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Bioconversion of car-3-ene by a dioxygenase of *Pleurotus sapidus*

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ABSTRACT

Mycelium of the basidiomycete *Pleurotus sapidus* known to contain a novel dioxygenase was used for the bioconversion of car-3-ene [I]. After 4 h of incubation 25.3 mg L^{-1} car-3-en-5-one [V], 5.4 mg L^{-1} car-3-en-2-one [VII], and 7.3 mg L^{-1} car-2-en-4-one [XV] accumulated as major oxidation products. The identity of the respective carenones and their corresponding alcohols was confirmed by comparison with MS and NMR spectral data obtained for synthesized authentic compounds. The peak areas of oxidation products were at least five times higher as compared with autoxidation. A radical mechanism similar to lipoxygenase catalysis was proposed and substantiated with detailed product analyses. The reduction of assumed car-3-ene hydroperoxides to the corresponding alcohols evidenced the radical initiated formation of hydroperoxides and confirmed the regio- and stereo-selectivity of the dioxygenase. The introduction of molecular oxygen into the bicyclic car-3-ene [I] molecule occurred at allylic positions of a cyclic isopentenyl moiety with a pronounced preference for the position adjacent to the non-substituted carbon atom of the C–C-double bond. This co-factor independent selective oxygenation presents an alternative to P450 mono-oxygenase based approaches for the production of terpene derived flavor compounds, pharmaceuticals and other fine chemicals.

1. Introduction

Terpenoids (isoprenoids) encompass more than 40,000 structures and form the largest class of plant metabolites. They are widely used as industrially important chemicals, including pharmaceuticals, flavors, fragrances, pesticides and disinfectants, and as large-volume feedstock for chemical industries (Bohlmann and Keeling, 2008). The bicyclic monoterpene (+)-car-3-ene (3,7,7-trimethyl bicyclo [4.1.0] hept-3-ene, [I] is a constituent of the tropical pine trees *Pinus roxburghii* and *Pinus logifolia* (55–65%) (Pattekhan et al., 1997), some juniper species, and citrus trees. The (−)-car-3-ene enantiomer is found in the essential oil of the Scots pine (*Pinus silvestris*) (Breitmaier, 2005). Both enantiomers are natural, inexpensive and widely available raw materials and serve as appropriate synthons in the chemical synthesis of pharmaceuticals, agrochemicals, flavors and fragrances (Kuriata et al., 2010; Macaev and Malkov, 2006; Moreira and Nascimento, 2007). Terpene hydrocarbons present suitable precursors for a sustainable biotechnological production of antibiotics, hormones, vitamins, amino acids, and for flavor compounds (Antranikian and Heiden, 2006; Krings and Berger, 2010; Sijbesma and Schepens, 2003; Ulber and Sell, 2007).

As early as in 1962 car-3-ene [I] was used as a precursor in bioconversion studies with *Aspergillus niger* that resulted in the formation of low yields of a hydroxyketone (Prema and Bhattacharyya, 1962a,b). Another study described the microbial oxidation of (+)-car-3-ene [I] using *Mycobacterium smegmatis*, with (+)-chaminic acid, car-3-en-5-one [V] and 2-(3′-methylcyclohexa-3′,5′-dienyl)propane-2-ol as the products (Stumpf et al., 1990). The epoxidation of the cyclic double occurred during incubation of *Nicotiana tabacum* and *Catharanthus roseus* with car-3-ene [I] (Hirata et al., 1994; Miyazawa and Kano, 2010).

During the last decade the bioconversion of terpenes using higher fungi has attracted notice with a special emphasis on the production of natural flavors. The basidiomycete *Pleurotus sapidus* was shown to convert bicyclic terpene hydrocarbons regio- and stereo-specifically to valuable flavor compounds: α-pinene to verbenone (Krings et al., 2009) and valencene to the grapefruit aroma impact nootkatone (Kaspera et al., 2005; Kruegener et al., 2010). This study presents the regio- and stereo-selective oxidation of the bicyclic monoterpene car-3-ene [I] using a dioxygenase previously isolated and sequenced from mycelium homogenate of *P. sapidus* (Fraatz et al., 2009).

2. Materials and methods

2.1. Chemicals

(+)-Car-3-ene [I] (90%, Sigma Aldrich, Germany) and azeotropic pentane/diethyl ether (1:1.12) were distilled (>99% purity) before use. In (+)-car-3-ene no oxidations products were detectable. BHT

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(3,5-di-*tert*-butyl-4-hydroxytoluene, 99%) was from Fluka (Seelze, Germany). All other chemicals used were analytical grade.

2.2. Fungi

Pleurotus sapidus (DSMZ 8266) was obtained from the culture collections of DSMZ, Braunschweig. For maintenance on agar slants and submerged culture, the fungus was grown on glucose/L-asparagine/yeast extract medium as described elsewhere (Onken and Berger, 1999).

2.3. Fungal growth

The fungal cultures (1 cm^2 of a densely overgrown piece of agar) were inoculated into a 150 mL glucose/L-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). Experimental cultures (500 mL shake flasks, 225 mL medium volume) were inoculated with 25 mL 5-day-old pre-cultures grown on the same medium and homogenized prior to inoculation.

2.4. Mycelium homogenates (MH)

After 5 days of fungal growth the active biomass was harvested by centrifugation at 10,000 × *g* and separation of the supernatant. To 500 mg of fungal mycelium (wet weight) 1 mL of 200 mM HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethansulfonic acid) buffer, pH 5.5 with 5% glycerol was added and homogenized with an Ultraturrax (Miccra D-9) for 2 min at 16,000 rpm on ice.

2.5. Bioconversion

Conversion experiments were started by adding 300 mg L^{-1} of (+)-car-3-ene [I] to 500 mg fungal mycelium homogenized in 1 mL HEPES-buffer. The homogenates were incubated 4 h at 24 °C and 150 rpm on an orbital shaker (Multitron, Infors, Bottingen, Switzerland). Conversion products were extracted three times with 1.5 mL azeotropic pentane/diethyl ether (1:1.2, v/v). The combined and dried ($Na_2SO_{4sicc.}$) organic phases were concentrated to approx. 1 mL and after addition of 60 μg of the external standard BHT (3,5-di-tert-butyl-4-hydroxytoluene; response factor 1.0 assumed) and analyzed by means of thin layer chromatography (TLC) and high resolution gas chromatography mass spectrometry (HRGC–MS). Blanks were carried out by incubation of inactivated (30 min at 100 °C) homogenates with (+)-car-3-ene [I] in HEPES-buffer. Furthermore the solvent extracts were reacted with $NaBH_4$ to convert presumed hydroperoxides to the corresponding alcohols (Section 2.11).

2.6. High resolution gas chromatography (HRGC)/high resolution gas chromatography–mass spectrometry (HRGC–MS)

One μL of each concentrated sample was injected into a Fison GC MFC 800 equipped with a cool on-column injector, a Factor Four VF Wax ms (Varian, 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 ionization detector (fid, 230 °C) using a temperature program from 40 °C (3 min) to 200 °C with a rate of 3 °C min^{-1} and to 240 °C with a rate of 10 °C min^{-1} and hold 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 mass spectra with data from reference compounds or literature (Wiley 08/NIST 08, 2008; spectral libraries) and by linear retention index values 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, electron impact ionization (ei, 70 eV), scan range *m*/*z* 33–300 amu).

2.7. Thin layer chromatography (TLC)

TLC was carried out on aluminium sheets covered with silica gel 60 (Merck, Darmstadt, Germany) and azeotropic pentane/diethyl ether (1:1.12) as mobile phase. The TLC plates were dried and subsequently stained with either anise aldehyde (0.5 g anise aldehyde, 10 mL glacial acetic acid, 85 mL methanol, 5 mL concentrated sulphuric acid) (van der Heide, 1966) or the hydroperoxide specific Huber reagent (3.0 g α-naphthol, 150 mL methanol, 1350 mL H_2O, 0.5 g $K_2S_2O_5$, 20 mL acetic acid, 0.5 g $FeSO_4 \times 7H_2O$, 2.2 g 2-[(4-amino-3-methylphenyl)ethylamino]ethyl sulfate) (Huber and Fröhlke, 1972).

2.8. NMR

^1H and ^{13}C NMR were performed on a BRUKER Advance DRX-500 (^1H at 500 MHz, ^{13}C at 125 MHz) and samples were dissolved in $CDCl_3$. The chemical shift signals were referenced to the residual signal of the solvent.

2.9. Synthesis of carenones and carenols

Car-3-en-5-one [V], car-3-en-2-one [VII] and car-2-en-4-one [XV] were synthesized as described elsewhere (Chidambaram and Chandrasekaran, 1987). In brief, 3.87 mmol of (+)-car-3-ene [I] was dissolved in 12 mL dry benzene, 4 mmol 70% *tert*-butyl hydroperoxide and 4 mmol pyridinium dichromate were added and reacted with continuous agitation for 4 h at 25 °C. The reaction yielded a mixture of oxygenated products with car-3-en-5-on [V], car-3-en-2-one [VII], and car-2-en-4-one [XV] in a ratio of 2.3:1.3:1.0 as the main products. The carenones were separated from the reaction mixture using flash chromatography on a silica gel column with silica gel 60. For conditioning a portion of 50 g of the silica gel 60 was dried at 120 °C over 12 h and the activity was adjusted to II–III by adding 2.25 mL water and subsequent equilibration for 12 h in a rotary evaporator. The adjusted silica gel was slurried into a glass column (20 cm × 1.5 cm) with pentane/diethyl ether 9:1. The reaction mixture was introduced to the column in three parts. The respective carenones were eluted with pentane/diethyl ether mixtures (9:1, 3:1 and pure diethyl ether, each 200 mL) of increasing polarity. Fractions were collected and concentrated using a Vigreux column and re-dissolved in $CDCl_3$. Full separation of car-3-en-2-one [VII] and car-2-en-4-one [XV] was achieved by means of preparative gas chromatography. The structures of purified carenones were confirmed according to their respective spectral data (EI-MS, ^1H and ^{13}C NMR, Table 1). Reduction of the particular carenones with $NaBH_4$ yielded the corresponding carenols. For *cis*/*trans* ratios see Table 3.

2.10. Preparative gas chromatography

Preparative gas chromatography was accomplished on a Hewlett-Packard 5890 gas chromatograph. The system was equipped with a CIS 3 cold injection system (Gerstel, Mülheim, Germany), a Multi Column Switching System II (Gerstel, Mülheim, Germany) and a Hewlett Packard 7673 autosampler (Hewlett Packard, Palo Alto, USA). A pre-column (Optima Wax, 5 m × 0.53 mm × 2 μm) was connected with a preparative column (Optima Wax, 25 m × 0.53 mm × 2 μm) (Macherey-Nagel, Düren, Germany). The carrier gas was hydrogen at a flow rate of