VIP C–H activation Very Important Paper

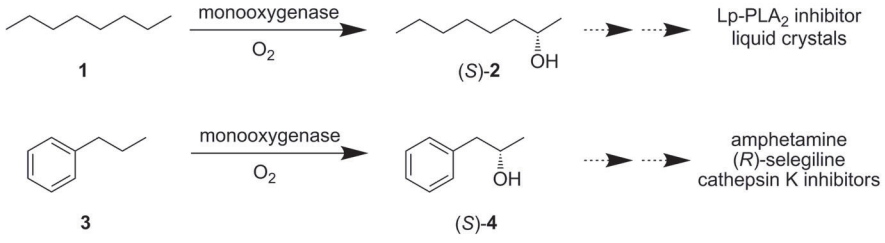
DOI: 10.1002/ange.201311091

Engineering of P450pyr Hydroxylase for the Highly Regio- and Enantioselective Subterminal Hydroxylation of Alkanes**

*Yi Yang, Ji Liu, and Zhi Li**

Abstract: *Terminal-selective cytochrome P450pyr has been successfully engineered through directed evolution for the subterminal hydroxylation of alkanes with excellent regio- and enantioselectivity. A sensitive colorimetric high-throughput screening (HTS) assay was developed for the measurement of both the regioselectivity and the enantioselectivity of a hydroxylation reaction. By using the HTS assay and iterative saturation mutagenesis, sextuple-mutant P450pyrSM1 was created for the hydroxylation of n-octane (**1**) to give (S)-2-octanol (**2**) with 98% ee and >99% subterminal selectivity. The engineered P450 is the first enzyme for this type of highly selective alkane hydroxylation, being useful for the C–H activation and functionalization of alkanes and the preparation of enantiopure alcohols. Molecular modeling provided structure-based understanding of the fully altered regioselectivity and the excellent enantioselectivity. Another sextuple-mutant P450pyrSM2 catalyzed the hydroxylation of propylbenzene (**3**) to afford (S)-1-phenyl-2-propanol (**4**) with 95% ee and 98% subterminal selectivity.*

Regio- and stereoselective hydroxylation at a non-activated carbon atom is a very useful reaction in organic chemistry for the functionalization of alkanes,[1] an abundant and cheap feedstock, and the production of enantiomerically pure alcohols, which are useful and valuable synthetic and pharmaceutical intermediates.[2] Despite some progress with transition-metal catalysts,[3] this type of reaction still remains a significant challenge in classical chemistry. On the other hand, nature offers an alternative solution for this reaction in the use of a monooxygenase, such as P450, sMMO, and alkB, as a green and efficient catalyst and molecular oxygen as a cheap and green oxidant.[1,4] However, native monooxygenases often show a narrow substrate range and unsatisfactory regio- and stereoselectivity in the hydroxylation of non-natural substrates for organic synthesis. Directed evolution has become a useful tool for the creation of new enzymes with improved catalytic performance.[5] By this approach, some monooxygenases have been engineered with new substrate specificity[6] and several with enhanced regio- and/or stereoselectivity[7] for the target hydroxylation reactions. For example, P450BM-3 mutants were engineered with excellent regio- and diastereoselectivity for the hydroxylation of testosterone[8] and artemisinin,[9] respectively. P450pyr[10] mutants were generated that showed excellent enantioselectivity for the hydroxylation of *N*-benzylpyrrolidine.[11] Nevertheless, it is a challenge to develop an enzyme for the nonterminal hydroxylation of alkanes with excellent regio- and enantioselectivity: Many monooxygenases, such as sMMO, alkB, and P450pyr, showed terminal selectivity;[4c,10e,12] P450BM-3 and P450cyp102A3 showed ω-1, ω-2, and ω-3 selectivity;[7a,13] engineered sMMO and alkB mutants demonstrated some subterminal selectivity;[4d,14] engineered P450BM-3 mutants gave enhanced subterminal selectivity,[15] with 89% regioselectivity and 65% enantioselectivity as the best results for the subterminal hydroxylation of *n*-octane; and engineered P450cam mutants showed subterminal hydroxylation of *n*-butane[16] and propylbenzene (78% regioselectivity),[6a] but no product *ee* values were reported.



Scheme 1. Monooxygenase-catalyzed highly regio- and enantioselective subterminal hydroxylation of alkanes for the functionalization of alkanes and preparation of chiral alcohols.

We are interested in developing enzymes for the highly regio- and enantioselective subterminal hydroxylation of alkanes. Monooxygenase-catalyzed subterminal hydroxylation reactions of *n*-octane (**1**) and propylbenzene (**3**) were selected as the target reactions (Scheme 1). These reactions could give simple access to (*S*)-2-octanol (**2**) and (*S*)-1-phenyl-2-propanol (**4**), respectively. Whereas (*S*)-**2** is useful for the synthesis of high-performance liquid crystals[17a] and a lipoprotein-associated phospholipase A_2 (Lp-PLA_2) inhibitor for the treatment of heart disease,[17b] (*S*)-**4** is a pharma-

[*] Y. Yang, Dr. J. Liu, Prof. Dr. Z. Li
Department of Chemical and Biomolecular Engineering
National University of Singapore
4 Engineering Drive 4, Singapore 117585 (Singapore)
E-mail: chelz@nus.edu.sg
Homepage: http://www.chbe.nus.edu.sg/people/faculty_liz.html

[**] This research was supported by GlaxoSmithKline (GSK) and Singapore Economic Development Board (EDB) through a Green and Sustainable Manufacturing grant (project No. 279-000-331-592). We thank Dr. Joseph P. Adams and Dr. Radka Snajdrova from GSK for helpful discussion.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201311091.

ceutical intermediate for the preparation of amphetamine,[18a] (*R*)-selegiline,[18b] and cathepsin K inhibitors.[18c] No enzyme could catalyze these hydroxylation reactions with high regio- and enantioselectivity.[6a,15a,c] Herein, we report the evolution of P450pyr hydroxylase to turn the terminal selectivity fully into subterminal selectivity and also gain excellent enantioselectivity for the hydroxylation of alkanes **1** and **3**.

The evolution studies started with the identification of the key amino acid residues by docking *n*-octane (**1**) onto the X-ray crystal structure of P450pyr hydroxylase; 22 residues located either within 6 Å of the substrate or in the substrate-accessing channel and "big loop" were selected (see Figure S1 in the Supporting Information). Iterative saturation mutagenesis (ISM) was applied for the evolution.[5c] P450pyr variants at each selected site were generated by PCR using NNK degenerate codon and screened for the subterminal hydroxylation of **1** on a microtiter plate by high-throughput screening (HTS). Variants with enhanced regio- and/or enantioselectivity were selected for individual biotransformation in a shake flask to confirm the selectivity, and the best mutant was used as the template for the subsequent round of evolution.

In the first three rounds of evolution, a HTS assay based on the hydroxylation of 4-nitrophenetole was used.[13,15c] Whereas wild-type P450pyr showed only terminal hydroxylation of *n*-octane (**1**), the screening of 4136 clones in the first round, 3948 clones in the second round, and 3760 clones in the third round led to the identification of the P450pyr triple mutant N100S/F403I/T186I with 40% subterminal selectivity (i.e., regioselectivity in favor of 2-hydroxylation) and 44% of the activity of wild-type P450pyr for terminal hydroxylation.

The limited improvement of the subterminal selectivity in the first three rounds of evolution is possibly due to the use of an artificial substrate in the HTS assay. To solve this problem and also to monitor enantioselectivity during evolution,[11a,19] a novel colorimetric HTS assay was developed to determine both the regio- and enantioselectivity of the subterminal hydroxylation of *n*-octane (**1**). Figure 1A illustrates the principle of the HTS assay. Three NAD^+-dependent alcohol dehydrogenases (ADHs) that are highly specific for the oxidation of **5**, (*S*)-**2**, and (*R*)-**2**, respectively, are used to separately oxidize the product mixture from a P450pyr-variant-catalyzed hydroxylation of **1** in three parallel experiments. In the presence of NAD^+, NBT, and PMS, the concentrations of **5**, (*S*)-**2**, and (*R*)-**2** can be obtained on the basis of the UV absorption of formazan at 580 nm. The subterminal selectivity and the enantioselectivity can be calculated by using Equation (1) and (2), respectively. To prove the concept, we screened a number of ADHs and found CpSADH from *Candida parapsilosis*,[20] PfODH from *Pichia finlandica*,[21] and YAD from *Saccharomyces cerevisiae*[7a] to be highly specific for the oxidation of (*S*)-**2**, (*R*)-**2**, and **5**, respectively. Whereas YAD is commercially available, CpSADH and PfODH were prepared by engineering *Escherichia coli* expressing the corresponding His-tagged enzyme, growing the cells, and purifying the enzymes with affinity chromatography. To validate the assay, 81 samples containing **5**, (*S*)-**2**, and (*R*)-**2** in different ratios with a total concentration of 1.0 mM were separately oxidized with YAD, CpSADH, and PfODH for 30 min. The UV absorption at 580 nm was readily determined by the use of a microtiter-plate reader and found to be proportional to the concentration of **5**, (*S*)-**2**, and (*R*)-**2**, respectively (see Figure S7A). The determined content of **2** and the *ee* value of (*S*)-**2** were very close to the true values (Figure 1B). Further experiments showed that the assay was independent of the total concentration of **5**, (*S*)-**2**, and (*R*)-**2** (see Figure S7B).

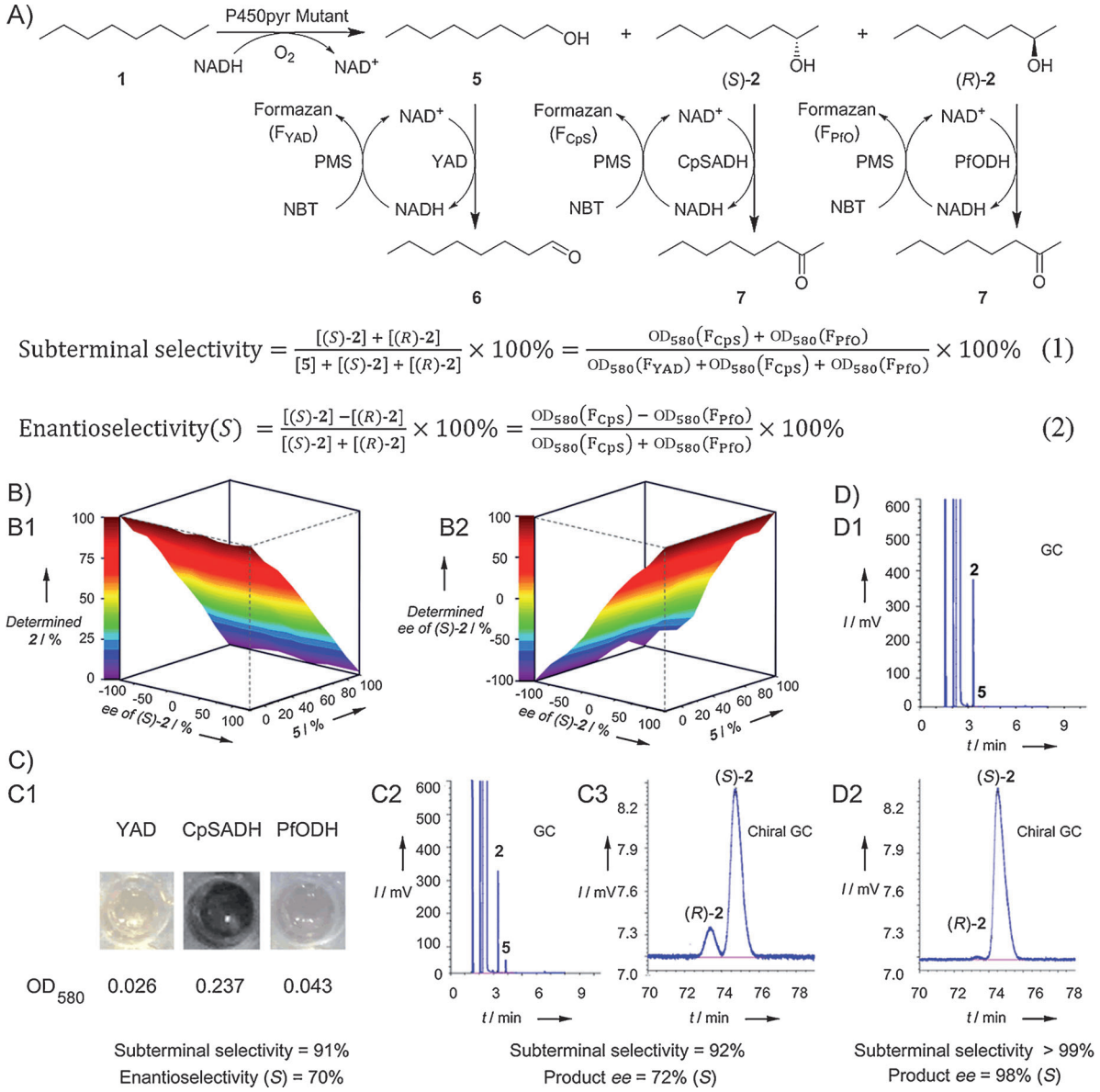
The colorimetric HTS assay was used for the 4th–6th rounds of evolution of P450pyr. After biotransformation of the variants on a 96-well plate, the supernatants were divided into three plates for the HTS assay. The developed colorimetric HTS assay was proven to be accurate and applicable for the evolution. Figure 1C shows a representative example of the determination of the subterminal selectivity and enantioselectivity of a P450pyr mutant for the hydroxylation of *n*-octane (**1**) during evolution. The values determined from the HTS assay are nearly the same as those obtained by GC analysis of the product of biohydroxylation with the same mutant in a shake flask.

The best mutant from the 4th round, N100S/F403I/T186I/L302V, increased the subterminal selectivity to 92% and gave (*S*)-**2** with 72% *ee* (Table 1). The best mutant from the 5th round, I83F/N100S/F403I/T186I/L302V, displayed higher than 99% subterminal selectivity to provide (*S*)-**2** with an

Table 1: Directed evolution of P450pyr hydroxylase for the regio- and enantioselective subterminal hydroxylation of *n*-octane (**1**) to (*S*)-2-octanol (**2**).

Round	No. of sites saturated	No. of clones screened	No. of positive clones identified	Best mutant	Subterminal selectivity [%][a]	*ee* of (*S*)-**2** [%][b]	Activity [U (g cdw)$^{-1}$][c]	Relative activity [%][d]
WT[e]	nil	nil	nil	nil	0	nil	1.8	100
1	22	4136	10	N100S	5	ND[e]	0.9	49
2	21	3948	20	N100S/F403I	33	ND	2.0	110
3	20	3760	40	N100S/T186I/F403I	40	56	0.8	44
4	19	3572	40	N100S/T186I/L302V/F403I	92	72	1.7	94
5	18	3384	100	I83F/N100S/T186I/L302V/F403I	>99	95	1.8	97
6	17	3196	120	A77Q/I83F/N100S/T186I/L302V/F403I	>99	98	1.7	90

[a] The subterminal selectivity of the best mutant in each round was determined by GC analysis of the products of the biotransformation of *n*-octane (**1**; 5 mM) with *E. coli* cells (2 g cdw L^{-1}) expressing the P450pyr mutant in potassium phosphate buffer (100 mM, pH 8.0; 10 mL) containing glucose (2%, w/v) at 30 °C and 250 rpm for 4 h. [b] The *ee* value of (*S*)-**2** was determined by GC analysis on a chiral stationary phase. [c] The activity is the specific activity determined for the first 30 min of the biotransformation. [d] The activity of the mutant is expressed as a percentage relative to the activity of *E. coli* cells expressing P450pyr for the terminal hydroxylation of *n*-octane (**1**) under the same conditions. [e] ND: not determined, WT: wild type.



$$\text{Subterminal selectivity} = \frac{[(S)\text{-}2] + [(R)\text{-}2]}{[5] + [(S)\text{-}2] + [(R)\text{-}2]} \times 100\% = \frac{OD_{580}(F_{CpS}) + OD_{580}(F_{PfO})}{OD_{580}(F_{YAD}) + OD_{580}(F_{CpS}) + OD_{580}(F_{PfO})} \times 100\% \quad (1)$$

$$\text{Enantioselectivity}(S) = \frac{[(S)\text{-}2] - [(R)\text{-}2]}{[(S)\text{-}2] + [(R)\text{-}2]} \times 100\% = \frac{OD_{580}(F_{CpS}) - OD_{580}(F_{PfO})}{OD_{580}(F_{CpS}) + OD_{580}(F_{PfO})} \times 100\% \quad (2)$$

Figure 1. A) Principle of the colorimetric HTS assay to measure both the subterminal selectivity and enantioselectivity of P450pyr variants for the hydroxylation of *n*-octane (**1**). The concentrations of the possible hydroxylation products **5**, (*S*)-**2**, and (*R*)-**2** are determined on the basis of the UV absorption of formazan at 580 nm in the oxidation of the biohydroxylation mixture with the highly selective alcohol dehydrogenase YAD, CpADH, and PfODH, respectively. The subterminal selectivity and enantioselectivity of the P450pyr variant are calculated by using Equation (1) and (2). NAD^+ = nicotinamide adenine dinucleotide, NBT = nitroblue tetrazolium, PMS = phenazine methosulfate; OD = optical density. B) Colorimetric HTS assay to measure the amount of **2** and *ee* value of (*S*)-**2** for 81 samples containing **5**, (*S*)-**2**, and (*R*)-**2** in different ratios with a total concentration of 1.0 mM. B1) Determined proportion of **2** versus the real proportion of **2** in the samples; B2) Determined *ee* value of (*S*)-**2** versus the real *ee* value of (*S*)-**2** in the samples. C) Determination of the subterminal and enantioselectivity of a P450pyr mutant (N100S/T186I/L302V/F403I) for the hydroxylation of *n*-octane (**1**) during directed evolution: C1) by the colorimetric HTS assay; C2) by GC analysis; C3) by GC analysis on a chiral stationary phase. D) Analysis of the product from the biohydroxylation of *n*-octane (**1**) with P450pyrSM1 (the best mutant created in the directed evolution): D1) by GC analysis; D2) by GC analysis on a chiral stationary phase.

impressive *ee* value of 95%. Finally, an excellent mutant was created in the 6th round, A77Q/I83F/N100S/F403I/T186I/L302V (P450pyrSM1), which gave higher than 99% subterminal selectivity and provided (*S*)-**2** with 98% *ee* (Figure 1D). The hydroxylation activity of P450pyrSM1 remained at 90% of the terminal hydroxylation activity of P450pyr. P450pyrSM1 showed a k_{cat} value of 5.9 min^{-1} and a K_m value of 2.187 mM. The catalytic efficiency (k_{cat}/K_m) is very close to the value of P450pyr for the terminal hydroxylation of **1** (see Figure S12 and Table S4). Thus, the P450pyrSM1 mutant is catalytically as efficient as P450pyr.

To gain an understanding of the stereochemical outcome of the reaction, we investigated the hydroxylation of *n*-octane (**1**) with P450pyr and P450pyrSM1 by molecular dynamics and docking simulation. A reshaping of the binding pocket that is responsible for the subterminal selectivity and

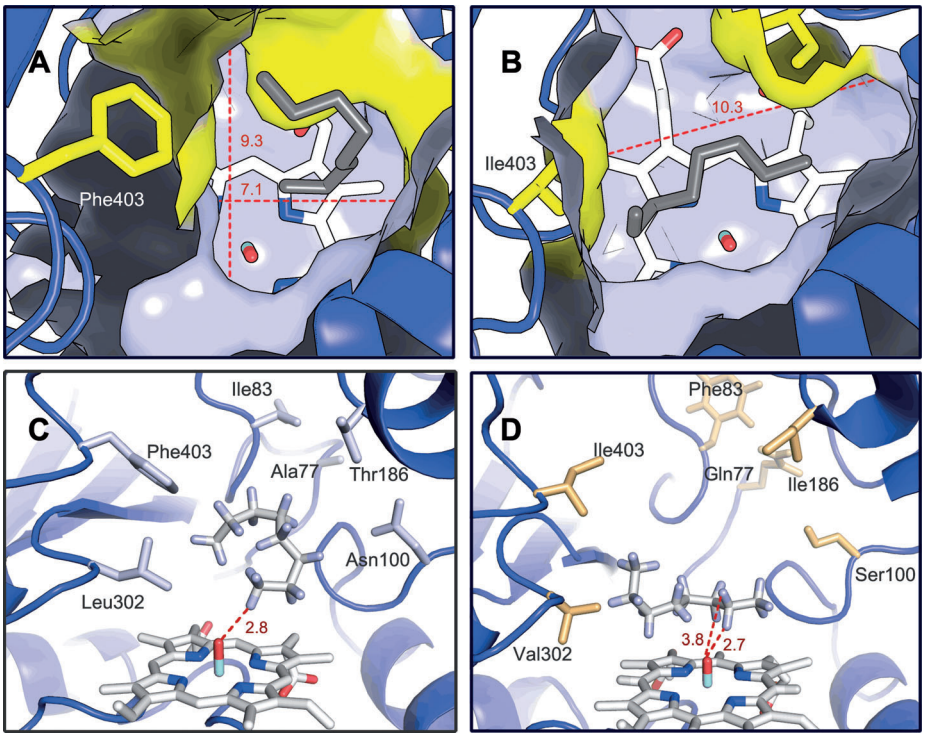


Figure 2. Substrate **1**–P450pyr enzyme binding pose. A, B) Top view of the surface structure of *n*-octane–P450pyr (A) and *n*-octane–P450pyrSM1 (B). Hydrophobic clusters are shown in yellow. The heme-iron–oxygen unit is shown in aqua–red. C, D) Side view of the binding pose of *n*-octane–P450pyr (C) and *n*-octane–P450pyrSM1 (D). The distances between the heme oxygen atom and nearby hydrogen atoms of *n*-octane are denoted by dashed lines.

enantioselectivity was observed for the enzyme mutant. As shown in Figure 2A, the binding pocket of P450pyr is very compact (9.3 Å long, I102–T259 and 7.1 Å wide, G255–L302), and the substrate stacks with a big hydrophobic cluster comprising the northeast and west residues, thus taking a vertical binding pose. The distance between the terminal C–H and the heme O atom is the shortest (2.8 Å; Figure 2C); thus, terminal hydroxylation occurs. In P450pyrSM1, the hydrophobic cluster is disrupted by the F403I and L302V mutation (Figure 2B). The binding pocket consequently extends westwards to reach a width of 10.3 Å. The substrate adopts a horizontal binding pose along the heme plane, in an east–west orientation. This special geometry of the binding pocket clearly leads to a catalytic preference for subterminal hydroxylation to form the *S* alcohol (Figure 2D): The distance between the subterminal C–H_S atom and the heme O atom is 2.7 Å, whereas the distance between the subterminal C–H_R atom and the heme O atom is 3.8 Å.

The positive variants obtained in six rounds of evolution of P450pyr for the subterminal hydroxylation of *n*-octane (**1**) were examined for the subterminal hydroxylation of propylbenzene (**3**) to produce (*S*)-1-phenyl-2-propanol (**4**; Table 2). The best mutant obtained from the 6th round, I83F/N100S/T186I/L251V/L302V/F403I (P450pyrSM2), gave 98% subterminal selectivity for the hydroxylation of **3**, and the formation of (*S*)-**4** with 95% *ee*. These selectivities are clearly confirmed in Figure 3.

In summary, a terminal-selective cytochrome P450pyr has been successfully evolved for the subterminal hydroxylation of alkanes at a non-activated carbon atom. The resulting sextuple mutant P450pyrSM1 showed excellent subterminal and enantioselectivity towards *n*-octane and is the first enzyme to be found for this type of highly selective alkane hydroxylation. The generation of P450pyrSM1 in this study is the first successful example of the full alteration of enzyme regioselectivity and simultaneous establishment of high enantioselectivity for biohydroxylation by directed evolution. Subterminal hydroxylation with the engineered P450pyr hydroxylase enabled the regio- and enantioselective functionalization of alkanes, a useful and challenging reaction in classical chemistry. P450pyrSM1 catalyzed the hydroxylation of *n*-octane (**1**) to produce (*S*)-2-octanol (**2**) with 98% *ee* and higher than 99% subterminal selectivity. Another sextuple mutant P450pyrSM2 hydroxylated propylbenzene (**3**) to give (*S*)-1-phenyl-2-propanol (**4**) with 95% *ee* and 98% subterminal selectivity. Both subterminal alcohols (*S*)-**2** and (*S*)-**4** are useful and valuable intermediates for chemical and pharma-

Table 2: Regio- and enantioselective subterminal hydroxylation of propylbenzene (**3**) to (*S*)-1-phenyl-2-propanol (**4**) with engineered P450pyr hydroxylase.

Round	No. of clones tested[a]	Best mutant	Subterminal selectivity [%][b]	*ee* of (*S*)-**4** [%][c]	Activity [U (g cdw)$^{-1}$][d]
WT	nil	nil	17	40	4.2
1	10	N100S	47	59	2.1
2	20	N100S/F403I	70	45	5.0
3	40	N100S/T186I/F403I	85	18	3.4
4	40	N100S/T186I/L302V/F403I	92	17	3.8
5	100	I83F/N100S/T186I/L302V/F403I	96	88	4.0
6	120	I83F/N100S/T186I/L251V/L302V/F403I	98	95	2.2

[a] The clones tested were the positive clones identified during the evolution of P450pyr hydroxylase for the hydroxylation of *n*-octane (**1**). [b] Subterminal selectivity was determined by GC analysis of the products of the biotransformation of **3** (5 mM) with *E. coli* cells (2 g cdw L^{-1}) expressing the P450pyr mutant in potassium phosphate buffer (100 mM, pH 8.0; 10 mL) containing glucose (2%, w/v) at 30 °C and 250 rpm for 4 h. [c] The *ee* value of (*S*)-**4** was determined by GC analysis on a chiral stationary phase. [d] The activity is the specific activity determined for the first 30 min of the biotransformation.

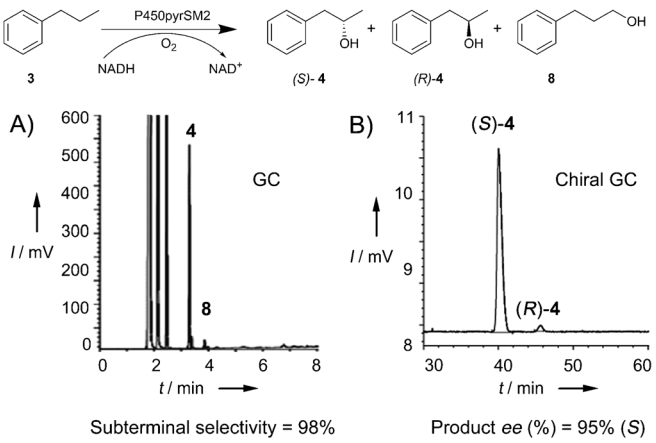


Figure 3. Analysis of the products of the regio- and enantioselective subterminal hydroxylation of propylbenzene (**3**) with P450pyrSM2: A) by GC chromatography; B) by GC chromatography on a chiral stationary phase.

ceutical synthesis. A novel, accurate, sensitive, and simple colorimetric HTS assay was developed for the measurement of both the regioselectivity and enantioselectivity of a hydroxylation reaction. This assay provided a solid basis for the successful evolution of a regio- and enantioselective P450pyr hydroxylase for subterminal hydroxylation. The colorimetric HTS assay could be generally applicable to the discovery of other type of enzymes or chemical catalysts for regio- and stereoselective hydroxylation reactions. Molecular modeling of the hydroxylation of *n*-octane (**1**) with P450pyr and the P450pyrSM1 mutant provided insight into the structural basis and the role of key mutations for the full alteration of the terminal selectivity to give subterminal selectivity and the establishment of excellent enantioselectivity. The knowledge and information obtained could be useful for guiding the future engineering of other P450 enzymes for selective oxidation reactions.

Received: December 20, 2013
Published online: February 19, 2014

Keywords: biohydroxylation · C—H activation · directed evolution · high-throughput screening · P450 enzymes

[1] a) J. C. Lewis, P. S. Coelho, F. H. Arnold, *Chem. Soc. Rev.* **2011**, *40*, 2003 – 2021; b) Z. Li, D. Chang, *Curr. Org. Chem.* **2004**, *8*, 1647 – 1658.

[2] S. Panke, M. Wubbolts, *Curr. Opin. Chem. Biol.* **2005**, *9*, 188 – 194.

[3] K. Kamata, K. Yonehara, Y. Nakagawa, K. Uehara, N. Mizuno, *Nat. Chem.* **2010**, *2*, 478 – 483.

[4] a) Z. Li, J. B. van Beilen, W. A. Duetz, A. Schmid, A. de Raadt, H. Griengl, B. Witholt, *Curr. Opin. Chem. Biol.* **2002**, *6*, 136 – 144; b) C. J. C. Whitehouse, S. G. Bell, L.-L. Wong, *Chem. Soc. Rev.* **2012**, *41*, 1218 – 1260; c) M. Merkx, D. A. Kopp, M. H. Sazinsky, J. L. Blazyk, J. Müller, S. J. Lippard, *Angew. Chem.* **2001**, *113*, 2860 – 2888; *Angew. Chem. Int. Ed.* **2001**, *40*, 2782 – 2807; d) D. J. Koch, M. M. Chen, J. B. van Beilen, F. H. Arnold, *Appl. Environ. Microbiol.* **2009**, *75*, 337 – 344.

[5] a) M. T. Reetz, *J. Org. Chem.* **2009**, *74*, 5767 – 5778; b) U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185 – 194; c) M. T. Reetz, *Angew. Chem.* **2011**, *123*, 144 – 182; *Angew. Chem. Int. Ed.* **2011**, *50*, 138 – 174; d) P. C. Cirino, F. H. Arnold, *Curr. Opin. Chem. Biol.* **2002**, *6*, 130 – 135.

[6] a) C. J. Whitehouse, S. G. Bell, H. G. Tufton, R. J. Kenny, L. C. Ogilvie, L. L. Wong, *Chem. Commun.* **2008**, 966 – 968; b) A. Dennig, N. Lulsdorf, H. Liu, U. Schwaneberg, *Angew. Chem.* **2013**, *125*, 8617 – 8620; *Angew. Chem. Int. Ed.* **2013**, *52*, 8459 – 8462.

[7] a) O. Lentz, A. Feenstra, T. Habicher, B. Hauer, R. D. Schmid, V. B. Urlacher, *ChemBioChem* **2006**, *7*, 345 – 350; b) H. Venkataraman, S. B. A. de Beer, D. P. Geerke, N. P. E. Vermeulen, J. N. M. Commandeur, *Adv. Synth. Catal.* **2012**, *354*, 2172 – 2184.

[8] S. Kille, F. E. Zilly, J. P. Acevedo, M. T. Reetz, *Nat. Chem.* **2011**, *3*, 738 – 743.

[9] K. Zhang, B. M. Shafer, M. D. Demars, H. A. Stern, R. Fasan, *J. Am. Chem. Soc.* **2012**, *134*, 18695 – 18704.

[10] a) D. Chang, B. Witholt, Z. Li, *Org. Lett.* **2000**, *2*, 3949 – 3952; b) Z. Li, H. J. Feiten, D. Chang, W. A. Duetz, J. B. van Beilen, B. Witholt, *J. Org. Chem.* **2001**, *66*, 8424 – 8430; c) D. L. Chang, H. J. Feiten, K. H. Engesser, J. B. van Beilen, B. Witholt, Z. Li, *Org. Lett.* **2002**, *4*, 1859 – 1862; d) D. L. Chang, H. J. Feiten, B. Witholt, Z. Li, *Tetrahedron: Asymmetry* **2002**, *13*, 2141 – 2147; e) W. Zhang, W. L. Tang, Z. S. Wang, Z. Li, *Adv. Synth. Catal.* **2010**, *352*, 3380 – 3390.

[11] a) W. L. Tang, Z. Li, H. M. Zhao, *Chem. Commun.* **2010**, *46*, 5461 – 5463; b) S. Q. Pham, G. Pompidor, J. Liu, X. D. Li, Z. Li, *Chem. Commun.* **2012**, *48*, 4618 – 4620.

[12] a) D. Böttcher, U. T. Bornscheuer, *Curr. Opin. Microbiol.* **2010**, *13*, 274 – 282; b) D. Scheps, S. Honda Malca, H. Hoffmann, B. M. Nestl, B. Hauer, *Org. Biomol. Chem.* **2011**, *9*, 6727 – 6733.

[13] E. T. Farinas, U. Schwaneberg, A. Glieder, F. H. Arnold, *Adv. Synth. Catal.* **2001**, *343*, 601 – 606.

[14] K. H. Halsey, L. A. Sayavedra-Soto, P. J. Bottomley, D. J. Arp, *J. Bacteriol.* **2006**, *188*, 4962 – 4969.

[15] a) P. Meinhold, M. W. Peters, M. M. Chen, K. Takahashi, F. H. Arnold, *ChemBioChem* **2005**, *6*, 1765 – 1768; b) A. Glieder, E. T. Farinas, F. H. Arnold, *Nat. Biotechnol.* **2002**, *20*, 1135 – 1139; c) M. W. Peters, P. Meinhold, A. Glieder, F. H. Arnold, *J. Am. Chem. Soc.* **2003**, *125*, 13442 – 13450.

[16] S. G. Bell, J. A. Stevenson, H. D. Boyd, S. Campbell, A. D. Riddle, E. L. Orton, L. L. Wong, *Chem. Commun.* **2002**, 490 – 491.

[17] a) A. Liese, T. Zelinski, M.-R. Kula, H. Kierkels, M. Karutz, U. Kragl, C. Wandrey, *J. Mol. Catal. B* **1998**, *4*, 91 – 99; b) Y. Hu, E. C. K. Lin, L. M. Pham, J. Cajica, C. M. Amantea, E. Okerberg, H. E. Brown, A. Fraser, L. Du, Y. Kohno, J. Ishiyama, J. W. Kozarich, K. R. Shreder, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 1553 – 1556.

[18] a) J. M. Wagner, C. J. McElhinny, Jr., A. H. Lewin, F. I. Carroll, *Tetrahedron: Asymmetry* **2003**, *14*, 2119 – 2125; b) S. K. Talluri, A. Sudalai, *Tetrahedron* **2007**, *63*, 9758 – 9763; c) F. X. Tavares, D. N. Deaton, A. B. Miller, L. R. Miller, L. L. Wright, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3891 – 3895.

[19] a) Z. Li, L. Butikofer, B. Witholt, *Angew. Chem.* **2004**, *116*, 1730 – 1734; *Angew. Chem. Int. Ed.* **2004**, *43*, 1698 – 1702; b) Y. Z. Chen, W. L. Tang, J. Mou, Z. Li, *Angew. Chem.* **2010**, *122*, 5406 – 5411; *Angew. Chem. Int. Ed.* **2010**, *49*, 5278 – 5283.

[20] H. Yamamoto, K. Kawada, A. Matsuyama, Y. Kobayashi, *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1051 – 1055.

[21] H. Yamamoto, M. Kudoh, *Appl. Microbiol. Biotechnol.* **2013**, *97*, 8087 – 8096.

