Supporting Information

Highly Selective Hydroxylation of Gaseous Alkanes at Terminal Position by Wild-type CYP153A33 Assisted by Decoy Molecules

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1. Cloning, expression, and quantification of purified proteins

Gene cloning & Protein expression of CYP153A33.

The gene of CYP153A33 was purchased from Eurofins. The cDNA was amplified by PCR and inserted into the expression plasmid pET28a(+) harboring His_6-tagged using the SLiCE method. The plasmid was used to transform competent *E. coli* Arctic Express (DE3) cells. Cells from a single colony were pre-inoculated into LB broth containing kanamycin 25 μg/mL, and gentamycin 20 μg/mL and grown overnight at 37 ºC. The following day, the culture was inoculated into LB broth (1:100) supplemented with kanamycin 25 μg/mL and gentamycin 20 μg/mL, *δ*-aminolevulinic acid (0.5 mM final concentration), and $FeCl_3 \cdot 6HCl$ (0.1 mM final concentration). Cultures were grown at 30 ºC, 85 rpm until OD ≈ 0.6-0.8 and then induced by isopropyl *β*-D-1-thiogalactopyranoside (IPTG, 0.1 mM final concentration). After induction, the temperature was lowered to 20 ºC and growth was allowed to continue for a total time of 24 h. Cells were harvested by centrifugation (5,500 rpm, 20 min, 4 ºC).

Protein Purification and Quantification of CYP153A33.[1]

Cells expressing His_6-tagged CYP153A33 (15 g) were resuspended in 50 mL lysis buffer (100 mM Tris-HCl buffer (pH 7.4 at 4 ºC) containing 300 mM NaCl, 20 % ethylene glycol, 1 % cholic acid, 0.1 % sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethylsulfonyl fluoride, 50 mg lysozyme chloride and 3.4 μL DNase) and disrupted using an ultrasonicator. After removing the cell debris (20 min, 17,500 rpm, 4 ºC, 2 times), the cell-free extract was loaded by cOmplete Hi-Tag Purification Resin nickel-affinity column (Sigma-Aldrich) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.4 at 4 ºC) containing 300 mM NaCl and washed with this buffer. After washing, His6-tagged CYP153A33 was eluted with 20 mM Tris-

HCl buffer (pH 7.4 at 4 ºC) containing 300 mM NaCl and 300 mM imidazole. After eluting, the buffer was exchanged to 20 mM Tris-HCl buffer (pH 7.4 at 4 ºC) by an ÄKTA system (GE Healthcare) equipped with a multiwavelength detector (set at 280/415 nm) onto a HiPrep 26/10 Desalting Column (GE Healthcare). The sample was then loaded by a TOSOH BioAssist (TOSOH) equipped with a DEAE650S (GE Healthcare) in 20 mM Tris-HCl buffer (pH 7.4 at 4 ºC) over a KCl concentration gradient ranging from 20 mM to 80 mM. The eluted fraction containing CYP153A33 was pooled, desalted, and concentrated with Amicon Ultra Centrifuge Filter Ultracel ® (Millipore, Co.) with an MWCO of 30 kDa. Whereafter the protein was applied to a Sephacryl S-300HR gel-filtration column pre-equilibrated in 20 mM Tris-HCl buffer (pH 7.4 at 25 ºC) containing 100 mM KCl. CYP153A33 fractions were collected, pooled, and concentrated, followed by an estimation of the CYP153A33 concentration by the pyridine heamochromogen assay.

Gene cloning & Protein expression of PdR, Pdx.

The gene of PdR and Pdx was allocated from Prof. Shimada (University of Hyogo). The cDNA was amplified by PCR and inserted into the expression plasmid pQE30 harboring His_6-tagged using the restriction enzymes. The plasmid was used to transform competent E. coli JM109 cells. Cells from a single colony were pre-inoculated into LB broth containing ampicillin 100 µg/mL and grown overnight at 37 ºC. The following day, the culture was inoculated into LB broth (1:100) supplemented with ampicillin 100 µg/mL. Cultures were grown at 37 ºC (PdR) or 30 ºC (Pdx), 85 rpm for a total time of 12 h. Cells were harvested by centrifugation (5,500 rpm, 20 min, 4 ºC).

Protein Purification and Quantification of PdR, Pdx.

Cells expressing His_6-tagged PdR or Pdx (10 g) were resuspended in 20 mM Tris-HCl buffer (pH 7.4 at 4 ºC) containing 300 mM NaCl (100 mL) and disrupted using an ultrasonicator. After removing the cell debris (20 min, 17,500 rpm, 4 ºC, 2 times), the cell-free extract was loaded by an ÄKTA system (GE Healthcare) equipped with a HiPrep Chelating HP cobalt-affinity column (Sigma-Aldrich) in 20 mM Tris-HCl buffer (pH 7.4 at 25 ºC) containing 300 mM NaCl over an imidazole concentration gradient ranging from 10 mM to 250 mM. After eluting, the buffer was exchanged to 20 mM Tris-HCl buffer (pH 7.4 at 4 ºC) containing 100 mM KCl by an ÄKTA system (GE Healthcare) equipped with HiPrep 26/10 Desalting Column (GE Healthcare). PdR or Pdx fractions were collected, pooled, and concentrated using Amicon Filters with an MWCO 30 kDa (PdR) or 3 kDa (Pdx), followed by estimation of the PdR or Pdx concentration by UV/vis spectrophotometry on the basis of the following extinction coefficient: ε_{455} = 10.0 mM^{-1} cm^{-1} (PdR) or 10.4 mM^{-1} cm^{-1}.

2. Hydroxylation of lauric acid by CYP153A33 for screening of reaction conditions

Hydroxylation reaction was carried out in 0.5 mL of lauric acid saturated 20 mM Tris-HCl (pH = 7.4) buffer containing 100 mM KCl at 25 °C for 6 h in the presence of X μM CYP153A33, Y μM Pdx, Z μM PdR (X, Y, Z are shown in Figure S1), and 5 mM NADH in a glass vial. Reaction mixture was stirred vigorously. After a 6 h reaction, reaction solution was extracted with ethyl acetate. The hydroxylated products in corrected organic phase were derivatized by BSTFA-TMCS (*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide with 1% Trimethylchlorosilane) for 30 min under dark condition. After derivatization, the organic phase was directly analyzed by GC-MS 2010SE (Shimazu, DI 2010) equipped with an CylsoSil-β (GL Science Inc.).

3. **Docking simulation of methane in PFC9-Ala-bound CYP153A33.[2]**

Docking simulation of methane to the active site of PFC9-Ala-bound CYP153A33 was performed using AutoDock Vina.[2] The crystal structure of the lauric acid-bound form of CYP153A33 (PDB code: 5FYG) was used as a rigid acceptor for the docking of PFC9-Ala. Nine conformations were generated, and docked conformations whose cavities were occupied by methane were selected as possible conformations. These results were visualized by using the PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.

4. Supplementary Figures

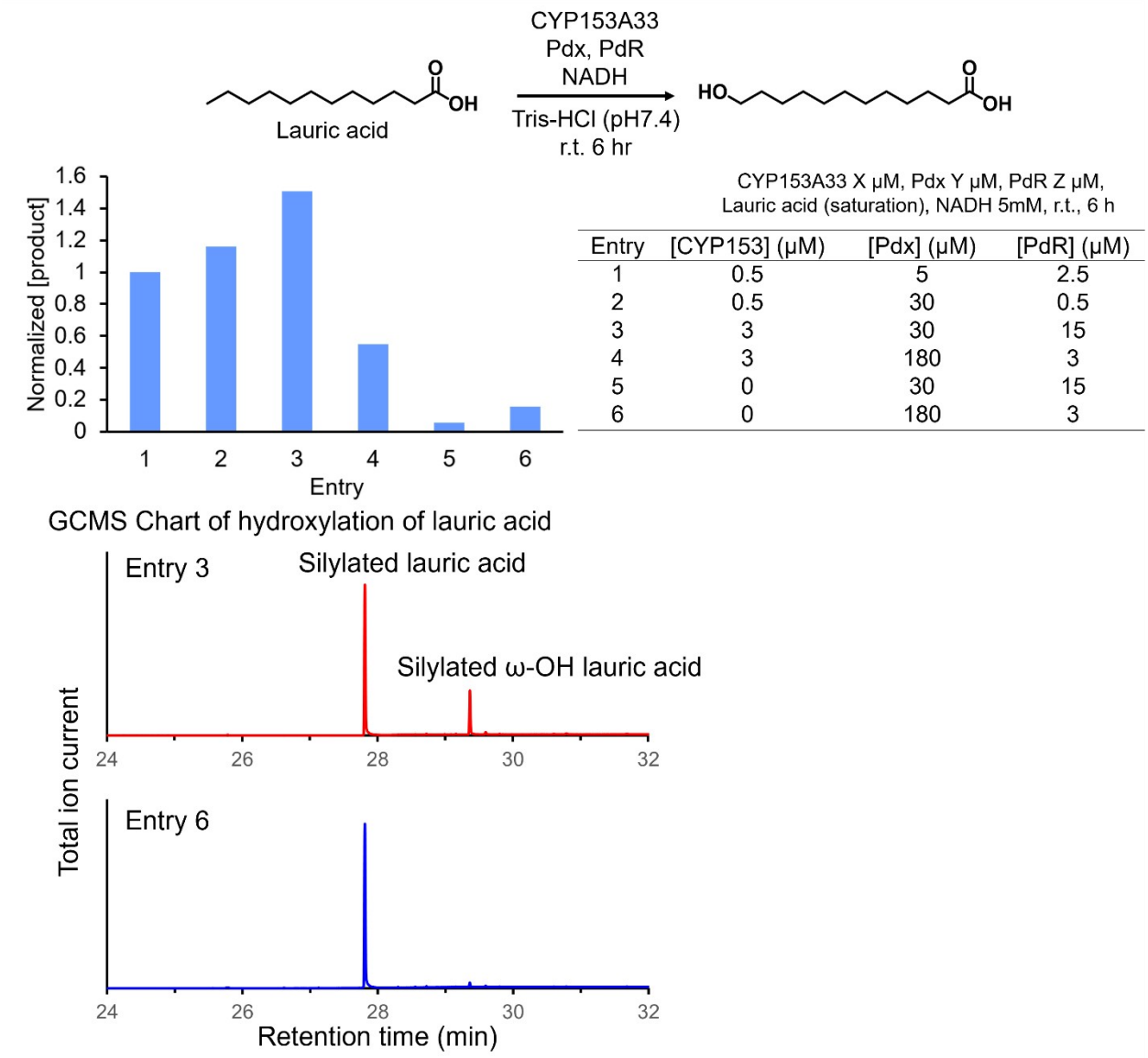

Figure S1. Reaction condition search using hydroxylation of lauric acid. Reaction conditions: CYP153A33 (0.5 or 3 μM), Pdx (5-180 μM), PdR (0.5-15 μM), lauric acid (saturation), NADH (5 mM), Tris-HCl buffer (20 mM Tris-HCl, 100 mM KCl, pH 7.4), at room temperature for 6 h.

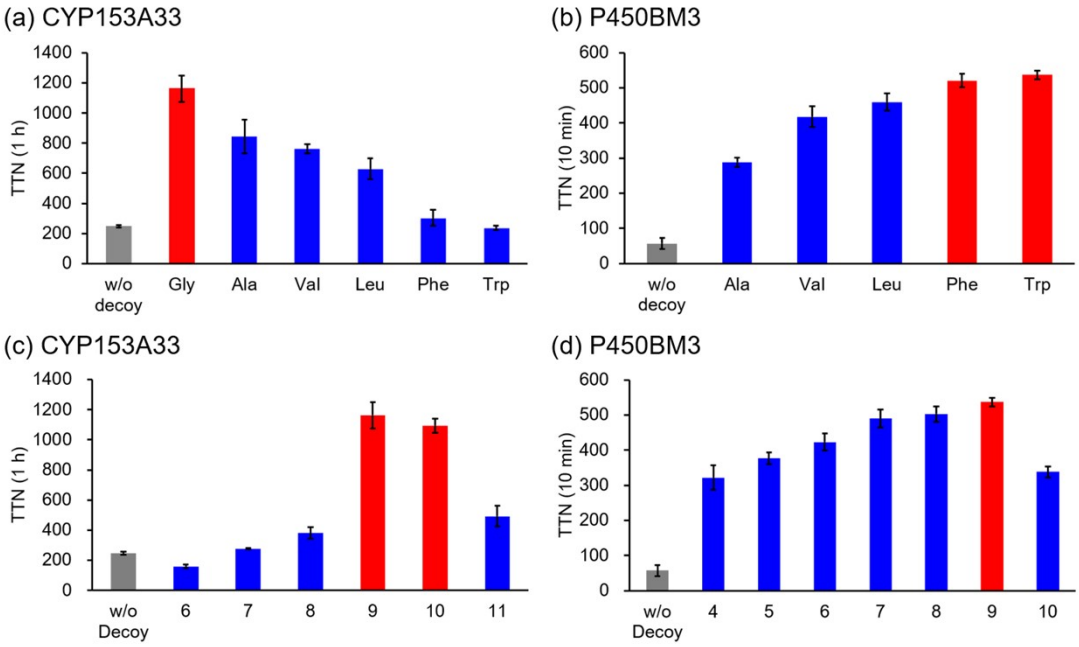

Figure S2. Comparison of trends of 2nd generation decoy molecules in propane hydroxylation by CYP153A33 or P450BM3. Reaction conditions for CYP153A33: CYP153A33 (1 μM), Pdx (10 μM), PdR (5 μM), propane (5 MPa), decoy molecules (100 μM), NADH (5 mM), Tris-HCl buffer (20 mM Tris-HCl, 100 mM KCl, pH 7.4), at room temperature for 1 h. Reaction conditions for P450BM3: P450BM3 (0.5 μM), propane (5 MPa), decoy molecules (100 μM), NADPH (5 mM), Tris-HCl buffer (20 mM Tris-HCl, 100 mM KCl, pH 7.4), at room temperature for 10 min.[3]

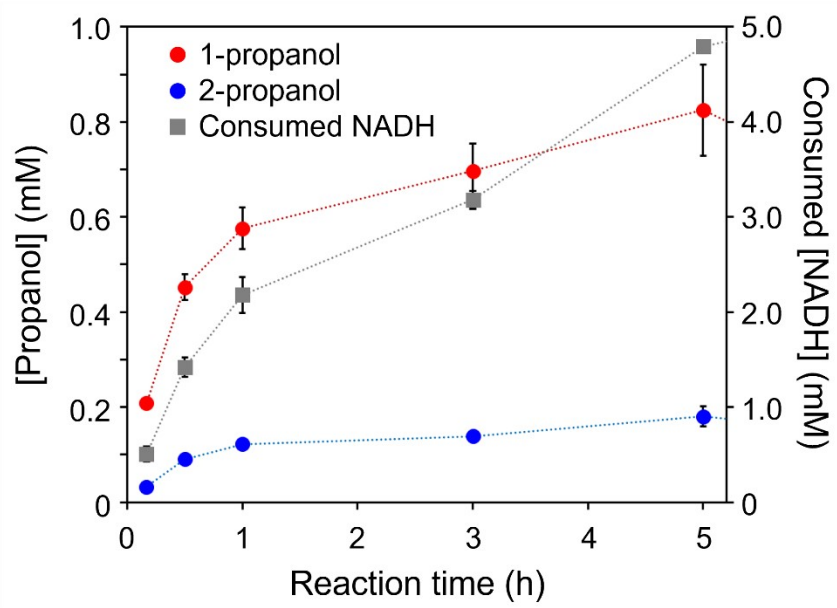

Figure S3. Time course of propane hydroxylation by CYP153A33. Reaction conditions for CYP153A33: CYP153A33 (1 µM), Pdx (10 µM), PdR (5 µM), propane (5 MPa), PFC9-Gly (100 µM), NADH (5 mM), Tris-HCl buffer (20 mM Tris-HCl, 100 mM KCl, pH 7.4), at room temperature for 5 h.

References

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2. O. Trott and A. J. Olson, *J. Comput. Chem.*, 2010, **31**, 455-461.

3. S. Ariyasu, Y. Kodama, C. Kasai, Z. Cong, J. K. Stanfield, Y. Aiba, Y. Watanabe and O. Shoji, *ChemCatChem*, 2019, **11**, 4709-4714.