

Conversions of Tricyclic Antidepressants and Antipsychotics with Selected P450s from *Sorangium cellulosum* So ce56[§]

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ABSTRACT

Human cytochromes P450 (P450s) play a major role in the biotransformation of drugs. The generated metabolites are important for pharmaceutical, medical, and biotechnological applications and can be used for derivatization or toxicological studies. The availability of human drug metabolites is restricted and alternative ways of production are requested. For this, microbial P450s turned out to be a useful tool for the conversion of drugs and related derivatives. Here, we used 10 P450s from the myxobacterium *Sorangium cellulosum* So ce56, which have been cloned, expressed, and purified. The P450s were investigated concerning the conversion of the antidepressant drugs amitriptyline, clomipramine, imipramine, and promethazine; the antipsychotic drugs carbamazepine, chlorpromazine, and thioridazine, as well as their precursors, iminodibenzyl and phenothiazine.

Amitriptyline, chlorpromazine, clomipramine, imipramine, and thioridazine are efficiently converted during the *in vitro* reaction and were chosen to upscale the production by an *Escherichia coli*-based whole-cell bioconversion system. Two different approaches, a whole-cell system using M9CA medium and a system using resting cells in buffer, were used for the production of sufficient amounts of metabolites for NMR analysis. Amitriptyline, clomipramine, and imipramine are converted to the corresponding 10-hydroxylated products, whereas the conversion of chlorpromazine and thioridazine leads to a sulfoxidation in position 5. It is shown for the first time that myxobacterial P450s are efficient to produce known human drug metabolites in a milligram scale, revealing their ability to synthesize pharmaceutically important compounds.

Introduction

Cytochromes P450 (P450s) are heme-thiolate monooxygenases. They are present in nearly all organisms and belong to one of the largest superfamilies of enzyme proteins (Nelson, 2011). P450s are involved in the degradation and detoxification of drugs and xenobiotics, as well as in the metabolism of steroid hormones, lipids, and antibiotics (Bernhardt, 2006; Bernhardt and Urlacher, 2014). In general, P450s catalyze the insertion of a molecular oxygen atom into organic molecules while the other oxygen atom is reduced to water. Beside the hydroxylation, they catalyze a broad range of reactions such as sulfoxidation, epoxidation, deamination, dehalogenation, and N-, O-, and S-dealkylation (Sono et al., 1996; Bernhardt and Urlacher, 2014). To catalyze such reactions, these enzymes are dependent on redox partners, either homologous or heterologous, which provide electrons from NAD(P)H via an electron transfer chain (Hannemann et al., 2007).

Human P450s play a major role in the metabolism of drugs. The human liver P450s CYP3A4/5, CYP2D6, CYP2C9, CYP1A2, and CYP2C19 are responsible for the conversion of about 80% of all drugs (Zanger and Schwab, 2013). One important group of pharmaceuticals is psychotherapeutic drugs. Antipsychotic drugs and tricyclic antidepressants are used for the treatment of psychiatric disorders. In 1952, these psychotherapeutic drugs started with the discovery of chlorpromazine and since that time many drugs, mainly based on phenothiazine or iminodibenzyl, have been discovered and are used

for psychiatric medication (Shen, 1999; Owens, 2014). The involvement of human liver P450s in the phase I biotransformation of these psychotherapeutics is well studied (Tanaka and Hisawa, 1999). Most of the drug metabolites are produced by P450s (see Table 1), with the exception of N-oxide products that are formed by the flavin-containing monooxygenases (Ziegler, 1993). Since some of the drug metabolites might have adverse effects, the U.S. Food and Drug Administration (FDA) published the guidance for safety testing of drug metabolites. Any human metabolite representing >10% of the parent drug exposure at steady state has to be tested in safety studies (FDA, 2008). However, this FDA guidance is superseded by the guidelines of the International Conference on Harmonization (Frederick and Obach, 2010; Haglund et al., 2014). Concerning these guidelines, further safety testing is recommended for human metabolites that are observed at exposures >10% of total drug-related exposure and at significantly greater levels in humans than the maximum exposure seen in toxicity studies (European Medicines Agency, 2009). As a result, ways for the production of these drug metabolites are demanded by the pharmaceutical industry. Production of such metabolites for toxicological studies and further derivatization can be achieved either by chemical synthesis or biocatalysis. The enzymatic production has many benefits such as high selectivity or the absence of employing toxic chemicals (Koeller and Wong, 2001). Microbial enzymes play an increasing role in the production of known human drugs and secondary metabolites, which are used for drug development (Demain, 1999; Patel, 2002). In this respect, microbial P450s are often involved in the production of these metabolites (Urlacher and Girhard, 2012) due to their ability to hydroxylate inactive carbon-hydrogen bonds in complex molecules.

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ABBREVIATIONS: Adx₄₋₁₀₈, adrenodoxin (truncated form); FDA, Food and Drug Administration; Fpr, ferredoxin-NADP⁺ reductase; GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; P450, cytochrome P450; RT, retention time.

TABLE 1
Overview of psychotherapeutic drugs, their metabolites, and the human P450s capable for the metabolism

Drug	Human P450	Main metabolite	Reference
Amitriptyline	CYP1A2, CYP3A4, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6	2-Hydroxyamitriptyline 3-Hydroxyamitriptyline 2,11-Dihydronoritriptyline 10-Hydroxyamitriptyline 10-,11-Dihydroxyamitriptyline 10-Oxy-amitriptyline Amitriptyline- <i>N</i> -oxide Amitriptyline dihydrodiol Desmethylamitriptyline	Prox and Breyer-Pfaff, 1987; Venkatakrishnan et al., 2001
Carbamazepine	CYP1A2, CYP2A6, CYP3A4, CYP2B6, CYP2C8, CYP2E1	10-,11-Epoxycarbamazepine 2-Hydroxycarbamazepine 3-Hydroxycarbamazepine	Kerr et al., 1994; Pearce et al., 2002
Chlorpromazine	CYP1A2, CYP3A4, CYP2B6, CYP2C19, CYP2D6	7-Hydroxychlorpromazine Chlorpromazine- <i>N</i> -oxide Chlorpromazine sulfoxide Desmethylchlorpromazine Didesmethylchlorpromazine	Murray, 2006; Wójcikowski et al., 2010
Clomipramine	CYP1A2, CYP3A4, CYP2C19, CYP2D6	2-Hydroxyclopmipramine 8-Hydroxyclopmipramine 10-Hydroxyclopmipramine Clomipramine- <i>N</i> -oxide Desmethylclomipramine	Nielsen et al., 1996; Yokono et al., 2001
Imipramine	CYP1A2, CYP3A4, CYP2C9, CYP2D6	2-Hydroxyimipramine 10-Hydroxyimipramine Desmethylimipramine Didesmethylimipramine Imipramine- <i>N</i> -oxide	Singh, 2012
Promethazine	CYP2B6, CYP2D6	3-Hydroxypromethazine 4-Hydroxypromethazine Desmethylpromethazine Promethazine sulfoxide	Nakamura et al., 1996
Thioridazine	CYP2D6	<i>N</i> -desmethylthioridazine 7-Hydroxythioridazine Mesoridazine Thioridazine-5-sulfoxide	Daniel et al., 2000

Myxobacteria are known for the production of pharmaceutically and chemically important compounds, which attracted the attention of the pharmaceutical industry (Weissman and Müller, 2010). In 2007, the myxobacterium *Sorangium cellulosum* So ce56 was sequenced and 21 P450 genes were identified (Schneiker et al., 2007; Khatri et al., 2010b). Although, the physiologic roles of those P450s are still not known, some of them are able to convert exogenous substrates such as terpenes and terpenoids or fatty acids (Khatri et al., 2010b; Ly et al., 2012). However, the potential for the conversion of pharmaceutically interesting compounds has not yet been tested.

In this study, 10 P450s (CYP109C1, CYP109C2, CYP109D1, CYP260A1, CYP260B1, CYP264A1, CYP264B1, CYP266A1, CYP267A1, and CYP267B1) from *S. cellulosum* So ce56 were selected and employed for the in vitro conversion of seven psychotherapeutic drugs and their precursors. Compounds showing significant in vitro conversion were further chosen for the conversion with an *Escherichia coli*-based whole-cell biotransformation system to upscale the production and facilitate structure elucidation via NMR spectroscopy. The purified compounds were compared with known human metabolites to check for specific derivatives and to elucidate possible new metabolites, which could be useful for further drug development.

Materials and Methods

Chemicals. Amitriptyline and thioridazine were provided by Dr. Stephan Lütz (Novartis, Basel, Switzerland). Isopropyl β -D-1-thiogalactopyranoside and 5-aminolevulinic acid were purchased from Carbolution Chemicals (Saarbruecken, Germany). Bacterial media were purchased from Becton Dickinson (Heidelberg, Germany). All other chemicals were obtained from standard sources in the highest purity available.

Strains. The *E. coli* strain Top 10 for cloning purpose was obtained from Invitrogen (San Diego, CA). The *E. coli* strain BL21(DE3) for the heterologous expression of the P450s and BL21-Gold(DE3) for the whole-cell conversions were purchased from Agilent Technologies (Santa Clara, CA).

Molecular Cloning and Preparation of Expression Vectors. The 10 P450s from *S. cellulosum* So ce56 (CYP109C1, CYP109C2, CYP109D1, CYP260A1, CYP260B1, CYP264A1, CYP264B1, CYP266A1, CYP267A1, and CYP267B1) were selected for this study. The genes of those P450s were cloned in pCWori⁺ plasmids as described elsewhere (Khatri et al., 2010b). To improve the expression yield, the genes of the P450s (CYP109C1, CYP109C2, CYP109D1, CYP260A1, CYP260B1, CYP264A1, and CYP264B1) were excised from their corresponding pCWori⁺ plasmids and cloned into a pET17b plasmid (Novagen, Darmstadt, Germany) (Ringle et al., 2013). Likewise, the genes of CYP266A1, CYP267A1, and CYP267B1 were excised and cloned into a pET22b plasmid (Novagen).

For the expression of the redox partners ferredoxin-NADP⁺ reductase (Fpr) and adrenodoxin (truncated form) (Adx₄₋₁₀₈) in the *E. coli*-based whole-cell system, the pCDFDuet-1 (Merck, Darmstadt, Germany) vector with a streptomycin resistance marker gene was used. The DNA fragment encoding Adx₄₋₁₀₈ was amplified by Polymerase chain reaction (forward primer, 5'- AAT GAC ATG CTT GAT CTG GCC TAT GGA CTA ACA GAT AGA T -3', and reverse primer, 5'- ATC TAT CTG TTA GTC CAT AGG CCA GAT CAA GCA TGT CAT T-3') using pKKHC_Adx₄₋₁₀₈ as a template (Uhlmann et al., 1994). Then, the DNA fragment was inserted between NcoI and HindIII of the multiple cloning site MS-I of pCDFDuet-1 (Merck). The forward primer was used to delete the NdeI restriction site. The fragment encoding Fpr was amplified by Polymerase chain reaction (forward primer, 5'- CAT ATG GCT GAT TGG GTA ACA GGC AAA GTC ACT AAA GTG CAG AAC TGG -3', and reverse primer, 5'- CGG GGT ACC TTA CCA GTA ATG CTC CGC TGT CAT GTG CGC CGG TCG GC-3') using the pET16_Fpr plasmid as a template (Girhard et al., 2010). The fragment was subcloned in the pCR4 blunt vector and finally inserted between NdeI and KpnI, resulting in the pCDF_dFA expression vector.

Media and Buffers. For the heterologous expression of the related P450s, terrific broth medium (24 g yeast extract, 12 g peptone, 4 ml glycerol, 2.31 g K_2HPO_4 , and 12.54 g KH_2PO_4 per liter H_2O) was used. The whole-cell conversion was performed in M9CA medium (6 g Na_2HPO_4 , 3 g KH_2PO_4 , 0.5 g NaCl, 1 g NH_4Cl , 4 g casamino acids, 4 g glucose, 50 μ l 1M $CaCl_2$, 2 ml 1 M $MgSO_4$, 2 ml of trace elements solution per liter H_2O ; Trace elements solution contained 2.5 g EDTA, 250 mg $FeSO_4$, 25 mg $ZnCl_2$ and 5 mg $CuSO_4$ per 50 ml H_2O). The conversion with resting cells was done in 50 mM potassium phosphate buffer (pH 7.4) containing 2% glycerol as the carbon source.

Expression and Purification of the Enzymes. The corresponding P450s were expressed and purified as described previously (Khatri et al., 2010b). The electron transfer partners Adx_{4-108} and adrenodoxin reductase from *Bos taurus* were expressed and purified as described elsewhere (Sagara et al., 1993; Uhlmann et al., 1994).

In Vitro Conversions. A reconstituted in vitro system containing the corresponding P450 (0.5 μ M), adrenodoxin reductase (1.5 μ M), Adx_{4-108} (10 μ M), $MgCl_2$ (1 mM), and a cofactor regenerating system with glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (2 U ml^{-1}) in the end volume of 250 μ l of potassium phosphate buffer (20 mM, pH 7.4) was used. The compounds (10 mM), except carbamazepine (MeOH), phenothiazine (EtOH), and iminodibenzyl (EtOH), were dissolved in water and added to an end-concentration of 200 μ M. The reaction was started by the addition of NADPH (500 μ M). After 3 hours at 30°C the reaction was quenched by adding chloroform (500 μ l). The aqueous phase was extracted twice with chloroform (2 \times 500 μ l). A negative control in the absence of P450 in the reaction sample was employed for each substrate to verify the P450-dependent reaction.

Whole-Cell System Using M9CA. The experiments were performed with *E. coli* BL21(DE3) gold cells. The cells were transformed with two plasmids, one for the corresponding P450 (pET17b or pET22b) and the other one for the redox partners Fpr and Adx_{4-108} (pCDF_dFA). The overnight culture was prepared in nutrient broth containing ampicillin (100 μ g/ml) and streptomycin (50 μ g/ml). The main culture with M9CA medium was inoculated with the overnight culture (dilution 1:100) and incubated at 37°C. The induction of the corresponding genes was initiated by adding 1 mM isopropyl β -D-1-thiogalactopyranoside and 0.5 mM 5-aminolevulinic acid when the optical density reached 0.9–1 and the culture was grown further at 28°C. After 21 hours of expression, the temperature was set to 30°C and the substrates [stock solution 50 mM in water except for carbamazepine (MeOH), phenothiazine (EtOH) and iminodibenzyl (EtOH)] were added to a final concentration of 200 μ M. To permeabilize the cells, EDTA was also added to a final concentration of 20 mM. The reaction was carried out for 48 hours under the same conditions. Samples were harvested, quenched with the same volume of chloroform and extracted twice. The organic phase was collected, pooled, and evaporated to dryness. The extracts were stored at $-20^\circ C$ until purification.

Whole-Cell System Using Resting Cells. The overnight culture was prepared as described before. Terrific broth medium was inoculated with the overnight culture (dilution 1:100). The expression was induced by adding 1 mM isopropyl β -D-1-thiogalactopyranoside and 0.5 mM 5-aminolevulinic acid at an optical density of 0.7–0.8. After 24 hours of expression at 28°C, the cells were harvested by centrifugation for 20 minutes at 4500g. The cell pellet was resuspended and washed in the buffer [20 mM potassium phosphate buffer (pH 7.4) containing 2% glycerol] following the centrifugation step. The 2.4 g cell mass was suspended in 100 ml buffer containing 2% glycerol. The substrates (200 μ M) and EDTA (20 mM) were added and the cells were incubated for 24 hours at 30°C. The reaction was stopped by adding the same volume of chloroform. The extraction was done as described previously. Until purification, the extracts were stored at $-20^\circ C$.

Analysis of the In Vitro and Whole-Cell Conversions via High-Performance Liquid Chromatography (HPLC)-Diode Array Detector. HPLC analysis was performed on a system consisting of a PU-2080 HPLC pump, an AS-2059-SF autosampler, and a MD-2010 multi wavelength detector (Jasco, Gross-Umstadt, Germany). A Nucleodur 100-5 C18 column (125 \times 4 mm; Macherey-Nagel, Düren, Germany) was used at 40°C. The mobile phase consisted of water containing 0.1% trifluoroacetic acid (A) and acetonitrile containing 0.1% trifluoroacetic acid (B). A gradient from 20% to 80% of B with a flow rate of 1 ml/min was used for the separation of the compounds. The detection wavelengths were substrate dependent: amitriptyline (215 nm), carbamazepine (288 nm), chlorpromazine

(256 nm), clomipramine (252 nm), iminodibenzyl (288 nm), imipramine (252 nm), phenothiazine (252 nm), promethazine (252 nm), and thioridazine (264 nm).

Purification of the Products. The extracts produced by the whole-cell system were purified via HPLC with a Nucleodur 100-5 C18 column (250 \times 5 mm; Macherey-Nagel). For the preparative column, a gradient from 20% to 80% of B was also used but the flow rate was increased to 1.5 ml/min. The fractions containing product were collected, pooled, and treated with the same volume of 1 M glycine buffer (pH 11.4). After four times of extraction with chloroform, the combined organic fractions were evaporated to dryness and prepared for the NMR analysis.

Analysis of the Products via Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS analysis was performed on a system consisting of an AI/AS 3000 autosampler, a DSQII quadrupole, a Focus GC column oven (Thermo Fisher Scientific, Waltham, MA), and a DB-5 column with a length of 30 m, 0.25 mm i.d., and 0.25 μ m film thickness (Agilent Technologies). The compounds were analyzed in an m/z range of 30–450. The starting oven temperature was 100°C for 1 minute, and then the temperature was ramped to 320°C for 20°C/min and held for 10 minutes with a flow rate of 1 ml/min. The EI-mass spectra were compared with the NIST Mass Spectral Library (version 2.0; Gaithersburg, MD).

NMR Analysis. The structures of the products were analyzed by NMR spectroscopy (Institut für Pharmazeutische Biologie, Universität des Saarlandes). The 1H and ^{13}C NMR were recorded on a Bruker (Rheinstetten, Germany) 500 NMR spectrometer. Two-dimensional NMR spectra were recorded as gs-HH-COSY, gs-HSQC, and gs-HMBC. All chemical shifts are relative to $CDCl_3$ ($\delta = 77.00$ for ^{13}C -NMR; $\delta = 7.24$ for 1H -NMR) using the standard δ notion in parts per million.

Results

In Vitro Conversions. Initially, 10 P450s were used for the in vitro conversion of seven drugs (amitriptyline, carbamazepine, chlorpromazine, clomipramine, imipramine, promethazine, and thioridazine) and two precursors (iminodibenzyl and phenothiazine) to evaluate their ability for the conversion of those compounds. As shown in Table 2, CYP260A1, CYP264A1, CYP267A1, and CYP267B1 were able to convert some of the compounds; however, the remaining six enzymes did not show any conversion. CYP264A1 converts four substrates (amitriptyline, chlorpromazine, clomipramine, and imipramine) with appropriate yields. The main products of amitriptyline, chlorpromazine, and imipramine formed by CYP264A1 and CYP267B1 are identical. However, none of the P450s were able to convert carbamazepine. In general, CYP260A1 and the CYP267 family show lower conversions for the substrates compared with CYP264A1.

The obtained product patterns were different for each substrate. Besides the expected hydrophilic products, several hydrophobic products were also found. CYP260A1, CYP267A1, and CYP267B1 showed different product patterns for the conversion of promethazine. Four products were observed with CYP260A1 and CYP267A1, whereas CYP267B1 gave five products, in which two products [retention time (RT) = 11.2 and 11.6 minutes] were much more hydrophobic than the substrate (RT = 7.6 minutes). We observed that the most hydrophobic product (RT = 11.6 minutes) showed the same RT as that of phenothiazine (see Fig. 1). The conversion of chlorpromazine by CYP260A1 also forms a hydrophobic product (RT = 11.2 minutes). Likewise, low amounts of a hydrophobic product (RT = 12.4 minutes) were also found using CYP267A1 for thioridazine conversion (RT = 9.0 minutes). All other products were more hydrophilic compared with the substrates.

In accordance with these in vitro results, amitriptyline, chlorpromazine, clomipramine, and imipramine were chosen for the *E. coli*-based whole-cell biotransformation by CYP264A1. Amitriptyline and chlorpromazine were chosen for the conversion by CYP267B1. Thioridazine was chosen for the conversion by CYP267A1 to upscale the production for structure elucidation of the products.

TABLE 2

In vitro conversions of the drugs with the corresponding myxobacterial P450s (the observed products are arranged according to their RTs) Dashes mark that there is no conversion.

Substrate	Conversion by			
	CYP260A1	CYP264A1	CYP267A1	CYP267B1
RT [min]	RT [min]	RT [min]	RT [min]	RT [min]
Amitriptyline 8.2	—	P1: 70%/6.2 P2: 3%/6.8 P3: 18%/7.0	—	P1: 15%/6.2
Carbamazepine 7.7	—	—	—	—
Chlorpromazine 8.5	P1: 9%/11.2	P1: 62%/6.2	—	P1: 39%/6.2
Clomipramine 8.7	—	P1: 69%/7.0 P2: 23%/7.4 P3: 2%/8.5	—	Traces of product
Imipramine 8.0	—	P1: 52%/6.1 P2: 10%/6.5 P3: 4%/7.6	—	P1: 9%/6.1
Iminodibenzyl 12.1	—	P1: 3%/8.4	—	P1: 31%/9.3
Phenothiazine 11.6	—	P1: 4%/6.5	—	P1: 8%/6.5
Promethazine 7.6	—	—	—	P1: 4%/6.5
	P2: 15%/7.4 P3: 5%/7.9	—	P2: 2%/7.4 P3: 3%/7.9	P2: 23%/7.4
	P5: 17%/11.2 P6: 20%/11.6	—	P5: 4%/11.2 P6: 5%/11.6	P4: 3%/10.8 P5: 3%/11.2 P6: 3%/11.6
Thioridazine 9.0	—	—	P1: 34%/6.9 P2: 5%/7.9 P3: 4%/12.4	—

Whole-Cell Conversions Using M9CA Medium and Resting Cells.

Two different systems for the whole-cell conversions, either using M9CA medium or applying resting cells in buffer, were used to obtain larger amounts of products for NMR characterization. Interestingly, these two systems showed a clear difference concerning substrate conversion. A close correlation between the tested P450s and the conversion of the compounds was not observed (see Fig. 2). CYP264A1 showed higher product yields from amitriptyline, chlorpromazine, and clomipramine with resting cells, whereas a higher yield of the imipramine product was obtained in M9CA medium. In contrast, the yields of the whole-cell conversions with members of the CYP267 family were always higher in M9CA medium. Nevertheless, the ability of CYP267 to convert tricyclic compounds showed their potential pharmaceutical importance to generate derivatives from such compounds.

Product Purification via Preparative HPLC. The products of the corresponding whole-cell conversions were purified via preparative HPLC. The purity of the isolated products was further verified by an additional HPLC measurement before employing it for the NMR measurement. The chromatograms of the purified products and the pure substrates are shown in Supplemental Fig. 1, confirming the successful purifications of the corresponding products. All products were obtained with high purity and sufficient amounts (5–25 mg) for the structure elucidation via NMR spectroscopy.

Structure Elucidation via NMR Spectroscopy and GC-MS. The main products of the CYP264A1-dependent conversion of amitriptyline, chlorpromazine, clomipramine, and imipramine as well as the CYP267A1-dependent conversion of thioridazine were purified and analyzed by NMR spectroscopy. To substantiate the NMR results, the products were additionally analyzed by GC-MS. The ^1H NMR,

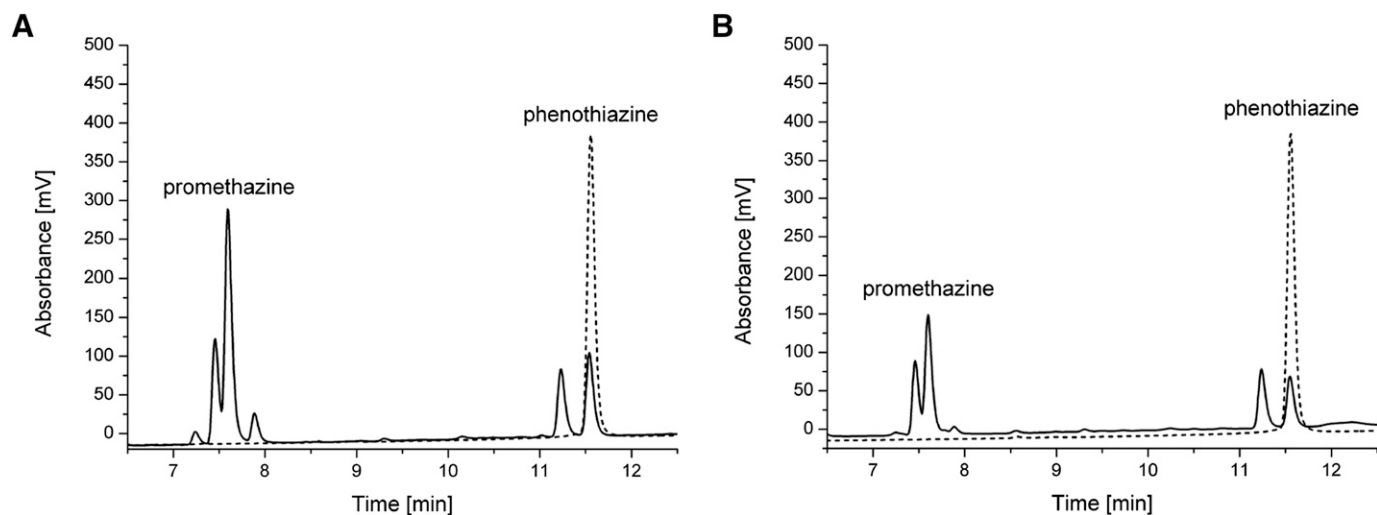


Fig. 1. HPLC chromatograms of the conversions of promethazine (solid lines) with CYP260A1 (A) and CYP267A1 (B) as well as pure phenothiazine (dashed lines). The hydrophobic product at 11.6 minutes shows the same RT as phenothiazine.

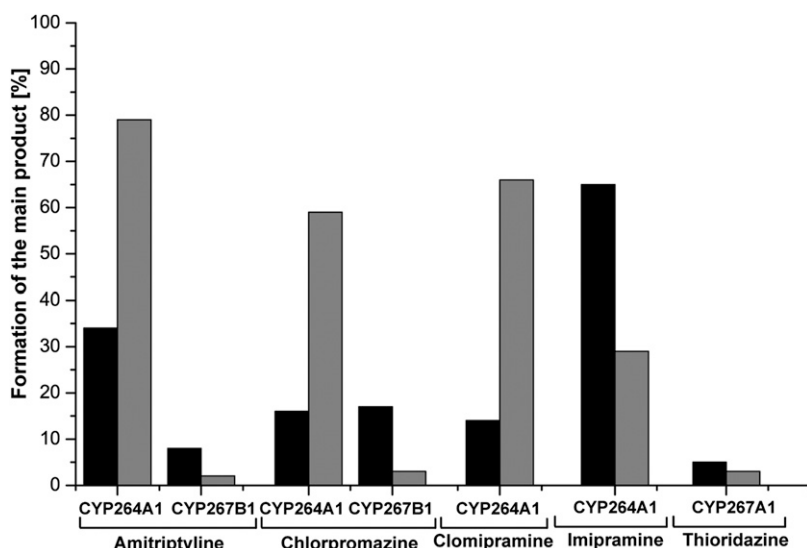


Fig. 2. Conversions of the tricyclic substrates to the corresponding main products. The conversions using M9CA medium (black bar) and resting cells (gray bar) are shown for each substrate.

^{13}C NMR, and GC-MS data are shown in the Supplemental Material (section 2: ^1H -NMR, ^{13}C NMR, and GC-MS data).

We observed that the EI-spectra showed a peak with m/z of 58, which is correlated to the tertiary ammonium moiety of the dimethylamino propyl group (Frigerio et al., 1972). This peak indicated that the dimethylamino group was intact and a demethylation can be excluded for amitriptyline, chlorpromazine, clomipramine, and imipramine. For clomipramine and imipramine conversion the database comparison of the fragmentation pattern showed the highest identity to the corresponding 10-hydroxy products, whereas chlorpromazine conversion showed the highest identity to chlorpromazine 5-sulfoxide. For the product of thioridazine, an ion with m/z of 386 was detected, indicating the insertion of a single oxygen atom. The corresponding 5-sulfoxidated thioridazine product was not available in the NIST database, and therefore it was only elucidated by NMR spectroscopy.

In summary, we showed that the myxobacterial P450s were able to oxidize the heterocyclic ring of the tricyclic compounds (see Fig. 3), in which CYP264A1 and CYP267B1 convert amitriptyline and imipramine to the corresponding 10-hydroxy products, whereas CYP264A1 additionally converts clomipramine to the 10-hydroxy product. In addition, chlorpromazine is sulfoxidated at position 5 by CYP264A1 and CYP267B1. Furthermore, CYP267A1 converts thioridazine to thioridazine 5-sulfoxide. The structures of the products are illustrated in Fig. 3.

Discussion

For the past few years, we have been engaged in the study of novel P450s from myxobacteria, and some P450s from *S. cellulorum* So ce56 have shown a potential for industrial and biotechnological applications (Khatri et al., 2010a,b, 2013, 2014; Ly et al., 2012; Ringle et al., 2013). We were also able to develop an efficient *E. coli*-based whole-cell bioconversion system for some myxobacterial P450s (Ringle et al., 2013). However, the potential applications of those P450s for the production of novel or known drug-related compounds have not yet been studied. Therefore, 10 myxobacterial P450s were investigated concerning their ability to produce human-like or novel drug metabolites from tricyclic psychotherapeutic drugs.

The production of human drug metabolites is an important challenge in the pharmaceutical industry. Drug metabolites formed at greater than 10% of the parent drug systemic exposure at steady state (FDA, 2008) or total drug-related exposure (European Medicines

Agency, 2009) need to be tested in toxicological studies, whereby the International Conference on Harmonization guidelines take precedence over the FDA guidelines (Frederick and Obach, 2010; Haglund et al., 2014). For such studies, small (μg) to large quantities (in a gram scale) of these drug metabolites are necessary to provide standards for analytical and toxicological studies, respectively. Utilization of human liver or human liver microsomes is restricted due to limited availability of this human organ. Therefore, alternative ways of producing such metabolites have been investigated. Several approaches, including cell cultures and different microbial systems expressing human P450s as whole-cell biocatalysts to convert drugs, have been described (Crespi et al., 1993; Döhmer, 2001; Drăgan et al., 2011; Geier et al., 2012). However, the low activity and stability of the mammalian P450s compared with the bacterial and fungal P450s are limiting their application at an industrial scale (Julsing et al., 2008; Sakaki, 2012). In addition, the membrane association of the mammalian P450s hinders a simple handling of these enzymes for biotechnological applications, in contrast to the soluble bacterial P450s (Urlacher et al., 2004; Bernhardt and Urlacher, 2014). As a result, bacterial P450s become an alternative method to produce the identical metabolites as those provided by mammalian P450s (Yun et al., 2007; Caswell et al., 2013). Using wild-type microorganisms such as *Cunninghamella* ssp. or *Streptomyces* ssp. for the conversion of drugs is another possibility to obtain sufficient amounts of metabolites (Asha and Vidyavathi, 2009; Murphy, 2015). However, employing such microbial organisms for the production of drug metabolites can be a time-consuming process because of the lack of information regarding the involving enzyme in the biotransformation of the corresponding drug. Therefore, improvements of such a system to obtain higher amounts of a specific product are limited. This can be overcome by the utilization of specific bacterial P450s efficiently expressed in a bacterial whole-cell system as a biocatalyst. In this regard, one of the best characterized bacterial P450s is CYP102A1 (P450BM3) from *Bacillus megaterium*, which has been the target of most engineering efforts to improve activity, selectivity, and the range of substrates (Jung et al., 2011; Whitehouse et al., 2012). However, screening a variety of bacterial P450s for their ability to convert various drugs will certainly lead to a broad range of substrates with high structural diversity to be converted by these P450s. Therefore, we investigated 10 P450s from *S. cellulorum*—a bacterium typically found in soil (Shimkets et al., 2006), where it is exposed to a variety of xenobiotics—for their ability to convert tricyclic drugs.

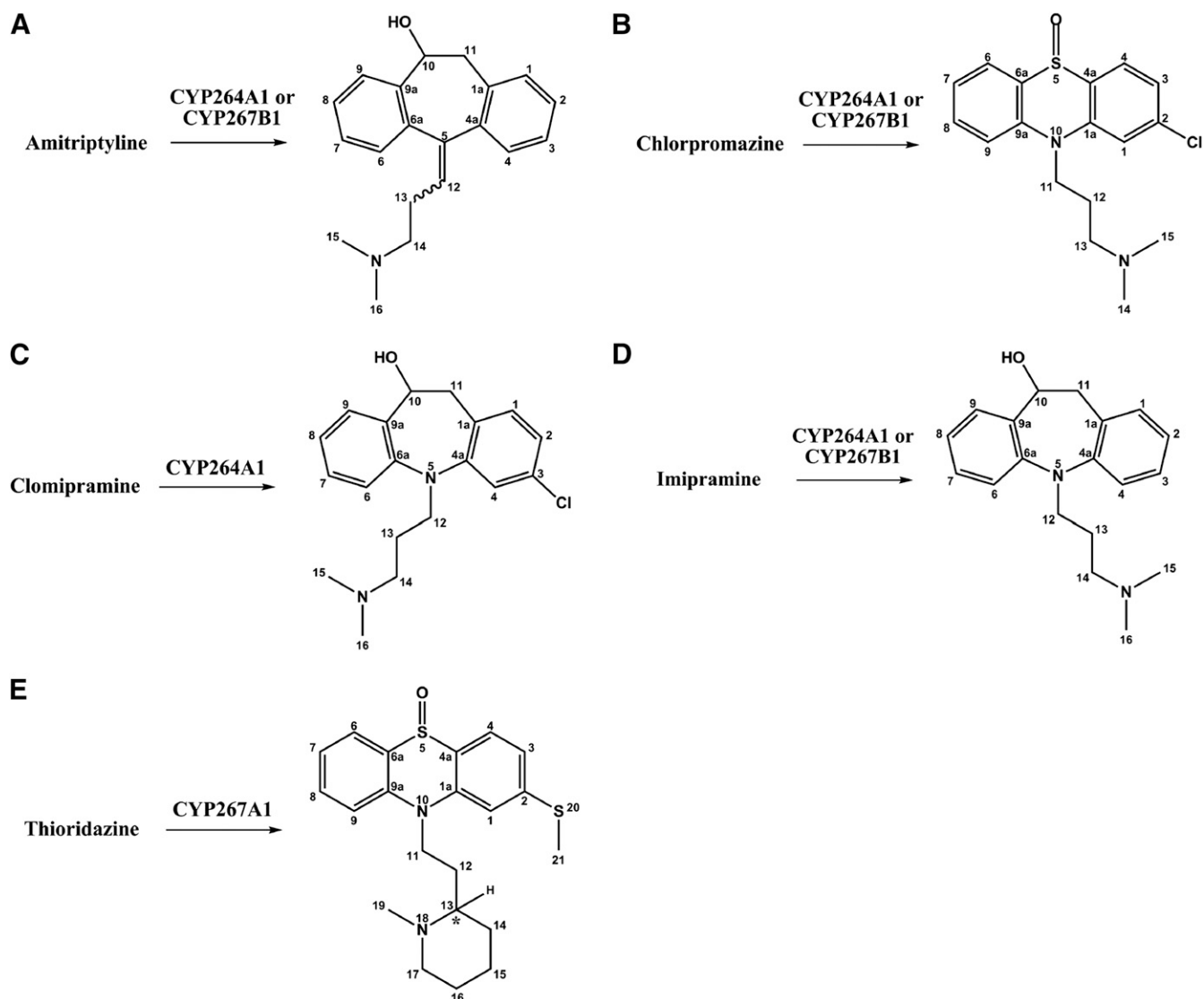


Fig. 3. Conversions of the substrates amitriptyline (A), chlorpromazine (B), clomipramine (C), imipramine (D), and thioridazine (E) by the myxobacterial P450s.

Terpenes and terpenoids as well as fatty acids have already been identified as substrates for these wild-type P450s in previous studies (Khatri et al., 2010a,b, 2014; Ly et al., 2012; Schiffrin et al., 2014). Moreover, 4-methyl-3-phenyl-coumarin, a tricyclic molecule, was identified as a substrate for CYP264A1 (Ringle et al., 2013). Here, we can demonstrate that all tricyclic compounds except carbamazepine are converted in vitro by the myxobacterial P450s CYP260A1, CYP264A1, CYP267A1, and CYP267B1. To obtain sufficient amounts of products for structural elucidation, we used an *E. coli*-based whole-cell biotransformation system. The obtained products of amitriptyline, chlorpromazine, clomipramine, imipramine, and thioridazine were purified and characterized by NMR spectroscopy. It is remarkable that these products were indeed the same compounds as those obtained by human liver P450s. The 10-hydroxy products of amitriptyline and imipramine are predominantly formed by CYP2D6, whereas CYP1A2 and CYP3A4 are mainly capable of sulfoxidations of chlorpromazine and thioridazine. Moreover, the myxobacterial P450s show high selectivity for the production of a single major product, whereas the human P450s produce several other side products (see Table 1). Therefore, the ability to produce pure metabolites in a large

quantity by the myxobacterial P450s is more suitable compared with the application of human P450s producing several metabolites.

Although 4-methyl-3-phenyl-coumarin has been previously identified as the only substrate for CYP264A1 (Ringle et al., 2013), its product has neither pharmaceutical nor chemical interest. However, the tricyclic moiety of this substrate indicated that CYP264A1 probably needs this structure for substrate recognition. This suggestion was supported by our observation that amitriptyline, chlorpromazine, clomipramine, and imipramine—all containing a tricyclic moiety—were converted by this P450. In addition, CYP264A1 is sensitive to the side chain moiety of the selected tricyclic compounds, since promethazine shows no conversion, whereas chlorpromazine is converted. In this regard, the dimethylamino propyl side chain seems to be necessary for the conversion. In agreement with this assumption, the two precursors, iminodibenzyl and phenothiazine, lacking the side chain, were also nearly not converted. Likewise, thioridazine containing a sterically demanding side chain is also not converted. However, amitriptyline, which possesses a side chain with reduced flexibility caused by the double bond, is converted. Comparing imipramine and clomipramine, there is no significant difference between their conversions, suggesting that the halogen

group at the aromatic ring does not seem to play a notable role in the reaction. Unexpectedly, the homolog of this enzyme from the same strain, CYP264B1, which shows no activity toward the tested tricyclic compounds (see Table 2), acts as a norisoprenoid and sesquiterpene hydroxylase (Ly et al., 2012).

Interestingly, we also observed the formation of products being more hydrophobic than the substrate from the conversion of promethazine by CYP260A1 as well as CYP267A1 and CYP267B1. One single hydrophobic product showing an identical RT as phenothiazine has been identified, suggesting that the side chain of promethazine might have been cleaved. Although the oxidative thermal degradation of promethazine has been previously described (Underberg, 1978), the dealkylation of promethazine by P450s has not yet been described. In addition, CYP260A1 also forms a more hydrophobic product from chlorpromazine. However, the formation of N-oxide products could also lead to more hydrophobic products, although these products are mainly formed by flavin-containing monooxygenases (Ziegler, 1993). In addition, ring-opening products are also unlikely due to the fact that these products are obviously more hydrophilic compared with the substrate. However, because of the very low yield we were not able to characterize these products. Interestingly, although tricyclic substrates for CYP267A1 and CYP267B1 were not known before, we identified the psychotherapeutic drugs as substrates for these P450s for the first time. Despite being homologous to each other, CYP267A1 and CYP267B1 showed quite different affinity for the tested substrates. Promethazine is the only common substrate converted by both P450s. However, the ability of CYP267B1 to convert most of the selected drugs makes this enzyme a promising candidate for the conversion of other drugs or drug-related derivatives. Furthermore, improvements of these P450s by protein engineering could lead to higher yields or an increased substrate range (Gillam, 2008).

We also observed that the conversion of tricyclic compounds by the myxobacterial CYP264A1 showed identical main products as those observed by human CYP2D6. There are no crystal structures for human CYP2D6 with the tested substrates or for the myxobacterial P450s available. Therefore, we performed an alignment of CYP2641 and CYP267A1 with human CYP2D6 to determine the substrate binding residues that have been shown in the crystal structure of CYP2D6 (Rowland et al., 2006) and the recent docking study of imipramine with CYP2D6.1 (Handa et al., 2014). Interestingly, the conserved Phe120 and Val374 of CYP2D6, which control the orientation of the substrate, are conserved as Phe61 and Val279, respectively, in CYP264A1, but not in CYP267A1 (see Supplemental Fig. 2). It is assumed that this observation might be the reason for the similar conversion patterns of both CYP264A1 and CYP2D6.

In our study carbamazepine is the only compound that has not been converted by any of the tested myxobacterial P450s. The presence of a double bond in the middle ring forms a higher electron density, which might subsequently hinder the oxidation at this specific position. In addition, the presence of the urea moiety may influence the hydrogen bonds of the substrate-enzyme interactions, which can lead to an incorrect substrate orientation in the active site of the P450. Taken together, our results showed the potential of the myxobacterial P450s as an efficient source for the production of drug metabolites or their derivatives. The products could be used as analytical standards or for toxicological investigations showing their significance for pharmaceutical studies.

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Authorship Contributions

Participated in research design: Litzenburger, Kern, Bernhardt.

Conducted experiments: Litzenburger, Kern.

Performed data analysis: Litzenburger, Kern.

Wrote or contributed to the writing of the manuscript: Litzenburger, Kern, Khatri, Bernhardt.

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