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Selective oxidation of carotenoid-derived aroma compounds by CYP260B1 and CYP267B1 from *Sorangium cellulosum* So ce56

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Abstract Due to their bioactive properties as well as their application as precursors in chemical synthesis, hydroxylated isoprenoids and norisoprenoids are very valuable compounds. The efficient hydroxylation of such compounds remains a challenge in organic chemistry caused by the formation of a variety of side products and lack of overall regio- and stereoselectivity. In contrast, cytochromes P450 are known for their selective oxidation under mild conditions. Here, we demonstrate for the first time the ability of myxobacterial CYP260B1 and CYP267B1 from *Sorangium cellulosum* So ce56 to oxidize such carotenoid-derived aroma compounds. A focused library of 14 substrates such as ionones, damascones, as well as some of their isomers and derivatives was screened in vitro. Both P450s were capable of an efficient oxidation of all tested compounds. CYP260B1-dependent conversions mainly formed multiple products, whereas conversions by CYP267B1 resulted predominantly in a single product. To identify the main products by NMR spectroscopy, an *Escherichia coli*-based whole-cell system was used. CYP267B1 showed a hydroxylase activity towards the formation of allylic alcohols. Likewise, CYP260B1 performed the allylic hydroxylation of β-damascone [(E)-1-(2,6,6-trimethylcyclohex-1-enyl)but-2-en-1-one] and δ-damascone [(E)-1-(2,6,6-trimethylcyclohex-3-enyl)but-2-en-1-one]. Moreover, CYP260B1 showed an epoxidase activity towards β-ionone [(E)-4-(2,6,6-trimethylcyclohex-1-enyl)but-3-en-2-one] as well as the methyl-substituted α-ionone derivatives raldeine [(E)-1-(2,6,6-trimethylcyclohex-2-enyl)pent-1-en-3-one] and isoraldeine [(E)-3-methyl-4-(2,6,6-trimethylcyclohex-2-enyl)but-3-en-2-one]. In addition, to known products, also novel products such as 2-OH-δ-damascone [(E)-1-(5-hydroxy-2,6,6-trimethylcyclohex-3-enyl)but-2-en-1-one], 3-OH-allyl-α-ionone [(E)-1-(4-hydroxy-2,6,6-trimethylcyclohex-2-enyl)hepta-1,6-dien-3-one], and 4-OH-allyl-β-ionone [(E)-1-(3-hydroxy-2,6,6-trimethylcyclohex-1-enyl)hepta-1,6-dien-3-one] were identified during our studies.

Keywords Cytochromes P450 · Ionone · Damascone · CYP260B1 · CYP267B1 · Norisoprenoids

Introduction

Cytochromes P450 (P450s) are heme-containing monooxygenases that are present in all domains of life (Nelson 2011). They catalyze the oxidation of various classes of compounds such as xenobiotics, steroids, terpenes, fatty acids, and others (Bernhardt 2006). The activation of non-activated C-H bonds by the insertion of a single oxygen atom from molecular oxygen is catalyzed by these enzymes. Besides that hydroxylation, they are able to perform diverse reactions including epoxidation, alcohol oxidation, *N*-, *S*-, and *O*-dealkylation or the cleavage of C–C bonds (Sono et al. 1996). The electrons, required for such reactions, are provided by NAD(P)H and delivered via an electron transfer chain (Hannemann et al. 2007).

Mammalian P450s are mainly involved in the degradation of xenobiotics and drugs as well as in the metabolism of steroids and lipids (Bernhardt and Urlacher 2014). In contrast, the physiological role of many microbial P450s is not characterized yet. However, it is known that they play an important role in the production of secondary metabolites (Urlacher et al. 2004). For that reason, the discovery of their functions and

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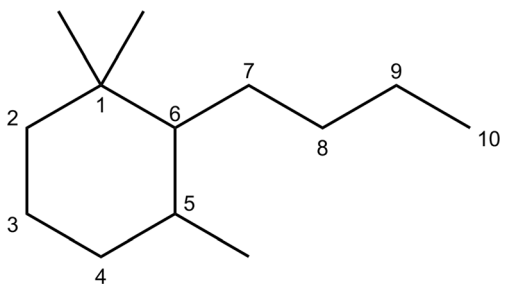
activities is a promising field of research (Kelly and Kelly 2013). Myxobacteria are top producers of microbial secondary metabolites which attract the attention of the pharmaceutical industry (Weissman and Müller 2010). The genome of the myxobacterium *Sorangium cellulosum* So ce56 was sequenced in 2007 (Schneiker et al. 2007) and 21 P450s were identified (Khatri et al. 2010b). Since that time, various exogenous substrates like terpenes, fatty acids, or drugs were characterized for these P450s (Khatri et al. 2015; Litzenburger et al. 2015; Schifrin et al. 2015). Likewise, α- and β-ionones were identified as substrates of CYP109D1 and CYP264B1 (Khatri et al. 2010a; Ly et al. 2012).

Ionones and their regioisomers damascones (or the so-called rose ketones) belong to the group of norisoprenoids (carotenoid-derived aroma compounds). These secondary metabolites of carotenoids occur in many essential oils and play an important role for the flavor and fragrance industry due to their olfactory properties as well as their low odor threshold. Several derivatives of ionones such as dihydro-ionones, ionols, or irones occur in nature. Besides their natural derivatives, there are also some synthetic derivatives like allyl-α-ionone, isomethyl-α-ionone (isoraldeine), or n-methyl-α-ionone (raldeine) which are commercially of even greater importance. From a structural point of view, all compounds possess a megastigmane carbon skeleton as shown in Scheme 1 (Ohloff et al. 2012; Winterhalter and Rouseff 2002).

Hydroxylated and epoxidized derivatives of ionones and related compounds are of significant interest since they are used as precursors for chemical syntheses (Barakat et al. 2008; Brenna et al. 2002; Tu et al. 2014), for pharmaceutical applications (Gerhäuser et al. 2009; Zhou et al. 2009), or for the investigation of their potential allelopathic properties (Kato-Noguchi et al. 2010a, b; Macias et al. 2008; Macias et al. 2004). Furthermore, these oxyfunctionalized compounds are of interest due to their olfactory properties as reported for 3-hydroxy-α-ionone and its tobacco-like odor (Yamazaki et al. 1988). In addition, such compounds might be used as fly attractants (Ishida et al. 2008).

The first production of oxidized ionones was described in 1950 by isolating 4-oxo-β-ionone and 4-oxo-β-ionol from rabbit urine after feeding the animals with β-ionone (Prelog and Meier 1950). Since the 1980s and 1990s, there are several studies showing the formation of oxyfunctionalized products by microorganisms like *Streptomyces*, *Botrytis cinera*, *Lasiodiplodia theobromae*, or *Aspergillus niger* (Krasnobajew and Helmlinger 1982; Lutz-Wahl et al. 1998; Mikami et al. 1981; Mikami et al. 1978; Schoch et al. 1991; Yamazaki et al. 1988). However, such approaches lack the information on which specific enzyme is capable of the desired reaction. Several studies about specific bacterial P450s able to perform the regio- and stereoselective hydroxylation of α-ionone and β-ionone were published in the last decade (Celik et al. 2005; Girhard et al. 2010; Hall and Bell 2015; Ma et al. 2011; Zhang et al. 2015), likewise CYP109D1 and CYP264B1 from *Sorangium cellulosum* So ce56 (Khatri et al. 2010a; Ly et al. 2012). However, CYP260B1 and CYP267B1 from So ce56 were never tested for their ability to convert such substrates. Hence, we screened these P450s concerning the conversion of α-ionone (**1**), β-ionone (**2**), α-irone (**3**), 7,8-dihydro-α-ionone (**4**), 7,8-dihydro-β-ionone (**5**), α-ionol (**6**), β-ionol (**7**), α-damascone (**8**), β-damascone (**9**), δ-damascone (**10**), β-damascenone (**11**), as well as the synthetic derivatives isomethyl-α-ionone (**12**), *n*-methyl-α-ionone (**13**), and allyl-α-ionone (**14**). Those substrates showing a high selective product formation during our in vitro screening were further investigated in an *Escherichia coli*-based whole-cell system to obtain sufficient amounts for product identification via NMR spectroscopy.



Scheme 1 Structure and numbering of the megastigmane carbon skeleton

Material and methods

Chemicals

Isoraldeine (isomethyl-α-ionone), α-damascone, β-damascone, δ-damascone, and β-damascenone were provided by Bell Flavors and Fragrances (Leipzig, Germany). Isopropyl β-D-1-thiogalactopyranoside and 5-aminolevulinic acid were purchased from Carbolution Chemicals (Saarbruecken, Germany). Bacterial media were purchased from Becton Dickinson (Heidelberg, Germany). All other chemicals were obtained from standard sources in the highest purity available.

Expression and purification of the enzymes

CYP260B1 and CYP267B1 were expressed and purified by a method previously described (Khatri et al. 2010b). The electron transfer partners $Adx_{4\text{-}108}$ and AdR from *Bos taurus* were expressed and purified as described elsewhere (Sagara et al. 1993; Uhlmann et al. 1994).

In vitro screening

A reconstituted in vitro system containing the corresponding P450 (0.5 μM), AdR (1.5 μM), $Adx_{4\text{-}108}$ (10 μM), $MgCl_2$

(1 mM), and a cofactor regenerating system with glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (1 U) in a final volume of 250 μl of potassium phosphate buffer (20 mM, pH 7.4) was used. All substrates were dissolved in EtOH (50 mM) and added to a final concentration of 200 μM. The reaction was started by the addition of NADPH (500 μM). After 1 h at 30 °C, the reaction was quenched by adding ethyl acetate (500 μl). The aqueous phase was extracted twice with ethyl acetate (2 × 500 μl). A negative control in the absence of cytochrome P450 in the reaction sample was employed for each substrate to verify the P450-dependent reaction.

Whole-cell experiments

All experiments were performed with *E. coli* BL21(DE3) gold cells (Agilent Technologies; Santa Clara, USA). The cells were transformed with two plasmids, one for the corresponding P450 (pET17b for CYP260B1 or pET22b for CYP267B1) and the other one for the redox partners Fpr and $Adx_{4\text{-}108}$. The overnight culture was prepared in nutrient broth containing ampicillin (100 μg/ml) and streptomycin (50 μg/ml). The main culture was carried out in 5 × 1 l sealed baffled flasks containing 200 ml modified M9CA medium (Litzenburger et al. 2015). The medium was inoculated with 1 % (*v*/*v*) of the overnight culture and incubated at 37 °C. The induction of the corresponding genes was initiated by adding 1 mM isopropyl β-D-1-thiogalactopyranoside and 0.5 mM 5-aminolevulinic acid when the optical density reached 0.8–1.0 and the culture was grown further at 28 °C. After 21 h of expression, the temperature was set to 30 °C and the substrates (stock solution 50 mM in EtOH) were added to a final concentration of 200 μM. After 48 h, the reaction was quenched with the same volume of ethyl acetate and extracted twice. Organic phases were collected and pooled, and the organic solvent was removed by vacuum distillation. The extracts were stored in a dark refrigerator at 4 °C until purification.

Analysis of the conversions via HPLC-DAD

HPLC analyses were performed on a system consisting of a PU-2080 HPLC pump, an AS-2059-SF autosampler, and a MD-2010 multi wavelength detector (Jasco, Gross-Umstadt, Germany). A Nucleodur 100–5 C18 column (125 × 4 mm, Macherey–Nagel, Düren, Germany) was used at 40 °C. The mobile phases consisted of water with 10 % acetonitrile (A) and pure acetonitrile (B). A gradient from 20 to 80 % of B with a flow rate of 1 ml/min was used for the separation of the compounds.

Analysis of the conversions and products via GC-MS

Gas chromatography mass spectrometry (GC-MS) analyses were performed on a system consisting of an AI/AS 3000 autosampler, a DSQII quadrupole, a Focus GC column oven (Thermo scientific, Waltham, USA), and a DB-5 column (Agilent) with a length of 25 m, 0.32 mm ID, and 0.52 μm film thickness. The compounds were analyzed in a *m*/*z* range of 20–350. The starting oven temperature was 50 °C for 1 min and then the temperature was ramped to 220 °C by 10 °C/min and held for 3 min with a flow rate of 1 ml/min. The EI-mass spectra were compared with the NIST mass spectral library (version 2.0).

Purification of the products

The extracts were purified using silica gel chromatography. Mixtures of hexane and ethyl acetate either 8:2 (epoxidized products) or 6:4 (hydroxylated products) were used as a mobile phase. Fractions were collected and analyzed via thin-layer chromatography (TLC). TLC plates were stained with anisaldehyde (4-methoxybenzaldehyde (0.5 % *v*/*v*) in sulfuric acid, acetic acid, and MeOH 5:10:85) for visualization of the products. Fractions containing single products were pooled, evaporated, and prepared for NMR analysis.

NMR analysis

NMR spectra were recorded on a Bruker (Rheinstetten, Germany) 500 NMR spectrometer. A combination of ^{1}H, ^{13}C, COSY, HSQC, and HMBC experiments was used for structure elucidation. The *cis*/*trans*-configuration of the products between H3 and H6 was carried out by NOESY experiments. All chemical shifts are relative to $CHCl_3$ (δ = 7.24 for ^{1}H NMR) or $CDCl_3$ (δ = 77.00 for ^{13}C NMR) using the standard δ notion in parts per million (ppm).

NMR and GC-MS data

Data for 5,6-epoxy-β-ionone (15) *m*/*z* = 208.3; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.99 (*d*, *J* = 15.6 Hz, 1H, H7), 6.26 (*d*, *J* = 15.6 Hz, 1H, H8), 2.25 (*s*, 3H, Me5), 1.92–1.84 (*m*, 1H, H4a), 1.77–1.70 (*m*, 1H, H4b), 1.48–1.37 (*m*, 3H, H3; H2a), 1.12 (*s*, 3H, Me5), 1.11 (*s*, 3H, Me1a), 1.08–1.02 (*m*, 1H, H2b), 0.91 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 197.53 (C9), 142.63 (C7), 132.44 (C8), 70.59 (C6), 65.86 (C5), 35.46 (C2), 33.53 (C1), 29.74 (C4), 28.26 (C10), 25.87 (Me1a), 25.81 (Me1b), 20.81 (Me5), 16.85 (C3).

Data for 7,11-epoxymegastigma-5,6-en-9-one (17) *m*/*z* = 208.1; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 5.22–5.16 (*m*, 1H, H7), 4.49 (ddt, *J* = 12.0; 5.2; 1.3 Hz, 1H, H11a), 4.37–4.33 (*m*, 1H, H11b), 2.69 (dd, *J* = 2.9; 14.8 Hz, 1H, H8a), 2.58

(dd, J = 9.8; 14.8 Hz, 1H, H8b), 2.20 (s, 3H, H10), 1.96–1.84 (*m*, 2H, H4), 1.73–1.65 (*m*, 2H, H3), 1.50–1.44 (*m*, 1H, H2a), 1.40–1.32 (*m*, 1H, H2b), 1.03 (*s*, 3H, Me1a), 1.02 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 207.46 (C9), 139.16 (C5), 132.27 (C6), 82.48 (C7), 76.27 (C11), 50.54 (C8), 40.07 (C2), 31.05 (C1), 30.91 (C10), 27.95 (Me1a), 27.93 (Me1b), 22.25 (C4), 19.06 (C3).

Data for 4,5-epoxy-isomethyl-α-ionone (20) m/z = 222.2; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.63 (dq, J = 10.9; 1.35 Hz, 1H, H7), 3.07 (*q*, J = 1.6 Hz, 1H, H4), 2.46 (*d*, J = 10.9 Hz, 1H, H6), 2.34 (*s*, 3H, H10), 2.01–1.95 (*m*, 1H, H3a), 1.93–1.85 (*m*, 1H, H3b), 1.82 (*d*, J = 1.4 Hz, 3H, Me8), 1.45–1.38 (*m*, 1H, H2a), 1.21 (*s*, 3H, Me5), 1.02–0.96 (*m*, 1H, H2b), 0.92 (*s*, 3H, Me1a), 0.72 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 200.23 (C9), 141.89 (C7), 138.89 (C8), 59.79 (C4), 59.43 (C5), 47.62 (C6), 31.89 (C1), 28.96 (C2), 28.37 (Me1a), 26.64 (Me1b), 25.73 (C10), 24.30 (Me5), 21.74 (C3), 11.95 (Me8).

Data for 4,5-epoxy-*n*-methyl-α-ionone (21) m/z = 222.3; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.72 (dd, J = 16.1; 10.2 Hz, 1H, H7), 6.09 (*d*, J = 16.1 Hz, 1H, H8), 3.07 (*t*, J = 1.9 Hz, 1H, H4), 2.63 (*q*, J = 7.3 Hz, 2H, H10), 2.05 (*d*, J = 10.2 Hz, 1H, H6), 2.01–1.94 (*m*, 1H, H2a), 1.92–1.84 (*m*, 1H, H2b), 1.44–1.36 (*m*, 1H, H2a), 1.23 (*s*, 3H, Me5), 1.09 (*t*, J = 7.3, 3H, H11), 1.02–0.96 (*m*, 1H, H2b), 0.90 (*s*, 3H, Me1a), 0.73 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 201.33 (C9), 144.94 (C7), 133.00 (C8), 59.44 (C4), 58.79 (C5), 52.46 (C6), 32.38 (C10), 31.19 (C1), 28.54 (C2), 27.89 (Me1a), 27.41 (Me1b), 24.01 (Me5), 21.73 (C3), 8.23 (C11).

Data for 4-hydroxy-β-damascone (22) m/z = 208.1; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.75 (dq, J = 6.9; 15.8 Hz, 1H, H9), 6.13 (dq, J = 1.7; 15.7 Hz, 1H, H8), 3.97 (*t*, J = 4.9 Hz, 1H, H4), 2.01–1.93 (*m*, 1H, H3a), 1.90 (dd, J = 1.6; 4.8 Hz, 3H, H10), 1.77–1.70 (*m*, 1H, H3b), 1.67–1.62 (*m*, 1H, H2a), 1.62 (*s*, 3H, Me5), 1.45–1.39 (*m*, 1H, H2b), 1.01 (*s*, 6H, Me1a; Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 200.90 (C7), 146.47 (C9), 143.55 (C5), 133.90 (C8), 130.97 (C6), 68.98 (C4), 34.61 (C2), 33.95 (C1), 28.79 (Me1a), 28.60 (C3), 27.61 (Me1b), 18.43 (C10), 17.93 (Me5).

Data for *trans*-2-hydroxy-δ-damascone (23) m/z = 208.4; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.87 (dq, J = 6.9; 15.6 Hz, 1H, H9), 6.22 (dq, J = 1.7; 15.6 Hz, 1H, H8), 5.76 (ddd, J = 2.4; 5.1; 9.9 Hz, 1H, H3), 5.70 (dd, J = 2.0; 10.0 Hz, 1H, H4), 3.43 (*d*, J = 5.0 Hz, 1H, H2), 2.79 (*d*, J = 10.5 Hz, 1H, H6), 2.63–2.54 (*m*, 1H, H5), 1.89 (dd, J = 1.6; 6.9 Hz, 3H, H10), 0.96 (*s*, 3H, Me1a), 0.86 (*s*, 3H, Me1b), 0.86 (*d*, J = 7.0 Hz, 3H, Me5); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 203.44 (C7), 142.29 (C9), 136.93 (C4), 134.76 (C8), 125.40 (C3), 73.27 (C2), 53.65 (C6), 37.62 (C1), 31.72 (C5), 25.07 (Me1a), 20.14 (Me1b), 19.39 (Me5), 18.27 (C10).

Data for 3-hydroxy-α-irone (24a) m/z = 222.1; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.59 (dd, J = 16.0; 9.8 Hz, 1H, H7), 6.03 (*d*, J = 15.9 Hz, 1H, H8), 5.62–5.58 (*m*, 1H, H4), 4.14–4.09 (*m*, 1H, H3), 2.41 (*d*, J = 9.6, 1H, H6), 2.22 (*s*, 3H, H10), 1.76–1.70 (*m*, 1H, H2), 1.59 (*t*, J = 1.1 Hz, 3H, Me5), 0.95 (*s*, 3H, Me1a), 0.94 (*d*, J = 7.7, 3H, Me2), 0.85 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 198.18 (C9), 147.05 (C7), 135.41 (C5), 133.18 (C8), 125.50 (C4), 68.58 (C3), 54.90 (C6), 39.10 (C2), 35.86 (C1), 27.12 (C10), 25.92 (Me1a), 24.37 (Me1b), 22.61 (Me5), 9.77 (Me2).

Data for 3-hydroxy-α-irone (24b) m/z = 222.2; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.56 (dd, J = 16.1; 10.9 Hz, 1H, H7), 6.11 (*d*, J = 15.5 Hz, 1H, H8), 5.56–5.53 (*m*, 1H, H4), 3.76–3.71 (*m*, 1H, H3), 2.58 (*d*, J = 9.9, 1H, H6), 2.24 (*s*, 3H, H10), 1.54 (*q*, J = 3.0; 1.4 Hz, 3H, Me5), 1.30–1.26 (*m*, 1H, H2), 1.01 (*d*, J = 6.8 Hz, 3H, Me2), 0.85 (*s*, 3H, Me1a), 0.72 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 197.88 (C9), 147.58 (C7), 134.76 (C8), 134.35 (C5), 126.83 (C4), 71.41 (C3), 55.86 (C6), 47.02 (C2), 37.83 (C1), 27.00 (C10), 26.46 (Me1a), 22.53 (Me5), 15.24 (Me1b), 10.86 (Me2).

Data for *trans*-3-hydroxy-α-damascone (25) m/z = 208.2; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.87 (dq, J = 6.9; 15.5 Hz, 1H, H9), 6.20 (dq, J = 1.6, 15.4 Hz, 1H, H8), 5.69–5.66 (*m*, 1H, H4), 4.34–4.29 (*m*, 1H, H3), 3.10 (*s*, 1H, H6), 1.91 (dd, J = 5.8; 13.7 Hz, 1H, H2a), 1.88 (dd, J = 1.6; 6.9 Hz, 3H, H10), 1.60 (*s*, 3H, Me5), 1.37 (dd, J = 5.4; 13.6 Hz, 1H, H2a), 1.09 (*s*, 3H, Me1a), 0.85 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 200.50 (C7), 142.91 (C9), 134.16 (C5), 132.57 (C8), 126.64 (C4), 65.43 (C3), 60.95 (C6), 43.56 (C2), 33.30 (C1), 30.50 (Me1a), 25.80 (Me1b), 22.71 (Me5), 18.29 (C10).

Data for *trans*-3-hydroxy-7,8-dihydro-α-ionone (26) m/z = 210.1; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 5.43 (*t*, J = 1.2 Hz, 1H, H4), 4.17–4.12 (*m*, 1H, H3), 2.55–2.37 (*m*, 2H, H8), 2.10 (*s*, 3H, H10), 1.79–1.71 (*m*, 2H, H2a; H7a), 1.70 (*s*, 3H, Me5), 1.59 (*t*, J = 5.1 Hz, 1H, H6), 1.51–1.43 (*m*, 1H, H7b), 1.32 (dd, J = 5.2, 13.7 Hz, 1H, H2b), 0.99 (*s*, 3H, Me1a), 0.86 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 208.69 (C9), 139.17 (C5), 124.53 (C4), 65.38 (C3), 48.56 (C6), 43.95 (C8), 43.79 (C2), 33.39 (C1), 29.97 (C10), 29.74 (Me1a), 24.89 (Me1b), 23.18 (Me5), 22.63 (C7).

Data for 4-hydroxy-7,8-dihydro-β-ionone (27) m/z = 210.1; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 3.87 (*t*, J = 4.5 Hz, 1H, H4), 2.48 (*t*, J = 8.3 Hz, 2H, H8), 2.31–2.18 (*m*, 2H, H7), 2.11 (*s*, 3H, H10), 1.86–1.79 (*m*, 1H, H3a), 1.68 (*s*, 3H, Me5), 1.66–1.54 (*m*, 2H, H2a; H3b), 1.36–1.30 (*m*, 1H, H2b), 0.99 (*s*, 3H,

Me1a), 0.93 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 208.47 (C9), 140.98 (C6), 129.42 (C5), 70.04 (C4), 43.75 (C6), 35.47 (C1), 34.45 (C2), 29.75 (C10), 28.54 (C3), 28.33 (Me1a), 26.83 (Me1b), 22.28 (C7), 16.69 (Me5).

Data for *trans*-3-hydroxy-isomethyl-α-ionone (28) *m/z* = 222.1; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.31 (dd, *J* = 1.3; 10.2 Hz, 1H, H7), 5.56 (*s*, 1H, H4), 4.28–4.22 (*m*, 1H, H3), 2.86 (dd, *J* = 0.5; 11.2 Hz, 1H, H6), 2.28 (*s*, 3H, H10), 1.84 (dd, *J* = 5.7; 13.1 Hz, 1H, H2a), 1.81 (*d*, *J* = 1.4 Hz, 3H, Me8), 1.54 (br *s*, 3H, Me5), 1.39 (dd, *J* = 7.4; 13.1 Hz, 1H, H2b), 0.95 (*s*, 3H, Me1a), 0.84 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 199.74 (C9), 143.06 (C7), 139.41 (C5), 136.09 (C8), 125.58 (C4), 65.54 (C3), 49.94 (C6), 44.77 (C2), 34.86 (C1), 29.59 (Me1a), 25.68 (C10), 23.62 (Me1b), 22.36 (Me5), 11.62 (Me8).

Data for *trans*-3-hydroxy-*n*-methyl-α-ionone (29) *m/z* = 222.1; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.54 (dd, *J* = 10.2; 15.8 Hz, 1H, H7), 6.09 (*d*, *J* = 15.7 Hz, 1H, H8), 5.59 (br *m*, 1H, H4), 4.26–4.21 (*m*, 1H, H3), 2.55 (*q*, *J* = 7.3 Hz, 2H, H10), 2.45 (*d*, *J* = 10.2 Hz, 1H, H6), 1.81 (dd, *J* = 6.2; 13.3 Hz, 1H, H2a), 1.60–1.57 (br *s*, 3H, Me5), 1.37 (dd, *J* = 13.5; 6.4 Hz, 1H, H2b), 1.08 (*t*, *J* = 7.3 Hz, 3H, H11), 0.99 (*s*, 3H, Me1a), 0.85 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 200.60 (C9), 145.76 (C7), 135.51 (C5), 132.48 (C8), 125.71 (C4), 65.46 (C3), 54.26 (C6), 43.87 (C2), 33.83 (C1), 33.49 (C10), 29.28 (Me1a), 24.56 (Me1b), 22.62 (Me5), 8.09 (C11).

Data for *trans*-3-hydroxy-allyl-α-ionone (31) *m/z* = 248.1; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.55 (dd, *J* = 10.2; 15.8 Hz, 1H, H7), 6.10 (*d*, *J* = 15.8 Hz, 1H, H8), 5.86–5.76 (*m*, 1H, H12), 5.59 (br *s*, 1H, H12), 4,99 (ddq, *J* = 1.5; 8.9; 23.7 Hz, 2H, H13), 4.26–4.21 (*m*, 1H, H3), 2.63 (*t*, *J* = 7.7 Hz, 2H, H10), 2.46 (*d*, *J* = 10.0 Hz, 1H, H6), 2.38–2.29 (*m*, 2H, H11), 1.81 (dd, *J* = 6.0; 13.4 Hz, 1H, H2a), 1.59 (*s*, 3 H, Me5), 1.37 (dd, J = 6.5; 13.5 Hz, 1 H, H2b), 0.99 (s, 3 H, Me1a), 0.86 (s, 3 H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 199.26 (C9), 146.24 (C7), 137.13 (C12), 135.49 (C5), 132.66 (C8), 125.69 (C4), 115.26 (C13), 65.49 (C3), 54.26 (C6), 43.82 (C2), 39.36 (C10), 33.86 (C1), 29.28 (Me1a), 28.13 (C11), 24.59 (Me1b), 22.66 (Me5).

Data for 4-hydroxy-allyl-β-ionone (32) *m/z* = 248.1; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.20 (*d*, *J* = 16.3 Hz, 1H, H7) 6.13 (*d*, *J* = 16.3 Hz, 1H, H8), 5.88–5.78 (*m*, 1H, H12), 5.01 (ddq, *J* = 1.3; 10.2; 26.8 Hz, 2H, H13), 4.00 (*t*, *J* = 4.9 Hz, 1H, H4), 2.67 (*t*, *J* = 7.7 Hz, 2H, H10), 2.42–2.36 (*m*, 2H, H11), 1.94–1.86 (*m*, 1H, H3a), 1.82 (*s*, 3H, Me5), 1.74–1.66 (*m*, 2H, H2a; H3b), 1.46–1.40 (*m*, 1H, H2b), 1.04 (*s*, 3H, Me1a), 1.02 (*s*, 3H, Me1b); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 199.57 (C9), 141.78 (C7), 139.61 (C6), 137.17 (C12), 133.66 (C5), 132.04 (C8), 115.28 (C13), 69.94 (C4), 39.83 (C10), 34.65 (C1), 34.56 (C2), 28.83 (C3), 28.25(C11), 28.23(Me1a), 27.43 (Me1b), 18.51 (Me5).

Results

In vitro screening with CYP260B1 and CYP267B1

CYP260B1 and CYP267B1 were screened for their ability to convert the substrates α-ionone (**1**), β-ionone (**2**), α-irone (**3**), dihydro-α-ionone (**4**), dihydro-β-ionone (**5**), α-ionol (**6**), β-ionol (**7**), α-damascone (**8**), β-damascone (**9**), δ-damascone (**10**), β-damascenone (**11**), isomethyl-α-ionone (**12**), *n*-methyl-α-ionone (**13**), and allyl-α-ionone (**14**).The conversions were analyzed by HPLC-DAD as well as GC-MS. **4**–**6** were solely analyzed by GC-MS, due to their low UV absorbance. Both P450s were able to catalyze the conversion of all tested substrates. CYP260B1 converted most of the substrates by 90–95 % under the conditions applied, whereas CYP267B1 converted most of them by 80–95 %, with the exception of damascones (**8**–**10**) and allyl-α-ionone (**14**). These compounds were converted in lower amounts by both P450s (50–75 %). CYP267B1 showed a high selectivity for the formation of a single product (except for **11**) as exemplified in Fig. 1b. The side-products were formed to less than 10 % of the total conversion, except for **10** (~15 %). In contrast, CYP260B1 was able to perform the selective conversions of **2** (including two side products), **9**, and **10** (including one side product), whereas the other CYP260B1-dependent conversions showed multiple products (see Fig. 1a). Nevertheless, the main products of the conversions of **9** and **10** were identical for both P450s. The CYP260B1-dependent conversion of **2** resulted in one main product (~75 %) as well as two side products (~15 % in total). The main product of this conversion showed an absorption maximum shifted to shorter wavelengths indicating the lack of a conjugated double bond. One of the side products had an identical retention time as the main product of the CYP267B1-dependent conversion. GC-MS analyses identified all products as mono-oxidized products, and the database comparison of the fragment patterns showed the highest probabilities for 5,6-epoxy-β-ionone (**15**) as the main product as well as 7,11- epoxymegastigma-5,6-en-9-one (**17**) and 3-hydroxy-β-ionone (**19**) as the side products. 3-Hydroxy-α-ionone (**18**), 3-hydroxy-β-ionone (**19**), and 4-hydroxy-β-ionone (**16**) were available as standards, since these products were characterized during previous studies in our group (Khatri et al. 2010a; Ly et al. 2012). The comparison of the retention times and mass spectra identified the side product of the CYP260B1-dependent conversion of **2**, therefore, as **16**. Further database comparisons of CYP260B1-dependent conversions indicated the most apolar product (~20 %) of the conversion of **12** as the corresponding 4,5-

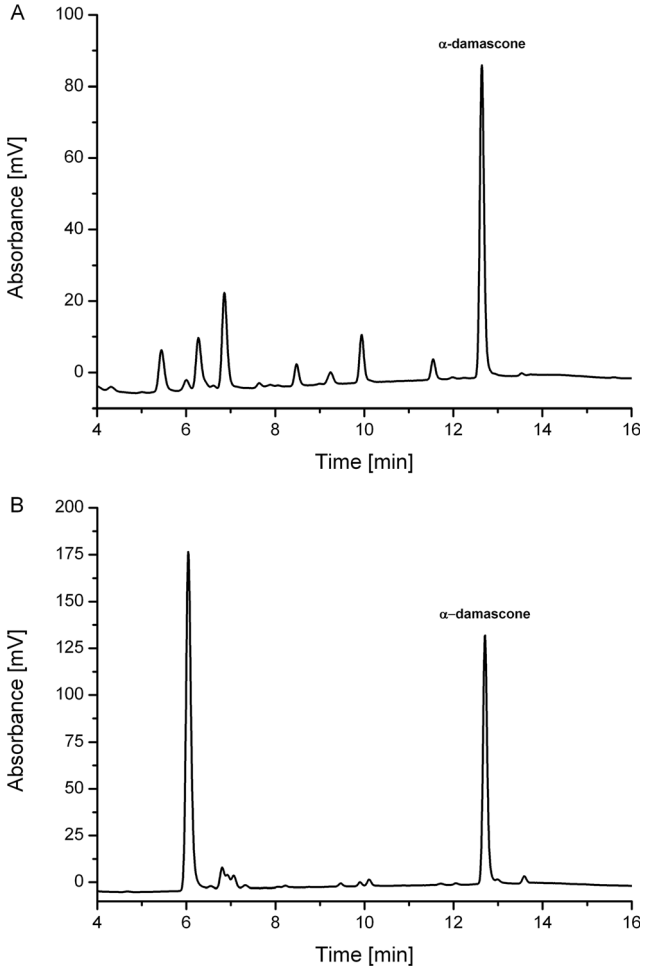


Fig. 1 HPLC chromatograms of in vitro conversions of α-damascone (8) by CYP260B1 (**a**) and CYP267B1 (**b**). CYP260B1 shows an unselective product formation, whereas the CYP267B1-dependent conversion results predominantly in one main product

epoxy isomethyl-α-ionone (**20**). The other products did not show a reliable hit. Likewise, the main products of CYP267B1-dependent conversions were not further characterized by database comparisons. However, the main products of the conversions of **1** and **2** by CYP267B1 were compared with the standards and, therefore, identified as **16** and **18**, respectively.

Whole-cell conversions with CYP260B1 and CYP267B1

Higher amounts of products are required for structure elucidation via NMR spectroscopy. Therefore, an *E. coli*-based whole-cell system harboring the corresponding P450 as well as the electron transfer proteins $Adx_{4\text{-}108}$ and Fpr was applied. **9** and **10** were used in the whole-cell system harboring CYP260B1 to characterize the corresponding products. Additionally, **2**, **12**, and **13** were investigated to verify the epoxidase activity of CYP260B1. The remaining substrates were not further investigated due to the formation of multiple

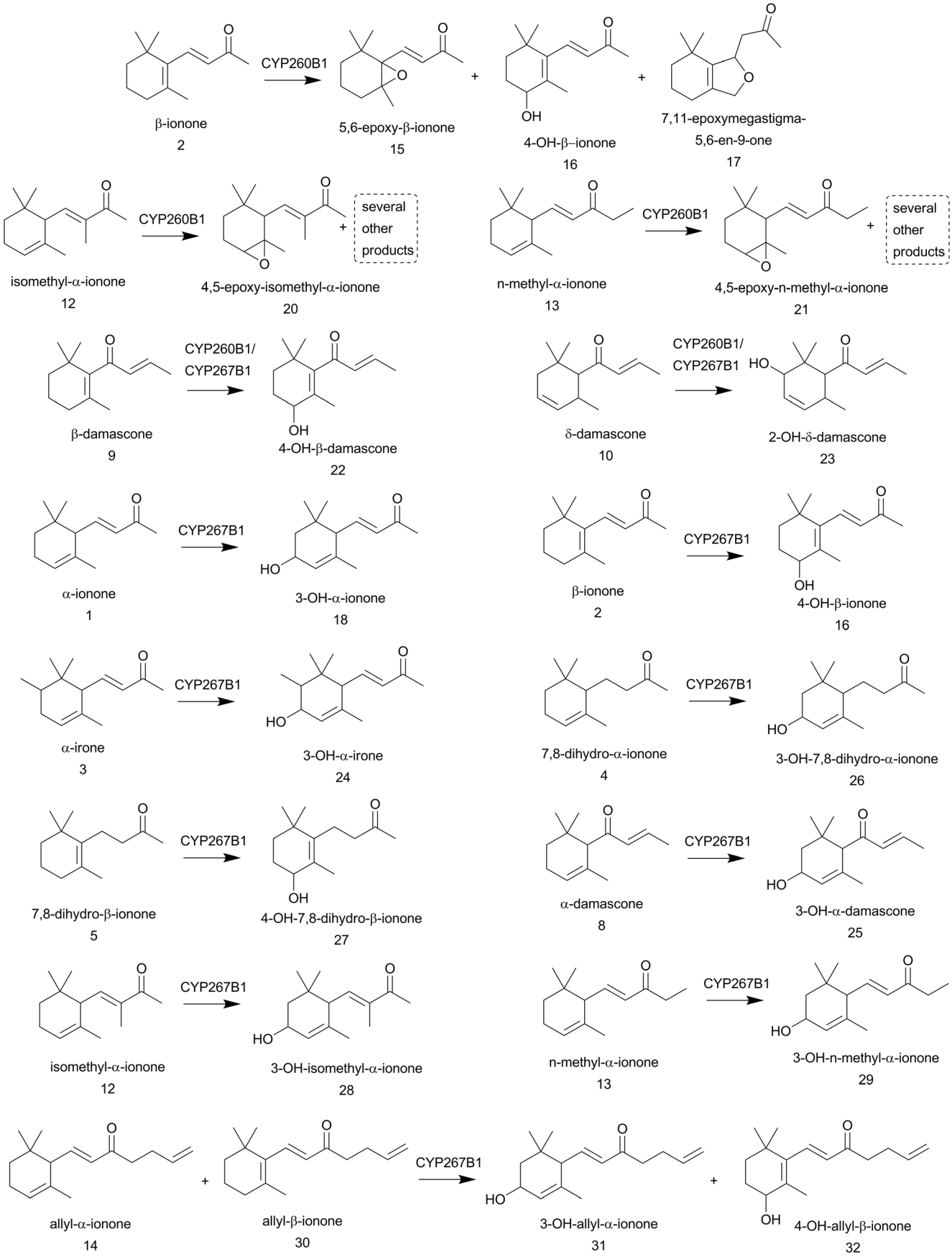
Scheme 2 Structures of the purified products formed by CYP260B1 and CYP267B1. The structures were elucidated by NMR spectroscopy; except for (16) and (18), which were elucidated by GC-MS via standards ▶

products by CYP260B1. In contrast, CYP267B1 showed a high selectivity for the formation of a single product, except for **11** which formed multiple products. In addition, the conversion of **14** resulted in two products. **1** and **2** were not investigated since the corresponding products were elucidated by GC-MS via standards. The other substrates were applied to the whole-cell system to obtain sufficient amounts of products for NMR characterization. **6** and **7** showed different main products during the whole-cell conversions compared with the in vitro reactions. This observation might be explained by the higher reactivity of the ionols caused by their alcohol group. As a result, the formation of such products is P450-independent or an *E. coli* caused consecutive reaction occurred. For that reason, these products were not further characterized. The remaining nine substrates were applied to the whole-cell system and sufficient amounts of products (2–20 mg/l) were obtained after purification. All purified products were analyzed via GC-MS and the mass spectra indicated all compounds as mono-oxidized products.

Product identification via NMR spectroscopy

The structures of the corresponding products were identified by different NMR techniques like ^{1}H, ^{13}C, COSY, HSQC, and HMBC. The main products of **9** and **10** formed by CYP260B1-dependent conversions were identified as compounds hydroxylated in allylic position (**22** and **23**). The conversion of **2** resulted in three products. **16** was elucidated via comparison with a standard and the formation of **15** as well as **17** was verified by NMR analyses. The conversion of the methyl-substituted α-ionone derivatives **12** and **13** led to the 4,5-epoxy products **20** and **21** proving an epoxidase activity of CYP260B1 for α-ionone derivatives. The other products of the CYP260B1-dependent conversions were not further characterized. The CYP267B1-dependent conversions resulted predominantly in a single main product. All products formed by this enzyme were identified as allylic alcohols (**16**, **18**, **22**–**29**, **31**, **32**). The structures of the products are illustrated in Scheme 2.

However, the conversion of **14** resulted in two products. The product obtained in higher amounts was identified as 3-hydroxy-allyl-α-ionone (**31**), whereas the side product was identified as 4-hydroxy-allyl-β-ionone (**32**). This observation can be explained by the composition of the substrate. Allyl-β-ionone (**30**) is mentioned as major impurity (13 %) by Sigma Aldrich and, therefore, served as potential substrate for CYP267B1 in our experiments. As shown for the other substrates, **30** was hydroxylated in allylic position at C4.

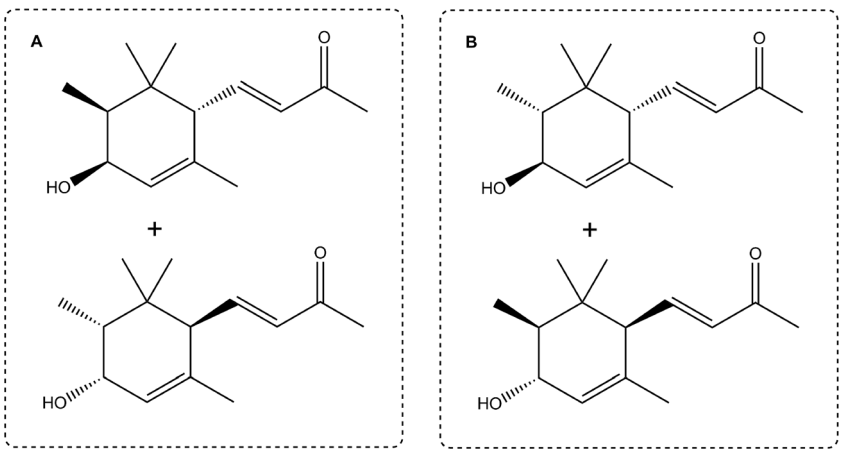

CYP260B1
β-ionone
2
5,6-epoxy-β-ionone
15
4-OH-β–ionone
16
7,11-epoxymegastigma-5,6-en-9-one
17
isomethyl-α-ionone
12
several other products
4,5-epoxy-isomethyl-α-ionone
20
n-methyl-α-ionone
13
4,5-epoxy-n-methyl-α-ionone
21
CYP260B1/ CYP267B1
β-damascone
9
4-OH-β-damascone
22
δ-damascone
10
2-OH-δ-damascone
23
CYP267B1
α-ionone
1
3-OH-α-ionone
18
4-OH-β-ionone
16
α-irone
3
3-OH-α-irone
24
7,8-dihydro-α-ionone
4
3-OH-7,8-dihydro-α-ionone
26
7,8-dihydro-β-ionone
5
4-OH-7,8-dihydro-β-ionone
27
α-damascone
8
3-OH-α-damascone
25
isomethyl-α-ionone
12
3-OH-isomethyl-α-ionone
28
n-methyl-α-ionone
13
3-OH-n-methyl-α-ionone
29
allyl-α-ionone
14
allyl-β-ionone
30
3-OH-allyl-α-ionone
31
4-OH-allyl-β-ionone
32

The *cis*/*trans*-geometry of known products was elucidated by comparison with published data (Barakat et al. 2008; Schwab and Schreier 1991; Yamazaki et al. 1988), whereas those products where no data are available were elucidated by NOESY experiments. The epoxides **20** and **21** produced by CYP260B1 show *trans*-geometry between Me5 and the corresponding side chain at position C6. Moreover, the CYP260B1-dependent conversion of **10** resulted in the 2-hydroxy-*trans*-product (**23**). CYP267B1 showed a high regio- and diastereoselectivity for such compounds, since all 3-hydroxy-α-products formed by this enzyme were identified as *trans*-isomers. Likewise, **23** resulted in a *trans*--configuration as shown for CYP260B1. Interestingly, none of the substrates led to *cis*-configurated products despite their application as racemic mixtures.

HPLC and GC analyses of pure α-irone (**3**) showed two peaks which represent the diastereomers of this compound (methyl at C2 and butenone at C6). The conversion of **3** by CYP267B1 resulted in two main products despite the introduction of a third stereogenic center caused by the hydroxylation at position C3. These two compounds were not successfully separated; nevertheless, the corresponding structures were identified by 2D-NMR techniques and their data matched those in the literature (Yamazaki et al. 1986). As shown in Scheme 3, one product (**24a**) shows *cis*-geometry between H2 and H3, whereas the other product (**24b**) shows *trans*-geometry. However, both compounds show *trans*-geometry between H3 and H6 verifying the high diastereoselectivity of CYP267B1 for the formation of 3,6-*trans*-products.

Discussion

Since a couple of years, we are investigating the functions, properties, and activities of P450s from *Sorangium cellulosum* So ce56. An efficient *E. coli*-based whole-cell system for some of these P450s was established (Ringle et al. 2013), and several substrate classes like terpenes, drugs, or fatty acids were already identified (Khatri et al. 2015; Litzenburger et al. 2015; Schifrin et al. 2015). Likewise, α- and β-ionone (**1**, **2**) serve as substrates for CYP109D1 and CYP264B1 (Khatri et al. 2010a; Ly et al. 2012). In contrast, about potential substrates for CYP260B1 and CYP267B1, which are members of new P450 families found in this myxobacterium, only little is known. However, knowledge about substrates is necessary to characterize these enzymes in more detail. For that reason, we screened their ability to convert carotenoid-derived aroma compounds like ionones, damascones, as well as some of their isomers and derivatives (**1**–**14**). As shown here, both P450s are capable of the efficient in vitro conversions of all tested substrates. CYP267B1 shows a high selectivity towards the formation of a single product, whereas CYP260B1 mainly forms multiple products. Furthermore, based on our whole-cell experiments, we were able to identify several main products formed by these P450s. CYP267B1 is mostly responsible for the regioselective hydroxylation in allylic position. Likewise, CYP260B1 hydroxylates **9** and **10** as well as small quantities of **2** in this specific position. The formation of allylic alcohols is preferred by many P450s as shown by several studies (Celik et al. 2005; Hall and Bell 2015; Khatri et al. 2010a; Ly et al. 2012; Ma et al. 2011; Venkataraman et al. 2012; Zhang et al. 2015). This observation can be explained by the bond strength of the C–H bonds. The bond strength of the allylic C–H bond is about 10 kcal/mol lower than that of a secondary C–H bond (Ortiz de Montellano 2010). As a result, the allylic position is more reactive and, therefore, preferred for hydroxylation. Moreover, free energy-related calculations of the stereoselective hydroxylation of α-ionone (**1**) by P450 BM3 mutants revealed that the *trans*-isomers show the most favorable free energies in solution (de Beer et al. 2012). As shown here, CYP267B1 predominantly forms such energetically preferred diastereomers, despite the application of racemic mixtures as substrate. Previous studies of the conversion of **1** by *Streptomyces* strains also revealed the formation of *trans*-isomers out of a racemic mixture (Lutz-Wahl et al.



Scheme 3 Relative geometry of the 3-hydroxy-α-irone main products 24a (**a**, *left side*) and 24b (**b**, *right side*) formed by CYP267B1

1998). In contrast, several other bacterial P450s produce mixtures of *cis*- and *trans*-isomers (Hall and Bell 2015; Ma et al. 2011; Venkataraman et al. 2012). The selectivity for the production of a specific isomer is, therefore, considerably lower. As a result, CYP267B1 is a suitable candidate for the diastereoselective production of several norisoprenoids.

Interestingly, during all previous studies published in the literature, only **1**, **2**, and **9** were applied as substrates for P450s, and none of their isomers or derivatives was investigated. Both P450s investigated in this study were able to convert all tested substrates in vitro. Thereby, the selectivity of CYP260B1 towards a single product is lower compared with CYP267B1. The formation of multiple compounds hinders, therefore, the product characterization. Nevertheless, **2**, **9**, and **10** are converted by CYP260B1 with high selectivity, whereby the main products of **9** and **10** are identical to those obtained with CYP267B1. In addition, CYP260B1 shows an epoxidase activity towards **2** as well as the methyl-substituted α-ionone derivatives **12** and **13**, albeit in lower amounts. CYP260B1 hydroxylates **9** in the allylic position at C4, whereas **2** is mainly epoxidized between C5 and C6. This shows that the keto-group either at position C7 (damascones) or C9 (ionones) seems to alter the substrate orientation in the active site of this enzyme and, hence, the regioselectivity as well as the type of reaction. In addition, the location of the double bond in the cyclohexene ring has also a high impact on the product formation. The conversions of **9** and **10** by CYP260B1 lead predominantly to single products of allylic alcohols, whereas the conversion of **8** resulted in multiple products. The location of the double bond is thereby responsible for the conformation of the cyclohexene ring in the active site, and this way affects the hydroxylation selectivities. This sensitivity towards the moieties of these compounds might be explained by a low flexibility of the active site. On the contrary, CYP267B1 selectively hydroxylates all tested substrates in allylic position. Its active site might be more flexible and allows the required substrate orientation for allylic hydroxylation. In addition, the catalyzed reactions seem to be thermodynamically controlled, since predominantly 3,6-*trans*-products were formed. The conversion of **3** is an interesting example which substantiates this assumption due to the formation of the thermodynamically most stable products (**24a** and **24b**). A more detailed assertion about the conformation of the active site or substrate orientation can only be given after the crystal data of these enzymes become available. Furthermore, improved analytical methods to elucidate the absolute configuration of the products as well as computational methods to estimate the binding affinities are necessary to understand the detailed mechanistic of the stereoselectivity. Nevertheless, this study gives first insights in the substrate range as well as variety of reactions catalyzed by theses P450s. CYP267B1 shows the typical for P450s allylic hydroxylase activity, whereas CYP260B1 is additionally able to epoxidize some of the substrates. This epoxidase activity is of interest, since neither CYP109D1 nor CYP264B1 or CYP267B1 from So ce56 are known for such an activity. Hence, CYP260B1 expands the "toolbox" towards oxyfunctionalized norisoprenoids, a group of valuable compounds known for their wide range of applications.

Hydroxylated and epoxidized norisoprenoids are utilized as building blocks for the organic chemistry, like for the syntheses of odorants (Fráter et al. 1998) or carotenoids (Brenna et al. 2002) and their derivatives (Tu et al. 2014). Furthermore, they are investigated for their potential medical applications (Gerhäuser et al. 2009) or used as precursors for pharmaceuticals (Colombo et al. 1992; Zhou et al. 2009). There are several studies indicating the potential growth inhibiting and allelopathic properties of such compounds, especially those with a keto- or hydroxyl-function at position C3 (Walter and Strack 2011). Aside from these examples, there are several other possible applications for these products including their utilization as standard (Venkataraman et al. 2012). This wide range of applications proves the significance of such products for diverse fields of research and novel compounds with altered properties are very valuable and might provide new possibilities for application. During our studies, we were able to identify several known as well as some novel products like **23**, **31**, and **32**. These novel compounds might be investigated for their olfactory properties or used as standards to simplify the product characterization of other P450s. However, higher amounts of these products in a multimillgram to gram scale are required to apply them as building blocks for synthetic routes. The whole-cell system used in this study yielded up to 20 mg/l under non-optimized conditions. Improvements, such as the utilization of alternative redox partners, applying resting cells in buffer or protein engineering towards higher activities can be used to establish a system towards preparative scale.

Taken together, the carotenoid-derived aroma compounds were identified as substrates for CYP260B1 and CYP267B1 for the first time. The utilization of a whole-cell system facilitates the product identification via NMR spectroscopy and provides, therefore, first insights in the substrate range and reaction diversity of these myxobacterial P450s. The high regio- and diastereoselectivity of CYP267B1 as well as the epoxidase activity of CYP260B1 reveal the potential of these P450s for the oxyfunctionalization of norisoprenoids.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

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