Supporting Information

Highly Stereoselective Biocatalytic Synthesis of Key Cyclopropane Intermediate to Ticagrelor

Kari E. Hernandez[†], *Hans Renata*[†,§], *Russell D. Lewis*[‡], *S. B. Jennifer Kan*[†], *Chen Zhang*[¶], *Jared Forte*[¶], *David Rozzell*[¶], *John A. McIntosh*[†,#], *and Frances H. Arnold*[†,‡,*]

[†]Division of Chemistry and Chemical Engineering and [‡]Biology and Biological Engineering, California Institute of Technology, Pasadena, CA, 91125, United States

[§]Current address: Department of Chemistry, Scripps Research Institute, Jupiter, FL, 33458, United States

[¶]Provivi, Inc., Santa Monica, CA, 90404, United States

[#]Current address: Merck, Kenilworth, NJ, 07033, United States

Materials and Methods

All chemicals and reagents, unless otherwise stated, were purchased from chemical suppliers (Sigma-Aldrich, Fischer Scientific, Combi-Blocks) and used without purification. Silica gel chromatography purifications were carried out using AMD Silica Gel 60, 230-400 mesh. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Prodigy 400 MHz instrument and are internally referenced to the residual solvent peak (chloroform). Data for ^{1}H NMR are reported in the conventional form: chemical shift (δ ppm), integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (Hz). Data for ^{13}C are reported in terms of chemical shift (δ ppm). High-resolution mass spectra were obtained with a JEOL JMS-600H High Resolution Mass Spectrometer at the California Institute of Technology Mass Spectral Facility. Sonication was performed using a Qsonica Q500 sonicator. Chemical reactions were monitored using thin layer chromatography (Merck 60 silica gel plates) and a UV-lamp for visualization.

Plasmid pET22b was used as a cloning vector, cloning was performed using Gibson assembly.[1] Cells were grown using Luria-Bertani medium or HyperBroth (AthenaES) with 100 μg/mL ampicillin (LB_{amp} or HB_{amp}). Primer sequences are available upon request. Electrocompetent *Escherichia coli* cells were prepared following the protocol of Sambrook *et al.*[2] T5 exonuclease, Phusion polymerase, and *Taq* ligase were purchased from New England Biolabs (NEB, Ipswich, MA). M9-N minimal medium (pH 7.4) was used as a buffering system for whole cells. Unless otherwise specified, M9-N minimal medium was used without a carbon source, and contains 47.7 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 8.6 mM NaCl, 2.0 mM $MgSO_4$, and 0.1 mM $CaCl_2$.

General Procedures

Cloning plasmids and library generation. A codon-optimized gBlock of *B. subtilis* truncated globin (UniProt ID: O31607)[3] was ordered from Integrated DNA Technologies (IDT) and used for library construction and reaction testing. The pET22b plasmid was used as a cloning vector. Site-saturation mutagenesis was performed using primers bearing the desired mutations using degenerate codons (NDT, VHG, TGG) ordered from IDT and used per the company's instructions. Generated sequences were sequenced by Laragen using primers specific to the T7 promotor. Unless noted otherwise, E. cloni® Express BL-21 (DE3) cells were used for cloning. The other two strains used in these sets of experiments were the OverXpress™ C41 (DE3) and OverXpress™ C43 (DE3) lines (all from Lucigen). Electroporation was carried out in 2 mm electroporation cuvettes using the Gene Pulser XCell Electroporation System (Bio-Rad).

Hemochrome assay. At room temperature, 1.75 mL pyridine was combined with 0.75 mL of 1M NaOH. The solution was vortexed for 30 seconds to ensure complete mixing then centrifuged for 1 minute at 5000 rpm to remove excess base from the pyridine solution. 0.75 mL of protein lysate in M9-N minimal medium was combined with 0.25 mL of the pyridine solution along with about 2.0 mg of sodium dithionite, the cuvette's contents were mixed with a pipet and sealed with parafilm. The content's absorbance was immediately read. The protein concentration was determined using an extinction coefficient of 196 mM^{-1} cm^{-1} at 420 nm. Baseline absorbance was determined through extrapolation of two points on either side of the hemochrome peak at 390 and 450 nm.

Growth and induction in flasks. Overnight cultures were prepared in LB_{amp} medium (100 μg/mL ampicillin) from glycerol stocks of *B. subtilis* truncated globin variants. The overnight cultures were incubated at 37 °C for 12 hours. Flasks containing HB_{amp} medium (100 μg/mL ampicillin) were inoculated with 1% volume of the overnight culture. Flasks were incubated at 37 °C until they reached OD_{600}=0.6-0.8. Flasks were cooled on ice for 20 minutes, then induced with 0.5 mM final concentration isopropyl β-D-1-thiogalactopyranoside (IPTG) and 1 mM final concentration 5-aminolevulinic acid (ALA). Flasks were incubated at 25 °C for 18-24 hours. Cells were harvested via centrifugation at 4000 rpm, 10 minutes, at 4 °C.

Small-scale biocatalytic reactions. Small-scale (400 μL) reactions took place in 2 mL glass crimp vials. Whole cells expressing the enzyme were resuspended in M9-N minimal medium to the OD_{600} specified in the reaction conditions. The headspace of the vials was purged during gas cycling of an anaerobic chamber. The open vials were allowed to shake in the anaerobic chamber for one minute before reagents were added. 10 μL of 3,4-difluorostyrene (DFS, either 800 mM or 2 M in ethanol) was added to the reactions followed by 10 μL of ethyl diazoacetate (EDA, either 1.6 M or 4 M in ethanol). Vials were crimp-sealed with silicone septa and removed from the anaerobic chamber and allowed to shake at 400 rpm at room temperature for 1 hour. After reaction completion, reactions to be screened with gas chromatography (GC) had 0.9 mL cyclohexane and 20 μL acetophenone (40 mM in cyclohexane) added to them. The vials were vortexed for 30 seconds two times and then centrifuged at 13000 rpm for 5 minutes at 20 °C. The supernatant was transferred to a clean 2 mL glass vial for analysis by GC. Reactions to be screened via supercritical fluid chromatography (SFC) had 0.9 ml of cyclohexane added to them then were vortexed twice for 30 seconds and centrifuged at 13000 rpm for 5 minutes at 20 °C. The supernatant was moved to a clean 2 mL glass vial for analysis on the SFC. Enantioselectivity was screened using SFC, while yield and diastereoselectivity were determined by GC. All reactions were performed in triplicate (unless otherwise noted).

Reaction screening in 96-well plates. Using sterile toothpicks, colonies were picked from site-saturation libraries on agar plates and used to inoculate a 96-well deep-well plate containing 400 μL of LB_{amp} media per well. The cultures were incubated at 37 ^{o}C overnight at 180 rpm and 80% relative humidity. After overnight growth, 30 μL of overnight culture was transferred to 1 mL of HB_{amp} media in a fresh 96-well deep-well plate using a multichannel pipette. The plates were incubated at 37 ^{o}C, 180 rpm, 80% relative humidity for 3 hours. The plates were then removed from the incubator and put on ice for 20 minutes. 3 μL of 1 M IPTG and 3 μL of 1 M ALA were added to the 96-well plate using a multichannel pipette. The plates were then incubated at 25 ^{o}C and 180 rpm for 18 hours. Cells were harvested by centrifugation at 4000 rpm, 4 °C for 10 minutes and resuspended in 350 μL M9-N minimal media. In the anaerobic chamber 50 μL of DFS (0.16 M in ethanol) and EDA (0.32 M in ethanol) were added to the whole cells in each well. The plate was sealed with a gas-impermeable membrane and allowed to shake in the anaerobic chamber at room temperature at 400 rpm for 1 hour. The plate was then

removed from the anaerobic chamber, and 1 mL of cyclohexane was added to each well using a multichannel pipette and mixed up and down three times. The plates were spun for 5 minutes at 4000 rpm and 20 ^{o}C to separate the organic and aqueous layers. 600 µL of the organic layer was transferred into a shallow 96-well plate and screened for enantioselectivity on the SFC.

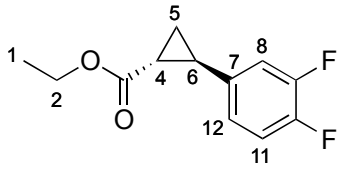
Large-scale biocatalytic reactions

One-pot preparative scale reactions. 19 mL of OD_{600}=80 whole-cell catalyst, resuspended in M9-N minimal medium, was added to a clean 100 mL round bottomed flask. The flask was then sealed with a rubber stopper, covered with parafilm, and the headspace was purged with argon for 10 minutes while the cell mixture was stirred with a 1 cm stir bar at ~100 rpm. The headspaces of a solution of DFS (800 mM in ethanol) and a solution of EDA (1.6 M in ethanol) were purged under argon for 2 minutes. The flask was placed on a stir plate and set to ~100 rpm. 0.5 mL of the DFS solution was added dropwise to the flask through a syringe over a period of ~1 minute. Then 0.5 mL of the EDA solution was added dropwise through a syringe into the flask over a period of ~1 minute. The reaction was allowed to stir for 15 hours at room temperature, after which the reaction was worked up according to procedure described below.

Reactions using slow addition of catalyst and EDA. An empty 100 mL round bottomed flask with a 1 cm stir bar sealed with a rubber stopper and parafilm was purged with argon for 10 minutes. 19 mL of OD_{600}=80 whole-cell catalyst resuspended in M9-N minimal medium was added to a 50 mL conical tube. The head space of the 50 mL conical tube was purged with argon for 10 minutes. The headspaces of a solution of DFS (800 mM in ethanol) and a solution of EDA (1.6 M in ethanol) were purged under argon for 2 minutes. 0.5 mL of the DFS solution was added to the flask. The flask was put onto a stir plate and set to ~100 rpm. 0.5 mL of EDA solution and 19 mL of whole cell-catalyst solution were loaded into separate syringes. Over the next 3 hours the EDA solution was added to the flask at a rate of 0.17 mL/hr, and the cell solution was added at a rate of 6.3 ml/hr to the flask containing the DFS using a syringe pump. The reaction was allowed to continue for 12 hours past the final addition of either catalyst or EDA for a total reaction time of 15 hours at room temperature. The reaction was worked up according to procedure described below.

Workup of large-scale reactions. The 20 mL reaction was poured into a falcon tube. 20 mL of cyclohexane was added to falcon tube. The tube was vortexed for 30 seconds two times and then spun at 5000 rpm and 20 °C for 30 minutes. 1 mL of the cyclohexane supernatant was removed for enantioselectivity analysis on the SFC. 377 µL of acetophenone (40 mM in cyclohexane) was added to the remainder of the reaction mixture, the mixture was again vortexed for 30 seconds two times and then spun at 5000 rpm 20 °C for 15 minutes. The supernatant was analyzed for yield and diastereoselectivity on the GC.

Chemical Synthesis, Characterization and NMR Spectra of Cyclopropane 5



Racemic ticagrelor cyclopropane *rac*-(**5**) was prepared according to the following procedure: To hemin (1.63 g, 2.5 mmol) in water (20 mL) under Ar was added 3,4-difluorostyrene (1.72 g, 10 mmol). Ethyl diazoacetate (2.42 mL, 20 mmol) in DCM (3 mL) was added dropwise to the vigorously stirred mixture, and the reaction was left to stir for 12 h. The reaction was extracted with hexane (20 mL x 3), and the organic extracts were washed with brine (50 mL), dried ($MgSO_4$) and concentrated *in vacuo*. Purification by silica flash chromatography (2 to 20% EtOAc in hexane) separated the desired *trans*-cyclopropane from its *cis*-isomer and gave *rac*-(**5**) as a colorless oil (1.43 g, 6.3 mmol, 63%).

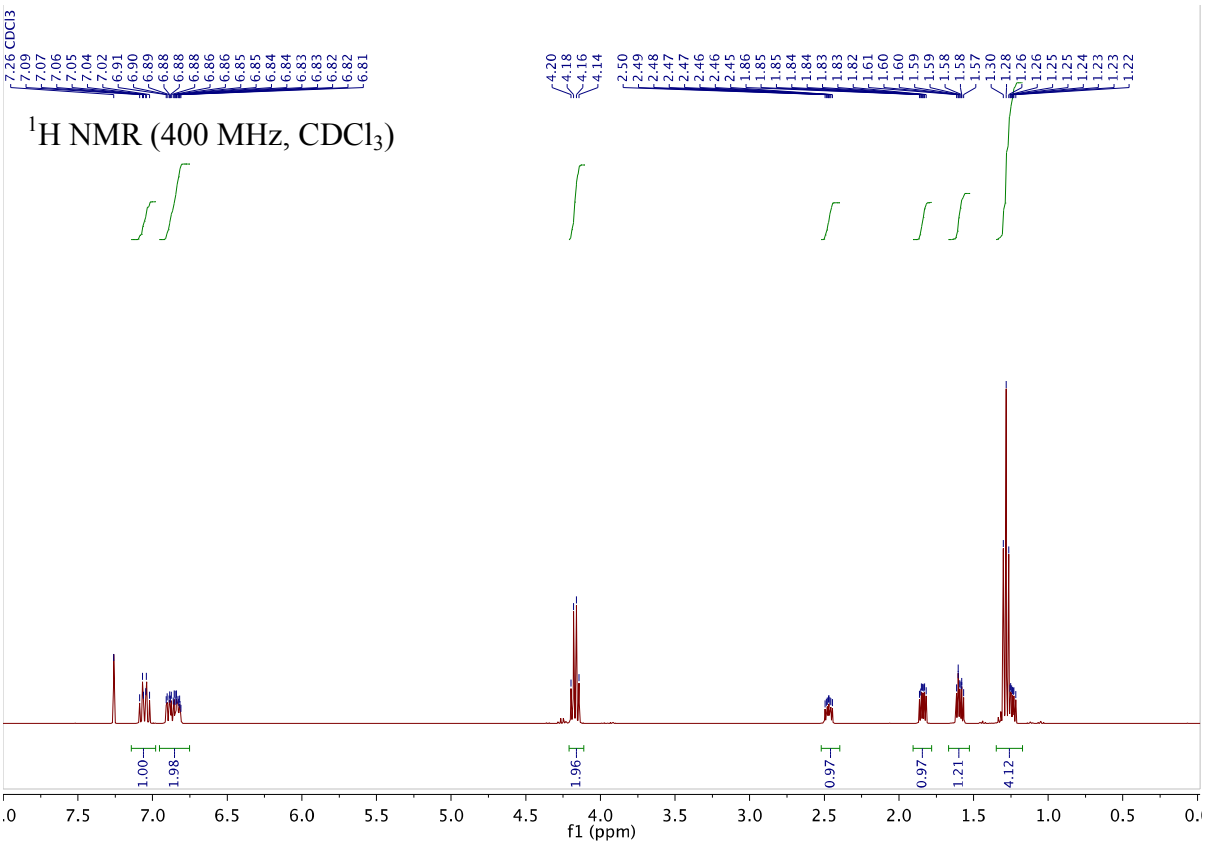
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 (1H, td, J = 10.3, 8.5 Hz, H_{11}), 6.94–6.74 (2H, m, H_8 and H_{12}), 4.17 (2H, q, J = 7.2 Hz, H_2), 2.47 (1H, ddd, J = 9.4, 6.4, 4.2 Hz, H_6), 1.84 (1H, ddd, J = 8.5, 5.3, 4.2 Hz, H_4), 1.59 (1H, ddd, J = 9.2, 5.3, 4.8 Hz, H_{5a}), 1.28 (3H, t, J = 7.1 Hz, H_1), 1.24 (1H, ddd, J = 8.4, 6.4, 4.8 Hz, H_{5b}).

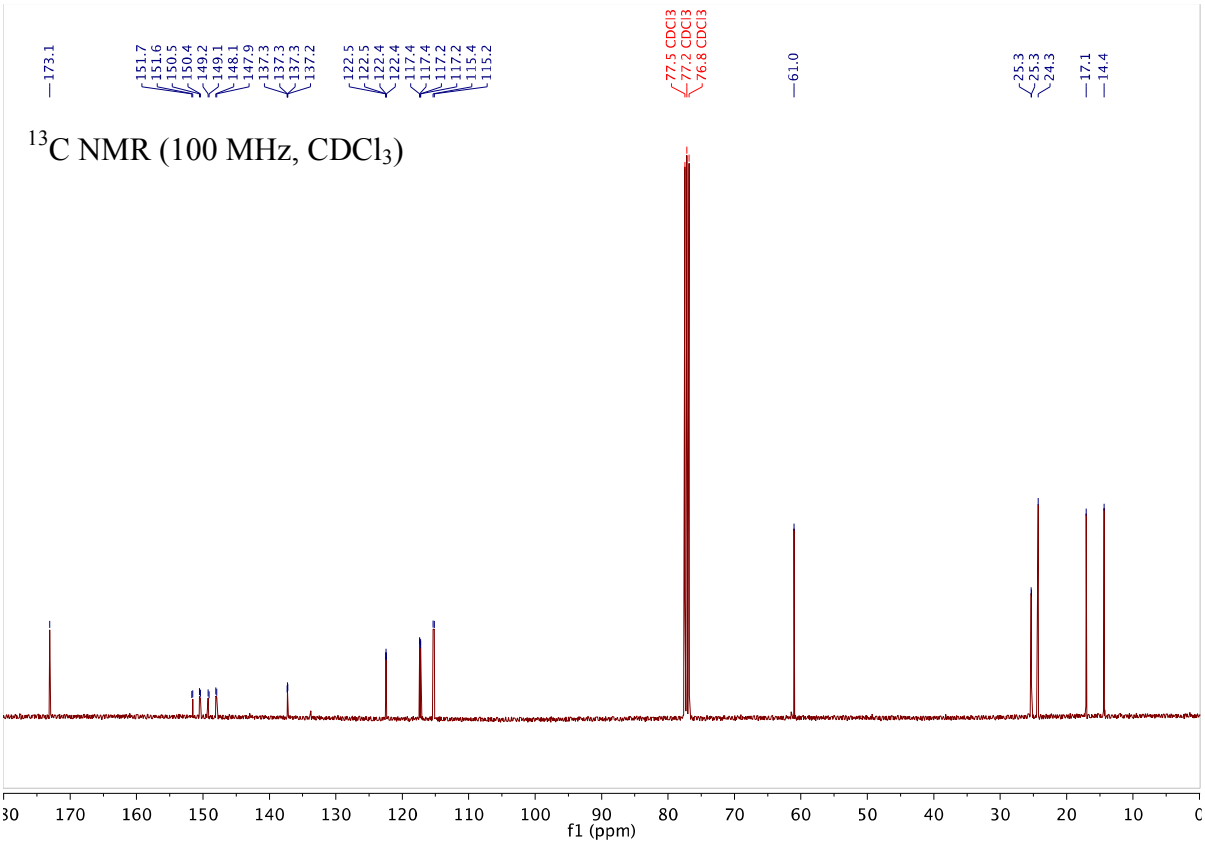
^{13}C NMR (100 MHz, $CDCl_3$) δ 173.1, 151.0 (dd, $^1J_{C–F}$ = 118.7 Hz, $^2J_{C–F}$ = 12.8 Hz), 148.6 (dd, $^1J_{C–F}$ = 117.5 Hz, $^2J_{C–F}$ = 12.7 Hz), 137.3 (dd, $^3J_{C–F}$ = 5.9 Hz, $^4J_{C–F}$ = 3.7 Hz), 122.5 (dd, $^3J_{C–F}$ = 6.2 Hz, $^4J_{C–F}$ = 3.4 Hz), 117.3 (dd, $^2J_{C–F}$ = 17.2 Hz, $^3J_{C–F}$ = 0.2 Hz), 115.3 (d, $^2J_{C–F}$ = 17.6 Hz), 61.0, 25.3 (d, $^4J_{C–F}$ = 1.7 Hz), 24.3, 17.1, 14.4.

HRMS (FAB) *m/z*: 227.0904 ($M+H^+$); calc. for $C_{12}H_{13}F_2O_2$: 227.0884.

$[\alpha_D]^{25}$ = −213.7 (*c* 0.55 in EtOH, 98% *ee*)

The absolution configuration of enzymatically synthesized cyclopropane (*R*,*R*)-**5** was determined by comparing (1) the optical rotation value of (*R*,*R*)-**5** and (2) the HPLC retention times of *rac*-**5** and (*R*,*R*)-**5** with that reported in the literature.[4]


^{1}H NMR (400 MHz, $CDCl_3$)
f1 (ppm)


^{13}C NMR (100 MHz, $CDCl_3$)
f1 (ppm)

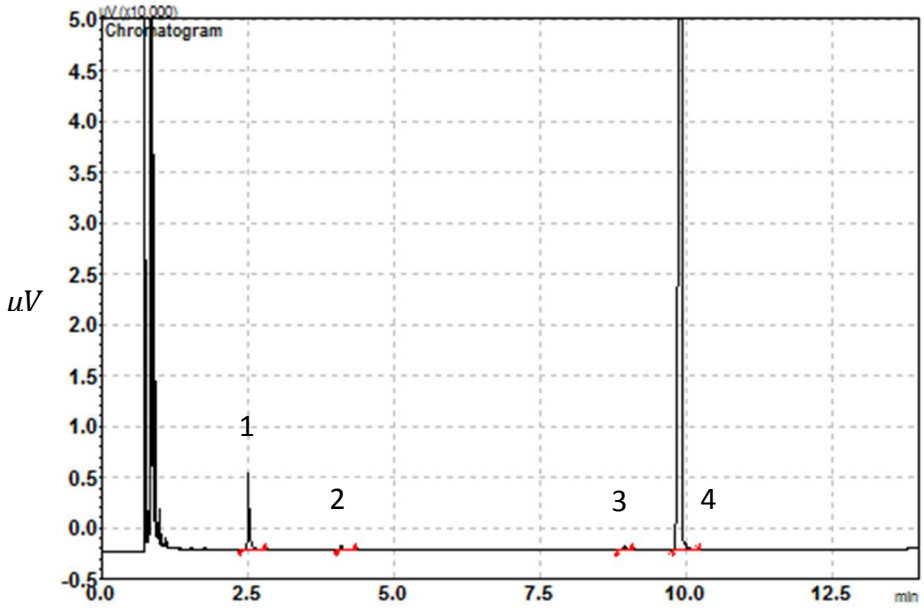
Gas Chromatography (GC) Method

Gas chromatography analyses were carried out using a Shimadzu GC-17A gas chromatograph, a FID detector, and a J&W HP-5 column (30m x 0.32 mm, 0.25 μm film). Acetophenone was used as an internal standard. The temperature program used is shown below:

Rate (°C/min)	Temp (°C)	Hold Time (min)
0	90	2
6	150	0
80	230	1

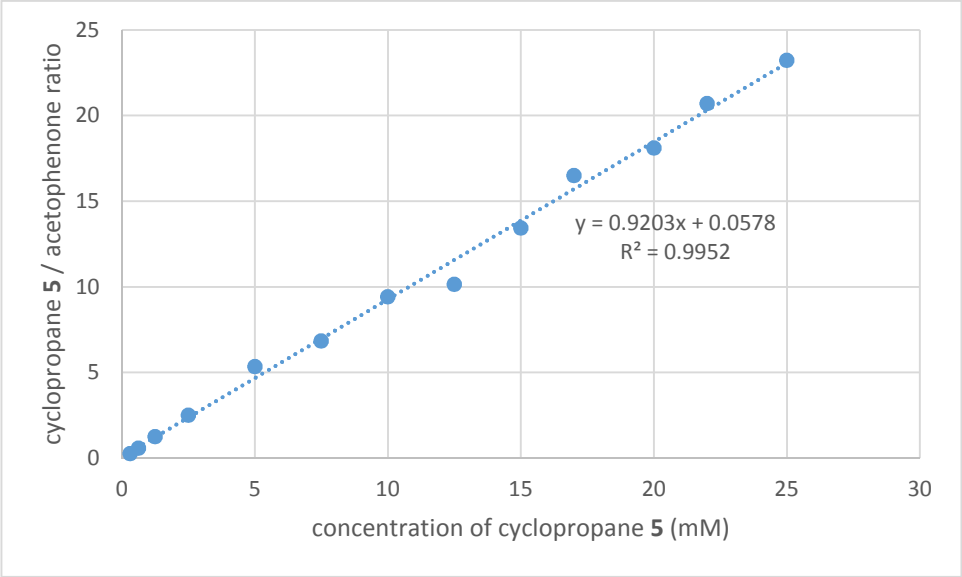
Total GC method time: 14 minutes

A representative example of a GC trace of cyclopropane **5**, 3,4-difluorostyrene, and acetophenone is shown below:



Peak	Retention time (min)	Identity
1	2.51	Acetophenone
2	4.09	3,4-difluorostyrene
3	8.96	*cis*-isomer of cyclopropane **5**
4	9.92	cyclopropane **5**

The GC standard curve for cyclopropane **5** is shown below:



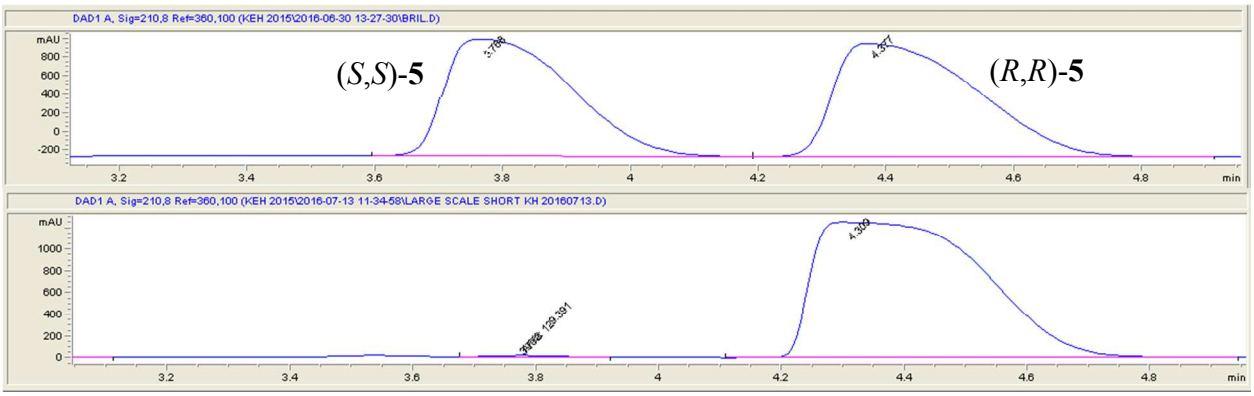
Sample preparation for GC standard curve. The GC standard curve was generated using known amounts of chemically synthesized racemic cyclopropane **5** and performing mock extraction on the same scale as the small-scale biocatalytic reactions. For the mock extractions, 375 µL of M9-N minimal medium, 25 µL of cyclopropane **5** at various concentrations (5 to 400 mM in cyclohexane), 0.9 mL of cyclohexane, and 20 µL of acetophenone (40 mM in cyclohexane) were mixed and vortexed for 30 seconds two times then spun at 14,000 rpm and 20 °C for 5 minutes. The cyclohexane supernatant was transferred to 2 mL glass vials and quantified using the GC. The average of duplicate extractions was used for the GC standard curve.

Supercritical Fluid Chromatography (SFC) Method

Analytical chiral supercritical fluid chromatography was performed with a JACSO 2000 series instrument using *i*-PrOH and supercritical CO_2 as the mobile phase, with visualization at 210 and 254 nm. The column used was AD-H (4.6 mm x 250 mm) from Chiral Technology.

The SFC traces for racemic and enzymatically synthesized cyclopropane **5** are shown below.

Chiralpak AD-H, 2% *i*-PrOH in CO_2, 2.5 mL/min, 210 nm

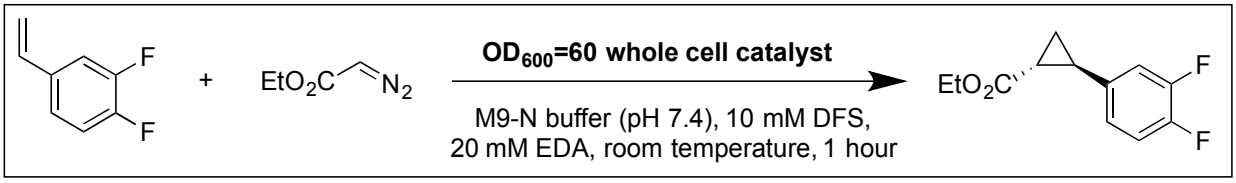


Area% report for *rac*-**5** and (*R*,*R*)-**5**:

rac-**5**			(*R*,*R*)-**5**		
Retention Time (min)	Area (mAU*s)	Area %	Retention Time (min)	Area (mAU*s)	Area %
3.768	17277.5	48.16	3.773	129.3	0.54
4.377	18598.3	51.84	4.309	23713.4	99.46
Total	35875.8	100.00	Total	23842.7	100.00

Supporting Tables

Table S1. Preliminary experiments with hemin or *E. coli* cells expressing various heme proteins

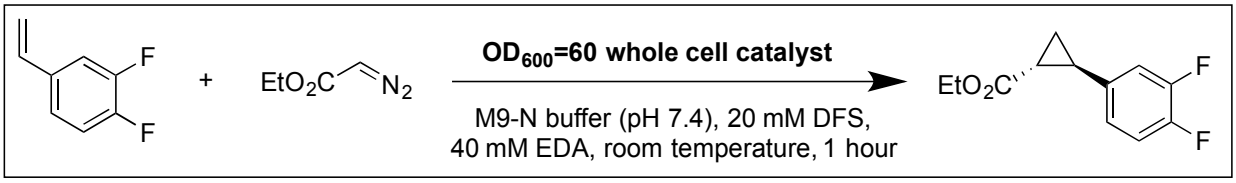


	yield %	dr (*trans*:*cis*)	% *ee* (*trans*)
Hemin (0.1 mM)	6	92:8	0
Rhodothermus marinus cyt *c* M100D V75T[5]	6	91:9	−13
Physeter macrocephalus Mb H64V V68A[6]	38	90:10	−93±3
Hydrogenobacter thermophilis cyt *c* M59A Q62A[5]	19±6	93:7	50±4
BM3 Hstar heme[7]	82±6	94:6	10
BM3 Hstar[7]	19	91:9	18
BM3 Hstar H92N H100N[8]	26	92:8	38

Reaction with hemin: A 1 mM solution of hemin was prepared in DMSO. 40 μL of 1 mM hemin solution was added to 340 μL of M9-N minimal medium. The reactions were performed at room temperature under anaerobic conditions with a final concentration of 10 mM DFS and 20 mM EDA for 1 hour. The reaction was performed in duplicate.

Whole-cell reactions: Cells were grown according to literature procedure (see references cited). Experiments were performed using whole-cell catalysts resuspended in M9-N minimal medium to OD_{600}=60. The reactions were performed at room temperature under anaerobic conditions with a final concentration of 10 mM DFS and 20 mM EDA for 1 hour. Reactions were performed in duplicate.

Table S2. Background reactions of different strains of *E. coli.*



	yield %	dr (*trans*:*cis*)	% *ee* (trans)
BL 21	44.70	92:8	−5
C 41*	34.84	93:7	−5
C 43*	27.77	91:9	−8

Cells with no pET22b plasmid were grown and induced according to procedure described in General Procedures using media with no antibiotics. Small-scale biocatalytic experiments were performed using whole-cell catalysts resuspended in M9-N minimal medium to OD_{600}=60. The reactions were performed at room temperature under anaerobic conditions with a final concentration of 20 mM DFS and 40 mM EDA for 1 hour. Reactions were performed in duplicate.

*Lucigen's C43(DE3) and C41(DE3) are derived from BL21(DE3) and have mutations in the lacUV5 promoter which lower the expression of T7RNA polymerase causing protein production to occur more slowly.[9] It is unclear how this improves the expression of cytosolic proteins.

Nucleotide and Amino Acid Sequences

B. subtilis truncated globin wild-type amino acid sequence
MGQSFNAPYEAIGEELLSQLVDTFYERVASHPLLKPIFPSDLTETARKQKQFLTQYLGGP
PLYTEEHGHPMLRARHLPFPITNERADAWLSCMKDAMDHVGLEGEIREFLFGRLELTAR
HMVNQTEAEDRSS

B. subtilis truncated globin wild-type nucleotide sequence
ATGGGTCAAAGTTTCAATGCACCATACGAAGCCATCGGGGAAGAGCTGCTCAGTCA
GCTCGTTGATACGTTCTATGAACGTGTGGCGTCTCATCCGCTGCTCAAACCGATTTTT
CCGTCTGATCTGACGGAAACCGCACGTAAACAGAAGCAGTTTCTGACGCAATATCTC
GGTGGTCCGCCGTTATACACTGAAGAACATGGTCACCCAATGCTTCGCGCGCGGCAC
CTCCCATTCCCGATCACCAATGAGCGTGCGGACGCTTGGCTCTCCTGTATGAAAGAC
GCAATGGATCACGTGGGACTGGAAGGTGAAATTCG
TGAATTCCTGTTTGGTCGTTTAGAACTGACGGCGCGCCACATGGTCAATCAGACCGA
AGCAGAAGACCGGAGCAGC

B. subtilis truncated globin T45A Q49A amino acid sequence
MGQSFNAPYEAIGEELLSQLVDTFYERVASHPLLKPIFPSDLTEAARKAKQFLTQYLGGP
PLYTEEHGHPMLRARHLPFPITNERADAWLSCMKDAMDHVGLEGEIREFLFGRLELTAR
HMVNQTEAEDRSS

B. subtilis truncated globin T45A Q49A nucleotide sequence
ATGGGTCAAAGTTTCAATGCACCATACGAAGCCATCGGGGAAGAGCTGCTCAGTCA
GCTCGTTGATACGTTCTATGAACGTGTGGCGTCTCATCCGCTGCTCAAACCGATTTTT
CCGTCTGATCTGACGGAAGCCGCACGTAAAGCGAAGCAGTTTCTGACGCAATATCTC
GGTGGTCCGCCGTTATACACTGAAGAACATGGTCACCCAATGCTTCGCGCGCGGCAC
CTCCCATTCCCGATCACCAATGAGCGTGCGGACGCTTGGCTCTCCTGTATGAAAGAC
GCAATGGATCACGTGGGACTGGAAGGTGAAATTCGTGAATTCCTGTTTGGTCGTTTA
GAACTGACGGCGCGCCACATGGTCAATCAGACCGAAGCAGAAGACCGGAGCAGC

B. subtilis truncated globin Y25L T45A Q49A amino acid sequence
MGQSFNAPYEAIGEELLSQLVDTFLERVASHPLLKPIFPSDLTEAARKAKQFLTQYLGGP
PLYTEEHGHPMLRARHLPFPITNERADAWLSCMKDAMDHVGLEGEIREFLFGRLELTAR
HMVNQTEAEDRSS

B. subtilis truncated globin Y25L T45A Q49A nucleotide sequence
ATGGGTCAAAGTTTCAATGCACCATACGAAGCCATCGGGGAAGAGCTGCTCAGTCA
GCTCGTTGATACGTTCCTGGAGCGTGTGGCGTCTCATCCGCTGCTCAAACCGATTTTT
CCGTCTGATCTGACGGAAGCCGCACGTAAAGCGAAGCAGTTTCTGACGCAATATCTC
GGTGGTCCGCCGTTATACACTGAAGAACATGGTCACCCAATGCTTCGCGCGCGGCAC
CTCCCATTCCCGATCACCAATGAGCGTGCGGACGCTTGGCTCTCCTGTATGAAAGAC
GCAATGGATCACGTGGGACTGGAAGGTGAAATTCGTGAATTCCTGTTTGGTCGTTTA
GAACTGACGGCGCGCCACATGGTCAATCAGACCGAAGCAGAAGACCGGAGCAGC

Alignment of *P. macrocephalus* Mb and *B. subtilis* TrHb

The mutations found to improve cyclopropanation activity of *P. macrocephalus* Mb by Fasan *et al.*[6] and their corresponding residues in *B. subtilis* TrHb are highlighted in red. Alignment was performed using the PROMALS3D web server,[10] using PDB files 1UX8 and 1A6K.

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B. subtilis TrHb          ---N-------APYEAIGEELLSQLVDTFYERVA-SHPLLKPIF-PS-------------
P. macrocephalus Mb       VLSEGEWQLVLHVWAK-VEADVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAEMKASE

B. subtilis TrHb          DLTETARKQKQFLTQYLGGPPLYTEEHGHP--MLRARHL-PFPITNERADAWLSCMKDAM
P. macrocephalus Mb       DLKKHGVTVLTALGAILKKKGHHE----AELKPLAQSHATKHKIPIKYLEFISEAIIHVL

B. subtilis TrHb          DHV-G--LEGEIREFLFGRLELTARHM---V----N
P. macrocephalus Mb       HSRHPGDFGADAQGAMNKALELFRKDIAAKYKELGY
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