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Stereoselectivity of the Generation of 3-Mercaptohexanal and 3-Mercaptohexanol by Lipase-Catalyzed Hydrolysis of 3-Acetylthioesters

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The enantioselectivity of the generation of 3-mercaptohexanal and 3-mercaptohexanol, two potent sulfur-containing aroma compounds, by lipase-catalyzed hydrolysis of the corresponding 3-acetylthioesters was investigated. The stereochemical course of the kinetic resolutions was followed by capillary gas chromatography using modified cyclodextrins as chiral stationary phases. The enzyme preparations tested varied significantly in terms of activity and enantioselectivity (*E*). The highest *E* value ($E = 36$) was observed for the hydrolysis of 3-acetylthiohexanal catalyzed by lipase B from *Candida antarctica* resulting in (*S*)-configured thiol products. Immobilization of the enzyme ($E = 85$) and the use of *tert*-butyl alcohol as cosolvent ($E = 49$) improved the enantioselectivity. Modification of the acyl moiety of the substrate (3-benzoylthiohexanal) had no significant impact. The sulfur-containing compounds investigated possess attractive odor properties, and only one of the enantiomers exhibits the pleasant citrus type note.

KEYWORDS: Thioester; thiol; lipase; enantioselectivity; 3-acetylthiohexanal; 3-acetylthiohexanol

INTRODUCTION

Lipases are well-established biocatalysts, which are widely used for regioselective and enantioselective biotransformations (*1*, *2*). For esters, alcohols, and acids, many examples of kinetic resolutions of enantiomers via hydrolysis, transesterification, and esterification have been described (*3*, *4*). Analogous reactions have been reported for thio acids and esters (*5*−*8*). Apart from a first communication on the lipase-catalyzed hydrolysis of 3-acetylthiocycloheptene (*9*), the exploitation of the stereoselectivity of enzyme-catalyzed reactions of sulfur-containing esters started rather late (*10*, *11*). In the meantime, various approaches have been described (*12*−*14*), many of them focusing on the enzymatic resolution of 2-arylpropionates, an important class of nonsteroidal antiinflammatory drugs (*15*−*17*).

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. In addition to the nonhydrolytic liberation of potent volatiles from precursors using β-lyases (*18*, *19*), lipase-catalyzed syntheses (*20*, *21*) as well as hydrolyses of thioesters (*22*, *23*) have been described as useful approaches.

Sulfur-containing volatiles are not only generated during the thermal treatment of foods (*24*) but are also biosynthesized in various plants, especially tropical fruits (*25*). For example, passion fruit's flavor is typically determined by sulfur-containing compounds (*26*). 3-Mercaptohexanol, first identified in yellow passion fruits (*27*) and later also described as a volatile constituent of wine (*28*) plays an important sensory role. The corresponding aldehyde, 3-mercaptohexanal, had been described as a synthetic intermediate (*29*). Later, it was reported as a flavor compound in cooked liver and was described as imparting "tropical fruit" type aroma notes (*30*). Synthesis via combinatorial approach and sensory evaluation by gas chromatography/olfactometry (GC/O) revealed this mercaptoaldehyde to have a citrus peel note (*31*).

Recently, the potential to use porcine liver esterase for the generation of 3-mercaptohexanal by hydrolysis of 3-acetylthiohexanal has been reported (*32*). However, the stereochemical course of the reaction had not been followed. The aim of this study was to screen lipases from different sources for their potential to generate 3-mercaptohexanal and 3-mercaptohexanol by hydrolysis of the corresponding thioesters and to investigate the degree of enantiodiscrimination related to these kinetic resolutions.

MATERIALS AND METHODS

Materials. *E*-2-Hexenal, thioacetic acid, thiobenzoic acid, *p*-chloromercuribenzoic acid, and L-cysteine (~99%) were obtained from Fluka Chemie AG (Germany).

Lipases from *Aspergillus niger* (catalog number, 62301; abbreviation, ANL), *Aspergillus oryzae* (95184; AOL), *Candida antarctica* (62299;

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CAL), *C. antarctica* lipase A (62287; CAL-A), *C. antarctica* lipase B (62288; CAL-B), *Penicillium roqueforti* (62308; PRL), and *Rhizopus oryzae* (80612; ROL) were purchased from Fluka Chemie AG. Lipases from *C. antarctica* lipase B (L4777; CAL-B resin), *Candida rugosa* (L1754; CRL), *Mucor javanicus* (L8906; MJL), *Mucor miehei* (L9031; MML), porcine pancreas (L3126; PPL), *Pseudomonas cepacia* (L9156; PCL), *Thermomyces lanuginosus* (L0902; TLL), wheat germ (L3001; WGL), and the esterase from porcine liver (E3019; PLE) were from Sigma-Aldrich Chemie GmbH (Germany).

Synthesis of the Thioester Substrates. Thioesters were synthesized by Michael type addition of thiocarboxylic acid to α,β-unsaturated carbonyls (*33*, *34*).

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 the excess of thioacetic acid was removed 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 ($M^{+\bullet}$; 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, C*H*$_3$−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 the reaction mixture was dissolved 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 it was dried over anhydrous sodium sulfate, dichloromethane was removed under reduced pressure at 40 °C. A 4.96 g (21 mmol) amount 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 ($M^{+\bullet}$; 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 sodium borohydride (50 mg, 1.3 mmol dissolved in 2 mL of water) was added dropwise 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 the combined extracts were washed (10 mL of distilled water) and dried (anhydrous sodium sulfate), dichloromethane was removed under reduced pressure at 40 °C. A 97.3 mg (0.55 mmol) amount 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), 158 (1), 176 ($M^{+\bullet}$; 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, C*H*$_3$−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).

Synthesis of 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 it was washed (two times 10 mL of distilled water) and dried (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 ($M^{+\bullet}$; 17).

Enzyme-Catalyzed Hydrolysis. A 50 μmol amount 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 (300 rpm) 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 cosolvent effects, 10−40 vol % of acetone and *tert*-butyl alcohol, respectively, was added to the buffer solution.

Separation of Remaining Substrate/Product and Conversion into 3-Mercaptohexanol. Kinetic resolution of 3-acetylthiohexanal (350 μmol substrate; reaction time 8 h) was performed as described above using CAL-B as the 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 sodium borohydride (3.7 mg, 98 μmol in 1 mL of distilled water) was added, 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 (*35*). 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 (*36*). 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 of 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.

GC Analysis. Capillary GC was performed on the following four GC systems. (I) A Carlo Erba MEGA2 gas chromatograph equipped with FID and FPD. Parallel detection was achieved by dividing the effluent of the column (DB-WAX, J&W; 60 m × 0.32 mm i.d.; film thickness 0.25 μm) via a chrom-fit connector and short pieces of deactivated fused silica capillaries to the two detectors. Split injection was performed at 215 °C, and column temperature was programmed from 40 (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 the carrier gas at 105 kPa.

(II) A Carlo Erba GC 6000 gas chromatograph with FID; the columns used were DB-1 (30 m × 0.25 mm i.d.; film thickness 1 μm) and SE-54 (15 m × 0.25 mm i.d.; film thickness 0.15 μm), respectively. Split injection was performed at 220 °C, and the column temperature was programmed from 50 (2 min hold) to 300 °C (5 min hold) at 4 °C/min. Hydrogen was used as the 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 (*37*) were used. A DB-5 fused silica column (28 m × 0.25 mm i.d.; film thickness 0.25 μm; J&W) was used as the precolumn, and a fused silica column (30 m × 0.25 mm i.d.) coated with 33% heptakis-(per-*O*-ethyl)-β-cyclodextrin in OV1701-vi (PerEt-β-CD) was used as the main column. Split injection was performed at 205 °C, and the precolumn temperature was programmed from 40 (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 (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 the 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 were managed with Chrom-Card for Windows software (Fisons Instruments) on a work station.

(IV) A Carlo Erba GC 6000 gas chromatograph equipped with FID; the column used was a fused silica column (30 m × 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 (2 min hold) to 200 °C (5 min hold) at 3 °C/min. Hydrogen was used as the 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 and from 150 to 180 °C (5 min hold) at 0.2 °C/min.

Capillary GC/O. GC/O was performed on the following two GC systems. (I) A DB-WAX column (55 m × 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 the column, the effluent was split 1:1 for FID and sniffing port with chromfit connector and deactivated fused silica tube. The column temperature was programmed from 60 (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 the sniffing port. Hydrogen was used as the 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 split to FID and a sniffing port as described above. The typical temperature program was from 40 (2 min hold) to 80 °C at 40 °C/min, 80 to 140 °C at 2 °C/min, and 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 the sniffing port. Hydrogen was used as the carrier gas at 70 kPa.

GC-MS. DB-WAXETR fused silica column (30 m × 0.25 mm i.d.; film thickness 0.5 μm; J&W) installed into a Finnigan GC8000 gas chromatograph equipped with a split/splitless injector and a Voyager mass spectrometer were used. The mass spectrometer was operated at scan mode at 20−400 amu, and the ionization voltage was 70 eV. The column temperature was programmed from 40 (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 the carrier gas at 75 kPa.

For the analysis of 3-benzoylthiohexanal, SE-54 fused silica column (15 m × 0.25 mm i.d.; film thickness 0.15 μm) was used, and the temperature program was from 50 (2 min hold) to 250 °C (5 min hold) at 4 °C/min. Helium was used as the carrier gas at 25 kPa.

NMR Spectroscopy. ^{1}H NMR and ^{13}C NMR spectra were recorded at 500.13 and 125.6 MHz with an AVANCE 500 spectrometer (Bruker Instruments, Germany). Two-dimensional COSY, HMQC, and HMBC experiments were performed according to standard Bruker software (XWINNMR 3.0). Samples were measured in 0.5 mL of [D]-chloroform. The sample temperature was 25 °C. Composite pulse decoupling was used in the ^{13}C NMR experiments. All signals were assigned by proton−proton and proton−carbon correlation experiments (COSY, HMQC, and HMBC).

RESULTS AND DISCUSSION

Synthesis of 3-Acetylthioesters. 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. The identities of the compounds were confirmed by means of GC-MS and NMR.

Table 1. Enzyme-Catalyzed Kinetic Resolution of 3-Acetylthiohexanal

enzyme	enantiomeric excess (%) ee_s	enantiomeric excess (%) ee_p	conversion[a] (%)	enantioselectivity *E*	preferred enantiomer
ROL	0.9	18.4	4.9	1.5	*R*
ANL	21.0	27.2	43.5	2	*R*
WGL	17.8	37.1	32.4	3	*R*
MJL	0.5	4.1	2.2	1.1	*S*
PRL	0.9	2.3	4.1	1.1	*S*
MML	0.5	9.1	2.1	1.2	*S*
PCL	2.1	29.7	6.7	2	*S*
PPL	4.3	29.6	12.6	2	*S*
CRL	8.2	50.2	14.1	3	*S*
PLE	32.0	41.2	43.7	3	*S*
AOL	5.6	55.0	9.3	4	*S*
TLL	30.4	76.3	28.5	10	*S*
CAL	2.1	45.0	4.6	3	*S*
CAL-A	21.1	66.1	24.2	6	*R*
CAL-B	51.1	91.1	35.9	36	*S*
CAL-B[b]	36.5	96.7	27.4	85	*S*

[a] Reaction time, 2 h; other conditions, see Materials and Methods. [b] Immobilized enzyme adsorbed on a macroporous resin.

Enzyme-Catalyzed Hydrolysis of 3-Acetylthiohexanal. Commercially available enzyme preparations (15 lipases and one esterase) of various origins (microbial, plant, and mammalian) were tested for their suitability to hydrolyze the thioester bond in 3-acetylthiohexanal. The conversion rate and stereochemical course of the reaction were followed by means of capillary GC. As shown in **Table 1**, 3-acetylthiohexanal (**1**) was accepted as substrate by all enzymes tested. The retention index and MS spectrum of the generated 3-mercaptohexanal (**2**) were identical to data from literature (*30*) 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 by means of capillary GC (**Figure 1**). This enabled the determination of the enantiomeric excesses of product (ee_p) and remaining substrate (ee_s) on the basis of the peak areas. The data obtained were used to calculate conversion rates (*c*) and enantioselectivities (*E*) applying equations previously described for kinetic resolutions (*38*).

The most pronounced enantiodiscrimination was observed for *Candida antarctica* lipase. The optically enriched product and remaining substrate obtained by the kinetic resolution using CAL-B (nonimmobilized) 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 (*39*). 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 2**. Because of the nonenantioselective 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** (ee 60%). The enantiomer **4a** obtained by alkaline

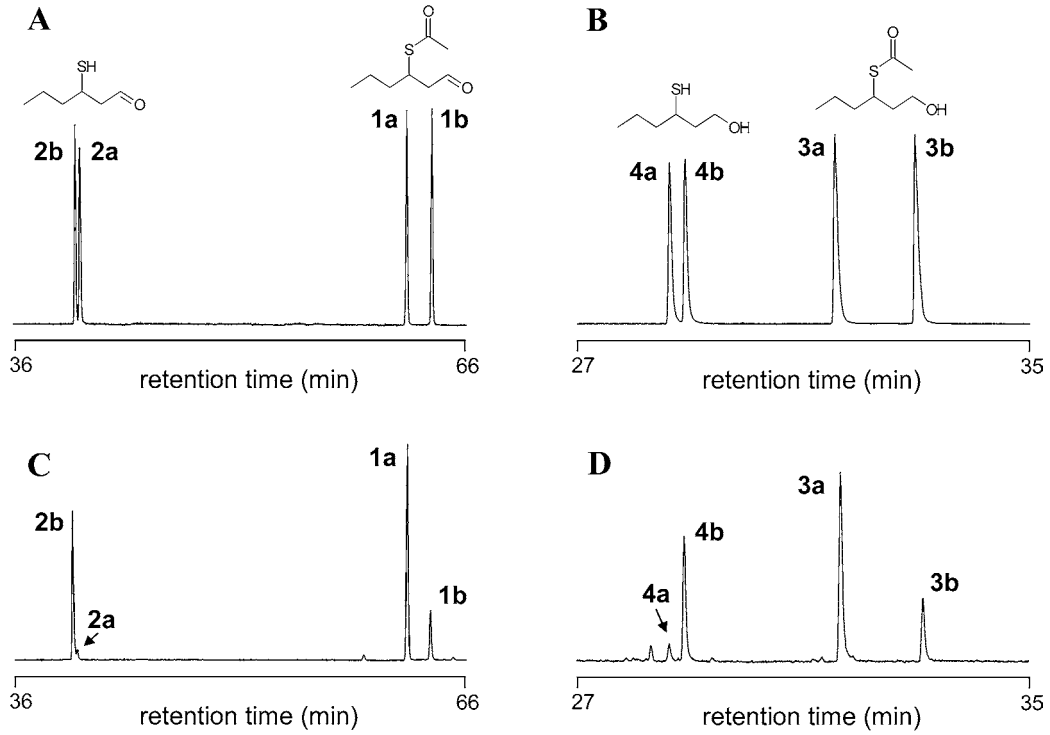

Figure 1. Capillary GC separation of (**A**) racemic 3-acetylthiohexanal (**1**) and 3-mercaptohexanal (**2**) (GC system III); (**B**) racemic 3-acetylthiohexanol (**3**) and 3-mercaptohexanol (**4**) (GC system IV); (**C**,**D**) enantiomerically enriched compounds obtained after kinetic resolution (CAL-B, 8 h, $c = 42\%$ (**C**) and 41% (**D**)). Enantiomers a, (*R*)-configuration; enantiomers b, (*S*)-configuration.

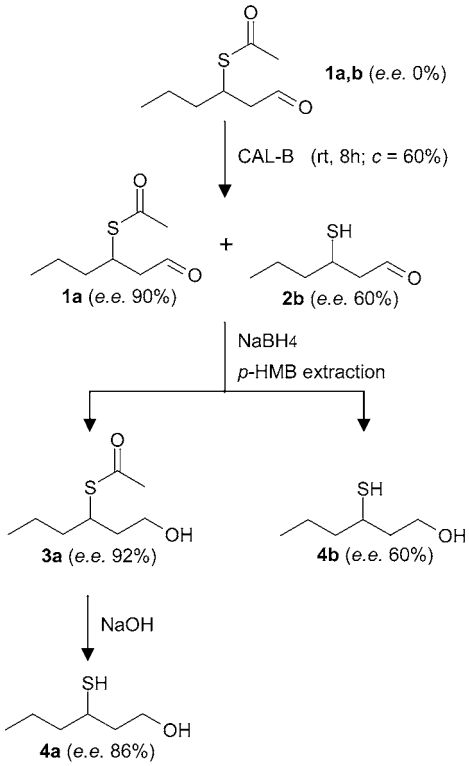

Figure 2. Sequence of reactions applied to convert product and substrate of the kinetic resolution into 3-mercaptohexanol in order to determine their absolute configurations.

hydrolytic cleavage of the remaining thioester **3a** (ee 92%) was shown to have the (*R*) configuration (ee 86%).

As shown in **Table 1**, the enzyme preparations tested differed strongly in terms of degree of enantiodiscrimination. 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 by Kazlauskas et al. (*40*, *41*) for esters of secondary alcohols, the substrates resolved most efficiently by lipase-catalyzed hydrolyses are those with substituents that differ significantly in size, and the enantiomer preferred by the enzyme can be predicted. This rule could also be confirmed for hydrolysis and interesterification, respectively, of corresponding esters of secondary thiols (*11*, *13*). For 3-acetylthiohexanal, the only slight differences in size between the substituents at the stereocenter do not allow a clear categorization and prediction according to the rule of Kazlauskas. The enantioselectivity observed for CAL-B must be based on factors other than solely the difference in size of the substituents.

It is noteworthy that the four commercial preparations of *Candida antarctica* lipase employed as catalysts differed significantly in their enantioselectivities. This yeast produces two different lipases (A and B), which have been purified and characterized (*42*). Both have been cloned and expressed in *A. oryzae* (*43*). 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 CAL-B obtained from recombinant *A. oryzae*. In contrast, the heterologously expressed lipase A (CAL-A) showed preference for the opposite (*R*) enantiomer ($E = 6$).

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 have been described for the lipase from *Candida cylindracea* immobilized on agarose and silica gel (*44*).

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 the enzyme in the immobilized form. Extraction of the removed resin with

Table 2. Effects of Cosolvents on the Enantioselectivity of the Hydrolysis of 3-Acetylthiohexanal Catalyzed by CAL-B

cosolvent	concentration (vol %)	enantiomeric excess (%) ee_s	enantiomeric excess (%) ee_p	conversion[a] (%)	enantioselectivity[b] *E*
		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] Reaction time, 2 h; other conditions, see Materials and Methods. [b] Preferred enantiomer, *S*.

Table 3. Enzyme-Catalyzed Kinetic Resolution of 3-Acetylthiohexanol and 3-Benzoylthiohexanal

enzyme	enantiomeric excess (%) ee_s	enantiomeric excess (%) ee_p	conversion[a] (%)	enantioselectivity *E*	preferred enantiomer
			3-Acetylthiohexanol		
ANL	93.9	6.3	93.8	3	*R*
WGL	82.0	38.0	68.4	5	*R*
TLL	1.3	19.1	6.2	2	*S*
PPL	2.9	52.4	5.2	3	*S*
PLE	64.8	24.5	72.6	3	*S*
CAL	0.7	35.9	1.9	2	*S*
CAL-A	0.2	6.8	3.5	1	
CAL-B	27.1	81.1	25.1	12	*S*
			3-Benzoylthiohexanal		
ANL	0.1	2.9	0.9	1	
PLE	6.9	71.3	10.1	6	*S*
CRL	0.7	54.6	1.2	3	*S*

[a] Reaction time, 2 h; other conditions, see Materials and Methods.

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.

Influence of Cosolvents on Enantioselectivity. 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, in particular acetone and *tert*-butyl alcohol (*45*). As shown in **Table 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 cosolvent actually resulted in a decrease of *E*. Addition of *tert*-butyl alcohol significantly improved the enantiodiscrimination up to a level of 20 vol %; higher proportions again resulted in lower enantioselectivity. These results are not fully consistent with the formerly reported effects of acetone and *tert*-butyl alcohol on the enantioselectivity of CAL-B (*45*). The mechanism underlying the influence of cosolvents on the enantioselectivity of the hydrolysis of 3-acetylthiohexanal remains unclear. NMR studies of CAL-B applied to the hydrolysis of 3-chloro-1-phenylmethoxy-2-propyl butanoate had shown that the conformation of the enzyme most likely remains unchanged upon addition of up to 50% acetone (*45*). The increased solubility of the liberated alcohol resulting from the addition of acetone to the reaction mixture had been discussed as possible explanation of the phenomenon (*46*).

Influence of Structural Modifications on Enantioselectivity. 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 3**. The preference of enantiomers remained the same as observed for the hydrolysis of 3-acetylthiohexanal. However, the lipases from TLL and CAL 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.

Menthylbenzoate has been reported as suitable starting material to obtain (−)-menthol via hydrolysis catalyzed by CRL (*47*). 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; K_1, 42.3; *R*, 1.13; 145 °C isothermal; hydrogen 31.4 cm/s). When using CAL, CAL-A, CAL-B, TLL, PPL, and WGL as biocatalysts, the conversion rates observed after 2 h were negligible (<0.1%) as compared to the data obtained for ANL, PLE, and CRL (**Table 3**). The replacement of the acetyl moiety by a bulky group drastically reduced the conversion rates without significant impact on the enantioselectivities.

Odor Descriptions. Odor descriptions of 3-acetylthiohexanal, 3-acetylthiohexanol, and 3-mercaptohexanal were determined by means of GC/O. As shown in **Table 4**, the sulfur-containing volatiles exhibited attractive citrus type notes. The odors of the stereoisomers differed significantly, and only one of the enantiomers possessed the pleasant fruity note. Quantitative studies on differences in odor thresholds of the enantiomers are in progress.

The enantiomers of 3-mercaptohexanol had been reported to possess the same odor properties (*48*). Structural modifications at the hydroxy moiety, e.g., 3-mercaptohexyl alkanoates (*49*) and 1-methoxyhexane-3-thiol (*50*), and at the thio group, e.g., 3-methylthiohexanol (*48*), 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.

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Table 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-acetylthiohexanal[b]	citrus peel, sulfurous, fruity	fruity, grapefruit, sulfurous	sulfurous, roasted, rubberlike
3-mercaptohexanal[c]	sulfurous, citrus peel	sulfurous, rubberlike	green, citrus peel, fruity

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

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