



Preparation of human drug metabolites using fungal peroxxygenases

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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 peroxxygenases (APOs; EC 1.11.2.1) secreted by the agaric fungi *Agrocybe aegerita* and *Coprinellus radians* catalyzed the H₂O₂-dependent selective monooxygenation of diverse drugs, including acetanilide, dextrophan, 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 H₂¹⁸O₂ resulted in almost complete incorporation of ¹⁸O into the corresponding products, thus establishing that these reactions are peroxxygenations. 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 peroxxygenases may be useful biocatalytic tools to prepare pharmacologically relevant drug metabolites.

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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 H₂O₂-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 peroxxygenases (APOs)¹ from the agaric basidiomycetes *Agrocybe aegerita* (AaeAPO) and *Coprinellus radians* (CraAPO) to produce diverse HDMs. These stable, secreted enzymes oxidize a wide range of substrates and are promising oxidoreductases for biotechnological applications [6,17–23].

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

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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, glycineylidide and monoethylglycineylidide from TLC PharmaChem., Inc. (Vaughan, Canada). 17 α -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. H₂¹⁸O₂ (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-[1-²H]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, –C₂H₂O), 110 (12, –C₄DH₆O), 109 (96, –C₃DH₂O₂), 108 (100, –C₃DH₆ON), 80 (16) and 43 (18).

The extracellular peroxygenase of *A. aegerita* (*AaeAPO*; 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 *AaeAPO* preparation was 117 U mg^{–1}, where 1 U represents the oxidation of 1 μ mol of 3,4-dimethoxybenzyl alcohol (veratryl alcohol) to 3,4-dimethoxybenzaldehyde (veratraldehyde) in 1 min at 23 °C [26]. The *Coprinellus radians* peroxygenase (*CraAPO*) was produced and purified as described previously [27]. It was homogeneous by SDS-PAGE, exhibited an *A*₄₁₈/*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 μ M), 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 H₂O₂ (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, H₂O₂ 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 μ m 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 μ m 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 μ m 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 μ M) 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 H₂O₂. 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] × 100%; yield: (*P*/*S*_c) × 100%; regioselectivity: (*P*/(*S*_c – *S*_s)) × 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 μ M peroxygenase (*AaeAPO*), 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 H₂O₂. 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 μ M *AaeAPO*, 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 H₂O₂ 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 ($\epsilon = 1100 \text{ M}^{-1} \text{ cm}^{-1}$) 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. ¹⁸O-labeling experiments

The reaction mixtures (0.20 ml, stirred at room temperature) contained 2 U ml⁻¹ (0.08 μM) of AaeAPO, 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) H₂¹⁸O₂. For reactions with carbamazepine, the same quantity of H₂¹⁸O₂ 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 AaeAPO (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 H₂O₂ (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 *Agroclybe aegerita* (AaeAPO) and *Coprinellus radians* (CraAPO) 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, AaeAPO 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]. CraAPO 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 AaeAPO 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 H₂¹⁸O₂ in place of H₂O₂, 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, H₂O₂ was the source of the introduced oxygen in both reactions.

Also using AaeAPO, 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 CraAPO-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 H₂¹⁸O₂ 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 H₂O₂ 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 AaeAPO. 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 AaeAPO 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 AaeAPO-catalyzed cleavage of *N*-(4-[1-²H]ethoxyphenyl)acetamide (phenacetin-*d*₁) resulted in a preponderance of [²H]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 AaeAPO 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, AaeAPO regioselectively *N*-dealkylated sildenafil (XII) at the tertiary N in its *N*-methyl piperazine ring to give *N*-

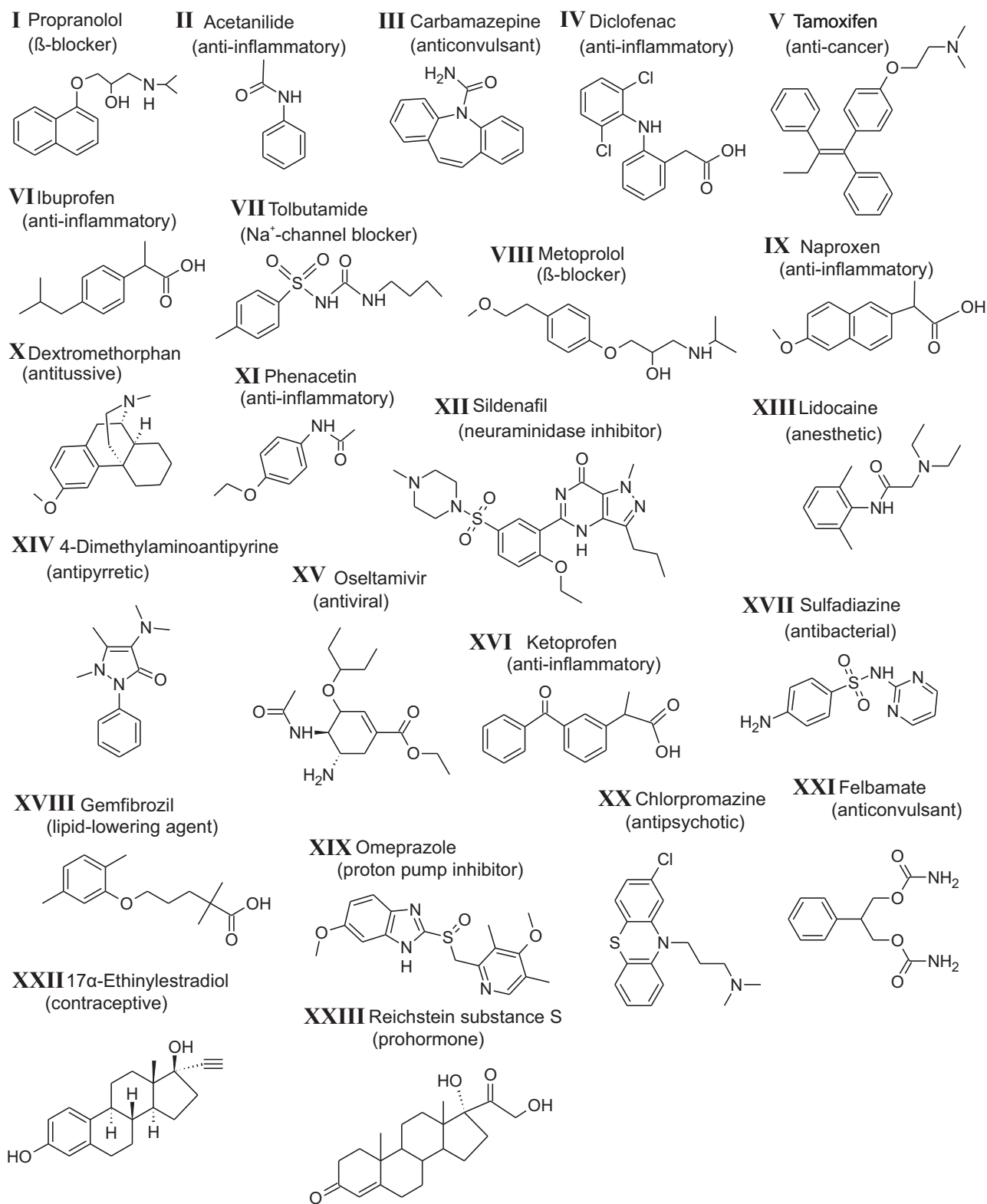


Fig. 1. Chemical structures of pharmaceuticals tested as peroxxygenase substrates.

desmethylsildenafil and formaldehyde in high yield. By contrast, *Cra*APPO was ineffective in this oxidation (Table 1). A quantitative analysis of *Aae*APPO-catalyzed sildenafil oxidation in the presence of the limiting H₂O₂ 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 peroxxygenase can be regarded as a special case of *O*-dealkylation. Thus, *Cra*APPO selectively cleaved oselta-

Table 1

Products identified by mass spectroscopy after oxidation of pharmaceuticals by AaeAPO and CraAPO in the presence of H₂O₂. 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.	<i>m/z</i>	AaeAPO (%)			CraAPO (%)		
				SC	Y	RS	SC	Y	RS
I	5-OH-Propranolol	[9,29]	[M+H] ⁺ 276	23	21	91	11	6	53
	4-OH-Propranolol	[9,29,46]	[M+H] ⁺ 276			–		2	13
	<i>N</i> -Desisopropyl-propranolol	[9,29]	[M+H] ⁺ 218			Traces		3	22
II	Acetaminophen	[47–49]	[M–H] [–] 150	89	80	90	20	13	65
	3-OH-Acetaminophen	[47]	[M–H] [–] 166		5	5		1	2
III^b	3-OH-Carbamazepine	[50]	[M+H] ⁺ 253	22	13	61	15	6	40
IV	4'-OH-Diclofenac	[51,52]	[M+H] ⁺ 312	78	68	87	15	5	30
V	4-OH-Tamoxifen	[53–55]	[M+H] ⁺ 388	25		n.d.			n.d.
	<i>N</i> -Desmethyltamoxifen	[53,54]	[M+H] ⁺ 358			n.d.			n.d.
	Endoxifen	[53,54]	[M+H] ⁺ 374			n.d.			n.d.
VI	2-OH-Ibuprofen	[56,57]	[M+H] ⁺ 223	87	21	24	98	74	75
	1-OH-Ibuprofen	[57]	[M+H] ⁺ 223		7	8			–
	1-Oxo-Ibuprofen	n.a.	[M+H] ⁺ 221			Traces			–
VII^a	4-OH-Tolbutamide	[58]	[M–H] [–] 287	25	15	60	20	13	62
VIII^a	<i>O</i> -Desmethylmetoprolol	[59]	[M+H] ⁺ 254	82	17	20	73	4	5
	α -OH-Metoprolol	[59]	[M+H] ⁺ 284		2	2		1	1
IX^b	<i>O</i> -Desmethylnaproxen	[60,61]	[M–H] [–] 215	60	57	95	10	9	85
X	Dextrorphan	[62,63]	[M+H] ⁺ 258	17	16	95	15	8	53
XI	Acetaminophen	[64,65]	[M+H] ⁺ 152	34	23	66	16	13	80
	3-OH-Acetaminophen	[64]	[M+H] ⁺ 168		2	5			–
XII	<i>N</i> -Desmethylsildenafil	[66]	[M+H] ⁺ 461	82	82	99	80	4	5
XIII	Monoethylglycinexylidide	[67]	[M+H] ⁺ 207	60	25	41	45	32	70
	Glycinexylidide	[67]	[M+H] ⁺ 179		18	30		5	11
XIV	4-Aminoantipyrine	[68]	[M+H] ⁺ 204	68	16	23	38	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 AaeAPO.

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**), 17 α -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 enantiomers 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

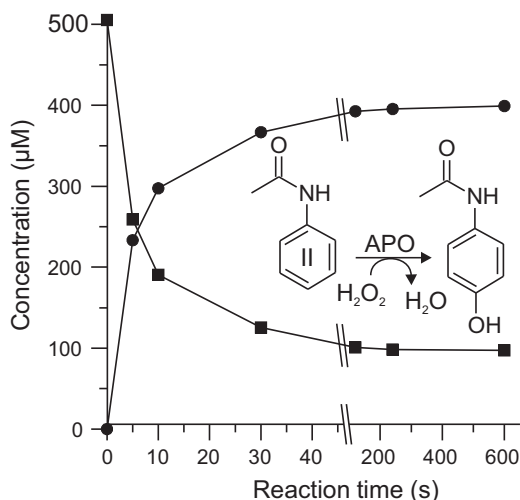


Fig. 2. Time course of *AaeAPO*-catalyzed *para*-hydroxylation of acetanilide (●, II) to acetaminophen (■).

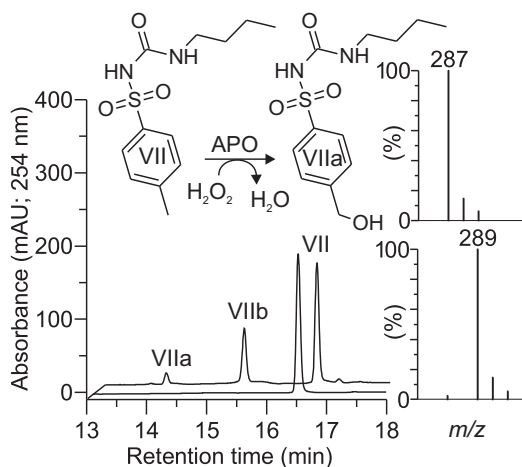


Fig. 3. HPLC elution profiles after conversion of tolbutamide (VII) in the presence of ascorbic acid: (front profile) control without enzyme; (background profile) completed reaction with *AaeAPO*. Insets of mass spectra showing the incorporation of ^{18}O from $\text{H}_2^{18}\text{O}_2$ into the alcohol group of 4-hydroxytolbutamide (VIIa) after hydroxylation of tolbutamide by *AaeAPO* are as follows. Upper: MS of the product obtained with natural abundance H_2O_2 . Lower: MS of the product obtained with 90 atom% $\text{H}_2^{18}\text{O}_2$. The peak VIIb showed a mass as expected for 4-oxo-tolbutamide, a potential oxidation product of 4-hydroxytolbutamide (see supplemental Table 2) [19].

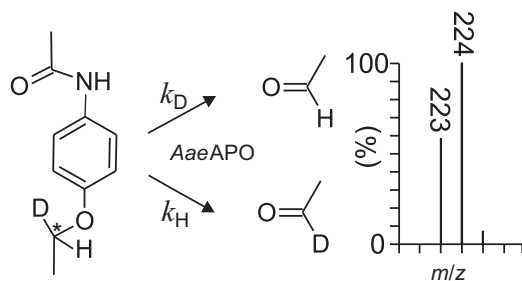


Fig. 4. Cleavage of *N*-(4-[1- ^2H]ethoxyphenyl)acetamide (phenacetin- d_1) by *AaeAPO*. The mass spectrum of the reaction mixture containing the products acetaldehyde 2,4-dinitrophenylhydrazone ($[\text{M}-\text{H}]^+$, 123) and [^2H] acetaldehyde 2,4-dinitrophenylhydrazone ($[\text{M}-\text{H}]^+$, 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 *AaeAPO*.

H_2O_2 added (μM)	<i>N</i> -Desmethylsildenafil produced (μM)	Ratio <i>N</i> -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 $\text{H}_2^{18}\text{O}_2$. 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 *AaeAPO*, in that our value of $(k_{\text{H}}/k_{\text{D}})_{\text{obs}}$ around 3 is close to the values of $(k_{\text{H}}/k_{\text{D}})_{\text{obs}}$ 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 *AaeAPO* probably does proceed via hydrogen abstraction, because it exhibits a much higher $(k_{\text{H}}/k_{\text{D}})_{\text{obs}}$ near 12 [18,40].

The kinetics data we report here for *AaeAPO* 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 $[k_{\text{cat}}/K_{\text{m}}]$ of *AaeAPO* for pharmaceutical oxidations lie in the same range as those of the P450s, as exemplified by our values for propranolol hydroxylation ($1.32 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$), metoprolol *O*-dealkylation ($4.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) and phenacetin *O*-dealkylation ($3.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$). The $k_{\text{cat}}/K_{\text{m}}$ value we obtained for acetanilide ($1.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) 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 *AaeAPO* 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, 17 α -ethinyloestradiol, 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 phylogenetic 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.

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