Experimental (Materials and Methods)

Materials

Unless otherwise noted, all reagents were obtained from commercial vendors and used without further purification. For expression of proteins in *E. coli*, Luria broth (LB), unlabeled amino acids, uracil, thiamine-HCl, nicotinic acid, biotin, ampicillin, chloramphenicol, IPTG, PMSF, and Triton X-100 were purchased from RPI corp. Nucleotide bases and 3-fluorotyrosine (3FY) were purchased from Alfa Aesar. Lysozyme was purchased from Gold Biotech, HEPES was purchased from Fischer Scientific, and [^{15}N] ammonium chloride was obtained from Cambridge Isotope Laboratories. ^{1}H, ^{13}C, and ^{19}F spectra were recorded at 500 MHz, 125 MHz, and 470 MHz respectively. MLL and pKID peptide sequences, synthesis, and characterization were reported by Pomerantz et al.[1]

Methods:

Unlabeled (UL), 3FY, 4FF, and 3FY/4FF-Labeled Protein Expression and Purification of His_6-KIX (586-672)

Expression

Protein expression using the same plasmid DNA for KIX was performed as previously described[1] with slight modifications. The protein expression plasmid was transformed into either the *E. coli* BL21(DE3) strain co-transformed with pRARE (Novagen) (unlabeled protein) or DL39(DE3) cells with the plasmid pRARE (3FY-, 4FF-, or 3FY/4FF-labeled proteins) and plated on an agar plate containing ampicillin (100 mg/L) and chloramphenicol (35 mg/L). 5 mL primary cultures of DL39 or BL21 cells transformed with the KIX expression plasmid supplemented with ampicillin (100 mg/L) and chloramphenicol (34 mg/L) were grown overnight (~12-14 h, 25 °C, 250 rpm) in an incubator shaker (New Brunswick Scientific Excella E25).

Primary cultures were added to fresh LB media (1 mL primary/50 mL media) to begin secondary culture growth. Secondary cultures were grown at (37 °C, 250 rpm) until they reached an OD_{600} of approximately 0.8 as evaluated by a Varian Cary 50 Bio UV/Vis spectrophotometer. The culture was centrifuged (8000 g, 10 min, 4 °C) to form a solid pellet of bacteria. The LB media was decanted, and the pellet was resuspended in defined media[2] containing the designated amount of aromatic or fluorinated aromatic amino acids (Table S1) at the original volume of LB media.. A recovery time of 30-120 min at 20 °C, 250 rpm was allowed prior to the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to induce protein overexpression overnight (15-20 h) Cultures were spun down (6000 g, 20 min, 4 °C). Media was decanted, and the pellet was stored at -20 °C.

Optimization of 4FF and 3FY/4FF KIX Expression Conditions

A range of conditions were varied to determine optimal expression conditions, including amount of 4FF, phenylalanine, and recovery time. Fluorine incorporation was calculated based on ESI-TOF data as described below and calculated using Equation 1. Results are summarized in the table below. Yields were not recorded for many early experiments since the first goal was to optimize label incorporation conditions. Bann and coworkers previously reported higher 4FF-labeld protein yields with the addition of low amounts of phenylalanine.[3] Initial results indicated that additional phenylalanine should be kept under 40 μM. Follow-up studies showed little to no improvement in label incorporation with higher amounts of 4FF. The next round of experiments determined that a 30 minute recovery time and 10 μM provided improved label incorporation. Longer recovery times were also explored and slightly lower amounts of phenylalanine were explored, determining that between 7.5 and 10 μM F and a 30 – 40 min.

recovery time yielded the best results. High labeling of the dual-labeled protein is also achievable by leaving out phenylalanine.

Table S1: Optimization of 4FF and 3FY/4FF KIX Conditions

4FF KIX			
4FF (μM)	F (μM)	Recovery Time (min)	% Fluorine Incorporation
160	160 80 40 0	30 min	32% 27% 28% 84%
160	40 20 0	30 min	33% 66% 75%
640 320	40	30 min	36% 27%
160	20 10 0	0 min	21% 32% 66%
160	20 10 0	30 min	73% 80% 75%
160	10 7.5 5	40 min	73% 84% 79%
160	10	60 min 120 min	68% 77%
3FY/4FF KIX			
160	0	40 min	95.5%

Purification via Fast Protein Liquid Chromatography (FPLC)

Following cell lysis (lysis buffer, lysozyme, β-mercaptoethanol, protease inhibitor phenylmethanesulfonylfluoride (PMSF, 2 mM), and sonication), proteins were purified on a GE Äktapurifier FPLC equipped with a Superloop for large volume injections. A column containing Ni-NTA beads (Qiagen) was used for affinity purification of the His-tagged protein. An elution gradient (30-400 mM) imidazole was used to initially bind and subsequently elute the His-tagged-protein from the Ni beads. Protein was then buffer exchanged into 50 mM HEPES and

100 mM NaCl, pH 7.2 using a HiPrep 26/10 Desalting column (GE Healthcare, Life Sciences) and stored at -20 °C or 4°C. During protein purification multiple aliquots of the protease inhibitor PMSF were added due to the instability of PMSF in aqueous solution.

UPLC/MS Conditions for Protein Characterization

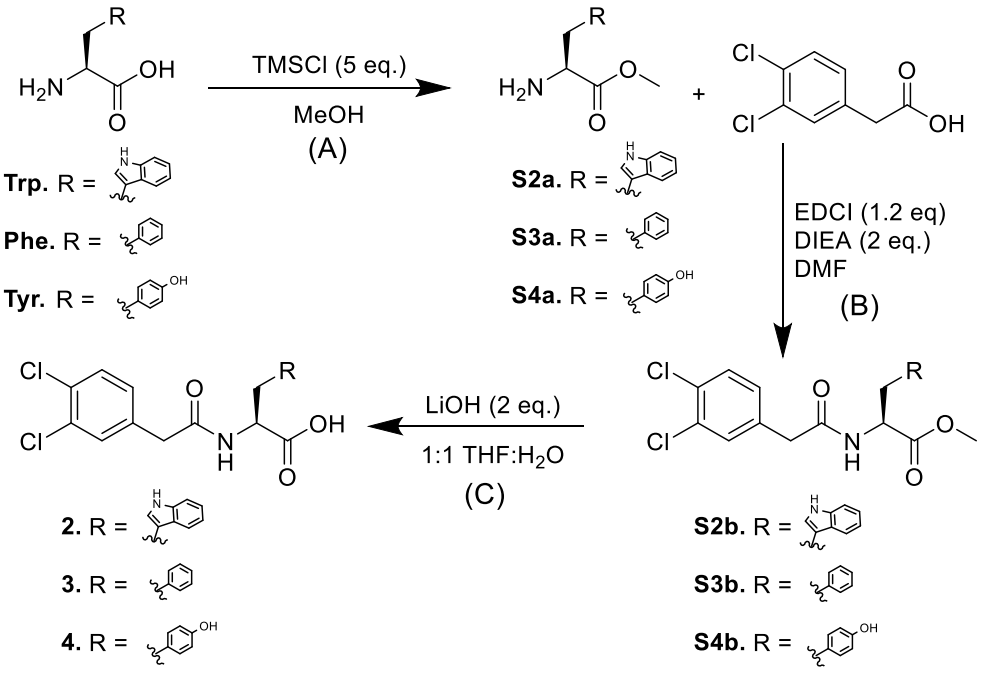
For UPLC/MS analysis of proteins, a Waters Acquity UPLC coupled to a Waters Synapt G2 HDMS quadrupole orthogonal acceleration time of flight mass spectrometer was used (Waters Corp., Milford, MA USA). A Waters Acquity UPLC Protein BEH C_4 2.1 mm x 100 mm column (1.7 μm diameter particles) at 35°C was used for the following 15 min linear gradient separation at a flow rate of 0.400 mL/min using A: water containing 0.1% formic acid and B: acetonitrile containing 0.1% formic acid: 3% B, 0 min to 3 min; 3% B to 97% B, 3 min to 9 min; 97% B, 9 min to 11 min; 97% B to 3% B, 11 min to 13 min; 3% B 13 min to 15 min. Mass spectra were collected in profile mode over the range *m/z* 300-2500 every 0.1 s during the chromatographic separation. MS parameters in positive electrospray ionization mode were as follows: capillary, 0.3 kV; sampling cone, 35.0 V; extraction cone, 4.0 V; desolvation gas flow, 800 L/h; source temperature, 100 °C; desolvation temperature, 350 °C; cone gas flow, 20 L/h; trap CE, off. Lockspray (on-the-fly mass calibration) configuration consisted of infusion of a 5 μg/mL solution of leucine-enkephalin and acquisition of one mass spectrum (0.2s scan, *m/z* 50-1200) every 10s. Three lockspray *m/z* measurements of protonated (positive ionization mode) leucine-enkephalin were averaged and used to apply a mass correction to measured *m/z* values during the course of the analysis. The m/z values were deconvoluted to calculate the mass of the protein.

Small Molecule Synthesis

Scheme 1 Methyl esterification of carboxylic acids

***General Procedure A*: *Methyl esterification of carboxylic acids*:** One of the compounds **1** or amino acids tryptophan, tyrosine, or phenylalanine, was dissolved in 5-10 mL MeOH. TMSCl (5 eq) was added, and the reaction was stirred at room temperature overnight. TLC indicated the reaction was complete. The solvent was evaporated *in vacuo*, and material was collected to carry on to the next step without further purification.

methyl 2-(3,4-dichlorophenyl)acetate (1-OMe)
3,4-dichlorophenylacetic acid (**1**) was converted to its methyl ester according to general procedure A on a 0.512 mmol scale. The purification of this compound deviated from the general procedure since it was the final compound. After removing the MeOH solvent, the material was dissolved in EtOAc, and the organic phase was washed three times with a $NaHCO_3$ solution, and then washed with brine. The organic layer was removed, dried over $MgSO_4$, filtered to remove the drying agent, and concentrated *in vacuo*, providing **1-OMe** as a white solid (80 mg/0.365 mmol, 71%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39 (d, *J* = 8.1 Hz, 1 H), 7.38 (d, *J* = 2.0 Hz, 1 H), 7.11 (dd, *J* = 8.1, 2.0 Hz, 1 H), 3.70 (s, 3 H), 3.57 (s, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.14, 134.10, 132.64, 131.51, 131.41, 130.58, 128.88, 52.40, 40.21; GC-MS calc'd m/z 218, found M [218]



Scheme 2 Analog synthesis for ligand development

General Procedure B*: *Amide bond formation Compound **1** and **S2a**, **S3a**, or **S4a** (1 eq) were dissolved in DMF with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (1.2 eq) and N,N-diisopropylethylamine (DIEA) (2 eq). Reactions were stirred at room temperature overnight. TLC indicated the completion of the reaction. The reaction mixture was then diluted with EtOAc and washed with water and brine. The organic layer was collected, dried over $MgSO_4$, and the solvent was evaporated *in vacuo*. The product was then purified by column chromatography and isolated as a solid.

General Procedure C: Hydrolysis of methyl ester to carboxylic acid. Compound **S2b**, **S3b**, or **S4b** (1 eq) were dissolved in a 1:1 THF:H_2O solution with LiOH (2 eq) and stirred for approximately 6 hours. TLC indicated the completion of the reaction. The final product was isolated using an acid/base extraction to separate starting materials and unreacted methyl esters from the free carboxylic acid. The final organic layer was dried over $MgSO_4$, filtered, and concentrated *in vacuo* to isolate a solid. It is important to note that although the stereochemistry of the final product (**2, 3, 4**) is indicated in the reaction scheme the degree to which the stereochemistry may have eroded during hydrolysis has not been evaluated.

<u>Tryptophan Analogs</u>

methyl tryptophanate (S2a)

Tryptophan (**Trp**) was converted to its methyl ester according to general procedure A on a 1.47 mmol scale in 10 mL of MeOH. Removal of solvent yielded **S2a** as a red-purple solid (436 mg/2 mmol, 135% yield). ^{1}H NMR (500 MHz, MeOD): δ 7.54 (d, *J* = 7.9 Hz, 1 H), 7.39 (d, *J* = 8.1 Hz, 1 H), 7.19 (s, 1 H), 7.15 (t, *J* = 7.6 Hz, 1 H), 7.07 (t, 7.6 Hz, 1 H), 4.32 (dd, *J* = 7.6, 5.5 Hz, 1 H), 3.80 (s, 3 H), 3.46 (dd, *J* = 15.1, 5.4, 1 H), 3.35 (dd, *J* = 15.1, 7.6 Hz, 1 H); ^{13}C NMR (125 MHz, MeOD): δ 170.86, 138.36 (low intensity), 128.17, 125.53, 123.00, 120.36, 118.79, 112.70, 107.49, 54.64, 53 65, 27.62; Hi-Res MS calc'd *m/z*, 218.10553, found M+H [219.11366]

methyl (2-(3,4-dichlorophenyl)acetyl)tryptophanate (S2b)

Compound **S2a** was coupled to **1** according to general procedure B on a 1.18 mmol scale. Following the removal of solvent *in vacuo*, the material was purified by column chromatography using a 3:2 EtOAc:Hexanes mobile phase. The final product (**S2b**) was isolated as a yellow solid (199 mg/0.491 mmol, 41.5% yield). ^{1}H NMR (500 MHz, MeOD): δ 7.47 (d, *J* = 7.9 Hz, 1 H), 7.30-7.34 (m, 3 H), 7.09 (t, *J* = 7.6 Hz, 1 H), 6.95-7.01 (m, 3H), 4.76 (dd, *j* = 8.3, 5.3 Hz 1 H), 3.68 (s, 3 H), 3.45 (s, 2 H), 3.3 (m, 1 H)*, 3.16 (dd, *J* = 14.7, 8.3 Hz, 1 H) *Signal obscured due to solvent overlap; ^{13}C NMR (125 MHz, MeOD): δ 173.82, 172.70, 138.02, 137.35, 133.08, 132.21, 131.69, 131.38, 129.98, 128.69, 124.36, 122.47, 119.87, 119.08, 112.36, 110.62, 54.98, 52.73, 42.16, 28.35; Hi-Res MS calc'd *m/z*, 404.06945 found M+Na [427.06065]

(2-(3,4-dichlorophenyl)acetyl)tryptophan (2)

Compound **S2b** was hydrolyzed according to general procedure C on a 0.10 mmol scale. The final compound **2** was isolated as a yellow oil (26 mg/, 66.5% yield). ^{1}H NMR (500 MHz, MeOD): δ 7.51 (d, *J* = 8.0 Hz, 1 H), 7.33 (d, *J* = 8.1 Hz, 1 H), 7.29 (s, 1 H), 7.28 (d, *J* = 8.1 Hz, 1 H), 7.09 (ddd, *J* = 7.9, 7.2, 1.0 Hz, 1 H), 7.03 (s, 1 H), 6.98 (ddd, *J* = 7.9, 7.2, 1.0 Hz, 1 H),

6.92 (dd, *J* = 8.2, 1.9 Hz, 1 H), 4.75 (dd, *J* = 8.3, 4.8 Hz, 1 H), 3.43 (s, 2 H), 3.37 (dd, *J* = 14.8, 4.7 Hz, 1 H), 3.17 (dd, *J* = 14.7, 8.3 Hz, 1 H); ^{13}C NMR (125 MHz, MeOD): δ 175.64, 172.54, 137.97, 137.36, 133.05, 132.27, 131.64, 131.37, 130.01, 128.93, 124.33, 122.36, 119.81, 119.23, 112.29, 111.04, 55.15, 42.35, 28.42; Hi-Res MS calc'd *m/z*, 390.05380 found M-H [389.04570]

Phenylalanine Analogs

methyl phenylalaninate (S3a)

Phenylalanine (**Phe**) was converted to its methyl ester according to general procedure A on a 1.82 mmol scale in 10 mL of MeOH. Removal of solvent yielded **S3a** as a white solid and used without further purification. ^{1}H NMR (500 MHz, MeOD): δ 7.38 (t, *J* = 7.3 Hz, 2 H), 7.33 (t, *J* = 7.3 Hz, 1 H), 7.26 (d, *J* = 7.3 Hz, 1 H), 4.32 (dd, *J* = 6.2, 7.3 Hz, 1 H), 3.80 (s, 3 H), 3.27 (dd, *J* = 14.4, 6.2 Hz, 1 H), 3.17 (dd, *J* = 14.4, 6.2 Hz, 1H); ^{13}C NMR (125 MHz, MeOD): δ 170.47, 135-27, 130.40, 130.20, 129.01, 55.20, 53.60, 37.42; Hi-Res MS calc'd *m/z*, 179.19463 found M+H [180.10942]

methyl (2-(3,4-dichlorophenyl)acetyl)phenylalaninate (S3b)

Compound **S3a** was coupled to **1** according to general procedure B on a 0.858 mmol scale. Following the removal of solvent *in vacuo*, the material was purified by column chromatography using a 2:1 EtOAc:Hexanes mobile phase. The final product (**S3b**) was isolated as a white solid (116 mg/0.317 mmol, 37.0 % yield). ^{1}H NMR (500 MHz, MeOD): δ 7.48 (d, *J* = 8.3 Hz, 1 H), 7.35 (d, *J* = 2.0 Hz, 1 H), 7.19-7.24 (m, 3 H), 7.13 (dd, *J* = 8.0, 1.7 Hz, 2 H), 7.06 (dd, *J* = 8.4, 2.0 Hz, 1 H), 4.68 (dd, *J* = 9.4, 5.3 Hz, 1 H), 3.71 (s, 3 H), 3.45 (s, 2 H), 3.17 (dd, *J* = 13.9, 5.2 Hz, 1 H), 2.94 (dd, *J* = 13.9, 9.6 Hz, 1 H); ^{13}C NMR (125 MHz, MeOD): δ 173.35, 172.69, 138.02, 137.44, 133.12, 132.16, 131.76, 131.45, 130.15, 130.05, 129.48, 127.89, 55.18, 42.77, 42.24, 38.23; Hi-Res MS calc'd *m/z*, 365.05855 found M+Na [388.05118]

(2-(3,4-dichlorophenyl)acetyl)phenylalanine (3)

Compound **S3b** was hydrolyzed according to general procedure C on a 0.082 mmol scale. The final compound **3** was isolated as a white solid though a small contamination does not allow for accurate yield calculations). ^{1}H NMR (500 MHz, MeOD): δ 7.37 (d, *J* = 8.3 Hz, 1 H), 7.34 (d, *J* = 2.0 Hz, 1 H), 7.18-7.24 (m, 3 H), 7.13 (dd, *J* = 8.0, 1.1 Hz, 2 H), 7.04 (dd, *J* = 8.4, 2.0 Hz, 1 H), 4.66 (dd, *J* = 9.4, 5.0 Hz, 1 H), 3.45 (d, *J* = 2.0 Hz, 2 H), 3.22 (dd, *J* = 14.0, 4.9 Hz, 1 H), 2.93 (dd, *J* = 14.0, 9.4 Hz, 1 H); ^{13}C NMR (125 MHz, MeOD): δ 174.80 (low intensity), 172.55, 138.37, 137.53, 133.11, 132.21, 131.73 (low intensity), 131.42, 130.22, 130.07, 129.39, 127.74, 55.14, 42.38, 38.35; Hi-Res MS calc'd *m/z*, 351.04290, found M-H [350.02350]

Tyrosine Analogs

methyl tyrosinate (S4a)

Tyrosine (**Tyr**) was converted to its methyl ester according to general procedure A on a 1.66 mmol scale in 10 mL of MeOH. Removal of solvent yielded **S4a** as a white solid (126.4 mg/0.648 mmol, 39% yield). ^{1}H NMR (500 MHz, $CDCl_3$/MeOD): δ 6.96 (d, *J* = 8.3 Hz, 2 H), 6.73 (d, *J* = 8.3 Hz, 2 H), 3.69 (s, 3 H), 3.65 (dd, *J* = 7.6, 5.4 Hz, 1 H), 2.97 (dd, *J* = 13.7, 5.2 Hz, 1 H), 2.78 (dd, *J* = 13.7, 7.6 Hz, 1 H); ^{13}C NMR (125 MHz, $CDCl_3$/MeOD): δ 175.28, 155.79, 130.37, 127.61, 115.65, 55.56, 52.18, 39.78; Hi-Res MS calc'd *m/z*, 195.08954 found M+H [196.09711]

methyl (2-(3,4-dichlorophenyl)acetyl)tyrosinate (S4b)

Compound **S4a** was coupled to **1** according to general procedure B on a 0.647 mmol scale. Following the removal of solvent *in vacuo*, the material was purified by column chromatography using a 79:1 DCM:MeOH mobile phase. The final product (**S4b**) was isolated as a white solid (44.9 mg/0.117 mmol, 18.0 % yield). ^{1}H NMR (500 MHz, MeOD): δ 7.39 (d, *J* = 8.3, 1 H), 7.37 (d, *J* = 2.0 Hz, 1 H), 7.03 (dd, *J* = 8.2, 2.0 Hz, 1 H), 6.95 (d, *J* = 8.5 Hz, 2 H), 6.67 (d, *J* = 8.6 Hz, 2 H), 4.62 (dd, *J* = 9.2, 5.4 Hz, 1 H), 3.70 (s, 3 H), 3.47 (s, 2 H), 3.07 (dd, *J* = 14.1, 5.4 Hz, 1 H), 2.85 (dd, *J* = 14.1, 9.2 Hz, 1 H) ^{13}C NMR (125 MHz, MeOD): δ 173.50, 172.69, 157.47, 137.47, 133.14, 132.21, 131.75, 131.45, 131.16, 130.01, 128.59, 116.27, 55.48, 52.70, 42.22, 37.50; Hi-Res MS calc'd *m/z*, 381.05346 found M-H [380.04781]

(2-(3,4-dichlorophenyl)acetyl)tyrosine (4)

Compound **S4b** was hydrolyzed according to general procedure C on a 0.045 mmol scale. An excess of LiOH was used in this reaction (~400 eq). The final compound **4** was isolated as a white solid, though a small contamination does not allow for accurate yield calculations. ^{1}H NMR (500 MHz, MeOD): δ 7.40 (d, *J* = 8.3 Hz, 1 H), 7.38 (d, *J* = 2.0 Hz, 1 H), 7.02 (dd, *J* = 8.3, 2.0 Hz, 1 H), 6.97 (d, *J* = 8.5 Hz, 2 H), 6.66 (d, *J* = 8.5 Hz, 2 H), 4.60 (dd, *J* = 9.27, 4.82 Hz, 1 H), 3.47 (d, *J* = 5.61 Hz, 2 H), 3.12 (dd, *J* = 14.0, 4.9 Hz, 1 H), 2.85 (dd, *J* = 14.1, 9.2 Hz, 1 H) ^{13}C NMR (125 MHz, MeOD): δ 175.06 (low intensity), 172.53, 157.33, 137.55, 133.13, 132.26, 131.71, 131.43, 131.23, 130.04, 129.02, 116.19, 55.47, 42.38, 37.60; Hi-Res MS calc'd *m/z*, 367.03781 found M-H [366.03161]

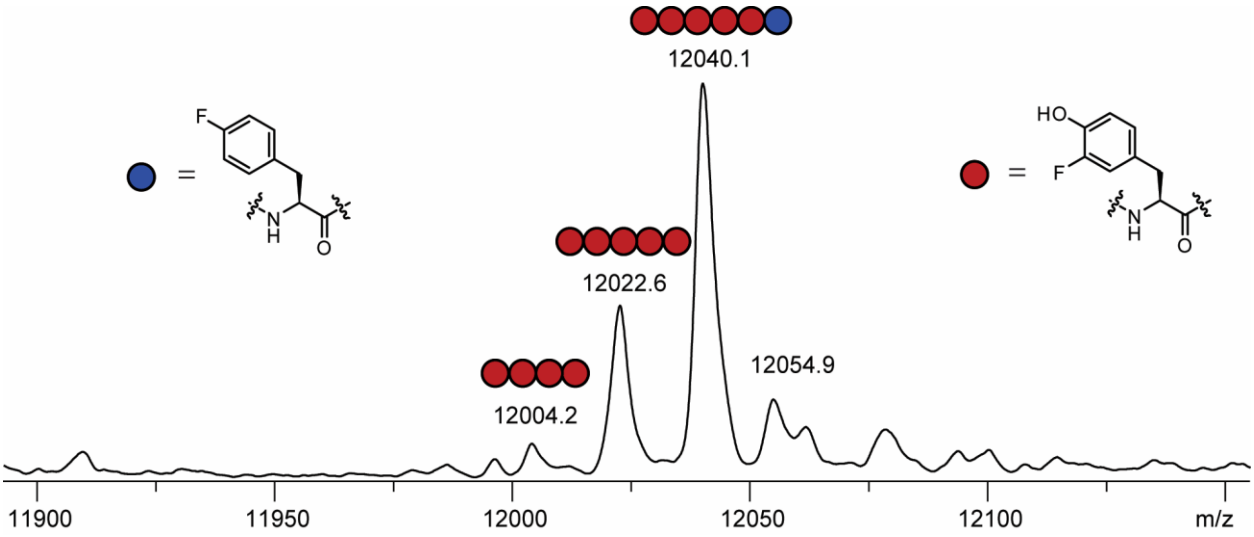
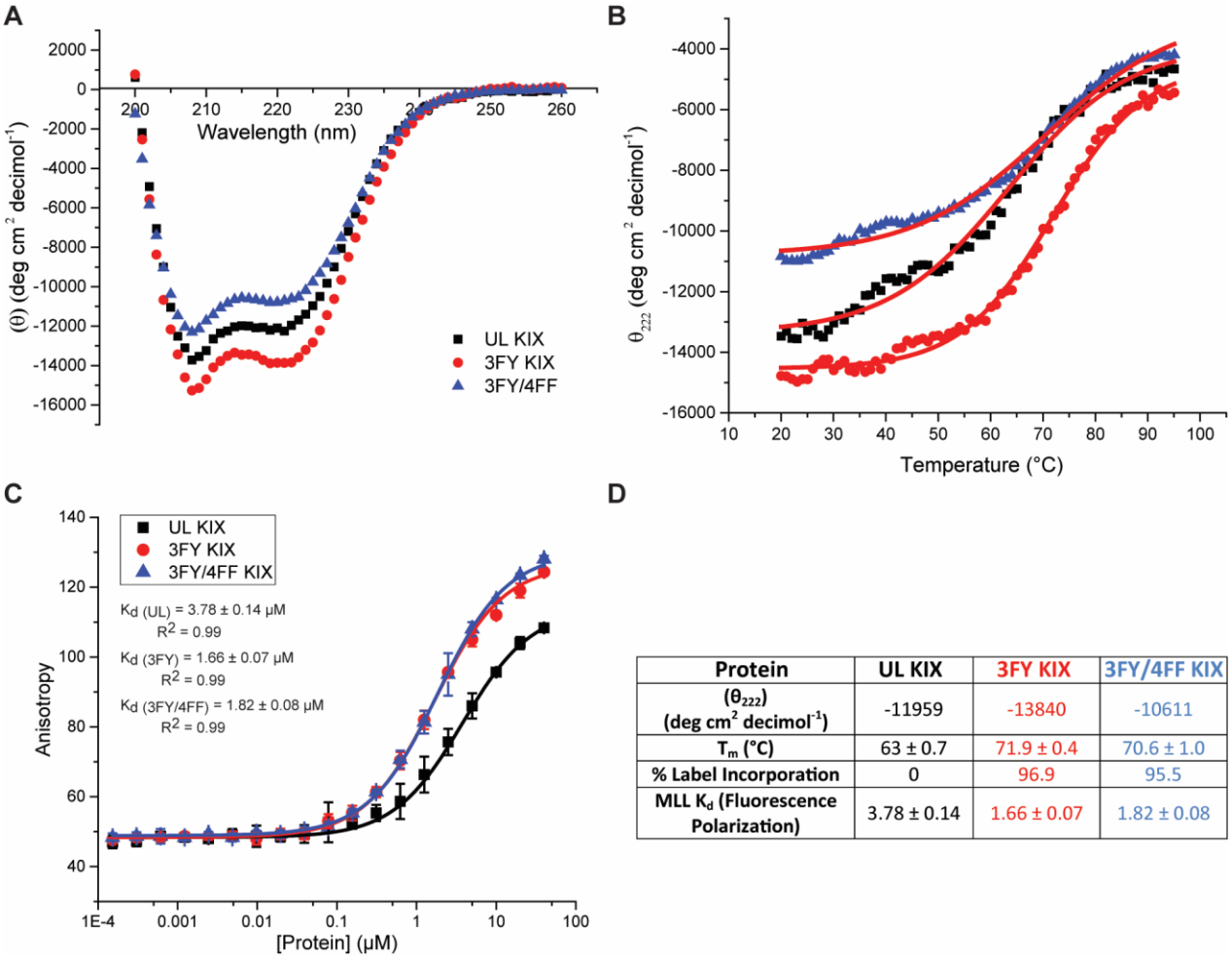


Figure S1: The deconvoluted ESI-mass spectrogram of a 3FY/4FF KIX sample demonstrating high fluorine incorporation. The peak at 12004.2 is a 67% labeled protein, the peak at 12022.6.6 is an 83% labeled protein, the peak at 12040.1 is the 100% labeled protein containing all six fluorinated residues, and the peak at 12054.9 is consistent with a small percentage of an oxidized product that was presumed to occur from methionine oxidation. Percent labeling is calculated with the equation below, using the integration values of the various deconvoluted mass peaks as the values for F KIX.

$$\% \, Fluorine \, Incorporation = \frac{(0F \; KIX)*0+(1F \; KIX)*1+(2F \; KIX)*2\ldots(6F \; KIX)*6}{(0F \; KIX)*6+(1F \; KIX)*1+(2F \; KIX)*6\ldots(6F \; KIX)*6} \quad (1)$$



Protein	UL KIX	3FY KIX	3FY/4FF KIX
(θ_{222}) (deg cm^2 decimol^{-1})	-11959	-13840	-10611
T_m (°C)	63 ± 0.7	71.9 ± 0.4	70.6 ± 1.0
% Label Incorporation	0	96.9	95.5
MLL K_d (Fluorescence Polarization)	3.78 ± 0.14	1.66 ± 0.07	1.82 ± 0.08

Figure S2: Structure and function validation of dual labeled protein. A) CD Spectra, B) thermal melt, and C) MLL affinity comparisons of unlabeled (UL), 3FY-labeled, and 3FY/4FF-labeled KIX. Unlabeled protein is plotted in black. 3FY protein is plotted in red. 3FY/4FF protein is plotted in blue. D) Summary table of structural and functional data.

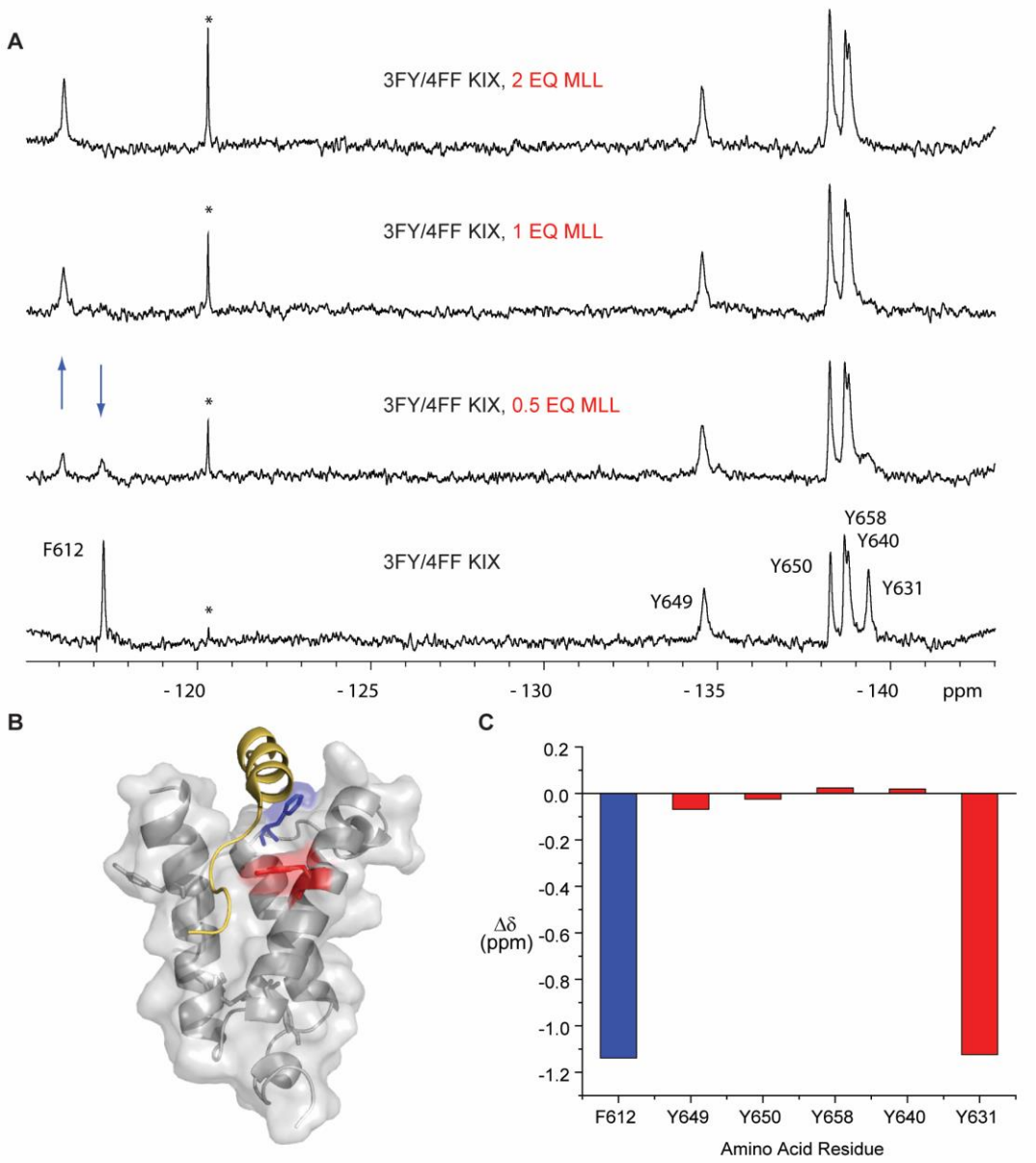


Figure S3: PrOF NMR results with 3FY/4FF KIX and MLL A) Stacked PrOF NMR spectra of 3FY/4FF KIX and increasing concentrations of MLL. Y631 and F612 are the only two resonance that are perturbed. MLL also demonstrates slow exchange kinetics, as noted by the unbound and bound states being resolved at 0.5 EQ MLL. ***** This resonance is an artifact that does not originate in the sample. B) Structure of KIX:MLL complex (2AGH) with Y631 highlighted in red and F612 highlighted in blue. Both are in close proximity to the native MLL peptide. C) Chemical shift perturbations of 3FY/4FF KIX with 2eq MLL.

Table S2: 3FY/4FF-KIX ^{19}F NMR chemical shift perturbations at varying MLL concentrations.							
[MLL]	Chemical shift (ppm)	F612	Y649	Y650	Y658	Y640	Y631
0	Chemical shift (ppm)	-117.2887	-134.6191	-138.2642	-138.6706	-138.7750	-139.3630
2 EQ	Δδ (ppm)	-1.1385	-0.0680	-0.0250	0.0236	0.0189	-1.1238

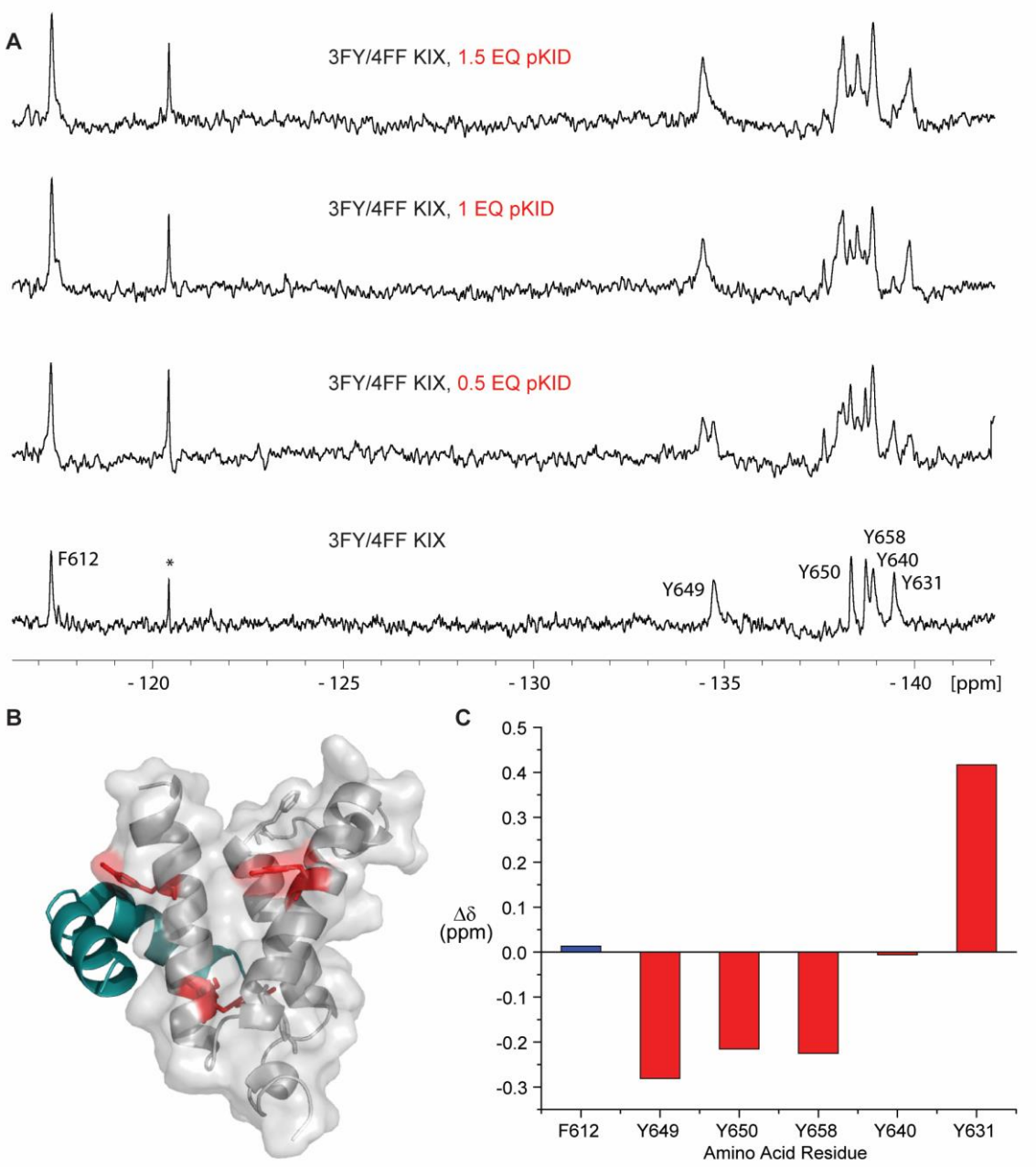


Figure S4: PrOF NMR results with 3FY/4FF KIX and pKID A) Stacked PrOF NMR spectra of 3FY/4FF KIX and increasing concentrations of pKID. Y649, Y650, and Y658 are perturbed by direct contact while Y631 is allosterically perturbed. ***** This resonance is an artifact that does not originate in the sample. B) Structure of KIX:MLL complex (1KDX) with Y649, Y650, Y658, and Y631 highlighted in red. C) Chemical shift perturbations of 3FY/4FF KIX with 1.5 eq pKID.

Table S3: 3FY/4FF-KIX ^{19}F NMR chemical shift perturbations at varying pKID concentrations.							
[pKID]	Chemical shift (ppm)	F612	Y649	Y650	Y658	Y640	Y631
0	Chemical shift (ppm)	-117.3396	-134.7238	-138.3410	-138.7253	-138.9210	-139.4710
1.5 EQ	Δδ (ppm)	0.0136	-0.2809	-0.2155	-0.2253	-0.0061	0.4167

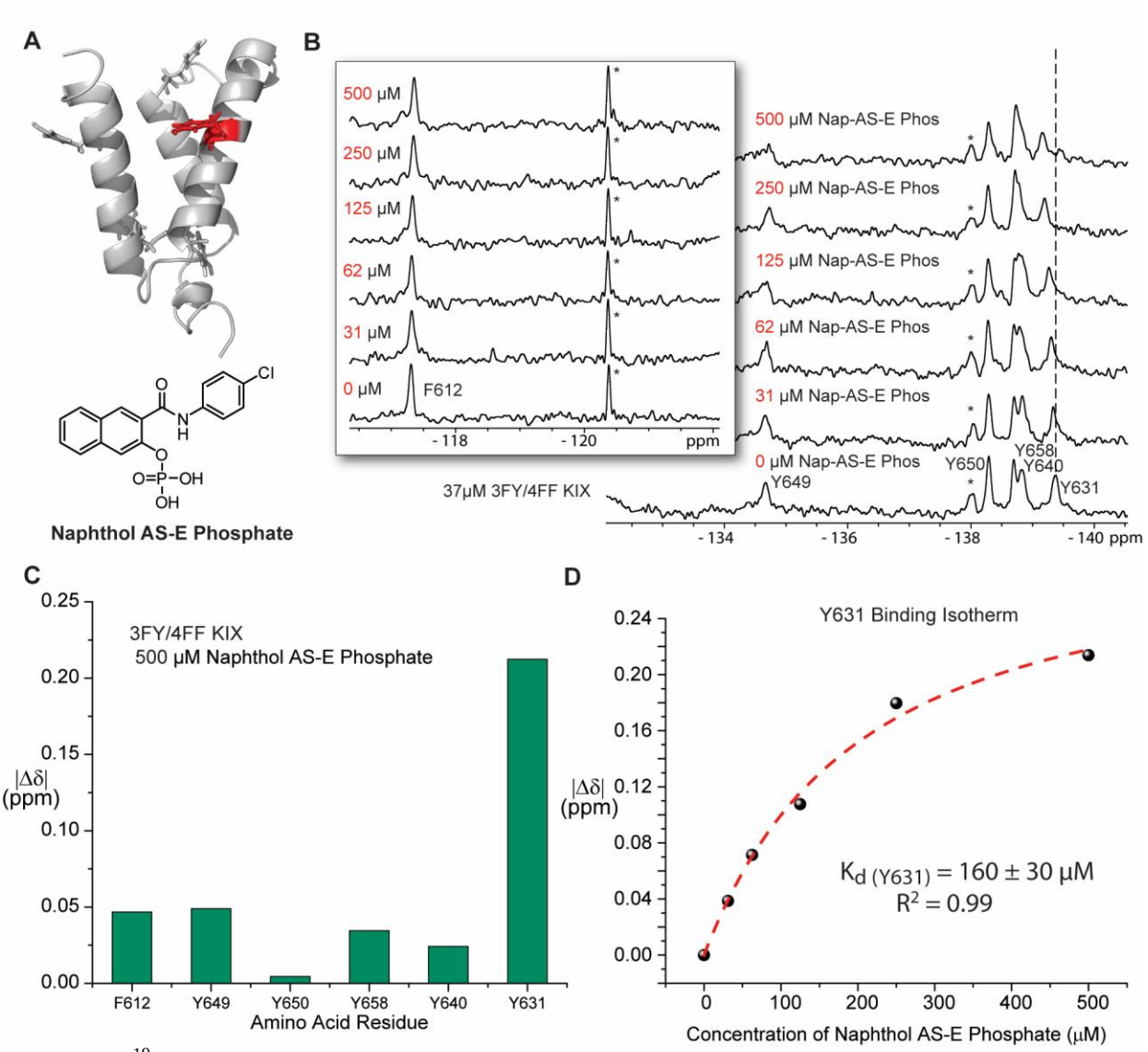


Figure S5: ^{19}F NMR spectral analysis of 3FY/4FF KIX in the presence of increasing concentrations of Naphthol AS-E Phosphate. A) Ribbon diagram of KIX (PDB Code: 1KDX) with tyrosine side chains indicated as sticks. Residues that were significantly perturbed and approached saturation are highlighted in red. ***** This resonance is an artifact that does not originate in the sample. B) Stacked spectra of 3FY/4FF KIX with increasing concentrations of Naphthol AS-E Phosphate. Inset) Resonances from F612. C) Absolute value of chemical shift perturbations for all 3FY/4FF KIX resonances at 500 µM Naphthol AS-E Phosphate. D) Binding isotherm of Y631 perturbation for the titration of Naphthol AS-E Phosphate.

Table S4: 3FY/4FF-KIX ^{19}F NMR chemical shift perturbations at varying Naphthol AS-E Phosphate concentrations.

[Nap]	Chemical shift (ppm)	F612	Y649	Y650	Y658	Y640	Y631
0	Chemical shift (ppm)	-117.311	-134.6720	-138.2915	-138.6966	-138.8242	-139.3750
31.25	|Δδ| (ppm)	0.0084	0.0065	0.0022	0.0060	0.0106	0.0386

62	|Δδ| (ppm)	0.0090	0.0189	0.0114	0.0043	0.0351	0.0714
125	|Δδ| (ppm)	0.0225	0.0220	0.0101	0.0794	0.0482	0.1076
250	|Δδ| (ppm)	0.1114	0.0593	0.0119	0.0202	0.0552	0.1796
500	|Δδ| (ppm)	0.0467	0.0490	0.0045	0.0344	0.0242	0.2138

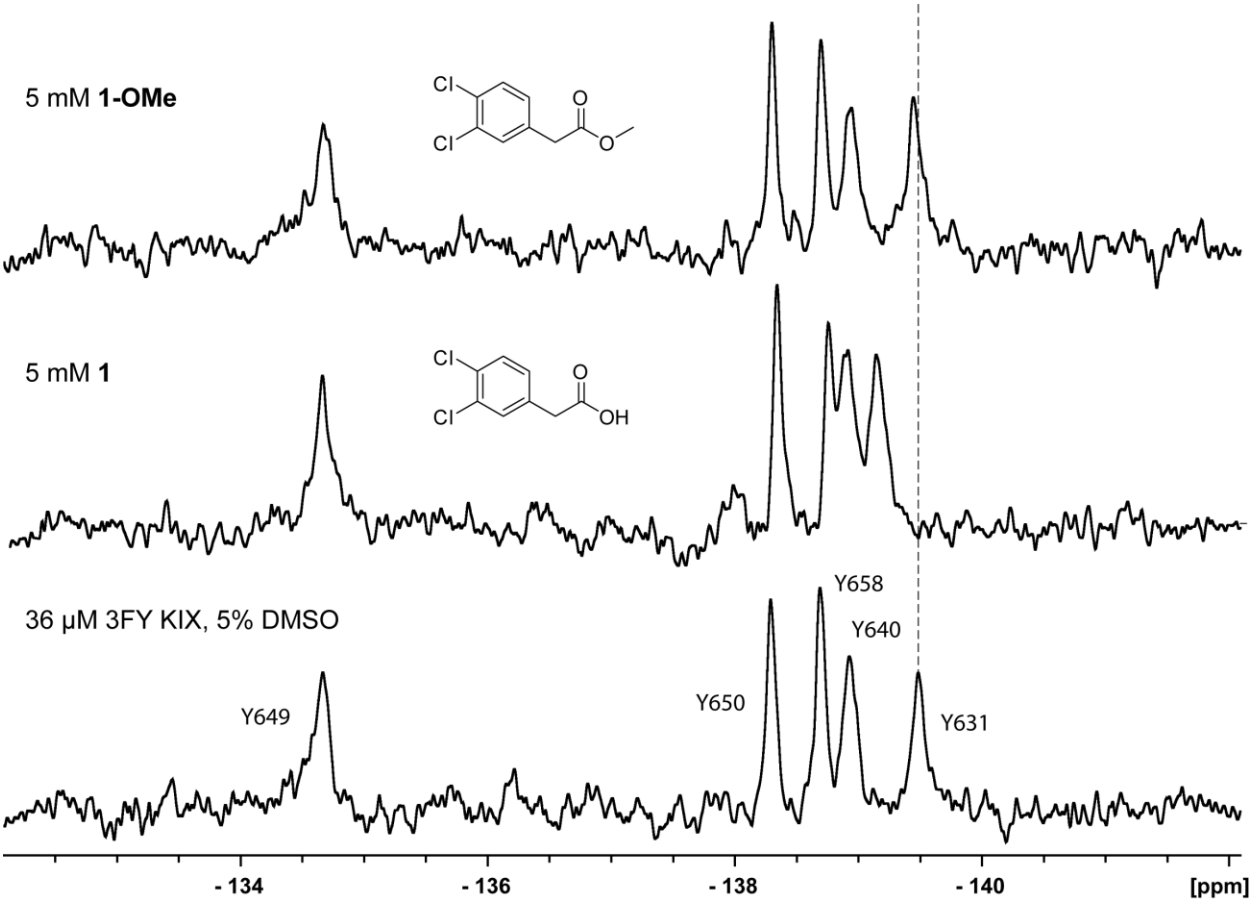


Figure S6: PrOF NMR data comparing **1** and **1-OMe** which indicates that the methyl ester eliminates the activity of the parent compound based on the observation that Y631 is no longer perturbed.

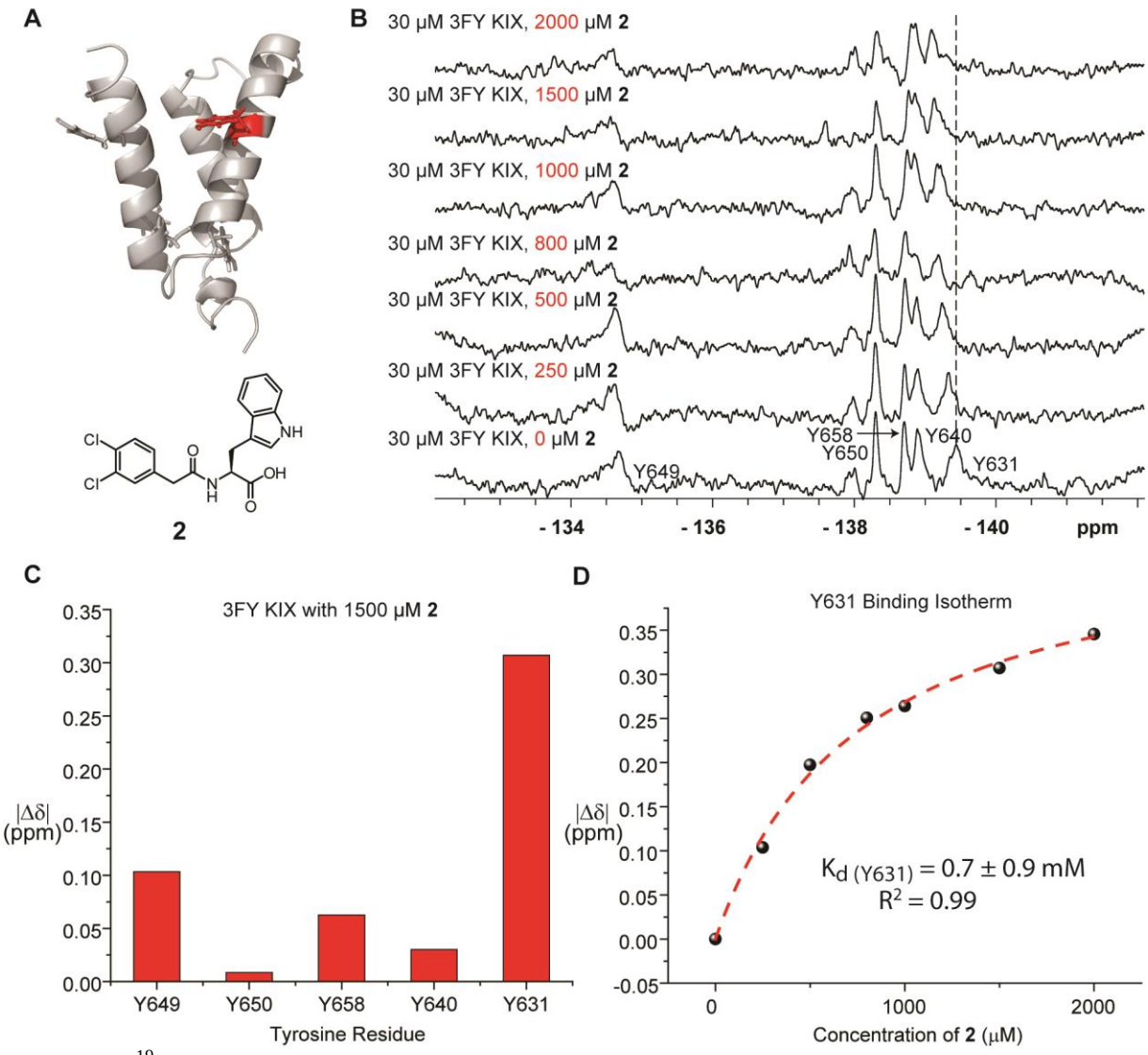


Figure S7: ^{19}F NMR spectral analysis of 3FY KIX in the presence of increasing concentrations of **2**. A) Ribbon diagram of KIX (PDB Code: 1KDX) with tyrosine side chains indicated as sticks. Residues that were significantly perturbed and approached saturation are highlighted in red. B) Stacked spectra of 3FY KIX with increasing concentrations of **2** C) Absolute value of chemical shift perturbations for all 3FY KIX resonances at 1500 μM **2**. D) Binding isotherm of Y631 perturbation for the titration of **2**

Table S5: 3FY-KIX ^{19}F NMR chemical shift perturbations at varying **2** concentrations.						
[**2**] (μM)	Chemical shift (ppm)	Y649	Y650	Y658	Y640	Y631
0	Chemical shift (ppm)	-134.6638	-138.3012	-138.7056	-138.8887	-139.437
250	|Δδ| (ppm)	0.0451	0.0048	0.0061	0.0099	0.1038
500	|Δδ| (ppm)	0.0503	0.0019	0.0110	0.0060	0.1974
800	|Δδ| (ppm)	0.0910	0.0120	0.0181	0.0250	0.2507
1000	|Δδ| (ppm)	0.0840	0.0014	0.0410	0.0428	0.2641
1500	|Δδ| (ppm)	0.1034	0.0085	0.0626	0.0301	0.3071
2000	|Δδ| (ppm)	0.0657	0.0186	0.1008	0.0231	0.3456

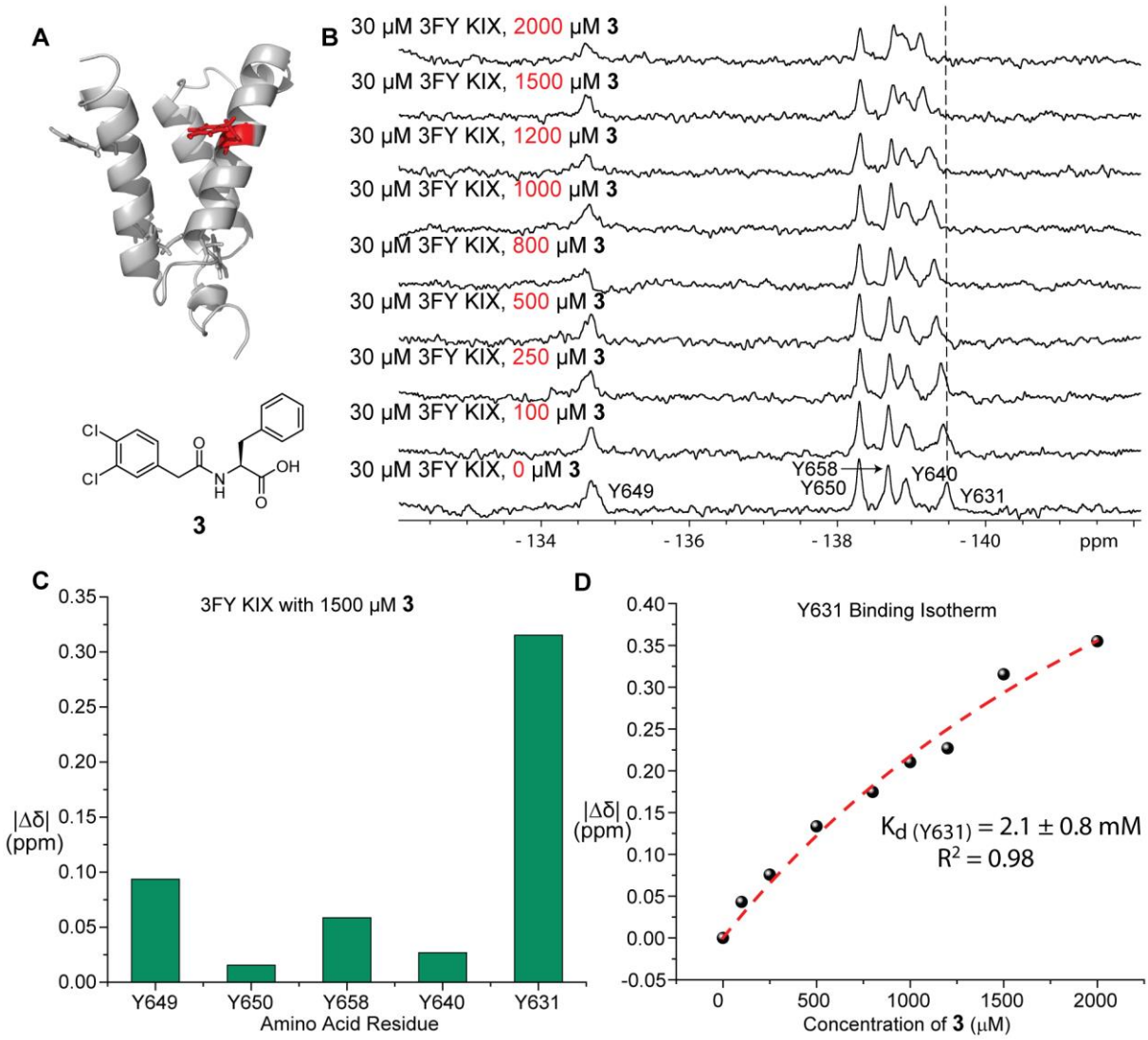


Figure S8. ^{19}F NMR spectral analysis of 3FY KIX in the presence of increasing concentrations of **3**. A) Ribbon diagram of KIX (PDB Code: 1KDX) with tyrosine side chains indicated as sticks. Residues that were significantly perturbed and approached saturation are highlighted in red. B) Stacked spectra of 3FY KIX with increasing concentrations of **3** C) Absolute value of chemical shift perturbations for all 3FY KIX resonances at 1500 μM **3**. D) Binding isotherm of Y631 perturbation for the titration of **3**

Table S6: 3FY-KIX ^{19}F NMR chemical shift perturbations at varying **3** concentrations.						
[**3**] (μM)	Chemical shift (ppm)	Y649	Y650	Y658	Y640	Y631
0	Chemical shift (ppm)	-134.6927	-138.2924	-138.6904	-138.9366	-139.4730
100	|Δδ| (ppm)	0.0111	0.0045	0.0009	0.0132	0.0431
250	|Δδ| (ppm)	0.0212	0.0114	0.0139	0.0094	0.0757
500	|Δδ| (ppm)	0.0170	0.0073	0.0138	0.0335	0.1336
800	|Δδ| (ppm)	0.0357	0.0053	0.0208	0.0270	0.1747
1000	|Δδ| (ppm)	0.0397	0.0101	0.0335	0.0081	0.2104
1200	|Δδ| (ppm)	0.0959	0.0180	0.0394	0.0140	0.2269
1500	|Δδ| (ppm)	0.0936	0.0156	0.0587	0.0269	0.3154
2000	|Δδ| (ppm)	0.0956	0.0157	0.0719	0.0554	0.3549

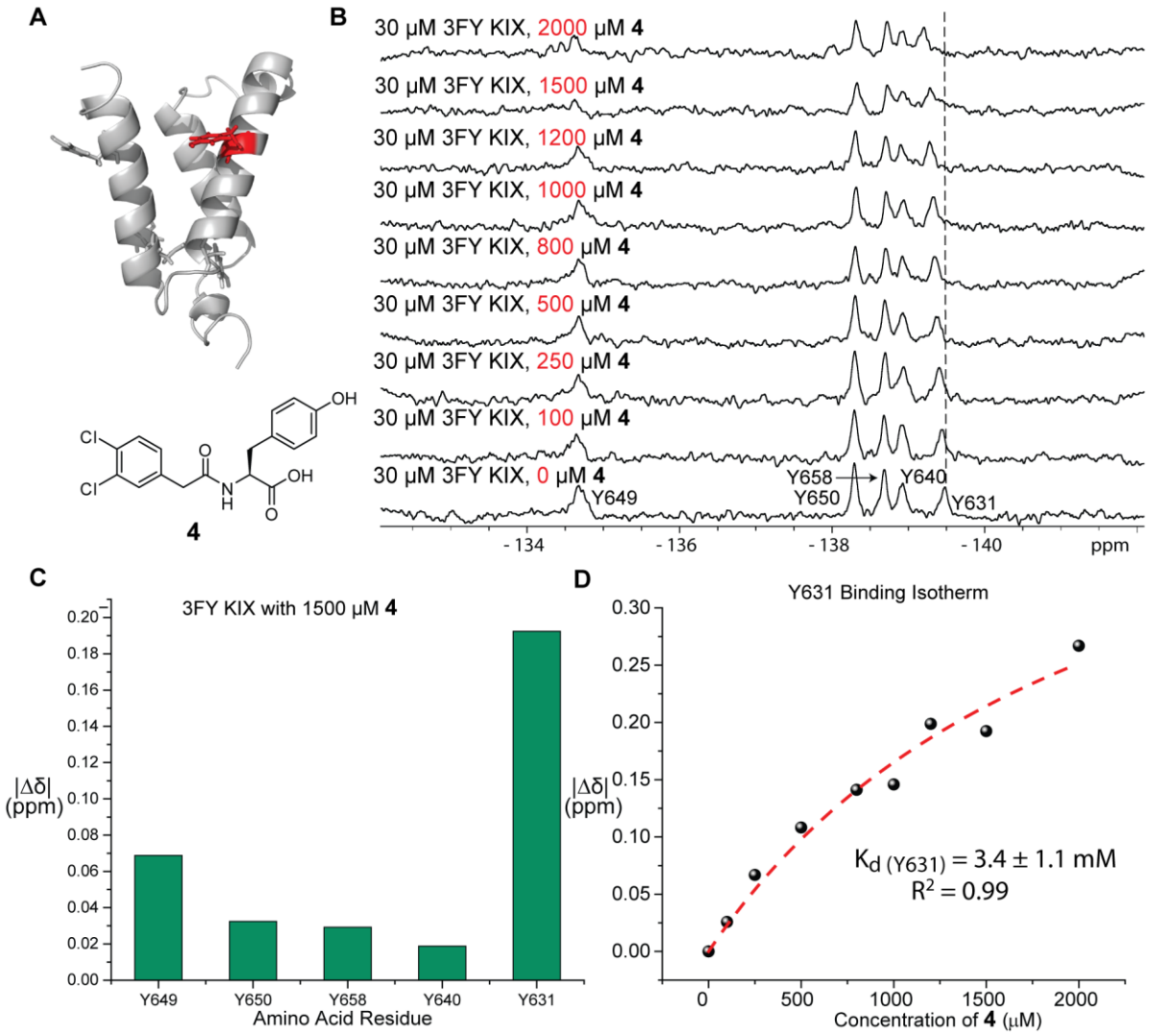


Figure S9 ^{19}F NMR spectral analysis of 3FY KIX in the presence of increasing concentrations of **3**. A) Ribbon diagram of KIX (PDB Code: 1KDX) with tyrosine side chains indicated as sticks. Residues that were significantly perturbed and approached saturation are highlighted in red. B) Stacked spectra of 3FY KIX with increasing concentrations of **4** C) Absolute value of chemical shift perturbations for all 3FY KIX resonances at 1500 μM **4**. D) Binding isotherm of Y631 perturbation for the titration of **4**

Table S7: 3FY-KIX ^{19}F NMR chemical shift perturbations at varying **4** concentrations.						
[**4**] (μM)	Chemical shift (ppm)	Y649	Y650	Y658	Y640	Y631
0	Chemical shift (ppm)	-134.6927	-138.2924	-138.6904	-138.9366	-139.4730
100	|Δδ| (ppm)	0.0471	0.0008	0.0098	0.0125	0.0258
250	|Δδ| (ppm)	0.0167	0.0067	0.0083	0.0026	0.0668
500	|Δδ| (ppm)	0.0124	0.0128	0.0027	0.0067	0.1082
800	|Δδ| (ppm)	0.0042	0.0116	0.0146	0.0099	0.1411
1000	|Δδ| (ppm)	0.0147	0.0267	0.0192	0.0063	0.1458
1200	|Δδ| (ppm)	0.0325	0.0186	0.0224	0.0307	0.1987
1500	|Δδ| (ppm)	0.0688	0.0324	0.0292	0.0188	0.1924
2000	|Δδ| (ppm)	0.0739	0.0198	0.0331	0.0040	0.2669

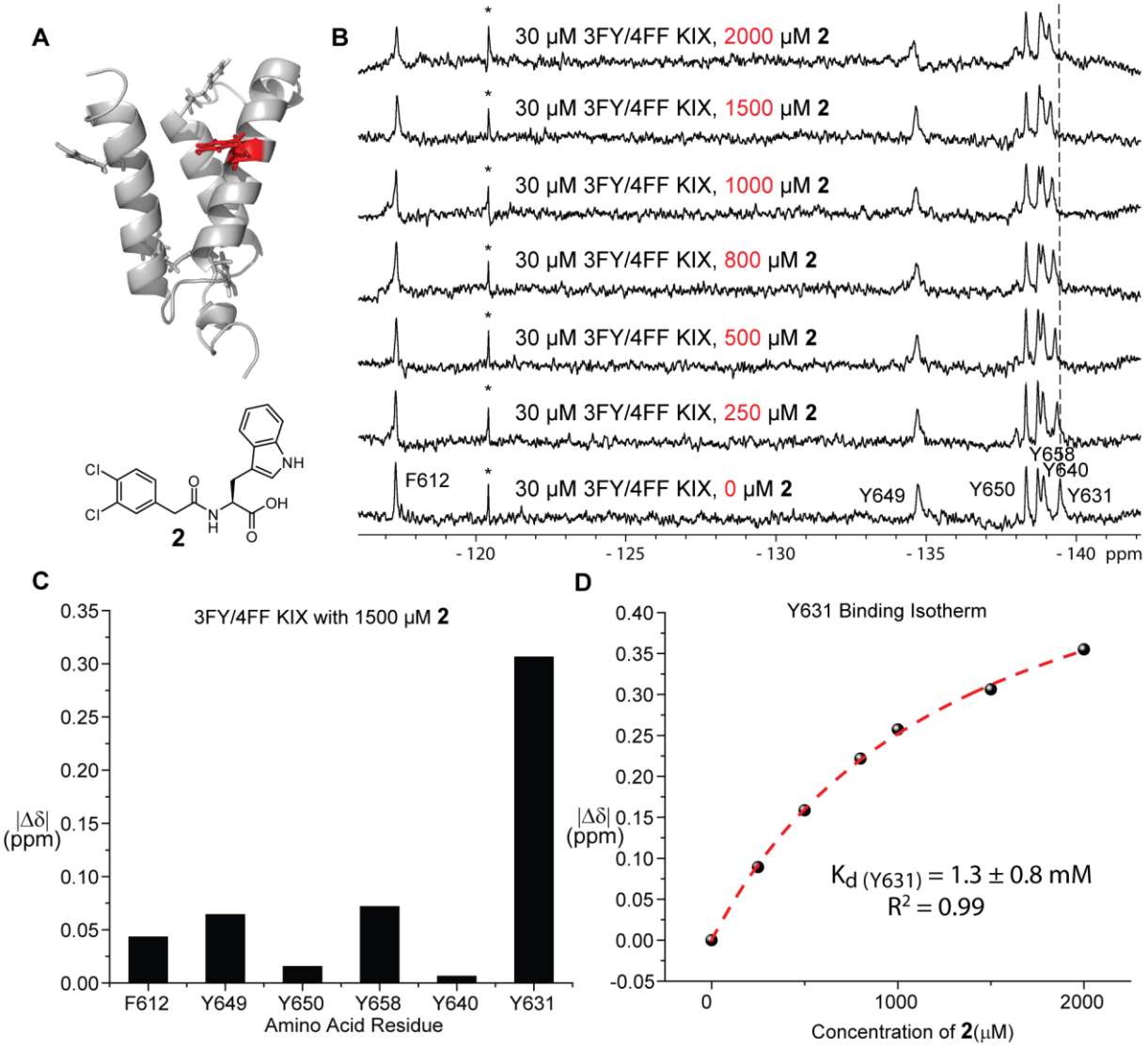


Figure S10 ^{19}F NMR spectral analysis of 3FY/4FF KIX in the presence of increasing concentrations of **2**. A) Ribbon diagram of KIX (PDB Code: 1KDX) with tyrosine side chains indicated as sticks. Residues that were significantly perturbed and approached saturation are highlighted in red. ***** This resonance is an artifact that does not originate in the sample. B) Stacked spectra of 3FY/4FF KIX with increasing concentrations of **2** C) Absolute value of chemical shift perturbations for all 3FY/4FF KIX resonances at 1500 μM **2**. D) Binding isotherm of Y631 perturbation for the titration of **2.**

Table S8: 3FY/4FF-KIX ^{19}F NMR chemical shift perturbations at varying **2** concentrations.							
[**2**]	Chemical shift (ppm)	F612	Y649	Y650	Y658	Y640	Y631
0	Chemical shift (ppm)	-117.3300	-134.7212	-138.3184	-138.7131	-138.8843	-139.4560
250	|Δδ| (ppm)	0.0187	0.0361	0.0030	0.0100	0.0119	0.0892
500	|Δδ| (ppm)	0.0260	0.0278	0.0180	0.0351	0.0143	0.1586
800	|Δδ| (ppm)	0.0428	0.0352	0.0177	0.0396	0.0100	0.2218
1000	|Δδ| (ppm)	0.0362	0.0605	0.0146	0.0568	0.0056	0.2573
1500	|Δδ| (ppm)	0.0433	0.0643	0.0155	0.0718	0.0063	0.3063
2000	|Δδ| (ppm)	0.0376	0.0739	0.0153	0.0884		0.3551

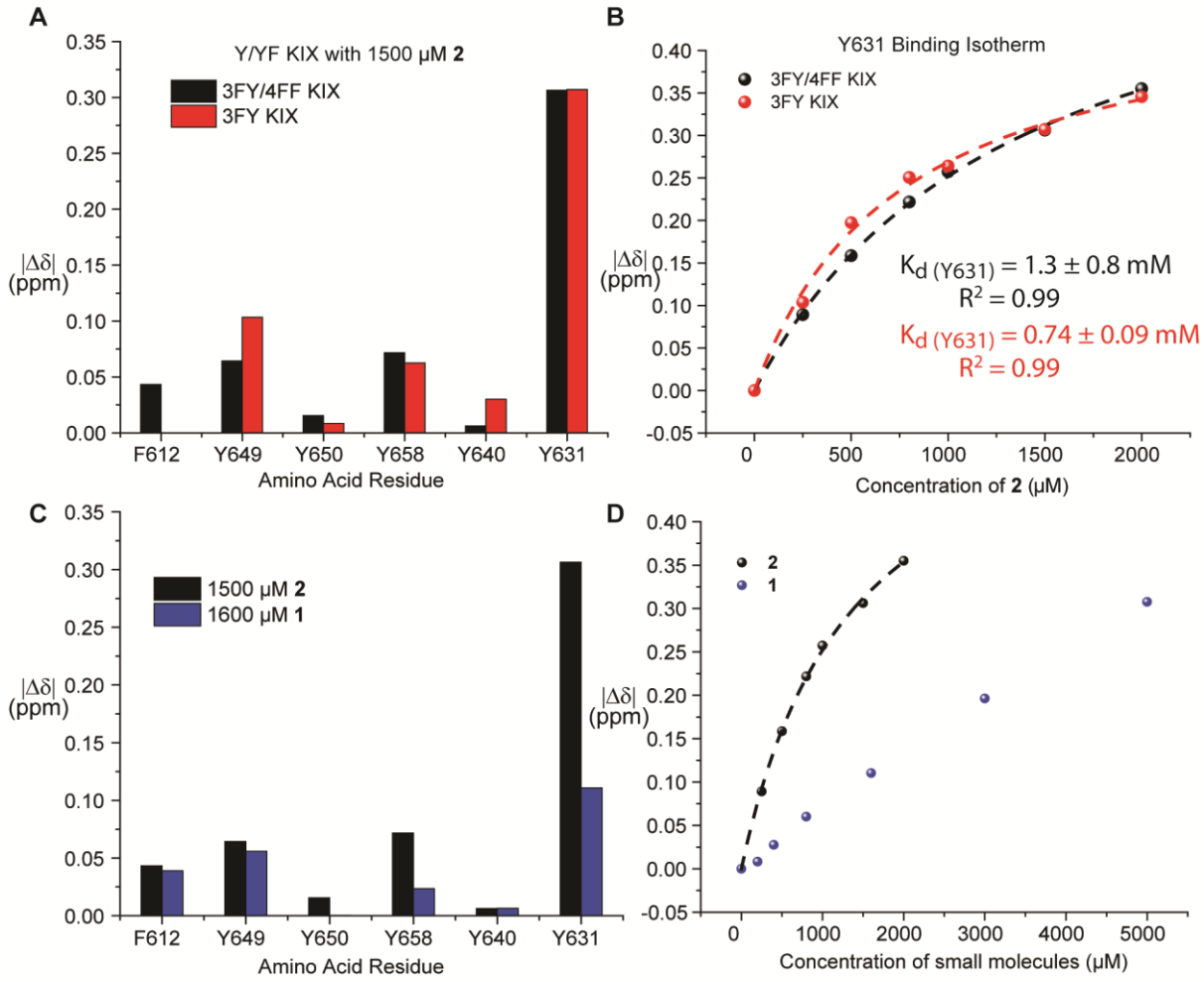


Figure S11: Comparison of binding data with **2**. A) Chemical shift perturbations of **2** with 3FY KIX and 3FY/4FF KIX demonstrating very similar perturbations. B) Binding isotherm of Y631 perturbations for the titrations of **2** against 3FY KIX and 3FY/4FF KIX. C) Chemical shift perturbations of **1** and **2** against 3FY/4FF KIX. Y631 is perturbed more significantly by **2** than it is by **1.** D) Binding isotherm of Y631 perturbations for the titrations of **1** and **2** against 3FY/4FF KIX.

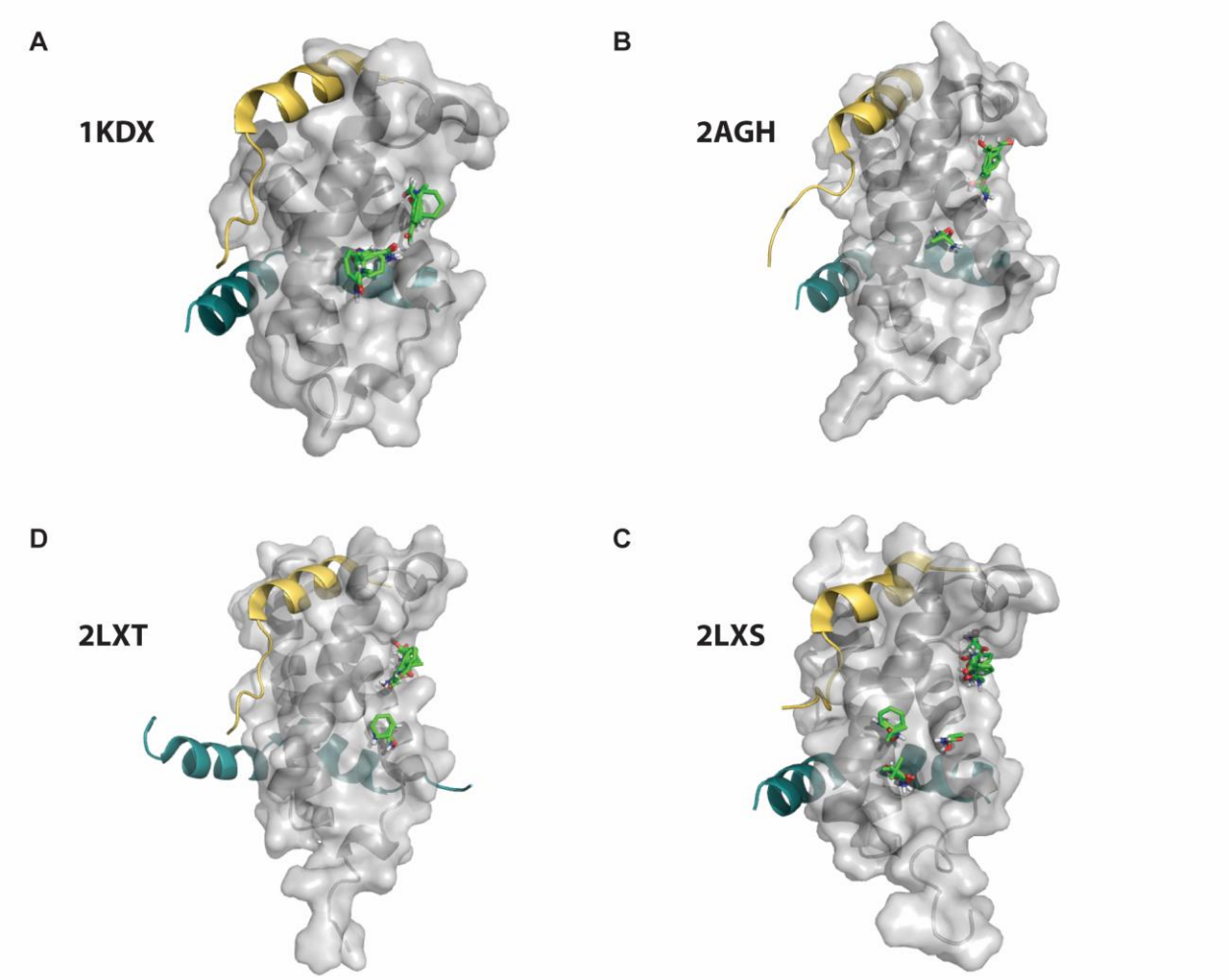


Figure S12: FTMap comparison of multiple KIX structures. KIX ribbon structures are shown in gray with the native MLL peptide shown in gold, and the pKID peptide shown in teal. FTMap solvent probe clusters that do not occupy either the MLL or pKID site are shown in green, indicating the possibility of a new, previously unreported binding site.

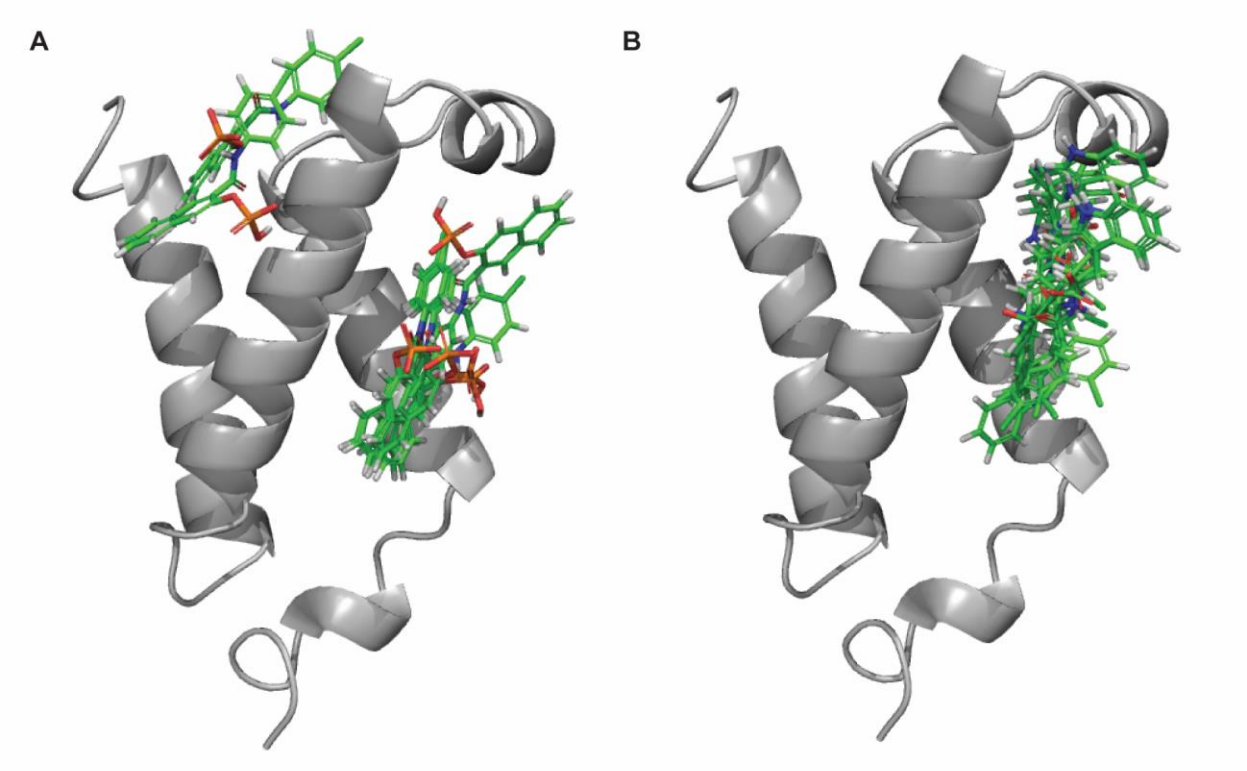


Figure S13 Glide docking pose comparisons of small molecule ligands against four different KIX PDBs (1KDX, 2AGH, 2LXT, 2LXS). Two poses per PDB are presented. A) Naphthol AS-E phosphate binding poses predicted by Glide docking. Six poses place Naphthol AS-E phosphate in the new binding site while the remaining two are placed in the MLL site. B) Binding poses of **2** predicted by Glide docking. All eight predicted poses are in the new binding site, and no poses are predicted for the MLL site.

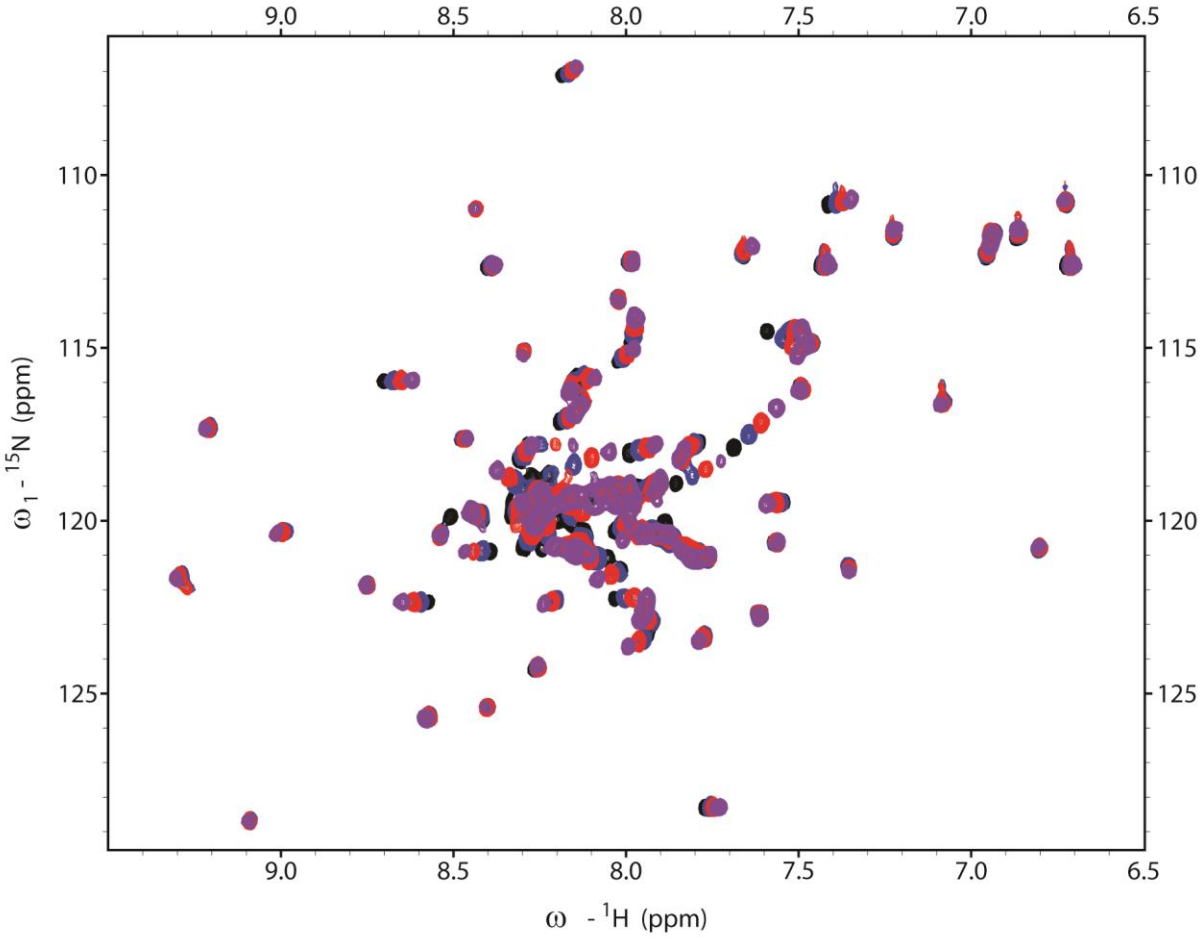


Figure S14. ^{1}H-^{15}N HSQC of **2** binding to KIX. ^{15}N KIX is shown in black, KIX + 0.5 mM **2** is shown in blue, KIX + 1 mM **2** is shown in red, and KIX + 2 mM **2** is shown in purple .

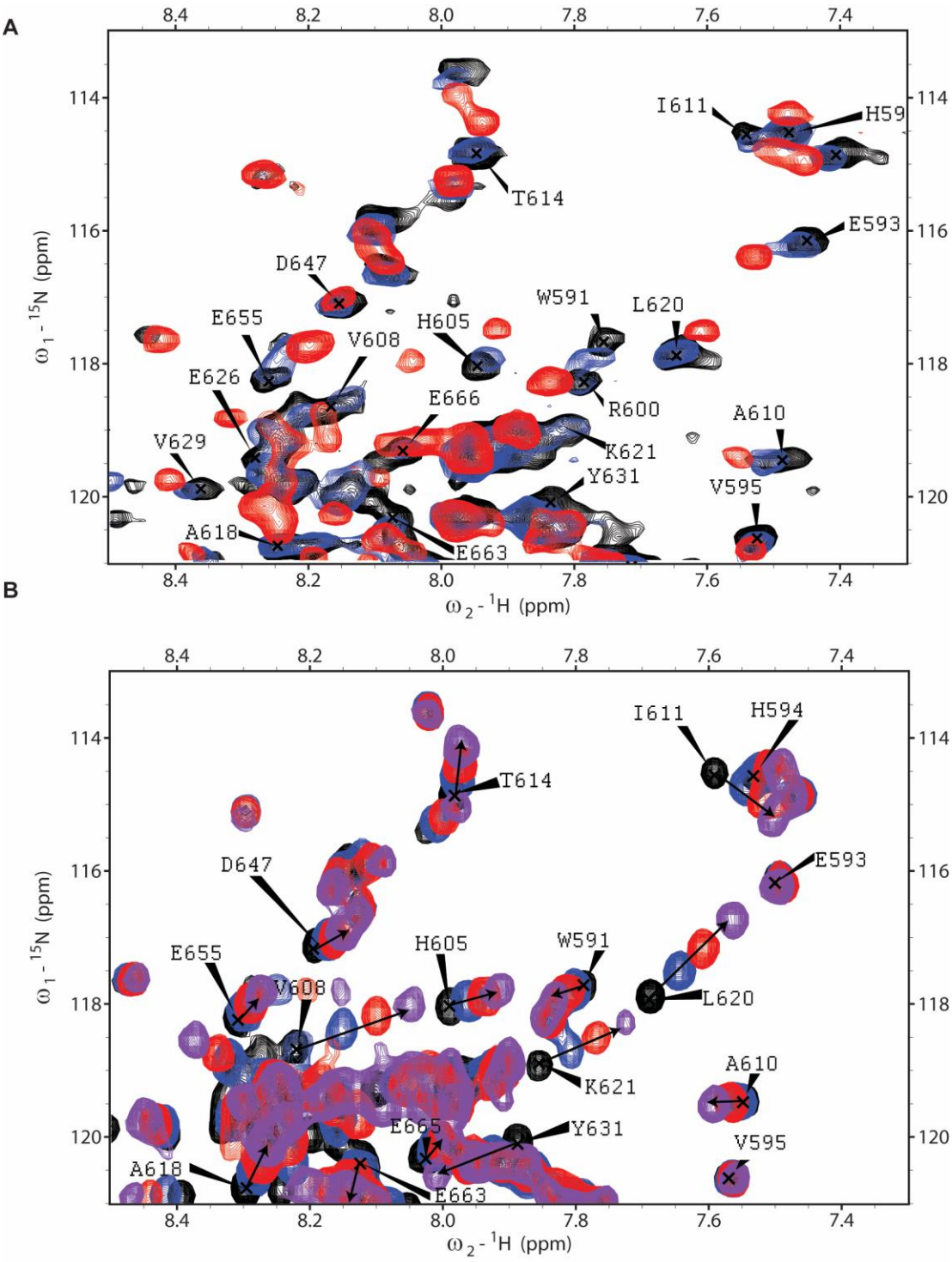


Figure S15: Comparison of ^{1}H-^{15}N HSQC/HMQC data from KIX A) Select region of 1H-15N HSQC NMR spectrum with **1**. Native protein is shown in black. 2 mM **1** is shown in blue. 5 mM **2** is shown in red. B) Select region of 1H-15N HMQC NMR spectrum with **2**. Distinctly, the chemical shift perturbations in the presence of **2** consistently exhibit a linear change in chemical shift. Native protein is shown in black. 0.5 mM **2** is shown in blue. 1 mM **2** is shown in red. 2 mM **2** is shown in purple. Assignments previously obtained by Majmudar et al.[4]

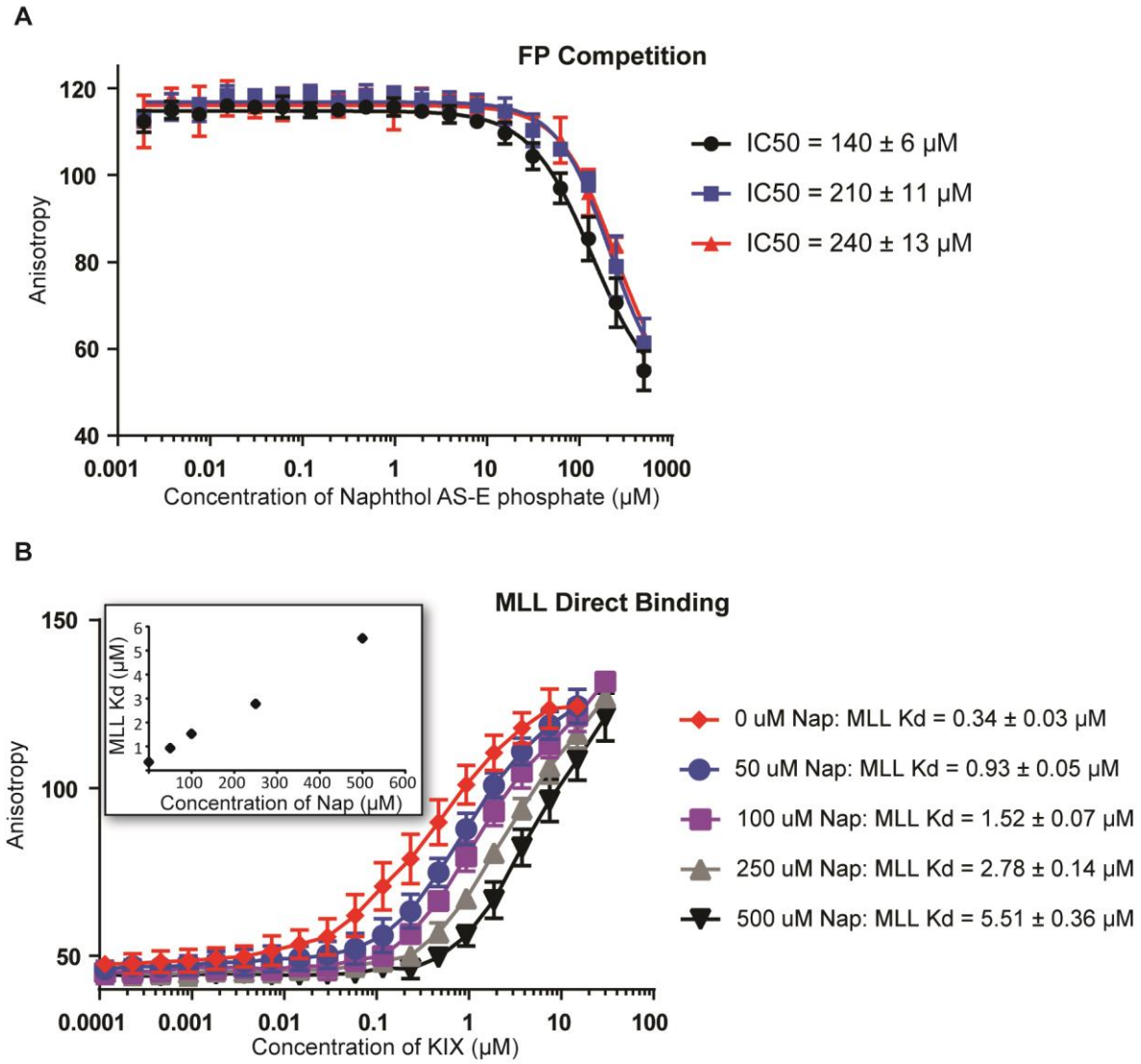
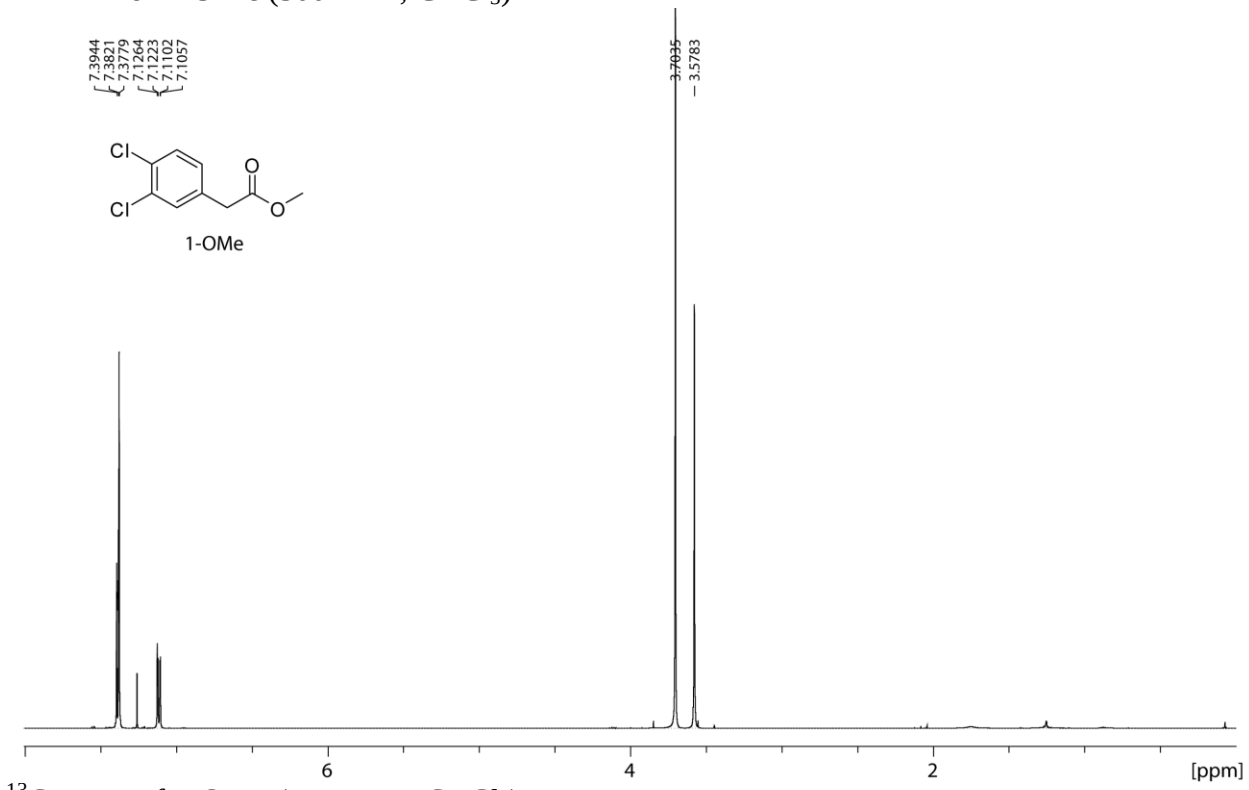
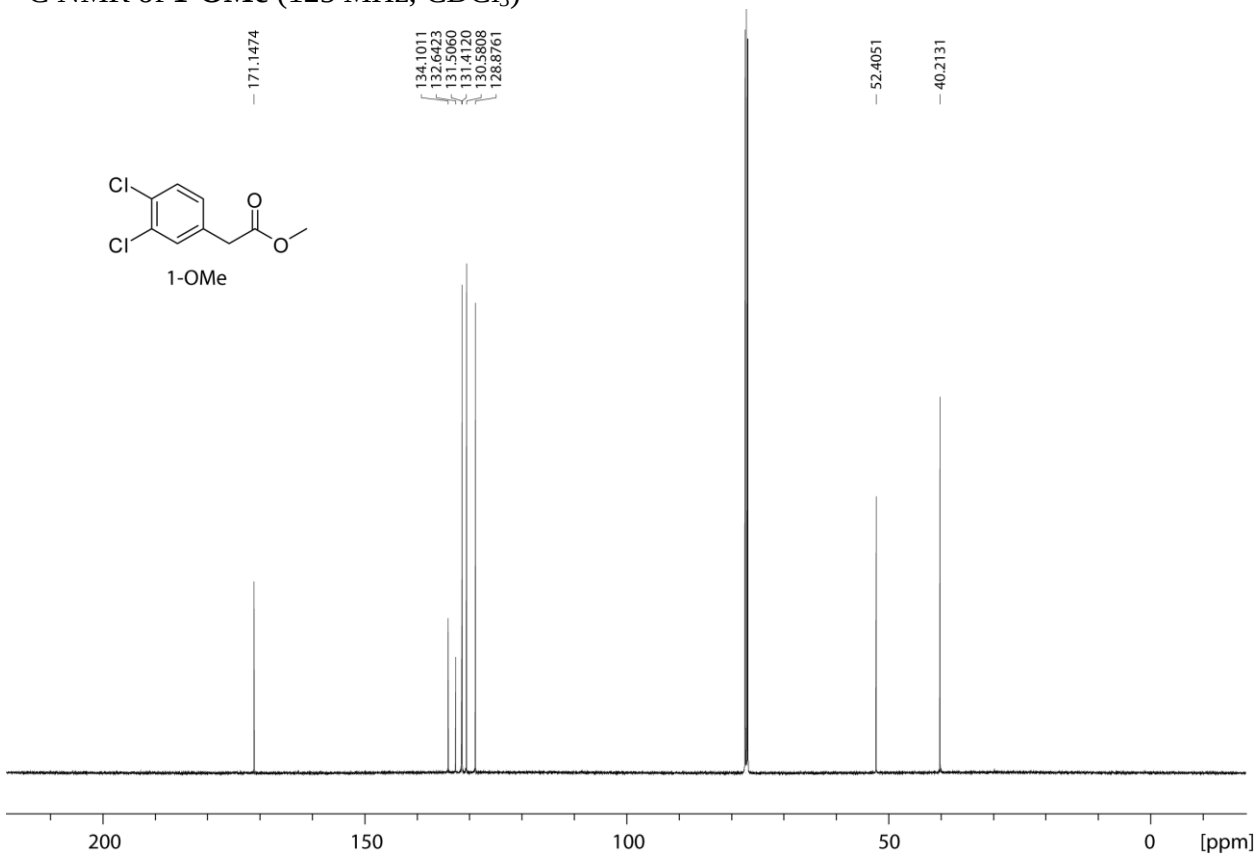


Figure S16: FP studies to profile ligand binding A) Replicate trials for an FP competition experiment with Naphthol AS-E phosphate to inhibit the MLL:KIX interaction, demonstrating only partial inhibition, relative to the inhibition of the pKID:KIX interaction. Data was constrained at the bottom to B) Direct binding experiments of MLL in the presence of Naphthol AS-E phosphate (Nap) demonstrating attenuated MLL binding at higher concentrations of Naphthol AS-E phosphate. Inset) Scatter plot of MLL Kd vs Naphthol AS-E phosphate concentration, showing a linear attenuation of MLL affinity

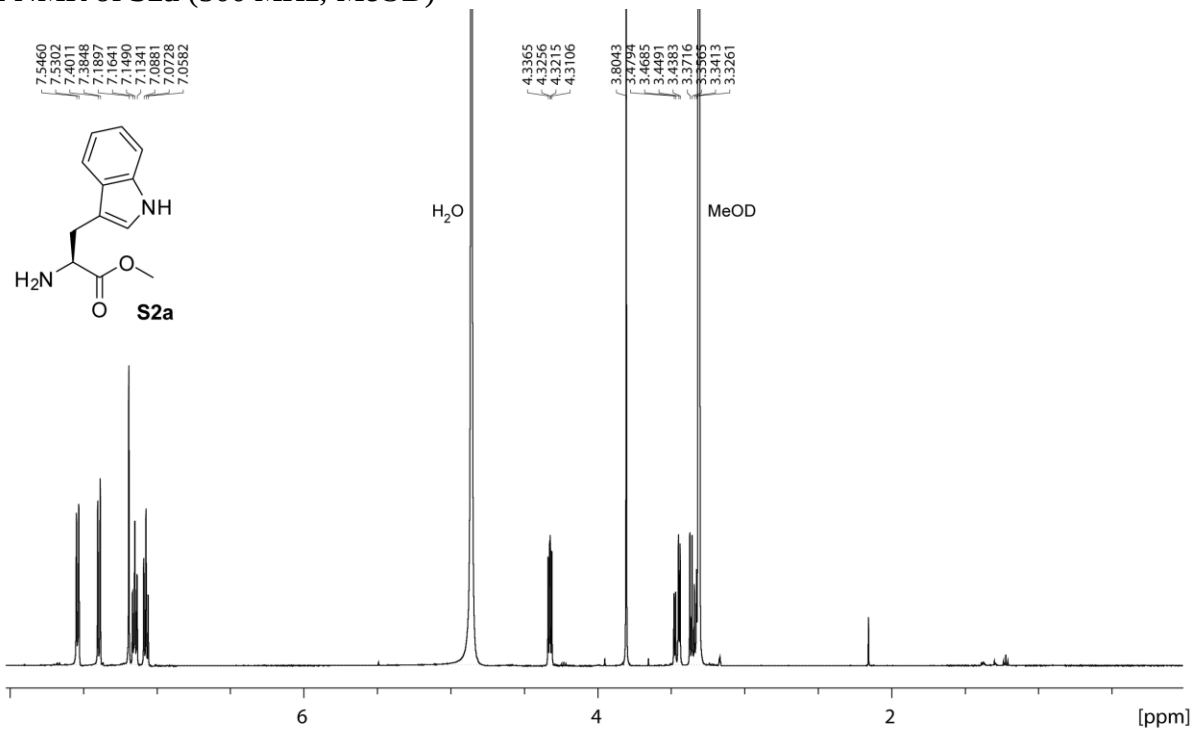
^{1}H NMR of **1-OMe** (500 MHz, $CDCl_3$)



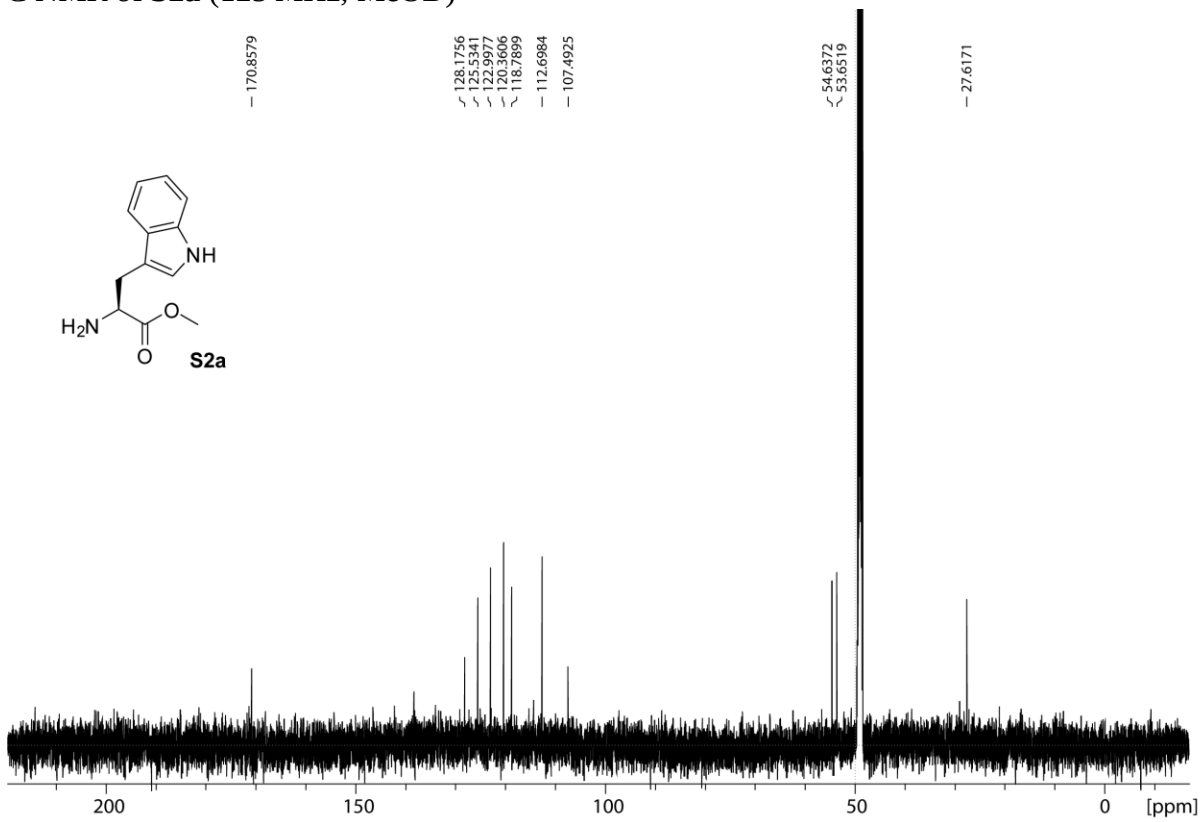
^{13}C NMR of **1-OMe** (125 MHz, $CDCl_3$)



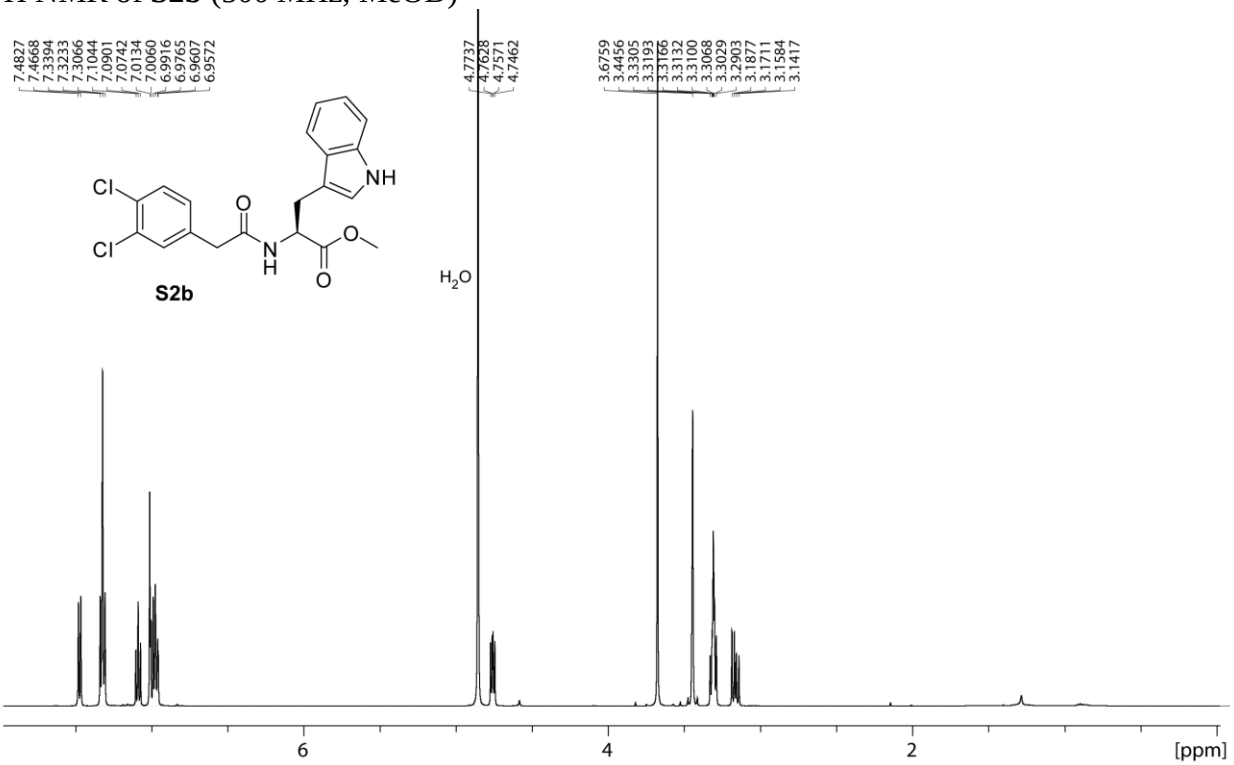
^{1}H NMR of **S2a** (500 MHz, MeOD)



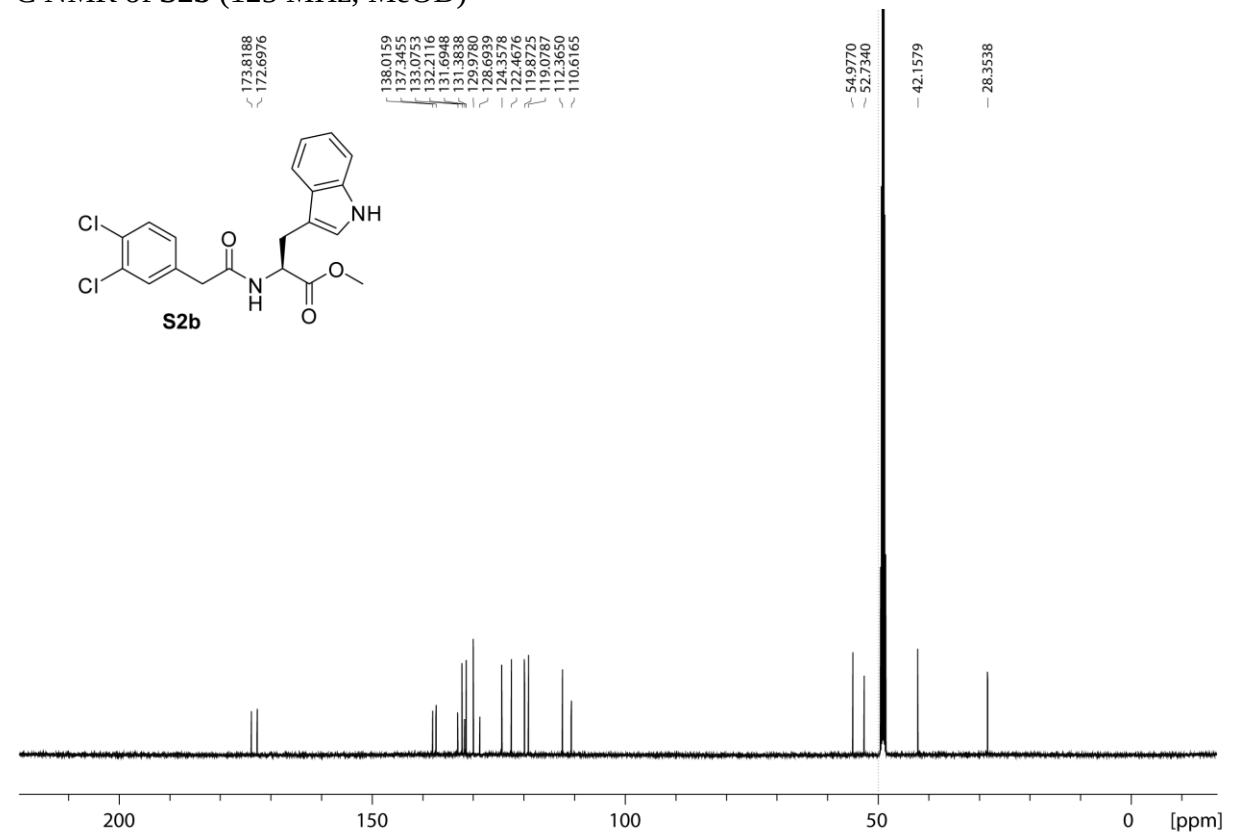
^{13}C NMR of **S2a** (125 MHz, MeOD)



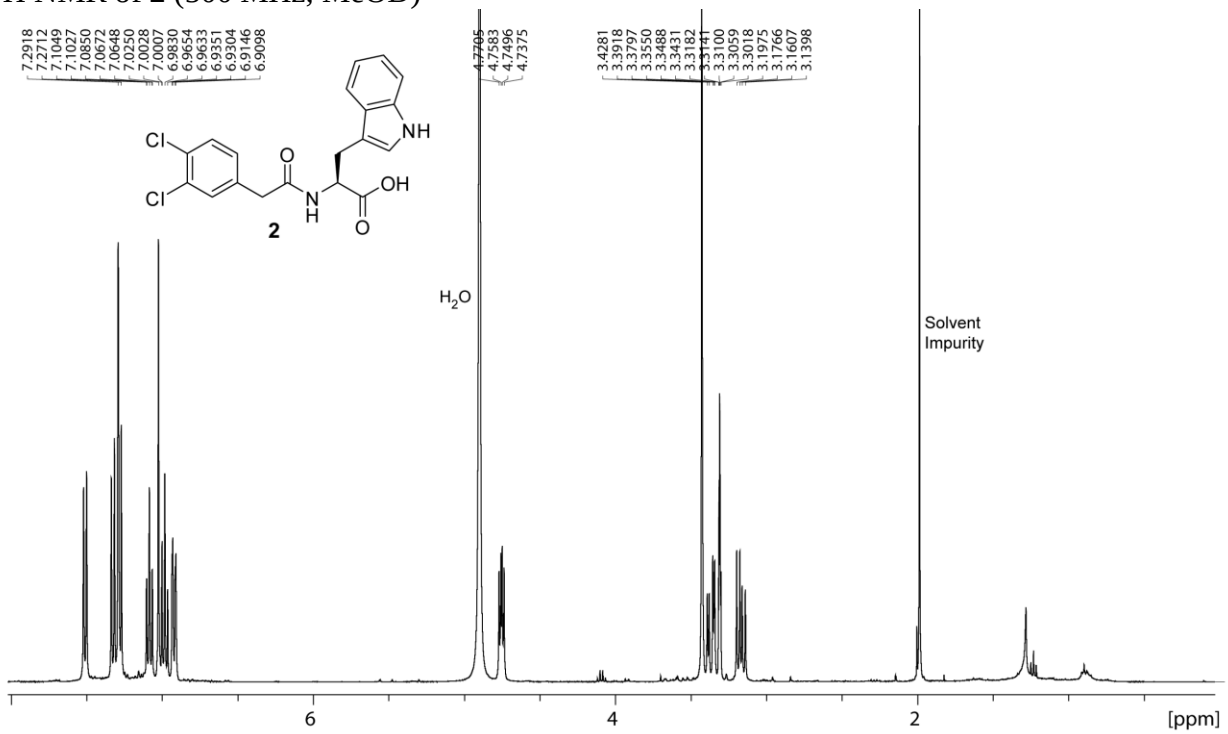
^{1}H NMR of **S2b** (500 MHz, MeOD)



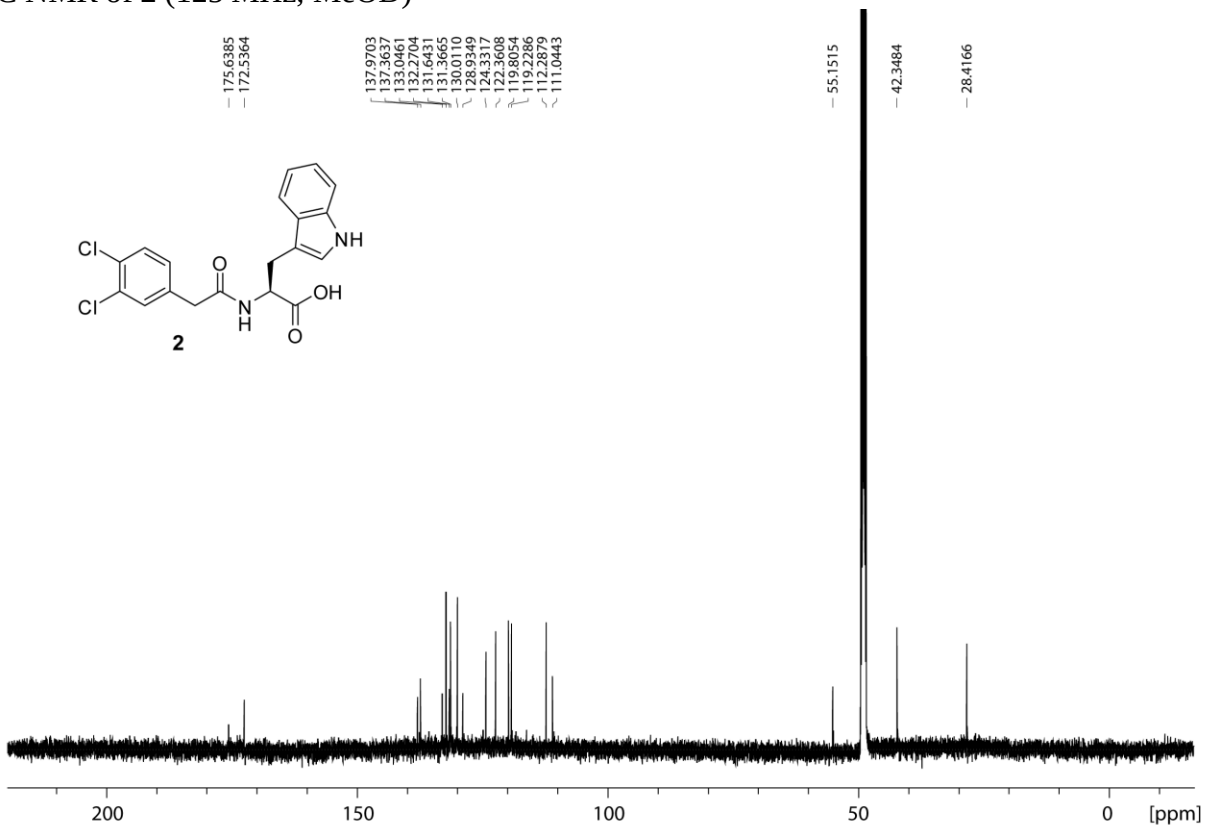
^{13}C NMR of **S2b** (125 MHz, MeOD)



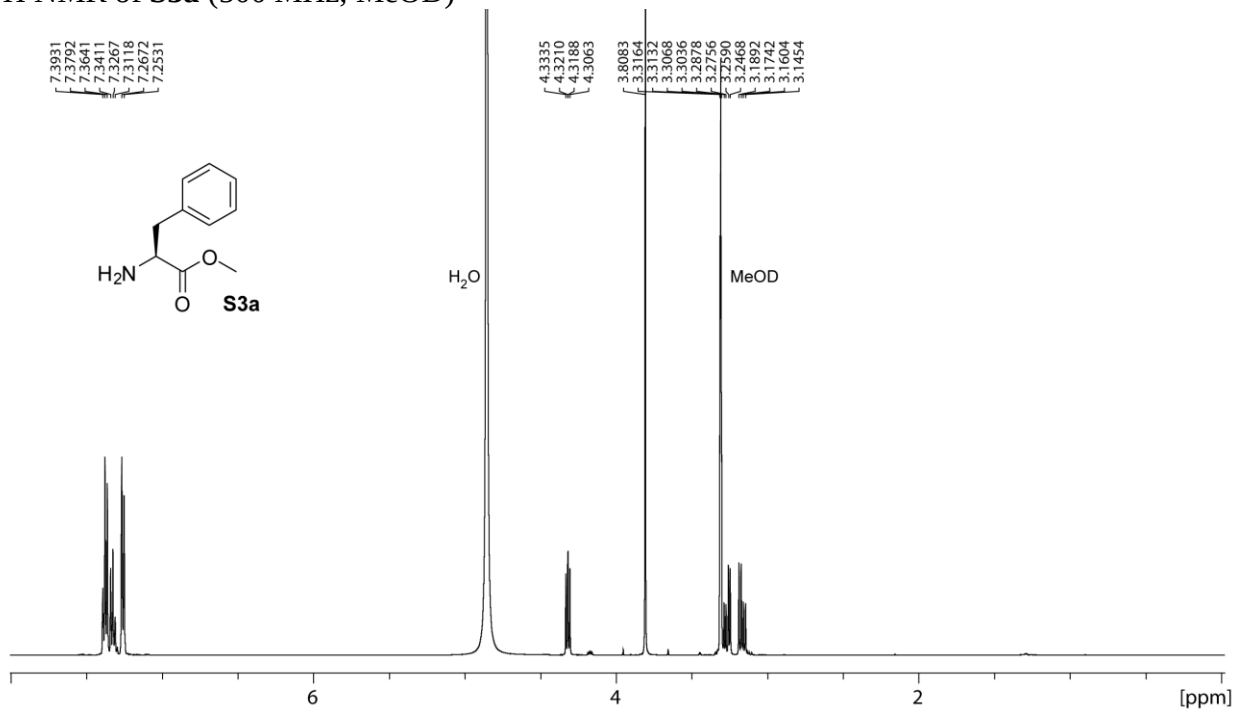
^{1}H NMR of **2** (500 MHz, MeOD)



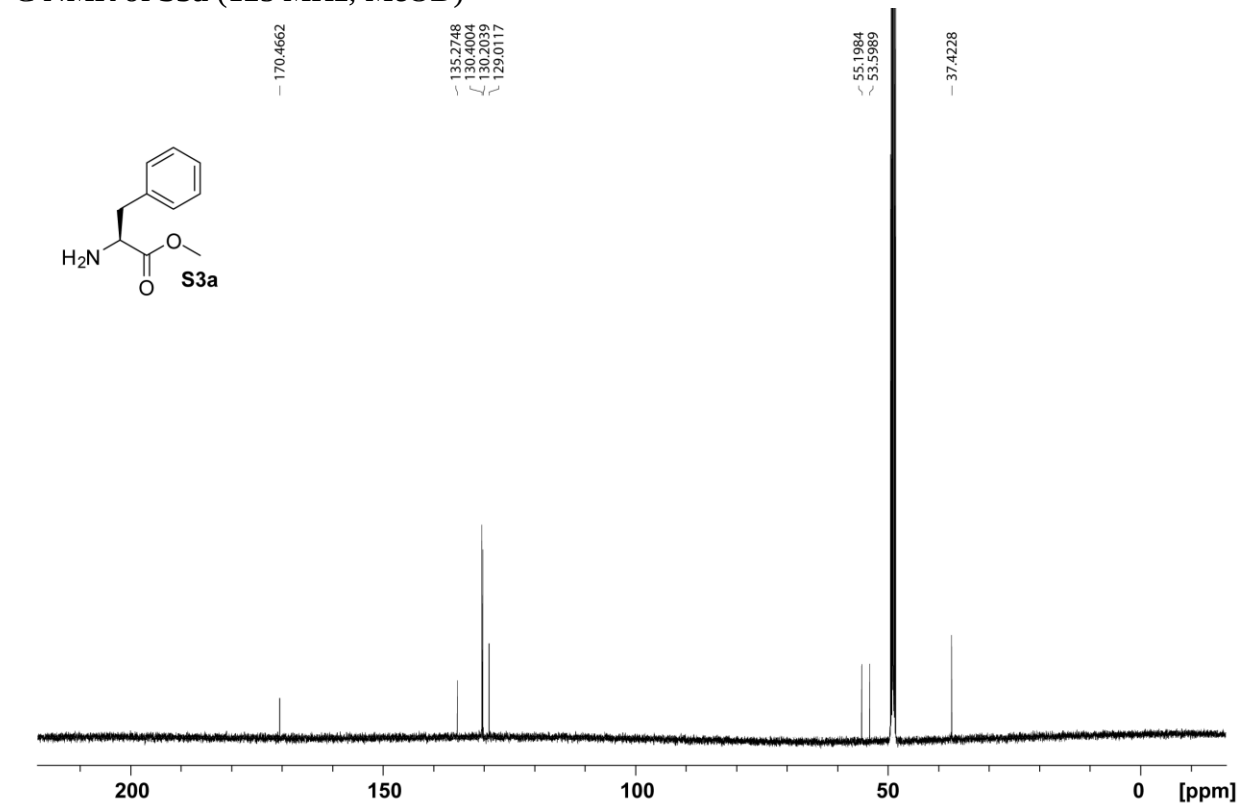
^{13}C NMR of **2** (125 MHz, MeOD)



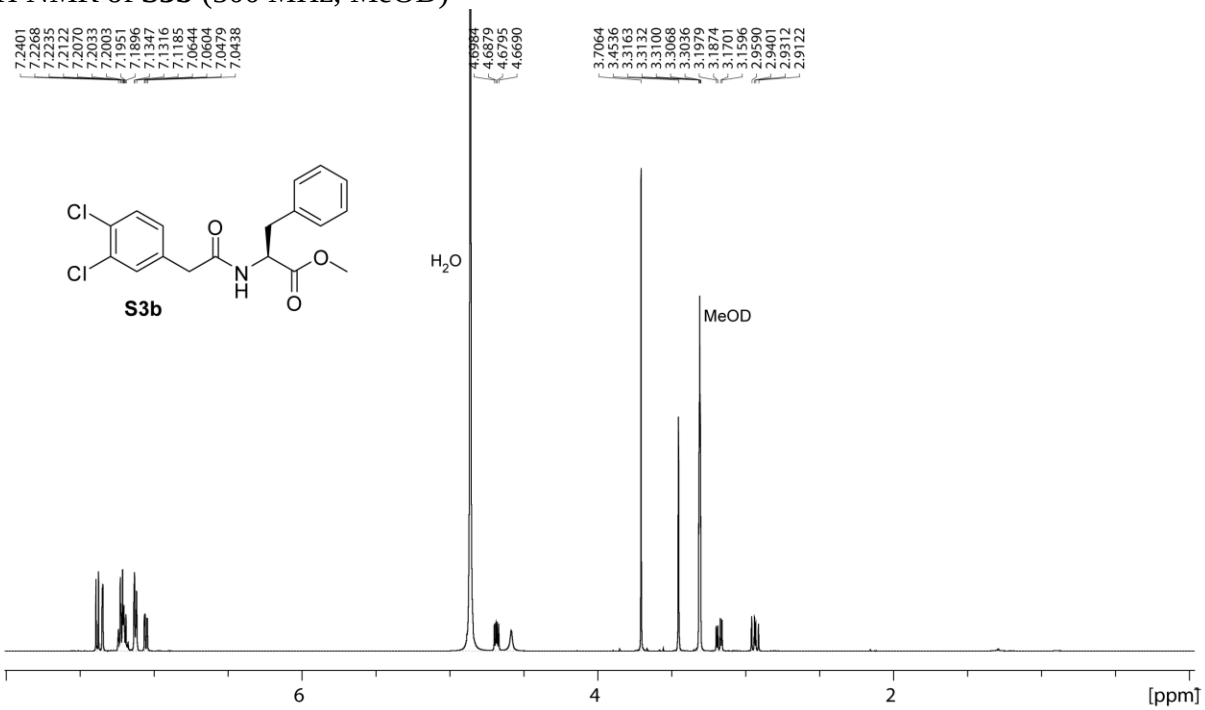
^{1}H NMR of **S3a** (500 MHz, MeOD)



^{13}C NMR of **S3a** (125 MHz, MeOD)



^{1}H NMR of **S3b** (500 MHz, MeOD)



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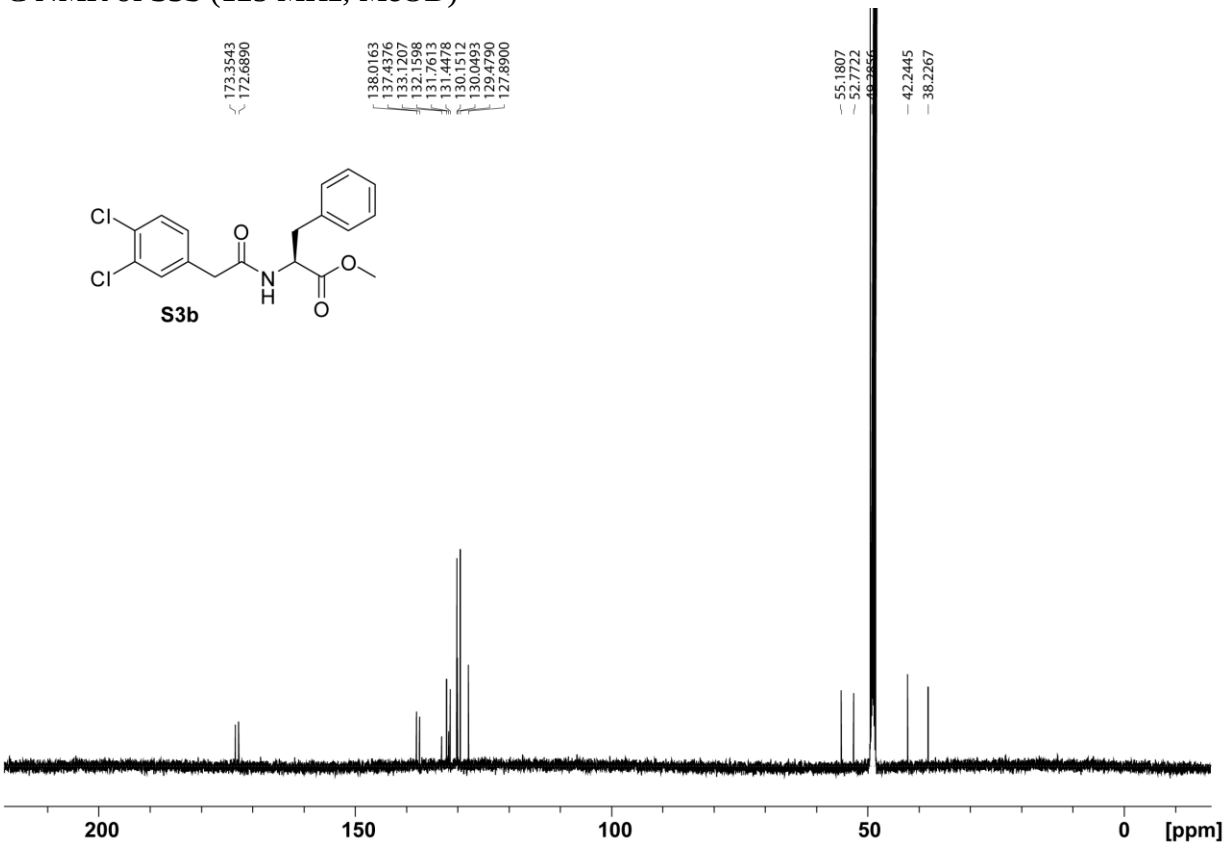
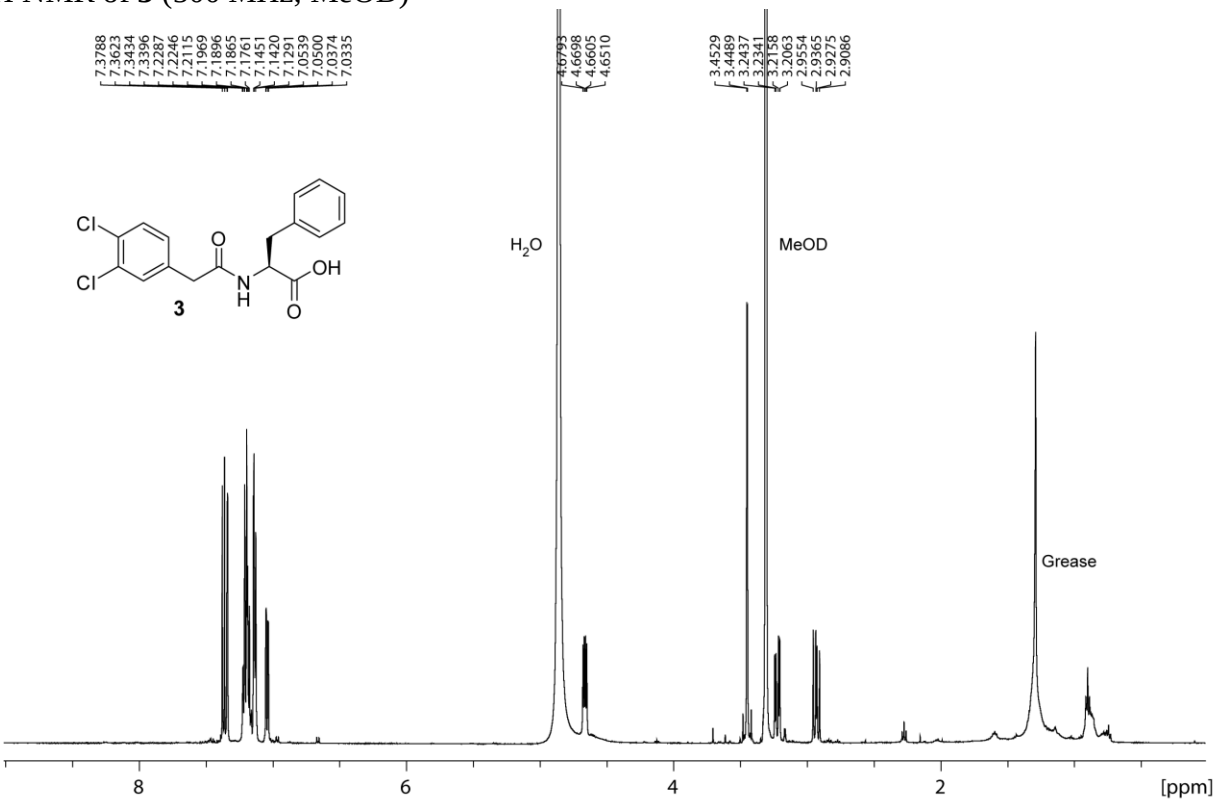


^{1}H NMR of **3** (500 MHz, MeOD)



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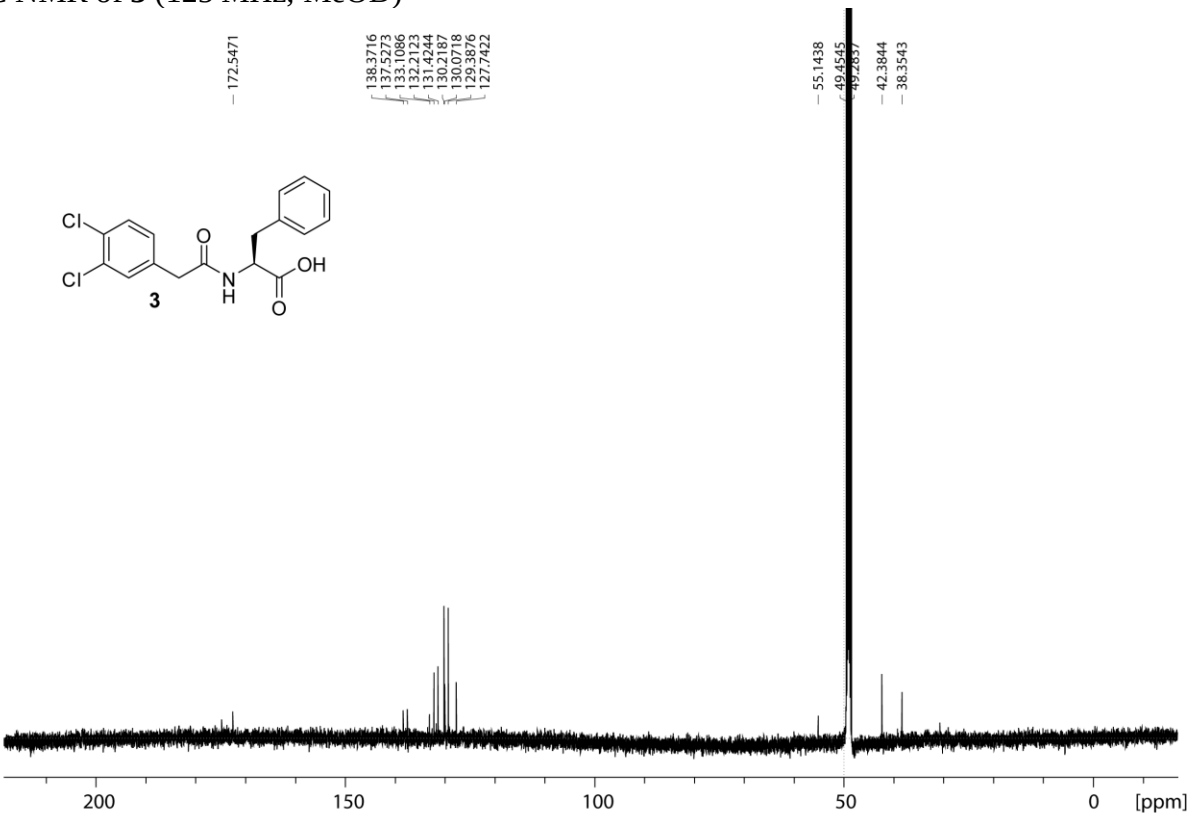
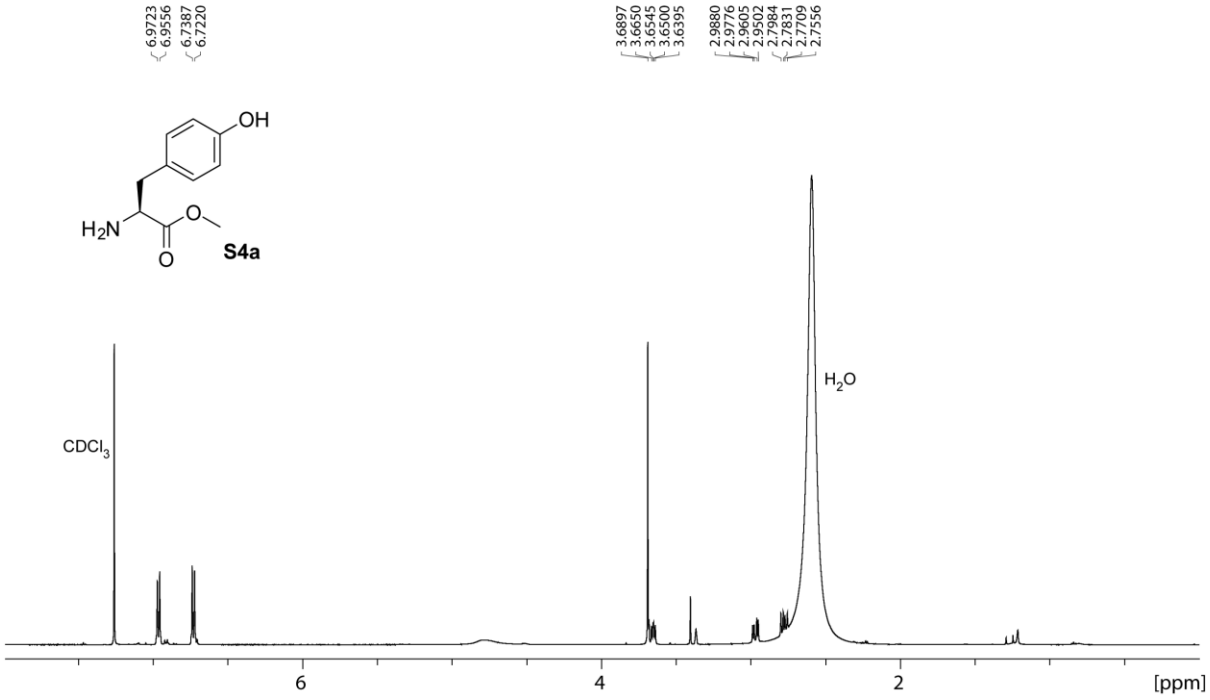
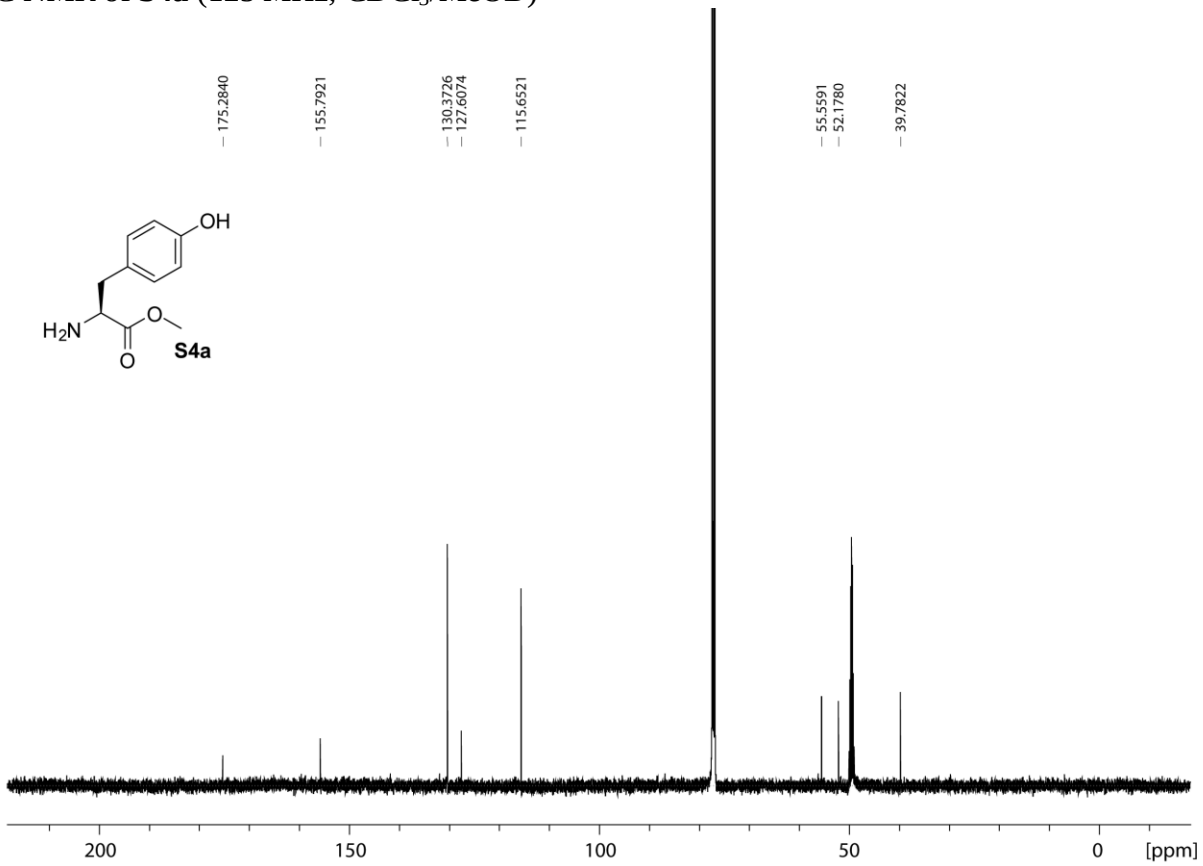


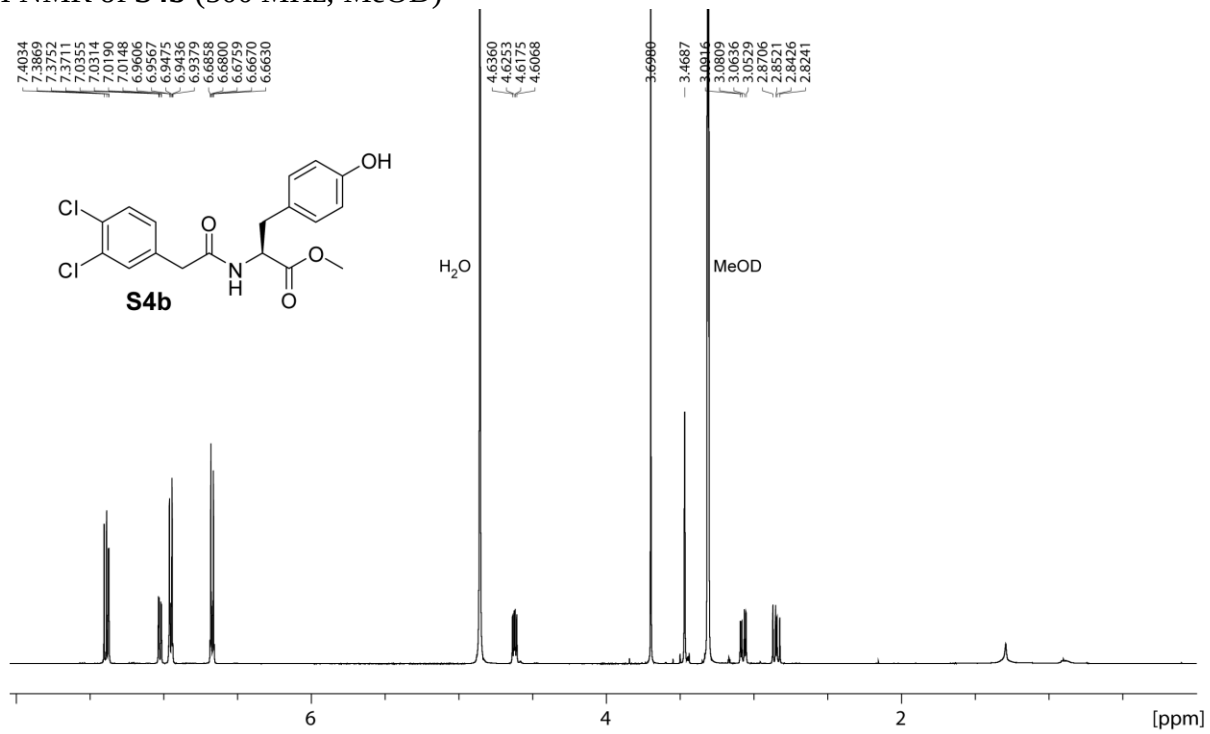
^{1}H NMR of **S4a** (500 MHz, $CDCl_3$/MeOD)



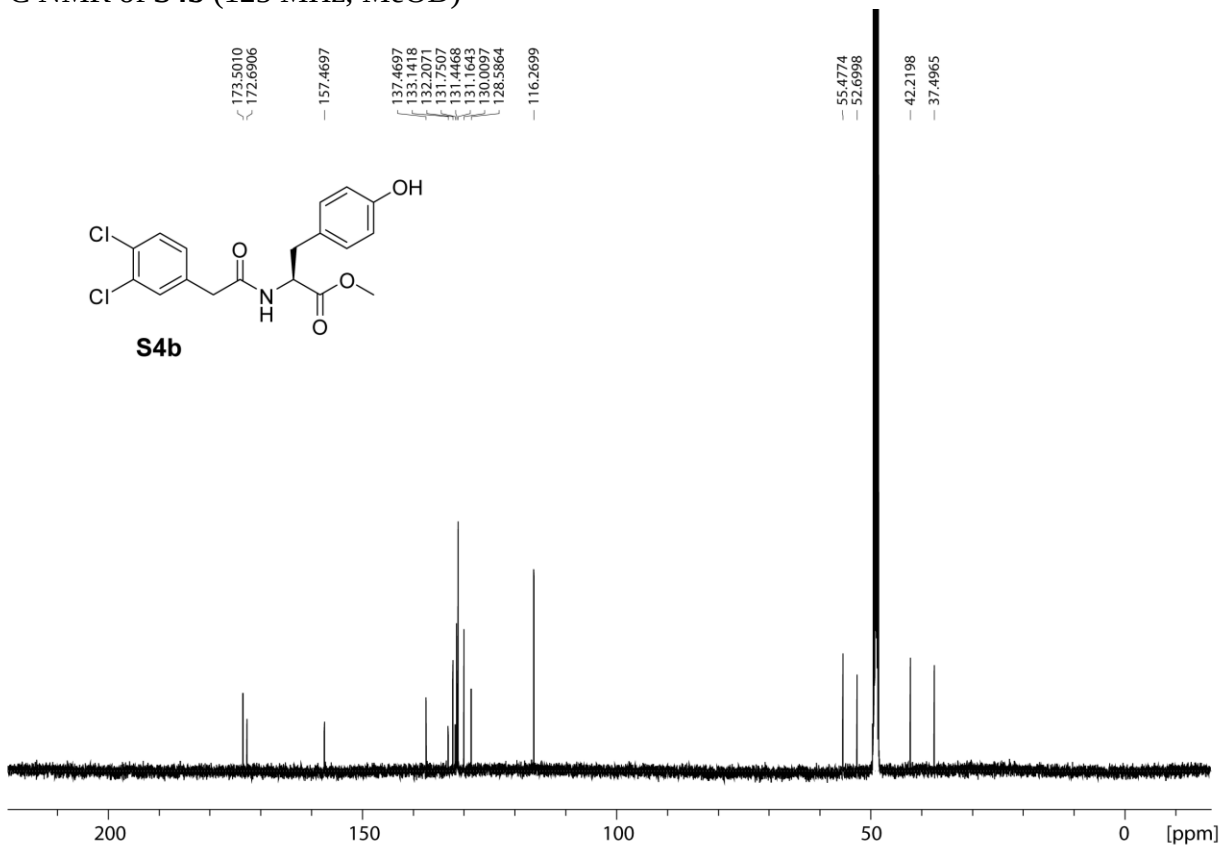
^{13}C NMR of **S4a** (125 MHz, $CDCl_3$/MeOD)



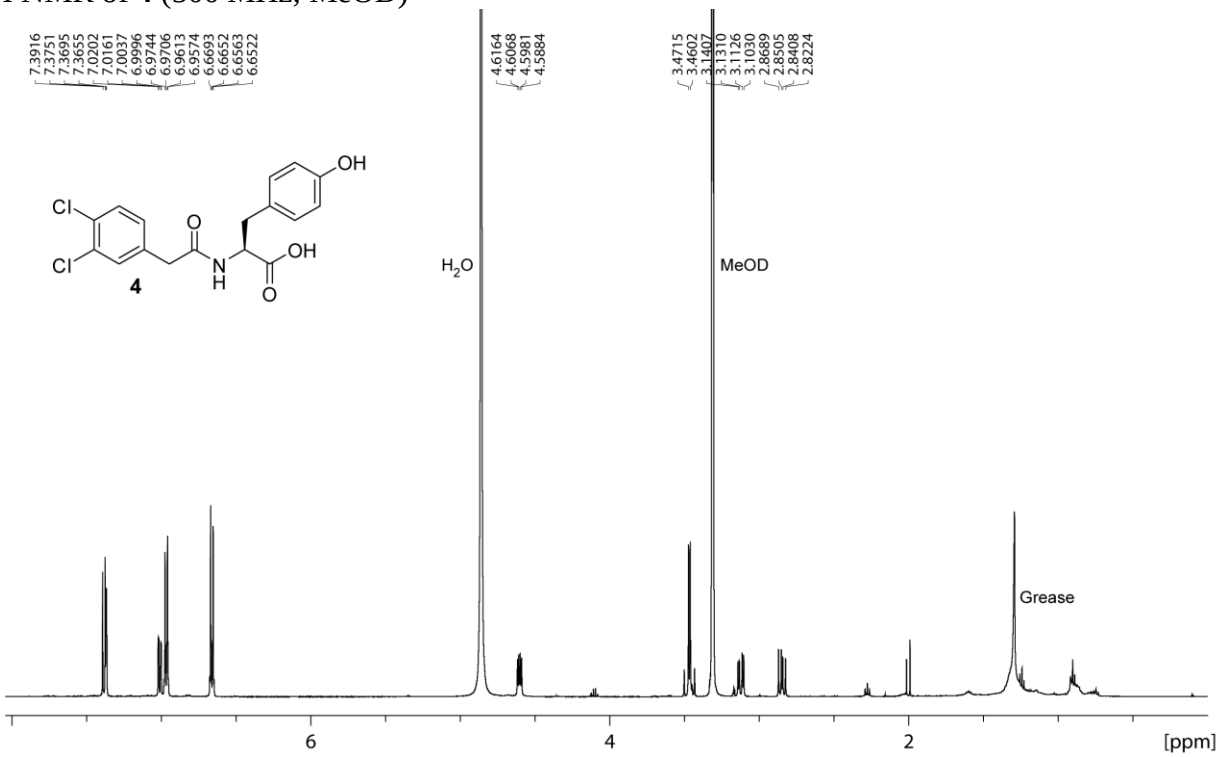
^{1}H NMR of **S4b** (500 MHz, MeOD)



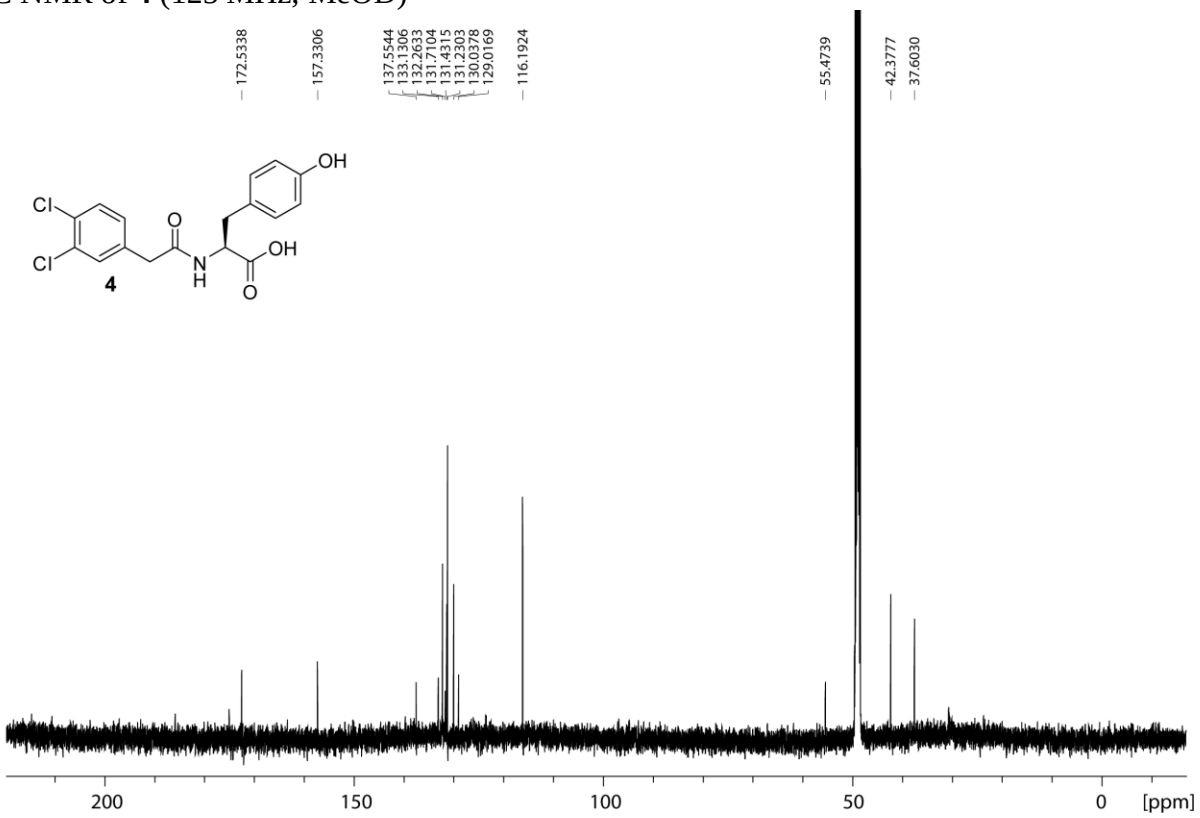
^{13}C NMR of **S4b** (125 MHz, MeOD)



^{1}H NMR of **4** (500 MHz, MeOD)



^{13}C NMR of **4** (125 MHz, MeOD)



[1] W. C. Pomerantz, N. Wang, A. K. Lipinski, R. Wang, T. Cierpicki, A. K. Mapp, *ACS Chem. Biol.* **2012**, *7*, 1345-1350.
[2] a) D. C. Muchmore, L. P. McIntosh, C. B. Russell, D. E. Anderson, F. W. Dahlquist, *Methods Enzymol.* **1989**, *177*, 44-73; b) C. T. Gee, K. E. Arntson, A. K. Urick, N. K. Mishra, L. M. L. Hawk, A. J. Wisniewski, W. C. K. Pomerantz, *Nat. Protoc.* **2016**, *11*, 1414-1427.
[3] C. Frieden, S. D. Hoeltzli, J. G. Bann, *Methods Enzymol.* **2004**, *380*, 400-415.
[4] C. Y. Majmudar, J. W. Højfeldt, C. J. Arevang, W. C. Pomerantz, J. K. Gagnon, P. J. Schultz, L. C. Cesa, C. H. Doss, S. P. Rowe, V. Vásquez, G. Tamayo-Castillo, T. Cierpicki, C. L. Brooks, D. H. Sherman, A. K. Mapp, *Angew. Chem. Int. Ed.* **2012**, *51*, 11258-11262.