

RESEARCH ARTICLE

Cytochrome P450-catalysed arene-epoxidation of the bioactive tea tree oil ingredient *p*-cymene: indication for the formation of a reactive allergenic intermediate?

R. J. W. Meesters^{1,2}, M. Duisken², and J. Hollender^{2,3}

¹Laboratories of Neuro-Oncology/Clinical and Cancer Proteomics, Department of Neurology, Erasmus University Medical Center, Rotterdam, the Netherlands, ²Institute of Hygiene and Environmental Medicine, RWTH Aachen University, Aachen, Germany, and ³Swiss Federal Institute of Aquatic Science and Technology (Eawag), Dübendorf, Switzerland

Abstract

1. The cytochrome P450-mediated metabolism of the tea tree oil ingredient *p*-cymene (*p*-isopropyltoluene) was studied by the application of *in vitro* enzymatic assays using different recombinant human cytochrome P450 enzymes.
2. In total, four enzymatic products were identified by gas chromatography-mass spectrometry. The enzymatic products identified were: thymol (2-isopropyl-5-methylphenol), *p*-isopropylbenzyl alcohol, *p*, α , α -trimethylbenzyl alcohol, and *p*-isopropylbenzaldehyde.
3. The enzymatic products of *p*-cymene resulted from catalysed enzymatic arene-epoxidation and hydroxylation reactions by the studied cytochrome P450 enzymes.
4. An *in vivo* study could only confirm the formation of one enzymatic product, namely thymol. Thymol was identified after enzymatic hydrolysis of glucuronide and sulphate conjugates in collected blood and urine samples.
5. The obtained results may help to increase the understanding of cases where skin sensitization and irritation by tea tree oil-containing products that are involved with allergic reactions of users of these products. The results also indicate that skin sensitization and irritation reactions not only can be explained by the frequently in literature reported auto-oxidation of tea tree resulting in bioactive oxidized products, but also now by the formation of epoxide intermediates resulting from catalysed arene-epoxidation reactions by selected human cytochrome P450 enzymes which are also located in different organs in humans.

Keywords: Tea tree oil; *p*-cymene; arene-epoxidation; thymol; gas chromatography-mass spectrometry (GC-MS); National Institutes of Health (NIH) shift; allergens

Introduction

The use of raw plant materials such as herbs, roots, leaves, and spices to manufacture natural remedies, also named complementary and alternative medicine, is very old and dates back thousands of years. The earliest evidence of its use by humans dates to the time of Neanderthal humans (Kleiner 1995). Approximately 15 years ago, already 30% of modern medicines were estimated to contain raw plant materials (Kleiner 1995) as (active) ingredients.

Moreover, very popular among the population in Europe and the United States is the use of complementary and alternative medicine. This due to the revival of a 'back-to-nature' movement. Tea tree oil (TTO) is a frequently used complementary and alternative medicine manufactured by steam distillation from the leaves of the tree *Maleleuca alternifolia*. TTO is widely incorporated as active ingredient in topical formulations that are used to treat several different cutaneous diseases. It has anti-microbiological (Carson et al. 2006), antifungal (Hammer et al. 2003, 2006) and cancer-suppressive

Address for Correspondence: R. J. W. Meesters, Laboratories of Neuro-Oncology/Clinical and Cancer Proteomics, Department of Neurology, Erasmus University Medical Center, Dr. Molewaterplein 50, NL-3015 GE Rotterdam, the Netherlands. Tel: 31-10-7043320. Fax: 31-10-7044365. E-mails: r.meesters@erasmusmc.nl or rjwmeesters@gmail.com

(Received 22 February 2009; revised 21 April 2009; accepted 22 April 2009)

properties (Knight & Hausen 1994; Papadopoulos et al. 2006; Edris 2007). Although many studies have been undertaken, the (bioactive) mechanism of TTO is still not (entirely) understood (Valero & Salmeron 2003; Chorianopoulos et al. 2004; Nostro et al. 2004; Schelz et al. 2006).

In contrast to its medicinal healing properties, TTO is also reported to cause allergic skin-contact dermatitis (Apted 1991; Knight & Hausen 1994; Bhushan & Beck 1997; Rubel et al. 1998; Rutherford et al. 2007; Aberer 2008), intoxication (Woolf 1999), ataxia, and even coma (Hammer et al. 2006). Allergic contact dermatitis is a disease frequently observed in patients with (skin) allergic reactions to (cosmetic) products containing plant extracts or raw plant ingredients (Thomson & Wilkinson 2000; Larsen et al. 2001; Rastogi et al. 2001; Jappe et al. 2005).

The chemical composition of TTO is only partly known. Fifteen substances have been identified: twelve are monoterpenes and the other three are sesquiterpenes (Knight & Hausen 1994). Monoterpenes such as γ -terpinene, 1,8-cineole, *p*-cymene, α -pinene (Carson and Riley 2001), and their oxidation products like monoterpene epoxides and hydroperoxides (Hausen et al. 1999; Skold et al. 2004; Johansson et al. 2008) are under suspicion as being the causes of this contact dermatitis. TTO contains several monoterpenes where under *p*-cymene, its concentration varies depending on the cultivation methods used and is, according to ISO4730, to be between 0.5% and 12% (International Organisation for Standardisation (ISO) 1996). Recently, the present authors reported the detection of monoterpene oxidation products (alcohols) in urine samples of humans after ingestion of a cough syrup containing monoterpenes (Meesters et al. 2008). Due to their chemical activity, oxidation products may bind to skin proteins and produce haptens with bioactive properties, as reported by Nilsson et al. (2001), that may result in an allergic reaction of the skin. We reported the enzymatic formation of bioactive allergens by CYP, Δ^3 -carene-epoxide, an enzymatic product (CYP1A2) from the monoterpene Δ^3 -carene (Duisken et al. 2005a) and the enzymatic intermediate 6,7-epoxylinolool (CYP2D6) (Meesters et al. 2007) from the monoterpene alcohol linolool. The CYP-catalysed metabolism of monoterpenes, enzymes specialized in the detoxification of exogenous substances (Baron & Merk 2001; Guengerich 2003), is for many monoterpenes still unknown. From the elucidation of specific enzymatic reaction products one can obtain information about potential bioreactive oxidation products which could help in an understanding of the observed allergic reactions caused in humans after the application of TTO.

The aim of present study was (1) the elucidation of the CYP-catalysed metabolism of *p*-cymene by human recombinant CYP, (2) the possible confirmation of

in vitro enzymatic products by an *in vivo* experiment by a volunteer with an orally intake of complementary and alternative medicine containing 1% TTO, and (3) obtaining of results that probably will give an indication about the formed enzymatic products and/or enzymatic intermediates that may possibly exhibit bioactive properties which can result in the formation of allergens.

Materials and methods

Chemicals and enzymes

Chemicals, unless specified, were different from analytical grade quality or for biochemical use. Ethanol (100%, EtOH) and thymol (97%, 2-isopropyl-5-methylphenol, IMP) were purchased from Sigma Aldrich (Taufkirchen, Germany), *p*-cymene (99%, *p*-isopropyltoluene, IPT), *p*-isopropylbenzyl alcohol (97%, IPBA) and *p*, α , α -trimethylbenzyl alcohol (99%, TBA), *p*-isopropylbenzaldehyde (98%, IBA), hydrochloric acid (HCl) and trichloroacetic acid (TCA) were from Acros (Geel, Belgium). Glucose 6-phosphate potassium salt (G6P), the enzyme glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and nicotinamide adenine dinucleotide phosphate sodium salt (NADP, 98%) were purchased from Roche Diagnostics (Basel, Switzerland), ethyl acetate (EtOAc) was of residue analysis quality and was purchased from LGC Promochem (Wesel, Germany). Purified water (approximately 18 M Ω) produced by a Milli-Q water purification system (Millipore, Eschborn, Germany) was applied for the preparation of phosphate buffers. Di-sodium hydrogen phosphate dihydrate (Na₂HPO₄·2H₂O), sodium acetate (NaAc) and potassium dihydrogen phosphate trihydrate (KH₂PO₄·3H₂O) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Chemical structures of the substrate *p*-cymene, identified enzymatic products and other monoterpenes are presented in Figure 1.

Recombinant P450 enzymes and NADPH-regeneration system

Recombinant human CYP enzymes (EC 1.14.14.1); CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP4A11 (approximately 1 nmol) with co-expressed CYP-reductase in *Escherichia coli* were obtained from Cypex (Dundee, UK) and stored at -80°C until usage. The enzyme G6PDH (1.5 U ml⁻¹) in combination with NADP (0.5 mM)/G6P (4.7 mM) prepared in phosphate buffer (pH 7.2) was used as an NADPH-generation system in the CYP-mediated enzymatic assays. Phosphate buffer was prepared by a mixture (61:39, v/v%) of Na₂HPO₄ (0.05 M) and KH₂PO₄ (0.02 M). The NADPH-generation solution was prepared fresh on a daily base.

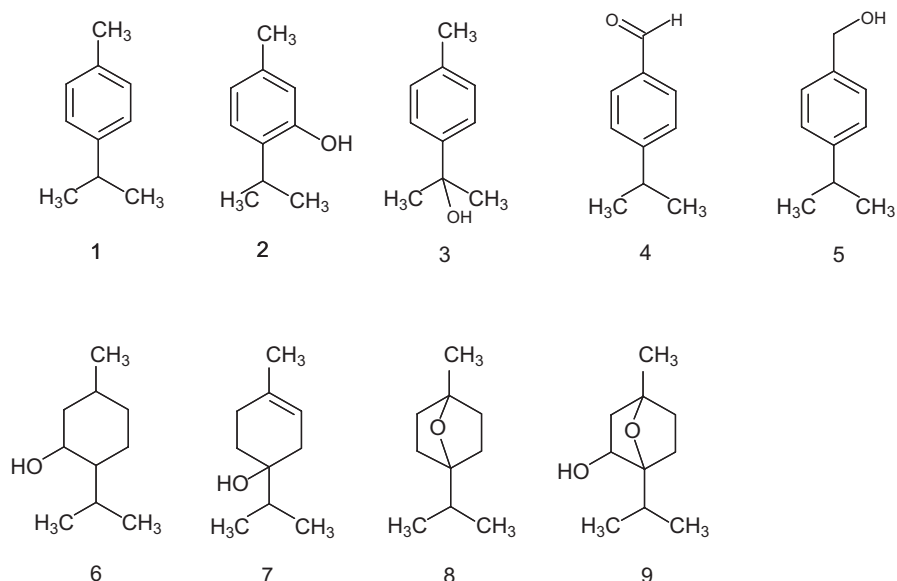


Figure 1. Chemical structures of *p*-cymene (1), 2-isopropyl-5-methylphenol (2), *p*, α , α -trimethylbenzyl alcohol (3), *p*-isopropylbenzaldehyde (4), *p*-isopropylbenzyl alcohol (5), ($\pm$)-menthol (6), terpinen-4-ol (7), 2-hydroxycineole (8), and 1,8-cineole (9).

P450-mediated enzymatic assays and gas chromatography-mass spectrometry

The gas chromatographic separation and mass spectrometric determination (GC-MS) as well as application and study of three different enzymatic assays (time dependency, enzyme concentration dependency and substrate dependency) were previously described by Meesters et al. (2007).

In short, the substrate *p*-cymene was diluted in phosphate buffer and the CYP enzyme and NADPH-regeneration solution were added and the mixture incubated at 37°C. The enzymatic reactions were stopped by addition of 1 ml ethyl acetate and enzymatic products were extracted by vigorously shaking the solution with a Vortex® shaker. The organic ethyl acetate layer was removed and transferred into a brown-coloured glass vial and injected into the GC-MS system. The applied enzymatic assay is illustrated in Figure 2.

In the present paper, enzyme kinetics were determined only for CYP1A2, CYP2A6, CYP2D6 and CYP3A5. Other CYPs were only used to study the formation of the enzymatic products by each CYP. The applied CYP concentrations in the enzymatic assays are presented in Table 1.

Determination of enzymatic product concentrations and calibration curves

Enzymatic products and substrate stock solutions were prepared at a concentration of 6.67 M in EtOAc. Calibration solutions for the preparation of calibration curves were made by serial dilutions of the stock solution (6.67 M) with EtOAc at concentrations of 0.16, 0.32, 0.40, 0.80 and 1.6 μ M containing IMP, IPT, IPBA, TBA and

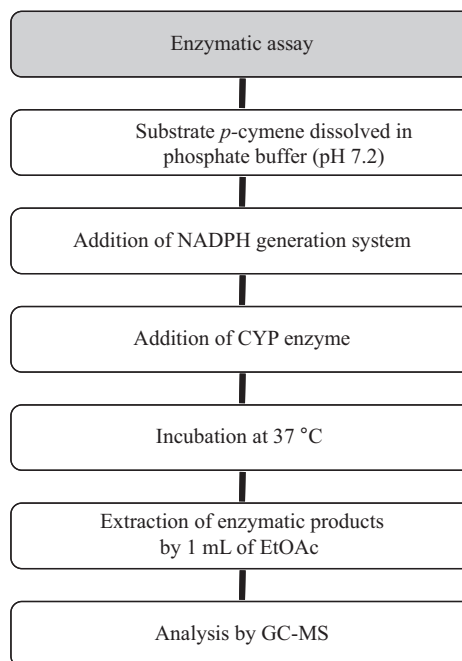


Figure 2. Cytochrome P450 enzymatic assay.

IBA. The calibration curves were constructed by plotting measured peak areas against known concentrations and linear regression of the calibration points was carried out. All calibration curves were shown to be linear in a selected concentration range between 0.16 and 1.6 μ M. Regression coefficients (r^2) for the enzymatic products TBA, IBA, IMP and IPBA were 0.993, 0.998, 0.996 and 0.999, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) for all enzymatic products were determined using a signal/noise ratio of 3:1 and

Table 1. Identified enzymatic products from the substrate *p*-cymene (marked by (+))

Cytochrome P450 (CYP)	Concentration (pmol ml ⁻¹)	Thymol (IMP)	<i>p</i> -Isopropylbenzyl-alcohol (IPBA)	<i>p</i> , α , α -Trimethylbenzylalcohol (TBA)	<i>p</i> -Isopropylbenzaldehyde (IBA)
CYP1A1	15.7	(+)	-	-	-
CYP1A2	104.9	-	(+)	(+)	(+)
CYP2A6	21.0	(+)	(+)	-	-
CYP2B6	35.0	(+)	(+)	-	-
CYP2C8	174.8	-	(+)	(+)	-
CYP2C9	104.9	(+)	(+)	-	-
CYP2C19	78.7	(+)	(+)	-	-
CYP2D6	52.4	(+)	(+)	-	(+)
CYP2E1	69.9	(+)	-	-	-
CYP3A4	73.4	(+)	(+)	-	-
CYP3A5	26.2	(+)	-	-	-
CYP4A11	125.9	(+)	-	-	-

10:1 and were for all enzymatic products, 0.07 $\mu\text{mol L}^{-1}$ and 0.21 $\mu\text{mol L}^{-1}$, respectively.

In vivo study

A volunteer subject after an overnight fast of 12 h swallowed 35 tablets containing 1% TTO from biological cultivation (Aurica, Schwabach, Germany) with tap water over 2 h. A control blood and urine sample before administration (-10 min) was collected and at fixed time intervals after administration of the TTO pills (at 60, 130 and 145 min) urine samples were collected, while blood was only collected at -10 and 130 min after the start of the oral intake of the TTO pills by the volunteer subject. Blood samples were drawn from an arm vein, and urine samples were collected into sterile plastic vessels. Urine samples were directly frozen and stored at -20°C until analysis. Blood samples (4 ml) were collected using a Vacuette blood-collecting tube containing lithium heparin (Greiner bio-one, Solingen, Germany). Plasma was isolated by centrifugation of the lithium heparin tubes for 10 min at 4000 rpm in a cooled centrifuge at 4°C. Approximately 1.5 ml plasma were isolated and transferred into safety lock micro-tubes (2.0 ml) and plasma was deproteinized by the addition of 0.15 ml of a TCA solution (3.1 M) and homogenized by a Vortex mixer for 1 min and finally stored until analysis in a freezer at -80°C.

Enzymatic hydrolysis of blood and urine samples

Urine samples were thawed at room temperature and enzymatic hydrolysis of the glucuronide and sulphate conjugates of the IPT metabolites was carried out by a enzyme mixture (Merck, Darmstadt, Germany) containing β -glucuronidase (EC 3.2.1.31, 30 U ml⁻¹) and arylsulphatase (EC 3.1.3.1, 60 U ml⁻¹) both from *Helix pomatia* as described previously by Meesters et al. (2008). Therefore, 0.5 ml of urine or deproteinized plasma were transferred into a 2 ml safe-lock micro-tube

and mixed with 0.5 ml of a NaAC solution (pH 5.0, 0.1 M) and the pH of the sample was adjusted to a pH 5.0 with HCl solution (0.05 M). Then, 25 μl of the enzyme mixture were added and samples were incubated and shaken at a temperature of 37°C for approximately 24 h on an incubation shaker. After 4 h of the start of the incubation, a surplus of 25 μl of the enzyme mixture was added to ensure complete conjugate hydrolysis. Enzymatic hydrolysis was stopped by the addition of 1.0 ml EtOAc. Hydrolysed enzymatic products were extracted by vigorously mixing of the samples for 30 min and the EtOAc layer was separated and transferred into brown-coloured glass GC vials with septum screw caps.

Results

Identification of enzymatic products from in vitro experiments

The enzymatic products from incubation assays of *p*-cymene (Figure 1, (1)) and CYP enzymes were extracted using the organic solvent EtOAc and an aliquot of 40 μl was injected into the GC-MS system. The GC-MS chromatogram shown in Figure 3b illustrates the chromatogram of a control incubation of the substrate *p*-cymene (IPT) with an NADPH-generation system, but without the addition of CYP enzyme. The chromatogram in Figure 3a illustrates the formation of enzymatic products of the substrate by catalysed enzymatic reactions of CYP1A2 enzyme.

The chromatogram illustrates the formation of three different enzymatic products. Although the application of other CYPs revealed the formation of thymol (Figure 1, (2)), a fourth enzymatic product (Table 1). The enzymatic formation of the enzymatic product thymol was not catalysed by the CYP1A2 enzyme. Thymol was identified as the main formed enzymatic product. Ten out of twelve studied CYPs catalysed the formation

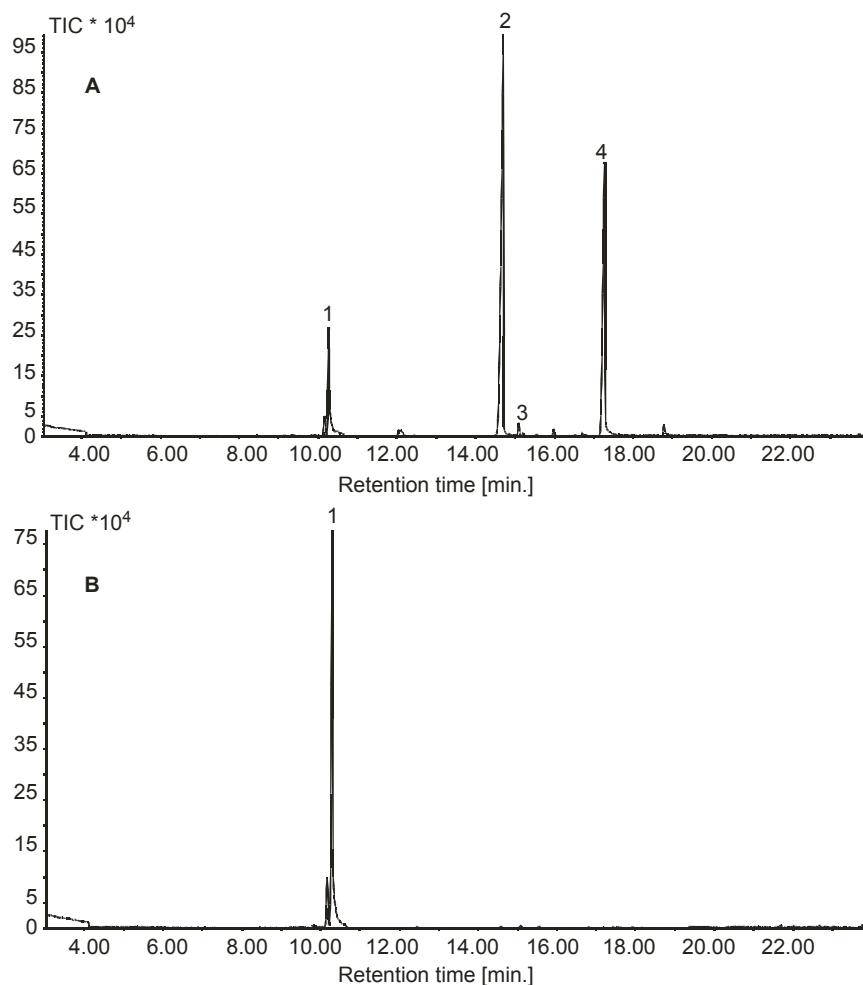


Figure 3. Gas chromatography-mass spectrometry (GC-MS) total ion current (TIC) chromatogram after 40 min of incubation of *p*-cymene: (A) with CYP1A2 (5.5 nmol ml⁻¹), where (1) = *p*-cymene, (2) = *p*, α , α -trimethylbenzyl alcohol, (3) = *p*-isopropylbenzaldehyde, and (4) = *p*-isopropylbenzyl alcohol; and (B) without CYP1A2 but in the presence of the β -nicotinamide adenine dinucleotide phosphate (NADPH)-regeneration system, where (1) = *p*-cymene.

of thymol, while eight CYPs catalysed the formation of *p*-isopropylbenzylalcohol (Figure 1, (5)) and just two out of twelve CYPs catalysed the formation of *p*, α , α -trimethylbenzylalcohol (Figure 1, (3)) and/or *p*-isopropylbenzaldehyde (Figure 1, (4)), respectively. Identification of the enzymatic products was performed by comparison of retention times and mass spectra of the formed enzymatic products with retention times and mass spectra of the purchased reference substances and by a library search using the GC-MS mass spectra library.

CYP-mediated enzyme kinetics

The formation of enzymatic products of *p*-cymene by the different CYPs was studied using incubation time and enzyme and substrate dependency enzymatic assays as previously described by Meesters et al. (2007). The enzymatic assay showed that the time-dependent enzymatic formation of all four enzymatic products was linear

($r^2=0.93$, standard deviation (SD)=0.03) with the studied CYP concentrations. Concentrations of enzymatic products above the limit of quantitation (LOQ) of 0.21 μ M were calculated by the prepared calibration curves.

Increasing the CYP concentration by a factor of two resulted in a doubling of enzymatic activity, which was observed with all CYP enzymes (mean $r^2=0.98$, SD=0.02). The concentration dependency of IPT on the enzymatic formation of the products was studied by Lineweaver-Burk analysis and was found to be linear for all CYP-catalysed formation of enzymatic products (mean $r^2=0.82$, SD=0.03). The Lineweaver-Burk analysis was also used to calculate K_m and V_{max} values. The calculated specific enzyme properties of studied CYP enzymes are presented in Table 2.

In vivo experiments

By means of GC-MS analysis, only thymol was identified in both blood and urine samples. In contrast, in the urine

Table 2. Michaelis–Menten values of studied recombinant human cytochrome P450 (CYP).

CYP	Enzymatic product	K_m mM	v_{max} mmol min ⁻¹ mmol ⁻¹	v_{max}/K_m 1 mmol ⁻¹ min ⁻¹	K_{cat} s ⁻¹	K_{cat}/K_m s ⁻¹ mmol ⁻¹
CYP1A2	Thymol	0.15	1.00	6.67	1.6×10^2	1.10×10^3
CYP2A6	Thymol	0.12	0.45	3.75	3.6×10^2	3.00×10^3
CYP2D6	Thymol	0.50	0.42	0.84	1.30×10^2	2.70×10^2
CYP3A5	Thymol	0.10	1.10	11.0	7.00×10^2	7.00×10^3
CYP1A2	<i>p</i> -Isopropylbenzylalcohol	1.50	15.1	10.1	2.40×10^3	1.60×10^3
CYP2A6	<i>p</i> -Isopropylbenzylalcohol	0.12	0.80	6.67	6.00×10^2	5.30×10^3
CYP2D6	<i>p</i> -Isopropylbenzylalcohol	1.10	22.1	20.1	7.00×10^3	6.40×10^3
CYP3A5	<i>p</i> -Isopropylbenzylalcohol	0.13	1.30	10.0	8.30×10^2	6.40×10^3
CYP1A2	<i>p</i> -Isopropylbenzylaldehyde	0.12	1.00	8.33	1.60×10^2	1.30×10^3
CYP2D6	<i>p</i> -Isopropylbenzylaldehyde	0.15	0.45	3.0	1.40×10^2	9.5×10^2

samples collected at 60 and 145 min, other monoterpenes were identified by the GC-MS library such as menthol (Figure 1 (6)), terpinen-4-ol (Figure 1 (7)), and 2-hydroxycineole (Figure 1 (9)). The origin of these monoterpenes might be the TTO pills themselves because these monoterpenes were previously identified in TTO (ISO 1996). The thymol urine concentration increased from 0.47 mol l⁻¹ measured at 60 min to 1.08 µM l⁻¹ in the urine sample collected at 145 min. The measured thymol concentration in blood was 0.87 µM l⁻¹ (60 min). Control blood and urine samples were measured and no thymol (concentration less than the limit of detection (LOD)) was detected.

Discussion

Results from enzymatic assays showed that enzymatic reactions catalysed by the different applied CYP resulted in the formation of four different enzymatic products. The enzymatic products were identified by the GC-MS library and by comparison of retention times and mass spectra of purchased reference substances. The main enzymatic product formed was thymol followed by *p*-isopropylbenzylalcohol, *p*, α , α -trimethylbenzylalcohol and *p*-isopropylbenzaldehyde (Table 1).

Enzymatic hydroxylation reactions by CYP are the most common reported reactions that result in the formation of hydroxylated enzymatic products (Wrighton et al. 1990; Bylund et al. 1998). The formation of both hydroxylated products is the result of a hydroxylation substitution reaction of one of the methyl groups of the isopropyl group (*p*, α , α -trimethylbenzylalcohol) or the methyl group at situated at the 1'-carbon atom (*p*-isopropylbenzylalcohol). The next identified enzymatic product isopropylbenzaldehyde may result from a cooperative enzymatic reaction mechanism of the CYP enzymes and the co-expressed CYP reductase enzyme (Goeptar et al. 1995) which catalyse reduction reactions leading to the formation of the enzymatic product *p*-isopropylbenzaldehyde probably from the

enzymatic product *p*-isopropylbenzylalcohol. Catalysed hydroxylation activities for the enzymatic conversion of *p*-cymene to thymol and *p*-isopropylbenzylalcohol by CYP (CYP1A2, CYP2A6, CYP2D6 and CYP3A5) are presented in Table 2. Comparison of the hydroxylation activities for the different enzymes shows that CYP2A6 had the highest enzymatic affinity for the *p*-cymene substrate, while CYP1A2 had the lowest enzymatic affinity. Moreover, CYP3A5 and CYP2D6 were shown to have the highest catalytic efficiency (K_{cat}/K_m) for the formation of *p*-isopropylbenzylalcohol (Table 2). For the enzymatic product thymol, the enzymatic affinities were almost equal for CYP1A2, CYP2A6 and CYP3A5, while for CYP2D6 the affinity was just 20%. The highest catalytic efficiency had CYP3A5, while the catalytic efficiency of CYP1A2 was approximately 14% of that of CYP3A5 (Table 2).

Enzymatic affinities for the enzymatic product *p*-isopropylbenzaldehyde were in the same range, but the enzymatic catalytic efficiency of the CYP1A2 was approximately 30% higher than CYP2D6 (Table 2). The measured enzymatic activities were in approximately the same range as the previously reported enzymatic hydroxylation activities by other CYPs (Miyazawa & Shindo 2001; Duisken et al. 2005b). Accordingly, the formation of thymol may be the result of an enzymatic reaction mechanism that can occur by two different catalysed enzymatic reactions (Guengerich 2003): (1) epoxidation of one of the π -double bonds in the benzene moiety followed by an NIH shift or (2) by binding of the benzene moiety of the *p*-cymene molecule to oxo-Fe-heme of the CYP followed by a rearrangement of the bound and NIH shift (Figure 4).

Confirmation of *in vitro* results was performed by an *in vivo* experiment with a volunteer taking orally a complementary and alternative medicine containing 1% TTO. Results of the collected blood and urine samples showed that the main formed enzymatic product thymol could be identified in both urine and blood samples after enzymatic hydrolysis of its glucuronide/sulphate conjugate. Beside the metabolite thymol, other

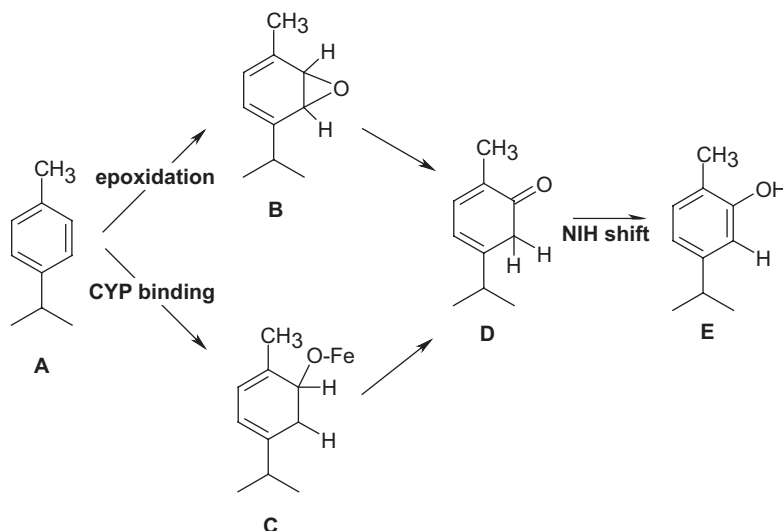


Figure 4. Postulated enzymatic reaction mechanism for the formation of thymol from the substrate *p*-cymene by arene-epoxidation or by binding of the substrate to the oxo-Fe group of the cytochrome P450 (CYP) enzyme followed by an NIH shift.

substances were also identified such as terpinen-4-ol and 2-hydroxycineole. Both substances were only identified in urine samples but not in any blood sample. The high concentration of terpinen-4-ol (Carson et al. 2006) in TTO might explain the presence of this substance in urine and blood samples. With highest possibility both substances were present in the urine samples as the glucuronide or sulphate conjugate. We concluded that both substances originated from the intake of the TTO pills because they are like *p*-cymene which is an ingredient of TTO. Moreover, 2-hydroxy-cineole is an enzymatic metabolite of 1,8-cineole (Figure 1 (8)) previously reported by Duisken et al. (2005b).

Although there are no data about the metabolism of this monoterpene by human CYP reported in literature to our knowledge, until now the metabolism of *p*-cymene was studied only in rabbits (Ishida et al. 1981; Matsumoto et al. 1992), possums (Southwell et al. 1980), rats and guinea pigs (Walde et al. 1983). Southwell et al. (1980) identified the metabolite *p*-cresol (*p*-methylphenol) in possums, while another *in vivo* study by Walde et al. (1983) with guinea pigs and rats reported the formation of a total of 18 urinary metabolites. Some metabolites were ring-hydroxylated such as carvacrol (*p*-cymene) and hydroxycarvacrol (guinea pigs). Matsumoto et al. (1992) identified three carboxylated and one hydroxylated metabolite. The formation of the carboxylated metabolite in rabbits was confirmed by Ishida et al. (1981). The present study only identified three hydroxylated enzymatic products and one aldehyde. Carboxylated enzymatic products were not identified because GC-MS was applied for the identification of enzymatic products. Thereby carboxylated products can only identified if they are derivatized before GC-MS analysis.

However, we suggest that because of the strong polar properties of these carboxylated enzymatic products, the allergenic properties are very low or even absent. Therefore, we choose not to detect and identify carboxylated enzymatic products.

As discussed above, the enzymatic transition of *p*-cymene into thymol may occur by intermediate formation of epoxides that can easily react with nucleophilic groups of (skin) proteins leading to the formation of haptens which may cause (skin) allergic reactions in the host. The second possible enzymatic formation reaction of thymol is the formation by binding of *p*-cymene to the oxo-Fe group of the CYP, which may have a direct but temporary influence on the catalytic properties of the CYP enzyme. That this reaction occurred was not directly confirmed, but all the results from the enzymatic assays indicate that no inactivation of the studied human recombinant CYP enzymes was observed not by the substrate nor by one of the formed enzymatic products, so we concluded that there was no mechanism-based inactivation of the CYPs as reported for olefins (Murray & Reidy 1990).

Conclusion

The results obtained confirm that with the enzymatic transformation of *p*-cymene by human CYP, an enzymatic product may be formed by arene-hydroxylation resulting in an epoxide that is rearranged by an NIH shift to form a ring-hydroxylated enzymatic product, thymol. However, the enzymatic product can also be the result of a reversible binding of the *p*-cymene to the oxo-Fe group of the CYP enzyme followed by the

equal rearrangement reaction as for the formation of the epoxide. In case arene-hydroxylation is the major enzymatic reaction, an electrophilic intermediary is formed that may cause sensitization and irritational skin reactions after binding to skin proteins with a topical application of TTO. No mechanism-based inactivation of CYP was observed, indicating that the formation of thymol might also be the result of binding of *p*-cymene to the oxo-Fe group.

Acknowledgements

This work was supported by grant number BL 340/3-1 from the German Research Foundation (DFG). The authors thank Wolfgang Dott of the RWTH Aachen for continuous support and Brunhilde Blömeke for helpful discussions.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

- Aberer W. (2008). Contact allergy and medicinal herbs. *J Ger Soc Dermatol* 6:15–24.
- Apted JH. (1991). Contact dermatitis associated with the use of tea-tree oil. *Aust J Dermatol* 32:177.
- Baron JM, Merk HF. (2001). Drug metabolism in the skin. *Curr Opin Allerg Clin Immunol* 1:287–91.
- Bhushan M, Beck MH. (1997). Allergic contact dermatitis from tea tree oil in a wart paint. *Contact Dermat* 36:117–18.
- Bylund J, Kunz T, Valmsen K, Oliw EH. (1998). Cytochromes P450 with bisallylic hydroxylation activity on arachidonic and linoleic acids studied with human recombinant enzymes and with human and rat liver microsomes. *J Pharmacol Exp Ther* 284:51–60.
- Carson CF, Hammer KA, Riley TV. (2006). Melaleuca alternifolia (tea tree) oil: a review of antimicrobial and other medicinal properties. *Clin Microbiol Rev* 19:50–62.
- Carson CF, Riley TV. (2001). Safety, efficacy and provenance of tea tree (Melaleuca alternifolia) oil. *Contact Dermat* 45:65–7.
- Chorianopoulos N, Kalpoutzakis E, Aligiannis N, Mitaku S, Nychas GJ, Haroutounian SA. (2004). Essential oils of Satureja, Origanum, and Thymus species: chemical composition and antibacterial activities against foodborne pathogens. *J Agric Food Chem* 52:8261–7.
- Duisken M, Benz D, Peiffer TH, Blomeke B, Hollender J. (2005a). Metabolism of Delta(3)-carene by human cytochrome p450 enzymes: identification and characterization of two new metabolites. *Curr Drug Metab* 6:593–601.
- Duisken M, Sandner F, Blomeke B, Hollender J. (2005b). Metabolism of 1,8-cineole by human cytochrome P450 enzymes: identification of a new hydroxylated metabolite. *Biochim Biophys Acta Gen Subj* 1722:304–11.
- Edris AE. (2007). Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. *Phytother Res* 21:308–23.
- Goeptar AR, Scheerens H, Vermeulen NP. 1995. Oxygen and xenobiotic reductase activities of cytochrome P450. *Crit Rev Toxicol* 25:25–65.
- Guengerich FP. (2003). Cytochrome P450 oxidations in the generation of reactive electrophiles: epoxidation and related reactions. *Arch Biochem Biophys* 409:59–71.
- Hammer KA, Carson CF, Riley TV. (2003). Antifungal activity of the components of Melaleuca alternifolia (tea tree) oil. *J Appl Microbiol* 95:853–60.
- Hammer KA, Carson CF, Riley TV, Nielsen JB. (2006). A review of the toxicity of Melaleuca alternifolia (tea tree) oil. *Food Chem Toxicol* 44:616–25.
- Hausen BM, Reichling J, Harkenthal M. (1999). Degradation products of monoterpenes are the sensitizing agents in tea tree oil. *Am J Contact Dermat* 10:68–77.
- International Organisation for Standardisation (ISO) (1996). ISO 4730: Oil of melaleuca, terpinen-4-ol type (tea tree oil). Geneva: International Organisation for Standardisation.
- Ishida T, Asakawa Y, Takemoto T, Aratani T. (1981). Terpenoids biotransformation in mammals III: Biotransformation of alpha-pinene, beta-pinene, pinane, 3-carene, carane, myrcene, and p-cymene in rabbits. *J Pharmaceut Sci* 70:406–15.
- Jappe U, Schnuch A, Uter W. (2005). Rosacea and contact allergy to cosmetics and topical medicaments — retrospective analysis of multicentre surveillance data 1995–2002. *Contact Dermat* 52:96–101.
- Johansson S, Gimenez-Arnau E, Grotli M, Karlberg AT, Borje A. (2008). Carbon- and oxygen-centered radicals are equally important haptens of allylic hydroperoxides in allergic contact dermatitis. *Chem Res Toxicol* 21:1536–47.
- Kleiner SM. (1995). The true nature of herbs. *Phys Sportsmed* 23:13–14.
- Knight TE, Hausen BM. (1994). Melaleuca oil (tea tree oil) dermatitis. *J Am Acad Dermatol* 30:423–7.
- Larsen W, Nakayama H, Fischer T, Elsner P, Frosch P, Burrows D, Jordan W, Shaw S, Wilkinson J, Marks J, Jr., Sugawara M, Nethercott M, Nethercott J. (2001). Fragrance contact dermatitis: a worldwide multicenter investigation (Part II). *Contact Dermat* 44:344–6.
- Matsumoto T, Ishida T, Yoshida T, Terao H, Takeda Y, Asakawa Y. (1992). The enantioselective metabolism of p-cymene in rabbits. *Chem Pharmaceut Bull* 40:1721–6.
- Meesters RJ, Duisken M, Hollender J. (2007). Study on the cytochrome P450-mediated oxidative metabolism of the terpene alcohol linalool: indication of biological epoxidation. *Xenobiotica* 37:604–17.
- Meesters RJ, Duisken M, Jahnigen H, Hollender J. (2008). Sensitive determination of monoterpene alcohols in urine by HPLC-FLD combined with ESI-MS detection after online-solid phase extraction of the monoterpene-coumarincarbamate derivatives. *J Chromatograph B Analyt Tech Biomed Life Sci* 875:444–50.
- Miyazawa M, Shindo M. (2001). Biotransformation of 1,8-cineole by human liver microsomes. *Natural Prod Lett* 15:49–53.
- Murray M, Reidy GF. (1990). Selectivity in the inhibition of mammalian cytochromes P-450 by chemical agents. *Pharmacol Rev* 42:85–101.
- Nilsson AM, Gafvert E, Salvador L, Luthman K, Bruze M, Gruvberger B, Nilsson JL, Karlberg AT. (2001). Mechanism of the antigen formation of carvone and related alpha, beta-unsaturated ketones. *Contact Dermat* 44:347–56.
- Nostro A, Blanco AR, Cannatelli MA, Enea V, Flamini G, Morelli I, Sudano Roccaro A, Alonzo V. (2004). Susceptibility of methicillin-resistant staphylococci to oregano essential oil, carvacrol and thymol. *FEMS Microbiol Lett* 230:191–5.
- Papadopoulos CJ, Carson CF, Hammer KA, Riley TV. (2006). Susceptibility of pseudomonads to Melaleuca alternifolia (tea tree) oil and components. *J Antimicrob Chemother* 58:449–51.
- Rastogi SC, Heydorn S, Johansen JD, Basketter DA. (2001). Fragrance chemicals in domestic and occupational products. *Contact Dermat* 45:221–5.
- Rubel DM, Freeman S, Southwell IA. (1998). Tea tree oil allergy: what is the offending agent? Report of three cases of tea tree oil allergy and review of the literature. *Australasian J Dermatol* 39:244–7.
- Rutherford T, Nixon R, Tam M, Tate B. (2007). Allergy to tea tree oil: retrospective review of 41 cases with positive patch tests over 4.5 years. *Australasian J Dermatol* 48:83–7.
- Schelz Z, Molnar J, Hohmann J. (2006). Antimicrobial and antiplasmod activities of essential oils. *Fitoterapia* 77:279–85.

- Skold M, Borje A, Harambasic E, Karlberg AT. (2004). Contact allergens formed on air exposure of linalool. Identification and quantification of primary and secondary oxidation products and the effect on skin sensitization. *Chem Res Toxicol* 17:1697-705.
- Southwell IA, Flynn TM, Degabriele R. (1980). Metabolism of alpha- and beta-pinene, p-cymene and 1,8-cineole in the brushtail possum, *Trichosurus vulpecula*. *Xenobiotica* 10:17-23.
- Thomson KF, Wilkinson SM. (2000). Allergic contact dermatitis to plant extracts in patients with cosmetic dermatitis. *Br J Dermatol* 142:84-8.
- Valero M, Salmeron MC. (2003). Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. *Int J Food Microbiol* 85:73-81.
- Walde A, Ve B, Scheline RR, Monge P. (1983). p-Cymene metabolism in rats and guinea-pigs. *Xenobiotica* 13:503-12.
- Woolf A. 1999. Essential oil poisoning. *J Toxicol Clin Toxicol* 37:721-7.
- Wrighton SA, Brian WR, Sari MA, Iwasaki M, Guengerich FP, Raucy JL, Molowa DT, Vandenbranden M. (1990). Studies on the expression and metabolic capabilities of human liver cytochrome P450III_{A5} (HLP3). *Mol Pharmacol* 38:207-13.