Microbial flavoprotein monooxygenases as mimics
of mammalian flavin-containing monooxygenases
for the enantioselective preparation of drug metabolites

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Abbreviations: BVMO, Baeyer-Villiger monooxygenase; CD, circular dichroism; CHMO, cyclohexanone monooxygenase; CYP450, cytochrome P450; e.e., enantiomeric excess; FAD, flavin adenine dinucleotide; FMO, flavin-containing monooxygenase; HILIC, hydrophilic interaction chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; LB medium, lysogeny broth medium; LC, liquid chromatography; MS/MS, tandem mass spectrometry; NADPH, reduced nicotinamide adenine dinucleotide phosphate; OD, optical density; PAMO, phenylacetone monooxygenase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPE, solid phase extraction; SRM, selected reaction monitoring.
Abstract

Mammalian flavin-containing monooxygenases are difficult to obtain and study while they play a major role in detoxifying various xenobiotics. In order to provide alternative biocatalytic tools to generate FMO-derived drug metabolites, a collection of microbial flavoprotein monooxygenases, sequence-related to human flavin-containing monooxygenases (FMOs), was tested for their ability to oxidize a set of xenobiotic compounds. For all tested xenobiotics (nicotine, lidocaine, 3-(methylthio)aniline, albendazole, and fenbendazole), one or more monooxygenases were identified capable of converting the target compound. Chiral LC-MS/MS analyses of the conversions of 3-(methylthio)aniline, albendazole and fenbendazole revealed that the respective sulfoxides are formed in good to excellent enantiomeric excess by several of the tested monooxygenases. Intriguingly, depending on the chosen microbial monooxygenase, either the (R)- or (S)-sulfoxide was formed. For example, when using a monooxygenase from *Rhodococcus jostii* the (S)-sulfoxide of albendazole (ricobendazole) was obtained with an e.e. of 95%, while a fungal monooxygenase yielded the respective (R)-sulfoxide in 57% e.e. For nicotine and lidocaine, monooxygenases could be identified that convert the amines into their respective N-oxides. This study shows that recombinantly expressed microbial monooxygenases represent a valuable toolbox of mammalian FMO mimics that can be exploited for the production of FMO-associated xenobiotic metabolites.
Introduction

Metabolism of xenobiotics in humans and other mammals often starts with oxidation of the target molecule. Most of the Phase I metabolism reactions are catalyzed by cytochrome P450 monooxygenases (CYP450s) (Cashman et al., 2005). However, apart from CYP450s, recent studies have shown that the so-called flavin-containing monooxygenases (FMOs) also play a crucial role in the biotransformation of a large variety of xenobiotics, including pharmaceuticals and natural products. Mammals typically employ several FMO isoforms. The human proteome contains 5 isoforms, FMO1-FMO5, all of which have their typical tissue-dependent expression patterns and roles in metabolism (Cashman and Zhang, 2006). FMOs have been shown to be involved in the oxygenation of heteroatom-containing compounds, such as amines and sulfides (Cashman and Zhang, 2006; Cashman et al., 2004; Krueger and Williams, 2005). Different from CYP450s, which contain a heme cofactor, FMOs utilize a flavin cofactor for oxidations which also translates into a different oxidative mechanism. Furthermore, to discriminate between metabolism by human FMOs or CYP50s, often differences in stability and specific inhibitors can be used (Taniguchi-Takizawa et al., 2015). FMO enzymes require NADPH for reducing the FAD flavin cofactor. The reduced flavin subsequently reacts with molecular oxygen resulting in the formation of a reactive 4α-hydroperoxyflavin. This reactive flavin intermediate is able to perform a variety of oxygenation reactions, for example sulfoxidations and N-hydroxylations (see (Cashman et al., 2005; Cashman and Zhang, 2006; Krueger and Williams, 2005; van Berkel et al., 2006; Ziegler et al., 1993; Malito et al., 2004; Ziegler et al., 1990) for mechanistic details).

While it has been established that human FMOs are essential in oxidizing a variety of xenobiotics, biochemical and metabolic studies on these enzymes are hampered by their poor availability. Human FMOs (hFMOs) and their mammalian orthologs are typically membrane associated and often thermolable which appear to be the major reasons for their problematic
isolation from tissue (Cashman et al., 1995; Wu et al., 2004) and inefficient recombinant production. While human FMOs can be studied using microsomal preparations and some human FMOs were expressed as functional enzymes in heterologous hosts (Motika et al., 2009; Balke et al., 2012; Geier et al., 2015; Shimizu et al., 2015), these enzyme preparations involve costly and cumbersome isolation procedures, and often suffer from low activity and stability (Cashman and Zhang, 2006; Cashman et al., 1992). Sequence comparison studies have revealed that FMOs are part of a large family of monooxygenases, the so-called Class B flavoprotein monooxygenases (Reddy et al., 2010). Intriguingly, many bacteria and fungi contain sequence-related Class B flavin-containing monooxygenases (Mascotti et al., 2015) that are typically involved in catalyzing Baeyer-Villiger oxidations forming a subfamily of Baeyer-Villiger monooxygenases (BVMOs). Biocatalytic studies on these microbial monooxygenases confirmed that they employ the same catalytic mechanism as FMOs (Pazmiño et al., 2008) and, interestingly, are also able to catalyze oxygenations of heteroatom containing compounds. In contrast to hFMOs, many microbial BVMOs are soluble enzymes and can be easily produced in recombinant form (de Gonzalo et al., 2010).

Inspired by the observation that microbial BVMOs are sequence-related to human FMOs and exhibit similar activities, we set out to explore their use as mammalian FMO mimics. By testing a panel of xenobiotic compounds, including drug molecules, with a collection of microbial BVMOs, we discovered that these biocatalysts may serve as tools to prepare metabolites. By choosing the proper monooxygenase, all tested xenobiotics (nicotine, lidocaine, 3-(methylthio)aniline, albendazole, and fenbendazole, see Figure 1) could be converted. Chiral LC-MS/MS analysis showed that sulfides were converted to the corresponding sulfoxides with excellent and complementary enantioselectivities. This study reveals that recombinant microbial BVMOs,
which are relatively easy to produce and robust as biocatalysts, represent attractive alternatives to mammalian FMOs for the preparation of FMO-related metabolites.

**Materials and Methods**

**Materials**

3-(Methylthio)aniline, albendazole, ricobendazole (racemic albendazole sulfoxide), fenbendazole, lidocaine, nicotine, 1,4-dioxane and Tris were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Acetaminophen was purchased from Fluka, ultra-pure HPLC grade acetonitrile and HPLC grade methanol were purchased from Biosolve (Valkenswaard, The Netherlands). Catalase was purchased from Fluka while phosphite dehydrogenase was prepared using an established protocol (Dudek *et al.*, 2011). Ultrapure water was obtained from a Milli-Q Advantage A10 Water Purification system (Millipore Corp., Billerica, MA, USA). Oasis HLB 30 mg solid phase extraction (SPE) cartridges were purchased from Waters (Manchester, UK).

**Recombinant expression of BVMOs and preparation of cell extracts**

The enzymes were overexpressed in *Escherichia coli* using previously established conditions and protocols. CHMOAc, PAMOM446G and BVMOBj24 were expressed using the pCRE2 expression vector (Pazmiño *et al.*, 2009), yielding the enzyme fused to His-tagged phosphite dehydrogenase which facilitates cofactor regeneration. His-tagged PAMO and Strep-tagged FMOBjE were expressed as described previously (Fraaije *et al.*, 2005) while for expressing BVMOMt1, a pET_SUMO vector was used. Precultures were grown overnight at 37 °C with shaking (180 rpm) in lysogeny broth (LB) medium containing ampicillin (50 µg/mL). The exception was BVMOMt1 for which cells were grown in the presence of kanamycin (100 µg/mL). Flasks containing 200 mL TB medium with the respective antibiotic were inoculated 1:100 (v/v) using the preculture
and grown for another 4 h at 30 °C. After that, each flask was supplemented with inducer: 1.0 mM isopropyl β-D-thiogalactopyranoside (IPTG) for BVMO_Mt1, and 0.02% arabinose for PAMO, PAMO_M446G, and CHMO_Ac, and 0.002% arabinose for the remaining enzymes. After 48 h of growth at 24 °C with shaking (130 rpm) cells were harvested by centrifugation at 4 °C, 17,000 g and resuspended in 50 mM Tris buffer pH 8. Cells were diluted to an OD₆₀₀ of 212 for TB samples and an OD₆₀₀ of 98 for LB samples. Subsequently, the cells were disrupted by sonication for 90 s using 2 s sonication pulses and 2 s breaks, while on ice. The prepared cell extracts were supplemented with glycerol (15%), aliquoted (100 µL in Eppendorf tubes), frozen in liquid nitrogen, and stored at -80 °C. As negative control E. coli cells were grown without expression plasmid and used for the preparation of cell extract as described above. Overexpression of the enzymes was confirmed with SDS-PAGE by analyzing OD-normalized samples from bacterial cultures.

**Monooxygenase-catalyzed conversions**

For conversions, cell extracts (100 µL) were supplemented with 1.0 mM substrates (except for 3- (methylthio)aniline: 3.0 mM was used) using 1,4-dioxane as a cosolvent (1% v/v for all substrates except for 3-(methylthio)aniline (0.6% v/v)), 100 µM NADPH, 20 mM phosphite, 5.0 µM phosphite dehydrogenase, 20 mU catalase and 50 mM Tris-HCl (pH 8.5) in a total volume of 300 µL. In order to increase the solubility of albendazole and fenbendazole, 9.6 mM β-cyclodextrine was added. Negative control experiments were performed by incubating substrates with cell extracts that did not contain any expressed monooxygenase. All the conversions were performed in duplicate. After 135 min of incubation at room temperature, a 100 µL sample was taken and proteins were precipitated by adding 300 µL acetonitrile containing 0.2% formic acid.
Samples were vortexed for 30 s and centrifuged at 13000 rpm for 6.5 min. After centrifugation, 200 µL of the supernatants was evaporated to dryness under nitrogen prior to solid phase extraction (SPE). SPE was performed on Oasis HLB 30 mg cartridges that were wetted with acetonitrile and equilibrated with H$_2$O/acetonitrile (95:5). Dried samples were dissolved in 200 µL water and loaded onto the cartridge. Water (3 x 250 µL) was used to wash the cartridges and the final elution was performed with acetonitrile (4 x 250 µL). For the LC-MS/MS analysis, samples were 10x diluted in water containing 10 µM acetaminophen, as an internal standard for LC-MS/MS signal normalization.

**Chiral LC-MS/MS in the Selected Reaction Monitoring (SRM) mode**

LC-MS/MS analyses in the SRM mode were carried out on an HPLC system with an Accela Autosampler and a Surveyor Pump coupled to a TSQ Quantum AM triple quadrupole mass spectrometer (Thermo Finnigan, San José, CA) with an ESI interface in the positive mode (Supplemental Table 1 and Supplemental Table 2). 3-(Methylthio)aniline, albendazole, fenbendazole and their chiral sulfoxide products were separated with an amylose tris(3-chlorophenylcarbamate)-based chiral column (Chiralpak ID, 5 µm particle size, 2.1x150 mm; Chiral Technologies Europe, Illkirch, France) at a flow rate of either 100 or 200 µL/min. The LC separation of lidocaine and its products was performed with a C$_{18}$ reversed-phase column (GraceSmart RP 18, 5 µm particle size, 2.1x150 mm; Grace Davison, Lokeren, Belgium) at a flow rate of 250 µL/min. The LC separation of nicotine and its products was performed with a hydrophilic interaction (HILIC) column (Xbridge amide, 3.5 µm particle size, 2.1x150 mm; Waters, Milford, MA, USA) at a flow a rate of 250 µL/min. The following set of solvents was used for the separations: solvent A (H$_2$O with 0.1% formic acid), solvent B (acetonitrile with
0.1% formic acid), solvent C (H₂O with 20 mM ammonium bicarbonate (pH 9, adjusted with NH₃)), solvent D (acetonitrile) and solvent E (H₂O with 10 mM ammonium formate, pH 5.5). Separation of the two 3-(methylthio)aniline sulfoxide enantiomers was performed isocratically at 100 μL/min (20 min) with 90% solvent C / 10% solvent D. The albendazole sulfoxide enantiomers were separated isocratically with 50% solvent C / 50% solvent D at 200 μL/min (20 min) and fenbendazole sulfoxides were separated with 40% solvent C / 60% solvent D at 200 μL/min (15 min), using the Chiralpak column. The LC-MS/MS analysis of lidocaine and its N-oxide was performed by reversed-phase LC applying a linear gradient starting from 5% to 95% solvent B in solvent A over 11 min which was held for 1 min. Solvent B was decreased rapidly to 5% in 20 s and the column re-equilibrated at 5% solvent B for 4 min. The LC-MS/MS analysis of nicotine and its N-oxide was performed by HILIC applying a linear gradient starting from 10% to 90% solvent E in solvent D (acetonitrile) over 8 min which was held for 2 min. Solvent E was decreased rapidly to 10% in 20 s and finally the column re-equilibrated at 10% solvent E for 3 min. Acetaminophen was used as internal standard to normalize the peak areas across LC-MS/MS runs.

**Circular Dichroism (CD)**

In order to assign the absolute configuration of the products, samples were analyzed by CD spectroscopy. Samples were purified with SPE, dried by evaporation of acetonitrile, and dissolved in methanol to a nominal concentration of 0.75 mM. For CD analysis samples were further diluted 4 times in methanol. CD spectra were recorded on a J-810 spectropolarimeter (JASCO, Tokyo, Japan) using a 1 mm quartz cell cuvette and scanning from 200 to 350 nm at 25 °C; methanol was used as a blank.
Results

Conversion of the sulfide 3-(methylthio)aniline

We selected seven microbial flavoprotein monooxygenases originating from three different microorganisms. Besides three well-studied BVMOs, phenylacetone monooxygenase (PAMO) from Thermobifida fusca (Fraaije et al., 2005), the Met446Gly PAMO mutant (PAMO\textsubscript{M446G}) (de Gonzalo et al., 2012) and cyclohexanone monooxygenase from Acinetobacter calcoaceticus (CHMO\textsubscript{Ac}) (Pazmiño et al., 2009), two recently discovered BVMOs from Rhodococcus jostii (BVMO\textsubscript{Rj4} and BVMO\textsubscript{Rj24}) (Riebel et al., 2013) and a BVMO from the fungus Myceliophthora thermophila (BVMO\textsubscript{Mt1}) were included in this study. Furthermore, we also included a representative of a newly discovered distinct subfamily of microbial monooxygenases, the so-called Type II FMOs, that share characteristics of both BVMOs and FMOs: FMO\textsubscript{RjE} from \textit{R. jostii} (Riebel et al., 2013). All studied monooxygenases belong to the Class B flavoprotein monooxygenases and, hence, are distantly related to mammalian FMOs as evidenced by significant sequence identities (20-40\%) and highly conserved sequence motifs (Krueger and Williams, 2005). Also the obtained crystal structures of a bacterial FMO and several BVMOs have confirmed that Class B flavoprotein monooxygenases share structural and mechanistic features (Malito et al., 2004). CHMO\textsubscript{Ac} and PAMO display complementary and broad substrate acceptance profiles (Pazmino & Fraaije, 2008), while the other monooxygenases have been hardly explored for their substrate scope. Therefore, we anticipated that by studying such a large panel of different microbial monooxygenases, several targeted compounds could be converted by one or more monooxygenases.

All monooxygenases were produced in \textit{E. coli} as expression host. SDS-PAGE gel analysis confirmed high and quantitatively comparable overexpression in soluble form of all investigated enzymes. In all cell extracts, the expressed monooxygenase was the most prominent protein band.
on the SDS PAGE gel. Because *E. coli* does not contain any endogenous enzymes with similar activity, BVMO- or FMO-type monooxygenases, no enzyme purification step was required and the cell extracts were used for performing the conversions. As a first test substrate, 3-(methylthio)aniline was used. PAMO and PAMO mutants have been shown to be able to efficiently convert aromatic sulfides (de Gonzalo *et al*., 2005). Human FMOs are also known for their ability to perform sulfoxidations of aromatic sulfides or thioureas (Cashman and Zhang, 2006; Motika *et al*., 2007). Depending on the type of sulfide and FMO isoform, various enantioselectivities by mammalian FMOs have been described (Hai *et al*., 2009; Virkel *et al*., 2004; Moroni *et al*., 1995). Conversion of this relatively simple aromatic thioether was probed with all 7 studied monooxygenases. For the conversions, 3.0 mM 3-(methylthio)aniline was incubated with cell extracts supplemented with phosphite, NADPH and phosphite dehydrogenase to regenerate the reduced coenzyme. After 135 min incubation, product analysis was performed by chiral LC-MS/MS in the SRM mode. The absolute configuration of the observed sulfoxides was determined by CD spectroscopy of the isolated enantiomers. Control reactions also resulted in formation of low amounts of sulfoxides due to spontaneous reaction with molecular oxygen. These reactions are not enantioselective, and the observed amounts in the enzymatic conversions were corrected for the background oxidation level. All tested monooxygenases produced significant amounts of sulfoxides but with markedly different enantioselectivity (Figure 2). CD analysis of the 3-(methylthio)aniline sulfoxides formed in the BVMO*Rj*4 and BVMO*Mt*1 samples gave $[\alpha]_{D}^{25}$ values of +20.8 and -16.0, respectively (Supplemental Figure 1). Based on comparison with literature data for (R)-3-(methylthio)aniline we assign the first eluting enantiomer (at 8.4 min) to (+)-(R)-3-(methylthio)aniline sulfoxide and the second eluting enantiomer (at 11.2 min) to (-)-(S)-3-(methylthio)aniline sulfoxide (Slack *et al*., 2012; Folli *et al*., 1973).
Most monooxygenases have a preference for forming the (+)-(R)-3-(methylthio)aniline sulfoxide (e.e. values 66-88%). However, BVMO_{Rj4} produced the (-)-(S)-3-(methylthio)aniline sulfoxide with an e.e. of > 99.5% showing that this set of monooxygenases allows the synthesis of both sulfoxide enantiomers in very good to excellent enantiomeric excess. Only BVMO_{Rj4} and BVMO_{Mt1} produced an additional sulfone product at less than 2% of the amount of the sulfoxide. The standard addition method was used to quantify the conversion of 3-(methylthio)aniline after 135 min incubation. The conversion reached 72% for BVMO_{Rj24} and 97% for BVMO_{Mt1} (Supplemental Figures 2-and 4). This indicates that with the current approach 4-5 mg of enantiopure sulfoxide metabolite is produced in 1 h using a cell extract from a 1 L culture.

Conversion of the thioether drugs, albendazole and fenbendazole

Two drugs that are commonly used to treat worm infestations in mammals, albendazole and fenbendazole, are known to be converted in an enantioselective manner into their sulfoxides by mammalian FMOs (Moroni et al., 1995). Testing the panel of microbial monooxygenases revealed that three monooxygenases (CHMO_{Ac}, BVMO_{Rj24} and BVMO_{Mt1}) converted albendazole, yielding sulfoxides in significant enantiomeric excess (Figure 3). Only BVMO_{Mt1} was able to catalyze the sulfoxidation of fenbendazole. Determination of the [\alpha]_{D}^{25} values by CD analysis could not be performed, because the yields of the albendazole and fenbendazole sulfoxides were too low. However, the sulfoxide enantiomers of albendazole and fenbendazole have been characterized using the same chiral column and a similar solvent system by Materazzo et al. (2014), allowing us to assign the first eluting enantiomers to (R)-albendazole sulfoxide (3.1 min) and (R)-fenbendazole sulfoxide (3.5 min), respectively, and the second eluting enantiomers to (S)-albendazole sulfoxide (5.5 min) and (S)-fenbendazole sulfoxide (7.1 min), respectively. Chiral LC-MS/MS in the SRM mode showed that CHMO_{Ac} and BVMO_{Mt1} formed the same
product as mammalian FMOs, (R)-albendazole sulfoxide (32 and 55% e.e., respectively), whereas BVMO$_{Rj24}$ enzyme produced (S)-albendazole sulfoxide with an enantiomeric excess of 95%. The yields of albendazole sulfoxide were determined with standard addition and reached 55% for BVMO$_{Rj24}$ and 25% for BVMO$_{Mt1}$ (Supplemental Figures 5 and 7). This corresponds to 1-2 mg/h and per L of bacterial culture. Less than 1% of the sulfone product was formed by BVMO$_{Rj24}$, BVMO$_{Mt1}$ and CHMO$_{Ac}$ upon conversion. No other side products were detected. Chiral LC-MS/MS in the SRM mode showed that conversion of fenbendazole by BVMO$_{Mt1}$ yields (S)-fenbendazole sulfoxide in 96% e.e. (Figure 4).

Conversion of the amines, lidocaine and nicotine

Lidocaine and nicotine contain a regular and a cyclic tertiary amine group, respectively. Lidocaine is a widely-used local anesthetic while nicotine is a plant alkaloid which acts as a stimulant. Both drugs are known to be oxidized by mammalian FMOs into their corresponding N-oxides. LC-MS/MS analysis showed that significant conversion of lidocaine into the N-oxide is performed by BVMO$_{Rj24}$, while nicotine is converted by CHMO$_{Ac}$ (Figure 5). With the applied conditions, the degree of conversion for both substrates was rather low, below 10%. For lidocaine no other products (specifically the Cytochrome P450-catalyzed N-dealkylation or aromatic hydroxylation products) were observed in significant amounts. The N-oxide of lidocaine can be distinguished from other monoxygenation products by its specific SRM transition (m/z 251/130). Additionally, its retention time was confirmed using a chemically oxidized lidocaine N-oxide standard.

LC-MS/MS analysis of the nicotine conversion samples showed two N-oxidation products which could be assigned on the basis of their fragmentation patterns; the SRM transition of m/z 179/117 is unique for nicotine-1`-N-oxide (oxidation of nitrogen on the pyrrolidine ring) and the m/z
179/148 transition is unique for nicotine-1-N-oxide (oxidation of nitrogen on the pyridine ring) (Piller et al., 2014; Smyth et al., 2007). The nicotine-1'-N-oxide product was the major N-oxide formed by CHMOAc. The low amount of nicotine-1-N-oxide was similar to the amount formed in the control reaction.

Discussion

Human FMOs and other mammalian FMOs play a crucial role in degrading a wide range of xenobiotics, including many drugs. While they are known for their chemo- and enantioselective oxidations, mammalian FMOs are notoriously difficult to obtain or to use as isolated biocatalysts. To provide an alternative for the biocatalytic production of FMO-derived metabolites, we explored the use of the microbial Class B flavoprotein monooxygenases which are all sequence-related to FMOs. Besides sequence similarities, members of the Class B flavoprotein monooxygenases all share a similar structural fold. They are composed of FAD-binding domain with a tightly bound FAD as a prosthetic group and a NADPH binding domain which binds NADPH as coenzyme during catalysis (Krueger and Williams, 2005). In addition, kinetic and mechanistic studies on FMOs and BVMOs have revealed that these flavoprotein monooxygenases also share a common catalytic mechanism. This is also reflected in the type of oxygenation reactions that are catalyzed by members of both monooxygenase groups: they overlap and include N-oxygenations, sulfoxidations and Baeyer-Villiger oxidations (Fiorentini et al., 2016). The catalytic cycle starts with binding of the reduced coenzyme NADPH which results in reduction of the flavin cofactor. Through a subsequent fast reaction with molecular oxygen, the per oxyflavin intermediate is formed that is key to catalyze substrate oxygenation (Beaty and Ballou, 1981; Pazmiño et al., 2008). The reactive per oxyflavin is stabilized through interactions with active site residues and awaits entry of a suitable substrate in the active pocket. The
accessibility, character and size of the active site pocket determines the substrate specificity and the enantio- and regioselectivity of each monooxygenase. As a consequence and different from many other flavoprotein monooxygenases and CYP450, formation of the reactive oxygenating enzyme intermediate is not dependent on binding of a substrate. Many Class B monooxygenases, including human FMOs, have been shown to display a relaxed substrate acceptance profile. This triggered our study to explore the catalytic potential of microbial flavoprotein monooxygenases, that are sequence related to mammalian FMOs, for the conversion of FMO substrates. One of the advantages of using such enzymes for in vitro conversion of FMO-targeted xenobiotics is the ease of production of the microbial enzymes at high levels and in soluble form in E. coli. Upon growth of the recombinant bacteria, the cell extracts could be immediately used for conversion of the targeted xenobiotics. Another advantage of this approach is the fact that in the last decade a large number of recombinant microbial flavoprotein monooxygenases have become available. For example, we have generated an in-house library of >30 different microbial flavoprotein monooxygenases (Fraaije et al., 2005; Riebel et al., 2013). For our study we decided to explore a set of seven monooxygenases that are known to display dissimilar substrate acceptance and oxygenation selectivity profiles.

Five different xenobiotics (3-(methylthio)aniline, albendazole, fenbendazole, lidocaine and nicotine) were chosen to examine enantio-, region- and chemoselective oxygenation by using a panel of seven different recombinant microbial flavoprotein monooxygenases. Chiral LC-MS/MS in the SRM mode was instrumental in establishing activity and selectivity of each enzyme towards each test compound. The enzymes that were found to be able to convert albendazole and fenbendazole formed the corresponding sulfoxides with very good enantiomeric excess. Both enantiomers of albendazole sulfoxide were produced in enantiomeric excess (CHMOAc and BVMOM21 for the (R)-sulfoxide and BVMOR24 for the (S)-sulfoxide). Previously,
it has been shown that mammalian FMOs have a preference for forming (R)-albendazole sulfoxide from albendazole (Virkel et al., 2004; Moroni et al., 1995). For fenbendazole, only one active enzyme (BVMO Mt1) was identified which preferentially forms the (S)-sulfoxide. Fenbendazole has been shown to be converted into the (R)-sulfoxide by mammalian FMOs with significant enantiomeric excess (Virkel et al., 2004). The observation that only one out of seven enzymes was active on fenbendazole may reflect the fact that fenbendazole differs from albendazole in having a phenyl moiety replacing a propyl moiety making it more bulky, sterically hindering oxidation of the thioether. It is worth mentioning that, except for the formation of low amounts of sulfones, no other oxidation products were formed upon conversion of the tested thioethers which demonstrates that the monooxygenases are chemo-, regio- and enantioselective. Monooxygenases that form the N-oxides of nicotine (CHMO Ac) and lidocaine (BVMO Rj24) were also identified. In the literature it was reported that human FMO3 is responsible for selective formation of nicotine-1`-N-oxide (Cashman et al., 1992), which was also the major N-oxide formed by CHMO Ac. This shows that the microbial monooxygenases can also be used for chemo- and regioselective N-oxidations of xenobiotics.

Our study illustrates that sequence-related microbial monooxygenases can be used for the production of FMO-related metabolites. As for mammalian FMOs (Wu et al., 2004; Cashman et al., 1992), each tested oxidation was very specific and no side products were formed in considerable amounts. This makes them interesting biocatalysts for the production of pharmaceutically relevant drug metabolites.
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Authorship Contributions:

Participated in research design: Gul, Krzek, Permentier, Fraaije and Bischoff

Conducted experiments: Gul and Krzek

Performed data analysis: Gul, Krzek and Permentier

Wrote or contributed to the writing of the manuscript: Gul, Krzek, Permentier, Fraaije and Bischoff
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demethylation by cytochrome P450 enzymes in liver microsomes from rats, dogs, monkeys, and humans. *Drug Metab Pharmacokinet* **30**:64–69.


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Legends for Figures:

**Figure 1:** Substrates used in microbial monooxygenase-catalyzed conversions.

**Figure 2:** Chiral LC-MS/MS in the SRM mode of 3-(methylthio)aniline sulfoxide (3-MTA-SO) (SRM transition: 156/93). 3-(methylthio)aniline was incubated for 135 min in the presence of the following microbial monooxygenases: a) PAMO<sub>M446G</sub>, b) CHMO<sub>Ac</sub>, c) PAMO, d) FMO<sub>RjE</sub>, e) BVMO<sub>Rj4</sub>, f) BVMO<sub>Rj24</sub> and g) BVMO<sub>Mt1</sub>. Based on CD analysis and data reported in the literature, the first eluting enantiomer at 8.4 min was assigned to (R)-3-methylthioaniline sulfoxide and the second eluting enantiomer at 11.5 min to (S)-3-methylthioaniline sulfoxide (Slack *et al.*, 2012). The enantiomeric excess is given as *e.e.*.

**Figure 3:** Chiral LC-MS/MS in the SRM mode of albendazole sulfoxide (ABZ-SO) (SRM transition: 282/240). Albendazole was incubated for 135 min in the presence of the following monooxygenases: a) CHMO<sub>Ac</sub>, b) BVMO<sub>Rj24</sub>, c) BVMO<sub>Mt1</sub>. The first eluting enantiomer at 3.1 min was assigned to (R)-albendazole sulfoxide and the second eluting enantiomer at 5.5 min to (S)-albendazole sulfoxide based on literature data (Materazzo *et al.*, 2014).

**Figure 4:** Chiral LC-MS/MS in the SRM mode of fenbendazole sulfoxide (FBZ-SO) (SRM transition: 316/284). Fenbendazole was incubated for 135 min in the presence of the studied monooxygenases of which only BVMO<sub>Mt1</sub> showed activity. The first eluting enantiomer at 3.5 min was assigned to (R)-fenbendazole sulfoxide and the second eluting enantiomer at 7.1 min to (S)-fenbendazole sulfoxide based on literature data (Materazzo *et al.*, 2014).
Figure 5: Relative yields of the N-oxide products of lidocaine (top) and nicotine (bottom) in the presence of different bacterial monooxygenases as determined by LC-MS/MS in the SRM mode. Experiments were performed in duplicate.
3-(methylthio)aniline
albendazole
fenbendazole
nicotine
lidocaine

Figure 1
Figure 2

A) $1.6 \times 10^6$ PAMO$_{M446G}$

B) $1.6 \times 10^6$ CHMO$_{Ac}$

C) $1.6 \times 10^6$ PAMO

D) $1.6 \times 10^6$ FMO$_{RjE}$

E) $1.6 \times 10^6$ BVMO$_{Rj4}$

F) $1.6 \times 10^6$ BVMO$_{R24}$

G) $1.6 \times 10^6$ BVMO$_{Mt1}$

Time (min)

Intensity

$ee = 85 \pm 0.2$ (R)

$ee = 71 \pm 1.2$ (R)

$ee = 70 \pm 1.2$ (R)

$ee = 66 \pm 0.6$ (S)

$ee \geq 99.5 \pm 0.03$ (R)

$ee = 36 \pm 2.9$ (R)

$ee = 88 \pm 0.2$ (R)
Figure 3

A) CHMOAc

Intensity

B) BVMOR24

Intensity

C) BVMOMt1

Intensity

Time (min)

Figure 3
Figure 4

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Figure 5