Supporting Information for

Cytochrome P450-catalyzed insertion of carbenoids into N-H bonds

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I. 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. The diazo substrate for product **7** (2-diazo-N,N-diethylacetamide) was synthesized by the method described by Chanthamath.[1] 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 either a Varian Mercury 300 spectrometer (300 MHz and 75 MHz, respectively), or a Varian Inova 500 MHz (500 MHz and 125 MHz, respectively), and are internally referenced to residual solvent peak for chloroform. Data for ^{1}H NMR are reported in the conventional form: chemical shift (δ ppm), multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad), coupling constant (Hz), integration. High-resolution mass spectra were obtained with a JEOL JMS-600H High Resolution Mass Spectrometer at the California Institute of Technology Mass Spectral Facility. Reactions were monitored using thin layer chromatography (Merck 60 silica gel plates) using an UV-lamp, $KMnO_4$ or I_2 for visualization. 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. Gas chromatography mass spectrometry (GC-MS) analyses were carried out using a Shimadzu TQ8030 GC-MS with ion count detector and J&W HP-5 column (30 m x 0.32 mm, 0.25 μM film).

Plasmids pCWori[BM3] and pET22 were used as cloning vectors. Site-directed mutagenesis was accomplished by standard overlap mutagenesis using primers bearing desired mutations (IDT, San Diego, CA). Restriction enzymes BamHI, EcoRI, XhoI, Phusion polymerase, and T4 ligase were purchased from New England Biolabs (NEB, Ipswich, MA). Alkaline phosphatase was obtained from Roche (Nutley, NJ). The 1,000x trace metal mix used in expression cultures contained: 50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM $MnSO_4$, 10 mM $ZnSO_4$, 2 mM $CoSO_4$, 2 mM $CuCl_2$, 2 mM $NiCl_2$, 2 mM Na_2MoO_4, and 2 mM H_3BO_3.

II. General Procedures

CO binding assay. Two cuvettes containing P450 of unknown concentration were prepared. Carbon monoxide was gently bubbled through one solution for 30 seconds and $Na_2S_2O_4$ (1-3 mg) was added immediately. $Na_2S_2O_4$ (1-3 mg) was added to the other cuvette as well and both were sealed with parafilm. P450 concentration was determined from ferrous CO binding difference spectra between the two samples using extinction coefficients of $\varepsilon_{450\text{-}490}$ = 91 mM^{-1} cm^{-1} for cysteine-ligated BM3.[3]

P450 expression and purification. For the enzymatic transformations, P450 variants were used in purified form. One liter TB_{amp} was inoculated with an overnight culture (25 mL, TB_{amp}) of recombinant *E. coli* BL21 cells harboring a pCWori or pET22 plasmid encoding the P450 variant under the control of the *tac* promoter. The cultures were shaken at 200 rpm at 37 °C for roughly 3.5 h or until an optical of density of 1.2-1.8 was reached. The temperature was reduced to 25 °C and the shake rate was reduced to 180 rpm for 20 min, then the cultures were induced by adding IPTG and aminolevulinic acid to a final concentration of 0.5 mM. The cultures were allowed to continue for another 20 hours at this temperature. Cell were harvested by centrifugation (4 °C, 15 min, 3,000xg), and the cell pellet was stored at -20 °C for at least 2 h.

For the purification of untagged proteins, the thawed cell pellet was resuspended in 25 mM Tris.HCl buffer (pH 7.5 at 25 °C) and cells were lysed by sonication (2x1 min, output control 5, 50% duty cycle; Sonicator, Heat Systems - Ultrasonic, Inc.). Cell debris was removed by centrifugation for 20 min at 4 °C and 27,000xg and the supernatant purified using anion exchange chromatography on a Q Sepharose column (HiTrapTM Q HP, GE Healthcare, Piscataway, NJ) on an AKTAxpress purifier FPLC system (GE healthcare). The P450 was eluted from the Q column by running a gradient from 0 to 0.5 M NaCl over 10 column volumes (P450 elutes at 0.35 M NaCl). The P450 fractions were collected and concentrated using a 30 kDa molecular weight cut-off centrifugal filter and buffer-exchanged with 0.1 M phosphate buffer (pH = 8.0). 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, 0.5 mL/gcw) and lysed by sonication (2x1 min, output control 5, 50% duty cycle). The lysate was centrifuged at 27,000xg for 20 min at 4 °C to remove cell debris. The collected supernatant was first 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 30 kDa molecular weight cut-off centrifugal filter. Protein concentrations were determined by CO-assay as described above. For storage, proteins were portioned into 300 µL aliquots and stored at -80 °C.

Synthesis of product standards. N-H insertion product standards were prepared independently from reaction of diazo compounds and amines in the presence of hemin using 1% catalyst loading and compared to known ^1H NMR data.[2] A typical procedure is as follows:

A large crimp vial containing hemin (30 µL of 50 mM stock solution) and 300 µL DMSO was flushed with Ar for 5 min. A solution of sodium dithionite (30 mg in 3 mL phosphate buffer, 0.1 M, pH 8) was simultaneously purged with Ar by bubbling for the same amount of time. The dithionite solution was transferred via syringe to the crimp vial containing hemin. The amine substrate and EDA were added neat, and the mixture was shaken gently at 35 rpm for

at least 4 h. The crude mixture was extracted with 50% ethyl acetate, 50% hexanes mixture (3x, 1.5 mL each). The organics were filtered through a silica plug to remove residual hemin, washed with water, dried over sodium sulfate, then concentrated. The crude product was purified by chromatography on EtOAc/hexanes. The purity of the isolated product was confirmed by ^{1}H NMR and GC. Comparison of the retention times of the standards synthesized by this method were used to confirm the identity of enzyme-prepared N-H insertion products.

Small-scale protein reactions. Small-scale (400 µL) reactions were carried out in 2 mL glass crimp vials (Agilent Technologies, San Diego, CA). To an unsealed crimp vial, 60 µL of a P450 solution (67 or 133 µM) was added. The vials were sealed by crimping with a silicone septum. 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. The headspace of the 2 mL vials containing P450 solution were flushed with argon (no bubbling). If multiple reactions were being carried out in parallel, a maximum of 8 vials were connected via cannulae and degassed in series. The buffer/dithionite solution (360 µL) was then added to each reaction vial via syringe, and the gas lines were disconnected from the vials. Via a glass syringe, 10 µL of an 800 mM stock of aniline was added, followed by 10 µL of a 340 mM stock of EDA (both stocks in EtOH). The reaction vials were then placed in a tray on a plate shaker and left to shake at 40 rpm. The final concentrations of the reagents were typically: 20 mM aniline, 8.5 mM EDA, 10 mM $Na_2S_2O_4$, and 10 or 20 µM P450. After 12 h at 25 °C, the vials were removed from the shaker and uncapped and 1 mL of cyclohexane was added, followed by 20 µL of a 20 mM solution of phenethyl alcohol (internal standard). The mixture was transferred to a 1.5 mL Eppendorf tube, vortexed, and centrifuged (13,000x rpm, 1 min.). The organic layer was dried over sodium sulfate if necessary then analyzed by GC or GC-MS.

Preparative scale reactions. 250 µL of a 400 µM solution of H2-5-F10 heme domain 6XHis (0.1% protein loading) was added to a 6 mL crimp vial and degassed by flowing with argon. 3.7 mL of phosphate buffer containing 17.4 mg of sodium dithionite was bubbled with argon for 15 minutes, then added to the vial containing the protein via syringe. Via a glass syringe, 9.3 µL of neat aniline was added, followed by 13.4 µL of an 85% EDA solution. Final concentrations: 25 µM H2-5-F10, 25 mM aniline and EDA, 25 mM dithionite. The reaction was shaken overnight at 25 °C then uncapped and extracted with ethyl acetate (3x, 3 mL each). The organics were concentrated and chromatographed on SiO_2, eluting with 8-10% EtOAc/hexanes to afford white flaky solids (11.7 mg, 65% yield).

Small scale whole cell reactions. E. coli (BL21) cells coding for H2-5-F10 holo were grown from glycerol stock overnight (37 °C, 250 rpm) in 5 ml Hyperbroth medium (1 L Hyperbroth prepared from powder from AthenaES©, 0.1 mg mL^{-1} ampicillin). The pre-culture was used to inoculate 45 mL of Hyperbroth medium in a 125 mL Erlenmeyer flask and this culture was incubated at 37 °C, 200 rpm for 2 h and 30 min. At OD_{600} = 1.2, the cultures were cooled to 25 °C and the shaking was reduced to 160 rpm before inducing with IPTG (0.25 mM) and δ-aminolevulinic acid (0.25 mM). Cultures were harvested after 20 h and resuspended (OD_{600} = 30) in nitrogen-free M9 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 $CaCl_2$, 1 mL micronutrients). The micronutrient solution contains 0.15 mM $(NH_4)_6Mo_7O_{24}$, 20.0 mM H_3BO_3, 1.5 mM $CoCl_2$, 0.5 mM $CuSO_4$, 4.0 mM $MnCl_2$, and 0.5 mM

$ZnSO_4$. Aliquots of the cell suspension were used for determination of the P450 expression level (4 mL) after lysis.

E. coli cells (OD_{600} = 30, 425 µL, 7.1 µM P450) were made anaerobic by bubbling argon through the cell suspension in a crimped 2 mL vial. A degassed solution of glucose (50 µL, 20 mM) was added to the cells before adding EDA (12.5 µL of a 400 mM solution in MeOH) and olefin (12.5 µL of a 1.2 M solution in EtOH). The reactions were stirred at room temperature for 8 h after which, 20 µL of the internal standard (20 mM 2-phenylethanol) was added, followed by 1 mL of cyclohexane. The mixture was transferred to a 1.5 mL Eppendorf tube, vortexed, and centrifuged (13,000x rpm, 1 min). The organic layer was dried over Na_2SO_4 before analyzing the product mixture by GC. P450 concentration in crude lysate was determined by CO-assay (6.0 µM) and used to determine turnover number. Product **1** was formed in 38% yield and 538 TTN.

N-H insertion using variants of P450-BM3. All proteins described herein were expressed as the holo protein containing heme and fused reductase domains. Reactions were performed following the general procedure for small scale reactions using a 133 µM stock solution, such that the final protein concentration is 20 µM. CO controls were conducted by bubbling CO for 1 min through the protein and dithionite solution prior to addition of substrates, while under anaerobic atmosphere. Aerobic controls were conducted without sealing the crimp vials.

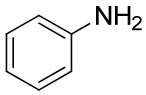
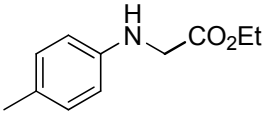
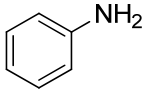
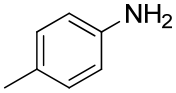
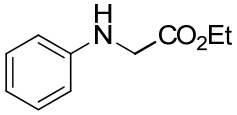
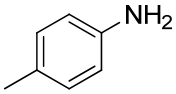
Reductant screen. The reactions performed herein were all conducted with variant BM3-CIS, following the general procedure for small scale reactions using a 67 µM stock solution, such that the final protein concentration was 10 µM. When NAD(P)H was used as a reductant, dithionite was omitted and general procedure was modified such that a solution of NAD(P)H (12.5 mM) in phosphate buffer was degassed then used, with the final concentration of NAD(P)H at 10 mM. For reaction with only 0.25 equivalents of $Na_2S_2O_4$, the $Na_2S_2O_4$ stock solution was diluted 4x then degassed and used as described previously.

Table S.1. Screen of reductants for N-H insertion.

Reductant	Final conc	Yield
$Na_2S_2O_4$	10 mM	43%
$Na_2S_2O_4$	2.5 mM	32%
NADPH	10 mM	2%
NADH	10 mM	2%

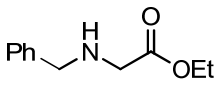
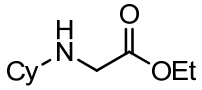
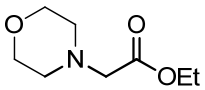
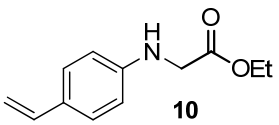
Substrate inhibition. These reactions were all conducted with BM3-CIS holo or H2-5-F10 heme domain 6XHis as listed, in Table S.2 following the general procedure for small scale reactions. For substrate inhibition experiments with BM3-CIS, 1.0 equiv of ethyl 2-(*p*-tolylamino)acetate was added to a mixture of aniline, EDA, $Na_2S_2O_4$ and protein, and the mixture was then shaken at room temperature for 4 h. For substrate inhibition experiments with H2-5-F10, ethyl 2-(phenylamino)acetate was added to a mixture of toluidine, EDA, $Na_2S_2O_4$ and protein, and the mixture was then shaken at room temperature for 4 h.

Table S.2. Screen for substrate inhibition in P450-catalyzed N-H insertion.

Protein	Substrate	Additive	Yield
BM3-CIS			48%
BM3-CIS		none	43%
H2-5-F10			69%
H2-5-F10		none	70%

Investigation of substrate scope. The reactions performed herein were all conducted with H2-5-F10 heme domain 6XHis following the general procedure for small scale reactions. Products **1-4** and **7** were analyzed with the following method: 90 °C (hold 2 min), 90-190 °C (6 °C/min), 190-230 °C (40 °C/min). Products **5, 6**, **8**, and **10** were analyzed with the following method: 100 °C (hold 1 min), 100-140 °C (6 °C/min), 140-260 °C (20 °C/min), 260 °C (hold 3 min). Elution time and turnover number of each product is shown in Table S.3. The yield and TTN of product **9** was determined by NMR after preparative scale reaction.

Table S.3. Elution times and turnover numbers of N-H insertion products.

Product	Elution Time	TTN	Product	Elution Time	TTN
1	9.8 min	291	**5**	9.9 min	157
2	10.1 min	354	**6**	10.4 min	251
3	10.7 min	130	**7**	14.2 min	214
4	11.6 min	296	**8**	11.3 min	155
	10.1 min	14		15.6 min	0
	13.7 min	6	 **10**	10.3 min	317

Products **1-8** have previously been reported and characterized.[1,2] Product **10** was made on preparative scale following general procedure for preparative scale reactions as a pale yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.27 (m, 2H), 6.61 (dd, J = 17.6, 10.9 Hz, 1H), 6.58 – 6.55 (m, 2H), 6.23 (s, 1H), 5.54 (dd, J = 17.6, 1.0 Hz, 1H), 5.03 (dd, J = 10.9, 1.0 Hz, 1H), 4.25 (qd, J = 7.1, 3.0 Hz, 2H), 3.91 (dd, J = 3.1, 1.3 Hz, 2H), 1.31 (td, J = 7.1, 6.2 Hz, 3H). ^{13}C NMR (126 MHz, cdcl3) δ 171.1, 165.4, 146.9, 136.6, 129.9, 128.1, 127.5, 113.0, 110.1, 61.5, 61.4, 45.9, 14.3, 14.2.

Double versus single insertion: 10 µL of a 100 mM solution of hemin in DMSO (1% catalyst loading) was added to an additional 500 µL of DMSO in a 1.5 mL crimp vial and degassed by bubbling with argon. In a crimp vial, a solution of 17.4 mg of dithionite in 500 µL of phosphate buffer was degassed by bubbling with argon, then added to the hemin solution in DMSO via syringe. Via a glass syringe, 9.3 µL (0.1 mmol) of aniline was added neat, followed by 13.4 µL (0.1 mmol) of an 85% solution of EDA. The reaction was shaken overnight at 25 ^{o}C, then uncapped and extracted with a 50% ethyl acetate, 50% hexanes mixture (3x, 1.5 mL each). The organics were filtered through a silica plug, washed with water, dried over sodium sulfate, then concentrated. ^{1}H NMR of the crude mixture was used to determine the ratio of single and double addition products, using the diagnostic peaks at 3.90 ppm (s, 2 H) and 4.13 (s, 4 H), respectively. For product **1**, the products were formed in with a single:double insertion ratio of 3.5:1 with 50% conversion. For product **7**, the ratio of single:double insertion was 2.5:1. The single insertion products (**1** and **7**) were isolated specifically using column chromatography on SiO_2, eluting with hexanes/ethyl acetate.

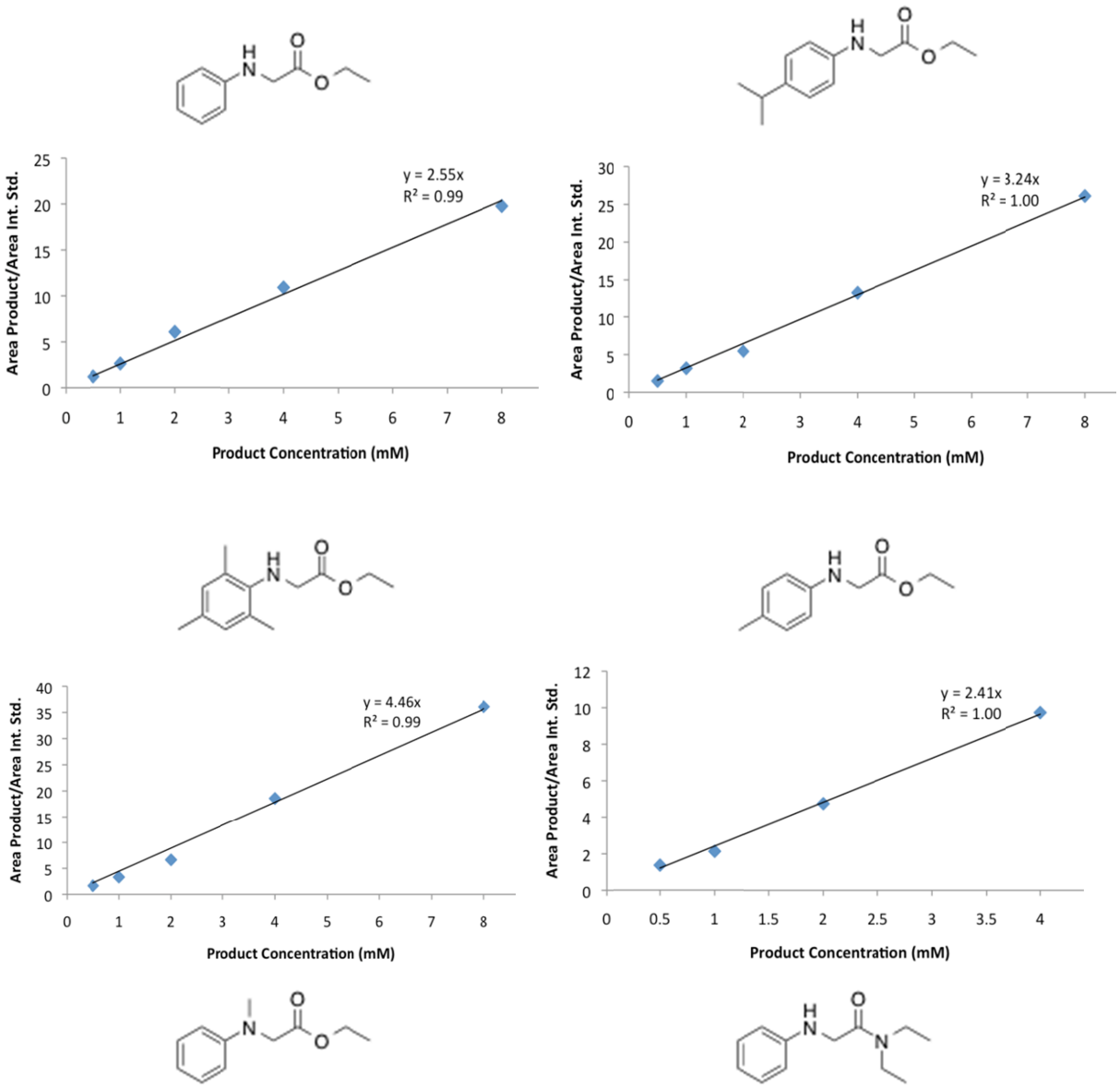
Competition experiments with cyclopropanation. Following the general procedure for small scale reactions, H2-5-F10 heme domain 6XHis was used. EDA, aniline, and styrene were all added in a 1:1:1 ratio such that the final concentration of each substrate was 10 µM. Two reactions were performed in parallel at room temperature for 1 h and 2 h. GC using calibration curves for **1** and ethyl 2-phenylcyclopropanecarboxylate (cyclopropane product) were used to determine conversions for N-H insertion and cyclopropanation, respectively. The relative rate of N-H insertion to cyclopropanation was determined by taking a ratio of the yield of **1** to the yield of ethyl 2-phenylcyclo-propanecarboxylate.[4]

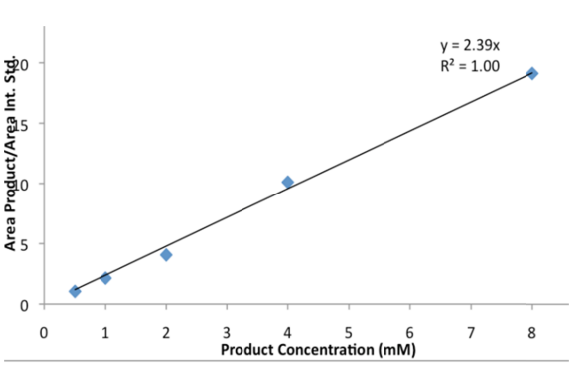
Table S.4. Competition experiment using aniline and styrene. The yield of N-H insertion versus cyclopropanation product formed was used to determine the relative rate of reaction.

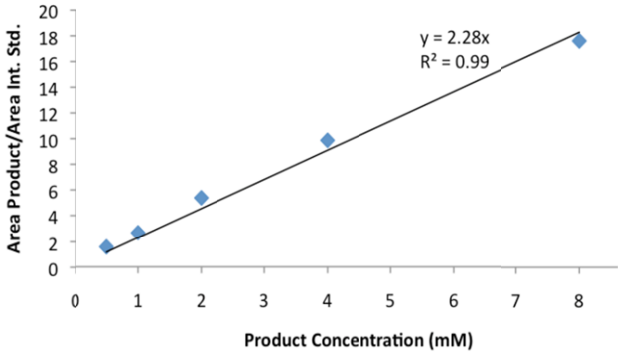
Time of Reaction	Yield of **1** (N-H insertion)	Yield of cyclopropane product (cyclopropanation)
1 h	63%	9%
2 h	60%	9%

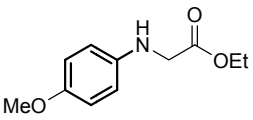
Calibration curves. Yields of N-H insertion products were determined using calibration curves made with independently synthesized standards. Two stock solutions of product were made at 160 mM and 40 mM. To 4-5 samples containing 380 µL of buffer, product was added from either of the stock stolutions such that a final concentration of 0.5-8.0 mM in 400 µL was obtained. To each Eppendorf tube, 20 µL of internal standard was added, followed by 1 mL of

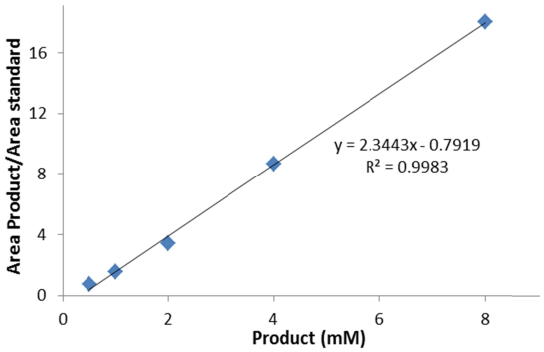
cyclohexane was added. The tubes were vortexed and centrifuged (13,000 x rpm, 30 seconds). The organic layer was then analyzed by GC. The ratio of the areas under the internal standard and product peaks was plotted against the concentration for each solution (0.5, 1, 2, 4, and 8 mM).

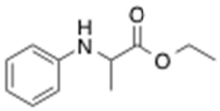


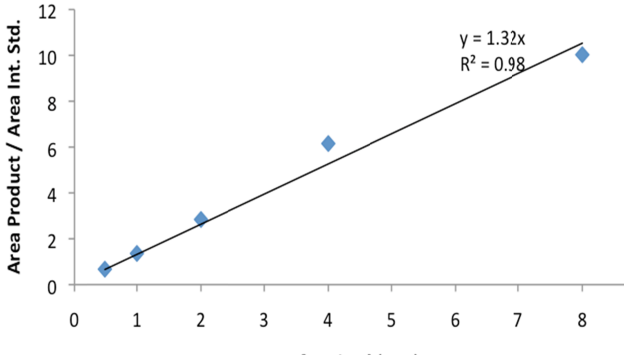












III. Amino Acid Sequences

Table S.5. Amino acid sequences of mutants, relative to wild type BM3 (WT). All mutations listed below are for the heme domain. There are no mutations present in the reductase domain relative to wild type.

Enzyme	Amino acid substitution with respect to WT
T268A	T268A
P411-T268A	T268A, C400S
9-10A TS	V78A, P142S, T175I, A184V, S226R, H236Q, E252G, A290V, L353V, I366V, E442K
BM3-CIS	**9-10A TS** + F87V, T268A
P411-CIS	**9-10A TS** + F87V, T268A, C400S
H2-A-10	**9-10A TS** + F87V, L75A, L181A, T268A

H2-4-D4	**9-10A TS** + F87V, L75A, M177A, L181A, T268A, L437A,
H2-5-F10	**9-10A TS** + F87V, L75A, I263A, T268A, L437A

IV. References

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