Synthesis of oxyfunctionalized NSAID metabolites by microbial biocatalysts

Jan M. Klenk [a], Lisa Kontny [a]*, Bernd A. Nebel [a], Bernhard Hauer [a]#

[a]Institute of Biochemistry and Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany

Running Head: NSAID oxyfunctionalization by microbial biocatalysts

#Address correspondence to Bernhard Hauer, bernhard.hauer@itb.uni-stuttgart.de; phone: +49 711 685 63193; fax: +49 711 685 63196.

*Present address: Lisa Kontny, Marabu GmbH, Tamm, Germany.

Abbreviations: GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; NSAID, non-steroidal anti-inflammatory drug; PEG, potato-extract-glucose; P450, cytochrome P450 monooxygenase; P450 BM3, cytochrome P450 from *Bacillus megaterium*; P450 RhF, cytochrome P450 from *Rhodococcus* sp. strain NCIMB 9784.

Abstract

The synthesis of valuable metabolites and degradation intermediates of drugs, like non-steroidal anti-inflammatory drugs (NSAIDs), are substantially for toxicological and environmental studies, but efficient synthesis strategies and the metabolite availability are still challenging aspects. To overcome these bottlenecks filamentous fungi as microbial biocatalysts were applied. Different NSAIDs like diclofenac, ibuprofen, naproxen and mefenamic acid could be oxyfunctionalized to produce human metabolites in isolated yields of up to 99% using 1 g L^{-1} of substrate. Thereby the biotransformations using *Beauveria bassiana*, *Clitocybe nebularis* or *Mucor hiemalis* surpass previous reported chemical, microbial and P450-based routes in terms of efficiency. In addition to different hydroxylated compounds of diclofenac, a novel metabolite, 3',4'-dihydroxydiclofenac, has been catalyzed by *B. bassiana* and the responsible P450s were identified by proteome analysis. The applied filamentous fungi present an interesting alternative, microbial biocatalysts platform for the production of valuable oxyfunctionalized drug metabolites.

Importance

The occurrence of pharmaceutically active compounds, such as diclofenac and its metabolites, in the environment, in particular in aquatic systems, is of increasing concern because of the increased application of drugs. Standards of putative metabolites are therefore necessary for environmental studies. Moreover, pharmaceutical research and development requires assessment of the bioavailability, toxicity and metabolic fate of potential new drugs to ensure its safety for users and the environment. Since most of the reactions in the early pharmacokinetics of drugs are oxyfunctionalizations catalysed by

P450s, oxyfunctionalized metabolites are of major interest. However, to assess these metabolites chemical synthesis often suffer from multistep reactions, toxic substances, polluting conditions and achieve only low regioselectivity. Biocatalysis can contribute to this by using microbial cell factories. The significance of our research is to complement or even exceed synthetic methods for the production of oxyfunctionalized drug metabolites.

Keywords: Biotransformation; Filamentous fungi; Microbial biocatalysts; NSAIDs; Oxyfunctionalized metabolites

Introduction

Next to chemical processes, biotechnology routes can play a significant role for the access to pharmaceutically active substances and their metabolites. Metabolites and degradation intermediates can have major environmental implications, which can lead to unwanted side reactions in nature and mammalians. Due to the fact that numerous drug metabolites are detected in higher amounts in different habitats, thus detailed environmental and toxicological studies are necessary. These findings induced that the FDA (Food and Drug Administration) issued guidelines for metabolites in drug testing, setting a limit of 10% in 2008.

Approximately 80% of all reactions in the early pharmacokinetics of drugs (phase I reactions) are P450-catalyzed (1, 2). Therefore, oxyfunctionalized metabolites are often major degradation intermediates of potential toxic endogenous and exogenous compounds in mammalians and nature. For further approval of new drugs, knowledge about potential metabolism intermediates and their availability for toxicological studies are obligatory (3–

5). As a very present example, a molecule like diclofenac (**1**), a widespread non-steroidal anti-inflammatory drug (NSAID), and its already known hydroxylated degradation metabolites 4'- and 5-hydroxydiclofenac (**2** and **3**) pose an environmental problem due to their low degradability (6, 7). Thereby, the request for particular drug metabolites increases simultaneously with the number of newly developed drugs, to study pharmacokinetic and toxic effects caused by metabolism (8, 9). Due to limited availability and often time as well as cost elaborating chemical synthesis routes, the value of drug metabolites can reach up to several hundred dollars per milligram. As mentioned above, the chemical synthesis of human metabolites such as 4'- and 5-hydroxydiclofenac (**2** and **3**) for toxicological studies is tedious and has only been achieved in low yields linked with high by-product formation (10–12). To complement synthetic methods, microbial processes with heterologous expressed P450s and wild-type organisms have emerged as an valuable alternative to chemical syntheses (13, 14).

Most of the previous studies focused on heterologous expressed human P450s which can produce the corresponding metabolites detectable in the human body (15). One example is CYP2C9 that was recombinant produced in different hosts and finally used for the production of 468 mg L^{-1} of 4'-hydroxydiclofenac (**2**) (14, 16). Furthermore, the low activities and selectivities of the wild type P450 BM3 were improved by directed evolution resulting in variants which led to a significantly enhanced oxidation capability of NSAIDs (17–20). In this context we recently published the scale-up of the self-sufficient P450 RhF (21). The enzyme was heterologous expressed and implemented as whole cell system, whereby 5-hydroxydiclofenac (**3**) was produced exclusively in high titers of up to 357 mg L^{-1}. However, high yields with NSAIDs are rather the exception, because often the

product yields obtained with P450s or microorganisms are in the lower milligram range (17, 22–26). Therefore, the biotechnological synthesis of such oxyfunctionalized compounds for environmental and toxicological studies remains expandable.

Based on our previous hydroxylation experiment studies with diclofenac (**1**) as substrate, we further extended the biocatalyst platform for NSAID oxyfunctionalizations by a spectrum of available biocatalysts. In this work, we intended to identify new microbial biocatalysts able to catalyze NSAID metabolites and degradation intermediates in high yields, to provide those molecules for further drug development and toxicological studies.

Materials and Methods

Materials

The chemicals and media used in this study were obtained from Fluka (Buchs, Switzerland), Sigma-Aldrich (St. Louis, Missouri, USA) Alfa-Aesar (Ward Hill, Massachusetts, USA) and Carl-Roth (Karlsruhe, Germany) in highest available purity degrees. The KOD HS polymerase was from Novagene Inc. (Madison, Wisconsin, USA).

Isolation of genomic DNA from eukaryotes

For the isolation of genomic DNA from eukaryotes, precultures of the various filamentous fungi were cultured on PEG broth (potato-extract-glucose broth) as medium for about 3 days at 27°C and 180 rpm. An initial cell lysis was achieved by centrifugation of the cultures, freezing at -80°C for 10 min and crushing the frozen mycelia with a mortar. Subsequently, the samples were isolated according to the manufacturer's instructions using

the ZR Fungal/Bacterial DNA Microprep ™ kit (Zymo Research Corp., Irvine, California, USA). The elution of the DNA was carried out with 25 µL ddH_2O.

Polymerase chain reaction (PCR) for the amplification of DNA fragments

The PCR was used to selectively amplify DNA fragments of genomic DNA based on the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') or LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-TCCTGAGGGAAACTTCG-3') as described by Schoch and coworkers in 2012 (27). For the elongation KOD HS Polymerase was used in 50 µL total volume using the components and programs as shown in TABLE S1 and S2.

The success of the PCR reactions was controlled by an agarose gel and the DNA fragments were then directly isolated (Figure S1). Therefore, the DNA fragments were visualized under UV light (366 nm) and cut out of the agarose gel with a scalpel, weighed and treated with the Zymoclean Gel DNA Recovery Kit (Zymo Research Corp., Irvine, California, USA) according to the manufacturer's instructions. The isolated DNA was subsequently eluted with 20 µL ddH2O. For the analysis of the DNA samples, a volume of 20 µL and a concentration of 50 - 70 ng μL^{-1} were used. The sequencing was done by GATC Biotech AG (Konstanz, Germany) using the primers of the PCR amplification.

Proteome analysis of Beauveria bassiana

For the cell disruption of *Beauveria bassiana*, liquid cultures were centrifuged and the mycelia subsequently washed with ddH_20 over a filter paper and dried. In a Petri dish, the

132 samples were lyophilized (Alpha 2-4 LD plus, Martin Christ Gefriertrocknungsanlagen
133 GmbH, Osterode am Harz, Germany), to rub this later as a first partial disruption with the
134 mortar. 35-50 mg of powder was mixed with an equivalent volume of glass beads (0.1-
135 0.25 mm) and 1 mL urea buffer (28) (25 mM Tris/HCl pH 6.8, 9 M urea, 1% SDS, 1 mM
136 EDTA, direct before use 0.7 M DTT). Subsequently, the cell disruption samples were
137 heated to 95°C for 2 min, shaken for 1 min by vortexing and heated again at 95°C for
138 1 min. After separation of the glass beads by short centrifugation, the DNA was sheared
139 by sonication (Branson Sonifier 250 equipped with a microtip: 1/8 "diameter, Danbury,
140 Connecticut, USA, pulse: output 2, duty cycle: 35%) for 30 s.
141 A 3-day PEG broth preculture was used to inoculate 100 mL PEG broth with 1/75 volume
142 of preculture. The biotransformations were started after 24 h incubation with the addition
143 of 0.5 g L^{-1} of the substrates or glucose as a reference. The reactions were stopped after
144 first products were detected (48 h for (*R*)-2-phenoxypropionic acid and 68 h for diclofenac
145 (**1**)). The cells were then lysed as described above and the proteins of the samples analyzed
146 and diluted as a whole cell suspension on a 10% and a 15% SDS-polyacrylamide gel.
147 The proteome analysis was carried out at the mass spectrometry service unit in Hohenheim
148 (Germany, group of Dr. Pfannstiel) using an ACQUITY nano-UPLC system (Waters
149 GmbH, Milford, USA) directly coupled to a LTQ-Orbitrap XL hybrid mass spectrometer
150 (Thermo Fisher Scientific, Bremen, Germany) with following minor changes as described
151 previously (29). Tryptic digests were separated on a 25 cm x 75 µm x 1.7 µm BEH 130 C_{18}
152 reversed phase column (Waters GmbH) with gradient elution performed from 1% ACN to
153 50% ACN in 0.1% formic acid within 120 min. Identification of the proteins was based on
154 a global NCBI database search applying the MASCOT search algorithm. The mass

spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (30) partner repository with the dataset identifier PXD009664 and 10.6019/PXD009664.

Preparative biotransformations

The filamentous fungi applied in this work were cultured in PEG broth, in which a better growth could be achieved in comparison to sabouraud medium. On PEG-agar, the hyphae were transferred once a month and incubated at 27°C for several days (*M. hiemalis*: 3 days, *B. bassiana*: 4 days, *C. nebularis*: 5 days). *B. bassiana* used in this work was modified by UV radiation to allow yeast-like growth in liquid culture (31). 50 mL of PEG broth was once inoculated with a loop tip, whereas *M. hiemalis* and *C. nebularis* were inoculated several times with hyphae to slightly disperse and therefore minimize lumpy mycelial growth. The fungi were cultured for 4 days at 27°C and 180 rpm and then transferred to 50 – 400 ml main cultures which were inoculated with 1/75 volume (*B. bassiana*) or with the filtered mycelia (*M. hiemalis* and *C. nebularis*) of the preculture. After 24 h of incubation, the biotransformations were started with 1 g L^{-1} substrate (50 g L^{-1} stock solution in DMSO) or in the case of diclofenac (**1**) with 0.6 g L^{-1} for 72 h or 144 h, respectively. At regular intervals, 500 µL samples were taken, centrifuged and the supernatant was used for analysis by GC-FID, HPLC-DAD or LC-MS. The biotransformations were carried out in biological duplicates starting from different agar plates.

178 *HPLC and LC-MS analytic*

179 Diclofenac (**1**) and its metabolites were analyzed by HPLC and LC-MS as described
180 elsewhere (21).

181

182 *GC analytic*

183 Naproxen (**6**), ibuprofen (**8**) and mefenamic acid (**12**) were analyzed by gas
184 chromatography. For this purpose, the samples (500 µL) were centrifuged and 250 µL of
185 the supernatant initially acidified by 10 µL HCl prior extraction with the same volume of
186 MTBE. The organic phase was then evaporated on a Genevac EZ-2 Plus Evaporator
187 (Ipswich, UK) and resuspended in a mix of 50% MTBE and 50% BSTFA + TCMS (99: 1).
188 The derivatization of the samples (150 µL) was carried out in GC vials at 70°C for 30 min.
189 GC analysis was performed on a Shimadzu GC-2010 equipped with an AOC-20i
190 autoinjector (Shimadzu, Nakagyo-ku, Japan). The samples were injected with a split of 20
191 (1 µL injection volume, injector temperature 250°C, carrier gas H_2, 30 cm s^{-1}) and
192 separated by a DB-5 column (30 m x 0.25 mm x 0.25 µm, Agilent Technologies, Santa
193 Clara, California, USA). The analytes were detected by a flame ionization detector (FID,
194 detector temperature 330°C). For **6** and **12** the column temperature was maintained at
195 150°C for 1 min, increased to 280°C at a rate of 10°C min^{-1} and held for 1 min, raised to
196 320°C at a rate of 65°C min^{-1} and held for 3 min. For **8** the column temperature was
197 maintained at 90°C for 1 min, increased to 280°C at a rate of 12°C min^{-1} and held for 1 min,
198 raised to 320°C at a rate of 65°C min^{-1} and held for 3 min.
199 For initial product identification, the samples were run on GC-2010 GC-MS system
200 equipped with Shimadzu GCMS-QP2010 detector and AOC-5000 autoinjector (Shimadzu,

Nakagyo-ku, Japan) and helium as carrier gas (linear velocity 30 cm s^{-1}). The analytes were separated on a DB-5 column and measured with identical temperature programs for GC-FID analysis. For the recording of the mass spectra, an ionization (EI, Electron Ionization) of 70 eV, an interface temperature of 250°C and an ion source temperature of 200°C was used. The detection of the mass fragments was finally carried out in the scan mode from 40 to 600 m/z.

Product purification and identification

For product purification, the supernatant of the duplicates (2 x 50 mL or 2 x 400 mL) from preparative biotransformations were extracted twice with the identical volume of MTBE. In the case of biotransformations with substrate **6**, **8** and **12**, the supernatant was additionally acidified with HCl and afterwards extracted with MTBE. The organic phases were combined, evaporated to dryness and the residues were dissolved in 10 mL of acetonitrile and stored at 4°C for further use. The batches were finally purified by reverse phase semi-preparative HPLC on an Agilent 1200 System (St. Clara, California, USA) equipped with a G1311A quaternary pump, a HIP AS G1367B autosampler (1200 µL loop), a G1315D DAD detector and an analytical G1364C fraction collector. The separation of the analytes (injection volume: 1000 µL) was ensured using a Trentec Reprosil® 100-5 C18 column (250 × 20 mm, 5 µm) from Dr. Maisch GmbH (Ammerbuch-Entringen, Germany). The mobile phases A and B were composed of water containing 0.1% formic acid and acetonitrile, respectively. Elution was done in a gradient mode at a flow rate of 5 mL min^{-1} and a column temperature of 20°C using following program: 85% A (0 min), 20% A (63 min), 20% A (67 min), 85% A (67.01 min), 85% A (83 min).

The elution of substrates and products were followed spectrophotometrically at a wavelength of 272 nm for **1**, 232 nm for **6**, 224 nm for **8** and 275 nm for **12**. Identical product fractions were combined, the solvent mixture of acetonitrile and water removed on a rotary evaporator and completely dried by a nitrogen gas flow. The remaining solid products were finally dissolved in *d*-methanol or *d*-DMSO and analyzed by ^{1}H- and ^{13}C-NMR to clarify the chemical structures. The purity of all samples were determined by GC or HPLC.

NMR measurements

For the characterization of the purified products, NMR spectroscopy was used. ^{1}H-, ^{1}H-COSY- and ^{13}C-NMR spectra were recorded using a Bruker Avance 500 spectrometer at 500.15 and 125.76 MHz, respectively, or a Bruker Ascend 700 ™ spectrometer at 700.36 and 176.10 MHz respectively (both Bruker, Billerica, Massachusetts, USA). The chemical shift δ was measured in ppm (parts per million) and referred to TMS (tetramethylsilane) δ = 0 ppm as a standard. Unless otherwise stated, 10 mg of the product was used for NMR analysis. The NMR spectra are given in the supplemental material (Figures S2-S9).

5-Hydroxydiclofenac: ^{1}H-NMR (500 MHz, CD_3OD): δ (ppm) 3.71 (s, 2H; C-7), 6.37 (d, J = 8.8 Hz, 1H; C-3), 6.55 (dd, J = 8.8, 2.5 Hz, 1H; C-4), 6.72 (d, J = 2.5 Hz, 1H; C-6), 6.96 (t, J = 7.8 Hz, 1H; C-4'), 7.34 (d, J = 7.8 Hz 2H; C-3' and 5').

^{13}C-NMR (126 MHz, CD_3OD): δ (ppm) 39.2 (C7), 115.3 (ArCH-4), 118.3 (ArCH-6), 121.9 (ArCH-3), 124.1 (ArCH-4'), 129.2 (ArC-2), 129.3 (ArC-1'), 130.1 (2ArCH-3' and 5'), 136.4 (ArC-1), 140.5 (2CCl), 154.1 (C-OH), 175.8 (COOH).

5-Hydroxyquinoneimine: ^{1}H-NMR (500 MHz, $(CD_3)_2SO$): δ (ppm) 3.69 (s, 2H; C-7), 6.59 (dd, J = 10.1, 2.5 Hz, 1H; C-4), 6.74 (d, J = 10.1 Hz, 1H; C-3), 6.80 (d, J = 2.5 Hz, 1H; C-6), 7.25 (t, J = 8.2 Hz, 1H; C-4'), 7.58 (d, J = 7.6 Hz, 2H; C-3' and 5').

^{13}C-NMR (126 MHz, $(CD_3)_2SO$): δ (ppm) 36.3 (C7), 123.1 (ArC), 126.6 (ArC), 128.6 (ArC), 129.1 (ArC), 133.1 (ArC), 133.5 (ArC), 143.4 (ArC-1'), 145.1 (ArC-1), 160.4 (ArC-2), 170.6 (COOH), 186.9 (C=O).

4'-Hydroxydiclofenac: ^{1}H-NMR (500 MHz, CD_3OD): δ (ppm) 3.7 (s, 2H, C-7), 6.26 (d, 1H, J = 8.0 Hz, C-3), 6.8 (t, 1H, J = 7.4, C-5), 6.88 (s, 2H, C-3` and C-5`), 7.02 (t, 1H, J = 7.5, C-4), 7.17 (d, 1H, J = 7.4, C-6).

^{1}H-COSY (500 MHz, CD_3OD): (7.17) δ (ppm) 3.7, 6.8, (7.02) δ (ppm) 6.26, 6.8.

^{13}C-NMR (126 MHz, CD_3OD): δ (ppm) 39.2 (C7'), 116.4 (ArC), 116.9 (2ArC), 121.3 (ArC), 124.2 (ArC), 128.9 (ArC), 130.5 (ArC), 131.9 (2ArC), 133.7 (ArC), 145.5 (ArC), 156.3 (ArC-4`), 175.9 (COOH).

3',4'-Dihydroxydiclofenac: ^{1}H NMR (700 MHz, CD_3OD): δ (ppm) 3.72 (s, 2H, C-7), 6.29 (d, 1H, J = 8.1 Hz, C-3), 6.80 (t, 1H, J = 7.6, C-5), 6.86 (s, 1H, C-5`), 7.02 (t, 1H, J = 7.9 Hz, C-4), 7.17 (d, 1H, 3.5 Hz, C-6).

6-*o*-Desmethylnaproxen: ^{1}H-NMR (500 MHz, CD_3OD): δ (ppm) 1.50 (d, 3H, J = 7.0 Hz, C-3), 3.81 (q, 1H, J = 7.2 Hz, C-2), 7.03 - 7.09 (m, 2H, C-8 and C-10) 7.35 (d, 1H, J = 8.5 Hz, C-11), 7.59 (d, 1H, J = 8.4 Hz, C-13), 7.64 (s, 1H, C-5), 7.67 (d, 1H, J = 8.8 Hz, C-6).

^{13}C-NMR (126 MHz, CD_3OD): δ (ppm) 15.4 (C7), 113.2 (ArC), 114.5 (ArC), 117.9 (ArC), 127.3 (ArC), 127.5 (ArC), 128.7 (ArC), 133.3 (ArC), 134.9 (ArC), 135.1 (ArC), 135.2 (ArC), 141.6 (ArC), 150.2 (ArC), 172.1 (COOH).

2-Hydroxyibuprofen: ^{1}H-NMR (500 MHz, CD_3OD): δ (ppm) 1.16 (s, 6H, C-12 and C-13), 1.43 (d, 3H, J = 7.2 Hz, C-3), 2.72 (s, 2H, C-10), 3.68 (q, 1H, J = 7.2 Hz, C-2), 7.18 (d, 2H, J = 8.0 Hz, C-5 and C-9), 7.22 (d, 2H, J = 7.9 Hz, C-6 and C-8).

^{13}C-NMR (126 MHz, CD_3OD): δ (ppm) 19.1 (C3), 29.2 (C12 and C13), 46.4 (C2), 50.3 (C10), 71.8 (C11), 128.0 (2x ArC), 131.9 (2 x ArC), 138.5 and 140.3 (C4 and C7), 178.6 (COOH).

1-Hydroxyibuprofen: ^{1}H-NMR (700 MHz, $(CD_3)_2SO$): δ (ppm) 0.73 (d, 3H, J = 6.8 Hz, C-12) 0.85 (d, 3H, J =6.7 Hz, C-13), 1.32 (d, 3H, J = 7.1 Hz, C-3), 1.77 (oct, 1H, J = 16.4 Hz, C-11), 3.58 (q, 1H, J = 7.2 Hz, C-2), 4.19 (d, 1H, J= 6.2, C-10), 7.19 (d, 2H, J = 8.5 Hz, C-5 and C-9), 7.21 (d, 2H, J = 8.6 Hz, C-6 and C-8).

^{13}C-NMR (176 MHz, $(CD_3)_2SO$): δ (ppm) 17.4 (C3), 18.2 and 18.5 (C12 and C13), 34.3 (C11), 44.4 (C2), 76.8 (C10), 125.9 and 126.1 (4 x ArC), 139.6 and 142.6 (C4 and C7), 175.3 (COOH).

1,2-Dihydroxyibuprofen: ^{1}H-NMR (500 MHz, CD_3OD): δ (ppm) 1.12 (s, 6H, C-12 and C-13), 1.43 (d, 3H, J = 3.4 Hz, C-3), 3.69 (q, 1H, J = 7.2 Hz, C-2), 4.43 (s, 1H, C-10), 7.26 (d, 2H, J = 7.8 Hz, C-5 and C-9), 7.34 (d, 2H, J = 8.0 Hz, C-6 and C-8).

^{13}C-NMR (126 MHz, CD_3OD): δ (ppm) 19.1 (C3), 25.3 and 26.0 (C12 and C13), 46.7 (C2), 74.0 (C11), 81.6 (C10), 127.8 (2 x ArC), 129.2 (2 x ArC), 141.7 and 141.8 (C4 and C7). COOH group is barely visible.

3'-Hydroxymethylmefenamic acid: ^{1}H-NMR (500 MHz, $(CD_3)_2SO$): δ (ppm) 2.11 (s, 3H, C-8'), 4.53 (s, 2H, C-7'), 6.68 – 6.73 (m, 2H, C-2 and C-4), 7.18 – 7.25 (m, 3H, C-4', 5' and 6'), 7.31 (t, 1H, J = 7.5 Hz, C-3), 7.89 (d, 1H, J = 7.5 Hz, C-5).

290 ^{13}C-NMR (126 MHz, $(CD_3)_2SO$): δ (ppm) 12.4 (C8‘), 61.3 (C7`), 111.6 (ArC), 112.9
291 (ArC), 116.3 (2ArC-3 and 4‘), 122.6 (ArC), 123.5 (ArC), 125.9 (ArC), 129.9 (ArC), 131.6
292 (ArC), 134.0 (ArC), 138.2 (ArC), 141.8 (ArC), 170.0 (COOH).
293 3’-Carboxymefenamic acid: ^{1}H-NMR (500 MHz, CD_3OD): δ (ppm) 2.43 (s, 3H, C-8’),
294 6.71 (t, 1H, *J* =7.5 Hz, C-4), 6.75 (d, 1H, *J* = 4.3 Hz, C-2), 7.28 (q, 2H, *J* = 7.3 Hz, C-3,
295 and C-5’), 7.46 (d, 1H, *J* = 7.8 Hz, C-4’), 7.62 (d, 1H, *J* = 7.8 Hz, C-6’), 7.98 (d, 1H, *J* =
296 8.0 Hz, C-5).
297 ^{13}C-NMR (126 MHz, CD_3OD): δ (ppm) 15.4 (C8‘), 113.2 (ArC), 114.5 (ArC), 117.9
298 (ArC), 127.3 (ArC), 127.5 (ArC), 128.7 (ArC), 133.3 (ArC), 134.9 (ArC), 135.1 (ArC),
299 135.2 (ArC), 141.6 (ArC), 150.2 (ArC), 172.1 (2 x COOH).
300
301 **Results and Discussion**
302 *Identification of microbial biocatalysts for NSAID oxyfunctionalizations*
303 In addition to the already published organism *Beauveria bassiana*, which is able to perform
304 one of the most effective hydroxylation processes in biocatalysis, the production of (*R*)-2-
305 (4-hydroxyphenoxy)propionic acid and a variety of other hydroxylation reactions (31, 32),
306 we identified two other filamentous fungi as further novel NSAID accepting biocatalysts.
307 One of these strains was isolated from soil samples close to Ludwigshafen (Germany)
308 during previous diclofenac (**1**) degradation experiments (33). The third filamentous fungus
309 was isolated by coincident as a contamination strain out of a 5-hydroxydiclofenac (**3**)
310 standard, diluted in M9 minimal medium, after 1.5 years of aerobic incubation. Both fungi
311 were assigned by the universal biomarkers LSU (28S nuclear ribosomal large subunit
312 rRNA gene) and ITS (internal transcribed spacer). Sequence matching with the fungal

313 barcoding and the NCBI database revealed that one strain was > 99% consistent with the
314 S1 organism *Clitocybe nebularis* (confirmed by LSU and ITS biomarkers). For the other
315 organism, the LSU biomarker found a > 98% similarity with the S1 organism
316 *Mucor hiemalis*.

317 *Biotransformations with diclofenac*

318 The fungi were cultivated in 400 mL PGE broth and the biotransformations were done in
319 duplicates with a starting concentration of 0.6 g L^{-1} diclofenac (**1**). The reaction was
320 monitored over time by HPLC-DAD and stopped after 144 h (Figure 1). All products were
321 purified by semi-preparative HPLC for further detailed structure identification by ^{1}H and
322 ^{13}C NMR. Similar to the distribution of products in humans, the 4'-position (**2**) was
323 favoured in the biotransformations with the fungi, which was also observed with P450s
324 and microorganisms in previous studies (Scheme 1, Table 1). In comparison to
325 *C. nebularis* which is able to catalyze a mixture of the metabolites 4'-hydroxydiclofenac
326 (**2**, 60%), 5-hydroxydiclofenac (**3**, 30%) and the corresponding quinoneimine (**4**, 10%)
327 with 50.6% isolated yield, *M. hiemalis* selectively oxyfunctionalized diclofenac (**1**)
328 yielding 61.7% of 4'-hydroxydiclofenac (**2**) after purification. High isolated yields of
329 55.3% were additionally achieved using *B. bassiana*. Next to the major product
330 4'-hydroxydiclofenac (**2**, 90%), a 3',4'-dihydroxydiclofenac (**5**) metabolite was identified
331 that has not been characterized and published yet. In studies concerning the fungi
332 *Epicoccum nigrum* and *Cunninghamella elegans*, preparative biotransformations were
333 performed resulting in product titers of 16 and 57 mg L^{-1}, respectively (34, 35). In contrast,
334 the filamentous fungi identified in the present work were able to obtain significantly higher
335 values comparable with biotransformations using P450 RhF and P450 BM3 (Table 1).

Proteome analysis of B. bassiana

A common question that is often raised as a negative element for the use of microbial cell factories is which enzymes are involved in the detected biotransformation. In this respect, we aimed to identify 450s, which are responsible for the oxyfunctionalization of diclofenac (**1**) and (*R*)-2-phenoxypropionic acid in *B. bassiana* using omics studies. As a proof of concept, we analyzed biotransformation samples whether we can identify differences by a proteome analysis depending on the substrate. Therefore, samples of biotransformations with the corresponding substrates and glucose as negative control were processed to obtain the organisms proteome. By applying the proteome analysis clear evidence of P450s (implied by the number of detected and assigned fragments) which were upregulated with the specific substrates, were achieved drastically reducing the high number of 83 putative P450s in *B. bassiana* (36). With diclofenac (**1**) as substrate two P450s, CYP548A5 and CYP51F1, were exclusively expressed compared to the samples exposed with the other substrates (Table 2). In case of (*R*)-2-phenoxypropionic acid as substrate, CYP52A10 seems to be strongly upregulated while CYP684A2 was induced exclusively. In eukaryotes, only one bifunctional NADPH-reductase serves as electron delivering partner that was also found in all samples. In this work, the proteome analysis has thus proved to be a targeted method for identifying involved proteins in eukaryotes that can subsequently lead to valuable new heterologous expressed biocatalysts.

NSAID biotransformations applying the identified microbial biocatalysts

To investigate the potential of the microbial biocatalysts further we increased the NSAID substrate scope of the fungi strains to oxyfunctionalize naproxen (**6**), ibuprofen (**8**) and mefenamic acid (**12**). In some cases, high titers and full conversion towards the investigated

359 NSAIDs were achieved. For example, *M. hiemalis* demethylated naproxen (**6**) to the human
360 metabolite 6-*o*-desmethylnaproxen (**7**) with an isolated yield of over 99% using 1 g L^{-1} of
361 substrate (Scheme 2). In comparison the *in vitro* biotransformations with P450 BM3
362 variants and the strain *A. niger* showed much lower selectivity and isolated yield (Table 1)
363 (19, 20, 37).
364 Furthermore, *M. hiemalis* almost completely hydroxylated 1 g L^{-1} ibuprofen (**8**) to the
365 metabolites 2-hydroxyibuprofen (**9**, 90%) 1-hydroxyibuprofen (**10**, 2%), and the double
366 hydroxylated secondary product 1,2-dihydroxyibuprofen (**11**, 8%) (Scheme 2). The highest
367 product titer so far was achieved by a P450 BM3 variant *in vitro* with 0.4 g L^{-1} of
368 2-hydroxyibuprofen (**9**) (Table 1) (17, 20, 38). Microorganisms such as
369 *Trametes versicolor* and *Nigospora sphaerica* or the P450s CYP2C9, CYP267A1 and B1
370 had significantly lower product yields (> 30 g L^{-1}) (25, 39–41). Using the anthranilic acid
371 derivative mefenamic acid (**12**) as substrate, the highest activity was achieved by
372 *B. bassiana* with an isolated yield of 48% at 1 g L^{-1} substrate concentration (Scheme 2).
373 Next to the 3'-hydroxymethylmefenamic acid (**13**) as main product (68%), the product
374 3'-carboxymefenamic acid (**14**) was catalyzed. In humans, **12** is mainly converted to **13** by
375 CYP2C9 which undergoes further oxidation to the carboxy metabolite **14** (42). An evolved
376 P450 BM3 variant generated besides 3'-hydroxymethylmefenamic acid (**13**) the other
377 human P450 catalyzed metabolites 4'- and 5-hydroxymefenamic acid (**20** and **21**) which
378 are hydroxylated at the aromatic rings (18, 43). In this case, *in vitro* product titers of
379 216 mg L^{-1} were below those obtained with *B. bassiana* (Table 1). In degradation
380 experiments, the wild-type organism *Phaenerochaete sordida* (white-rot fungus) also

produced different mefenamic acid metabolites with significantly lower yields of less than 3 mg L^{-1} (26).

Overall, the filamentous fungi of the present study achieved high product titers in the biotransformations with the NSAIDs. Applying a semi-preparative HPLC purification method, all NSAID metabolites could be isolated in high yields and high purities. Moreover, reproducible product titers were obtained at 50 mL or 400 mL scale exhibiting the possibility to further increase the yields by improving the cultivation and biotransformation procedure. One drawback of filamentous fungi as catalyst for biotransformations is their mycelial growth. This particular cell shape minimizes the possible surface to the medium and thus the mass transfer of substrate solubilized in the medium and the cells. Especially cells located inside the mycelia may therefore be unable to participate in biocatalysis, but against our expectation, the clump-forming organism *M. hiemalis* has achieved the highest yields so far.

Compared to already published biotechnological processes the total process time with the filamentous fungi of 4 days or 7 days for diclofenac (**1**), respectively, were in accordance with most of the studies (Table 1 and 2). However, a simple cultivation and biotransformation setup using fungi with less steps are main benefits, besides the achieved high product yields. In detail, compared to P450-based reactions, less requirements on the process were necessary since issues associated with an often complex P450 system like biocatalyst expression, stability or cofactor dependency are negligible.

In conclusion, the results of the present work demonstrate the potential of microbial biocatalysts for NSAID oxyfunctionalizations. Often, low activity, difficult handling, or

404 high by-product formation are illustrated as criteria against the use of microorganisms (40).
405 In contrast, the microorganisms identified in this work are easy to cultivate, inexpensive
406 and are able to produce high valued metabolites in promising yields. The applied and
407 identified filamentous fungi serve with their versatile metabolism as an interesting platform
408 for novel cell factories to produce valuable NSAID metabolites in an effective and cheap
409 way or can help to develop new strategies in drug design.
410

411 **Acknowledgements**

412 The research leading to these results has received funding from the European Union's
413 Seventh Framework Programme for research, technological development and
414 demonstration under grant agreements no 613849 (BIOOX). We also gratefully
415 acknowledge financial support from the Landesgraduiertenförderung (LSFG) Baden-
416 Württemberg. We thank Berit Würtz and Dr. Jens Pfannstiel for conduction and helpful
417 advice concerning the proteome analysis.
418

419 **References**

420 1. Bernhardt R. 2004. Cytochrome P450 : versatile Enzymsysteme mit Anwendungen
421 in der Biotechnologie und Medizin. Mag Forsch 1:10–16.
422 2. Guengerich FP. 2008. Cytochrome P450 and Chemical Toxicology Cytochrome
423 P450 and Chemical Toxicology. Chem Res Toxicol 21:70–83.
424 3. Guengerich F. 2006. Cytochrome P450s and other enzymes in drug metabolism and
425 toxicity. AAPS J 8:E101–E111.
426 4. Naisbitt DJ, Sanderson LS, Meng X, Stachulski A V., Clarke SE, Park BK. 2007.

427 Investigation of the immunogenicity of diclofenac and diclofenac metabolites.
428 Toxicol Lett 168:45–50.
429 5. den Braver MW, Zhang Y, Venkataraman H, Vermeulen NPE, Commandeur JNM.
430 2016. Simulation of interindividual differences in inactivation of reactive para-
431 benzoquinone imine metabolites of diclofenac by glutathione S-transferases in
432 human liver cytosol. Toxicol Lett 255:52–62.
433 6. Langford K, Thomas K V. 2011. Input of selected human
434 pharmaceuticalmetabolites into the Norwegian aquatic environment. J Environ
435 Monit 13:416–421.
436 7. Richardson SD, Kimura SY. 2016. Water Analysis: Emerging Contaminants and
437 Current Issues. Anal Chem 88:546–582.
438 8. Guengerich FP, MacDonald JS. 2007. Applying mechanisms of chemical toxicity to
439 predict drug safety. Chem Res Toxicol 20:344–369.
440 9. Daily EB, Aquilante CL. 2009. Cytochrome P450 2C8 pharmacogenetics: a review
441 of clinical studies. Pharmacogenomics 10:1489–1510.
442 10. Waterhouse I. 1999. Synthesis of deuterium labelled 4'-Hydroxydiclofenac. J Label
443 Compd Radiopharm 42:1075–1083.
444 11. Kim SH, Kwon JH, Yoon SH. 2010. An improved synthesis of 4'-
445 hydroxydiclofenac. Bull Korean Chem Soc 31:3007–3009.
446 12. Kenny JR, Maggs JL, Meng X, Sinnott D, Clarke SE, Park BK, Stachulski A V.
447 2004. Syntheses and characterization of the acyl glucuronide and hydroxy
448 metabolites of diclofenac. J Med Chem 47:2816–2825.
449 13. Urlacher VB, Girhard M. 2012. Cytochrome P450 monooxygenases: an update on

450 perspectives for synthetic application. Trends Biotechnol 30:26–36.

451 14. Drăgan CA, Peters FT, Bour P, Schwaninger AE, Schaan SM, Neunzig I, Widjaja
452 M, Zapp J, Kraemer T, Maurer HH, Bureik M. 2011. Convenient gram-scale
453 metabolite synthesis by engineered fission yeast strains expressing functional
454 human P450 systems. Appl Biochem Biotechnol 163:965–980.

455 15. Geier M, Braun A, Emmerstorfer A, Pichler H, Glieder A. 2012. Production of
456 human cytochrome P450 2D6 drug metabolites with recombinant microbes – a
457 comparative study. Biotechnol J 7:1346–1358.

458 16. Vail RB, Homann MJ, Hanna I, Zaks A. 2005. Preparative synthesis of drug
459 metabolites using human cytochrome P450s 3A4, 2C9 and 1A2 with NADPH-P450
460 reductase expressed in Escherichia coli. J Ind Microbiol Biotechnol 32:67–74.

461 17. Tsotsou GE, Sideri A, Goyal A, Di Nardo G, Gilardi G. 2012. Identification of
462 mutant Asp251Gly/Gln307His of cytochrome P450 BM3 for the generation of
463 metabolites of diclofenac, ibuprofen and tolbutamide. Chem - A Eur J 18:3582–
464 3588.

465 18. Venkataraman H, Verkade-Vreeker MCA, Capoferri L, Geerke DP, Vermeulen
466 NPE, Commandeur JNM. 2014. Application of engineered cytochrome P450
467 mutants as biocatalysts for the synthesis of benzylic and aromatic metabolites of
468 fenamic acid NSAIDs. Bioorganic Med Chem 22:5613–5620.

469 19. Ren X, Yorke JA, Taylor E, Zhang T, Zhou W, Wong LL. 2015. Drug Oxidation by
470 Cytochrome P450BM3 : Metabolite Synthesis and Discovering New P450 Reaction
471 Types. Chemistry 21:15039–15047.

472 20. Rentmeister A, Brown TR, Snow CD, Carbone MN, Arnold FH. 2011. Engineered

473 Bacterial Mimics of Human Drug Metabolizing Enzyme CYP2C9. ChemCatChem
474 3:1065–1071.
475 21. Klenk JM, Nebel BA, Porter JL, Kulig JK, Hussain SA, Richter SM, Tavanti M,
476 Turner NJ, Hayes MA, Hauer B, Flitsch SL. 2017. The self-sufficient P450 RhF
477 expressed in a whole cell system selectively catalyses the 5-hydroxylation of
478 diclofenac. Biotechnol J 12:1–8.
479 22. Xu LH, Ikeda H, Liu L, Arakawa T, Wakagi T, Shoun H, Fushinobu S. 2015.
480 Structural basis for the 4'-hydroxylation of diclofenac by a microbial cytochrome
481 P450 monooxygenase. Appl Microbiol Biotechnol 99:3081–3091.
482 23. Marco-Urrea E, Pérez-Trujillo M, Cruz-Morató C, Caminal G, Vicent T. 2010.
483 Degradation of the drug sodium diclofenac by Trametes versicolor pellets and
484 identification of some intermediates by NMR. J Hazard Mater 176:836–842.
485 24. Ferrando-Climent L, Collado N, Buttiglieri G, Gros M, Rodriguez-Roda I,
486 Rodriguez-Mozaz S, Barceló D. 2012. Comprehensive study of ibuprofen and its
487 metabolites in activated sludge batch experiments and aquatic environment. Sci
488 Total Environ 438:404–413.
489 25. Borges KB, De Oliveira ARM, Barth T, Jabor VAP, Pupo MT, Bonato PS. 2011.
490 LC-MS-MS determination of ibuprofen, 2-hydroxyibuprofen enantiomers, and
491 carboxyibuprofen stereoisomers for application in biotransformation studies
492 employing endophytic fungi. Anal Bioanal Chem 399:915–925.
493 26. Hata T, Kawai S, Okamura H, Nishida T. 2010. Removal of diclofenac and
494 mefenamic acid by the white rot fungus Phanerochaete sordida YK-624 and
495 identification of their metabolites after fungal transformation. Biodegradation

21:681–689.

27. Schoch CL, Seifert K a., Huhndorf S, Robert V, Spouge JL, Levesque C a., Chen W, Consortium FB, Bolchacova E, Voigt K, Crous PW, Miller a. N, Wingfield MJ, Aime MC, An K-D, Bai F-Y, Barreto RW, Begerow D, Bergeron M-J, Blackwell M, Boekhout T, Bogale M, Boonyuen N, Burgaz a. R, Buyck B, Cai L, Cai Q, Cardinali G, Chaverri P, Coppins BJ, Crespo A, Cubas P, Cummings C, Damm U, De Beer ZW, de Hoog GS, Del-Prado R, Dentinger B, Dieguez-Uribeondo J, Divakar PK, Douglas B, Duenas M, Duong T a., Eberhardt U, Edwards JE, Elshahed MS, Fliegerova K, Furtado M, Garcia M a., Ge Z-W, Griffith GW, Griffiths K, Groenewald JZ, Groenewald M, Grube M, Gryzenhout M, Guo L-D, Hagen F, Hambleton S, Hamelin RC, Hansen K, Harrold P, Heller G, Herrera C, Hirayama K, Hirooka Y, Ho H-M, Hoffmann K, Hofstetter V, Hognabba F, Hollingsworth PM, Hong S-BS-BS-BS-B, Hosaka K, Houbraken J, Hughes K, Huhtinen S, Hyde KD, James T, Johnson EM, Johnson JE, Johnston PR, Jones EBG, Kelly LJ, Kirk PM, Knapp DG, Koljalg U, Kovacs GM, Kurtzman CP, Landvik S, Leavitt SD, Liggenstoffer a. S, Liimatainen K, Lombard L, Luangsa-ard JJ, Lumbsch HT, Maganti H, Maharachchikumbura SSN, Martin MP, May TW, McTaggart a. R, Methven a. S, Meyer W, Moncalvo J-M, Mongkolsamrit S, Nagy LG, Nilsson RH, Niskanen T, Nyilasi I, Okada G, Okane I, Olariaga I, Otte J, Papp T, Park D, Petkovits T, Pino-Bodas R, Quaedvlieg W, Raja H a., Redecker D, Rintoul TL, Ruibal C, Sarmiento-Ramirez JM, Schmitt I, Schussler A, Shearer C, Sotome K, Stefani FOP, Stenroos S, Stielow B, Stockinger H, Suetrong S, Suh S-O, Sung G-H, Suzuki M, Tanaka K, Tedersoo L, Telleria MT, Tretter E, Untereiner W a.,

519 Urbina H, Vagvolgyi C, Vialle A, Vu TD, Walther G, Wang Q-M, Wang Y, Weir
520 BS, Weiss M, White MM, Xu J, Yahr R, Yang ZL, Yurkov A, Zamora J-C, Zhang
521 N, Zhuang W-YW-Y, Schindel D. 2012. Nuclear ribosomal internal transcribed
522 spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad
523 Sci U S A 109:1–6.
524 28. Osherov N, May GS. 1998. Optimization of protein extraction from Aspergillus
525 nidulans for gel electrophoresis. Fungal Genet Newsl 45:38–40.
526 29. Voolstra O, Beck K, Oberegelsbacher C, Pfannstiel J, Huber A. 2010. Light-
527 dependent phosphorylation of the Drosophila transient receptor potential ion
528 channel. J Biol Chem 285:14275–14284.
529 30. Vizcaíno JA, Csordas A, Del-Toro N, Dianes JA, Griss J, Lavidas I, Mayer G, Perez-
530 Riverol Y, Reisinger F, Ternent T, Xu QW, Wang R, Hermjakob H. 2016. 2016
531 update of the PRIDE database and its related tools. Nucleic Acids Res 44:D447–
532 D456.
533 31. Dingler C, Ladner W, Krei GA, Cooper B, Hauer B. 1996. Preparation of (R)-2-(4-
534 Hydroxyphenoxy) propionic Acid by Biotransformation. Pestic Sci 46:33–35.
535 32. Grogan GJ, Holland HL. 2000. The biocatalytic reactions of Beauveria spp . J Mol
536 Catal B Enzym 9:1–32.
537 33. Facey SJ, Nebel BA, Kontny L, Allgaier M, Hauer B. 2018. Rapid and complete
538 degradation of diclofenac by native soil microorganisms. Environ Technol Innov
539 10:55–61.
540 34. Webster R, Pacey M, Winchester T, Johnson P, Jezequel S. 1998. Microbial
541 oxidative metabolism of diclofenac: Production of 4’-hydroxydiclofenac using

Epicoccum nigrum IMI354292. Appl Microbiol Biotechnol 49:371–376.

35. Quinn L, Dempsey R, Casey E, Kane A, Murphy CD. 2015. Production of drug metabolites by immobilised Cunninghamella elegans: from screening to scale up. J Ind Microbiol Biotechnol 42:799–806.

36. Xiao G, Ying S-H, Zheng P, Wang Z-L, Zhang S, Xie X-Q, Shang Y, St Leger RJ, Zhao G-P, Wang C, Feng M-G. 2012. Genomic perspectives on the evolution of fungal entomopathogenicity in Beauveria bassiana. Sci Rep 2:483.

37. He A, Rosazza JPN. 2003. Microbial transformations of S-naproxen by Aspergillus niger ATCC 9142. Pharmazie 58:420–422.

38. Di Nardo G, Dell'Angelo V, Catucci G, Sadeghi SJ, Gilardi G. 2015. Subtle structural changes in the Asp251Gly/Gln307His P450 BM3 mutant responsible for new activity toward diclofenac, tolbutamide and ibuprofen. Arch Biochem Biophys 602:106–115.

39. Marco-Urrea E, Pérez-Trujillo M, Vicent T, Caminal G. 2009. Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by Trametes versicolor. Chemosphere 74:765–772.

40. Kern F, Khatri Y, Litzenburger M, Bernhardt R. 2016. CYP267A1 and CYP267B1 from sorangium cellulosum so ce56 are highly versatile drug metabolizers. Drug Metab Dispos 44:495–504.

41. Neunzig I, Göhring A, Drăgan CA, Zapp J, Peters FT, Maurer HH, Bureik M. 2012. Production and NMR analysis of the human ibuprofen metabolite 3-hydroxyibuprofen. J Biotechnol 157:417–420.

42. Venkataraman H, Den Braver MW, Vermeulen NPE, Commandeur JNM. 2014.

565 Cytochrome P450-mediated bioactivation of mefenamic acid to quinoneimine
566 intermediates and inactivation by human glutathione S-transferases. Chem Res
567 Toxicol 27:2071–2081.
568 43. Capoferri L, Leth R, ter Haar E, Mohanty AK, Grootenhuis PDJ, Vottero E,
569 Commandeur JNM, Vermeulen NPE, Jørgensen FS, Olsen L, Geerke DP. 2016.
570 Insights into regioselective metabolism of mefenamic acid by cytochrome P450
571 BM3 mutants through crystallography, docking, molecular dynamics, and free
572 energy calculations. Proteins Struct Funct Bioinforma 84:383–396.
573 44. Osorio-Lozada A, Surapaneni S, Skiles GL, Subramanian R. 2008. Biosynthesis of
574 drug metabolites using microbes in hollow fiber cartridge reactors: Case study of
575 diclofenac metabolism by actinoplanes species. Drug Metab Dispos 36:234–240.
576 45. Tavanti M, Porter JL, Sabatini S, Turner NJ, Flitsch SL. 2018. Panel of New
577 Thermostable CYP116B Self-Sufficient Cytochrome P450 Monooxygenases that
578 Catalyze C−H Activation with a Diverse Substrate Scope. ChemCatChem 10:1042–
579 1051.

580

581 **Table legends**

582

583 TAB 1

584 Summary of biotechnological processes to produce NSAID metabolites.
585 4'-hydroxydiclofenac (**2**); 5-hydroxydiclofenac (**3**); 5-hydroxyquinoneimine (**4**);
586 3',4'-dihydroxydiclofenac (**5**); 4',5-dihydroxydiclofenac (**15**); 6-*o*-desmethylnaproxen (**7**);
587 2-hydroxyibuprofen (**9**); 1-hydroxyibuprofen (**10**); 1,2-dihydroxyibuprofen (**11**);

588 3'-hydroxymethylmefenamic acid (**13**); 3'-carboxymefenamic acid (**14**); 2-Acetyl-6-
589 methoxynaproxen (**16**); 7-hydroxynaproxen (**17**); 3',5-dihydroxymefenamic acid (**18**);
590 3',6'-dihydroxymefenamic acid (**19**); 4'-hydroxymefenamic acid (**20**);
591 5-hydroxymefenamic acid (**21**).
592
593
594
595
596
597
598
599
600
601

Biocatalyst	Product(s)	Process time[a] / Scale	Product titer	Reference
Diclofenac (1)				
E. nigrum	**2** (> 90%)	2 d / 20 L (Reactor)	16 mg L^{-1} [b]	(34)
CYP267B1 (*E. coli*)	**2**	3 d / 50 mL	24 mg L^{-1}	(40)
CYP105D7 (*E. coli*)	**2**	1.5 d / 0.5 mL	44 mg L^{-1}	(22)
C. elegans	**2**	18 d / 45 mL	57 mg L^{-1} [b]	(35)
B. bassiana *C. nebularis* *M. hiemalis*	**2**, **3**, **4**, **5**	7 d / 400 mL	320 – 390 mg L^{-1} [b]	This study
CYP2C9 (*S. pombe*)	**2**	6 d / 1000 mL	468 mg L^{-1} [b]	(14)
P450 BM3 variants [c]	**2** (42%), **3** (58%)	-	512 mg L^{-1} [b]	(19)
P450 RhF (*E. coli*)	**3**	2 d / 800 mL (Reactor)	357/ max. 580 mg L^{-1}	(21)
Actinoplanes sp.	**2** (72%), **3** (18%), **15** (10%)	5 d / 100 mL	848 mg L^{-1} [b]	(44)
CYP116B64 (*E. coli*)	**3**	2 d / 100 mL	1600 mg L^{-1}	(45)
Naproxen (6)				
P450 BM3 variants [c]	**7** (66%)**, 16** (34%)	-	Max. 144 mg L^{-1} [b]	(19)(20)
A. niger	**7** (73%), **17** (27%)	2.5 d / 200 mL	220 mg L^{-1} [b]	(37)
M. hiemalis	**7**	4 d / 50 mL	980 mg L^{-1} [b]	This study
Ibuprofen (8)				
Microorganisms and heterologous expressed P450s	**9**, **10**, **11**	-	< 30 mg L^{-1} [b]	(41)(39) (40)(25)
P450 BM3 variants [c]	**9**	-	400 mg L^{-1} [b]	(20)
M. hiemalis	**9** (90%), **10** (2%), **11** (8%)	4 d / 50 mL	990 mg L^{-1} [b]	This study
Mefenamic acid (12)				
P. sordida	**13** (67%), **14** (13%), **18** 17%), **19** (3%)	7 d / 200 mL	> 3 mg L^{-1} [b]	(26)
P450 BM3 variants [c]	**13** (40%), **20** (55%), **21** (5%)	-	216 mg L^{-1} [b]	(18)(43)
B. bassiana	**13** (68%), **14** (32%)	4 d / 50 mL	520 mg L^{-1} [b]	This study

[a] The duration of the process includes cultivation of the main culture, expression (recombinant produced P450s) and subsequent biotransformation in days (d).

[b] The product titer was calculated from isolated yields.

[c] Purified enzyme (*in vitro*).

602

603

TAB 2

Results of the proteome analysis of the biotransformations with *Beauveria bassiana* carried out with different substrates. The numbers stand for the quantity of clearly attributable peptide fragments to a protein. A higher number contributes to a higher probability that the protein is actually present in the sample. A sample with glucose as substrate served as a reference.

	Glucose	Diclofenac (**1**)	(*R*)-2-phenoxypropionic acid
NADPH-CYP-Reductase	**14**	**9**	**10**
CYP52A10	**2**	0	**11**
CYP52A13	**5**	0	0
CYP548A5	0	**5**	0
CYP51F1	0	**1**	0
CYP684A2	0	0	**2**

620 **Figure legends**

621

622 FIG 1

623 Diclofenac (**1**) biotransformations with the filamentous fungi *Beauveria bassiana*,
624 *Clitocybe nebularis* and *Mucor hiemalis*. The reactions were carried out in 400 mL of PEG
625 broth with 0.6 g L^{-1} substrate for 144 h at 27°C and 180 rpm. **Red**: negative control without
626 cells; **Magenta**: standard of 4'-hydroxydiclofenac (**2**), 5-hydroxydiclofenac (**3**) and
627 5-hydroxyquinoneimine (**4**); **Green**: *Beauveria bassiana*; **Blue**: *Mucor hiemalis*; **Ocher**:
628 *Clitocybe nebularis*. The retention times for the different metabolites are: 11.9 min (**5**);
629 13.1 min (**4**); 13.3 min (**2** and **3**); 15.5 min (**1**).

630

631 SCHEME 1

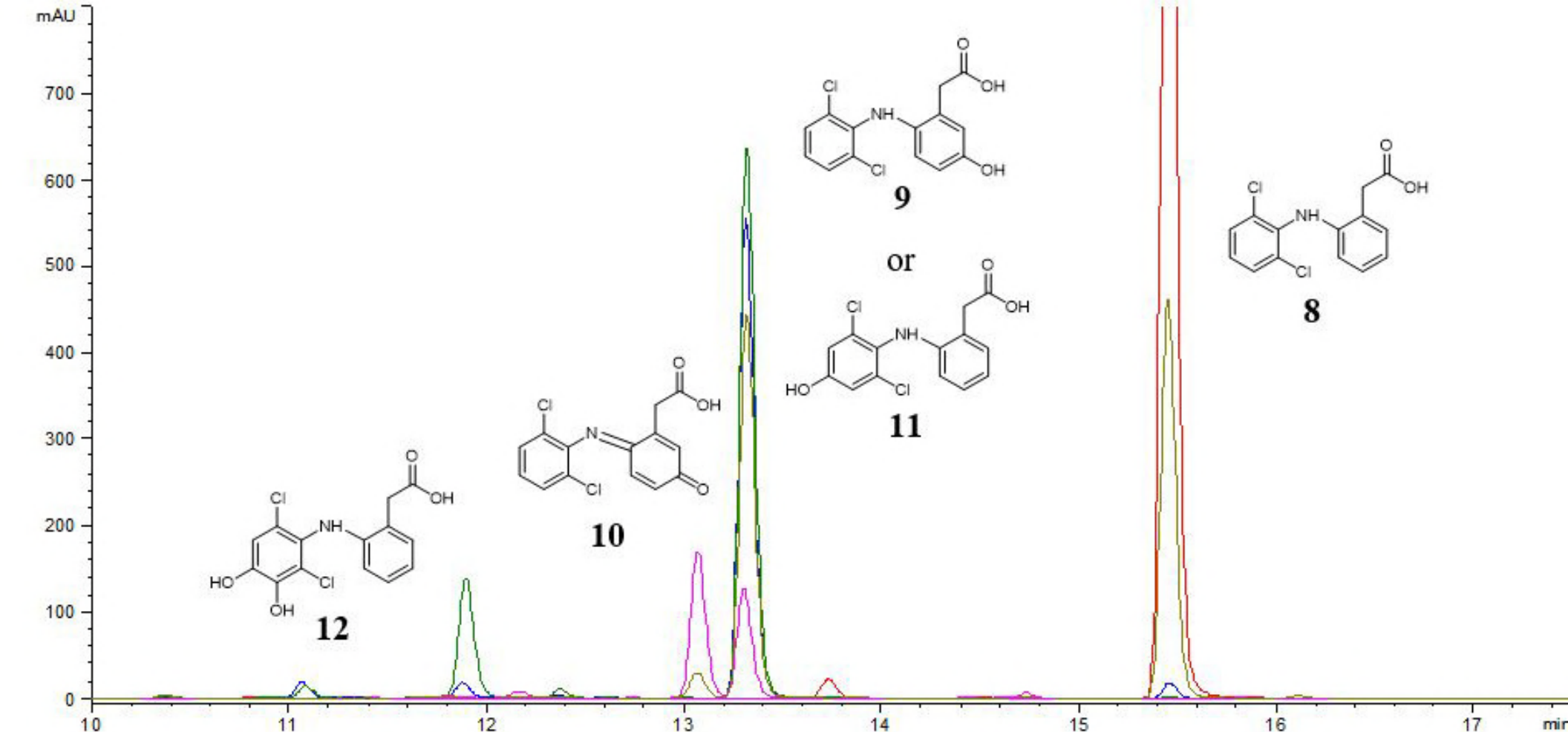
632 Diclofenac metabolites (**2** – **5**) synthesized by different filamentous fungi as biocatalyst
633 using 0.6 g L^{-1} substrate. The isolated yields after extraction and purification *via* semi-
634 preparative HPLC and the product distribution in brackets are given. The metabolites
635 4'-hydroxydiclofenac (**2**), 5-hydroxydiclofenac (**3**), 5-hydroxyquinoneimine (**4**),
636 3',4'-dihydroxydiclofenac (**5**) were identified by NMR. [a)]Not yet observed diclofenac
637 metabolite.

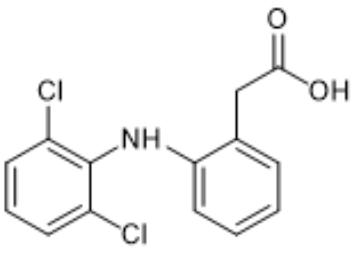
638

639 SCHEME 2

640 NSAID-metabolites synthesized by different filamentous fungi as biocatalyst using 1 g L^{-1}
641 substrate. The isolated yields after extraction and purification *via* semi-preparative HPLC
642 and the product distribution in brackets are given. The metabolites 6-*o*-desmethylnaproxen

643 (**7**), 2-hydroxyibuprofen (**9**), 1-hydroxyibuprofen (**10**), 1,2-dihydroxyibuprofen (**11**),
644 3'-hydroxymethylmefenamic acid (**13**) and 3'-carboxymefenamic acid (**14**) were identified
645 by NMR. [a)]Products were not purified; hence no isolated yield and exact products are given.
646

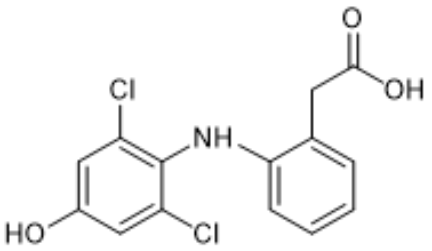

mAU
700
600
500
400
300
200
100
0
10
11
12
13
14
15
16
17
min
9
or
11
8
10
12



1

M. hiemalis

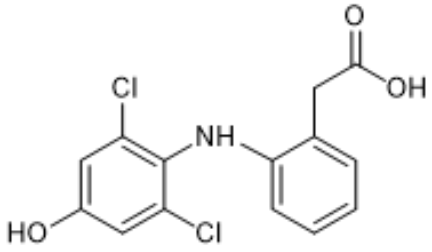
61.7%



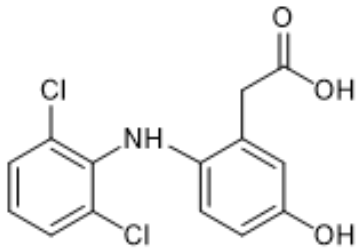
2 (> 99%)

C. nebularis

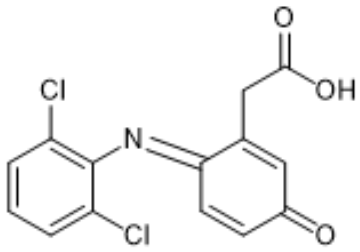
50.6%



2 (60%)



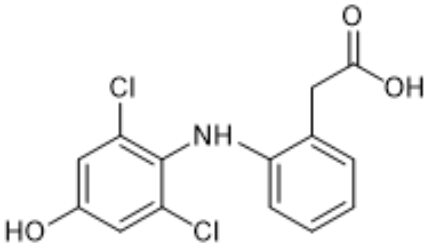
3 (30%)



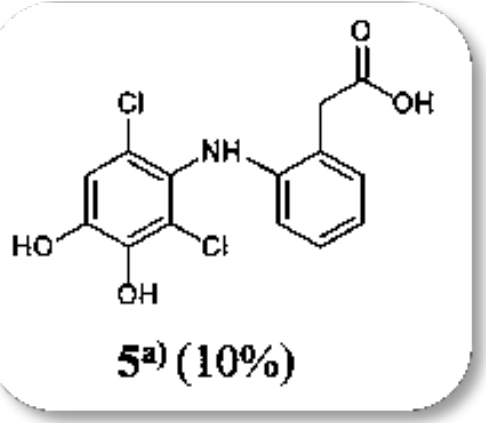
4 (10%)

B. bassiana

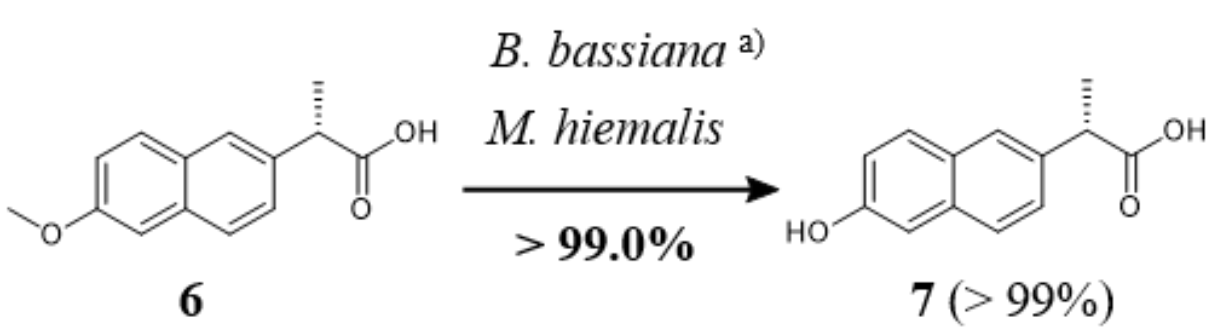
55.3%



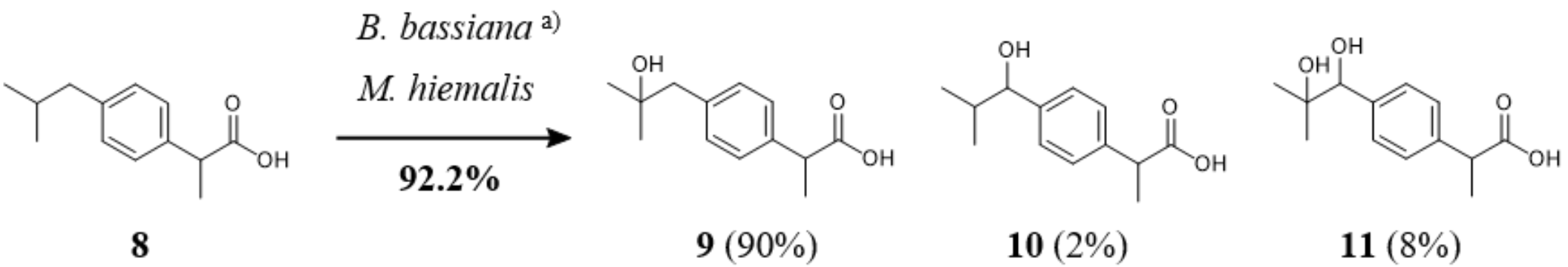
2 (90%)



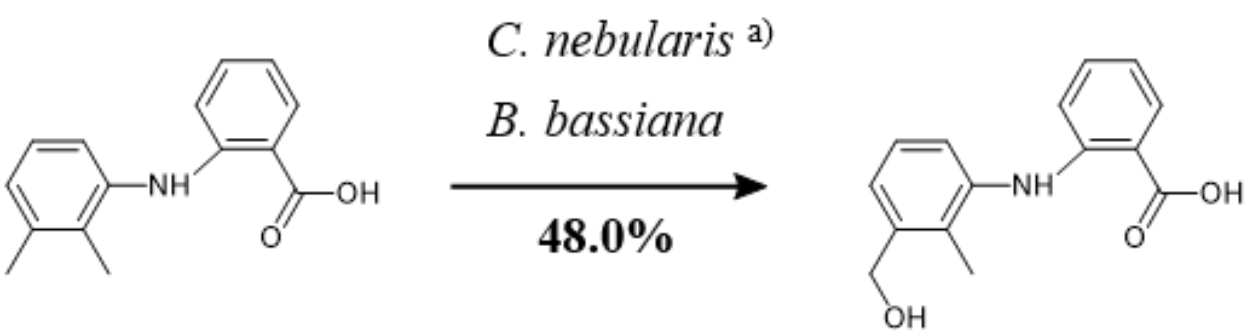
5[a] (10%)



6 **7** (> 99%)



8 **9** (90%) **10** (2%) **11** (8%)



12 **13** (68%) **14** (32%)