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Highly Productive Continuous Flow Synthesis of Di- and Tripeptides in Water

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Supporting Information

Contents

1. Approximate heat flow and consumption of base during dipeptide formation

Understanding the reaction heat flow and stoichiometry are important. Since a conventional calorimeter was unsuitable for this reaction, the bespoke reactor shown in Section 7.2 was used as a rudimentary calorimeter with lagged walls to minimise heat loss. Furthermore the rapid reaction and 300 mL volume facilitated heat flow measurement. The titration of base to maintain pH 10.2 provided understanding of the main- and side reactions.

(i) As a control, without stirring or any addition the temperature of the reaction mass initially at 0 °C rose to 2.2 °C over 10 minutes, ie a temperature difference of 20 °C caused the reactor water temperature to rise at 0.22 °C/min.

(ii) A fed-batch control reaction was run to establish the temperature rise due to the high shear stirrer at 4000rpm and the addition of 18ml KOH at ambient over 10 minutes. This caused the water temperature to rise at 0.85 °C/min.

(iii) The reactor was set-up as a solid NCA fed-batch. To the reactor was added 300 mL of the reaction solvent (0.1M $Na_2B_4O_7$ aq. or water/base) containing 60mmol L-Phe the pH was adjusted to pH10.2 by addition of base as necessary. The reaction mass was stirred (4000 rpm) and actively cooled to 0 ℃. Once at 0℃ the cooling was switched-off and to the reactor was then fed the required L-alanine-NCA solid (69 mmol, 1.15 eq.) over 8 minutes, and base titrated, at a rate necessary to maintain the reaction solution at pH 10.2, as in Figure S2. The internal reaction temperature was recorded every 30s and plotted as the observed temperature minus a cumulative 0.85°C giving a temperature rise due to the reaction of NCA and L-Phe of 0.54 °C/min. The graph shows after the end of the additions at 8 minutes, and for a further 7 minutes there is little heat flow,

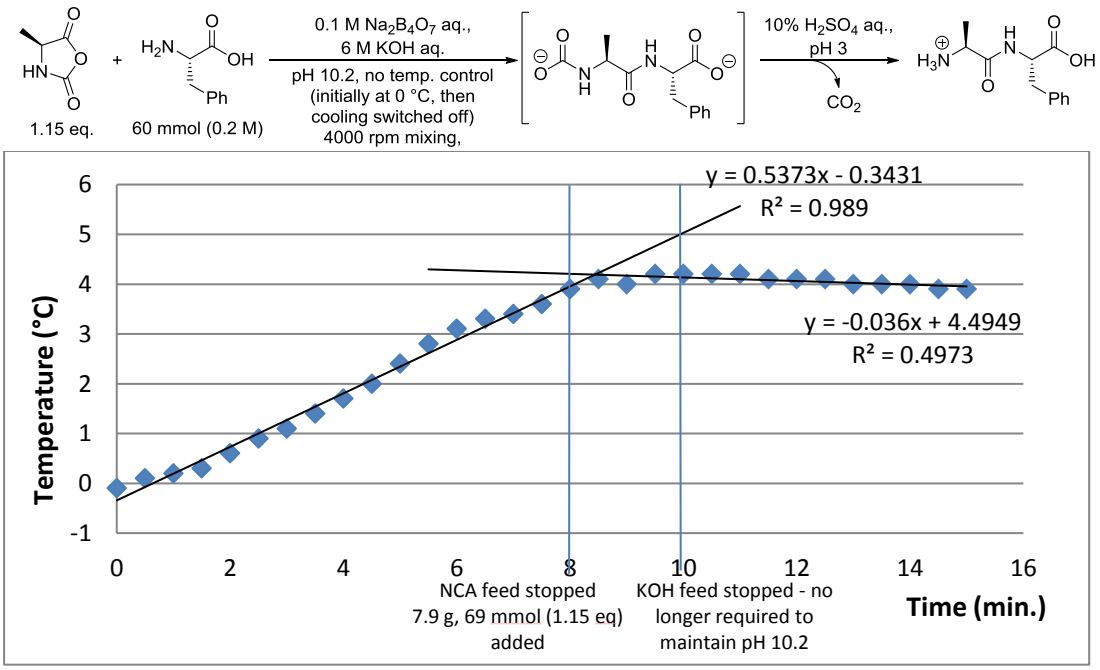


Figure S1. Temperature rise for addition of L-alanine-NCA (69 mmol, 1.15 eq.) to L-phenylalanine (60 mmol) at constant volume 300mL (fed batch). pH maintained at 10.2 with addition of 6 M KOH aq. as necessary. The temperature rise due to mixer 4000rpm and KOH addition at room temperature has been subtracted.

therefore calculation of the exotherm is valid over this short time. At the end of the reaction the mass was acidified with 3M sulfuric acid to allow decarboxylation of the carbamate intermediate and analysed to determine conversion by NMR and HPLC.

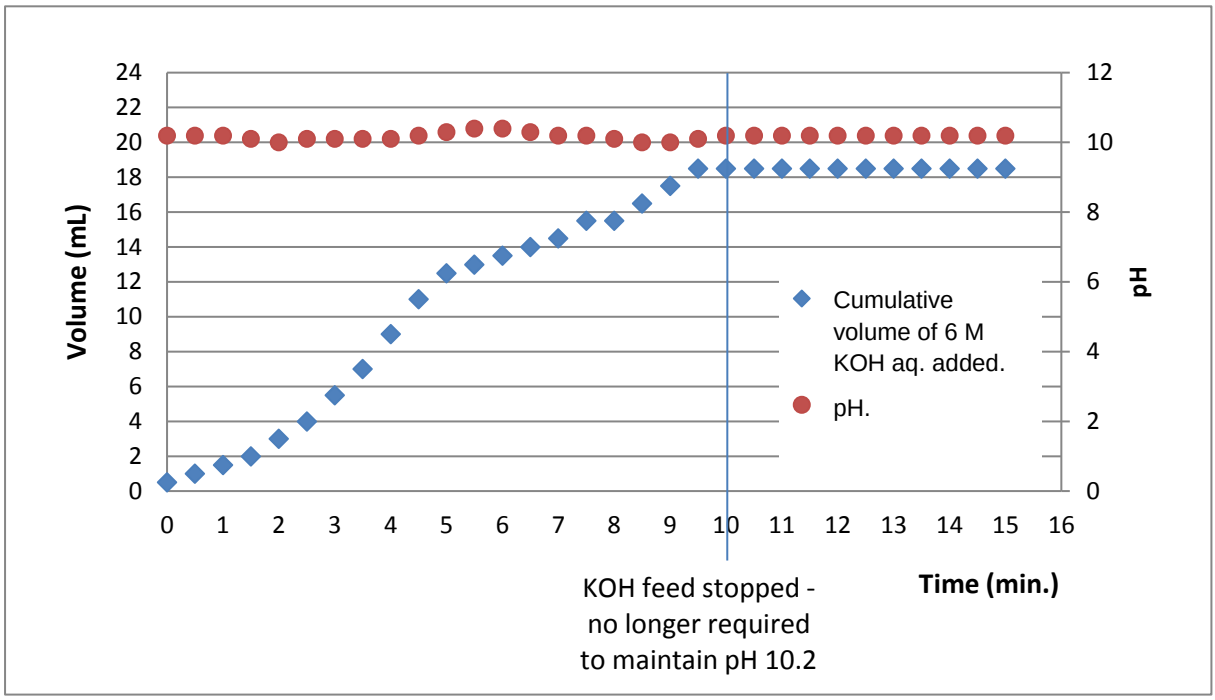
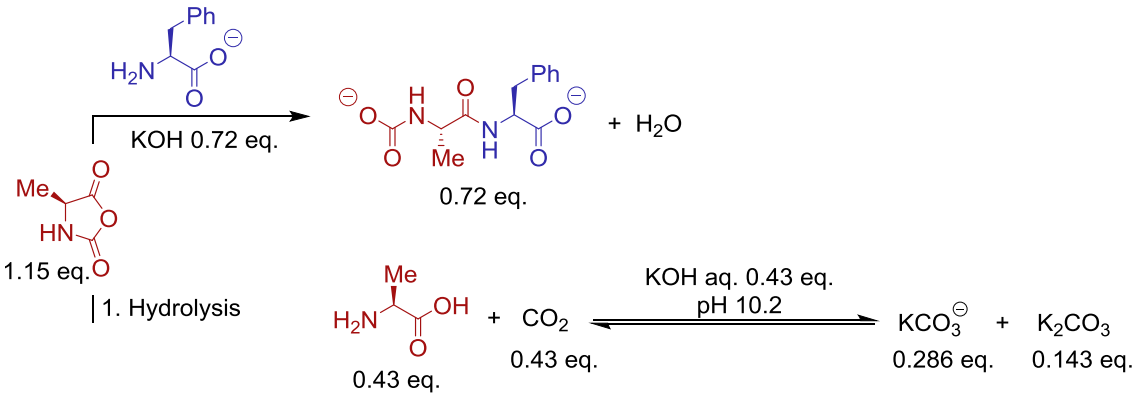


Figure S2. Volume of KOH used for addition of L-alanine-NCA (69 mmol, 1.15 eq.) to L-phenylalanine (60 mmol) at constant volume 300mL (fed batch). pH maintained at 10.2 with addition of 6 M KOH aq. as necessary. Temperature rise due to mixer 4000rpm, KOH addition and room temperature atmosphere has been subtracted.

60 mmol L-phenylalanine, 69 mmol L=alanyl NCA, total KOH used = 111 mmol
^{1}H NMR showed 72% conversion of phenylalanine to L-ala-L-phe (43.2 mmol L-ala-L-phe). 43.2 mmol of KOH and L-alanyl NCA used for formation of 43.2 mmol L-ala-L-phe. Remaining 67.8 mmol KOH reacts with remaining 25.8 mmol L-alanyl NCA. Since hydrolysis with hydroxide and decarboxylation release in theory 1 mole equivalent of acid, 67.8-25.8 mmol leaves 42 mmol base that react with 25.8 mmol CO_2 to form at pH 10.2 a 63:37 mixture of carbonate: bicarbonate giving a pKa of 10.6 in this solution of high ionic strength compared to the literature pKa = 10.3 in pure water.

Scheme S1. Proposed consumption of KOH



2. Steady state and residence time distribution determination

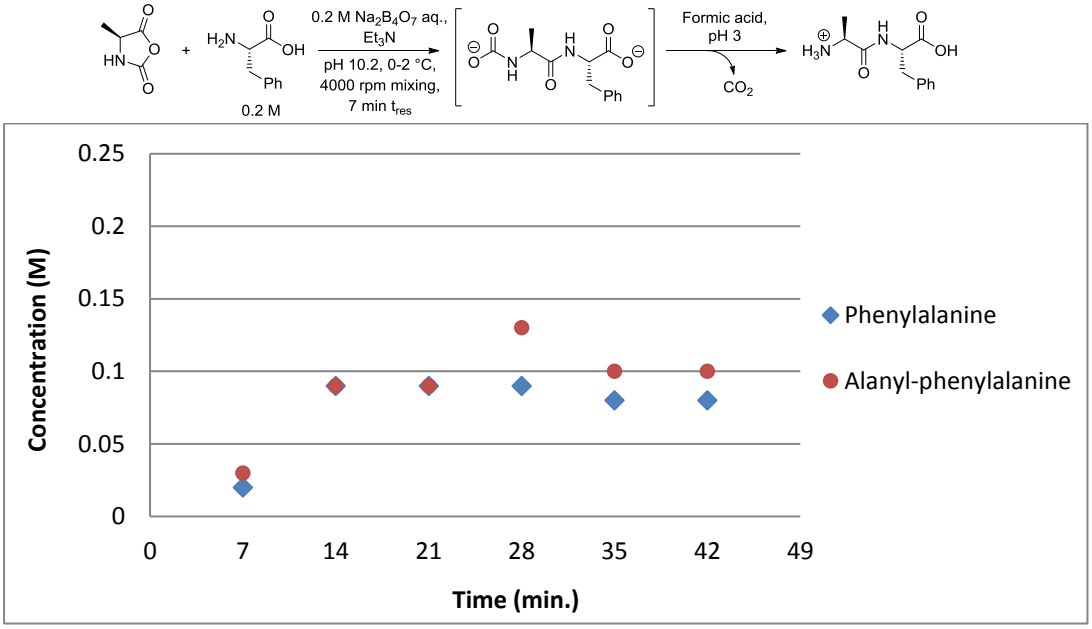


Figure S3. Concentration of phenylalanine and alanylphenylalanine over time during continuous dipeptide formation. Concentrations determined by HPLC by comparison with biphenyl external standard.

The concentration of starting material and dipeptide product stabilises from the second residence time (14 min.). Apparent steady state is achieved after 2 residence times.

The residence time distribution for the bespoke reactor shown in section 7 was determined colorimetrically using a pulse of food dye with fractions collected every minute and flow rate of 43mL/min to give a theory residence time of 7 minutes. The measured average residence time $t^*(E_t)$ taken from integration of the curve in Figure S4 was 8.6 minutes.

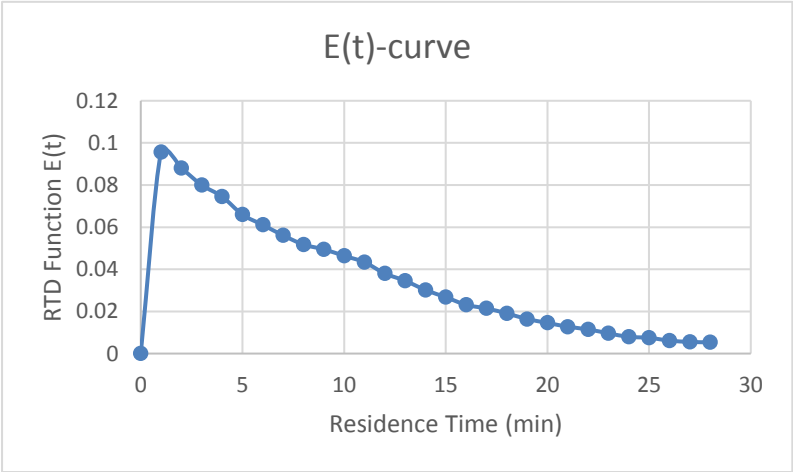


Figure S4. Residence time distribution curve based on converting food dye absorbance to the RTD function E(t) as a function of time with fractions collected every minute.

3. Effect of slow mixing speed (1200 rpm) on reaction

Figure S5. Build-up of NCA on surface of reaction solution

4. Comparison of crude reaction product for formation of L-val-phe with 1.1 and 1.5 eq. L-valyl NCA.

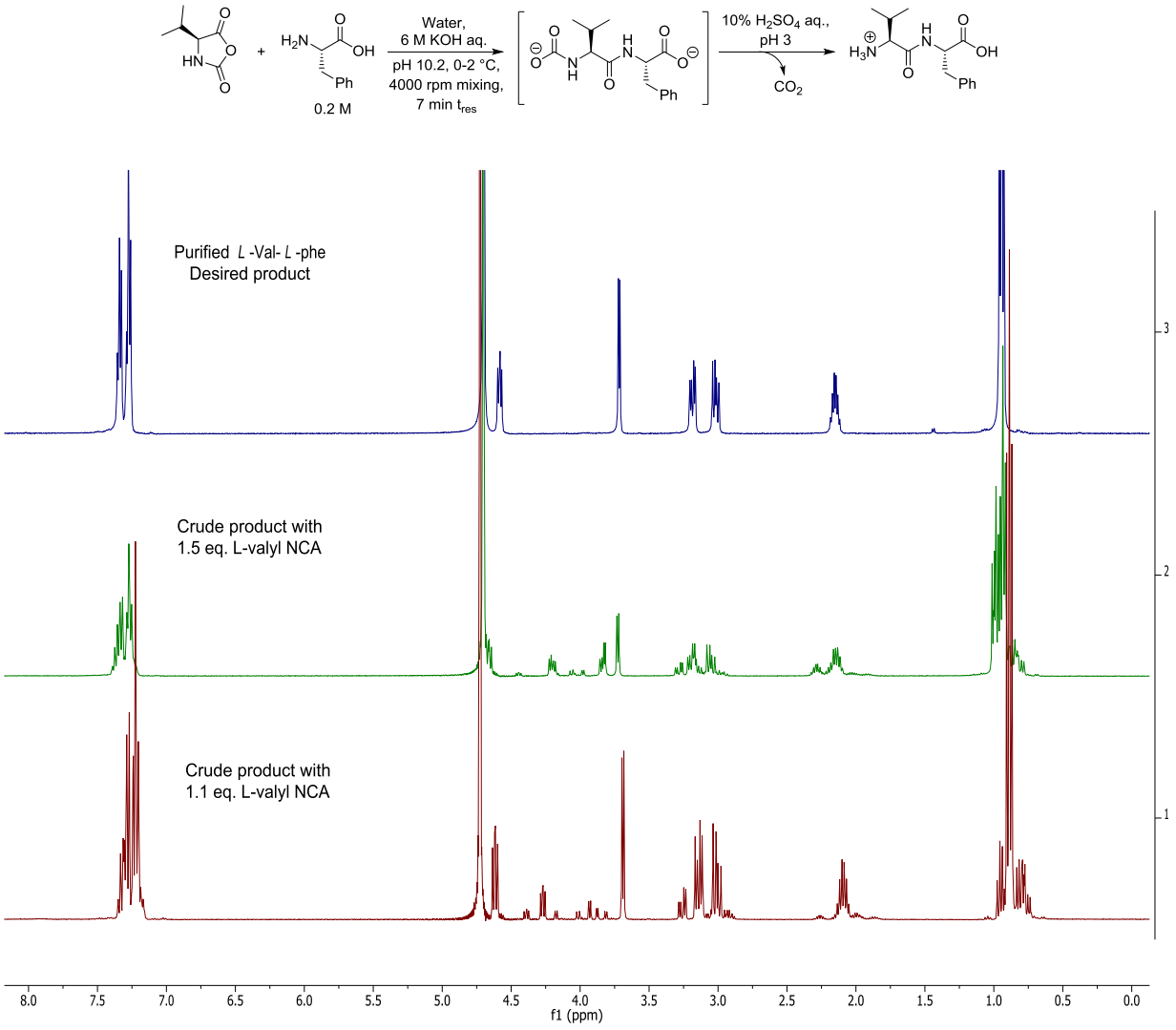
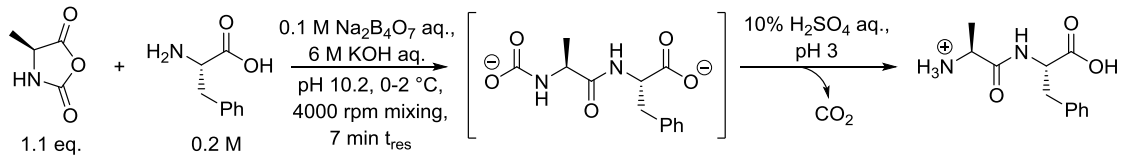


Figure S6. ^{1}H NMR spectra for formation of L-val-L-phe with 1.1 and 1.5 eq. NCA.

5. Mass Balance



Mass directed auto preparative chromatography (for method see Experimental section):

10 mL of crude reaction purified. Fractions containing L-phe (reagent), L-ala-L-phe (product) and L-ala-L-ala-L-phe (side product) were combined and dried.

L-Phe: 40 mg, 0.24 mmol.

L-Ala-L-phe: 400 mg, 1.7 mmol.

L-Ala-L-ala-L-phe: 20 mg, 0.065 mmol.

Total moles L-phe (present in 3 compounds) = 2 mmol. In 10 mL = 0.2 M.

Concentration of L-phe reagent solution = 0.2 M.

6. Reaction metrics

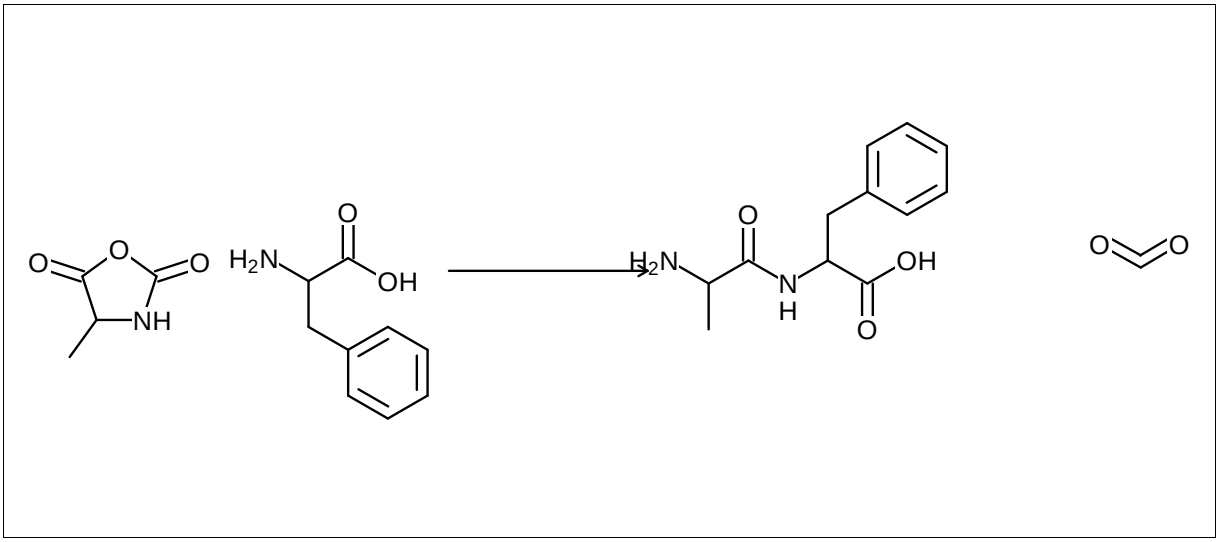
Metric	SPPS[1]	Solution phase synthesis (batch)[3]	Continuous un-buffered solution phase synthesis[a]	Continuous peptide formation[2]
Peptide	L-Leu-L-Ala[a]	L-Ala-L-Phe	L-Val-L-Phe	N-Boc-D-phenylgly-O-allyl-L-phe
Yield (%)	50	70	82	97
Space time yield ($g.L^{-1}h^{-1}$)	0.014[b]	0.35	60	
Total Mass intensity:	3165	686	28	840
Reaction mass efficiency (%)	4	57	67	44.9
Atom economy (%)	10	84	86	57.3
Health and safety issues	DMF	$Na_2B_4O_7$	None	DMF

[a]See main paper, Figure 3.

Metrics were calculated according to recent literature.[4]

6.1 Solution phase dipeptide synthesis (batch)

Hirschmann et al. J. Org. Chem. 1967, **32**, 3415-3425.



Status: First pass metrics entry

Procedure

Step 1

Reactor types: Batch;

Conditions: 0 ℃; 4:00 (hh:mm); 1 bar; under Air

Reactants: **L-Alanine-NCA** (2.53 g, 22 mmol); **L-phenylalanine**‡ (3.3 g, 20 mmol);

Reagents: **potassium tetraborate tetrahydrate** (61.1 g, 200 mmol); **KOH** (2.24 g, 40 mmol);

Solvents: **H_2O** (200 mL);

Workup

Techniques: Quenching; Filtration;

Chemicals: **Sulphuric acid** (5.52 g); **Carbon** (330 g);

Solvents: **H_2O** (1.3 L); **Acetic Acid** (120 mL); **acetone** (300 mL);

Products

L-alanyl-L-phenylalanine† (3.31 g, 14 mmol)

Carbon Dioxide (616 mg, 14 mmol)

Name	Value/Reason	Flag
Yield	70%	
Conversion	100%	
Selectivity	70%	

Reaction Mass Efficiency	56.7
Atom Economy	84.3
Solvents	
Health & Safety	
Mass intensity: Total	686
Mass intensity: Reaction	81.4
Mass intensity: Reaction chemicals	20.9
Mass intensity: Reaction solvents	60.5
Mass intensity: Workup	604
Mass intensity: Workup chemicals	101
Mass intensity: Workup solvents	604
Catalysts used	
Catalysts recovered	
Reactor	
Elements	
Energy	
Workup	

Productivity calculation
3.31 g produced in 4 h* in 2325 ml reaction volume
= 0.356 g/L/h

* based on similar experiment carried out in our laboratories

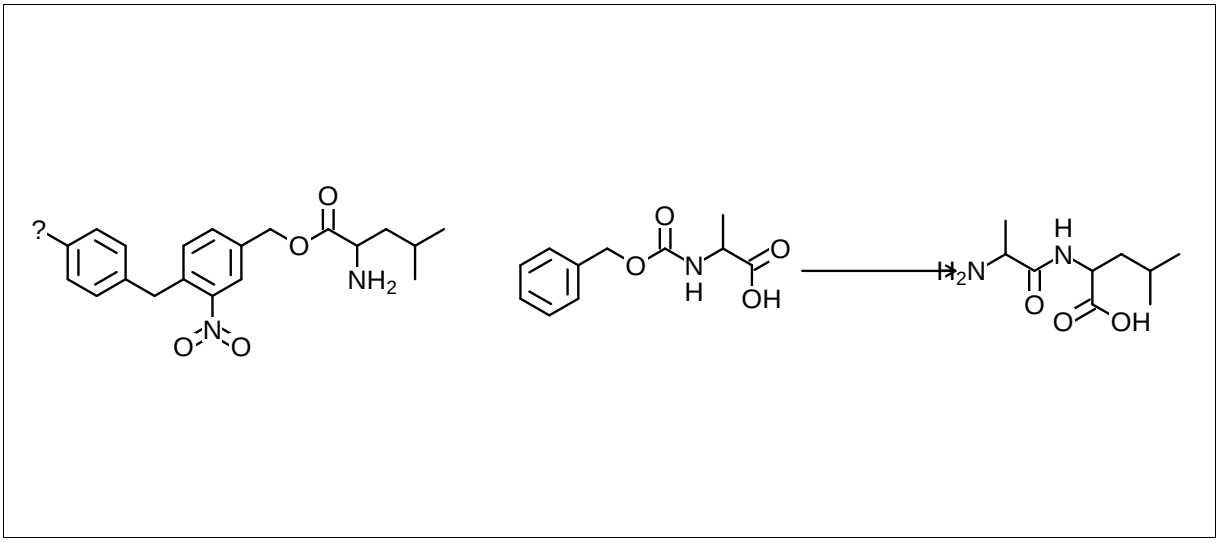
6.2 Solid phase synthesis (batch)

Merrifield J. Am. Chem. Soc. 1963, 85, 2149-2154

Preparation described for L-leucyl-L-alanylglycyl-L-valine. Calculation of green metrics based on hypothetical process to make L-leucyl-L-alanine: solid-phase reaction of protected L-alanine with solid supported L-leucine, deprotection and cleavage from the resin.

- Yield of tetrapeptide reported as 265 mg, 0.74 mmol, 13%; therefore assume 50% dipeptide yield 0.5^3 = 0.125); 2.86 mmoles * 202 = 0.575g
- Reaction time (excluding purification) for tetrapeptide = 85 hours; for dipeptide = 23 + 6 + 2.5 = 31.5 h

- In the published procedure, volumes were not given for solvent used in wash steps. For the metrics calculations we have assumed 50 mL solvent for each wash.



Status: First pass metrics entry

Procedure

Step 1

Reactor types: Batch;

Conditions: 25 ℃; 30:00 (hh:mm); under Air

Reactants: **N-carbobenzoxy-valyl-nitropolymer**‡ (10 g, 5.6 mmol); **N-carbobenzoxy-leucine** (4.46 g, 16.8 mmol);

Reagents: **Acetic Acid** (343 g, 5.71 mol); **HBr** (4.47 g, 55.2 mmol); **1,3-dicyclohexylcarbodiimide** (3.47 g, 16.8 mmol); **Et3N** (2.9 g, 28.7 mmol); **1M HCl** (9.15 g, 251 mmol); **NaOH** (2.1 g, 52.5 mmol);

Solvents: **Dimethylformamide** (495 mL); **EtOH** (390 mL); **H_2O** (50 mL);

Workup

Techniques: Evaporation; Filtration;

Product

alanyl-leucine† (575 mg, 2.84 mmol)

Metrics

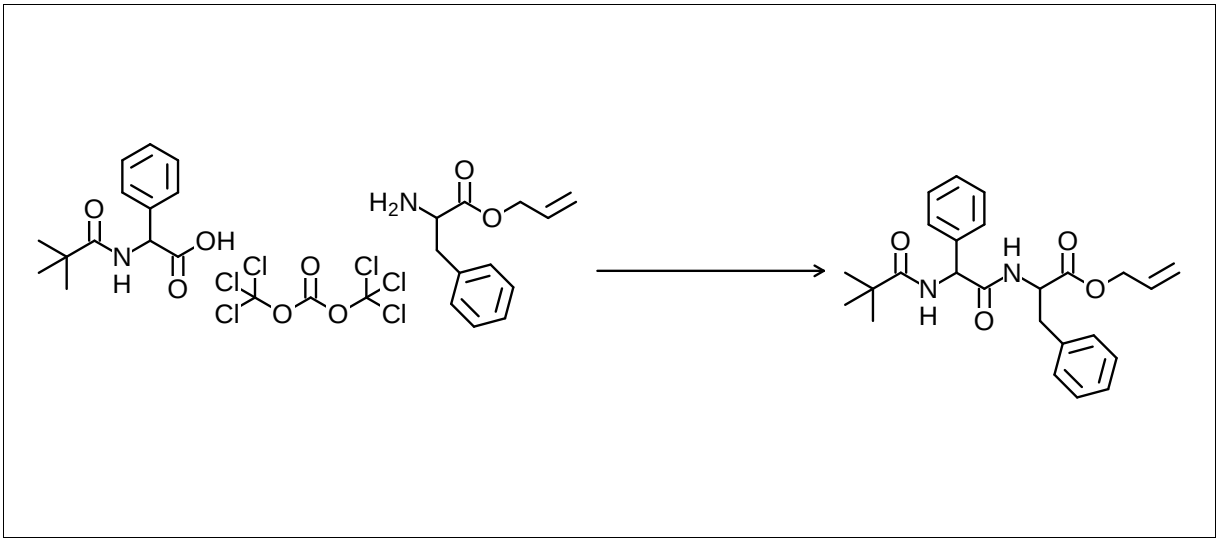
Name	Value/Reason	Flag
Yield	50%	
Conversion	100%	

Selectivity	50%
Reaction Mass Efficiency	3.98
Atom Economy	9.86
Solvents	
Health & Safety	
Mass intensity: Total	3165
Mass intensity: Reaction	2095
Mass intensity: Reaction chemicals	660
Mass intensity: Reaction solvents	1435
Mass intensity: Workup	1070
Mass intensity: Workup chemicals	0
Mass intensity: Workup solvents	1070
Catalysts used	
Catalysts recovered	
Reactor	
Elements	
Energy	
Workup	

Productivity calculation
0.575g produced in 30 h in 1314 ml reaction volume
= 0.014 g/L/h

6.3 Solution phase dipeptide synthesis (flow: microflow tube reactor)

Fuse et al. Angew. Chem. Int. Ed. 2014, 53, 851-855



Status: First pass metrics entry

Procedure

Step 1

Reactor types: Flow;

Conditions: under Air

Reactants: **(2,2-dimethylpropanamido)(phenyl)acetic acid** (108 mg, 0.46 mmol); **Triphosgene**‡ (22 mg, 0.074 mmol); **prop-2-en-1-yl 2-amino-3-phenylpropanoate** (36.9 mg, 0.18 mmol);

Reagents: **DIPEA** (59.5 mg, 0.46 mmol);

Solvents: **MeCN** (2.1 mL); **Dimethylformamide** (1.3 mL);

Workup

Techniques: Filtration; Evaporation; Chromatography; Aqueous workup;

Chemicals: **Ammonium Chloride** (1 g); **Magnesium sulfate** (2 g); **sodium bicarbonate (aq. saturated)** (11.5 g);

Solvents: **Saturated brine** (10 mL); **H_2O** (10 mL); **Chloroform** (15 mL);

Product

prop-2-en-1-yl 2-[2-(2,2-dimethylpropanamido)-2-phenylacetamido]13-phenylpropanoate† (73.9 mg, 0.175 mmol)

Metrics

Name	Value/Reason	Flag
Yield	97%	
Conversion	100%	
Selectivity	97%	

Reaction Mass Efficiency	44.2
Atom Economy	57.3
Solvents	
Health & Safety	
Mass intensity: Total	840
Mass intensity: Reaction	42
Mass intensity: Reaction chemicals	3.06
Mass intensity: Reaction solvents	38.9
Mass intensity: Workup	798
Mass intensity: Workup chemicals	196
Mass intensity: Workup solvents	798
Catalysts used	
Catalysts recovered	
Reactor	
Elements	
Energy	
Workup	

6.4 Solution phase dipeptide synthesis (flow: CSTR)

Continuous dipeptide formation without buffer and with automated pH control

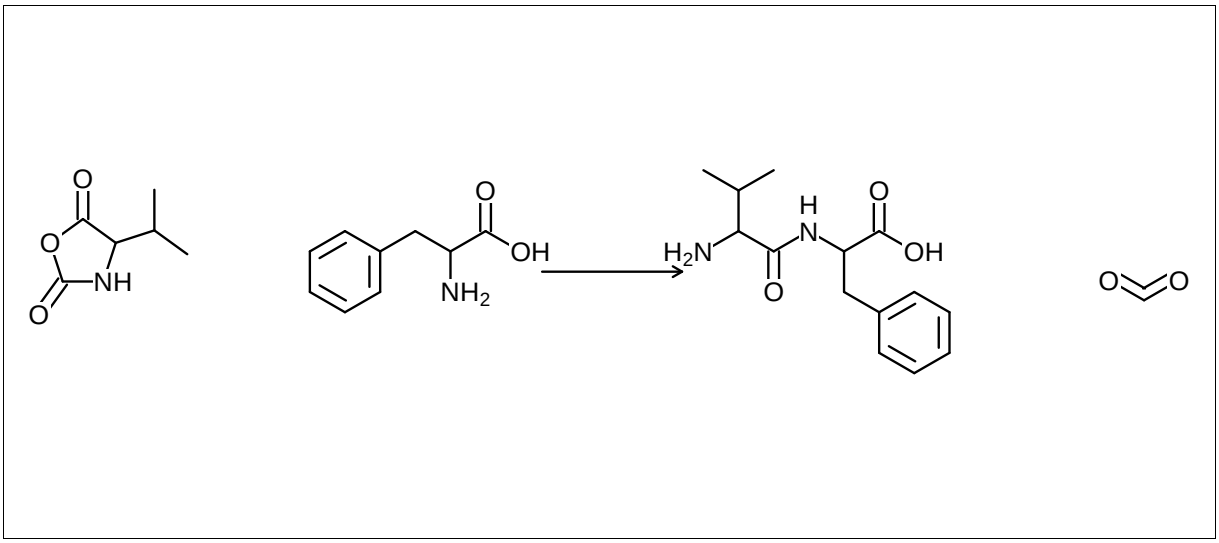
- Reaction carried out at 0-2 ℃
- Residence time = 7 min. Reaction run for 35 min. (5 residence times)
- Yield of product is 82% based on representative purified sample (see below)

    - Purification
      Mass directed prep:
      To purify 9.9 mL of reaction solution: 11 injections of 900 µL
      8 min. run time per injection, 20 mL/min flow rate
      5-95% MeOH in water with 1 % formic acid. Per injection: 160 mL solvent (79.2 mL MeOH, 79.2 mL Water, 1.6 mL formic acid)
    - Column chromatography:
      To purify 20 mL of reaction solution:

Ion exchange chromatography first: Estimated amounts: ~50 g DOWEX 50WX4 100-200 mesh, 500 mL eluent (aqueous at pH 5, 5.5 and 6)

Followed by silica gel flash chromatography:

Estimated amounts: ~50 g silica, 500 mL eluent (DCM:MeOH:18% NH_4OH aq. 30:60:10).



Status: First pass metrics entry

Procedure

Step 1

Reactor types: Flow;

Conditions: under Air

Reactants: **L-valyl-NCA** (47.2 g, 330 mmol); **L-phenylalanine**‡ (49.6 g, 300 mmol);

Reagents: **KOH** (30.3 g, 540 mmol); **KOH** (33.7 g, 600 mmol); **H_2SO_4** (10 mL, 188 mmol);

Solvents: **H_2O** (1.7 L);

Products

L-val-L-phe† (65 g, 246 mmol)

Carbon Dioxide (10.8 g, 246 mmol)

Metrics

Name		Value/Reason	Flag
Yield	82%		
Conversion	100%		
Selectivity	82%		
Reaction Mass Efficiency	67.2		

Atom Economy	85.7
Solvents	
Health & Safety	
Mass intensity: Total	28.4
Mass intensity: Reaction	28.4
Mass intensity: Reaction chemicals	2.29
Mass intensity: Reaction solvents	26.1
Mass intensity: Workup	0
Mass intensity: Workup chemicals	0
Mass intensity: Workup solvents	0
Catalysts used	
Catalysts recovered	
Reactor	
Elements	
Energy	
Workup	

Productivity calculation
65g produced in 35min in 1840 ml reaction volume
= 60.5 g/L/h

7. Experimental

7.1 Chemicals and instrumentation

Commercially available chemicals used in this work were obtained from Sigma Aldrich and Fluorchem of reagent or reagent plus grade. L-Alanyl NCA and L-valyl NCA were obtained from Isochem and were stored under N_2 at -20 °C.

NMR: Bruker DPX-300 (300 MHz), Bruker Ascend 400 (400 MHz) or Bruker DRX-500 (500 MHz) instruments using D_2O solvent.

HPLC: Agilent 11oo Series HPLC with Chemstation software for processing

LCMS: Bruker HCT Ion trap mass spectrometer coupled with Agilent 1100 HPLC

HRMS: Bruker Maxis Impact sprectrometer with Ultimate 3000 UPLC

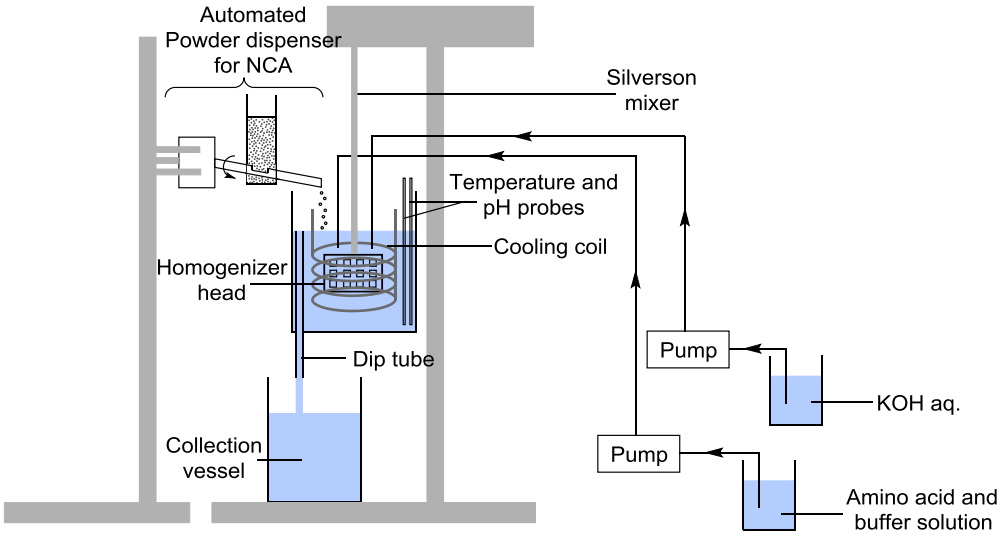
IR: Bruker ALPHA FT-IR spectrometer

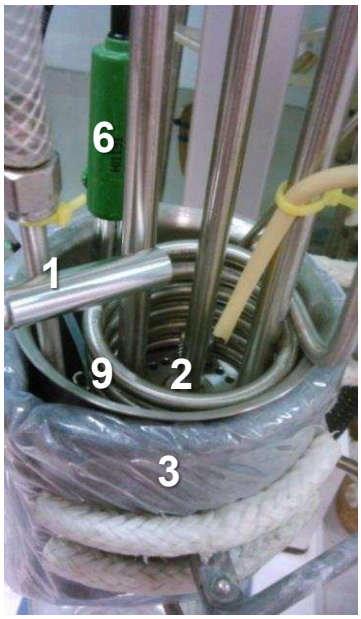
Melting point: Stuart Melting Point Aparatus SMP30

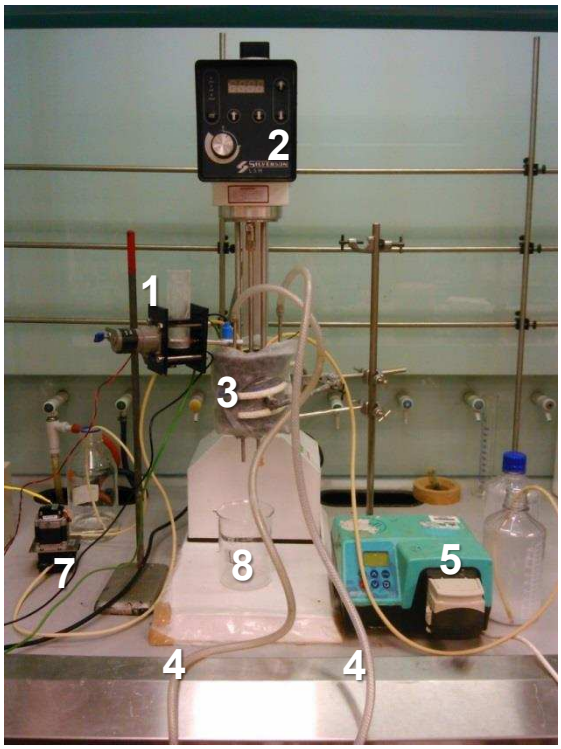
Optical rotation: Schmidt and Haensch Polartronic H 352 with 1 dm cell.

Mass Directed Automated preparative chromatography (MDAP): Agilent Technologies 2120 Quadrupole mass spectrometer with Agilent Technologies 1260 Infinity HPLC.

7.2 Experimental set up







1: Powder dispenser for NCA addition
2: Silverson mixer
3: Stainless steel reactor (600 mL) with aerogel insulation jacket
4: Coolant feeds
5: Pump for buffer/amino acid solution
6: pH and temperature probes
7: Pump for KOH aq. feed
8: Collection vessel
9: Cooling coil

7.3 Equipment information

Mixer: Silverson mixer with a 50 mm diameter homogeniser.

Pumps: Watson Marlow SCI 323 peristaltic pump with Watson Marlow marprene tubing 1.6 mm bore diameter, 2.4 mm wall thickness.

250 Series stepper motor pump, SMB driver, 4 rollers. 1.6 mm bore Norprene tubing.

Reactor vessel: 600 mL stainless steel beaker

Modifications made in-house.

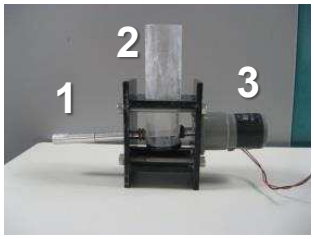
- Adjustable height overflow pipe added
- Drain pipe added

1. Drain
2. Overflow pipe

Powder dispenser: Lymann E-ZEE Flo Universal adjustable powder trickler

Modifications made in-house

- Base removed
- Material reservoir replaced
- Motor added to feed pipe



1. Feed pipe
2. Solid container
3. Motor

Chiller: Huber Ministat 125

Insulation for reactor vessel: Aerogel thermal insulation

Cooling coil: stainless steel pipe 6 mm OD.

The pH controller was made in-house and specially tailored for the application from the following elements

- Arduino UNO development board
- Atlas Scientific EZO pH Circuit
- Atlas Scientific scientific grade silver / silver chloride pH Probe
- Atlas Scientific 125 mL calibration solutions (pH 4, 7 and 10)
- Atlas Scientific Female BNC Spec. Thread size ¼" – 36 UNS Thread type
- Atlas Scientific Inline Voltage Isolator
- Adafruit Analog Output K-Type Thermocouple Amplifier – AD8495 Breakout
- Thermosense Mineral Insulated k-type Thermocouple Sensor with Pot Seal
- Adafruit RGB LCD Shield

A hardware serial communication was established between the microprocessor and the Atlas Scientific EZO chip to access the pH readings, being the ground of this chip isolated from the pump's using the Inline Voltage Isolator to prevent electrical noise affecting the pH readings.

The pH probe was calibrated using three point calibration with the calibration solutions at room temperature.

The temperature is acquired by the k-type thermocouple, amplifying its signal with the thermocouple amplifier chip and reading its value using the built-in ADC in the board. This value is further processed to calculate the temperature in Celsius. Since the temperature will change during operation, its effect on the pH readings is compensated using the equation below

$$pH_{CORRECTED} = 7.0 + (pH - 7.0) * {}^{T}/_{T_0}$$

Where pH is the value of the pH read by the probe, T is the temperature of operation in Kelvin read by the thermocouple and T_0 is the temperature of calibration, room temperature (298.15 K) in the current case.

A PI control loop was programmed in the microprocessor, being the corrected pH process variable. The PI control parameters were estimated using lambda tuning from step input experiments information and then modified manually to achieve a robust control.

In each call, the PI algorithm computes a new output signal that controls the speed of the 250 Series Stepper Motor Pump, which feeds the KOH aq. solution to the reactor in order to reach the desired pH Setpoint.

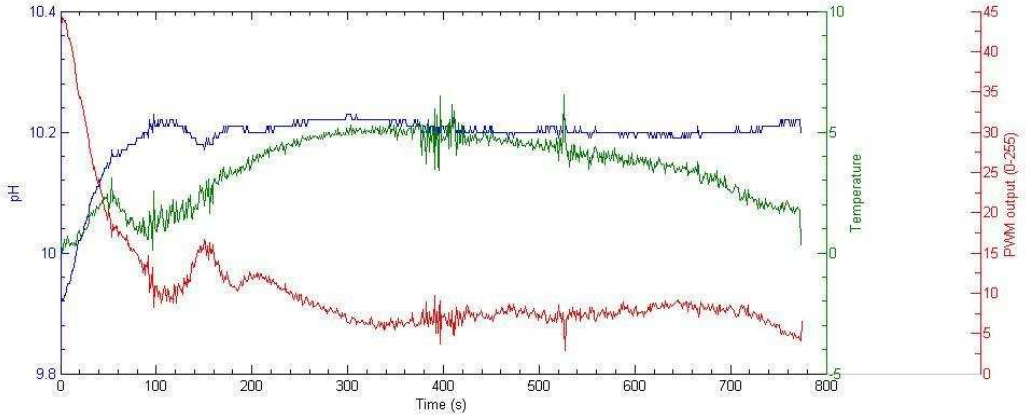


Figure S7. pH (blue), temperature (green) and control algorithm output (red) signals for the first 800s of operation. Once the pH setpoint (10.2) is reached, its value remains in a margin of +/- 0.02

Furthermore, an additional serial communication was set to share the temperature, pH, and output signal values with a computer via USB connection, which made possible to track the values of temperature and pH in the reactor during operation.

An LCD screen was installed to show the user the pH and temperature values at all times.

7.4 General experimental procedure

The equipment was set-up as in the diagram and photos above and the overflow pipe was set at the 300 mL level. To the reactor was added 300 mL of the reaction solvent (0.1M $Na_2B_4O_7$ aq. or water/base) and the pH was adjusted to pH10.2 by addition of base as necessary. The solvent was stirred (4000 rpm) and cooled to 0 °C. Once at 0°C, to the reactor was then fed 0.2 M, pH 10.2 aqueous amino acid solution (41 mL/min, 8.2 mmol/min.), the required NCA (9 mmol/min.) and base, if required, at a rate necessary to maintain the reaction solution at pH 10.2. The reaction was stirred at 4000 rpm and the temperature maintained between 0-2 °C.

The reaction solution was collected via the overflow pipe. During the reaction, the reaction temperature, pH and flow rate of base were recorded each minute. After 7 min. (1 residence time), 300 mL (1 reactor volume) of solution had been collected. The collection vessel was replaced and the process repeated until the required number of reactor volumes had been collected. Once collected, each reactor volume was acidified to allow decarboxylation of the collected product. The products from each individual reactor volume were analysed as below:

NMR: 1 mL of the reaction solution was dried by rotary evaporation. A portion of the obtained solid was analysed by NMR in D_2O. For the initial reactions, a known mass of NaOAc was added to the NMR sample as an external standard however results from this were found to be inconsistent and were not used.

HPLC: 20 µL of the reaction solution is added to 10 µL of 0.1M biphenyl in MeOH (external standard). To this is added 500 µL MeOH and 470 µL H_2O. Grace Davidson Vydac C18(218TP) 250 x 4.6 mm, pore size 300 Å, particle size 5 µm, pH 2-7.5; 210 nm; 25 °C column oven; 10 µL injection volume; 1 mL/min.; solvent A: Water + 0.1% TFA; solvent B: MeCN + 0.1% TFA; Gradient: 0 min 5% B, 16 min 40% B, 18 min 40% B, 20 min 80% B, 27 min 80% B, 28 min 5% B.

Retention times:
L-Phenylalanine: 7.2 min.
L-Alanyl-L-phenylalanine: 8.9 min.
DL-Alanyl-DL-phenylalanine: 8.9 and 10.6 min.
L-Alanyl-L-alanyl-L-phenylalanine: 14.0 min.
L-Valyl-L-phenylalanine: 14.7 min.
L-Alanyl-L-leucine: 10.2 min.
L-Leucine: 7.0 min.
L-Valyl-L-alanyl-L-leucine: 17.3
L-Valyl-L-aspartyl-L-phenylalanine: 18.0 min.
L-aspartame: 16.2 min.
Biphenyl (external standard): 25.7 min.

7.5 Purification

Mass directed auto preparative chromatography (MDAP)

Small quantities (5-10 mL) of the crude reaction solutions were purified by MDAP to give pure samples of the desired reaction products for characterisation (see 4.5 Peptide products).
MDAP method: Xbridge Prep C18 5 µm OBD™ 19x100 mm. 5-95% MeOH in water with 0.1% formic acid over 8 min., 20 mL/min flow rate.

Ion exchange Chromatography

The ion exchange resin (Dowex 50WX4 100-200 mesh) was washed with water. 20 mL of the crude reaction solution was basified to pH 5.5 with 1 M KOH aq. was loaded onto the column. The products were eluted with firstly a pH 5.5 aqueous solution, followed by pH 5.7 and finally pH 6. The fractions collected were analysed by TLC (Si gel, $ChCl_3$:MeOH:NH_4OH 18% aq. 50:40:10, visulaisation by UV and PMA dip.)

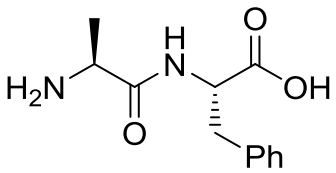
This method was found to successfully remove salts and tri-, tetra-, etc. peptide side products from buffer free reactions. For L-alanyl-L-phenylalanine formation, the dipepteide was

successfully separated from residual phenylalanine starting material. For the formation of other dipeptides, the dipeptide and amino acid were not separated and subsequent silica gel flash chromatography was required.

Flash chromatography

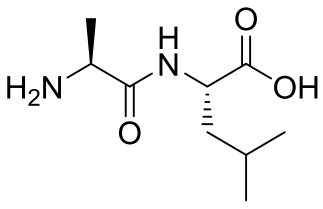
Following ion exchange chromatography, the dipeptide/amino acid mixture was loaded onto the silica gel column. The products were eluted with DCM:MeOH:NH_4OH aq. 18% 30:60:10. Fractions were analysed by TLC using the same solvent system and visualised by UV and PMA dip. For L-val-L-phe preparation, the dipeptide was obtained as an enriched mixture along with a reduced amount of phenylalanine than obtained from the ion exchange chromatography alone.

7.6 Characterisation data for peptide products



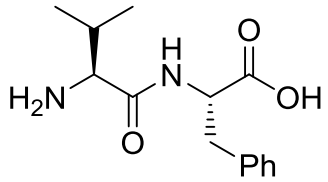
L-Alanyl-L-Phenylalanine

Mp 242-243°C; $[\alpha]_D^{20}$ +39.3 (589 nm, c 2, water) (lit.[5] $[\alpha]_D^{25}$ + 38.8 (L,L)); IR ν_{max} 3229.8, 2923.7, 1668.0, 1522.6, 1222.5, 1076.8, 739.4, 697.5, 608.7 cm^{-1}; ^1H NMR (500 MHz, D_2O) ^1H NMR (501 MHz, Deuterium Oxide) δ 7.43 (t, J = 7.3 Hz, 3H, CHAr), 7.38 – 7.31 (m, 4H, CHAr), 4.65 (dd, J = 8.9, 5.6 Hz, 1H, $CHCH_2Ar$), 4.03 (q, J = 7.1 Hz, 2H, $CHCH_3$), 3.29 (dd, J = 14.0, 5.6 Hz, 1H, CH^aH^bAr), 3.09 (dd, J = 14.1, 9.0 Hz, 2H, CH^aH^bAr), 1.53 (d, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, D_2O) 175.78 (C=O), 170.37 (C=O), 137.11 (CiHAr), 129.30 (2 x C_oHAr), 128.83 (2 x C_mHAr), 127.19 (C_pHAr), 55.36 ($CHCH_2$), 49.01 ($CHCH_3$), 36.78 (CH_2), 16.49 (CH_3); LRMS m/z (ESI) 237.3 (M^+ + H); HRMS m/z (ESI): M^+ + H, 237.12342 $C_{12}H_{17}N_2O_3$ requires M 237.12337.



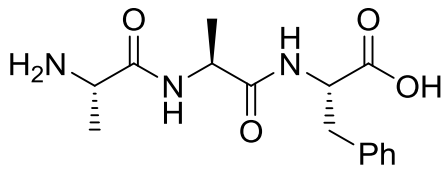
L-Alanyl-L-Leucine

Mp 226-227°C; $[\alpha]_D^{20}$ -30.2 (589 nm, c 1, water); IR ν_{max} 3218.1, 3067.3, 2956.8, 1667.4, 1520.7, 1049.5, 606.6 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 4.42 (t, J=7.3 Hz, 1H, $CHCH_2CH(CH_3)_2$), 4.15 (q, J=7.1 Hz, 1H, $CHCH_3$), 1.73-1.72 (m, 3H, CH_2 and $CH(CH_3)_2$ overlapping), 1.60 (d, J=7.0 Hz, 3H, CH_3), 0.97 (dd, J=17.8, 6.3 Hz, 6H, $CH(CH_3)_2$); ^{13}C NMR (125 MHz, D_2O) 176.82 (C=O), 170.85 (C=O), 52.20 ($CHCH_2(CH_3)_2$), 49.02 ($CHCH_3$), 39.41 (CH_2), 24.57 ($CH(CH_3)_2$, 22.22 (CH_3), 20.72 (CH_3), 16.57 (CH_3); LRMS m/z (ESI) 203.4 (M^+ + H); HRMS m/z (ESI): M^+ + H, 203.13897 $C_9H_{19}N_2O_3$ requires M 203.13902.



L-Valyl-L-Phenylalanine

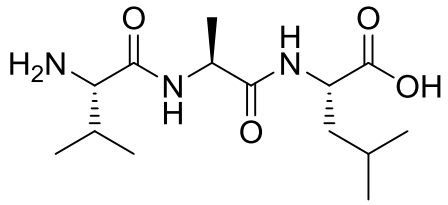
Mp 233-234°C; $[\alpha]_D^{20}$ +70 (589 nm, c 1, water); IR ν_{max} 3218.1, 3067.3, 2956.8, 1667.4, 1520.7, 1049.5, 606.6 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ7.44-7.41 (m, 2H, CHAr), 7.38 – 7.35 (m, 3H, CHAr), 4.67 (dd, J = 8.6 Hz, 1H, $CHCH_2Ar$), 3.80 (d, J = 5.6 Hz, 1H, $CHCH(CH_3)_2$), 3.27 (dd, J = 14.1, 5.8 Hz, 1H, CH^aH^bAr), 3.10 (dd, J = 14.1, 8.7 Hz, 1H, CH^aH^bAr), 2.28 – 2.18 (m, 1H, $CH(CH_3)_2$), 1.05 – 1.01 (m, 6H, $(CH_3)_2$); ^{13}C NMR (125 MHz, D_2O) 175.78 (C=O), 168.93 (C=O), 137.07 (C_iHAr), 129.29 (2 x C_oHAr), 128.85 (2 x C_mHAr), 127.20 (C_pHAr), 58.50 ($CHCH_2CH(CH_3)_2$), 55.54 ($CHCH(CH_3)_2$), 36.88 (CH_2), 30.09 ($CH(CH_3)_2$), 17.69 (CH_3CHCH_3), 16.69 (CH_3CHCH_3); LRMS m/z (ESI) 265.3 (M^+ + H); HRMS m/z (ESI): M^+ + H, 265.15510 $C_{14}H_{21}N_2O_3$ requires M 265.15467.



L-Alanyl-L-Alanyl-L-Phenylalanine

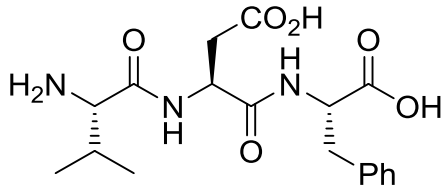
Mp 209-210 °C; $[\alpha]_D^{20}$ -13.6 (589 nm, c 1, water); IR ν_{max} 3261.8, 3063.9, 2935.8, 1632.8, 1529.9, 1077.7, 958.5, 696.5, 575.8 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ7.42-7.30 (m, 2H, CHAr), 4.63-4.54 (m, 1H, $CHCH_2Ar$), 4.34 (q, J = 7.2 Hz, 1H, $CHCH_3$), 4.08 (q, J = 7.1 Hz, 1H, $CHCH_3$), 3.24 (dd, J = 13.8, 5.4 Hz, 1H, CH^aH^bAr), 3.08 (dd, J = 13.8, 7.9 Hz, 1H, CH^aH^bAr), 1.51 (d, J = 7.0 Hz, 3H, CH_3), 1.37 (d, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, D_2O) 176.13

(C=O), 173.87 (C=O), 170.50 (C=O), 137.00 (CiHAr), 129.47 (2 x C_oHAr), 128.74 (2 x C_mHAr), 127.10 (C_pHAr), 55.13 ($CHCH_2Ar$), 49.82 ($CHCH_3$), 49.00 ($CHCH_3$), 37.17 (CH_2), 16.69 (CH_3), 16.65 (CH_3); LRMS m/z (ESI) 308.4 ($M^+ + H$); HRMS m/z (ESI): $M^+ + H$, 308.16069 $C_{15}H_{22}N_3O_4$ requires M 308.16048.



L-Valyl-L-Alanyl-L-Leucine

Mp 341-342 °C; $[\alpha]_D^{20}$ -93.4 (589 nm, c 1, water); IR ν_{max} 3295.1, 3065.6, 2961.1, 1644.7, 1541.2, 1232.1, 1160.8, 1044.2, 605.1 cm^{-1}; ^{1}H NMR (500 MHz, D_2O) δ4.48 (q, J = 7.2 Hz, 1H, $CHCH_3$), 4.41-4.35 (m, 1H, $CHCH_2$), 3.85 (d, J = 5.9 Hz, 1H, $CHCH(CH_3)_2$), 2.30-2.21 (m, 1H, $CH(CH_3)_2$), 1.74-1.69 (m, 3H, CH_2 and $CH(CH_3)_2$ overlapping), 1.45 (d, J = 7.2 Hz, 3H, CH_3), 1.08-1.06 (m, 6H, $(CH_3)_2$, 0.98 (d, J = 6.3 Hz, 3H, $(CH_3)_2$, 0.93 (d, J = 6.3 Hz, 3H, $(CH_3)_2$); ^{13}C NMR (125 MHz, D_2O) 177.11 (C=O), 174.27 (C=O), 168.89 (C=O), 58.45 ($CHCH(CH_3)_2$), 52.04 ($CHCH_2(CH_3)_2$), 49.58 ($CHCH_3$), 39.69 (CH_2), 30.10 ($CH_2CH(CH_3)_2$), 24.52 ($CHCH(CH_3)_2$), 22.32 (CH_3), 20.71 (CH_3), 17.65 (CH_3), 16.94 (CH_3), 16.50 (CH_3); LRMS m/z (ESI) 302.4 ($M^+ + H$); HRMS m/z (ESI): $M^+ + H$, 302.20749 $C_{14}H_{28}N_3O_4$ requires M 302.20743.



L-Valyl-L-Aspartyl-L-Phenylalanine

Mp 184-186 °C; $[\alpha]_D^{20}$ -5.6 (589 nm, c 1, water); IR ν_{max} 3193.3, 3066.0, 1713.8, 1660.3, 1517.5 1030.3, 581.8 cm^{-1}; ^{1}H NMR (500 MHz, D_2O) δ7.37-7.14 (m, 5H, CHAr in tripeptide and 2H CHAr in impurity), 4.69-4.67 (m, 1H, $CHCH_2CO_2H$), 4.49 (dd, J = 8.0, 5.3 Hz, $CHCH_2Ar$), 4.40-4.38 (m, 0.4H, impurity), 4.14-4.12 (m, 0.4H, impurity), 3.69 (d, J = 5.9 Hz, 1H, $CHCH(CH_3)_2$), 3.16 (ddd, J = 38.2, 13.8, 4.6 Hz, 1.4H, $CH^aH^bCO_2H$ and impurity), 2.98-2.95 (m, 1.4H,

$CH^aH^bCO_2H$ and impurity), 2.77 (dd, J = 16.7, 5.0 Hz, 1H, CH^aH^bAr), 2.64 (dd, J = 16.8, 8.8 Hz, 1H, CH^aH^bAr), 2.10-2.07 (m, 0.5H, impurity), 2.04-1.99 (m, 1H, $CH(CH_3)_2$), 0.85 (t, J = xx Hz, 6H, $CH(CH_3)_2$); ^{13}C NMR (125 MHz, D_2O) Complex spectra obtained due to unidentified impurity. Peaks could not be assigned; LRMS m/z (ESI) 380.4 (M^+ + H); HRMS m/z (ESI): M^+ + H, 380.18241 $C_{18}H_{26}N_3O_6$ requires M 380.18161.

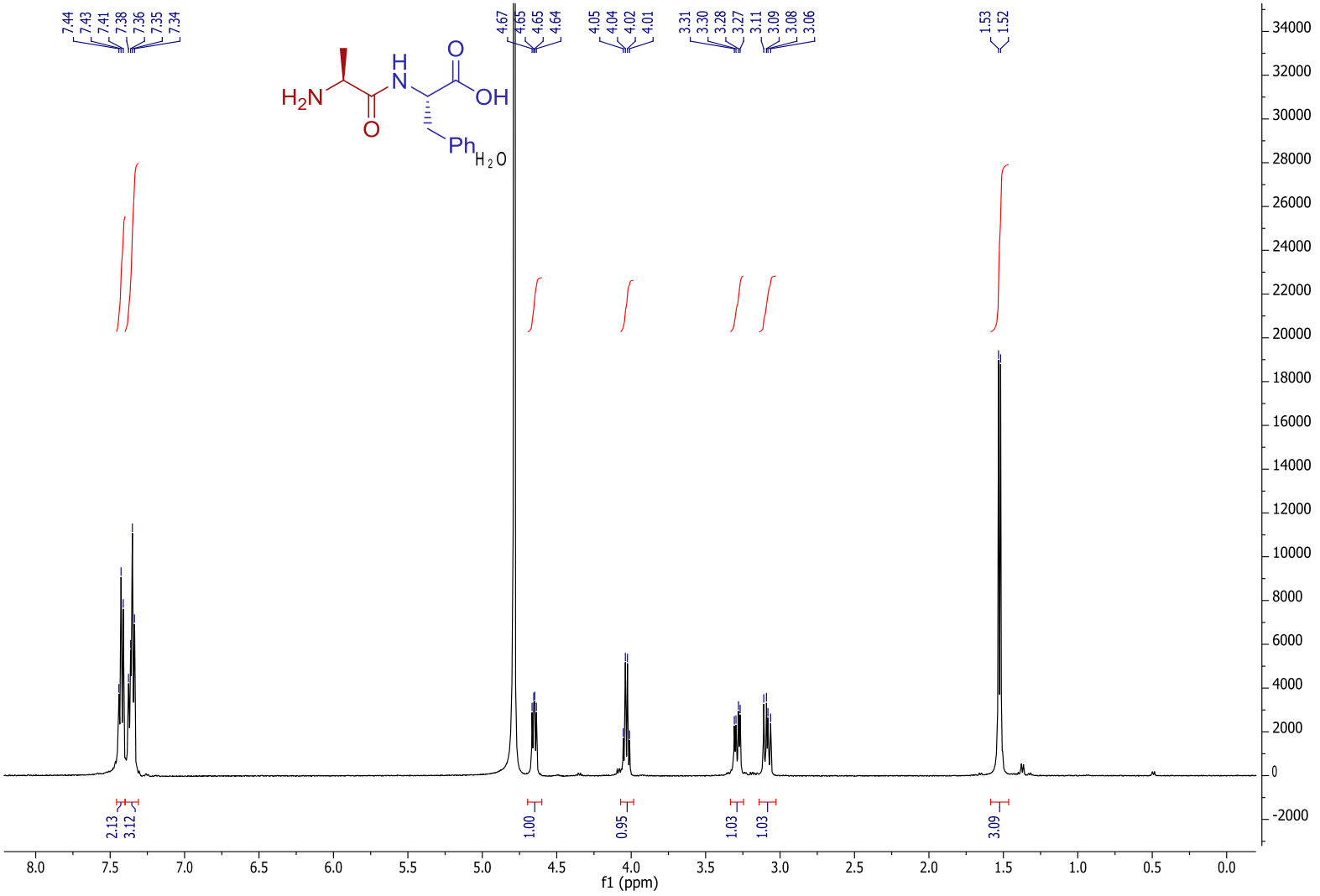
8. Spectra and chromatograms

^1H NMR and ^{13}C NMR in D_2O were obtained for the di- and tripeptide products prepared. Products were isolated from the crude reaction solution by mass directed automated preparative chromatography as detailed above for characterisation.

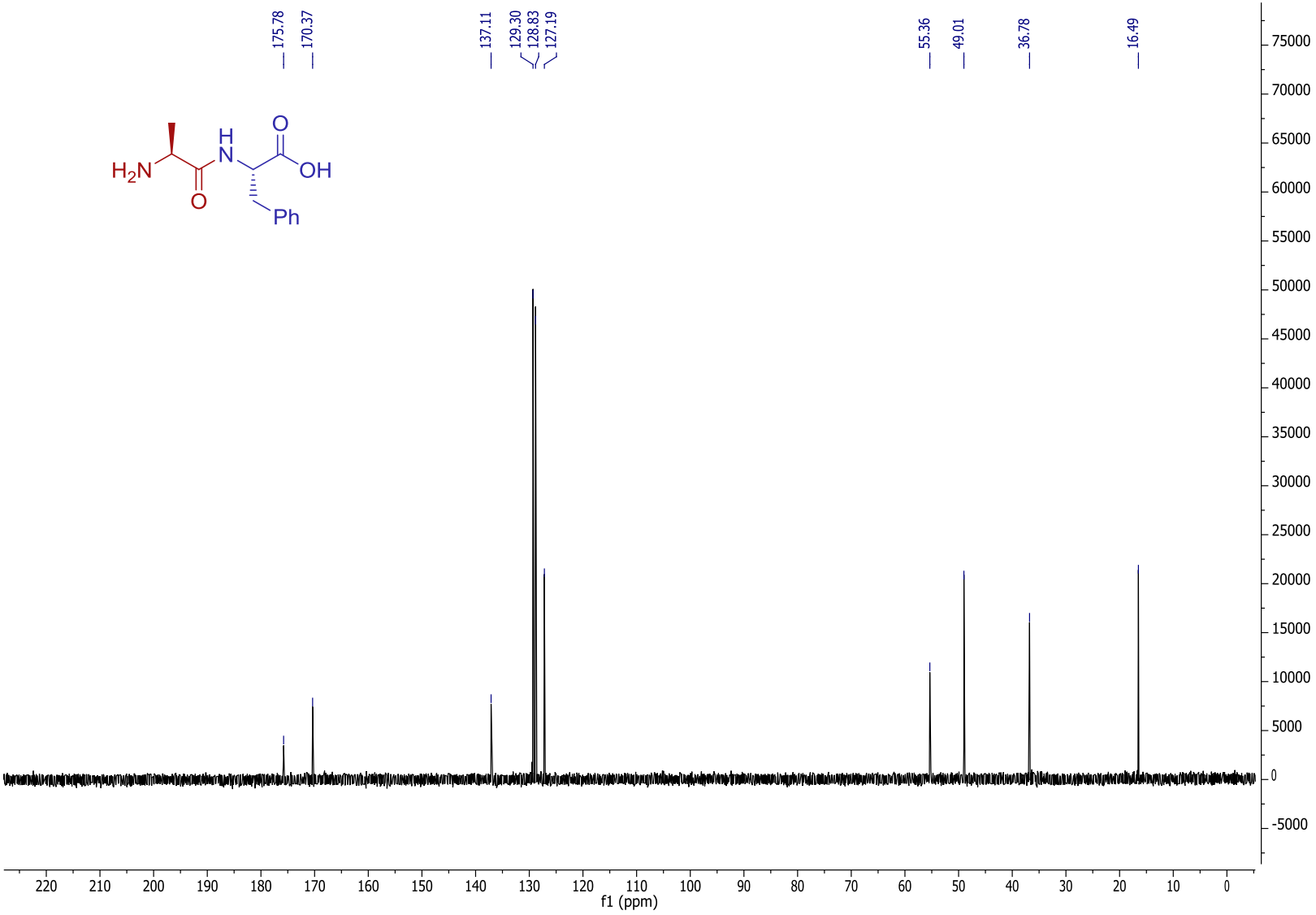
HPLC data was obtained for crude reaction solutions using the sample preparation detailed above.

9.1 NMR spectra for isolated reaction products

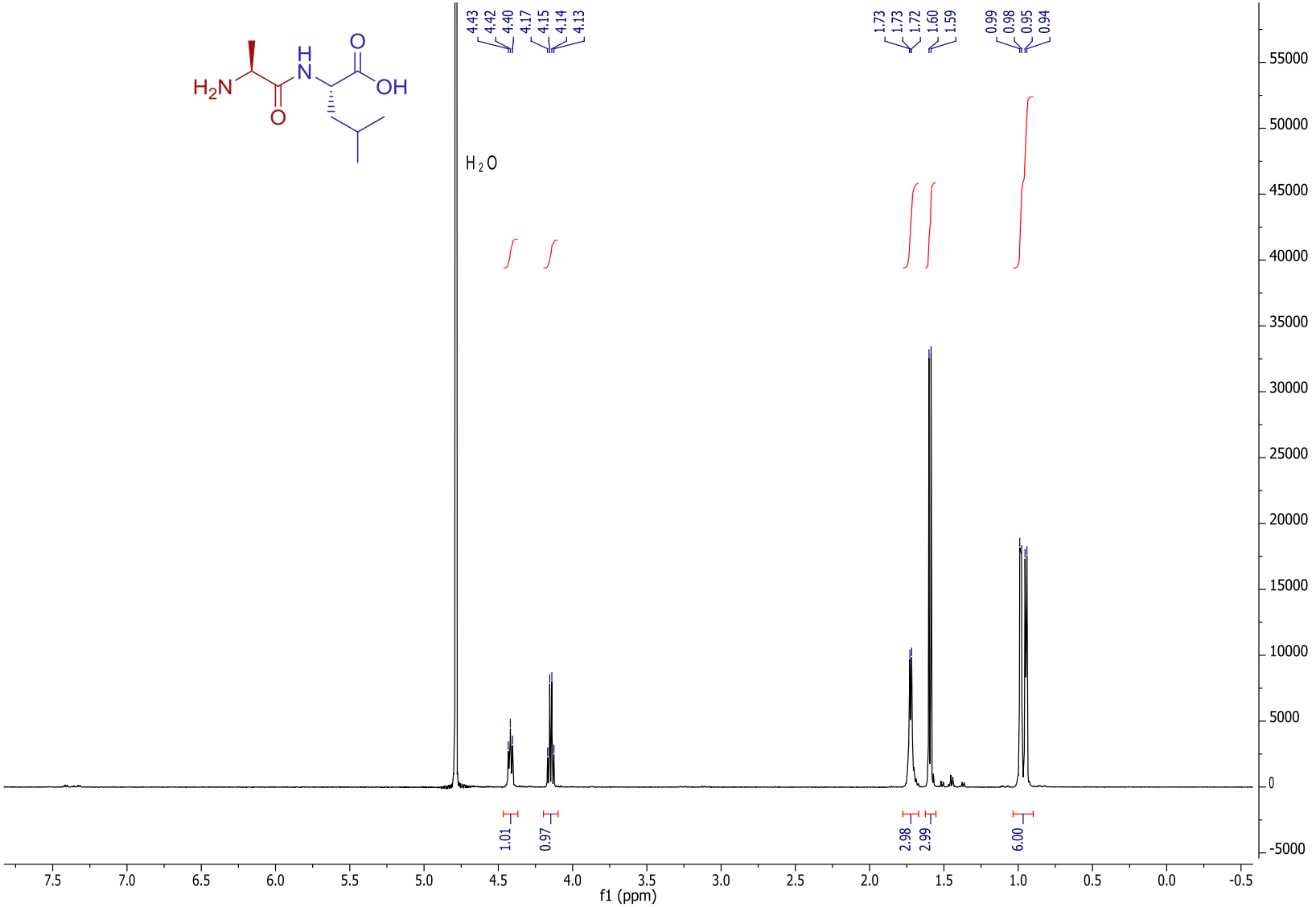
L-Alanyl-L-phenylalanine ^{1}H NMR (500 MHz, D_2O)



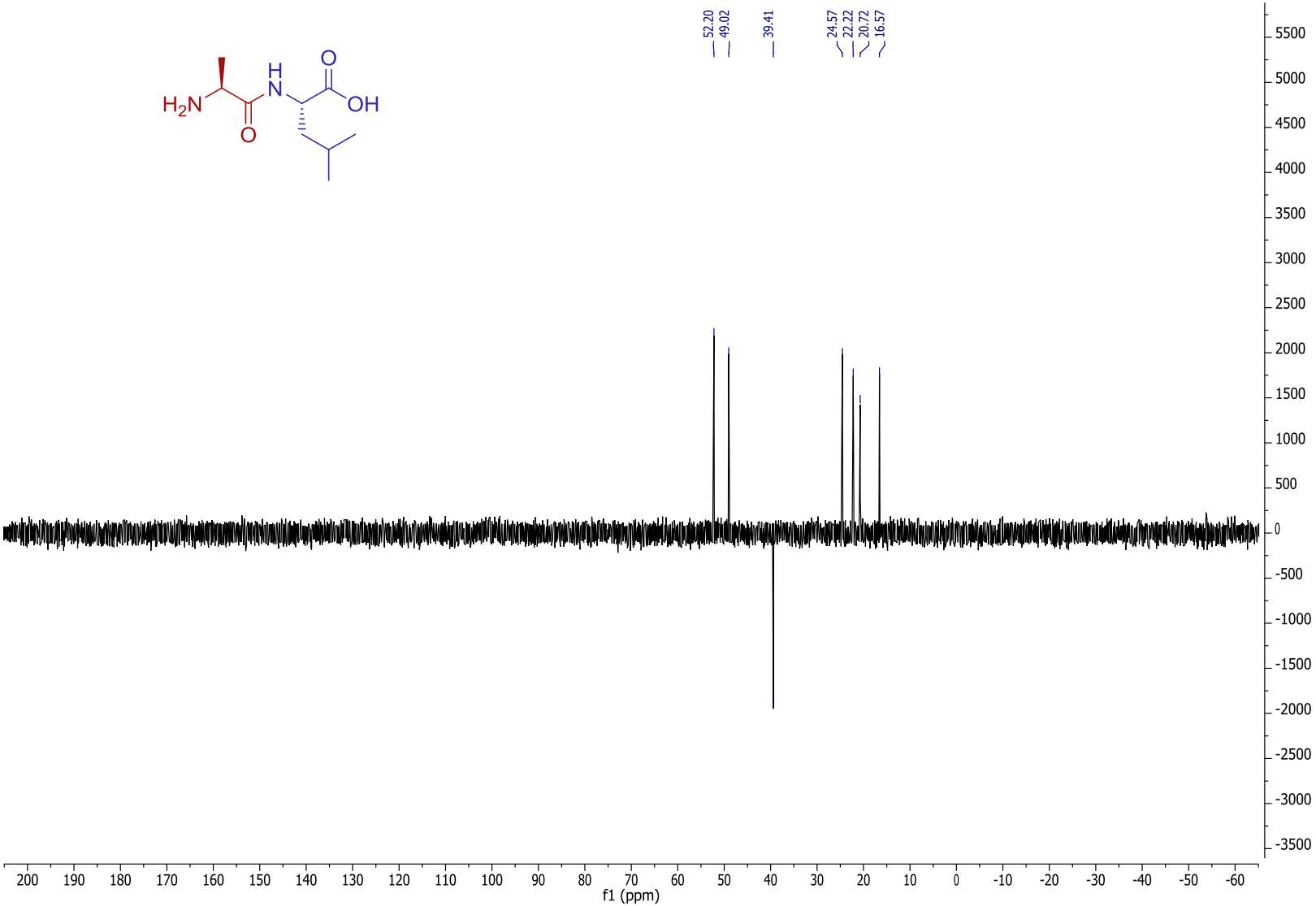
L-Alanyl-L-phenylalanine ^{13}C NMR (125 MHz, D_2O)



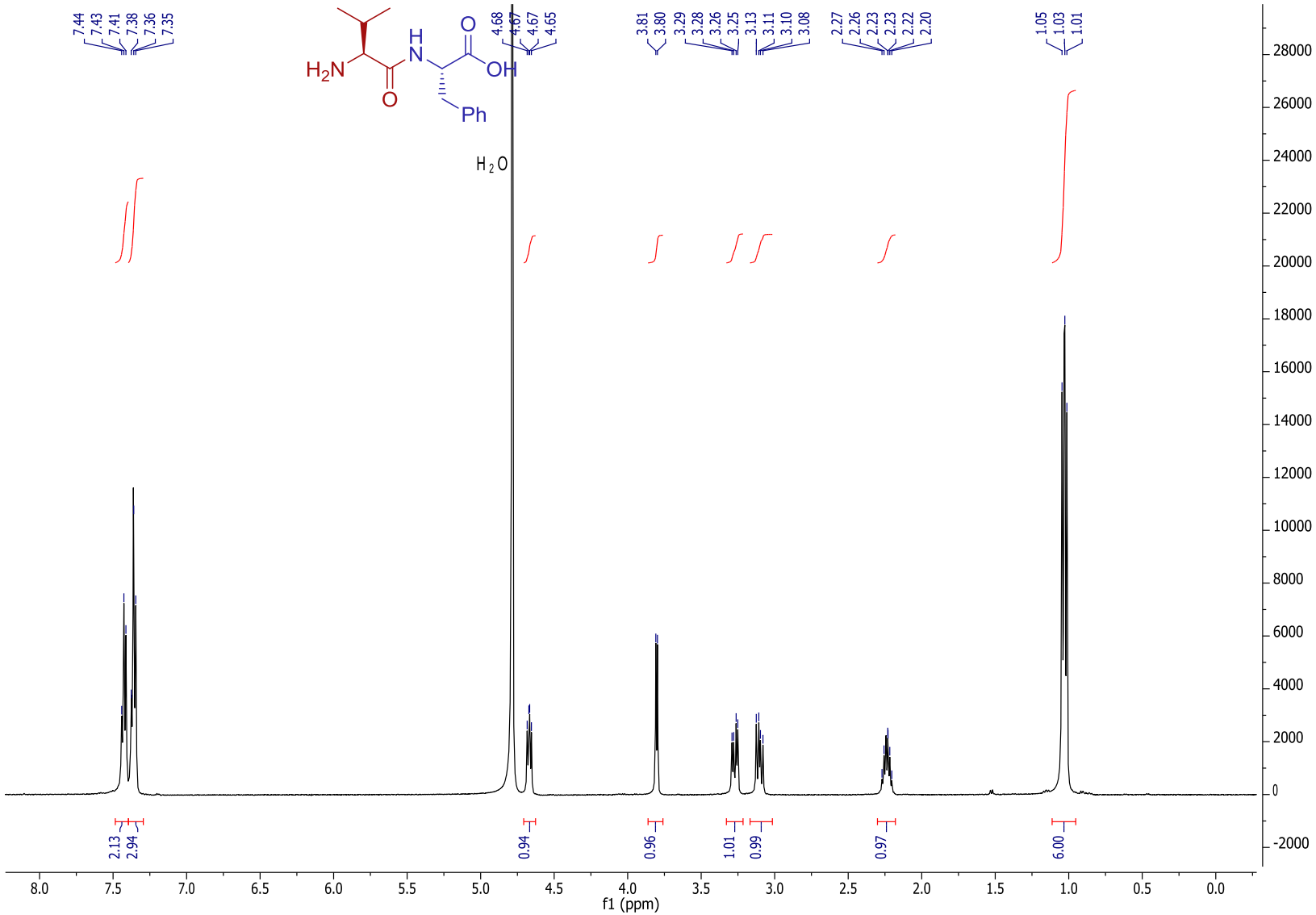
L-Alanyl-L-leucine ^{1}H NMR (500 MHz, D_2O)



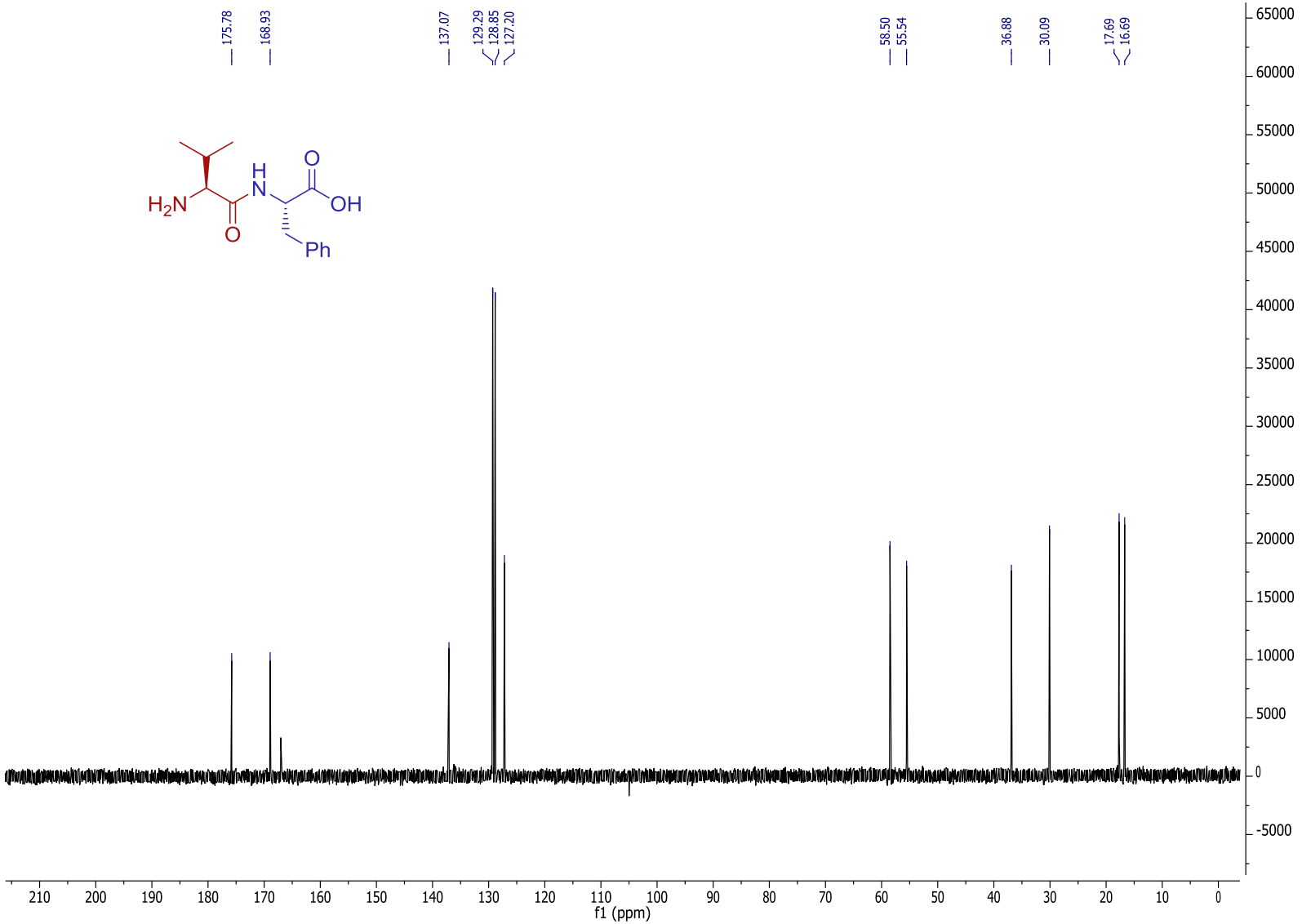
L-Alanyl-L-leucine ^{13}C NMR (125 MHz, D_2O)



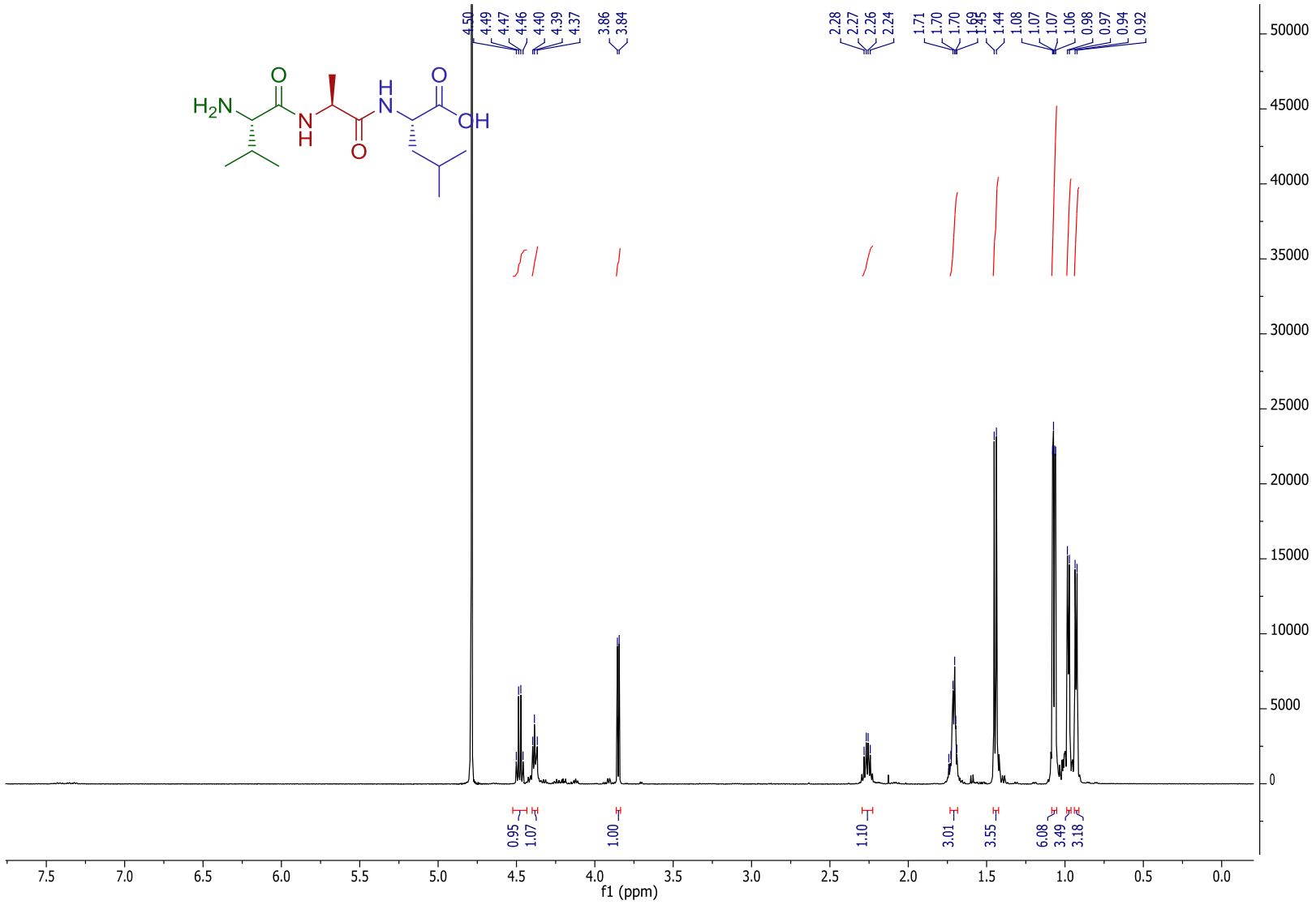
L-Valyl-L-phenylalanine ^{1}H NMR (500 MHz, D_2O)



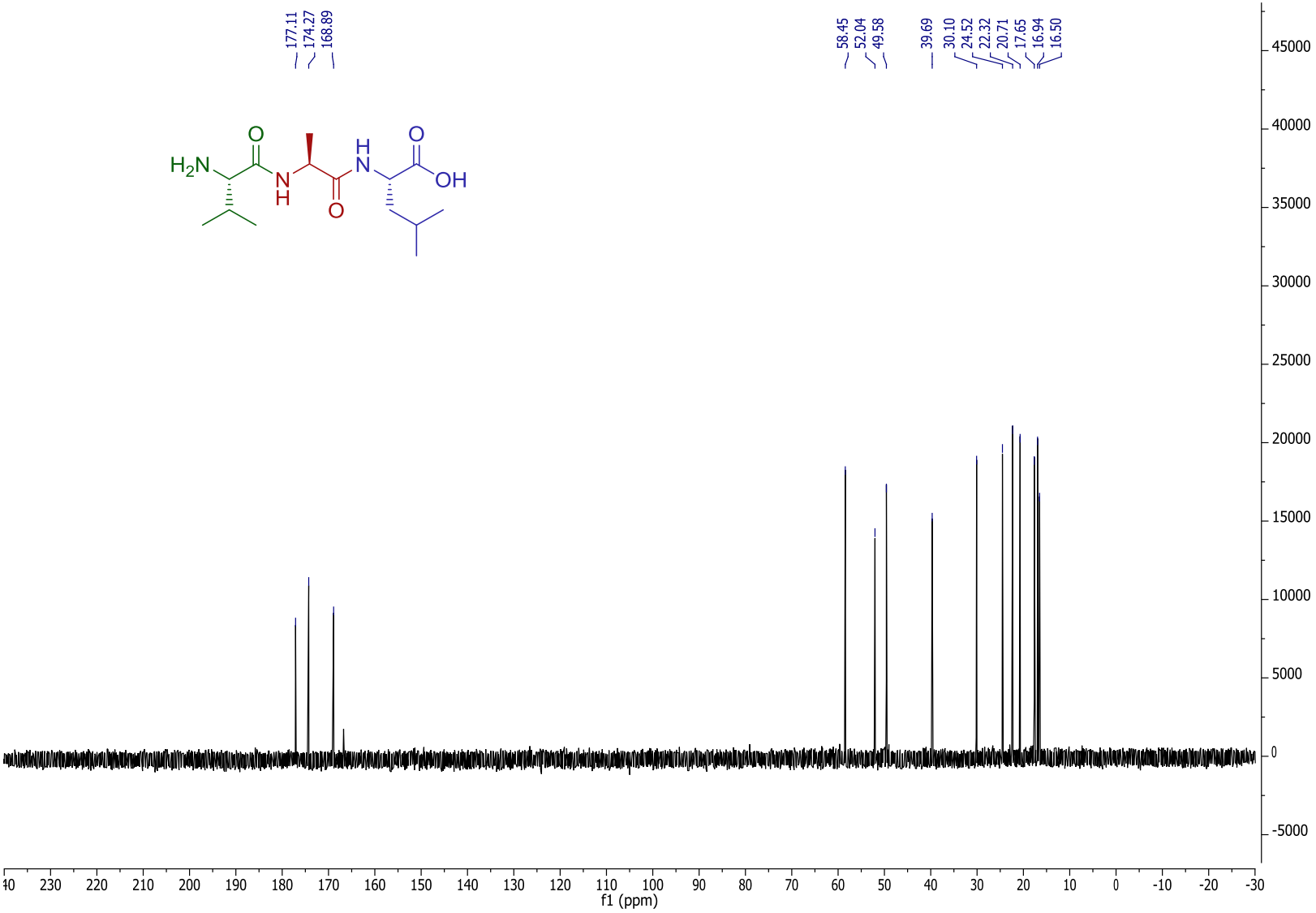
L-Valyl-L-phenylalanine ^{13}C NMR (125 MHz, D_2O)



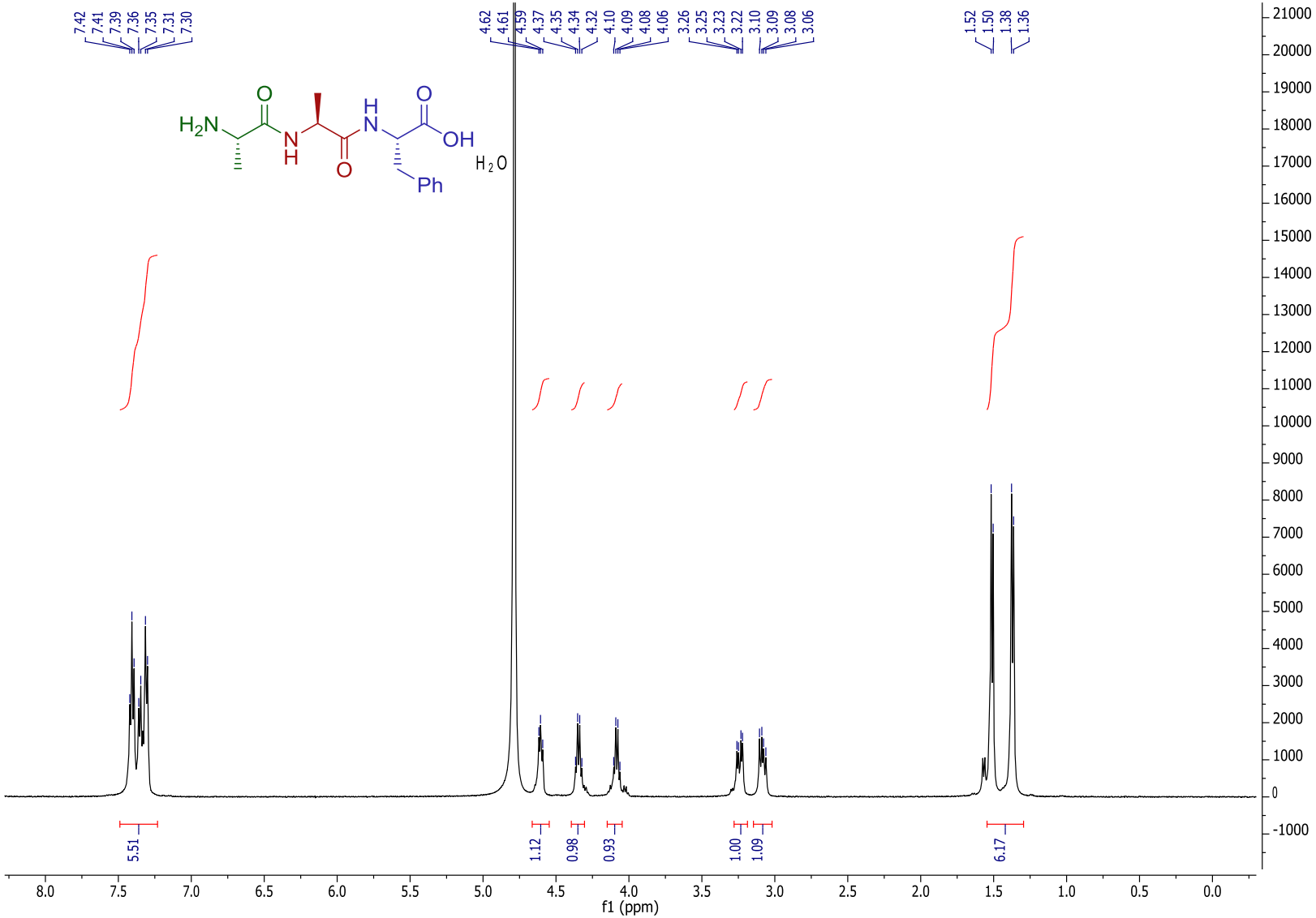
L-Valyl-L-alanyl-L-leucine ^{1}H NMR (500 MHz, D_2O)



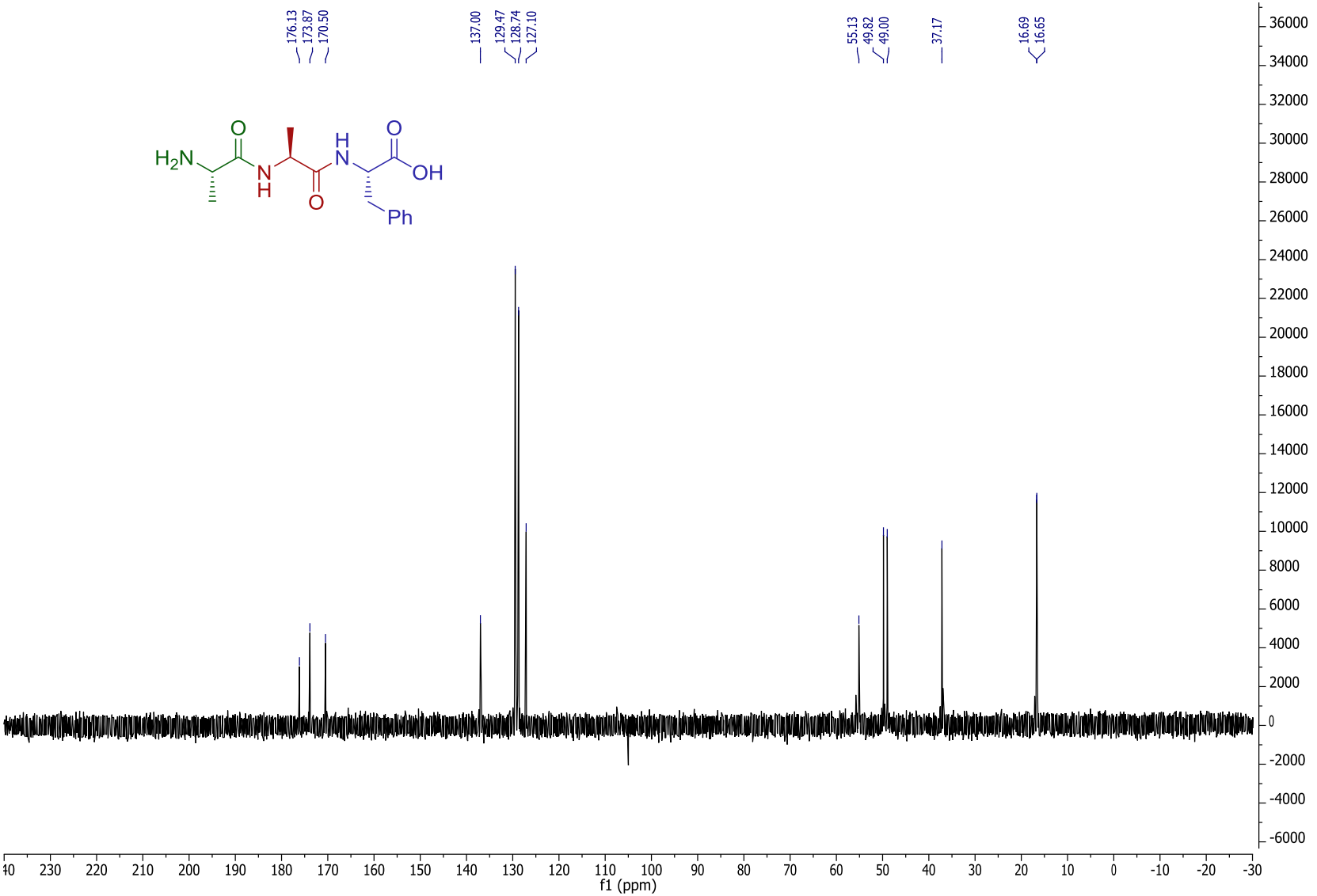
L-Valyl-L-alanyl-L-leucine ^{13}C NMR (125 MHz, D_2O)



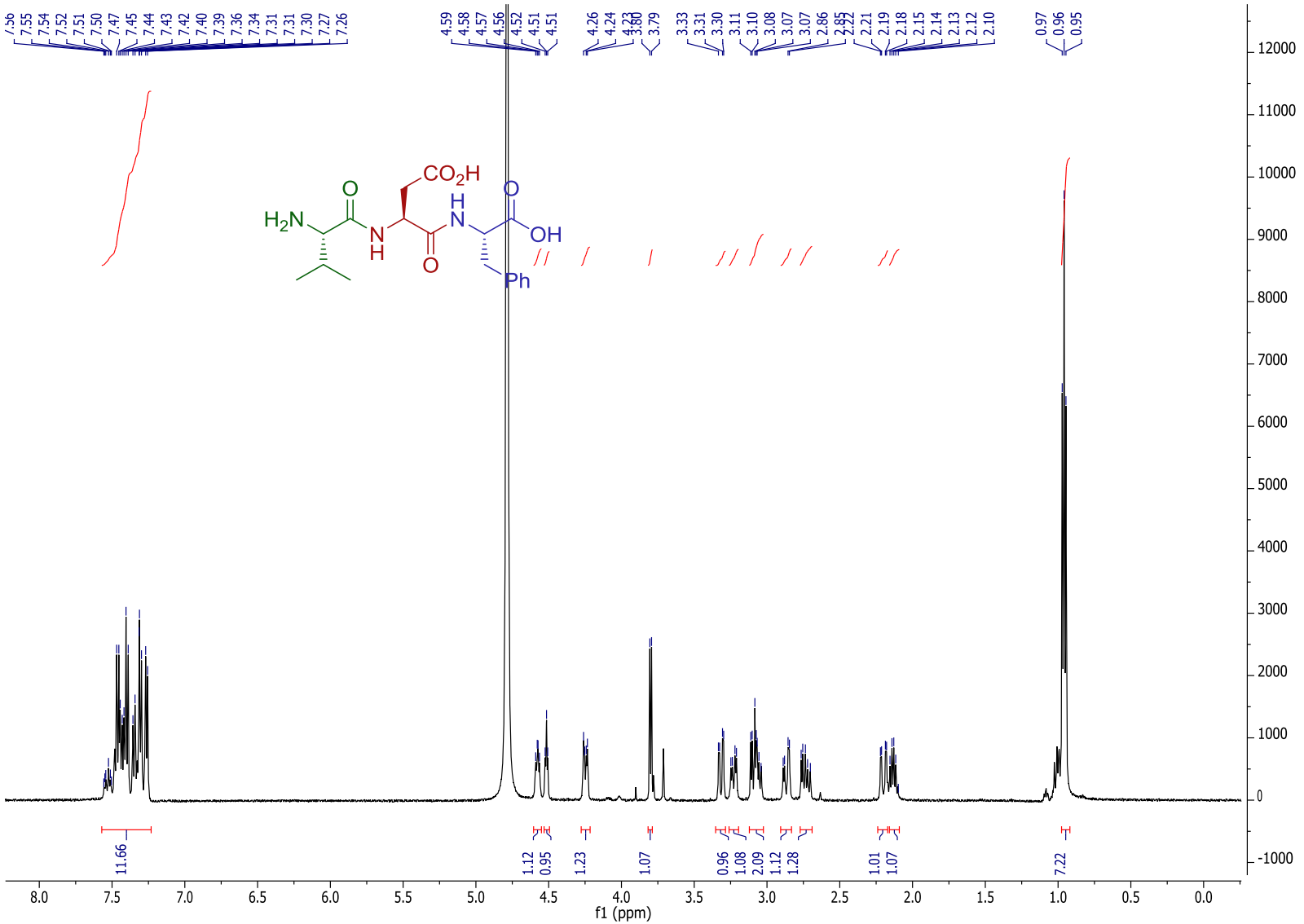
L-Alanyl-L-alanyl-L-phenylalanine ^{1}H NMR (500 MHz, D_2O)



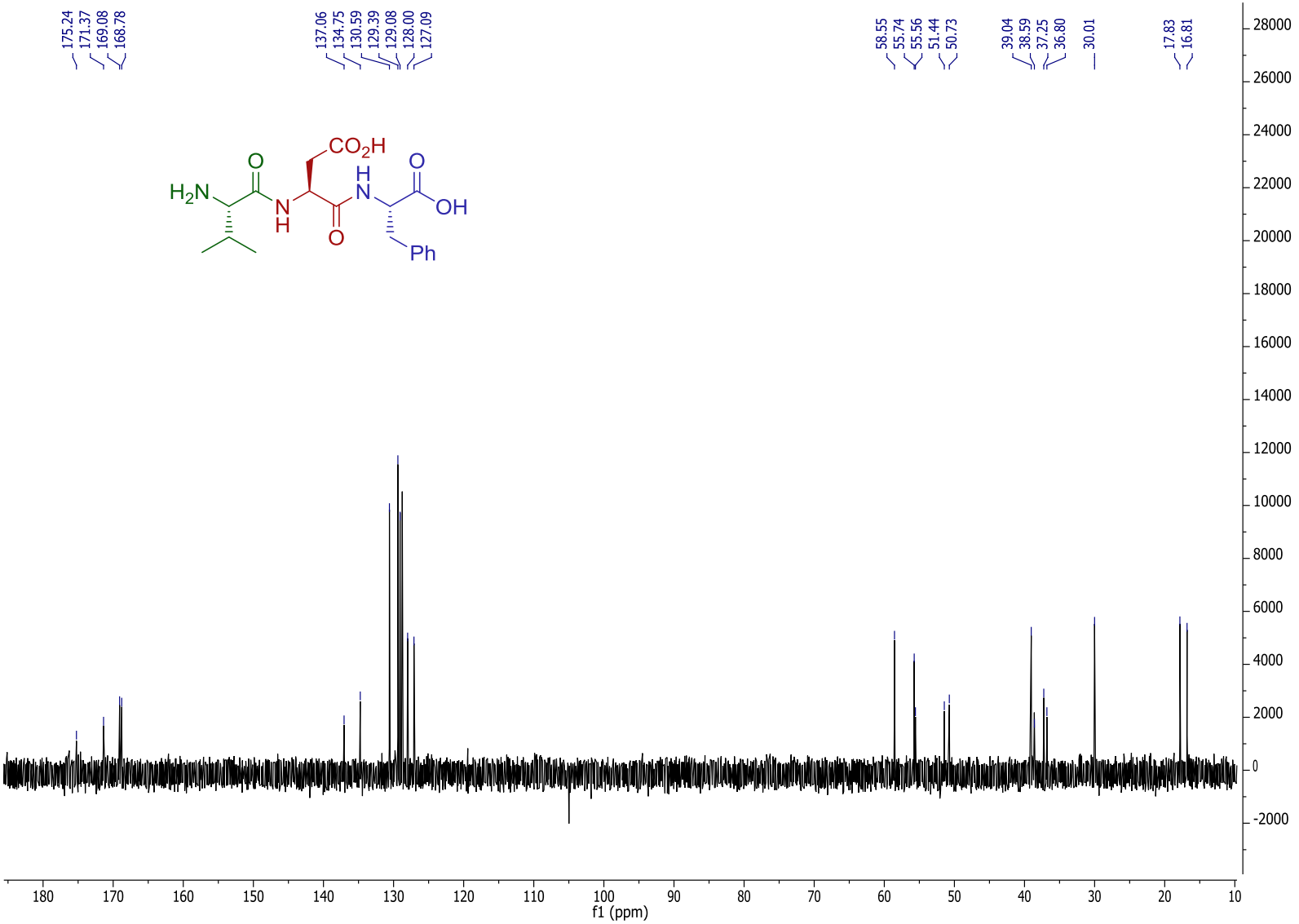
L-Alanyl-L-alanyl-L-phenylalanine ^{13}C NMR (125 MHz, D_2O)



L-Valyl-L-aspartyl-L-phenylalanine ^{1}H NMR (500 MHz, D_2O)

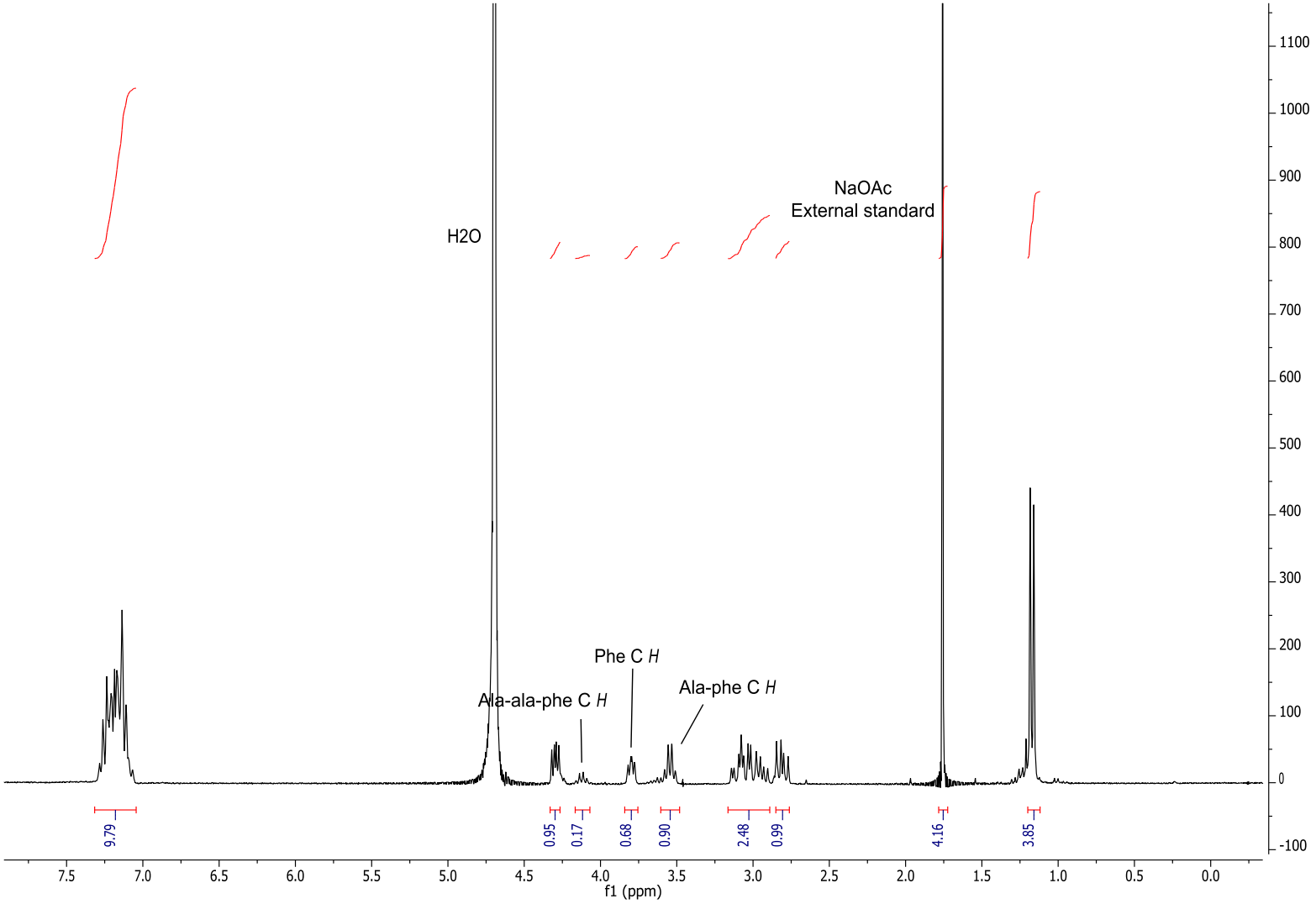


L-Valyl-L-aspartyl-L-phenylalanine ^{13}C NMR (500 MHz, D_2O)

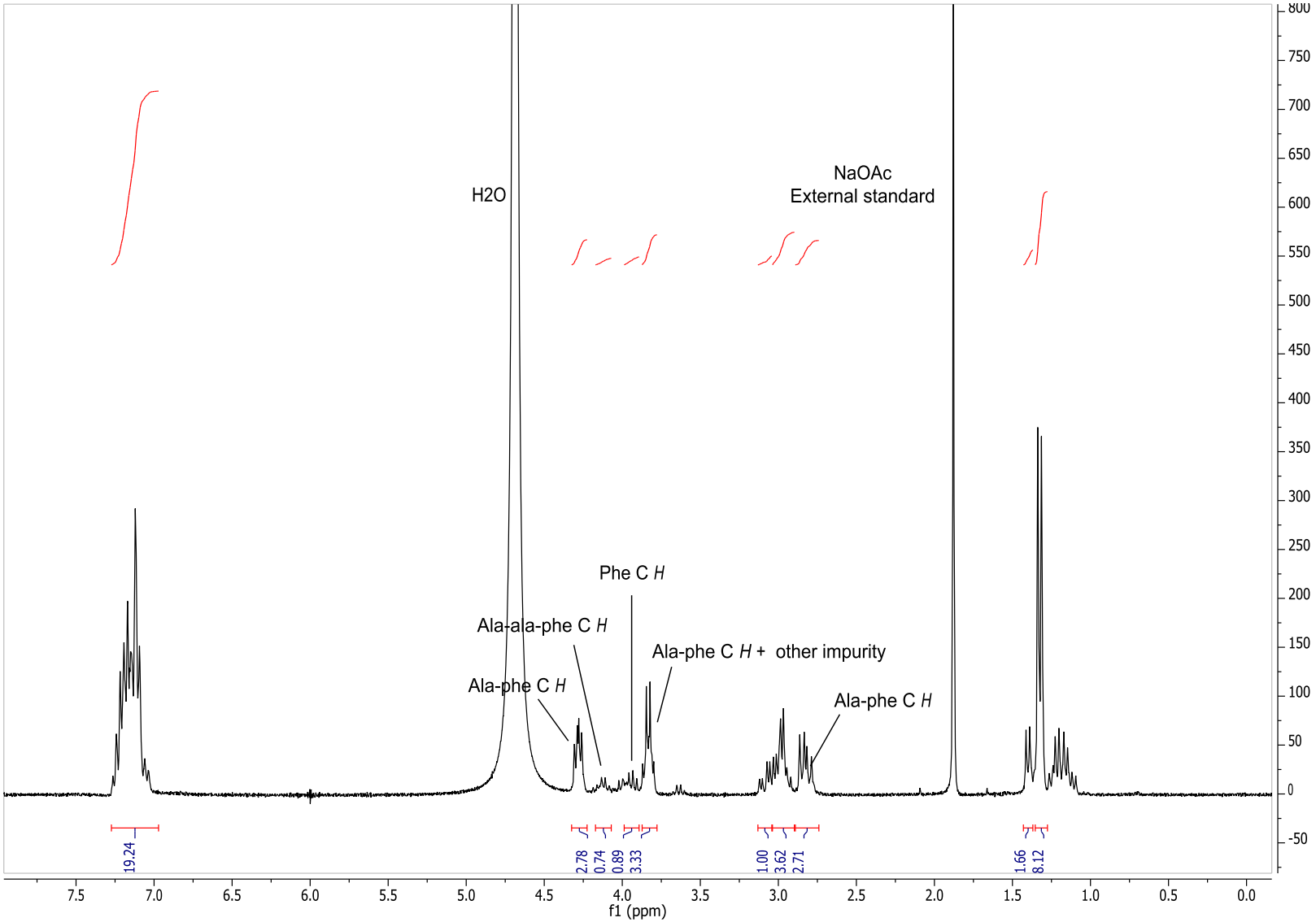


9.2 NMR spectra for crude reaction solutions at steady state during

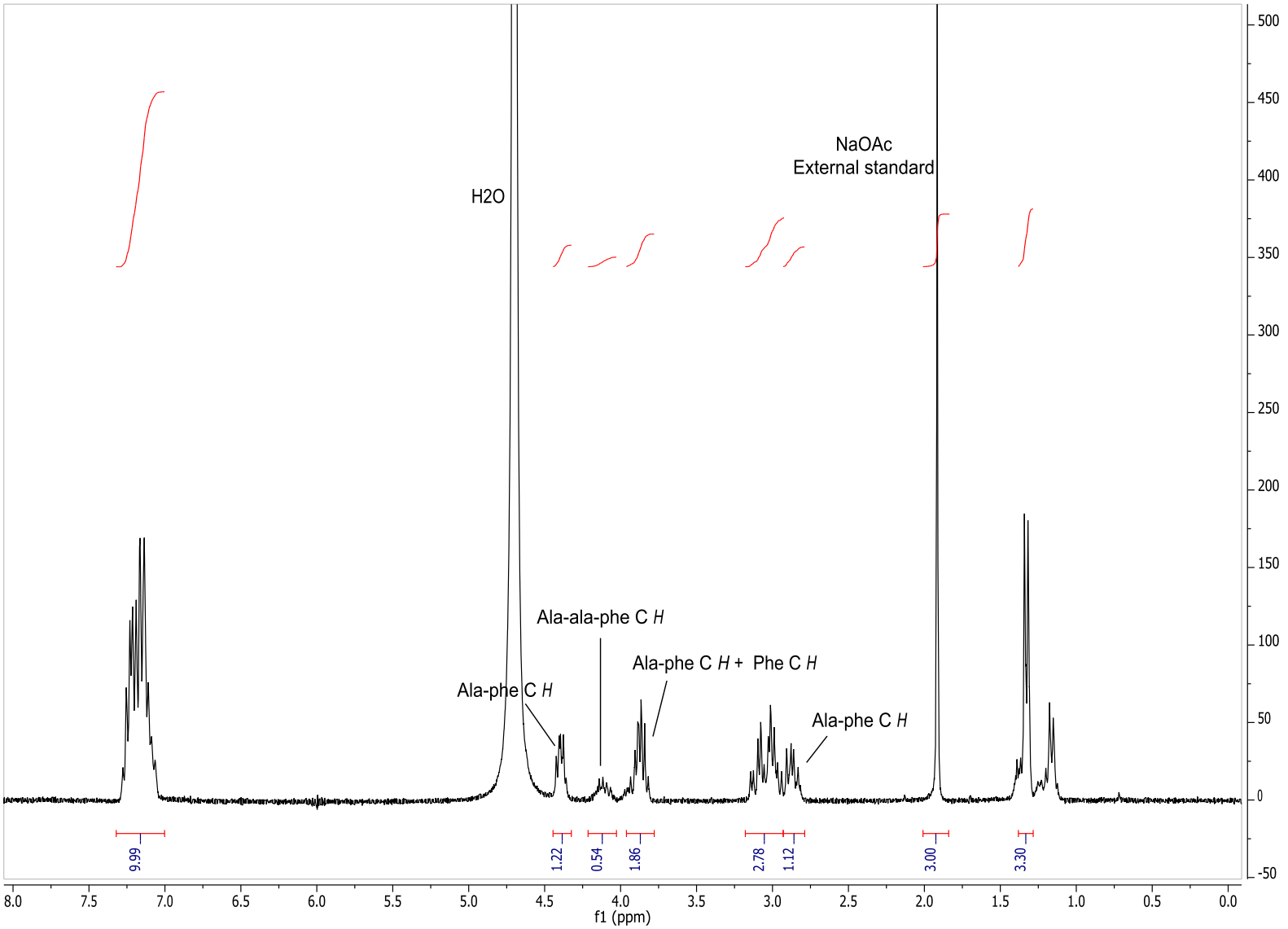
Formation of L-alanyl-L-phenylalanine (Main paper: Table 1, entry 1) ^{1}H NMR (300 MHz, D_2O)



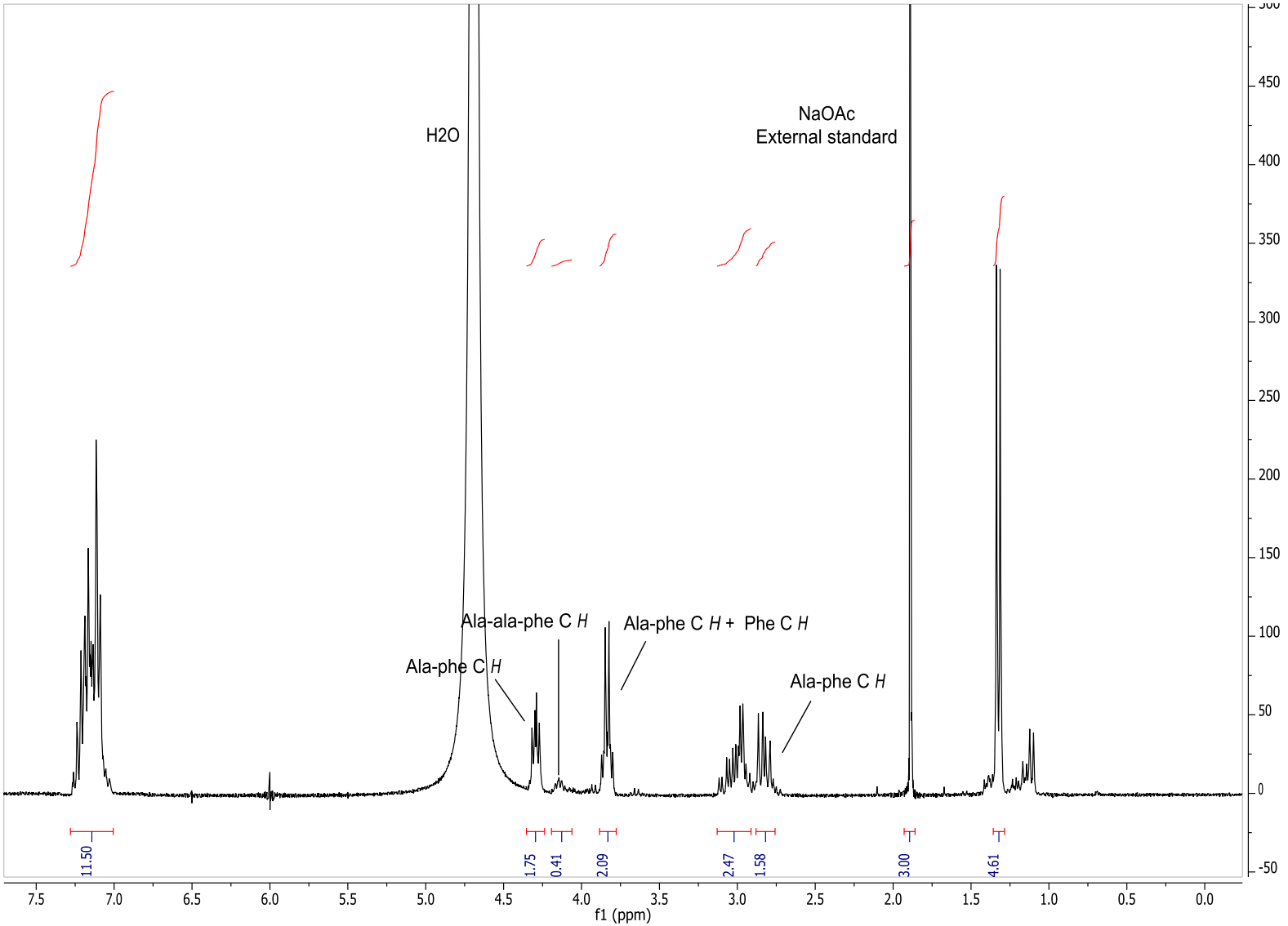
Formation of L-alanyl-L-phenylalanine (Main paper: Table 1, entry 2 and Figure 3) ^{1}H NMR (300 MHz, D_2O)



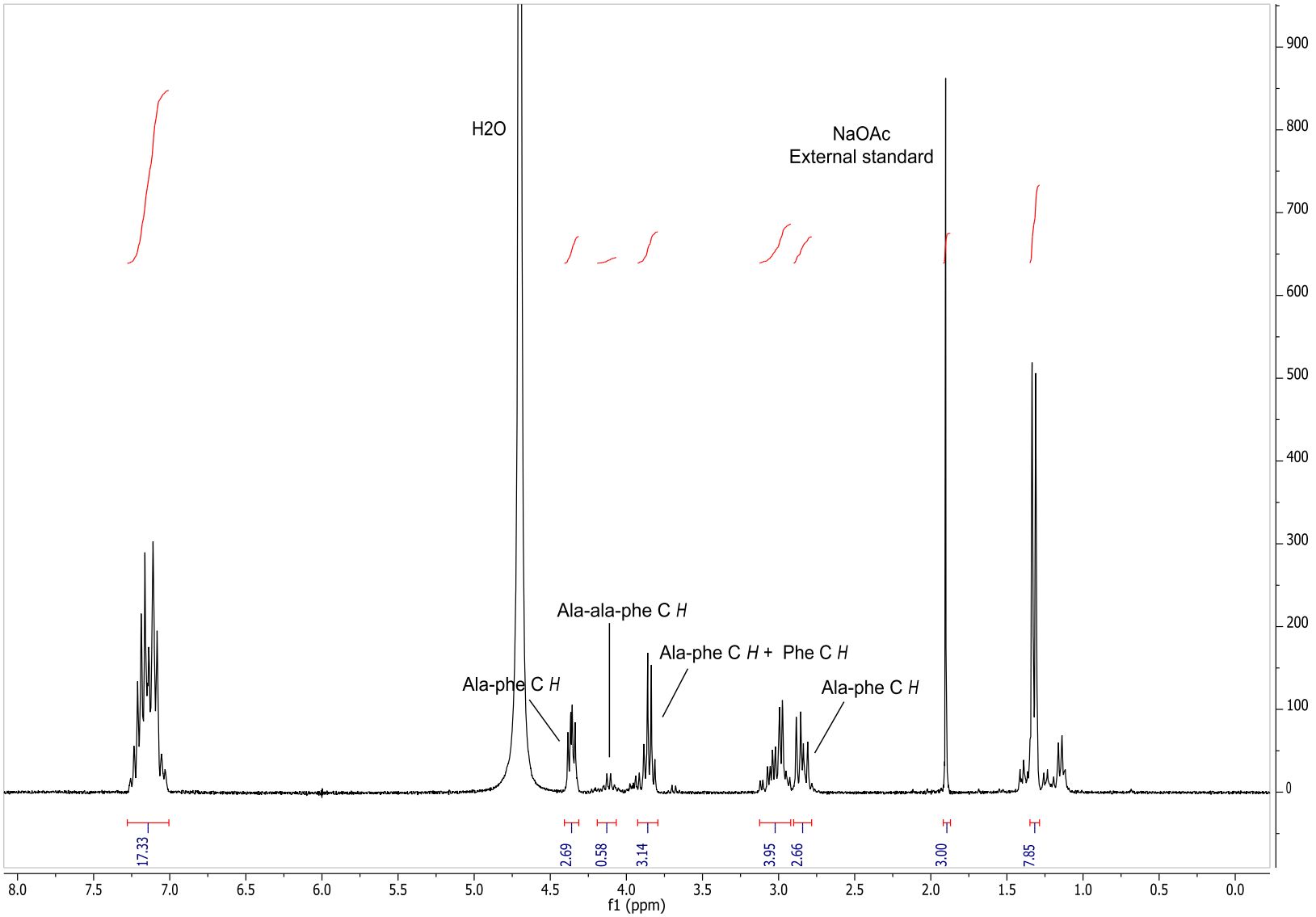
Formation of L-alanyl-L-phenylalanine (Main paper: Table 1, entry 3) ^{1}H NMR (300 MHz, D_2O)



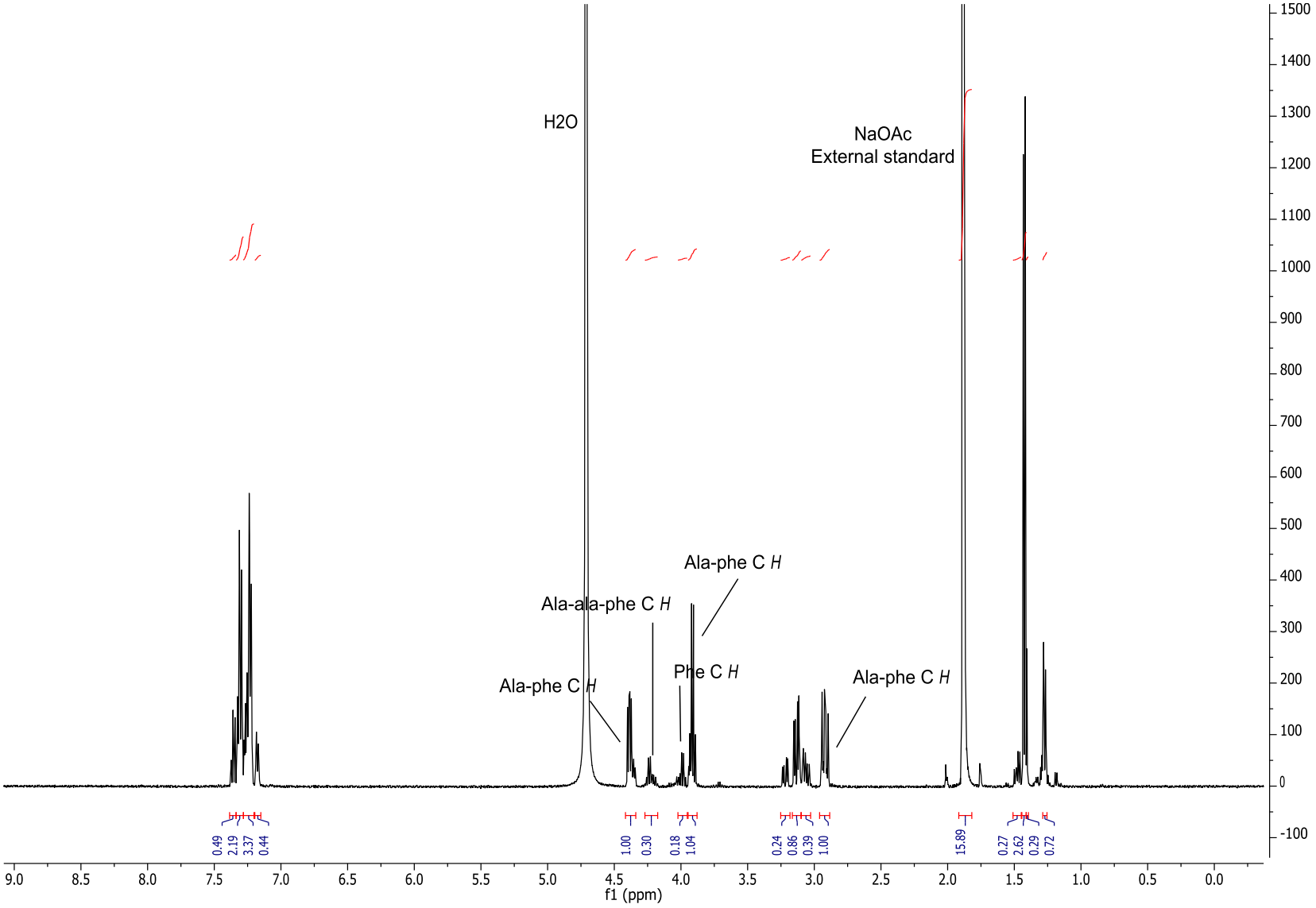
Formation of L-alanyl-L-phenylalanine (Main paper: Table 1, entry 4) ^{1}H NMR (300 MHz, D_2O)



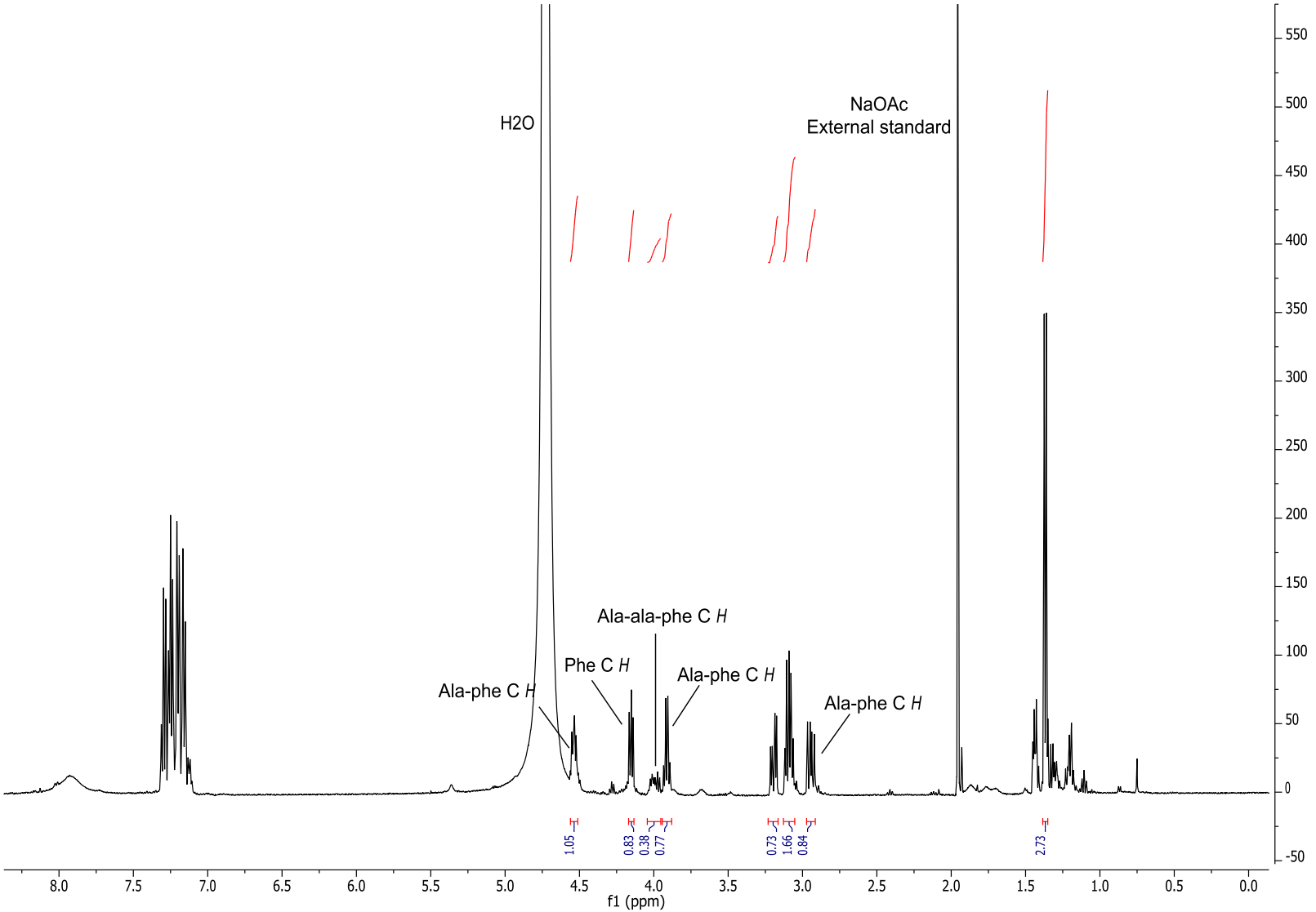
Formation of L-alanyl-L-phenylalanine (Main paper: Table 1, entry 5) ^{1}H NMR (300 MHz, D_2O)



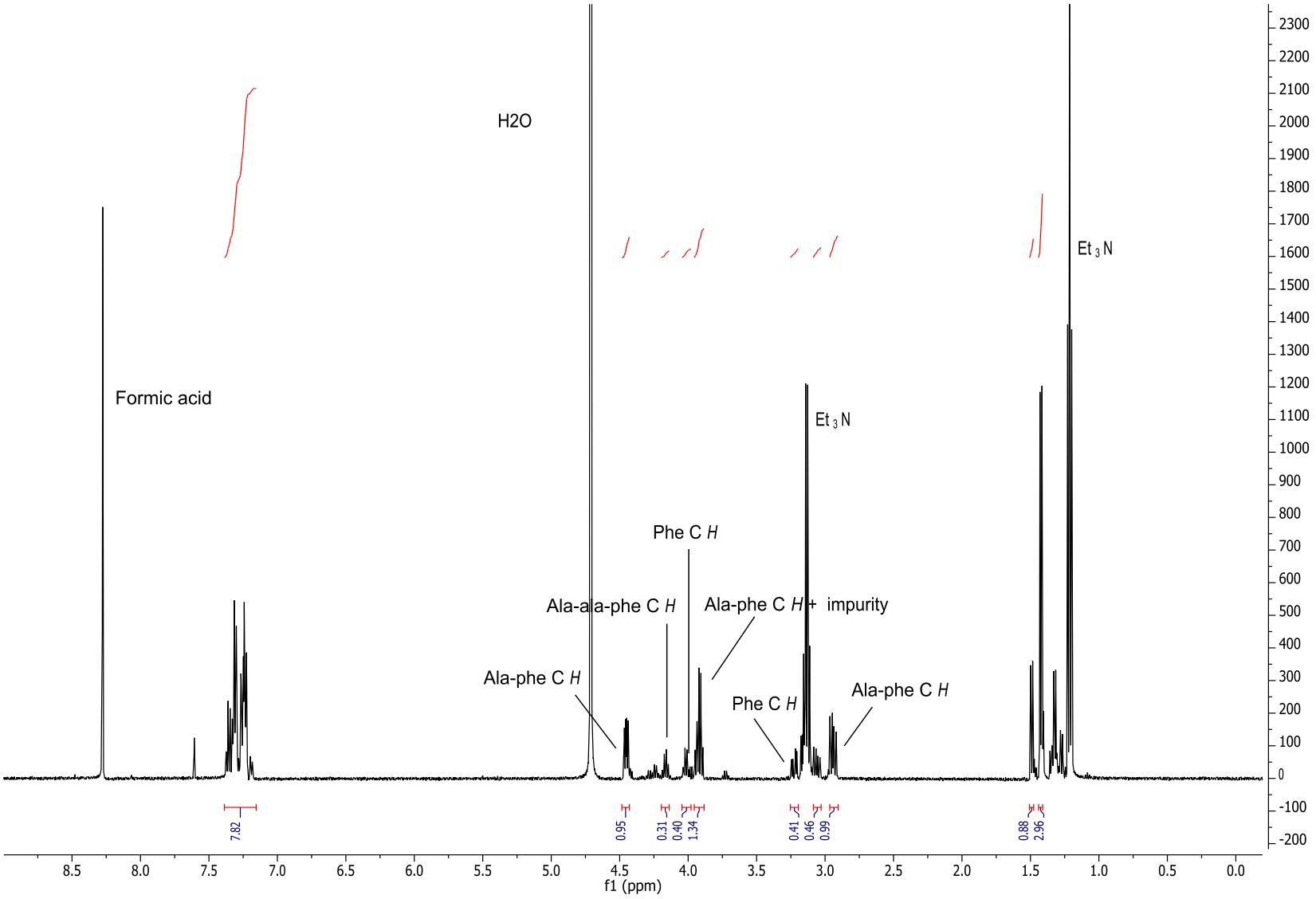
Formation of L-alanyl-L-phenylalanine (Main paper: Table 1, entry 6) ^{1}H NMR (500 MHz, D_2O)



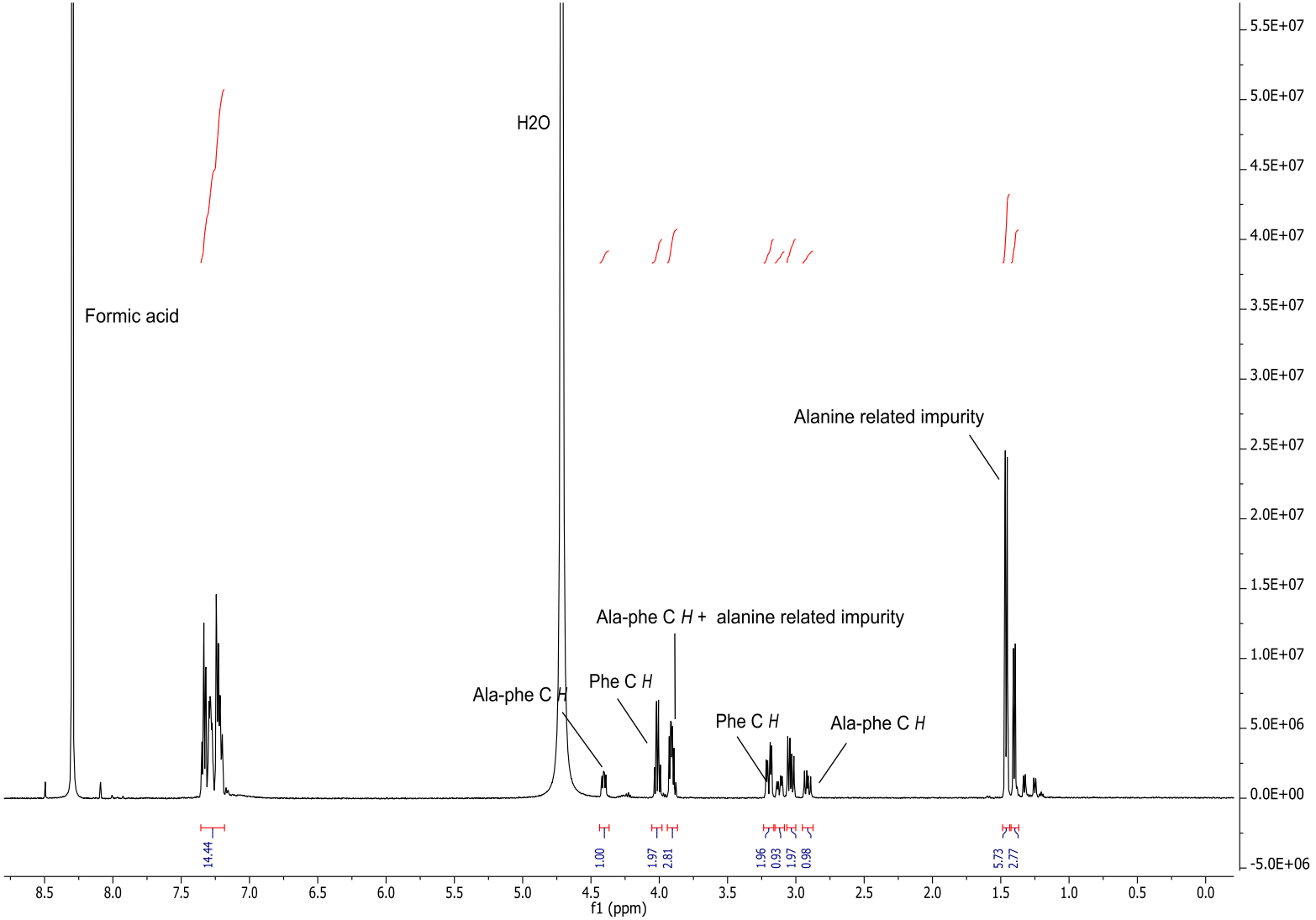
Formation of L-alanyl-L-phenylalanine (Main paper: Table 1, entry 7) ^{1}H NMR (500 MHz, D_2O)



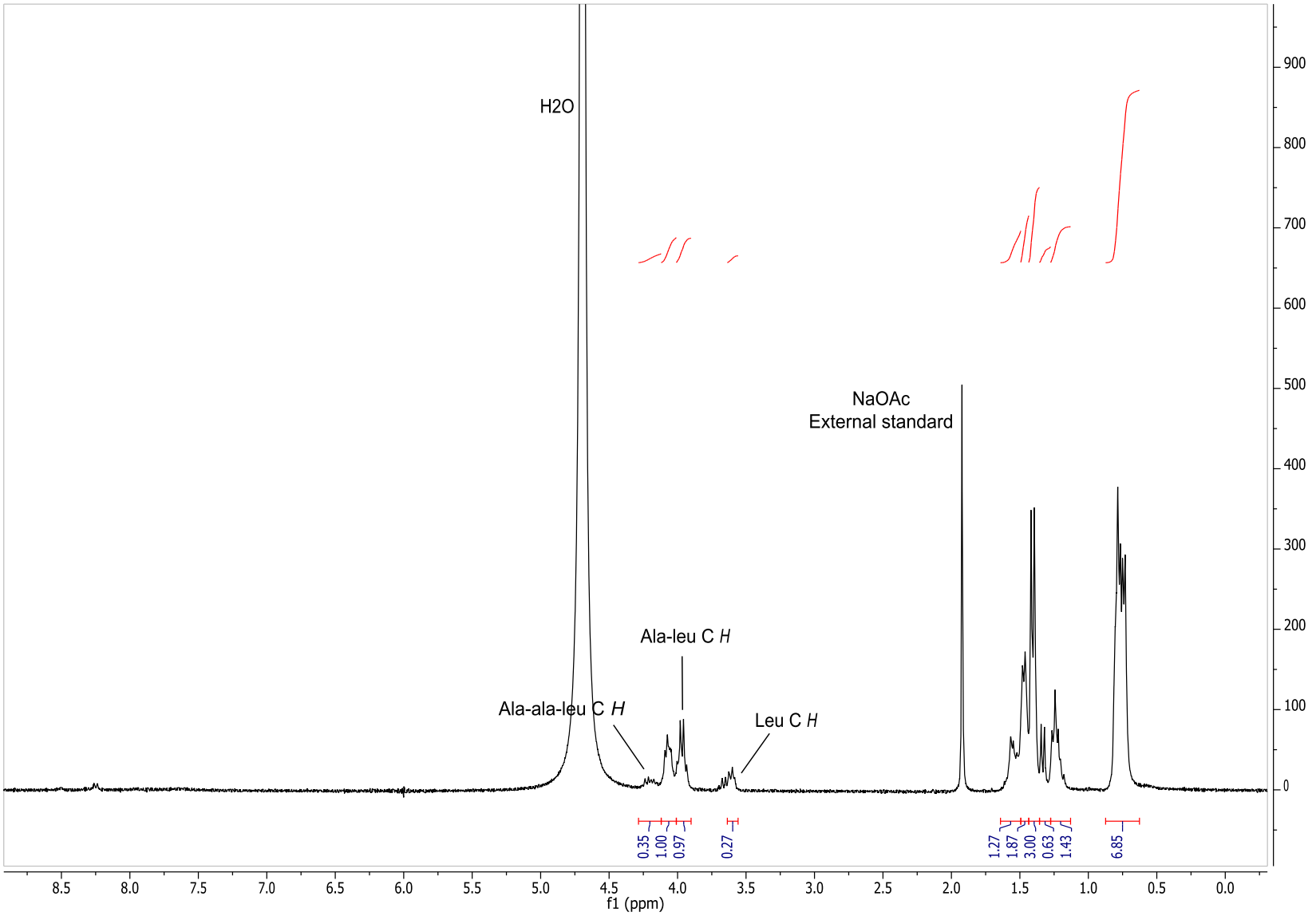
Formation of L-alanyl-L-phenylalanine (Main paper: Table 1, entry 8) ^{1}H NMR (500 MHz, D_2O)



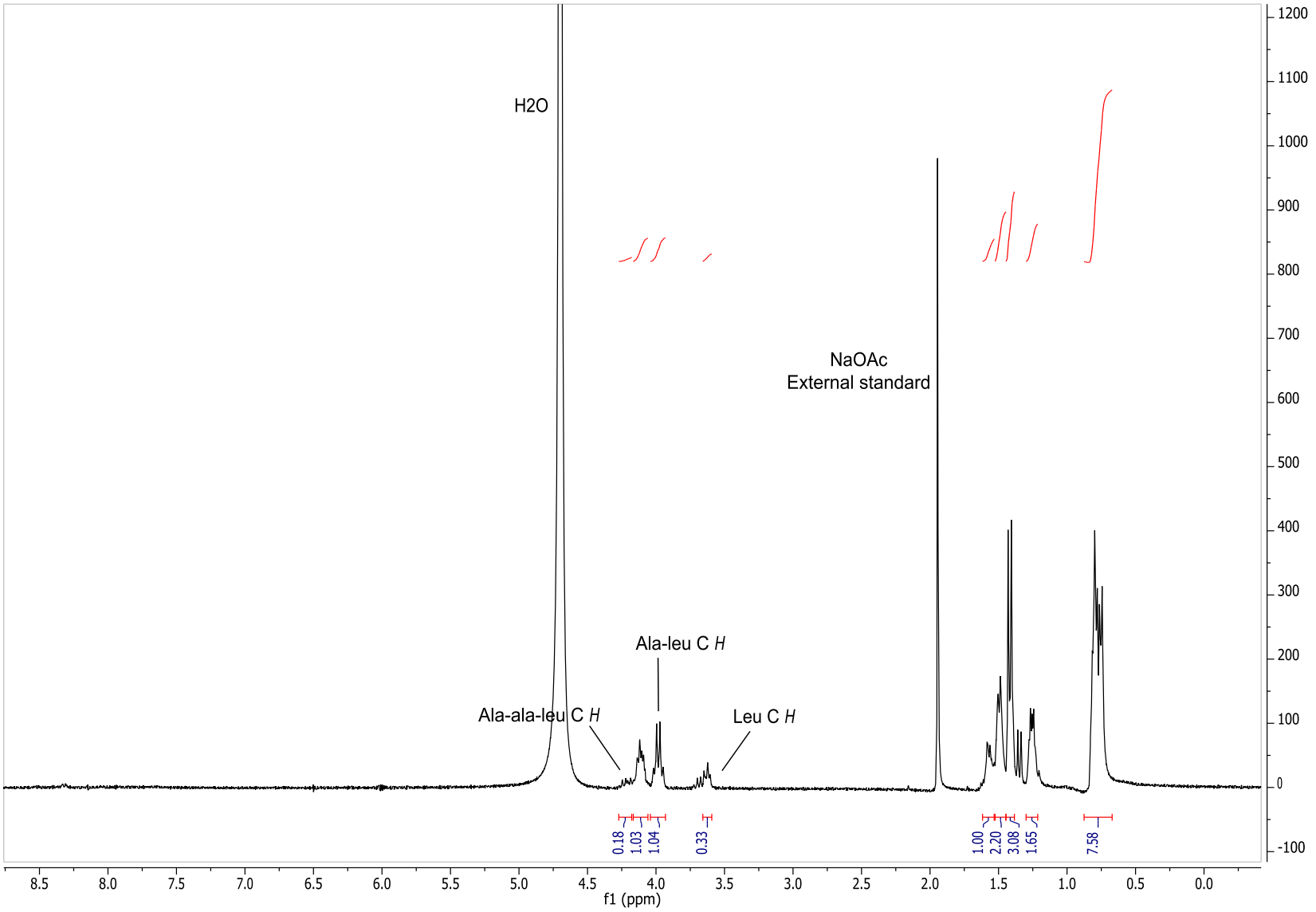
Formation of L-alanyl-L-phenylalanine (Main paper: Table 1, entry 9) ^{1}H NMR (500 MHz, D_2O)



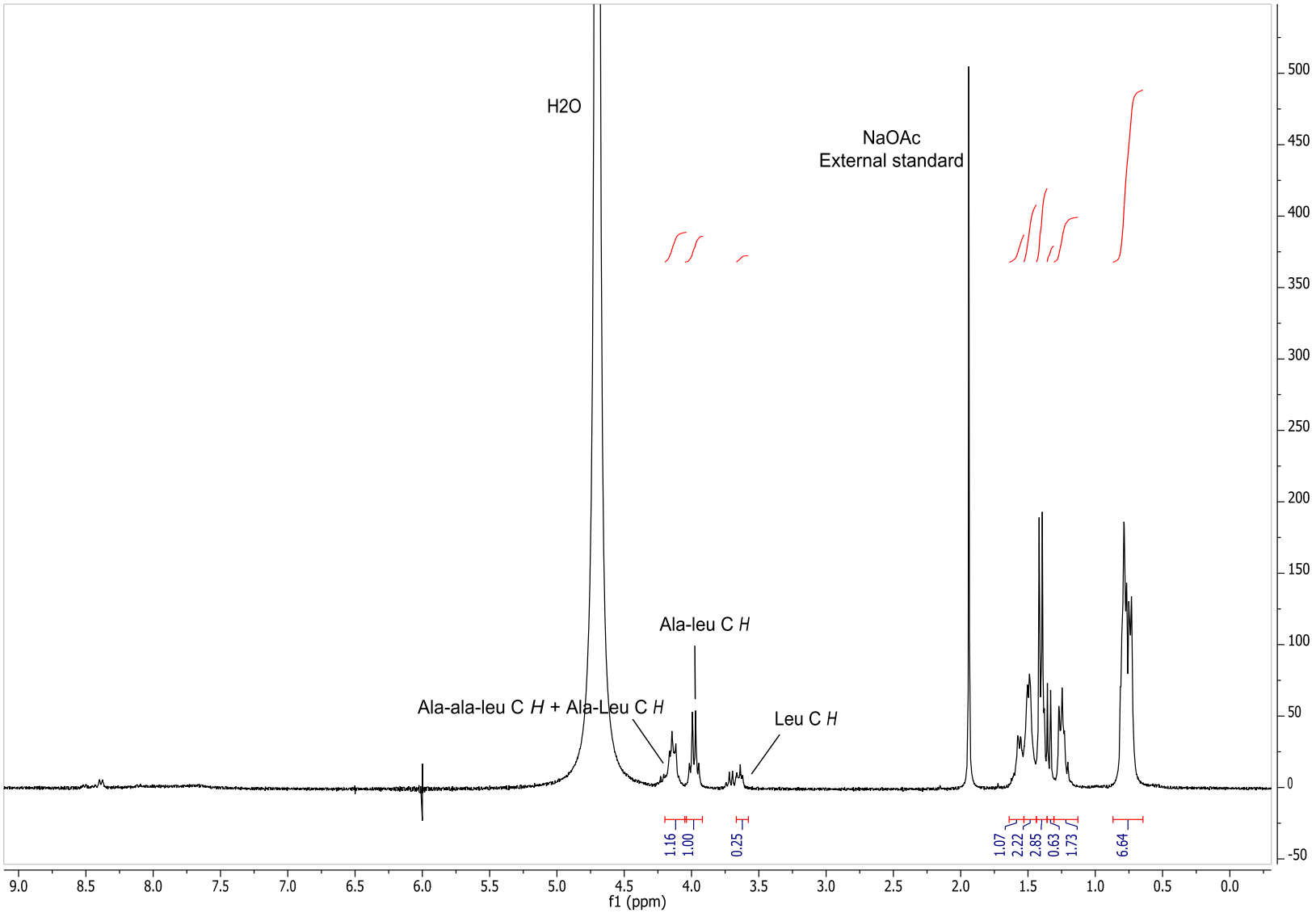
Formation of L-alanyl-L-leucine (Main paper: Table 1, entry 10) ^{1}H NMR (300 MHz, D_2O)



Formation of L-alanyl-L-leucine (Main paper: Table 1, entry 11, Figure 3) ^{1}H NMR (300 MHz, D_2O)



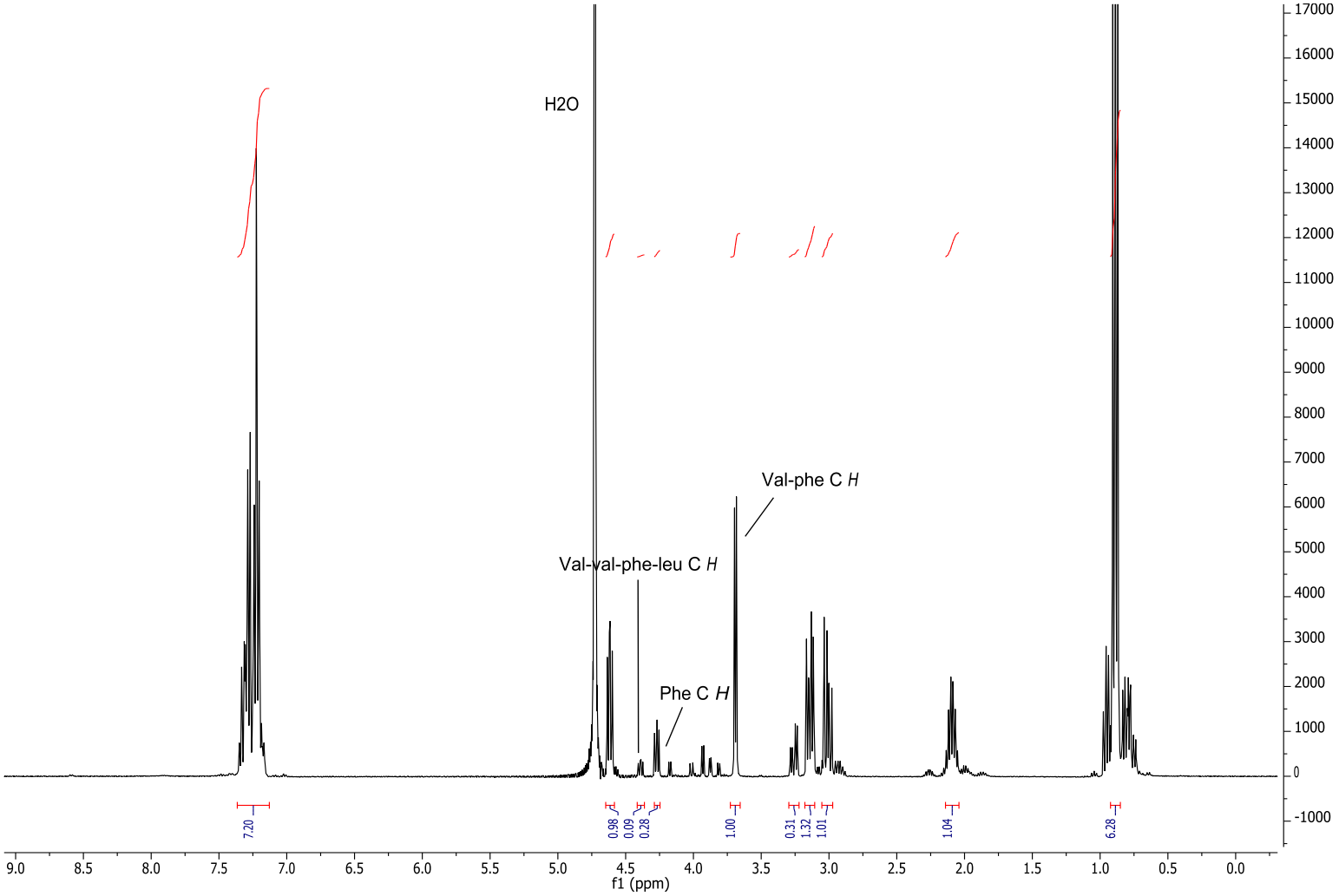
Formation of L-alanyl-L-leucine (Main paper: Table 1, entry 12) ^{1}H NMR (300 MHz, D_2O)



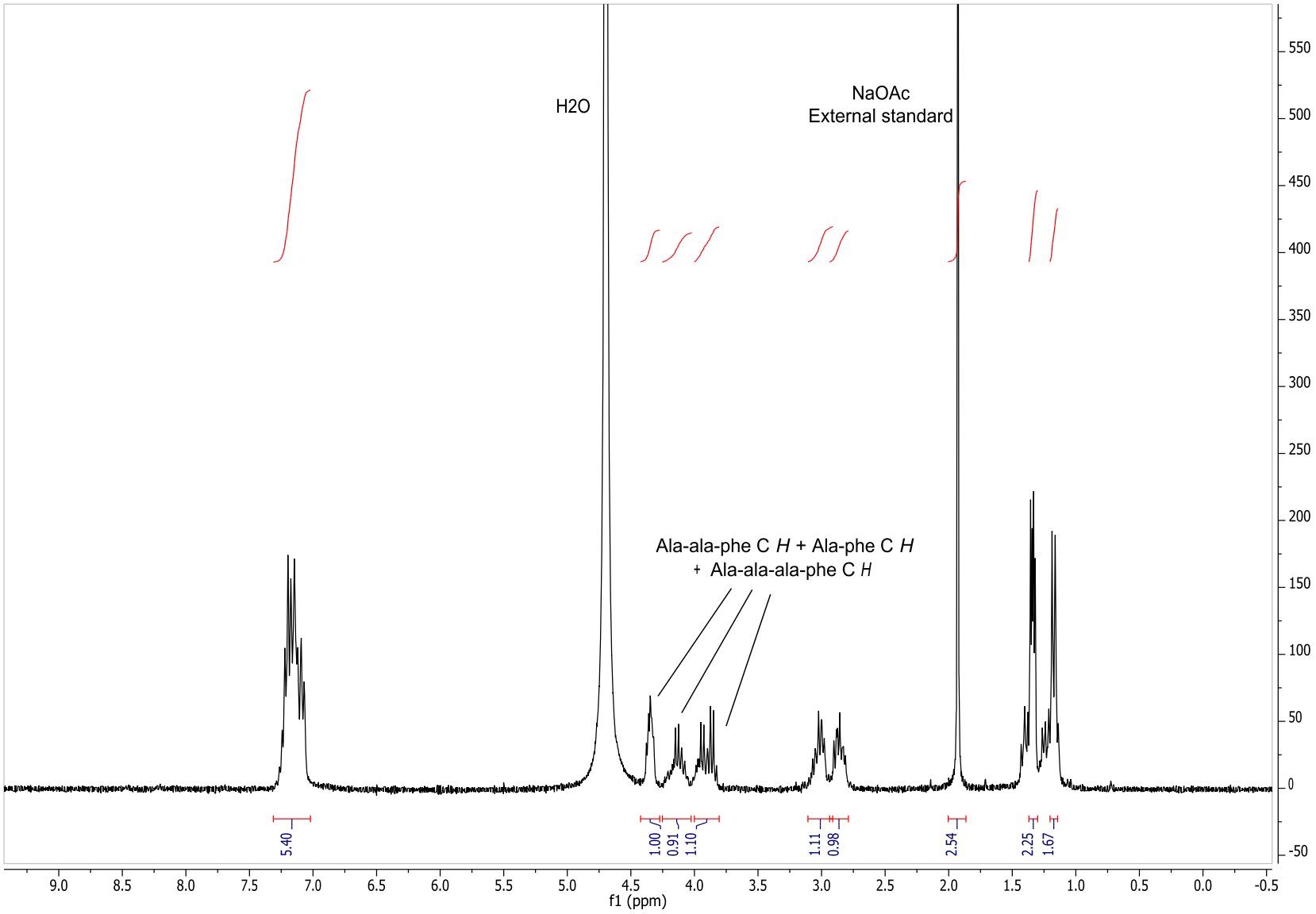
9.3 Examples of ^{1}H NMR spectra at steady state for di- and tri-peptide products under optimum reaction conditions

For formation of L-alanyl-L-phenylalanine and L-alanyl-L-leucine see section 9.2.

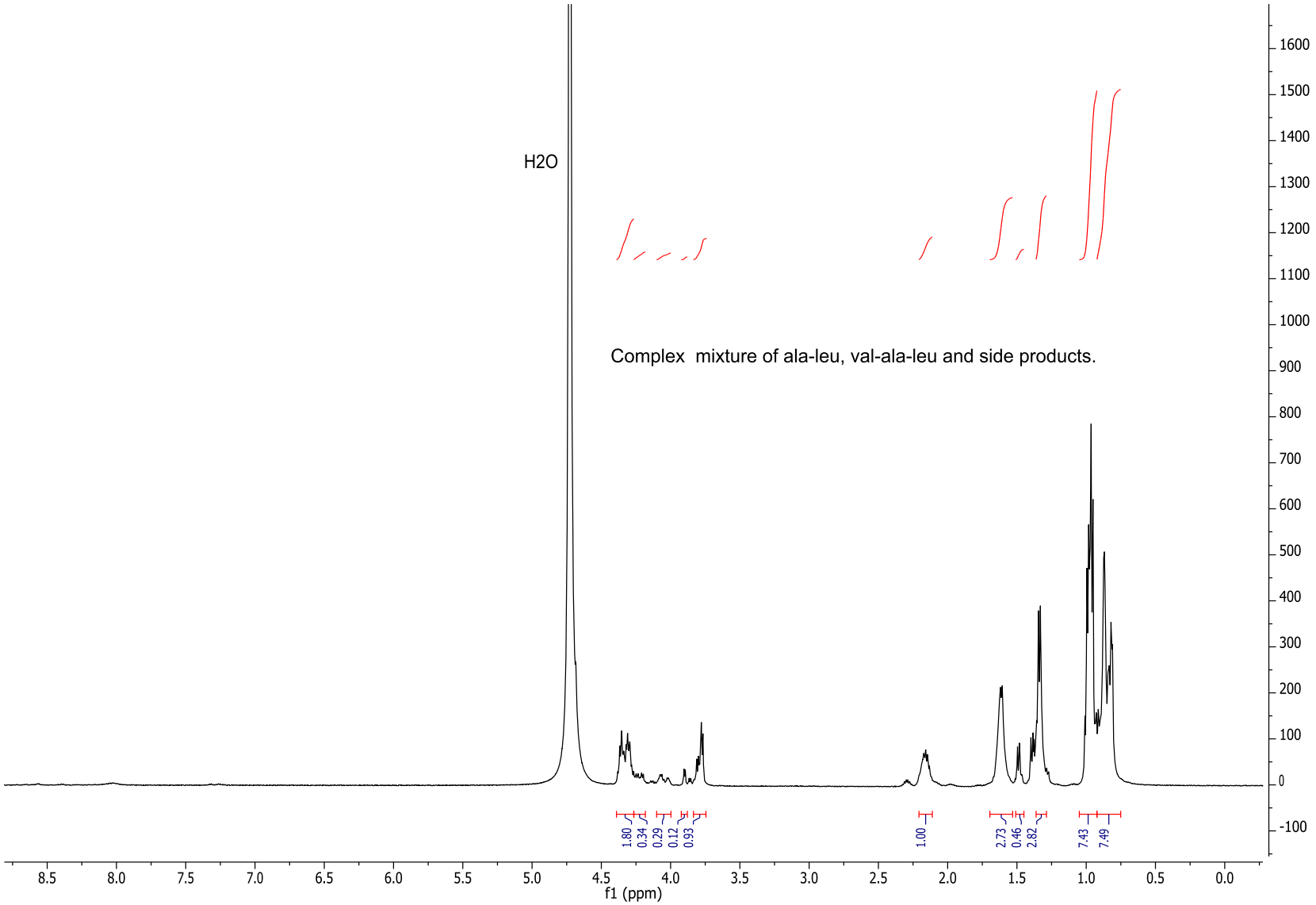
Formation of L-valyl-L-phenylalanine (Main paper: Figure 3) ^{1}H NMR (400 MHz, D_2O)



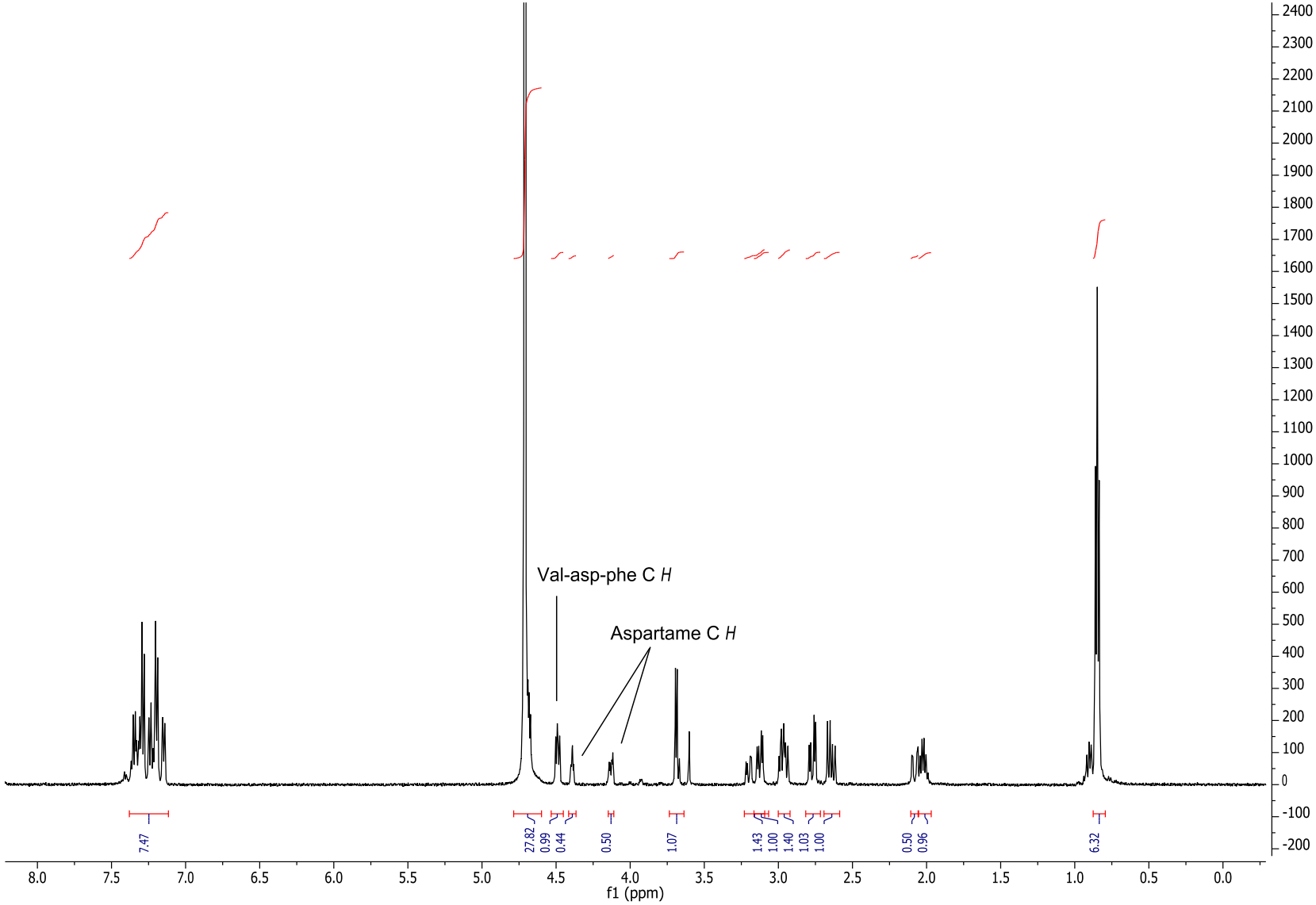
Formation of L-alanyl-L-alanyl-L-phenylalanine (Main paper: Figure 3) ^{1}H NMR (300 MHz, D_2O)



Formation of L-valyl-L-alanyl-L-leucine (Main paper: Figure 3) ^{1}H NMR (500 MHz, D_2O)

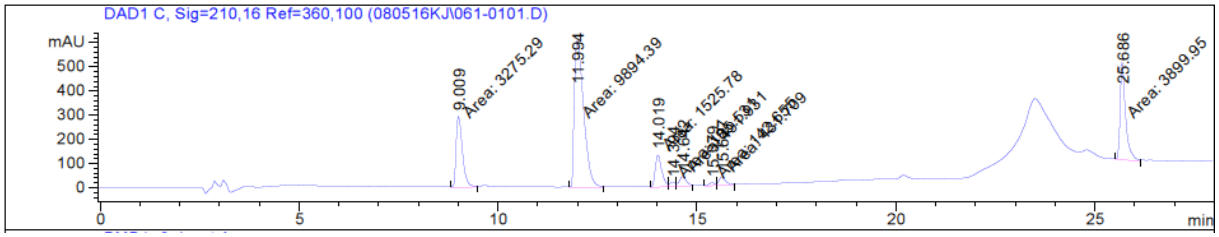


Formation of L-valyl-L-aspartyl-L-phenylalanine (Main paper: Figure 3) ^{1}H NMR (500 MHz, D_2O)



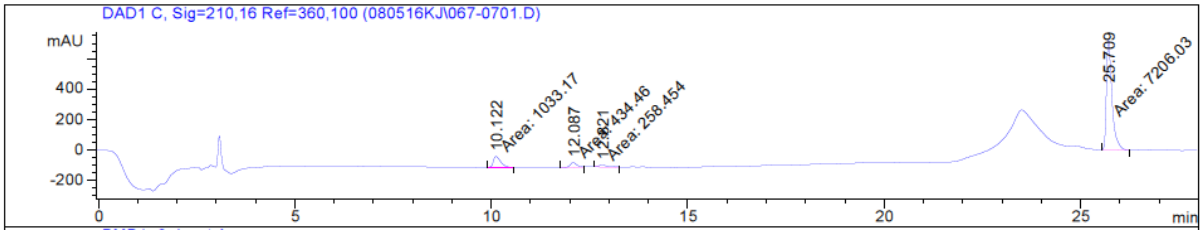
9.4 HPLC chromatograms for crude reaction solutions

Formation of L-Alanyl-L-phenylalanine



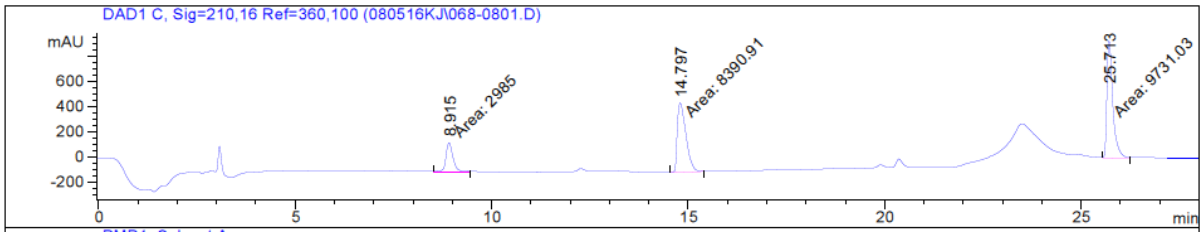
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	9.009	MM	0.1862	3275.28540	293.21326	16.5025
2	11.994	MM	0.2715	9894.38867	607.40411	49.8527
3	14.019	MF	0.1913	1525.78223	132.91330	7.6876
4	14.394	MF	0.1893	185.53064	16.33751	0.9348
5	14.642	FM	0.1977	491.93100	41.46343	2.4786
6	15.379	MF	0.1891	142.65488	12.57442	0.7188
7	15.627	FM	0.1961	431.70895	36.68488	2.1752
8	25.686	MM	0.1611	3899.95288	403.37393	19.6499
Totals :				1.98472e4	1543.96483	

Formation of L-Alanyl-L-leucine



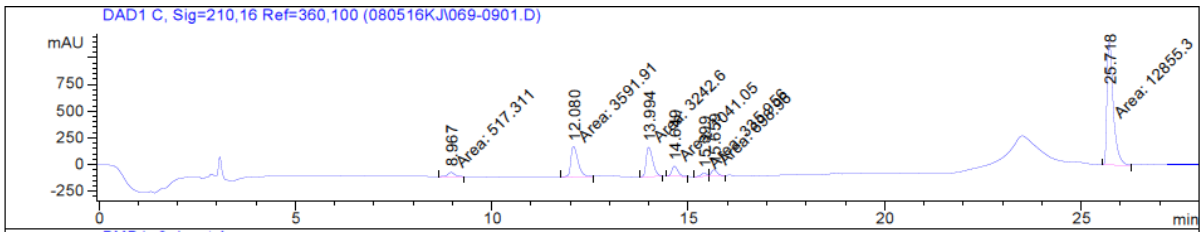
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	10.122	MM	0.2347	1033.17004	73.37262	11.5669
2	12.087	MM	0.2225	434.46036	32.54597	4.8640
3	12.821	MM	0.3183	258.45361	13.53321	2.8935
4	25.709	MM	0.1659	7206.03467	723.91724	80.6755
Totals :				8932.11868	843.36903	

Formation of L-Valyl-L-phenylalanine



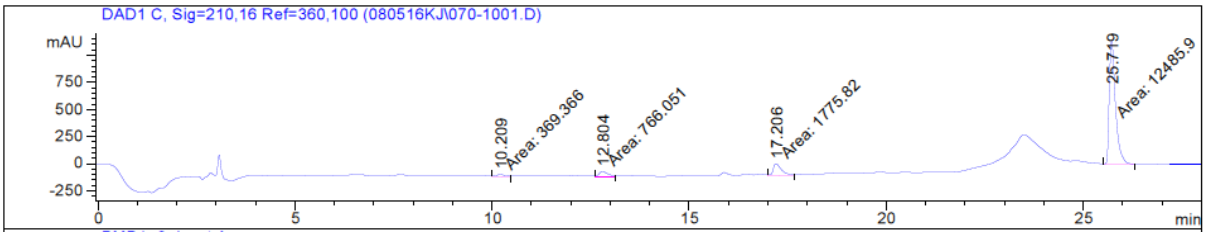
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.915	MM	0.2141	2984.99512	232.37984	14.1422
2	14.797	MM	0.2549	8390.91016	548.63324	39.7543
3	25.713	MM	0.1747	9731.03027	928.27277	46.1035
Totals :				2.11069e4	1709.28584	

Formation of L-Alanyl-L-alanyl-L-phenylalanine



Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.967	MM	0.2124	517.31085	40.59197	2.3215
2	12.080	MM	0.2109	3591.91431	283.83630	16.1195
3	13.994	MM	0.1967	3242.60303	274.78009	14.5519
4	14.649	MM	0.1844	1041.05396	94.06982	4.6719
5	15.399	MF	0.1734	335.95642	32.29836	1.5077
6	15.659	FM	0.1885	698.95972	61.78560	3.1367
7	25.718	MM	0.1857	1.28553e4	1154.06506	57.6908
Totals :				2.22831e4	1941.42720	

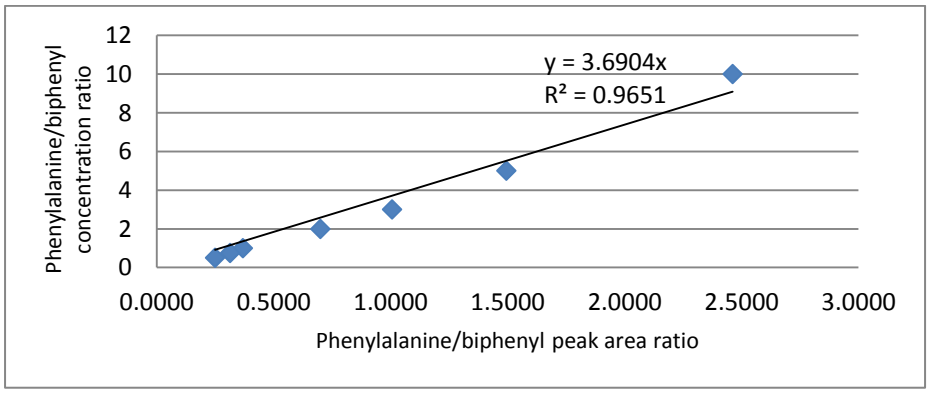
Formation of L-Valyl-L-alanyl-L-leucine



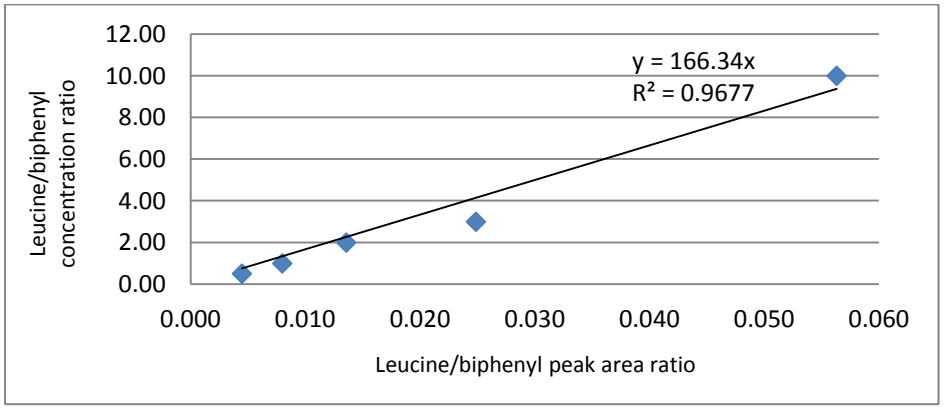
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	10.209	MM	0.2682	369.36618	22.94930	2.3989
2	12.804	MM	0.2593	766.05103	49.23340	4.9753
3	17.206	MM	0.2640	1775.82202	112.09115	11.5335
4	25.719	MM	0.1836	1.24859e4	1133.52039	81.0923
Totals :				1.53971e4	1317.79424	

9.5 HPLC calibration data

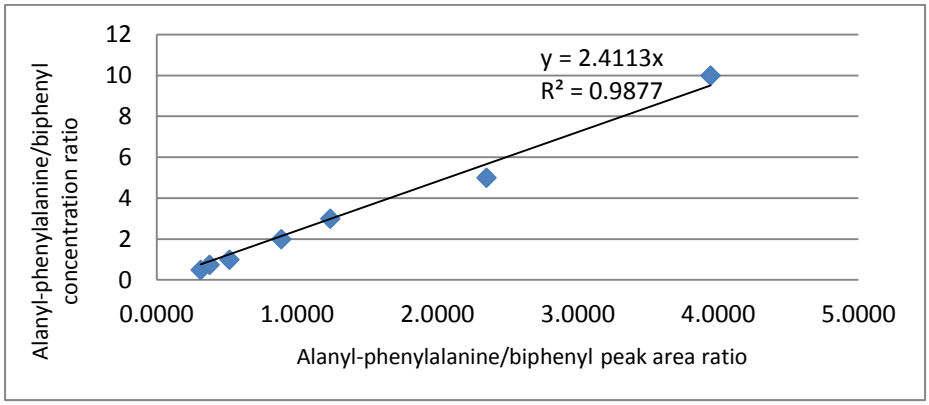
L-Phenylalanine



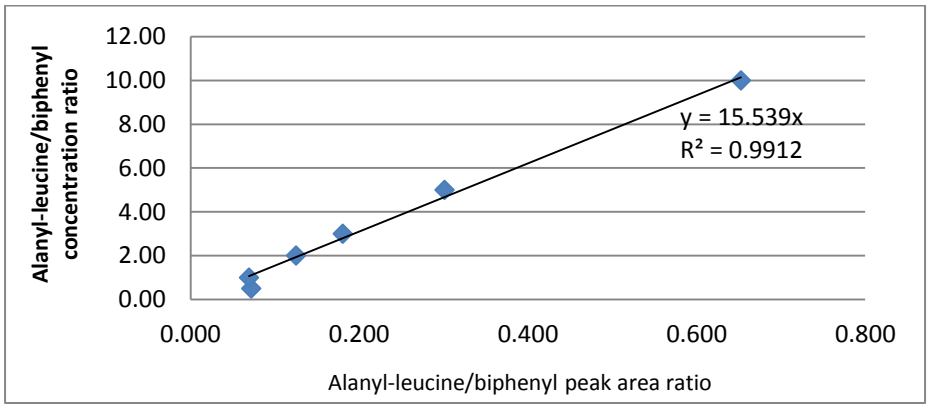
L-Leucine



L-Alanyl-L-phenylalanine

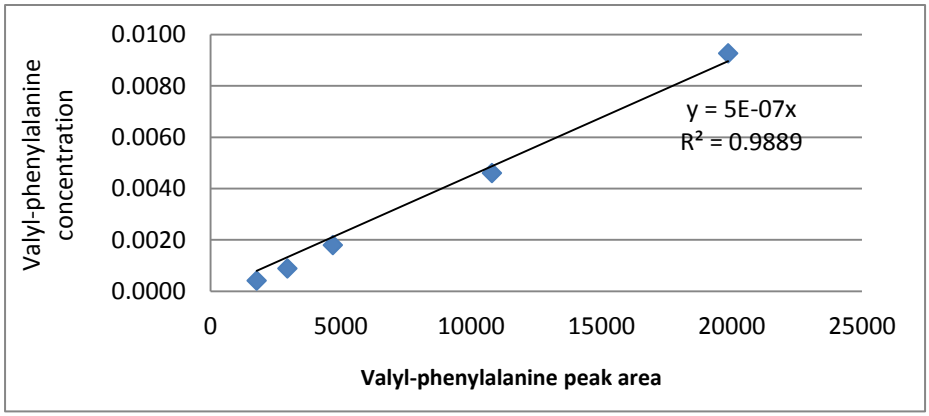


L-Alanyl-L-leucine

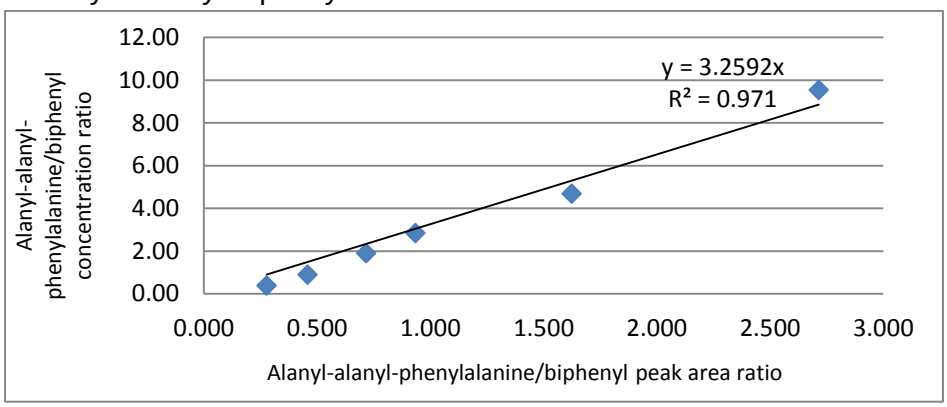


L-Valyl-L-phenylalanine

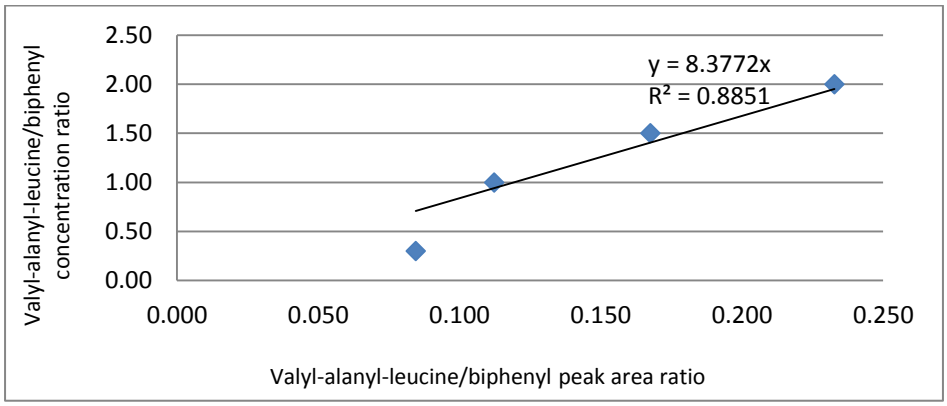
Quantification of valyl-phenylalanine using biphenyl external standard gave results inconsistent with the NMR data obtained for reaction solutions. Instead, the valyl-phenylalanine formed in reaction solutions was quantified using a plot of valyl-phenylalanine standard concentration vs. valyl-phenylalanine standard peak area (below).



L-Alanyl-L-alanyl-L-phenylalanine



L-Valyl-L-alanyl-L-leucine



9. References

[1] R. B. Merrifield, J. Am. Chem. Soc. **1963**, 85, 2149-2154.

[2] S. Fuse, Y. Mifune, T. Takahashi, Angewandte Chemie International Edition **2014**, 53, 851-855.

[3] R. Hirschmann, R. G. Strachan, H. Schwam, E. F. Schoenewaldt, H. Joshua, B. Barkemeyer, D. F. Veber, W. J. P. Jr, T. A. Jacob, T. E. Beesley, R. G. Denkewalter, The Journal of Organic Chemistry **1967**, 32, 3415-3425.

[4] C. R. McElroy, A. Constantinou, L. C. Jones, L. Summerton, J. H. Clark, Green Chemistry **2015**, 17, 3111-3121.

[5] R. U. Lemieux, M. A. Barton, Canadian Journal of Chemistry **1971**, 49, 767-776.