

Hydroxylation of the triterpenoid dipterocarpol with CYP106A2 from *Bacillus megaterium*

Daniela Schmitz¹, Josef Zapp² and Rita Bernhardt¹

¹ Department of Biochemistry, Saarland University, Saarbruecken, Germany

² Department of Pharmaceutical Biology, Saarland University, Saarbruecken, Germany

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Correspondence

R. Bernhardt, Department of Biochemistry, Saarland University, Campus, B 2.2, 66123 Saarbruecken, Germany
Fax: +49 681 302 4739
Tel: +49 681 302 4241
E-mail: ritabern@mx.uni-saarland.de

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The bacterial steroid-hydroxylase CYP106A2 from *Bacillus megaterium* ATCC 13368 hydroxylates a variety of 3-oxo- Δ -4-steroids and has recently been shown to catalyse regioselective hydroxylation of the diterpene abietic acid, as well as the pentacyclic triterpene 11-keto- β -boswellic acid. The broad substrate spectrum of this enzyme makes it an excellent candidate for biotechnological application. Because the natural substrate of this enzyme is not known, we assumed that the whole substrate spectrum might not yet be fully discovered. The difference spectroscopy method was used to screen a natural product library of 502 compounds. Screening of the library resulted in the identification of twelve hits, among them eight potential and four known substrates for CYP106A2. Interestingly, when testing the potential substrates, product formation was obtained only with triterpenes, namely dipterocarpol and betulin. Dipterocarpol is the most promising compound for biotechnological application because it is a dammarane-type triterpenoid, as are the major bioactive compounds of ginseng. The dipterocarpol hydroxylation products were analysed by NMR and identified as 7 β -hydroxydipterocarpol and 7 β ,11 α -dihydroxydipterocarpol. To investigate the putative bioactive properties of these novel compounds, *in vitro* cytotoxicity assays with HeLa and COS-1 cells were performed. The substrate dipterocarpol and the dihydroxylated product did not show cytotoxic activity in our study. By contrast, the 7 β -hydroxylated product was found to be cytotoxic to both tested cell lines. This study highlights the potency of CYP106A2 as a versatile biocatalyst for the bioconversion of natural products into pharmaceutically relevant bioactive products.

Introduction

Cytochrome P450 enzymes (CYPs) constitute a superfamily of haem-thiolate proteins capable of converting a wide range of endogenous and exogenous substrates [1]. A wide range of reactions is also catalysed by these enzymes, i.e. hydroxylations, epoxidations and dealkylations [2]. Their ability to insert hydroxyl groups into even nonactivated carbon bonds is unique. To date, > 18 000 CYP genes have been discovered ([http://](http://www.ncbi.nlm.nih.gov/gene?term=cytochrome%20p450)

www.ncbi.nlm.nih.gov/gene?term=cytochrome%20p450), with representatives in all kingdoms of life [3], reflecting the essential roles these enzymes play in metabolism. For example, they are involved in the biosynthesis of cholesterol and cholesterol-derived sterols, prostaglandins, vitamins and eicosanoids, as well as in the metabolism of drugs and xenobiotics [4]. Moreover, mainly in plants and fungi, but also in

Abbreviation

CYP, cytochrome P450.

bacteria and animals, they take part in the biosynthesis and tailoring of important secondary metabolism products. The broad substrate range and the increasing number of CYP family members make these enzymes potent candidates for biotechnological applications [5].

However, CYPs are rarely employed as biocatalysts in industrial processes for several reasons. For example, a major disadvantage of human CYPs is that they are membrane-bound proteins and therefore difficult to express and handle, which limits productivity [5,6]. In addition, CYPs involved in degradation processes convert a broad range of substrates and this may lead to unspecific reactions. The use of soluble, bacterial enzymes, which can often be expressed more easily, provides a solution to overcome the limits of expression and expand the substrate spectrum compared with the human enzymes [7]. However, the identification and characterization of CYP substrates and their respective products precedes any possible biotechnological application. Screening techniques are required for the quick and easy identification of novel substrates. Hence, a multitude of screening systems have been developed, often based on the measurement of CYP activity, for example, by monitoring the formation of a fluorescent product [8]. These systems are suitable for screening mutant libraries for more active CYP variants or small-molecule libraries to identify new enzyme inhibitors. LC [9], LC-MS [10] GC-MS or MS [11] analyses remain the methods of choice for efficient substrate identification. Unfortunately, these techniques are often time-consuming and expensive. Other approaches rely on measuring the consumption of cosubstrates like NADPH and molecular oxygen to indirectly monitor substrate conversion. These methods have been used successfully for self-sufficient cytochromes like P450 BM3 (CYP102A1) that show very stringent coupling [12], but they remain challenging for cytochrome substrate interactions that show low coupling efficacy.

Fortunately, the chromophoric haem group of CYP can be used to analyse the enzymes' interaction with potential substrates or inhibitors, even at a high throughput [13]. Compounds that bind to the active site and thereby induce a high-spin shift in the haem iron are often substrates for these enzymes, whereas ligands that stabilize the enzyme in the low-spin state are more often inhibitors than substrates. The absorbance spectra display a shift in the *soret* band to higher wavelengths for low-spin enzymes (from 417 to ~425 nm; type II spectrum), and to lower wavelengths for high-spin enzymes (from 417 to ~380 nm; type I spectrum) [14]. Thus, difference spectroscopy is a

promising method for the cheap, fast and easy identification of putative CYP substrates.

The bacterial steroid hydroxylase CYP106A2 has been investigated extensively in our laboratory, and has been shown to be an excellent candidate for biotechnological applications [15,16]. The enzyme is able to convert a variety of substrates, especially triterpenes, usually with high regio- and stereoselectivity. Recently, we reported the role of this enzyme as a regioselective diterpene allylic hydroxylase, utilizing difference spectroscopy to screen a library containing 16 671 synthetic organic compounds [13]. Because the natural substrate of CYP106A2 is not known, we assumed that the substrate spectrum of this enzyme may not yet be fully discovered. Therefore, a natural product library was screened to identify pharmaceutically relevant new reaction products of CYP106A2.

Results

Natural product library screen

The purpose of this study was the functionalization of pharmaceutically relevant natural products by a bacterial CYP enzyme. Starting with the identification of new potential substrates, a commercially available natural product library (Biomol International, Enzo Life Sciences GmbH, Lörrach, Germany) comprising 502 organic molecules (flavonoids, terpenoids, peptolides, coumarines, synthetic derivatives) was screened using difference spectroscopy. Difference spectroscopy was adapted to the 96-well format and optimized to increase sensitivity. Optimizations were performed using betulinic acid [17] and cholestenone [18] as type I substrates, imidazole as a type II ligand, pregnenolone and 11-deoxycorticosterone as type 0 ligands [19]; the solvent dimethylsulfoxide was used as a blank. The enzyme concentrations ranged from 0 to 10 μM , and the compound concentration ranged from 0 to 50 μM . Optimal results were achieved using 5 μM enzyme and 5 μM substrate (Fig. 1). Pregnenolone and dimethylsulfoxide showed no spin-shift spectra; in both cases, only a decrease in the absorbance at 420 nm was observed. Imidazole induced a typical type II spectrum with a ΔA (440–420 nm) value of 0.053. Clear type I spectra were obtained with betulinic acid and cholestenone, with a ΔA (380–420 nm) value of 0.1017 for betulinic acid and 0.0214 for cholestenone, respectively (Fig. 1). Surprisingly, a type I shift with a ΔA of 0.047 was also achieved by using 11-deoxycorticosterone as substrate, which, to the best of our knowledge, has never been described causing a type I shift before (Fig. 1B).

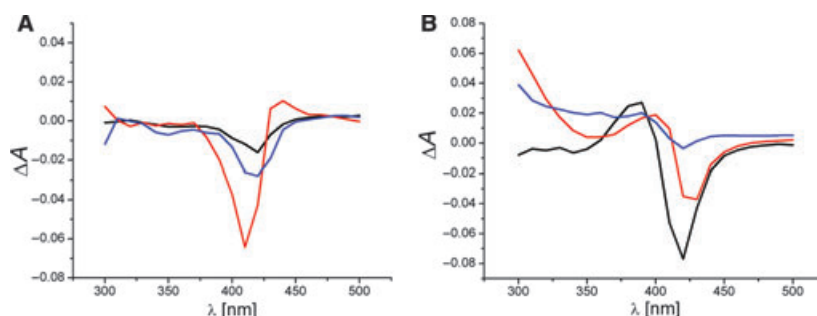


Fig. 1. Optimized screening conditions. Difference spectra of CYP106A2 (5 μ M) in 50 mM potassium phosphate buffer (250 μ L, pH 7.4) recorded in a 96-well plate. Absorbance scans performed from 300 to 500 nm in 10-nm steps and 100 reads per well. After recording the ligand-free spectra, 5 μ M compound (dissolved in 0.5 μ L dimethylsulfoxide) was added and incubated for 15 min at room temperature. Difference spectra were achieved by subtracting the ligand-free from the ligand-bound form. (A) Pregnenolone was chosen as the type 0 control (blue line), imidazole as the type II inhibitor (red line) and dimethylsulfoxide (black line) as the blank. (B) Betulinic acid (black line), cholesterone (blue line) and 11-deoxycorticosterone (red line) served as type I ligands.

After optimization, the screening was successfully applied to the natural product library and resulted in twelve hits (Fig. 2 and Scheme 1): four triterpenoid carbon acids (betulinic acid, oleanolic acid, ursolic acid and glycyrrhizic acid), four triterpenes (sarsasapogenin, panaxadiol, betulin and dipterocarpol), two flavonoids (rhamnetin and geraldol), one alkaloid (noscaphine) and one oxytetracycline derivative (α -apo-oxytetracycline).

The screening results were confirmed by recording the spectra in a two-beam photometer.

In vitro conversion assays

Because binding to the active site is an essential, but not the only, requirement for catalytic activity, verification of the screening hits as substrates was done

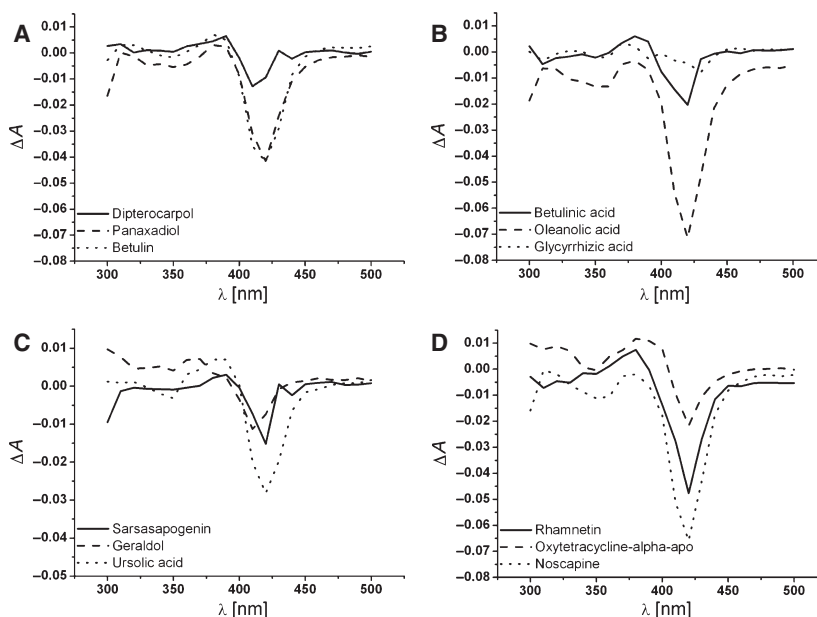
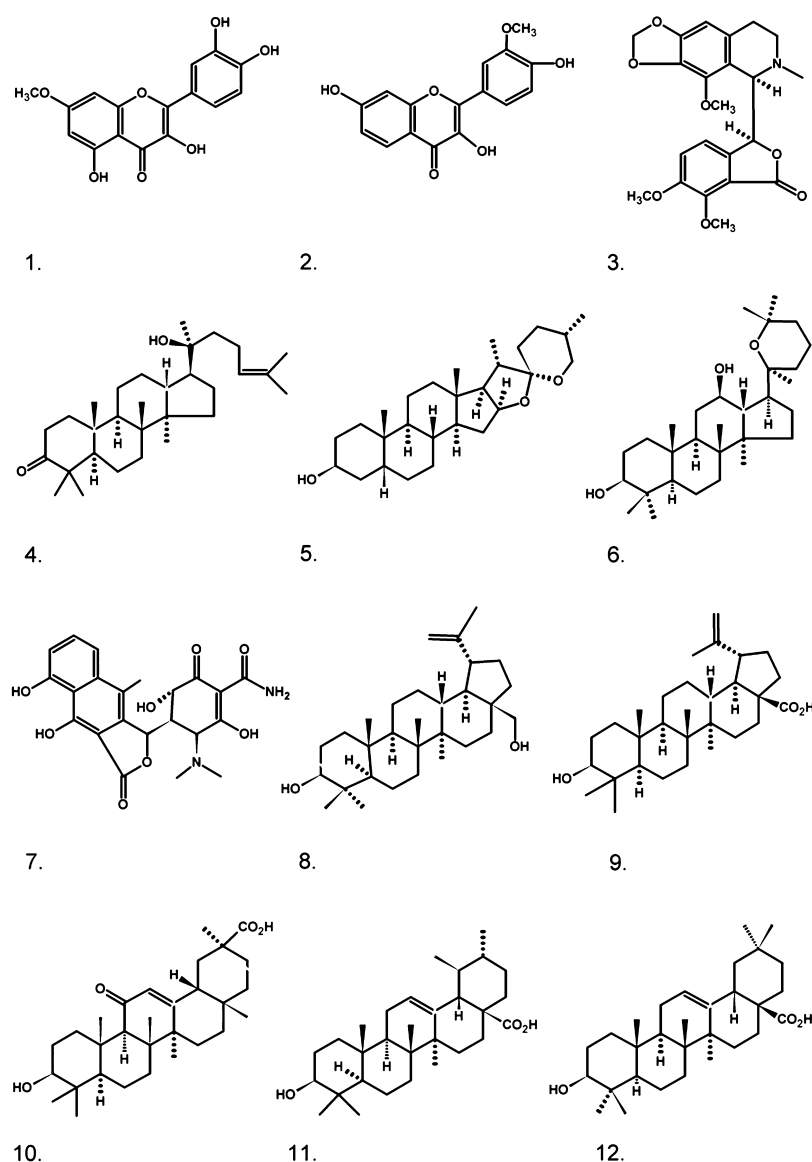


Fig. 2. Screening hits. Difference spectroscopy-based screening performed with 5 μ M CYP106A2 in 50 mM potassium phosphate buffer (250 μ L, pH 7.4) in 96-well plates. Absorbance scans were performed from 300 to 500 nm in 10-nm steps and 100 reads per well. After recording the ligand-free spectra, 5 μ M of compound (dissolved in 0.5 μ L dimethylsulfoxide) was added and incubated for 15 min at room temperature. Difference spectra were achieved by subtracting the ligand-free from the ligand-bound form. (A) Dipterocarpol (solid line), panaxadiol (dashed line) and betulin (spotted line). (B) Betulinic acid (solid line), oleanolic acid (dashed line) and glycyrrhizic acid (spotted line). (C) Sarsasapogenin (solid line), geraldol (dashed line) and ursolic acid (spotted line). (D) Rhamnetin (solid line), oxytetracycline- α -apo (dashed line) and noscaphine (spotted line).



Scheme 1. Structures of the screening hits: (1) rhamnetin, (2) geraldol, (3) alpha-apo-oxytetracycline, (4) dipterocarpol, (5) sarsasapogenin, (6) panaxadiol, (7) noscapine, (8) betulin, (9) betulinic acid, (10) glycyrrhizic acid, (11) ursolic acid and (12) oleanolic acid.

using an *in vitro* conversion assay. The *in vitro* reconstituted system consisted of CYP106A2, truncated bovine adrenodoxin and bovine adrenodoxin reductase in a molar ratio of 1 : 2 : 20. For the sufficient supply of electrons, a glucose-6-phosphate dehydrogenase-based NADPH regenerating system was used.

Four triterpenoic carbon acids were found (glycyrrhizic, oleanolic, ursolic and betulinic acid) among the screening hits; these have recently been identified as substrates for CYP106A2 [17] and are investigated in detail elsewhere. The remaining eight screening hits depicted novel binders for CYP106A2 which were tested for *in vitro* product formation. For this, 50 μM compound was converted for 10 min at 30 $^{\circ}\text{C}$ with 0.25 μM enzyme. No *in vitro* product formation was

observed using rhamnetin, sarsasapogenin, noscapine and panaxadiol. Geraldol and alpha-apo-oxytetracycline were not tested because both compounds are scarce and also not promising with regard to potential biotechnological applications. Successful *in vitro* conversion was achieved using betulin and dipterocarpol, confirming both as novel substrates for CYP106A2. Whereas the dipterocarpol conversion resulted in the formation of two major products [R_f 0.62 (P1) and 0.21 (P3)] and one minor product [R_f 0.42 (P2)] (Fig. 3A), betulin was specifically converted to just one product (R_f 0.28) (Fig. 3B).

Dipterocarpol was further examined as a substrate for CYP106A2 because the compound has structural similarities with the leading bioactive compounds of

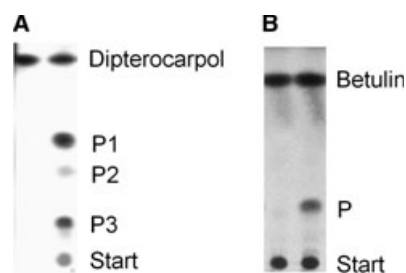


Fig. 3. *In vitro* conversion of dipterocarpol (A) and betulin (B). TLC plates after single development with hexane/ethylacetate (50 : 50) and staining with anisaldehyde. Substrate (100 μM) was converted for 10 min at 30 $^{\circ}\text{C}$. No additional spots were detected in the negative controls (left lanes). Dipterocarpol was converted into three products (R_f 0.62, 0.42 and 0.21) and betulin into one product (R_f 0.28).

ginseng. The bioactive ginseng component dipterocarpol is a dammarane-type triterpene, which differs only in the position and number of the hydroxyl groups, indicating a high probability of increasing the bioactivity of dipterocarpol by functionalization with a CYP enzyme. The binding affinity of dipterocarpol to CYP106A2 was determined using difference spectroscopy, by titrating 10 μM of enzyme with various amounts of the substrate. The equilibrium dissociation constant (K_D) was calculated to be $2.82 \pm 0.35 \mu\text{M}$ (Fig. 4). The *in vitro* catalytic activity of CYP106A2 towards dipterocarpol was analysed by Michaelis–Menten kinetics using standard assay conditions for CYP106A2 as detailed in the literature [20,21]. Exceptionally high reaction velocities were obtained for the overall reaction. Individual analysis of

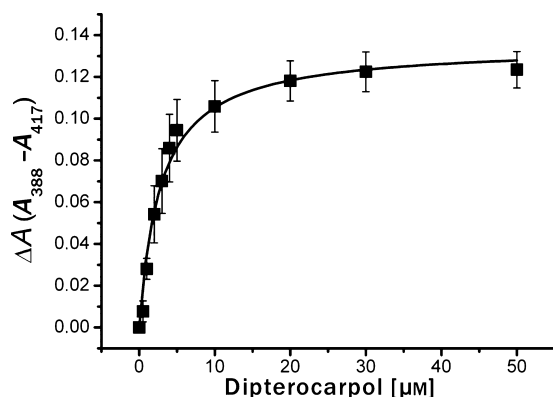


Fig. 4. Binding affinity. The dissociation constant K_D of dipterocarpol was analysed by titrating 10 μM enzyme with 0–50 μM dipterocarpol dissolved in dimethylsulfoxide. The assay was performed in tandem cuvettes at 25 $^{\circ}\text{C}$. Peak to trough differences ΔA ($A_{380} - A_{417}$) were plotted in reverse to the final substrate concentration (μM) and the dissociation constant was calculated by hyperbolic fitting of the resulting diagram.

the products showed that saturation was only achieved for the reaction leading to product 3 (R_f 0.21), resulting in an apparent V_{max} of $18.72 \pm 1.16 \text{ nmol product/nmol P450/min}^{-1}$ and K_m of $75.40 \pm 8.21 \mu\text{M}$. Down-scaling of the reactions to saturate the reactions leading to product 1 and 2 was not feasible within the detection limits.

Whole-cell conversion of dipterocarpol

The next step was identification of the dipterocarpol reaction products. Whole-cell conversions were performed to obtain sufficient amounts of the products for NMR analysis and further investigations. Several studies have demonstrated that the host strain of CYP106A2, *B. megaterium* ATCC 13368, can be used for the preparative scale whole-cell production of CYP106A2 reaction products [16,22]. This strain has the advantages that it is easy to cultivate and does not encode other CYPs in its genome. *In vivo* conversion of dipterocarpol with the wild-type *B. megaterium* resulted in the formation of six *in vivo* products, two main products with R_f values of 0.6 (P1) and 0.214 (P2), and four minor products with R_f values 0.4, 0.38, 0.142 and 0.00285. Compared with the *in vitro* reaction product pattern, more minor products were obtained. Nevertheless, the R_f values of the main products were in accordance with those of products P1 and P3 of the *in vitro* reaction (Fig. 3A). A *cyp106a2*-knockout strain was used to confirm that the observed reactions are CYP106A2 dependent. No reaction products were detected using the knockout strain (Fig. 5).

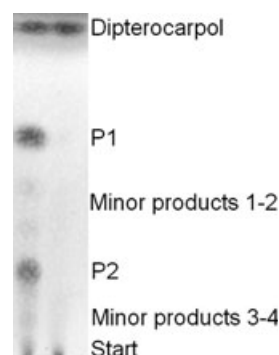


Fig. 5. *In vivo* conversion of dipterocarpol. TLC plate after single development with hexane/ethylacetate (50 : 50) and staining with anisaldehyde. Dipterocarpol (100 μM) was incubated with *B. megaterium* ATCC 1338 and the corresponding knockout strain lacking the capacity to express CYP106A2. Dipterocarpol was metabolized into two major and four minor products in the wild-type strain (left), whereas no products were obtained from the knockout strain lacking the capacity to express CYP106A2 (right).

For the preparative scale of dipterocarpol conversion, 150 mg of substrate were added to 1 L of *B. megaterium* culture and incubated for 25 h at 30 °C and 150 r.p.m.. The conversion resulted in a relatively low yield of 6.66% for P1 (R_f 0.6) and 1.33% for P3 (R_f 0.214). Because the product yield might be limited by the toxic effects of dipterocarpol, we checked whether growth inhibition occurs by incubating the cells with the substrate. We therefore monitored the growth of *B. megaterium* in the presence and absence of dipterocarpol for 30 h. Only the solubilizer ethanol was added to the control culture. Both the substrate and pure ethanol were added 4 h after inoculation of the culture (end of initial-lag phase). The addition of dipterocarpol had a considerable impact on the growth of the *B. megaterium* cells, resulting in the D_{585} value being half (9.68) that obtained with the control culture (19.49) (Fig. 6). To increase the product yield, conversion of dipterocarpol was done by stepwise addition of substrate to 1-L cultures, followed by cultivation for 48 h. Substrate (300 mg) was dissolved in 50 mL ethanol and added to the culture in 10-mL portions with a 1 h break between individual additions. After three time extractions and subsequent chromatography via a silica gel column, 46 mg of P1 and 33 mg of P3 (15 and 11% yield, respectively) were obtained. To investigate whether the observed minor products are CYP106A2 metabolites, the isolated products were used in the *in vitro* conversion assay. P1 was thereby converted into P3, whereas P3 was not metabolized by CYP106A2 (Fig. S1).

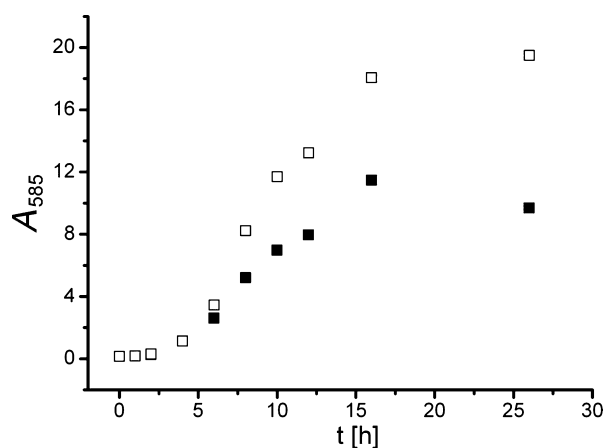


Fig. 6. Growth curve of *B. megaterium* ATCC 13368: 50 mL of soytone-containing media was inoculated with 500 μ L of a cryogenic culture. Cells were cultivated at 30 °C with vigorous shaking. After 4 h incubation, 2.5 mL of ethanol was added to one culture (empty dots) and 2.5 mL of an ethanolic solution of dipterocarpol (final concentration 200 μ L) was added to the other (solid dots). D_{585} was monitored over 26 h.

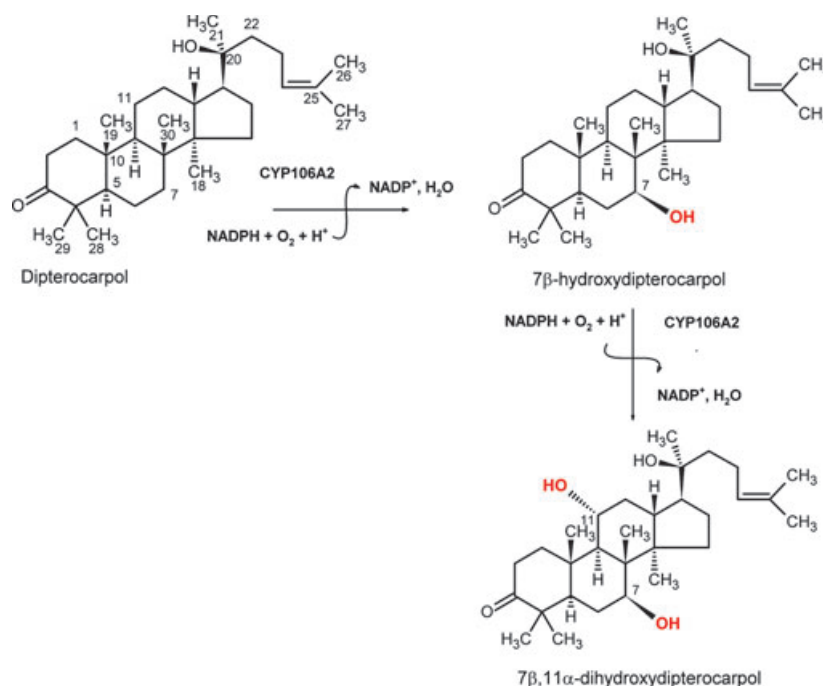
NMR characterization of the dipterocarpol reaction products

The purified *in vivo* reaction products were of adequate amount and purity to be characterized by NMR spectroscopy. In contrast to dipterocarpol, the ^{13}C NMR of its first biotransformation product showed the signal of an additional secondary alcohol function at δ_{C} 74.52. Its carbinol proton at δ_{H} 3.81 was correlated to C-5, C-6, C-8, C-9, C-14 and C-30 in the HMBC spectrum and was thus assigned to C-7. The stereochemistry at C-7 could not be deduced from the coupling pattern of its proton. Unfortunately, H-7 was part of an ABX spin system, in which H-6a (δ_{H} 1.60) and H-6b (δ_{H} 1.59) represented the AB part. Therefore, the coupling pattern for H-7 was of a higher order and any form of first-order analysis would lead to wildly incorrect structure interpretations. Finally, the NOESY spectrum revealed the axial position of H-7 with correlations to the axial H-5, H-9 and methyl group H-18. Therefore, the hydroxyl group at C-7 was equatorial and in the β -orientation (Scheme 2).

The second biotransformation product was found to be a dihydroxylated dipterocarpol derivative. The NMR data again showed signals for a β -orientated hydroxyl group at C-7 (δ_{H} 3.79 *m* and δ_{C} 74.25). The additional secondary hydroxyl function could be located at C-11 (δ_{C} 70.69) because of correlations between its carbinol proton (δ_{H} 3.79 *ddd*) with H-9, H-12a and H-12b in the HHCOSY, as well as with C-8, C-9, C-10 C-12 and C-13 in the HMBC. The relative position of H-11 followed directly from the analysis of its six-line spin system. This indicated a special case of a doublet of doublet of doublets, in which two of three coupling constants were equal with $J = 11$, 11 and 5 Hz. Thus H-11 was in axial position, showing diaxial couplings to H-9 and H-12ax and an axial–equatorial coupling to H-12eq. The position of the hydroxyl group at C-11 was therefore equatorial and in an α -orientation (Scheme 2).

Cytotoxicity assays with dipterocarpol and its biotransformation products

To confirm the assumption that insertion of a hydroxyl group into dipterocarpol influences the bioactive behaviour of the compound, cytotoxicity assays were performed using two different cell lines. Assays were conducted in 96-well tissue culture plates with HeLa and COS-1 cells. To ensure logarithmic growth, 10 000 cells were seeded per well and doubled overnight in a 95% air incubator. Dipterocarpol, 7 β -hydroxydipterocarpol and 7 β ,11 α -dihydroxydipterocarpol were added to the cells



Scheme 2. CYP106A2-catalysed hydroxylation of dipterocarpol in *B. megaterium* ATCC 13368.

at final concentrations ranging from 0 to 250 μM . A XTT-based assay was used to investigate potential cytotoxicity. The substrate dipterocarpol and the dihydroxylated product P3 were not cytotoxic to the tested cell lines. By contrast, the monohydroxylated P1 showed cytotoxicity against both tested cell lines, with a IC_{50} of $24.58 \pm 14.66 \mu\text{M}$ using HeLa cells (Fig. 7A) and $62.36 \pm 2.75 \mu\text{M}$ using COS-1 cells (Fig. 7B).

Discussion

The aim of this study was the identification and characterization of new pharmaceutically relevant substrates

for CYP106A2. Difference spectroscopy was used to screen a library of 502 bioactive natural products. Twelve hits were identified (Scheme 1), among them eight novel potential substrates of this enzyme and four compounds (namely betulinic acid, oleanolic acid, ursolic acid, and glycyrrhizic acid) that had previously been identified as type I substrates of CYP106A2 in our laboratory, supporting the feasibility of the screening system used here.

Triterpenoids, namely dipterocarpol, panaxadiol, sarsasapogenin and betulin, were found among the novel type I ligands detected. These compounds match the substrate spectrum of CYP106A2 perfectly,

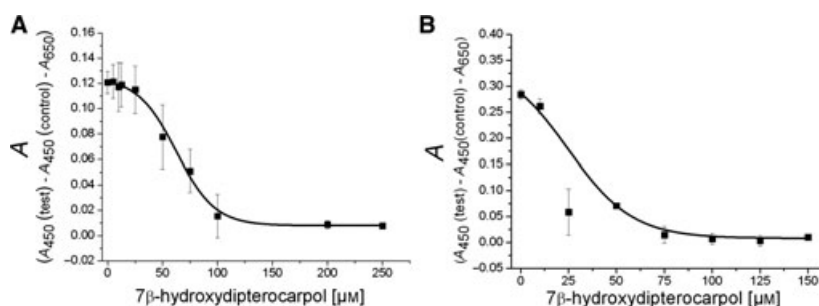


Fig. 7. XTT assay. (A) COS-1 cells, (B) HeLa cells. Cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal bovine serum and 100 U streptomycin/penicillin. Cells (10 000 per well) were seeded into 96-well microtiter plates and doubled overnight in a 95% air incubator. 7β-Hydroxydipterocarpol was added to the cells at different concentrations (0–250 μM). After further incubation for 48 h, the medium was changed to Phenol Red- and serum-free Dulbecco's modified Eagle's medium and 20% XTT solution was added. After an additional 4-h incubation at 37 °C, specific absorbance was measured and plotted against the final concentration of 7β-hydroxydipterocarpol. The IC_{50} value was determined by sigmoidal fitting of the resulting plot.

because the main substrates for this enzyme identified to date are penta- and tetracyclic triterpenes, like the abovementioned carbon acids. Furthermore, CYP106A2 was one of the first discovered bacterial steroid hydroxylases and the conversion of different steroids has been characterized in detail [18,20,21]. To verify these novel binders as substrates for CYP106A2, *in vitro* reaction assays were performed and reaction products were obtained for two of the four potential substrates, specifically dipterocarpol and betulin. However, in addition to the triterpenoids, four hits were identified during the screening that are structurally rather different from the CYP106A2 substrates known to date. Two of these are flavonoids (rhamnetin and geraldol), one is a morphine-type alkaloid (noscapine) and one a polyketide (alpha-apo-oxytetracycline). Despite the observed binding, *in vitro* reaction assays of rhamnetin and noscapine with CYP106A2 did not result in any detectable product formation.

The most promising novel CYP106A2 substrate identified in this study is dipterocarpol, a tetracyclic triterpene found in the exudate of dipterocarpaceae (dammar resin). A strong binding behaviour of dipterocarpol was observed, with a K_D of $2.82 \pm 0.35 \mu\text{M}$. The catalytic activity of CYP106A2 towards dipterocarpol was investigated using Michaelis–Menten kinetic tests. Unfortunately, saturation was achieved only for the reaction leading to $7\beta,11\alpha$ -dihydroxydipterocarpol (one of the main products, R_f 0.21, see Fig. 3A). Determination of K_m and $V_{\max}$ for the overall reaction was therefore obsolete. In this context, it is noteworthy that dipterocarpol belongs to the group of oxidized triterpenes of medium polarity, implying poor solubility in polar and nonpolar solvents. However, the kinetic constants of the reaction leading to $7\beta,11\alpha$ -dihydroxydipterocarpol were calculated as an apparent $V_{\max}$ of 18.72 ± 1.16 (nmol-product·nmol P450⁻¹·min⁻¹) and a K_m of $75.40 \pm 8.21 \mu\text{M}$.

The dipterocarpol *in vivo* conversion with the host strain of CYP106A2, *B. megaterium*, resulted in the formation of two main products, whose R_f values on TLC matched those of the *in vitro* reaction products P1 and P3. In contrast to the *in vitro* reactions, additional more-hydrophilic minor products were seen. Interestingly, the isolated 7β -hydroxydipterocarpol was metabolized by CYP106A2 *in vitro* to $7\beta,11\alpha$ -dihydroxydipterocarpol, whereas no conversion was observed using $7\beta,11\alpha$ -dihydroxydipterocarpol as the substrate (Fig. S1). Additional LC-MS analysis detected only the masses of the monohydroxylated dipterocarpol [m/z 459 (positive ion mode)] and dihydroxylated dipterocarpol [m/z 475 (positive ion mode)], no polyhydroxylated products were

observed. Likewise, no polyhydroxylated dipterocarpol products were observed with LC-MS analysis of the *in vivo* conversion. These results indicate that the reaction product P3 is not metabolized further by CYP106A2, prompting the assumption that the observed minor products of the *in vivo* conversion are P450-independent side products of *B. megaterium* metabolism.

Further, the presented results do not give information about the natural function of CYP106A2 and the endogenous substrate remains unknown. However, the fact that CYP106A2 metabolizes a huge variety of triterpenoids, and deletion of the gene is not lethal to *B. megaterium*, leads to the presumption that the enzyme plays a role in detoxification processes, similar to mammalian liver CYP enzymes.

Nonetheless, preparative scale conversion of dipterocarpol succeeded in the production of the two main products, which could be identified as dammarane-type triterpenoids, which to the best of our knowledge, have not been described in the literature to date. Dammarane triterpenoids are known to be biologically active components of ginseng, one of the most used herbal drugs worldwide. Today, more than 30 different dammarane-type saponins of ginseng, called ginsenosides, are described, displaying a vast range of pharmacological effects including antioxidant, antiviral and anti-tumour, as well as effects on the cardiovascular, endocrine and immune systems [22,23]. This diversity in bioactivity is due to different physiochemical properties of these compounds. It has been demonstrated that the cytotoxic effects of these compounds are inversely proportional to the number of sugar molecules and the number of hydroxyl groups [24].

However, despite their pharmacological potency, ginsenosides have not yet been developed for clinical medicine because obtaining large amounts remains challenging. By contrast, dipterocarpol can be isolated from various plants of the Dipterocarpaceae family and is therefore affordably obtained at adequate purity. Employing CYP106A2 as an efficient dipterocarpol hydroxylase, we now show that it is possible to biotechnologically produce 7β -hydroxydipterocarpol and $7\beta,11\alpha$ -dihydroxydipterocarpol with high yield. The insertion of O-functional groups into dipterocarpol provides new chemical properties, which might be accompanied by changes in bioactivity [25]. To examine the bioactivity of dipterocarpol compared with its hydroxylated derivatives, a XTT-based *in vitro* cytotoxicity assay was performed with HeLa and COS-1 cells. Previously conducted MTT-based (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) cytotoxicity tests displayed that dipterocarpol had potent activity against leukaemia cells (HL60) with an

IC₅₀ value of 19.2 μM , whereas the compound showed no cytotoxic effects on melanoma (CRL1579) cells [26]. In our study, dipterocarpol did not show any cytotoxicity against HeLa cells or COS-1 cells.

By contrast, 7 β -hydroxydipterocarpol showed a dose-dependent cytotoxic effect on both tested cell lines, with IC₅₀ values of 24 μM for HeLa and 60 μM for COS-1 cells. A cytotoxicity of 100% was obtained after 48 h using 100 μM 7 β -hydroxydipterocarpol with the HeLa cells and 200 μM 7 β -hydroxydipterocarpol with the COS-1 cells. The measured cytotoxicity of 7 β -hydroxydipterocarpol is lower than that of the ginsenoside precursor panaxadiol observed with HeLa cells (IC₅₀ = 1.2 μM) [27]. The cytotoxicity is comparable with that of other dammarane triterpenes tested with HeLa cells, for example 3 β ,30(S),24(S)-tryhydroxydammar-25-enol (IC₅₀ $\sim$ 30 μM) [28]. Little data of cytotoxic activity of dammarane triterpenoids on COS cells is available, but it has been observed that ginsenoside-Rh2 arrests the cell cycle of SK-HEP-1 cells at the G₁/S boundary and also exhibits strong effects on the viability of COS and HeLa cells [29].

The 7 β ,11 α -dihydroxydipterocarpol did not show any cytotoxicity at the tested concentrations. However, the compound is interesting chemically, because the combination of a 7 β - and 11 α -hydroxyl-groups seems to be very uncommon in dammarane triterpenes. Naturally occurring dammaranes are mainly hydroxylated at position 6, 12 or both, whereas there is little data regarding 7-hydroxylated compounds and none displaying the combination of 7 and 11 [30]. Instead of being cytotoxic, this compound might have other interesting effects which are worth investigating.

In summary, the screening of 502 compounds containing a natural product library succeeded in the identification of dipterocarpol as a very interesting new substrate for CYP106A2. The bioconversion of this compound resulted in the first description of two new dammarane triterpenoids, namely 7 β -hydroxy- and 7 β ,11 α -dihydroxydipterocarpol. Furthermore, 7 β -hydroxydipterocarpol emerged as a pharmaceutically relevant compound, exerting cytotoxic effects on two mammalian cell lines. This study again highlights the capability of CYP enzymes in biotechnological and drug-development processes.

Material and methods

Enzymes used

The following enzymes were used: CYP106A2 [EC 1.14.15.8](#), adrenodoxin reductase [EC 1.18.1.2](#) and glucose-6-phosphate dehydrogenase [EC 1.1.1.49](#).

Reagents and chemicals

The natural product library was purchased from Biomol International as deep frozen dimethylsulfoxide stocks (1 mg/0.5 mL⁻¹). All other chemicals were from standard sources and of the highest purity available.

Bacteria strains and cultivation

Bacillus megaterium ATCC13368, the host strain of CYP106A2 and the *cyp106A2* knockout strain, were both kind gifts of R. Rauschenbach (Schering AG, Berlin, Germany). *Bacillus megaterium* cultivation was performed as described previously [17]. The *cyp106a2* DNA was a kind gift from R. Rauschenbach (Schering AG). Expression and purification of the enzyme were performed as described previously [31]. Truncated bovine adrenodoxin (4–108) and bovine adrenodoxin reductase were expressed and purified as described elsewhere [32].

Difference spectroscopy-based screening

The screening was conducted with 5 μM enzyme and 5 μM compound in potassium phosphate buffer (50 mM, pH 7.4). Medium throughput screening assays were performed with flat-bottomed, transparent, pure grade polystyrene 96-well plates from Brand (Wertheim, Germany). The assay volume was 250 μL . Absorbance spectra were recorded using a Tecan Sapphire II (Tecan Group Ltd, Maennedorf, Germany) (100 reads per well, 0.3 ms between move and read, 10-nm steps). Absolute spectra were recorded before and after addition of the compound, at which the final amount of dimethylsulfoxide did not exceed 1% (v/v). The mixtures were incubated at 25 °C for 15 min. Difference spectra were obtained by subtracting the substrate-free spectrum from the substrate-bound spectrum using Microsoft EXCEL 2003 (Microsoft Corp., Redmond, CA, USA). The accuracy of the spectra was affirmed by comparison with spectra recorded in a two-beam spectrophotometer.

Determination of the dissociation constant

The K_D of dipterocarpol was determined by titration of 10 μM enzyme solution with dipterocarpol (0–50 μM) in tandem cuvettes. Equal amounts of dipterocarpol were added to the reference cuvette in the enzyme free chamber. All assays were performed in triplicate. K_D was determined by plotting the peak-to-trough difference ΔA ($A_{387} - A_{417}$) versus the substrate concentration and subsequent hyperbolic fitting. Hyperbolic regression was performed using ORIGIN LAB 7.5 (OriginLab Corp., Northampton, MA, USA) with a regression coefficient of 0.98.

***In vitro* conversion and catalytic activity**

Substrate conversions with CYP106A2 were performed with an *in vitro* reconstituted system consisting of truncated bovine adrenodoxin (5 μ M), bovine adrenodoxin reductase (0.5 μ M), CYP106A2 (0.25 μ M) and a NADPH regenerating system consisting of glucose-6-phosphate-dehydrogenase (1 U), glucose-6-phosphate (5 mM) and MgCl_2 (1 mM). Reactions were performed in Hepes buffer (pH 7.4, 50 mM) supplemented with 0.05% (v/v) Tween 20, in a total reaction volume of 500 μ L. Reactions were started by the addition of 100 μ M NADPH to prewarmed reaction mixtures and performed under continuous mixing with an eppendorf thermomixer (Eppendorf, Hamburg, Germany) at 750 r.p.m. and 30 °C for 10 min. Reactions were quenched and extracted with 500 μ L ethylacetate. The organic phases were evaporated to dryness and subsequently chromatographed. The residues were redissolved in acetonitrile or methanol (100 μ L) for HPLC analysis and in acetone (50 μ L) for TLC analysis.

To measure the catalytic activity of CYP106A2 towards dipterocarpol, reactions were performed in 250 μ L total volume and started by addition of 500 μ M NADPH. Reactions were quenched with 500 μ M ethylacetate after 2 min reaction time and subsequently extracted. Organic phases were evaporated to dryness and redissolved in 25 μ L ethylacetate. The substrate concentration was varied from 10 to 300 μ M. Product amounts were determined using relative spot intensity and the respective activity was calculated. Kinetic constants were determined by plotting the amount of products formed ($\text{nmol-products-nmol P450}^{-1}\text{min}^{-1}$) against the substrate concentration (μ M) followed by hyperbolic fitting with the ORIGIN LAB 7.5 software.

***In vivo* conversion**

Dipterocarpol *in vivo* conversions were performed with *B. megaterium*. After inoculation of the main culture, cells were cultivated for 16 h prior to the addition of substrate. Dipterocarpol conversion was investigated using 500- μ L aliquots of fresh culture and conversion was performed in 1.5-mL reaction tubes at 30 °C, 1 h and under continuous mixing with an eppendorf thermomixer (Eppendorf, Hamburg, Germany) at 1000 r.p.m. Reactions were quenched and concomitantly extracted with 500 μ L ethylacetate. Organic phases were evaporated to dryness and chromatographed using TLC.

For the preparative scale conversion of dipterocarpol, 1-L cultures were used in baffled shake flasks, inoculated with 1 mL of a cryogenic culture and cultivated for 16 h at 30 °C. Dipterocarpol was added as ethanolic solution and the *in vivo* conversions were performed at 30 °C. The reaction was quenched with 500 mL ethylacetate and extracted three times with 500 mL ethylacetate. The resulting organic phase was dried and chromatographed via a silica gel (60)

column, with hexane/ethylacetate 50 : 50. Resulting homogeneous fractions were evaporated to dryness and analysed by NMR spectroscopy.

HPLC and TLC analysis

Compounds that were not detectable with a UV-Vis detector were analysed by TLC with subsequent anisaldehyde staining (betulin, panaxadiol, sarsasapogenin, dipterocarpol). Compounds that had conjugated double bonds or other UV-active moieties were analysed by HPLC. Reversed-phase liquid chromatography was performed using a Jasco system (Pu-980 HPLC pump, an AS-950 sampler, a UV-975 UV/visible detector, and a LG-980-02 gradient unit; Jasco, Gross-Umstadt, Germany). The mobile phase was methanol/water 85 : 15 for rhamnetin and acetonitrile/water 30 : 70 with 0.1% (v/v) trifluoroacetic acid for noscapine. The respective detection wavelengths were 254 and 240 nm. The following measurement parameters were used: temperature, 40 °C; flow rate, 1 $\text{mL}\cdot\text{min}^{-1}$; injection volume, 5 μ L.

For the TLC the samples were spotted onto TLC aluminium plates (4 $\times$ 8 cm; silica 60; layer thickness, 0.2 mm; Roth, Karlsruhe Germany), and developed once in a rectangular solvent tank containing hexane/ethylacetate 50 : 50. Spots were detected with an anisaldehyde dipping bath (4-methoxybenzaldehyde, 5 mL; sulfuric acid, 2.5 mL; glacial acetic acid, 3.75 mL; ethanol, 478.75 mL), the colour reaction was started by heating the plates with a hot air gun.

NMR characterization of the dipterocarpol hydroxylation products

All NMR spectra were recorded in CDCl_3 at 300 K with a Bruker DRX 500 NMR spectrometer. The chemical shifts were given relative to CHCl_3 at δ 7.24 (^1H NMR) or CDCl_3 at δ 77.00 (^{13}C NMR) using the standard δ notation in parts per million (p.p.m.). The 2D NMR spectra were recorded as gs-HHCOASY, gs-NOESY, gs-HSQCED and gs-HMBC.

7 β -Hydroxydipterocarpol

^1H NMR: (CDCl_3 , 500 MHz), δ 0.90 s (3H, 3 $\times$ H-19), 0.93 s (3H, 3 $\times$ H-18), 0.98 s (3H, 3 $\times$ H-30), 1.03 s (3H, 3 $\times$ H-29), 1.07 s (3H, 3 $\times$ H-28), 1.14 s (3H, 3 $\times$ H-21), 1.22 m (H-12a), 1.30 m (H-15a), 1.31 m (H-9), 1.37 m (H-11a), 1.39 m (H-1a), 1.46 m (H-5), 1.46 m (2H, 2 $\times$ H-22), 1.50 m (H-11b), 1.51 m (H-16a), 1.59 m (H-6a), 1.60 m (H, H-6b), 1.61 m (H-13), 1.61 s (3H, 3 $\times$ H-27), 1.67 m (H-15b), 1.67 s (3H, 3 $\times$ H-26), 1.69 m (H-17), 1.77 m (H-16b), 1.86 m (H-12b), 1.87 m (H-1b), 2.04 m (2H, 2 $\times$ H-23), 2.43 m (H-2a), 2.46 m (H-2b), 3.81 m (H-7), 5.10 tsep (J = 7 and 1.5 Hz, H-24). ^{13}C NMR (CDCl_3 , 125 MHz), δ 9.51

(C-30), 15.94 (C-19), 16.16 (C-18), 17.69 (C-27), 20.98 (C-29), 21.98 (C-11), 22.54 (C-23), 25.22 (C-16), 25.66 (C-21), 25.66 (C-26), 26.72 (C-28), 27.33 (C-12), 28.98 (C-6), 33.93 (C-2), 34.60 (C-15), 36.64 (C-10), 39.44 (C-1), 40.19 (C-22), 43.18 (C-13), 45.89 (C-8), 47.04 (C-4), 48.83 (C-17), 49.55 (C-9), 49.58 (C-14), 52.72 (C-5), 74.52 (C-7), 75.30 (C-20), 125.58 (C-24), 131.72 (C-25), 217.35 (C-3).

7 β , 11 α -dihydroxydipterocarpol

¹H NMR: (CDCl₃, 500 MHz), δ 0.98 s (3H, 3 $\times$ H-18), 1.00 m (3H, 3 $\times$ H-30), 1.04 s (3H, 3 $\times$ H-19), 1.05 m (3H, 3 $\times$ H-29), 1.08 m (3H, 3 $\times$ H-28), 1.15 s (3H, 3 $\times$ H-21), 1.34 m (H-15a), 1.39 m (H-12a), 1.47 m (2H, 2 $\times$ H-22), 1.48 m (H-9), 1.51 m (H-16a), 1.59 m (H-6a), 1.61 m (H-6b), 1.61 m (3H, 3 $\times$ H-27), 1.62 m (H-5), 1.62 m (H-15b), 1.66 m (H-1a), 1.67 m (3H, 3 $\times$ H-26), 1.73 m (H-17), 1.78 m (H-13), 1.79 m (H-16b), 2.04 m (2H, 2 $\times$ H-23), 2.16 ddd (J = 12, 5 and 3 Hz, H-12b), 2.37 ddd (J = 15, 8.3 and 7 Hz, H-2a), 2.48 ddd (J = 15, 9 and 5.7 Hz, H-2b), 2.65 ddd (J = 14, 8.3 and 5.7 Hz, H-1b), 3.79 m (H-7), 4.01 ddd (J = 11, 11 and 5 Hz, H-11), 5.10 *tsep* (J = 7 and 1.5 Hz, H-24). ¹³C NMR (CDCl₃, 125 MHz), δ 10.32 (C-30), 15.94 (C-18), 16.80 (C-19), 17.72 (C-27), 20.71 (C-29), 22.55 (C-23), 25.55 (C-16), 25.71 (C-26), 25.95 (C-21), 27.54 (C-28), 29.12 (C-6), 34.05 (C-2), 34.45 (C-15), 38.19 (C-10), 2 $\times$ 39.66 (C-12 and C-22), 41.50 (C-13), 41.59 (C-1), 46.14 (C-8), 47.36 (C-4), 48.74 (C-17), 49.35 (C-14), 52.37 (C-5), 54.60 (C-9), 70.69 (C-11), 74.25 (C-7), 75.17 (C-20), 124.46 (C-24), 131.88 (C-25), 218.09 (C-3).

In vitro cytotoxicity assay

The cytotoxicities of 7 β -hydroxydipterocarpol and 7 β ,11 α -dihydroxydipterocarpol were determined using HeLa and COS-1 cell lines. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 mL L⁻¹ penicillin/streptomycin solution and 10% (v/v) fetal bovine serum. To maintain logarithmic growth, 1×10^4 cells per well were seeded and into 96-well plates in 100 μ L medium. After 24 h of incubation at 37 °C and 5% CO₂ the products of the CYP106A2-dependent conversions were added (final concentration 0–250 μ M) and the cells were treated for 48 h. Afterwards, the cells were washed once with 100 μ L NaCl/P_i before adding 80 μ L of serum-free Dulbecco's modified Eagle's medium without Phenol Red. Cell viability was checked with a XTT reaction kit (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) [33]. Twenty microlitres of XTT reagent were added and the mixture was incubated for 4 h at 37 °C. The absorbance at 450 nm (specific wavelength for formazan) and 690 nm (unspecific wavelength) was measured with a Tecan Saphire II plate reader. The specific absorbance of treated cells was calculated mathematically as follows: $A_{450}(\text{test}) - A_{450}(\text{blank}) - A_{690}$

(test). IC₅₀ values were calculated by plotting the resulting specific absorbance against the concentration of the test-compound and subsequent sigmoidal fitting.

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Supporting information

The following supplementary material is available:

Fig. S1. *In vitro* conversion assays using 7 β -hydroxy-dipterocarpol and 7 β ,11 α -dihydroxydipterocarpol as substrates for CYP106A2.

This supplementary material can be found in the online version of this article.

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