SUPPORTING INFORMATION

Rational design and synthesis of orally bioavailable peptides guided by NMR amide temperature coefficients

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Supporting Methods and Materials

General

Fmoc-protected amino acids and coupling agents were purchased from ChemImpex International and ChemPep, and all other reagents were purchased from Auspep, Merck and Sigma, and used without further purification. Resins were also purchased from ChemImpex International.

Peptide synthesis

Cyclic peptides were synthesized on 2-chlorotrityl chloride (2CTC) resin. In general, couplings were performed using 4 equiv. Fmoc-protected amino acid, 3 equiv. HATU (*O*-(7-azabenzotriazol-1-y1)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate) or HCTU (*O*-(1*H*-6-chlorobenzotriazol-1-y1)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate) and 6 equiv. *N,N*-diisopropylethylamine (DIPEA) in dimethylformamide (DMF) (0.1 M in amino acid) for 0.5-2 h. Fmoc deprotection was carried out with 30% (v/v) piperidine in DMF. After each coupling and deprotection step, the resin was washed with DMF (3×), dichloromethane (DCM) (3×) and DMF (3×). Peptides were cleaved from the resin using 1% (v/v) TFA (trifluoroacetic acid) in DCM (dichloromethane). Head-to-tail cyclization was performed in DMF with 3 equiv. HATU and 6 equiv. DIPEA. Following removal of the solvent, side-chain protecting groups were removed in 94:3:3 TFA/TIPS (triisopropylsilane)/water. After cyclization, the peptides were purified by reverse-phase preparative HPLC. Purity of fractions was assessed using ESI-MS and analytical HPLC.

NMR spectroscopy and structure determination

All spectra were recorded on a Bruker ARX 500 MHz spectrometer at 298 K. Spectra were processed using CCPNMR (1). Assignment of resonances for each amino acid was accomplished using 1D, TOCSY and ROESY experiments. TOCSY spectra were recorded with a mixing time of 80 ms, and ROESY spectra with a mixing time of 200-250 ms. Chemical shifts were internally referenced to DSS at 0 ppm. Distance restraints were derived from cross-peaks in ROESY spectra. Backbone dihedral angle restraints were derived from $^{3}J_{HNH\alpha}$ coupling constants measured from splitting of peaks in a 1D spectrum. Structure calculations were performed using the program CYANA (2).

Hydrogen-deuterium exchange studies

All NMR spectra for the hydrogen-deuterium exchange studies were recorded on a Bruker ARX 500 MHz spectrometer at 298 K. Spectra were referenced to DSS at 0 ppm. Lyophilized peptide was initially solubilized in 30% v/v CD_3CN, 70% v/v H_2O and the pH adjusted to 3 using concentrated solutions of HCl and NaOH. The pH of the sample was measured using a micro pH combination electrode (Sigma-Aldrich). The peptide was then lyophilized. Lyophilized peptide was dissolved in the appropriate solvent (30% v/v

CD_3CN, 70% v/v D_2O) and immediately placed in the spectrometer for measurement. 1D and TOCSY spectra were recorded at specific time points over a 24 h period. Hydrogen-deuterium exchange rates were measured by integrating each exchangeable amide resonance separately.

Amide temperature coefficient studies

The temperature dependence of amide proton resonances was derived from 1D and TOCSY spectra recorded on a Bruker ARX 500 MHz spectrometer. Spectra were measured between 288 K to 308 K in 5 K increments and referenced to DSS at 0 ppm. Assignment of the spectra was performed using the program rNMR (3) and/or CCPNMR (1).

PAMPA for determining passive cell permeability

A 96-well donor and accepter plate system pre-loaded with artificial lipid membrane (0.4 μm Polyvinylidene fluoride [PVDF] membrane) was used. 300 μL of 50 μM peptide (5% v/v DMSO in Hank's Balanced Salt Solution [HBSS]) was added to the donor wells and 200 μL of buffer (5% v/v DMSO in HBSS) was added in the acceptor wells. The acceptor plate was lowered onto the donor plate so that the artificial membrane was in contact with the peptide solution below. A cover plate was placed on the acceptor plate and the assay plate covered with foil. Incubation of peptides was carried out at room temperature for 5 h.

Acceptor and donor well concentrations were measured by LC-MS using positive ion mode. As the concentration differences between acceptor and donor wells are significant they were analysed separately, Dilution series of each peptide sample were prepared and injected into the LC-MS to generate a standard curve for quantification. 5 μL from donor or 40 μL from acceptor wells were injected into the LC-MS and the analyte peak areas were recorded. The limit of detection of the peptides vary; however most gave a value of approximately 0.05 μM. Percentage permeability was calculated relative to the equilibrium concentration.

Caco-2 permeability studies

Caco-2 cells were cultured in an atmosphere of 5% CO_2 and 90% relative humidity in T75 flasks (Nunc) with 10 ml of media (10% foetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 1% penicillin/streptomyocin). Cells were passaged 1:10 every five days, or when 80-90% confluent, using 0.25% trypsin-EDTA (Invitrogen, Gibco Laboratories).

Caco-2 cells were seeded onto each membrane of the transwell permeable support 12-well plate insert to give 50,000 cells/well. The individual feeding tray wells received 1.5 ml of cell culture medium. The cell culture medium was changed every second day. Caco-2 cell monolayers were used for experimentation 21 days post seeding.

To prepare the cells for transport studies (apical to basolateral, A to B) cell culture media was removed from both compartments and the cells were washed once with HBSS in both the A and B compartments. The washing buffer was removed and 0.75 ml of fresh HBSS placed in compartment A and 1.5 ml of HBSS in compartment B for pre-incubation, carried out at 37°C for 30 min under agitation. Transepithelial electrical resistance (TEER) was measured across the cell membranes at the beginning of the experiment. HBSS was subsequently removed from compartment A, replaced with 0.75 ml of the peptide solution prepared in HBSS. Monolayers were incubated and continuously shaken. 100 µL samples were taken from the A and B compartments at 45 min and 90 min intervals, and the buffer volume replenished. After 90 min, 200 µL of compartment A and B was also collected for Lucifer Yellow (LY) measurements. Amount of LY permeated was measure on a Tecan plate reader (excitation 485 nm, emission 530 nm). At the conclusion of the experiment TEER was measured again to confirm the integrity of the Caco-2 monolayers (TEER >300 Ω cm^2). Acceptor and donor well concentrations were measured by LCMS as per the Pampa Assay.

The permeability coefficient (P_{app}) values were calculated according to the Equation (1).

$$P_{app} = \frac{V_A}{t} \times \frac{C_A}{C_0} \times \frac{1}{A} \qquad (1)$$

Where C_A is the sampled concentration in the acceptor compartment, V_A is the volume in the acceptor compartment, t is the incubation time, C_0 is the initial concentration in the donor compartment, and A is the area of the filter of the transwell plate.

Animals

Male Wistar rats (250 ± 20 g) were bred at, and obtained from, the Australia Animal Resource Centre (Canning Vale, WA). Following Australian ethical standard animal air transport, animals were housed at The University of Queensland Biological Resources (UQBR) Animal Facility at The Australian Institute for Bioengineering and Nanotechnology at The University of Queensland, Australia in a 12 h light/dark cycle according to the standard of holding facility with food and water provided. All experiments were approved by the animal ethics committee of The University of Queensland. A period of at least 48 h habituation in the UQBR facility was provided prior to any experimental intervention. After experimentation, animals were humanely euthanized by CO_2 inhalation as stipulated by the ethical agreements.

***In vivo* pharmacokinetic studies**

Rats were surgically implanted with a jugular vein catheter and fasted overnight, as previously described (4). Blood samples (200 µL) were collected from the indwelling catheter of an unanaesthetised, unrestrained rat,

5 min prior to peptide administration (10 mg/kg p.o. in olive oil (100 μg/100 μL), or 1 mg/kg in neat DMSO (300 μg/100 μL)). Oral dosing was performed by gavage (75 mm soft-tipped polyethylene 18G), i.v. dosing was directly via the indwelling catheter using a 100 μL glass Hamilton syringe. Further blood samples were collected 5 min, 15 min, 30 min and at given time points from 1 to 8 h, post administration. Volumes collected were replaced with sterile heparinised saline (20 U/mL). Blood samples were centrifuge (8000 rpm, 5 min), plasma extracted and snap frozen on dry ice. Rats were fed after the 4 h sample was collected.

Plasma compound concentration (C_p) was determined by extraction using simple acetonitrile-based techniques. Briefly, thawed plasma was diluted 1:3 in acetonitrile (9:1 ultrafiltered water), vortexed, sonicated and then centrifuged (13,000 rpm, 5 min). Spiked samples of known concentrations of peptide (i.e. 1 pg – 1 ng/mL) were prepared in both acetonitrile and clean plasma using the same technique for standard curve preparation. The supernatant of all centrifuged samples was used directly for LC/MS analysis (ABSCIEX 4000 QTRAP Triple Quadrupole, Linear Ion-Trap LCMS mass spectrometer). Chromatography was carried out on a C18 column (Phenomenex, 5 μm, 2.1 × 50mm) using a linear gradient (2–80% Buffer B in 12 minutes, flow rate 0.3 mL/min). Buffer A was 0.1% v/v formic acid (aq) and Buffer B was 90% v/v acetonitrile/0.1% v/v formic acid (aq).

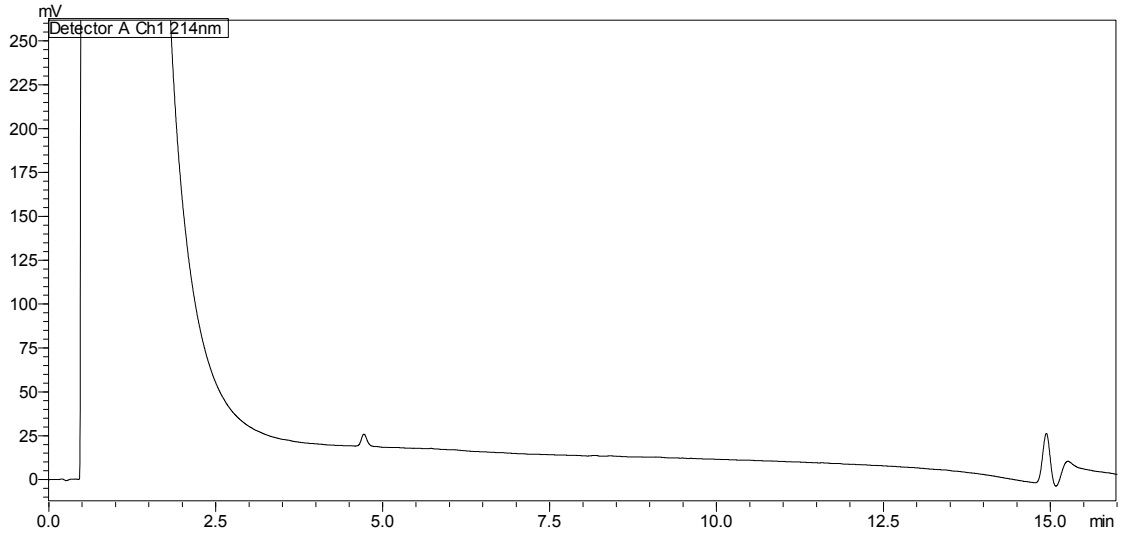
LC/MS data was analyzed for area under curve (AUC) of peaks corresponding to both protonated (H^+; i.e. peptide MW+1) and sodium (Na^+; i.e. peptide MW+23) moieties. These peaks were summed to give a total AUC, which corresponded to the total concentration of compound in the original plasma sample. These AUC were compared to those obtained from the plasma standard samples for conversion to concentration. The peptide fraction absorbed (i.e. oral bioavailability; F%) was determined using Equation (2).

$$F\% = 100 \times \frac{AUC_{p.o.}}{AUC_{i.v.}} \times \frac{Dose_{i.v.}}{Dose_{p.o.}} \qquad (2)$$

Supporting Results

HPLC: DMSO (control)

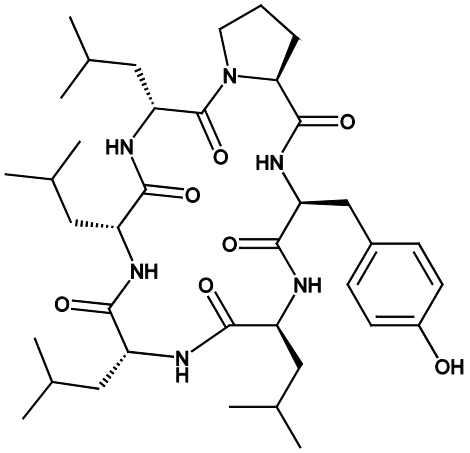
All peptides were analysed for purity by RP-HPLC. The peptides were solubilized in DMSO at approximately 0.1 mM concentration. A control HPLC trace with an injection of DMSO only has been included here as a reference.



The peptides presented in this publication were all determined to be 95% pure or greater.

The TOCSY spectra presented in this Supplementary material was acquired at 298 K in 30% (v/v) acetonitrile-d3.

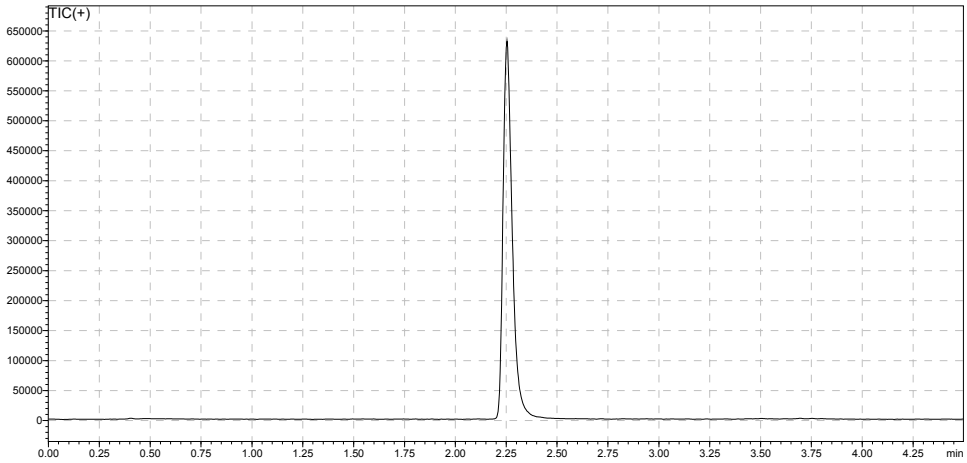
Characterization of Peptide 1



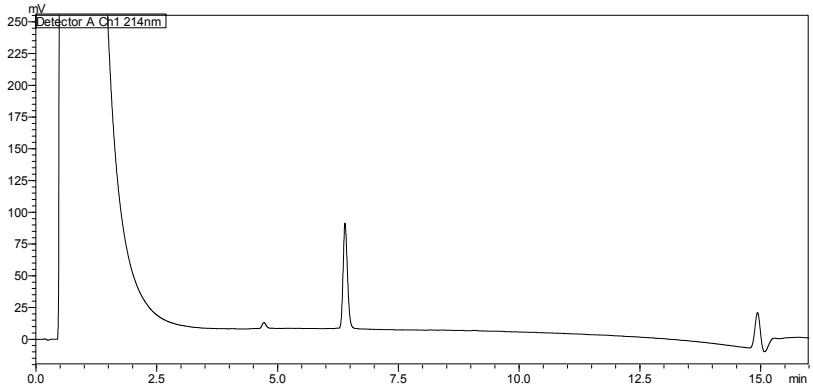
Chemical Formula: $C_{38}H_{60}N_6O_7$

Exact Mass: 712.45

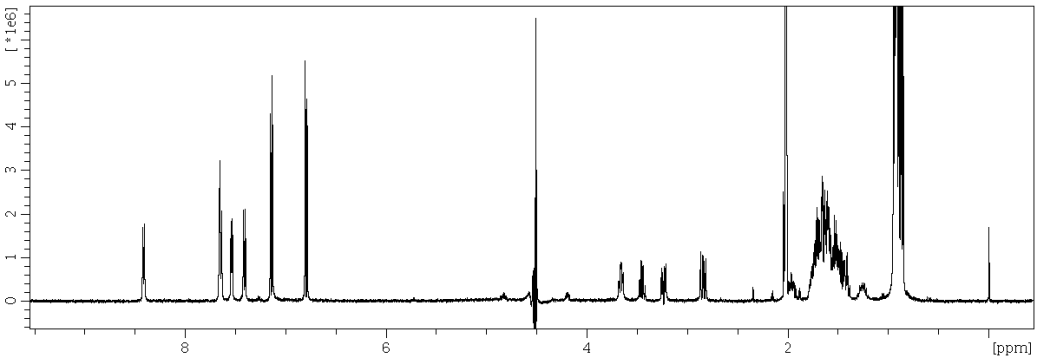
ESI-MS (*m/z*): 713.35 $[M + H]^+$



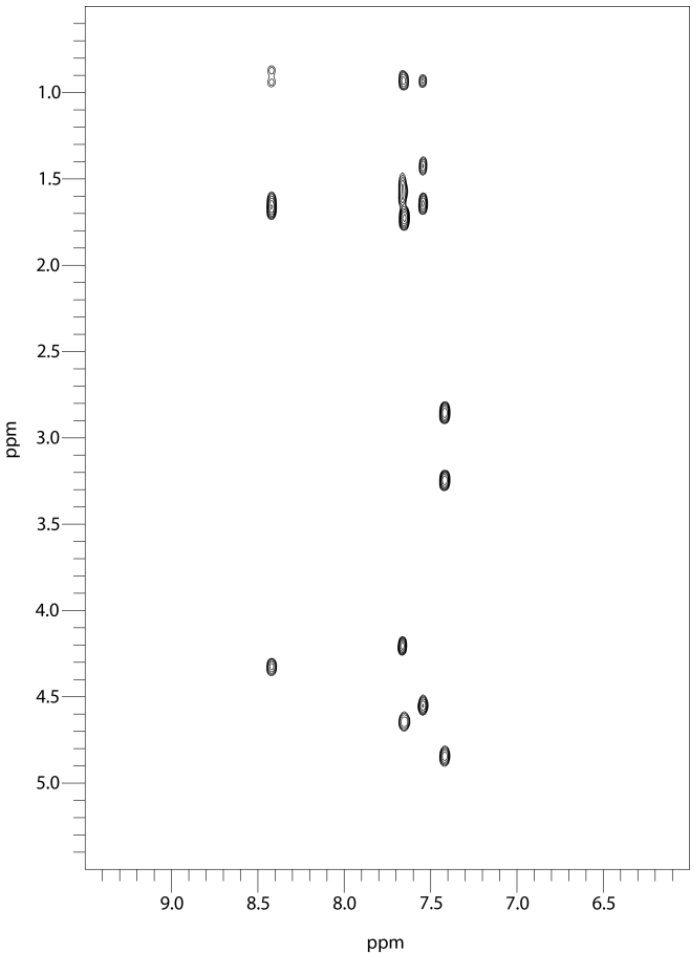
Peptide 1: ESI-MS TIC (source: PAMPA analysis)



Peptide 1: RP-HPLC trace

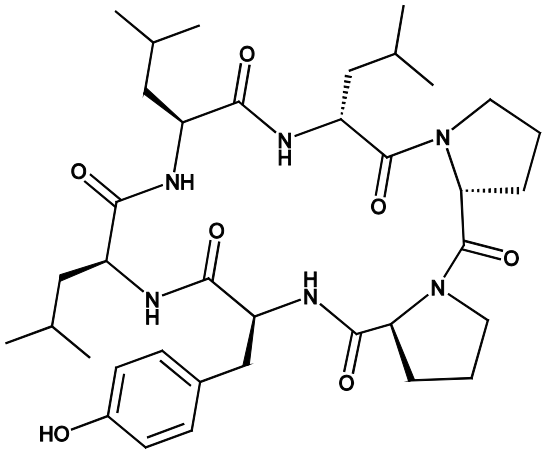


Peptide 1: ^{1}H NMR



Peptide 1: TOCSY

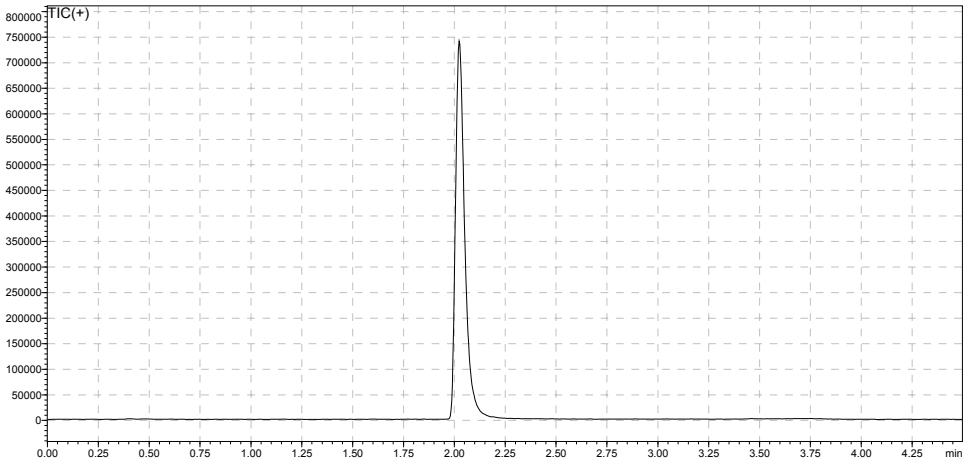
Characterization of Peptide 2



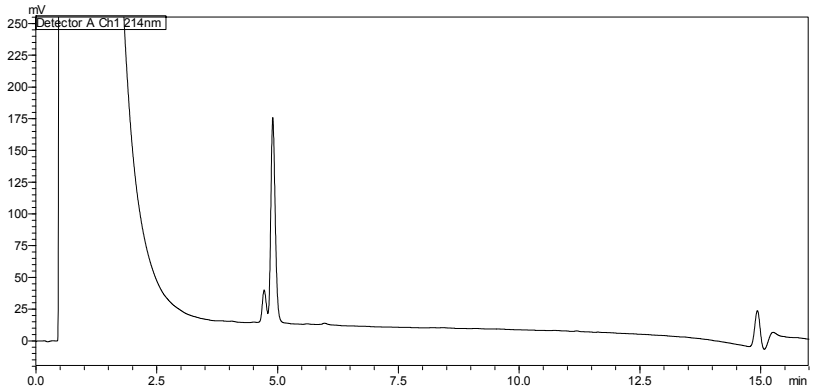
Chemical Formula: $C_{37}H_{56}N_6O_7$

Exact Mass: 696.42

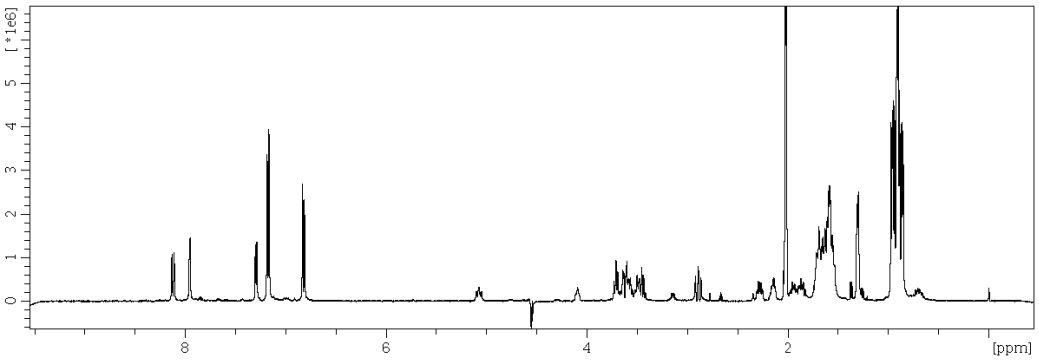
ESI-MS (*m/z*): 697.35 $[M + H]^+$



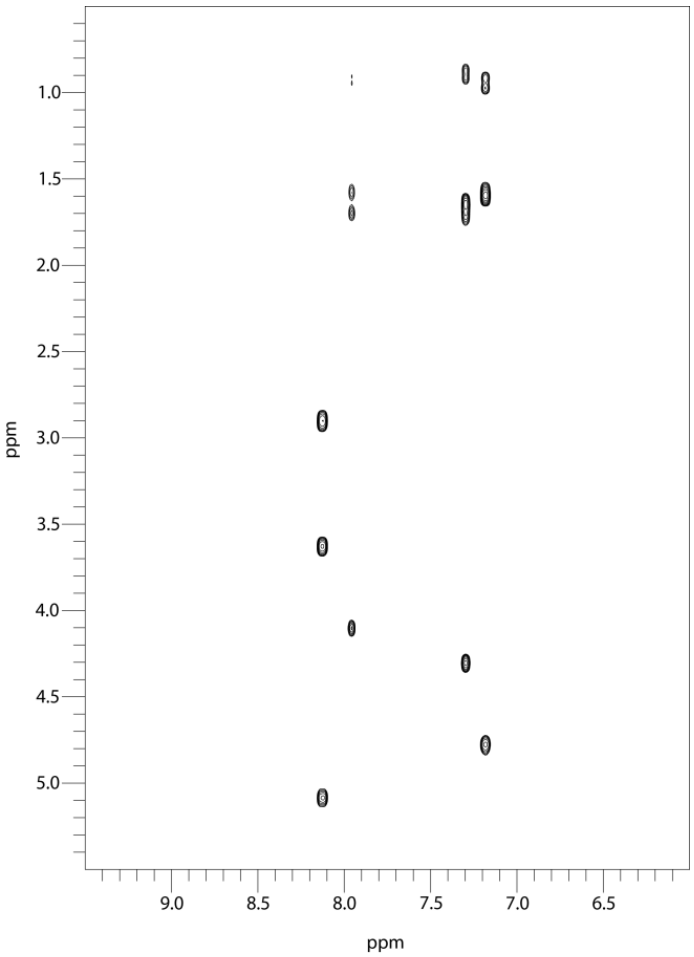
Peptide 2: ESI-MS TIC (source: PAMPA analysis)



Peptide 2: RP-HPLC trace

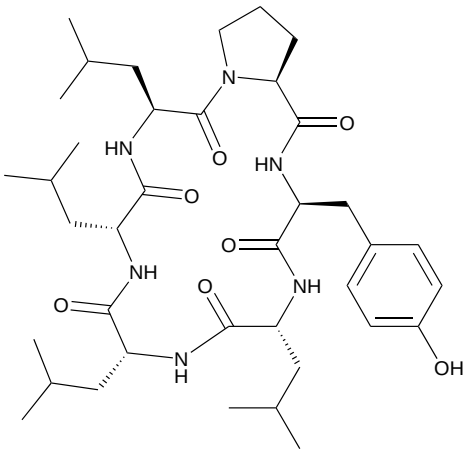


Peptide 2: ^{1}H NMR



Peptide 2: TOCSY

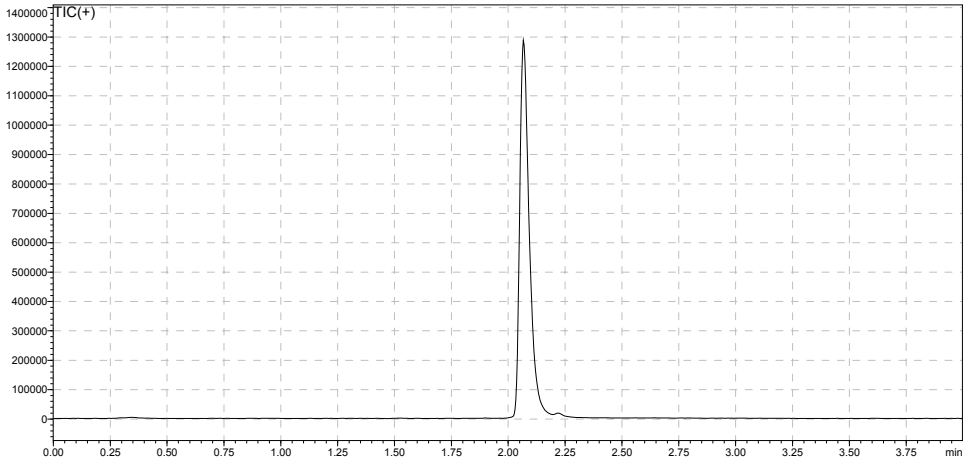
Characterization of Peptide 3



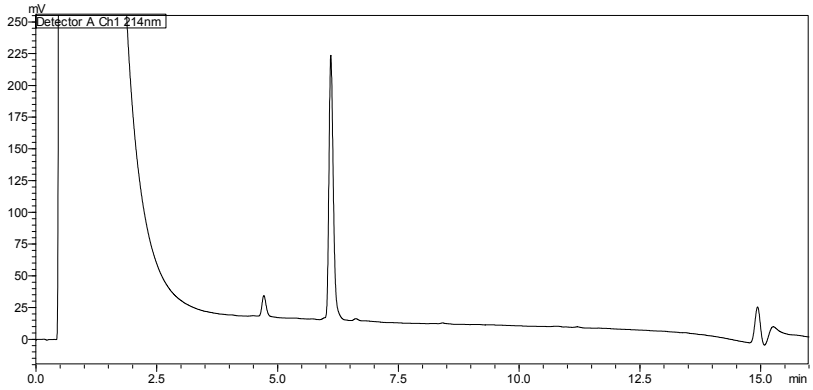
Chemical Formula: $C_{38}H_{60}N_6O_7$

Exact Mass: 712.45

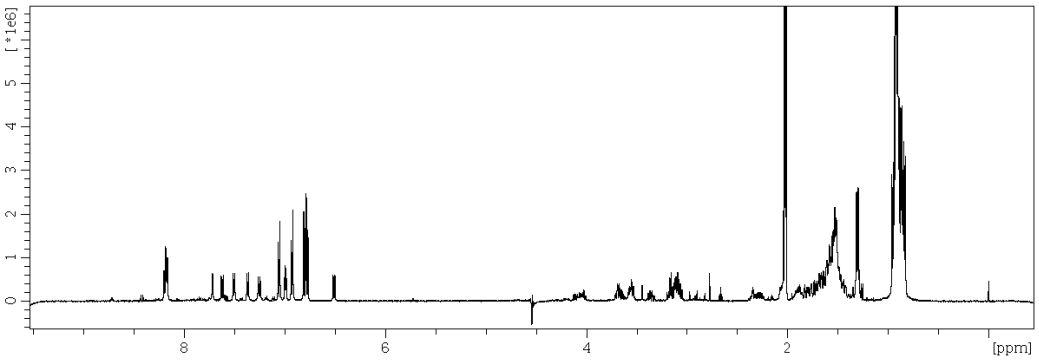
ESI-MS (*m/z*): 713.35 $[M + H]^+$



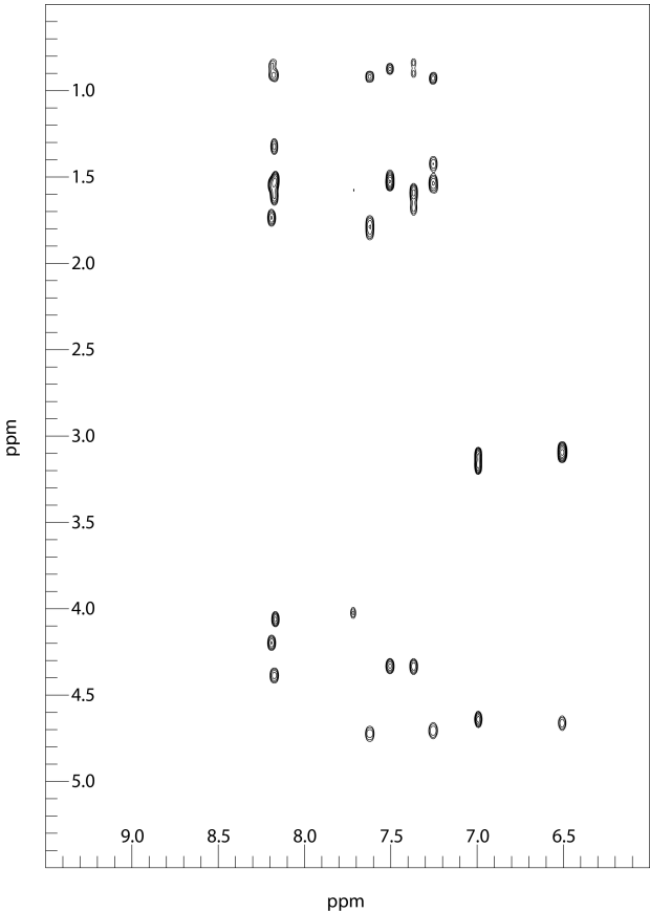
Peptide 3: ESI-MS TIC (source: PAMPA analysis)



Peptide 3: RP-HPLC trace

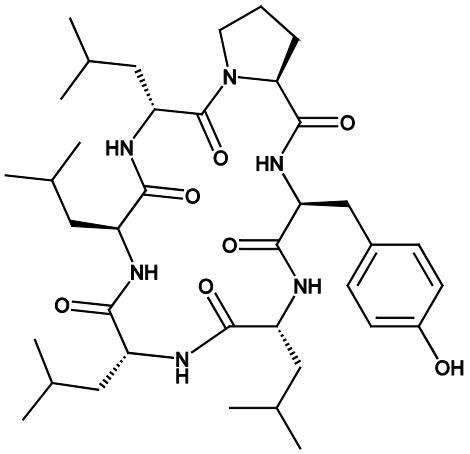


Peptide 3: ^{1}H NMR



Peptide 3: TOCSY

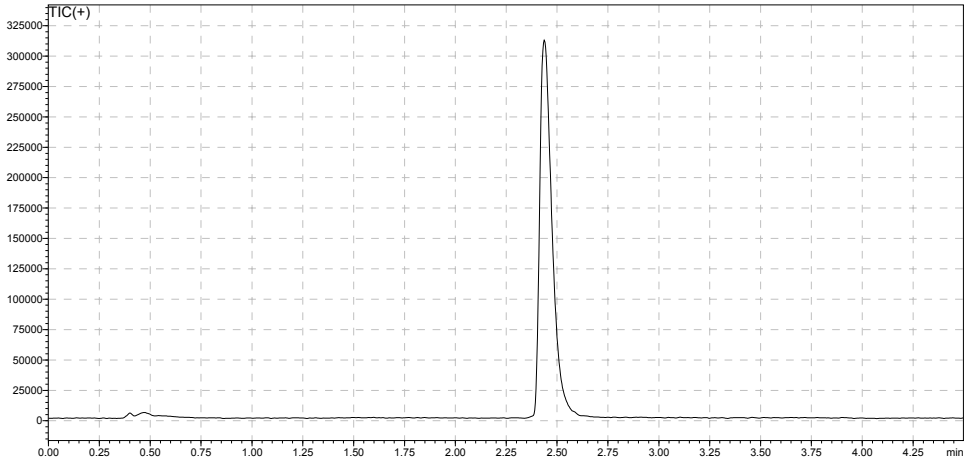
Characterization of Peptide 4



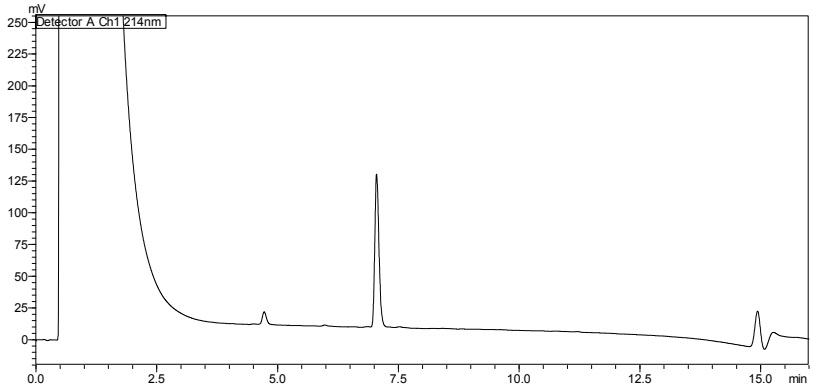
Chemical Formula: $C_{38}H_{60}N_6O_7$

Exact Mass: 712.45

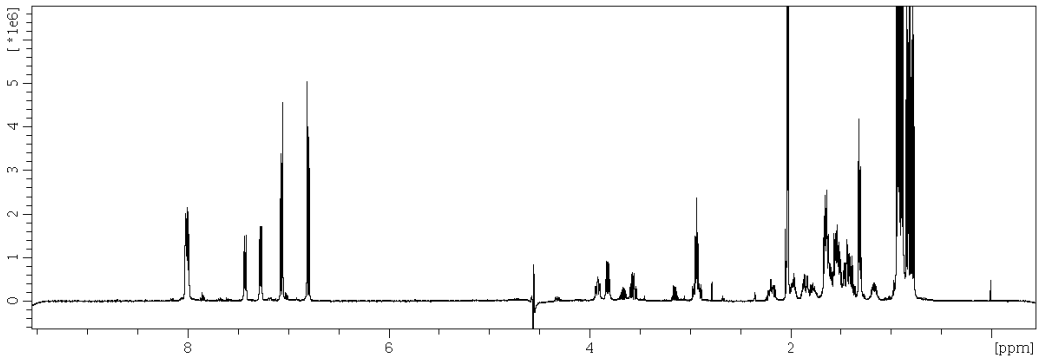
ESI-MS (*m/z*): 713.35 $[M + H]^+$



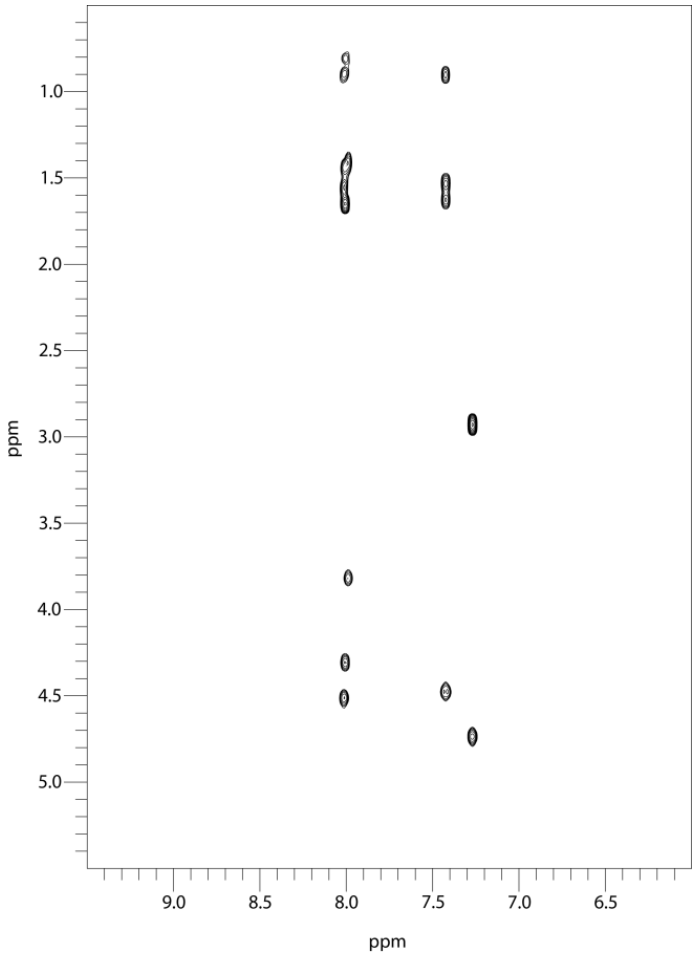
Peptide 4: ESI-MS TIC (source: PAMPA analysis)



Peptide 4: RP-HPLC trace

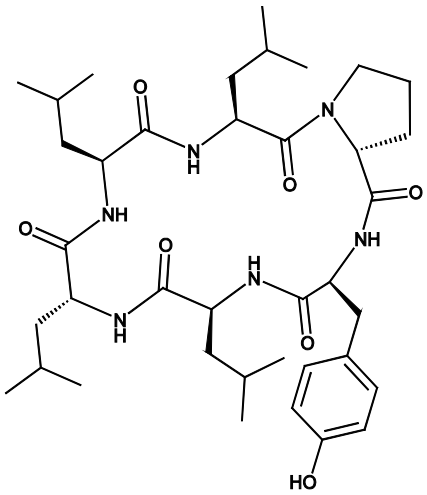


Peptide 4: ^{1}H NMR



Peptide 4: TOCSY

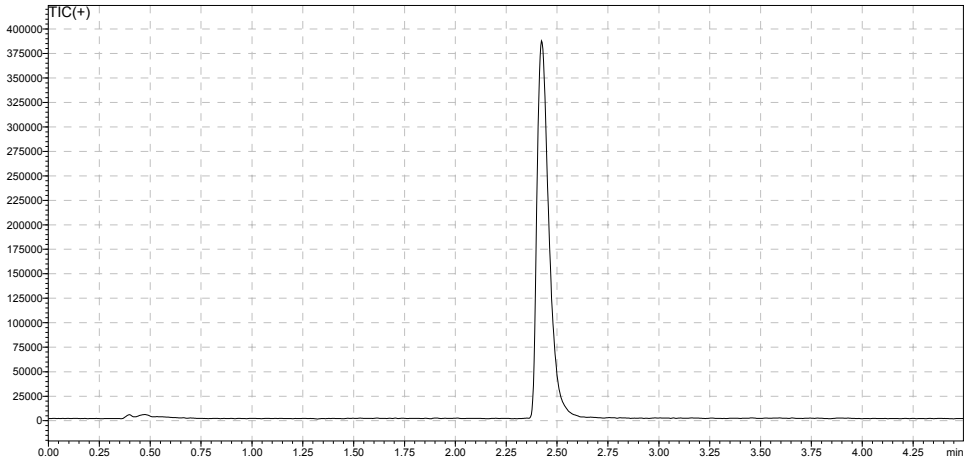
Characterization of Peptide 5



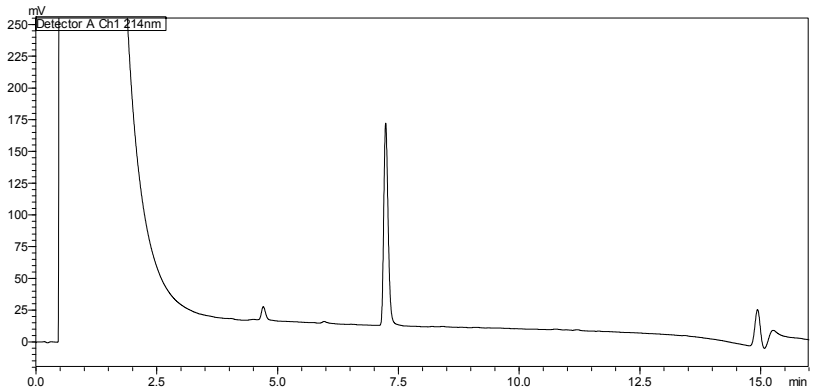
Chemical Formula: $C_{38}H_{60}N_6O_7$

Exact Mass: 712.45

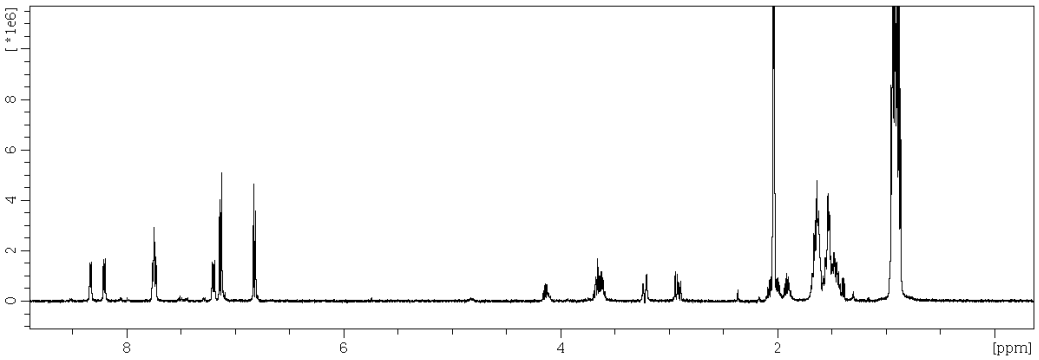
ESI-MS (*m/z*): 713.40 $[M + H]^+$



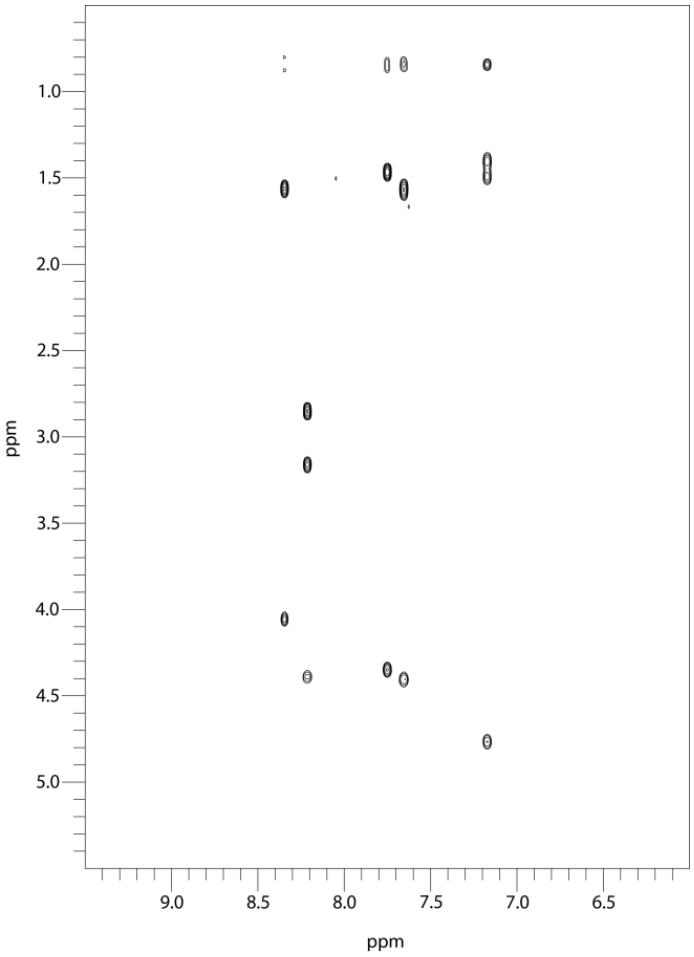
Peptide 5: ESI-MS TIC (source: PAMPA analysis)



Peptide 5: RP-HPLC trace

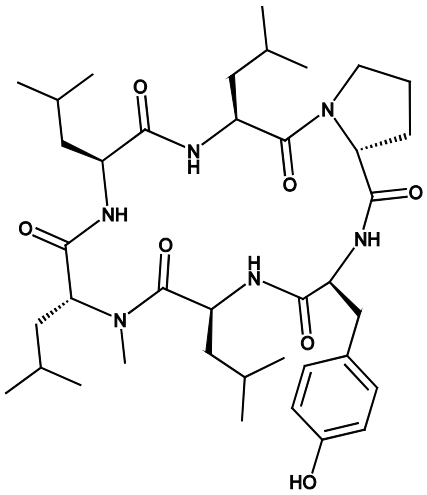


Peptide 5: ^{1}H NMR



Peptide 5: TOCSY

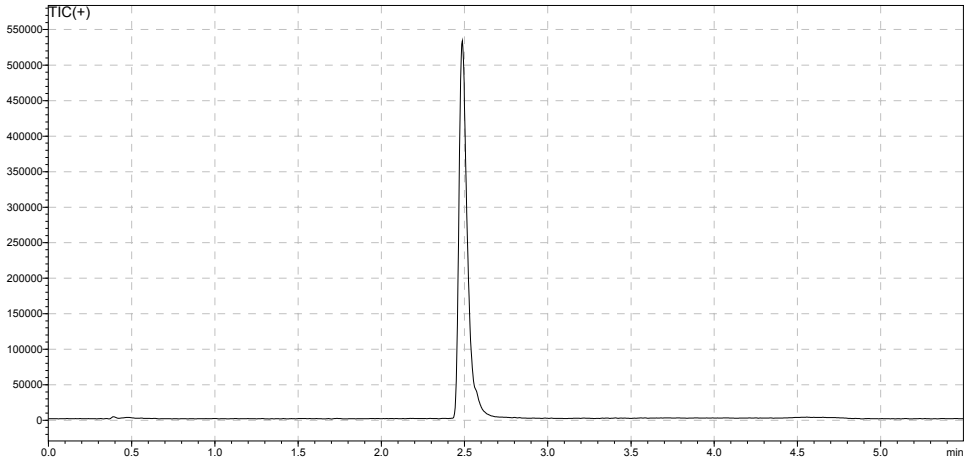
Characterization of Peptide 6



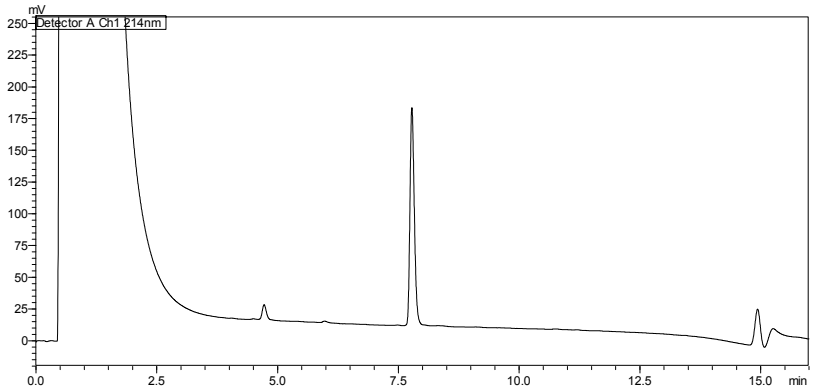
Chemical Formula: $C_{39}H_{62}N_6O_7$

Exact Mass: 726.47

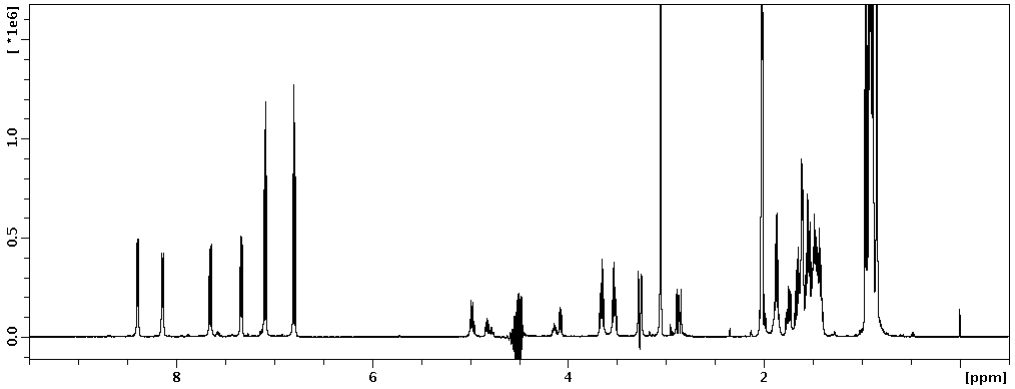
ESI-MS (*m/z*): 727.40 $[M + H]^+$



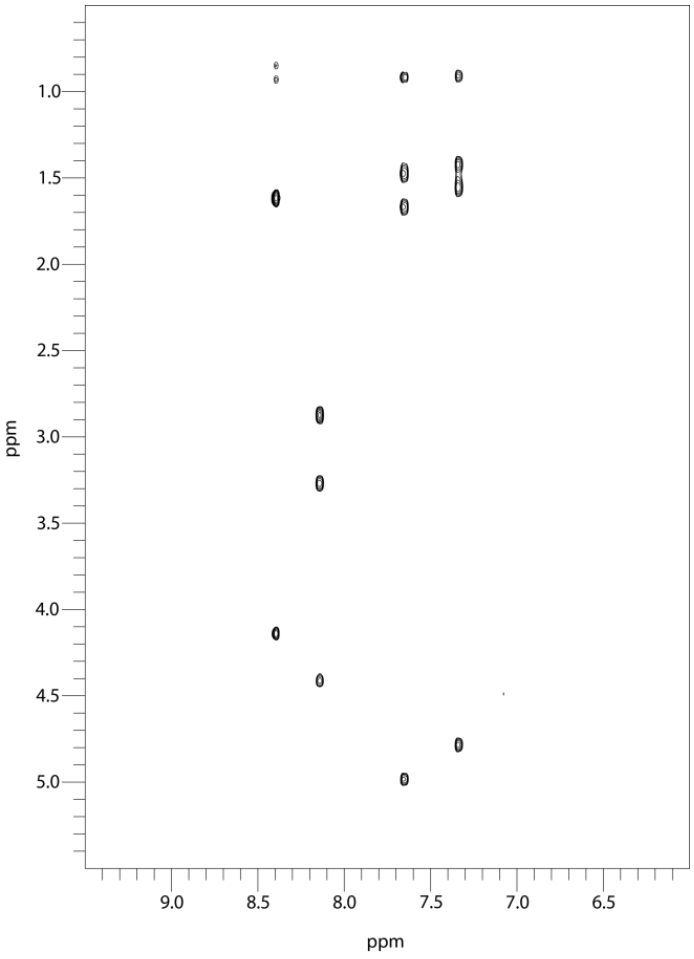
Peptide 6: ESI-MS TIC (source: PAMPA analysis)



Peptide 6: RP-HPLC trace

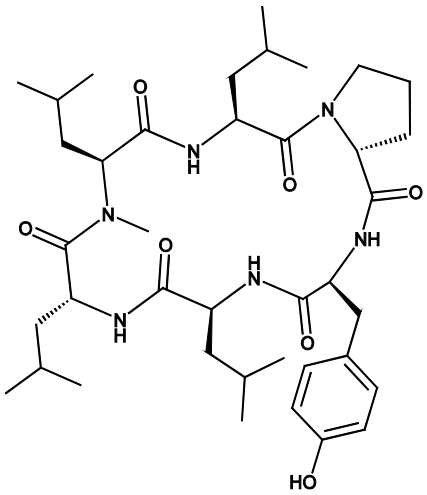


Peptide 6: ^{1}H NMR



Peptide 6: TOCSY

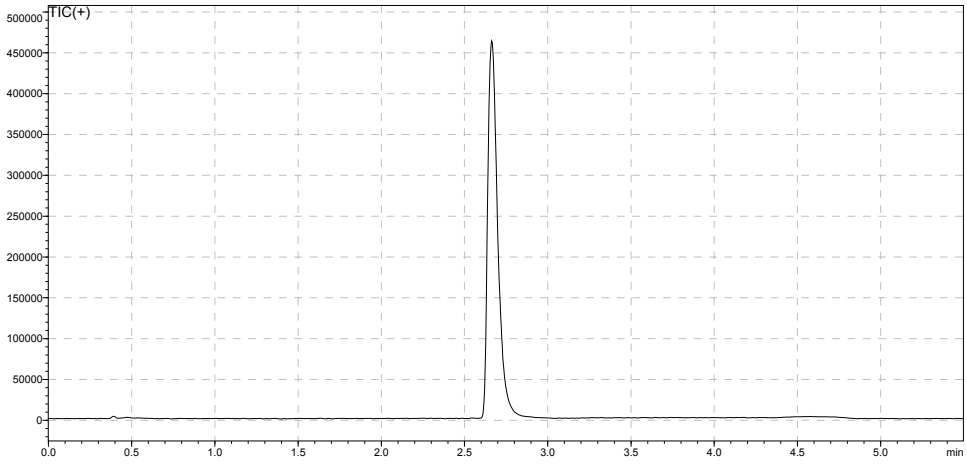
Characterization of Peptide 7



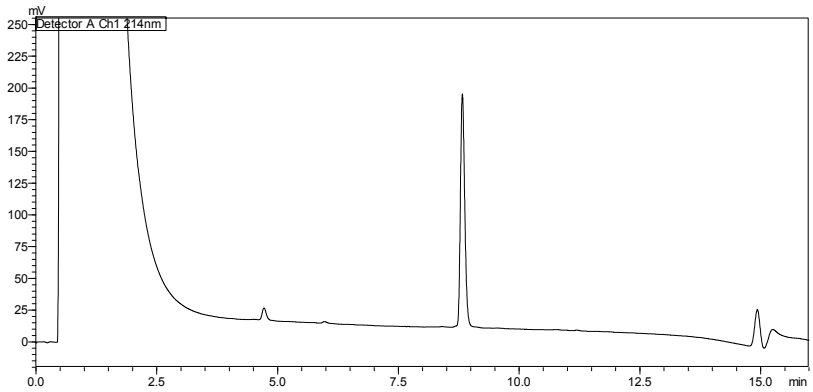
Chemical Formula: $C_{39}H_{62}N_6O_7$

Exact Mass: 726.47

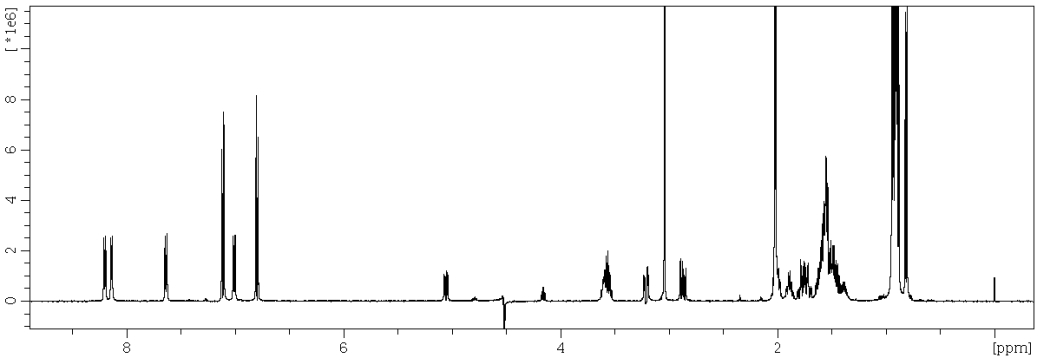
ESI-MS (*m/z*): 727.40 $[M + H]^+$



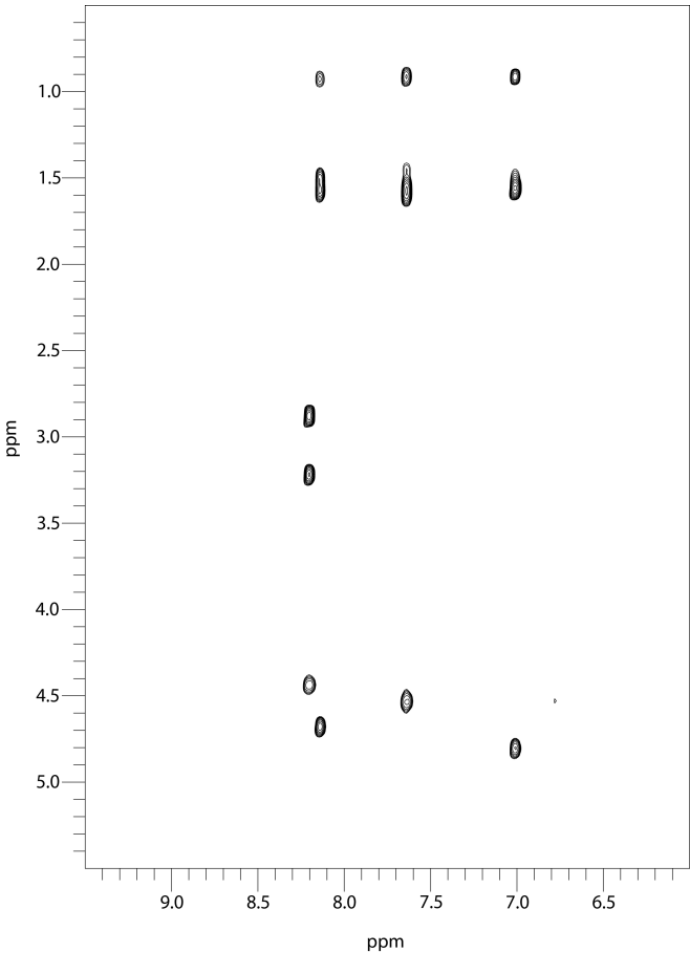
Peptide 7: ESI-MS TIC (source: PAMPA analysis)



Peptide 7: RP-HPLC trace

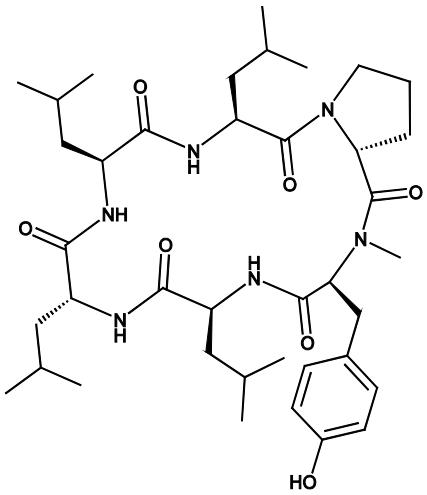


Peptide 7: ^{1}H NMR



Peptide 7: TOCSY

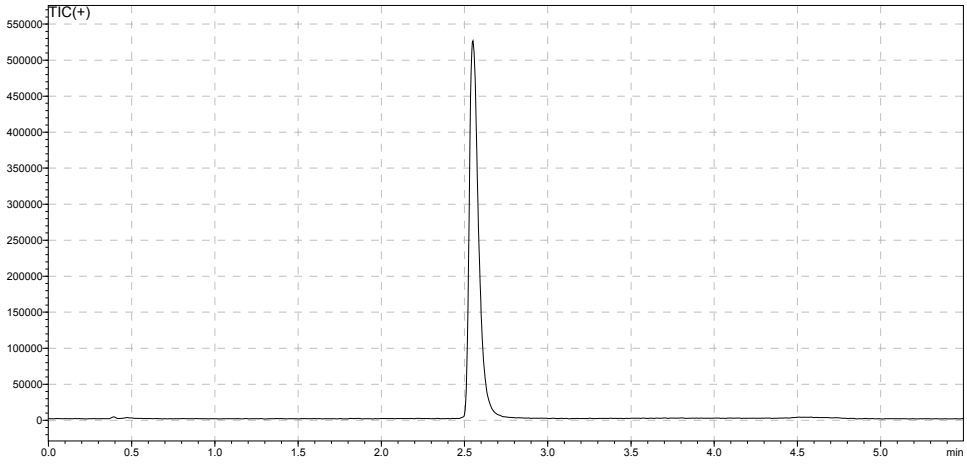
Characterization of Peptide 8



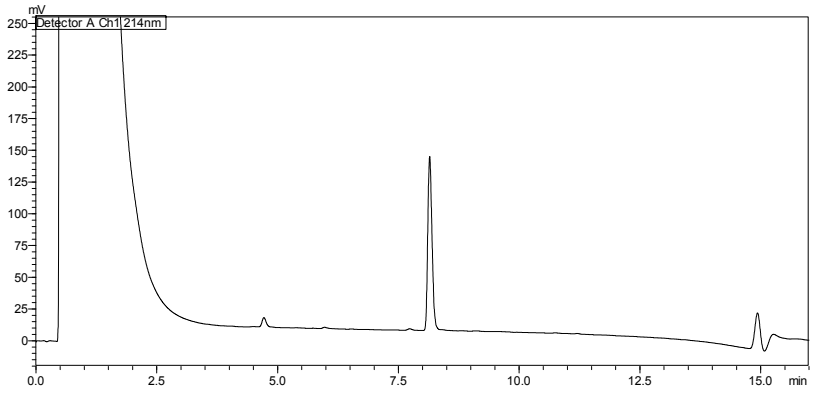
Chemical Formula: $C_{39}H_{62}N_6O_7$

Exact Mass: 726.47

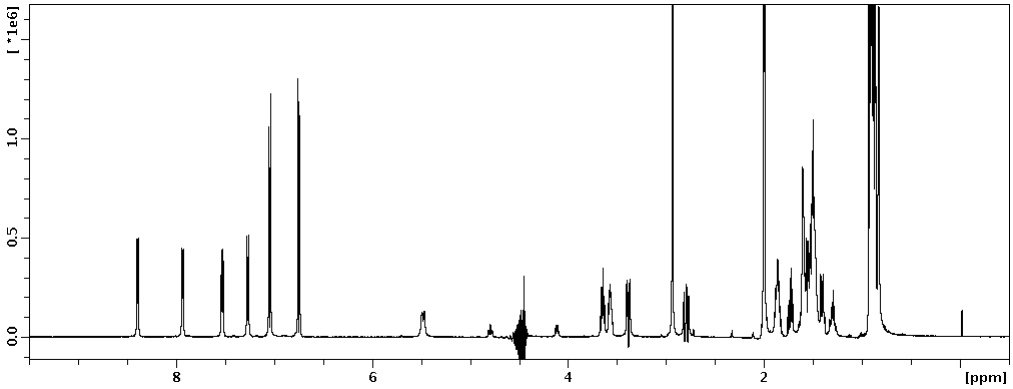
ESI-MS (*m/z*): 727.40 $[M + H]^+$



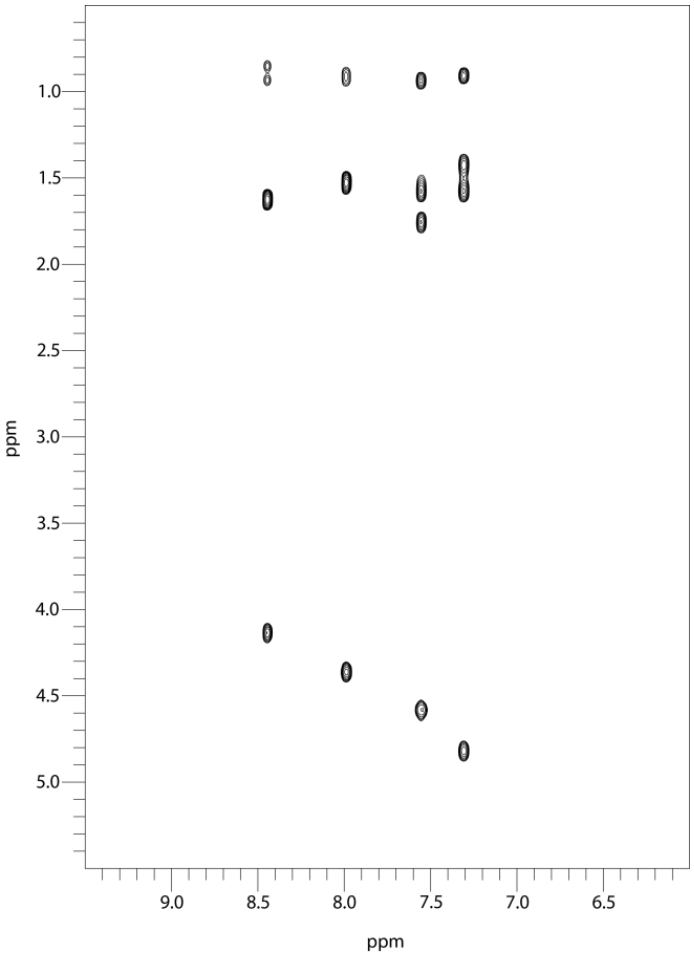
Peptide 8: ESI-MS TIC (source: PAMPA analysis)



Peptide 8: RP-HPLC trace

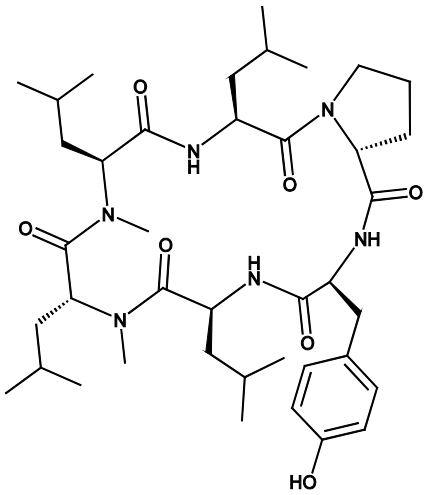


Peptide 8: ^{1}H NMR



Peptide 8: TOCSY

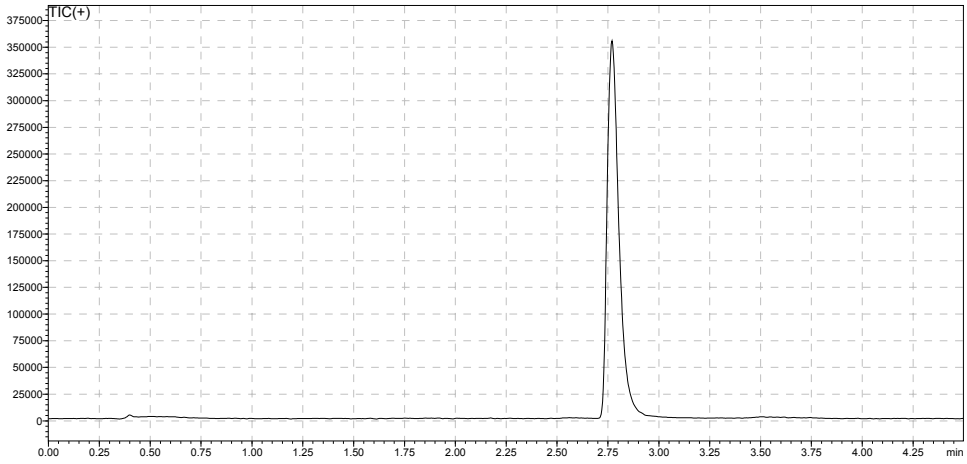
Characterization of Peptide 9



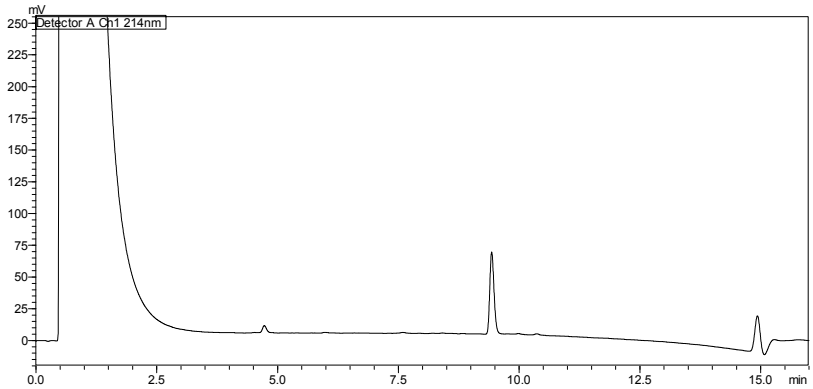
Chemical Formula: $C_{40}H_{64}N_6O_7$

Exact Mass: 740.48

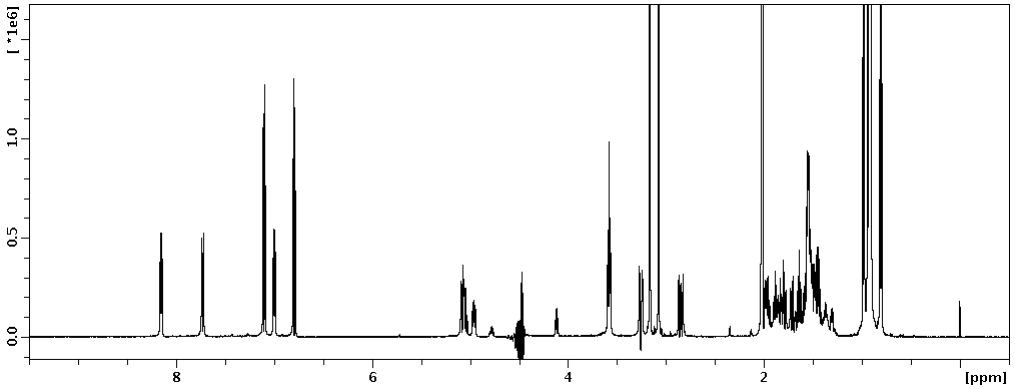
ESI-MS (*m/z*): 741.45 $[M + H]^+$



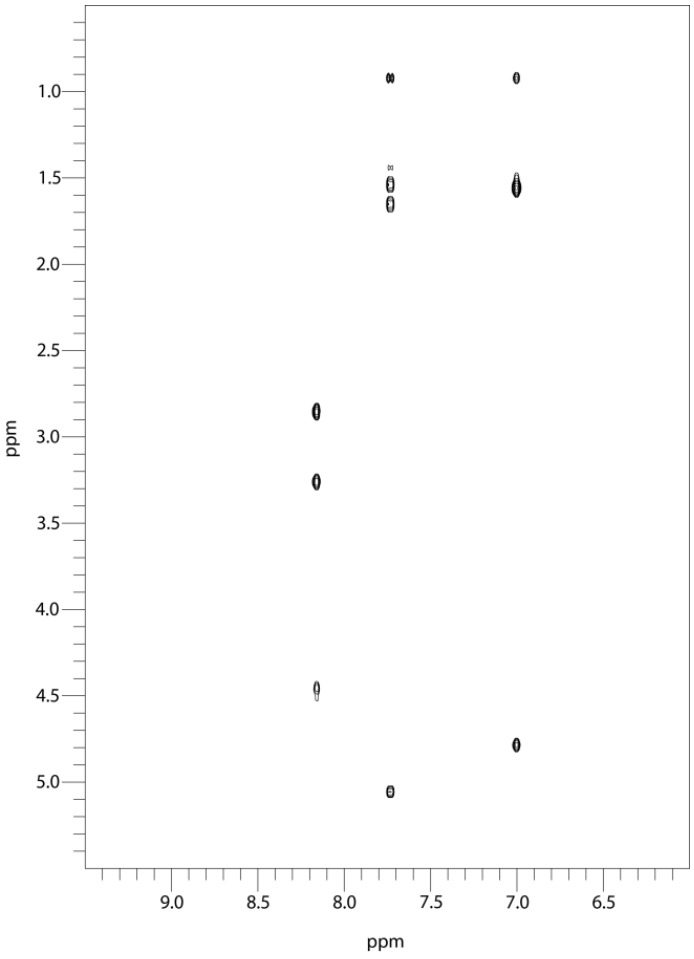
Peptide 9: ESI-MS TIC (source: PAMPA analysis)



Peptide 9: RP-HPLC trace

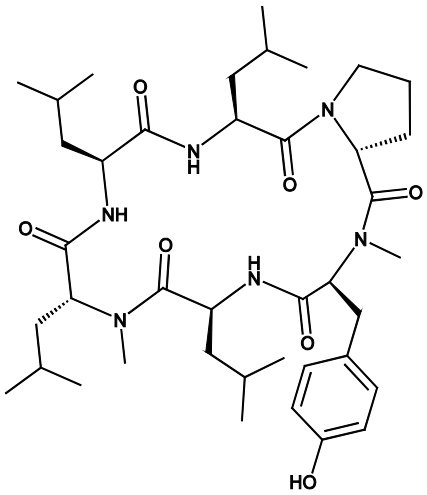


Peptide 9: ^{1}H NMR



Peptide 9: TOCSY

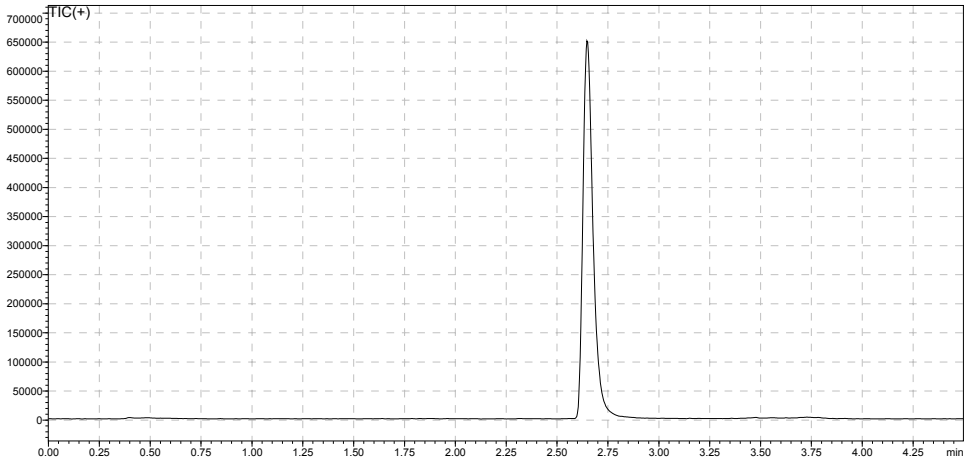
Characterization of Peptide 10



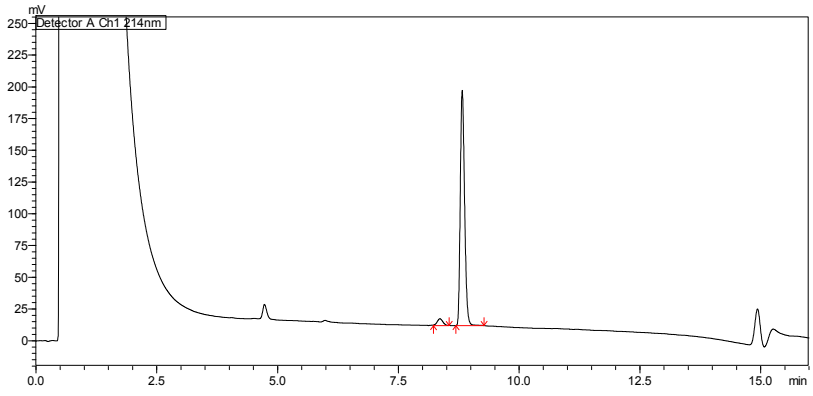
Chemical Formula: $C_{40}H_{64}N_6O_7$

Exact Mass: 740.48

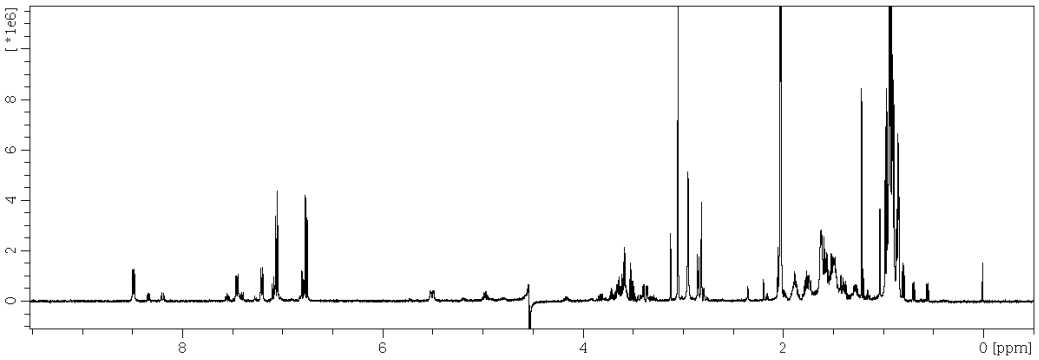
ESI-MS (*m/z*): 741.45 $[M + H]^+$



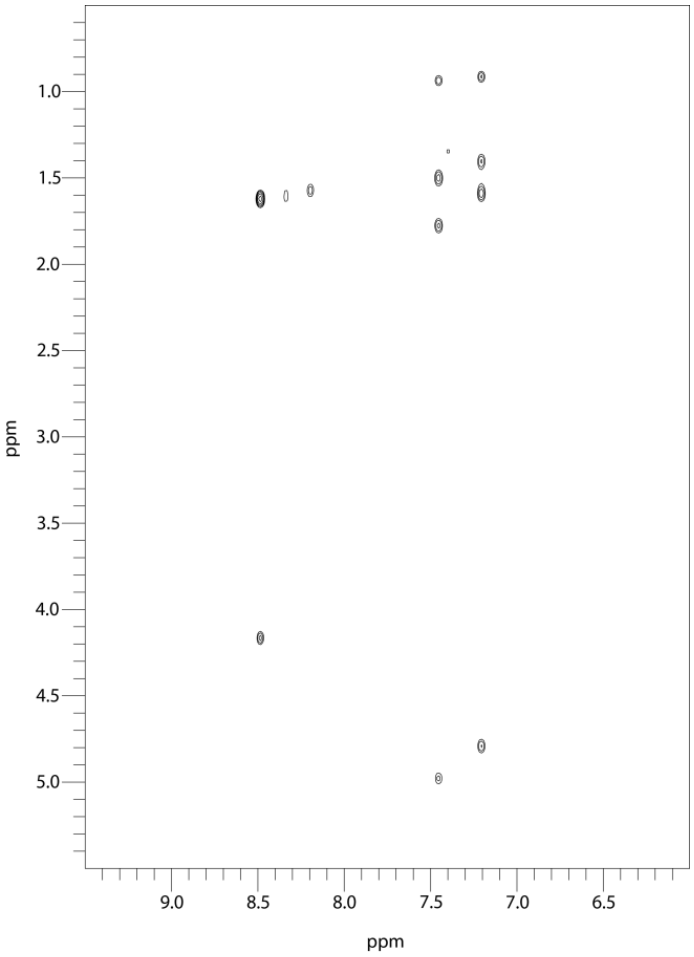
Peptide 10: ESI-MS TIC (source: PAMPA analysis)



Peptide 10: RP-HPLC trace

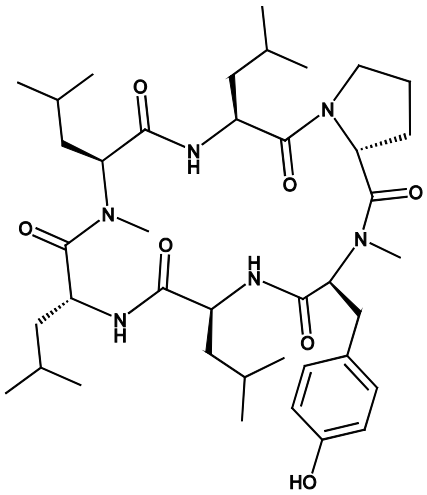


Peptide 10: ^{1}H NMR



Peptide 10: TOCSY

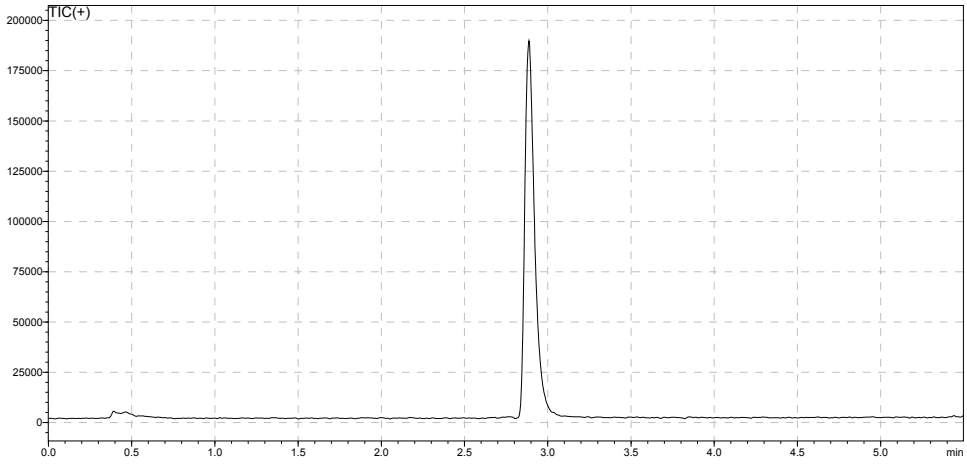
Characterization of Peptide 11



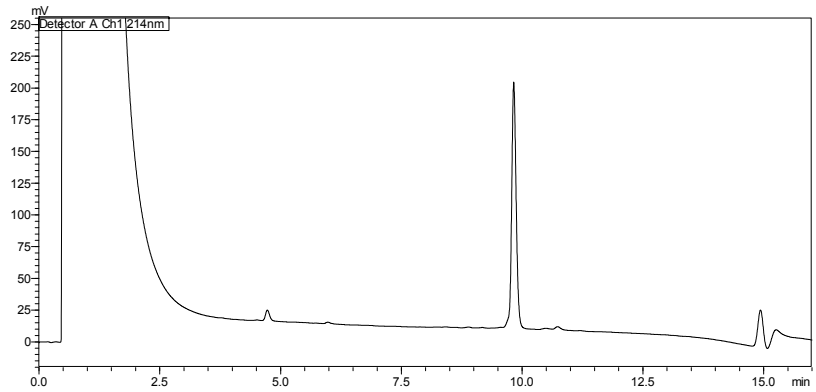
Chemical Formula: $C_{40}H_{64}N_6O_7$

Exact Mass: 740.48

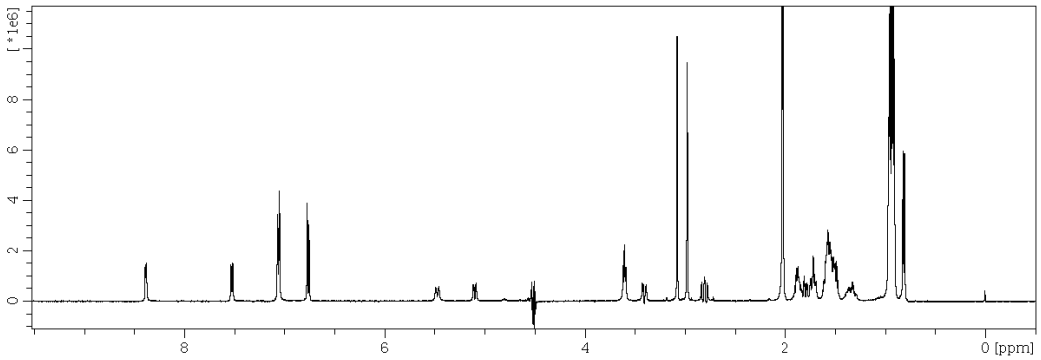
ESI-MS (*m/z*): 741.45 $[M + H]^+$



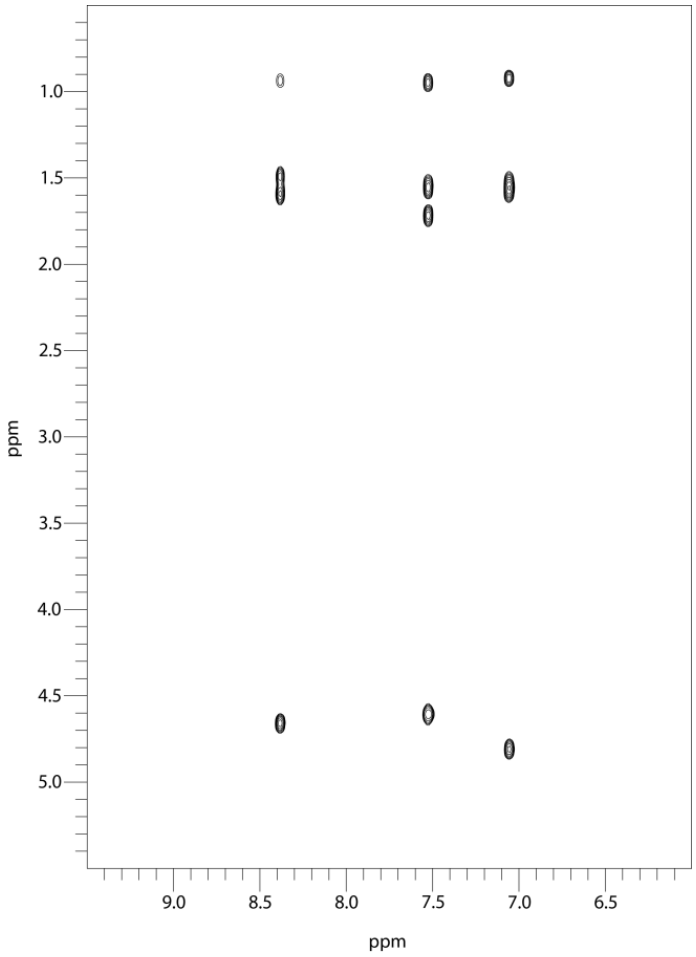
Peptide 11: ESI-MS TIC (source: PAMPA analysis)



Peptide 11: RP-HPLC trace

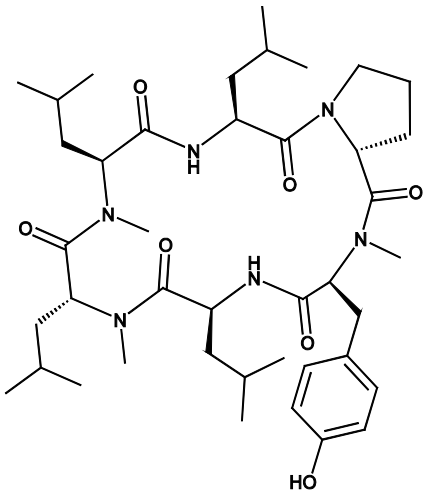


Peptide 11: ^{1}H NMR



Peptide 11: TOCSY

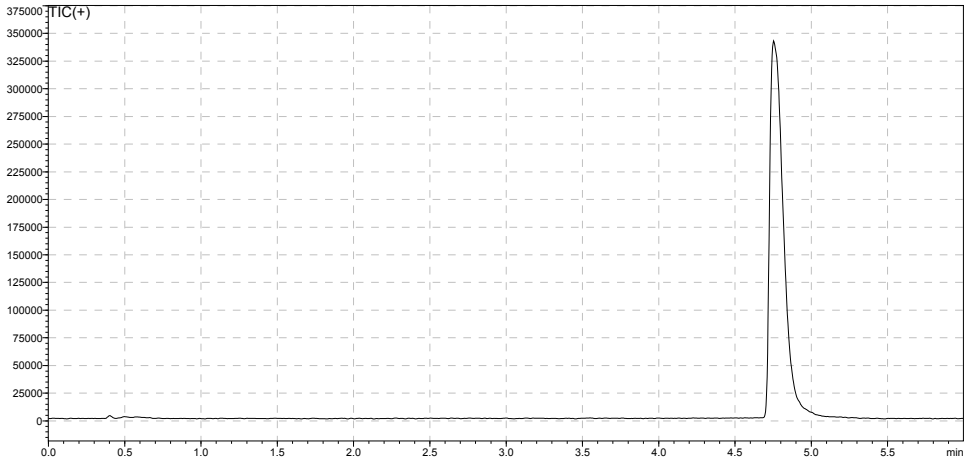
Characterization of Peptide 12



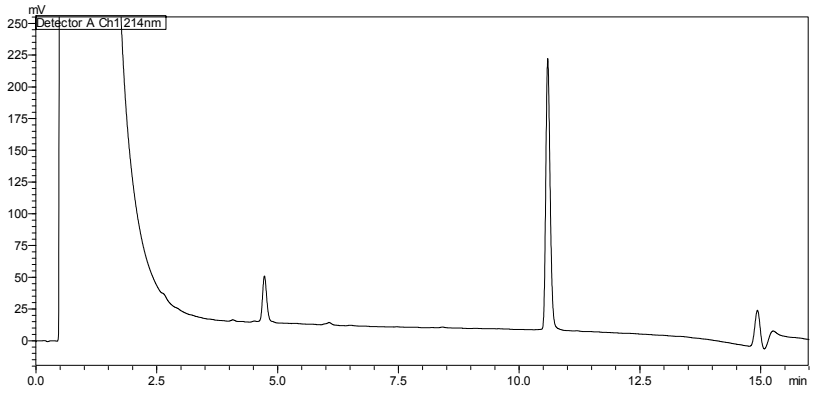
Chemical Formula: $C_{41}H_{66}N_6O_7$

Exact Mass: 754.50

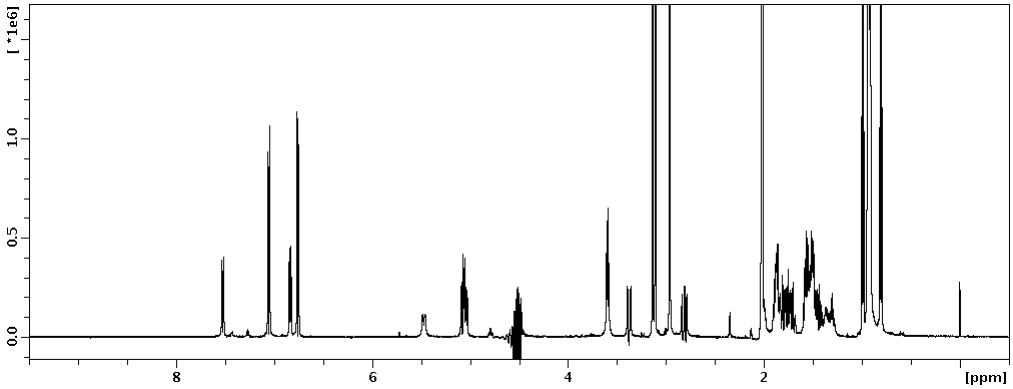
ESI-MS (*m/z*): 755.45 $[M + H]^+$



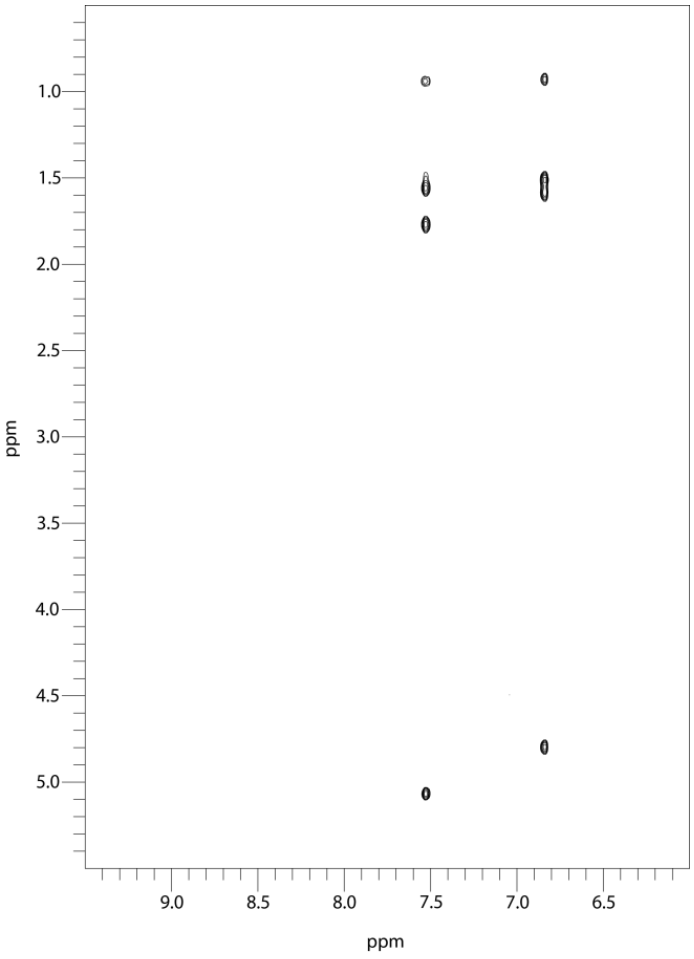
Peptide 12: ESI-MS TIC (source: PAMPA analysis)



Peptide 12: RP-HPLC trace

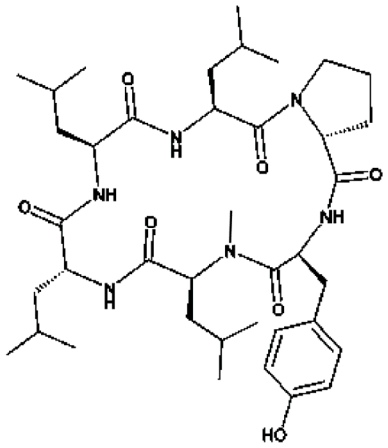


Peptide 12: ^{1}H NMR



Peptide 12: TOCSY

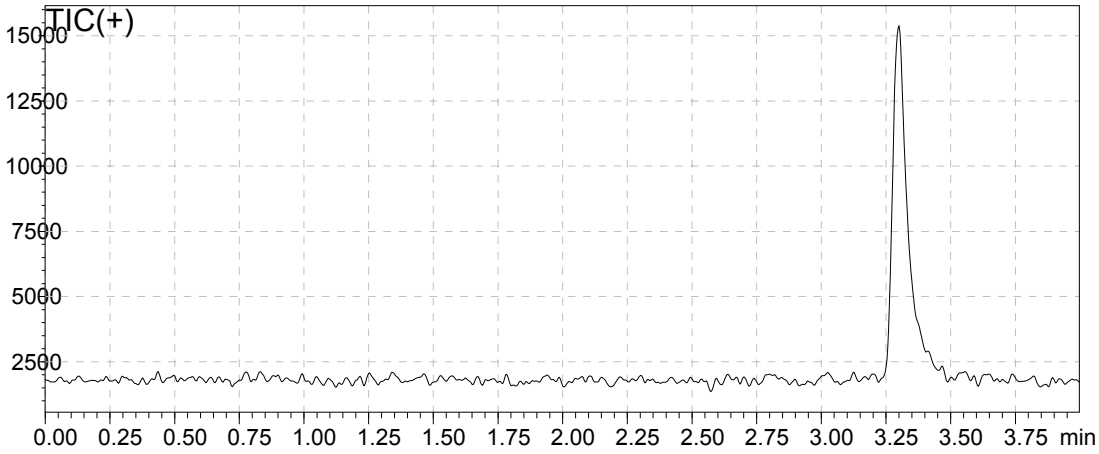
Characterization of Peptide 13



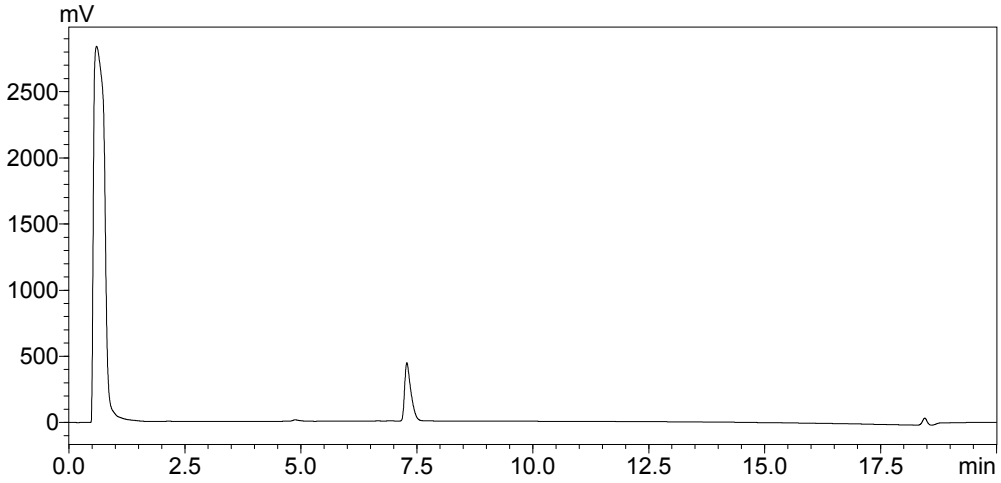
Chemical Formula: $C_{39}H_{62}N_6O_7$

Exact Mass: 726.47

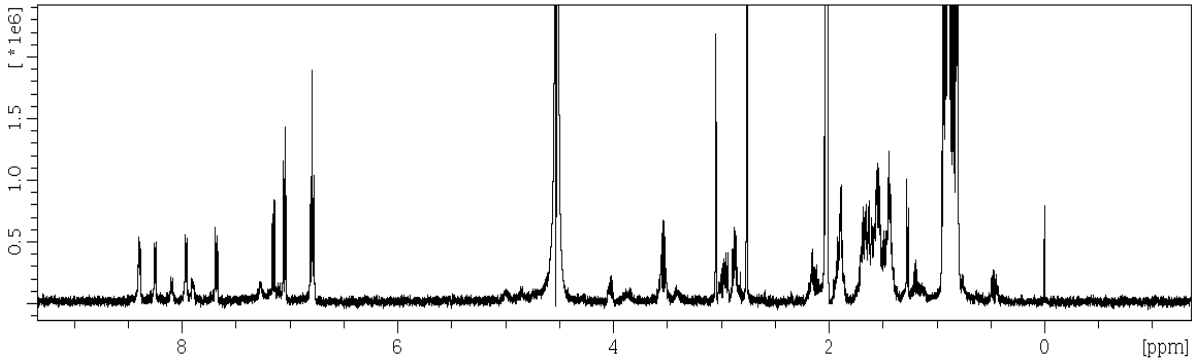
ESI-MS (*m/z*): 727.40 $[M + H]^+$



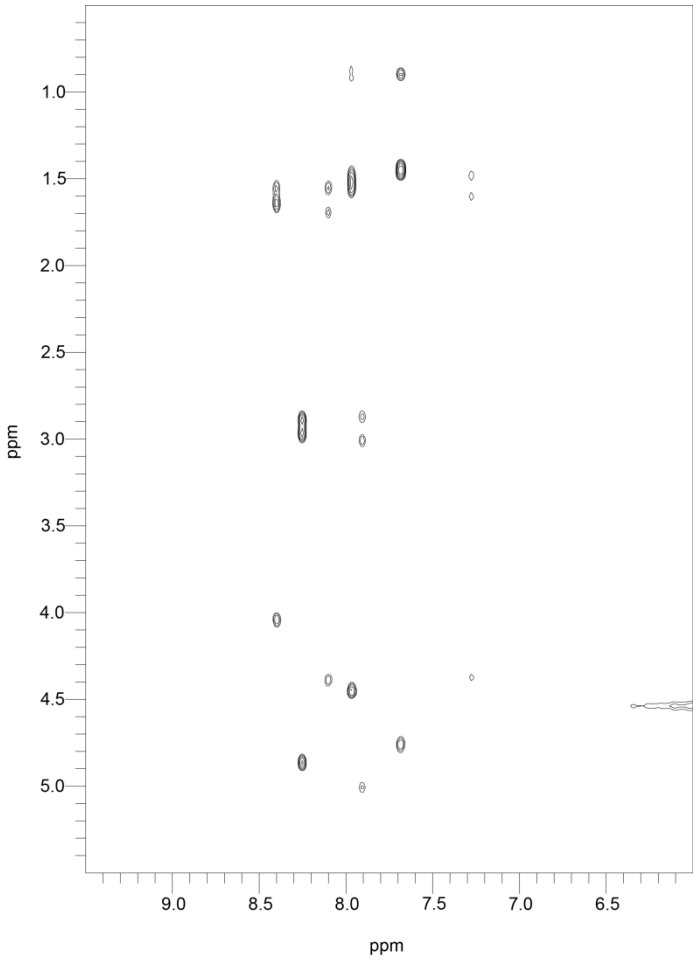
Peptide 13: ESI-MS TIC (source: Caco-2 analysis)



Peptide 13: RP-HPLC trace

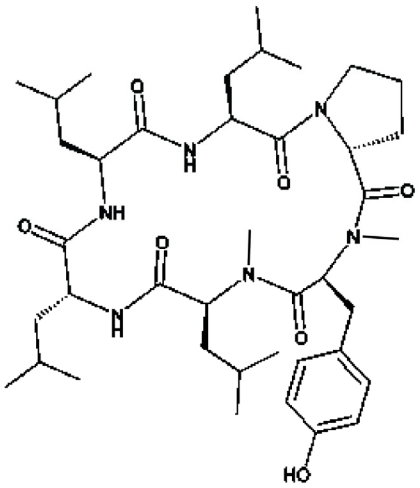


Peptide 13: ^{1}H NMR



Peptide 13: TOCSY

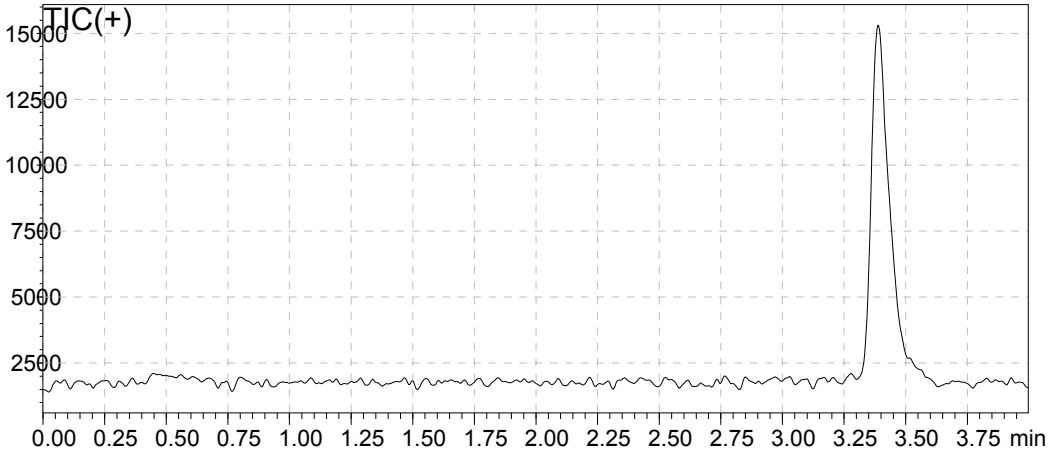
Characterization of Peptide 14



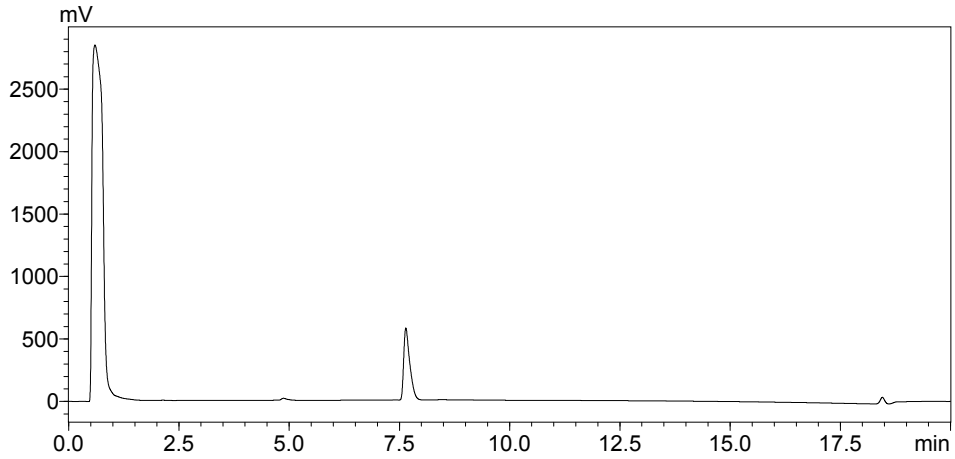
Chemical Formula: $C_{40}H_{64}N_6O_7$

Exact Mass: 740.48

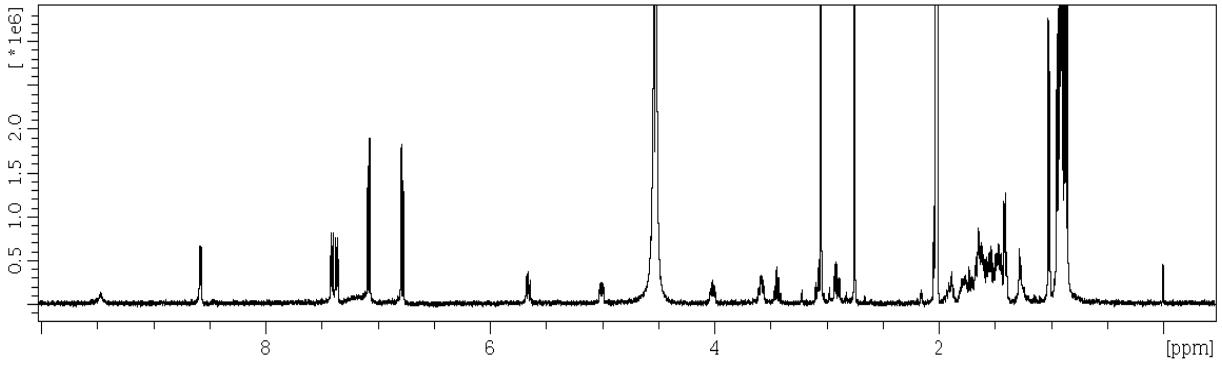
ESI-MS (*m/z*): 741.45 $[M + H]^+$



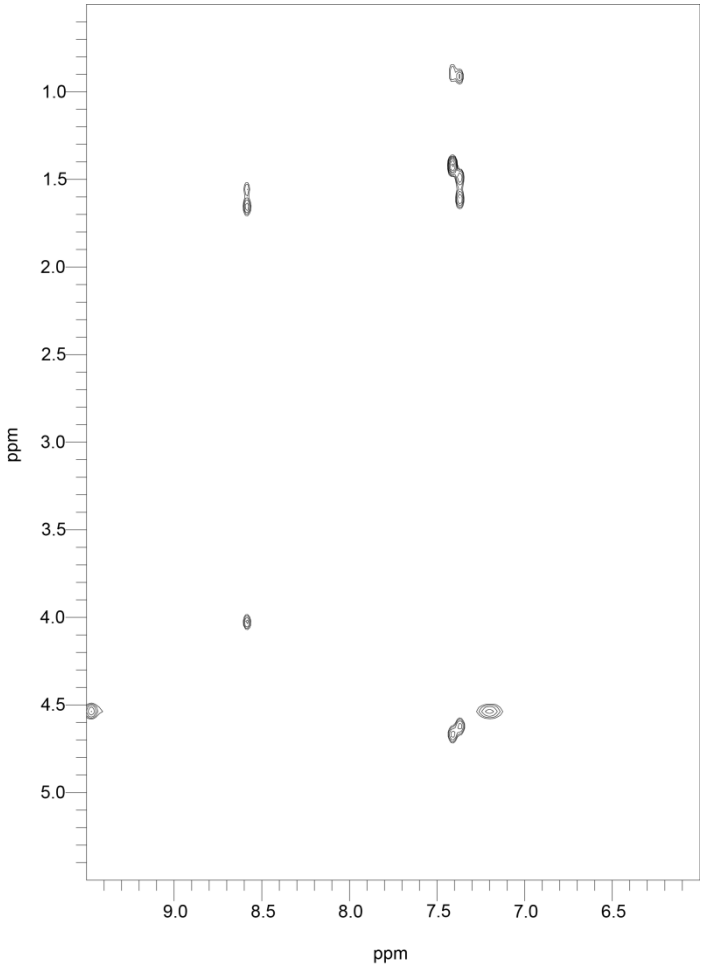
Peptide 14: ESI-MS TIC (source: Caco-2 analysis)



Peptide 14: RP-HPLC trace

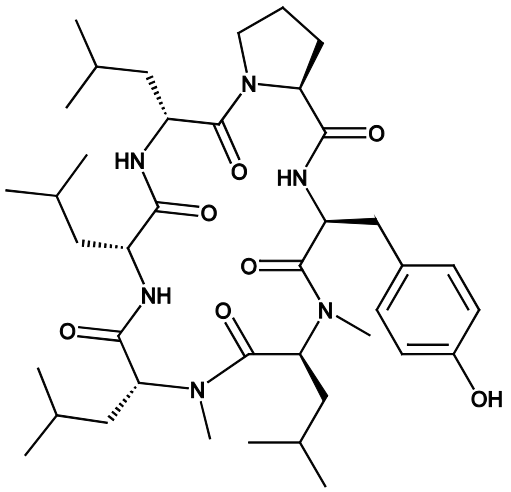


Peptide 14: ^{1}H NMR



Peptide 14: TOCSY

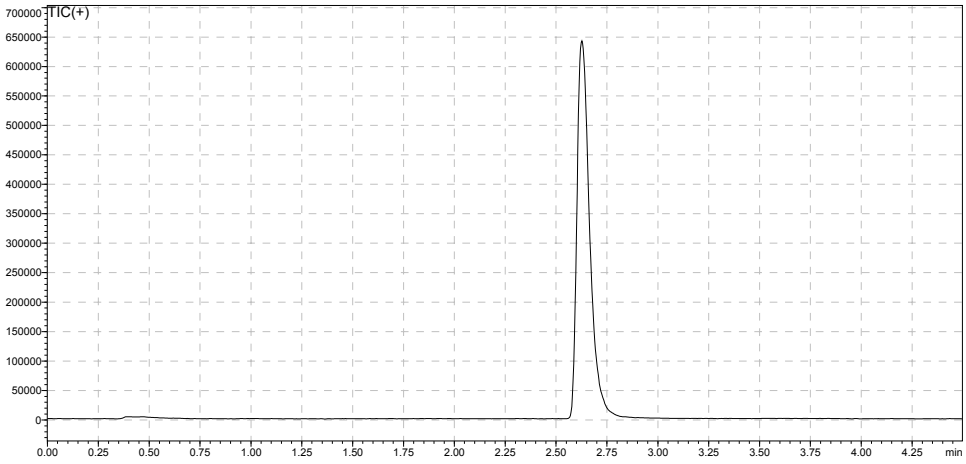
Characterization of Peptide 15



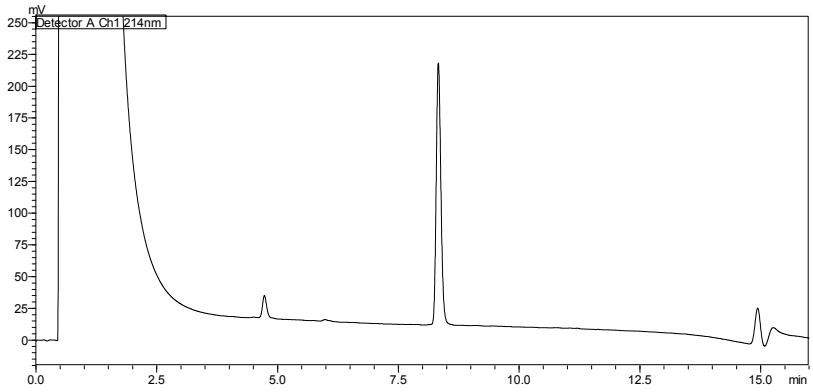
Chemical Formula: $C_{40}H_{64}N_6O_7$

Exact Mass: 740.48

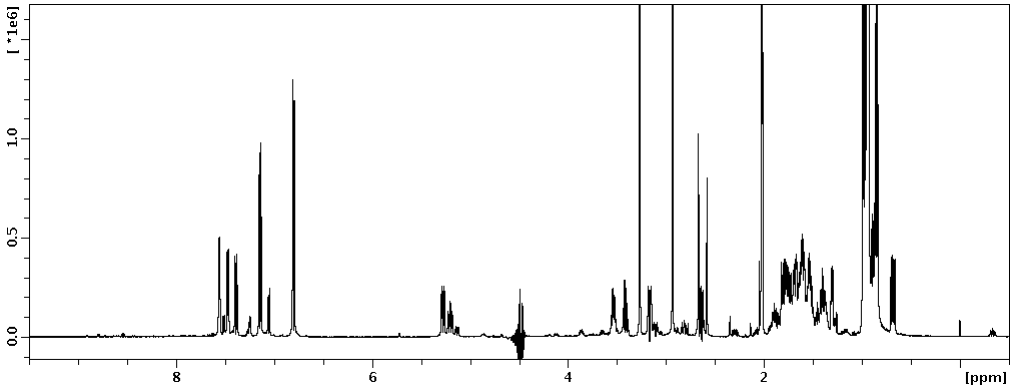
ESI-MS (*m/z*): 741.40 $[M + H]^+$



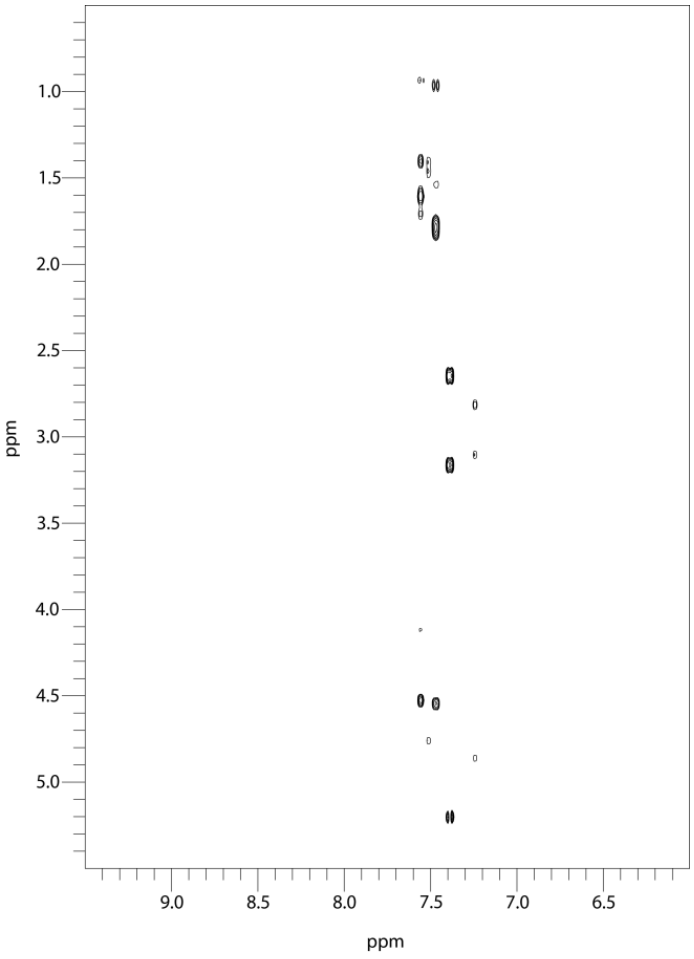
Peptide 15: ESI-MS TIC (source: PAMPA analysis)



Peptide 15: RP-HPLC trace

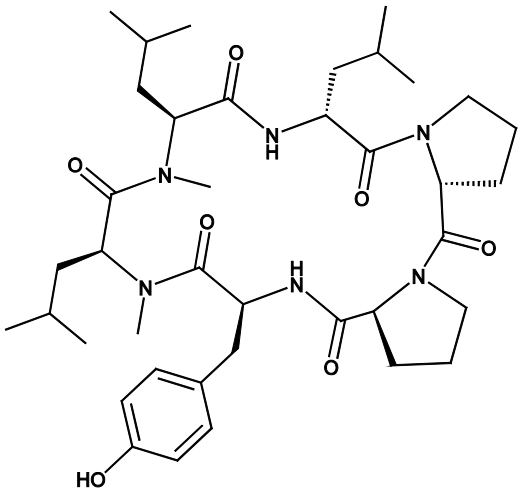


Peptide 15: ^{1}H NMR



Peptide 15: TOCSY

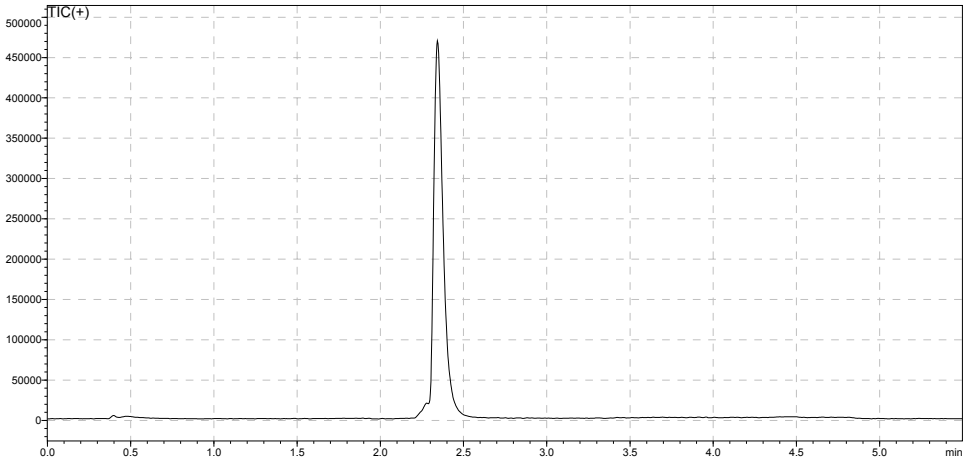
Characterization of Peptide 16



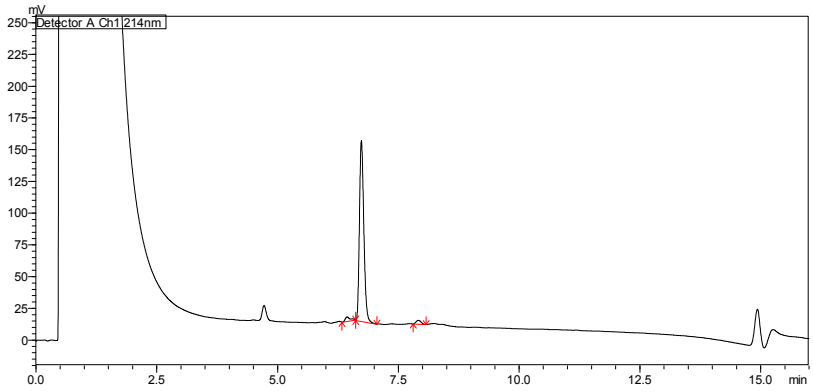
Chemical Formula: $C_{39}H_{60}N_6O_7$

Exact Mass: 724.45

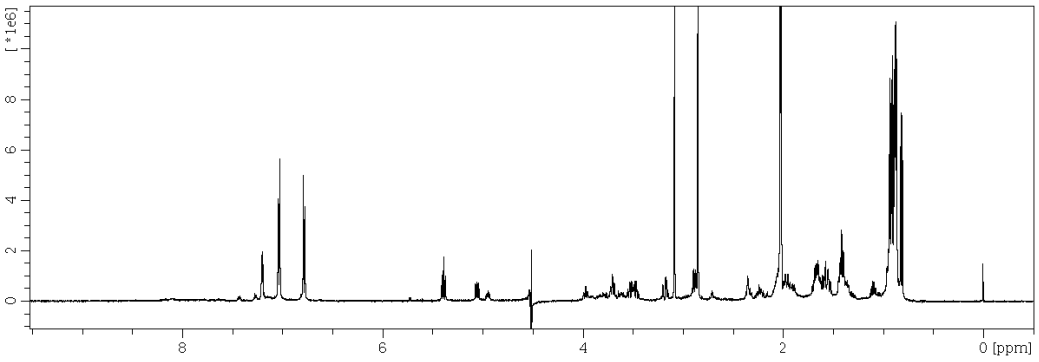
ESI-MS (*m/z*): 725.35 $[M + H]^+$



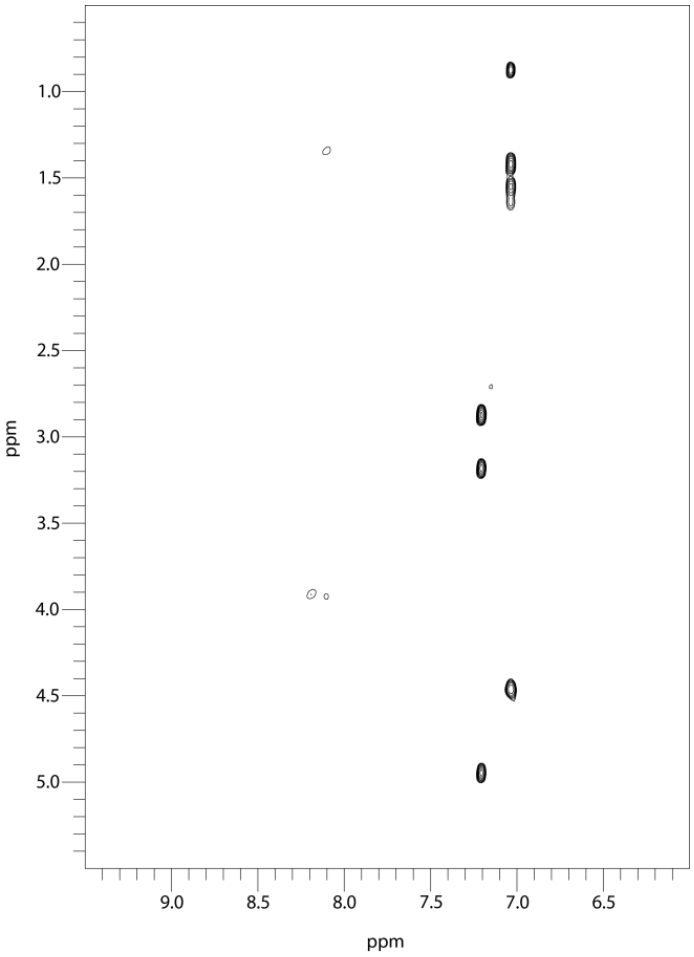
Peptide 16: ESI-MS TIC (source: PAMPA analysis)



Peptide 16: RP-HPLC trace

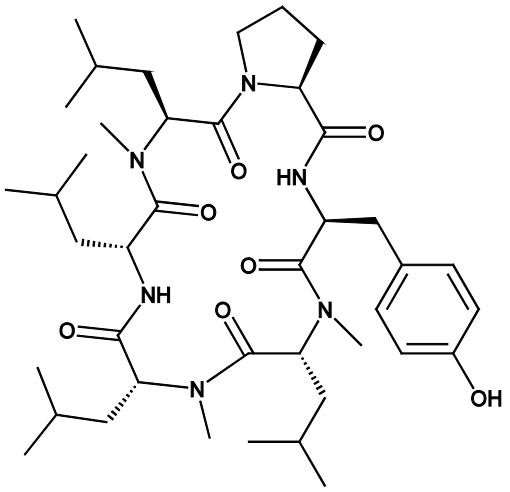


Peptide 16: ^{1}H NMR



Peptide 16: TOCSY

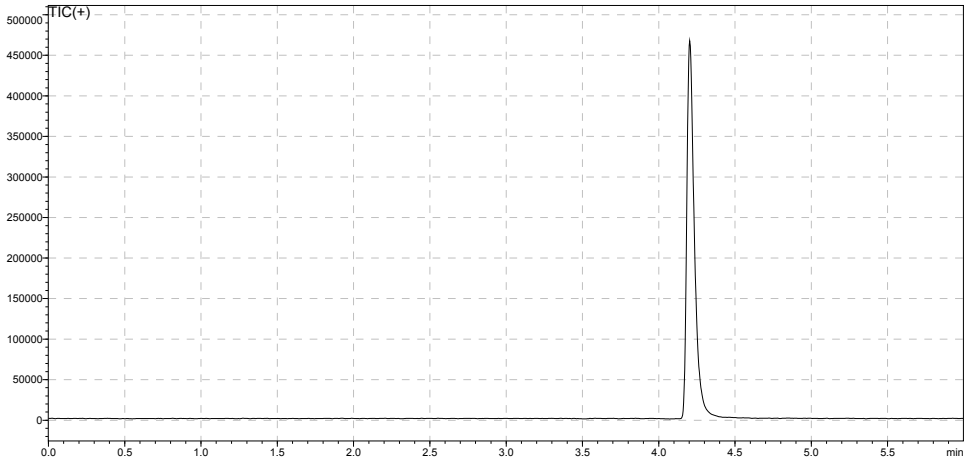
Characterization of Peptide 17



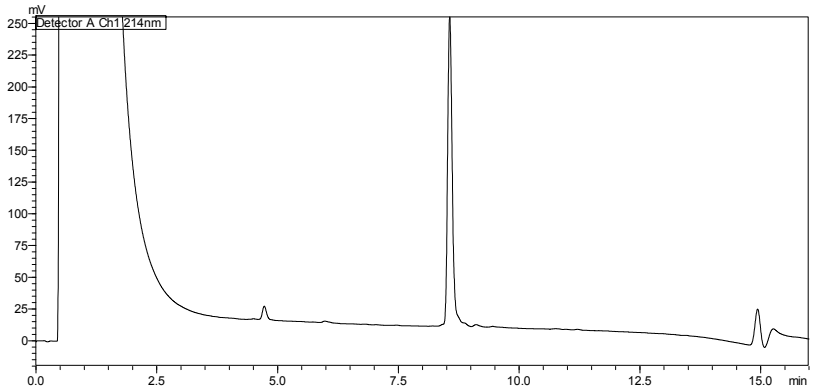
Chemical Formula: $C_{41}H_{66}N_6O_7$

Exact Mass: 754.50

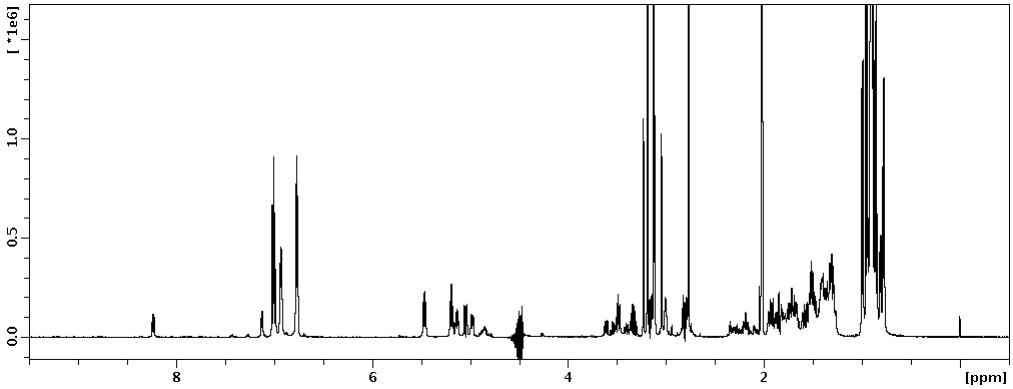
ESI-MS (*m/z*): 755.45 $[M + H]^+$



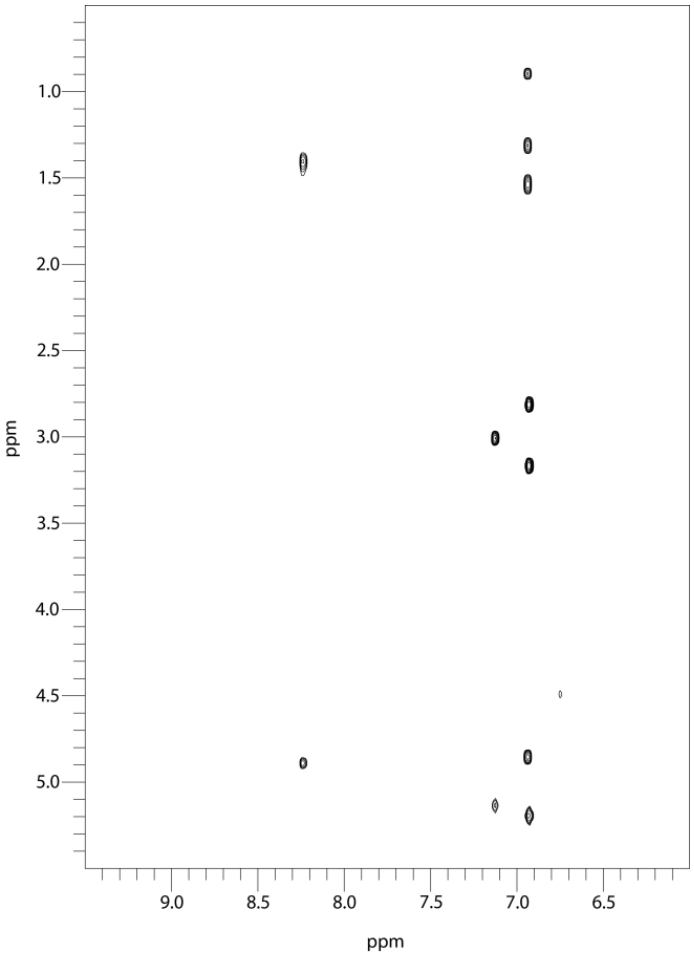
Peptide 17: ESI-MS TIC (source: PAMPA analysis)



Peptide 17: RP-HPLC trace

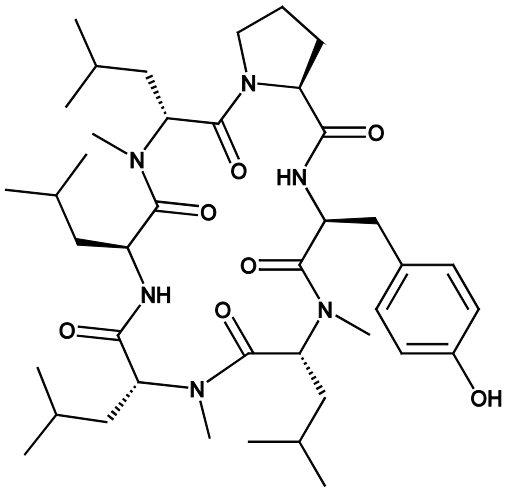


Peptide 17: ^{1}H NMR



Peptide 17: TOCSY

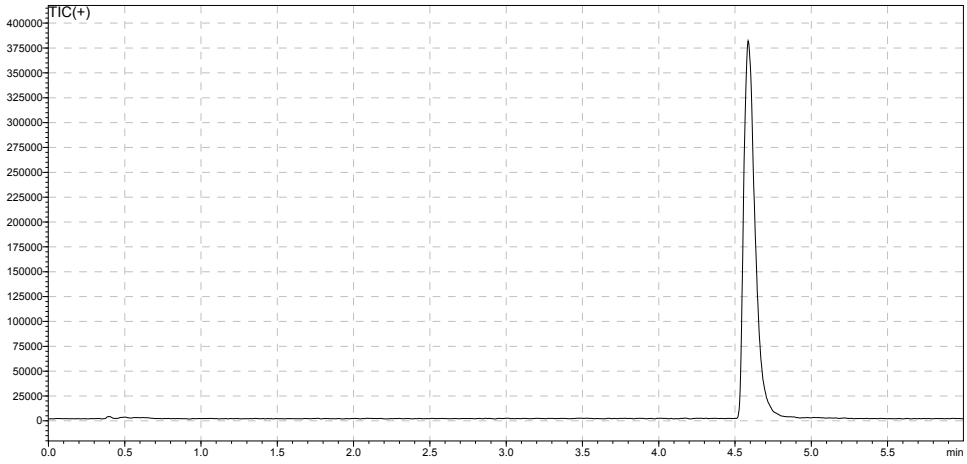
Characterization of Peptide 18



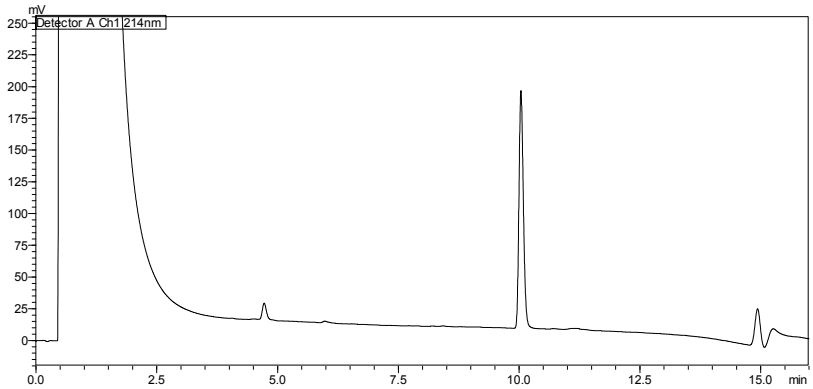
Chemical Formula: $C_{41}H_{66}N_6O_7$

Exact Mass: 754.50

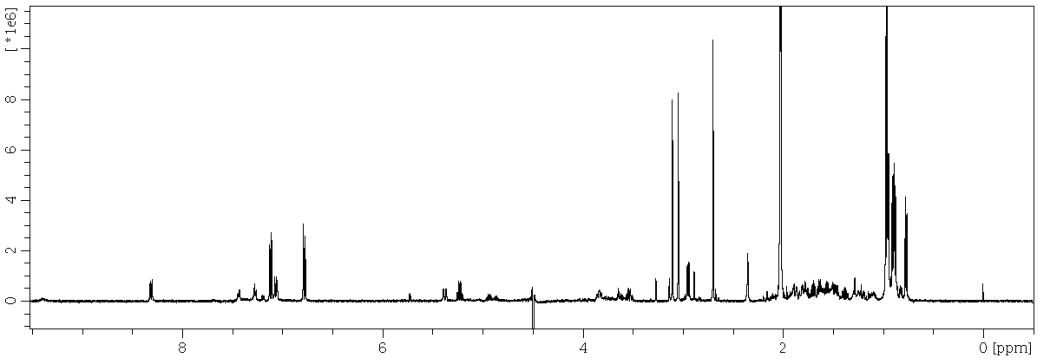
ESI-MS (*m/z*): 755.45 $[M + H]^+$



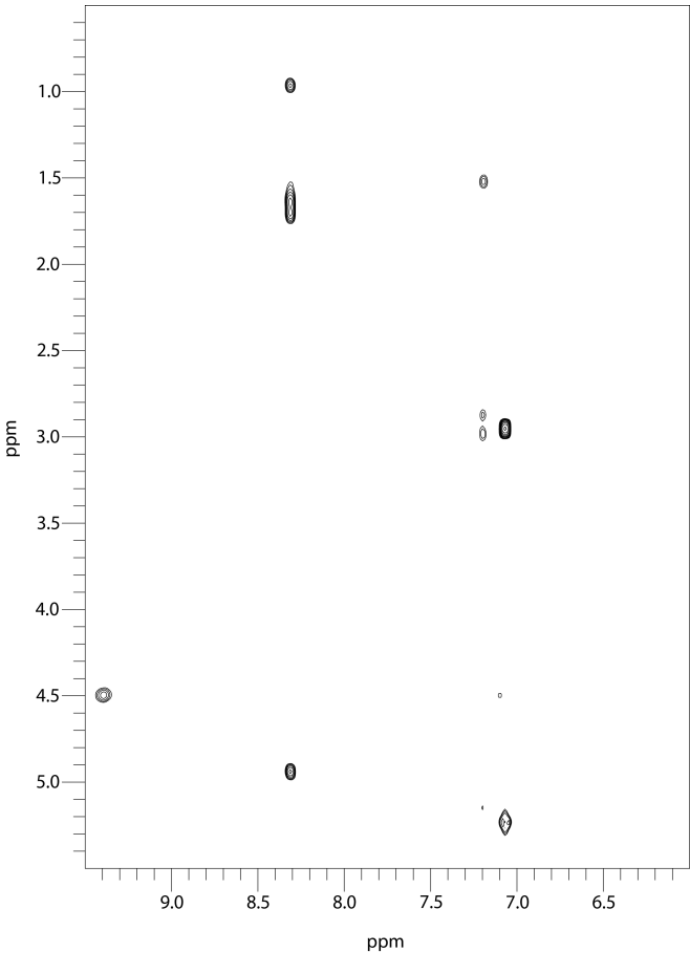
Peptide 18: ESI-MS TIC (source: PAMPA analysis)



Peptide 18: RP-HPLC trace



Peptide 18: ^{1}H NMR



Peptide 18: RP-HPLC trace

Supplementary Table S1: Rat pharmacokinetic data for peptide 15

	Intravenous administration			Oral administration		
Peptide	**CL (ml min^{-1} kg^{-1})**	**V_d (l kg^{-1})**	**$t_{1/2}$ (min)**	**AUC (ng h ml^{-1})**	**C_{max} (ng ml^{-1})**	**% F**
15	55.0	1.1	29	1003	117	33.0

Supplementary Table S2: Hα chemical shifts of peptides 1–5

		Solvent					
		acetonitrile[a]			**trifluroethanol**[a]		**chloroform**
$\delta_{H\alpha}$		*(polar aprotic)*			*(polar protic)*		*(non-polar)*
Residue		**30%**	**90%**	**100%**	**30%**	**100%**	**100%**
Peptide 1							
1	**L**	4.20	4.14	4.05	4.23	4.12	3.66
2	**l**	4.32	4.30	4.32	4.39	4.48	4.83
3	**l**	4.64	4.58	4.40	4.68	4.58	3.73
4	**l**	4.54	4.49	4.44	4.61	4.58	4.33
5	**P**	4.33	4.32	4.34	4.37	4.44	4.38
6	**Y**	4.83	4.75	4.61	4.91	4.80	4.51
Peptide 2							
1	**L**	4.11	4.05	4.03	4.12	3.92	4.06
2	**L**	4.31	4.31	4.27	4.40	4.62	4.58
3	**l**	4.79	4.75	4.72	4.81	4.84	4.71
4	**p**	4.61	4.57	4.57	4.60	4.63	4.49
5	**P**	4.53	4.50	4.82	4.57	4.67	4.80
6	**Y**	5.08	5.03	4.95	5.16	5.10	4.84
Peptide 3 (conformation 1)							
1	**l**	4.06	4.07	4.71	4.12	4.84	4.84
2	**l**	4.20	4.14	3.95	4.16	4.10	4.10
3	**l**	4.34	4.32	4.32	4.42	4.62	4.61
4	**L**	4.39	4.42	4.68	4.51	4.80	4.67
5	**P**	4.29	4.08	4.05	4.32	4.19	3.95
6	**Y**	4.61	4.59	4.60	4.73	4.79	4.84
Peptide 3 (conformation 2)							
1	**l**	4.72	4.72	4.71	4.79	4.84	4.84
2	**l**	4.03	3.99	3.95	4.12	4.10	4.10
3	**l**	4.34	4.34	4.32	4.44	4.62	4.61
4	**L**	4.70	4.70	4.68	4.72	4.80	4.67
5	**P**	4.12	4.22	4.05	4.18	4.19	3.95
6	**Y**	4.66	4.60	4.60	4.71	4.79	4.84
Peptide 4							
1	**l**	3.81	3.90	3.91	3.94	4.02	4.05
2	**l**	4.30	4.32	4.55	4.41	4.63	4.86
3	**L**	4.48	4.49	4.49	4.52	4.60	4.53
4	**l**	4.51	4.46	4.43	4.43	4.48	4.28
5	**P**	4.32	4.28	4.25	4.35	4.36	4.37
6	**Y**	4.73	4.67	4.53	4.78	4.71	4.54
Peptide 5							
1	**L**	4.40	4.45	4.36	4.52	4.53	4.38
2	**l**	4.35	4.39	4.37	4.47	4.38	4.23
3	**L**	4.06	4.08	4.08	4.13	4.14	4.74
4	**L**	4.76	4.80	4.74	4.88	4.89	3.93
5	**p**	4.09	4.08	4.08	4.14	4.14	4.49
6	**Y**	4.39	4.40	4.32	4.51	4.47	4.63

[a] organic solvent mixtures with H_2O (v/v)

Supplementary Table S3: HN chemical shifts of peptides 1–5

		Solvent					
		acetonitrile[b]			**trifluroethanol**[b]		**chloroform**
δ_{HN}		*(polar aprotic)*			*(polar protic)*		*(non-polar)*
Residue		**30%**	**90%**	**100%**	**30%**	**100%**	**100%**
Peptide 1							
1	**L**	7.68	7.27	6.91	7.60	6.83	7.62
2	**l**	8.45	7.40	6.98	8.31	7.25	6.45
3	**l**	7.66	7.48	7.36	7.67	7.49	7.47
4	**l**	7.56	7.14	6.88	7.31	6.76	7.96
5	**P**[a]						
6	**Y**	7.42	7.25	7.14	7.45	7.23	7.26
Peptide 2							
1	**L**	7.96	7.39	6.93	7.73	6.30	6.49
2	**L**	7.30	6.72	6.29	7.05	6.75	6.39
3	**l**	7.18	7.05	7.00	7.26	7.49	7.16
4	**p**[a]						
5	**P**[a]						
6	**Y**	8.13	8.07	8.04	8.27	8.15	8.08
Peptide 3 (conformation 1)							
1	**l**	8.17	7.57	7.43	7.94	7.58	7.45
2	**l**	8.19	7.66	6.80	8.14	6.70	5.88
3	**l**	7.50	7.28	6.40	7.60	6.68	6.33
4	**L**	8.18	7.66	7.01	8.03	7.38	6.98
5	**P**[a]						
6	**Y**	6.99	6.85	5.89	7.06	5.86	5.57
Peptide 3 (conformation 2)							
1	**l**	7.62	7.50	7.34	7.66	6.72	7.45
2	**l**	7.72	7.20	6.49	7.56	7.11	5.88
3	**l**	7.37	6.81	6.86	7.16	7.23	6.33
4	**L**	7.25	7.11	6.94	7.31	7.16	6.98
5	**P**[a]						
6	**Y**	6.51	6.19	7.04	6.17	7.41	5.57
Peptide 4							
1	**l**	7.99	7.50	7.34	7.79	6.72	7.47
2	**l**	8.01	7.59	6.49	7.94	7.11	6.23
3	**L**	7.42	7.14	6.86	7.46	7.23	6.82
4	**l**	8.01	7.46	6.94	7.91	7.16	7.12
5	**P**[a]						
6	**Y**	7.27	7.17	7.04	7.34	7.41	7.25
Peptide 5							
1	**L**	7.71	7.78	7.67	7.83	7.94	7.61
2	**l**	7.81	7.45	7.00	7.69	7.15	7.39
3	**L**	8.40	7.77	6.88	8.08	7.41	6.78
4	**L**	7.23	6.89	6.35	7.00	6.87	5.88
5	**p**[a]						
6	**Y**	8.27	7.62	6.96	7.92	7.07	6.10

[a] no NH proton present in Pro

[b] organic solvent mixtures with H_2O (v/v)

Supplementary Table S4: Chemical shifts of peptide 15 in acetonitrile

Residue		30% acetonitrile[b]		100% acetonitrile[b]	
		Hα	**HN (H_{Me})**	**Hα**	**HN (H_{Me})**
1	**L**	4.68	(3.26)	4.39	(3.28)
2	**l**	5.28	(2.93)	5.32	(2.82)
3	**l**	4.55	7.47	4.25	7.42
4	**l**	4.53	7.56	4.42	7.21
5	**P**[a]	4.40		4.35	
6	**Y**	5.20	7.39	5.06	7.02

[a] no NH proton present in Pro

[b] organic solvent mixtures with H_2O (v/v)

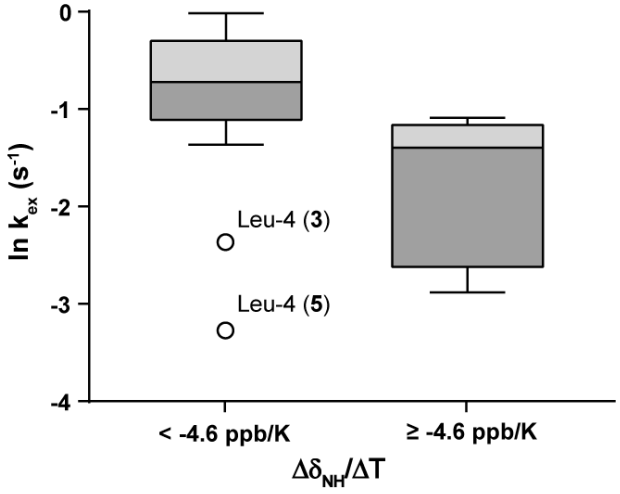
Supplementary Table S5: Distance restraints for peptide 15

Inter-proton distance		d_{upp} [Å]
NME-D-LEU-2 HA	NME-D-LEU-2 HB2	3.92
NME-D-LEU-2 HA	NME-D-LEU-2 HB3	3.08
NME-D-LEU-2 HA	NME-D-LEU-2 HG	5.25
NME-D-LEU-2 HA	NME-D-LEU-2 HG	4.76
NME-D-LEU-2 HA	NME-D-LEU-2 QD1	4.28
NME-D-LEU-2 HA	NME-D-LEU-2 QD1	4.43
NME-D-LEU-2 QN	NME-D-LEU-2 HA	5.46
NME-D-LEU-2 QN	NME-D-LEU-2 HA	5.89
NME-D-LEU-2 QN	NME-D-LEU-2 HB2	4.84
NME-D-LEU-2 QN	NME-D-LEU-2 HB3	4.99
NME-D-LEU-2 QN	NME-D-LEU-2 HG	5.77
NME-D-LEU-2 QN	NME-D-LEU-2 QD1	7.77
D-LEU-3 H	NME-D-LEU-2 HA	3.11
D-LEU-3 H	NME-D-LEU-2 HB2	5.50
D-LEU-3 H	NME-D-LEU-2 QN	4.90
D-LEU-3 H	D-LEU-3 HA	3.14
D-LEU-3 H	D-LEU-3 HB2	3.48
D-LEU-3 H	D-LEU-3 HB3	4.07
D-LEU-3 H	D-LEU-3 HG	5.50
D-LEU-3 H	TYR-6 HB2	4.60
D-LEU-3 H	NME-LEU-1 HA	4.01
D-LEU-3 HA	D-LEU-3 HB2	3.14
D-LEU-3 HA	D-LEU-3 HB3	3.33
D-LEU-3 HA	D-LEU-3 HG	4.38
D-LEU-3 HA	D-LEU-3 QD1	5.62
D-LEU-3 HA	D-LEU-3 QD2	4.29
D-LEU-3 HA	D-LEU-4 H	3.02
D-LEU-3 HB3	D-LEU-4 H	3.02
D-LEU-3 HG	D-LEU-4 H	4.85
D-LEU-4 H	D-LEU-4 HA	3.02
D-LEU-4 H	D-LEU-4 HB2	5.50
D-LEU-4 H	D-LEU-4 HB3	3.21
D-LEU-4 H	D-LEU-4 HG	4.14
D-LEU-4 H	D-LEU-4 QD1	6.52
D-LEU-4 HA	D-LEU-4 HB2	3.27
D-LEU-4 HA	D-LEU-4 HB3	3.21
D-LEU-4 HA	D-LEU-4 HG	4.23
D-LEU-4 HA	D-LEU-4 QD2	3.45
D-LEU-4 HA	PRO-5 HD2	2.86
D-LEU-4 HA	PRO-5 HD3	2.71
D-LEU-4 HA	TYR-6 H	3.92

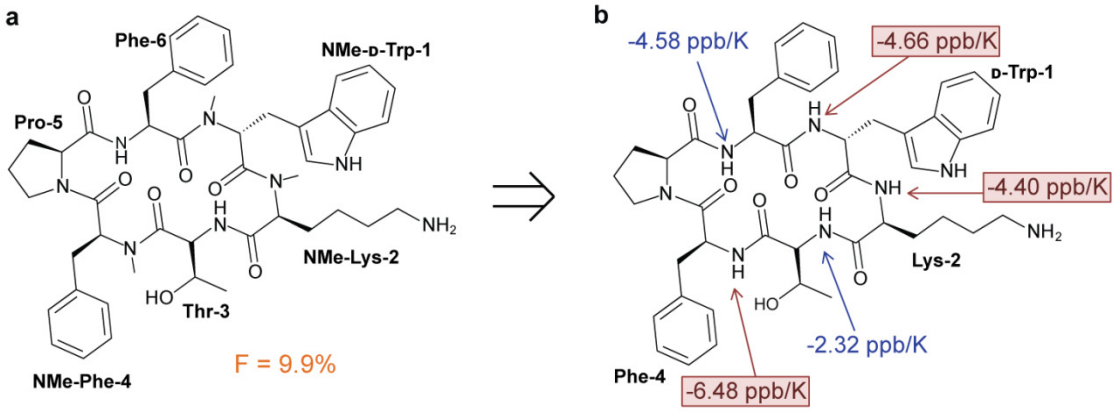
Inter-proton distance		d_{upp} [Å]
D-LEU-4 HB2	PRO-5 HD2	4.23
PRO-5 HA	TYR-6 H	3.86
PRO-5 HB2	PRO-5 HA	3.17
PRO-5 HB2	PRO-5 HD2	5.38
PRO-5 HB2	PRO-5 HD3	5.50
PRO-5 HB3	PRO-5 HA	3.52
PRO-5 HB3	PRO-5 HD2	5.50
PRO-5 HB3	PRO-5 HD3	5.50
PRO-5 HD2	PRO-5 HA	4.54
PRO-5 HD3	PRO-5 HA	4.35
PRO-5 HD3	TYR-6 H	4.42
PRO-5 HD3	TYR-6 QD	6.98
PRO-5 HG2	PRO-5 HA	5.50
PRO-5 HG2	PRO-5 HD2	5.50
PRO-5 HG2	PRO-5 HD3	4.51
PRO-5 HG3	PRO-5 HA	5.50
PRO-5 HG3	PRO-5 HD2	3.42
PRO-5 HG3	PRO-5 HD3	5.50
TYR-6 H	TYR-6 HA	2.80
TYR-6 H	TYR-6 HB2	3.24
TYR-6 H	TYR-6 HB3	5.50
TYR-6 HA	TYR-6 HB2	3.55
TYR-6 HA	TYR-6 HB2	3.67
TYR-6 HA	TYR-6 HB3	3.08
TYR-6 HA	TYR-6 HB3	2.96
TYR-6 HA	TYR-6 QD	5.68
TYR-6 HA	NME-LEU-1 QN	3.63
TYR-6 HA	NME-LEU-1 QN	3.72
TYR-6 QD	TYR-6 HB2	5.86
TYR-6 QD	TYR-6 HB3	6.48
NME-LEU-1 HA	NME-D-LEU-2 QN	3.63
NME-LEU-1 HA	NME-LEU-1 HB2	3.42
NME-LEU-1 HA	NME-LEU-1 HB3	3.55
NME-LEU-1 HA	NME-LEU-1 HG	4.26
NME-LEU-1 HA	NME-LEU-1 QD1	4.03
NME-LEU-1 HB2	NME-D-LEU-2 QN	4.90
NME-LEU-1 QN	NME-LEU-1 HA	4.37
NME-LEU-1 QN	NME-LEU-1 HB2	5.46
NME-LEU-1 QN	NME-LEU-1 HB3	4.50
NME-LEU-1 QN	NME-LEU-1 HG	4.90

Supplementary Figure S1: Amide temperature coefficients and hydrogen-deuterium exchange rates for selected peptides.

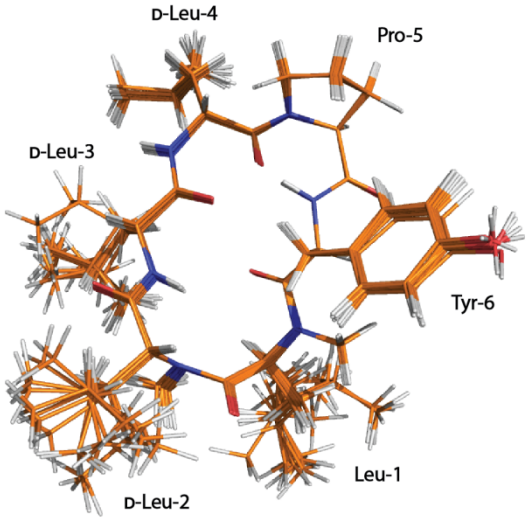
A box-plot representation comparing the hydrogen-deuterium exchange rates (in 30% v/v deuterated acetonitrile, 70% v/v deuterium oxide) and amide temperature coefficients (in 30% v/v deuterated acetonitrile, 70% v/v water) for peptides **1**–**5**. Two outliers (i.e. Leu-4 of **3** and Leu-4 of **5**) are labeled (see text for explanation).



Supplementary Figure S2: Amide temperature coefficient analysis of the Veber-Hirschmann peptide. (a) The N-methylated peptide has a reported oral bioavailability, F, of 9.9%. (b) The non-methylated form was synthesized. The amide temperature coefficients for each amide are indicated; values for amides which need to be N-methylated are highlighted using the color red.

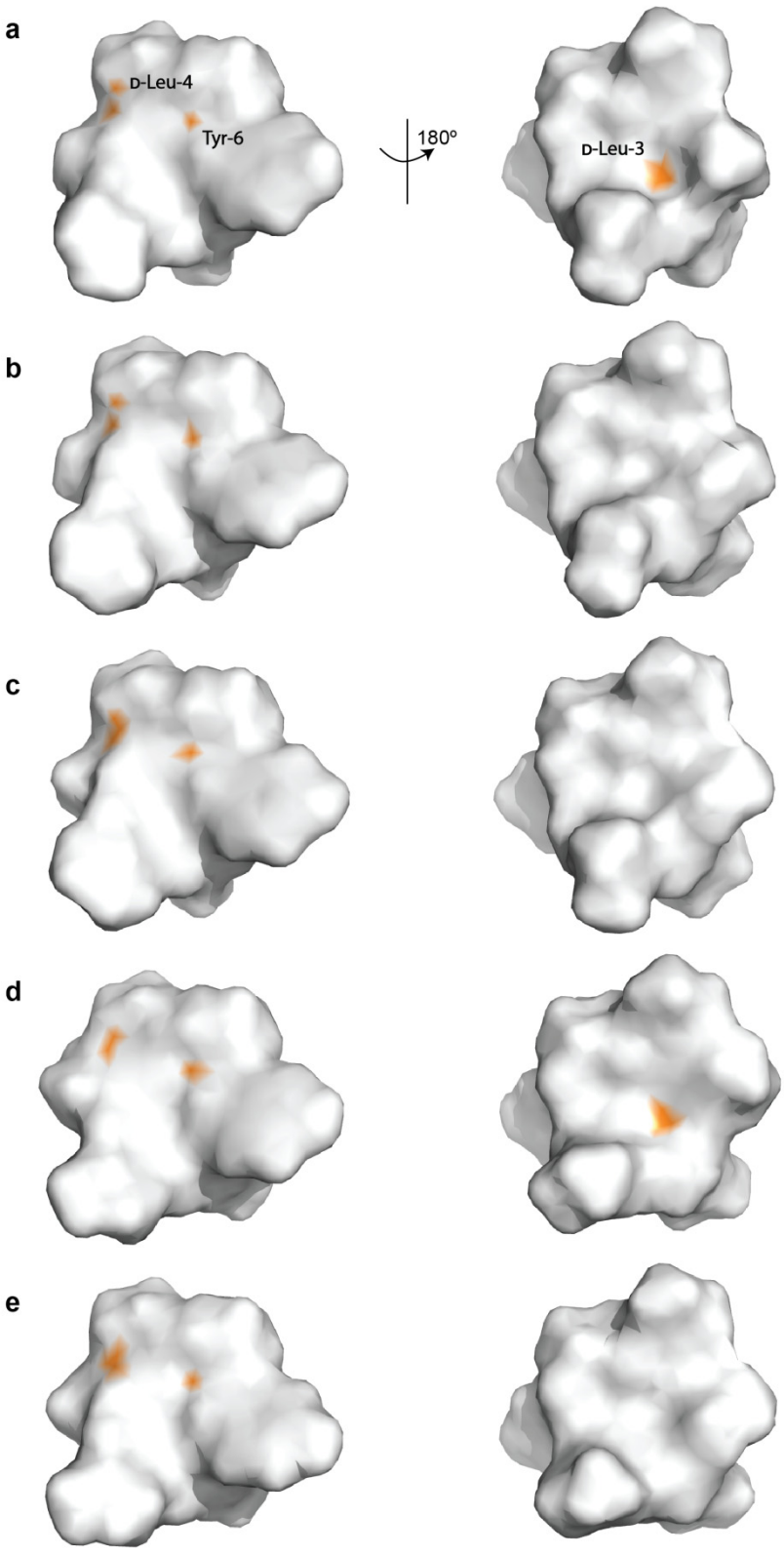


Supplementary Figure S3: Superposition of the ten lowest energy structures of peptide 15



Supplementary Figure S4: Surface representations of the five lowest energy structures of peptide 15

Exposed surface of backbone amides highlighted in orange.



References

1. Vranken WF*, et al.* (2005) The CCPN data model for NMR spectroscopy: development of a software pipeline. *Proteins* 59(4):687-696.

2. Guntert P, Mumenthaler C, & Wuthrich K (1997) Torsion angle dynamics for NMR structure calculation with the new program DYANA. *J Mol Biol* 273(1):283-298.

3. Lewis IA, Schommer SC, & Markley JL (2009) rNMR: open source software for identifying and quantifying metabolites in NMR spectra. *Magn Reson Chem* 47 Suppl 1:S123-126.

4. Lohman RJ*, et al.* (2012) An antagonist of human protease activated receptor-2 attenuates PAR2 signaling, macrophage activation, mast cell degranulation, and collagen-induced arthritis in rats. *FASEB J* 26(7):2877-2887.