Supporting information: Pharmacological characterisation of mouse calcitonin and calcitonin receptor-like receptors reveals differences compared to human receptors

Michael L. Garelja[1,2], Rebekah L Bower[2], Margaret A. Brimble[2,3,4], Shanan Chand[2], Paul W.R. Harris[2,3,4], Muhammad Aqfan Jamaluddin[2], Jakeb Petersen[2], Andrew Siow[2,4], Christopher S. Walker[2,3], Debbie L. Hay*[1,2,3].

1. Department of Pharmacology and Toxicology, University of Otago, Dunedin, 9016, New Zealand
2. School of Biological Sciences, University of Auckland, Auckland, 1010, New Zealand
3. Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland, 1010, New Zealand
4. School of Chemical Sciences, University of Auckland, Auckland, 1010, New Zealand

Figure count: 29 (22 Figures, 7 Schemes)

Contents

Supplemental Biology

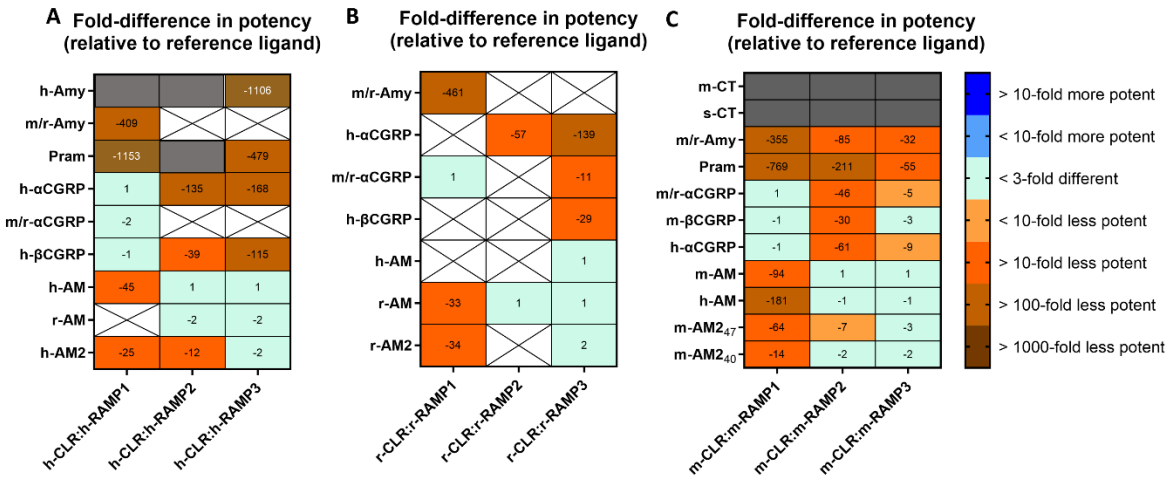


Figure S 1. Heat-maps showing the relative potency of different peptides at human and rat CLR-based receptors. Panel **A** shows results from cells transfected with human CLR and human RAMPs, while panel **B** shows results from cells transfected with rat CLR and rat RAMPs. Panel **C** shows results from this publication. Results presented as fold difference relative to a reference ligand. A positive number indicates that the peptide is more potent than the reference ligand (species matched αCGRP at CLR:RAMP1, and species matched AM at CLR:RAMP2 and CLR:RAMP3), while a negative number indicates the peptide has a lower potency than reference ligand. White boxes displaying a cross indicate that the ligand/receptor combination has not been tested, while a grey box indicates that no curve could be fit to this ligand:receptor combination. Human and rat results are fold-difference between the mean pEC_{50} values derived from multiple previously published studies using transfected cells, values were recently compiled in (Hay et al., 2018) and (Garelja et al., 2020).

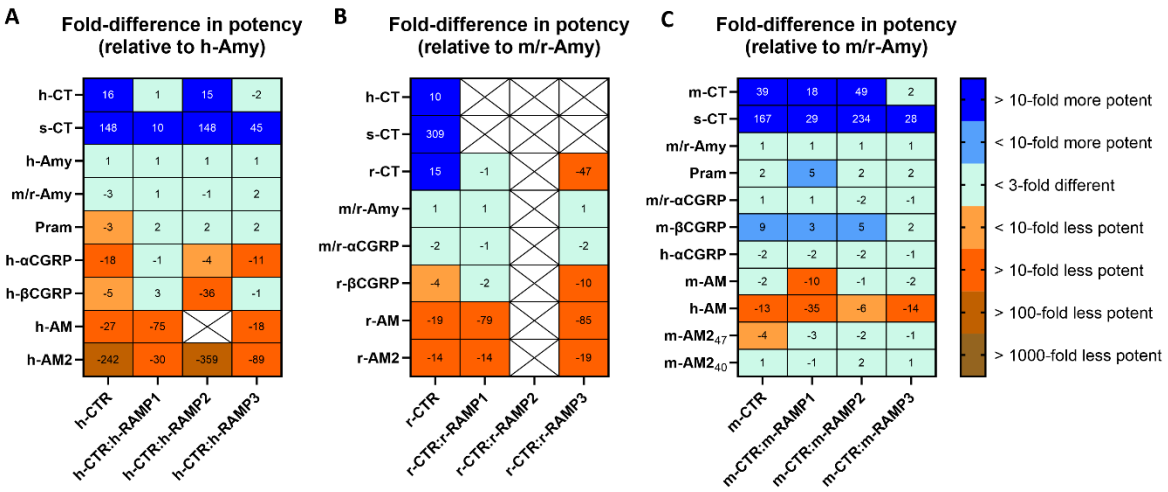


Figure S 2 Heat-maps showing the potencies of peptides at CTR-based receptors, relative to the species-matched Amy (comparisons within receptors). Panel **A** shows results from cells transfected with human receptor components, while panel **B** shows the results from cells transfected with rat receptor components. Panel **C** shows results from this publication. Results presented as fold-difference between the named peptide and the species matched Amy. A positive number indicates that the peptide is more potent than Amy, while a negative number indicates the peptide has a lower potency than Amy. White boxes displaying a cross indicate that the ligand/receptor combination has not been tested. Human and rat results are fold-difference between the mean pEC_{50} values derived from multiple previously published studies using transfected cells, values were recently compiled in (Hay et al., 2018).

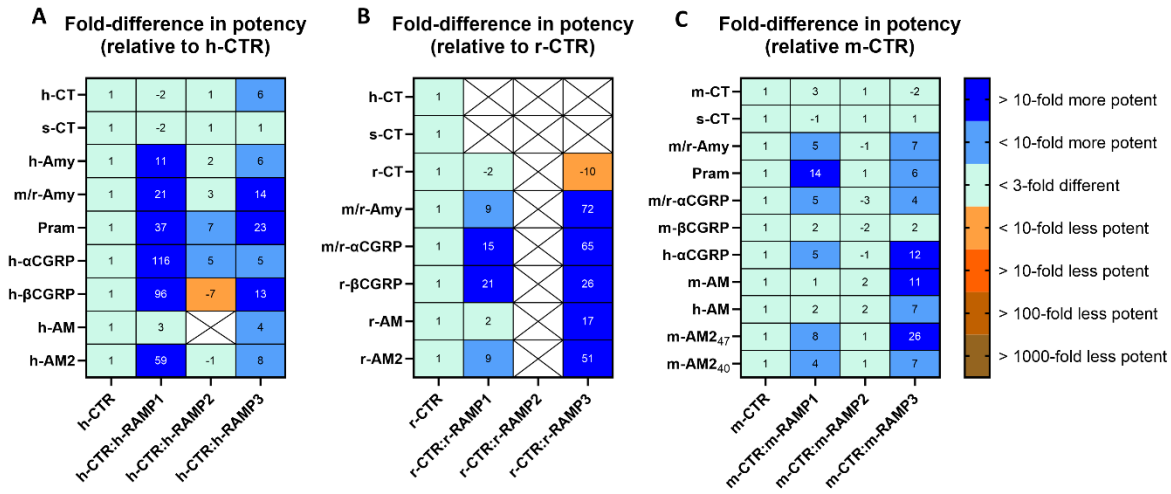


Figure S 3. Heat-maps showing the relative potency of peptides at CTR:RAMP complexes compared to CTR alone. Panel **A** shows results from cells transfected with human CTR and human RAMPs, while panel **B** shows results from cells transfected with rat CTR and rat RAMPs. Panel **C** shows results from this publication. Results presented as fold change relative to the potency at CTR alone. A positive number indicates an increase in peptide potency when cells were transfected with a RAMP in combination with CTR compared to cells transfected with CTR alone. White boxes displaying a cross indicate that the ligand/receptor combination has not been tested. Human and rat results are fold-difference between the mean pEC_{50} values derived from multiple previously published studies using transfected cells, values were recently compiled in (Hay et al., 2018).

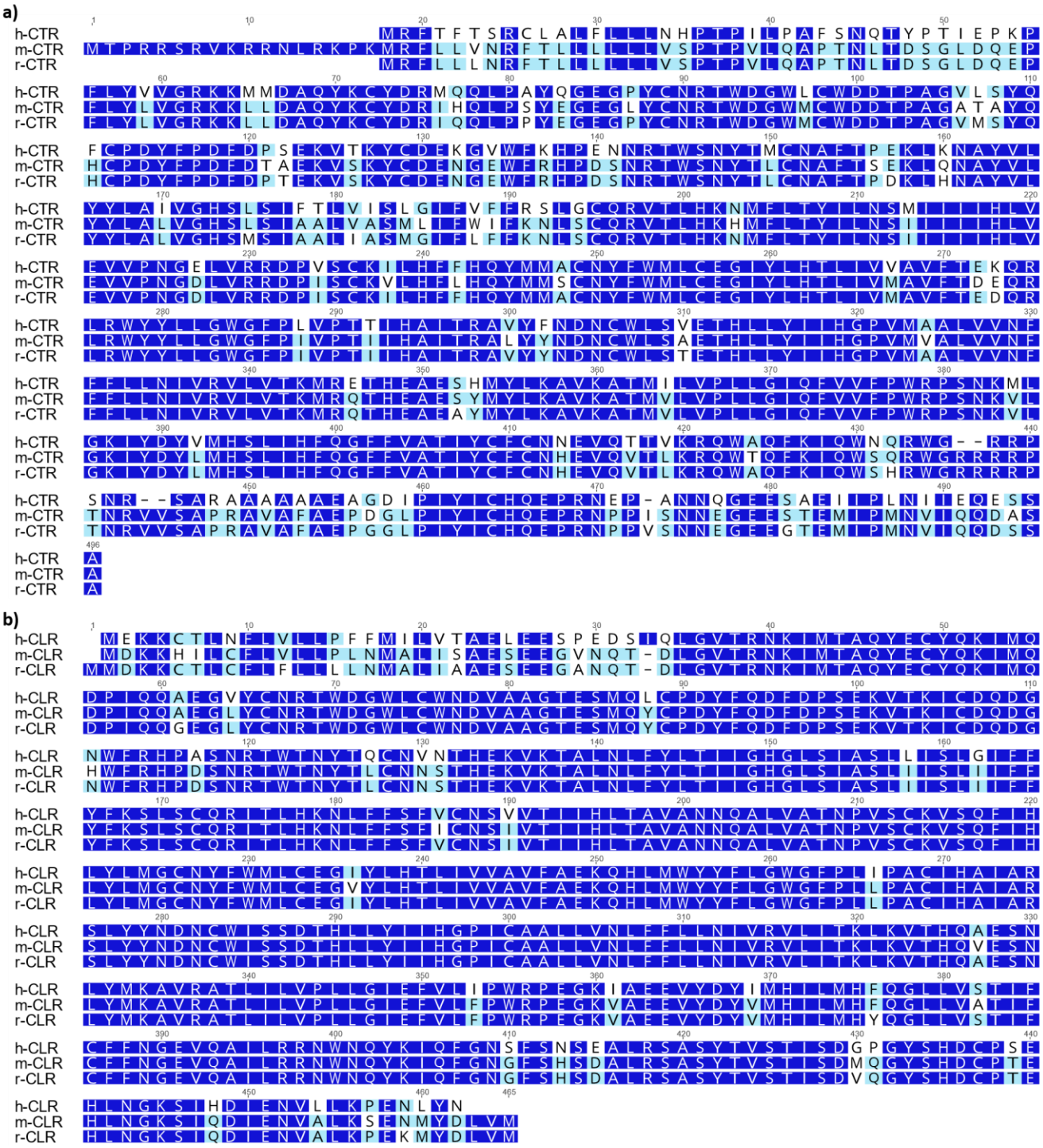


Figure S 4. Amino acid sequence alignment of human, mouse, and rat (a) $CTR_{(a)}$ and (b) CLR. Sequence alignment created using Geneious Prime. Dark blue background with white text indicates an exact match, medium blue background indicates greater than 80% similarity at a given position, and light blue background indicates a similarity between 60% and 80% at a given position.

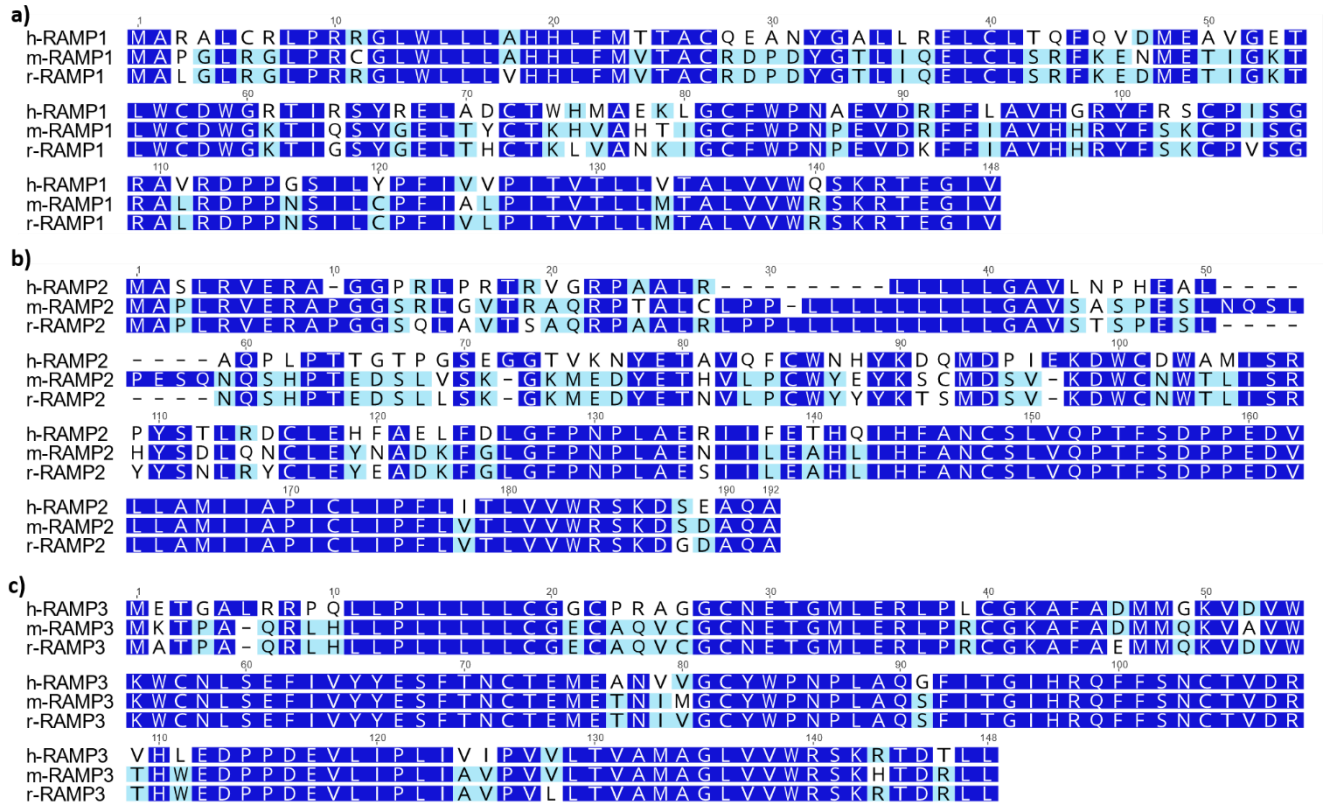


Figure S 5. Amino acid sequence alignment of human, mouse, and rat (a) RAMP1, (b) RAMP2, and (c) RAMP3. Sequence alignment created using Geneious Prime using the uniprot accepted sequences. Dark blue background with white text indicates an exact match, medium blue background indicates greater than 80% similarity at a given position, and light blue background indicates a similarity between 60% and 80% at a given position. Note that the m-RAMP2 used in this study encodes a threonine in position ten.

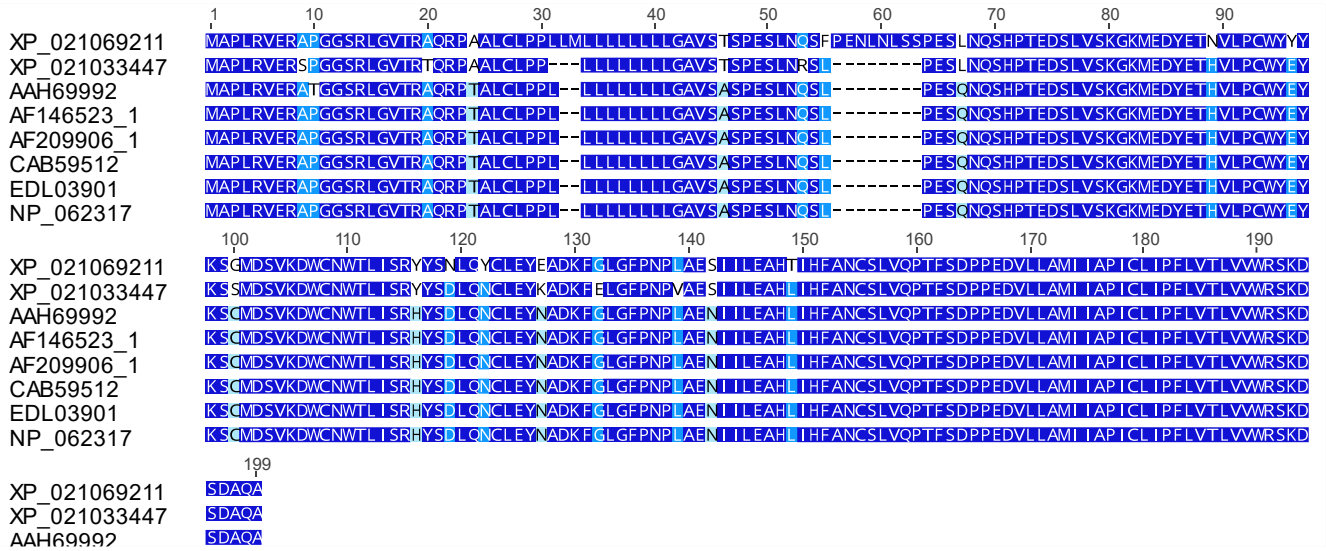


Figure S 6. Amino acid sequence of m-RAMP2 from different studies. NCBI protein accession numbers are listed on the left. Sequences alignment performed in Geneious Prime. Dark blue background with white text indicates an exact match, medium blue background indicates greater than 80% similarity, and light blue indicates a similarity between 60% and 80%. The sequence used in this study was AAH69992.

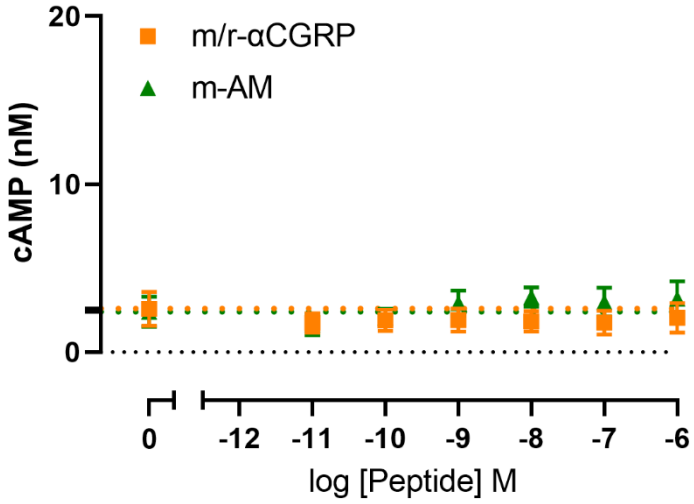


Figure S 7 cAMP production in Cos7 cells transfected with m-CLR:pcDNA and stimulated with m/r-αCGRP or m-AM. Results are the mean ± s.e.m. of 3 independent experiments performed in duplicate. The black dotted line indicates 0, while the colour coded lines indicate the mean of the unstimulated point for each peptide.

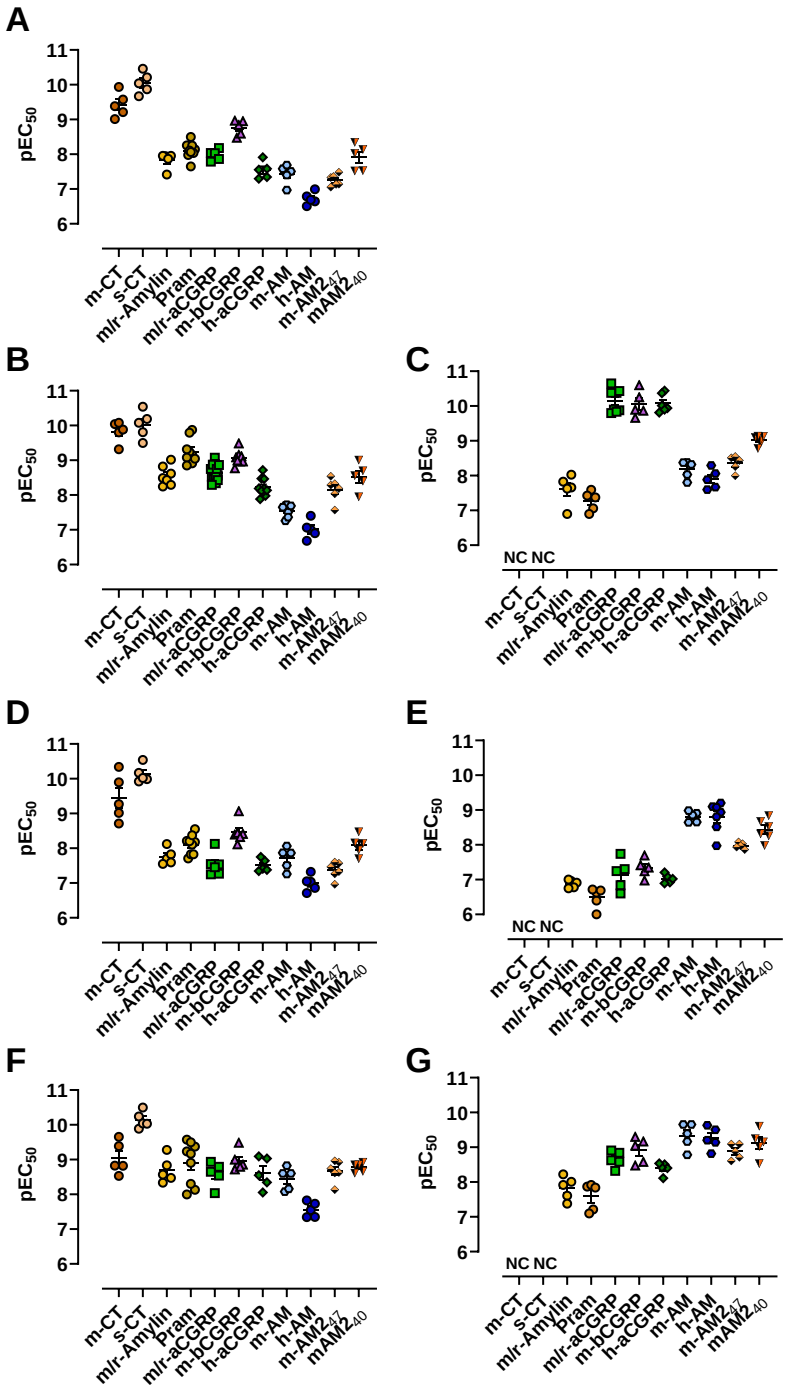


Figure S 8. Distribution of pEC_{50} values from agonist concentration-response experiments in Cos7 cells transfected with m-CTR:pcDNA (**A**), m-CTR:m-RAMP1 (**B**), m-CTR:m-RAMP2 (**D**), m-CTR:m-RAMP3 (**F**), m-CLR:m-RAMP1 (**C**), m-CLR:m-RAMP2 (**E**), or m-CLR:m-RAMP3 (**G**). NC indicates no curve could be fit to the data. Each individual point represents an independent experiment. Bars show the mean ± s.e.m. of experiments. Significance determined using one-way ANOVA with Tukey's post-hoc test comparing peptides within a receptor. For presentation, only differences to a reference agonist are shown in this figure (m-CT at m-CTR:pcDNA, m/r-Amy for m-CTR in complex with m-RAMP1, 2, or 3, m/r-αCGRP at m-CLR:m-RAMP1, and m-AM at m-CLR:m-RAMP2 or 3). See also **Table 1**.

Supplemental Chemistry

General Procedure

All reagents were purchased as reagent grade and used without further purification. *N*,*N*-Diisopropylethylamine (DIPEA), piperidine, *N*,*N*′-diisopropylcarbodiimide (DIC), 1,2-ethanedithiol (EDT), triisopropylsilane (TIPS) and 4-methylmorpholine (NMM) were purchased from Sigma-Aldrich (St. Louis, Missouri). *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH (Pbf = 2,2,4,6,7-pentamethyIdlhydrobenzofuran-5-sulfonyl), Fmoc-Asn(Trt)-OH (Trt = triphenylmethane), Fmoc-Asp(*t*Bu)-OH (*t*Bu = *tert*-butyl), Fmoc-Cys(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-lle-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH (Boc = *tert*-butoxycarbonyl), Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH and 4-[(2,4-Dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid (Rink amide linker) were purchased from CS Bio (Shanghai, China). 6-Chloro-1-hydroxybenzotriazole (6-Cl-HOBt), Fmoc-Ser(*t*Bu)-Ser(Psi(Me,Me)pro)-OH, Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH, Fmoc-Leu-Thr(Psi(Me,Me)pro)-OH, Fmoc-Asp-Ser(Psi(Me,Me)pro)-OH, Fmoc-Gly-Thr(Psi(Me,Me)pro)-OH were purchased from Apptec (Louisville, Kentucky). Aminomethyl polystyrene resin was purchased from Rapp Polymere (Tübingen, Germany). Guanidine hydrochloride (Gu·HCl) was purchased from AK Scientific (Union City, California). Yields refer to chromatographically homogeneous materials. Microwave reactions were carried out on a Biotage® Initiator+ Alstra™ (Uppsala, Sweden) automated peptide synthesizer. Semi-preparative/analytical RP-HPLC was performed on a Thermo Scientific (Waltham, MA) Dionex Ultimate 3000 HPLC equipped with a four channel UV detector at 210, 225, 254 and 280 nm using either an analytical column (Waters (Milford, MA) XTerra® MS C18, (5 μm; 4.6 × 150 mm) at a flow rate of 1 mL min^{-1}, or a Phenomenex® semi-preparative column (Torrance, CA), Gemini C18, (5 μm; 10 × 250 mm) at a flow rate of 4 mL min^{-1}. A suitably adjusted gradient of 5% B to 95% B was used, where solvent A was 0.1% TFA in H_2O and B was 0.1% TFA in acetonitrile. LC-MS spectra were acquired using Agilent Technologies (Santa Clara, CA) 1260 Infinity LC equipped with an Agilent Technologies 6120 Quadrupole mass spectrometer. An analytical column (Agilent C3, 3.5 μm; 3.0 × 150 mm) was used at a flow rate of 0.3 mL min^{-1} using a linear gradient of 5% B to 95% B over 30 min, where solvent A was 0.1% formic acid in H_2O and B was 0.1% formic acid in acetonitrile.

General Methods

Method 1: General procedure for attachment of Fmoc Rink amide to the resin:

To aminomethyl polystyrene resin (80 mg, 0.1 mmol, loading: 1.26 mmol/g) pre-swollen in CH_2Cl_2 (5 mL, 20 min), was added 4-[(2,4-dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid (Rink amide linker) (220.2 mg, 4 equiv., 0.4 mmol) and 6-Cl-HOBt (70.0 mg, 3.5 equiv., 0.35 mmol) dissolved in DMF (1.5 mL) followed by addition of DIC (62 µL, 4 equiv., 0.4 mmol). The reaction mixture was gently agitated at room temperature for 24 h. The resin was filtered and washed with DMF (3 × 3 mL) after which a negative ninhydrin test confirmed successful coupling**.**

Method 2: General procedure for removal of N^{α}-Fmoc-protecting group:

Peptidyl resin was treated with a solution of 20 vol % piperidine in DMF (*v*/*v*, 4 mL) and the mixture was agitated on the Biotage® Initiator Alstra for 2 × 5 min at room temperature. The resin was filtered and washed with DMF (3 × 3 mL).

Method 3: General coupling procedure for Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH Fmoc-Gly-OH, Fmoc-lle-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH:

Couplings were performed using the Biotage® Initiator Alstra with the appropriate Fmoc-protected amino acid (0.2 M, DMF, 5 equiv.), HATU (0.5 M, DMF, 4.75 equiv.) and NMM in DMF (2 M, 8equiv.) using a single coupling cycle at 75 °C, 110 W for 5 min. The resin was filtered and washed with DMF (3 × 3 mL).

Method 4: General coupling procedure for Fmoc-Arg(Pbf)-OH:

Double coupling cycles of Fmoc-Arg(Pbf)-OH were carried out with the Biotage® Initiator Alstra using Fmoc-Arg(Pbf)-OH (0.2 M, DMF, 5 equiv.), HATU (0.5 M, DMF, 4.75 equiv.) and NMM in DMF (2 M, 8 equiv.) with first coupling at room temperature for 25 min, followed by a second coupling cycle at 72 °C, 110 W for 5 min. The resin was filtered and washed with DMF (3 × 3 mL).

Method 5: General coupling procedure for Fmoc-Cys(Trt)-OH and Fmoc-His(Trt)-OH:

Double coupling cycles of Fmoc-Cys(Trt)-OH and Fmoc-His(Trt)-OH were carried out with the Biotage® Initiator Alstra using Fmoc protected amino acid (0.2 M, DMF, 5 equiv.), HATU (0.5 M, DMF, 4.75 equiv.) and NMM in DMF (2 M, 8 equiv.) with first coupling at room temperature for 15 min, followed by 43 °C, 110 W for 10 min. The resin was filtered and washed with DMF (3 × 3 mL).

Method 6: Coupling of Fmoc-dipeptide(Psi(Me,Me)pro)-OH:

Deprotected resin was treated with the appropriate Fmoc-dipeptide(Psi(Me,Me)pro)-OH (5 equiv., 0.5 mmol) and HATU (171.1 mg, 4.5 equiv., 0.45 mmol) in 1.5 mL DMF followed by addition of NMM (88 µL, 8 equiv., 0.8 mmol) and agitated for 30 min, before being filtered, washed with DMF (3 × 3 mL) and the procedure repeated again with fresh reagents. The resin was filtered and washed with DMF (3 × 3 mL).

Method 7: General procedure for the capping of free amino groups:

Fmoc-protected peptidyl resin was treated with 5 M Ac_2O in DMF (0.47 mL, 2.5 equiv.) and NMM in DMF (2 M, 8 equiv.) using the Biotage® Initiator Alstra at room temperature for 10 min. The resin was filtered and washed with DMF (3 × 3 mL).

Method 8: General procedure for TFA-mediated resin cleavage and global deprotection:

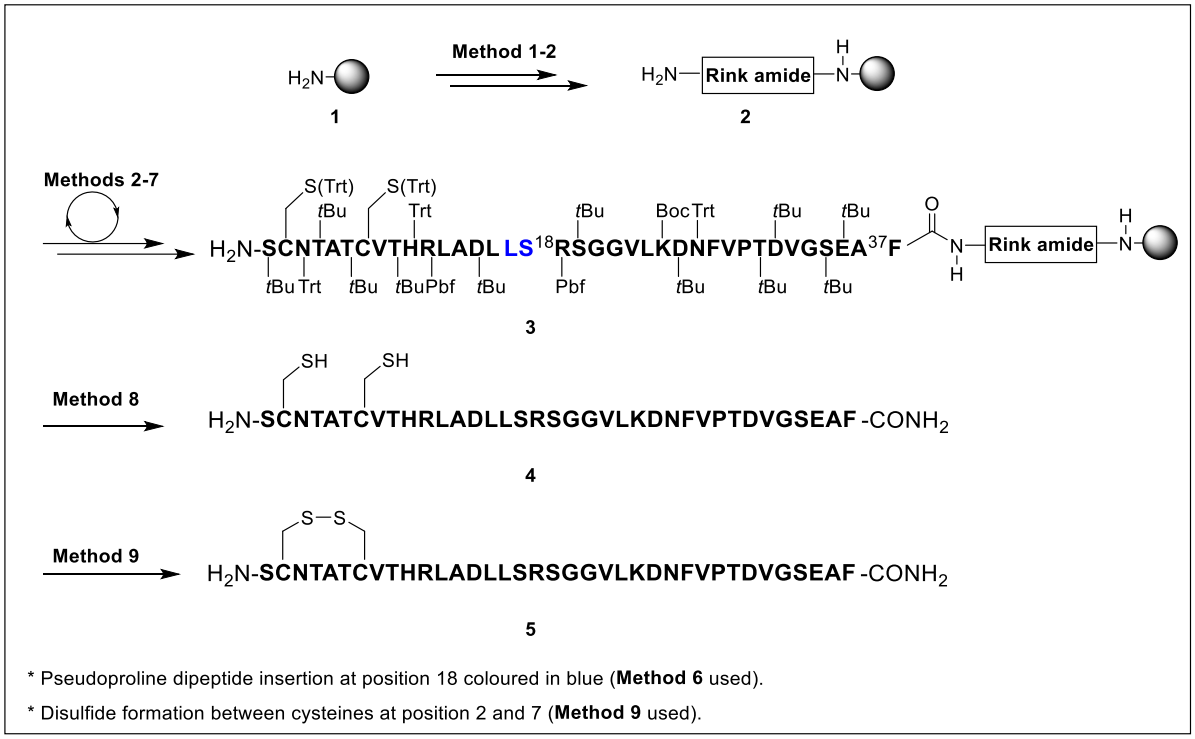
Peptidyl resin was treated with a mixture of TFA/H_2O/TIPS/EDT (94:2.5:2.5:1, *v/v/v/v*, 10 mL) for 120 min. The filtrate was partially concentrated under a gentle stream of N_2, then cold diethyl ether was then added to form a precipitate. The mixture was centrifuged, and the solution was carefully decanted off and discarded, before dissolving the solid pellet in H_2O:acetonitrile containing 0.1% TFA (1:1, *v*/*v*, 25 mL) and lyophilised.

Method 9: General procedure for oxidation between cysteines at position 2 and 7:

To purified peptide was added a pre-made solution of (6 M Gu·HCl/ 0.2 M Na_2HPO_4/ 0.1 mM EDTA/ 8 mM cysteine/ 1 mM cysteine) in H_2O adjusted to pH 8.2- 8.3 using 5 M HCl and 10 M NaOH to make a peptide concentration of 1 mM. Once the peptide had been added and dissolved the solution was subsequently diluted with H_2O to a final peptide concentration 0.25 mM and the resulting solution was sonicated for 1 min then agitated for 3 h at room temperature. The crude reaction mixture was quenched with 5 M HCl (40 µL) and the crude solution of peptide was immediately purified batchwise by semi-preparative RP-HPLC.

Synthesis of m-βCGRP using Fmoc-SPPS.

Scheme 1. Synthesis of m-βCGRP.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmoc-removal using **Method 2**. Direct attachment of Fmoc-Phe-OH at position 37 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α}-Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ^{18}Arg indicated in **Scheme 1** using **Method 3**. All Fmoc-Arg(Pbf)-OH residues are coupled using **Method 4**. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using **Method 5**. Capping of free amino groups was conducted throughout the synthesis using **Method 7**. Coupling of Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH dipeptide at position 18 was achieved using **Method 6**. Linear synthesis of the peptide was continued up to Ser at position 1 using appropriate **Methods** (**2**-**5**, **7**). Peptide **3** was liberated from resin using **Method 8** affording **4**. Crude **4** was purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18, (10 × 250 mm, 5 µm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford **4** as a white amorphous solid (90 mg, 24% yield based on 0.1 mmol scale). Oxidation of **4** (40 mg, 1.0×10^{-5} mol) was achieved using **Method 9**. The crude **5** reaction mixture was subsequently quenched with 5 M HCl (40 µL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18 column (10 × 250 mm, 5 µm) using a linear gradient of 5% to 95% over 95 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford the compound **5** (m-βCGRP) as a white amorphous solid (11 mg, 28% yield based on 1.0×10^{-5} mol), t_R = 34.4 min, >98% purity by HPLC (**Figure S 9**, **Figure S10**).

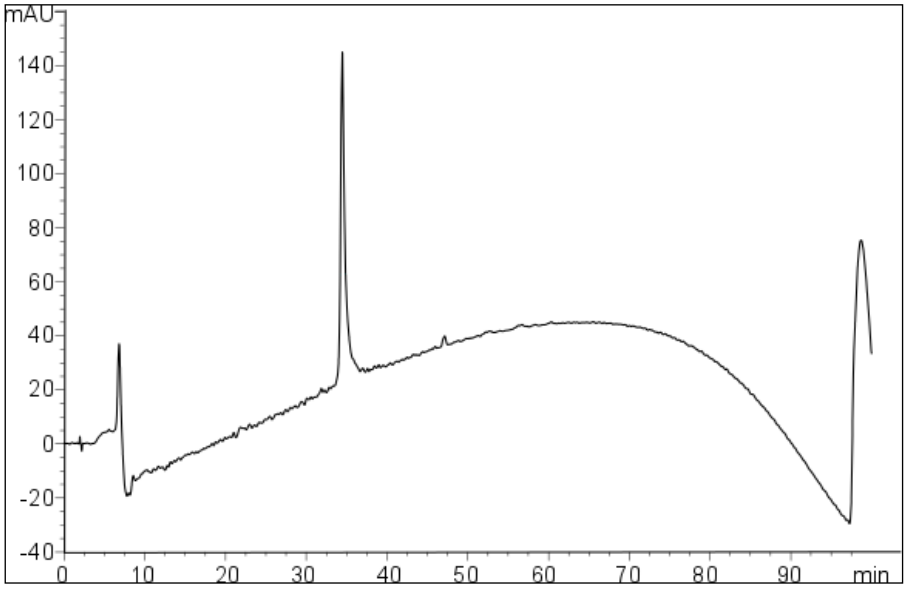


Figure S 9. Analytical RP-HPLC chromatogram of purified peptide **5**, t_R = 34.4 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 μm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

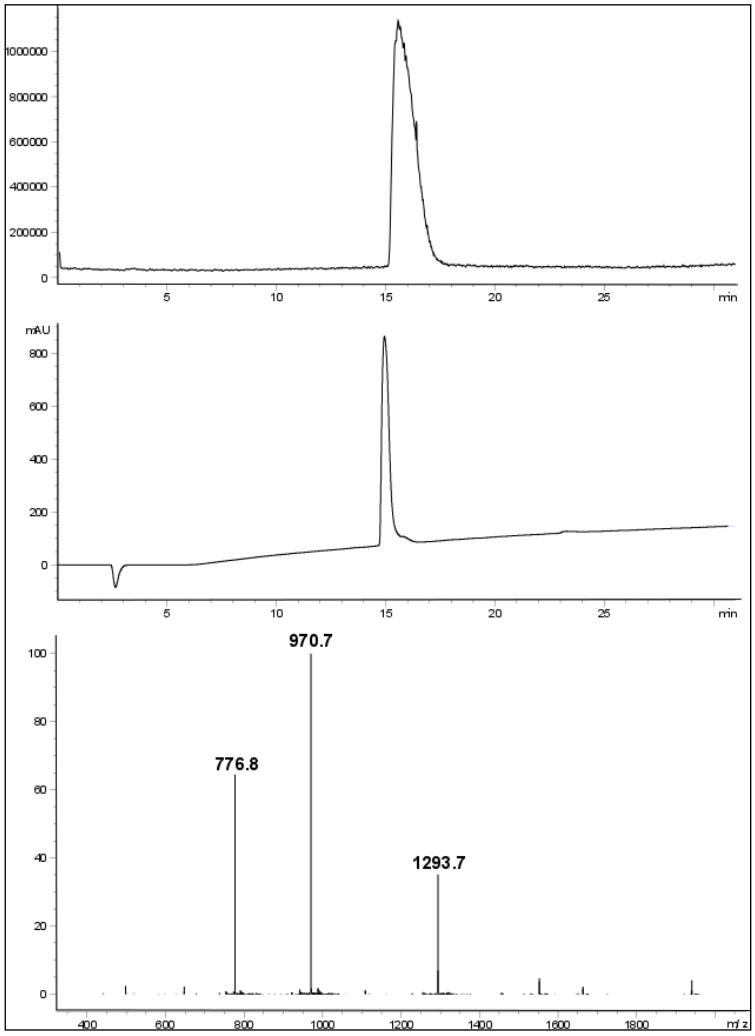
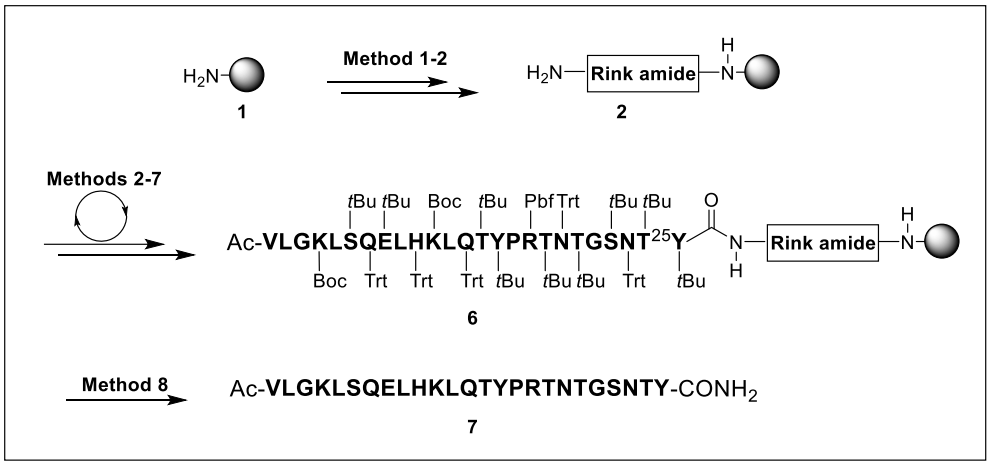


Figure S 10. LC-MS profile of purified peptide **5**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS. (*m/z* $[M+3H]^{3+}$ calcd: 1294.1; found: 1293.7; $[M+4H]^{4+}$ calcd: 970.8; found: 970.7; $[M+5H]^{5+}$ calcd: 776.8; found: 776.8. Mass deconvolution calculated at 3878.63 Da with standard deviation of 0.47; theoretical mass calculated at 3879.34 Da.

Synthesis of AC187 using Fmoc-SPPS.

Scheme 2. Synthesis of AC187.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmoc-removal using **Method 2**. Direct attachment of Fmoc-Tyr(*t*Bu)-OH at position 25 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α}-Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ^{1}Val indicated in **Scheme S2** using appropriate **Methods** (**2**-**5**, **7**). All Fmoc-Arg(Pbf)-OH residues are coupled using **Method 4**. All Fmoc-His(Trt)-OH residues are coupled using **Method 5**. Capping of free amino groups was conducted throughout the synthesis using **Method 7**. Peptide **6** was liberated from resin using **Method 8** which was subsequently purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18, (10 × 250 mm, 5 μm) using a linear gradient of 5% to 95% over 95 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford the compound **7** (AC187) as a white amorphous solid (84 mg, 29% yield based on 0.1 mmol scale), t_R = 32.3 min, >99% purity by HPLC; (**Figure S 11**, **Figure S 12**).

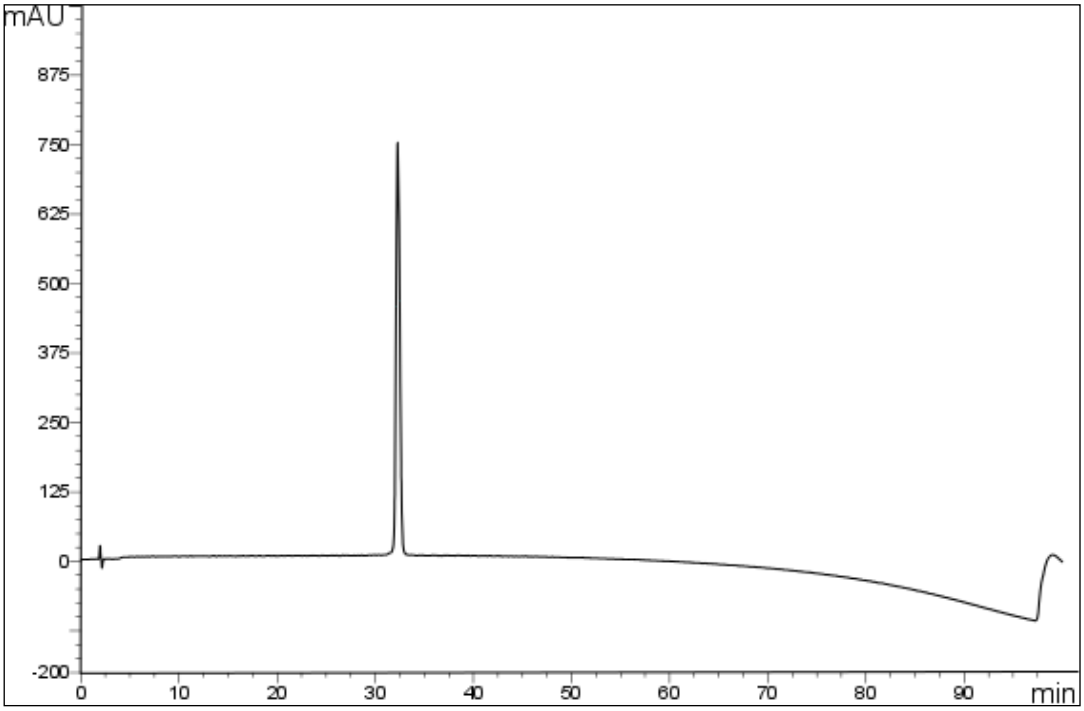


Figure S 11. Analytical RP-HPLC chromatogram of purified peptide **7**, t_R = 32.3 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 μm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

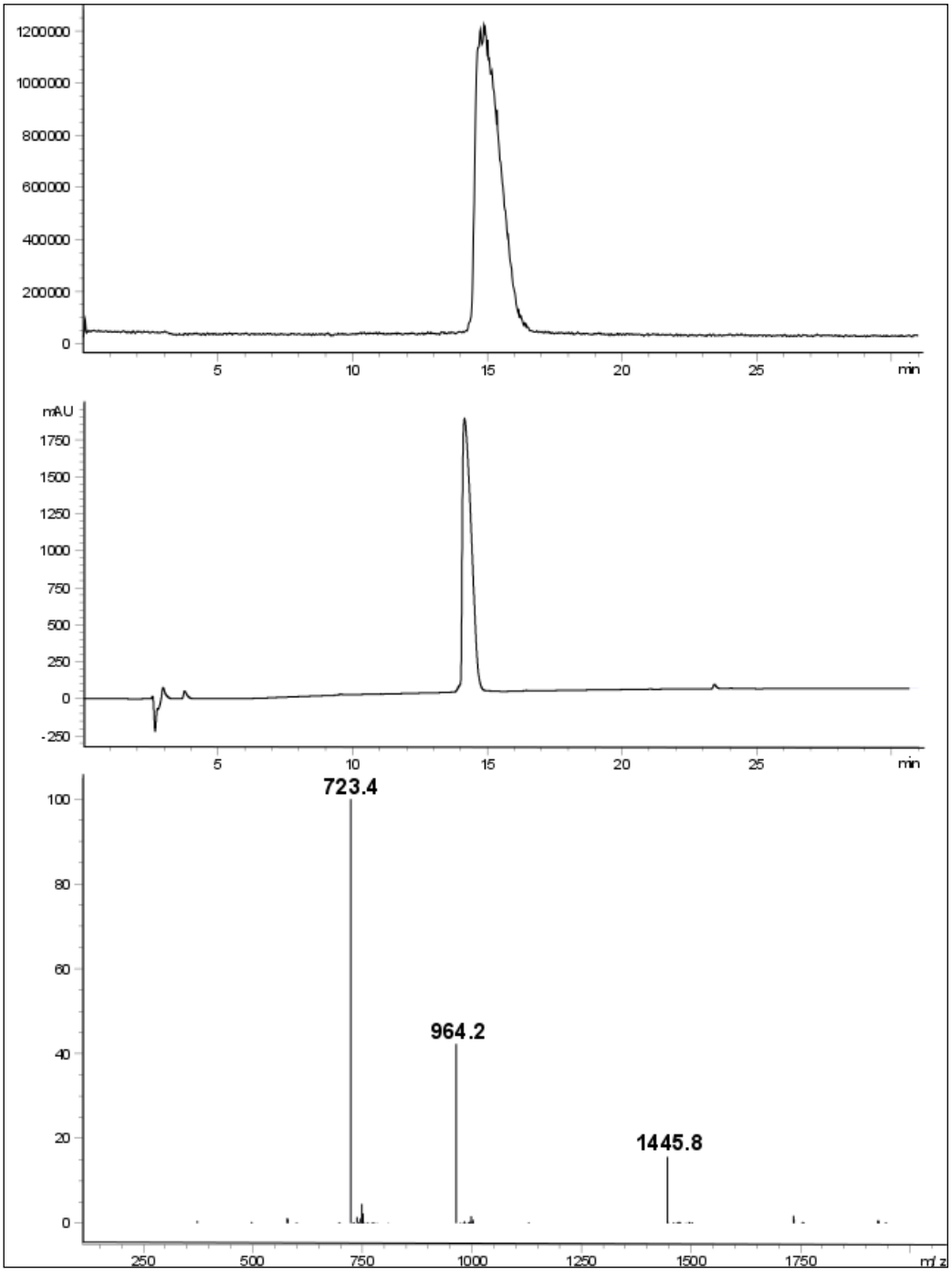
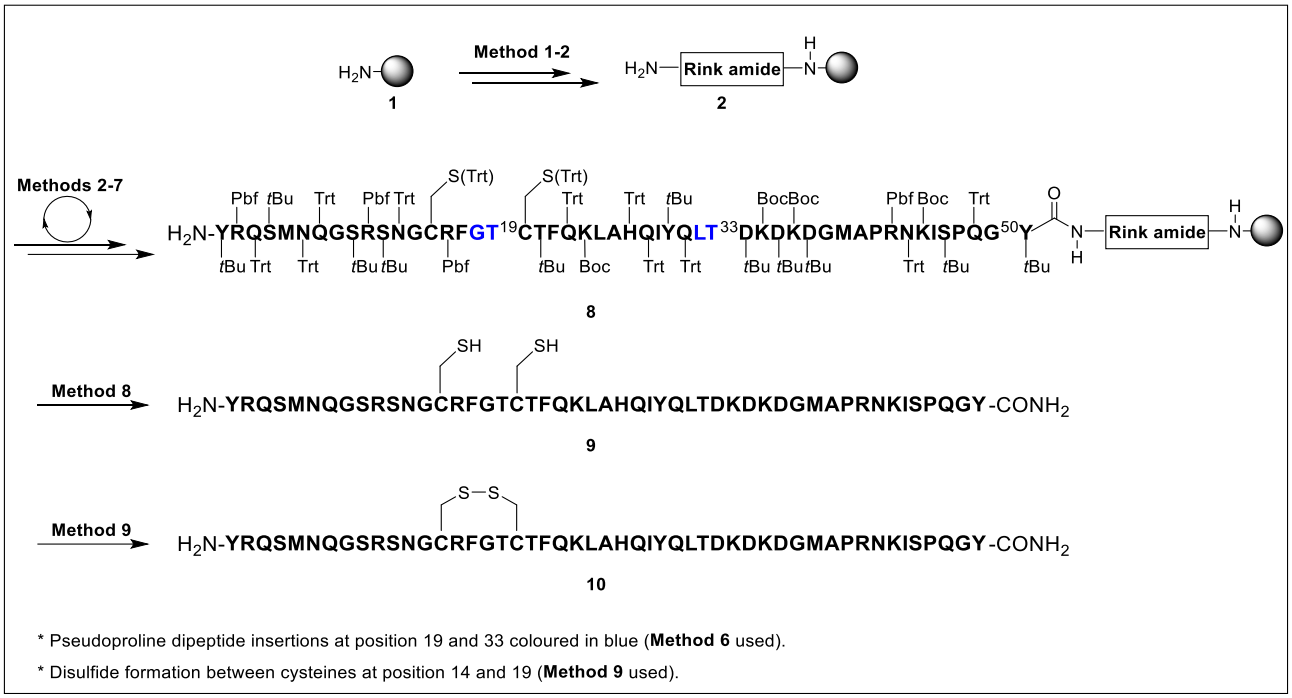


Figure S 12. LC-MS profile of purified peptide **7**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS (*m/z* $[M+2H]^{2+}$ calcd: 1446.13; found: 1445.8; $[M+3H]^{3+}$ calcd: 964.4; found: 964.2; $[M+4H]^{4+}$ calcd: 723.6; found: 723.4. Mass deconvolution calculated at 2889.60 Da with standard deviation of 0.00; theoretical mass calculated at 2890.26 Da.

Synthesis of m-AM using Fmoc-SPPS.

Scheme 3 Synthesis of m-AM.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmoc-removal using **Method 2**. Direct attachment of Fmoc-Tyr(*t*Bu)-OH at position 50 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α}-Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ^{33}Asp indicated in **Scheme S3** using **Method 3**. All Fmoc-Arg(Pbf)-OH residues are coupled using **Method 4**. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using **Method 5**. Capping of free amino groups was conducted throughout the synthesis using **Method 7**. Coupling of Fmoc-Leu-Thr(Psi(Me,Me)pro)-OH dipeptide at position 33 was achieved using **Method 6**. Linear synthesis of the peptide was continued up to Cys at position 19 using appropriate **Methods** (**2-5**, **7**). Coupling of Fmoc-Gly-Thr(Psi(Me,Me)pro)-OH dipeptide at position 19 was achieved using **Method 6**. Linear synthesis of the peptide was continued up to Tyr at position 1 using appropriate **Methods** (**2-5, 7**). Peptide **8** was liberated from resin using **Method 8** affording **9**. Crude **9** was purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18, (10 × 250 mm, 5 µm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford **9** as a white amorphous solid (60 mg, 10% yield based on 0.1 mmol scale). Oxidation of **9** (40 mg, 6.9×10^{-6} mol) was achieved using **Method 9**. The crude **10** reaction mixture was subsequently quenched with 5 M HCl (40 µL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18 column (10 × 250 mm, 5 µm) using a linear gradient of 5% to 95% over 95 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford the compound **10** (m-AM) as a white amorphous solid (8 mg, 20% yield based on 6.9×10^{-6} mol), t_R = 30.4 min, >97% purity by HPLC; (**Figure S 13**, **Figure S 14)**

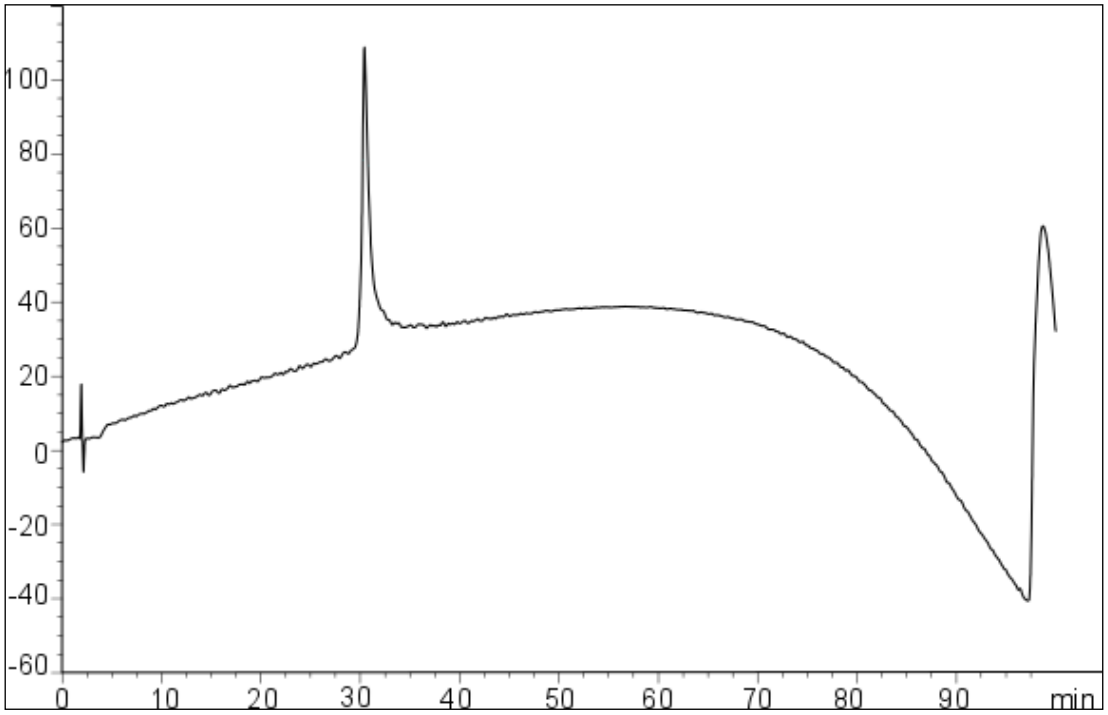


Figure S 13. Analytical RP-HPLC chromatogram of purified peptide **10**, t_R = 30.4 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 μm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

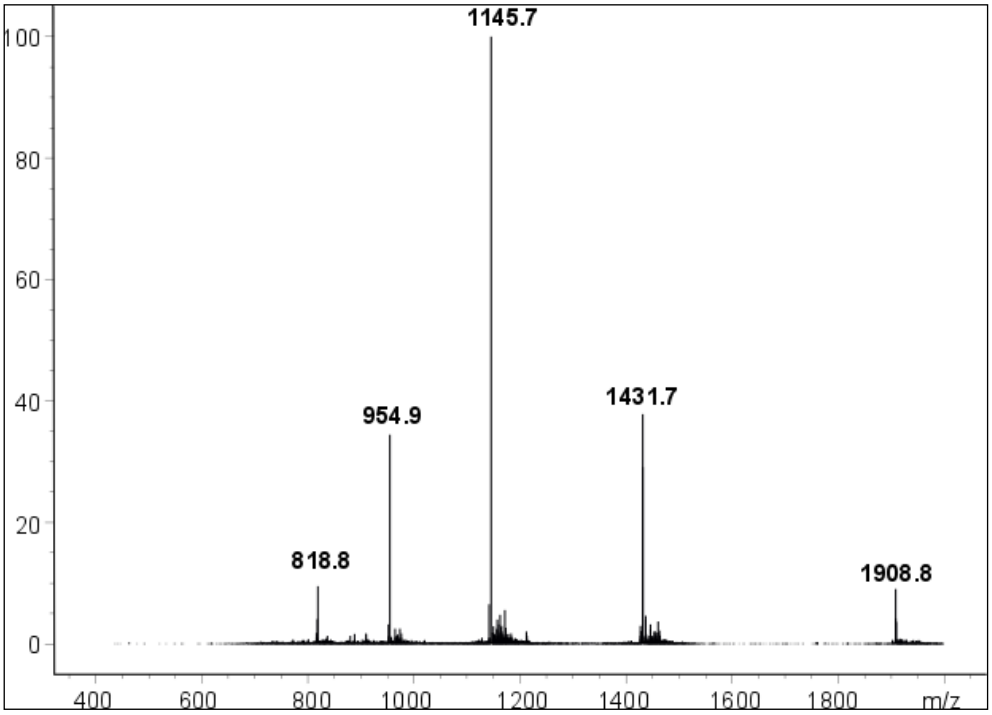
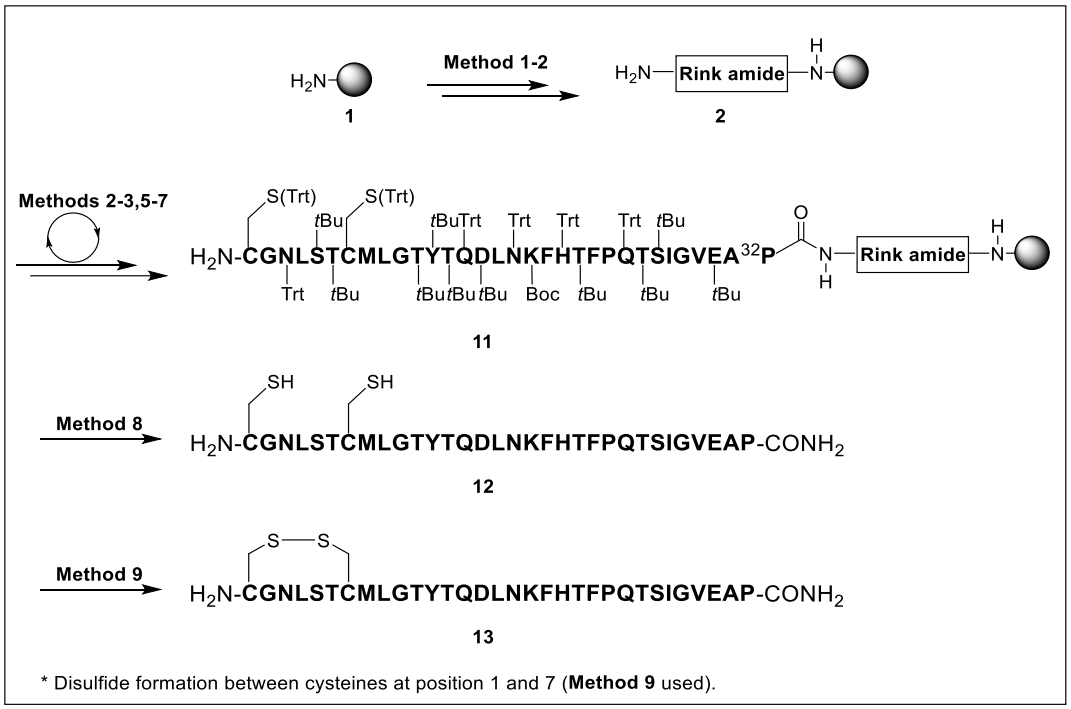


Figure S 14. ESI-MS profile of purified peptide **10**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS (*m/z* $[M+3H]^{3+}$ calcd: 1909.1; found: 1908.8; $[M+4H]^{4+}$ calcd: 1432.1; found: 1431.7; $[M+5H]^{5+}$ calcd: 1145.8; found: 1145.7; $[M+6H]^{6+}$ calcd: 955.0; found: 954.9; $[M+7H]^{7+}$ calcd: 818.7; found: 818.8. Mass deconvolution calculated at 5723.54 Da with standard deviation of 0.65; theoretical mass calculated at 5724.44 Da.

Synthesis of m-CT using Fmoc-SPPS.

Scheme 4. Synthesis of m-CT.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmoc-removal using **Method 2**. Direct attachment of Fmoc-Pro-OH at position 32 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α}-Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ^{1}Cys indicated in **Scheme S4** using **Method 3**. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using **Method 5**. Capping of free amino groups was conducted throughout the synthesis using **Method 7**. Peptide **11** was liberated from resin using **Method 8** affording **12**. Crude **12** was purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18, (10 × 250 mm, 5 μm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford **12** as a white amorphous solid (47 mg, 13% yield based on 0.1 mmol scale). Oxidation **12** (47 mg, 1.3×10^{-5} mol) was achieved using **Method 9**. The crude **13** reaction mixture was subsequently quenched with 5 M HCl (40 μL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18 column (10 × 250 mm, 5 μm) using a linear gradient of 5% to 95% over 95 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford the compound **13** (m-CT) as a white amorphous solid (14.7 mg, 31% yield based on 1.3×10^{-5} mol), t_R = 32.2 min, >99% purity by HPLC (**Figure S 15**, **Figure S 16**).

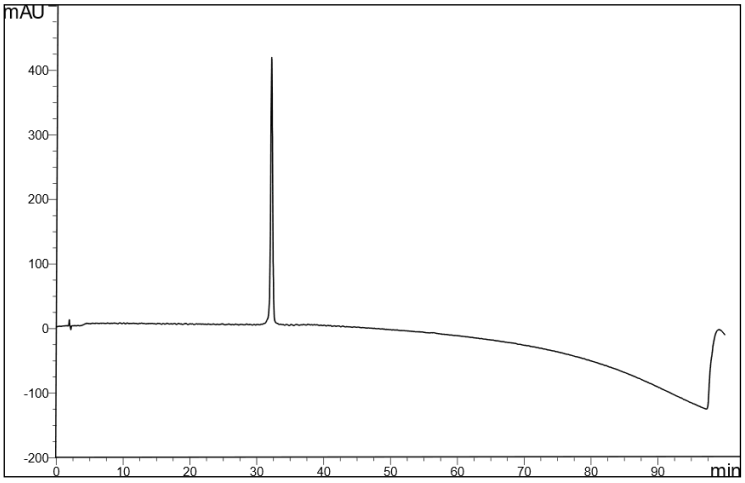


Figure S 15. Analytical RP-HPLC chromatogram of purified peptide **13**, t_R = 32.2 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 μm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

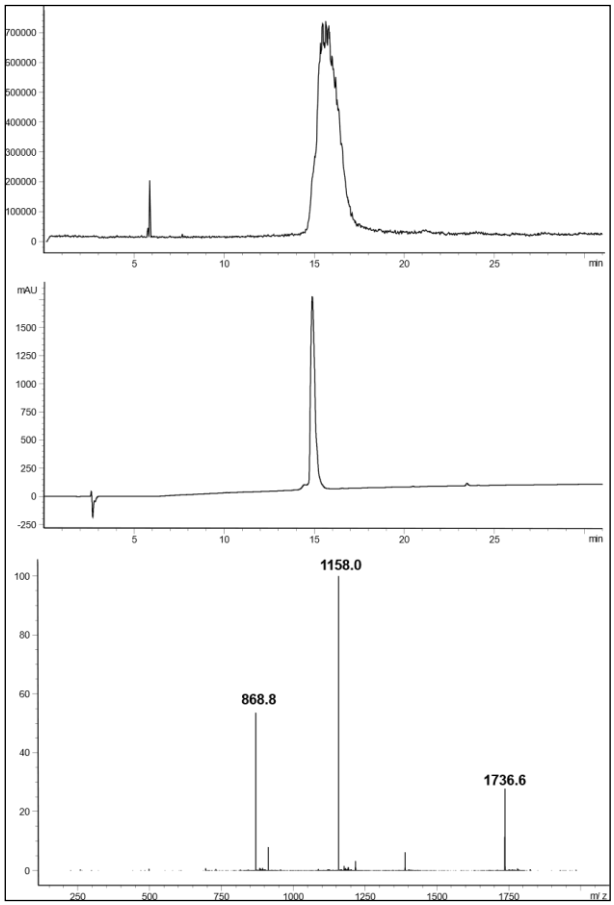
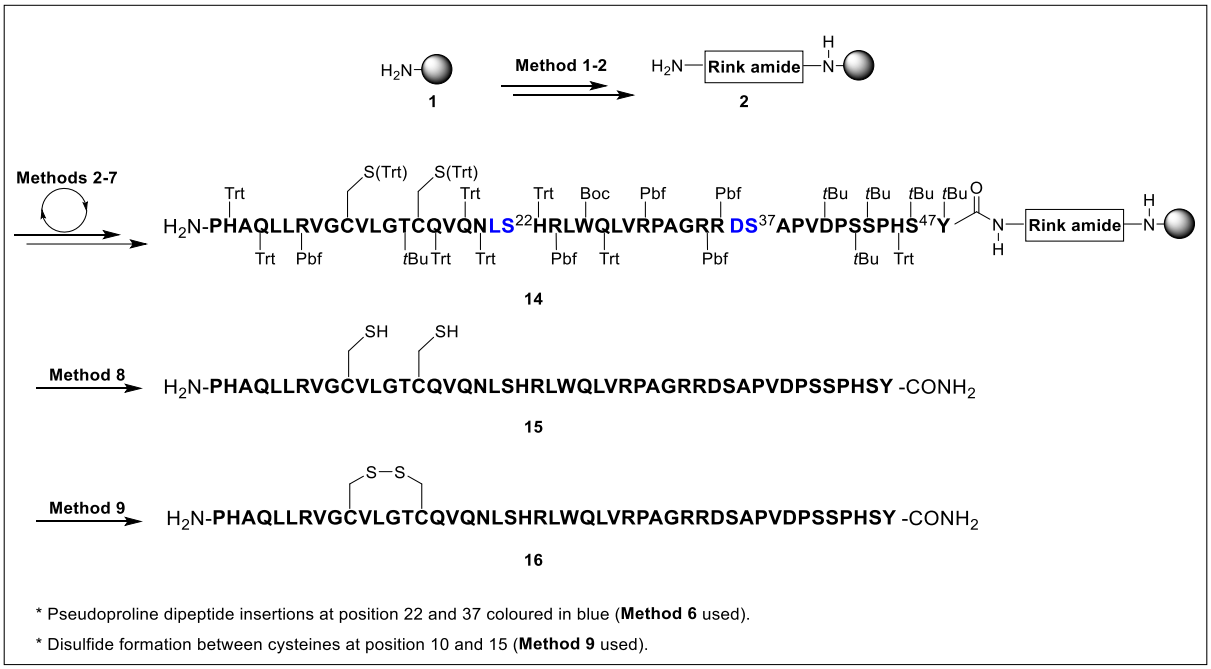


Figure S 16. LC-MS profile of purified peptide **13**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS (*m/z* $[M+2H]^{2+}$ calcd: 1736.9; found: 1736.6; $[M+3H]^{3+}$ calcd: 1158.3; found: 1158.0; $[M+4H]^{4+}$ calcd: 868.9; found: 868.8. Mass deconvolution calculated at 3471.13 Da with standard deviation of 0.12; theoretical mass calculated at 3471.93 Da.

Synthesis of m-AM2$_{47}$ mouse using Fmoc-SPPS.

Scheme 5 Synthesis of m-AM2$_{47}$.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmoc-removal using **Method 2**. Direct attachment of Fmoc-Tyr(*t*Bu)-OH at position 47 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α}-Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ^{37}Ala indicated in **Scheme S5** using **Method 3**. All Fmoc-Arg(Pbf)-OH residues are coupled using **Method 4**. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using **Method 5**. Capping of free amino groups was conducted throughout the synthesis using **Method 7**. Coupling of Fmoc-Asp-Ser(Psi(Me,Me)pro)-OH dipeptide at position 37 was achieved using **Method 6**. Linear synthesis of the peptide was continued up to His at position 22 using appropriate **Methods** (**2-5**, **7**). Coupling of Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH dipeptide at position 22 was achieved using **Method 6**. Linear synthesis of the peptide was continued up to Pro at position 1 using appropriate **Methods** (**2-5, 7**). Peptide **14** was liberated from resin using **Method 8**. Crude **15** was purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18, (10 × 250 mm, 5 µm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford **15** as a white amorphous solid (50 mg, 10% yield based on 0.1 mmol scale). Oxidation of **15** (50 mg, 9.6 × 10^{-6} mol) was achieved using **Method 9**. The crude **16** reaction mixture was subsequently quenched with 5 M HCl (40 µL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18 column (10 × 250 mm, 5 µm) using a linear gradient of 5% to 95% over 95 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford the compound **16** (m-AM2$_{47}$) as a white amorphous solid (15 mg, 30% yield based on 9.6 × 10^{-6} mol), t_R = 32.6 min, >98% purity by HPLC (**Figure S 17, Figure S 18**).

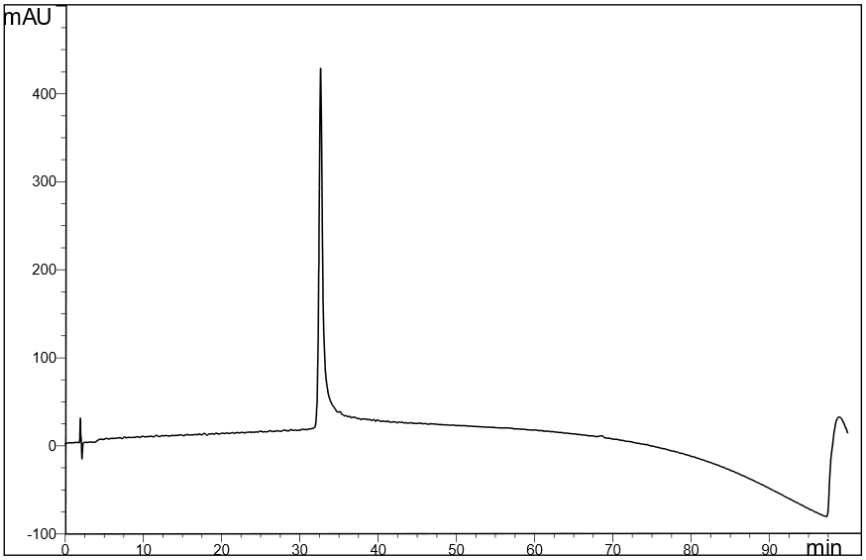


Figure S 17. Analytical RP-HPLC chromatogram of purified peptide **16**, t_R = 32.6 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

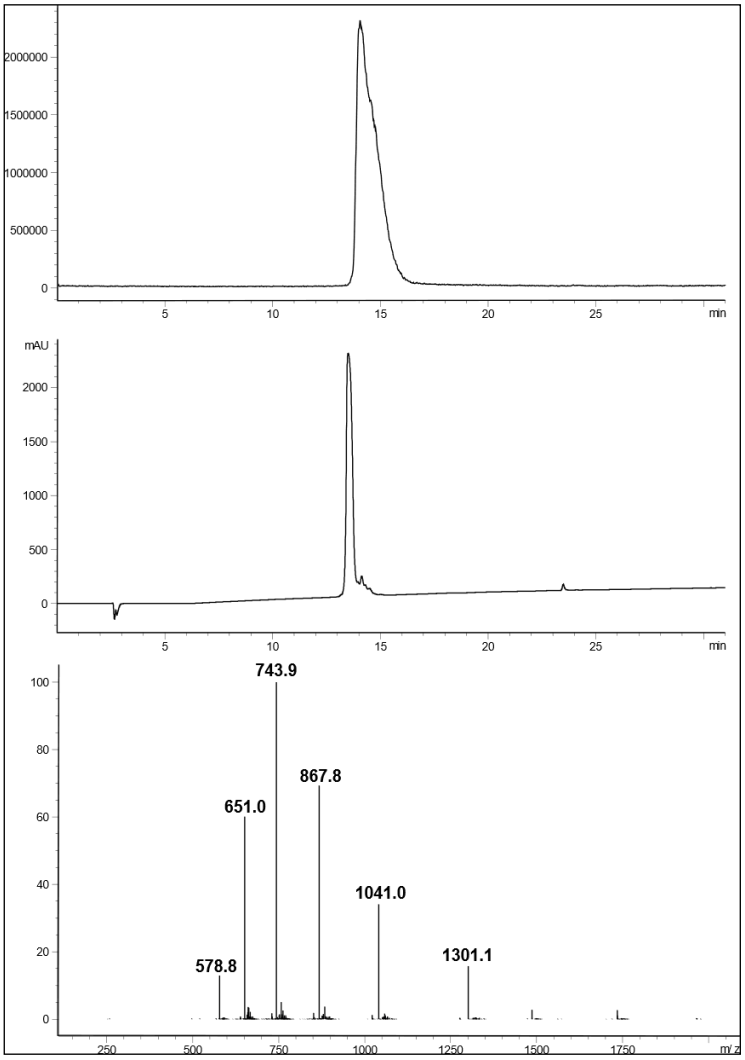
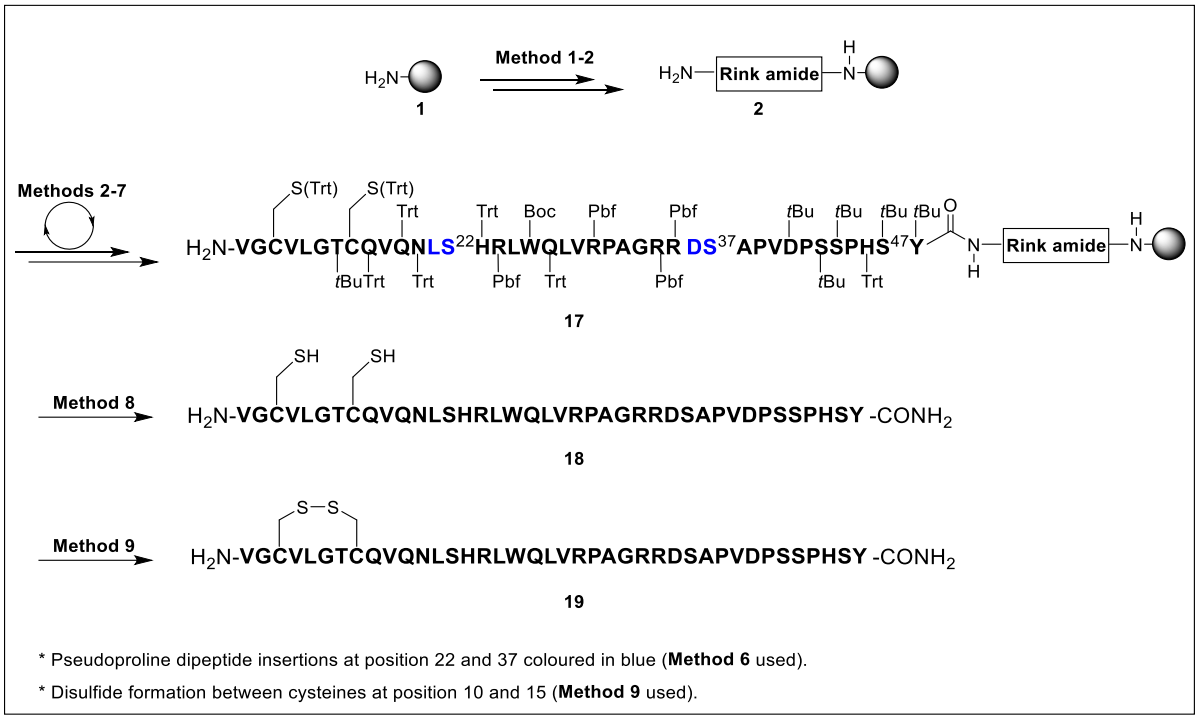


Figure S 18. LC-MS profile of purified peptide **16**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS (*m/z* $[M+4H]^{4+}$ calcd: 1301.1; found: 1301.1; $[M+5H]^{5+}$ calcd: 1041.1; found: 1041.0; $[M+6H]^{6+}$ calcd: 867.7; found: 867.8; $[M+7H]^{7+}$ calcd: 743.9; found: 743.9; $[M+8H]^{8+}$ calcd: 651.1; found: 651.0 $[M+9H]^{9+}$ calcd: 578.8; found: 578.8. Mass deconvolution calculated at 5200.28 Da with standard deviation of 0.30; theoretical mass calculated at 5200.45 Da.

Synthesis of m-AM2$_{40}$ mouse using Fmoc-SPPS.

Scheme 6. Synthesis of m-AM2$_{40}$.



Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmoc-removal using **Method 2**. Direct attachment of Fmoc-Tyr(*t*Bu)-OH at position 47 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α}-Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ^{37}Ala indicated in **Scheme S6** using **Method 3**. All Fmoc-Arg(Pbf)-OH residues are coupled using **Method 4**. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using **Method 5**. Capping of free amino groups was conducted throughout the synthesis using **Method 7**. Coupling of Fmoc-Asp-Ser(Psi(Me,Me)pro)-OH dipeptide at position 37 was achieved using **Method 6**. Linear synthesis of the peptide was continued up to His at position 22 using appropriate **Methods** (**2**-**5**, **7**). Coupling of Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH dipeptide at position 22 was achieved using **Method 6**. Linear synthesis of the peptide was continued up to Val at position 8 using appropriate **Methods** (**2**-**5, 7**). Peptide **17** was liberated from resin using **Method 8**. Crude **18** was purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18, (10 × 250 mm, 5 µm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford **18** as a white amorphous solid (75 mg, 17% yield based on 0.1 mmol scale). Oxidation of **18** (75 mg, 1.7 × 10^{-5} mol) was achieved using **Method 9**. The crude **19** reaction mixture was subsequently quenched with 5 M HCl (40 µL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18 column (10 × 250 mm, 5 µm) using a linear gradient of 5% to 95% over 95 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford the compound **19** (m-AM2$_{40}$) as a white amorphous solid (30 mg, 40% yield based on 1.7 × 10^{-5} mol), t_R = 32.3 min, >98% purity by HPLC (**Figure S 19**, **Figure S 20**).

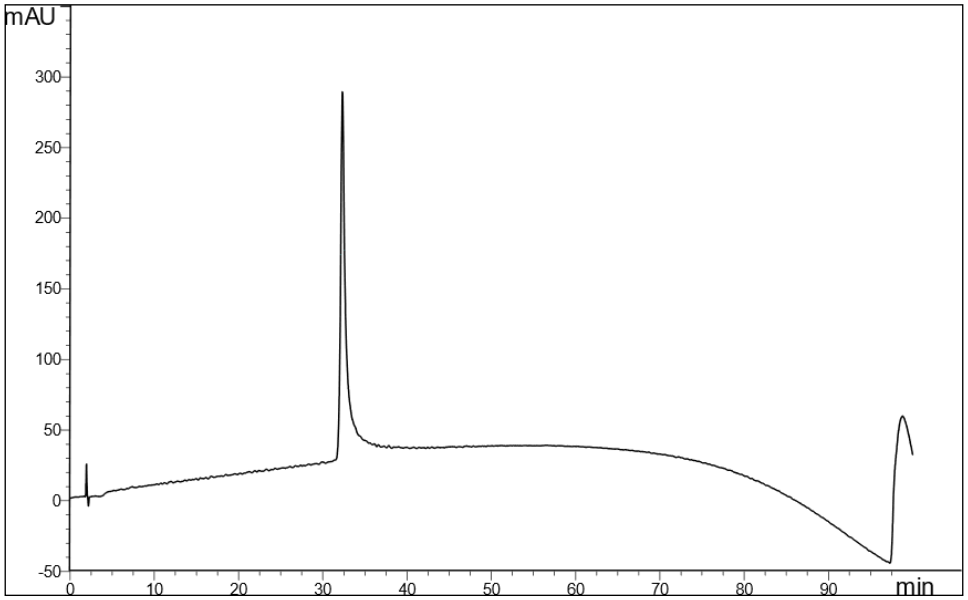


Figure S 19. Analytical RP-HPLC chromatogram of purified peptide **19**, t_R = 32.3 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 μm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

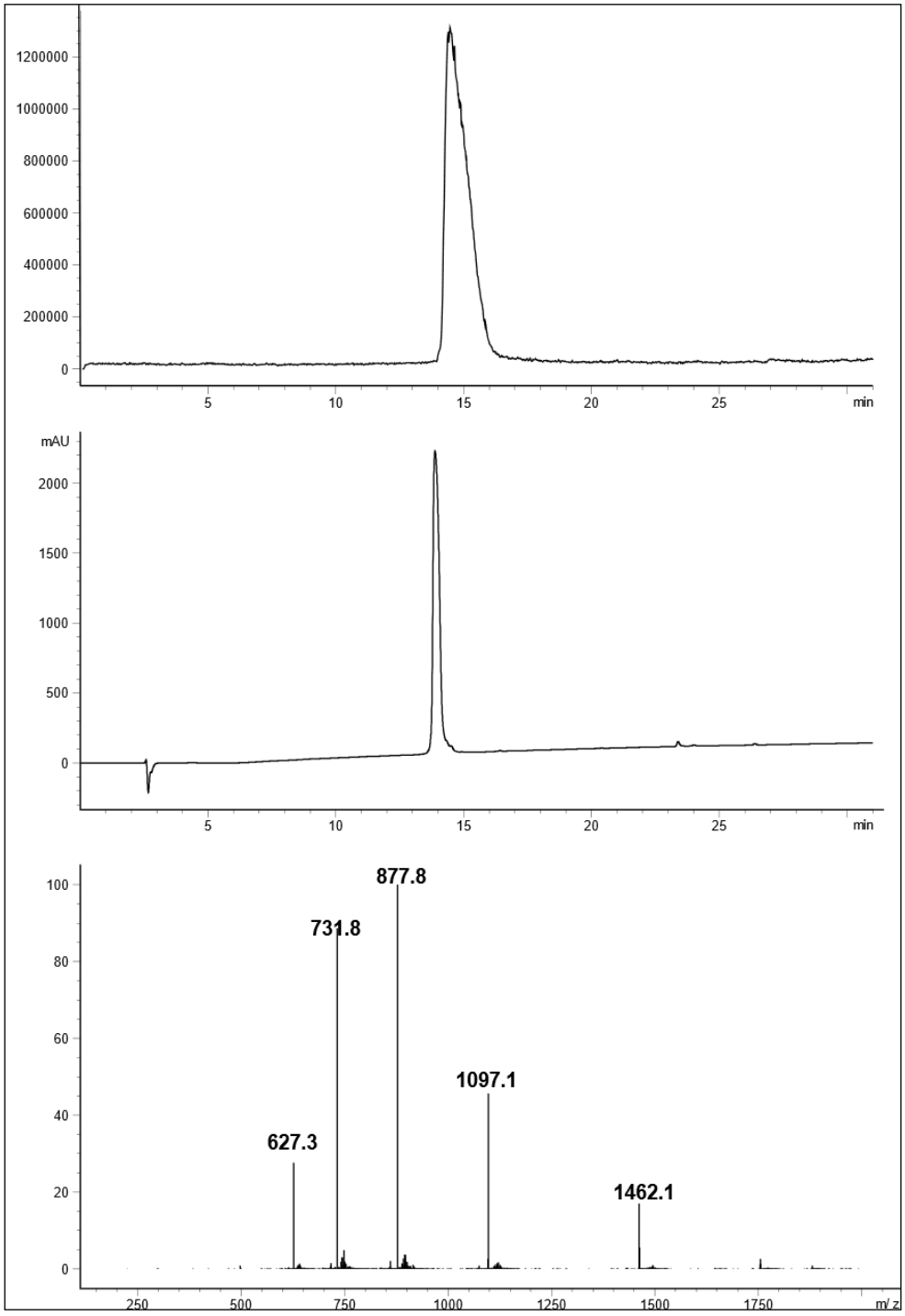
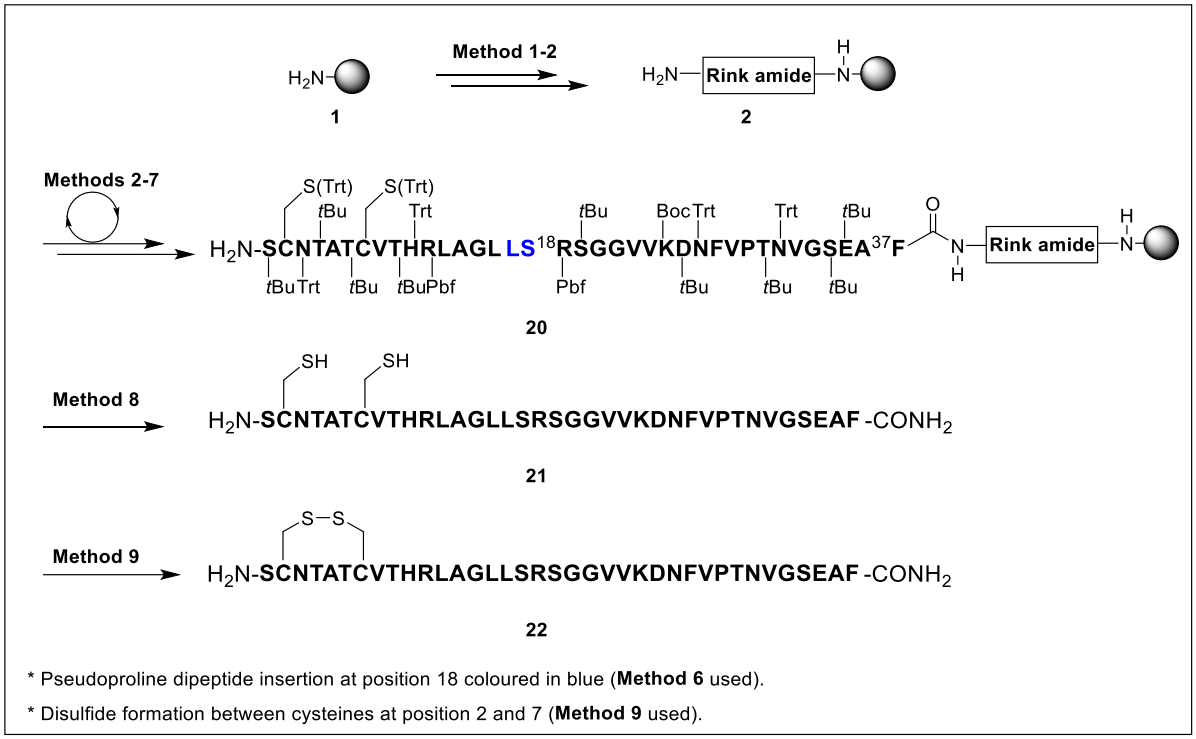


Figure S 20. LC-MS profile of purified peptide **19**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS (*m/z* $[M+3H]^{3+}$ calcd: 1462.6; found: 1462.1; $[M+4H]^{4+}$ calcd: 1097.2; found: 1097.1; $[M+5H]^{5+}$ calcd: 877.9; found: 877.8; $[M+6H]^{6+}$ calcd: 731.8; found: 731.8; $[M+7H]^{7+}$ calcd: 627.4; found: 627.3. Mass deconvolution calculated at 4384.12 Da with standard deviation of 0.55; theoretical mass calculated at 4384.98 Da.

Synthesis of m/r-αCGRP using Fmoc-SPPS.

Scheme 7 Synthesis of m/r-αCGRP



Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmoc-removal using **Method 2**. Direct attachment of Fmoc-Phe-OH at position 37 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α}-Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ^{18}Arg indicated in **Scheme 7** using **Method 3**. All Fmoc-Arg(Pbf)-OH residues are coupled using **Method 4**. All Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH residues are coupled using **Method 5**. Capping of free amino groups was conducted throughout the synthesis using **Method 7**. Coupling of Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH dipeptide at position 18 was achieved using **Method 6**. Linear synthesis of the peptide was continued up to Ser at position 1 using appropriate **Methods** (**2-5**, **7**). Peptide **20** was liberated from resin using **Method 8** affording **21**. Crude **21** was purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18, (10 × 250 mm, 5 µm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford **21** as a white amorphous solid (74 mg, 19% yield based on 0.1 mmol scale). Oxidation of **21** (20 mg, 5.2×10^{-6} mol) was achieved using **Method 9**. The crude **22** reaction mixture was subsequently quenched with 5 M HCl (40 µL) and purified batchwise by semi-preparative RP-HPLC on a Phenomenex® Gemini C18 column (10 × 250 mm, 5 µm) using a linear gradient of 5% to 95% over 95 min (*ca.* 1% B/min) with a flow rate of 4 mL/min. Fractions were collected at 0.2 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with correct *m*/*z* were combined and lyophilised to afford the compound **22** (m/r-αCGRP) as a white amorphous solid (3 mg, 15% yield based on 5.2×10^{-6} mol), t_R = 31.1 min, >99% purity by HPLC (**Figure S 21, Figure S 22**).

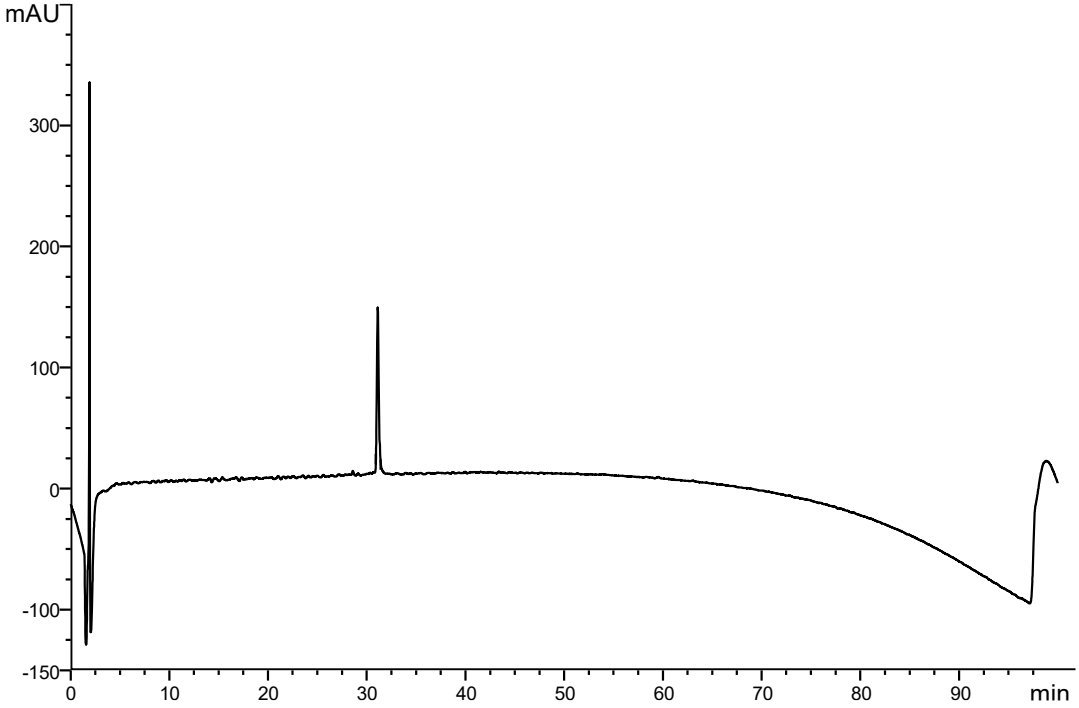


Figure S 21. Analytical RP-HPLC chromatogram of purified peptide **22**, t_R = 31.1 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 μm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

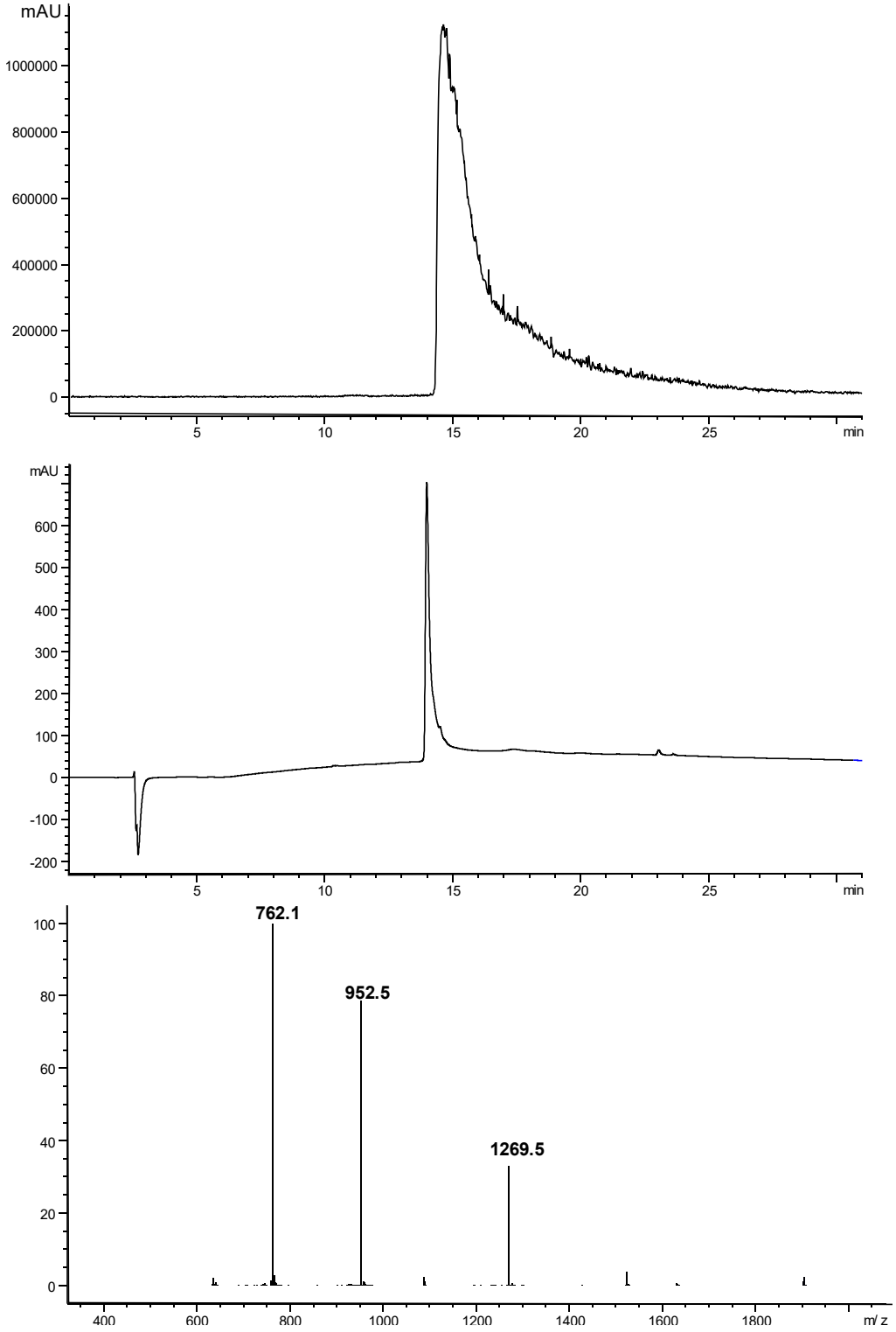


Figure S 22. LC-MS profile of purified peptide **22**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS. (*m/z* $[M+3H]^{3+}$ calcd: 1269.7; found: 1269.5; $[M+4H]^{4+}$ calcd: 952.6; found: 952.5; $[M+5H]^{5+}$ calcd: 762.2; found: 762.1. Mass deconvolution calculated at 3805.67 Da with standard deviation of 0.29; theoretical mass calculated at 3806.30 Da.

References

Garelja, M. L., Au, M., Brimble, M. A., Gingell, J. J., Hendrikse, E. R., Lovell, A., Prodan, N., Sexton, P. M., Siow, A., Walker, C. S., Watkins, H. A., Williams, G. M., Wootten, D., Yang, S. H., Harris, P. W. R., & Hay, D. L. (2020, Apr 10). Molecular Mechanisms of Class B GPCR Activation: Insights from Adrenomedullin Receptors. *ACS Pharmacol Transl Sci*, *3*(2), 246-262. https://doi.org/10.1021/acsptsci.9b00083

Hay, D. L., Garelja, M. L., Poyner, D. R., & Walker, C. S. (2018, Jan). Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. *Br J Pharmacol*, *175*(1), 3-17. https://doi.org/10.1111/bph.14075