



**HAL**  
open science

## Preparation of human drug metabolites using fungal peroxygenases

Marzena Poraj-Kobielska, Matthias Kinne, René Ullrich, Katrin Scheibner, Gernot Kayser, Kenneth E. Hammel, Martin Hofrichter

► **To cite this version:**

Marzena Poraj-Kobielska, Matthias Kinne, René Ullrich, Katrin Scheibner, Gernot Kayser, et al.. Preparation of human drug metabolites using fungal peroxygenases. *Biochemical Pharmacology*, 2011, 82 (7), pp.789. 10.1016/j.bcp.2011.06.020 . hal-00723636

**HAL Id: hal-00723636**

**<https://hal.science/hal-00723636v1>**

Submitted on 12 Aug 2012

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

Title: Preparation of human drug metabolites using fungal peroxygenases

Authors: Marzena Poraj-Kobielska, Matthias Kinne, René Ullrich, Katrin Scheibner, Gernot Kayser, Kenneth E. Hammel, Martin Hofrichter



PII: S0006-2952(11)00403-5  
DOI: doi:10.1016/j.bcp.2011.06.020  
Reference: BCP 10944

To appear in: *BCP*

Received date: 28-3-2011  
Revised date: 14-6-2011  
Accepted date: 14-6-2011

Please cite this article as: Poraj-Kobielska M, Kinne M, Ullrich R, Scheibner K, Kayser G, Hammel KE, Hofrichter M, Preparation of human drug metabolites using fungal peroxygenases, *Biochemical Pharmacology* (2010), doi:10.1016/j.bcp.2011.06.020

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

## Preparation of human drug metabolites using fungal peroxygenases

1  
2  
3  
4  
5  
6 Marzena Poraj-Kobielska<sup>1</sup>, Matthias Kinne<sup>1\*</sup>, René Ullrich<sup>1</sup>, Katrin Scheibner<sup>2</sup>, Gernot  
7  
8 Kayser<sup>1</sup>, Kenneth E. Hammel<sup>3</sup> and Martin Hofrichter<sup>1</sup>  
9

10  
11  
12  
13  
14  
15  
16 <sup>1</sup>International Graduate School of Zittau (IHI Zittau)

17  
18 Department of Bio- and Environmental Sciences, Markt 23, 02763 Zittau, Germany  
19

20  
21 <sup>2</sup>Section of Biotechnology, Chemistry and Process Engineering, Lausitz University of Applied  
22  
23 Sciences, Großhainer Straße 57, 01968 Senftenberg, Germany  
24

25  
26  
27 <sup>3</sup>USDA Forest Products Laboratory, Madison, WI 53726, USA, and Department of  
28  
29 Bacteriology, University of Wisconsin, Madison, WI 53706, USA  
30

31  
32  
33  
34 \*Address correspondence to: Matthias Kinne, International Graduate School of Zittau, Markt  
35  
36 23, Zittau, 02763, Tel.: 00493583612723, Fax: 00493583612734, E-mail: kinne@ihi-zittau.de  
37  
38

39  
40  
41 The synthesis of hydroxylated and *O*- or *N*-dealkylated human drug metabolites (HDMs) via  
42  
43 selective monooxygenation remains a challenging task for synthetic organic chemists. Here  
44  
45 we report that aromatic peroxygenases (APOs; EC 1.11.2.1) secreted by the agaric fungi  
46  
47 *Agrocybe aegerita* and *Coprinellus radians* catalyzed the H<sub>2</sub>O<sub>2</sub>-dependent selective  
48  
49 monooxygenation of diverse drugs, including acetanilide, dextrorphan, ibuprofen, naproxen,  
50  
51 phenacetin, sildenafil and tolbutamide. Reactions included the hydroxylation of aromatic rings  
52  
53 and aliphatic side chains, as well as *O*- and *N*-dealkylations and exhibited different  
54  
55 regioselectivities depending on the particular APO used. At best, desired HDMs were  
56  
57 obtained in yields greater than 80% and with isomeric purities up to 99%. Oxidations of  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

tolbutamide, acetanilide and carbamazepine in the presence of H<sub>2</sub><sup>18</sup>O<sub>2</sub> resulted in almost complete incorporation of <sup>18</sup>O into the corresponding products, thus establishing that these reactions are peroxygenations. The deethylation of phenacetin-*d*<sub>1</sub> showed an observed intramolecular deuterium isotope effect [(*k*<sub>H</sub>/*k*<sub>D</sub>)<sub>obs</sub>] 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.

**Keywords:** Peroxidase, peroxygenation, hydroxylation, *O*-dealkylation, *N*-dealkylation, cytochrome P450

## 1. Introduction

1  
2  
3  
4 Human drug metabolites (HDMs) are valuable chemicals needed for the development of safe  
5  
6 and effective pharmaceuticals. They are frequently used as substrates and authentic standards  
7  
8 in studies of drug bioavailability, pharmacodynamics and pharmacokinetics[1-3]. *In vivo*, the  
9  
10 C-H bonds of pharmaceuticals are predominantly oxygenated by liver cytochrome P450-  
11  
12 monooxygenases (P450s) to yield more polar HDMs that are excreted directly or as  
13  
14 conjugates [4]. This directed incorporation of an oxygen atom into a complex organic  
15  
16 structure is one of the most challenging reactions in synthetic organic chemistry [5] and thus  
17  
18 low yields and the need for laborious removal of byproducts have prevented the cost-effective  
19  
20 preparation of HDMs by purely chemical methods [6].  
21  
22  
23  
24  
25  
26  
27

28 Another approach is the *in vitro* preparation of HDMs with enzymes. The obvious route is to  
29  
30 use isolated human P450s, but these complex multiprotein systems are membrane-bound,  
31  
32 poorly stable, cofactor-dependent, and generally exhibit low reaction rates [7, 8]. More  
33  
34 promising results have been reported for laboratory-evolved bacterial P450s, which are  
35  
36 capable of catalyzing the H<sub>2</sub>O<sub>2</sub>-dependent hydroxylation of pharmaceuticals via the "peroxide  
37  
38 shunt" pathway [9]. Recent studies, however, have demonstrated that this approach needs  
39  
40 further optimization [10]. Alternatively, modified hemoproteins such as microperoxidases  
41  
42 might be used to catalyze hydroxylations by a P450-like oxygen transfer mechanism, but so  
43  
44 far these catalysts do not exhibit the necessary performance and selectivity [11-16].  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Here we have adopted a recently developed approach, using aromatic peroxygenases (APOs)<sup>1</sup> 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].

## 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),  $\alpha$ -hydroxymetoprolol, glycineylidide and monoethylglycineylidide from TLC PharmaChem. Inc. (Vaughan, Canada). 17 $\alpha$ -Ethinylestradiol, (*R*)-naproxen, 4-hydroxypropranolol, 5-hydroxypropranolol, (*S*)-*N*-desisopropylpropranolol and *O*-desmethylnmetoprolol 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

---

<sup>1</sup> 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).

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Co., Ltd. (Zhejiang, China), respectively.  $\text{H}_2^{18}\text{O}_2$  (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- $^2\text{H}$ ]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, -  $\text{C}_2\text{H}_2\text{O}$ ), 110 (12, -  $\text{C}_4\text{DH}_6\text{O}$ ), 109 (96, -  $\text{C}_3\text{DH}_2\text{O}_2$ ), 108 (100, -  $\text{C}_3\text{DH}_6\text{ON}$ ), 80 (16), 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  $117 \text{ U mg}^{-1}$ , where 1 U represents the oxidation of 1  $\mu\text{mol}$  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  $A_{419}/A_{280}$  ratio of 1.04 and had a specific activity of  $25.8 \text{ U mg}^{-1}$  in the above assay.

## 2.2. Product identification

Typical reaction mixtures (0.2-1.0 ml) contained purified peroxygenase ( $1.0\text{-}2.0 \text{ U ml}^{-1} = 0.04\text{-}0.08 \mu\text{M}$ ), 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

1 that were released [6, 17]). The reactions were started by the addition of limiting H<sub>2</sub>O<sub>2</sub> (2.0-  
2 4.0 mM) and stirred at room temperature. They were stopped by addition of sodium azide (1  
3 mM) or trichloroacetic acid (TCA) (5%) after 3 min. In some cases, H<sub>2</sub>O<sub>2</sub> was added  
4 continuously with a syringe pump and the reactions were stopped after 4-6 h, when  
5 chromatographic analyses showed that product formation was complete.  
6  
7  
8  
9

10  
11  
12  
13 Reaction products of interest were obtained, generally with baseline resolution, by high  
14 performance liquid chromatography (HPLC) using an Agilent Series 1200 instrument  
15 equipped with a diode array detector (DAD) and an electrospray ionization mass spectrometer  
16 (MS) (Agilent Technologies Deutschland GmbH, Böblingen, Germany). Unless otherwise  
17 stated, reverse phase (RP) chromatography of reaction mixtures was performed on a Luna  
18 C18 column (2 mm diameter by 150 mm length, 5 µm particle size, Phenomenex  
19 (Aschaffenburg, Germany), which was eluted at 0.35 ml min<sup>-1</sup> and 40 °C with aqueous 0.01%  
20 vol/vol ammonium formate (pH 3.5)/acetonitrile, 95:5 for 5 min, followed by a 25-min linear  
21 gradient to 100% acetonitrile. For the experiments with acetaminophen, acetanilide and  
22 diclofenac, a Synergi 4µ Fusion RP-80A column (4.6 mm diameter by 150 mm length, 4 µm  
23 particle size, Phenomenex) was used. The column was eluted at 40 °C and a flow rate of 1 ml  
24 min<sup>-1</sup> with a mixture of aqueous phosphoric acid solution (15 mM, pH 3) and acetonitrile,  
25 95:5, for 5 min, followed by a 20-min linear gradient to 100% acetonitrile. For metoprolol, a  
26 Gemini-Nx 3µ 110A C18 reverse phase column (2 mm diameter by 150 mm length, 3 µm  
27 particle size, Phenomenex) was used. The column was eluted at 45 °C and a flow rate of 0.3  
28 ml min<sup>-1</sup> with a mixture of aqueous 0.2% vol/vol ammonium (pH 10.5) and acetonitrile, 95:5,  
29 for 1 min, followed by a 20-min linear gradient to 80% acetonitrile, followed by 21-min linear  
30 gradient to 100% acetonitrile. Chiral separation was done using the HPLC apparatus above,  
31 but using a Whelk-O 5/100 Kromasil column (4.6 mm diameter by 250 mm length, Regis  
32 Technologies (Morton Grove, USA). The isocratic mobile phase consisted of 80% vol/vol  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

methanol and 20% of 15 mM phosphate buffer. The columns were operated at 40°C and 1 ml min<sup>-1</sup> 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<sup>-1</sup> (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<sub>2</sub>O<sub>2</sub>.

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]<sup>+</sup> or [M - H]<sup>-</sup> 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:  $(P/(S_c - S_s)) \times 100\%$  ( $S_c$  - substrate concentration of the control,  $S_s$  - substrate concentration after reaction with the enzyme,  $P$ - product concentration).

### 2.3. Enzyme kinetics

1 The kinetics of propranolol hydroxylation as well as metoprolol and phenacetin *O*-  
2 dealkylation were analyzed in stirred microscale reactions (0.10 ml, 23 °C) that contained  
3  
4 0.40  $\mu\text{M}$  peroxygenase (*Aae*APO), potassium phosphate buffer (50 mM, pH 7.0), ascorbic  
5  
6 acid (4 mM) and 0.010-5.000 mM substrate. The reactions were initiated with 2.0 mM  $\text{H}_2\text{O}_2$ .  
7  
8 Reactions with propranolol or phenacetin were stopped with 0.010 ml of 50% TCA solution  
9  
10 after 5 seconds, and reactions with metoprolol were stopped with 0.2 volume of 0.1% 2,4-  
11  
12 dinitrophenylhydrazine solution in 0.6 N HCl after 10 seconds. The resulting products were  
13  
14 quantified by HPLC as described above using external standard curves prepared with  
15  
16 authentic standards.  
17  
18  
19  
20  
21  
22  
23  
24  
25

26 The kinetics of acetanilide hydroxylation were analyzed in stirred reactions (0.5 ml, 23 °C)  
27  
28 that contained 0.08  $\mu\text{M}$  *Aae*APO, potassium phosphate buffer (50 mM, pH 7.0), ascorbic acid  
29  
30 (4 mM) and 0.010-5.000 mM substrate. The reactions were initiated with 2.0 mM  $\text{H}_2\text{O}_2$  and  
31  
32 stopped with 0.050 ml of sodium azide solution (1 mM) after 5 seconds. The initial velocity of  
33  
34 acetaminophen formation was then determined from the increase in absorbance at 290 nm ( $\epsilon =$   
35  
36  $1,100 \text{ M}^{-1} \text{ cm}^{-1}$ ) using a Cary 50 UV/visible spectrophotometer. The kinetic parameters were  
37  
38 determined by nonlinear regression using the Lineweaver-Burk model in the ANEMONA  
39  
40 program [28].  
41  
42  
43  
44  
45  
46  
47

#### 48 **2.4. $^{18}\text{O}$ -labeling experiments**

49  
50  
51  
52

53 The reaction mixtures (0.20 ml, stirred at room temperature) contained 2 U  $\text{ml}^{-1}$  (0.08  $\mu\text{M}$ ) of  
54  
55 *Aae*APO, potassium phosphate buffer (50 mM, pH 7.0) and 0.5 mM substrate (tolbutamide,  
56  
57 acetanilide, or carbamazepine). Reactions with tolbutamide or acetanilide were initiated with a  
58  
59 single addition of 2.0 mM (final concentration)  $\text{H}_2^{18}\text{O}_2$ . For reactions with carbamazepine, the  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

same quantity of H<sub>2</sub><sup>18</sup>O<sub>2</sub> was added continuously with a syringe pump over 6 hours. 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, 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<sup>-1</sup>, 0.08 μM), potassium phosphate buffer (50 mM, pH 7.0), phenacetin-*d*<sub>1</sub> and ascorbic acid (4.0 mM). The reaction was started by the addition of H<sub>2</sub>O<sub>2</sub> (2.0 mM) and stirred at room temperature. The reaction was stopped by the addition of 10% sodium azide (10 mM) after 10 seconds. 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

1  
2  
3  
4 The peroxygenases from *Agrocybe aegerita* (*Aae*APO) and *Coprinellus radians* (*Cra*APO)  
5 oxidized diverse pharmaceuticals (**I-XX**, Fig. 1), showing different selectivity in some cases.  
6  
7 The products, most of which we obtained with baseline resolution by HPLC, were  
8  
9 monooxygenated or dealkylated compounds that correspond in most cases to previously  
10 identified HDMs (Table 1). Below, we have classified the major reactions according to the  
11 chemical moiety that was oxidized on the targeted drug. In many cases, a single oxidation  
12 predominated but, as noted in Table 1, more than one of these reactions sometimes occurred  
13 on a single substrate. A complete overview of all reactants investigated, confirmed products  
14 and hypothetical products for which we lacked authentic standards (drawn in brackets), is  
15 presented in Supplementary Table 2.  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30

#### 3.1. Aromatic hydroxylation

31  
32  
33  
34  
35 Both APOs catalyzed the hydroxylation of aromatic rings, in some cases with high  
36 regioselectivity. For example, *Aae*APO preferentially oxidized propranolol (**I**) to 5-OH-  
37 propranolol [17], acetanilide (**VI**) to acetaminophen (paracetamol), carbamazepine (**III**) to 3-  
38 OH-carbamazepine and diclofenac (**IV**) to 4'-OH-diclofenac [17]. *Cra*APO catalyzed the  
39 same reactions but with lower regioselectivity. Both enzymes also oxidized tamoxifen (**V**) to  
40 produce low yields of 4-OH-tamoxifen and other products, which we were unable to resolve  
41 sufficiently by HPLC to permit quantifications.  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54

55 Using *Aae*APO and two of the above substrates, we investigated the source of the oxygen  
56 introduced during hydroxylation. When the oxidation of acetanilide (**II**) was conducted with  
57  $\text{H}_2^{18}\text{O}_2$  in place of  $\text{H}_2\text{O}_2$ , mass spectral analysis of the resulting acetaminophen showed that  
58  
59  
60  
61  
62  
63  
64  
65

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  $\mu M$  and the  $k_{cat}$  was 59  $s^{-1}$ ; for acetanilide, the constants were 1,310  $\mu M$  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 phenoxy 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  $\mu\text{M}$   
and an apparent  $k_{\text{cat}}$  of 96  $\text{s}^{-1}$ .

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  $\alpha$ -  
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- $^2\text{H}$ ]ethoxyphenyl)acetamide  
(phenacetin- $d_1$ ) resulted in a preponderance of [ $^2\text{H}$ ]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  
[( $k_{\text{H}}/k_{\text{D}}$ ) $_{\text{obs}}$ ] from three experiments was  $3.1 \pm 0.2$ . The apparent  $K_m$  of *Aae*APO for  
phenacetin was 998  $\mu\text{M}$  and the apparent  $k_{\text{cat}}$  was 33  $\text{s}^{-1}$ .

### 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*-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  $\text{H}_2\text{O}_2$  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 oseltamivir (**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**), 17 $\alpha$ -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 extracellular APOs share characteristics with intracellular P450s, which also catalyze H<sub>2</sub>O<sub>2</sub>-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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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 <sup>18</sup>O-labeled when the experiment was conducted with H<sub>2</sub><sup>18</sup>O<sub>2</sub>.

Moreover, the stoichiometrical result we obtained using sildenafil as the substrate - one equivalent of *N*-desmethylsildenafil formed per equivalent of H<sub>2</sub>O<sub>2</sub> 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  $(k_H/k_D)_{\text{obs}}$  around 3 is close to the values of  $(k_H/k_D)_{\text{obs}}$  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 *Aae*APO probably does proceed via hydrogen abstraction, because it exhibits a much higher  $(k_H/k_D)_{\text{obs}}$  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  $\mu\text{M}$ , they generally exhibit relatively low  $k_{\text{cat}}$  values in the vicinity of  $0.2 \text{ s}^{-1}$  or less [40, 41]. As a result, the catalytic efficiencies [ $k_{\text{cat}}/K_m$ ] of *Aae*APO for pharmaceutical oxidations lie in the same range as those of the P450s, as exemplified by our values for propranolol hydroxylation ( $1.32 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ), 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  $k_{\text{cat}}/K_m$  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 [42].

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<sub>cam</sub> (CYP101A1) resulted in total conversions between 15 and 44% and yields around 10% [43]. By contrast, wild-type *Aae*APO gave a total conversion of 78% and a yield of 68% for this reaction. In addition,

1 fungal APOs may be better than P450s in practical applications for several other reasons: (i)  
2 they utilize an inexpensive co-substrate, H<sub>2</sub>O<sub>2</sub>; (ii) they do not require costly co-reactants such  
3 as pyridine nucleotides, flavin reductases, or ferredoxins; (iii) they are secreted enzymes and  
4 thus can be cost-effectively produced; and (iv) they are stable and water-soluble due to their  
5 high degree of glycosylation [44, 45].  
6  
7  
8  
9

10  
11  
12  
13 There are also disadvantages associated with APO-catalyzed oxidations. One is that the  
14 enzymes have relatively narrow catalytic clefts (albeit considerably wider than those of other  
15 heme peroxidases), that prevent access of markedly bulky substrates to the active site [46].  
16  
17  
18  
19

20  
21 The negative results we obtained with felbamate, 17 $\alpha$ -ethinylestradiol, and Reichstein  
22 substance S may reflect this limitation. Another problem is the high general peroxidase  
23 activity of APOs, which necessitates the inclusion of a radical scavenger such as ascorbate in  
24 reactions when the desired products are phenols. Ultimately, protein engineering of APOs  
25 may address these limitations and the recent crystallization of an APO provides the first  
26 information needed to begin this work [44, 46]. In the meantime, the most fruitful approach is  
27 likely to involve empirical comparisons of APOs to determine their individual substrate  
28 specificities. Recent phylogenic investigations have shown that APOs are widespread in the  
29 fungal kingdom [45] and some new representatives have already been isolated from  
30 *Coprinopsis verticillata*, *Marasmius rotula* and other fungi [27]. The above developments  
31 open the possibility that APOs may serve as a “monooxygenation toolbox” for the selective  
32 production of HDMs and other fine chemicals.  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52

### 53 **Acknowledgments**

54  
55  
56

57 We thank M. Kluge, C. Dolge, A. Karutz, N. Lemanska, M. Brandt, U. Schneider and A.  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Social Fund (project 080935557), the German Environmental Foundation (DBU, project 13225-32) and the German Ministry for Education and Research (BMBF, Cluster Integrierte Bioindustrie Frankfurt) is gratefully acknowledged.

## 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 Microb 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.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [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<sub>2</sub>O<sub>2</sub>-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<sub>2</sub>O<sub>2</sub>-driven oxygen incorporation in aromatic hydrocarbons catalyzed by microperoxidase-8. *Eur J Biochem* 1998;253:659-568.
- [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.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [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<sub>2</sub>O<sub>2</sub> 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. ipcom. USA: Syngenta Participations AG, 2010.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [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.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [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 P450BS $\beta$ . *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;39:11319-29.
- [41] Uptagrove AL, Nelson WL. Importance of amine pKa 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] Liu G, Gelboin HV, Myers MJ. Role of cytochrome P450 IA2 in acetanilide 4-hydroxylation as determined with cDNA expression and monoclonal antibodies. *Arch of Biochem and Biophys* 1991;284:400-6.
- [43] 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.
- [44] Ullrich R, Liers C, Schimpke S, Hofrichter M. Purification of homogeneous forms of fungal peroxygenase. *Biotechnol J* 2009;4:1619-26.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [45] 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.
- [46] 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 66:693-8.
- [47] Fitzgerald JD, O'Donnell SR. Pharmacology of 4-hydroxypropranolol, a metabolite of propranolol. Br J Pharmacol 1971;43:222-35.
- [48] Greenberg LA, Lester D. The metabolic fate of acetanilid and other aniline derivates. J Pharmacol Exp Ther 1946;88:87-98.
- [49] 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.
- [50] 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.
- [51] 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.
- [52] 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.
- [53] 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.



- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [54] 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.
- [55] 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.
- [56] 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.
- [57] Hamman MA, Thompson GA, Hall SD. Regioselective and stereoselective metabolism of ibuprofen by human cytochrome P450 2C. *Biochem Pharmacol* 1997;54:33-41.
- [58] 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.
- [59] Hansen LL, Brøsen K. Quantitative determination of tolbutamide and its metabolites in human plasma and urine by high-performance liquid chromatography and UV detection. *Therap Drug Monitor* 1999;21:664.
- [60] Hoffmann K-J, Gyllenhaal O, Vessman J. Analysis of  $\alpha$ -hydroxy metabolites of metoprolol in human urine after phosgene/trimethylsilyl derivatization. *Biol Mass Spectrom* 1987;14:543-8.
- [61] Segre EJ. Naproxen metabolism in man. *J Clin Pharmacol* 1975;15:316-23.
- [62] Bougie D, Aster R. Immune thrombocytopenia resulting from sensitivity to metabolites of naproxen and acetaminophen. *Blood* 2001;97:3846-50.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- [63] Jacqz-Aigrain E, Funck-Brentano C, Cresteil T. CYP2D6- and CYP3A-dependent metabolism of dextromethorphan in humans. *Pharmacogenetics* 1993;3:197-204.
- [64] 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.
- [65] 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.
- [66] 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.
- [67] 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.
- [68] 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.
- [69] Banerjee NC, Miller GE, Stowe CM. Excretion of aminopyrine and its metabolites into cows' milk. *Toxicol Appl Pharmacol* 1967;10:604-12.
- [70] McClellan K, Perry CM. Oseltamivir: A review of its use in influenza. *Drugs* 2001;61:263-83.

## Legends:

1  
2  
3  
4 Table 1. Products identified by mass spectroscopy after oxidation of pharmaceuticals by  
5  
6 *Aae*APO and *Cra*APO in the presence of H<sub>2</sub>O<sub>2</sub>. The *m/z* value for the major observed  
7  
8 diagnostic ion, consumed substrate (SC), the yield (Y) and the regioselectivity (RS) of the  
9  
10 product is shown in each case.  
11  
12  
13

14  
15  
16 Table 2. Stoichiometry of sildenafil (**XII**) oxidation by *Aae*APO  
17  
18

19  
20 Figure 1. Chemical structures of pharmaceuticals tested as peroxygenase substrates.  
21  
22

23  
24  
25 Figure 2. Time course of *Aae*APO-catalyzed *para*-hydroxylation of acetanilide (●, **II**) to  
26  
27 acetaminophen (■).  
28  
29  
30

31  
32 Figure 3. HPLC elution profiles after conversion of tolbutamide (**VII**) in the presence of  
33  
34 ascorbic acid: (front profile) Control without enzyme; (background profile) completed  
35  
36 reaction with *Aae*APO. Insets of mass spectra showing the incorporation of <sup>18</sup>O from H<sub>2</sub><sup>18</sup>O<sub>2</sub>  
37  
38 into the alcohol group of 4-hydroxytolbutamide (**VIIa**) after hydroxylation of tolbutamide by  
39  
40 *Aae*APO are as follows. *Upper*: MS of the product obtained with natural abundance H<sub>2</sub>O<sub>2</sub>.  
41  
42 *Lower*: MS of the product obtained with 90 atom % H<sub>2</sub><sup>18</sup>O<sub>2</sub>. The peak **VIIb** showed a mass as  
43  
44 expected for 4-oxo-tolbutamide, a potential oxidation product of 4-hydroxytolbutamide (see  
45  
46 Supplemental Table 2) [19].  
47  
48  
49  
50  
51

52  
53  
54 Figure 4. Cleavage of *N*-(4-[1-<sup>2</sup>H]ethoxyphenyl)acetamide (phenacetin-*d*<sub>1</sub>) by *Aae*APO. The  
55  
56 mass spectrum of the reaction mixture containing the products acetaldehyde 2,4-  
57  
58 dinitrophenylhydrazone ([M - H]<sup>-</sup>, 123) and [<sup>2</sup>H] acetaldehyde 2,4-dinitrophenylhydrazone  
59  
60  
61

([M - H]<sup>-</sup>, 126) obtained from the oxidation of phenacetin-*d*<sub>1</sub> is shown. The mass spectrum is one of three used to calculate the observed mean intramolecular isotope effect.

Supplementary Material (see attached files):

Supplementary Table 1. HPLC methods for reaction products investigated.

Supplementary Table 2. Substrates and products investigated.

Accepted Manuscript

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

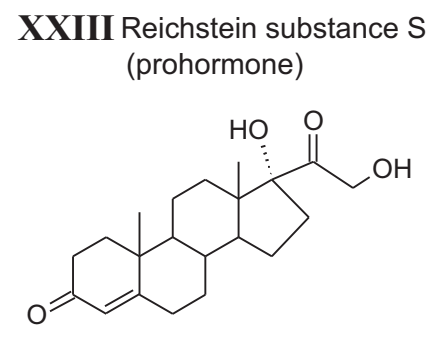
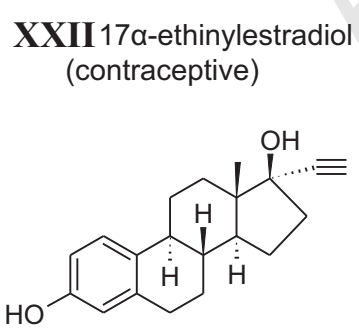
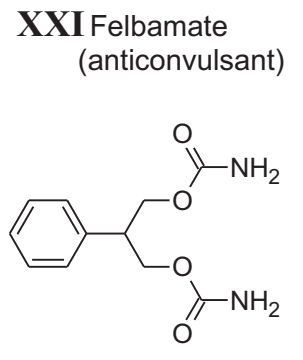
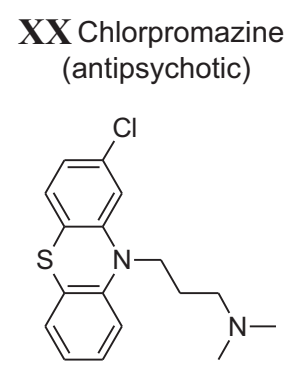
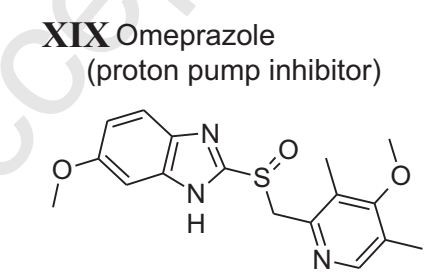
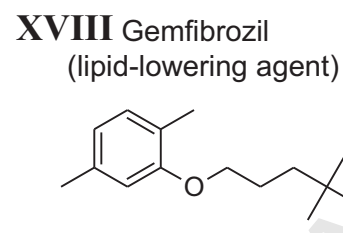
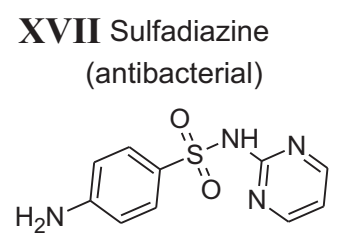
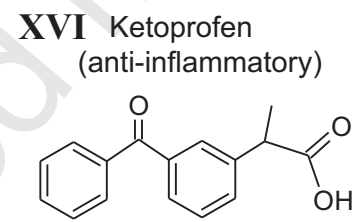
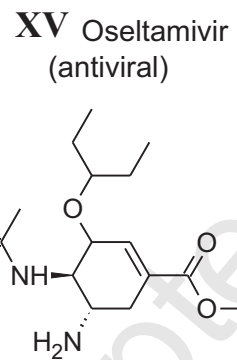
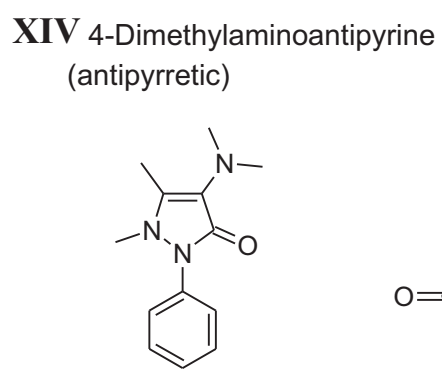
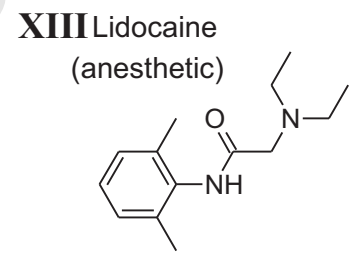
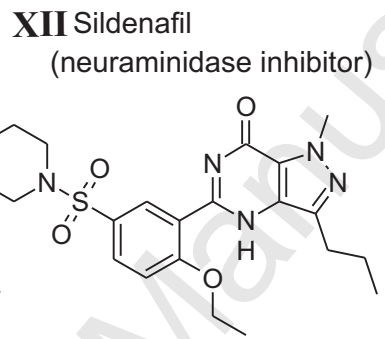
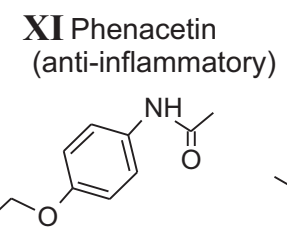
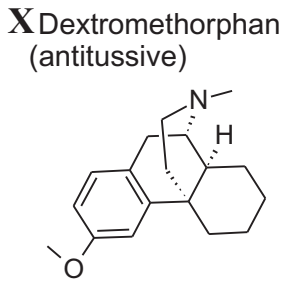
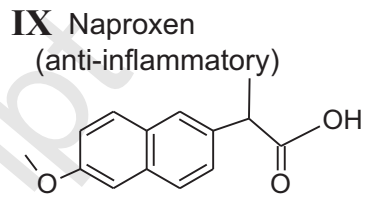
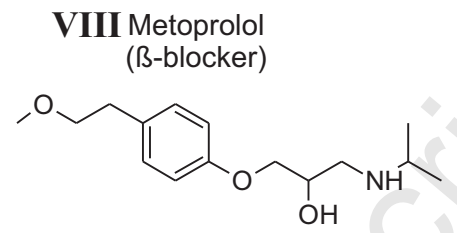
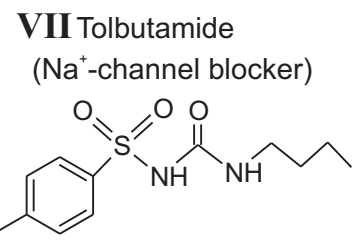
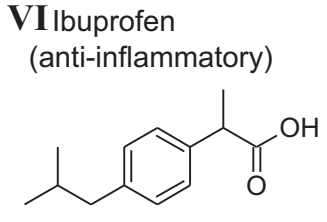
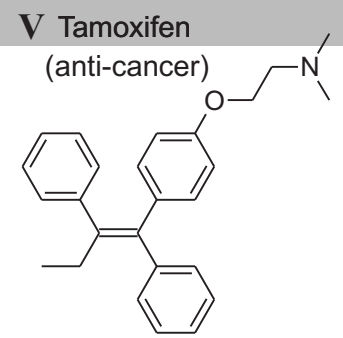
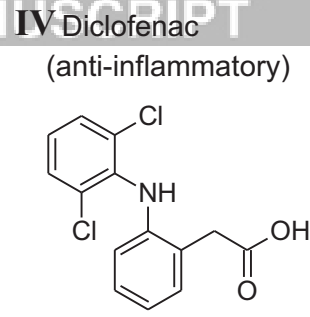
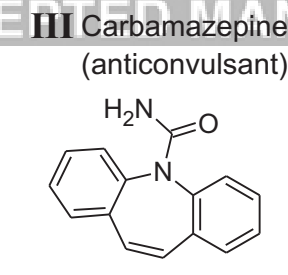
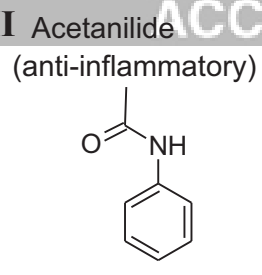
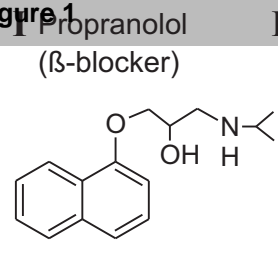
<sup>a</sup>Mass spectral data indicate the formation of corresponding carbonyls as described previously [19] (see Supplementary Material)

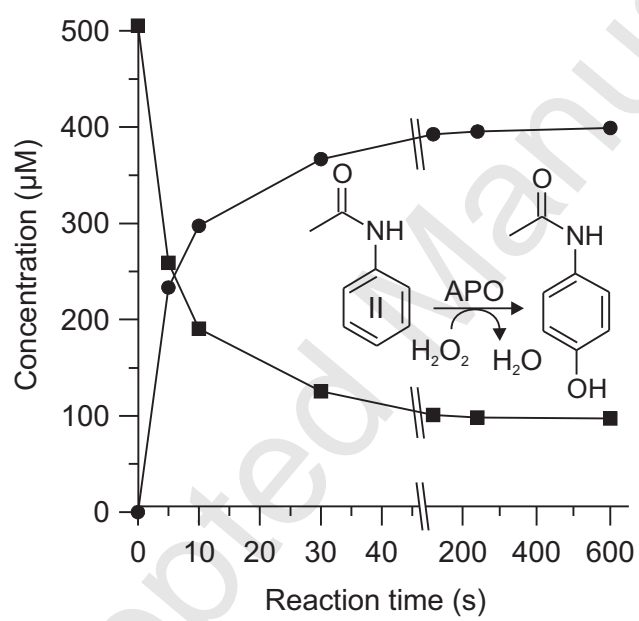
<sup>b</sup>A syringe pump was used for hydrogen peroxide supply  
(n.d.) = not determined due to poor resolution, (–) = not detected  
(n.a.) = not applicable

H <sub>2</sub> O <sub>2</sub> added	<i>N</i> -desmethylsildenafil produced	<i>Ratio</i> <i>N</i> -desmethylsildenafil/H <sub>2</sub> O <sub>2</sub>
	μM	
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

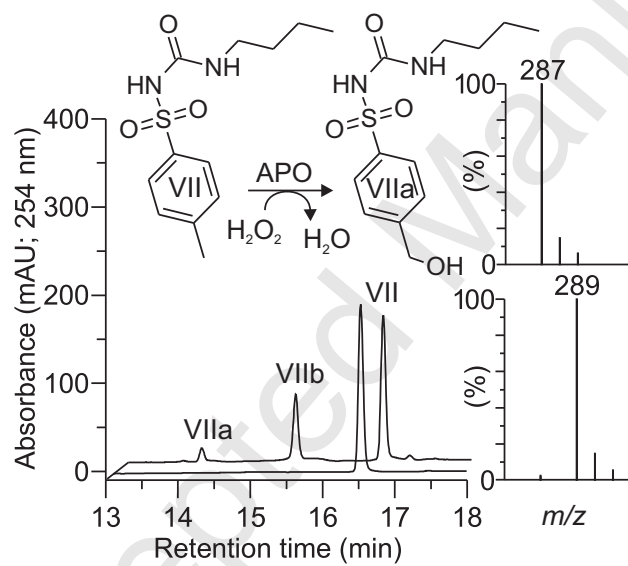
The initial sildenafil concentration was 250 μM.

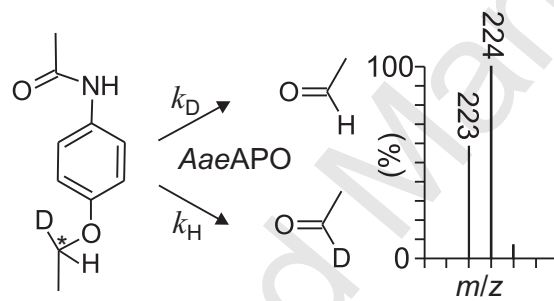
Accepted Manuscript

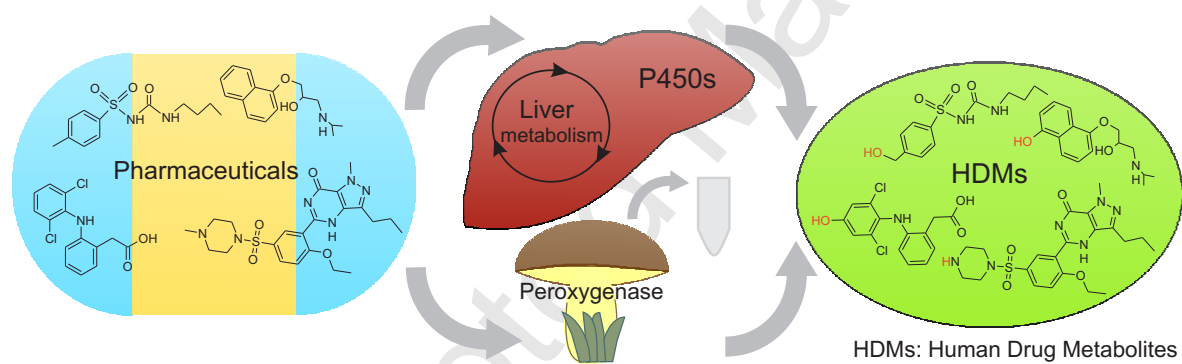












Supplementary Table 1. HPLC methods for reaction products investigated.

Product	Retention Time (min)	HPLC Column	Eluants	Conditions
4-OH-Tamoxifen	14.8	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
<i>N</i> -Desmethyltamoxifen	15.7	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
Endoxifen (E/Z) <sup>b</sup>	14.6,14.7	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
2-OH-Ibuprofen	14.5	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
1-OH-Ibuprofen	15.4	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
1-Oxo-Ibuprofen	16.3	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
5-OH-Propranolol	11.6	Luna C18;	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B) 15mM phosphoric acid pH 3 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
	12.1	Synergi 4μ Fusion		40°C, 1.00 ml min <sup>-1</sup> . 5% B for 5 min, followed by linear gradient to 100% B in 15 min.
4-OH-Propranolol	12.4	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
<i>N</i> -Desisopropylpropranolol	13.0	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
Oseltamivir carboxylate	11.1	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
<i>N</i> -Desmethylsildenafil	13.4	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
Acetaminophen	5.4	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.

	5.6	Synergi 4 $\mu$ Fusion	5mM phosphoric acid pH 3 (A)/acetonitrile (B)	40°C, 1.00 ml min <sup>-1</sup> . 5% B for 5 min, followed by linear gradient to 100% B in 15 min.
3-OH-Acetaminophen	3.5	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
	4.1	Synergi 4 $\mu$ Fusion	5mM phosphoric acid pH 3 (A)/acetonitrile (B)	40°C, 1.00 ml min <sup>-1</sup> . 5% B for 5 min, followed by linear gradient to 100% B in 15 min.
3-OH-Carbamazepine	14.2	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
4'-OH-Diclofenac	16.5	Luna C18;	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
	18.5	Synergi 4 $\mu$ Fusion	5mM phosphoric acid pH 3 (A)/acetonitrile (B)	40°C, 1.00 ml min <sup>-1</sup> . 5% B for 5 min, followed by linear gradient to 100% B in 15 min.
4-OH-Tolbutamide	14.3	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
<i>O</i> -Desmethylnetoprolol	12.4	Gemini- Nx 3 $\mu$ 110A C18	0.02% ammonium pH 10.5 (A)/acetonitrile (B)	45°C, 0.30 ml min <sup>-1</sup> . Isocratic at 5% B for 1 min, followed by linear gradient to 80% B in 20 min, and then to 100% B in 1 min.
$\alpha$ -OH-Metoprolol	12.2	Gemini- Nx 3 $\mu$ 110A C18	0.02% ammonium pH 10.5 (A)/acetonitrile (B)	45°C, 0.30 ml min <sup>-1</sup> . Isocratic at 5% B for 1 min., followed by linear gradient to 80% B in 20 min, and then to 100% B in 1 min.
<i>O</i> -Desmethylnaproxen	14.7	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
Dextrophan	11.5	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
Monoethylglycinexylidide	6.2	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
Glycinexylidide	4.6	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.
4-Aminoantipyrine	11.5	Luna C18	0.01% ammonium formate pH 3.5 (A)/acetonitrile (B)	40°C, 0.35 ml min <sup>-1</sup> . Isocratic at 5% B for 5 min, followed by linear gradient to 100% B in 20 min.

<sup>b</sup>stereoisomers