Supplementary Materials for

Unnatural Biosynthesis by an Engineered Microorganism with Heterologously Expressed Natural Enzymes and an Artificial Metalloenzyme

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I. Supplementary Methods

1. General methods

Unless otherwise noted, the chemicals, salts, and solvents used were reagent grade and used as received from commercial suppliers without further purification. Oligonucleotides were obtained from Integrated DNA Technologies. Enzymes and reagents used for cloning were obtained from New England BioLabs and Thermo Fisher Scientific. pChuA was a gift from Alan Jasanoff (Addgene plasmid 42539). pHug21 was a gift from Prof Douglas Henderson in the University of Texas Permian Basin. All expression media and buffers were prepared using ddH_2O (MilliQ A10 Advantage purification system, Millipore). All expression media were sterilized using either an autoclave (30 min, 121 ºC) or a sterile syringe filter (0.22 μm). The synthetic procedures of Ir(Me)MPIX were reported previously.[1]

2. Plasmids and site-directed mutagenesis

The plasmids used in this study are listed in the table below. The sequences of CYP119,[2] heme transport protein ChuA (Addgene_42539), and heme utilization system HUG[3] were described elsewhere and cloned into the respective vectors. Primer sequences are available upon request. Site-directed mutagenesis was used to introduce mutations into pJHA110. Phusion High-Fidelity DNA polymerase was used to amplify the parent plasmid with primers containing mutations at target sites. After DpnI digestion, the PCR product was purified with a QIAGEN gel purification kit and ligated with T4 ligase and T4 PNK (polynucleotide kinase). All pBb plasmids in the table below were constructed in previous study.[4]

Name	Marker	Origin	Vector backbone	Genes
pCYP119-141[1]	Amp	ColE1	2BT(Addgene _29666)	CYP119-141 mutant (C317G,T213G,V254L,L155W) with T7 promoter
pJHA147	Kan	p15A	pBbA5a	ChuA with lacUV5 promoter
pHug21[3]	Amp	SC101	-	*hug* operon
pJHA029	Amp	ColE1	pBbE5a	CYP119-141 mutant with lacUV5 promoter
pJHA108	Kan	ColE1	pBbE1K	CYP119-141 mutant with trc promoter
pJHA110	Kan	ColE1	pBbE5K	CYP119-141 mutant with lacUV5 promoter
pJHA047	Kan	SC101	pBbS8K	*hug* operon
pJHA135	Kan	SC101	pBbS8K	*hug* operon and CYP119 mutant (C317G,T213G,V254L,L155W) with trc promoter
pJHA170	Kan	SC101	pBbS8K	*hug* operon and CYP119 mutant (C317G,T213G,V254A,L155W,R256W) with trc promoter
pJHA184	Amp	SC101	pBbS5A	*chuA*, *tonB, exbB* and *exbD* cloned from *E. coli* O6:K5:H1[5]
pBbS5A	Amp	SC101	-	RFP with lacUV5 promoter
pBbE7A	Amp	ColE1	-	RFP with T7 promoter
pJBEI6409[6]	Cm	p15A	pBbA5c	genes for limonene synthesis
pJBEI6410[6]	Amp	p15A	pBbA5a	genes for limonene synthesis

3. Media preparation

Preparation of M9-rich media: Salts (47.7 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 8.6 mM NaCl, 1 g/L NH_4Cl) were dissolved in 1 L ddH_2O and autoclaved to give a medium with pH ~7. Solutions of glucose (20 w/v%), casein hydrolysate (20 w/v%), $MgSO_4$ (1 M), antibiotics and $CaCl_2$ (1 M) were sterilized by filter. The following amounts of the listed solutions were added per liter of sterilized salt solution: 40 mL glucose, 10 mL casein hydrolysate, 2 mL $MgSO_4$, 100 µL $CaCl_2$.

Preparation of M9-N reaction buffer: Salts (47.7 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 8.6 mM NaCl) were dissolved in 1 L ddH_2O and autoclaved to give a medium with pH ~7.4. Solutions of $MgSO_4$ (1 M), $CaCl_2$ (1 M) and glucose (20 w/v%) were added to give a final concentration of 2.0 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.4 w/v% glucose.

4. *In vivo* expression of Ir-CYP119

A plasmid containing CYP119 genes was co-transformed with a plasmid encoding the corresponding heme transport system (ChuA, HUG or truncated HUG system) into chemically competent BL21(DE3) cells (NEB). Individual colonies from freshly transformed plates (incubated at 30 °C) were inoculated into 2 mL M9-rich media supplemented with 100 µg/mL carbenicillin and 50 µg/mL kanamycin (for low copy plasmids with SC101 origin, the concentration of the antibiotics was reduced by half). The cultures were grown for 18 h at 30 °C, 200 rpm. In a 50 mL glass culture tube, 10 mL of M9-rich media was inoculated with 100 µL of overnight starter culture and incubated at 37 °C, 200 rpm to an OD_{600} of ~1. Unless otherwise indicated, the cultures were then induced by adding final concentration of 50 µM IPTG and 0.1 µM Ir(Me)MPIX (2 µL of 0.5 mM stock solution in DMSO). Upon induction, the incubation temperature was reduced to 30 °C, and expressions were allowed to continue for 18 h at 200 rpm. After harvesting the cells by centrifugation (RT, 1 min, 10,000 rpm), the cell pellets were resuspended in M9-N reaction buffer to an OD_{600} of ~10.

5. Whole-cell reactions

The cell suspension in M9-N reaction buffer (350 µL, OD_{600} ~10) was transferred into a 2 mL screw-capped GC vial, and 4 mM substrate ((-)-carvone or (-)-limonene) and 20 mM EDA were added. Both stock solutions were prepared in ethanol and added to the cell culture to give a final ethanol concentration of 1% (v/v). The vials were capped and shaken at 30 °C and 200 rpm for 4 h. The reaction was quenched by adding 24 µL of 2 N HCl, followed by 160 µL saturated NaCl and 800 µL ethyl acetate (containing 160 mg/L dodecane as internal standard). The mixture was subjected to vortex at 2500 rpm for 4 min, transferred to a 1.7 mL microcentrifuge tube, and centrifuged at 5,000 rpm for 2 min. After separation of the layers, approximately 500 µL of the aqueous phase was removed from the bottom of the vial by pipette. The remaining contents of the vial were neutralized by the addition of saturated $NaHCO_3$ (160 µL). The microcentrifuge tube was then vortexed at 2500 rpm for 1 min and centrifuged at 15,000 rpm for 4 min. The organic layer was then transferred to a separate vial for GC analysis.

6. Limonene biosynthesis

Chemically competent BL21(DE3) (NEB) cells were transformed with a plasmid coding for MEV pathway enzymes to produce limonene from glucose in *E. coli* (pJBEI6410 or pJBEI6409). Individual colonies from freshly transformed plates (incubated at 30 °C) were inoculated into 2 mL M9-rich media supplemented with 100 µg/mL carbenicillin or 25 µg/mL chloramphenicol,

respectively. The cultures were grown overnight at 30 °C and 200 rpm for 18 h. In a 50 mL glass culture tube, 10 mL of M9-rich media was inoculated with 100 µL of overnight starter culture and incubated at 37 °C and 200 rpm to an OD_{600} of ~1. Unless otherwise indicated, the cultures were then induced by adding 50 µM IPTG (final concentration), and 10% (v/v) dodecane was added to trap the limonene produced. Upon induction, the temperature was reduced to 30 °C and expressions were allowed to continue for 24 h at 200 rpm. An aliquot of the 100 µL dodecane overlay was diluted with 900 µL ethyl acetate containing 40 mg/L beta-pinene (beta-pinene as internal standard) and analyzed by GC-MS.

7. Artificial pathway for the production of cyclopropyl limonene

General procedure: BL21(DE3) cells were transformed with a plasmid (pJBEI6410) coding for enzymes in limonene biosynthetic pathway and a plasmid (pJHA135 or pJHA170) encoding both the heme transport system HUG and the corresponding CYP119 mutant. Individual colonies from freshly transformed plates (incubated at 30 ºC) were inoculated into 2 mL M9-rich media supplemented with 100 µg/mL carbenicillin and 25 µg/mL kanamycin. The cultures were grown overnight at 30 °C, 200 rpm for 18 h. In a 50 mL glass culture tube, 10 mL of M9-rich media was inoculated with 100 µL of overnight starter culture and incubated at 37 °C, 200 rpm to an OD_{600} of ~1. The cultures were then induced by adding 50 µM IPTG, at which time 2 µl of a 0.5 mM solution of Ir(Me)MPIX in DMSO was added to give a final Ir(Me)MPIX concentration of 0.1 µM. Upon induction, the incubation temperature was reduced to 30 °C, and expression was allowed to continue for 18 h at 200 rpm. To the 10 mL culture was added 21.2 µl EDA dissolved in 100 µl ethanol to give a final EDA concentration of 20 mM and a final ethanol concentration of 1% (v/v), and the mixture was further incubated at 30 ºC, 200 rpm for 4.5 h. To analyze the titer of cyclopropyl limonene, aliquots of cell culture (1.4 mL) were removed from the tube and extracted with an equal volume of ethyl acetate as described in the section of whole-cell reaction. The organic layer (1 mL) was concentrated to 90 µL with a Speedvac, and to this concentrate was added 10 µL of ethyl acetate containing 1 g/L (-)-carvone (carvone used as internal standard). The final solution was analyzed by GC-MS.

8. EDA consumption in the cell culture expressing the artificial pathway

E. coli cells co-transformed with pJBEI6410 and pJHA170 were grown as described in **7**, except for the following steps. After addition of 50 µM IPTG and 0.1 µM Ir(Me)MPIX, the cultures were incubated at 30 °C, 200 rpm for 12 h. To the cultures were added 100 µL EDA stock solution (0.2 M in EtOH) to give a final concentration of 2 mM, and the mixture was further incubated at 30 ºC, 200 rpm. Aliquots of cell culture (600 µL) were removed from the tube at each two-hour interval over 10 h. The aliquots were extracted with ethyl acetate (600 µL, containing 80 ng/L dodecane as internal standard). The organic phase was analyzed by GC-FID.

9. Cell viability test with CFU (colony forming units) assay

E. coli cells co-transformed with pJBEI6410 and pJHA170 were grown as described in **7**, except for the following steps. After addition of 50 µM IPTG and 0.1 µM Ir(Me)MPIX, the cultures were incubated at 30 °C, 200 rpm for 12 h. A portion of cell culture (1.4 mL) was transferred into Falcon 14 mL round-bottom tube. The stock solutions of EDA in ethanol were added to the cell culture to give final concentration of 0 mM, 2 mM, 5 mM, 20 mM EDA and 1% (v/v) ethanol. For the control group, only sterile ddH_2O (1%, v/v) was added. The cultures were further incubated at 30 ºC, 200 rpm for 4 h. Then the cultures were diluted 10^5 times. Aliquots of

diluted cell suspension (50 μL) were plated onto LB agar plates. The plates were incubated at 30 °C for 24 h. The colonies grown on the plates were manually counted and the percentage of viable cells in each group was calculated by normalizing the number of colonies grown in the control group as 100%.

10. Cytotoxicity of Ir(Me)MPIX and cyclopropyl limonene

Ir(Me)MPIX: pJHA047 (containing *hug* operon) or pBbS5K (empty vector) was transformed into chemically competent BL21(DE3) (NEB) cells. Individual colonies from freshly transformed plates (incubated at 30 °C) were inoculated into 2 mL M9-rich media supplemented with appropriate antibiotics. A 1:100 dilution of overnight cultures into 300 μL M9-rich media was performed in a 48-well plate. To the culture in the wells of this plate were added 1.5 μL of solutions of Ir(Me)MPIX in DMSO varying in concentration (0 mM, 0.02 mM, 0.2 mM, 2 mM) to give final concentration of 0 μM, 0.1 μM, 1 μM, 10 μM Ir(Me)MPIX. The OD_{600} values were recorded by a plate reader (F200 or F200pro, Tecan) set at 30 °C.

Cyclopropyl limonene: BL21(DE3) cells co-transformed with pHug21 (coding for HUG system) and pJHA110 (coding for CYP119 mutant) were grown and cultivated as described above. The stock solution of cyclopropyl limonene in ethanol was added to the cell culture to give final concentration of 0, 0.2, 1, 5, 25 mg/L cyclopropyl limonene and 1% (v/v) ethanol. The OD_{600} values were recorded by a plate reader (F200 or F200pro, Tecan) set at 30 °C.

11. Sample preparation for ICP-MS measurement

(a) Cell fractionation (10 mL culture):[7]

A plasmid containing CYP119 mutant (pJHA110) was co-transformed with a plasmid (pHug21) encoding the heme transport system HUG or an empty vector backbone (pBbS5a) into chemically competent BL21(DE3) (NEB) cells. Individual colonies from freshly transformed plates were inoculated into 4 mL M9-rich media supplemented with 100 μg/mL carbenicillin and 25 μg/mL kanamycin. The cultures were grown overnight at 37 °C and 200 rpm. In a 50 mL Falcon tube, 10 mL of M9-rich media was inoculated with 100 μL of overnight starter culture and incubated at 37 °C and 200 rpm to an OD_{600} of 0.7~0.8. The cultures were then induced by adding 50 μM IPTG, at which time 2 μL of a 0.5 mM solution of Ir(Me)MPIX in DMSO was added to give a final Ir(Me)MPIX concentration of 0.1 μM. Upon induction, the incubation temperature was reduced to 30 °C, and expression was allowed to continue for 18 h at 200 rpm. Cells were harvested by centrifugation (4000 rpm, 15 min, 4 °C) and the supernatants were carefully removed by pipette.

Periplasmic extraction: The cell pellet was suspended in 1 mL of spheroplast buffer (0.1 M TRIS, 500 mM sucrose, 0.5 mM EDTA, pH 8.0), incubated on ice for 5 min, and centrifuged (10000 rpm, 5 min, 4 °C). The supernatant was carefully removed. The pellet was then resuspended in 1 mL of hypotonic solution (0.1 mM $MgCl_2$) and the sample was incubated for 5 min on ice. After centrifugation, the recovered supernatant was combined with the supernatant from the sucrose wash. The resulting periplasmic fraction (2 mL) was carefully transferred to a new tube and the pellet was incubated on ice for subsequent cytoplasmic extraction.

Cell disruption: The pellet from previous step was suspended in 5 mL lysis buffer (50 mM TRIS, 100 mM NaCl, pH 8.0). The suspension was sonicated (5 s on, 5 s off, 10 min, 70% power) on ice using a Qsonica Q125 sonicator. After centrifugation at 10000 rpm for 30 min, the

supernatant was transferred to a new tube as the cytoplasmic fraction and the pellet, resuspended in 5 mL TRIS lysis buffer, was reserved as the insoluble fraction.

(b) Protein purification (100 mL culture):
A plasmid containing CYP119 mutant (pJHA110) was co-transformed with a plasmid (pHug21) encoding the heme transport system HUG, or an empty vector backbone (pBbS5a) into chemically competent BL21(DE3) (NEB) cells. Individual colonies from freshly transformed plates were inoculated into 4 mL M9-rich media supplemented with 100 μg/mL carbenicillin and 25 μg/mL kanamycin. The cultures were grown overnight at 37 °C and 200 rpm. In a 500 mL Erlenmeyer flask, 100 mL of M9-rich media was inoculated with 1 mL of overnight starter culture and incubated at 37 °C and 200 rpm to an OD_{600} of 0.7~0.8. The cultures were then induced by adding 50 μM IPTG, at which time 20 μL of a 0.5 mM solution of Ir(Me)MPIX in DMSO was added to give a final Ir(Me)MPIX concentration of 0.1 μM. Upon induction, the incubation temperature was reduced to 30 °C, and expressions were allowed to continue for 18 h at 200 rpm. Cells were harvested by centrifugation and stored at -20 °C until purification.

Ni-NTA affinity chromatography: Cell pellets were thawed at room temperature, resuspended in 10 mL lysis buffer (50 mM TRIS, 100 mM NaCl, pH 7.5), and lysed on ice by sonication (5 s on, 5 s off, 10 min, 70% power). Cell debris was removed by centrifugation (10000 rpm, 45 min, 4 °C). The supernatants were incubated with Ni-NTA agarose (50% suspension, 4 mL) on a tube rotator (15 min, 4 °C, 20 rpm) and poured into glass frits (coarse, 50 mL). The resin was washed (3×35 mL) with Ni-NTA lysis buffer (50 mM NaPi, 250 mM NaCl, 10 mM Imidazole, pH 8.0). The desired protein was eluded with 15 mL Ni-NTA elusion buffer (50 mM NaPi, 250 mM NaCl, 250 mM Imidazole, pH 8.0) and concentrated to 1 mL with a 30 kDa molecular weight cut-off Amicon centrifugal filtration device (EMD Millipore).

(c) ICP-MS analysis: Conc. HNO_3 was added to each sample (1 mL) in to make a final concentration of 4% (v/v). After microwave digestion (140 °C, 30 min), the samples were analyzed by ICP-MS (Elemental Analysis Core, Oregon Health & Science University).

12. Reactions catalyzed by Ir-CYP119 reconstituted *in vitro*
Apo-CYP119 was expressed and purified as described earlier.[1] The apo-protein and Ir(Me)MPIX cofactor were combined in a 2.5 : 1 molar ratio. The buffer of the protein solution was subsequently exchanged to TRIS buffer (20 mM, pH 7.5) using a NAP-10 column (GE Healthcare). On the bench, 1 mL of catalyst stock solution (1.8 mg/mL CYP119, 40% [Ir]) was added to a 4 mL glass vial equipped with a micro stirring bar. To the reaction vial, 10 μL of 0.125 M stock solution of limonene in EtOH was added. The reaction vial was sealed with a cap containing a septum. The syringes of a multichannel syringe pump were loaded with a stock solution of EDA in DMSO (20 μL of 0.25 M solution of EDA in DMSO), and the EDA solution was added to the reaction over 1.5 h. The reaction was quenched upon the conclusion of the addition of EDA by adding 100 μL of 2 N HCl and extracted with 1 mL dodecane stock solution (0.1% (v/v) in ethyl acetate). The organic layer was then transferred to a separate vial for GC analysis.

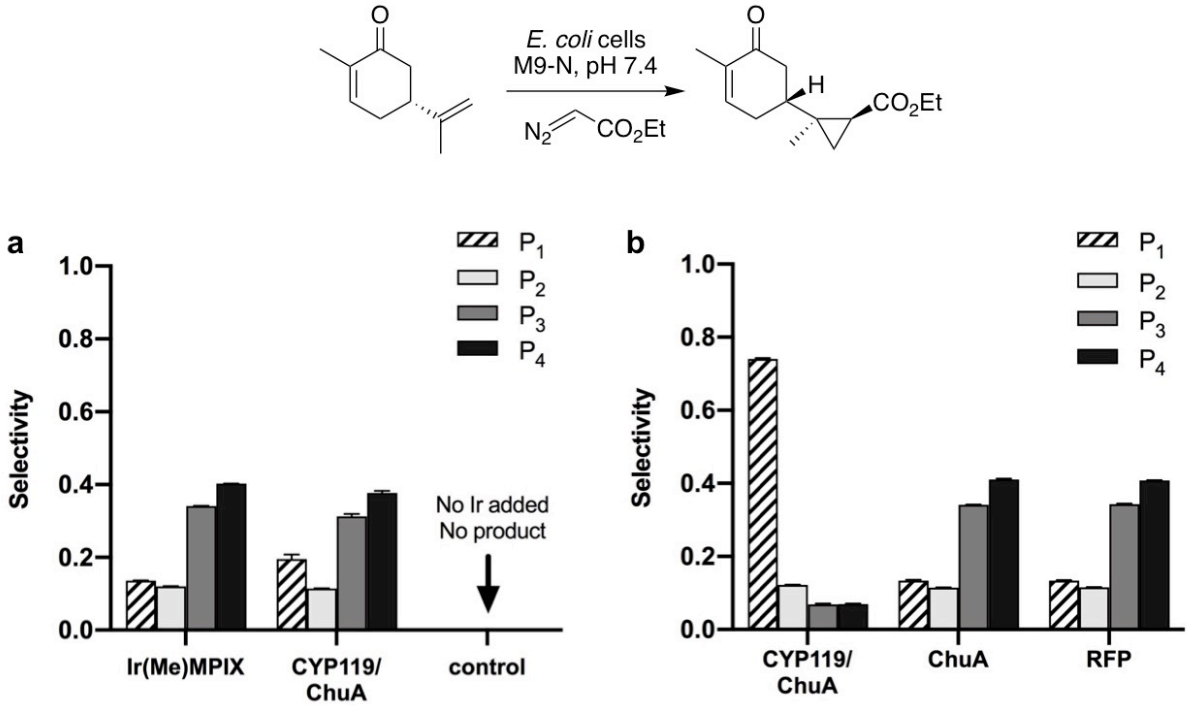
13. General procedure for the synthesis of cyclopropyl limonene standard

To a solution of alkene (0.2 M) and Ir(Me)MPIX (0.5 mol% with respect to alkene) in dry DCM (CH_2Cl_2), a solution of ethyl diazoacetate (1 M) in dry DCM was added slowly while the reaction mixture was vigorously stirred. After complete addition of EDA, the reaction was stirred for 1 h. Then, the volatile materials were evaporated under reduced pressure, and the residue was purified by column chromatography on silica gel, with a mixture of hexane and ethyl acetate (100:0 to 95:5 gradient) as the eluent. Fractions of the pure product(s) were combined, and the solvent evaporated, yielding cyclopropanation products.

14. Binding affinity of apo-CYP119 towards Ir(Me)MPIX/hemin

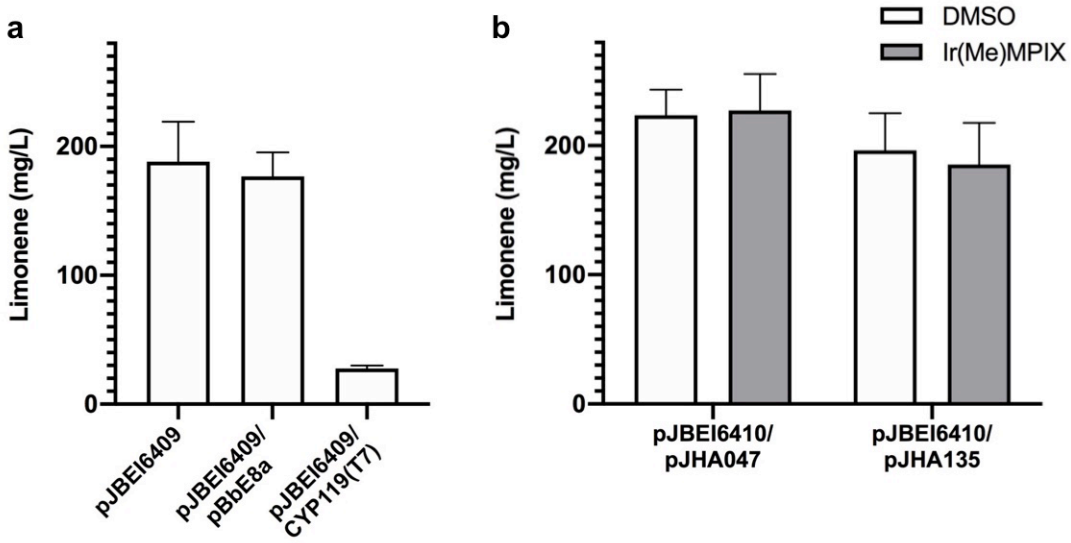
Intrinsic fluorescence quenching:[8] Modified from a reported procedure, aliquots (10 μL) of metalloporphyrin stock solutions (in DMSO) were added to a solution containing 0.15 mg/L apo-CYP119 (CYP119-141 mutant, 800 μL, 10 mM TRIS buffer, pH 8.0). This addition caused the concentration of the protein to decrease from its initial value to a final concentration of 0.148 mg/L in the process. After each addition, the emission spectrum of CYP119 was recorded upon excitation at 294 nm. In quenching experiments, the concentration of the chromophore (CYP119 or porphyrin) was kept sufficiently low (<0.15) to ensure uniform light absorption throughout the sample and reduced inner filter effects. The fluorescence emission intensity was calculated as the integral of the emission spectrum in the recorded wavelength range (305–450 nm). The corrected emission intensity was analyzed with the Benesi–Hildebrand equation.

II. Supplementary Figures

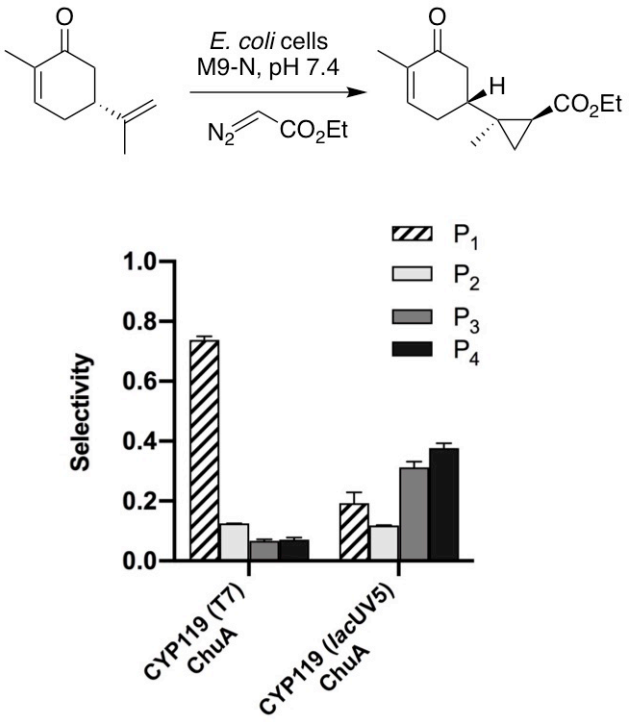


Supplementary Fig. 1 | The cyclopropanation of carvone catalyzed by whole cells. a, The diastereoselectivity of the cyclopropanation of (-)-carvone catalyzed by *E. coli* cells harboring Ir-CYP119 was similar to that of the reaction catalyzed by the free cofactor Ir(Me)MPIX when 10 μM Ir(Me)MPIX was added to cell culture. Left: reaction catalyzed by Ir(Me)MPIX; Middle:

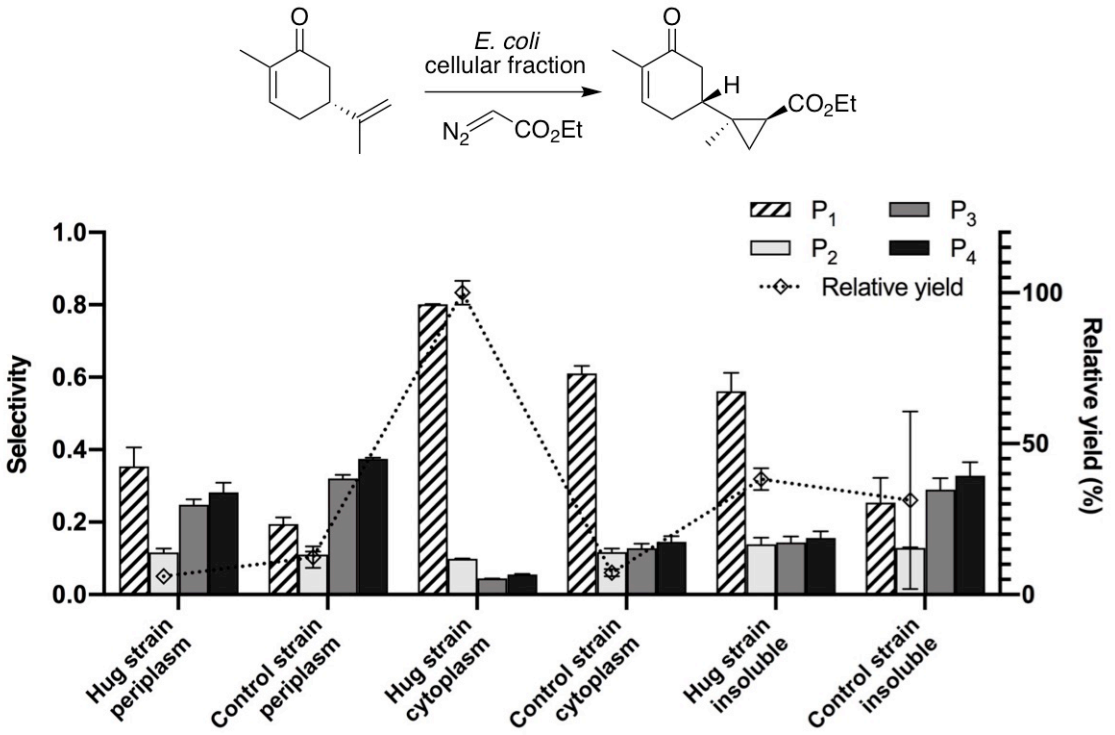
reaction catalyzed by *E. coli* transformed with pCYP119-141 (coding for CYP119 mutant) and pJHA147 (coding for ChuA). Protein expression was induced with 0.5 mM IPTG, and 10 μM Ir(Me)MPIX was added upon induction; Right: reaction catalyzed by *E. coli* cells cultivated in the absence of Ir(Me)MPIX. **b,** The expression of CYP119 is required for the high diastereoselectivity of the cyclopropanation of (-)-carvone catalyzed by *E. coli* cells. From left to right, reactions were catalyzed by *E. coli* cells transformed with 1) pCYP119-141 and pJHA147 2) pJHA147 3) pBbE7A (coding for RFP, red fluorescent protein). Protein expression was induced with 0.5 mM IPTG and 0.1 μM Ir(Me)MPIX was added concurrently. Reaction conditions: 4 mM (-)-carvone, 20 mM EDA, 1% ethanol, 350 μL M9-N buffer. P_1-P_4 are the four diastereomeric products numbered in the order of elution by GC. All data from whole-cell reactions are shown as the average from three biological replicates, with error bars indicating 1 standard deviation.



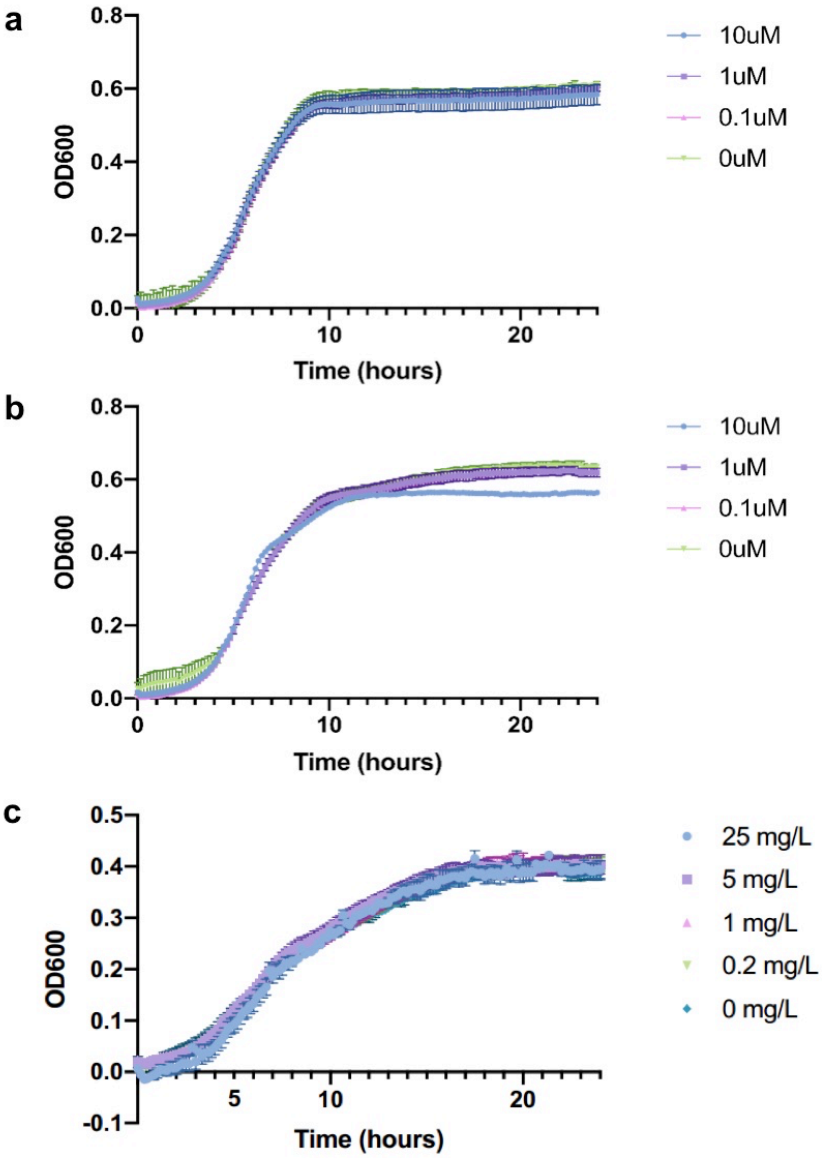
Supplementary Fig. 2 | The effect of CYP119 co-expression and Ir(Me)MPIX addition on limonene titer. a, High-level expression of CYP119 decreased the limonene titer sharply. From left to right, limonene was produced from *E. coli* cells expressing 1) pJBEI6409 (plasmid encoding limonene biosynthetic pathway) 2) pJBEI6409 and pBbE8a (empty vector) 3) pJBEI6409 and pCYP119-141 with T7 promoter. **b,** The addition of 0.1 μM Ir(Me)MPIX in DMSO (DMSO alone as a control) had little impact on the titers of limonene. From left to right, limonene was produced from *E. coli* cells co-transformed with 1) pJBEI6410 (plasmid encoding limonene biosynthetic pathway) and pJHA047 (plasmid encoding HUG system) 2) pJBEI6410 and pJHA135 (plasmid encoding both the HUG system and CYP119 mutant). Limonene production was induced with 0.5 mM IPTG and 10% (v/v) dodecane was added to trap limonene. After 24 h, limonene titer was determined by GC-MS. All data are shown as the average from three biological replicates, with error bars indicating 1 standard deviation.



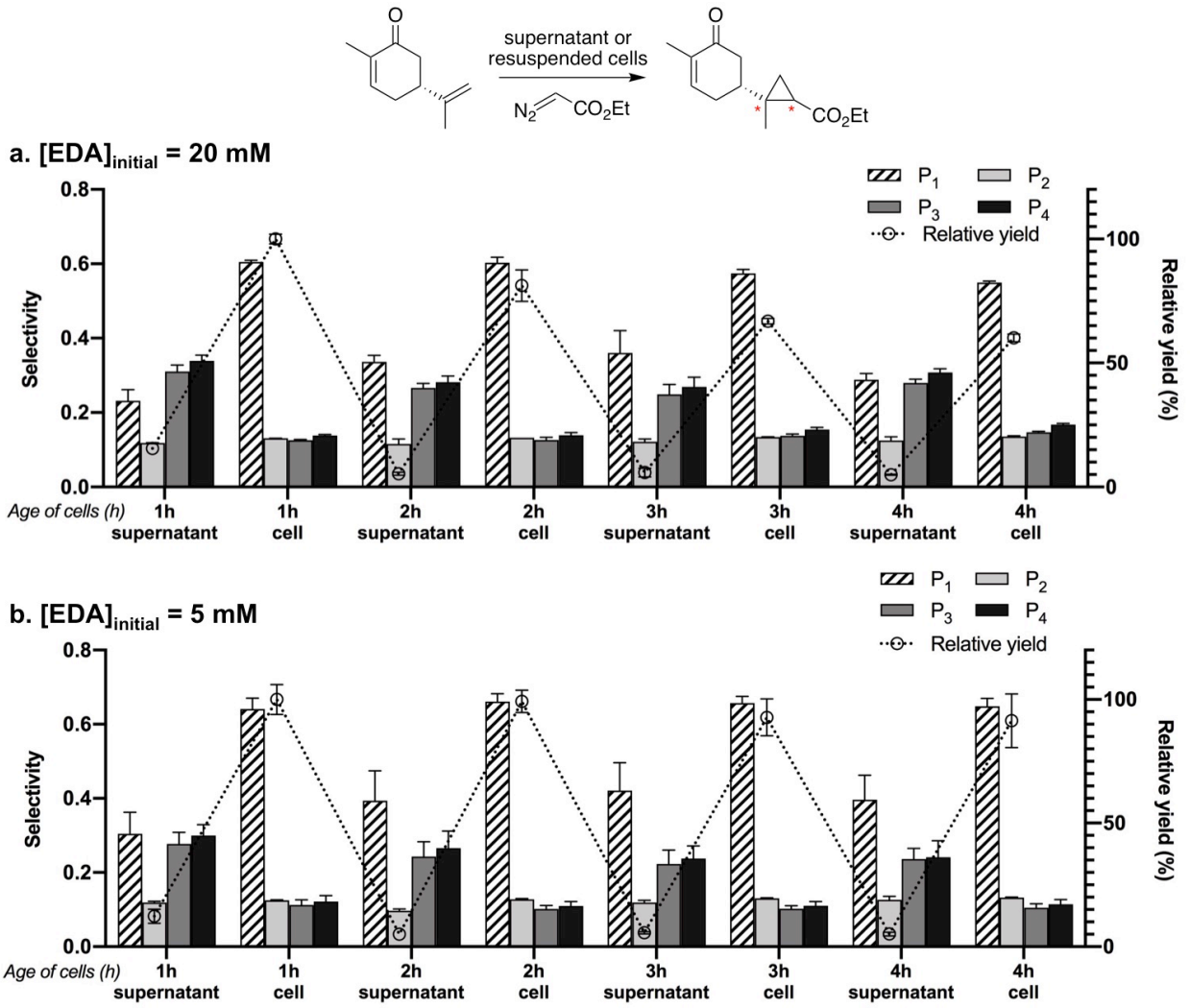
Supplementary Fig. 3 | High-level expression of CYP119 was essential for obtaining high diastereoselectivity for the whole-cell reaction when using ChuA as a transporter. From left to right, reactions were catalyzed by *E. coli* cells transformed with 1) pJHA147 (encoding ChuA) and pCYP119-141 (encoding CYP119 with T7 promoter) and 2) pJHA147 and pJHA029 (encoding CYP119 with *lac*UV5 promoter), respectively. All strains were induced with 0.5 mM IPTG and 0.1 μM Ir(Me)MPIX was added concurrently. Reaction conditions: 4 mM (-)-carvone, 20 mM EDA, 1% ethanol, 350 μL M9-N buffer. P_1-P_4 are the four diastereomeric products numbered in the order of elution by GC. All data are shown as the average from three biological replicates, with error bars indicating 1 standard deviation.



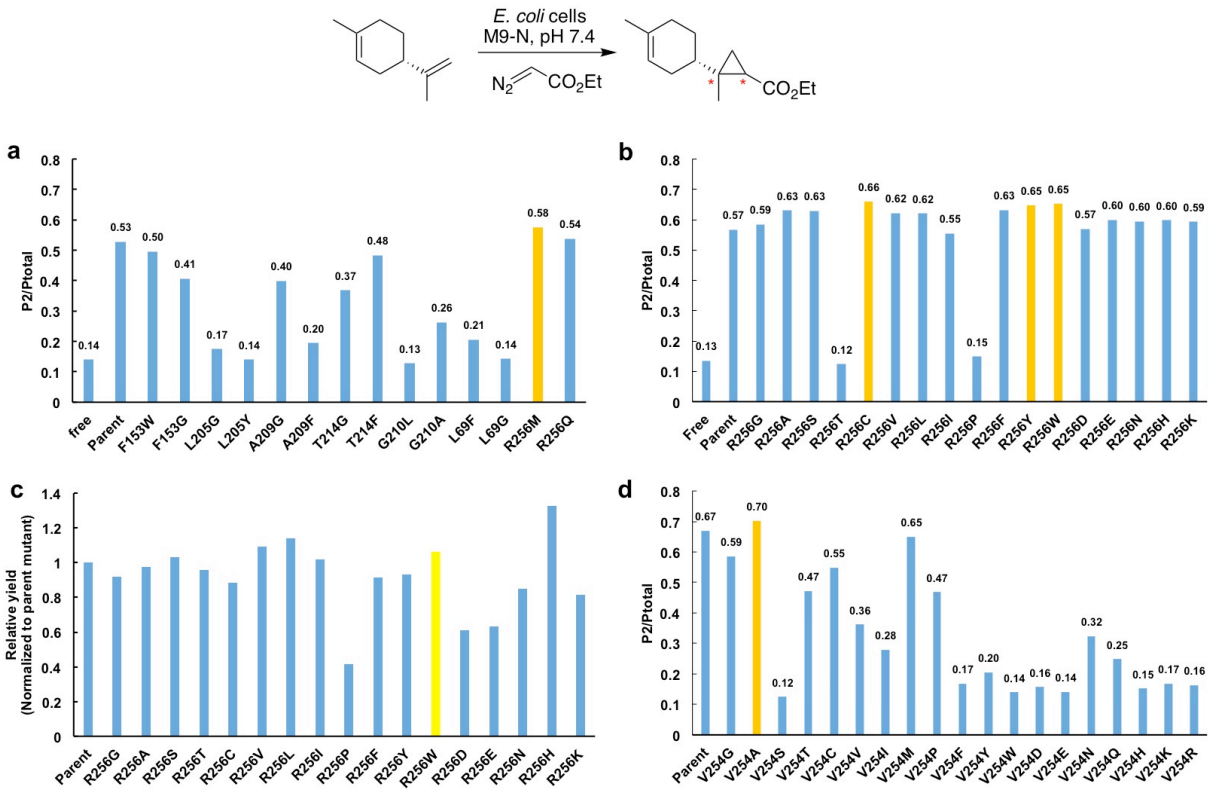
Supplementary Fig. 4 | The diastereoselectivity and relative yield of the cyclopropanation of (-)-carvone catalyzed by different cellular fractions isolated from *E. coli* cells. Hug strain: *E. coli* transformed with pHug21 (encoding HUG system) and pJHA110 (encoding CYP119 mutant with *lac*UV5 promoter); Control strain: *E. coli* transformed with pBbS5a (encoding RFP protein) and pJHA110. Reaction conditions: 4 mM (-)-carvone, 20 mM EDA, 1% ethanol, 500 μL cellular fraction. The relative yields of these reactions were calculated by normalizing the yield of the reaction catalyzed by the cytoplasmic fraction from Hug strain as 100%. All data are shown as the average from three biological replicates, with error bars indicating 1 standard deviation.



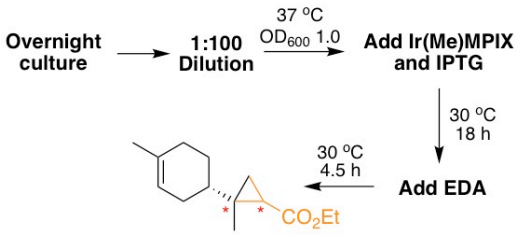
Supplementary Fig. 5 | Evaluation of the effect of Ir(Me)MPIX and cyclopropyl limonene on cell growth. a, The addition of Ir(Me)MPIX has little effect on the growth of BL21(DE3) cells transformed with an empty vector pBbS5K under the range of concentrations tested. **b,** The addition of Ir(Me)MPIX has little effect on the growth of BL21(DE3) cells expressing the HUG system under the range of concentrations tested. **c,** The presence of cyclopropyl limonene has little effect on the growth of BL21(DE3) cells co-transformed with pHug21 and pJHA110 (encoding CYP119) under the range of concentrations tested. Error bars represent 1 standard deviation of three biological replicates.



Supplementary Fig. 6 | The cyclopropanation of (-)-carvone catalyzed by supernatants and resuspended cell pellets. The *E. coli* cultures expressing the artificial biosynthetic pathway were supplemented with 20 mM (**a**) or 5 mM (**b**) EDA to initiate the production of cyclopropyl limonene. Aliquots were removed from the cell culture and centrifuged to separate the media and cells at each one-hour interval. Cell pellets were resuspended in fresh M9-rich medium. Then the supernatants and resuspended cell pellets were used to catalyze the reaction of (-)-carvone with EDA. Reaction conditions: supernatant or resuspended cell pellets, 4 mM (-)-carvone, 20 mM EDA, 1% ethanol, 350 μL M9-rich medium, 30 °C, 2 h. The relative yields of these reactions were calculated by normalizing the reactivity of the cells that were separated one hour after the addition of EDA as 100%. All data are shown as the average from three biological replicates, with error bars indicating 1 standard deviation.

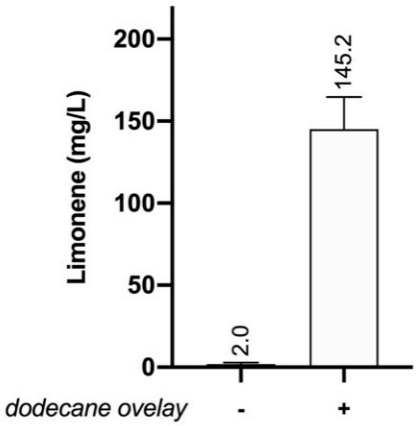


Supplementary Fig. 7 | Directed evolution of CYP119 mutants for the diastereoselective cyclopropanation of (-)-limonene. a, Site-directed mutagenesis of the CYP119 parent mutant was performed at F153, L205, A209, T214, G210, L69 and R256 and mutants were screened for higher diastereoselectivity. **b-c,** Site-saturation mutagenesis was performed at R256 and mutants were screened for higher diastereoselectivity (**b**) and higher yield (**c**). **d,** Site-saturation mutagenesis was performed at V254 and mutants were screened for higher diastereoselectivity. The cyclopropanation of (-)-limonene was catalyzed by *E. coli* cells containing pHug21 and a plasmid coding for CYP119 mutants. Parent designates CYP119 mutant (C317G, T213G, V254L, L155W) in **a-c** and mutant (C317G, T213G, V254L, L155W, **R256W**) in **d**. Free: Ir(Me)MPIX. P2/Ptotal is the ratio of the peak area of the second diastereomer in the order of elution by GC to the peak area of all four diastereomeric products. Ptotal represents the peak area of all four diastereomers. The relative yield in **c** was calculated by normalizing the yield of the reaction catalyzed by the parent mutant as 1.

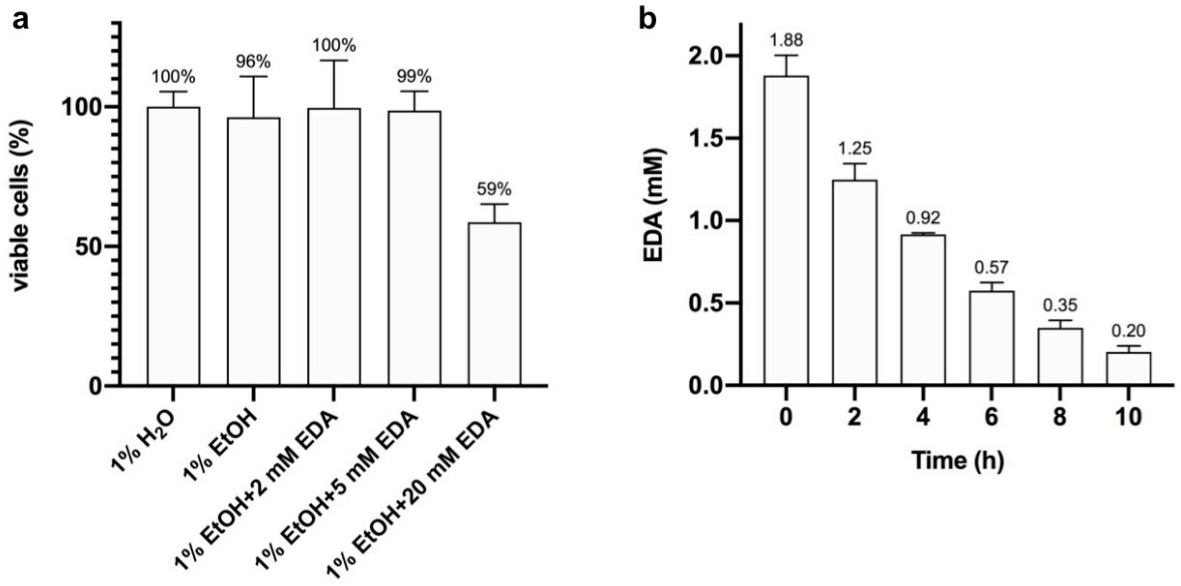


dr (P_2/P_{total})	59±3%
Titer (mg/L)	0.26±0.04

Supplementary Fig. 8 | Process to evaluate the evolved CYP119 mutant (C317G, T213G, V254A, L155W, R256W) and resulting dr and titer using this mutant. *E. coli* strain co-transformed with pJBEI6410 (encoding limonene pathway) and pJHA170 (plasmid coding for both the HUG system and evolved CYP119 mutant) was used to produce cyclopropyl limonene. The process is the same as the experiment in Fig. 3d. All data are shown as the average from three biological replicates.

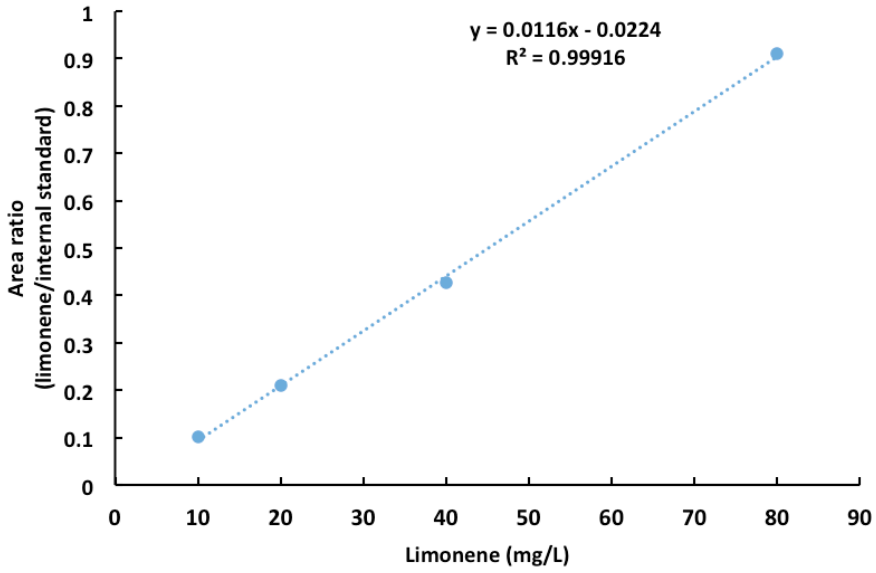


Supplementary Fig. 9 | The titer of limonene from the *E. coli* culture without dodecane overlay was much lower than that with an overlay. Limonene was produced from *E. coli* cells expressing the artificial biosynthetic pathway (pJBEI6410+pJHA170) and the titer was determined 18 hours after the induction of protein expression. For the cell culture without dodecane overlay, aliquots were removed and extracted with equal volume of ethyl acetate containing internal standard. The limonene titer was analyzed by GC-MS. All data are shown as the average from three biological replicates, with error bars indicating 1 standard deviation.

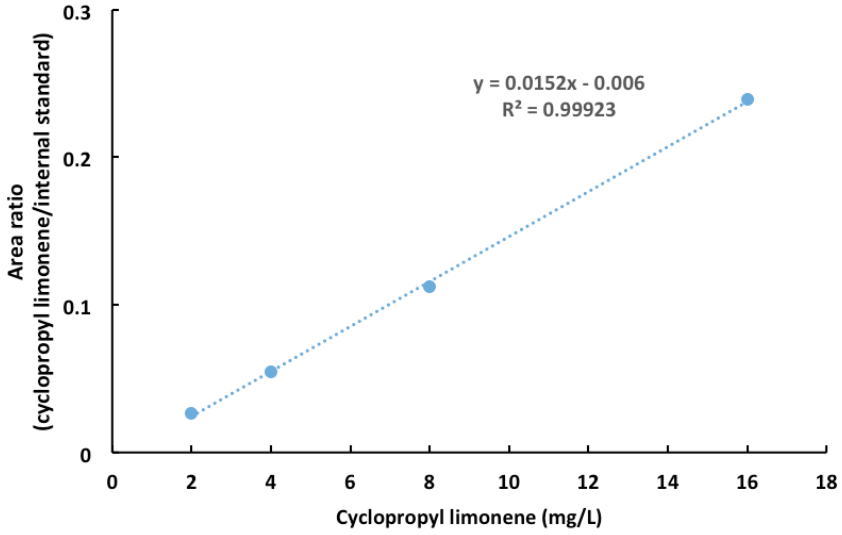


Supplementary Fig. 10 | The toxicity and consumption rate of EDA in the artificial pathway. **a,** High concentration of EDA is toxic to *E. coli* cells. The figure shows the percentages of viable cells in the *E. coli* culture expressing the artificial biosynthetic pathway (pJBEI6410+pJHA170) after exposing to different concentrations of EDA for 4 h. The percentage of viable cells was calculated by normalizing the number of colonies grown in the control group (with 1% ddH_2O) as 100%. **b,** The rate of EDA consumption in the *E. coli* culture expressing the artificial biosynthetic pathway (pJBEI6410+pJHA170). All data are shown as the average from three biological replicates, with error bars indicating 1 standard deviation.

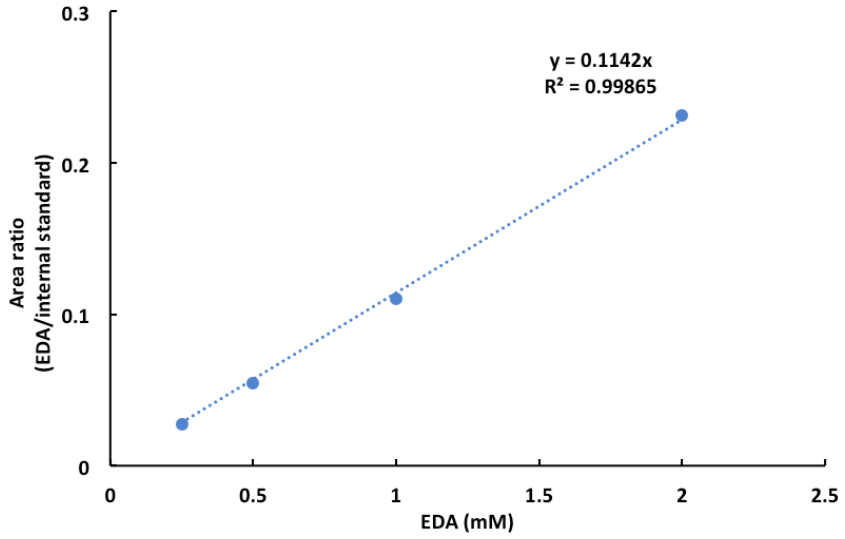
(A)



(B)

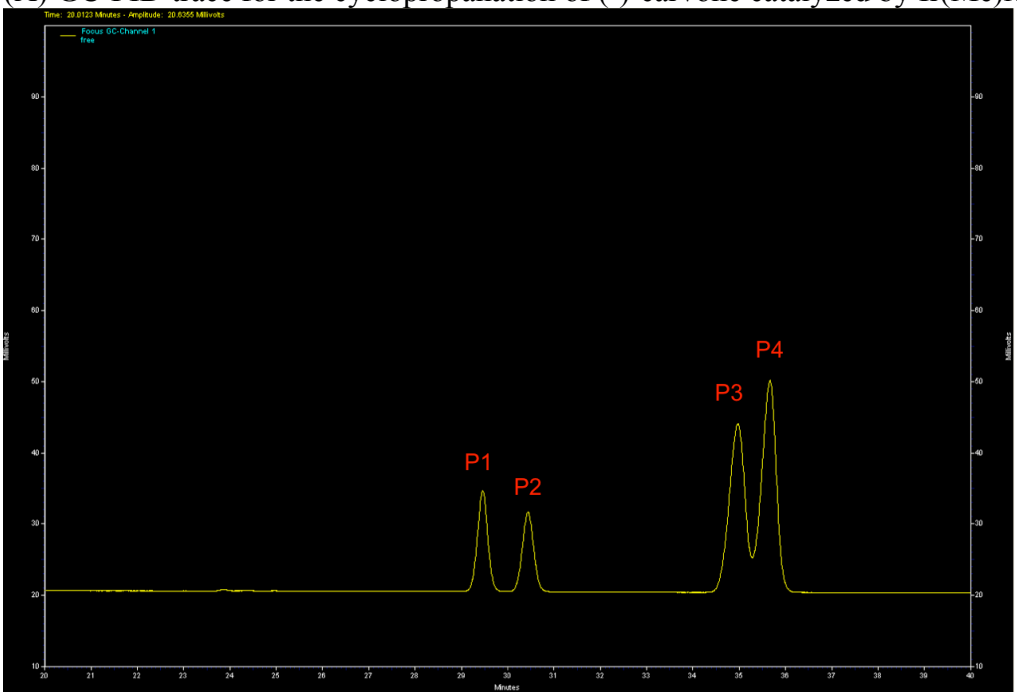


(C)

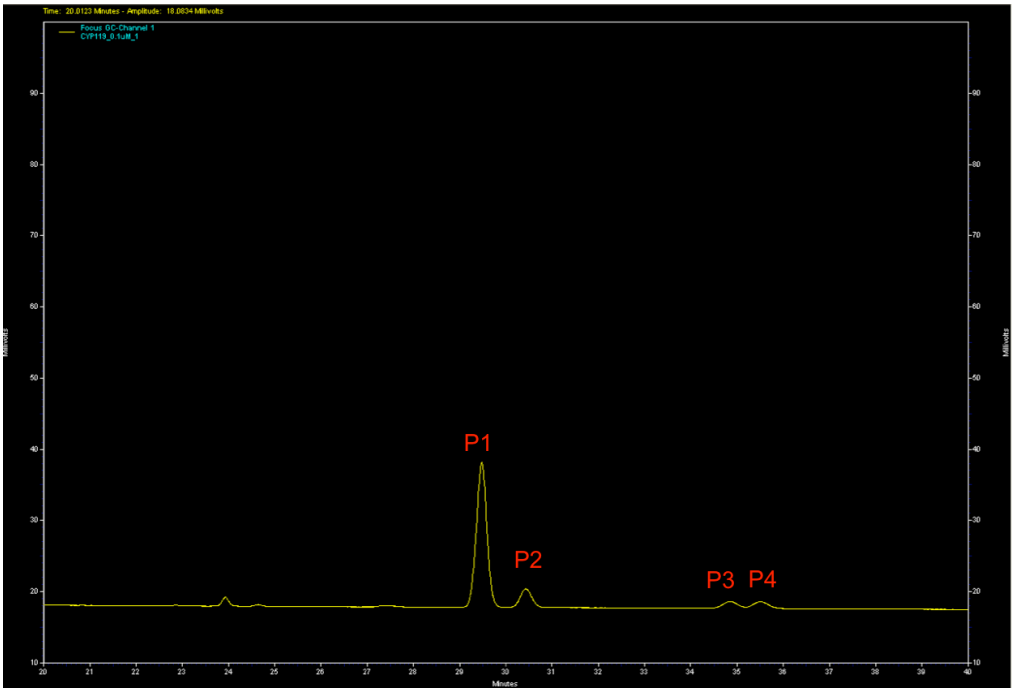


Supplementary Fig. 11 | Calibration curves for (A) limonene (GC-MS) and (B) cyclopropyl limonene (GC-MS) and (C) EDA (GC-FID).

(A) GC-FID trace for the cyclopropanation of (-)-carvone catalyzed by Ir(Me)MPIX.

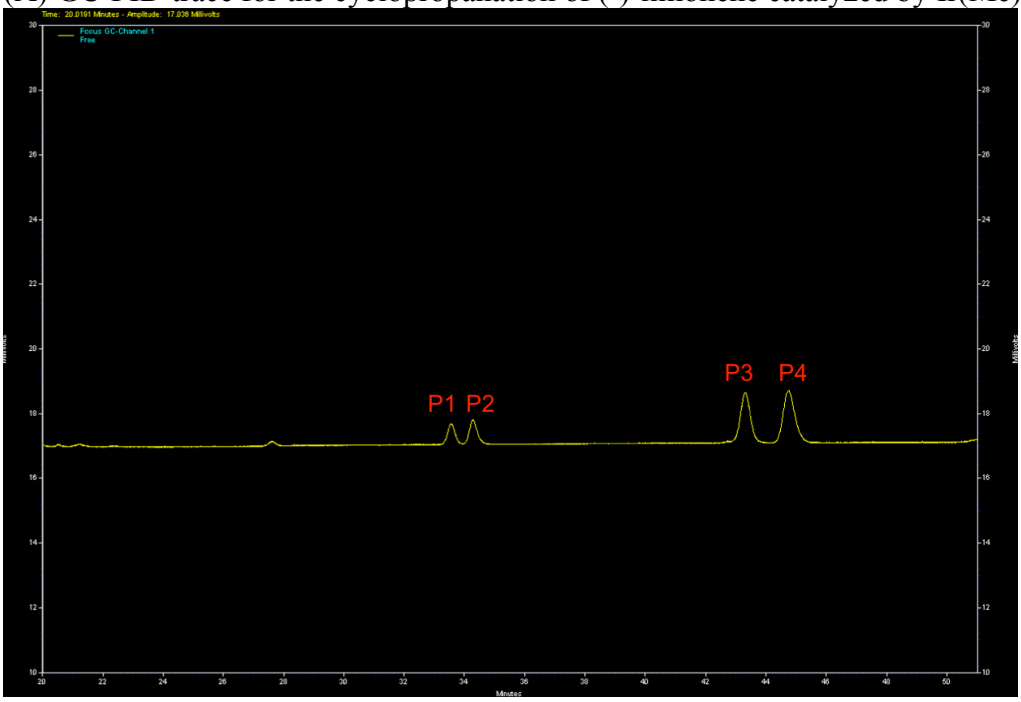


(B) GC-FID trace for the cyclopropanation of (-)-carvone catalyzed by *E. coli* harboring Ir-CYP119 mutant.

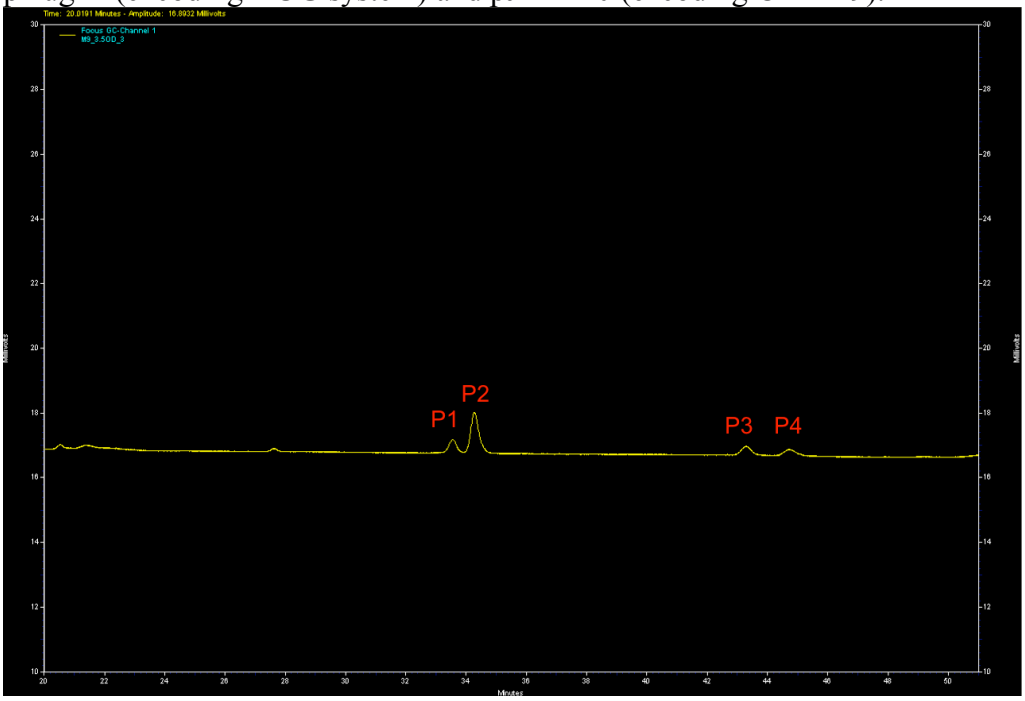


Supplementary Fig. 12 | Representative GC-FID (with DB wax column) traces showing the product mixtures enriched in the P1 diastereomer from whole-cell cyclopropanation of (-)-carvone catalyzed by Ir-CYP119.

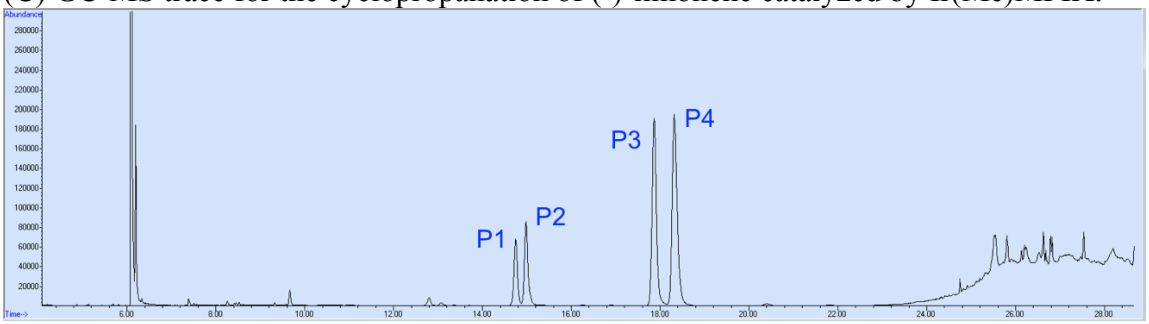
(A) GC-FID trace for the cyclopropanation of (-)-limonene catalyzed by Ir(Me)MPIX.



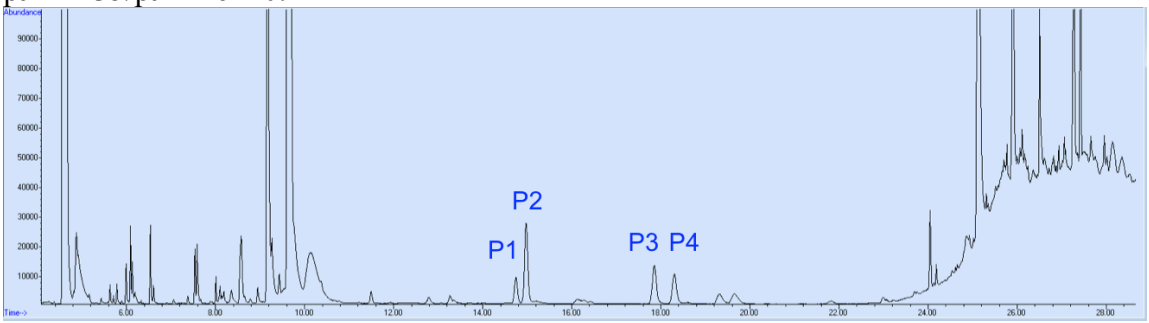
(B) GC-FID trace for the cyclopropanation of (-)-limonene catalyzed by *E. coli* containing pHug21 (encoding HUG system) and pJHA110 (encoding CYP119).



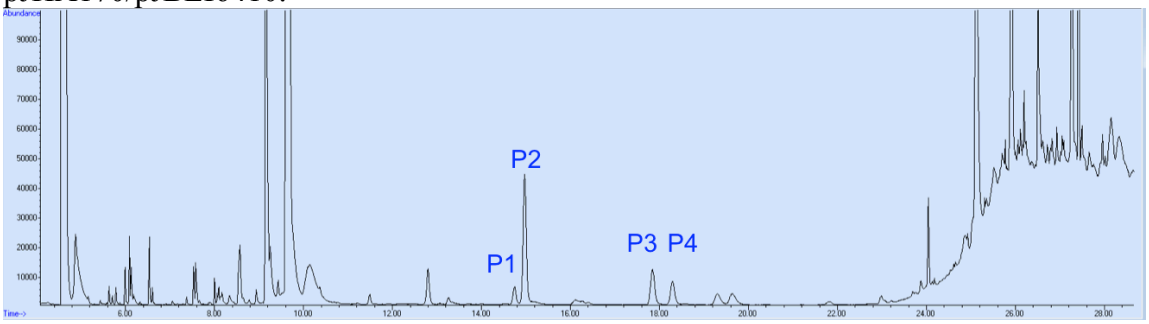
(C) GC-MS trace for the cyclopropanation of (-)-limonene catalyzed by Ir(Me)MPIX.



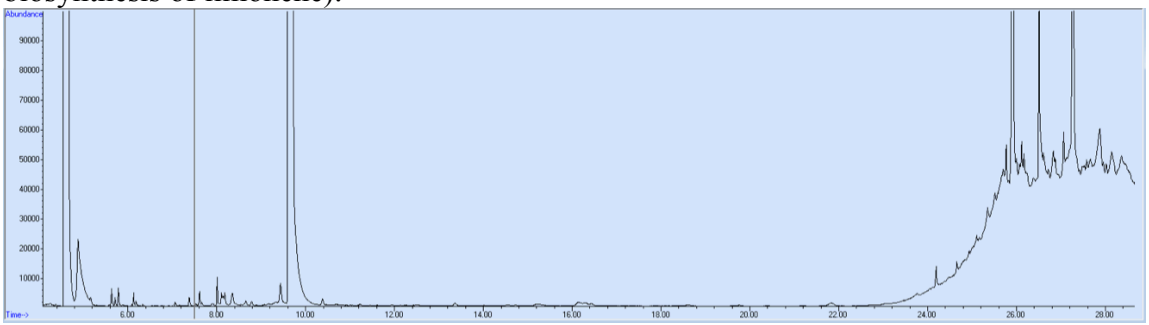
(D) GC-MS trace of the cyclopropyl limonene produced from *E. coli* containing pJHA135/pJBEI6410.



(E) GC-MS trace of the cyclopropyl limonene produced from *E. coli* containing pJHA170/pJBEI6410.



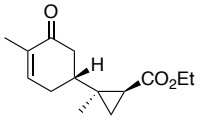
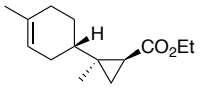
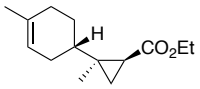
(F) GC-MS trace for the negative control with *E. coli* expressing pJHA135/pBbA5a (no biosynthesis of limonene).



Supplementary Fig. 13 | Representative GC-FID/GC-MS (GC equipped with CycloSil-B column) traces showing the product mixtures enriched in the P2 diastereomer from whole-cell cyclopropanation of (-)-limonene catalyzed by Ir-CYP119.

III. Supplementary Tables

Supplementary Table 1. GC Methods used to separate diastereomers of the cyclopropanation products and biosynthesized products.

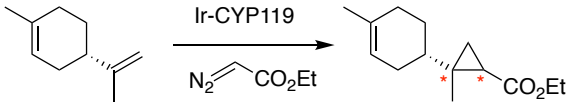
Products[a]	Instrument	Column	Method	Retention Times
	GC-FID	DB-wax (15 m × 0.32 mm × 0.25 μm)	40 °C, 5 °C/min to 140 °C; hold for 20 min; 30 °C/min to 245 °C, hold 3 min	P1: 29.472 min (major) P2: 30.432 min P3: 34.848 min P4: 35.507 min
	GC-FID	CycloSil-B (30 m × 0.25 mm × 0.25μm)	40 °C, 15 °C/min to 150 °C; hold 43 min; 30 °C/min to 245 °C, hold 3 min	P1: 33.563 min P2: 34.288 min (major) P3: 43.310 min P4: 44.733 min
	GC-MS EIC: 93, 121 and 222	CycloSil-B (30 m × 0.25 mm × 0.25 μm)	40 °C, 15 °C/min to 150 °C; hold 43 min; 30 °C/min to 245 °C, hold 3 min	P1: 14.741 min P2: 14.967 min (major) P3: 17.846 min P4: 18.304 min
Ethyl diazoacetate	GC-FID	DB-wax (15 m × 0.32 mm × 0.25 μm)	40 °C, 10 °C/min to 80 °C; hold 0 min; 30 °C/min to 245 °C, hold 2 min	2.572 min
(-)-limonene	GC-MS EIC: 93,121 and 136	HP-5 (30 m × 0.25 mm × 0.25 μm)	80 °C, hold 1 min; 20 °C/min to 120 °C; 50 °C/min to 250 °C.	3.074 min

[a]The configuration of the major diastereomeric product of the cyclopropanation of (-)-carvone was determined in the prior publication.[1] The configuration of the major diastereomeric product of the cyclopropanation of (-)-limonene is proposed by analogy to the cyclopropane product of (-)-carvone.

Supplementary Table 2. Summary of the binding constants for the formation of the Fe(Cl)PPIX and Ir(Me)MPIX complexes with apo-CYP119, yielded by the Benesi–Hildebrand analysis of fluorescence quenching data.

Cofactor	K_b (M^{-1})	K_d (μM)
Fe(Cl)PPIX	$6.7(0.7) \times 10^5$	1.5(0.2)
Ir(Me)MPIX	$4.2(0.3) \times 10^5$	2.4(0.2)
Binding data of hemoproteins from literature [9]		
Cofactor	Protein	K_d
Fe(Cl)PPIX	apo-ascorbate peroxidase	0.19 μM
Fe(Cl)PPIX	apo-myoglobin	0.60 μM
Reduced Fe(Cl)PPIX	apo-myoglobin	3.1 fM

Supplementary Table 3. The comparison of the diastereoselectivity of the cyclopropanation of (-)-limonene catalyzed by Ir-CYP119 mutants reconstituted *in vitro* and whole cells harboring Ir-CYP119 mutants.



CYP119 mutants	*in vivo* diastereoselectivity (P_2/P_{total})[a]	*in vitro* diastereoselectivity (P_2/P_{total})[b]
P (C317G, T213G, V254L, L155W)	53%	20%
P/256W	63%	48%
P/256W/254A	72%	60%

[a]The data are shown as the average from three biological repeats. [b]The data are shown as the average from two replicate experiments.

Supplementary Table 4. (A) ICP-MS measurement of CYP119 purified from *E. coli* co-expressing the heme transport system and CYP119 (pJHA110).[a]

RFP/CYP119[b]	protein yield (mg)	protein yield (μmol)	Ir (nmol)[c]	%Ir recovery[d]
Replicate 1	1.88	0.042	0.59	5.9%
Replicate 2	2.55	0.057	0.62	6.2%
Replicate 3	2.36	0.052	0.66	6.6%
			Average	6.3±0.4%
HUG/CYP119[e]				
Replicate 1	3.63	0.081	3.15	31.5%
Replicate 2	3.65	0.081	3.66	36.6%
Replicate 3	2.96	0.066	3.08	30.8%
			Average	33.0±3.1%
ChuA/Ton/CYP119[f]				
Replicate 1	2.52	0.056	0.83	8.3%
Replicate 2	2.23	0.050	0.69	6.9%
Replicate 3	2.22	0.049	0.87	8.7%
			Average	8.0±0.9%

[a]The cell culture (100 mL) was cultivated and the CYP119 was purified following the protocol described in *section 11 (b)*. [b]*E. coli* cells were transformed with pBbS5a (plasmid encoding RFP as control) and pJHA110 (plasmid encoding CYP119 with *lac*UV5 promoter). [c]The data show the total amount of Ir present in the purified protein. [d]The percentages of added iridium (0.1 μM) that were recovered from the purified protein. [e]*E. coli* cells were transformed with pHug21 (plasmid encoding the HUG system) and pJHA110 (plasmid encoding CYP119 with *lac*UV5 promoter). [f]*E. coli* cells were transformed with pJHA184 (plasmid encoding ChuA and Ton complex) and pJHA110 (plasmid encoding CYP119 with *lac*UV5 promoter).

Supplementary Table 4. (B) ICP-OES measurement of CYP119 purified from *E. coli* co-expressing HUG (pHug21) and CYP119 (pJHA108).[a]

HUG/CYP119	protein yield (mg)	protein yield (μmol)	Fe (μmol)[b]	Fe:CYP119[c]	Ir (μmol)[d]	Ir:CYP119[e]
Replicate 1	16.26	0.36	0.031	8.5%	0.040	11.1%
Replicate 2	17.26	0.38	0.035	9.1%	0.041	10.8%
Replicate 3	15.81	0.35	0.044	12.4%	0.039	11.0%

[a]The cell culture (700 mL) was cultivated and the CYP119 was purified following the protocol described in *section 11 (b)* except that 0.2 mg/L Ir(Me)MPIX was supplemented upon the induction of protein expression. The purified protein was dialyzed against 10 mM TRIS buffer (pH 8) before concentration and acid digestion to remove the residual iron in Ni-NTA elution buffer. [b]The data show the total amount of Fe present in the purified protein. The concentration of Fe in ICP-OES (Inductively coupled plasma - optical emission spectrometry) samples were measured at an emission wavelength of 259 nm. [c]Fe:CYP119 is calculated as the molar ratio of iron and CYP119. The value represents the percentage of heme-containing CYP119 in the purified protein sample by assuming that all Fe exists in the form of heme and binds to CYP119. [d]The data show the total amount of Ir present in the purified protein. The concentration of Ir in ICP-OES samples was measured at an emission wavelength of 212 nm. [e]Ir:CYP119 is calculated as the molar ratio of iridium and CYP119. The value represents the percentage of Ir(Me)MPIX-containing CYP119 in the purified protein sample by assuming that all Ir exists in the form of iridium porphyrin and binds to CYP119.

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