Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Enhancing the Gastrointestinal Stability of Salmon Calcitonin through Peptide Stapling

Hiba Ghareeb[1] and Norman Metanis[1,2,3,*]

[1]Institute of Chemistry, [2]The Center for Nanoscience and Nanotechnology, [3]Casali Center for Applied Chemistry, The Hebrew University of Jerusalem, Jerusalem 9190401, Israel.

Contents

1. Materials and Methods

All Fmoc-amino acids were obtained from CS Bio Co. (Menlo Park, CA) or Matrix innovation (Quebec City, Canada), with the following side chain protecting groups: Arg(Pbf), Asn(Trt), Gln(Trt), Glu(OtBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Tyr(tBu) and Cys(Trt). Fmoc-Glu(OAll)-OH, Fmoc-Lys(Alloc)-OH, and Fmoc-Lys(Tfa)-OH was obtained from Iris biotech and Fmoc-Sec(4-MeBzl)-OH was synthesised as reported previously.[1] TentaGel® R RAM resin (loading 0.19 mmol/g) was purchased from Rapp Polymere GmbH (Germany). *N,N,N',N'*-Tetramethyl-O-(6-chloro-1H-benzotriazol-1-yl)uronium hexafluorophosphate (HCTU), 1-[Bis(dimethylamino)methylene]-*1H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) and Ethyl cyano(hydroxyimino)acetate (OxymaPure) were purchased from Luxembourg Biotechnologies Ltd. (Rehovot, Israel). 2,2`-Dithiobis (5-nitropyridine) (DTNP), triisopropylsilane (TIPS), Diiodomethane, Hexafluoroisopropanol (HFIP), Phenylsilane, Tetrakis(triphenylphosphine) palladium ($Pd(PPh_3)_4$), and oxidized *L*-Glutathione (GSSG) were purchased from Merck (Jerusalem, Israel). All solvents: *N,N*-dimethylformamide (DMF), dichloromethane (DCM), acetonitrile (MeCN), *N,N*-diisopropylethyl amine (DIEA), piperidine (Pip), diethyl ether (Et_2O), Dimethyl sulfoxide (DMSO), formaldehyde (HCHO) and trifluoroacetic acid (TFA) were purchased from Bio-Lab (Jerusalem, Israel) and were peptide synthesis, HPLC or ULC-grade. Pepsin endopeptidase from porcine gastric mucosa and pancreatin from porcine pancreas was purchased from Sigma-Aldrich (Rehovot, Israel). Buffers for all the reactions were prepared by using MilliQ water (Millipore, Merck).

2. High Performance Liquid Chromatography (HPLC)

The analytical analyses and semi-preparative RP-HPLC were performed on a reverse-phase Waters Alliance HPLC with UV detector (220 nm and 280 nm) using an X-Bridge C4 column (300 Å, 3.5 µm, 4.6 ×150 mm) and C18 (3.5 µm, 130 Å, 4.6 x 150 mm). Preparative RP-HPLC was performed on a Waters LCQ150 system (XBridge C 4 column, 5µM, 19 x 250mm). Linear gradients of MeCN with 0.1% TFA (buffer B) and water with 0.1% TFA (buffer A) were used for all systems to elute peptides. The flow rates were 1 mL/min (analytical), 3.34 mL/min (semi-preparative) and 10 mL/min (C4 preparative).

3. Mass spectrometry (MS) and HR-MS

ESI-MS was performed on an LCQ Fleet Ion Trap mass spectrometer (Thermo Scientific) in the positive mode. The HR-MS were recorded on a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific) with a ESI source and 140'000 FWHM, a method that the AGC target was set to 1E6, and scan range was 400-2800 m/z. The raw data were deconvoluted using MagTran v1.03.

4. Experimental section

4.1. Peptide synthesis

General procedure for Fmoc-SPPS

Peptides were prepared by using an automated peptide synthesizer (CS136XT, CS Bio Inc. CA) typically on 0.25 mmol scale. Fmoc-amino acids (1 mmol in 5 mL DMF, 4 equiv) were activated with HCTU (1 mmol in 5 mL DMF, 4 equiv) and DIEA (2 mmol in 5 mL of DMF, 8 equiv) for 5 min and coupled for 25 min, with constant shaking. Fmoc deprotection step was carried out with 20% piperidine in DMF for 2 x 10 min, and DMF was used for washing the resin. Fmoc-Sec(4-MeBzl)-OH was coupled manually using DIC/OxymaPure activation method.[2] The resulting resins were washed with DMF (3×), DCM (3×) and dried.

The peptide was cleaved off resin using a TFA: triisopropylsilane (TIPS): H_2O (95:2.5:2.5) cocktail with 2 equiv DTNP for 2.5 h at room temperature. The cleavage mixture was filtered, and the resin was washed with TFA. The combined solutions were concentrated by N_2 bubbling, to which cold ether was added. The precipitated crude peptides were centrifuged, ether was removed, and the crude peptides were dissolved in MeCN: water (1:1) containing 0.1% TFA and water and lyophilized.

4.1.1 Synthesis of sCT

CSNLSTCVLG KLSQELHKLQ TYPRTNTGSG TP-NH2

The synthesis of sCT was carried out on Rink amid resin (0.19 mmol/g, 0.25 mmol scale) on an automated peptide synthesizer. The resin was swelled in DMF for 1 h and treated with 20% of piperidine for Fmoc deprotection. Following the SPPS the peptide was cleaved and deprotected using TFA: TIPS: water (95: 2.5: 2.5) cocktail to give 400 mg crude material (46%). The peptide

was oxidized by dissolving it in 20 mL phosphate buffer (100 mM, pH 8) in the presence of 5 equiv (64 mg) GSSG and then purified using RP-HPLC (C4 column) to give 80 mg sCT (20% yield), which was used to prepare MT-CT, KaY-1, and KaY-2 (Figure S1, S2).

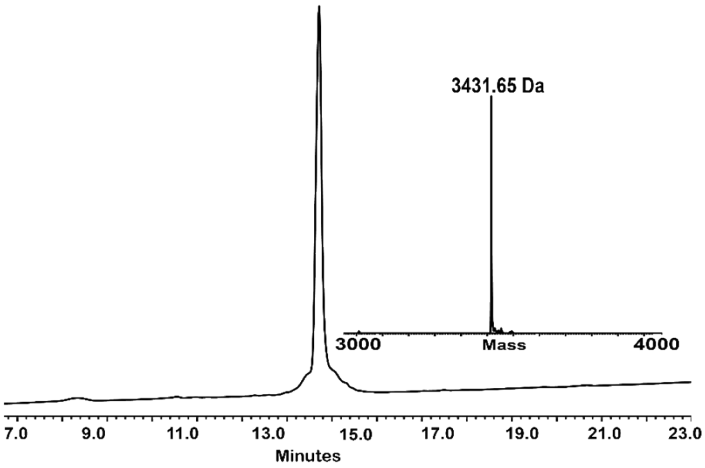


Figure S1. Characterization of sCT by HPLC and ESI-MS, obs. 3431.65 Da, calc. 3431.89 Da.

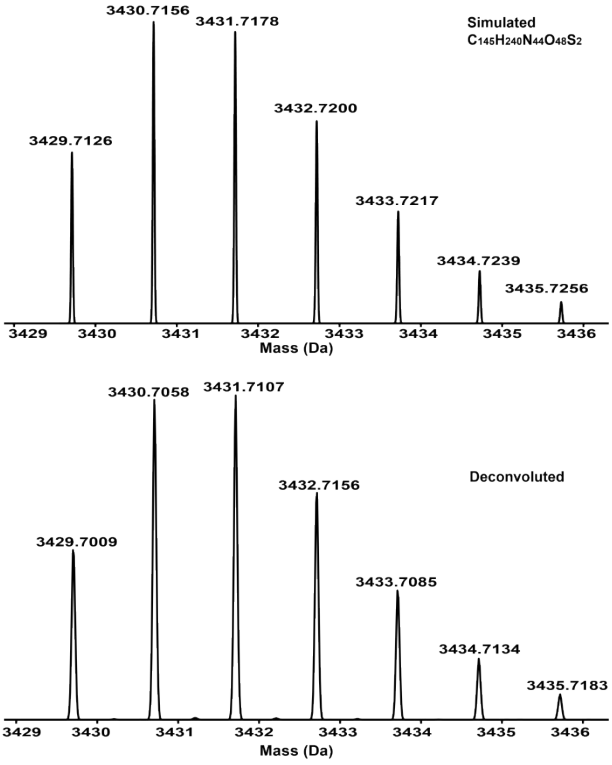


Figure S2. HR-MS analysis of sCT. *Top*: the simulated HR-MS of sCT with chemical formula $C_{145}H_{240}N_{44}O_{48}S_2$, and *bottom*: the experimental deconvoluted spectrum.

4.1.2 Synthesis of MT-CT

20 mg of sCT (2 mM) dissolved in 3 mL phosphate buffer (200 mM, 6 M Gn-HCl, pH 8) and 1.5 equiv TCEP were added to ensure it is fully reduced. After the reaction was completed (followed by HPLC and MS), 100 equiv diiodomethane (CH_2I_2) in DMSO were added, and the reaction was mixed overnight at 37 ºC. The reaction was quenched using 0.1% TFA in water and purified by Prep HPLC (XBridge C4 column, 5 μM, 19 x 250 mm) using a gradient of 25-50% for 55 min of solvent A in B (solvent A is ACN with 0.1%TFA, while B is water with 0.1%TFA), to give 12 mg, 60% isolated yield (Figure S3 and S4).

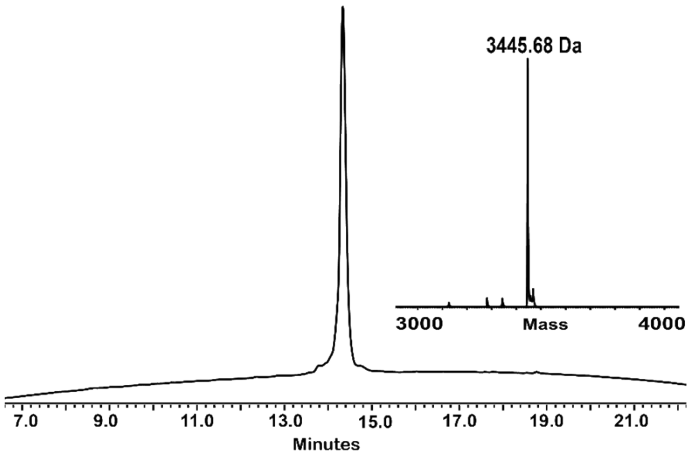


Figure S3. Characterization of MT-CT by HPLC and ESI-MS, obs. 3445.68 Da, calc. 3445.92 Da.

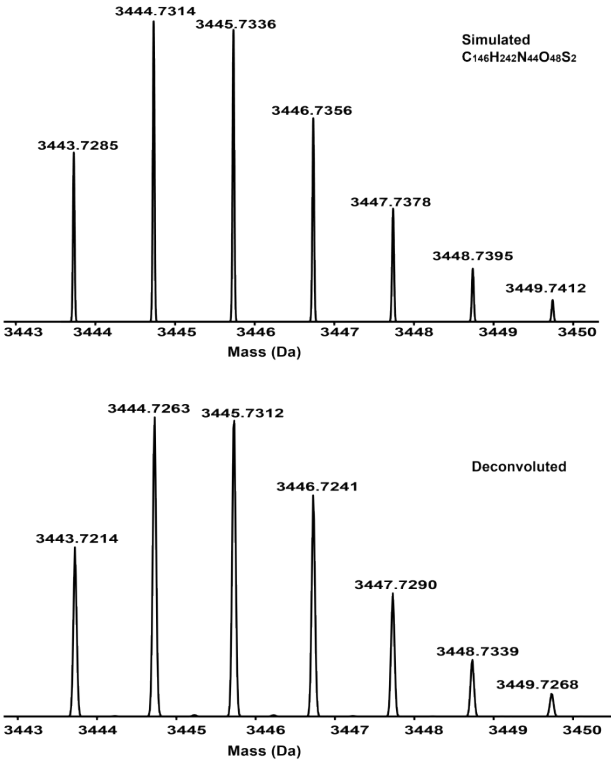


Figure S4. HR-MS analysis of MT-CT. *Top*: the simulated HR-MS of MT-CT with chemical formula $C_{146}H_{242}N_{44}O_{48}S_2$; *bottom*: the experimental deconvoluted spectrum.

4.1.3 Synthesis of Se-CT

The synthesis of Se-CT was carried out on Rink amid resin (0.19 mmol/g, 0.25 mmol scale) on an automated peptide synthesizer. The resin was swelled in DMF for 1 h and treated with 20% of piperidine for Fmoc deprotection, and Sec1 and Sec 7 were manually coupled using 2 equiv (166 mg) of Fmoc-Sec(4-MeBzl)-OH using 2 equiv of Oxyma pure (71 mg) with 3 equiv (93.3 μL) of DIC for 2 h. Subsequently, half of the peptide resin was cleaved using DTNP (2 equiv) in the cleavage cocktail TFA: TIPS: H_2O (95%: 2.5%: 2.5%) giving 200 mg crude peptide (45%), which were purified (same column and gradient as before), giving 50 mg (25% isolated yield) (Figure S5, S6).

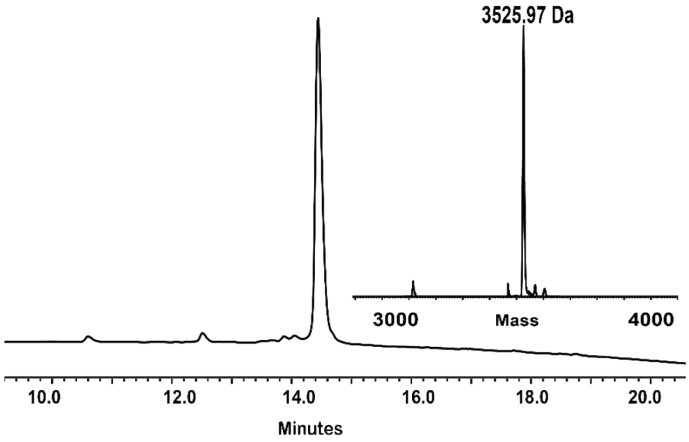


Figure S5. Characterization of Se-CT by HPLC and ESI-MS, obs. 3525.97 Da, calc. 3525.72 Da.

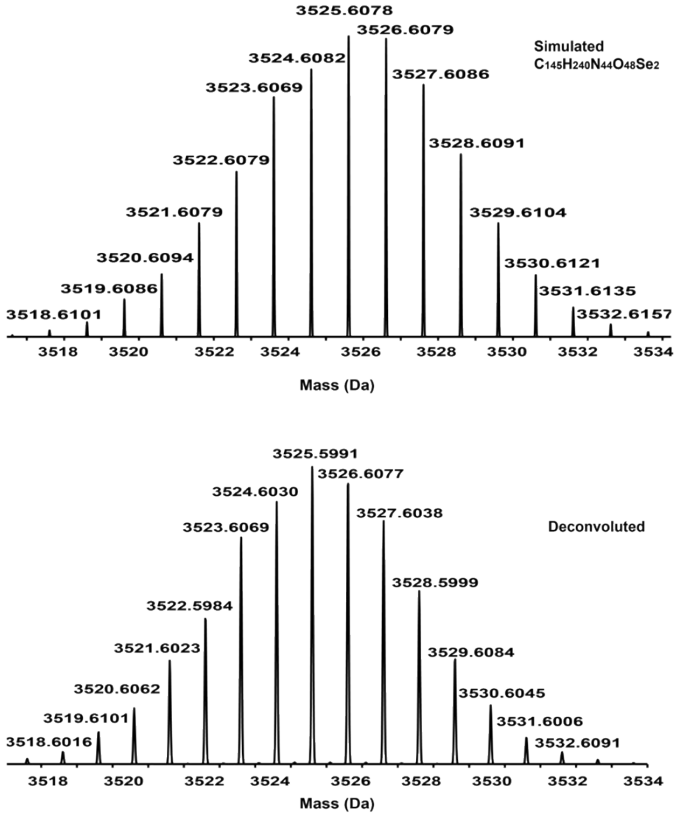


Figure S6. HR-MS analysis of Se-CT. *Top*: the simulated HR-MS of Se-CT with chemical formula $C_{145}H_{240}N_{44}O_{48}Se_2$; *bottom*: the experimental deconvoluted spectrum.

4.1.4 Synthesis of CT(E_{15}-K_{18})

The synthesis of CT(E_{15}-K_{18}) was carried out on Rink amid resin (0.19 mmol/g, 0.25 mmol scale) on an automated peptide synthesizer. The resin was swelled in DMF for 1 h and treated with 20%

of piperidine for Fmoc deprotection. Fmoc-Glu(OAll)-OH and Fmoc-Lys(Alloc)-OH were used at the site of cyclization, Glu15 and Lys18, respectively. When synthesis was completed (without removing the N-terminal Fmoc group), one third of the peptide-resin were taken and the Alloc/Ally groups were selectively removed by Pd-catalysis. For that, the resin was swelled in DCM for 30 min, then 20 equiv of $PhSiH_3$ and 0.35 equiv $Pd[PPh_3]_4$ in DCM were added and mixed for 2 x 3 h. Subsequently, the resin was washed with 0.5 M DIEA in DMF and 20 mM of sodium diethyldithiocarbamate in DMF and DCM for 3 x 2 min.[3] Next, the lactam formation between the liberated carboxyl and amine group using 5 equiv (160 mg) HATU and 10 equiv (110 µL) DIEA in 5 mL DMF (2 x 2 h), and the completion of the cyclization reaction was confirmed by ninhydrin test. Subsequently the N-terminal Fmoc was deprotected by 20% piperdine (2 x 5 mL) and the peptide-resin was cleaved. After lyophilization we obtained 30 mg of crude peptide which was oxidized using 5 equiv GSSG and then purified by Prep HPLC (same column and gradient) to yield 10 mg (33%) of in CT(E_{15}-K_{18}) (Figure S7, S8).

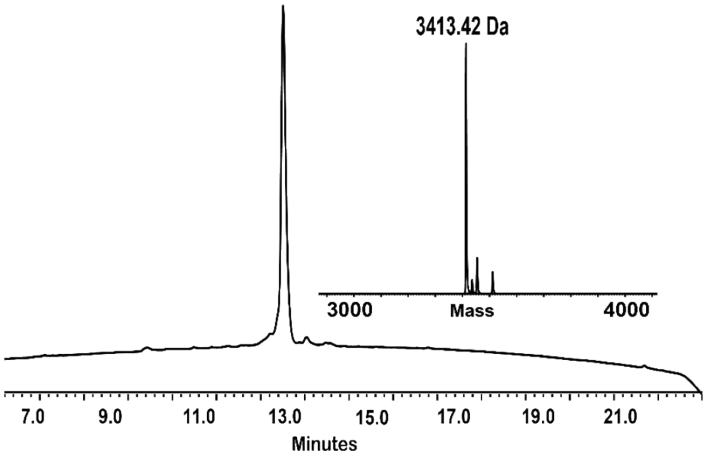


Figure S7. Characterization of pure CT(E_{15}-K_{18}) by HPLC and ESI-MS, obs. 3413.42 Da, calc. 3413.88 Da.

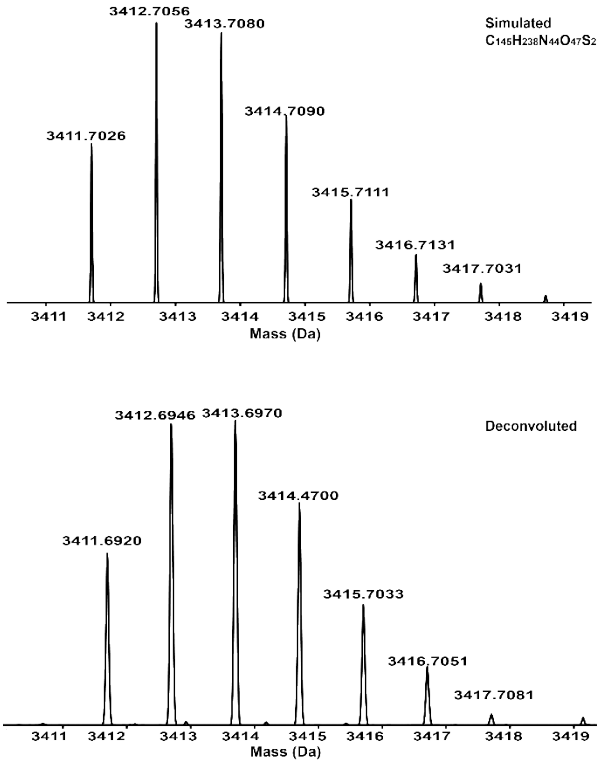
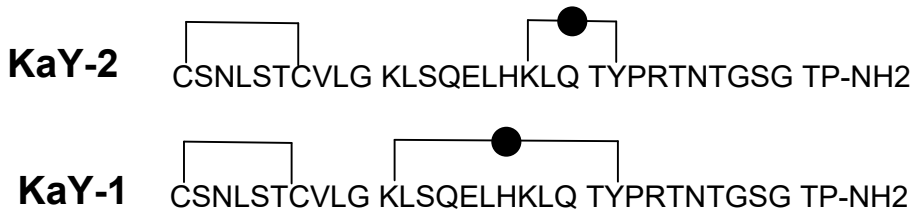


Figure S8. HR-MS analysis of CT(E15-K18). *Top*: the simulated HR-MS of CT(E15-K18) with chemical formula $C_{145}H_{238}N_{44}O_{47}S_2$; *bottom*: the experimental deconvoluted spectrum.

4.1.5 Synthesis of KaY-1, KaY-2



sCT (20 mg, 6 μmol, 1 equiv) was dissolved in 5 mL HFIP, and 3 equiv of HCOH and 3 equiv of DIEA and the reaction mixture was stirred at 37 ºC for 5 h. Then 20 μL of the mixture was taken and precipitated using ether. The precipitate was dissolved in H_2O: ACN (containing 0.1%TFA) and analyzed by HPLC and ESI-MS, the mass of KaY-1 (r.t. 8.9 min) and KaY-2 (r.t. 9.2 min) was 3443.63 Da, indicated the insertion of one methylene group between Tyr and the Lys residues. This synthesis provided 1 mg KaY-1 and 5 mg of KaY-2 (Figure S9, S10).

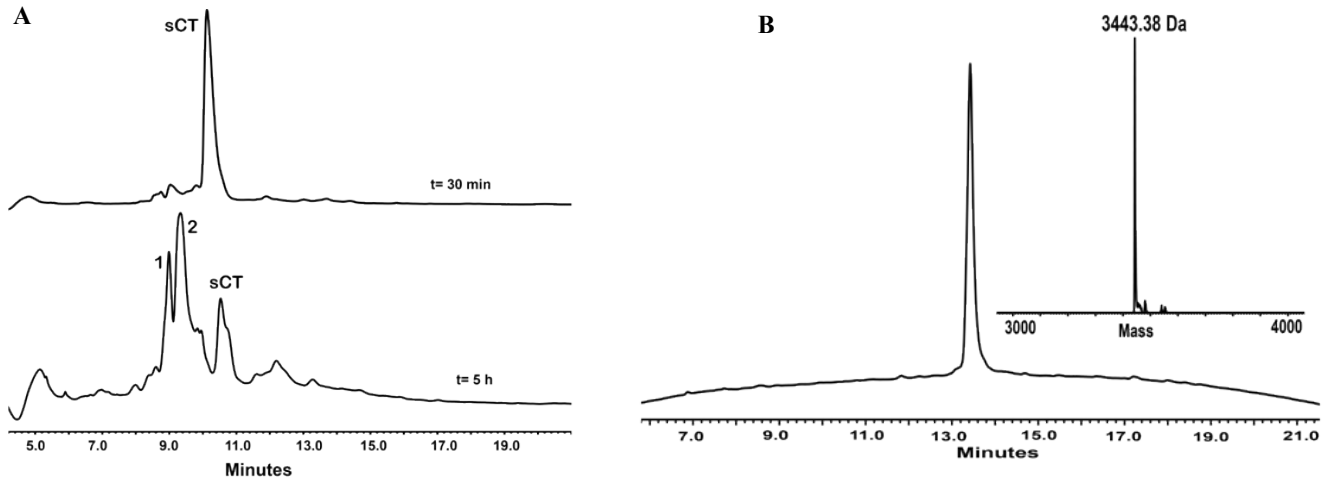


Figure S9. Characterization of KaY-1 and KaY-2 formation. (A) Analytical HPLC of the cross-linking reaction forming two main peaks labeled as 1 and 2, for KaY-1 and KaY-2, respectively. (B) Pure HPLC and ESI-MS of KaY-2, obs. 3443.38 Da, calc. 3443.91 Da, a different gradient was used (25-50% for 55 min of ACN with 0.1% TFA).

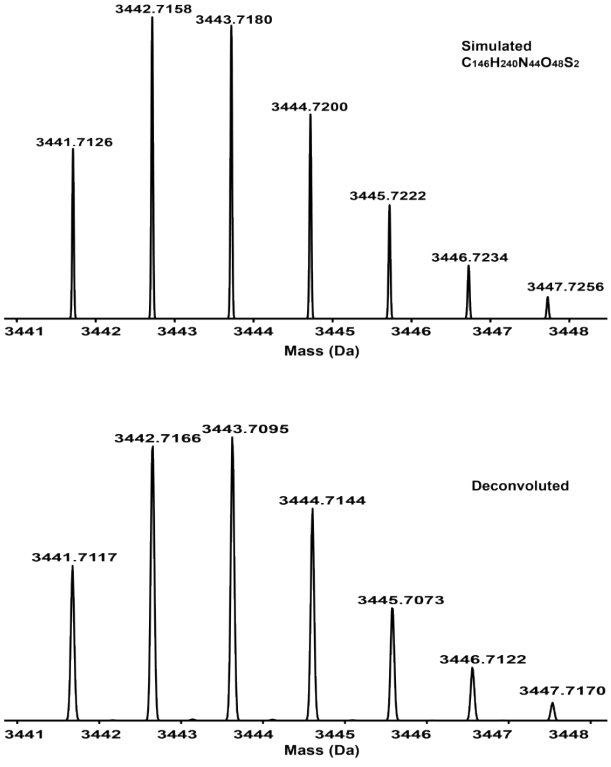


Figure S10. HR-MS analysis of KaY-2. *Top*: the simulated HR-MS of KaY-2 with chemical formula $C_{146}H_{240}N_{44}O_{48}S_2$; *bottom*: the experimental deconvoluted spectrum.

4.1.6 Synthesis of KaY-1

The synthesis of sCT(Lys18-Tfa) was carried out on Rink amid resin (0.19 mmol/g, 0.25 mmol scale) on an automated peptide synthesizer as with sCT (see above). For this synthesis, Lys18 was manually coupled using 2 equiv Fmoc-Lys(Tfa)-OH, activated with 2 equiv HATU and 4 equiv DIEA, and coupled for 2 h, while Lys11 was coupled with the standard Fmoc-Lys(Boc)-OH. Following the SPPS, half of the peptide-resin was cleaved and deprotected using TFA: TIPS: water (95: 2.5: 2.5) cocktail to give 200 mg (23%). Then 100 mg of the peptide was oxidized by dissolving it in 20 mL phosphate buffer (100 mM, pH 8) in the presence of 5 equiv (16 mg) GSSG (for 2 hours) and then purified using RP-HPLC (C4 column) to give 30 mg of pure sCT(Lys18-Tfa). Subsequently, the peptide was reacted with formaldehyde as described above, and after reaction completion, the Tfa protecting group was removed with 100 mM phosphate buffer pH 13 to give 5 mg KaY-1 (16% isolated yield). Figure S11, S12. Furthermore, we co-injected this peptide with peak 1 from previous synthesis with sCT, and recored its CD spectra. These results (Fig. S13) confirmed the identity of the peptide, i.e. the methylene bridge is between Lys11 and Tyr22.

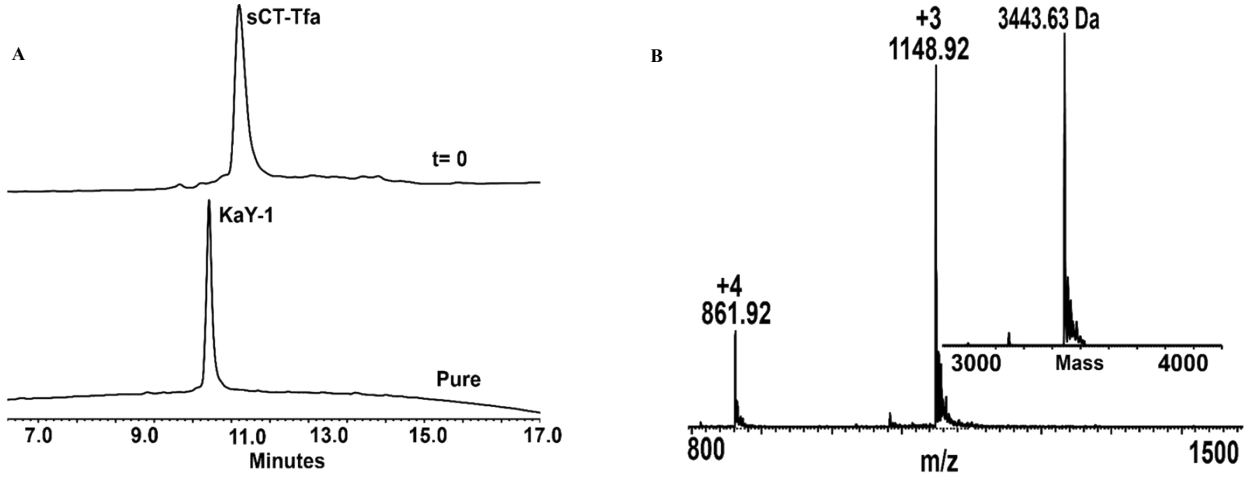


Figure S11. HPLC chromatograms for the KaY-1 stapling using 3 equiv HCOH, 3 equiv DIEA in HFIP [4]. (**A**) Stapling reaction on sCT with protected Lys18 with Tfa provided KaY-1. (**B**) ESI-MS of KaY-1, obs. 3443.38 Da, calc. 3443.91 Da, a different gradient was used (25-50% for 55 min of ACN with 0.1% TFA).

Figure S12. HR-MS analysis of KaY-1. *Top*: the simulated HR-MS of KaY-1 with chemical formula $C_{146}H_{240}N_{44}O_{48}S_2$; *bottom*: the experimental deconvoluted spectrum.

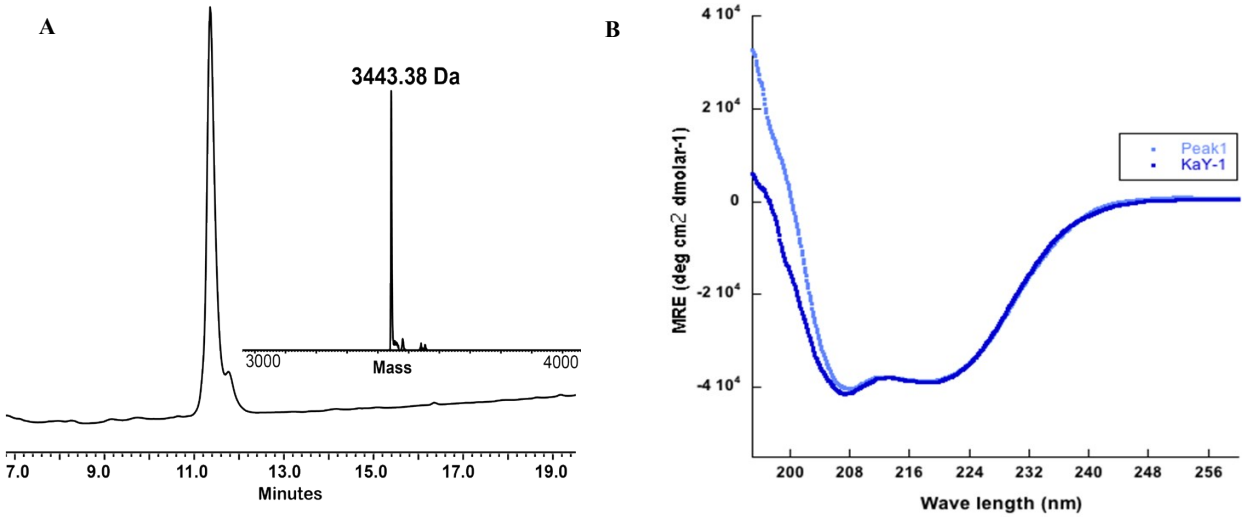
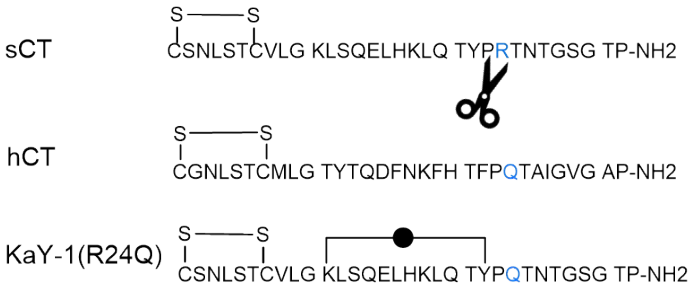


Figure S13. HPLC (**A**) and CD spectra (**B**) for the characterization of KaY-1. Co-injection of peak 1 from the reaction of sCT with 3 equiv HCOH, 3 equiv DIEA in HFIP[4] and the selective

preparation of KaY-1 using orthogonal protecting group (Tfa) on Lys18. These data confirm that KaY-1 has a methylene crosslink between Lys11 and Tyr22.

4.1.7 Synthesis of KaY-1(R24Q)

The synthesis of CT(Lys18-Tfa)(R24Q) was carried out on Rink amid resin (0.19 mmol/g, 0.25 mmol scale) on an automated peptide synthesizer, as the case of sCT(Lys18-Tfa). After peptide synthesis completion, half of the peptide-resin was cleaved and deprotected using TFA: TIPS: water (95: 2.5: 2.5) cocktail to give 280 mg (32%). Then 100 mg of the peptide was oxidized by dissolving it in 20 mL phosphate buffer (100 mM, pH 8) in the presence of 5 equiv (15 mg) GSSG and then purified using RP-HPLC (C4 column) to give 20 mg of pure CT(Lys18-Tfa)(R24Q). Subsequently, it was reacted with formaldehyde as described above, and after reaction completion, the Tfa protecting group was removed with 100 mM phosphate buffer pH 13 to give 2 mg (10% isolated yield) KaY-1(R24Q) (Figure S14, S15).



Scheme S1: Rational design of hybrid analogue KaY-1(R24Q). sCT has Arg residue at position 24, which we found to be one of the main cleavage sites by pancreatin enzymes,[5] so we decided to prepare a new analogue of KaY-1 in which Arg24 was replaced by glutamine found in hCT.

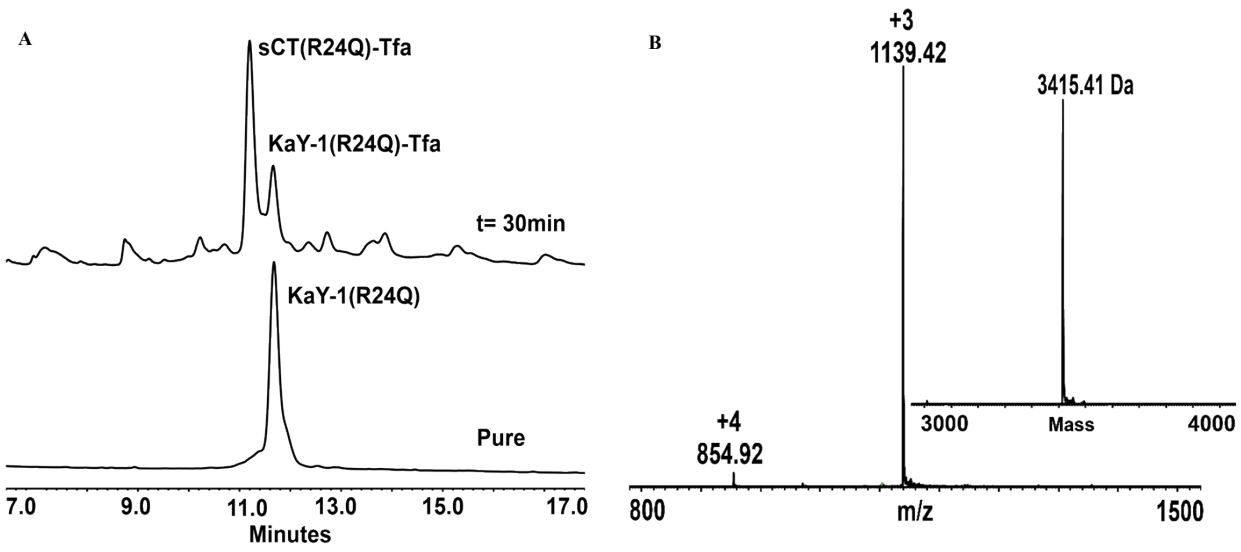


Figure S14. (**A**) Preparation of KaY-1(R24Q) starting from sCT(R24Q). (**B**) ESI-MS of KaY-1(R24Q), obs. 3415.41 Da, calc. 3415.91 Da, a different gradient was used (25-50% for 55 min of ACN with 0.1% TFA).

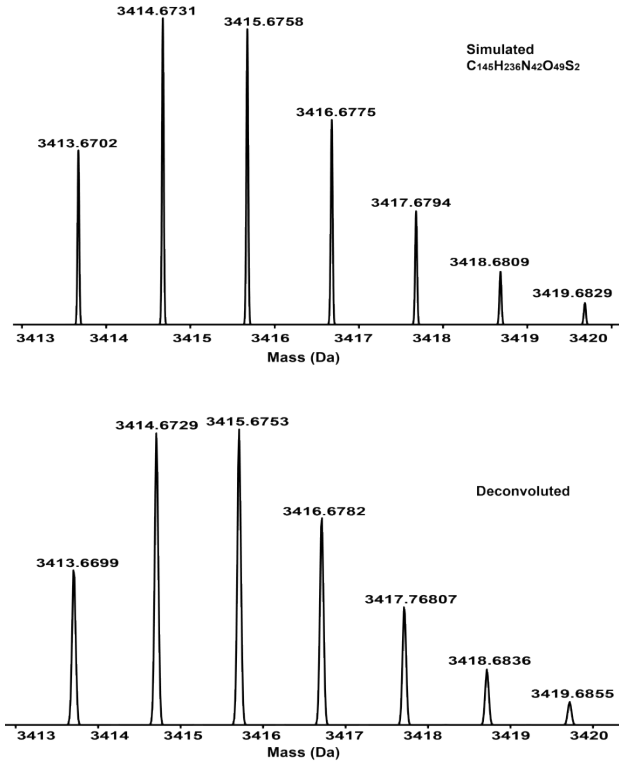


Figure S15. HR-MS analysis of KaY-1(R24Q). *Top*: the simulated HR-MS of KaY-1(R24Q) with chemical formula $C_{146}H_{240}N_{44}O_{48}S_2$; *bottom*: the experimental deconvoluted spectrum.

4.2. Circular Dichroism spectroscopy

Far ultraviolet spectra were obtained from 195-260 nm on J-180 spectropolarimeter (Jasco) using 10-30 μM of sCT and all analogues in 10 mM phosphate buffer pH 7 and in the presence of sodium dodecyl sulphate (SDS), which were left for 2 h to allow folding. Each spectrum represents the average of 3 scans, and θ_{MRE} was calculated according to the following equation

$$\theta_{MRE} = \frac{\theta(mdeg)}{10 \times l(cm) \times c(M) \times N(\#\ amide\ bond)}$$

Percentage helicity of peptides (Table 1) was calculated according to this equation:

$$\%helicity = 100 \times \left(\frac{\emptyset 222}{-39500 \times \left(1 - \frac{2.57}{n}\right)} \right)$$

. Where, n is the number of amino acids in the peptide.[6]

Table 1. Summary of % α-helicity for all analogues.

CT analogue	% α- Helicity
sCT	98.6
MT-CT	98.5
Se-CT	99.2
CT(E_{15}-K_{18})	62.9
KaY-2	56.2
KaY-1	93.7
KaY-1(R24Q)	97.4

4.3. Digestion of sCT and analogues in SGF

The stock solution of all analogues was prepared by dissolving 2 mg of the peptide in 1 mL water. The stock solution of pepsin enzyme was prepared by dissolving 5 mg of pepsin in 5 mL of 10 mM HCl (1 mg/mL), and then was diluted to a 0.2 mg/mL with 10 mM HCl. [7]

For degradation, 34 μL of pepsin (0.2 mg/mL) was added to 100 μL of peptide (2 mg/mL) at pH 1.3 and the reaction was incubated at 37 ºC. To monitor the degradation, 10 μL of the reaction aliquots were taken and quenched with 10 μL of 10 mM NaOH every 30 min for several hours and

analyzed by HPLC using XSelect C18 column (3.5 µm, 130 Å, 4.6 × 150 mm) with a gradient of 5-70% B (0.1% TFA in MeCN) over 20 min at 220 nm. The results are shown in Figure S16.

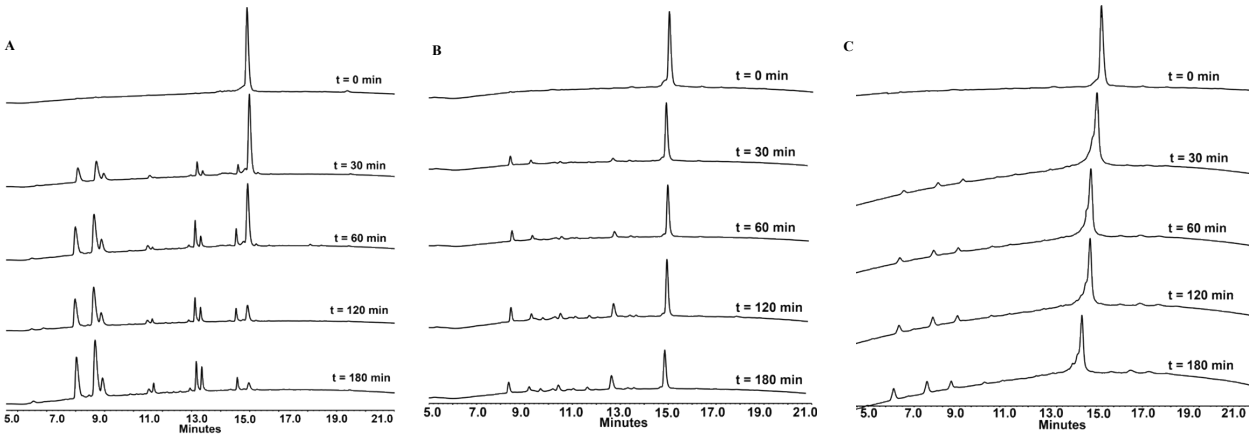


Figure S16. Degradation assay of (**A**) sCT, (**B**) KaY-1 and (**C**) KaY-1(R24Q), using stimulated gastric fluid (SGF).

4.4. Digestion of sCT KaY-1, and KaY-1(R24Q) in SIF

Simulated intestinal fluid was prepared by dissolving 68 mg KH_2PO_4 in 500 µL H_2O, 800 µL of 0.2 M NaOH and 10 mg porcine pancreatin. The volume was adjusted to 5 mL with water and the pH was adjusted to 6.8 to obtain 2 mg/mL. [7]

For degradation, 3.5 µL of pancreatin (2 mg/mL) was added to 100 µL of peptide (2 mg/mL) at pH 6.8 and the reaction was incubated at 37 ºC. To monitor degradation progress, 10 µL of the reaction aliquots were removed and quenched with 10 µL of 10 mM HCl every 30 min for several hours and were analyzed by HPLC using XSelect C18 column (3.5 µm, 130 Å, 4.6 × 150 mm) with a gradient of 5-70% B (0.1% TFA in MeCN) over 20 min at 220 nm. The results are shown in Figure S17.

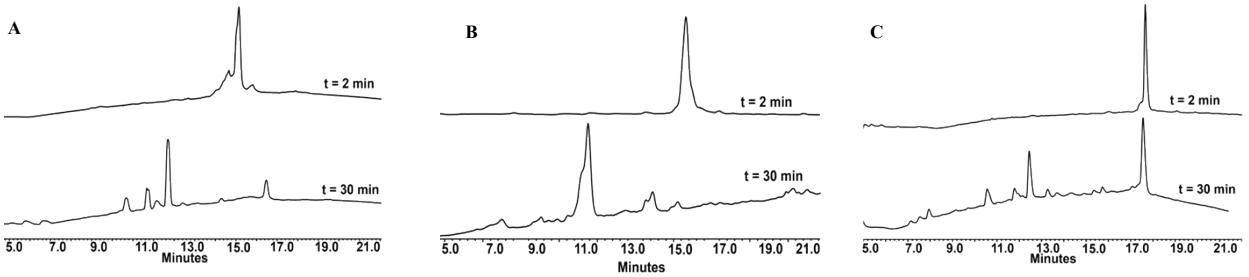


Figure S17. Degradation assay of (**A**) sCT (**B**) KaY-1 and (**C**) KaY-1(R24Q), using stimulated intestinal fluid (SIF). Cleavage of the proteins were observed over time.

4.5. Calcitonin receptor function assay, Agonist effect

This experiment was performed by Innoprot SL (Innovative technologies in biological systems, Dario, Spain) as follow: [8]

4.5.1. Reagents and Equipment

- U2OS Red $_{cAMP}$Nomad-CALCR (Innoprot P70521)
- HEK293 CALCR-HiTSeeker (Innoprot P30136)
- Human calcitonin (Sigma-Aldrich T3535)
- DMEM-F12 (Sigma-Aldrich D6421)
- DMEM high glucose (Sigma-Aldrich D6429)
- Opti-MEM (Thermo-Fisher scientific 31985070)
- FBS (Sigma-Aldrich F2442)
- PBS (Sigma-Aldrich D8537)
- Flat bottom black 96-well plates (Becton Dickinson 353219)
- Fluo-4 NW calcium assay kit (Thermo-Fisher scientific F36206)
- Synergie II Microplate reader (Biotek)

4.5.2. Compound dissolution

All test peptides were dissolved in DMSO at a final concentration of 1 mM. The working concentrations of each peptide were prepared in Opti-MEM for the cAMP assay or in the commercial kit buffer for the Ca^{2+} assay.

4.5.3 Methods

4.5.3.1 cAMP assay

- Day 1: U2OS Red $_{cAMP}$Nomad-CALCR cell line was thawed (2x106 cells per T25).
- Day 2: cells were plated into 96-well plates with a density of 20,000 cells (+/-2,000 cells) per well. Cells were maintained in DMEM-F12 medium supplemented with 10% FBS for 24 h at 37°C in a humidified 5% CO_2 atmosphere.
- Day 3: cells were incubated with the test compounds diluted in Opti-MEM overnight. Treatments were carried out in triplicate.
- Day 4: fluorescence intensity changes were quantified in living cells as follows.

- The medium was replaced with 100 µL of PBS to perform the fluorescence intensity acquisition. For tFP650 detection the filters were 590/20 and 665/8 nm for excitation and emission, respectively.
- GraphPad Prism 9 was used for EC_{50} calculation.

U2OS Red $_{cAMP}$Nomad-CALCR cell line was treated with human calcitonin and the test peptides. Figure S18, show the dose-response curves performed with the calcitonin and test compounds.

4.5.3.2 Ca^{2+} assay

- Day 1: HEK293 CALCR-HiTSeeker cell line was thawed (2x106 cells per T25).
- Day 2-4: Cells were maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere.
- Day 5: cells were plated into 96-well plates with a density of 40,000 cells (+/-2,000 cells) per well. Cells were maintained in DMEM medium supplemented with 10% FBS for 24 h at 37 °C in a humidified 5% CO2 atmosphere.
- Day 7: cells were incubated with the test compounds following calcium kit manufacturer´s instructions.
- GraphPad Prism 9 was used for EC_{50} calculation.

HEK293 CALCR-HiTSeeker cell line was treated with human calcitonin and the test compounds. Figure S19 show the dose-response curves performed in the experiments.

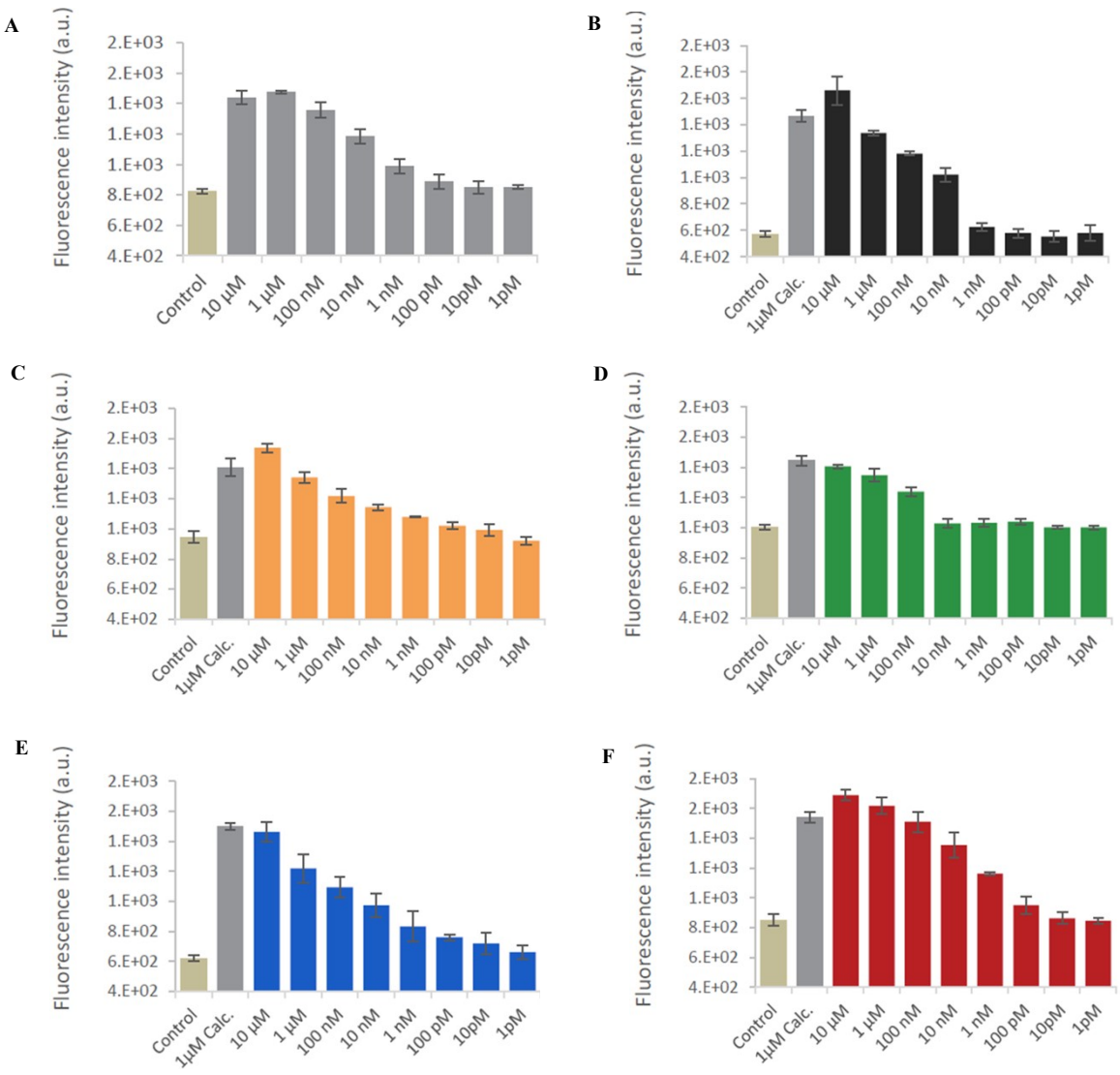


Figure S18 Dose-response curve for the tested peptides in the cAMP assay. Cells were treated with 1 µM calcitonin as positive control and the tested peptides at serial concentrations. (**A**) hCT, (**B**) sCT, (**C**) MT-CT, (**D**) Se-CT, (**E**) KaY-1, and (**F**) KaY-1(R24Q). Results are expressed as the fluorescence intensity (arbitrary units) of the red $_{cAMP}$Nomad biosensor. Data points represent the mean ± SD for each condition for a single experiment performed in triplicate.

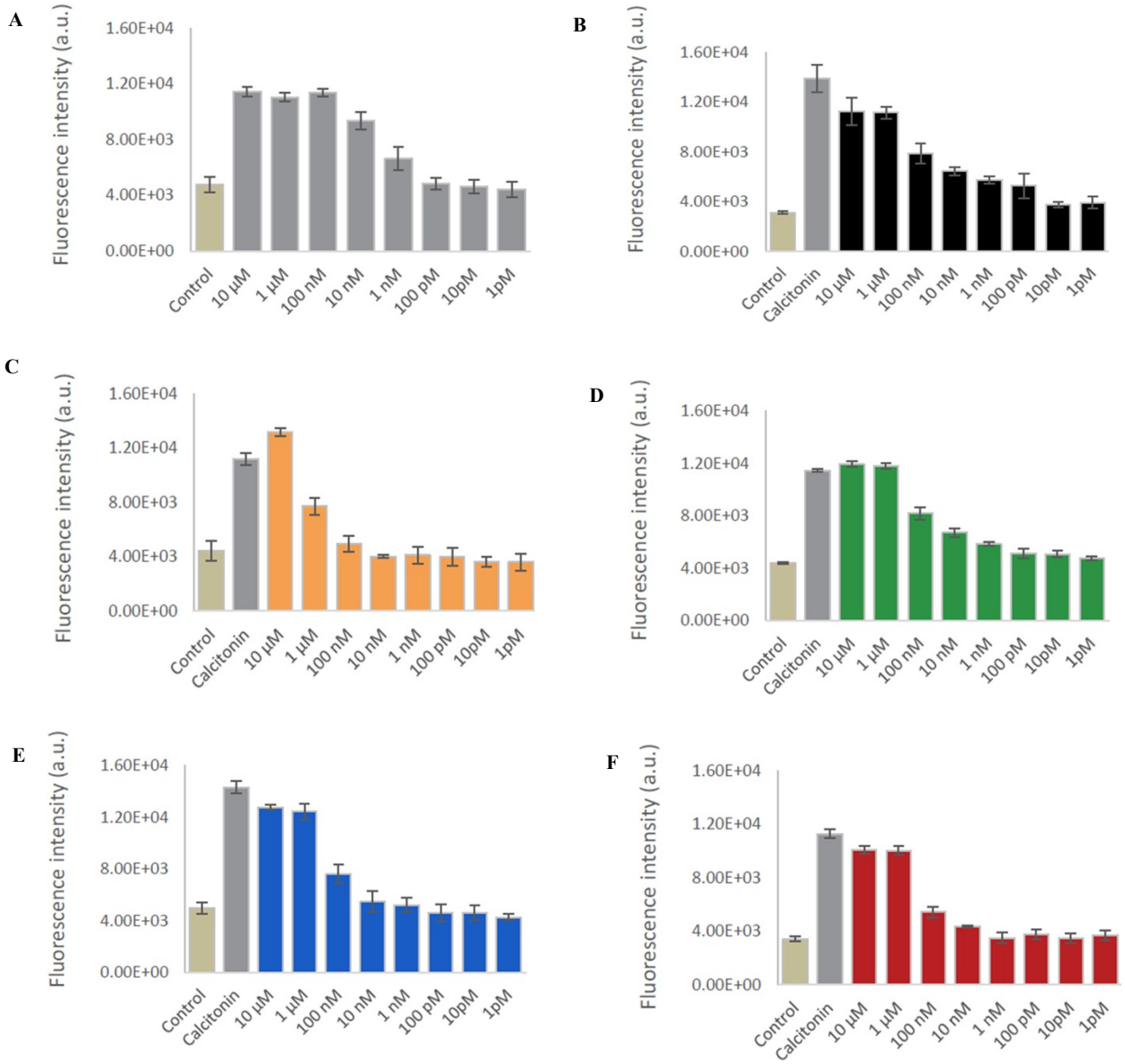


Figure S19. Dose-response curve for the test compounds in the Ca^{2+} assay. Cells were treated with calcitonin and analogues at serial concentrations. (**A**) hCT, (**B**) sCT, (**C**) MT-CT, (**D**) Se-CT, (**E**) KaY-1, and (**F**) KaY-1(R24Q). Results are expressed as the fluorescence intensity (arbitrary units) of the Fluo-4 calcium flux indicator. Data points represent the mean ± SD for each condition for a single experiment performed in triplicate.

References

(1) Quaderer, R.; Sewing, A.; Hilvert, D. Selenocysteine-mediated native chemical ligation. *Helvetica Chimica Acta* **2001**, *84* (5), 1197-1206.
(2) Reddy, P. S.; Dery, S.; Metanis, N. Chemical Synthesis of Proteins with Non-Strategically Placed Cysteines Using Selenazolidine and Selective Deselenization. *Angewandte Chemie* **2016**, *128* (3), 1004-1007.
(3) Thieriet, N.; Gomez-Martinez, P.; Guibé, F. Tandem deprotection-coupling of Nα-Alloc-amino acids by use of ternary systems Pd cat./PhSiH3/carboxy-activated amino acid. *Tetrahedron letters* **1999**, *40* (13), 2505-2508.
(4) Li, B.; Tang, H.; Turlik, A.; Wan, Z.; Xue, X. S.; Li, L.; Yang, X.; Li, J.; He, G.; Houk, K. N. Cooperative stapling of native peptides at lysine and tyrosine or arginine with formaldehyde. *Angewandte Chemie International Edition* **2021**, *60* (12), 6646-6652.
(5) Whitcomb, D. C.; Gorry, M. C.; Preston, R. A.; Furey, W.; Sossenheimer, M. J.; Ulrich, C. D.; Martin, S. P.; Gates Jr, L. K.; Amann, S. T.; Toskes, P. P. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nature genetics* **1996**, *14* (2), 141-145.
(6) Sommese, R. F.; Sivaramakrishnan, S.; Baldwin, R. L.; Spudich, J. A. Helicity of short E-R/K peptides. *Protein Science* **2010**, *19* (10), 2001-2005.
(7) Wang, J.; Yadav, V.; Smart, A. L.; Tajiri, S.; Basit, A. W. Toward oral delivery of biopharmaceuticals: an assessment of the gastrointestinal stability of 17 peptide drugs. *Molecular pharmaceutics* **2015**, *12* (3), 966-973.
(8) Aiyar, N.; Disa, J.; Stadel, J. M.; Lysko, P. G. Calcitonin gene-related peptide receptor independently stimulates 3′, 5′-cyclic adenosine monophosphate and Ca2+ signaling pathways. *Molecular and cellular biochemistry* **1999**, *197* (1), 179-185. Gee, K. R.; Brown, K.; Chen, W. U.; Bishop-Stewart, J.; Gray, D.; Johnson, I. Chemical and physiological characterization of fluo-4 Ca2+-indicator dyes. *Cell calcium* **2000**, *27* (2), 97-106. Clister, T.; Mehta, S.; Zhang, J. Single-cell analysis of G-protein signal transduction. *Journal of Biological Chemistry* **2015**, *290* (11), 6681-6688.