# Peptide Data

## General

All amino acids and coupling reagents were obtained from commercial sources and used as received without further purification. *N,N'*-dimethylformamide, dichloromethane, diethyl ether, piperidine and acetic anhydride were obtained from commercial suppliers and used without further purification.

Peptides were synthesised manually using a Merrifield bubbler, attached to a vacuum line, a nitrogen line and a round-bottom flask for waste. Peptides were synthesised in an automated manner on a Gyros Protein Technologies Prelude X Peptide Synthesiser.

Centrifugation was carried out using an Eppendorf 5702 centrifuge. Reverse-phase HPLC analysis of peptides was conducted using an Agilent Technologies 1260 Infinity system using a Machery-Nagel, EC 4.6 x 250 mm, Nucleodur C18 Gravity, 5 µm column. Analysis was performed using a gradient method ranging from 5-95% MeCN (containing 0.1% TFA)/$H_2O$ (containing 0.1% TFA)/ over 31 minutes with a flow rate of 1 mL/min and UV monitoring at 214 nm.

Low-resolution mass spectra were obtained using an Agilent Technologies 1200 series instrument with a 6130 single quadropole LC/MS using a poroshell EC-C18 column. Analysis was performed using a gradient method, eluting with 5–95% MeCN (containing 5nM formic acid)/$H_2O$ (containing 5nM formic acid) over 18 minutes at a flow rate of 1 mL/min, with UV monitoring at 214 nm.

## Peptide Synthesis

All peptides were assembled onto Wang resin. The first amino acid was converted to the symmetrical amino acid anhydride by dissolving the amino acid (5 equiv.) in a minimum volume of anhydrous DCM then DIC (2.5 equiv.) dissolved in a minimum volume of anhydrous DCM was added to the flask and the mixture stirred at 0 °C for 10 minutes. The reaction mixture was then allowed to warm to room temperature and stirred for a further 10 minutes. The solvent was removed *in vacuo* and the anhydride was redissolved in DMF for manual loading onto the resin using a Merrifield bubbler, attached to a vacuum line, a nitrogen line and a round-bottom flask for waste (Table 1).

Table 1: Manual Procedure for Coupling of First Amino Acid Residue onto Wang Resin

| Step | Solvent/Reagents | Volume (mL) | Time (min) | Mixing | Iterations |
|---|---|---|---|---|---|
| **Swell** | DCM | 10 | 10 | - | |
| **Wash** | DMF | 10 | 0.5 | $N_2$ bubbling | 5 |
| **Esterification** | Amino acid anhydride (5 equiv.), DMAP (1 equiv.) in DMF | 10 | 60 | $N_2$ bubbling | 1 |
| **Wash** | DMF | 10 | 0.5 | $N_2$ bubbling | 5 |
| **Wash** | DCM | 10 | 0.5 | $N_2$ bubbling | 5 |
| **Wash** | $Et_2O$ | 10 | 0.5 | $N_2$ bubbling | 5 |
| **Vacuum Dry** | - | - | 30 | - | 1 |
| **Loading Test** | 20 % (v/v) piperidine in DMF | 10 | 15 | Sonication | 2 |
| **Swell** | DCM | 10 | 10 | - | 1 |
| **Wash** | DMF | 10 | 0.5 | $N_2$ bubbling | 5 |

| | | | | | |
|---|---|---|---|---|---|
| **Capping** | Acetic anhydride (10 equiv.), pyridine (1 equiv.) in DMF | 10 | 30 | $N_2$ bubbling | 1 |
| **Wash** | DMF | 10 | 0.5 | $N_2$ bubbling | 5 |
| **Wash** | DCM | 10 | 0.5 | $N_2$ bubbling | 5 |
| **Wash** | $Et_2O$ | 10 | 0.5 | $N_2$ bubbling | 5 |
| **Vacuum Dry** | - | - | 30 | - | 1 |

The manual loading of each amino acid residue was assessed by an Fmoc loading test. The resin (~10 mg) was added to a 10 mL volumetric flask (x2) and dissolved in a solution of 20% (v/v) piperidine in DMF then sonicated for 15 minutes. The sample absorption was measures by UV against a blank solution of 20% (v/v) piperidine in DMF. The resin loading was then calculated using an equation derived from the Beer-Lambert law and an average taken of the two calculated values.

$$L = \frac{A \times 10}{m \times 7.8}$$

Where, L = loading; A = absorbance at 302 nm; m = mass of resin used.

The resin loading value determined was used to calculate the requires masses for the subsequent amino acid residues for assembly of the desired peptide, which was carried out through both manual (Tables 2-4) and automated (Tables 5-7) processes.

Table 2: Manual Procedure for Coupling of Second Amino Acid Residue

| Step | Solvent/Reagents | Volume (mL) | Time (min) | Mixing | Iterations |
|---|---|---|---|---|---|
| **Swell** | DCM | 5 | 15 | $N_2$ bubbling | 1 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling | 5 |
| **Deprotection** | 20% (v/v) piperidine in DMF | 5 | 5 | $N_2$ bubbling | 3 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling | 5 |
| **Coupling** | Amino acid, PyAOP, DIPEA in DMF | 5 | * | $N_2$ bubbling | 1 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling | 5 |

*coupling times 2 hours for unoptimised synthesis and as stated in table 8 for optimised synthesis

Table 3: Manual Procedure for Coupling of Subsequent Amino Acid Residues

| Step | Solvent/Reagents | Volume (mL) | Time (min) | Mixing | Iterations |
|---|---|---|---|---|---|
| **Deprotection** | 20% (v/v) piperidine in DMF | 5 | 5 | $N_2$ bubbling | 3 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling | 5 |
| **Coupling** | Amino acid, PyAOP, DIPEA in DMF | 5 | * | $N_2$ bubbling | 1 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling | 5 |

*coupling times 2 hours for unoptimised synthesis and as stated in table 8 for optimised synthesis

Table 4: Manual Procedure for Coupling of Final Amino Acid Residue

| Step | Solvent/Reagents | Volume (mL) | Time (min) | Mixing | Iterations |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| **Deprotection** | 20% (v/v) piperidine in DMF | 5 | 5 | $N_2$ bubbling | 3 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling | 5 |
| **Coupling** | Amino acid, PyAOP, DIPEA in DMF | 5 | * | $N_2$ bubbling | 1 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling | 5 |
| **Wash** | DCM | 5 | 0.5 | $N_2$ bubbling | 5 |
| **Wash** | $Et_2O$ | 5 | 0.5 | $N_2$ bubbling | 5 |
| **Vacuum dry** | - | - | 30 | - | 1 |

*coupling times 2 hours for unoptimised synthesis and as stated in table 8 for optimised synthesis

Table 5: Automated Procedure for Coupling of Second Amino Acid Residue

| Step | Solvent/Reagents | Volume (mL) | Time (min) | Mixing | Iterations |
|---|---|---|---|---|---|
| **Swell (Top Wash)** | DCM | 5 | 15 | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Top Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 4 |
| **Deprotection** | 20% (v/v) piperidine in DMF | 5 | 5 | $N_2$ bubbling and shaking (350 rpm) | 3 |
| **Top Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 4 |
| **Coupling** | Amino acid (0.25 M, 2 mL), PyAOP (0.25 M, 2 mL), DIPEA (1.0 M, 1 mL) in DMF | 5 | * | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Top Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 4 |

*coupling times 2 hours for unoptimised synthesis and as stated in table 8 for optimised synthesis

Table 6: Automated Procedure for Coupling of Subsequent Amino Acid Residues

| Step | Solvent/Reagents | Volume (mL) | Time (min) | Mixing | Iterations |
|---|---|---|---|---|---|
| **Deprotection** | 20% (v/v) piperidine in DMF | 5 | 5 | $N_2$ bubbling and shaking (350 rpm) | 3 |

| | | | | | |
|---|---|---|---|---|---|
| **Top Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 4 |
| **Coupling** | Amino acid (0.25 M, 2 mL), PyAOP (0.25 M, 2 mL), DIPEA (1.0 M, 1 mL) in DMF | 5 | * | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Top Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 4 |

*coupling times 2 hours for unoptimised synthesis and as stated in table 8 for optimised synthesis

Table 7: Automated Procedure for Coupling of Final Amino Acid Residue

| Step | Solvent/Reagents | Volume (mL) | Time (min) | Mixing | Iterations |
|---|---|---|---|---|---|
| **Deprotection** | 20% (v/v) piperidine in DMF | 5 | 5 | $N_2$ bubbling and shaking (350 rpm) | 3 |
| **Top Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 4 |
| **Coupling** | Amino acid (0.25 M, 2 mL), PyAOP (0.25 M, 2 mL), DIPEA (1.0 M, 1 mL) in DMF | 5 | * | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Top Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Wash** | DMF | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 4 |
| **Top Wash** | DCM | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 1 |
| **Wash** | DCM | 5 | 0.5 | $N_2$ bubbling and shaking (350 rpm) | 4 |

*coupling times 2 hours for unoptimised synthesis and as stated in table 8 for optimised synthesis

After SPPS, a trial cleave was conducted for each peptide to determine if the synthesis was successful. 10 mg of each resin was added to 250 µL of the cleaving solution TFA/$H_2O$/TIS (95:2.5:2.5

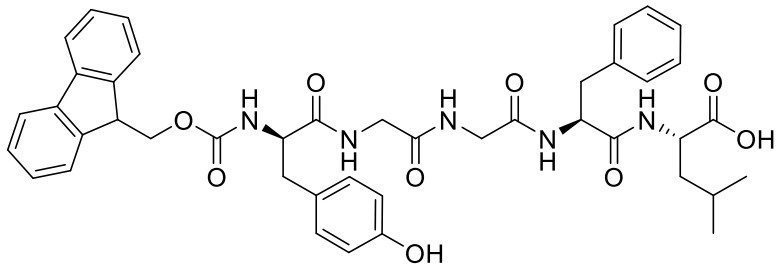
v/v). The solution was stirred at room temperature for 1 hour then decanted into cold $Et_2O$ to precipitate the peptide. The suspension was centrifuged for 3 minutes at 4400 rpm and the liquid discarded. This was repeated 3 times then the remaining solid peptide was dissolved in a MeCN/$H_2O$ solution for analysis by HPLC and LCMS.

Once the success of the synthesis was confirmed, a full cleave was performed for each peptide. The resin was suspended in 10 mL of the cleaving solution TFA/$H_2O$/TIS (95:2.5:2.5 v/v) and the solution was shaken at room temperature for 4 hours then added dropwise to cold $Et_2O$ to precipitate the peptide. The suspension was centrifuged for 3 minutes at 4400 rpm and the liquid discarded. This was repeated 5 times then the remaining solid peptide was freeze dried before analysis by HPLC and LCMS.

Table 8: Optimised Coupling Times

| Sequence | SPPS Cycle | Optimised Coupling Time (min) |
|---|---|---|
| **YGGFL** | 1st | 50 |
| | 2nd | 70 |
| | 3rd | 45 |
| | 4th | 45 |
| **YAibAibFL** | 1st | 45 |
| | 2nd | 16 |
| | 3rd | 75 |
| | 4th | 60 |
| **KLLQDILDA** | 1st | 60 |
| | 2nd | 50 |
| | 3rd | 15 |
| | 4th | 40 |
| | 5th | 80 |
| | 6th | 110 |
| | 7th | 95 |
| | 8th | 55 |

## YGGFL

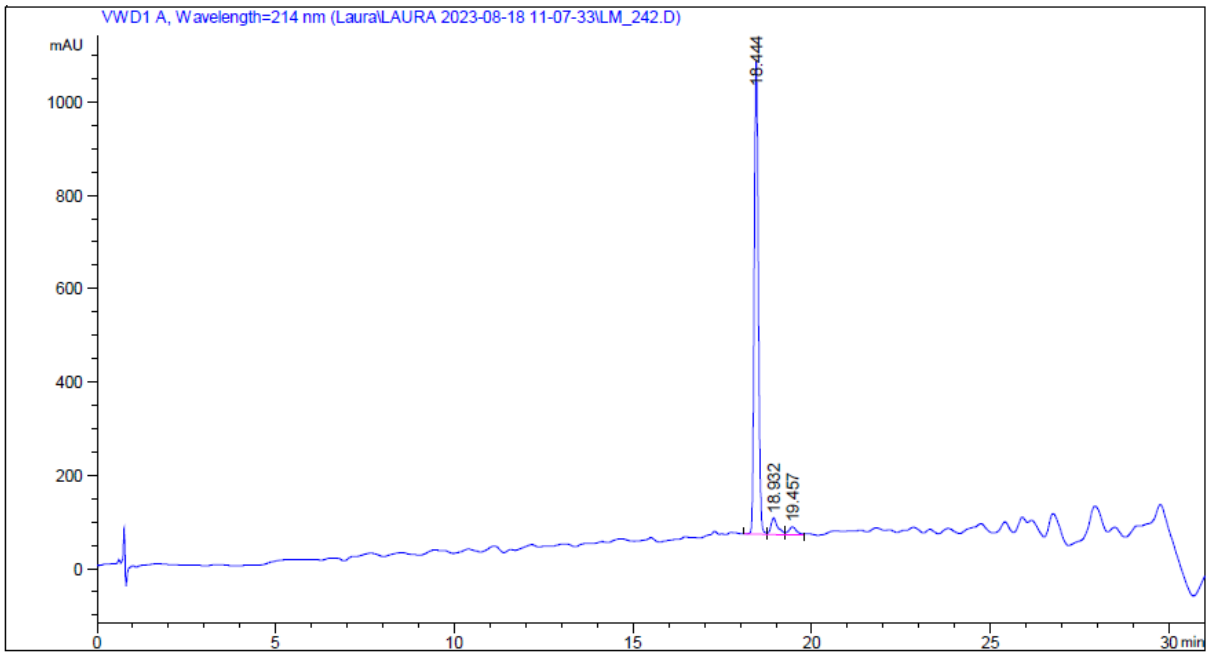


### Unoptimised

Using a resin loading value of 0.546 mmol/g, Leu-loaded Wang resin (183 mg, 0.1 mmol) was added to a 50 mL Merrifield bubbler and the title peptide was synthesised according to tables 2-4 with a coupling time of 2 hours for each residue. Full cleavage yielded Fmoc-YGGFL (59.1 mg, 76 µmol, 76%) as a white solid.

HPLC purity: 91%

LRMS m/z: $[M-H]^-$ calculated for $C_{43}H_{46}N_5O_9$ 776.3296; found 776.2

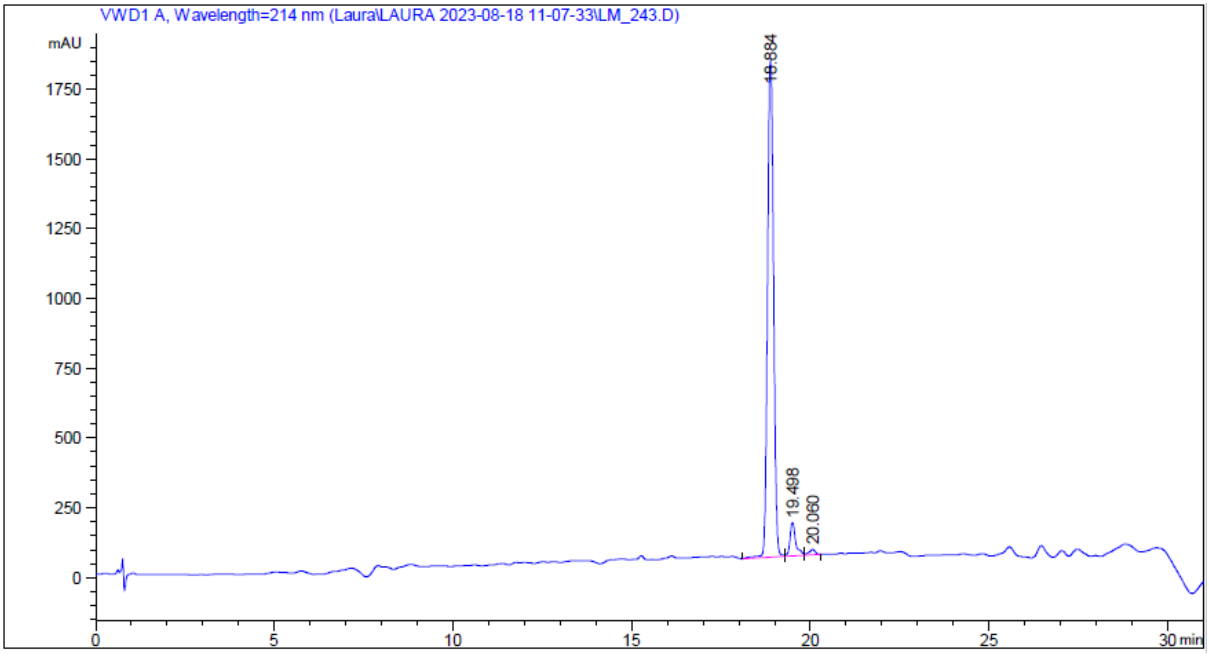



## Optimised

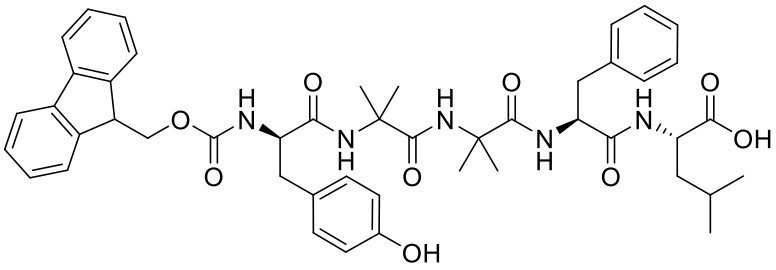
Using a resin loading value of 0.546 mmol/g, Leu-loaded Wang resin (183 mg, 0.1 mmol) was added to a 50 mL Merrifield bubbler and the title peptide was synthesised according to tables 2-4 with coupling times as stated in table 8. Full cleavage yielded Fmoc-YGGFL (59.6 mg, 77 µmol, 77%) as a white solid.

HPLC purity: 93%

LRMS m/z: $[M-H]^-$ calculated for $C_{43}H_{46}N_5O_9$ 776.3296; found 776.2




## YAibAibFL

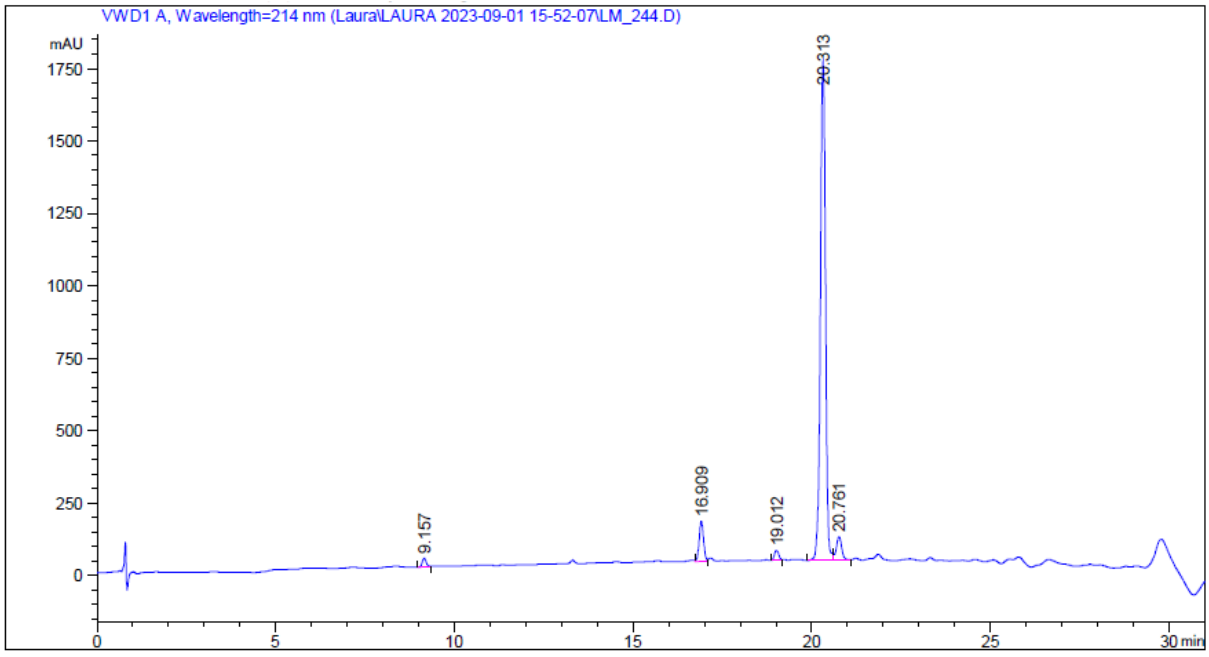


Unoptimised

Using a resin loading value of 0.546 mmol/g, Leu-loaded Wang resin (183 mg, 0.1 mmol) was added to a 50 mL Merrifield bubbler and the title peptide was synthesised according to tables 2-4 with a coupling time of 2 hours for each residue. Full cleavage yielded Fmoc-YAibAibFL (79.8 mg, 96 µmol, 96%) as a white solid.

HPLC purity: 87%

LRMS m/z: $[M-H]^-$ calculated for $C_{47}H_{54}N_5O_9$ 832.3922; found 832.3

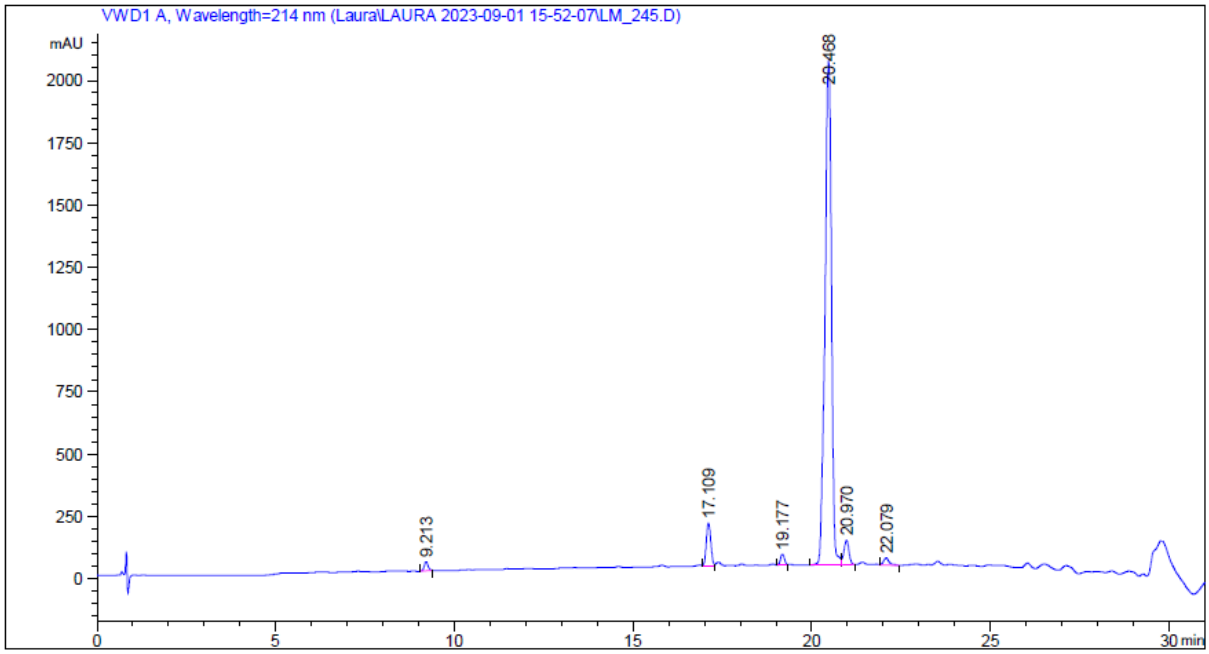



Optimised

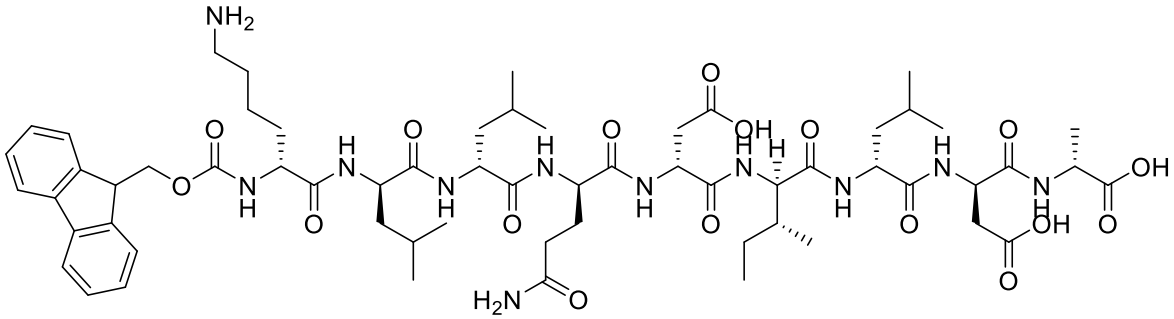
Using a resin loading value of 0.546 mmol/g, Leu-loaded Wang resin (183 mg, 0.1 mmol) was added to a 50 mL Merrifield bubbler and the title peptide was synthesised according to tables 2-4 with coupling times as stated in table 8. Full cleavage yielded Fmoc-YAibAibFL (79.5 mg, 95 µmol, 95%) as a white solid.

HPLC purity: 88%

LRMS m/z: $[M-H]^-$ calculated for $C_{47}H_{54}N_5O_9$ 832.3922; found 832.3




## KLLQDILDA:

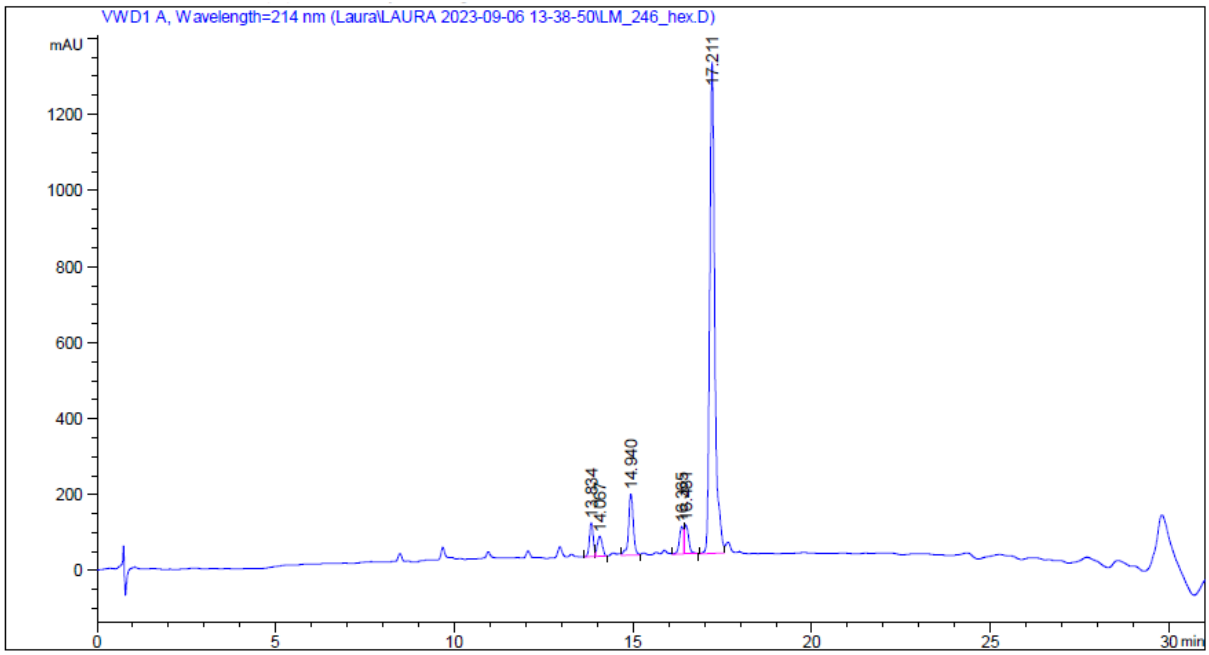


## Unoptimised (Manual)

Using a resin loading value of 0.559 mmol/g, Ala-loaded Wang resin (179 mg, 0.1 mmol) was added to a 50 mL Merrifield bubbler and the title peptide was synthesised according to tables 2-4 with a coupling time of 2 hours for each residue. Full cleavage followed by trituration with hexane yielded Fmoc-KLLQDILDA (106.4 mg, 85 µmol, 85%) as a white powder.

HPLC purity: 77%

LRMS m/z: $[M-H]^-$ calculated for $C_{61}H_{90}N_{11}O_{17}$ 1248.6516; found 1248.7

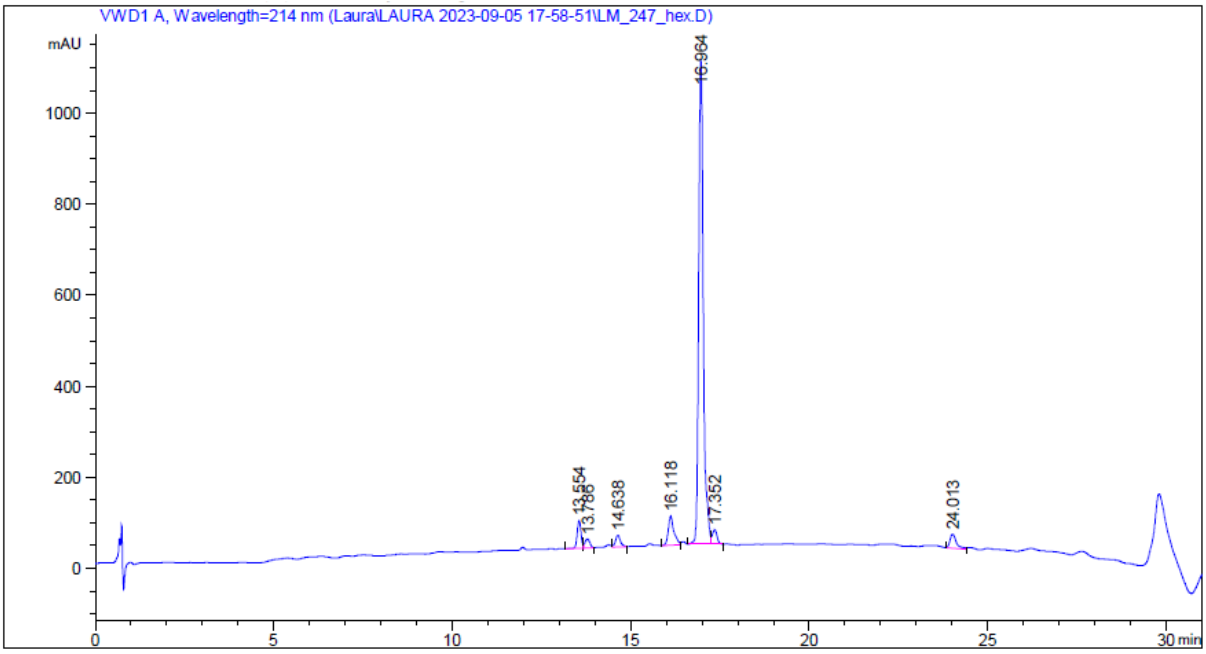



## Optimised (Manual)

Using a resin loading value of 0.559 mmol/g, Ala-loaded Wang resin (179 mg, 0.1 mmol) was added to a 50 mL Merrifield bubbler and the title peptide was synthesised according to tables 2-4 with coupling times as stated in table 8. Full cleavage followed by trituration with hexane yielded Fmoc-KLLQDILDA (101 mg, 81 µmol, 81%) as a white powder.

HPLC purity: 81%

LRMS m/z: $[M-H]^-$ calculated for $C_{61}H_{90}N_{11}O_{17}$ 1248.6516; found 1248.5



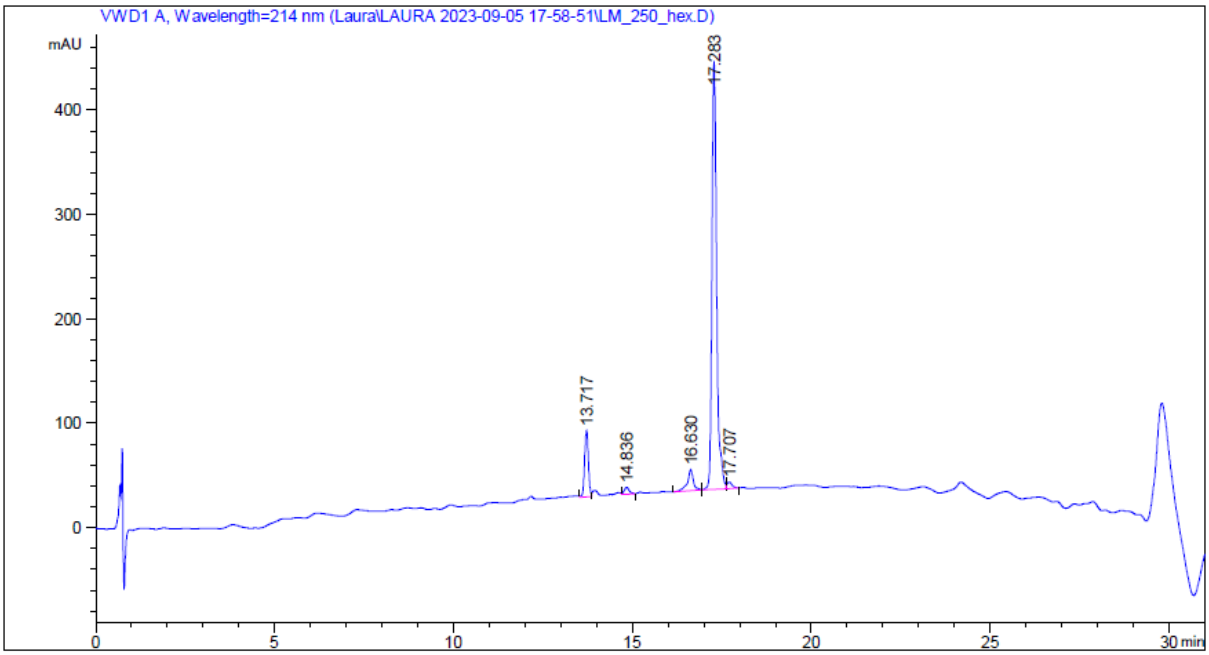

## Unoptimised (Automated)

Using a resin loading value of 0.559 mmol/g, Ala-loaded Wang resin (179 mg, 0.1 mmol) was added to a 10 mL plastic RV and the title peptide was synthesised according to tables 5-7 with a coupling

time of 2 hours for each residue. Full cleavage followed by trituration with hexane yielded Fmoc-KLLQDILDA (121.8 mg, 97 µmol, 97%) as a white powder.

HPLC purity: 82%

LRMS m/z: $[M-H]^-$ calculated for $C_{61}H_{90}N_{11}O_{17}$ 1248.6516; found 1248.5

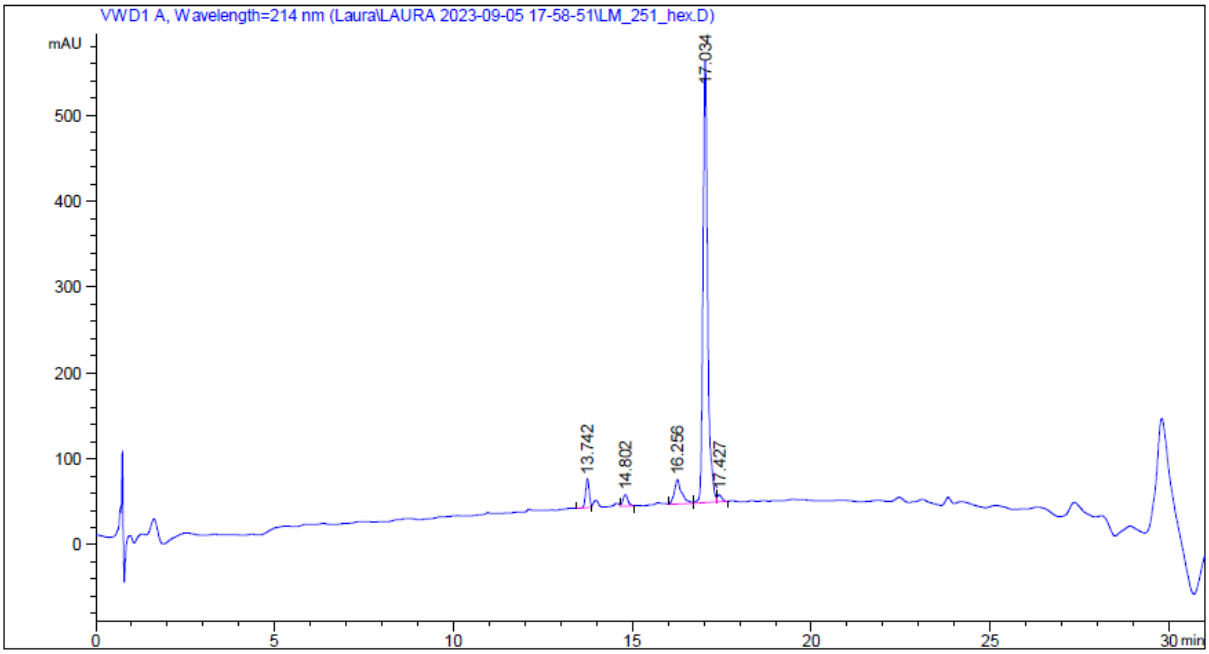



## Optimised (Automated)

Using a resin loading value of 0.559 mmol/g, Ala-loaded Wang resin (179 mg, 0.1 mmol) was added to a 10 mL plastic RV and the title peptide was synthesised according to tables 5-7 with coupling times as stated in table 8. Full cleavage followed by trituration with hexane yielded Fmoc-KLLQDILDA (120.2 mg, 96 µmol, 96%) as a white powder.

HPLC purity: 85%

LRMS m/z: $[M-H]^-$ calculated for $C_{61}H_{90}N_{11}O_{17}$ 1248.6516; found 1248.6




Summary (as per Table 1 in manuscript)

| **Peptide** | **Unoptimized Yield (HPLC Purity)** | **Optimized Yield (HPLC Purity)** | **Optimized Yield (HPLC Purity) AUTOMATED** |
|---|---|---|---|
| YGGFL | 76 (91) | 77 (93) | |
| YAibAibFL | 96 (87) | 95 (88) | |
| KLLQDILDA | 85 (77) | 81 (81) | 96 (85) |