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ARTICLE

Autoxidation versus Biotransformation of α-Pinene to Flavors with *Pleurotus sapidus*: Regioselective Hydroperoxidation of α-Pinene and Stereoselective Dehydrogenation of Verbenol

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The enzymatic conversion of α-pinene to verbenols, verbenone, and minor volatile flavors was studied using submerged cultured cells, lyophilisate, and microsomal fractions of the edible basidiomycete *Pleurotus sapidus*. The similarity of the product range obtained by the bioconversions with the range of products found after autoxidation of α-pinene at 100 °C suggested similar initial pinene radicals. Extracts of the bioconversions were analyzed using thin layer chromatography with hydroperoxide staining and cool on-column capillary gas chromatography−mass spectrometry. Two isomer α-pinene hydroperoxides were identified as the key intermediates and their structures confirmed by comparison with synthesized reference samples and by microchemical reduction to (*Z*)- and (*E*)-verbenol. When the biocatalysts were supplemented with one of the verbenols, only the (*Z*)-isomer was oxidized, indicating the activity of a highly stereospecific monoterpenol dehydrogenase. The structural comparison of subunits shows that fungal oxifunctionalization reactions of some common terpene substrates, such as (+)-limonene or (+)-valencene, might likewise be catalyzed by dioxygenases rather than by CYP450 enzymes, as previously assumed.

KEYWORDS: *Pleurotus sapidus*; autoxidation; α-pinene dioxygenase; hydroperoxides; stereoselective verbenol dehydrogenase

INTRODUCTION

Terpenoids (isoprenoids) encompass more than 40000 structures and form the largest class of known plant metabolites. They are widely used as industrially important chemicals, including pharmaceuticals, flavors, fragrances, pesticides, and disinfectants and as large-volume feedstocks for chemical industries (*1*). Many terpene hydrocarbons are abundant in nature, for example, (+)-limonene and the pinenes. Due to their chemical instability and poor sensory impact, they are not qualified as flavorings, but represent an ideal starting material for biocatalytic oxyfunctionalization leading to natural terpenoid flavor and fragrance compounds (*2*). The oxygenation of hydrocarbons with dioxygen occurs formally along four possible mechanisms of reductive activation of oxygen (*3*):

(i) Liquid-phase oxidation reactions, particularly conversion of unsaturated hydrocarbons, are most often proceeded by an autoxidation mechanism. The reaction is initiated by the abstraction of a hydrogen atom in allylic position to form free radicals from the substrate. Dioxygen (triplet oxygen) is activated through attachment to an alkyl radical with formation of a hydroperoxide radical.

(ii) In terms of photo-oxidation a double bond is directly attacked by light-excited dioxygen (singlet oxygen) to give the respective hydroperoxides.

(iii) If the system contains a coreducer that is more readily oxidizable by oxygen into a peroxide than the substrate, then the overall process will be a conjugate reaction (co-oxidation). The unsaturated hydrocarbon will react with the primarily generated peroxide by a homolytic or heterolytic mechanism.

(iv) Enzymatic oxidations are regarded as a special class of conjugated reactions. The high selectivity of substrate conversion is due to transition metal complexes, the ligands of which participate in electron transfer and control the stereochemistry of the process.

One of the earliest publications on microbial terpene oxyfunctionalization described the biotransformation of α-pinene **1** by *Aspergillus niger*. The culture broth contained verbenone **4**, (*E*)-sobrerol **11**, hydroxycarvotanacetone, and (*Z*)-verbenol **3b** as main products (*4*). Since that time, verbenone **4**, an impact compound of rosemary oil, and its precursor verbenol **3** have been frequently described as the main products of biotransformations of

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α-pinene with bacteria, yeasts, and fungi (*2*, *5*). The oxyfunctionalization reactions are usually attributed to cytochrome P450 monooxygenases. However, only a few microbial terpene transforming P450 proteins were purified. Among the few exceptions are P450cam from *Pseudomonas putida* and P450BM-3 from *Bacillus megaterium*, which converted (+)-valencene to a number of terpenoids of the nootkatone family (*6*). This paper describes the biotransformation of α-pinene with *Pleurotus sapidus*. On the basis of the identification of novel intermediates and products, the reaction mechanism was elucidated, and two enzymes, a regiospecific α-pinene dioxygenase and a stereoselective (*Z*)-verbenol dehydrogenase, were described for the first time to be responsible for the microbial formation of verbenone **4**.

MATERIALS AND METHODS

Fungi. *Pleurotus sapidus* (DSM 8266), *Chaetomium globosom* (DSM 1962), *Stemphylium botryosum* (DSM 62928), *Pleurotus eryngii* (CBS 458.79), and *Penicillium solitum* (CBS 424.89) were obtained from the culture collections of DSMZ, Braunschweig, Germany, and Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands. For maintenance on agar slants and submerged culture, the fungi were grown on glucose/asparagine/yeast extract medium as described elsewhere (*7*). The flasks were sealed with cellulose plugs and autoclaved for 20 min at 121 °C.

Fungal Growth. *Submerged Cultivation* (*SB*). The fungal cultures (1 cm^2 of a densely overgrown piece of agar) were inoculated into a 100 mL glucose/asparagine/yeast extract medium, homogenized using an Ultraturrax homogenizer (Jahnke & Kunkel, Staufen Germany), and grown aerobically at 24 °C and 150 rpm on an orbital shaker (Multitron, Infors, Bottingen, Switzerland) in the dark. Experimental cultures (500 mL shake flasks, 250 mL medium volume) were inoculated with 25 mL of 3-day-old precultures grown on the same medium and homogenized prior to inoculation.

Mycelium Concentrates (*MC*), *Lyophilisates* (*Lyo*), *and Microsomal Fraction* (*MF*). After 3 days of fungal growth, the active biomass was harvested by centrifugation at 10000*g* and withdrawal of the supernatant. In terms of MC to approximately 50 g of fungal mycelium (wet weight) was added 50 mL of fresh liquid medium before the addition of α-pinene **1**. For mycelium lyophilisates the fungal biomass was transferred into a 400 mL beaker, shock frozen using liquid nitrogen, and lyophilized (Valco2, Zirbus Technology, 37539 Bad Grund, Germany) at room temperature, 2×10^{-5} Pa, and sterile conditions. The MF was obtained from harvested biomass after cell disruption using a ball mill. To a portion of 30 g of frozen fungal mycelium were added 80 mL of beads and 25 mL of 200 mM HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl)ethansulfonic acid) buffer, pH 5.5, with 5% glycerol, and the mixture was cooled to −16 °C. During the milling process the temperature did not exceed 4 °C. The suspension was separated from the beads by filtration (linen) and subsequently centrifuged for 90 min at 100000*g*. The microsomal pellet was redissolved in 15 mL of HEPES buffer containing 0.5% OBAS and 2.0% sodium cholate (MF) and centrifuged again at 100000*g*.

Chemicals. α-Pinene (96%, Fluka, Germany) and azeotropic pentane/diethyl ether (1:1.12) were distilled before use. BHT (3,5-di-*tert*-butyl-4-hydroxytoluene, 99%), myrtenol (>99%), myrtenal (>95%), (*E*)-pinocarveol (98%), (*E*)-sobrerol (95%), (*Z*)-verbenol (>95%), verbenone (>99%), α-pinene epoxide (<97%), and sodium cholate (purum) were from Fluka (Seelze, Germany), and OBAS (4-octylbenzyl amidosulfobetaine, purum) was provided from Chalbiochem (San Diego, CA). All other chemicals used were of analytical grade.

Synthesis of (*E*)- *and* (*Z*)-α-*Pinene Hydroperoxide*. (*E*)- and (*Z*)-α-pinene hydroperoxides were synthesized according to the method by Moore (*8*). In brief, 100 g of α-pinene was heated in a stream of oxygen (purity = 3.5) at 100 °C. The reaction yielded a mixture of products with α-pinene epoxide, (*E*)-verbenol, verbenone, and the assumed (*E*)-verbenyl hydroperoxide as the main products in a ratio of 4:4:1:1; (*Z*)-verbenyl hydroperoxide (10% intensity of (*E*)-verbenyl hydroperoxide) was found as a minor product, only. After reduction of the mixture with Na_2S in the presence of NaOH, the concentration of products remained constant except for verbenyl hydroperoxides, which completely disappeared for the benefit of (*E*)- and (*Z*)-verbenol.

(*E*)-*Verbenyl hydroperoxide:* GC-MS ($RI_{CW\ 20M}$ = 2132); [*m*/*z* (% of base peak)] 168 [M^+ (<0.1)], 153 [$M^+ - CH_3$ (<0.1)], 150 [$M^+ - H_2O$ (2.0)], 137 (9.0), 134 (10), 121 (12), 119 (35), 109 (82), 94 (57), 91 (100), 81 (61), 55 (49), 43 (65), 41 (89).

(*Z*)-*Verbenyl hydroperoxide:* GC-MS ($RI_{CW\ 20M}$ = 2103); [*m*/*z* (% of base peak)] 168 [M^+ (<0.1)], 150 [$M^+ - H_2O$ (<0.1)], 137 (10.0), 134 (11), 121 (7), 119 (42), 109 (72), 94 (95), 91 (91), 79 (60), 67 (53), 59 (74), 43 (62), 41 (100).

Synthesis of (*E*)-*Verbenol*. The reaction mixture of α-pinene hydroperoxides after reduction with Na_2S was fractionated using silica gel (silica gel 60; 0.063−0.100 mm, Merck KGaA, Darmstadt, Germany) and pentane/diethyl ether (80:20, v/v) as the eluent yielding (*E*)-verbenol of >95% purity (GC): GC-MS ($RI_{CW\ 20M}$ = 1681); [*m*/*z* (% of base peak)] 152 [M^+ (1.0)], 137 [$M^+ - CH_3$ (10.8)], 134 [$M^+ - H_2O$ (11.5)], 123 (8.9), 121 (13.4), 119 (38.2), 117 (8.2), 110 (9.8), 109 (88.4), 107 (20.4), 105 (15.3), 95 (39.2), 94 (60.3), 93 (20.6), 92 (33.8), 91 (100), 81 (58.0), 79 (40.4), 69 (40.0), 67 (45.2), 55 (47.6), 43 (54.3), 41 (77.8).

The trans orientation of the hydroxyl group was confirmed by NMR (nuclear Overhauser effect, NOE) spectrometry (Bruker Advance DRX-500, ^{1}H-NOE difference spectrum, 500 MHz, C_6D_6). An NOE-signal of the proton at C4 and the methyl protons at C9 was observed with (*Z*)-verbenol only.

α-Pinene Bioconversion. Conversion experiments were started by adding 1 mmol of α-pinene **1** to 100 mL (SB), 50 mL (MC) medium after 3 days of fungal growth, to 15 mL of 0.1 M MOPS buffer, pH 7.0 (Lyo), or 0.2 mmol of HEPES buffer, pH 5.5 (MF), respectively. Different incubation times at 24 °C and 150 rpm on an orbital shaker (Multitron, Infors, Bottingen, Switzerland) in the dark are referred to in the text. For each α-pinene biotransformation a blank was performed using exactly the same conditions and the respective heat-inactivated biocatalyst.

Product Isolation and Identification. Conversion products of α-pinene **1** were extracted three times with 20 mL of azeotropic pentane/diethyl ether (1:1.2, v/v). The combined and dried (Na_2SO_{4sicc}) organic phases were concentrated to approximately 1 mL and injected into a cool on-column after the addition of 60 μg of the external standard BHT.

One microliter 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) 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 FID (230 °C) using a temperature program from 40 °C (3 min) to 230 °C at a rate of 3 °C min^{-1} to 250 °C and held for 5 min. Quantification was performed according to the standard BHT.

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 the literature (Wiley 08/NIST 08, 2008; 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 ionization, 70 eV; scan range, *m*/*z* 33−300 amu).

Thin layer chromatography (TLC) was carried out on aluminum sheets covered with silica gel 60 (Merck, Darmstadt, Germany) and azeotropic pentane/diethyl ether (1:1.12) as the mobile phase. The TLC plates were dried and subsequently stained with either anisaldehyde or the hydroperoxide specific Huber reagent (3.0 g of α-naphthol, 150 mL of methanol, 1350 mL of H_2O, 0.5 g of $K_2S_2O_5$, 20 mL of acetic acid, 0.5 g of $FeSO_4 \cdot 7H_2O$, 2.2 g of 2-[(4-amino-3-methylphenyl)ethylamino]ethyl sulfate) (*9*).

RESULTS AND DISCUSSION

Biotransformation. Some ascomycetes and basidiomycetes have been found to tolerate millimolar concentrations of the cytotoxic bicyclic monoterpene α-pinene **1**. Five strains—known for their potential to convert terpenes (*10*−*12*)—were submerged cultured and supplied with 1 mmol of α-pinene **1** to produce natural verbenone **4**. Some 20 mg of (*E*)-verbenol **3a** and up to 4 mg of verbenone **4** were found in the culture liquid after 48 h (**Table 1**). However, control experiments with inactivated biocatalysts under the same conditions resulted in the same product

Table 1. Fungal Biotransformation of α-Pinene **1** to (*E*)-Verbenol **3a** and Verbenone **4**

fungus/ incubation time	(*E*)-verbenol[a] (mg L^{-1})	verbenone[a] (mg L^{-1})	ratio (*E*)-verbenol/ verbenone
P. eryngii (SB)[b]/48 h	<0.5	15.0	<1
S. botryosum (SB)/48 h	10.0	1.9	5.3
P. solitum (SB)/48 h	10.2	1.5	6.8
C. globosum (SB)/48 h	13.0	4.0	3.3
P. sapidus (SB)/48 h	19.9	3.1	6.0
P. sapidus (MC)[b]/20 h	39.3	9.8	4.0
P. sapidus (Lyo)[b]/20 h	155.6	61.9	2.5
P. sapidus (MF)[b]/20 h	152.2	149.3	1.0
blank (Lyo)[c]/20 h	9.8	1.8	6.1

[a] Mean of at least three independent measurements, CV not exceeding 10%. [b] SB, submerged culture; MC, mycelium concentrate; Lyo, lyophilisate; MF, microsomal fraction. [c] Similar concentrations found with SB and MF.

spectrum (**Tables 1** and **2**) as with the active biocatalysts. This raised the question as to what extent α-pinene **1** transformation observed was the result of an enzymatic reaction. The difference in product yields of (*E*)-verbenol **3a** and verbenone **4** from the control was largest with *P. sapidus* and doubled approximately the yields obtained with the chemical blank (**Table 1**). Therefore, further experiments were carried out using *P. sapidus*. Interestingly, *P. eryngii* produced verbenone **4** as the main biotransformation product and did not accumulate any detectable amounts of (*E*)-verbenol **3a**, the main product of α-pinene autoxidation (blank) and biotransformation with the other fungi.

In concurrent transformation studies it was found that mycelium concentrates and lyophilisates were superior to submerged cultured fungi in terms of terpene oxidation (*13*−*15*). Consequently, mycelium concentrates and lyophilisated mycelium of *P. sapidus* responded to the presence of α-pinene **1** with a significant increase of the concentration of (*E*)-verbenol **3a**, verbenone **4**, and, to a minor extent, pinene epoxide **10**. Other volatile products stayed at the low concentrations observed with the respective blanks (autoxidation). This confirmed that, besides the unavoidable autoxidation, a regioselective enzymatic oxidation of α-pinene **1** took place. This is supported further by the relative decrease of the (*E*)-verbenol **3a** to verbenone **4** ratio from 6.1 (autoxidation) to 2.5 (lyophilisate) and 1.0 (microsomal fraction) (**Table 1**).

The increased generation of α-pinene epoxide **10**, (*E*)-verbenol **3a**, and verbenone **4** might be interpreted as a cytochrome P450 monooxygenase catalyzed oxidation of α-pinene **1** (**Figure 1**). In eukaryotic organisms the P450 activities are associated with membranes, whereas in bacteria they are found to be soluble (*16*, *17*). To separate the assumed P450 monooxygenase(s) from the fungal mycelium the lyophilisate was resuspended in buffer and solubilized using a combination of two detergents, OBAS and sodium cholate, which was found best to mantain a maximum of α-pinene oxidizing activity (data not shown). The cell debris was removed by filtration, and the filtrate was centrifuged at 100000*g*, but neither the resulting microsomal pellet nor the supernatant showed roughly the same high activity as the resuspended lyophilisate. However, recombination of the supernatant and the microsomal pellet (microsomal fraction, MF) restored the activity completely (data not shown). Using this MF almost the same peak concentration of (*E*)-verbenol **3a** and a significant increase of the verbenone **4** concentration was achieved. A very weak Soret band, which should have indicated the presence of the heme group of a P450 enzyme in the CO difference spectrum of the MF (data not shown) and the missing corresponding alcohol, 3-pinanol **12**, render P450 monooxygenase

Table 2. Volatile Product Spectrum of α-Pinene **1** Biotransformation (Lyophilisate of *P. sapidus*) and Autoxidation (Inactivated Lyophilisate); 20 h Incubation Time

product	identification[a]	RI (CW 20 M)
α-pinene epoxide **10**	MS, ref	1608
myrtenal[b] **7**	MS, ref	1612
(*E*)-pinocarveol[b] **9**	MS, ref	1652
(*Z*)-verbenol[b] **3b**	MS, ref	1658
(*E*)-verbenol **3a**	MS, Syn, NMR	1681
verbenone **4**	MS, ref	1703
myrtenol[b] **6**	MS, ref	1792
(*E*)-carveol[b]	MS, ref	1841
α-(*Z*)-verbenyl hydroperoxide **2b**	MS, Syn	2103
α-(*E*)-verbenyl hydroperoxide **2a**	MS, Syn	2132
(*E*)-sobrerol[b] **11**	MS, ref	2225

[a] MS, reference mass spectrum; LRI, retention index (polar GC-column, Carbowax 20M) according to Van den Dool and Katz method (*26*); ref, reference compound co-analyzed; Syn, reference compound synthesized; NMR, stereochemistry confirmed by NMR data. [b] No significant differences found in blanks and biotransformations, concn < 1.5 mg L^{-1}.

catalysis unlikely; instead, a lipoxygenase-like absorption band with $\lambda_{max} = 418$ nm was observed. However, a minor contribution of a P450 activity to α-pinene oxidation cannot be ruled out. The enzyme-catalyzed oxidation of α-pinene **1** seemed to proceed along a similar mechanism as autoxidation, but with a clear regioselective preference toward the allylic oxidation at C4 of α-pinene **1**.

Autoxidation. To distinguish between autoxidation and a dioxygenase activity a detailed look at the steps of α-pinene **1** autoxidation is needed. The reaction kinetics follow a radical chain reaction and result in numerous products (**Tables 1** and **2**). Among other minor products the main products found in several studies were (*E*)-verbenol **3a** and verbenone **4** (*8*, *18*, *19*). At ambient temperatures and atmospheric conditions, the autoxidation of pine and fir tree resins, with α-pinene **1** being the major constituent, produces significant amounts of (*E*)-verbenol **3a**, an aggregation pheromone for many species of bark beetles (*20*). Peroxides might work as initiators of the radical chain reactions in the liquid-phase olefin autoxidations. The rapid decomposition of alkylhydroperoxides in hydrocarbon solution at elevated temperature and/or in the presence of trace amounts of transition metal ions is a well-known reaction. It is worth to noting that (*Z*)-verbenol **3b** was not found or found in traces only among the degradation products. Depending on time, temperature, and oxygen flow rate the molar ratio of (*E*)-verbenol **3a** to verbenone **4** decreases with increasing values of these three parameters. At ambient temperature, however, a huge excess of (*E*)-verbenol **3a** over verbenone **4** was found (*8*, *21*). The initiation step of α-pinene **1** autoxidation is the abstraction of a hydrogen atom. The activation energy of this reaction is estimated from Arrhenius plot to be 81.3 kJ mol^{-1} (*19*). The propagation of the chain reaction is characterized by oxygen consumption and the generation of further radicals. The first detectable stable products are the corresponding hydroperoxides, which are prone to cleavage, especially at elevated temperature or in presence of metal ions. α-Pinene **1** contains three allylic positions (a tertiary at C1, a secondary at C4, and a primary at C10), which are prone to an attack by triplet oxygen or radicals already present.

At first sight one of the most likely positions for the formation of a radical is the tertiary carbon atom at C1 (radical A, **Figure 2**), but this carbon atom is not favored because a resonance of the unshared electron along the double bond is not feasible. In the rigid structure of α-pinene **1** the four-membered ring is puckered, and five carbons of the six-membered ring (including olefinic

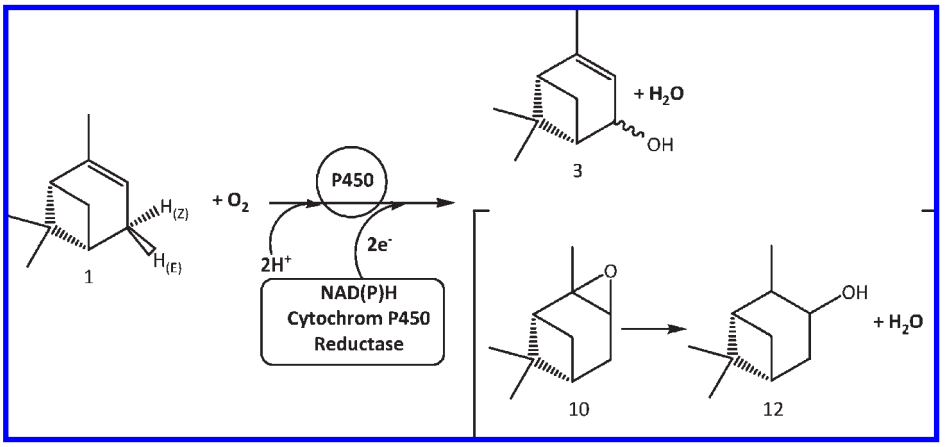

Figure 1. Hydroxylation of α-pinene **1** with the two-component system P450 monooxygenase/reductase (adapted from ref *27*).

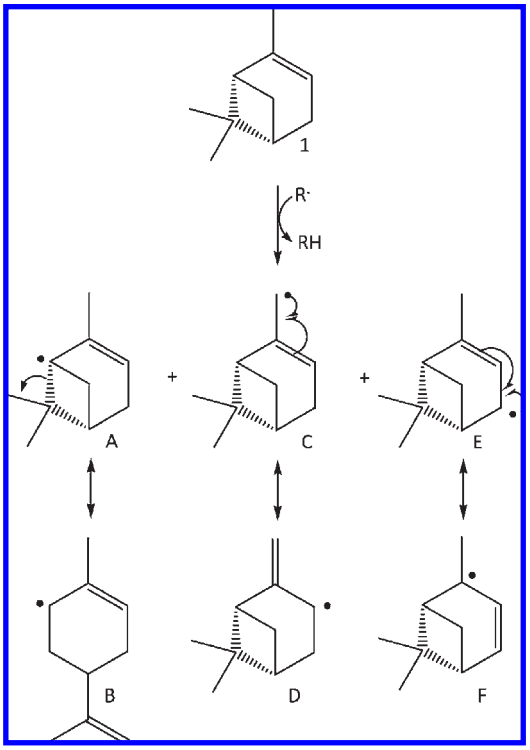

Figure 2. Initial radicals of α-pinene **1** autoxidation.

carbons) are approximately in-plane. This configuration does not allow the C1 to change from sp^3 to sp^2 hybridization without C−C-bond cleavage. Reaction products (hydroperoxide or alcohol) of the tertiary radical were not detected; however, the respective hydroperoxide can decompose under ring scission to give radical B (**Figure 2**) and subsequently the monocyclic (*E*)-carveol (*8*), which was found as a minor product in the controls and transformations of α-pinene **1** with *P. sapidus* (**Table 1**; **Figure 3**). The primary radical (radical C, **Figure 2**) at C10 can be stabilized along the double bond to give the secondary resonance radical (radical D, **Figure 2**) at C3. Products of both radicals were found. Myrtenol **6** and myrtenal **7** are reaction products of the primary myrtenyl hydroperoxide **5**, whereas (*E*)-pinocarveol **9** is the decomposition product of hydroperoxide **8** (**Figure 3**). The dominating products in the blank and the biotransformation assays were (*E*)-verbenol **3a** and verbenone **4**, which correspond to the secondary radical at C4 (radical E, **Figure 2**). This unshared electron can rearrange to result in the most stable tertiary resonance radical at C2 (radical F, **Figure 2**). However, the expected tertiary alcohol was not detected. The putative hydroperoxide at C2 might undergo a Schenk rearrangement to give verbenyl hydroperoxide **2** (*22*), which would result in verbenol **3** and verbenone **4**. This explains satisfactorily the main autoxidation products of α-pinene at ambient temperature, but does not explain the exclusive generation of (*E*)-verbenol **3a**. However, the failure to detect (*Z*)-verbenol **3b** does not necessarily indicate that only (*E*)-verbenol hydroperoxide **2a** was formed during the autoxidation of α-pinene **1**. As α-pinene **1** is a rigid structure with two secondary allylic hydrogens, pro *E* and pro *Z* (**Figure 1**) being at about 45° angle to this plane, it is unlikely that the steric hindrance caused by one of the methyl groups of the out-of-plane carbon atom (C7) of the four-membered ring of α-pinene **1** would entirely exclude the formation of (*Z*)-isomers. (*Z*)-verbenol **3b** is known to be less stable to heat and acid than the (*E*)-form (*8*). Autoxidation of other terpenes such as valencene showed a similar preference for one isomer, in this case 88% α- compared to 12% *β*-nootkatol (*23*). The approach of $^3O_2$ at the *β*-face of the intermediate allylic α-pinene radical is less hindered (*24*). One of the two methyl groups of the quaternary carbon atom is directed toward the ring plane, thus causing a steric hindrance for the approaching $^3O_2$. Furthermore, a Schenk rearrangement is a nondissociative mechanism (sigmatropic [2,3]-rearrangement (*24*)), therefore maintaining the (*E*)-configuration of the first generated hydroperoxide. Verbenone **4** becomes more dominating at elevated temperature or at higher hydroperoxide concentrations at advanced autoxidation (*25*). Interestingly, in the presence of molecular oxygen and catalytic amounts of Co^{2+} complexes at 80 °C the oxidation of verbenol **3** to verbenone **4** was successful in the case of (*E*)-verbenol **3a**, only, whereas (*Z*)-verbenol **3b** was assumed to further react to some rearrangement products. Therefore, (*Z*)-verbenol **3b** is not a direct precursor of verbenone **4** in the autoxidation of α-pinene **1** (*21*).

The origin of α-pinene epoxide **10**, detected in the control and biotransformation, during the radical chain reaction in the liquid phase is not yet clear. Ancel et al. (*19*) described the formation of verbenol **3** (stereochemistry not given) and α-pinene epoxide **10** when α-pinene hydroperoxides **2** were added to α-pinene **1** in high concentration and/or at elevated temperature. These conditions of α-pinene epoxide **10** generation might have occurred during the biotransformation of α-pinene **1**, or during the subsequent product separation and concentration, and definitely existed in GC analysis of the products. It was reported that verbenyl hydroperoxide **2** was unstable during GC analysis, and only the product of its decomposition was observed. In the presence of residual α-pinene **1** the decomposition products of verbenyl hydroperoxides **2** in the GC system were verbenol **3** and

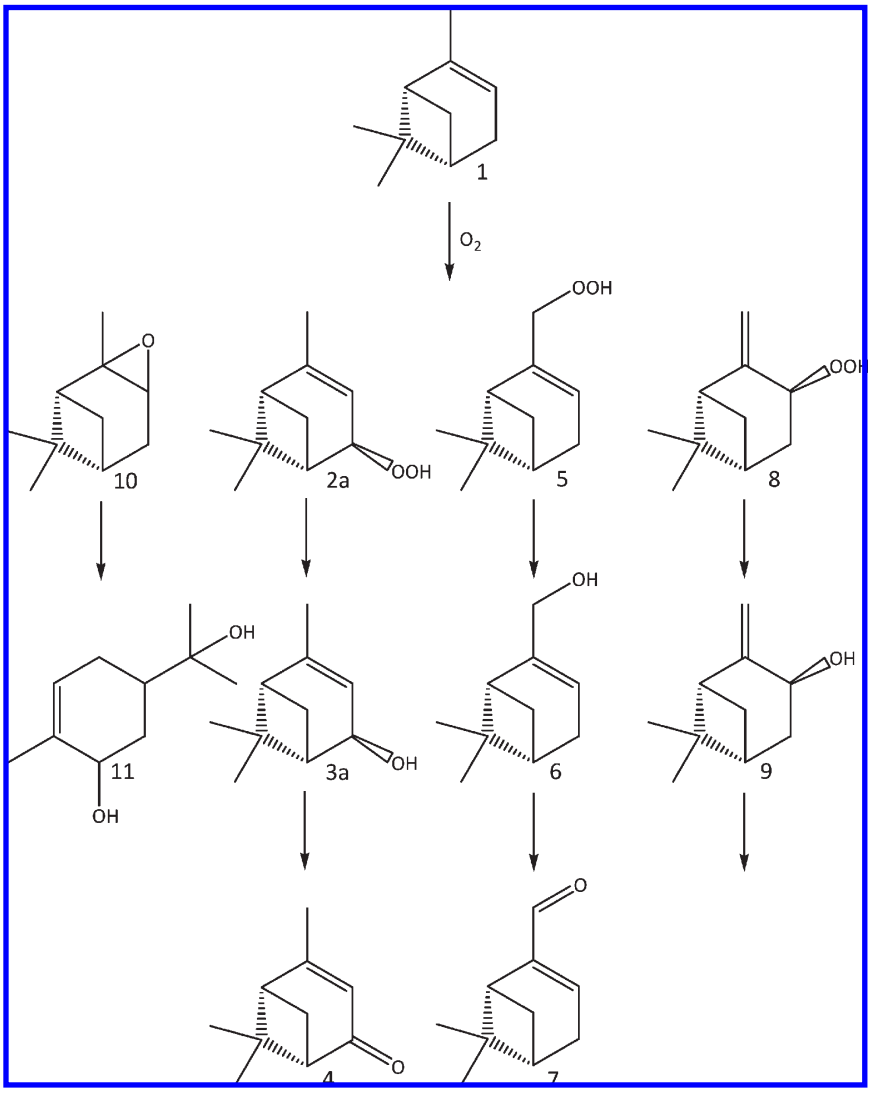

Figure 3. Reaction scheme and products of α-pinene **1** autoxidation.

Table 3. Biotransformation of (*Z*)-Verbenol **3b** to Verbenone **4**

fungus/ incubation time	(*Z*)-verbenol **3b** (%)[a]	(*E*)-verbenol **3a** (%)[a]	verbenone **4** (%)[a]
P. eryngii (SB)[b]/48 h	48	5	47
P. eryngii (MC)[b]/48 h	<0.5	<0.5	98
P. sapidus (Lyo)[b]/48 h	<0.5	1.5	98
P. sapidus (MF)[b]/20 h	<0.5	2.5	97
blank (Lyo)/48 h	93	7	<0.2

[a] Molar percentage of (*Z*)-verbenol (5.3 mM) fed. [b] SB, submerged culture; MC, mycelium concentrate; Lyo, lyophilisate; MF, microsomal fraction.

α-pinene epoxide **10** (*3*, *19*). Detection of sorbreol **11**, a known product of the hydrolysis of α-pinene epoxide **10** (*8*), gave additional evidence that at least in part α-pinene epoxide **10** was already generated during the autoxidation of α-pinene **1** (**Figure 3**).

Enzyme-Catalyzed Formation of (*E*)-Verbenol 3a and Verbenone 4. Although the qualitative product spectrum of α-pinene **1** biotransformation was almost identical to chemical blanks (inactivated lyophilisate, **Table 2**) the selective increase of (*E*)-verbenol **3a** (15-fold) and verbenone **4** (30−100-fold) using lyophilisate or microsomal fraction of *P. sapidus* mycelium proved the action of oxidoreductases. In a study using the same basidiomycete and (+)-valencene as substrate a dioxygenase was identified that catalyzed a similar transformation, the allylic oxidation to nootkatol and nootkatone via the respective hydroperoxides (*14*). The thermally unstable hydroperoxides were not amenable to GC analysis but were separated and detected via TLC using a hydroperoxide selective staining (*14*). The same procedure was successfully carried out with the solvent extract of α-pinene **1** biotransformation and autoxidation (data not shown). Cool on-column GC analysis of the hydroperoxide spots resulted in the known products of α-pinene **1** autoxidation, with α-pinene-epoxide **10** and (*E*)-verbenol **3a** as the main products and verbenone **4** as a minor compound. (*Z*)-Verbenol **3b** was found in traces, only. Additionally, two major unknown compounds eluted (RI 2103 and 2132). Both showed a considerable fronting and mass spectra similar to one another and to verbenol **3** and verbenone **4**. These two compounds were assumed to be (*Z*)-**2a** and (*E*)-**2b** verbenyl hydroperoxide. The fronting of the peaks indicated the thermal decomposition during GC analysis (and not during cool on-column injection) with increasing elution temperature. According to the method of Moore (*8*) a reductive treatment of the hydroperoxides with an alkaline solution of Na_2S should yield the corresponding alcohols. Reanalysis showed that the two peaks at RI 2103 and 2132 disappeared in favor of (*E*)-**3a** and (*Z*)-verbenol **3b** newly formed. The peak area of (*E*)-verbenol **3a** was increased to the extent the peak area of the larger peak disappeared. The same was found for the minor

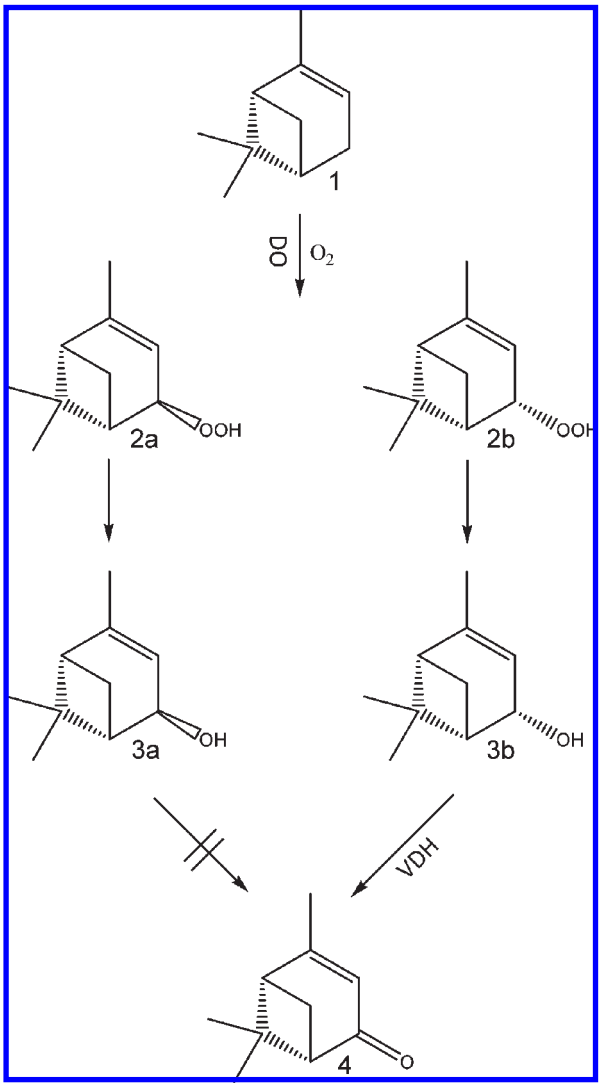

Figure 4. Reaction scheme of enzymatic generation of verbenone **4** with *P. sapidus*. DO, dioxygenase; VDH, (*Z*)-verbenol dehydrogenase.

hydroperoxide and (*Z*)-verbenol **3b**. From these results we concluded that the compounds eluting at RI 2103 and 2132 were (*Z*)-verbenol hydroperoxide **2b** and (*R*)-verbenol hydroperoxide **2a**, respectively, which decomposed partially during GC analysis.

Both hydroperoxides were found in the biotransformation assays with *P. sapidus* lyophilisate and MF as well. In contrast to the clear preference for (*E*)-verbenyl hydroperoxide **2a** by autoxidation, the putative α-pinene dioxygenase generated both verbenyl hydroperoxides, **2a** and **2b**. During autoxidation of α-pinene **1** it was shown that added (*Z*)-verbenol **3b** was not oxidized to verbenone **4**, not even at elevated temperature and Co^{2+} catalysis (*21*). Both precursor alcohols were added separately to the active biocatalyst to examine the enzymatic conversion to verbenone **4** by an assumed dehydrogenase. With (*E*)-verbenol **3a** no conversion to verbenone **4** or other conversion products was observed. The blank of (*Z*)-verbenol **3b** showed a slow isomerization to the thermally more stable (*E*)-isomer. Depending on the biocatalysts used (*P. eryngii* or *P. sapidus*) (*Z*)-verbenol **3b** was reacted to verbenone **4** in a molar yield of 47% (SB) to 98% (Lyo, MC, and MF) (**Table 3**). In contrast to autoxidation the fungal formation of verbenone **4** followed the reaction sequence as shown in **Figure 4**: Catalyzed by an α-pinene dioxygenase and in the presence of molecular oxygen, α-pinene

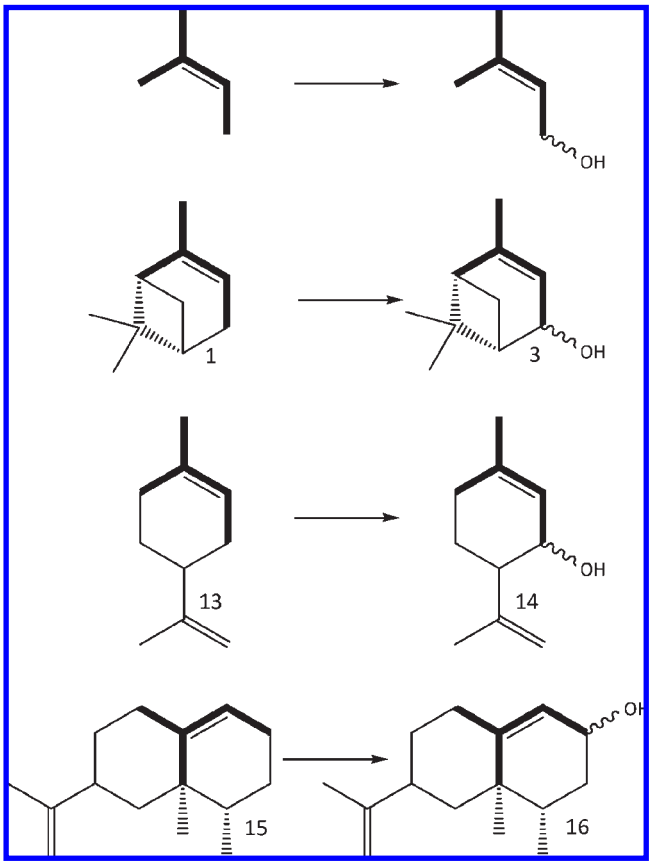

Figure 5. Substrate specificity of the putative α-pinene dioxygenase of *P. sapidus*.

1 was oxidized regioselectively to (*Z*)- and (*E*)-verbenyl hydroperoxides **2a** and **2b**. The stereoselectivity of the oxygenation could not be determined because of the instability of the hydroperoxides. Homolytic scission of the labile peroxo-bond led to both corresponding alcohols. (*Z*)-Verbenol **3b** was then oxygenated by a diastereoselective (*Z*)-verbenol dehydrogenase to verbenone **4**, whereas (*E*)-verbenol **3a** accumulated. The ratio of 1:1 of (*E*)-verbenol **3a** to verbenone **4** upon biotransformation with MF suggested that the α-pinene dioxygenase produced a diastereomeric mixture of verbenyl hydroperoxides **2a** and **2b**. The same regioselectivity as with the α-pinene dioxygenase was observed in biotransformations of other terpenes: (i) (+)-valencene **15** to nootkatol **16** (*14*) and (ii) (+)-limonene **13** to isopiperitenol **14** (*10*). The enzyme presumably requires a structural unit with an olefinic tertiary ring carbon atom, which is reacted in allylic position with $^3O_2$ (**Figure 5**). Further biotransformations using the dioxygenase of *P. sapidus* will be carried out using structure-related compounds to characterize the substrate specificity of the enzyme in more detail.

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