Supplementary Material for Chemical Communications

Supporting Information

Stereoselective hydroxylation of an achiral cyclopentanecarboxylic acid derivative using engineered P450s BM-3

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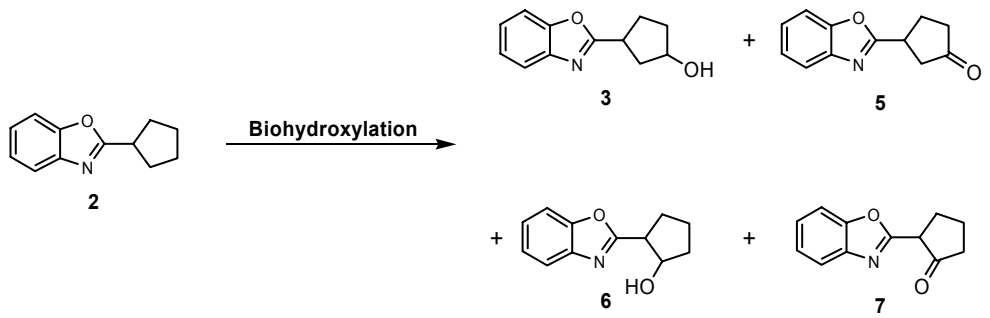
Chemicals: For the Caltech laboratory: Unless noted otherwise, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). NADPH was from Biocatalytics, Inc. (Pasadena, CA). Chloroform was purchased from Mallinchrodt (Paris, KY).For the TU-Graz laboratory: All chemicals were purchased from either Sigma-Aldrich / Fluka or Merck. If required, chemicals and solvents were purified according to Perrin and Armarego.[1] Optical rotations were measured on a Perkin Elmer Polarimeter 341. ^{1}H and ^{13}C NMR were recorded on either a Gemini 200 (Varian) or UNITY / INOVA 500 (Varian). HETCOR, DEPT and COSY experiments were carried out as required. $CDCl_3$ was used as solvent and as internal standard. Before use, the $CDCl_3$ was filtered through a short plug of basic alumina to remove traces of acid. The minor isomer is given in italics. Chiral HPLC was determined with a Agilent 1100 Series HPLC system containing column unity, degasser, quarternary pump, autosampler, UV and RI detector. A Lichrosorb SJ 60, 10 µm normal phase precolumn was used. LC was performed on Silica gel 60 (Merck, 70 - 230 mesh) using mixtures of ethyl acetate and petroleum ether unless otherwise stated. GC analysis was carried out on an Agilent 6890 N network GC system with a 7683 series autosampler, FID and a Agilent 19091J-413 HP 5 column (30 m). TLC was performed on Silica gel 60 F254 aluminium plates (Merck) and compounds detected with UV (254 nm) and dipping into either reagent A (1 g vanillin, 140 ml ethanol or methanol, 20 ml concentrated H_2SO_4) or reagent B (10% H_2SO_4, 10% $(NH_4)_6Mo_7O_{24} \times 4\ H_2O$ and 0.8% $Ce(SO_4)_2 \times 4\ H_2O$ in water). The TLC plates were then developed by heating with a heat gun. Mixtures of petroleum ether/ethyl acetate were used as eluent. Centrifugation was carried out with a Beckman Coulter Avanti J-20 XP centrifuge with a JA-10 rotor. For the biotransformations a Memmert BE 600 incubator was used.



Scheme 1 Biohydroxylation of substrate **2**

Synthesis of 2-cyclopentylbenzoxazole 2 (TU-Graz). The biotransformation substrate **2** was synthesised using published methods.[2]

Synthesis of Product Standards (TU-Graz). For the identification of the peaks in HPLC and GC, for example for the determination of the *ee/de* of the hydroxylated products **3**, reference compounds were chemically synthesised.

Racemic ketone 5. The title compound was obtained in 34% yield from reacting racemic 3-oxocyclopentanecarboxylic acid (1.07 g, 8.35 mmol) with 2-aminophenol (0.91 g, 8.35 mmol) as described (Method B) in the published synthesis for benzoxazoles.[2] The desired ketone **5** was purified by column chromatography (pet. ether : ethyl acetate = 5:1 to 3:1). Physical data are identical to literature.[2]

Product 3 Standards. For the determination of *ee/de* values of product **3** obtained from the biohydroxylation, three different reference mixtures were used.

reference 1 [(*S,S*)-**3**, 79% *ee*, 95% *de*]. The title compound was obtained from biohydroxylation of **2** with *Sphingomonas* sp. HXN-200.[3] The absolute configuration of this sample was determined by comparing optical rotation ($[\alpha]_D^{20}$ = +20.1°, c = 2.1 in CH_2Cl_2) to the published value.[2]

reference 2: A mixture of all four diastereoisomers of **3,** [(*S,R*)-**3** and (*R,S*)-**3**] : [(*S,S*)-**3** and (*R,R*)-**3**] = 5:1 (NMR), was also required for reference purposes. The mixture was obtained by reducing racemic ketone **5** with $NaBH_4$ in methanol. Accordingly, 1.5 eq of $NaBH_4$ were suspended in 2 ml of methanol. After 30 seconds, a solution of 80 mg (0.40 mmol) of ketone **5** in 1 ml of methanol was added to the stirred suspension. After five minutes the reaction was complete (TLC). To the reaction mixture, 5 ml of CH_2Cl_2 were added, the solution was washed with equal amounts of 5% aqueous HCl, saturated aqueous $NaHCO_3$ and water, dried over Na_2SO_4, filtered and concentrated down under reduced pressure. Column chromatography (pet. ether : ethyl acetate = 2:1) gave 65 mg of **3** (80% yield) as a colourless syrup.

^{1}H-NMR ($CDCl_3$): δ = 1.71-2.46 (m, 7H), 3.49, *3.72* (m, *p*, 1H), 4.46, *4.58* (2 × bm, 1H), 7.28, 7.45, 7.63 (3 × m, 2H, 1H, 1H). ^{13}C-NMR ($CDCl_3$): δ = *29.0*, 30.1, *35.1*, 35.8, *36.7*, 37.3, 40.4, *40.9*, *73.4*, 73.5, *110.4*, 110.5, 119.6, *124.2*, 124.4, *124.6*, 124.7, 141.2, 150.9, *170.3*, 171.3.

reference 3: A mixture of (*S,S*)-**3** and (*S,R*)-**3** was also prepared. The mixture was obtained by the oxidation of **reference 1** with pyridinium chlorochromate (PCC) in CH_2Cl_2 and subsequent reduction of the isolated ketone **5** to alcohol **3** with $NaBH_4$ in methanol. For the oxidation, 1.5 eq of PCC were suspended in 1 ml of CH_2Cl_2 and a solution of 45 mg (0.22 mmol) of **3** (79% *ee*, 95% *de*) in 1 ml of CH_2Cl_2 was added. The colour of the reaction mixture changed from brown to dark grey. After 1 h the reaction was complete (TLC); the reaction mixture was immediately put onto silica gel and, after column chromatography (pet. ether : ethyl acetate = 1:1), 36 mg (81% yield) of ketone **5** were obtained as white crystals. Physical data were identical to literature.[2] Subsequently, 25 mg of **5** were reduced as described for **reference 2**. 20 mg (80% yield) of the desired mixture were obtained. In this case, chromatography was not necessary.

Cultivation of BM-3 mutants for whole cell biotransformations (TU-Graz). The following mutants were tested: 35-4, 139-3, 139-29, 139-37, A, B, J, L, 9-10A, 9-10A-A328V, 9-10A-A-82L, 1-12G as well as the wild type. (see Table 1 for mutations).

Table 1 Amino acid substitutions in P450 BM-3 mutants

Position (wt)	DNA mutation	35-4	139-3	139-29[5]	139-37	A	B	L	J	9-10A	9-10A-A82L	9-10A-A328V	1-12G
E5	A15G						E[1]						
R47	C142T									C	C	C	C
V78	T236C	A	A		A	A	A	A	A	A	A	A	A
A82	247-249[2]										L		L
K94	A284T									I	I	I	I
K99	A297G						K[1]						
F107	C324T		F[1]		F[1]	F[1]	F[1]	F[1]	F[1]	F[1]	F[1]	F[1]	F[1]
A112	A336G							A[1]					
H138	C415T		Y										
P142	C427T									S	S	S	S
G157	G472T					C							
N159	C480T				N[1]								
F162	T487C							L					
D168	G505A							N					
T175	C527T		I		I	I	I	I	I	I	I	I	I
V178	G535A		I										
A184	553-555[3]					I	I						
A184	C554T		V		V			V	V	V	V	V	V
N186	A559G				D								
R203	C612T				R[1]								
F205	T617G								C	C	C	C	C
D217	A653T				V								
D223	T669A					E	E						
S226	C681G								R	R	R	R	R
G227	G682A					S	S						
H236	709-711[4]					R							
H236	T711G	Q	Q		Q		Q	Q	Q	Q	Q	Q	Q
E252	A758G	G	G		G	G	G	G	G	G	G	G	G
R255	C766A		S						S	S	S	S	S
V281	T845C					A							
A290	C872T		V		V	V	V	V	V	V	V	V	V
A295	G886A		T										
P303	A912C				P[1]								
Q308	A924G						Q[1]						
A328	C986T											V	V
L353	C1060G		V		V	V	V	V	V	V	V	V	V
G396	G1189A				M								
E372	A1119G								E[1]	E[1]	E[1]	E[1]	E[1]
Q397	G1194A		Q[1]										
T411A	A1234G							A					
P525P	G1575C						P[1]						

[1] synonymous mutation
[2] A82L DNA mutation was GCA to CTT
[3] A184I DNA mutation was GCA to ATA

[4] H236R DNA mutation was CAT to CGG
[5] not sequenced

E. coli DH5α, transformed with these plasmids, was used for the biotransformations. All experiments were conducted in parallel. All mutants were cultivated and stored on LBamp[4] agar plates. For the stage 1 cultures 10 mL Greiner tubes were used. 2 mL LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl. These were dissolved in distilled H_2O and made up to 1L) together with 100mg/L ampicillin were inoculated with the respective *E. coli* cells which had been previously grown on agar. These stage 1 cultures were then incubated over night (18.5 h) at 100 rpm and 30°C. For stage 2 cultures 1L Erlenmeyer flasks were used. 150 mL TB medium (solution 1: 12 g tryptone, 24 g yeast extract and 4 mL glycerine. These were dissolved in distilled H_2O and made up to 900 mL. solution 2: 100 mL buffer consisting of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4. Each solution was sterilised separately and then mixed. The pH value should lie between 6 and 8.) together with 100 mg/L ampicillin and, in one run, 1.5 mL/L trace element solution (0.5 g $CaCl_2{\times}2H_2O$, 0.18 g $ZnSO_4{\times}7H_2O$, 0.1 g $MnSO_4{\times}H_2O$, 20.1 g Na_2-EDTA, 16.7 g $FeCl_3{\times}6H_2O$, 0.16 g $CuSO_4{\times}5H_2O$, 0.18 g $CoCl_2{\times}6H_2O$ in 1L distilled H_2O) were inoculated with 1 mL stage 1 culture. They were then incubated at 100 rpm and 30°C for 8h.

Substrate addition (TU-Graz). After the previously mentioned 8h, substrate **2** (3 mM/L) was dissolved in EtOH (84 mg **2** in 1 mL abs. EtOH) and this added to the stage 2 culture. In addition, 50 mg/L ampicillin was also added. After incubation for a further 15h, additional ampicillin (50 mg/L) was added. Each experiment was stopped (see the following section) after a final 21h of incubation.

Sample processing and purification (TU-Graz). Each experiment was stopped by the following procedure: the respective culture was centrifuged (4400 g) and the supernatant liquid extracted three times with ethyl acetate. The organic phases were collected, dried with Na_2SO_4, filtered and concentrated down under reduced pressure. The crude extracts were purified with column chromatography (pet. ether : ethyl acetate; 10:1 followed by 2:1). During chromatography, priority was placed on not influencing the *de* of **3**.

Analytics (TU-Graz). The determination of the *ee*/*de* of the products **3** from the whole cell biotransformation was achieved with reference compounds/mixtures (all 4 diastereoisomers of **3**) and chiral HPLC (Daicel Chiralpak AD-H, 1mL/min, *n*-heptane:ethanol = 95.5:4.5, 230 nm; retention times: 23.3 min (*S,S*)-**3**, 25.9 min (*R,R*)-**3**, 27.9 min (*S,R*)-**3**, 29.8 min (*R,S*)-**3**).

Summary of results for phase one experiments

Mutant	Yield	Main Isomer	*ee*	*de*	Recovered 2	Product 3
wild type	0.4%	*S,S*	1.5%	87%	42.2 mg	0.4 mg
35-4	5%	*S,S*	72%	96%	33.7 mg	4.7 mg
139-3	4%	*S,S*	79%	96%	35.0 mg	3.5 mg
139-29	4%	*S,S*	77%	96%	33.3 mg	3.3 mg
139-37	1%	*S,S*	75%	96%	31.8 mg	1.2 mg
A	5%	*S,S*	76%	95%	39.5 mg	4.1 mg
B	15%	*S,S*	82%	96%	42.1 mg	13.4 mg
J	4%	*S,S*	75%	94%	30.3 mg	3.2 mg
L	6%	*S,S*	69%	96%	33.2 mg	5.3 mg
9-10A	2%	*S,S*	78%	95%	30.8 mg	1.6 mg
9-10A-328V	2%	*S,S*	49%	97%	31.8 mg	1.8 mg
9-10A-82L	2%	*S,S*	21%	81%	40.7 mg	1.5 mg
1-12G	3%	*R,R*	89%	94%	30.3 mg	2.3 mg

Table 2 Biohydroxylation of **2** with modified whole cell systems. Yields are reported as mol % product **3** from starting material **2** and do not take into account recovered starting material. Data are from one set of experiments.

Additional remarks (TU-Graz). Biotransformations with *E. coli* expressing BM-3 mutants 35-4, 139-3, 139-29, 139-37, A, B, J, L as well as the wild-type were carried out 3 times in total. In the first two "runs", trace element solution was not used. With one of these "runs", with the last addition of ampicillin, 0.5 mM IPTG was also added in order to induce enzyme production. However, the addition of IPTG was found to give inferior results, according to TLC, compared with "non-induced" cultures. In one case, with mutant 139-3, the main product was isolated and *ee*/*de* values determined. The addition of IPTG was not found to influence these parameters. Consequently, this line of work was no

longer investigated. Results discussed in the communication are from experiments were trace element solution was used. Yields, in general, were found to be low. In addition, on account of the small amounts involved, these yields were difficult to compare. Furthermore, the product was not obtained totally "clean" as priority in column chromatography was placed on not influencing the *de* of **3**. Concerning *ee/de* values: Regardless of which experimental method was used, in all three "runs", *ee* and *de* values were found to be comparable.
In this communication, the wild type as well as the most active variants (139-3, B and L) were tested with unprotected substrate **1** and found to be "inactive". This means that, within the limits of detection, activity could not be perceived employing the methods described. The experiments in this regard were carried out with cyclopentanecarboxylic acid **1** as substrate in the same way as the transformations with substrate **2**. For product detection, TLC (petroleum ether : ethyl acetate = 1:2) and GC (2 min 50°C, 10°C/min, 5 min 300°C, injector temperature: 250°C, detector temperature: 250°C, carrier gas: hydrogen, 1 ml/min) were used.

Expression and purification of P450 BM-3 (Caltech). P450 BM-3 wt and mutants 139-3, B and 1-12G were expressed as described previously.[4] The enzymes were purified following published procedures.[5] Enzyme concentrations were measured in triplicate from the CO-difference spectra.[6]

Hydroxylation reactions of 2-cyclopentylbenzoxazole 2 (Caltech). Reactions for the determination of enantioselectivity and total turnover number were carried out similarly to those described for octane.[4] Hydroxylation reactions were performed in 20 mL glass vials and stirred at low speed using magnetic stirring bars. Purified protein was added to 4.65 mL of 0.1 M potassium phosphate buffer, pH 8.0, such that the total protein concentration would equal 25 nM (mutants 139-3, B and 1-12G) or 100 nM (wt) in a final volume of 5 mL. The substrate was added to this solution as 50 µL of a 100 mM substrate in ethanol solution to give 1 mM total substrate and 1% ethanol. The mixture was incubated for two minutes before 500 µL of 10 mM NADPH was added to start the reaction. After 12 hours of stirring at room temperature, a 1.5 mL aliquot of the reaction was removed from the vial and quenched with 300 µL of chloroform in a 2 mL microcentrifuge tube. An internal standard containing 15 µL of 20 mM coumarin in ethanol was added to the tube. The tube was vortexed and then centrifuged at 10,000 g for 2 minutes in a microcentrifuge. The chloroform layer was removed with a pipette and analyzed by gas chromatography to determine total turnover numbers and product distributions. Control reactions performed by repeating these steps without the addition of substrate revealed no detectable background levels of these specific products.

Product formation rates (Caltech). To determine formation rates of **3** (measured at 21°C), 5 mL reactions containing 100 nM (139-3, B, 1-12G) or 200 nM (wt) protein were set up as described above. Aliquots (1.4 mL) were removed from the reaction at 20 second intervals for one minute and quenched in 2 mL microcentrifuge tubes containing 300 µL chloroform, 100 µL 6 N HCl, and 14 µL 20 mM coumarin in ethanol. The tubes were vortexed and centrifuged at ~10,000 g for 2 minutes to separate the layers. The chloroform layer was removed and analyzed by gas chromatography. Rates were determined in triplicate.

By-product formation: The ketone product of **3** was detected at very low concentrations (1 to 1.5% of **3**) regardless of the protein variant. One or two other by-products visible in the gas chromatogram could not be identified but are most likely products hydroxylated at other positions of the cyclopentane ring.

Gas chromatography (Caltech). Analysis of the hydroxylation products of **2** was performed using authentic standards and a 5-point calibration curve with coumarin as an internal standard. The injection volume was 1 µL. Analysis was performed on a Hewlett-Packard 5890 Series II Plus gas chromatograph connected to a FID detector using a CycloSil-B column (J&W Scientific, 30 m length, 0.320 mm ID, 0.25 µm film thickness) for chiral analysis or a HP-5MS column (Agilent Technologies, 30 m length, 0.25 mm ID, 0.25 µM film thickness) for the determination of rates and total turnover numbers. The temperature program for the CycloSil-B column was: 100°C injector, 250°C detector, 100° oven for 3 min, 10°C/min gradient to 170°C, 170°C for 10 min, 25°C/min gradient to 200°C, and then 200°C for 8 min. The temperature program for the HP-5MS column was: 100°C injector, 250°C detector, 100° oven for 3 min, 10°C/min gradient to 210°C, 25°C/min gradient to 250°C, and then 250°C for 3 min.

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