Generation of a Cell-Permeable Cycloheptapeptidyl Inhibitor against Peptidyl-Prolyl Isomerase Pin1

Walaa Bedewy, Hui Liao, Nageh A. Abou-Taleb, Sherif F. Hammad, Tamer Nasr, and Dehua Pei

Supplementary Information

Materials and General Methods. Reagents for peptide synthesis and Rink amide resin (100-200 mesh, 0.54 mmol/g) were purchased from Chem-Impex (Wood Dale, IL). Fmoc-D-Thr-(PO(OBzl)OH)-OH was purchased from NovaBiochem (La Jolla, CA). Cell culture media (DMEM) was purchased from Life Technologies. Fetal bovine serum and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA), while 0.25% trypsin-EDTA and DPBS were purchased from Sigma. The purity of the peptides was assessed by analytical HPLC and the identity was confirmed by MALDI-TOF mass spectrometric analyses on a Bruker UltrafleXtreme MALTI-TOF-TOF (Campus Chemical Instrument Center, The Ohio State University) instrument (unless noted otherwise).

Synthesis of Peptides 2-14. Each peptide was manually synthesized on 100 mg of Rink amide resin (0.54 mmol/g). The resin was swollen in DMF for 20 min and Fmoc group was then removed by treatment with 20% piperidine in DMF for 5 min (twice). 4-(4-Hydroxymethyl-3-methoxyphenoxy)butyric acid (HMPBA) (4 eq) was coupled using HBTU/HOBt/DIPEA (4, 4, 8 equiv, respectively) for 1 h. The resin was washed with DMF/DCM/Dry DCM 3 times each. Fmoc-Nal was coupled to HMPBA by incubating the beads with Fmoc-Nal-OH/DIC/4-dimethyaminopyridine (DMAP) (4, 4, 0.1 equiv, respectively) in dry DCM for 2 h (twice). The resin was washed with DMF/DCM/DMF, 3 times each. The next amino acid residues were coupled by standard Fmoc/HATU chemistry and the coupling reaction was monitored by ninhydrin test after each position. After coupling of Fmoc-D-pThr, any unreacted free amine was capped with acetic anhydride/DIPEA (15, 15 equiv.) in DCM for 15 min and the resin was washed with DCM and DMF 3 times each. After coupling the last amino acid residue, Fmoc group was removed by 20% piperidine. The resin was washed with DMF, DCM, and DMF (3 times each) and incubated with 1 M HOBt for 30 min. The linear peptides were released from the beads by treating the resin with 2% TFA and 2% triisopropylsilane (TIPS) in DCM for 1 h, followed by 2.5% TFA and 2% TIPS in DCM for 1 h (twice). The combined filtrates were evaporated and the peptide was cyclized in solution overnight using PyBOP/HOBt/DIPEA (10, 10, 30 equiv. respectively) in 300 mL of DCM. Next day, DCM was evaporated and the residue was treated with 95% TFA, 2.5% H_2O and 2.5% TIPS for 2 h. After evaporation of the solvents, the crude peptides were triturated with cold diethylether 3 times and purified by reversed-phase HPLC on a semi-preparative C_{18} column. The HPLC tracings and MS spectra for each peptide are shown in Fig. S1.

Synthesis of FITC-Labeled Peptides 15 and 16. In order to label peptides **4** and **6** with FITC, the D-pThr residue was replaced with a Gln and a dipeptide Asp-Lys was added to the Gln side chain. The resulting peptides (**15** and **16**, respectively) were synthesized on Rink amide resin (0.54 mmol/g) using Fmoc/HATU chemistry. Synthesis began with the attachment of the side chain of Fmoc-Glu-OAll to the resin. After completion of the linear sequence, the allyl group on the α-carboxyl group of D-Glu was removed by treating with tetrakis(triphenylphosphine)palladium/phenylsilane (0.5, 5 equiv., respectively) in DCM for 15 min (3 times). The Fmoc group on the N-terminal Fmoc-Pip-OH was removed by 20% piperidine. After washing with DMF, DCM, and

DMF (3 times each), the peptides were incubated with 1 M HOBt for 30 min. Peptide cyclization was effected by incubating the resin with PyBOP/HOBt/DIPEA (10, 10, 30 equiv. respectively) for 30 min (twice). The cyclic peptides were cleaved by treating with 92.5% TFA, 2.5% H_2O, 2.5% TIPS, and 2.5% dimethoxybenzene (DMB) for 3 h. After solvent evaporation, the crude peptides were triturated with cold diethylether and purified by HPLC. The purified peptide (~0.5 mg) was dissolved in 50 μL of DMSO and the pH was adjusted to ~9 with the addition of 1 M $NaHCO_3$. Ten μL of FITC (100 mg/mL) in DMSO was added to the peptide and the mixture was incubated for 1 h. The labeled peptide was purified again by reversed-phase HPLC on a C_{18} column. The HPLC tracings and MS spectra for each peptide are shown in Fig. S1.

IC_{50} Determination by Fluorescence Anisotropy. The competition assay was carried out by incubating 50 nM FICT-labeled peptide **1** with 100-400 nM MBP-Pin1 in PBS containing 2 mM TCEP for 30 min. The mixture was then added to serially diluted peptide (0-25 μM) and incubated for 90 min. The FA values were recorded on a Molecular Devices Spectramax M5 spectrofluorimeter, with excitation and emission wavelengths at 470 and 530 nm, respectively. The IC_{50} was determined by plotting FA values against the peptide concentration and data fitting using the four-parameter dose response inhibition equation (Prism 6, GraphPad).

Flow Cytometry. HeLa cells were seeded in 6-well plates at a density 1.5 x 10^5 cells per well overnight. Next day, 5 μM FITC-labeled peptide **15** or **16** was added in DMEM media supplemented with 1% FBS and 1% penicillin/streptomycin sulfate for 2 h. After 2 h, the media was removed, and the cells were washed with cold DPBS and harvested by incubating with 0.25% trypsin for 5 min. The detached cells were washed with DPBS, suspended in DPBS, and analyzed by flow cytometry (BD FACS Aria III), with excitation at 535 nm.

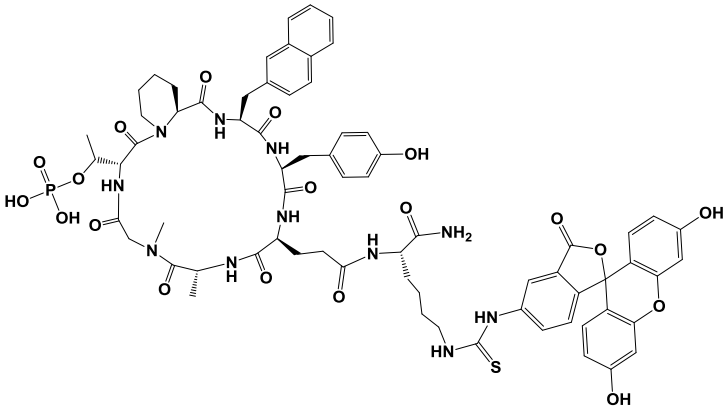
MTT Cell Viability Assay. HeLa cells were seeded in a 96-well plate at a density of 5000 cell/well (100 μL in each well) in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin sulfate overnight. Next day, peptide was added to the cells and incubated in 5% CO_2 incubator at 37 ^oC for 72 h, during which the growth media was replaced with fresh media containing the peptide every 24 h. 10 μL of MTT solution (5 mg/mL) was added to each well and incubated for 2 h. 100 μL of the SDS-HCl solubilizing buffer was added to each well and the contents were carefully mixed by pipetting up and down. The plate was incubated for 4 h and the absorbance of the solubilized formazan was measured on a Molecular Devices Spectramax M5 plate reader at 570 nm.

Immunoblotting. HeLa cells were seeded in a 6-well plate with standard DMEM supplemented with 10% FBS and 1% penicillin-streptomycin sulfate at 37 ℃ in 5% CO_2 to reach 90% confluence. The cells were treated with peptide **11** (0-10 μM), peptide **2** (10 μM) or 1% DMSO (vehicle control) in serum-free DMEM for 2 h. FBS was then added to a final concentration as 10% and cells were grown for additional 22 h. The cells were harvested by washing twice with cold PBS, detaching by treatment with 0.25% Trypsin-EDTA solution, and combining all of the fractions. After centrifugation in a microcentrifuge (5000 rpm, 5 min), cell pellets were lysed on ice for 30 min in IP lysis buffer containing protease and phosphatase inhibitors. Cell lysates were centrifuged at 15000 rpm for 10 min, and the extracted proteins in the supernatant were collected. Protein concentrations were measured by using BCA Protein Assay Kit (Thermo, 23235), and equal amounts of protein were loaded onto a 10% SDS-PAGE gel. The proteins were transferred

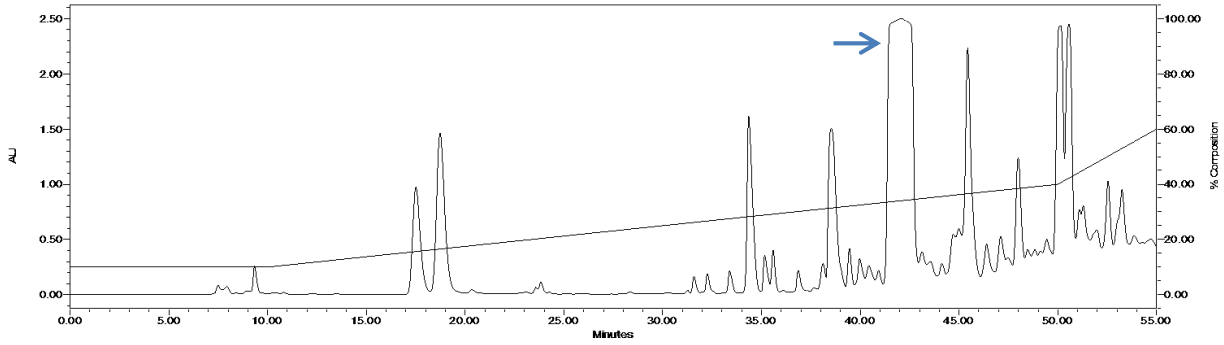
electrophoretically to a nitrocellulose membrane at 4 ℃. The membrane was first blocked with 10% nonfat dry milk in TBST (0.1% (v/v) Tween 20 in 1x TBS) for 1 h, followed by incubating with anti-PML (Santa Cruz sc-5621) or anti-β-actin (Sigma A5441) overnight at 4 ℃. The membranes were washed three times with TBST and incubated with corresponding HRP-conjugated secondary antibodies for 2 h at room temperature, followed by washing with TBST three times and proceeding to detection immediately. The signals were detected by using Chemiluminescent HRP Antibody Detection Reagent (Denville, E-2500) by following the manufacturer's protocol.

Fig. S1. Peptide purification by reversed-phase HPLC (semi-preparative), purity assessment by analytical HPLC, and MALDI-TOF mass spectrometry.

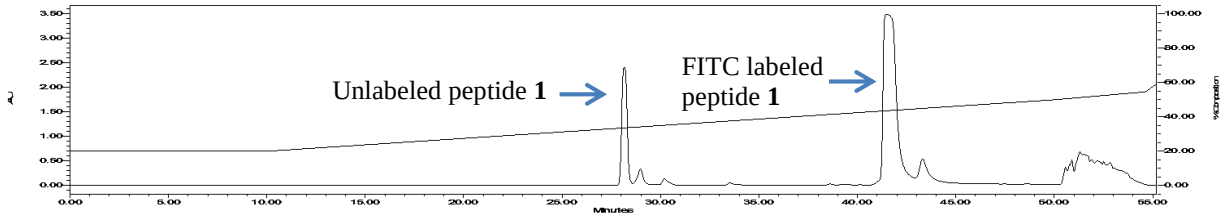
FITC-labeled peptide **1**:



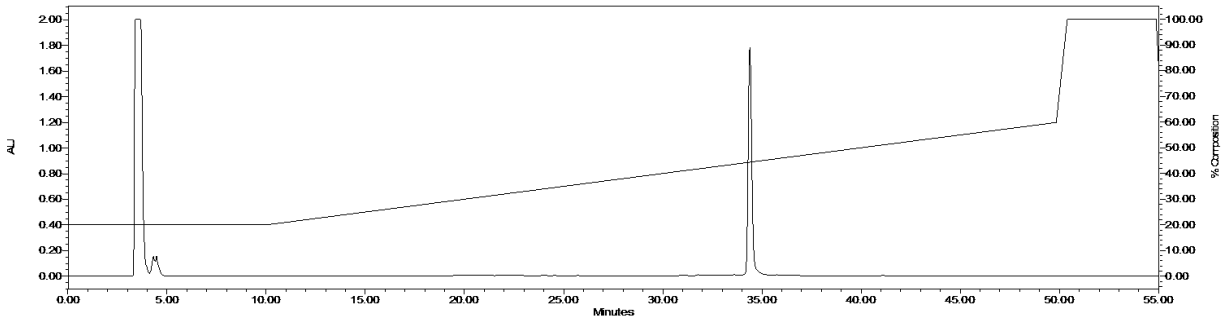
Crude peptide **1** before labeling with FITC (semi preparative reversed-phase HPLC)



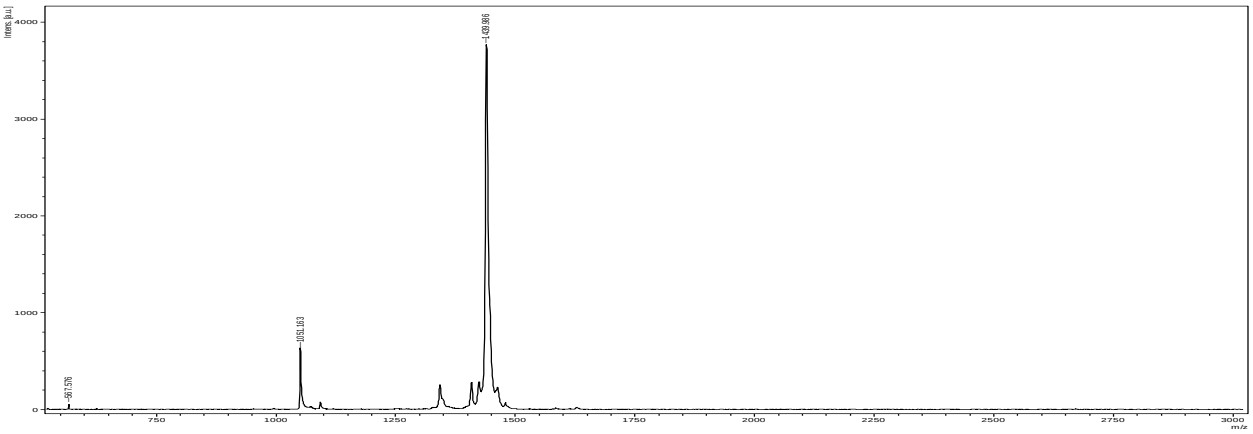
Crude FITC-labeled peptide **1** (semi preparative reversed-phase HPLC)



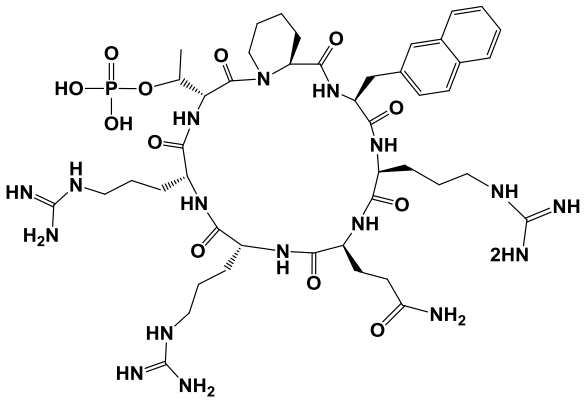
Purity assessment of FITC-labeled Peptide **1** (analytical reversed-phase HPLC)



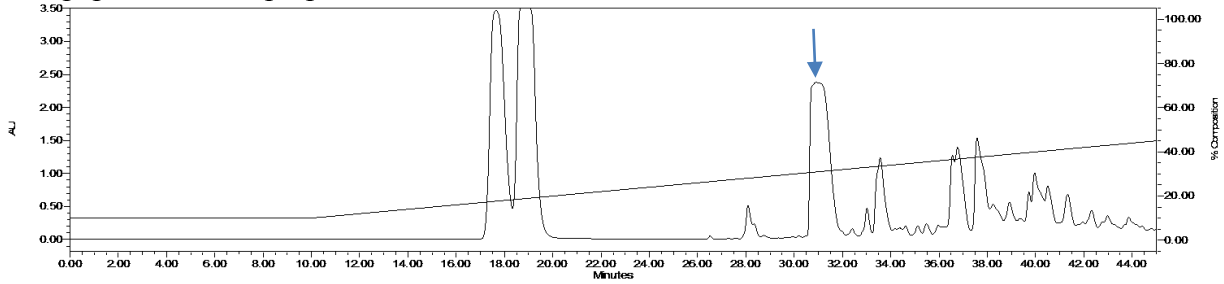
MALDI-TOF MS analysis of FITC-labeled peptide **1** (low-resolution)
Expected for $M+H^+$: 1440.491; Found 1439.986



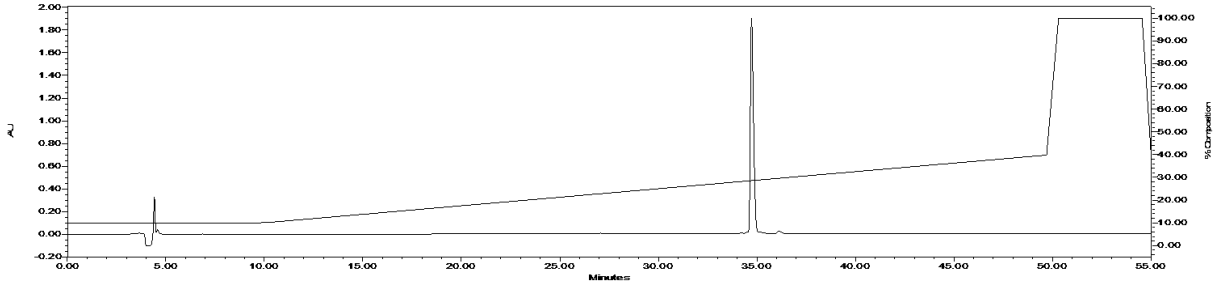
Peptide **2**:



Crude peptide on semipreparative C18 column

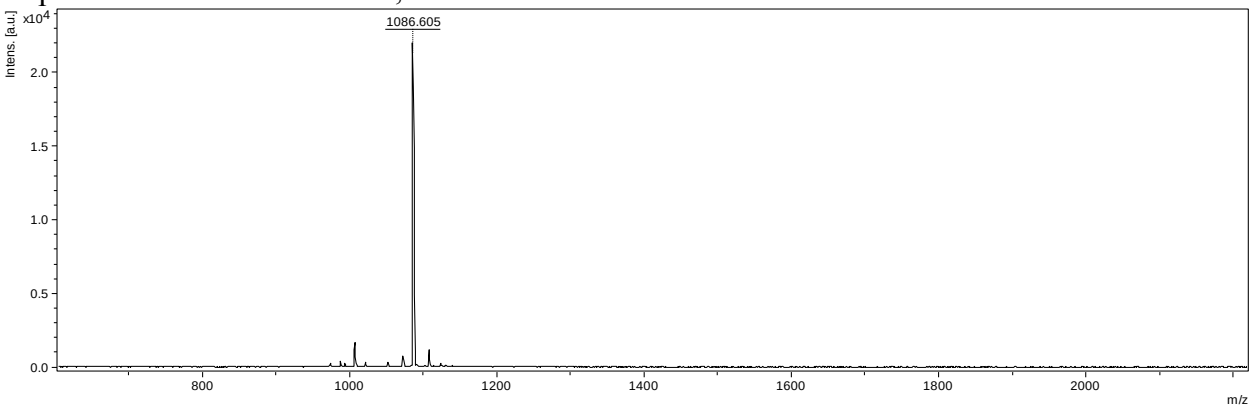


Purity assessment of peptide **2** (analytical reversed-phase HPLC)

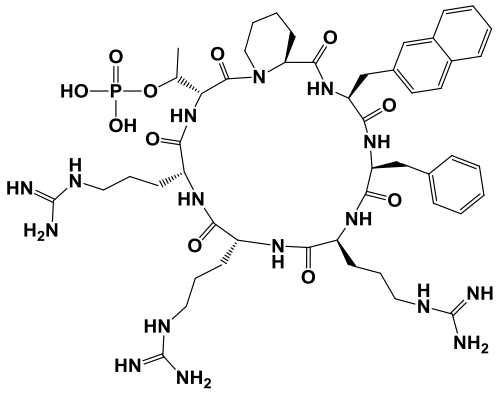


MALDI-TOF MS analysis

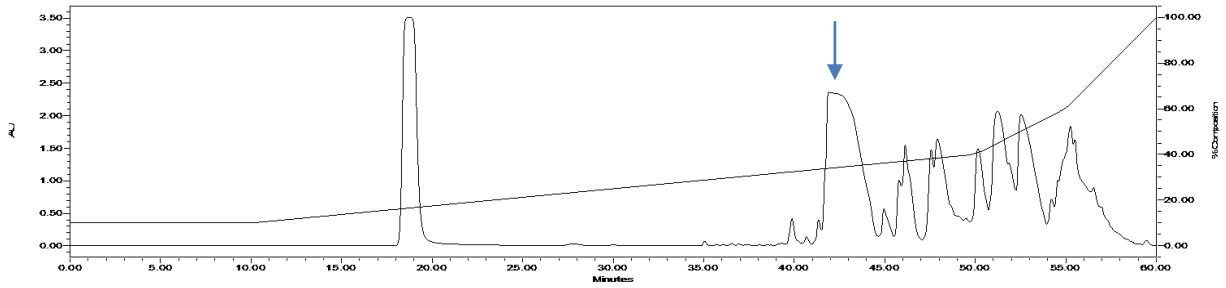
Expected for $M+H^+$: 1086.531; Found 1086.605



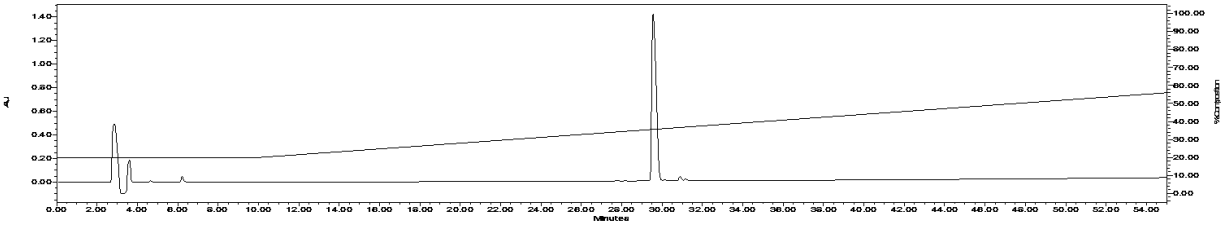
Peptide **3**:



Crude peptide on semipreparative reversed-phase HPLC

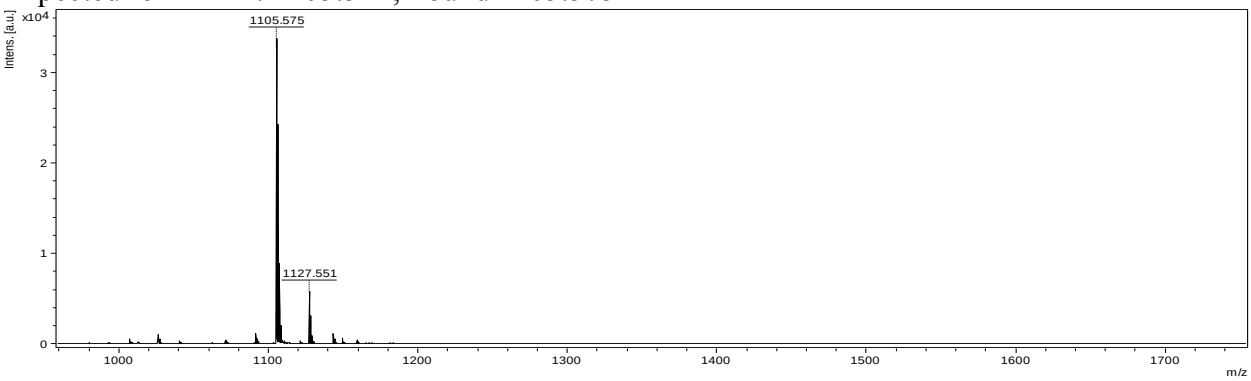


Peptide **3** purity assessment (analytical reversed-phase HPLC)

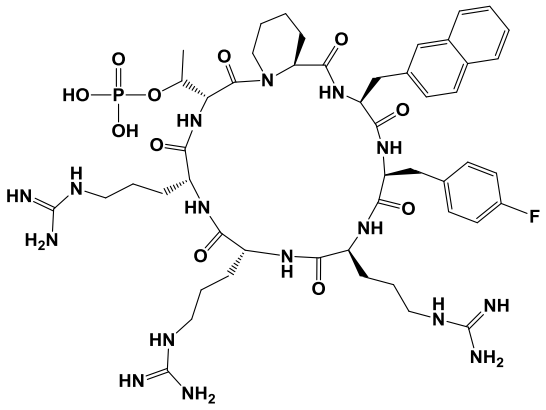


MALDI-TOF MS analysis

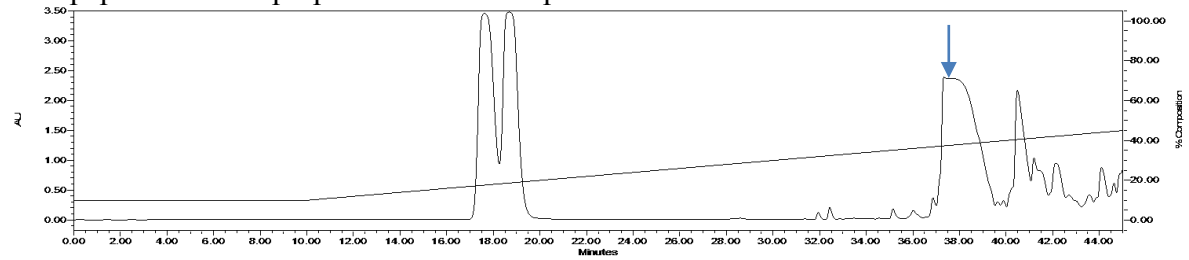
Expected for $M+H^+$: 1105.541; Found 1105.575



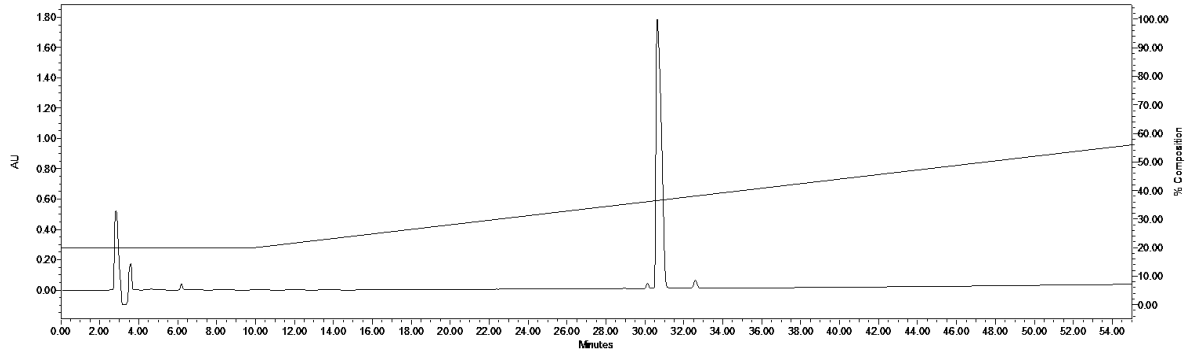
Peptide **4**:



Crude peptide on semipreparative reversed-phase HPLC

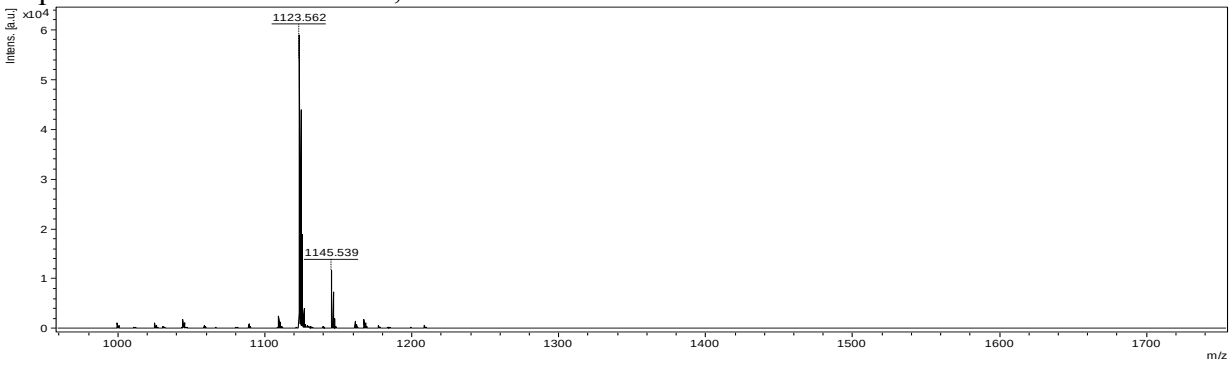


Peptide **4** purity check (analytical reversed-phase HPLC)

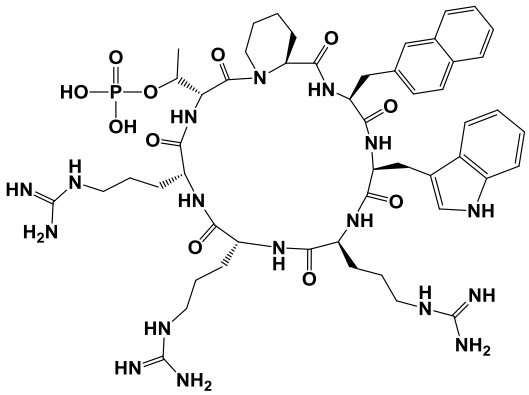


MALDI-TOF MS analysis

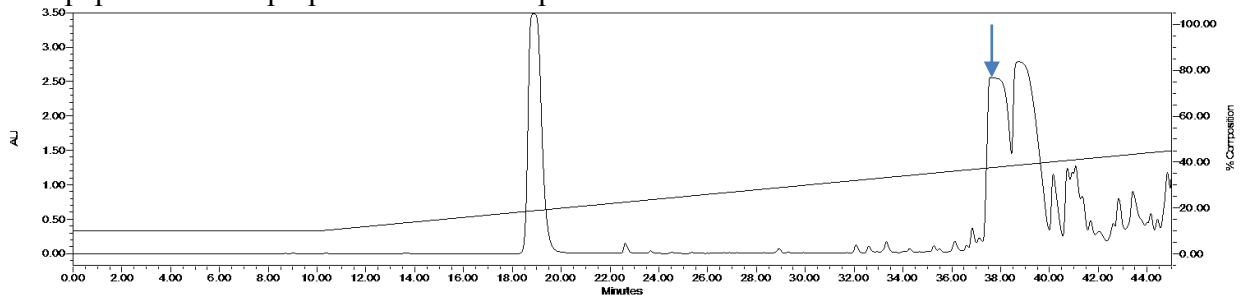
Expected for $M+H^+$: 1123.531; Found 1123.562



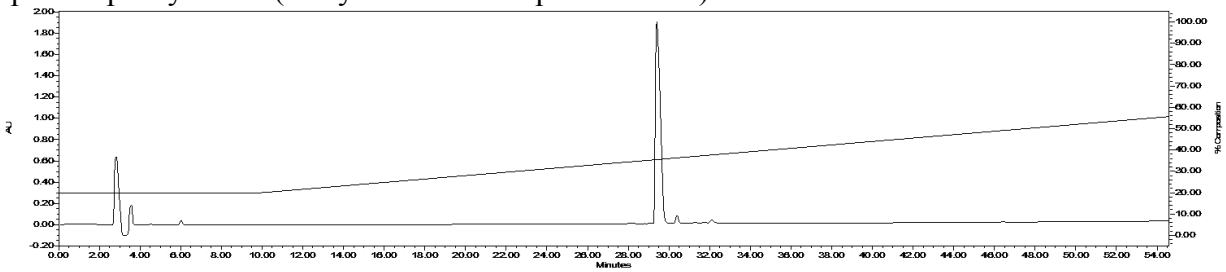
Peptide **5**:



Crude peptide on semipreparative reversed-phase HPLC

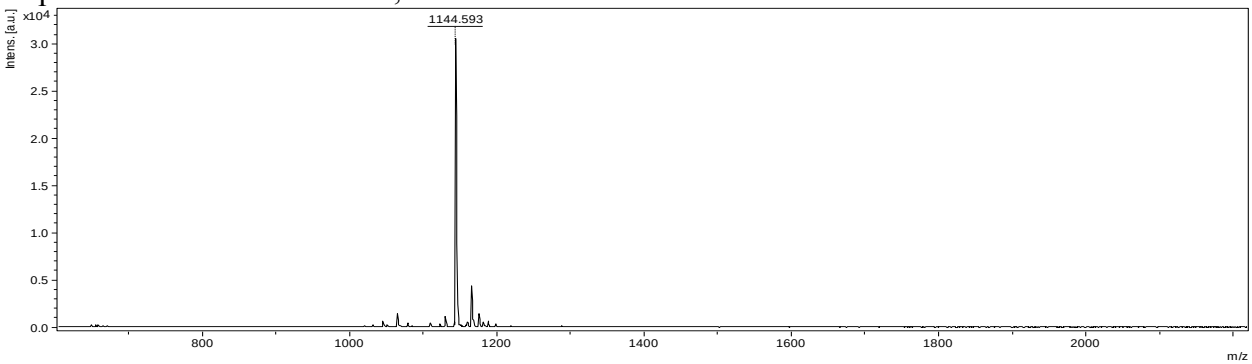


Peptide **5** purity check (analytical reversed-phase HPLC)

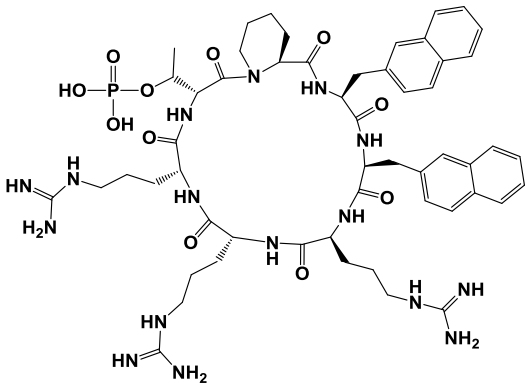


MALDI-TOF MS analysis

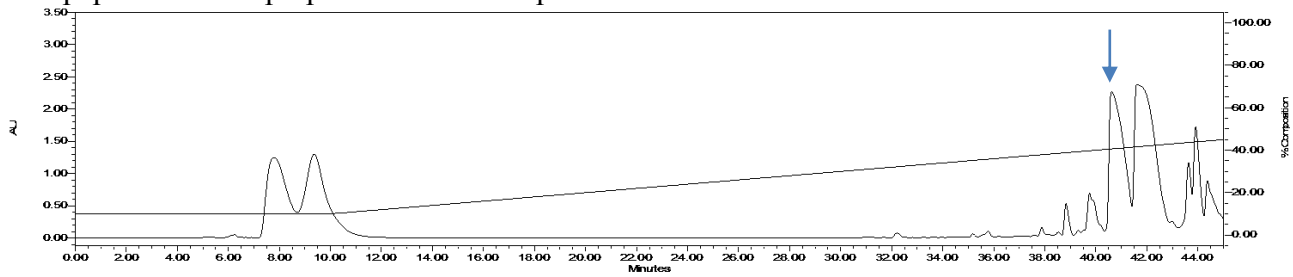
Expected for $M+H^+$: 1144.551; Found 1144.593



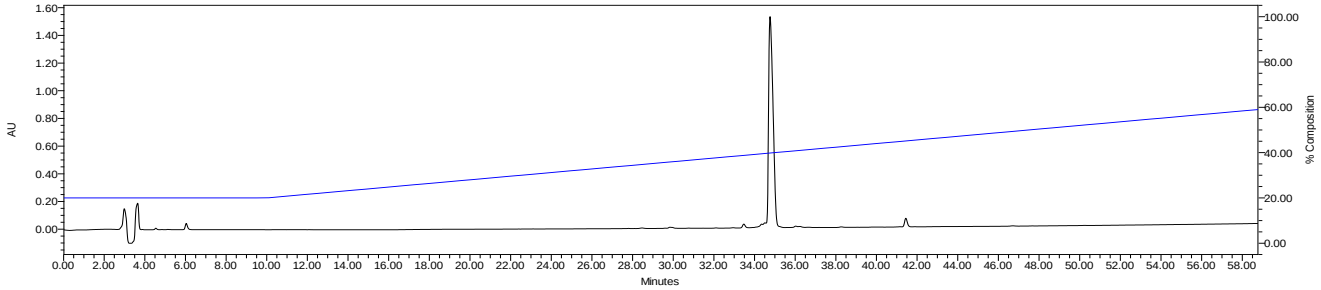
Peptide **6**:



Crude peptide on semipreparative reversed-phase HPLC

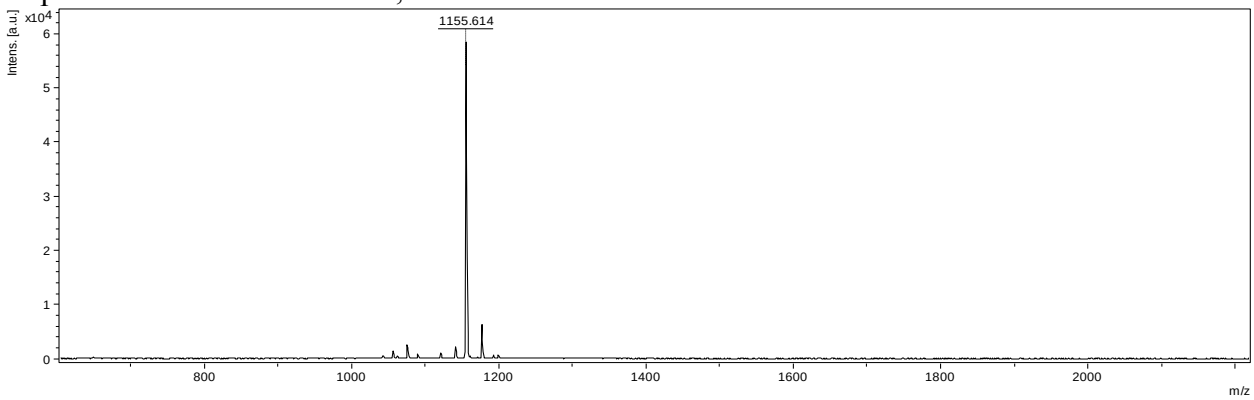


Peptide **6** purity check (analytical reversed-phase HPLC)

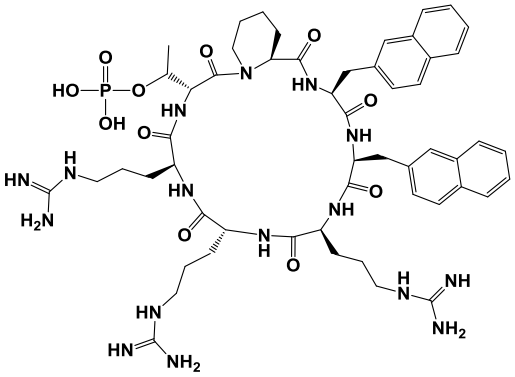


MALDI-TOF MS analysis

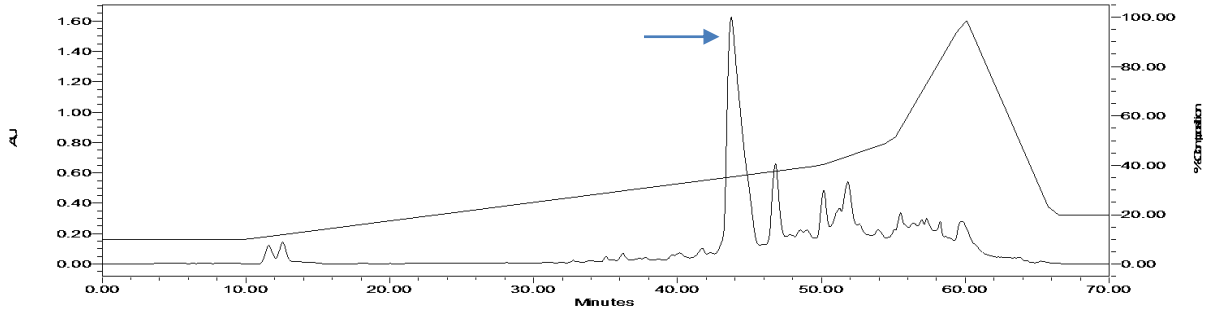
Expected for $M+H^+$: 1155.551; Found 1155.614



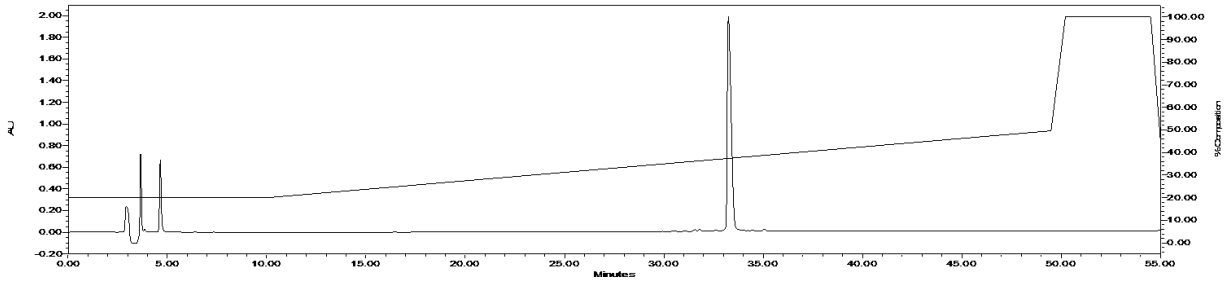
Peptide **7**:



Crude peptide on semipreparative reversed-phase HPLC

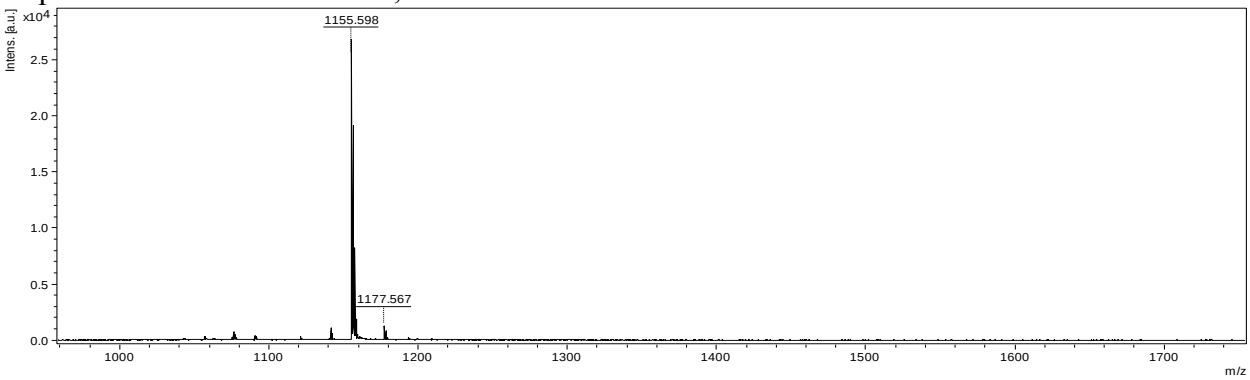


Peptide **7** purity check (analytical reversed-phase HPLC)

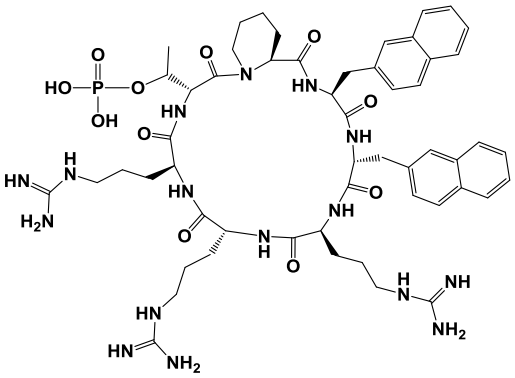


MALDI-TOF MS analysis

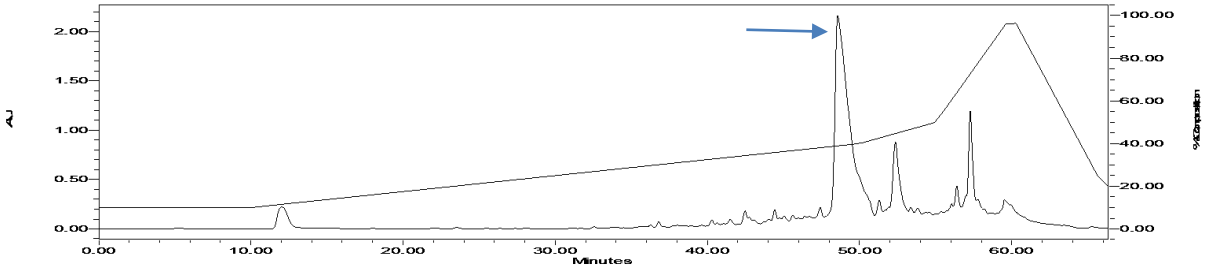
Expected for $M+H^+$: 1155.551; Found 1155.598



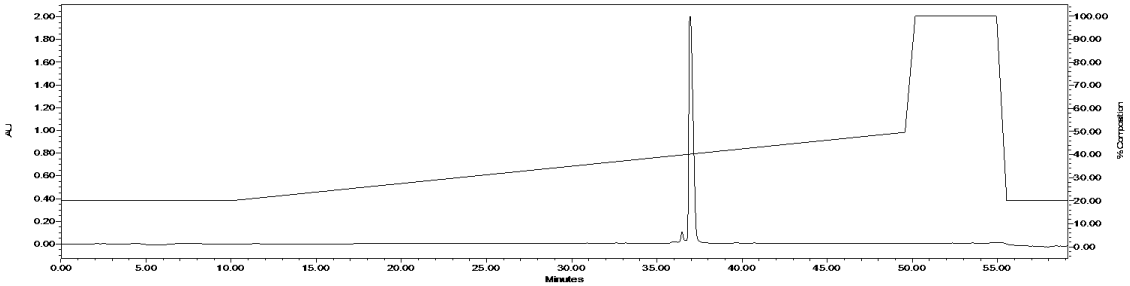
Peptide **8**:



Crude peptide on semipreparative reversed-phase HPLC

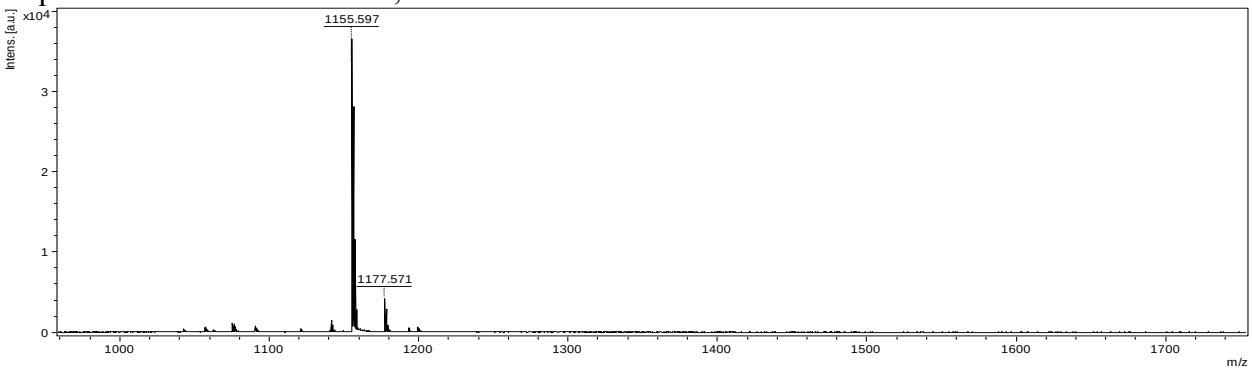


Peptide **8** purity check (analytical reversed-phase HPLC)

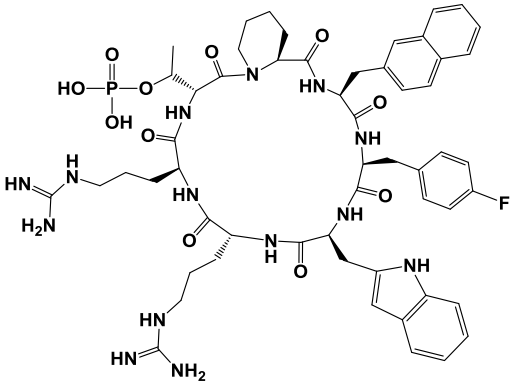


MALDI-TOF MS analysis

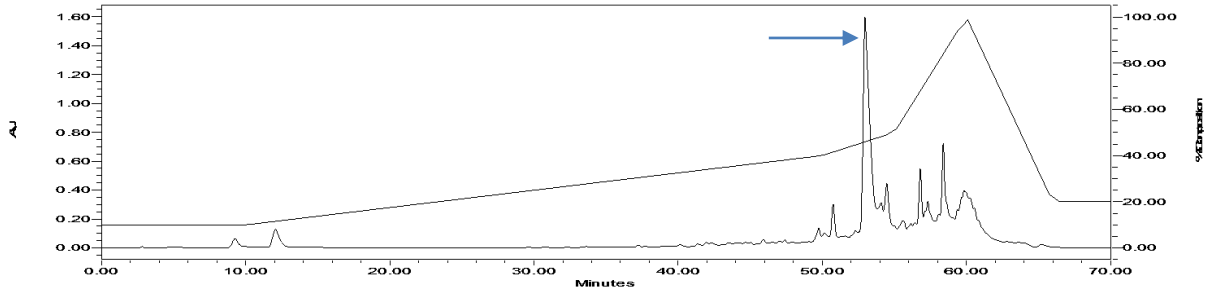
Expected for $M+H^+$: 1155.551; Found 1155.597



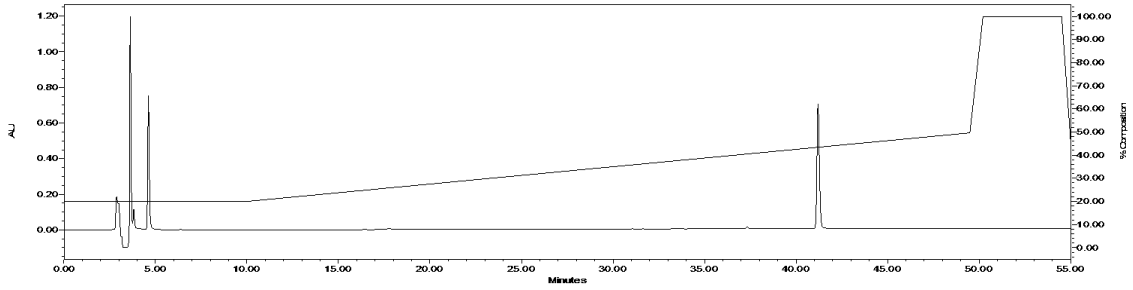
Peptide **9**:



Crude peptide on semipreparative reversed-phase HPLC

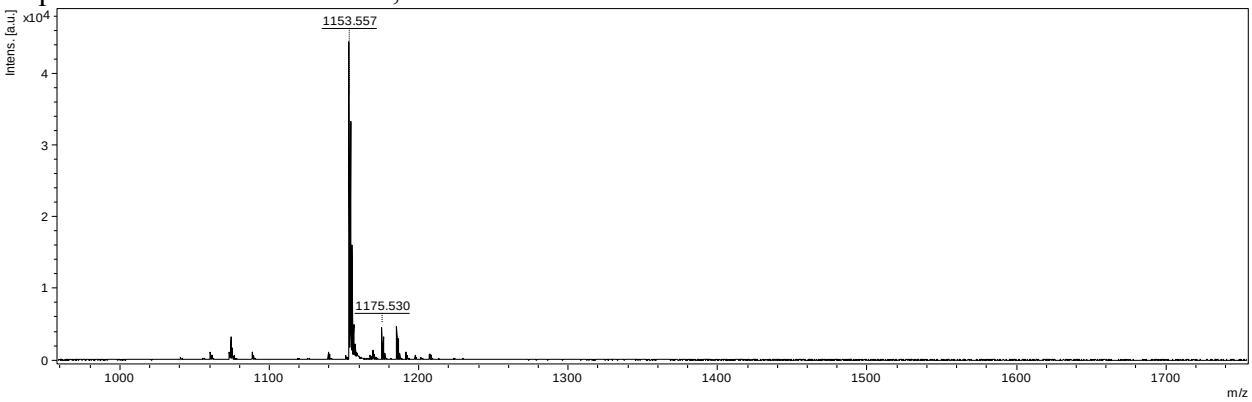


Peptide **9** purity check (analytical reversed-phase HPLC)

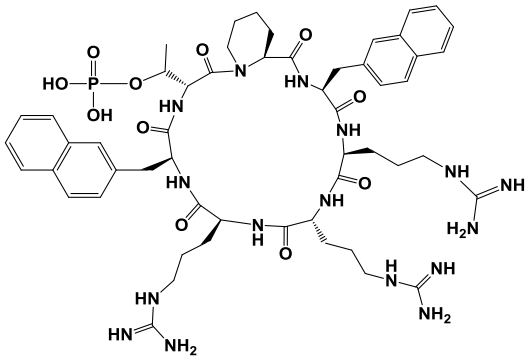


MALDI-TOF MS analysis

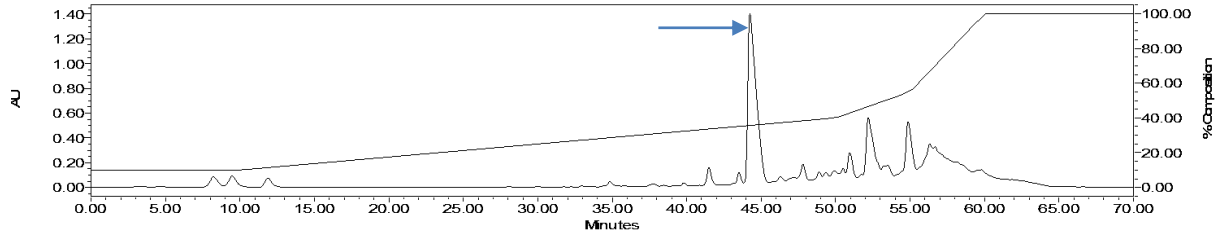
Expected for $M+H^+$: 1153.511; Found 1153.557



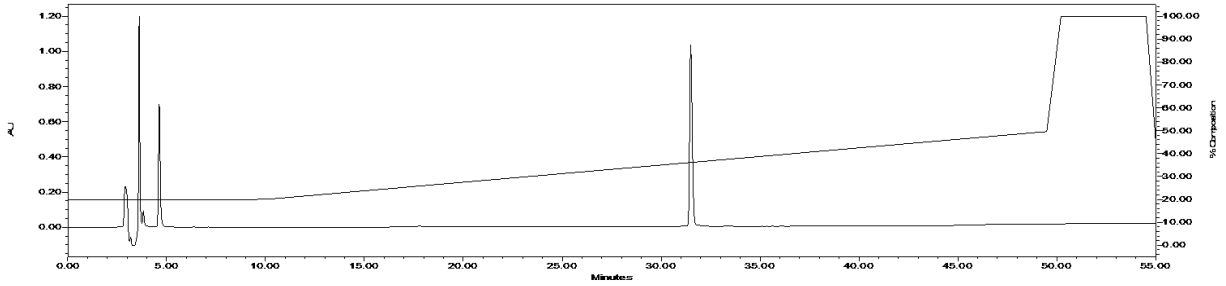
Peptide **10**:



Crude peptide on semipreparative reversed-phase HPLC

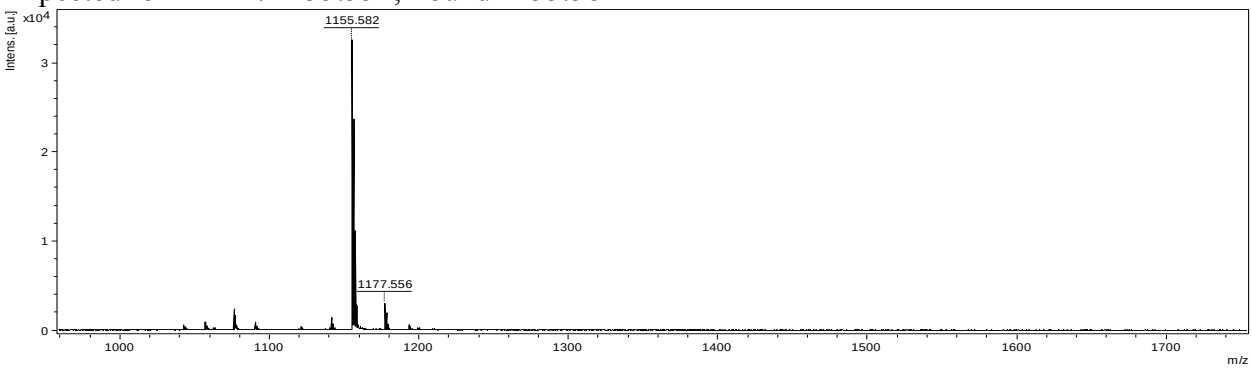


Peptide **10** purity check (analytical reversed-phase HPLC)

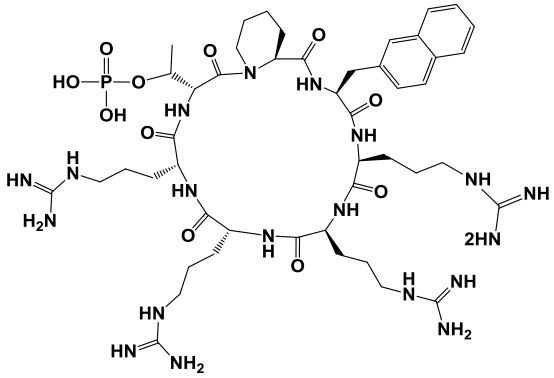


MALDI-TOF MS analysis

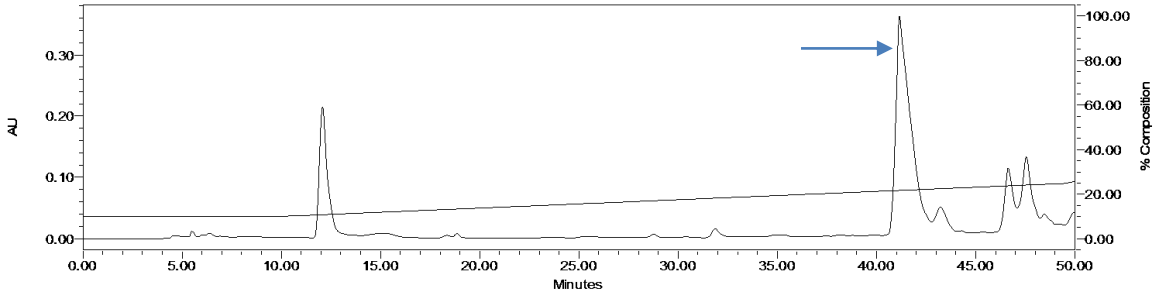
Expected for $M+H^+$: 1155.551; Found 1155.582



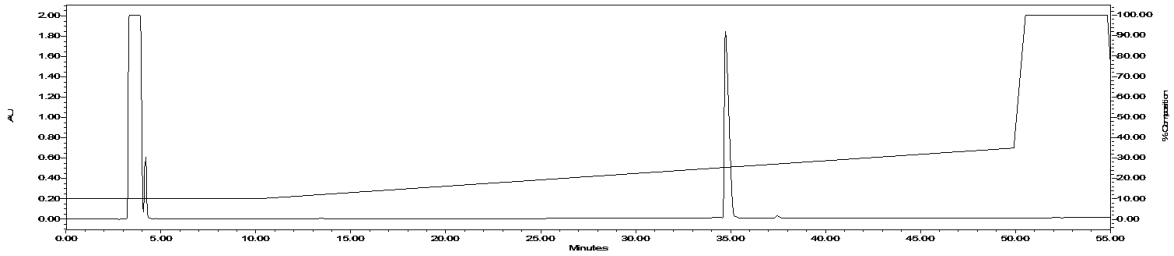
Peptide **11**:



Crude peptide on semipreparative reversed-phase HPLC

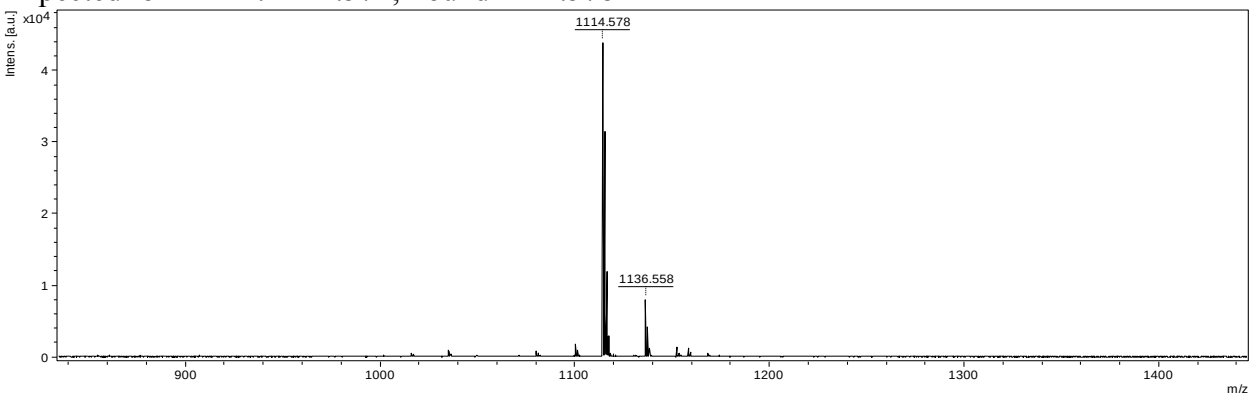


Peptide **11** purity check (analytical reversed-phase HPLC)

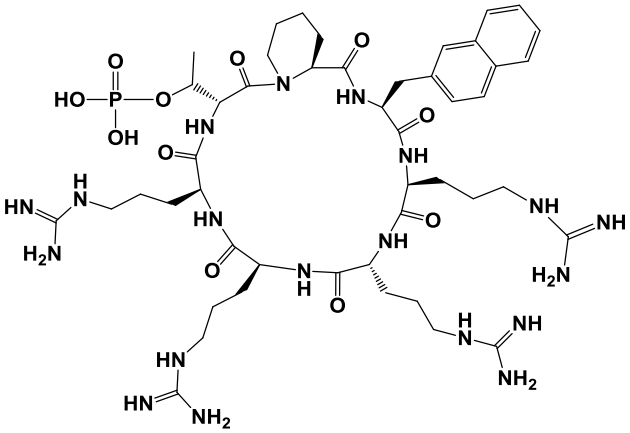


MALDI-TOF MS analysis

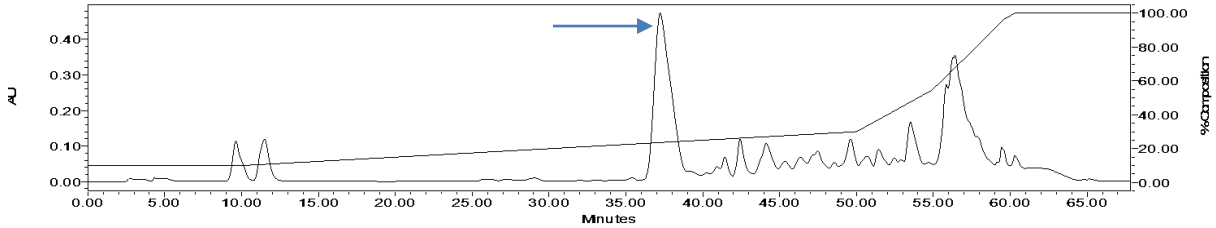
Expected for $M+H^+$: 1114.571; Found 1114.578



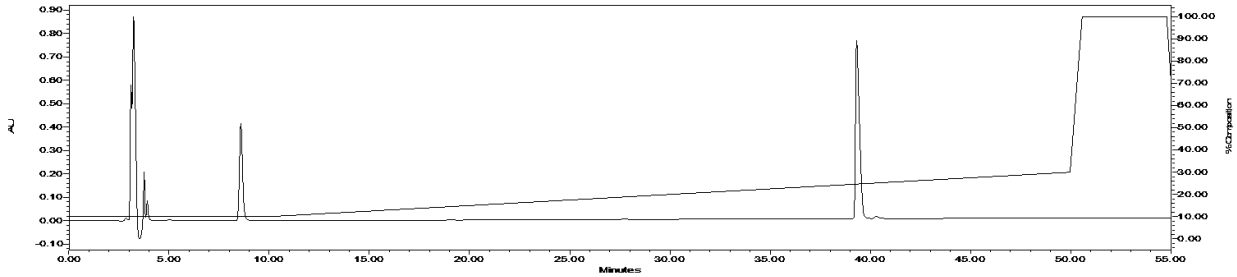
Peptide **12**:



Crude peptide on semipreparative reversed-phase HPLC

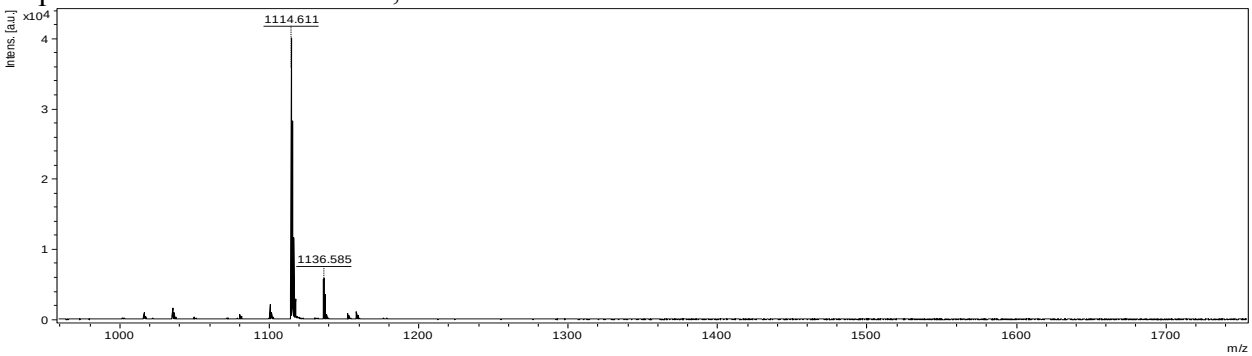


Peptide **12** purity check (analytical reversed-phase HPLC)

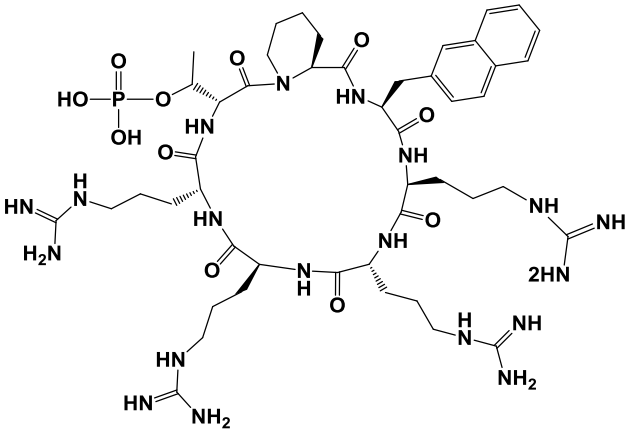


MALDI-TOF MS analysis

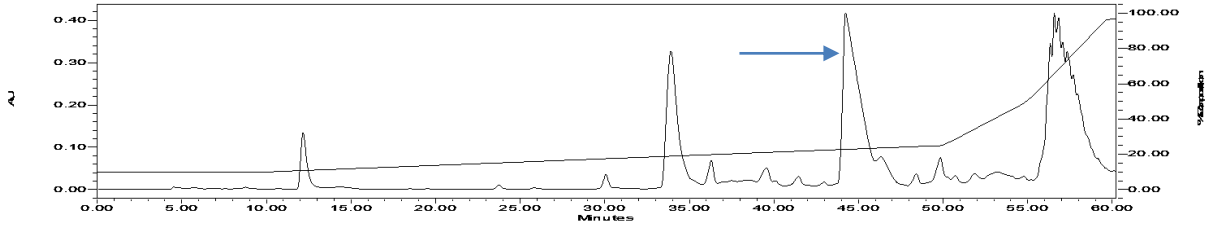
Expected for $M+H^+$: 1114.571; Found 1114.611



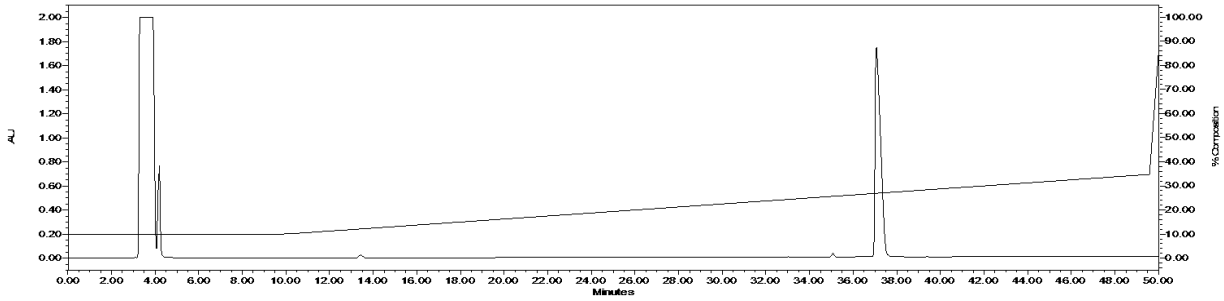
Peptide **13**:



Crude peptide on semipreparative reversed-phase HPLC

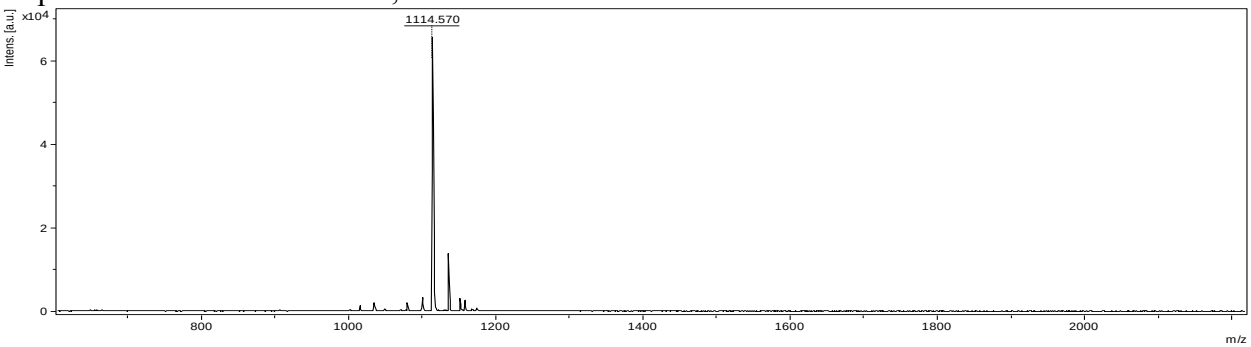


Peptide **13** purity check (analytical reversed-phase HPLC)

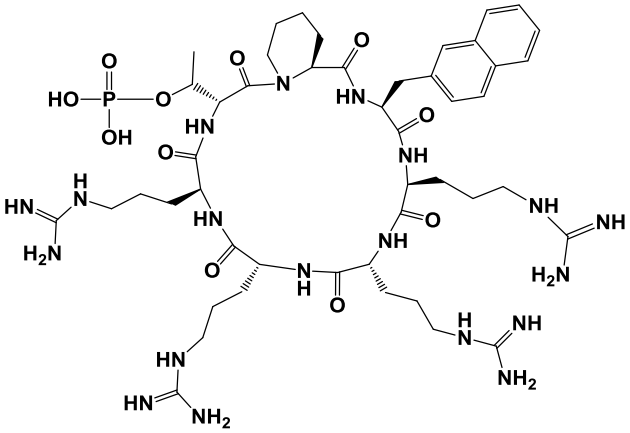


MALDI-TOF MS analysis

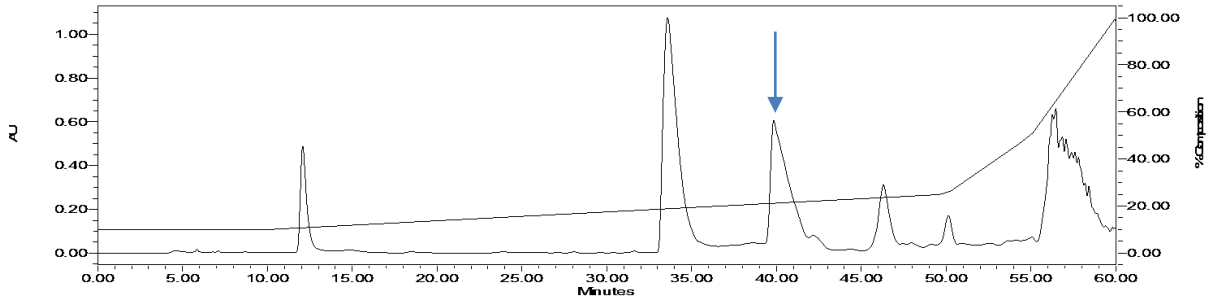
Expected for $M+H^+$: 1114.571; Found 1114.570



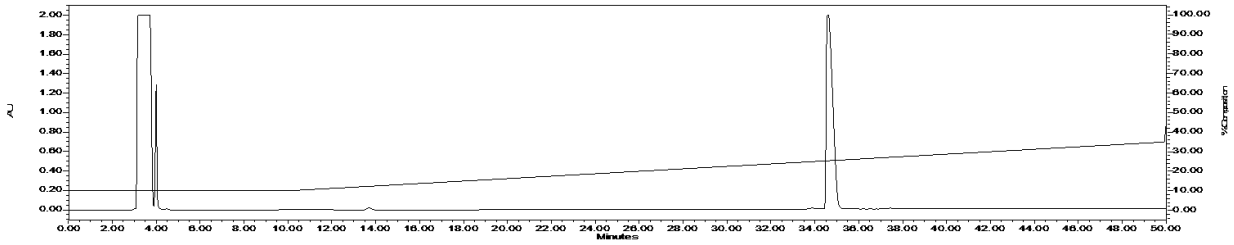
Peptide **14**:



Crude peptide on semipreparative reversed-phase HPLC

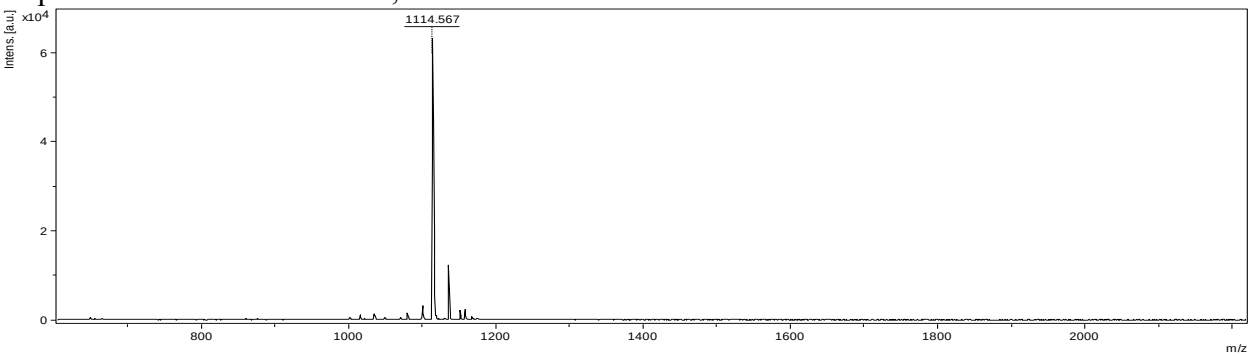


Peptide **14** purity check (analytical reversed-phase HPLC)

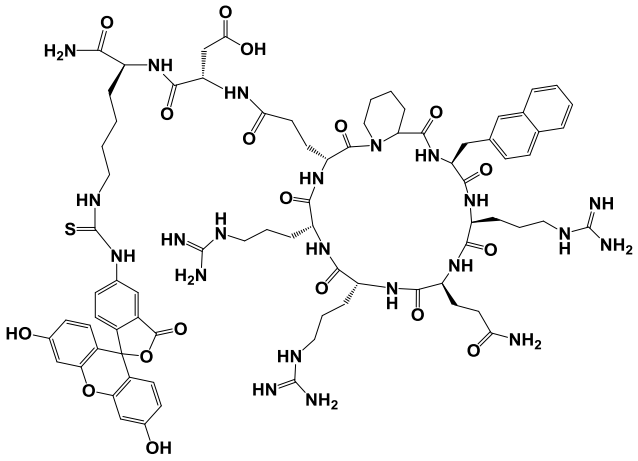


MALDI-TOF MS analysis

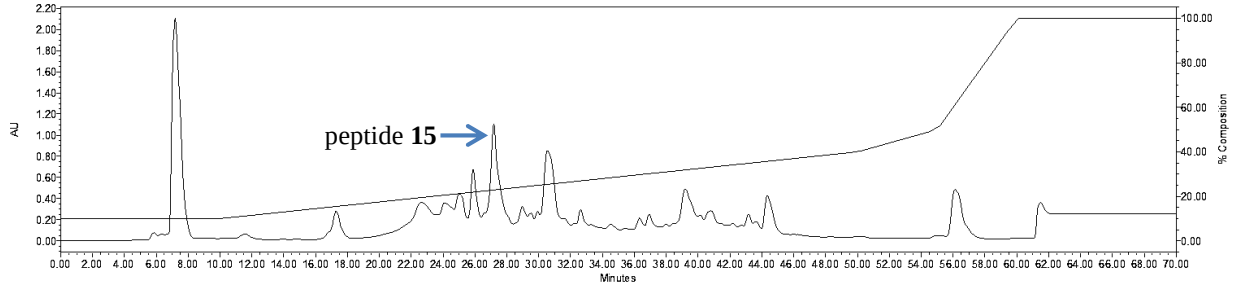
Expected for $M+H^+$: 1114.571; Found 1114.567



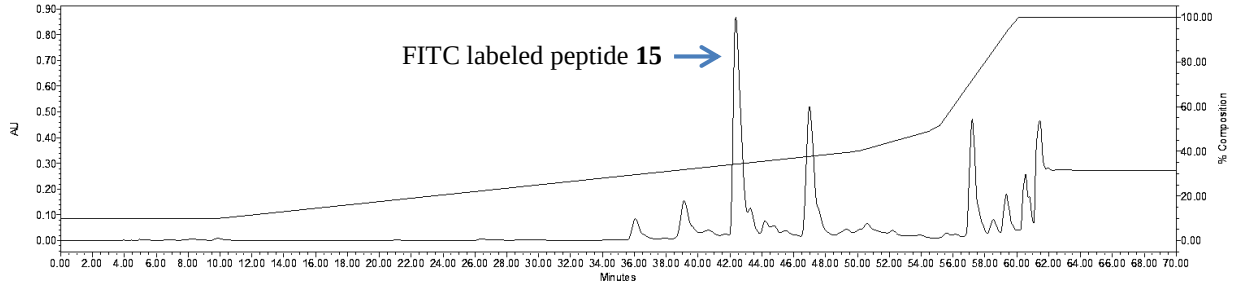
Peptide **15 (**FITC-Labeled Peptide **2** analog):



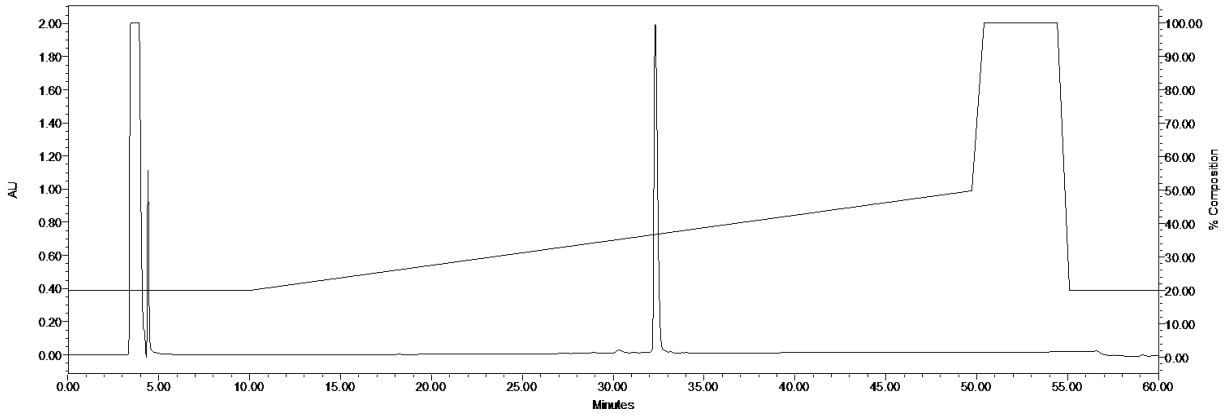
Crude peptide **15** before FITC labeling (on semipreparative reversed-phase HPLC)



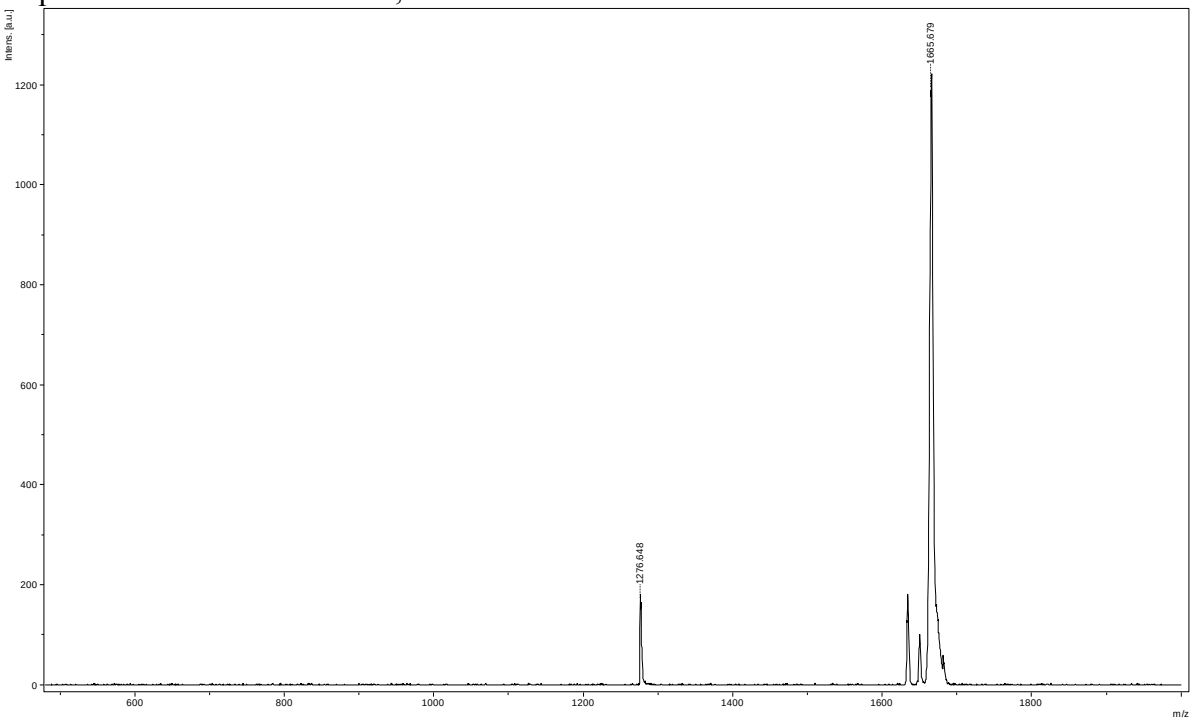
FITC-labeled peptide **15** on semipreparative reversed-phase HPLC



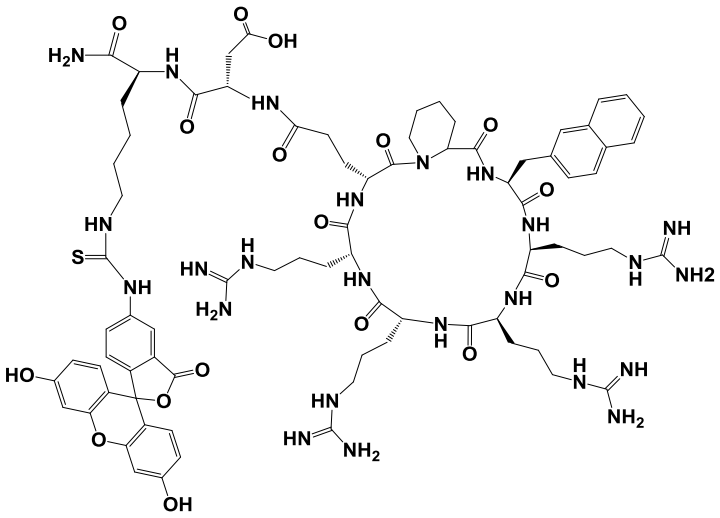
Purity assessment of peptide **15**:



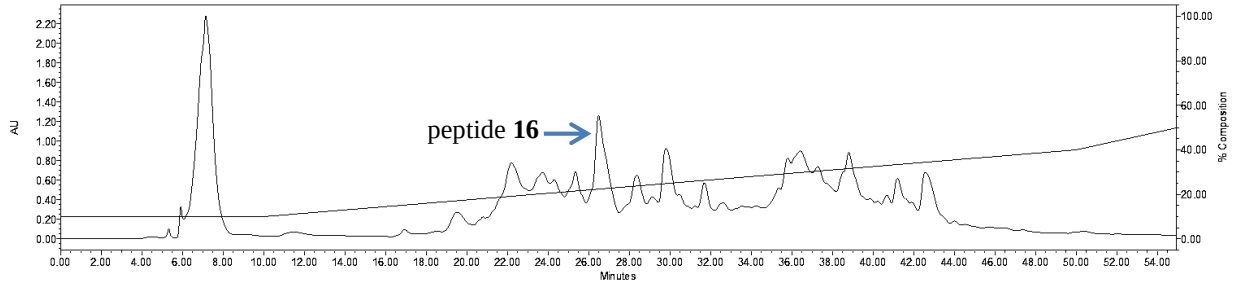
MALDI-TOF MS analysis (low-resolution)
Expected for $M+H^+$: 1665.731; Found 1665.679



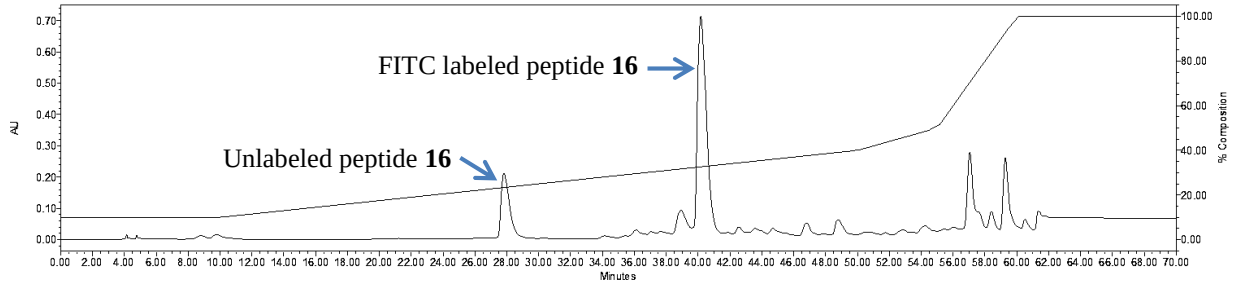
Peptide **16 (**FITC-Labeled Peptide **11** analog):



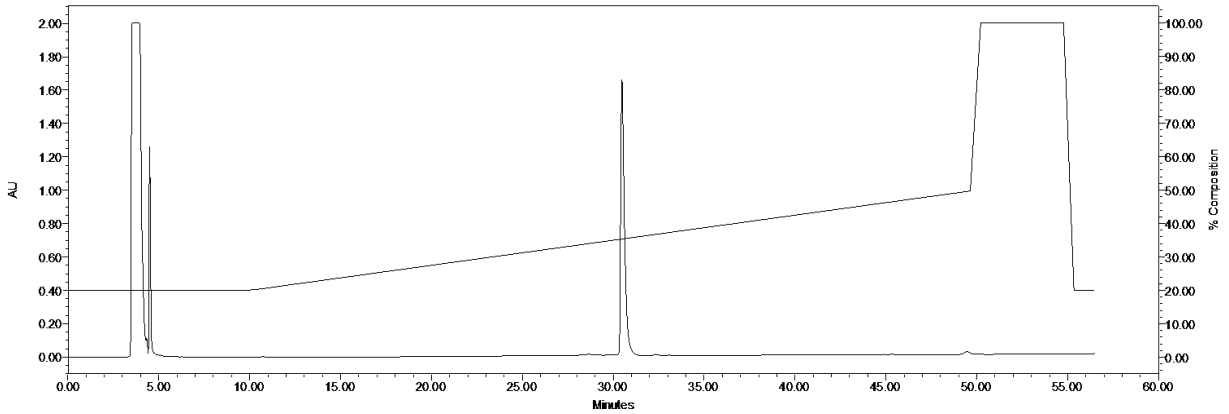
Crude peptide **16** before FITC labeling (on semipreparative reversed-phase HPLC)



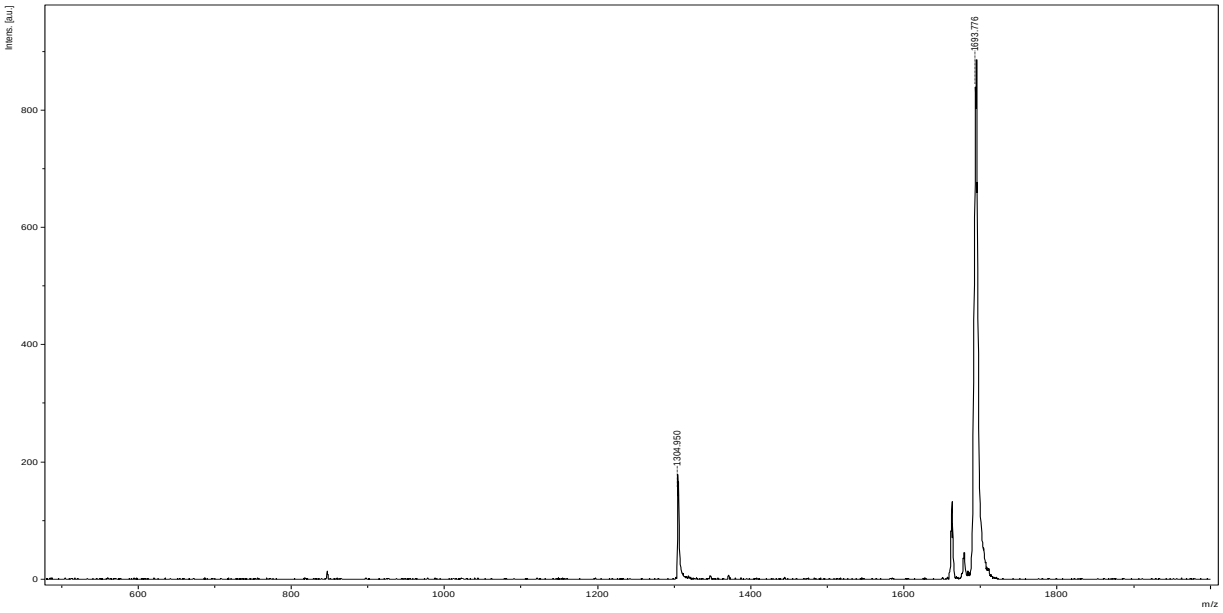
FITC-labeled peptide **16** on semipreparative reversed-phase HPLC



Purity assessment of peptide **16**:



MALDI-TOF MS analysis (low-resolution)
Expected for $M+H^+$: 1693.771; Found 1693.776



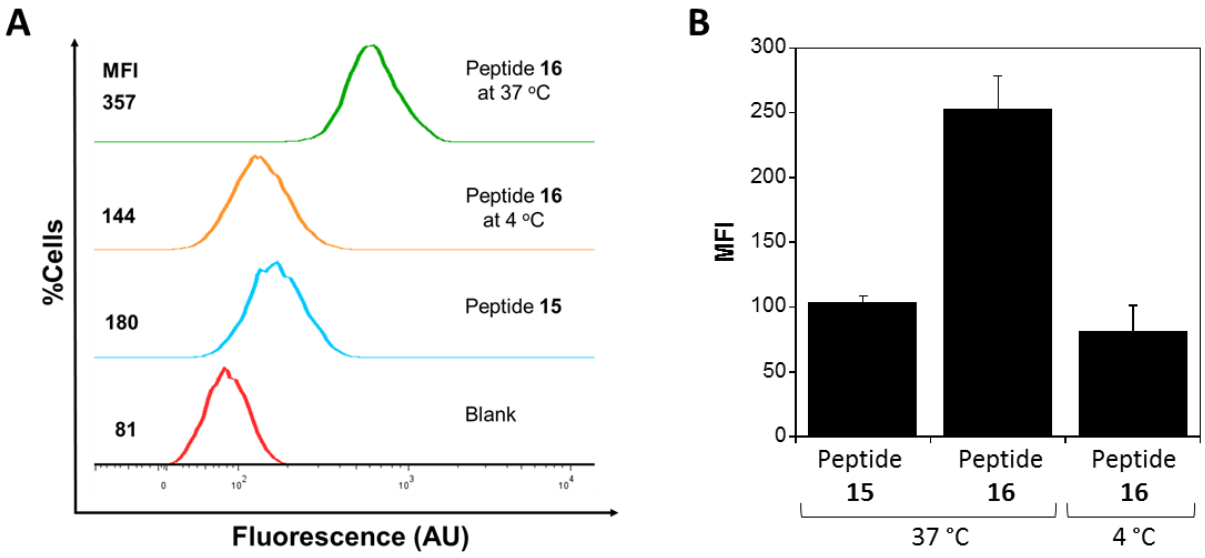


Fig. S2. Cellular entry efficiency. (A) Representative flow cytometry data for untreated HeLa cells (blank) and HeLa cells after treatment with 5 μM FITC-labeled peptide for 2 h at 37 °C (and peptide **16** at 4 °C). (B) Comparison of the cellular uptake efficiencies of FITC-labeled peptides **15** and **16** at 4 or 37 ºC. Data presented are the mean ± SD from three independent experiments (after subtraction of background signal derived from "blank"). MFI, fluorescence intensity.