Two-step Screening for Identification of Drug-metabolizing Bacterial Cytochromes P450 with Diversified Selectivity

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The evaluation of drug metabolites is compulsory during drug development. Since recently, bacterial cytochromes P450 and their mutated variants have attracted considerable interest as an alternative to hepatic P450s for the synthesis of human drug metabolites. Thus, straightforward screening approaches are required that enable rapid identification and evaluation of drug-metabolizing bacterial P450s with different product selectivities. Herein, we report a two-step screening method for discovery and characterization of new P450s from actinomycetes that enable oxidation of various drugs. In the first step, substrate profiling with three structurally different model drugs, ritonavir, testosterone, amitriptyline, allowed us to select CYP105D and CYP107Z from Streptomyces platensis DSM 40041 that accepted all model substrates and produced human-like drug metabolites. In the second step, activity tests with an array of 25 structurally-related molecules and derivatives of the three model compounds revealed a correlation between structural variations in the target drugs and the enzyme chemo- and regioselectivity.

Introduction

Low efficiency of some existing therapies, population ageing, and the occurrence of new diseases make the development of new medicines indispensable.[1] Assessment of the efficacy, safety and therapeutic effect of a new drug includes the elucidation of its metabolic fate.[2] Thus, production of drug metabolites for their structural characterization, toxicological evaluation and drug-drug interactions tests remains an important task in the drug development process. Traditionally, hepatocytes, liver microsomes and recombinant human drug-metabolizing enzymes have been used for this purpose.[3] The major drug-metabolizing enzymes in the human body are heme-containing cytochrome P450 monoxygenases (CYP or P450), which have been reported to oxidize about two-third of all drugs.[4]

In the presence of molecular oxygen, P450s catalyze a variety of oxidation reactions on chemically diverse compounds including aliphatic and aromatic hydroxylation, C=C bond epoxidation, N- and S-oxidation, N- and S-dealkylation and many others.[5] For their activity P450s require electrons that are derived from the nicotine amide cofactors NADH or NADPH and are transferred via redox partner proteins to the heme iron.

Generally, human drug-metabolizing P450s possess an extraordinary broad substrate spectrum and form distinct oxidation products, which are difficult to access via chemical synthesis.[5] However, application of membrane bound human P450s is somewhat limited by their low expression titers, activity and process stability.[6] For this reason, bacterial P450s with high activity and stability, easily expressible in recombinant hosts, have been subjected to rational protein design and/or directed evolution to achieve production of drug metabolites. As a result, engineered variants of the well-studied P450 BM3 from Bacillus megaterium (CYP102A1) were constructed which are not only able to oxidize various drugs but also form human drug metabolites.[6c,7] Nevertheless, the correlation between metabolite patterns produced by human and engineered bacterial P450s still remains a challenge for protein engineering, which often relies on extensive screening rounds.

Complementary to recombinant human P450 based platforms and engineered bacterial P450s, pharmaceutical companies use microbial biotransformation of drugs, especially when larger amounts of metabolites must be prepared.[8] Microbial strain collections used for this purpose usually include many strains from the phylum actinobacteria. Recent genome sequencing projects have revealed large collections of P450 genes in those strains.[9] P450s from actinomycetes have been found to often mimic activities of human drug-metabolizing P450s and thus are considered as worthwhile candidates for metabolite production.[10] For example, an array consisting of 250 recombinant P450s from actinomycetes has been established in E. coli and successfully applied for drug biotransformation in a 96-well format.[11] Many P450s from actinomycetes belong to the largest bacterial subfamilies CYP105 and CYP107, which are known to accept and oxidize a variety of chemically diverse scaffolds e.g. for the diversification of natural products or the degradation of xenobiotics.[9] The examples include among others CYP105D7 from Streptomyces avermitilis, which
catalyzes oxidation of the natural sesquiterpenoid pentalenic acid[15] and of the non-physiological substrate daidzein;[16] CYP107L from Streptomyces platensis, which combines activities against the drugs amodiaquine, ritonavir, amitriptyline and thioridazine;[14] as well as CYP107 (PldB) and CYP105 (PsmA) that perform selective hydroxylations during the pladieniolide D synthesis in recombinant Streptomyces platensis.[15]

Nevertheless, the identification of activities similar to human P450s cannot easily be deduced from the sequence or even structural similarity of P450s. Consequently, extensive screenings are required to identify a desired P450. Testing P450s towards one or few chemically related drug compounds like steroids[14,16] or tricyclic antidepressants[17] reveals a correlation between the enzyme activity/selectivity and the particular drug compound’s structure. This focused approach has a limitation as it does not necessarily distinguish between P450s with narrow and broad substrate spectra. A randomized screening with various drug compounds facilitates discovery of P450s with broad substrate spectra as for example described for CYP267 from S. cellulosum or P450 BM3 variants.[18] But in this case the effect of small changes in drug structure on the enzyme activity and selectivity cannot be deduced.

In this study we combined the advantages of both screening methods aiming at identification and characterization of drug-metabolizing P450 enzymes from Streptomyces platensis DSM 40041 and Pseudonocardia autotrophica DSM 535. Both strains are utilized by pharmaceutical companies for the oxidation of various drugs.[14,19] Using substrate profiling with three model drugs namely ritonavir, testosterone and amitriptyline in the first step, two P450s from S. platensis with broad substrate spectra and complementary selectivities were selected. In the second step, these two P450s classified as CYP105D and CYP107Z were subsequently evaluated against an array of structurally-related drugs and derivatives of the model compounds. Besides their extraordinary broad substrate promiscuity, the newly identified P450s produced human drug metabolites. Following this two-step screening approach, we recognized how small variations in structure of the target drugs influence chemo- and regioselectivity of the selected P450s. This correlation will help to reduce future screening efforts for the identification of a P450 to furnish a certain drug metabolite.

Results and Discussion

Identification of drug-metabolizing P450s

The recently published genomes of Pseudonocardia autotrophica DSM 535 and Streptomyces platensis DSM4004129[15] contain 70 cyp genes. 33 of them were found in P. autotrophica and 37 cyp genes in S. platensis. According to the online P450 database CYPED[20] 31 genes belong to the CYP105 and the CYP107 families. Fourteen of those genes (13 of S. platensis and 1 of P. autotrophica) have been investigated in previous studies.[14,21] One cyp gene in P. autotrophica has a length of only 378 bp and was not considered further. The remaining 16 so far uncharacterized cyp genes were cloned and expressed in E. coli C43 (DE3) (Table S4). Judging from the CO-difference spectra that is used as indicator for functional P450 enzymes, all P450s could be produced in soluble form at concentrations varying from 13 to 1036 nmolP450/gCDW (corresponding to 0.6–48 mgP450/gCDW, Figures S1 and S2).

Thirteen P450s expressed in E. coli at concentrations of above 100 nmolP450/gCDW were tested in vitro against three model drugs: the anti-HIV agent ritonavir (1), the steroid testosterone (7) and the tricyclic antidepressant amitriptyline (16) (Figure 1). These drugs of various size and shape belong to different chemical classes. P450 mediated oxidation of the model drugs was supported by heterologous redox partners, either flavodoxin YkUN from B. subtilis and flavodoxin reductase from E. coli (Fdr) or putidaredoxin (Pdx) and putidaredoxin reductase (Pdr) from P. putida (Figures S3 and S4). Oxidized products were identified by LC/MS analysis (Figures S5–S36; Tables S7–S31).

Ritonavir (1) is the drug with the highest molecular weight (720.9 g/mol). This peptide analogue contains many sites for functional P450s and is therefore a suitable target for the screening with recombinant P450s. In this study, three ritonavir analogues with different molecular weights were used: D-dasatinib (spl) which is a derivative of dasatinib, a dual BCR-ABL and ABL kinase inhibitor, and two further analogues with a mass of 900 and 800 Da, respectively (Figures S5). The oxidations of ritonavir and the two analogues were compared to identify the activities of a P450 in vitro (Figure 1).

**Figure 1.** Conversions of the model drugs 1, 7 and 16 and product patterns observed with active P450s. Only P450s showing activities against at least one drug are shown. The hydroxylation products of substrate 7 are different for different P450s.
oxidation and is metabolized by human P450s including CYP3A family members mainly via N-dealkylation leading either to the loss of the thiazolyl carbamate moiety or of the isopropylthiazolylmethyl moiety, N-demethylation, and hydroxylation of the isopropyl group of the isopropylthiazolyl moiety (Scheme 1). The second model drug testosterone (7) has a lower molecular weight (288.4 g/mol). It can be hydroxylated by human P450s like CYP3A4 or CYP2D6 at different positions often in both, α- and β-configuration (Scheme 1), and is often used for evaluation of P450 activity and selectivity. The last model compound, the tricyclic antidepressant amitriptyline (16) (277.4 g/mol) is primarily oxidized by human CYP2C19 at the triazol moiety to the demethylated product nortriptyline (21) followed by the hydroxylation to 10-hydroxy-amitriptyline in (E)-configuration catalyzed mainly by human CYP2D6 and CYP3A4 (Scheme 1). Other reactions include didemethylation to desmethylnortriptyline or N-oxidation to amitriptyline N-oxide.

The in vitro activity screening revealed seven P450 enzymes that demonstrated activity against at least one drug compound (Figure 1). Among them, four P450s from S. platensis (SPL_00625, SPL_01896, SPL_03767 and SPL_06346) accepted at least two model drugs as substrates. The active P450s exhibited different product selectivities. Ritonavir (1) was oxidized by SPL_00625 and SPL_01896 to the same main hydroxylated product with >80% regioselectivity. SPL_03767 and SPL_06346 converted 1 with lower activities to form product mixtures with higher amounts of dealkylated and demethylated metabolites. Also during oxidation of testosterone (7) SPL_00625 formed one main hydroxylation product with a regioselectivity of 70%, while SPL_06346 was less selective and formed a product mixture (Figure S16). Both P450s were quite active and enabled almost full conversion of 7. With amitriptyline (16), SPL_00625 showed again the highest regioselectivity of 80% towards nortriptyline (21) followed by SPL_03767, which formed besides the monodemethylated nortriptyline (56%) also the didemethylated product (17%). Since SPL_00625 and SPL_06346 catalyzed oxidation of all model substrates with different regioselectivities, their activity and selectivity were further evaluated in the second step using a focused substrate library. According to the CYP nomenclature by Prof. David Nelson, these P450s were classified as CYP105D (SPL_00625) and CYP107Z (SPL_06346) (Table S5).

Library design for systematic substrate screening

In order to elucidate how variations in structure and size of the target drug compounds influence enzymes’ activity and selectivity, we followed a systematic screening with either structurally related compounds for the target drug (anti-HIV compounds 1–6) or derivatives with differences in functional groups (steroids 7–15 and tricyclic antidepressants 16–25). Overall 25 compounds were tested (Scheme 2).

Screening results for anti-HIV compounds

The tested anti-HIV agents 1–6 are bulky, have a high molecular weight (505.6–720.9 g/mol) and contain a hydroxyethylene group which is responsible for the inhibition of HIV protease. CYP105D and CYP107Z accept all tested compounds 1–6 (Figure 2A). The highest conversion with both P450s was observed for saquinavir 5 (71% with CYP105D and 97% with CYP107Z). CYP105D demonstrated its lowest activity with darunavir 3 (7% conversion), while the lowest activity of CYP107Z was measured with atazanavir 4 (21% conversion). In previous studies CYP267B1 and P450 BM3 variants were reported that enabled in vitro oxidation of anti-HIV compounds including ritonavir 1 and saquinavir 5. For ritonavir 1 very low conversions of 1.4% were observed with CYP267B1, while saquinavir 5 was most efficiently oxidized by the P450 BM3 variant MT35 with 35% conversion. Thus, the activities of CYP105D and CYP107Z are equal or at least 2–3 times higher than those of previously reported bacterial P450s. Besides, they accept a broader spectrum of anti-HIV agents.

Analysis of the product patterns revealed that CYP105D generally seemed more selective but less active than CYP107Z (Figures S5–S13). One major hydroxylated product (50–85% of the product ratio) was detected after turnover of the anti-HIV drugs (except for 2) catalyzed by CYP105D. In contrast, CYP107Z produced with the corresponding drugs not only hydroxylated but also dealkylated metabolites at higher ratios (up to 69% of the product ratio). For example, ritonavir metabolites 1b (Δm: −14 Da), 1c (Δm: −139 Da) and 1d (Δm: −141 Da) had characteristically smaller masses compared to the substrate.

Recently, our group described nine metabolites of ritonavir biotransformed by the parent organism of both P450s Streptomyces platensis DSM 40041. Metabolites were analyzed by LC/MS and LC/MS-MS analysis. The observed mass differences and retention times for 1b–d correspond to the N-dealkylated

Scheme 1. Chemical structures of the model drugs. Major oxidation sites of human P450s are highlighted with an arrow. The color code displays either hydroxylation (red), demethylation (blue), dealkylation (green) or alcohol oxidation (grey).
and N-demethylated metabolites described there (Table S7). CYP107Z was able to furnish N-demethylated (13% of 1b) and N-dealkylated (6% of 1c and 5% of 1d) metabolites more efficiently compared to CYP105D (Table 1). However, a single hydroxylation product was best accessed with CYP105D (58%). This product was identified as hydroxy ritonavir (1a) as deduced from a spiking experiment with an authentic standard (Figure S6).

In conversions with amprenavir (2) and darunavir (3) CYP107Z formed one metabolite (36% and 44%, respectively) with the same m/z of 339 (Table S8 and S9). By co-elution with an authentic standard, this metabolite was identified as 4-amino-N-((2R,3S)-3-amino-2-hydroxy-4-phenylbutyl)-N-isobutylbenzenesulfonamide (2a) (Figure S8 and S10). This metabolite is formed through N-dealkylation.

Scheme 2. Substrate library based on derivatives and functionally related compounds of the model drugs ritonavir (1), testosterone (7) and amitriptyline (16). Differences in structures of the model drugs are highlighted in red.
All identified metabolites are formed as major metabolites by human P450s including CYP3A isoforms and CYP2D6.[22,27]

**Screening results for steroids and glucocorticoids**

The tested steroids 7–15 possess a tetracyclic core structure. They differ in the number and position of hydroxy-, keto-, and methyl groups and double bonds. Synthetic glucocorticoids like 9, 11–15 have more substituting groups than natural compounds like 7, 8 or 10 which often leads to increased drug efficiency.[28] In human drug metabolism, CYP3A4 is one of the most frequently investigated P450s to form steroid metabolites.[29]

Also in case of steroids, the substrate scope of CYP107Z is generally broader compared to that of CYP105D (Figure 2B, Figures S14–S25). CYP105D converted 7, 8 and 9 to one major product (≥66% of the product ratio) each with high activity (≥85% conversion), but barely or not accepted 10–15 as substrates. CYP107Z oxidized 7, 8 and 9 nearly completely
Table 1. Overview of identified metabolite structures in this study. The product percentage was calculated based on the observed peak areas related to the sum of product peaks areas. The color code highlights the type of P450 reaction depicted in the figures before.

| Substrate                  | Reaction type | Identified metabolite structure | Product percentage [%] |
|----------------------------|---------------|---------------------------------|------------------------|
|                           |               |                                 | CYP105D | CYP107Z |
| Anti-HIV agents            |               |                                 |          |        |
|                           |               | hydroxylation 1a: Δm: +16 Da    | 86       | 30     |
|                           |               | demethylation 1b: Δm: −14 Da    | 0        | 32     |
| Ritonavir (1)             |               | dealkylation 1c: Δm: −139 Da    | 8        | 14     |
|                           |               | dealkylation 1d: Δm: −141 Da    | 0        | 13     |
| Steroids and glucocorticoids |           |                                 |          |        |
|                           |               | hydroxylation 7a: Δm: +16 Da    | 0        | 6      |
| Testosterone (7)          |               | hydroxylation 7b: Δm: +16 Da    | 70       | 5      |
(≥ 96% conversion), but was less selective than CYP105D. The corresponding main product accounted for 38–54% of the total products (Figure S16–S18). In contrast to CYP105D, the substrates 10–13 were clearly oxidized by CYP107Z, though with low activity (2–32% conversion). Only one main product of each compound could be detected (Figure S19–S22). Remarkable is the difference in activity of CYP107Z towards dexamethasone (14) with a 16α-methyl group at C16 and its epimer betamethasone (15) carrying the methyl group at C16 in β-position. Dexamethasone (14) was converted to 60% with 97% selectivity to one major product. The activity against betamethasone was more than six times lower (9% conversion).

NMR analysis revealed that testosterone (7) was oxidized by CYP105D mainly to 2β-hydroxytestosterone (7b) (Table 1, Figure S37), which was confirmed later by spiking with the authentic reference (Figure S14). This product was also formed by CYP107Z, but only in traces (Figure S15). The major human metabolite of 14 is 6β-hydroxydexamethasone (14a) (Table 1), which is also formed by CYP107Z as it was deduced from spiking experiments (Figure S23).246 The preference for the 6β-position (97%) of 14 with a side chain increased significantly compared to the initial model compound 7 (only 6% 6β-hydroxytestosterone 7a).

Other bacterial P450s including P450 BM3 variants, CYP106A1, and CYP106A2 were also tested for testosterone and dexamethasone oxidation. Dexamethasone and prednisolone were converted by CYP106A1 and CYP106A2 with different selectivities. While CYP106A2 was selective for the 15β-position, CYP106A1 oxidized these compounds at position 6β, 15β and 11.246 Conversions between 33% and 40% were achieved with CYP106A1. Compared to the reported CYP106A1, CYP107Z oxidized dexamethasone with higher activity and selectivity for the position 6β. Considering the different selectivity of CYP106A2, the activity and selectivity of CYP107Z against dexamethasone 14 complement and expand the possibilities for drug metabolite syntheses starting with other glucocorticoids.

P450 BM3 wild type does not accept testosterone, but variants thereof carrying up to 20 mutations were reported to perform stereo- and regioselective oxidations of steroids like testosterone at position 2β, 15β and 16β.24,30–31

Table 1. continued

| Substrate              | Reaction type | Identified metabolite structure | Product percentage [%] |
|------------------------|---------------|--------------------------------|------------------------|
|                        |               |                                | CYP105D | CYP107Z |
| Dexamethasone (14)     | hydroxylation | 14a: Δm: + 16 Da               | 0       | 97     |
| Tricyclic antidepressants | hydroxylation | 16a: Δm: + 16 Da               | 15      | 12     |
| Amitriptyline (16)    | demethylation | 21: Δm: −14 Da                 | 79      | 55     |
| Chlorpromazine (19)   | sulfoxidation | 19b: Δm: + 16 Da               | 72      | 36     |
Although significant advances have been achieved to reduce screening efforts among P450 BM3 variants, the herein described P450s might represent valuable alternatives and new targets for protein engineering as they naturally convert steroids with quite high activity and moderate selectivity.

Screening results for tricyclic antidepressants

Tricyclic antidepressants including 16–25 can be grouped either according to the ring arrangement or to the respective side chain.[32] Several human P450s including CYP3A4 and CYP2D6 oxidize these compounds mainly via N-demethylation and hydroxylation as summarized elsewhere.[34]

CYP105D and CYP107Z accepted nearly all tested tricyclic antidepressants (Figure 2C). Only carbamazepine (23) and oxcarbazepine (24), both missing a long alkyl chain, were not accepted by any P450. Also during oxidation of tricyclic antidepressants CYP105D was less active than CYP107Z. Generally, trialkylated amines were better substrates than dialkylated amines. CYP107Z oxidized the trialkylated amines 16–20 nearly completely (>95% conversion), while CYP105D showed 2 to 10 times lower activity (10–45% conversion) (Figures S26–S36). Dialkylated antidepressants 21 and 22 were moderately oxidized by CYP107Z (30–70% conversion), while activity of CYP105D against these substrates was almost not present (max. 2% conversion). Both, CYP105D and CYP107Z, were able to produce demethylated metabolites of 16, 17, 18 and 20, which have a characteristic mass difference of ~14 m/z compared to the substrates (Table S22–S28). CYP107Z additionally produced didemethylated products. Starting from 16, 53% nortriptyline (21) and 7% desmethylnortriptyline (16b) were produced as it was confirmed by co-elution with authentic standards (Figure S26, Table 1).

Chlorpromazine (19) is an example of a diarylthioether. Besides demethylation, 19 was converted by both enzymes to another product having a mass difference of +16 m/z compared to the substrate (Table S25). By co-elution with an authentic standard, this product was identified as chlorpromazine sulfoxide (19b) (Figure S30).

Also with 16, 17, 18 and 21 a single oxidation product was formed with both enzymes showing a Δm = +16 Da. This product was absent after conversions of 20 and 22. In comparison to the other tested antidepressants, cyclobenzaprine (20) and protriptyline (22) carry an additional double bond in the central ring between C10 and C11, which has been reported to hinder the oxidation there.[17] This might indicate that the hydroxylation of 16, 17, 18 and 21 occurs at C10, which was indeed confirmed for amitriptyline (16) conversion via co-elution with an authentic standard (Figure S26). The hydroxy group at C10 of (+)-(E)-10-Hydroxymitriptyline (16a) is (E)-configured and matches the preferred position and configuration achieved with human P450s.[23b] The ability to perform hydroxylation, sulfonation, demethylation, dealkylation and didemethylation on tricyclic antidepressants has been reported for other bacterial P450s including P450 BM3 variants or CYP267B1 and CYP267A1 from Sorangium cellulosum So ceS6.[24,17–26] The selectivities and activities to form human drug metabolites of the described enzymes there are similar to CYP105D and CYP107Z. However, the screening developed in this study helps to differentiate P450 enzymes in terms of activity, chemo- and regioselectivity, thus facilitating the pre-selection of an appropriate enzyme candidate for other drug compounds with different modifications of the lead structures.

Conclusions

In summary, a two-step screening approach was applied to identify P450 candidates that on the one hand accept a broad range of drugs of various size and chemical structure and on the other hand demonstrate different selectivities leading to various metabolites. The identified CYP105D and CYP107Z both from S. platensis DSM 40041 are promiscuous bacterial cytochrome P450 monooxygenases showing complementary activities and selectivities on chemically diverse drugs. Whereas CYP105D generally possesses a narrower substrate spectrum and lower activity than CYP107Z, it shows a higher regioselectivity and allows to produce one main hydroxylated metabolite of many drugs. For instance 2β-hydroxytestosterone was formed in the reaction with CYP105D with 70% regioselectivity after complete conversion, while hydroxyl ritonavir was formed with 86% regioselectivity after a conversion of 70%. CYP107Z demonstrates a higher preference for N-dealkylation than CYP105D and thus provide an access to demethylated drug metabolites. Additionally, CYP107Z catalyzed steroid hydroxylation with a rather high regioselectivity of up to 97%, for instance for 6β-hydroxydexamethasone. The design of a focused substrate library pointed out not only the differences between both enzymes but also indicated a remarkable overlap with the substrate and product spectra of human hepatic P450s. Importantly, the screening of closely related compounds allowed us to evaluate how small variations in chemical structures of target drugs can change the respective enzyme activity and selectivity. This knowledge might reduce future screening efforts for drugs from the same chemical groups, which have not been tested with these P450s yet.

Experimental section

Bacterial strains, enzymes and chemicals: E. coli strains DH5α and BL21 (DE3) were purchased from Clontech, OverExpress C43(DE3) from Lucigen, Phusion High-Fidelity DNA-polimerase, restriction endonucleases (Fast Digest variants), FastAP thermosensitive alkaline phosphatase and T4 DNA-ligase were obtained from Thermo Scientific. Catalase from bovine liver was obtained from Sigma Aldrich. Unless indicated otherwise, all chemicals were of analytical grade or higher and purchased from Sigma Aldrich, VWR, AppliChem, Carl Roth, BD BioSciences and Grüssing. Suppliers of all tested drugs and respective reference metabolites are listed in Table S1.

Construction of expression vectors: Genes encoding P450s from S. platensis were amplified from genomic DNA and integrated with conventional cloning methods in expression vectors pET22b,
pET24b or pET28a using the primers listed in Table S2. The cyps from P. autotrophica were cloned in pET24b after amplification from genomic DNA using primers for PCR allowing vector integration via Gibson Assembly.[11] All existing GTG start codons were replaced by the start codon ATG in the primer sequences. The sequence for the N-terminal His6-tag of cyp105D was inserted after the start codon following the instructions of the Q5 site-directed mutagenesis kit (New England Biolabs). The construction of pCOLADuet-PP was performed by integrating camA and camB in MSCI of pCOLADuet-1 via Gibson Assembly.[12] DNA Sequences were confirmed by sequencing (GATC Biotech, Germany).

Gene expression and enzyme preparation: All genes are integrated in vectors allowing expression using the T7-expression system. P450 genes were expressed in recombinant E. coli C43 (DE3), while redox partner proteins and GDHV were produced in E. coli BL21 (DE3). Detailed information on expression and purification is provided in the Supporting Information (SI).

Enzyme assays: The P450 concentrations in crude cell extracts or purified fractions were calculated from CO difference spectra using the extinction coefficient ε450=91 mM−1 cm−1 as published elsewhere.[13] The concentration of tested redox partner proteins was estimated spectrophotometrically. Concentration of heterologous redox partners were determined as described in the SI.

Substrate screening: During initial screening, reactions were performed in 200 μL 50 mM KPi, pH 7.5. The tested P450ds were typically applied in form of crude cell extracts, while the tested redox partner proteins were purified before. Reaction mixtures contained 2 μM P450, 2 μM Fdr or Pdx, 20 μM YkuN or Pdx, 0.2 mM NAD(P)+ and 200 μM substrate dissolved in either in 2% (v/v) DMSO (anti-HIV drugs, oxcarbazepine, opipramol and carbamazepine) or 2% (v/v) ethanol (steroids) or deionized water (tricyclic antidepressants). For NAD(P)H cofactor regeneration, 5 U/mL GDH was typically applied in form of crude cell extracts, while the tested redox partner proteins were purified before. Reaction mixtures contained 2 μM P450, 2 μM YkuN or Pdx, 0.2 mM NAD(P)+ and 200 μM substrate dissolved in either in 2% (v/v) DMSO (anti-HIV drugs, oxcarbazepine, opipramol and carbamazepine) or 2% (v/v) ethanol (steroids) or deionized water (tricyclic antidepressants). For NAD(P)H cofactor regeneration, 5 U/mL GDH with 20 mM glucose was used, while 600 U/mL catalase from bovine (Sigma Aldrich) was added to eliminate possibly formed hydrogen peroxide due to uncoupling. The samples were incubated at 25°C in 2 mL reaction tubes with open lids in a thermomixer (Eppendorf) with a shaking speed of 300 min−1. After 4 h or 20 h incubation, 4 μL of internal standard (10 mM stock solution in methanol, DMSO or water) was added. For conversions with tricyclic antidepressants and Anti-HIV agents, 300 μL 0.1 M sodium carbonate buffer, pH 10 was supplemented to alkalize the solution for better extraction. Then an appropriate volume of ethyl acetate was added, the samples vigorously shaken for 5 min and centrifuged for 5 min at 12300 g. The organic phase was transferred into a new reaction tube and evaporated under reduced pressure. The dried samples were resuspended in methanol (Anti-HIV drugs, steroids) or water with 20% acetonitrile and 0.1% formic acid (tricyclic antidepressants) and used for LC/MS-analysis. The given conversion values are based on substrate depletion with an internal standard referred to a control reaction with crude cell extract of E. coli pET24b harboring no P450. If conversions were below 5%, the sum of product and substrate peaks was used to calculate conversions. Product distribution was calculated from the sum of peak areas in the MS or the absorption at 254 nm. Due to the complex product pattern in some cases, single ion monitoring analysis was used additionally during MS analysis to distinguish the product peaks. All measurements represent mean values from technical duplicates.

Product analysis: Substrate biotransformations were analyzed by liquid chromatography coupled to mass spectrometry (LC/MS) on a Prominance/LCM5 2020 device (Shimadzu). A Chromolith® Performance RP-18e column (100×4.6 mm, Merck) was used for Anti-HIV agents and steroids whereas a Chromolith® Performance RP-8e column (100×4.6 mm, Merck) was applied for tricyclic antidepressants. Solvent A was always ddH2O with 0.1% formic acid, while solvent B was either methanol (MeOH) or acetonitrile (ACN). 1 μL of each sample was injected and analytes were separated with a flow rate of 0.5–1 mL/min at 30°C. The separated compounds were ionized by electron spray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in a dual ionization mode. Mass compounds were detected in positive scan mode in a range between 150–1000 m/z (Anti-HIV agents), 200–950 m/z (tricyclic antidepressants) and 100–500 m/z (steroids). A detailed overview about the chromatographic conditions is given in Table S6. Products were identified based on retention times, MS spectra and by spiking of authentic reference compounds to reaction solutions.

NMR-analysis (1D and 2D-spectra) was performed on a Bruker Avance III-600 spectrometer (1H-NMR: 600 MHz; 13C-NMR: 150 mHz). Chemical shifts (δ) are given in ppm and were referred to the solvent CDCl3 (δIC: 77.2 ppm; δIH: 7.26 ppm). Coupling constants (J) are stated in Hz. The NMR data were in accordance with literature data.[14]

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Conflict of Interest

The authors declare no conflict of interest.

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