Supplementary Materials for

Assembly and Evolution of Artificial Metalloenzymes within *E. coli* Nissle 1917 for Enantioselective and Site-Selective Functionalization of C–H and C=C Bonds

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I. Materials and Methods

1. General methods

Unless otherwise noted, all chemicals, salts, and solvents 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. Mutaflor® was purchased from Pharma-Zentrale GmbH. 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 °C) or a sterile syringe filter (0.22 µm). The synthetic procedures of Ir(Me)MPIX were reported previously.[1]

2. *E. coli* strains and expression plasmids

Chemically competent EcN cells were prepared from Mutaflor® following a standard $CaCl_2$/glycerol protocol.[2] Competent EcN cells were stored at −80 °C for no longer than three months. Chemically competent BL21(DE3) cells were obtained from New England Biolabs. The plasmids used in this study are listed in the table below. The sequences of CYP119 and outer-membrane transporters (pE1-pE15) were cloned into the respective vectors. Primer sequences are available upon request.

Name	Marker	Origin	Vector backbone	Genes
3-105-3	Cam	f1	pASK-IBA3C	CYP119 mutant for enantioselective C-H activation of phthalan **1**
3-82-1	Cam	f1	pASK-IBA3C	CYP119-M_{L1} mutant for *meta*-selective C-H activation of phthalan **3a** and **3b**
3-82-2	Cam	f1	pASK-IBA3C	CYP119-P_{L1} mutant for *para*-selective C-H activation of phthalan **3a**, **3b** and **3c**
3-105-1	Cam	f1	pASK-IBA3C	CYP119-P_{L2} mutant for *para*-selective C-H activation of phthalan **3d**
3-105-2	Cam	f1	pASK-IBA3C	CYP119-M_{L2} mutant for *meta*-selective C-H activation, used as a parent plasmid for diastereoselective cyclopropanation of (+)-nootkatone
pJHA190-1	Kan	colE1	pBbE1K	CYP119 mutant for enantioselective C-H activation of phthalan **1** (with trc promoter)
pE1-15	Amp	p15A	pBbS5A	Transporters on EcN

CYP119 mutant sequences (with mutated positions highlighted)

3-105-3
MKSSHHHHHHENLYFQSNYDWFSEMRKKDPVYYDGNIWQVFSYRYTKEVLNNFSKFSSD
LTGYHERLEDLRNGKIRFDIPTRYTMLTSDPPLHDELRSMSADIFSPQKLQTLETFIRE
TTRSLLDSIDPREDDIVKKLAVPLPIIVISKILGLPIEDKEKFKEWSDLVLFRLGKPGE
IFELGKKYLELIGYVKDHLNSGTEVVSRVVNSNLSDIEKLGYIILLLIAGNEGTTNLIS
NSVIDFTRFNLWQRIREENLYLKAIEEALRYSSPAMRTVRKTKERVKLGDQTIEEGEYV
RVWIASANRDEEVFHDGEKFIPDRNPNPHLSFGSGIHLGLGAPLARLEARIAIEEFSKR
FRHIEILDTEKVPNEVLNGYKRLVVRLKSNESAWSHPQFEK
3-82-1
MYDWFSEMRKKDPVYYDGNIWQVFSYRYTKEVLNNFSKFSSDLTGYHERLEDLRNGKIR
FDIPTRYTMLTSDPPLHDELRSMSADIFSPQKLQTLETFIRETTRSLLDSIDPREDDIV

KKLAVPLPIIVISKILGLPIEDKEKFKEWSDLVLFRLGKPGEIFELGKKYLELIGYVKD
HLNSGTEVVSRVVNSNLSDIEKLGYIILLLIAGNEGTTNLISNSVIDFTRFNLWQRIRE
ENLYLKAIEEALRYSPPAMRTVRKTKERVKLGDQTIEEGEYVRVWIASANRDEEVFHDG
EKFIPDRNPNPHLSFGSGIHLGLGAPLARLEARIAIEEFSKRFRHIEILDTEKVPNEDL
NGYKRLVVRLKSNESAWSHPQFEK

3-82-2

MYDWFSEMRKKDPVYYDGNIWQVFSYRYTKEVLNNFSKFSSDLTGYHERLEDLRNGKIR
FDIPTRYTMLTSDPPLHDELRSMSADIFSPQKLQTLETFIRETTRSLLDSIDPREDDIV
KKLAVPLPIIVISKILGLPIEDKEKFKEWSDLVAFRFGKPGEIFELGKKYLELIGYVKD
HLNSGTEVVSRVVNSNLSDIEKLGYIILLLIAGNEGTTNLISNSVIDFTRFNLWQRIRE
ENLYLKAIEEALRYSPPLMRTVRKTKERVKLGDQTIEEGEYVRVWIASANRDEEVFHDG
EKFIPDRNPNPHLSFGSGIHLGLGAPLARLEARIAIEEFSKRFRHIEILDTEKVPNEVL
NGYKRLVVRLKSNESAWSHPQFEK

3-105-1

MKSSHHHHHHENLYFQSNYDWFSEMRKKDPVYYDGNIWQVFSYRYTKEVLNNFSKFSSD
LTGYHERLEDLRNGKIRFDIPTRYTMLTSDPPLHDELRSMSADIFSPQKLQTLETFIRE
TTRSLLDSIDPREDDIVKKLAVPLPIIVISKILGLPIEDKEKFKEWSDLVFFRLGKPGE
IFELGKKYLELIGYVKNHLNSGTEVVSRVVNSNLSDIEKLGYIILLLIAGNEGTTNLIS
NSVIDFTRFNLWQRIREENLYLKAIEESLRYSPPLMRTVRKTKERVKLGDQTIEEGEYV
RVWIASANRDEEVFHDGEKFIPDRNPNPHLSFGSGIHLGLGAPLARLEARIAIEEFSKR
FRLIEILDTEKVPNEVLNGYKRLVVRLKSNESAWSHPQFEK

3-105-2

MKSSHHHHHHENLYFQSNYDWFSEMRKKDPVYYDGNIWQVFSYRYTKEVLNNFSKFSSD
LTGYHERLEDLRNGKIRFDIPTRYTMLTSDPPLHDELRSMSADIFSPQKLQTLETFIRE
TTRSLLDSIDPREDDIVKKLAVPLPIIVISKILGLPIEDKEKFKEWSDLVLFRLGKPGE
IFELGKKYLELIGYVKNHLNSGTEVVSRVVNSNLSDIEKLGYIILLLIAGNEGTTNLIS
NSVIDFTRFNLWQRIREENLYLKAIEESLRYSPPAMRTVRKTKERVKLGDQTIEEGEYV
RVWIASANRDEEVFHDGEKFIPDRNPNPHLSFGSGIHLGLGAPLARLEARIAIEEFSKR
FRLIEILDTEKVPNEVLNGYKRLVVRLKSNESAWSHPQFEK

pJHA190-1

MKSSHHHHHHENLYFQSNYDWFSEMRKKDPVYYDGNIWQVFSYRYTKEVLNNFSKFSSD
LTGYHERLEDLRNGKIRFDIPTRYTMLTSDPPLHDELRSMSADIFSPQKLQTLETFIRE
TTRSLLDSIDPREDDIVKKLAVPLPIIVISKILGLPIEDKEKFKEWSDLVLFRLGKPGE
IFELGKKYLELIGYVKDHLNSGTEVVSRVVNSNLSDIEKLGYIILLLIAGNEGTTNLIS
NSVIDFTRFNLWQRIREENLYLKAIEEALRYSSPAMRTVRKTKERVKLGDQTIEEGEYV
RVWIASANRDEEVFHDGEKFIPDRNPNPHLSFGSGIHLGLGAPLARLEARIAIEEFSKR
FRHIEILDTEKVPNEVLNGYKRLVVRLKSNE

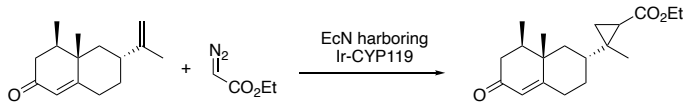
3. Preparation of CYP119 mutant library

Combinatorial screening: Combinatorial codon mutagenesis libraries were prepared based on a procedure described by Belsare et al.[3] The CYP119 template was amplified from plasmid 3-105-2. A library targeting 10 active site residues was prepared with a pool of 10 forward and reverse primers containing degenerate NNK codons at the targeted residues. The full-length genes were obtained from fragment and joining PCRs, purified using agarose gel electrophoresis and cloned into a pASK-IBA3C vector using standard Golden Gate assembly methods.

First round	Primer sequence (5'-3')

insert_for	GCTGTTGGTCTCGAATGAAATCTTCTCACCATCACC
insert_rev	GATGTTGGTCTCAGCGCTCTCATTGCTCTTCAG
69_rev	CGGGTCGCTGGT**MNN**CATGGTATAACG
69_for	CGTTATACCATG**NNK**ACCAGCGACCCG
87_rev	CTTCTGCGGGCT**MNN**GATATCCGCGCTC
87_for	GAGCGCGGATATC**NNK**AGCCCGCAGAAG
152_rev	CCCCAACGAAA**MNN**AACCAGATCGC
152_for	GCGATCTGGTT**NNK**TTTCGTTGGGG
153_rev	GGTTTACCCCAACG**MNN**CGCAACCAGATCG
153_for	CGATCTGGTTGCG**NNK**CGTTGGGGTAAACC
205_rev	CCGCAATCAGCAG**MNN**AATGATGTAACCCA
205_for	TGGGTTACATCATT**NNK**CTGCTGATTGCGG
209_for	GCTGCTGATT**NNK**GGCAACGAAGG
209_rev	CCTTCGTTGCC**MNN**AATCAGCAGC
254_rev	AACGGTACGCAT**MNN**CGGCGGGCTATA
254_for	TATAGCCCGCCG**NNK**ATGCGTACCGTT
282_for	CGTGTTTGG**NNK**GCGAGCGCGAACC
282_rev	GGTTCGCGCTCGC**MNN**CCAAACACG
310_for	GCACCTGAGC**NNK**GGTAGCGGCAT
310_rev	ATGCCGCTACC**MNN**GCTCAGGTGC
353_for	AGGTTCCGAATGAA**NNK**CTGAATGGTTACAAGCGT
353_rev	ACGCTTGTAACCATTCAG**MNN**TTCATTCGGAACCT

In the first found of screening, we targeted all 10 sites at once, and the gene library was directly transformed into EcN. Approximately 400 colonies were picked and cultured for whole-cell screening. As a positive control, the parent mutant was also included in the screening and cultured under the identical conditions. The whole-cell catalyst containing the original mutant, on average, catalyzed the formation of the major diastereomeric product with 40% diastereoselectivity. Therefore, only the colonies that produced the cyclopropyl nootkatone with diastereoselectivity higher than or equal to 40% were selected and sequenced. Out of the four 96-well plates, we obtained 16 mutants that led to improved selectivity. Among these 16 "hits," nine were the parent mutants, and the mutant that afforded the highest selectivity contains an I282L mutation.



4 diastereomeric products
Free cofactor (15% : 14% : 38% : 33%)

	Selectivity (P_2/P_{total})	Sequence
Hit 1	41%	parent
Hit 2	41%	parent
Hit 3	**57%**	**parent+282L**
Hit 4	46%	parent+353G

Hit 5	40%	parent+153I,353A
Hit 6	42%	parent
Hit 7	42%	parent
Hit 8	46%	parent
Hit 9	47%	parent
Hit 10	43%	parent
Hit 11	45%	parent
Hit 12	52%	parent+282M,353N
Hit 13	44%	parent
Hit 14	43%	parent+153G
Hit 15	43%	parent+153G
Hit 16	40%	parent+153G

Targeted screening: Site-directed mutagenesis was used to introduce mutations into the most selective mutant (P/I282L) identified from the first round of screening. 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, concentrated and transformed into Nissle 1917 cells for screening.

Second round	Primer sequence (5'-3')
152-153_for	GAATGGAGCGATCTGGTT**NNKNNK**CGTCTGGGTAAACCG
152-153_rev	CGGTTTACCCAGACG**MNNMNN**AACCAGATCGCTCCATTC
152-155_for	GCGATCTGGTT**NNK**TTTCGT**NNK**GGTAAACCGG
152-155_rev	CCGGTTTACC**MNN**ACGAAA**MNN**AACCAGATCGC

In the second round of screening, around 200 colonies were picked from each library (152/153 NNK and 152/155 NNK). Similarly, the parent mutant (P/282L) was also included in the screening and cultured under the identical conditions as a positive control. For this round, only the colonies that produced the cyclopropyl nootkatone with a diastereoselectivity higher than or equal to 60% were selected and sequenced. Out of the four 96-well plates, we obtained 8 mutants reacting with this selectivity. Among these 8 "hits," the mutant P/282L/152N/153I appeared twice and afforded the highest selectivity.

	Library	Selectivity (P_2/P_{total})	Mutations
Hit 1	**152/153 NNK**	**69%**	**152N,153I**
Hit 2	152/153 NNK	61%	152P,153R
Hit 3	**152/153 NNK**	**72%**	**152N,153I**
Hit 4	152/155 NNK	60%	152S,153L
Hit 5	152/155 NNK	61%	152A,155L
Hit 6	152/155 NNK	62%	152Y,155G
Hit 7	152/155 NNK	63%	152L,155E
Hit 8	152/155 NNK	62%	152S,155P

Third round	Primer sequence (5'-3')
254-256_for	GTTATAGCCCGCCG**NNK**ATG**NNK**ACCGTTCGTAAAAC
254-256_rev	GTTTTACGAACGGT**MNN**CAT**MNN**CGGCGGGCTATAAC

In the third round of screening, the mutant P/282L/152N/153I was selected as the template, and a new library was created containing mutations at the 254/256 positions. For this round of evolution, only the colonies that produced the cyclopropyl nootkatone with a diastereoselectivity higher than or equal to 70% were selected and sequenced. Out of around 200 colonies, we obtained 5 mutants reacting with this selectivity. The mutants (P/282L/152N/153I/256S) afforded the highest selectivity.

	Library	Selectivity (P_2/P_{total})	Mutations
Hit 1	254/256 NNK	70%	254T,256A
Hit 2	254/256 NNK	73%	254C,256S
Hit 3	254/256 NNK	79%	256S
Hit 4	254/256 NNK	74%	256M
Hit 5	254/256 NNK	76%	256G

Note: The reaction results obtained in the screening were slightly different from the results reported in Fig. 4 in the main text due to the difference in the scale of culture. The reactions (1 mL culture) during the screening were only performed once. To validate the "hits", the selected mutants were grown in a larger culture (4 mL). Three biological replicates were performed and the validated results were reported in Fig. 4.

4. 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/w%), casamino acids (20 w/w%), $MgSO_4$ (1 M), antibiotics and $CaCl_2$ (1 M) were sterilized by filter (PES membrane, 0.45 μm pore size). The following amounts of the listed solutions were added per liter of sterilized salt solution: 20 mL glucose, 10 mL casamino acids, 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/w%) were added to give a final concentration of 2.0 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.8 w/w% glucose.

5. *In vivo* expression of Ir-CYP119 in *E. coli* Nissle 1917

A pASK-IBA3C plasmid containing CYP119 genes was transformed into $CaCl_2$ competent EcN cells. Individual colonies from freshly transformed plates were inoculated into 4 mL M9-rich media supplemented with 50 μg/mL chloramphenicol. The cultures were grown overnight at 37 °C and 250 rpm for 18 h. In a Falcon® 14 mL round bottom culture tube, 4 mL of fresh M9-rich medium was inoculated with 40 μL of overnight starter culture and incubated at 37 °C and 250 rpm to an OD_{600} of 0.6~0.8. The cultures were then induced by adding final concentration of 200 ng/mL anhydrotetracycline hydrochloride and 0.1 mg/L Ir(Me)MPIX (4 μL of 0.1 mg/mL stock solution in DMSO). Upon induction, the incubation

temperature was reduced to 30 °C, and expressions were allowed to continue for 18 h at 250 rpm. After harvesting the cells by centrifugation (4 °C, 10 min, 4000 rpm), the cell pellets were resuspended in 600 µL M9-N reaction buffer.

6. *In vivo* expression of Ir-CYP119 in *E. coli* BL21(DE3) with co-expression of outer-membrane transporter

A plasmid containing CYP119 genes (pJHA190-1) was co-transformed with a plasmid encoding the corresponding outer-membrane transporter (pE1-pE15) into chemically competent BL21(DE3) cells (NEB). Individual colonies from freshly transformed plates were inoculated into 4 mL M9-rich media supplemented with 100 µg/mL carbenicillin and 50 µg/mL kanamycin. The cultures were grown overnight at 37 °C and 250 rpm for 18 h. In a Falcon® 14 mL round bottom culture tube, 4 mL of M9-rich medium was inoculated with 40 µL of overnight starter culture and incubated at 37 °C and 250 rpm to an OD_{600} of 0.6~0.8. The cultures were then induced by adding final concentration of 50 µM IPTG and 0.1 mg/L Ir(Me)MPIX (4 µL of 0.1 mg/mL stock solution in DMSO). Upon induction, the incubation temperature was reduced to 30 °C, and expressions were allowed to continue for 18 h at 250 rpm. After harvesting the cells by centrifugation (4 °C, 10 min, 4000 rpm), the cell pellets were resuspended in 600 µL M9-N reaction buffer.

7. Large-scale expression for protein purification and ICP measurement

Protein expression: E. coli containing the respective plasmid(s) was grown at 37 °C in 100 mL M9-rich medium supplemented with appropriate antibiotics for Ir-CYP119 production. The cultures were incubated to an OD_{600} of 0.6~0.8. The cultures were then induced by adding 200 ng/mL anhydrotetracycline hydrochloride or 50 µM IPTG, at which time 10 µL of a 1 mg/mL solution of Ir(Me)MPIX in DMSO was added to give a final Ir(Me)MPIX concentration of 0.1 mg/L. The cells were further incubated for 18 h at 30 °C. After the cells were harvested, the pellets were frozen at −80 °C.

Protein purification: Cell pellets were thawed at room temperature, suspended in 5 mL TRIS 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) with a Qsonica Q125 sonicator. Cell debris was removed by centrifugation (10000 rpm, 45 min, 4 °C). The supernatants were briefly incubated with Ni-NTA agarose (50% suspension, 5 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 eluted 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). UV−vis spectroscopy was performed using a NanoDrop 2000c (Thermo Scientific).

ICP-MS/ICP-OES analysis: Conc. HNO_3 was added to each sample (protein, supernatant, or cell debris) to make a final concentration of 4 v/v%. After microwave digestion (140 ºC, 30 min), the samples were analyzed by ICP-MS (Elemental Analysis Core, Oregon Health & Science University) or ICP-OES (Microanalytical Facility, College of Chemistry, UC Berkeley)

8. Cell growth with different concentrations of Ir(Me)MPIX

Plasmid 3-105-3 (containing CYP119 genes) was transformed into chemically competent Nissle 1917 cells. Individual colonies from freshly transformed plates were inoculated into 4 mL M9-rich media supplemented with 50 μg/mL chloramphenicol. The cultures were grown overnight at 37 °C and 250 rpm for 18 h. In a 50 mL centrifuge tube, 10 mL of M9-rich medium was inoculated with 25 μL of overnight starter culture. To the cultures were added 10 μL of solutions of Ir(Me)MPIX in DMSO varying in concentration (0, 0.1, 0.5, 1 mg/mL) to give final concentrations of 0, 0.1, 0.5, 1 mg/L Ir(Me)MPIX. The cell cultures were incubated at 37 °C and 250 rpm, and the OD_{600} values were recorded every hour using NanoDrop 2000c (Thermo Scientific).

9. Whole-cell reactions

The cell suspension in M9-N (300 μL, OD_{600} ~30 for Nissle 1917, OD_{600} ~20 for BL21(DE3)) was transferred into a 4 mL screw-capped glass vial, and 2 μmol substrate (4 μL of 0.5 M stock solution in DMSO) and 16 μmol EDA (16 μL of 1 M stock solution in DMSO) were added. The vials were capped and shaken at 30 °C and 250 rpm for 4 h. The reaction was quenched by adding 50 μL of 2 M HCl, followed by 300 μL ethyl acetate (containing 0.1 v/v% dodecane as internal standard). The mixture was vortexed for 10 s, transferred to a 1.7 mL microcentrifuge tube, and centrifuged at 10000 rpm for 4 min. After separation of the layers, the organic layer was transferred to a separate vial for GC analysis.

Whole-cell screening with 96-well plates: The CYP119 gene library was transformed into $CaCl_2$ competent Nissle 1917 cells. Individual colonies were picked, arrayed into 96-deep-well plates (2 mL) and grown in 1 mL M9-rich medium with 50 μg/mL chloramphenicol. Cells were grown overnight at 37 °C, 250 rpm. Aliquots (10 μL) of overnight cultures were transferred into new 96-deep-well plates (2 mL) containing 1 mL M9-rich medium in each well. The plate was incubated at 37 °C, 250 rpm for 3 h to a final OD_{600} of 0.6~1.0. The gene expression was induced by adding 2 μL of anhydrotetracycline hydrochloride stock solution (0.1 mg/mL in EtOH) and 2 μL of Ir(Me)MPIX stock solution (0.05 mg/mL in DMSO), and the cultures were allowed to grow for another 20 h at 30 °C, 250 rpm. Cells were then pelleted (4000 rpm, 15 min, 4 °C) and resuspended in 250 μL M9-N reaction buffer. To each well of the plate, (+)-nootkatone (4 μL, 0.5 M in DMSO) and EDA (10 μL, 1 M in DMSO) stock solutions were added using a multi-channel pipette. The plate was then sealed with aluminum film and shaken at 30 °C, 250 rpm for 4 h. The reactions were quenched by adding 50 μL of 2 M HCl, followed by 250 μL ethyl acetate (containing 0.1 v/v% dodecane as internal standard). The suspensions were carefully mixed by pipetting up and down using a multi-channel pipette. The plate was then centrifuged at 4000 rpm for 15 min and frozen at -80 °C overnight. The organic layers were transferred to a 96-well plate assembled with glass vials for GC analysis.

10. 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 (0.45 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 substrate in DMSO 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 HCl (50 μL, 2 M) and extracted with 1 mL dodecane stock solution (0.1 v/v% in EA). The organic layer was then transferred to a separate vial for GC analysis.

11. Nissle 1917 gene disruption

The gene knockouts in Nissle 1917 were performed with a lambda red recombineering system.[4] EcN cells carrying a pKD46 plasmid were grown in 10 mL LB medium with 100 μg/mL carbenicillin to an OD_{600} of 0.2~0.3 at 30 °C. The cell culture was supplemented with 5 mM L-arabinose and further incubated at 30 °C to a final OD_{600} of ~0.6. The culture was then made electrocompetent by concentrating 10-fold, washing four times with ice-cold 10% glycerol and freezing at -80 °C in 300 μL ice-cold 10% glycerol. A kanamycin resistance gene surrounded by FRT sites was amplified from plasmid pKD13 by using primers with 50 bp tails that have homology with the target genes on EcN. PCR products were digested with DpnI and gel-purified. The electroporation was performed by using a MicroPulser electroporator (BIO-RAD) with 50 μL of cells and 100 ng of PCR product. Shocked cells were added to 1 mL SOC medium, incubated for 1.5 h at 37 °C, concentrated to 100 μL and spread onto agar to select kanamycin-resistant transformants. To remove pKD46, the colonies were streaked on LB agar plate with no antibiotics and incubated at 42 °C. The individual colonies were then picked and tested that they were sensitive to ampicillin. After removal of pKD46, the mutant EcN strain was transformed with plasmid pCP20 and cured of kanamycin marker. The plasmid pCP20 was removed in the same way as pKD46, and the cells were sensitive to all antibiotics.

	Primer sequence (5'-3')
*ΔchuA*_for	ATTGTCTCTCTTCCTTCCAGATAAATGCCCCGAAATATCGGGGCAGATG Aattccggggatccgtcgacc
*ΔchuA*_rev	GTATTAACCCAGGCAGCCAGCATTTTTTATTTTCCTCATGGAGAATCGCTt gtaggctggagctgcttcg
*ΔchuUchuV*_for	TAACGGAGTTTTGCGTGCTCAAGGATTCACTCTCTTCCGCCAGGGTCTT Tgtgtaggctggagctgcttc
*ΔchuUchuV*_rev	CTGTATAGTTATTTATTCGCAGGAGTACCAGTGCGGTACTCCTGTTGGTT attccggggatccgtcgacc

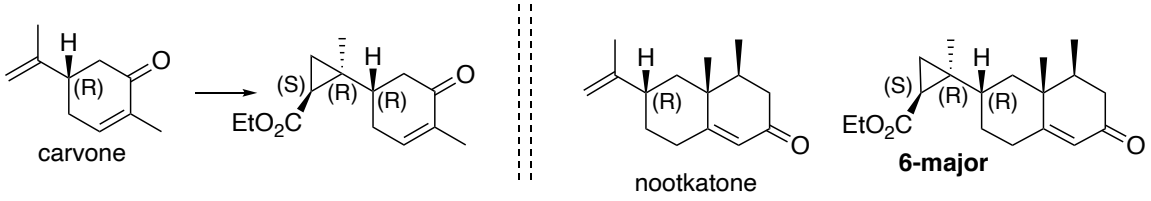
PCR verification: A freshly isolated colony was suspended in 10 μL ddH_2O, boiled at 98 °C for 10 min and used as the template DNA for colony PCR. The test primers for *ΔchuA* strain are p1 (GAACTATCGCCATCATCGACTTTGTCG) and p2 (GTATTAACCCAGGCAGCCAGCA), with which the new mutant-specific fragment occurred at ~1.4 kb after gel analysis of the PCR product. The test primers for *ΔchuUchuV* strain are p3 (GCTGATGATGATCATGCGTAACGC) and p4 (AGCTAATCTGTATAGTTATTTATTCGCAGGAGTAC), with which the new mutant-specific fragment occurred at ~1.5 kb after gel analysis of the PCR product.

12. General procedure for the synthesis of authentic products

The synthesis of **2**, **4a-4d** follows the reported procedure.[5]

*Synthesis of cyclopropyl nootkatone **6**:* To a solution of nootkatone (0.2 M) and $Rh_2(OAc)_4$ (0.5 mol% with respect to EDA) 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 85:15 gradient) as the eluent. Fractions of the pure product(s) were combined, and the solvent evaporated, yielding cyclopropanation products (a mixture of four diastereomers, 35% yield).

To isolate the major diastereomeric product from Ir-CYP119-catalyzed reactions, 32 mg of Ir-CYP119 mutant (P+282L/152N/153I/256S, CYP119:Ir(Me)MPIX = 2.5:1) was prepared in TRIS buffer (20 mM, pH 7.5, 72 mL). A solution of nootkatone (1 mL, 100 mM solution in EtOH:DMSO 4:1) was added to the catalyst solution. To the mixture, a solution of EDA (3 mL, 267 mM in EtOH) was slowly added via syringe pump over 3 h. The reaction was stirred at room temperature for 6 h, quenched by adding HCl solution (5 mL, 1 M) and extracted with ethyl acetate (3×50 mL). The organic phase was concentrated and dried The pure product (10 mg, 33%) was isolated by preparative TLC (20% ethyl acetate in hexane). The configurations of the new stereogenic centers in the product cannot be determined by NMR spectroscopy due to free rotation about the inter-ring C-C bond. However, if the configurations at the two new centers are controlled by the enzyme with the same sense of chirality as for carvone shown below,[1] then structure **6-major** below would be the major stereoisomer.



^{1}H NMR (400 MHz, $CDCl_3$) δ 5.74 (s, 1H), 4.25 – 4.05 (m, 2H), 2.49 (dddd, J = 15.4, 13.4, 5.1, 2.0 Hz, 1H), 2.37 (ddd, J = 15.0, 4.3, 2.4 Hz, 1H), 2.27 – 2.16 (m, 2H), 1.98 (dqd, J = 13.4, 6.8, 5.0 Hz, 1H), 1.87 (dt, J = 12.6, 3.1 Hz, 1H), 1.83 – 1.76 (m, 1H), 1.59 – 1.49 (m, 2H), 1.49 – 1.39 (m, 1H), 1.29 (t, J = 7.1 Hz, 3H), 1.26 – 1.19 (m, 1H), 1.16 (dd, J = 5.6, 4.5 Hz, 1H), 1.00 (s, 3H), 0.97 (s, 3H), 0.94 – 0.86 (m, 4H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 199.69, 172.37, 170.84, 124.68, 60.41, 42.17, 42.02, 40.46, 39.26, 34.37, 33.25, 30.19, 29.95, 27.28, 21.89, 19.42, 16.69, 14.82, 14.51.

IR (neat, cm^{-1}): 2969, 2936, 1721, 1669, 1175.

HRMS (ESI) calc. for $C_{19}H_{28}O_3$: m/z= 304.2038, found: m/z=304.2041.

II. Supplementary figures and tables

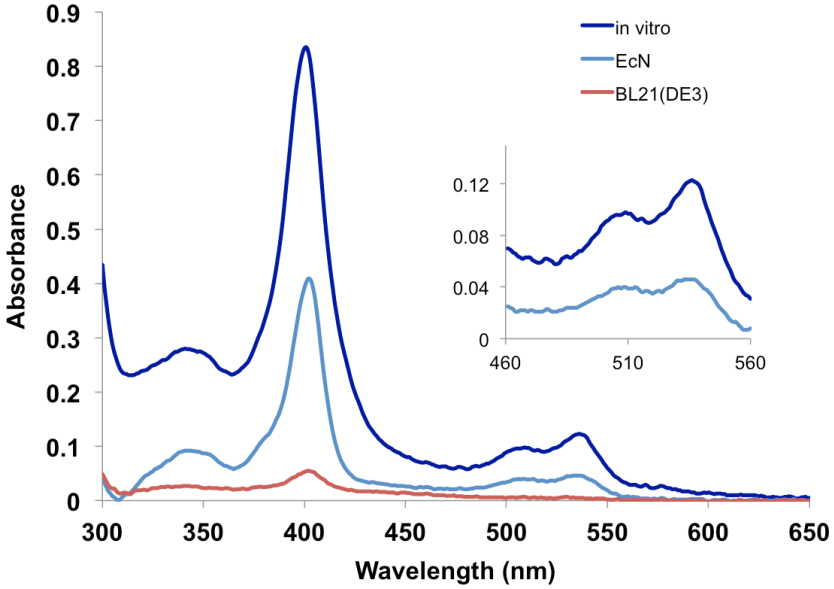
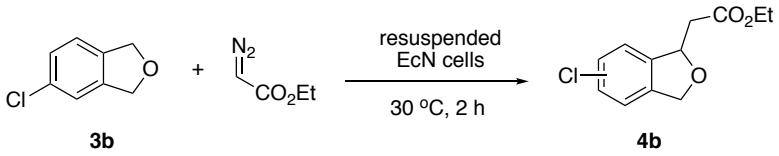
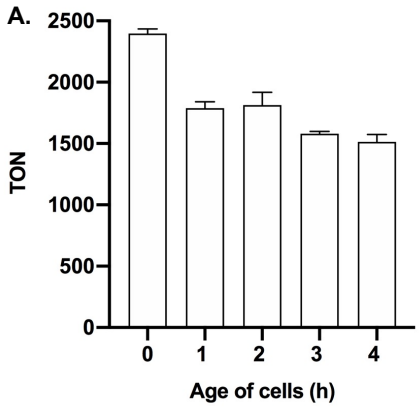
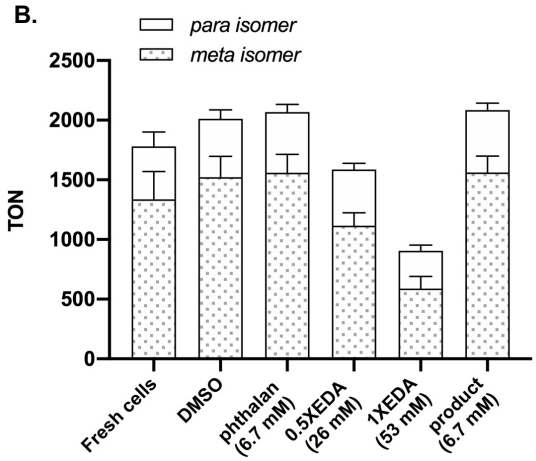


Figure S1. UV-vis spectra of Ir-CYP119 (from top to bottom): reconstituted *in vitro*; purified from Nissle 1917, [CYP119] = 0.4 mg/mL; purified from BL21(DE3), [CYP119] = 0.4 mg/mL. For *in vivo* assembly of Ir-CYP119, *E. coli* cultures were supplemented with 0.5 ppm Ir(Me)MPIX during protein expression.







C.

CFU	Replicate 1	Replicate 2	Replicate 3	Average
H_2O	31	37	42	37±6
DMSO	29	28	22	26±4
26 mM EDA	4	2	3	3±1
53 mM EDA	0	0	0	0

Figure S2. (A) The C-H activation of 4-chlorophthalan **3b** catalyzed by resuspended cell pellets. *Compared with Fig. 3b, lower concentration of EDA led to a higher reactivity of resuspended cell pellet (8 μmol EDA vs. 16 μmol EDA in Fig. 3b).* The suspension of EcN cells harboring Ir-CYP119 (C317G, A152L, T213G, P252S, V254A) was treated with phthalan **1** (2 μmol), and EDA (8 μmol), DMSO (20 μL) and incubated at 30 °C, 300 rpm for 0-4 h. The reaction mixture was centrifuged at each one-hour interval. The cells obtained after centrifugation were resuspended in fresh M9-N buffer and used for a second round of catalysis. Reaction conditions: Resuspended EcN cell pellets, 4-chlorophthalan **3b** (1 μmol), EDA (8 μmol), DMSO (10 μL), M9-N (300 μL), 30 °C, 2 h. All data from whole-cell reactions are shown as the average from three biological replicates, with error bars indicating 1 standard deviation. (B) The C-H activation of 4-chlorophthalan **3b** catalyzed by resuspended cell pellets. The suspension of EcN cells (OD_{600} ~30) harboring Ir-CYP119 (C317G, A152L, T213G, P252S, V254A) was treated with different reagents

and incubated for 2 h (30 °C, 300 rpm). From left to right, 1) none; 2) 20 µL DMSO; 3) 20 µL of 0.1 M phthalan **1** stock solution in DMSO; 4) 20 µL of 0.4 M EDA stock solution in DMSO; 5) 20 µL of 0.8 M EDA stock solution in DMSO; 6) 20 µL of 0.1 M stock solution of **2** (C-H activation product). The cells obtained after centrifugation were resuspended in fresh M9-N buffer and used for a second round of catalysis. Reaction conditions: Resuspended EcN cell pellets, 4-chlorophthalan **3b** (1 µmol), EDA (8 µmol), DMSO (10 µL), M9-N (300 µL), 30 °C, 2 h. All data from whole-cell reactions are shown as the average from three biological replicates, with error bars indicating 1 standard deviation. (C) High concentration of EDA is toxic to EcN cells. The suspension of EcN cells (OD_{600} ~30) harboring Ir-CYP119 (C317G, A152L, T213G, P252S, V254A) was treated with different reagents (20 µL ddH2O, 20 µL DMSO, 20 µL of 0.4 M EDA stock solution in DMSO and 20 µL of 0.8 M EDA stock solution in DMSO, respectively) and the mixtures were incubated at 30 °C, 300 rpm for 4.5 h. The suspension was then diluted by a factor of 2.5×10^6, and aliquots of the diluted mixtures (50 µL) were plated onto LB agar plates. The number of viable cells was estimated by counting the number of colonies formed on the plates. CFU: colony forming unit.

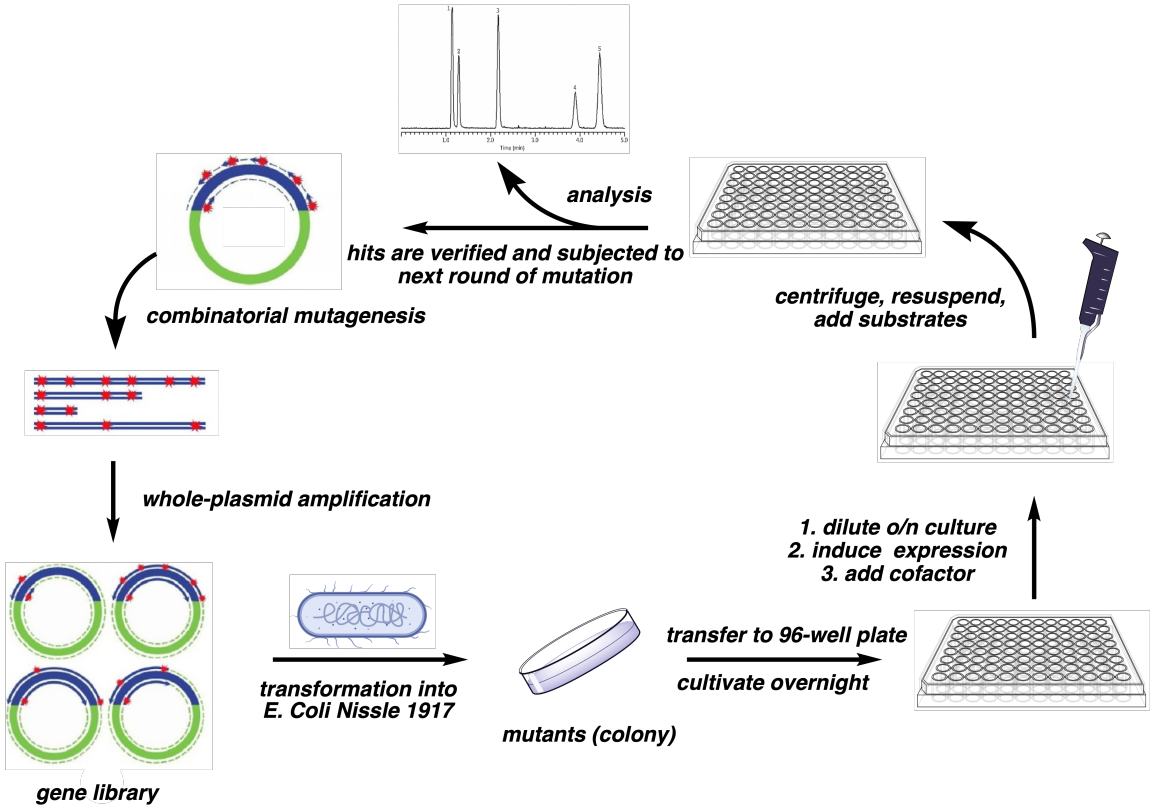


Figure S3. Directed evolution of Ir-CYP119 with Nissle 1917 as a whole-cell screening platform.

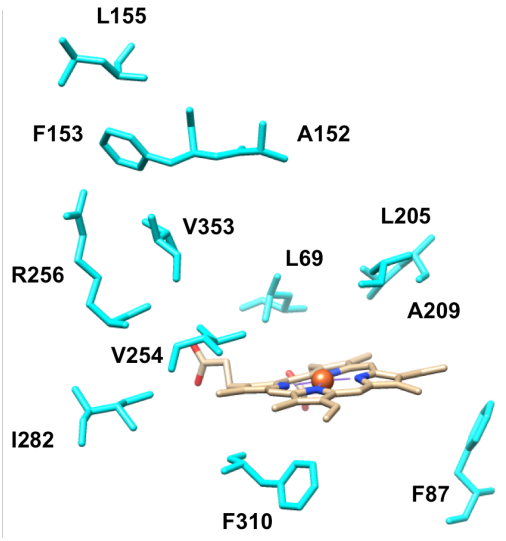


Figure S4. Amino acid residues targeted for screening during the directed evolution of Ir-CYP119.

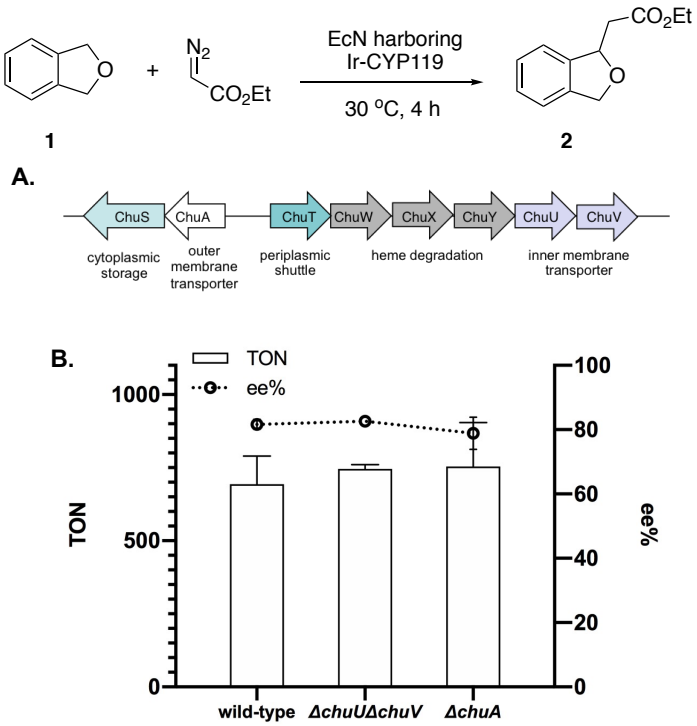


Figure S5. Whole-cell C-H activation of phthalan **1** with knock-out strains of EcN. (A) A graphic presentation of *chu* operon.[6] (B) Reaction conditions: EcN expressing CYP119 mutant (C317G, A152L, T213G, P252S, V254A), OD_{600} ~30, phthalan **1** (2 μmol), EDA (16 μmol), DMSO (20 μL), M9-N (300 μL), 30 °C, 4 h.

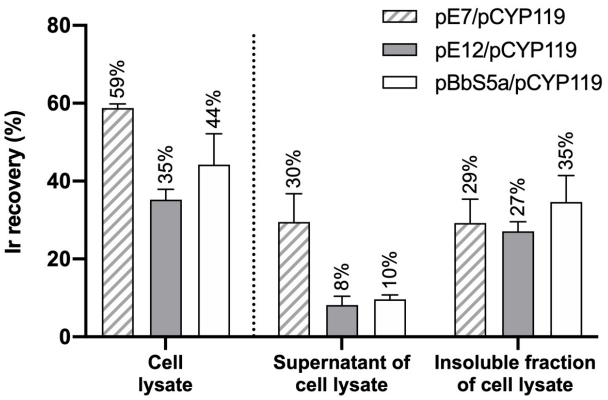


Figure S6. The percentages of added iridium that were recovered in the cell lysates, the supernatants of cell lysates, and the insoluble fractions of cell lysates from BL21(DE3) cells co-expressing CYP119 and outer-membrane transporters. BL21(DE3) cells were co-transformed with a plasmid containing CYP119 genes (pJHA190-1, abbreviated as pCYP119) and a plasmid coding for the transporters (pE7 or pE12), and the protein expression was induced by adding 50 μM IPTG and 0.1 ppm Ir(Me)MPIX. In the control group, BL21(DE3) cells were co-transformed with pCYP119 and an empty vector lacking the transporter genes (pBbS5a), and cultivated under identical conditions. The ICP-OES data are shown as the average from three biological replicates, with error bars indicating 1 standard deviation.

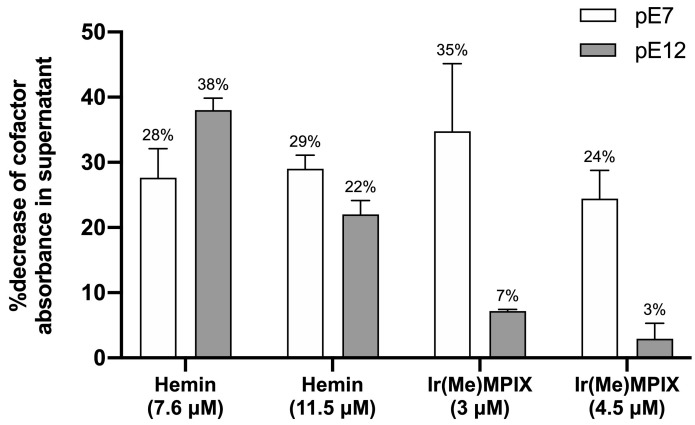
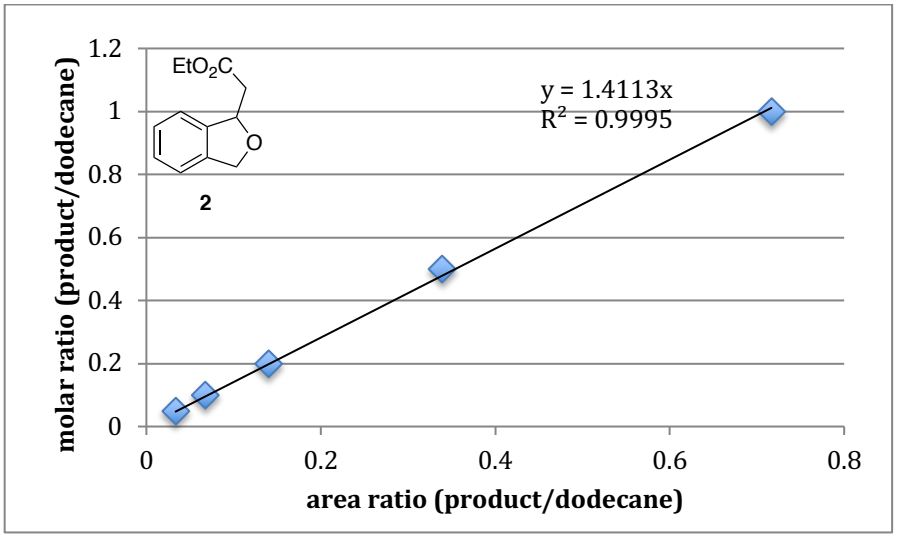


Figure S7. Binding of *E. coli* cells to metalloporphyrins. BL21(DE3) cells expressing pE7 or pE12 and cells containing the empty vector were grown in M9-rich medium. The gene expression was induced by 50 μM IPTG at OD_{600} 0.50~0.55. The cultures were incubated at 30 ºC, 250 rpm for 4 h. Aliquots of cultures (1 mL) were centrifuged, and the harvested cells were resuspended in 950 μL M9-N buffer. Stock solutions of metalloporphyrins in DMSO (50 μL) were added to each set of cell suspensions with a final concentration of 7.6 μM hemin, 11.5 μM hemin, 3 μM Ir(Me)MPIX and 4.5 μM Ir(Me)MPIX, respectively. The mixtures were incubated at 37 ºC for 1 h and centrifuged, and the UV-vis absorbance of the supernatants was measured (395 nm for hemin and 397 nm for Ir(Me)MPIX). Cells containing the vector alone (pBbS5a) were incubated under the same conditions and used as control. The percent decrease of UV absorbance of the supernatant is calculated by setting the absorbance of the control group as 100%.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area ratio (product/dodecane)
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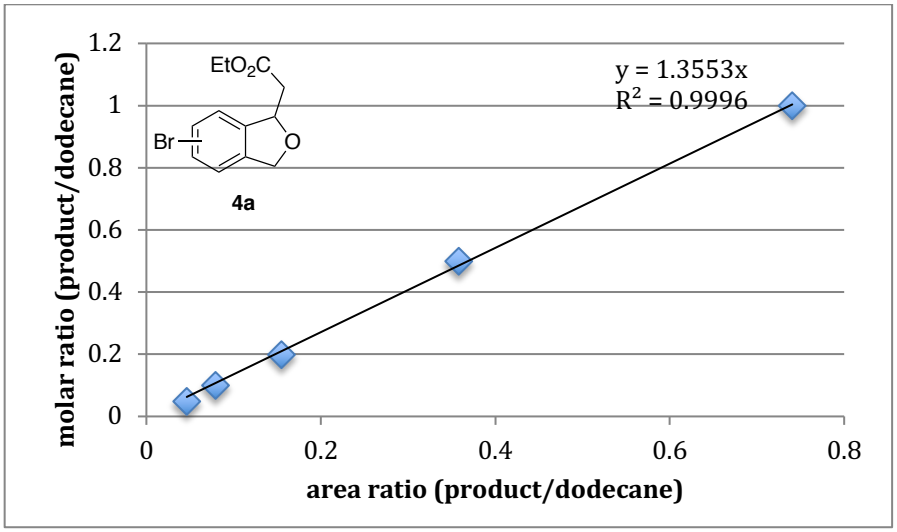
R² = 0.9995
EtO2C
O
2
0
0.2
0.4
0.6
0.8
1
1.2


molar ratio (product/dodecane)
area ratio (product/dodecane)
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R² = 0.9996
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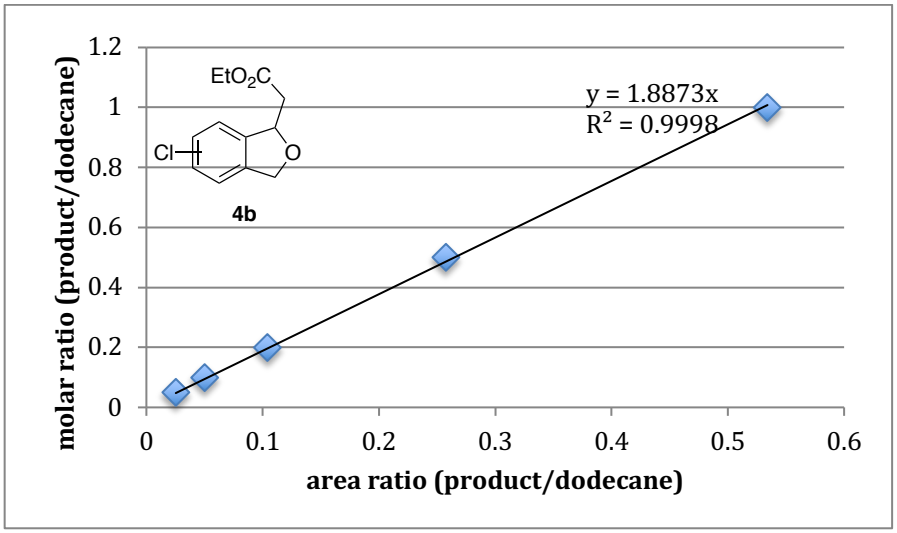
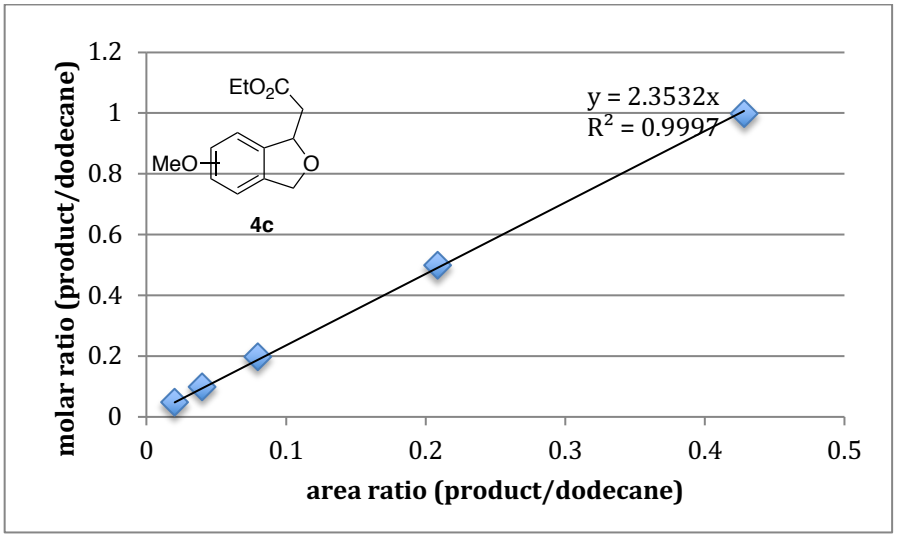
Br
O
4a
0
0.2
0.4
0.6
0.8
1
1.2


molar ratio (product/dodecane)
area ratio (product/dodecane)
y = 1.8873x
R² = 0.9998
EtO2C
Cl
O
4b
0
0.1
0.2
0.3
0.4
0.5
0.6
1
1.2


molar ratio (product/dodecane)
area ratio (product/dodecane)
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R² = 0.9997
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MeO
O
4c
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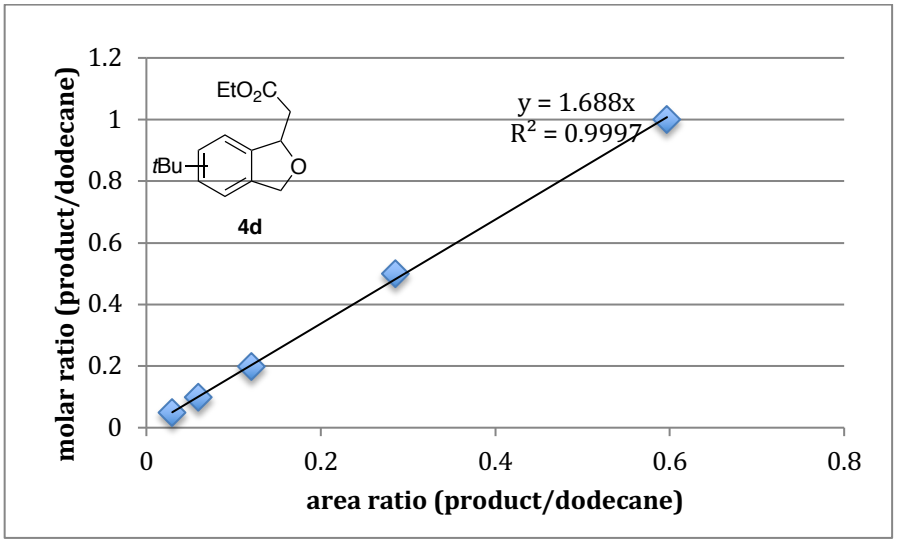
0.2
0.4
0.6
0.8
1
1.2
0.1
0.3
0.5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area ratio (product/dodecane)
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R² = 0.9997
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tBu
O
4d
0
0.2
0.4
0.6
0.8
1
1.2

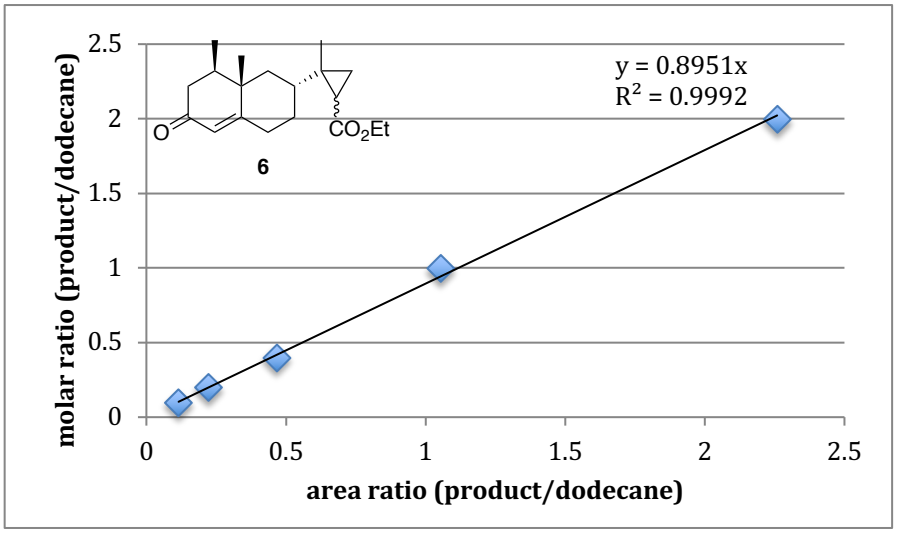

molar ratio (product/dodecane)
area ratio (product/dodecane)
y = 0.8951x
R² = 0.9992
O
CO2Et
6
0
0.5
1
1.5
2
2.5

Figure S8. Calibration curves for GC analysis of compound **2**, **4a**-**4d** and **6**.

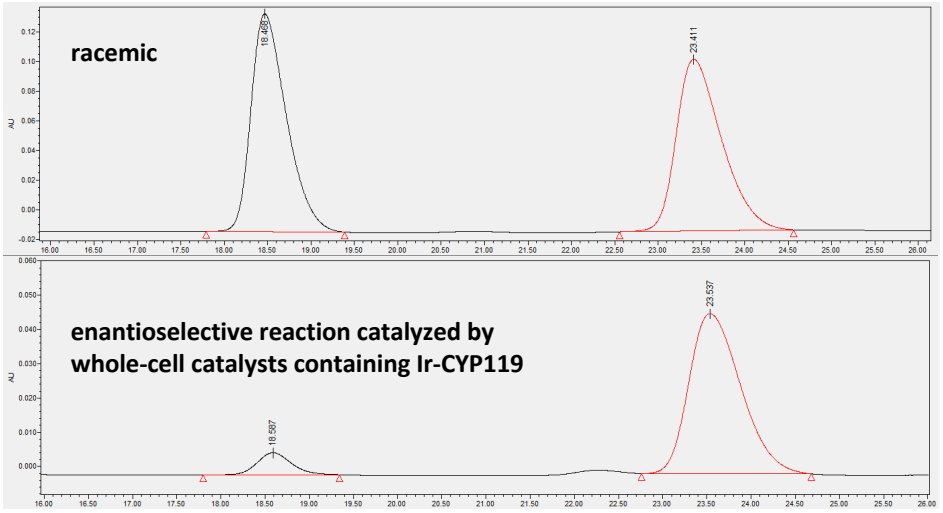
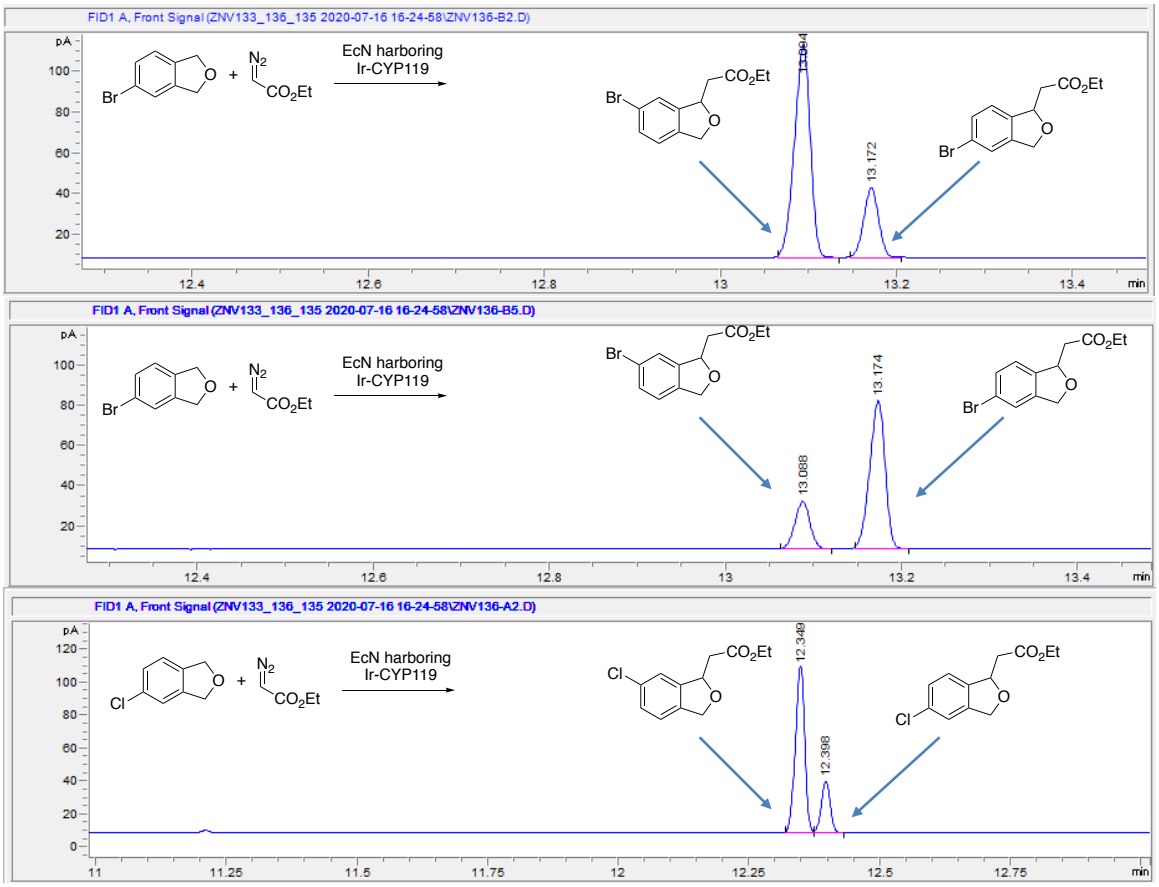


Figure S9. HPLC chromatograms for enantioselective C-H activation of phthalan 1.



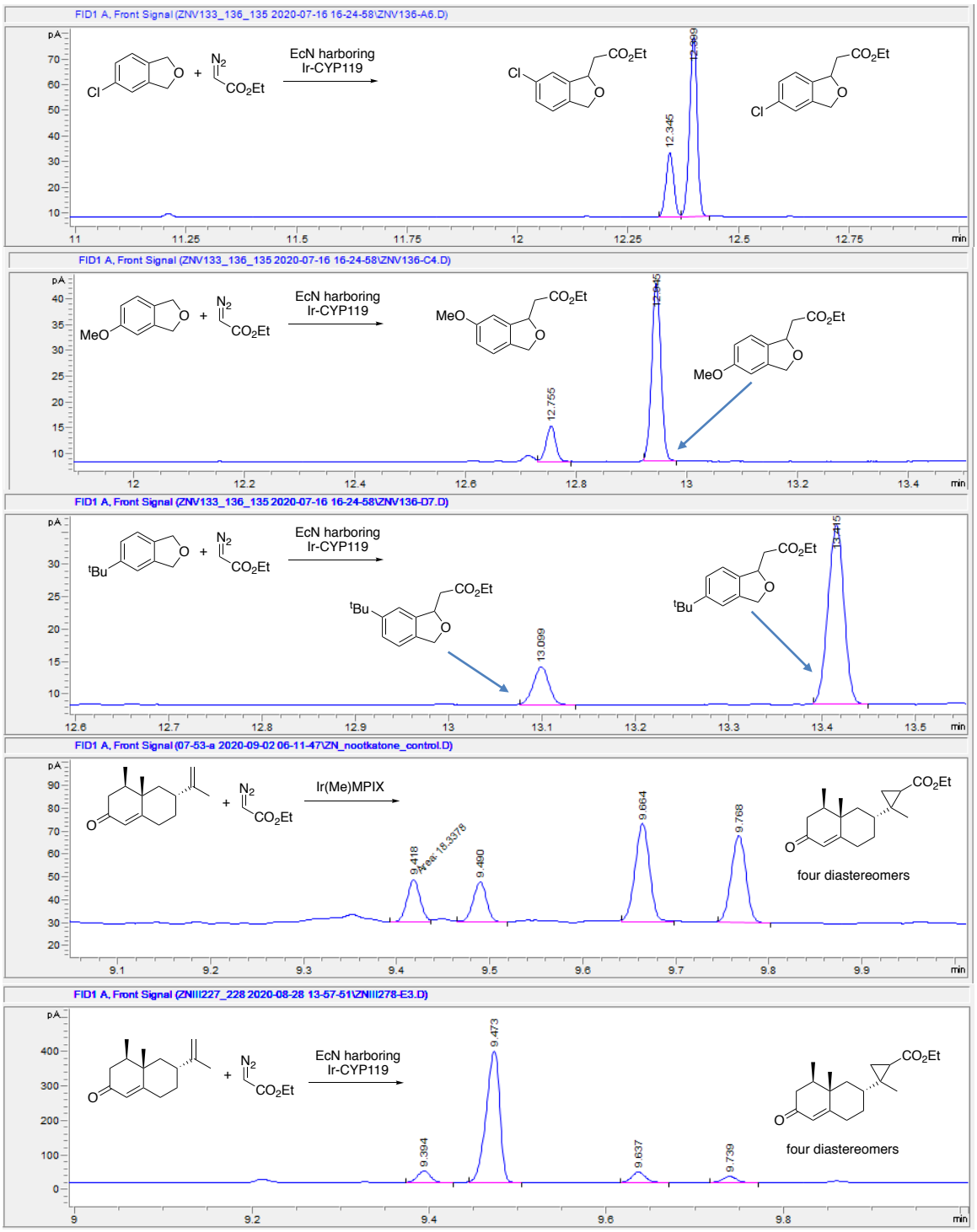


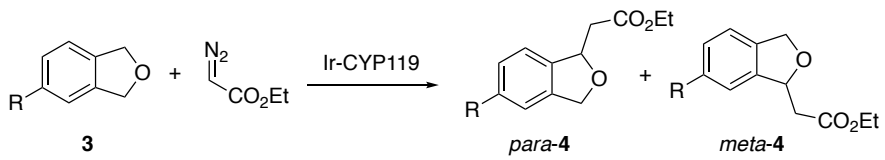
Figure S10. GC chromatograms for site-selective C-H activation of phthalan derivatives 3a-3d and diastereoselective cyclopropanation of (+)-nootkatone 5.

Table S1. The percentage of purified CYP119 proteins that contain the iridium porphyrin.[a]

	EcN		BL21(DE3)	
0.5 ppm Ir(Me)MPIX	Protein yield (mg/L)	%Ir-CYP119[b]	Protein yield (mg/L)	%Ir-CYP119[b]
Replicate 1	3.5	18.4	6.9	2.4
Replicate 2	5.8	14.3	4.7	2.6
Replicate 3	4.3	24.5	4.5	2.9
Average	4.5±1.2	19.1±5.1	5.4±1.3	2.6±0.2

[a]The cell culture (100 mL) was cultivated and the CYP119 was purified following the protocol described in section *7* except that 0.5 mg/L Ir(Me)MPIX was supplemented upon the induction of protein expression. The concentration of Ir in protein samples was measured by ICP-OES at an emission wavelength of 212 nm. [b]The value represents the percentage of CYP119 containing Ir(Me)MPIX in the purified protein sample by assuming that all Ir exists in the form of iridium porphyrin and binds to CYP119.

Table S2. Site-selective C-H activation of 4-substituted phthalans catalyzed by Ir-CYP119 reconstituted *in vitro*.

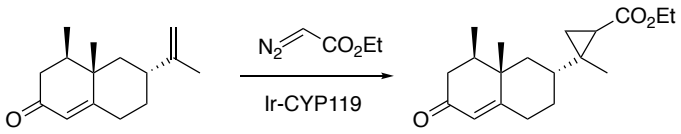


3 *para*-**4** *meta*-**4**

Substrate	Mutant	*para*:*meta*[a]	TON[b]
R=Br (**3a**)	CYP119-P_{L1}	1.7:1	201
R=Br (**3a**)	CYP119-M_{L1}	1:2.2	172
R=Cl (**3b**)	CYP119-P_{L1}	2.0:1	279
R=Cl (**3b**)	CYP119-M_{L1}	1:1.6	224
R=OMe (**3c**)	CYP119-P_{L1}	3.9:1	167
R=^{t}Bu (**3d**)	CYP119-P_{L2}	6.0:1	132

Reaction condition: 5 mM phthalan 70 mM EDA (14 equiv., slow addition over 1.5 h), 0.1 mol% Ir-CYP119, 1 mL NaPi buffer (100 mM NaPi, 100 mM NaCl, pH 6). [a]The site-selectivity was analyzed by GC-FID. [b]The TONs were measured with dodecane as internal standard.

Table S3. Diastereoselective cyclopropanation of (+)-nootkatone catalyzed by Ir-CYP119 reconstituted *in vitro*.



CYP119 mutant	Selectivity[a]				Yield[b]	TON
	P_1	P_2	P_3	P_4		
P/282L/152N/153I	10%	56%	18%	17%	43%	134
P/282L/152N/155L	9%	56%	18%	16%	38%	117
P+282L/152N/153I/254A/256S	8%	70%	13%	10%	46%	145

Reaction condition: 1.25 mM alkene, 6.25 mM EDA (5 equiv., slow addition over 1.5 h), 0.32 mol% Ir-CYP119, 1 mL TRIS buffer (20 mM, pH 7.5). [a]The diastereoselectivity was analyzed by GC-FID. [b]The yields were measured with dodecane as internal standard.

Table S4. Protein sequences of outer-membrane receptors on Nissle 1917 that share homology with ChuA.

Plasmid	Query cover	%Identity	Accession number	Description
pE1	94%	24.96%	WP_000489277.1	catecholate siderophore receptor CirA
pE2	21%	30.18%	WP_001034892.1	siderophore enterobactin receptor FepA
pE3	12%	35.71%	WP_001304440.1	TonB-dependent receptor
pE5	21%	33.11%	WP_001305033.1	TonB-dependent vitamin B12 receptor BtuB
pE6	51%	30.53%	WP_001704245.1	TonB-dependent receptor
pE7	33%	30.34%	WP_000966628.1	TonB-dependent receptor
pE8	21%	29.27%	WP_001222189.1	siderophore salmochelin receptor IroN
pE10	45%	26.92%	WP_000973516.1	ferric aerobactin receptor IutA
pE11	26%	33.33%	WP_024199380.1	bifunctional siderophore receptor/adhesin Iha
pE12	100%	100%	WP_000089583.1	TonB-dependent heme/hemoglobin receptor ChuA
pE13	38%	30.71%	WP_000784549.1	siderophore yersiniabactin receptor FyuA
pE14	46%	27.95%	WP_000430015.1	catecholate siderophore receptor Fiu
pE15	69%	30.67%	WP_001240663.1	TonB-dependent siderophore receptor

Table S5. Protein sequence alignment of known *E. coli* outer-membrane transporters with the target protein (E7).

UniProt ID	Protein name	E-value	Score	Percent identity
P17315	Colicin I receptor	1e-33	355	25.8%
P05825	Ferrienterobactin receptor	1.9e-23	273	23.9%
Q1R3U1 Q0TA90 A1AIE8	Vitamin B12 transporter BtuB	1.6e-17	223	32.3%
Q8X714	Vitamin B12 transporter BtuB	2.9e-17	221	31.9%
P76115	Probable TonB-dependent receptor YncD	3.4e-17	221	20.9%
P06129 A8A774 A7ZUI6	Vitamin B12 transporter BtuB	3.8e-17	220	31.9%
Q8CVJ0	Vitamin B12 transporter BtuB	5e-17	219	31.9%
Q93SE0	Vitamin B12 transporter BtuB	7e-14	193	29.8%
P14542	Ferric aerobactin receptor	2.2e-12	181	22.6%
Q1REC0 P75780 Q8CW90 Q8X7W7	Catecholate siderophore receptor Fiu	1.5e-5	124	29.5%
P13036	Fe(3+) dicitrate transport protein FecA	5.7e-5	119	26.8%
P06971	Ferrichrome outer membrane transporter/phage receptor	2.9e-4	113	26.6%
P16869	FhuE receptor	8.1e-1	84	23.8%

Table S6. Methods used to separate enantiomers, constitutional isomers or diastereomers of the reaction products.

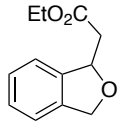
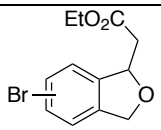
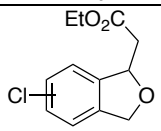
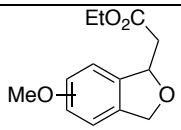
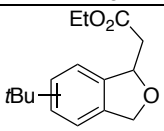
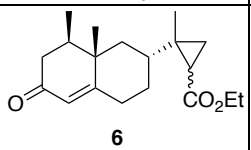
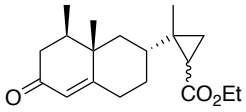
Products	Instrument	Column	Method	Retention Times
 **2**	HPLC	CHIRALCEL OD-H (5μm, 4.6mm×250mm)	98% hexane, 2% isopropyl alcohol, 1 mL/min	t_1=18.5 min; t_2=23.5 min
 **4a**	GC	HP-5 (25m×200μm×0.33μm)	100 °C, 3 min; 100 °C to 300 °C, ramp=14 °C/min; 300 °C, 4 min	t_1=13.09 min; t_2=13.17 min
 **4b**	GC	HP-5 (25m×200μm×0.33μm)	100 °C, 3 min; 100 °C to 300 °C, ramp=14 °C/min; 300 °C, 4 min	t_1=12.35 min; t_2=12.40 min
 **4c**	GC	HP-5 (25m×200μm×0.33μm)	100 °C, 3 min; 100 °C to 300 °C, ramp=14 °C/min; 300 °C, 4 min	t_1=12.76 min; t_2=12.94 min
 **4d**	GC	HP-5 (25m×200μm×0.33μm)	100 °C, 3 min; 100 °C to 300 °C, ramp=14 °C/min; 300 °C, 4 min	t_1=13.10 min; t_2=13.42 min
 **6**	GC	HP-5 (25m×200μm×0.33μm)	100 °C, 3 min; 100 °C to 300 °C, ramp=40 °C/min; 300 °C, 5 min	t_1=9.39 min; t_2=9.47 min; t_3=9.64 min; t_4=9.74 min

Table S7. Nonlinear fit of Nissle 1917 growth curves into Gompertz growth model.[a]
($Y = Y_M*(Y_0/Y_M)^{(exp(-K*X))}$)

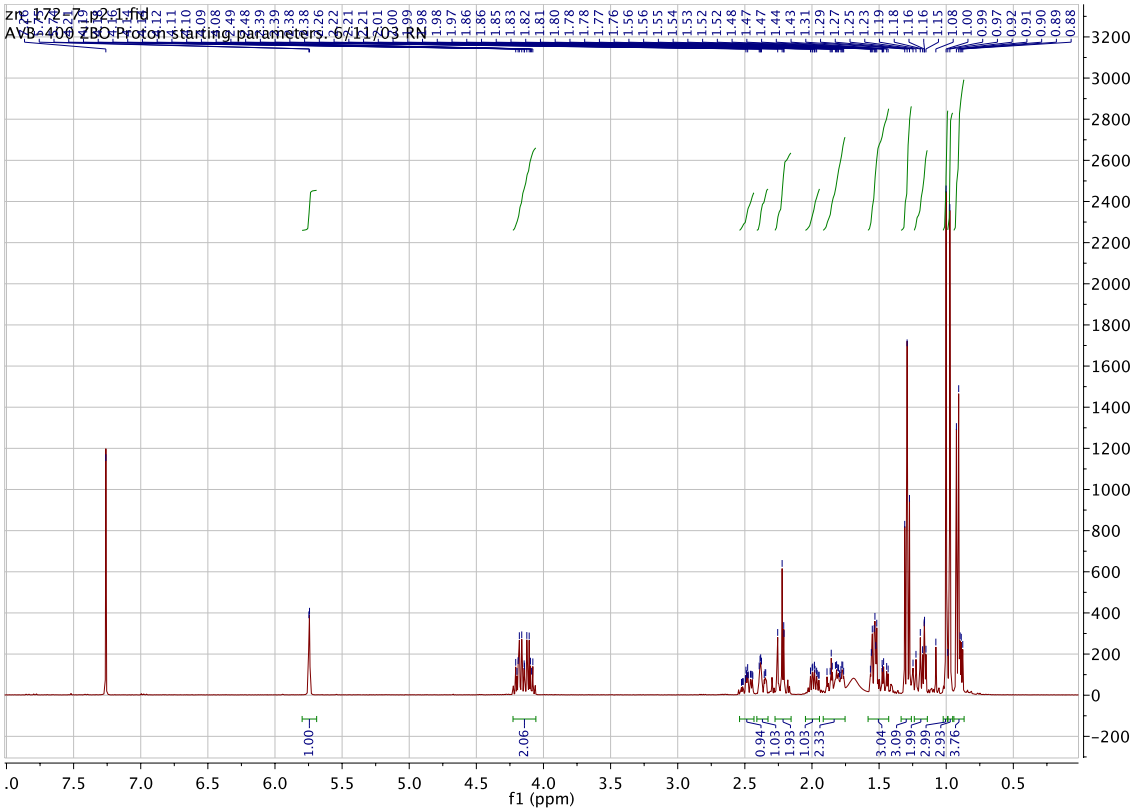
	0 ppm	0.1 ppm	0.5 ppm	1 ppm
Gompertz growth				
Best-fit values				
Y_M	2.461	2.494	2.624	2.582
Y_0	5.79E-05	9.37E-05	8.40E-06	7.30E-07
K	0.513	0.4845	0.5137	0.5523
1/K	1.949	2.064	1.947	1.811
95% CI (profile likelihood)				
Y_M	2.377 to 2.555	2.400 to 2.602	2.566 to 2.688	2.536 to 2.631
Y_0	2.657e-006 to 0.0004927	5.291e-006 to 0.0007018	8.871e-007 to 4.996e-005	5.915e-008 to 5.540e-006
K	0.4600 to 0.5720	0.4330 to 0.5415	0.4802 to 0.5494	0.5210 to 0.5854
1/K	1.748 to 2.174	1.847 to 2.309	1.820 to 2.083	1.708 to 1.920
Goodness of Fit				
Degrees of Freedom	30	30	30	30
R squared	0.9945	0.9943	0.998	0.9985
Sum of Squares	0.1468	0.1491	0.06106	0.04566
Sy.x	0.06996	0.07051	0.04511	0.03901
Constraints				
Y_0	$Y_0 > 0$	$Y_0 > 0$	$Y_0 > 0$	$Y_0 > 0$
K	K > 0	K > 0	K > 0	K > 0
Number of points				
# of X values	33	33	33	33
# Y values analyzed	33	33	33	33

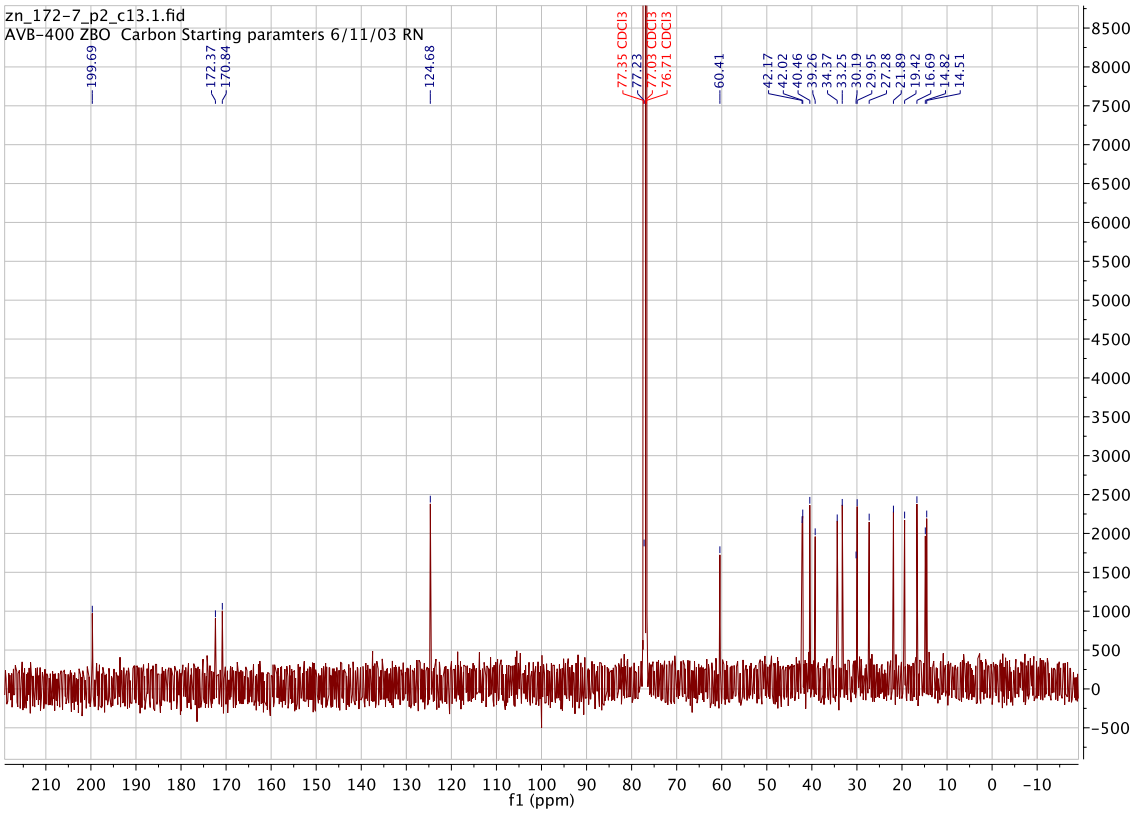
[a] Fits were performed with GraphPad Prism. GraphPad, www.graphpad.com (accessed December 10, 2021) version 8.0.0 for macOS, GraphPad Software, San Diego, California USA

III. NMR Spectra



6





IV. References

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