Identification of Mechanism-Based Inactivation in P450-Catalyzed Cyclopropanation Facilitates Engineering of Improved Enzymes

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SUPPLEMENTARY MATERIAL

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General materials and methods

Unless otherwise noted, all chemicals and reagents for chemical reactions were obtained from commercial suppliers (Sigma-Aldrich, Acros) and used without further purification. Sonication was performed using a Qsonica Q500 sonicator. For UV-vis analyses, spectra were collected between 650 and 390 nm on a UV1800 Shimadzu spectrophotometer. Circular dichroism (CD) analyses were performed using Aviv Biomedical Model 410 CD spectrophotometer. Gas chromatography (GC) analyses were carried out using a Shimadzu GC-17A gas chromatograph with FID detector and J&W HP-5 column (30 m x 0.32 mm, 0.25 μm film) and 2-phenylethanol as an internal standard. Analytical SFC was performed with a Mettler SFC supercritical CO_2 analytical chromatography system with isopropanol and liquid CO_2 as the mobile phase. Enantiomers were separated using a Chiralpak AS column (4.6 mm x 25 cm) obtained from Daicel Chemical Industries, Ltd. High resolution mass spectrometry (HRMS) was performed with Agilent 6200 Series TOF with an Agilent G1978A multimode source in electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), or mixed (MM) ionization mode. Enzymes (DpnI, Phusion polymerase) were purchased from New England Biolabs (NEB, Ipswich, MA).

Generation of enzyme variants

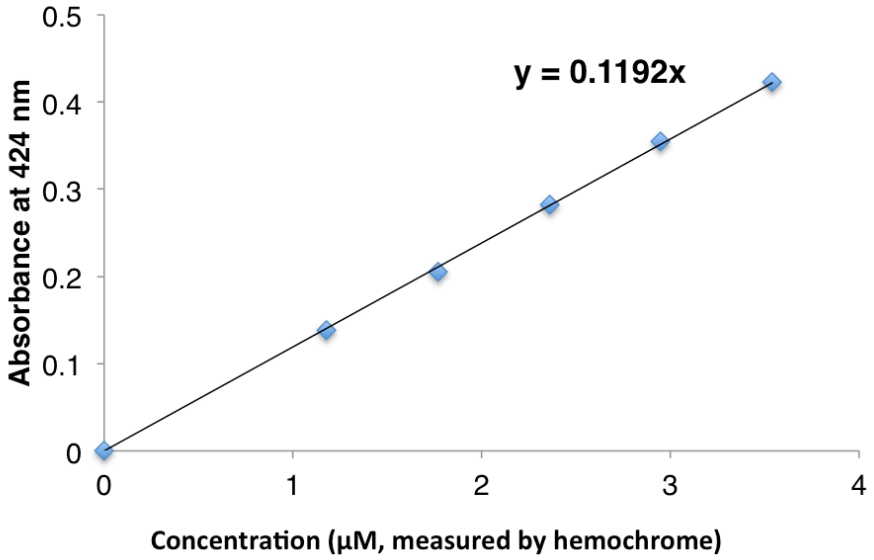
Plasmid pET22b(+) was used as a cloning and expression vector for all enzymes used in this study. Site-directed mutagenesis was accomplished by modified QuikChange™ protocol using primers bearing the desired mutations (IDT, San Diego, CA). The PCR products were gel purified, digested with DpnI, repaired using Gibson Mix™, and used directly to transform *E. coli* strain BL21(DE3). Variants were stored as glycerol stocks at –80 ºC.

Hemochrome binding assay

A solution of pyridine was made by combining 1.75 mL pyridine with 0.75 mL 1 M NaOH. The solution mixed at room temperature was centrifuged for 30 s at 5000 rcf to remove excess aqueous base. To a cuvette containing 0.75 mL of protein solution in phosphate buffer (0.1 M, pH 8.0), 0.25 mL of the pyridine solution was added followed by a few grains (less than 2.0 mg) of sodium dithionite. The cuvette was sealed with parafilm and a UV-vis spectrum was recorded immediately. P450 concentration was determined from the absorbance of the hemochrome complex using extinction coefficient ε_{418} = 196 mM^{-1} cm^{-1}. Absorbance was assigned as the difference between the peak max at 418 nm and the baseline at 420 nm as determined by extrapolating from two points on either side of the hemochrome peak.

Determination of ε_{max} for ferrous histidine-ligated P450 enzymes

BM3-HStar holoprotein with known concentration (determined by hemochrome binding assay) was used for extinction coefficient determination. An appropriate amount of the protein was transferred to a semi-micro cuvette fitted with a screw septum cap (Starna Cells, product number 9-SOG-10-GL14-S). The cuvette was made anaerobic by flushing with argon. In 6 mL crimp-sealed vials, a phosphate buffer solution (0.1 M, pH = 8.0) and a 12.5 mM solution of sodium dithionite in phosphate buffer (0.1 M, pH = 8.0) were degassed by bubbling with argon. To the semi-micro cuvette, 700 μL of the degassed phosphate buffer and 250 μL of the degassed $Na_2S_2O_4$ solution were added, and then the UV-vis spectrum was recorded. Absorbance was assigned as the difference between the peak max at 424 nm and the baseline as determined by extrapolating from two points on either side of the ferrous peak (390 nm and 460 nm). The measurement was repeated for five different enzyme concentrations and a calibration curve was constructed to determine the ferrous ε_{max}.



From the above calibration curve, we determine ε_{max} = 119 $mM^{-1}cm^{-1}$

Ferrous concentration assay

Ferrous concentration assay was used to determine the concentration of His-ligated P450-BM3 variant in crude lysate. To an anaerobic cuvette containing 800 μL of P450 lysate of unknown concentration was added 200 μL of degassed $Na_2S_2O_4$ solution (2 mg/mL), and the UV-vis spectrum was recorded. Absorbance was assigned as the difference between the peak max at 424 nm and the baseline as determined by extrapolating from two points on either side of the ferrous peak (390 nm and 460 nm). P450 concentration was determined from the absorbance of the ferrous species using an extinction coefficient of ε = 119 mM^{-1} cm^{-1} for histidine-ligated P450-BM3.

P450 expression and purification

One liter $Hyperbroth_{amp}$ (AthenaES©) was inoculated with an overnight culture (25 mL, LB_{amp}) of recombinant *E. coli* BL21(DE3) cells harboring a pET22b(+) plasmid encoding the P450 variant. The cultures were shaken at 200 rpm at 37 °C for roughly 3 h or until an optical of density of 0.7–1.0 was reached. Cultures were cooled on ice (20 min) and then induced by adding IPTG and aminolevulinic acid to a final concentration of 0.5 mM and 1.0 mM, respectively. The cultures were allowed to continue for another 20 hours at 20 ºC and shaking at 200 rpm. Cell were harvested by centrifugation (4 °C, 15 min, 3,000xg), and the cell pellet was stored at –20 °C or below for at least 2 h.

Purification was performed with an AKTA purifier FPLC system (GE Healthcare). For the purification of 6XHis tagged P450s, the thawed cell pellet was resuspended in Ni-NTA buffer A (25 mM Tris.HCl, 200 mM NaCl, 25 mM imidazole, pH 8.0, 4 mL/g of cell wet weight) and lysed by sonication (2x1 min, 30% duty cycle). The lysate was centrifuged at 27,000xg for 20 min at 4 °C to remove cell debris. The collected supernatant was subjected to a Ni-NTA chromatography step using a Ni Sepharose column (HisTrap-HP, GE healthcare, Piscataway, NJ). The P450 was eluted from the Ni Sepharose column using 25 mM Tris.HCl, 200 mM NaCl, 300 mM imidazole, pH 8.0. Ni-purified protein was buffer exchanged into 0.1 M phosphate buffer (pH = 8.0) using a 10 kDa MW cut-off centrifugal filter. Protein concentrations were determined by hemochrome binding assay described above. For storage, proteins were portioned into 300 μL aliquots and stored at –80 °C.

Small-scale whole-cell bioconversions

E. coli BL21(DE3) cells with plasmid coding for a P450 mutant were grown from glycerol stock overnight (37 °C, 250 rpm) in 5 ml LB_{amp}. A portion of the pre-culture (1.25 mL) was used to inoculate 50 mL of Hyperbroth medium (1 L Hyperbroth prepared from powder from AthenaES©, 0.1 mg mL^{-1} ampicillin) in a 250 mL Erlenmeyer flask and this culture was incubated at 37 °C, 200 rpm for approximately 3 h. At OD_{600} = 0.7–1.0, the cultures were cooled on ice (20 min) before inducing with IPTG (0.5 mM final concentration) and δ-aminolevulinic acid (1.0 mM final concentration). Shaking was continued at 20 ºC, 200 rpm. Cultures were harvested after 20 h and resuspended in nitrogen-free M9-N medium (1 L: 31 g Na_2HPO_4, 15 g KH_2PO_4, 2.5 g NaCl, 0.24 g $MgSO_4$, 0.01 g $CaCl_2$) until the indicated OD_{600} was reached (OD_{600} = 30 unless otherwise noted). Aliquots of the cell suspension were used for determination of the P450 expression level (2–3 mL) after lysis.

Anaerobic reaction conditions: E. coli cells of the appropriate OD_{600} were transferred to a crimped 6 mL vial and degassed with argon for 5-10 min. In parallel, a solution of glucose in M9-N (250 mM) was degassed with argon for the same period of time. All solutions were uncapped and transferred to an anaerobic chamber. Glucose solution (50 μL, 250 mM) was added to 2 mL crimp vials, followed by cell suspension (425 µL), the olefin substrate (12.5 µL of a 400 mM solution in EtOH), and EDA (12.5 µL of a 800 mM solution in EtOH). The vials were sealed, removed from the anaerobic chamber and shaken on a tabletop shake plate at room temperature for 1 h at 350 rpm. The reactions were quenched by addition of 20 µL of 3 M HCl, followed by 20 µL of the internal standard (20 mM 2-phenylethanol solution in cyclohexane) and 1 mL cyclohexane. The mixture was transferred to a 1.7 mL Eppendorf tube, vortexed

and then centrifuged (10,000x rcf, 30 s). The organic layer was removed and analyzed by GC to determine yield and chiral SFC to determine enantioselectivity.

Small-scale cyclopropanation reactions with purified enzymes (anaerobic)

Small-scale (400 μL) reactions were carried out in 2 mL glass crimp vials (Agilent Technologies, San Diego, CA). P450 solution (60 μL, 67 μM) was added to an unsealed crimp vial. In parallel, a 12.5 mM solution of sodium dithionite in phosphate buffer (0.1 M, pH = 8.0) was degassed by bubbling with argon in a 6 mL crimp-sealed vial. All solutions were uncapped and transferred to an anaerobic chamber. The buffer/dithionite solution (320 μL) was added to each reaction vial containing P450 solution, followed by the olefin substrate (10 μL, 400 mM in EtOH) and EDA (10 μL, 800 mM in EtOH). The reaction vials were capped, removed from the anaerobic chamber, and then placed in a tray on a plate shaker and left to shake at 350 rpm for 2 h at room temperature. The final concentrations of the reagents were typically: 10 olefin, 20 mM EDA, 10 mM $Na_2S_2O_4$, and 10 μM purified enzyme. The reaction was quenched by the addition of 3 M HCl (20 μL), followed by 20 μL of the internal standard (20 mM 2-phenylethanol solution in cyclohexane) and 1 mL cyclohexane. The mixture was transferred to a 1.7 mL Eppendorf tube and vortexed and centrifuged (10,000x rcf, 30 s). The organic layer was then analyzed by GC to determine yield and chiral SFC to determine enantioselectivity.

Whole-cell reaction of 1 with EDA catalyzed by BM3-HStar variants

Following the procedure for small-scale whole-cell bioconversions under anaerobic conditions (page SI-5), whole cells expressing BM3-HStar, BM3-HStar H92N, BM3-HStar H100N, and BM3-HStar H92N H100N were grown and resuspended to OD_{600} = 10. To determine the P450 concentration of each sample, 2 mL of each cell stock was removed and then analyzed by ferrous concentration assays following the general procedure outlined above.

Small-scale reactions were performed following the general procedure with 12.5 μL **1** at 400 mM and 12.5 μL EDA at 800 mM. Reactions were shaken for 2 h at room temperature. GC analysis of product **2** was performed using J&W HP-5 column (30 m x 0.32 mm, 0.25 μM film) with the method 90 °C hold 2 min, 90-110 at 6 °C/min, 110-190 at 40 °C/min, 190-280 at 20 °C/min, 280 °C hold 1 min, 12.8 min total): internal standard (3.55 min), trans-**2** (9.55 min), and cis-**2** (9.69 min). Yields of cyclopropanation products were determined using calibration curves previously described in reference 1.

Analytical SFC of product **2** was performed on a Chiralpak AS column, eluting with 2% iPrOH at 2.5 mL/min: λ = 210 nm, t_R (min): major = 8.50, minor = 9.48.

Reaction of acrylamide 1 with EDA and purified enzymes

Small-scale reactions with isolated proteins were performed with purified BM3-HStar variants following the general procedure outlined on page SI-6, with 10 μM enzyme concentration, 10 mM of **1**, and 20 mM of EDA at room temperature. Yields of the reactions were determined by GC and enantioselectivities were determined by chiral SFC using the methods described above for whole-cell reactions.

Analysis of diazo-induced inactivation

Small-scale reaction mixtures with whole cells expressing the appropriate enzyme variants or purified enzymes were prepared according to the general procedures outlined above. To the reaction vial was added 10 μL of EDA solution (800 mM in EtOH). After the specified pre-incubation time, 10 μL of olefin solution (400 mM in EtOH) was added. For determination of olefin-induced inactivation, the order of addition was reversed. The reactions were shaken on a tabletop shake plate at room temperature for 1 h at 350 rpm. The reactions were quenched by addition of 20 µL of 3 M HCl, followed by 20 µL of the internal standard (20 mM 2-phenylethanol solution in cyclohexane) and 1 mL cyclohexane. The mixture was transferred to a 1.7 mL Eppendorf tube, vortexed and then centrifuged (10,000x rcf, 30 s). The organic layer was removed and analyzed by GC to determine yield and by chiral SFC to determine enantioselectivity.

Liquid chromatography-mass spectrometry (LCMS) analysis of cofactor modification

To a 6 mL crimp-sealed vial was added BM3-HStar (final concentration = 10 μM) and the vial was made anaerobic by degassing the headspace with argon for 10 min. In a separate 6 mL crimp-sealed vial, a 12.5 mM solution of $Na_2S_2O_4$ in phosphate buffer (0.1 M, pH = 8.0) was degassed by bubbling with argon for 10 min. The buffer/dithionite solution (1.8 mL) was then added to the reaction vial via syringe, followed by 50 μL of 200 mM EDA solution. For investigation of reductant effects, degassed phosphate buffer solution (1.8 mL) was added in place of buffer/dithionite solution. The reaction was quenched at the appropriate time points by adding 100 μL of 3 M HCl, followed by 2.5 mL of 9:1 dichloromethane:MeOH. The mixture was transferred to a 15 mL Falcon tube, vortexed and centrifuged (5000 rpm, 5 min). The organics were collected, and the extraction procedure was repeated one more

time. The organics were combined, concentrated *in vacuo*, redissolved in MeCN, and submitted for LCMS analysis. For reaction progress monitoring, the analyses were performed at 15 s and 1 min time points.

Isolation of modified porphyrin cofactor

Cofactor was extracted from the reaction mixture of isolated BM3-HStar and EDA using the procedure outlined above. Following extraction, the crude organics were concentrated *in vacuo* and then purified by preparative TLC (10:90:0.1 MeOH:DCM:AcOH), isolating the red fluorescent band (R_f = 0.3). The isolated porphyrin was dissolved in MeOH and then submitted to HRMS analysis. UV-vis analysis was performed using a solution of isolated porphyrin in DCM. For UV-vis analysis of Zn^{2+}-complexed porphyrin, isolated porphyrin was dissolved in 0.5% $ZnCl_2$ solution in 5:95 MeOH/DCM and submitted for analysis.

Analysis of enzyme modification by intact protein mass determination

Small-scale reactions were performed following the general procedure for catalysis using purified enzyme. The protein samples were analyzed on an LC-MSD SL 1100 series (Agilent) liquid chromatography mass spectrometry system. The samples were subjected to reverse phase chromatography on a 2.1 x 150 mm Zorbax 300SB-C3 column (Agilent) using a gradient consisting of 0.2% formic acid (solvent A) and 0.2% formic acid in acetonitrile (89.8%) and methanol (10%) (solvent B). Eluted intact proteins were ionized using electrospray ionization and measured in the single quadrupole mass spectrometer and quantified.

Alkylation analysis by bottom-up proteomics

Small-scale reactions were performed following the general procedure for catalysis using purified enzyme. Appropriate amounts of the reaction aliquot were lyophilized to afford 10–20 μg of proteins. Dried pellets were dissolved in 40 μL of 8 M urea/100 mM Tris-HCl (pH 8.5) mixture. Proteins were reduced by addition of 0.25 μL of 500 mM TCEP (tris(2-carboxyethyl)-phosphine hydrochloride) and alkylated by addition of 0.9 μL of 500 mM iodoacetamide. For sample digestion, 1 μL of 0.1 gL^{-1} of lysyl endopeptidase (from *A. lyticus*) in 100 mM Tris-HCl (pH 8.5) was added, and the samples were incubated at room temperature for 4 h in the dark. The samples were diluted with 120 μL of 100 mM Tris-HCl (pH 8.5) and 1.6 μL of 100 mM $CaCl_2$, followed by addition of 1 μL of 0.5 gL^{-1} of trypsin.

After overnight incubation at room temperature, the digested mixture was diluted to 5% formic acid concentration, desalted and lyophilized, and then resuspended in 0.2% formic acid solution for analysis. In-solution digested samples were subjected to LC-MS/MS analysis on a nanoflow LC system, EASY-nLC II, (Thermo Fisher Scientific) coupled to a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Nanospray Flex ion source (Thermo Fisher Scientific).
For the EASY-nLC II system, solvent A consisted of 97.8% H2O, 2% ACN, and 0.2% formic acid and solvent B consisted of 19.8% H2O, 80% ACN, and 0.2% formic acid. Samples were directly loaded onto a 16-cm analytical HPLC column (75 mm ID) packed in-house with ReproSil-Pur C_{18AQ} 3 μm resin (120A° pore size, Dr. Maisch, Ammerbuch, Germany). The column was heated to 45° C. The peptides were separated with a 90 min gradient at a flow rate of 350 nL/min. The gradient was as follows: 2–30% Solvent B (90 min), 30–100% B (1 min), and 100% B (9 min). Eluted peptides were then ionized using a standard coated silica tip (New Objective, Woburn, MA) as an electrospray emitter and introduced into the mass spectrometer. The LTQ Orbitrap was operated in a data-dependent mode, automatically alternating between a full-scan (m/z 300-1700) in the Orbitrap and subsequent MS/MS scans of the 15 most abundant peaks in the linear ion trap (Top15 method). Data acquisition was controlled by Xcalibur 2.0.7 and Tune 2.4 software (Thermo Fisher Scientific).

Raw files were searched using MaxQuant (v 1.5.1.2) using default parameters except where noted. UniProt *E. coli* entries along with expressed protein sequence and a contaminant database were considered in the search. A decoy database was generated and searched in parallel to estimate the false discovery rate (FDR). Trypsin was specified as the digestion enzyme with up to two missed cleavages allowed. Precursor mass tolerance was 4.5 ppm after recalibration and fragment ion mass tolerance was 0.5 Da. Variable modifications included carbamidomethylation of cysteine (+57.0215 Da), oxidation of methionine (+15.9949 Da), protein N-terminal acetylation (+42.0106 Da), and the custom modification (+86.0368 Da) on lysine, cysteine, histidine, serine, and arginine. FDRs of Peptide Spectrum Matches (PSM), Protein, and site modifications were all less than 1% as estimated by the target-decoy approach.

Circular dichroism analysis

Small-scale reactions were performed following the general procedure for catalysis using the heme domain of the appropriate enzyme variants after isolation and purification by HisTrap-HP column (final concentrations: 10 μM protein, 2 mM substrates, 2 mM $Na_2S_2O_4$). CD samples were obtained by mixing 20 μL aliquot of the reaction with 1.0 mL of 10 mM phosphate buffer (pH 8), and 2.0 mL of H_2O. CD

spectra were recorded in the far UV region, 190–260 mm, using a quartz cell with 1 cm optical path length. Measurements were taken every 1 nm at a scan rate of 1 nm/sec.

Amino Acid Sequences

Table S1. Amino acid sequences of mutants, relative to wild type BM3 (WT). All mutations listed below are in the heme domain. There are no mutations present in the reductase domain.

Enzyme	Amino acid substitution with respect to WT
BM3-HStar	V78M, L181V, T268A, C400H, L437W
BM3-HStar H92N	V78M, H92N, L181V, T268A, C400H, L437W
BM3-HStar H100N	V78M, H100N, L181V, T268A, C400H, L437W
BM3-HStar H92N H100N	V78M, H92N, H100N, L181V, T268A, C400H, L437W

Table S2. Total turnover numbers (TTN), diastereoselectivities, and enantioselectivities of whole-cell bioconversions of 1 and EDA.[a]

Variant	***TTN***	***%trans***	***%ee***
BM3-Hstar	7400	91	61
Hstar H92N	10900	91	82
Hstar H100N	12300	95	69
Hstar H92N H100N	20100	96	83

[a]Experiments were performed at room temperature using whole cells expressing the BM3-HStar variant, resuspended to OD_{600} = 10, with 10 mM 1, and 20 mM EDA. Results are the average of experiments performed with three independent batches of *E. coli* cultures, each used to perform three independent chemical reactions (total of 9 reactions for each enzyme variant). ee = enantiomeric excess.

Table S3. Total turnover numbers (TTN), diastereoselectivities, and enantioselectivities of reaction of 1 and EDA with purified enzymes.[a]

Variant	TTN	%trans	%ee
BM3-Hstar	310	92	10
Hstar H92N	360	93	61
Hstar H100N	420	91	57
Hstar H92N H100N	560	98	89

[a]Results are the average of three independent chemical reactions, performed with 10 μM enzyme concentration, 10 mM of **1**, 20 mM of EDA, and 10 mM of $Na_2S_2O_4$ at room temperature.

Table S4. Enantioselectivities of whole-cell bioconversions with varying concentrations of 1 and EDA.[a]

Variant	1 (mM)	EDA (mM)	% ee
BM3-Hstar	10	20	92
BM3-Hstar	20	40	86
BM3-Hstar	50	100	50
BM3-Hstar	150	300	0
Hstar H92N H100N	10	20	98
Hstar H92N H100N	20	40	90
Hstar H92N H100N	50	100	49
Hstar H92N H100N	150	300	0

[a]Experiments were performed at room temperature using whole cells expressing the BM3-HStar variant, resuspended to OD_{600} = 60, with reactant concentrations described in the table.

Whole-cell reaction of styrene with EDA catalyzed by BM3-HStar variants

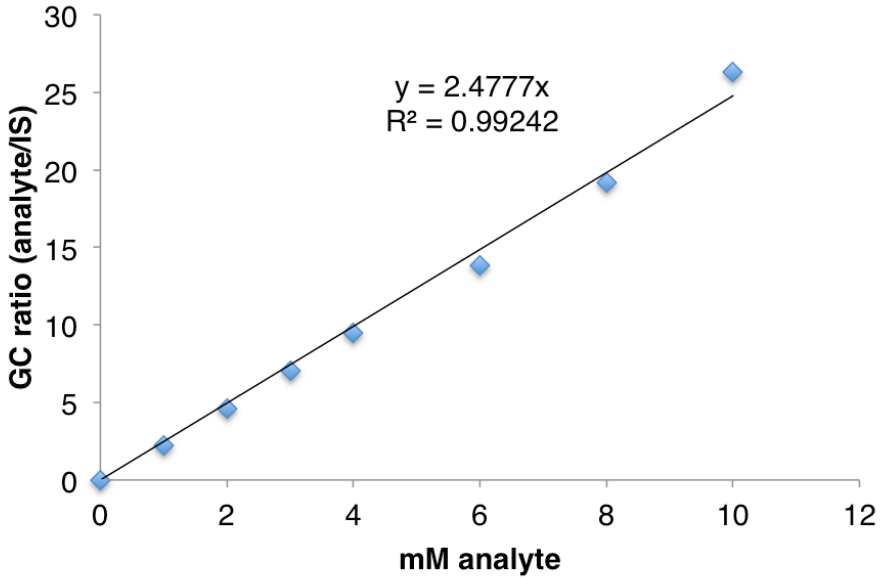
Following the procedure for small-scale whole-cell reactions under anaerobic conditions, *E. coli* expressing BM3-HStar, and BM3-HStar H92N H100N were grown and resuspended to OD_{600} = 10. To determine the P450 concentration of each sample, 2 mL of each cell stock was removed and analyzed by ferrous concentration assays following the general procedure outlined above. Small-scale reactions were performed following the general procedure with 10 mM final concentrations each of styrene and EDA. Reactions were shaken for 2 h at room temperature. GC analysis of product **2** was performed using J&W HP-5 column (30 m x 0.32 mm, 0.25 μM film) with the method 90 °C hold 2 min, 90-190 at 6 °C/min, 190-230 °C at 40 °C/min, 230 °C hold 2 min (internal standard: 3.19 min, *Z* product 8.97 min, *E* product

9.99 min). Yields of cyclopropanation products were determined using calibration curves previously described in reference 1.

Whole-cell reaction of aniline with EDA catalyzed by BM3-HStar variants

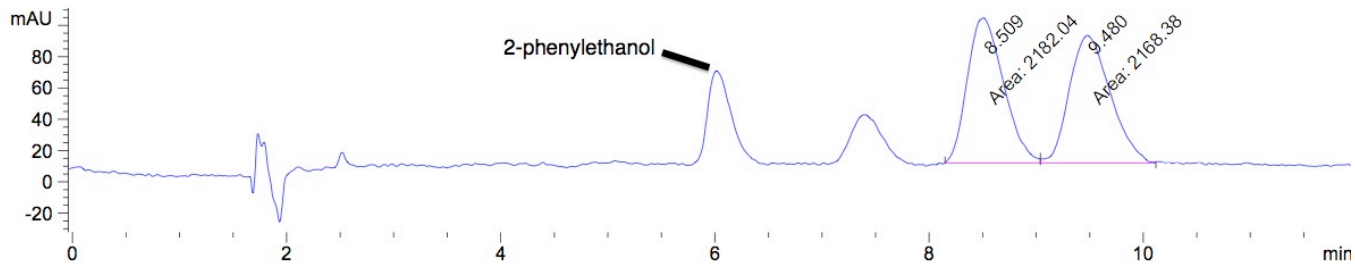
Following the procedure for small-scale whole-cell reactions under anaerobic conditions, *E. coli* expressing BM3-HStar, and BM3-HStar H92N H100N were grown and resuspended to OD_{600} = 10. To determine the P450 concentration of each sample, 2 mL of each cell stock was removed and analyzed by ferrous concentration assays following the general procedure outlined above. Small-scale reactions were performed following the general procedure with 20 mM and 10 mM final concentrations of aniline and EDA, respectively. Reactions were shaken for 2 h at room temperature. GC analysis of product **2** was performed using J&W HP-5 column (30 m x 0.32 mm, 0.25 μM film) with the method 90 °C hold 2 min, 90-190 at 6 °C/min, 190-230 °C at 40 °C/min, 230 °C hold 2 min (internal standard: 3.19 min, product 9.84 min). Yields of N–H insertion product were determined using calibration curves described below.

Calibration curve for carbenoid N–H insertion into aniline

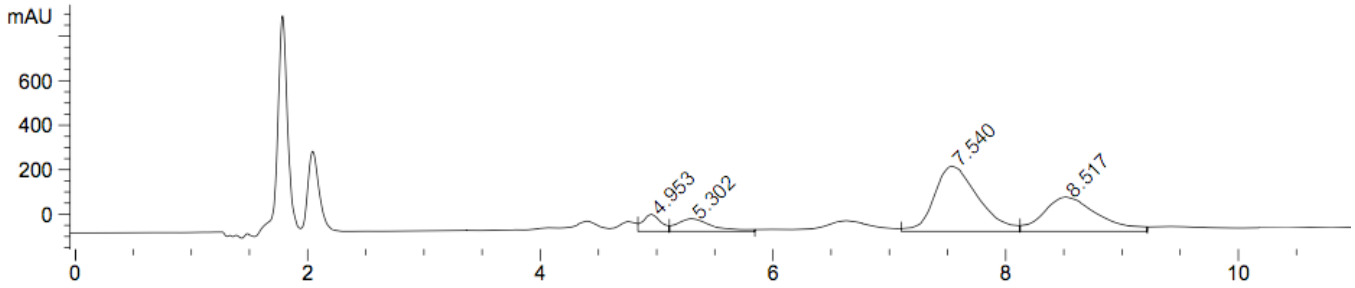


Enantioselectivity for cyclopropanation of N,N-diethyl-2-phenylacrylamide (1) catalyzed by BM3-HStar variants

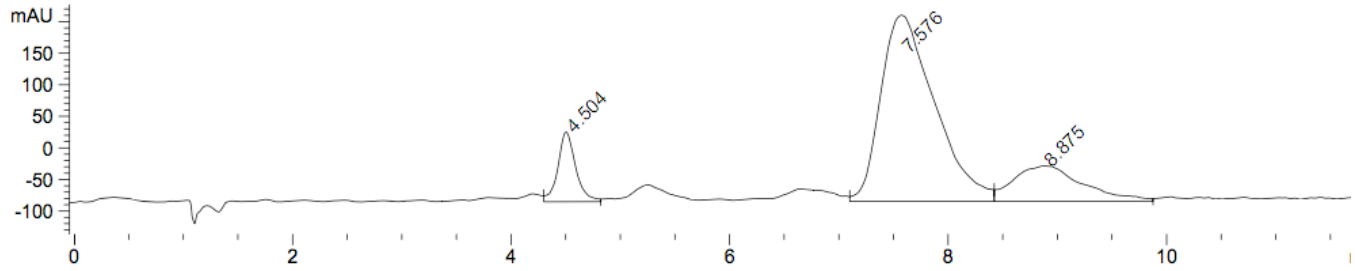
Racemic mixture



Purified BM3-HStar



Purified HStar H92N



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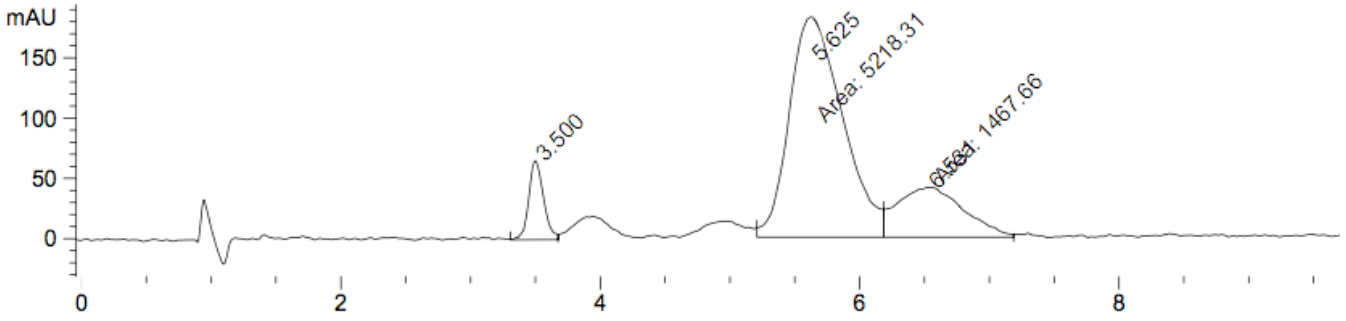
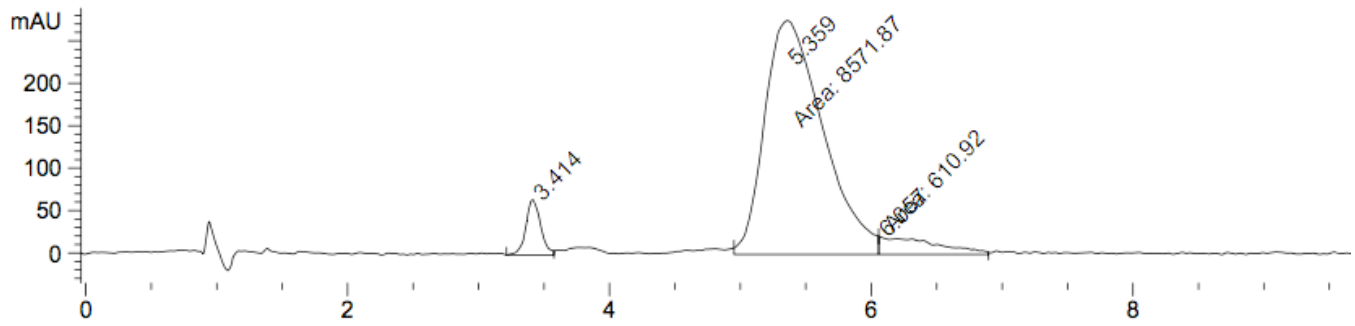
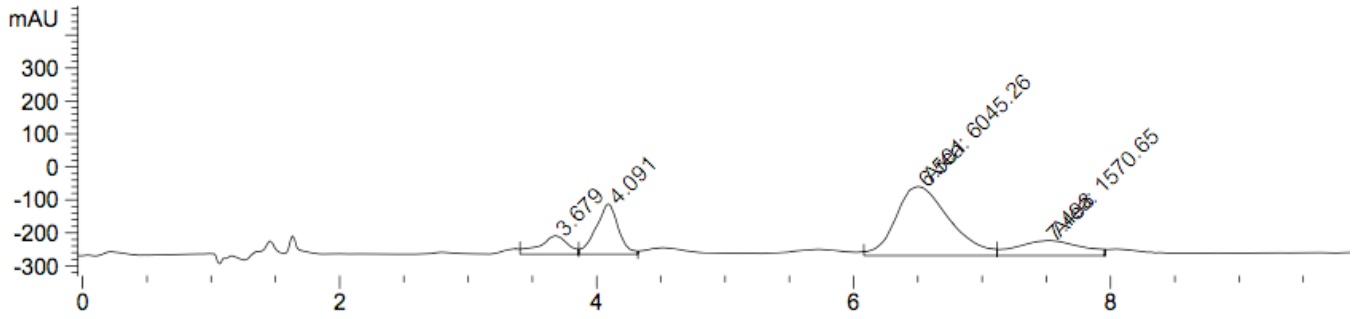


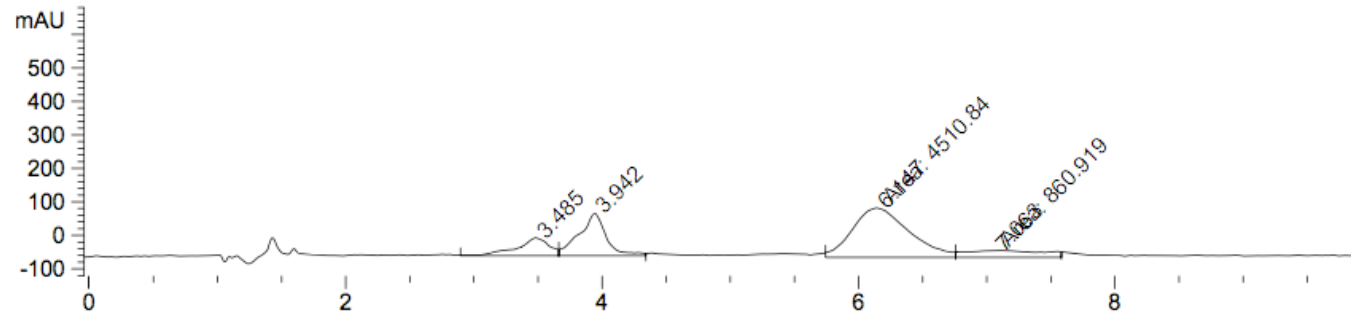
Purified HStar H92N H100N



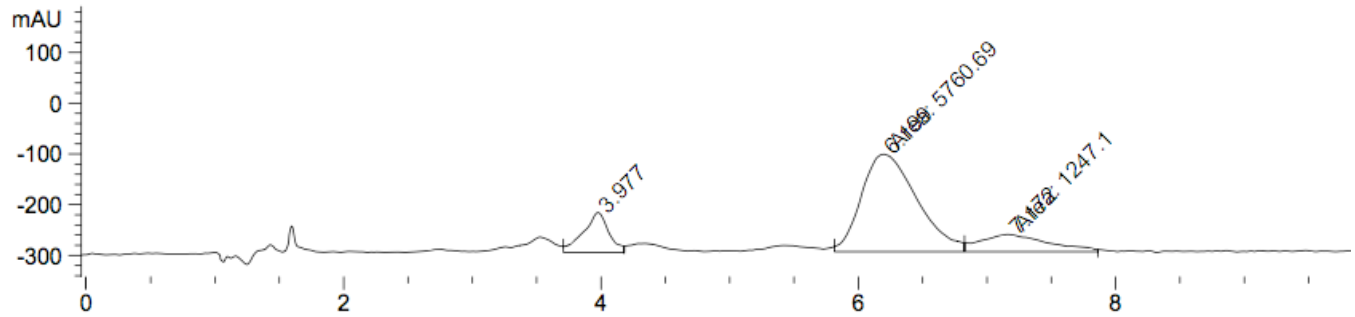
BM3-HStar (whole cell, OD_{600} = 10)



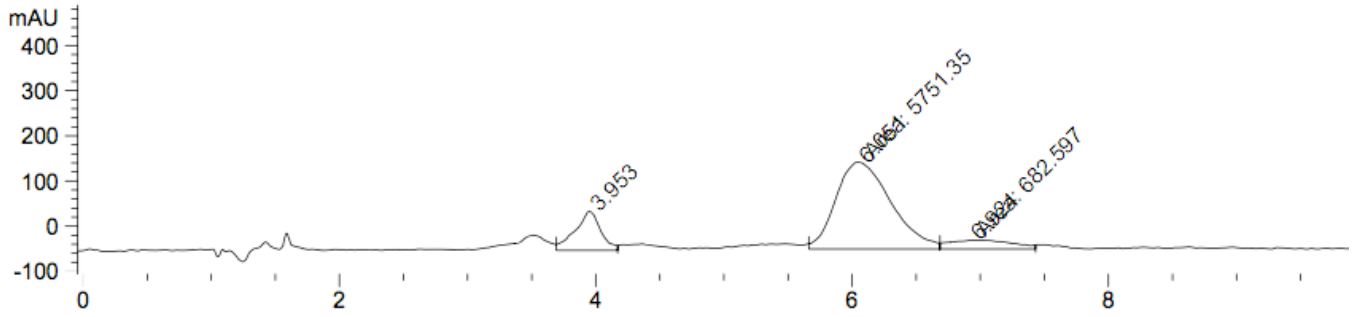
HStar H92N (whole cell, OD_{600} = 10)



HStar H100N (whole cell, OD_{600} = 10)



HStar H92N H100N (whole cell, OD_{600} = 10)



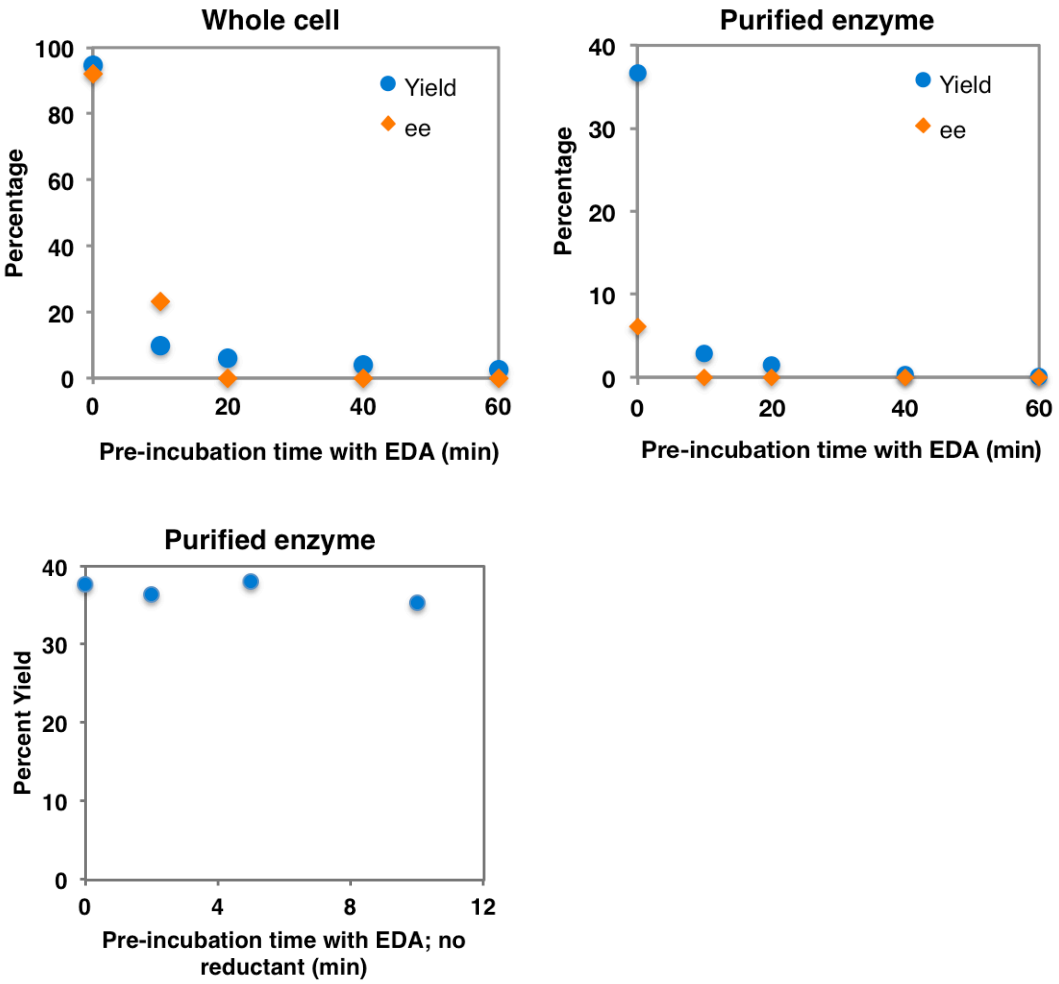
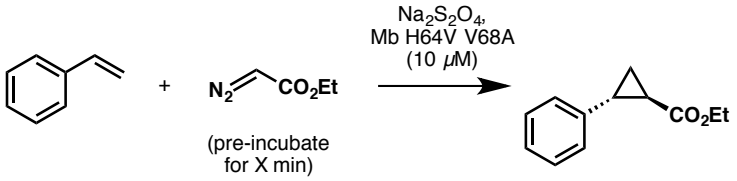
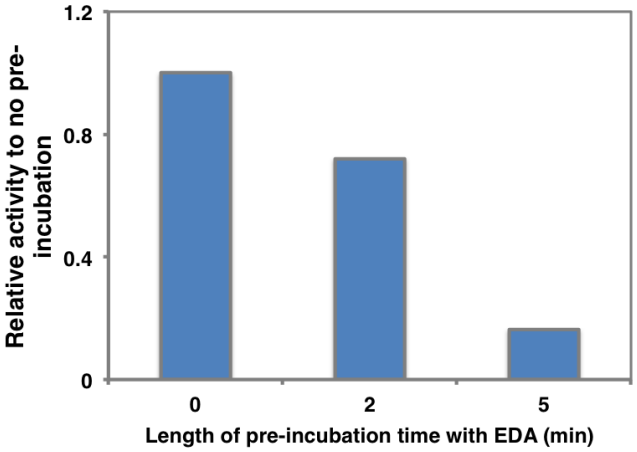
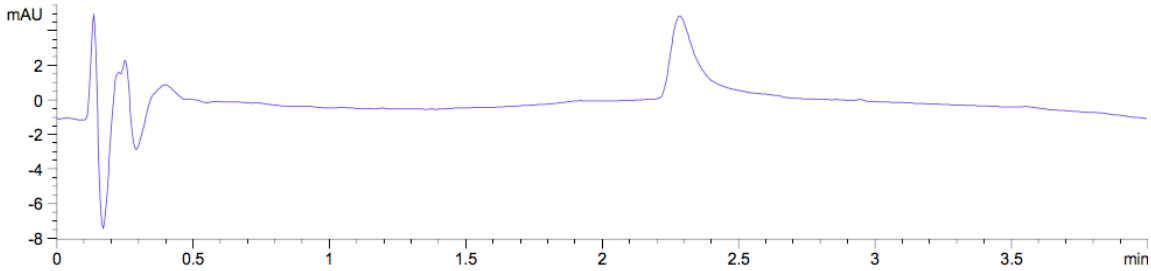


Figure S1. Pre-incubation experiments with EDA (top left, reaction with whole cells expressing the enzyme; top right, reaction with purified enzyme) showed rapid loss in activity and enantioselectivity, suggesting EDA-induced inactivation. For whole-cell experiments, reactions were performed with whole *E. coli* cell suspension (OD600 = 60), 10 mM of **1**, and 20 mM of EDA. For experiments with purified enzyme, reactions were performed with purified BM3-HStar (10 µM), 10 mM of **1**, and 20 mM of EDA. Pre-incubation experiments with EDA in the absence of $Na_2S_2O_4$ (bottom graph) showed that the enzyme maintained its activity in the absence of reductant; reaction conditions: purified BM3-HStar (10 µM), 10 mM of **1**, and 20 mM of EDA, with $Na_2S_2O_4$ solution added concurrently with **1**.





Before reaction



After reaction

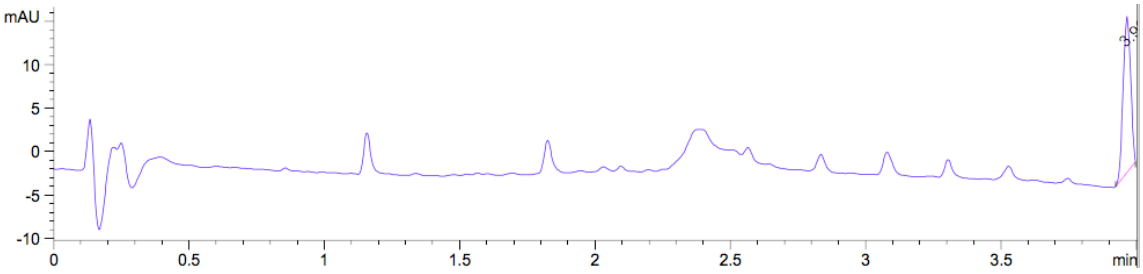
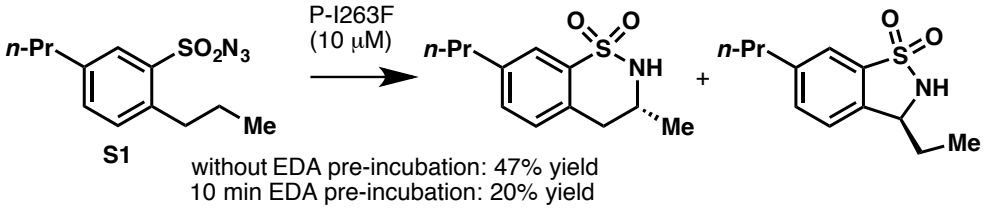
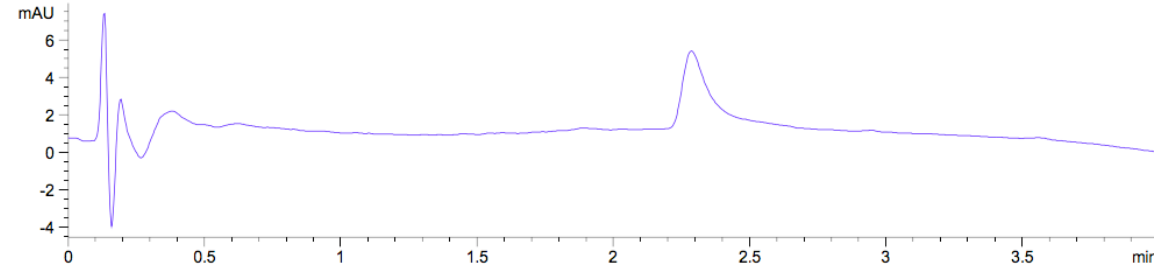


Figure S2. Decrease in cyclopropanation activity of Mb H64V V68A after pre-incubation with EDA, and LC-MS analysis of heme content of the protein, before and after treatment with EDA. Reaction was performed at room temperature, with 10 μM of purified Mb H64V V68A, 10 mM of styrene, and 20 mM of EDA.



Before reaction



After reaction

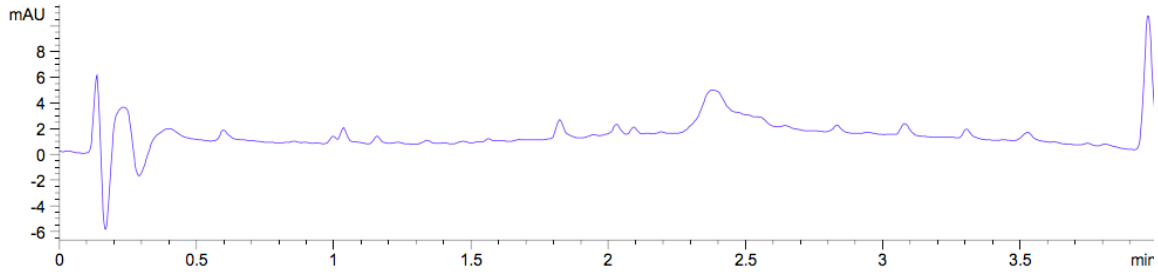


Figure S3. Outcome of intramolecular C–H amination reaction of **S1**, with and without pre-incubation with EDA, and LC-MS monitoring of heme content of the protein, before and after treatment with EDA. P-I263F = P450-BM3 V78A F87V T175I A184V S226R H236Q E252G I263F T268A A290V L353V I366V C400S T438S E442K, this enzyme was previously reported by our laboratory to catalyze regioselective intramolecular C–H amination on **S1** (see Hyster, T. K.; Farwell, C. C.; Buller, A. R.; Arnold, F. H. *J. Am. Chem. Soc.* **2014**, *136*, 15505–15508). Reaction was performed at room temperature with 10 μM of purified P-I263F, 10 mM of EDA, 2.5 mM of **S1**, and 10 mM of NADPH.

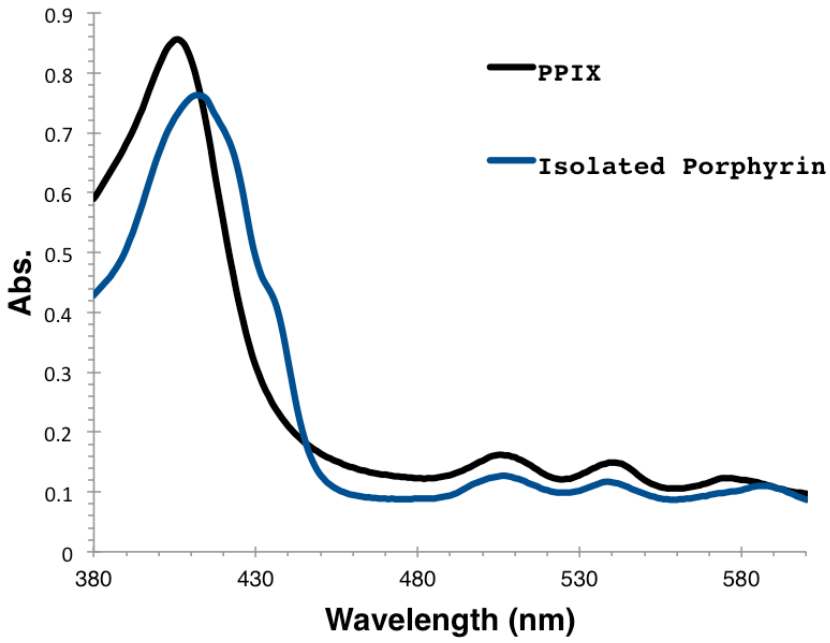


Figure S4. Comparison of absorption spectra of isolated porphyrin species from BM3-HStar after cyclopropanation reaction with that of authentic protoporphyrin IX (PPIX) standard.

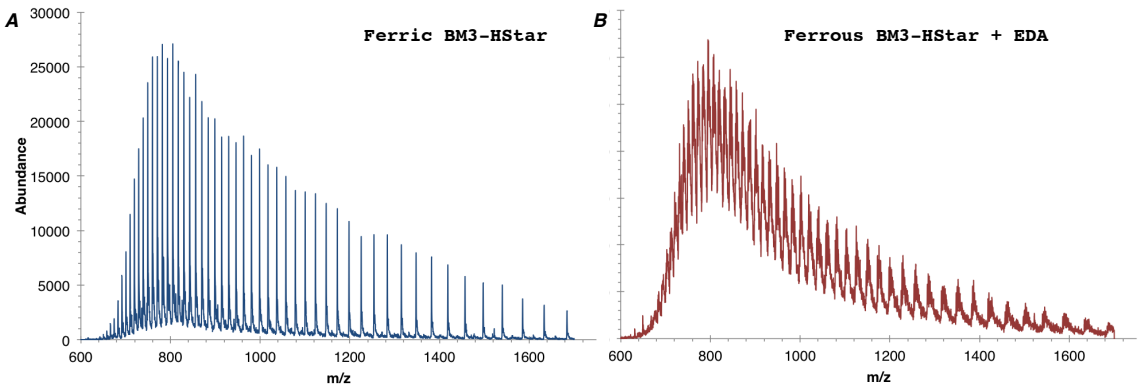


Figure S5. Intact mass spectra of unreacted BM3-HStar heme domain (left) and at the end of reaction with **1** and EDA (right), exhibiting extreme heterogeneity. Mass spectra can be deconvoluted to reveal the presence of multiply-modified protein species.

Table S5. Representative deconvolution of purified, intact protein[a]

Expected mass (Da)	*Observed mass (Da)*	*ppm error*	*EDA equivalents*
53,866	53,871	93	0

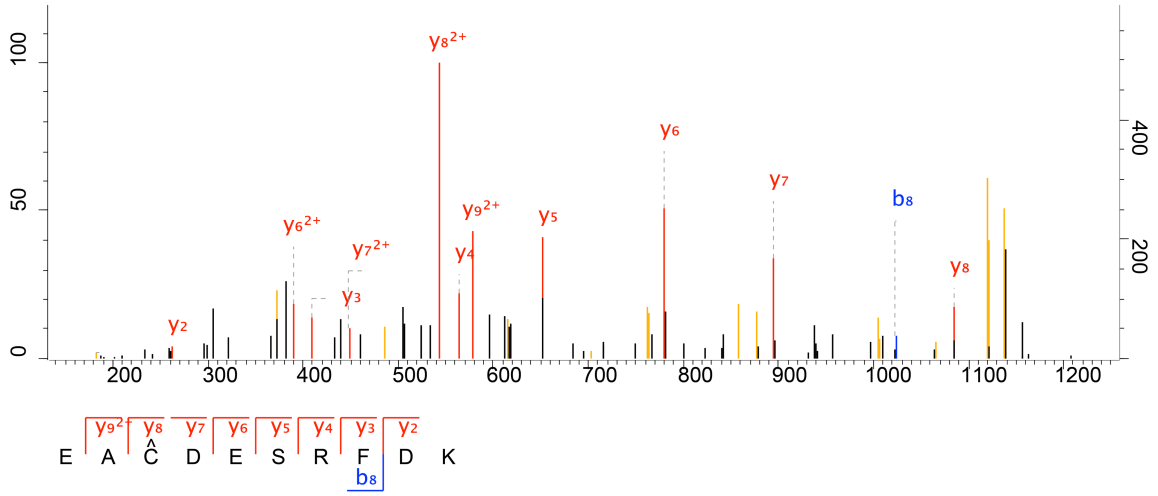
[a] Error on the LC-MSD SL 1100 series is approximately 100 ppm, or ± 5.4 Da in a 54000 Da protein.

Table S6. Representative deconvolution of EDA-treated, intact protein[a]

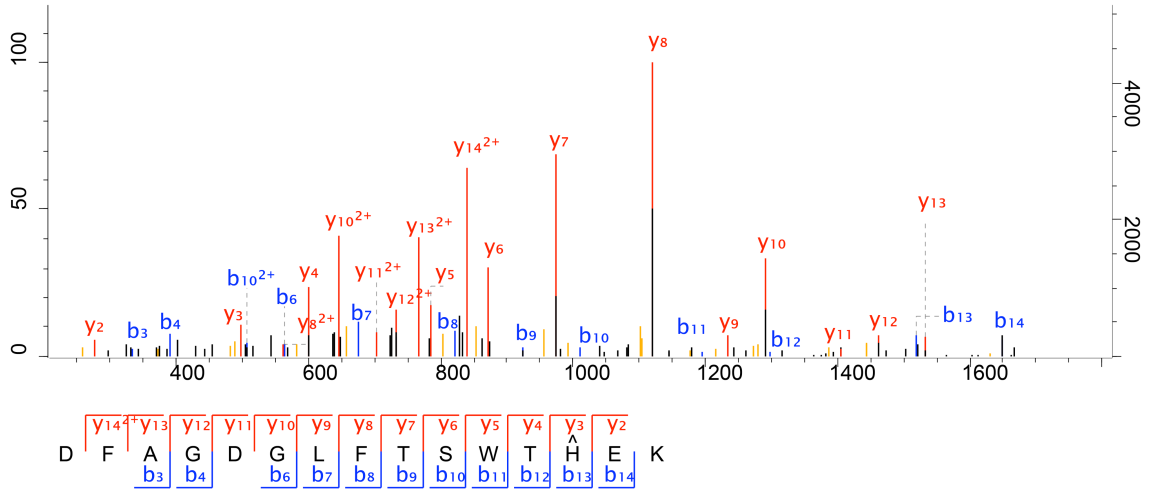
Expected mass (Da)	*Observed mass (Da)*	*ppm error*	*EDA equivalents*
53,866	53,870	74	0
53,953	53,953	3	1
54,039	54,040	17	2
54,125	54,125	4	3
54,211	54,212	24	4
54,297	54,300	64	5
54,383	not detected	not determined	6
54,469	54,477	147	7

[a] Error on the LC-MSD SL 1100 series is approximately 100 ppm, or ± 5.4 Da in a 54000 Da protein.

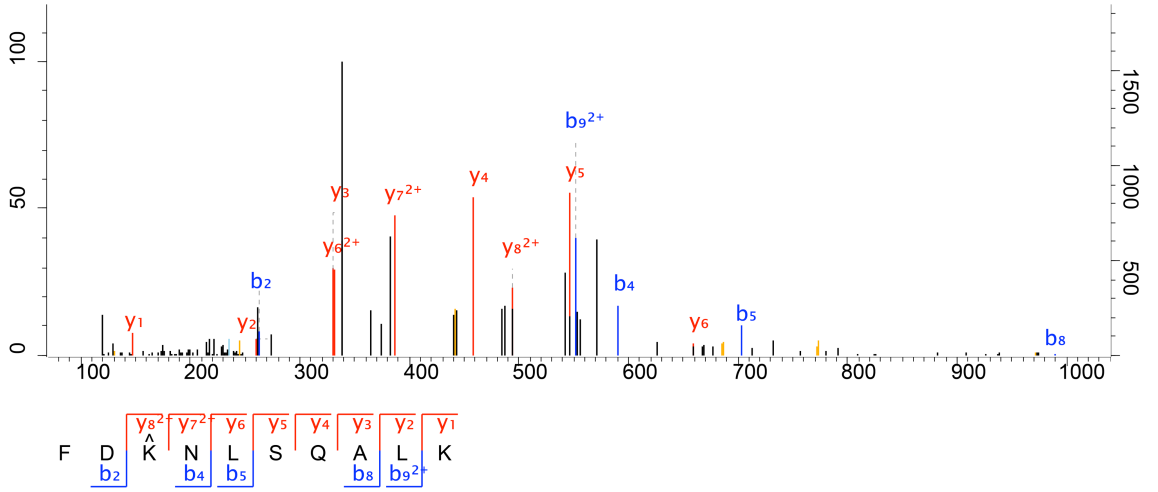
C62 modification



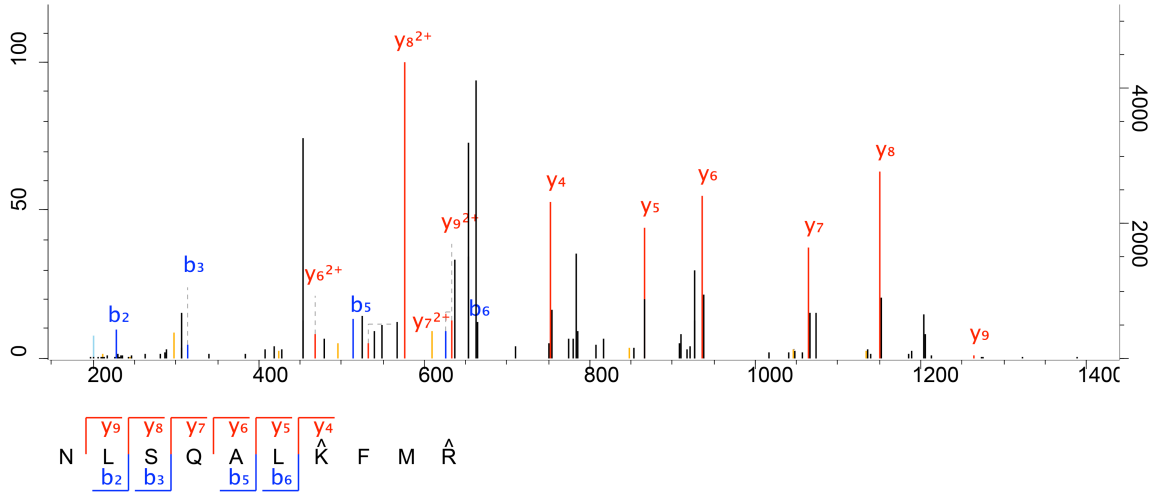
H92 modification



K69 modification



K76 and R79 modifications



C62, K69, and S72 modifications

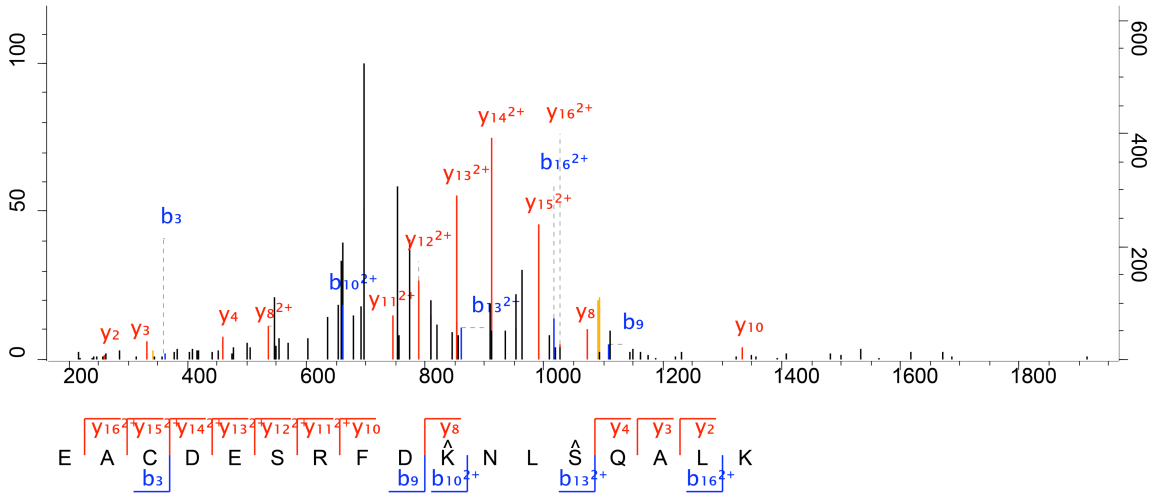


Figure S6. MS/MS spectra of modified peptide fragments from the cyclopropanation reaction mixture with BM3-HStar after trypsin digestion. "□" indicates site of modification.

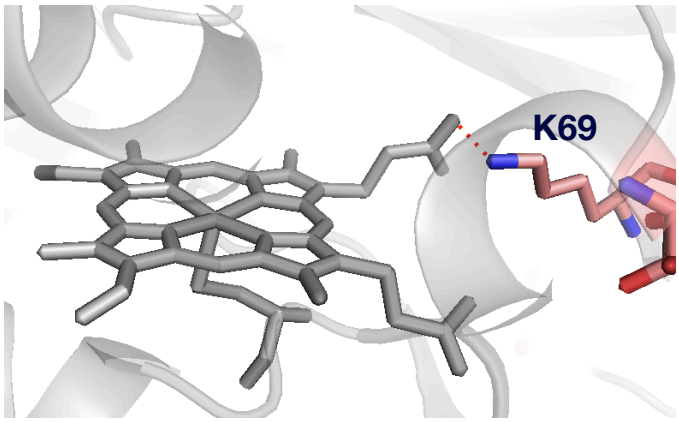


Figure S7. Polar contact between K69 and the propionate side-chain of the heme in P450-BM3.

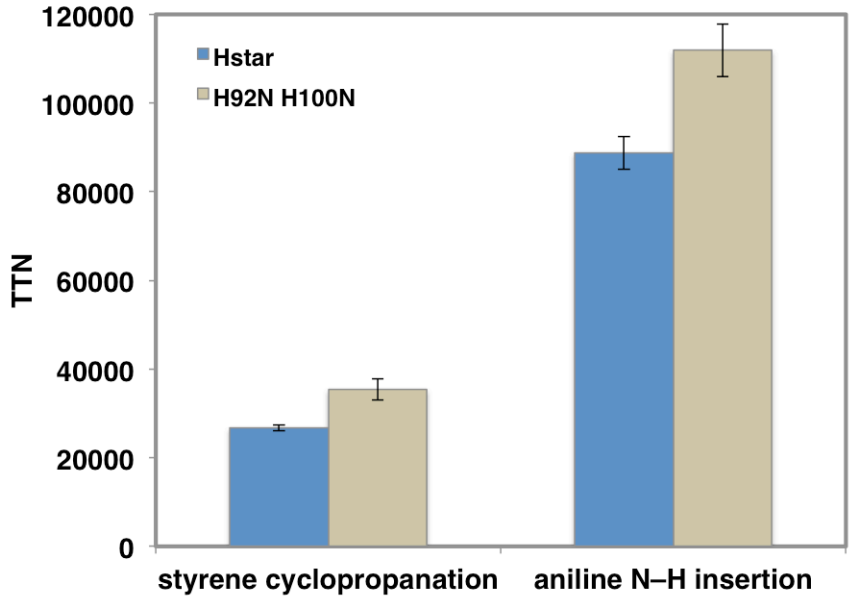


Figure S8. Comparison of TTNs for styrene cyclopropanation and carbenoid insertion into N–H bond of aniline between HStar and HStar H92N H100N. Reaction conditions for cyclopropanation: whole *E. coli* cells expressing the enzyme variant (suspension in M9-N, OD_{600} = 10), 10 mM of styrene, 10 mM of EDA, room temperature, 2 h. Reaction conditions for N–H insertion: whole *E. coli* cells expressing the enzyme variant (suspension in M9-N, OD_{600} = 10), 20 mM of aniline, 10 mM of EDA, room temperature, 2 h.

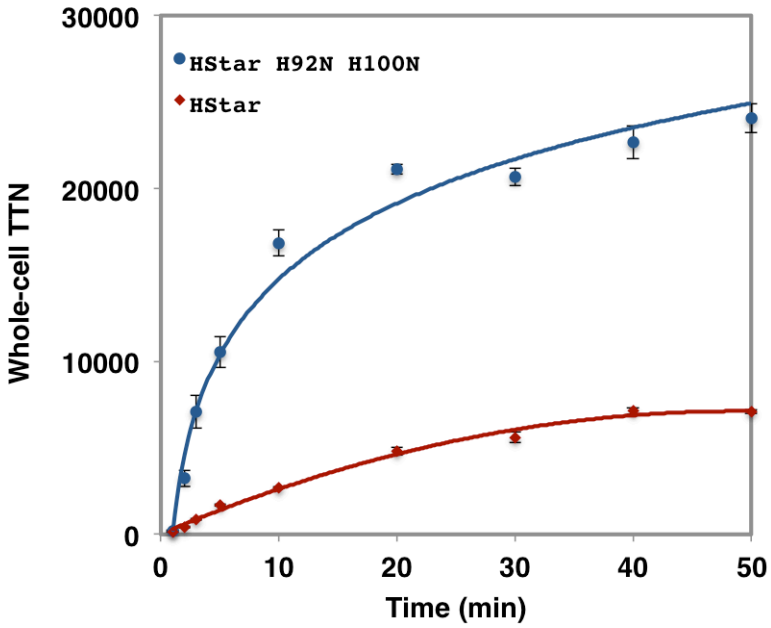


Figure S9. Time-course of cyclopropanation of **1** with EDA using whole *E. coli* cells expressing BM3-HStar variants. Reaction conditions: whole *E. coli* cells expressing enzyme variants (OD_{600} = 10, 0.2 μM enzyme), 10 mM of **1**, 20 mM of EDA, room temperature.

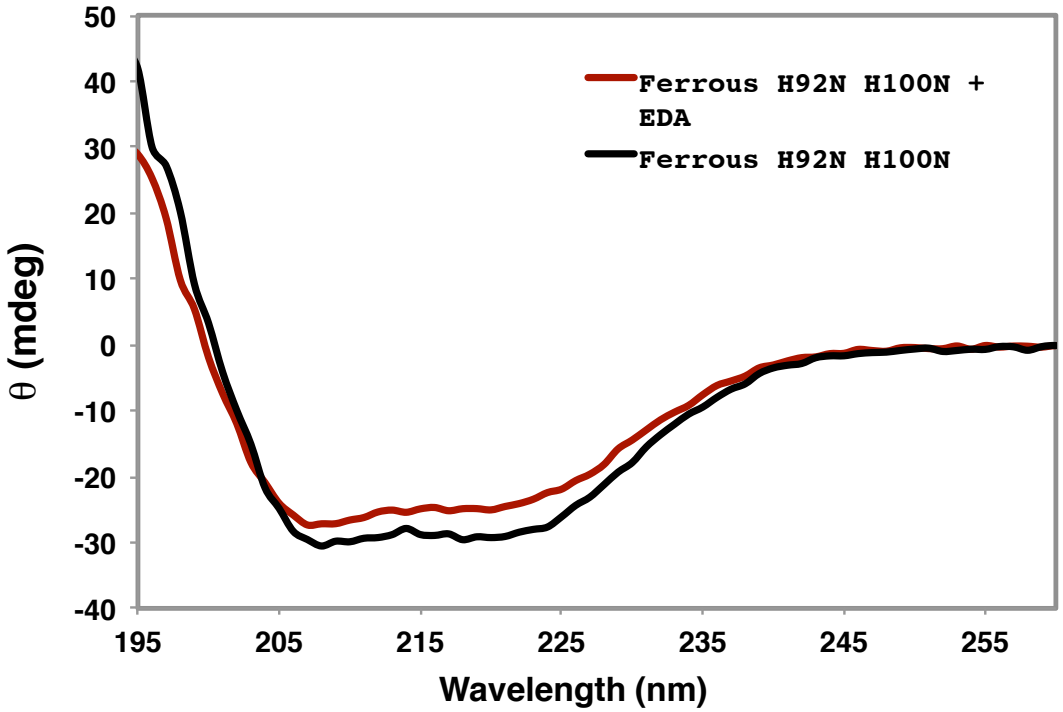
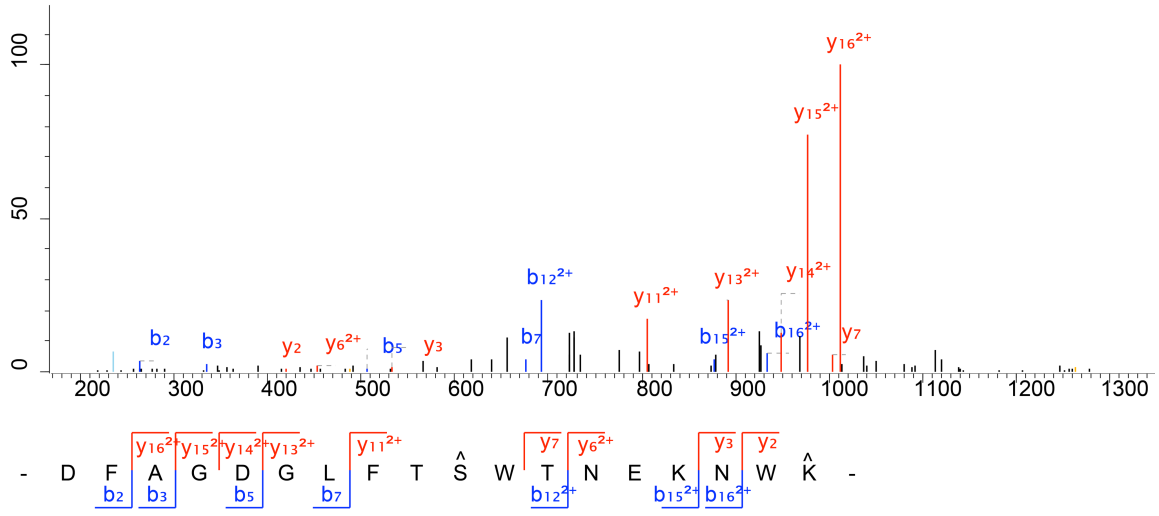
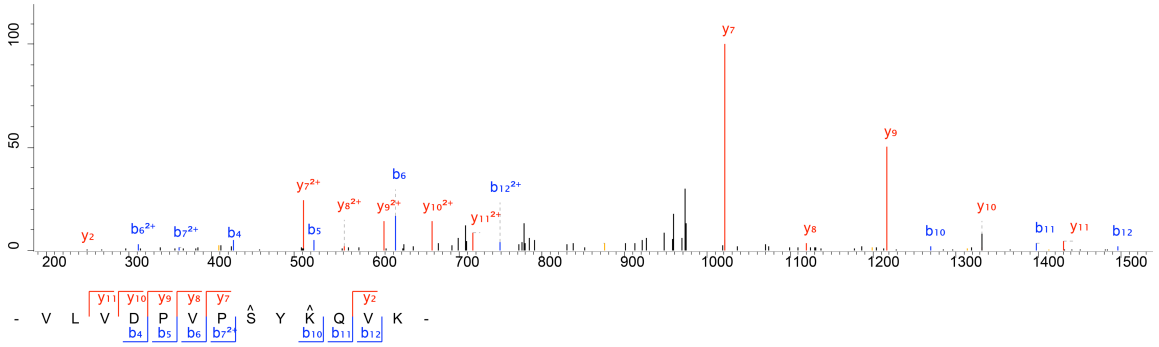


Figure S10. Circular dichroism (CD) spectra of ferrous BM3-HStar H92N H100N before (black) and after reaction with EDA (red).

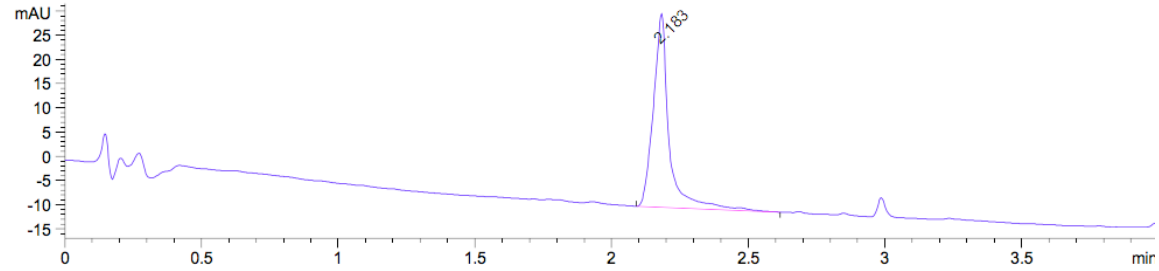
S89 and K97 modifications



S304 and K306 modifications



LCMS analysis of heme modification, before reaction



LCMS analysis of heme modification, after reaction

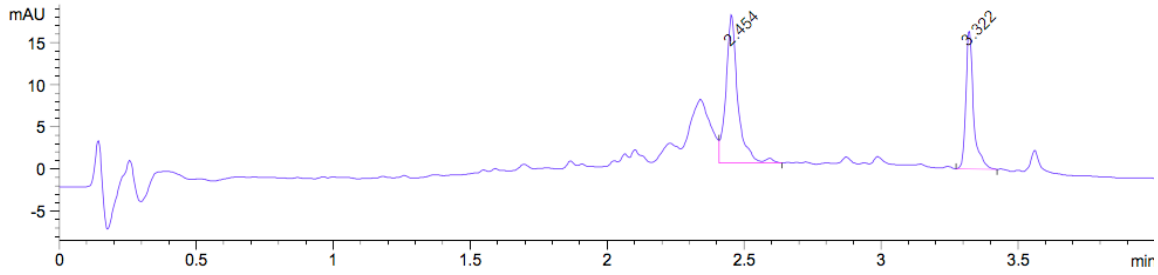


Figure S11. (Top panels) MS/MS spectraof modified peptide fragments from the cyclopropanation reaction mixture with BM3-HStar H92N H100N after trypsin digestion. New modification sites at residues S89, K97, S304, and K306 were observed even though residues N92 and N100 were not modified in the reaction. Additionally, modification of heme cofactor was also observed during the reaction. (Bottom panels) LC-MS chromatograms of the organic extract from BM3-HStar H92N H100N before and after reaction with EDA.

References

1. Wang, Z. J.; Renata, H.; Peck, N. E.; Farwell, C. C.; Coelho, P. S.; Arnold, F. H. *Angew. Chem. Int. Ed.* **2014**, *53*, 6810–6813.