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Preparation of human drug metabolites using fungal peroxygenases

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ABSTRACT

The synthesis of hydroxylated and *O*- or *N*-dealkylated human drug metabolites (HDMs) via selective monooxygenation remains a challenging task for synthetic organic chemists. Here we report that aromatic peroxygenases (APOs; EC 1.11.2.1) secreted by the agaric fungi *Agrocybe aegerita* and *Coprinellus radians* catalyzed the H_2O_2-dependent selective monooxygenation of diverse drugs, including acetanilide, dextrorphan, ibuprofen, naproxen, phenacetin, sildenafil and tolbutamide. Reactions included the hydroxylation of aromatic rings and aliphatic side chains, as well as *O*- and *N*-dealkylations and exhibited different regioselectivities depending on the particular APO used. At best, desired HDMs were obtained in yields greater than 80% and with isomeric purities up to 99%. Oxidations of tolbutamide, acetanilide and carbamazepine in the presence of $H_2{}^{18}O_2$ resulted in almost complete incorporation of ^{18}O into the corresponding products, thus establishing that these reactions are peroxygenations. The deethylation of phenacetin-d_1 showed an observed intramolecular deuterium isotope effect $[(k_H/k_D)_{obs}]$ of 3.1 ± 0.2, which is consistent with the existence of a cytochrome P450-like intermediate in the reaction cycle of APOs. Our results indicate that fungal peroxygenases may be useful biocatalytic tools to prepare pharmacologically relevant drug metabolites.

1. Introduction

Human drug metabolites (HDMs) are valuable chemicals needed for the development of safe and effective pharmaceuticals. They are frequently used as substrates and authentic standards in studies of drug bioavailability, pharmacodynamics and pharmacokinetics [1–3]. *In vivo*, the C–H bonds of pharmaceuticals are predominantly oxygenated by liver cytochrome P450-monooxygenases (P450s) to yield more polar HDMs that are excreted directly or as conjugates [4]. This directed incorporation of an oxygen atom into a complex organic structure is one of the most challenging reactions in synthetic organic chemistry [5] and thus low yields and the need for laborious removal of byproducts have prevented the cost-effective preparation of HDMs by purely chemical methods [6].

Another approach is the *in vitro* preparation of HDMs with enzymes. The obvious route is to use isolated human P450s, but these complex multiprotein systems are membrane-bound, poorly stable, cofactor-dependent, and generally exhibit low reaction rates [7,8]. More promising results have been reported for laboratory-evolved bacterial P450s, which are capable of catalyzing the H_2O_2-dependent hydroxylation of pharmaceuticals via the "peroxide shunt" pathway [9]. Recent studies, however, have demonstrated that this approach needs further optimization [10]. Alternatively, modified hemoproteins such as microperoxidases might be used to catalyze hydroxylations by a P450-like oxygen transfer mechanism, but so far these catalysts do not exhibit the necessary performance and selectivity [11–16].

Here we have adopted a recently developed approach, using aromatic peroxygenases (APOs)[1] from the agaric basidiomycetes *Agrocybe aegerita* (*Aae*APO) and *Coprinellus radians* (*Cra*APO) to produce diverse HDMs. These stable, secreted enzymes oxidize a wide range of substrates and are promising oxidoreductases for biotechnological applications [6,17–23].

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[1] In previous publications, the enzymes were also referred to as haloperoxidase-peroxygenases, mushroom/fungal peroxygenases, AaP (*Agrocybe aegerita* peroxidase/peroxygenase) or CrP (*Coprinellus radians* peroxygenase) [5,18,20,22]. In February 2011, they were classified in the Enzyme Nomenclature under EC 1.11.2.1 (unspecific peroxygenase).

2. Materials and methods

2.1. Reactants

Metoprolol, oseltamivir phosphate, 4-hydroxytolbutamide, 4′-hydroxydiclofenac, 5-hydroxydiclofenac, 3-hydroxyacetaminophen, omeprazole and sildenafil were obtained from Chemos GmbH (Regenstauf, Germany). 3-Hydroxycarbamazepine, 1-hydroxyibuprofen, 2-hydroxyibuprofen, 1-oxoibuprofen and *N*-desmethylsildenafil were purchased from Toronto Research Chemicals, Inc. (Toronto, Canada), α-hydroxymetoprolol, glycinexylidide and monoethylglycinexylidide from TLC PharmaChem., Inc. (Vaughan, Canada). 17α-Ethinylestradiol, (*R*)-naproxen, 4-hydroxypropranolol, 5-hydroxypropranolol, (*S*)-*N*-desisopropylpropranolol and *O*-desmethylmetoprolol were obtained from Fluka (St. Gallen, Switzerland), Shanghai FWD Chemicals, Ltd. (Shanghai, China), Biomol GmbH (Hamburg, Germany), SPIbio, Bertin Group (Montigny Le Bretonneux, France), ABX Advanced Biochemical Compounds (Radeberg, Germany) and Sandoo Pharmaceuticals & Chemicals Co., Ltd. (Zhejiang, China), respectively. $H_2{}^{18}O_2$ (90 atom%, 2% wt/vol) was a product of Icon Isotopes (New York, USA). All other chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany).

Phenacetin-d_1 (N-(4-[1-^{2}H]ethoxyphenyl)acetamide) was prepared from acetaminophen (*N*-(4-hydroxyphenyl)acetamide) and ethyl iodide-d_1 as described previously for phenacetin-d_3 [24]. The reaction product of the synthesis (phenacetin-d_1) was identified by comparison of retention time, UV absorption spectra, and mass spectra relative to authentic phenacetin [25]. Mass spectrum (*m*/*z*, %): 180 (100, −H), 138 (62, $-C_2H_2O$), 110 (12, $-C_4DH_6O$), 109 (96, $-C_3DH_2O_2$), 108 (100, $-C_3DH_6ON$), 80 (16) and 43 (18).

The extracellular peroxygenase of *A. aegerita* (*Aae*APO; isoform II, 45 kDa) was produced and purified as described previously [26]. The enzyme preparation was homogeneous by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and exhibited an A_{418}/A_{280} ratio of 1.75. The specific activity of the *Aae*APO preparation was 117 U mg^{-1}, where 1 U represents the oxidation of 1 μmol of 3,4-dimethoxybenzyl alcohol (veratryl alcohol) to 3,4-dimethoxybenzaldehyde (veratraldehyde) in 1 min at 23 °C [26]. The *Coprinellus radians* peroxygenase (*Cra*APO) was produced and purified as described previously [27]. It was homogeneous by SDS-PAGE, exhibited an A_{419}/A_{280} ratio of 1.04 and had a specific activity of 25.8 U mg^{-1} in the above assay.

2.2. Product identification

Typical reaction mixtures (0.2–1.0 ml) contained purified peroxygenase (1.0–2.0 U ml^{-1} = 0.4–0.8 μM), substrate to be oxidized (0.5–2.0 mM), potassium phosphate buffer (50 mM, pH 7.0) and ascorbic acid (4.0–6.0 mM, to inhibit further oxidation of any phenolic products that were released [6,17]). The reactions were started by the addition of limiting H_2O_2 (2.0–4.0 mM) and stirred at room temperature. They were stopped by addition of sodium azide (1 mM) or trichloroacetic acid (TCA) (5%) after 3 min. In some cases, H_2O_2 was added continuously with a syringe pump and the reactions were stopped after 4–6 h, when chromatographic analyses showed that product formation was complete.

Reaction products of interest were obtained, generally with baseline resolution, by high performance liquid chromatography (HPLC) using an Agilent Series 1200 instrument equipped with a diode array detector (DAD) and an electrospray ionization mass spectrometer (MS) (Agilent Technologies Deutschland GmbH, Böblingen, Germany). Unless otherwise stated, reverse phase (RP) chromatography of reaction mixtures was performed on a Luna C18 column (2 mm diameter by 150 mm length, 5 μm particle size, Phenomenex (Aschaffenburg, Germany), which was eluted at 0.35 ml min^{-1} and 40 °C with aqueous 0.01% vol/vol ammonium formate (pH 3.5)/acetonitrile, 95:5 for 5 min, followed by a 25-min linear gradient to 100% acetonitrile. For the experiments with acetaminophen, acetanilide and diclofenac, a Synergi 4 μ Fusion RP-80A column (4.6 mm diameter by 150 mm length, 4 μm particle size, Phenomenex) was used. The column was eluted at 40 °C and a flow rate of 1 ml min^{-1} with a mixture of aqueous phosphoric acid solution (15 mM, pH 3) and acetonitrile, 95:5, for 5 min, followed by a 20-min linear gradient to 100% acetonitrile. For metoprolol, a Gemini-Nx 3 μ 110A C18 reverse phase column (2 mm diameter by 150 mm length, 3 μm particle size, Phenomenex) was used. The column was eluted at 45 °C and a flow rate of 0.3 ml min^{-1} with a mixture of aqueous 0.2% vol/vol ammonium (pH 10.5) and acetonitrile, 95:5, for 1 min, followed by a 20-min linear gradient to 80% acetonitrile, followed by 21-min linear gradient to 100% acetonitrile. Chiral separation was done using the HPLC apparatus above, but using a Whelk-O 5/100 Kromasil column (4.6 mm diameter by 250 mm length, Regis Technologies (Morton Grove, USA). The isocratic mobile phase consisted of 80% vol/vol methanol and 20% of 15 mM phosphate buffer. The columns were operated at 40 °C and 1 ml min^{-1} for 30 min. Retention times for products are given in Supplemental Table 1.

Aliphatic aldehydes were analyzed as their 2,4-dinitrophenylhydrazones after addition of 0.2 volume of 0.1% 2,4-dinitrophenylhydrazine solution in 0.6 N HCl to each reaction mixture. The derivatized products were analyzed using the same HPLC apparatus as above, but the Luna C18 column was eluted with aqueous 0.1% vol/vol ammonium formate (pH 3.5)/acetonitrile, 70:30 for 5 min, followed by a 20-min linear gradient to 100% acetonitrile. Stoichiometrical experiments on sildenafil *N*-demethylation were conducted in stirred reactions (0.20 ml) that contained 2 U ml^{-1} (0.144 μM) peroxygenase, potassium phosphate buffer (50 mM, pH 7.0) and the substrate (0.25 mM). The reactions were initiated with 0.0023–0.037 mM H_2O_2.Mass spectroscopic determinations were made in positive or negative ESI mode (electrospray ionization) in a mass range from 70 to 500, step size 0.1, drying gas temperature 350 °C, capillary voltage 4000 V for positive mode and 5500 V for negative mode. The reaction products were identified relative to authentic standards, based on their retention times, UV absorption spectra, and mass spectral $[M+H]^+$ or $[M-H]^-$ ions.Quantifications of reactants and products were obtained by HPLC as described above, using a linear external standard curve ($R > 0.98$) of the respective compound. The parameters were calculated as follows: total conversion $[(S_c - S_s)/S_c) \times 100\%$; yield: $(P/S_c) \times 100\%$; regioselectivity: $(P/(S_c - S_s)] \times 100\%$ (S_c is the substrate concentration of the control, S_s is the substrate concentration after reaction with the enzyme and P is the product concentration).

2.3. Enzyme kinetics

The kinetics of propranolol hydroxylation as well as metoprolol and phenacetin *O*-dealkylation were analyzed in stirred microscale reactions (0.10 ml, 23 °C) that contained 0.40 μM peroxygenase (*Aae*APO), potassium phosphate buffer (50 mM, pH 7.0), ascorbic acid (4 mM) and 0.010–5.000 mM substrate. The reactions were initiated with 2.0 mM H_2O_2. Reactions with propranolol or phenacetin were stopped with 0.010 ml of 50% TCA solution after 5 s, and reactions with metoprolol were stopped with 0.2 volume of 0.1% 2,4-dinitrophenylhydrazine solution in 0.6 N HCl after 10 s. The resulting products were quantified by HPLC as described above using external standard curves prepared with authentic standards.

The kinetics of acetanilide hydroxylation were analyzed in stirred reactions (0.5 ml, 23 °C) that contained 0.08 μM *Aae*APO, potassium phosphate buffer (50 mM, pH 7.0), ascorbic acid (4 mM) and 0.010–5.000 mM substrate. The reactions were initiated with

2.0 mM H_2O_2 and stopped with 0.050 ml of sodium azide solution (1 mM) after 5 s. The initial velocity of acetaminophen formation was then determined from the increase in absorbance at 290 nm (ε = 1100 M^{-1} cm^{-1}) using a Cary 50 UV/visible spectrophotometer. The kinetic parameters were determined by nonlinear regression using the Michaelis–Menten model in the ANEMONA program [28].

2.4. ^{18}O-labeling experiments

The reaction mixtures (0.20 ml, stirred at room temperature) contained 2 U ml^{-1} (0.08 μM) of *Aae*APO, potassium phosphate buffer (50 mM, pH 7.0) and 0.5 mM substrate (tolbutamide, acetanilide, or carbamazepine). Reactions with tolbutamide or acetanilide were initiated with a single addition of 2.0 mM (final concentration) $H_2{}^{18}O_2$. For reactions with carbamazepine, the same quantity of $H_2{}^{18}O_2$ was added continuously with a syringe pump over 6 h. A portion of each completed reaction was then analyzed by HPLC/MS as described in Section 2.2. For each *m*/*z* value, the average total ion count within the metabolite (4-hydroxytolbutamide, acetaminophen and 3-hydroxycarbamazepine) peak was used after background correction to generate the ion count used for mass abundance calculations [18].

2.5. Determination of intramolecular isotope effect

The reaction mixture (0.2 ml) contained purified *Aae*APO (2 U ml^{-1}, 0.08 μM), potassium phosphate buffer (50 mM, pH 7.0), phenacetin-d_1 and ascorbic acid (4.0 mM). The reaction was started by the addition of H_2O_2 (2.0 mM) and stirred at room temperature. The reaction was stopped by the addition of 10% sodium azide (10 mM) after 10 s. The reaction products were analyzed as described above. For each *m*/*z* value, the average total ion count within the acetaminophen peak was used after background correction to generate the ion count used for mass abundance calculations.

3. Results

The peroxygenases from *Agrocybe aegerita* (*Aae*APO) and *Coprinellus radians* (*Cra*APO) oxidized diverse pharmaceuticals (**I**–**XX**, Fig. 1), showing different selectivity in some cases. The products, most of which we obtained with baseline resolution by HPLC, were monooxygenated or dealkylated compounds that correspond in most cases to previously identified HDMs (Table 1). Below, we have classified the major reactions according to the chemical moiety that was oxidized on the targeted drug. In many cases, a single oxidation predominated but, as noted in Table 1, more than one of these reactions sometimes occurred on a single substrate. A complete overview of all reactants investigated, confirmed products and hypothetical products for which we lacked authentic standards (drawn in brackets), is presented in Supplementary Table 2.

3.1. Aromatic hydroxylation

Both APOs catalyzed the hydroxylation of aromatic rings, in some cases with high regioselectivity. For example, *Aae*APO preferentially oxidized propranolol (**I**) to 5-OH-propranolol [17], acetanilide (**VI**) to acetaminophen (paracetamol; Fig. 2), carbamazepine (**III**) to 3-OH-carbamazepine and diclofenac (**IV**) to 4′-OH-diclofenac [17]. *Cra*APO catalyzed the same reactions but with lower regioselectivity. Both enzymes also oxidized tamoxifen (**V**) to produce low yields of 4-OH-tamoxifen and other products, which we were unable to resolve sufficiently by HPLC to permit quantifications.

Using *Aae*APO and two of the above substrates, we investigated the source of the oxygen introduced during hydroxylation. When the oxidation of acetanilide (**II**) was conducted with $H_2{}^{18}O_2$ in place of H_2O_2, mass spectral analysis of the resulting acetaminophen showed that the principal $[M-H]^-$ ion had shifted from its natural abundance *m*/*z* of 150 to *m*/*z* 152. Likewise, the hydroxylation of 3-hydroxycarbamazepine under these conditions resulted in a shift of the $[M+H]^+$ ion from its natural abundance *m*/*z* of 253 to *m*/*z* 255. Accordingly, H_2O_2 was the source of the introduced oxygen in both reactions.

Also using *Aae*APO, we determined apparent kinetic constants for two of these aromatic hydroxylations. For propranolol, the K_m was 442 μM and the k_{cat} was 59 s^{-1}; for acetanilide, the constants were 1,310 μM and 1,925 s^{-1}.

3.2. Aliphatic hydroxylation

Both peroxygenases catalyzed the hydroxylation of aliphatic side chains. Thus, ibuprofen (**IV**) was predominantly oxidized to 2-hydroxyibuprofen, with the *Cra*APO-catalyzed reaction exhibiting a higher yield and regioselectivity. Similarly, tolbutamide (**VII**) was oxidized to 4-hydroxytolbutamide (Table 1). A mass spectral experiment with tolbutamide and $H_2{}^{18}O_2$ as the substrates showed that the resulting 4-hydroxytolbutamide exhibited a principal $[M-H]^-$ ion with an *m*/*z* of 289 rather than 287, again showing that H_2O_2 was the source of the introduced oxygen (Fig. 3).

3.3. O-Dealkylation

Both APOs cleaved alkyl aryl ether linkages in various drugs to yield phenols [18]. Thus, metoprolol (**VIII**) was selectively demethylated to *O*-desmethylmetoprolol, naproxen (**IX**) to *O*-desmethylnaproxen and dextromethorphan (**X**) to dextrorphan. Yields and regioselectivities were generally greater with *Aae*APO. The above phenolic reaction products all tended to undergo further oxidation to quinones and/or coupling products because APOs exhibit general peroxidase activity that generates phenoxyl radicals from phenols [5,6,17,18], but this undesired reaction was partially inhibitable via addition of the radical scavenger ascorbate to the reactions. DNPH derivatization of the reaction mixtures showed that the methyl group was released as formaldehyde in each case. A kinetics analysis done with *Aae*APO and one of the substrates, metoprolol, showed that the reaction exhibited an apparent K_m for it of 2,330 μM and an apparent k_{cat} of 96 s^{-1}.

Analogously to the demethylations discussed above, phenacetin (**XI**) was de-ethylated to give acetaminophen and acetaldehyde (Table 1). Since phenacetin has a symmetrical site at its α-carbon, it is a suitable substrate to determine whether a catalyzed etherolytic reaction exhibits an intramolecular deuterium isotope effect. LC/MS analysis of DNPH-derivatized reactions showed that the *Aae*APO-catalyzed cleavage of *N*-(4-[1-^{2}H]ethoxyphenyl)acetamide (phenacetin-d_1) resulted in a preponderance of [^{2}H]acetaldehyde 2,4-dinitrophenylhydrazone (*m*/*z* 224, $[M-H]^-$) over natural abundance acetaldehyde 2,4-dinitrophenylhydrazone (*m*/*z* 223, $[M-H]^-$) (Fig. 4). The observed mean intramolecular deuterium isotope effect $[(k_H/k_D)_{obs}]$ from three experiments was 3.1 ± 0.2. The apparent K_m of *Aae*APO for phenacetin was 998 μM and the apparent k_{cat} was 33 s^{-1}.

3.4. N-Dealkylation

The APOs catalyzed the oxidative *N*-dealkylation of several drugs that contain secondary or tertiary amine groups. For example, *Aae*APO regioselectively *N*-dealkylated sildenafil (**XII**) at the tertiary N in its *N*-methyl piperazine ring to give *N*-

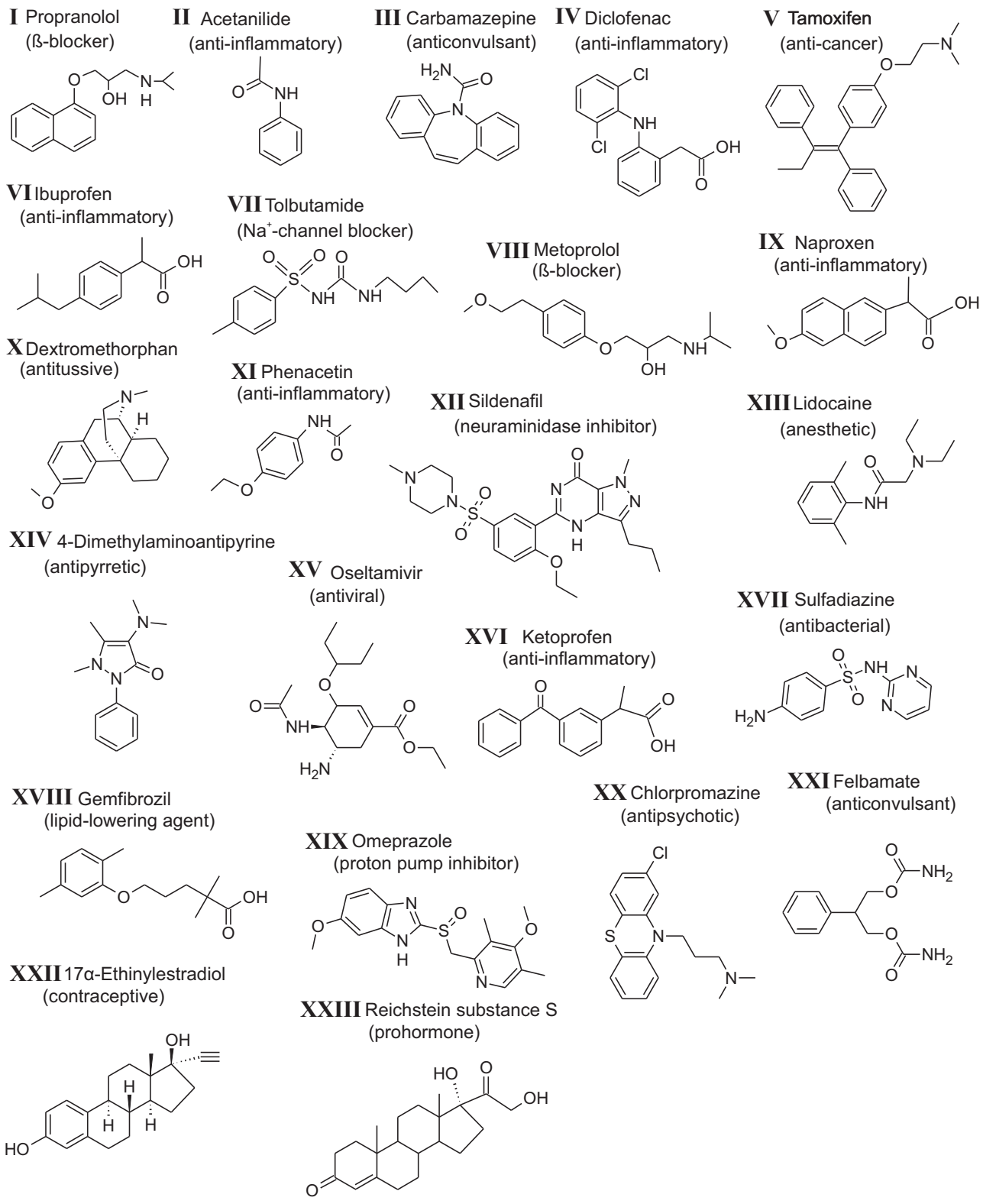

Fig. 1. Chemical structures of pharmaceuticals tested as peroxygenase substrates.

desmethylsildenafil and formaldehyde in high yield. By contrast, *Cra*APO was ineffective in this oxidation (Table 1). A quantitative analysis of *Aae*APO-catalyzed sildenafil oxidation in the presence of the limiting H_2O_2 showed that one equivalent of *N*-desmethylsildenafil was formed per equivalent of oxidant supplied (Table 2). Among the other *N*-dealkylations listed in Table 1 are the conversion of lidocaine (**XIII**) to monoethylglycinexylidide and glycinexylidide and of 4-dimethylaminoantipyrine (**XIV**) to 4-aminoantipyrine.

3.5. Ester cleavage

Ester cleavage by a peroxygenase can be regarded as a special case of *O*-dealkylation. Thus, *Cra*APO selectively cleaved oselta-

Table 1
Products identified by mass spectroscopy after oxidation of pharmaceuticals by *Aae*APO and *Cra*APO in the presence of H_2O_2. The *m*/*z* value for the major observed diagnostic ion, consumed substrate (SC), the yield (*Y*) and the regioselectivity (RS) of the product is shown in each case.

Substrate	Reaction product	Discussed as HDM in Ref no.	*m*/*z*	*Aae*APO (%)			*Cra*APO (%)		
				SC	*Y*	RS	SC	*Y*	RS
I				23			11		
	5-OH-Propranolol	[9,29]	$[M+H]^+$ 276		21	91		6	53
	4-OH-Propranolol	[9,29,46]	$[M+H]^+$ 276		–			2	13
	N-Desisopropyl-propranolol	[9,29]	$[M+H]^+$ 218		Traces			3	22
II				89			20		
	Acetaminophen	[47–49]	$[M-H]^-$ 150		80	90		13	65
	3-OH-Acetaminophen	[47]	$[M-H]^-$ 166		5	5		1	2
III[b]				22			15		
	3-OH-Carbamazepine	[50]	$[M+H]^+$ 253		13	61		6	40
IV				78			15		
	4′-OH-Diclofenac	[51,52]	$[M+H]^+$ 312		68	87		5	30
V				25					
	4-OH-Tamoxifen	[53–55]	$[M+H]^+$ 388		n.d.			n.d.	
	N-Desmethyltamoxifen	[53,54]	$[M+H]^+$ 358		n.d.			n.d.	
	Endoxifen	[53,54]	$[M+H]^+$ 374		n.d.			n.d.	
VI				87			98		
	2-OH-Ibuprofen	[56,57]	$[M+H]^+$ 223		21	24		74	75
	1-OH-Ibuprofen	[57]	$[M+H]^+$ 223		7	8		–	
	1-Oxo-Ibuprofen	n.a.	$[M+H]^+$ 221		Traces			–	
VII[a]				25			20		
	4-OH-Tolbutamide	[58]	$[M-H]^-$ 287		15	60		13	62
VIII[a]				82			73		
	O-Desmethylmetoprolol	[59]	$[M+H]^+$ 254		17	20		4	5
	α-OH-Metoprolol	[59]	$[M+H]^+$ 284		2	2		1	1
IX[b]				60			10		
	O-Desmethylnaproxen	[60,61]	$[M-H]^-$ 215		57	95		9	85
X				17			15		
	Dextrorphan	[62,63]	$[M+H]^+$ 258		16	95		8	53
XI				34			16		
	Acetaminophen	[64,65]	$[M+H]^+$ 152		23	66		13	80
	3-OH-Acetaminophen	[64]	$[M+H]^+$ 168		2	5		–	
XII				82			80		
	N-Desmethylsildenafil	[66]	$[M+H]^+$ 461		82	99		4	5
XIII				60			45		
	Monoethylglycinexylidide	[67]	$[M+H]^+$ 207		25	41		32	70
	Glycinexylidide	[67]	$[M+H]^+$ 179		18	30		5	11
XIV				68			38		
	4-Aminoantipyrine	[68]	$[M+H]^+$ 204		16	23		19	48
XV[b]				–			80		
	Oseltamivir carboxylate	[69]	$[M+H]^+$ 285		–			71	88

[a] Mass spectral data indicate the formation of corresponding carbonyls as described previously [19] (see supplementary Material).
[b] A syringe pump was used for hydrogen peroxide supply. (n.d.) = not determined due to poor resolution; (–) = not detected; (n.a.) = not applicable.

mivir (**XV**) to oseltamivir carboxylate and acetaldehyde in high yield. Interestingly, oseltamivir was not converted by *Aae*APO.

3.6. Additional observations

For some products of APO-catalyzed oxidations no authentic standards were available. However, the mass shifts we observed after these reactions (see supplementary material) indicate the regioselective oxidation of ketoprofen (**XVI**), sulfadiazine (**XVII**), gemfibrozil (**XVIII**), omeprazole (**XIX**) and chlorpromazine (**XX**).

We also noted some limitations on substrates for the APOs. No product formation was observed from felbamate (**XXI**), 17α-ethinylestradiol (**XXII**), or Reichstein substance S (**XXIII**). Moreover, we found that the APOs generally failed to discriminate between chiral centers in the pharmaceutical substrates. For example, chiral HPLC separation of the two *O*-desmethylnaproxen entantiomers that resulted from naproxen oxidation showed that neither one predominated in the end-product mixture (data not shown).

4. Discussion

Our results show that two fungal peroxygenases (APOs) catalyzed the hydroxylation or dealkylation of diverse pharmaceuticals, in some cases with high regioselectivity. Furthermore, most of the oxidations matched those catalyzed by human liver P450s, e.g. propranolol to 5-hydroxypropranolol (CYP2D6) [29], tolbutamide to 4-hydroxytolbutamide (CYP2C9) [30], naproxen to *O*-desmethylnaproxen (CYP1A2) [31], acetanilide to acetaminophen (CYP1A2) [31,32] and sildenafil to *N*-desmethylsildenafil (CYP3A4) [33]. The catalyzed reactions: aromatic hydroxylation, aliphatic hydroxylation, *O*-dealkylation of ethers and esters and *N*-dealkylation of amines, are all typical of P450s [34,35]. Our data agree with previous work that suggests the

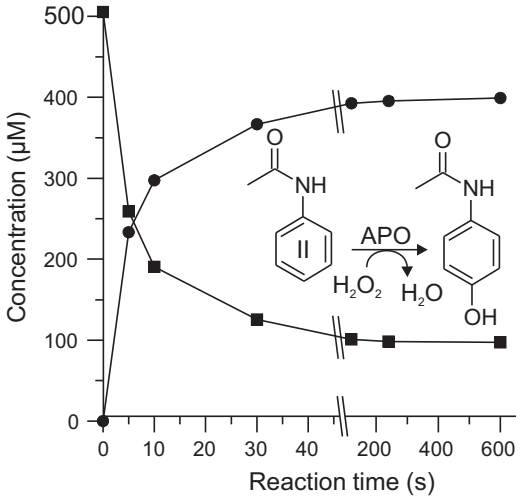

Fig. 2. Time course of *Aae*APO-catalyzed *para*-hydroxylation of acetanilide (●, **II**) to acetaminophen (■).

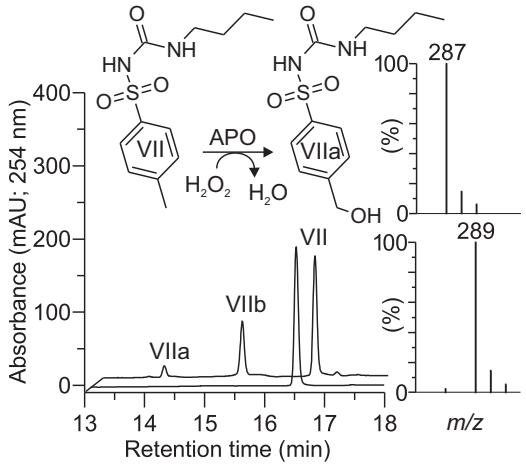

Fig. 3. HPLC elution profiles after conversion of tolbutamide (**VII**) in the presence of ascorbic acid: (front profile) control without enzyme; (background profile) completed reaction with *Aae*APO. Insets of mass spectra showing the incorporation of ^{18}O from $H_2{}^{18}O_2$ into the alcohol group of 4-hydroxytolbutamide (**VIIa**) after hydroxylation of tolbutamide by *Aae*APO are as follows. *Upper*: MS of the product obtained with natural abundance H_2O_2. *Lower*: MS of the product obtained with 90 atom% $H_2{}^{18}O_2$. The peak **VIIb** showed a mass as expected for 4-oxo-tolbutamide, a potential oxidation product of 4-hydroxytolbutamide (see supplemental Table 2) [19].

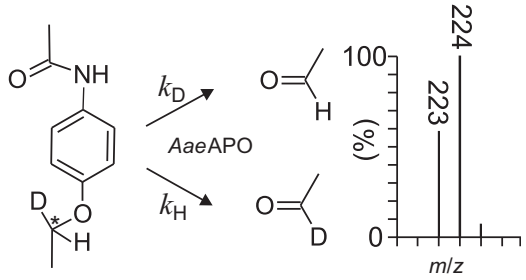

Fig. 4. Cleavage of *N*-(4-[1-^{2}H]ethoxyphenyl)acetamide (phenacetin-d_1) by *Aae*APO. The mass spectrum of the reaction mixture containing the products acetaldehyde 2,4-dinitrophenylhydrazone ($[M-H]^-$, 123) and [^{2}H] acetaldehyde 2,4-dinitrophenylhydrazone ($[M-H]^-$, 126) obtained from the oxidation of phenacetin-d_1 is shown. The mass spectrum is one of three used to calculate the observed mean intramolecular isotope effect.

Table 2
Stoichiometry of sildenafil (**XII**) oxidation by *Aae*APO.

H_2O_2 added (μM)	*N*-Desmethylsildenafil produced (μM)	Ratio *N*-desmethylsildenafil/H_2O_2
2.3	2.12	0.92
4.6	4.27	0.93
9.2	8.75	0.96
18.3	20.49	1.12
36.6	32.37	0.88

The initial sildenafil concentration was 250 μM.

extracellular APOs share characteristics with intracellular P450s, which also catalyze H_2O_2-dependent oxidations by a pathway termed the peroxide shunt [36–38]. These peroxygenations are thought to be initiated when the enzyme heme is oxidized by H_2O_2 to give an iron species (oxo-ferryl iron, compound I) that carries one of the peroxide oxygens, which subsequently oxidizes a C–H bond in the substrate [39].

According to the above model, oxygen incorporation from H_2O_2 should be quantitative when the substrate is oxidized. Our data on APO-catalyzed pharmaceutical oxidations agree with this picture since 100% of the oxygen present in newly generated phenolic or benzylic reaction products – e.g. in the phenol group of acetaminophen or in the alcohol moiety of 4-hydroxytolbutamide – was ^{18}O-labeled when the experiment was conducted with $H_2{}^{18}O_2$. Moreover, the stoichiometrical result we obtained using sildenafil as the substrate – one equivalent of *N*-desmethylsildenafil formed per equivalent of H_2O_2 supplied – agrees with the two-electron oxidation expected from a peroxygenative mechanism (Table 2).

Also consistent with a P450-like mechanism is the intramolecular deuterium isotope effect we observed for phenacetin-d_1 oxidation by *Aae*APO, in that our value of $(k_H/k_D)_{obs}$ around 3 is close to the values of $(k_H/k_D)_{obs}$ near 2 that have been observed for the P450-catalyzed *O*-dealkylation of this substrate. A value of 3 is considerably smaller than the intrinsic isotope effect near 10 expected for a hydrogen abstraction mechanism, and may indicate that phenacetin oxidation by APOs proceeds instead via electron transfer, as proposed earlier for the P450-catalyzed reaction [40]. By contrast, the *O*-dealkylation of 1,4-dimethoxybenzene-d_3 by *Aae*APO probably does proceed via hydrogen abstraction, because it exhibits a much higher $(k_H/k_D)_{obs}$ near 12 [18,40].

The kinetics data we report here for *Aae*APO action on a variety of pharmaceuticals suggest that APOs may be useful alternatives to P450s for the regioselective preparation of HDMs. Although some P450s are known to bind pharmaceutical substrates more strongly than APOs, exhibiting K_m values between 1 and 70 μM, they generally exhibit relatively low k_{cat} values in the vicinity of 0.2 s^{-1} or less [40,41]. As a result, the catalytic efficiencies [k_{cat}/K_m] of *Aae*APO for pharmaceutical oxidations lie in the same range as those of the P450s, as exemplified by our values for propranolol hydroxylation (1.32×10^5 M^{-1} s^{-1}), metoprolol *O*-dealkylation (4.1×10^4 M^{-1} s^{-1}) and phenacetin *O*-dealkylation (3.3×10^4 M^{-1} s^{-1}). The k_{cat}/K_m value we obtained for acetanilide (1.5×10^6 M^{-1} s^{-1}) is much higher than the corresponding value found for the P450-catalyzed reaction [32].

APOs have some advantages over P450s, including currently available laboratory-evolved P450s, where reaction yields are concerned [17]. For example, the transformation of diclofenac to 4-hydroxydiclofenac by mutants of $P450_{cam}$ (CYP101A1) resulted in total conversions between 15 and 44% and yields around 10% [42]. By contrast, wild-type *Aae*APO gave a total conversion of 78% and a yield of 68% for this reaction. In addition, fungal APOs may be better than P450s in practical applications for several other reasons: (i) they utilize an inexpensive co-substrate, H_2O_2; (ii) they do not require costly co-reactants such as pyridine nucleotides,

flavin reductases, or ferredoxins; (iii) they are secreted enzymes and thus can be cost-effectively produced; (iv) they are stable and water-soluble due to their high degree of glycosylation [43,44].

There are also disadvantages associated with APO-catalyzed oxidations. One is that the enzymes have relatively narrow catalytic clefts (albeit considerably wider than those of other heme peroxidases), that prevent access of markedly bulky substrates to the active site [45]. The negative results we obtained with felbamate, 17α-ethinylestradiol, and Reichstein substance S may reflect this limitation. Another problem is the high general peroxidase activity of APOs, which necessitates the inclusion of a radical scavenger such as ascorbate in reactions when the desired products are phenols. Ultimately, protein engineering of APOs may address these limitations and the recent crystallization of an APO provides the first information needed to begin this work [43,45]. In the meantime, the most fruitful approach is likely to involve empirical comparisons of APOs to determine their individual substrate specificities. Recent phylogenic investigations have shown that APOs are widespread in the fungal kingdom [44] and some new representatives have already been isolated from *Coprinopsis verticillata*, *Marasmius rotula* and other fungi [27]. The above developments open the possibility that APOs may serve as a "monooxygenation toolbox" for the selective production of HDMs and other fine chemicals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.06.020.

References

[1] Nelson SD. Metabolic activation and drug toxicity. J Med Chem 1982;25:753–65.
[2] Baillie TA, Cayen MN, Fouda H, Gerson RJ, Green JD, Grossman SJ, et al. Drug metabolites in safety testing. Toxicol Appl Pharmacol 2002;182:188–96.
[3] Peters FT, Dragan CA, Wilde DR, Meyer MR, Zapp J, Bureik M, et al. Biotechnological synthesis of drug metabolites using human cytochrome P450 2D6 heterologously expressed in fission yeast exemplified for the designer drug metabolite 4′-hydroxymethyl-alpha-pyrrolidinobutyrophenone. Biochem Pharmacol 2007;74:511–20.
[4] Gibson GG, Skett P. Introduction to drug metabolism. Cheltenham, UK: Nelson Thornes Publishers; 2001.
[5] Ullrich R, Hofrichter M. Enzymatic hydroxylation of aromatic compounds. Cell Mol Life Sci 2007;64:271–93.
[6] Kinne M, Ullrich R, Hammel KE, Scheibner K, Hofrichter M. Regioselective preparation of (*R*)-2-(4-hydroxyphenoxy)propionic acid with a fungal peroxygenase. Tetrahedron Lett 2008;49:5950–3.
[7] Urlacher VB, Lutz-Wahl S, Schmid RD. Microbial P450 enzymes in biotechnology. Appl Microbiol Biotechnol 2004;64:317–25.
[8] Eiben S, Kaysser L, Maurer S, Kuhnel K, Urlacher VB, Schmid RD. Preparative use of isolated CYP102 monooxygenases—a critical appraisal. J Biotechnol 2006;124:662–9.
[9] Otey CR, Bandara G, Lalonde J, Takahashi K, Arnold FH. Preparation of human metabolites of propranolol using laboratory-evolved bacterial cytochromes P450. Biotechnol Bioeng 2006;93:494–9.
[10] Sawayama AM, Chen MM, Kulanthaivel P, Kuo MS, Hemmerle H, Arnold FH. A panel of cytochrome P450 BM3 variants to produce drug metabolites and diversify lead compounds. Chemistry 2009;15:11723–9.
[11] Osman AM, Koerts J, Boersma MG, Boeren S, Veeger C, Rietjens IM. Microperoxidase/H_2O_2-catalyzed aromatic hydroxylation proceeds by a cytochrome-P-450-type oxygen-transfer reaction mechanism. Eur J Biochem 1996;240:232–8.
[12] Veeger C. Does P450-type catalysis proceed through a peroxo-iron intermediate?. A review of studies with microperoxidase. J Inorg Biochem 2002;91:35–45.
[13] Caputi L, Di Tullio A, Di Leandro L, De Angelis F, Malatesta F. A new microperoxidase from *Marinobacter hydrocarbonoclasticus*. Biochim Biophys Acta 2005;1725:71–80.
[14] Prieto T, Marcon RO, Prado FM, Caires AC, Di Mascio P, Brochsztain S, et al. Reaction route control by microperoxidase-9/CTAB micelle ratios. Phys Chem Chem Phys 2006;8:1963–73.
[15] Dorovska-Taran V, Posthumus MA, Boeren S, Boersma MG, Teunis CJ, Rietjens IM, et al. Oxygen exchange with water in heme-oxo intermediates during H_2O_2-driven oxygen incorporation in aromatic hydrocarbons catalyzed by microperoxidase-8. Eur J Biochem 1998;253:568–659.
[16] Dallacosta C, Monzani E, Casella L. Reactivity study on microperoxidase-8. J Biol Inorg Chem 2003;8:770–6.
[17] Kinne M, Poraj-Kobielska M, Aranda E, Ullrich R, Hammel KE, Scheibner K, et al. Regioselective preparation of 5-hydroxypropranolol and 4′-hydroxydiclofenac with a fungal peroxygenase. Bioorg Med Chem Lett 2009;19:3085–7.
[18] Kinne M, Poraj-Kobielska M, Ralph SA, Ullrich R, Hofrichter M, Hammel KE. Oxidative cleavage of diverse ethers by an extracellular fungal peroxygenase. J Biol Chem 2009;284:29343–9.
[19] Kinne M, Zeisig C, Ullrich R, Kayser G, Hammel KE, Hofrichter M. Stepwise oxygenations of toluene and 4-nitrotoluene by a fungal peroxygenase. Biochem Biophys Res Commun 2010;397:18–21.
[20] Hofrichter M, Ullrich R, Pecyna M, Liers C, Lundell T. New and classic families of secreted fungal heme peroxidases. Appl Microbiol Biotechnol 2010;87:871–97.
[21] Kluge M, Ullrich R, Dolge C, Scheibner K, Hofrichter M. Hydroxylation of naphthalene by aromatic peroxygenase from *Agrocybe aegerita* proceeds via oxygen transfer from H_2O_2 and intermediary epoxidation. Appl Microbiol Biotechnol 2009;81:1071–6.
[22] Aranda E, Kinne M, Kluge M, Ullrich R, Hofrichter M. Conversion of dibenzothiophene by the mushrooms *Agrocybe aegerita* and *Coprinellus radians* and their extracellular peroxygenases. Appl Microbiol Biotechnol 2008;82:1057–66.
[23] Aranda E, Ullrich R, Hofrichter M. Conversion of polycyclic aromatic hydrocarbons, methyl naphthalenes and dibenzofuran by two fungal peroxygenases. Biodegradation 2009;21:267–81.
[24] Garland WA, Hsiao KC, Pantuck EJ, Conney AH. Quantitative determination of phenacetin and its metabolite acetaminophen by GLC-chemical ionization mass spectrometry. J Pharm Sci 1977;66:340–4.
[25] Nottebaum LJ, McClure TD. Methods, systems, and computer program products for producing theoretical mass spectral fragmentation patterns of chemical structures. USA: Syngenta Participations AG/ipcom; 2010.
[26] Ullrich R, Nüske J, Scheibner K, Spantzel J, Hofrichter M. Novel haloperoxidase from the agaric basidiomycete *Agrocybe aegerita* oxidizes aryl alcohols and aldehydes. Appl Environ Microbiol 2004;70:4575–81.
[27] Anh DH, Ullrich R, Benndorf D, Svatos A, Muck A, Hofrichter M. The coprophilous mushroom *Coprinus radians* secretes a haloperoxidase that catalyzes aromatic peroxygenation. Appl Environ Microbiol 2007;73:5477–85.
[28] Hernandez A, Ruiz MT. An EXCEL template for calculation of enzyme kinetic parameters by non-linear regression. Bioinformatics 1998;14:227–8.
[29] Masubuchi Y, Hosokawa S, Horie T, Suzuki T, Ohmori S, Kitada M, et al. Cytochrome P450 isozymes involved in propranolol metabolism in human liver microsomes. The role of CYP2D6 as ring-hydroxylase and CYP1A2 as N-desisopropylase. Drug Metab Dispos 1994;22:909–15.
[30] Miners JO, Birkett DJ. Use of tolbutamide as a substrate probe for human hepatic cytochrome P450 2C9. Methods Enzymol 1996;272:139–45.
[31] Miners JO, Coulter S, Tukey RH, Veronese ME, Birkett DJ. Cytochromes P450, 1A2, and 2C9 are responsible for the human hepatic *O*-demethylation of R- and S-naproxen. Biochem Pharmacol 1996;51:1003–8.
[32] Liu G, Gelboin HV, Myers MJ. Role of cytochrome P450 IA2 in acetanilide 4-hydroxylation as determined with cDNA expression and monoclonal antibodies. Arch Biochem Biophys 1991;284:400–6.
[33] Ku HY, Ahn HJ, Seo KA, Kim H, Oh M, Bae SK, et al. The contributions of cytochromes P450 3A4 and 3A5 to the metabolism of the phosphodiesterase type 5 inhibitors sildenafil, udenafil, and vardenafil. Drug Metab Dispos 2008;36:986–90.
[34] Ortiz de Montellano P. Cytochrome P450—structure, mechanism and biochemistry. New York: Kluwer Academic/Plenum Publishers; 2005.
[35] Guengerich FP. Oxidative cleavage of carboxylic esters by cytochrome P-450. J Biol Chem 1987;262:8459–62.
[36] Shoji O, Fujishiro T, Nakajima H, Kim M, Nagano S, Shiro Y, et al. Hydrogen peroxide dependent monooxygenations by tricking the substrate recognition of cytochrome P450BSβ. Angew Chem Int Ed 2007;46:3656–9.
[37] Guengerich FP. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. Chem Res Toxicol 2001;14:611–50.
[38] Ortiz de Montellano PR, de Voss JJ. Substrate oxidation by cytochrome P450 enzymes. New York: Kluwer Academic/Plenum Publishers; 2005.
[39] Rittle J, Green MT. Cytochrome P450 compound **I**: capture, characterization, and C–H bond activation kinetics. Science 2010;330:933–7.
[40] Yun CH, Miller GP, Guengerich FP. Rate-determining steps in phenacetin oxidations by human cytochrome P450 1A2 and selected mutants. Biochemistry 2000;39:11319–2.
[41] Upthagrove AL, Nelson WL. Importance of amine pK_a and distribution coefficient in the metabolism of fluorinated propranolol derivatives. Preparation, identification of metabolite regioisomers, and metabolism by CYP2D6. Drug Metab Dispos 2001;29:1377–88.

[42] Weis R, Winkler M, Schittmayer M, Kambourakis S, Vink M, Rozzell JD, et al. A diversified library of bacterial and fungal bifunctional cytochrome P450 enzymes for drug metabolite synthesis. Adv Synth Catal 2009;351: 2140–6.
[43] Ullrich R, Liers C, Schimpke S, Hofrichter M. Purification of homogeneous forms of fungal peroxygenase. Biotechnol J 2009;4:1619–26.
[44] Pecyna MJ, Ullrich R, Bittner B, Clemens A, Scheibner K, Schubert R, et al. Molecular characterization of aromatic peroxygenase from *Agrocybe aegerita*. Appl Microbiol Biotechnol 2009;84:885–97.
[45] Piontek K, Ullrich R, Liers C, Diederichs K, Plattner DA, Hofrichter M. Crystallization of a 45 kDa peroxygenase/peroxidase from the mushroom Agrocybe aegerita and structure determination by SAD utilizing only the haem iron. Acta Crystallogr Sect F Struct Biol Cryst Commun 2010;66:693–8.
[46] Fitzgerald JD, O'Donnell SR. Pharmacology of 4-hydroxypropranolol, a metabolite of propranolol. Br J Pharmacol 1971;43:222–35.
[47] Greenberg LA, Lester D. The metabolic fate of acetanilid and other aniline derivates. J Pharmacol Exp Ther 1946;88:87–98.
[48] Berthou F, Flinois JP, Ratanasavanh D, Beaune P, Riche C, Guillouzo A. Evidence for the involvement of several cytochromes P-450 in the first steps of caffeine metabolism by human liver microsomes. Drug Metab Dispos 1991;19:561–7.
[49] Yoshitomi S, Ikemoto K, Takahashi J, Miki H, Namba M, Asahi S. Establishment of the transformants expressing human cytochrome P450 subtypes in HepG2, and their applications on drug metabolism and toxicology. Toxicol In Vitro 2001;15:245–56.
[50] Pearce RE, Vakkalagadda GR, Leeder JS. Pathways of carbamazepine bioactivation in vitro. I. Characterization of human cytochromes P450 responsible for the formation of 2- and 3-hydroxylated metabolites. Drug Metab Dispos 2002;30:1170–9.
[51] Bort R, Macé K, Boobis A, Gómez-Lechón M-J, Pfeifer A, Castell J. Hepatic metabolism of diclofenac: role of human CYP in the minor oxidative pathways. Biochem Pharmacol 1999;58:787–96.
[52] Bort R, Ponsoda X, Jover R, Gomez-Lechon MJ, Castell JV. Diclofenac toxicity to hepatocytes: a role for drug metabolism in cell toxicity. J Pharmacol Exp Ther 1999;288:65–72.
[53] Jin Y, Desta Z, Stearns V, Ward B, Ho H, Lee K-H, et al. CYP2D6 Genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. J Natl Cancer Inst 2005;97:30–9.
[54] Vitseva O, Flockhart DA, Jin Y, Varghese S, Freedman JE. The effects of tamoxifen and its metabolites on platelet function and release of reactive oxygen intermediates. J Pharmacol Exp Ther 2005;312:1144–50.
[55] Stearns V, Johnson MD, Rae JM, Morocho A, Novielli A, Bhargava P, et al. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 2003;95:1758–64.
[56] Hamman MA, Thompson GA, Hall SD. Regioselective and stereoselective metabolism of ibuprofen by human cytochrome P450 2C. Biochem Pharmacol 1997;54:33–41.
[57] Holmes E, Loo RL, Cloarec O, Coen M, Tang H, Maibaum E, et al. Detection of urinary drug metabolite (xenometabolome) signatures in molecular epidemiology studies via statistical total correlation (NMR) spectroscopy. Anal Chem 2007;79:2629–40.
[58] Hansen LL, Brøsen K. Quantitative determination of tolbutamide and its metabolites in human plasma and urine by high-performance liquid chromatography and UV detection. Ther Drug Monit 1999;21:664.
[59] Hoffmann K-J, Gyllenhaal O, Vessman J. Analysis of α-hydroxy metabolites of metoprolol in human urine after phosgene/trimethylsilyl derivatization. Biol Mass Spectrom 1987;14:543–8.
[60] Segre EJ. Naproxen metabolism in man. J Clin Pharmacol 1975;15:316–23.
[61] Bougie D, Aster R. Immune thrombocytopenia resulting from sensitivity to metabolites of naproxen and acetaminophen. Blood 2001;97:3846–50.
[62] Jacqz-Aigrain E, Funck-Brentano C, Cresteil T. CYP2D6- and CYP3A-dependent metabolism of dextromethorphan in humans. Pharmacogenetics 1993;3: 197–204.
[63] Broly F, Libersa C, Lhermitte M, Bechtel P, Dupuis B. Effect of quinidine on the dextromethorphan O-demethylase activity of microsomal fractions from human liver. Br J Clin Pharmacol 1989;28:29–36.
[64] Brodie BB, Axelrod J. The fate of acetophenetidin (phenacetin) in man and methods for the estimation of acetophenetidin and its metabolites in biological material. J Pharmacol Exp Ther 1949;97:58–67.
[65] Fura A, Shu Y-Z, Zhu M, Hanson RL, Roongta V, Humphreys WG. Discovering drugs through biological transformation: role of pharmacologically active metabolites in drug discovery. J Med Chem 2004;47:4339–51.
[66] Hyland R, Roe EGH, Jones BC, Smith DA. Identification of the cytochrome P450 enzymes involved in the N-demethylation of sildenafil. Br J Clin Pharmacol 2001;51:239–48.
[67] Orlando R, Piccoli P, De Martin S, Padrini R, Floreani M, Palatini P. Cytochrome P450 1A2 is a major determinant of lidocaine metabolism in vivo: effects of liver function. Clin Pharmacol Ther 2004;75:80–8.
[68] Banerjee NC, Miller GE, Stowe CM. Excretion of aminopyrine and its metabolites into cows' milk. Toxicol Appl Pharmacol 1967;10:604–12.
[69] McClellan K, Perry CM. Oseltamivir: a review of its use in influenza. Drugs 2001;61:263–83.