Supporting Information

Novel Fluorescently Labeled PACAP and VIP Highlight Differences between Peptide Internalization and Receptor Pharmacology

Tyla I. Alexander[1,2#], Zoe Tasma[2,3#], Andrew Siow[4], Tayla A. Rees[2,3], Margaret A. Brimble[2,4], Paul W. R. Harris[2,4], Debbie L. Hay[1,2], Christopher S. Walker[2,3]*

[1]Department of Pharmacology and Toxicology, The University of Otago, Dunedin, New Zealand

[2]Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Auckland, New Zealand

[3]School of Biological Sciences, The University of Auckland, Auckland, New Zealand

[4]School of Chemical Sciences, The University of Auckland, Auckland, New Zealand

[#]These authors contributed equally to the manuscript.

*Corresponding author: Christopher S Walker, E-mail: cs.walker@auckland.ac.nz

Table of Contents

Supporting Tables

Table S1. Summary of [Cy5^{21}]PACAP-38 and [Cy5^{34}]PACAP-38 peptide potency and maximal response (E_{max}) in receptor transfected Cos7 cells.

	PACAP-38			**[Cy5^{21}]PACAP-38**				**[Cy5^{34}]PACAP-38**			
	pEC_{50}	E_{max} (%)	n	pEC_{50}	E_{max} (%)	Fold Change (pEC_{50})	n	pEC_{50}	E_{max} (%)	Fold Change (pEC_{50})	n
PAC_{1n}	9.35 ± 0.15	100.0	5	9.08 ± 0.18	103.7 ± 21.6	1.7	5	9.10 ± 0.12	107.0 ± 25.5	1.8	5
PAC_{1s}	8.78 ± 0.14	100.0	5	9.03 ± 0.19	79.1 ± 2.9*	1.8	5	8.90 ± 0.15	69.9 ± 4.6*	1.3	5
$VPAC_1$	9.18 ± 0.15	100.0	5	8.71 ± 0.16	83.7 ± 4.2*	3.0	5	8.68 ± 0.22	71.1 ± 3.3*	3.2	5
$VPAC_2$	9.01 ± 0.05	100.0	5	8.77 ± 0.14	109.9 ± 22.4	1.7	5	8.56 ± 0.20	105.6 ± 20.2	2.8	5

Data points are mean ± SEM, combined from 5 independent experiments performed in duplicate or triplicate. E_{max} values are presented as a percentage of the unlabeled peptide. Differences in agonist potency to the unlabeled peptide were analyzed using a one-way ANOVA with Dunnett's test. Differences in peptide E_{max} were analyzed from the non-normalized data log-transformed followed by repeated measures one-way ANOVA with Dunnett's test. *$p < 0.05$.

Table S2. Summary of [Cy5^{21}]PACAP-27 peptide potency and maximal response (E_{max}) in receptor transfected Cos7 cells.

	PACAP-27			**[Cy5^{21}]PACAP-27**			
	pEC_{50}	E_{max} (%)	n	pEC_{50}	E_{max} (%)	Fold Change (pEC_{50})	n
PAC_{1n}	9.78 ± 0.11	100.0	5	9.22 ± 0.13*	106.8 ± 13.3	3.6	5
PAC_{1s}	9.56 ± 0.18	100.0	5	9.08 ±0.12*	121.3 ± 10.9	3.0	5
$VPAC_1$	9.75 ± 0.11	100.0	5	9.10 ± 0.08*	112.7 ± 13.3	4.5	5
$VPAC_2$	9.40 ± 0.24	100.0	5	8.76 ± 0.08*	108.7 ± 18.9	4.4	5

Data points are mean ± SEM, combined from 5 independent experiments performed in duplicate or triplicate. E_{max} values are presented as a percentage of the unlabeled peptide. Differences in agonist potency to the unlabeled peptide were analyzed using a paired two-tailed *t*-test. Differences in peptide E_{max} were analyzed from the non-normalized data by a ratio paired two-tailed *t*-test. *$p < 0.05$.

Table S3. Summary of [Cy5^{19}]VIP peptide potency and maximal response (E_{max}) in receptor transfected Cos7 cells.

	VIP			**[Cy5^{19}]VIP**			
	pEC_{50}	E_{max} (%)	n	pEC_{50}	E_{max} (%)	Fold Change (pEC_{50})	n
PAC_{1n}	7.83 ± 0.15	100.0	5	7.97 ± 0.14	69.5 ± 9.5*	1.4	5
PAC_{1s}	8.81 ± 0.12	100.0	5	8.23 ± 0.16	110.9 ± 11.3	3.8	5
$VPAC_1$	9.66 ± 0.10	100.0	5	9.68 ± 0.28	101.9 ± 11.5	1.1	5
$VPAC_2$	9.11 ± 0.16	100.0	5	9.26 ± 0.19	106.2 ± 12.8	1.4	5

Data points are mean ± SEM, combined from 5 independent experiments performed in duplicate or triplicate. E_{max} values are presented as a percentage of the unlabeled peptide. Differences in agonist potency to the unlabeled peptide were analyzed using a paired two-tailed *t*-test. Differences in peptide E_{max} were analyzed from the non-normalized data by a ratio paired two-tailed *t*-test. *p < 0.05.

Table S4. Degree of internalization (AUC) summary for each Cy5-labeled PACAP and VIP peptide in receptor transfected Cos7 cells.

Internalization								
	[Cy5^{21}]PACAP-38		**[Cy5^{34}]PACAP-38**		**[Cy5^{21}]PACAP-27**		**[Cy5^{19}]VIP**	
	Area under the curve (AUC)	n	Area under the curve (AUC)	n	Area under the curve (AUC)	n	Area under the curve (AUC)	n
PAC_{1n}	1358 ± 141.4	4	1009 ± 163.6	4	983.7 ± 140.4	4	148.1 ± 18.62*	4
PAC_{1s}	1541 ± 117.3	4	1332 ± 255.1	4	1434 ± 261.8	4	277.6 ± 57.57*	4
$VPAC_1$	1441 ± 231.2	4	864.3 ± 233.5	4	1188 ± 257.7	4	1030 ± 241.6	4
$VPAC_2$	1605 ± 212.0	4	1275 ± 303.3	4	1377 ± 214.7	4	1357 ± 232.3	4

Data points are mean ± SEM, combined from 4 independent experiments performed in duplicate. Differences in internalization (AUC) were analyzed using a one-way ANOVA with Tukey's test. *p < 0.05 compared to both the $VPAC_1$ and $VPAC_2$ receptors for [Cy5^{19}]VIP.

Supporting Figures

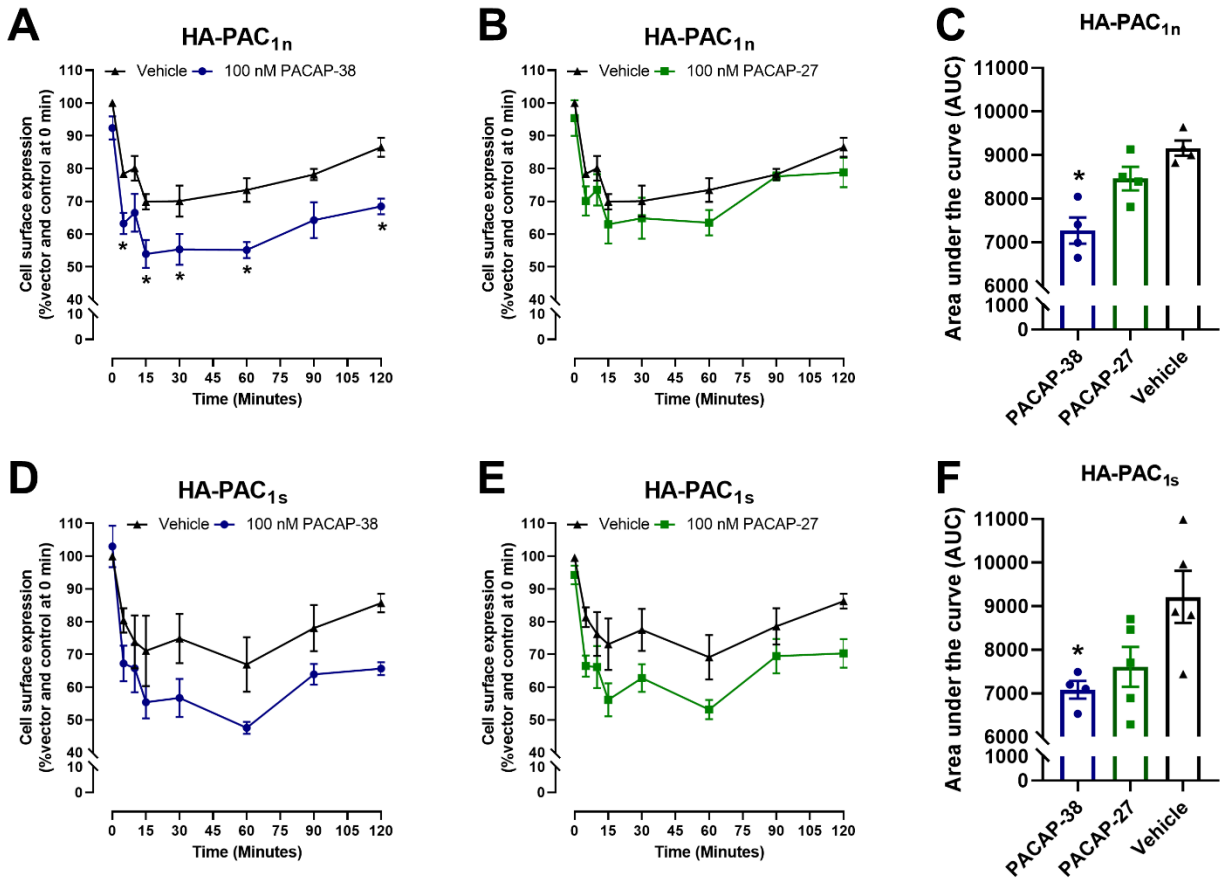


Figure S1. Change in cell surface expression of the A,B) HA-PAC_{1n} or D,E) HA-PAC_{1s} receptor in response to PACAP-38 or PACAP-27 stimulation in Cos-7 cells. Internalization data are summarized and analyzed as AUC for the C) HA-PAC_{1n} and F) HA-PAC_{1s} receptor. Data are mean ± SEM from 4-5 independent experiments performed in duplicate. * $p < 0.05$ compared to vehicle (media) control treated cells at each time point by A,B,D,E) repeated measures two-way ANOVA with post-hoc Bonferroni's test or C,F) Ordinary one-way ANOVA with post-hoc Dunnett's test.

Quantification of fluorescent peptide internalization was performed using spot counting in the Columbus software package. The cytoplasm of the cells and cell border were defined through staining by CellMask green. Representative images of staining with Cy5-labeled peptides are shown in Figures S1-S4.

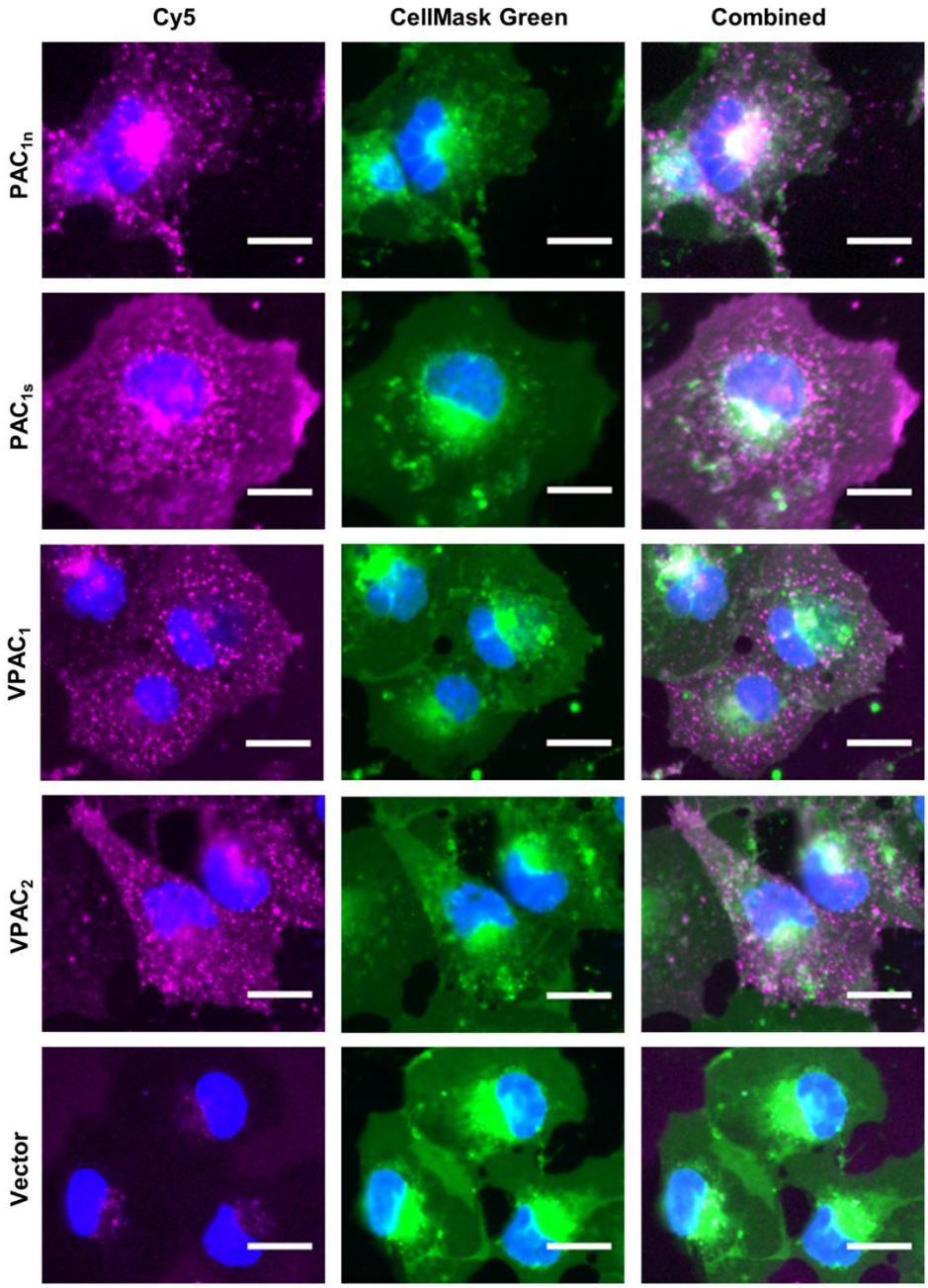


Figure S2. Internalization of [Cy5[21]]PACAP-38 (10 nM) at 15 minutes in Cos7 cells transfected with the human PAC_{1n}, PAC_{1s}, $VPAC_{1,}$ or $VPAC_2$ receptors colocalized with CellMask Green. [Cy5[21]]PACAP-38 fluorescence shown in magenta, CellMask Green staining in green and nuclear DAPI staining in blue. Images are representative of 4 independent experiments. Scale bar, 20 µm.

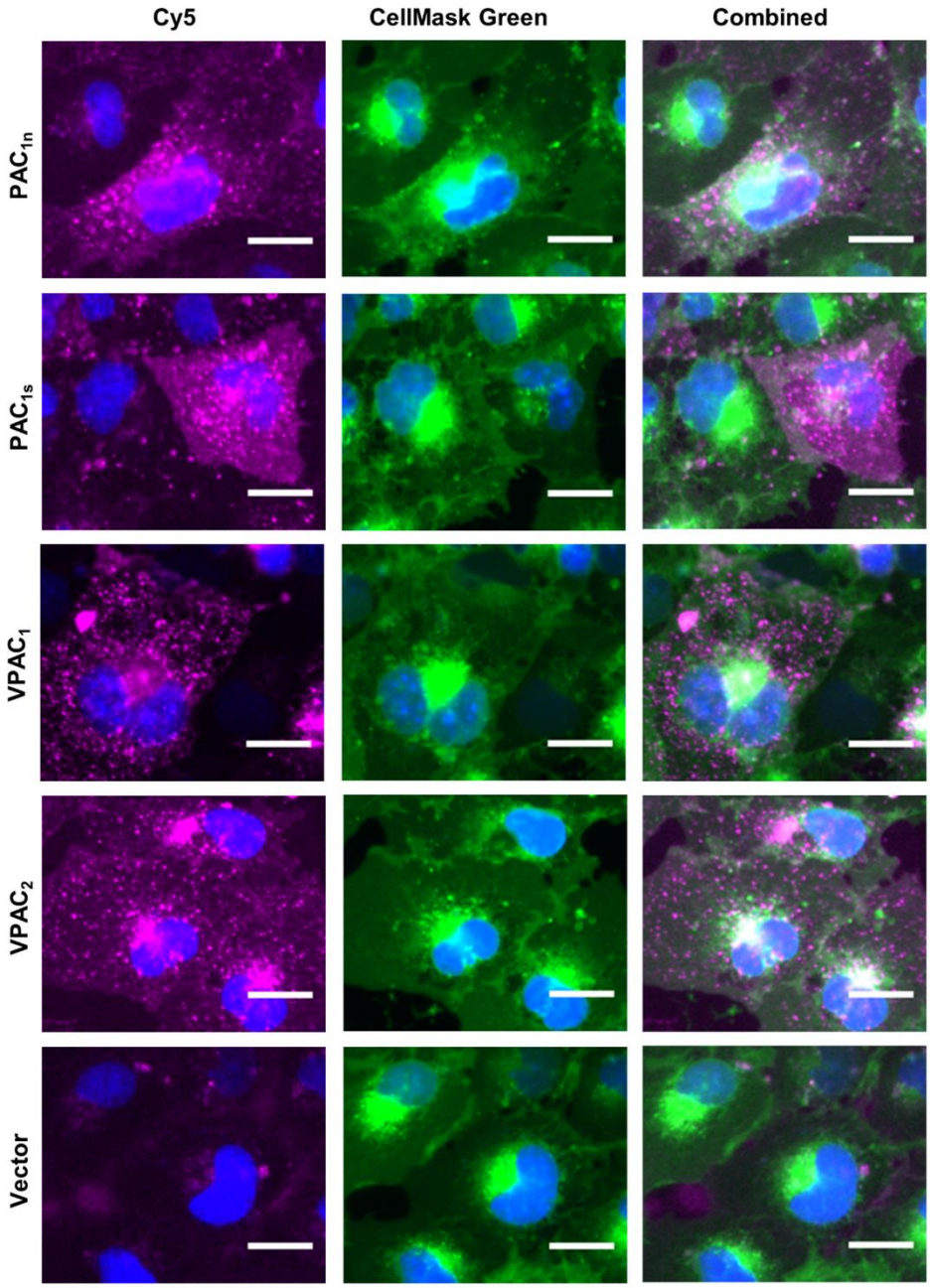


Figure S3. Internalization of [Cy5[34]]PACAP-38 (10 nM) at 15 minutes in Cos7 cells transfected with the human PAC_{1n}, PAC_{1s}, $VPAC_{1,}$ or $VPAC_2$ receptors colocalized with CellMask Green. [Cy5[34]]PACAP-38 fluorescence shown in magenta, CellMask Green staining in green and nuclear DAPI staining in blue. Images are representative of 4 independent experiments. Scale bar, 20 µm.

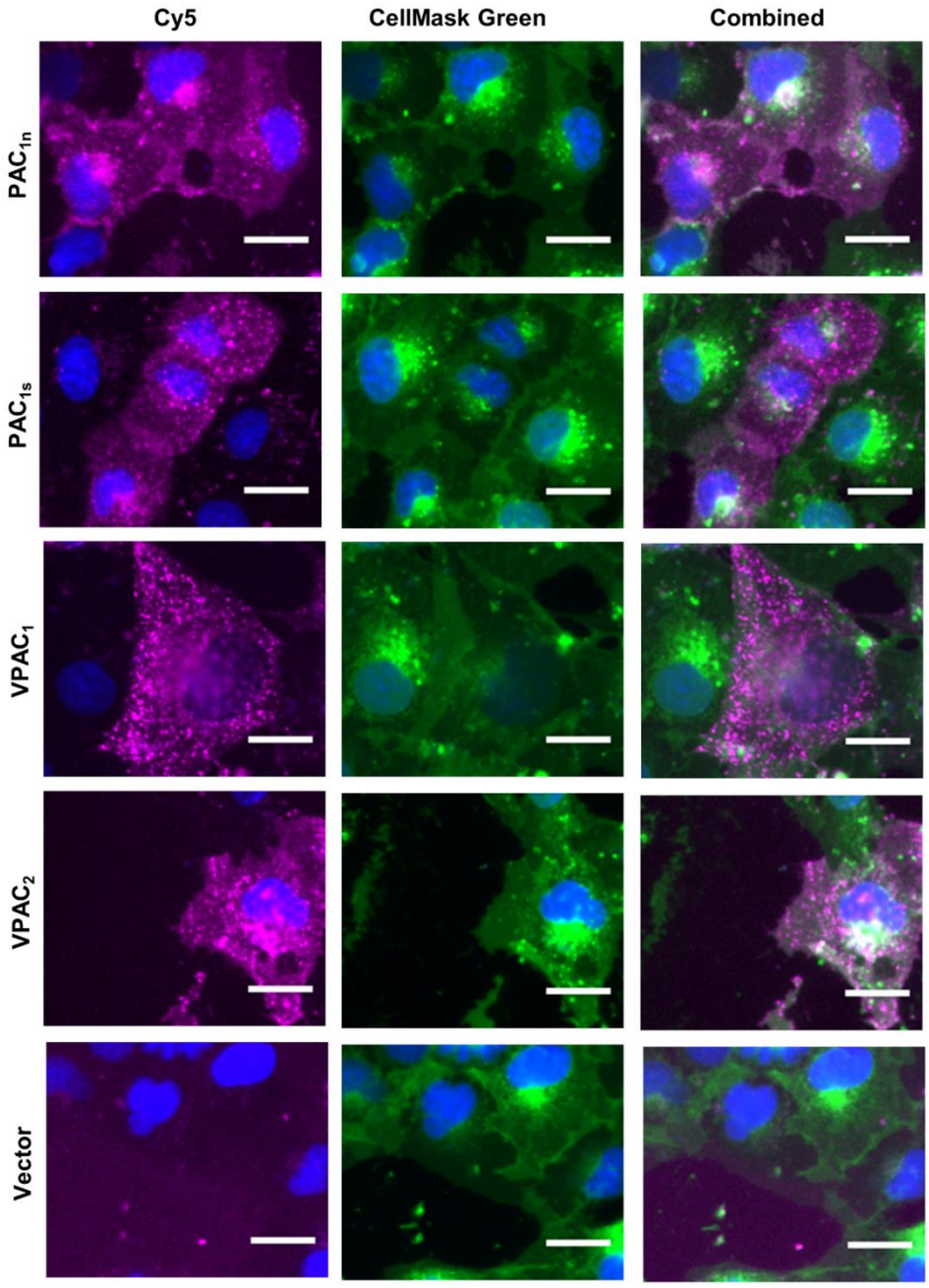


Figure S4. Internalization of [Cy5[21]]PACAP-27 (10 nM) at 15 minutes in Cos7 cells transfected with the human PAC_{1n}, PAC_{1s}, $VPAC_{1,}$ or $VPAC_2$ receptors colocalized with CellMask Green. [Cy5[21]]PACAP-27 fluorescence shown in magenta, CellMask Green staining in green and nuclear DAPI staining in blue. Images are representative of 4 independent experiments. Scale bar, 20 µm.

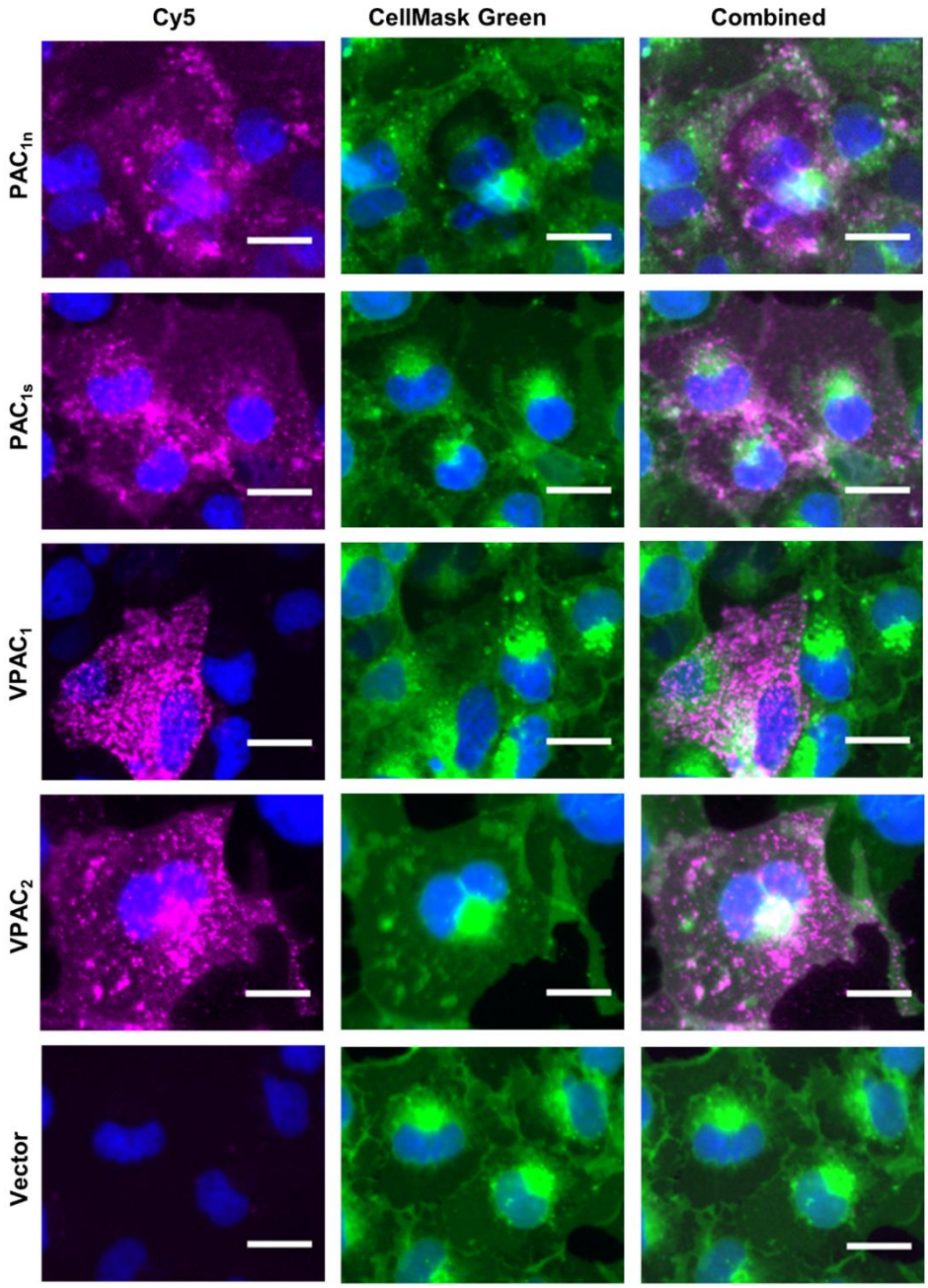


Figure S5. Internalization of [Cy5[19]]VIP (10 nM) at 15 minutes in Cos7 cells transfected with the human PAC_{1n}, PAC_{1s}, $VPAC_{1,}$ or $VPAC_2$ receptors colocalized with CellMask Green. [Cy5[19]]VIP fluorescence is shown in magenta, CellMask Green staining in green and nuclear DAPI staining in blue. Images are representative of 4 independent experiments. Scale bar, 20 µm.

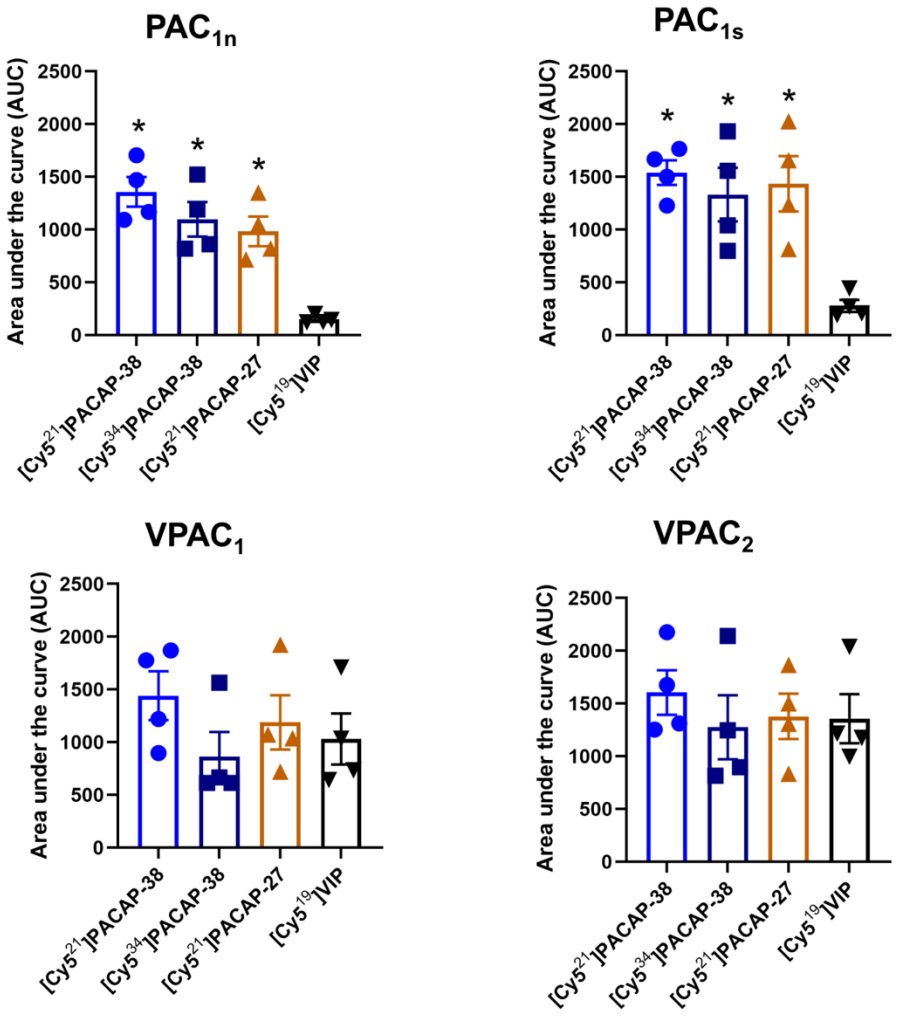


Figure S6. Comparison of the degree of Cy5-labeled peptide internalization at the PAC_{1n}, PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptors. Data are the mean ± SEM from 4 independent experiments. Statistical significance was determined by one-way ANOVA with Tukey's test. *$p < 0.05$ compared to [$Cy5^{19}$]VIP.

Supporting Biology
Supporting Methods

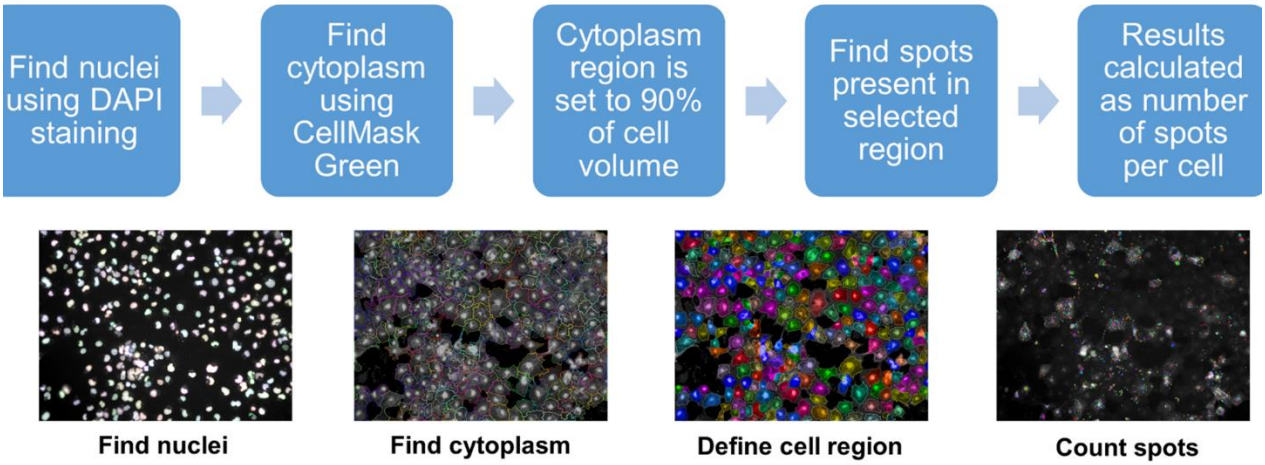


Figure S7. Workflow of spot analysis in Columbus software. Eight fields of view per well were imaged and used in spot analysis.

Supporting Chemistry

General Procedure

All reagents were purchased as reagent grade and used without further purification. *N*,*N*-Diisopropylethylamine (DIPEA), piperidine, hydroxylamine hydrochloride, imidazole, *N*,*N*′-diisopropylcarbodiimide (DIC), 1,2-ethanedithiol (EDT), triisopropylsilane (TIPS), piperazine and 4-methylmorpholine (NMM) were purchased from Sigma-Aldrich (St. Louis, Missouri). Fmoc-Lys(Dde)-OH (Dde = 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl), tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and guanidine hydrochloride (Gu·HCl) were purchased from AK Scientific (Union City, California). *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-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-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-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) and Fmoc-Tyr(*t*Bu)-Ser(Psi(Me,Me)pro)-OH were purchased from Apptec (Louisville, Kentucky). Aminomethyl polystyrene resin was purchased from Rapp Polymere (Tübingen, Germany). 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}, a Phenomenex® semi-preparative column (Torrance, CA), Gemini C18, (5 μm; 10 × 250 mm) at a flow rate of 4 mL min^{-1} or C18 prep column (Waters (Milford, MA), Xterra® prep C18 (10 um; 19 × 300 mm) at a flow rate of 8 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 ZORBAX 300SB-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 (220.2 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 Kaiser test confirmed successful coupling.[1]

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-Gly-OH, Fmoc-lle-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Dde)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-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, 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 using fresh reagents 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-His(Trt)-OH:

Double coupling cycles of 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 a second coupling using fresh reagents at 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 DMF (1.5 mL) 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 removal of N^{α}-Fmoc-protecting group from aspartimide prone sequences:

Peptidyl resin was treated with 5% piperazine in DMF (*w*/*v*) with 0.1 M 6-Cl-HOBt and the mixture was agitated on the Biotage® Initiator Alstra for 5 min at room temperature, filtered and repeated once for a further 15 min at room temperature. The resin was filtered and washed with DMF (3 × 3 mL).

Method 9: Removal of Dde side-chain protecting group: [2]

Peptidyl resin pre-swelled in CH_2Cl_2 for 30 min was treated with 3.5 mL of (2.56 mM $NH_2OH{\cdot}HCl$/1.93 mM imidazole) dissolved in NMP/CH_2Cl_2 (6:1,v/v, 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 10: Coupling of 2-azidoacetic acid to lysine side chain:

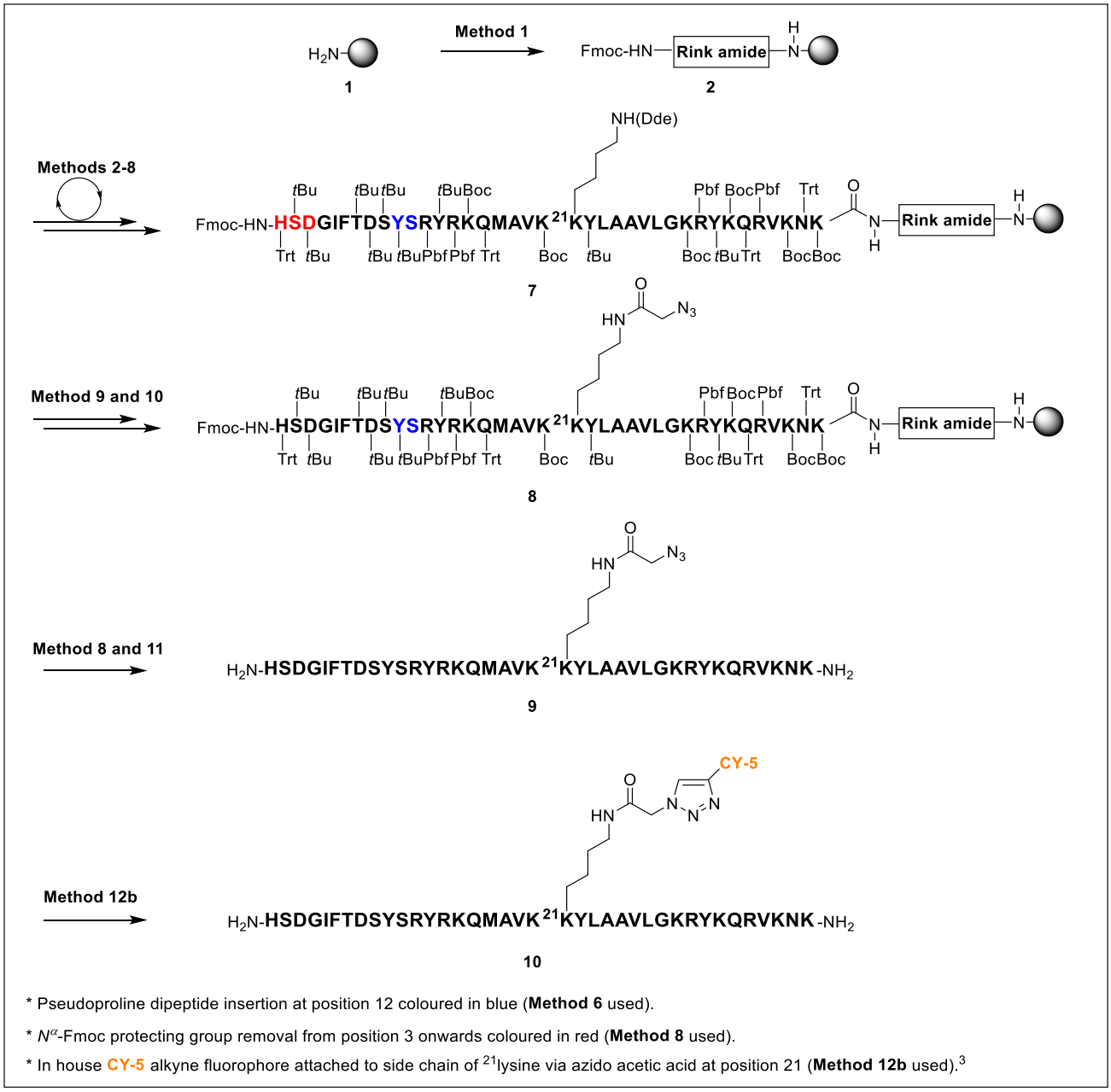
Peptidyl resin was treated with 2-azidoacetic acid (37 µL, 5 equiv., 0.5 mmol) and PyBOP (260 mg, 5 equiv., 0.5 mmol) in DMF (1.5 mL) followed by addition of NMM (110 µL, 10 equiv., 1 mmol). The reaction mixture was gently agitated at room temperature for 60 min, before being filtered, washed with DMF (3 × 3 mL) and the procedure repeated again with fresh reagents. The resin was filtered and washed with DMF (3 × 3 mL).

Method 11: General procedure for TFA-mediated resin cleavage and global deprotection:

Peptidyl resin was treated with a mixture of TFA/H_2O/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 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 (1:1, *v*/*v*, 25 mL) containing 0.1% TFA and lyophilised.

Synthesis of [Cy5^{21}]PACAP-38 using Fmoc-SPPS.

Scheme SC1. Synthesis of [Cy5^{21}]PACAP-38.



* Pseudoproline dipeptide insertion at position 12 coloured in blue (**Method 6** used).

* N^{α}-Fmoc protecting group removal from position 3 onwards coloured in red (**Method 8** used).

* In house **CY-5** alkyne fluorophore attached to side chain of ^{21}lysine via azido acetic acid at position 21 (**Method 12b** used).[3]

Method 12b: 1,3-Dipolar cycloaddition via click chemistry:

To azido peptide **9** (10.5 mg, 2.3 × 10^{-6} mol) was added a solution of (6 M Gu·HCl/0.2 M Na_2HPO_4/20 mM TCEP/20 mM $CuSO_4$·$5H_2O$) in H_2O (758 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 mg, 3.7 × 10^{-6} mol, 1.7 equiv.) 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 **10** was immediately purified batchwise by semi-preparative RP-HPLC.

Synthesis of PACAP (1-38) CY-5 at 21 (**10**).

Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmoc-removal using **Method 2**. Direct attachment of Fmoc-Lys(Boc)-OH at position 38 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α}-Fmoc removals up to position ^{3}Asp. Subsequent N^{α}-Fmoc removals from position 3 to 1 utilised **Method 8**. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ^{12}Arg indicated in **Scheme SC1** using **Method 3**. 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**. Coupling of Fmoc-Tyr(*t*Bu)-Ser(Psi(Me,Me)pro)-OH dipeptide at position 12 was achieved using **Method 6**. Linear synthesis of the peptide was continued up to His at position 1 using appropriate **Methods** (**2-5**, **7-8**). Selective Dde removal of **7** was achieved using **Method 9** followed by coupling of 2-azidoacetic acid on the side-chain amine group of lysine at position 21 using **Method 10** affording peptide **8**. Peptide **8** was then subjected to a final Fmoc removal using **Method 8** and then liberated from resin using **Method 11** affording **9**. Crude **9** was purified batchwise by semi-preparative RP-HPLC on a Xterra® Prep MS C18 column, (19 × 300 mm, 10 µm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 8 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* **9** as a white amorphous solid (40 mg, 9% yield based on theoretical 0.1 mmol scale). 1,3-Dipolar cycloaddition of fluorophore CY-5 with **9** (10.5 mg, 2.3×10^{-6} mol) was achieved using **Method 12b**. The crude **10** 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* **10** as a blue amorphous solid (3 mg, 26% yield, t_R = 32.9 min, > 99% purity); (**Figure SC1** and **SC2**).

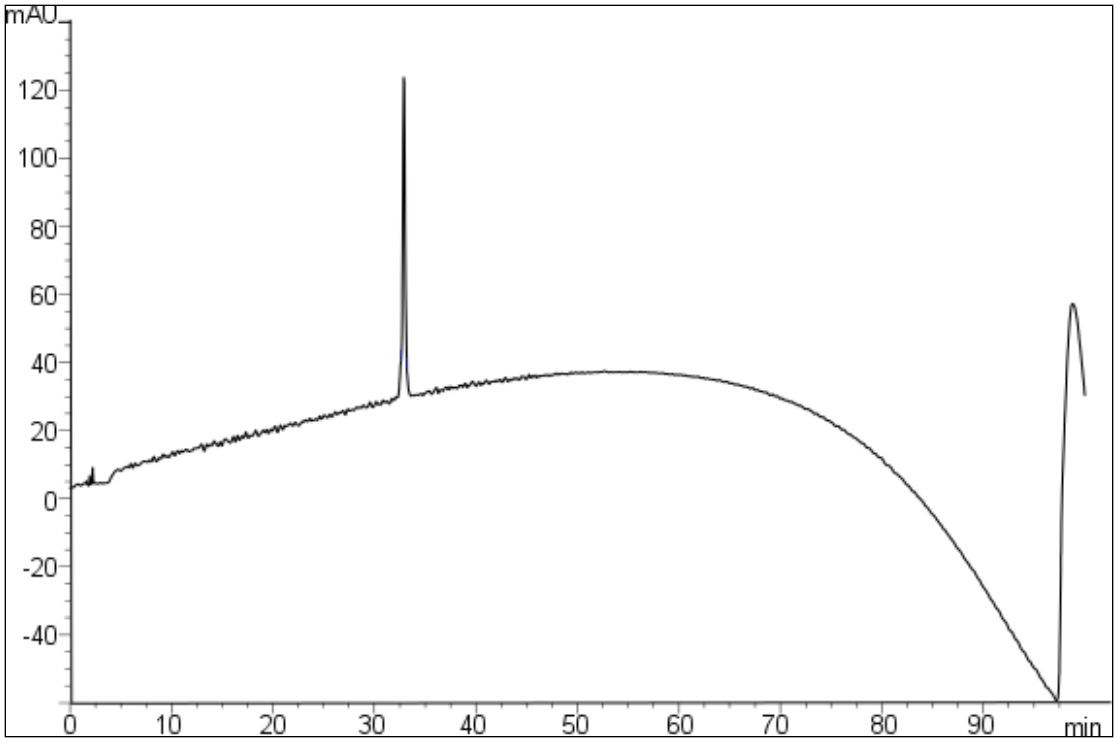


Figure SC1: Analytical RP-HPLC chromatogram of purified peptide **10**, t_R = 32.9 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 μm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 1% B per min at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

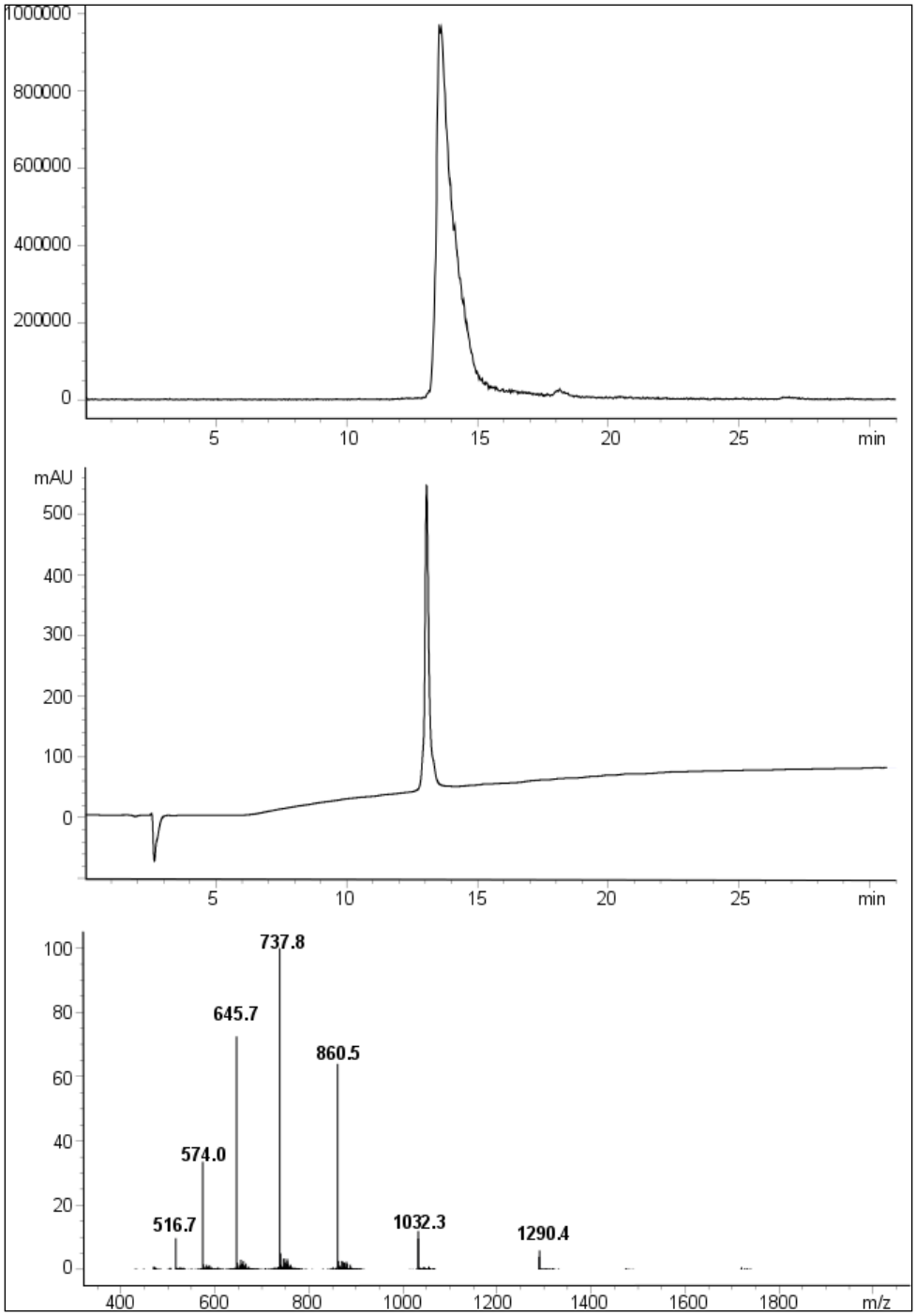
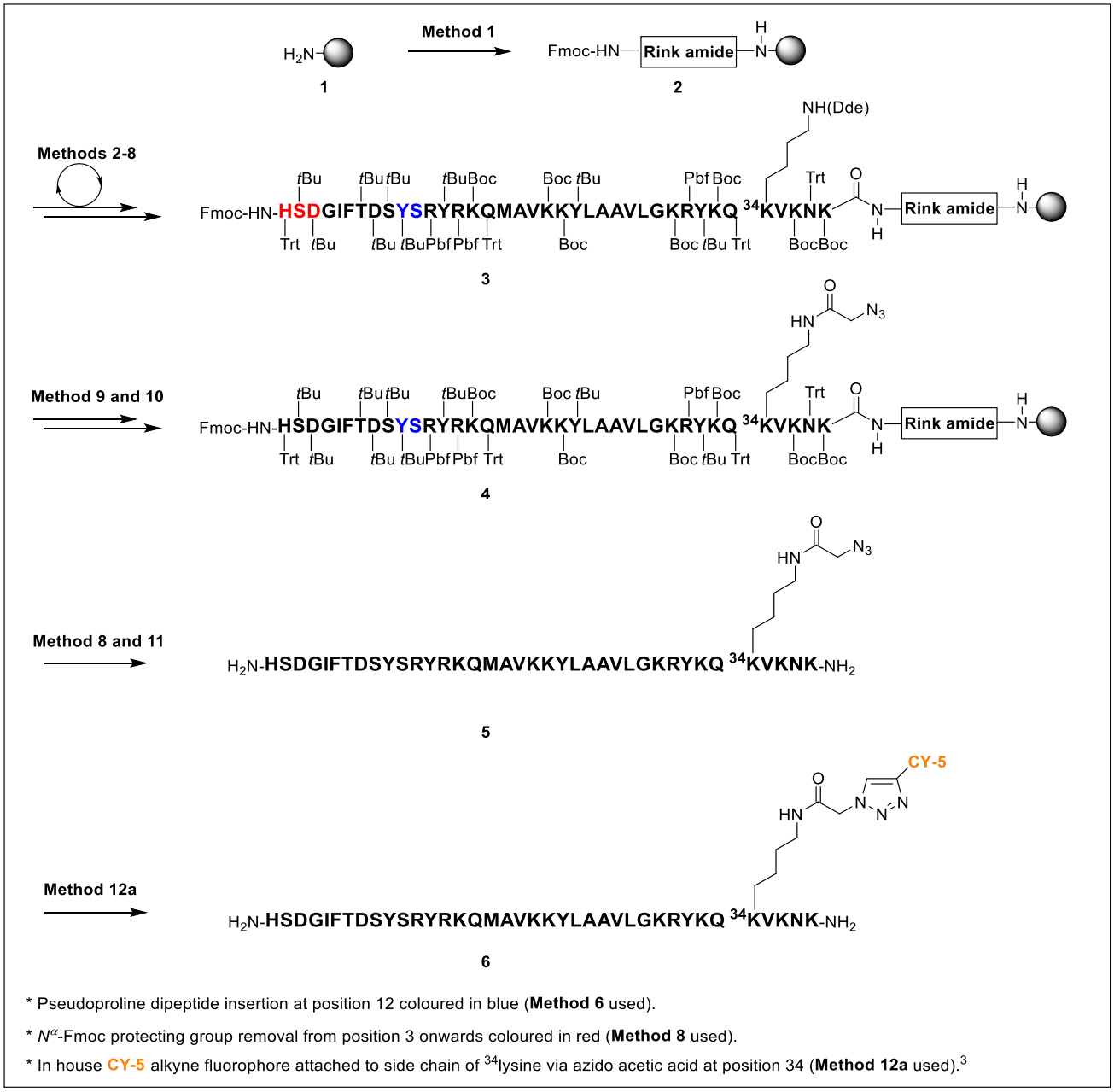


Figure SC2: LC-MS profile of purified peptide **10**; ion polarity positive operating at a nominal accelerating voltage of 200 eV. ESI-MS (m/z $[M+4H]^{4+}$ calcd: 1290.7; found: 1290.4; $[M+5H]^{5+}$ calcd: 1032.8; found: 1032.3; $[M+6H]^{6+}$ calcd: 860.8; found: 860.5; $[M+7H]^{7+}$ calcd: 738.0; found: 737.8; $[M+8H]^{8+}$ calcd: 645.9; found: 645.7; $[M+9H]^{9+}$ calcd: 574.2; found: 574.0; $[M+10H]^{10+}$ calcd: 516.9; found: 516.7. Mass deconvolution calculated at 5157.22 Da with standard deviation of 0.46; theoretical mass calculated at 5159.09 Da.

Synthesis of [Cy5^{34}]PACAP-38 using Fmoc-SPPS.

Scheme SC2. Synthesis of [Cy5^{34}]PACAP-38.



* Pseudoproline dipeptide insertion at position 12 coloured in blue (**Method 6** used).

* N^{α}-Fmoc protecting group removal from position 3 onwards coloured in red (**Method 8** used).

* In house CY-5 alkyne fluorophore attached to side chain of ^{34}lysine via azido acetic acid at position 34 (**Method 12a** used).[3]

Method 12a: 1,3-Dipolar cycloaddition via click chemistry:

To azido peptide **5** (5.3 mg, 1.1 × 10^{-6} mol) was added a solution of (6 M Gu·HCl/0.2 M Na_2HPO_4/20 mM TCEP/20 mM $CuSO_4{\cdot}5H_2O$) in H_2O (384 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 (1.1 mg, 1.9 × 10^{-6} mol, 1.7 equiv.) 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 **6** was immediately purified batchwise by semi-preparative RP-HPLC.

Synthesis of PACAP (1-38) CY-5 at 34 (**6**).

Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmoc-removal using **Method 2**. Direct attachment of Fmoc-Lys(Boc)-OH at position 38 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α}-Fmoc removals up to position ^{3}Asp. Subsequent N^{α}-Fmoc removals from position 3 to 1 utilised **Method 8**. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ^{12}Arg indicated in **Scheme SC2** using **Method 3**. 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**. Coupling of Fmoc-Tyr(*t*Bu)-Ser(Psi(Me,Me)pro)-OH dipeptide at position 12 was achieved using **Method 6**. Linear synthesis of the peptide was continued up to His at position 1 using appropriate **Methods** (**2-5**, **7-8**). Selective Dde removal of **3** was achieved using **Method 9** followed by coupling of 2-azidoacetic acid on the side-chain amine group of lysine at position 34 using **Method 10** affording peptide **4**. Peptide **4** was then subjected to a final Fmoc removal using **Method 8** and then liberated from resin using **Method 11** affording **5**. Crude **5** was purified batchwise by semi-preparative RP-HPLC on a Xterra® Prep MS C18 column, (19 × 300 mm, 10 µm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 8 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* **5** as a white amorphous solid (35 mg, 8% yield based on theoretical 0.1 mmol scale). 1,3-Dipolar cycloaddition of fluorophore CY-5 with **5** (5.3 mg, 1.1×10^{-6} mol) was achieved using **Method 12a**. The crude **6** 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* **6** as a blue amorphous solid (2.1 mg, 35% yield, t_R = 33.2 min, > 99% purity); (**Figure SC3** and **SC4**).

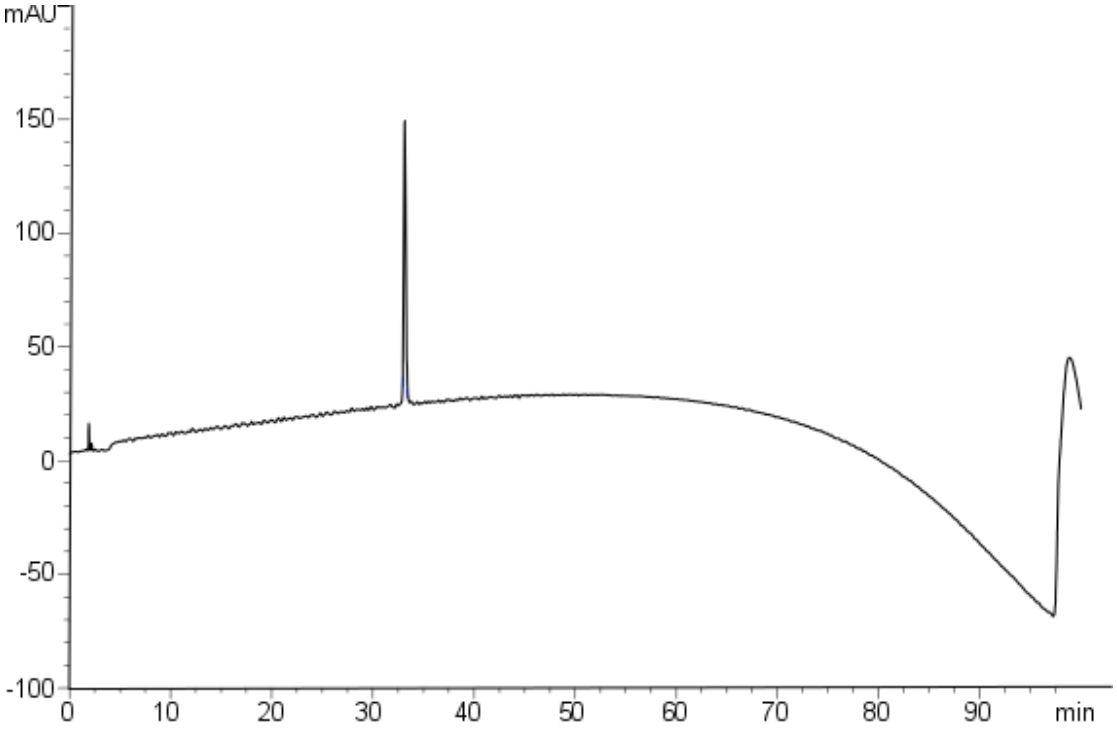


Figure SC3: Analytical RP-HPLC chromatogram of purified peptide **6**, t_R = 33.2 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 µm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 90 min at room temperature, *ca*. 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*).

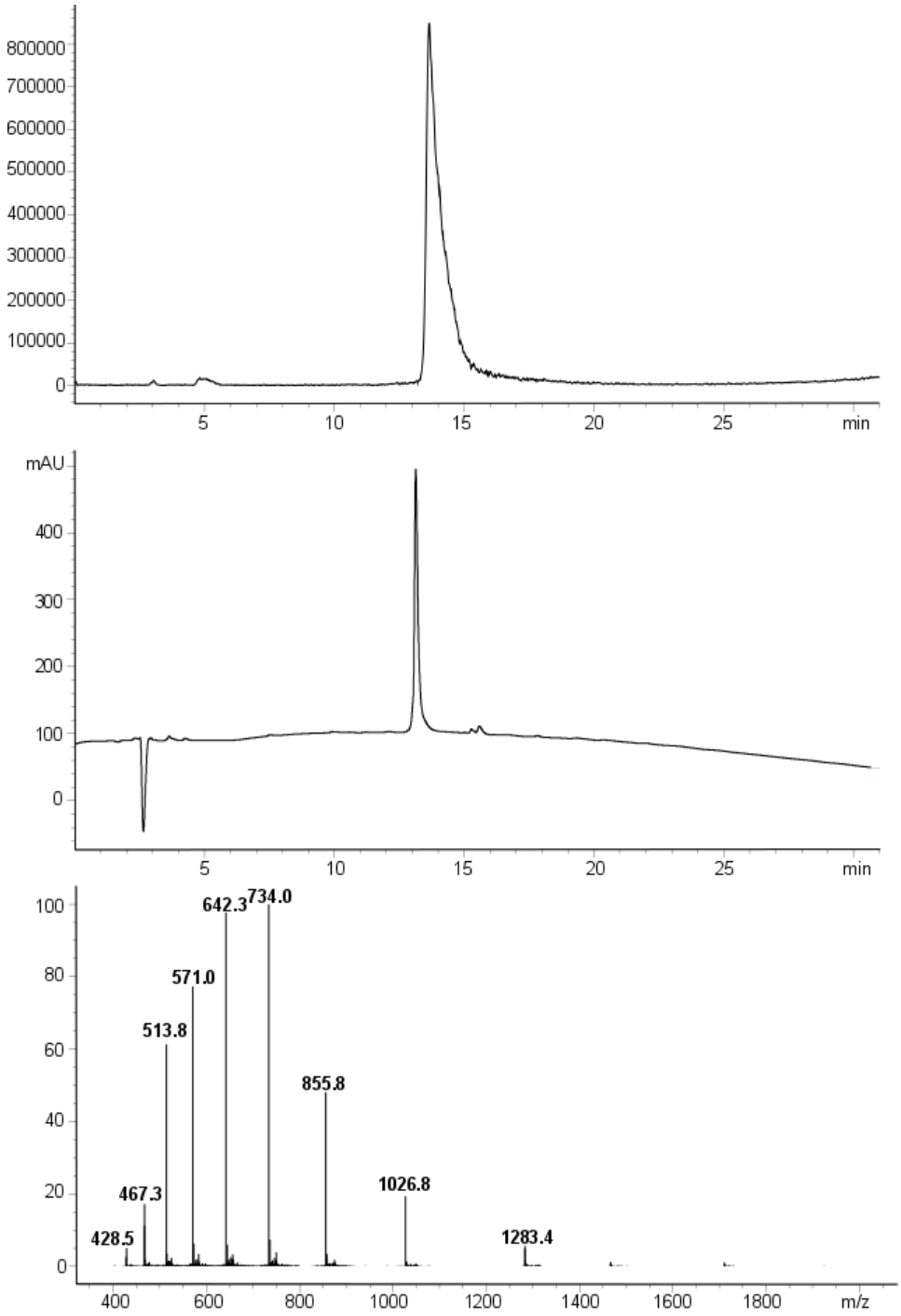
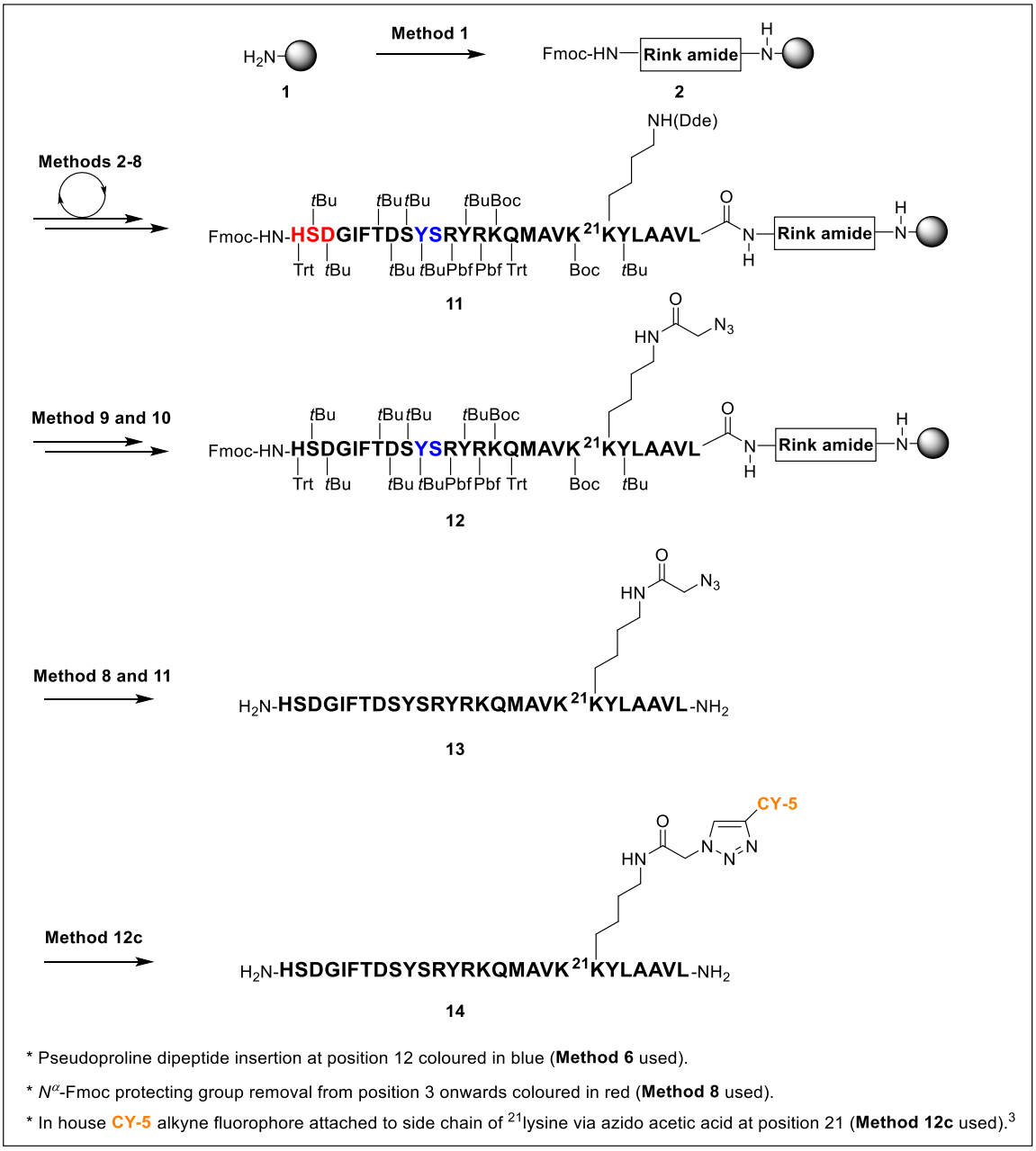


Figure SC4: LC-MS profile of purified peptide **6**; ion polarity positive operating at a nominal accelerating voltage of 200 eV. ESI-MS (*m/z* $[M+4H]^{4+}$ calcd: 1283.7; found: 1283.4; $[M+5H]^{5+}$ calcd: 1027.2; found: 1026.8; $[M+6H]^{6+}$ calcd: 856.2; found: 855.8; $[M+7H]^{7+}$ calcd: 734.0; found: 734.0; $[M+8H]^{8+}$ calcd: 642.4; found: 642.3; $[M+9H]^{9+}$ calcd: 571.1; found: 571.0; $[M+10H]^{10+}$ calcd: 514.1; found: 513.8; $[M+11H]^{11+}$ calcd: 467.5; found: 467.3; $[M+12H]^{12+}$ calcd: 428.6; found: 428.5. Mass deconvolution calculated at 5129.80 Da with standard deviation of 0.84; theoretical mass calculated at 5131.08 Da.

Synthesis of [Cy5^{21}]PACAP-27 using Fmoc-SPPS.

Scheme SC3. Synthesis of [Cy5^{21}]PACAP-27.



Method 12c: 1,3-Dipolar cycloaddition via click chemistry:

To azido peptide **13** (8.1 mg, 2.5 × 10^{-6} mol) was added a solution of (6 M Gu·HCl/0.2 M Na_2HPO_4/20 mM TCEP/20 mM $CuSO_4 \cdot 5H_2O$) in H_2O (835 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 (2.3 mg, 4.3 × 10^{-6} mol, 1.7 equiv.) 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 **14** was immediately purified batchwise by semi-preparative RP-HPLC.

Synthesis of PACAP (1-27) CY-5 at 21 (**14**).

Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmoc-removal using **Method 2**. Direct attachment of Fmoc-Leu-OH at position 27 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α}-Fmoc removals up to position ^{3}Asp. Subsequent N^{α}-Fmoc removals from position 3 to 1 utilised **Method 8**. Linear elongation of the peptide chain was achieved by coupling appropriate Fmoc-amino acids up to ^{12}Arg indicated in **Scheme SC2** using **Method 3**. 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**. Coupling of Fmoc-Tyr(*t*Bu)-Ser(Psi(Me,Me)pro)-OH dipeptide at position 12 was achieved using **Method 6**. Linear synthesis of the peptide was continued up to His at position 1 using appropriate **Methods** (**2-5**, **7-8**). Selective Dde removal of **11** was achieved using **Method 9** followed by coupling of 2-azidoacetic acid on the side-chain amine group of lysine at position 21 using **Method 10** affording peptide **12**. Peptide **12** was then subjected to a final Fmoc removal using **Method 8** and then liberated from resin using **Method 11** affording **13**. Crude **13** was purified batchwise by semi-preparative RP-HPLC on a Xterra® Prep MS C18 column, (19 × 300 mm, 10 µm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 8 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* **13** as a white amorphous solid (23 mg, 7% yield based on theoretical 0.1 mmol scale). 1,3-Dipolar cycloaddition of fluorophore CY-5 with **13** (8.1 mg, 2.5×10^{-6} mol) was achieved using **Method 12c**. The crude **14** 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* **14** as a blue amorphous solid (3.3 mg, 35% yield, t_R = 15.5 min, > 99% purity); (**Figure SC5-SC7**).

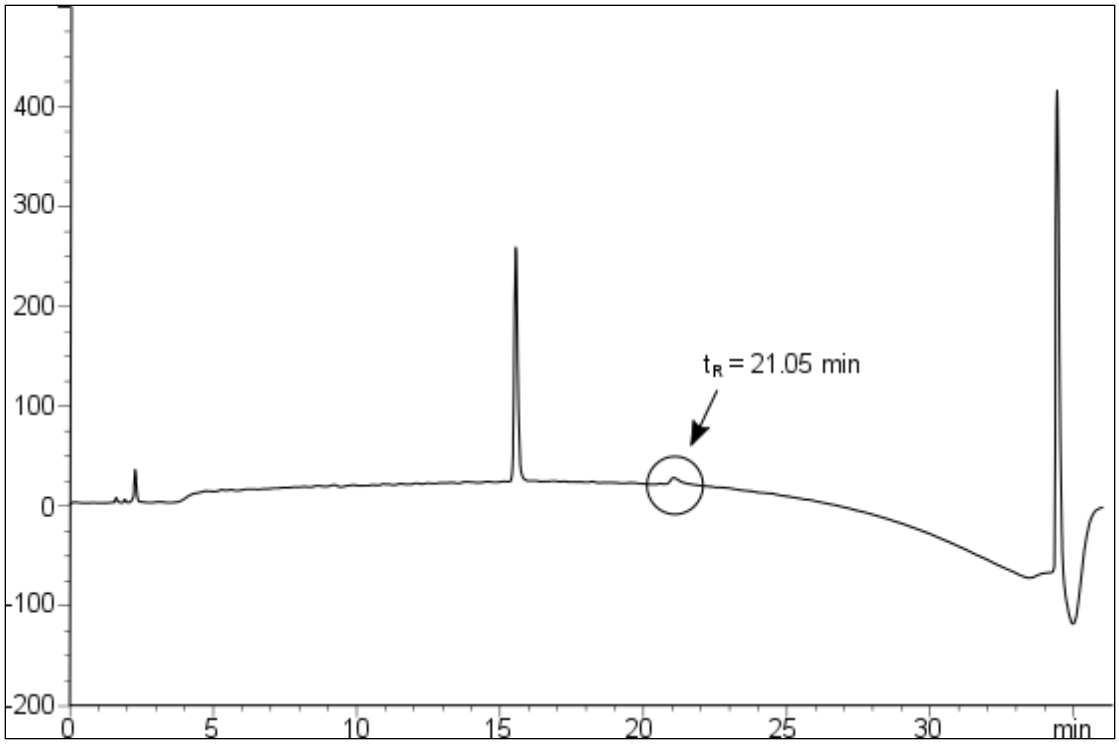


Figure SC5: Analytical RP-HPLC chromatogram of purified peptide **14**, t_R = 15.5 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 μm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 35 min at room temperature, *ca*. 3% 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*).

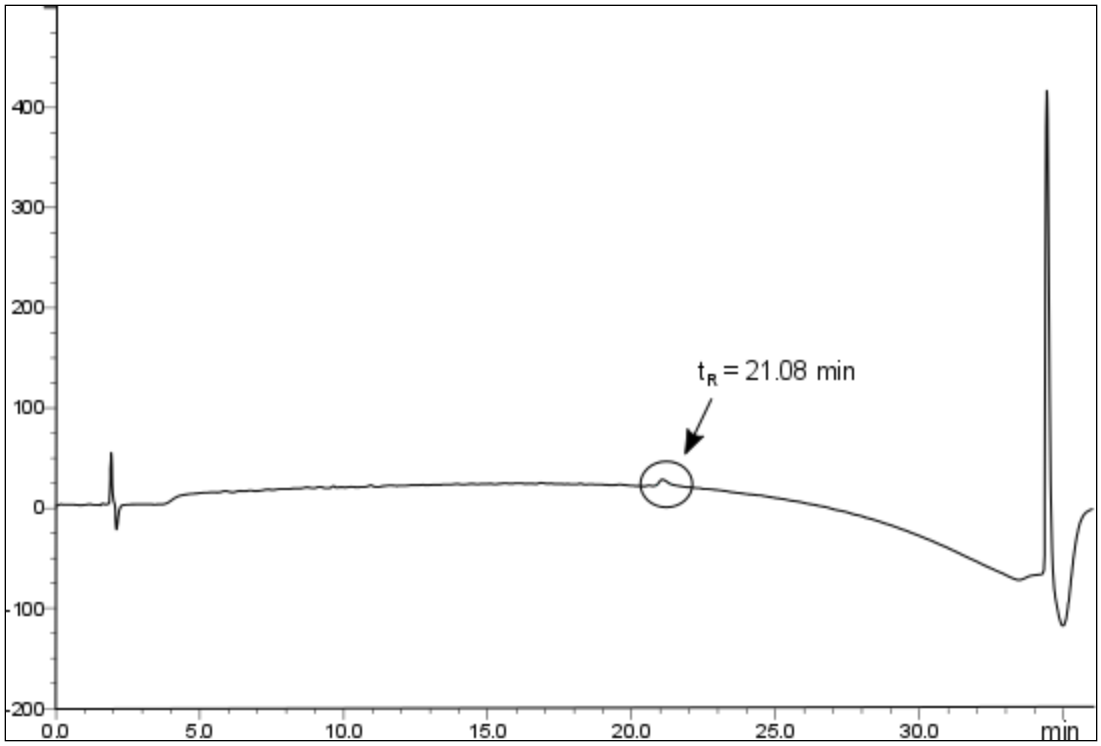


Figure SC6: Analytical RP-HPLC chromatogram of blank sample, column artifact present at t_R = 21.08 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 μm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 35 min at room temperature, *ca*. 3% 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*).

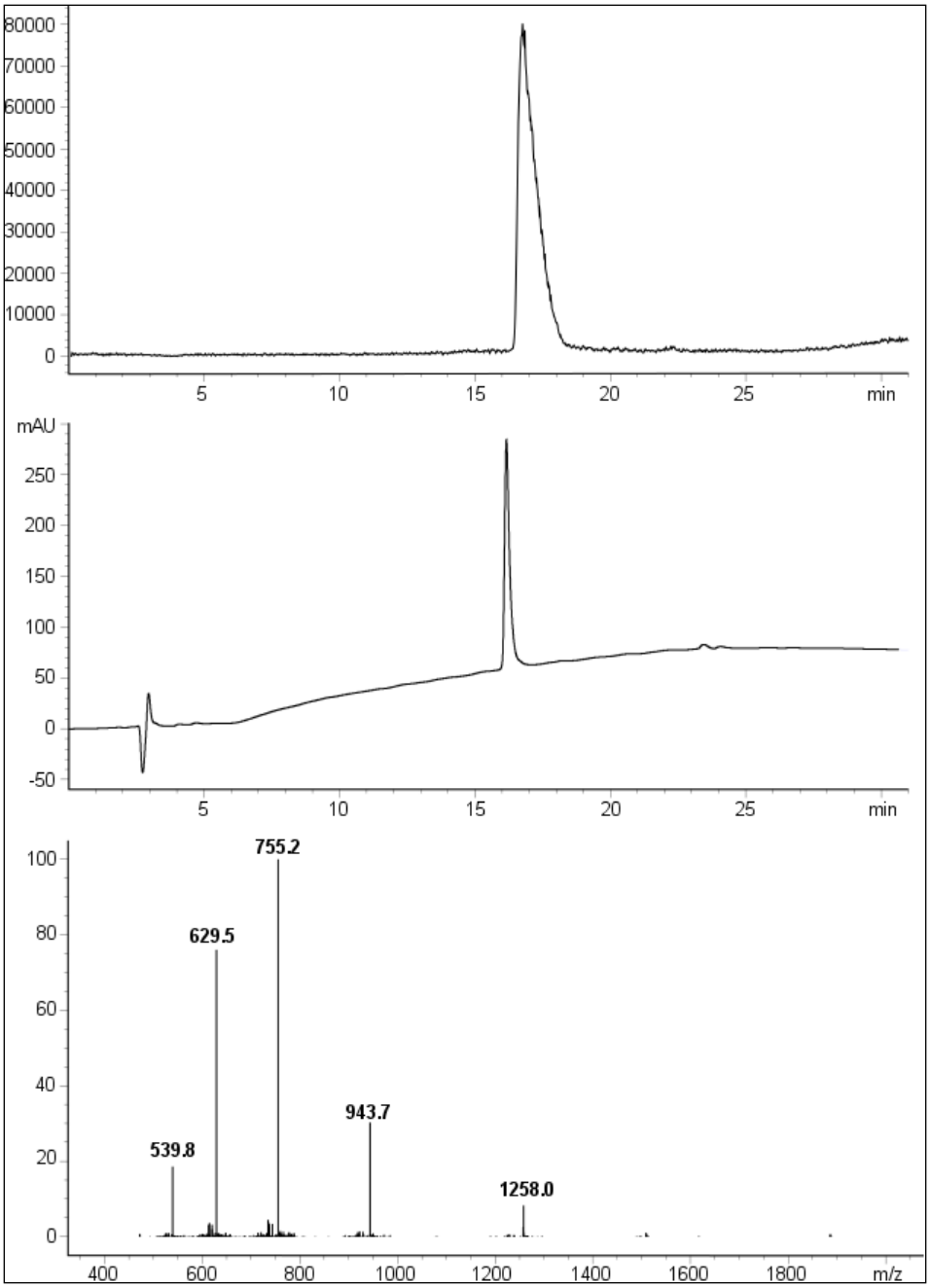
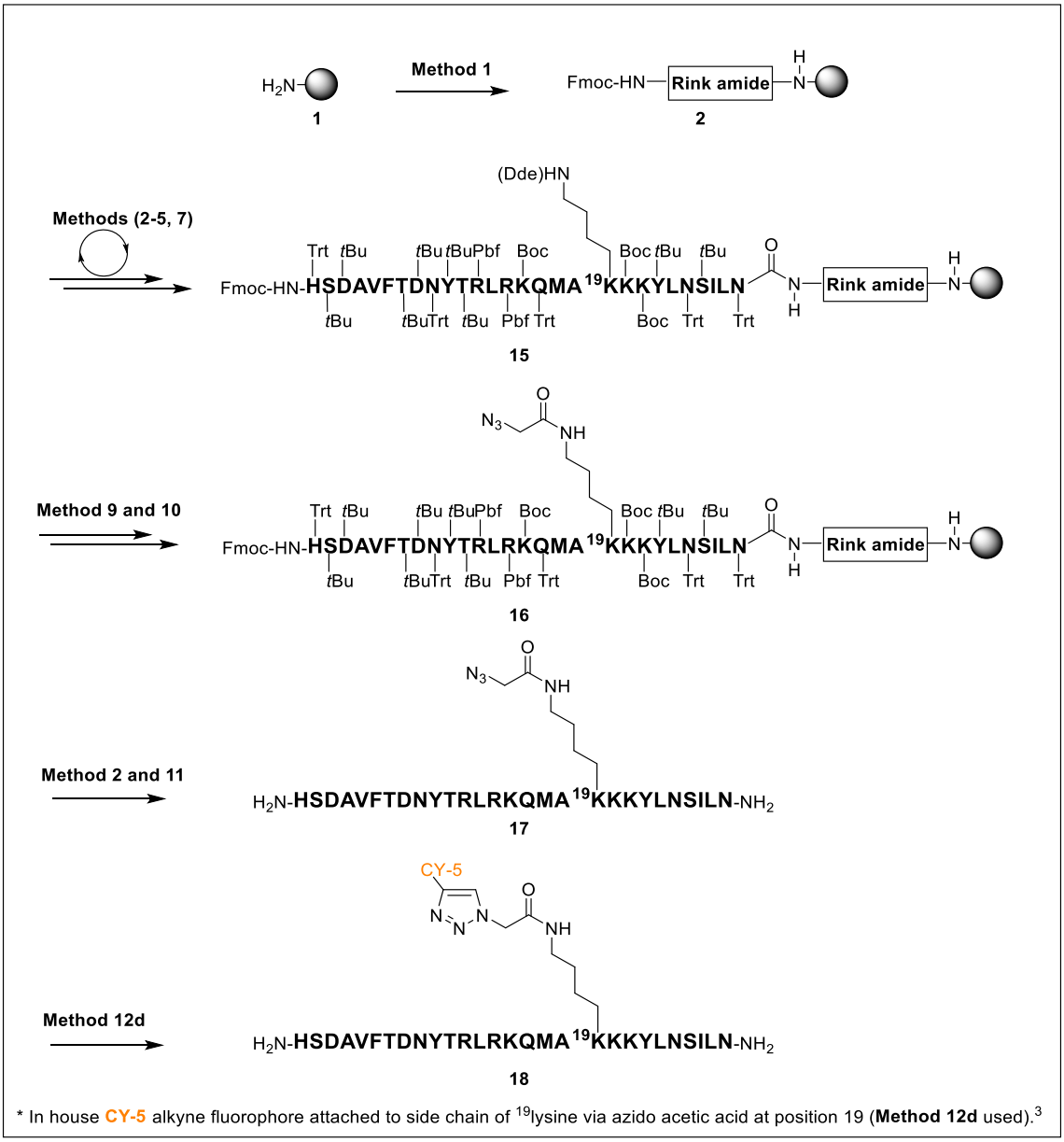


Figure SC7: LC-MS profile of purified peptide **14**; ion polarity positive operating at a nominal accelerating voltage of 200 eV. ESI-MS (*m/z* $[M+3H]^{3+}$ calcd: 1258.4; found: 1258.0; $[M+4H]^{4+}$ calcd: 944.1; found: 943.7; $[M+5H]^{5+}$ calcd: 755.5; found: 755.2; $[M+6H]^{6+}$ calcd: 629.7; found: 629.5; $[M+7H]^{7+}$ calcd 539.9; found: 539.8. Mass deconvolution calculated at 3771.08 Da with standard deviation of 0.30; theoretical mass calculated at 3772.42 Da.

Synthesis of [Cy5[19]]VIP using Fmoc-SPPS.

Scheme SC4. Synthesis of [Cy5[19]]VIP.



Method 12d: 1,3-Dipolar cycloaddition via click chemistry:

To azido peptide **17** (10 mg, 2.9 × 10^{-6} mol) was added a solution of (6 M Gu·HCl/0.2 M Na_2HPO_4/20 mM TCEP/20 mM $CuSO_4 \cdot 5H_2O$) in H_2O (970 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 (2.7 mg, 4.9 × 10^{-6} mol, 1.7 equiv.) 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 **18** was immediately purified batchwise by semi-preparative RP-HPLC.

Synthesis of the VIP CY-5 at 19 (**18**).

Fmoc-Rink amide was attached to aminomethyl polystyrene resin **1** using **Method 1** followed by Fmoc-removal using **Method 2**. Direct attachment of Fmoc-Asn(Trt)-OH at position 28 to resin bound Rink amide **2** was achieved using **Method 3**. **Method 2** was used for all subsequent N^{α}-Fmoc removals up to position ^{1}His. Linear synthesis of the peptide was continued up to His at position 1 using appropriate **Methods** (**2-5**, **7-8**) as indicated in **Scheme SC4**. 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**. Selective Dde removal of **15** was achieved using **Method 9** followed by coupling of 2-azidoacetic acid on the side-chain amine group of lysine at position 19 using **Method 10** affording peptide **16**. Peptide **16** was then subjected to a final Fmoc removal using **Method 8** and then liberated from resin using **Method 11** affording **17**. Crude **17** was purified batchwise by semi-preparative RP-HPLC on a Xterra® Prep MS C18 column, (19 × 300 mm, 10 μm) using a linear gradient of 5% to 95% over 90 min (*ca.* 1% B/min) with a flow rate of 8 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* **17** as a white amorphous solid (18.3 mg, 5% yield based on theoretical 0.1 mmol scale). 1,3-Dipolar cycloaddition of fluorophore CY-5 with **17** (10 mg, 2.9×10^{-6} mol) was achieved using **Method 12d**. The crude **18** 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* **18** as a blue amorphous solid (4 mg, 35% yield, t_R = 34.5 min, > 98% purity); (**Figure SC8** and **SC9**).

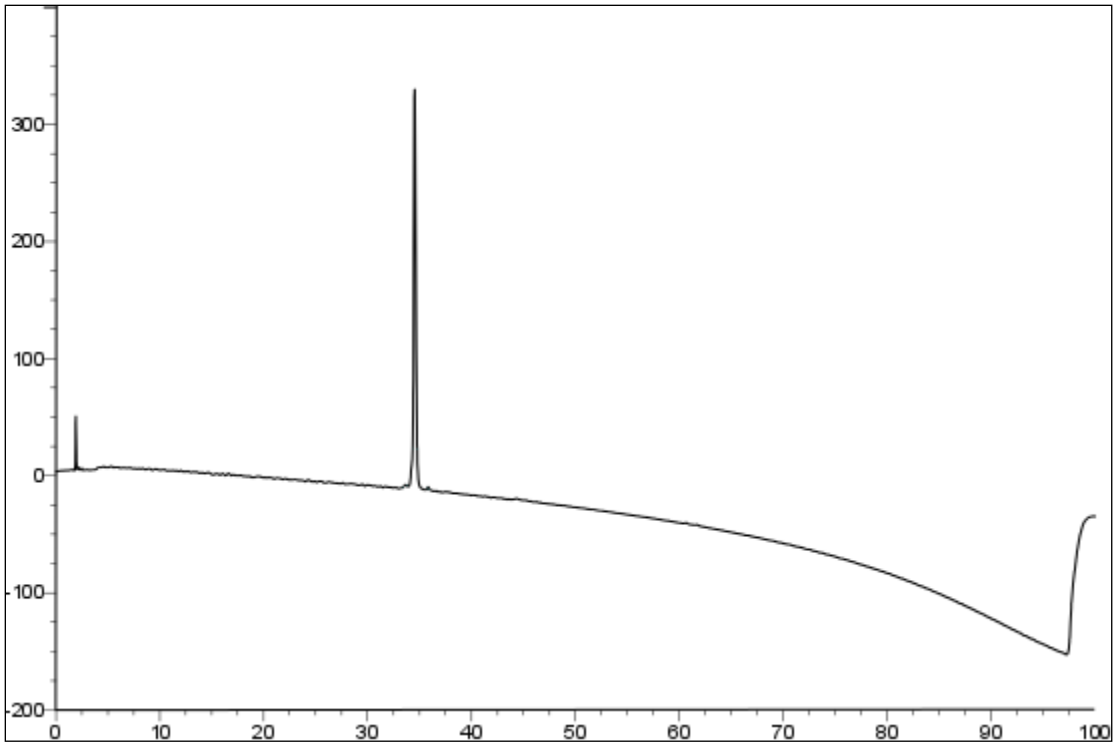


Figure SC8: Analytical RP-HPLC chromatogram of purified peptide **18**, t_R = 34.5 min. Chromatographic separations were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC using a XTerra® MS C-18 column (5 μm; 4.6 × 150 mm) and a linear gradient of 5-95% B in 95 min at room temperature, *ca*. 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*).

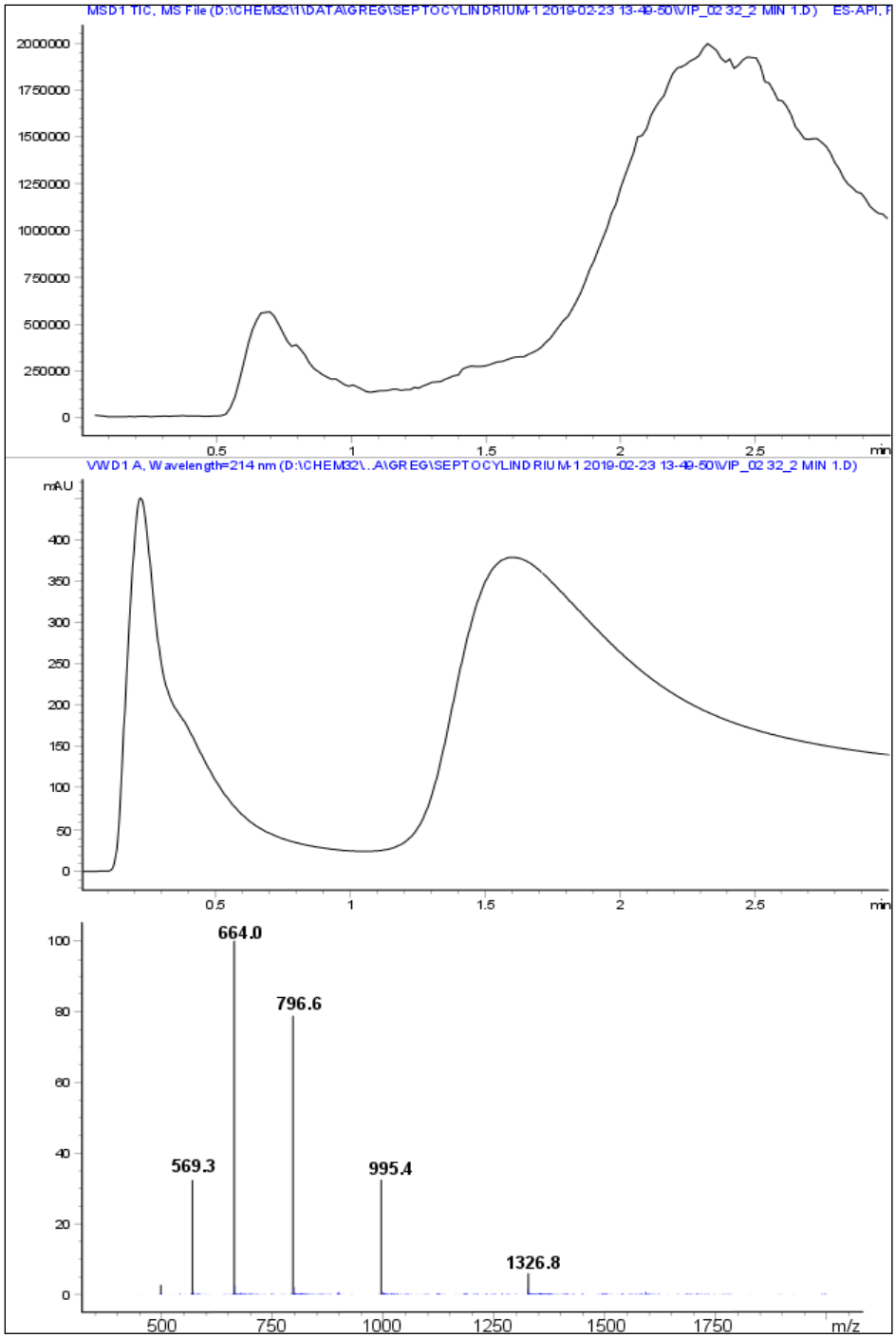


Figure SC9: FI-MS profile of purified peptide **18**; ion polarity positive operating at a nominal accelerating voltage of 70 eV. ESI-MS. (*m/z* $[M+3H]^{3+}$ calcd: 1327.5; found: 1326.8; $[M+4H]^{4+}$ calcd: 995.9; found: 995.4; $[M+5H]^{5+}$ calcd: 796.9; found: 796.6; $[M+6H]^{6+}$ calcd: 664.3; found: 664.0; $[M+7H]^{7+}$ calcd 569.5; found: 569.3. Mass deconvolution calculated at 3977.82 Da with standard deviation of 0.30; theoretical mass calculated at 3979.62 Da.

References

(1) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34(2)*, 595-598.
(2) Díaz-Mochón, J. J.; Bialy, L.; Bradley, M. *Org. Lett*. **2004**, *6*, 1127-1129.
(3) Zhao, C.; Fernandez, A.; Avlonitis, N.; Velde, V. G.; Bradley, M.; Read, N. D.; Vendrell, M. *ACS Comb. Sci.* **2016**, *18*, 689-696.