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Odor-Active Alcohols from the Fungal Transformation of α-Farnesene

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Submerged microbial cultures were screened for their potential to oxifunctionalize α-farnesene. The major oxidation product in all transforming cultures, 3,7,11-trimethyldodeca-1,3(*E*),5(*E*)10-tetraen-7-ol, showed a pleasant citrus-like odor and peak concentrations of 170 mg L^{-1}. An *Aspergillus niger* isolate from mango generated another two terpene alcohols identified as diastereomeric menth-1-en-3-[2-methyl-1,3-butadienyl]-8-ol, a new natural compound with an apricot-like odor. The regiospecificity of the oxygen attack with concurrent lack of stereoselectivity suggested that the initial step of the bioconversion resembled the chemical autoxidation starting with the generation of an intermediate resonance-stabilized carbon-centered radical or carbocation.

KEYWORDS: Fungal bioconversion; α-farnesene; mycelium concentrate; *p*-menth-1-en-3-[2-methyl-1,3-butadienyl]-8-ol; flavor

INTRODUCTION

The characteristic and pleasant odor impressions of citrus fruits, herbs and spices are mainly imparted by volatile terpenes. The ecological tasks of the terpenes are fragmentarily established so far. Under consideration are bioactivities as repellents, attractants, elicitors, and pheromones (*1*). The sensory properties of terpenoids are extensively applied in the flavor and fragrance industry, whereas their precursor hydrocarbons, due to their weak odor and chemical instability, are separated from the essential oils. Some terpene hydrocarbons are used as parent substances for chemosyntheses, but they have also been suggested for selective biotransformations by microorganisms. The enzymatic introduction of oxygen preferably at the allylic position of the terpene hydrocarbon yielded flavor compounds, for example, verbenone from α-pinene, carvone from limonene, and nootkatone from valencene (*2*−*4*). α-Farnesene, a minor constituent (up to 1%) of several essential oils such as that of apple, grapefruit, lime peel, orange, mandarin, and pepper, has not been extensively investigated as a substrate in microbial biotransformations. An attempt has become known to produce the highly sought after flavor compound α-sinensal, a potent orange flavor impact compound with a very low odor threshold of 50 ng kg^{-1}, but bacterial strains such as *Arthrobacter* or *Pseudomonas* gave low product yields (*5*, *6*).

Autoxidation of α-farnesene occurs naturally on the surface wax of apples and pears and is causal for superficial scald, a physiological disorder of fruit resulting in blackening of the skin during cold storage. A number of α-farnesene-derived autoxidation products, among them hydroperoxids, epoxides, and alcohols, have been identified (*7*−*9*). This paper describes a screening of terpene-converting fungi from commercial collections and strain isolates from terpene-containing habitats for their potential to oxifunctionalize α-farnesene and the identification and characterization of major odorous fungal oxidation products.

MATERIALS AND METHODS

Microrganisms. Three fungal strains, *Aspergillus niger* 737, *Botrytis cinerea* 877, and *Chaetomium globosum* 1962, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. Nineteen fungal strains were isolated from Brazilian habitats and deposited in the culture collection of the Laboratory of Bioflavors, State University Campinas, UNICAMP, 13083−862 Campinas, SP, Brazil).

Five fungal strains were isolated from overripe fruits in Hannover, Germany, and deposited in the culture collection of the Institut für Lebensmittelchemie, Wunstorfer Str.14, D-30453 Hannover, Germany. The strains were maintained on yeast malt agar slants.

Strain Identification. LB 2025, an isolate from a ripe Brazilian mango, was identified as *Aspergillus niger* by PCR and the morphology of the mycelium and ascospores. On the basis of an alignment of mitochondrial 18S rRNA, 5.8S rRNA, and 28S rRNA gene sequences of different *Aspergillus* species, conserved sequences were used for primer construction to amplify the ITS1 and ITS2 regions (internal transcribed spacer). The PCR products (both 470 bp) were subcloned

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and sequenced and showed 100% sequence identity to *A. niger* and to two further *Aspergillus* species, *A. awamori* and *A. foetidus* (EMBL database, http://www.ebi.ac.uk, using the FASTA algorithm). The strain was deposited in the culture collection of the Brazilian "Fundação Andre Tosello" (http://www.fat.org.br/) under # CCT 7449.

Medium and Culture Conditions. *Submerged Cultivation.* The fungal cultures were inoculated into a glucose/asparagine/yeast extract medium described elsewhere (*11*) and grown aerobically at 24 °C and 150 rpm on an orbital shaker (Multitron, Infors, Bottingen, Switzerland). Experimental cultures (500-mL shake flasks, 200-mL medium volume, cotton plug) were inoculated with 10 mL of 3-day-old precultures grown on the same medium and homogenized using an Ultraturrax homogenizer (Jahnke & Kunkel, Staufen Germany) prior to inoculation. The evaporative loss of α-farnesene in 24 h was negligible. Each culture was performed in duplicates.

Mycelium Concentrates. After 3 days of fungal growth the active biomass was harvested by centrifugation at 10 000*g* and withdrawal of the supernatant. To approximately 20 g of fungal mycelium (wet weight) was added 10 mL of fresh liquid medium. Conversion experiments were started by adding 0.5% (v/v) (24.5 mM) α-farnesene (70% α-, 30% *β*-farnesene, Treatt, Bury St. Edmunds, Suffolk, UK) to the 300-mL flask after the addition of 10 mL of fresh medium (pH 6.0) and resuspension of the mycelium for 30 min. The bioconversion kinetic of **3a** was monitored for 3 days using 2.5-fold of active biomass compared to the screening experiment. The pH of the aqueous phase of the biphasic medium did not change during this time. Bioconversion of *β*-farnesene did not result in the formation of measurable volatile intermediates with any of the strains tested (data not shown).

Isolation and Purification of Transformation Products. Twenty-four and 96 h after addition of α-farnesene the culture broth (medium and biomass or the chemical blank without active biomass at the same pH) was completely transferred into a separatory funnel and extracted three times with 20 mL of azeotropic pentane/Et_2O (1:1.12 v/v). The combined extracts were dried over dry sodium sulfate and concentrated (42 °C) to a volume of 1 mL using a Vigreux column. Thymol (60 *μ*g) was added as an internal standard right after the extract was concentrated to the final volume.

Prior to GC−HRMS and NMR measurements, major transformation products were isolated by flash chromatography using a silica gel column and gradient elution with pentane/Et_2O. Silica gel 60 (63−200 *μ*m, Merck, Germany) was conditioned at 100 °C for 2 h. After return to ambient temperatures, 4% of deionized water was added and equilibrated overnight. A slurry in pentane was used to fill empty SPE filtration columns (J.T. Baker, 7121-06) to yield a column of 5.0 cm × 1.3 cm i.d. A 1-mL portion of concentrate was applied to the conditioned column and the compounds were each eluted successively with 5 mL of pentane and pentane/Et_2O 80:20, 60:40, 40:60, 20:80. The resulting five fractions were reconcentrated by distillation using a Vigreux column. Pure compounds were finally achieved by means of preparative GC performed on a MCS Gerstel Series II instrument (Gerstel, Mühlheim/Ruhr, Germany) with a Gerstel MCS control and data acquisition system, a Gerstel KAS-3 cold injection system, and a HP 7673 autosampler. A CW 20 M precolumn (5 m × 0.53 mm i.d. × 2 *μ*m film thickness) was connected to a CW 20 M fused silica column (25 m × 0.53 mm i.d. × 2 *μ*m film thickness) and hydrogen as the carrier gas (5.0 mL min^{-1}). Two ports (PC1 and PC2, respectively), allowing the removal of undesired components from the sample, were located between the cold injection system and the precolumn on one side and the precolumn and the main column. The oven was temperature programmed as follows: 100 °C isothermal for 3 min, linear gradient to 160 °C at 5 °C min^{-1}, a second linear gradient to 220 °C at 3 °C min^{-1}, and isothermal at 220 °C for 5 min. Other temperatures were FID 250 °C, supply pipe and distributor 240 °C, trap cooling 0 °C. Compounds were rinsed from the traps using $CDCl_3$.

Characterization of Transformation Products. NMR (^{1}H-, ^{13}C-, ^{1}H,^{1}H-COSY, HMBC, HMQC, ROESY) identification was performed on a BRUKER Avance DRX-500.

GC and GC−MS. One microliter of each concentrated sample was injected into a Fisons GC 8360 equipped with a cool on-column injector, a J&W CW 20 M fused silica capillary column (30 m × 0.32 mm i.d. × 0.25 *μ*m film thickness), hydrogen as the carrier gas (52 cm s^{-1}), and a FID (230 °C) using a temperature program from 100 °C (2 min) to 160 °C with a rate of 5 °C min^{-1} to 230 °C with 3 °C min^{-1} hold for 5 min. Quantification was performed according to the internal standard thymol.

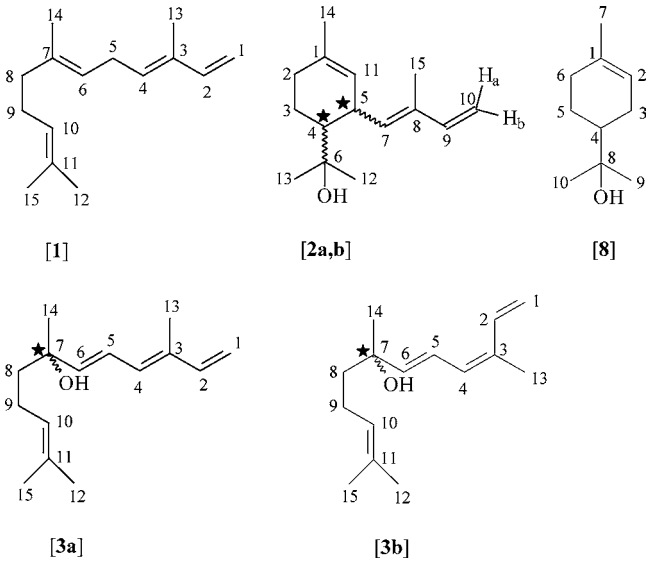

Figure 1. Structures of α-farnesene, α-terpineol, and identified bioconversion products.

GC−MS and GC−high-resolution MS analysis were 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 the literature (Wiley and NIST spectral libraries, 9,12-16) 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* 33−400 amu).

HR-EIMS data were collected on a GC−MS instrument at 70 eV consisting of an Agilent GC 6890N coupled to an AMD M 40-QuAS^3-AR (AMD Intectra GmbH, Germany) double-focusing sector field mass spectrometer in the positive ion mode (interface 230 °C, ion source 200 °C) and the same chromatographic conditions as for GC-FID analysis. Accurate masses (peak matching mode) were measured using perfluorotributylamine (PFTBA) as the calibration gas. Chemical ionization was carried out using methane at 5 mbar source pressure as the reactant gas. TMSi of sesquiterpene alcohols were obtained in pentane/Et_2O (azeotrop, 1:1,12 v/v) using *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylsilane (Sigma Aldrich) as the reactant.

Odor impressions and thresholds were determined by means of GC−olfactometry using a Satochrom GC equipped with a cold on-column injector, a J&W CW 20M fused silica capillary column (30 m × 0.32 mm i.d. × 0.25 *μ*m film thickness) splitted (1:1) into a sniff-port (230 °C) and a FID (230 °C) and the same chromatographic conditions as for GC-FID analysis. A panel of five persons was used to note the odor impression induced by eluting compounds. Characteristic odor impressions were considered valid if at least 50% of the judges reproducibly signaled an sensory perception. Odor thresholds were determined by aroma extract dilution analysis (*17*) using 1:10 dilution steps.

Enantiomeric distribution of isomers was measured using a double oven gas chromatograph (Sichromat 2-8, Siemens) equipped with a PTV (Programmable Thermal Vaporizer), a CW 20M capillary column (Macherey&Nagel, 30 m × 0.32 mm i.d. × 0.25 *μ*m 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 *μ*m film thickness, J&W Scientific) column (CW 20M column with the same chromatographic conditions as for GC-FID analysis; chiral column at 100 °C, 50 min, 1 °C min^{-1}; 200 °C, 10 °C min^{-1}; 220 °C, 15 min) and hydrogen as the carrier gas (5 mL min^{-1}).

Identification of Conversion Products (Figures 1**and 3).** *p-Menth-1-en-3-[2-methyl-1,3-butadienyl]-8-ol (**2a,b**).* For NMR data of **2a**, see

Table 3. **2a**, RI 2150 (CW 20M): EI-MS *m*/*z* 220 $[M]^+$ (2.0), 202 ($-H_2O$, 10), 187 (14), 159 (30), 147 (37), 134 (20), 119 (76), 106 (26), 105 (46), 93 (32), 91 (49), 79 (28), 59 (100); EI-MS of TMSi *m*/*z* 292 $[M - Si(CH_3)_3]^+$ (1.5), 277 (2), 202 (10), 187 (9), 159 (16), 131 (100), 73 (57); CI-MS *m*/*z* 221 $[MH]^+$ (2), 220 (3), 219 (2.5), 203 $[MH - H_2O]^+$ (75), 163 (70). 109 (100); HR-EIMS *m*/*z* 220.1828 (calcd for $C_{15}H_{24}O$, 220.1827), racemic mixture. **2b**, RI 2110 (CW 20M): EI-MS *m*/*z* 220 $[M]^+$ (2.0), 202 ($-H_2O$, 10), 187 (20), 159 (54), 147 (32), 134 (20), 119 (66), 106 (24), 105 (48), 93 (30), 91 (51), 79 (30), 59 (100); EI-MS of TMSi *m*/*z* 292 $[M - Si(CH_3)_3]^+$ (1.5), 277 (2), 202 (11), 187 (9), 159 (15), 131 (100), 73 (60); CI-MS *m*/*z* 221 $[MH]^+$ (1), 220 (3), 219 (3), 203 $[MH - H_2O]^+$ (100), 163 (85), 109 (95); HR-EIMS *m*/*z* 220.1821 (calcd for $C_{15}H_{24}O$, 220.1827), racemic mixture.

*3,7,11-Trimethyldodeca-1,3(E),5(E),10-tetraen-7-ol (**3a**)*, RI 2294 (CW 20M): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.58 (dd, $^3J = 10.0$ Hz, 1H, C8), 6.41 (dd, $^3J = 10.0$ Hz, 1H, C11), 6.08 (d, $^3J = 11.0$ Hz, 1H, C9), 5.80 (d, $^3J = 15$ Hz, 1H, C7), 5.04/5.21 (dd, $J = 10.0$ Hz, (*E*)-H, C12, d, $J = 17.0$ Hz, (*Z*)-H, C12), 5.13 (t, $J = 7.0$ Hz, 1H, C13), 2.00 (m, 2H, C4), 1.88 (s, 3H, C13), 1.68 (s, 3H, C1), 1.62 (m, 2H, C5), 1.60 (s, 3H, C15), 1.32 (s, 3H, C14); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 142.0 (CH, C7), 141.9 (CH, C11), 135.8 (C, C10), 132.7 (C, C2), 131.5 (CH, C9), 125.0 (CH, C3), 124.4 (CH, C8), 113.2 (CH_2, C12), 74.2 (COH, C6), 43.2 (CH_2, C5), 29.1 (CH_3, C14) 26.4 (CH_3, C1), 23.7 (CH_2, C4), 18.4 (CH_3, C15), 12.7 (CH_3, C13). EI-MS *m*/*z* 220 $[M]^+$ (1.0), 205 ($-CH_3$, 4.3), 202 ($-H_2O$, 9.1), 187 (4.7), 175 (8.5), 162 (45), 159 (30), 137 (14), 133 (13), 119 (18), 105 (27), 95 (30), 93 (39), 91 (32), 69 (41), 55 (43), 43 (100), 41 (50); A TMSi derivative was not obtained using BSTFA; CI-MS *m*/*z* 243 $[M - H_2O + C_3H_5]^+$ (0.5), 231$[M - H_2O + C_2H_5]^+$ (2), 221 $[MH]^+$ (3), 220 (3), 219 (3), 203 $[MH - H_2O]^+$ (100), 163 (45), 147 (55), 109 (100); HR-EIMS *m*/*z* 220.1819 (calcd for $C_{15}H_{24}O$, 220.1827), racemic mixture.

*3,7,11-Trimethyldodeca-1,3(Z),5(E),10-tetraen-7-ol (**3b**)*, RI 2273 (CW 20M): EI-MS *m*/*z* 220 $[M]^+$ (0.1), 205 ($-CH_3$, 1.0), 202 ($-H_2O$, 3.0), 187 (2.0), 175 (1.0), 162 (14), 159 (12), 137 (10), 133 (8), 119 (18), 105 (24), 95 (32), 93 (37), 91 (30), 69 (30), 55 (42), 43 (100), 41 (50); A TMSi derivative was not obtained using BSTFA; CI-MS *m*/*z* 243 $[M - H_2O + C_3H_5]^+$ (0.2), 231 $[M - H_2O + C_2H_5]^+$ (2), 221 $[MH]^+$ (3), 220 (3), 219 (7), 203 $[MH - H_2O]^+$ (100), 163 (20), 147 (25) 109 (25).

*6-Methyl-hept-5-en-2-one (**7**)*, RI 1344 (CW20M): EI-MS *m*/*z* 126 $[M]^+$ (8), 111 (11), 108 (34), 93 (14), 83 (8), 69 (35), 55 (41), 43 (100), 41 (50).

*p-Menth-1-en-8-ol (α-Terpineol) (**8**):* ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.38 (m, $J = 6.2$ Hz, 1H, H2), 2.20 (m, 1H, H3), 1.80 (m, 1H, H3), 2.05 (m, 1H, H6), 1.95 (m, 1H, H6), 1.90 (m, 1H, H5), 1.25 (m, 1H, H5), 1.67 (s, 3H, H7), 1.52 (dddd, $J = 12.05$; $J = 11.24$; $J = 4.79$; $J = 2.41$ 1H, H4), 1.19 (s, 3H, H9) 1.16 (s, 3H, H10); ^{13}C NMR (CDCl3, 100 MHz) δ 134.0 (C, C1), 120.5 (CH, C2), 72.7 (C-OH, C8), 45.0 (CH, C4), 31.0 (CH_2, C6), 27.4 (CH_3, C9), 26.9 (CH_2, C3), 26.2 (CH_3, C10), 23.9 (CH_2, C5), 23.3 (CH_3, C7).

RESULTS AND DISCUSSION

When exposed to elevated temperatures, reactive oxygen species or light trigger the rapid onset of a multitude of alterations of α-farnesene, such as rearrangements, oxidation, and degradation (*8*, *15*, *18*, *19*). In the present screening, many submerged cultures of microorganisms fed with α-farnensene yielded oxidation products in concentrations in the range of the chemical blank (data not shown). After increasing the ratio of active biomass to liquid medium (mycelium concentrate), several cultures produced oxifunctionalized metabolites of α-farnesene as the major volatiles observed in gas chromatograms (**Figure 1**). Summed amounts of microbial α-farnesene bioconversion products after subtraction of the chemical blank are listed in **Table 1**. Low actual concentrations of α-farnesene in essential-oil-bearing plant tissues associated with chemical instability seem to have impeded the evolutionary development of highly active enzymes for handling this particular substrate.

Table 1. Screening of α-Farnesene Transforming Fungi (products sampled on the first and fourth day after α-farnesene supplementation)

strain/isolate	volatile α-farnesene conversion products[c] 1 d/4 d [mg L^{-1}]
chemical blank	8.8/1.7
A. niger	29.3/n.d.
B. cinerea	13.1/19.0
C. globosum	47.2/30.0
isolate from kiwi[a]	28.4/n.d.
isolate from mandarine 3[a]	28.0/n.d.
isolate from grapefruit 4[a]	28.0/n.d.
isolate from soil (LB 2000)[b]	28.8/n.d.
isolate from mango (LB 2010)[b]	36.5/41.0
isolate from umbu (LB 2015)[b]	30.2/21.5
isolate from ripe mango (LB 2025)[b], identified as *A. niger*	67.6/7.8
isolate from acerola (LB 2038)[b]	45.0/15.5
isolate from tamarine (LB 2047)[b]	36.4/17
isolate from serigula (LB 2060)[b]	13.7/7.2
isolate from jenipago (152 B[b])	35.0/11.0
isolate from jaca (2383[b])	22.0/10.0
isolate from soil (*Scopulariopsis* sp. [b])	26.0/4.0
isolate from grape (Asp.11068[b])	25.0/3.0
isolate from ripe mango (2036[b])	36.0/3.0
isolate from coconut (2402[b])	22.0/4.0
isolate from cashew (2111[b])	36.0/2.0
isolate from sapoti (2242[b])	22.0/9.0
isolate from cashew (M-2-B[b])	20.0/5.0
isolate from grape (2395[b])	18.0/4

[a] Isolates from terpene-rich habitats, Hannover, Germany. [b] Isolates from terpene-rich habitats, Campinas, Brazil. [c] MW 218, 220, or 236, chemical blank subtracted. nd = not detectable.

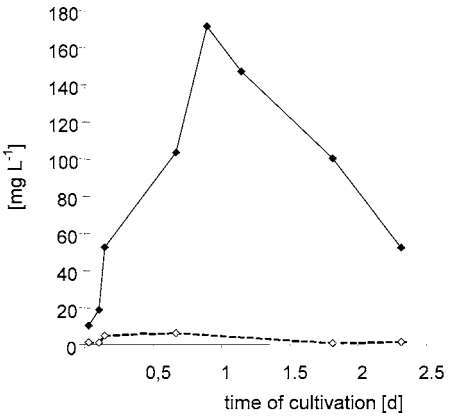

Figure 2. Course of 7-hydroxyfarnesene (**3a**) generation using mycelium concentrates of *C. globosum* (24 mM α-farnesene): ◆, *C. globosum*; ◇, chemical blank.

For the majority of the transforming strains the product concentrations peaked 1 day after addition of α-farnesene and then decreased. In some cases conversion products were not detectable at all on the fourth day (**Table 1**). The overall pictures of the GC chromatograms of concentrated solvent extracts of the respective culture broths were similar for most of the transforming strains showing one dominating bioconversion product. A representative kinetic of formation of this major conversion product, later identified as **3a**, is shown in **Figure 2**. The oxidation of α-farnesene started right after the mycelium concentrate of *C. globosum* was supplemented with α-farnesene, increased considerably over a period of 24 h, and then decreased rapidly. After 24 h, **3a** was more rapidly attacked by a fungal (exo)enzyme than its precursor α-farnesene.

The solvent extracts of all strains were investigated by means

Table 2. Odor Impressions and Thresholds of Major α-Farnesene Transformation Products of *Aspergillus niger* Wild Strain

compd	RI (CW 20M)	odor impression	odor threshold (ng/sniff-port GC-O)
p-menth-1-en-3-[2-methyl-1,3-butadienyl]-8-ol (**2a**)	2110	citrus, fruity	<50
p-menth-1-en-3-[2-methyl-1,3-butadienyl]-8-ol (**2b**)	2150	apricot, peach	<50
2,6,10-trimethyldodeca-2,7,9,11-tetraen-6-ol (**3b**)	2273	citrus, flowery	<50
2,6,10-trimethyldodeca-2,7,9,11-tetraen-6-ol (**3a**)	2294	citrus, flowery	<50

of GC−olfactometry (data not shown). Two strains were investigated in more detail. A culture isolated from overripe mango and identified as a strain of *A. niger* was selected, because it exhibited the most versatile and attractive flavor profile (**Table 2**). *C. globosum* was chosen because it produced the highest peak concentrations of the main conversion product **3a** with a pleasant citrus-like odor.

The most abundant conversion product **3a** was extracted from the culture medium of *C. globosum*, purified by means of a preparative GC, and identified as 3,7,11-trimethyl dodeca-1,3-(*E*),5(*E*),10-tetraen-7-ol (**3a**) by MS and NMR. The molecular mass of the sesquiterpene alcohol was confirmed by EI- and CI-mass spectra. The elemental composition $C_{15}H_{24}O$ was deduced from the accurate mass determination (5.0 ppm definition) of the molecular ion using the peak matching mode. The fragmentation pattern of the EI mass spectrum of **3a** was in good accordance with published data (*14*, *16*) but did not match with other data published recently (*9*, *20*). In the latter papers a more stable M^+-ion and a base peak at *m*/*z* 82 were found for **3a**, whereas the ion at *m*/*z* 162 was missing. The ^{1}H and ^{13}C NMR data for **3a**, however, were in excellent agreement (*9*, *12*, *14*, *16*, *20*). The magnitude of the coupling constant $J_{5,6}$ and NOESY experiments confirmed the (*E*) configuration of the new conjugated double bond (C5−6). Reanalyzing the pure NMR sample of **3a** by means of GC−MS yielded again the EI-mass spectrum as listed in the experimental section. The introduction of oxygen at C7 of α-farnesene under rearrangement of the 6,7-double bond introduces a new chiral center into the molecule. Separation and quantification of the enantiomers by chiral GC yielded an almost racemic mixture. Chemical controls without active biomass unequivocally demonstrated that the oxidation of α-farnesene was not caused by autoxidation, but enzyme-catalyzed oxidation proceeded at a low rate (**Figure 2**).

Another compound with a similar EI-mass spectrum and odor impression eluted from the GC column close to the main transformation product. The EI-mass spectrum of this compound matched with the mass spectrum of **3b** (*16*), the 3,4 (*Z*) isomer of **3a**. The concentration ratio was determined to be 87% *E*,*E* and 13% *Z*,*E* respectively, similar to the ratio of 90%:10% reported for α-farnesene autoxidation (*16*). The reaction mechanism of the bioconversion and chemical autoxidation seem to be similar in this respect. Bohlmann and Grenz (*21*) reported **3b** as a natural minor constituent of the roots of *Eupatorium perfoliatum* L. Anet et al. (*18*) isolated and characterized the conjugated trienes **3a,b**, after reduction of the corresponding hydroperoxides, from autoxidized samples of α-farnesene.

In the culture broth of an *A. niger* wild strain two further compounds with pleasant odor impressions at RI 2110 (citrus, fruity) and RI 2150 (apricot, peach), respectively, were detected. These two compounds were solely found in the culture media of this ascomycete. The EI-mass spectra of both conversion products were almost identical. The EI/CI-mass spectra as well as the trimethylsilyl derivatives indicated monooxygenated α-farnesenes with a molecular mass of 220 amu. The elemental composition $C_{15}H_{24}O$ for both was deduced from the accurate mass determination (5.0 ppm definition) of the molecular ion using the peak matching mode. The EI-mass spectra suggested **2a,b** as possible structures. The intensive ion at *m*/*z* 59 gave evidence that the introduction of a hydroxyl group occurred at the isopropylene moiety of α-farnesene. A hydroxylation at the terminal C12 yielding 3,7,11-trimethyl-1,3,6,10-tetraen-12-ol was ruled out, because the EI-mass spectrum did not match with the mass spectrum reported for this compound (*15*). However, the weak intensity of the molecular ion ($M^+ = 220$ amu) together with the base peak at *m*/*z* 59 were in good accordance with a hydroxyl group at the tertiary carbon atom of the terminal isopropyl group. A water addition at the 10,11-double bond of α-farnesene would agree with a base peak at *m*/*z* 59, but this would result in a molecular mass of 222 amu. A chemical structure able to explain the observed fragmentation pattern would be a monocycle. The more abundant compound (RI 2150, ratio 2:1) of the two related sesquiterpenols, **2a** (with a peak concentration of 20 mg L^{-1}), was isolated by preparative GC (approximately 1 mg) and subjected to NMR (**Table 3**). The ^{13}C spectrum showed a monocyclic molecule with 15 carbon atoms and three double bonds at position 1,11; 7,8; and 9,10, respectively, indicated by the large chemical shift of six C-atoms (111 to 142 ppm). Three C-atoms at 72.8 ppm (C6), 132.2 ppm (C8), and 134.2 ppm (C1), of which the latter two were located at double bonds, were identified as quaternary C-atoms. They did not show any coupling to a H-atom in two-dimensional HMQC experiments. This indicates a cyclic structure for this conversion product with three double bonds and two quaternary C-atoms located at double bonds. The ^{1}H-shifts were assigned to the corresponding C-atoms by spin−spin correlation. The side chain (C7−C10) data of **2a** showed conjugated double bonds at C9,10 and C7,8. The proton at C7 correlated with C15, $^3J = 7.9$ Hz, and also there was a ^3J (10.7 Hz) coupling of the

Table 3. ^{1}H and ^{13}C NMR Data of *p*-Menth-1-en-3-[2-methyl-1,3-butadienyl]-8-ol (**2a**)

position[a]	δ ^{1}H (multiplicity, *J*)	δ ^{13}C	function
1	–	134.2	C
2	2.05 (m)	31.5	CH_2
3	1.72, 1.81 (m)	19.3	CH_2
4	1.72 (m)	48.3	CH
5	3.30 (m)	35.8	CH
6	–	72.8	COH
7	5.51 (br d, 10.5)	132.6	CH
8	–	132.2	C
9	6.35 (ddd, 17.8, 10.7, 0.75)	141.6	CH
10a	5.11 (ddd, 17.8, 0.95, 0.60)	111.4	CH_2
10b	4.95 (ddd, 10.7, 0.95, 0.78)		
11	5.15 (dq, 7.3, 1.5)	122.9	CH
12	1.21 (s)	28.0	CH_3
13	1.22 (s)	28.5	CH_3
14	1.71 (br s)	23.4	CH_3
15	1.82 (d, 1.3)	11.9	CH_3

[a] According to **Figure 1**.

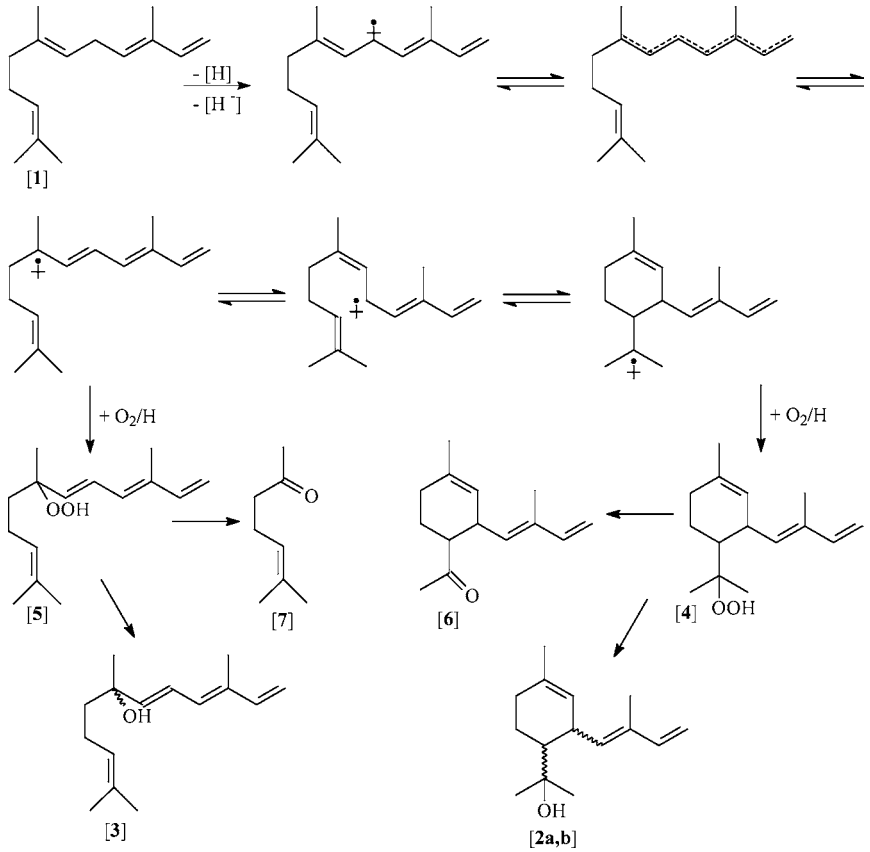

Figure 3. Proposed pathway of fungal α-farnesene bioconversion.

proton at C7 with the proton at C9. These vicinal couplings cannot unequivocally determine the configuration at the C7,8 double bond. Therefore, ROESY spectra were acquired to elucidate the spatial proximities determined by the double bond C7,8. The proton at C7 showed a strong ROE connectivity to the proton at C9, while the proton at C5 had a strong ROE cross peak to the CH_3-15 methyl group. This clearly indicated that the C7,8 double bond must have been (*E*) configured. The proton at C5 coupled with the proton at C11 and the proton at C4. These couplings agree with a ring closure from C4 to C5. The position of the hydroxyl group was deduced from the HMBC spectra. The hydroxy group is attached to C6, and ^{1}H and ^{13}C NMR data are in good agreement with those of α-terpineol **8**, a monoterpenol with *p*-menth-1-en-8-ol structure. The protons at the two terminal methyl groups as well as the tertiary carbon atom exhibited the same chemical shift (1.21/1.22 and 72.8 ppm, respectively) caused by the hydroxyl group located at the tertiary carbon atom of the isopropyl group. These spectral features require a structure of *p*-menth-1-en-3-[2-methyl-1,3-butadienyl]-8-ol, a new natural compound, for **2a**. The new alcohols possess the humbertiane skeleton, a relatively rare type of sesquiterpenes. Weyerstahl et al. isolated four isomeric isohumbertiols structurally related to the alcohols **2a,b** from the wood of *Humbertia madagascariensis* Lam.; however, the NMR data (^{1}H, ^{13}C) differ significantly from the data obtained for the new sesquiterpene alcohols (*22*).

The ring closure affords two new chiral centers at C4 and C5. Chiral GC of the compounds at RI 2110 and RI 2150 yielded racemic mixtures for both compounds. Because both alcohols possessed identical EI-mass spectra, it is most likely that the minor compound **2b** is the diastereomer of **2a**.

Chemical blanks of the bioconversion experiments clearly indicated that the formation of the cyclic sesquiterpene alcohols was not caused by chemical autoxidation but required an enzymatic step like the formation of **3a,b**. The cyclic sesquiterpenes **2a,b** were neither detected in the control experiments during this study nor described as α-farnesene autoxidation products or from other sources (*7*−*9*, *18*−*19*).

The main constituent in all bioconversion experiments was **3a**. This compound was detected to a minor extent in the chemical blanks and known as the major autoxidation product of α-farnesene causing superficial scald (*7*−*9*). The initial step in the autoxidation was suggested to be a hydrogen abstraction at the diallylic position at C5 of the α-farnesene molecule resulting in a carbon radical that is stabilized along the three double bonds. An analogue mechanism via the formation of an intermediate carbocation was suggested by Fielder (*9*). Fungal redox-enzymes, such as laccases, are known for their ability to attack substrates by several mechanisms including radical formation. A proposed oxidation pathway for α-farnesene via a radical-initiated mechanism or, alternatively, via an intermediate carbon cation is shown in **Figure 3**. Neither the alcohol at the diallylic position of α-farnesene nor one of the isomer alcohols at C1, C3 nor the corresponding hydroperoxides were detected in chemical blanks or in the culture media. Three allylic alcohols (with a conjugated diene structure) were found after photo-oxidation of α-farnesene using Rose Bengal as photosensitizer (*15*). Fielder et al. (*9*) reported that under acid-catalyzed conditions the three alcohols at C1, C3, C5 (obtained from chemical synthesis) rearranged easily to **3a,b** within 6 h at 20 °C in THF. The initial *E*/*Z* ratio of the alcohols after their synthesis (>95:5) was not altered during these rearrangements, indicating that despite the assumed intermediate delocalized carbocation, a new orientation of the double bonds was not achieved. Therefore, it is logically consistent that no cyclization products were detected during autoxidation, because cyclization requires the isomerization of the 6,7-(*E*)-double bond of α-farnesene. Obviously, this isomerization occurred catalyzed

by an enzyme of the *A. niger* strain. A cisoid radical/cation can attain the necessary geometry for cyclization (**Figure 3**). An analogous enzyme-assisted isomerization is required for the bioconversion of geranyl pyrophosphate to cyclic terpenes (*1*). In the case of *A. niger* the cyclization showed no distinct stereochemical orientation, and, after oxygen uptake, the two diastereomeric alcohols, both racemic, were formed (**2a,b**). Such nonspecific conversion reactions are not unusual for fungal terpene bioconversions. *Pleurotus sapidus*, for example, converted enantiopure (*R*)-(+)-limonene to both carvone enantiomers (almost racemic) through all four possible carveols (*23*). The postulated intermediate hydroperoxides **4** and **5** of both hydroxylation products **2** and **3** were not detected. They might have been immediately reduced to the corresponding alcohols **2** and **3** by fungal peroxygenases or degraded by a lyase-catalyzed cleavage of the hydroperoxides to the corresponding methyl ketones **6** and **7**. This cleavage would represent an enzymatic counterpart to the Hock cleavage. 6-Methyl-5-hepten-2-one (*7*), the cleavage product of **5**, was detected at a peak concentration of 10 mg L^{-1} and identified in the culture medium by its RI (1344, CW 20M) and EI-mass spectrum. The corresponding methyl ketone **6** of hydroperoxide **4** was not detected. Compound **6** is not described in the literature; thus, no spectral data are available. An involvement of lipid peroxidation in the degradation of nonphenolic lignin model compounds by a manganese peroxidase of the fungus *Stropharia coronilla* was described by Kapich et al. (*24*). A co-oxidation of the target nonphenolic lignin compound was initiated by linoleic acid peroxide radicals. The present bioconversion mechanism was different, because neither the addition of (i) linoleic acid, (ii) linoleic acid and manganese, nor (iii) the direct addition of linoleic acid hydroperoxides to the culture medium of *A. niger* and *C. globosum* resulted in an increase of the product concentration (data not shown). Further investigations will be required to confirm the detailed conversion mechanism.

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LITERATURE CITED

(1) Breitmaier, E. *Terpene Aromen, Düfte, Pharmaka, Pheromone*; Wiley-VCH: Weinheim, Germany, 2005.

(2) Schrader, J., Berger, R. G. Biotechnological production of terpenoid flavor and fragrance compounds. In *Biotechnology*, Rehm, H. J., Ed.; Wiley-VCH: Weinheim, Germany, 2001; Vol. 10: Special Processes, pp 374−422.

(3) Demyttenaere, J. C. R. Biotransformation of terpenoids by microorganisms. In *Studies in natural product chemistry;* Atta-ur-Rahman, H. E. J., Ed.; Elsevier Science Ltd.: New York, 2001; Vol. 25, Part F, pp 125−178.

(4) Kaspera, R.; Krings, U.; Nanzad, T.; Berger, R. G. Bioconversion of (+)-valencene in submerged cultures of the ascomycete *Chaetomium globosum. Appl. Microbiol. Biotechnol.* **2005**, *67*, 477−483.

(5) Abraham, W.-R.; Arfmann, H.-A.; Giersch, W. Microbial hydroxylation of precursors of sinensal. *Z. Naturforsch.* **1992**, *47c*, 851−858.

(6) Ohloff, G. *Scent and Fragrances*; Springer-Verlag: Berlin, 1994.

(7) Rowan, D. D.; Hunt, M. B.; Fielder, S.; Norris, J.; Sherburn, M. S. Conjugated triene oxidation products of α-farnesene induce symptoms of superficial scald on stored apples. *J. Agric. Food Chem.* **2001**, *49*, 2780−2787.

(8) Whitaker, B. D.; Saftner, R. A. Temperature-dependant autoxidation of conjugated trienols from apple peel yields 6-methyl-5-hepten-2-one, a volatile implicated in induction of scald. *J. Agric. Food Chem.* **2000**, *48*, 2040−2043.

(9) Fielder, S.; Rowan, D. D.; Sherburn, M. S. Synthesis of sesquiterpene polyene hydroperoxides by regio- and stereoselective transposition reactions. *Tetrahedron* **1998**, *54*, 12907−12922.

(10) Henry, T.; Iwen, P. C.; Hinrichs, S. H. Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. *J. Clin. Microbiol.* **2000**, *38*, 1510−1515.

(11) Onken, J.; Berger, R. G. Effects of *R*-(+)-limonene on submerged cultures of the terpene transforming basidiomycete *Pleurotus sapidus. J. Biotechnol.* **1999**, *69*, 163−168.

(12) Brimble, M. A.; Rowan, D. D.; Spicer, J. A. Synthesis of conjugated trienes and related oxidation products of α-farnesene. *Aust. J. Chem.* **1994**, *47*, 1979−1988.

(13) Fielder, S.; Rowan, D. D.; Sherburn, M. S. Synthesis of α-farnesene hydroperoxides. *Synlett* **1996**, 349−350.

(14) Rowan, D. D.; Allen, J. M.; Fielder, S.; Spicer, J. A.; Brimble, M. A. Identification of conjugated triene oxidation products of α-farnesene in apple skin. *J. Agric Food Chem.* **1995**, *43*, 2040−2045.

(15) Spicer, J. A.; Brimble, M. A.; Rowan, D. D. Oxidation of α-farnesene. *Aust. J. Chem.* **1993**, *46*, 1929−1939.

(16) Whitaker, B. D.; Solomos, T.; Harrison, D. J. Quantification of α-farnesene and its conjugated trienol oxidation products from apple peel by C_{18}-HPLC with UV detection. *J. Agric. Food Chem.* **1997**, *45*, 760−765.

(17) Grosch, W. Detection of potent odorants in foods by aroma extract dilution analysis. *Trends Food Sci. Technol.* **1993**, *4*, 513−518.

(18) Anet, E. F. L. J. Autoxidation of α-farnesene. *Aust. J. Chem.* **1969**, *22*, 2403−2410.

(19) Cavill, G. W. K.; Coggiola, I. M. Photosensitized oxygenation of α-farnesene. *Aust. J. Chem.* **1971**, *24*, 135−42.

(20) Rueda, A.; Zubía, E.; Ortega, M. J.; Salvá J. New acyclic sesquiterpenes from the Caribbean gorgonian *Plexaurella grisea. J. Nat. Prod.* **2001**, *64*, 401−405.

(21) Bohlmann, F.; Grenz, M. Über neue Inhaltsstoffe aus Vertretern der *Eupatorium-Gruppe. Chem. Ber.* **1977**, *110*, 1321−1329.

(22) Weyerstahl, P.; Christiansen, C.; Marshall, H. Isolation and synthesis of isohumbertiol, the first naturally occurring sesquiterpene alcohol with a humbertian skeleton. *Liebigs Ann. Chem.* **1992**, 1325−1328.

(23) Kaspera, R.; Krings, U.; Pescheck, M.; Sell, D.; Schrader, J.; Berger, R. G. Regio- and stereoselective fungal oxyfunctionalisation of limonenes. *Z. Naturforsch.* **2005**, *60c*, 45−466.

(24) Kapich, A. N.; Steffen, K. T.; Hofrichter, M.; Hatakka, A. Involvement of lipid peroxidation of a non-phenolic lignin model compound by manganese peroxidase of the litter-decomposing fungus *Stropharia coronilla. Biochem. Biophys. Res. Commun.* **2005**, *330*, 371−377.

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