cysteine conjugates of pulegone in plants systems, e.g. buchu leaves, have not studied yet. Based on the data gathered so far, it may be hypothesized that a reaction cascade comprising (i) the addition of glutathione to (1*S*)-pulegone, (ii) the cleavage of glutathione conjugates by γ-glutamyltranspeptidase, and (iii) the cleavage of the intermediate 8-L-cysteinyl-*p*-menthan-3-one by β-lyase eventually results in *cis*-configured (1*S*,4*R*)-8-mercapto-*p*-menthan-3-one. The contribution of these reactions and their stereochemical courses *in vivo* should be investigated in future studies.
In the described screening for additional β-lyases sources only acetone powders were used because of the limited access to fresh plant materials. Even this preliminary approach revealed that in addition to animals and microorganisms, plants constitute a huge reservoir to be exploited for selections of the suitable biocatalysts enabling the targeted generation of important sulfur-containing flavor compounds.

4.1.2. Cysteine conjugates of C_6-compounds

4.1.2.1. Syntheses and structural elucidations

Reaction between E-2-hexenal and L-cysteine

According to literature (*Tominaga et al., 1998b; Tominaga and Dubourdieu, 2000; Peyrot des Gachons et al., 2000*), 3-*S*-cysteinyl-1-hexanol can be synthesized by reaction of *E*-2-hexenal and L-cysteine and subsequent reduction of the resulting intermediate with sodium borohydride (Fig. 4.1.11)

E-2-hexenal + L-cysteine → [intermediate] → ($NaBH_4$) → 3-*S*-cysteinyl-1-hexanol

Figure 4.1.11 Synthesis pathway of 3-*S*-cysteinyl-1-hexanol as postulated in literature (*Tominaga et al., 1998b; Tominaga and Dubourdieu, 2000; Peyrot des Gachons et al., 2000)*

The product obtained from the reaction of *E*-2-hexenal and L-cysteine had been named as *S*-3-(hexan-1-al)-L-cysteine (*Tominaga et al., 1998b; Tominaga and Dubourdieu, 2000; Peyrot des Gachons et al., 2000*). However, no evidence has been provided for the existence of this intermediate. Therefore, the first goal of the present studies was to elucidate the structure of the conjugate generated by the reaction of *E*-2-hexenal and L-cysteine.

Investigations by GC and GC-MS (trimethylsilyl derivative), LC-MS, IR as well as by ^1H and ^{13}C NMR revealed 2-(2-*S*-L-cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid **1** (Figure 4.1.12) as product of the reaction between *E*-2-hexenal and L-cysteine.

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*)	(*S*)/(*R*)	(*R*)
C	0.8	*cis*	(*R*)	(*R*)	(*R*)/(*S*)	(*R*)
D	0.5	*cis*	(*R*)	(*R*)	(*S*)/(*R*)	(*R*)

Figure 4.1.12 Diastereoisomeric products resulting from the reaction between *E*-2-hexenal and L-cysteine.

The reaction does not stop at the level of the mono-adduct 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 as shown in Figure 4.1.12. This route has been described for other α,β-unsaturated aldehydes, such as acroleine and crotonaldehyde (*Esterbauer et al., 1976*).
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 (Fig. 4.1.12). 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}).

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 4.1.9). 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-$7_{A\text{-}D}$) 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 4.1.9). 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 (*Pesek, 1978*). NOESY experiments allowed to determine 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 4.1.9). 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 (*Restelli et al., 1990*). 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 < 13 Hz for *trans* (i.e., 11.2 Hz for isomer A and 12.3 Hz for isomer B, respectively) and around 16 Hz for cis configuration (15.7 Hz for isomer C and 16.1 Hz for isomer D, respectively).

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 (Fig. 4.1.12).

Table 4.1.9 NMR data of **1** (diastereoisomeric forms A, B, C and D)

position	chemical shifts			coupling constant	correlation pattern	
	δ^1H, ppm	$\delta^{13}C$, ppm		J_{HH}, Hz	COSY	NOESY
		observed	predicted[a]			
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, 7c, 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					
	3.72	56.0				
	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				4_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

Synthesis of 3-S-L-Cysteinylhexanol

E-2-hexenal + Boc-cysteine →

$NaBH_4$ →

HCl / Dioxane →

5

Figure 4.1.13 Synthesis of 3-*S*-L-cysteinyl-1-hexanol **5**

3-*S*-L-Cysteinyl-1-hexanol **5** was synthesized by Michael-type addition of Boc-L-cysteine to *E*-2-hexenal, reduction with sodium borohydride and subsequent acidolysis (Fig. 4.1.13). The GC-MS data of the TMS-derivative were in agreement with those previously reported (*Tominaga et al., 1998b*). The applied synthetic route, i.e. the addition of Boc-L-cysteine, allowed for the first time the isolation of the purified product in an amount sufficient for full structural elucidation via LC-MS, IR, ^{1}H and ^{13}C NMR. For the ^{13}C NMR signals of C-1, C-2, C-3, C-5 and C-7, splitting of signals (0.03 – 0.1 ppm) was observed under complete decoupling conditions. The signals of C-3 position appearing at 43.18 ppm and 43.28 ppm were completely separated. These signals revealed the presence of mixture of two diastereoisomers; from the signal intensities molar ratios of about 52 % : 48 % were estimated.

Synthesis of 3-S-(N-Acetylcysteinyl)hexanal

E-2-hexenal N-acetylcysteine 3

Figure 4.1.14 Synthesis of 3-*S*-(*N*-acetylcysteinyl)hexanal **3**

N-Acetyl-L-cysteine was used as reaction partner in the Michael addition to *E*-2-hexenal (Fig. 4.1.14) in order to avoid the formation of the thiazolidine derivative **1**. NMR and FT-IR analysis confirmed the presence of the aldehyde moiety in the resulting 3-*S*-(*N*-acetyl-L-cysteinyl)hexanal **3** (^{1}H NMR: 9.72 ppm; ^{13}C NMR: 201.5 ppm; FT-IR: 1720 cm^{-1}). Because of the unstability of **3** under the conditions of trimethylsilylation, the synthesized aldehyde was converted to the corresponding alcohol 3-*S*-(*N*-acetyl-L-cysteinyl)hexanol for further confirmation of the structure.

4.1.2.2. Enzymatic cleavage

Enzyme-catalyzed transformations of 2-(2-S-L-cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid ***1***

Adduct **1** was used as substrate for the same enzyme preparations which had been employed for the transformation of the cysteine conjugates of pulegone: (i) a commercially available tryptophanase from *E. coli* and (ii) a cell free extract obtained from *E. limosum*. As shown in Table 4.1.10, the application of both β-lyase sources 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.

Table 4.1.10 β-Lyase-catalyzed formation of 3-mercaptohexanal from 2-(2-*S*-L-cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid **1** [a]

enzyme	substrate (n mol)	3-mercaptohexanal (n mol)		conversion (%)	$E^{b,c}$	
		(*R*)	(*S*)		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 *Chen et al.* (*1982*).
[c] based on an exess of 10 % (*R*) (I) or 10 % (*S*) (II) at position 7 of substrate **1**.

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 mono-adducts and eventually the parent aldehydes (*Esterbauer et al., 1976*). Such an equilibrium has also been postulated for the formation of 2-furfuryl alcohol from the cysteine-furfural conjugate by baker´s yeast (*Huynh-Ba et al., 2003*). Therefore, it is likely that the formation of 3-mercaptohexanal proceeds via routes **a** and **b** outlined in Figure 4.1.15.
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 order of elution was determined by analysing an (*S*)-enriched sample obtained by a lipase-catalyzed kinetic resolution (see chapter 4.2.2). The data obtained were used to calculate conversion rates (*c*), enantiomeric excesses of product (ee_p) and enantioselectivities (*E*) applying the previously described equations for kinetic resolutions developed by *Chen et al.* (*1982*). 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.

Figure 4.1.15 Hypothetical pathways involved in the transformation of **1** by β-lyase and yeast, respectively, in aqueous media.

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

Enzyme-catalyzed cleavage of 3-S-(N-acetyl-L-cysteinyl)hexanal ***3***

In order 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. The resulting 3-*S*-(*N*-acetyl-L-cysteinyl)hexanal **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 (*Tomisawa et al., 1984; Tateishi et al., 1978*). However, after addition of an acylase to the reaction mixture (Fig. 4.1.16), i.e. an enzyme catalyzing a deacetylation (*Giardina et al., 1997*), a release of 3-mercaptohexanal was observed (Table 4.1.11).

Figure 4.1.16 Enzymatic cleavage of 3-*S*-(*N*-acetyl-L-cysteinyl)hexanal **3**

Table 4.1.11 Enzymatic formation of 3-mercaptohexanal from 3-*S*-(*N*-acetyl-L-cysteinyl)hexanal **3** by acylase I and β-lyases, according to Figure 4.1.16 [a]

enzyme	substrate (n mol)	3-mercaptohexanal (n mol)		conversion (%)	E^b	preferred product
		(*R*)	(*S*)			
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	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 *Chen et al.* (*1982*).

The amounts of products 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-cysteinyl-1-hexanol ***5***

3-*S*-L-cysteinyl-1-hexanol **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 agreement with those previously reported (*Tominaga et al., 1998b*). ^{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 4.1.12, **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.

Table 4.1.12 β-Lyase-catalyzed formation of 3-mercaptohexanol from 3-*S*-L-cysteinyl-1-hexanol **5** [a]

enzyme	substrate (n mol)	3-mercaptohexanol (n mol)		conversion (%)	E^{b}	preferred product
		(*R*)	(*S*)			
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 (*Chen et al., 1982*).

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. The order of elution was determined by analysing an (*S*)-enriched sample obtained by a lipase-catalyzed kinetic resolution (see chapter 4.2.2). 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 4.1.17). 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.

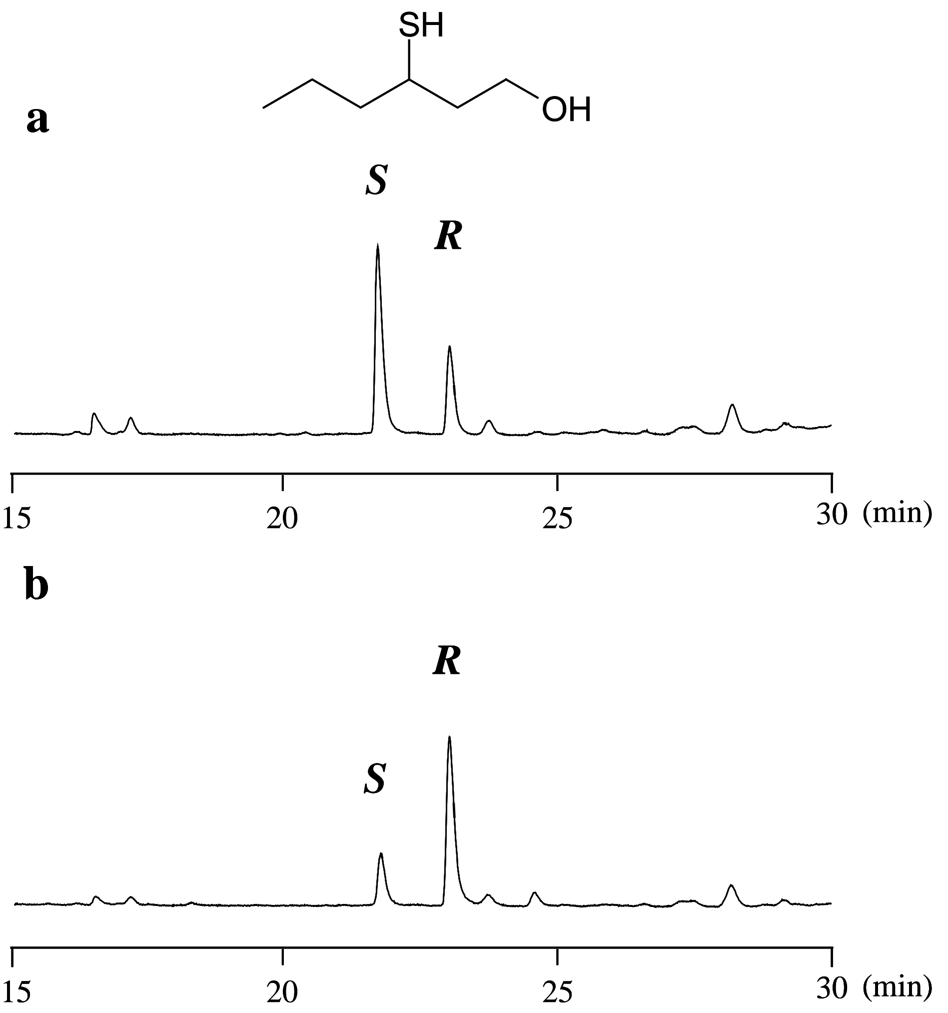


Figure 4.1.17 Enantiodifferentiation of 3-mercaptohexanol generated from 3-*S*-L-cysteinyl-1-hexanol **5** by tryptophanase (**a**) and a cell free extract from *E. limosum* (**b**).

(GC system V; 2500 nmol of substrate; for other conditions, see Materials and Methods.)

Enzyme-catalyzed cleavage by yeast

1 was accepted as substrate by different types of yeast (Table 4.1.13). Surprisingly, the reaction product was not 3-mercaptohexanal but 3-mercaptohexanol. Yeasts are well known to possess reductase activities, e.g. alcohol dehydrogenases (*Bränden et al., 1975*). Thus, it seems plausible that routes **c** or **d** outlined in Figure 4.1.15 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 (*Tominaga et al., 1998b*). The formation of 3-mercaptohexanol from **1** proceeded without preference of one of the enantiomers.

Table 4.1.13 Enzymatic transformation of 2-(2-*S*-L-cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid **1** and 3-cysteinyl-1-hexanol **5** by yeasts

enzyme source	temp. (°C)	time (day)	3-mercaptohexanol (n mol)		conversion (%)
			(*R*)	(*S*)	
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.

3-*S*-L-cysteinylhexanol has been proposed as precursor of 3-mercaptohexanol in wine (*Tominaga et al., 1998b; Peyrot des Gachons et al., 2000*). The wine yeast tested in this study exhibited only low activity towards **5** and showed no significant differences in the rates of release for the two enantiomers (Table 4.1.13).

4.1.2.3. Discussion

The data obtained demonstrate that stereoselectivity plays an important role in the formation of cysteine conjugates of α,β-unsaturated aldehydes and their cleavage into thiols by β-lyases. Taking into account the results reported for grapes (*Peyrot des Gachons et al., 2002*), the cysteine conjugates themselves seem to be breakdown products resulting from the enzyme-catalyzed degradation of the corresponding glutathione conjugates as outlined in Figure 4.1.18. S-3-(Hexan-1-ol)-glutathione has been identified in must from *Vitis vinifera* L. cv. Sauvignon blanc (*Peyrot des Gachons et al., 2002*). Upon treatment of the must with γ-glutamyltranspeptidase, the degradation of *S*-3-(hexan-1-ol)-glutathione into *S*-3-(hexan-1-ol)-cysteinylglycine and eventually *S*-3-(hexan-1-ol)-cysteine could be demonstrated. The role of *S*-3-(hexan-1-ol)-glutathione as precursor was also confirmed by reaction of the synthesized conjugate with γ-glutamyltranspeptidase. Assuming that the reaction sequence leading to the thiol starts with the addition of glutathione to *E*-2-hexenal, it remains unclear at which level the reduction step necessary to obtain the final mercaptoalcohol takes place. The facts that (i) *S*-3-(hexan-1-ol)-glutathione and *S*-3-(hexan-1-ol)-cysteine have been reported to occur naturally in grape and passion fruits, respectively, and (ii) 3-mercaptohexanal has not been identified in these plants indicate the reduction to take place already at the first step (**b** in Figure 4.1.18).

Nevertheless, the data obtained by transforming 2-(2-*S*-L-cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid, which is in equilibrium with the free 3-*S*-L-cysteinylaldehyde, by yeast cells demonstrate that under reducing conditions the alcohol 3-mercaptohexanol in principle could be formed from the

aldehyde precursor.

Data on the naturally occurring enantiomeric composition of 3-mercaptohexanol are only available for passion fruits. The thioalcohol has consistently been reported to be present as (*S*)-enantiomer; however, the enantiomeric excess varies considerably. The proportions of (*S*)-3-mercaptohexanol range from 64 % up to 93 % in fresh yellow passion fruits. In the purple variety an enantiomeric purity of 63 % (*S*) has been described (*Weber et al., 1994; Werkhoff et al., 1998*). These ratios are decreased down to 64 – 67 % (*S*) in yellow passion fruits concentrate and 58 – 61 % in nectar (*Weber et al., 1994*). On the other hand, the biogenetically related 3-methylthiohexanol, the 3-mercaptohexyl- and 3-methylthiohexyl esters, and the oxathiane have been reported to occur with ratios of the (*S*)-enantiomer higher than 90 % (*Weber et al., 1995*). One hypothesis forwarded to explain these at first glance contradicting results assumes that 3-mercaptohexanol is first biosynthesized in high enantiomeric excess of (*S*)-enantiomer, but this enantiomer is subsequently used for the highy stereoselective generation of the esters and the oxathiane. Consequently, the enantiomeric purity of the remaining 3-mercaptohexanol is decreased (*Weber et al., 1995*).

The data described for the kinetic resolution of 3-*S*-L-cysteinyl-1-hexanol **5** by tryptophanase and the cell free ectract from *E. limosum* demonstrate for the first time that in principle β-lyases are able to liberate 3-mercaptohexanol stereoselectively from the cysteine precursor. Further *in vivo* studies on the diastereoisomeric composition of 3-*S*-L-cysteinyl-1-hexanol and the stereoselectivities of β-lyases involved in their cleavage are necessary.

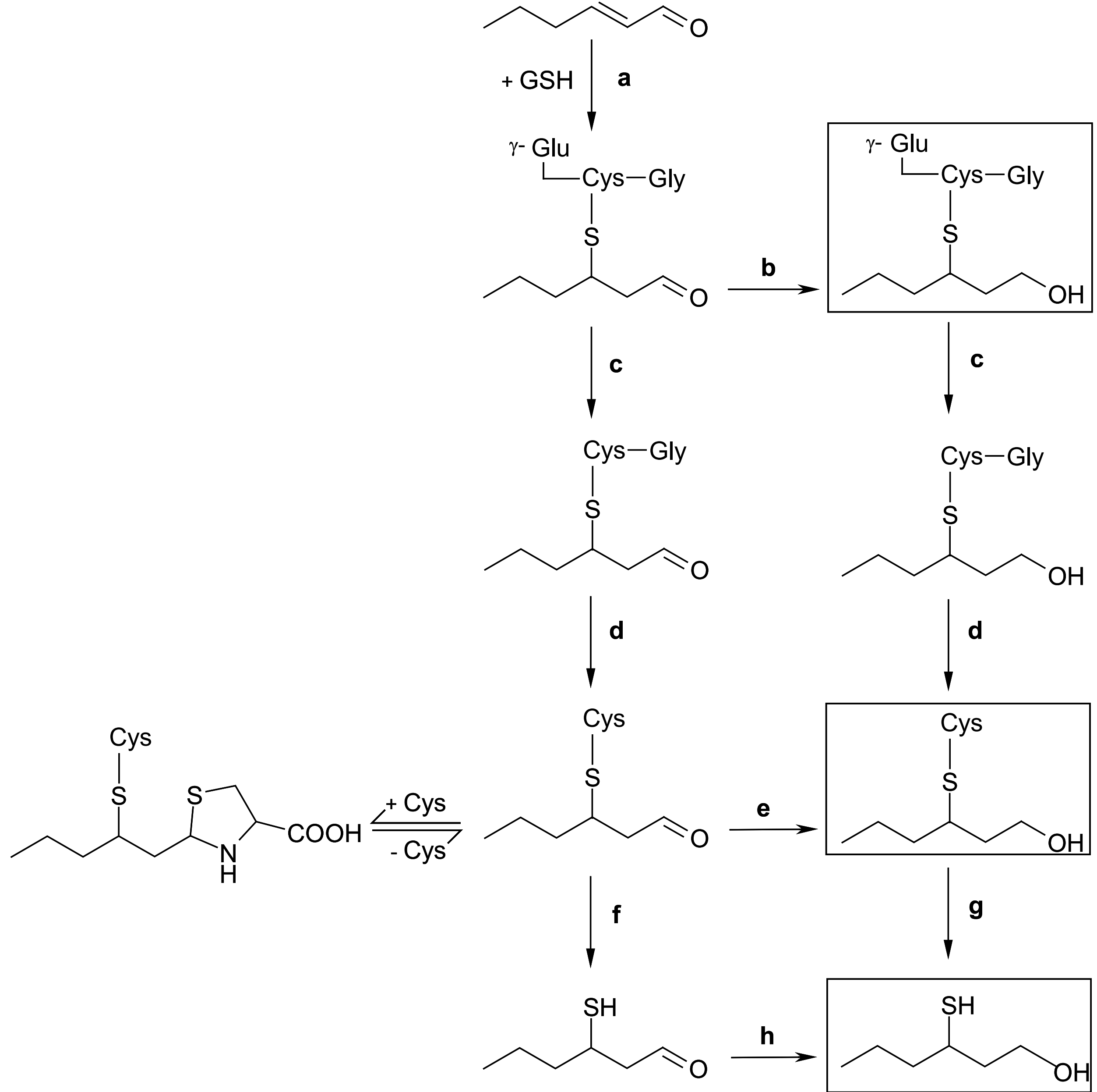


Figure 4.1.18 Reactions potentially involved in the transformation of sulfur-containing conjugates of *E*-2-hexenal into 3-mercaptohexanol in passion fruits and grape must.
(The compounds in boxes have been shown to occur naturally in the plant systems)
a: chemically and/or glutathione transferase-catalyzed;
b, **e**, **h**: reductase; **c**: γ-glutamyltransferase; **d**: dipeptidase;
f, **g**: cysteine conjugate β-lyase

4.1.3. β-Lyase-catalyzed transformations of other substrates

In chapters 4.1.1. and 4.1.2., the activities and selectivities of β-lyases towards cysteine conjugates of pulegone and C_6-compounds as substrates have been demonstrated. To get an idea on structural features determining the acceptance of cysteine conjugates by β-lyases, a spectrum of precursors having structures related to 3-*S*-cysteinyl-1-hexanol **1** and 8-*S*-cysteinyl-*p*-menthane-3-one **3** were synthesized and employed as substrates.

4.1.3.1. Syntheses and structural elucidations

The structures of the synthesized compounds are shown in Figure 4.1.19.
The structural modifications were guided by the following considerations:

(i) By moving the binding position of cysteine, the secondary alkylcysteine *S*-conjugate **1** was changed to the positional isomer 6-*S*-cysteinyl-1-hexanol **2**.

(ii) 4-*S*-Cysteinyl-4-methyl-2-pentanone **4** was chosen as one of the structural elements in 8-*S*-cysteinyl-*p*-menthane-3-one **3**. This basic skeleton was modified by demethylation (4-*S*-cysteinyl-2-pentanone **5**), chain elongation (4-*S*-cysteinyl-2-heptanone **6**, 4-*S*-cysteinyl-2-octanone **7** and 4-*S*-cysteinyl-2-nonanone **8**) and cyclization (3-*S*-cysteinylcyclo-hexanone **9**).

(iii) To test the importance of the keto-function, the alcohols 3-*S*-cysteinylcyclo-hexanol **10** and 2-*S*-cysteinylcyclohexanol **11** were synthesized.

(iv) *S*-Benzylcysteine **12** has been widely used as standard substrate to test for activities of β-lyases. To study the importance of the aromatic moiety and the cyclic structure of this substrate, *S*-(cyclohexylmethyl)cysteine **13** and *S*-*n*-heptylcysteine **14** were synthesized.

(v) Baker's yeast has been reported to show β-lyase-like activity towards 2-substituted thiazolidine-4-carboxylic acids. For example, 2-phenyl-1,3-thiazolidine-4-carboxylic acid **15** and 2-furyl-1,3-thiazolidine-4-carboxylic acid **16** have been shown to be converted into benzylmercaptane and

furfurylmercaptane, respectively, by baker's yeast (*Huynh-Ba et al., 1998; 2003*). To determine whether these substrates would be accepted by other β-lyases, these two thiazolidine compounds as well as derivatives with non-aromatic (2-cyclohexyl-1,3-thiazolidine-4-carboxylic acid **17**) and non-cyclic (2-*n*-hexyl-1,3-thiazolidine-4-carboxylic acid **18**) moieties were synthesized as substrates.

4-*S*-Cysteinyl-4-methyl-2-pentanone **4** was synthesized by addition of cysteine to mesityl oxide, as described previously (*Tominaga et al., 1998b*; *Starkenmann, 2003*). The other substrates related to this structure, i.e. **5**, **6**, **7** and **8** were also obtained by Michael addition to the corresponding α,β-unsaturated precursors. The cyclic conjugate **9** was derived via Michael addition from 2-cyclohexenone; **10** was obtained by subsequent reduction. The conjugates **2**, **11**, **13** and **14** were synthesized via nucleophilic substitution reactions (*Vince and Wadd, 1969*). The 2-substituted thiazolidine-4-carboxylic acids **17** and **18** were obtained by cyclic condensations as described by *Huynh-Ba et al.* (*2003*). The gas chromatographic and mass spectrometric data of these conjugates are given in chapter 3.2.1; except for **4**, these data are reported for the first time.

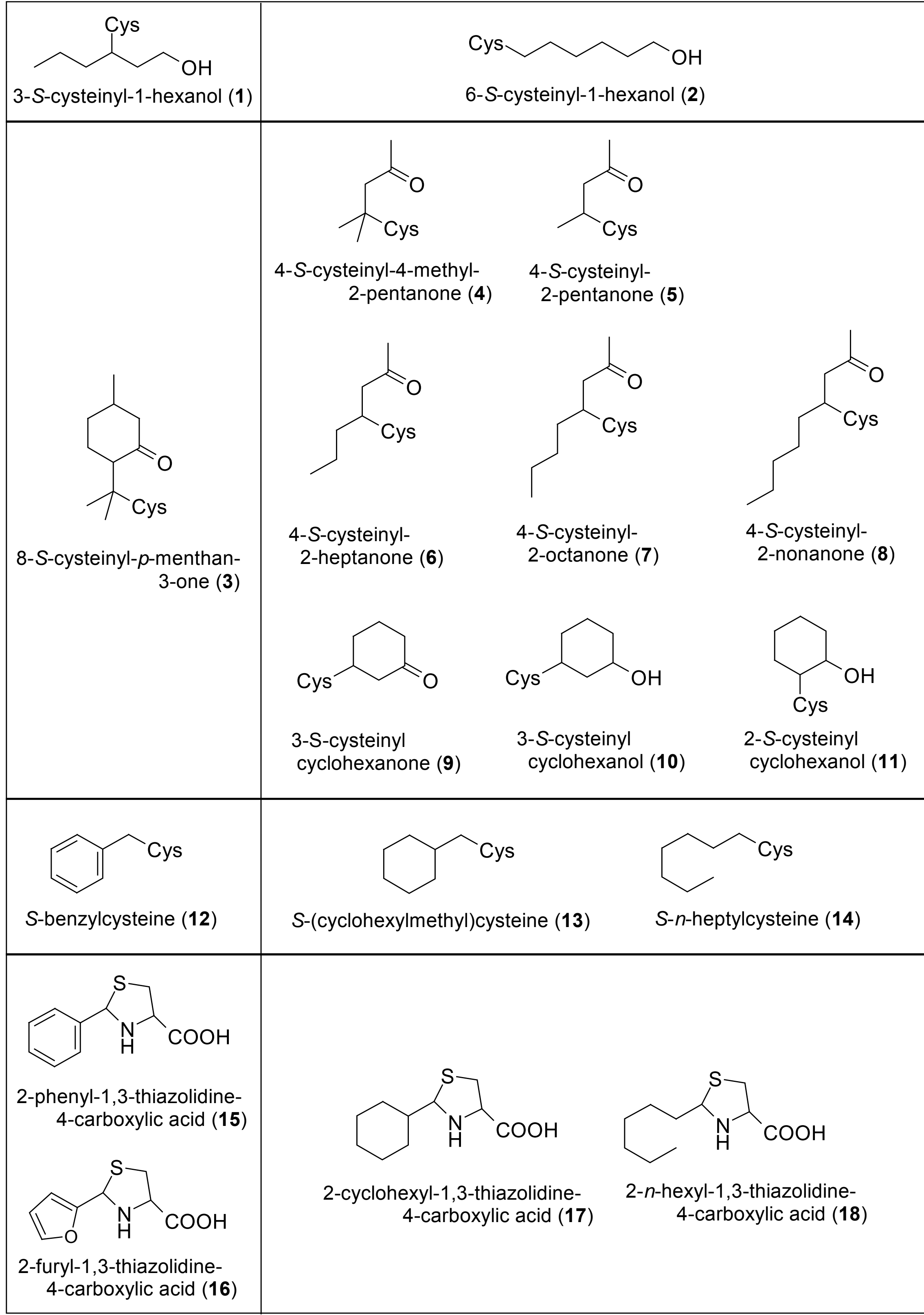


Figure 4.1.19 Substrates synthesized for studying structural effects on the activities of β-lyases

4.1.3.2. Enzymatic transformations

The influence of structural modifications of the substrates on the activities of β-lyases is shown in Tables 4.1.14 and 4.1.15.

The activity of tryptophanase observed for the secondary alkylcysteine *S*-conjugate 3-*S*-cysteinyl-1-hexanol **1** was lower than that for the primary 6-*S*-cysteinyl-1-hexanol **2**. An increase of the hydrophobicity of **2** (replacement of the hydroxy moiety by a methyl group resulting in *S*-*n*-heptylcysteine **14**) had no significant impact on the activity.

The activity of tryptophanase towards 4-*S*-cysteinyl-4-methyl-2-pentanone **4** was significantly lower than the one observed for 8-*S*-cysteinyl-*p*-menthan-3-one **3**. Apparently, the 4-*S*-cysteinyl-4-methyl-2-pentanone skeleton is not sufficient to mimic **3** as substrate for tryptophanase. Surprisingly, the activity of tryptophanase towards the demethylated secondary cysteine *S*-conjugate 4-*S*-cysteinyl-2-pentanone **5** was even slightly higher than that towards **3**. This effect was not observable for the enzyme preparation of *E. limosum*. The acceptance by tryptophanase of 4-*S*-cysteinyl-2-heptanone **6**, 4-*S*-cysteinyl-2-octanone **7** and 4-*S*-cysteinyl-2-nonanone **8** resulting from elongation of the aliphatic chain of **5** was drastically reduced. The formation of a ring structure (3-*S*-L-cysteinylcyclohexanone **9**) increased the acceptance by tryptophanase compared to the acyclic substrates **6** - **8**. The essential role of the keto function becomes obvious when comparing the activities determined for **9** and the respective hydroxy derivative **10**. Surprisingly, a change of the position of the hydroxy group from 3 to 2 (2-*S*-L-cysteinylcyclohexanol **11**) re-increased the acceptance as substrate by tryptophanase.

In contrast to the effects observed for tryptophanase, the use of the simplified 4-*S*-L-cysteinyl-2-pentanone skeletone (**5**) decreased the activity of the *E. limosum* extract compared to 8-*S*-cysteinyl-*p*-menthan-3-one **3**. Neither elongation of the aliphatic chain (**6** - **8**) nor formation of a cyclic structure (**9**) could significantly improve the acceptance of these substances as substrates by the *E. limosum* extract.

Comparison of the activities observed for *S*-benzyl-L-cysteine **12** to those determined for the non-aromatic substrate **13** and the acyclic homologue **14**

demonstrates the importance of the benzyl moiety for tryptophanase-catalyzed cleavages. This effect was even more pronounced for baker's yeast; **13** and **14** were not accepted as substrates at all.

2-Substituted-1,3-thiazolidine-4-carboxylic acid-type substrates were not accepted as substrates by tryptophanase. The reported generation of thiols by yeast-catalyzed transformations of **15** and **16** (*Huynh-Ba et al., 1998; 2003*) could not be fully confirmed. Under the conditions applied in the present study, only **16** was accepted by baker's yeast. The modified substrates **17** and **18** were not accepted as substrates at all.

Table 4.1.14 Effect of structural modifications of substrates on the activities of β-lyases

Substrate[a]	Generated thiol (n mol)	
	tryptophanase	*E. limosum*
3-*S*-L-cysteinyl-1-hexanol (**1**)	23.4	38.1
6-*S*-L-cysteinyl-1-hexanol (**2**)	48.0	-[b]
8-*S*-L-cysteinyl-*p*-menthan-3-one (**3**)	121.9	106.0
4-*S*-L-cysteinyl-4-methyl-2-pentanone (**4**)	32.3	-
4-*S*-L-cysteinyl-2-pentanone (**5**)	157.8	20.5
4-*S*-L-cysteinyl-2-heptanone (**6**)	28.1	33.2
4-*S*-L-cysteinyl-2-octanone (**7**)	40.0	36.8
4-*S*-L-cysteinyl-2-nonanone (**8**)	15.6	23.8
3-*S*-L-cysteinylcyclohexanone (**9**)	79.1	18.5
3-*S*-L-cysteinylcyclohexanol (**10**)	11.8	-
2-*S*-L-cysteinylcyclohexanol (**11**)	80.6	-

[a] amount of substrate: 2500 nmol (except for the reactions of (**9**): 440 nmol, (**10**): 380 nmol, (**11**): 670 nmol)

[b] not determined

for other conditions, see Materials and Methods

Table 4.1.15 Effect of structural modifications of substrates on the activities of β-lyases

Substrate[a]	Generated thiol (n mol)		
	Tryptophanase	Baker's yeast	Beer yeast
S-benzyl-L-cysteine (**12**)	92.5	5.6	5.9
S-(cyclohexylmethyl)-L-cysteine (**13**)	45.0	0	-[b]
S-*n*-heptyl-L-cysteine (**14**)	50.4	0	-
2-phenyl-1,3-thiazolidine-4-carboxylic acid (**15**)[c]	0	0	3.1
2-furyl-1,3-thiazolidine-4-carboxylic acid (**16**)[c]	0	21.0	-
2-cyclohexyl-1,3-thiazolidine-4-carboxylic acid (**17**)[c]	0	0	-
2-*n*-hexyl-1,3-thiazolidine-4-carboxylic acid (**18**)[c]	0	0	-

[a] amount of substrate : 2500 nmol
[b] not determined
[c] diastereomeric mixture of (2*R*,4*R*) and (2*S*,4*R*)
for other conditions, see Materials and Methods

4.1.3.3. Discussion

The data demonstrate that a broad spectrum of cysteine conjugates may act as substrates for β-lyases. On the other hand, the clear differences observed between tryptophanase and the extract from *E. limosum* regarding the generation of thiols from the series of 4-*S*-cysteinyl-2-alkanones showed that β-lyases from different sources may have very specific activity profiles. Considering the lack of systematic studies, the suitability of β-lyase preparations for the cleavage of specific substrates would have to be tested on a case-by-case basis.

The stereochemical course of these reactions has not been followed in these experiments. Taken into account the data shown for the cysteine conjugates of pulegone and *E*-2-hexenal, this is expected to be a further decisive criterion differentiating enzyme preparations and qualifying them for specific purposes.

The fact that the results demonstrated for the generation of thiols from 2-phenyl-1,3-thiazolidine-4-carboxylic acid **15** and 2-furyl-1,3-thiazolidine-4-carboxylic acid **16** by yeast (*Huynh-Ba et al., 1998; 2003*) could not be fully

confirmed might be explained by the use of different yeast strains. The strict limitation of the activity of baker's yeast to **16** observed under the conditions of this study indicates the strong structural influence on the course of the enzyme-catalyzed cleavage of this class of substrates.

Tryptophanase showed no activity for the thiazolidine compounds **15**-**18**, at all. This indicates that the reported activities of yeasts for thiazolidine compounds (*Huynh-Ba et al., 1998; 2003*) might be caused by enzymes other than C-S β-lyase. Considering the importance of yeast as "food-grade" biocatalyst, the investigation of this question should deserve further attention.

4.2. Lipase-catalyzed transformations of thioesters

4.2.1. Syntheses of thioesters

3-Acetylthiohexanal **1** was synthesized by Michael-type addition of thioacetic acid to *E*-2-hexenal. 3-Acetylthiohexanol **3** was obtained by subsequent reduction with sodium borohydride under controlled pH-conditions to prevent alkaline hydrolysis of the thioester moiety and the formation of by-products as described by *Rowe and Tangel* (*1999*). These reactions are outlined in Fig. 4.2.1. The identities of the compounds were confirmed by means of GC-MS and NMR.

O + HS O → S O O **1**

S O O $NaBH_4$ buffer (pH7.4) → S O OH **3**

Figure 4.2.1. Synthesis of 3-acetylthiohexanal **1** and 3-acetylthiohexanol **3**

4.2.2. Lipase-catalyzed kinetic resolutions of thioesters

4.2.2.1. Activities and enantioselectivities of lipase preparations

Commercially available enzyme preparations (fifteen lipases and one esterase) of various origins (microbial, plant, mammalian) were tested for their suitability to hydrolyze the thioester bond in 3-acetylthiohexanal as shown in Figure 4.2.2.

lipase / H_2O

Figure 4.2.2 Lipase-catalyzed enantioselective hydrolysis of 3-acetylthiohexanal **1**

Conversion rate and sterochemical course of the reaction were followed by means of capillary gas chromatography. As shown in Table 4.2.1, 3-acetylthiohexanal **1** was accepted as substrate by all enzymes tested. Retention index and MS spectrum of the generated 3-mercaptohexanal **2** were identical to data from literature (*Werkhoff et al., 1998*) and to those obtained from a synthesized reference compound. Formation of the product by chemical cleavage of substrate could be ruled out by incubation under the same conditions without enzymes.

Using heptakis(per-*O*-ethyl)-β-cyclodextrin (PerEt-β-CD) and heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin (DiMe-β-CD) as chiral stationary phases, the enantiomers of 3-mercaptohexanal **2a,b**, 3-acetylthiohexanal **1a,b** and 3-acetylthiohexanol **3a,b** could be separated for the first time (Fig. 4.2.3). This enabled the determination of the enantiomeric excesses of product (ee_p) and remaining substrate (ee_s) by means of capillary GC. The data obtained were used to calculate conversion rates (c) and enantioselectivities (E) applying the equations developed for kinetic resolutions (*Chen et al., 1982*) as shown in eq. 6.

$$E = \frac{\ln\left[\dfrac{1-ee_S}{1-\dfrac{ee_S}{ee_P}}\right]}{\ln\left[\dfrac{1-ee_S}{1+\dfrac{ee_S}{ee_P}}\right]} \qquad \text{(eq. 6)}$$

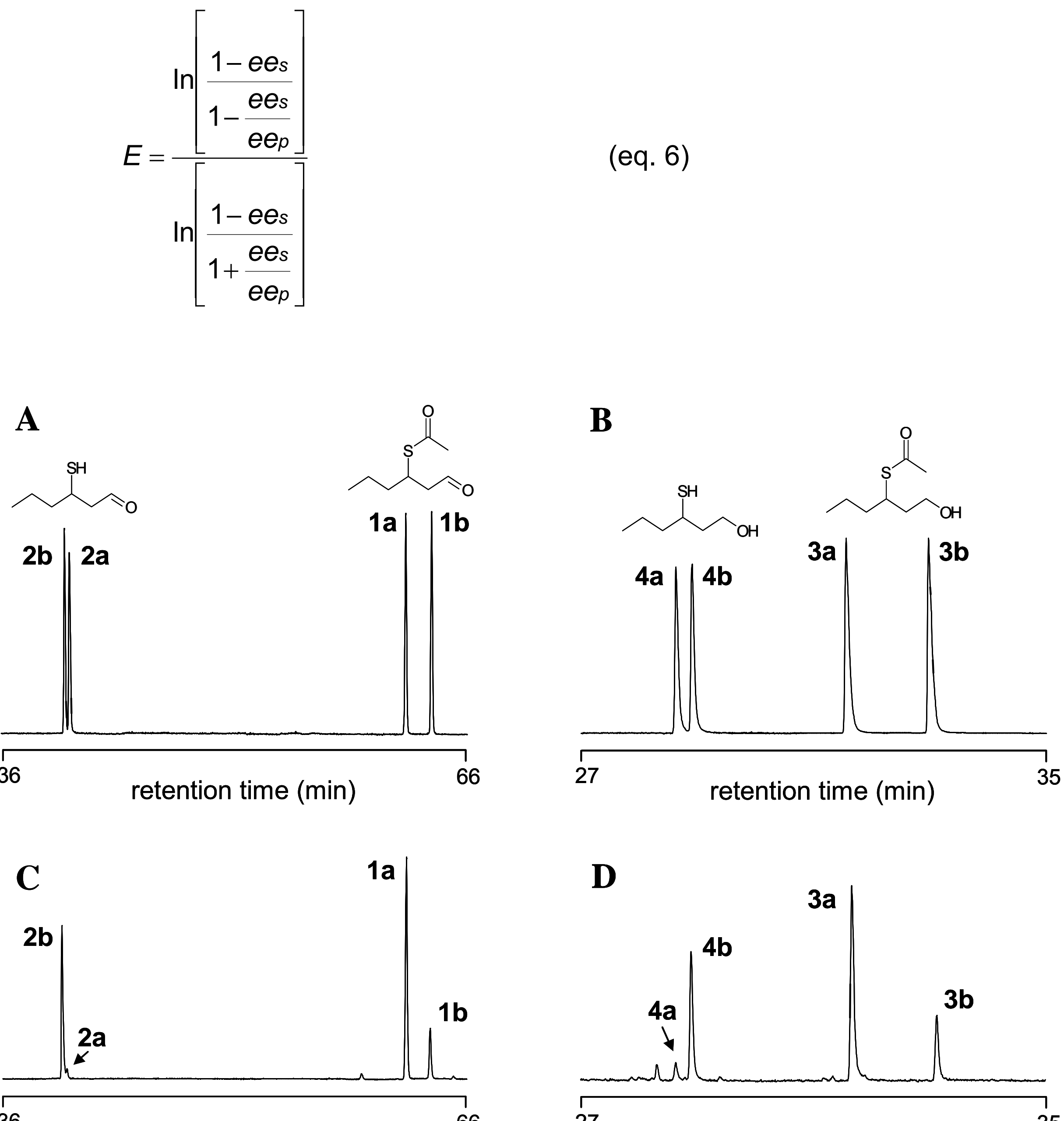


Figure 4.2.3 Capillary GC separation of **A**: racemic 3-acetylthiohexanal **1** and 3-mercaptohexanal **2** (GC system III); **B**: racemic 3-acetylthiohexanol **3** and 3-mercaptohexanol **4** (GC system IV); **C**,**D**: enantiomerically enriched compounds obtained after kinetic resolution (CAL-B, 8h).
Enantiomers **a**: (*R*)-configuration; enantiomers **b**: (*S*)-configuration.

As shown in Table 4.2.1, the enzyme preparations tested differed strongly in terms of degree of enantiodiscrimination.

Table 4.2.1 Enzyme-catalyzed kinetic resolution of 3-acetylthiohexanal **1**

enzyme	enantiomeric excess (%)		c^a (%)	E^b	preferred enantiomer
	ee_s	ee_p			
Rhizopus oryzae lipase	0.9	18.4	4.9	1.5	(*R*)
Aspergillus niger lipase	21.0	27.2	43.5	2	(*R*)
Wheat germ lipase	17.8	37.1	32.4	3	(*R*)
Mucor javanicus lipase	0.5	4.1	2.2	1.1	(*S*)
Penicillium roqueforti lipase	0.9	2.3	4.1	1.1	(*S*)
Mucor miehei lipase	0.5	9.1	2.1	1.2	(*S*)
Pseudomonas cepacia lipase	2.1	29.7	6.7	2	(*S*)
Porcine pancreas lipase	4.3	29.6	12.6	2	(*S*)
Candida rugosa lipase	8.2	50.2	14.1	3	(*S*)
Porcine liver esterase	32.0	41.2	43.7	3	(*S*)
Aspergillus oryzae lipase	5.6	55.0	9.3	4	(*S*)
Thermomyces lanuginosus lipase	30.4	76.3	28.5	10	(*S*)
Candida antarctica lipase	2.1	45.0	4.6	3	(*S*)
Candida antarctica lipase A	21.1	66.1	24.2	6	(*R*)
Candida antarctica lipase B	51.1	91.1	35.9	36	(*S*)
Candida antarctica lipase B[c]	36.5	96.7	27.4	85	(*S*)

[a] conversion rate
[b] enantioselectivity
[c] immobilized enzyme adsorbed on a macroporous resin.

The fact that there was no consistent preference of the same enantiomer may be explained by the structure of the substrate. According to a rule established for esters of secondary alcohols, the substrates resolved most efficiently by lipase-catalyzed hydrolyses are those having substituents which differ significantly in size, and the enantiomer preferred by the enzyme can be predicted (*Kazlauskas et al., 1991; Cygler et al., 1994*). When the alcohol is

drawn with the hydroxyl group pointing backward, the favored enantiomer bears a large substituent on the left, e.g. phenyl, and a medium substituent on the right, e.g. methyl. This rule is shown in Figure 4.2.4.

lipase
Pseudomonas cepacia
Cholesterol esterase
Candida rugosa (R>C3)
(R) + (S)
L: large
M: medium

Figure 4.2.4 Enantioselective course of lipase-catalyzed kinetic resolution of esters according to rule established by Kazlauskas et al., 1991

This rule could also be confirmed for hydrolysis and interesterification, respectively, of corresponding esters of secondary thiols (*Baba et al., 1990; Öhrner et al., 1996*). For 3-acetylthiohexanal, however, the difference in size between the substituents at the asymmetric center seems not to be sufficient to induce such a strict course of enantioselection.

It is noteworthy that the four commercial preparations of *Candida antartica* lipase employed as catalysts differed significantly in their enantioselectivities. This yeast produces two different lipases (A and B) which have been purified and characterized (*Patker et al., 1993*). Both have been cloned and expressed in *Aspergillus oryzae* (*Høegh et al., 1995*). The original lipase preparation from the yeast (CAL) exhibited only low selectivity for the (*S*)-configured substrate ($E = 3$). This preference was significantly enhanced ($E = 36$) when using the lipase component B (CAL-B) obtained from recombinant *Aspergillus oryzae*. In contrast, the heterologously expressed lipase A (CAL-A) showed preference for the opposite (*R*)-enantiomer ($E = 6$).

4.2.2.2. Determination of absolute configurations

Determination of the absolute configurations of compounds is one of the most difficult and important steps when studying the stereoselectivity of reactions. Recently an elegant method for determining the absolute configuration of 2- and 3-sulfanyl-1-alkanols has been proposed (*Weckerle et al., 2001*). They used the 9-anthroate chromophore for the derivatization of sulfanyl-1-alkanols and determined the configurations by circular dichroism measurement.

On the other hand, the conversion of the target compound into a product of known configuration via stereochemically defined reactions can also be used to assign the conformation.

The most pronounced enantiodiscrimination was observed for *Candida antartica* lipase. The optically enriched product and remaining substrate obtained by the kinetic resolution using CAL-B as catalyst were used to assign the absolute configurations. The order of elution of the enantiomers of 3-mercaptohexanol on DiMe-β-CD had been determined previously (*Weber et al., 1994*). Thus, the orders of elution for the enantiomers of 3-mercaptohexanal, 3-acetylthiohexanal and 3-acetythiohexanol could be assigned by transforming the enantiomerically enriched compounds into 3-mercaptohexanol, using the series of reactions outlined in Figure 4.2.5. Due to the non-enantioselective course of these reactions, the enantiomeric ratios determined after each step were in accordance with the starting ratios (apart from a slight racemization observed for the alkaline hydrolysis). 3-Mercaptohexanol obtained by reduction of the mixture obtained after the enzyme-catalyzed resolution with sodium borohydride and subsequent selective extraction of the thiol using *p*-hydroxymercuribenzoate proved to be the (*S*)-enantiomer **4b** (*e.e.* 60 %). The enantiomer **4a** obtained by alkaline hydrolytic cleavage of the remaining thioester **3a** (*e.e.* 92 %) was shown to have the (*R*)-configuration (*e.e.* 86 %).

O
S
O
1a,b (*e.e.* 0%)

CAL-B (rt, 8h; *c* = 60%)

O
S
O
1a (*e.e.* 90%)

+

SH
O
2b (*e.e.* 60%)

$NaBH_4$
p-HMB extraction

O
S
OH
3a (*e.e.* 92%)

SH
OH
4b (*e.e.* 60%)

NaOH

SH
OH
4a (*e.e.* 86%)

Figure 4.2.5 Sequence of reactions applied to convert product and substrate of the kinetic resolution of 3-acetylthiohexanal into 3-mercaptohexanol in order to determine their absolute configurations.

4.2.2.3. Influence of immobilization

The most pronounced discrimination (E = 85) was observed for the enzyme preparation with CAL-B immobilized on a macroporous acrylic resin. This enhancement of enantioselectivity may be explained by the increased rigidity of the enzyme conformation due to interactions with the polymer. Similar phenomena had been described for the lipase from *Candida cylindracea* immobilized on agarose and silica gel (*Sánchez et al., 1996*).

In practice, immobilization of enzymes may be useful to overcome problems such as stability against auto-oxidation, self-digestion and denaturation by the solvent or to allow repeated use of enzymes which is important to ensure their economic application.

A partial adsorption of the thiol product on the resin (from 20 to 75 %, depending on enzyme and substrate concentrations) turned out to be a disadvantage of using CAL-B in the immobilized form. Extraction of the removed resin with dichloromethane revealed that the adsorbed 3-mercaptohexanol had the same enantiomeric composition as the portion still present in the buffer solution. Therefore, the increased enantioselectivity obtained by using immobilized CAL-B is not due to enantiodiscriminating phenomena involved in adsorption / desorption.

The influence of immobilizations on the stereospecificity of enzymes has not been studied in detail. Therefore, at present, predictions about the effects of different types of immobilization are difficult to make.

4.2.2.4. Influence of co-solvent

It had been reported that the enantioselectivity of CAL-B in the hydrolysis of esters can be enhanced by addition of water-miscible organic solvents; by using acetone and *tert*-butanol, the *E*-value could be raised from 7 to 220 (*Hansen et al., 1995*). As shown in Table 4.2.2, the enantioselectivity of the hydrolysis of 3-acetylthiohexanal was not influenced by the presence of acetone at a level of 10 vol %; higher concentrations of this co-solvent actually resulted in a decrease

of *E*. Addition of *tert*-butanol significantly improved the enantiodiscrimination up to a level of 20 vol %; higher proportions again resulted in lower enantioselectivity.

Table 4.2.2 Effects of co-solvents on the enantioselectivity of the hydrolysis of 3-acetylthiohexanal catalysed by CAL-B.

Co-solvent	concentration (vol %)	enantiomeric excess (%)		conversion (%)	E^a
		ee_s	ee_p		
-	-	51.1	91.1	35.9	36
acetone	10	36.3	92.5	28.2	35
	20	18.6	89.2	17.3	20
	40	9.9	87.1	10.2	16
tert-butanol	10	47.0	93.5	33.5	47
	20	45.9	93.8	32.9	49
	40	22.2	91.8	19.5	29

[a] enantioselectivity

It is well-known that lipases change their configurations at lipophilic interfaces and show higher activity beyond critical micellar concentration, called "interfacial-activation". It is easy to imagine that the lipophilicity of the medium affects the configuration of lipases which in turn relates to the selectivity of enzyme for certain reactions.

An impressive example for this effect is the kinetic resolution of mesifuran (2,5-dimethyl-4-methoxy-3(2*H*)-furanone), one of the key volatiles found in strawberries. The (+)-enantiomer, reported to have a more intensive and fruity note than the racemate (*Fischer and Hammerschmidt, 1992; Ochi et al., 1995*), was prepared from racemic mesifuran using an enzyme-catalyzed reaction. Among 49 commercially available lipases, the lipase from *C. antarctica* gave the best result (47.9 % e.e.); by using 50 % diisopropylether as co-solvent the enantiomeric excess of the product could be increased up to 96 % (*Nozaki et al., 2000*).

4.2.2.5. Influence of structural modifications

The effects of the replacement of the aldehyde function in the thioester substrate by an alcoholic group on enzyme activities and enantioselectivities are summarized in Table 4.2.3. The preference of enantiomers remained the same as observed for the hydrolysis of 3-acetylthiohexanal. However, the lipases from *Thermomyces lanuginosus* and *Candida antarctica* lipases exhibited conversion rates as well as enantioselectivities significantly lower than those for the aldehyde substrate. CAL-A even showed no enantiodiscrimination when catalyzing the hydrolysis of 3-acetylthiohexanol.

Table 4.2.3 Enzyme-catalyzed kinetic resolution of 3-acetylthiohexanol and 3-benzoylthiohexanal.

enzyme	enantiomeric excess (%)		c^a (%)	E^b	preferred enantiomer
	ee_s	ee_p			
3-acetylthiohexanol					
Aspergillus niger lipase	93.9	6.3	93.8	3	(*R*)
Wheat germ lipase	82.0	38.0	68.4	5	(*R*)
Thermomyces lanuginosus lipase	1.3	19.1	6.2	2	(*S*)
Porcine pancreas lipase	2.9	52.4	5.2	3	(*S*)
Porcine liver esterase	64.8	24.5	72.6	3	(*S*)
Candida antarctica lipase	0.7	35.9	1.9	2	(*S*)
Candida antarctica lipase A	0.2	6.8	3.5	1	-
Candida antarctica lipase B	27.1	81.1	25.1	12	(*S*)
3-benzoylthiohexanal					
Aspergillus niger lipase	0.1	2.9	0.9	1	-
Porcine liver esterase	6.9	71.3	10.1	6	(*S*)
Candida rugosa lipase	0.7	54.6	1.2	3	(*S*)

[a] conversion rate
[b] enantioselectivity

Menthylbenzoate has been reported as suitable starting material to obtain (-)-menthol via hydrolysis catalyzed by *C. rugosa* lipase (*Gatfield et al., 2002*). In order to study the effect of a bulkier acyl residue on the kinetic resolution, 3-benzoylthiohexanal was employed as substrate. The synthesis was performed by addition of thiobenzoic acid to *E*-2-hexenal. Capillary GC separation of the enantiomers was achieved on DiMe-β-CD as chiral stationary phase (α: 1.02; K1: 42.3; R: 1.13; 145 °C isothermal; hydrogen 31.4 cm/sec). When using CAL, CAL-A, CAL-B, TLL, PPL and WGL as biocatalysts the conversion rates observed after 2 h were negligible (< 0.1 %). Data obtained for ANL, PLE and CRL are shown in Table 4.2.3. The replacement of the acetyl moiety by a bulky group drastically reduced the conversion rates without significant impact on the enantioselectivities.

4.2.3. Sensory properties of thioesters and thiols

Odor descriptions of 3-acetylthiohexanal, 3-acetylthiohexanol and 3-mercaptohexanal were determined by means of gas chromatography/ olfactometry (GC/O). As shown in Table 4.2.4, the sulfur-containing volatiles exhibited attractive tropical citrus-type notes.

The 1,3-oxygen-sulfur position has been discussed as the essential structural feature resulting in tropical odor (*Rowe and Tangel 1999; Rowe, 2002*). This basic sensation is modified towards specific notes such as savory, vegetable or catty by variation of the substituents of the olfactophore skeleton, as shown in Figure 4.2.6.

Table 4.2.4 Odor properties of 3-acetylthiohexanal, 3-acetylthiohexanol and 3-mercaptohexanal, determined by GC/O.

compound	racemic mixture	(*R*)-enantiomer	(*S*)-enantiomer
3-acetylthiohexanal [a]	grapefruit, citrus peel, sweet	sulfurous, roasted, citrus peel	fruity, sweet, grapefruit
3-acetylthio-1-hexanol [b]	citrus peel, sulfurous, fruity	fruity, grapefruit, sulfurous	sulfurous, roasted, rubber like
3-mercaptohexanal [c]	sulfurous, citrus peel	sulfurous, rubber like	green, citrus peel, fruity

amounts at GC sniffing port: [a] 0.1 µg (racemic mixture) and 1.0 µg (enantiomers); [b] 0.07 µg (racemic mixture) and 0.3 µg (enantiomers); [c] 0.01 µg (racemic mixture) and 0.04 µg (enantiomers). GC/O systems I (racemic mixture) and II (enantiomers) were used.

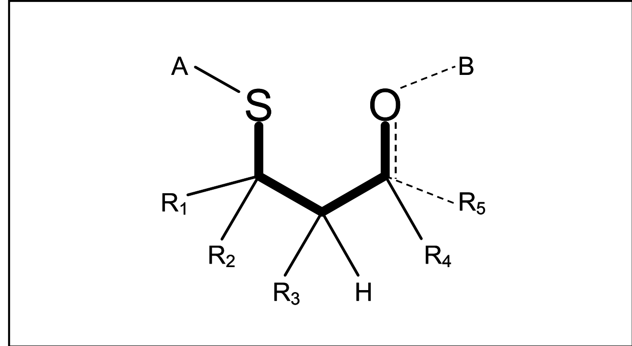


Figure 4.2.6 The "tropical" olfactophore according to *Rowe, 2002*
A: H, SCH_3, ring; **B**: H, CH_3, acyl, absent if carbonyl;
R_1, **R_2**: H, alkyl; **R_3**; H, alkyl, ring; **R_4**: H, CH_3, ring, OR;
R_5: H, absent if carbonyl

The sensory properties determined for 3-acetylthiohexanal and 3-acetylthio-1-hexanol demonstrate for the first time that the requirements for the “tropical” olfactophore are also fulfilled if the substituent A constitutes an acetyl group.

However, there is a significant impact of the configuration at position 3 on the sensory properties. As shown in Table 4.2.4, the odors of the stereoisomers differed significantly, only one of the enantiomers possessed the pleasant fruity note. Interestingly, for 3-acetylthiohexanal and 3-mercaptohexanal the (*S*)-enantiomers exhibited a more fruity and pleasant odor, whereas for 3-acetylthiohexanol, the (*R*)-enantiomer showed these odor qualities.

The enantiomers of 3-mercaptohexanol had been reported to possess the same odor properties (*Heusinger and Mosandl, 1984*). Structural modifications at the hydroxy moiety, e.g. 3-mercaptohexyl alkanoates (*Weber et al., 1992*) and 1-methoxyhexane-3-thiol (*van de Waal et al., 2002*), and at the thio group, e.g. 3-methylthiohexanol (*Heusinger and Mosandl, 1984*), resulted in significant sensory differences between enantiomers.

These sensory data demonstrate that it is worthwhile to invest in methods to obtain enantiomers of this group of sulfur-containing flavor compounds and to exploit the enantioselectivity of enzyme-based approaches.

4.2.4. Discussion

Candida antarctica lipase B turned out to exhibit the highest enantioselectivity in the course of the hydroysis of 3-acetylthiohexanal.

For many lipases the existence of two isoforms (isoenzymes), usually called A and B, has been demonstrated (e.g. lipases from *Penicillium cyclopium* (*Iwai et al., 1975*), *Rhodotorula pilimanae* (*Muderhwa et al., 1986*), *C. antarctica* (*Patker et al., 1993*) and *C. rugosa* (*Lundell et al., 1998*)). As shown in Table 4.2.5, lipase A (CAL-A) and lipase B (CAL-B) isolated from *C. antarctica* show rather different properties (*Patkar et al., 1993; Martinelle et al., 1995*). CAL-A is an extremely thermostable protein, keeping its complete activity after 120 min incubation at 60 °C. The two lipases also differ in substrate specificity: CAL-A is

active in a non-specific manner towards triglycerides and has only low activity towards simple esters; CAL-B is less active to large triglycerides but very active to a broad range of esters, amides and thiols (*Anderson et al., 1998*). These two lipases have been cloned and expressed in *Aspergillus oryzae* (*Høegh et al., 1995*).

Table 4.2.5 Characterization of CAL-A and CAL-B

	CAL-A	CAL-B
molecular weight (kD)	45	33
isoelectric point (pI)	7.5	6.0
pH optimum	7	7
specific activity (LU/mg)	420	435
thermostability at 60 °C[a]	100 [100]	15 [0]
pH stability	6-9	7-10
interfacial activation	yes (marginal)	no

[a] residual activities after incubation at 60 °C in 0.1 M tris buff. (pH 7.0) for 20 min and [120 min].

The structures of lipases from several bacteria and animals have been determined. About 30 lipases have been cloned and 12 X-ray structures are available. For example, CAL-B consists of 317 amino acid residues and has the common structure with a β-sheet core surrounded by α-helices (*Uppenberg et al., 1995*). Comparable to other lipases, CAL-B contains a catalytic triad consisting of Ser, Asp and His residues. X-ray crystallographic studies revealed that a very limited amount of space is available in the active site pocket of CAL-B as compared to other lipases (*Uppenberg et al., 1995*). This fact seems to be one of the reasons for the high degree of selectivity of CAL-B. The crystal structures of covalent complexes between *C. rugosa* lipase (CRL) and (*R*)- and (*S*)-menthyl ester transition state analogues revealed that only the fast-reacting enantiomer could bind to the enzyme with an intact hydrogen bond (*Cygler et al., 1994*).

Molecular modeling studies carried out with *Rhizomucor miehei* (RML) indicated

that for stereoselectivity to occur, the formation of an essential hydrogen bond network at the catalytic triad is inevitable. Only one enantiomer of the investigated substrate was able to form the relevant hydrogen bonds while binding as the first tetrahedral intermediate in ester hydrolysis (*Yagnik et al., 1997*). A prediction of enantioselectivity has also been possible by molecular modeling studies calculating the lowest energy between substrate and CAL-B (*Hæffner et al., 1998*).

The interactions between esters of secondary alcohols and both RML and CRL have been studied (*Botta et al., 1997*). They used racemic arylpropionic esters as the substrates, and found π-interaction between the aromatic ring of Trp 88 in RML or Phe 296 in CRL and the aromatic ring of the substrates only in the case of preferred enantiomers.

Ema et al. (*1998*) proposed to use the thermodynamic stability of the transition state calculated by the semiempirical molecular orbital calculation (MNDO-PM3) as a criterion to rationalize the stereoselectivity of RML.

Protein engineering, i.e. modifications of the sequence to increase and/or alter activity and specificity, and to improve resistance to heat, pH or organic solvents will definitely be a useful tool to adapt lipases for kinetic resolutions of sulfur-containing compounds. In addition to hydrolysis, esterification and transesterification performed in organic media should be tested as approaches to obtain enantiomers of sensorially active thio-compounds.

5. Summary

The potentials of C-S β-lyases and lipases to generate sensorially active thiols from non-volatile sulfur-containing precursors were investigated. The substrates were synthesized by Michael-type addition of nucleophiles (cysteine, thioacetic acid) to α,β-unsaturated carbonyls (pulegone, *E*-2-hexenal). Their structures were elucidated by means of GC-MS, LC-MS and ^{1}H/^{13}C NMR. A cell-free extract obtained from *Eubacterium limosum*, a commercially available tryptophanase preparation from *E. coli*, and yeast (*Saccharomyces cerevisiae*) were used as sources for C-S β-lyases. For the lipase-catalyzed hydrolyses commercially available enzyme preparations of microbial, plant and animal origin were employed. The stereochemical course of the reactions was followed by capillary gas chromatography using modified cyclodextrins as chiral stationary phases.

The addition of cysteine to the double bond of pulegone resulted in the preferred formation of the *trans*-configured diastereoisomeric products, irrespective of the configuration at position C1 of the starting material. 8-*S*-L-cysteinyl-*p*-menthan-3-one was accepted as substrate by the three β-lyase sources tested resulting in the liberation of 8-mercapto-*p*-menthan-3-one, a powerful flavoring substance exhibiting a “cassis”-type odor note. The cleavage was shown to proceed with only low enantioselectivity; a preference of the (1*S*,4*S*)-stereoisomer was observed for tryptophanase. Diastereoselectivity was more pronounced; tryptophanase and the extract from *E. limosum* exhibited a preference of the (1*R*,4*S*)- and (1*S*,4*R*)-diastereoisomers.

Screening of β-lyases from other sources, e.g. plants in which thiols play important sensory roles, revealed cysteine conjugate β-lyase activities in passion fruits and in the leaves of blackcurrant and box tree.

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. Treatment of the conjugate with tryptophanase from *E. coli* and the enzyme extract from *E. 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 as substrate for β-lyases was demonstrated by *in situ* generation of this compound from 3-*S*-(*N*-acetyl-L-cysteinyl)hexanal using acylase. Opposite enantioselectivities were observed for the liberation of 3-mercaptohexanol, the key aroma compound occurring in yellow passion fruits and various grape musts, from 3-*S*-L-cysteinylhexanol by the enzyme preparation from *E. limosum* and tryptophanase, respectively. Various yeasts produced 3-mercaptohexanol starting from 2-(2-*S*-L-cysteinylpentyl)-1,3-thiazolidine-4-carboxylic acid as well as from 3-*S*-L-cysteinylhexanol. The reactions proceeded without preferential formation of one of the enantiomers.

Structural effects on the activities of C-S β-lyases were investigated by employing synthesized analogues as substrates.

The hydrolysis of 3-acetylthiohexanal was catalyzed by all lipases tested. The enzyme preparations varied significantly in terms of activity and enantioselectivity. The most pronounced enantioselectivity was observed for the hydrolysis of 3-acetylthiohexanal catalyzed by lipase B from *Candida antarctica* resulting in the (*S*)-configured thiol-product. Immobilization of the enzyme and the use of *tert*-butanol as co-solvent improved the enantioselectivity. Modification of the acyl moiety of the substrate by a bulkier moiety (3-benzoylthiohexanal) reduced conversion rates but had no significant impact on enentioselectivities. For most of the enzymes tested, activities and enantioselectivities for the hydrolysis of 3-acetylthiohexanol were significantly lower than those for the aldehyde substrate. The 3-acetylthio-compounds investigated possess attractive sensory properties. The odors of the stereoisomers differed significantly; only one of the enantiomers exhibited the pleasant citrus-type note.

The data elaborated demonstrate that it is worthwhile to invest in methods to obtain optically pure (enriched) stereoisomers of sulfur-containing flavor compounds and to exploit the stereoselectivity of enzyme-based approaches. The properties revealed for β-lyases and lipases in the course of kinetic resolutions should be useful for preparative purposes as well as for further biogenetic studies.

6. Zusammenfassung

Das Potential von C-S β-Lyasen und Lipasen zur Freisetzung sensorisch aktiver Thiole aus nichtflüchtigen schwefelhaltigen Vorstufen wurde untersucht. Die Substrate wurden durch Michael-Addition von Nucleophilen (Cystein, Thioessigsäure) an α,β-ungesättigte Carbonylverbindungen (Pulegon, *E*-2-Hexenal) synthetisiert. Ihre Strukturen wurden mittels GC/MS, LC-MS und $^{1}H/^{13}C$ NMR untersucht. Als Quellen für C-S β-Lyasen wurden ein aus *Eubacterium limosum* gewonnener zellfreier Extrakt, eine kommerziell erhältliche Tryptophanase aus *E. coli* sowie Hefe (*Saccharomyces cerevisiae*) verwendet. Für die Lipase-katalysierten Hydrolysen wurden kommerziell verfügbare Enzympräparate mikrobiellen, pflanzlichen und tierischen Ursprungs eingesetzt. Der stereochemische Verlauf der Umsetzungen wurde mittels kapillargaschromatographischer Untersuchungen auf chiralen stationären Phasen verfolgt.

Die Addition von Cystein an die Doppelbindung von Pulegon resultierte in der bevorzugten Bildung der *trans*-konfigurierten diastereoisomeren Produkte, unabhängig von der Konfiguration an Position C1 des Ausgangsmaterials. Die eingesetzten β-Lyasen akzeptierten 8-*S*-L-Cysteinyl-*p*-menthan-3-on als Substrat und setzten 8-Mercapto-*p*-menthan-3-on, eine intensiv nach Cassis riechende Verbindung frei. Die Spaltung verlief mit nur geringer Enantioselektivität; für Tryptophanase war eine Bevorzugung des (1*S*,4*S*)-Stereoisomers zu beobachten. Die Diastereoselektivität war stärker ausgeprägt; Tryptophanase und der Extrakt aus *E. limosum* zeigten eine Bevorzugung der (1*R*,4*S*)- und (1*S*,4*R*)- Diastereoisomere.

Ein Screening von β-Lyasen aus anderen Quellen, z.B. Pflanzen, in denen Thiole wichtige Aromastoffe darstellen, zeigte, dass Passionsfrüchte sowie die Blätter von Schwarzer Johannisbeere und Buchsbaum Cysteinkonjugat-β-Lyase-Aktivitäten aufwiesen.

Das aus der Reaktion von *E*-2-Hexenal und L-Cystein resultierende Produkt konnte als 2-(2-*S*-L-Cysteinylpentyl)-1,3-thiazolidin-4-carbonsäure identifiziert werden. Die Umsetzung dieses Konjugats mit Tryptophanase aus *E. coli* bzw. dem Extrakt aus *E. limosum* führte zur Bildung von 3-Mercaptohexanal. Die

Reaktion verlief unter leichter Bevorzugung des (*S*)-konfigurierten Produkts, jedoch mit nur geringer Umsatzrate. Die Rolle von 3-*S*-L-Cysteinylhexanal als Substrat für β-Lyasen wurde durch *in situ* Bildung dieser Komponente aus 3-*S*-(*N*-acetyl-L-cysteinyl)hexanal mittels Acylase aufgezeigt. Entgegengesetzte Enantioselektivitäten wurden für die Freisetzung von 3-Mercaptohexanol, der in Passionsfrüchten und verschiedenen Traubenmosten vorkommenden Schlüsselaromakomponente, aus 3-*S*-L-Cysteinylhexanol durch den Extrakt aus *E. limosum* bzw. Tryptophanase beobachtet. Verschiedene Hefen bildeten 3-Mercaptohexanol ausgehend sowohl von 2-(2-*S*-L-Cysteinylpentyl)-1,3-thiazolidin-4-carbonsäure als auch von 3-*S*-L-Cysteinylhexanol. Die Reaktionen verliefen ohne bevorzugte Bildung eines Enantiomers.

Strukturelle Einflüsse auf die Aktivitäten von β-Lyasen wurden durch Umsetzung synthetisierter Substratanaloge untersucht.

Die Hydrolyse von 3-Acetylthiohexanal wurde durch alle getesteten Lipasen katalysiert. Die Enzympräparate zeigten deutliche Unterschiede hinsichtlich Aktivität und Enantioselektivität. Die Lipase B aus *Candida antarctica* zeigte die am stärksten ausgeprägte Enantioselektivität und setzte die (*S*)-konfigurierte Thiol-Verbindung frei. Durch Immobilisierung des Enzyms und Einsatz von *tert.*-Butanol als zusätzliches Lösungsmittel konnte die Enantioselektivität gesteigert werden. Eine Modifizierung des Acylrestes im Substrat durch eine voluminösere Gruppe (3-Benzoylthiohexanal) verringerte die Umsatzrate, hatte jedoch keinen signifikanten Einfluss auf die Enanatioselektivität. Für die meisten der getesteten Enzympräparate waren Aktivität und Enantioselektivität für die Hydrolyse von 3-Acetylthiohexanol deutlich geringer als für die des Aldehydsubstrats. Die untersuchten 3-Acetylthioverbindungen besitzen attraktive sensorische Eigenschaften. Die Stereoisomere zeigten deutliche Unterschiede; nur eines der Enantiomere besaß die angenehme Zitrus-Note.

Die erarbeiteten Daten zeigen, dass es lohnenswert ist, in Methoden zur Gewinnung optisch reiner (angereicherter) Stereoisomere schwefelhaltiger Aromastoffe zu investieren und die Stereoselektivität Enzym-katalysierter Reaktionen zu nutzen. Die für β-Lyasen und Lipasen im Zuge kinetischer Racematspaltungen aufgezeigten Eigenschaften sollten sowohl für präparative Anwendungen als auch für weitere biogenetische Studien von Nutzen sein.

7. References

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Presentations

Weurman Symposium (2002) (oral)
β-Lyase-catalyzed biotransformations of sulphur-containing flavour precursors.

GDCh (2002) (poster)
β-Lyase-catalyzed biotransformations of sulfur-containing flavor precursors.

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