Supporting Information

SAR and molecular mechanics reveal the importance of ring entropy in the biosynthesis and activity of a natural product

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I. Plasmid complementation

A plasmid complementation system was used to produce WT thiocillin and thiocillin mutants. The thiocillin prepeptide gene, tclE, was PCR amplified from WT *Bacillus cereus* ATCC 14579 and ligated into the BamHI and AatII sites of pHT01 (MoBiTec GmbH, Goettingen, Germany), an IPTG-inducible Bacillus expression plasmid, to create pHT01-tclE. Use of this plasmid for expressing thiocillin mutants have previously been shown.[1] Cloning and plasmid preparation was performed in *E. coli* XL10-Gold cells. *B. cereus* ΔtclE-H (KO cells), a strain lacking the endogenous thiocillin prepeptide gene, was generously provided by the Walsh lab (Harvard Medical School, Boston, MA). The plasmid was then electroporated into KO cells and selected on LB agarose plates containing 5 μg/mL chloramphenicol. Electroporation protocol for *B. cereus* ATCC 14579 has previously been reported.[2] 3 mL cultures were grown in LB with 10 μg/mL chloramphenicol +/- 1 mM IPTG for 72 hours at 30°C. WT and KO cells were grown in LB. Methanolic extracts of the pellet were dried and resuspended in 100 μL of 1:2 B:A (Solvent A = water/0.1% TFA; Solvent B = acetonitrile/0.1% TFA). 10 μL was loaded onto a Waters XBridge C18 3.5 μm 1x150 mm column with a flow rate of 50 μL/min and a linear gradient of 30-65% B over 24 min (Waters, Milford, MA).

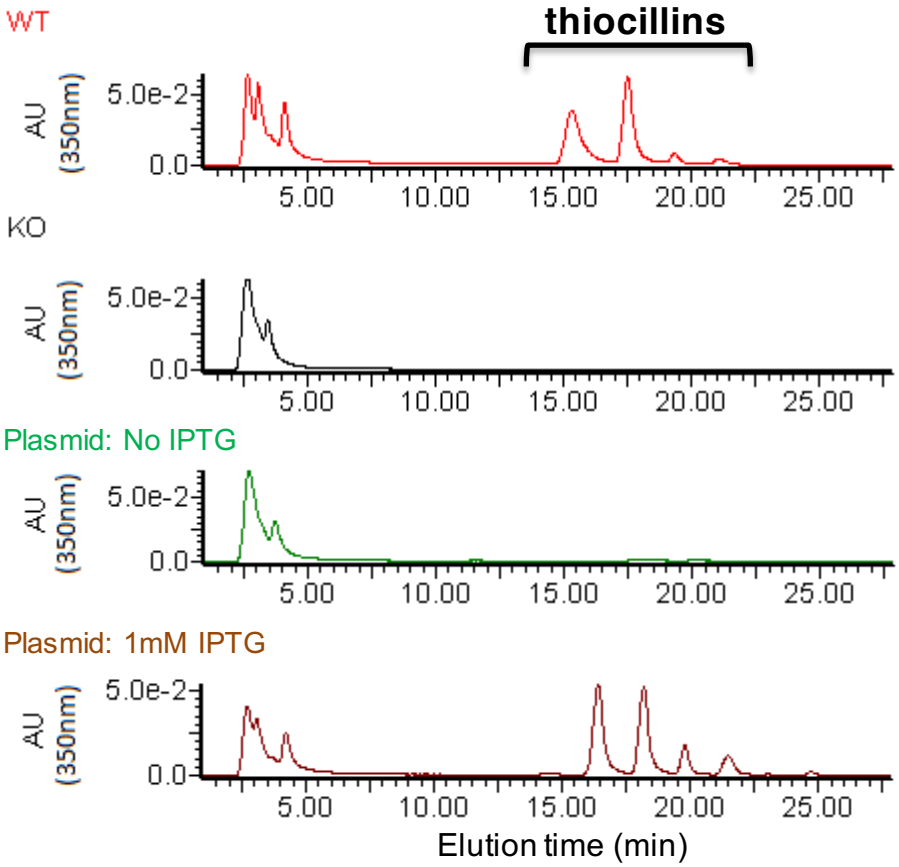

Figure S1: Plasmid complementation shows rescue of thiocillin production under IPTG control.

II. Library construction

The thiocillin saturation mutagenesis library was constructed by individually mutating each of the macrocycle residues 2-9 to the remaining 19 amino acids. pHT01-tclE was restriction digested with AfeI and SmaI (New England Biolabs, Ipswich, MA) to create a linear vector. An oligo template for each mutant tclE construct was ordered from Integrated DNA Technologies (IDT, Coralville, IA) containing the full 14AA core sequence (**Table S1**). Primers tclE-ins-F and tclE-ins-R was used to amplify the oligo templates to create DNA inserts. Gibson cloning was used to assemble the insert and vector into a plasmid. Plasmids were cloned in *E. coli* XL10-Gold and sequenced.

Combinatorial double mutant library was developed using Künkel mutagenesis. To develop the Künkel plasmid, an F1 origin sequence was inserted into the XhoI site of pHT01-tclE to create pHT08. Site-directed mutagenesis was used to replace residues 6-8 with a stop codon and SpeI cleavage site 'taaactagt' to prevent WT background in our screen. Primer 68NNK (**Table S1)** was used as a Kunkel template to randomize residues 6 and 8 with NNK codons. The library was purified and electroporated directly into *B. cereus* ΔtclE-H.

Name	Sequence
tclE-ins-F	ggttgatgttgatgctatgccagaaaacgaagcgcttgaaattatgggagcgtca
tclE-ins-R	gctcattaggcgggctgccccggggacgtctcaagttgtacaacaact
C2A	tgaaattatgggagcgtcaGCTacgacatgcgtatgtacatgcagttgttgtacaacttg
C2D	tgaaattatgggagcgtcaGATacgacatgcgtatgtacatgcagttgttgtacaacttg
C2E	tgaaattatgggagcgtcaGAAacgacatgcgtatgtacatgcagttgttgtacaacttg
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C2Q	tgaaattatgggagcgtcaCAAacgacatgcgtatgtacatgcagttgttgtacaacttg
C2R	tgaaattatgggagcgtcaCGTacgacatgcgtatgtacatgcagttgttgtacaacttg
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C2T	tgaaattatgggagcgtcaACAacgacatgcgtatgtacatgcagttgttgtacaacttg
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T3C	gaaattatgggagcgtcatgtTGTacatgcgtatgtacatgcagttgttgtacaacttga
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C9W	attatgggagcgtcatgtacgacatgcgtatgtacaTGGagttgttgtacaacttgagac
C9Y	attatgggagcgtcatgtacgacatgcgtatgtacaTATagttgttgtacaacttgagac

68NNK	ggacgtctcaagttgtacaacaactgcaMNNacaMNNgcatgtcgtacatgacgctccc

Table S1: Primers used to generate all mutants in this study. Capital letters in sequence indicate mutation.

III. Expression and extraction of thiocillin analogs

Plasmids containing thiocillin mutants were electroporated into *B. cereus* ΔtclE-H cells and selected on chloramphenicol selective plates. For small-scale expression, colonies were grown in 1.5 mL LB Lennox + 10 μg/mL chloramphenicol + 1 mM IPTG in 2.2 mL deep well blocks with shaking at 30°C for 72 hours. Cells were pelleted and decanted. Compounds were extracted twice with 800 μL methanol. Methanol extracts were dried by Genevac and resuspended in 50 μL DMSO for downstream analysis.

For large-scale preparations of thiocillin analogs, fresh colonies were grown overnight in 30 mL LB Lennox + 10 μg/mL chloramphenicol at 30°C and 200 rpm. 7.5 mL overnight culture was innoculated into 1.5 L LB Lennox in 2.8 L baffled flask and were grown at 30°C and 200 rpm. 5 μg/mL chloramphenicol was added at 0, 24, and 48 hrs. 1 mM IPTG was added at 3 hr and 0.5 mM IPTG was added at 24 and 48 hrs. Cells were pelleted and decanted. 50 mL of methanol and 20 g of anhydrous sodium sulfate were added to the pellet and vortexed vigorously. The methanol was filtered with a Whatman no. 1 filter. A second methanol extraction was performed on the pellet and filtered. The methanol was removed by vacuum to leave a dark yellow residue. Ethyl acetate extractions was used to further purify the compounds. The residue was redisolved in 40 mL of 1:1 EtOAc:saturated NaCl solution and shaken in a separatory funnel. The EtOAc layer was collected. The aqueous layer was washed twice with 20 mL EtOAc. EtOAc layers were combined, dried with anhydrous Na_2SO_4 and filtered. EtOAc was removed by rotovap. Residue was dissolved in 1 mL DMSO, filtered, and purified by preparative HPLC. 500 μL was injected onto a Waters XBridge Prep C18 5 μm OBD 19x50 mm column with a flow rate of 20 mL/min. Solvent A = water/0.05% formic acid. Solvent B = methanol/0.05% formic acid. The method started at 50% B for 2 min, followed by a linear gradient from 50-75% B over 12 min, then a linear gradient to 100% B for 1 min, static at 100% B for 2 min, and then static at 50% B for 1 min. Major peaks with 350 nm absorption were collected and vacuumed to dryness. Mass of dried residue was used to determine the yield. Compounds with yields greater than 0.1 mg were kept for further analysis.

[^{15}N]-labelled thiocillin was prepared for NMR analysis. Large-scale preparation protocol was followed exchanging the media with M9 minimal media containing [^{15}N]-ammonium chloride (Cambridge Isotope Laboratories, Tewksbury, MA) as the nitrogen source and supplemented with ATCC trace mineral supplement (ATCC) and BME vitamins (Sigma-Aldrich). WT *B. cereus* was grown without antibiotics or IPTG. The protocol described above was used to isolate [^{15}N]-thiocillin. The compound was dissolved in d_6-DMSO (Cambridge Isotope Laboratories) for NMR analysis.

IV. LC/MS macrocyclization assay

To assay for macrocyclization of thiocillin mutants, an LC/MS assay was used to detect the pyridine ring formed during macrocyclization (350 nm absorption) and the mass of the product. Samples were analyzed on a Waters 2795 HPLC with a Waters 2996 UV detector and a Waters ZQ-4000 quadrapole ESI mass spectrometer. 20 μL of the DMSO stocks from the small scale expression, described in Section III, were injected onto a Waters XBridge C18 3.5 μm 4.6x50 mm column with a flow rate of 1 mL/min and a linear gradient of 5-95% B:A over 8 min (Solvent A = water/0.1% TFA; Solvent B = methanol/0.1% TFA).

V. Overlay activity assay

To screen for active antibiotics, an overlay assay was used. Cultures of engineered *B. cereus* containing each tclE mutant from our saturation mutagenesis library were grown overnight in 96-well plates in LB media with 10 μg/mL chloramphenicol. A strain producing WT tclE via plasmid and a strain with an empty vector were used as positive and negative controls, respectively. After overnight growth, cultures were diluted 100-fold into LB media and spotted on LB agarose plates containing 10 μg/mL chloramphenicol and 1 mM IPTG. Colonies were allowed to grow for 30 hours at 30°C. Our "victim" strain for the overlay assay is *B. subtilis* BG2864 ATCC 47096 (a kanamycin resistant strain) which we transformed with pHT01 expressing LacZ (a β-galactosidase) to distinguish cell types in our assay when LacZ degrades X-gal to form a blue pigment. The overlay was prepared by cooling 25 mL of molten Top Agar (LB + 0.7% agarose) to 42°C and adding 10 μL of 50 mg/mL kanamycin (to prevent further growth of *B. cereus* colonies), 20 μL of 5 μg/mL chloramphenicol, 25 μL of 100 mM IPTG, 300 μL of 20 mg/mL X-gal dissolved in DMSO, and 25 μL of dense "victim" strain. The Top Agar solution was poured over the colonies and allowed to solidify. Plates were placed in a 37°C incubator overnight. Kill zones indicate a colony producing an active antibiotic.

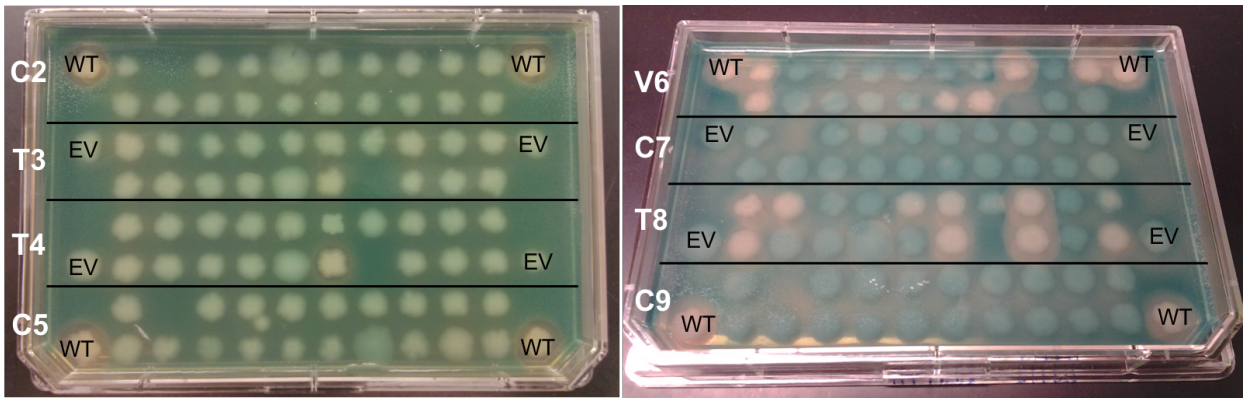


Figure S2: Overlay assay for active antibiotics. Kill zones show colonies producing an active antibiotic. WT = *B. cereus* ΔtclE-H + pHT01-tclE. EV = *B. cereus* ΔtclE-H + pHT01-Empty Vector.

VI. Minimal inhibitory concentration assay

A dense culture of *B. subtilis* 168 was diluted to an OD_{600} of 0.001 in LB media. 96-well plates were filled to 147 μL per well. A 2-fold serial dilution series was made in DMSO (400 μg/mL - 0.39 μg/mL) for each purified compound. 3 μL of the serial dilution DMSO stock was added to the wells. The plates were sealed with foil and incubated for 16 hrs at 37°C with shaking at 280 rpm. OD_{600} of cultures were measured by plate reader. MIC is designated as the lowest concentration required to inhibit growth. Inhibited growth is defined as cultures showing less than 10% increases in OD_{600} over kanamycin (25 μg/mL) treated control.

A mixture of all WT thiocillin variants was used as a positive control. This was necessary to obtain the literature reported MIC value of 0.5 μg/mL.[3] T4A mutant was included as an inactive negative control mutant. The results for the single point mutant library are shown in Table 1 and double mutants in Table S2.

Mutant	Yield (mg/L)	MIC (μg/mL)	Modifications
WT	3.9	0.5	All variants
V6A-T8I1	1.0	0.5	
V6A-T8V1	1.0	0.25	
V6A-T8Y1	0.7	4	
V6I-T8A	ND	ND	
V6I-T8M	ND	ND	
V6I-T8S	ND	ND	
V6I-T8V	ND	ND	
V6L-T8I	ND	ND	
V6L-T8M	ND	ND	
V6L-T8S	ND	ND	
V6L-T8V	ND	ND	
V6M-T8I1	0.5	0.5	
V6M-T8M	ND	ND	
V6M-T8V1	0.9	1	M6-OH
V6M-T8V2	0.7	1	
V6M-T8Y1	0.4	>8	M6-OH
V6M-T8Y2	0.7	>8	
V6T-T8F	ND	ND	

Table S2: MIC table of active double mutants. ND = not determined.

VII. LC/MS and HRMS structural characterization

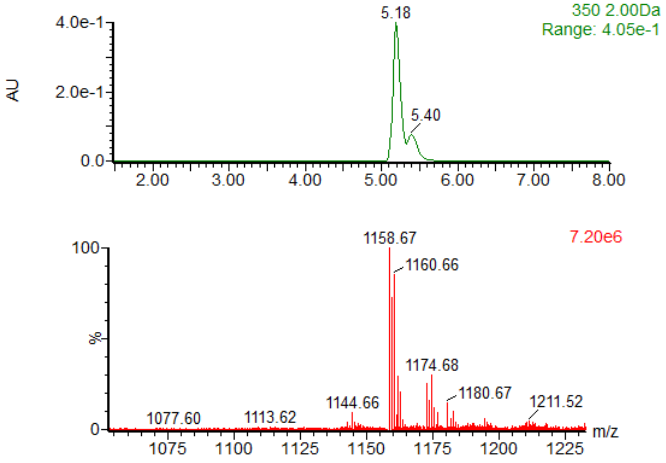
LC/MS analysis was performed on all purified compounds as described in Section IV. High resolution mass spectrometry (HRMS) data was collected on a Q Exactive Plus (ThermoFisher Scientific) with resolution set to 140,000 at *m/z* 200. Purified compounds were diluted to 500 nM in 30% acetonitrile in water with 0.1% formic acid. 1.5 μL of the samples were direct injected into the mass spectrometer at a rate of 500 nL/min for analysis. HRMS and retention time data helped provide insight on PTMs. LC/MS and HRMS data is shown in **Figure S3**. Summary of HRMS data is shown in **Table S3**.

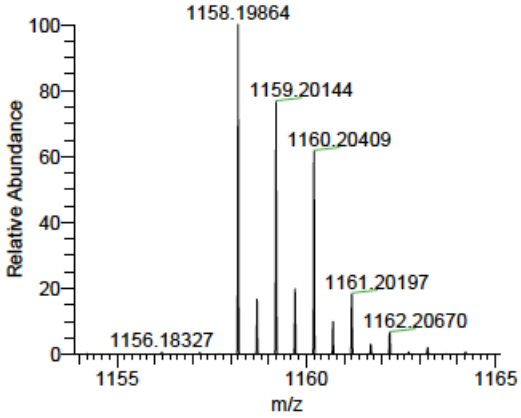
Mutant	Modifications	Chemical Formula	Expected Mass [M+H]	Observed Mass	Figure
WT	All variants	C48H47N13O10S6	1158.19659	1158.19864	S3A
T3S1	V6-OH	C47H45N13O10S6	1144.18094	1144.18205	S3B
T3S2		C47H45N13O9S6	1128.18602	1128.18843	S3C
T3S3	T8-CH3	C48H47N13O9S6	1142.20167	1142.20401	S3D
T3S4	T3-Dha	C47H43N13O8S6	1110.17546	1110.17857	S3E
T4A1	V6-OH	C47H47N13O10S6	1146.19659	1146.19836	S3F
T4A2		C47H47N13O9S6	1130.20167	1130.20404	S3G
T4S1	T4-Dha; V6-OH	C47H45N13O10S6	1144.18094	1144.18208	S3H
T4S2	T4-Dha; V6-OH; T8-CH3	C48H47N13O10S6	1158.19659	1158.19797	S3I
T4S3	T4-Dha	C47H45N13O9S6	1128.18602	1128.18795	S3J
V6A1		C46H43N13O9S6	1114.17037	1114.17171	S3K
V6A2	T8-CH3	C47H45N13O9S6	1128.18602	1128.18689	S3L
V6I1	I6-OH	C49H49N13O10S6	1172.21224	1172.21319	S3M
V6I2		C49H49N13O9S6	1156.21732	1156.21847	S3N
V6L1	L6-OH; T8-CH3	C50H51N13O10S6	1186.22789	1186.22864	S3O
V6L2	T8-CH3	C50H53N13O9S6	*1172.24862	1172.25000	S3P
V6M1	M6-OH; T8-CH3	C49H49N13O10S7	1204.18431	1204.18549	S3Q
V6M2	T8-CH3	C49H49N13O9S7	1188.18939	1188.19066	S3R
V6S1	T8-CH3	C47H45N13O10S6	1144.18094	1144.18291	S3S
V6T1	T8-CH3	C48H47N13O10S6	1158.19659	1158.19936	S3T
T8A1	V6-OH	C47H45N13O9S6	1128.18602	1128.18840	S3U
T8C1	V6-OH; C8-CH3	C48H47N13O9S7	1174.17374	1174.17637	S3V
T8F1		C53H51N13O8S6	*1190.23806	1190.24049	S3W
T8G1	V6-OH	C46H43N13O9S6	1114.17037	1114.17190	S3X
T8G2		C46H43N13O8S6	1098.17546	1098.17732	S3Y
T8I1	V6-OH	C50H51N13O9S6	1170.23297	1170.23432	S3Z
T8L1	V6-OH	C50H51N13O9S6	1170.23297	1170.23375	S3AA
T8L2		C50H53N13O8S6	*1156.25371	1156.25448	S3BB
T8M1	V6-OH	C49H49N13O9S7	1188.18939	1188.19226	S3CC
T8M2		C49H51N13O8S7	*1174.21013	1174.21150	S3DD
T8S1	V6-OH	C47H45N13O10S6	1144.18094	1144.18211	S3EE
T8S2	V6-OH; S8-Dha	C47H43N13O9S6	1126.17037	1126.17223	S3FF
T8V1	V6-OH	C49H49N13O9S6	1156.21732	1156.21959	S3GG

T8V2		C49H51N13O8S6	*1142.23806	1142.23920	S3HH
T8Y1	V6-OH	C53H49N13O10S6	1220.21224	1220.21448	S3II
T8Y2		C53H51N13O9S6	*1206.23297	1206.23292	S3JJ
V6A-T8I1		C48H47N13O8S6	1126.20676	1126.20849	S3KK
V6A-T8V1		C47H45N13O8S6	1112.19111	1112.19422	S3LL
V6A-T8Y1		C51H45N13O9S6	1176.18602	1176.18810	S3MM
V6M-T8I1		C50H53N13O8S7	*1188.22578	1188.22679	S3NN
V6M-T8V1	M6-OH	C49H49N13O9S7	1188.18939	1188.19102	S3OO
V6M-T8V2		C49H49N13O8S7	1172.19448	1172.19607	S3PP
V6M-T8Y1	M6-OH	C53H49N13O10S7	1252.18431	1252.18618	S3QQ
V6M-T8Y2		C53H49N13O9S7	1236.18939	1236.19122	S3RR

Table S3: HRMS data for all compounds in this study. Expected [M+H] are shown for for compounds containing an aminoacetone residue at position 14. * indicates an expected [M+H] for an aminoisopropanol residue at position 14.

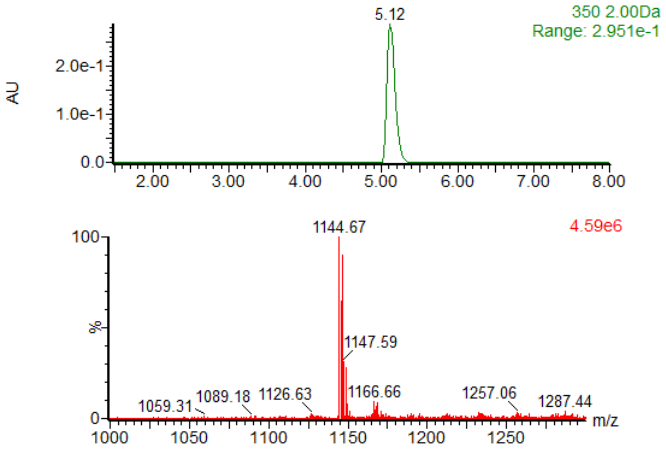
A) WT Thiocillin Mixture

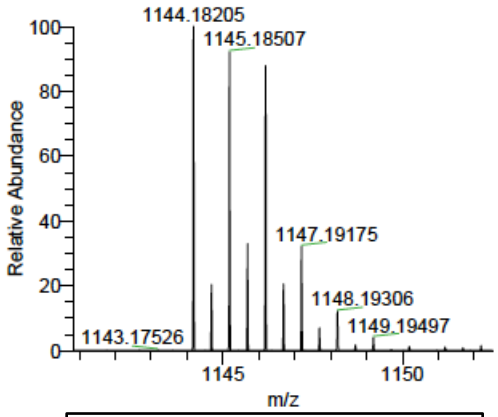




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1158.19659

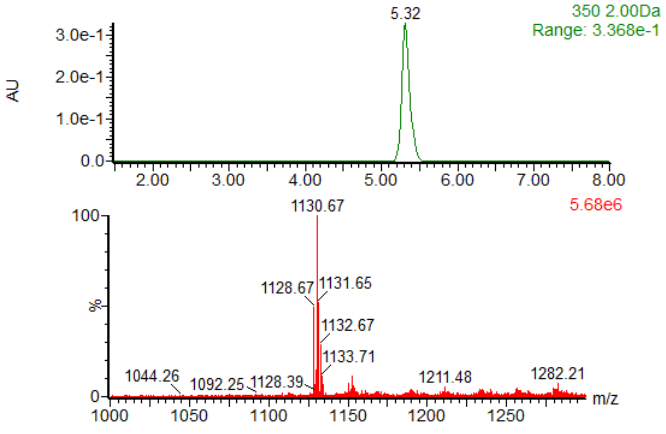
B) T3S1

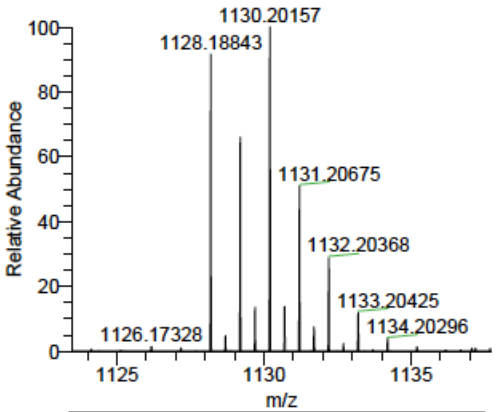




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1144.18094

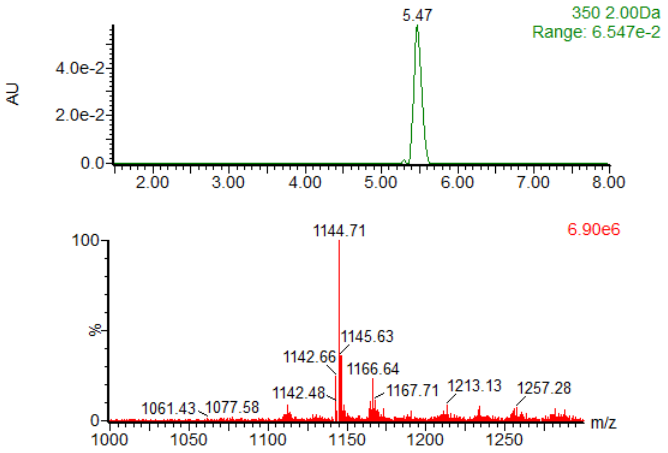
C) T3S2

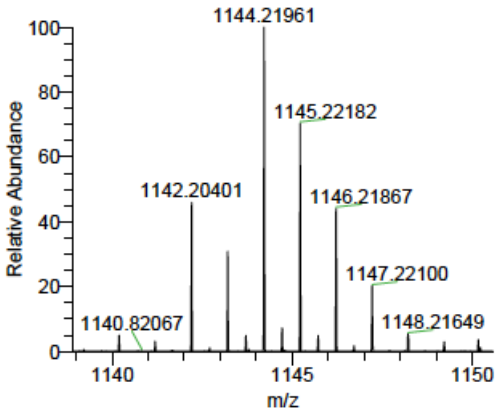




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1128.18602

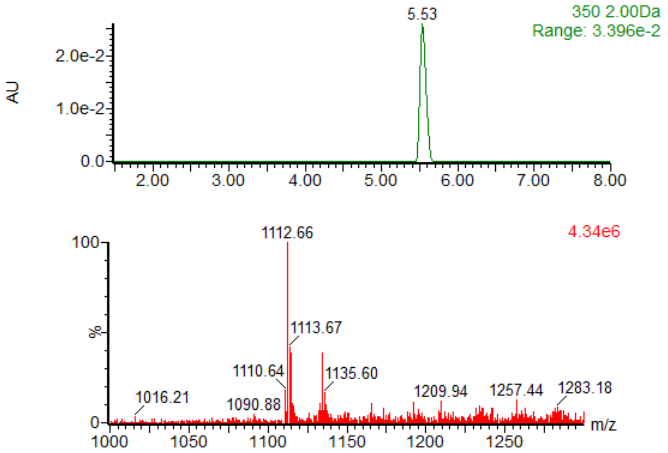
D) T3S3

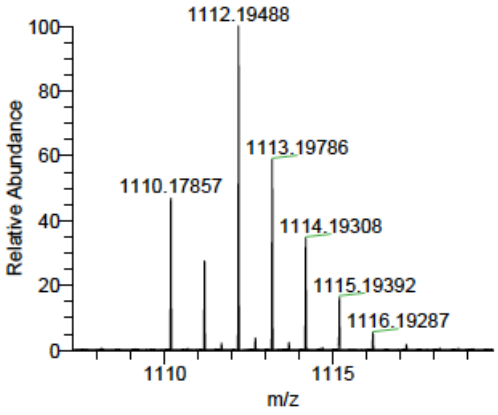




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1142.20167

E) T3S4





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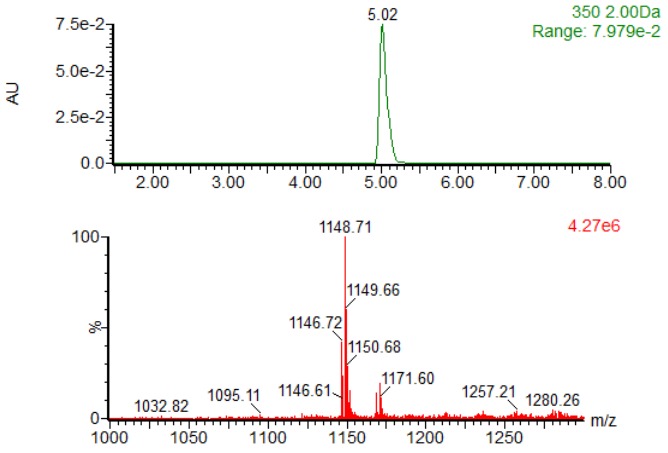
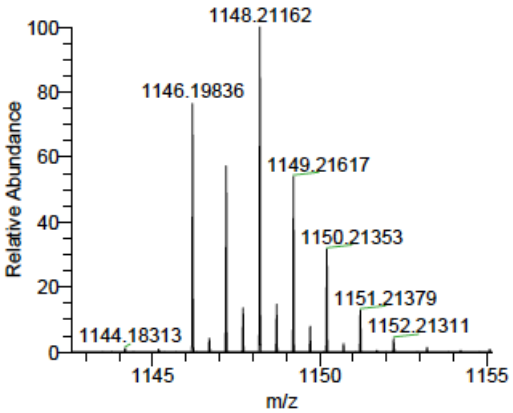
Theoretical [M+H] = 1110.17546

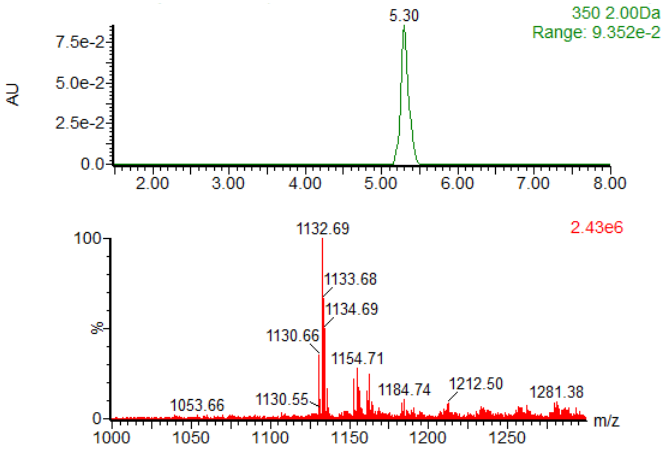
F) T4A1

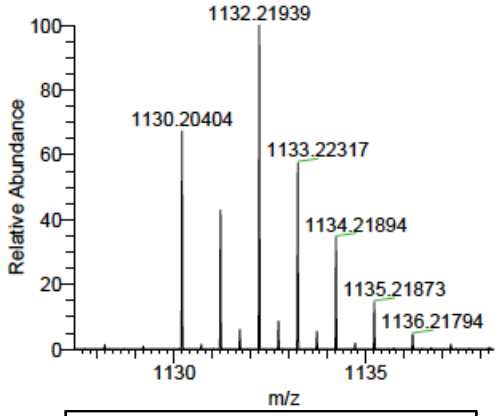




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1146.19659

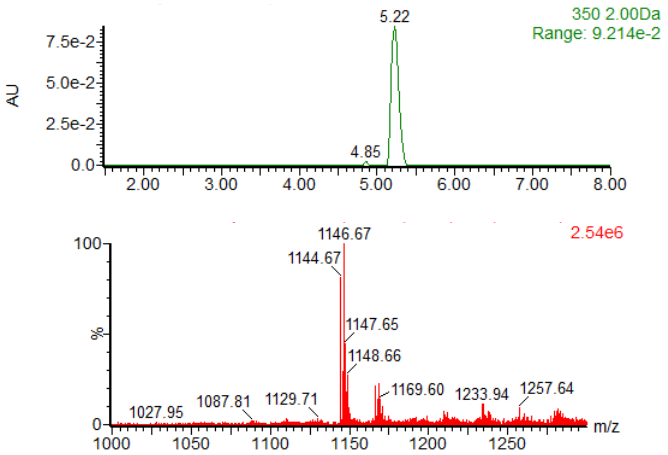
G) T4A2

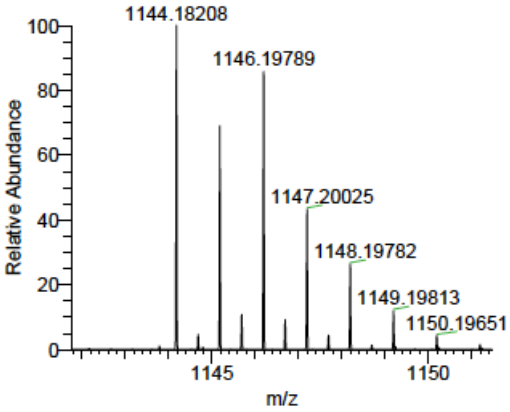




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1130.20167

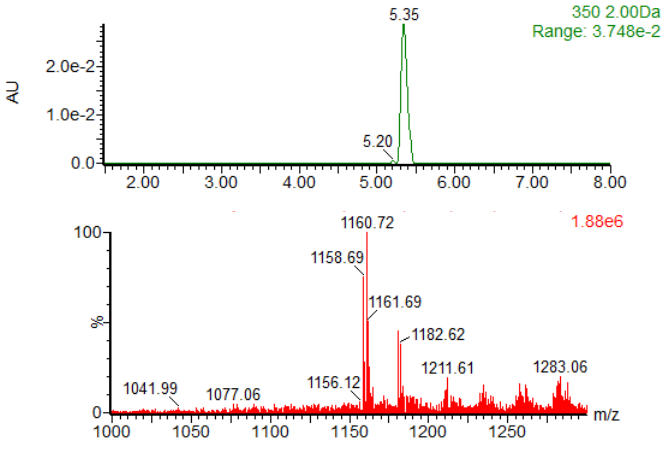
H) T4S1

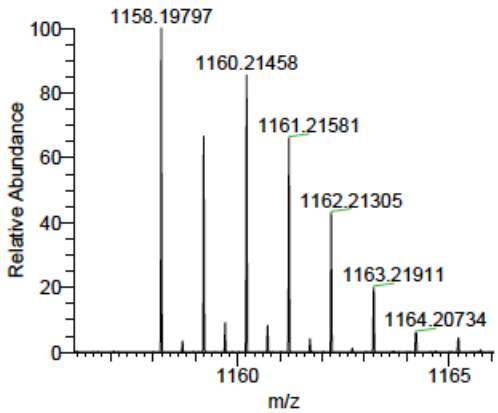




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1144.18094

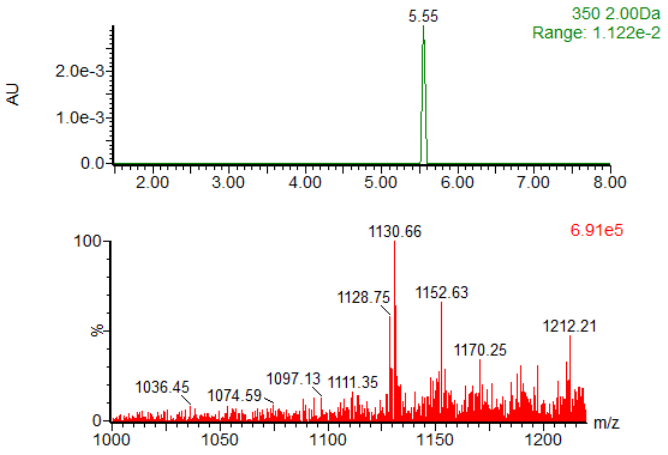
I) T4S2

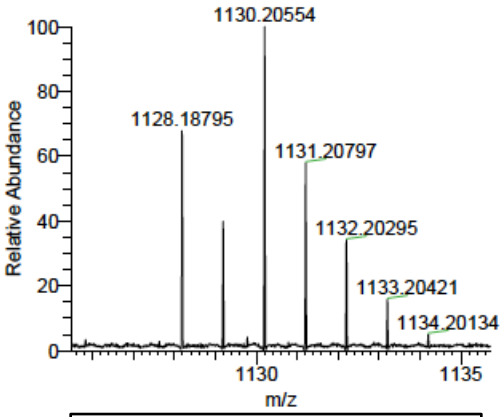




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1158.19659

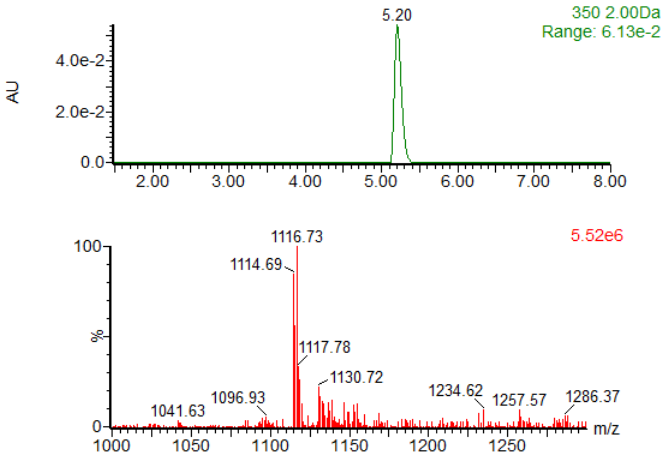
J) T4S3

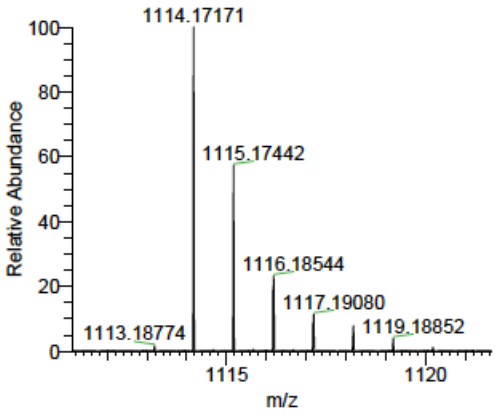




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1128.18602

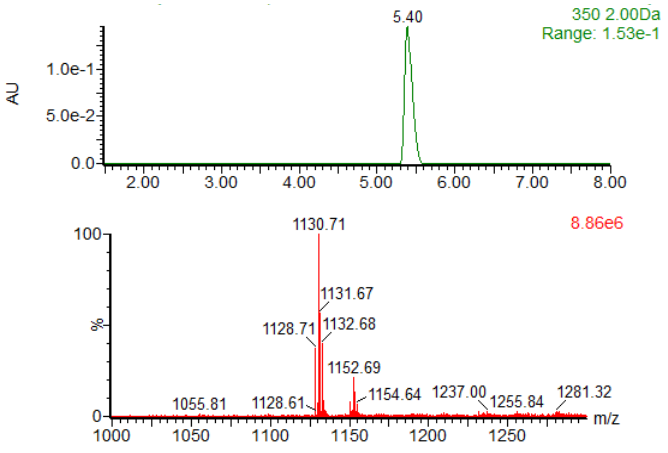
K) V6A1

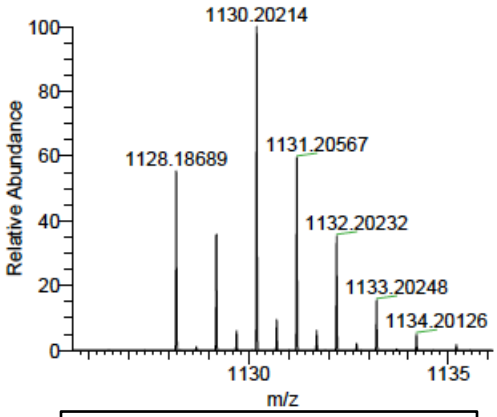




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1114.17037

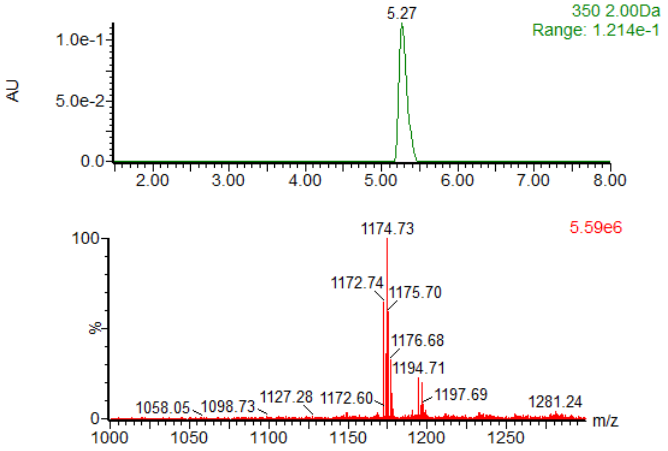
L) V6A2

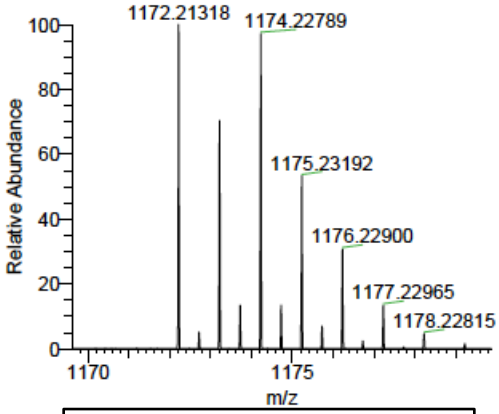




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1128.18602

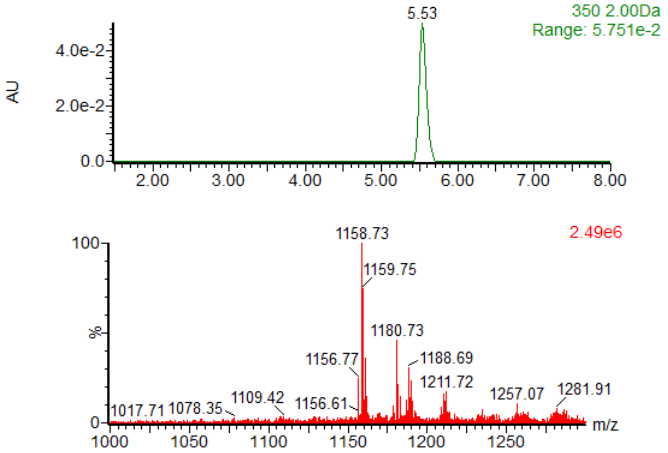
M) V6I1

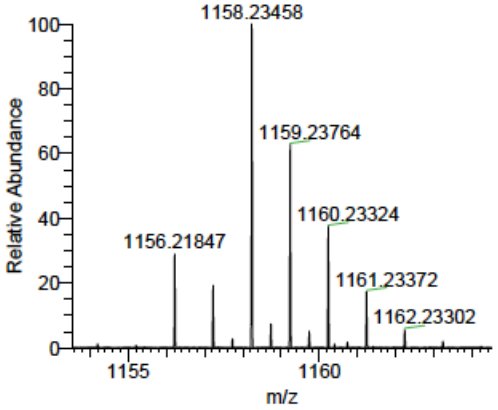




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1172.21224

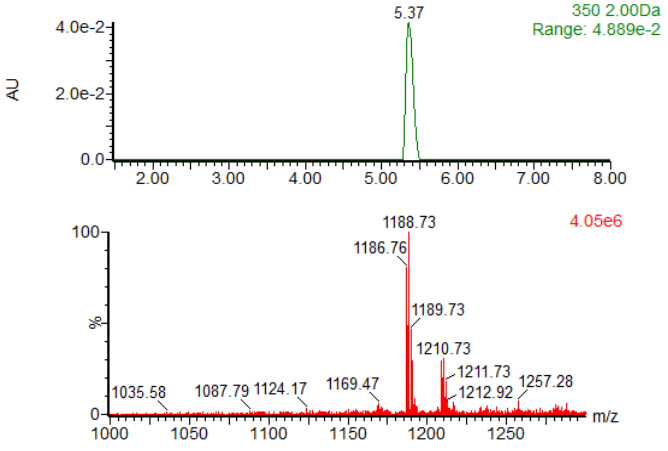
N) V6I2

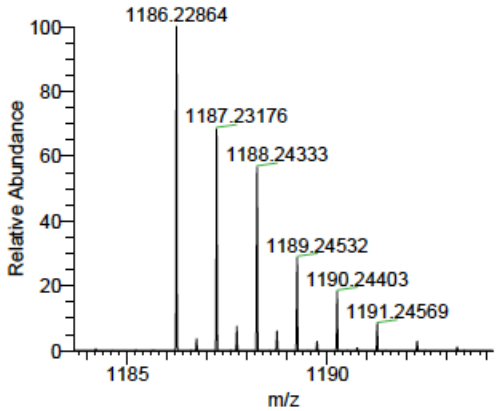




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1156.21732

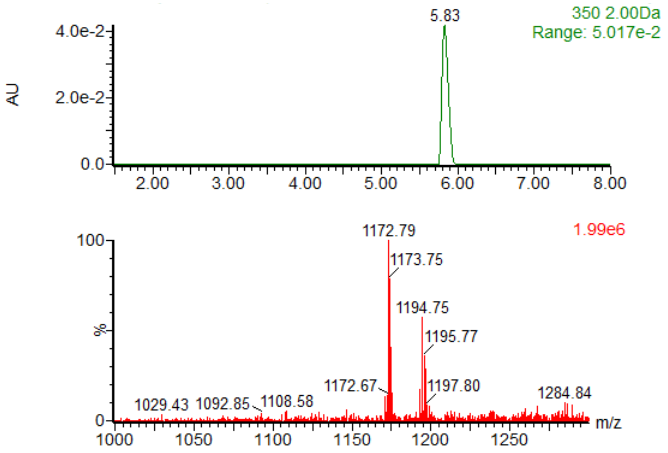
O) V6L1

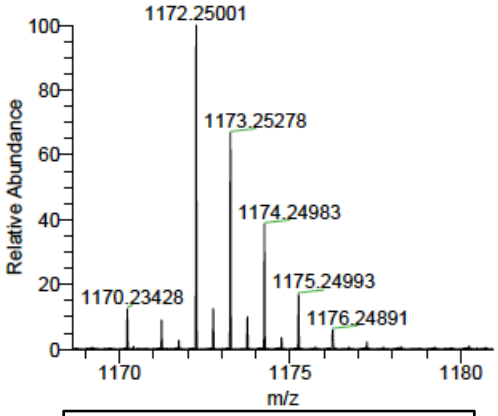




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1186.22789

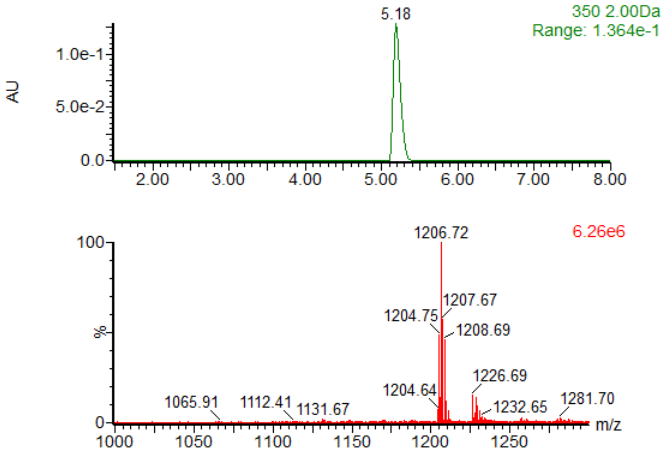
P) V6L2

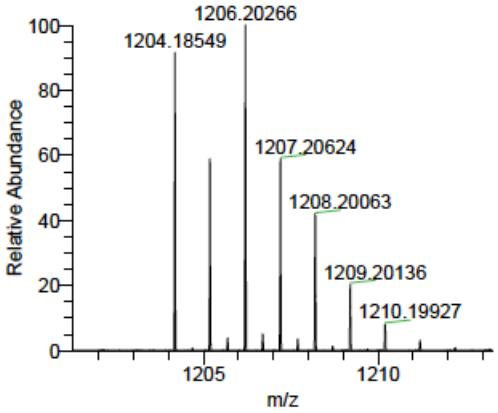




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1172.24862

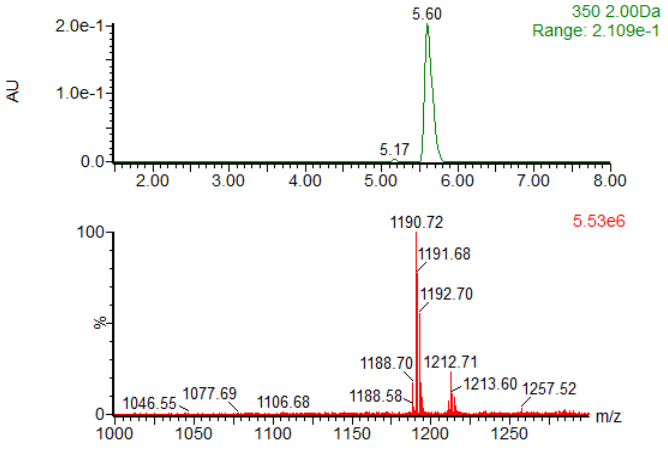
Q) V6M1

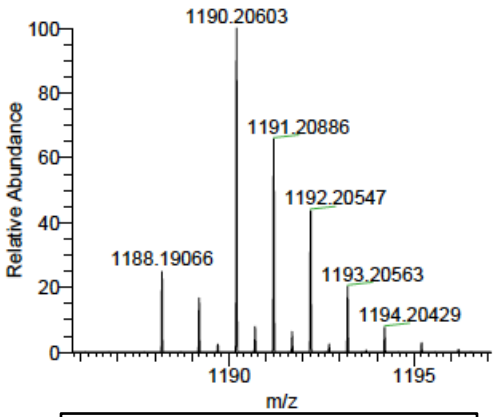




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1204.18431

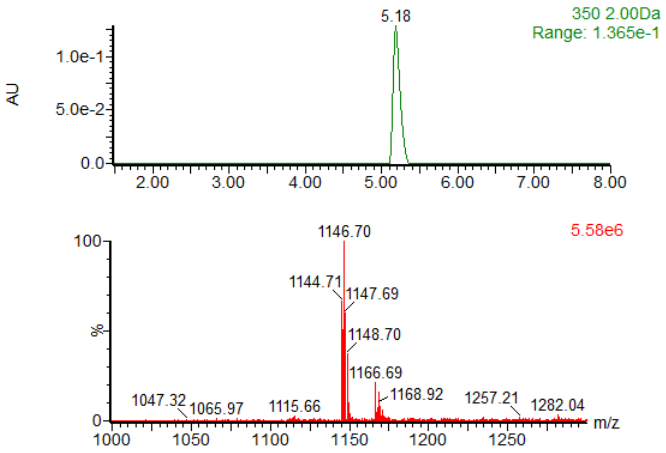
R) V6M2

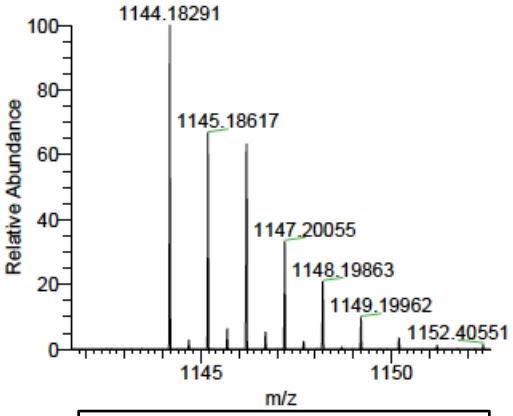




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1188.18939

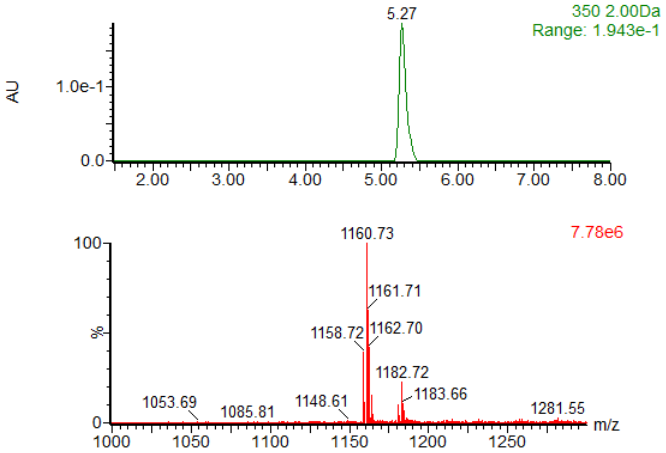
S) V6S1

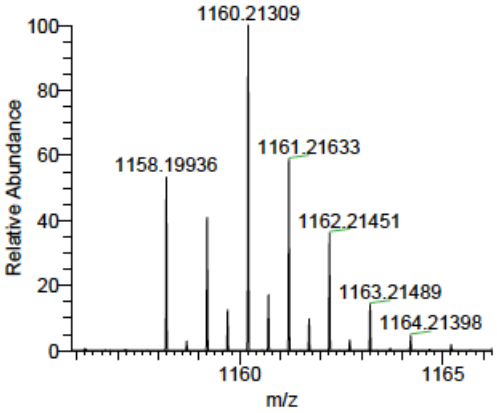




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1144.18094

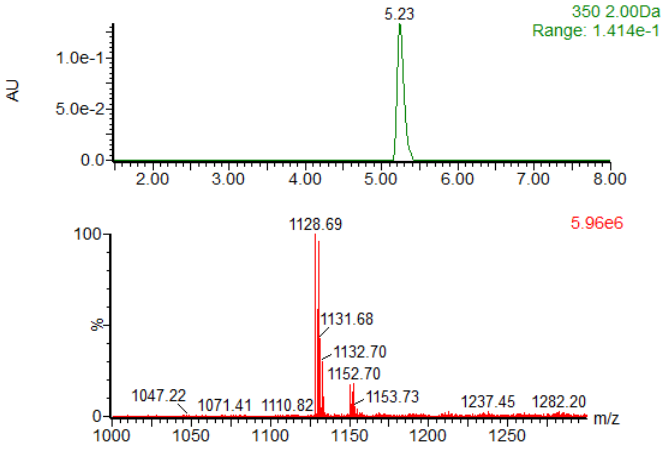
T) V6T1

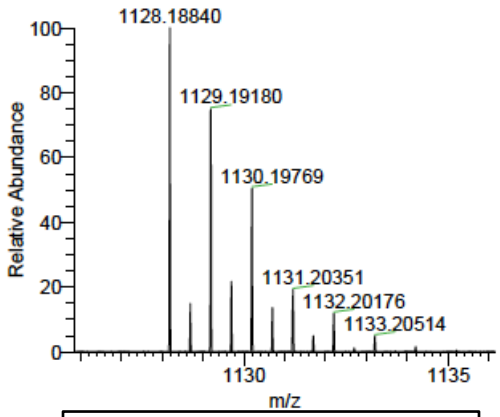




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1158.19659

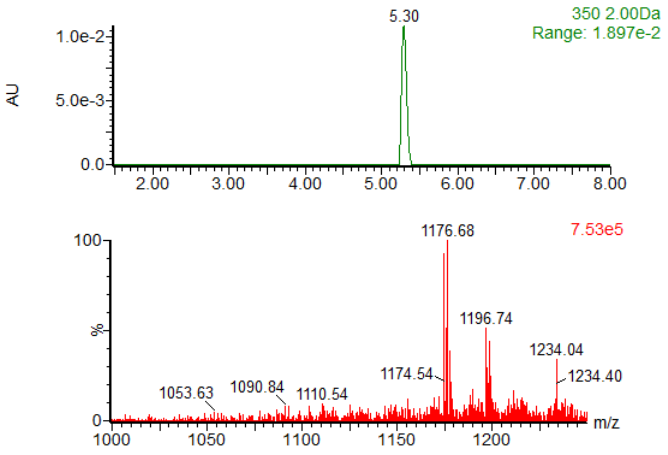
U) T8A1

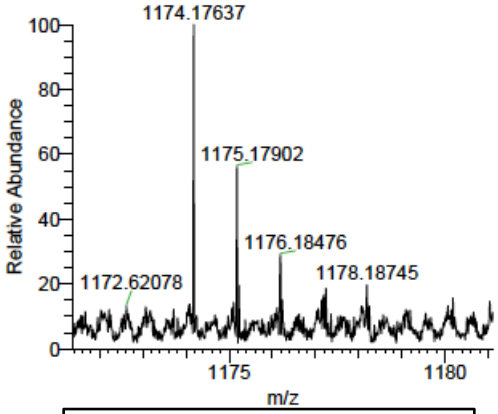




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1128.18602

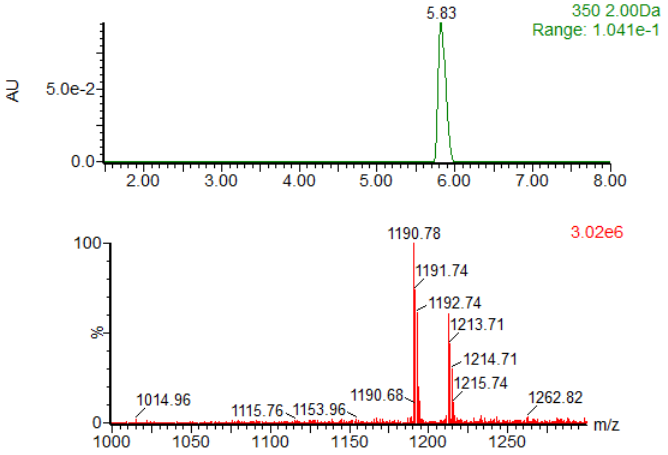
V) T8C1

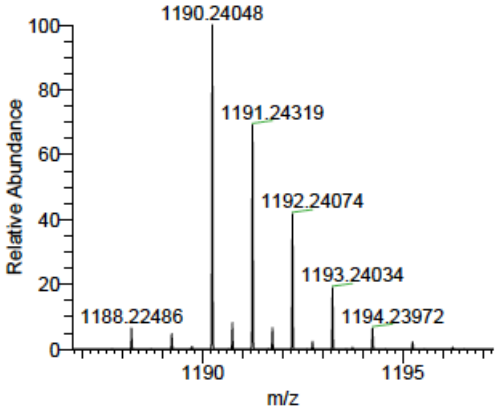




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1174.17374

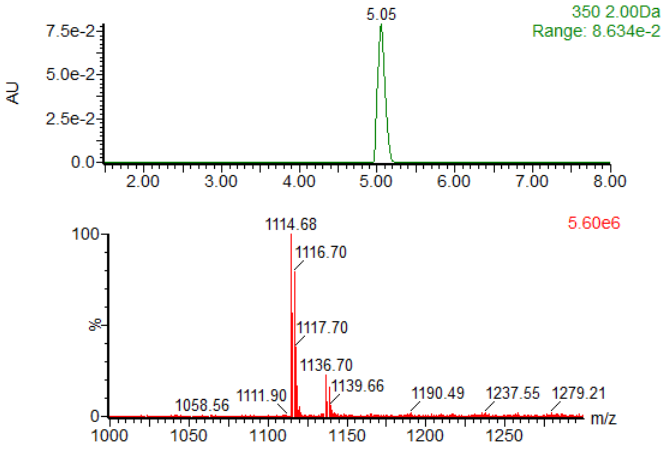
W) T8F1

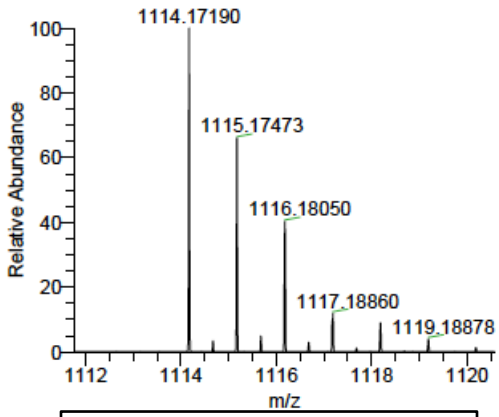




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1190.23806

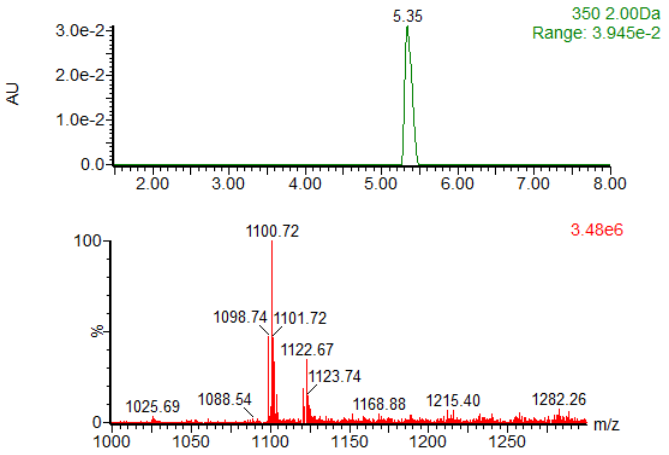
X) T8G1

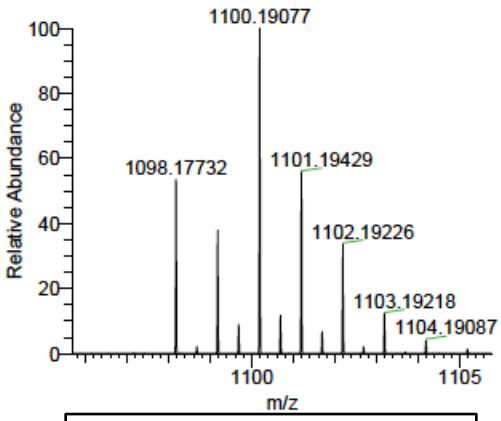




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1114.17037

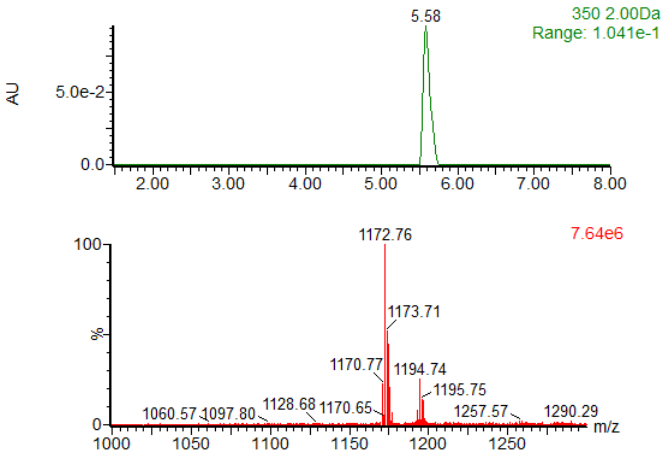
Y) T8G2

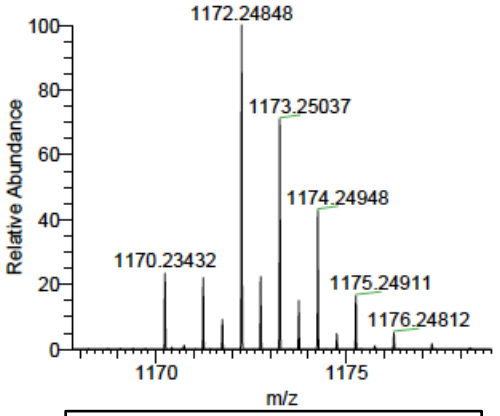




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1098.17546

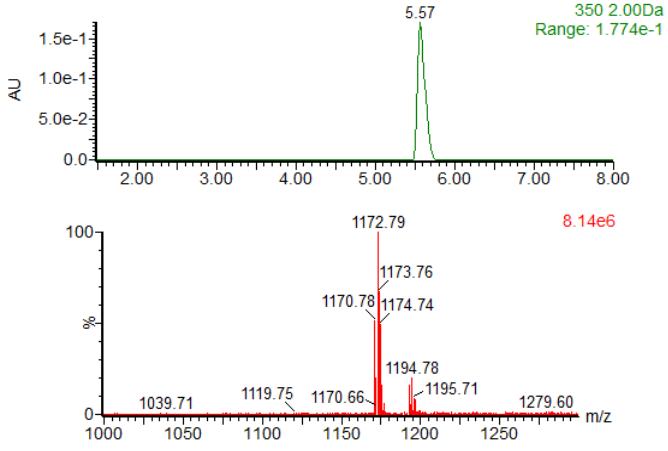
Z) T8I1

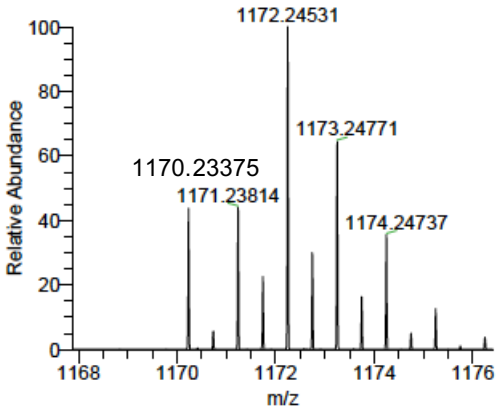




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1170.23297

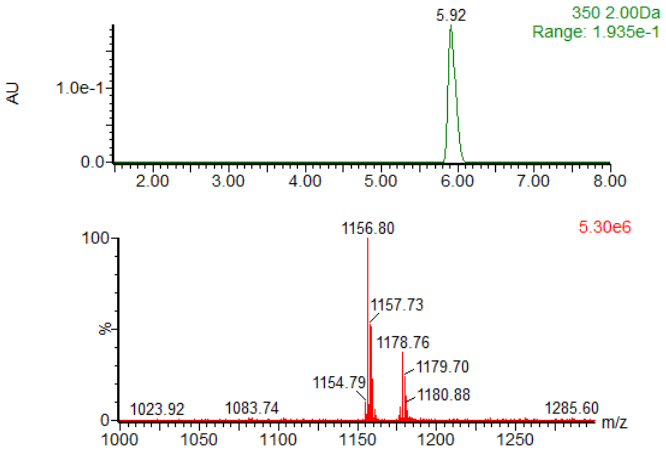
AA) T8L1

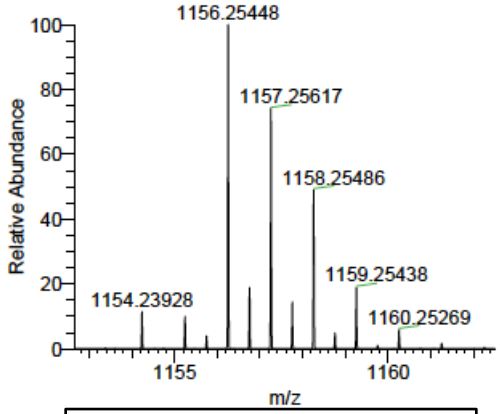




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1170.23297

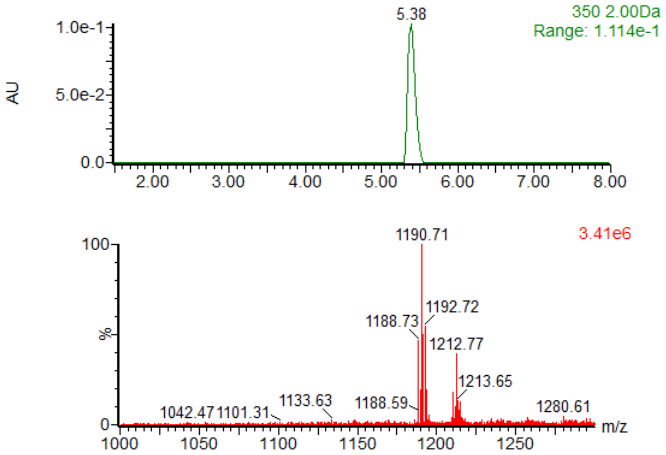
BB) T8L2

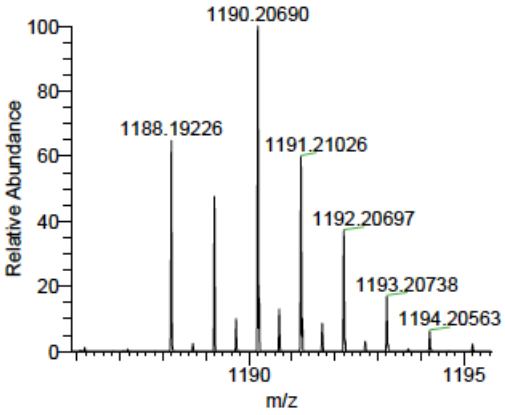




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1156.25371

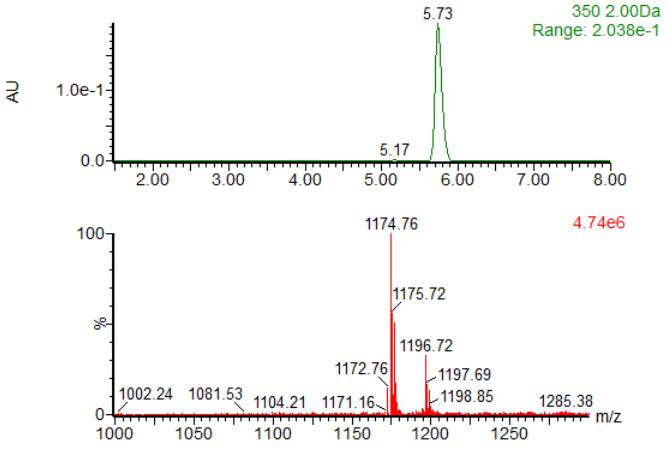
CC) T8M1

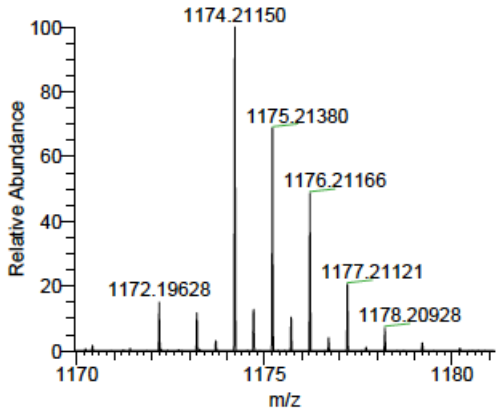




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1188.18939

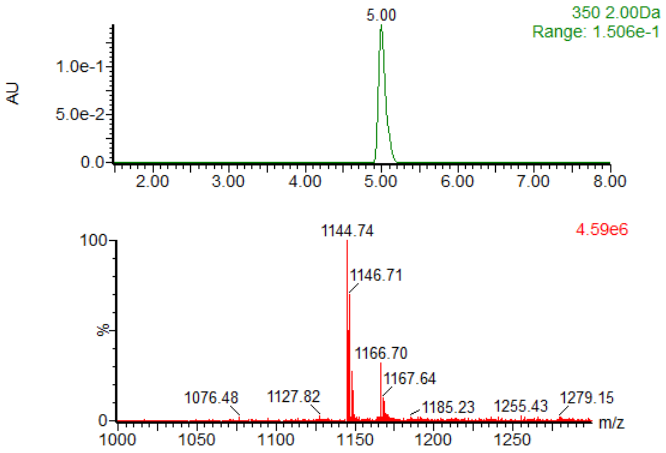
DD) T8M2

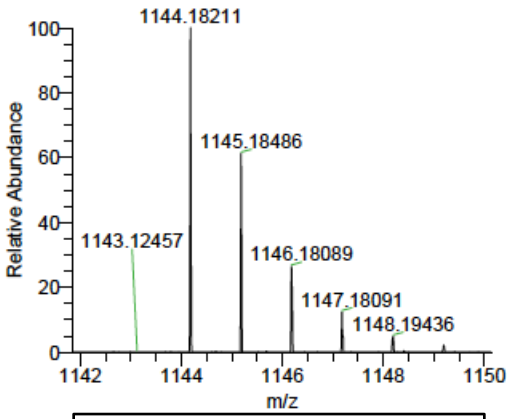




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1174.21013

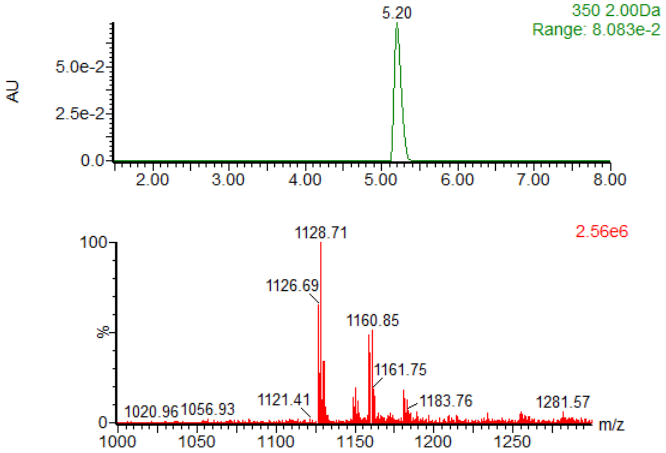
EE) T8S1

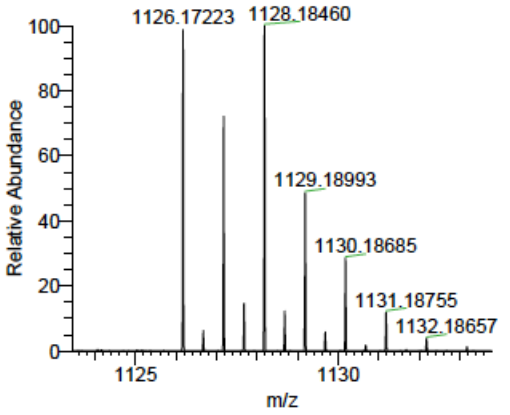




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1144.18094

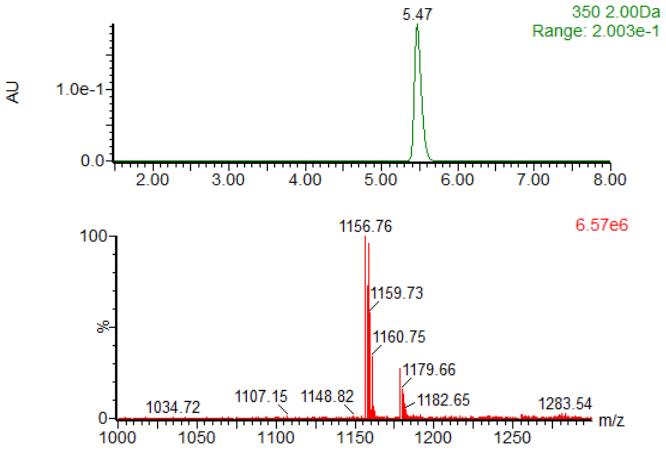
FF) T8S2

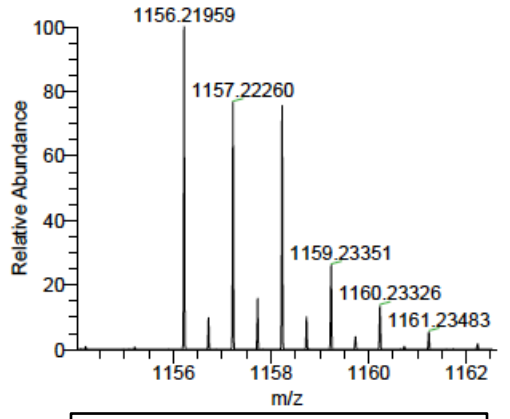




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1126.17037

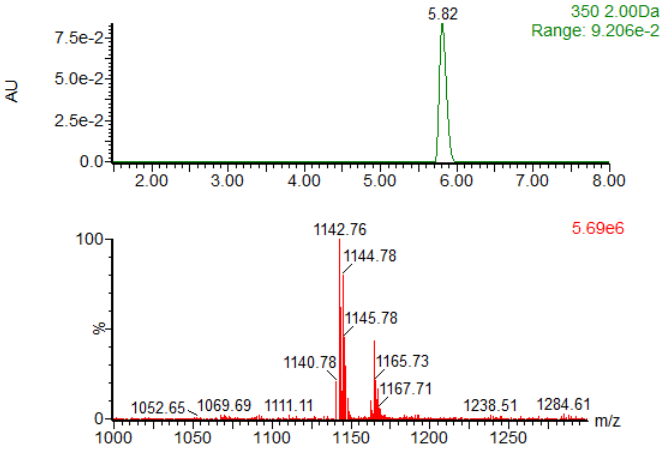
GG) T8V1

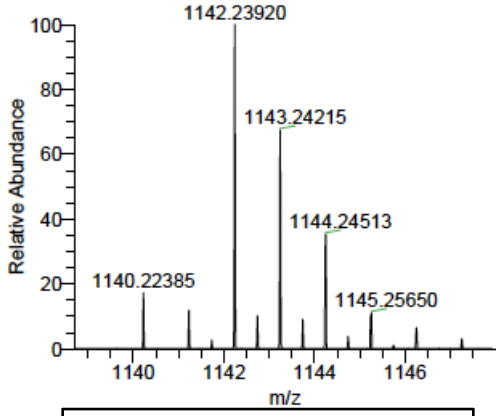




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1156.21732

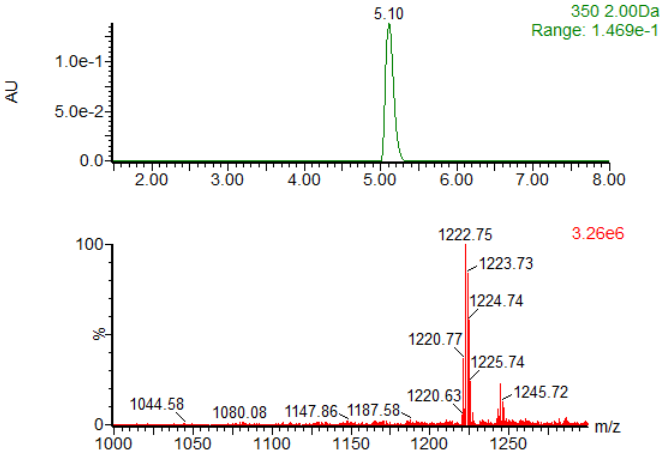
HH) T8V2

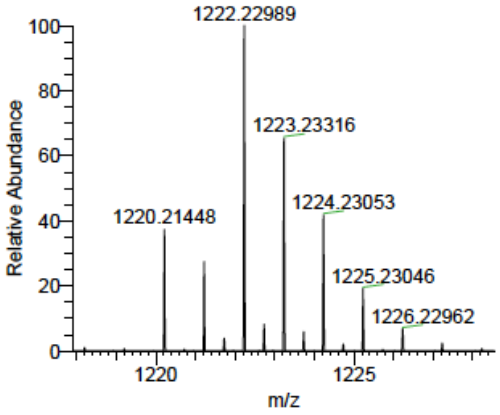




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1142.23806

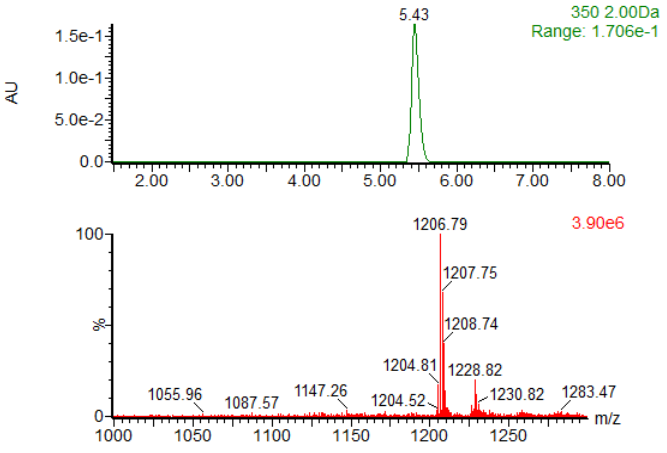
II) T8Y1

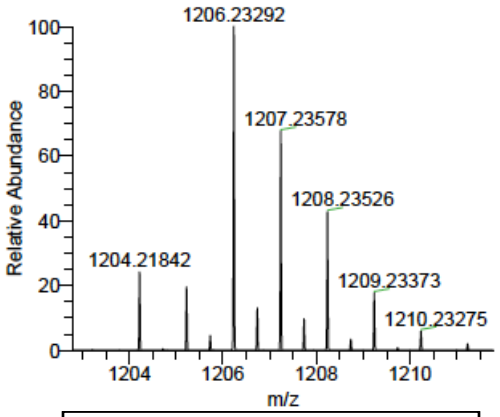




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1220.21224

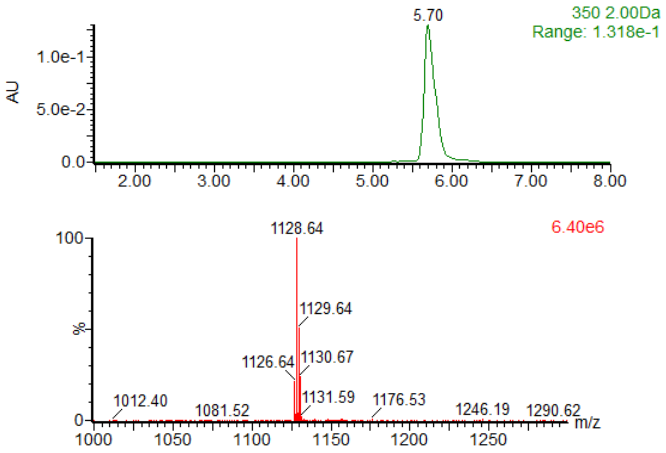
JJ) T8Y2

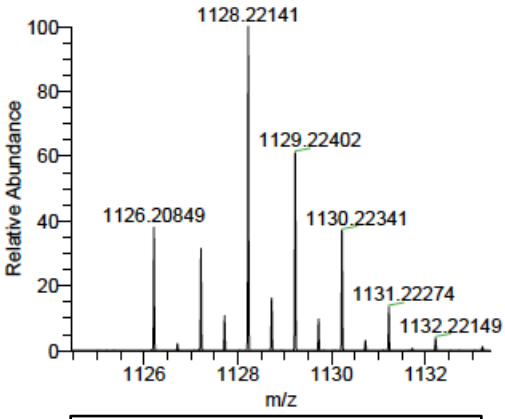




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1206.23297

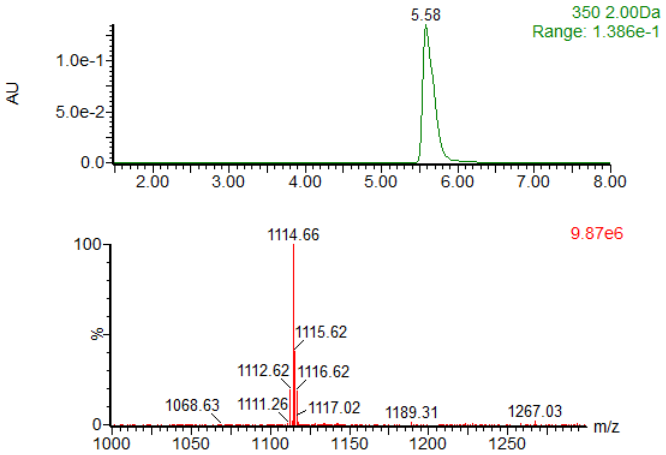
KK) V6A-T8I1

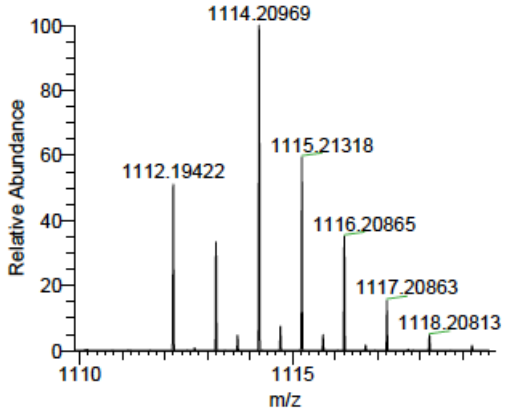




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1126.20676

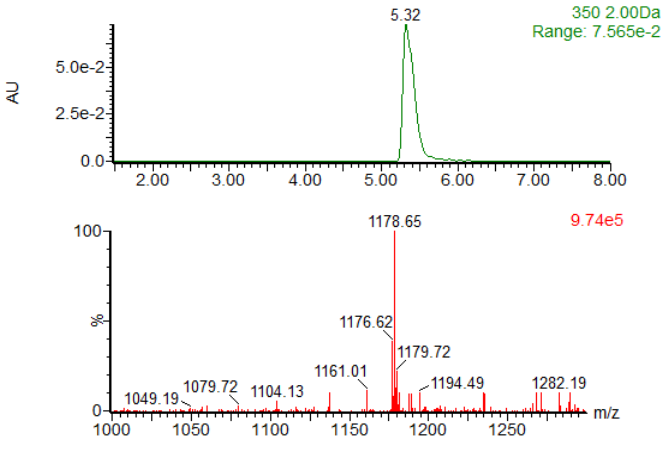
LL) V6A-T8V1

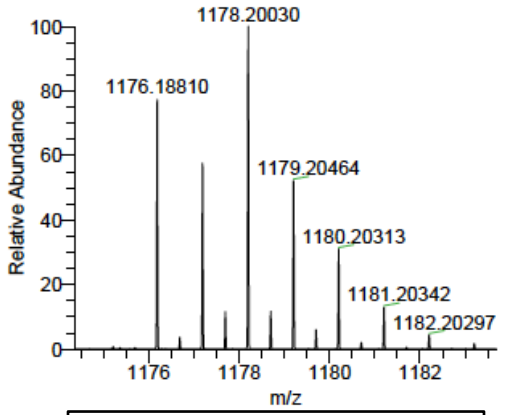




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1112.19111

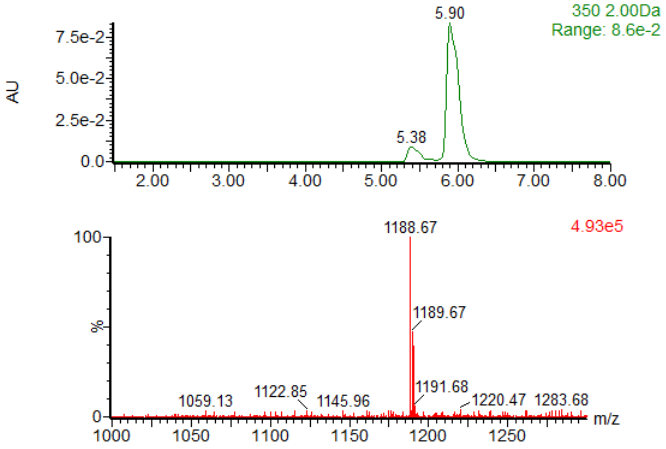
MM) V6A-T8Y1

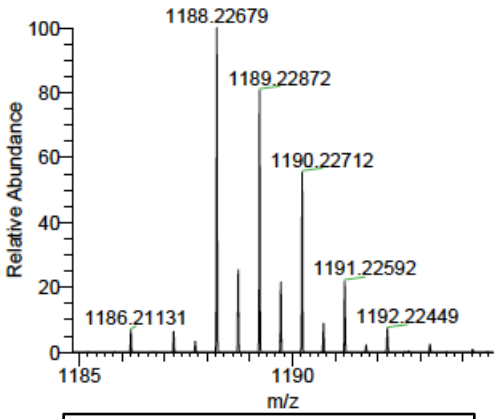




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1176.18602

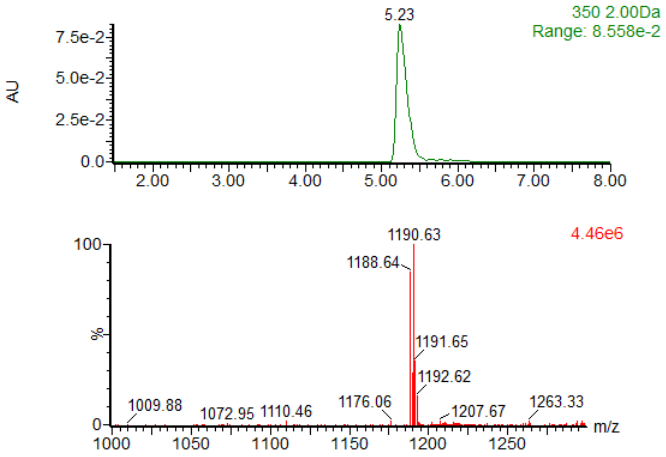
NN) V6M-T8I1

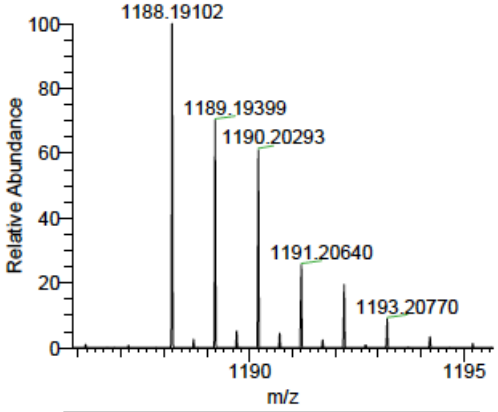




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1188.22578

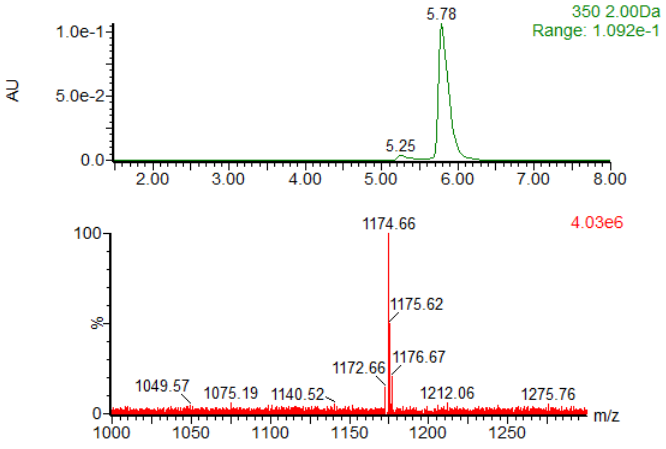
OO) V6M-T8V1

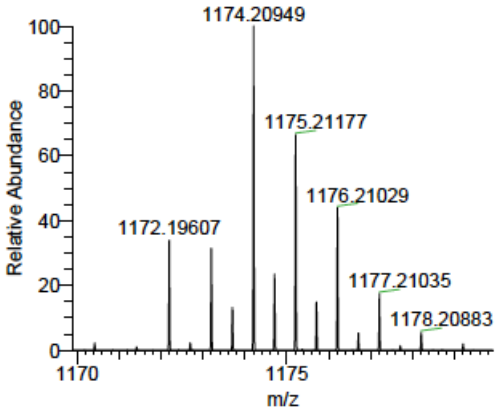




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1188.18939

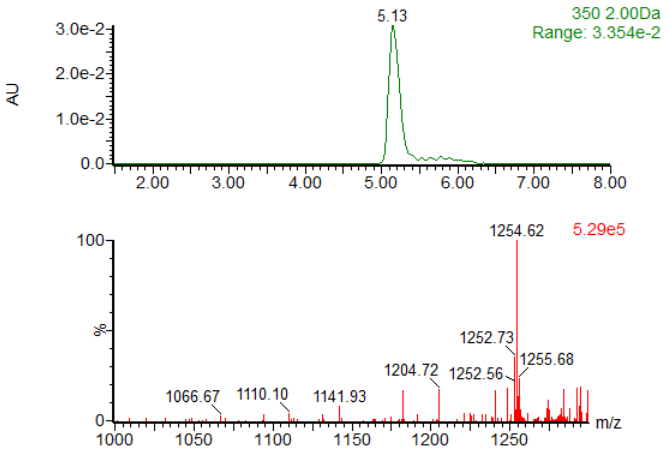
PP) V6M-T8V2

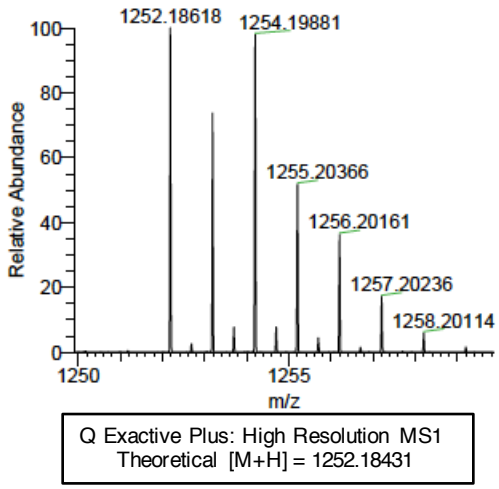




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1172.19448

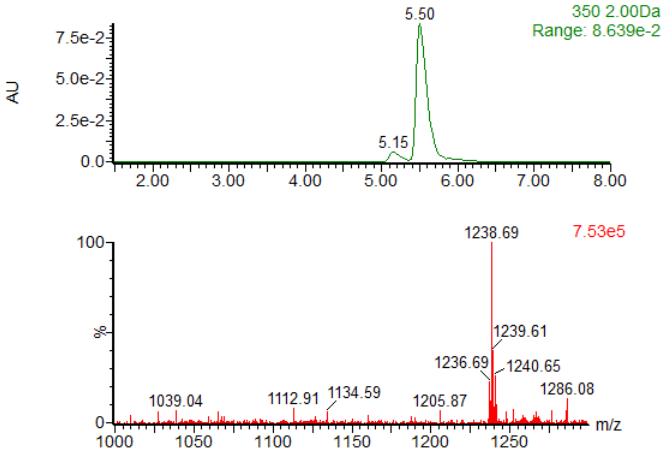
QQ) V6M-T8Y1

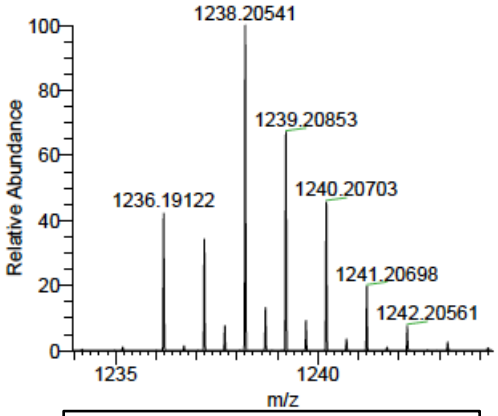




Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1252.18431

RR) V6M-T8Y2





Q Exactive Plus: High Resolution MS1
Theoretical [M+H] = 1236.18939

Figure S3: **A-RR)** Left panel: LC (green) and MS (red) data for each analog in this study. Right panel: High Resolution MS1 data and theoretical [M+H].

VIII. NMR structural characterizaion

All NMR experiments were performed on a Bruker Avance 800 MHz equipped with a cryoprobe at the UCSF NMR Facility. All data was collected at 298K. WT thiocillin YM-266183 variant, T4S1(Dha), and T8Y1 were dissolved to approximately 2-10 mg/mL in DMSO-d_6 (Cambridge Isotope Laboratories) and placed in 5 mm DMSO-matched SHIGEMI NMR tubes (Catalog #: DMS-005TB, SHIGEMI Co., Tokyo, Japan) or 5 mm Wilmad Thin Wall Precision NMR sample tubes (Catalog #: 535-PP-8, Wilmad LabGlass, Vineland, NJ). 1D ^{1}H NMR, 2D ^{1}H-^{13}C HSQC, 2D ^{1}H-^{13}C HMBC, and 2D ^{1}H-^{1}H ROESY spectra were taken for these samples. Chemical shift assignments are shown below (**Table S4, Figure 16,** and **Figure 21**). {^{1}H-^{15}N} HSQC was taken on an [^{15}N]-labelled WT sample for ^{1}H and ^{15}N assignments. For 9 additional analogs, 1D ^{1}H NMR and 2D TOCSY spectra were taken including an additional COSY spectra for V6A2 (**Figures S22-S40**).

Further 3D structural characterization was performed on WT thiocillin. 3D HNHA experiment was used to determine the homonuclear three-bond $^{3}J_{HNH_\alpha}$ coupling constants (**Figure S12**). The coupling constants for Thr-3, Val-6, and Thr-8 were used as phi angle restraints in BRIKARD modelling. **Table S5** shows the phi angles and the percent occupancy from the top 5 NMR models. **Figure S4a** shows the ensemble of the top 5 NMR models and **Figure S4b** shows the most predominant conformation, WT_1.

Residue	Group	δ ^{1}H (ppm)	δ ^{13}C (ppm)	δ ^{15}N (ppm)
Thr 3	NH	7.98		107.65
	CH (α)	3.09	47.40	
	CH (β)	3.71	65.49	
	CH_3 (γ)	1.03	21.68	
Dhb 4	NH	9.59		117.34
	C (α)		131.07	
	CH (β)	6.51	128.41	
	CH_3 (γ)	1.70	14.04	
Val 6 (β-OH)	NH	8.35		115.84
	CH (α)	5.48	57.87	
	C (β)		71.77	
	CH_3 (γ1)	1.23	27.94	
	CH_3 (γ2)	1.25	26.43	
Thr 8	NH	8.42		116.73
	CH (α)	5.06	57.22	
	CH (β)	3.96	67.55	
	CH_3 (γ)	1.02	21.45	
Dhb 13	NH	9.65		116.67
	C (α)		130.69	
	CH (β)	6.59	129.63	
	CH_3 (γ)	1.73	14.04	
Thr 14	NH	8.37		103.73
(aminoacetone)	CH_2 (α)	3.88	50.05	
	CO (β)		205.59	
	CH_3 (γ)	2.09	27.55	

Table S4: Chemical shift assignments for WT thiocillin YM-266183.

Model	Thr 3	Val 6	Thr 8	Occupancy
$^{3}J_{HNHA}$	7.0	9.2	8.6	
$^{3}J_{HNHA}$ Torsion	±142	±165	±157	
WT_1	158.4	-129.1	176.8	37%
WT_2	148.4	-134.1	138.6	16%
WT_3	63.6	-141.6	53.2	16%
WT_4	163.2	-72.3	65.3	9%
WT_5	161.1	-29.0	163.9	7%

Table S5: Experimentally determined NMR $^{3}J_{HNHA}$ coupling constants (Hz) and the corresponding torsion angles (°) calculated from the Karplus equation. Three macrocycle phi angles (°) and percent occupancy are listed for the top five computationally constrained models of thiocillin.

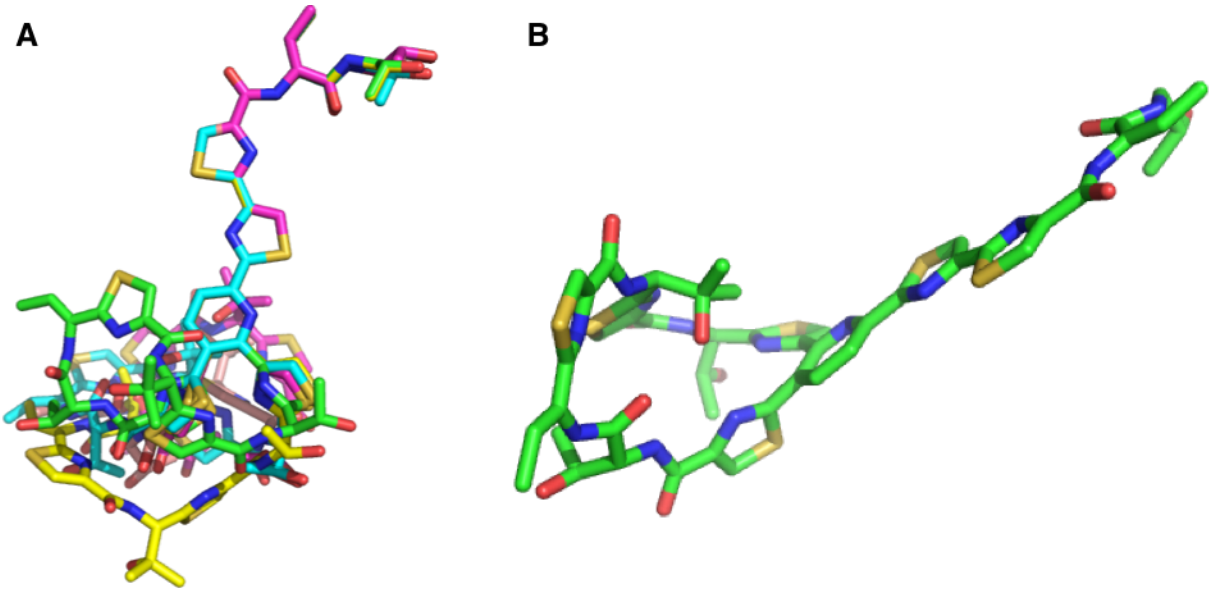


Figure S4: 3D structure of WT thiocillin **A)** Ensemble of conformations as modeled by BRIKARD with NMR restraints. **B)** Structure of WT_1, the predominant 3D NMR solution structure (37% occupancy).

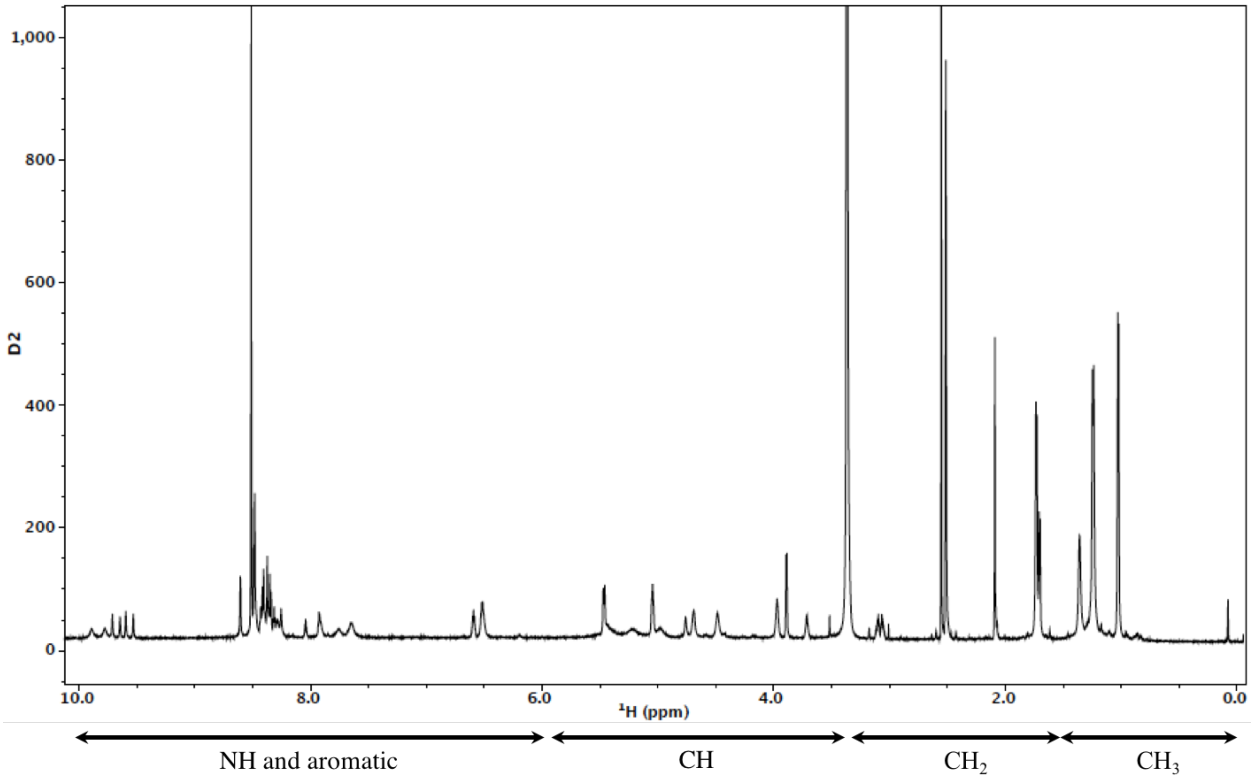


Figure S5: 1D ^{1}H NMR spectrum of [^{15}N] WT Thiocillin YM-266183.

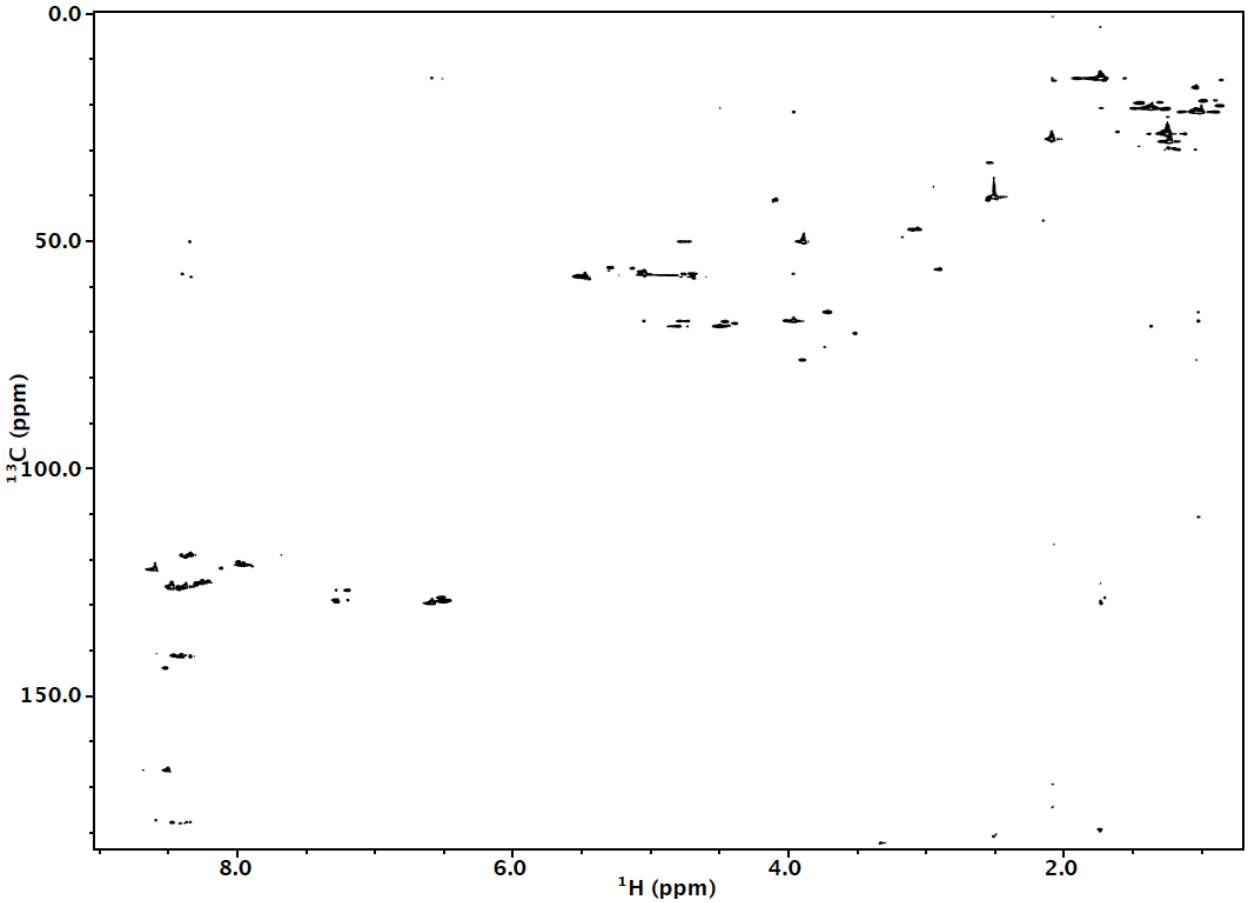


Figure S6: 2D ^{1}H-^{13}C HSQC of WT Thiocillin YM-266183.

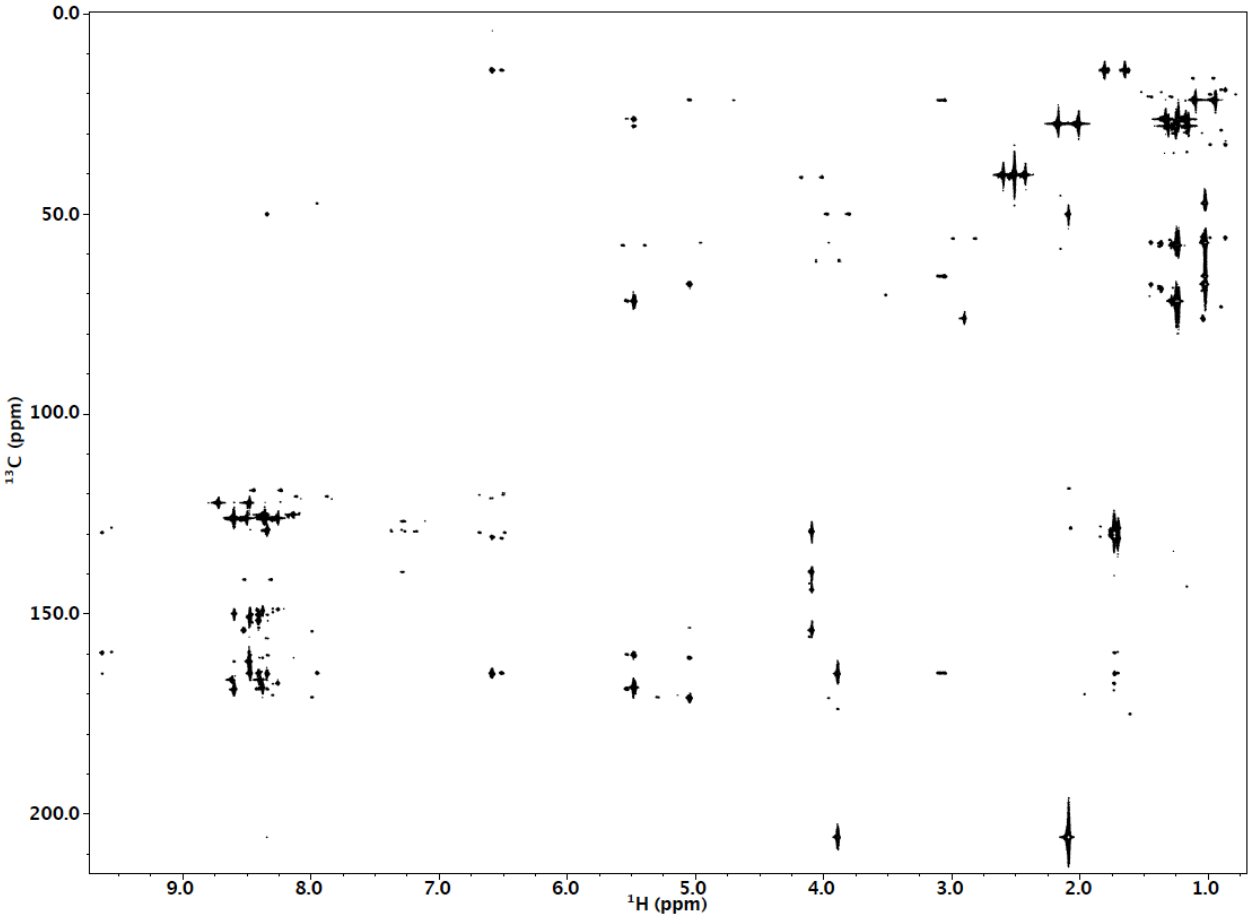


Figure S7: 2D ^{1}H-^{13}C HMBC of WT Thiocillin YM-266183.

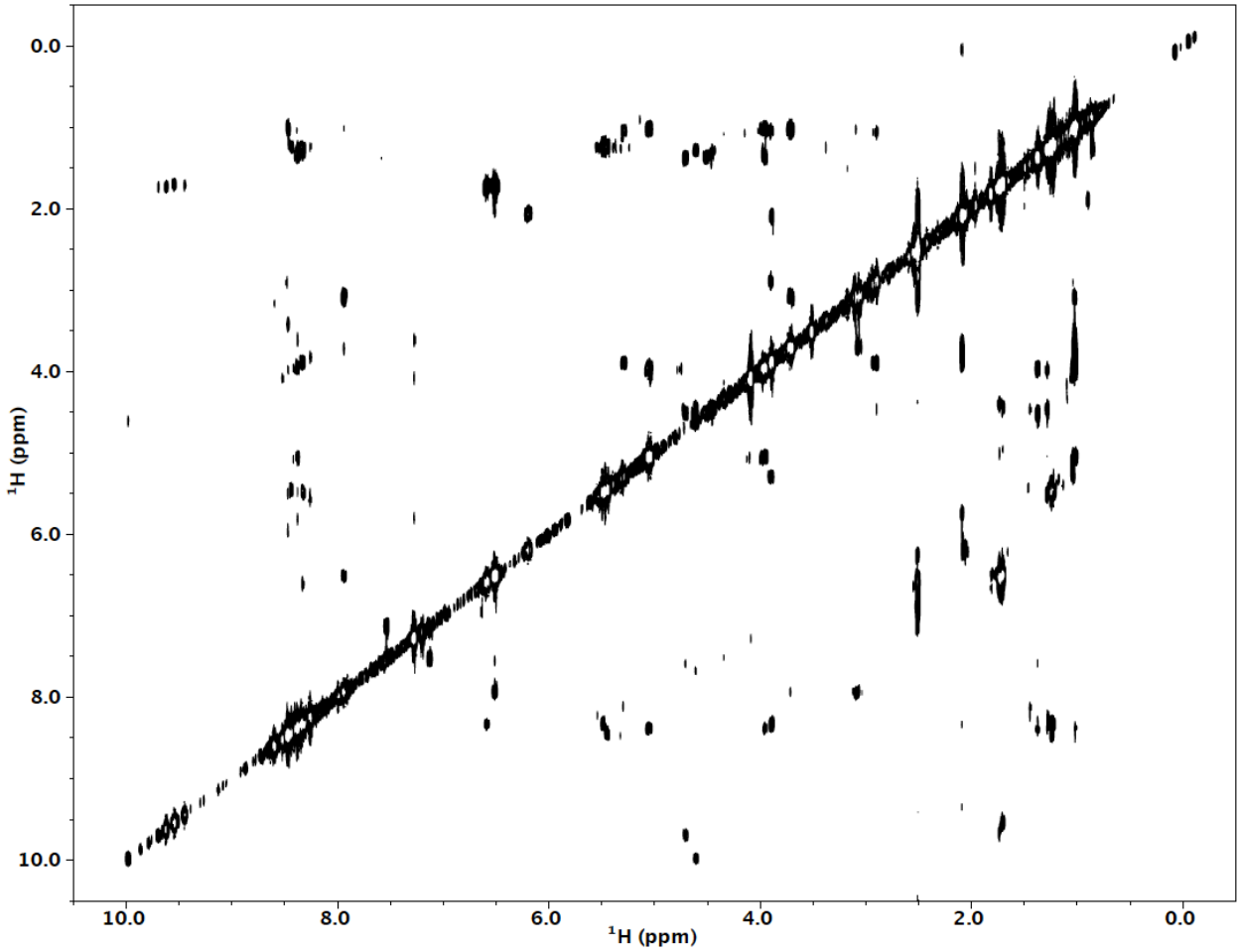


Figure S8: 2D ^{1}H-^{1}H ROESY spectra of WT Thiocillin YM-266183 with a 300ms mixing time.

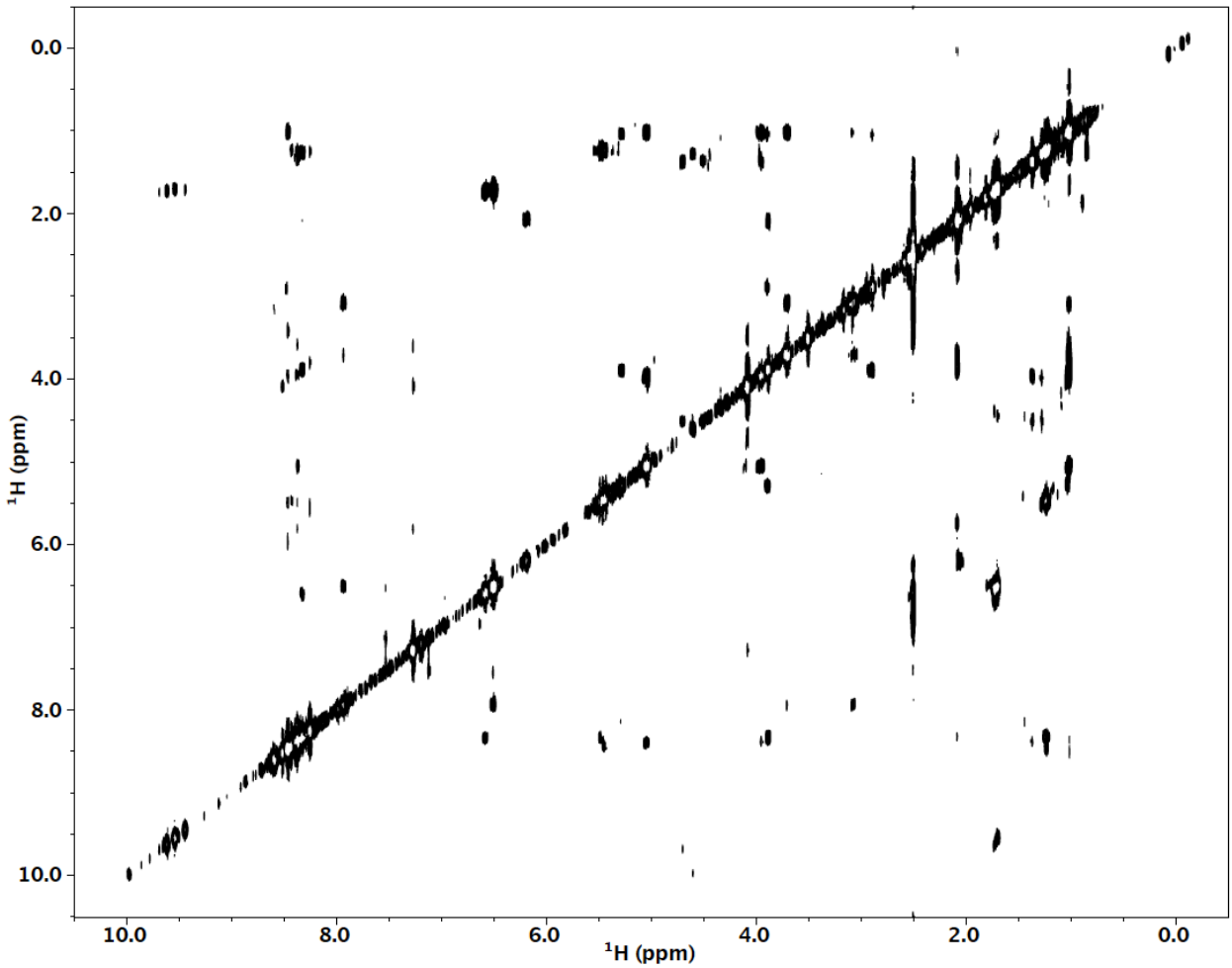


Figure S9: 2D ^{1}H-^{1}H ROESY spectra of WT Thiocillin YM-266183 with a 500ms mixing time.

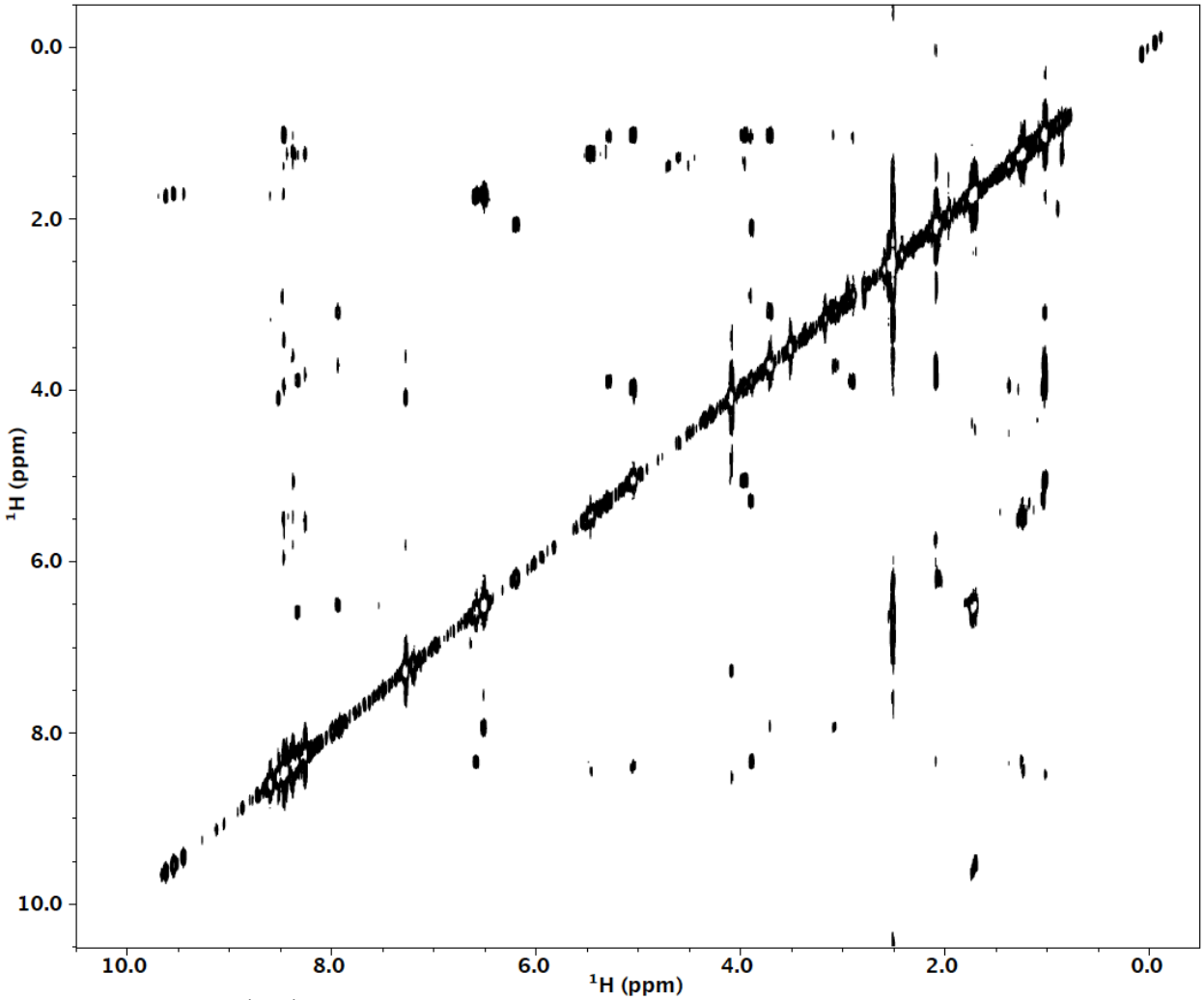


Figure S10: 2D ^{1}H-^{1}H ROESY spectra of WT Thiocillin YM-266183 with an 800ms mixing time.

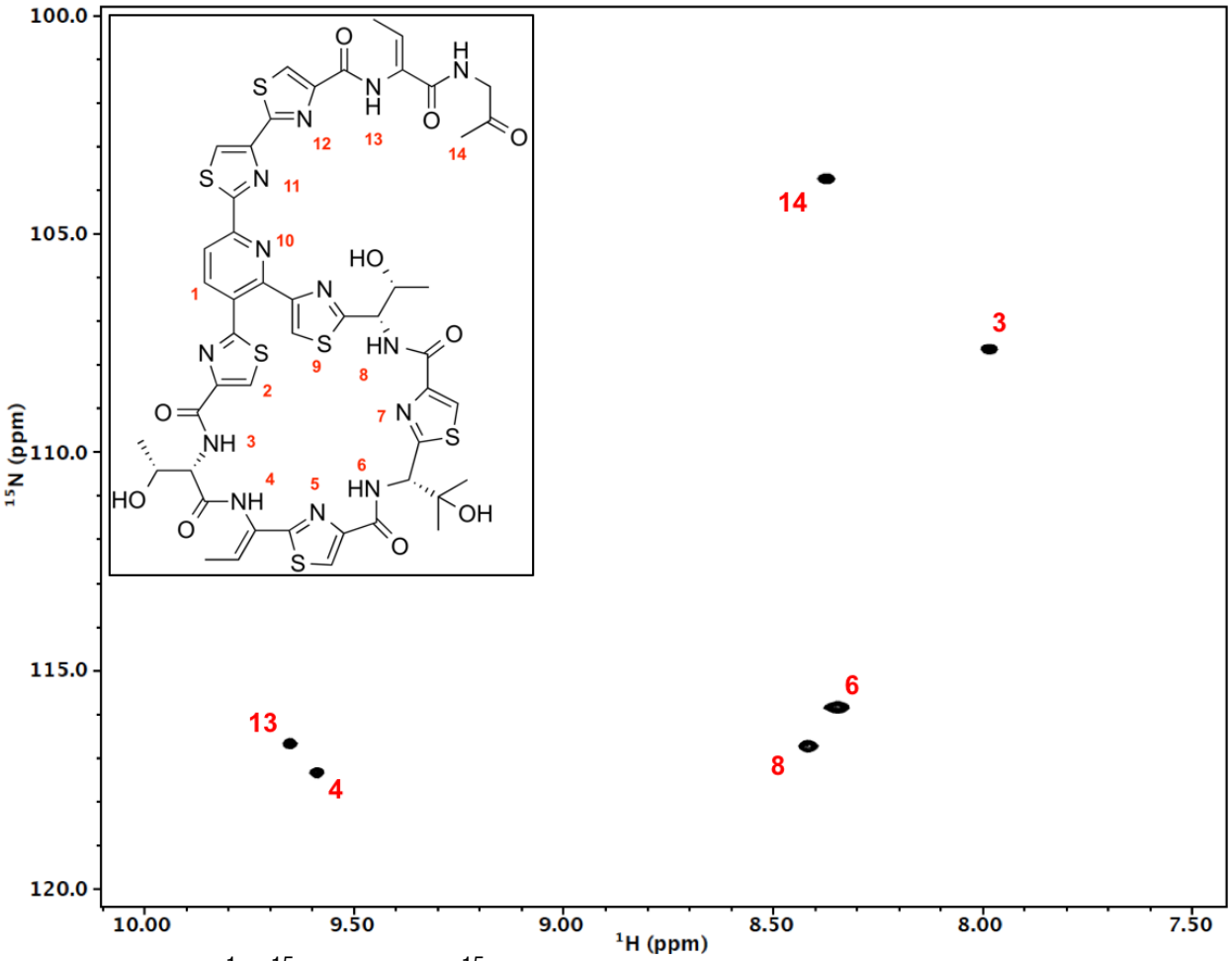


Figure S11: 2D ^{1}H-^{15}N HSQC of ^{15}N WT Thiocillin YM-266183.

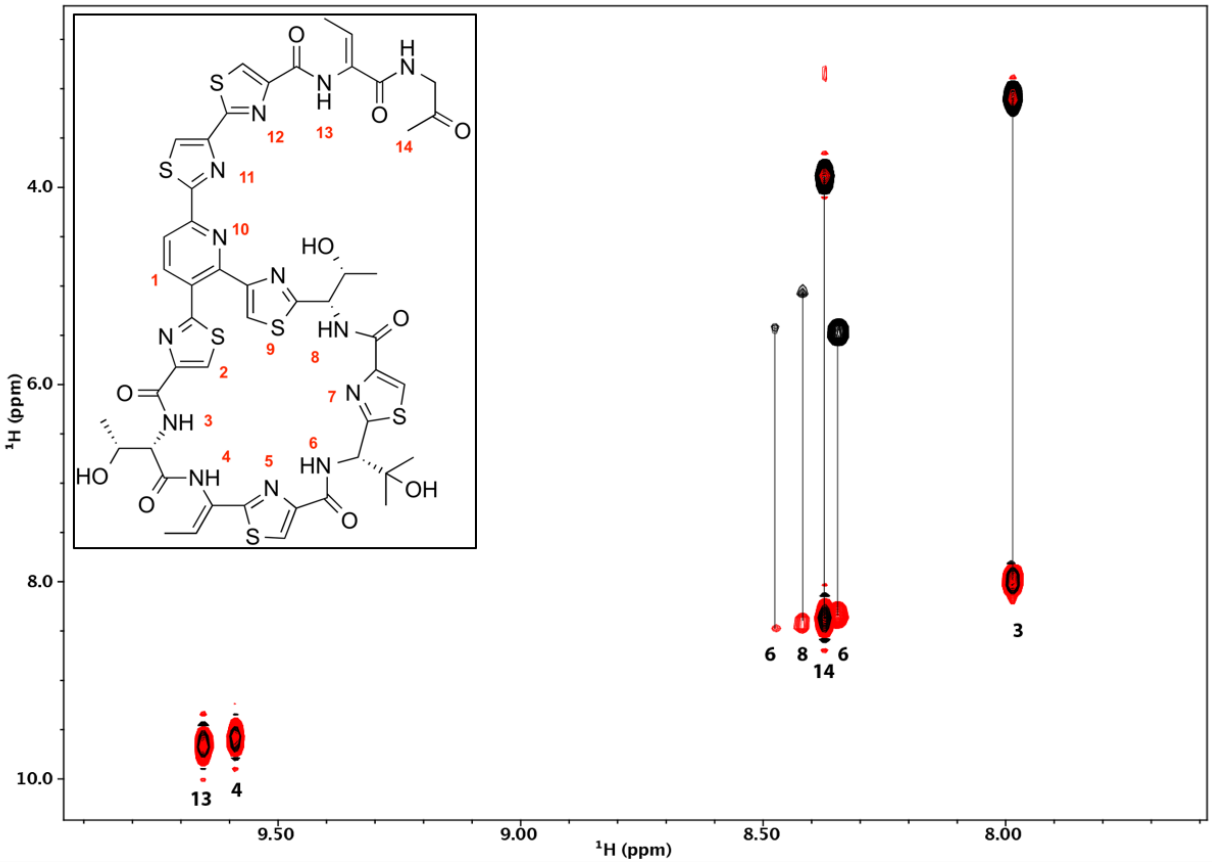


Figure S12: 3D HNHA of [^{15}N] WT Thiocillin YM-266183 shown in 2D collapsed along the ^{15}N-axis.

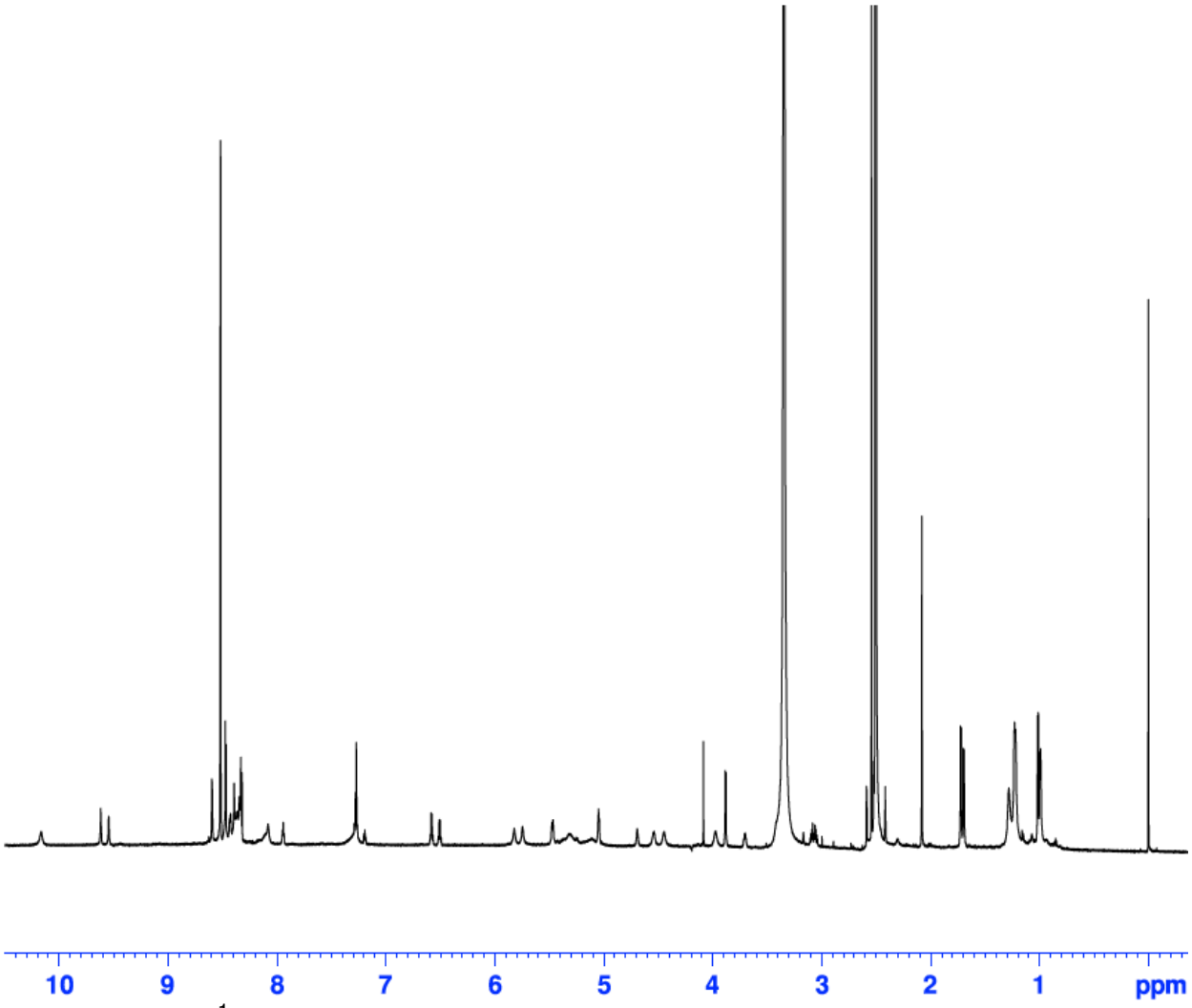


Figure S13: 1D ^{1}H NMR of T4S1.

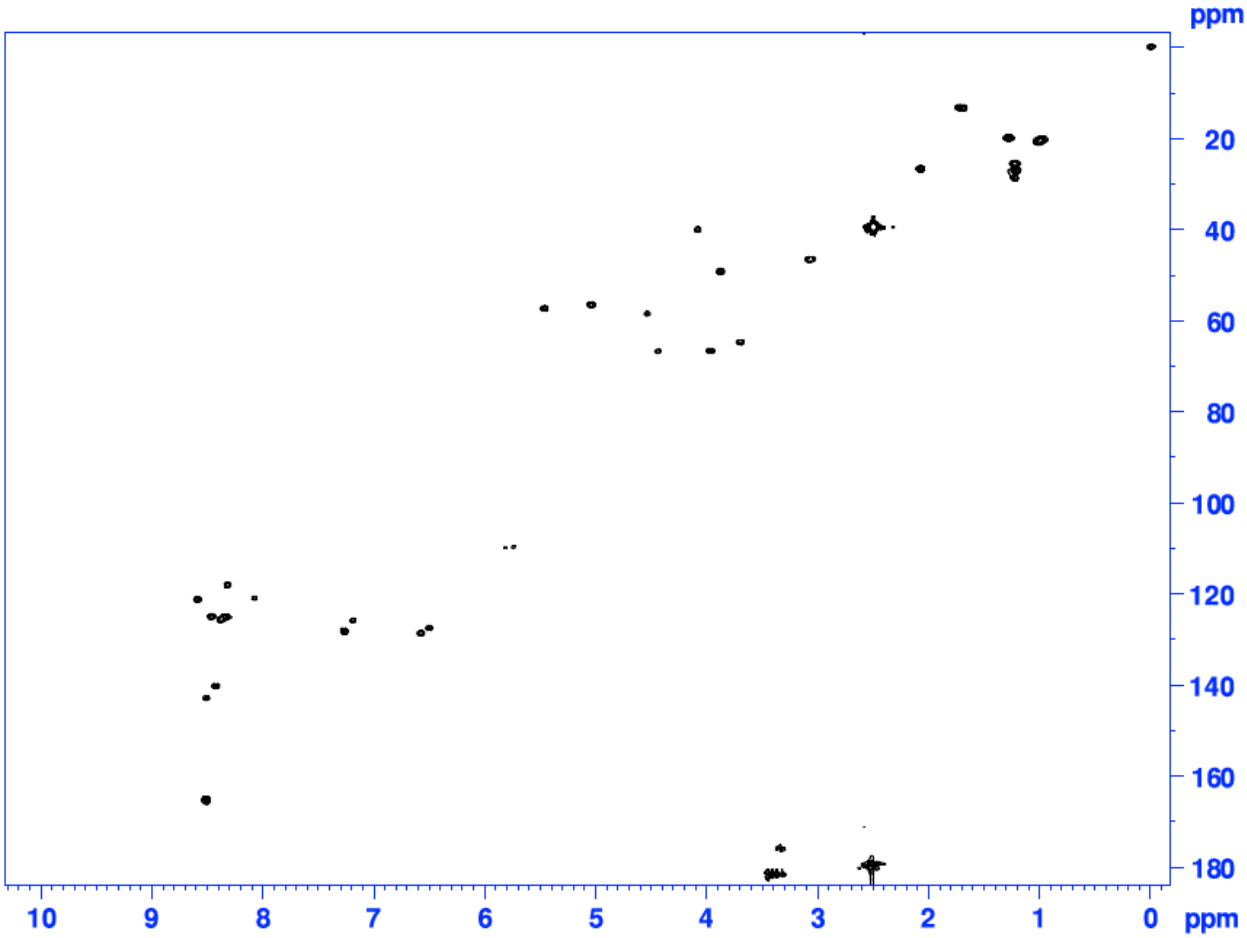


Figure S14: 2D ^{1}H-^{13}C HSQC of T4S1.

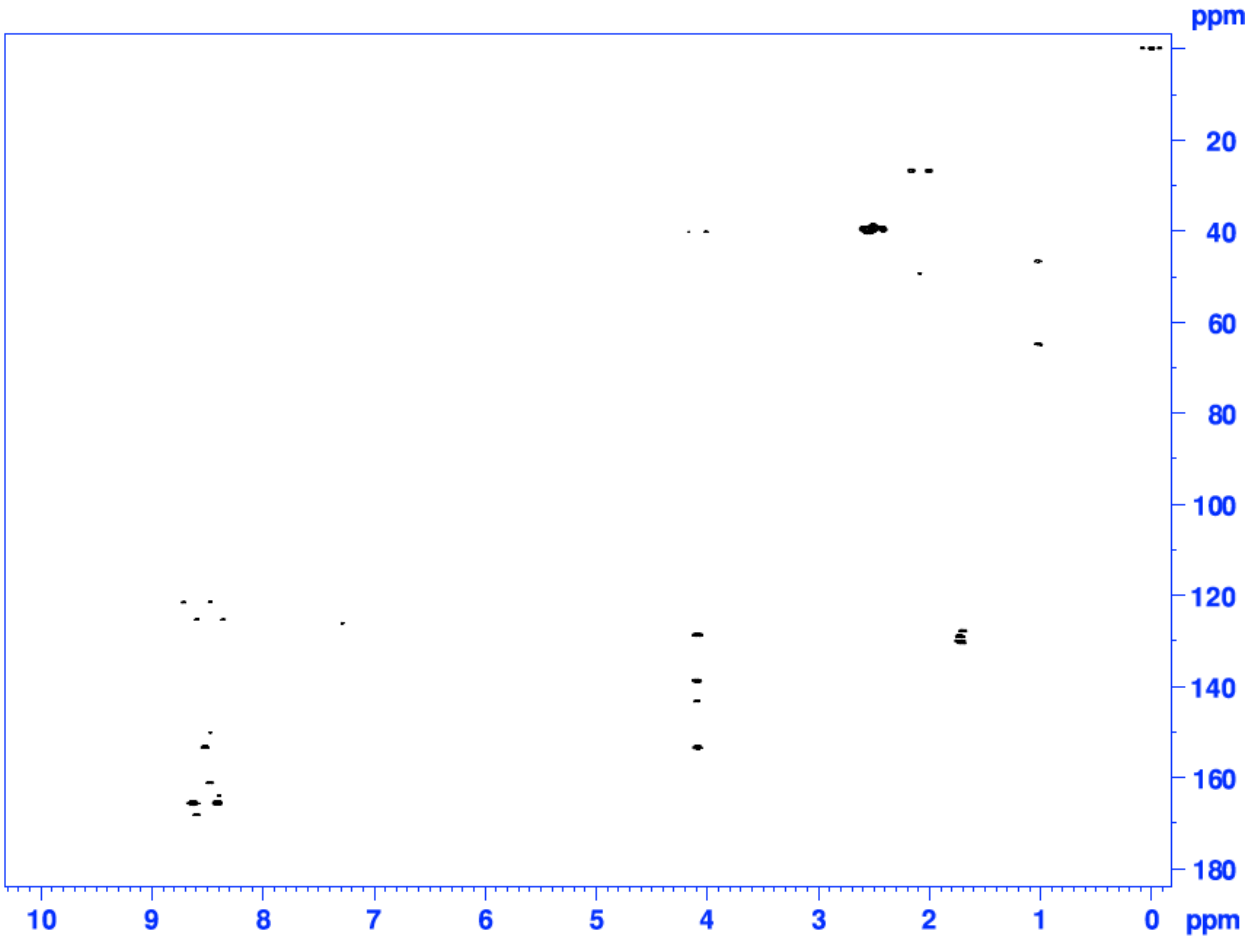


Figure S15: 2D ^{1}H-^{13}C HMBC of T4S1.

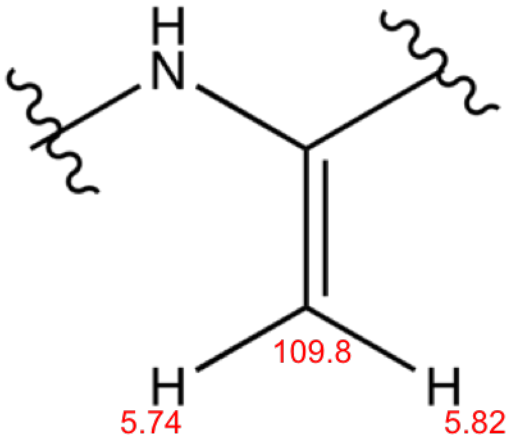


Figure S16: Observed ^{1}H and ^{13}C chemical shifts for the dehydroalanine at residue 4.

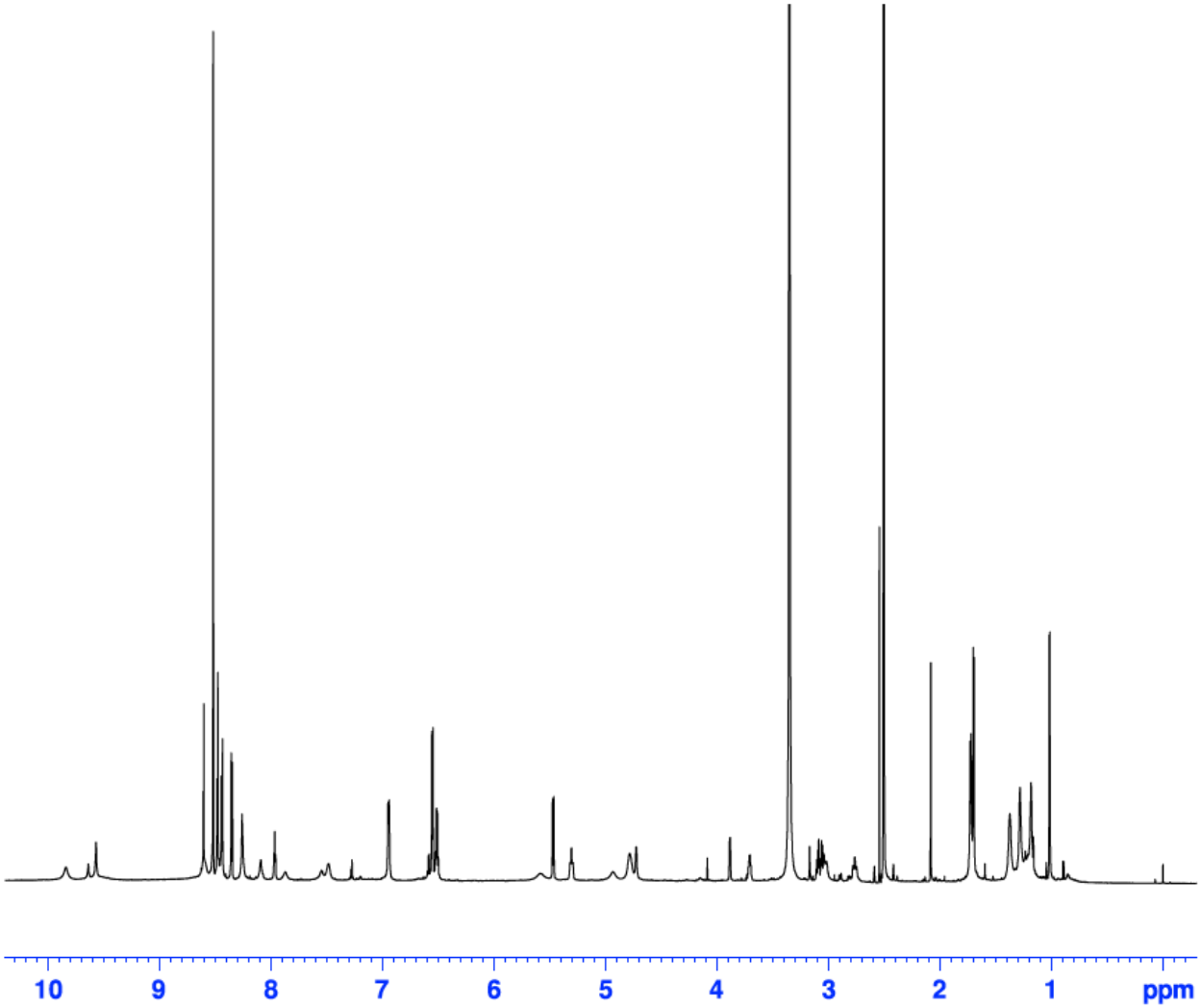


Figure S17: 1D ^{1}H NMR of T8Y1.

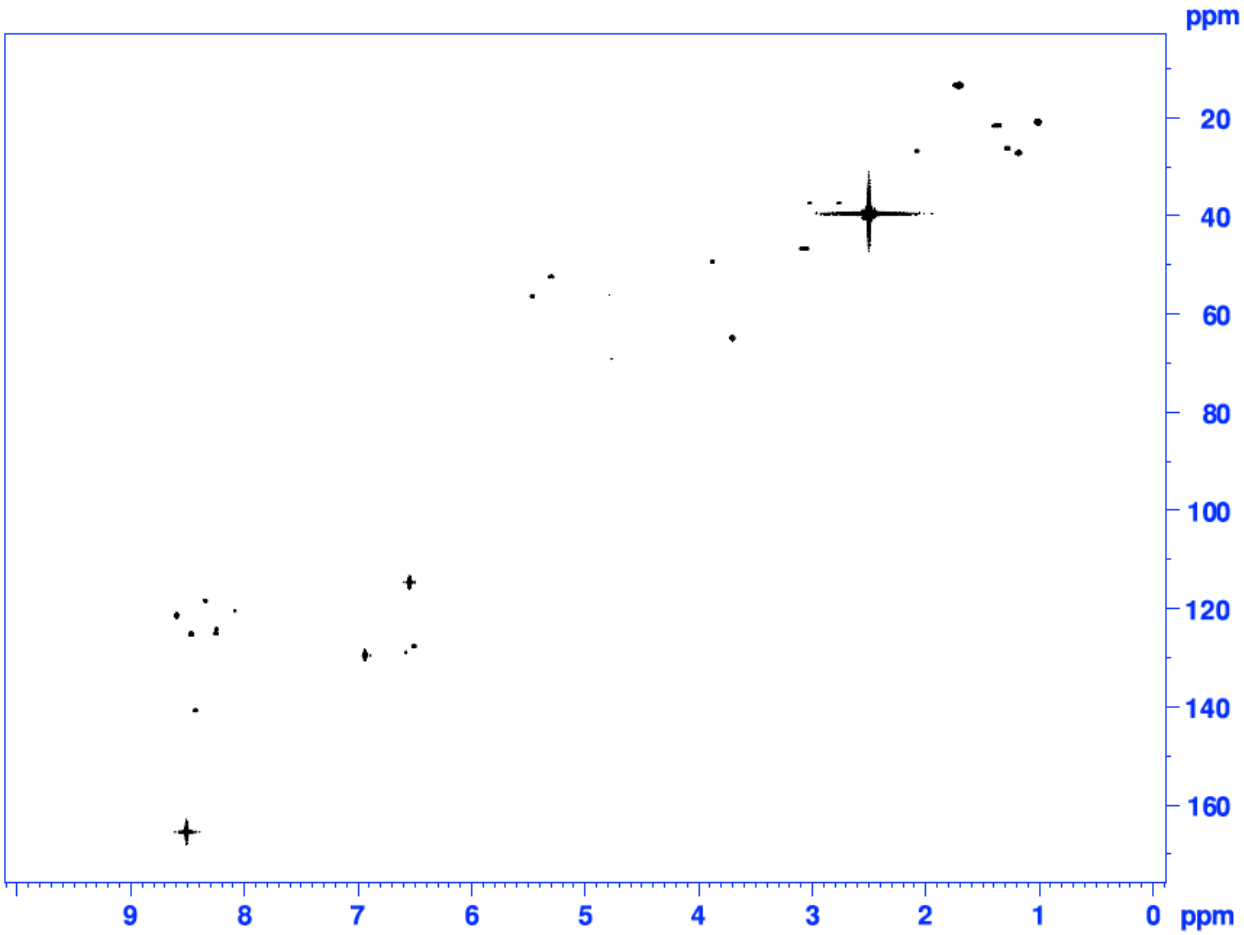


Figure S18: 2D ^{1}H-^{13}C HSQC of T8Y1.

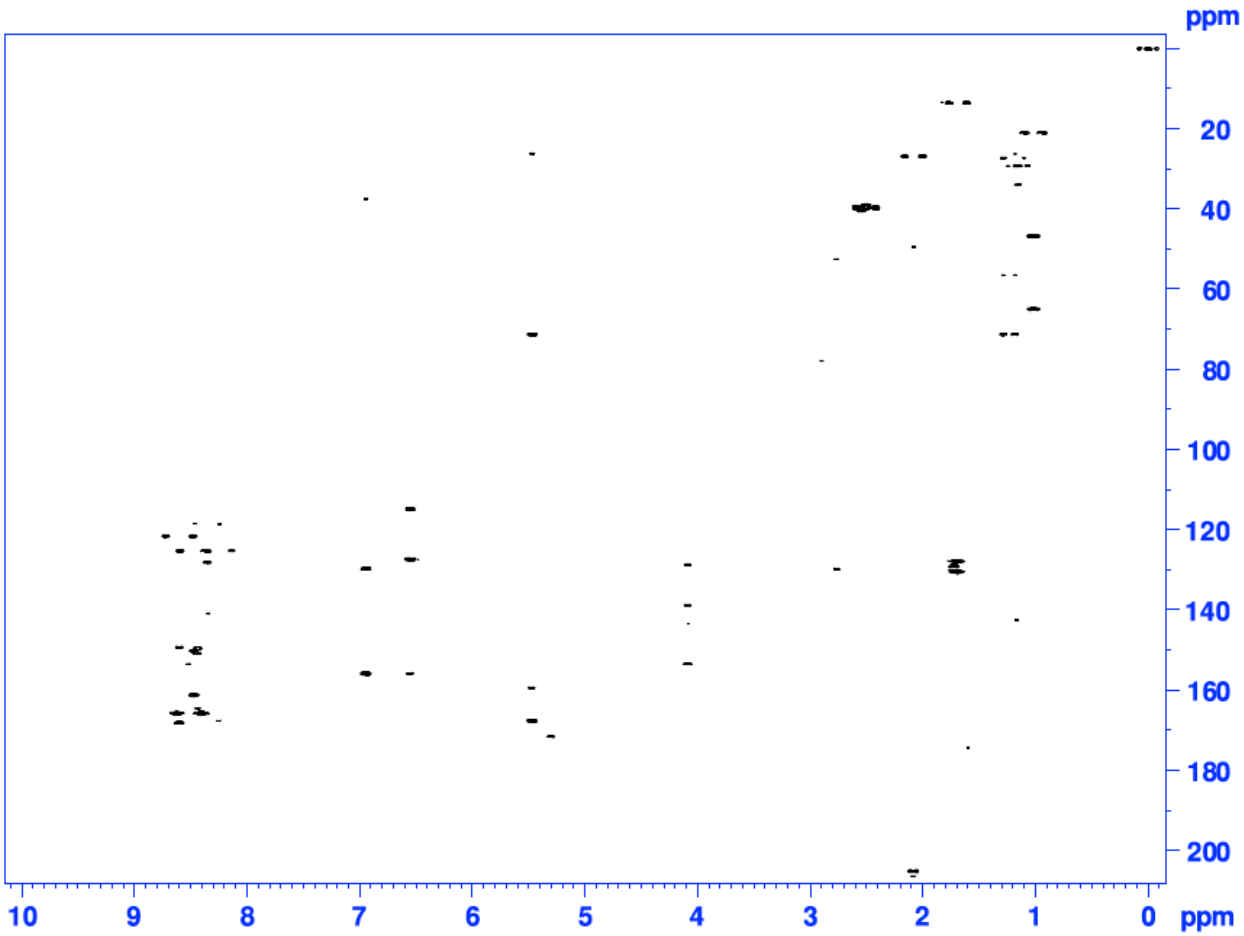


Figure S19: 2D ^{1}H-^{13}C HMBC of T8Y1.

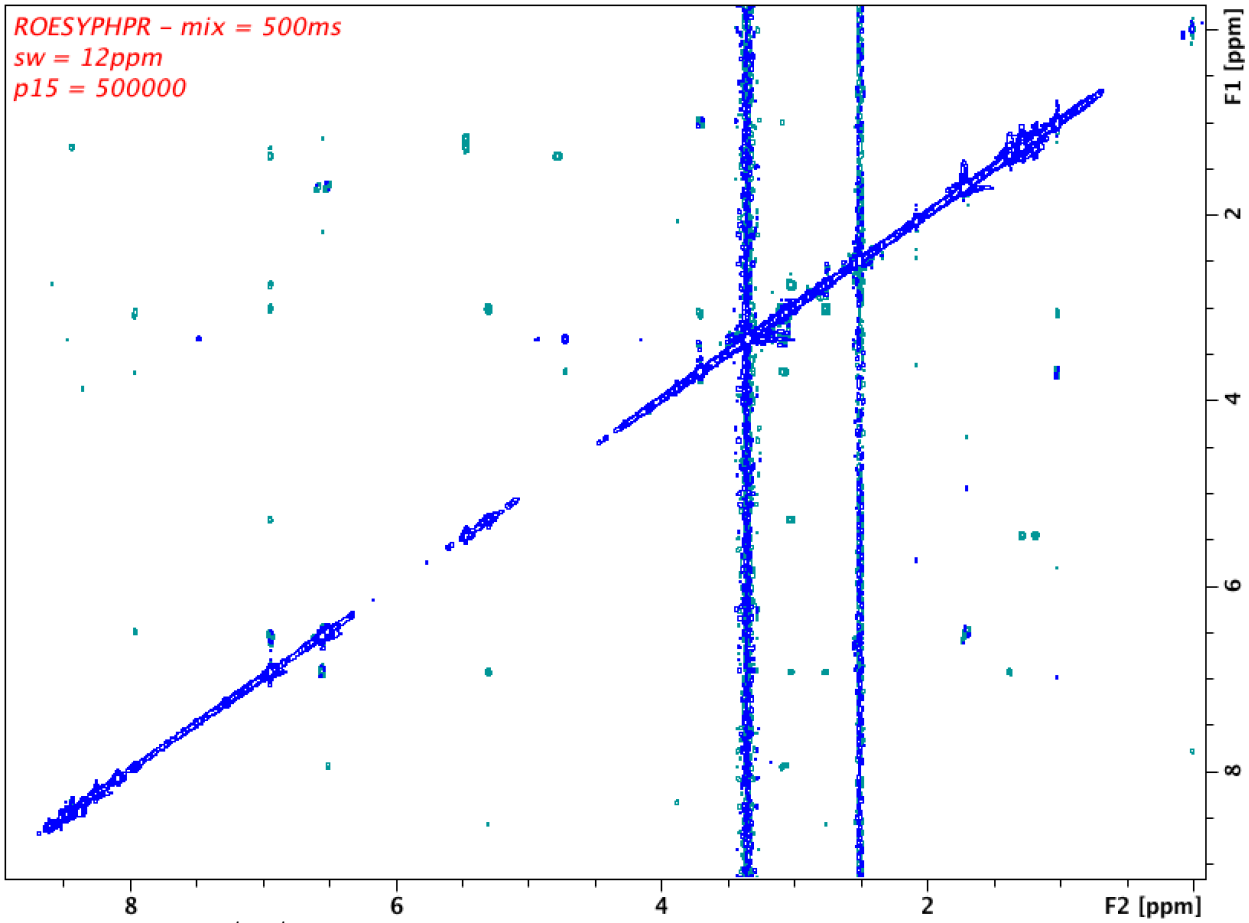


Figure S20: 2D ^{1}H-^{1}H ROESY spectra of T8Y1 with a 500ms mixing time.

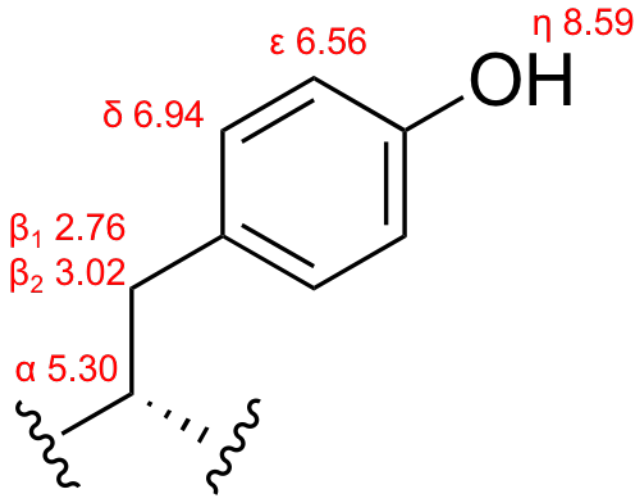


Figure S21: Proton chemical shift assignments for tyrosine residue in T8Y1.

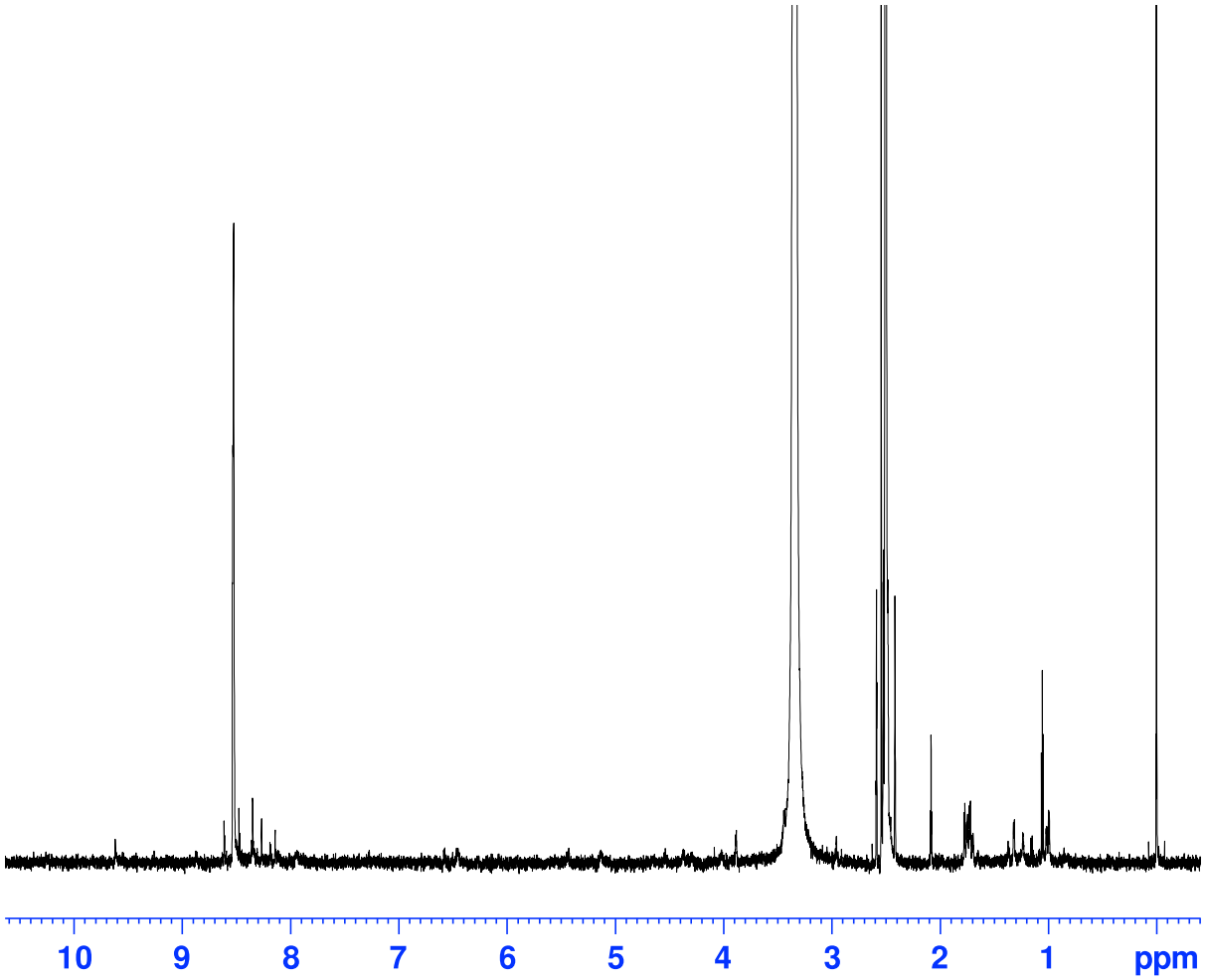


Figure S22: 1D ^{1}H NMR of V6A1.

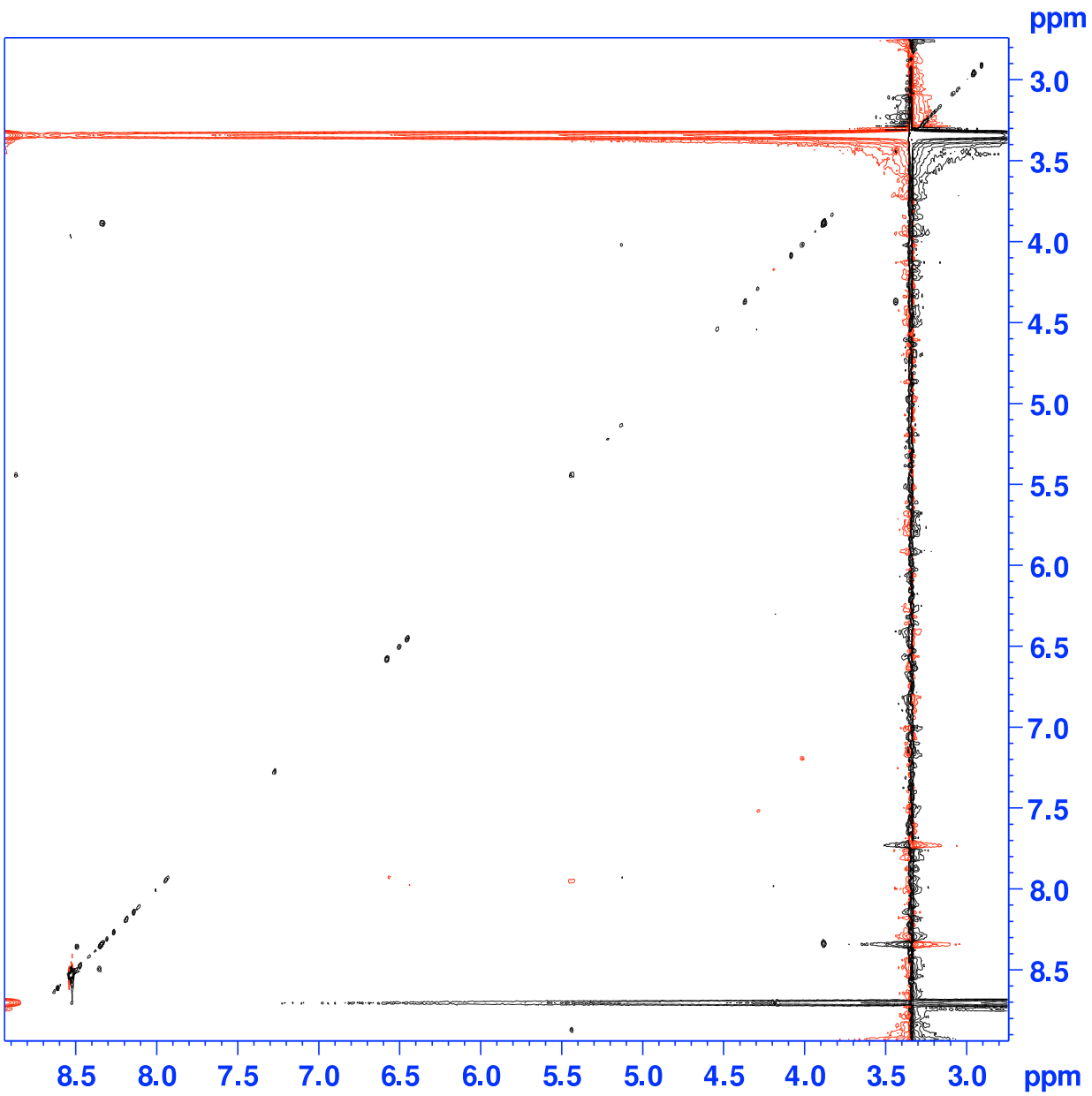


Figure S23: ^{1}H-^{1}H TOCSY spectra of V6A1. No chemical shift assignments were made due to low sample concentration.

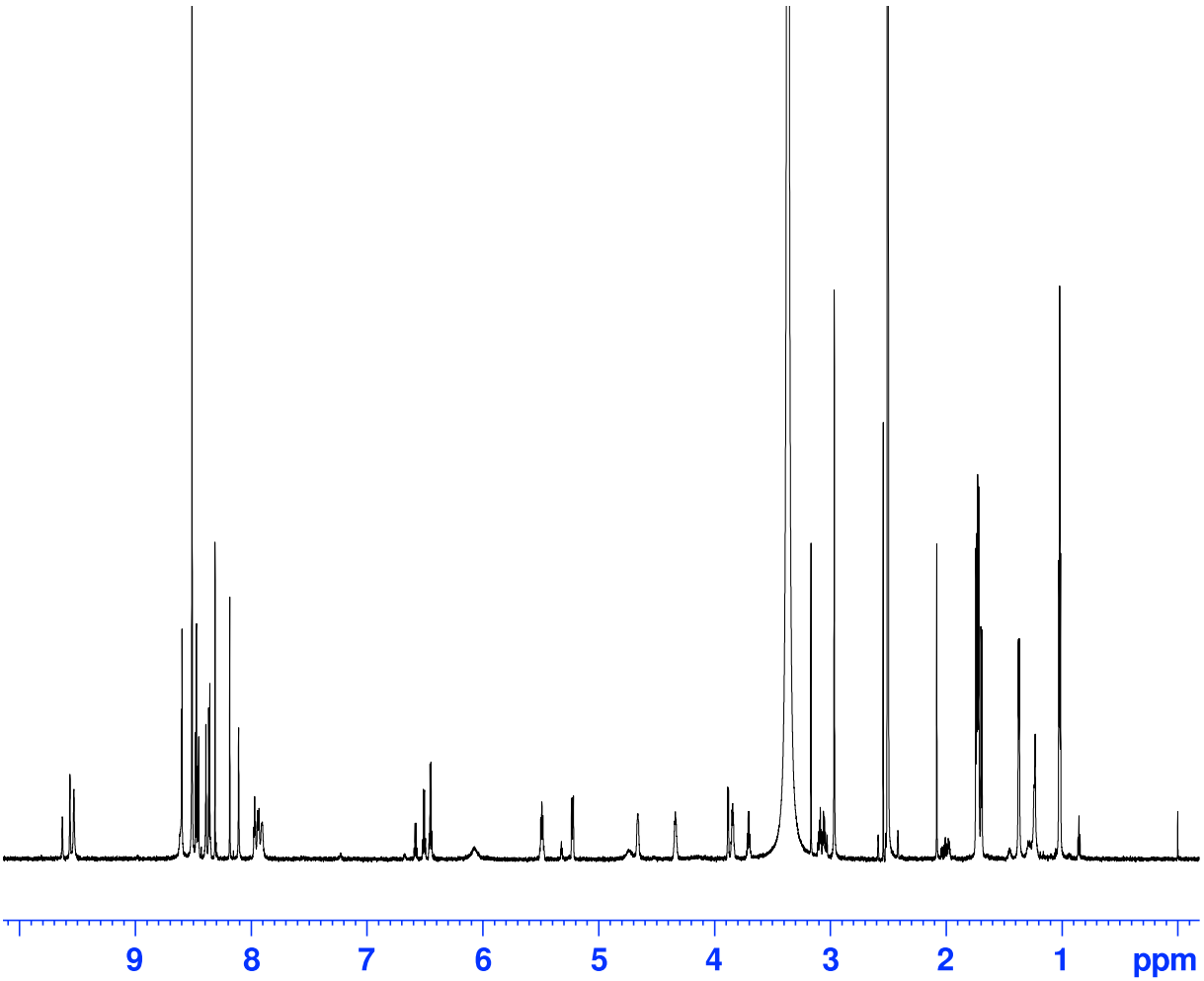


Figure S24: 1D ^{1}H NMR of V6A2.

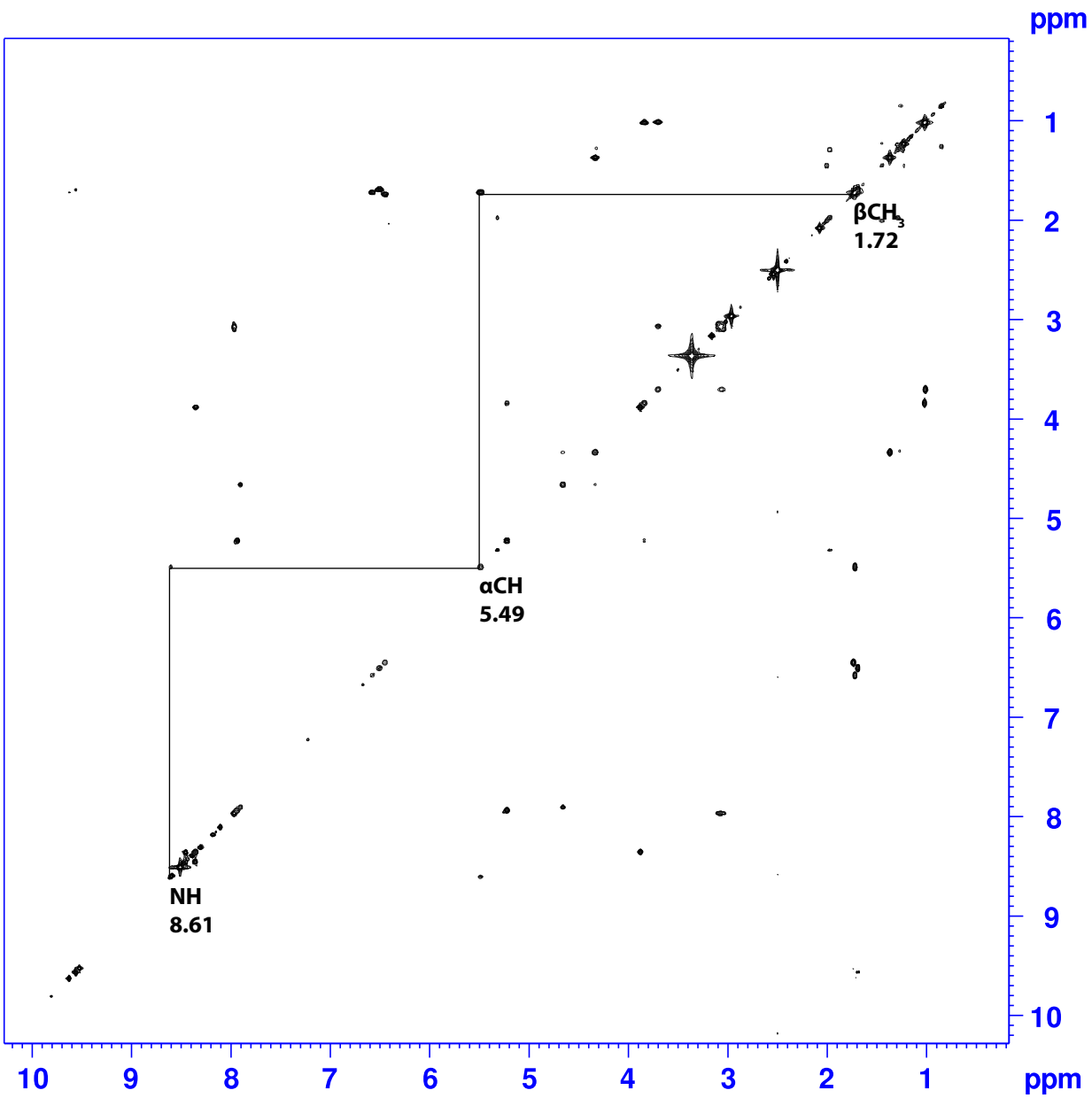


Figure S25: 2D ^{1}H-^{1}H COSY spectra of V6A2. Proton chemical shift assignments for alanine at residue 6 are shown.

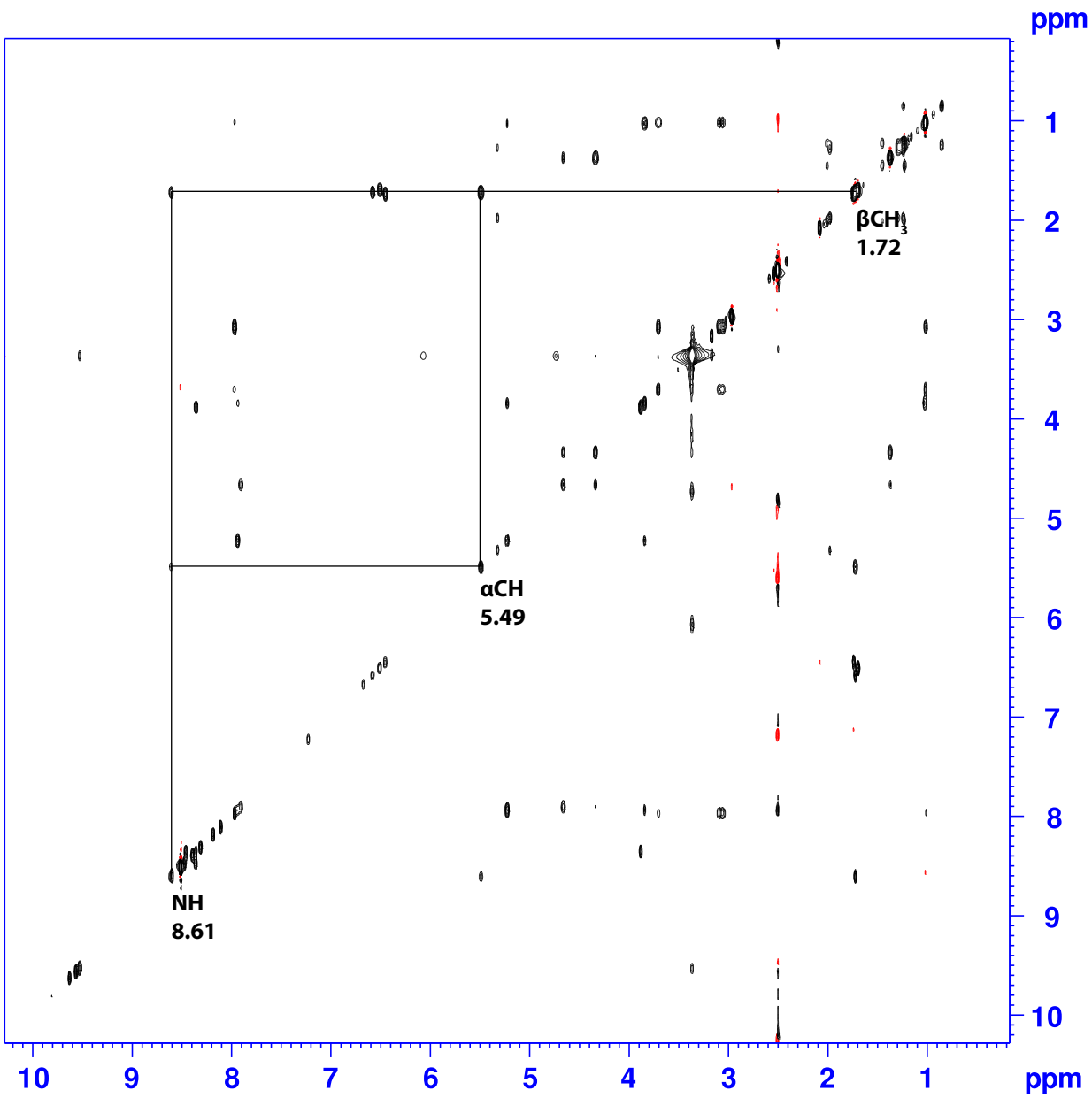


Figure S26: 2D ^{1}H-^{1}H TOCSY spectra of V6A2. Proton chemical shift assignments for alanine at residue 6 are shown.

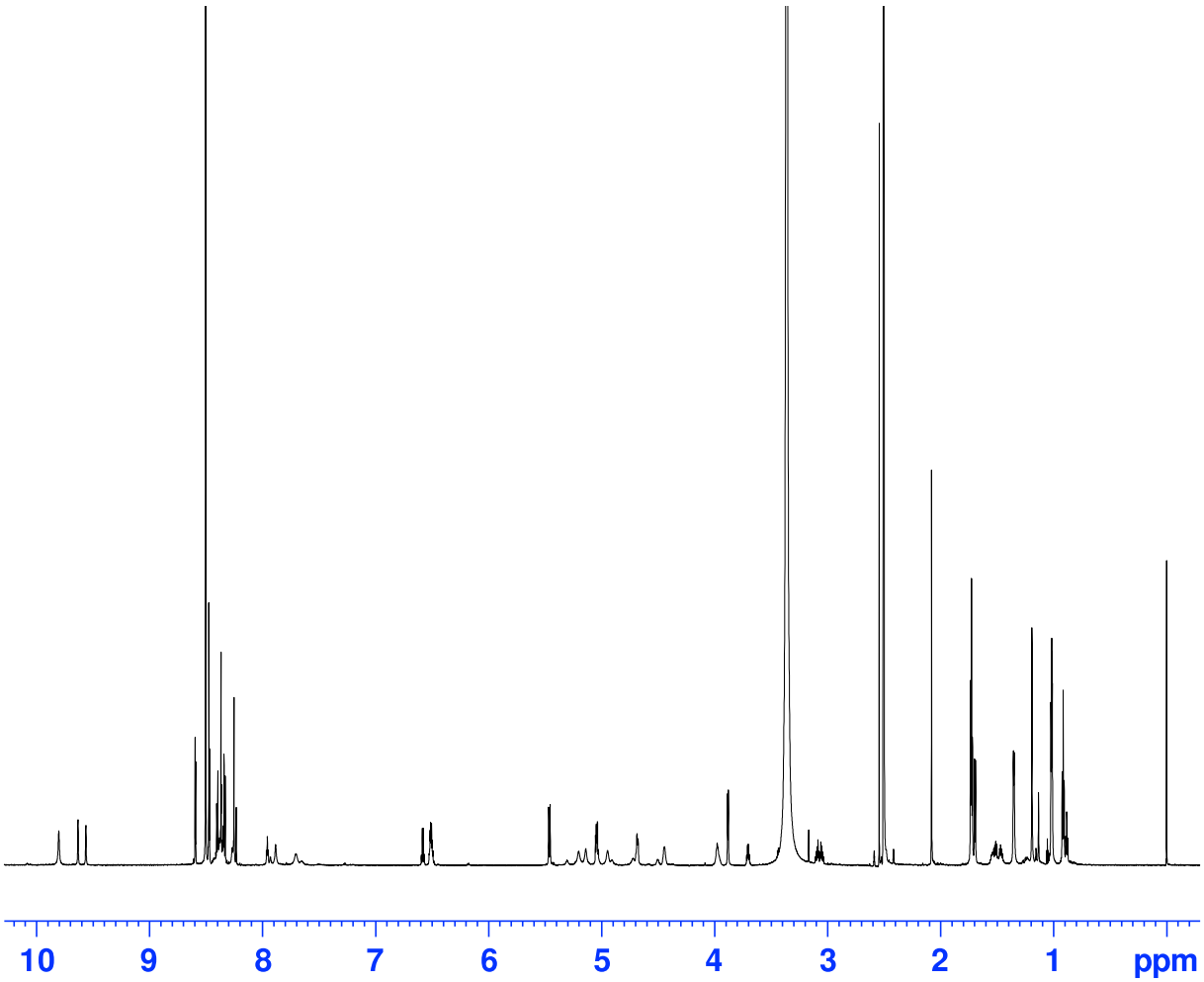


Figure S27: 1D ^{1}H NMR of V6I1.

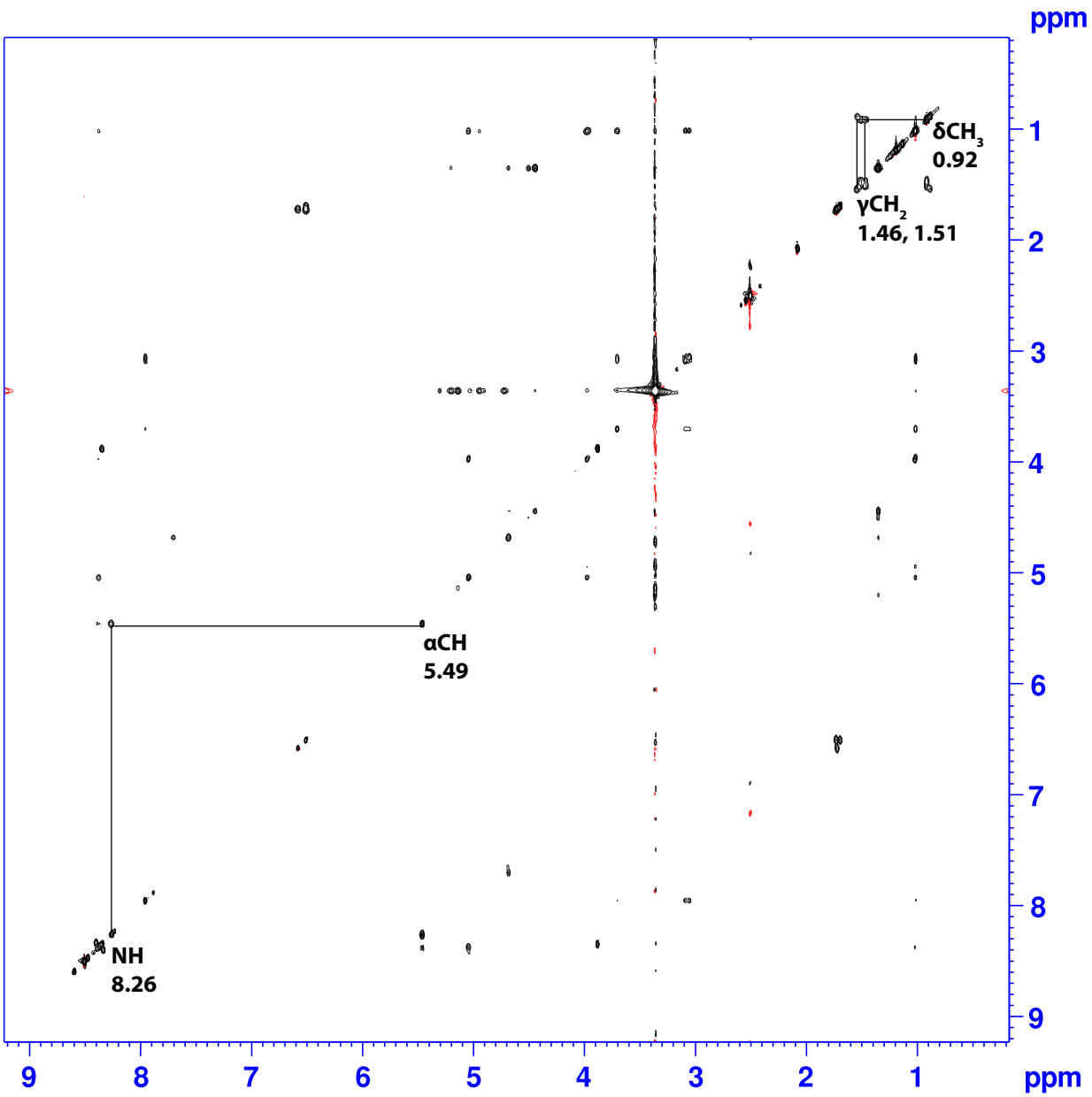


Figure S28: 2D ^{1}H-^{1}H TOCSY spectra of V6I1. Proton chemical shift assignments for β-hydroxy-isoleucine at residue 6 are shown.

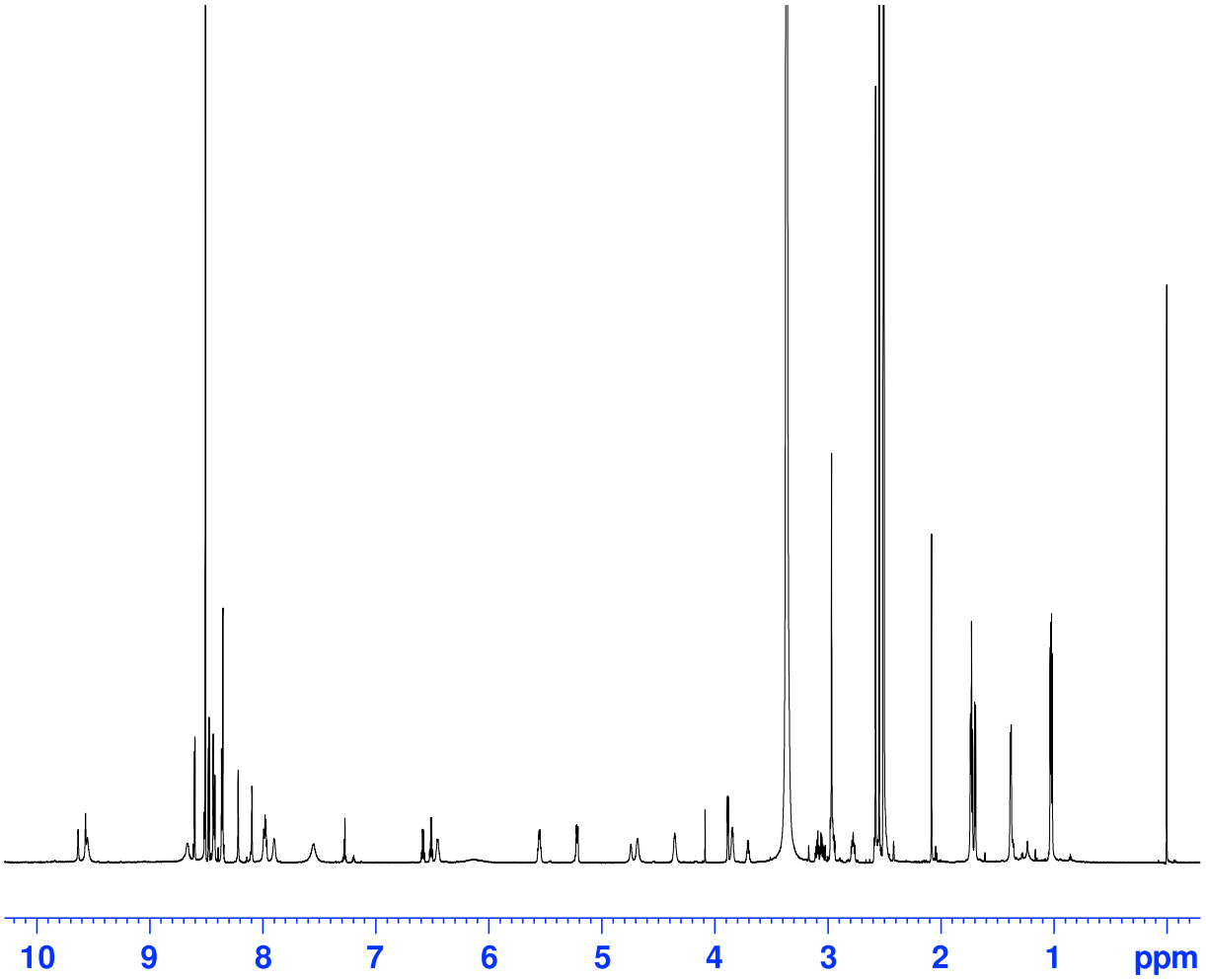


Figure S29: 1D ^{1}H NMR of V6M1.

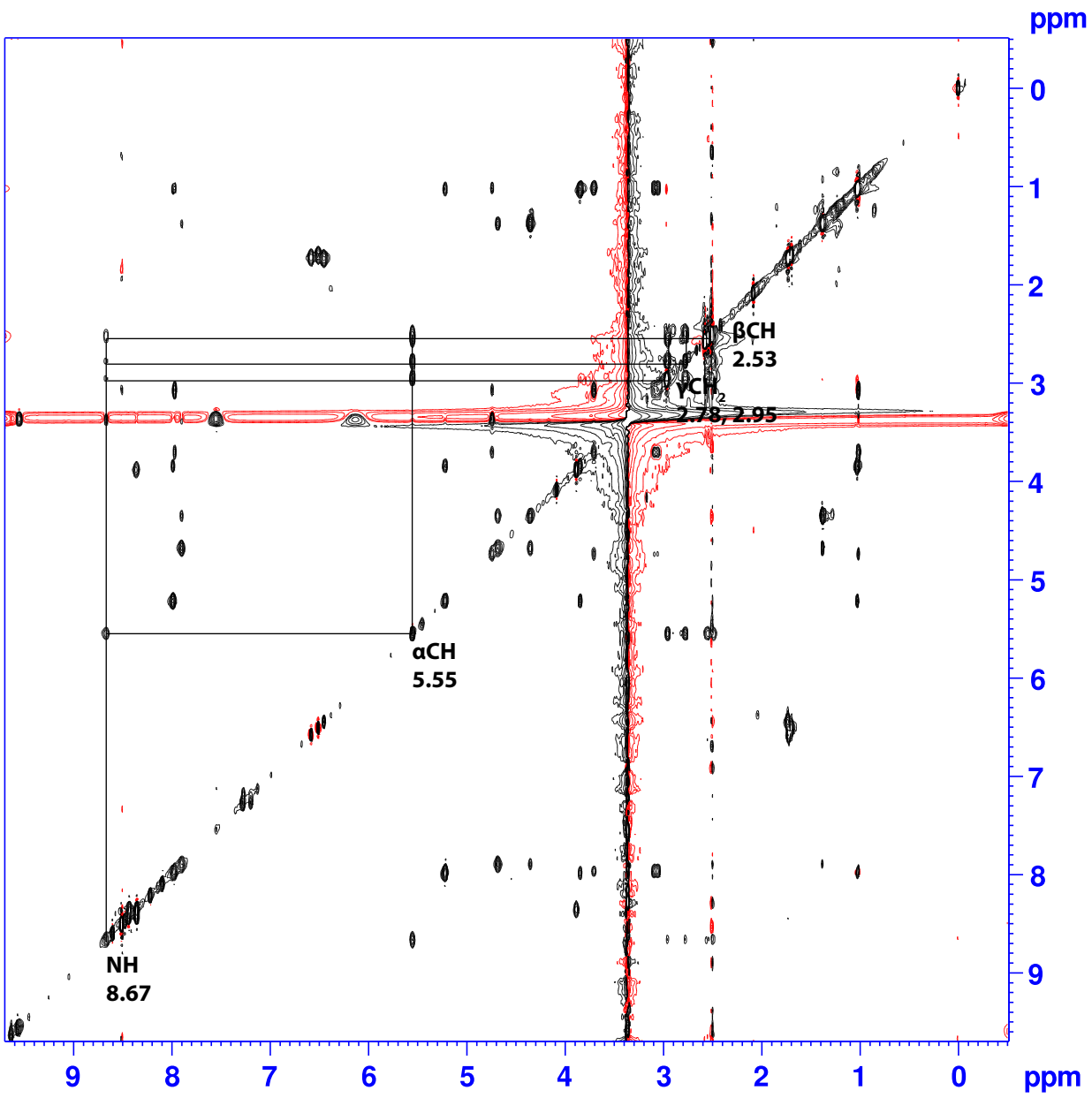


Figure S30: 2D ^{1}H-^{1}H TOCSY spectra of V6M1. Proton chemical shift assignments for β-hydroxy-methionine at residue 6 are shown.

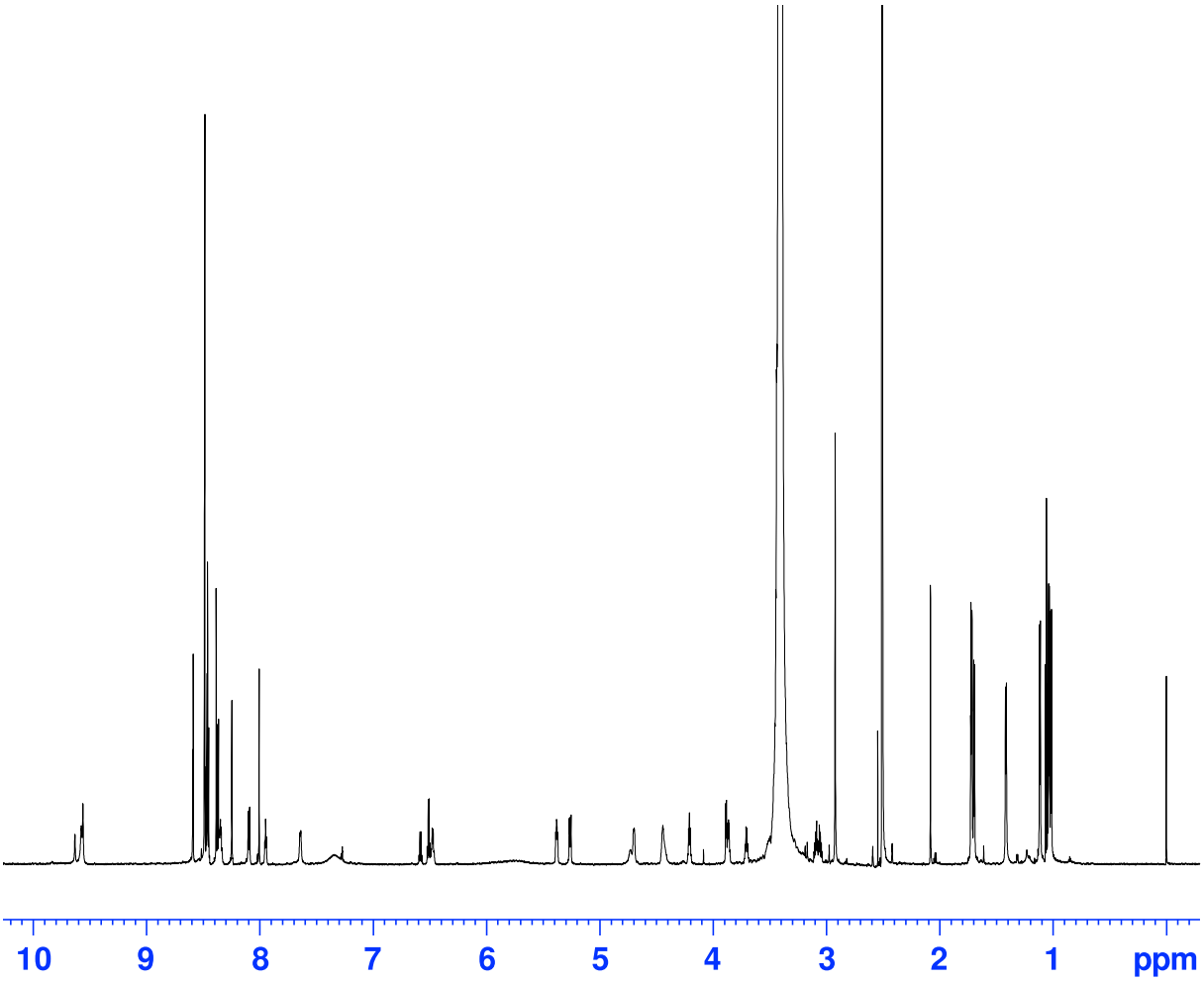


Figure S31: 1D ^{1}H NMR of V6T1.

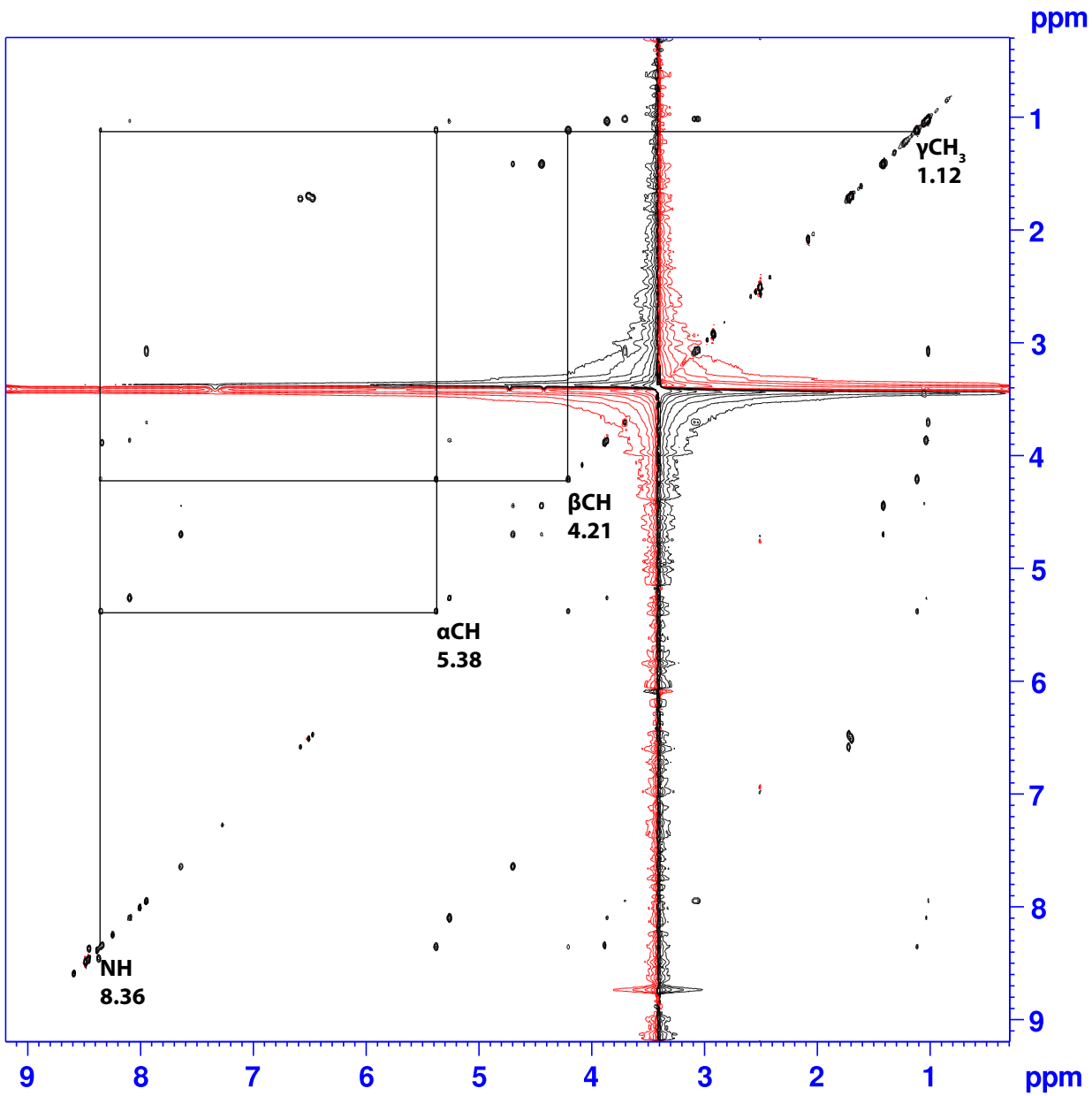


Figure S32: 2D ^{1}H-^{1}H TOCSY spectra of V6T1. Proton chemical shift assignments for threonine at residue 6 are shown.

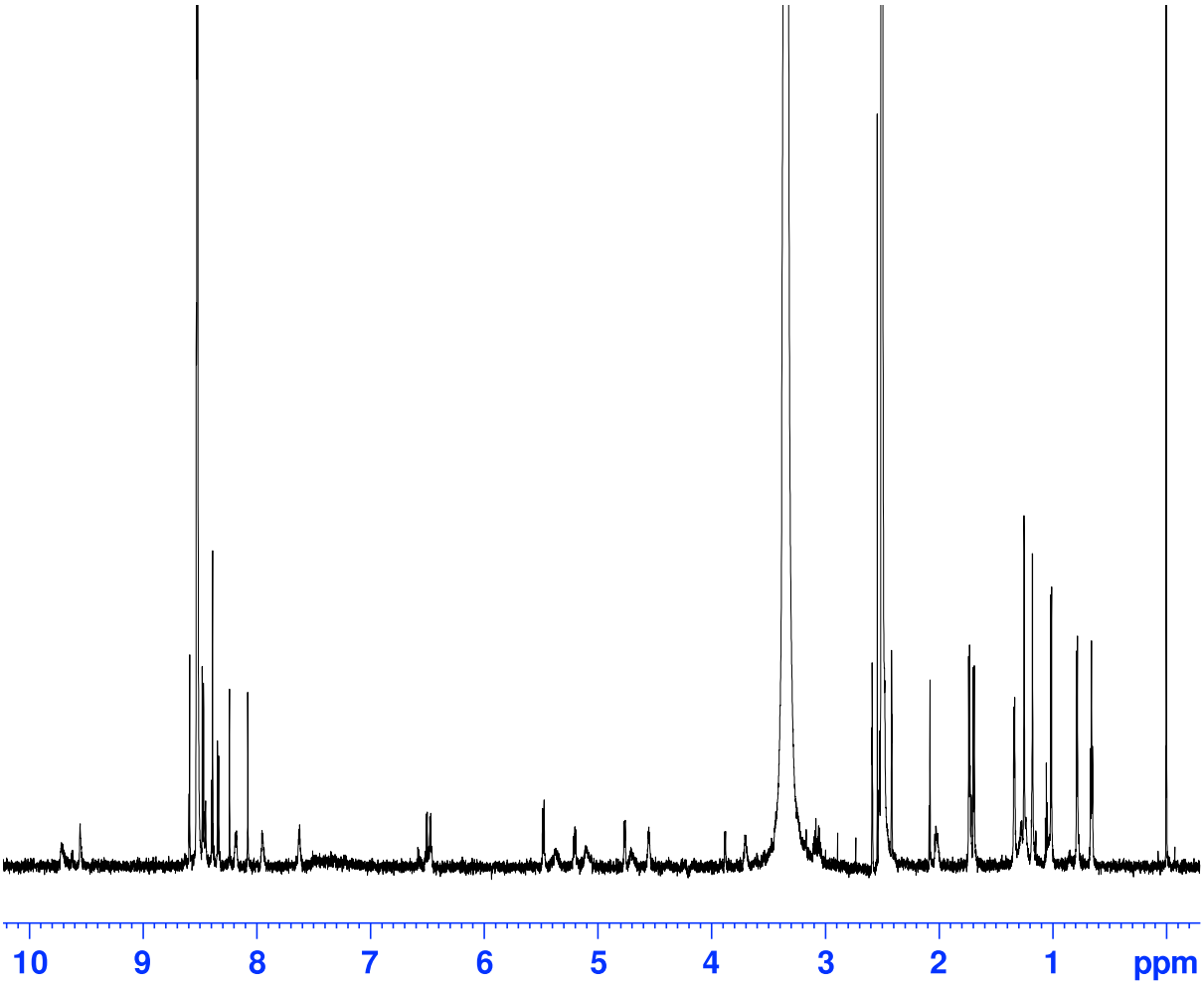


Figure S33: 1D ^{1}H NMR of T8I1.

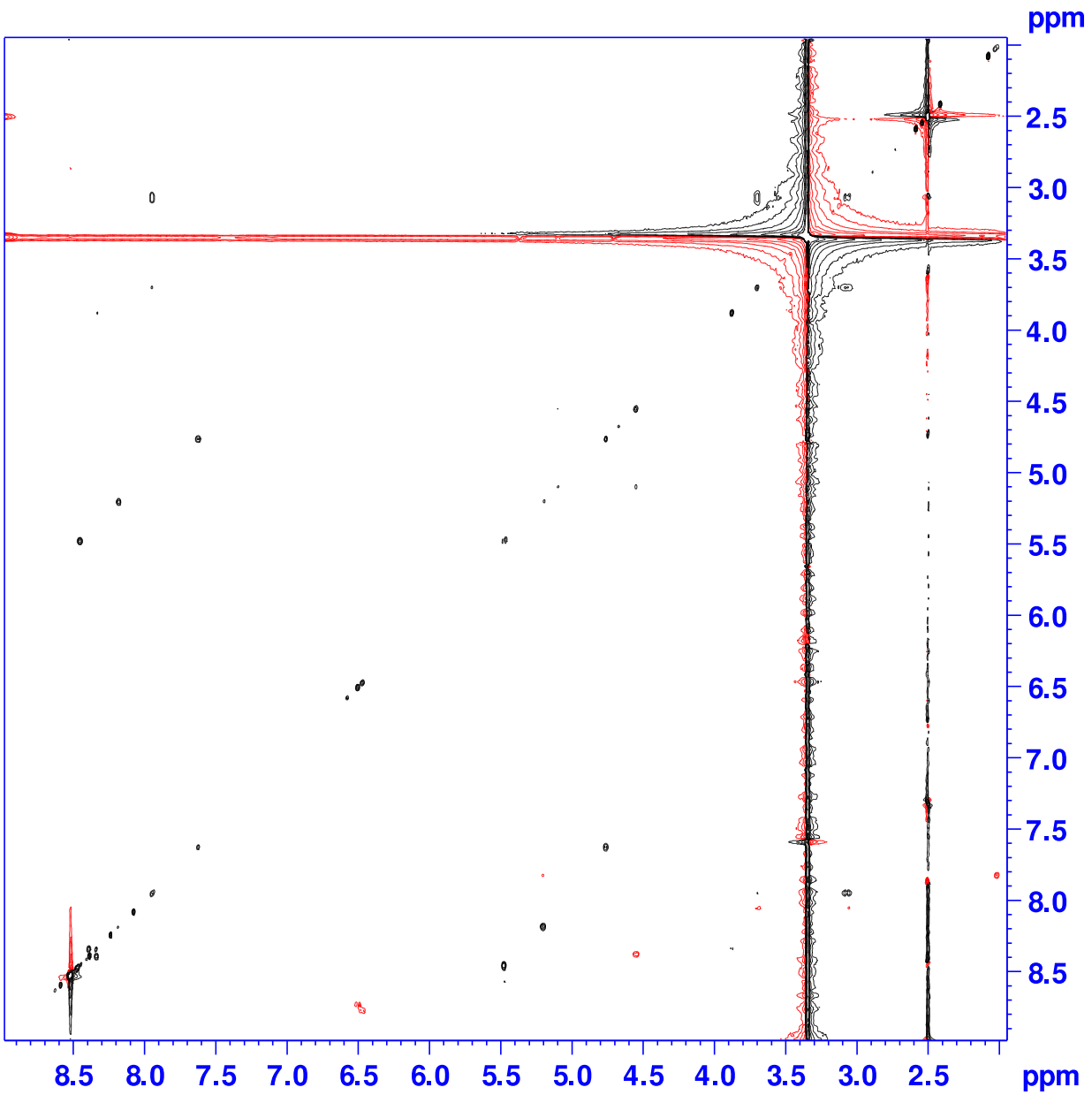


Figure S34: 2D ^{1}H-^{1}H TOCSY spectra of T8I1. No chemical shift assignments were made due to low sample concentration.

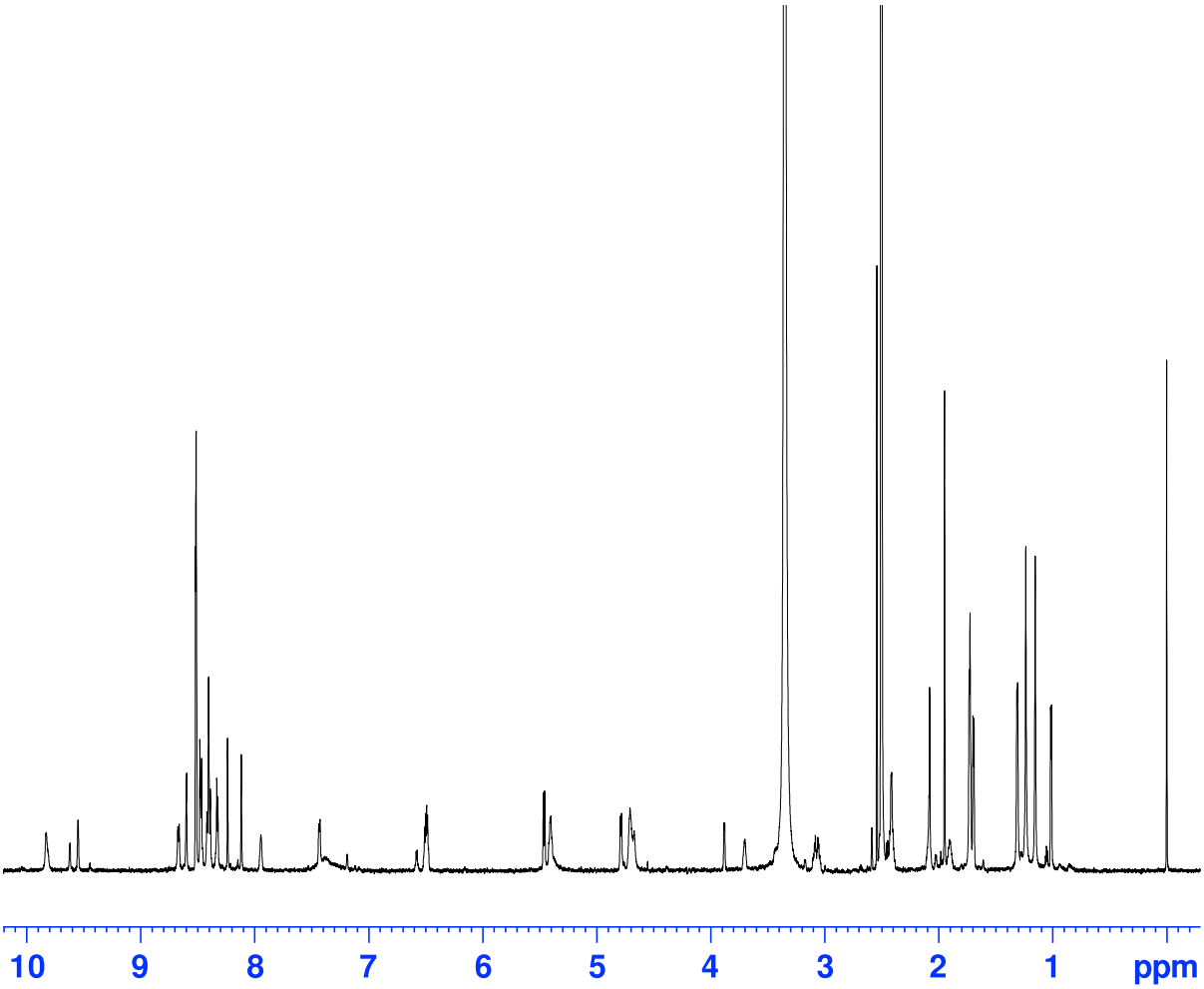


Figure S35: 1D ^{1}H NMR of T8M1.

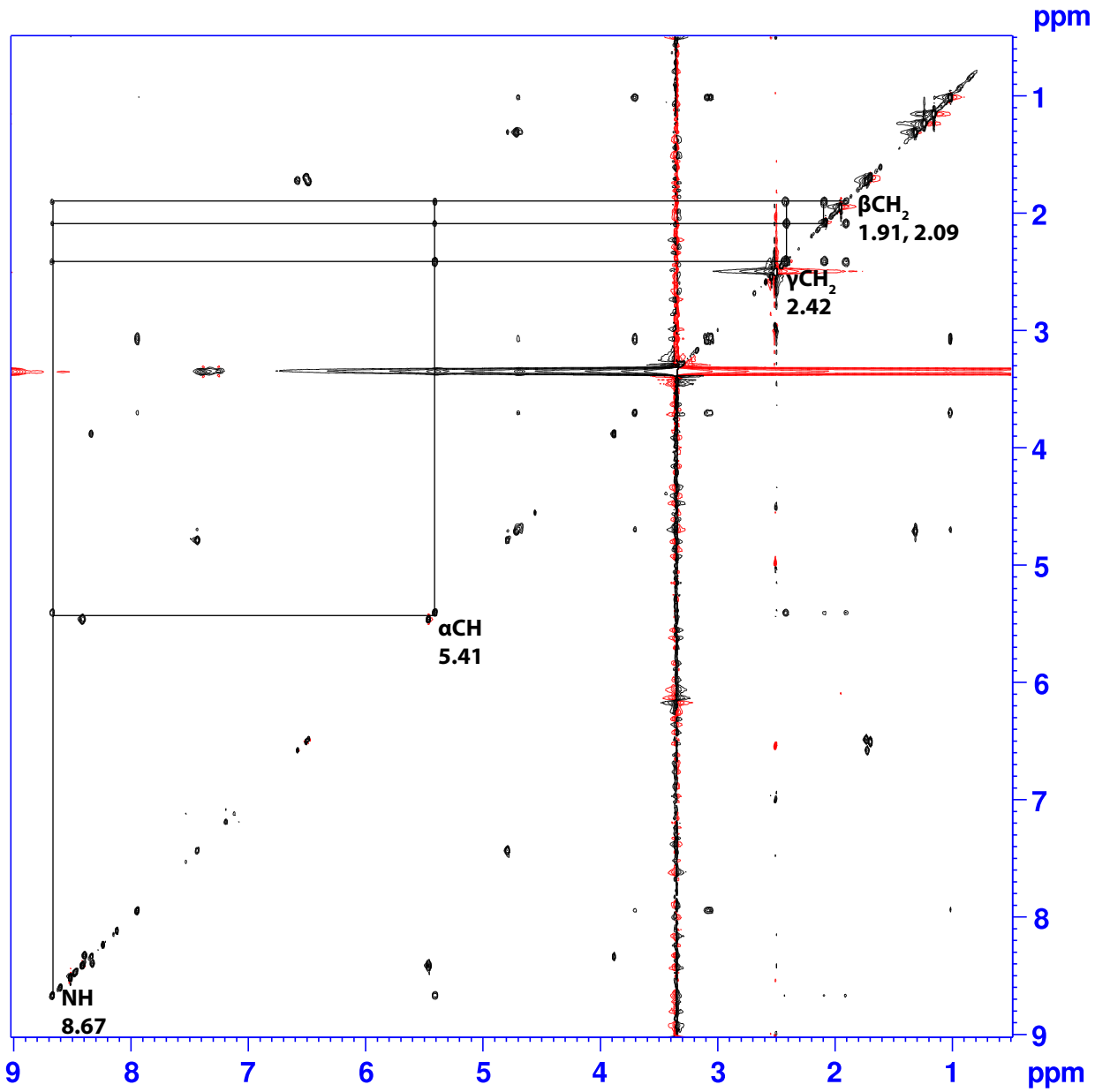


Figure S36: 2D ^{1}H-^{1}H TOCSY spectra of T8M1. Proton chemical shift assignments for methionine at residue 8 are shown.

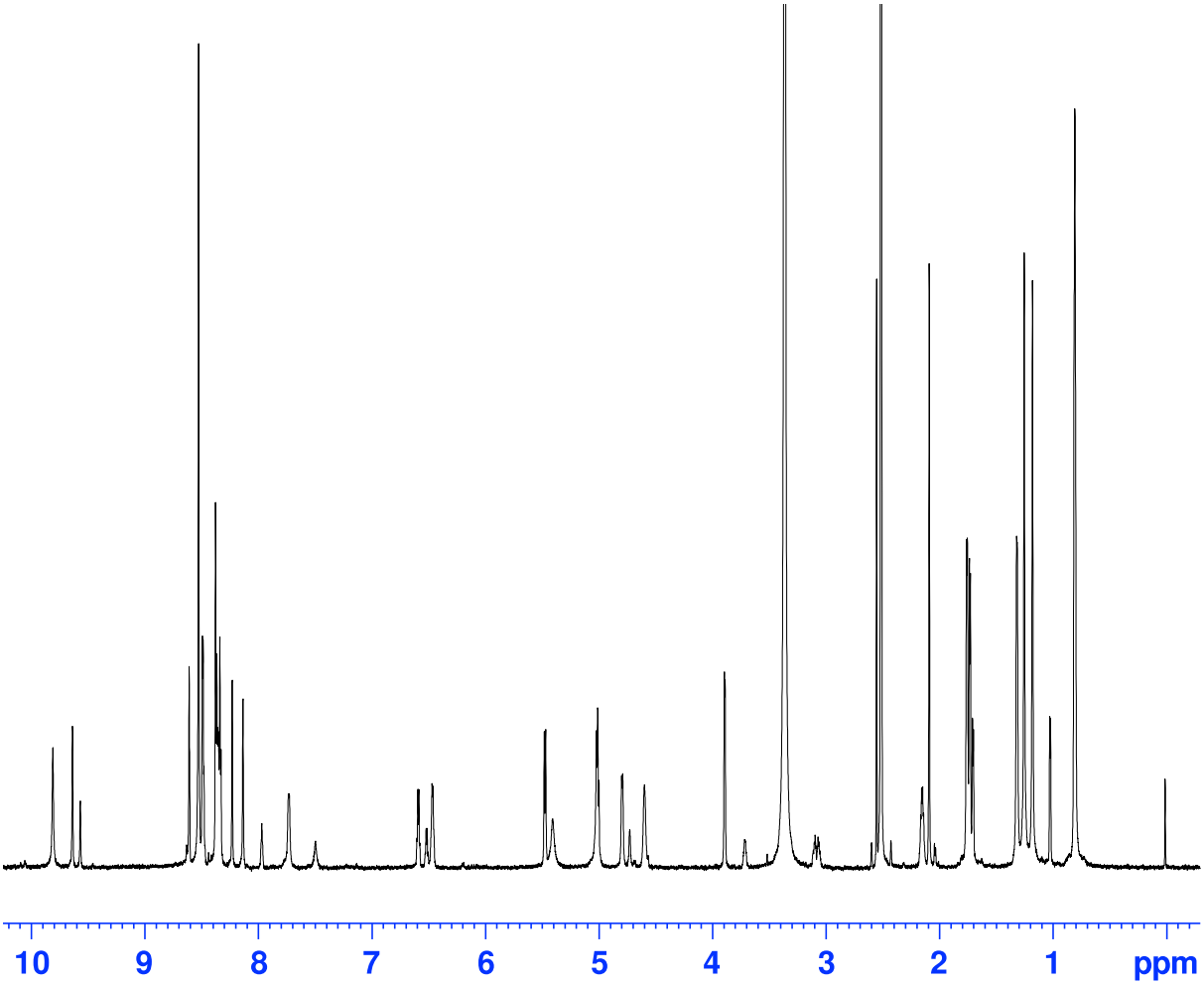


Figure S37: 1D ^{1}H NMR of T8V1.

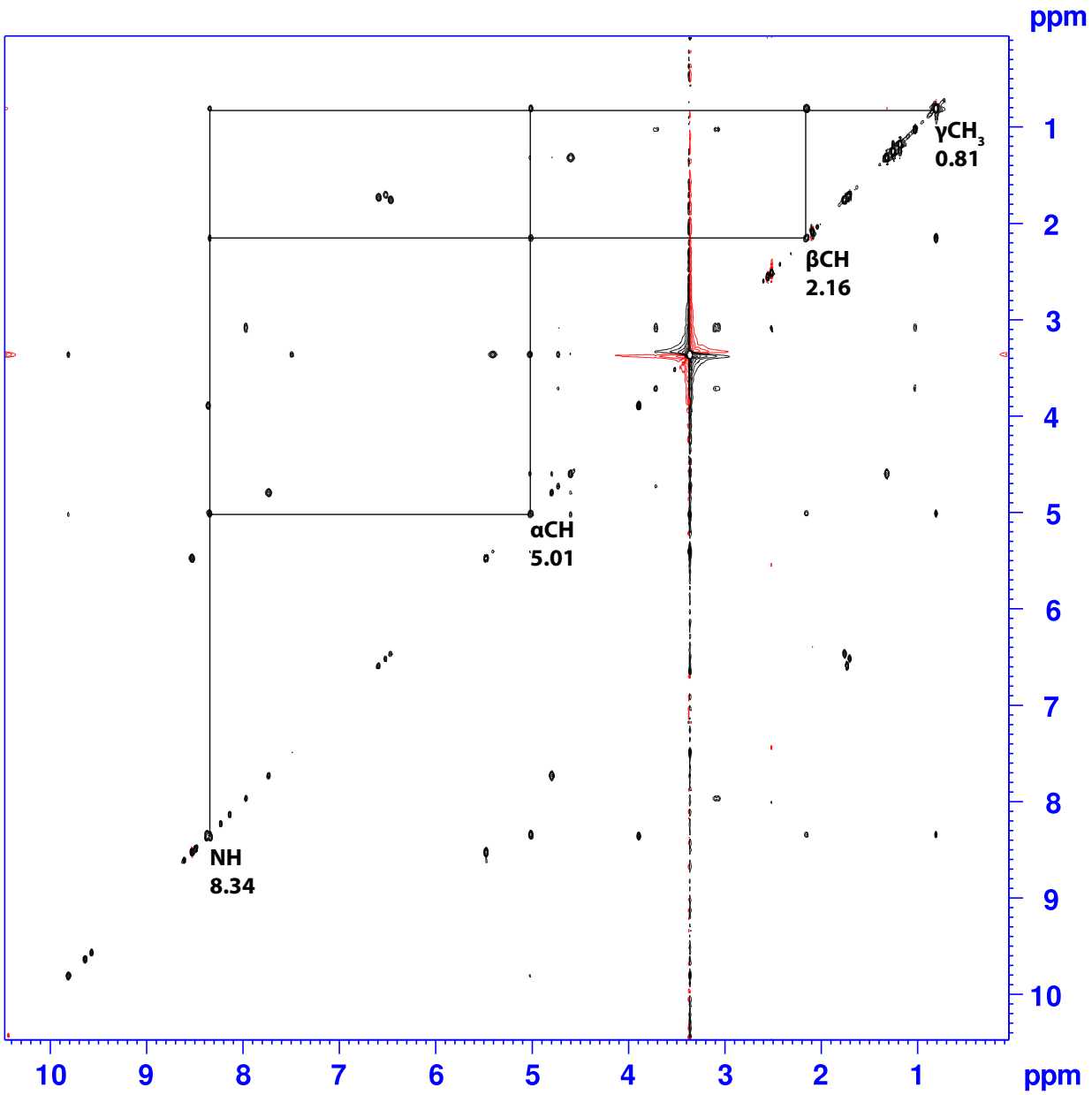


Figure S38: 2D ^{1}H-^{1}H TOCSY spectra of T8V1. Proton chemical shift assignments for valine at residue 8 are shown.

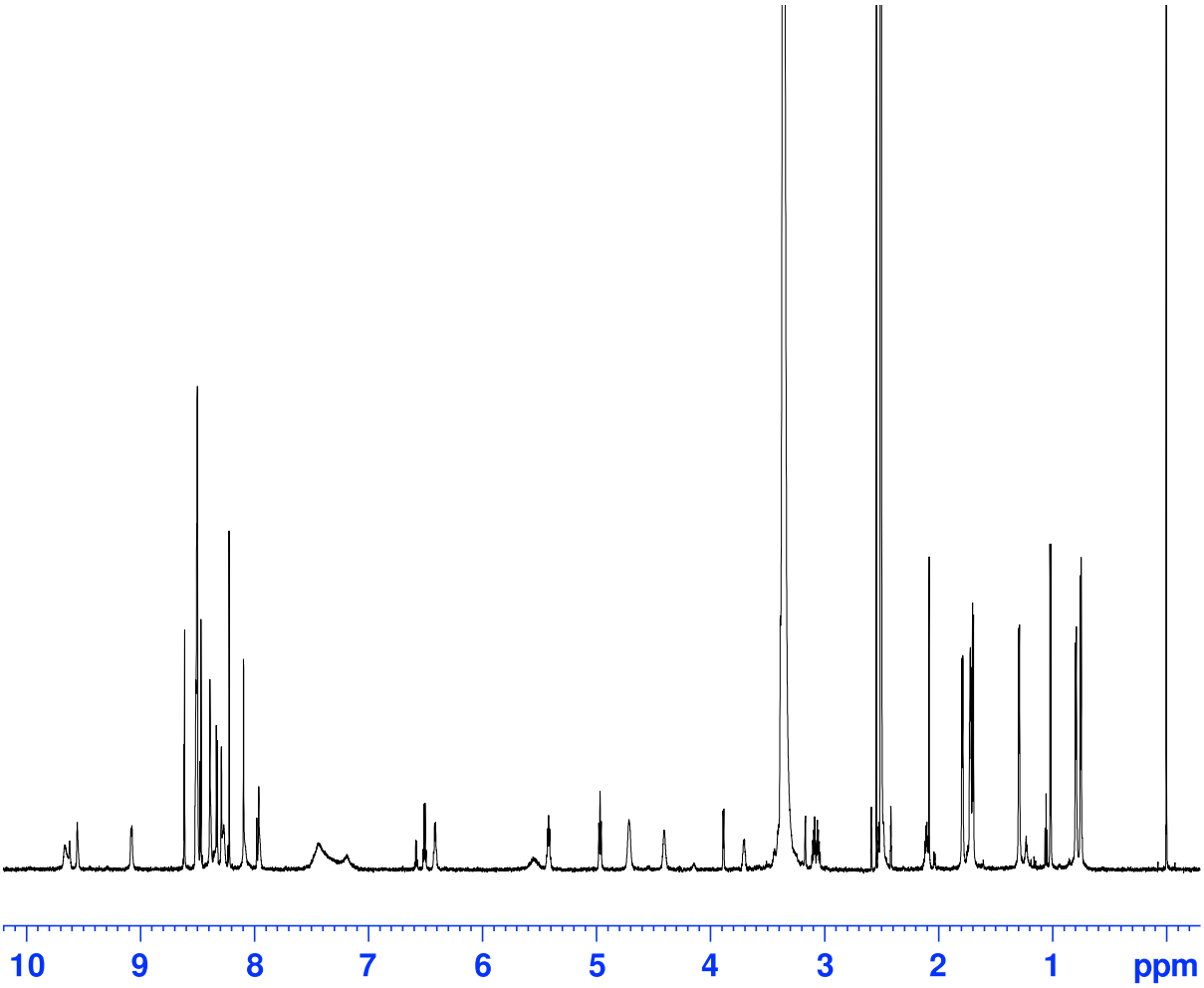


Figure S39: 1D ^{1}H NMR of V6A-T8V1.

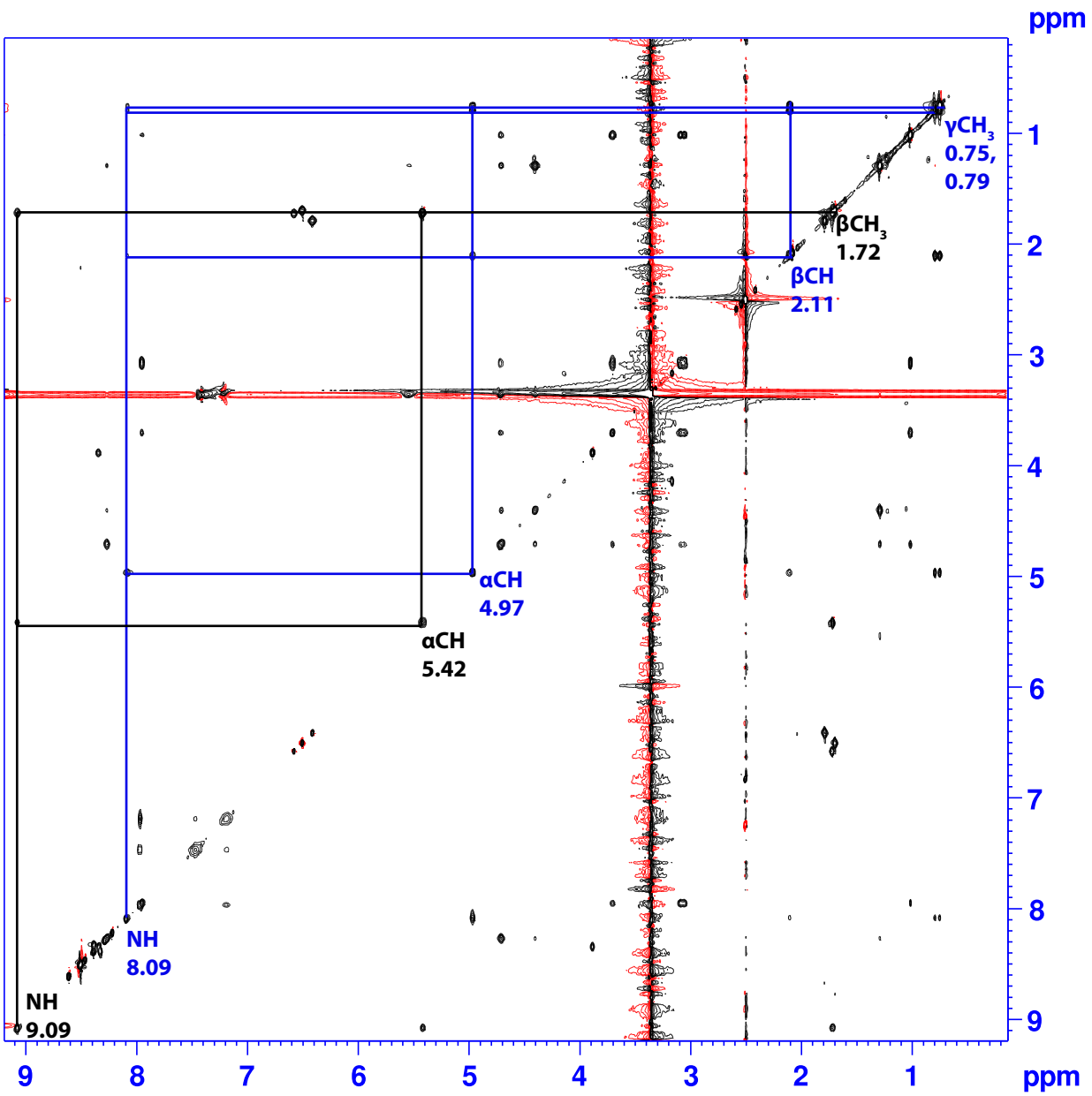


Figure S40: 2D ^{1}H-^{1}H TOCSY spectra of double mutant V6A-T8V1. Proton chemical shift assignments for alanine at residue 6 are shown in black and valine at residue 8 are shown in blue.

IX. Computational details

A single input structure is required to run BRIKARD. We tested our approach to using BRIKARD to thoroughly sample experimentally relevant conformations using thiostrepton and nosiheptide; two thio-containing macrocycles with literature data.[4] These two molecules were used to benchmark the number of iterations required to generate sufficient conformational diversity for our purposes and the best clustering cut-off. Since our questions in this study focused solely on conformational diversity and the underlying conformational entropy of each mutant, we used a shortened version of the published BRIKARD protocol, in which 1000 iterations were performed for each compound of interest.[5]

An extended, linear pre-cursor peptide was built for the wild-type structure, the cycle was formed, and the resultant structure was minimized in Maestro. An opls2005-derived force field file describing the non-bonded and bonded terms for the compound was generated with the hetgrp_ffgen utility in the Schrodinger package. This was used as the input into BRIKARD to generate an initial conformational ensemble of 1000 PLOP-minimized structures. Energy-based clustering was performed with the tools available in BRIKARD. Further details into specific methods underlying BRIKARD and BRIKARD with PLOP are available in the citation Coutsias *et al* 2016. We identified an RMSD threshold of 0.25 Å to be optimum for clustering conformations into population families. These conformations were compared with the experimental data to ensure that a near-native state was contained within our final structural ensembles.

A similar build procedure was followed for thiocillin. All mutants of interest were built by making the corresponding backbone and/or side chain change to the initial wild type input structure. The conformation of the long, rigid tail on thiocillin (residues 11-14) was based on our observations of the similar extended tail seen in nosiheptide (elongated, rigid but slightly bent). These input structures each had a force field file generated for the compound of interest and were subjected to 1000 iterations of BRIKARD with PLOP minimization. The output was clustered to identify the extent of conformational rigidity.

To explore the NMR ensemble of wild type thiocillin conformations, BRIKARD was run with restraints placed on the phi angles sampled on residues 3, 6, and 8. These restraints were derived from the ^{3}J couplings and enforced as follows: ±142°±5, ±165°±5, ±157°±5. We found no conformations satisfied all of these restraints absolutely and simultaneously. Instead, our best approximation of the NMR ensemble came from loosening the RMSD threshold to 0.75 Å and shifting the restraints slightly to ±142±5 & 22°±5 for phi3, ±160°±5 for phi6, and ±153°±5 for phi8 to enable successful sampling. No structures exactly matched all three constraints at once, but we were able to identify low-energy structures that approximately matched one or two of the ^{3}J coupling-derived restraint. This may be because the NMR results represent the average of a rapidly interconverting conformational ensemble rather than one single structure.

X. Supplemental references

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(3) Acker, M. G.; Bowers, A. A.; Walsh, C. T. *J Am Chem Soc* **2009**, *131*, 17563.
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