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Preparation of labeled human drug metabolites and drug-drug interaction-probes with fungal peroxygenases†

Marzena Poraj-Kobielska,[a*] Jens Atzrodt,[b] Wolfgang Holla,[b] Martin Sandvoss,[b] Glenn Gröbe,[c] Katrin Scheibner,[c] and Martin Hofrichter[a]

Enzymatic conversion of a drug can be an efficient alternative for the preparation of a complex metabolite compared with a multi-step chemical synthesis approach. Limitations exist for chemical methods for direct oxygen incorporation into organic molecules often suffering from low yields and unspecific oxidation and also for alternative whole-cell biotransformation processes, which require specific fermentation know-how. Stable oxygen-transferring biocatalysts such as unspecific peroxygenases (UPOs) could be an alternative for the synthesis of human drug metabolites and related stable isotope-labeled analogues. This work shows that UPOs can be used in combination with hydrogen/deuterium exchange for an efficient one-step process for the preparation of 4′-OH-diclofenac-d6. The scope of the reaction was investigated by screening of different peroxygenase subtypes for the transformation of selected deuterium-labeled substrates such as phenacetin-d3 or lidocaine-d3. Experiments with diclofenac-d7 revealed that the deuterium-labeling does not affect the kinetic parameters. By using the latter substrate and $H_2^{18}O_2$ as cosubstrate, it was possible to prepare a doubly isotope-labeled metabolite (4′-^{18}OH-diclofenac-d6). UPOs offer certain practical advantages compared with P450 enzyme systems in terms of stability and ease of handling. Given these advantages, future work will expand the existing 'monooxygenation toolbox' of different fungal peroxygenases that mimic P450 *in vitro* reactions.

Keywords: Isotopic labeled synthesis; deuterium; 4′OH-diclofenac; H/D exchange; paracetamol; phenacetin; lidocaine; unspecific/aromatic peroxygenase; EC 1.11.2.1; human drug metabolites

Introduction

Although xenobiotic metabolism should serve as biochemical detoxification process,[1] pharmacologically active metabolites or molecules or molecules with intrinsic chemical reactivity may also be generated in this process. These particular compounds may have the potential to cause serious adverse events.[2] As a consequence, the US Food and Drug Administration and International Conference on Harmonization guidance require identification and characterization[3] of metabolites as well as further safety assessment[4] for those metabolites formed at greater than 10% systemic exposure of parent drug at steady state.[5] For evaluation of the potential safety risks associated with drug metabolism, pharmacological testing, and safety studies and sometimes even detailed pharmacokinetic analysis and toxicological studies may be necessary.[6] In addition, reference standards of drug metabolites are needed as authentic samples for their structural confirmation and for liquid chromatography–mass spectrometry (LC/MS) method validation. If a relevant metabolite needs to be monitored in clinical studies, stable isotope labeling might be used to compensate the effect of ion-suppression, and hence, to facilitate quantification by bioanalytical LC-MS/MS assays.[7]

Quantitative LC-MS/MS methods are also applied for the investigation of the drug-drug-interaction (DDI) potential for new drug candidates.[8] Therefore, the potential of a new chemical entity to inhibit or to induce the formation of a specific metabolite from isoform-selective cytochrome P450 (CYP) probe substances is evaluated.[9] For validation of the CYP inhibition screening method, stable labeled versions of each CYP probe and its major metabolite are needed as internal standards.[10]

A more thorough evaluation for potential side effects and DDIs has driven an increased interest to prepare metabolites

[a]*Department of Biological and Environmental Sciences, TU Dresden - International Institute Zittau, Markt 23, 02763 Zittau, Germany*

[b]*Isotope Chemistry & Metabolite Synthesis, DSAR-DD, Sanofi-Aventis Deutschland GmbH, Industriepark Höchst G876, 65926 Frankfurt am Main, Germany*

[c]*Faculty of Science, Lausitz University of Applied Sciences, Großenhainer Straße 57, 01968 Senftenberg, Germany*

**Correspondence to: Marzena Poraj-Kobielska, Department of Biological and Environmental Sciences, TU Dresden - International Institute Zittau, Markt 23, 02763 Zittau, Germany.*
E-mail: poraj@ihi-zittau.de

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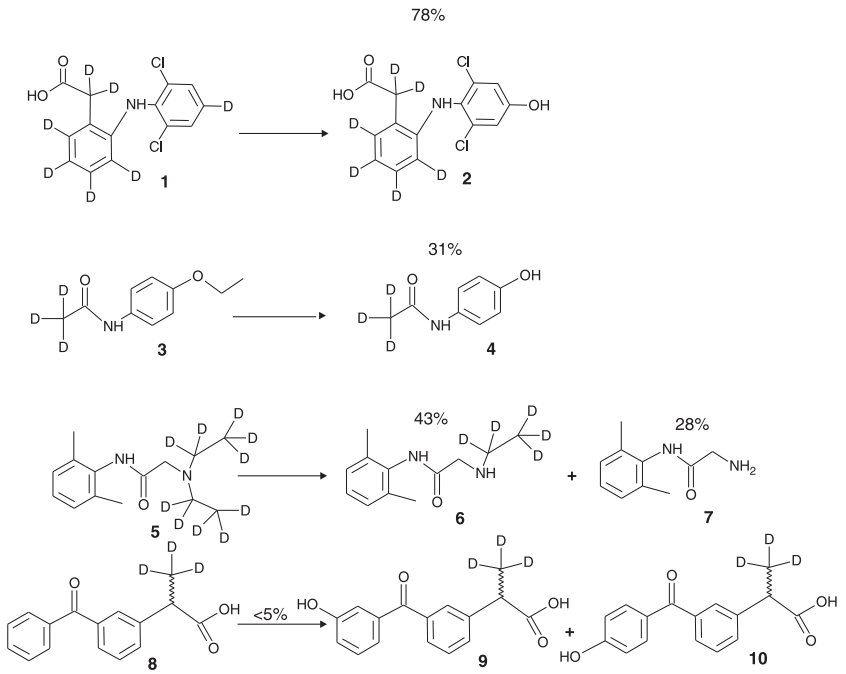
and their stable isotope-labeled analogues that support these studies. Internal standards of drug metabolites can be prepared by conventional synthesis starting from commercially available labeled precursors. However, classical approaches are highly dependent on the synthetic complexity of metabolite structure, which can be very time and resource intensive.[11] The direct single-step transformation of parent drugs can be an efficient, alternative approach for the preparation of difficult to synthesize metabolites. In addition to preparative scale, chemical oxidation[12] and biomimetic approaches,[13] whole-cell biotransformation processes[14] have been applied for the oxyfunctionalization of drug molecules. In combination with the recently developed hydrogen/deuterium (H/D)-exchange[15] protocols, oxidative biotransformation proved to be a suitable and inexpensive approach for the preparation of stable labeled internal standards of key probes for *in vitro* DDI investigations.[16]

To further improve the efficiency of metabolite preparation and stable labeled DDI probe synthesis, we studied here recently discovered unspecific peroxygenase enzymes (UPOs, EC 1.11.2.1)[17] from agaric basidiomycetes as biocatalysts for oxyfunctionalization reactions. The reactions catalyzed by UPOs are very similar to P450 transformations[18] and human drug metabolism.[19] However, in contrast to the membrane-bound and poorly stable human P450s, UPOs do not require cofactors like NAD(P)H or electron-transport proteins (flavin reductases and ferridoxines).[20] The enzymes can be isolated and stored for several weeks or even months without losing catalytic activity. Because of their extracellular nature and high stability over a large pH range and even in acetonitrile/water mixtures, UPOs can be handled without specific fermentation know-how or equipment, just like a standard organic reagent.[21] First examples suggest a broad scope as synthesis tools for metabolite preparation[19,20] and other synthetic applications[22,24d] as demonstrated by the selective conversion of widely used pharmaceuticals (e.g., propranolol and diclofenac) to their respective (human) metabolites. Regioselective hydroxylations and dealkylations were the preferred reactions catalyzed and in many cases, the products were identical to those of the human drug metabolism.[19]

Agrocybe aegerita unspecific peroxygenase (*Aae*UPO)[23] was the first enzyme of this type discovered in 2004. It has a very broad reaction spectrum that includes, for example, the epoxidiation/hydroxylation of mono and polyaromatics (e.g., benzene, toluene, naphthalene, and pyrene), sulfoxidations (e.g., of dibenzothiophene and thioanisole), *N*-oxidations (e.g., of pyridine derivatives), the *O*-dealkylation of diverse ethers (e.g., of tetrahydrofuran, diethyl ether, and alkyl aryl ethers), the *O*-demethylenation of benzodioxoles, *N*-dealkylations (e.g., of secondary and tertiary amines), aliphatic hydroxylations (e.g., of *n*-alkanes from C_3-C_{16}), and the epoxidation of diverse alkenes.[19–24] *Coprinellus radians* unspecific peroxygenase (*Cra*UPO) is a second peroxygenase of this type that was first described in 2007. *Cra*APO was found to regioselectively hydroxylate toluene and naphthalene, oxidize heterocyclic sulfur, and various PAHs as well as to hydroxylate and *O*-/*N*-dealkylate diverse pharmaceuticals.[19,24,25] *Marasmius rotula* unspecific peroxygenase (*Mro*UPO) is a third peroxygenase, which is found in the Pinwheel mushroom. *Mro*APO oxidizes all typical peroxygenase substrates and additionally bulky molecules such as steroids.[26,27]

In this communication, we present the application of fungal UPOs in combination with H/D exchange for the synthesis of stable labeled metabolites with particular focus on the preparation of deuterated 4′-hydroxydiclofenac **2**. In order to investigate the scope of the enzymatic transformation, four different deuterated substrates have been selected representing three different reaction pathways, namely aromatic hydroxylation via initial epoxidation (diclofenac-d7 **1** and ketoprofen-d3 **7**), *O*-dealkylation via hemiacetal formation (phenacetin-d3 **3**), and *N*-dealkylation via a hemiaminal intermediate (lidocaine-d3 **5**).[19,28] Time consuming,



Scheme 1. Deuterated compounds studied, expected products of human phase I metabolism and products obtained with UPOs. Percentages given represent the maximum theoretical product yields (**2**, **4**, **6**, and **7**) and in case of **8**, the total conversion.

low-yielding multi-step syntheses have been reported so far for the preparation of stable isotope-labeled diclofenac **1**,[29] phenacetin **3**,[30] lidocaine **5**,[31] and their respective main human metabolites 4′-hydroxydiclofenac **2**,[32] acetaminophen (paracetamol) **4**[33], and glycine-exylidine **7**.[34]

Results and discussion

For preparation of the deuterated-labeled substrates, different H/D exchange methods were recently developed and utilized in our group.[35] Deuteration of diclofenac **1** was achieved by the combination of acid-catalyzed and homogeneous metal-catalyzed H/D exchange reactions, resulting in a stepwise introduction of seven deuterium atoms into the molecule.[16] Considerable efficiency gains were noted in comparison to reported conventional syntheses.[32] Hydride activated H/D exchange under standard microwave conditions (160 °C for 2 h)[36] with Pd/C (10% on charcoal) was applied for labeling of phenacetin **3**. The deuterium incorporation occurred predominantly in the methyl group of the acetamide moiety accompanied by some minor exchange at the adjacent aromatic *ortho*-amide hydrogen. After crystallization, the deuterated phenacetin **3** was obtained in 68% yield with the [D_3]-isotopologue being the most abundant. Lidocaine **5** was labeled by Shvo catalyzed H/D exchange utilizing the 'borrowing hydrogen' methodology as recently reported.[37] However, because of a reduced reaction temperature (160 °C vs. 170 °C as used in the original procedure), the deuterium incorporation in both the α-position and β-position to the nitrogen atom was slightly lower.

Deuterated substrates **1**, **3**, **5**, and **8** (racemic ketoprofen-d3 **8** was purchased from a Sigma-Aldrich [Schnelldorf, Germany]) were screened with available UPOs for their ability to produce the expected human drug metabolites (Scheme 1). In addition to the three wild-type enzymes *Aae*UPO, *Cra*UPO, and *Mro*UPO, a recombinant enzyme from Novozymes A/S Copenhagen (*rNovo*)[38] was also tested. Detailed results are summarized in supplemental Table 1. Different catalytic activities were observed for the UPO subtypes leading to differences with respect to regioselectivity, product pattern and substrate spectrum. These findings were consistent with our previous studies.[19] *Aae*UPO was the most active enzyme for the transformation of **1** and after optimization of the reaction parameters, the desired **2** was obtained in up to 78% yield. The reaction may proceed via a previously described epoxidation mechanism leading to the

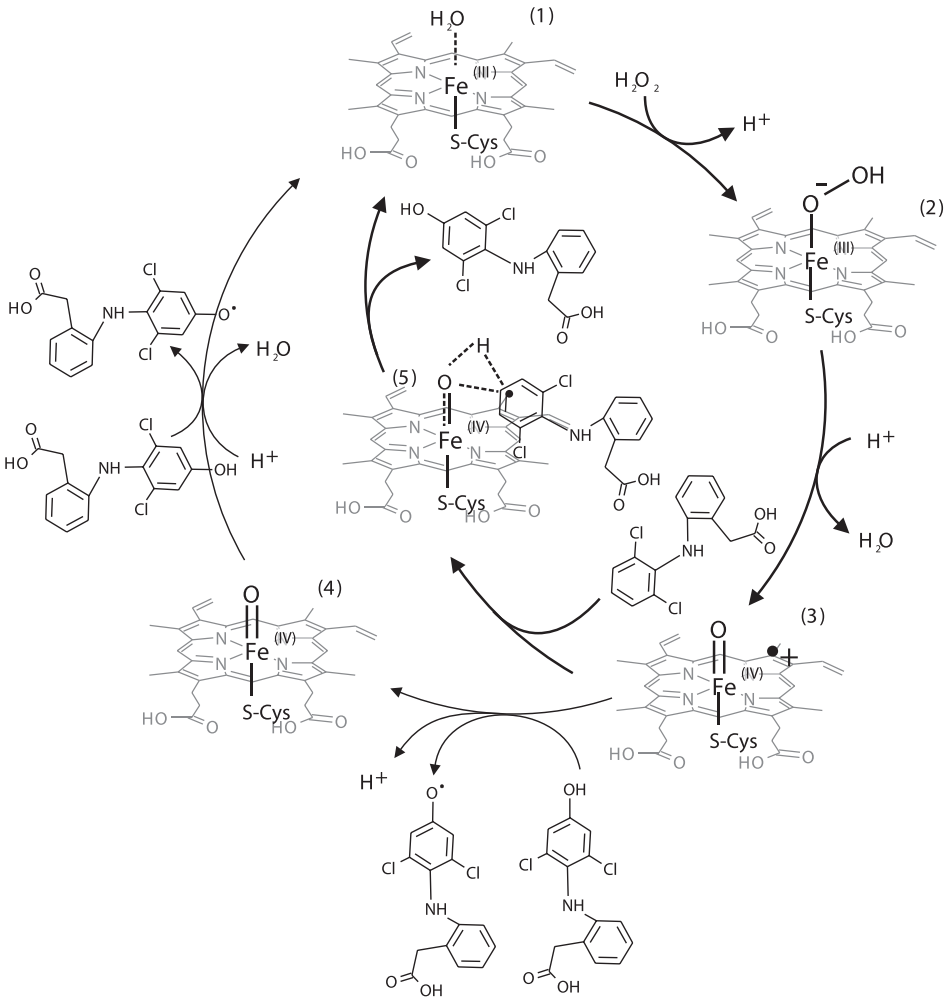


Figure 1. Catalytical cycle of unspecific peroxygenases illustrated by the hydroxylation of diclofenac by *Aae*UPO.[40] (1) native (hydro)ferric enzyme, (2) iron(III)-peroxide complex (Compound 0), and (3) Compound I.[41] The reaction of Compound I (3) with aromatic carbon of diclofenac leads to a hypothetic transitional state (5) that releases 4′-hydroxydiclofenac (very probably via an epoxide intermediate); this reaction is a two-electron oxidation. Compound I (3) can oxidize 4′-hydroxydiclofenac to a phenoxyl radical while it is reduced to Compound II (4) that in turn oxidizes a second 4′-hydroxydiclofenac molecule (two one-electron oxidations). To prevent the coupling of phenoxyl radicals formed, they can be re-reduced to 4′-hydroxydiclofenac by addition of ascorbic acid.[20,26]

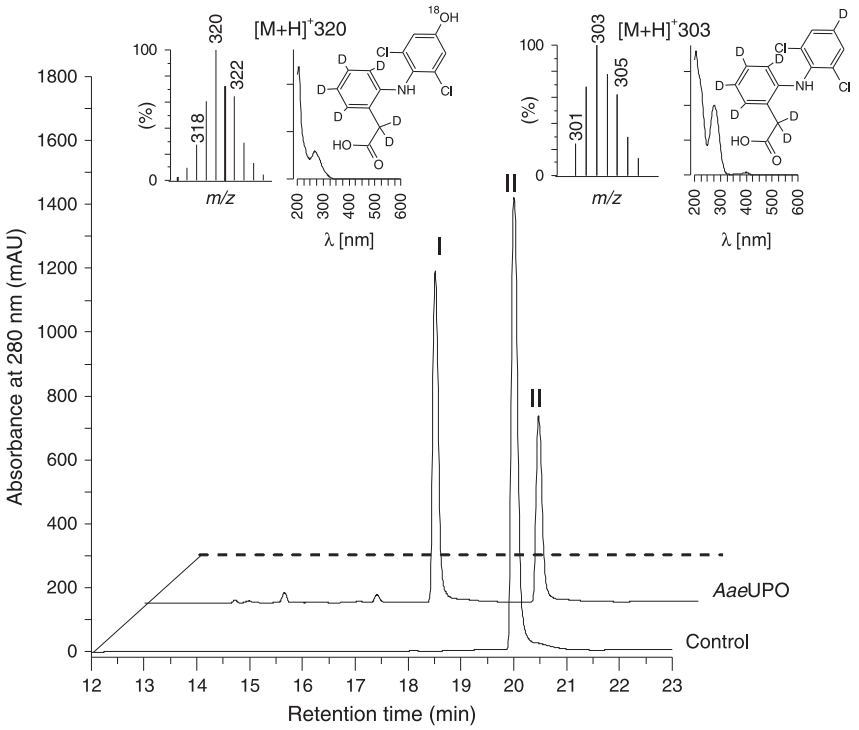


Figure 2. HPLC elution profile of diclofenac-d7 conversion by *Aae*UPO in the presence of $H_2^{18}O_2$; insets show the ultraviolet and mass spectra as well as the chemical structures. I–4′-^{18}OH-diclofenac-d6, II–diclofenac-d7.

preferred hydroxylation in *para*-position.[39] The reaction mechanism of UPOs that combines elements of classic heme peroxidases and P450-type monooxygenases is shown in the Figure 1 by the example of diclofenac oxidation.

The *O*-dealkylation of **3** catalyzed by *Aae*UPO[21,29,36] is proposed to proceed via an unstable hemiacetal intermediate (as shown earlier for diverse ethers) and yields the expected acetaminophen-d3 (paracetamol-d3) **4** in 31%. Alternatively, **4** can be also prepared, with even higher yields, by the UPO-catalyzed hydroxylation of acetanilde. The hydroxylation of the latter substance was reported to give up to 80% paracetamol under comparable reaction conditions.[19]

Best results for the single *N*-deethylation of **5** were achieved with a recombinant UPO from *rNovo* yielding 43% of monoethylglycinexylidide **6**, whereas the peroxygenase of *Cra*UPO gave the highest yield (28%) of completely *N*-deethylated lidocaine (glycinexylidide) **7**. In contrast to *N*-dealkylations catalyzed by human P450s that are supposed to proceed via attack of the nitrogen and aminium radical intermediates, or via direct C-H abstraction hydroxylation[42,43] adjacent to the nitrogen (or combination thereof), fungal UPOs apparently involve hemiaminal intermediates similar to *O*-dealkylations (deduced from the characteristic aldehyde products formed).

Ketoprofen **8** was not a good substrate for all UPOs (≤5% conversion), and the traces of two oxygenated metabolites (m/z + 16) could not be identified because of lack of standards. The keto group of **8** might be the reason for its limited conversion as it has been observed in other contexts that related compounds (acetophenone, acetone, and DMSO) are poor substrates or inhibitors of UPOs.[44]

Because our main focus was the development of an optimized process for the preparation of **2**, we further studied the *Aae*UPO-catalyzed hydroxylation of **1**. The kinetic experiments with diclofenac and diclofenac-d7 did not show significant differences with regard to the conversion rate. The kinetic parameters for diclofenac and respectively diclofenac-d7 were as follows: K_m-690 and 652 μM, k_{cat}-651 and 652 s^{-1} as well as $k_{cat}/K_m = 9.42 \times 10^5\ M^{-1}\ s^{-1}$ and $k_{cat}/K_m = 9.62 \times 10^5\ M^{-1}\ s^{-1}$.

Figure 2 shows the HPLC elution profile of diclofenac-d7 conversion by *Aae*UPO. Simply replacing normal hydrogen peroxide ($H_2^{16}O_2$) by ^{18}O-labeled hydrogen peroxide ($H_2^{18}O_2$) provided a simple route to obtain a doubly isotope-labeled metabolite (4′-^{18}OH-diclofenac-d6). Such an approach could be of interest for mechanistic investigations.

For preparative applications, an upscaling of the *Aae*UPO-catalyzed transformation of **1** was necessary. The reaction conditions were analogously to screening conditions after upscaling from 0.2 to 200 ml, except for a slightly longer reaction time (15 min) and hydrogen peroxide addition (final concentration of 6 mM, 4 steps 1.5 mM/min^{-1}). Under this optimized conditions (pH 7, KPP 50 mM, 6 mM H_2O_2, 4 mM ascorbic acid, and reaction time of 15 min), 100 mg of **1** were incubated with *Aae*UPO affording after prep HPLC purification 30 mg of 4′-OH-diclofenac-d6 **2** (i.e., 30% real yield).

Conclusion

We have developed a practical and efficient one-step process for the preparation of 4′-OH-diclofenac-d6 **2** through applying the recently discovered and produced UPO enzymes in combination with H/D exchange. Compared with the existing whole-cell biotransformations, isolated UPO enzymes allow for a much easier handling without the need for specific fermentation know-how and equipment. Further screening of different reaction types (aromatic and alkyl hydroxylation, *O*-dealkylation, and *N*-dealkylation) revealed the general applicability of fungal peroxygenases as promising biocatalysts to prepare labeled human drug metabolites. Compared with the chemical

syntheses, the selectivity of peroxygenases can reduce the number of synthesis and purification steps, which results in a shorter preparation time and lower costs. New wild-type peroxygenases have been found in several basidiomycetes and ascomycetes, for example, in *Coprinopsis verticillata*, *Agrocybe parasitica*, *Auricularia auricula-judae*, *Morchella elata*, and *Giberella* sp.[45] Furthermore, first attempts to produce recombinant peroxygenases in suitable hosts (e.g., *Aspergillus* spp. and *Saccharomyces cerevisiae*) have been successful.[46] These recent developments raise the possibility to extend the peroxygenase-based 'monooxygenation toolbox' for the production of a broad spectrum of labeled human drug metabolites at a scale that is required in pharmacological research.

Experimental

Compounds used for enzymatic conversion

General

The $H_2^{18}O_2$ (90 atom % and 2%wt/vol) was purchased from Icon Isotopes (New York, USA). Diclofenac and DMSO-d6 (99.5% D) were purchased from ABCR (Karlsruhe, Germany). All other chemicals were purchased from Sigma-Aldrich.

The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DRX 500 (500 MHz) or on a Bruker Avance 300 (300 MHz) nuclear magnetic resonance spectrometer. ^{1}H-NMR data were checked against an authentic sample of the corresponding unlabeled compound. Mass spectra were recorded on a Bruker Esquire 3000 ion-trap mass spectrometer. Final HPLC controls were performed on either Waters Alliance 2695 or Agilent 1200 Series HPLC systems each equipped with diode array detector (DAD) or variable wavelength ultraviolet detectors, respectively.

Synthesis of deuterated substrates

Diclofenac-d7 (**1**) was prepared as described previously.[16] Under argon, 7 ml MeOD and 3.3 ml NaOD (2 M in D_2O) were added to 0.9 g (3.16 mmol) dichlofenac, the Biotage microwave vial sealed, and the mixture irradiated in the microwave at 130 °C for 10 s. MeOD was removed and the remaining suspension taken up in 50 ml water. The pH was adjusted to 3–4 by dropwise addition of 2 M HCl and the precipitated solid isolated by filtration. The crude product was recrystallised from *tert*-butylmethyl ether/heptane (1:1, v/v). Yield: 0.62 g (2.04 mmol, 65 %) colorless solid. A final HPLC control resulted in a purity of 99.7 % by area. Conditions: column: Waters SunFire C18, 150 × 3 mm, 3.5 μm. Eluent A: 0.1 % formic acid (aq, v/v). Eluent B: 0.1 % formic acid in acetonitrile (v/v). Flow rate: 0.5 ml/min. UV detection wavelength: 280 nm. Injection volume: 10 μl. Sample solvent/concentration: 0.5 mg/ml in acetonitrile. Column temperature: 30 °C. Autosampler temperature: 8 °C. Gradient profile: 0 min/30% eluent B, 10 min/90% eluent B, 14 min/90% eluent B, 15 min/30% eluent B, and 20 min/30% eluent B. Retention time: 9.8 min. ^{1}H-NMR (500 MHz, DMSO-d_6) = 12.67 (s, 1 H), 7.52 (s, 2 H), 7.27 (s, 1NH), 7.20 (s, 0.1 H), 7.18 (m 0.1 H), 7.05 (s, 0.1 H) ppm, 6.85 (s, 0.1 H), 6.28 (s, 0.04 H), and 3.68 (s, 0.29 H_2). MS *m/z* (ESI^+) $(M+H)^+ = 303$. *m/z* (ESI^-) $(M-H)^- = 301$.

Phenacetin-d3 (**3**) was prepared as described previously.[36] A pressure tube filled with argon was charged with 180 mg (1 mmol of phenacetin), 10 weight-% catalyst, 5 mol-% $NaBD_4$ (98 % D), and 6 mL D_2O (99 % D). The mixture was stirred for approximately 30 sec, and the tube was sealed (note: the reaction vessel was not closed until effervescence had stopped) and heated to 150 °C for 2 h in a microwave. The mixture was cooled at room temperature and 3 mL acetonitrile were added. The catalyst was separated by filtration and the solvent evaporated. The residue was purified by column chromatography with ethyl acetate/heptane. Yield: 122 mg (0.67 mmol, 68%) colorless crystals with a purity of 99.9% by area (HPLC control). Conditions: column: Supelco Ascentis Express C18, 150 × 4.6 mm, 2.7 μm. Eluent A: water/acetonitrile/TFA 900:100:1 (v/v/v). Eluent B: water/acetonitrile/TFA 100:900:0.75. Flow rate: 1.0 ml/min. UV detection wavelength: 254 nm. Injection volume: 5 μl. Sample solvent/concentration: 0.5 mg/ml in 50% acetonitrile. Column temperature: 20 °C. Autosampler temperature: 5 °C. Gradient profile: 0 min/5% eluent B, 20 min/30% eluent B, 22 min/95% eluent B, 26 min/95% eluent B, 27 min/5% eluent B, and 30 min/5% eluent B. Retention time: 11.0 min. ^{1}H-NMR (300 MHz, DMSO-d6) δ 9.73 (s, 1NH), 7.44 (m, 0.62H_2), 6.83 (m, 2H), 3.96 (q, 2H $^2J = 14.0$ Hz and $^3J = 6.9$ Hz), 1.99 (s, 0.11H_3), and 1.30 (t, 3H $^3J = 6.9$ Hz). MS *m/z* (ESI^+) $(M+H)^+ = 183$.

Lidocaine-d3 (**5**) was prepared as described previously.[37] A microwave vial was equipped with a magnetic stirring bar and 27.1 mg of Shvo's catalyst (0.025 mmol). After evacuating and purging the reaction vessel with argon for five times, 58.6 mg (0.25 mmol) lidocaine, 1911 μL *i*-PrOH-d_8, and 500 μL toluene were added under an argon atmosphere. The vial was fitted with a Teflon cap and heated in a microwave (CEM Corporation: Discover SP) for 2 h at 160 °C. After cooling the reaction mixture at room temperature, the organic layer was separated, and the water phase was extracted three times with small portions (ca. 1 mL) of MTBE. The solvent of the combined organic layers was then removed *in vacuo*, and the crude product was purified by column chromatography. Yield: 49 mg (0.21 mmol, 84%);Slightly lower deuterium incorporations observed here resulted from a reduction of the reaction temperature from 170 °C as reported to 160 °C. ^{1}H-NMR (300 MHz, DMSO-d6) δ 9.16 (s, 1NH), 7.09 (s, 3H), 3,12 (s, 0.84H_2), 2.61 (q, 0.75 H_2, $^2J = 14.2$ Hz and $^3J = 7.1$ Hz), 2.13 (s, 6H), 1.07 (t, 0.85H_6, $^3J = 7.1$ Hz). ^{13}C-NMR (75 MHz, DMSO-d6) δ 169.5 (CO), 135.2 (C), 135.1 (C), 127.6 (CH), 126.3 (CH), 56.82 (CH2), 48.0 (CH2)18.2 (CH3), and 12.2 (CH3). MS *m/z* (ESI^+) $(M+H)^+ = 237$.

Ketoprofen-d3 (**8**), 2-(3-benzoylphenyl)-propanoic acid[$^2H_3$], and racemic mixture was purchased from Sigma-Aldrich (Schnelldorf, Germany); the $[M+H]^+$ was 258 nm, and λ_{max} was 205 and 255 nm.

Enzymatic conversions

Enzymes

*Aae*UPO, *Cra*UPO, and *Mro*UPO were produced and purified as described previously.[27,42,47] The specific activities were 97 U/mg^{-1}, 29 U/mg^{-1}, and 25 U/mg^{-1}, respectively; 1 U represents the oxidation of 1 μmol from 3,4-dimethoxybenzyl alcohol to 3,4-dimethoxybenzaldehyde in 1 min at 23 °C at pH 7.[23] *rNovo* was kindly provided by Novozymes A/S (Copenhagen, Denmark) with a specific activity of 15 U/mg^{-1}.

General procedure for enzymatic screening without continuous addition of H_2O_2 (transformations of diclofenac-d3 (1) and phenacetin-d3 (3)

The reaction mixtures (0.2 ml) contained purified peroxygenase (2.0 U/ml^{-1} = 0.25 μM), substrate (diclofenac-d7 or phenacetin-d3, 0.5 mM), potassium phosphate buffer (50 mM, pH 7.0), and ascorbic acid (4.0 mM, to prevent further oxidation of phenolic products that were released.[18,20] The reactions were started by the addition of limiting H_2O_2 (2.0 mM) and stirred at room temperature. The resulting products were quantified by HPLC as described previously using external standard curves prepared with authentic standards. 4′-OH-Diclofenac-d6, 2-[(2,6-dichloro-4′-hydroxyphenyl) amino]-benzene [$^2H_6$] acetic acid–LC/MS m/z 318 $[M+H]^+$318, UV λ_{max} 204, 278 nm and paracetamol-d3, acetaminophen-*N*-(4-hydroxyphenyl)acetamide[$^2H_3$] – LCMS m/z 155 $[M+H]^+$, and UV λ_{max} 200, 244 nm.

General procedure for enzymatic screening with continuous addition of H_2O_2 (transformation of lidocaine-d3 (6) and ketoprofen-d3 (8)

The reaction mixtures (0.2 ml) contained purified peroxygenase (2.0 U/ml^{-1} = 0.25 μM), substrate (lidocaine-d3 or ketoprofen-d3, 0.5 mM), potassium phosphate buffer (50 mM, pH 7.0), and ascorbic acid (4.0 mM). The reactions were prepared by continuously adding H_2O_2 (2.0 mM) with a syringe pump, stirred at room temperature and, and stopped after 2 h, when chromatographic analyses showed that product formation was complete or no conversion was observed, respectively. The resulting

products were quantified by HPLC as described in the succeeding text using external standard curves prepared with authentic standards.

Analytical methods

Liquid chromatography–mass spectrometry (LC/MS) analyses were performed using an Agilent Series 1200 instrument (Waldbronn, Germany) equipped with a diode array detector and mass spectrometry detection electrospray ionization mass spectrometer. Reversed phase chromatography was performed on a Kinetex XB-C18 column (4.6 diameter by 150 mm length, 5 μm particle size [Phenomenex, Aschaffenburg, Germany]), which was eluted at 1 ml/min^{-1} and 40 °C with aqueous 0.01% vol/vol ammonium formate (pH 3.6, and by experiments with Lidocaine pH 9.3)/acetonitrile, 95:5 for 5 min, followed by a 17-min linear gradient to 100% acetonitrile. Mass spectrometric determinations were made in the positive electrospray ionization mode in a mass range from 70 to 500, at a step size of 0.1, a drying gas temperature of 350 °C, a capillary voltage of 4000 V for the positive mode, and 5500 V for the negative mode. Products were identified relative to authentic standards (except ketoprofen-d3 products), based on their retention times, UV absorption spectra, and $[M+H]^+$ ions.

Enzyme kinetics

Kinetic measurements with diclofenac and diclofenac-d7 were performed under identical conditions. Hydroxylation in 4′-position was analyzed in stirred reactions (0.2 ml, 23 C) that contained 0.25 μM of *Aae*UPO, potassium phosphate buffer (50 mM, pH 7), 4 mM of ascorbic acid, and 0.010–2.000 mM of the substrate. The reactions were initiated with 2 mM H_2O_2 and stopped with 10% of 1.3 mM sodium azide solution after 5 s. The resulting amounts of 4′-OH-diclofenac or 4′-OH-diclofenac-d6 were quantified by LC/MS method as described previously. The apparent K_m values for diclofenac and diclofenac-d7 were obtained by nonlinear regression using the Michaelis-Menten model in the ANEMONA program.[48]

^{18}O-Labeling experiment

The reaction mixture (0.20 ml, stirred at room temperature) contained 2 U/ml^{-1} (0.25 μM) of *Aae*UPO, potassium phosphate buffer (50 mM, pH 7.0), 4 mM ascorbic acid, and 0.5 mM diclofenac-d7. The reaction was initiated by a single addition of 2.0 mM $H_2^{18}O_2$ (final concentration). A portion of the completed reaction was then analyzed by LC/MS method as described previously. For *m/z* value determination, the average total ion count within the metabolite (4′-OH-diclofenac-d6) peak was used after background correction to generate the ion count used for mass abundance calculations.

Semi-preparative preparation of 4′-OH-diclofenac-d6 (2)

Reaction mixtures (200 ml) contained *Aae*UPO (2 U/ml^{-1}), potassium phosphate buffer (50 mM, pH 7), 4 mM ascorbic acid, and diclofenac-d7 **1** (100 mg). The reaction was initiated with addition of H_2O_2 (final concentration of 6 mM, 4 steps 1.5 mM/min^{-1}) and run over 15 min. In the course of the reaction, small samples (20 μl) were collected and analyzed by LC/MS method as described previously. After about 50% conversion, 4′-OH-diclofenac was separated: the reaction mixtures were twice extracted with ethyl acetate/methanol (9:1, v/v). The pH of the remaining aqueous phase was adjusted to 3 with 1 N hydrochloric acid and extracted three times with ethyl acetate. The combined organic phases were dried over Na_2SO_4 and evaporated under vacuum with a rotating evaporator. The solid was then subjected to semi-preparative chromatography under following conditions:

Phenomenex Luna C18 column (150 × 21.2 mm, 5 μm), eluent A-0.1% formic acid (aq, v/v)/acetonitrile 90:10 (v/v), eluent B-0.1% formic acid (aq, v/v)/acetonitrile 10:90 (v/v), flow rate-7.0 ml/min, UV detection at 280 and 254 nm, injection volume-1 ml, sample/solvent concentration-8 mg/ml in water/acetonitrile (1:2, v/v), column temperature-25 °C, and gradient profile-0 min/20% eluent B, 1 min/20% eluent B, 20 min/90% eluent B, 24 min/90% eluent B, and 25 min/20% eluent B.

The acetonitrile was evaporated from the combined fractions and after addition of water, the solution was submitted to lyophilisation to yield 30 mg and 25 mg of a white solid with a purity of 99.4% and respectively 99.7% by area. Following conditions were used:

Phenomenex Kinetex C18 column (100 Å, 150 × 4.6 mm, 2.6 μm), eluent A-0.1% formic acid (aq, v/v)/acetonitrile 90:10 (v/v), eluent B-0.1% formic acid (aq, v/v)/acetonitrile 10:90 (v/v), flow rate-1.0 ml/min, UV detection at 280 nm, injection volume-10 μl, sample/solvent concentration-0.5 mg/ml in acetonitrile, column temperature-30 °C, sampler temperature-8 °C, and Gradient profile-0 min/20% eluent B, 20 min/90% eluent B, 24 min/90% eluent B, 25 min/20% eluent B, 30 min/20% eluent B, and retention time-8.9 min. ^{1}H-NMR (300 MHz, DMSO-d6) δ 12.5 (br s, OH), 10.2 (br s, OH), 7.12 (s ~0.08H), 6.99 (s ~0.07H), 6.94 (s, 2H,), 6.87 (br s, NH), 6.73 (s ~0.11H), 6.11 (s, 0.03H), and 3.63 (s, 0.27H_2); ^{13}C-NMR (125 MHz, DMSO-d6) δ 173.2 (CO), 155.4 (COH),144.1 (CNH), 133.1 (CCl), 130.3 (m, CD), 128.1 (CNH), 127.1 (m, CD), 121.6 (C), 118.5 (m, CD), 115.8 (CH), 113.1 (m, CD), and 37.1 (m, CD_2). NMR purity ca. 95%: minor CH signal at 7.44 or 129.0 ppm coupling to a CD at 7.04 ppm observed. MS *m/z* (ESI$^+$) $(M+H)^-$ = 318 and $(M+Na)^+$ = 340; *m/z* (ESI$^-$) $(M-H)^-$ = 316.

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Conflicts of Interest

The authors did not report any conflict of interest.

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