Supporting Information For

Ligand-Induced Conformational Heterogeneity of Cytochrome P450 CYP119 Identified by 2D NMR Spectroscopy with the Unnatural Amino Acid ^{13}C-*p*-Methoxyphenylalanine

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Experimental Section

Chemicals.

All chemicals used were of analytical grade and obtained from commercial sources. Solvents were HPLC grade and all water used was deionized, distilled Milli-Q quality. Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Synthesis of ^{13}C-p-methoxyphenylalanine.

General

Moisture-sensitive reactions were performed in flame-dried glassware under a positive pressure of nitrogen or argon. Air- and moisture-sensitive materials were transferred by a syringe under an argon atmosphere. Solvents were either ACS reagent grade or HPLC grade. *N*,*N*-Dimethylformamide was dried over 4 Å molecular sieves for at least 1 week prior to use. K_2CO_3 was dried in an oven at 150 ^oC overnight and cooled to room temperature under argon. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) using 0.25 mm Alltech precoated silica gel plates. ^1H NMR spectra were recorded at 400 MHz on a Varian Gemini spectrometer using TMS as the internal reference. Chemical shifts are reported in parts per million (δ) and coupling constants (*J* values) are given in hertz (Hz). The following abbreviations are used to explain the multiplicities: s = singlet; d = doublet. LC/MS was performed on Waters system equipped with an XTerra MS C18 reverse phase column (3.5 μm, 2.1x50 mm).

Boc-Tyr(O-$^{13}CH_3$)-OCH_3

To a solution of Boc-Tyr-OCH_3 (4 g, 13.6 mmol) in anhydrous DMF (15 mL) was added anhydrous K_2CO_3 (4 g, 29 mmol) and ^{13}C-iodomethane (0.944 mL, 14.9 mmol, 1.1 equiv). The reaction mixture was stirred under argon at room temperature overnight (16 h) and the completeness of the reaction was monitored by TLC (hexane/EtOAc, 4/1). The reaction mixture was poured into CH_2Cl_2 (100 mL) and water (300 mL). The aqueous solution was further extracted with CH_2Cl_2 (100 mL x 3). The extractions were combined, washed with water (100 mL), and dried over Na_2SO_4. After evaporation of the solvent *in vacuo*, the resulting yellow oil was purified by silica gel flash chromatography (hexane/EtOAc, 4/1) to give a white solid (3.9 g, 93%).^1H NMR (400 MHz, CD_3OD) δ; LC-MS (ESI+).

Boc-Tyr(O-$^{13}CH_3$)-OH

To a solution of Boc-Tyr(O-$^{13}CH_3$)-OCH_3 (3.9 g, 12.6 mmol) in methanol (30 mL) was added LiOH (1 N, 20 mL). The reaction mixture was stirred at room temperature for 5 h and the completeness of the reaction was monitored by TLC (hexane/EtOAc, 2/1). HCl (1 N) was added to adjust to pH ~ 5, followed by removal of methanol *in vacuo*. The residue was taken up in EtOAc (300 mL), washed with saturated NaCl (aq), and dried over Na_2SO_4. Evaporation of the solvent gave the product as a white foamy solid (3.6 g, 97%).^{1}H NMR (400 MHz, CD_3OD) δ; LC-MS (ESI+).

HCl·H-Tyr(O-$^{13}CH_3$)-OH

Boc-O-$^{13}CH_3$-Tyr-OH (3.6 g, 12.1 mmol) was treated with 25% (v/v) TFA in CH_2Cl_2 (45 mL) for 1 h at room temperature. After evaporation of solvents, the residue was dissolved in 40% methanol/water (100 mL) containing 0.5 N HCl and methanol was then removed *in vacuo*. The aqueous solution was subjected to lyophilization to give a white solid (2.8 g, 100%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.17 (d, J = 8 Hz, 2H), 6.89 (d, J = 8 Hz, 2H), 4.12 (dd, J = 8, 5.2 Hz, 1H), 3.75 (d, J = 144 Hz, 3H), 3.19 (dd, J = 14.4, 5.2 Hz, 1H), 3.04 (dd, J = 14.4, 8 Hz, 1H); LC-MS (ESI+) 197.08 (MH^+, 100%), 180.10 (MH^+-NH_3, 48%).

Construction of CYP119 Mutants.

The CYP119 mutants were constructed using the Stratagene QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Briefly, the forward and reverse oligonucleotides containing the desired TAG stop codon mutation were used to amplify CYP119-pCWori using the polymerase chain reaction. *Dpn*I was then used to digest the parental DNA. Transformation of the remaining DNA resulted in colonies that were screened by DNA sequencing.

Expression and Purification of ^{13}C-labeled CYP119-F162-MeOF.

Initially, pSup-OTyrRS/pCWOri$^+$-CYP119-F162-MeOF transformed DH10B *E. coli* were grown in a 50 mL LB starter culture containing 25 μg/mL chloramphenicol/ 50 μg/mL ampicillin overnight. 5 mL of this starter culture was then used to inoculate 0.5 L of 2X YT expression media containing 25 μg/mL chloramphenicol and 100 μg/mL ampicillin. The expression culture was incubated at 37 ^{o}C with shaking at 225 rpm until it reached log phase (0.6 OD_{600}; 2-4 h). Protein expression was

induced by simultaneous addition of 1 mM IPTG and 1 mM ^{13}C-*p*-methoxyphenylalanine. The incubation temperature was reduced to 30 ^{o}C and the cultures were allowed to continue to incubate with shaking at 160 rpm for 36 hr. The mutant CYP119-F162-O-^{13}CMeF was purified according to the standard protocol used for CYP119-wt. Briefly, cells were harvested by centrifugation in a Sorvall RC-5B centrifuge at 5,000 rpm and 5 ^{o}C for 20 min. The cell pellet was resuspended in 4 vol (4 mL/g cell paste) of 50 mM Tris-HCL, pH 7.0, 4 mg/mL lysozyme, 16 U/mL DNase, 4 U/mL RNase, and 1 mM PMSF. The cells were allowed to stir on ice for 1 h to ensure complete resuspension and promote lysis. Next, the entire resuspension was lysed using a Branson sonicator (3 x 1-min bursts at 50% power, with 1-min cooling in an ice bath between each burst). Cellular debris was separated from recombinant protein by centrifugation in a Sorvall Ultracentrifuge at 100,000*g* for 1 h at 4 ^{o}C. The protein containing supernatant was collected, pooled and loaded directly onto a pre-equilibrateed Q Sepharose column. The column was washed with ~10 column volumes of 50 mM Tris-HCL, pH 7.0 containing 1 mM PMSF. The protein was eluted with a salt gradient of 0-250 mM NaCl in 50 mM Tris-HCL, pH 7.0 buffer. All of the reddish fractions were combined and dialyzed overnight against 50 mM Tris-HCL, pH 7.0 buffer. The protein was then loaded onto a PBE94 column pre-equilibrated with 50 mM Tris-HCL, pH 7.0 buffer, washed, and eluted with Polybuffer 74 at pH 5.0. CYP119 eluted at ~ pH 6.6. The polybuffer was removed by precipitation with 80% ammonium sulfate, and the protein was concentrated and stored in 100 mM KP_i, pH 7.4, 20% glycerol, 1 mM EDTA at –80 ^{o}C until needed for experiments. The protein was judged to be greater than 90% pure by SDS-PAGE gel electrophoresis and Gelcode Blue staining. Final protein concentration was determined using the method of Omura and Sato, with an extinction coefficient of E_{450} = 100 $mM^{-1}cm^{-1}$. In the absence of ^{13}C-*p*-methoxyphenylalanine, no protein was produced.

Whole-protein MS characterization of ^{13}C-labeled CYP119-F162-MeOF.

Reverse phase separations were performed on an Eldex MicroPro capillary HPLC system (Napa, CA) equipped with a 100 µM x 150 mm monolithic C18 column (Phenomenex, Torrance, CA). Mobile

phase A consisted of 0.1% formic acid in water while mobile phase B consisted of 0.1% formic acid in acetonitrile. After equilibration of the column at 35% B for 7 min, 5 pmol of protein was injected at a flow rate of 0.5 µl/min. The column was loaded and washed at this mobile phase concentration for 10 min before the proteins were eluted by raising the mobile phase concentration to 50% for 10 min. The eluent from the reverse phase column was coupled on-line to a microspray ion source attached to a QSTAR-XL mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) operated in positive ion mode. MS scans were acquired through the tune page over a m/z range of 500-2000. Charge state envelopes were deconvoluted using the Bayesian Protein Reconstruct algorithm in the Analyst QS version 1.1, Bioanalyst extension pack (Applied Biosystems/MDS Sciex, Foster City, CA).

Ligand K_d Determination using Optical Difference Spectroscopy.

In order to determine wether or not the CYP119-^{13}CMeOF mutant proteins were indeed active, , UV-visible difference spectra were acquired for a variety of ligands using a dual beam OLIS/Aminco DW2a spectrophotometer (OLIS, Bogart, GA). Both sample and reference chambers contained 1 mL of 0.5 µM CYP119-^{13}CMeOY in 100 mM KP_i, pH 7.4. Prior to initiating the titration, a baseline was recorded between 350 and 500 nm. Subsequently, 1 µL aliquots of a concentrated stock solution of ligand was added to the sample cuvette while an equal volume of vehicle solvent was added to the reference cuvette. Care was taken so that the final concentration of added solvent in each cuvette did not exceed 1.5%. Difference spectra were acquired after a 1 min equilibration time. All spectra were recorded at 22 °C (room temperature). Data were imported into the Origin v.7.5 software package (OriginLab Corp., Northampton, MA) for analysis. The absolute change in absorbance between the peak vs. the trough was plotted as a function of ligand concentration. To obtain an accurate binding affinity constant, data were fit to a single site binding model using the hyperbolic Michaelis-Menten equation.

^{1}H,^{13}C-HSQC NMR Measurements.

$^{1}H,^{13}C$-HSQC spectra were acquired on a Varian Inova 600 MHz spectrometer equipped with a $^{1}H/^{13}C/^{15}N$ triple resonance Coldprobe^{TM} with actively shielded pulse-field gradients at 298 K. Spectra were acquired with 40 scans using spectral widths of 8385 Hz (^{1}H) and 1600 Hz (^{13}C), 128 complex points in 1, and a 1 s recycle time. Each NMR sample consisted of 250 μL of a 0.35 mM concentrated solution of labeled CYP119-^{13}C-*p*-methoxyphenylalanine protein in a 100 mM potassium phosphate (pH 7.4) buffer with 10% D_2O added as a lock solvent placed in a Shigemi tube. For the 4-phenylimidazole titration experiments, aliquots of a 25 mM stock solution of 4-phenylimidazole were added sequentially to reach the desired final concentrations for each experiment, and then data were recorded using the above conditions. Data processing and analysis were performed in the program NMRPipe using 2D Rance-Kay acquisition mode.

Results Section

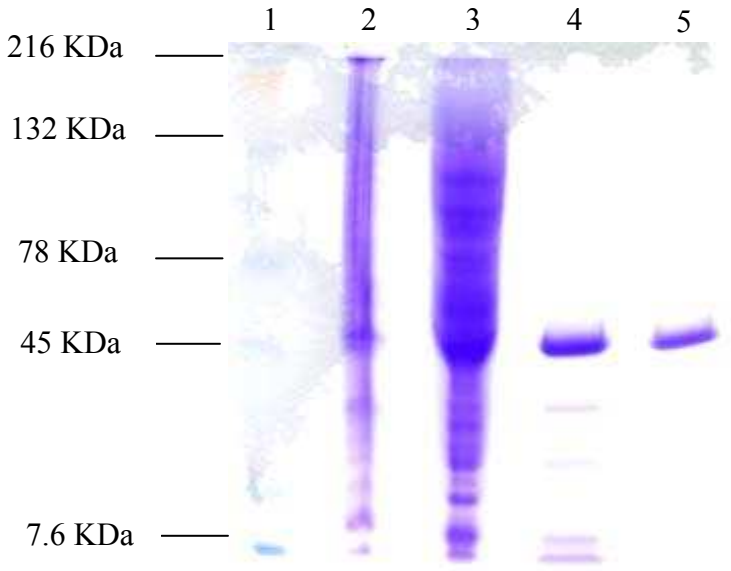
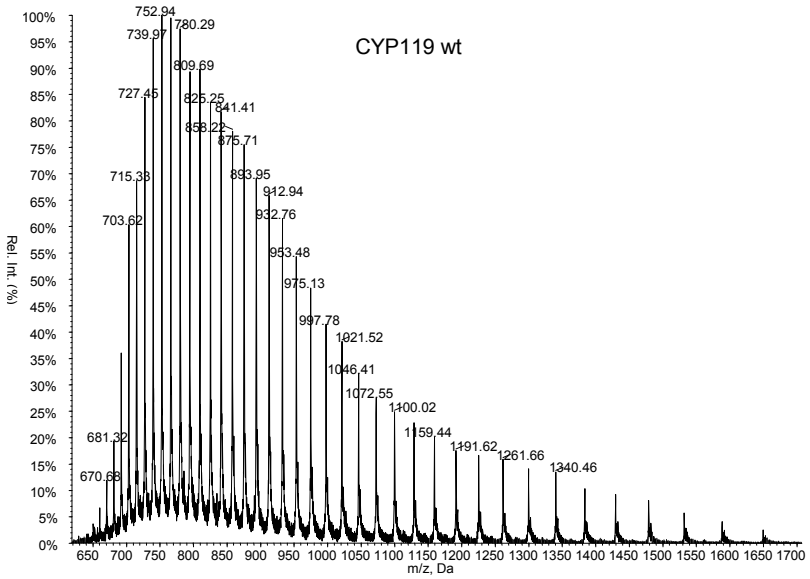


Figure S1. SDS-PAGE analysis of CYP119-F162-^{13}C-*p*-methoxyphenylalanine expression in DH10B *E. coli*. Lane 1: protein MW markers, lane 2: DH10B *E. coli* cells post-expression, lane 3: high-speed centrifugation supernatant of *E. coli* cell homogenate, lane 4: post-Q Sepharose column purification, lane 5: post-PBE94 column purification.

(A)



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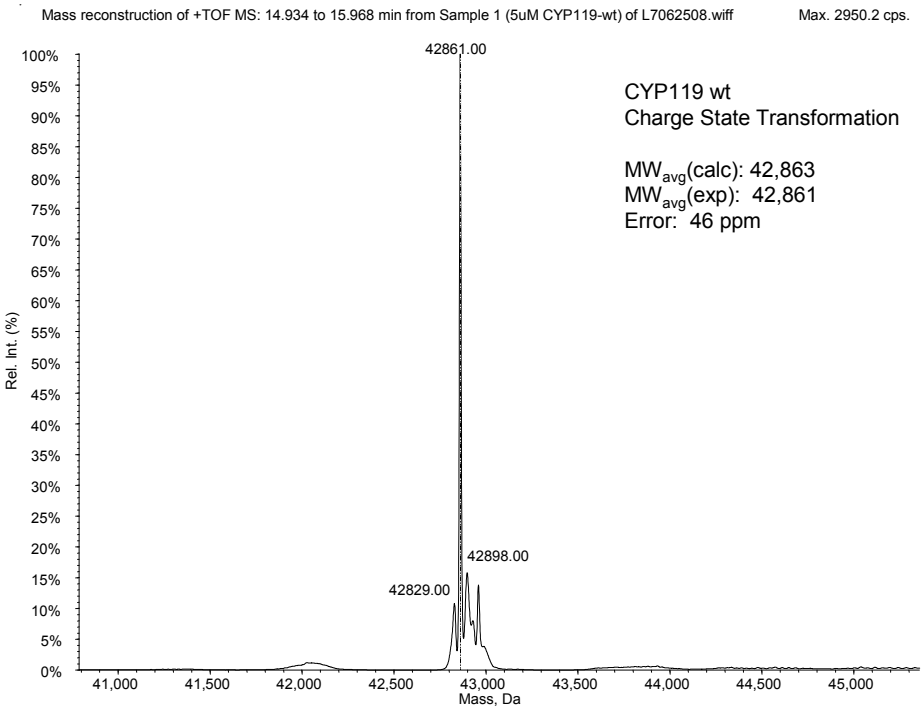
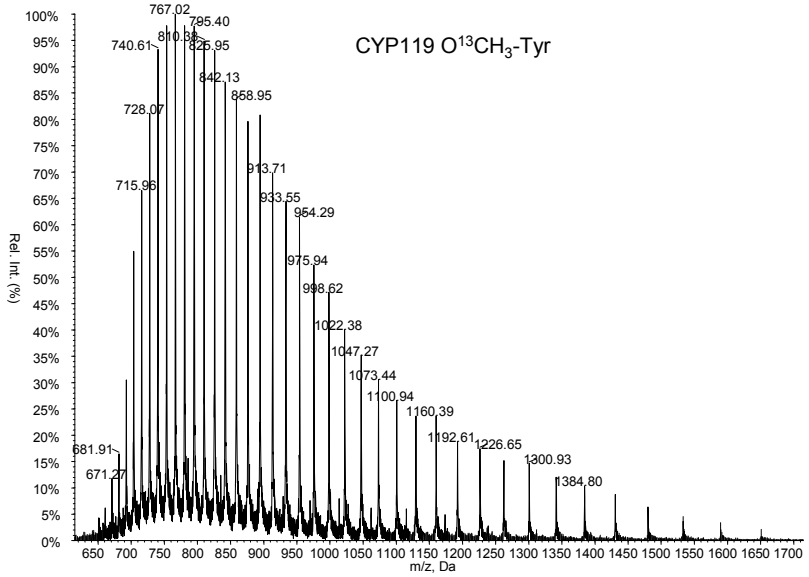


Figure S2. (A) TOF charge envelope for CYP119-wt. (B) Deconvoluted whole-protein MS spectra of CYP119-wt.

(A)



(B)

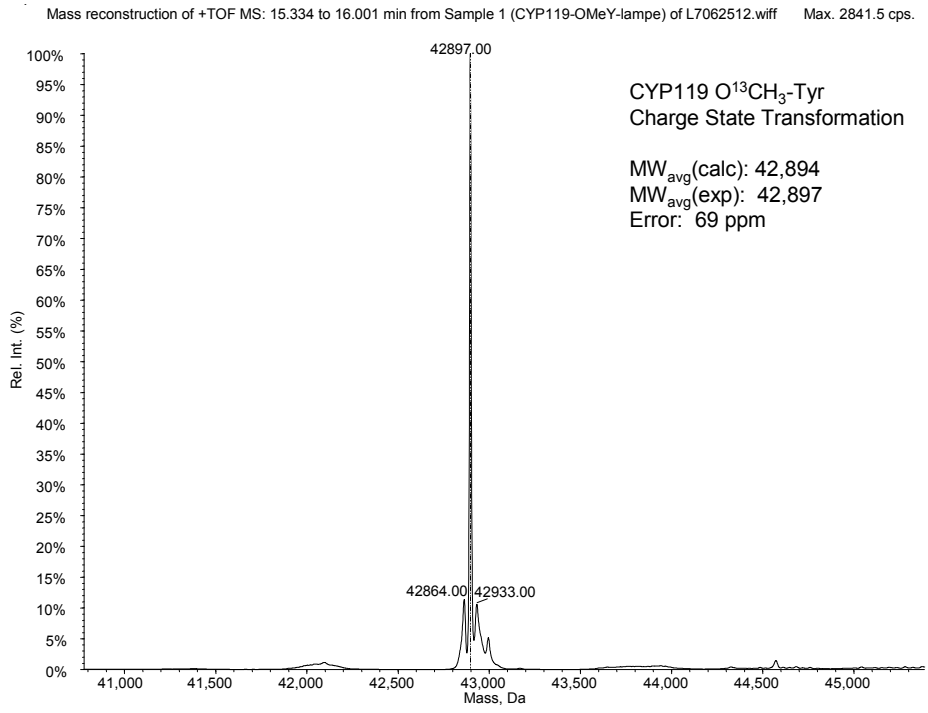


Figure S3. (A) TOF charge envelope for CYP119-F162-^{13}C-*p*-methoxyphenylalanine. (B) Deconvoluted whole-protein MS spectra of CYP119-F162-^{13}C-*p*-methoxyphenylalanine.

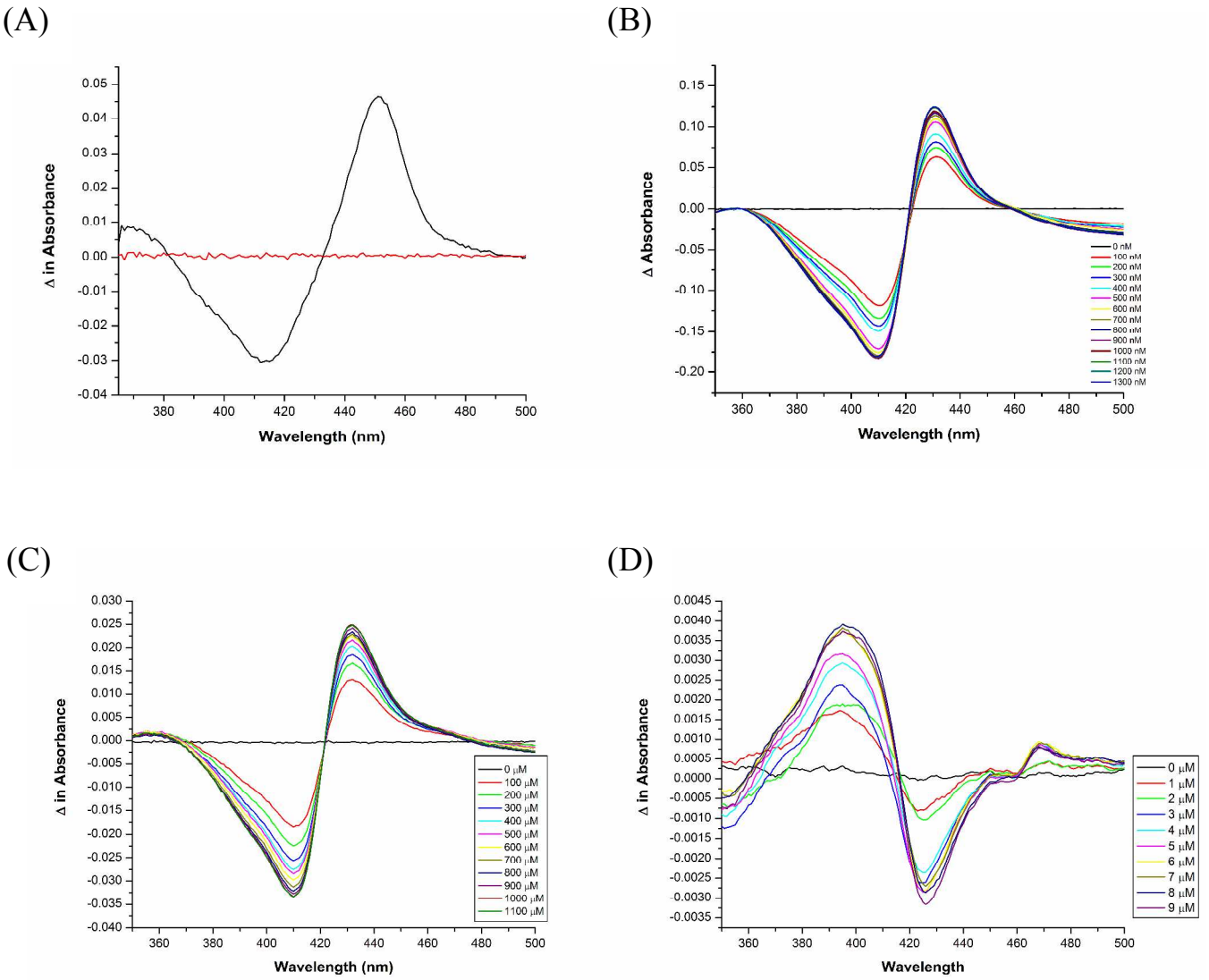


Figure S4. (A) Reduced CO-difference spectrum for CYP119-F162-^{13}CMeOF. (B) Optical binding titration for 4-phenylimidazole binding to CYP119-F162^{13}CMeOF. (C) Optical binding titration for Imidazole binding to CYP119-F162^{13}CMeOF. (D) Optical binding titration for lauric acid binding to CYP119-F162^{13}CMeOF. Note the appearance of a Type I spectrum, typical of substrate binding.

Ligand	***K*$_{s(app)}$ for CYP119-wt**	***K*$_{s(app)}$ for CYP119-F162-^{13}CMeOF**
4-Phenylimidazole	120 nM	176 nM
Imidazole	145 μM	137 μM
Lauric Acid	1 μM	2.4 μM

Table S1. Constants for the binding of 4-phenylimidazole, imidazole, and lauric acid to CYP119-F162-^{13}CMeOF. Spectral data from Figure S4 was fit to a single site Michaelis-Menten hyperbolic binding model to obtain the individual binding constants. $K_{s(app)}$ refers to the apparent dissociation constant as determined under these conditions.

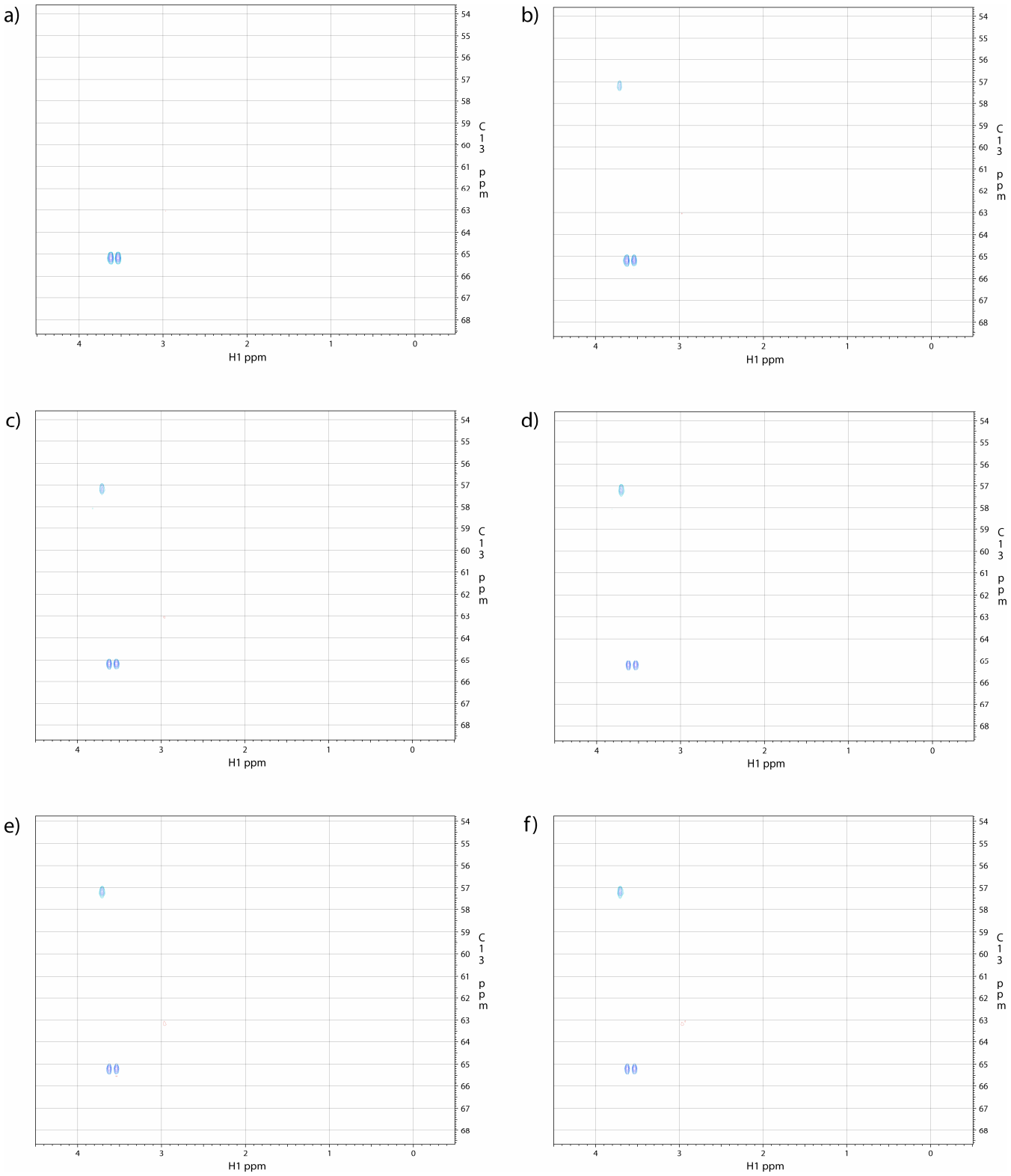


Figure S5. Full $^{1}H,^{13}C$-HSQC spectrum of the titration of CYP119-F162MeOF with 4-phenylimidazole (A) 0 M equivalents, (B) 0.3 M equivalents, (C) 0.6 M equivalents, (D) 0.9 M equivalents, (E) 1.2 M equivalents, and (F) 1.5 M equivalents.

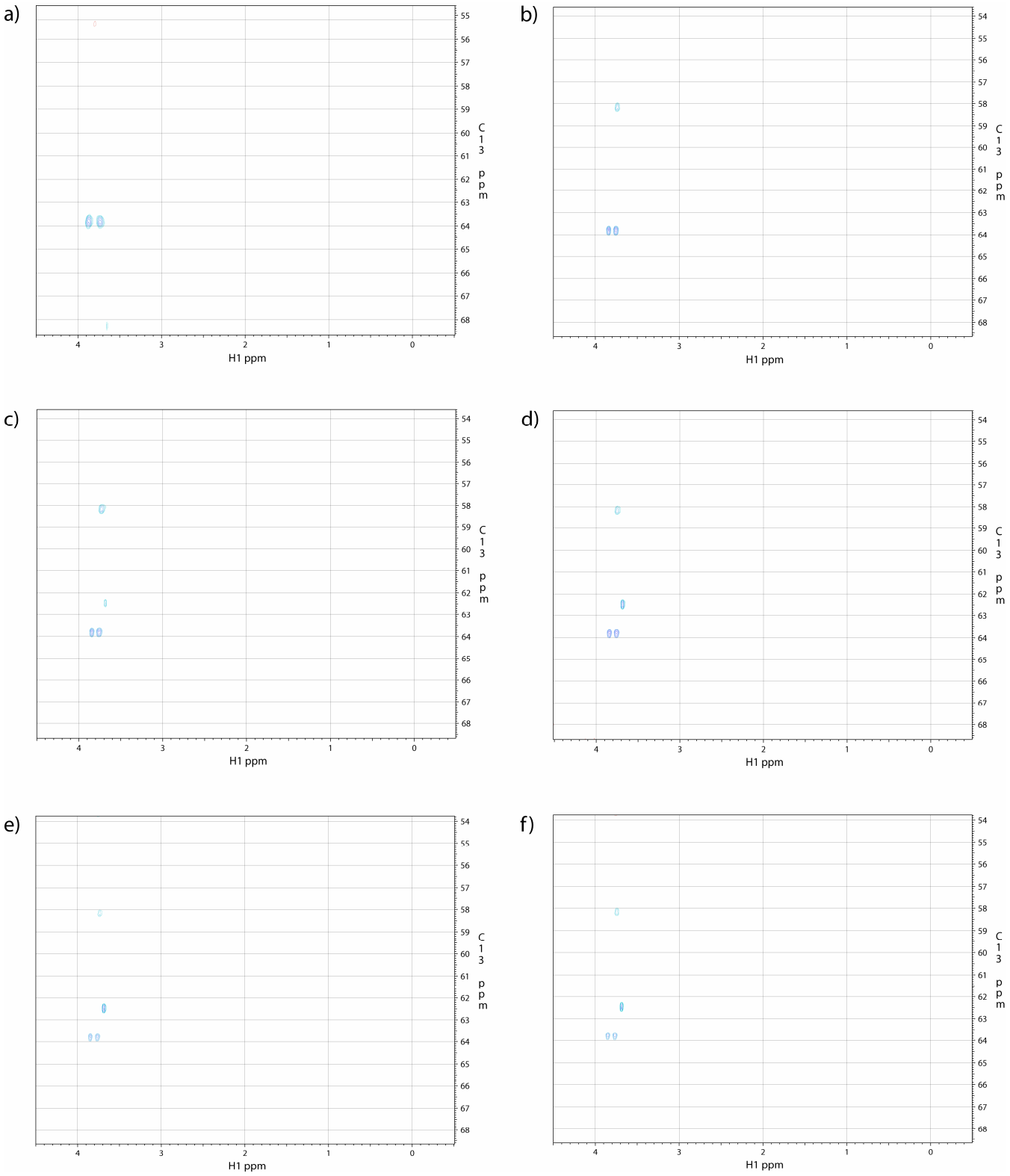


Figure 6. Full $^{1}H,^{13}C$-HSQC spectrum of the titration of CYP119-F153MeOF with 4-phenylimidazole (A) 0 M equivalents, (B) 0.3 M equivalents, (C) 0.6 M equivalents, (D) 0.9 M equivalents, (E) 1.2 M equivalents, and (F) 1.5 M equivalents.

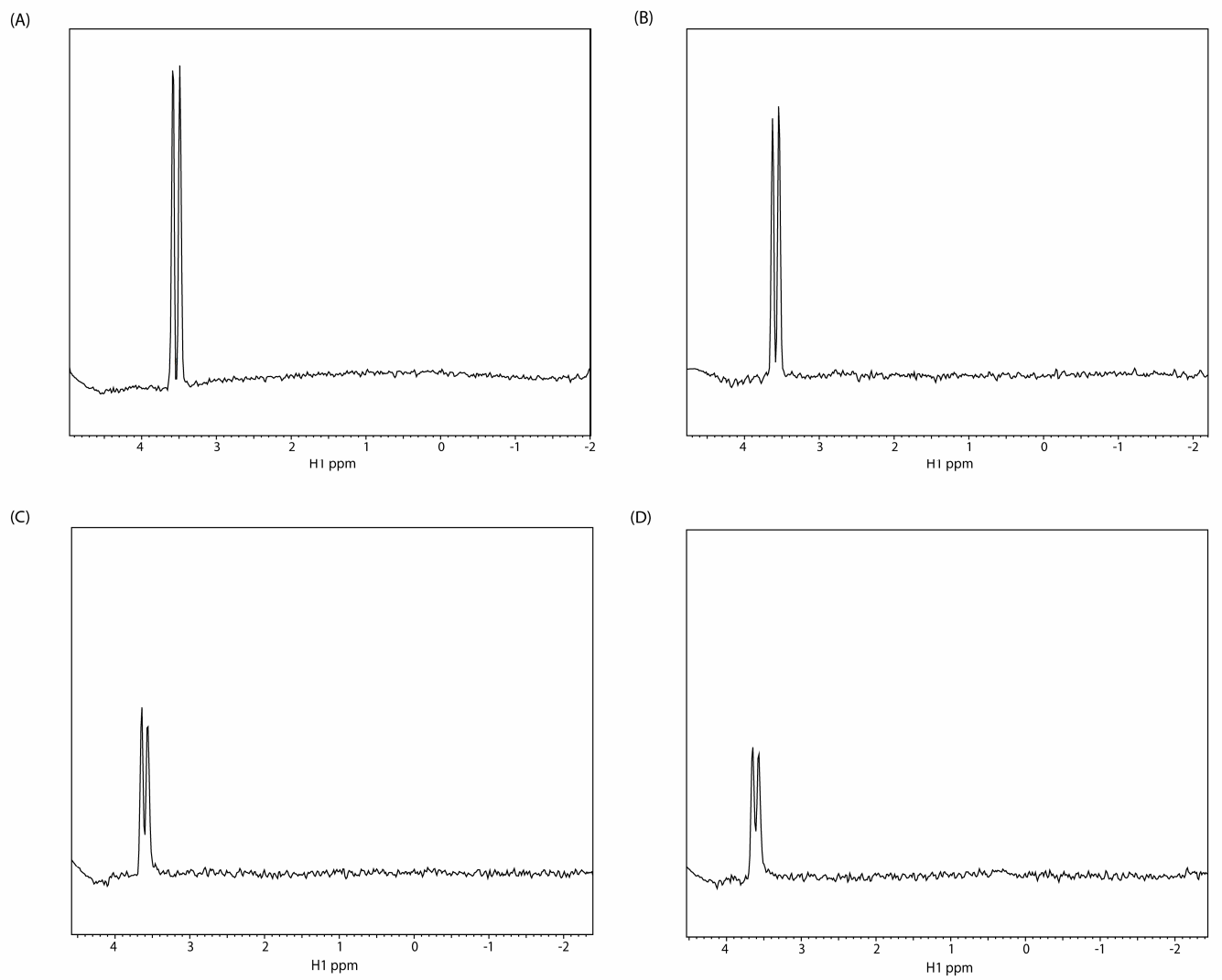


Figure S7. Effect of increasing temperature on the $^{1}H,^{13}C$ HSQC spectra of ligand-free CYP119-F162-^{13}C-MeOF. (A) 5 °C, (B) 25 °C, (C) 45 °C, (D) 55 °C.

Full Reference 9: Cellitti, S. E.; Jones, D. H.; Lagpacan, L.; Hao, X.; Zhang, Q.; Hu, H.; Brittain, S. M.; Brinker, A.; Caldwell, J.; Bursulaya, B.; Spraggon, G.; Brock, A.; Ryu, Y.; Uno, T.; Schultz, P. G.; Geierstanger, B. H. *J. Am. Chem. Soc.* **2008**, *130*, 9268-9281..