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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

Table 1
Spectral data of carenones.

C/H	δ ^{1}H (ppm)	δ ^{13}C (ppm)	*m*/*z*/(%) of bp
(a) Car-3-en-5-one [V]			
8	1.06 (s, 3H)	14.39	[M$^+$], 150 (81)
7		22.56	135 (39)
10	1.89 (s, 3H)	23.69	122 (16)
1	1.46 (t, 1H, $^3J_{1-6} = {}^3J_{1-2} = 8.2$ Hz)	25.83	109 (34)
2	2.34 (d, 1H, $^2J = 20.9$)	27.86	108 (62)
2	2.65 (dd, 1H, $^2J = 21.2$, $^3J_{2-1} = 8.1$)		107 (100)
9	1.20 (s, 3H)	28.43	95 (24)
6	1.58 (d, 1H, $^3J_{6-1} = 7.9$)	32.84	91 (76)
4	5.84 (s, 1H)	126.36	80 (20)
3		158.91	79 (55)
5		196.75	77 (30)
(b) Car-3-en-2-one [VII]			
8	1.10 (s, 3H)	14.3	[M$^+$], 150 (100)
10	1.79 (s, 3H)	16.1	135 (59)
7		21.9	121 (12)
5	2.46 (d, 1H, $^2J = 21.4$)	23.1	109 (27)
5	2.71 (dd, 1H, $^2J = 20.8$, $^3J_{5-6} = 8.3$),		108 (70)
6	1.44 (t, 1H, $^3J_{6-1} = {}^3J_{6-5} = 8.0$)	26.4	107 (96)
9	1.20 (s, 3H),	28.5	95 (18)
1	1.66 (d, 1H, $^3J_{1-6} = 7.9$)	34.4	91 (72)
3		135.1	79 (58)
4	6.42 (s, 1H)	142.6	67 (59)
2		196.6	65 (18)
(c) Car-2-en-4-one [XV]			
8	0.85 (s, 3H)	13.4	[M$^+$], 150 (59)
10	1.81 (s, 3H)	16.0	135 (30)
6	1.37 (t, 1H, $^3J_{6-1} = {}^3J_{6-5} = 8.0$)	23.4	122 (9)
1	1.52 (s, 3H)	24.1	109 (19)
7		24.9	108 (95)
9	1.26 (s, 3H)	27.6	107 (100)
5	2.61 (d)	33.3	105 (17)
5	2.63 (dd)		93 (25)
3		133.2	91 (83)
2	6.84 (d, 1H, $^2J = 7.8$)	144.5	80 (19)
4		196.7	79 (75)

bp, base peak (100%).

8.9 mL min^{-1} at 100 °C and the detector was a flame ionization detector (FID, 230 °C). The following temperature program was used: 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.

2.11. Reduction of bioconversion products/synthetics

Isolated bioconversion products or synthesized reference substances were re-dissolved in 0.5 mL MeOH (water free) and treated with $NaBH_4$ for 1 h on ice with subsequent addition of 0.5 mL water. The reaction mixture was evaporated to dryness and re-dissolved in pentane/diethyl ether (1:1.12) and submitted to HRGC–MS.

3. Results

Homogenates of *P. sapidus* were incubated with 300 mg L^{-1} of (+)-car-3-ene [I]. After 4 h of agitating several car-3-ene [I] derived oxidation products were detected in concentration up to 25.3 mg L^{-1} for the major product 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]. It is known that, after contact with molecular oxygen, (+)-car-3-ene [I] is prone to autoxidation and readily polymerizes above 40 °C (Rothenberg and Sasson, 1998). In fact, longer incubation times were found to direct the transformation towards car-3-ene [I] polymerization products (data not shown) which had an adverse impact on both concentration of primary oxygenated products and on HR–GC analysis. Because most of the mono-oxygenated car-3-enes [I] are not commercially available and HRGC–MS data in literature are inconsistent, car-3-en-5-one [V], car-3-en-2-one [VII], and car-3-en-5-one [XV] have been synthesized to confirm the respective structures. Spectral data (^{1}H/^{13}C NMR and MS) are presented Table 1. In contrast to EI-MS data NMR data are in good accordance with published data (Kolehmainen et al., 1993; Maas et al., 1984; Menini et al., 2008).

Table 2
Car-3-ene derived oxidation products of *P. sapidus* (4.4 μmol in 1 mL buffer + 500 mg homogenized mycelium, 4 h).

Product	Bioconversion (mg L^{-1})	Autoxidation (mg L^{-1})	Identification
Car-3-en-5-one [V]	25.3	0.9	MS, Syn, NMR
Car-3-en-2-one [VII]	5.4	0.4	MS, Syn, NMR
Car-2-en-4-one [XV]	7.3	0.2	MS, Syn, NMR
cis/*trans*-Car-3-en-5-ol [IV]	8.1	1.5	MS, Syn
cis/*trans*-Car-3-en-2-ol [VI]	1.6	0.4	MS, Syn
cis/*trans*-Car-2-en-4-ol [XIV]	2.4	0.7	MS, Syn
α-Phellandren-8-ol [XVI]	2.9	1.9	MS, RI
m-Mentha-4,6-dien-8-ol [XII]	0.36	0.1	MS, RI
4-Cymen-8-ol [XVII]	0.8	0.10	MS, RI
3-Cymen-8-ol [XIII]	n.d.	<0.1	MS, RI

MS, mass spectrum; Syn, synthesis; NMR, nuclear magnetic resonance; n.d., not detected.
Analyses were performed in duplicates; all standard deviations were below 10%.

Table 2 shows the peak concentration of car-3-ene [I] derived mono-oxygenated products. Both, bioconversion and autoxidation of caren-3-ene [I] showed a similar product spectrum, but differed in terms of regio-chemistry and quantity. The carenones V, VII, and XV were the predominating products in bioconversion batches, whereas the major autoxidation product found was α-phellandren-8-ol [XVI]. Generally, the concentration of oxygenated products was considerably higher – five times averaged over all products – in the bioconversion trials compared to car-3-ene autoxidation. This ratio was much higher than average for the three carenones [V, VII, XV], especially for the car-3-en-5-one [V] supporting a predominant enzyme catalyzed formation of the respective ketone. The samples were concentrated up to 24 mg L^{-1} (car-3-en-5-one [V], 24 g L^{-1} (car-3-en-2-one [VII] and 11.5 g L^{-1} (car-2-en-4-one [XV]), but no odor impression was detected during GC-olfactometry (data not shown).

Thin layer chromatography with subsequent peroxide selective staining (*Huber*'s reagent) indicated that both biochemical and autoxidative generation of oxygenated products were initiated by the formation of allylic radicals of car-3-ene [I] which either instantly added molecular oxygen, or underwent re-arrangements before the respective terpene hydroperoxide was built. Comparing biochemical and autoxidative car-3-ene [I] oxygenation not only the reaction rate differed, but the regio-chemical orientation of the oxygenation products was different, too. Table 3 (a and b) shows the relative ratios of carenones [V, VII, XV], carenols [IV, VI, XIV] and their respective *cis*/*trans* ratios. For autoxidation and bioconversion the initial oxidative attack took place predominantly at the C5 of car-3-ene [I] to give [V] and [VI] and to a minor extent at C2 to give [VI] and [VII], respectively, but a significant formation of car-2-en-4-one [XV] occurred during the bioconversion only (Table 3a). Autoxidation and bioconversion yielded the corresponding carenols in almost the same ratio as the carenones. Of the detected carenols the respective *trans*-isomers prevailed in all cases (Table 3b). In order to measure the amount of intact carene hydroperoxides the concentrated solvent extract of the bioconversion was treated with a surplus of $NaBH_4$. Most of each carenone [VII] and [XV] was reduced to the corresponding carenols and increased their amount proportionally. As the $NaBH_4$ reduction of pure references yielded preferably the *cis* configurated carenols (Kuhn and Fischer, 2009), the ratio turned to *cis* carenols in the

Table 3
Product ratios after bioconversion and autoxidation of car-3-ene with *P. sapidus*: (a) carenones and corresponding carenols; (b) *cis*/*trans* ratios of the respective carenols before and after $NaBH_4$ reduction (4.4 μmol in 1 mL buffer + 500 mg homogenized mycelium (=300 mg L^{-1}), 4 h).

	Car-3-en-5-one [V]	Car-3-en-2-one [VII]	Car-2-en-4-one [XV]
(a) Carenones and corresponding carenols			
Bioconversion	4.7	1.0	1.4
Autoxidation	1.6	1.0	0.3
	Car-3-en-5-ol [IV]	Car-3-en-2-ol [VI]	Car-2-en-4-ol [XIV]
(a) Carenones and corresponding carenols			
Bioconversion	1.4	1.0	0.7
Autoxidation	1.2	1.0	0.6
	cis/*trans*	*cis*/*trans*	*cis*/*trans*
b) *cis*/*trans* ratios			
Bioconversion	*trans*	0.15/1.0	0.15/1.0
Autoxidation	*trans*	0.3/1.0	0.5/1.0
Bioconversion (red.)[a]	0.34/1.0	2.0/1.0	2.4/1.0
Carenones (red.)[a]	4.0/1.0	2.5/1.0	2.4/1.0

[a] Reduced with $NaBH_4$; *trans*, *trans*-isomer detected only; n.d., not definable.

reduced bioconversion trial. However, the increase of the response of the isomeric car-3-en-5-ols [IV] was almost twice as much than calculated from the reduction of the corresponding car-3-en-5-one [V] alone with an unexpected surplus of *trans*-car-3-en-5-ol (Table 3b). An accurate calculation was not feasible due to incomplete reduction of the ketones, rearrangements and partial integrity of the presumed car-3-en-5-hydroperoxide [II] during reduction.

4. Discussion

The first introduction of oxygen is usually the rate limiting step in the catabolism of non-polar compounds, such as terpenes. Especially cytochrome P450 systems, constitutive of all living beings and representing one of the largest and oldest gene super-families, may act as terminal mono-oxygenases in a range of reactions. However, for an industrial application of co-factor dependent enzymes, several crucial obstacles have to be surmounted. Therefore, co-factor independent specific oxygenation of terpene precursors would encounter particular interest of both research and industry (Krings and Berger, 2010). Recently, a novel dioxygenase from *P. sapidus* was characterized and the structural gene sequenced. The dioxygenase, with best homologies to lipoxygenases from ascomycetes, catalyzed regio- and stereo-specifically the allylic hydroperoxygenation of (+)-valencene to the valuable flavor compound nootkatone (Fraatz et al., 2009; Kruegener et al., 2010) and of α-pinene to verbenone (Krings et al., 2009). In this study (+)-car-3-ene [I] was used as another bicyclic terpene substrate to confirm the assumed substrate specifity and stereo-selectivity of the enzyme.

For the above mentioned mono- and sesquiterpene it was shown that both autoxidation and bioconversion took place along the same radical mediated pathway. Thus, enzyme catalyzed oxygenation and autoxidation needed to be carefully distinguished. This was likewise found for the bioconversion of (+)-car-3-ene [I]. The incubation time was restricted to 4 h, as the dioxygenase initiated reaction ran out of control in terms of selectivity with increasing incubation time because of the high reactivity of both (+)-car-3-ene [I] and the hydroperoxide intermediates.

4.1. Autoxidation

As confirmed by TLC with hydroperoxide specific staining the initial introduction of molecular triplet oxygen during autoxidation yielded several hydroperoxides of (+)-car-3-ene [I] which was in good agreement with earlier work on car-3-ene autoxidation (McGraw et al., 1999; Rothenberg and Sasson, 1998). Based on a detailed product analysis a substantiated reaction pathway was deduced which is shown in Fig. 1. The first attack occurred at the two allylic ring positions (C2, C5) of (+)-car-3-ene [I] and afforded the two radicals [Ia, Ib] which either reacted directly with triplet oxygen to give the respective hydroperoxides [II, III] or underwent diverse rearrangements. While maintaining the bicyclic backbone, oxygen uptake gave the hydroperoxides [IX] and [X]. The cleavage of the cyclopropyl ring shifted the reaction towards the formation of menthadiene hydroperoxides [VIII, XI]. As known for other terpene hydroperoxides (Krings et al., 2009; Kruegener et al., 2010; Kuznetsova et al., 2005) carene hydroperoxides were unstable during GC-analysis. Their occurrence was confirmed by TLC with selective peroxide staining and concluded from their respective degradation products. The structures of the corresponding alcohols and ketones were unambiguously elucidated (Tables 1 and 2). Though tertiary radical [Id] is likely the most stable carbon centered radical, degradation products of the corresponding hydroperoxide [IX] were not found. Tertiary hydroperoxides in conjugation to a double bond are known to undergo a fast rearrangement, and this afforded hydroperoxide [II] (Schenck et al., 1958). The same rearrangement was described for (+)-valencene autoxidation (Kruegener et al., 2010).

The approach of triplet oxygen to the intermediate free radicals of car-3-ene [I] took place preferably on the opposite side of the cyclopropyl ring and afforded the *trans*-configurated carene hydroperoxides [II], [III], and [X]. The energy minimized (CS Chem 3D Ultra 7.0) conformation of car-3-ene (Fig. 2) shows the almost planar cyclohexenyl ring with the methyl group (at C3) in plane and the bis-methylated cyclopropyl ring out of plane hampering the free access of oxygen from this side.

4.2. Enzyme catalysis

The enzyme catalyzed oxygenation of car-3-ene [I] showed a significantly accelerated product formation (Table 2). For the respective carenones, at first glance, the regio-selectivity seemed to be similar to autoxidation. However, the true regio-chemical orientation of the dioxygenase catalyzed oxygenation was revealed after reduction of the respective carene hydroperoxides and carenones. After treating the product mixture of the bioconversion with $NaBH_4$ the measured amount of *trans*-car-3-en-5-ol [IV] was much higher than expected from the total of genuine car-3-en-5-ol [IV] and

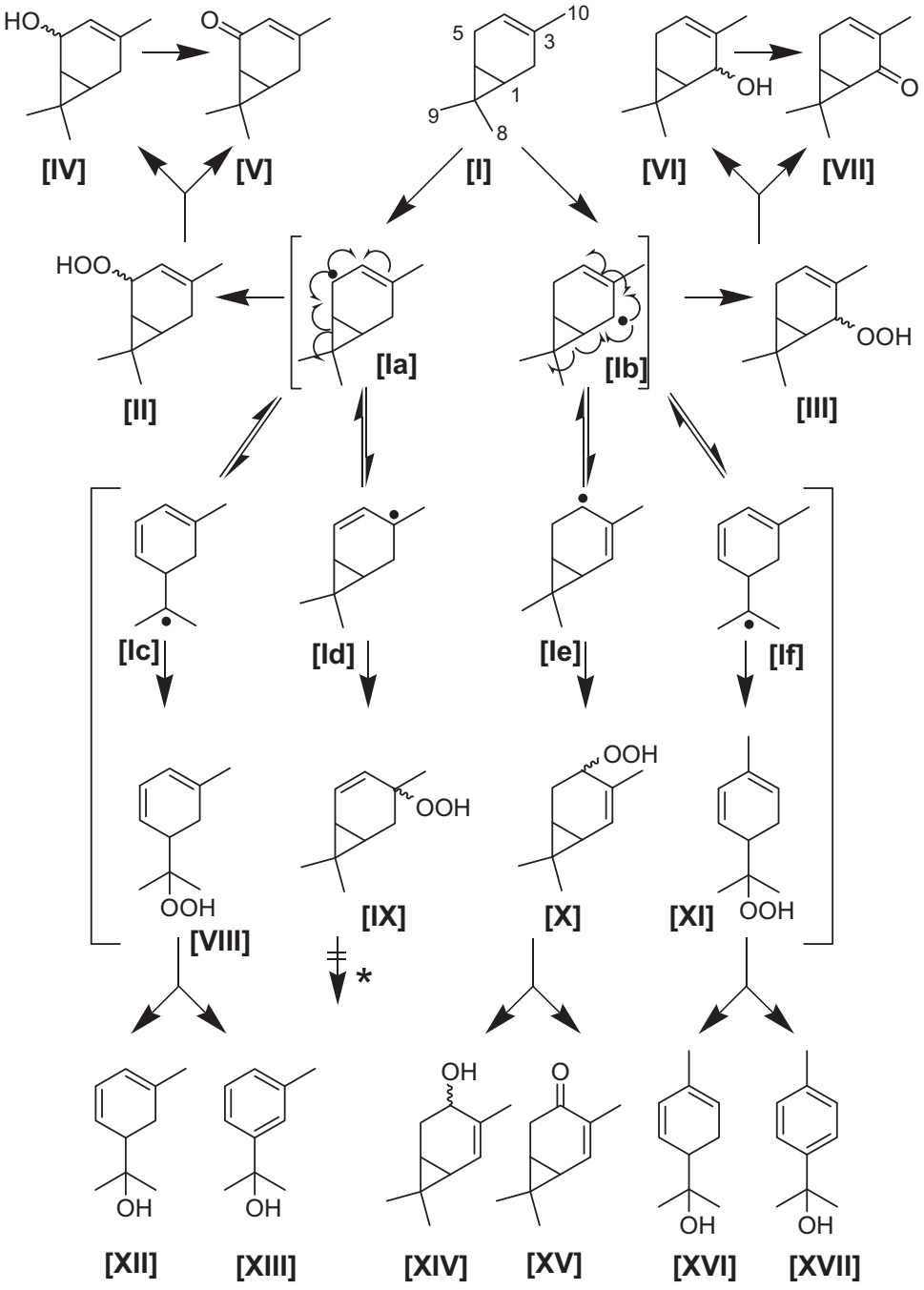

Fig. 1. Reaction scheme of bioconversion and autoxidation of car-3-ene [I] (* rearrangement of [IX] afforded hydroperoxide [II]; Schenck et al., 1958).

the calculated amount of [IV] obtained after the reduction of car-3-en-5-one [V] that resulted in a preferred *cis*-orientation of the respective carenol. This was not observed for the two other carenols [VI, XIV]. This gives evidence for a preferred enzyme catalyzed formation of *trans*-car-3-en-5-hydroperoxide [II] that remained during the bioconversion. After reduction of the bioconversion extract, the ratio of *cis*/*trans* for both car-3-en-5-ol [IV] and car-2-en-4-ol [XIV] was found inverted, whereas *trans*-car-3-en-5-ol [IV] was still prevailing (Table 3b). This strongly suggests that in agreement to car-3-ene [I] autoxidation the dioxygenase catalyzed introduction of oxygen occurred on the opposite side of the bulky bis-methylated cyclopropyl ring but in contrast to autoxidation predominately at the allylic C5 position. With increasing incubation time hydroperoxides accumulate. This may start to inhibit the activity of the dioxygenase and also deliver starters for non-selective autoxidation.

The radical mediated reaction mechanism as well as the regio- and stereo-selective orientation of the dioxygenase catalyzed oxidation of car-3-en [I] is in line with results obtained for the bioconversion of valencene using the same enzyme. However, in the case of the oxygenation of valencene even autoxidation showed an apparent regio-selectivity. Owed to allylic rearrangements of the respective allylic "valencene hydroperoxides" oxidation products were found at C7 only (Davies and Davison, 1989; Kruegener et al., 2010; Schenck et al., 1958). The dioxygenase catalyzed reaction showed a pronounced preference towards the formation of 6(*R*)-Isopropenyl-4(*R*),4a(*S*)-dimethyl-2,3,4,4a,5,6,7,8-octahydro-naphthalen-2(*R*)-yl-hydroperoxide (introduction of O_2

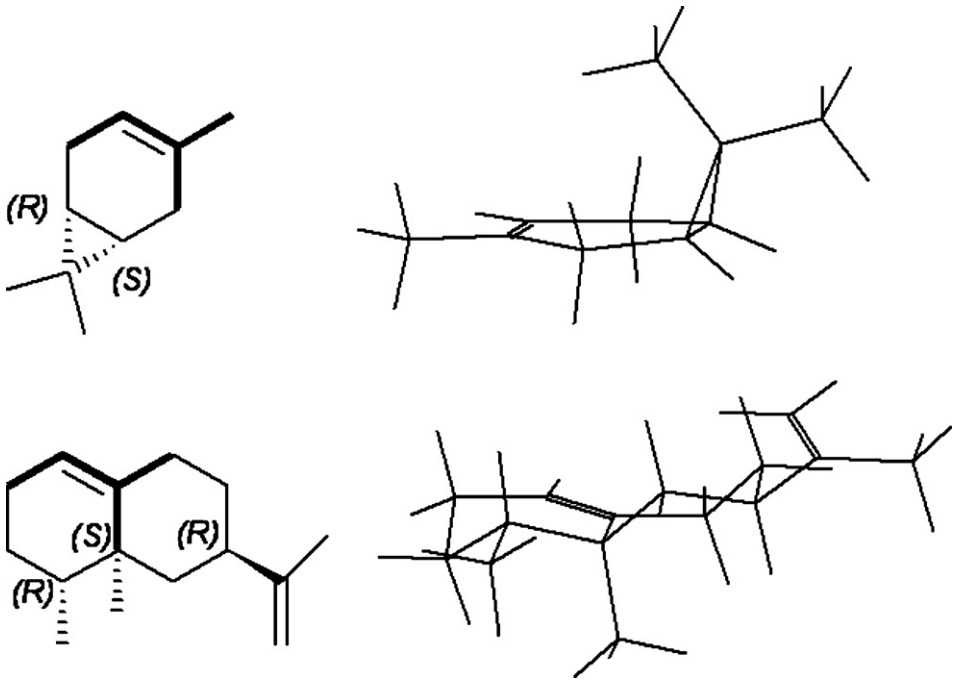

Fig. 2. Structure and energy minimized (CS Chem 3D Ultra 7.0) conformation of car-3-ene and (+)-valencene.

opposite to both methyl groups and on the same side as the distant isopropylene group), whereas the autoxidation resulted simply in a slight excess of the same diastereomer (mean steric hindrance due to the two methyl groups, Fig. 2).

In conclusion, the dioxygenase of *P. sapidus* catalyzed the regio- and stereo-selective introduction of molecular oxygen into mono- and sesquiterpenes at allylic positions of cyclic isopentenyl moieties with a pronounced preference for the allylic position adjacent to the non-substituted carbon atom of the C–C-double bond. In contrast to P450-enymes the dioxygenase did not require any redox-cofactor which is an important advantage for possible industrial applications. However, because of the reactivity of the hydroperoxides intermediates the bioconversion was not easily controlled, and autoxidation competed on the longer run.

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