Bioconversion of mono- and sesquiterpenoids by recombinant human cytochrome P450 monooxygenases

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Abstract
Cytochrome P450 monooxygenases play an important role in the biosynthesis and metabolism of terpenoids. We explored the potential of recombinant human liver cytochrome P450 monooxygenases CYP1A2, CYP2C9, and CYP3A4, heterologously expressed in *E. coli*, to convert mono- and sesquiterpenoids to human metabolites. This natural product group is a diverse class of secondary metabolites and includes several industrially and pharmaceutically interesting compounds. Incubation of cedrol with CYP3A4 resulted in a bioconversion of 74 % (± 8.9 %) after 1 hour of the unknown metabolites 2-hydroxycedrol and 4-hydroxycedrol, which have been structurally elucidated by H/C-NMR and GC-MS. We conclude that recombinant human cytochrome P450 enzymes can be useful tools in a combinatorial biosynthesis strategy for the production of new natural products and for *in vitro* metabolism studies.
Introduction

Cytochrome P450 monooxygenases are important enzymes in the biosynthesis of plant secondary metabolites such as terpenoids. Terpenoids originate from general aliphatic precursors like geranyl diphosphate and farnesyl diphosphate and the first committed step is cyclization by terpene synthases. In subsequent biosynthetic steps, introducing modifications like oxygenation or hydroxylation, cytochrome P450 monooxygenases are often involved. As example, in the biosynthetic pathway leading to the antineoplastic agent paclitaxel, the precursor taxadiene is converted by eight cytochrome P450 enzymes [58]. Another example is the biosynthesis of artemisinin, a potent antimalarial drug, in which a complex cytochrome P450 dependent hydroxylation is assumed [43]. Recently the cDNA clone CYP71AV1 encoding a cytochrome P450 enzyme has been isolated from Artemisia annua that is capable of catalyzing the hydroxylation of amorphadiene to artemisinic alcohol and additionally further oxidation to artemisinic aldehyde and artemisinic acid [41]. Isolation and identification of plant enzymes especially cytochromes and corresponding genes is laborious and the presence of many isoenzymes with highly specialised substrate specificity - as known for Arabidopsis thaliana with more than 100 cytochrome (CYP) genes have been found [179] – hampers the search for a specific biocatalyst. That is why there is need for alternative biological systems to convert selected structures in order to mimic plant biosynthetic steps. As an interesting side effect this biosynthesis studies allow to modify pathways to design new structures that are not known in nature.

Modern biotechnology techniques and the availability of suitable organisms as host cells offer the possibility to combine the potential of enzymatic bioconversions of different organisms. In the class of the polyketide antibiotics the strategy of combinatorial biosynthesis has already led to new lead compounds. Recent work shows the successful production of terpenoids using the endogenous or engineered biosynthetic pathway of host cells like Escherichia coli [12, 32] or Saccharomyces cerevisiae [13, 14, 180].

In contrast to plant cytochrome P450 monooxygenases, human liver cytochrome P450 enzymes are known to metabolize a broad variety of exogenous compounds. Over 65% of administered drugs are metabolized in the liver by the cytochrome P450 enzymes CYP3A4, CYP2C9 and CYP1A2 [181]. In most cases this involves hydroxylation and O-dealkylation reactions. Since human cytochrome P450 genes have been sequenced and expressed in E. coli [182], the activity of the enzymes can be tested in vitro for any substrate available. By exploiting the broad substrate specificity of this class of enzymes, new compounds of commercial or pharmaceutical interest may be obtained or produced on a larger scale. In contrast to most plant enzymes the gene sequences of most of the human cytochrome P450 enzymes are well known and can be used for genetic engineering. It is an interesting approach to use recombinant human cytochrome P450 enzymes directly without spending tremendous efforts to clone and express cytochrome P450 genes from distinct plants.

The biotransformation of terpenoids by mammals, microorganisms, and plant cultures has recently been reviewed [183]. As model compounds with relation to food products or herbal medicines cedrol and several mono- and sesquiterpenoids were chosen to investigate the in vitro metabolism by human cytochrome P450 enzymes. Especially the biotransformation of cedrol (Fig. 1), an oxygenated sesquiterpenoid isolated from the volatile oil of Juniperus sp., is previously well described for rabbits [184], dogs [185], and several microorganisms [186-194]. According to these publications a high variety of oxygenated compounds was detected. The aims of the present study were, first, to explore...
the biosynthetic potential of three human cytochrome P450 enzymes, CYP3A4, CYP2C9, and CYP1A2, heterologously expressed in *E. coli* DH5α to mimic plant related bioconversion, secondly, to study the selectivity of these enzymes in comparison to plant cytochrome P450s, and in conclusion, to give a proof of concept that heterologous cytochromes may replace plant cytochromes for distinct bioconversion concepts.

![Chemical structures of cedrol and the metabolites 2-hydroxycedrol and 4-hydroxycedrol](image)

**Fig. 1**: Chemical structures of cedrol and the metabolites 2-hydroxycedrol and 4-hydroxycedrol

## Materials and Methods

### Chemicals

Cedrol was obtained from Fluka (Zwijndrecht, the Netherlands), all other terpenoids were from commercial source (Fluka, Aldrich, USA, and Extrasynthese, France). Ethyl acetate, *n*-hexane, diethyl ether and methanol were from Merck (HPLC grade, Darmstadt, Germany). Culture medium components were from Duchefa (Haarlem, the Netherlands). All other chemicals were purchased from Sigma (USA). Carbon monoxide gas was obtained from Hoekloos (the Netherlands).

### Bacterial strains and plasmids

The human genes encoding CYP1A2, CYP2C9, and CYP3A4 were cloned together with a NADPH-P450 reductase gene into a bicistronic pCW vector [182]. The used vector allowed independent expression of the monooxygenase and the reductase gene. Plasmids without a monooxygenase encoding gene were used for control experiments and were revered to as control plasmid. All described plasmids were a kind gift from F.P. Guengerich (Vanderbilt University School of Medicine, Nashville). Expression was performed in *E. coli* DH5α (Gibco BRL, USA).

### Expression of cytochrome P450 enzymes

*E. coli* DH5α containing the expression plasmid was grown overnight (37 °C, 250 rpm) in 10 ml LB-medium (100 µg ml⁻¹ of ampicillin). This culture was used for inoculation
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(1:100) of 30 ml TB-medium supplemented with 1% glucose, 100 µg ml⁻¹ of ampicillin and 0.25 ml of a solution of trace elements [195] that was additionally grown (37 °C, 200 rpm) to an OD600 of 0.5. Expression was induced by adding IPTG (1.0 mM), thiamine (1.0 mM) and δ-aminolevulinic acid (0.5 mM) followed by another 20 h of growth (30 °C, 200 rpm). Cells were harvested by centrifugation (10 min, 2500x g, 4 °C) after the determination of the OD600, and washed with potassium phosphate buffer (0.1 M, pH 7.4). Cells were resuspended to an OD600 of 15.0 in potassium phosphate buffer (0.1 M, pH 7.4) and were used for bioconversion assays directly.

**CO-saturation difference analysis**

In order to determine the amount of active cytochrome P450 enzyme in the bacterial cells, potassium phosphate buffer (0.1 M, pH 7.4) was gently mixed with cell culture (1:1) in a 1 ml cuvette. A small amount of sodium hydrosulphite was added and carefully mixed. After 1 min the baseline absorption (400-600 nm) was recorded. Then the mixture was gassed through with CO for 30 s, with a gas flow of 1-2 bubbles s⁻¹, and again the absorption spectrum at 400-600 nm was recorded. The concentration of protein was calculated by extracting the extinction at 490 nm from 450 nm using an extinction coefficient of 91 mM⁻¹ cm⁻¹ [196]. Measurements have been performed on a Unicam UV500 Thermo spectronics spectrophotometer.

**Bioconversion assays**

Assays were performed in potassium phosphate buffer (0.1 M, pH 7.4) containing glucose (12.5 mM). Cells with expressed cytochrome P450 enzyme were added to the buffer (200 µl ml⁻¹) together with the one of the substrates (0.2 mM), NADP (1 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (1 u ml⁻¹). Finally, MgCl₂ (30 mM) was added, followed by incubation for 2 h (37 °C, 250 rpm). The substrates were dissolved in methanol (20 mM).

Bioconversions were performed in a final volume of 1 ml. After incubation the reaction mixture was extracted twice with 2 ml ethyl acetate. The combined organic layers were dried over anhydrous sodium sulphate and evaporated to a volume of 150 µl. This residue was submitted to GC and GC-MS analysis. For HPLC the ethyl acetate was evaporated to dryness and the residue dissolved in 150 µl methanol. For the isolation and purification of the products from the cedrol bioconversion large scale assays were performed with a total volume of 50 ml in 500 ml glass Erlenmeyer flasks. After extraction the ethyl acetate fractions were pooled and evaporated to a suitable small volume for further isolation.

**Isolation and purification**

The concentrated product solution was purified by flash chromatography. A glass column (30 x 2.0 cm) with silica gel 60 (ICN; 63-200 mesh, 600 nm) was used. The products were eluted with a mixture of n-hexane with 10% diethyl ether. Fractions of 100 ml were collected and checked by GC. After cedrol was eluted (200 ml), the diethyl ether concentration was raised to 25% to elute the two unknown sesquiterpenoid alcohols The fraction containing the bioconversion products was evaporated to dryness and the residue dissolved in a mixture of n-hexane and diethyl ether (1:1) and placed at 4 °C for precipitation.
**HPLC**

For analysis of the control bioconversions performed with phenacetin (CYP1A2), tolbutamide (CYP2C9) and testosterone (CYP3A4) a Shimadzu-VP system (Shimadzu, the Netherlands) consisting of a LC-10ATvp pump, a Kontron 360 auto sampler, a SPD-M10Avp DAD detector (200 – 340 nm, band with: 4 nm), a FCV-10A1vp low pressure gradient mixer, a SCL-10A1vp system controller, a FIATron systems CH-30 column heater (USA), and CLASS-VP software, version 6.12SP4. ProFill 25 mm syringe HPLC filters, nature, PTFE, pore size 0.45 µm (Alltech Applied Science Group, the Netherlands) were used, together with 2 ml autosampler vials (Brown Chromatography Supplies, Germany). As crimp seals, 11 mm with rubber/PTFE septa (Brown Chromatography Supplies, Germany) were used.

The column we used was a Luna C18(2), 250 x 4.6 mm, 5µm, together with a Phenomenex guard cartridge C18 (ODS, 4x3 mm). (Phenomenex, USA). The injection volume was 20 µl with a flow rate of 1 ml min\(^{-1}\) using a time programme of 30 minutes consisting of 5 min 95% solvent A (5 mM ammonium formate (0.05% formic acid) : ACN = 800 : 156 (w/w)) and 5% solvent B (ACN : MeOH (0.05% formic acid) : 5 mM ammonium formate (0.05% formic acid) = 585:40:200 (w/w)), followed by a gradient to 100% solvent B in 19 min, 2 min 100% solvent B, a gradient back to 5 % solvent B in 2 min and remaining on this final concentration for another 2 min (based on Walsky, et al. 2004 [197]). All peaks, substrates and metabolites, were identified by injecting the individual reference compounds. Detection of the compounds was performed at 220 nm.

**Gas Chromatography**

GC analysis was performed on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a 7673 injector and a Hewlett Packard 3365 Series II Chemstation. For a rapid control of the different fractions, a WCOTT fused-silica CP-Sil 5CB, (10 m x 0.25 mm i.d.; film thickness, 0.12 µm, Varian (Chrompack), USA) was used. Oven temperature programme: 50 °C rising to 200 °C at 5 °C min\(^{-1}\); injector temperature: 250 °C; detector temperature (FID): 300 °C. Carrier gas: He; inlet pressure: 5 bar; linear gas velocity: 27 cm s\(^{-1}\); split ratio: 95:1; injected volume: 2 µl.

Retention indices (RI) were performed with the same equipment and under the following conditions: column, WCOT fused-silica (J & W Scientific, Folsom, USA) DB-5 (30 m x 0.26 mm; film thickness 0.25 µm); oven temperature programme, 60 °C-290 °C at 3 °C min\(^{-1}\); injector temperature, 250 °C; detector (FID) temperature, 300 °C; carrier gas, He; inlet pressure, 18 bar; linear gas velocity, 31.8 cm s\(^{-1}\); split ratio, 56:1; injected volume, 1.0 µL.

**Gas Chromatography-Mass Spectrometry**

A Shimadzu GCMS QP5000 system was used equipped with a GC-17A gas chromatograph, an AOC-20ii auto injector, and GCMS solution version 1.10 software. The GC conditions were: column, WCOT fused-silica (J & W Scientific, USA) DB-5 (30 m x 0.26 mm; film thickness 0.25 µm); oven temperature programme, 50-250 °C at 5 °C min\(^{-1}\); injector temperature, 275 °C; carrier gas, helium; inlet pressure, 75 pKa; linear gas velocity, 81.4 cm s\(^{-1}\); column flow, 2.5 ml min\(^{-1}\); total flow, 56.7 ml min\(^{-1}\); split ratio, 21:1; injected
volume, 1.0 μL. MS conditions: ionization energy, 70 eV; ion source temperature, 250 °C; interface temperature, 250 °C; scan speed, 3 scans s⁻¹; mass range, 34-300 u.
The identity of the components was assigned by comparison of the retention indices (RI), relative to C₉-C₂₀ n-alkanes, mass spectral databases, and data from literature[198, 199]. The percentages of the components were calculated from the GC peak areas, using the normalization method.

**NMR-analysis**
The spectra were acquired using an AVANCE 600 NMR spectrometer (14.1 T) Bruker BioSpin GmbH, Germany) with a 5 mm BBI probe and a DMX 400 NMR spectrometer (9.4 T, Bruker) with a 5 mm TBI probe.
The ¹H NMR spectrum was acquired at 14.1 T (Larmor frequency of 600.2 MHz) using a 30° pulse, a pre-acquisition delay of 12 μs, 64 k data points (corresponding to an acquisition time of 4.6 s at a spectral width of 7184 Hz), a relaxation delay of 15 s, and a total of 16 scans. Fourier transformation was done after zero filling the data to 64 k time domain points and exponential filtering (em command) of 0.3 Hz.
The ¹³C NMR spectrum was recorded at 14.1 T (Larmor frequency of 150.9 MHz) using a 90° pulse with ¹H decoupling during the acquisition time. The acquisition of the spectrum was set by a pre-acquisition delay of 12 μs, 96 k data points (corresponding to an acquisition time of 1.6 s at a spectral width of 30303 Hz), a relaxation delay of 20 s, and a total of 2568 scans. Fourier transformation was done after zero filling the data to 128 k time domain points and exponential filtering of 1.0 Hz.
The ¹³C DEPT was acquired at 9.4 T (Larmor frequency of 100.6 MHz) using a 135° read pulse with ¹H decoupling during acquisition. For acquisition a pre-acquisition delay of 30 μs, 128 k data points (corresponding to an acquisition time of 3.0 s at a spectral width of 22124 Hz), a relaxation delay of 6 s, and a total of 7726 scans. Fourier transformation was done after zero filling the data to 128 k time domain points and exponential filtering of 1.0 Hz.
The HMQC was acquired at 14.1 T using 256 data points for the direct dimension (¹H, spectral width of 1202 Hz)) and 177 data points for the indirect (¹³C, spectral width of 18112 Hz), a relaxation delay of 1.5 s, and a total of 32 scans. Fourier transformations were done to both directions after zero filling to 1 k domain points for the direct as well as the indirect dimension.
The HMBC was acquired at 14.1 T using 1 k data points for the direct dimension (¹H, spectral width of 1796 Hz) and 154 data points for the indirect (¹³C, spectral width 18116 Hz), a relaxation delay of 1.5 s, and a total of 256 scans. Fourier transformations were done in both directions after zero filling to 2 k domain points (direct dimension) and 1 k domain points (indirect dimension). All 2D spectra were printed as magnitude spectra. The sample was dissolved in dimethylsulfoxide-d₆ (DMSO, Merck, D > 99.96 %). All spectra were calibrated to the resonance lines of DMSO (δ (¹H) = 2.49 ppm, δ (¹³C) = 39.50 ppm).
Results and Discussion

Expression of CYP1A2, CYP2C9, and CYP3A4

Initially, growth and expression levels of the transformed *E. coli* strains were examined. All strains showed a similar growth curve after induction with IPTG. Genetic modified strains expressing CYP450 reached a final optical density that was between 20 and 50% lower as compared to the control strain (Fig. 2A). All transformed strains showed expression of the cytochrome P450 gene with a maximum level of recombinant enzyme 20 h after induction (Fig. 2B). The protein concentrations differed, transformed hosts expressing CYP1A2 showed highest levels (33.4 pmol/ml/OD\(_{600\text{ nm}}\)), followed by CYP3A4 (22.1 pmol/ml/OD\(_{600\text{ nm}}\)) and CYP2C9 (8.87 pmol/ml/OD\(_{600\text{ nm}}\)). During the bioassay protein concentration and activity has been controlled for all grown cultures over time.

To document activity of the heterologous proteins, reference substrates that are specifically metabolised and give defined metabolic products were used. For CYP1A2, CYP2C9, CYP3A4 metabolisation studies with phenacetine, tolbutamide, and testosterone, respectively, were carried out and paracetamol, hydroxytolbutamide, and 6β-hydroxytestosterone, respectively, were determined as known metabolites. No other metabolites were detected.

Bioconversion experiments

Small scale assays with a total volume of 1 ml were performed to determine the bioconversion of a list of terpenoids of pharmaceutical interest. The enzymes CYP1A2 and CYP2C9 did not display any activity to the tested terpenoids. Mono- and sesquiterpenoids, like cedrol, (-)-aristolene, (+)- and (-)-carvon, caryophyllene oxide, and nootkatone, underwent bioconversion and showed an interesting metabolic profile in the presence of CYP3A4. Especially the bioconversion of cedrol will be discussed in detail. α-Bisabolol, (+)-δ-cadinene, camphor, caryophyllene alcohol, (-)-α-cedrene, (+)-β-cedrene, (-)-α-copaene, (-)-α-cubebebe, (α)-humulene, (β)-humulene, (γ)-humulene, R-(+)-limonene, and S-(-)-limonene were not metabolised either by CYP3A4, CYP1A2 or CYP2C9. Most of the metabolites were identified by retention indices, mass spectral data, database search and in house standards.

Bioconversion of cedrol

Cedrol was chosen as model compound, because of the commercial interest from industry and its bioconversion is well described [184-194]. As initial orientating experiment cedrol was added as substrate to transformed *E. coli* cells with a total volume of 1 ml. Where CYP1A2 and CYP2C9 did not lead to any bioconversion, cedrol added to cells containing CYP3A4 resulted in two extra peaks with retention indices (RI) of 1769 (structure I) and 1789 (structure II), whereas the non metabolised cedrol showed a retention index of 1592. Different incubation times of CYP3A4 and cedrol resulted in 51.7% (± 5.14%) bioconversion after 20 min and in a maximum of 74% (± 8.9%) conversion of cedrol already obtained after 1 hour of incubation. Prolongation of the incubation time to 2, 8, and 30 hours did not result in a significant increase of the percentage bioconversion, showing respectively 74.6% (± 10.9%), 74.1% (± 6.0%), and 83.0% (± 7.9%) bioconversion. In
Fig. 2: Growth curves of *E. coli* DH5α strains expressing the human cytochrome P450 enzymes (A) and levels of the expressed cytochrome P450 enzymes during bacterial growth based on CO-saturation spectra and corrected for the amount of cells (OD600) (B). (control (●); CYP1A2 (○); CYP2C9 (▼); CYP3A4 (Δ))
parallel, enhancement of the protein or cedrol concentration did not increase the absolute amount of products formed. Both products always appeared in a ratio of 3.23 : 1 (± 0.15) for structure I to structure II, independently of modified assay conditions like the amount of cedrol added, protein concentration or time of incubation. From databank search it appeared that the detected compounds were not known. GC-MS analysis indicated that the molecular weight for both products was m/z 238 (C_{15}H_{26}O_2), whereas cedrol has a molecular weight of m/z 222 (C_{15}H_{26}O). The mass fragmentation pattern for structure I: m/z (rel. int.): 238[M]^+(0); 220(4); 205(4); 187(2); 177(3); 162(12); 149(16); 135(12); 121(11); 107(17); 93(26); 79(11); 69(10); 55(13); 43(100); and for structure II: m/z (rel. int.): 238[M]^+(0); 223(2); 205(2); 187(2); 177(4); 162(13); 151(58); 133(47); 121(12); 107(28); 93(100); 79(13); 69(12); 55(21); 43(80).

To isolate the unknown compounds and to allow structure elucidation, bioconversion was scaled up to a volume of 50 ml per batch. After fractionation of the reaction mixture and isolation on a silica gel column, 4.7 mg of residue containing both bioconversion products was obtained. Final fractionation resulting in two pure components was not possible and therefore structure elucidation was performed with the mixture as starting material with sophisticated spectroscopic techniques.

Various NMR techniques were needed to elucidate the structure of the sample. In the one dimensional (1D) $^1$H NMR spectrum (Table 1) eight resonance lines in the range of 0.8 ppm up to 1.26 ppm could be identified as proton signals of methyl (CH$_3$) groups. These signals can be grouped into two components: four intense lines at 0.93 ppm, 1.01 ppm, 1.13 ppm, and 1.18 ppm (structure I) and four weak ones at 0.83 ppm, 1.03 ppm, 1.11 ppm, and 1.26 ppm (structure II). The rough intensity ratio is 3:1. Furthermore, the signal at 3.83 ppm was assigned as the proton of a methylidyne (CH) group of structure II bonded to a hydroxyl (OH), a methylene (CH$_2$), and a CH group, based on the shift value and the coupling pattern consisting of eight signals with similar intensities. This assignment was checked by further two dimensional (2D) NMR experiments. Further assignments were not possible due to overlapping and superposition of the $^1$H resonance lines.

The 1D $^{13}$C NMR spectrum shows 30 well separated signals; 15 for structure I and 15 for structure II. Moreover an additional $^{13}$C DEPT spectrum was obtained using a flip angle $\theta = 135^\circ$ to distinguish CH/CH$_3$ and CH$_2$ lines phased “up” and “down”, respectively. By comparing the 1D $^{13}$C with the DEPT spectrum the quaternary carbon species could be identified, too. All $^{13}$C chemical shifts are presented in Table 1. Due to the downfield shifts of 71.51 ppm, 72.51 ppm, 72.52 ppm, and 77.60 ppm it is concluded that four OH groups exist. Two of those belong to structure I and two to structure II. Moreover, the chemical shift values of all proton signals as well as the correlations between proton and carbon atoms over one bond were determined by $^1$H-$^{13}$C two dimensional NMR spectra (HMBC), which are presented in Table 1. Additional HMBC experiments were used to observe all $^1$H-$^{13}$C correlations over 2 and 3 bonds.

In summary, the NMR investigations verified two very similar structures with the only difference in the position of one OH group. The main component (structure I) is 2-hydroxycedrol (2,6,6,8-tetramethyl-tricyclo[5.3.1.0$^{1,5}$]undecan-2,8-diol) whereas the minor component (structure II) is 4-hydroxycedrol (2,6,6,8-tetramethyltricyclo[5.3.1.0$^{1,5}$]undecan-4,8-diol) (Fig. 1).
Bioconversion of terpenoids by rh-CYP450s

Table 1: Chemical shifts (1H- and 13C-NMR) of the bioconversion products 2-hydroxycedrol and 4-hydroxycedrol.

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* in ppm

Bioconversion of (-)-aristolene

(-)-Aristolene (RI= 1284) showed after bioconversion with CYP3A4 one extra peak with a RI of 1748. This peak was identified by its retention time and mass spectral data as aristolone. The mass fragmentation pattern for the detected peak was: m/z (rel.int.): 218 [M]⁺ (3); 203(68); 189(3); 175(28); 161(28); 147(54); 133(32); 119(44); 109(41); 91(64); 77(34); 67(22); 55(36); 43(39); 41(100), which stands in good correlation with mass data for aristolone.

Bioconversion of caryophyllene oxide

Caryophyllene oxide (RI=1574), showed four additional peaks after bioconversion with CYP3A4. Peak 1 with a RI of 1739 showed a mass fragmentation pattern m/z (rel.int.): 221 (<1); 193(<1); 179 (1); 163(2); 145(2); 133(4); 121(8); 108(15); 93(25); 79(22); 67(19); 55(33); 43(100); 41(60). Peak 2 (RI = 1754), peak 3 (RI = 1761), as well as peak 4 (RI = 1794) have almost the same fragmentation pattern as peak 1, with only small quantitative differences. No clear M⁺ ion could be observed but the molecular weight can be 234 and/or 236.
Bioconversion of nootkatone

Nootkatone (RI = 1800) showed one extra peak (RI = 1939) after bioconversion with CYP3A4. The mass fragmentation pattern was: m/z (rel. int.): 220(2); 216 (25); 206(11); 187(2); 176(45); 161(45); 147(25); 135(38); 119(7); 105(45); 91(70); 79(36); 67(27); 55(45); 43(100); 41(93). The mass fragmentation pattern of this structure indicates the presence of a sesquiterpene alcohol, but the structure does not stand in accordance with known nootkatol metabolites from literature [200].

Bioconversion of (+)- and (-)-carvone

(+)- and (-)-Carvone (RI = 1240) gave identical bioconversion products after conversion with CYP3A4. The metabolites were identified as the stereoisomers cis- and trans-dihydrocarvone (RI = 1190 and 1197, respectively). CYP3A4 showed a different specificity for these substrates. (+)- Carvone was converted into cis- and trans-dihydrocarvone in a ratio of 1:1, whereas for the conversion of (-)-carvone resulted the ratio was 10:1. The fragmentation pattern of both isomers was almost the same: m/z (rel. int.): 152 [M]+(15); 137(9); 123(3); 109(14); 95(71); 81(26); 67(100); 55(41); 41(81). Interestingly, carvone showed the same efficient bioconversion to dihydrocarvone with and without the expressed CYP3A4 enzyme. Our presumption is that not the recombinant expressed CYP3A4 is responsible for the bioconversion, but that the genuine *E. coli* cells themselves are responsible for this metabolisation step.

We found the same reaction mechanism for (-)-aristolene bioconversion, but the rate of bioconversion is much lower in the mixture using the control strain without CYP3A4.

Conclusions

Biotransformation of natural compounds using whole microorganisms often result in a complex mixture of products. In addition, the incubation time is often long and the enzymatic conversion not efficient. Heterologously expressed human CYP3A4 provides a fast and defined enzymatic system with a broad substrate specificity resulting in a smaller number of metabolites. This makes it more suitable for commercial applications.

Metabolic engineering is an important tool to improve the production of desired natural products in this context. With a full understanding of the biosynthetic pathway and identification of the responsible genes, it may be possible to bioengineer cell cultures for high and commercially sustainable production rates of plant based drugs. On the other hand, little is known about the precise sequence of plant cytochromes in most biosynthesis and alternative pathways and branched biosynthesis are major problems in the identification and elucidation of plant cytochromes. Using recombinant human cytochromes may show potential application to overcome this problem and to be an alternative production system.

So far, metabolization has been considered as drug detoxification in humans but no communication does describe recombinant human cytochromes for biosynthesis strategies. Since CYP3A4 has the broadest substrate specificity of all human liver cytochromes, it is obvious that this enzyme converted several terpenoid structures, where the other two enzymes tested showed no activity towards these substrates.

Using the heterologously expressed human CYP3A4 the sesquiterpene cedrol was converted into new compounds. The chemical structures of both products were identified by GC-MS and NMR analysis. The bioconversion assay using whole *E. coli* cells shows...
that this is an interesting approach in combinatorial biosynthesis to obtain known metabolites but also new compounds. On the other hand plant cytochromes have a different function in metabolism and ought to be more specific in the product formation whereas our results with the human enzyme showed that new metabolites like 2-hydroxycedrol or 4-hydroxycedrol can be formed. In conclusion, recombinant human cytochromes will not replace plant cytochromes in defined biosynthetic routes, but they have a great potential in combinatorial biosynthesis to show up an alternative route in drug discovery for seminatural products with improved pharmacological activities. Furthermore, using genetic techniques in the near future the known three dimensional structures of human cytochromes in in silico and directed evolution strategies will be of high importance to predict specificity of metabolization by rational changes of amino acid in the protein sequence.

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