Distinct patterns of internalization of different calcitonin gene-related peptide receptors

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Supplementary Biology

Methods

Spot Counting – Time courses
Spot counting was performed with the Columbus software package (Perkin-Elmer, Waltham, MA, USA) (Figure S5). The cell border was defined with cell mask blue, and the intensity of anti-myc-Alexa-555 fluorescence was then measured in each cell. Cells with an Alexa-555 fluorescence intensity above pcDNA transfected cells were selected as “myc-positive”. The cytoplasm region was then set as 90% of cell volume; this was determined by measuring the percentage of overlap of cell mask blue and cell mask green (a plasma membrane stain) (Figure S6), to ensure fluorescent peptide at the cell surface was not counted. Spots of fluorescent peptide were quantified within the cytoplasm region of the “myc-positive” cells in each field of view. Data are an average of 8 fields of view for each well and output as number of spots per “myc-positive” cell.

Spot Counting – Concentration-response curves
HEK293S cells were stimulated with varying concentrations of [Cy5]-hαCGRP, fixed with 4% PFA for 15-20 minutes and washed 2x with PBS. Cells were then stained with HCS Cell Mask Blue in PBS (#H32720). Spot counting was performed with the Columbus software package (Perkin-Elmer) (Figure S5). The cell border was defined with cell mask blue. The cytoplasm region was then set as 90% of cell volume; this was determined by measuring the percentage of overlap of cell mask blue and cell mask green (a plasma membrane stain) (Figure S6), to ensure fluorescent peptide at the plasma membrane was not counted. Spots of fluorescent peptide were quantified within the cytoplasm region of the cells in each field of view. Data are an average of 8 fields of view for each well and output as number of spots per cell.

Confocal Microscopy
Immunofluorescence staining was performed in HEK293S cells as described for the widefield microscopy experiments with the exception that DAPI was used in place of HCS Cell Mask Blue. Confocal Microscopy was performed on a Zeiss LSM 710 laser scanning confocal microscope using a 63x Plan-Apochromat oil immersion objective (NA 1.4) (Zeiss, Oberkochen, Germany). Images were captured as Z-stacks 0.5 µm apart; single sections are shown. Images were saved as 12-bit files. For presentation images were processed in FIJI, a 2-pixel median filter was applied and images were adjusted for color and brightness. Within each multi panel figure the images all use the same settings.

Cell Signaling Assays - ERK1/2 and CREB phosphorylation
Extracellular signal-regulated kinase 1/2 (ERK1/2) and cAMP response element-binding protein (CREB) phosphorylation assays were performed as previously described1. Briefly, Cos-7 cells were serum starved in assay media (DMEM + 0.1% BSA) for 4 hours at 37 °C/5% CO2. Peptides were then serially diluted in assay media and cells were incubated with peptide, media alone or control, forskolin for phosphorylated (p) CREB and FBS (50%) pERK1/2, for 15 minutes at 37 °C. Media was then aspirated and pERK1/2 and pCREB were detected using the AlphaLISA SureFire Ultra pERK1/2 (Thr202/Tyr204) or the AlphaLISA SureFire Ultra pCREB (Ser133) assay kits (PerkinElmer) as per the manufacturer’s protocol.
Results: Tables and Figures

Figure S1: cAMP production at calcitonin family receptors for unmodified and fluorescently labelled CGRP and pramlintide peptides in Cos-7 cells. Data were normalized to unmodified CGRP or pramlintide, with the exception of the AM₁ and AM₂ receptor where hAM₁-52 was used. Data are mean ± s.e.m. from 5 independent experiments, as detailed in Table S1.
**Table S1:** Summary of peptide potency and log(τ/K_A) values from cAMP assays in Cos-7 cells. Data are the mean ± s.e.m. from 5 independent experiments. *

< 0.05 by unpaired Student’s t-test comparing the pEC 50 or log(τ/K_A) value of the fluorescently labelled peptide with that of the unmodified control peptide at each receptor. For the CGRP data sets, #p < 0.05 by one-way ANOVA with post hoc Dunnett’s test, comparing the pEC50 or log(τ/K_A) of the peptide at each receptor to the CGRP receptor. For the pramlintide data sets, ¥p < 0.05 by one-way ANOVA with post hoc Dunnett’s test, comparing the pEC 50 or log(τ/K_A) of the peptide at each receptor to the AMY1 receptor.

**Figure S2:** Heat map of peptide log(τ/K_A) values from cAMP assays. Mean log(τ/K_A) data plotted from Table SB1 to provide a visual aid.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>CGRP</th>
<th>[Cy5³]-h°CGRP</th>
<th>hαCGRP</th>
<th>[Cy5²¹]-h°CGRP</th>
<th>Pramlintide</th>
<th>[Cy5²¹]-Pramlintide</th>
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<tr>
<td></td>
<td>pEC₅₀</td>
<td>Log(τ/K_A)</td>
<td>pEC₅₀</td>
<td>Log(τ/K_A)</td>
<td>pEC₅₀</td>
<td>Log(τ/K_A)</td>
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<tr>
<td>Receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGRP</td>
<td>9.95 ± 0.26</td>
<td>9.70 ± 0.26</td>
<td>6.96 ± 0.13</td>
<td>9.44 ± 0.13</td>
<td>9.89 ± 0.15</td>
<td>6.65 ± 0.11*</td>
</tr>
<tr>
<td>AM1</td>
<td>6.92 ± 0.26†</td>
<td>6.61 ± 0.26†</td>
<td>8.09 ± 0.30**</td>
<td>7.89 ± 0.33†</td>
<td>6.80 ± 0.07†</td>
<td>6.42 ± 0.08†</td>
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<tr>
<td>AM2</td>
<td>6.99 ± 0.10†</td>
<td>6.75 ± 0.08†</td>
<td>8.43 ± 0.18**</td>
<td>8.13 ± 0.21†</td>
<td>6.89 ± 0.09†</td>
<td>6.61 ± 0.07†</td>
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<tr>
<td>CT</td>
<td>6.98 ± 0.09†</td>
<td>6.43 ± 0.09†</td>
<td>7.35 ± 0.15†</td>
<td>6.91 ± 0.18†</td>
<td>6.98 ± 0.09†</td>
<td>6.43 ± 0.09†</td>
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<tr>
<td>AMY1</td>
<td>9.33 ± 0.41</td>
<td>9.15 ± 0.40</td>
<td>9.35 ± 0.20</td>
<td>9.07 ± 0.22</td>
<td>9.09 ± 0.20†</td>
<td>9.05 ± 0.19</td>
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<td>AMY2</td>
<td>7.76 ± 0.11†</td>
<td>7.44 ± 0.11†</td>
<td>7.95 ± 0.16†</td>
<td>7.65 ± 0.16†</td>
<td>7.76 ± 0.11†</td>
<td>7.44 ± 0.11†</td>
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<tr>
<td>AMY3</td>
<td>7.49 ± 0.33†</td>
<td>7.07 ± 0.37†</td>
<td>7.96 ± 0.34†</td>
<td>7.50 ± 0.42†</td>
<td>7.49 ± 0.33†</td>
<td>7.07 ± 0.37†</td>
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</table>

Table S1: Summary of peptide potency and log(τ/K_A) values from cAMP assays in Cos-7 cells. Data are the mean ± s.e.m. from 5 independent experiments. *p < 0.05 by unpaired Student’s t-test comparing the pEC50 or log(τ/K_A) value of the fluorescently labelled peptide with that of the unmodified control peptide at each receptor. For the CGRP data sets, †p < 0.05 by one-way ANOVA with post hoc Dunnett’s test, comparing the pEC50 or log(τ/K_A) of the peptide at each receptor to the CGRP receptor. For the pramlintide data sets, ¥p < 0.05 by one-way ANOVA with post hoc Dunnett’s test, comparing the pEC50 or log(τ/K_A) of the peptide at each receptor to the AMY1 receptor.
**Figure S3:** Phosphorylation of ERK1/2 (A,B) and CREB (C,D) at the CGRP (A,C) and AMY1 (B,D) receptors for [Cy5\(^{3}\)]-h\(\alpha\)-CGRP and unmodified CGRP in Cos-7 cells. Data were normalized to unmodified CGRP and expressed as mean ± s.e.m. from 4-5 independent experiments, as detailed in Table S2.

### Table S2: Summary of peptide pEC\(_{50}\) and log(\(\tau/\kappa_{A}\)) values from ERK1/2 and CREB phosphorylation assays in Cos-7 cells.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Signaling pathway</th>
<th>haCGRP (\text{pEC}_{50})</th>
<th>haCGRP (\text{log}(\tau/\kappa_{A}))</th>
<th>[Cy5(^{3})]-haCGRP (\text{pEC}_{50})</th>
<th>[Cy5(^{3})]-haCGRP (\text{log}(\tau/\kappa_{A}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP</td>
<td>pERK1/2</td>
<td>8.72 ± 0.27</td>
<td>8.66 ± 0.27</td>
<td>8.65 ± 0.15</td>
<td>8.58 ± 0.16</td>
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<tr>
<td></td>
<td>pCREB</td>
<td>9.92 ± 0.40</td>
<td>9.71 ± 0.40</td>
<td>9.75 ± 0.16</td>
<td>9.54 ± 0.14</td>
</tr>
<tr>
<td>AMY1</td>
<td>pERK1/2</td>
<td>8.80 ± 0.17</td>
<td>8.72 ± 0.17</td>
<td>8.93 ± 0.10</td>
<td>8.70 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>pCREB</td>
<td>9.97 ± 0.61</td>
<td>9.82 ± 0.59</td>
<td>9.34 ± 0.53</td>
<td>9.27 ± 0.53</td>
</tr>
</tbody>
</table>

Data are the mean ± s.e.m. from 4-5 independent experiments. Unpaired Student’s t-test was used to compare the \(\text{pEC}_{50}\) or \(\text{log}(\tau/\kappa_{A})\) of [Cy5\(^{3}\)]-haCGRP with that of haCGRP at each receptor. Unpaired Student’s t-test was used to compare the \(\text{pEC}_{50}\) or \(\text{log}(\tau/\kappa_{A})\) of the peptide at the AMY1 receptor to the CGRP receptor. There were no significant differences.
Figure S4: Live cell time course of 100 nM [Cy5\(^{21}\)]-haCGRP at the CGRP and AMY\(_1\) receptor in HEK293S cells. Images are representative from 3 independent experiments performed in duplicate. Scale bar = 20 µM.

Figure S5: Workflow of spot analysis in Columbus software. Images are representative from 5 independent experiments in HEK293S cells.
Figure S6: Analysis to determine the cytoplasm area of a cell to prevent fluorescent peptide bound at the cell surface from contributing to the spot count. (A) Analysis to determine the percentage of cell area occupied by both cell mask blue (cytoplasm stain) and cell mask green (plasma membrane) stains. Results are from two independent experiments and show that the plasma membrane occupies approximately 9-10% of the cell volume in HEK293S cells as shown in the representative images (B).
**Figure S7:** Time course of 10 nM of [Cy5]-haCGRP in vector (pcDNA3.1+) transfected HEK293S cells (normalized to the fluorescence intensity in AMY1 receptor transfected cells). Images are a representative from 5-10 independent experiments performed in duplicate. Scale bar = 20 µM.

**Figure S8:** Quantification of co-localization between Cy5 labeled peptides and receptors or EEA1 using Pearson’s correlation coefficient in HEK293S cells. (A) Co-localization of [Cy5]-haCGRP with HA-CLR or HA-CTR at the CGRP and AMY1 receptor. (B) Co-localization of [Cy5]-haCGRP with EEA1 at the CGRP and AMY1 receptor. (C) Co-localization of [Cy52]-pramlintide with HA-CTR or EEA1 at the AMY1 receptor. Data are mean ± s.e.m. from 3-4 independent experiments. * p < 0.05 by unpaired Student’s t-test. ns, not significant.
Figure S9: [Cy5³]-hαCGRP (10 nM) shows robust internalization into CGRP receptor transfected HEK293S cells. Confocal microscopy of [Cy5³]-hαCGRP fluorescence (red) and EEA1 staining (cyan) in CGRP receptor transfected cells after 5 and 30 minutes incubation with peptide. [Cy5³]-hαCGRP is primarily intracellular showing colocalization with the early endosomal marker EEA1. Examples of overlapping spots are indicated by white arrows. Nuclei were stained with DAPI (blue). Images are representative of 3 independent experiments.

Figure S10: Change in cell surface expression of CGRP receptor components in response to CGRP stimulation in Cos-7 cells. Data are mean ± s.e.m. from 5 independent experiments performed in duplicate or triplicate. * p < 0.05 (myc) and † p < 0.05 (HA) compared to vehicle control treated cells (data not shown) at each time point by repeated measures two-way ANOVA with post-hoc Bonferroni’s test.
**Figure S11:** [Cy5]-hαCGRP (10 nM) is not robustly internalised into AMY₁ receptor transfected HEK293S cells. Confocal microscopy of [Cy5]-hαCGRP fluorescence (red) and EEA1 staining (cyan) of AMY₁ receptor transfected cells after 5 and 30 minutes incubation with peptide. [Cy5]-hαCGRP is primarily localised to the cell membrane showing very little colocalization with the early endosomal maker EEA1. Nuclei were stained with DAPI (blue). Images are representative of 3 independent experiments.

**Figure S12:** Cell surface expression of AMY₁ receptor components in response to (A) hαCGRP and (B) hAmy in Cos-7 cells. Data are mean ± s.e.m. from 3-5 independent experiments performed in duplicate or triplicate. myc and HA were compared to vehicle control treated cells (data not shown) at each time point by repeated measures two-way ANOVA with a post-hoc Bonferroni’s test; there were no significant differences.
Figure S13: Time course of 10 nM [Cy521]-Pramlintide in vector (pcDNA3.1+) transfected HEK-293S cells (normalized to the fluorescence intensity in AMY1 receptor transfected cells). Images are a representative from 5 independent experiments performed in duplicate. Scale bar = 20 µM.
Figure S14: [Cy5^{21}]-pramlintide (10 nM) is not robustly internalised into AMY₁ receptor transfected HEK293S cells. Confocal microscopy of [Cy5^{21}]-pramlintide fluorescence (red) and EEA1 staining (cyan) in AMY₁ receptor transfected cells. [Cy5^{21}]-pramlintide is primarily at the cell membrane, showing little colocalization with the early endosomal marker EEA1. White arrows indicate occasional overlapping spots. Nuclei were stained with DAPI (blue). Images are representative of 3 independent experiments.

Figure S15: Comparison of myc-RAMP1 expression in cells transfected with the CGRP or AMY₁ receptor. (A) Number of cells expressing myc-RAMP1 in unstimulated HEK293S cells, detected using immunofluorescence. Cell surface expression of myc-RAMP1 in (B) HEK293S and (C) Cos-7 cells, detected using ELISA. Data are mean ± s.e.m. from 5 independent experiments performed in duplicate or triplicate. ns, not significant by Student’s t-test. For (A), mean of 8 fields per well, with 20 wells for each receptor for each independent experiment. For (B) and (C), mean of 6-8 wells for each independent experiment.
Figure S16: Comparison of spots in HEK293S cells transfected with tagged or untagged AMY1 receptor for (A) [Cy5]-haCGRP or (B) [Cy521]-Pramlintide. Data are mean ± s.e.m. from 3 independent experiments performed in duplicate. *p < 0.05 by repeated measures two-way ANOVA with post-hoc Bonferroni’s test comparing spots of [Cy5]-haCGRP at the tagged and untagged AMY1 receptor at each time-point. Analysis was as per Figure S5. For untagged receptors, an Abcam antibody against RAMP1 (Ab156575, 1:500 dilution) was used, with selection for cells expressing RAMP1-like immunoreactivity above pcDNA values. We have performed in-house immunofluorescence characterisation of this antibody to confirm its ability to detect RAMP1.

Figure S17: (A) cAMP production by [Cy514]-hCT at the CT receptor (Cos-7). (B) Number of spots in HEK293S cells transfected with the CT receptor with 100 nM of [Cy514]-hCT (B) or 10 nM [Cy521]-pramlintide (C). Data are mean ± s.e.m. from 5 independent experiments performed in duplicate. *p < 0.05 by repeated measures two-way ANOVA with post-hoc Bonferroni’s test comparing each fluorescent peptide to vector at each time-point.
Figure S18: Time course for 100 nM of [Cy5^{14}]-hCT at CT receptor transfected HEK293S cells. Images are a representative from 5 independent experiments performed in duplicate. Scale bar = 20 µM.

Figure S19: Comparison of spots of fluorescent peptide at the AMY_{1} and CT receptor in HEK293S cells. [Cy5^{3}]-hαCGRP and [Cy5^{21}]-pramlintide time course data replotted from Figure 3, 4. (A) and (B) compare data for the CT receptor using [Cy5^{21}]-pramlintide or [Cy5^{14}]-hCT, respectively. Data are mean ± s.e.m. from 5 independent experiments performed in duplicate. Spots of [Cy5^{3}]-hαCGRP at the AMY_{1} receptor (* p < 0.05) and spots of [Cy5^{21}]-pramlintide at the AMY_{1} receptor († p < 0.05) compared to spots at the CT receptor by repeated measures two-way ANOVA with post-hoc Bonferroni’s test.
Supplementary 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), hydrazine hydrate, and 4-methylmorpholine (NMM) were purchased from Sigma-Aldrich (St. Louis, Missouri). \(O-(7\text{-Azabenzotriazol-1-yl})-N,N,N',N'\text{-tetramethyluronium hexafluorophosphate (HATU)}\), Fmoc-Pro-OH, Fmoc-Val-OH, Fmoc-Phe-OH, Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Glu(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Glu(tBu)-OH, Fmoc-Met-OH, Fmoc-Asp(tBu)-OH and Fmoc-Tyr(tBu)-OH were purchased from GL Biochem (Shanghai, China). Fmoc-Rink amide linker was purchased from CS Bio (Shanghai, China). 6-Chloro-1-hydroxybenzotriazole (6-Cl-HOBt), Fmoc-Ser(tBu)-Ser(Psi(Me,Me)pro)-OH, Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH and Boc-Lys(Fmoc)-OH were purchased from Apptec (Louisville, Kentucky). Aminomethyl polystyrene resin was purchased from RAPP polymere (Tübingen, Germany).

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), Fmoc-Lys(Dde)-OH and guanidine hydrochloride (Gu·HCl) were purchased from AK Scientific (Union City, California). Boc-Ala-OH was purchased from Polypeptide (Strasbourg, France). 4-(Dimethylamino)pyridine (DMAP) was purchased from AK Scientific (Union City, California). Yields refer to chromatographically homogeneous materials. Microwave reactions were carried out in a Biotage Alstra peptide synthesizer. Semi-preparative 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 x 150 mm) at a flow rate of 1 mL min⁻¹, a semi-preparative column (Phenomenex® Torrance, CA, Gemini C18, (5 μm; 10 x 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 H₂O and B was 0.1% TFA in acetonitrile. LC-MS spectra were acquired using Agilent Technologies 1260 Infinity LC equipped with an Agilent Technologies 6120 Quadrupole mass spectrometer. An analytical column (Agilent C3, 3.5 μm; 3.0 x 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 H₂O 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 CH₂Cl₂ (5 mL, 20 min), was added 4-[(2,4-dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid (Rink amide linker) (220 mg, 4 equiv, 0.4 mmol) and 6-Cl-HOBt (70 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 Nα-Fmoc-protecting group:**

Peptidyl resin was treated with a solution of 20 vol % piperidine in DMF (v/v, 4 mL) and the mixture 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-Pro-OH, Fmoc-Val-OH, Fmoc-Phe-OH, Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Glu(OrBu)-OH, Fmoc-Met-OH, Fmoc-Asp(OrBu)-OH, Fmoc-Tyr(tBu)-OH:**

Couplings were performed using the Biotage® Initiator Alstra with 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, 8 equiv) 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: General coupling procedure for Fmoc-Lys(Dde)-OH:**

Peptidyl resin was treated with Fmoc-Lys(Dde)-OH (266 mg, 5 equiv, 0.5 mmol) and HATU (180 mg, 4.75 equiv, 0.47 mmol) dissolved in DMF (2 mL) followed by addition of NMM (88 µL, 8 equiv, 0.8 mmol). The reaction mixture was gently agitated at room temperature for 25 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 coupling procedure for Boc-AA-OH:**

Peptidyl resin was treated with the appropriate Boc-AA-OH (5 equiv, 0.5 mmol) and HATU (180 mg, 4.75 equiv, 0.47 mmol) dissolved in DMF (1.5 mL) followed by addition of NMM (88 µL, 8 equiv, 0.8 mmol). The reaction mixture was gently agitated at room temperature for 25 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 8: 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 9: General procedure for the capping of free amino groups:

Fmoc-protected peptidyl resin was treated with 5 M Ac₂O 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 10a: Removal of Dde side-chain protecting group:

Peptidyl resin was treated with hydrazine hydrate in DMF (1:50, v/v, 5 mL). The reaction mixture was gently agitated at room temperature for 5 min, before being filtered, washed with DMF (3 × 3 mL), and the procedure repeated twice with fresh reagents. The resin was filtered and washed with DMF (3 × 3 mL).

Method 10b: Removal of Dde side-chain protecting group:

Peptidyl resin pre-swelled in CH₂Cl₂ for 30 min was treated with 3.5 mL of (2.56 mM NH₂OH·HCl/1.93 mM imidazole) dissolved in NMP/CH₂Cl₂ (6:1, 7 mL). The reaction mixture was gently agitated at room temperature for 5 h. The resin was filtered and washed with DMF (3 × 3 mL).

Method 11a: Coupling of 2-azidoacetic acid to lysine side chain:

Peptidyl resin was treated with 2-azidoacetic acid (37 µL, 5 equiv, 0.5 mmol) and PyBOP (260 mg, 5 equiv, 0.5 mmol) in DMF (1.5 mL) followed by addition of NMM (110 µL, 10 equiv, 1 mmol). The reaction mixture was gently agitated at room temperature for 60 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 11b: Coupling of 2-azidoacetic acid to lysine side chain:

Peptidyl resin was treated with 2-azidoacetic acid (37 µL, 5 equiv, 0.5 mmol) and DMAP (1.2 mg, 0.1 equiv, 0.01 mmol) in DMF (1 mL) followed by addition of DIC (47 µL, 3 equiv, 0.3 mmol). The reaction mixture was irradiated at 25 W at 75 °C for 5 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 12: General procedure for TFA-mediated resin cleavage and global deprotection:

Peptidyl resin was treated with a mixture of TFA/H₂O/EDT (95:2.5:2.5, v/v/v, 10 mL) for 100 min, followed by addition of TIPS (250 µL) for a further 5 min. The filtrate was partially concentrated under a gentle stream of N₂, 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 H₂O:acetonitrile containing 0.1% TFA (1:1, v/v, 25 mL) and lyophilised.
Method 13a: 1,3-Dipolar cycloaddition via click chemistry:

To azido peptide 6 (3 mg, 0.7 \times 10^{-6} \text{ moles}) was added a solution of (6 M Gu·HCl/0.2 M Na2HPO4/20 mM TCEP/20 mM CuSO4·5H2O) in H2O (256 µL) and the resulting solution was degassed with Argon, and adjusted to pH 7.35 - 7.41 using 5 M HCl and 10 M NaOH, followed by gentle heating at 40 °C for 5 min. A solution of alkyn Cy-5 fluorophore (0.7 mg, 1.7 equiv, 1.2 \times 10^{-6} \text{ moles}, 0.02 M) in DMSO (50 µL) was then added and the resulting solution was sonicated for 1 min then agitated at room temperature for 1 h. The reaction mixture was quenched with 5 M HCl (60 µL) and the crude solution of peptide 7 was immediately purified batchwise by semi-preparative RP-HPLC.
Synthesis of the human α-CGRP (7).

Fmoc-Rink amide was attached to aminomethyl 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 3 was achieved using Method 3. Method 2 was used for all subsequent N-terminal Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to 18Arg indicated in Scheme S1 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 9. Coupling of Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH dipeptide at position 18 was achieved using Method 8. Linear synthesis of the peptide was continued up to 4Thr using appropriate Methods (2, 3, 4, 5 and 9). Fmoc-Lys(Dde)-OH was attached to deprotected resin bound 4Thr using Method 6. The peptide chain was further elongated to 2Cys using Method 5. Boc-Ala-OH was coupled at position 2 using Method 7 affording resin-bound 4. Selective Dde removal of 4 was achieved using Method 10a followed by coupling of 2-azidoacetic acid on the side-chain amine group of lysine at position 3 using Method 11a affording peptide 5. Peptide 5 was liberated from resin using Method 12 affording deprotected 6 (150 mg). Crude 6 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 6 as a white amorphous solid (40 mg, 13% yield based on 0.1 mmol scale, tR = 12.9 min, >99% purity); (Figure S20 and S21). 1,3-Dipolar cycloaddition of fluorophore Cy-5 with 6 was achieved using Method 13a. The crude 7 reaction mixture was subsequently quenched with 5 M HCl (60 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 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 the title compound 7 as a blue amorphous solid (1.5 mg, 43% yield, tR = 15.9 min, >99% purity); (Figure S22 and S23).

**Figure S20**: Analytical RP-HPLC chromatogram of purified peptide 6, tR = 12.9 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 30 min at room temperature, ca. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H2O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).
Figure S21: LC-MS profile of purified peptide 6. Ion polarity positive. ESI-MS ($m/z$) 
[M+3H]$^+$ calcd: 1296.8; found 1296.5; [M+4H]$^+$ calcd: 972.8; found: 972.6; 
[M+5H]$^+$ calcd: 778.5; found: 778.3; [M+6H]$^+$ calcd: 648.9; found: 648.8. Mass deconvolution calculated at 
3886.55 Da with standard deviation of 0.17; theoretical mass calculated at 3887.51 Da.
Figure S22: Analytical RP-HPLC chromatogram of purified peptide 7, $t_R = 15.9$ 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 30 min at room temperature, ca. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H$_2$O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).
Figure S23: LC-MS profile of purified peptide 7; Ion polarity positive. ESI-MS (m/z [M+3H]+ calcd: 1476.7; found: 1476.2; [M+4H]++ calcd: 1107.8; found 1107.3; [M+5H]+++ calcd: 886.4; found: 886.1; [M+6H]++++ calcd: 738.8; found: 738.6; [M+7H]+++++ calcd: 633.4; found: 633.2. Mass deconvolution calculated at 4425.46 Da with standard deviation of 0.17; theoretical mass calculated at 4427.21 Da.
Synthesis of α-CGRP (human) with fluorophore conjugated on lysine at position 21 using Fmoc-SPPS.

Scheme S2. Synthesis of α-CGRP (Human) with CY-5 fluorophore conjugated on 21Lys.

Method 13b: 1,3-Dipolar cycloaddition via click chemistry:

To azido peptide 10 (23 mg, 5.8 × 10⁻⁶ moles) was added a solution of (6 M Gu HCl/0.2 M Na₂HPO₄/20 mM TCEP/20 mM CuSO₄·5H₂O) in H₂O (1960 µL) and the resulting solution was degassed with Argon, and adjusted to pH 7.35 - 7.41 using 5 M HCl and 10 M NaOH, followed by gentle heating at 40 °C for 5 min. A solution of alkyne Cy-5 fluorophore (5.3 mg, 9.7 × 10⁻⁶ moles, 1.7 equiv, 0.09 M) in DMSO (100 µL) was then added and the resulting solution was sonicated for 1 min then agitated at room temperature for 1 h. The reaction mixture was quenched with 5 M HCl (60 µL) and the crude solution of peptide 11 was immediately purified batchwise by semi-preparative RP-HPLC.
Synthesis of the human α-CGRP (11).

Fmoc-SPPS was used for the synthesis of the human α-CGRP (11) (Scheme S2). Fmoc-Rink amide was attached to aminomethyl 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 3 was achieved using Method 3. Method 2 was used for all subsequent Nα-Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to 22Val indicated in Scheme S2 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 by using Method 9. Fmoc-Lys(Dde)-OH was attached to deprotected resin bound 22Val using Method 6.

Peptide chain elongation to 18Arg was conducted using appropriate Methods (2, 3, 4 and 9). Coupling of Fmoc-Leu-Ser(Psi(Me,Me)pro)-OH dipeptide at 18Arg was achieved using Method 8. Linear synthesis of the peptide was continued up to 2Cys using appropriate Methods (2, 3, 4, 5 and 9). Boc-Ala-OH was coupled on resin bound cysteine at position 2 using Method 7 affording resin-bound 8. Selective Dde removal of 8 was achieved using Method 10a followed by coupling of 2-azidoacetic acid to the side-chain amine of lysine at position 21 using Method 11a affording peptide 9. Peptide 9 was liberated from resin using Method 12 affording deprotected 10 (163 mg). Crude 10 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 10 as a white amorphous solid (51 mg, 13% yield based on 0.1 mmol scale, tR = 12.8 min, >99% purity); (Figure S24 and S25).

1,3-Dipolar cycloaddition of fluorophore Cy-5 with 10 was achieved using Method 13b. The reaction mixture was subsequently quenched with 5 M HCl (60 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 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 the title compound 11 as a blue amorphous solid (10.2 mg, 39% yield, tR = 15.1 min, >99% purity); (Figure S26 and S27).

**Figure S24:** Analytical RP-HPLC chromatogram of purified peptide 10, tR = 12.8 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 30 min at room temperature, ca. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H2O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).
Figure S25: LC-MS profile of crude peptide 10; Ion polarity positive. ESI-MS (m/z [M+3H]⁺⁺⁺ calcd: 1316.1; found 1315.8; [M+4H]⁺⁺⁺ calcd: 987.4; found: 987.2; [M+5H]⁺⁺⁺ calcd: 790.1; found: 789.9; [M+6H]⁺⁺⁺ calcd: 658.6; found: 658.5. Mass deconvolution calculated at 3944.68 Da with standard deviation of 0.28; theoretical mass calculated at 3945.55 Da.
Figure S26: Analytical RP-HPLC chromatogram of purified peptide 11, $t_r = 15.1$ 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 30 min at room temperature, ca. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H₂O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).
Figure S27: LC-MS profile of purified peptide 11; Ion polarity positive. ESI-MS (m/z [M+3H]^{3+} calcd: 1496.0; found: 1495.3; [M+4H]^{4+} calcd: 1122.3; found: 1121.8; [M+5H]^{5+} calcd: 898.0; found: 897.7; [M+6H]^{6+} calcd: 748.5; found: 748.2; [M+7H]^{7+} calcd: 641.7; found: 641.5. Mass deconvolution calculated at 4483.26 Da with standard deviation of 0.25; theoretical mass calculated at 4485.24 Da.
Synthesis of pramlintide with fluorophore conjugated on lysine at position 21 using Fmoc-SPPS.

Scheme S3. Synthesis of pramlintide with CY-5 fluorophore conjugated on 21Lys.

Method 13c: 1,3-Dipolar cycloaddition via click chemistry:

To azido peptide 14 (12 mg, 2.9 × 10⁻⁶ moles) was added a solution of (6 M Gu-HCl/0.2 M Na₂HPO₄/20 mM TCEP/20 mM CuSO₄·5H₂O) in H₂O (980 µL) and the resulting solution was degassed with Argon, and adjusted to pH 7.35 - 7.41 using 5 M HCl and 10 M NaOH, followed by gentle heating at 40 °C for 5 min. A solution of alkyne Cy-5 fluorophore (2.72 mg, 5.0 × 10⁻⁶ moles, 1.7 equiv, 0.07 M) in DMSO (70 µL) was then added and the resulting solution was sonicated for 1 min then agitated at room temperature for 1 h. The reaction mixture was quenched with 5 M HCl (60 µL) and the solution of crude peptide 15 was immediately purified batchwise by semi-preparative RP-HPLC.
Synthesis of the human pramlintide (15).

Fmoc-SPPS was used for the synthesis of pramlintide (15) (Scheme S3). Fmoc-Rink amide was attached to aminomethyl resin 1 using Method 1 followed by Fmoc-removal using Method 2 to afford 3. Direct attachment of Fmoc-Tyr-OH at position 37 to resin bound Rink amide 3 was achieved using Method 3. Method 2 was used for all subsequent Nδ-Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to 32Asn 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 utilised throughout the synthesis using Method 9. Fmoc-Lys(Dde)-OH was attached to deprotected resin bound 32Asn using Method 6. Coupling of Fmoc-Ser-Ser(Psi(Me,Me)pro)-OH dipeptide at 31Lys was achieved using Method 8. Linear synthesis of the peptide was continued up to 37Cys using appropriate Methods (2, 3, 4, 5 and 9). Boc-Lys(Fmoc)-OH was coupled on resin bound cysteine at position 2 using Method 7 affording resin-bound 12. Selective Dde removal of peptide 12 was achieved using Method 10b followed by coupling of 2-azidoacetic acid to the side-chain amine of lysine at position 21 using Method 11a affording peptide 13. Peptide 13 side-chain Fmoc group was removed using Method 2, then liberated from resin using Method 12 affording deprotected 14 (120 mg). Crude 14 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 14 as a white amorphous solid (50 mg, 12.3% yield based on 0.1 mmol scale, tR = 14.4 min, >99% purity); (Figure S28 and S29). 1,3-Dipolar cycloaddition of alkyne fluorophore Cy-5 with 14 was achieved using Method 13c. The reaction mixture was subsequently quenched with 5 M HCl (60 µL) and 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 the title compound 15 as a blue amorphous solid (8.2 mg, 60% yield, tR = 41.9 min, >99% purity); (Figure S30 and S31).
Figure S29: LC-MS profile of purified peptide 14; Ion polarity positive. ESI-MS (m/z [M+3H]+ calcd: 1350.5; found: 1350.3; [M+4H]+ calcd: 1013.1; found: 1013.0; [M+5H]+ calcd: 810.7; found: 810.5; [M+6H]+ calcd: 675.7; found: 675.7; [2M+5H]+ calcd: 1620.4; found: 1620.4; [2M+7H]+ calcd: 1157.7; found: 1157.6; [3M+8H]++ calcd: 1519.2; found: 1519.1; [3M+7H]+ calcd: 1736.1; found: 1735.9. Mass deconvolution calculated at 4047.90 Da with standard deviation of 0.29; theoretical mass calculated at 4048.57 Da.

* Represent dimer and trimer charge states induced during LC-MS analysis.
Figure S30: Analytical RP-HPLC chromatogram of purified peptide 15, $t_r = 41.9$ 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 90 min at room temperature, ca. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H$_2$O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).
Figure S31: LC-MS profile of purified peptide 15; Ion polarity positive. ESI-MS (m/z [M+3H]^3+ caleld: 1530.4; found: 1529.8; [M+4H]^4+ caleld: 1148.0; found: 1147.6; [M+5H]^5+ caleld: 918.6; found: 918.3; [M+6H]^6+ caleld: 765.7; found: 765.3. Mass deconvolution calculated at 4586.28 Da with standard deviation of 0.32; theoritical mass calculated at 4588.28 Da.
Synthesis of human calcitonin with fluorophore conjugated on lysine at position 14 using Fmoc-SPPS.

Scheme S4. Synthesis of human calcitonin with Cy-5 fluorophore conjugated on 14Lys.

Method 13d: 1,3-Dipolar cycloaddition via click chemistry:

To azido peptide 18 (12.7 mg, 3.6 × 10^-6 moles) was added a solution of (6 M Gu-HCl/0.2 M Na2HPO4/20 mM TCEP/20 mM CuSO4·5H2O) in H2O (1208 µL) and the resulting solution was degassed with Argon, and adjusted to pH 7.35 - 7.41 using 5 M HCl and 10 M NaOH, followed by gentle heating at 40 °C for 5 min. A solution of alkyne Cy-5 fluorophore (2.72 mg, 5.0 × 10^-6 moles, 1.7 equiv, 0.05 M) in DMSO (100 µL) was then added and the resulting solution was sonicated for 1 min then agitated at room temperature for 1 h. The reaction mixture was quenched with 5 M HCl (60 µL) and the crude peptide 19 solution was immediately purified batchwise by semi-preparative RP-HPLC.
Synthesis of the human calcitonin (19).

Fmoc-SPPS was used for the synthesis of human calcitonin (19) (Scheme S4). Fmoc-Rink amide was attached to aminomethyl resin 1 using Method 1 followed by Fmoc-removal using Method 2 to afford 3. Direct attachment of Fmoc-Pro-OH at position 32 to resin bound Rink amide 3 was achieved using Method 3. Method 2 was used for all subsequent Nα-Fmoc removals where appropriate. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to 15Asp 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 by using Method 9. Fmoc-Lys(Dde)-OH was attached to deprotected resin bound 15Asp using Method 6. Coupling of Fmoc-Tyr-Thr(Psi(Me,Me)pro)-OH dipeptide at 14Lys was achieved using Method 8. Linear synthesis of the peptide was continued up to 16Cys using appropriate Methods (2, 3, 5 and 9) affording resin-bound 16. Selective Dde removal of peptide 16 was achieved using Method 10b followed by coupling of 2-azidoacetic to the side-chain amine of lysine at position 14 using Method 11b affording peptide 17. Peptide 17 Fmoc-group was removed using Method 2 and was then liberated from resin using Method 12 affording deprotected 18 (80 mg). 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 (12.7 mg, 4% yield based on 0.1 mmol scale, tR = 13.4 min, >99% purity); (Figure S32 and S33). 1,3-Dipolar cycloaddition of alkyne fluorophore Cy-5 with 18 was achieved using Method 13d. The reaction mixture was subsequently quenched with 5 M HCl (60 µL) and 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 the title compound 19 as a blue amorphous solid (1.3 mg, 9% yield, tR = 40.0 min, >99% purity); (Figure S34 and S35).

Figure S32: Analytical RP-HPLC chromatogram of purified peptide 18, tR = 13.4 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a X Terra® MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 30 min at room temperature, ca. 3% B per min at a flow rate of 1.0 mL/min. Buffer A: H2O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).
Figure S33: LC-MS profile of purified peptide 18. Ion polarity positive. ESI-MS (m/z [M+2H]^2+ calcd: 1752.5; found: 1751.8; [M+3H]^3+ calcd: 1168.6; found: 1168.4; [M+4H]^4+ calcd: 876.7; found: 876.5. Mass deconvolution calculated at 3501.93 Da with standard deviation of 0.31; theoretical mass calculated at 3502.99 Da.
Figure S34: Analytical RP-HPLC chromatogram of purified peptide 19, $t_R = 40.0$ min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a X Terra® MS C-18 column (5 μm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 90 min at room temperature, ca. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H$_2$O containing 0.1% TFA (v/v); Buffer B: acetonitrile containing 0.1% TFA (v/v).
Figure S35: LC-MS profile of purified peptide 19; ion polarity positive. ESI-MS (m/z [M+3H]^3+ calcd: 1348.5; found: 1347.8; [M+4H]^4+ calcd: 1011.6; found: 1011.2; [M+5H]^5+ calcd: 809.5; found: 809.2. Mass deconvolution calculated at 4040.73 Da with standard deviation of 0.31; theoretical mass calculated at 4042.69 Da.

References.
