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P450-catalyzed regio- and stereoselective oxidative hydroxylation of disubstituted cyclohexanes: creation of three centers of chirality in a single CH-activation event

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This paper is dedicated to the memory of Harry H. Wasserman

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ABSTRACT

Wild-type P450-BM3 is able to catalyze in a highly regio- and diastereoselective manner the oxidative hydroxylation of non-activated disubstituted cyclohexane derivatives lacking any functional groups, including *cis*- and *trans*-1,2-dimethylcyclohexane, *cis*- and *trans*-1,4-dimethylcyclohexane, and *trans*-1,4-methylisopropylcyclohexane. In all cases except chiral *trans*-1,2-dimethylcyclohexane as substrate, the single hydroxylation event at a methylene group induces desymmetrization with simultaneous creation of three centers of chirality. Certain mutants increase selectivity, setting the stage for future directed evolution work.

1. Introduction

Enzymes are being increasingly used as catalysts in the production of fine chemicals, stereoselectivity often being the focus of interest.[1] Of particular importance are those transformations, which are difficult or impossible using state of the art synthetic transition metal catalysts or organocatalysts. The toolbox of organic chemists is greatly enriched by the development of complementary methods.[1,2] Until recently the traditional limitations of enzymes as catalysts in synthetic organic chemistry and biotechnology have prevented broad application, but fortunately the advent of directed evolution[3] as a viable protein engineering technique has changed the situation so that even retrosynthetic approaches based on enzymes are now emerging.[4] One of the current challenges in synthetic organic methodology development is regio- and stereoselective CH-activating oxidative hydroxylation of simple and complex organic compounds RH → ROH. Significant progress using synthetic catalysts or reagents has been achieved in this exciting area,[5] but a number of problems remain unsolved. A complementary approach is to use cytochrome P450 monooxygenases,[6] and indeed a number of industrial processes utilizing natural forms (wild-type, WT) are known.[7]

Whenever WT P450 monooxygenases fail to be regio- and stereoselective in a transformation of synthetic interest, which is required for applications, two strategies are possible to solve this problem: (1) Test mutants generated previously for other substrates and hope that a good catalyst will be found.[8] (2) Apply directed evolution,[3] which has a higher probability of success.[9] Directed evolution has been applied to P450 enzymes for more than decade, *but the control of both regio- and stereoselectivity remained elusive for a long time.* In early work, Arnold and co-workers reported the application of directed evolution to the P450-catalyzed oxidative hydroxylation of linear alkanes such as octane or nonane, which resulted in some improvement of regioselectivity, but poor to moderate enantioselectivity.[9c] Later Zhao et al. utilized saturation mutagenesis in order to improve and invert the enantioselectivity of P450(pyr) as a catalyst in the hydroxylation of *N*-benzylpyrrolidine at the 2-position, wild-type (WT) being slightly (*S*)-selective (43% ee) and the best mutants leading to 65% ee (*S*) and 83% ee (*R*).[9d] Later Li et al. performed directed evolution in order to boost (*S*)-selectivity to 98% ee, but enhanced (*R*)-selectivity was not reported.[9f] Recently we reported laboratory evolution of a P450 monooxygenase

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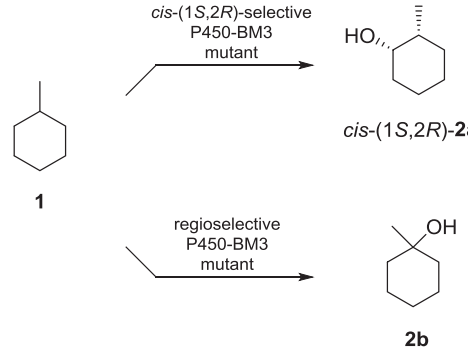
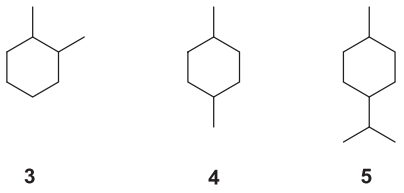
enabling both pronounced regio- and enantioselectivity in oxidative hydroxylation, both (*R*)- and (*S*)-selective mutants being evolved.[10] Using cyclohexene carboxylic acid methyl ester as substrate, highly (*R*)- and (*S*)- selective (>95% ee) mutants of P450-BM3[11] were evolved using iterative saturation mutagenesis (ISM),[2,12] essentially complete regioselectivity in favor of the 3-hydroxy products being observed.[10] In another recent study, >95% regio- and >95% diastereoselectivity in the oxidative hydroxylation of steroids was ensured by 2β- and 15β-selective mutants generated by directed evolution on the basis of ISM.[13] A number of other recent studies likewise addressed the challenge of regio- and stereoselectivity, rational design or directed evolution being used to generate appropriate mutants.[9] In many cases only one of the two possible enantiomers was accessed by protein engineering. Complete control of regio- and stereoselectivity as well as access to both stereoisomers on an optional basis remains a challenge for many types of substrates, as in the case of such simple molecules as *1-methylcyclohexene*.[9l]

P450 monoxygenases are Fe-heme dependent enzymes, the catalytically active high-spin intermediate [heme-Fe=O] (Compound I) inducing H-atom abstraction of the substrate RH with formation of the radical R• followed by rapid C–O bond formation and release of the product ROH.[6,14] This means that high regio- and stereoselectivity requires the compound to be held in a single specific pose with the respective C–H moiety pointing to the catalytically active species at an optimal angle of 130°.[14b,c] Substrates having functional groups may undergo H-bonding as one of the determining factors in positioning the compound in the large binding pockets of CYPs, which means that selective hydroxylation of substrates lacking such anchor points can be expected to be difficult, 1-methylcyclohexene being an example.[9l]

In order to address these challenges, directed evolution has been applied to small molecules, which are devoid of any functional groups (not even olefinic bonds), the hydroxylation of achiral methylcyclohexane **1** being a rare successful example.[15] Two different P450-BM3 mutants were evolved utilizing ISM, which led to regioselective hydroxylation with favored formation of *cis*-(1*S*,2*R*)-**2a** (71% regioselectivity; 94% diastereoselectivity; 92% enantioselectivity), and **2b** (71% regioselectivity), respectively.[15] In the case of desymmetrization **1** → *cis*-(1*S*,2*R*)-**2a**, two chiral centers are created in a single activation step, a phenomenon that is of considerable synthetic value.

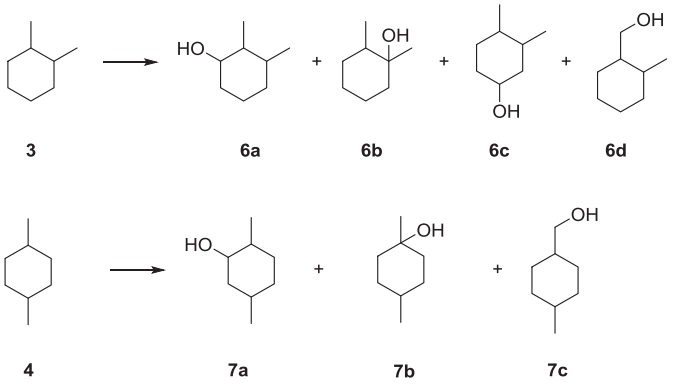


We were interested in the question whether a single oxidative hydroxylation can lead to the creation of *three* new centers of chirality and therefore considered disubstituted cyclohexanes as model compounds in this challenging endeavor. The selective oxidative functionalization of 1,2-dimethylcyclohexane (**3**) and 1,4-dimethylcyclohexane (**4**) using P450-BM3 as catalyst has not been attempted thus far. We also surmised that the regioselective P450-mediated oxidation of 1,4-methylisopropylcyclohexane (**5**) may constitute a novel access to biologically active compounds such as menthol or stereoisomers thereof. The present study constitutes an exploratory investigation of testing P450-BM3 as a catalyst in the oxidation of substrates **3**–**5**.



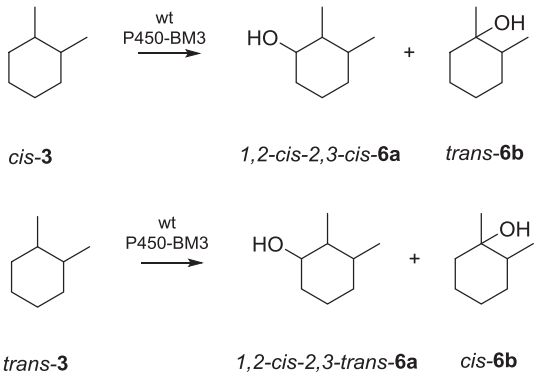
2. Results and discussion

In initial experiments, WT P450-BM3 was used as the biocatalyst in the oxidation of substrates **3** and **4**, which can lead to different regio-, distereo-, and enantiomers as indicated in Scheme 1. Hydroxylation at the methylene groups gives rise to molecules, which have three chiral centers. This ensures greater complexity and therefore added value to the products, provided some degree of stereoselectivity is achieved. This would be a highly desirable feature, but it also makes unambiguous stereochemical assignments more difficult.

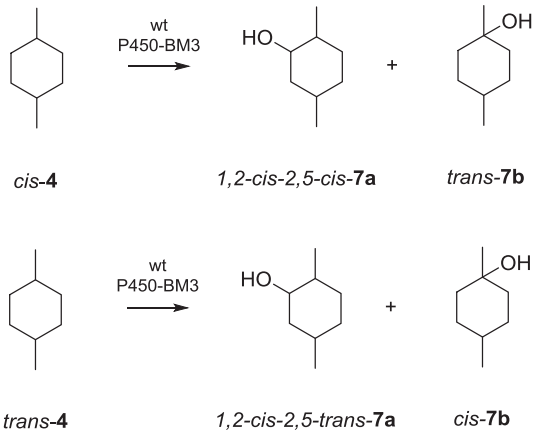


Scheme 1. Possible oxidation products by WT P450-BM3 catalyzed oxidation of substrates **3** and **4**.

By GC–MS analyses, we have identified all products arising from the P450-BM3 catalyzed oxidation of 1,2-dimethylcyclohexane (**3**) and 1,4-dimethylcyclohexane (**4**) (Schemes 2 and 3) Subsequently, the products were fully characterized by comparison with authentic commercially available or separately prepared samples. Initially, we used mixtures of *cis*- and *trans*-**3**, but the GC chromatograms of the crude product mixtures proved to be too complex



Scheme 2. Oxidation products obtained by WT P450-BM3 catalyzed oxidation of substrates *cis*-**3** and *rac-trans*-**3**.



Scheme 3. Oxidation products obtained by WT P450-BM3 catalyzed oxidation of achiral substrates *cis*- and *trans*-**4**.

due to several overlapping peaks. Therefore, *cis*- and *trans*-**3** were reacted separately. When achiral *cis*-**3** was used as starting material, WT P450-BM3 proved to be cis-selective with respect to the adjacent methyl group, in accord with the previously observed reaction pattern of methylcyclohexane[15] [Scheme 2; turnover frequency (TOF) 2.6; total turnover number (TTN) 400]. Upon subjecting racemic *trans*-**3** to WT P450-BM3 catalyzed oxidation, high diastereoselective preference for the formation of 1,2-*cis*-2,3-*trans* **6a** resulted (Table 1; TOF 3.2; TTN 530). The fact that the product is not quite racemic (5% ee) may be due to incomplete conversion and some degree of kinetic resolution. The oxidations of achiral *cis*- and *trans*-**4** also proceeded regio- and diastereoselectively, although with poor enantioselectivity (Table 1; TOF 6.1; TTN 970 and TOF 9.4; TTN 1900 using as starting materials *cis*- and *trans*-**4**, respectively). Of the four possible hydroxylation products, only compounds **6a**,**b** and **7a**,**b** were detected. In this sense P450-BM3 is much more diastereo- and regioselective than other catalysts.[5e] Similar results were observed when subjecting *cis*- and *trans*-**4** to P450-BM3 catalyzed oxidative hydroxylation (Table 1).

additionally led to small amounts of dihydroxylated products as in the case of 1-methylcyclohexene.[9l]

It was also of interest to test compound **5**. The use of menthane as substrate for P450-BM3 catalyzed hydroxylation has been mentioned in the literature, but no details about the products.[16] In our study, the reaction products were established by performing experiments using WT P450-BM3 as catalyst (TOF 2.9; TTN 500) and by comparison with commercial or separately synthesized chemical controls. To clearly assign the reaction products, and to easily assign the relative configuration, we used only *trans*-1,4-menthane (**5**) as the achiral starting material. The results of this analytical work are summarized in Fig. 1, which features the GC chromatogram of the crude product mixture.

The major product proved to be *cis*-2-isopropyl-5-methylcyclohexan-1-ol (**8c**) (40%), known as isomenthol, the crude reaction product containing significant amounts of tertiary alcohols (**8a**—3% and *cis*-**8b**—27%) (Fig. 1, top and Scheme 4). It is interesting to note that the relative configuration of the product **8c** is cis in accord with hydroxylation of substrates **3** and **4** and with oxidation of substrate **1**. However, the product proved to be racemic. Several mutants previously evolved for the regio- and stereoselective hydroxylation of compound **1**[15] were tested in the oxidation of substrate **5**, but only a moderate increase in selectivity for neomenthol **8c** was observed.

From the mutants screened, only A328F, a well-known variant,[9] proved to be considerably more selective than WT P450-BM3, the GC chromatogram of the crude product mixture indicating only the presence of only one compound, namely 2-(4-methylcyclohexyl)propan-2-ol **8e** (Fig. 1, bottom). In order to obtain enough products for structural analysis, reaction **5**→**8** was performed using 180 mg of substrate employing mutant A328F (TOF 1.7; TTN 150), pure products being successfully isolated following column chromatography (5–10% yield). The procedure was not optimized for preparative purposes. It should be noted that excessively longer reaction times when using WT or mutants allow the formation of the dihydroxylated species as observed by GC–MS analysis.

Table 1
Oxidative hydroxylation of 1,2- and 1,4-dimethylcyclohexanes (**3** and **4**) catalyzed by WT P450-BM3[a]

Entry	P450 Variant	Substrate		
1	WT	*cis*-**3**	**6a**, 23%, dr 83:17 (1,2-trans/1,2-*cis*)[b]	**6b**, 77%, trans (er=56:42)
2	WT	*trans*-**3**	**6a**, 89%, dr 98:2 (1,2-cis/1,2-trans), (*er*=55: 45)	**6b**, 11%, cis (er=53:47)
3	WT	*cis*-**4**	**7a**, 30%, dr 80:20 (1,2-*cis*/2,5-*cis*)[b]	**7b**, 70%, trans
4	WT	*trans*-**4**	**7a**, 81%, dr >99 (1,2-*cis*/2,5-trans), (er=64: 36)	**7b**, 19%, cis

[a] Values obtained from average of at least three independent experiments performed with resting cells using $NADP^+$ 50 μM. %-values refer to regioselectivity of hydroxylation. Owing to the tendency of **3** and **4** to evaporate under the reaction conditions, it is difficult to measure the exact %-conversion, which may vary when the reaction is performed in plastic or glass plates.
[b] Enantiomeric ratio (er) not measured.

In an attempt to increase regio- and enantioselectivity, some of our libraries of P450-BM3 mutants evolved for methylcyclohexane[15] were tested for substrates **3** and **4** without performing additional mutagenesis experiments. Approximately 300 transformants were screened, but better catalyst in terms of enhanced regio- and enantioselectivities were not discovered, although several mutants showed similar selectivities. Owing to the tendency of **3** and **4** to evaporate under the reaction conditions, as in the case of methylcyclohexane,[15] it is difficult to measure the exact %-conversion, which may vary considerably when the reaction is performed in plastic or glass plates. Reactions were allowed to run for 3–5 h, while longer reaction times (18 h)

3. Conclusions and perspectives

It is well-known that achieving high regio- and stereoselectivity in P450-catalyzed oxidative hydroxylation of 'small' molecules lacking any functional groups is a daunting task,[9l,15] as it is when attempting to apply synthetic catalysts.[5] In the present study we have identified all products arising from the P450-BM3 catalyzed oxidation of cyclic disubstituted alkanes **3**–**5**, including the assignment of relative stereochemistry. Several of them contain three new centers of chirality. Whereas bioprocess optimization was not performed for increasing isolated yields, the findings set the stage for future directed evolution work in the quest to control this type of

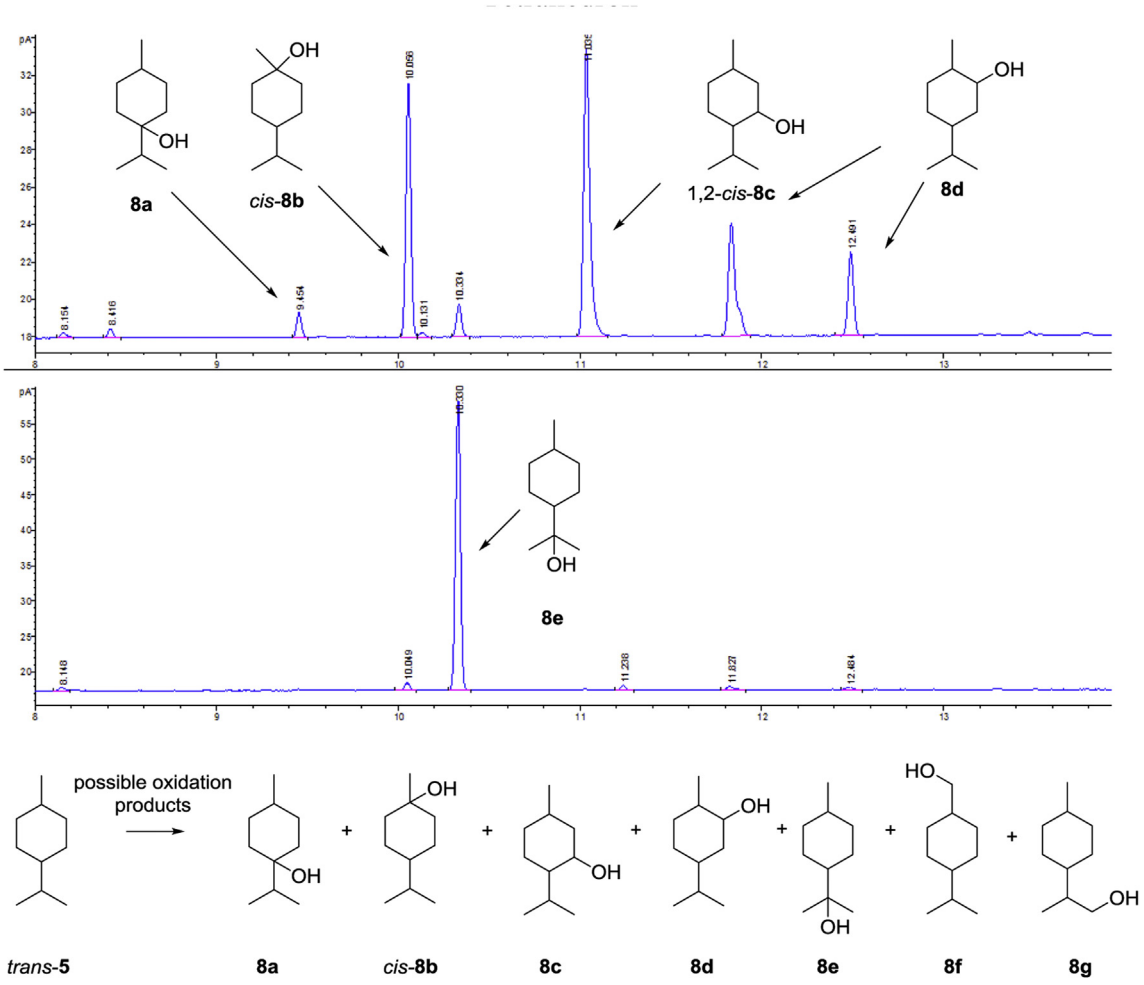
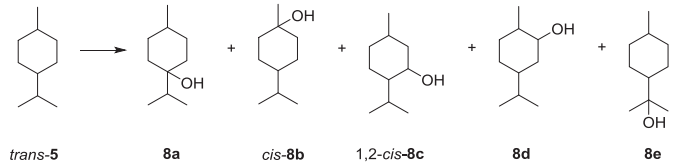


Fig. 1. GC chromatograms of the *trans*-**5** oxidation catalyzed by WT P450-BM3 (top) and mutant A328F (bottom) using resting cells (3 h of reaction time). GC conditions: 30 m DBWaxetr, inner diameter 0.25 mm; pressure: 0.8 bar H_2; injector: 230 °C; oven: temperature gradient: from 60 to 180 °C with 6 °C/min, then from 180 °C to 250 °C with 18 °C/min FID detector: 350 °C.



Scheme 4. Oxidation products formed by WT P450-BM3 catalyzed oxidation of substrate *trans*-**5**.

desymmetrization more precisely. The creation of two, three or more centers of chirality by a single oxidative hydroxylation of functionalized or non-functionalized substrates in desymmetrization events with formation of value-added products opens a new door in asymmetric catalysis. It will also be interesting to see if synthetic catalysts can be designed that make such transformations possible.

4. Experimental section

4.1. Molecular biology

4.1.1. Reagents. E. coli BOU730 cells used are described elsewhere.[9l] Electro-competent cells were prepared in-house according to standard protocols.[17] Scaled up biohydroxylation using P450 mutants was performed as described previously.[15] Turnover frequency; TOF (calculated as mol of product/nmol of protein/min during the first hour of reaction); and total turnover number; TTN (calculated as mol of product/nmol of after 1200 min of reaction) have been calculated as previously reported[15] in reactions containing 1.5 nmol of P450-BM3 and different amounts of starting materials **3**, **4** or **5** (20, 20 or 10 μmol, respectively).

4.2. Chemistry

4.2.1. General remarks. cis-, *trans-*, and *cis*+*trans*-1,2-dimethylcyclohexane, *cis-*, *trans-*, and *cis*+*trans*-1,4-dimethylcyclohexane, *trans*-1-isopropyl-4-methylcyclohexane, (−)-terpinen-4-ol, α-terpineol, (+)-neomenthol, (+)-menthol, (±)-menthol, (−)-menthone, and 3,4-dimethylcyclohexan-1-ol were purchased from Sigma–Aldrich, Alfa Aesar, ABCR or TCI and used without further purification. NMR spectra were recorded on a Bruker Avance 300 (^{1}H: 300 MHz, ^{13}C: 75 MHz) spectrometer using TMS as internal standard (d=0). Analytical thin layer chromatography was performed on Merck silica gel 60 F254 while for column chromatography Merck silica gel 60 was used. Conversion and enantiomeric excess were determined by achiral and chiral gas chromatography. Determination of the relative configuration was performed after comparison with commercial or separately prepared racemic samples. Diastereoisomeric mixtures of *cis*+*trans*-1-isopropyl-4-methylcyclohexan-1-ol (**8a**),[18] *cis*+*trans*-4-isopropyl-1-methylcyclohexan-1-ol (**8b**),[19] *cis*-(1*S*,2*S*,5*R*)+

trans-(1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexan-1-ol (**8c**),[20] *cis*-(1*R*,2*R*,5*S*)-2-isopropyl-5-methylcyclohexan-1-ol, and *trans*-(1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexan-1-ol (**8c**)[20] were prepared according to described literature protocols. (1*R*,2*S*,5*S*)-5-Isopropyl-2-methylcyclohexan-1-ol (**8d**) and (1*S*,2*S*,5*S*)-5-isopropyl-2-methylcyclohexan-1-ol (**8d**) were prepared according to a protocol described by Negoro et al.[21] and Pavia et al.[22] starting from (*S*)(+)-carvone and (*R*)(+)-carvone. Their spectroscopic data are identical to the published ones. Authentic samples of known products were obtained as follows:

*4.2.2. 1,2-cis-2,3-trans-2,3-Dimethylcyclohexan-1-ol (**6a**).* A commercial available mixture of four diastereoisomers (TCI) was separated on column chromatography to afford *1,2-cis-2,3-trans*-**6a** as crude oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.80–3.77 (m, 1H), 1.89–1.74 (m, 1H), 1.68–0.76 (10H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 71.9, 42.9, 35.1, 33.6, 32.2, 20.3, 20.7, 16.4.

*4.2.3. cis+trans-1,2-Dimethylcyclohexan-1-ol (**6b**).* A mixture of *cis*+*trans*-1,2-dimethylcyclohexan-1-ol was prepared starting from 2-methylcyclohexan-1-one and CH_3MgCl in dry THF according to a published protocol used for the synthesis of 1-isopropylcyclohexan-1-ol.[23] The crude reaction product was loaded on a column chromatography and *trans*-1,2-dimethylcyclohexan-1-ol separated. Spectroscopic data are in agreement with published data.[24] *cis*+*trans* **6b**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.77–1.52 (m, 4H), 1.44 (m, 4H), 1.22 (m, 3H), 0.90 (m, 3H). *cis* **6b**: ^{13}C NMR (75 MHz, $CDCl_3$) δ 73.1, 42.4, 41.5, 32.2, 25.4, 24.3, 21.0, 15.4. *trans* **6b**: ^{13}C NMR (75 MHz, $CDCl_3$) δ 71.1, 40.4, 40.1, 30.7, 28.7, 26.0, 22.2, 15.3.

*4.2.4. 1,2-cis-2,5-trans-2,5-Dimethylcyclohexan-1-ol (**7a**).* Compound *1,2-cis-2,5-trans-1,4-dimethylcyclohexan-1-ol* **7a** was separated from a commercial diastereoisomeric mixture and characterized by NMR. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.80 (s, 1H), 1.83 (d, ^3J=13.6 Hz, 1H), 1.73–164 (s, 2H), 1.55–1.04 (m, 6H), 0.91 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 71.3, 42.3, 36.1, 34.9, 28.2, 25.7, 22.4, 18.3.

*4.2.5. trans-1,4-Dimethylcyclohexan-1-ol (**7b**).* A mixture of *cis*+*trans*-1,4-dimethylcyclohexan-1-ol was prepared starting from 4-methylcyclohexan-1-one and CH_3MgCl in dry THF according to a published protocol used for the synthesis of 1-isopropylcyclohexan-1-ol.[23] The crude reaction product was loaded on a column chromatography and *trans*-1,4-dimethylcyclohexan-1-ol separated. Spectroscopic data are in agreement with published data.[25] ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.77–1.52 (m, 4H), 1.44 (m, 4H), 1.22 (m, 3H), 0.90 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 70.9, 39.9, 32.4, 31.9, 26.1, 21.6.

*4.2.6. cis+trans-2-(4-Methylcyclohexyl)propan-2-ol (**8e**).* α-Terpineol (300 mg, 1.94 mmol) was reduced using the same protocol as above, to afford a mixture of *cis*+*trans* 2-(4-methylcyclohexyl)propan-2-ol. A GC sample of the crude reaction product was compared with products resulting from hydroxylation reaction of *trans*-1-isopropyl-4-methylcyclohexane catalyzed by WT P450. *trans*-**8e** was obtained after hydroxylation reaction of *trans*-**5**, catalyzed by mutant P450-A328F, and was scaled-up according to a general procedure described elsewhere.[5e] After extraction and concentration using a rotavap, the crude reaction product was purified by column chromatography (EA/PE=1:4, R_f=0.55) to afford compound *trans*-**8e** as a colorless oil, yield <10%. ^{13}C NMR (75 MHz, $CDCl_3$) δ 71.8, 49.0, 35.5 (2C), 27.5 (2C), 27.1 (2C), 22.7, 14.3.

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References and notes

1. (a) *Enzyme Catalysis in Organic Synthesis*, 3rd ed.; Drauz, K., Gröger, H., May, O., Eds.; Wiley-VCH: Weinheim, Germany, 2012; (b) Faber, K. *Biotransformations in Organic Chemistry*, 6th ed.; Springer: Heidelberg, Germany, 2011; (c) Wells, A.; Meyer, H.-P. *ChemCatChem* **2014**, *6*, 918.
2. Reetz, M. T. *J. Am. Chem. Soc.* **2013**, *135*, 12480.
3. Reviews of directed evolution: (a) Directed Evolution Library Creation In *Methods in Molecular Biology*, 2nd ed.; Gillam, E. M. J., Copp, J. N., Ackerley, D. F., Eds.; Humana: Totowa, 2014; (b) Widersten, M. *Curr. Opin. Chem. Biol.* **2014**, *21*, 42; (c) Siloto, R. M. P.; Weselake, R. J. *Biocatal. Agric. Biotechnol.* **2012**, *1*, 181; (d) Goldsmith, M.; Tawfik, D. S. *Curr. Opin. Struct. Biol.* **2012**, *22*, 406; (e) Cobb, R. E.; Chao, R.; Zhao, H. *AIChE J.* **2013**, *59*, 1432; (f) Reetz, M. T. Directed Evolution of Enzymes In*Drauz, K., Dröge, H., May, O., Eds.. Enzyme Catalysis in Organic Synthesis*; Wiley-VCH: Weinheim, Germany, 2012; Vol. 1, pp 119–190; (g) Bommarius, A. S.; Blum, J. K.; Abrahamson, M. J. *Curr. Opin. Chem. Biol.* **2011**, *15*, 194; (h) Quin, M. B.; Schmidt-Dannert, C. *ACS Catal.* **2011**, *1*, 1017; (i) Brustad, E. M.; Arnold, F. H. *Curr. Opin. Chem. Biol.* **2011**, *15*, 201; (j) Jäckel, C.; Hilvert, D. *Curr. Opin. Biotechnol.* **2010**, *21*, 753; (k) Turner, N. J. *Nat. Chem. Biol.* **2009**, *5*, 567; (l) Lutz, S.; Bornscheuer, U. T. *Protein Engineering Handbook*; Wiley-VCH: Weinheim, Germany, 2009, Vols. 1 and 2.
4. Turner, N. J.; O'Reilly, E. *Nat. Chem. Biol.* **2013**, *9*, 285.
5. Recent representative studies of oxidative hydroxylation utilizing synthetic reagents or catalysts for the preparation of simple and complex organic compounds: (a) Newhouse, T.; Baran, P. S. *Angew. Chem., Int. Ed.* **2011**, *50*, 3362; (b) White, M. C. *Science* **2012**, *335*, 807; (c) Neufeldt, S. R.; Sanford, M. S. *Acc. Chem. Res.* **2012**, *45*, 936; (d) Adam, W.; Zhao, C.-G.; Saha-Möller, R. R.; Jakka, K. *Oxidation of Organic Compounds with Dioxiranes*; Wiley-VCH: New York, NY, 2009; (e) Kamata, K.; Yonehara, K.; Nakagawa, Y.; Uehara, K.; Mizuno, N. *Nat. Chem.* **2010**, *2*, 478; (f) Periana, R. A. *Acc. Chem. Res.* **2012**, *45*, 885; (g) Sun, M.; Zhang, J.; Putaj, P.; Caps, V.; Lefebvre, F.; Pelletier, J.; Basset, J. M. *Chem. Rev.* **2014**, *114*, 981; (h) Roduner, E.; Kaim, W.; Sarkar, B.; Urlacher, V. B.; Pleiss, J.; Gläser, R.; Einicke, W.-D.; Sprenger, G. A.; Beifuß, U.; Klemm, E.; Liebner, C.; Hieronymus, H.; Hsu, S.-F.; Plietker, B.; Laschat, S. *ChemCatChem* **2013**, *5*, 82.
6. Reviews of structure, mechanism and applications of P450 enzymes: (a) Ortiz de Montellano, P. R. *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 3rd ed.; Springer: Berlin, Germany, 2005; (b) Isin, E. M.; Guengerich, F. P. *Biochim. Biophys. Acta Gen. Subj.* **2007**, *1770*, 314; (c) Munro, A. W.; Girvan, H. M.; McLean, K. J. *Nat. Prod. Rep.* **2007**, *24*, 585; (d) Ortiz de Montellano, P. R. *Chem. Rev.* **2010**, *110*, 932; (e) Shaik, S.; Cohen, S.; Wang, Y.; Chen, H.; Kumar, D.; Thiel, W. *Chem. Rev.* **2010**, *110*, 949; (f) Urlacher, V. B.; Eiben, S. *Trends Biotechnol.* **2006**, *24*, 324; (g) Kadish, K. M.; Smith, K. M.; Guilard, R. *Handbook of Porphyrin Science*; World Scientific Publishing, 2010, Vol. 5; (h) Meunier, B.; de Visser, S. P.; Shaik, S. *Chem. Rev.* **2004**, *104*, 3947; (i) Whitehouse, C. J. C.; Bell, S. G.; Wong, L.-L. *Chem. Soc. Rev.* **2012**, *41*, 1218; (j) Lewis, J. C.; Coelho, P. S.; Arnold, F. H. *Chem. Soc. Rev.* **2011**, *40*, 2003; (k) O'Reilly, E.; Köhler, V.; Flitsch, S. L.; Turner, N. J. *Chem. Commun.* **2011**, 2490; (l) Fasan, R. *ACS Catal.* **2012**, *2*, 647; (m) Khatri, Y.; Hannemann, F.; Girhard, M.; Kappl, R.; Meme, A.; Ringle, M.; Janocha, S.; Leize-Wagner, E.; Urlacher, V. B.; Bernhardt, R. *Biotechnol. Appl. Biochem.* **2013**, *60*, 18; (n) *Fifty Years of Cytochrome P450 Research*; Yamazaki, H., Ed.; Springer: Berlin, Germany, 2014.
7. Review of early steroid oxidations practiced by: (a) Upjohn Company/USA: Hogg, J. A. *Steroids* **1992**, *57*, 593; (b) Schering Corporation: Herzog, H.; Oliveto, P. *Steroids* **1992**, *57*, 617.
8. See for example: Landwehr, M.; Hochrein, L.; Otey, C. R.; Kasrayan, A.; Bäckvall, J.-E.; Arnold, F. H. *J. Am. Chem. Soc.* **2006**, *128*, 6058.
9. Recent accounts of protein engineering of P450 enzymes:[6] (a) Jung, S. T.; Lauchli, R.; Arnold, F. H. *Curr. Opin. Biotechnol.* **2011**, *22*, 809; (b) Yang, Y.; Liu, J.; Li, Z. *Angew. Chem., Int. Ed.* **2014**, *53*, 3147; (c) Peters, M. W.; Meinwald, P.; Glieder, A.; Arnold, F. H. *J. Am. Chem. Soc.* **2003**, *125*, 13442; (d) Tang, W. L.; Li, Z.; Zhao, H. *Chem. Commun.* **2010**, 5461; (e) Urlacher, V. B.; Girhard, M. *Trends Biotechnol.* **2012**, *30*, 26; (f) Li, X. D.; Li, Z. *Chem. Commun.* **2012**, 4618; (g) Dennig, A.; Marienhagen, J.; Ruff, A. J.; Guddat, L.; Schwaneberg, U. *ChemCatChem* **2012**, *4*, 771; (h) Weber, E.; Seifert, A.; Antonovici, M.; Geinitz, C.; Pleiss, J.; Urlacher, V. B. *Chem. Commun.* **2011**, 944; (i) Venkataraman, H.; de Beer, S. B. A.; van Bergen, L. A. H.; van Essen, N.; Geerke, D. P.; Vermeulen, N. P. E.; Commandeur, J. N. M. *ChemBioChem* **2012**, *13*, 520; (j) Lewis, J. C.; Mantovani, S. M.; Fu, Y.; Snow, C. D.; Komor, R. S.; Wong, C. H.; Arnold, F. H. *ChemBioChem* **2010**, *11*, 2502; (k) Zhang, K.; Damaty, S. E.; Fasan, R. *J. Am. Chem. Soc.* **2011**, *133*, 3242; (l) Roiban, G.-D.; Agudo, R.; Reetz, M. T. *Tetrahedron* **2013**, *69*, 5306; (m) Roiban, G.-D.; Agudo, R.; Ilie, A.; Lonsdale, R.; Reetz, M. T. *Chem. Commun.* **2014**, 14310; (n) Bogazkaya, A. M.; von Bühler, C. J.; Kriening, S.; Busch, A.; Seifert, A.; Pleiss, J.; Laschat, S.; Urlacher, V. B. *Beilstein J. Org. Chem.* **2014**, *10*, 1347; (o) Bernhardt, R.; Urlacher, V. B. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 6185; (p) General review of oxidative hydroxylation using P450 monooxygenases and other enzymes: Holtmann, D.; Fraaije, M. W.; Arends, I. W. C. E.; Opperman, D. J.; Hollmann, F. *Chem. Commun.* **2014**, *50*, 13180; (q) Neufeld, K.; Henßen, B.; Pietruszka, J. *Angew. Chem., Int. Ed.* **2014**, *53*, 13253; (r) Kolev, J. N.; O'Dwyer, K. M.; Jordan, C. T.; Fasan, R. *ACS Chem. Biol.* **2014**, *9*, 164; (s) Zhang, K.; Shafer, B. M.; Demars, M. D., II; Stern, H. A.; Fasan, R. *J. Am. Chem. Soc.* **2012**, *134*, 18695; (t) Li, A.; Wu, S.; Adams, J. P.; Snajdrova, R.; Li, Z. *Chem. Commun.* **2014**, 8771.(u) General review of oxidative hydroxylation using P450 monooxygenases: Roiban, G.-D.; Reetz, M. T. *Chem. Commun.* http://dx.doi.org/10.1039/c4cc09218j.
10. Agudo, R.; Roiban, G.-D.; Reetz, M. T. *ChemBioChem* **2012**, *13*, 1465.
11. (a) Narhi, L. O.; Fulco, A. J. *J. Biol. Chem.* **1986**, *261*, 7160; (b) Munro, A. W.; Leys, D. J.; McLean, K. J.; Marshall, K. R.; Ost, T. W. B.; Daff, S.; Miles, C. S.; Chapman, S.

K.; Lysek, D. A.; Moser, C. C.; Page, C. C.; Dutton, P. L. *Trends Biochem. Sci.* **2002**, *27*, 250; (c) Jovanovic, T.; Farid, R.; Friesner, R. A.; McDermott, A. E. *J. Am. Chem. Soc.* **2005**, *127*, 13548; (d) Clodfelter, K. H.; Waxman, D. J.; Vajda, S. *Biochemistry* **2006**, *45*, 9393; (e) Schwaneberg, U.; Sprauer, A.; Schmidt-Dannert, C.; Schmid, R. D. *J. Chromatogr., A* **1999**, *848*, 149.
12. (a) Reetz, M. T. *Angew. Chem., Int. Ed.* **2011**, *50*, 138; (b) Acevedo-Rocha, C. G.; Kille, S.; Reetz, M. T. Iterative Saturation Mutagenesis: A Powerful Approach to Engineer Proteins by Simulating Darwinian Evolution In *Directed Evolution Library Creation: Methods and Protocols*, 2nd ed.; Ackerley, D., Copp, J., Gillam, E., Eds.; Methods in Molecular Biology; Humana: Totowa, NJ, 2014; 1179, p 103.
13. Kille, S.; Zilly, F. E.; Acevedo, J. P.; Reetz, M. T. *Nat. Chem.* **2011**, *3*, 738.
14. (a) Shaik, S.; Kumar, D.; de Visser, S. P.; Altun, A.; Thiel, W. *Chem. Rev.* **2005**, *105*, 2279; (b) Lonsdale, R.; Harvey, J. N.; Mulholland, A. J. *J. Phys. Chem. Lett.* **2010**, *1*, 3232; (c) Lonsdale, R.; Harvey, J. N.; Mulholland, A. J. *J. Phys. Chem. B* **2010**, *114*, 1156.
15. Roiban, G.-D.; Agudo, R.; Reetz, M. T. *Angew. Chem., Int. Ed.* **2014**, *53*, 8659.
16. Seifert, A.; Antonovici, M.; Hauer, B.; Pleiss, J. *ChemBioChem* **2011**, *12*, 1346.
17. Sambrook, J.; Russell, D. W. *The Condensed Protocols From Molecular Cloning: a Laboratory Manual*; CSHL: New York, NY, 2006.
18. Crabtree, R. H.; Davis, M. W. *J. Org. Chem.* **1986**, *51*, 2655.
19. Hueckel, W.; Heinzelmann, P. *Liebigs Ann. Chem.* **1965**, *687*, 82.
20. Tada, M.; Chiba, K.; Izumiya, K.; Tamura, M. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 3532.
21. Takaki, K.; Okada, M.; Yamada, M.; Negoro, K. *J. Org. Chem.* **1982**, *47*, 1200.
22. Accrombessi, G.; Geneste, P.; Olive, J.-L.; Pavia, A. A. *Tetrahedron* **1981**, *37*, 3135.
23. Imamoto, T.; Takiyama, N.; Nakamura, K.; Hatajima, T.; Kamiya, Y. *J. Am. Chem. Soc.* **1989**, *111*, 4392.
24. Bianchini, G.; Crucianelli, M.; Canevali, C.; Crestini, C.; Morazzoni, F.; Saladino, R. *Tetrahedron* **2006**, *62*, 12326.
25. Kunzelmann, A.; Lauterbach, G.; Potekhin, V. M.; Pritzkow, W.; Schmidtrenner, W.; Vasina, L. F. *J. Prakt. Chem.* **1986**, *328*, 772.