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Stereo- and regioselectivity in the P450-catalyzed oxidative tandem difunctionalization of 1-methylcyclohexene

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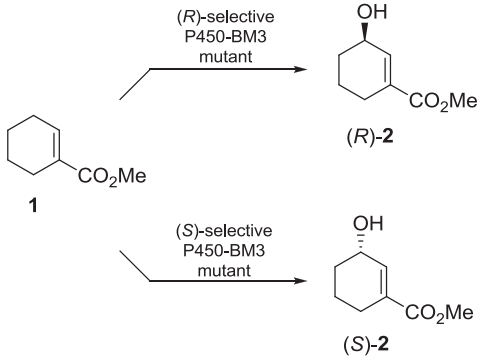
ABSTRACT

The selective partial oxidation of small non-functionalized molecules using biocatalysis based on P450 monooxygenases is known to be difficult due to the expected poor regio- and stereoselectivity, but in this study it was nevertheless attempted. 1-Methylcyclohexene was subjected to oxygen-mediated biocatalytic oxidation using P450-BM3 as the catalyst. Both oxidative hydroxylation and epoxidation were observed, in some cases leading to hydroxy epoxides with high diastereo- and enantioselectivity especially when employing BM3 mutants.

1. Introduction

Transition metal catalyzed chemo-, regio-, and stereoselective oxidative functionalization of simple and complex organic compounds continues to be an area of intense interest, CH-activation with selective introduction of hydroxy groups being a prominent example.[1] Alternatively, oxidative hydroxylation can be catalyzed by cytochrome P450 enzymes,[2] which are also capable of olefin epoxidation. In these biocatalytic reactions oxygen (air) serves as the oxidant. These ubiquitous enzymes are Fe—heme-dependent with very large binding pockets, catalyzing, for example, the regio- and stereoselective hydroxylation of precursors of terpenes, alkaloids, and steroids at late stages of biosynthesis. When 'foreign' lipophilic compounds enter organisms, P450-catalyzed detoxication occurs by way of solubilization as a result of hydroxylation, which need not be regio- or stereoselective.

When a P450 catalyst fails to be selective in the partial oxidation of a substrate of practical interest in synthetic organic chemistry or biotechnology, the wild-type (WT) can in principle be tuned by the methods of directed evolution.[3,4] However, the control of both regio- and stereoselectivity is a particularly difficult goal, which has not been achieved until recently using steroids as model compounds.[5] The mutations introduced by this form of protein engineering cause the substrate to be bound in an appropriate pose above the catalytically active high-spin heme—Fe=O species, which ensures regio- and stereoselective radical H-abstraction followed by rapid C—O bond formation. In these and other studies, P450-BM3 is often used,[6] because it contains a covalently fused NADPH-domain and has proven to be a viable catalyst for a number of transformations. More recently, directed evolution has also been applied to considerably smaller molecules, which also bear functional groups, 1-cyclohexene carboxylic acid ester **1** being an example for which two different P450-BM3 mutants were evolved leading to regioselective hydroxylation with optional formation of (*R*)-**2** and (*S*)-**2**, respectively.[7] Another impressive example is a P450-BM3 mutant, which hydroxylates *N*-benzyl pyrrolidine regioselectively at the 3-position leading to the *S*-configurated alcohol.[8] It is likely that H-bonds and/or electrostatic as well as hydrophobic effects contribute to the formation of active poses of these relatively small molecules in the large P450-BM3 binding pocket.



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It can be surmised that the difficulty in controlling both regio- and stereoselective CH-activating hydroxylation increases when subjecting small substrates devoid of functional groups to P450-catalysis. In the complete absence of functional groups capable of undergoing hydrogen bonding or electrostatic effects, various catalytically active poses are likely leading to many different oxidation products. Indeed, in protein engineering attempts to control both regio- and stereoselectivity of P450 enzymes as catalysts in the hydroxylation of such structurally simple alkanes as *n*-pentane or *n*-hexane have not been very successful to date.[9] The selective oxidative functionalization of 1-methylcyclohexene (**3**) using P450-catalysis has not been attempted thus far, but it is also likely to be a difficult task. The only functional group in this substrate is the olefinic double bond, which is less likely to undergo specific binding modes. Indeed, one can anticipate several active poses, including those that lead to epoxidation. Tandem oxidation leading to hydroxyl-epoxides is also possible, which would be of particular synthetic interest.

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The present study constitutes an exploratory investigation of using P450-BM3 as a catalyst in the partial oxidation of substrate **3**. The initial goal was to obtain a complete fingerprint of all oxidation products, including stereochemical assignments. Should this oxidation turn out to be unselective with formation of many different products, as expected, we planned to test some of the best mutants specifically evolved for the regio- and enantioselective hydroxylation of the more functionalized substrate **1**. Using this strategy we hoped to gain information for future directed evolution optimization, especially in the quest to control selectivity in tandem difunctionalization.

2. Results and discussion

In initial experiments, WT P450-BM3 was used as the biocatalyst in the oxidation of substrate **3** under different reaction conditions (variation of temperature, buffer pH, reaction time as well as $NADP^+$ concentration). In order to measure P450 activity, several small scale hydroxylation experiments as well as medium and higher scale experiments were conducted (final experimental conditions are listed in the Experimental section). Reactions performed with resting cells proved to be cleaner than those using crude lysate. Furthermore, resting cells do not need the addition of exogenous $NADP^+$, since similar conversion values and selectivities were found using concentrations, which ranged from 0 to 0.5 mM of $NADP^+$. All reaction products were first identified by GC–MS and subsequently fully characterized by comparison with authentic samples (Fig. 1, top).

It can be seen that the product mixture is the result of a fairly indiscriminate oxidation process, regio- and stereoselectivity being poor. In addition to unselective oxidation, subsequent reactions occur to a small degree under the reaction conditions, namely rearrangement of allylic alcohol **4** to **8** and ring-opening hydrolysis of epoxide **5** with formation of *trans*-**10** (see also Table 1). Thus, biocatalysis using WT P450-BM3 in the oxidation

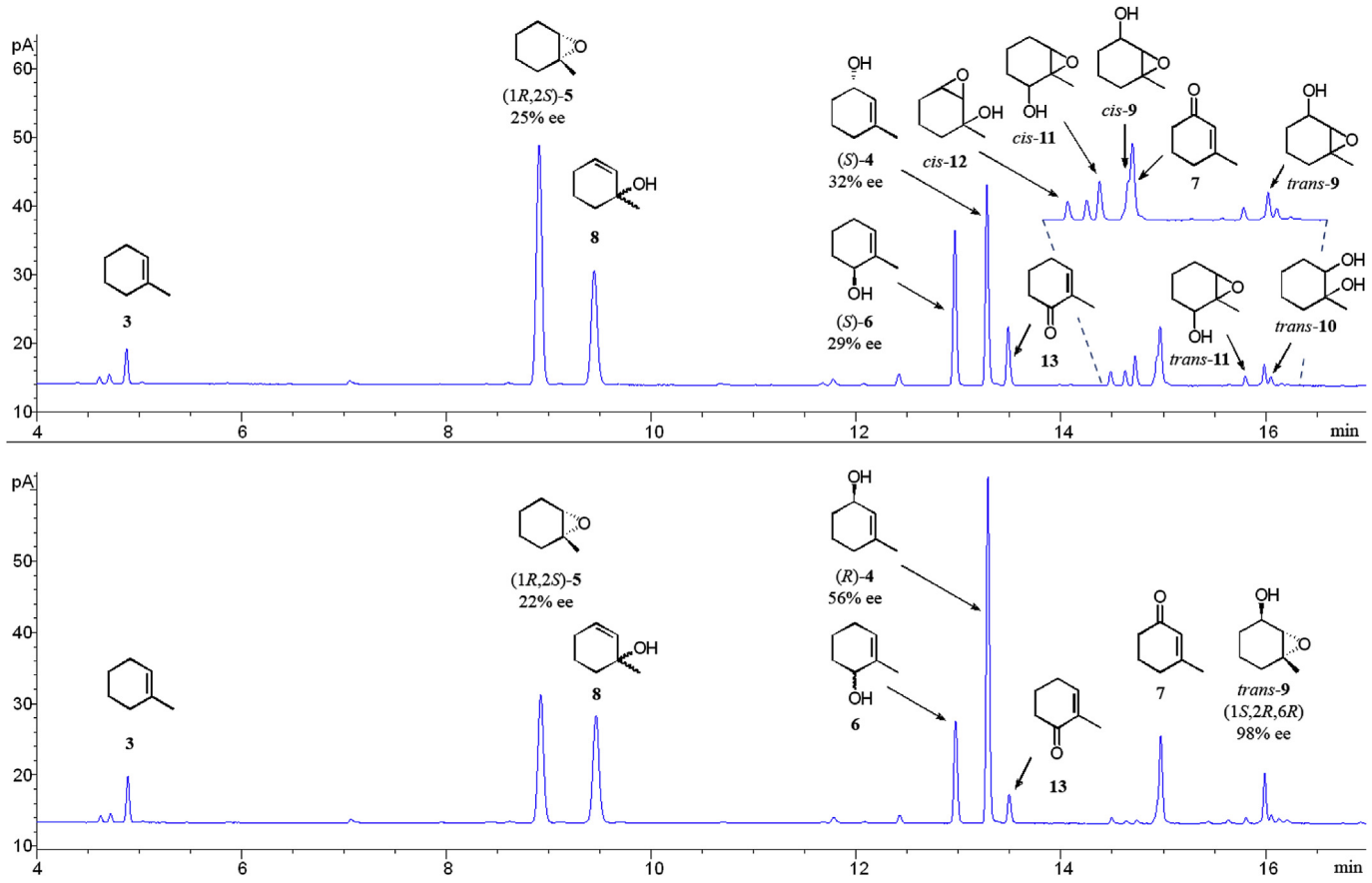


Fig. 1. GC chromatograms of the oxidation products of 1-methylcyclohexene (**3**) catalyzed by WT P450-BM3 (top) and mutant F87I/A328V (down) using resting cells (3 h of reaction time). GC conditions: 30 m DB1, inner diameter 0.25 mm; pressure: 0.5 bar H_2; injector: 230 °C; oven: temperature gradient: 50 °C isothermic 10 min, then from 50 to 205 °C with 12 °C/min, FID detector: 350 °C.

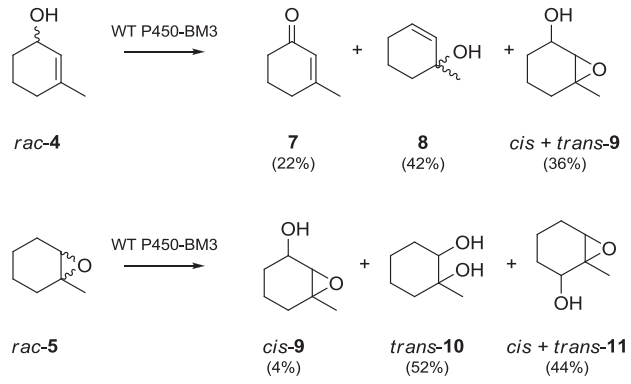
Table 1
Performance of WT P450-BM3 and mutant F87I/A328V here serving as catalysts in the oxidative hydroxylation of substrates **3**, *rac*-**4**, and *rac*-**5**[a]

Entry	Substrate	Mutation	Products (conversion (%)/configuration)										Substrate concentration (%)	Conversion (%)	Time (h)
			4	**5**	**6**	**7**	**8**[b]	**9**	**10**[c]	**11**	**12**	**13**			
1	**3**	WT	16/32(*S*)	34/25(1*R*,2*S*)	13/29(*S*)	5	17	4/cis+trans (60:40)	<1	4/cis+trans (77:23)	1/cis	5	38 mM	>97	3
2	**3**	F87I/A328V	32/56(*R*)	21/22(1*R*,2*S*)	10/0	10	19	4/trans (1*S*,2*R*,6*R*) (98% ee)	<1	—	—	4	38 mM	>97	3
3	*rac*-**4**	WT	—	—	—	22	42	36/cis+trans (15:85)	—	—	—	—	13 mM	28	21
4	*rac*-**4**	F87I/A328V	—	—	—	39	37	24/trans (1*S*,2*R*,6*R*) (72% ee)	—	—	—	—	13 mM	54	21
5	*rac*-**5**	WT	—	—	—	—	—	4/cis	52	44/cis+trans (97:3)	—	—	13 mM	46	21
6	*rac*-**5**	F87I/A328V	—	—	—	—	—	16/cis	76	8/cis	—	—	13 mM	9	3[d]

[a] Values obtained from average of atleast three independent experiments performed with resting cells using $NADP^+$ 50 μM.
[b] Alcohol was racemic in all the cases.
[c] Only the trans-diastereoisomer detected in all cases.
[d] Reaction time of 21 h leads to high amount of impurities.

of 1-methylcyclohexene **3** is quite different from the analogous reaction of 1-cyclohexene carboxylic acid ethyl ester (**1**). The latter substrate resulted in the formation of (*R*)-**2** (34% ee) and other regioisomeric alcohols in a ratio of 84:16, but epoxides were not formed. Since epoxidation is an electrophilic process, the electron-poor olefinic bond in this substrate is not epoxidized. These results are in accord with the observation that P450-BM3-catalyzed oxidation of unsubstituted cyclohexene results primarily in epoxidation (85%) in addition to allylic alcohols (15%).[10]

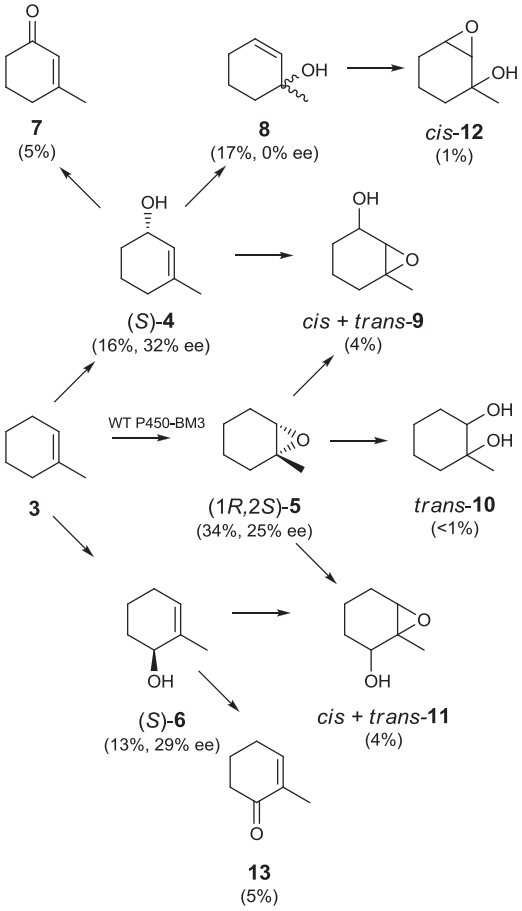
The reaction pathways of 1-methylcyclohexene (**3**) were established by performing additional experiments using WT P450-BM3 as the catalyst and racemic 3-methylcyclohex-2-en-1-ol (*rac*-**4**) and 1-methyl-1,2-epoxycyclohexane (*rac*-**5**) as substrates (Scheme 1). These experiments led to six products, namely **7**, **8**, and *cis*- and *trans*-**9** for the alcohol **4** and *cis*-**9**, *trans*-**10**, and *cis*- and *trans*-**11**, respectively, in the case of epoxide **5**, which had been observed in the initial oxidation of substrate **3**.



Scheme 1. Products obtained as a result of WT P450-BM3 catalyzed oxidation of substrates *rac*-**4** and *rac*-**5**.

As before, the products were identified by comparison with authentic samples. The results allow a complete reaction scheme to be established, which defines the origin of all products (Scheme 2).

Due to the lack of selectivity of WT P450-BM3 as a catalyst in the model reaction, several mutants previously evolved for the regio- and stereoselective allylic hydroxylation of the ester **1** were tested in the oxidation of substrate **3** (see Experimental section for the complete data). BM3 mutant F87I/A328V proved to be considerably more selective than WT, the GC chromatogram of the crude product mixture indicating fewer peaks (Fig. 1, bottom). Analysis of the data shows that regio- and stereoselectivity in the biotransformation of **3** have improved to a notable extent relative to the results of the



Scheme 2. Primary and secondary oxidative processes in the reaction of 1-methylcyclohexene (**3**) using WT P450-BM3 as catalyst, secondary rearrangements, and hydrolyzes being included in the description concerning the origin of all products.

oxidation using WT BM3 (Table 1). Especially diastereo- and enantioselectivity in the formation of the hydroxy-epoxide *trans*-**9** are high (98% ee in favor of the (1*S*,2*R*,6*R*)-stereoisomer). Although conversion to this product is still low, the result is remarkable, and also of potential synthetic interest. Indeed, the preparation of trans-configurated diastereoisomers of this type starting from substituted olefins is known to be tedious and occurs with lower selectivities, generally cis-products being favored.[11]

In small scale experiments performed with mutant F87I/A328V, enantioselectivity of *trans*-**9** reached 97–99% ee. However, upon

up-scaling, enantioselectivity decreases to 81% ee. Similar effects have been observed in other systems.[7] It is interesting to note that *trans*-**9** is formed only via alcohol **4**. When *rac*-**5** was subjected to oxidation using mutant F87I/A328V, the only diastereoisomer detected was *cis*-**9** (Table 1, entry 5). Moreover, when *rac*-**4** was used as the starting material in a reaction catalyzed by mutant F87I/A328V, epoxide *trans*-**9** was obtained with a moderate enantioselectivity (72% ee in favor of the (1*S*,2*R*,6*R*)-stereoisomer). This suggests that only the (*R*)-enantiomer of the alcohol is the preferred substrate in the next oxidation step, while the (*S*)-enantiomer is oxidized to ketone **7**. Furthermore, control measurements using alcohol *rac*-**4** and mutant F87I/A328V showed that the starting material remains racemic, suggesting a double kinetic resolution: the initially formed alcohol (*R*)-**4** formed reacts further to form *trans*-**9**, while alcohol (*S*)-**4** is oxidized to ketone **7**.

Reaction **3**→*trans*-**9** was up-scaled employing mutant F87I/A328V and using 480 mg (5 mmol) of starting material, pure product being successfully isolated following column chromatography (24 mg).

3. Conclusions and perspectives

We have identified all products arising from the P450-BM3 catalyzed oxidation of 1-methylcyclohexene (**3**), including the assignment of absolute and relative stereochemistry. No less than a dozen oxidation products were formed in this biocatalytic process, conversion to epoxy alcohols being of special synthetic interest. Unfortunately, such tandem bifunctionalization using WT P450-BM3 occurred only to a minor extent, stereoselectivity also being poor. However, the use of a P450-BM3 mutant previously evolved for the highly regio- and stereoselective oxidative hydroxylation of 1-cyclohexene carboxylic acid methyl ester (**1**) showed an intriguing degree of selectivity in this bifunctionalization to provide the *trans* epoxy alcohol with high diastereo- and enantioselectivity, although with low conversion. These findings set the stage for future directed evolution work in the quest to optimize tandem bifunctionalization of olefin **3** and of related substrates.

4. Experimental section

4.1. Molecular biology

4.1.1. Reagents. Escherichia coli BOU730 cells used are described elsewhere.[7] Electro-competent cells were prepared in-house according to standard protocols.[12] DNAse I and lysozyme were obtained from AppliChem. $NADP^+$ was purchased from Calbiochem. Luria–Bertani (LB) medium as a powder mixture, kanamycin (kan) and carbenicillin were obtained from Roth. *iso*-Propyl-β-D-thiogalactoside (IPTG) was purchased from Fermentas. Terrific Broth TB medium contained yeast extract (24 g/L), peptone (12 g/L), glycerol (4 mL/L), KH_2PO_4 (17 mM), and K_2HPO_4 (72 mM).

4.1.2. Small scale biohydroxylation using P450 mutants. Initially, WT P450-BM3 was tested for optimizing biohydroxylation reaction conditions of compound **3**. For small scale reactions, an individual BOU730 colony harboring plasmid pRSF-P450-BM3[7] was inoculated in 5 mL of LB with kan (50 μg/mL). After 5 h of incubation at 37 °C with shaking, this preculture was transferred to 50 mL of TB with kan (50 μg/mL). Cultures were grown at 30 °C until O.D. of 0.8–0.9 at 600 nm was reached, then IPTG was added to a final concentration of 0.2 mM and the cultures were allowed to grow at 30 °C with vigorous agitation for 20 h. Cells were collected by dividing the culture in 1 mL aliquots, which were centrifuged at 10 000 rpm for 6 min at room temperature, supernatant discarded and pellets stored at −20 °C for subsequent use. For biohydroxylation, aliquots were resuspended in 480 μL lysis buffer [phosphate buffer (pH 7–8, 100 mM), lysozyme (14 mg/mL), and DNAse I (6 U/mL)] and incubated at 37 °C during 20 min at 700 rpm in a thermomixer. After this time, tubes were centrifuged at 10 000 rpm at 4 °C for 15 min to pull down the cellular debris. Supernatant (c.a. 500 μL) was transferred to a new 1.5 mL tube, containing 500 μL reaction buffer [phosphate buffer (pH 7–8, 100 mM), $NADP^+$ (50–1000 μM) and glucose (100 mM). Reaction started after addition of 1 μL of compound **3** (8 mM final concentration, 8 μmol)]. Samples were incubated at 25 °C–37 °C for 20 h at 700 rpm in a thermomixer. After incubation time, 700 μL of reaction was extracted with ethyl acetate (700 μL) and the organic layer subjected to GC analysis. Best results were achieved using buffer pH 7.4, $NADP^+$ 500 μM, and 25 °C as incubation temperature.

Different P450-BM3 mutants were tested for biohydroxylation of compound **3** (single mutants F81R, A82L, A82M, F87D, F87G, F87I, L181E, I263G, and double mutants F87I/A328V, F87V/A328N, F87V/A328V, I263G/A328S). Individual BOU730 colonies harboring mutated plasmids were inoculated in 1 mL of LB with kan (50 μg/mL). After 5 h of incubation at 37 °C with shaking, this preculture was transferred to 15 mL of TB with kan (50 μg/mL). Cultures were grown as above, and an aliquot of 10 mL was pelleted and resuspended in 430 μL lysis buffer [phosphate buffer (pH 7.4, 100 mM), lysozyme (14 mg/mL), and DNAse I (6 U/mL)] and incubated at 37 °C during 20 min at 700 rpm. Tubes were centrifuged at 10 000 rpm at 4 °C for 20 min and 425 μL of supernatant were mixed with glucose (100 mM final concentration), $NADP^+$ (500 mM final concentration), and compound **3** (33 mM final concentration, 17 μmol). Samples were incubated at 37 °C for 20 h with 700 rpm in a thermomixer. Reaction volume was extracted with 500 μL of ethyl acetate and subjected to GC analysis. Best results (enantio- and stereoselectivities) were obtained using mutant F87I/A328V.

4.1.3. Medium scale biohydroxylation using P450 mutants. Biohydroxylation reaction with best mutant F87I/A328V was performed using resting cells or lysate. An Erlenmeyer flask (100 mL) containing LB (15 mL) and kan (50 μg/mL) was inoculated with a colony from BOU730 cells expressing corresponding P450-BM3 mutant and incubated 5 h at 37 °C with shaking. An aliquot of this preculture (5 mL) was inoculated into TB (50 mL) containing kan (50 μg/mL) and allowed to grow at 30 °C until O.D. of 0.8–0.9 at 600 nm was reached, then IPTG was added to a final concentration of 0.2 mM. Culture was grown at 30 °C during 20 h with gentle agitation and cells were pelleted by centrifugation (6 min, 4000 rpm at 4 °C). For experiments performed with lysate cells, pellets were resuspended in 10 mL of lysis buffer [phosphate buffer (pH 7.4, 100 mM), lysozyme (14 mg/mL), and DNAse I (6 U/mL)], incubated at 37 °C 45 min and centrifuged 20 min at 4000 rpm. Supernatants (c.a. 9 mL) were mixed with glucose (100 mM final concentration), $NADP^+$ [0–100 μL (0–0.5 mM final concentration)], and compound **3** (38 mM, 380 μmol). For experiments carried out with resting cells, pellets were resuspended in reaction buffer [phosphate buffer (pH 7.4, 100 mM)], $NADP^+$ [0–100 μL (0–0.5 mM final concentration)], glucose (100 mM), and compound **3** (38 mM, 380 μmol). Reactions were carried out in 100 mL flasks with tight closure using rubber caps at 25 °C, for 3–21 h with mild agitation. At different time points, 700 μL of reaction mixtures were extracted with 700 μL ethyl acetate and subjected to GC analysis. Resting cells did not need addition of exogenous $NADP^+$ (similar results were found adding either 0 or 0.5 mM of $NADP^+$) to perform the reaction. In contrast, this was necessary when using lysate cells. Results shown in Table 1 belong to experiments performed with resting cells using $NADP^+$ 50 μM and incubated for 21 h. Reactions with *rac*-**4** and *rac*-**5** were carried out using the protocol described above

for resting cells employing $NADP^+$ 50 μM and adding 130 μmol (13 mM final concentration) of starting material in each case.

4.1.4. High scale biohydroxylation using P450 mutants. Reaction between methylcyclohexene (**3**) and P450 mutant F87I/A328V was up-scaled according to the published protocol.[7] Briefly, an Erlenmeyer flask (100 mL) containing LB (20 mL) and kan (50 μg/mL) was inoculated with a colony from BOU730 cells expressing P450 mutant F87I/A328V and incubated for 6 h at 37 °C with shaking. This preculture was inoculated into TB (500 mL) containing kan (50 μg/mL) and allowed to grow at 30 °C until O.D. of 0.8–0.9 at 600 nm was reached, then IPTG was added to a final concentration of 0.2 mM and the culture grown at 30 °C during 16–20 h with gentle agitation. Cells were pelleted by centrifugation (15 min, 4000 rpm at 4 °C), and the pellet was resuspended in 100 mL of reaction buffer phosphate buffer (pH 7.4, 100 mM), $NADP^+$ (300 μM), and glucose (100 mM). The mixture was transferred to a 500 mL Erlenmeyer, and compound **3** (480 mg, 50 mM final concentration, 5 mmol) was added to start reaction, which was carried out at 25 °C for 24 h with mild agitation. After completion, the reaction mixture was extracted with ethyl acetate, organic phase dried, concentrated, and subjected to column chromatography.

4.2. Chemistry

4.2.1. General remarks. Compounds 1-methylcyclohexene, 3-methylcyclohex-2-en-1-one, 3-methylcyclohex-2-en-1-ol, *trans*-1-methylcyclohexane-1,2-diol, and all other GC controls were purchased from Acros, Sigma–Aldrich and Alfa and used without further purification. NMR spectra were recorded on a Bruker Avance 300 or DRX 400 (^{1}H: 300 MHz or 400 MHz, ^{13}C: 75 MHz or 101 MHz) spectrometer using TMS as internal standard (d=0). High-resolution mass spectra recorded in APCI mode were performed on a ThermoScientific LTQ-FT spectrometer. Conversion and enantiomeric excess were determined by achiral and chiral gas chromatography. Analytical thin layer chromatography was performed on Merck silica gel 60 F254q while for column chromatography Merck silica gel 60 (230–400 mesh ASTM) was used. Determination of the relative configuration was performed after comparison with commercial available racemic samples. Racemic 1,2-epoxy-1-methylcyclohexane (**5**) was prepared by oxidation with *m*-CPBA and its analytical data correspond to those published in the literature.[13] Absolute configuration of epoxide (**5**) was determined after reaction with limonene epoxide hydrolase (LEH) (see below). Racemic **8** was obtained following the same synthetic route as that reported by O'Brien et al.[14] Its spectroscopic data were found to be identical with the ones described in the literature.[15] A racemic mixture of *cis*-**9** [(1*S*,2*S*,6*R*)-**9** and its enantiomer (1*R*,2*R*,6*S*)-**9**] was obtained by *m*-CPBA oxidation of commercially available alcohol *rac*-**4**. Its spectroscopic data were found to be identical with ones described in literature.[11a] GC analyses showed a ratio of 97:3 (*cis*-**9**/*trans*-**9**). A racemic mixture of *cis*-**12** [(1*S*,2*R*,6*S*)-**12** and its enantiomer (1*R*,2*S*,6*R*)-**12**] and *trans*-**12** [(1*R*,2*R*,6*R*)-**12** and its enantiomer (1*S*,2*S*,6*S*)-**12**] were obtained by oxidation with *m*-CPBA using as starting material *rac*-**8** (97:3; *cis*-**12**/*trans*-**12**). A racemic mixture of *cis*-**11** [(1*S*,2*S*,6*R*)-**11** and its enantiomer (1*R*,2*R*,6*S*)-**11**] and *trans*-**11** [(1*R*,2*S*,6*S*)-**11** and its enantiomer (1*S*,2*R*,6*R*)-**11**] were obtained by oxidation with *m*-CPBA using as starting material **6** (85:15; *cis*-**11**/*trans*-**11**).

*4.2.2. Synthesis of 3-methylcyclohex-2-en-1-ol (R)-**4**.* Reaction of 1-methylcyclohexene catalyzed by mutant F87I/A328V was up-scaled as described above. After extraction with ethyl acetate and concentration, the crude reaction mixture was subjected to column chromatography to afford compound (*R*)-**4** (56% ee). The compound was identified after comparison with racemic 3-methylcyclohex-2-en-1-ol (**4**) purchased from Sigma–Aldrich its analytical data corresponds to the published ones.[16] The absolute configuration was assigned by optical rotation comparison with an authentic sample described by Okamura and Wu.[17] Chiral GC conditions: 25 m IVADEX 1, inner diameter 0.25 mm film thickness 0.15 μm; pressure: 0.5 bar H_2; injector: 220 °C; oven: 35 min isothermic at 75 °C; FID detector: 320 °C; (*S*)-**4** 13.55 min, (*R*)-**4** 17.00 min.

*4.2.3. Absolute configuration determination of 1,2-epoxy-1-methyl cyclohexane (**5**).* A single colony of *E. coli* BL-21 Gold (DE3) colony harboring plasmid pETLEH[18] (limonene epoxide hydrolase) was picked and inoculated in 10 mL of TB containing carbenicillin (50 ng/μL) and 0.5% lactose and incubated over night at 28 °C. After incubation, this preculture was added to 100 mL of TB containing carbenicillin (50 μg/mL) and 0.5% lactose, and incubated at 28 °C for 20 h with gentle agitation. Expression of LEH was confirmed by SDS-PAGE analysis (Data not shown). After incubation, 10 mL of culture were pelleted by centrifugation (15 min, 4000 rpm at room temperature) then pellet was resuspended in 1 mL of lysis buffer [phosphate buffer (pH 7.4, 100 mM), lysozyme (14 mg/mL), and DNAse I (6 U/mL)] and incubated at 37 °C during 20 min at 700 rpm in a thermomixer. The sample was centrifuged at 10 000 rpm at 4 °C for 30 min to pull down the cellular debris. Supernatant (c.a. 1 mL) was transferred to a new 1.5 mL tube and 3 μL of *rac*-**5** were added (27 mM final concentration). Reaction was incubated at 30 °C for 5 min at 700 rpm in a thermomixer. At different reaction times, aliquots of 100 μL were harvested, extracted with 100 μL of ethyl acetate, and subjected to chiral GC analysis for monitoring reaction progress. Enantiomers assignment was based on the fact that LEH digests enantiomer (1*R*,2*S*)-**5** faster than enantiomer (1*S*,2*R*)-**5**.[18] Chiral GC conditions: 25 m IVADEX 1, inner diameter 0.25 mm, film thickness 0.15 μm; pressure: 0.4 bar H_2; injector: 220 °C; oven: temperature ramp 40 °C–60 °C with 1 °C/min ramp, 25 min; FID detector: 320 °C; (1*S*,2*R*)-**5** 14.64 min, (1*R*,2*S*)-**5** 15.16 min.

*4.2.4. Synthesis of 2-methylcyclohex-2-en-1-ol (S)-**6**.* The reaction of 1-methylcyclohexene catalyzed by mutant F87I/A328V was up-scaled as described above. After extraction with ethyl acetate and concentration, the crude reaction was purified by column chromatography (ethyl acetate/petroleum ether 1:4) to afford compound 2-methylcyclohex-2-en-1-ol (*S*)-**6**, which contained traces of alcohol **5**. Its structure was proven after comparison of its analytical data (^{1}H, ^{13}C NMR) with those reported by Craig et al.[19] Absolute configuration was assigned by optical rotation comparison with an authentic sample, according to Craig et al.[19] Chiral GC conditions: 25 m IVADEX 1, inner diameter 0.25 mm film thickness 0.15 μm; pressure: 0.8 bar H_2; injector: 220 °C; oven: 70 min isothermic at 40 °C; FID detector: 320 °C; (*R*)-**6** 59.18 min, (*S*)-**6** 63.48 min.

*4.2.5. Synthesis of 3-methyl-trans-2,3-epoxycyclohexan-1-ol trans-(1S,2R,6R)-**9**.* The reaction of 1-methylcyclohexene catalyzed by mutant F87I/A328V was up-scaled as described above using 480 mg of methylcyclohexene. After extraction and concentration, the crude reaction was purified by column chromatography (ethyl acetate/petroleum ether 1:1) to afford 3-methyl-*trans*-2,3-epoxycyclohexan-1-ol *trans*-(1*S*,2*R*,6*R*)-**9** (24 mg, 4% yield). Its spectroscopic data were found to be identical with the ones described in the literature.[21] (R_f=0.43, ethyl acetate/petroleum ether 1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ=4.00 (t, ^3J=7.2 Hz, 1H), 2.92 (s, 1H), 2.00–1.82 (m, 2H), 1.80–1.62 (m, 2H), 1.59–1.06 (m, 2H), 1.34 (s, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ=67.01, 63.47, 58.90, 30.25, 29.70, 23.45, 15.89 ppm; HRMS ($APCI^+$) calcd for $C_7H_{13}O_2$ $[M+H]^+$: 129.0910; found: 129.0911; (1*S*,2*R*,6*R*)-**9**, 97–99% ee (small scale), 81% ee (high scale). The absolute configuration was determined after a sample of (*R*)-**5** (56% ee) was reacted with *m*-CPBA (GC vial, CH_2Cl_2, room temperature, 24 h) to afford a mixture of cis and

trans-diastereoisomers (87/13, cis/trans) according to GC–MS analysis. After chiral GC comparison with *trans*-**9** the major peak of the *trans*-diastereoisomer was assigned to (1*S*,2*R*,6*R*)-**9** while the minor peak of the trans-diastereoisomer was assigned to (1*R*,2*S*,6*S*)-**9**. Chiral GC conditions: 25 m Lipodex G, inner diameter 0.25 mm; pressure: 0.5 bar H_2; injector: 230 °C; oven: temperature gradient from 60 °C to 100 °C with 1 °C/min ramp then from 100 °C to 220 °C 12 °C/min; FID detector: 350 °C; (1*S*,2*R*,6*R*)-**9** 32.89 min, (1*R*,2*S*,6*S*)-**9** 33.70 min.

*4.2.6. Synthesis of trans-1-methylcyclohexane-1,2-diol (**10**).* The reaction of 1-methylcyclohexene catalyzed by mutant F87I/A328V-P450 was up-scaled as described above. After extraction with ethyl acetate and concentration, the crude reaction was purified by column chromatography (ethyl acetate/petroleum ether 1:1) to afford compound *trans*-**10** (R_f=0.16). Its spectroscopic data were found to be identical with ones described in literature.[20] In addition, *trans*-**10** was compared with authentic commercial *trans*-1-methylcyclohexane-1,2-diol. Relative configuration of *trans*-**10** was further confirmed after comparison with *cis*-**10**, sample obtained by reaction of metylcyclohexene with OsO_4 according to literature protocols.[20]

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References and notes

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