Metabolism of Δ^3-Carene by Human Cytochrome P450 Enzymes: Identification and Characterization of Two New Metabolites

Mike Duisken[a], Dorina Benz[a], Thomas H. Peiffer[b], Brunhilde Blömeke[c] and Juliane Hollender[a,*]

[a]*Institute of Hygiene and Environmental Medicine, RWTH Aachen, Pauwelsstr. 30, 52074 Aachen, Germany,* [b] *Institute for Organic Chemistry, RWTH Aachen, Professor-Pirlet-Str. 1, 52074 Aachen, Germany and* [c]*Department of Ecotoxicology and Toxicology, Am Wissenschaftspark 25-27, 54296 Trier, Germany*

Abstract: The metabolism of the bicyclic monoterpene Δ^3-carene was investigated *in vitro* using human liver microsomes as well as human smoker/non-smoker lung microsomes and 12 different recombinant cytochrome P450 enzymes coexpressed with human CYP-reductase in *Escherichia coli* cells. We detected two metabolites using GC-MS analysis. The mass fragmentation indicated for one metabolite hydroxylation in the allyl position and for the other metabolite epoxidation at the double bond. For clear identification the suggested metabolites were synthesized in a four-step reaction. Comparison of GC retention times and mass spectra lead to the identification of the metabolites as Δ^3-carene-10-ol ((1*S*, 6*R*)-7,7-Dimethylbicyclo[4.1.0]hept-3-en-3-yl-methanol) and Δ^3-carene-epoxide ((1*S*, 3*S*, 5*R*, 7*R*)-3,8,8-Trimethyl-4-oxatricyclo[5.1.0.0^{3,5}]octane). Δ^3-carene-10-ol was formed by human liver microsomes and recombinant human CYP2B6, CYP2C19 and CYP2D6. Δ^3-Carene-epoxide was obviously catalyzed only by CYP1A2. In both cases there was a clear correlation between the metabolite formation, incubation time and enzyme concentration, respectively. Further kinetic analysis revealed that CYP2B6 exhibited the highest activity for Δ^3-carene 10-hydroxylation. *Michaelis-Menten* K_m and V_{max} for oxidation of Δ^3-carene were 0.6 mM and 28.4 nmol/min/nmol P450 using human CYP2B6. For the formation of Δ^3-carene-epoxide 98.2 mM and 3.9 nmol/min/nmol P450 were determined as K_m and V_{max} by using human CYP1A2. To our knowledge, this is the first time that Δ^3-carene-10-ol and Δ^3-carene-epoxide are described as human metabolites of Δ^3-carene.

Key Words: Metabolism, Δ^3-Carene, Cytochrome P450, Δ^3-Carene-10-ol, Δ^3-Carene-epoxide.

INTRODUCTION

The bicyclic monoterpene Δ^3-carene is a component of oleoresin which naturally occurs in pine trees and certain bushes. Distillation of oleoresin leads to a volatile oily fraction, namely turpentine. The major substances are α-pinene, β-pinene and Δ^3-carene with yields varying with origin, season and species. Turpentine was used widely as a solvent in industrial and nonindustrial environments, but hydrocarbon solvents have recently replaced its use. Although occupational exposure to turpentine has declined, monoterpenes are released from different wood processes in mg/m^3-concentrations [1-3]. In the wood industry monoterpenes are thought to be one of the causative agents for irritation symptoms [4]. Subjects exposed to turpentine or Δ^3-carene in an exposure chamber experienced discomfort in the throat and airways and airway resistance was increased at the end of turpentine exposure [5, 6].

In addition, some monoterpenes irritate skin and mucous membranes and can be causes of allergic and nonallergic contact dermatitis [4]. Effects of low Δ^3-carene concentrations were examined *in vitro* on alveolar macrophages finding a clear dose-dependent relationship for the cell viability [7].

*Address correspondence to this author at the Environmental Chemistry, Eawag, Überlandstrasse 133, CH-8600 Dübendorf, Switzerland; Tel: +41 448235487; E-mail: Juliane.Hollender@eawag.ch

It is not clear, whether Δ^3-carene itself, photo-oxidation products, chemical oxidation products or mammalian metabolites cause the observable symptoms. Hellerström *et al*. [8, 9] supposed that the eczematogenic component is attached to photo- or chemical oxidation products of Δ^3-carene such as hydroperoxides. Examples of the thermal degradation products of Δ^3-carene are 4-hydroxy-2-methyl-2-cyclohexanone, p-cymene, 3-carene-2-one and 3-carene oxid [10].

Little is known about the metabolism of Δ^3-carene in mammalians. The toxicokinetics of Δ^3-carene were studied in human volunteers exposed by inhalation. The mean uptake determined by blood analysis was 68 % while less than 0.001% was eliminated unchanged *via* urine and approximately 3 % *via* the lungs [5]. This indicates that Δ^3-carene is readily metabolized but no metabolites have been identified in humans until now. However, after feeding rabbits with Δ^3-carene urinary metabolites such as 3-caren-9-ol, 3-caren-9-carboxylic, 3-caren-9,10-dicarboxylic, chamic acid and 3-caren-10-ol-9-carboxylic acid were reported [11]. Previous studies have demonstrated that several terpenes, such as 1,4-cineole, 1,8-cineole, and (+)- and (-)-limonenes are catalyzed in rat and human liver by cytochrome P450 enzymes (CYPs) to their respective oxidation products [12-16]. These metabolites could also be detected in the urine of exposed persons [16-18].

In order to identify metabolites which might contribute to the adverse effects of Δ^3-carene, in this study we investigated the human metabolism of Δ^3-carene *in vitro* with human lung microsomes, human liver microsomes and 12 different hu-

1389-2002/05 $50.00+.00

man recombinant CYPs and determined which P450 species are the major enzymes for this metabolism. Identification of the metabolites was confirmed by chemical synthesis.

METHODS

Chemicals

Δ^3-carene was purchased from Fluka (Buchs, CH) and was used without further purification. The purity of this compound found to be >99% based on GC-MS analysis. $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Roche (Basel, CH). n-Butyllithium was purchased as 15% solution in n-hexane from Merck (Darmstadt, Germany). Other reagents and chemicals were of the highest qualities commercially available. The synthesis of Δ^3-caren-10-ol is described later**.** All solvents were reagent grade or dried before use.

Enzymes

Human liver microsomes and human lung microsomes were purchased from Xenotech (Kansas, USA). They are prepared from at least 30 donors. Recombinant human CYP's and human CYP-reductase coexpressed in *Escherichia coli* were obtained from Cypex (Dundee, UK); the P450 amounts in the assays were chosen as recommended by the manufacturer.

GC/MS Analysis for Product Formation

A Hewlett-Packard model 5860 Series II (Waldbronn, Germany) equipped with a Gerstel programmable temperature vaporizer (PTV)/ Gerstel MPS large volume sampler (CIS 3, Gerstel, Mühlheim a. d. Ruhr, Germany) was combined with direct coupling to a Hewlett-Packard 5972 mass spectrometer. The metabolites were separated by a RTX-5SIL MS (Restek, Bad Homburg, Germany) silica capillary column (0.28 mm × 30 m, 0.25µm film thickness) using helium (at 0.7 ml/min) as carrier gas. The column temperature was increased from 37 to 200 °C at a rate of 6°C/min and from 200 to 330°C at a rate of 15°C/min. Forty µl aliquots were injected (injection speed 29 µl/min) into the liner of the PTV containing silanized glass wool (93 mm ×1 mm I.D., Gerstel) in the solvent vent mode with stop flow (vent flow, 200 ml /min helium). After purging of the organic solvent over 0.5 min at 20°C and further 0.5 min isotherm the injector was heated at 600°C/min to 300°C and was kept 2 min isotherm in the split less mode. The GC-MS transfer line was held at 300°C resulting in an ion source temperature of 180°C, a quadrupole temperature of 180°C and an ionization voltage of 70 eV. For detection the scan mode (45-300 amu) was used [17]. The values of metabolite formation products were determined by external calibration with the synthesized products.

Analysis of the Synthesis Products

For GC a Varian model CP 3800 was used. The injector temperature was held at 280 °C and the FID temperature was set at 300 °C. A Sil-8 (fused silica, 25 m x 0.25 mm ID) capillary column was used. ^{1}H NMR spectra were recorded as indicated on a Varian Mercury 300 (300 MHz) or a Varian Inova (400 MHz). ^{13}C NMR spectra were recorded at 75 MHz or 100 MHz as indicated. The IR spectra were recorded on a Perkin Elmer FT/IR 1760. Mass spectra were recorded on a Finnigan SSQ 7000. For high resolution mass spectra a Finnigan MAT 95 was used. Furthermore all yields are for purified compounds. All reactions were performed under an inert atmosphere (dry argon).

Oxidation of Δ^3-Carene by Human Liver Microsomes

Oxidation of Δ^3-carene by human liver microsomes was determined as follows. Ten µl Δ^3-carene were dissolved in 10 ml potassium phosphate buffer (100 mM, pH 7.2) and stirred vigorously before use. Standard reaction mixtures containing human liver microsomes (0.4 mg of protein/ml) and 80 µM Δ^3-carene in a final volume of 500 µl potassium phosphate buffer (100 mM, pH 7.2) with a NADPH-generating system (0.1 mM $NADP^+$, 0.8 mM glucose-6-phosphate and 1.0 units of glucose-6-phosphate dehydrogenase/ml) were used. Incubations were carried out at 37°C for various time points and the reaction was terminated by the addition of 1.0 ml ethylacetate. After centrifugation for 10 min at 20800 g (Eppendorf Centrifuge, Hamburg, Germany) the recovered organic phase was used for GC-MS analysis.

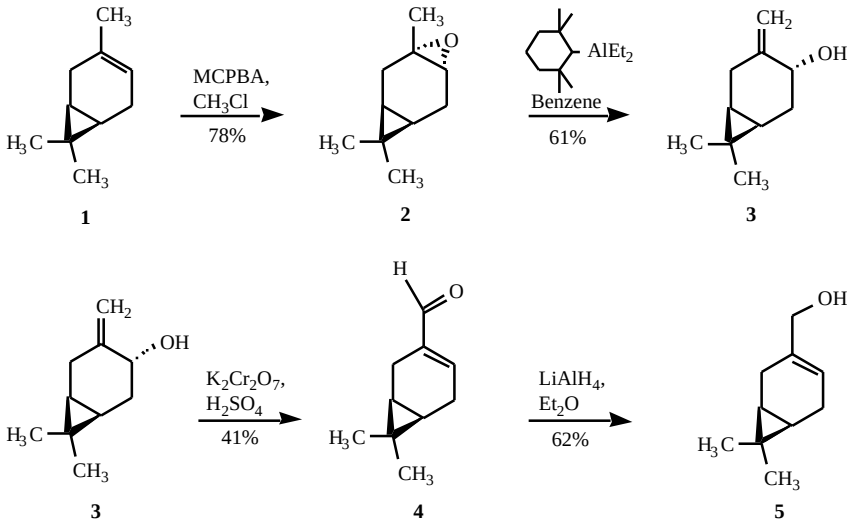
The oxidation of Δ^3-carene with human lung microsomes and the different human recombinant CYPs was performed as described above.

Synthesis of Δ^3-Carene-10-ol and Δ^3-Carene-Epoxide

Δ^3-carene-10-ol (5) and Δ^3-carene-epoxide (2), the metabolites of Δ^3-carene were not commercially available. Therefore, we synthesized both within four steps starting from Δ^3-carene (Scheme (**1**)). The synthesis route is described here for the first time with full identification of the intermediates. The overall yield is 12%, but reaction conditions were not optimized.

*(+)-(1**S**, 3**S**, 5**R**, 7**R**)-3,8,8-Trimethyl-4-oxa-tricyclo [5.1.0.0^{3,5}]octane (Δ^3-carene-epoxide) (2)*

In accordance with the procedure of Brown and Suzuki [19], 6.40 g (50 mmol) of Δ^3-carene **1** in 100 ml $CHCl_3$ was epoxidized with 13.32 g (54 mmol) m-chloroperbenzoic acid in 140 ml $CHCl_3$. Distillation provided 5.95g (78%) of **2** as colorless oil: $[\alpha]_D^{-}$ = +10.0, (c = 1.03, $CHCl_3$); **DC:** R_f = 0.45 (Et_2O:*n*-pentane, 1:5); **GC:** R_t = 4.05 min (Sil-8, 80-10-300); **bp:** 88-92 °C (p = 22 mbar); **^{1}H NMR** (300 MHz, $CDCl_3$)**:** δ = 0.40-0.56 (m, 2H, H-4,H-3), 0.73 (s, 3H, H-9), 1.01 (s, 3H, H-8), 1.26 (s, 3H, H-10), 1.49 (dd, *J* = 16.33 Hz, *J* = 2.24 Hz, 1H, H-2), 1.65 (dt, *J* = 16.32 Hz, *J* = 2.23 Hz 1H, H-5), 2.14 (dd, *J* = 16.08 Hz, *J* = 8.91 Hz, 1H, H-2´), 2.30 (ddd, *J* = 16.32 Hz, *J* = 8.90 Hz, *J* = 1.98 Hz, 1H, H-5´), 2.83 (d, 1H, *J* = 1.98 Hz, H-6) ppm; **^{13}C NMR** (75 MHz, $CDCl_3$)**:** δ = 13.86 (C-3), 14.60 (C-9), 16.00 (C-4), 19.22 (C-5), 23.09 (C-10), 23.34 (C-2), 27.75 (C-8), 55.85 (C-1), 58.17 (C-6) ppm; **IR** (kapillar)**:** $\tilde{\nu}$ = 2917 (vs), 2734 (w), 1713 (w), 1446 (s), 1378 (m), 1302 (w), 1208 (m), 1138 (w), 1066 (m), 1030 (w), 994 (w), 942 (w), 841 (s), 808 (w), 761 (w), 728 (w), 669 (w), 579 (w), 474 (w) cm^{-1}; **MS** (EI, 70 eV)**:** *m/z* [%] = 152 ($M^{+\cdot}$, 20), 137 (60), 134 (21), 123 (23), 119(69), 110 (15), 109 (100), 108 (11), 107 (11), 105 (13), 95 (26), 94(26), 93 (30), 92 (15), 91 (49), 82 (21), 81 (50), 79 (41), 77 (28), 74 (30), 68 (16), 67 (80), 65 (17), 59 (49), 55 (12), 53 (31), 51 (31), 45 (26).



Scheme (1). Synthesis of Δ³-carene-10-ol in a four-step reaction starting from Δ³-carene.

Anal. Calc. for $C_{10}H_{16}O$ (152.12); C, 78.90; H, 10.59. Found: C, 78.69; H, 9.80.

(−)-(1R, 3R, 6S)-4-Methylen-7,7-diimethylbicyclo[4.1.0] heptan-3-ol (3)

n-Butylithium (41.6 ml of 1.6 M in hexane, 66.5 mmol) was added to a solution of 2,2,6,6-tetramethylpiperidine (11.3 ml, 66.6 mmol) and benzene (50 ml) at 0°C. After 20 min a solution of diethylaluminium chloride (68.3 ml of 1 M in hexane, 68.3 mmol) was introduced and stirred for 30 min at 0°C. A cooled solution of **2** (5.05 g, 33.2 mmol) in 15 ml abs. benzene was added slowly within 30 min and stirred for another 30 min. The reaction mixture was quenched with 10% hydrochloric acid (150 ml) and extracted with ether (3 x 100 ml) [20]. The combined organic phases were dried and evaporated to provide brown oil. Flash chromatography (ether:n-pentane 1:5) provides 3.09 g (61%) of colorless crystals. $[\alpha]_D^{..}$ = −113.2 (c = 1.01, $CHCl_3$)**; DC:** R_f = 0.52 (Et_2O:*n*-Pentan, 1:2); **GC:** R_t = 4.98 min (Sil-8, 80-10-300); **bp.:** 52 °C; **^{1}H NMR** (300 MHz, $CDCl_3$)**:** δ = 0.70 (m, 1H, H-4), 0.81 (m, 1H, H-3), 0.89 (s, 3H, H-9), 1.02 (s, 3H, H-8), 1.55 (m, 1H, H-5), 2.00 (s, 1H, OH), 2.21 (m, 1H, H-5´), 2.26 (m, 1H, H-2), 2.76 (m, 1H, H-2´), 4.09 (m, 1H, H-6), 4.75 (m, 1H, C-10), 4.82 (m, 1H, C-10´) ppm; **^{13}C NMR** (75 MHz, $CDCl_3$)**:** δ = 14.28 (C-9), 15.51 (C-4), 18.11 (C-7), 20.57 (C-3), 24.72 (C-2), 28.67 (C-8), 28.89 (C-5), 70.95 (C-6), 109.12 (C-10), 149.32 (C-1) ppm; **IR** (KBr)**:** ν̃ = 3857 (w), 3840 (w), 3753 (w), 3713 (w), 3677 (w), 3653 (m), 3631 (m), 3324 (vs), 3072 (m), 3011 (s), 2980 (s), 2941 (vs), 1793 (w), 1650 (m), 1433 (s), 1404 (m), 1351 (w), 1298 (s), 1245 (m), 1209 (w), 1178 (m), 1132 (m), 1101 (w), 1070 (m), 1042 (vs), 993 (m), 966 (w), 934 (m), 892 (s), 871 (vs), 798 (w), 758 (m), 711 (m), 678 (m), 540 (m), 508 (w), 460 (w) cm^{-1}; **MS** (EI, 70 eV)**:** *m/z* [%] = 152 ($M^{+\cdot}$, 7), 137 (23), 134 (47), 123 (8), 119 (43), 110 (8), 109 (54), 107 (6), 105 (15), 96 (14), 95 (48), 94 (8), 93 (26), 92 (100), 91 (76), 85 (8), 83 (50), 82 (15), 81 (40), 79 (39), 77 (21), 70 (25), 69 (33), 67 (40), 65 (14), 56 (8), 55 (94), 53 (29), 51 (23).

Anal. Calc for $C_{10}H_{16}O$ (152.120): C, 78.90; H, 10.59. Found: C, 78.96%; H, 10.68.

(−)-(1S, 6R)-7,7-Dimethylbicyclo[4.1.0]hept-3-en-3-carboxaldehyd (4)

In accordance with the procedure of Gollnick and Schade [21], a solution of 2.50 g (16.4 mmol) **3** was treated with a solution of 5.33 g $K_2Cr_2O_7$, 10 ml H_2O and 5.07 g H_2SO_4 and stirred for 2 h at 60 °C. After extraction with ether, evaporating and purification by flash chromatography (ether:n-pentane 1:10) the yield of the colorless oil **4** was 41 % (1.01 g). $[\alpha]_D^{..}$ = −28.4 (c = 1.02, $CHCl_3$)**; DC:** R_f = 0.50 (Et_2O:*n*-Pentan, 1:10), **GC:** R_t = 12.19 min (Sil-8-CB, 50/ 4 min.iso-10-260); **^{1}H NMR** (400 MHz, $CDCl_3$)**:** δ = 0.70 (s, 3H, H-9), 0.77-0.87 (m, 2H, H-4,H-3), 1.07 (s, 3H, H-8), 2.12-2.20 (m, 1H, H-2), 2.27-2.36 (m, 1H, H-5), 2.44-2.54 (m, 1H, H-2'), 2.65-2.75 (m, 1H, H-5'), 6.70-6.74 (m, 1H, H-6), 9.40 (s, 1H, H-10) ppm; **^{13}C NMR** (100 MHz, $CDCl_3$)**:** δ = 13.44 (C-9), 16.56 (C-5), 17.08 (C-4), 17.61 (C-3), 17.68 (C-7), 22.42 (C-2), 28.14 (C-8), 139.64 (C-1), 150.25 (C-6), 193.71 (C-10) ppm; **IR-Spectrum** (kapillar)**:** ν̃ = 3000 (m), 2941 (m), 2873 (m), 2814 (m), 2709 (w), 1685 (vs), 1653 (s), 1420 (m), 1384 (w), 806 (w), 746 (m) cm^{-1}; **MS** (EI, 70 eV)**:** *m/z* [%] = 150 ($M^{+\cdot}$, 5), 135 (9), 121 (19), 108 (17), 107 (100), 106 (8), 105 (17), 93 (11), 91 (25), 80 (6), 79 (65), 78 (6), 77 (28), 67 (9), 66 (8), 65 (6), 55 (5), 53 (12), 51 (9).

Anal. Calc for $C_{10}H_{14}O$ (150.10): C, 79.96; H, 9.39. Found: C, 79.87%; H, 9.36.

(+)-(1R, 6S)-7,7-Dimethylbicyclo[4.1.0]hept-3-en-3-yl-methanol (Δ³-carene-10-ol) (5)

A solution of 0.60 g (3.99 mmol) Carenal **4** in 5 ml abs. Et_2O was added to a suspension of 0.24 g (6.3 mmol) lithium aluminium hydrid in 40 ml abs. Et_2O at 0 °C. The reaction mixture was stirred for 60 min at room temperature. After addition of 10 ml buffer (pH 7) the aqueous phase was extracted with dichloromethane (3x20 ml) [21]. The combined organic phases were dried and evaporated. Flash chromatography (ether:n-pentane 1:4) provided 62 % (0.375 g) of a colorless oil **5**. $[\alpha]_D^{..}$ = + 6.73 (c = 0.99, $CHCl_3$) **; DC:** R_f = 0.38 (Et_2O:*n*-Pentan, 1:2) ; **GC:** R_t = 7.91 min (Sil-8, 60-10-300); **^{1}H NMR** (300 MHz, C_6D_6)**:** δ = 0.56 (m, 1H, H-

4), 0.69 (m, 1H, H-3), 0.79 (s, 3H, H-9), 0.99 (s, 3H, H-8), 1.93, 2.26 (kB, 4H, H-2, H-5), 2.65 (s, 1H, OH), 3.83 (s, 2H, H-10), 5.47 (m, 1H, H-6) ppm; **^{13}C NMR** (75 MHz, C_6D_6)**:** δ = 13.51 (C-9), 17.05 (C-7), 17.60 (C-4), 18.38 (C-3), 20.72, (C-5), 20.78 (C-2), 28.52 (C-8), 67.38 (C-10), 120.93 (C-6), 135.84 (C-1) ppm; **IR-Spectrum** (kapillar): $\tilde{\nu}$ = 3327 (vs), 2997 (s), 2868 (vs), 2829 (s), 2734 (w), 1681 (w), 1432 (s), 1377 (m), 1215 (w), 1142 (m), 1036 (s), 1000 (s), 941 (m), 827 (w), 789 (m), 723 (w), 564 (w) cm^{-1}; **MS** (EI, 70 eV)**:** *m/z* [%] = 152 ($M^{+\cdot}$, 12), 137 (5), 134 (9), 121 (33), 120 (9), 119 (22), 109 (11), 108 (18), 107 (13), 105 (14), 96 (5), 95 (11), 94 (5), 93 (26), 92 (20), 91 (59), 85 (5), 83 (5), 91 (12), 80 (9), 79 (100), 78 (7), 77 (23), 69 (8), 67 (20), 66 (5), 65 (8), 55 (16), 53 (11), 51 (7).

Anal. Calc for $C_{10}H_{16}O$ (152.12): C, 78.90; H, 10.59. Found: C, 78.96%; H, 10.72.

MS m/z ($C_{10}H_{16}O$): calc.: 152.12012; obsd: 152.12013

RESULTS

Identification of the Hydroxylated Metabolite of Δ^3-Carene Formed by Human Liver Microsomes

Initially, the metabolism of Δ^3-carene was examined by human liver microsomes in the presence of a NADPH-generating system. After incubation and extracting with ethylacetate as described in the *Methods* section only one metabolite in the GC-MS chromatograms was detected (Fig. (**1**)). The mass spectrum of this metabolite was not found in the library (NBS75K, Hewlett-Packard). Compared to Δ^3-carene the molecular mass of the metabolite was increased from 136 to 152 indicating hydroxylation. The dehydration peak (134, $[M\text{-}H_2O]^+$) confirmed that a hydroxyl group was introduced into the molecule of Δ^3-carene. The mass fragment 108 can be explained as $[M\text{-}C_3H_7]^+$, which does not exist for Δ^3-carene. The base peak with m/z 79 can be formed by loss of $\text{-}CH_2OH$ and $\text{-}C_3H_6$. Based on the observed fragmentation pattern we postulated hydroxylation in the allyl position at the free methyl group forming Δ^3-carene-10-ol (5). The fragmentation and the retention time of the gas chromatographic analysis was in complete agreement with those of Δ^3-carene-10-ol synthesized as described in the methods.

Kinetic Analysis of Δ^3-Carene 10-Hydroxylation by Human Microsomes

The formation of Δ^3-carene-10-ol in the presence of human liver microsomes with an NADPH-generating system varied depending on incubation time, P450 amounts and

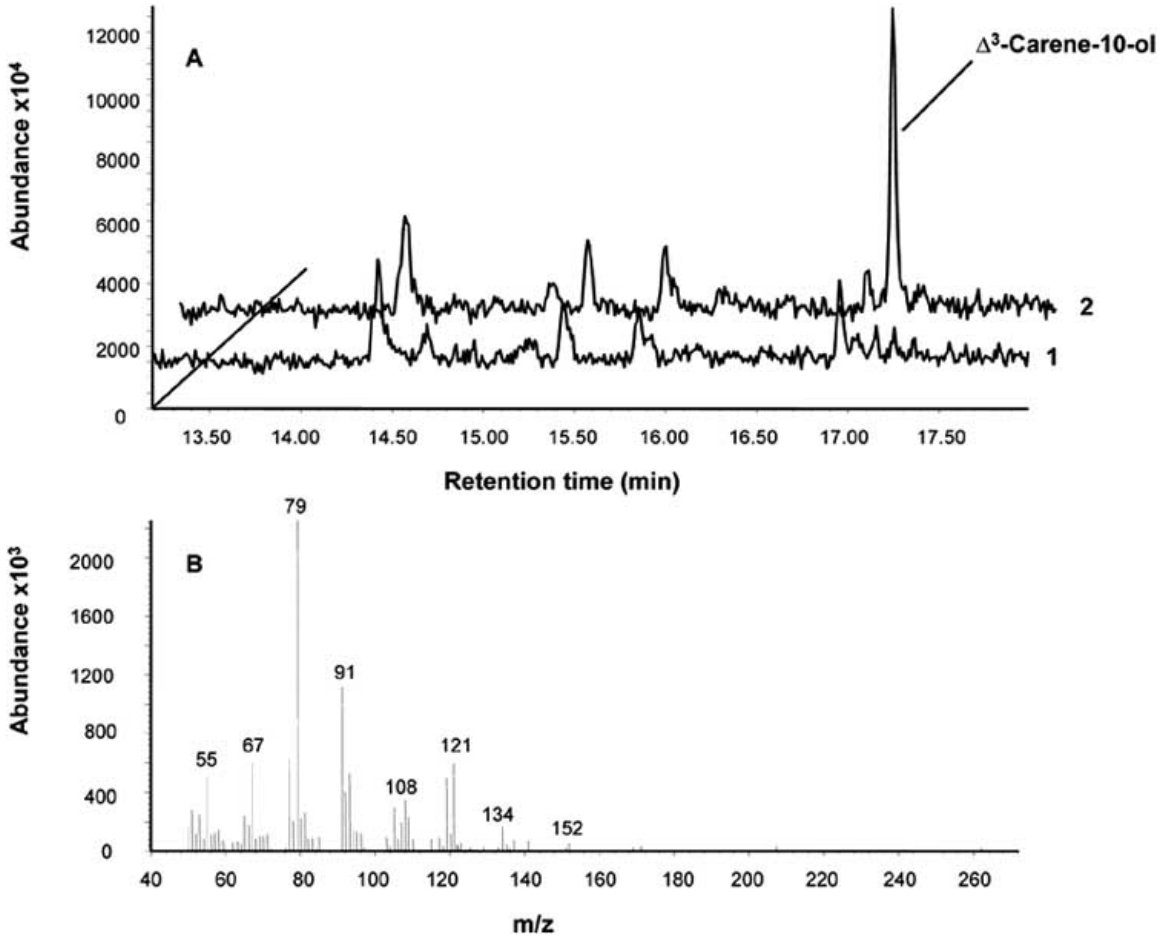


Fig. (1). GC-MS analysis of Δ^3-carene and its transformation product. Panel (A) shows the GC-MS chromatogram (electron impact) of the metabolite with human liver microsomes in the presence of an NADPH-generating system (2) and without liver microsomes (1). Panel (B) shows the full scan mass spectrum of the peak identified as Δ^3-carene-10-ol with the retention time of 16.8 min.

substrate concentration (Fig. (**2**)). Using different incubation times, we found linearity of the Δ³-carene 10-hydroxylation activities catalyzed by human liver microsomes up to 40 min. Increasing the P450 enzyme concentration or the Δ³-carene concentration lead to increasing Δ³-caren-10-ol formation. In the absence of human liver microsomes no Δ³-carene 10-hydroxylation was detected. In the presence of human lung microsomes of smokers and nonsmokers we found no Δ³-carene 10-hydroxylation (data not shown).

Kinetic analysis of the Δ³-carene 10-hydroxylation catalyzed by human liver microsomes were determined. The *Michaelis-Menten* K_m value for this reaction was 0.39 μM, the V_{max} value was 0.2 nmol/min/nmol P450. Finally, the efficiency of catalysis (V_{max}/K_m value) was determined to be 0.51 ml/min/nmol P450.

Metabolism of Δ³-Carene by Recombinant Human P450 Enzymes

Recombinant human P450 enzymes expressed in *Escherichia coli* cells were tested for their catalytic activities to transform Δ³-carene. Among 12 human P450 enzymes tested, CYP2B6, 2C19, and 2D6 were found to have the highest activities to hydroxylate Δ³-carene. Other P450 enzymes including CYP1A1, 1A2, 1B1, 2C8, 2C9, 2E1, 3A4, 3A5 and 4A11 had activities below the limit of detection [17].

CYP1A2 did not form Δ³-carene-10-ol but another signal was detected in the GC chromatogram. It could be identified by comparison of retention time and mass spectra with the synthesized reference compound as Δ³-carene-epoxide (Fig. (**3**)). CYP1A2 was the only of the tested recombinant human P450 enzymes found to form Δ³-carene-epoxide above the limit of detection.

Kinetic Analysis of Δ³-Carene 10-Hydroxylation and Epoxidation by Human P450 Enzymes

Kinetic analysis (Fig. (**4**) and (**5**), respectively) of the Δ³-carene 10-hydroxylation catalyzed by recombinant human P450 enzymes showed that apparent K_m values were 0.6 mM for CYP2B6, 0.8 mM for CYP2C19, and 1.5 mM for

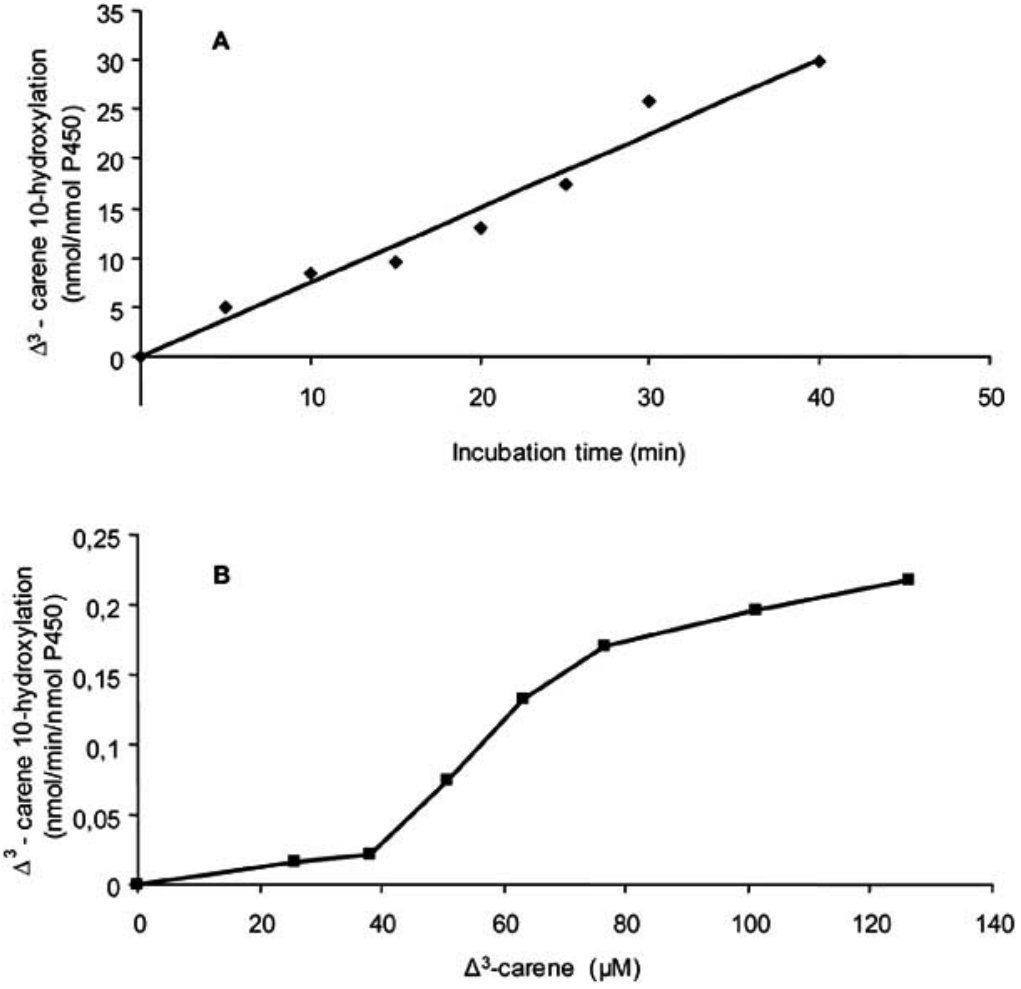


Fig. (2). Product dependence of Δ³-carene 10-hydroxylation catalyzed by human liver microsomes on incubation time (A) and Δ³-carene concentration (B).

(A) The concentrations of Δ³-carene and P450 enzymes were 80 μM and 134 pmol/ml, respectively.

(B) The incubation time and concentration of P450 enzymes was 30 min and 134 pmol/ml, respectively.

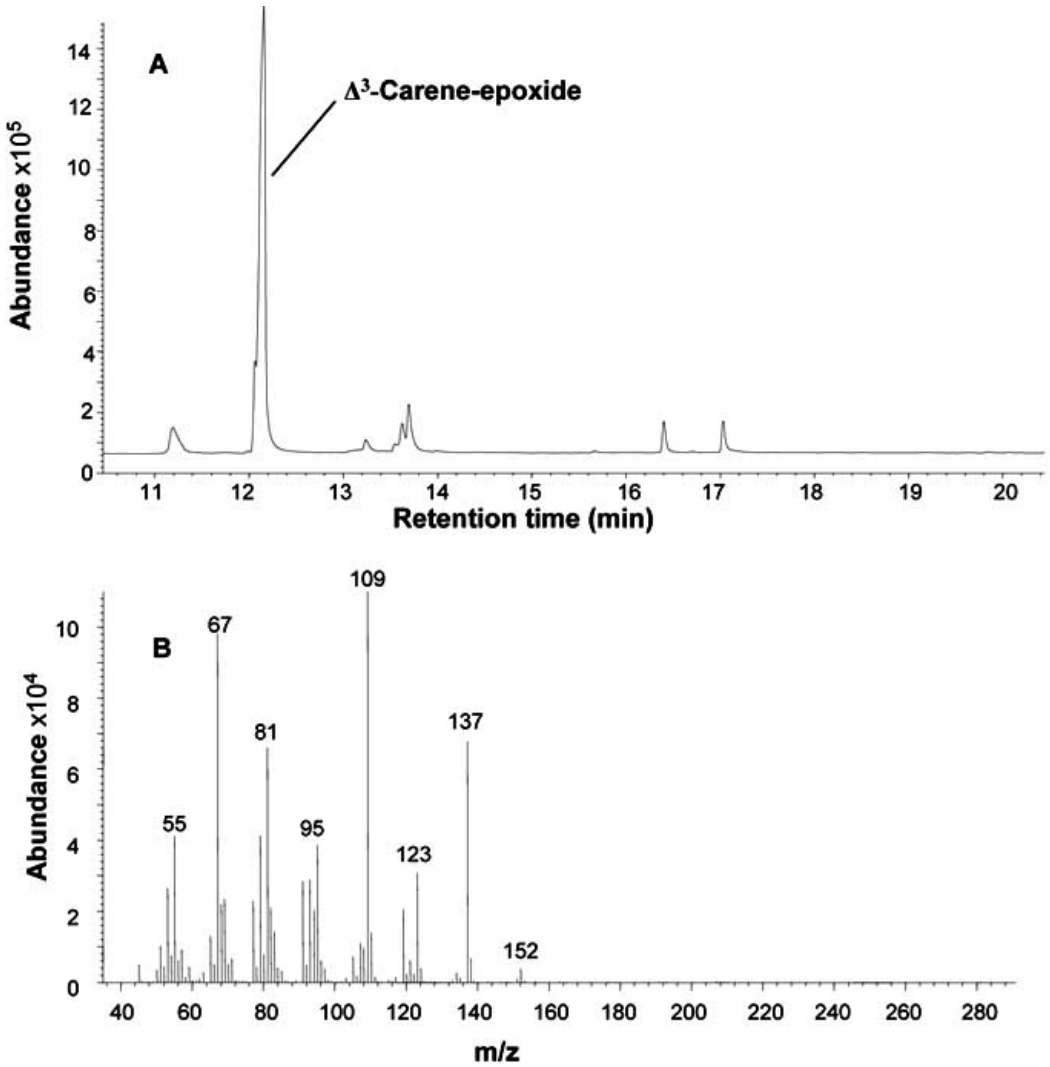


Fig. (3). GC-MS analysis of Δ^3-carene and its transformation product. Panel (A) shows the GC-MS chromatogram (electron impact) of the metabolite with human CYP1A2 in the presence of an NADPH-generating system. Panel (B) shows the full scan mass spectrum of the peak identified as Δ^3-carene-epoxide with the retention time of 12.1 min.

CYP2D6. The V_{max} values determined were 28.4 nmol/min/nmol P450 for CYP2B6, 1.0 nmol/min/nmol P450 for CYP2C19, and 1.4 nmol/min/nmol P450 for CYP2D6, respectively (Fig. (**4**)). The ratio of V_{max} and K_m was 50.0 $nM^{-1}min^{-1}$ for CYP2B6 and thus much higher then the ratios for CYP2C19 and CYP2D6 which were 1.3 $nM^{-1}min^{-1}$ and 0.9 $nM^{-1}min^{-1}$. For the epoxidation of Δ^3-carene by human recombinant human CYP1A2 the K_m value was found to be 98.2 mM, the V_{max} value was 3.9 nmol/min/nmol.

DISCUSSION

Various monoterpenes are metabolized by liver microsomes of different mammalian species. The results indicate that natural environmental compounds are differentially oxidized in several species. The structures of these chemicals determine at which position the attack by P450 enzymes takes place [16, 22].

Until now the human metabolism of Δ^3-carene was unclear. Based on our results we propose that Δ^3-carene is converted in humans to Δ^3-carene-10-ol and Δ^3-carene-epoxide (Scheme (**2**)). Δ^3-Carene-10-ol is formed by hydroxylation in allyl position in accordance with the known limonene oxidation to the perillyl alcohol [14]. Metabolic epoxidation of a monoterpene was recently described by Nilsson *et al*. [23]. Confirmation of the metabolite identity was performed by comparison of mass spectra and retention time of the GC-MS analysis with synthesized reference compounds. To our knowledge, we describe the first human metabolites of Δ^3-carene. In contrast to these results the study of Ishida *et al*. [11] described several other metabolites in rabbits.

After identification of both metabolites their formation was studied by human liver and lung microsomes as well as by 12 different recombinant human P450 enzymes in detail. Hydroxylation to Δ^3-carene-10-ol took place in the presence of human liver microsomes and three CYPs. CYP2B6 had the highest rate for Δ^3-carene 10-hydroxylation, CYP2C19 and CYP2D6 exhibited only one-fiftieth of this activity. Human lung microsomes of smoker and nonsmoker showed

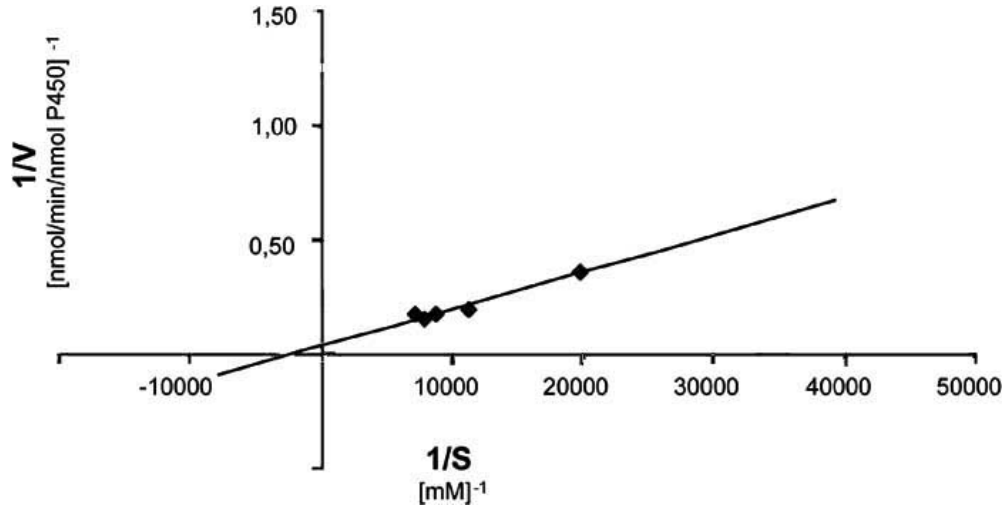


Fig. (4). Kinetic analysis of Δ³-carene 10-hydroxylation catalyzed by recombinant human CYP2B6 expressed in *Escherichia coli* cells.

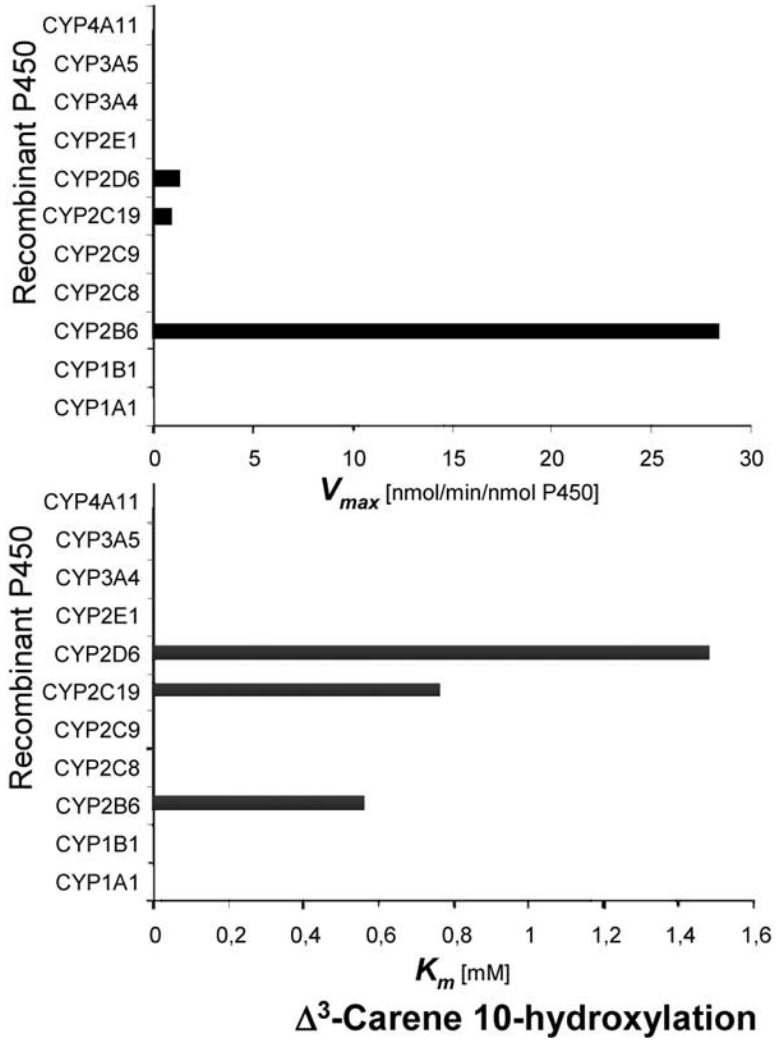
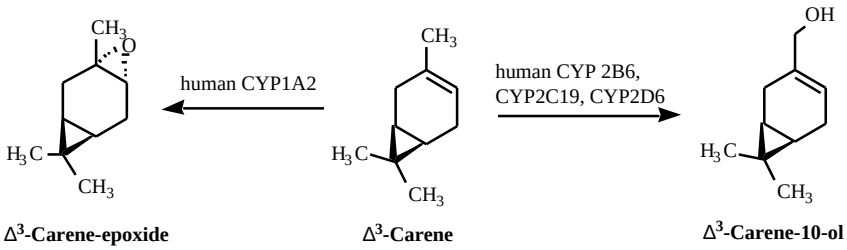


Fig. (5). Δ³-carene 10-hydroxylation K_m and V_{max} by recombinant human P450 enzymes expressed in *Escherichia coli* cells. Data are averages of duplicate determinations.



Scheme (2). Proposed metabolism of Δ^3-carene by human liver microsomes and recombinant human CYP1A2, CYP2B6, 2C19, and 2D6.

no activity at all under the same assay conditions probably caused by the low catalytic activity of the microsomes.

Epoxidation of Δ^3-carene to Δ^3-carene-epoxide was observed only with CYP1A2. For example this enzyme catalyzes also the epoxidation of AFB_1 [24]. For liver and lung microsomes which contain CYP1A2 we found no activity. We suppose that the transformation of Δ^3-carene to Δ^3-carene-epoxide was below the limit of detection because the CYP1A2 content is not high enough. In addition the formed epoxide might partially react fast with proteins in the assay so that it is not detectable in the measurements. However, Δ^3-carene-epoxide is a relative stable epoxide because it could be synthesized and isolated with relative high yields in this study. Reactivities and fates of different epoxides can differ significantly as described by Guengerich [24]. Nilsson *et al*. [23] proposed that reactive epoxides formed in the skin from the prohaptene (5R)-5-isopropenyl-2-methyl-1-methylen-2-cyclohexene are able to induce allergic contact dermatitis (ACD). They concluded that conjugated dienes can be metabolized in the skin to strong contact allergens, most likely epoxides, which are able to sensitize control animals after only one dermal exposure. We describe a similar reaction for Δ^3-carene, which is not a conjugated diene.

Johansson *et al.* found effects of low Δ^3-carene concentrations on alveolar macrophages [7]. The expression of different CYPs in the human lung is described in literature [25-28]. Wei *et al*. described CYP1A2 typical catalytic activities of human lung S9 [29] as well as induction of CYP1A2 in different human lung specimens [30]. Furthermore Mace *et al*. confirmed the expression of CYP1A2 in peripheral lung tissues on protein level [31]. Since we found conversion of Δ^3-carene to Δ^3-carene-epoxide by human CYP1A2, it can be assumed that Δ^3-carene might be metabolized in the human lung and the formed epoxide might cause allergic effects.

In addition Yengi *et al*. described expression of CYP2B6, 2C19 and 2D6 in the human skin [32]. We found that these three enzymes transform Δ^3-carene to Δ^3-carene-10-ol. Therefore we suppose that this metabolite can be formed in human skin.

Kinetic analysis of the Δ^3-carene 10-hydroxylation activities showed low K_m (0.4 µM), V_{max} (0.2 nmol/min/nmol P450), and V_{max}/K_m (0.5 ml/min/nmol P450) values for human liver microsomes in comparison to the K_m and V_{max} values of other monoterpenoids metabolised by P450 enzymes. For example for verbenone 10-hydroxylation K_m values between 0.6 and 2.8 mM were found for liver microsomes of different human samples. The V_{max} values were between 2.1 and 4.6 nmol/min/nmol P450 [33]. The K_m and V_{max} values determined for 1,8-cineole 2- and 3-hydroxylation by human liver microsomes of human sample were 50 µM and 91 nmol/min/nmol P450. This extremely high V_{max} value is explained with a high level of CYP3A enzymes in the used human sample [34]. This lead to the assumption that the P450 enzymes responsible for the Δ^3-carene 10-hydroxylation are not highly represented in our human liver microsomes used in these studies.

This was confirmed by the results we got with recombinant human P450 enzymes. Kinetic analysis of 12 different recombinant human P450 enzymes showed hydroxylation activities only for CYP2B6, 2C19, and 2D6. It is obviously that CYP2B6 shows the highest activity to transform Δ^3-carene to Δ^3-carene-10-ol followed by CYP2C19 and CYP2D6 (Fig. (**5**)). The CYP2B6 V_{max} value of 28.4 nmol/min/nmol P450 for the carene-10-hydroxylation is in the same range as the value described by Miyazawa [22] for (-)-verbenone 10-hydroxylation (21 nmol/min/nmol P450). However, the K_m value of 568 µM differs from the value described for (-)-verbenone 10-hydroxylation (91 µM).

For the transformation of (+)- and (-)-limonene to carveol and perillyl alcohol CYP2C19 is described as one of the major enzymes involved [14]. The value for the enzyme effiency (V_{max}/K_m ratio) is between 2.81 and 13.0 $nM^{-1}min^{-1}$. The value we found for Δ^3-carene 10-hydroxylation is lower (1.3 $nM^{-1}min^{-1}$). Perhaps steric effects play a role in this biotransformation. The monocyclic limonene might reach the active site of the enzyme better than the bicyclic Δ^3-carene.

The *Michaelis-Menten*-value K_m for Δ^3-carene epoxidation was in the same range as described by Miyzawa for (-)-verbenone 10-hydroxylation (91 mM for recombinant human CYP2B6) and V_{max} were lower for the epoxidation than the values for 10-hydroxylation of Δ^3-carene.

Further work is needed to investigate whether both identified metabolites can cause biological and toxicological responses, especially regarding allergic contact dermatitis or chronic lung function impairment. Additionally, *in vivo* relevance of Δ^3-carene-10-ol formation is interesting because it might be used as urinary marker of occupational exposure for Δ^3-carene in the same way as verbenol for α-pinene exposure [1, 3, 17, 35].

ACKNOWLEDGMENT

This work was supported by a grant from the Deutsche Forschungsgemeinschaft. We thank Nicole Fricke, Mathias

Christmann and Manfred Möller for technical assistance as well as Wolfgang Dott for continuous support.

ABBREVIATIONS

P450 = General term for cytochrome P450;

CYP = Individual isoform of P450;

GC = Gas chromatography;

MS = Mass spectrometer;

SPE = Solid phase extraction

REFERENCES

[1] Eriksson, K. and Levin, J.O. (1990) *Int. Arch. Occup. Environ. Health*, **62**(5), 379-383.
[2] Hedenstierna, G.; Alexandersson, R.; Wimander, K. and Rosen, G. (1983) *Int. Arch. Occup. Environ. Health*, **51**(3), 191-198.
[3] Eriksson, K.A.; Levin, J.O.; Sandstrom, T.; Lindstrom-Espeling, K.; Linden, G. and Stjernberg, N.L. (1997) *Scand. J. Work Environ. Health*, **23**(2), 114-120.
[4] Kasanen, J.P.; Pasanen, A.L.; Pasanen, P.; Liesivuori, J.; Kosma, V.M. and Alarie, Y. (1999) *J. Toxicol. Environ. Health A*, **57**(2), 89-114.
[5] Falk, A.; Lof, A.; Hagberg, M.; Hjelm, E.W. and Wang, Z. (1991) *Toxicol. Appl. Pharmacol.*, **110**(2), 198-205.
[6] Falk-Filipsson, A.F. (1996) *Occup. Environ. Med.*, **53**(2), 100-105.
[7] Johansson, A. and Lundborg, M. (1997) *Toxicology*, **120**(2), 99-104.
[8] Hellerstrom, S.; Thyresson, N.; Blohm, S.G. and Widmark, G. (1955) *J. Invest. Dermatol.*, **24**, 217-224.
[9] Pirila, V. and Siltanen, E. (1958) *Dermatologica*, **117**, 1-8.
[10] McGraw, G.W.; Hemingway, R.W.; Ingram, J.L.L.; Canady, C.S. and McGraw, W.B. (1999) *Environ. Sci. Technol.*, **33**(22), 4029-4033.
[11] Ishida, T.; Asakawa, Y.; Takemoto, T. and Aratani, T. (1981) *J. Pharm. Sci.*, **70**(4), 406-415.
[12] Miyazawa, M. and Shindo, M. (2001) *Nat. Prod. Lett.*, **15**(1), 49-53.
[13] Miyazawa, M.; Shindo, M. and Shimada, T. (2001) *Xenobiotica*, **31**(10), 713-723.
[14] Miyazawa, M.; Shindo, M. and Shimada, T. (2002) *Drug Metab. Dispos.*, **30**(5), 602-607.
[15] Miyazawa, M.; Shindo, M. and Shimada, T. (2002) *Chem. Res. Toxicol.*, **15**(1), 15-20.
[16] Duisken, M.; Sandner, F.; Blomeke, B. and Hollender, J. (2005) *Biochim. Biophys. Acta (BBA) - General Subjects*, **1722**(3), 304-311.
[17] Sandner, F.; Fornara, J.; Dott, W. and Hollender, J. (2002) *J. Chromatogr. B*, **780**(2), 225-230.
[18] Eriksson, K. and Levin, J.O. (1996) *J. Chromatogr. B. Biomed. Appl.*, **677**(1), 85-98.
[19] Brown, H.C.S., A. (1967) *J. Am. Chem. Soc.*, **89**, 1933.
[20] Paquette, L.A.R., Robert J.; Shi, Y.-J. (1989) *J. Org. Chem.*, **55**, 1589.
[21] Gollnick, K. and Schade, G. (1966) *Tetrahedron*, **22**, 133.
[22] Miyazawa, M.; Sugie, A. and Shimada, T. (2003) *Drug Metab. Dispos.*, **31**(8), 1049-1053.
[23] Nilsson, A.M.; Bergstrom, M.A.; Luthman, K.; Nilsson, J.L. and Karlberg, A.T. (2005) *Chem. Res. Toxicol.*, **18**(2), 308-316.
[24] Ueng, Y.F.; Shimada, T.; Yamazaki, H. and Guengerich, F.P. (1995) *Chem. Res. Toxicol.*, **8**(2), 218-225.
[25] Hukkanen, J., *Xenobiotic-Metabolizing Cytochrome P450 Enzymes in Human Lung*, in *Department of Pharmacology and Toxicology*. (2000), University of Oulu: Oulu. pp. 69.
[26] Anttila, S.; Hukkanen, J.; Hakkola, J.; Stjernvall, T.; Beaune, P.; Edwards, R.; Boobis, A.; Pelkonen, O. and Raunio, H. (1997) *Am. J. Respir. Cell Mol. Biol.*, **16**(3), 242-249.
[27] Hukkanen, J.; Pelkonen, O.; Hakkola, J. and Raunio, H. (2002) *Crit. Rev. Toxicol.*, **32**(5), 391-411.
[28] Hukkanen, J.; Vaisanen, T.; Lassila, A.; Piipari, R.; Anttila, S.; Pelkonen, O.; Raunio, H. and Hakkola, J. (2003) *J. Pharmacol. Exp. Ther.*, **304**(2), 745-752.
[29] Wei, C.; Cacavale, R.J.; Kehoe, J.J.; Thomas, P.E. and Iba, M.M. (2001) *Cancer Lett.*, **164**(1), 25-32.
[30] Wei, C.; Caccavale, R.J.; Weyand, E.H.; Chen, S. and Iba, M.M. (2002) *Cancer Lett.*, **178**(1), 25-36.
[31] Mace, K.; Bowman, E.D.; Vautravers, P.; Shields, P.G.; Harris, C.C. and Pfeifer, A.M. (1998) *Eur. J. Cancer*, **34**(6), 914-920.
[32] Yengi, L.G.; Xiang, Q.; Pan, J.; Scatina, J.; Kao, J.; Ball, S.E.; Fruncillo, R.; Ferron, G. and Roland Wolf, C. (2003) *Analyt. Biochem.*, **316**(1), 103-110.
[33] Miyazawa, M.; Sugie, A. and Shindo, M. (2002) *Biosci. Biotechnol. Biochem.*, **66**(11), 2458-2460.
[34] Miyazawa, M.; Shindo, M. and Shimada, T. (2001) *Drug Metab. Dispos.*, **29**(2), 200-205.
[35] Eriksson, K.A.; Stjernberg, N.L.; Levin, J.O.; Hammarstrom, U. and Ledin, M.C. (1996) *Scand. J. Work Environ. Health*, **22**(3), 182-190.