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A labeling study on the formation of perillene by submerged cultured oyster mushroom, *Pleurotus ostreatus*

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Abstract The conversion of β-myrcene to the furanoid flavour compound perillene by *Pleurotus ostreatus* was investigated using trideutero β-myrcene, trideutero α-(*Z*)-acaridiol and non-labeled 1,2- and 3,10-epoxy-β-myrcene, α,α-acarilactol, and perillene as substrates. Myrcene diols were formed from the cleavage of myrcene epoxides, but only α-(*Z*)-acaridiol, a 1,4-butanediol derivative most likely generated through a base-catalysed epoxide opening, was a suitable precursor of perillene. Once formed, this key intermediate was rapidly oxidised and the resulting cyclic lactol was dehydrated to yield perillene. Bioconversion of the supplemented perillene to α,α-acariolide indicated that perillene was another intermediate of the pathway and prone to further oxidative degradation. The data suggest that the fungus converted the cytotoxic β-myrcene in its environment into a metabolically useable carbon source along this route.

Keywords *Pleurotus ostreatus* · β-myrcene · Bioconversion · Perillene · Labeling study

Introduction

Perillene (3-(4-methyl-3-pentenyl)-furan) is a trace constituent of numerous plant-essential plant oils and imparts a strong and fresh citrus-flowery note (Ohloff 1994). Like other monoterpenoid and norisoprenoid furans, such as rosefuran (3-methyl-2-(3-methyl-2-buten-1-yl)-furan) or solanofuran (6-methyl-5-(4-methyl-2-furanyl)-2-heptanone), data on its biogenetic origin are lacking. Yuba et al. (1996) compared hybrids of different chemo-types of *Perilla frutescens* and suggested a hypothetical pathway starting from geranial.

A decade ago it was found that submerged cultures of some basidiomycetes tolerated millimolar levels of monoterpenes, such as β-myrcene, and oxidised the hydrocarbons to flavour compounds (Krings and Berger 1998; Schrader and Berger 2001; Schrader 2007). In the nutrient medium of a *Pleurotus* strain, perillene was identified as a minor bioconversion product (<1 mg L^{-1}) (Busmann and Berger 1994). Krings et al. (in press), in an orientating study, screened several *Pleurotaceae* and identified numerous volatile conversion products but did not observe a formation of perillene after addition of geranial or related monoterpenes. Perillene was only found in the nutrient medium of *Pleurotus ostreatus* when supplemented with β-myrcene. *P. ostreatus* is wide-spread in temperate forests throughout the world and a primary decomposer of wood. In its natural habitats, this saprophyte is frequently exposed to β-myrcene as this monoterpene hydrocarbon occurs abundantly in the resin of coniferous wood.

A pathway for the formation of perillene was suggested starting with the epoxidation of the conjugated double bonds of β-myrcene followed by hydrolysis of the 1,2- and 3,10 β-myrcene epoxides, which, besides the corresponding vicinal diols, could give α-(*Z*)-acaridiol (**4a** in Fig. 1) (Krings et al. in press). Subsequent oxidation and cyclisation of this 1,4-diol would yield perillene, α,α- and α,β-acariolide (Fig. 1). This work used trideutero-labeled precursors and *P. ostreatus* as a microbial model organism to confirm the postulated pathway, to distinguish enzymatic from chemical reactions and to discover the rate-limiting steps of the formation of perillene.

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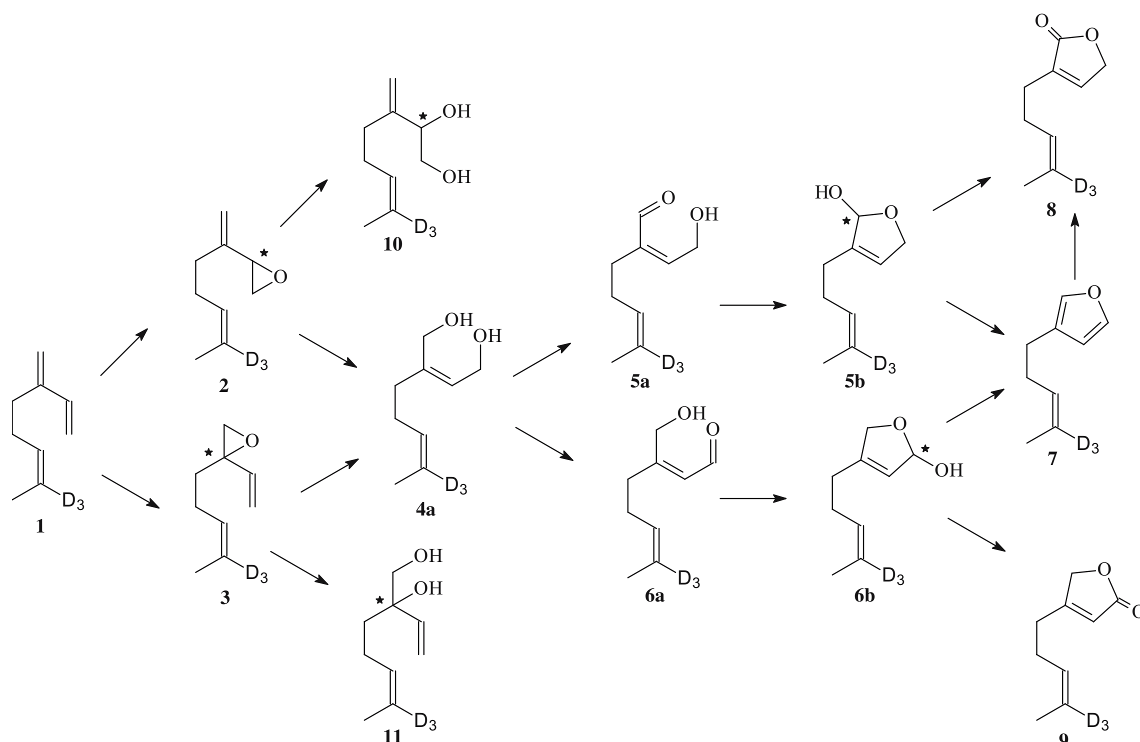

Fig. 1 Conversion of *β*-myrcene by *P. ostreatus*: **1**, *β*-myrcene; **2**, 1,2-epoxy myrcene; **3**, 3,10-epoxymyrcene; **4a**, *α*-(*Z*)-acaridiol; **5**, *α*,*α*-acarilactol; **6**, *α*,*β*-acarilactol; **7**, perillene; **8**, *α*,*α*-acariolide; **9**, *α*,*β*-acariolide; **10**, 1,2-myrcenedihydrodiol; **11**, 3,10-myrcenedihydrodiol

Materials and methods

Fungus P. ostreatus (DSMZ 1020) was obtained from the collection of the DSMZ, (Deutsche Sammlung von Mikroorganismen und Zellkulturen) Braunschweig, Germany.

For maintenance on agar slants and submerged culture, the fungus was grown on glucose/asparagine/yeast extract medium as described elsewhere (Onken and Berger 1999). The flasks were sealed with methyhydroxyethylcellulose-bonded cellulose plugs and autoclaved for 20 min at 121°C.

Chemicals β-Myrcene (>90%, Fluka, Germany) and azeotropic pentane/diethyl ether (1:1.12) were distilled before use.

Cultivation and precursor studies Pre-cultures (200 mL medium in 500 mL flask) were grown aerobically at 24°C and 150 rpm on an orbital shaker (Multitron, Infors, Bottmingen, Switzerland). Experimental cultures (200 mL medium in 500 mL flask) were inoculated with 20 mL of 5-day-old pre-cultures which had been grown on the same medium and homogenised using an Ultra Turrax Homogeniser (Janke & Kunkel, Germany) prior to inoculation. Biotransformation was started after 5 days of incubation by feeding the respective Gas Chromatography (GC)-pure precursor (0.15 to 0.53 mmol 100 mL^{-1}, as afforded by the respective syntheses) in independent experiments. Chemical blanks were carried out for each of the precursor feedings under the same conditions (medium, pH, time) with heat inactivated mycelium (boiling for 15 min). The chemical hydrolysis of 1,2- and 3,10-epoxymyrcene was performed in a 0.1 M Tris–HCl buffer containing L-asparagine (4.5 g L^{-1}).

Isolation and purification of conversion products After addition of the respective precursor, a daily volume of 20 mL culture broth (medium and biomass) was separated from the mycelium by filtration (folded filter, 240 mm, Roth, Karlsruhe, Germany), completely transferred into a separatory funnel and extracted three times with 30 mL azeotropic pentane/Et_2O (1:1.12, v/v). The combined extracts were dried over dry sodium sulphate and concentrated (42°C) to a volume of 1 mL using a Vigreux column. *n*-Nonadecane (43.8 µg) was added as a standard.

Separated fungal mycelium was re-suspended in 70 mL methanol and intracellular metabolites were isolated after fungal cell disruption using a glass bead mill (Dyno-Mill, Bachofen, Basel, Switzerland) as described elsewhere (Taskova et al. 2006). After centrifugation, the supernatant was extracted as described above.

High resolution gas chromatography (HRGC), chiral GC and HRGC-Mass spectroscopy (MS) analyses One µL of each concentrated sample was injected into a CE Instruments Trace GC 2000 equipped with a cool on-column injector, a

Zebron ZB-WAX (Phenomenex, USA) fused silica capillary column (30 m×0.32 mm i.d.×0.25 μm film thickness), hydrogen as the carrier gas (40 cm s^{-1}), and a flame ionisation detector (FID) (230°C) using a temperature program from 40°C (3 min) to 230°C with a rate of 3°C min^{-1} to 250°C held for 5 min. Quantification was performed according to the standard *n*-nonadecane.

Enantiomeric distribution of isomers was measured using a double oven gas chromatograph (Sichromat 2-8, Siemens) equipped with a PTV (Programmable Thermal Vaporiser), a CW 20 M capillary column (Macherey & Nagel, 30 m×0.32 mm i.d.×0.25 μm film thickness) in one oven and a life T-switching device to cut onto a chiral *β*-cyclodextrin (Cyclosil-B., 25 m×0.32 mm i.d.×0.25 μm film thickness, J&W Scientific) column (CW 20M column: the same chromatographic conditions as for GC-FID analysis; chiral column: 70°C—35 min, 0.5°C min^{-1}, 150°C, 1.0°C min^{-1}, 220°C, 220°C, 15 min) and hydrogen as the carrier gas (1.5 mL min^{-1}).

GC-MS analysis was carried out using the same chromatographic conditions as for GC-FID analysis and helium as the carrier gas (38 cm s^{-1}). Identification of transformation products was achieved by comparison of EI mass spectra with data from reference compounds or literature (Wiley 6 for MassLab 1996; NIST 02, June 2002 spectral libraries) using a Fisons GC 8000 gas chromatograph and a Fisons MD 800 mass selective detector (interface: 230°C, ion source: 200°C, quadrupole: 100°C, EI ionisation (70 eV), scan range *m*/*z* 33–300 amu).

Synthesis of perillene **7** *precursors* 1,2-, 6,7- and 3,10-*β*-epoxy myrcene, *α*-(*Z*)-acaridiol, *α,α*-acarilactol and perillene were synthesised as described (Krings et al. in press).

Trideutero-*β*-myrcene

Trideutero linalool (1st step) To a solution of pre-cooled (−70°C) 4-trideuteromethyl-3-pentenyliodide (117.8 mmol) in diethyl ether (73.6 mL), 141.9 mmol *n*-butyllithium dissolved in 93 mL *n*-hexane were added drop-wise under continuous stirring, and the reaction mixture was kept for 60 min at −70°C and then for a further 2 h at 0°C. After cooling once again to −70°C, a solution of 117.8 mmol 4-trideuteromethyl-3-pentenyliodide in diethyl ether (100 mL) was slowly added and stirred over night at room temperature. After addition of 100 mL of a pre-cooled (−20°C) saturated solution of NH_4Cl, the mixture was stirred for a further 1 h and after phase separation, the aqueous layer was extracted four times with 30 mL diethyl ether and the combined organic phases were dried over Na_2SO_4 sicc. After removal of the solvent, the crude trideutero linalool was separated to purity using silica 60 (0.063–0.200 mm, Merck, Darmstadt) column chromatography. The yield of *E*/*Z*-trideutero linalool was 15.4 mmol (13%).). 4-Trideuteromethyl-3-pentenyliodide was obtained according to Biernacki and Facile (1980) by reacting cyclopropylmethylketone with trideutero methylmagnesiumiodide (Fluka, Germany) in diethyl ether.

Trideutero-β-myrcene (2nd step) Trideutero linalool (2.7 mmol) was dissolved in water-free THF (5 mL) and added drop-wise in a N_2-atmosphere to a 10 mL solution of Burgess reactant (8.2 mmol) in dry THF. After addition, the reaction mixture was heated to 50°C and stirred for 1 h then cooled to 0°C and 15 mL ice water was added and then extracted three times with 10 mL *n*-pentane. The combined pentane fractions were washed with saturated NaCl-solution (20 mL) twice and dried over dry Na_2SO_4. After removal of *n*-pentane, a product mixture of five trideutero-labeled compounds resulted after column chromatography with silica gel 60. The mixture consisted of the trideutero-labeled compounds *β*-myrcene, *E*/*Z*-ocimene, limonene and 1-methyl-4-(1-methylethyliden)-cyclohexane (2:1:1:2:1). The yield of labeled *β*-myrcene **1b** was 0.23 mmol.

Trideuteromyrcene, MS, *m*/*z* (%):139, M^+ (2), 124 (2), 121 (2) 110 (2), 94 (11), 93 (100), 92 (12), 91 (26), 80 (13), 79 (20), 77 (18), 72 (18), 55 (9), 53 (9), 43 (77), 41 (52), 39 (29).

Prior to use, the mixture of the five labeled hydrocarbons was mixed with non-labeled *β*-myrcene **1** equal to the amount of labeled β-myrcene **1b**. This 50% labeling of *β*-myrcene enabled the identification of *β*-myrcene derived conversion products from conversion products of the other constituents of the mixture (negligible isotope effects assumed).

Trideutero α,α-acariolide, trideutero α,α-acarilactol, and (Z)-α-acaridiol The three compounds were synthesised to obtain analytical reference compounds. The syntheses were as for the non-labeled counterparts (Krings et al. in press), but introducing the label using 4-trideuteromethyl-3-pentenyliodide.

Trideutero *α,α*-acariolide, RI (ZB-WAX) 2186, MS, *m*/*z* (%): 169, M^+ (12), 154 (2), 124 (7), 99 (32), 98 (47), 91 (6), 79 (5), 72 (100) 55 (5), 53 (5), 44 (12), 43 (31), 42 (26), (14).

Trideutero *α*-(*Z*)-acaridiol, RI (ZB-WAX) 2555, MS, *m*/*z* (%): 173, M^+ (trace), 155 (2), 142 (6), 125 (5), 124 (7), 122 (4), 109 (9), 93 (8), 85 (21), 83 (15), 81 (7), 79 (7), 72 (100), 70 (22), 55 (24), 53 (20), 43 (57), 42 (45), 41 (25).

Trideutero *α,α*-acarilactol, RI (ZB-WAX) 2146, MS, *m*/*z* (%):171, M^+ (trace), 156 (trace), 153 (14) 138 (7), 135 (7), 107 (8), 99 (32), 98 (8), 91 (3), 86 (5), 85(12) 83 (15), 82, (21) 81 (30), 79 (11), 73 (15) 72 (100) 67 (17), 55 (8), 53 (12), 43 (45), 42 (38), 41 (19).

Results

Cultivation in the presence of labeled precursors A recent study has shown that submerged cultures of *P. ostreatus* converted millimolar concentrations of exogenous β-myrcene to a number of odorous compounds, among them perillene (Busmann and Berger 1994). A hypothetical biogenetic pathway was derived from the structures of the main conversion products (Krings et al. in press). For the present study, trideutero-labeled β-myrcene and α-(*Z*)-acaridiol were synthesised and added separately to submerged cultures of *P. ostreatus* to prove the proposed pathway. With the exception of 3-hydroxymethyl-7-methyl-octa-2,6-dienal **6a**, all of the postulated bioconversion intermediates of β-myrcene were found with the characteristic 50% abundance of the trideutero label in the isopropyl moiety, as it was present in the precursor mixture (Fig. 1).

Because of the low transient concentration of α-(*Z*)-acariolal **5a** and the acarilactols **5b**,**6b** their identification as intermediates of the perillene pathway of *P. ostreatus* was confirmed in a separate experiment adding trideutero α-(*Z*)-acaridiol. The hydroxyaldehyde **6a**, a positional isomer to **5a**, was not detected, but its transient formation was indirectly deduced from the presence of the corresponding lactol **6b** and lactone **9**, both found with the 50% deutero-label. β-Myrcene-1,10-peroxide, another facultative precursor of perillene **7**, was not found.

Supplementation of 1,2- and 3,10-epoxy-β-myrcene Because of the high volatility of both β-myrcene epoxides the conversion time was reduced to 5 h. Addition of 1,2-epoxymyrcene **2** to the nutrient medium of *P. ostreatus* resulted in the formation of the respective vicinal β-myrcene dihydrodiol, 3-methylene-7-methyl-6-octene-1,2-diol **10**, and three further β-myrcene diols (Figs. 1 and 2). The chemical controls (heat inactivated liquid culture medium and L-asparagine buffer) gave the same conversion products but in lower concentrations. The other diols were identified as (*E*)-2-(4-methyl-3-pentenyl)-2-butene-1,4-diol **4b** and the epimeric (*E*/*Z*)-*p*-menth-1-(7)-ene-2,8-diols **12a,b** according to their 70 eV electron impact (EI) mass spectra and retention indices compared with authentic standards **4b** and literature data (Bohlmann et al. 1985).

Similar observations were made after the addition of 3,10-epoxymyrcene **3**. Besides the vicinal β-myrcene diol, 6-methyl-2-ethenyl-5-hepten-1,2-diol **11** (Fig. 1), four additional products of hydrolysis were identified as (*E*/*Z*)-2-(4-methyl-3-pentenyl)-2-butene-1,4-diol **4a**,**b**, *p*-menth-1-ene-7,8-diol (7-hydroxyterpineol) **13** and its oxidation product 8-hydroxyphellandral **14** according to their 70 eV EI mass spectra and retention indices compared with authentic standards **4a**,**b** and literature data (Ohloff and Giersch 1980; Miyazawa and Ohsawa 2002). With the exception of **14**, all products were also found in the chemical blanks. Possible pathways to the β-myrcene dihydrodiols are compiled in Fig. 2.

Both the chemosynthetic and the biotic formation of the respective β-myrcene epoxides resulted in almost racemic mixtures (Table 2); a small enantiomeric excess was obtained only for the biocatalysed formation of 3,10-epoxymyrcene. Hydrolysis of epoxymyrcenes in sterile culture medium at pH 6.8 to the corresponding racemic diols started immediately. Supplementation of the racemic epoxides to the culture medium of *P. ostreatus*, however, resulted in ee values of around 40% for the diol products (Table 2). This suggested that stereoselective epoxide hydrolases were active in the fungal cells. Hence, the ee values of the respective vicinal β-myrcene diols (Table 2)

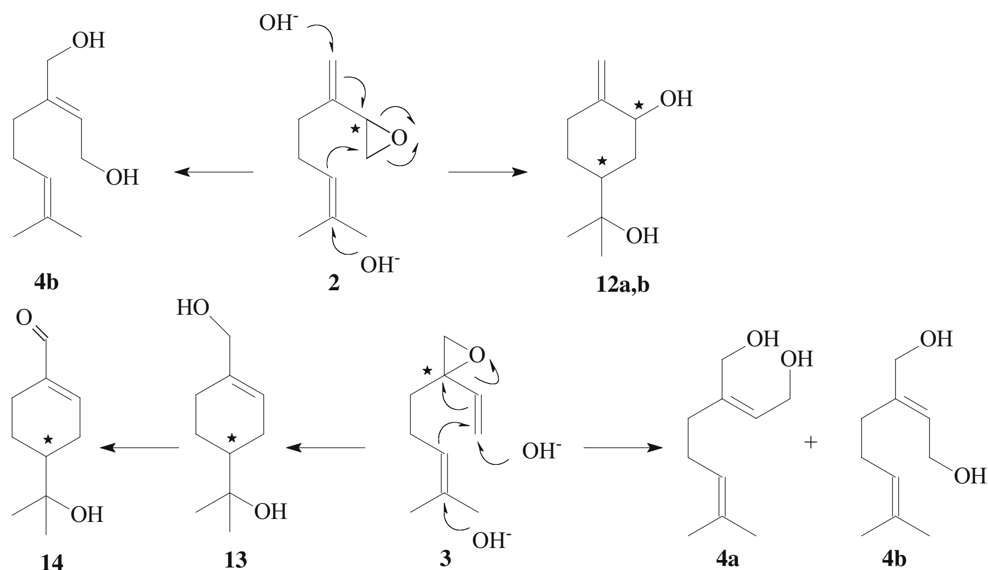

Fig. 2 Conversion products of β-myrcene epoxides additionally to the favoured generation of the respective vicinal diols (Fig. 1), **2,** 1,2-epoxymyrcene; **3**, 3,10-epoxymyrcene; **4a**, α-(*Z*)-acaridiol, **4b**, α-(*E*)-acaridiol; **12a**,**b**, (*E*/*Z*)-*p*-menth-1-(7)-ene-2,8-diol; **13**, 7-hydroxyterpineol; **14,** 8-hydroxyphellandral

represent the competing chemical and enzymatic hydrolyses. Biotic α-(*Z*)-acaridiol **4a** was obtained in low concentrations exclusively after hydrolysis of 3,10-epoxy β-myrcene added to the cells (Table 1). Intermediates following α-(*Z*)-acaridiol were not detected upon epoxide addition.

Supplementation of α-(Z)-acaridiol To prove if the concentration of α-(*Z*)-acaridiol **4a** was too low to allow for the formation of detectable concentrations of the subsequent metabolites, the nutrient medium, with or without active cells, was supplemented with this diol (Table 1). After 2 days of incubation, a noticeable portion of the diol remained unchanged in the cell-free culture broth. In this chemical blank, about 77% of the initially added compound was recovered, and this was just the recovery rate obtained in solvent extracts (three times azeotropic pentane diethyl ether). Besides a slow isomerisation to the (*E*)-isomer, α-(*Z*)-acaridiol was chemically stable under conditions, and no further volatile conversion products were detected.

In the presence of cells of *P. ostreatus*, by contrast, only one third (24.5% of 77%) of the precursor was recovered after 2 days of incubation. With the exception of 3-hydroxymethyl-7-methyl-octa-2,6-dienal **6a**, all of the expected metabolites, α-(*Z*)-acariolal **5a**, α,α- and α,β-acarilactol **6a**,**b**, both acariolides **8**,**9**, and the target compound perillene **7** were detected in the nutrient medium (Fig. 1, Table 1). The well water soluble acariolides **8**,**9** were found in a ratio of 1:8 (α,α:α,β), suggesting that the oxidation of α-(*Z*)-acaridiol along the "α,β-pathway" was preferred (compounds **6** and **9** in Fig. 1). The primary oxidation product of both terminal hydroxyl groups, (*E*/*Z*)-2-(4-methyl-3-pentenyl)-butanedial (α-acaridial), was not found.

Supplementation of α,α-(Z)-acarilactol Addition of 379 mg L^{-1} of α,α-acarilactol resulted in maximum concentrations of perillene of 24.0 (*P. ostreatus*) or 14.5 (blank) mg L^{-1} within 5 h (Table 1). α,α-Acariolide was found in the nutrient medium of *P. ostreatus* to a much larger amount than in the chemical blank, while in the blank, α,α-acariolide and α-(*Z*)-acaridiol occurred at about the same concentrations. In the presence of *P. ostreatus*, α-(*Z*)-acaridiol was not found because α-(*Z*)-acaridiol was rapidly re-oxidised and converted to α,α-acarilactol and perillene, as shown above.

Supplementation of perillene A chemical blank of perillene **7** in nutrient medium showed no volatile conversion products; perillene completely evaporated during 48 h. When submerged cultures of *P. ostreatus* were supplemented with perillene, a significant concentration of α,α-acariolide **8** was built up, but neither α,β-acariolide **9** nor the respective acarilactols **5**,**6** were found (Table 1).

Table 1 Maximum product concentration after supplementation of intermediates of the perillene pathway of *P. ostreatus*

Product [mg L^{-1}]	Precursor									
	1,2-Epoxymyrcene[a]		3,10-Epoxymyrcene[a]		α-(*Z*)-Acaridiol[b]		α,α-Acarilactol[b]		Perillene[b]	
	Blank	*P. ostreatus*	Blank	*P. ostreatus*	Blank	*P. ostreatus*	Blank	*P. ostreatus*	Blank	*P. ostreatus*
1,2-Myrcenedihydrodiol	3.8	9.6	–	–	–	–	–	–	–	–
3,10-Myrcenedihydrodiol	–	–	25.7	26.7	–	–	–	–	–	–
p-(*E*/*Z*)-Mentha-1-(7)-en -2,8-diol	5.8	14.6	–	–	–	–	–	–	–	–
7-Hydroxyterpineol	–	–	5.1	2.7	–	–	–	–	–	–
8-Hydroxyphellandral	–	–	–	3.0	–	–	–	–	–	–
α-(*Z*)-Acaridiol	<0.1	<0.1	3.5	1.6	658	224	3.5	<0.1	–	–
α-(*E*)-Acaridiol	3.3	1.3	<0.1	0.3	7.0	1.6	–	–	–	–
α,α-Acarilactol	–	–	–	–	–	0.1	<0.1	1.8	–	–
α,β-Acarilactol	–	–	–	–	–	–	–	–	–	–
Perillene	–	–	–	–	–	3.8	14.5	24.0	<0.1	<0.1
α,α-Acariolide	–	–	–	–	–	4.6	3.5	53.1	–	5.3
α,β-Acariolide	–	–	–	–	–	35.5	–	–	–	–

– Not detectable
[a] After 5 h
[b] After 48 h

Discussion

Supplementation of precursors Some of the structurally confirmed intermediates (Fig. 1) were added in separate experiments to the culture medium of *P. ostreatus* (Table 1). This was expected to reveal bottle necks of the formation of perillene and to allow to distinguish between enzyme-catalysed and chemical reaction steps. For all experiments, major gaps of the mass balance were found. This is partly explained by the use of cellulose plugs on the culture flasks, necessary to cover the demand of oxygen of the growing fungi. The high vapour pressure of both precursor and some of the intermediates caused losses through evaporation. Another possible source of losses, the formation of non-volatile bioconversion products, was not investigated. Nevertheless, additional information about details of the conversion of β-myrcene was deduced from these experiments.

The perillene pathway The precursor studies confirm a novel pathway toward the rare furanoid monoterpene perillene in the fungus *P. ostreatus* (Fig. 1). It can be envisaged that the key reaction steps, epoxide formation, hydrolysis, partial diol oxidation, lactolisation and aromatisation may as well occur in plants. However, genetic analyses of essential oil variants of *Perilla frutescens* pointed to a perillene formation along the mevalonate pathway *via* geranyl diphosphate and geranial (Yuba et al. 1996).

Chemical epoxide hydrolysis can be either acid- or base-catalysed. In either case, the ring opens along an S_N2-pathway with inversion of configuration at the reacting carbon atom (Schneider 2006). Therefore, the chemical hydrolysis of racemic epoxides results in a racemic mixture, as shown for the β-myrcene diols (Table 2). Most epoxide hydrolases belong to the α,β-hydrolase fold family which also encompasses many lipases, esterases and dehalogenases. Because of their prevalent stereoselectivity (often ee >90%), epoxide hydrolases can be either used to prepare enantiopure epoxides by kinetic resolution and/or to furnish vicinal diols of distinct stereochemistry (Steinreiber and Faber 2001; de Vries and Janssen 2003). The ee values of the three myrcene dihydrodiols suggest the contribution of an epoxide hydrolase of *P. ostreatus* to their formation (Table 2).

The enzymatic two-step cleavage of an epoxide starts with the attack of an aspartate group, reacting as a nucleophile to yield a covalent "glycol-monoester" intermediate. The electrophilic character of the oxiran ring is increased by proton-delivering amino acids. X-ray structures of epoxide hydrolases have shown that two tyrosine moieties are responsible for substrate activation. In the second catalytic step, a water molecule is activated by histidine and finally hydrolyses the monoester intermediate to furnish the vicinal glycol as a product (Fig. 3a; Steinreiber and Faber 2001; Smit 2004).

Table 2 Enantiomeric distribution (ee values) of β-myrcene epoxides and hydrolysis products thereof

	Chemosynthetic	Biotic product[a]
1,2-Epoxymyrcene **2**	0.2	0.8
3,10-Epoxymyrcene **3**	0.3	15.8
6,7-Epoxymyrcene	0.1	0.3
1,2-Myrcene dihydrodiol **10**	3.7[b]	47.0
3,10-Myrcene dihydrodiol **11**	0.8[b]	44.4
6,7-Myrcene dihydrodiol	0.2	39.6

[a] Found 3 days after feeding of β-myrcene (5.9 mmol 100 mL^{-1}) to submerged cultures of *P. ostreatus*

[b] Hydrolysis of the respective epoxymyrcene (synthetic, 25 mg L^{-1}) in sterile culture medium.

A similar but non-enzymatic activation of an enal-system has been described for the conversion of citral (Fig. 3b; Wolken et al. 2000). During the bioconversion of the pure isomers of citral (geranial and neral), a spontaneous chemical isomerisation has been observed in samples containing boiled cell extracts, whereas both isomers have been found to be chemically stable in phosphate and Tris buffer of the same pH. Isomerisation and subsequent degradation of both neral and geranial to 6-methyl-5-hepten-2-one has been catalysed by amino acids, with D,L-asparagine being the most efficient catalyst. It has also shown that the isomerisation is pH dependent. The separation of charge in the citral molecule over four positions requires stabilisation of both the intermediate negative charge at the ammonium nitrogen and the positive charge at the carboxy function (Fig. 3b, position 4). Furthermore it has been shown that both functionalities must be provided by the same molecule.

Formation of acaridiols An analogous observation was made for α-(*Z*)-acaridiol **4a**: it is found in the culture liquid (in the presence or absence of heat-inactivated fungal cells and in L-asparagine-containing buffer), but not in amino acid-free Tris–HCl buffer of the same pH of 6.8. The main nitrogen source of the nutrient medium used here is L-asparagine, the amino acid with the highest activity found for citral isomerisation (Wolken et al. 2000). The terminal oxiran ring in conjugation to a double bond of the two epoxymyrcenes **2**,**3** represents a structural moiety similar to the α,β-unsaturated citral. Therefore, a similar transition state, shown exemplary for 3,10-epoxymyrcene, may be passed through (Fig. 3c). The cleavage of the oxiran ring of **2** or **3** is facilitated by protonation. The less stable terminal carbocation is stabilised by the carboxyl group of the catalytic amino acid. This may explain the formation of the (*E*/*Z*)-2-(4-methyl-3-pentenyl)-2-butene-1,4-diols (α-acari-

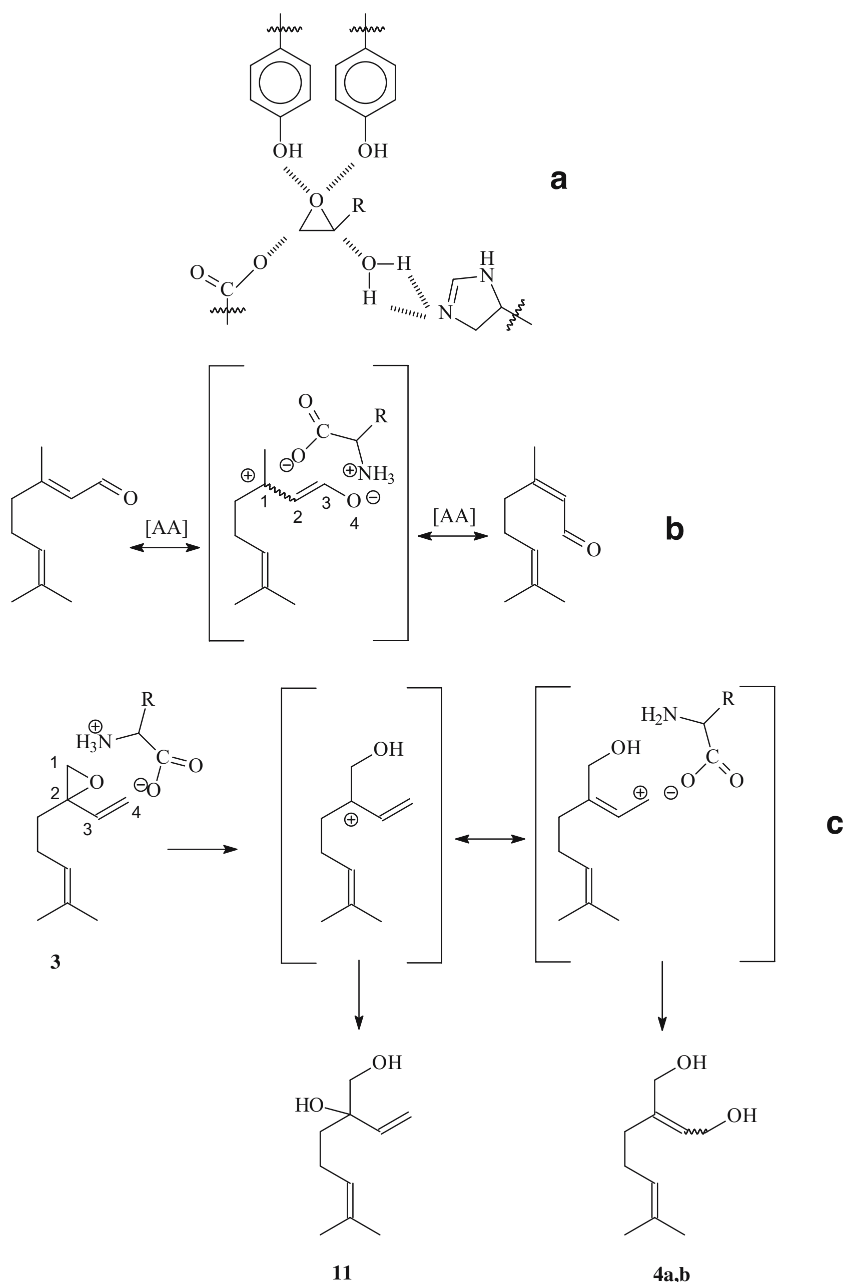

Fig. 3 Proposed transition states. **a** Hydrolysis of monosubstituted epoxides by *Aspergillus niger* epoxide hydrolases (according to (Smit 2004)). **b** Reversible isomerisation of citral isomers according to (Wolken et al. 2000); *AA* amino acid. **c** Postulated amino acid catalysed hydrolysis of 3,10-epoxymyrcene **3**

diols) **4a**,**b** besides the favoured hydrolysis to vicinal diols. A close contact of the epoxymyrcenes with the catalytic amino acid would allow a simultaneous oxiran ring cleavage and water addition at the intermediate terminal carbocation and explain the preferred formation of the (*Z*)-isomer **4a**.

A chemical generation of 1,4-butene diols using transition metal oxide catalysis at elevated temperatures (>80°C) has been described in a German patent application (Pinkos et al. 1994), but is not suitable to explain the formation of α-(*Z*)-acaridiol under moderate microbiological conditions. On the other hand, a contribution of common epoxide hydrolases to the formation of α-(*Z*)-acaridiol is unlikely as well because epoxide hydrolases are known to yield vicinal diols (Faber 2004).

Steps down-stream of α-(Z)-acaridiol Among all myrcene diols, α-(*Z*)-acaridiol **4a** is the only reasonable immediate precursor of perillene (Fig. 1). Its low intermediate concentrations may result from either slow formation or rapid consumption by formation of the hydroxyaldehydes **5a**,**6a** and the lactols **5b**,**6b**. Hydroxyl groups are easily oxidised by alcohol dehydrogenases which are amply cofactor supplied by the growing basidiomycete cells. The equilibria from **5a** to **6a** and from **5b** to **6b** are shifted to the lactols by dehydration and formation of perillene **7**.

Upon supplementation of α,α-(*Z*)-acarilactol, α,α-acariolide was present in the nutrient medium of *P. ostreatus* in much higher concentrations than in the chemical blank where α,α-acariolide and α-(*Z*)-acaridiol occurred in the same concentration (Table 1). Diol formation requires the reduction of α,α-acarilactol, a reaction highly unlikely in a cell-free nutrient medium. The occurrence of both compounds in equal amounts was explained by a disproportionation reaction (Fig. 4).

Fate of perillene α,α-Acariolide **8** was the only volatile product produced by the fungal cells in the presence of excess perillene (Table 1) indicating that the dehydration of the acarilactols was irreversible. In contrast, α,β-acariolide **9** was mostly likely generated via the oxidation or disproportionation of the corresponding α,β-acarilactol **6b** (analogous to **5b** in Fig. 4). Lactone intermediates are not unusual in microbial metabolism of terpenes, as nucleophilic opening and metabolic activation of the hence-formed hydroxy acid conjugate is a primrose pathway to further degradation by β-oxidation.

Chemical vs. biochemical conversion The conversion of β-myrcene to perillene requires at least two enzymatic steps, first the introduction of oxygen at the 1,2 or 3,10-double bonds of β-myrcene to yield the respective epoxides, and second the oxidation of the intermediary cis-1,4-butenediol at (either) one of the hydroxyl groups. Epoxidations are usually catalysed by cytochrome P450 monooxygenases and are believed to be the initial step of the detoxification of cytotoxic compounds, such as hydrocarbons (Faber 2004). However, a true P450 enzyme should have exhibited more stereoselectivity (Table 2).

The generation of the key intermediate α-(*Z*)-acaridiol was most likely an asparagine-catalysed chemical hydrolysis and the rate-limiting step in perillene generation. The cyclisation of the respective hydroxy aldehydes to the corresponding lactols followed by the thermodynamically-favoured elimination of water resulted in the heteroaromatic target compound perillene, obviously without involving enzyme catalysis. Another enzymatic activity, the selective oxidation of the heteroaromatic ring to α,α-acariolide, decreased the yields of perillene.

Environmental aspects The industrial production of flavours and fragrances from essential oils generates wastes of terpene hydrocarbons such as limonene, pinenes, terpinene and myrcene. The bioconversion of these hydrocarbons to bioactive and aroma compounds, such as perillene, could therefore represent a contribution to a sustainable future processing.

Since its first discovery back in 1919, perillene was found as an aroma compound in the essential oil of several species of plants (Suzuki et al. 1986; Başer et al. 2003). However, bioconversion products of β-myrcene described in this study are not only of interest as attractive aroma compounds. Perillene, for example, from the secretion of mandibular glands of the formicine ant, *Lasius fuliginosus LATR*., and the "acari"-compounds of thrips and mites were identified in several animals. It was assumed that these compounds serve as pheromones which would agree with their high volatility (Suzuki et al. 1986).

Acarid mites were recorded in Japan as an economically important agricultural pest. The opisthonotal gland exudation of these mites served as alarm, aggregation and sex pheromones (Leal et al. 1989; Tarui et al. 2002; Mizoguchi et al. 2003). Constituents of the mites' secretions (source of the prefix "acari" of the trivial names) were identified as oxygenated β-myrcene derivatives. Acariolals, acaridials, and acariolides were discovered. While the function of α,α- and α,β-acariolide is still unknown, α-acaridial possessed strong antifungal properties, in addition to the pheromone activity which was also established for the acariolals (Shimizu et al. 2003). The usage of these compounds as natural attractants in pest control or as agrochemical fungicides was considered (Kuwahara 1990).

Prospects The study of the bioconversion of the volatile, cytotoxic, and chemically unstable (Busmann and Berger 1994) β-myrcene to the volatile product perillene poses technical and analytical problems. Experiments using closed aeration gas systems, as demonstrated for limonene

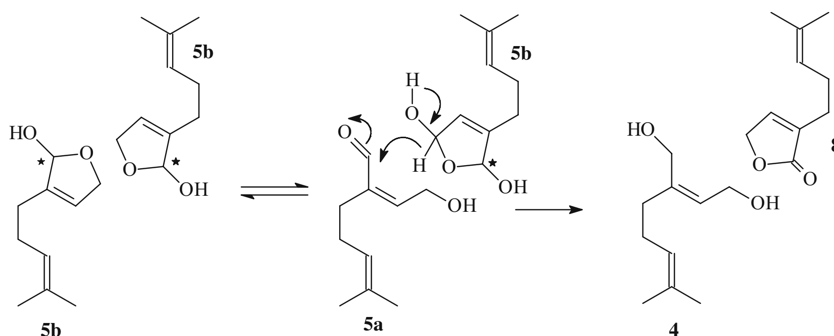

Fig. 4 Proposed disproportionation of α,α-acarilactol **5b 4a**, α-(*Z*)-acaridiol; **5b**, α,α-acarilactol; **8**, α,α-acariolide

conversion with *P. digitatum* (Schewe et al. 2006), or in situ product recovery (Rito-Polomares et al 2001; Hua et al. 2007), will reduce losses due to evaporation and contribute to close the gaps of the mass balance, as will the search for non-volatile metabolites. Work on the enzyme level will be required to establish a "white biotechnology" process delivering industrially acceptable yields.

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