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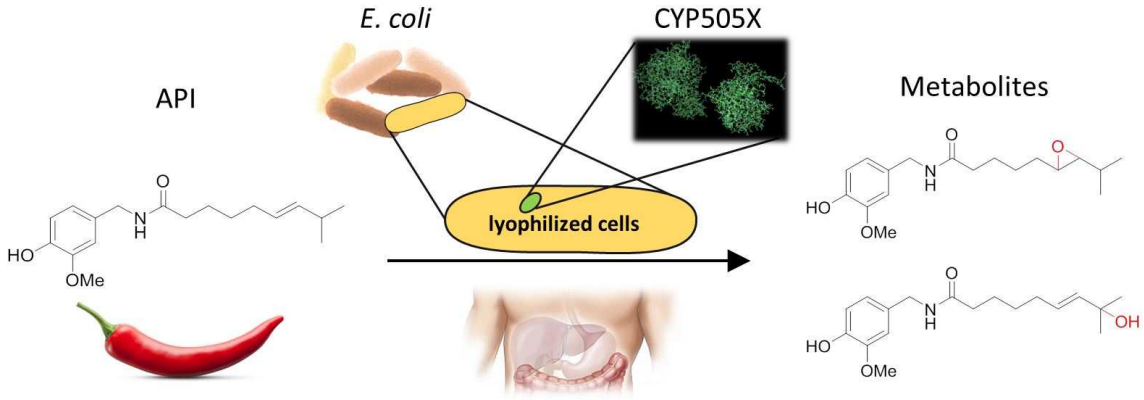

Aliphatic hydroxylation and epoxidation of capsaicin by Cytochrome P450 CYP505X

Anna K. Migglautsch[a], Melissa Willim[b], Bettina Schweda[a], Anton Glieder[c], Rolf Breinbauer[a, b], Margit Winkler [b*]

[a]*Institute of Organic Chemistry, Graz University of Technology, NAWI Graz, 8010 Graz, Austria*
[b]*Austrian Centre of Industrial Biotechnology (ACIB), Petersgasse 14/4, 8010 Graz, Austria*
[c]*bisy e.U., Hofstaetten, Austria*

*Corresponding author
E-mail address: margit.winkler@acib.at

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Abstract

Microbial cytochrome P450 enzymes (CYPs) are able to mimic the metabolism of human CYPs. One challenge is to identify the respective drug metabolites and to compare substrate specificities to those of the human enzymes. In this study, a class VIII self-sufficient CYP from *Aspergillus fumigatus* (CYP505X) and variants of this enzyme were heterologously expressed in *E. coli.* The substrate scope of the variants was determined using active pharmaceutical ingredients (APIs) and (hetero)cyclic compounds. Capsaicin – the active compound in chili peppers – was oxidized most efficiently (4.36 µM/min) in a whole cell mediated biotransformation. The products were isolated, purified and their structures elucidated by 1D and 2D NMR. The two major metabolites showed modifications on the lipophilic side chain. Specifically, capsaicin was hydroxylated at position 8 to give *(E)*-8-Hydroxy-*N*-(4-hydroxy-3-methoxybenzyl)-8-methylnon-6-enamide and epoxidized at the double bond to give *N*-(4-Hydroxy-3-methoxybenzyl)-5-(3-isopropyloxiran-2-yl)-pentanamide.

1. Introduction

Capsaicin (**1**) (8-methyl-*N*-vanillyl-6-nonenamide, Scheme 1) is the major pungent component of chilli peppers. Among other capsaicinoids, **1** is especially well known for its sensory irritant properties, and therefore finds application in pepper sprays[1] and in crop protection agents.[2] As an active pharmaceutical ingredient, **1** is used in heat wraps for pain relief. Moreover, capsaicin has substantial therapeutic significance including antioxidant, anticancer and anti-inflammatory activities and is in Phase III clinical trials as an agent for the treatment of arthritis, postoperative pain, acute and chronic neuropathic and musculoskeletal pain.[3] In the drug development process, authentic standards of human drug metabolites are needed for structure elucidation and toxicological tests. The challenge is to identify the molecular structure of these metabolites and to prepare significant amounts of each compound (milligram to gram quantities). For capsaicin(oids), human metabolites were recently identified.[4] A typical method to identify the metabolites is LC-MS/MS analysis of samples from incubations with disrupted tissue samples (e.g. human liver homogenate) or microsomal preparations thereof,[5] or recombinant drug metabolizing enzyme containing bactosomes[6] or supersomes.[7] Increasingly, human enzymes become available as recombinant catalysts, and their application in preparative syntheses has already been demonstrated for several metabolites of APIs.[8] However, many human enzymes are more complex in comparison to microbial counterparts, they are less easily accessible from microbial host systems and they may show less activity and stability. In case of cytochrome P450 enzymes (CYPs), human enzymes are membrane associated. Like other CYPs

they rely on an electron transport system that provides the electrons required for oxygen activation and substrate oxidation.[9] The redox partner of all drug and xenobiotic metabolizing CYPs is the NADPH-cytochrome P450 reductase (CPR). Microbial CYPs may be used as mimics of human CYPs.[10] CYP505X from *Aspergillus fumigatus* (*Af*CYP505X), for example, is a self-sufficient class VIII CYP, which is characterized by the fusion of the reductase domain to the CYP domain.[11] From the overall structure, it is similar to the well investigated CYP102 from *Bacillus megaterium*, commonly known as CYP BM3.[12] The advantage of self-sufficient CYPs is on the one hand their intrinsically balanced ratio of CYP to redox partner of 1:1 and on the other hand their soluble nature, which typically leads to higher expression levels as compared to membrane associated CYPs. Herein, we investigated wild-type *Af*CYP505X and a number of variants thereof heterologously expressed in *E. coli.* The resulting biocatalysts were evaluated for their potential to oxidize APIs and heterocyclic molecules with the aim to provide alternatives to the repertoire of oxidative human enzymes.

2. Results and Discussion

2.1 Screening of CYP505X for oxidation of APIs and heterocycles

Wild-type *Af*CYP505X and ten rationally designed variants were heterologously expressed in *E. coli* and used as lyophilized whole cell biocatalysts. A substrate profile was established using active pharmaceutical ingredients (APIs) and (hetero)cyclic compounds as listed in 4.1 as the substrates. Chlorzoxazone (**2**), Clopidogrel (**3**), Estriol (**4**), Ibuprofen (**5**), Metoprolol (**6**) 2-naphthol (**7**), Progesterone (**8**) and Tolbutamide (**9**) were oxidized by CYP505X variants (Tab. 1). Notably, wild-type CYP505X only oxidized capsaicin and chlorzoxazone (Tab. 1, entry 1), whereas a variant with five mutations (Tab. 1, entry 9) was capable to oxidize all nine compounds to some extent. Of all tested compounds, capsaicin – the active compound in chili peppers which is frequently used as an API in heat wraps for pain relief – was oxidized most efficiently. According to mass spectrometry, two or more different products were formed and we asked whether CYP505X produces human-like metabolites of **1** or different compounds.

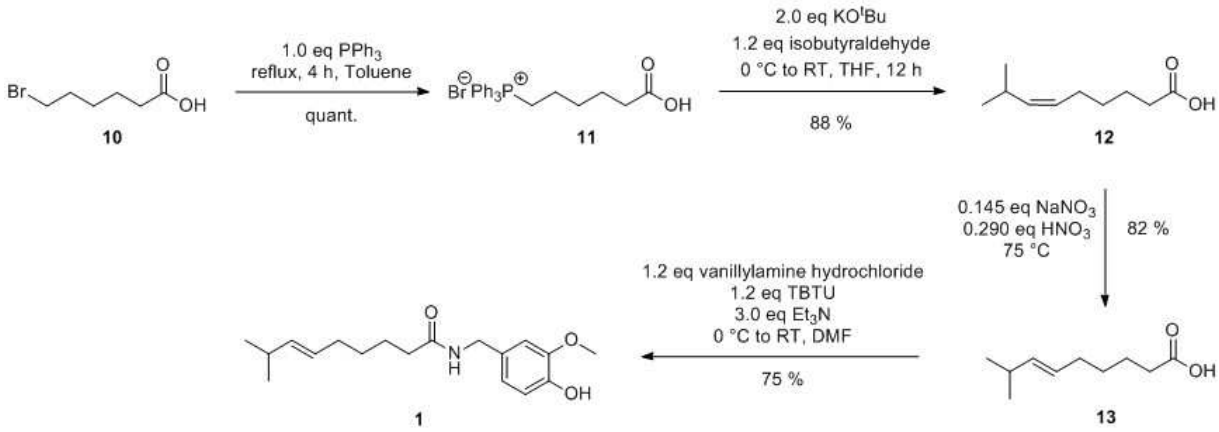
Tab. 1: Results of screening CYP505X variants on their ability to oxidize APIs. Conversions were calculated by area normalization and represent the average of at least two separate measurements.

		Conversion (%)								
entry	CYP505X variants	**1**[a]	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	CYP505X bsy01 (wild-type)	85[a]	7	0	0	0	0	0	0	0
2	CYP505X bsy02	72[a]	2	0	0	0	0	0	0	0
3	CYP505X bsy03	7	0	0	0	<1	0	0	0	0
4	CYP505X bsy04	20[a]	0	0	0	<1	0	12	0	0
5	CYP505X bsy05	28[a]	<1	0	1	<1	0	0	0	<1
6	CYP505X bsy06	28[a]	10	0	0	1	0	0	0	1
7	CYP505X bsy07	10[a]	<1	0	0	1	0	0	0	1
8	CYP505X bsy08	5	<1	0	0	1	0	0	0	<1
9	CYP505X bsy09	53[a]	25	5	2	15[a]	1	6	12[a]	1
10	CYP505X bsy10	54[a]	13	2	0	19[a]	<1	2	11[a]	2
11	CYP505X bsy11	17[a]	0	0	0	1	0	0	0	0

[a]HPLC-MS indicated the formation of 2-4 products

2.2 Synthesis of capsaicin

Capsaicin was produced *via* a four-step-synthesis, which is outlined in Scheme **1**. The first three steps were carried out according to Kaga *et al.* starting from 6-bromohexanoic acid **10**.[13] **10** was transformed to the corresponding Wittig salt **11**, which was then deprotonated using potassium *tert*-butoxide in dry THF and converted with isobutyraldehyde to the elongated acid **12**. In the following step, the acid, which is mainly present in its *Z*-form was isomerized. Fatty acids are known to isomerize upon treatment with HNO_2, generated *in situ* from $NaNO_2$ and a suitable acid.[14–17] An aqueous mixture of HNO_3 and $NaNO_2$ was used to obtain **13** in an E/Z ratio of 9:1 according to NMR analysis. Finally, TBTU-mediated amide coupling of acid **13** with vanillylamine produced capsaicin (**1**) in the same isomer ratio (E/Z 9:1) as a viscous oil, which crystallized under high-vacuum and freezing in liquid nitrogen. Eventually, **1** was furnished as an off-white power in 51 % overall yield.[18]



Scheme 1: Synthetic route to Capsaicin

2.3 Characterization of whole cell biotransformations of capsaicin

Capsaicin oxidation by wild-type *Afc*YP505X was studied further using a resting cell approach. After harvesting, the cell pellet was washed twice with buffer to remove CYP inhibiting compounds.[19] 'Intact' frozen and thawed cells in combination with citrate and $NADP^+$ were used to support cofactor regeneration.[20,21] The reaction progress was monitored by HPLC-MS. Therefore, separate analytical reactions on the 1 mL scale were started and three of the reactions were terminated at each given time point as depicted in Fig. 1. The first emerging and finally most prominent product according to the UV chromatogram showed a mass signal of 304 in the mass selective detector in positive mode, suggesting that the product might be M1, M4 or M9 (Fig. 2) as described by Reilley *et al.*[4] A second metabolite with a mass of 322 emerged, indicating insertion of an oxygen, for example on the aromatic ring or the lipophilic side chain (M5/M7 and M2/M3, as defined by Reilley *et al.*[4] respectively; Fig 2). The third product emerged in minor amounts and showed a mass signal of 338, indicating the consecutive insertion of two oxygen atoms. To identify appropriate conditions that would lead to high metabolite titers, 0.1-25 mM of **1** were used as the substrate concentration. Fig. 3 indicates that the initial reaction rates with substrate concentrations ≥2.5 mM – 10 mM were highly similar. Specifically, the activities were 4.07 µM/min at 2.5 mM of **1**, 4.36 µM/min at 5 mM and 4.29 µM/min at 10 mM. The highest total amount of metabolites was observed with 10 mM substrate. However, 10 mM substrate concentration did not only show slightly lower initial rate activity but also led to only 43% consumption of **1** after 24 h, whereas it was 79% for 5 mM and 90% for 2.5 mM. This concentration was therefore chosen for preparative scale reactions as described in 2.4.

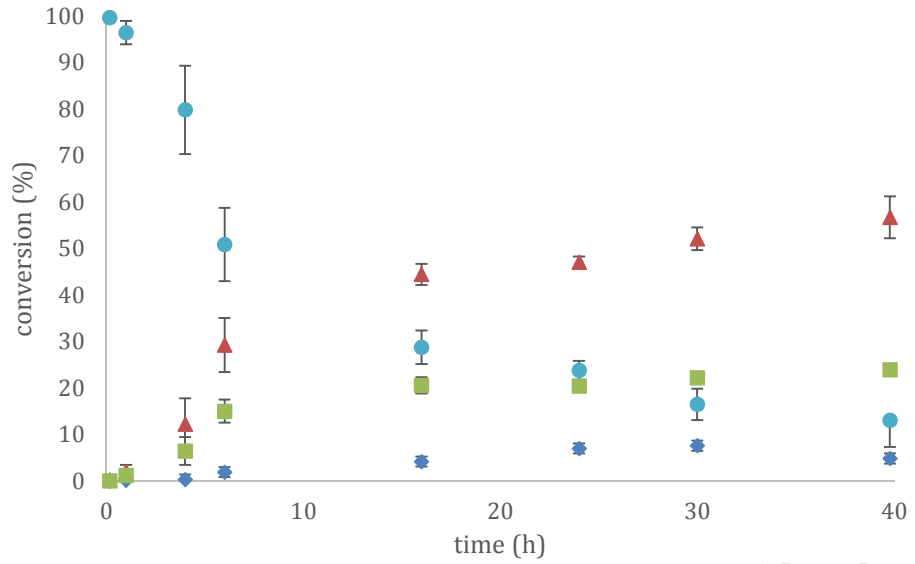


Fig. 1: Time dependent oxidation of capsaicin catalyzed by resting cells of *E. coli* DH5α expressing *A. fum.* CYP505X wild-type. Blue circles: capsaicin (molecular mass: 305 g/mol); Red triangles: Metabolite **1a** (molecular mass: 303 g/mol); Green squares: Metabolite **1b** (molecular mass: 321 g/mol); Black dashes: Metabolite **1c** (molecular mass: 337 g/mol)

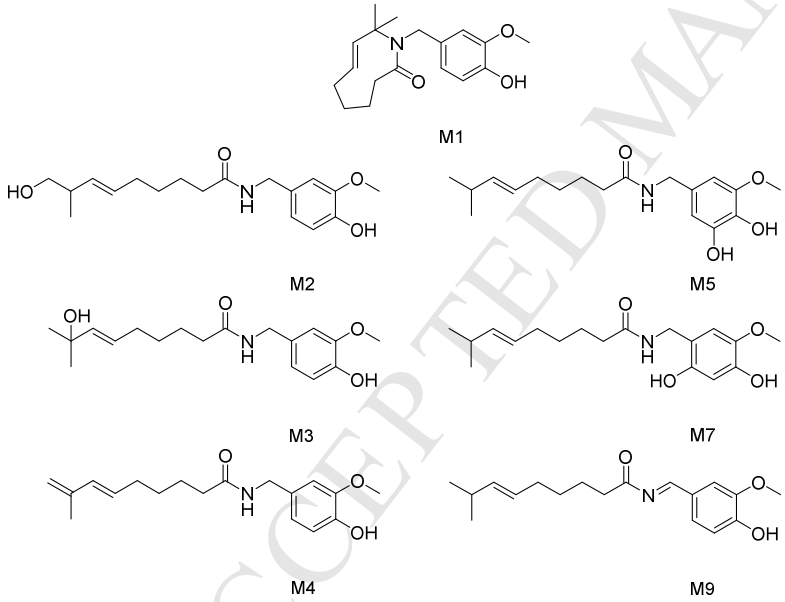


Fig. 2. Selected phase I metabolites as proposed by Reilley *et al.*[4] Figure adapted from Fig. 7 in Ref [4].

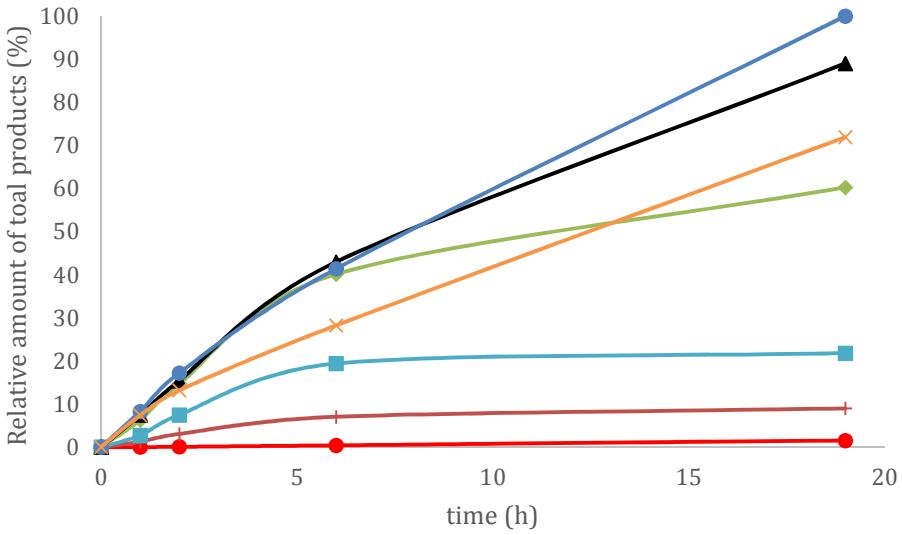
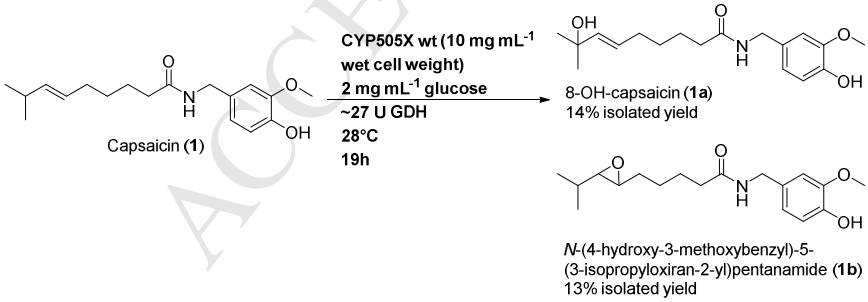


Fig. 3. Total of oxidation products of different capsaicin (**1**) concentrations catalyzed by resting cells of *E. coli* DH5α expressing *A. fum.* CYP505X wild-type. Red circles: 0.1 mM; Pink plus: 0.5 mM; light blue squares: 1 mM; Green diamonds: 2.5 mM; black triangles: 5 mM; Dark blue circles 10 mM; Orange crosses: 25 mM. Each time point represents biological duplicates of technical triplicates. Error bars were omitted for clarity.

2.4 Capsaicin metabolite preparation

On the multi-mg scale, two major (**1a** and **1b**) and one minor (**1c**) metabolite were formed (Fig. 1 and Scheme 2). The reaction did not proceed any further after approximately 16 h and was terminated after 19 h. The two major metabolites could be isolated in 14 and 13% yield, respectively, whereas the amount of **1c** was negligible and could not be used for structure elucidation. Unreacted capsaicin was re-isolated in 37% yield. According to NMR, **1a** resembles metabolite M3 (Fig. 2) that is formed by human CYP3A4 and 2C8. As a tertiary alcohol, the compound is prone to dehydration, which may explain the compounds mass peak at 304 upon HPLC-MS detection. **1b** is a capsaicinoid that has not yet been explicitly associated with CYP activity, however, it was observed as a metabolite when **1** was incubated in the presence of *Penicillium janthinellum* AS 3.510.[22] The cytotoxic potential against cancer cells has been evaluated using synthetic **1b**, and oxidant based action in human breast carcinoma cells was ascribed to this compound.[23] CYP505X used herein was annotated as a putative fatty acid hydroxylase. This may explain the high activity of the wild-type enzyme for the hydroxylation of the lipophilic side-chain of capsaicin.



Scheme 2: Biooxidation of **1**

3. Conclusion

Self-sufficient Cytochrome P450 enzyme CYP505X from *Aspergillus fumigatus* AF293 was evaluated as a biocatalyst for the biooxidation of APIs and heterocyclic compounds. Whereas a variant with five mutations in the amino acid sequence was able to oxidize 9 xenobiotics, the wild-type CYP505X was most efficient for the metabolization of capsaicin using a resting cell biotransformation approach. This reaction was studied in detail and suitable substrate

concentration (5 mM) and reaction time (<24 h) were determined in view of upscaling the reaction to preparative scale. Two products were isolated, purified by preparative HPLC and their chemical structures determined by NMR (Supporting Information). One metabolite was a product hydroxylated at the ω-1 position of the aliphatic chain of capsaicin, which has previously been described as metabolites of human CYP 3A4 and 2C8. The second metabolite was characterized by epoxidation of the double bond in the aliphatic chain. This compound has not been described as a human CYP metabolite, but it was observed upon incubation with fungal cells, possibly as a product of the action of a microbial CYP.

4. Experimental section

4.1 Strains and chemicals

Escherichia coli DH5α harboring the pMS470_CYP505-M1 plasmid was used. The gene of interest codes for the wild-type protein sequence of *Aspergillus fumigatus* Af293 CYP505X with the protein accession number EAL92660. Ten variants (designated pMS470_CYP505-bsy01 to pMS470_CYP505-bsy011) with up to seven amino acid mutations were similarly expressed in *E. coli* DH5α or *E. coli* BL21 (DE3).
Capsaicin (**1**) was synthesized as described in section 4.5 or purchased from Sigma Aldrich (Order Nr. M2028, ≥95% purity). Benzydamine hydrochloride, Caffeine, Chlorzoxazone (**2**), Clopidogrel (**3**) bisulfate, Dextrometorphane, Sodium diclofenac, Ethionamide, Flumetsulam, Harmine, Hydrocortisol, Lidocain, Metoprolol (**6**) tartrate, 2-naphthol (**7**), Naproxen, Phenacetine, Piperine, Progesterone (**8**), Propranolol hydrochloride, quinazoline, Salbutamol hemisulfate, Testosterone, Tolbutamide (**9**), and Trifluoperazine dihydrochloride were purchased from Sigma Aldrich/Fluka. Estriol (**4**) Nebivolol hydrochloride, Simvastatine, Famciclovir and Ibuprofen (**5**), were obtained from TCI (Tokyo, Japan) and Vardenafil hydrochloride trihydrate from USP (Rockville, USA). Moclobemide was ordered from AvaChem Scientific (San Antonio, USA). DMF was purchased as absolute solvent from Acros Organics. Toluene (Sigma Aldrich, 99.7%) was dried in an aluminum oxide column under inert conditions and stored in Schlenk bottle over 4A molecule sieves under argon atmosphere. Tetrahydrofuran was dried at reflux temperature under argon atmosphere over sodium until benzophenone indicated dryness by a deep blue color. All applied starting materials were commercially available from Alfa Aesar and Sigma Aldrich and were used as received. Silica gel chromatography was performed with Acros Organics silica gel 60 (35-70 µM). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE III 300 spectrometer (^{1}H: 300.36 MHz; ^{13}C: 75.53 MHz) and chemical shifts are referenced to residual protonated solvent signals as internal standard. Melting points were determined with the apparatus "Mel-Temp®" from Electrothermal with an integrated microscopical support. GDH.002 was obtained from DSM Innovative Synthesis BV.

TB Medium consists of 12 g tryptone, 24 g yeast extract, 4 mL glycerol in 900 mL of ddH_2O. TB-buffer consists of 23.1 g KH_2PO_4 and 125.4 g K_2HPO_4 in 1 L of ddH_2O (pH 7.6). Trace salt solution consists of 2.45 g Fe(III)citrate, 0.121g $ZnCl_2$, 0.2 g $CoCl_2 \cdot 6\ H_2O$, 0.1 g $NaMoO_4 \cdot 2\ H_2O$, 0.127 g $CuCl_2 \cdot 2\ H_2O$ and 0.05 g H_3BO_3 per 100 mL.

4.2 Preparation of resting-cell biocatalyst

Pre culture: One cell colony was used to inoculate 30 mL of TB-media supplemented with 100 mg/L ampicillin. The cells were grown at 37 °C on an orbital shaker at 120 rpm overnight.

Main culture: 900 mL of TB-medium, 100 mL of TB-buffer, 250 µL trace salt solution, 125 µL 1M $MgCl_2$ and 1 mL ampicillin 100 mg/L were mixed. 400 mL of this culture medium in 2 L Erlenmayer flask without baffles were inoculated to OD_{600} of 0.05. The cells were agitated at 37 °C in an orbital shaker at 120 rpm until an OD_{600} of 0.6-0.8 was reached. The flasks were incubated at 4 °C for 1 h. 1 mM IPTG was added to induce protein expression in an orbital shaker at 28 °C and 120 rpm for 24 h.

Cell harvest: The cell suspension was centrifuged for 15 min at 4 °C and 4,000 rpm in a Beckman Coulter Avanti J-20 centrifuge using a JA10 rotor. The wet cell pellet was washed twice with 50 mM PS-buffer and centrifuged each time for 20 min at 14,000 rpm in an Eppendorf centrifuge 5810R. The cell pellet was resuspended in about 10 mL 100 mM PS-buffer and stored at -20°C.

4.3 Biooxidation assay

Frozen cells were thawed and suspended in PS buffer (100 mM potassium phosphate, pH 7.4, 8.5% w/v sucrose) in 24-well plates. To 825 µL of cell suspension, 50 µL of trisodium citrate (1 M in ddH_2O), 50 µL of $NADP^+$ (1 mM in ddH_2O), 10 µL of $MgCl_2$ (1 M in ddH_2O), 10 µL of glucose (1 M in ddH_2O), and 5 µL GDH (10 mg mL^{-1} in ddH_2O). The reaction was started by the addition of 50 µL of capsaicin (100 mM in DMSO). The final cell density at 600 nm (OD_{600}) was 100 in a total volume of 1 mL. The plate was sealed with gas permeable adhesive seal and incubated at 30 °C and 120 rpm in an orbital shaker, typically for 16 h. The reactions were terminated by the addition of 1 mL of MeOH/ACN 1:1 v/v. After mixing, 1000 µL of the reaction mix was transferred into 1.5 mL Eppendorf vessels. Solids were removed by centrifugation for 5 min at 4 °C at 13,400 rpm in a tabletop centrifuge. 200 µL of the supernatant were transferred into wells of polypropylene microtiter plates and the conversion was determined by HPLC-MS measurement. These were performed on a 1200 HPLC Series equipped with G1379B degasser, G1312B binary pump, SL G1367C HiP-ALS SL autosampler, G1314C VWD SL UV detector, G1316B TCC SL column oven and G1956B mass selective detector (MSD) with a Kinetex 50x4.6 mm; 2.6u; C18; 100 Å HPLC column (Phenomenex) equipped with a UHPLC C18 Security Guard ULTRA cartridge (Phenomenex). The mobile phases were 0.1% v/v formic acid in water, and acetonitrile (ACN) at a flow rate of 1.00 mL/min. A stepwise gradient was used at 25 °C: 0% ACN (0-1 min), 0-100% ACN (1-4 min), 100% ACN (4-5 min) and 0% ACN (5-6 min) re-equilibration. The compounds were detected at 254 nm (DAD). The mass spectrometer was operated in positive electrospray ionization mode. The drying gas flow was 12 L/min at 350 °C, and the capillary voltage was 3 kV. Selected-ion monitoring or full-scan product ion spectra were collected. MS spectra supported data evaluation. For calculations, peak areas of the UV chromatograms recorded at 254 nm were used. Retention times were 3.5 min for **1c**, 3.7 min for **1a**, 4.0 min for **1b** and 4.4 min for **1**.

4.4 Kinetic characterization

Time dependence of capsaicin oxidation was monitored as described in 4.3. The concentration of **1** in the reaction was 5 mM. For every time point, three parallel reactions were started and terminated individually. Four of 8 time points were carried out in technical triplicates and the other four in biological duplicates of technical triplicates. Error bars represent standard deviations.

To determine the optimal substrate concentration for a scale up, time dependence of capsaicin oxidation was monitored in two independent experimental series as described in 4.3, however, different concentrations of **1** were used (0.2-25 mM). For every time point, three parallel reactions were started and terminated individually.

4.5 Capsaicin synthesis

3.5.2. (6-Carboxyhexyl)triphenylphosphonium bromide (**11**)

In a 100 mL triple-necked round-bottom flask 5.01 g (25.7 mmol, 1.0 eq.) 6-bromohexanoic acid and 6.71 g (25.6 mmol, 1.0 eq.) triphenylphosphine were dissolved in 30 mL dry toluene. The reaction mixture was heated to 120 °C and stirred for 17 h. After cooling to room temperature, the product was collected by filtration and washed with toluene (2 x 20 mL) and Et_2O (2 x 20 mL) before drying under reduced pressure. **11** was obtained as colorless powder in quantitative yield. m.p. = 201 – 205°C; ^{1}H-NMR (300.36 MHz; $CDCl_3$) δ = 9.12 (s, 1H), 7.74 (m,

15H), 3.60 (s, 2H), 2.34 (s, 2H), 1.64 (s, 6H); ^{13}C-NMR (75.53 MHz; $CDCl_3$) δ = 175.9, 135.1, 133.7, 133.6, 130.7, 130.5, 34.27, 29.6, 24.0, 21.9, 22.2.

3.5.3. (*Z*)-8-Methyl-6-nonenoic Acid (**12**)

In a 250 mL twin-necked round-bottom flask 3.00 g (6.57 mmol, 1.0 eq.) **11** and 1.53 g (13.89 mmol, 2.0 eq.) KO^{t}Bu were dissolved in 80 mL dry THF and cooled to 0 °C using an ice bath. The reaction mixture turned bright red while the compounds were dissolved. A solution of 720 µL (7.89 mmol, 1.2 eq.) isobutyraldehyde in 5 mL dry THF was slowly added to the cooled reaction mixture, which turned white immediately. After the addition was completed, the reaction mixture was warmed to room temperature and stirred for 12 h. The reaction mixture was quenched by addition of 80 mL H_2O at 0 °C. After acidification to pH 2, the reaction mixture was extracted with EtOAc (3 x 80 mL) and the combined organic layers were washed with brine (1 x 100 mL) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure. Purification via flash chromatography (125 g silica gel, cyclohexane/EtOAc = 4/1 + 0.5% glacial acetic acid, fraction size: 70 mL) gave **12** as a colorless oil (0.99 g, 88 %). ^{1}H NMR (300.36 MHz; $CDCl_3$) δ = 10.51 (s, 1H), 5.30 (m, 2H), 2.57 (q, $^3J_{HH}$ = 13.6, 6.8 Hz, 1H), 2.36 (t, $^3J_{HH}$ = 7.4 Hz, 2H), 2.03 (m, $^3J_{HH}$ = 7.08 Hz, 2H), 1.65 (m, 2H), 1.40 (m, 2H), 0.95 (d, $^3J_{HH}$ = 6.1 Hz, 6H) ^{13}C NMR (75.53 MHz; $CDCl_3$) δ = 180.3, 138.2, 126.7, 34.1, 29.4, 27.0, 26.6, 24.4, 23.3.

3.5.4. (*E*)-8-Methyl-6-nonenoic Acid (**13**)

In a 25 mL round-bottom flask with 0.99 g (5.82 mmol, 1.0 eq.) **12**, 420 µL (0.145 eq.) 2 M $NaNO_2$ and 280 µL (0.289 eq.) 6 M HNO_3 were added. The reaction mixture was heated to 75 °C and stirred for 35 min. The yellow reaction mixture was cooled down to room temperature and diluted with 10 mL Et_2O. The organic layer was washed with 10 mL H_2O. The aqueous phase was acidified to a pH value of 2 and extracted with 10 mL Et_2O. The combined organic layers were washed with brine (1 x 10 mL), dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. Purification via flash chromatography (50 g silica gel, cyclohexane/EtOAc = 7/1 + 0.5% glacial acetic acid, fraction size: 20 mL) gave **13** as yellowish oil in 82 % yield (0.81 g). ^{1}H NMR (300.36 MHz; $CDCl_3$) δ = 5.31 (m, 2H), 2.56, 2.21 (m, 1H), 2.35 (t, $^3J_{HH}$ = 7.4 Hz, 2H), 2.02 (m, 2H), 1.65 (m, 2H), 1.40 (m, 2H), 0.95 (d, $^3J_{HH}$ = 6.2 Hz, 6H) ^{13}C NMR (75.53 MHz; $CDCl_3$) δ = 180.1, 138.3, 126.5, 34.1, 32.2, 31.1, 29.1, 24.3, 22.8 (2C)

3.5.5. Capsaicin (**1**)

A 100 mL round-bottom flask equipped with Schlenk adapter and stirring bar was charged with 750 mg (4.41 mmol, 1.0 eq.) **13** and 20 mL dry DMF were added. After cooling the reaction mixture to 0 °C, 1.83 mL (13.22 mmol, 3.0 eq.) Et_3N, 1.7 g (5.29 mmol, 1.2 eq.) TBTU and 1.0 g (5.29 mmol, 1.2 eq.) vanillylamine hydrochloride were added successively. The yellowish suspension was stirred at RT for 2 h. The reaction mixture was quenched by the addition of 100 mL brine. A colorless precipitate formed which dissolved in water by the addition of water. The reaction solution was extracted with EtOAc (3 x 70 mL) and the combined organic layers were washed with 1 M HCl (1 x 50 mL), saturated $NaHCO_3$-solution (1 x 50 mL), and brine (1 x 50 mL). The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The product was purified via flash chromatography (75 g silica gel, cyclohexane/EtOAc = 2/1, fraction size: 50 mL) yielding a sticky oil which crystallized under high vacuum and freezing in liquid nitrogen. (583 mg, 82 %) m.p. = 58 – 61°C
^{1}H NMR (300.36 MHz; $CDCl_3$) δ = 6.80 (m, 3H), 5.70 (s, 2H), 5.30 (m, 2H), 4.34 (d, $^3J_{HH}$ = 5.5 Hz, 2H), 3.86 (s, 3H), 2.23 (m, 2H), 1.99 (m, 2H), 1.66 (m, 2H), 1.37 (m, 2H), 0.93 (d, $^3J_{HH}$ = 6.2 Hz, 6H,) ^{13}C NMR (75.53 MHz; $CDCl_3$) δ = 173.0, 146.9, 145.3, 130.5, 138.2, 126.6, 120.9, 114.5, 110.8, 56.1, 43.7, 36.8, 32.4, 31.1, 29.4, 25.4, 22.8

4.6 Preparative scale oxidation of capsaicin

For the bioconversions, 1 L shaking flasks without baffles were used. The flasks were closed with cloth to allow oxygen access. Approximately 3 g of wet cells were suspended in 50 mM PS buffer (pH 7.4) in a volume of 165 mL. Successively, 10 mL trisodiumcitrate dihydrate (1 M), 2 mL glucose (1 M), 1 mL GDH solution (10 mg/mL), 2 mL $MgCl_2$*6 H_2O (1 M), 150 mg **1** in 4 mL DMSO and finally 10 mL $NADP^+$ (1 mM) were added. The reaction mixture was agitated at 120 rpm at 28 °C. Samples were withdrawn and analyzed periodically. After 19 h, unreacted **1** and the metabolites were extracted with EtOAc. Centrifugation was used to facilitate phase separation. The combined organic layers were washed with brine, dried over Na_2SO_4, and the solvent was removed under reduced pressure. Compounds were separated via preparative HPLC on a Thermo Scientific UltiMate 3000 system. Detection was accomplished with a Dionex ULtiMate Diode Array Detector. The separations were carried out on a Macherey-Nagel 125/21 Nucleodur® 100-5 C18EC (125 x 21 mm, 5.0 µm) column. As eluents acetonitrile and water with 0.01% HCOOH were used. Two methods were used: Method 1: 0.0 min 40% MeCN, 0.1 – 5.0 min linear increase to 60% MeCN, 5.1 – 7.0 60% MeCN isocratic, 7.1 – 9.0 min linear increase to 100% MeCN, 9.1 – 11 min 100% MeCN isocratic, 11.1 – 13.0 min linear decrease to 40% MeCN, 13.1 – 15.0 min 40% MeCN isocratic, 12 mL/min, 30 °C.

Method 2: 0.0 min 2% MeCN, 0.1 – 20 min linear increase to 100% MeCN, 20.1 – 22.0 min 100% MeCN isocratic, 22.1 – 24.0 min linear decrease to 2% MeCN, 12 mL/min, 30 °C.

Metabolite **1a**: *(E)*-8-Hydroxy-*N*-(4-hydroxy-3-methoxybenzyl)-8-methylnon-6-enamide. Yield: 20.9 mg (0.065 mmol, 13%), colorless oil, $C_{18}H_{27}NO_4$ [321.42 g/mol], t_R = 4.66 min (preparative HPLC).
^{1}H NMR (300.36 MHz; DMSO) δ = 8.83 (bs, 1H), 8.17 (bs, 1H), 6.68 (m, 3H, H_{arom}), 5.46 (m, 2H), 4.42 (s, 1H), 4.14 (d, $^3J_{HH}$ = 5.5 Hz, 2H, CH_2-NH), 3.73 (s, 3H, OCH_3), 2.11 (t, $^3J_{HH}$ = 6.9 Hz, 2H), 1.95 (m, 2H), 1.52 (m, 2H), 1.31 (m, 2H), 1.14 (s, 6H)

^{13}C NMR (75.53 MHz; DMSO) δ = 171.9, 147.4, 145.4, 139.4, 130.5, 124.9, , 119.7, 115.2, 111.6, 68.8, 55.5, 41.8, 35.2, 31.4, 30.1(2C), 29.0, 25.5.

Metabolite **1b**:[23] *N*-(4-Hydroxy-3-methoxybenzyl)-5-(3-isopropyloxiran-2-yl)-pentanamide. Yield: 20.1 mg (0.069 mmol, 14%), yellowish oil, $C_{18}H_{27}NO_4$ [321.42 g/mol], t_R = 5.18 min (preparative HPLC).
^{1}H NMR (300.36 MHz; DMSO) δ = 8.47 (bs, 1H), 8.21 (bs, 1H), 6.69 (m, 3H), 4.13 (d, $^3J_{HH}$ = 5.6 Hz, 2H), 3.72 (s, 3H), 2.84-2.67 (m, 1H), 2.42 (m, 1H) 2.11 (t, $^3J_{HH}$ = 6.6 Hz, 2H), 1.55 (m, 2H), 1.39 (m, 5H), 0.89 (m, 6H)

^{13}C NMR (75.53 MHz; DMSO) δ = 171.9, 147.5, 145.5, 130.4, 119.7, 115.2, 111.7, 63.2, 56.7, 55.6, 41.8, 35.3, 31.3, 29.9, 25.3, 25.1, 18.9, 18.2.

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