Positional screening and NMR structure determination of side-chain-to-side-chain cyclized β^3-peptides[†,‡]

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Contents

Additional data as separate files from the author:

-Parameter files: parallhdg_beta.pro, topallhdg_beta.pro, toph19_beta.pep

-Restraint files: C18-noe2calc_2.tbl (distance calibration), C18-noe2_2.tbl (bin calibration), C18-hbo.tbl (hydrogen bond restraints)

-Pdb files with the final ensemble of 20 structures: 8b_bin-vacuo.pdb, 8b_bin_water.pdb, 8b_calib_water.pdb

1. General information.

1-D NMR experiments were acquired on a Varian Mercury 400 or a Varian Inova 600 spectrometer and processed with XWINNMR. LC-ESI-MS was carried out by using an Agilent 1100 series binary pump together with a reversed-phase HPLC C18 column (Macherey-Nagel) and a Finnigan Thermoquest LCQ. Purification of β-peptides was performed with an Agilent 1100 Series with a Nucleodur C18 gravity column (Macherey-Nagel) and flow rate of 25 mL/min. UV spectra were obtained in a Varian Cary 100 Bio spectrophotometer. CD spectra were recorded on a JASCO J-815 spectropolarimeter. Reactions under microwave irradiation were carried out in a CEM Explorer microwave, a monomodal synthesis reactor equipped with a 300 W (max) power source, an infrared sensor for temperature control and an automated synthesis workstation module.

Tenta Gel R PHB resin with a loading of 0.22 mmol/g was purchased from Rapp Polymere GmbH. D_2O was purchased from Deutero GmbH and CD_3OH from Aldrich. PyBOP® was obtained from Novabiochem. Commercial Fmoc protected β^3-*L*-amino acids were obtained from Aldrich-Sigma and Novabiochem. All other reagents were purchased from Aldrich-Sigma, Fluka and Acros.

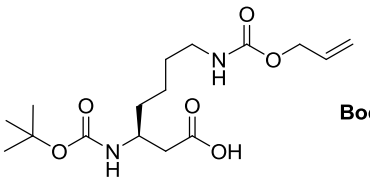
2-D NMR experiments with β3-peptide samples were performed on a Varian Inova 600 spectrometer, equipped with a triple resonance probe with shielded Z gradients. Peptides were dissolved either in CD_3OH or in aqueous buffer (10 mM NaPi [pH 7.4]) with 90:10 H_2O/D_2O at a concentration of 1-2 mM and measured in a temperature range from 25°C to -6°C. Homonuclear two-dimensional experiments were recorded following the Varian NMR suite with typically 2048 x 512 data points. NMR spectra were converted to Bruker format and processed with XWINNMR and evaluated and plotted with Aurelia.[1] The assignment strategy was based on the identification of individual resonance spin systems from homonuclear TOCSY experiments following the sequential

path with 2D ROESY spectra similarly as described.[2] Mixing times in TOCSY experiments were around 70 ms. Mixing times in ROESY experiments were 150 and/or 300 ms. Typically, 2D measurements were recorded with F1 x F2 = 64 x 2048 data points with 8 scans each.

2. β-Peptide synthesis.

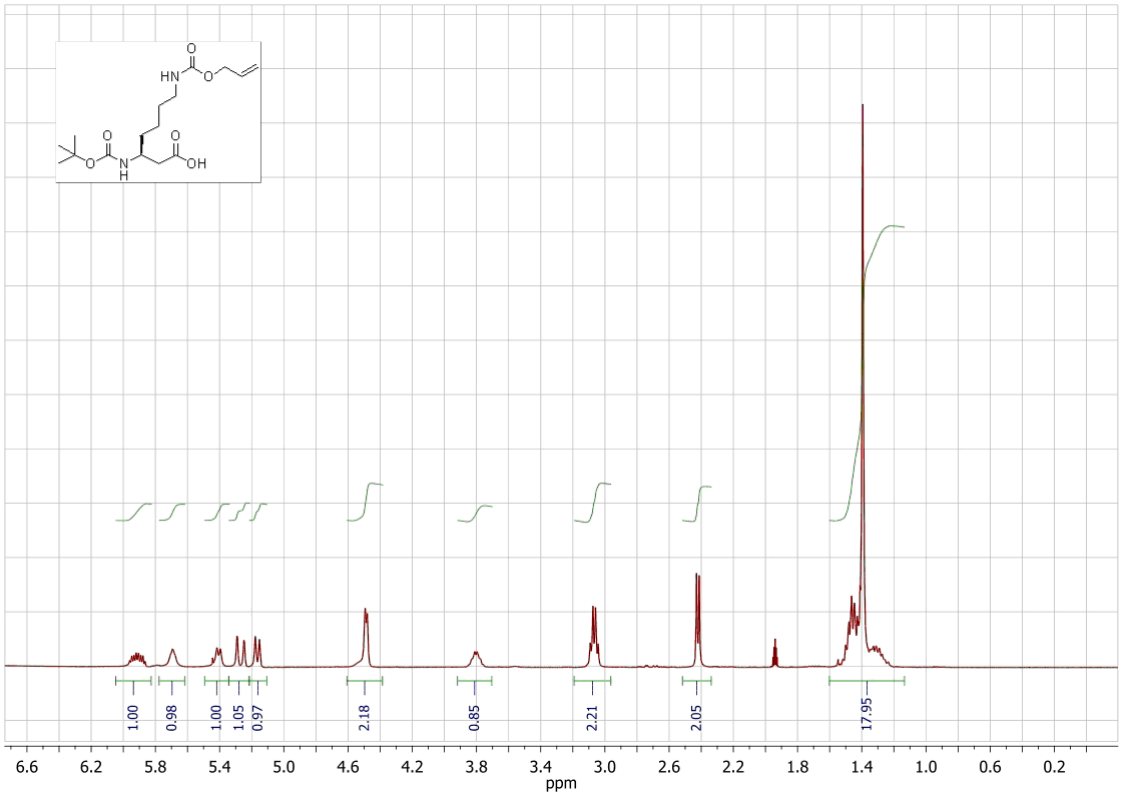
A. β-Amino acid building blocks

Not commercial Fmoc-protected and Boc-protected β^3-*L*-amino acid derivatives were prepared by homologation from the corresponding available α-*L*-amino acids as described previously.[3] Analytical data of β^3-*L*-amino acid derivatives newly used for this study are reported below.



Boc-β³Lys(Alloc)-OH
17

Boc-β^3Lys(Alloc)-OH (**17**): Yield: 85%. ^{1}H NMR (400 MHz, CD_3CN) δ ppm 5.92 (m, 1H, CH), 5.69 (bs, 1H, NH), 5.42 (d, *J* = 8.5 Hz, 1H, NH), 5.27 (d, *J* = 17.3 Hz, 1H, CH), 5.17 (dd, *J* = 10.5 Hz, 1H, CH), 4.49 (d, *J* = 5.0 Hz, 2H, CH_2), 3.81 (m, 1H, CH), 3.07 (q, *J* = 6.6 Hz, 2H, CH_2), 2.42 (d, *J* = 6.4 Hz, 2H, CH_2), 1.6-1.2 (m, 6H, CH_2), 1.39 (s, 9H, CH_3). ^{13}C NMR (100 MHz, CD_3CN) δ ppm 172.6 (s), 156.6 (s), 155.8 (s), 134.0 (d), 117.6 (t), 78.6 (s), 64.9 (t), 47.9 (d), 40.6 (t), 39.5 (t), 34.3 (t), 29.6 (t), 27.9 (q) 23.1 (t). ESI-MS: 344.92 $(M + H)^+$ (calc. mass 345.20). HRMS (ESI): calc for $(M + H)^+$ 345.2020; found 345.2021.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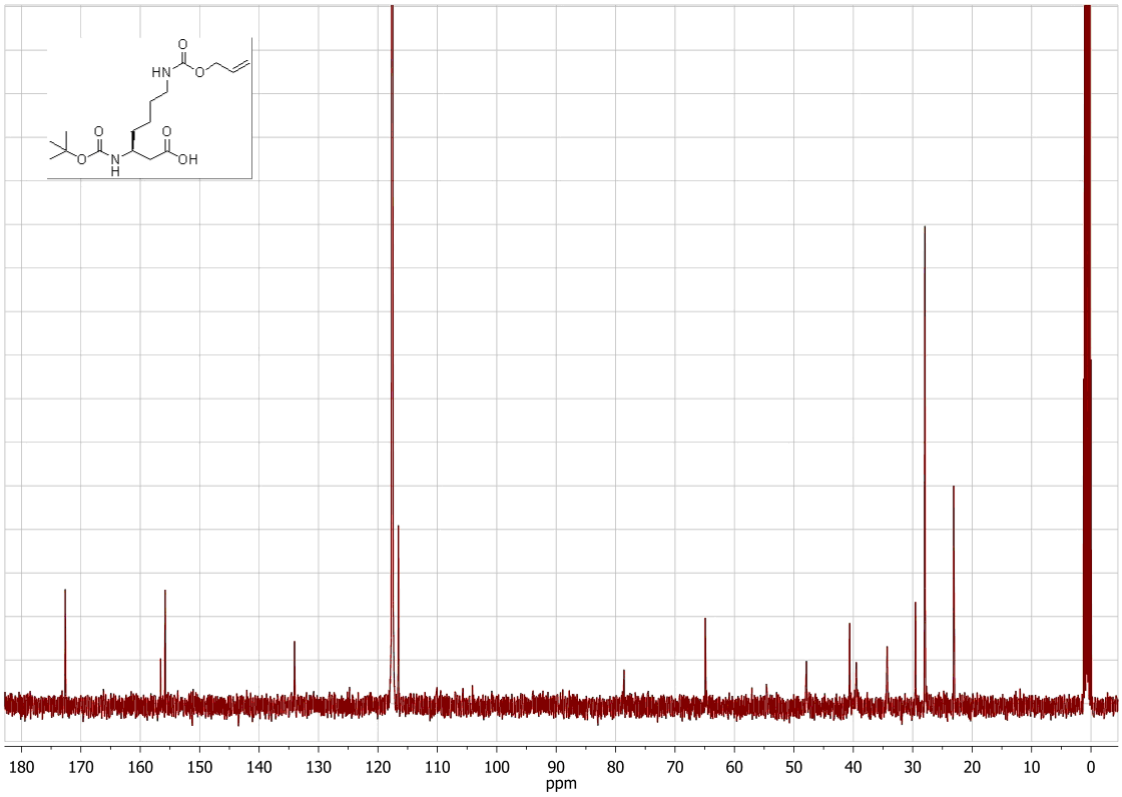
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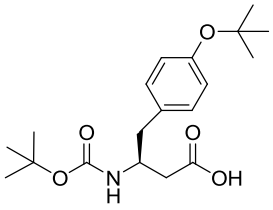
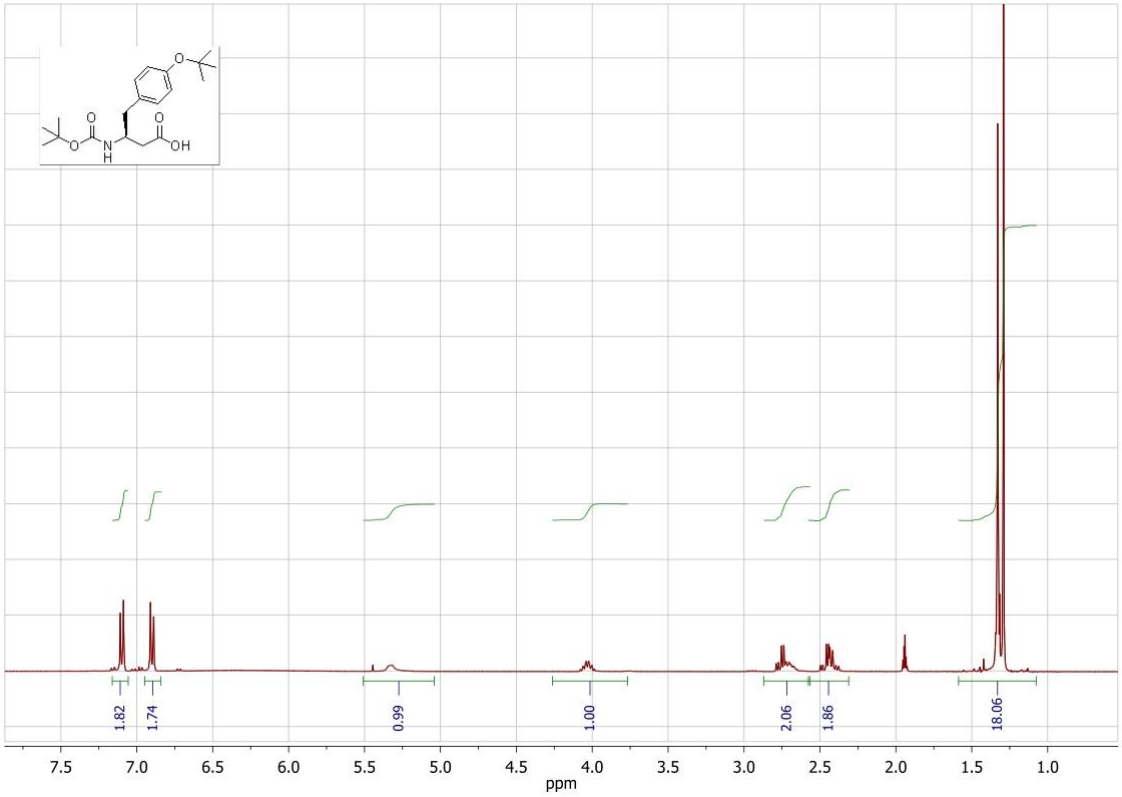
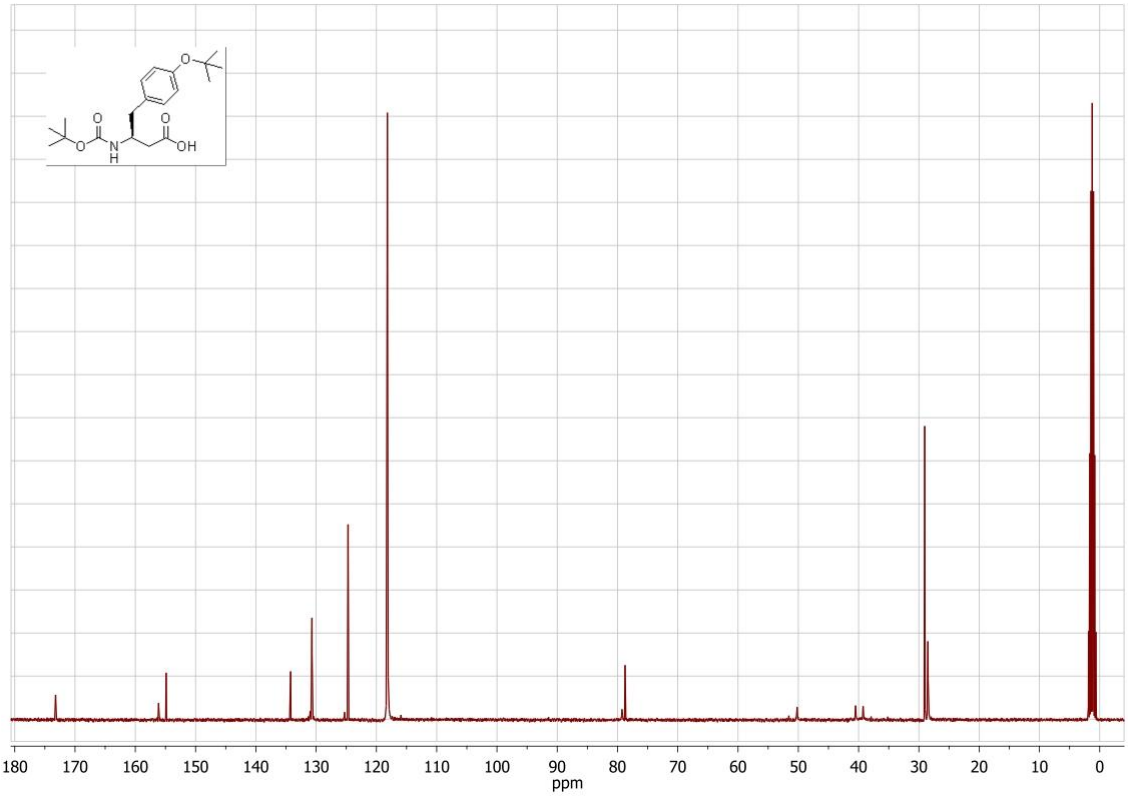


Boc-β^{3}Tyr(*t*Bu)-OH
18

Boc-β^{3}Tyr(tBu)-OH (**18**): Yield: 67%. ^{1}H NMR (400 MHz, CD_3CN) δ ppm 7.10 (d, *J* = 8.4 Hz, 2H, CH), 6.90 (d, *J* = 8.4 Hz, 2H, CH), 5.33 (bs, 1H, NH), 4.03 (m, 1H, CH), 2.8-2.6 (m, 2H, CH_2), 2.6-2.3 (m, 2H, CH_2), 1.33 (s, 9H, CH_3), 1.33 (s, 9H, CH_3), 1.29 (s, 9H, CH_3). ^{13}C NMR (100 MHz, CD_3CN) δ ppm 173.2 (s), 156.1 (s), 154.9 (s), 134.2 (s), 130.7 (d), 124.7 (d), 79.3 (s), 78.8 (s), 50.2 (d), 40.5 (t), 39.3 (t), 29.0 (q), 28.5 (q). ESI-MS: 351.80 $(M + H)^+$ (calc. mass 352.21).





B. β^3-Peptide synthesis.

Loading of the resin. Tenta Gel R PHB resin (1.0 g, 0.22 mmol/g resin initial loading) was swollen in dry CH_2Cl_2 (10.0 mL) for 30 min. A solution of Fmoc protected β^3-amino acid (3 equiv) and *N*,*N'*-diisopropylcarbodiimide (DIC) (46.7 μL, 1.5 equiv) in dry CH_2Cl_2 (1.5 mL) was stirred for 20 min at room temperature and then added to the resin, followed by 4-dimethylaminopyridine (DMAP) (36.7 mg, 1.5 equiv), and the suspension was stirred at room temperature for 21 h. The resin was washed with CH_2Cl_2 (5 x 30 s), *N*-methyl-2-pyrrolidone (NMP) (5 x 30 s), 90:10 NMP/MeOH (10 min), NMP (5 x 30 s), CH_2Cl_2 (5 x 30 s), shrunk down with diethyl ether (5 x 30 s) and dried under vacuum. Fmoc-determination by UV-spectrophotometry at 301 nm with a small sample of the resin (c.a. 2 mg) resulted in calculated loadings given in the Table S1. Unreacted sites of the resin were capped with a mixture of acetic anhydride (2.1 mL, 100 equiv), DMAP (29.6 mg, 1.1 equiv) and NMP (12 mL) by stirring

for 70 min at room temperature. The resin was subsequently washed with NMP (5 x 30 s), CH_2Cl_2 (5 x 30 s) and Et_2O (5 x 30 s). Synthesis of β-peptides **1-10** has been reported previously.[5]

Table S1. Calculated resin loadings (mmol/g resin).

β^3-peptide resin	**Loading (mmol/g resin)**
Fmoc-β^3Tyr(*t*Bu) resin	0.19
Fmoc-β^3Glu(Allyl) resin	0.20
Fmoc-β^3Asp(Allyl) resin	0.18

Microwave synthesis of β-peptides 11-16. β-Peptides were synthesized on a 38 μmol scale (200 mg of resin) using microwave irradiation. The Fmoc deprotection was carried out with 20% v/v piperidine/NMP (3 mL, 50 W maximum power, 60 ºC, ramp 2 min, hold 2 min), the mixture was filtered and the deprotection protocol was repeated once, then the resin was washed with NMP (5 x 30 s). Coupling of each unit was achieved *via* treatment of the deprotected resin with a cocktail containing Fmoc-β^3-amino acid (3 equiv), HBTU (43.2 mg, 0.114 mmol, 3 equiv), HOBt (17.5 mg, 0.114 mmol, 3 equiv) and DIEA (39.7 μL, 0.228 mmol, 6 equiv) in 3 mL of 0.8 M LiCl in NMP under microwave irradiation (50 W maximum power, 45 ºC, ramp 2 min, hold 4 min). After filtration the process was repeated.

These steps were repeated until the β^3-peptide sequence was complete. The Allyl and Alloc protecting groups were removed as described for α-peptides.[4] The resin was washed with NMP (5 x 30 s), CH_2Cl_2 (5 x 30 s), Et_2O (5 x 30 s) and dried under vacuum overnight. Then the resin was swollen with degassed CH_2Cl_2 (3 x 2 min) while Argon was bubbled through the mixture. A solution of $PhSiH_3$ (85.9 μL, 24 equiv) in 0.5 mL of CH_2Cl_2 was added, and stirred again with a flow of Argon for 2 min. Subsequently, a solution of $Pd(PPh_3)_4$ (8.4 mg, 0.25 equiv) in 1.5 mL of CH_2Cl_2 was added and after Argon was bubbled through the resin for 2 min, the reaction was shaken for 30 min under Argon. The peptide resin was washed with CH_2Cl_2 (3 x 2 min), NMP (3 x 2 min) and again with CH_2Cl_2 (4 x 2 min) and the process was repeated. Finally, the resin was washed with CH_2Cl_2 (3 x 2 min), NMP (3 x 2 min), a 0.02 M solution of Et_2NCS_2Na in NMP (3 x 2 min) and again with NMP (5 x 30 s).

Linear β^3-peptides (11-16a). The final Fmoc protecting group was removed as described above, the resin was washed with NMP (5 x 30 s), CH_2Cl_2 (5 x 30 s) and Et_2O (5 x 30 s) and dried under vacuum for 3 h. The resin was treated with a cleavage cocktail composed of 2% v/v water and 2% v/v triisopropylsilane (TIPS) in trifluoroacetic acid (TFA), and after 3 h, the resin was washed with TFA (3 x 15 s). The cleaved β^3-peptide was collected, concentrated by rotary evaporation into less than 1 mL solution and precipitated by addition of cold Et_2O (10 mL). The mixture was cooled in a liquid N_2 bath for 1 min, centrifuged (10000 rpm, 5 min, 4 °C) and the Et_2O was decanted from the pellet. Cold Et_2O was added again and the procedure was repeated twice. The crude peptide obtained was dissolved in H_2O/CH_3CN and lyophilized to dryness.

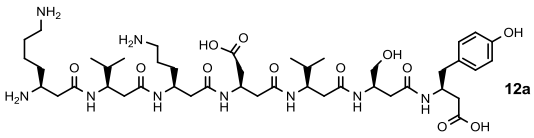
Cyclic β^3-peptides (11-16b). To the Alloc/Allyl deprotected β^3-peptide resin HATU (14.5 mg, 1 equiv) and DIEA (19.5 μL, 3 equiv) in 3 mL of NMP (or 1:1 NMP/THF for (**11-16b**) was added and shaken for 12 h to undergo cyclization *via* the free carboxylic acid side-chain of β^3-Glu/β^3-Asp and the free amino group side-chain of β^3-Lys/β^3-Orn. This process was repeated until a negative TNBS test resulted. After washing the resin with NMP (5 x 30 s), the final Fmoc protecting group was removed and the peptide was cleaved from the resin as described for the linear β^3-peptides. The precipitate obtained was dissolved in H_2O/CH_3CN and lyophilized to dryness.

β^3-Peptide purification and analysis. The crude reaction mixture of each β-peptide synthesized was analyzed by LC-MS. β-Peptides were then purified by reverse-phase HPLC on a Nucleodur C18 Gravity column (125 x 21 mm, Macherey-Nagel) with a linear gradient of A (0.1% HCOOH in H_2O) and B (0.1% HCOOH in CH_3CN) from 10% to 50% of B and flow rate of 25 mL min^{-1} and were detected at 214 nm using a diode array UV/VIS detector. The identities and purities of the purified β-peptides were assessed by LC-MS (ESI mass spectrometry) (Table S2). Following purification, all β-peptides were lyophilized and kept at –20 °C.

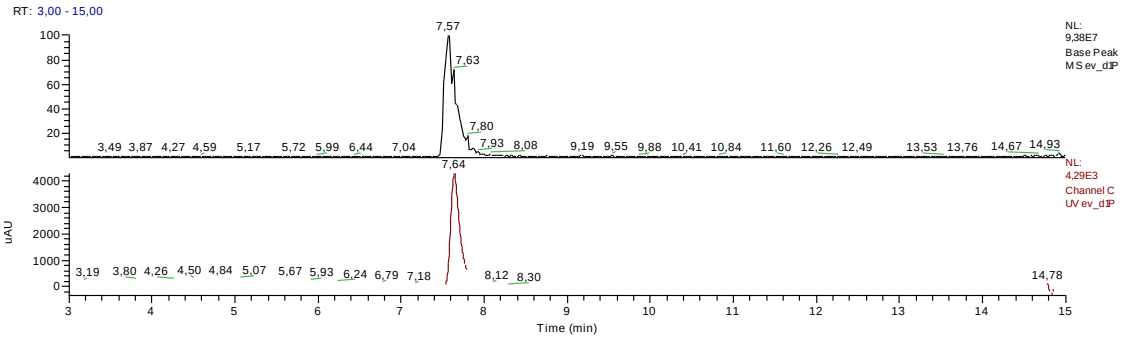
Table S2. Analytical data of synthesized β-peptides. Analytical data for β-peptides **1**-**10** have been previously reported.[3]

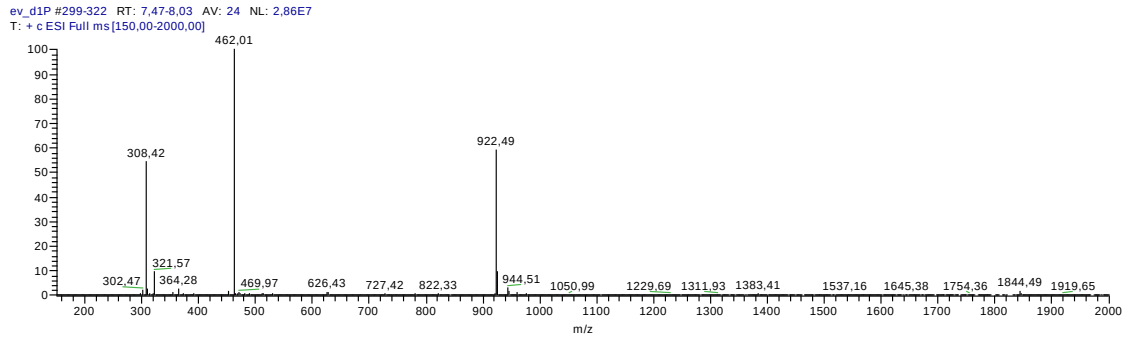
β^3-peptide	Calcd Mass $(M+H)^+$	Found Mass $(M+H)^+$	Purity	Yield
Lin(1,4)-LysGlu **11a**	936.58	936.53	> 99%	27%
Cy(1,4)-LysGlu **11b**	918.57	918.57	90%	5%
Lin(1,4)-LysAsp **12a**	922.56	922.51	> 99%	39%
Cy(1,4)-LysAsp **12b**	904.55	904.56	98%	11%
Lin(1,4)-OrnGlu **13a**	922.56	922.52	> 99%	26%
Cy(1,4)-OrnGlu **13b**	904.55	904.53	> 99%	6%
Lin(1,4)-OrnAsp **14a**	908.55	908.48	> 99%	40%
Cy(1,4)-OrnAsp **14b**	890.54	890.59	> 99%	7%
Lin(4,7)-LysGlu **15a**	936.58	936.53	> 99%	15%
Cy(4,7)-LysGlu **15b**	918.57	918.53	91%	1%
Lin(4,7)-LysAsp **16a**	922.56	922.52	> 99%	17%
Cy(4,7)-LysAsp **16b**	904.55	904.49	> 99%	5%

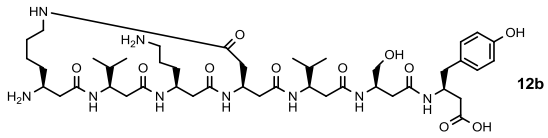
Representative LC-MS spectra of the linear β^3-peptides **12a**, **13a** and **16a** and cyclic β^3-peptides **12b**, **13b** and **16b** are shown.



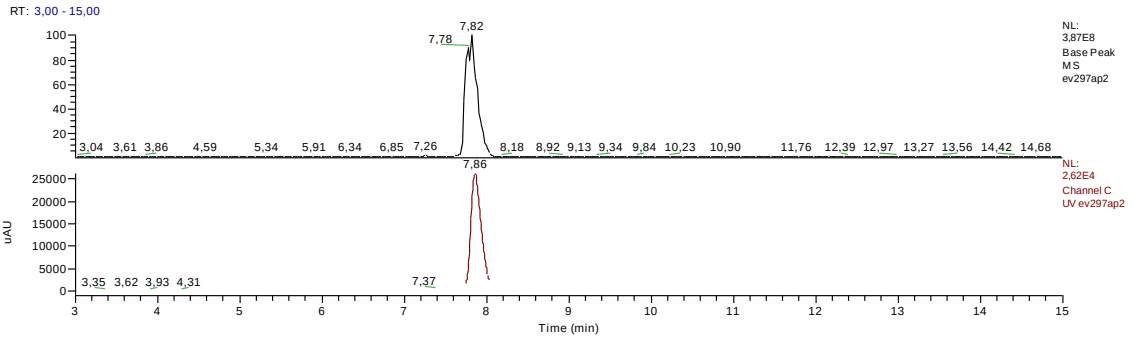
Lin(1,4)-LysAsp **12a**: calc. mass $(M+H)^+$ 922.56

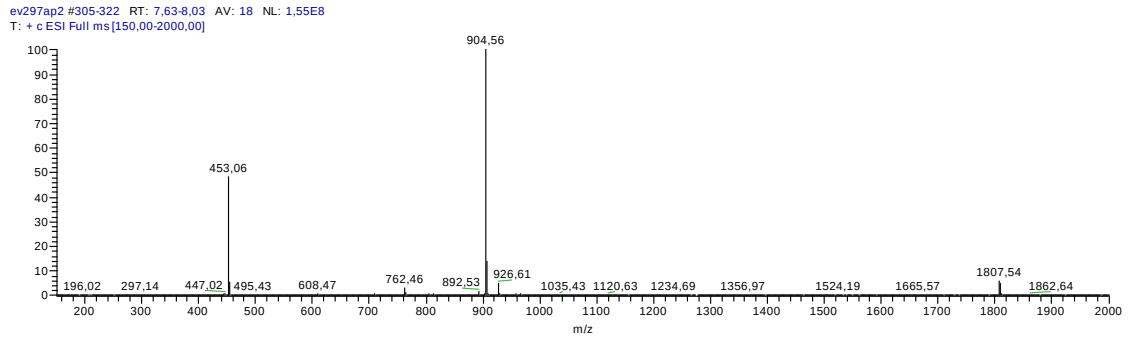


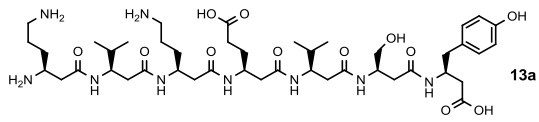




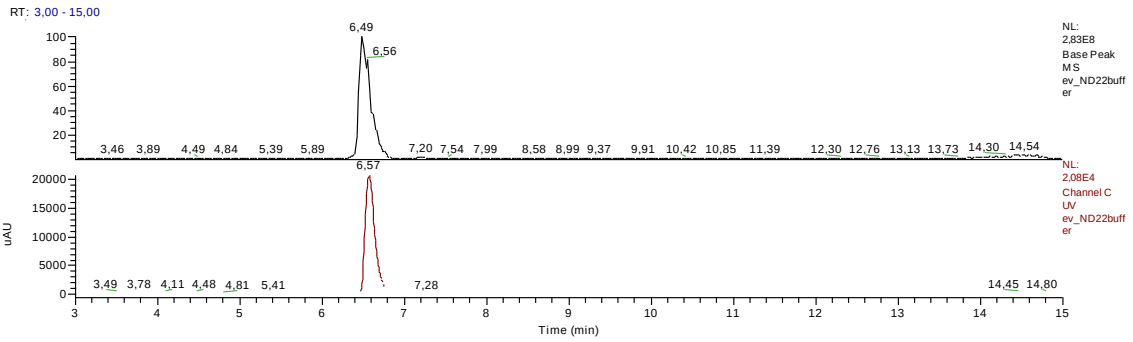
Cy(1,4)-LysAsp **12b**: calc. mass $(M+H)^+$ 904.55

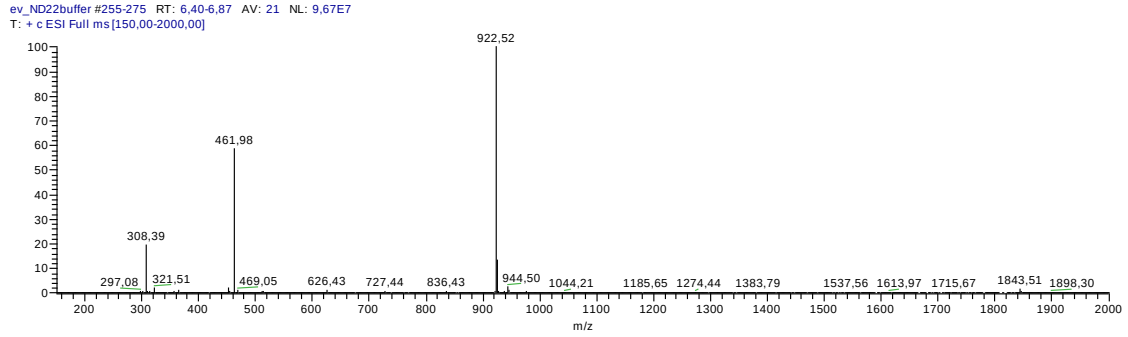


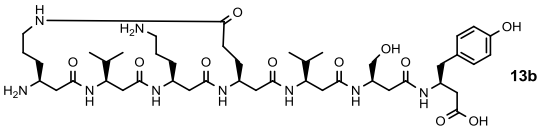




Lin(1,4)-OrnGlu **13a**: calc. mass $(M+H)^+$ 922.56

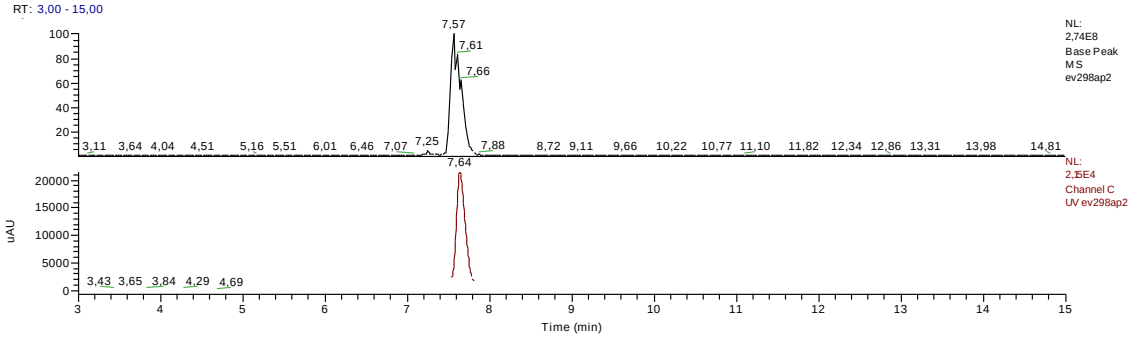


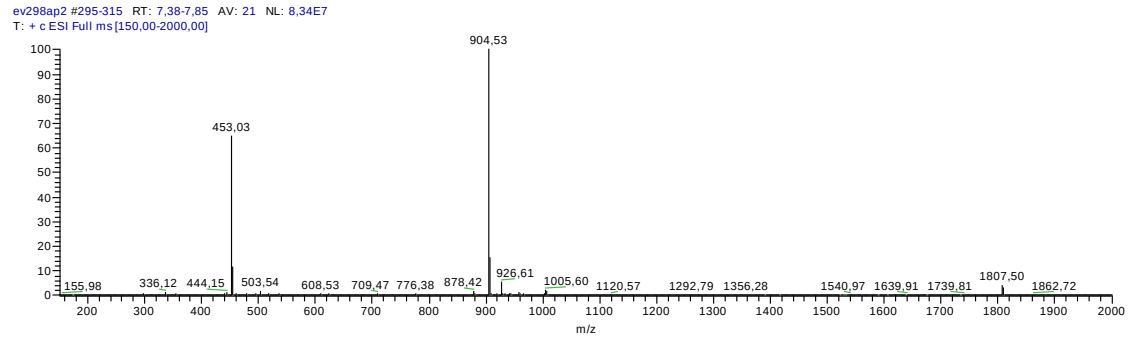


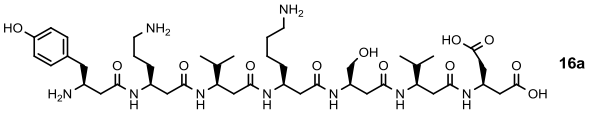


13b

Cy(1,4)-OrnGlu **13b**: calc. mass $(M+H)^+$ 904.55

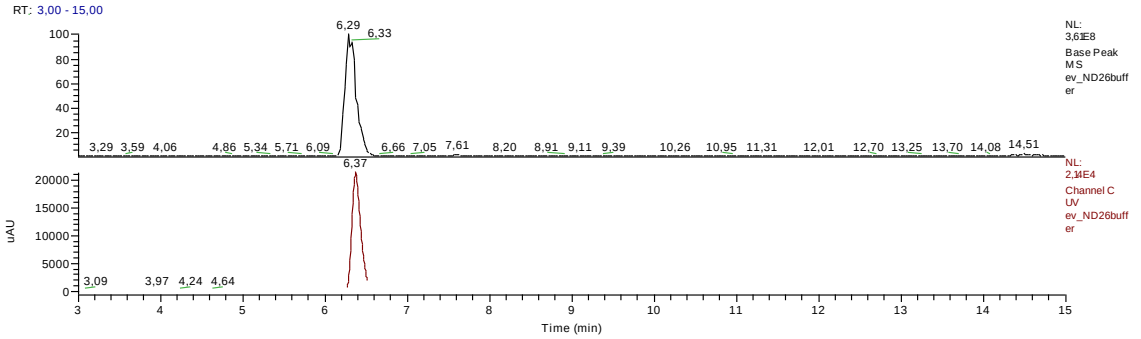


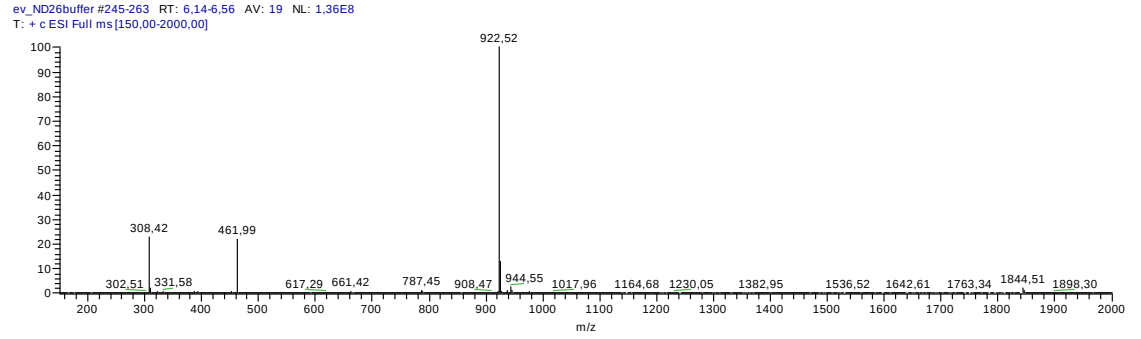


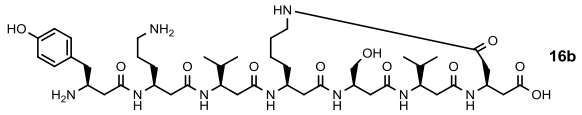


16a

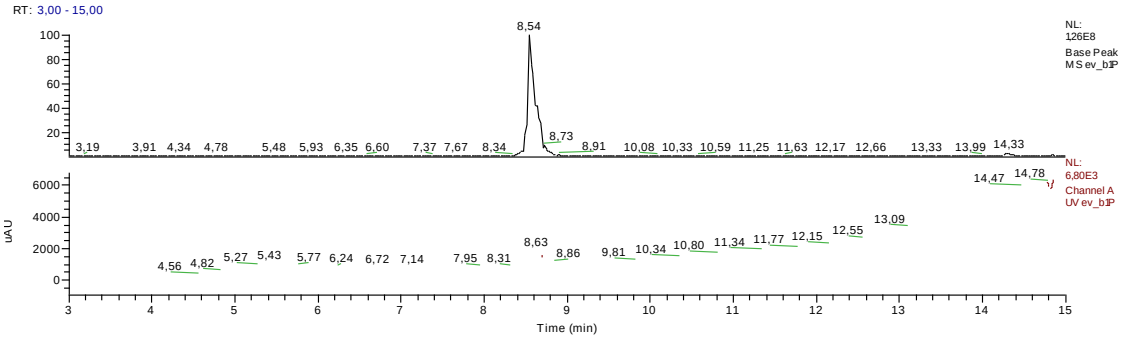
Lin(4,7)-LysAsp **16a**: calc. mass $(M+H)^+$ 922.56

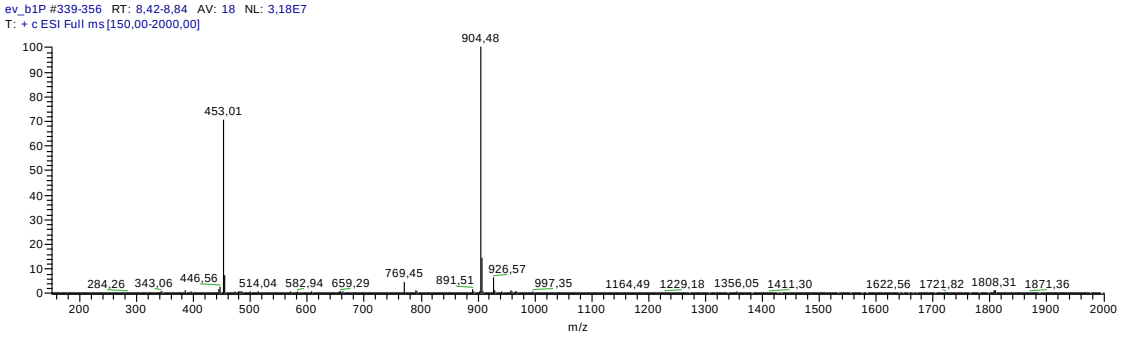






Cy(4,7)-LysAsp **16b**: calc. mass $(M+H)^+$ 904.55



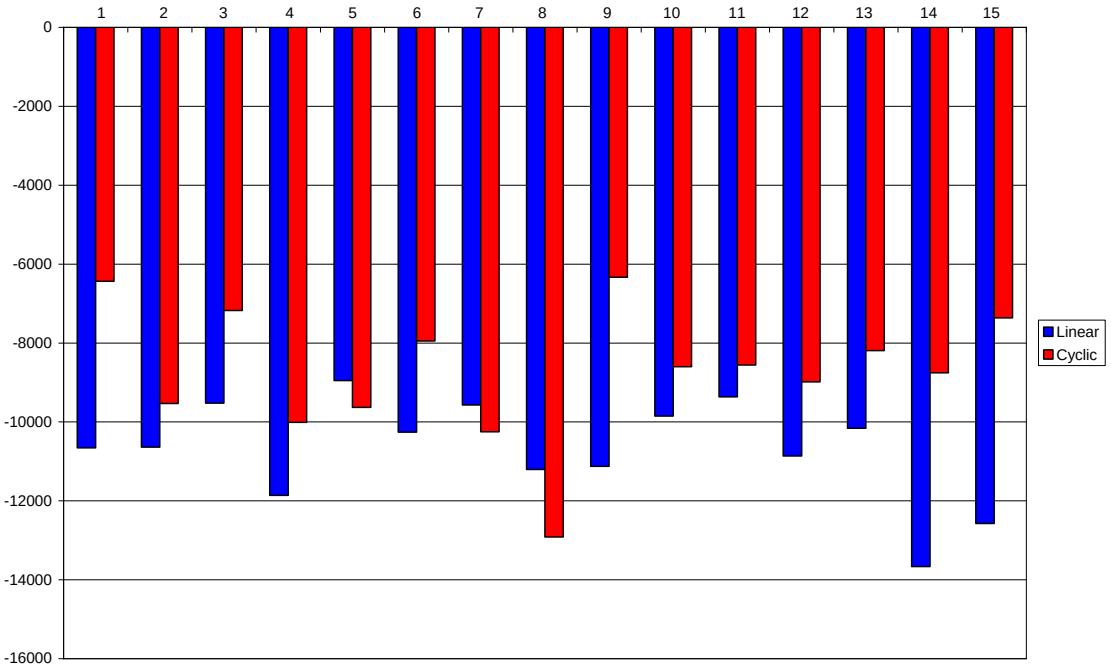


3. Circular Dichroism Spectroscopy

CD spectra were recorded on a JASCO-815 spectrometer at 20 ºC in MeOH or in a 10 mM sodium phosphate buffer (pH 7.4) using a 0.1 cm path length CD cell. For measurements at different pH, all data were obtained from solutions in 100 mM AcOK/AcOH buffer (pH 1.75 and pH 3.6) or 50 mM $NaHCO_3$/NaOH buffer (pH 9.6). For the evaluation of ionic strength effect in the structure stability, increasing concentrations of NaCl ranging from 0 to 1.6 M in 10 mM sodium phosphate buffer (pH 7.4) were prepared. Each peptide was analyzed at a concentration of 150-200 μM. The final concentration of the β-peptide in each MeOH and aqueous buffer solutions was determined immediately before obtaining the CD spectrum by the UV absorbance of the β^3-Tyr residue using the extinction coefficient (1420 cm^{-1} M^{-1} at 275 nm) of α-tyrosine.[5] Spectra from 250 to 190 nm represent the average of 10 scans (0.5 nm data pitch, continuous scanning mode, 20 nm min^{-1} scanning speed, 0.5 nm bandwidth, 1 s response) and were smoothed by Jasco software. Data were converted to mean-residue ellipticity MRE (in $deg \cdot cm^2 \cdot dmol^{-1} \cdot residue^{-1}$) according to the equation:

$$MRE = CD\ effect / C \cdot l \cdot n_{res}$$

where the CD effect is in millidegrees, the concentration (C) is in moles per liter, the path length (l) is in millimeters and n_{res} is the number of residues. Data for β-peptides **1**-**10** have been previously reported.[3]



Supporting Figure: Mean residue ellipticities obtained at 215 nm in methanol for both linear (a series) and cyclic (b series) β^3-peptides.

4. NMR characterization

NMR samples contained 1.0-2.0 mM β-peptide in CD_3OH or in 10 mM sodium phosphate buffer (pH 7.4) with 90:10 H_2O/D_2O. The assignment strategy was based on the identification of individual resonance spin systems from TOCSY experiments following NOE correlations with ROESY experiments (300 ms of mixing time, optimized).

Table S3. ^{1}H NMR chemical shifts of the cyclic β^3-heptapeptide Cy(3,6)-AspLys **6b** in aqueous solution at 283 K.

	H^N	H^{α}_{ax}	H^{α}_{eq}	H^{β}	H^{γ}	others
β^3-Val1	-	2.43	2.56	3.30	1.81	δCH_3: 0.84
β^3-Orn2	8.08	2.25	2.32	4.06	1.47, 1.52	δCH_2: 1.36; εCH_2: 2.82
β^3-Asp3	8.17	2.17	2.26	4.23	2.35, 2.37	
β^3-Val4	7.96	2.10	2.40	3.87	1.58	δCH_3: 0.72
β^3-Ser5	7.94	2.08	2.27	4.01	3.38	
β^3-Lys6	7.70	2.03	2.15	3.76	1.01, 0.92	δCH_2: 1.09, 1.14; εCH_2: 1.26 ζCH_2: 2.96, 3.11; ηNH: 7.98
β^3-Tyr7	7.82	2.18	2.24	4.24	2.39, 2.71	εH: 6.97; ζH: 6.65

Table S4. ^{1}H NMR chemical shifts of the cyclic β^3-heptapeptide Cy(3,6)-AspLys **6b** in MeOH solution at 283 K.

	H^N	H^{α}_{ax}	H^{α}_{eq}	H^{β}	H^{γ}	others
β^3-Val1	-	3.00	2.57	3.49	2.06	δCH_3: 1.07
β^3-Orn2	8.26	2.96	2.32	4.52	1.65	δCH_2: 1.54; εCH_2: 2.89
β^3-Asp3	8.94	3.12	2.50	4.46	2.19, 2.67	
β^3-Val4	8.32	2.62	2.39	4.25	1.76	δCH_3: 0.94
β^3-Ser5	8.17	2.37	2.44	4.42	3.48	
β^3-Lys6	7.60	2.18	2.40	4.29	1.20, 1.69	δCH_2: 1.34; εCH_2: 1.45 ζCH_2: 2.75, 3.79; ηNH: 7.98
β^3-Tyr7	7.46	2.11	2.40	4.50	2.59	εH: 6.95; ζH: 6.63

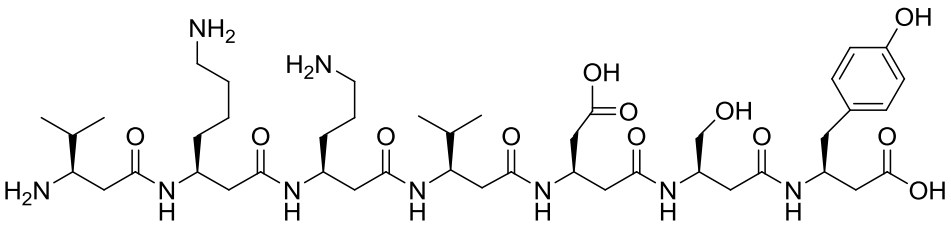
Table S5. ^{1}H NMR chemical shifts of the cyclic β^3-heptapeptide Cy(1,4)-LysAsp **12b** in MeOH at 283 K.

	H^N	H^{α}_{ax}	H^{α}_{eq}	H^{β}	H^{γ}	others
β^3-Lys1	-	2.98	2.61	3.71	1.36, 1.88	δCH_2: 1.64; εCH_2: 1.54 ζCH_2: 3.09, 3.48; ηNH: 8.09
β^3-Val2	8.24	2.82	2.47	4.17	1.79	δCH_3: 0.94
β^3-Orn3	8.23	2.53	2.44	4.25	1.62	δCH_2: 1.48; εCH_2: 2.85, 2.91
β^3-Asp4	8.55	2.79	2.52	4.61	2.27, 2.32	
β^3-Val5	8.25	2.40	2.40	4.16	1.69	δCH_3: 0.87
β^3-Ser6	7.87	2.30	2.50	4.29	3.35, 3.47	
β^3-Tyr7	7.70	2.15	2.43	4.46	2.60, 2.65	εH: 7.00; ζH: 6.64

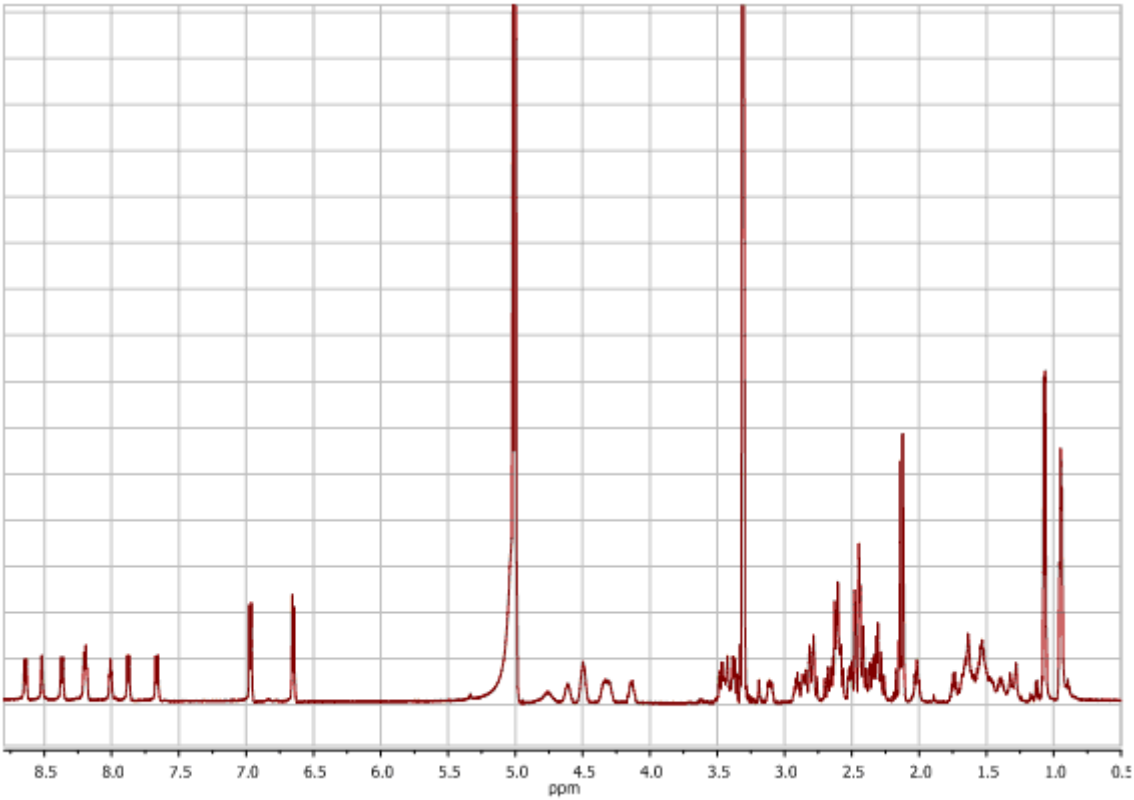
Table S6. ^{1}H NMR chemical shifts of the cyclic β^3-heptapeptide Cy(1,4)-LysAsp **12b** in MeOH at 271 K.

	H^N	H^{α}_{ax}	H^{α}_{eq}	H^{β}	H^{γ}	others
β^3-Lys1	-	2.99	2.61	3.71	1.36, 1.88	δCH_2: 1.63; εCH_2: 1.55 ζCH_2: 3.08, 3.49; ηNH: 8.16
β^3-Val2	8.27	2.82	2.49	4.17	1.79	δCH_3: 0.94
β^3-Orn3	8.28	2.54	2.45	4.25	1.62	δCH_2: 1.47; εCH_2: 2.85, 2.90
β^3-Asp4	8.58	2.79	2.51	4.60	2.28, 2.32	
β^3-Val5	8.25	2.35	2.41	4.14	1.68	δCH_3: 0.87
β^3-Ser6	7.95	2.30	2.50	4.28	3.34, 3.46	
β^3-Tyr7	7.79	2.15	2.42	4.45	2.59, 2.65	εH: 7.00; ζH: 6.63

Lin(2,5)-LysAsp **8a**

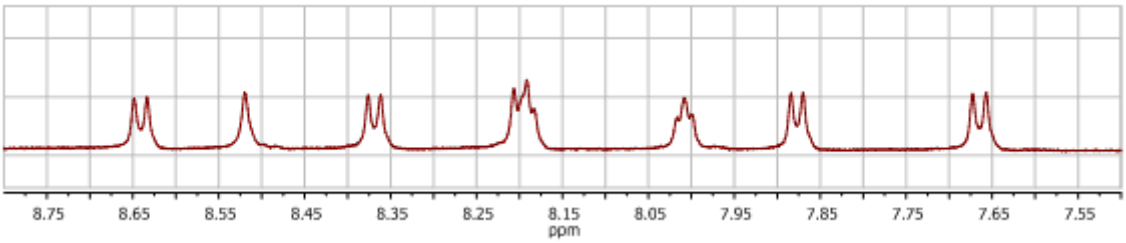


^{1}H-NMR (MeOH, 10 ºC)

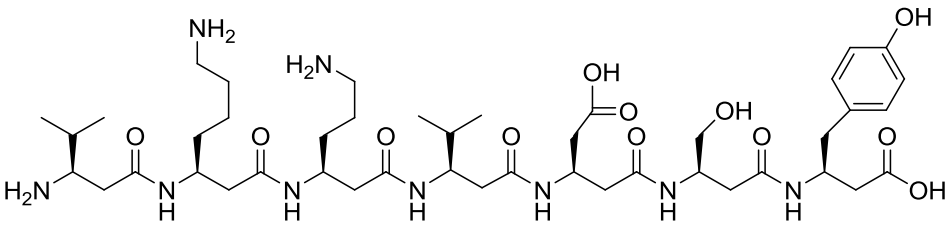


^{1}H-NMR (MeOH, 10 ºC)

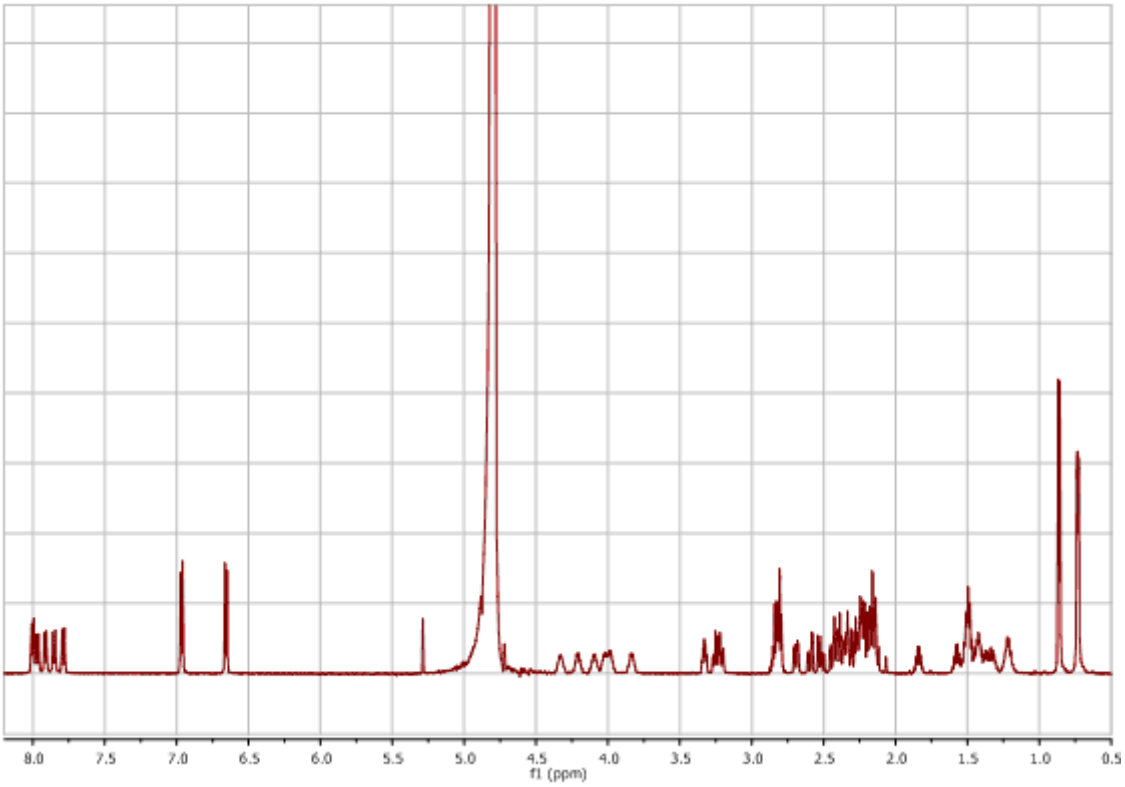
Amide region



Lin(2,5)-LysAsp **8a**

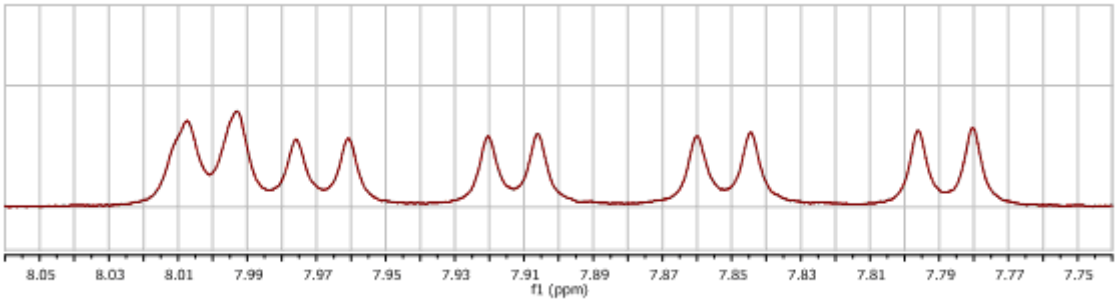


^{1}H-NMR (pH 7.4, 10 ºC)

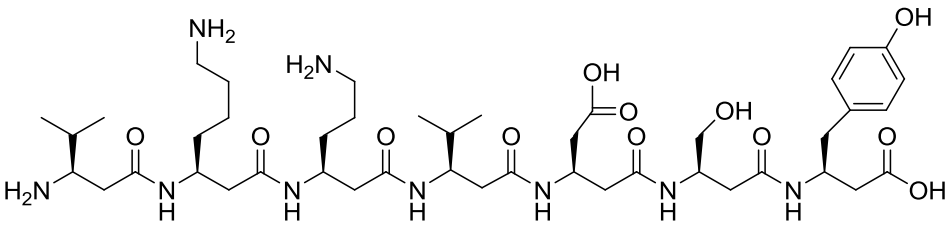


^{1}H-NMR (pH 7.4, 10 ºC)

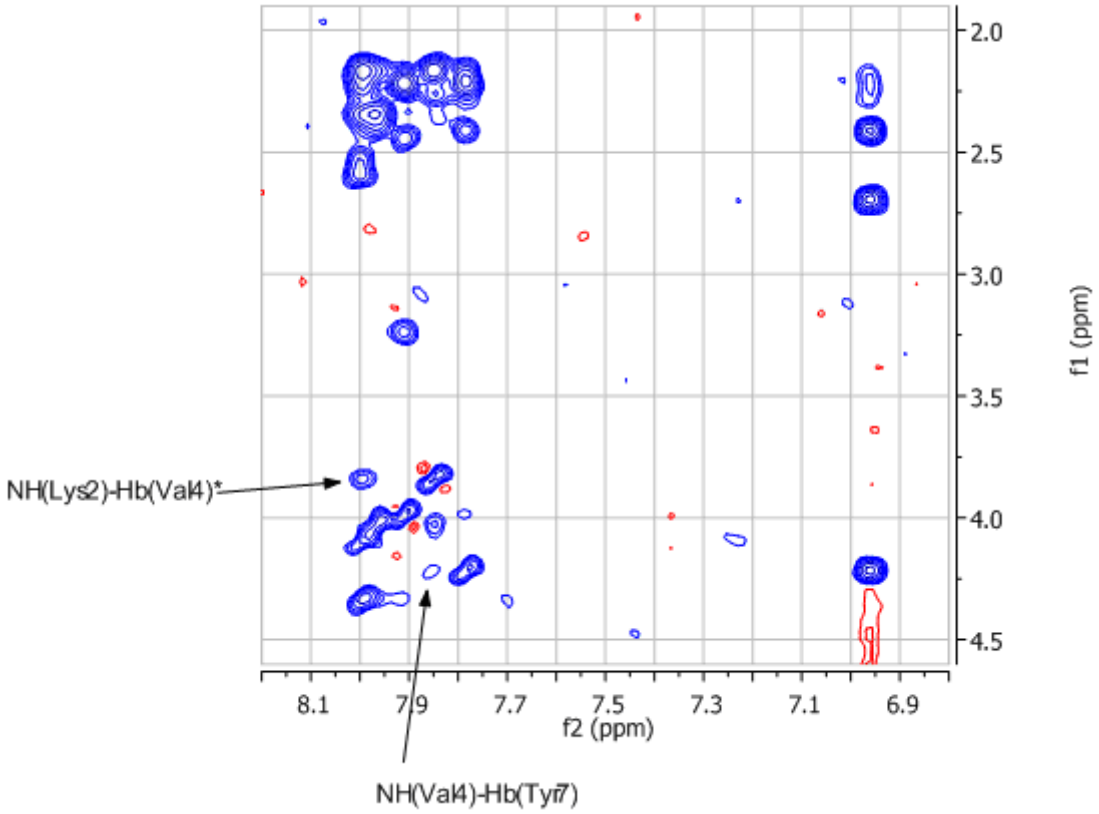
Amide region



Lin(2,5)-LysAsp **8a**

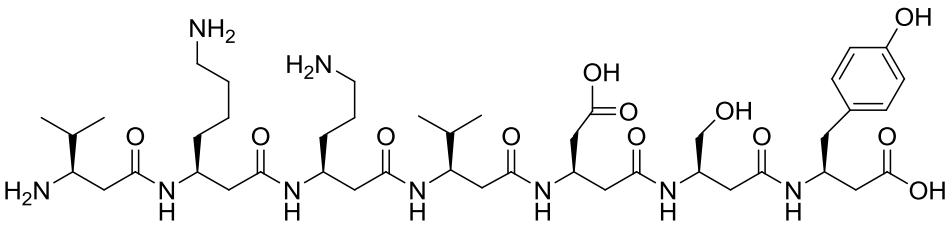


ROESY (pH 7.4, 10 ºC, 300ms mixing time)
Amide proton region

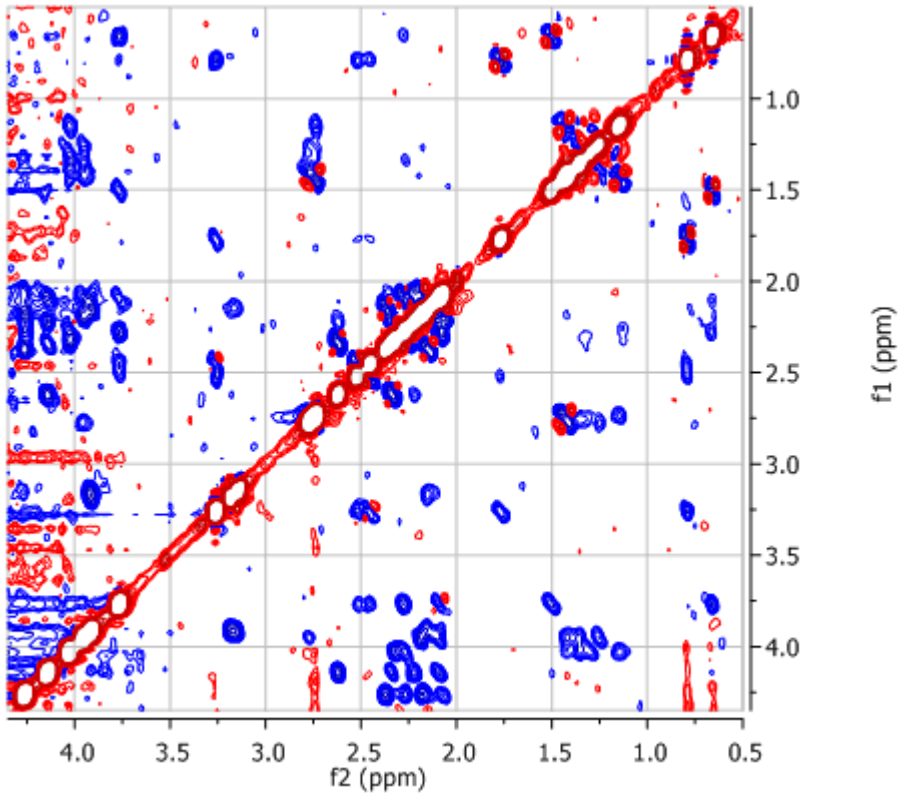


* ambiguously assigned.

Lin(2,5)-LysAsp **8a**



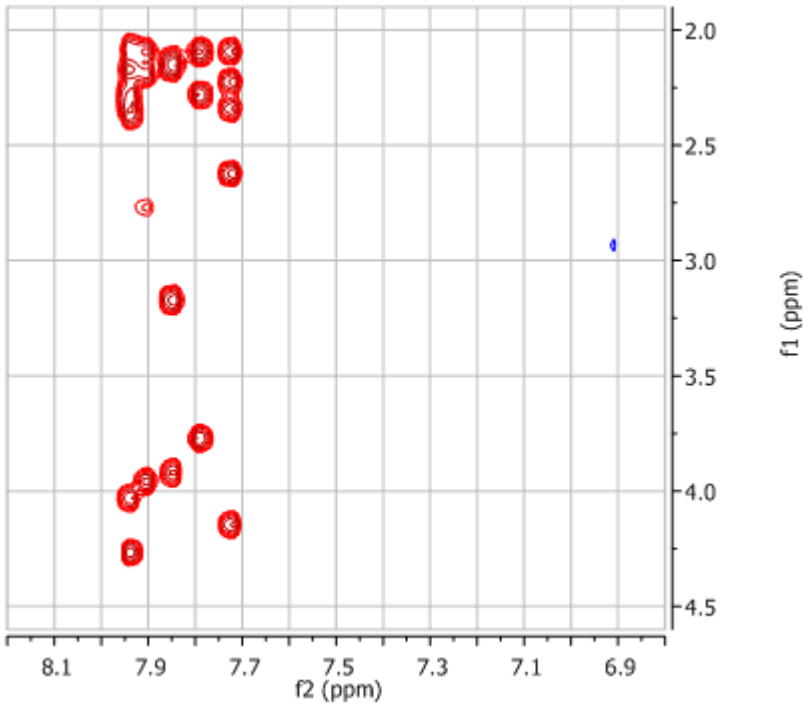
ROESY (pH 7.4, 10 ºC, 300ms mixing time)
Aliphatic region



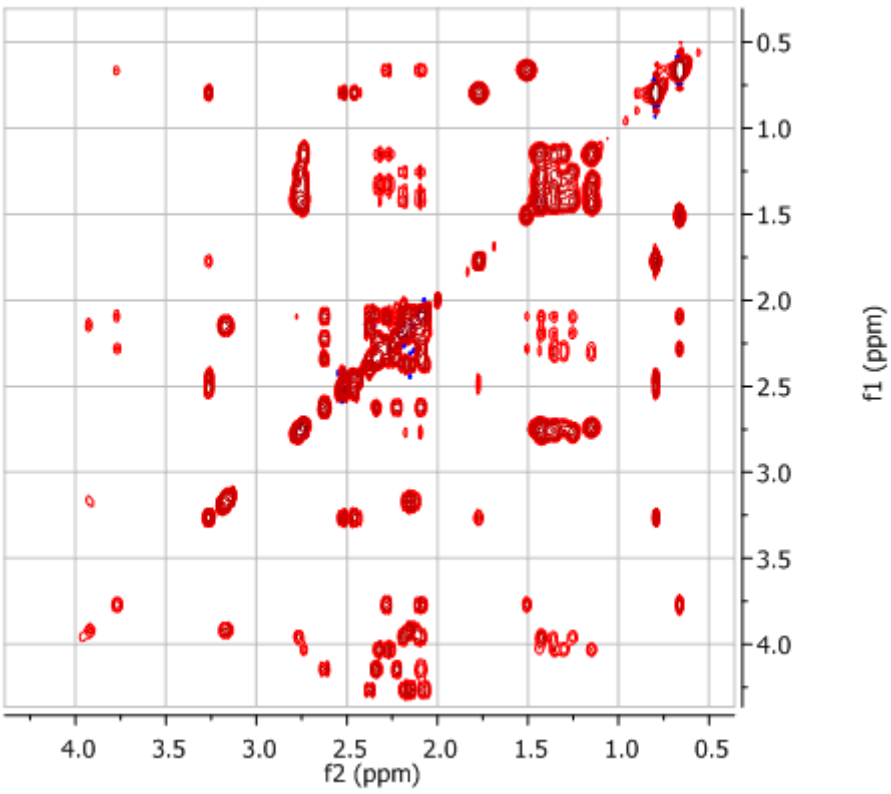
None of the NOE signals corresponded to long-range correlations between residues (i, i+2) or (i, i+3).

Lin(2,5)-LysAsp **8a**

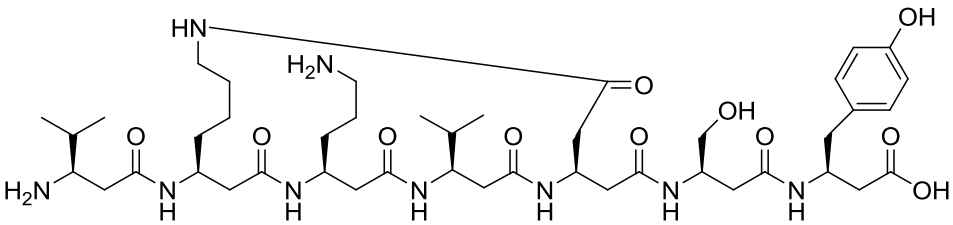
TOCSY (pH 7.4, 10 ºC) Amide proton region



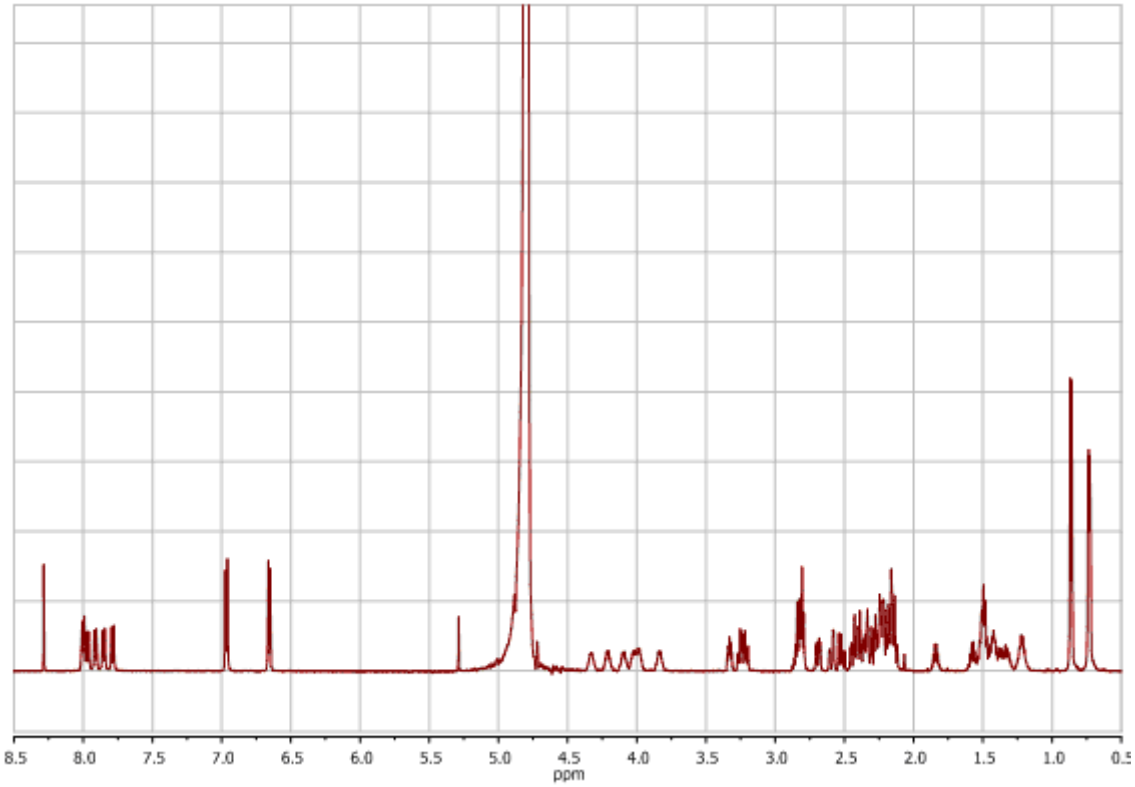
TOCSY (pH 7.4, 10 ºC) Aliphatic region



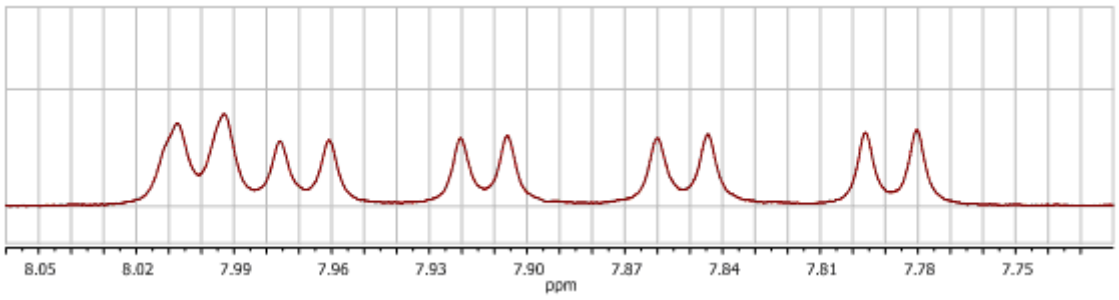
Cy(2,5)-LysAsp **8b**



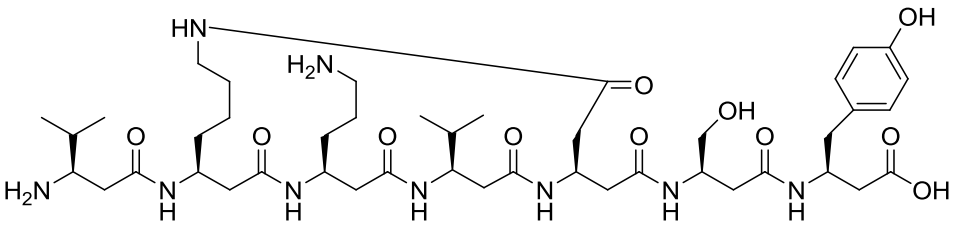
^{1}H-NMR (pH 7.4, 10 ºC)



^{1}H-NMR (pH 7.4, 10 ºC)
Amide proton region

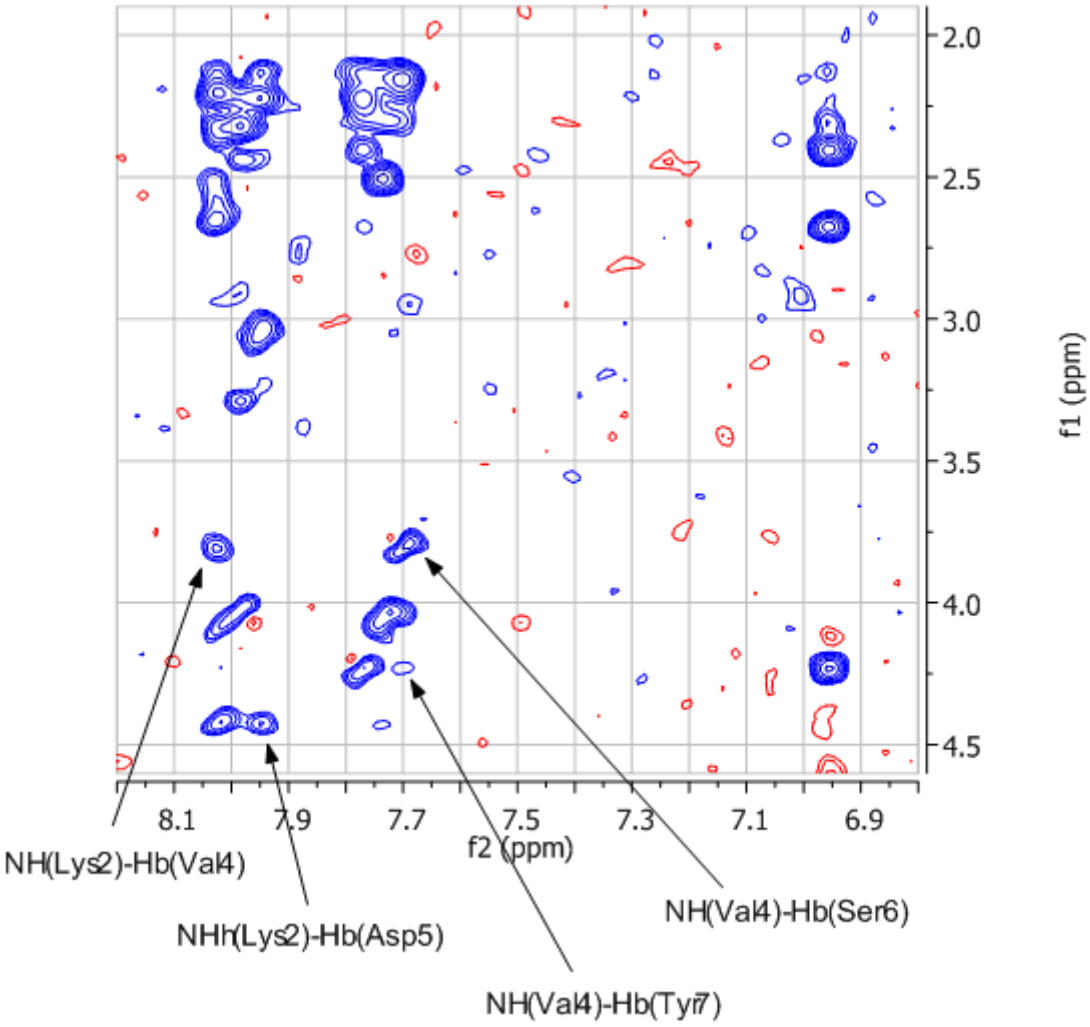


Cy(2,5)-LysAsp **8b**

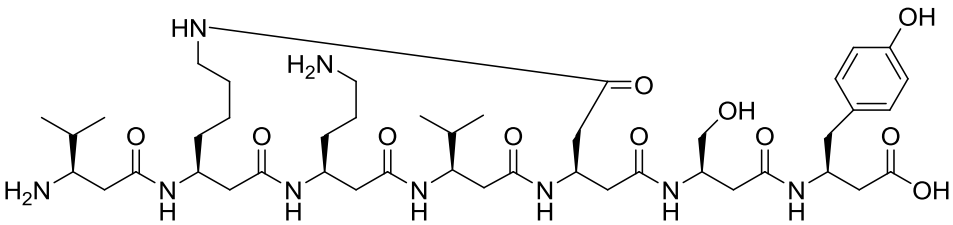


ROESY (pH 7.4, 10 ºC, 300ms mixing time)

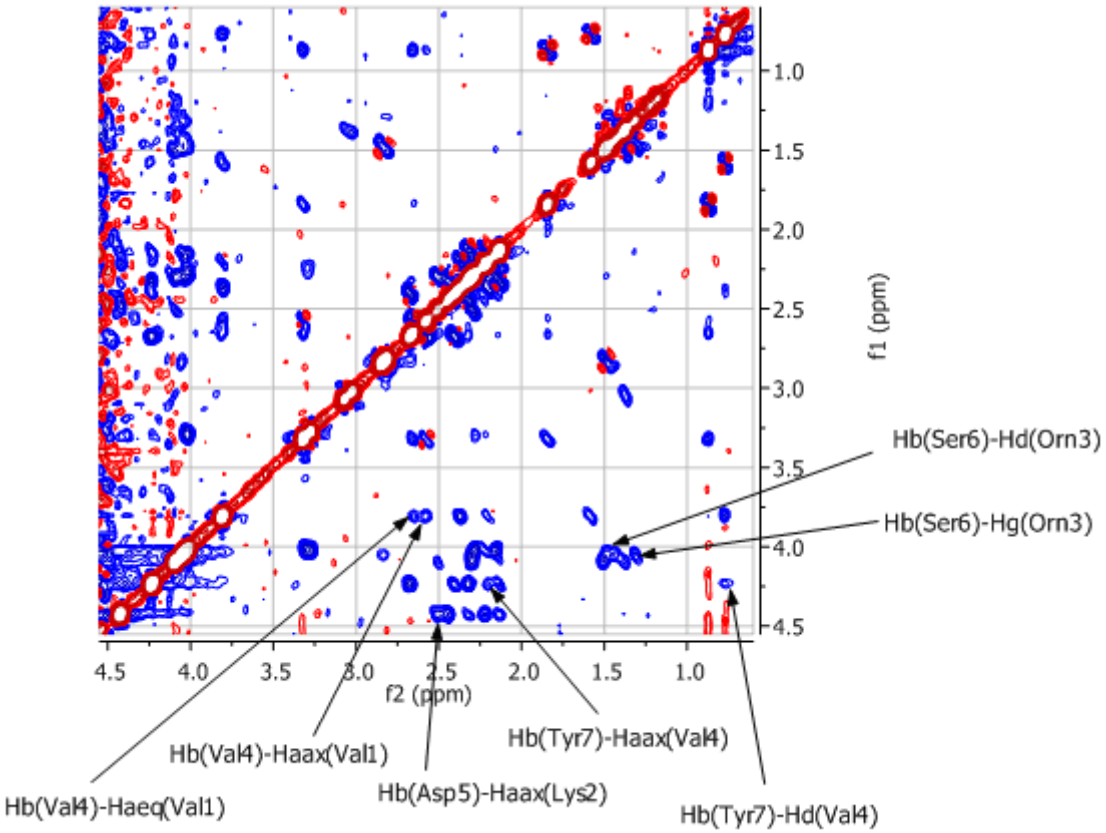
Amide proton region



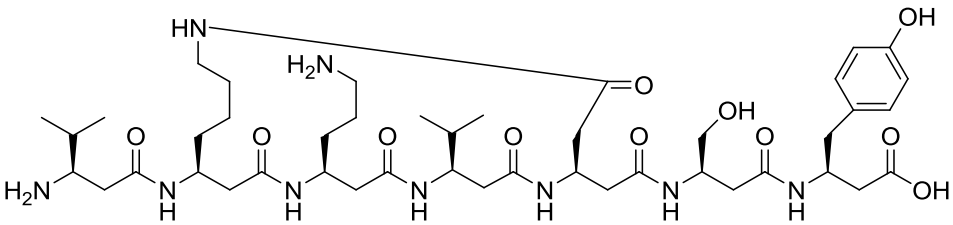
Cy(2,5)-LysAsp **8b**



ROESY (pH 7.4, 10 ºC, 300ms mixing time)
Aliphatic region

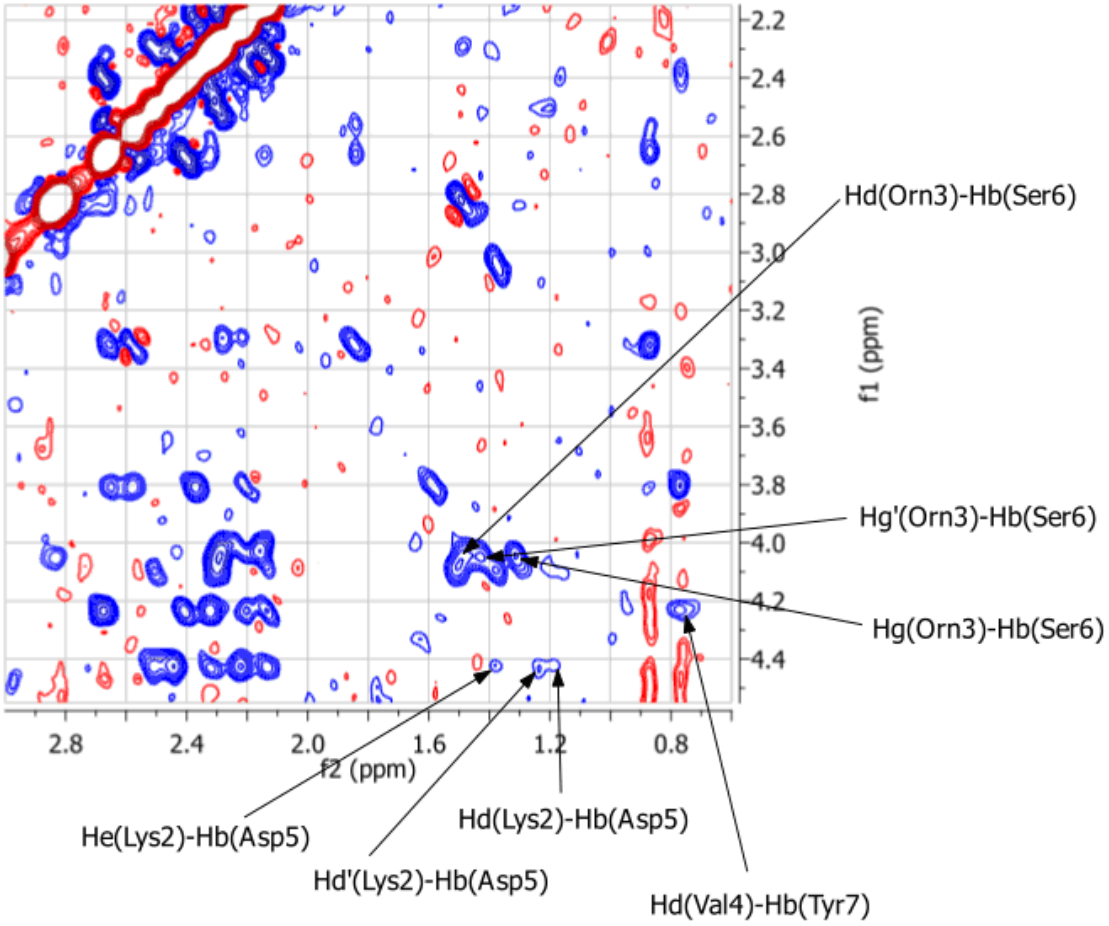


Cy(2,5)-LysAsp **8b**



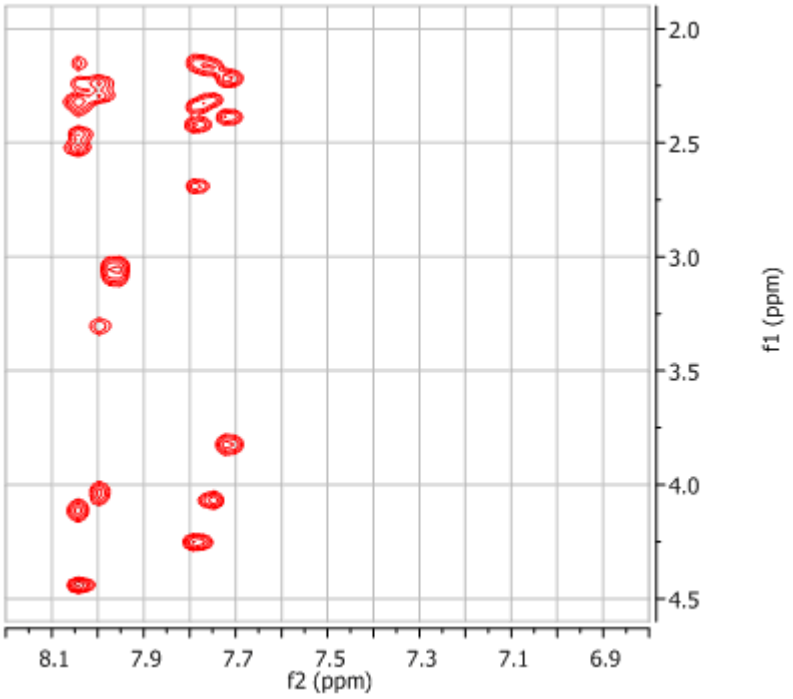
ROESY (pH 7.4, 10 ºC, 300ms mixing time)
Aliphatic region

(NOE correlations with the side-chains)

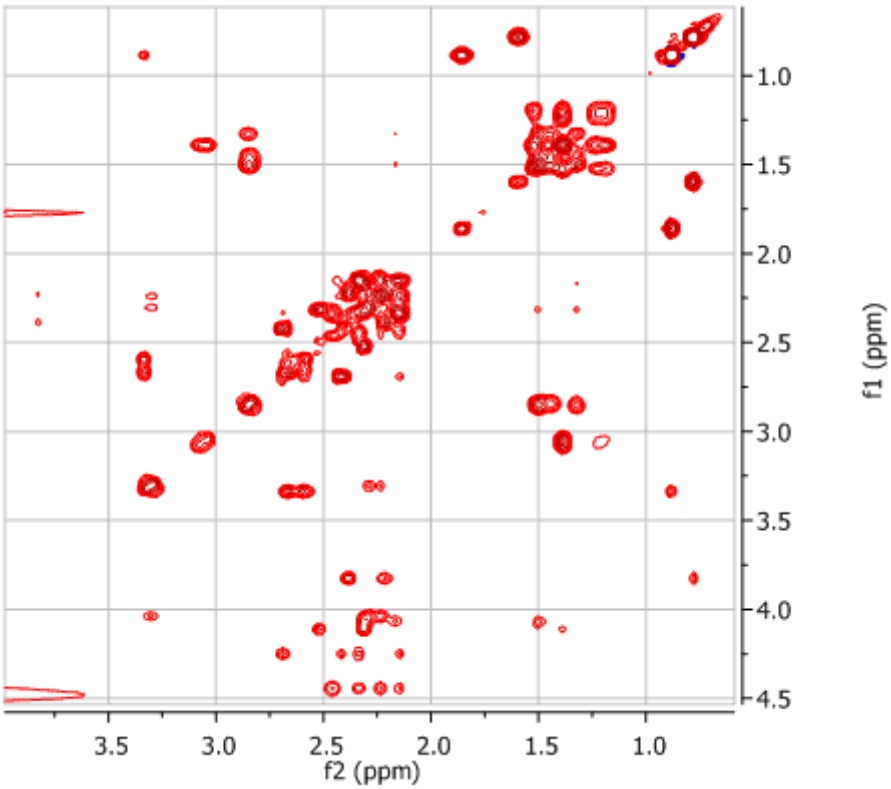


8b

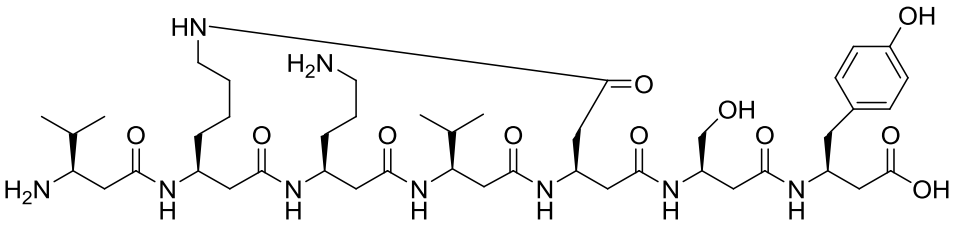
TOCSY (pH 7.4, 10 ºC) Amide proton region



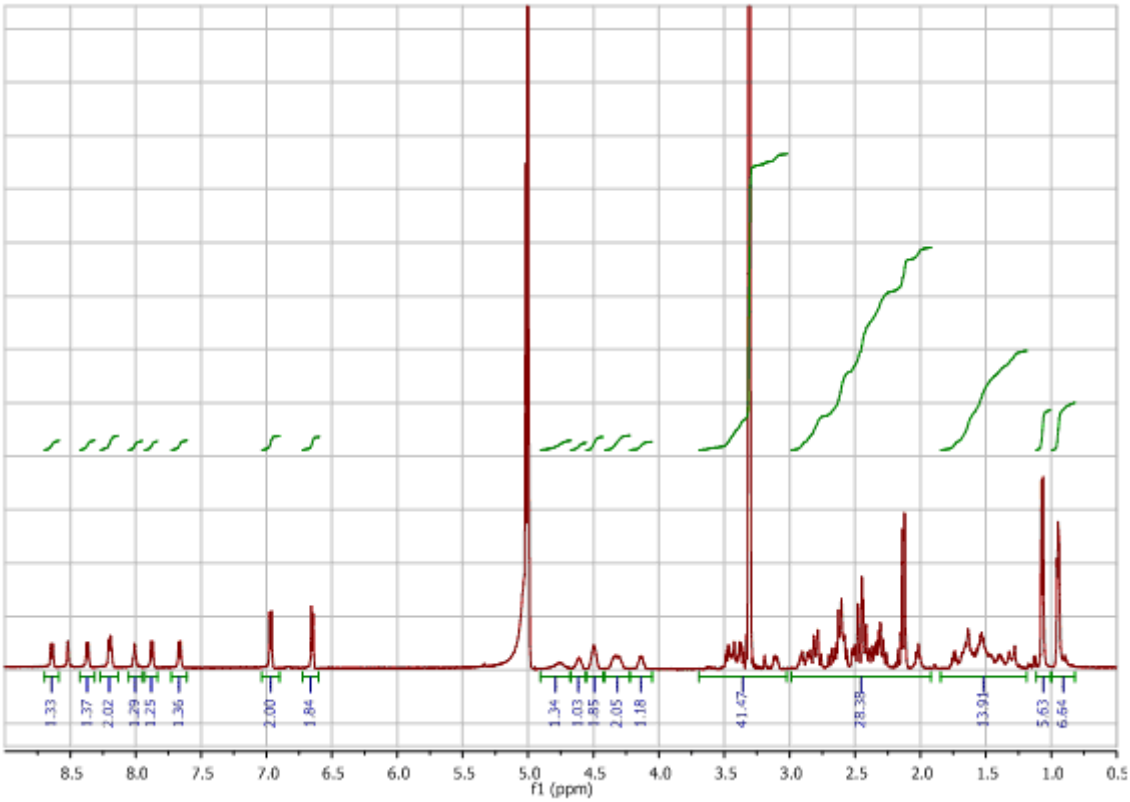
TOCSY (pH 7.4, 10 ºC) Aliphatic region



Cy(2,5)-LysAsp **8b**

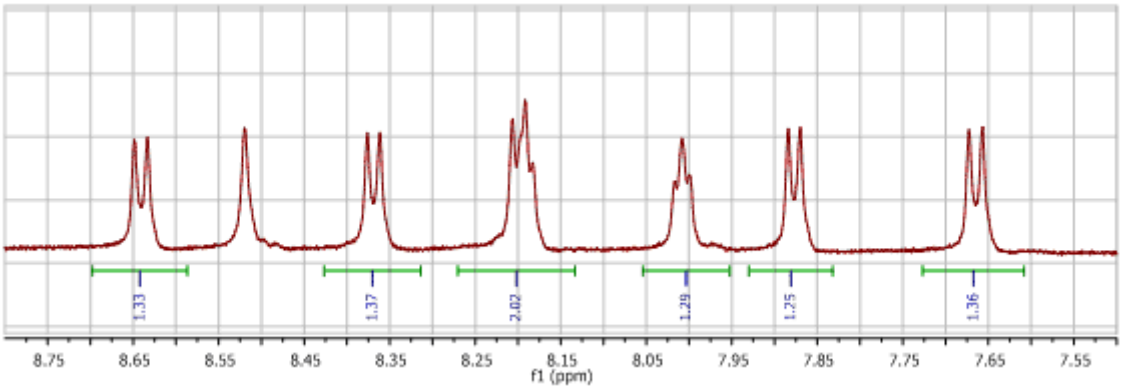


^{1}H-NMR (MeOH, 10 ºC)



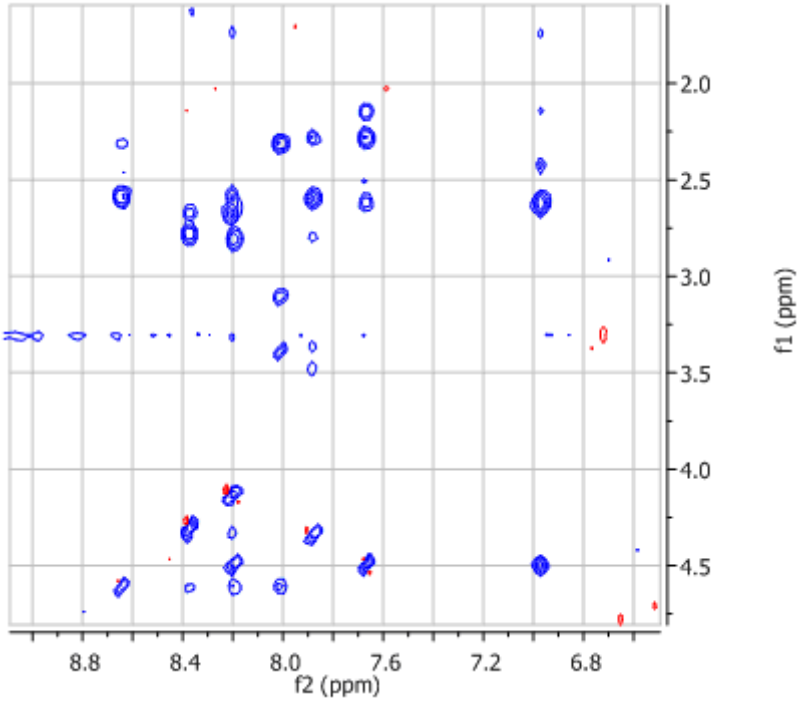
^{1}H-NMR (MeOH, 10 ºC)

Amide protons

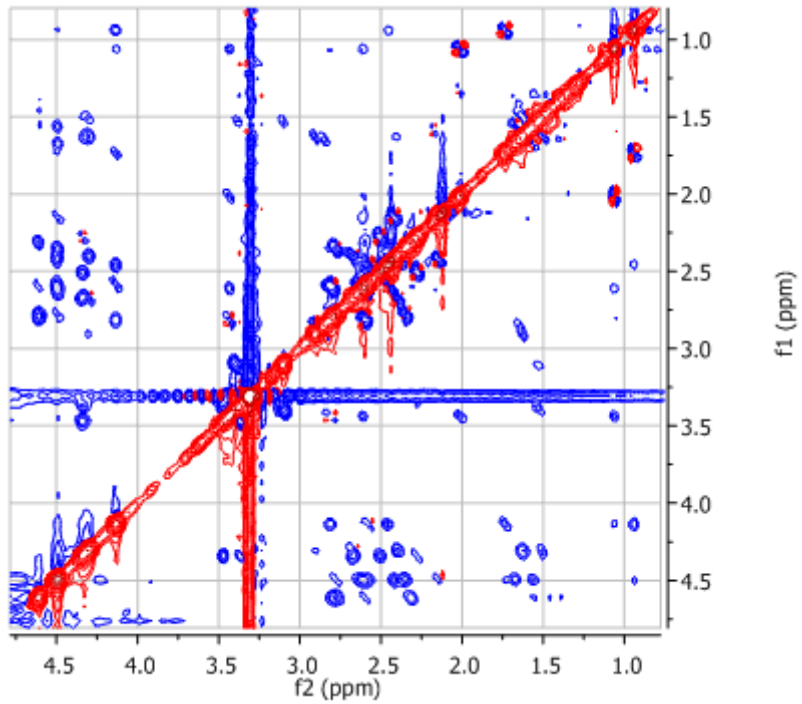


8b

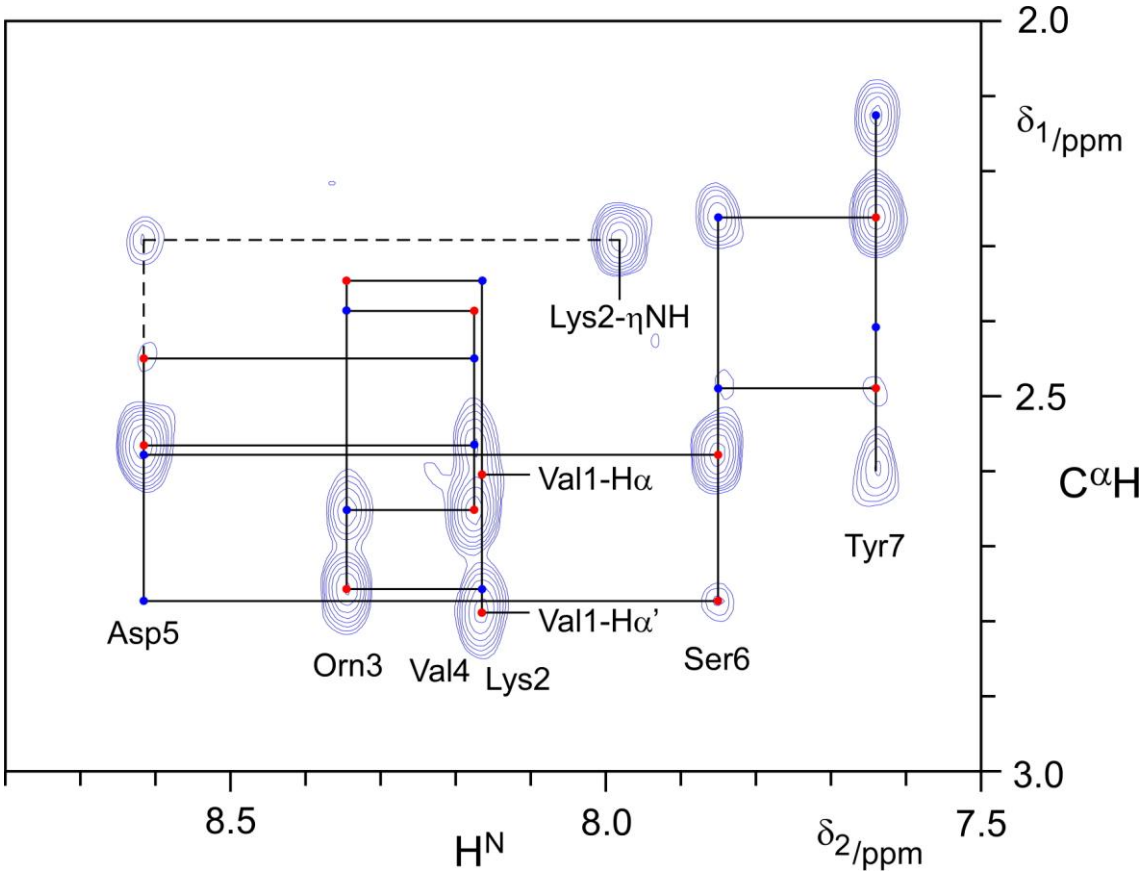
ROESY (MeOH, 10 ºC, 300ms mixing time)
Amide proton region



ROESY (MeOH, 10 ºC, 300ms mixing time)
Aliphatic region



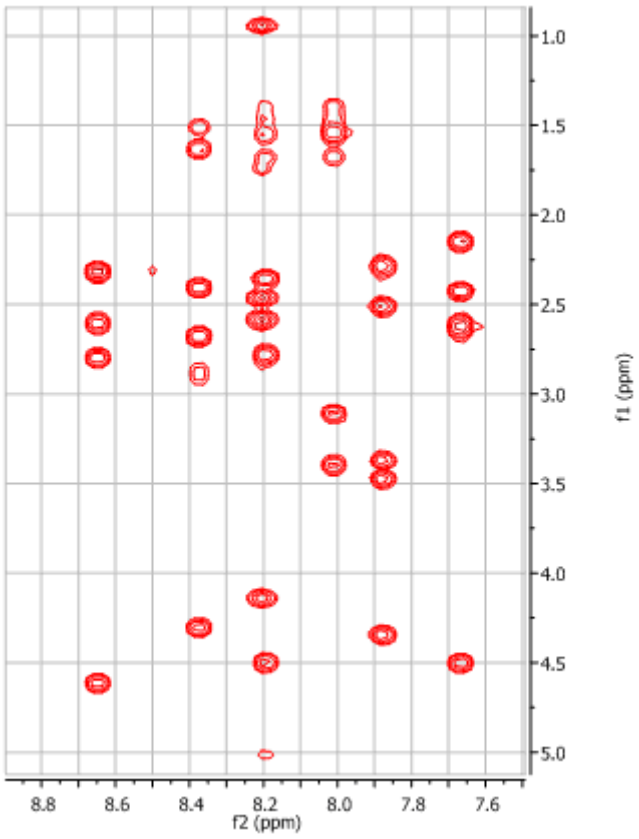
ROESY (MeOH, 10 ºC, 300ms mixing time)
Amide proton region [1]



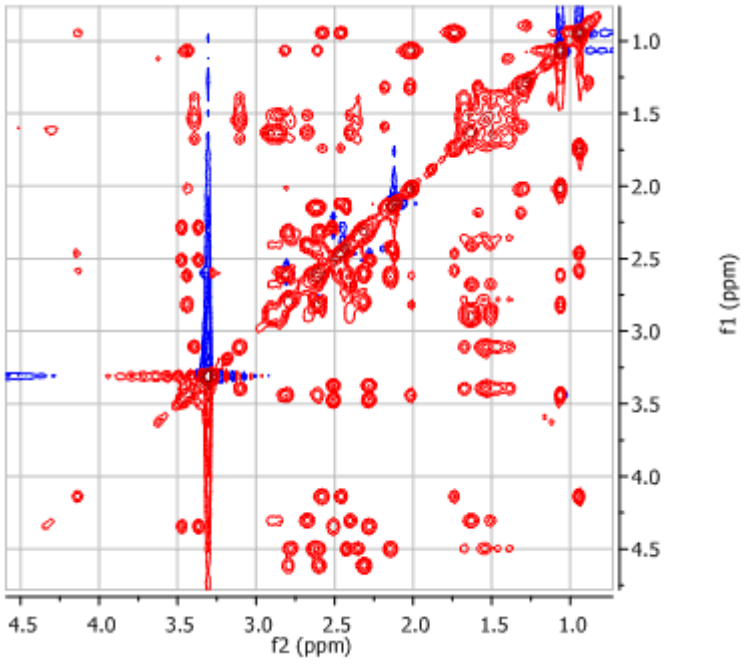
[1] At each H^N resonance line, blue dots indicate intra-residual (*i*,*i*) cross peaks, whereas red dots indicate inter-residual cross peaks that correspond to contacts of the H^N resonance to H^{α}_{axial} and $H^{\alpha}_{equatorial}$ atoms of the preceding (*i-1*,*i*) amino acid.

8b

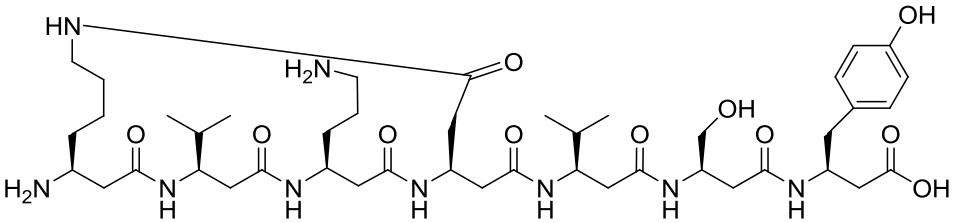
TOCSY (MeOH, 10 ºC) Amide proton region



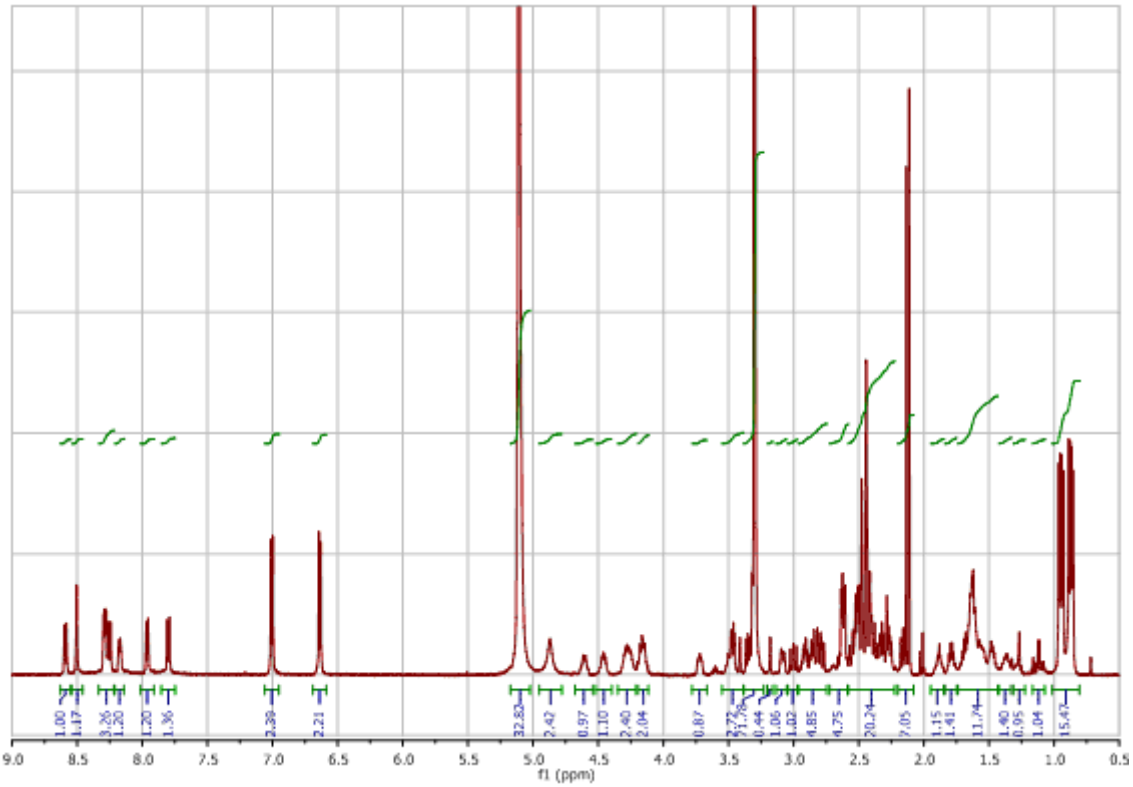
TOCSY (MeOH, 10 ºC) Aliphatic region



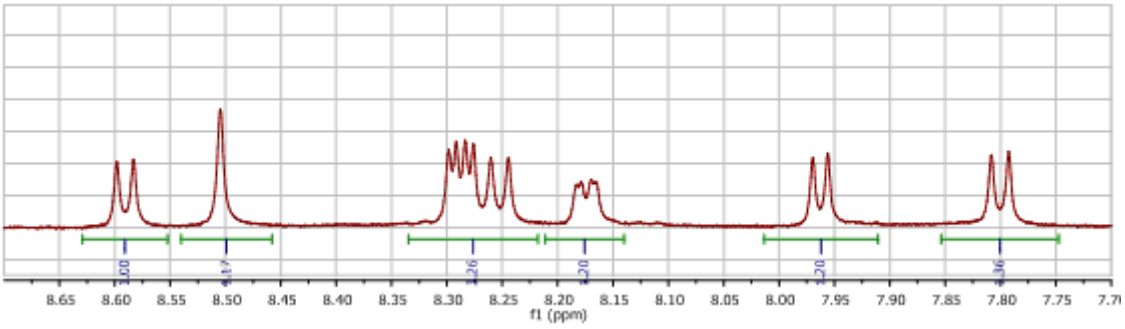
Cy(1,4)-LysAsp **12b**



^{1}H-NMR (MeOH, -2 ºC)

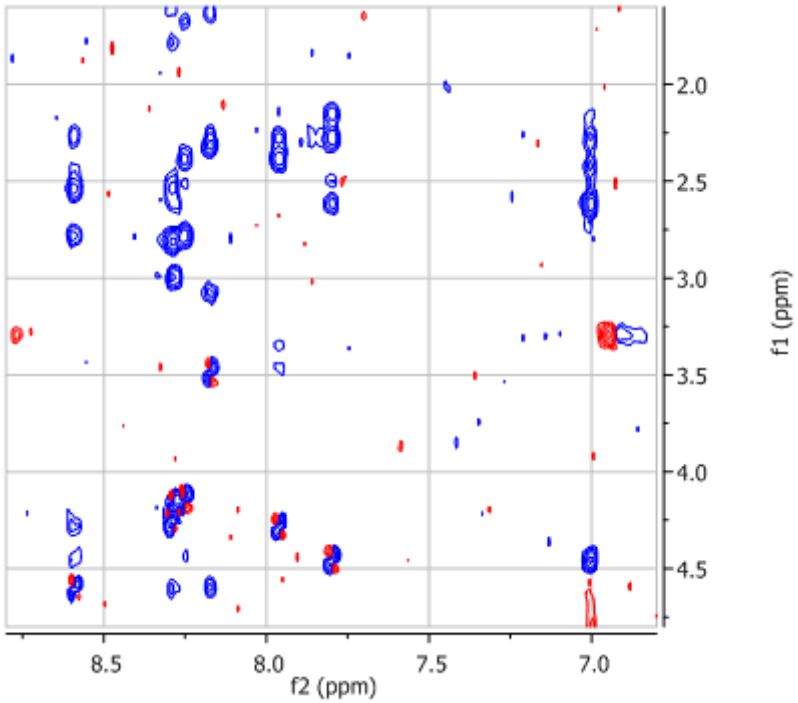


^{1}H-NMR (MeOH, 10 ºC)
Amide protons

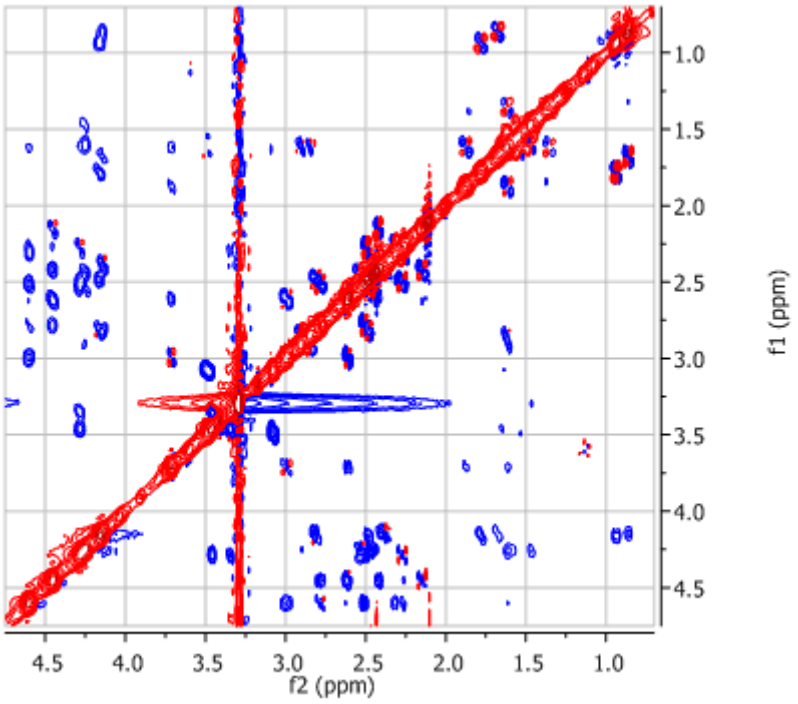


12b

ROESY (MeOH, -2 ºC, 300ms mixing time)
Amide proton region

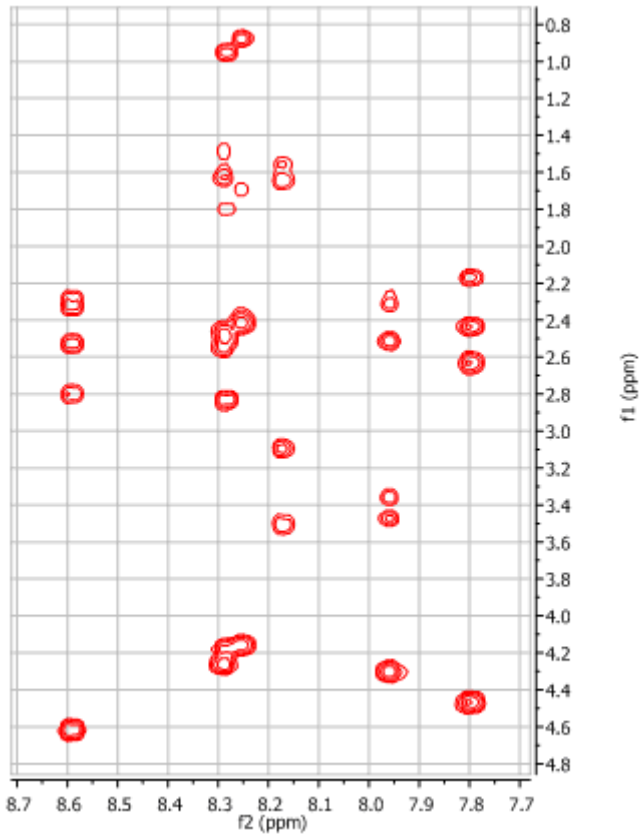


ROESY (MeOH, -2 ºC, 300ms mixing time)
Aliphatic region

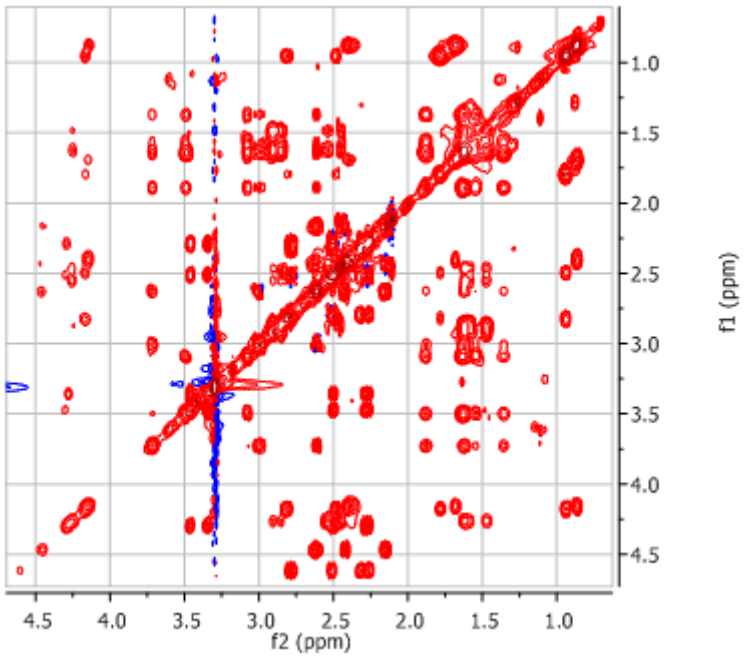


12b

TOCSY (MeOH, -2 ºC) Amide proton region



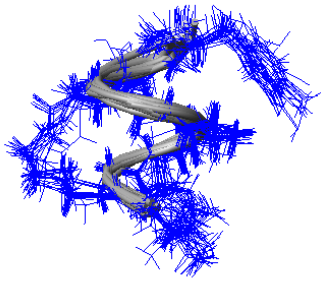
TOCSY (MeOH, -2 ºC) Aliphatic region



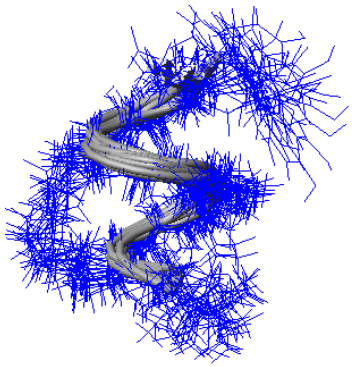
5. Structure calculations without H-bond restraints in vacuo

Statistics for 20 out of 200 calculated without hydrogen bond restraints:

a) calibrated: rmsd 0.42/0.86 backbone/heavy, no violations



b) bin: rmsd 0.72/1.40 backbone/heavy, no violations



6. References.

[1] Neidig, K. P., Geyer, M., Görler, A., Antz, C., Saffrich, R., Beneicke, W., and Kalbitzer, H. R. (1995) AURELIA, a program for computer-aided analysis of multidimensional NMR spectra, *J. Biomol. NMR* 6, 255-270
[2] a) Wüthrich, K. *NMR of Proteins and Nucleic Acids*, Wiley, New York, 1986. b) Etezady-Esfarjani, T., Hilty, C., Wüthrich K., Rueping, M., Schreiber, J. Seebach, D. *Helv. Chim. Acta* **2002**, *85*, 1197–1209.
[3] Vaz, E; Brunsveld, L. *Org. Lett.* **2006**, *8*, 4199-4202
[4] Grieco, P.; Gitu, P. M.; Hruby, V. J. *J. Pept. Res.* **2001**, *57*, 250-256
[5] Creighton, T. E. *Proteins: Structures and Molecular Principles*, 2nd ed.; W. H. Freeman and Company: New York, 1993; p14.