SUPPLEMENTARY INFORMATION

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Cross-Strand Interactions of Fluorinated Amino Acids in β-Hairpin Constructs

Ginevra A. Clark[†,‡], James D. Baleja[‡,§,*] and Krishna Kumar[†,§,*]

[†]*Department of Chemistry, Tufts University, 62 Talbot Avenue, Medford, MA 02155,* [‡]*Department of Biochemistry, Tufts University School of Medicine, Boston, MA 02111, and* [§]*Cancer Center, Tufts Medical Center, Boston, MA 02111*

Supplementary Information: Materials and Methods

Synthesis of Fmoc–Hexafluoroleucine

Flash column chromatography was performed on Kiesegel 60 silica gel (230–240 mesh, EM Science). Analytical thin–layer chromatography was performed using E. Merck silica gel Kiesegel 60 F_{254} (0.24 mm) plates. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DPX–300 instrument using standard deuterated solvents.

Boc–Hfl was synthesized according to previously reported procedures.[1,2] The purity of Boc–Hfl was confirmed by the presence of single peaks for OMe, Boc, and tBu protons in the ^{1}H NMR upon coupling to Ser(OtBu)OMe.[1] Boc–Hfl was treated with 1:1 CH_2Cl_2:TFA to form Hfl–HCl quantitatively. The Fmoc derivative was prepared from Hfl–HCl and Fmoc–OSu (0.354 g, 1.05 mmole) according to standard methods.[3] The crude product was purified via column chromatography with a 5:1 mixture of hexane:EtOAc with 0.1% acetic acid, followed by treatment to remove residual acetic acid.[4] The desired product was obtained in 63% yield.

Synthesis of Fmoc–@–Val–OH and Fmoc–@–Thr–OH

These amino acids were synthesized in accordance with previously published procedures and purified by flash chromatography.[5,6]

Scaffold I: Peptide Synthesis

NovaBiochem Novagel rink amide resin (0.63 mmole NH_2/gm) was used for peptide synthesis. To couple the Cys residues, 4 eq. Fmoc–

Cys(Trt)-OH, 3.6 eq. HBTU, 3.6 eq. HOBT, and 4 eq. of collidine were added to 3 mL of 1:1 CH_2Cl_2:DMF.[7] Fmoc-Hfl residues were coupled by the addition of 2 eq. of Fmoc-Hfl, 1.8 eq. HBTU, 1.8 eq. HOBt, and 2 eq. DIEA to 2 mL anhydrous DMF. Other residues were coupled by the addition of 4 eq. Fmoc-Amino acid, 3.6 eq. HBTU, and 4 eq. DIEA to 2 mL anhydrous DMF. Fmoc removal was accomplished by treating the resin four times with 5 mL of 2:8 solution of piperidine:DMF for 3 min. After removal of the final Fmoc group, the resin was capped with 5 mL of 90% DMF, 5% DIEA, and 5% acetic anhydride. Cleavage from the resin was perfomed with 94.5% TFA, 2.5% water, 2.5% EDT, and 1.0% TIS, according to typical procedures.

Peptide Purification

Peptides were purified by HPLC using a Vydac C18 column (22 mm × 250 mm, 300 Å pore size, 5μm) with a water/acetonitrile/TFA gradient. Molecular weights were confirmed by ESI-MS and purity was confirmed by analytical HPLC using a Vydac C18 column (4.6 mm × 250 mm, 100 Å pore size, 5μm).

Air Oxidation and Preparation of Stock Solutions

Peptides were dissolved in 0.1 M ammonium bicarbonate (0.25-0.5 g/mL) and stirred for 24 hours at RT in a vial that was open to air.[8] Peptides were purified by HPLC using a Vydac C18 column (22 mm × 250 mm, 300 Å pore size, 5μm) with a water/acetonitrile/TFA gradient. Molecular weights were confirmed by ESI-MS (110 V tube lens, 5.4 kV source voltage) on samples collected and injected directly from HPLC purification.

The oxidized peptides were analyzed for purity by analytical HPLC with a Vydac C18 column (4.6 mm × 250 mm, 100 Å pore size, 5μm). The pH was reduced to 2 with neat TFA and purified by HPLC. The purified peptides were freeze-dried, then re-dissolved in water and freeze-dried at least three times to remove residual TFA. All six peptides were obtained in 3.5-10 mg quantities.

Stock solutions were prepared in water and their concentrations were determined by amino acid analysis (AAA) at the Harvard Microchemistry and Proteomics Analysis Facility. For later experiments, concentrations were determined by measuring absorbance at 280 nm, using an extinction coefficient of 110 M^{-1}[9].

Scaffold I: CD Data Collection

CD spectra and T_m curves were obtained on a Jasco J-715 spectropolarimeter, where the temperature was controlled using a Jasco PTC-423S Peltier unit. Stock solutions were diluted to 125 μM with 20 mM Tris buffer, pH 8.0 in a 1 mm cuvette. The CD instrument was calibrated with CSA. Scans were performed from 250-180 nm, 0.5 nm pitch, at a scan rate of 10 nm/min, a 2 sec response time, and 1.0 nm bandwith. Two accumulations were obtained. Scans were performed at 20 °C and 90 °C. Molar ellipticities were calculated by the equation, $[\theta] = \theta_{obs} \times (MWR)/(10 \times l \times c)$ where θ_{obs} is the observed signal in millidegress, MWR is the peptide molecular weight per residue, l is the pathlength of the cell in cm, and c is the peptide concentration in mg/mL[10]. All peptides displayed similar CD

spectra (Figure S.1), and the spectra was unchanged at higher temperatures. This was consistent with results of Cochran and co-workers.

Scaffold I: Determination of C_{eff}

Preparation of Glutathione Stocks

A glutathione stock was prepared in water by mixing 3 mL of 0.2 M reduced glutathione with 1 mL of 0.1 M oxidized glutathione. The resultant solution was divided into 50 µL aliquots and stored at -80°C. The concentration of reduced glutathione was determined using Ellman's reagent.[8] The concentration of oxidized peptide was determined by UV absorbance.[9] The concentrations of reduced and oxidized glutathione were found to be 0.146 M and 0.0229 M, respectively to give a total concentration of 0.192M. This stock was used for all experiments performed at higher (375 µM) analyte peptide concentrations

A different was prepared to determine C_{eff} at lower concentrations of target peptide (18.75 and 37.5 µM). The ratio of oxidized:reduced glutathione was changed so that the ratio of oxidized:reduced peptide was approximately 1:1 under equilibrium conditions in the concentration range selected. Thus, glutathione stock was composed of 193 mM reduced glutathione and 6.8 mM oxidized glutathione. This stock was prepared by adding 3 mL of water to 12.5 mg of oxidized glutathione and 178.1 mg of reduced glutathione. For the experiments at 37.5 µM, 25 µL of stock was added to a 1.5 ml LoBind Eppendorf microcentrifuge tube. For the experiments at 18.75 µM, 25 µL of stock was added

to a 2.5 mL microcentrifuge tube. The concentration of reduced glutathione was determined using Ellman's reagent.[9] The concentration of oxidized peptide was determined by UV absorbance.[8] The concentration of reduced and oxidized glutathione was measured to be 0.146 M and 0.0229 M, respectively to give a total concentration of 0.192M.

Buffer Preparation

A buffer containing 0.2M Tris and 1 mM EDTA was prepared and the pH was adjusted to 8.0. An additional 80 µM Tris base was added to the buffer in order to titrate the glutathione to a pH of 8.1 ± 0.05 in the final reaction solution.[11]

Reaction Setup for Determining C_{eff}

The peptide stock solution (stored at −20° C) and the glutathione stock (stored at −80° C) were placed in a Ar-purged glove bag and allowed to warm to 20° C. The buffer solution (0.2M Tris, 1 mM EDTA, pH 8.0, plus 80 µM Tris base) was purged with Ar for 5 min, as was the water used in the experiments. The reaction was initiated upon addition of peptide. The reaction mixture was incubated at 20°C in an eppendorf shaker set to 300 RPM. The samples were stored on dry ice prior to HPLC analysis.

HPLC analysis for experiments at 375 µM peptide concentrations was performed using a Vydac C18 small pore analytical column (4.6 mm × 250 mm, 100 Å pore size, 5µm) at a flow rate of 1.5 mL/min. HPLC analysis for experiments at 18.75 and 37.5 µM peptide concentrations were performed with a Phalanx small pore

analytical column (4.6 mm × 250 mm, 100 Å pore size, 5μm). A gradient of solvent A (99% water, 1% acetonitrile, 0.1% TFA) and solvent B (90% acetonitrile, 10% water, 0.07% TFA) was used.

Determination of C_{eff} at 375 μM

To the solution of glutathione buffer was added water, then 250 μL buffer, then peptide stock sufficient to make a 375 μM solution. The amount of water added was calculated such that 100 μL of peptide stock plus water was added to each reaction. Three aliquots were quenched at < 1 min, 1.5 hours, and 4 hours. To perform the quench, 30 μL of solution was removed and added to 400 μL of 1% TFA. At least two aliquots at <1 min were analyzed. At least four aliquots at times 1.5 hour and 4 hours were analyzed (in most cases, five aliquots were analyzed). Glutathione and peptide concentrations were determined by the relationships described below:[11,12]

$$[GSH]+2[GSSG]=[GSH]_{init}+2[GSSG]_{init}$$ Equation 3.16

$$[GSSG]=([GSH]_{init}+2[GSSG]_{init})/[2+3.76(GSHarea/GSSGarea)]$$ Equation 3.17

$[peptide_{ox}]/[peptide_{red}]$ = (peak area ratio)/(absorbance ratio) Equation 3.18

Where $[GSH]_{init}+2[GSSG]_{init}$ are the initial glutathione concentrations, and 3.76 is the ratio of the HPLC response for the glutathione peaks. A sample HPLC under equilibrium conditions for at each concentration is provided in Figures S.2-4.

Determination of C_{eff} at 18.75 and 37.5 μM

To the solution of glutathione buffer (22.5 µL) was added water, then 112.6 µL buffer, then peptide stock sufficient peptide stock to make a 18.75 µM solution. The total reaction volume was 1,800 µL. Aliquots were quenched at < 10 min, 4 hours, and 7 hours. To perform the quench, 600 µL of solution was removed and added to 60 µL of 5% TFA. Three independent experiments were performed for each peptide, and standard deviations were obtained from the resulting six HPLC traces.

For the experiments at 37.5 µM, to the solution of glutathione buffer was added water, then 125 µL buffer, then peptide stock sufficient peptide stock to make a 37.5 µM solution. The amount of water added is calculated such that the total reaction volume was 1,000 µL. Three aliquots are quenched at < 10 min, 4 hours, and 7 hours. To perform the quench, 300 µL of solution is removed and added to 60 µL of 5% TFA plus 300 µL of water. Three independent experiments were performed for each peptide, and standard deviations were obtained from the resulting six HPLC traces.

Scaffold II: Peptide Synthesis

A NovaBiochem LL Wang resin, 0.44 mmole/gm) was used for peptide synthesis. The first residue was coupled using the MSNT method.[13] After coupling but before deprotection, the resin was capped with 5 mL of 90% DMF, 5% DIEA, 5% acetic anyhydride. To couple additional residues, a soultion of 4 eq. Fmoc-Aaa-OH, 3.6 eq. HBTU, 3.6 eq. HOBt, and 4 eq was used. To couple amino acids to secondary amines (D-Pro or @-residues), a solution of 4 eq. Fmoc-

Aaa, 3.6 eq. HATU, and 4 eq. DIEA was prepared. Mixture was pre-activated for 5 min prior to coupling to resin. Coupling was confirmed by the presence of a negative chloranil resin test, but did not exceed 1 hr, even if coupling was incomplete, where deletion sequences were removed by HPLC purification. Fmoc removal was accomplished by treating the resin four times with 5 mL of 2:8 solution of piperidine:DMF for 3 min.

Prior to cleavage, the resin was washed with 5×5mL DMF, 3×5mL CH_2Cl_2, 2×5mL MeOH, then dried overnight. The resin was transferred to a 10 ml RB flask and stirred for 2 hours in 1:1 CH_2Cl_2:TFA. The solvent was evaporated and the resin was dried *in vacuo* for several hours. The peptide was removed by addition of 3×2 mL of MeOH and filtration over a medium porosity fritted glass filter. The volume of MeOH was reduced to < 1.5 mL and the crude product was immediately purified by HPLC. HPLC was performed with a Vydac C18 prep column using a water/acetonitrile gradient (10% B for 5 min, 1 min to equilibrate to gradient, 25-75% gradient for 25 min, followed by 100% B).

Scaffold II: Determination of Chemical and Stereochemical Purity

Peptide molecular weights were verified by MALDI-TOF-MS. Purity was determined by Analytical HPLC using a Phalanx small pore analytical C18 column (4.6 mm × 250 mm, 100 Å pore size, 5μm) with a water/acetonitrile gradient. Two peptides were observed in the HH2 analytical HPLC and the MW of both peaks corresponded

to the product (1023). The first peak (retention time 11.0 min) had an area roughly 40% of the later peak (retention time 13.3 min). These peaks were isolated by HPLC using a Vydac C18 column (25 mm × 250 mm, 300 Å pore size, 5μm). The larger peak was used for CD analysis. Previous reports have suggested racemization of Hfl during Fmoc synthesis, and we suspected that this gave rise to the peaks observed by HPLC. Thus, the chiral purity of all scaffold II peptides was determined by hydrolysis of the peptide and subsequent treatment with Marpheys reagent (FDAA), and analysis by LC-ESI-MS.[14] Peptide hydrolysis was performed using deuterated solvents, so that racemization occurring during hydrolysis could be distinguished from racemization occurring during peptide synthesis. Racemization resulting from the hydrolysis reaction was labeled with deuterium, thus increasing its molecular weight by 1 Dalton, which can be easily distinguished by MS.

To perform this analysis, stock solutions of peptides in water (~0.05 mg of peptide) were added to a 5 mL Reacti-Vials™ adorned with a MiniNert™ valves. (The vials were cleaned by water and methanol washings, followed by pyrolysis at 500 °C for 5 hours.) The solvent was removed by freeze-drying then the vials were purged with Ar. To the Ar-purged samples was added 200 μL of a 1:1 DCl:AcOH(d4) solution. The vials were sealed and heated to 130 °C overnight. Upon cooling, the solutions were diluted 10-fold with water, and the solvent was removed by freeze-drying.

To the dried samples was added 50 μL of 1M $NaHCO_3$, then 100 μL of 38.7 mM FDAA in acetone. The samples were sealed and heated to 40 °C for 1 hr. Reactions were quenched by the addition of 100 μL of 1M HCl, and 250 μL of HPLC solvent A (see below). The solutions were then concentrated to ~100 μL to facilitate detection by LCMS. Two peptides, HH2 and GH2 did not yield sufficient LC/MS response. The analysis of these peptides was repeated using 0.15 mg of peptide and 400 μL of hydrolysis solution. Coupling to FDAA and LC/MS was performed in the same manner.

LC-ESI-MS analysis was performed using a Finnigan LTQ MS (tube lens 100 V, source voltage, 4 kV) and with a ThermoFinnigan Surveyor Pump, Autosampler, and PDA detector (UV absorbance was measured at 340 nm). Separation was achieved with an Aquasil C18 small pore analytical column (4.6 mm × 150 mm, 100 Å pore size, 3-5μm) using a gradient of solvent A and B. Solvent A is composed of 1% ACN, 1% MeOH, 0.1 % TFA, and 98% water. Solvent B is composed of 89% ACN, 1% MeOH, 0.07% TFA, and 10% water. Standards of the free D and L amino acids treated with FDAA were used to confirm the identity of each peak. D and L amino acids were well separated on the column, could be identified by both retention times and masses, and quantified using TIC from the MS analysis. All of the peptides used in this study were free of racemization. The presence of all racemized amino acids could be

attributed to the hydrolysis step, due to the observed increase in molecular weight. The HH2 sample with a retention time of 11.0 min was clearly the result of racemization of Hfl during peptide synthesis. Evidence of racemization was not observed in any other peptide samples. This suggests that for other peptides, either the racemized peptides were easily separated by prep HPLC or that racemization did not occur during syntheses.

Scaffold II: Determination of [Θ] and Temperature-Dependent CD

General Procedures

CD spectra and T_m curves were obtained on a Jasco J-715 spectropolarimeter, where the temperature was controlled using a Jasco PTC-423S Peltier unit. The CD instrument was calibrated using a CSA standard. Variable wavelength CD was performed from 320-240 nm, with a data pitch of 0.5 nm, at a scan rate of 20 nm/min, a 2 second response time, 2.0 nm bandwidth, and two accumulations per run. Variable temperature CD was performed with a 1 nm bandwith, 1°C pitch, 8 sec response time and rate of 2°C/min. Stock solutions of peptide were prepared in water and filtered with a 0.22 μm PVDF syringe filter. The concentration of the stock solution was determined by measuring the absorbance at 284 nm using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer using the reported extinction coefficient (22,200 M^{-1} cm^{-1}) in 10 mM phosphate buffer.

Preparation methanol/water and trifluoroethanol/water solutions and buffer solutions

For initial studies CD experiments were performed at 15, 37.5 and 60 μM concentrations in 10 mM phosphate buffer, pH 7.0, 25 °C.

The exact concentration of each solution was determined by measuring absorbance at 284 nm using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer. These results are given in Table S.1. For comparison studies in aqueous and organic solutions, CD was performed at 5 μM peptide concentrations, and the final peptide concentration was determined by measuring the absorbance at 280 nm using a Cary 100 Bio Spectrophotometer. Organic stocks were prepared so that when 20 μL of peptide sock was added to 630 μL of solution, a solution with the appropriate % (v/v) of methanol or trifluoroethanol was prepared. CD was performed in solutions of 0–60% trifluorethanol, and 0–90% methanol (Figures S.5 and S.6, respectively). Additional studies, including temperature-dependent CD, were performed in 60% trifluoroethanol and 90% methanol. Temperature-dependent CD data were fit to Equation S.6 using the nonlinear least-squares method implemented in Igor Pro v5.03. From the thermodynamic parameters show in Tables S.2–4, the changes in interaction entropies, enthalpies and heat capacities of unfolding were calculated and are shown in Tables S.5–7.

Scaffold II: Thermodynamic Analysis

Thermodynamic parameters of folding ΔH, ΔS, and ΔC_p were determined by measuring the change in CD signal at 284 nm with respect to temperature from 10-85 °C. The free energy and χ_β were related in Equation 6. In addition:[15]

$$\Delta G = \Delta H - T\Delta S, \qquad \text{Equation S.1}$$

where ΔH and ΔS are temperature-dependent, since ΔC_p is large:

$$\Delta H^\circ(T) = \int_{T_h}^{T} \Delta C_p dT \qquad \text{Equation S.2}$$

Where T_h is the temperature at which $\Delta H = 0$, and:

$$\Delta S^\circ(T) = \int_{T_s}^{T} \Delta C_p / T dT \qquad \text{Equation S.3}$$

Where T_s is the temperature at which $\Delta S = 0$, therefore:

$$\Delta H = \Delta H^\circ_{298} + \Delta C_p (T - 298) \qquad \text{Equation S.4}$$

and:

$$\Delta S = \Delta S^\circ_{298} + \Delta C^\circ_p \ln(T/298) \qquad \text{Equation S.5}$$

Where ΔH°_{298} and ΔS°_{298} are ΔH and ΔS at 298 °K, respectively.

Therefore:

$$\chi_\beta = [\exp(x/RT)]/[1 + \exp(x/RT)] \qquad \text{Equation S.6}$$

Where:

$$x = [T(\Delta S^\circ_{298} + \Delta C^\circ_p \ln(T/298) - (\Delta H^\circ_{298} + \Delta C^\circ_p (T - 298))] \qquad \text{Equation S.7}$$

Scaffold II: NMR Fitting Procedures

For 1D NMR experiments, samples of scaffold II peptides were prepared at 1 mM concentrations in water with 100 mM sodium phosphate buffer, pH 7.0 and 10% D_2O, 30% CD_3OD, or 10% H_2O and 30% CF_3CD_2OH. Spectra were collected at ambient temperature on a Bruker DPX-300 spectrometer with a proton frequency of 300.34 MHz. Water suppression was performed using pre-saturation.

For 2D NMR experiments, samples of scaffold II peptides were prepared at 1 mM concentration in water with 30% CD_3OD and 10 mM sodium phosphate buffer, pH 7.0. Samples in D_2O for 1D measurements were prepared in the same manner. Spectra were collected at 10 °C on a Bruker AMX-500 spectrometer with a proton frequency of 500.14 MHz. Two-dimensional ROESY, two-dimensional NOESY, total correlation spectroscopy (TOCSY), and double-quantum filtered COSY spectra were collected with 2048 points in t_2 and 512 time-proportional phase increments in t_1. ROESY spectra were collected with mixing times of 100 ms. Additional ROESY spectra were collected with mixing times of 200 msec, or NOESY spectra were collected with mixing times of 800 msec. TOCSY were collected with mixing times of 63 ms using an MLEV-17 sequence.[16] Final spectra were zero-filled to 2048 × 1024 (real) points. 1D measurements were also obtained using this spectrometer. Detailed procedures structure determination, proton chemical shift values, structures, 1D NMR spectra, and ROESY spectra are provided.

Proton assignments were made by following standard homonuclear methods.[17] and are given in Table S.8. Structure calculations were performed on all peptides by employing the CNS program

(version 1.21). Parameter and topology files for Hfl were written based on the parameters for Leu. The charges on CF_3 groups were set to +0.6 and −0.2 for C and F, respectively. Bond angles and distances were evaluated by building a model in Macromodel and minimizing using default minimization parameters. For the @-residue, models were built in Insight II and minimized using the default minimization criteria. From the resultant structural (pdb) file, parameter (param) and topology (top) files were generated using HIC-Up. Models of the tripeptide L@V were built in Macromodel and minimized using the mm2 force field with a Newton minimization. The files generated by HIC-Up were modified to reflect the minimization obtained using Macromodel and to include attachments to hydrogen atoms. From these files, an mtf file was generated for each peptide using the GENERATE_SEQUENCE script. The resultant mtf file was manually modified to include bonds, angles, and dihedral angles between the @-residue and the adjacent residues. Bonds to Hfl were incorporated by modification of the protein.link file and additional modification of the mtf file was not required. From this mtf file, a pdb file of the extended structure was generated using the GENERATE_EXTENDED script.

Folded structures were generated using the ANNEAL script. The NOE and dihedral constraints were input using the default force constants (75 kcal/Å^2 and 400 kcal/deg^2, respectively). High-temperature dynamics, and then a cooling cycle in torsion space (with K_{NOE} = 75 kcal/mol) and minimization (K_{NOE} = 75 kcal/mol), were performed with modifications as previously described.[18]

Briefly, (1) the cooling cycles in torsion space and in Cartesian space were each doubled in length. (2) The dihedral angle energy function was activated, and the chemical shift function was disabled. Then, (3) the E_{repel} function was replaced by a Lennard-Jones potential during the final Powell minimization. Ten accepted structures ware accepted using the default ACCEPT script. Accepted structures were refined using the ENSEMBLE script with high-temperature dynamics and a cooling cycle in Cartesian space. The number of cooling cycles in Cartesian space was doubled, and the E_{repel} function was replaced by a Lennard-Jones potential during the final Powell minimization. Resulting structures were visualized by using Chimera. Ramachandran analysis was performed using Sirius.

For structure determination, ROE cross-peak intensities were converted into distance restraints and calibrated using the distances between atoms that could be determined by an analysis of three-bond coupling constants (^3J) and ROE intensities.[19] ROEs were calibrated using the 100 msec spectra for each peptide. Where possible, backbone distances and intra-residue distances of well-ordered residues were used. Cross-peaks that were observed in 200 msec ROESY or 800 msec NOESY, but not 100 msec ROESY were given constraints of 1.5 to 5 Å. For the 100 msec ROESY, strong intensity, intra-residue cross-peaks were given a constraint of 1.5-3 Å. Strong-medium intensity inter-residue cross-peaks, and medium intensity intra-residue cross-peaks were given a constraint of 1.5-4 Å. Weak intensity cross-peaks were given constraints of 1.5-5 Å. Constraints were widened appropriately

for cross-peaks that could not be stereospecifically assigned. Three-bond coupling constants, $^{3}J_{HN,H\alpha}$ were measured from the 1D spectrum and converted to dihedral angles.[17] Where possible, $^{3}J_{H\alpha,H\beta}$ and other side chain coupling constants were measured from the spectra taken in D_2O. Otherwise, $^{3}J_{H\alpha,H\beta}$ and other side chain coupling constants were estimated from the splitting in the t_2 dimension of the DQF-COSY spectra. Most of the β-methylene or γ-methyl protons were stereospecifically assigned on the basis of coupling constants and NOE patterns.

In addition, backbone H-bonding constraints were added to structural refinements, based on the (1) the expected positions of H-bonds in a β-hairpin, and (2) the observed location of backbone amides in structures calculated in the absence of this constraint. Ensembles composed of 10 structures are provided for each peptide in Figures 6 and S.7. For LH2, the preferred conformation of the N-terminal residues was significantly altered upon addition of H-bonding constraints and these structures are provided (Figure S.8). This alteration resulted in a conformation of LH2 that was similar to LL2, HH2, and HL2.

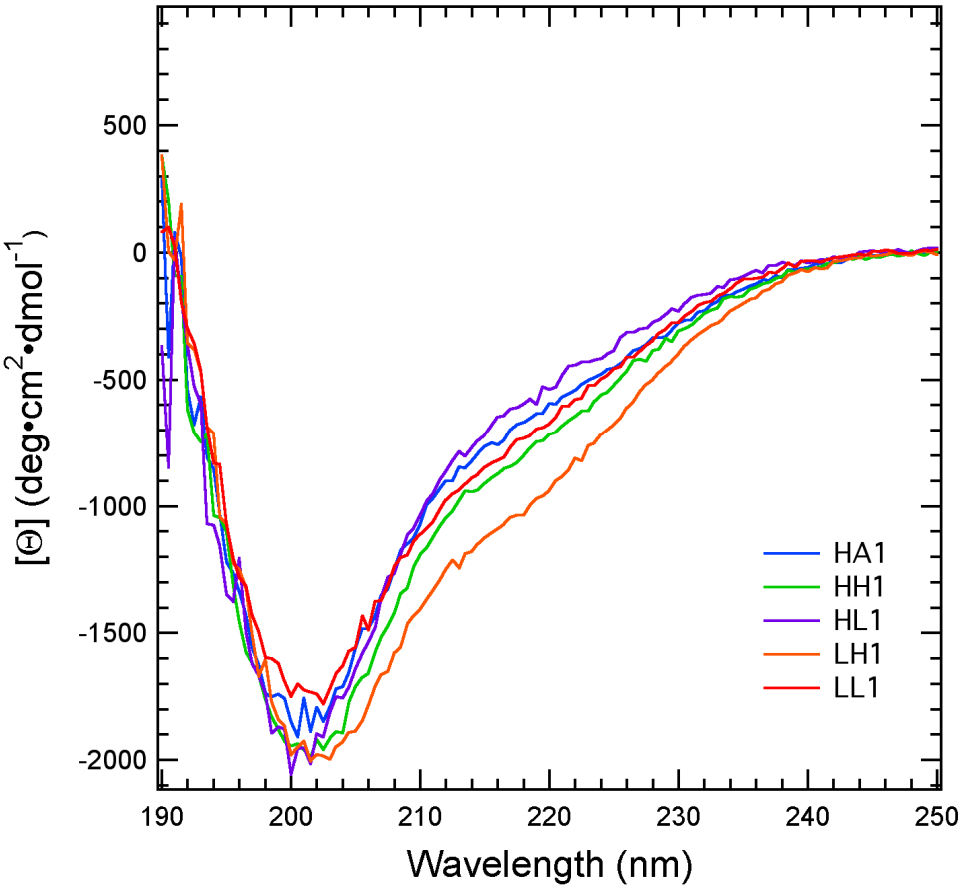

Figure S.1: Scaffold I: CD Spectra, 125 µM peptide, 20 mM Tris buffer, pH 8.0, 20 °C.

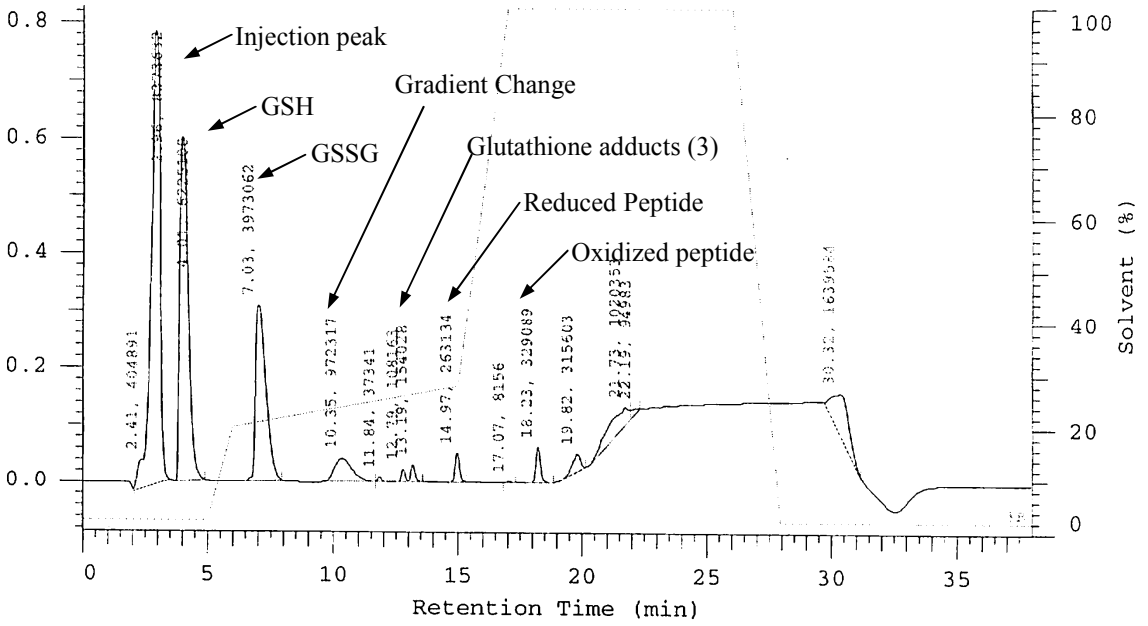

Figure S.2 Scaffold II: Sample HPLC of Determination of C_{eff} . Samples at 375 μM peptide concentrations were analyzed by HPLC with a Vydac C18 analytical column (4.6 mm × 250 mm, 5μm pore size). A water/acetonitrile/TFA gradient was used with a flow rate of 1.5 mL/min. Solvent "A" consisted of 99% water, 1% acetonitrile, and 0.1% trifluoroacetic acid. Solvent "B" consisted of 10% water, 90% acetonitrile, and 0.07% trifluoroacetic acid. To elute the oxidized and reduced glutathione, an initial gradient of 2–4% solvent B was used. The gradient used to elute the other peptides is given under each HPLC trace. In addition, the % solvent B is shown on the right axis.

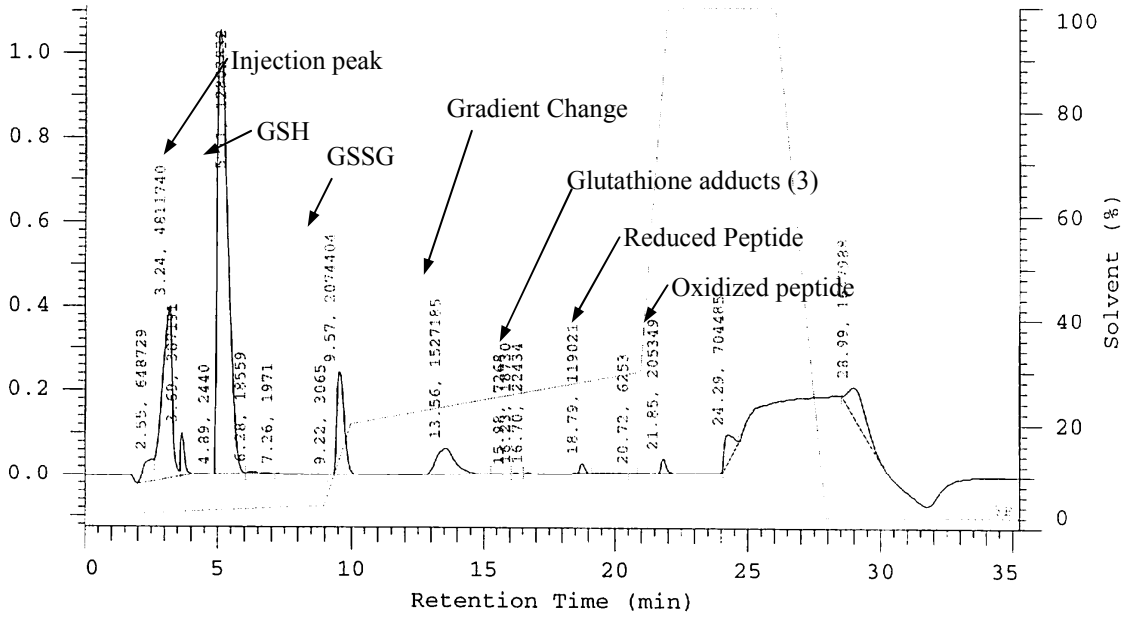

Figure S.3 Scaffold II: Sample HPLC of Determination of C_{eff} . Samples at 37.5 μM peptide concentrations were analyzed by HPLC with a Phalanx C18 analytical column (4.6 mm × 250 mm, 5μm pore size A water/acetonitrile/TFA gradient was used with a flow rate of 1.5 mL/min. Solvent "A" consisted of 99% water, 1% acetonitrile, and 0.1% trifluoroacetic acid. Solvent "B" consisted of 10% water, 90% acetonitrile, and 0.07% trifluoroacetic acid. To elute the oxidized and reduced glutathione, an initial gradient of 2-4% solvent B was used. The gradient used to elute the other peptides is given under each HPLC trace. In addition, the % solvent B is shown on the right axis.

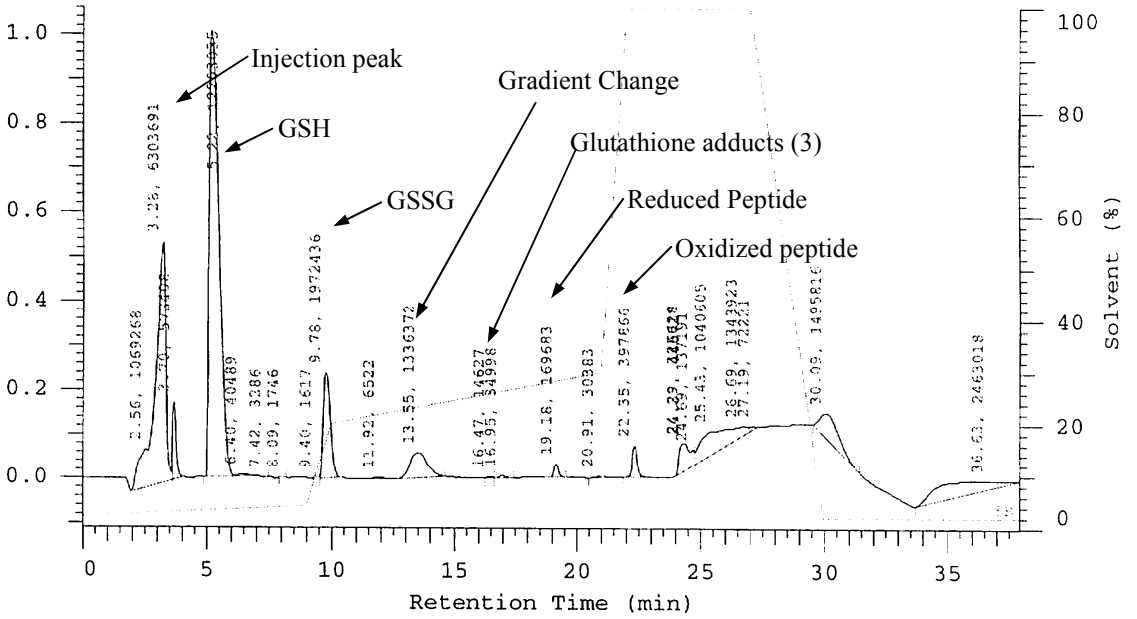

Figure S.4 Scaffold II: Sample HPLC of Determination of C_{eff} . Samples at 18.75 μM peptide concentrations were analyzed by HPLC with a Phalanx C18 analytical column (4.6 mm × 250 mm, 5μm pore size A water/acetonitrile/TFA gradient was used with a flow rate of 1.5 mL/min. Solvent "A" consisted of 99% water, 1% acetonitrile, and 0.1% trifluoroacetic acid. Solvent "B" consisted of 10% water, 90% acetonitrile, and 0.07% trifluoroacetic acid. To elute the oxidized and reduced glutathione, an initial gradient of 2-4% solvent B was used. The gradient used to elute the other peptides is given under each HPLC trace. In addition, the % solvent B is shown on the right axis.

Conc.	LH1	HH1	AH1	HL1	LL1	AL1
18.75 μM	0.0525 ± .0043	0.0416± .0040	0.0421± .0043	0.0539± .0035	0.0572± .0053	0.0339± .0039
37.5 μM	0.0522± .010	0.0424± .0073	0.0368± .0052	0.0458± .0090	0.0630± .0081	0.0297± .0042
375 μM	0.0600± .0013	0.0592± .001	0.0501± .0077	0.082± .024	0.0692± .0037	0.0486± .0013

Table S.1: Scaffold I: C_{eff} values at 18.75, 37.5, and 375 μM Peptide Concentrations. See Supplementary Information for experimental conditions. At 18.75 and 37.5 μM, error bars represent the 95% confidence interval from multiple comparison tests, where the data was collected from three independent experiments for a total of six HPLC traces. The error bars for experiments performed at 375 μM represent the standard deviations of three independent experiments, where the average of five HPLC traces were used for each experiment. Uniformity of the data at 18.75 μM and 37.5 μM was determined using Bartlett's test ($p = 0.05$).

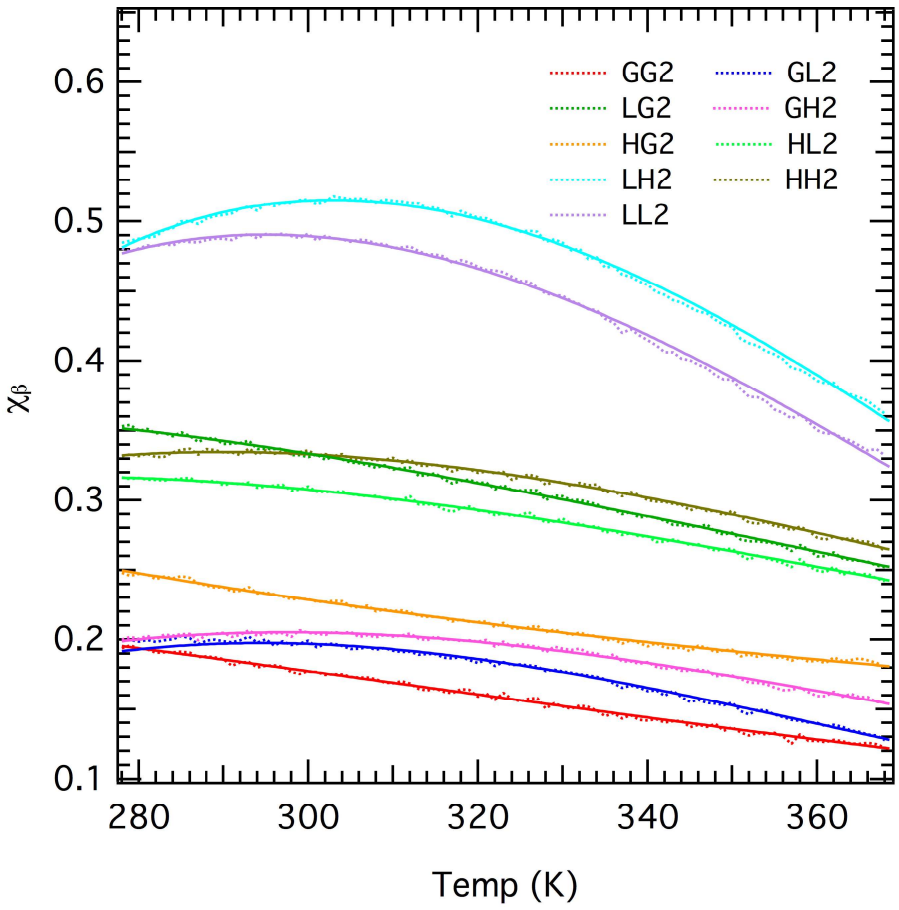

Figure S5a: Scaffold II : Thermal Denaturation of Peptides in Buffer. Average shown with dotted line. Global fit shown with solid line.

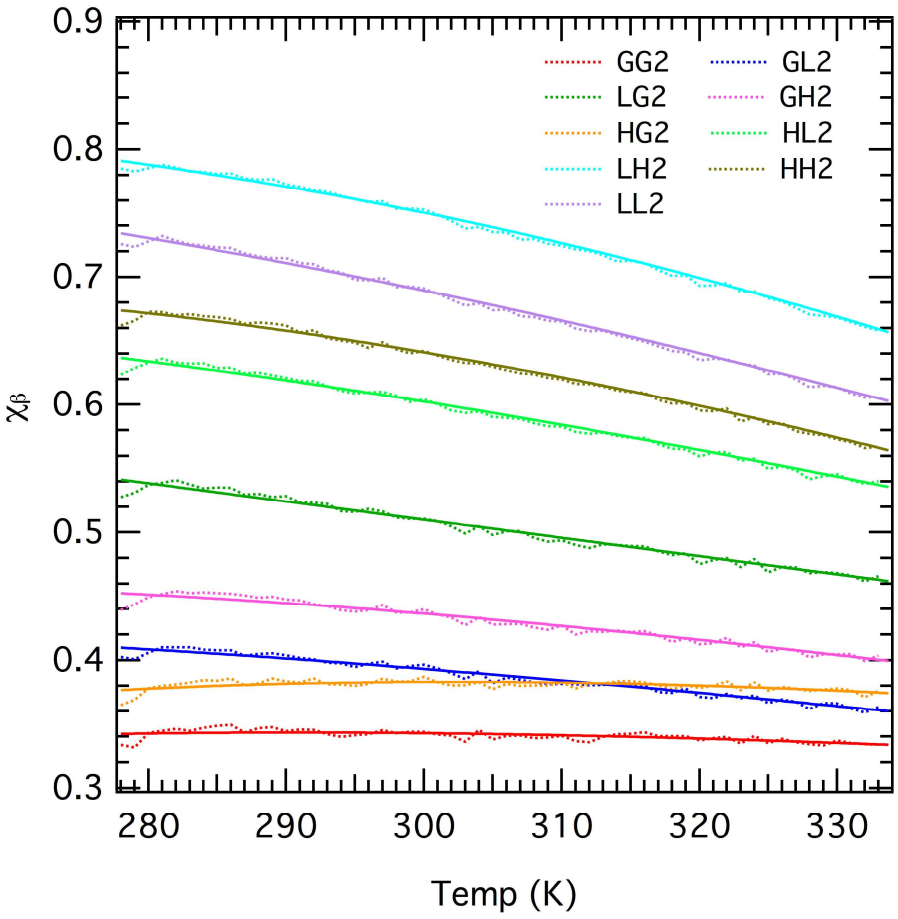

Figure S5b: Scaffold II:Thermal Denaturation of Peptides in 90% Methanol. Average shown with dotted line. Global fit shown with solid line.

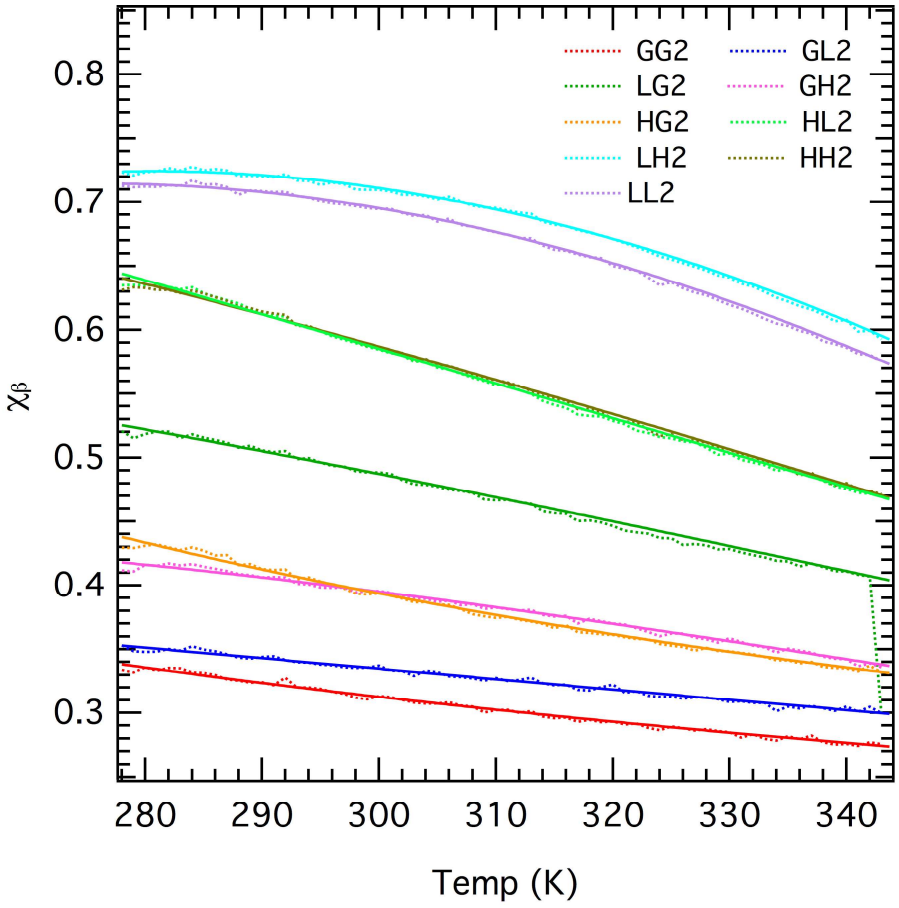

Figure S5c: Scaffold II: Thermal Denaturation of Peptides in 60% Trifluoroethanol. Average shown with dotted line. Global fit shown with solid line.

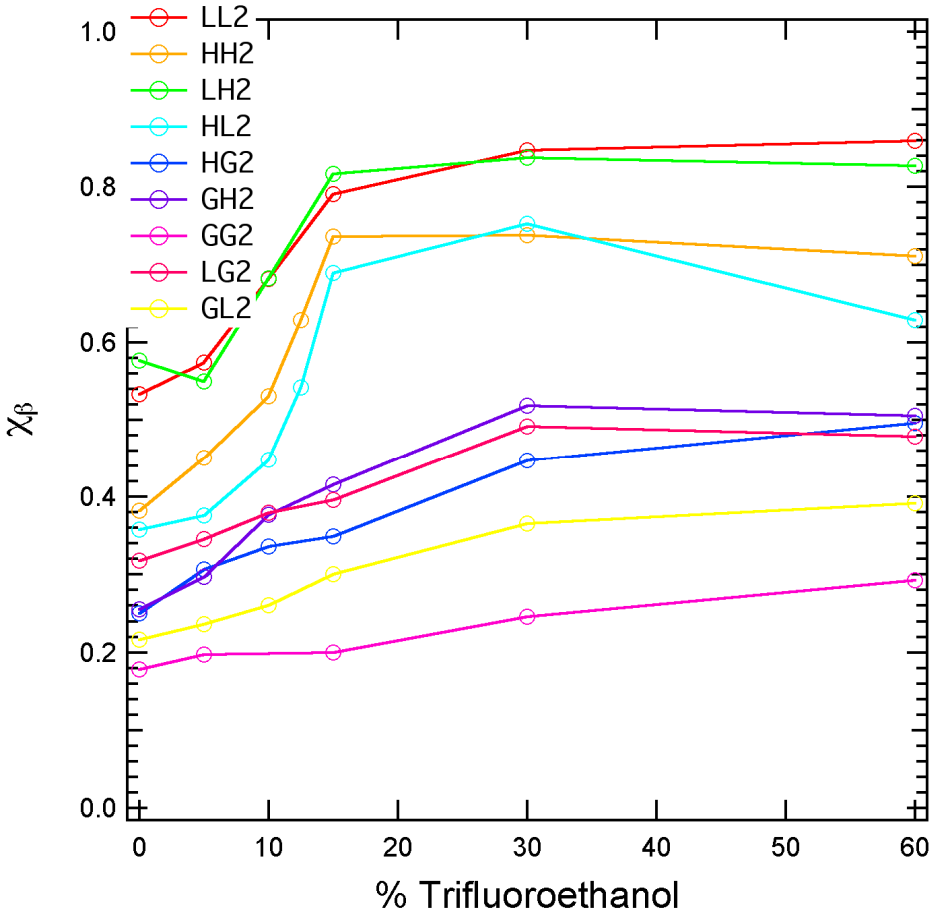

Figure S.6a Scaffold II: Trifluoroethanol Titration Curves. Conditions: 15 μM peptide, 10 mM phosphate buffer, pH 7.0, 20 °C

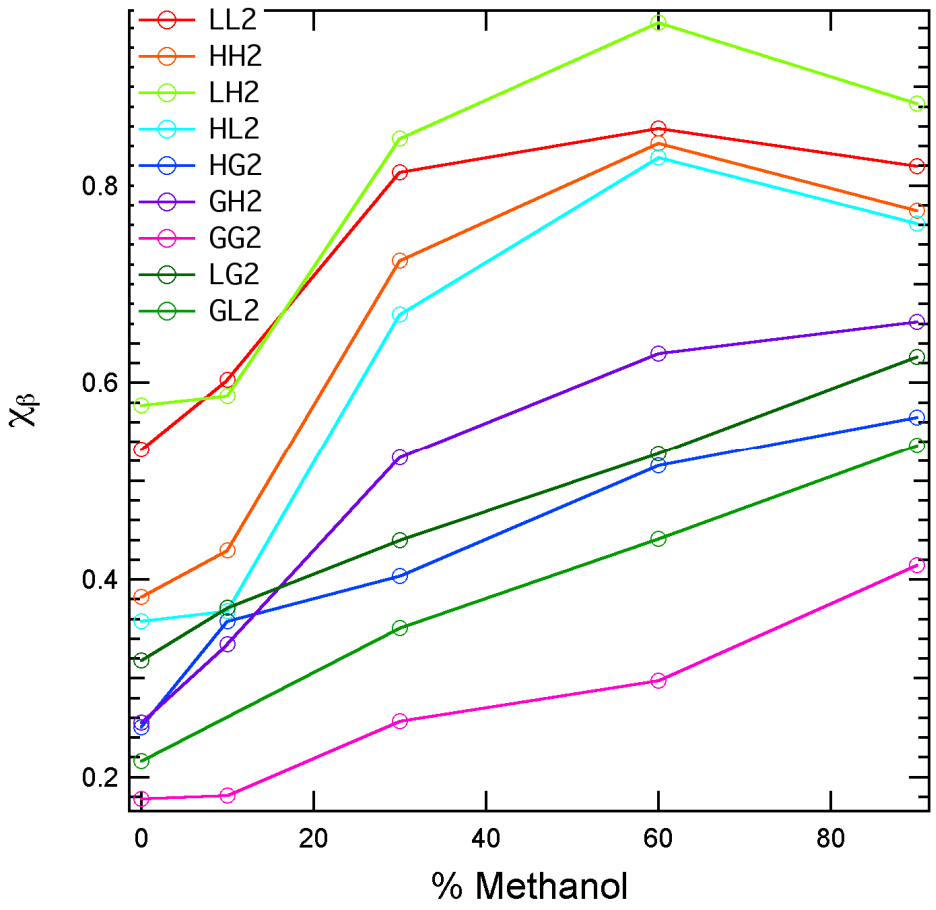

Figure S.6b Scaffold II: Methanol Titration Curves. Conditions: 15 μM peptide, 10 mM phosphate buffer, pH 7.0, 20 °C

Peptide ID	Conc.	[Θ] (deg/cm/dmol)*104	χβ
V-@-T	15	-2.54	0.00
	37.5	-2.73	0.01
	60	-2.60	0.00
	Average	-2.62	0.00
CY	15	-20.38	1.02
	37.5	-19.70	0.98
	60	-19.81	0.99
	Average	-19.96	1.00
LL2	15	-12.12	0.55
	37.5	-12.70	0.58
	60	-10.71	0.47
	Average	-11.85	0.53
LG2	15	-7.91	0.30
	37.5	-8.43	0.33
	60	-8.06	0.31
	Average	-8.13	0.32
GL2	15	-6.76	0.24
	37.5	-6.24	0.21
	60	-6.14	0.20
	Average	-6.38	0.22
HH2	15	-9.62	0.40
	37.5	-8.02	0.31
	60	-8.48	0.34
	Average	-8.71	0.35
LH2	15	-13.33	0.62
	37.5	-12.31	0.56
	60	-12.24	0.55
	Average	-12.62	0.58
HL2	15	-8.93	0.36
	37.5	-9.38	0.39
	60	-8.16	0.32
	Average	-8.82	0.36
HG2	15	-7.04	0.25
	37.5	-7.13	0.26
	60	-6.73	0.24
	Average	-6.96	0.25
GH2	15	-7.90	0.30
	37.5	-6.75	0.24
	60	-6.49	0.22
	Average	-7.05	0.26
GG2	15	-5.77	0.18
	37.5	-5.34	0.16
	60	-6.02	0.20

	Average	−5.71	0.18

Table S.2: Scaffold II:[Θ] and χ_β at Different Peptide Concentrations, 10 mM phosphate buffer, pH 7.0, 20 °C

peptide	ΔG° (cal/mol), aqueous buffer	ΔG° (cal/mol), 60% trifluoroethanol	ΔG° (cal/mol), 90% methanol
LL2	−75.66 ± 0.06	−477 ± 61	−479 ± 22
HH2	284.23 ± 0.10	−194 ± 17	−349 ± 14
LH2	−183.24 ± 0.04	−516 ± 53	−661 ± 53
HL2	346.81 ± 0.04	−188 ± 38	−256 ± 12
HG2	649.60 ± 0.01	267 ± 9	283 ± 7
GH2	634.09 ± 0.04	262± 39	152 ± 17
GG2	906.21 ± 0.02	476 ± 38	389 ± 12
LG2	452.22 ± 0.02	38 ± 29	−26 ± 15
GL2	761.83 ± 0.02	419 ± 84	257 ± 19

Table S3: Scaffold II: ΔG° values calculated from CD data.

Conditions: 15 μM peptide, 10 mM phosphate, pH 7.0, 20 °C

$\Delta G^\circ_{interact}$ (cal/mol)	LL2	HH2	LH2	HL2
aqueous buffer	−318 ± 58	−214 ± 58	−310 ± 58	−350 ± 58
90% methanol	−320 ± 15	−395 ± 15	−398 ± 15	−671 ± 15
60% trifluoroethanol	−457.7 ± 35	−246 ± 35	−338.98 ± 35	−511.4 ± 35

Table S4: Scaffold II: $\Delta G^\circ_{interact}$ values calculated from CD data.

Conditions: 15 μM peptide, 10 mM phosphate, pH 7.0, 20 °C

Peptide	ΔH°298 (cal/mol)	ΔS°298 (cal/mol/K)	ΔCp (cal/mol/K)
LL2	−191 ± 18	−0.718 ± 0.060	−60.66 ±0.75
HH2	−206 ± 15	−2.065 ± 0.050	−25.28 ± 0.63
LH2	336 ± 17	1.240 ± 0.055	−72.17 ± 0.68
HL2	−471 ±− 22	−3.180 ± 0.074	−17.36 ± 0.93
HG2	−892 ± 14	−5.390 ± 0.048	−1.54 ± 0.62
GH2	−17± 28	−2.747 ± 0.093	−33.06 ± 1.18
GG2	−1011± 25	−6.417 ± 0.083	−12.20 ± 1.10
LG2	−749 ± 19	−3.871 ± 0.062	−15.49 ± 0.79
GL2	−158 ± 71	−3.32 ± 0.24	−44.4 ± 3.1
YTV*	884	2.175	−270

* Hairpin reported by Maynard *et. al.*[15]

Table S.5 Scaffold II: Thermodynamic Parameters in Buffer. Conditions: 15 μM peptide, 10 mM phosphate buffer, pH 7.0, fit from 5–95 °C.

Peptide	ΔH°_{298} (cal/mol)	ΔS°_{298} (cal/mol/K)	ΔC_p (cal/mol/K)
LL2	−1867 ± 23	−4.647 ± 0.077	−22.3 ± 2.3
HH2	−1385 ± 18	−3.468 ± 0.062	−29.7 ± 1.9
LH2	−2082 ± 21	−4.761 ± 0.072	−34.1 ± 2.1
HL2	−1288 ± 18	−3.471 ± 0.060	−18.6 ± 1.8
HG2	60 ± 22	−0.745 ± 0.075	−15. 9 ± 2.3
GH2	−636 ± 19	−2.631 ± 0.064	−15.1 ± 2.0
GG2	−71 ± 21	−1.531 ± 0.071	−9.6 ± 2.2
LG2	−1026 ± 19	−3.345 ± 0.066	−6.8 ± 2.1
GL2	−634 ± 23	−2.976 ± 0.077	−11.9 ± 2.4
YTV*	-9200	-27	-2.6

* Hairpin reported by Maynard *et. al* in 50% MeOH.[15]

Table S.6 Scaffold II: Thermodynamic Parameters in 90% Methanol. Conditions: 15 μM peptide, 10% 10 mM phosphate buffer, pH 7.0, 90% trifluoroethanol, fit from 5–75 °C.

Peptide	ΔH°_{298} (cal/mol)	ΔS°_{298} (cal/mol/K)	ΔC_p (cal/mol/K)
LL2	−1235 ± 18	−2.481 ± 0.060	−56.5 ± 1.3
HH2	−1879 ± 17	−5.569 ± 0.056	−16.7 ± 1.2
LH2	−1077 ± 14	−1.803 ± 0.045	−62.61 ± 0.94
HL2	−1997 ± 20	−5.977 ± 0.067	−11.0 ± 1.4
HG2	−1350 ± 15	−5.362 ± 0.052	3.5 ± 1.2
GH2	−859 ± 15	−3.713 ± 0.052	14.5 ± 1.2
GG2	−889± 23	−4.531 ± 0.079	0.1 ± 1.8
LG2	−1306 ± 18	−4.460 ± 0.060	−14.3 ± 1.3
GL2	−656 ± 26	−3.552 ± 0.087	−5.4 ± 1.9

Table S.7 Scaffold II: Thermodynamic Parameters in 60% Trifluoroethanol. Conditions: 15 μM peptide, 40% 10 mM phosphate buffer, pH 7.0, 60% trifluoroethanol, fit from 5–65 °C.

Peptide ID:	LL2	HH2	LH2	HL2
$\Delta H^{\circ}_{\text{interact}}$ (cal/mol)	−295±80	−308±43	91±45	−431±80
$T\Delta S^{\circ}_{\text{interact}}$ (cal/mol)	16±80	−103±42	430±45	−265±80
$\Delta C^{\circ}_{p\ \text{interact}}$ (cal/mol/K)	−13±3.5	−2.9±1.8	−36±2	16±4

Table S.8a: Scaffold II: Thermodynamic Parameters at 25 °C for Side-Chain Interactions in Aqueous Media. Conditions: 15 μM peptide, 10 mM phosphate buffer, pH 7.0, fit from 5-95 °C.

Peptide ID:	LL2	HH2	LH2	HL2
$\Delta H^{\circ}_{\text{interact}}$ (cal/mol)	−278±43	−880±40	−490±40	−785±42
$T\Delta S^{\circ}_{\text{interact}}$ (cal/mol)	43±41	−484±41	−94±41	−382±42
$\Delta C^{\circ}_{p\ \text{interact}}$ (cal/mol/K)	−13±4	−8.4±4.2	−22±4	−0.4±4.4

Table S.8b: Scaffold II: Thermodynamic Parameters at 25 °C for Side-Chain Interactions in 90% Methanol. Conditions: 15 μM peptide, 10% 10 mM phosphate buffer, pH 7.0, 90% methanol, fit from 5-65 °C.

Peptide ID:	LL2	HH2	LH2	HL2
$\Delta H^{\circ}_{\text{interact}}$ (cal/mol)	−163±43	−558±36	199±36	−880±43
$T\Delta S^{\circ}_{\text{interact}}$ (cal/mol)	298±43	−305±36	548±36	−475±43
$\Delta C^{\circ}_{p\ \text{interact}}$ (cal/mol/K)	−37±3	−5.6±2.7	−33.7±2.7	−9.0±3.2

Table S.8c Scaffold II: Thermodynamic Parameters at 25 °C for Side-Chain Interactions in 60% Trifluoroethanol. Conditions: 15 μM peptide, 40% 10 mM phosphate buffer, pH 7.0, 60% trifluoroethanol, fit from 5-65 °C.

Residue	Proton	HH	HL	LH	LL
1, Leu or Hfl	Hα	4.473	4.28	4.555	4.537
	Hβ	2.12, 2.201	2.102, 2.028	1.7, 1.553	1.752, 1.554
	Hγ	3.615		1.674	1.674
	Hδ			0.946, 0.884	0.992, 0.928
2, -@-	Hα	5.49	5.492	5.546	5.566
	H6	4.153	4.214	4.139	4.175
3, Val	Hα	4.292	4.298	4.29	4.314
	Hβ	2.102	2.136	2.087	2.119
	Hγ	0.947	0.966	0.942	0.962
	HN	7.963	7.998	7.958	8.034
4, D-Pro	Hα	4.366	4.368	4.368	4.37
	Hβ	1.922, 2.281	1.931, 2.276	2.285, 1.925	1.92, 2.288
	Hγ	1.998, 2.13	2.116, 2.007	2.135, 2.011	2.13, 2.008
	Hδ	3.883, 3.631	3.864, 3.669	3.892, 3.605	3.894, 3.613
5, Ala	Hα	4.339	4.342	4.346	4.341
	Hβ	1.419	1.396	1.438	1.421
	HN	8.93	8.791	8.998	8.954
6, Val	Hα	4.176	4.153	4.166	4.143
	Hβ	2.238	2.201	2.258	2.253
	Hγ	0.905, 0.977	0.912, 0.966	0.903, 0.984	0.903, 0.977
	HN	8.237	8.194	8.263	8.238
7, Thr	Hα	4.842	4.667	4.9	4.727
	Hβ	4.08	4.075	4.058	4.035
	Hγ	1.179	1.199	1.185	1.223
	HN	8.355	8.29	8.405	8.404
8, Leu or Hfl	Hα	4.462	4.229	4.473	4.243
	Hβ	2.384, 1.947	1.514, 1.581	2.383, 1.963	1.542
	Hγ	3.17	1.537	3.127	1.474
	Hδ		0.832		0.824
	HN	8.589	8.274	8.668	8.365

Table S.9 Scaffold II: Table of ^{1}H Chemical Shift Values for @-tides.

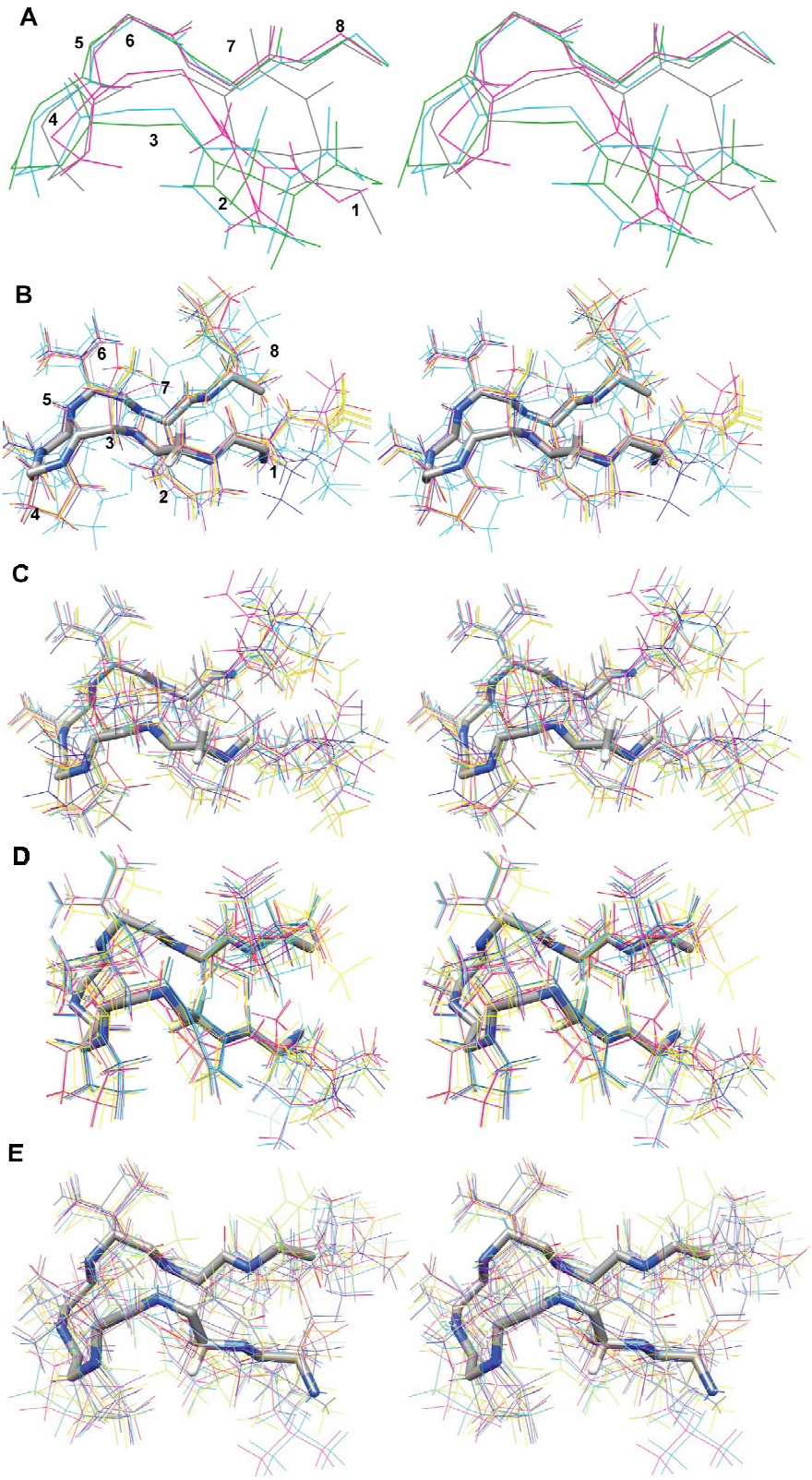

Figure S.7: (A) Scaffold II: Stereoview of overlay of Backbones for Peptides. The residue numbers are indicated on the structures. HH2 = green, HL2 = cyan, LH2 = magenta, LL = grey. Ensemble of 10 lowest energy structures for (B) HH2. (C) HL, (D) LH, (E) LL.

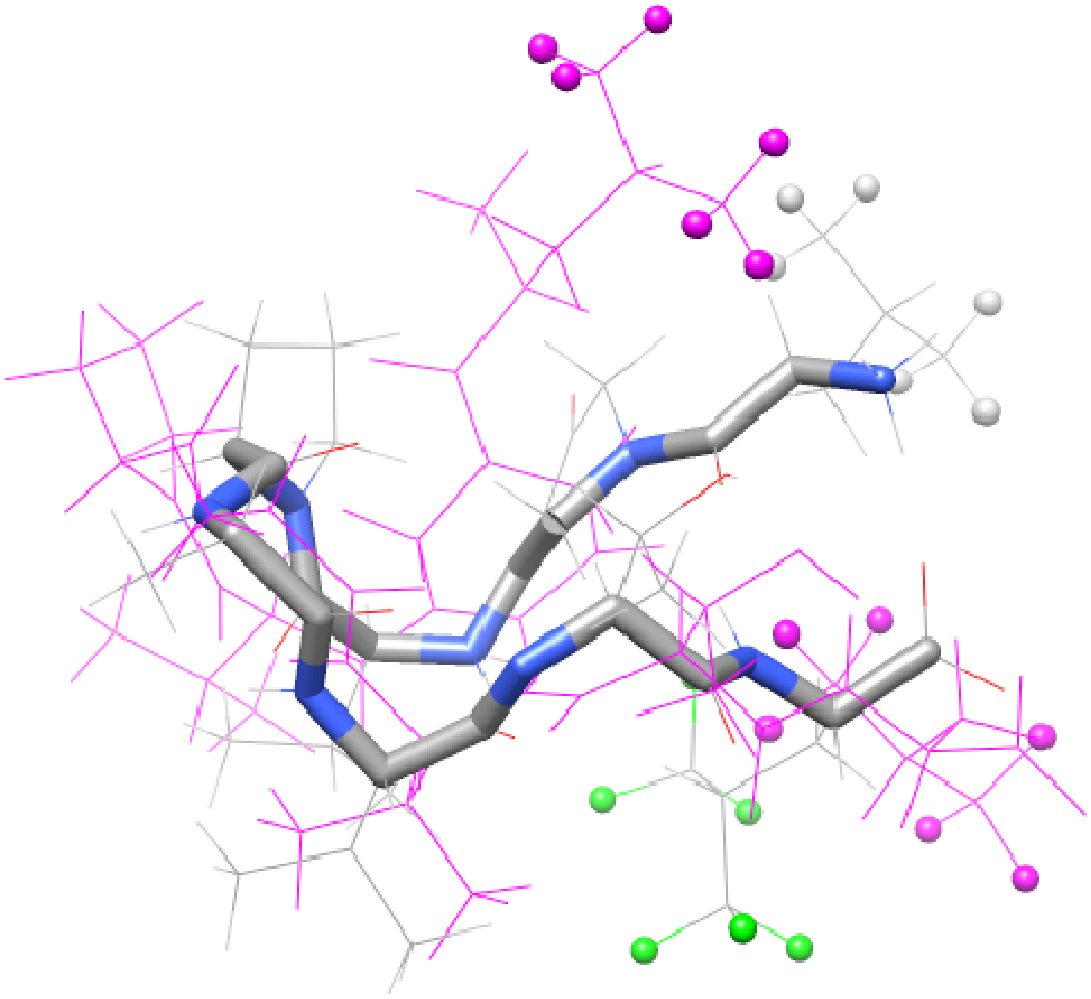

Figure S.8 Scaffold II: Comparison of data with and without H-bonding constraints LH peptide. The calculation using H-bonding is shown with N = blue, O = red, C = grey, F = green, H = light grey. The calculation without H-bonding data is shown in magenta. The terminal H and F atoms on residues 1 and 8 are depicted as spheres. (a) HH2 (b) HL2 (c) LH2 (d) LL2

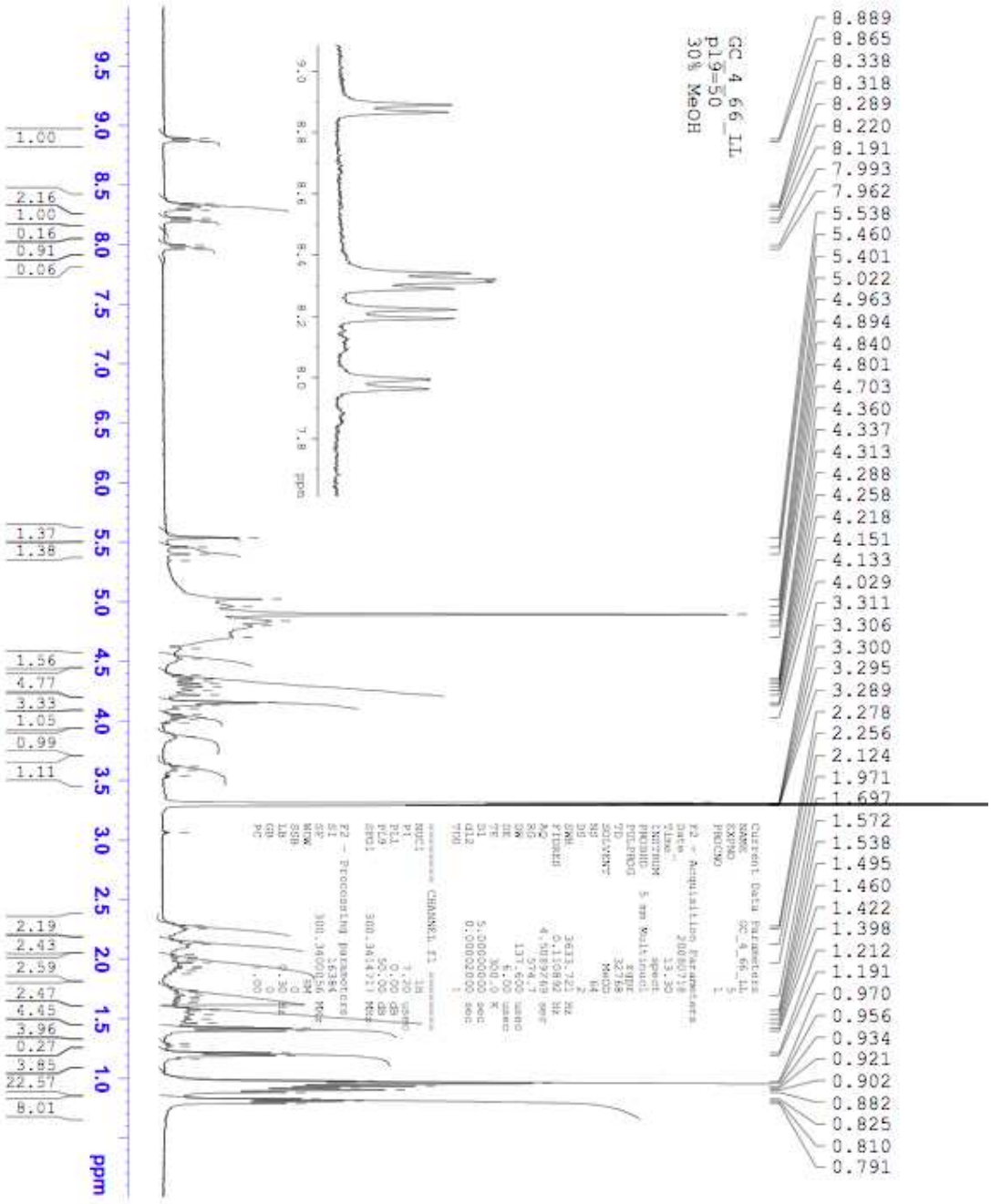

Figure S.9.a: 1D ^{1}H NMR, LL2 in 30% MeOH

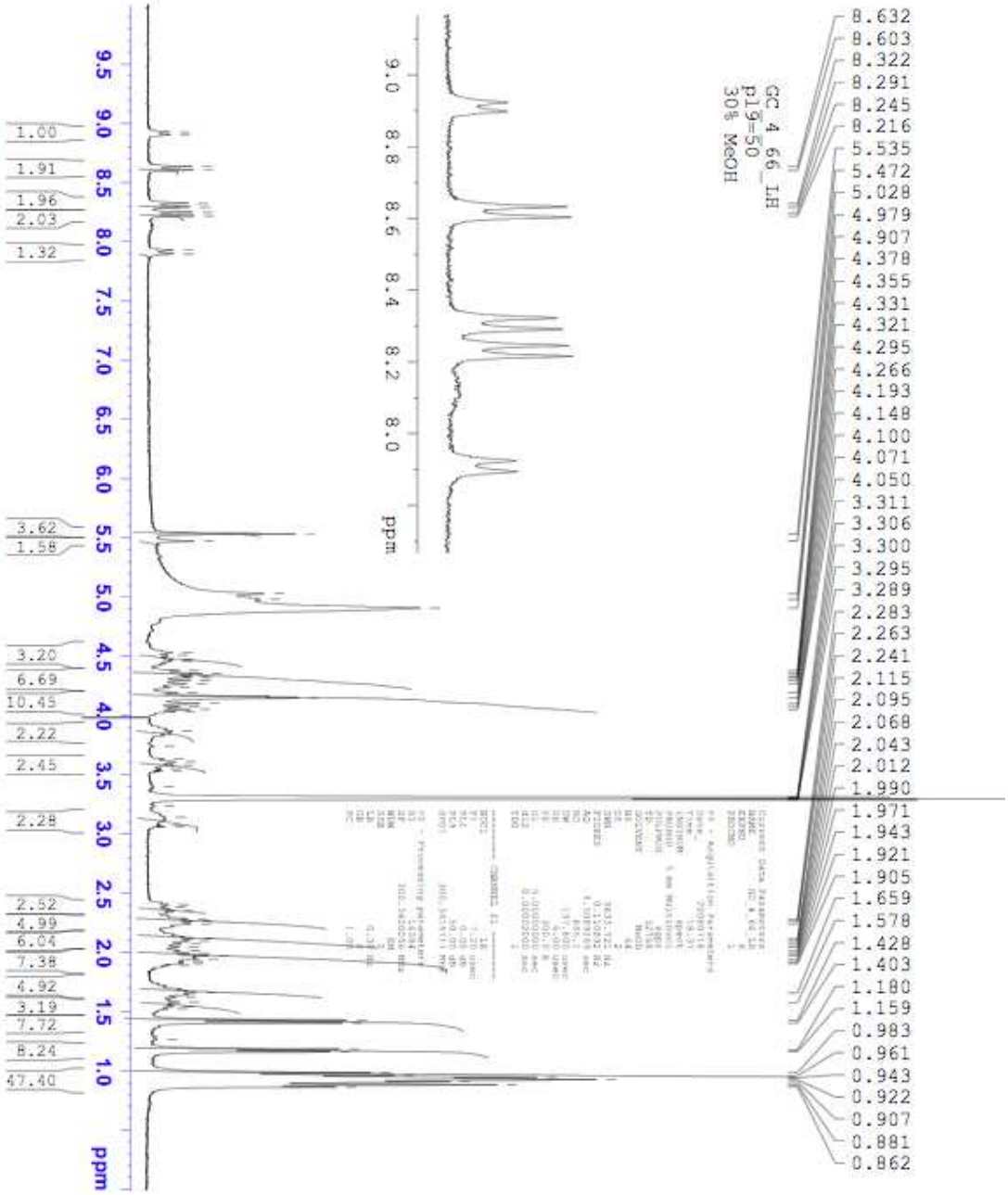

Figure S.9.b: 1D ^{1}H NMR, LH2 in 30% MeOH

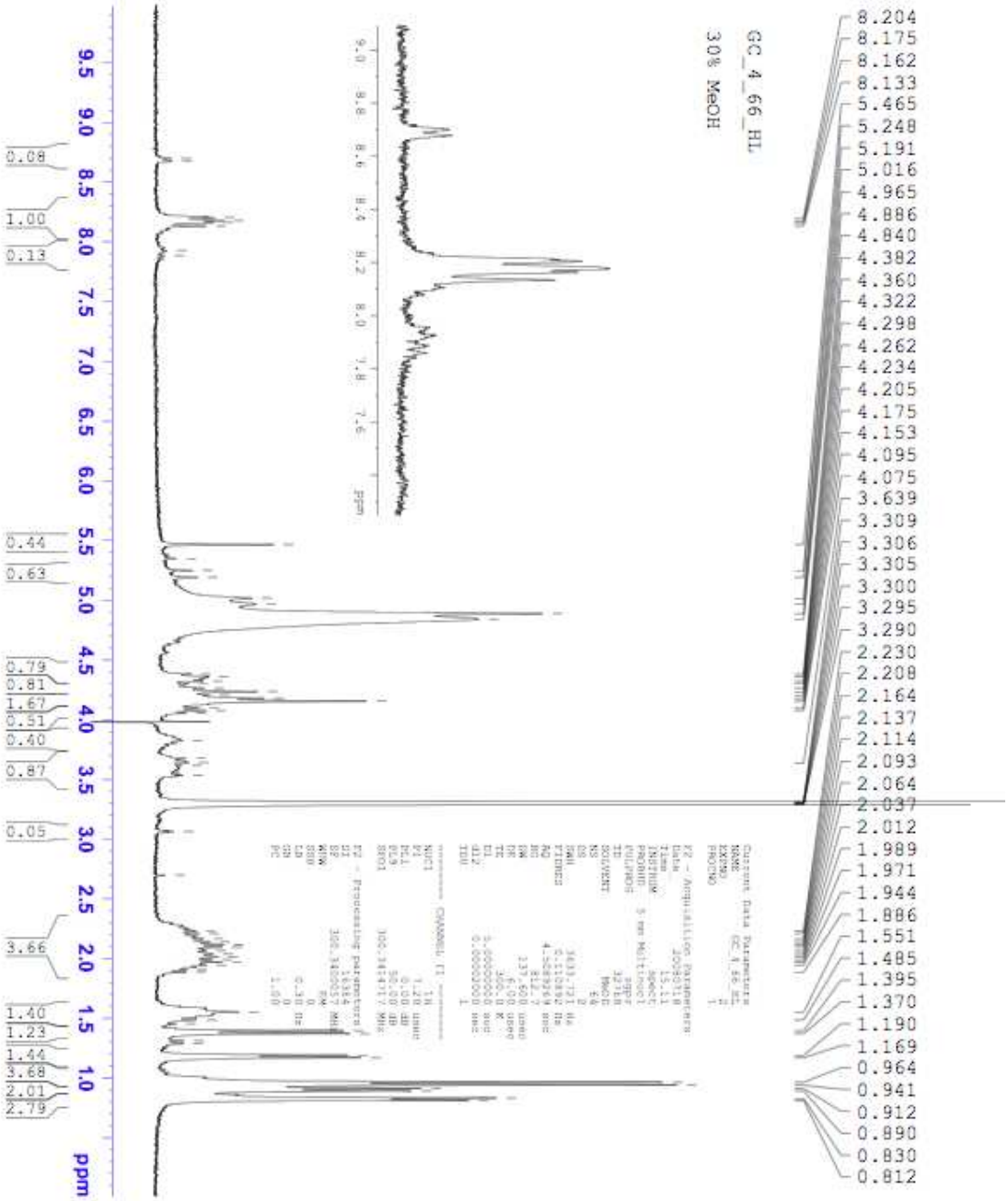

Figure S.9.c: 1D ^{1}H NMR, HL2 in 30% MeOH

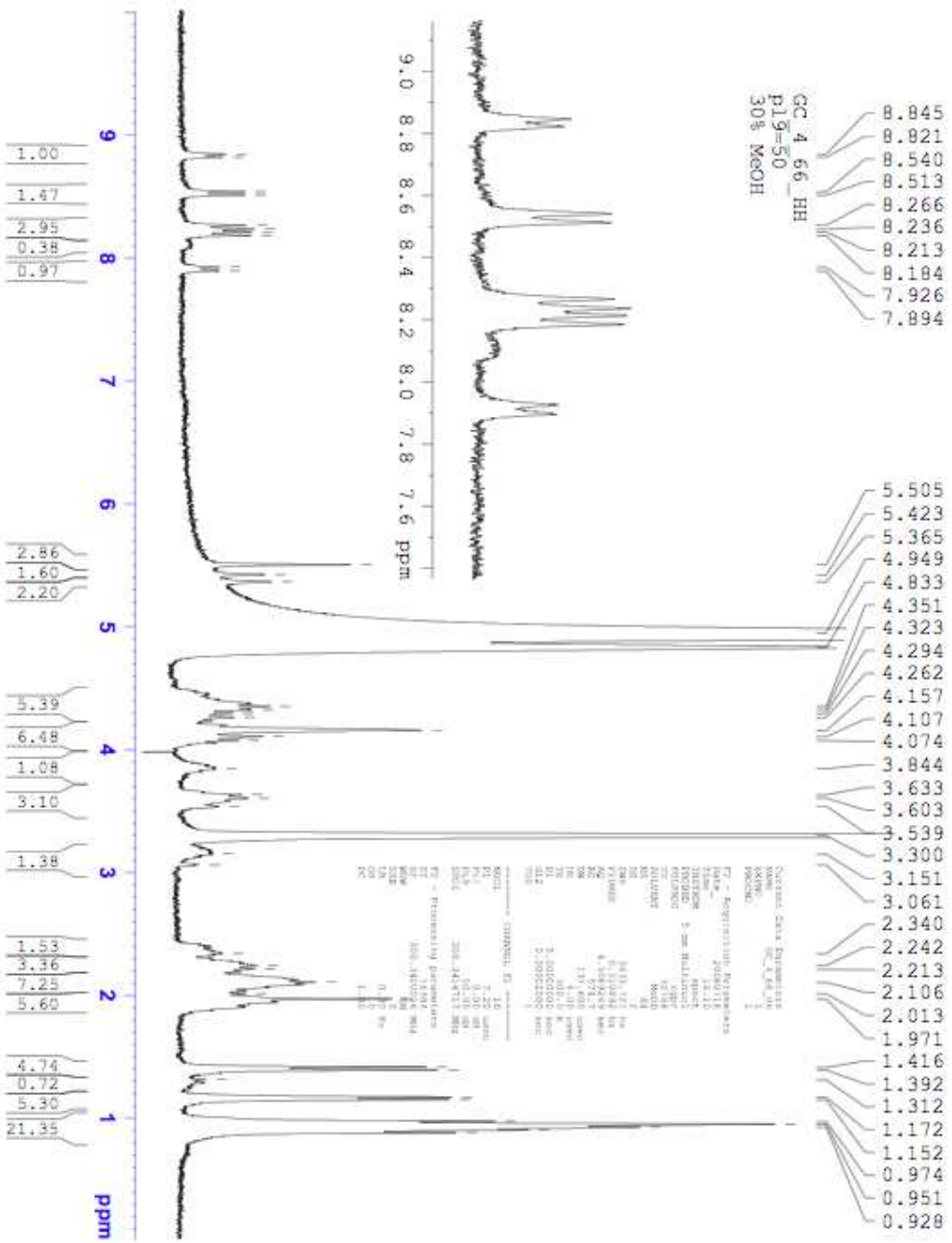

Figure S.9.d: 1D ^{1}H NMR, HH2 in 30% MeOH

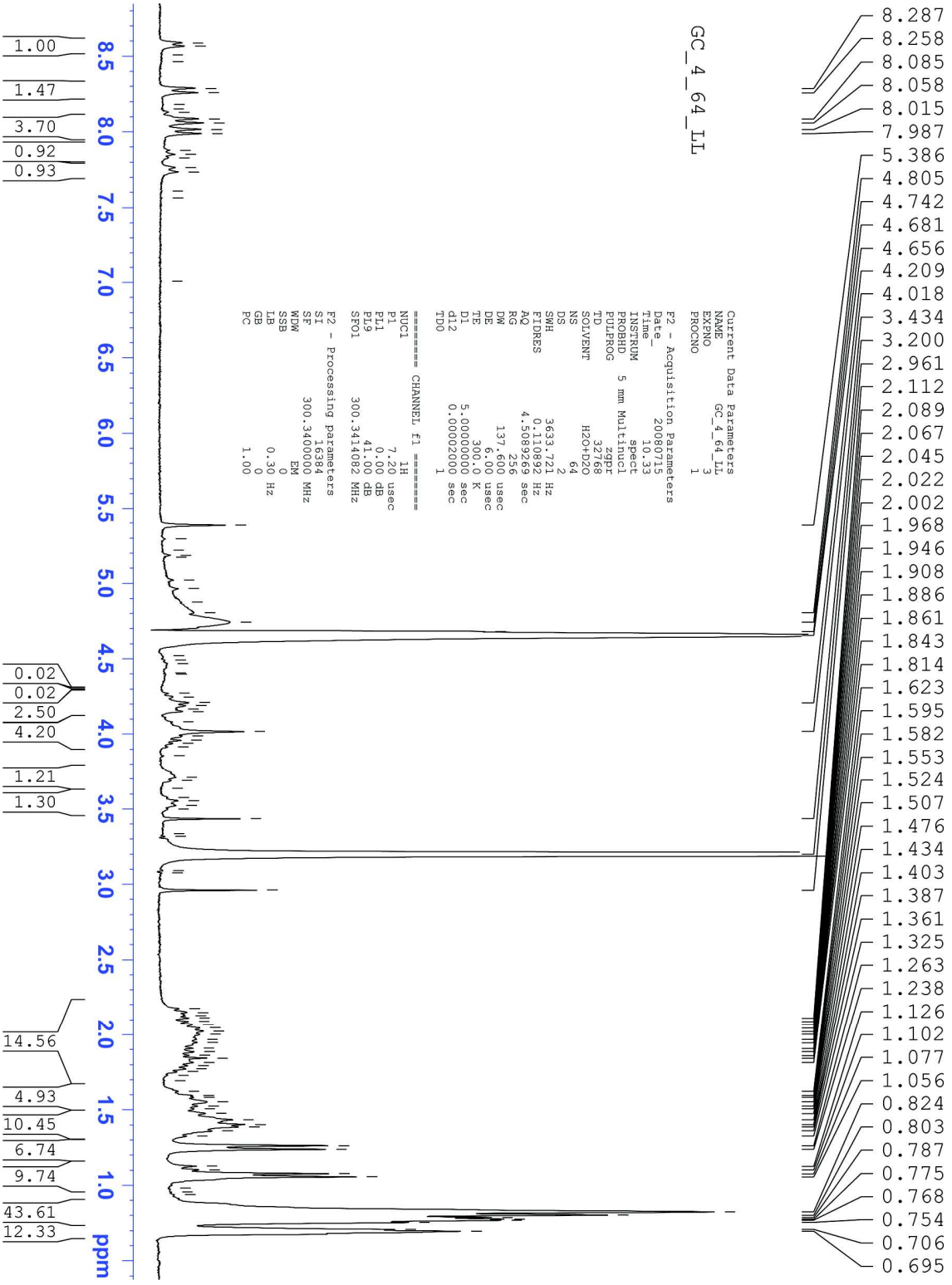

Figure S.9.e: 1D ^{1}H NMR, LL2 in Buffer

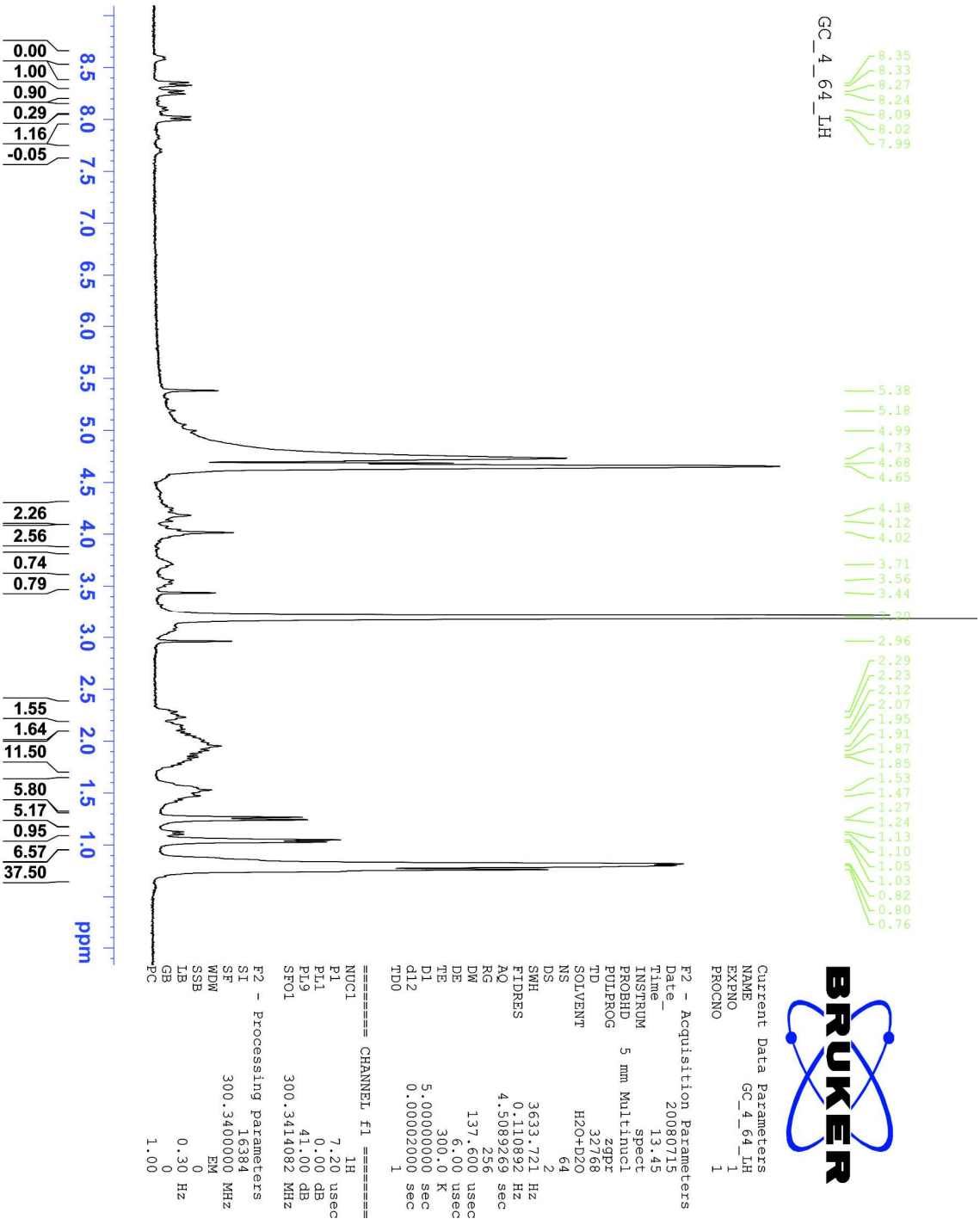

Figure S.9.f: 1D ^{1}H NMR, LH2 buffer

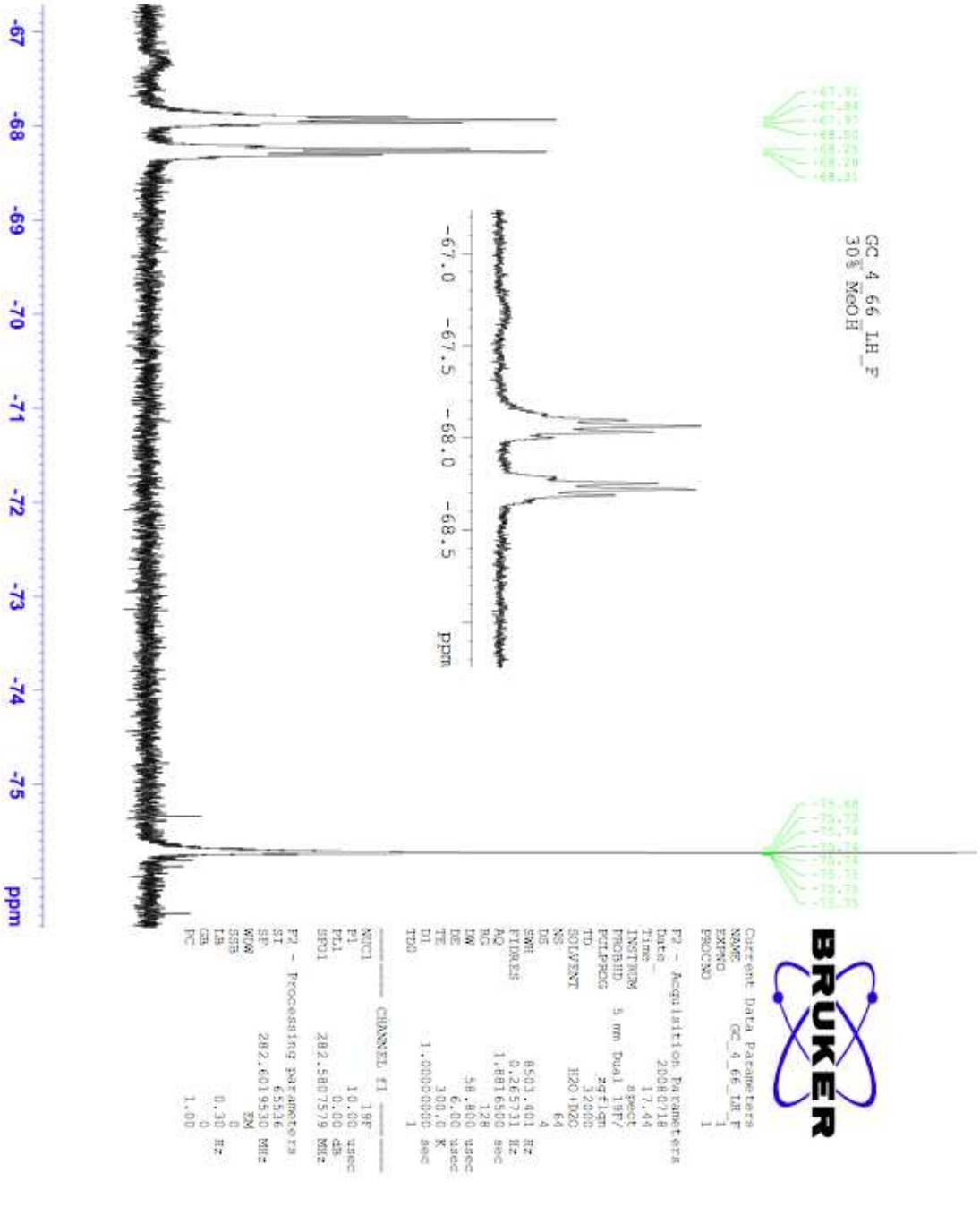

Figure S.10.a: 1D ^{19}F NMR, LH2 in 30% MeOH

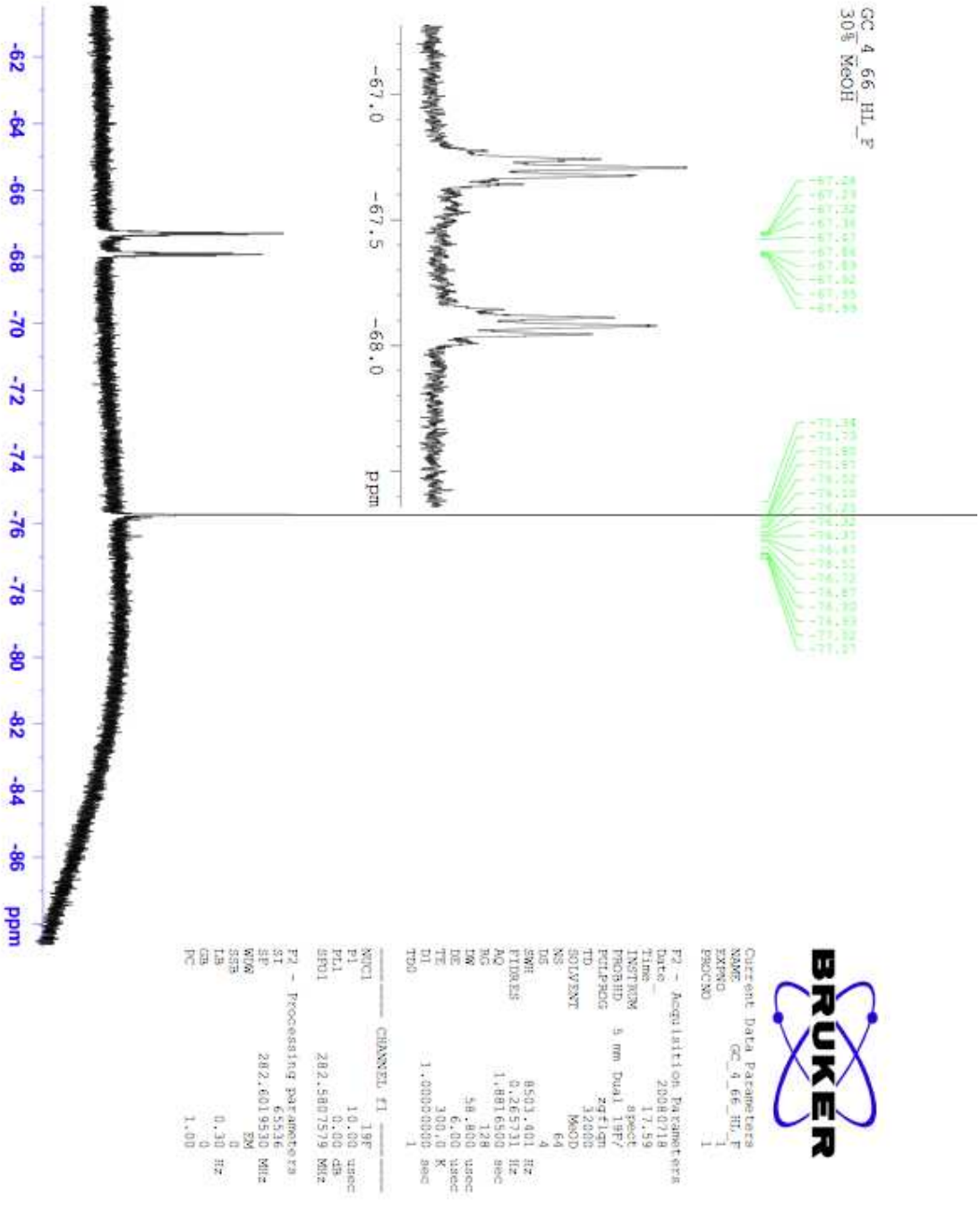

Figure S.10.b: 1D ^{19}F NMR, HL2 in 30% MeOH

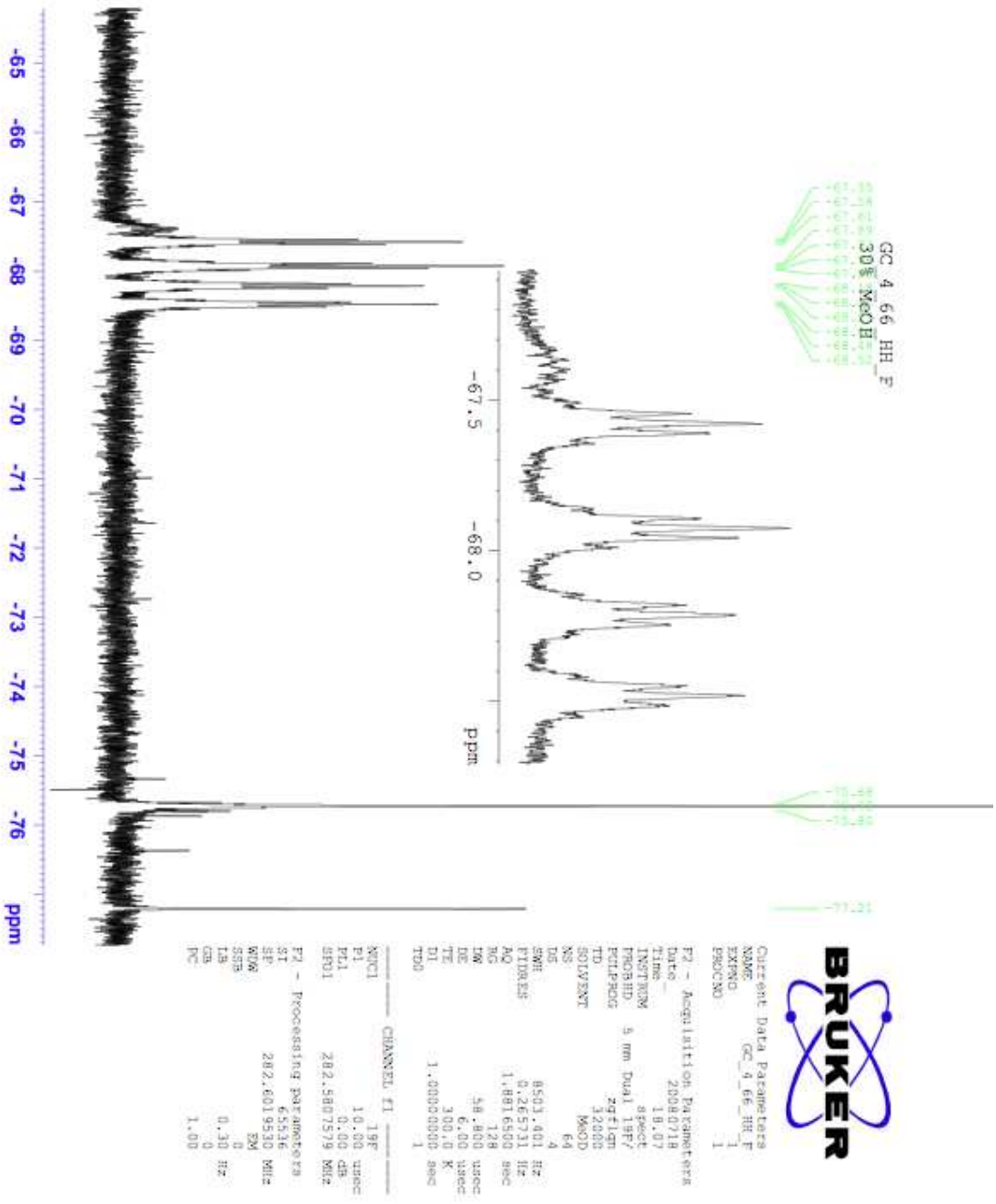

Figure S.10.c: 1D ^{19}F NMR, HH2 in 30% MeOH

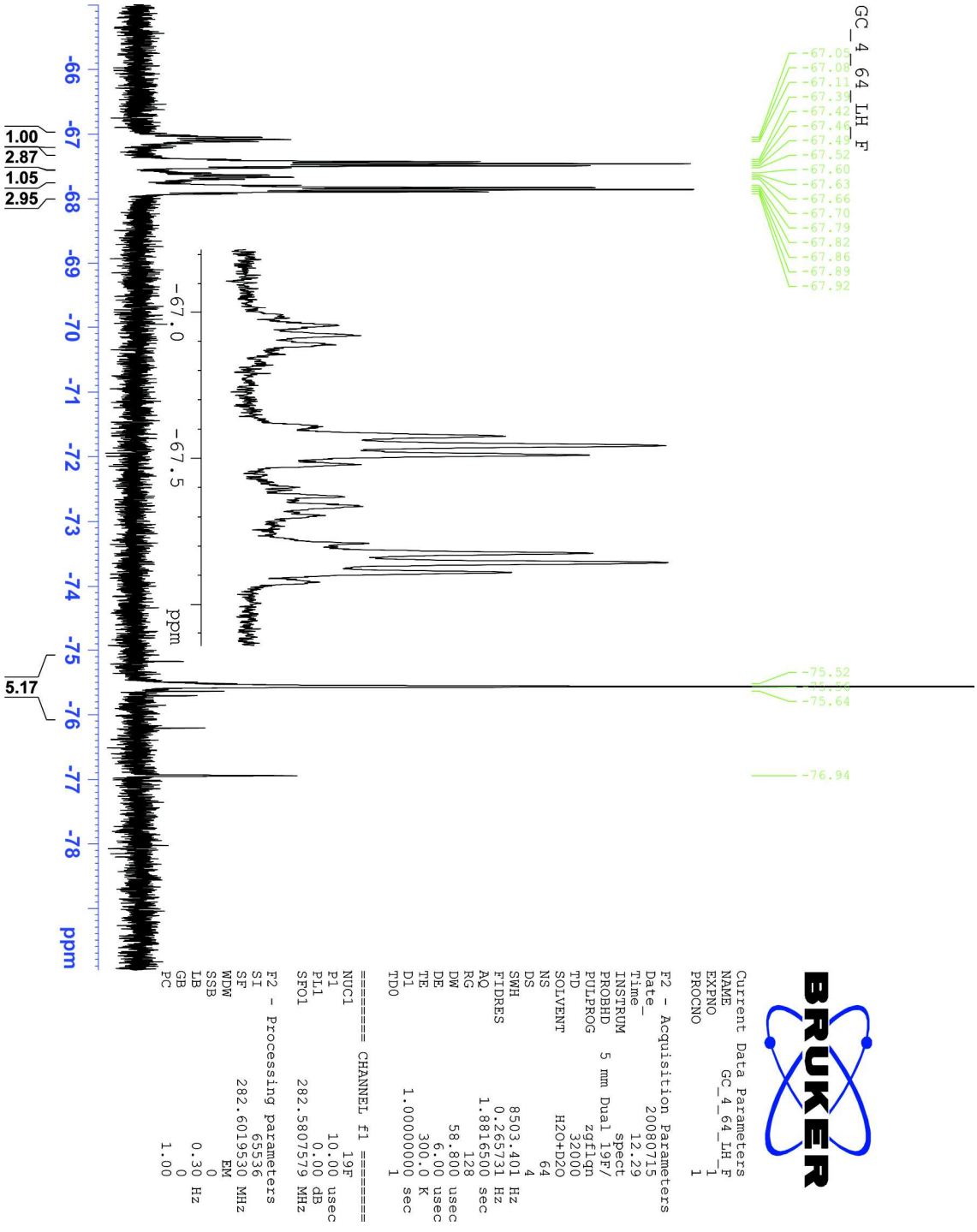

Figure S.10.d: 1D ^{19}F NMR, LH2 in Buffer

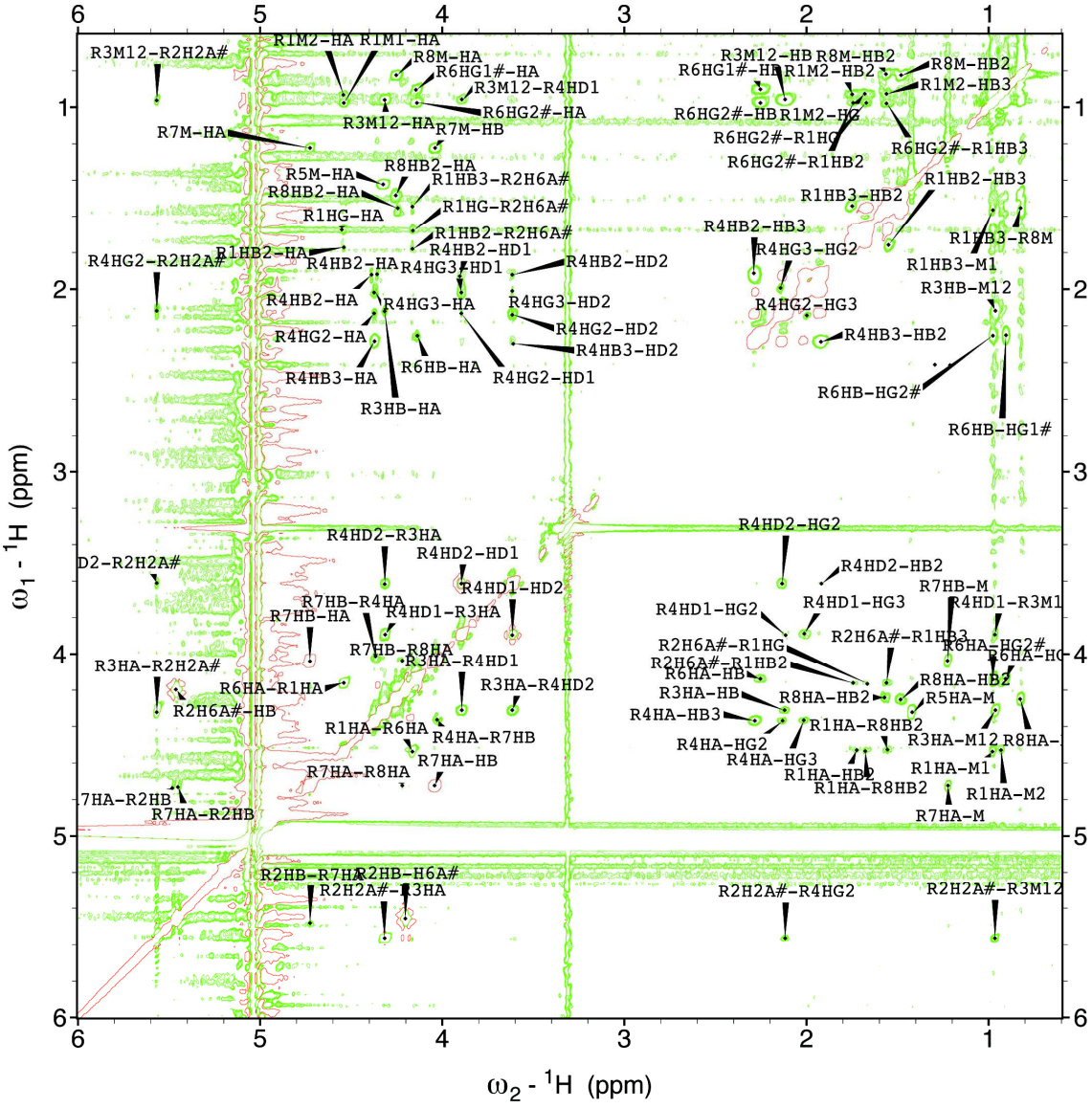

Figure S.11.a: LL2 ROESY spectra 6.0-0.6 ppm

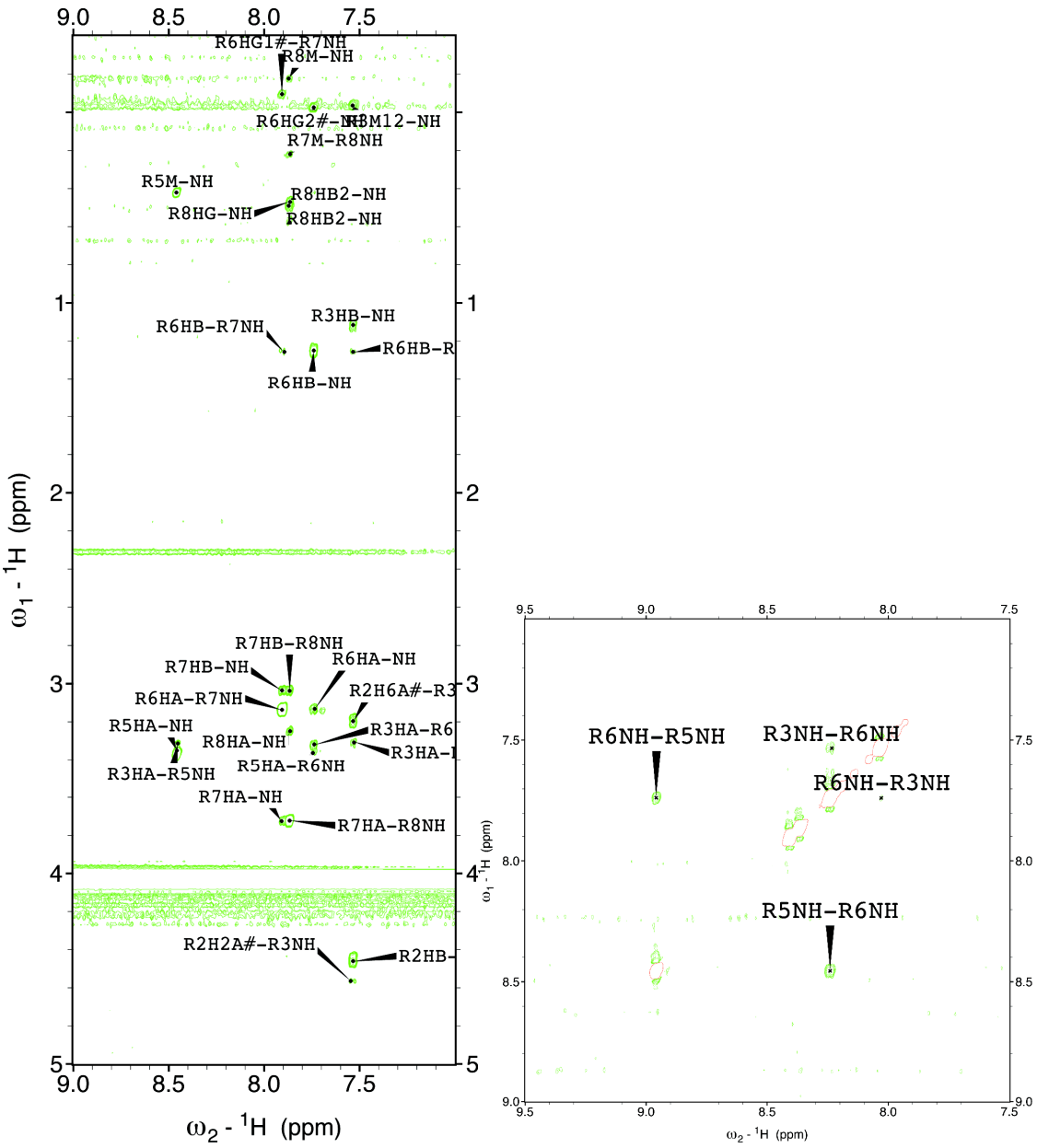

Figure S.11.b: LL2 ROESY 9.0-7.3 × 5.0-0.6 ppm, 9.0-7.5 ppm

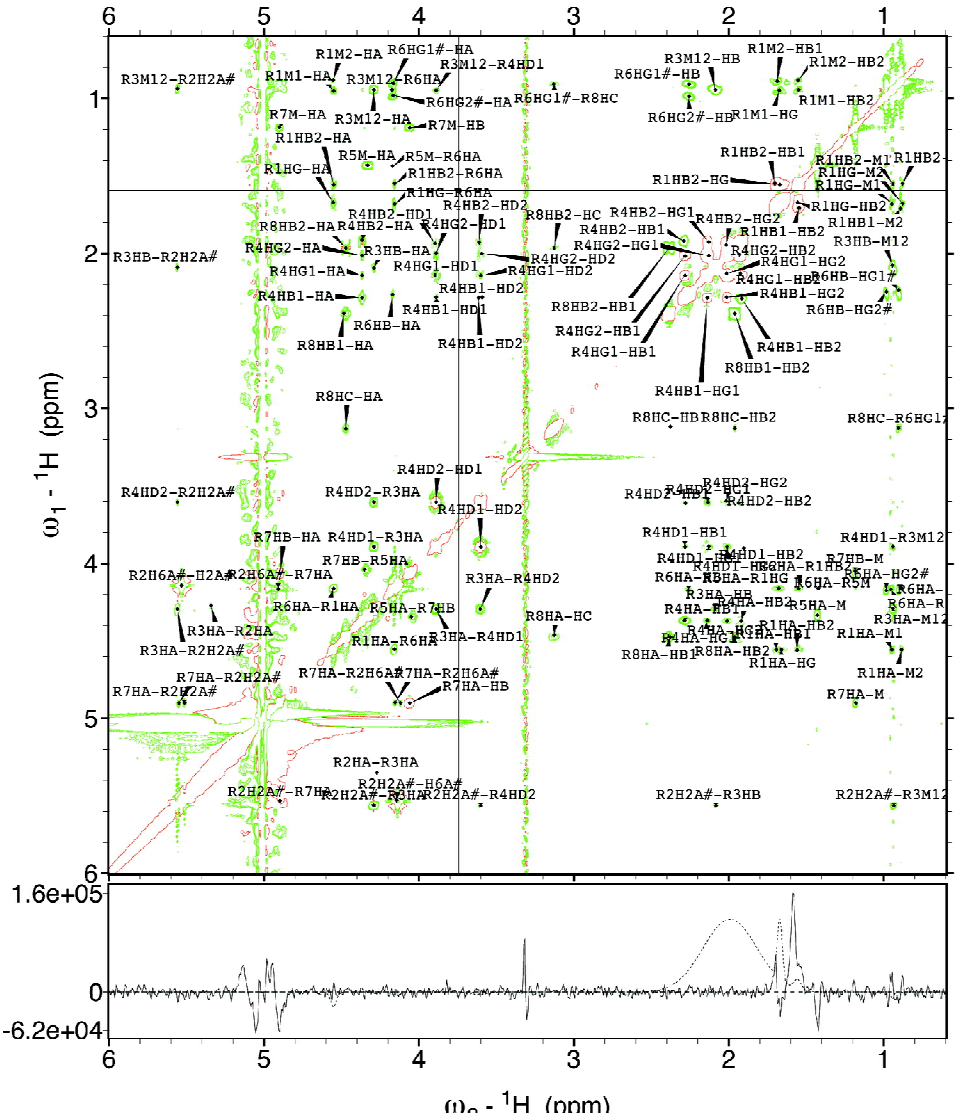

Figure S.11.c: LH2 ROESY 6.0-0.6 ppm

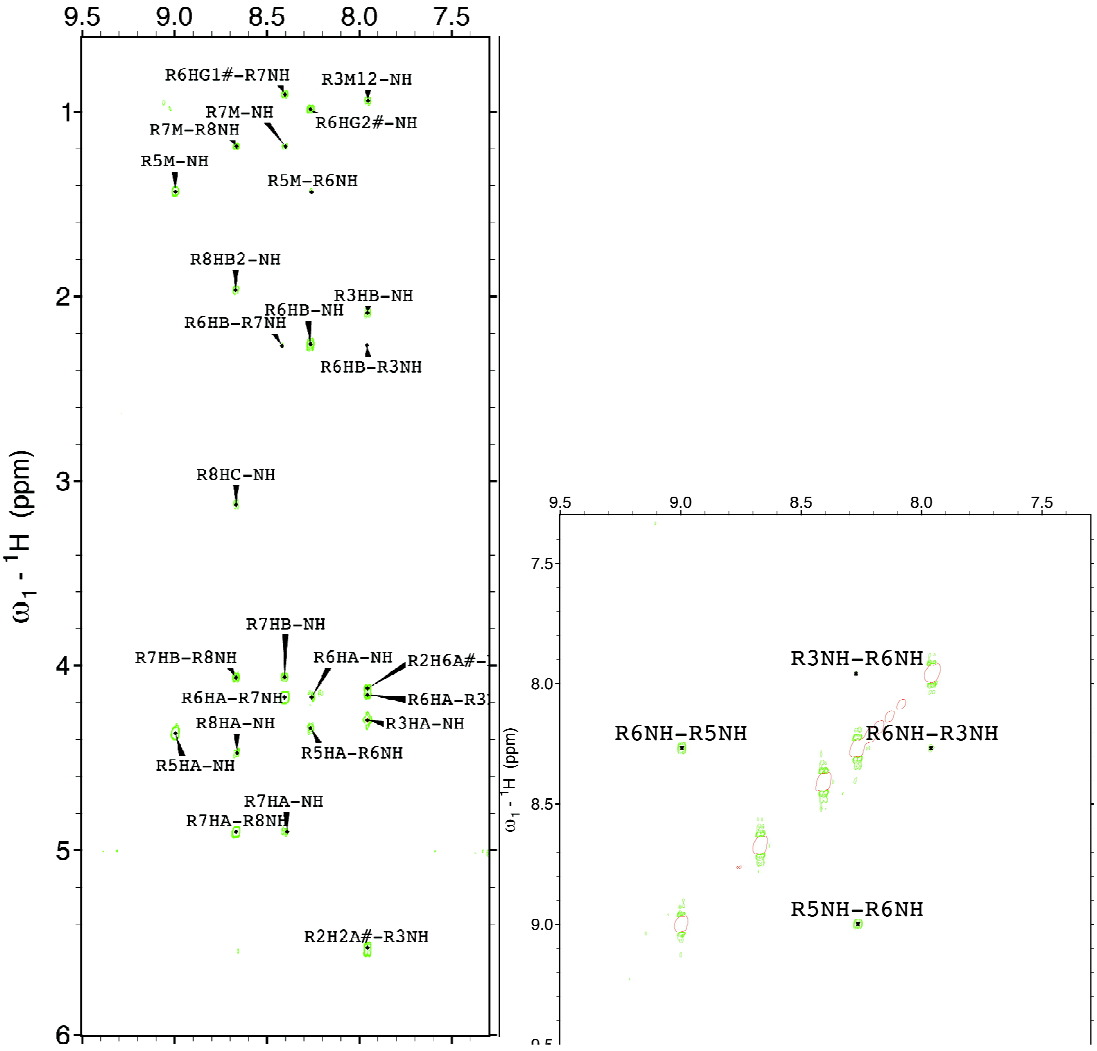

Figure S.11.d: LL2 ROESY 9.0-7.3 × 5.0-0.6 ppm, 9.0-7.5 ppm

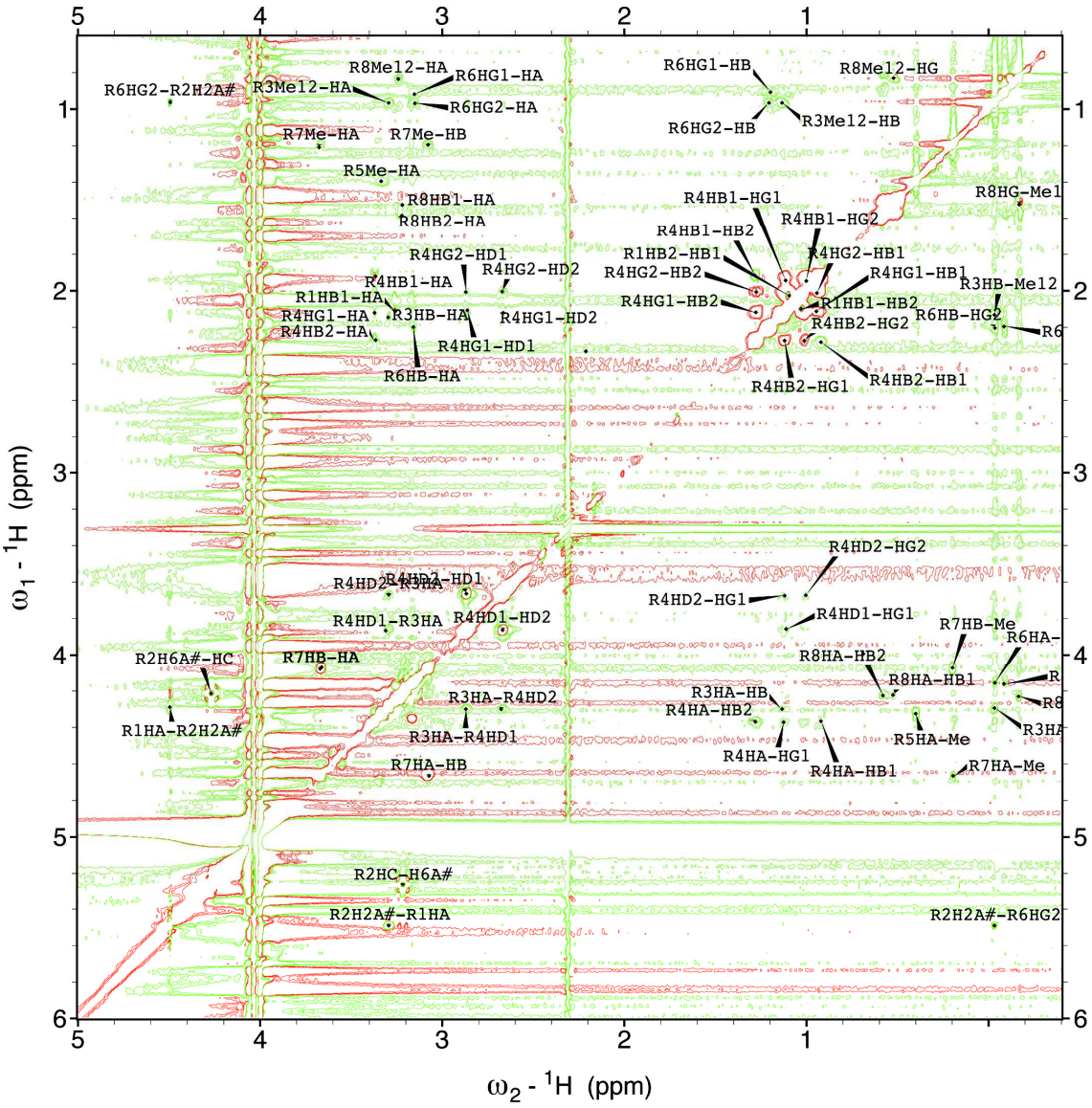

Figure S.11.e: HL2 ROESY 5.0-0.6 ppm

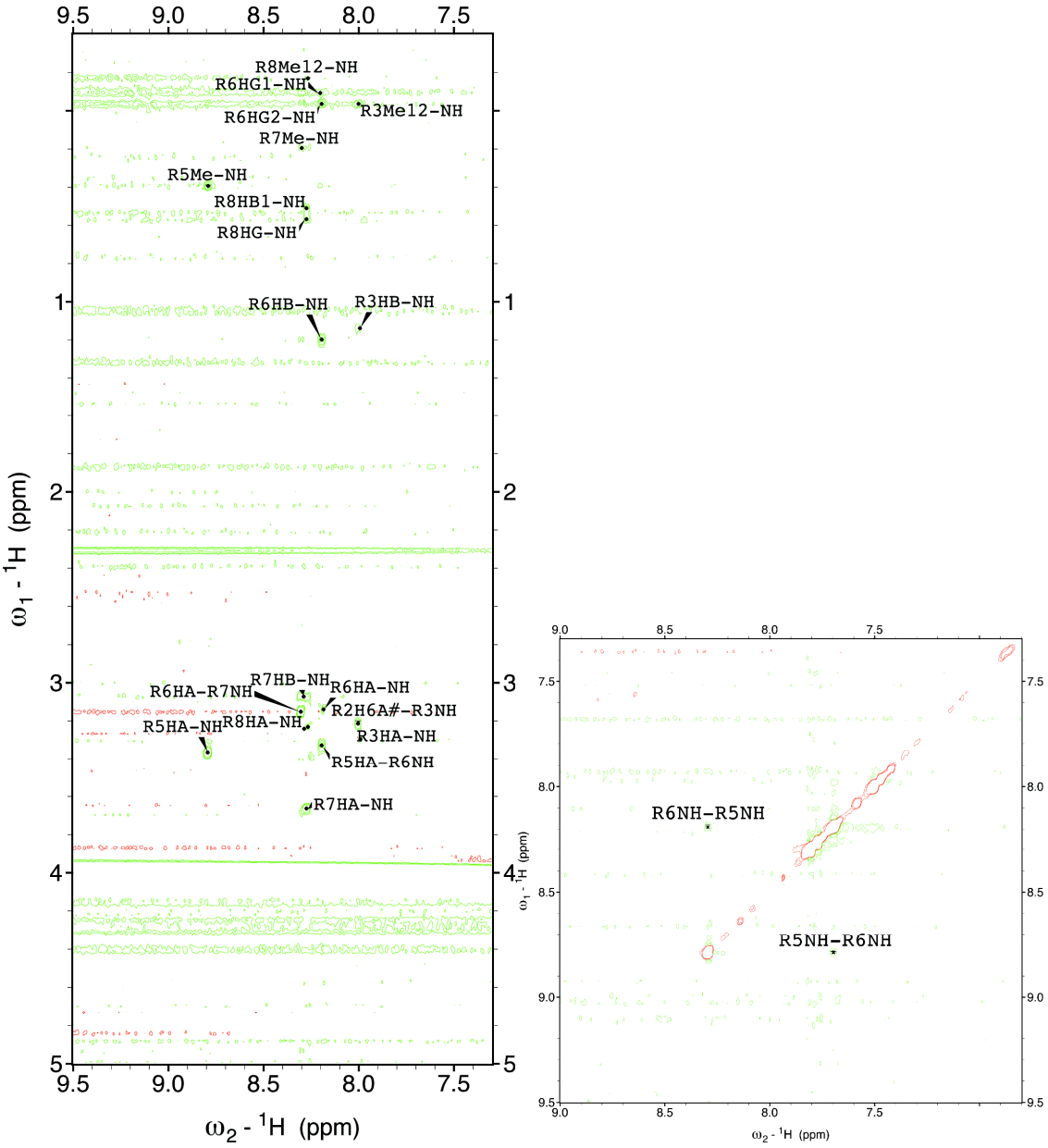

Figure S.11.f: HL2 ROESY 9.0-7.3 × 5.0-0.6 ppm, 9.0-7.5 ppm

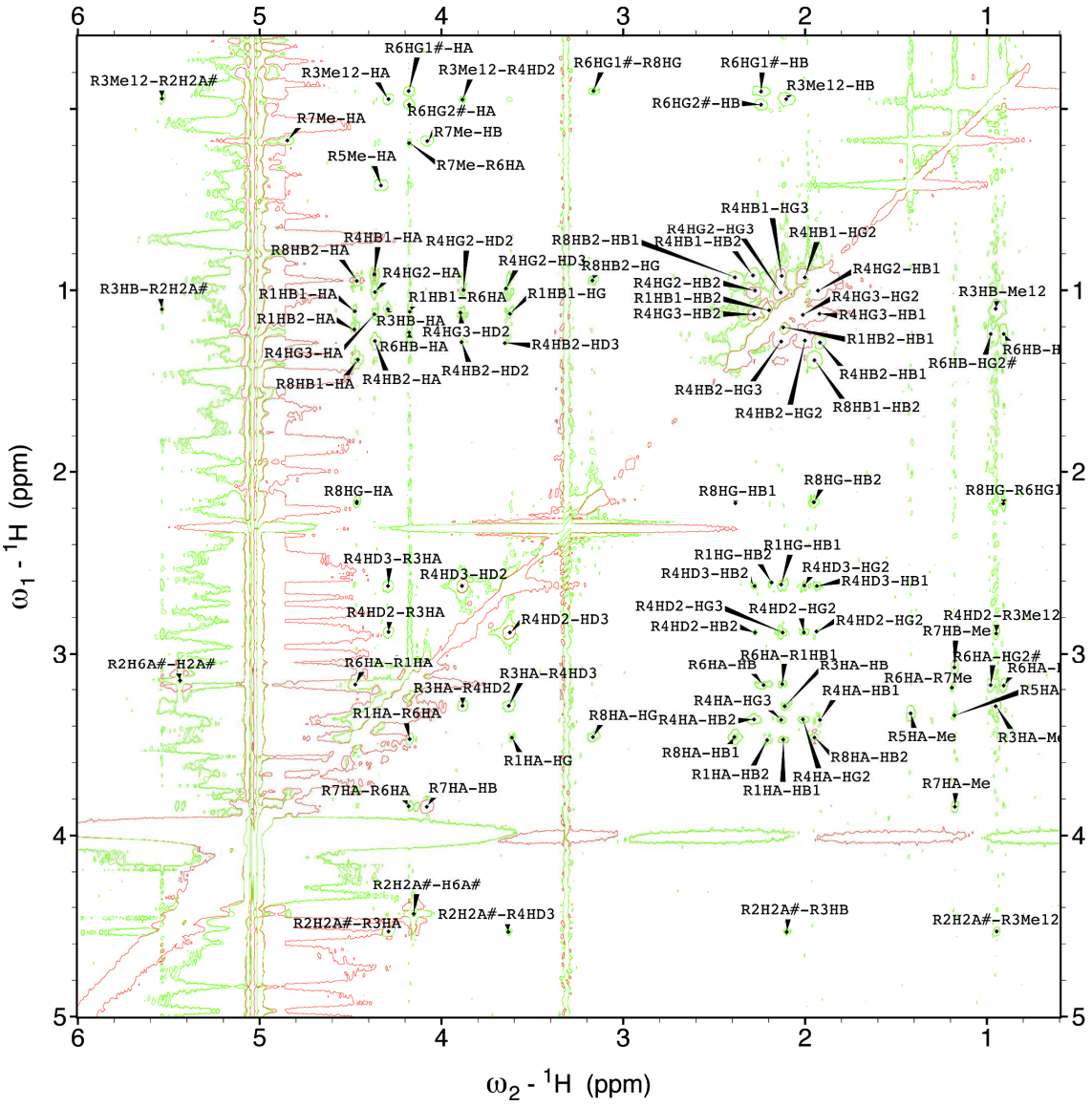

Figure S.11.g: HH2 ROESY 6.0-0.6 ppm

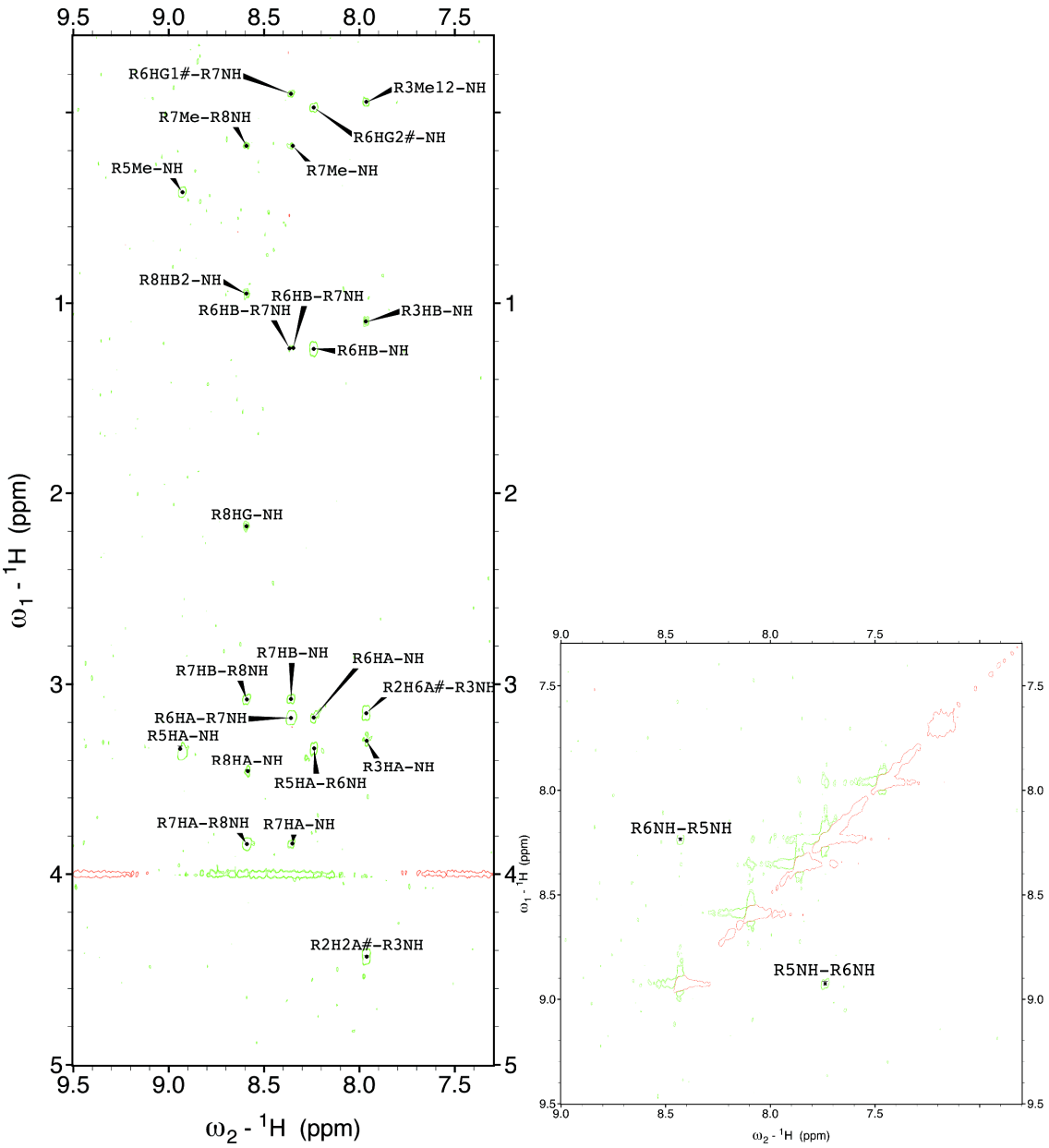

Figure S.11.h: HH2 ROESY 9.0-7.3 × 5.0-0.6 ppm, 9.0-7.5 ppm

REFERENCES

(1) Xing, X.; Fichera, A.; Kumar, K. *Organic Letters* **2001**, *3*, 1285-1286.

(2) Garner, P.; Park, J. M. *J. Org. Chem.* **1987**, *52*, 2361-2364.

(3) Fields, C. G.; Fields, G. B.; Noble, R. L.; Cross, T. A. *Int. J. Pept. Protein Res.* **1989**, *33*, 298-303.

(4) Clark, G. A., Tufts University, 2009.

(5) Phillips, S. T.; Piersanti, G.; Ruth, M.; Gubernator, N.; van Lengerich, B.; Bartlett, P. A. *Organic Letters* **2004**, *6*, 4483-4485.

(6) Testaferri, L.; Tiecco, M.; Tingoli, M.; Bartoli, D.; Massoli, A. *Tetrahedron* **1985**, *41*, 1373-1384.

(7) Han, Y. X.; Albericio, F.; Barany, G. *J. Org. Chem.* **1997**, *62*, 4307-4312.

(8) Crankshaw, M. W.; Grant, G. A. *Curr. Prot. Prot. Sci.* **2000**.

(9) Pace, C. N.; Vajdos, F.; Fee, L.; Grimsley, G.; Gray, T. *Protein Science* **1995**, *4*, 2411-2423.

(10) Greenfie.N; Fasman, G. D. *Biochemistry* **1969**, *8*, 4108-&.

(11) Russell, S. J.; Cochran, A. G. *J. Am. Chem. Soc.* **2000**, *122*, 12600-12601.

(12) Cochran, A. G.; Tong, R. T.; Starovasnik, M. A.; Park, E. J.; McDowell, R. S.; Theaker, J. E.; Skelton, N. J. *J. Am. Chem. Soc.* **2001**, *123*, 625-632.

(13) Phillips, S. T.; Piersanti, G.; Bartlett, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13737-13742.

(14) Goodlett, D. R.; Abuaf, P. A.; Savage, P. A.; Kowalski, K. A.; Mukherjee, T. K.; Tolan, J. W.; Corkum, N.; Goldstein, G.; Crowther, J. B. *J. Chromatogr. A* **1995**, *707*, 233-244.

(15) Maynard, A. J.; Sharman, G. J.; Searle, M. S. *J. Am. Chem. Soc.* **1998**, *120*, 1996-2007.

(16) Cavanagh, J. *Protein NMR Spectroscopy: Principles and Practice*; Academic Press: San Diego, 1996.

(17) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.

(18) Neidigh, J. W.; Fesinmeyer, R. M.; Prickett, K. S.; Andersen, N. H. *Biochemistry* **2001**, *40*, 13188-13200.

(19) Wagner, G.; Braun, W.; Havel, T. F.; Schaumann, T.; Go, N.; Wuthrich, K. *J. Mol. Biol.* **1987**, *196*, 611-639.