Supporting Information

Environmentally friendly SPPS I. Application of NaOH in 2-MeTHF/methanol for Fmoc removal

Adam Přibylka,[a] Viktor Krchňák, [a,b] and Eva Schütznerová[a*]

[a]Department of Organic Chemistry, Faculty of Science, Palacký University, 17. listopadu 12, 771 46 Olomouc, Czech Republic

[b]Department of Chemistry and Biochemistry, 251 Nieuwland Science Center, University of Notre Dame, Notre Dame, Indiana 46556, United States

Corresponding author. E-mail: eva.schutznerova@upol.cz

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Material and Methods

Solvents were used without further purification. The Rink amide resin (100-200 mesh, 1% DVB, 0.57 mmol/g) was used. Synthesis was carried out on Domino Blocks (www.torviq.com) in disposable polypropylene reaction vessels.

All reactions were carried out at ambient temperature (~21 °C) unless stated otherwise. The volume of wash solvent was 10 mL per 1 g of resin. For washing, resin slurry was shaken with the fresh solvent for at least 1 min before changing the solvent. After adding a reagent solution, the resin slurry was manually vigorously shaken to break any potential resin clumps. Resin-bound intermediates were dried by a stream of nitrogen for prolonged storage and/or quantitative analysis.

For the LC/MS analysis a sample of resin (~5 mg) was treated by 50% TFA in DCM, the cleavage cocktail was evaporated by a stream of nitrogen, and cleaved compounds extracted into 1 mL of MeOH. The LC/MS analysis were carried out using an instrument comprising a 3 x 50 mm C18 reverse phase column, 5 um particles. Mobile phases: 0.05% trifluoroacetic acid in HPLC grade water (A) and HPLC grade acetonitrile (B). A gradient was formed from 5% to 80% of B in 10 minutes, flow rate of 0.7 mL/min. The MS electrospray source operated at capillary voltage 3.5 kV and a desolvation temperature 300 ^{o}C.

All ^{1}H NMR experiments were performed at magnetic field strengths of 9.39 T (with operating frequencies 399.78 MHz for ^{1}H) at ambient temperature (20 °C). ^{1}H spectra spectra were referenced relative to the signal of DMSO (^{1}H δ = 2.49 ppm).

Solid-phase syntheses were performed in plastic reaction vessels (syringes, each equipped with a porous disk) using a manually operated synthesizer.(V. Krchnak, V. Padera, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 3261.) Commercially available Rink Amide AM resin (100–200 mesh, 0.57 mmol/g) was used. The volume of wash solvent was 10 mL per 1 g of resin in the preparative scale syntheses, and analytical experiments were performed with 1 mL of solvent per 20 mg of resin. For washing, the resin slurry was shaken with fresh solvent for at least 1

min before the solvent was changed. The yields of the crude products were calculated with respect to the loading of the first building block.

HPLC separation:

Instrumentation: Separation of diastereomeric dipeptides was performed using Agilent Infinity 1260 HPLC system equipped with diode array detector and autosampler. All separations were done in a mode of reverse phase ion-pair liquid chromatography. Chromatographic column Gemini C18, 4.6x150mm, 5 µm particles, was maintained at 40 °C. Isocratic elution was created by mobile phase consisting of MeOH/acetonitrile/ion-pair reagent (IPR) in a ratio of 20:20:60 with the flow rate 0.5 mL/min. IPR was formed by an aqueous mixture of 5 mM sodium heptanesulfonate and 15 mM KH_2PO_4. pH was adjusted with phosphoric acid at 3. The detection wavelength was set at 215 nm.

Sample preparation: Both diastereomeric dipeptides were characterized and quantified by NMR prior HPLC separation. Although compounds were lyophilized after NMR measurements, samples contain traces of DMSO-d_6. Stock solutions were prepared by dissolving of defined amount of compounds in MeOH. Calculated volume of each stock solution was pipetted out and subsequently diluted with mobile phase to get a sample concentration of 0.3 mg/mL. Injection volume was 10 µL.

Experimental Procedures

Green Fmoc removal by NaOH in 2-MeTHF/methanol:

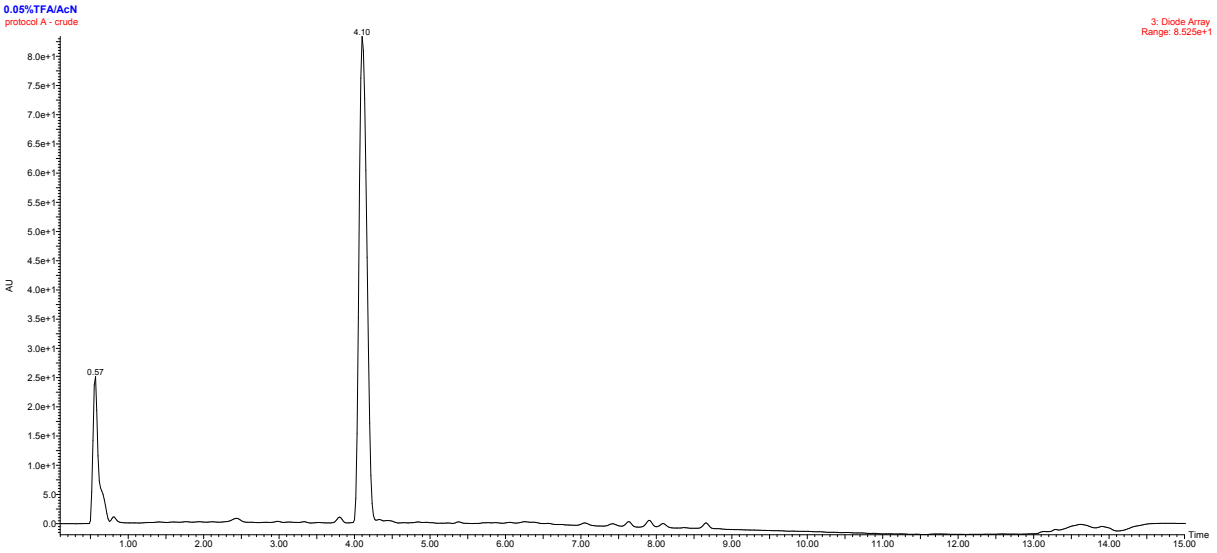
The resin (250 mg) was washed 3× with 2-MeTHF and treated with 0.2 M NaOH (25 µL of 20 M NaOH aq. solution) in MeOH/2-MeTHF (1:1, v/v; 2.5 mL) for 15 min at rt. The resin was then washed 5× with 2-MeTHF.

Acylation with Fmoc-amino Acid: the resin (250 mg) was washed 3× with the appropriate solvent depending on the procedure (A, B or C). A solution of Fmoc-amino acid (0.5 mmol), HOBt (0.5 mmol, 77 mg) and DIC (0.5 mmol, 78 µL) in 2.5 mL of the chosen solvent was added to the resin, and the reaction slurry was shaken for 1 h. The resin was washed according to protocol A, B or C.

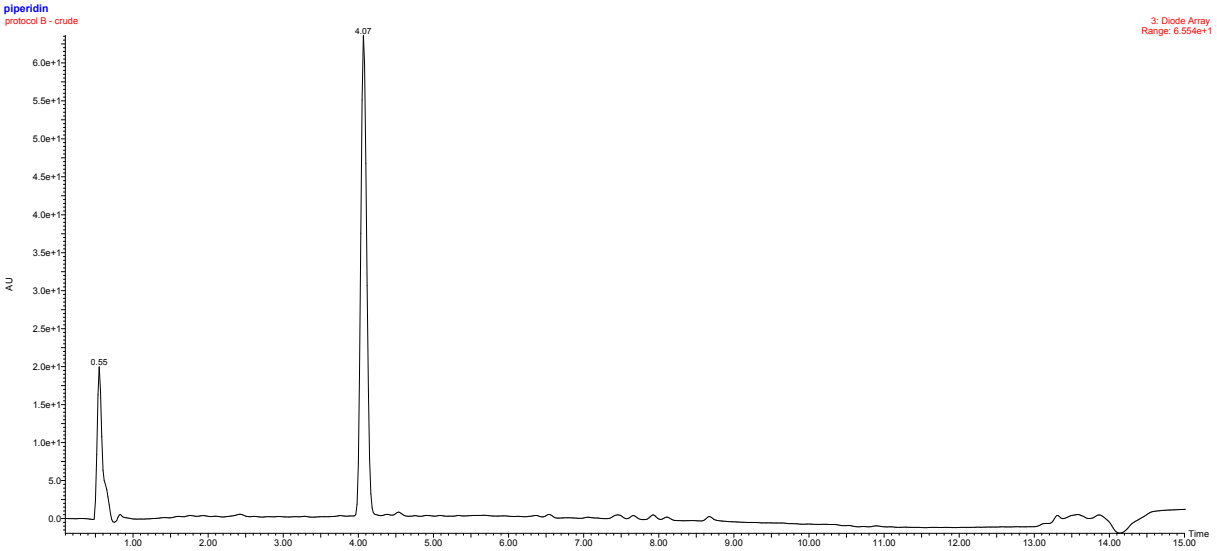
Final isolation:

250 mg of Fmoc-Leu-enkephalin pentapeptide was washed 3× with DCM, 3× with DMF and treated with 2.5 mL of 50% piperidine in DMF for 15 min. The resin was washed 5× with DMF, 3× with MeOH and 3× with DCM. The resin was treated with a solution of 50% TFA in DCM (3 mL) for 1.5 h. The TFA solution was collected, and the resin was washed 3× with 10% TFA/DCM. The combined extracts were concentrated under a stream of nitrogen, and the crude peptide was precipitated with Et_2O (3 mL) by ultrasound, filtered and lyophilized.

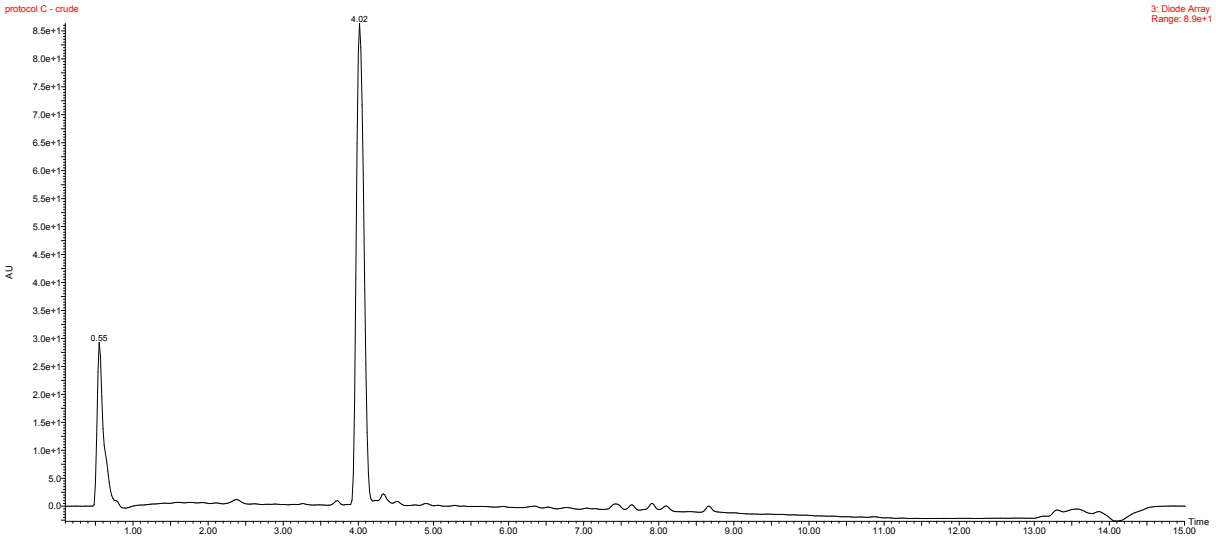
HPLC chromatogram of pentapeptide Leu-enkaphalin amide using protocol A:



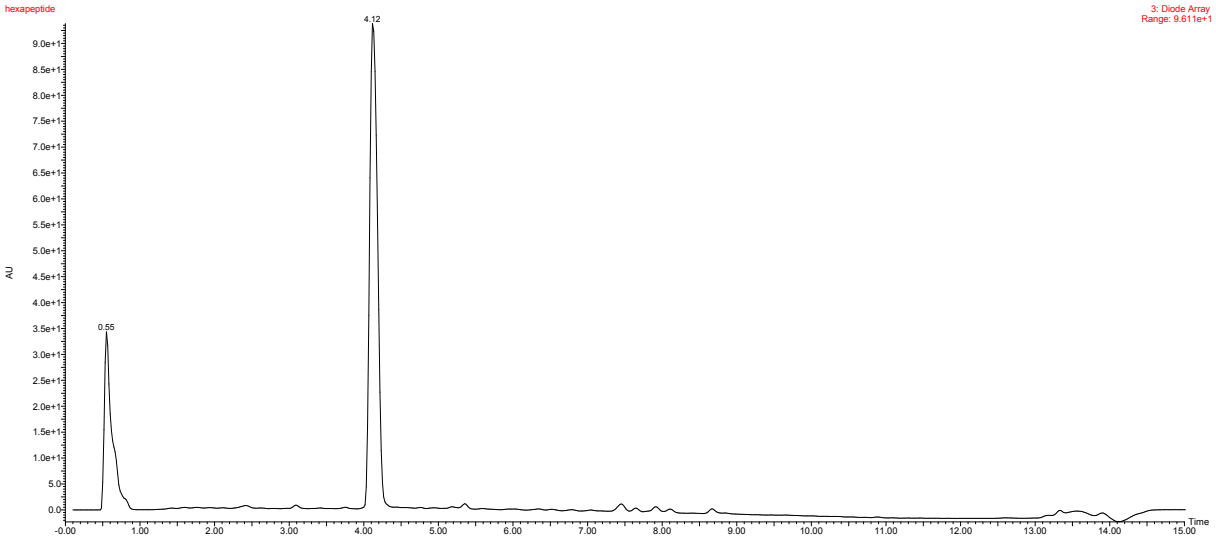
HPLC chromatogram of pentapeptide Leu-enkaphalin amide using protocol B:



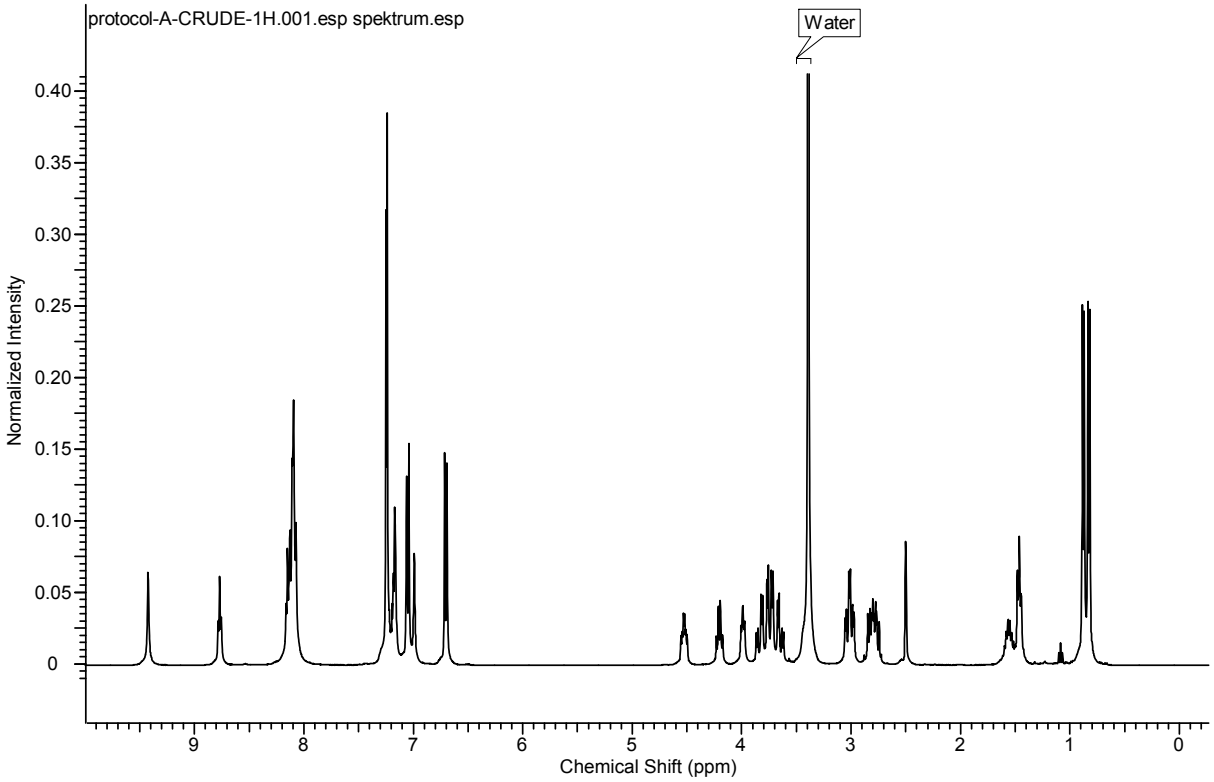
HPLC chromatogram of pentapeptide Leu-enkaphalin amide using protocol C:



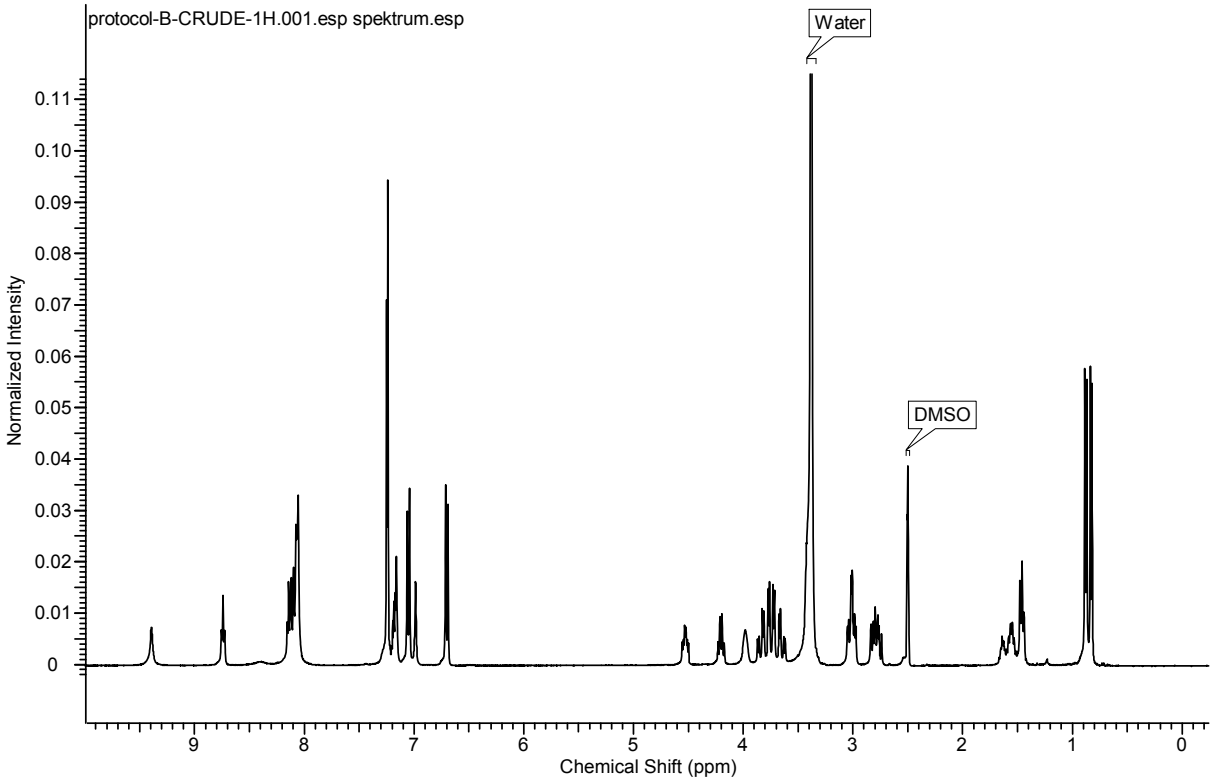
HPLC chromatogram of hexapeptide amide H-Ala-Tyr-Gly-Gly-Phe-Leu-NH_2 using protocol C, final Fmoc removal before isolation using 0.1 M NaOH 2-MeTHF/MeOH:



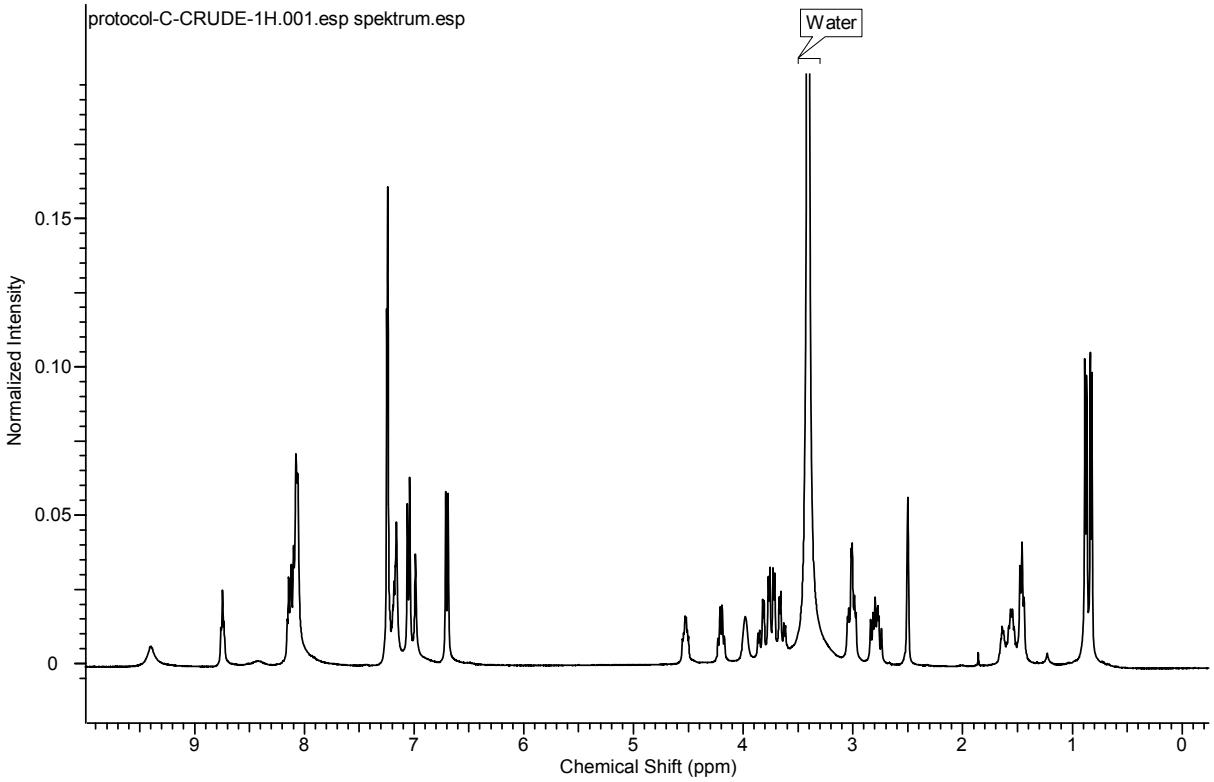
^{1}H NMR spectrum of pentapeptide Leu-enkaphalin amide using protocol A:



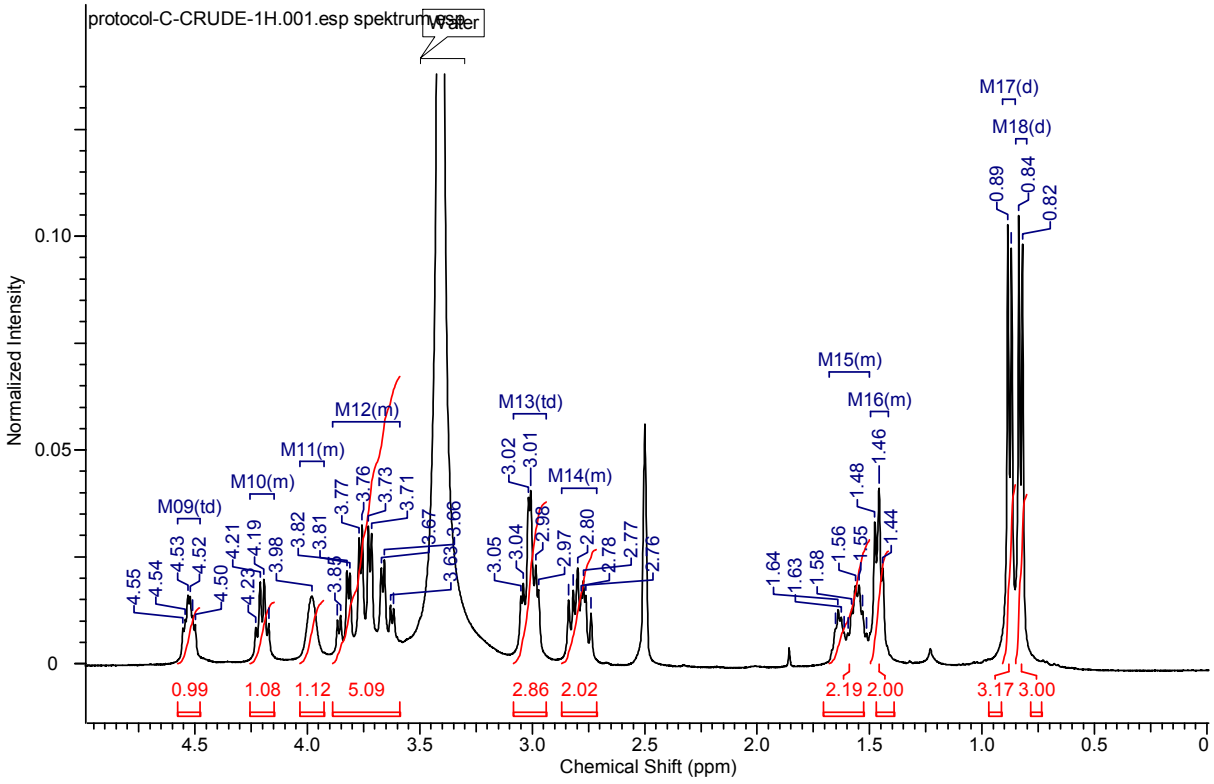
^{1}H NMR spectrum of pentapeptide Leu-enkaphalin amide using protocol B:

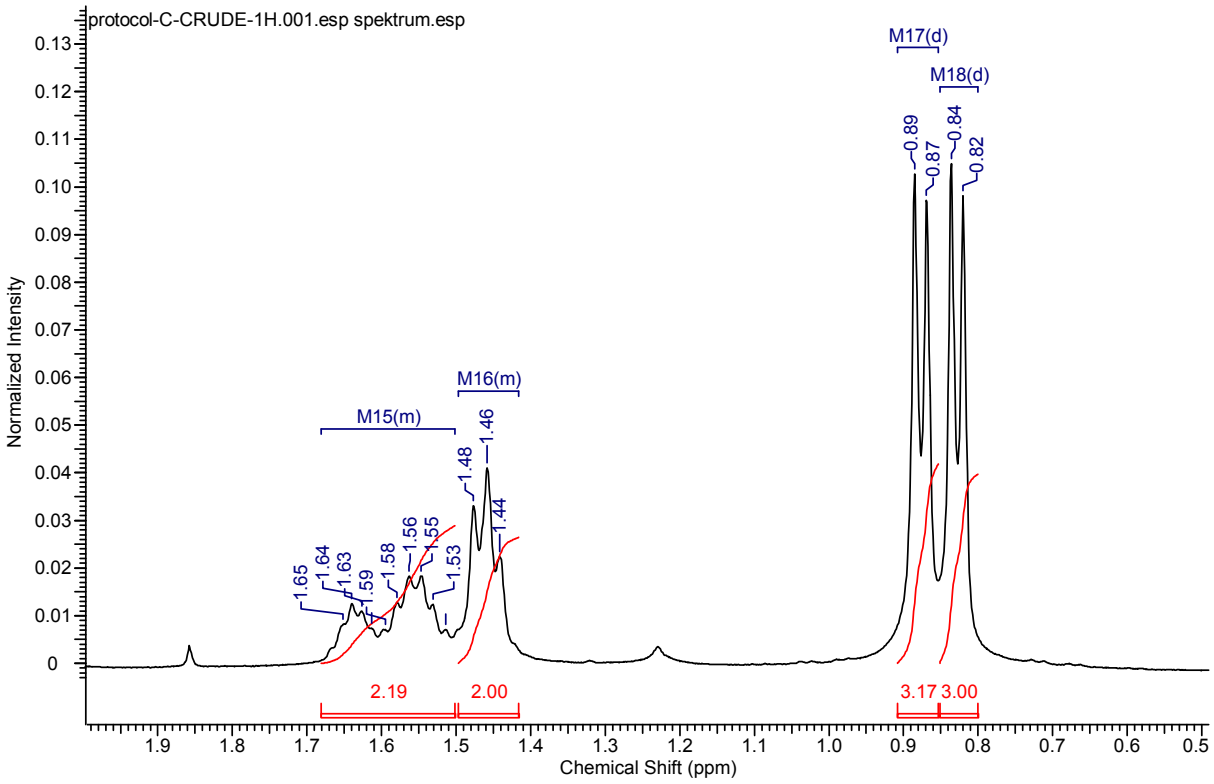


^{1}H NMR spectrum of pentapeptide Leu-enkaphalin amide using protocol C:

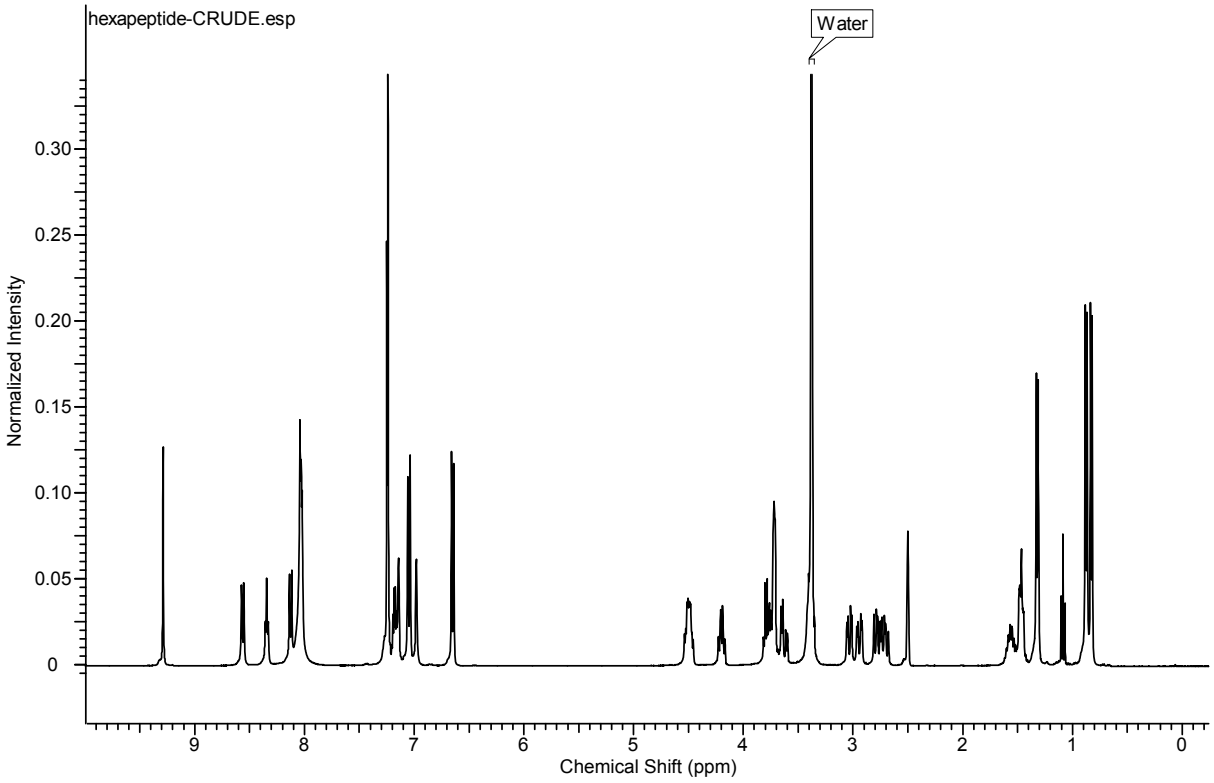


Zoomed aliphatical area of ^{1}H NMR spectrum of pentapeptide Leu-enkaphalin amide using protocol C showing no sign of epimerization (0 – 4.5 ppm and 0.5 - 1.9 ppm):

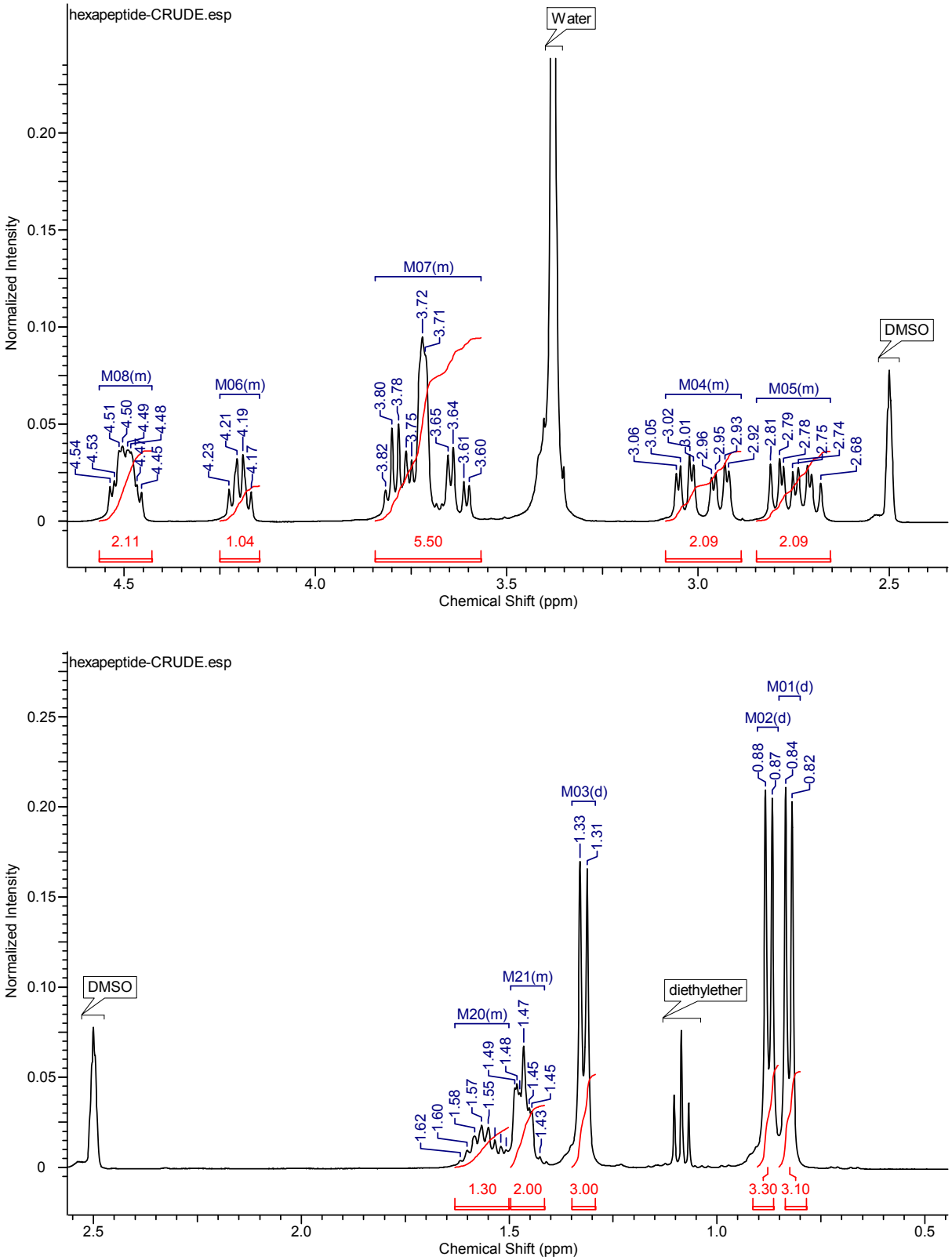




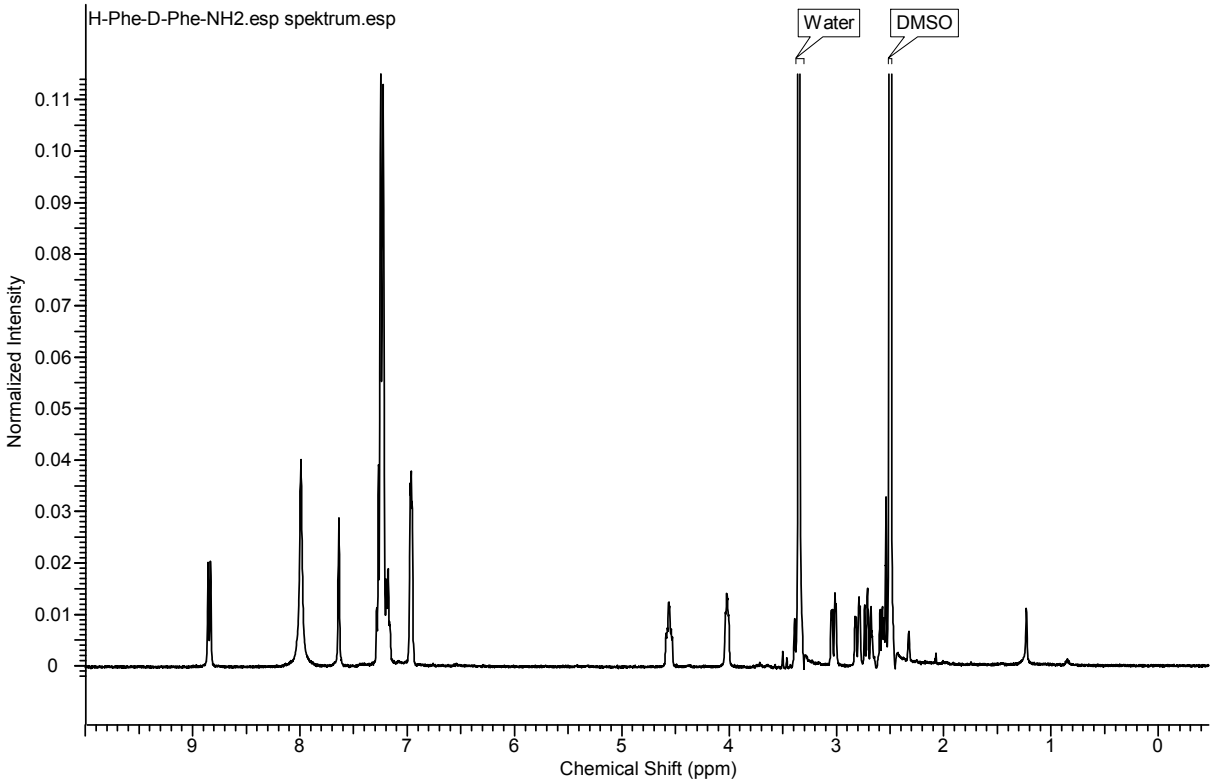
^{1}H NMR spectrum of hexapeptide amide H-Ala-Tyr-Gly-Gly-Phe-Leu-NH_2 using protocol C, final Fmoc removal before isolation using 0.1 M NaOH 2-MeTHF/MeOH:



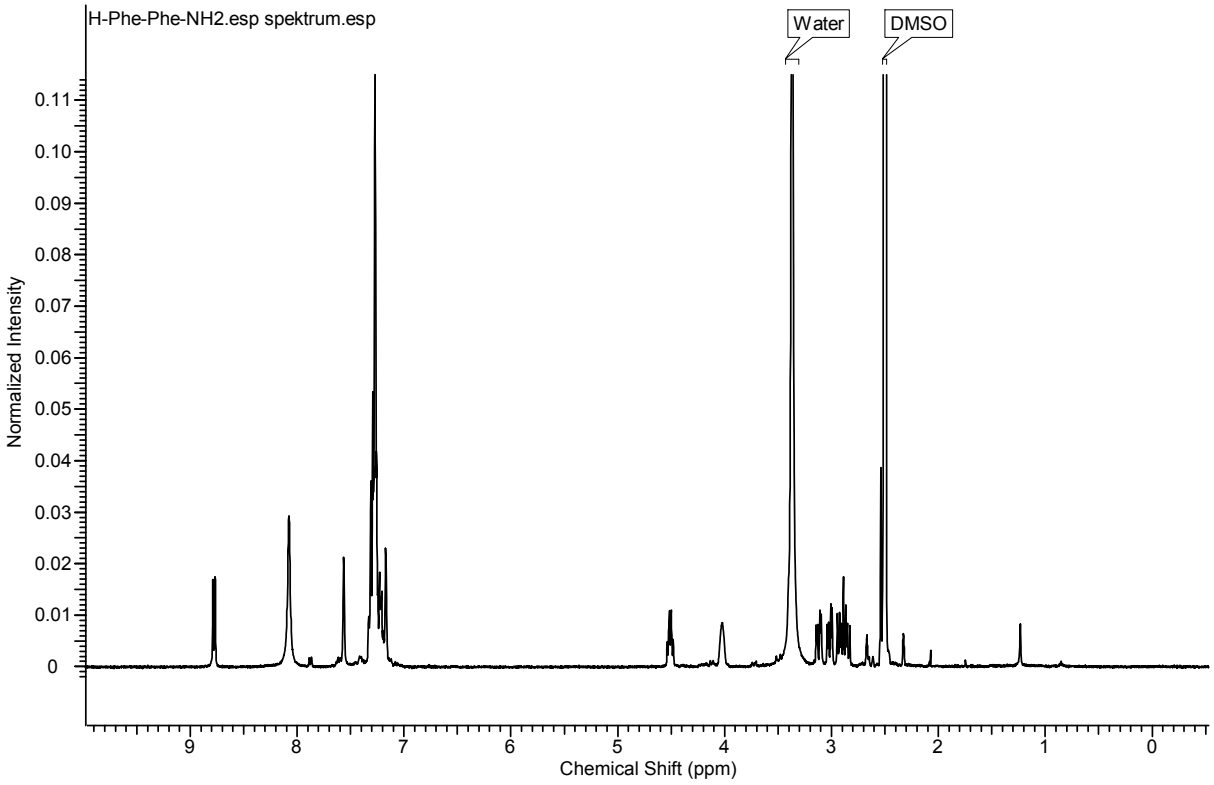
Zoomed aliphatical area of ^{1}H NMR spectrum of hexapeptide amide using protocol C showing no sign of epimerization (2.5 – 4.5 ppm and 0.5 – 2.5 ppm):



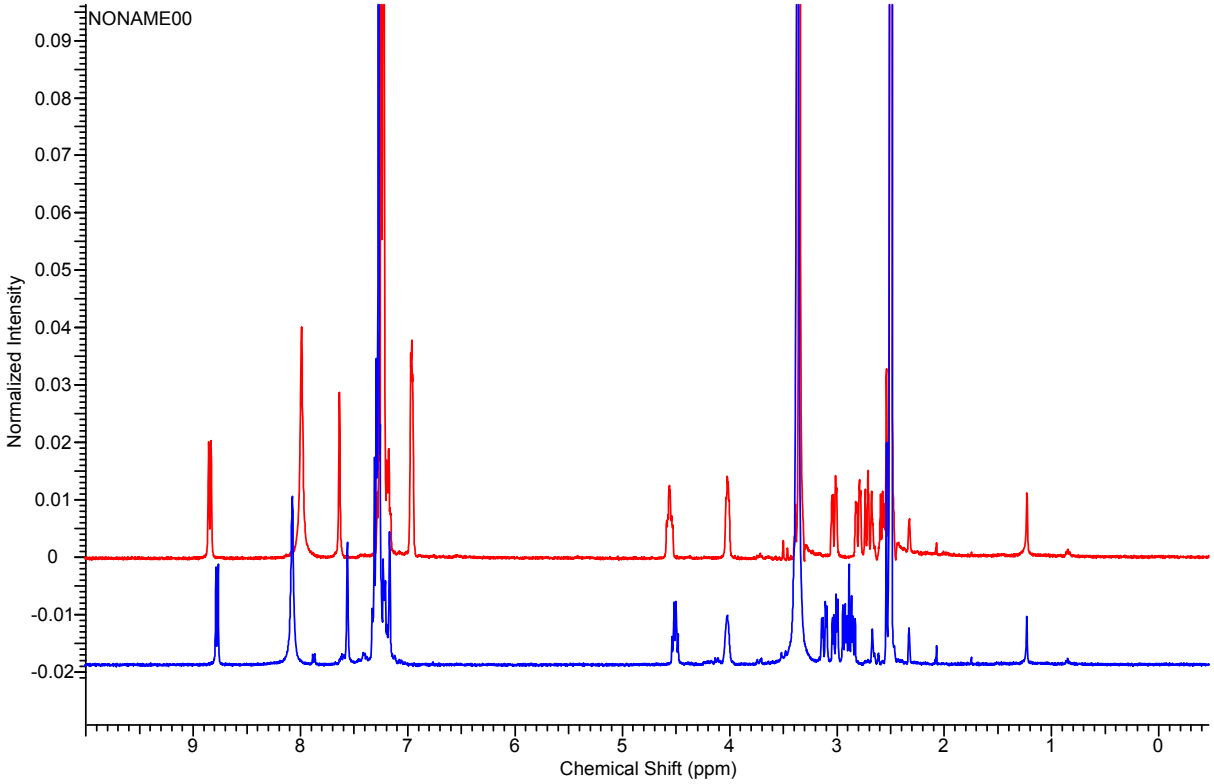
^{1}H NMR spectrum of dipeptide H-Phe-D-Phe-NH_2 amide using protocol C:



^{1}H NMR spectrum of dipeptide H-Phe-Phe-NH_2 amide using protocol C:

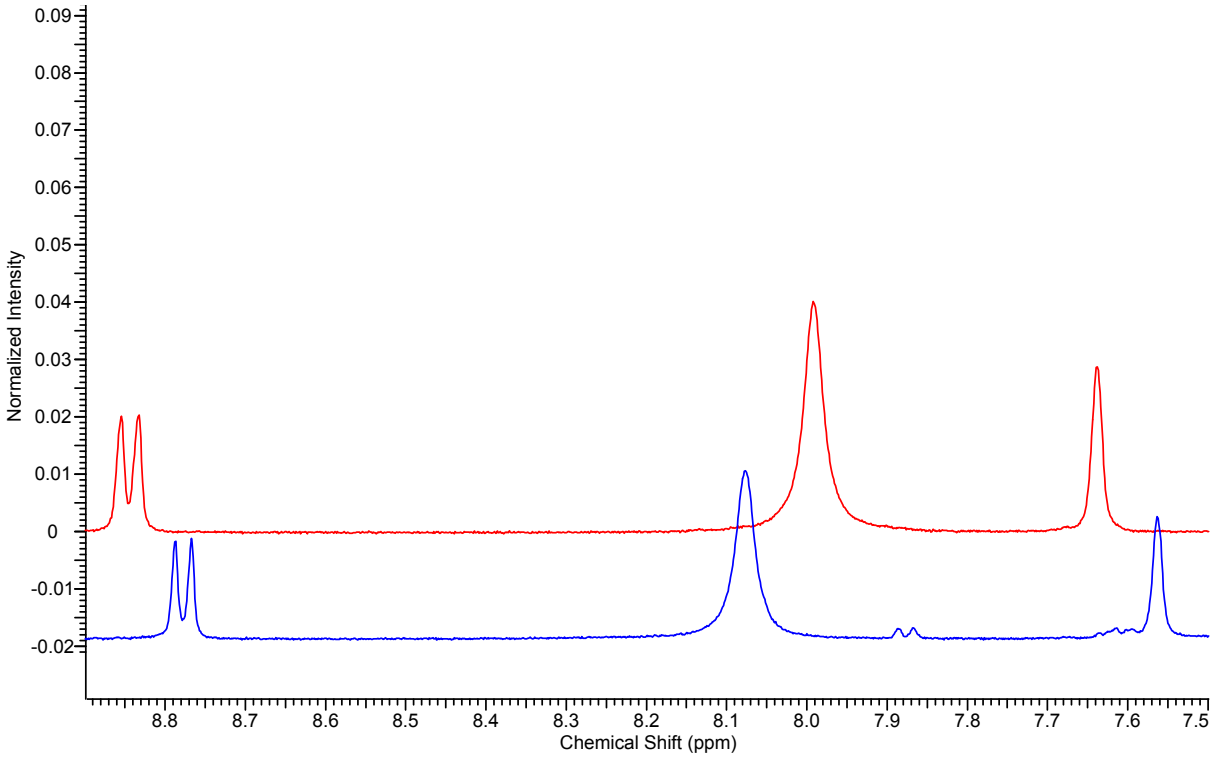


Overlap of ^{1}H NMR spectra of H-Phe-D-Phe-NH_2 (red) and H-Phe-Phe-NH_2 (blue)

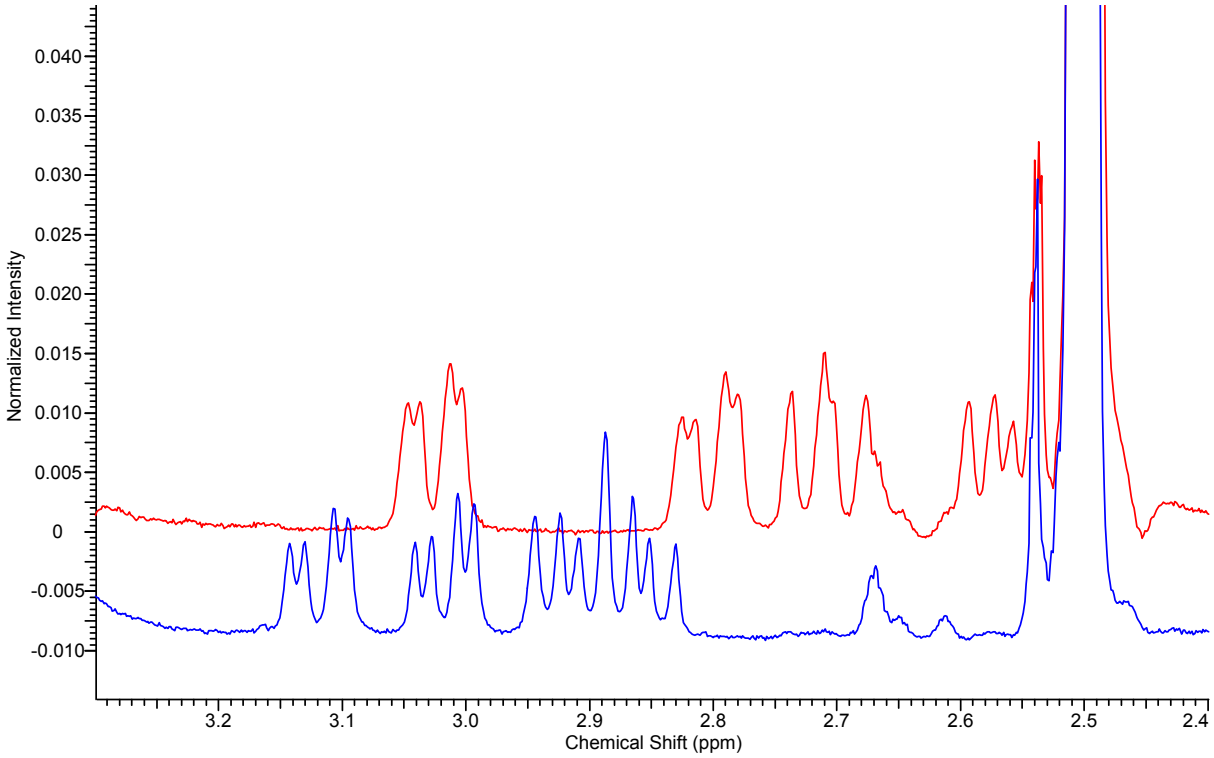


Zoomed areas of ^{1}H NMR spectra of H-Phe-D-Phe-NH_2 (red) and H-Phe-Phe-NH_2 (blue)

(7.5 – 8.9 ppm)



Zoomed areas of ^{1}H NMR spectra of H-Phe-D-Phe-NH_2 (red) and H-Phe-Phe-NH_2 (blue) (2.4 – 3.3 ppm)

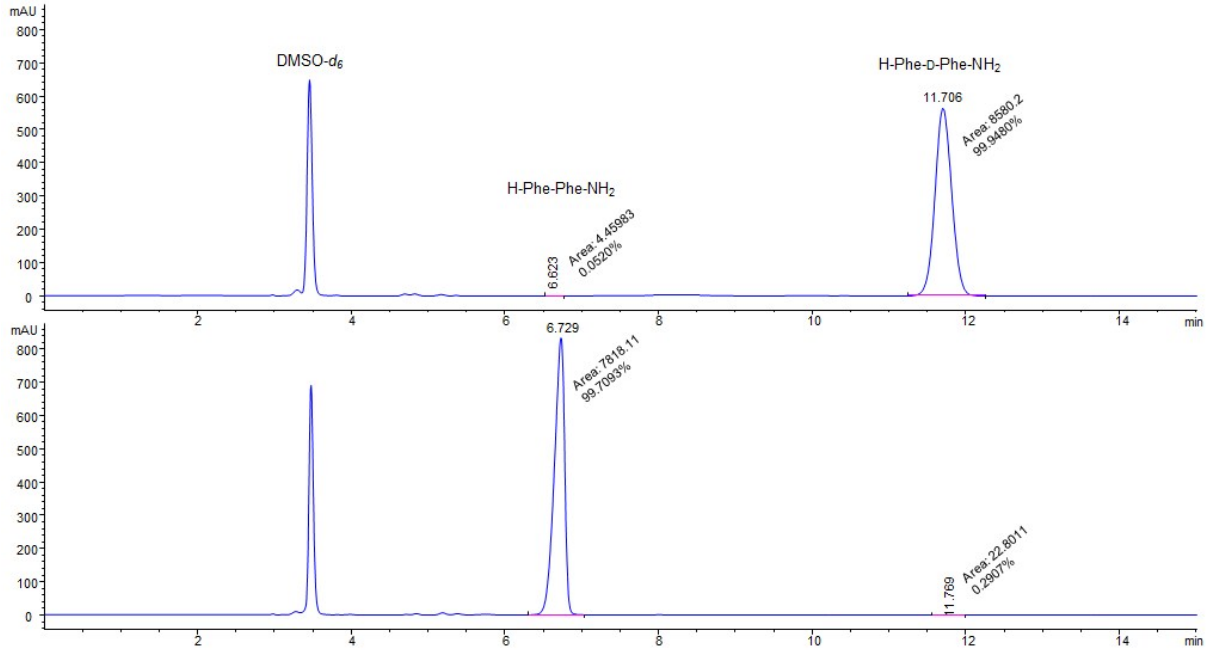


Swelling properties of PS-Rink resin in chosen solvents

Solvent	Volume [mL/g]
2-MeTHF	5.1
2-MeTHF/MeOH (1:1)	3.0
MeOH	2.1

Resin swelling was evaluated according to reference: R. Santini, M. C. Griffith, M. Qi, *Tetrahedron Lett.,* 1998, **39**, 8951.

HPLC separation of dipeptides H-Phe-D-Phe-NH_2 and H-Phe-Phe-NH_2 prepared using protocol C



Zoomed area of HPLC separation of dipeptides H-Phe-D-Phe-NH_2 and H-Phe-Phe-NH_2 prepared using protocol C (6 – 13 min)