Improved cyclopropanation activity of histidine-ligated cytochrome P450 enables enantioselective formal synthesis of levomilnacipran

*Z. Jane Wang[+], Hans Renata[+], Nicole E. Peck, Christopher C. Farwell, Pedro S. Coelho and Frances H. Arnold**

Contribution from the Division of Chemistry and Chemical Engineering, California Institute of Technology

1200 E California Blvd, MC 210-41, Pasadena, California 91125

SUPPLEMENTARY MATERIAL

Table of Contents

Materials and Methods....page SI-2
General Procedures....page SI-3
Spectrochemical Characterization of AxX Proteins....page SI-6
Monitoring Reaction of EDA with Styrene....page SI-10
Whole Cell Reaction of **1** with EDA....page SI-11
In Vitro Reaction of **1** with EDA....page SI-12
Calibration of **2**....page SI-12
Site Saturation Mutagenesis of T268A-AxH....page SI-13
Monitoring Reaction of **1** with EDA....page SI-14
Amino Acid and Nucleotide Sequences....page SI-15-16
Experimental Procedures for Formal Synthesis of Levomilnacipran....page SI-16–19
- Acrylamide **1**....page SI-16
- Preparative Whole Cell Synthesis of **2**....page SI-17
- Alcohol **3**....page SI-18

References....page SI-20
NMR Spectra of Compounds....page SI-21–27

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. 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 126 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 light for visualization and an acidic mixture of anisaldehyde, phosphomolybdic acid, or ceric ammonium molybdate, or basic aqueous $KMnO_4$ as developing agents. 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). Analytical SFC was performed with a Mettler SFC supercritical CO_2 analytical chromatography system utilizing Chiralpak AS column (4.6 mm x 25 cm) obtained from Daicel Chemical Industries, Ltd. Semi-preparative HPLC was performed using an Agilent 1200 series, a UV detector, and an Agilent XDB-C18 column (9.4 mm x 250 mm, 5 μm). Optical rotations were measured with a Jasco P-2000 polarimeter operating on the sodium D-line (589 nm), using a 100 mm path-length cell.

Plasmids pCWori[BM3] and pET22 were used as cloning vectors. Site-directed mutagenesis was accomplished by modified QuickChange protocol 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).

General Procedures

Hemochrome binding assay. A solution of pyridine was made by combining 1.75 mL pyridine with 0.75 mL 1 M NaOH. The solution was mixed at room temperature then 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. Hemoprotein concentration was determined from the absorbance of the hemochrome complex using extinction coefficients of 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.

CO binding assay. CO assay was used to determine the concentration in crude lysate. Two cuvettes containing hemoprotein of unknown concentration were prepared. Carbon monoxide was gently bubbled through one solution for 30 seconds and $Na_2S_2O_4$ (<2 mg) was added immediately. $Na_2S_2O_4$ (2 mg) was added to the other cuvette as well and both were sealed with parafilm. Hemoprotein concentration was determined from ferrous CO binding difference spectrum between the two samples using extinction coefficients of $\varepsilon_{450\text{-}490}$ = 91 mM^{-1} cm^{-1} for cysteine-ligated BM3[1] and $\varepsilon_{411\text{-}490}$ = 103 mM^{-1} cm^{-1} for serine-ligated BM3.[1] The extinction coefficients $\varepsilon_{450\text{-}490}$ for axial Ala, Met, His, and Tyr variants were independently determined as described below.

Enzyme expression and purification. For the enzymatic transformations, enzyme 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 enzyme 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 22 °C and the shake rate was reduced to 130-150 rpm for 20 min, then the cultures were induced by adding IPTG and aminolevulinic acid to a final concentration of 0.25 mM and 0.50 mM, respectively. The cultures were allowed to continue for another 20 hours at this temperature and shake rate. 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.

For the purification of 6XHis tagged enzymes, 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 enzyme 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.

Small-scale in vitro protein reactions (anaerobic). Small-scale (400 µL) reactions were carried out in 2 mL glass crimp vials (Agilent Technologies, San Diego, CA). Enzyme solution (60 µL, 67 µM) was added to an unsealed crimp vial before crimp sealing 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 the enzyme 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 (320 µL) was then added to each reaction vial via syringe, and the gas lines were disconnected from the vials. 10 µL of a stock solution of olefin (400 mM for acrylamide **1**) was added via a glass syringe, followed by 10 µL of a 400 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 35 rpm for 12 h at room temperature. The final concentrations of the reagents were typically: 10 olefin, 8.7 mM EDA, 10 mM $Na_2S_2O_4$, and 10 µM enzyme. The reaction was quenched by the addition of 3 M HCl (25 µL). The vials were uncapped and 1 mL of cyclohexane was added, followed by 20 µL of a 20 mM solution of 2-phenylethanol solution in cyclohexane (internal standard). The mixture was transferred to a 1.5 mL Eppendorf tube and vortexed and centrifuged (10,000x rcf, 30 s). The organic layer was then analyzed by GC.

Small scale whole cell reactions. E. coli (BL21) cells coding for appropriate enzyme variant were grown from glycerol stock overnight (37 °C, 250 rpm) in 5 ml TB_{amp}. The pre-culture was used to inoculate 45 mL of Hyperbroth medium (1 L Hyperbroth prepared from powder from AthenaES©, 0.1 mg mL^{-1} ampicillin) in a 125 mL Erlenmeyer flask and this culture was incubated at 37 °C, 200 rpm for approximately 3 h. At OD_{600} = 1.8, the cultures were cooled to 25 °C and the shaking was reduced to 140

rpm before inducing with IPTG (0.25 mM) and δ-aminolevulinic acid (0.50 mM). 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 $MgSO_4$, 0.010 g $CaCl_2$) until the indicated OD_{600} is obtained (usually OD_{600} = 30 or 60). Aliquots of the cell suspension were used for determination of the enzyme expression level (2–3 mL) after lysis.

Anaerobic conditions: E. coli cells (OD_{600} = 30 or 60) were transferred to a crimped 6 mL vial and made anaerobic by degassing with argon for 5-10 min. In parallel, glucose (50 μL, 25 mM) was added to 2 mL crimp vials that are sealed. The headspaces of these vials were purged with argon for 5-10 min. If multiple reactions were being carried out in parallel, a maximum of 8 vials were connected via cannulae and degassed in series. Cells (425 μL) were transferred to each vial via syringe and the olefin substrate was added (12.5 μL of a 800 mM solution of styrene in EtOH or a 400 mM solution of acrylamide **1** in EtOH), followed by EDA (12.5 μL of a 350 mM or 400 mM solution in EtOH). The reactions were shaken on a table top shake plate at room temperature for the indicated amount of time at 35 rpm. The reactions were quenched by addition of 25 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 2 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.

Aerobic conditions: Cell suspension was used without sparging with argon. Cells (425 μL, OD_{600} = 60) and glucose (50 uL, 25 mM) were combined in an unsealed 2 mL glass vial. The olefin substrate was added (12.5 μL, 400 mM in EtOH), followed by EDA (12.5 μL, 400 mM in EtOH). The vial was covered with foil then shaken at 35 rpm for 1 h. The reactions were quenched by addition of 25 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 2 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.

Mutant Library generation. For amino acid positions 263 and 438, site-saturation mutagenesis was performed using QuickChange PCR method with primers containing NNK codons at the desired positions. For positions 78, 87, 181 and 437, libraries were generated by employing the '22c-trick' method.[2] Primers containing NDT, VHG and TGG at the desired positions were mixed in 12:9:1 ratio

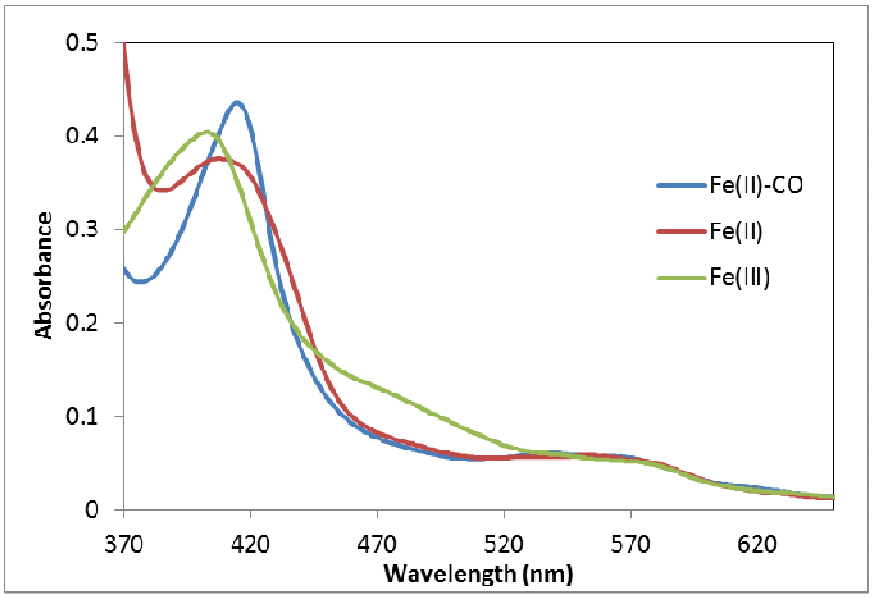
and then used for PCR using standard QuickChange protocol. *E. coli* libraries generated were stored as glycerol stocks at –80 ºC in 96-well plates.

Reaction screening in 96-well plate format. Glycerol stocks from the libraries generated above were used to inoculate a 96-well deep-well plate containing 300 µL TB_{amp} medium using a 96-pin stamp. The cells were incubated at 37 ºC, 200 rpm, and 80% relative humidity overnight. After 16 h, 200 µL of these overnight cultures were transferred into a 2 mL, deep-well plate containing Hyperbroth medium using a multichannel pipette. The cultures were incubated at 37 ºC, 200 rpm for 3 h. After reducing the temperature to 25 ºC, 15 µL of a stock solution containing 0.025 M IPTG and 0.05 M δ-aminolevulinic acid in H_2O was added to each well, and the cultures were allowed to continue for another 20 h at 25 ºC, 160 rpm. Cells were then pelleted and resuspended in 270 µL of nitrogen-free M9 medium (M9-N). A stock solution containing 1.0 mL of 1 M glucose, 1.0 mL of 400 mM acrylamide in EtOH, 1.0 mL of 400 mM EDA in EtOH, 4.0 mL of M9-N was made, and 80 µL of this stock solution was added to each well. To each well was then added 30 µL of glucose oxidase/catalase solution containing 14000 U/mL catalase and 1000 U/mL glucose oxidase. The plate was sealed with aluminum sealing tape and shaken at 35 rpm overnight. After the reaction, the sealing tape was removed and 25 µL of 3 M HCl was added to each well, followed by 700 µL of cyclohexane. The plate was vortexed and centrifuged (5000x rcf, 1 min) and 500 µL aliquots of the organic extracts were transferred into a shallow-well plate for analysis.

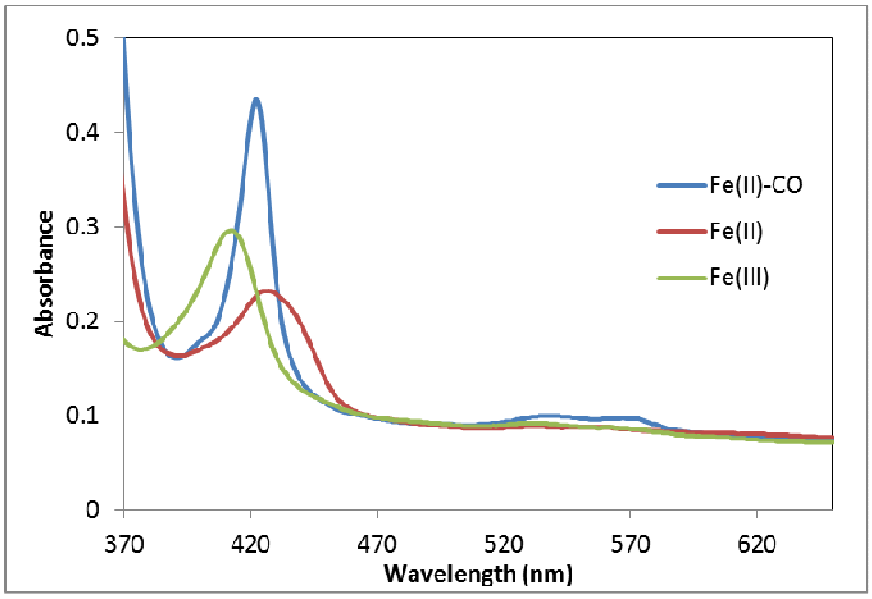
Spectrochemical Characterization of AxX proteins

UV-visible spectrum of AxX proteins: All spectra were recorded from 370-650 nm at protein concentrations of 1-4 µM. Fe(II) spectrum was recorded after addition of 1-2 mg sodium dithionite to a solution of protein in a cuvette. The cuvette was sealed with parafilm. The Fe(II)-CO spectrum was recorded after gently bubbling CO through the protein solution for 30 s, followed by addition of 1-2 mg sodium dithionite. Again, the cuvette was sealed with parafilm.

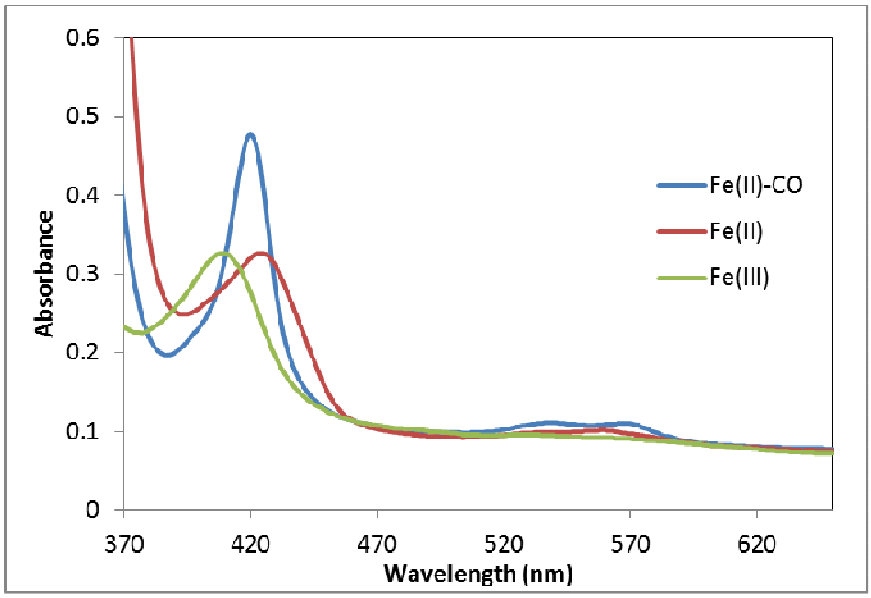
a) Peak max: Fe(III) = 407 nm, Fe(II) = 413 nm, Fe(II)-CO = 415 nm



b) Peak max: Fe(III) = 412 nm, Fe(II) = 427 nm, Fe(II)-CO = 422 nm



c) Peak max: Fe(III) = 409 nm, Fe(II) = 425 nm, Fe(II)-CO = 420 nm



d) Peak max: Fe(III) = 408 nm, Fe(II) = 425 nm, Fe(II)-CO = 417 nm

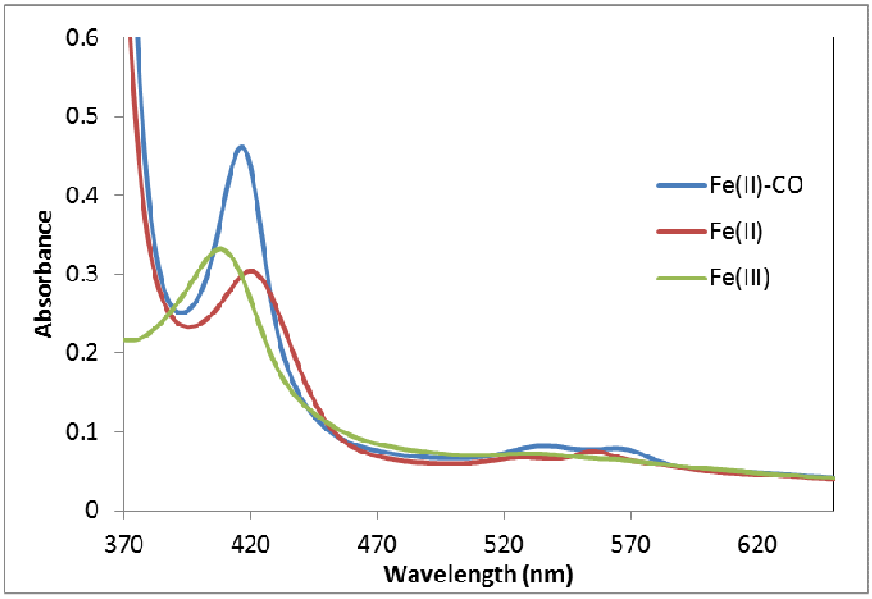


Figure S1. UV-vis spectra of Fe(III) resting state, Fe(II), and Fe(II)-CO complex for variants a) T268A-AxA b) T268A-AxH c) T268A-AxM and d) T268A-AxY.

Determination of $\varepsilon_{max-490}$ for AxX proteins: The holo T268A-AxX protein of each axial mutant (Ala, His, Met, and Tyr) were used. The CO binding difference $\varepsilon_{max-490}$ for each axial mutant was determined by CO binding assay, calibrated to the known enzyme concentration as determined by hemochrome assay. Each value measured is an average of 3 to 7 separate determinations at different enzyme concentrations.

Table S1. Determination of $\varepsilon_{max-490}$ for a) Axial alanine mutants (AxA) b) Axial histidine mutants (AxH) c) Axial methionine mutants (AxM) and d) Axial tyrosine mutants (AxY).

a) AxA mutants:

Hemochrome (A_{418})	[enzyme]	CO difference ($A_{max} - A_{490}$)	$\varepsilon_{max-490}$
0.298	0.002027	0.142	70.1
0.136	0.000925	0.068	73.5
0.076	0.000517	0.043	83.2
0.254	0.001727	0.129	74.7
0.131	0.000891	0.069	77.4
0.064	0.000435	0.040	91.9
		Average:	78.5 $\pm$ 7.9

b) AxH mutants:

Hemochrome (A_{418})	[enzyme]	CO difference ($A_{max} - A_{490}$)	$\varepsilon_{max-490}$
0.072	0.00042	0.094	223.9
0.033	0.000192	0.043	223.5
0.601	0.003884	0.601	154.7
0.306	0.001978	0.360	182.0
0.193	0.001196	0.176	147.2
0.085	0.000527	0.107	203.2
0.098	0.000607	0.131	215.8
		Average:	193 $\pm$ 32

c) AxM mutants:

Hemochrome (A_{418})	[enzyme]	CO difference ($A_{max} - A_{490}$)	$\varepsilon_{max-490}$
0.184	0.001251	0.167	133.5
0.086	0.000585	0.083	141.9
0.045	0.000306	0.048	156.8
		Average:	144 ± 12

d) AxY mutants:

Hemochrome (A_{418})	[enzyme]	CO difference ($A_{max} - A_{490}$)	$\varepsilon_{max-490}$
0.172	0.00117	0.168	143.6
0.086	0.000585	0.085	145.3
0.037	0.000252	0.042	166.9
0.156	0.001061	0.139	131.0
0.079	0.000537	0.079	147.0
0.037	0.000252	0.041	162.9
		Average:	149 ± 13

Monitoring reaction of EDA with styrene catalyzed by T268A-AxX mutants

Following the procedure for small scale whole cell reactions, whole cells expressing T268A-AxA, T268A-AxH, T268A-AxM, T268A-AxY, T268A (axial Cys) and T268A-P411 (axial Ser) were grown and resuspended to OD_{600} = 60. To determine the enzyme concentration of each sample, 1 mL of each cell stock was removed, diluted with 1 mL of phosphate buffer, and then analyzed by CO assays following the general procedure outlined above. The optical density of the cells was adjusted such that the final enzyme concentration of all T268A-AxX mutants was 2.0 μM and the concentration of T268A (axial Cys) and T268A (axial Ser) was 2.7 μM.

For each mutant, a series of reactions was set up following the general procedure for anaerobic whole cell reactions. The substrates (12.5 μL styrene at 800 mM and 12.5 μL EDA at 350 mM) were added to each sample at time 0 min. Time points were taken at 5, 10, 20, 30, and 45 and all measurements were performed in duplicate or triplicate. At each specified time point, the reaction was worked up using procedure described in the small scale whole cell reactions and then submitted for GC

analysis on J&W HP-5 column (30 m x 0.32 mm, 0.25 μM film) using 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, *cis* product 8.97 min, *trans* product 9.99 min). Error was determined based on the standard deviation of independent experiments at each time point.

Table S2. Percent yield for reaction of styrene and EDA catalyzed by T268A-AxX mutants as a function of time.

	% Average Yield					
Time (min)	T268A-AxA	T268A-AxH	T268A-AxM	T268A-AxY	T268A	T268A-AxS
5	1.3	9.9	1.1	2.8	0.3	0.9
10	3.7	19.7	1.9	4.4	0.4	2.6
15	7.9	37.8	2.7	8.7	0.5	4.1
30	11.4	53.2	2.4	9.1	0.8	7.0
45	12.9	61.1	4.6	12.9	1.4	12.3

Whole cell reaction of 1 with EDA catalyzed by T268A-AxX mutants

Following the procedure for small scale whole cell reactions under anaerobic conditions, whole cell expressing T268A-AxA, T268A-AxH, T268A-AxM, T268A-AxY, T268A (axial Cys) and T268A-AxS were grown and resuspended to OD_{600} = 30. To determine the enzyme concentration of each sample, 2 mL of each cell stock was removed and then analyzed by CO assays following the general procedure outlined above. The optical density of the cells was adjusted such that the final enzyme concentration of all T268A-AxX mutants was 1.0 μM.

Small scale reactions were performed following the general procedure with 12.5 μL **1** at 400 mM and 12.5 μL EDA at 350 mM. Reactions were shaken for 12 h at room temperature. GC 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). Analytical SFC of product **2** was performed on Chiralpak AS column, eluting with 2% iPrOH at 2.5 mL/min: λ = 210 nm, t_R (min): major = 8.50, minor = 9.48.

In vitro reaction of acrylamide 1 with EDA catalyzed by T268A-AxX mutants

Small scale reactions with isolated proteins were performed following the general procedure for *in vitro* catalysis using T268A-AxX, T268A, and T268A-AxS variants that are isolated and purified by HisTrap-HP column. Yields of the reactions were determined by GC and enantioselectivity determined by chiral SFC using the same methods as described above for whole cell reactions.

Table S3. Reaction of **1** and EDA catalyzed by purified enzymes.

Variant	% Yield	% ee
T268A-AxA	34	-5
T268A-AxH	94	46
T268A-AxM	46	3
T268A-AxY	45	0
T268A	4	0
T268A-AxS	33	0

Calibration of 2

Yields of cyclopropanation products were determined using calibration curves made with independently synthesized standards. Two stock solutions of product were made at 200 mM and 40 mM in ethanol. To 5 samples containing cells at OD_{600} = 30, product was added from either of the stock solutions such that a final concentration of 1.0-10.0 mM product was obtained. Additional ethanol was added such that the total volume of organics added to each tube was 25 μL. Next, 20 μL of a 20 mM stock solution of internal standard in cyclohexane was added to each Eppendorf tube, followed by 1 mL of cyclohexane. The Eppendorf tubes were vortexed and centrifuged (13,000 x rcm, 30 seconds). The organic layer was then analyzed by GC using J&W HP-5 column (30 m x 0.32 mm, 0.25 μM film: 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). The ratio of the areas under the internal standard and product peaks was plotted against the concentration for each solution (1, 2, 4, 6, and 10 mM).

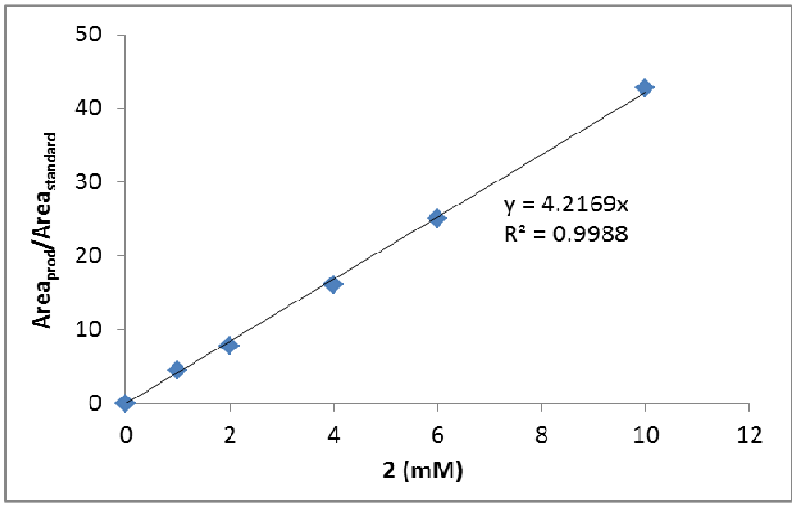


Figure S2. Calibration curve for product **2** for GC on HP-5 column.

Site saturation mutagenesis of T268A-AxH

Site saturation libraries were created as described above in the general procedure and screened in 96-well plate format for reaction of **1** with EDA. The organics extracts from these plate reactions were analyzed by chiral SFC on Chiralpak AS column, eluting with 4% iPrOH at 3.0 mL/min for 6.5 min: λ = 210 nm, t_R (min): major = 3.09, minor = 3.41. Whole cells that provided the product in enantioselectivities higher than those of the parent were sequenced and regrown on 50 mL scale. The reaction was repeated in triplicate using these cells following the general procedure for small scale whole cell reactions under anaerobic conditions to determine yield and confirm enantioselectivity. The results from two rounds of site saturation mutagenesis are summarized below.

Table S4. Top hits from site saturation mutagenesis of T268A-AxH for reaction of **1** with EDA**.**

Variant	% Yield	trans:cis	% ee
T268A-AxH (parent)	81	6:94	42
T268A-AxH-L437W	93	8:92	69
T268A-AxH-T438W	53	10:90	68
T268A-AxH-L437W-T438W	<20	n/a	23
T268A-AxH-L437W-L181V	65	6:94	75
T268A-AxH-L437W-V78M	80	3:97	87
T268A-AxH-L437W-V78M-L181V (BM3-Hstar)	92	2:98	92

Monitoring reaction of 1 with EDA catalyzed by T268A-AxH, BM3-Hstar, and T268A-AxS

Following the procedure for small scale whole cell reactions, whole cell expressing T268A-AxH, BM3-Hstar, and T268A-AxS were grown and resuspended to OD_{600} = 60. To determine the enzyme concentration of each sample, 1 mL of each cell stock was removed, diluted with 1 mL of phosphate buffer, and then analyzed by CO assays following the general procedure outlined above. The optical density of each sample was adjusted such that the final enzyme concentration of all mutants was 1.6 µM.

For each mutant, a series of reactions was set up following the general procedure for anaerobic whole cell reactions. The substrates (12.5 µL **1** at 400 mM and 12.5 µL EDA at 400 mM) were added to each sample at time 0 min. Time points were taken at 5, 10, 20, 30, 45, and 60 min. At each specified time point, the reaction was worked up using procedure described in the small scale whole cell reactions and then submitted for GC analysis.

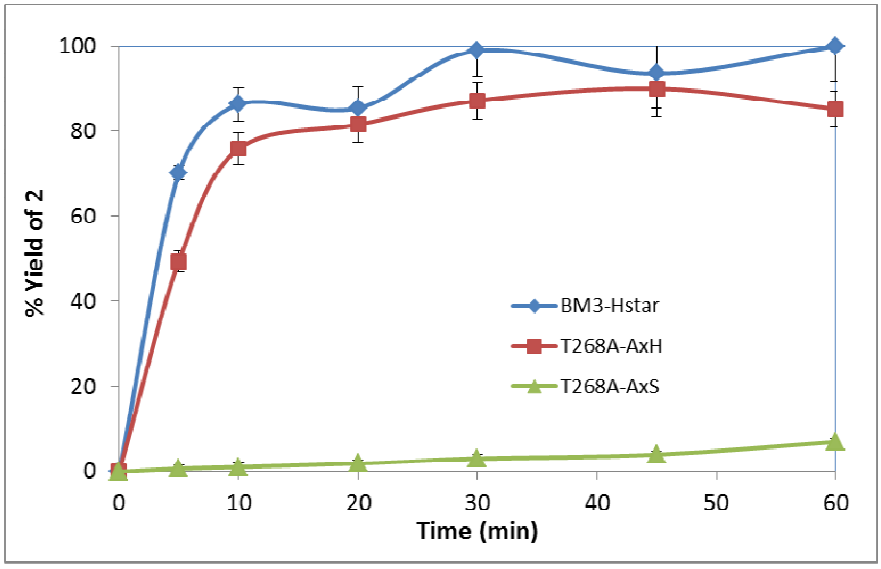


Figure S3. Reaction of **1** and EDA catalyzed by enzyme variants.

Amino Acid and Nucleotide Sequences

Table S5. List of mutations in enzyme variants, relative to wild type BM3 holoprotein (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
T268A-AxS	T268A, C400S
T268A-AxA	T268A, C400A
T268A-AxH	T268A, C400H
T268A-AxM	T268A, C400M
T268A-AxY	T268A, C400Y
BM3-HStar	V78M, L181V, T268A, C400H, L437W

The amino acid sequence for WT (holoprotein) is as follows:

>SEQ ID NO:1: gi|142798|gb|AAA87602.1| cytochrome P-450:NADPH-P-450 reductase precursor [*Bacillus megaterium*]

MTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIKEACDESRFDKNLSQALK
FVRDFAGDGLFTSWTHEKNWKKAHNILLPSFSQQAMKGYHAMMVDIAVQLVQKWERLNADEHIEVPEDMTRLTLDTI
GLCGFNYRFNSFYRDQPHPFITSMVRALDEAMNKLQRANPDDPAYDENKRQFQEDIKVMNDLVDKIIADRKASGEQS
DDLLTHMLNGKDPETGEPLDDENIRYQIITFLIAGHETTSGLLSFALYFLVKNPHVLQKAAEEAARVLVDPVPSYKQ
VKQLKYVGMVLNEALRLWPTAPAFSLYAKEDTVLGGEYPLEKGDELMVLIPQLHRDKTIWGDDVEEFRPERFENPSA
IPQHAFKPFGNGQRACIGQQFALHEATLVLGMMLKHFDFEDHTNYELDIKETLTLKPEGFVVKAKSKKIPLGGIPSP
STEQSAKKVRKKAENAHNTPLLVLYGSNMGTAEGTARDLADIAMSKGFAPQVATLDSHAGNLPREGAVLIVTASYNG
HPPDNAKQFVDWLDQASADEVKGVRYSVFGCGDKNWATTYQKVPAFIDETLAAKGAENIADRGEADASDDFEGTYEE
WREHMWSDVAAYFNLDIENSEDNKSTLSLQFVDSAADMPLAKMHGAFSTNVVASKELQQPGSARSTRHLEIELPKEA
SYQEGDHLGVIPRNYEGIVNRVTARFGLDASQQIRLEAEEEKLAHLPLAKTVSVEELLQYVELQDPVTRTQLRAMAA
KTVCPPHKVELEALLEKQAYKEQVLAKRLTMLELLEKYPACEMKFSEFIALLPSIRPRYYSISSSPRVDEKQASITV
SVVSGEAWSGYGEYKGIASNYLAELQEGDTITCFISTPQSEFTLPKDPETPLIMVGPGTGVAPFRGFVQARKQLKEQ
GQSLGEAHLYFGCRSPHEDYLYQEELENAQSEGIITLHTAFSRMPNQPKTYVQHVMEQDGKKLIELLDQGAHFYICG
DGSQMAPAVEATLMKSYADVHQVSEADARLWLQQLEEKGRYAKDVWAGHHHHHH

The nucleotide sequence for WT (holoprotein) is as follows:

ATGACAATTAAAGAAATGCCTCAGCCAAAAACGTTTGGAGAGCTTAAAAATTTACCGTTATTAAACACAGATAAACC
GGTTCAAGCTTTGATGAAAATTGCGGATGAATTAGGAGAAATCTTTAAATTCGAGGCGCCTGGTCGTGTAACGCGCT
ACTTATCAAGTCAGCGTCTAATTAAAGAAGCATGCGATGAATCACGCTTTGATAAAAACTTAAGTCAAGCGCTTAAA
TTTGTACGTGATTTTGCAGGAGACGGGTTATTTACAAGCTGGACGCATGAAAAAAATTGGAAAAAAGCGCATAATAT
CTTACTTCCAAGCTTCAGTCAGCAGGCAATGAAAGGCTATCATGCGATGATGGTCGATATCGCCGTGCAGCTTGTTC
AAAAGTGGGAGCGTCTAAATGCAGATGAGCATATTGAAGTACCGGAAGACATGACACGTTTAACGCTTGATACAATT
GGTCTTTGCGGCTTTAACTATCGCTTTAACAGCTTTTACCGAGATCAGCCTCATCCATTTATTACAAGTATGGTCCG
TGCACTGGATGAAGCAATGAACAAGCTGCAGCGAGCAAATCCAGACGACCCAGCTTATGATGAAAACAAGCGCCAGT

TTCAAGAAGATATCAAGGTGATGAACGACCTAGTAGATAAAATTATTGCAGATCGCAAAGCAAGCGGTGAACAAAGC
GATGATTTATTAACGCATATGCTAAACGGAAAAGATCCAGAAACGGGTGAGCCGCTTGATGACGAGAACATTCGCTA
TCAAATTATTACATTCTTAATTGCGGGACACGAAACAACAAGTGGTCTTTTATCATTTGCGCTGTATTTCTTAGTGA
AAAATCCACATGTATTACAAAAAGCAGCAGAAGAAGCAGCACGAGTTCTAGTAGATCCTGTTCCAAGCTACAAACAA
GTCAAACAGCTTAAATATGTCGGCATGGTCTTAAACGAAGCGCTGCGCTTATGGCCAACTGCTCCTGCGTTTTCCCT
ATATGCAAAAGAAGATACGGTGCTTGGAGGAGAATATCCTTTAGAAAAAGGCGACGAACTAATGGTTCTGATTCCTC
AGCTTCACCGTGATAAAACAATTTGGGGAGACGATGTGGAAGAGTTCCGTCCAGAGCGTTTTGAAAATCCAAGTGCG
ATTCCGCAGCATGCGTTTAAACCGTTTGGAAACGGTCAGCGTGCGTGTATCGGTCAGCAGTTCGCTCTTCATGAAGC
AACGCTGGTACTTGGTATGATGCTAAAACACTTTGACTTTGAAGATCATACAAACTACGAGCTCGATATTAAAGAAA
CTTTAACGTTAAAACCTGAAGGCTTTGTGGTAAAAGCAAAATCGAAAAAAATTCCGCTTGGCGGTATTCCTTCACCT
AGCACTGAACAGTCTGCTAAAAAAGTACGCAAAAAGGCAGAAAACGCTCATAATACGCCGCTGCTTGTGCTATACGG
TTCAAATATGGGAACAGCTGAAGGAACGGCGCGTGATTTAGCAGATATTGCAATGAGCAAAGGATTTGCACCGCAGG
TCGCAACGCTTGATTCACACGCCGGAAATCTTCCGCGCGAAGGAGCTGTATTAATTGTAACGGCGTCTTATAACGGT
CATCCGCCTGATAACGCAAAGCAATTTGTCGACTGGTTAGACCAAGCGTCTGCTGATGAAGTAAAAGGCGTTCGCTA
CTCCGTATTTGGATGCGGCGATAAAAACTGGGCTACTACGTATCAAAAAGTGCCTGCTTTTATCGATGAAACGCTTG
CCGCTAAAGGGGCAGAAAACATCGCTGACCGCGGTGAAGCAGATGCAAGCGACGACTTTGAAGGCACATATGAAGAA
TGGCGTGAACATATGTGGAGTGACGTAGCAGCCTACTTTAACCTCGACATTGAAAACAGTGAAGATAATAAATCTAC
TCTTTCACTTCAATTTGTCGACAGCGCCGCGGATATGCCGCTTGCGAAAATGCACGGTGCGTTTTCAACGAACGTCG
TAGCAAGCAAAGAACTTCAACAGCCAGGCAGTGCACGAAGCACGCGACATCTTGAAATTGAACTTCCAAAAGAAGCT
TCTTATCAAGAAGGAGATCATTTAGGTGTTATTCCTCGCAACTATGAAGGAATAGTAAACCGTGTAACAGCAAGGTT
CGGCCTAGATGCATCACAGCAAATCCGTCTGGAAGCAGAAGAAGAAAAATTAGCTCATTTGCCACTCGCTAAAACAG
TATCCGTAGAAGAGCTTCTGCAATACGTGGAGCTTCAAGATCCTGTTACGCGCACGCAGCTTCGCGCAATGGCTGCT
AAAACGGTCTGCCCGCCGCATAAAGTAGAGCTTGAAGCCTTGCTTGAAAAGCAAGCCTACAAAGAACAAGTGCTGGC
AAAACGTTTAACAATGCTTGAACTGCTTGAAAAATACCCGGCGTGTGAAATGAAATTCAGCGAATTTATCGCCCTTC
TGCCAAGCATACGCCCGCGCTATTACTCGATTTCTTCATCACCTCGTGTCGATGAAAAACAAGCAAGCATCACGGTC
AGCGTTGTCTCAGGAGAAGCGTGGAGCGGATATGGAGAATATAAAGGAATTGCGTCGAACTATCTTGCCGAGCTGCA
AGAAGGAGATACGATTACGTGCTTTATTTCCACACCGCAGTCAGAATTTACGCTGCCAAAAGACCCTGAAACGCCGC
TTATCATGGTCGGACCGGGAACAGGCGTCGCGCCGTTTAGAGGCTTTGTGCAGGCGCGCAAACAGCTAAAAGAACAA
GGACAGTCACTTGGAGAAGCACATTTATACTTCGGCTGCCGTTCACCTCATGAAGACTATCTGTATCAAGAAGAGCT
TGAAAACGCCCAAAGCGAAGGCATCATTACGCTTCATACCGCTTTTTCTCGCATGCCAAATCAGCCGAAAACATACG
TTCAGCACGTAATGGAACAAGACGGCAAGAAATTGATTGAACTTCTTGATCAAGGAGCGCACTTCTATATTTGCGGA
GACGGAAGCCAAATGGCACCTGCCGTTGAAGCAACGCTTATGAAAAGCTATGCTGACGTTCACCAAGTGAGTGAAGC
AGACGCTCGCTTATGGCTGCAGCAGCTAGAAGAAAAAGGCCGATACGCAAAAGACGTGTGGGCTGGGCTCGAGCACC
ACCACCACCACCACTGAGATCCGGCTGCTAACAAAGC

Experimental Procedures for Formal Synthesis of Levomilnacipran

Acrylamide 1: To a solution of atropic acid (11.1 g, 75 mmol) in DCM (150 mL, 0.2 M) at 0 ºC was added oxalyl chloride (7.63 mL, 90 mmol) dropwise, followed by DMF (0.1 mL). The resulting colorless solution was then warmed to rt and stirred for 2 h, upon which it turned slightly yellow. After cooling to 0 ºC, sat. $NaHCO_3$ (150 mL) was added, followed by Et_2NH (11.63 mL, 112.5 mmol) dropwise. The resulting solution was stirred vigorously (uniform emulsion) at rt overnight. The organic and aqueous layers were separated and the aqueous layer was extracted with Et_2O (2 x 150 mL). The combined organic layer was washed with H_2O (100 mL), and brine (100 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The resulting yellow oil (13.0 g, 85%) was used for the next step without further purification.

Physical state: yellow oil;

R_f = 0.33 (silica gel, 1:2 EtOAc:hexanes);

HRMS (*m/z*): calcd for $C_{13}H_{17}NO$, $[M+H]^+$, 204.1388; found, 204.1398;

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.47–7.40 (m, 2H), 7.38–7.27 (m, 3H), 5.69 (s, 1H), 5.32 (s, 1H), 3.50 (q, *J* = 7.1 Hz, 2H), 3.22 (q, *J* = 7.1 Hz, 2H), 1.22 (t, *J* = 7.1 Hz, 3H), 0.99 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR ($CDCl_3$, 126 MHz): δ 170.4, 145.6, 135.9, 128.9, 128.6, 125.7, 113.1, 42.9, 38.9, 14.1, 12.9.

Preparative-Scale Whole Cell Synthesis of 2:

E. coli (BL21(DE3)) cells were grown from glycerol stock overnight (37 ºC, 200 r.p.m.) in 100 ml TB medium. The preculture was used to inoculate three 350 mL volumes of Hyperbroth medium in three 500-mL Erlenmeyer flasks (using ca. 30 mL each), and the resulting cultures were incubated at 37 ºC, 200 r.p.m. for 3.5 h. At OD_{600nm} = 2.1, the cultures were cooled to 25 ºC, and the shaking was reduced to 140 r.p.m. Cultures were harvested after 18 h and resuspended (OD_{600nm} = 60) in nitrogen-free M9 medium (total volume = 80 mL). This corresponds to [BM3-Hstar] ~ 2.6 μM and a dry cell weight of 17-19 g/L. A solution of glucose (2.0 mL, 1 M) was added to the cells, followed by 2.0 mL EtOH, 158 mg acrylamide **1** (0.16 mL, 0.82 mmol), 119 mg of EDA (87% solution in DCM, 0.11 mL, 0.91 mmol). The reaction was stirred at room temperature under ambient atmosphere for 3 h. The crude mixture was portioned into three 50 mL Falcon tubes and extracted with cyclohexanes (25 mL to each tube) and centrifuged (5000 r.p.m., 5 min). The organics were collected, and this extraction sequence was performed two more times. The organics were combined, dried over Na_2SO_4 and then concentrated *in vacuo* to afford crude organics. NMR analysis indicated that this crude product consisted of a 1.0:0.07 ratio of cyclopropane **2** to acrylamide **1**. This ratio corresponds to 93% conversion to desired product **2**. The crude organic mixture could be used for the next step without further purification. To obtain an isolated yield, the crude mixture was purified by preparative scale HPLC (30 mm x 250 mm, 5 μm Agilent XDB-C18 column, detection at 230 nm, flow rate 50 mL/min, H_2O/MeCN, gradient: ramp 30-60% MeCN over 15 min, 100% MeCN for 3 min for a total run time of 18 min, t_R (min) = 13.1 min) to afford 204 mg of the desired diastereomer of **2** (86% yield). Representative traces of chiral SFC for determining enantiomeric excess are shown below.

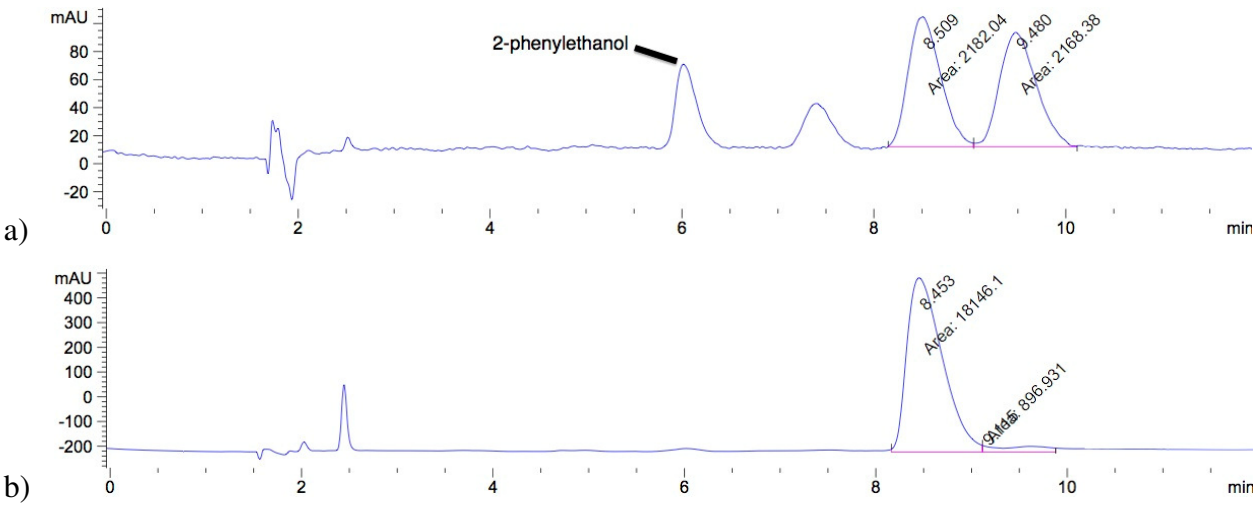


Figure S4. Representative SFC traces for a) a racemic mixture and b) enantiomeric mixture made by BM3-Hstar.

Physical state: clear oil;

R_f = 0.33 (silica gel, 1:2 EtOAc:hexanes);

$[\alpha]_D$ = -105.9° (c = 2.0, $CHCl_3$) at 23 °C;

HRMS (*m/z*): calcd for $C_{17}H_{23}NO_3$, $[M+H]^+$, 290.1756; found, 290.1755;

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.33–7.29 (m, 3H), 7.26–7.21 (m, 2H), 4.17 (qd, *J* = 7.1, 1.8 Hz, 2H), 3.56–3.40 (m, 2H), 3.19 (ddq, *J* = 38.5, 14.2, 7.1 Hz, 2H), 2.44 (dd, *J* = 8.4, 6.2 Hz, 1H), 2.18 (dd, *J* = 6.2, 4.9 Hz, 1H), 1.48 (dd, *J* = 8.4, 4.9 Hz, 1H), 1.28 (t, *J* = 7.1 Hz, 3H), 1.09 (t, *J* = 7.1 Hz, 3H), 0.76 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR ($CDCl_3$, 126 MHz): δ 170.8, 167.7, 139.0, 128.9, 128.9, 127.3, 126.4, 61.1, 41.5, 39.4, 39.0, 28.3, 21.3, 14.4, 13.1, 12.4.

Alcohol 3. To a solution of the crude mixture from the previous reaction containing 0.519 mmol of cyclopropane **2** in Et_2O (3.4 mL, 0.2 M) was added $LiBH_4$ (17 mg, 0.781 mmol, 1.5 equiv). The resulting mixture was stirred overnight. The reaction was diluted with Et_2O (5 mL) and quenched with H_2O (2.5 mL). The aqueous mixture was extracted with Et_2O (2 x 10 mL), and the combined organic layers were washed with saturated aq. NaCl (5 mL), dried with Na_2SO_4 and concentrated *in vacuo*. The crude mixture was purified by silica gel chromatography (4:1 to 1:1 hexanes:EtOAc) to afford alcohol **3** (113 mg, 88% yield). Spectral data obtained for **3** are in agreement with those previously reported in the literature.[3]

Physical state: clear oil;

R_f = 0.10 (silica gel, 1:2 EtOAc:hexanes);

$[\alpha]_D$ = +54.0º (c = 2.0, $CHCl_3$);

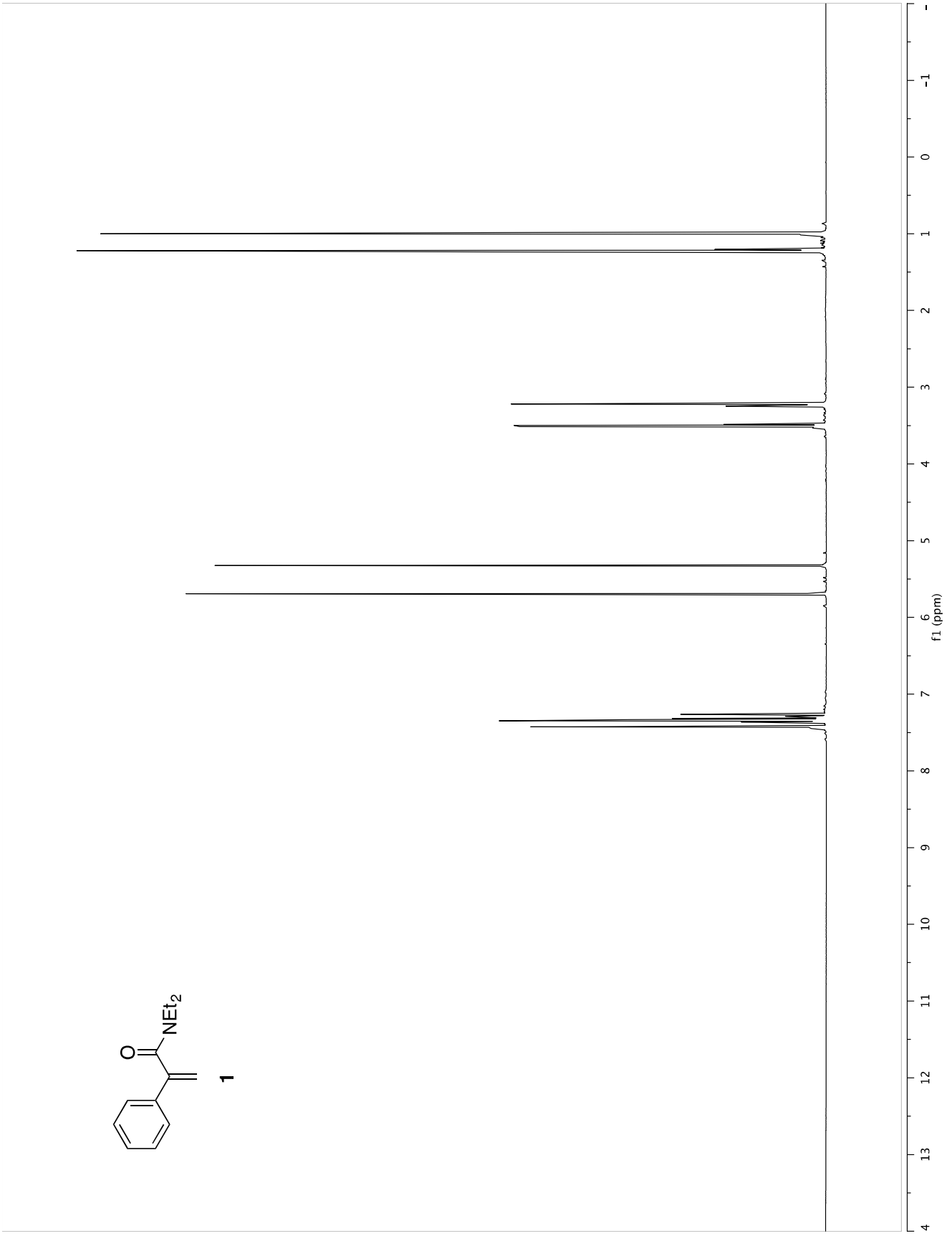
HRMS (*m/z*): calcd for $C_{15}H_{21}NO_2$, $[M+H]^+$, 248.1650; found, 248.1658;

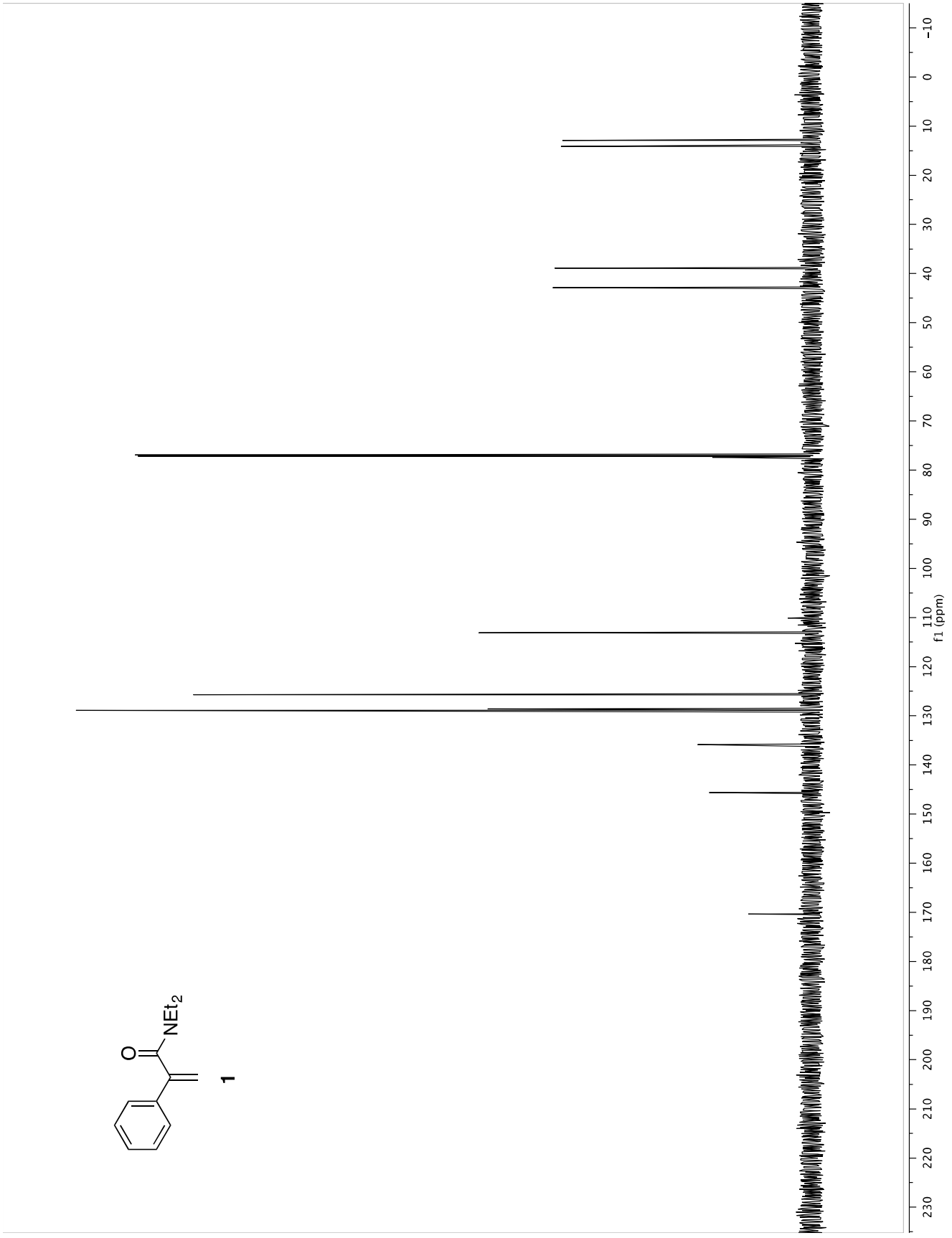
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.31–7.26 (m, 2H), 7.24–7.17 (m, 3H), 4.55 (brs, 1H), 4.04 (dd, *J* = 12.2, 4.8 Hz, 1H), 3.47–3.55 (m, 1H), 3.45–3.31 (m, 3H), 3.18 (dd, *J* = 12.2, 10.4 Hz, 1H), 1.64 (dd, *J* = 8.8, 5.3 Hz, 1H), 1.55 (dddd, *J* = 10.4, 8.8, 6.3, 4.8 Hz, 1H), 1.13 (t, *J* = 7.1 Hz, 3H), 1.08 (dd, *J* = 6.3, 5.3 Hz, 1H), 0.90 (t, *J* = 7.1 Hz, 3H).

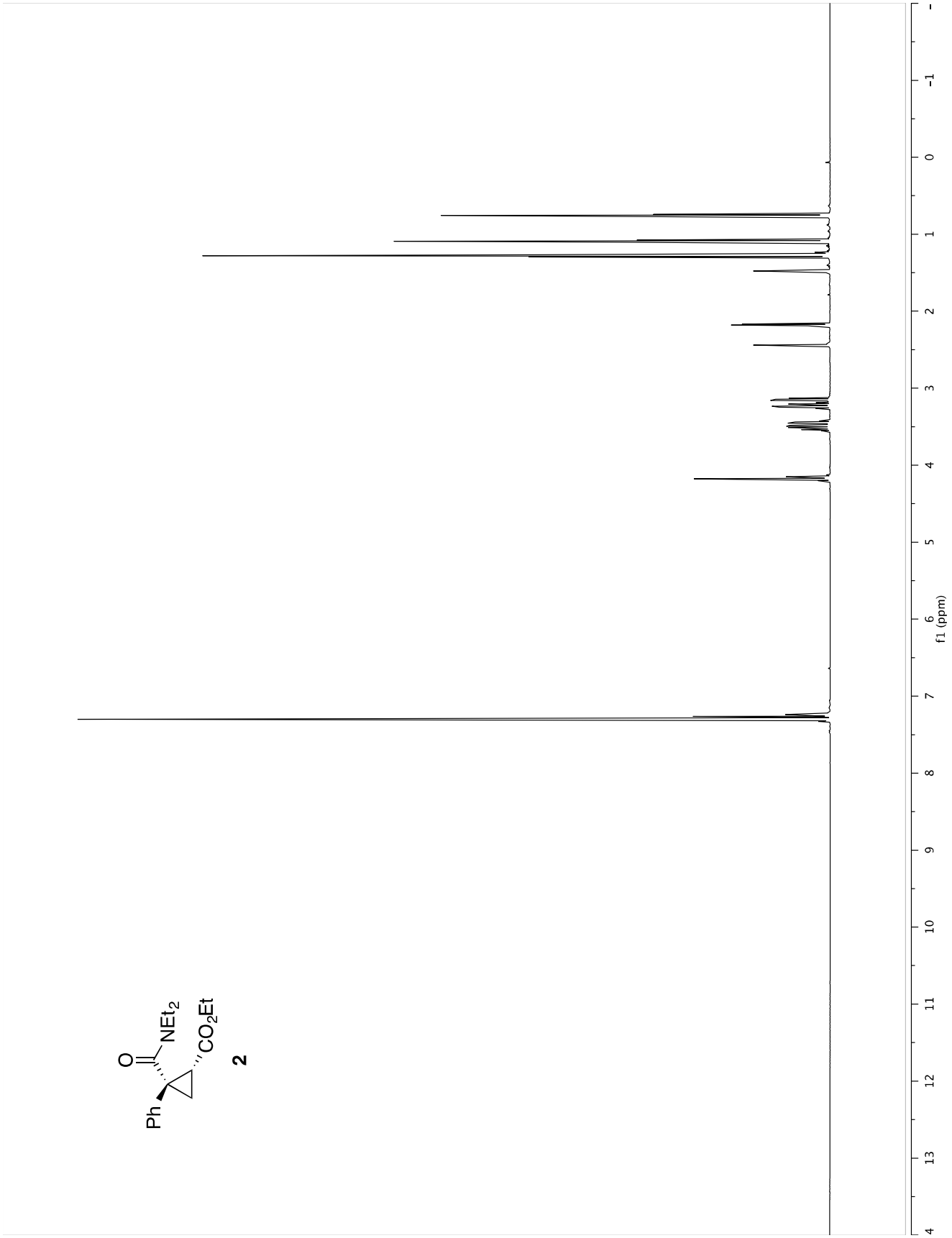
^{13}C NMR ($CDCl_3$, 126 MHz): δ 171.4, 140.4, 128.8, 128.8, 126.8, 125.9, 65.0, 42.2, 39.7, 34.5, 32.1, 16.9, 13.3, 12.5.

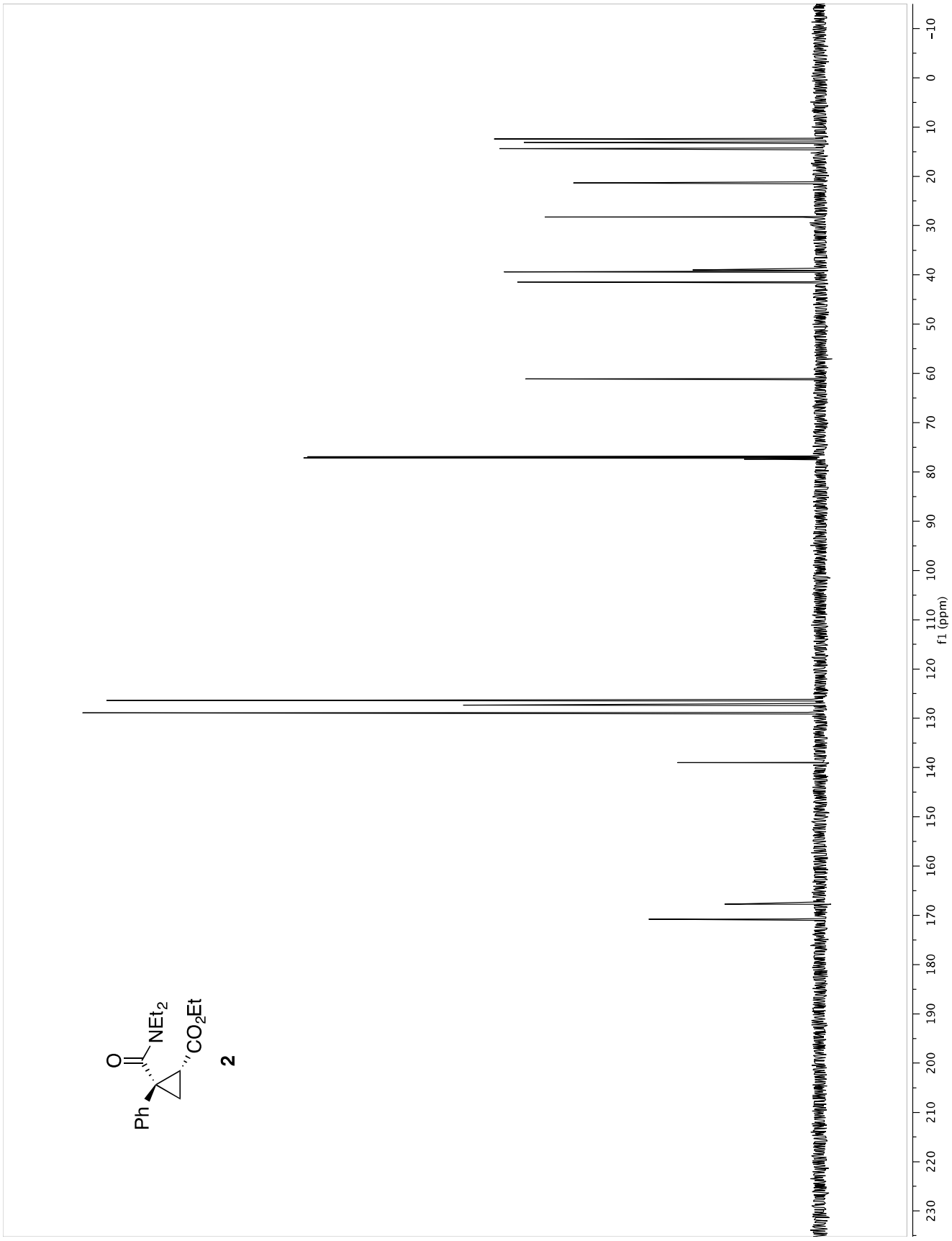
References

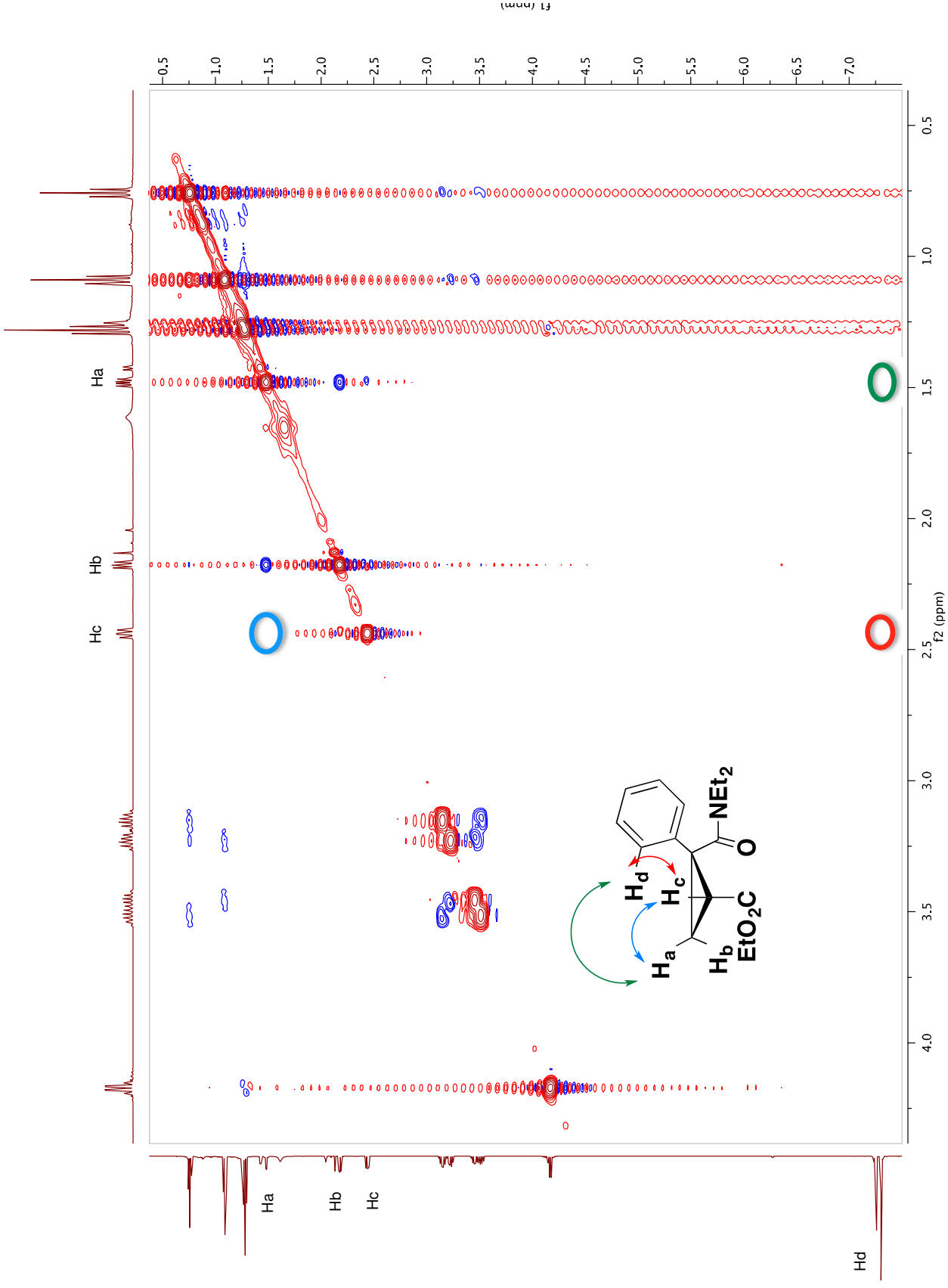
1. C. R. Otey, in *Methods in Molecular Biology: Directed Enzyme Evolution*, Arnold, F. H.; Georgiou, G. Eds. Humana Press: Totowa, NJ, 2003, vol. 230.
2. S. Kille, C. G. Acevedo-Rocha, L. P. Parra, Z.-G Zhang, D. J. Opperman, M. T. Reetz, J. P. Acevedo, *ACS Synth. Biol.* **2013**, *2*, 83–92.
3. J. Alliot, E. Gravel, F. Pillon, D.-A. Buisson, M. Nicolas, E. Doris, *Chem. Commun*. **2012**, *48*, 8111–8113.

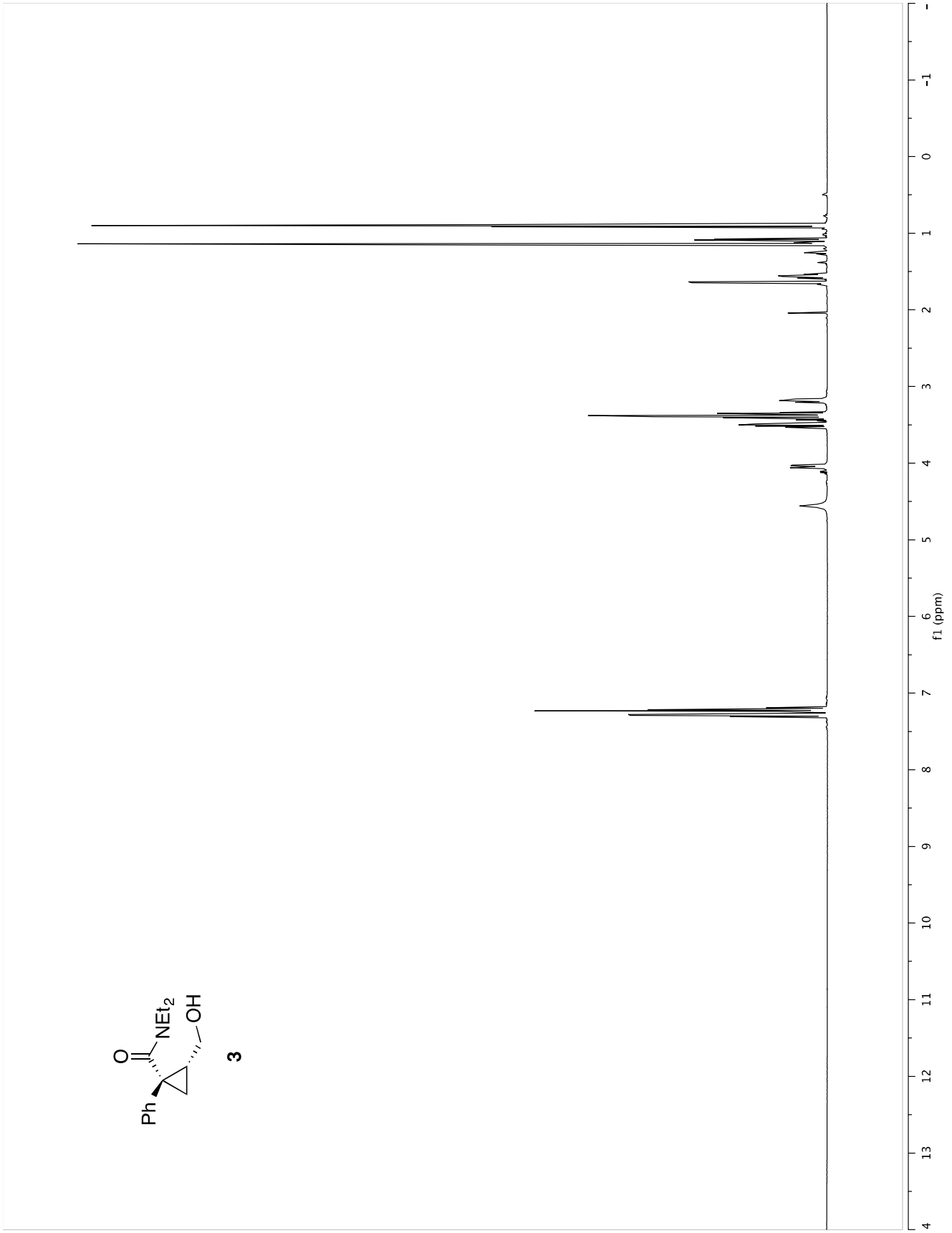

NEt2
O
1
f1 (ppm)

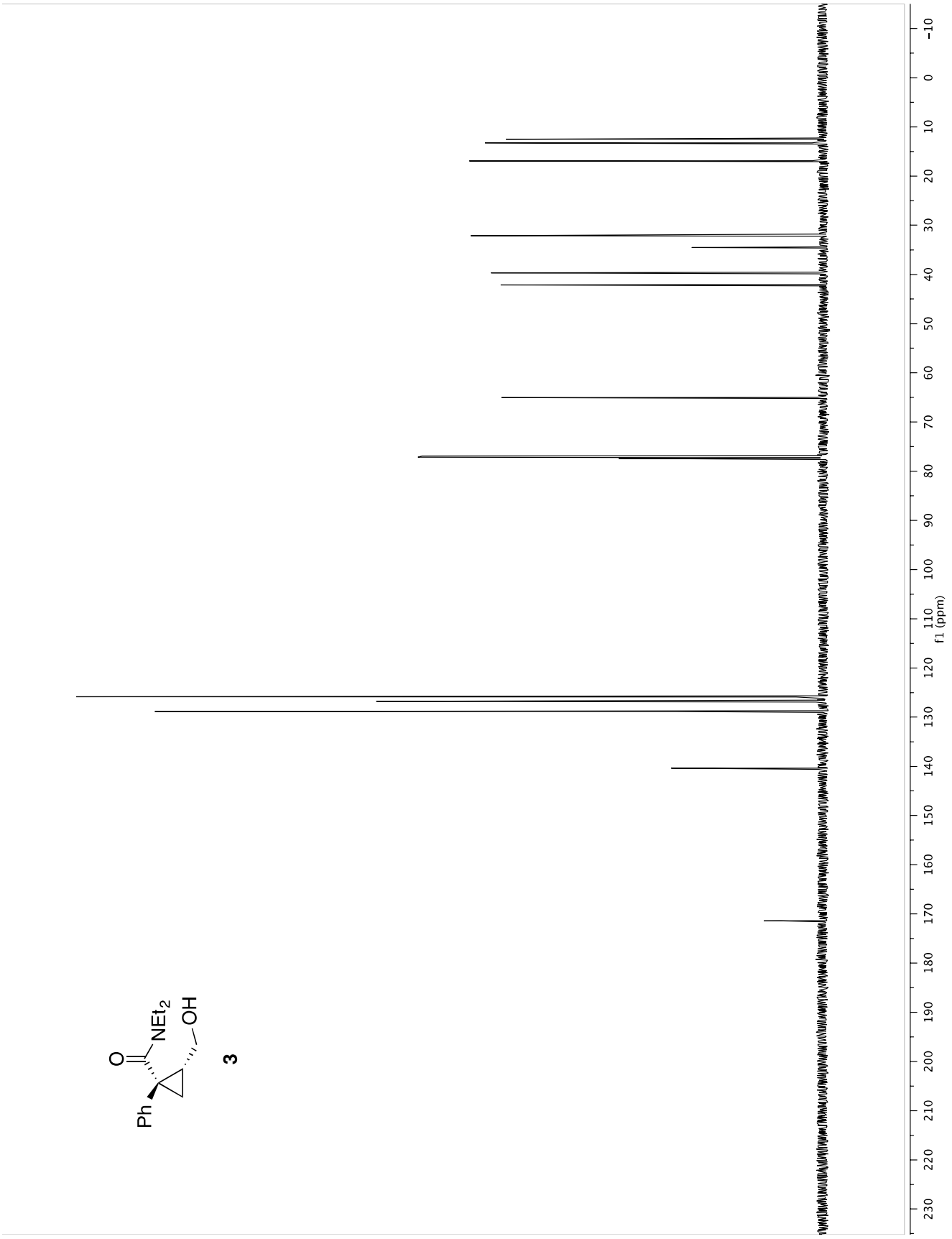

O
NEt2
1
f1 (ppm)


O
NEt2
CO2Et
Ph
2
f1 (ppm)


O
NEt2
CO2Et
Ph
2
f1 (ppm)


Ha
Hb
Hc
Hd
f2 (ppm)
NEt2
O
EtO2C
Hd
Hc
Ha
Hb


O
NEt2
OH
Ph
3
f1 (ppm)


Ph
O
NEt2
OH
3
f1 (ppm)