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Screening for Cytochrome P450 Reactivity by Harnessing Catalase as Reporter Enzyme

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Cytochrome P450 enzymes are known to catalyze a variety of reactions that are difficult to perform by standard organic synthesis, such as the oxidation of unactivated C–C bonds. Cytochrome P450 enzymes can also be used in artificial systems in which organic peroxides act as cosubstrates. To find substrates that are converted by a certain P450 catalyst in the presence of an organic peroxide, various screening assays have been established, however, most of them are limited to one or only a few specific substrates. Here, we report a simple and rapid screening assay that works independently of the nature of the substrate and utilizes a previously undescribed reactivity of catalase as reporter enzyme. In an initial demonstration of this assay, we screened 180 enzyme/peroxide/substrate combinations for potential bioconversions. As shown by subsequent verification of the screening results with liquid chromatography/multistage mass spectrometry (LC/MS^n), we were able to identify three new substrates for the enzyme CYP152A1 and at least two previously undescribed conversions by the enzyme CYP119.

Introduction

The superfamily of cytochrome P450 enzymes catalyzes a broad range of reactions, including oxygenation, hydroxylation and C–H activation. They are essential for drug metabolism in vivo and they are considered versatile biocatalysts for applications in biotechnology and biosensing.[1] To realize applications in biocatalysis, the development of high-throughput screening assays for the identification of active members of enzyme libraries and their substrate specificity is of major importance.[2] Owing to the broad range of reactivities of P450 enzymes, several screening approaches have been established, which are often based on specific fluorogenic or liquid chromatography/mass spectrometry (LC/MS) detectable substrates,[3] reactive cleavage products,[4] or the consumption of cofactors, such as NAD(P)H.[2h] With respect to the latter, it is noteworthy that the essential requirement of expensive NAD(P)H cofactors still represents a significant obstacle for the establishment of P450 enzymes as biocatalysts, although the successful implementation of NAD(P)H regeneration systems has been demonstrated in various cases.[5,6] Because some P450 enzymes accept hydrogen peroxide and organic peroxides as a source of activated oxygen, in a pathway referred to as peroxide shunt,[2k,7] Auclair and co-workers have recently emphasized that the use of organic peroxides in combination with condition engineering might help to overcome this limitation.[8] In comparison to a P450 enzyme system with NAD(P)H regeneration, the shunting of P450 enzymes with peroxides offers a less complex and thus easier controlled catalyst system for process engineering. While the enzymatic quantification of hydrogen peroxide by means of horseradish peroxidase-based assays is a standard method, which has been used to screen for P450 reactivity,[9] to the best of our knowledge, no assay is currently available for the high-throughput screening of organic peroxide-dependent P450 reactivity.[10] Here, we report the screening for cytochrome P450 reactivity, and harness the yet undescribed peroxidation of the commercial fluorogenic dye Amplex Red by organic peroxides using the enzyme catalase as reporter (Scheme 1). Since this peroxide-depletion assay is based on a simple multiwell-plate fluorescence read-out, it can be readily applied to screen for the conversion of large numbers of potential substrates by a given P450 enzyme in the presence of an organic peroxide. Here, we demonstrate the feasibility of this assay by screening 180 enzyme/peroxide/substrate combinations for potential conversion. Positive hits from this screen were then subjected to liquid chromatography/multistage mass spectrometry (LC/MS^n) measurements to validate the conversion and identify reaction products. The results clearly indicate that this assay is suitable for the screening of P450 reactivity against a broad range of potential substrates.

Results and Discussions

In the course of our studies of novel P450-based hybrid catalysts,[11] we accidentally observed that reaction mixtures containing Amplex Red (**1**; Scheme 1), organic peroxides, such as *tert*-butylhydroperoxide (**2**) or cumene hydroperoxide (**3**), and

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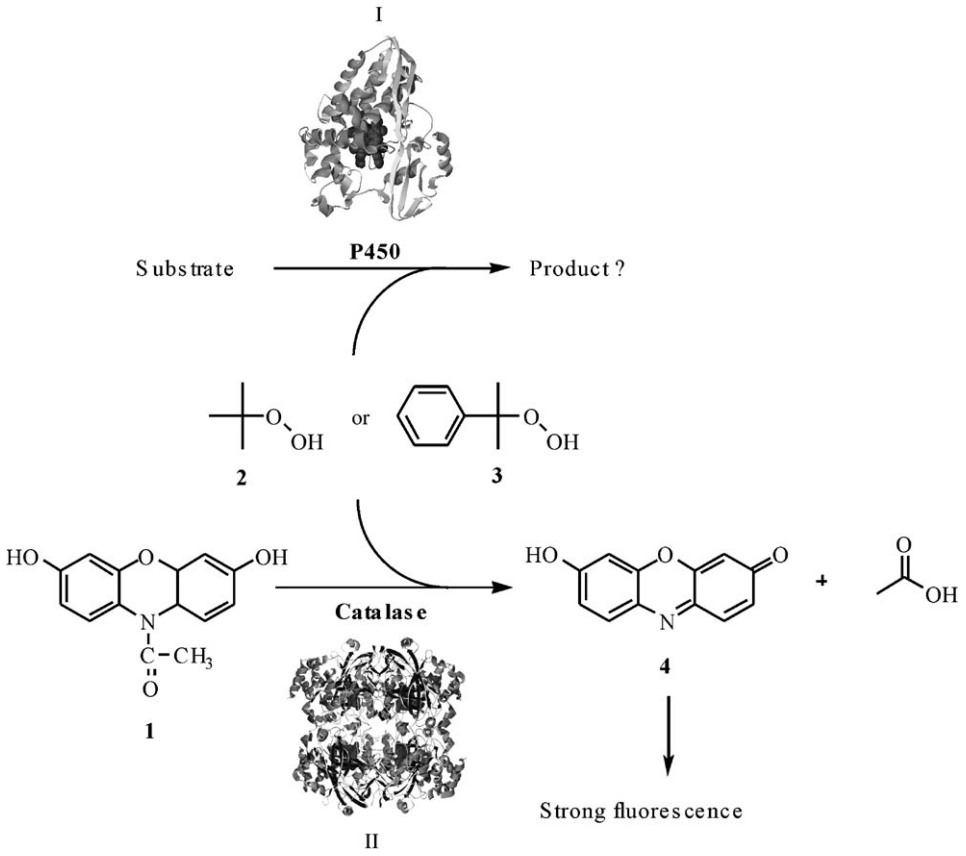


Scheme 1. Schematic representation of catalase-dependent screening for P450 reactivities. The assay is based on the quantification of organic peroxides (**2**, **3**), which are cosubstrates required for putative P450 reactivity against potential substrates (reaction I). Depletion of the hydroperoxide is measured in reaction II by addition of Amplex Red (**1**) and catalase and subsequent fluorescence detection of resorufin (**4**).

the enzyme catalase, rapidly developed strong fluorescence signals, owing to the formation of resorufin (**4**; Scheme 1). We reasoned that this signal development might have occurred due to the enzymatic peroxidation of **1** by catalase, and that this yet undescribed reaction might be useful to screen for organic peroxide-dependent reactivity of cytochrome P450 enzymes. In the proposed assay, the efficiency of P450 conversion of a certain substrate (Scheme 1, reaction I) determines the level of remaining organic peroxide, which is subsequently quantified by using the catalase reaction (Scheme 1, reaction II) and fluorescence detection of resorufin (**4**).

It has previously been observed that catalase decomposes[12] and forms adducts with methyl and ethyl hydrogen peroxide and peroxoacetic acid,[13,14] and this reactivity has been used for the oxidation of alcohols.[15–17] Although it has been described that catalase possesses oxidase activity, which requires molecular oxygen and utilizes electron donor substrates in the absence of hydrogen peroxide or any added cofactors,[18] and it has been used to prepare organic-phase enzyme electrodes (OPEE) for electrochemical detection of organic peroxides in nonaqueous media, such as toluene, *n*-decane or chloroform,[19,20] we could not find any reports on the peroxidation of Amplex Red by catalase in the presence of organic peroxides. To set up the coupled enzymatic assay depicted in Scheme 1, we initially carried out kinetic studies to elucidate the novel catalase reaction. We observed that the enzyme reveals Michaelis–Menten behavior in the conversion of **1** by using **2** (K_M = (1579 ± 160) μM, k_{cat} = (0.036 ± 0.002) s^{-1}) and **3** (K_M = (56 ± 3) μM, k_{cat} = (0.0150 ± 0.0005) s^{-1}) as the cosubstrate (Figure S2 in the Supporting Information). The kinetic study also showed that organic peroxides **2** and **3** can be quantified in the range of 1–100 μM by using regression curves (Figure 1). Thus, it was possible to quantitatively determine the depletion of peroxides **2** and **3** due to the P450-catalyzed conversion of a range of potential substrates.

To validate our assay, we screened three different enzymes, chloroperoxidase (CPO) from *Caldariomyces fumago*, and the bacterial P450 enzymes CYP152A1 from *Bacillus subtilis* and CYP119 from *Sulfolobus acidocaldarius*, against a panel of known substrates and inhibitors of P450 enzymes (Scheme 2, Figure 2). To limit the screen to the identification of efficient transformation reactions, a 2:1 stoichiometric ratio of substrate:peroxide was chosen instead of the normally applied large excess of peroxide.[11c] In a typical reaction, the substrates (200 μM) were allowed to react with either **2** or **3** (100 μM) in the presence of enzyme (200 nM) in 200 μL of a phosphate buffered solution.[21] Subsequent to overnight incubation, an aliquot (50 μL) was mixed with a solution containing Amplex Red and catalase to quantify the remaining peroxide. Controls were carried out in parallel and lacked either the substrate, the enzyme or both components. To ease the handling of the large body of fluorescence data created in the course of this screening, we divided the observed data into five categories (Figure 2A). Case A represents the clear evidence of a peroxide-dependent P450 substrate conversion, because considerable amounts of peroxide are consumed only when both substrate and enzyme were present in solution. This detection of "hits" led to the identification of several new substrates for the bacterial P450 enzymes (see below), which is the key purpose of this screening assay.

In case B, low amounts of peroxide were consumed even in the absence of the enzyme while in its presence peroxide depletion was almost complete (Figure 2A). This reaction profile indicates either that the peroxide reacts with the substrate in the absence of the P450 to a lower extent than in the P450-

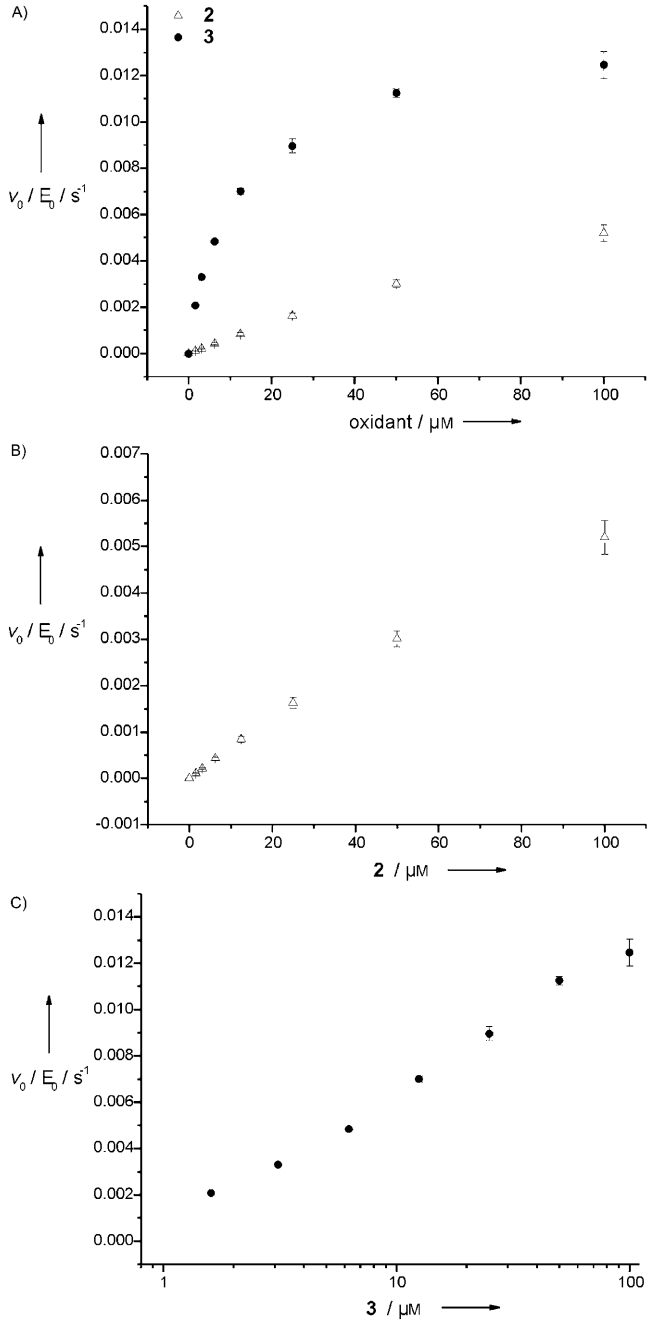


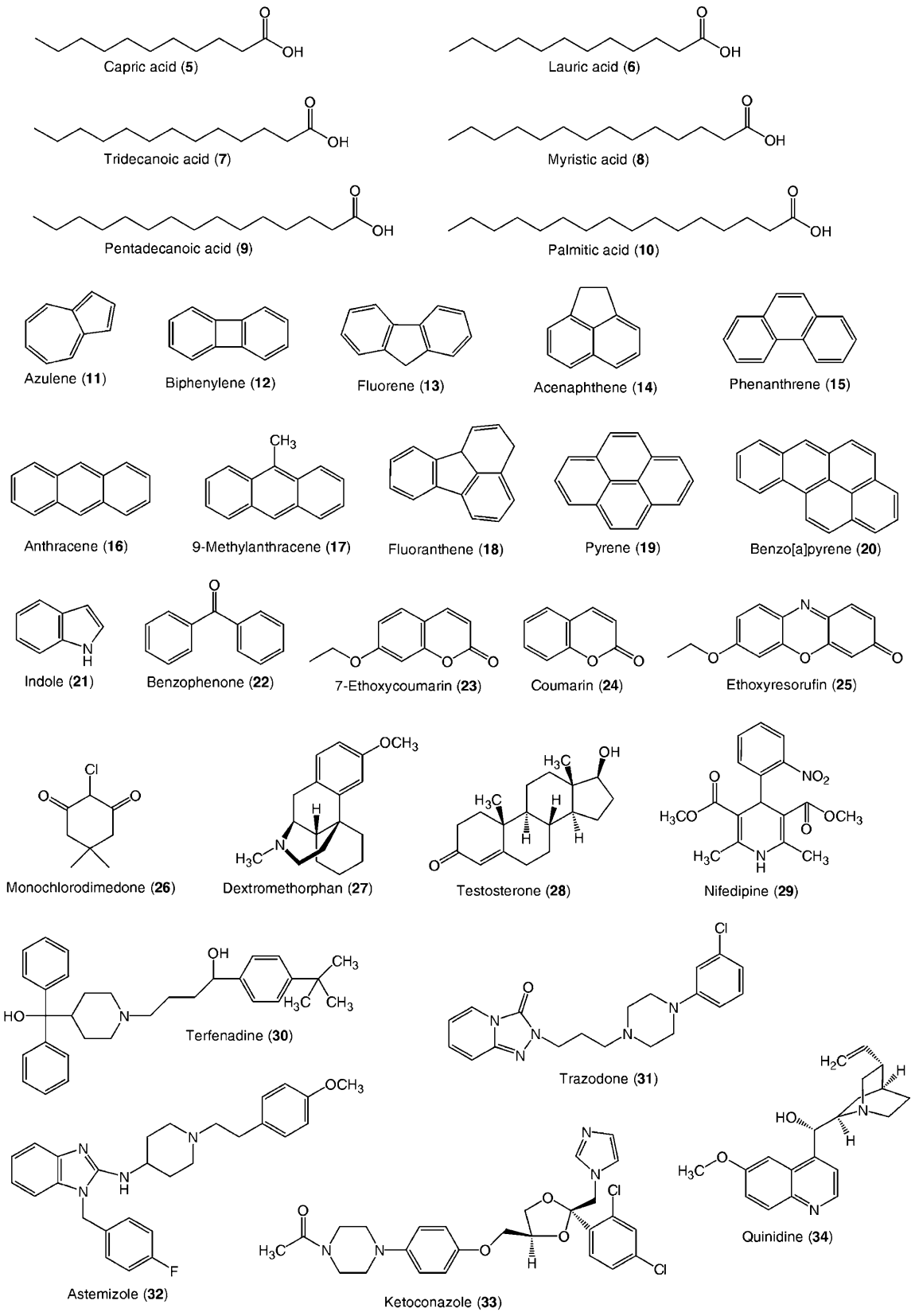
Figure 1. A) Calibration curves for the reaction of catalase and Amplex Red with up to 100 μM *tert*-butylhydroperoxide (**2**) or cumene hydroperoxide (**3**). The hydroperoxide-dependent signals of the reaction between catalase and Amplex Red were fitted to: B) a linear regression in the case of *tert*-butylhydroperoxide, and C) a logarithmic regression for cumene hydroperoxide.

catalyzed reaction, or that the catalase reaction (Scheme 1, reaction II) is inhibited by the substrate. As an augmentation of case B, almost the entire amount of peroxide was consumed in case C, even when no enzyme was present. This case indicates either efficient reaction between the substrate and the peroxide, or strong inhibition of the catalase. Indeed, we observed cases B and C several times in our screen and, due to the ambiguity of these reaction profiles, further elaboration using enzymatic and LC/MS^n analysis was carried out. This postassay analysis led to the identification of both undescribed P450 substrates and novel inhibitors for catalase (see below).

Case D, in which peroxide is consumed by the enzyme even in the absence of a substrate, indicates the decoupling of peroxide consumption and substrate oxygenation. This decoupling has been observed previously in H_2O_2-dependent P450 reactions.[22] Although this behavior does not provide information about the substrate specificity of the P450 enzyme, the assay presented here might help to optimize process conditions that favor less efficient decoupling. Finally, in case E no consumption of peroxide was detected in any of the reaction vessels, which indicates that no reaction occurred. In conclusion, the screening assay provides valuable information on: 1) new possible P450–substrate combinations (cases A/B), 2) P450 decoupling (case D), and 3) catalase inhibition (cases B/C).

In the course of our initial screen, 180 enzyme/peroxide/substrate combinations were analyzed and classified (Figure 2). Chloroperoxidase (CPO) does not belong to the P450 superfamily, and thus, it was considered as control of the assay's feasibility. In fact, CPO did not exhibit any peroxide conversion. However, the CPO screen clearly identified inhibitors of catalase (Figure 2, cases B/C). This was observed for the substrates indole (**21**), ethoxyresorufin (**25**), nifedinipine (**29**), trazodone (**31**) and ketoconazole (**33**). Because only the inhibition of catalase by indole has been described so far,[18] we cross-examined our screening data in separate experiments, which confirmed that compounds **21**, **25**, **29**, **31** and **33** inhibit the catalase reaction to various extents (Figure S3). Our screen also revealed that CYP152A1 shows a strong decoupling with both peroxides. In fact, no case E reactions were observed for this enzyme, but case D was found for all reactions that did not correspond to categories A, B or C.

As expected, the screen for P450 reactivity also revealed several clear-cut hits (Figure 2, cases A and B). For example, the CYP152A1-mediated conversion of azulene (**11**) with **2** and **3**, which has been described before,[11c] was also observed here. Interestingly, nifedipine (**29**), trazodone (**31**) and ketoconazole (**33**) were previously assigned as inhibitors of CYP152A1, because these compounds significantly reduced the conversion of the diagnostic substrate 9-methylanthracene (**17**).[11c] Since the present screen classified **29**, **31** and **33** as substrates, we reasoned that they are better substrates than **17** for CYP152A1, and thus inhibit the conversion of **17** by active-site competition. The inefficient conversion of **17** by CYP152A1 was also evident from the corresponding screening data (Figure 2). To verify the conversion of **29**, **31** and **33** by CYP152A1, LC/MS^n analyses were conducted to identify oxidation products. As exemplified for the conversion of **31** (Scheme 3), elemental composition determination by exact mass measurements and collisionally-induced dissociation (CID) experiments led to structural assignments, which were in agreement with earlier studies on the conversion of **31** with human liver microsomes.[23] Similar LC/MS^n analyses of the conversion of **29** and **33** also led to the identification of distinctive oxidation products (Table S4). Interestingly, our screen also led to the discovery of novel substrates of the thermostable P450



Scheme 2. Substances used in the P450 screen. The hydroxylation of fatty acids (**5**–**10**) have been described for CYP152A1[26] and CYP119.[27] Polyaromatic hydrocarbons (**11**–**22**) have been tested as substrates for CYP152A1.[11c] Coumarin and resorufin derivatives (**23**–**25**) have been frequently used as P450 substrates.[28] Monochlorodimedone (**26**) is the natural substrate of chloroperoxidase (CPO) in a H_2O_2-dependent reaction.[29] Dextromethorphan (**27**) and testosterone (**28**) are standard substrates for human P450 enzymes.[30] The remaining substances are classified as inhibitors of different human P450 enzymes.[31]

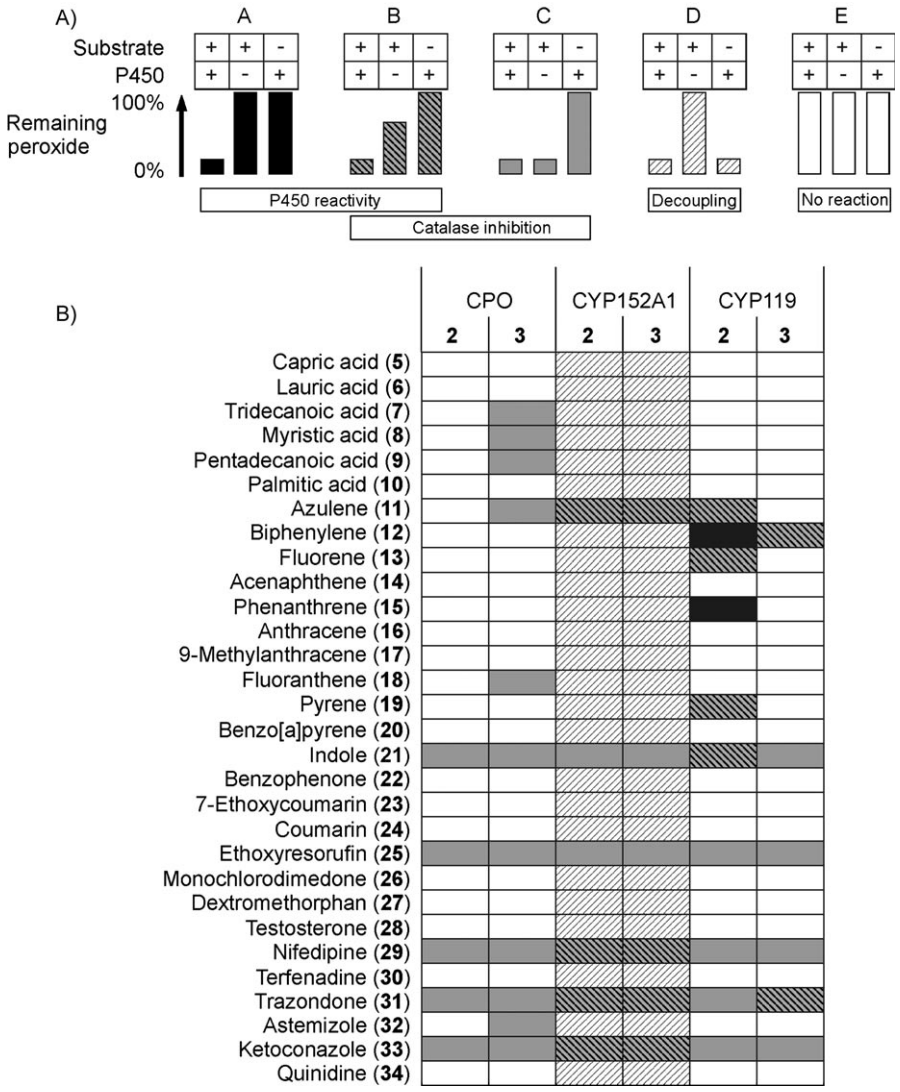


Figure 2. Classifications of the reactivities detected. A) Definition of the shading codes for the different reactivities (cases A–E). The heights of the bars represent the amount of remaining peroxide. B) Classification of screening reactions according to the shading code; see text for details. Structures of substrates **5**–**34** are shown in Scheme 2.

CYP119, such as biphenylene (**12**) or phenanthrene (**15**; Figure 2, Table S4). This is highly important, because this enzyme is considered to be a powerful biocatalyst for various applications, however, organic peroxide-mediated reactions have so far only been reported for styrene.[24, 11d]

Conclusions

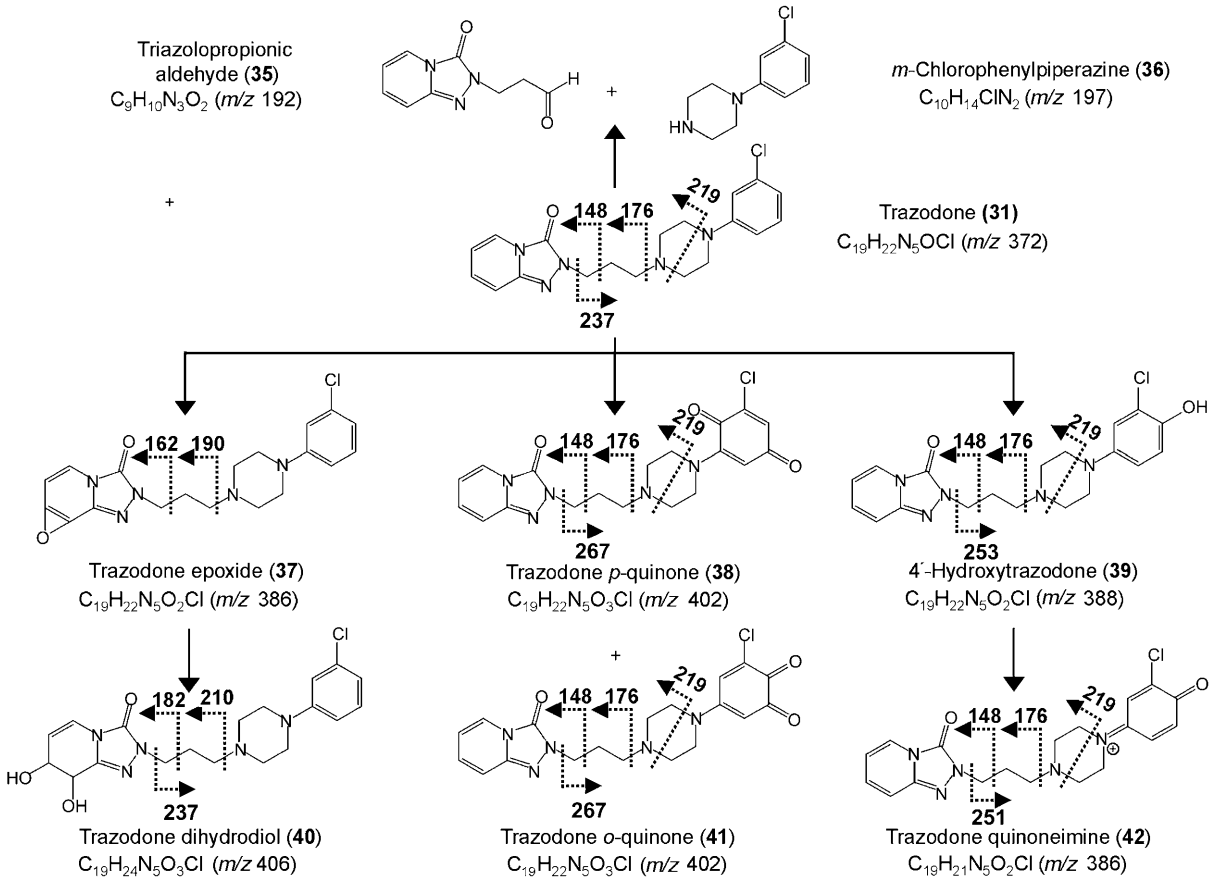
Here, we reported a novel assay for the high-throughput screening of organic peroxide-dependent P450 reactivity, and take advantage of the previously undescribed peroxidase activity of catalase. In a first demonstration, this assay allowed the straightforward identification of several new substrates of the bacterial P450 enzymes CYP152A1 and CYP119. Subsequent LC/MS^n measurements confirmed the novel reactivities found in the screen and led to initial insights on the structure of the products of the enzymatic conversion. As additional information, decoupling of P450 enzymes under the chosen reaction conditions could be identified and yet unknown inhibitors of catalase were discovered. Our assay should be well suited for robotic automation because it only requires successive pipetting steps. Thus, even large libraries of P450 enzymes should be screenable against libraries of chemically interesting compounds at low cost and with high-throughput.

Experimental Section

Chemicals: Chloroperoxidase, anthracene, indole, biphenylene, fluoranthene, fluorene and acenaphthene were purchased from Aldrich. Catalase, *tert*-butylhydroperoxide, 7-ethoxycoumarin, astemizole, coumarin, trazodone, testosterone, nifedipine, ketoconazole, ethoxyresorufin, monochlorodimedone, KH_2PO_4, K_2HPO_4, ammonium acetate and formic acid were obtained from Sigma. Cumene hydroperoxide, Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine, sold under the name of Ampliflu Red), 7-ethoxycoumarin, benzophenone, quinidine, capric acid, lauric acid, tridecanoic acid, myristic acid, pentadecanoic acid, palmitic acid were from Fluka. 9-Methylanthracene, azulene, pyrene, terfenadine, benzo[a]pyrene and phenanthrene were purchased from Acros Chemicals. Dextromethorphan was obtained from MPBiomedicals, LLC; the PeroXOquant kit was from Pierce. Acetonitrile and water (LC/MS grade) were purchased from Roth.

Kinetics of the peroxide-dependent reaction of catalase with Amplex Red: For the kinetic measurements a fixed concentration of catalase (100 nM) and Amplex Red (5 μM) was mixed with different concentrations of cumene- or *tert*-buthylhydroperoxide in KP_i buffer (100 mM, pH 7.0). The resulting fluorescence (due to the generation of resorufin) was recorded with a Synergy II microplate reader (BIO-TEK) at 25 °C. In all cases, the initial reaction velocity (v_0) was calculated from the linear phase of substrate formation, which was typically recorded in the initial phase of the reaction diagram when less than 10% of the maximum signal intensities was reached. All reactions were carried out at least in triplicate.

Recombinant P450BSß (CYP152A1): CYP152A1 was expressed in *Escherichia coli* M15 (pREP4) by using the plasmid pQE-30tBSb, which was kindly provided by Dr. Isamu Matsunaga.[25] The enzyme containing a C-terminal hexahistidine tail was overexpressed and purified by affinity chromatography, as reported earlier.[11a, c, 25]



Scheme 3. Proposed pathway of trazodone (**31**) oxidation based on LC/MS^n analysis (positive ionization mode). The elemental compositions were assigned by exact mass measurements of the detected protonated molecules $[M+H]^+$. Localization of modification sites was carried out by collisionally induced dissociation; the respective characteristic fragments (*m/z*) are included and indicated by arrows in the structures.

Recombinant CYP119: CYP119 was expressed in *E. coli* BL21(DE3) by using the plasmid pET-EXP42-CYP119. The enzyme containing a C-terminal hexahistidine tail was overexpressed and purified by affinity chromatography, as reported earlier.[11d]

Microtiterplate screening of P450 reactivity: All P450 reactions where carried out in 200 μL reaction volume, containing 100 μM of either cumene- or *tert*-butylhydroperoxide, the corresponding substrate (200 μM; obtained from 20 mM stock solutions in either acetonitrile or DMF) and enzyme (200 nM) in KP_i (100 mM, pH 7.0). After incubation at 37 °C for 24 h, an aliquot (50 μL) was withdrawn and mixed with 50 μL of a solution of Amplex Red (10 μM) and catalase (1000 nM) in KP_i (100 mM, pH 7.0). The initial reaction velocity (v_0) was calculated by recording the change in fluorescence as described above. For each substrate, control reactions lacking either the substrate, enzyme or peroxide were also performed. All reactions were performed as quadruplicates. Each microplate also contained a dilution series of known concentration of the peroxide in the same reaction buffer; this was used as standard for the determination of the remaining peroxide concentrations.

Analysis of oxidation products by liquid chromatography/multistage mass spectrometry (LC/MS^n): A reaction containing P450 enzyme (200 nM), substrate (2 mM) and the hydroperoxide (2 mM) in ammonium acetate buffer (50 mM) was prepared as described above. Chromatographic separations were performed by using a Surveyor MS pump and Surveyor autosampler (Thermo Fisher Scientific, San Jose, USA), equipped with reversed-phase C-18 column (Hypurity Aquastar, 150×1 mm, 3 μm, 190 Å). Two binary gradients consisting of acetonitrile and water containing formic acid (0.1%, *v/v*) were used for separation of reaction products of nonpolar and polar substrates, respectively. In particular, biphenylene and phenanthrene measurements were carried out by using 15% acetonitrile isocratic for 2 min, followed by 15 to 95% in 36 min, 95% isocratic for 7 min, from 95 to 15% in 3 min, and then 15% acetonitrile isocratic for 15 min. Trazodone, nifedipine and ketoconazole were measured by using 5% acetonitrile isocratic for 2 min, followed by 5 to 50% in 36 min, 50 to 95% in 2 min, 95% isocratic for 7 min, from 95 to 5% in 3 min, and then 5% acetonitrile isocratic for 15 min. The flow rate was 90 μL min^{-1} and the injection volume was set to 2 μL. The column temperature was set to 45 °C.

Mass spectrometric detection: MS spectrometry was carried out with an LTQ FT (Thermo Fisher Scientific, Bremen, Germany) Fourier transform ion cyclotron resonance hybrid mass spectrometer (FTICR-MS) by using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in the positive ionization mode. Briefly, the LTQ FT was set to automatically switch between MS and MS^n acquisition as already described in an earlier study.[11c] Survey MS spectra in the mass range *m/z* 100–800 were acquired in the FTICR with a resolution $r=25000$ (FWHM). The three most intense ions were sequentially isolated for accurate mass measurements by a FTICR "SIM scan" in a narrow mass window (±5 Da, $r=50000$). Subsequent fragmentation (MS^2, MS^3) was carried out in the linear ion trap by collisionally-induced dissociation (CID). ESI was used for the reaction mixtures of polar substrates, whereas

APCI was applied for nonpolar polycyclic aromatic hydrocarbons. All data were processed by using Qual Browser (Thermo Fisher Scientific, San Jose, USA), and the chemical formula calculator was used to obtain *m/z* values for probable oxidation products. These *m/z* values were used to generate reconstructed ion chromatograms to selectively screen for oxidation products.

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Keywords: biocatalysis · cytochrome P450 · enzymes · high-throughput screening · oxidases

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