SUPPORTING INFORMATION

Conformational Switch to a β-turn in a Staphylococcal Quorum Sensing Signal Peptide Causes a Dramatic Increase in Potency

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Contents

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Experimental procedures

Chemical reagents, strains, and general methods

All reagents and solvents were purchased from Sigma-Aldrich or Chem-Impex and used as obtained. Water (18 MΩ) was purified using a Barnstead Nanopure from Thermo Scientific. The majority of the peptides used in this study were previously synthesized in our laboratory,[1] and all were purified to homogeneity using established reverse-phase high performance liquid chromatography (RP-HPLC) methods on a Shimadzu instrument composed of SCL-10Avp controller, DGU-14A degasser, FCV-10ALvp solvent mixer, LC-10ATvp pump, CTO-10ASvp column oven with a 5 mL manual injection loop and Kromasil Eternity column (5 μm 100 Å C18 packing of 10 mm × 250 mm), SPD-M10Avp diode array detector, and FRC-10A fraction collector. Peptide masses were determined using a Bruker microflex LRF MALDI-TOF mass spectrometer. The *S. epidermidis* GFP agr group-I reporter strain AH3408[2] was generously donated by Prof. Alexander Horswill (University of Colorado Medical School) and cultured in Tryptic Soy Broth (TSB, from Sigma) supplemented with 10 μg/mL of erythromycin.

Solid-phase peptide synthesis methods

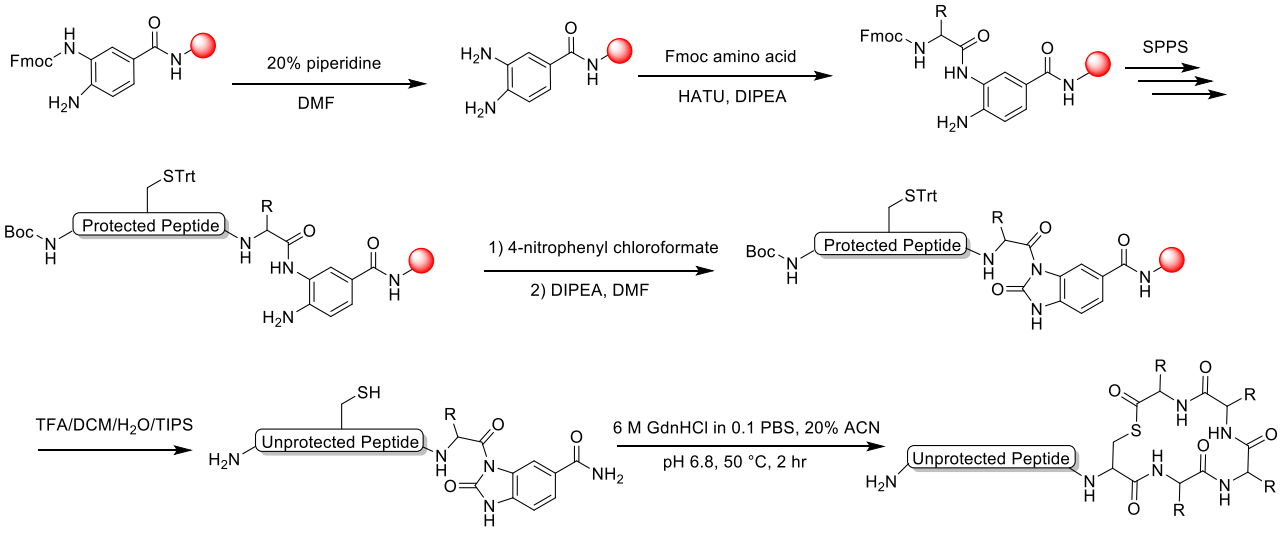
The β-turn modifying peptide analogs of AIP-I D1AS6A and AIP-I D1AV3AS6A (AAA), designed to examine the importance of the β-turn motif, were synthesized according to previously reported protocols (shown in Scheme S1).[1] Approximately 30 mg (0.0147 mmol, 1 eq.) of Fmoc Dawson Dbz AM resin was swelled in DCM for 40 min in a fritted reactor vessel. The resin was washed with DCM (2 mL ×3), DMF (2 mL ×3), and the Fmoc group deprotected with 20% piperidine in DMF (2 mL ×3; 5 min, 5 min, 10 min) while shaking. The deprotected resin was washed with DMF (2 mL ×3). N^{α}-Fmoc protected amino acid (4 eq.), HATU (4 eq.), and DIPEA (8 eq.) were dissolved in 2 mL DMF, incubated for 5 min, and added to the deprotected resin and shaken (1 h for the first amino acid, 0.5 h for the remaining amino acids). Deprotections and couplings were repeated to couple the remaining amino acids to the resin, with the final amino acid coupling using a Boc-protected amino acid instead of a Fmoc-protected amino acid.

Following the final Boc-protected amino acid coupling, the resin was washed with DMF (2 mL ×3) and DCM (2 mL ×3). Thereafter, 4-nitrophenyl chloroformate (4 eq.) dissolved in 2 mL of DCM was added to the vessel, and the slurry was shaken for 30 min. An additional 2 mL of 4-nitrophenyl chloroformate (4 eq.) in DCM was added to the vessel, and the slurry was shaken for another 30 min. The resin was washed with DCM (2 mL ×3) and treated with 2 mL of 5.5% DIPEA in DMF (10 min ×3). The resin was washed with DMF (2 mL ×3), DCM (2 mL ×3), and Et_2O (2 mL ×3), dried under N_2, and dried under vacuum. The peptide was cleaved from the resin (while also being deprotected) by treatment with 2 mL of 90/5/2.5/2.5 TFA/DCM/H_2O/triisopropylsilane (TIPS) and shaking for 2 h. The solution was filtered, mixed with 40 mL of Et_2O, chilled at -20 °C for 1 h to precipitate peptide, and centrifuged using a Beckman-Coulter Allegra 6R with a GH-3.8 rotor at 3500 rpm and 4 °C for 30 min. The supernatant was decanted, the pellet dissolved in 3 mL 50% ACN in H_2O, and lyophilized.

Lyophilized peptide was redissolved in 25% ACN in H_2O and purified by RP-HPLC. Fractions containing peptides were identified using MALDI MS and lyophilized. The lyophilized linear peptides were cyclized in 3 mL of cyclization buffer (20% ACN, 80% 6 M guanidinium chloride in 0.1 M Na_2PO_4, pH 6.8) while shaking at 50 °C for 2 h. The cyclized peptide was purified using RP-HPLC; fractions that contained cyclized peptide were identified by MALDI MS. Aliquots of the cyclized peptide fractions were submitted for high-resolution mass measurement and

analytical RP-HPLC to check purity, while the remaining portions of the fractions were lyophilized in pre-massed vials to obtain final isolated yields of approximately 12–23%.

Scheme S1. Solid-phase synthesis of β-turn modifying analogs.



Solid-phase N-methylated peptide synthesis methods

N-methylated analogs of AIP-I D1AS6A and AAA were synthesized and purified following the above peptide synthesis protocol with two exceptions. First, instead of using a standard N^{α}-Fmoc protected amino acid, an N^{α}-methyl, N^{α}-Fmoc protected amino acid was used to load the resin. Second, the coupling time to couple the following N^{α}-Fmoc protected amino acid was extended to 3 h. Final isolated yields of peptides ranged from approximately 5–16%.

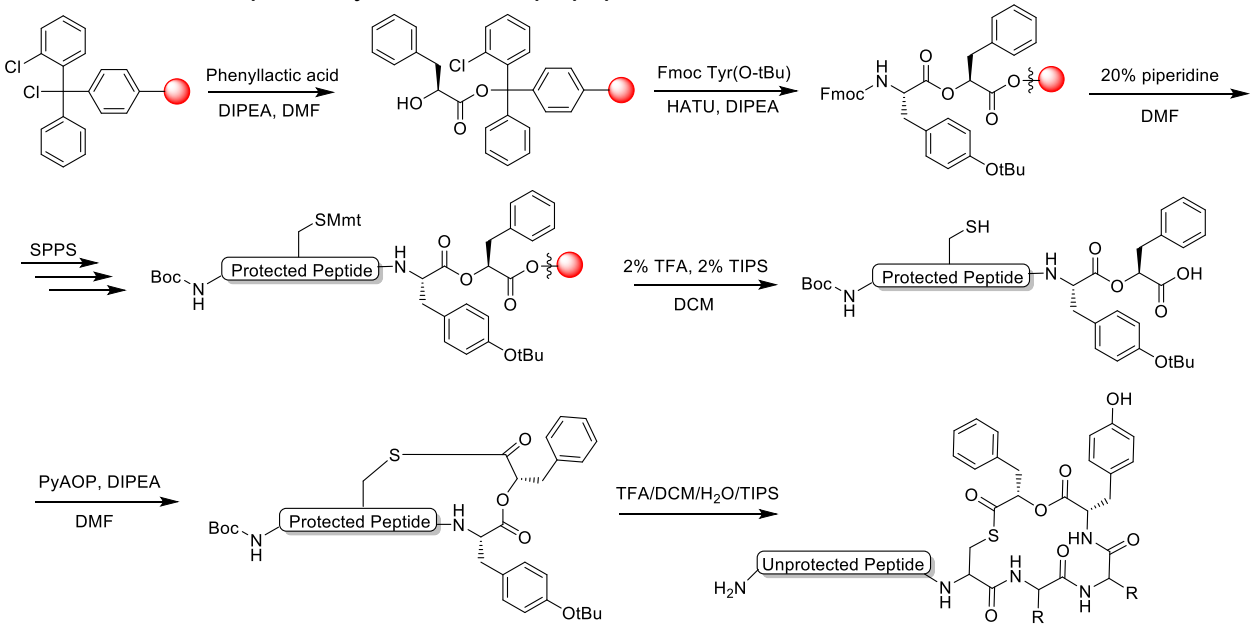
Solid-phase depsipeptide synthesis methods

Depsipeptide analogs of AIP-I D1AS6A and AAA were synthesized by replacing Phe8 with L-phenyllactic acid (PLA) (shown in Scheme S2). Approximately 100 mg (0.1 mmol, 1 eq.) of 2-chlorotrityl resin was swelled in DCM for 10 min in a fritted reactor vessel, and the resin was washed with DCM (2 mL ×3). To load PLA onto the resin, PLA (2 eq.) and DIPEA (4 eq.) were dissolved in 2 mL of DMF, added to the resin, and shaken for 30 min. The resin was washed with DCM (2 mL ×3), and then capped by incubating with 2 mL of a 15% MeOH/5% DIPEA in DCM solution for 10 min while shaking (×2). The resin was washed with DCM (2 mL ×3) and then with DMF (2 mL ×3). Thereafter, N^{α}-Fmoc-O-*t*Bu-L-tyrosine (4 eq.), HATU (4 eq.), and DIPEA (8 eq.) were dissolved in 2 mL of DMF, pre-incubated for 5 min, and then added to the PLA-loaded resin vessel and shaken for 16 h. The resin was then washed with DMF (2 mL ×3), then Fmoc deprotected with 2 mL of 20% piperidine in DMF ×3 (5 min, 5 min, 10 min) while shaking. The deprotected resin was washed with DMF (2 mL ×3). The next N^{α}-Fmoc protected amino acid (4 eq.), HATU (4 eq.), and DIPEA (8 eq.) were dissolved in 2 mL of DMF, pre-incubated for 5 min, and added to the deprotected resin and shaken for 30 min. Deprotections and couplings were repeated to couple the remaining amino acids to the resin, with the final amino acid coupling using a Boc-protected amino acid instead of a Fmoc-protected amino acid.

Following the final Boc-protected amino acid coupling, the resin was washed with DMF (2 mL ×3), DCM (2 mL ×3), MeOH (2 mL ×3), dried with N_2, and then dried under vacuum. The peptide was cleaved from the resin (while simultaneously deprotecting only the Cys4 thiol from its Mmt protecting group) using 5 mL of 2% TFA/2% TIPS in DCM solution, and shaking for 15 min. The solution was filtered into a 25 mL round-bottom flask, and the resin was treated with another 5 mL of 2% TFA/2% TIPS in DCM solution, shaken for another 15 min, and drained into a 25 mL round bottom flask. A small volume (~0.5 mL) of DMSO was added to the flask, and the DCM was removed under reduced pressure. The remaining approximately 0.5 mL of the peptide dissolved in DMSO was diluted with 25% ACN in water and purified on RP-HPLC. Fractions containing the partially-protected linear depsipeptides were identified by MALDI MS and lyophilized. Yields of partially-protected linear depsipeptides ranged from 1−2%.

The partially-protected linear depsipeptides (1 eq.) were cyclized with PyAOP (1.5 eq.) and DIPEA (2 eq.) in 0.5 mL DMF, stirring for 48 h in a 50 °C water bath. The solvent was removed by rotary evaporation under reduced pressure, and the cyclized, partially-protected depsipeptides were fully deprotected by stirring in 0.5 mL of a 90/5/2.5/2.5 TFA/DCM/H_2O/TIPS solution for 2 h. The solution was removed by rotary evaporation under reduced pressure, leaving a yellow oil. The oil then was diluted with water and purified by RP-HPLC, and fractions that contained cyclized depsipeptide were identified with MALDI MS. Aliquots of the cyclized depsipeptide fractions were submitted for high-resolution mass measurement and analytical RP-HPLC to check purity, while the remaining portions of the fractions were lyophilized in pre-massed vials to obtain isolated yields of approximately 21−58% (relative to linear starting material).

Scheme S2. Solid-phase synthesis of depsipeptides.



Fluorescent AgrC-I reporter assay protocol

Peptides were evaluated for agr activity using the *S. epidermidis* GFP agr group-I reporter strain AH3408.[2] A culture of AH3408 was grown overnight at 37 °C with shaking at 200 rpm, after which it was diluted 1:50 in fresh TSB medium. Peptide stocks (1 mM) were serially diluted in DMSO, and 2 µL aliquots were transferred to wells in triplicate within a black 96-well microtiter plate. For antagonism assays, 198 µL of the diluted AH3408 culture were added to each well. For agonism assays, 2 µL of a 2.5 µM AAA stock solution (final concentration in plate: 25 nM) were added to each well to block agr activity, and subsequently 196 µL of diluted AH3408 culture were transferred into each well. Separate from the test wells, each plate also included two controls: a vehicle control (wells containing only 2 or 4 µL DMSO with dilute AH3408 culture for agonism or antagonism assays, respectively), and a media control (wells containing TSB medium only). Plates were incubated with shaking (200 rpm) at 37 °C for 24 h. Fluorescence (excitation at 500 nm, emission at 540 nm) and the OD_{600} of each well were measured using a BioTek Synergy 2 plate reader. Measurements were processed by subtracting background fluorescence (TSB medium), correcting to OD_{600}, and then normalizing using the respective DMSO control for the agonism and antagonism assays. Non-linear regression curves were fitted to the data sets with GraphPad Prism software (version 7) by using variable slope (four-parameter) dose-response analysis to obtain potency, efficacy, and statistical information about the activity profiles of the tested peptides.

NMR methods and spectral analyses

To obtain solution structures of key peptides, NMR experiments (1-D proton, 2-D TOCSY, and 2-D ROESY) and subsequent analyses of the spectral data were carried out using our previously reported methods.[3] In brief, peptides were dissolved in H_2O:D_2O (95:5), except for AIP-I which was dissolved in 12.5% CD_3CN in H_2O and t-AIP-I which was dissolved in 30% CD_3CN in H_2O at pH 5. Final concentrations of each peptide were at least 700 µM. These NMR spectra were obtained on a Bruker Avance-III 750 MHz spectrometer equipped with a TXI cryoprobe using standard pulse sequences at ambient temperature. Solvent suppression of the water peak was achieved using excitation sculpting, and chemical shifts in the spectra were referenced to residual H_2O at 4.79 ppm or CD_3CN at 1.94 ppm.

All spectra were analyzed using MestReNova 10 NMR processing software, with important ^3J couplings and chemical shifts provided in Tables S1−S16. Resonance assignments for each peptide, calculations of interproton distances from ROESY cross peak volumes, and the three-dimensional structure calculations and refinements were completed according to established methods.[4-6] ROE constraint specifics are provided below.

An average structure for each peptide was determined from an ensemble of the 20 lowest-energy structures from a set of 100 total structures obtained by torsion angle simulated annealing in Xplor-NIH.[7-13] During refinement, an initial constraint allowance of ±20% of each calculated ROE was used to determine initial structures with a soft potential. Final refinement was done with a constraint allowance of ±10% of each calculated ROE plus an additional 0.5 Å using a hard potential for the final structures. The average structure was used as a comparative tool, from which a representative structure with a small RMS difference from the average structure was selected from the low-energy ensemble. These representative structures are shown in the main text (Figures 3−8). Additional images are provided in Figures S1–S9. Visual analysis, structure comparisons with alignment fitting, and presentation of the peptide structures were performed with PyMOL.[14] For consistent RMS fitting in PyMOL, degenerate hydrogens

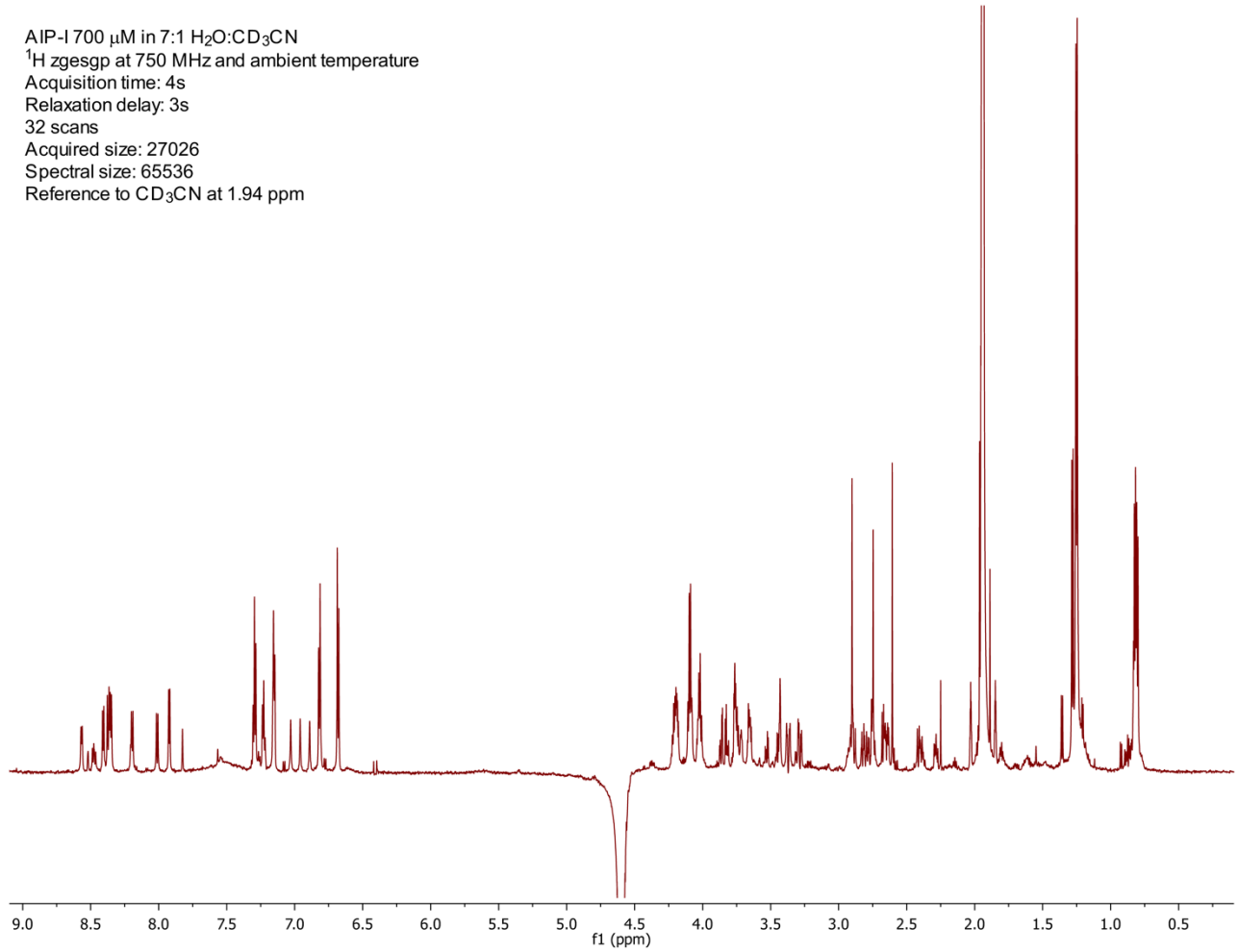
were numbered for consistent relative coordinates. Final representative structures were validated using MolProbity.[15-16]

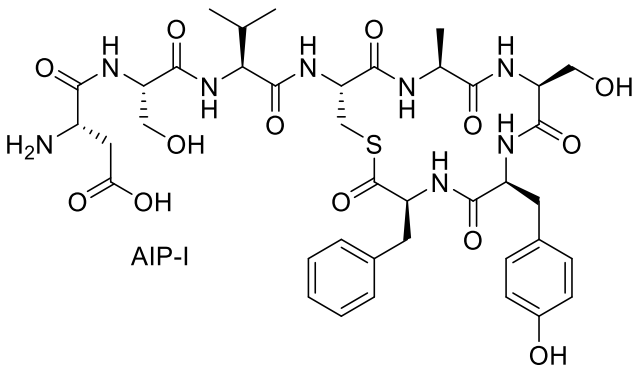
Additional NMR experiments (1-D proton, 2-D TOCSY, and 2-D ^{1}H-^{13}C HSQC) were performed to compare the chemical shifts of select peptides. Peptides were dissolved in D_2O at pH 5, with the exception of AIP-I which was dissolved in 12.5% CD_3CN in D_2O at pH 5. Final concentrations of each peptide were at least 700 µM. Spectra were obtained on a Varian 800 MHz spectrometer with a HCN cryoprobe using standard pulse sequences at ambient temperature. Chemical shifts within the spectra were referenced to a DMSO internal standard at 2.71 ppm (^{1}H) or 39.39 ppm (^{13}C). The chemical shift data for AIP-I and AIP-I D1AS6A were entered into the TALOS-N program in attempt to predict torsion angles (see additional discussion below).[17-18]

NMR spectra for 3-D solution-phase structures

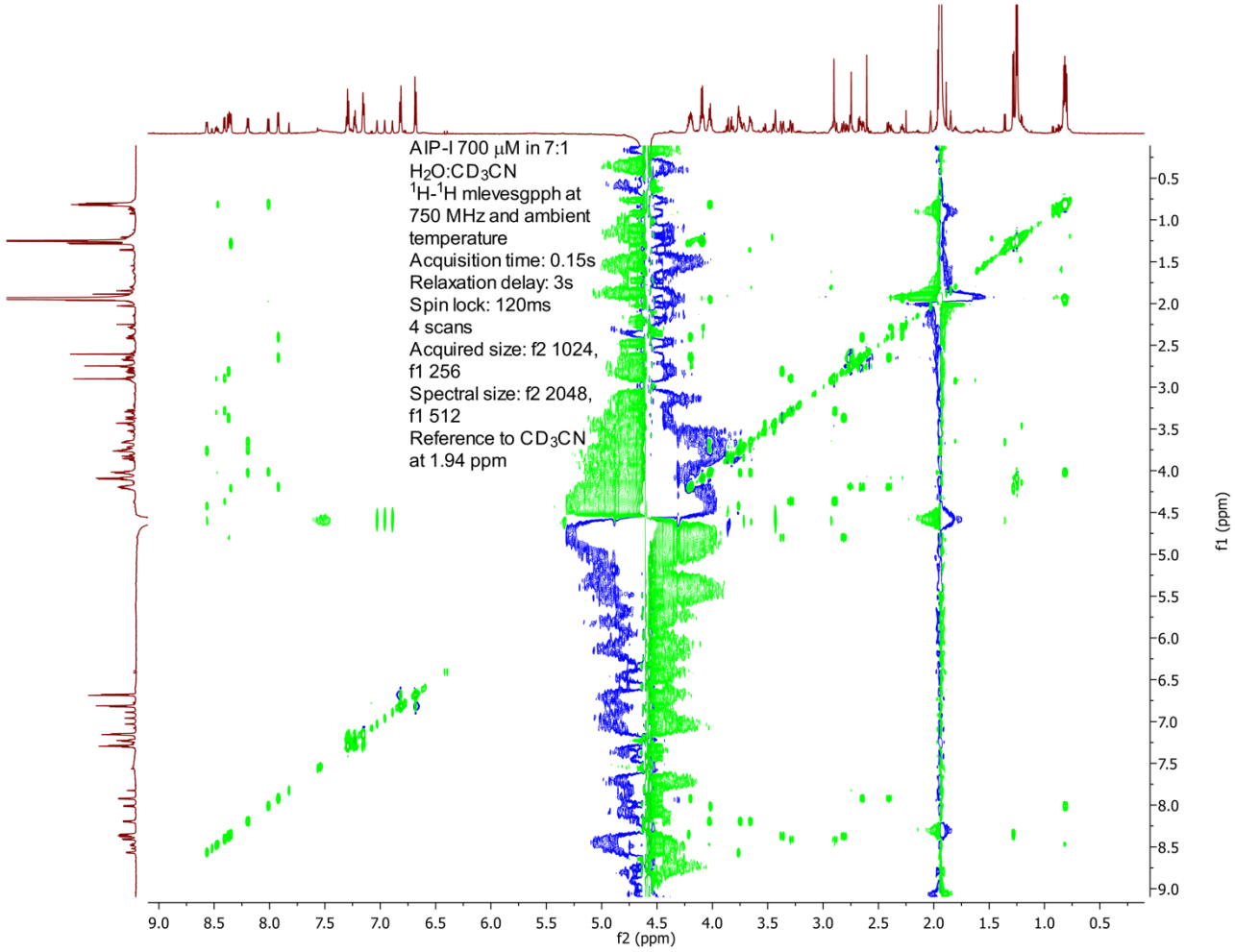
One-dimensional proton, 2-D TOCSY, and 2-D ROESY NMR experiments were performed for each peptide as described in the experimental procedures above. Selected parameters for each experiment are included in each spectrum. For the 2-D experiments, blue indicates negative peak intensity and green indicates positive peak intensity.

- *AIP-I ^{1}H NMR spectrum (700 μM dissolved in 12.5% CD_3CN in H_2O, pH ~6.5)*

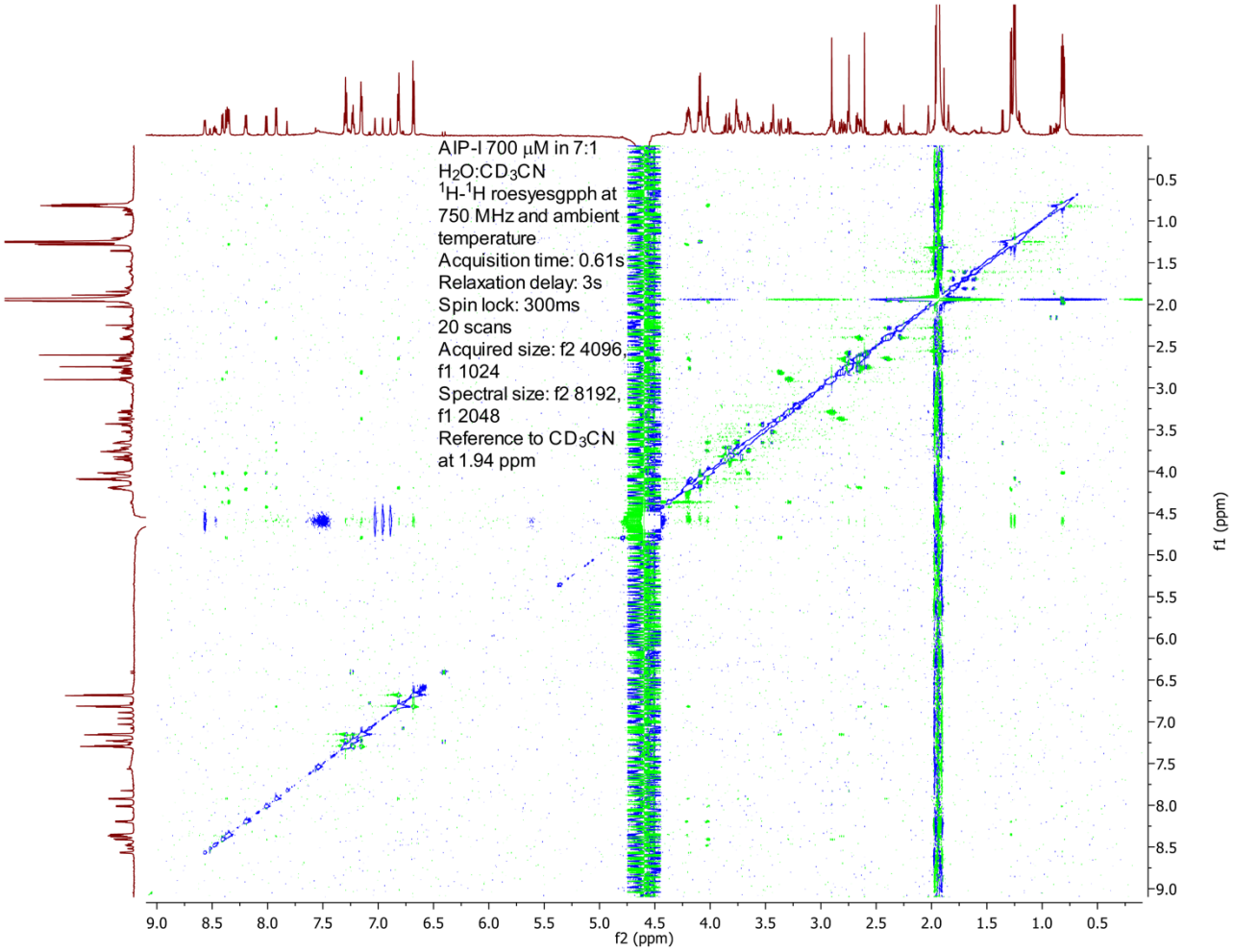




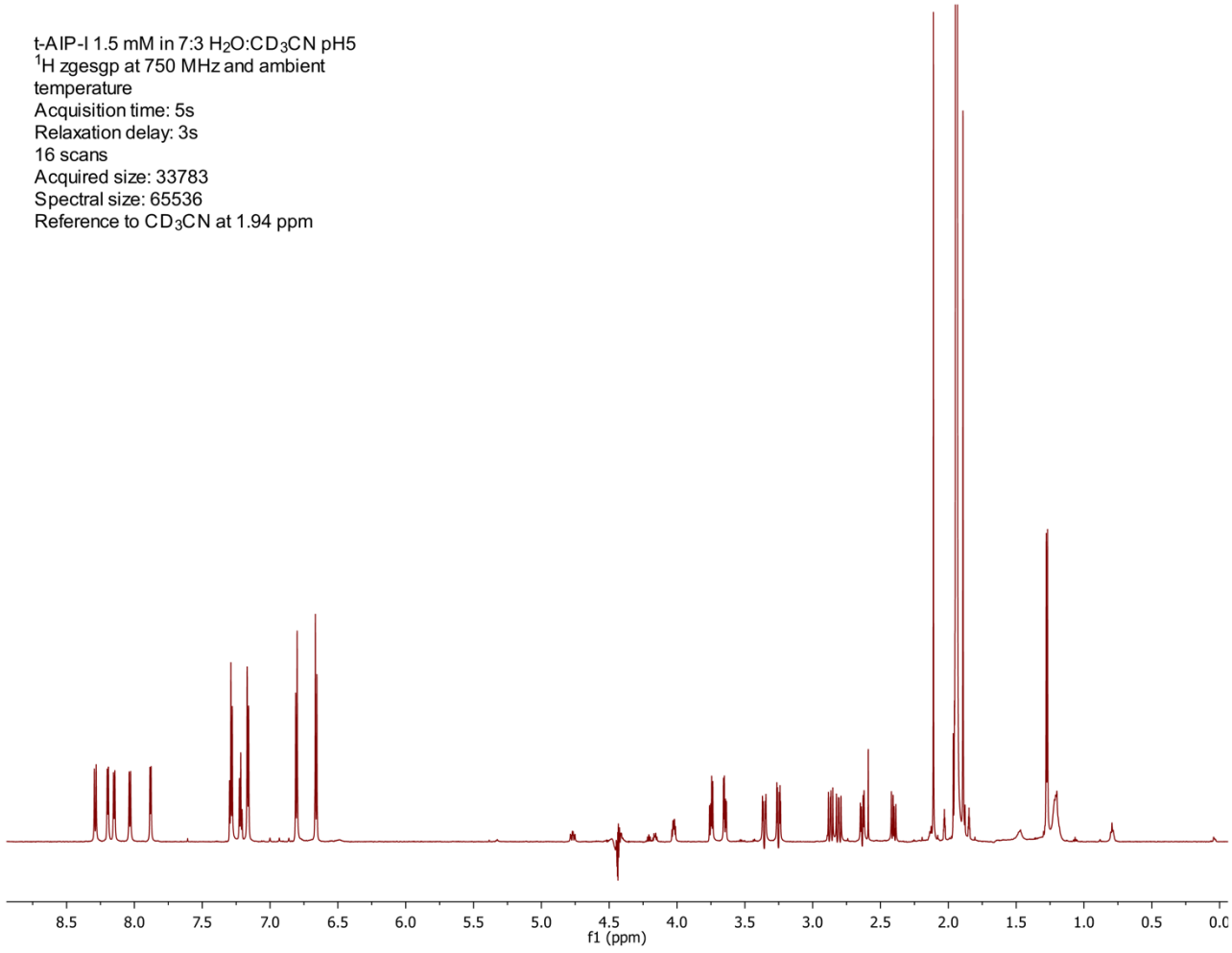
- *AIP-I ^{1}H-^{1}H TOCSY spectrum*

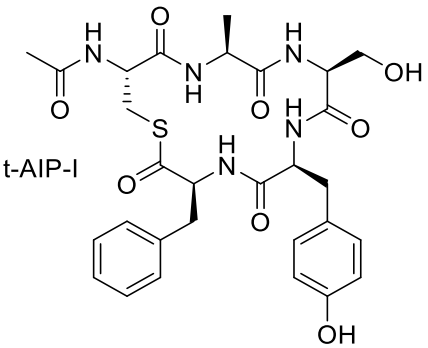


- *AIP-I $^{1}H-^{1}H$ ROESY spectrum*

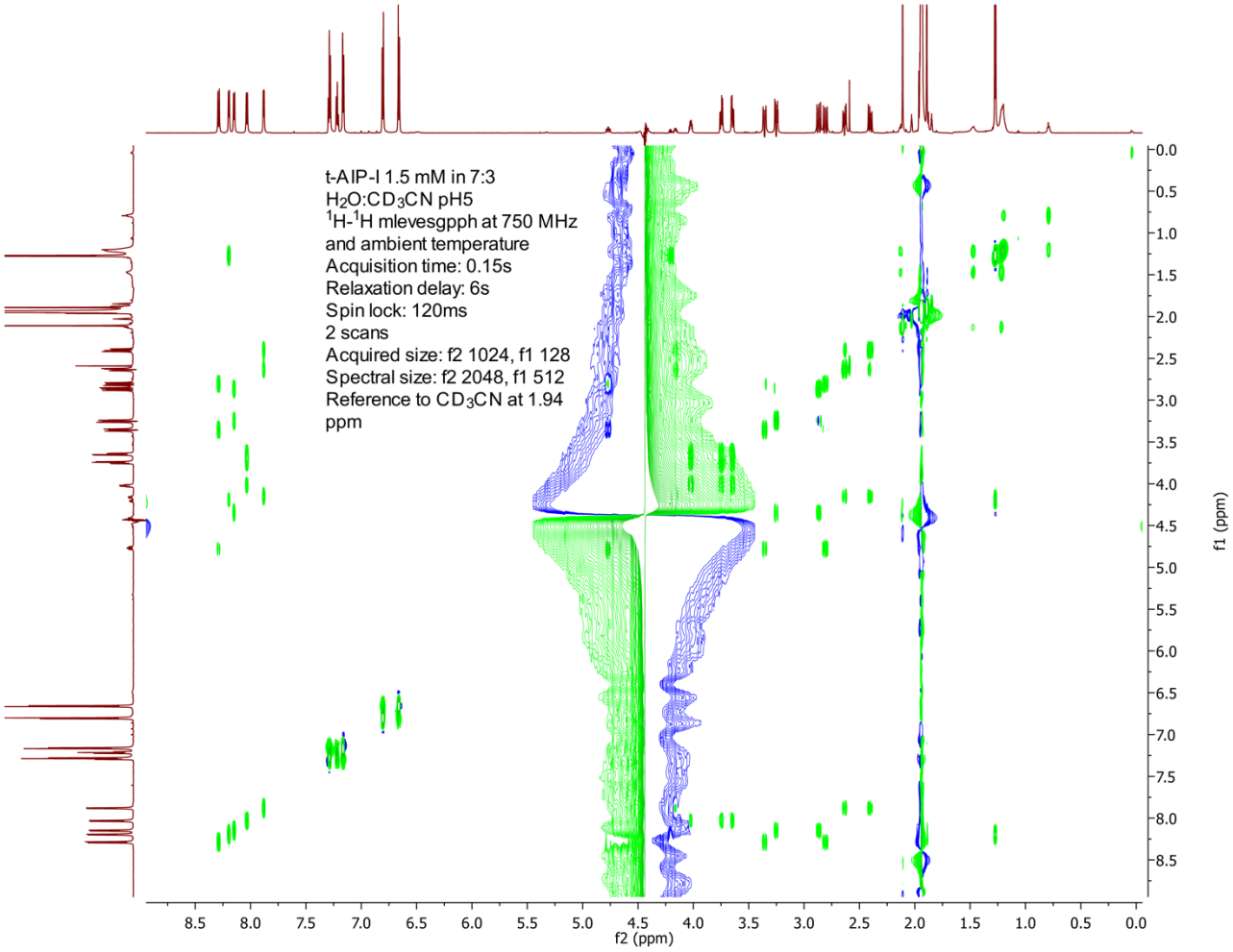


- *t-AIP-I ^{1}H NMR spectrum (1.5 mM dissolved in 30% CD_3CN in H_2O, pH ~5 with HCl)*

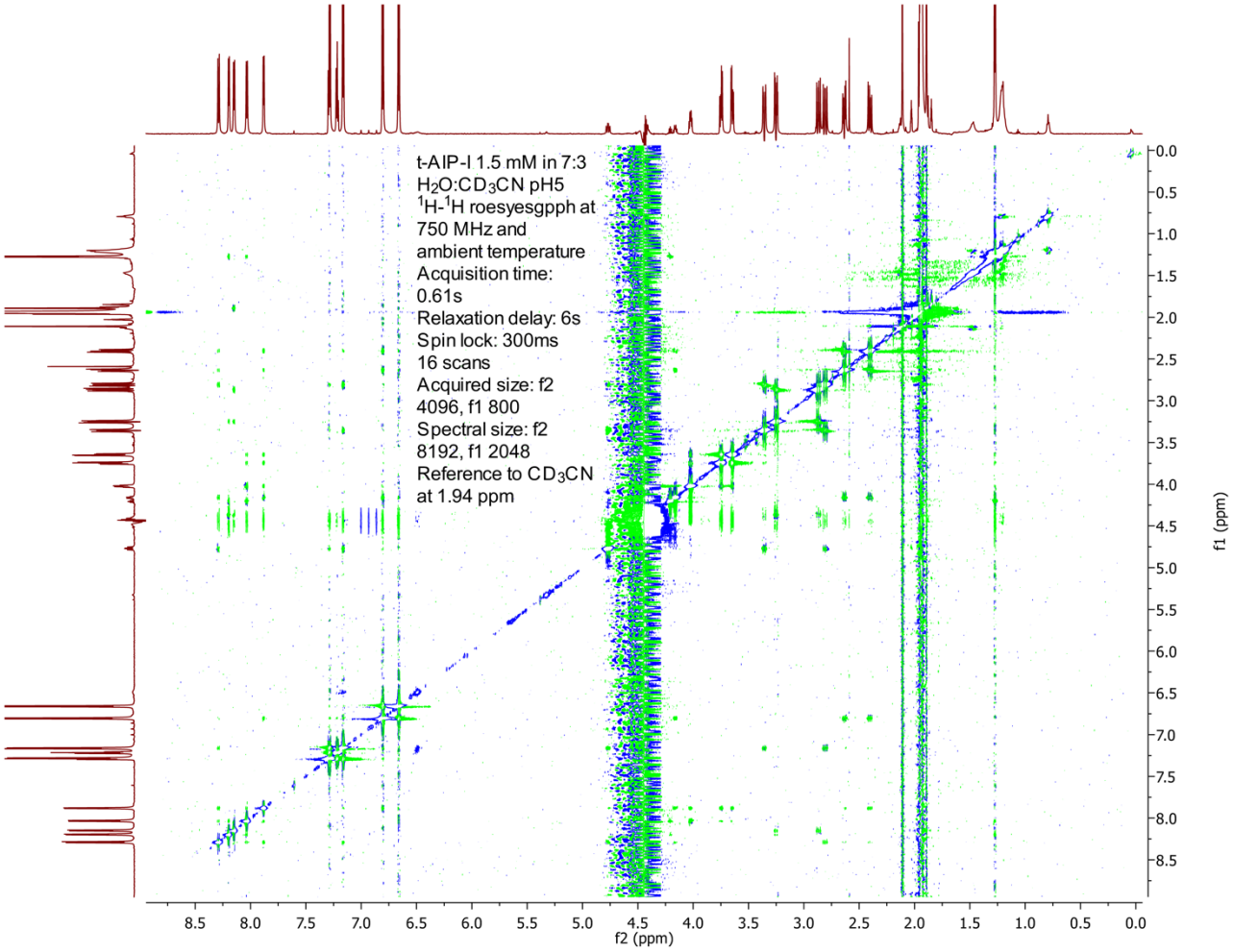




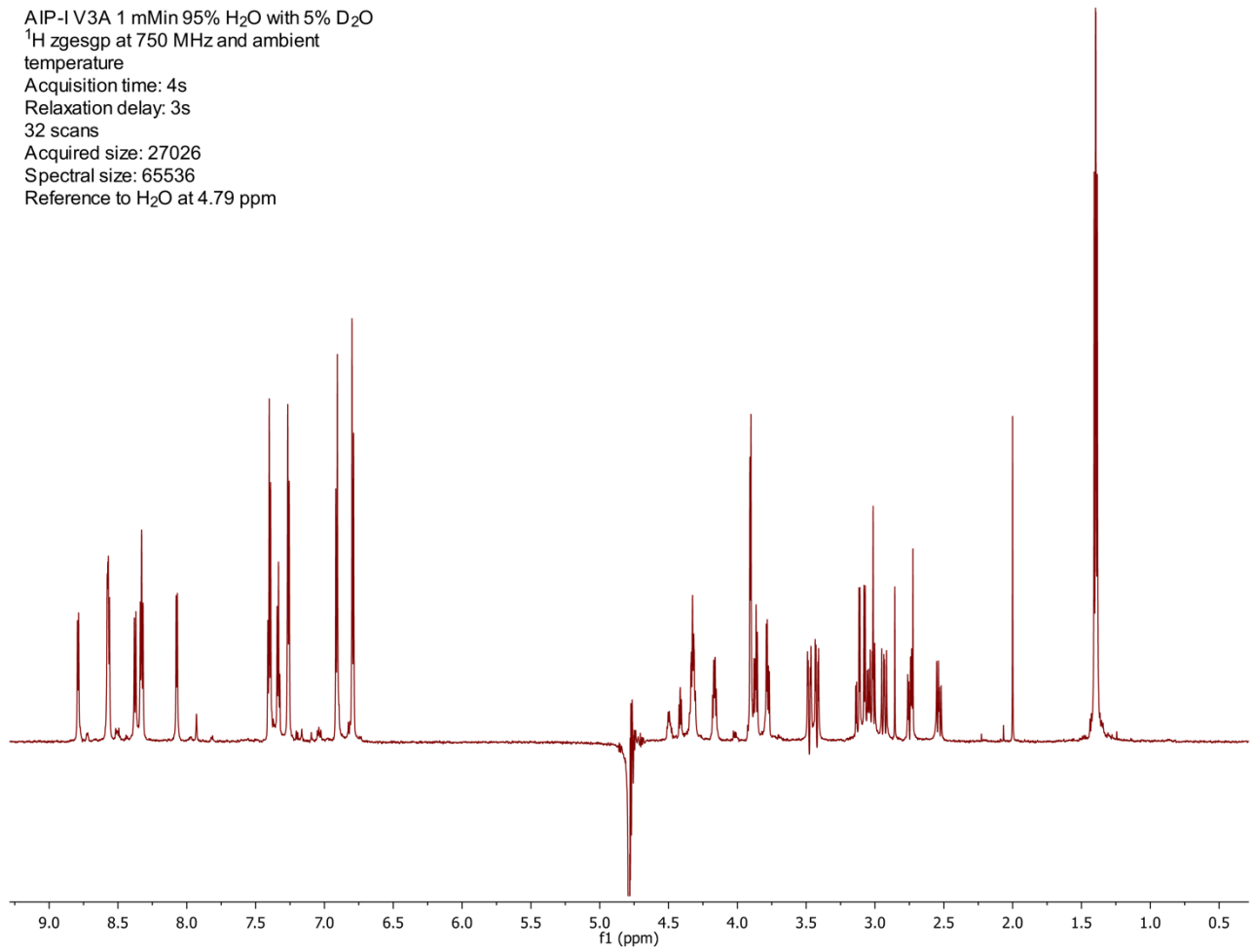
- *t-AIP-I $^{1}H-^{1}H$ TOCSY spectrum*

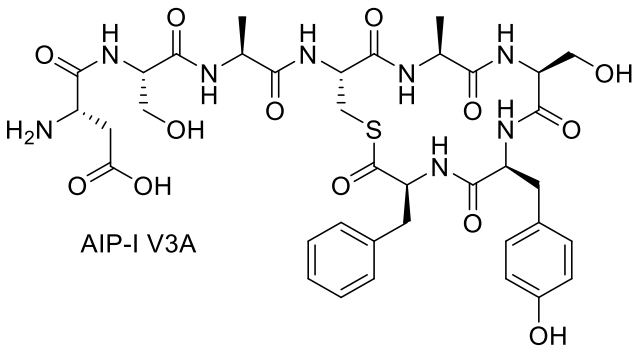


- *t-AIP-I ^{1}H–^{1}H ROESY spectrum*



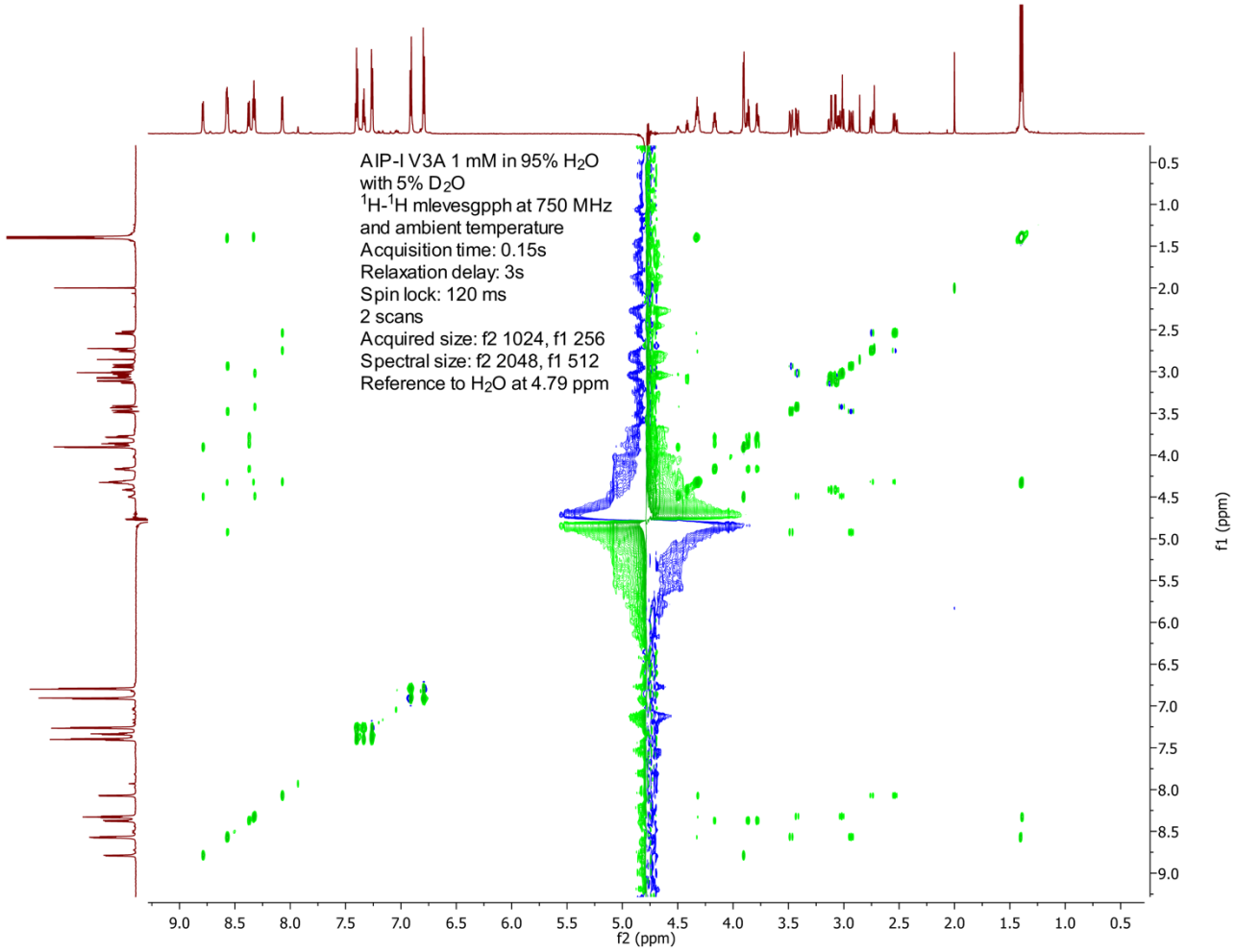
- *AIP-I V3A ^{1}H spectrum (1 mM dissolved in 95% H_2O with 5% D_2O, pH ~6.5)*





AIP-I V3A

- *AIP-I V3A ^{1}H–^{1}H TOCSY spectrum*



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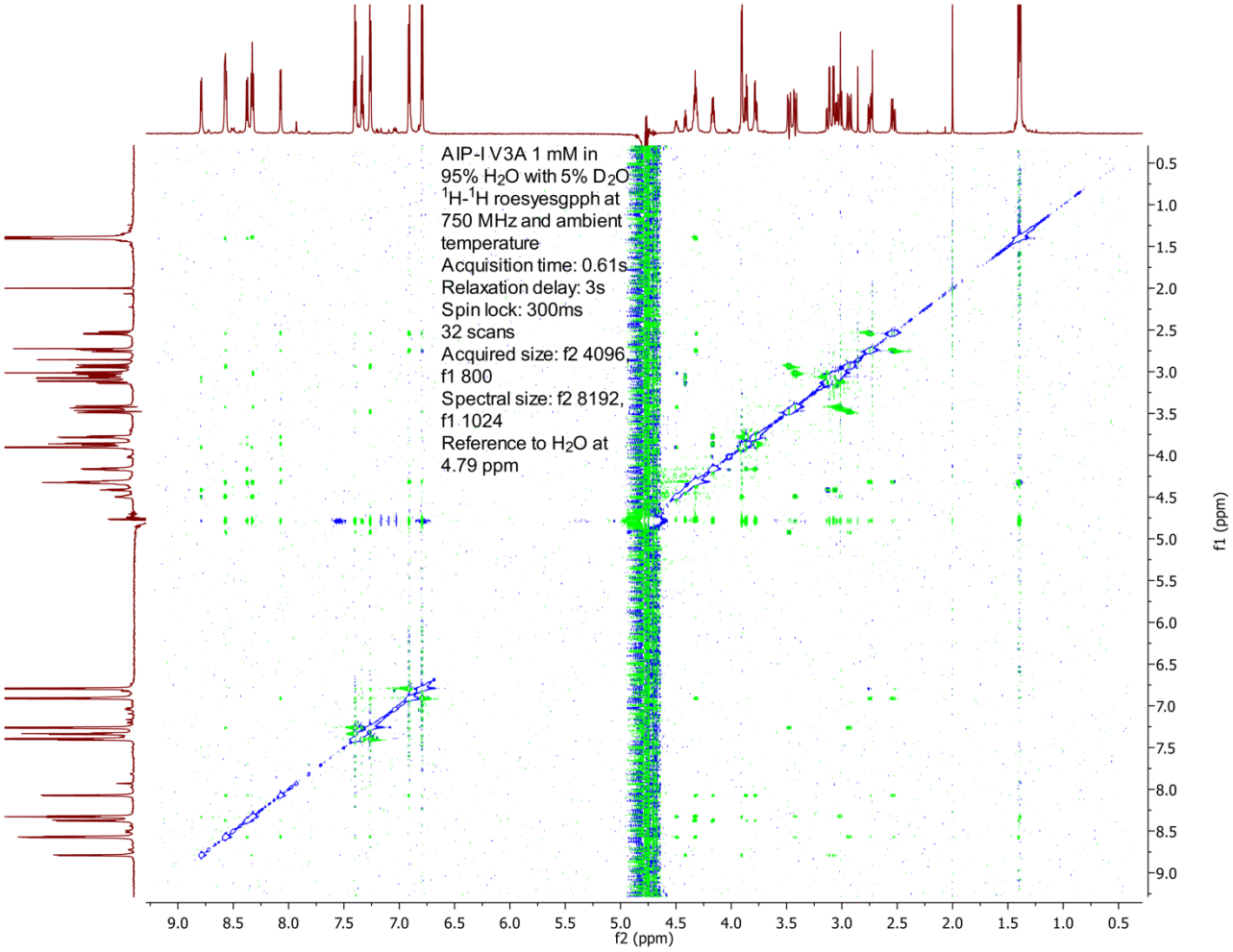
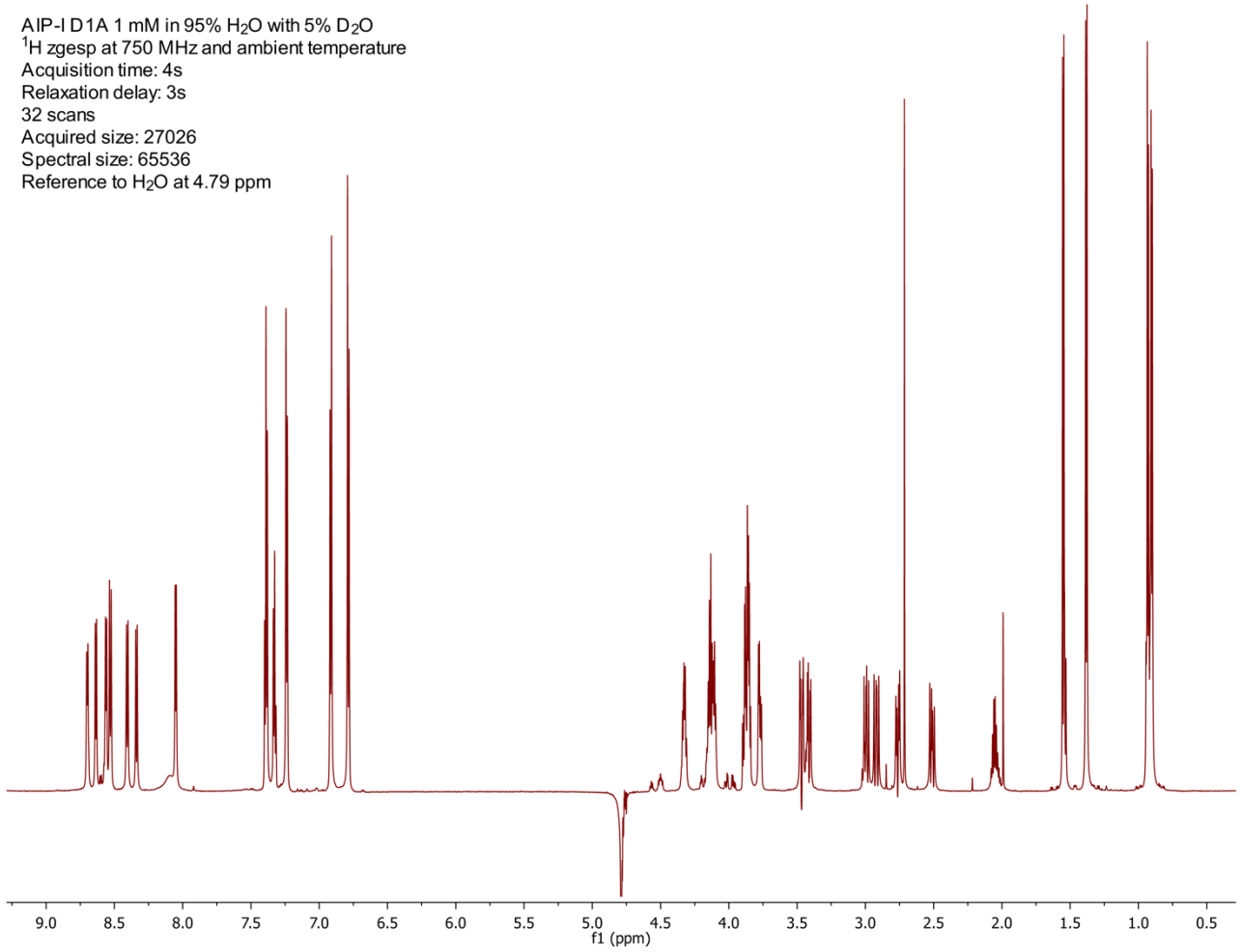
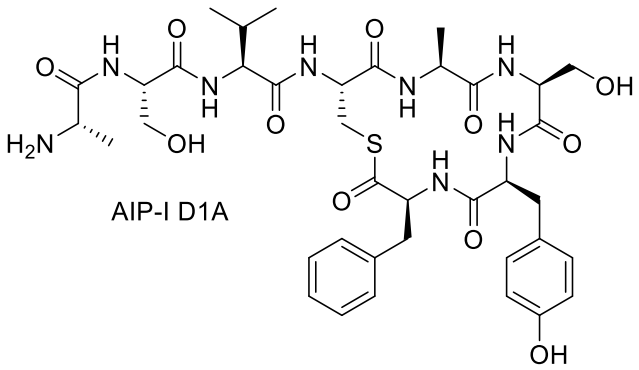


- *AIP-I D1A ^{1}H NMR spectrum (1 mM dissolved in 95% H_2O with 5% D_2O, pH ~6.5)*





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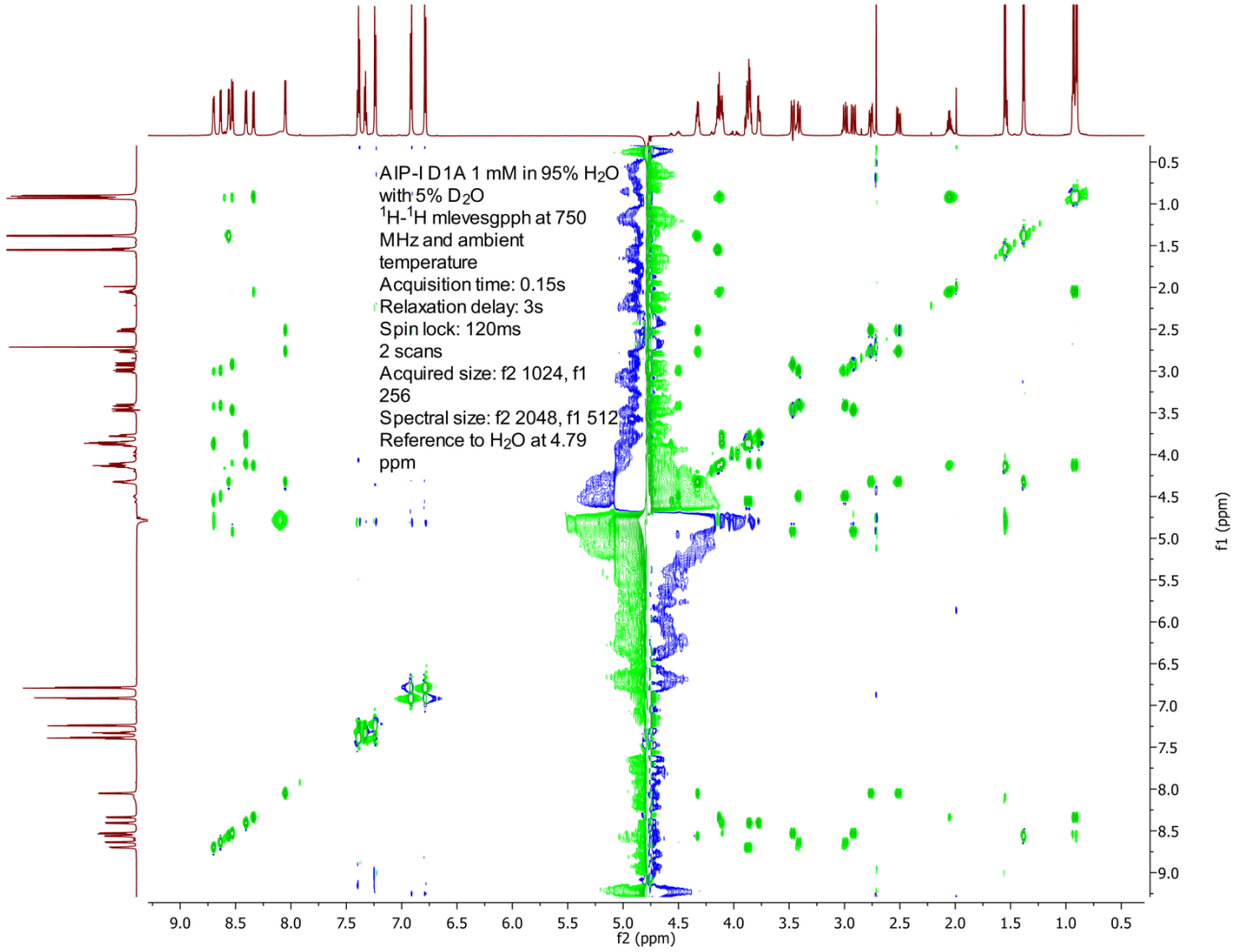

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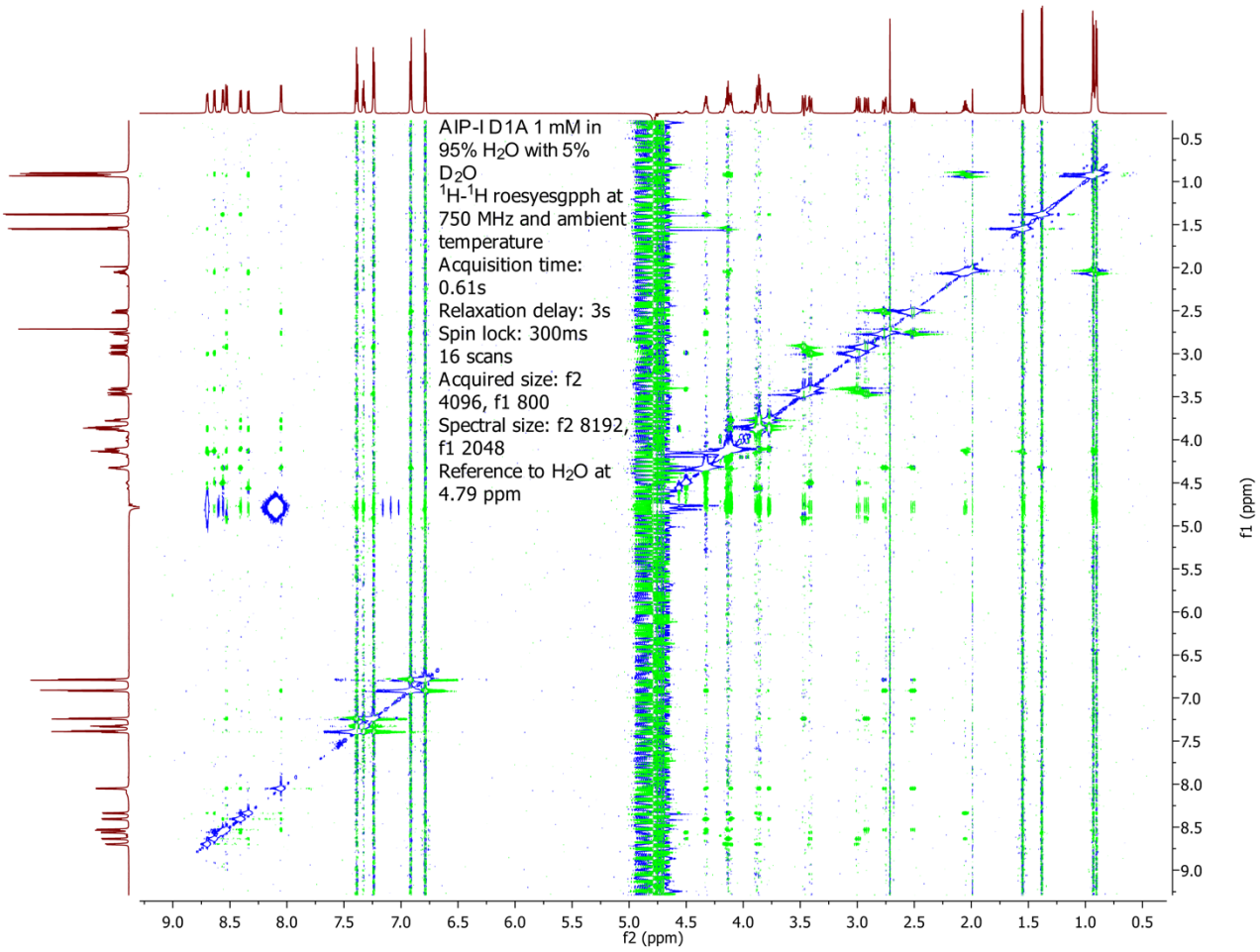
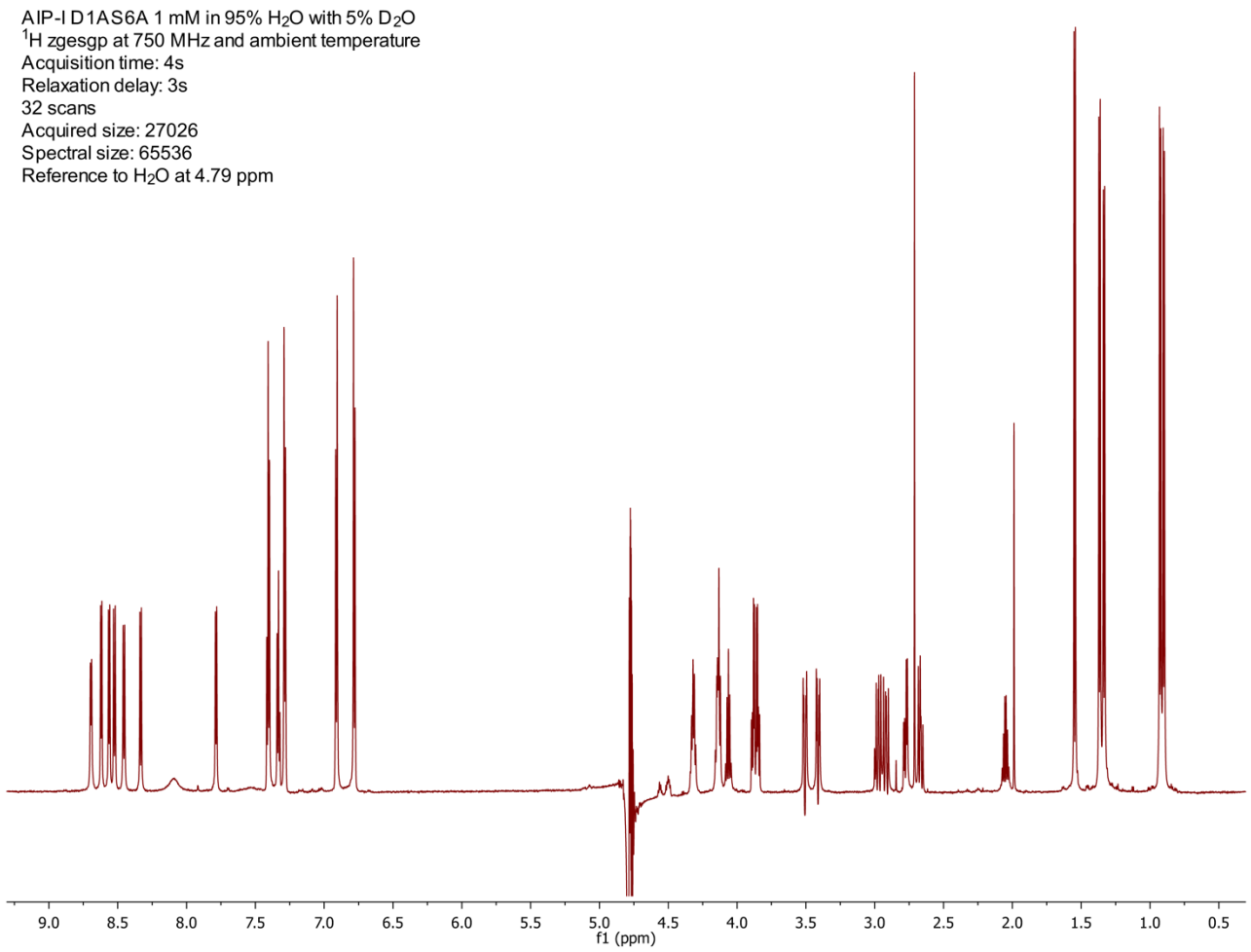
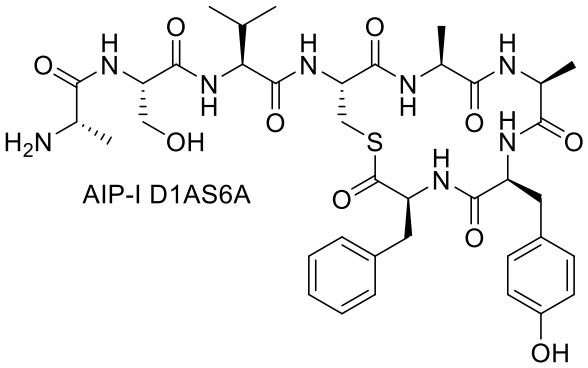
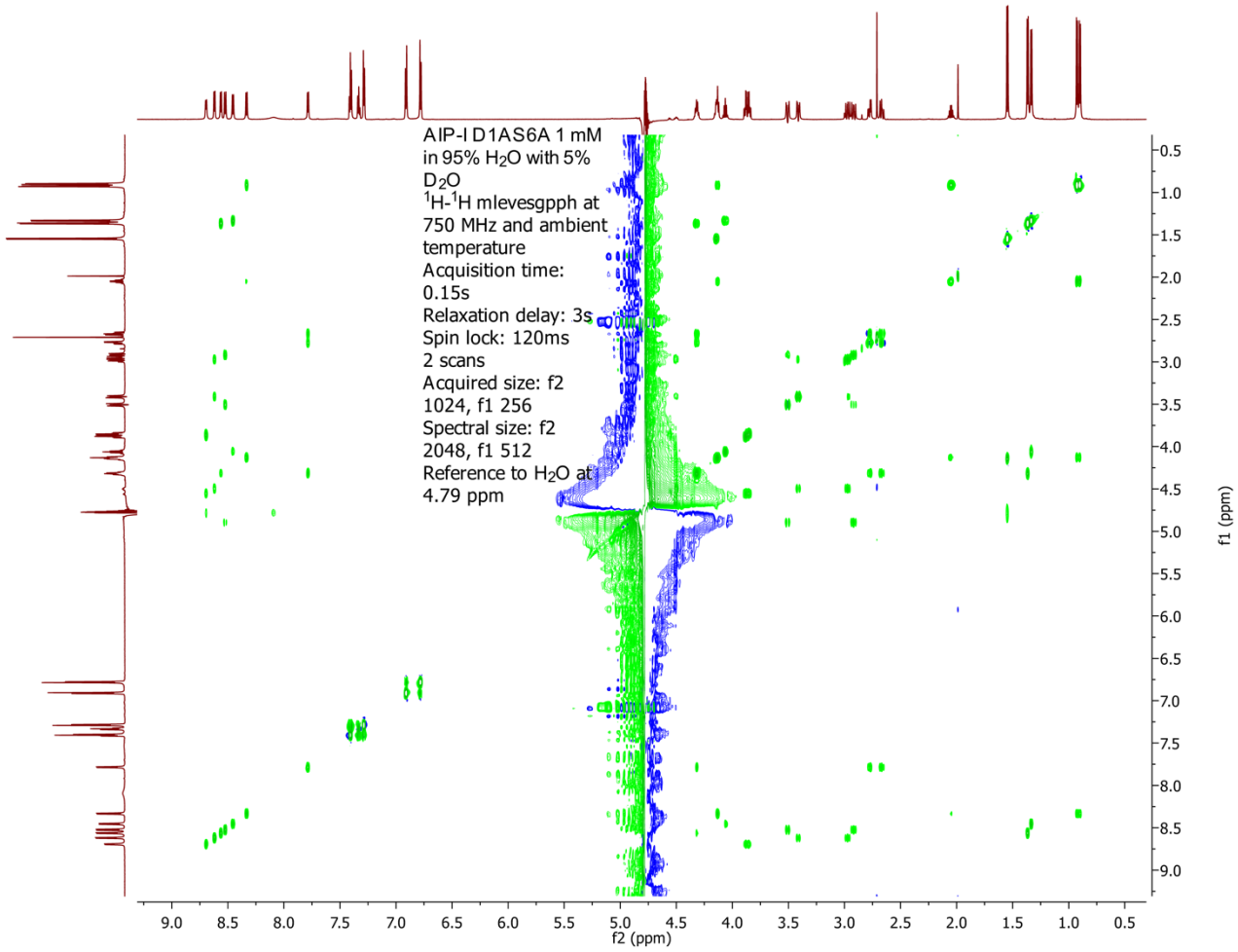


- *AIP-I D1AS6A ^{1}H NMR spectrum (1 mM dissolved in 95% H_2O with 5% D_2O, pH ~6.5)*

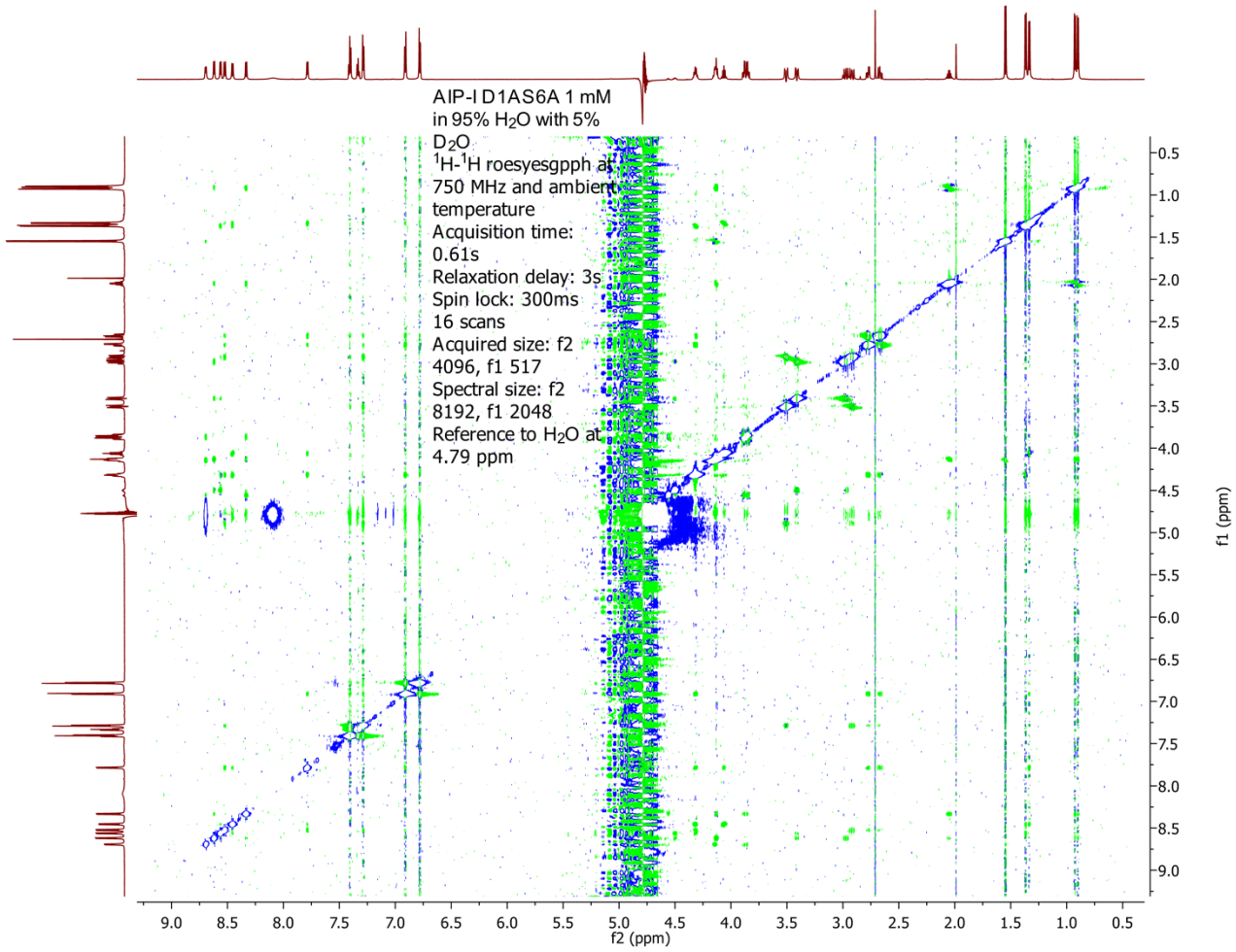




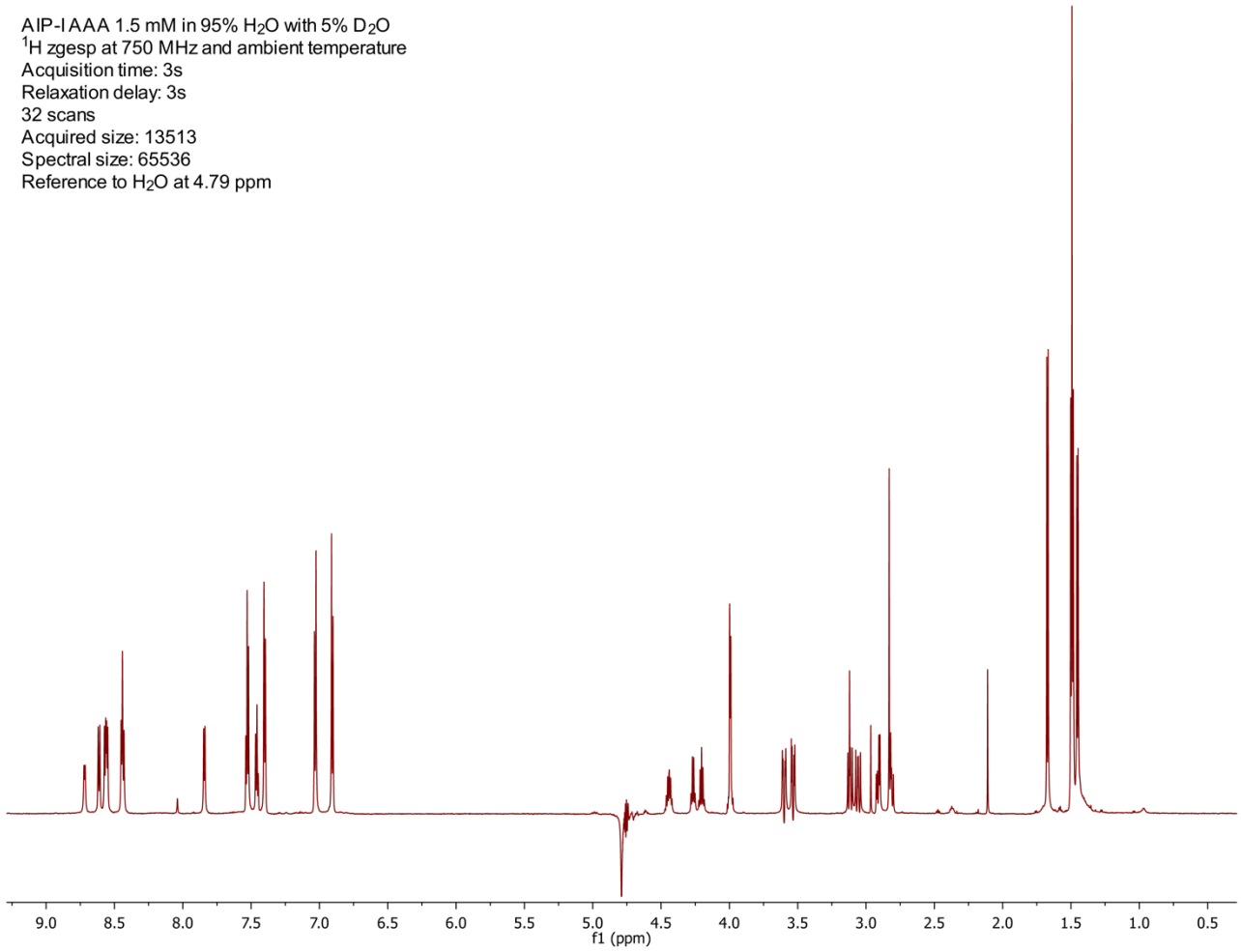
- *AIP-I D1AS6A ^{1}H–^{1}H TOCSY spectrum*

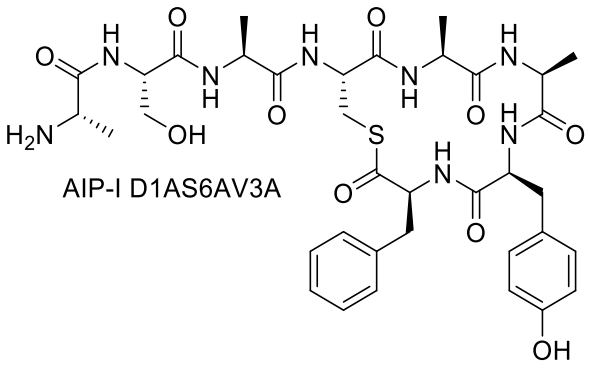


- *AIP-I D1AS6A ^{1}H–^{1}H ROESY spectrum*

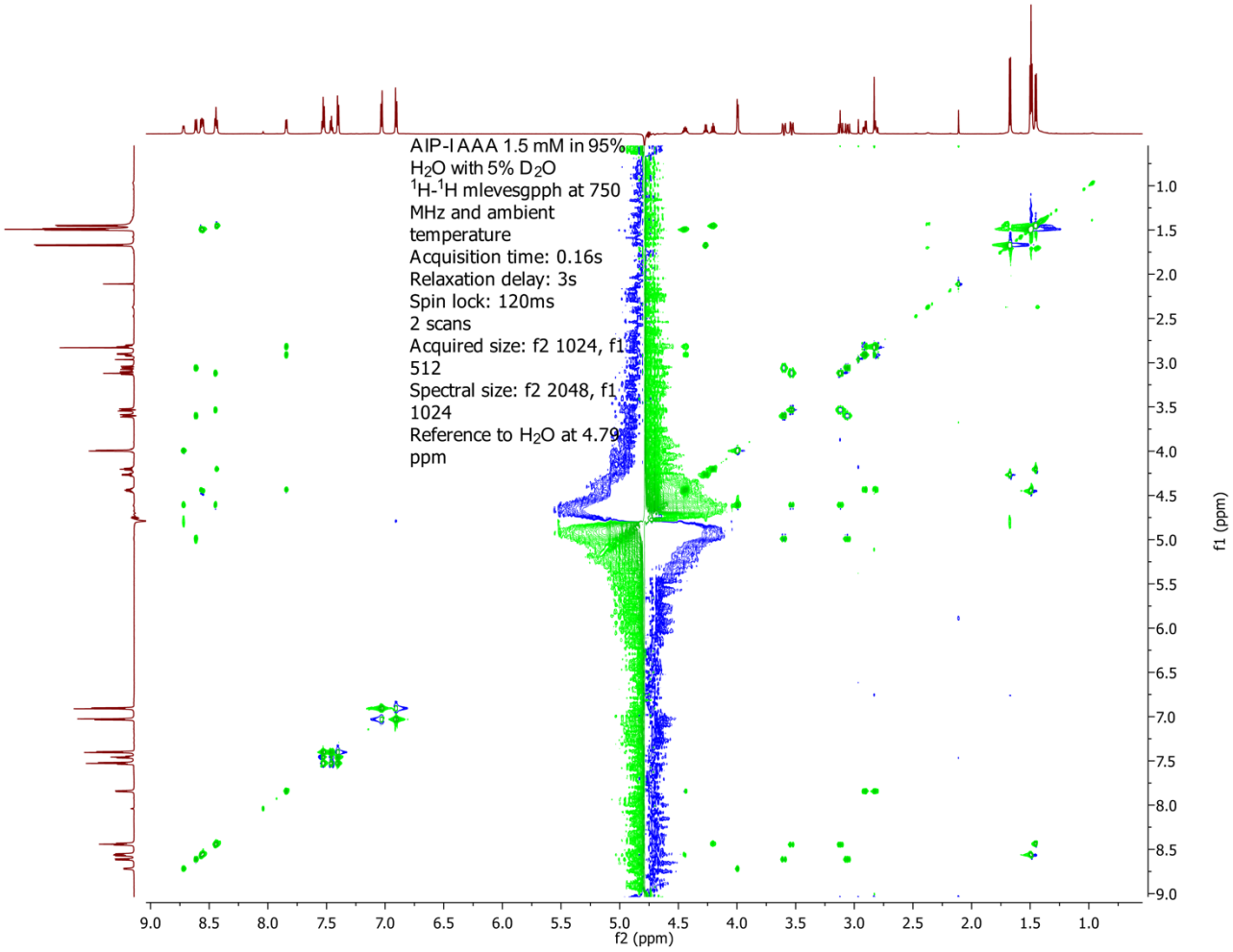


- *AIP-I D1AV3AS6A (AAA) ^{1}H NMR spectrum (1.5 mM dissolved in 95% H_2O with 5% D_2O, pH ~6.5)*





- *AAA ^{1}H–^{1}H TOCSY spectrum*



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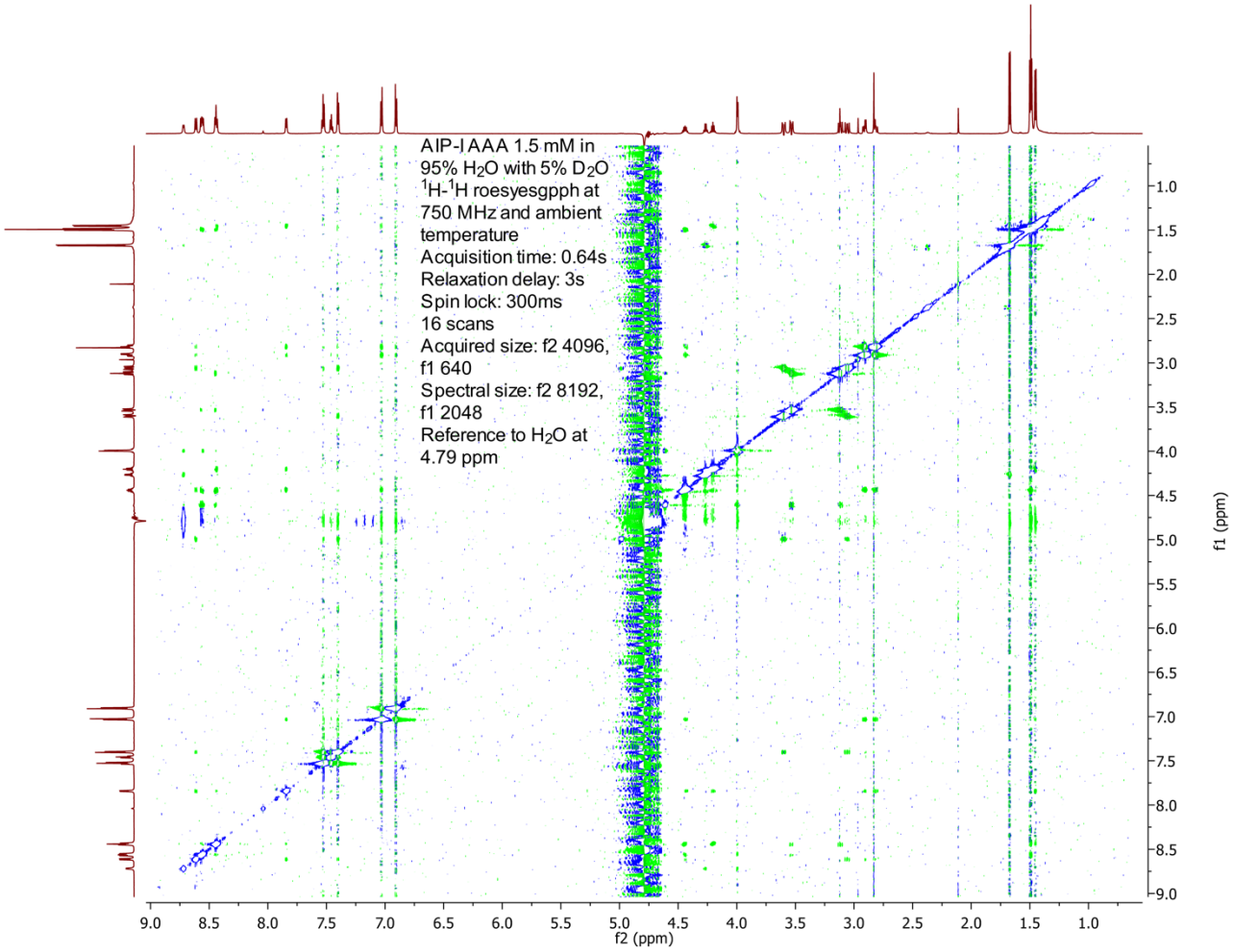
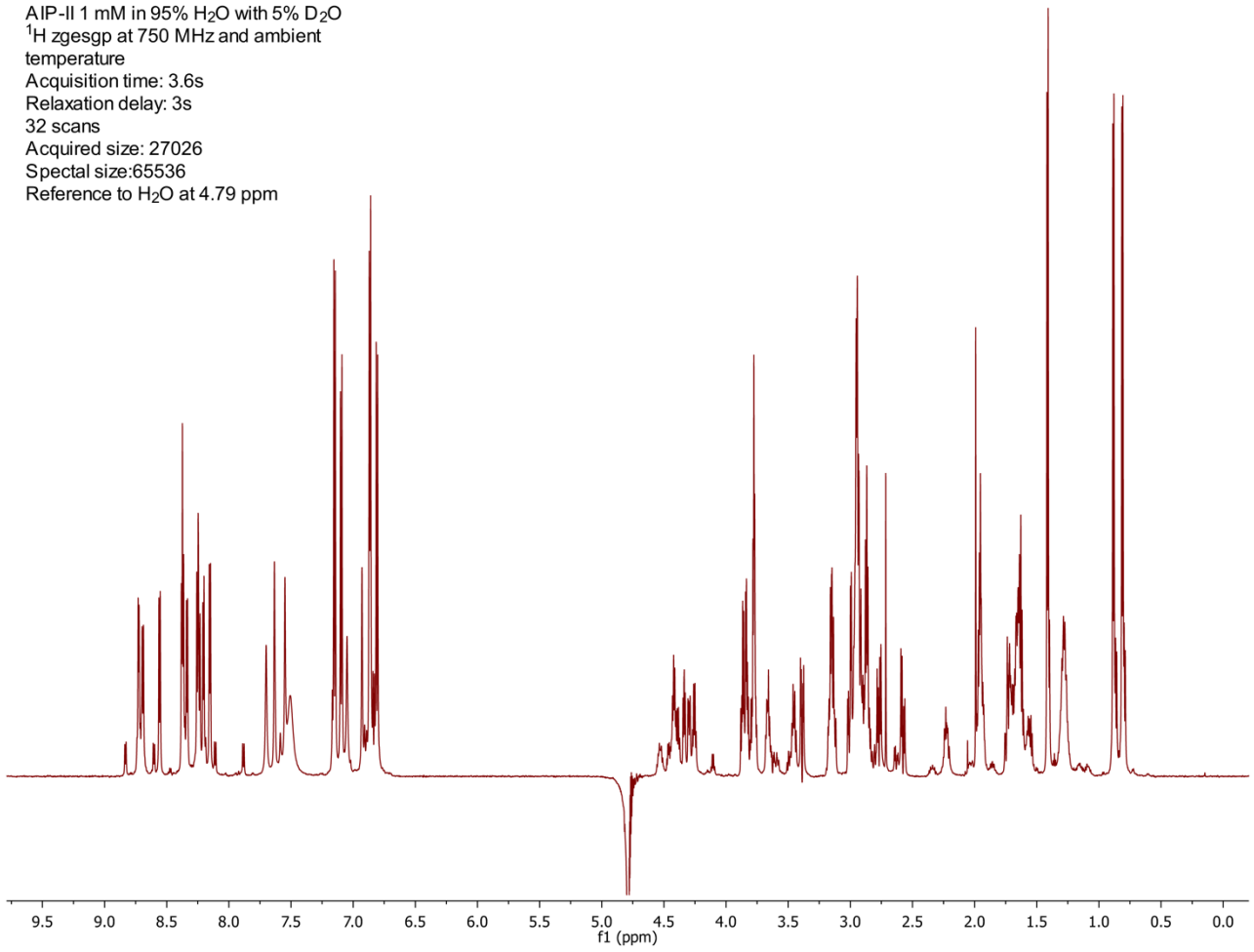
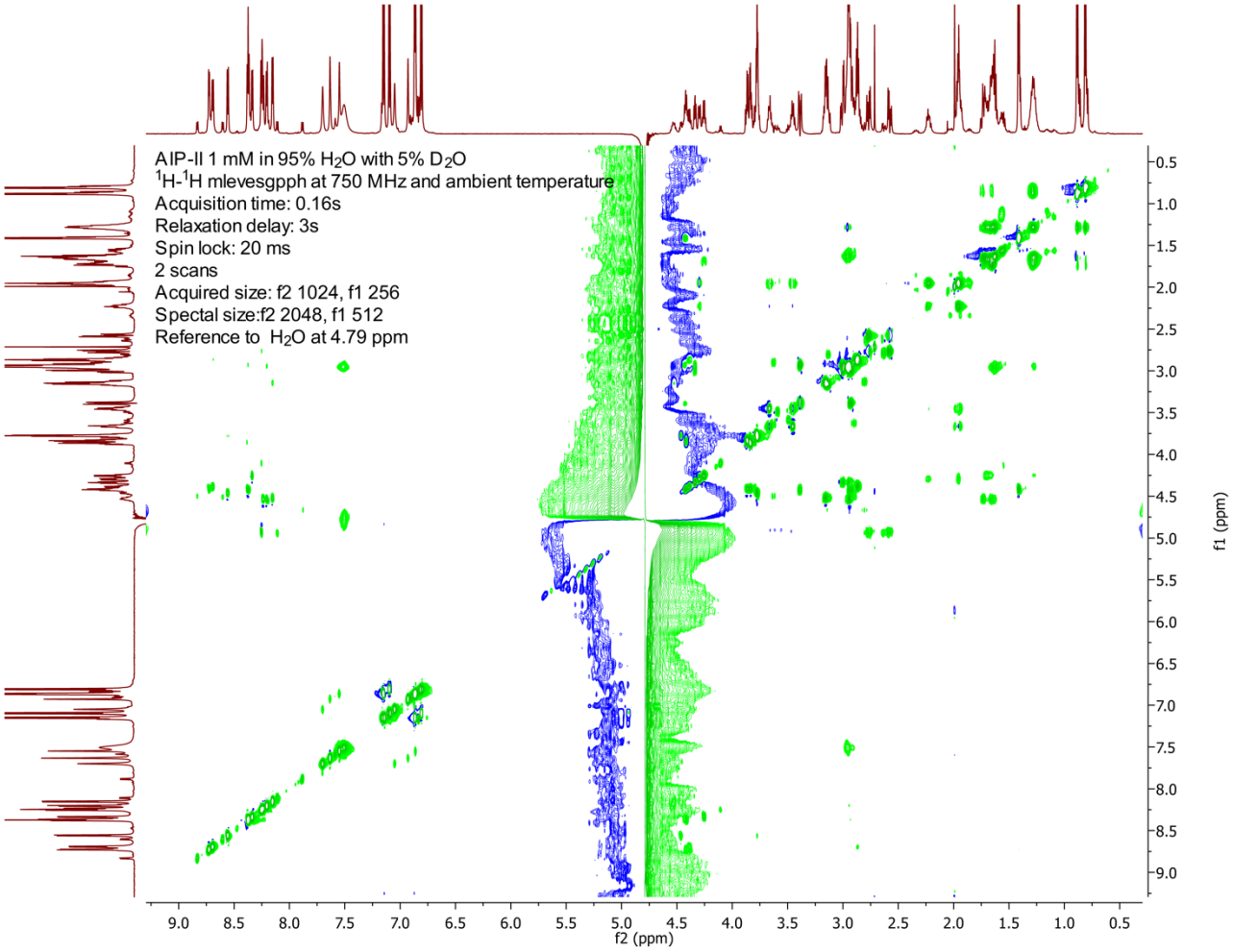


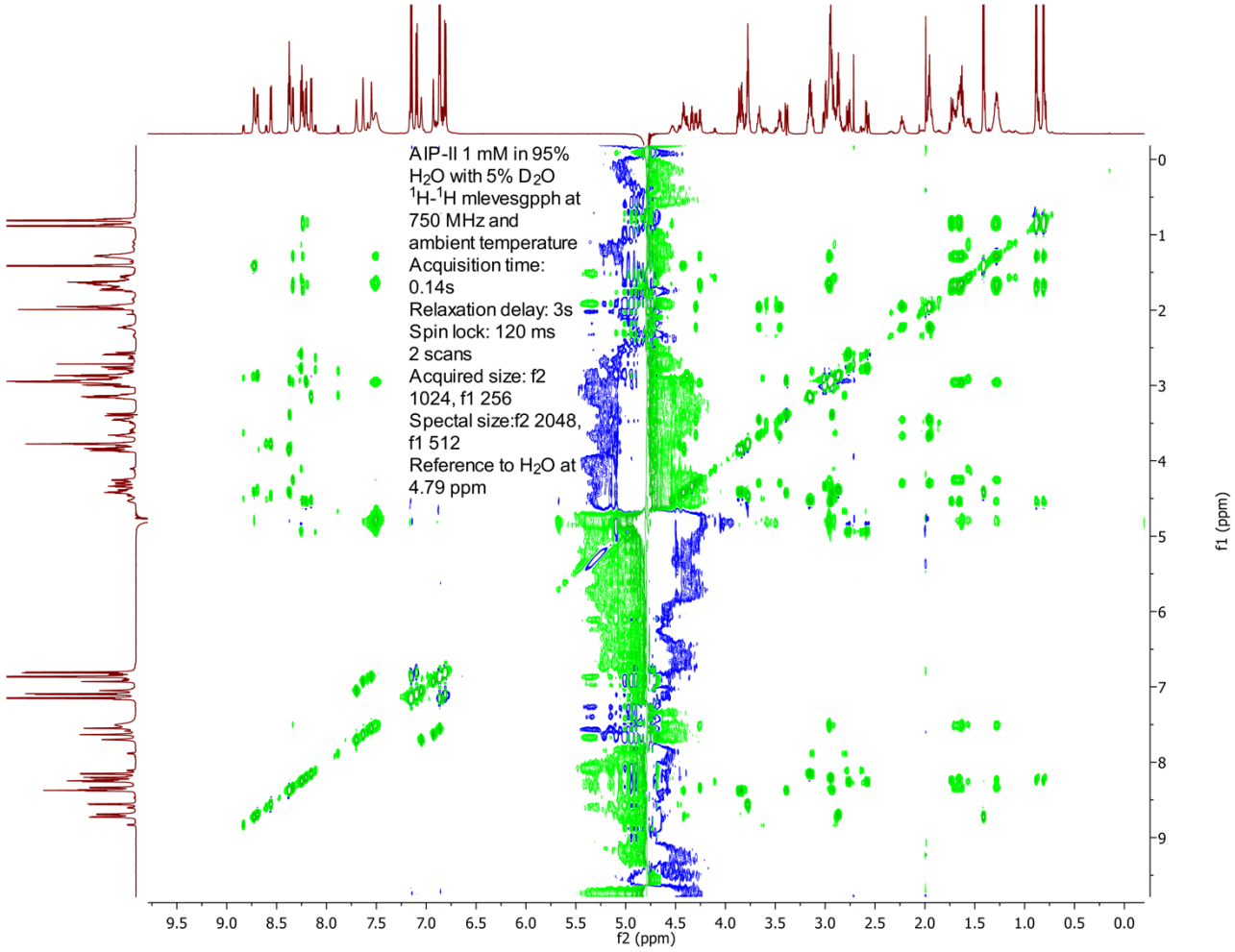
- *AIP-II ^{1}H NMR spectrum (1 mM dissolved in 95% H_2O with 5% D_2O, pH ~6.5)*



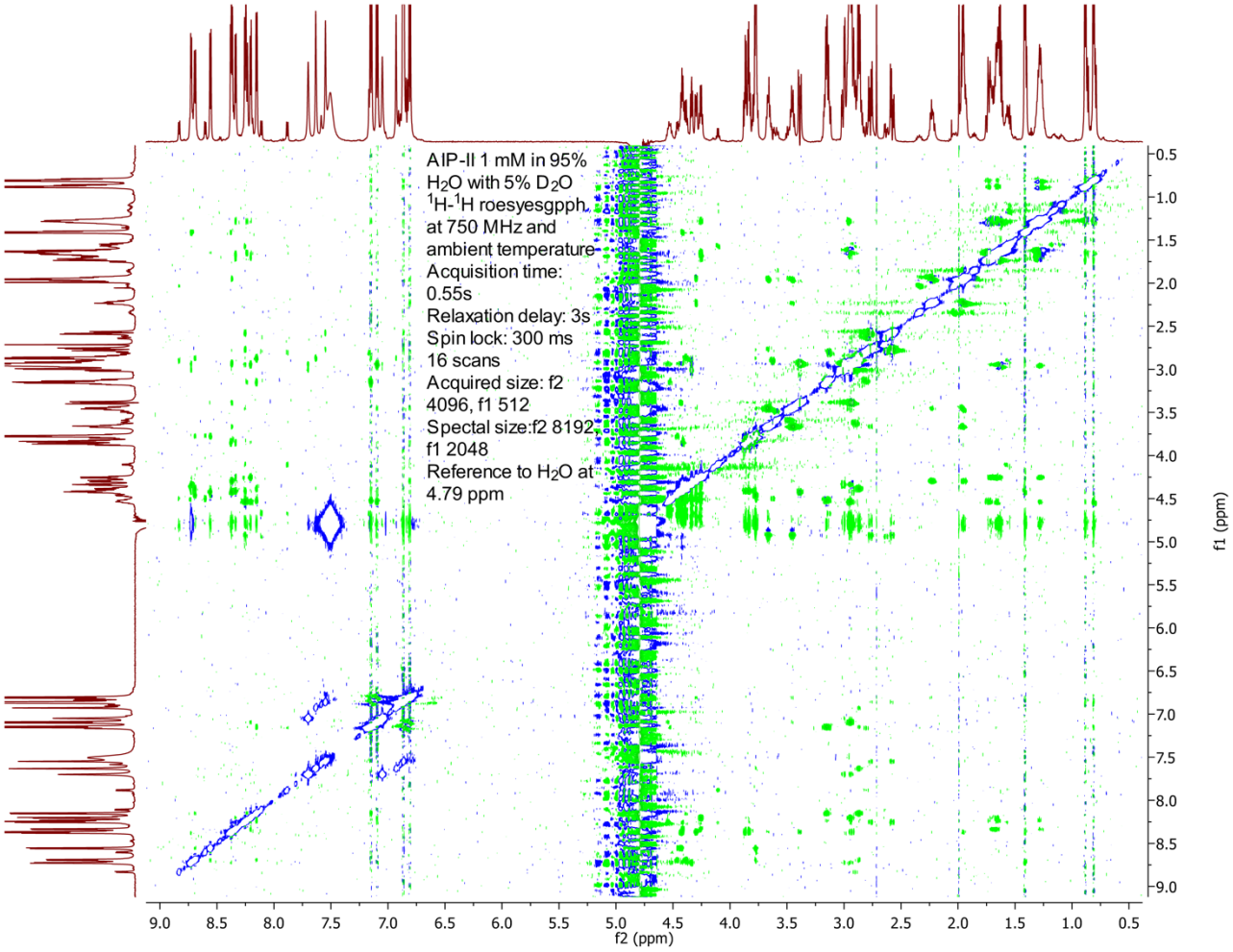
- *AIP-II $^{1}H-^{1}H$ TOCSY spectrum (20 ms spin lock)*



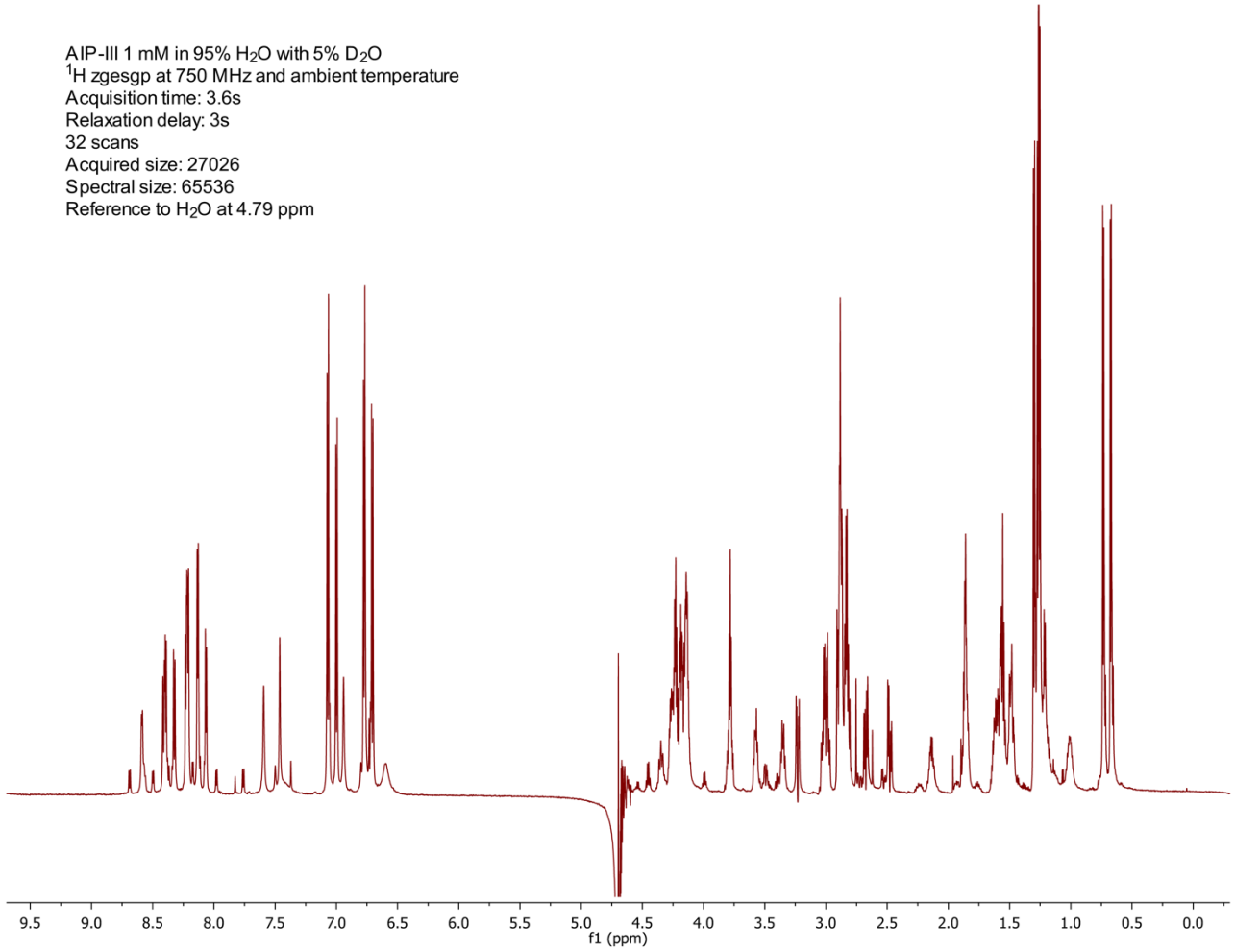
- *AIP-II $^{1}H-^{1}H$ TOCSY spectrum (120 ms spin lock)*



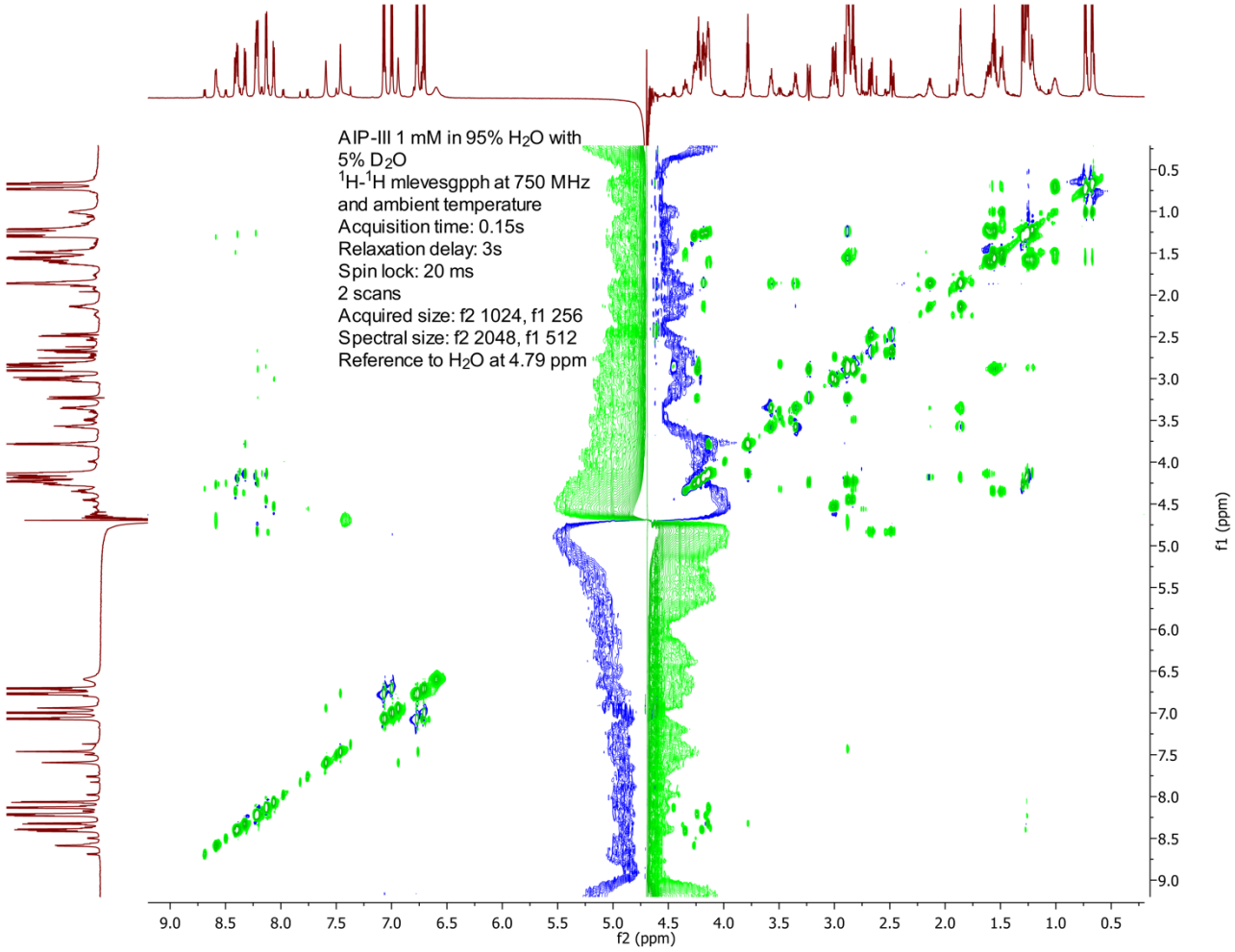
- *AIP-II $^{1}H-^{1}H$ ROESY spectrum*



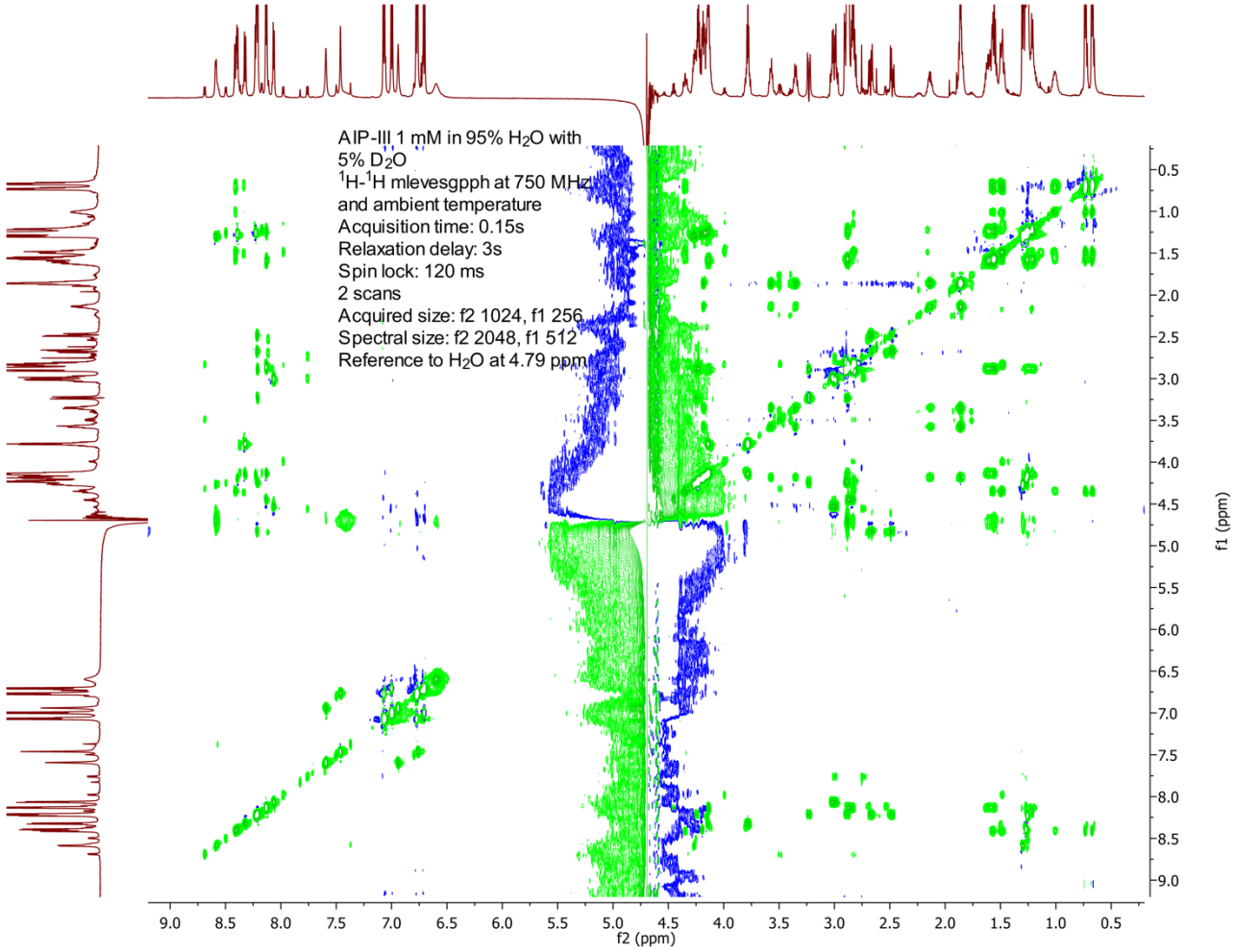
- *AIP-III ^{1}H NMR spectrum (1 mM dissolved in 95% H_2O with 5% D_2O, pH ~6.5)*



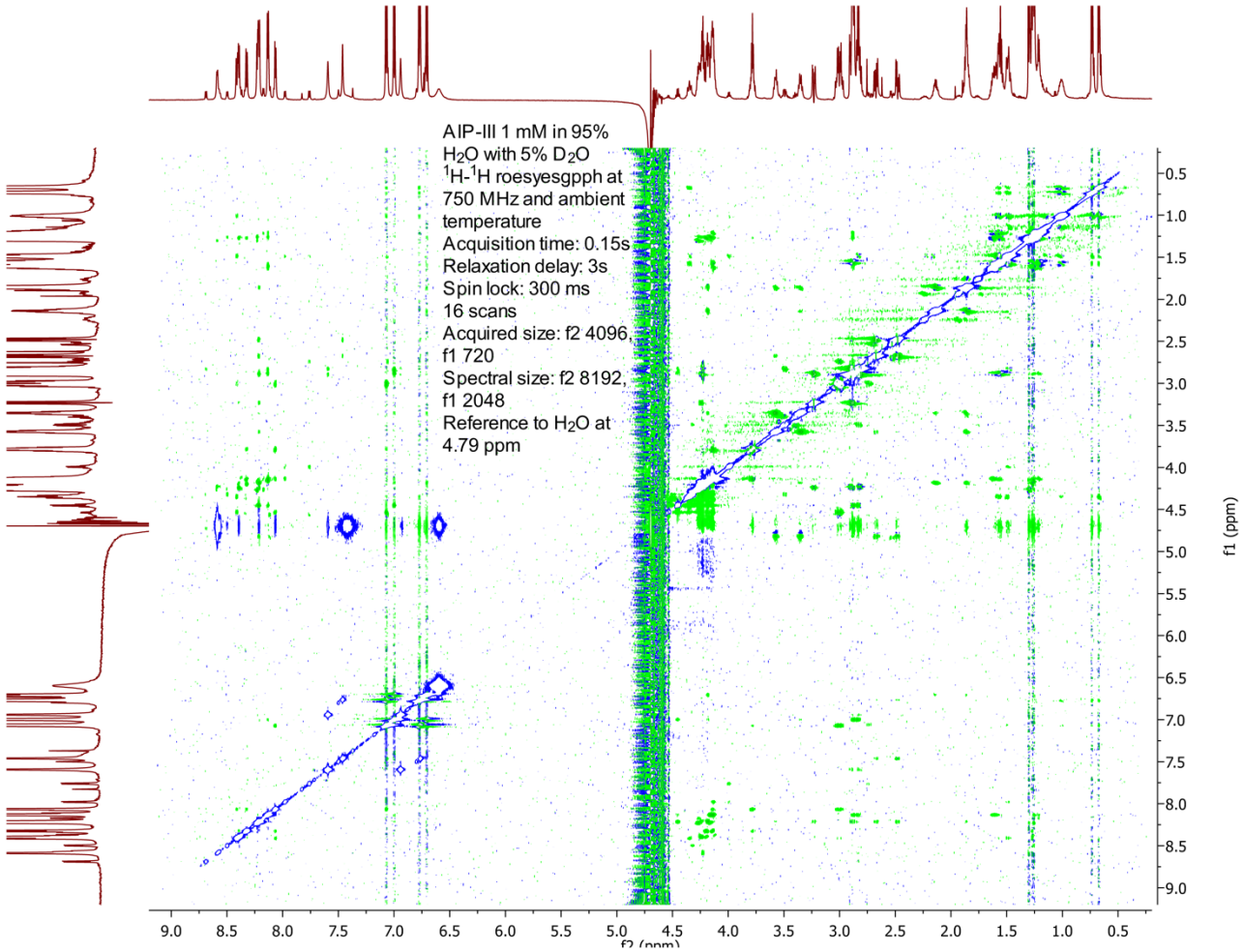
- *AIP-III ^{1}H–^{1}H TOCSY spectrum (20 ms spin lock)*



- *AIP-III ^{1}H–^{1}H TOCSY spectrum (120 ms spin lock)*



- *AIP-III ^{1}H–^{1}H ROESY spectrum*



^3J coupling constants for 3-D solution-phase structures

^3J coupling constants are listed below (if resolved from spectra).

Table S1: Important ^3J coupling constants of AIP-I in Hz

Residue	Hα−HN	Hα−Hβ$_1$	Hα−Hβ$_2$	Hβ$_1$−Hβ$_2$	Hα−Hβ	Hβ−Hγ1	Hβ−Hγ2
Ser2	7.1						
Val3	8.3				8.2	7.2	7.0
Cys4	7.4	11.2	4.7	14.4			
Ala5	7.0				7.5		
Ser6	8.5	4.8*	4.5*	11.6			
Tyr7	6.8	3.8	9.7	14.9			
Phe8	9.3	4.3	10.7	14.9			

** indicates stereochemistry ambiguous, not defined*

Table S2: Important ^3J coupling constants of t-AIP-I in Hz

Residue	Hα−HN	Hα−Hβ$_1$	Hα−Hβ$_2$	Hβ$_1$−Hβ$_2$	Hα−Hβ	Hβ−Hγ1	Hβ−Hγ2
Cys1	7.7	10.6	4.5	13.8			
Ala2	6.7				7.3		
Ser3	8.3	4.8*	6.5*	11.7			
Tyr4	6.5	5.7	9.5	14.8			
Phe5	9.6	4.5	11.0	14.8			

** indicates stereochemistry ambiguous, not defined*

Table S3: Important ^3J coupling constants of AIP-I V3A in Hz

Residue	Hα−HN	Hα−Hβ$_1$	Hα−Hβ$_2$	Hβ$_1$−Hβ$_2$	Hα−Hβ	Hβ−Hγ1	Hβ−Hγ2
Ser2	6.6						
Ala3	6.8				7.8		
Cys4	7.6	10.6	4.5	14.0			
Ala5	6.3				7.7		
Ser6	8.4	5.1*	6.7*	11.6			
Tyr7	6.4	6.2	9.6	14.8			
Phe8	9.4	4.3	11.0	14.9			

** indicates stereochemistry ambiguous, not defined*

Table S4: Important ^3J coupling constants of AIP-I D1A in Hz

Residue	Hα−HN	Hα−Hβ$_1$	Hα−Hβ$_2$	Hβ$_1$−Hβ$_2$	Hα−Hβ	Hβ−Hγ1	Hβ−Hγ2
Ser2	6.9	5.3*	6.9*	11.6			
Val3	8.1				7.9	7.1	7.1
Cys4	7.4	10.8	4.5	14.0			
Ala5	7.0				7.4		
Ser6	8.5	5.2*	6.8*	11.4			
Tyr7	6.7	5.9	9.6	14.9			
Phe8	9.4	4.4	11.0	14.9			

* *indicates stereochemistry ambiguous, not defined*

Table S5: Important ^3J coupling constants of AIP-I D1AS6A in Hz

Residue	Hα−HN	Hα−Hβ$_1$	Hα−Hβ$_2$	Hβ$_1$−Hβ$_2$	Hα−Hβ	Hβ−Hγ1	Hβ−Hγ2
Ser2	7.0	5.3*	6.5*	11.6			
Val3	8.2				8.3	7.2	7.2
Cys4	7.3	10.9	4.6	14.2			
Ala5	7.3				7.5		
Ala6	8.2				7.6		
Tyr7	7.4	6.3	10.0	14.4			
Phe8	9.4	4.0	11.1	15.0			

* *indicates stereochemistry ambiguous, not defined*

Table S6: Important ^3J coupling constants of AIP-I D1AV3AS6S (AAA) in Hz

Residue	Hα−HN	Hα−Hβ$_1$	Hα−Hβ$_2$	Hβ$_1$−Hβ$_2$	Hα−Hβ	Hβ−Hγ1	Hβ−Hγ2
Ser2	6.5						
Ala3	6.4				7.2		
Cys4	7.4	10.4	4.5	14.1			
Ala5	7.0				7.3		
Ala6	8.4				7.3		
Tyr7	7.2	6.2	9.6	14.4			
Phe8	9.3	4.2	10.9	14.9			

* *indicates stereochemistry ambiguous, not defined*

Table S7: Important ^{3}J coupling constants of AIP-II in Hz

Residue	Hα−HN	Hα−Hβ$_1$	Hα−Hβ$_2$	Hβ$_1$−Hβ$_2$	Hα−Hβ	Hγ−Hδ1	Hγ−Hδ2
Ala2	5.7				7.4		
Ser3	6.6	6.1*	5.5*	11.4			
Lys4	7.0						
Tyr5	7.3	7.8*	8.3*	13.9			
Asn6	8.2	7.3*	7.2*	16.0			
Pro7							
Cys8	7.2	11.1	4.7	14.2			
Ser9	8.0	6.3*	5.6*	12.0			
Asn10	8.1	7.8*	6.4*	15.6			
Tyr11	7.4	6.1	9.1	14.1			
Leu12	9.3					7.0	7.0

* *indicates stereochemistry ambiguous, not defined*

Table S8: Important ^{3}J coupling constants of AIP-III in Hz

Residue	Hα−HN	Hα−Hβ$_1$	Hα−Hβ$_2$	Hβ$_1$−Hβ$_2$	Hα−Hβ	Hγ−Hδ1	Hγ−Hδ2
Ala2	5.6				7.4		
Ala3	6.2				7.4		
Lys4	7.2						
Tyr5	7.2	8.4*	7.6*	14.2			
Asn6	7.5	7.5*	7.0*	16.1			
Pro7							
Cys8	7.6	11.3	4.4	14.0			
Ala9	6.8				7.4		
Ser10	8.6	6.5*	5.9*	11.5			
Tyr11	7.2	7.6*	6.8*	14.2			
Leu12	9.3					7.0	7.0

* *indicates stereochemistry ambiguous, not defined*

Chemical shift assignment for 3-D solution-phase structures

Table S9: Assignments for AIP-I

Residue	HN (ppm)	Hα (ppm)	Hβ (ppm)	Others (ppm)
Asp1		4.19	2.75, 2.75	
Ser2	8.57	4.43	3.76, 3.76	
Val3	8.01	4.02	1.97	Hγ 0.80, 0.82
Cys4	8.41	4.37	2.89, 3.28	
Ala5	8.35	4.21	1.28	
Ser6	8.20	4.03	3.65, 3.75	
Tyr7	7.92	4.20	2.40, 2.64	Hδ 6.82; Hε 6.68
Phe8	8.37	4.80	2.82, 3.37	Hδ 7.15; Hε 7.30; Hζ 7.23

Table S10: Assignments for t-AIP-I

Residue	HN (ppm)	Hα (ppm)	Hβ (ppm)	Others (ppm)
Ac		1.89		
Cys1	8.15	4.34	2.89, 3.25	
Ala2	8.20	4.21	1.27	
Ser3	8.03	4.02	3.65, 3.75	
Tyr4	7.88	4.16	2.40, 2.64	Hδ 6.81; Hε 6.66
Phe5	8.29	4.77	2.81, 3.36	Hδ 7.17; Hε 7.29; Hζ 7.22

Table S11: Assignments for AIP-I V3A

Residue	HN (ppm)	Hα (ppm)	Hβ (ppm)	Others (ppm)
Asp1		4.32	3.97, 3.03	
Ser2	8.70	4.41	3.81, 3.81	
Ala3	8.24	4.23	1.30	
Cys4	8.23	4.40	2.93, 3.33	
Ala5	8.48	4.24	1.31	
Ser6	8.38	4.08	3.69, 3.78	
Tyr7	7.98	4.32	2.45, 2.66	Hδ 6.82; Hε 6.70
Phe8	8.48	4.83	2.84, 3.39	Hδ 7.17; Hε 7.31; Hζ 7.24

Table S12: Assignments for AIP-I D1A

Residue	HN (ppm)	Hα (ppm)	Hβ (ppm)	Others (ppm)
Ala1		4.06	1.46	
Ser2	8.61	4.47	3.78, 3.78	
Val3	8.25	4.04	1.97	Hγ 0.81, 0.84
Cys4	8.55	4.41	2.91, 3.32	
Ala5	8.47	4.24	1.29	
Ser6	8.32	4.02	3.68, 3.77	
Tyr7	7.96	4.24	2.42, 2.67	Hδ 6.83; Hε 6.70
Phe8	8.44	4.83	2.83, 3.38	Hδ 7.15; Hε 7.30; Hζ 7.24

Table S13: Assignments for AIP-I D1AS6A

Residue	HN (ppm)	Hα (ppm)	Hβ (ppm)	Others (ppm)
Ala1		4.05	1.46	
Ser2	8.60	4.47	3.76, 3.79	
Val3	8.24	4.04	1.96	Hγ 0.81, 0.84
Cys4	8.53	4.41	2.88, 3.32	
Ala5	8.47	4.22	1.28	
Ala6	8.36	3.97	1.24	
Tyr7	7.69	4.22	2.58, 2.69	Hδ 6.82; Hε 6.69
Phe8	8.43	4.80	2.83, 3.42	Hδ 7.20; Hε 7.32; Hζ 7.24

Table S14: Assignments for AIP-I D1AV3AS6A (AAA)

Residue	HN (ppm)	Hα (ppm)	Hβ (ppm)	Others (ppm)
Ala1		4.18	1.58	
Ser2	8.63	4.52	3.91, 3.91	
Ala3	8.46	4.36	1.41	
Cys4	8.36	4.41	3.03, 3.45	
Ala5	8.48	4.35	1.40	
Ala6	8.35	4.12	1.36	
Tyr7	7.75	4.35	2.73, 2.82	Hδ 6.94; Hε 6.82
Phe8	8.52	4.90	2.97, 3.51	Hδ 7.31; Hε 7.44; Hζ 7.37

Table S15: Assignments for AIP-II

Residue	HN (ppm)	Hα (ppm)	Hβ (ppm)	Others (ppm)
Asn1		4.34	2.94, 3.01	Hδ 7.05, 7.50
Ala2	8.73	4.42	1.41	
Ser3	8.38	4.41	3.83, 3.87	
Lys4	8.34	4.26	1.65, 1.70	Hγ 1.28; Hδ 1.60, 1.66; Hε 2.96
Tyr5	8.20	4.54	2.93, 2.96	Hδ 7.10; Hε 6.81
Asn6	8.25	4.93	2.58, 2.77	Hδ 6.86, 7.55
Pro7		4.30	1.96, 2.23	Hγ 1.94, 1.98; Hδ 3.49, 3.63
Cys8	8.37	4.43	2.93, 3.39	
Ser9	8.56	4.46	3.77, 3.79	
Asn10	8.69	4.39	2.85, 2.89	Hδ 6.93, 7.63
Tyr11	8.15	4.52	3.12, 3.17	Hδ 7.15; Hε 6.87
Leu12	8.24	4.54	1.64, 1.74	Hγ 1.29; Hδ 0.81, 0.88

Table S16: Assignments for AIP-III

Residue	HN (ppm)	Hα (ppm)	Hβ (ppm)	Others (ppm)
Asn1		4.23	2.83, 2.89	Hδ 6.94, 7.59
Ala2	8.59	4.27	1.30	
Ala3	8.23	4.16	1.26	
Lys4	8.13	4.13	1.59, 1.63	Hγ 1.22, 1.26; Hδ 1.54, 1.57; Hε 2.88
Tyr5	8.13	4.45	2.84, 2.88	Hδ 7.00; Hε 6.70
Asn6	8.21	4.82	2.48, 2.67	Hδ 6.76, 7.46
Pro7		4.18	1.87, 2.14	Hγ 1.85, 1.88; Hδ 3.35, 3.57
Cys8	8.21	4.24	2.89, 3.23	
Ala9	8.39	4.20	1.27	
Ser10	8.32	4.14	3.78	
Tyr11	8.06	4.53	2.98, 3.02	Hδ 7.07; Hε 6.77
Leu12	8.41	4.35	1.49, 1.57	Hγ 1.01; Hδ 0.67, 0.74

ROE constraint statistics

AIP-I

```
================================================================
total NOEs in noe-sq.tbl residue 1 to residue 8: 66
================================================================
intra_residual (j-i = 0):  46
sequential  (j-i = 1):     20
medium range (j-i = 2 ):   0
medium range (j-i = 3 ):   0
medium range (j-i = 4 ):   0
long range (j-i >= 5 ):    0

residue 1 has 2 NOEs
residue 2 has 5 NOEs
residue 3 has 9 NOEs
residue 4 has 7 NOEs
residue 5 has 6 NOEs
residue 6 has 8 NOEs
residue 7 has 19 NOEs
residue 8 has 10 NOEs
```

t-AIP-I

```
================================================================
total NOEs in noe-sq.tbl residue 0 to residue 5: 63
================================================================
intra_residual (j-i = 0):  33
sequential  (j-i = 1):     25
medium range (j-i = 2 ):   2
medium range (j-i = 3 ):   1
medium range (j-i = 4 ):   2
long range (j-i >= 5 ):    0

residue 0 has 1 NOEs
residue 1 has 9 NOEs
residue 2 has 10 NOEs
residue 3 has 12 NOEs
residue 4 has 21 NOEs
residue 5 has 10 NOEs
```

AIP-I V3A

```
=====================================================================
total NOEs in noe-sq.tbl residue 1 to residue 8: 78
=====================================================================
intra_residual (j-i = 0):  41
sequential  (j-i = 1):     34
medium range (j-i = 2 ):   1
medium range (j-i = 3 ):   0
medium range (j-i = 4 ):   2
long range (j-i >= 5 ):    0

residue 1 has 7 NOEs
residue 2 has 9 NOEs
residue 3 has 5 NOEs
residue 4 has 9 NOEs
residue 5 has 6 NOEs
residue 6 has 10 NOEs
residue 7 has 20 NOEs
residue 8 has 12 NOEs
```

AIP-I D1A

```
=====================================================================
total NOEs in noe-sq.tbl residue 1 to residue 8: 88
=====================================================================
intra_residual (j-i = 0):  39
sequential  (j-i = 1):     39
medium range (j-i = 2 ):   6
medium range (j-i = 3 ):   0
medium range (j-i = 4 ):   4
long range (j-i >= 5 ):    0

residue 1 has 6 NOEs
residue 2 has 9 NOEs
residue 3 has 13 NOEs
residue 4 has 12 NOEs
residue 5 has 6 NOEs
residue 6 has 15 NOEs
residue 7 has 18 NOEs
residue 8 has 9 NOEs
```

AIP-I D1AS6A

```
================================================================
total NOEs in noe-sq.tbl residue 1 to residue 8: 74
================================================================
intra_residual (j-i = 0):  37
sequential  (j-i = 1):     30
medium range (j-i = 2 ):   2
medium range (j-i = 3 ):   1
medium range (j-i = 4 ):   4
long range (j-i >= 5 ):    0

residue 1 has 2 NOEs
residue 2 has 7 NOEs
residue 3 has 12 NOEs
residue 4 has 11 NOEs
residue 5 has 6 NOEs
residue 6 has 8 NOEs
residue 7 has 20 NOEs
residue 8 has 8 NOEs
```

AIP-I D1AV3AS6A (AAA)

```
================================================================
total NOEs in noe-sq.tbl residue 1 to residue 8: 72
================================================================
intra_residual (j-i = 0):  32
sequential  (j-i = 1):     31
medium range (j-i = 2 ):   1
medium range (j-i = 3 ):   1
medium range (j-i = 4 ):   7
long range (j-i >= 5 ):    0

residue 1 has 3 NOEs
residue 2 has 6 NOEs
residue 3 has 5 NOEs
residue 4 has 15 NOEs
residue 5 has 7 NOEs
residue 6 has 7 NOEs
residue 7 has 20 NOEs
residue 8 has 9 NOEs
```

AIP-II

```
=====================================================================
total NOEs in noe-sq.tbl residue 1 to residue 12: 158
=====================================================================
intra_residual (j-i = 0):  82
sequential  (j-i = 1):     56
medium range (j-i = 2 ):   14
medium range (j-i = 3 ):   5
medium range (j-i = 4 ):   0
long range (j-i >= 5 ):    1

residue 1 has 11 NOEs
residue 2 has 6 NOEs
residue 3 has 16 NOEs
residue 4 has 16 NOEs
residue 5 has 19 NOEs
residue 6 has 16 NOEs
residue 7 has 13 NOEs
residue 8 has 8 NOEs
residue 9 has 9 NOEs
residue 10 has 11 NOEs
residue 11 has 20 NOEs
residue 12 has 13 NOEs
```

AIP-III

```
=====================================================================
total NOEs in noe-sq.tbl residue 1 to residue 12: 137
=====================================================================
intra_residual (j-i = 0):  75
sequential  (j-i = 1):     52
medium range (j-i = 2 ):   8
medium range (j-i = 3 ):   0
medium range (j-i = 4 ):   2
long range (j-i >= 5 ):    0

residue 1 has 11 NOEs
residue 2 has 8 NOEs
residue 3 has 6 NOEs
residue 4 has 12 NOEs
residue 5 has 17 NOEs
residue 6 has 13 NOEs
residue 7 has 13 NOEs
residue 8 has 11 NOEs
residue 9 has 6 NOEs
residue 10 has 8 NOEs
residue 11 has 21 NOEs
residue 12 has 11 NOEs
```

Additional images of structure ensembles

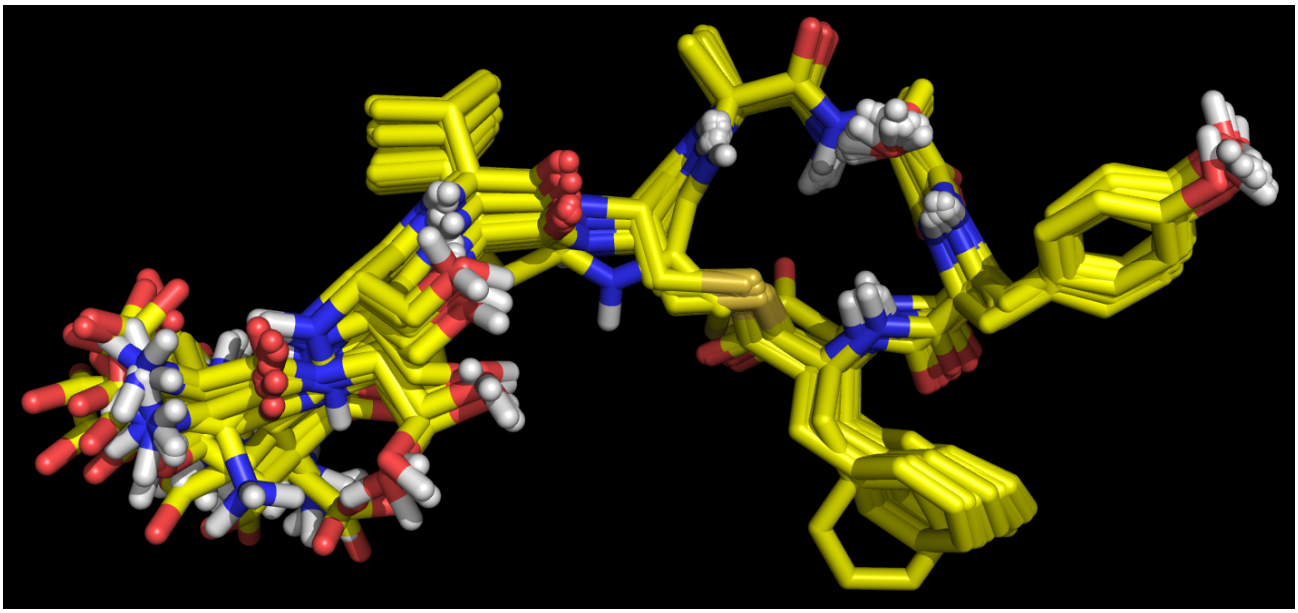


Figure S1: Heavy atom ensemble of the 20 lowest-energy structures for AIP-I. Backbone RMSD from average = 0.4 Å, heavy atom RMSD from average = 1.2 Å.

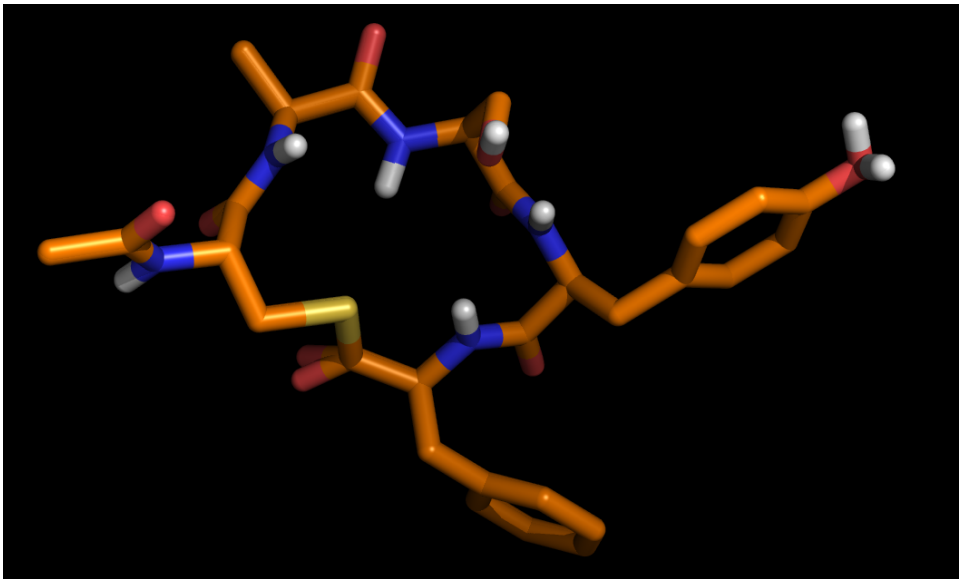


Figure S2: Heavy atom ensemble of the 20 lowest-energy structures for t-AIP-I. Backbone RMSD from average = 0.1 Å, heavy atom RMSD from average = 0.5 Å.

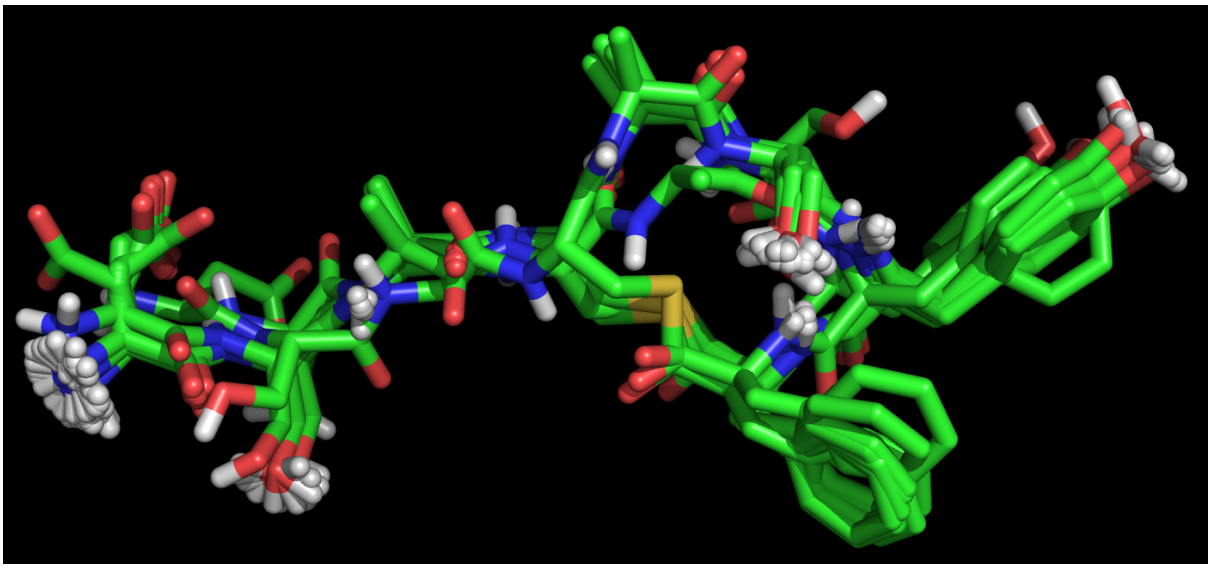


Figure S3: Heavy atom ensemble of the 20 lowest-energy structures for AIP-I V3A. Backbone RMSD from average = 0.3 Å, heavy atom RMSD from average = 1.0 Å.

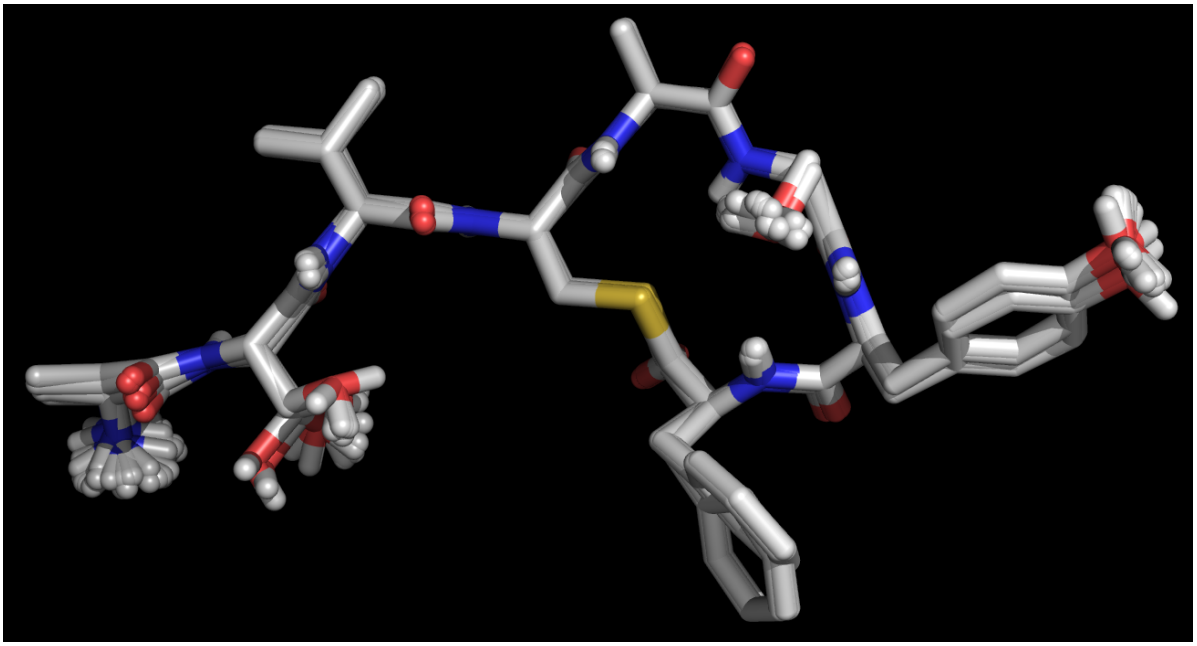


Figure S4: Heavy atom ensemble of the 20 lowest-energy structures for AIP-I D1A. Backbone RMSD from average = 0.1 Å, heavy atom RMSD from average = 0.7 Å.

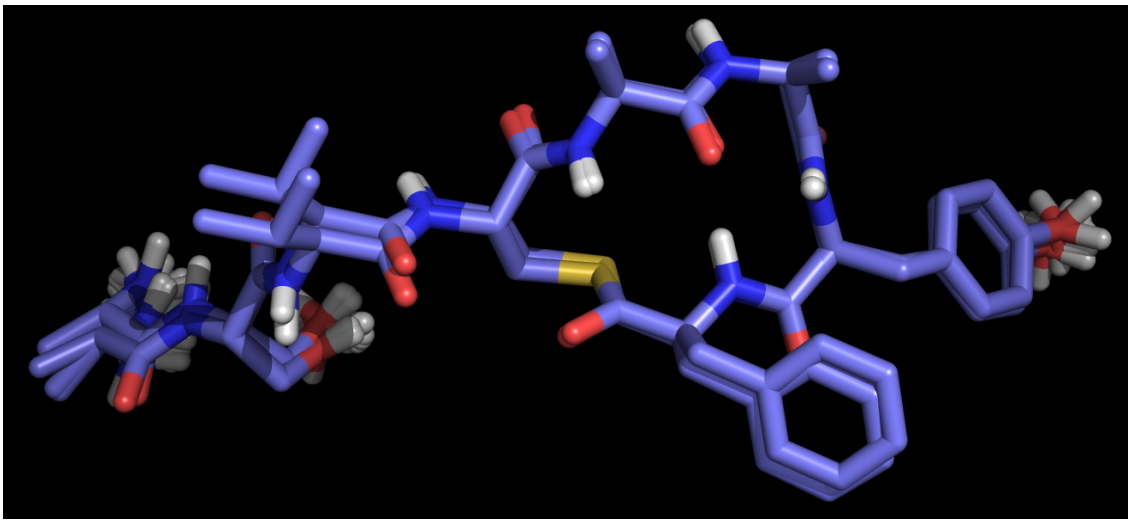


Figure S5: Heavy atom ensemble of the 20 lowest-energy structures for AIP-I D1AS6A. Backbone RMSD from average = 0.1 Å, heavy atom RMSD from average = 0.5 Å.

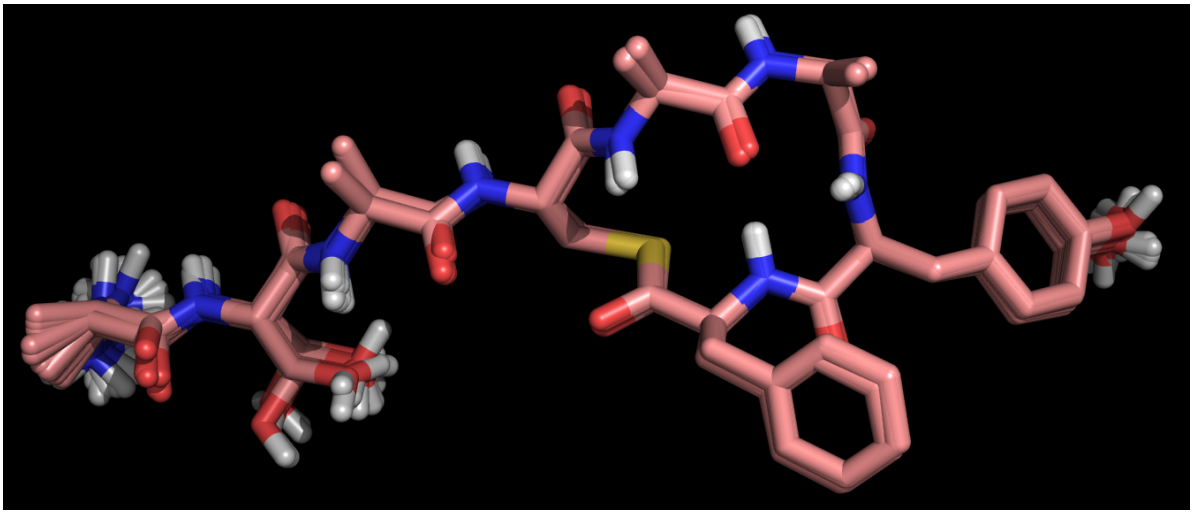


Figure S6: Heavy atom ensemble of the 20 lowest-energy structures for AIP-I D1AV3AS6A (AAA). Backbone RMSD from average = 0.2 Å, heavy atom RMSD from average = 0.6 Å.

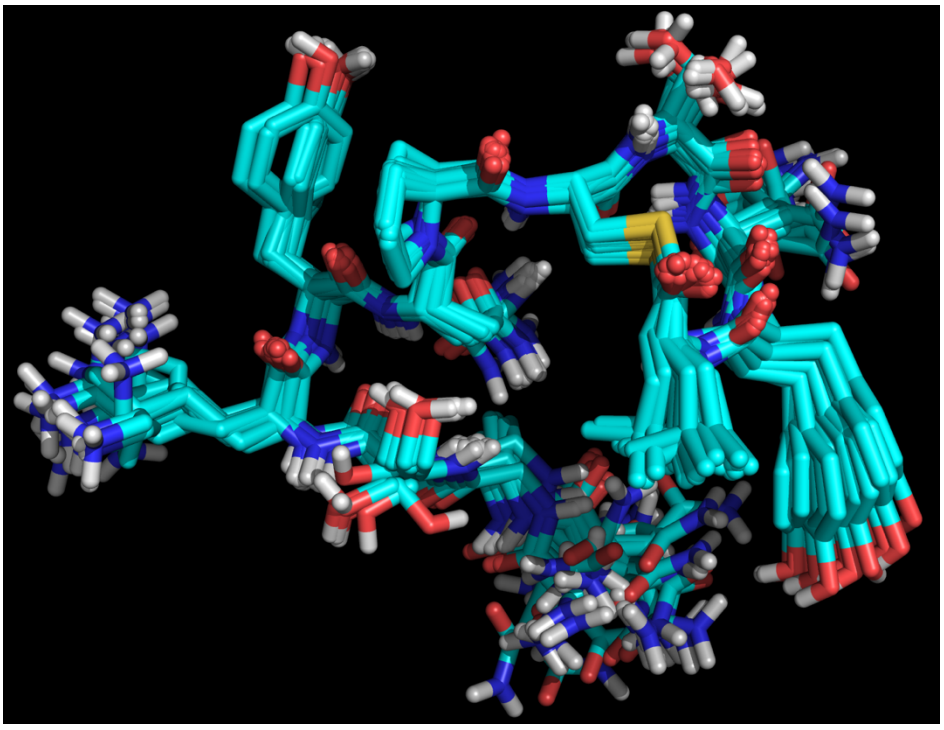


Figure S7: Heavy atom ensemble of the 20 lowest-energy structures for AIP-II. Backbone RMSD from average = 0.4 Å, heavy atom RMSD from average = 1.1 Å.

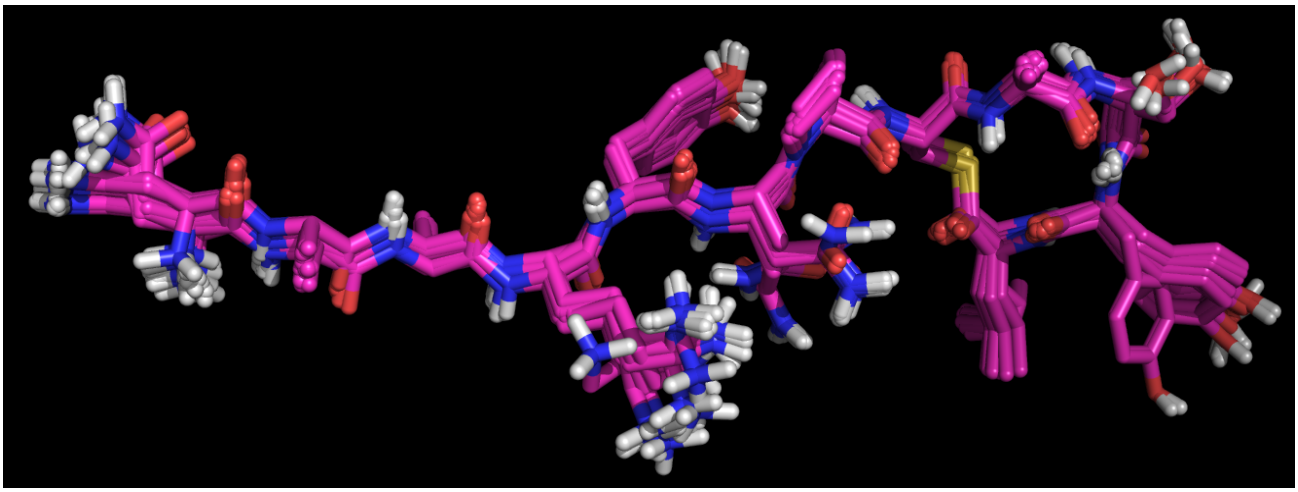


Figure S8: Heavy atom ensemble of the 20 lowest-energy structures for AIP-III. Backbone RMSD from average = 0.3 Å, heavy atom RMSD from average = 0.9 Å.

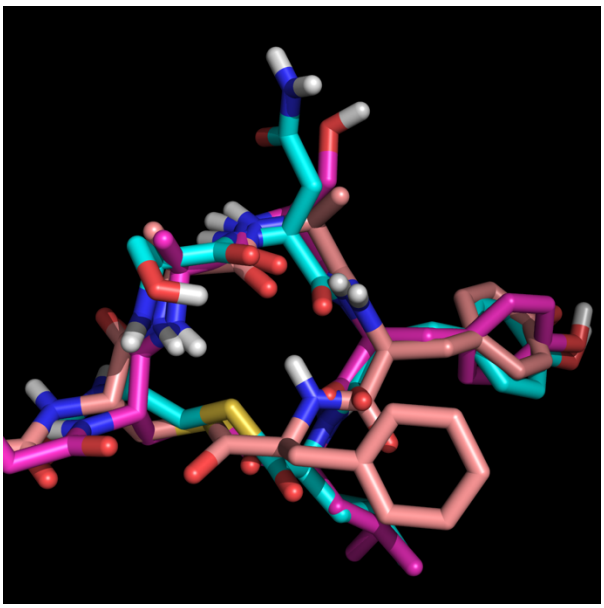


Figure S9: Overlay of the macrocycles from AAA, AIP-II, and AIP-III. The tails are removed for clarity. All-atom RMS difference of AIP-II to AAA = 1.9 Å, and all-atom RMS difference of AIP-III to AAA = 1.2 Å.

Validation using MolProbity

- *Analysis of AIP-I representative structure*

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All-Atom Contacts	Clashscore, all atoms:	0		100th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	0	0.00%	Goal: <0.3%
	Favored rotamers	7	100.00%	Goal: >98%
	Ramachandran outliers	1	16.67%	Goal: <0.05%
	Ramachandran favored	4	66.67%	Goal: >98%
	MolProbity score^	1.37		98th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 62	0.00%	Goal: 0%
	Bad angles:	1 / 83	1.20%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	0 / 0	0.00%	Expected: ≤1 per chain, or ≤5%

In the two column results, the left column gives the raw count, right column gives the percentage.

* 100th percentile is the best among structures of comparable resolution; 0th percentile is the worst. For clashscore the comparative set of structures was selected in 2004, for MolProbity score in 2006.

^ MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

#	Alt	Res	High B	Clash > 0.4Å	Ramachandran	Rotamer	Cβ deviation	Bond lengths	Bond angles	Cis Peptides
			Avg: 0.00	Clashscore: 0	Outliers: 1 of 6	Poor rotamers: 0 of 7	Outliers: 0 of 8	Outliers: 0 of 8	Outliers: 1 of 8	Non-Trans: 0 of 7
1		ASP	0	-	-	Favored (98.1%) *m-30* chi angles: 286.9,167.7	0.06Å	-	-	-
2		SER	0	-	Favored (24.33%) General / -153.6,146.4	Favored (46.9%) *t* chi angles: 180.2	0.03Å	-	-	-
3		VAL	0	-	Favored (70.89%) Ile or Val / -127.0,128.6	Favored (83.5%) *t* chi angles: 177	0.03Å	-	-	-
4		CYS	0	-	Favored (33%) General / -125.3,121.1	Favored (52.5%) *t* chi angles: 180.6	0.05Å	-	-	-
5		ALA	0	-	Allowed (1.67%) General / -135.0,61.5	-	0.04Å	-	-	-
6		SER	0	-	OUTLIER (0)%) General / -174.5,-97.4	Favored (69.9%) *p* chi angles: 58.9	0.02Å	-	-	-
7		TYR	0	-	Favored (81.51%) General / -68.0,-37.6	Favored (67%) *m-80* chi angles: 286.9,99.5	0.02Å	-	-	-
8		PHE	0	-	-	Favored (70.2%) *m-80* chi angles: 288.5,102.5	0.04Å	-	OUTLIER(S) worst is CA-CB-CG: 4.1 σ	-

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- *Analysis of AIP-II representative structure*

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All-Atom Contacts	Clashscore, all atoms:	5.46		92nd percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	1	9.09%	Goal: <0.3%
	Favored rotamers	9	81.82%	Goal: >98%
	Ramachandran outliers	3	30.00%	Goal: <0.05%
	Ramachandran favored	5	50.00%	Goal: >98%
	MolProbity score^	2.99		23rd percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 97	0.00%	Goal: 0%
	Bad angles:	0 / 131	0.00%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	0 / 1	0.00%	Expected: ≤1 per chain, or ≤5%

In the two column results, the left column gives the raw count, right column gives the percentage.

* 100th percentile is the best among structures of comparable resolution; 0th percentile is the worst. For clashscore the comparative set of structures was selected in 2004, for MolProbity score in 2006.

^ MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

#	Alt	Res	High B	Clash > 0.4Å	Ramachandran	Rotamer	Cβ deviation	Bond lengths	Bond angles	Cis Peptides
			Avg: 0.00	Clashscore: 5.46	Outliers: 3 of 10	Poor rotamers: 1 of 11	Outliers: 0 of 12	Outliers: 0 of 12	Outliers: 0 of 12	Non-Trans: 0 of 11
1		ASN	0	-	-	Favored (2.4%) *m-40* chi angles: 263.1,17.6	0.06Å	-	-	-
2		ALA	0	-	Favored (2.63%) General / -160.8,120.1	-	0.03Å	-	-	-
3		SER	0	0.40Å O with 5 TYR N	Favored (36.09%) General / -71.8,158.4	Favored (86.9%) *p* chi angles: 67.1	0.03Å	-	-	-
4		LYS	0	-	OUTLIER (0.01)%) General / -51.0,94.6	Favored (50.7%) *tttp* chi angles: 179.1,179.7,177.4,62.7	0.06Å	-	-	-
5		TYR	0	0.40Å N with 3 SER O	Favored (2.17%) General / 60.6,59.4	Favored (37.7%) *t80* chi angles: 193.4,258.6	0.03Å	-	-	-
6		ASN	0	-	Allowed (1.66%) Pre-Pro / -49.1,110.9	Allowed (1.2%) *p0* chi angles: 40.5,21.7	0.07Å	-	-	-
7		PRO	0	-	Favored (3.38%) Trans-Pro / -81.8,50.1	Favored (82.3%) *Cg_endo* chi angles: 30.5,330.4,16.7	0.05Å	-	-	-
8		CYS	0	-	Favored (35.81%) General / -136.7,133.1	Favored (23.4%) *t* chi angles: 173.3	0.04Å	-	-	-
9		SER	0	-	Allowed (0.48%) General / -69.9,84.3	Favored (7.3%) *p* chi angles: 49.1	0.04Å	-	-	-
10		ASN	0	-	OUTLIER (0.01)%) General / 62.9,127.7	Favored (98.9%) *m-40* chi angles: 289.4,342	0.02Å	-	-	-
11		TYR	0	-	OUTLIER (0.01)%) General / 44.6,90.9	OUTLIER (0.2%) chi angles: 239.9,256.5	0.01Å	-	-	-
12		LEU	0	-	-	Favored (5.8%) *mt* chi angles: 315,189.5	0.04Å	-	-	-

- *Analysis of AIP-III representative structure*

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All-Atom Contacts	Clashscore, all atoms:	0		100th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	3	33.33%	Goal: <0.3%
	Favored rotamers	6	66.67%	Goal: >98%
	Ramachandran outliers	0	0.00%	Goal: <0.05%
	Ramachandran favored	9	90.00%	Goal: >98%
	MolProbity score^	2.21		64th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 93	0.00%	Goal: 0%
	Bad angles:	0 / 126	0.00%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	0 / 1	0.00%	Expected: ≤1 per chain, or ≤5%

In the two column results, the left column gives the raw count, right column gives the percentage.

* 100th percentile is the best among structures of comparable resolution; 0th percentile is the worst. For clashscore the comparative set of structures was selected in 2004, for MolProbity score in 2006.

^ MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

#	Alt	Res	High B	Clash > 0.4Å	Ramachandran	Rotamer	Cβ deviation	Bond lengths	Bond angles	Cis Peptides
			Avg: 0.00	Clashscore: 0	Outliers: 0 of 10	Poor rotamers: 3 of 9	Outliers: 0 of 12	Outliers: 0 of 12	Outliers: 0 of 12	Non-Trans: 0 of 11
1		ASN	0	-	-	Favored (43.5%) *t0* chi angles: 189.1,359.7	0.06Å	-	-	-
2		ALA	0	-	Favored (17.96%) General / -156.1,141.9	-	0.04Å	-	-	-
3		ALA	0	-	Favored (33.97%) General / -149.2,151.2	-	0.04Å	-	-	-
4		LYS	0	-	Favored (39.71%) General / -106.7,119.6	Favored (51.5%) *tttp* chi angles: 181,178.6,177.5,62.4	0.04Å	-	-	-
5		TYR	0	-	Favored (47.11%) General / -118.8,124.4	Favored (89.2%) *t80* chi angles: 180,78.9	0.05Å	-	-	-
6		ASN	0	-	Favored (54.95%) Pre-Pro / -107.4,117.8	OUTLIER (0.2%) chi angles: 180.5,109.7	0.02Å	-	-	-
7		PRO	0	-	Favored (73.4%) Trans-Pro / -68.9,150.6	Favored (56.6%) *Cg_endo* chi angles: 26,326.1,28.2	0.04Å	-	-	-
8		CYS	0	-	Favored (25.3%) General / -99.1,113.2	Favored (45%) *t* chi angles: 177.4	0.04Å	-	-	-
9		ALA	0	-	Favored (29.37%) General / -119.8,117.9	-	0.04Å	-	-	-
10		SER	0	-	Allowed (0.15%) General / 69.8,-70.1	OUTLIER (0%) chi angles: 250.7	0.02Å	-	-	-
11		TYR	0	-	Favored (46.11%) General / -111.1,121.7	OUTLIER (0.2%) chi angles: 237.8,128.2	0.05Å	-	-	-
12		LEU	0	-	-	Favored (50.9%) *mt* chi angles: 285.9,174	0.04Å	-	-	-

- *Analysis of t-AIP-I representative structure*

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All-Atom Contacts	Clashscore, all atoms:	0		100th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	0	0.00%	Goal: <0.3%
	Favored rotamers	4	100.00%	Goal: >98%
	Ramachandran outliers	1	33.33%	Goal: <0.05%
	Ramachandran favored	1	33.33%	Goal: >98%
	MolProbity score^	1.55		94th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 43	0.00%	Goal: 0%
	Bad angles:	1 / 56	1.79%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	0 / 0	0.00%	Expected: ≤1 per chain, or ≤5%

In the two column results, the left column gives the raw count, right column gives the percentage.

* 100th percentile is the best among structures of comparable resolution; 0th percentile is the worst. For clashscore the comparative set of structures was selected in 2004, for MolProbity score in 2006.

^ MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

#	Alt	Res	High B	Clash > 0.4Å	Ramachandran	Rotamer	Cβ deviation	Bond lengths	Bond angles	Cis Peptides
			Avg: 0.00	Clashscore: 0	Outliers: 1 of 3	Poor rotamers: 0 of 4	Outliers: 0 of 5	Outliers: 0 of 6	Outliers: 1 of 6	Non-Trans: 0 of 5
0		ACE	0	-	-	-	-	-	-	-
1		CYS	0	-	-	Favored (44.8%) *t* chi angles: 177.4	0.04Å	-	-	-
2		ALA	0	-	Allowed (1.8%) General / -138.5,52.8	-	0.02Å	-	-	-
3		SER	0	-	OUTLIER (0.02)%) General / -159.3,-103.5	Favored (80.1%) *p* chi angles: 61.4	0.03Å	-	-	-
4		TYR	0	-	Favored (89.52%) General / -65.9,-38.8	Favored (38.8%) *m-80* chi angles: 282.5,111	0.03Å	-	-	-
5		PHE	0	-	-	Favored (41.7%) *m-80* chi angles: 285.4,114.7	0.03Å	-	OUTLIER(S) worst is CA-CB-CG: 4.1 σ	-

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- *Analysis of AIP-I D1A representative structure*

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All-Atom Contacts	Clashscore, all atoms:	0		100th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	0	0.00%	Goal: <0.3%
	Favored rotamers	6	100.00%	Goal: >98%
	Ramachandran outliers	1	16.67%	Goal: <0.05%
	Ramachandran favored	5	83.33%	Goal: >98%
	MolProbity score^	1.19		99th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 59	0.00%	Goal: 0%
	Bad angles:	1 / 79	1.27%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	0 / 0	0.00%	Expected: ≤1 per chain, or ≤5%

In the two column results, the left column gives the raw count, right column gives the percentage.
* 100th percentile is the best among structures of comparable resolution; 0th percentile is the worst. For clashscore the comparative set of structures was selected in 2004, for MolProbity score in 2006.
^ MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

#	Alt	Res	High B	Clash > 0.4Å	Ramachandran	Rotamer	Cβ deviation	Bond lengths	Bond angles	Cis Peptides
			Avg: 0.00	Clashscore: 0	Outliers: 1 of 6	Poor rotamers: 0 of 6	Outliers: 0 of 8	Outliers: 0 of 8	Outliers: 1 of 8	Non-Trans: 0 of 7
1		ALA	0	-	-	-	0.06Å	-	-	-
2		SER	0	-	Favored (21.9%) General / -157.1,148.3	Favored (33.9%) *t* chi angles: 181.8	0.02Å	-	-	-
3		VAL	0	-	Favored (51.59%) Ile or Val / -123.9,117.6	Favored (57.3%) *t* chi angles: 180.3	0.08Å	-	-	-
4		CYS	0	-	Favored (53.46%) General / -119.9,128.2	Favored (48.3%) *t* chi angles: 178.8	0.04Å	-	-	-
5		ALA	0	-	Favored (2.07%) General / -130.0,51.6	-	0.04Å	-	-	-
6		SER	0	-	OUTLIER (0.01)%) General / -171.0,-121.9	Favored (58.9%) *p* chi angles: 73.3	0.06Å	-	-	-
7		TYR	0	-	Favored (93.86%) General / -64.7,-39.5	Favored (93.6%) *m-80* chi angles: 296.8,102.1	0.03Å	-	-	-
8		PHE	0	-	-	Favored (89%) *m-80* chi angles: 292,99.6	0.04Å	-	OUTLIER(S) worst is CA-CB-CG: 4.2 σ	-

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- *Analysis of AIP-I V3A representative structure*

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All-Atom Contacts	Clashscore, all atoms:	0		100th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	0	0.00%	Goal: <0.3%
	Favored rotamers	5	83.33%	Goal: >98%
	Ramachandran outliers	0	0.00%	Goal: <0.05%
	Ramachandran favored	4	66.67%	Goal: >98%
	MolProbity score^	1.37		98th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 60	0.00%	Goal: 0%
	Bad angles:	1 / 80	1.25%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	0 / 0	0.00%	Expected: ≤1 per chain, or ≤5%

In the two column results, the left column gives the raw count, right column gives the percentage.

* 100th percentile is the best among structures of comparable resolution; 0th percentile is the worst. For clashscore the comparative set of structures was selected in 2004, for MolProbity score in 2006.

^ MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

#	Alt	Res	High B	Clash > 0.4Å	Ramachandran	Rotamer	Cβ deviation	Bond lengths	Bond angles	Cis Peptides
			Avg: 0.00	Clashscore: 0	Outliers: 0 of 6	Poor rotamers: 0 of 6	Outliers: 0 of 8	Outliers: 0 of 8	Outliers: 1 of 8	Non-Trans: 0 of 7
1		ASP	0	-	-	Favored (27.1%) *t0* chi angles: 200.1,8	0.06Å	-	-	-
2		SER	0	-	Favored (8.37%) General / -144.7,116.8	Allowed (1.8%) *m* chi angles: 275.9	0.07Å	-	-	-
3		ALA	0	-	Favored (22.4%) General / -149.9,140.4	-	0.03Å	-	-	-
4		CYS	0	-	Favored (26.67%) General / -125.5,118.8	Favored (53%) *t* chi angles: 180.8	0.05Å	-	-	-
5		ALA	0	-	Allowed (0.75%) General / -111.0,52.1	-	0.03Å	-	-	-
6		SER	0	-	Allowed (0.25%) General / -165.8,-155.8	Favored (2.8%) *p* chi angles: 87.5	0.05Å	-	-	-
7		TYR	0	-	Favored (68.91%) General / -60.4,-29.4	Favored (70%) *m-80* chi angles: 290.5,107.6	0.03Å	-	-	-
8		PHE	0	-	-	Favored (36.5%) *m-80* chi angles: 309.5,114.6	0.03Å	-	OUTLIER(S) worst is CA-CB-CG: 4.3 σ	-

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- *Analysis of AIP-I D1AS6A representative structure*

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All-Atom Contacts	Clashscore, all atoms:	0		100th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	0	0.00%	Goal: <0.3%
	Favored rotamers	4	80.00%	Goal: >98%
	Ramachandran outliers	1	16.67%	Goal: <0.05%
	Ramachandran favored	5	83.33%	Goal: >98%
	MolProbity score^	1.19		99th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 58	0.00%	Goal: 0%
	Bad angles:	1 / 78	1.28%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	0 / 0	0.00%	Expected: ≤1 per chain, or ≤5%

In the two column results, the left column gives the raw count, right column gives the percentage.

* 100th percentile is the best among structures of comparable resolution; 0th percentile is the worst. For clashscore the comparative set of structures was selected in 2004, for MolProbity score in 2006.

^ MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

#	Alt	Res	High B	Clash > 0.4Å	Ramachandran	Rotamer	Cβ deviation	Bond lengths	Bond angles	Cis Peptides
			Avg: 0.00	Clashscore: 0	Outliers: 1 of 6	Poor rotamers: 0 of 5	Outliers: 0 of 8	Outliers: 0 of 8	Outliers: 1 of 8	Non-Trans: 0 of 7
1		ALA	0	-	-	-	0.06Å	-	-	-
2		SER	0	-	Favored (43.41%) General / -149.9,157.7	Favored (93.8%) *p* chi angles: 66.3	0.06Å	-	-	-
3		VAL	0	-	Favored (18.58%) Ile or Val / -117.6,105.7	Favored (63.1%) *t* chi angles: 179.6	0.07Å	-	-	-
4		CYS	0	-	Favored (26.43%) General / -144.2,136.4	Favored (46.7%) *t* chi angles: 177.9	0.02Å	-	-	-
5		ALA	0	-	Favored (48.34%) General / -133.1,141.7	-	0.04Å	-	-	-
6		ALA	0	-	OUTLIER (0.03)%) General / 62.7,-88.2	-	0.02Å	-	-	-
7		TYR	0	-	Favored (8.47%) General / -125.1,16.0	Allowed (0.4%) *m-80* chi angles: 253.9,52.8	0.03Å	-	-	-
8		PHE	0	-	-	Favored (94.7%) *m-80* chi angles: 298,100.7	0.02Å	-	OUTLIER(S) worst is CA-CB-CG: 4.2 σ	-

- *Analysis of AIP-I AAA representative structure*

Viewing AAA-multi.table

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When finished, you should close this window.

Hint: Use File | Save As... to save a copy of this page.

All-Atom Contacts	Clashscore, all atoms:	0		100th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	0	0.00%	Goal: <0.3%
	Favored rotamers	3	75.00%	Goal: >98%
	Ramachandran outliers	1	16.67%	Goal: <0.05%
	Ramachandran favored	5	83.33%	Goal: >98%
	MolProbity score^	1.19		99th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 56	0.00%	Goal: 0%
	Bad angles:	1 / 75	1.33%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	0 / 0	0.00%	Expected: ≤1 per chain, or ≤5%

In the two column results, the left column gives the raw count, right column gives the percentage.

* 100th percentile is the best among structures of comparable resolution; 0th percentile is the worst. For clashscore the comparative set of structures was selected in 2004, for MolProbity score in 2006.

^ MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

#	Alt	Res	High B	Clash > 0.4Å	Ramachandran	Rotamer	Cβ deviation	Bond lengths	Bond angles	Cis Peptides
			Avg: 0.00	Clashscore: 0	Outliers: 1 of 6	Poor rotamers: 0 of 4	Outliers: 0 of 8	Outliers: 0 of 8	Outliers: 1 of 8	Non-Trans: 0 of 7
1		ALA	0	-	-	-	0.07Å	-	-	-
2		SER	0	-	Favored (28.91%) General / -149.1,147.8	Favored (46.7%) *t* chi angles: 180	0.03Å	-	-	-
3		ALA	0	-	Favored (40.65%) General / -136.7,138.1	-	0.05Å	-	-	-
4		CYS	0	-	Favored (27.58%) General / -143.8,136.8	Favored (48.4%) *t* chi angles: 178.4	0.03Å	-	-	-
5		ALA	0	-	Favored (50.03%) General / -130.3,137.6	-	0.03Å	-	-	-
6		ALA	0	-	OUTLIER (0.03)%) General / 62.2,-89.2	-	0.03Å	-	-	-
7		TYR	0	-	Favored (10.43%) General / -122.6,18.4	Allowed (0.4%) *m-80* chi angles: 253.5,52.4	0.04Å	-	-	-
8		PHE	0	-	-	Favored (93%) *m-80* chi angles: 299.9,95.6	0.03Å	-	OUTLIER(S) worst is CA-CB-CG: 4.4 σ	-

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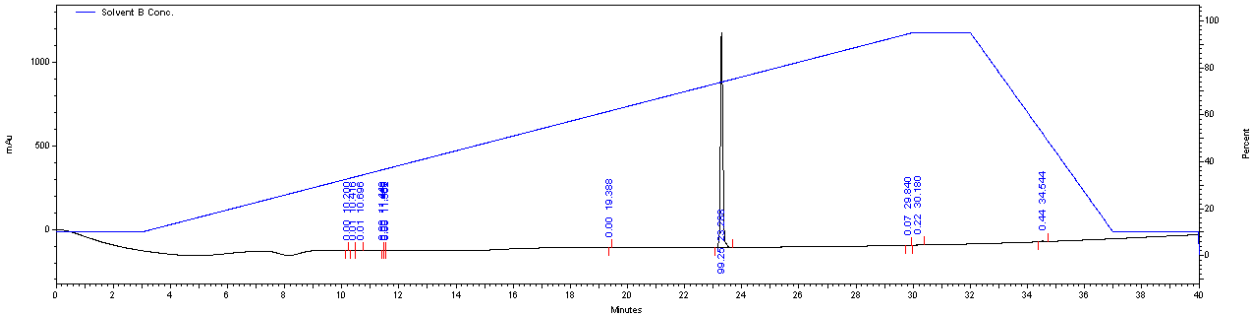
MS and analytical HPLC data for β-turn modifying analogs

Table S17: MS spectral data, HPLC retention times, and purity data for the β-turn modifying analogs. The m/z of each peptide is given as $[M+H]^+$.

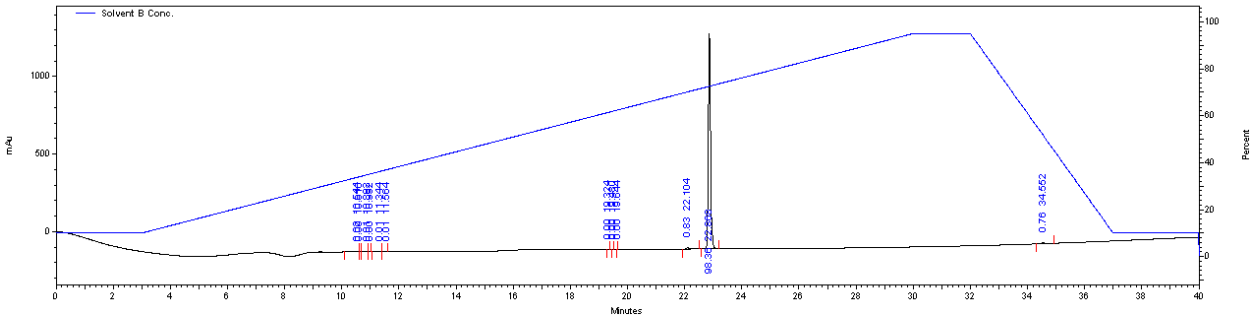
peptide	calculated m/z (Da)	observed m/z (Da)	Δ*m* (ppm)	retention time (min)	HPLC trace purity (%, 220 nm)
AIP-I D1AS6dA	813.3600	813.3597	0.4	23.29	>99
AIP-I D1AS6G	799.3443	799.3442	0.1	22.87	98.3
AIP-I D1AS6A [N-MeF8]	827.3756	827.3750	0.7	23.71	98.7
AIP-I D1AS6A [F8PLA]	814.3440	814.3444	0.5	24.09	95.4
AAdA	785.3287	785.3287	<0.1	22.62	99.0
AAG	771.3130	771.3129	0.1	22.72	96.1
AAA [N-MeF8]	799.3443	799.3438	0.6	23.10	96.6
AAA [F8PLA]	786.3127	786.3130	0.4	23.45	95.6

Analytical HPLC traces for β-turn modifying analogs

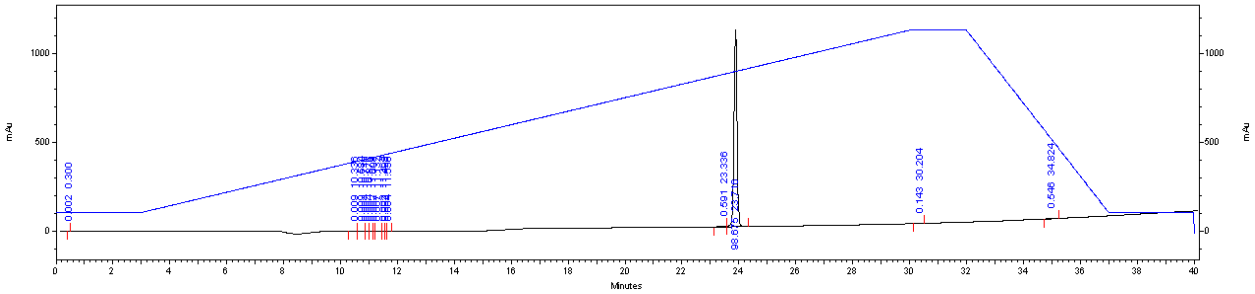
AIP-I D1AS6dA



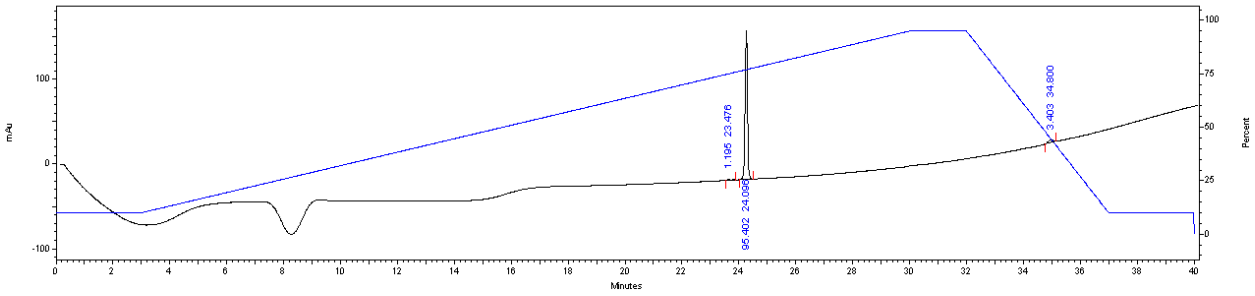
AIP-I D1AS6G



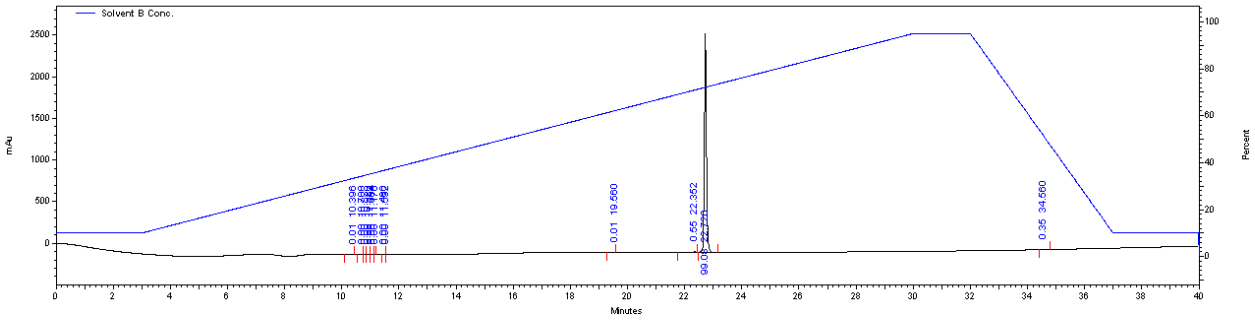
AIP-I D1AS6A [N-MeF8]



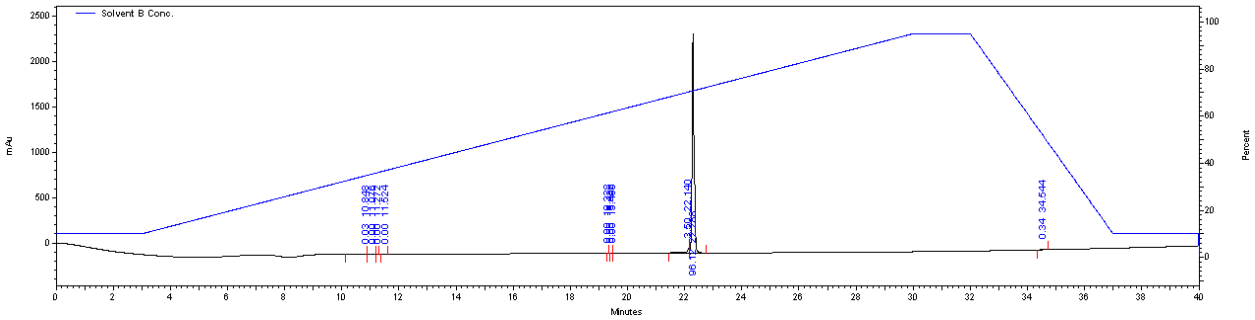
AIP-I D1AS6A [F8PLA]



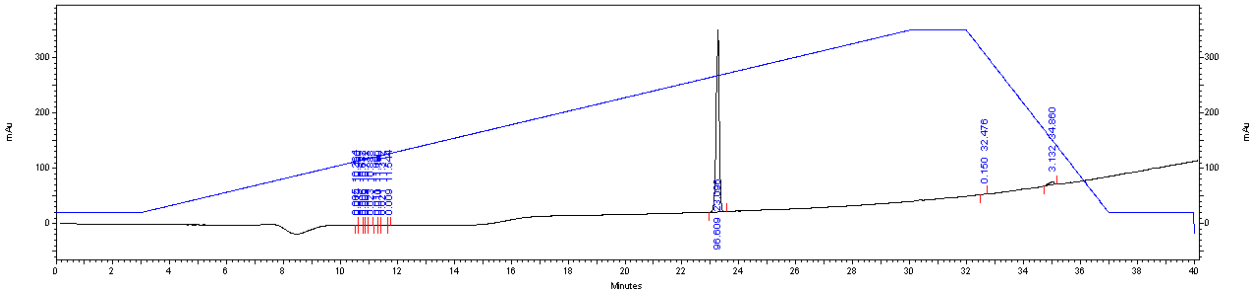
AAdA



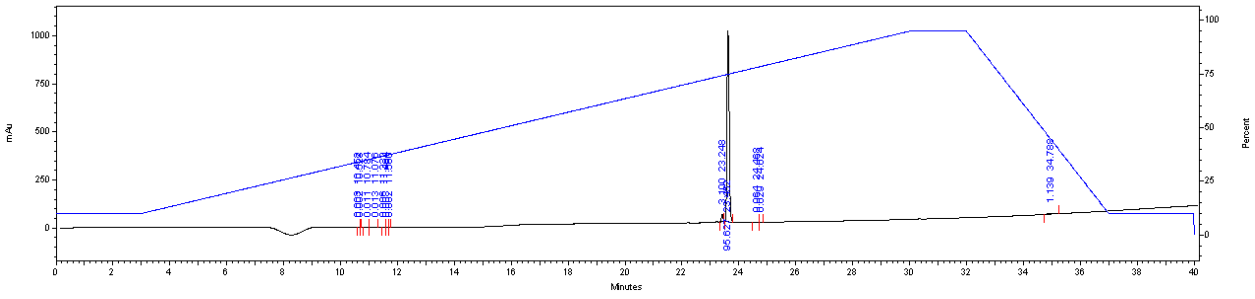
AAG



AAA [N-MeF8]



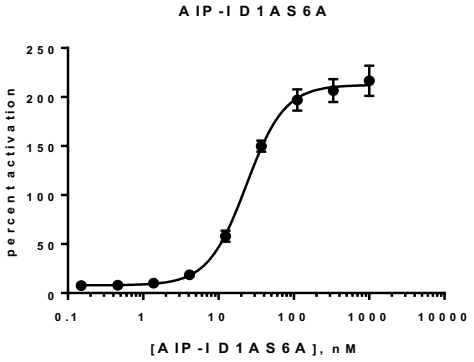
AAA [F8PLA]

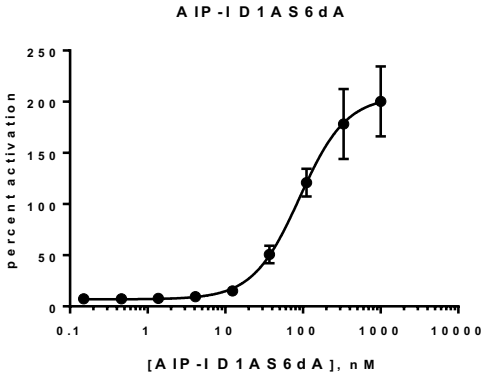


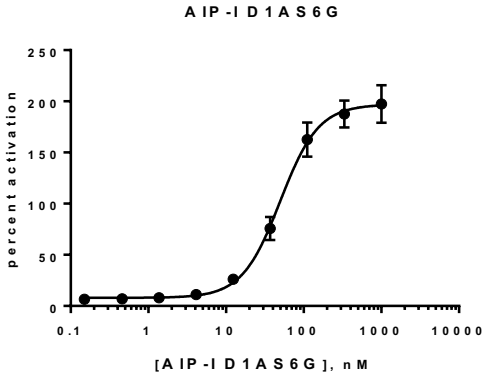
S. epidermidis **AgrC-I reporter dose-response activity curves for β-turn modifying analogs**

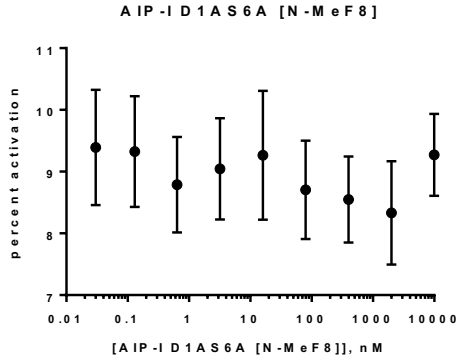
Error bars indicate standard deviation (SD). Three biological replicates were used to generate each curve. Curves for the parent agonist (AIP-I D1AS6A) and antagonist (AAA) are also provided. See assay protocol in the experimental procedures section above for details of method.

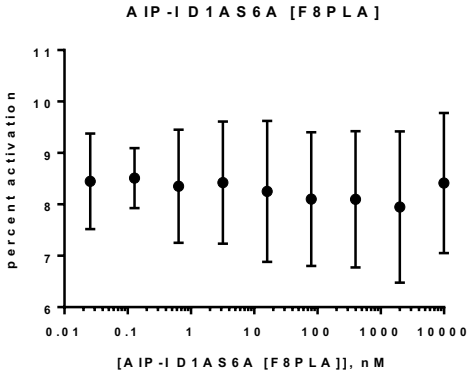
Agonists



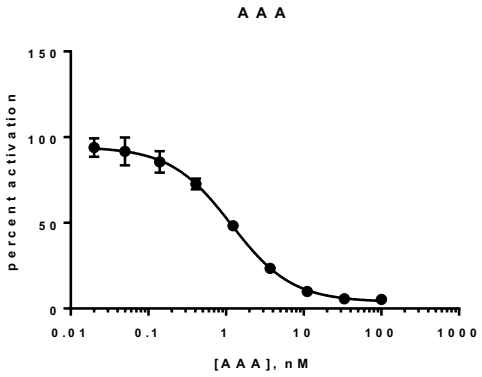


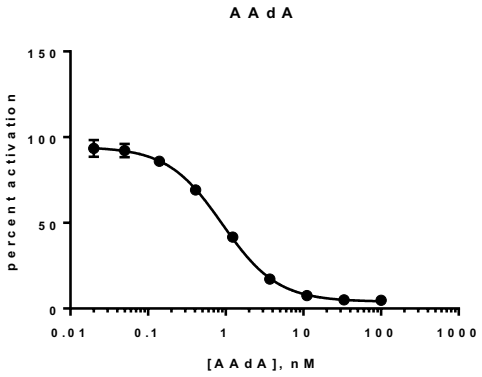


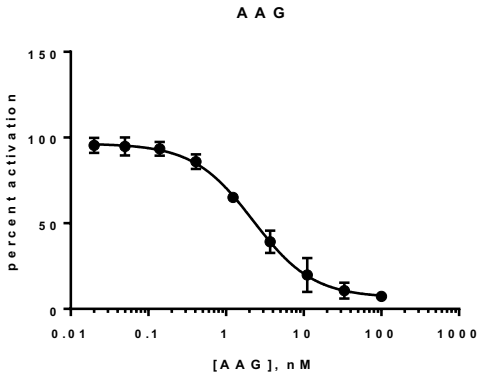


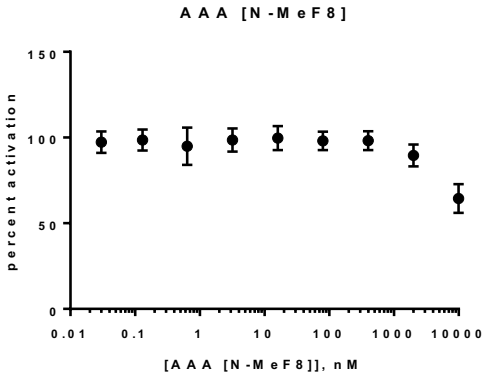


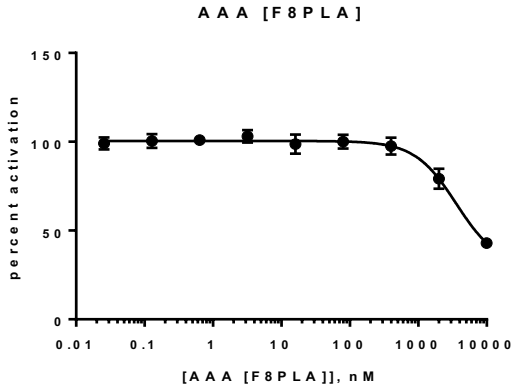
Antagonists











EC_{50} and IC_{50} values for β-turn modifying analogs

Table S18: Cell-based reporter activities in AgrC-I of agonist AIP-I D1AS6A, antagonist AAA, and the β-turn modifying peptide analogs.

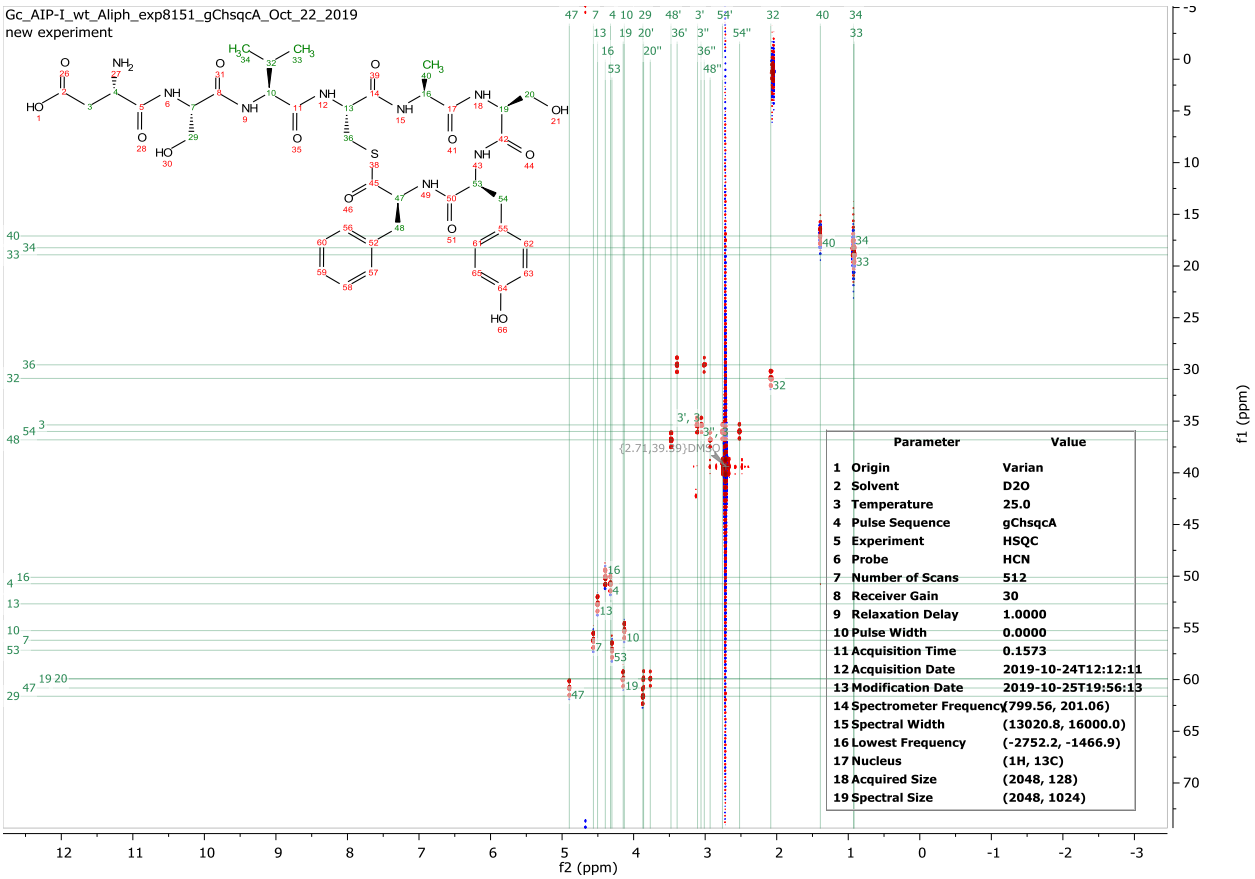
peptide name	sequence	EC_{50} in nM, (95% CI)	IC_{50} in nM, (95% CI)
AIP-I D1AS6A	A-S-V-(C-A-A-Y-F)	23.4 (21.9–25.0)	--
AIP-I D1AS6dA	A-S-V-(C-A-dA-Y-F)	90.6 (75.7–111)	--
AIP-I D1AS6G	A-S-V-(C-A-G-Y-F)	49.9 (45.7–54.6)	--
AIP-I D1AS6A [N-MeF8]	A-S-V-(C-A-A-Y-[N-MeF])	--	--
AIP-I D1AS6A [F8PLA]	A-S-V-(C-A-A-Y-PLA)	--	--
AAA	A-S-A-(C-A-A-Y-F)	--	1.16 (1.04–1.30)
AAdA	A-S-A-(C-A-dA-Y-F)	--	0.908 (0.852–0.967)
AAG	A-S-A-(C-A-G-Y-F)	--	2.23 (1.94–2.58)
AAA [N-MeF8]	A-S-A-(C-A-A-Y-[N-MeF])	--	>1000
AAA [F8PLA]	A-S-A-(C-A-A-Y-PLA)	--	>1000

CI = 95% confidence interval.

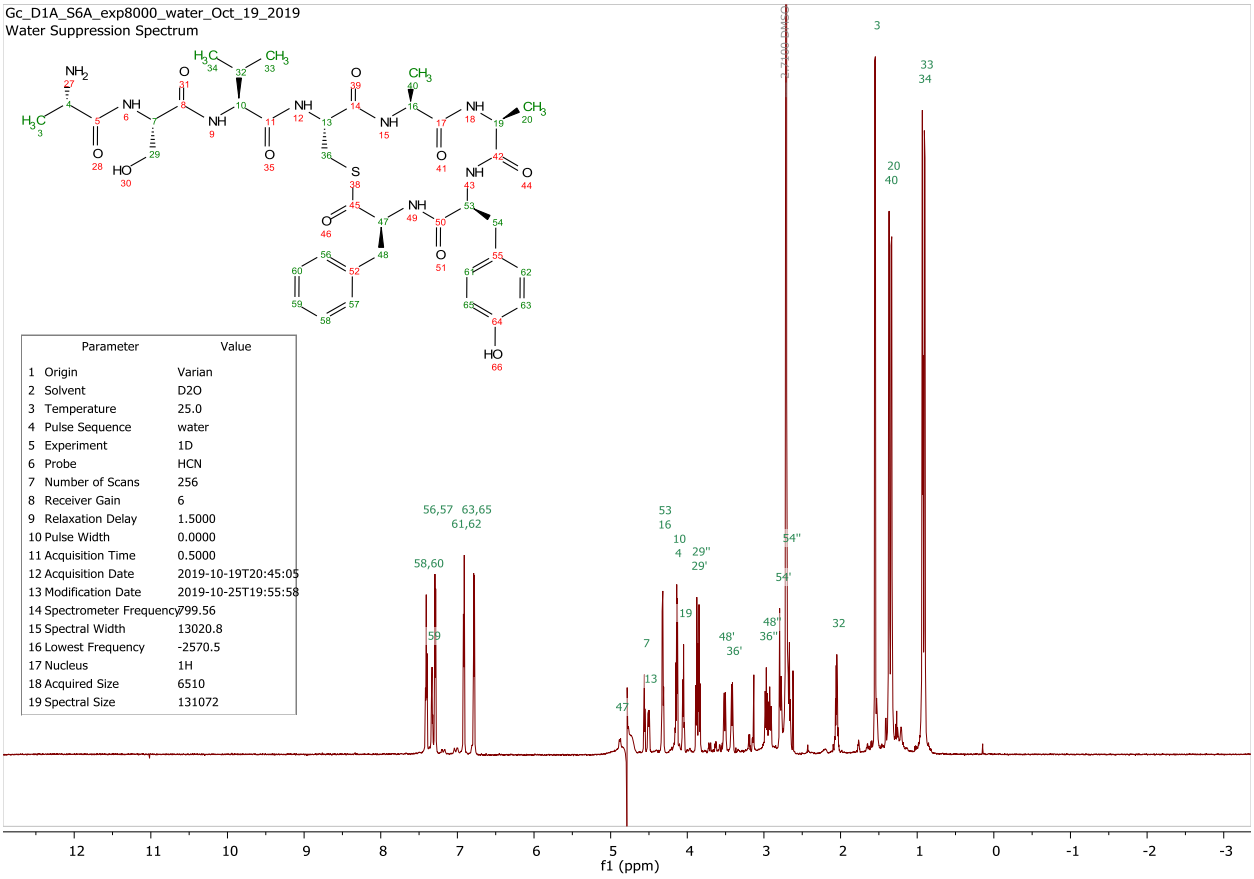
NMR spectra for select β-turn modifying analogs

Two-dimensional ^{1}H-^{13}C HSQC NMR experiments were performed for each peptide as described in the experimental procedures above, along with 1-D proton and 2-D TOCSY NMR experiments for select peptides. Selected parameters for each experiment are included in each spectrum. For the 2-D experiments, blue indicates negative peak intensity and red indicates positive peak intensity.

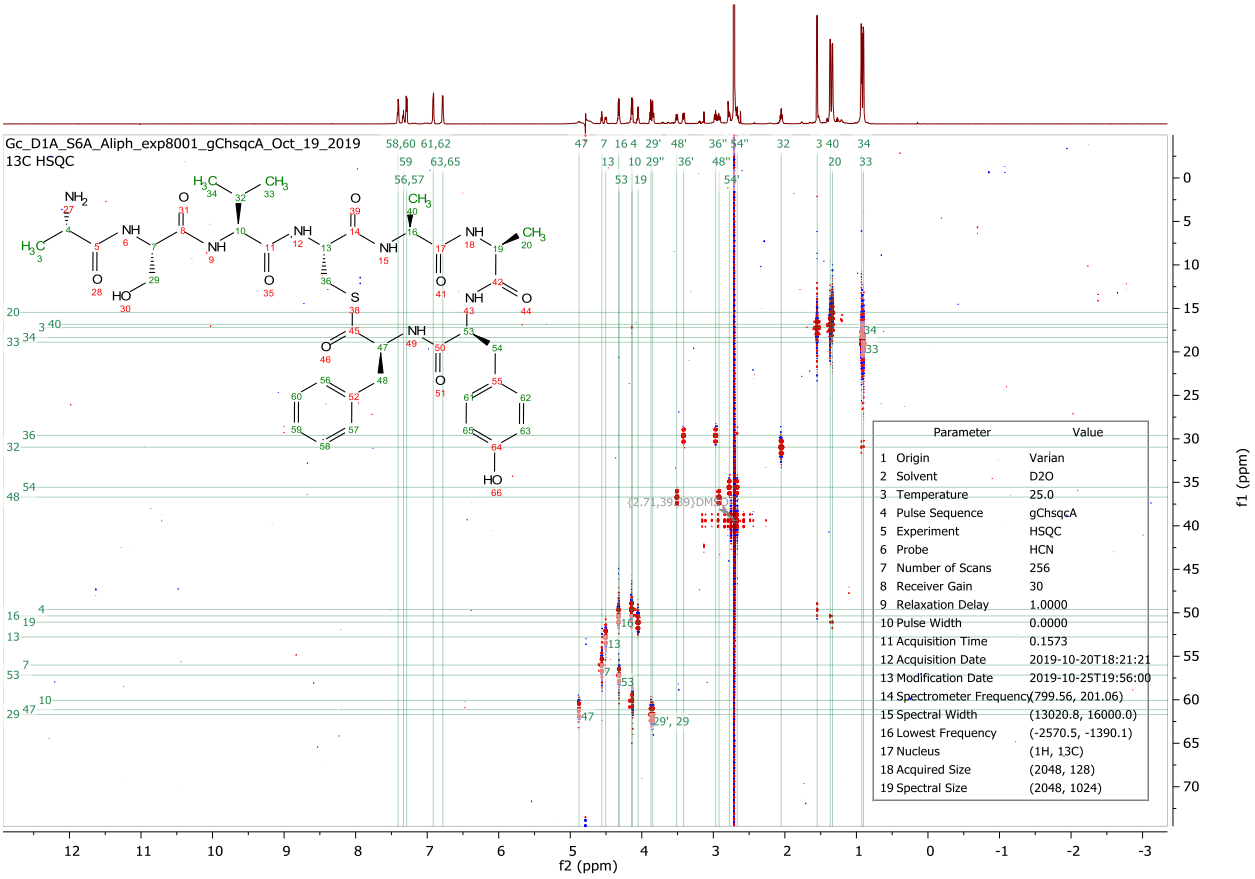
- *AIP-I ^{1}H-^{13}C HSQC NMR spectrum (700 μM dissolved in 12.5% CD_3CN in D_2O, pH ~5)*



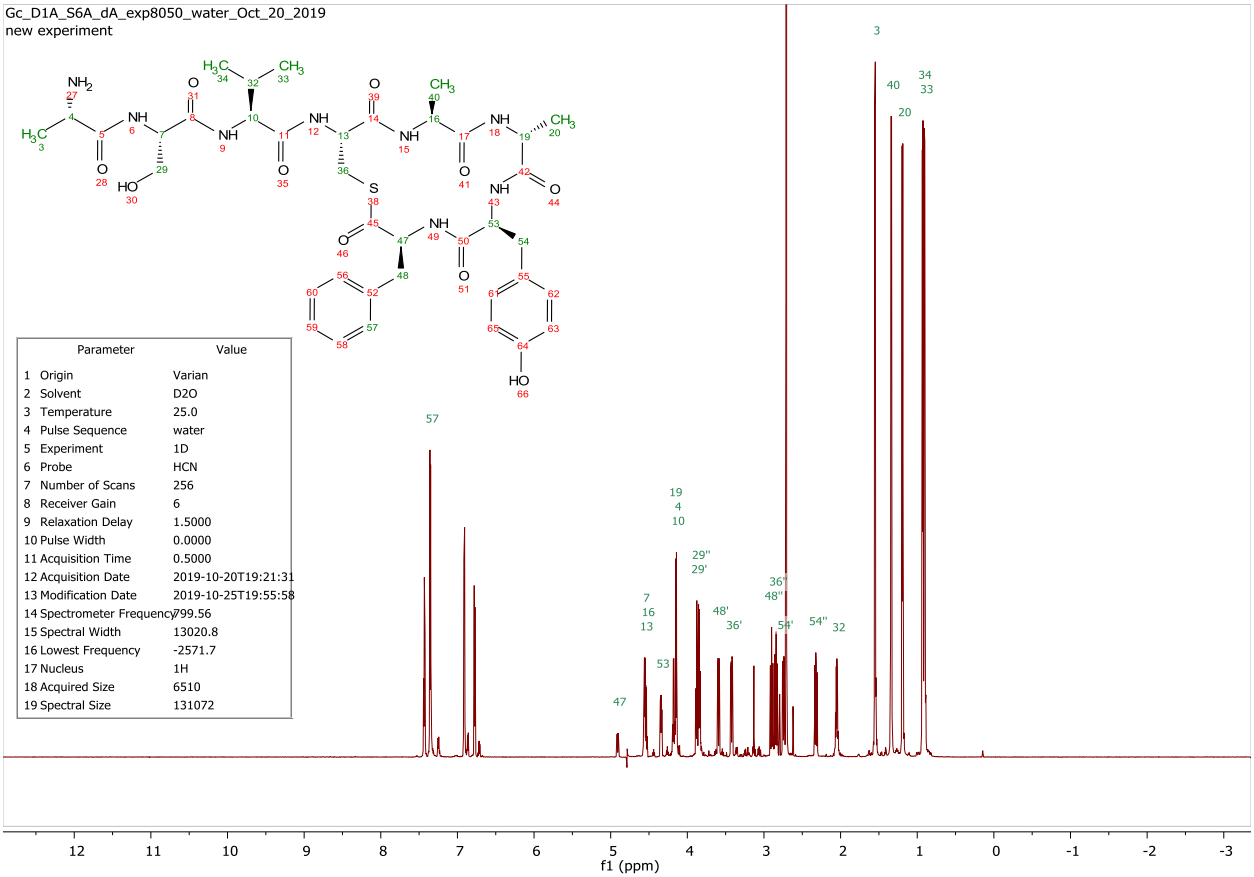
- *AIP-I D1AS6A ^{1}H NMR spectrum (1.43 mM dissolved in D_2O, pH ~5)*



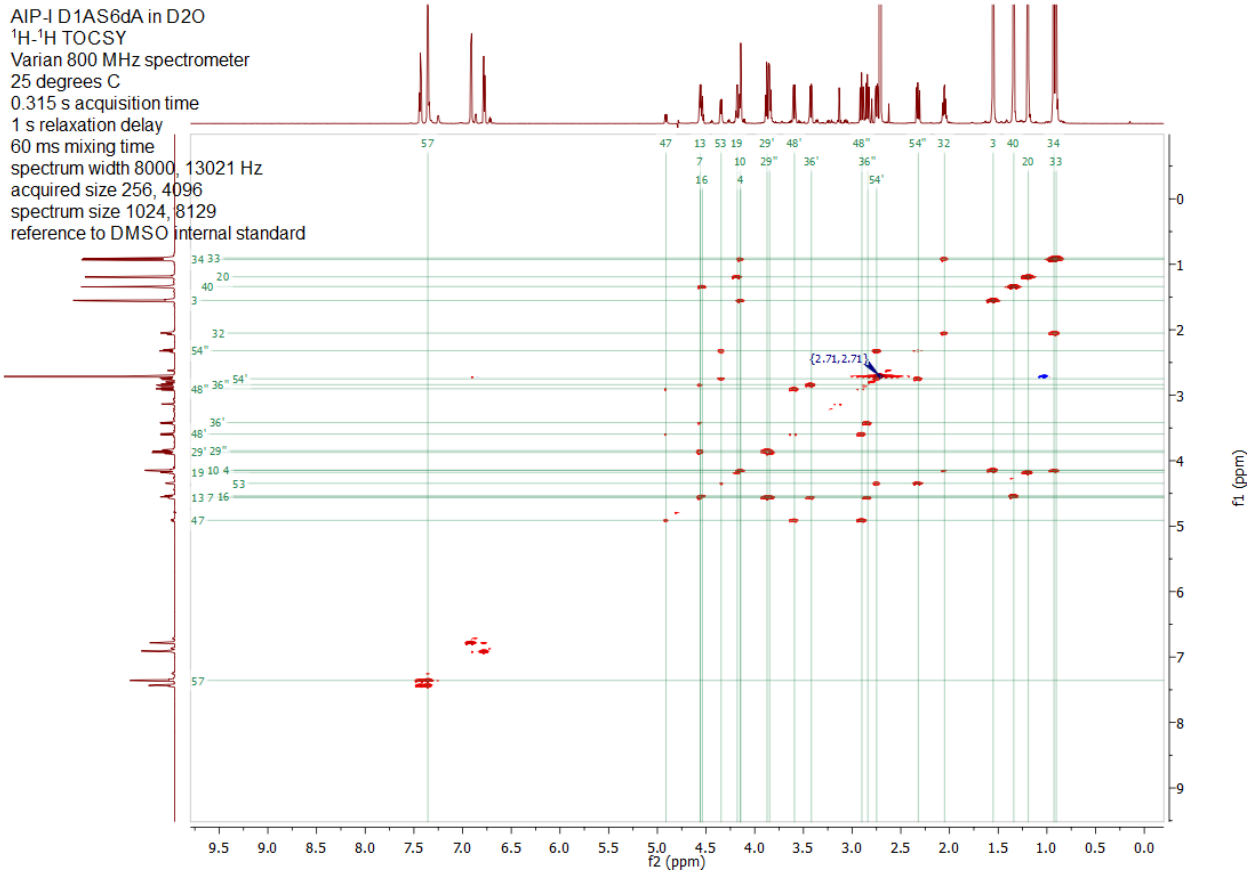
- *AIP-I D1AS6A ^{1}H-^{13}C HSQC NMR spectrum*



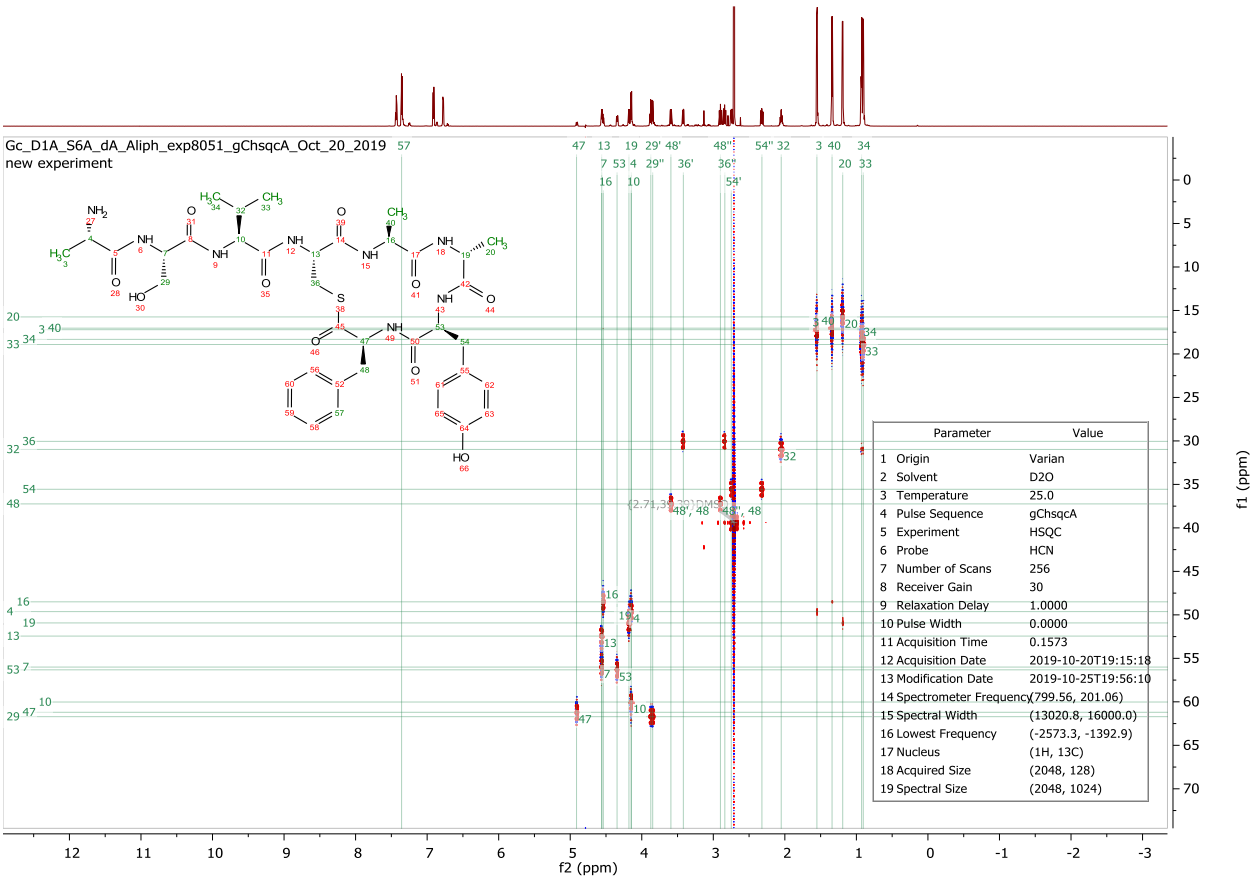
- *AIP-I D1AS6dA ^{1}H NMR spectrum (1.43 mM dissolved in D_2O, pH ~5)*



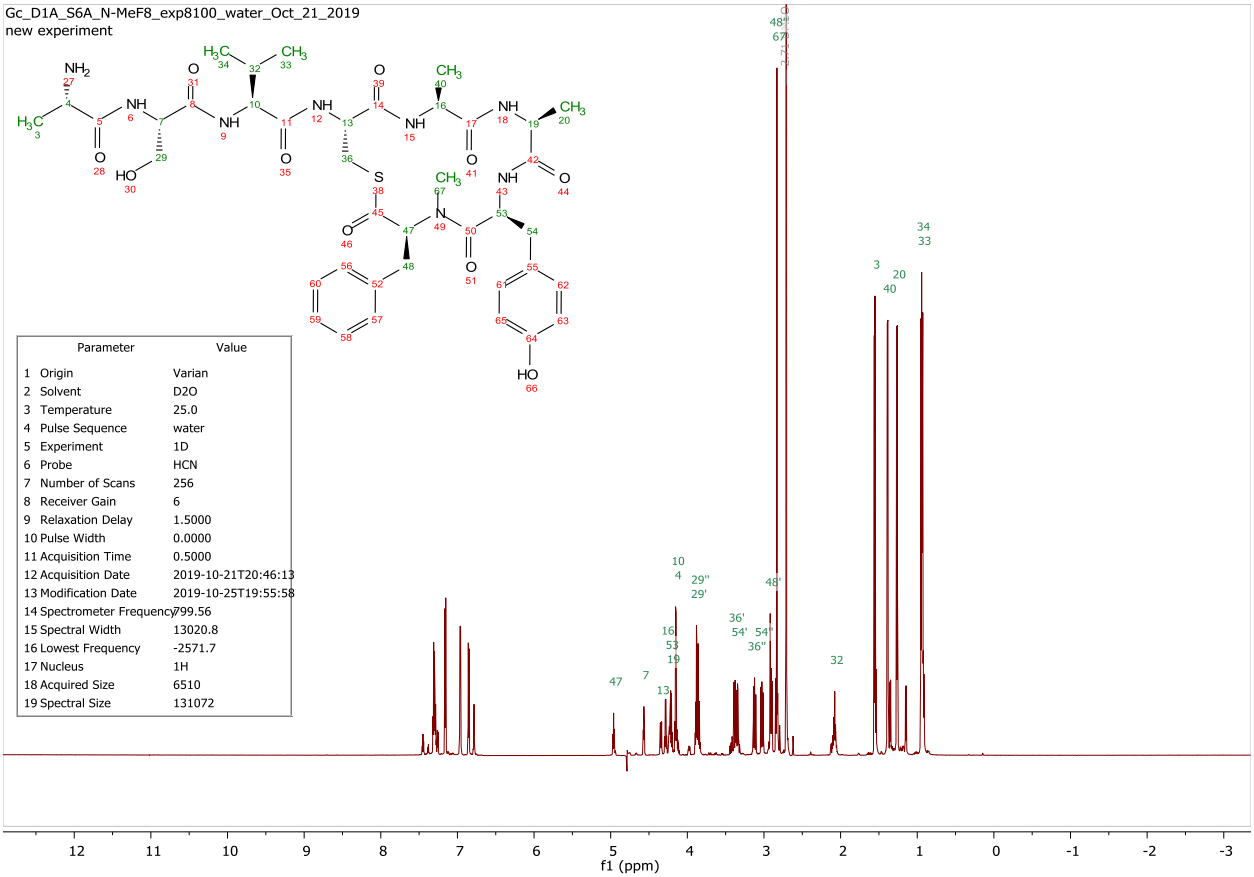
- *AIP-I D1AS6dA ^{1}H-^{1}H TOCSY NMR spectrum*



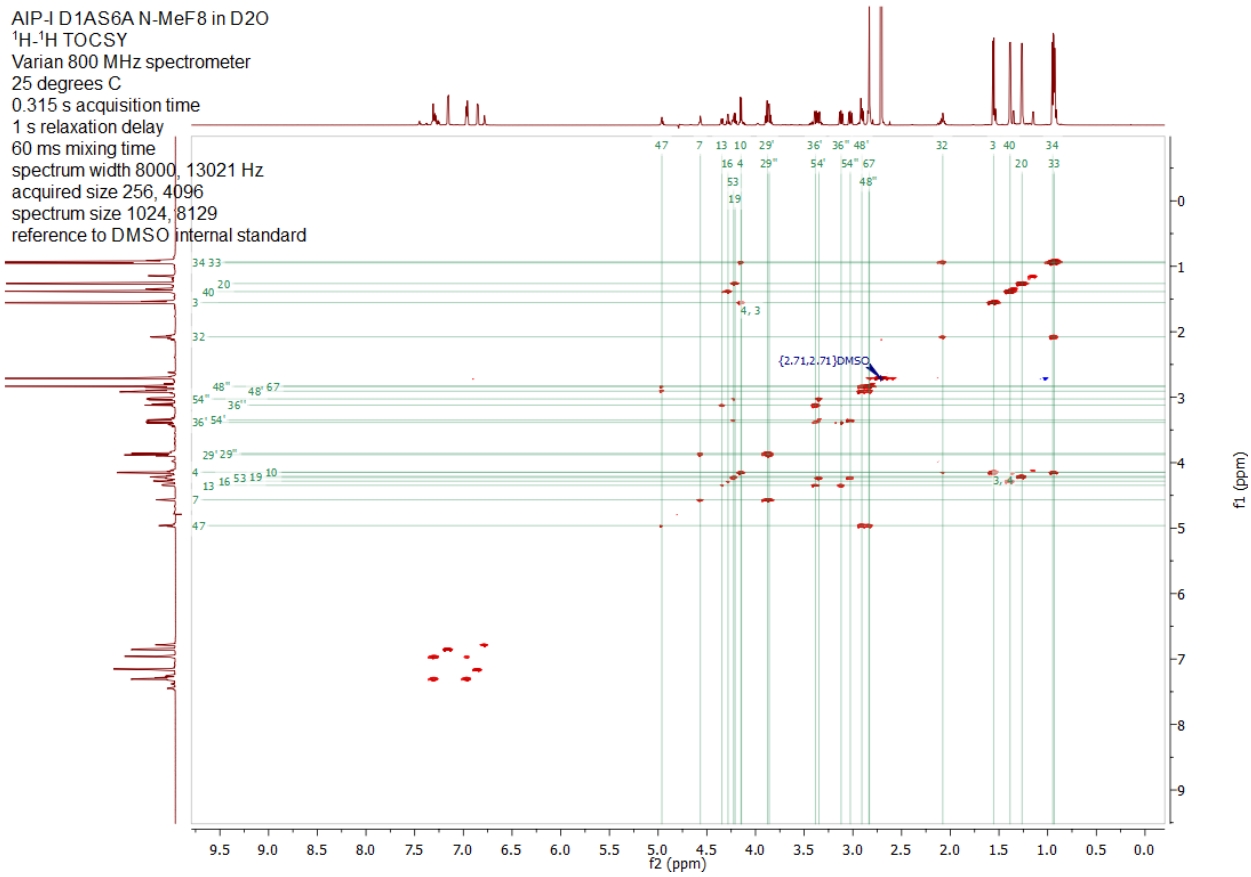
- *AIP-I D1AS6dA ^{1}H-^{13}C HSQC NMR spectrum*



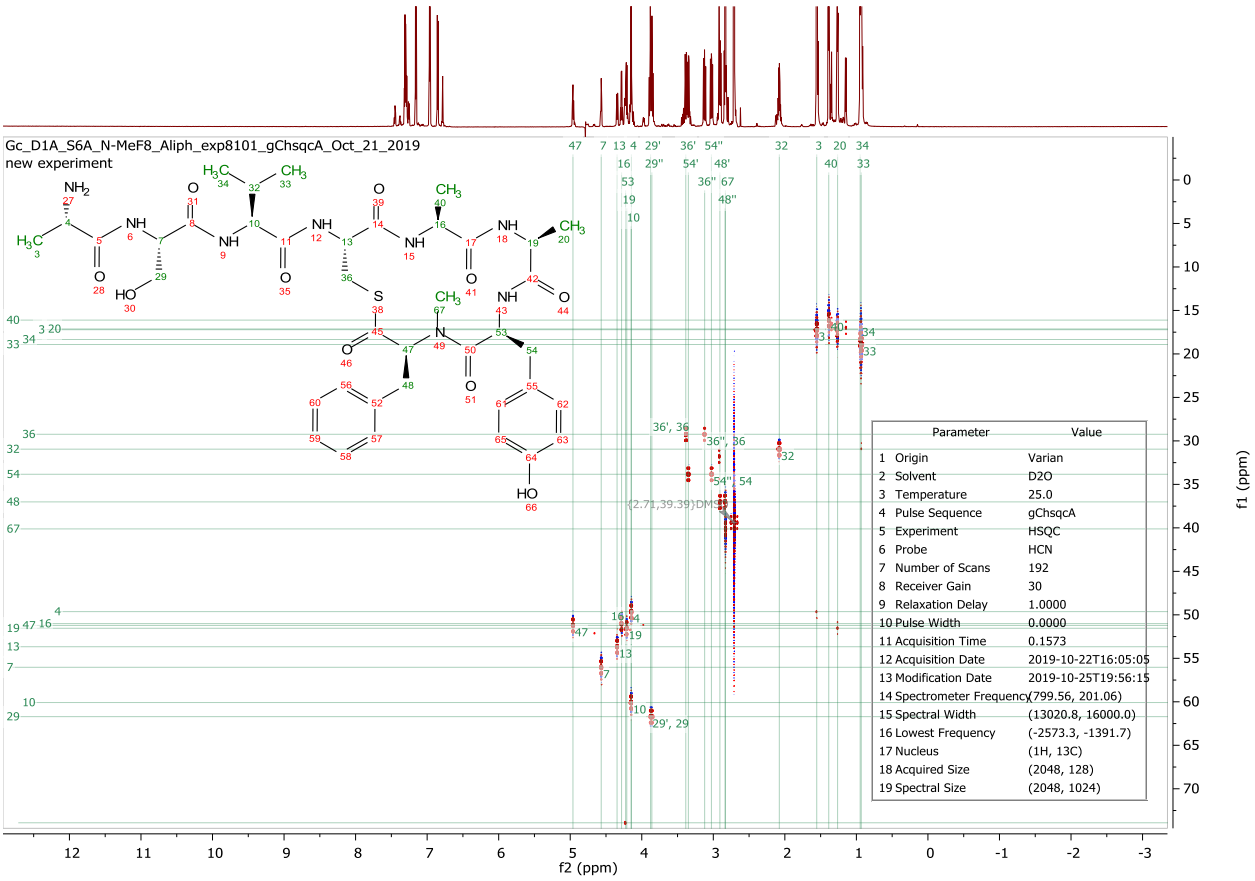
- *AIP-I D1AS6A [N-MeF8] ^{1}H NMR spectrum (1.43 mM dissolved in D_2O, pH ~5)*



- *AIP-I D1AS6A [N-MeF8] ^{1}H-^{1}H TOCSY NMR spectrum*



- *AIP-I D1AS6A [N-MeF8] ^{1}H-^{13}C HSQC NMR spectrum*



Chemical shift assignments for select β-turn modifying analogs

Table S19: Assignments for AIP-I

Residue	Hα (ppm)	Cα (ppm)	Hβ (ppm)	Cβ (ppm)	Others (ppm)
Asp1	4.32	50.73	3.06, 3.11	35.37	
Ser2	4.56	56.20	3.87	61.61	
Val3	4.13	55.26	2.08	30.87	Hγ 0.92, 0.93 and Cγ 18.92, 18.23
Cys4	4.50	52.67	3.01, 3.39	29.56	
Ala5	4.40	50.11	1.39	17.09	
Ser6	4.15	59.94	3.77, 3.86	59.91	
Tyr7	4.30	57.16	2.52, 2.76	36.00	
Phe8	4.90	60.81	2.93, 3.48	36.81	

Table S20: Assignments for AIP-I D1AS6A

Residue	Hα (ppm)	Cα (ppm)	Hβ (ppm)	Cβ (ppm)	Others (ppm)
Ala1	4.14	49.62	1.55	17.20	
Ser2	4.56	56.01	3.85, 3.87	61.69	
Val3	4.13	60.07	2.05	30.96	Hγ 0.90, 0.93 and Cγ 18.88, 18.34
Cys4	4.50	52.78	2.97, 3.42	29.60	
Ala5	4.32	50.36	1.37	16.83	
Ala6	4.05	51.08	1.33	15.46	
Tyr7	4.32	57.17	2.67, 2.78	35.57	
Phe8	4.88	61.14	2.92, 3.51	36.69	

Table S21: Assignments for AIP-I D1AS6dA

Residue	Hα (ppm)	Cα (ppm)	Hβ (ppm)	Cβ (ppm)	Others (ppm)
Ala1	4.15	49.63	1.55	17.21	
Ser2	4.56	56.00	3.85, 3.87	61.71	
Val3	4.15	60.02	2.05	30.98	Hγ 0.90, 0.93 and Cγ 18.91, 18.32
Cys4	4.56	52.44	2.84, 3.42	30.05	
Ala5	4.54	48.50	1.34	17.04	
dAla6	4.18	50.93	1.19	15.74	
Tyr7	4.35	56.32	2.32, 2.75	35.56	
Phe8	4.91	61.20	2.90, 3.59	37.24	

Table S22: Assignments for AIP-I D1AS6A [N-MeF8]

Residue	Hα (ppm)	Cα (ppm)	Hβ (ppm)	Cβ (ppm)	Others (ppm)
Ala1	4.14	49.63	1.55	17.22	
Ser2	4.57	56.03	3.86, 3.88	61.71	
Val3	4.15	60.08	2.08	30.95	Hγ 0.93, 0.95 and Cγ 18.92, 18.34
Cys4	4.35	53.67	3.12, 3.38	29.24	
Ala5	4.28	51.01	1.39	16.11	
Ala6	4.21	51.55	1.26	17.13	
Tyr7	4.23	73.91	3.03, 3.35	33.84	
N-MePhe8	4.96	51.22	2.83, 2.91	37.02	N-Me H 2.83, C 40.12

TALOS-N predictions for AIP-I and AIP-I D1AS6A

Table S23: Torsion angle analysis for AIP-I

Residue	*Predicted*			*Observed**		Ramachandran
	Phi	Psi	CLASS	Phi	Psi	
Asp1	none	none	none	none	45.393	N/A
Ser2	-103.427 ± 26.247	101.222 ± 63.095	warn	-153.616	146.409	MATCH
Val3	-103.571 ± 14.362	126.446 ± 5.802	strong	-126.956	128.62	MATCH
Cys4	-104.668 ± 14.482	130.147 ± 12.928	strong	-125.294	121.087	MATCH
Ala5	-104.113 ± 26.363	135.859 ± 24.183	generous	-135.013	61.471	MISMATCH
Ser6	-68.757 ± 15.126	-17.975 ± 17.111	warn	-174.485	-97.422	MISMATCH
Tyr7	-90.578 ± 11.683	0.962 ± 11.899	strong	-68.035	-37.567	MATCH
Phe8	none	none	none	-135.013	none	N/A

*As determined from the solution-phase NMR structure in this study.

Table S24: Torsion angle analysis for AIP-I D1AS6A

Residue	*Predicted*			*Observed**		Ramachandran
	Phi	Psi	CLASS	Phi	Psi	
Ala1	none	none	none	none	63.436	N/A
Ser2	-90.000 ± 13.261	129.065 ± 12.271	strong	-149.893	157.675	MATCH
Val3	-100.836 ± 14.319	122.150 ± 8.839	strong	-117.633	105.724	MATCH
Cys4	-111.628 ± 9.710	134.821 ± 10.895	strong	-144.247	136.439	MATCH
Ala5	-102.063 ± 28.363	133.140 ± 22.407	warn	-133.073	141.684	MATCH
Ala6	-87.544 ± 22.952	128.997 ± 32.590	warn	62.737	-88.195	MISMATCH
Tyr7	-104.505 ± 28.071	139.797 ± 14.313	warn	-125.077	15.963	MISMATCH
Phe8	none	none	none	-133.073	none	N/A

*As determined from the solution-phase NMR structure in this study.

Discussion of TALOS-N usage with AIPs and AIP analogs

The TALOS-N program (https://spin.niddk.nih.gov/bax/software/TALOS-N/) is an artificial neural network based system for the prediction of protein backbone φ/ψ torsion angles, sidechain χ1 torsion angles, and secondary structure using NMR chemical shift assignments. Along with angle predictions for specific residues, TALOS-N assigns a level of confidence to each prediction (indicated in the "CLASS" columns of Tables S23 and S24 above).[17-18] Predictions classified as "strong" indicate that the 25 best database matches to the residue are well-clustered on the Ramachandran map. If instead only the top 10 best database matches are well-clustered, the prediction is classified as "generous." All other cases are considered ambiguous and are designated as "warn." However, even with the "warn" status, the φ/ψ angle predictions can still be useful if clustered.

Although some of the TALOS-N predictions of the AIP-I and AIP-I D1AS6A residue torsion angles are "strong," just as many are classified as "generous" or "warn," and these do not always match the regions of the Ramachandran map in which these angles were determined experimentally to reside (via their solution-phase NMR structures; Tables S23 and S24). As these peptides look different from the natural proteins the TALOS-N database utilizes, especially in regard to the constrained thiolactone macrocycle, the lack of consistent, high quality predictions is perhaps not surprising. That said, the TALOS-N predictions appear consistent within the exocyclic tail regions (residues 1-3 in the peptides tested herein), most likely because being outside the macrocycle allows these residues to adopt conformations commonly found in proteins within the database that TALOS-N queries.

Comparative chemical shift analysis for select β-turn modifying analogs

Table S25: Calculated NMR chemical shift differences between AIP-I D1AS6A and either AIP-I D1AS6dA or AIP-I D1AS6A [N-MeF8]. Positive values indicate the shift of the analog's atom is further downfield than the corresponding shift in AIP-I D1AS6A, while negative values indicate the shift is further upfield. A standard method was used to determine the threshold for which chemical shift differences were deemed significant, and these shifts are indicated in yellow (see calculations below).[19] Briefly, the mean and standard deviation of the chemical shift difference magnitudes were calculated for each nucleus, and the significance threshold was set at any shift with a magnitude greater than two standard deviations above the mean.

	AIP-I D1AS6A vs. **AIP-I D1AS6dA**					AIP-I D1AS6A vs. **AIP-I D1AS6A [N-MeF8]**				
Residue	Hα (ppm)	Cα (ppm)	Hβ1 (ppm)	Hβ2 (ppm)	Cβ (ppm)	Hα (ppm)	Cα (ppm)	Hβ1 (ppm)	Hβ2 (ppm)	Cβ (ppm)
1	+0.01	+0.01	+0.00	-----	+0.01	+0.00	+0.01	+0.00	-----	+0.02
2	+0.00	-0.01	+0.00	+0.00	+0.02	+0.01	+0.02	+0.01	+0.01	+0.02
3	+0.02	-0.05	+0.00	-----	+0.02	+0.02	+0.01	+0.03	-----	-0.01
4	+0.06	-0.34	-0.13	+0.00	+0.45	-0.15	+0.89	+0.15	-0.04	-0.36
5	+0.22	-1.86	-0.03	-----	+0.21	-0.04	+0.65	+0.02	-----	-0.72
6	+0.13	-0.15	-0.14	-----	+0.28	0.16	+0.47	-0.07	-----	+1.67
7	+0.03	-0.85	-0.35	-0.03	-0.01	-0.09	+16.74	+0.36	+0.57	-1.73
8	+0.03	+0.06	-0.02	+0.08	+0.55	+0.08	-9.92	-0.09	-0.60	+0.33

Calculation of the mean for proton chemical shift differences:

$$\mu_H = \frac{\sum^N \left|\delta_{H[\text{AIP-I D1AS6A}]} - \delta_{H[\text{analogs}]}\right|}{N} = 0.09 \text{ ppm}$$

Standard deviation calculation for proton chemical shift differences:

$$\sigma_H = \sqrt{\frac{\sum^N \left(\delta_{H[\text{analog}]} - \mu_H\right)^2}{N-1}} = 0.14 \text{ ppm}$$

Determination of the cutoff value for significant proton shifts differences:

$$\text{Proton Cutoff} \geq \mu_H + 2\sigma_H = 0.37 \text{ ppm}$$

Calculation of the mean for carbon chemical shift differences:

$$\mu_C = \frac{\sum^N \left|\delta_{C[\text{AIP-I D1AS6A}]} - \delta_{C[\text{analogs}]}\right|}{N} = 1.20 \text{ ppm}$$

Standard deviation calculation for carbon chemical shift differences:

$$\sigma_C = \sqrt{\frac{\sum^N \left(\delta_{C[\text{analog}]} - \mu_C\right)^2}{N-1}} = 3.34 \text{ ppm}$$

Determination of the cutoff value for significant carbon shifts differences:

$$\text{Carbon Cutoff} \geq \mu_C + 2\sigma_C = 7.88 \text{ ppm}$$

Discussion of chemical shift differences for select β-turn modifying analogs

When comparing the ^{1}H and ^{13}C chemical shifts for analogous residues between AIP-I D1AS6A and either AIP-I D1AS6dA or AIP-I D1AS6A [N-MeF8], the ^{1}H and ^{13}C shifts of the exocyclic residues were nearly identical. The chemical shift differences were within 0.03 ppm for ^{1}H chemical shifts and 0.06 ppm for ^{13}C chemical shifts (see Table S25 above).

For the endocyclic residues, the differences were slightly larger than those in the exocyclic tails for similar residues (<0.25 or <1 ppm for most ^{1}H or ^{13}C shifts, respectively; see Table S25), unless a large difference in conformation was suspected. The most significant differences are commented on here. The first macrocyclic residue, Cys4, exhibits little variation in chemical shifts in most of the peptides, but there is a noticeable 0.2 ppm change in the Hα shift and a 1 ppm difference in the Cα shift of AIP-I D1AS6A [N-MeF8]. With Ala5, all three peptides exhibit small changes in Hα, Cα, and Cβ shifts relative to each other. For the sixth residue, either L-Ala or D-Ala fills this position, and the ^{1}H and ^{13}C shifts all differ between the peptides. Interestingly, AIP-I D1AS6A and AIP-I D1AS6A [N-MeF8] share the same amino acid at this residue (L-Ala) and at the flanking positions, yet there are ^{1}H and ^{13}C shift differences at Ala6 between the two peptides, suggestive of a conformational difference between these two peptides and that the β-turn structure could be perturbed in AIP-I D1AS6A [N-MeF8]. Of all the changes in chemical shifts between the three peptides, the largest deviation occurs in Tyr7. While the shifts of Tyr7 in AIP-I D1AS6A and AIP-I D1AS6dA are very close, and almost indistinguishable in some cases (differences of <0.05, <0.9, <0.4, <0.05, and <0.05 ppm for Hα, Cα, Hβ1, Hβ2, and Cβ respectively), there are large shift differences for AIP-I D1AS6A [N-MeF8]. Most notable are those that surpass two standard deviations above the average shift displacement, namely the Cα with a shift difference of >15 ppm and Hβ2 with a shift difference of >0.5 ppm. These large changes in chemical shifts for AIP-I D1AS6A [N-MeF8], alongside a smaller but still noticeable Cβ shift of 1.7 ppm, suggests a perturbation in the local conformation around this residue, presumably as a result of the *N*-methylation of Phe8. Lastly, while the final residues' shifts are fairly consistent between AIP-I D1AS6A and AIP-I D1AS6dA, there are significant shift differences in Hβ and Cα of AIP-I D1AS6A [N-MeF8] (0.6 and 10 ppm, respectively), again, presumably due to the *N*-methylation of Phe8 in this analog.

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