Convenient preparation of bactericidal hydrogels by covalent attachment of stabilized antimicrobial peptides using thiol-ene click chemistry

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Supporting Experimental Procedures

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. MilliQ grade water was used unless stated otherwise. Analytical HPLC runs were carried out on a Shimadzu HPLC system and preparative HPLC runs were performed on a Gilson HPLC workstation. Analytical HPLC runs were performed on Phenomenex Gemini C18 column (250 × 4.60 mm, particle size: 5 µm, pore size: 110Å) at a flow rate of 1.0 mL/min using a linear gradient of buffer B (0–100% in 45 min) in buffer A (buffer A: 0.1% TFA in H_2O/CH_3CN, 95:5, v/v, buffer B: 0.1% TFA in CH_3CN/H_2O, 95:5, v/v). Preparative HPLC runs were performed on a Phenomenex Gemini C18 column (250 × 20 mm, particle size: 10 µm, pore size: 110 Å), at a flow rate of 12.5 mL/min using an identical buffer system as described above. MALDI-TOF analysis was performed on a Kratos Axima CFR apparatus with hydroxycinnamic acid or sinapinic acid as matrices.

The coupling reagents 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), N-Hydroxy-benzotriazole

(HOBt), N-9- fluorenylmethyloxycarbonyl (Fmoc) and all protected amino acids were obtained from GL Biochem Ltd. (Shanghai, China), with the exception for Fmoc-D-Cys(Trt)-OH and Fmoc-D-Arg(Pbf)-OH, which were obtained from IrisBiotech GmbH (Marktredwitz, Germany). Methyl tert-butyl ether (MTBE), N,N-diisopropylethylamine (DIPEA), n-hexanes and trifluoroacetic acid (TFA) were purchased from Biosolve B.V. (Valkenswaard, The Netherlands). HPLC-grade N-methylpyrrolidone (NMP), acetonitrile and dichloromethane were purchased from Actu-All Chemicals (Oss, The Netherlands). MilliQ grade water was obtained using a MilliPore Gradient A10 system with a Quantum EX Ultrapure Organex cartridge. 10x PBS was obtained from Santa Cruz Biotechnology Inc. Pentaerythritol tetrakis(3-mercaptopropionate), Poly(ethylene glycol) diacrylate (Mn ~700), Triisopropylsilane (TIS), Tween80 and Ciprofloxacin were obtained from Sigma Aldrich. 2,2-Dimethoxy-2-phenylacetophenone (DMPA) was obtained from Acros Organics (Geel, Belgium).

Antimicrobial Peptide Synthesis

Typically, peptides were synthesized on a Rink Amide resin (0.24 mmol/g) (Rapp Polymere GmbH, Tübingen, Germany) on a 0.25 mmol scale. The peptide was assembled using an automatic ABI 433A Peptide Synthesizer, equipped with a UV-monitoring system, which was used to monitor the Fmoc removal step i.e. formation of the dibenzofulvene-piperidine adduct absorbing at 301 nm. ABI FastMoc 0.25 mmol protocols were applied, with the exception of a standard double coupling of 45 min.[1,2] : The resin was first washed with DCM and NMP (5 times). Subsequently, 1 mmol of the appropriate amino acid was dissolved in NMP (2mL), and HBTU/HOBt (1 mmol, 2.78 mL of 0.36 M in NMP) was added. To this mixture DiPEA (1 mL, 2 M in NMP) was added and the activated amino acid was then transferred to the reaction vessel. After 45 min, the reaction vessel was drained and the resin was washed with NMP (3 times) followed by addition of another batch of pre-activated amino acid, which was allowed to couple for another 45 min. Next, any of the remaining free amino groups were acetylated with an acetic anhydride capping solution (0.5 M Ac_2O, 0.125 M DiPEA and 0.015 M HOBt in NMP) for 15 min. After capping, the Fmoc protecting group was removed from the N-terminus by treatment with 20% piperidine in NMP

solution (2 times, 3 min and 7.6 min). The last coupling cycle was also followed by removal of the Fmoc-group by a 20% piperidine solution, washing the resin with NMP. Finally, the resin was washed with NMP (5 times 10 mL) and DCM (6 times, 10 mL), removed from the reaction vessel, washed with ether and dried *in vacuo*. The resin-bound peptide was deprotected and cleaved from the resin by treatment with TFA/H_2O/TIS (95/2.5/2.5, 15 mL) for 3 h at room temperature. The mixture was filtered and added dropwise to 90 mL MTBE/n-hexane (1/1, v/v) solution. The precipitate was collected by centrifugation (3000 rpm, 5 min), the supernatant was decanted and the pellet was re-suspended in MTBE/ n-hexane (1/1, v/v) (90 mL). The resin was washed again with TFA (10 mL), filtered and this TFA filtrate was added dropwise to the crude peptide suspension and centrifuged again. This procedure was repeated twice. Hereafter, the pellets were dissolved in MeCN/H_2O (1/1, v/v) (ca. 40 mL) and lyophilized to yield approximately 200 mg of the crude peptide as a white, fluffy solid.

The crude peptide (ca 200 mg) was dissolved in 30 mL buffer A, 10 mL buffer B and purified by preparative HPLC (Gemini C18, TFA buffers). Fractions containing the pure peptide were pooled and lyophilized to yield approximately 75 mg (25%) of pure peptide. The purity was established by analytical HPLC and characterization was carried out by MALDI-TOF.

Microbicidal activity; LC99.9 assay[3]

The microbicidal activity of purified peptides against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 49230 UAMS-1 (a human osteomyelitis clinical isolate[4] capable of biofilm formation[5]) and *Staphylococcus epidermidis* ATCC 35984 (a catheter sepsis isolate, produces polysaccharide adhesin) was quantified in a LC99.9 assay. The LC99.9 was defined as the lowest concentration of AMP at which <0.1% of an inoculum of 10^6 CFU/ml survived after 2 h or 24 h of exposure. Overnight cultures in trypticase soy broth (TSB; Difco) were diluted 100-fold in fresh TSB and cultured for 3 h at 37°C. Bacteria were washed twice with 10 mM phosphate buffer, pH 7.0, plus 1% (v/v) TSB (PT), the optical density at 620 nm was measured, and the bacteria were diluted to 2×10^6 colony forming units (CFU)/mL in PT, based on an established relationship between optical density and the number of CFU. TSB was used since in Mueller-Hinton

medium cationic peptides may aggregate[6]. Fifty-microliter aliquots of twofold serially diluted peptide in PT (60-0.94 µM) were prepared in a low-protein-binding polypropylene microtiter plate (Costar Corning). To each of the wells, 50 µL of the bacterial suspension was added. After 2 h and 24 h of incubation on a rotary shaker (300 rpm at 37 °C), duplicate 10-µL aliquots were plated on blood agar plates . The plates were inspected for growth after 24 h of incubation at 37 °C. All experiments were performed at least in duplicate. BP2M1[3], magainin II[7] and ciprofloxacin[8] were used as positive controls. Ciprofloxacin was used in a concentration range of 3.75-0.06 µM.

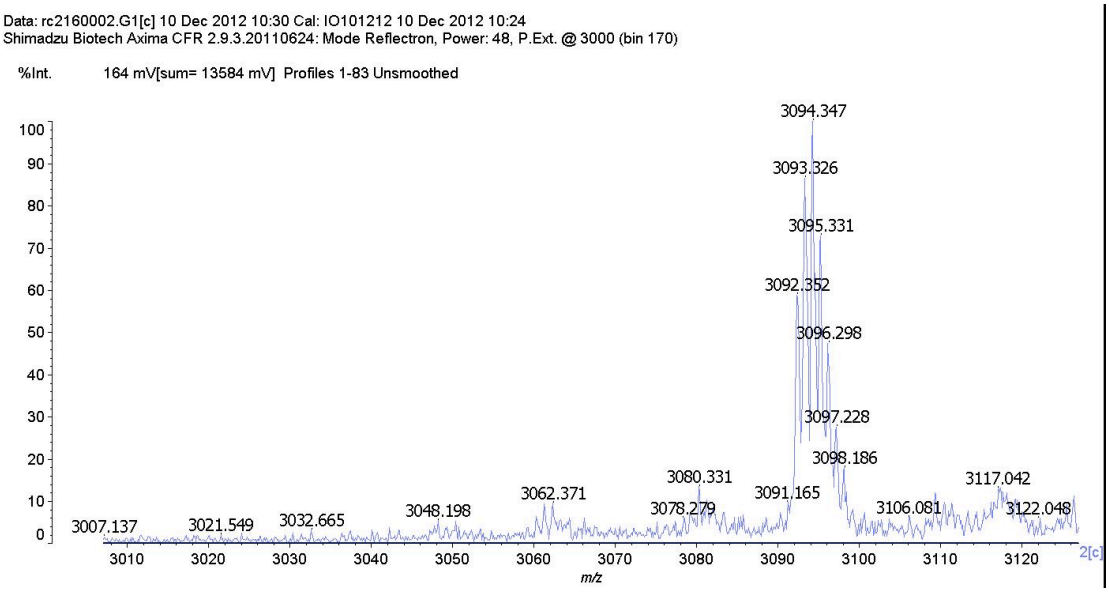


Figure SI 1. Inverso-CysHHC10 dimer MALDI-TOF spectrum. Mass calc.: 3091.757, mass found $[M+H]^+$: 3092.352

Hemolytic activity

The hemolytic activity of the peptides was determined using sheep blood alsever (Biotrading, Mijdrecht, The Netherlands). Prior to the assay, the erythrocytes were washed three times in saline solution (0.9% NaCl). The washed sheep blood alsever was diluted to an optical density at 414 nm of 0.3 in Phosphate Buffered Saline pH 7.4 (PBS). Serial 2-fold dilutions of the peptide solution (50 µL) in PBS were made in a 96-well plate, ranging from 250-3.9 µg/mL . For determination of the toxicity, 50 µL erythrocyte suspension was added to peptide dilution. The suspensions were incubated for 1 h at 37°C with gentle shaking. After cooling to room temperature and centrifugation at 2,000 rpm for

5-10 min, 25µL of the supernatant was transferred to a flatbottom microtiter plate and the optical density at 414 nm was determined (Bio-Tek µQuant, USA). Zero hemolysis (blank) and 100% hemolysis (control) were determined with cell suspensions incubated in PBS and 1% Triton X-100, respectively. Hemolysis at 15.6 µg/mL was used in article figure 1 to illustrate the hemolysis at 2.5-10 times the LC99.9 concentration.

Serum stability[9]

The serum stability of the peptides was determined in 25% (vol/vol) aqueous pooled human serum (Sanquin, Utrecht, The Netherlands). Peptides were dissolved in 1 ml 25% (vol/vol) aqueous pooled human serum to a final concentration of 150 µg/ml and incubated at 37°C. Aliquots of 95 µl taken after 0, 1, 2, 4, 8 and 24 h were precipitated by a mixture of acetonitrile, water, and formic acid (300 µl; 89:10:1 by volume). After 45 min. on ice, the samples were centrifuged (10 min, 14000 rpm, at rt), and the supernatants were concentrated *in vacuo* (Alpha RVC, Martin Christ GmbH, Germany). Addition of 200 µL of MeCN/H2O (5:95) + 0.1% TFA was followed by HPLC analysis (Gemini C18, TFA buffers). See **Figure SI 13** and **14.**

Hydrogel formation[10]

Pentaerythritol tetrakis(3-mercaptopropionate) (PTMP) (1.56 mL, 4,1 mmol), poly(ethylene glycol) diacrylate (PEGDA) (Mn 700, 6.25 mL, 10 mmol) and <0.1 wt% photoinitiator (DMPA) were mixed. Next, 0.97 mL of this mixture was diluted with MeOH (3.87 mL) and inverso-CysHHC10 (120 mg, 77.6 µmol) was dissolved in this mixture. Next, 1.5 mL of the mixture was transferred to a PET surface (Toyobo, grade A4300, 188µm) and rolled out using a K4 meter bar (Testing Machines Inc., New Castle, USA) and an area of ca. 300 cm^2 was prepared. The sample was then polymerized using a 365 nm wavelength lamp (UV-fusion Systems, D-bulb) for 5 consecutive runs (~15 sec) on conveyor (UV-Fusion Systems, DRS10/12 Conveyor Systems) to yield a clear hydrogel film.

HPLC leakage analysis

In order to assess leakage of non-incorporated peptide from the hydrogel, samples, the hydrogel coating on PET (20 x 20 mm) were transferred to a falcon tube (50 mL) and 15 mL demi-water (washing solution 1) was added and the tube was shaken for 16 hours at room temperature. After incubation, the samples were transferred to a new falcon tube. Shaking was continued in fresh demi-water to a total of 24 hours (washing solution 2). These steps were repeated after 48 and 72 hours (washing solutions 3 and 4, respectively). All washing solutions were then lyophilized separately and 1 mL of buffer A/buffer B (9:1) was added to each residue. HPLC analysis was carried out to detect non-incorporated peptide (Figure SI 2).

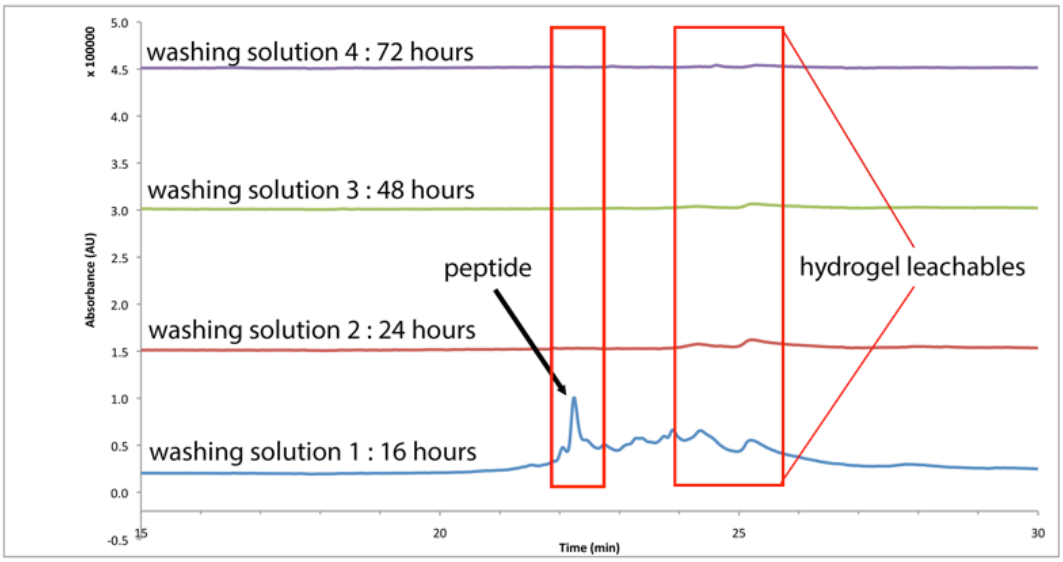


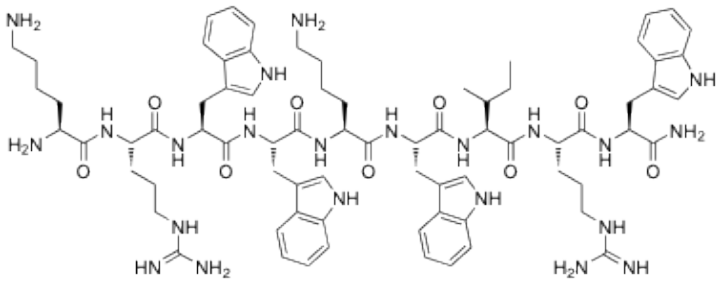
Figure SI 2. Combined HPLC chromatograms of washing solutions from hydrogels after 16, 24, 48 and 72 hours of washing of hydrogel containing 10 wt% inverso-CysHHC10.

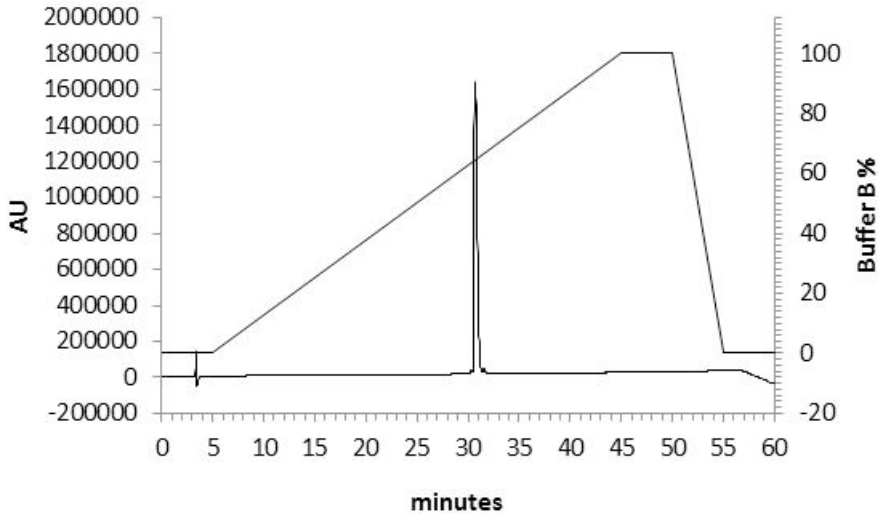
Hydrogel Antimicrobial activity

The Japanese Industrial Standard JIS Z 2801:2000[11] was used to evaluate the bactericidal activity of the hydrogel coatings (20 x 20 mm) against *S. aureus* ATCC 49230, *S. epidermidis* ATCC 35984 and *E. coli* ATCC 8739. All samples were washed to remove any non-bound peptide by shaking in 15 mL demi-water for at least 16 hours at 150 rpm at room temperature and were subsequently sterilized in 70% ethanol and dried in a sterile environment for at least 30 min prior to bacterial inoculation. Overnight cultures in TSB were diluted 100-fold in fresh

TSB and cultured for 3 h at 37°C, shaking. Bacteria were washed twice with PT, and diluted to 10^5 CFU/mL in PT. Sixty-four-microliters of this suspension, containing 6.4 x 10^3 CFU, was pipetted onto the hydrogel-coated PET samples. Sterilized parafilm (18 x 18 mm; i.e. slightly smaller than the coated surface) was placed on top of the inoculated hydrogel coatings in wells of a 6-well plate (Corning Inc., New York, USA) and incubated at 37 °C for 24 h. After incubation, 1.6 mL of 0.1 % Tween80 in phosphate buffered saline (PBS) was added to each well followed by sonication for 30 s and gently shaken for 2 min. This procedure does not affect bacterial viability. The sonicates were 10-fold serially diluted in PT and duplicate 10-µL aliquots of the undiluted and diluted suspensions were pipetted onto blood agar plates (Oxoid, Basingstoke, UK). The blood agar plates were inspected for growth after overnight incubation at 37 °C. Six parallel experiments were performed for each hydrogel coating against *S. aureus* and three against *S. epidermidis* and *E. coli*.

HHC10
H-KRWWKWIRW-NH_2
R_f : 30.7 min.
Formula: $C_{74}H_{102}N_{22}O_9$
Mass calc.: 1442.820
Mass found $[M+H]^+$: 1443.821
purity: 97.0%





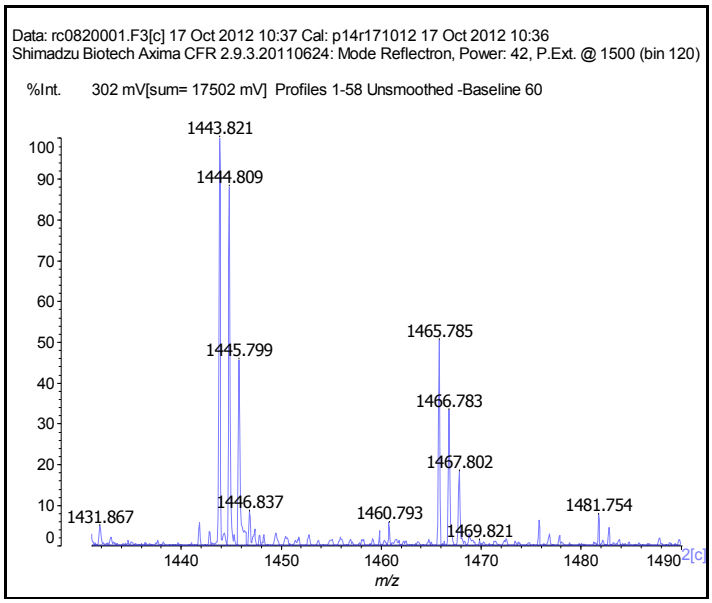
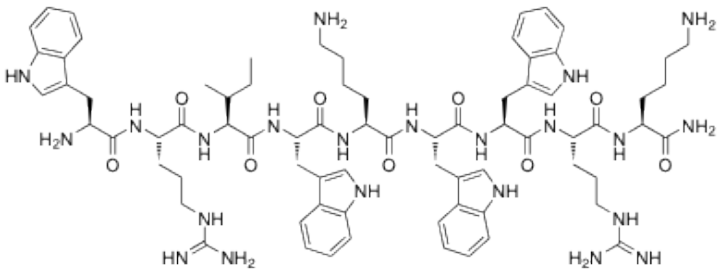
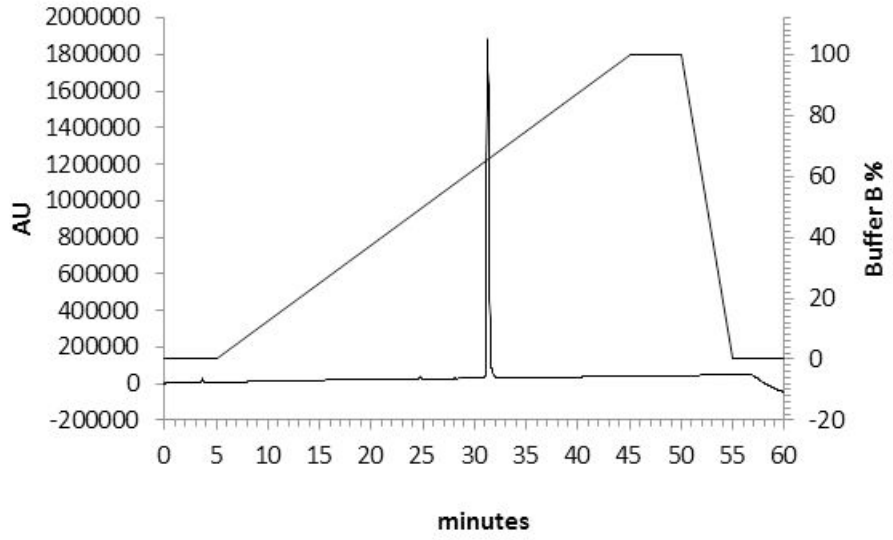


Figure SI 3. HHC10 peptide, HPLC chromatogram and MALDI-TOF spectrum.

retro-HHC10
H-WRIWKWWRK-NH_2
Formula: $C_{74}H_{102}N_{22}O_9$
R_f : 31.3 min.
Mass calc.: 1442.820
Mass found $[M+H]^+$: 1443.508
purity: >99%





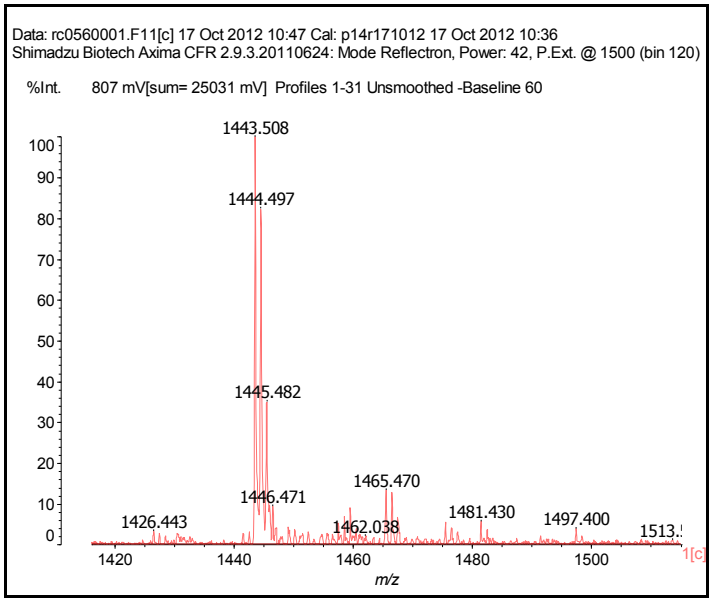
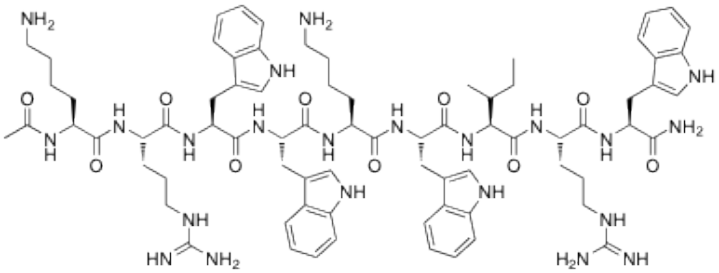
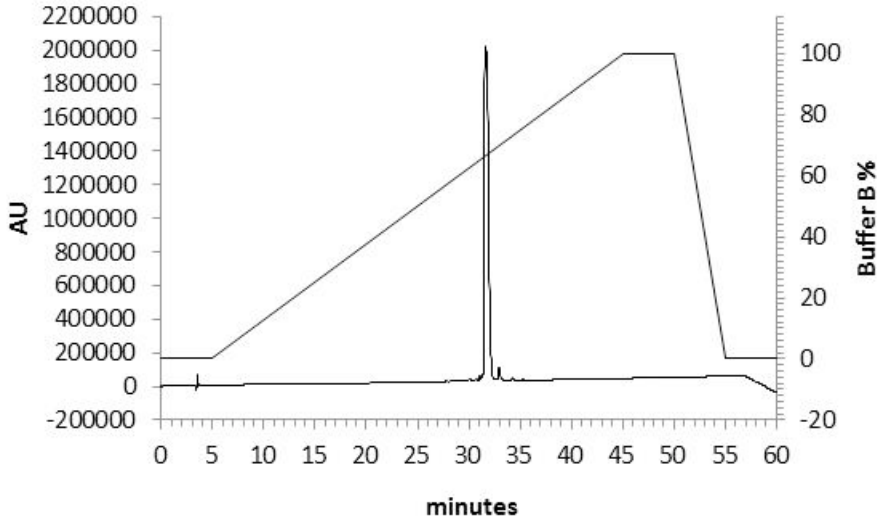


Figure SI 4. retro-HHC10 peptide HPLC chromatogram and MALDI-TOF spectrum.

Ac-HHC10
Ac-KRWWKWIRW-NH_2
Formula: $C_{76}H_{104}N_{22}O_{10}$
R_f : 31.7 min.
Mass calc.: 1484.831
Mass found $[M+H]^+$: 1485.694
purity: 96.6%





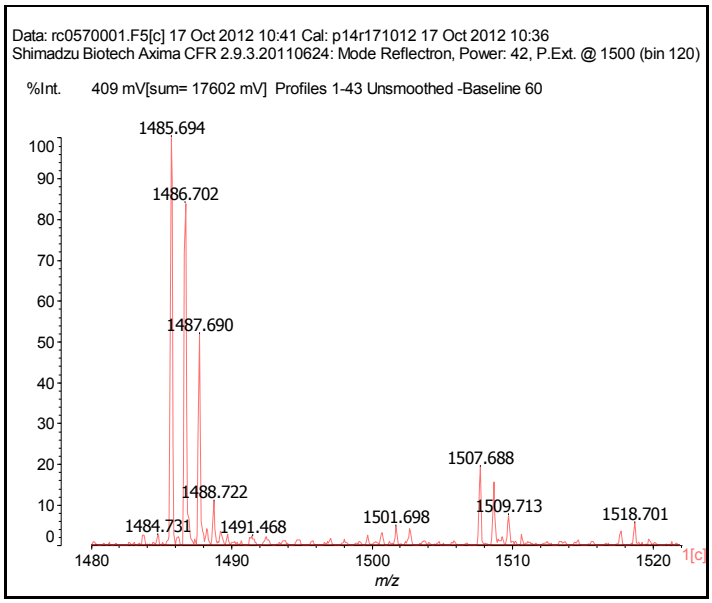
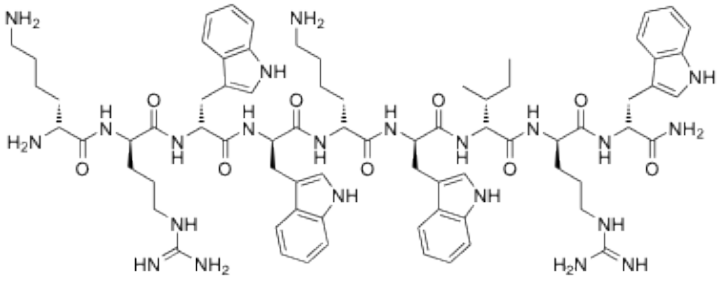
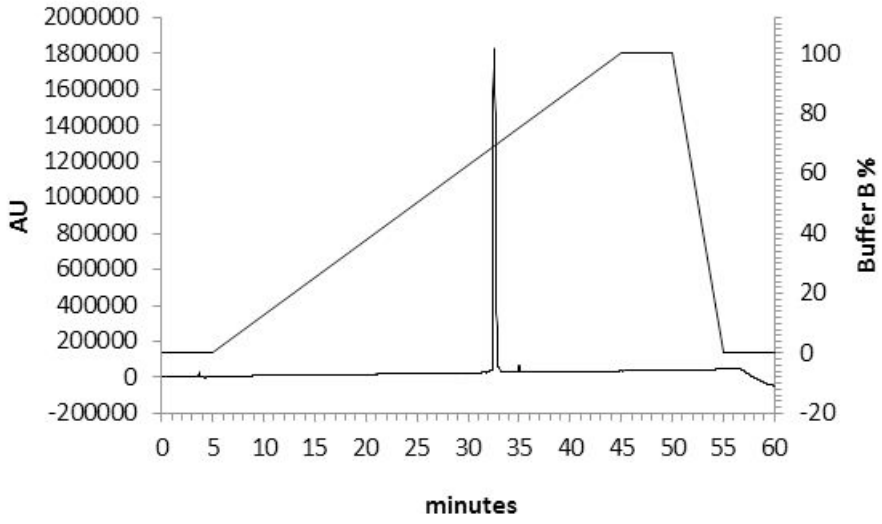


Figure SI 5. Ac-HHC10 peptide, HPLC chromatogram and MALDI-TOF spectrum.

Inverso-HHC10
H-krwwkwirw-NH_2
Formula: $C_{74}H_{102}N_{22}O_9$
R_f : 32.5 min.
Mass calc.: 1442.820
Mass found $[M+H]^+$: 1443.592
purity: 98.4%





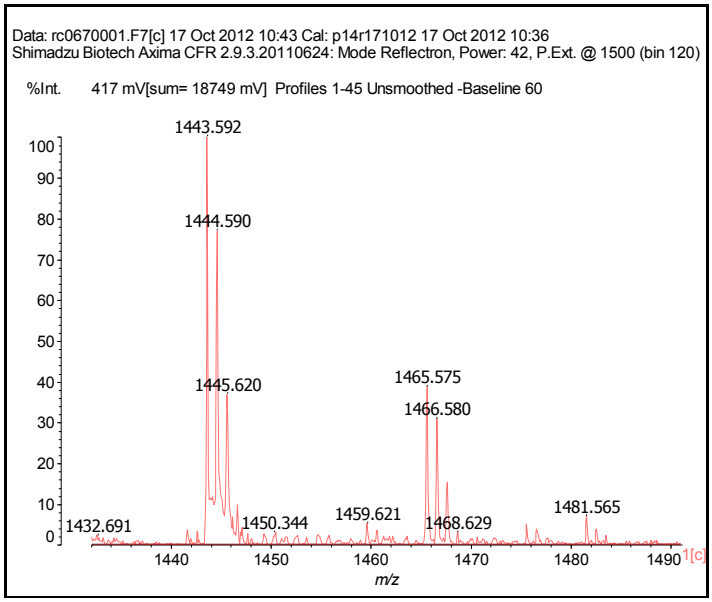
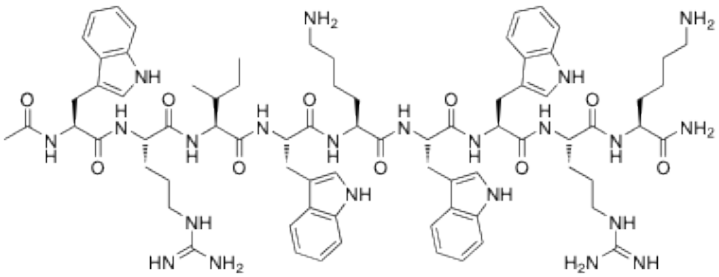
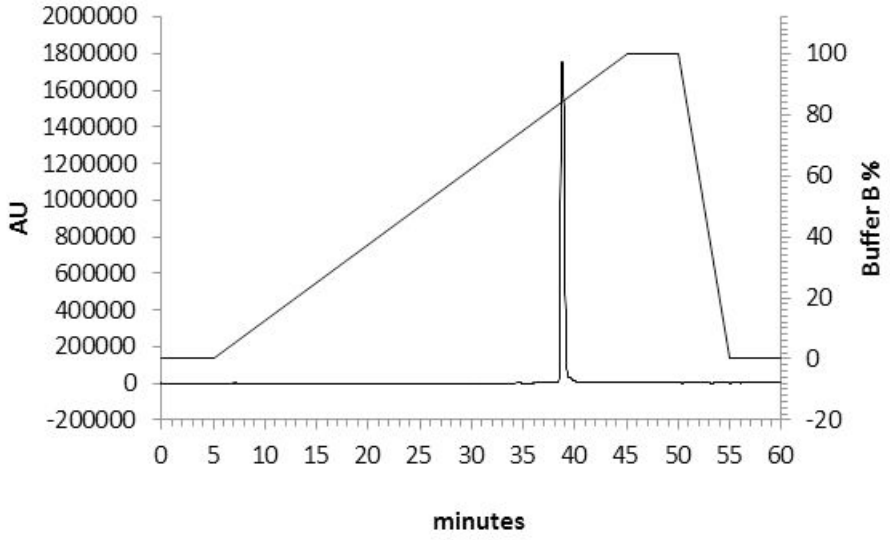


Figure SI 6. Inverso-HHC10 peptide, HPLC chromatogram and MALDI-TOF spectrum.

Ac-retro-HHC10
Ac- WRIWKWWRK -NH_2
Formula: $C_{76}H_{104}N_{22}O_{10}$
R_f : 38.8 min.
Mass calc.: 1484.831
Mass found $[M+H]^+$:
1485.412
purity: >99%





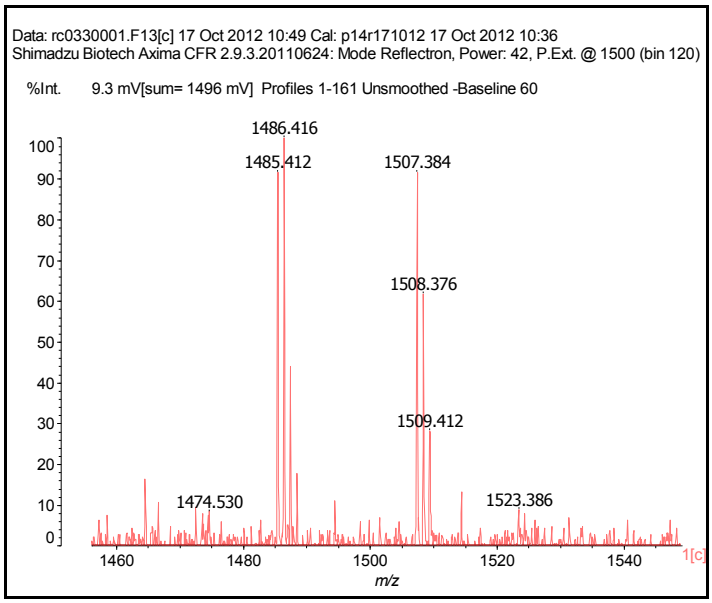
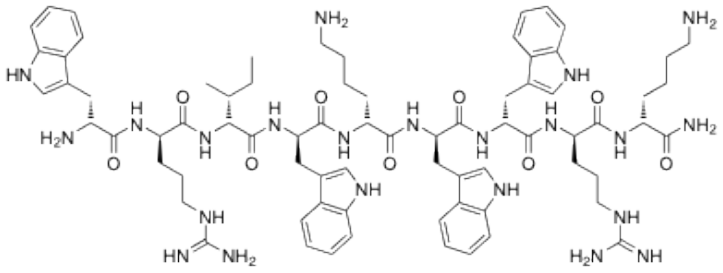
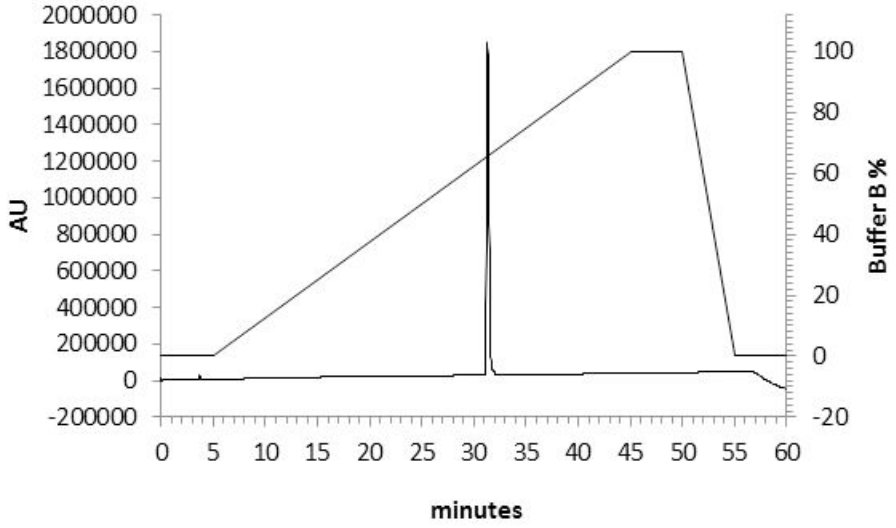


Figure SI 7. Ac-retro-HHC10 peptide, HPLC chromatogram and MALDI-TOF spectrum.

retro-inverso-HHC10
H-wriwkwwrk-NH_2
Formula: $C_{74}H_{102}N_{22}O_9$
R_f : 31.3 min.
Mass calc.: 1442.820
Mass found $[M+H]^+$:
1443.449
purity: >99%





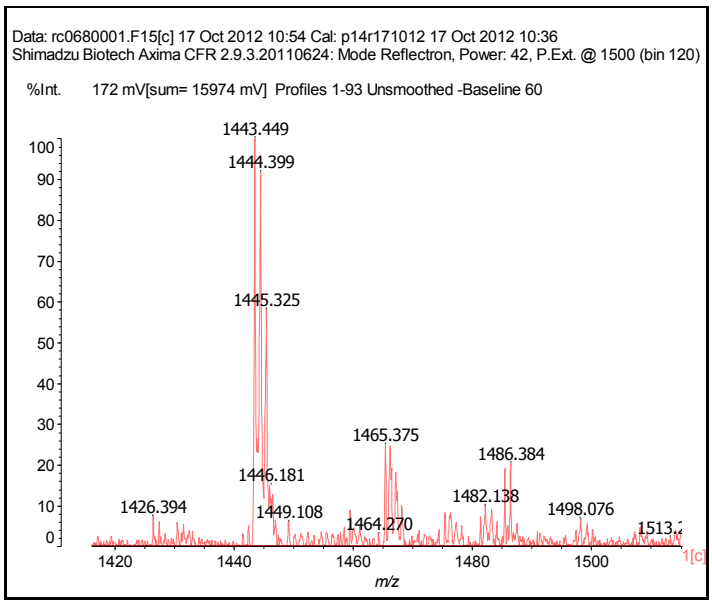
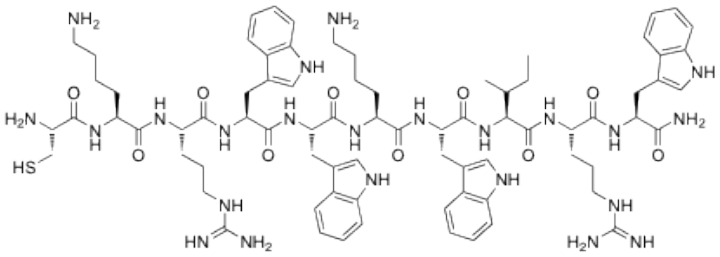
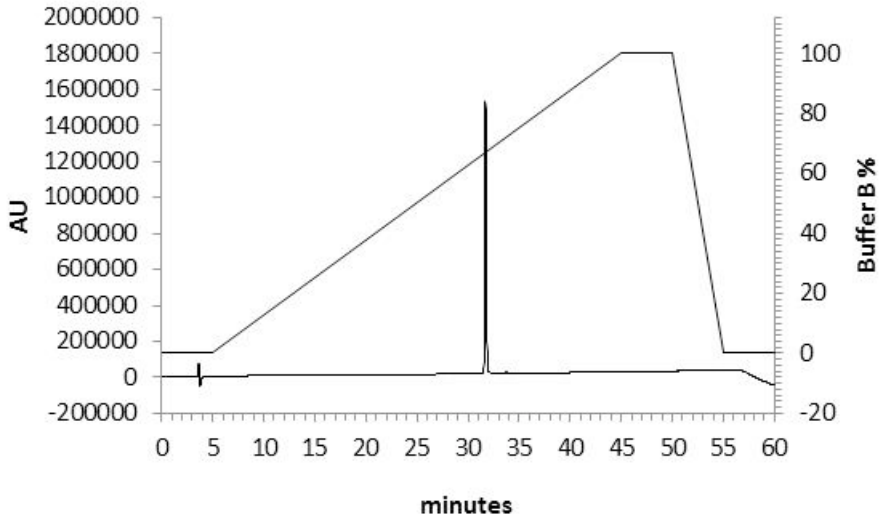


Figure SI 8. Retro-inverso-HHC10 peptide, HPLC chromatogram and MALDI-TOF spectrum.

Cys-HHC10
H-CKRWWKWIRW-NH_2
Formula:$C_{77}H_{107}N_{23}O_{10}S$
R_f : 31.7 min.
Mass calc.: 1545.829
Mass found $[M+H]^+$:
1546.216
purity: >99%





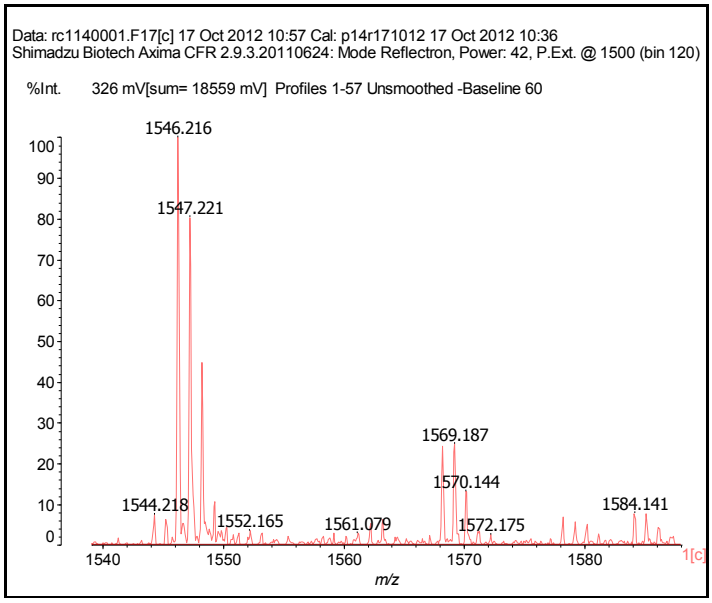
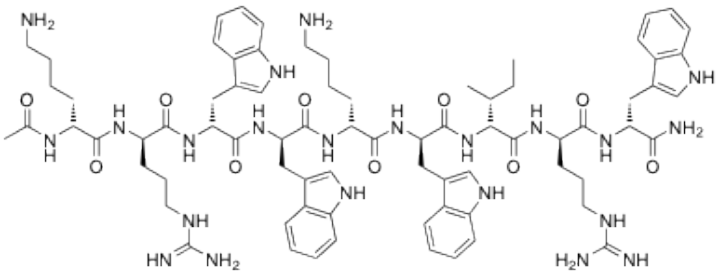
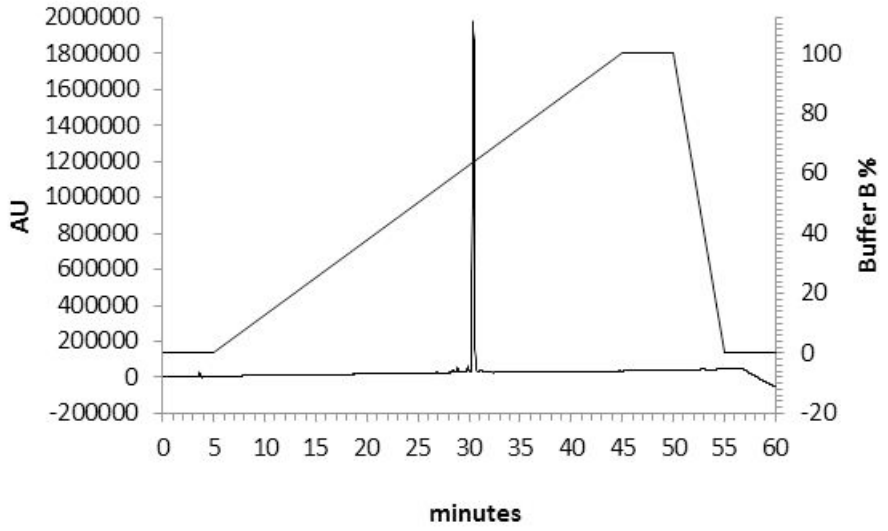


Figure SI 9. Cys-HHC10 peptide, HPLC chromatogram and MALDI-TOF spectrum.

Ac-inverso-HHC10
Ac-krwwkwirw-NH_2
Formula: $C_{76}H_{104}N_{22}O_{10}$
R_f : 30.4 min.
Mass calc.: 1484.831
Mass found $[M+H]^+$:
1485.548
purity: 95.3%





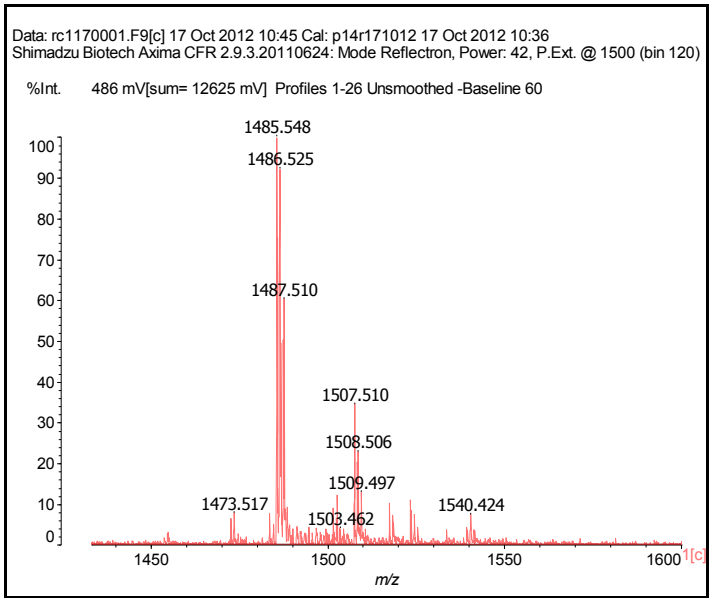
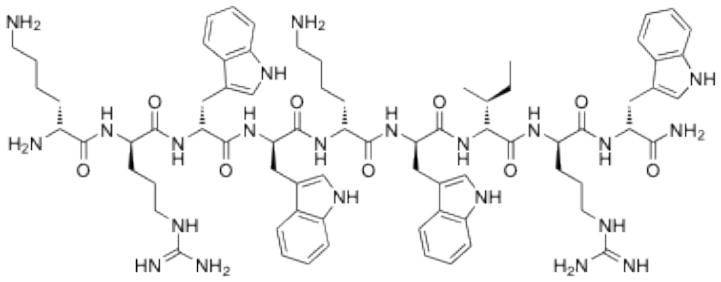
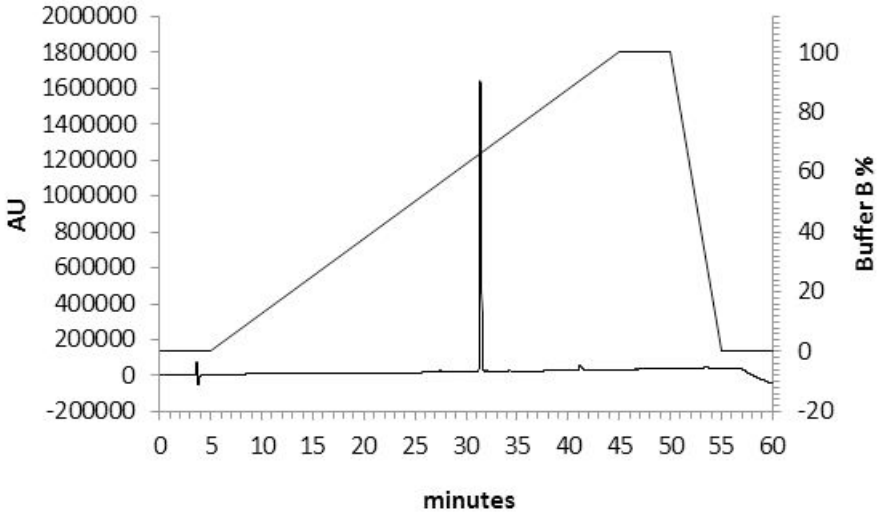


Figure SI 10. Ac-inverso-HHC10 peptide HPLC chromatogram and MALDI-TOF spectrum.

inverso-d-allo-HHC10
H-krwwkwirw-NH_2
Formula: $C_{74}H_{102}N_{22}O_9$
R_f : 31.4 min.
Mass calc.: 1442.820
Mass found $[M+H]^+$: 1443.209
purity: 95.4%





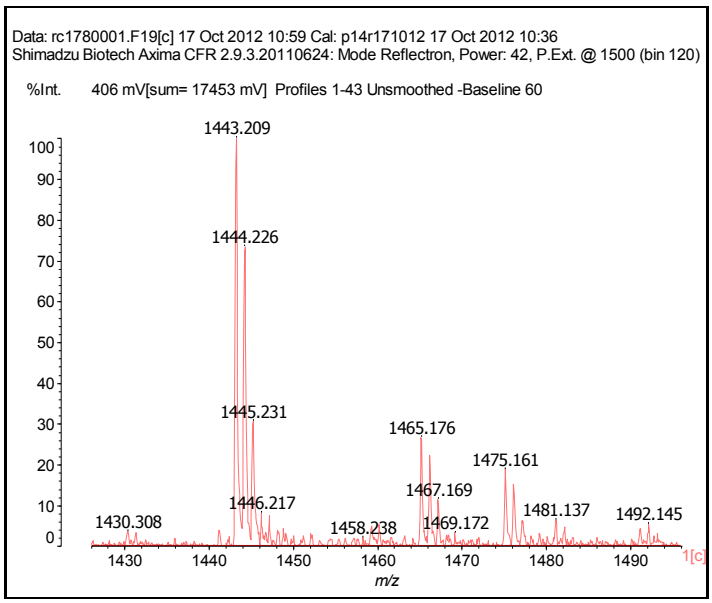
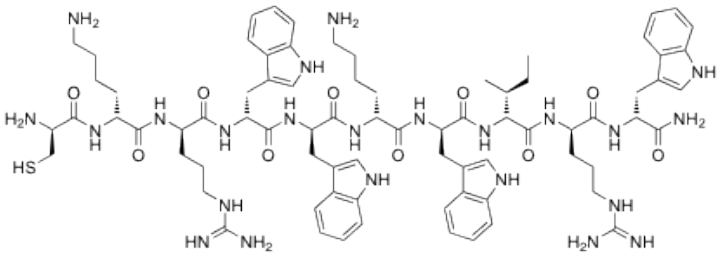
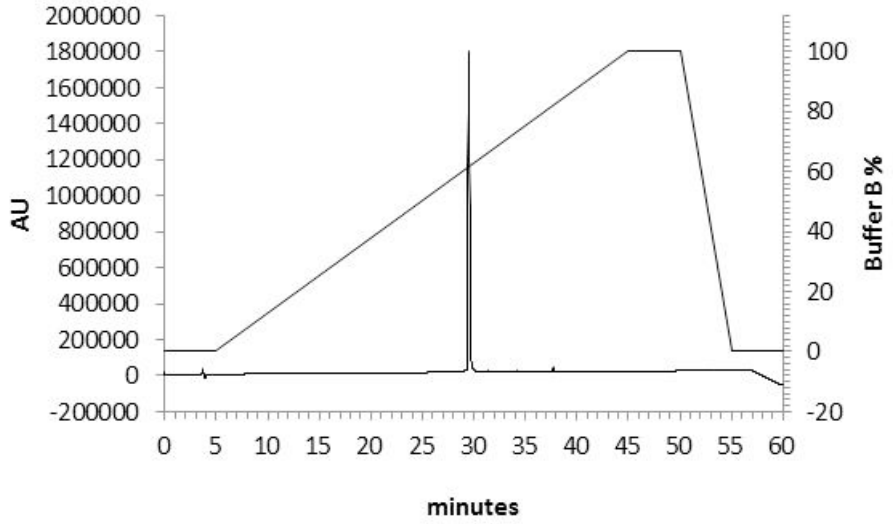


Figure SI 11. Inverso-D-allo-HHC10 peptide, HPLC chromatogram and MALDI-TOF spectrum.

inverso-CysHHC10
H-ckrwwkwirw-NH_2
Formula:$C_{77}H_{107}N_{23}O_{10}S$
R_f : 29.5 min.
Mass calc. : 1545.829
Mass found $[M+H]^+$:
1546.047
purity: >99%





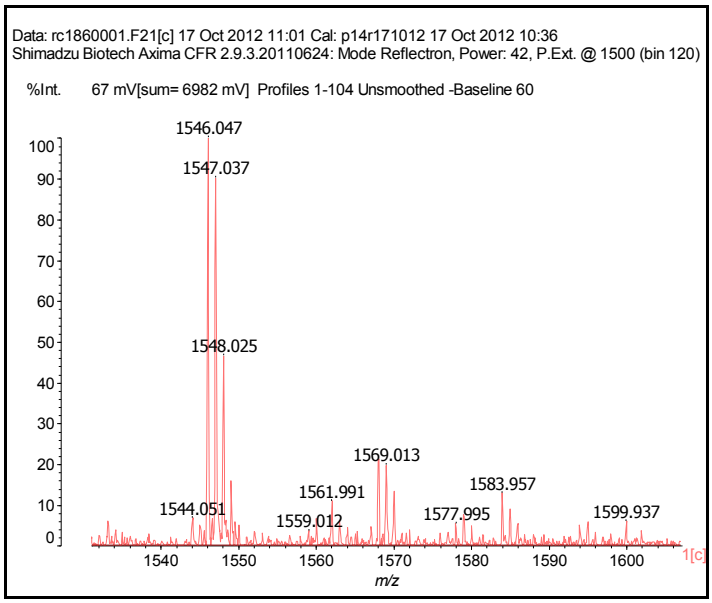


Figure SI 12. Inverso-CysHHC10 peptide, HPLC chromatogram and MALDI-TOF spectrum.

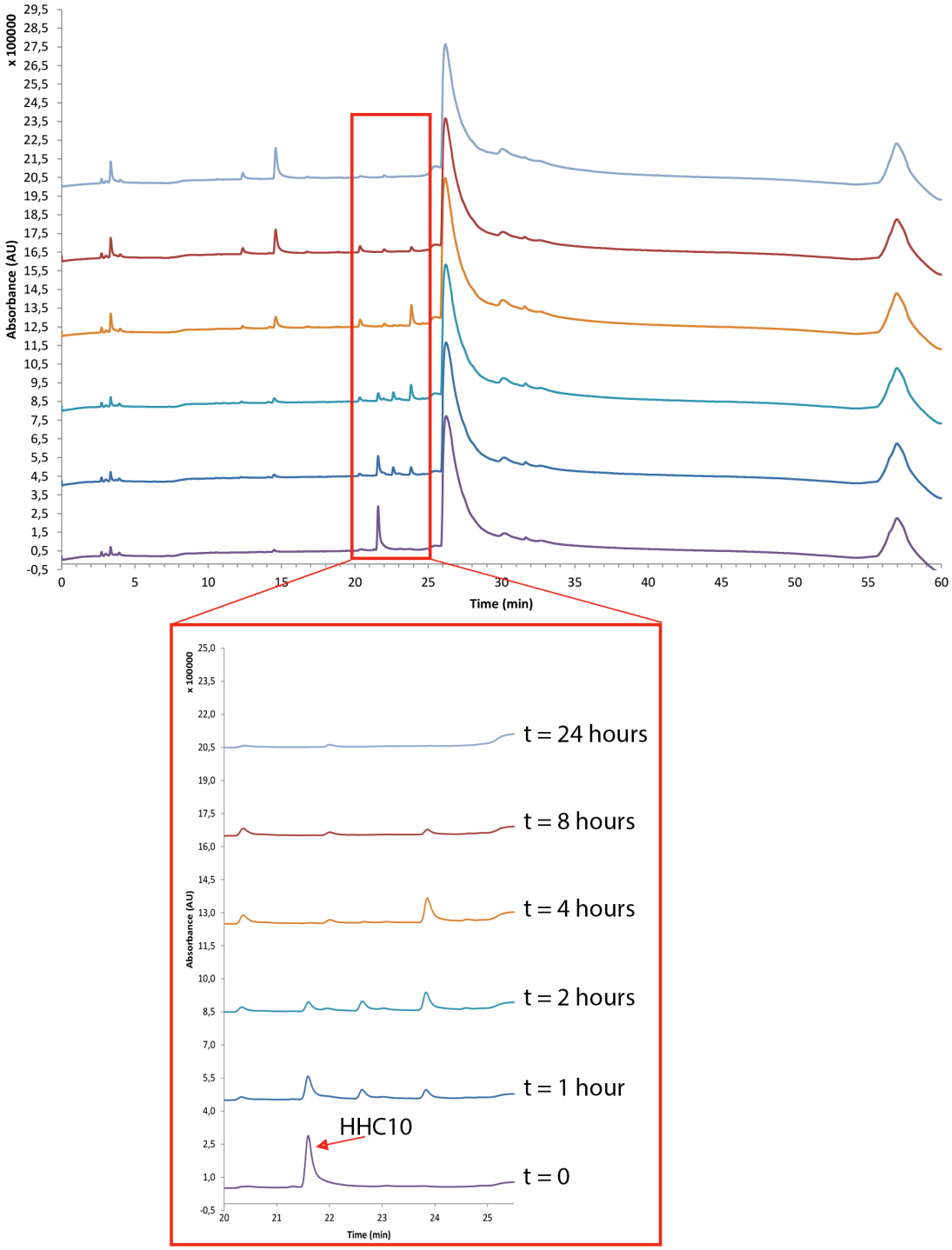


Figure SI 13. Stability of HHC10 in serum. Combined HPLC chromatograms of HHC10 after incubation in serum for 0, 1, 2, 4, 8 and 24 hours .

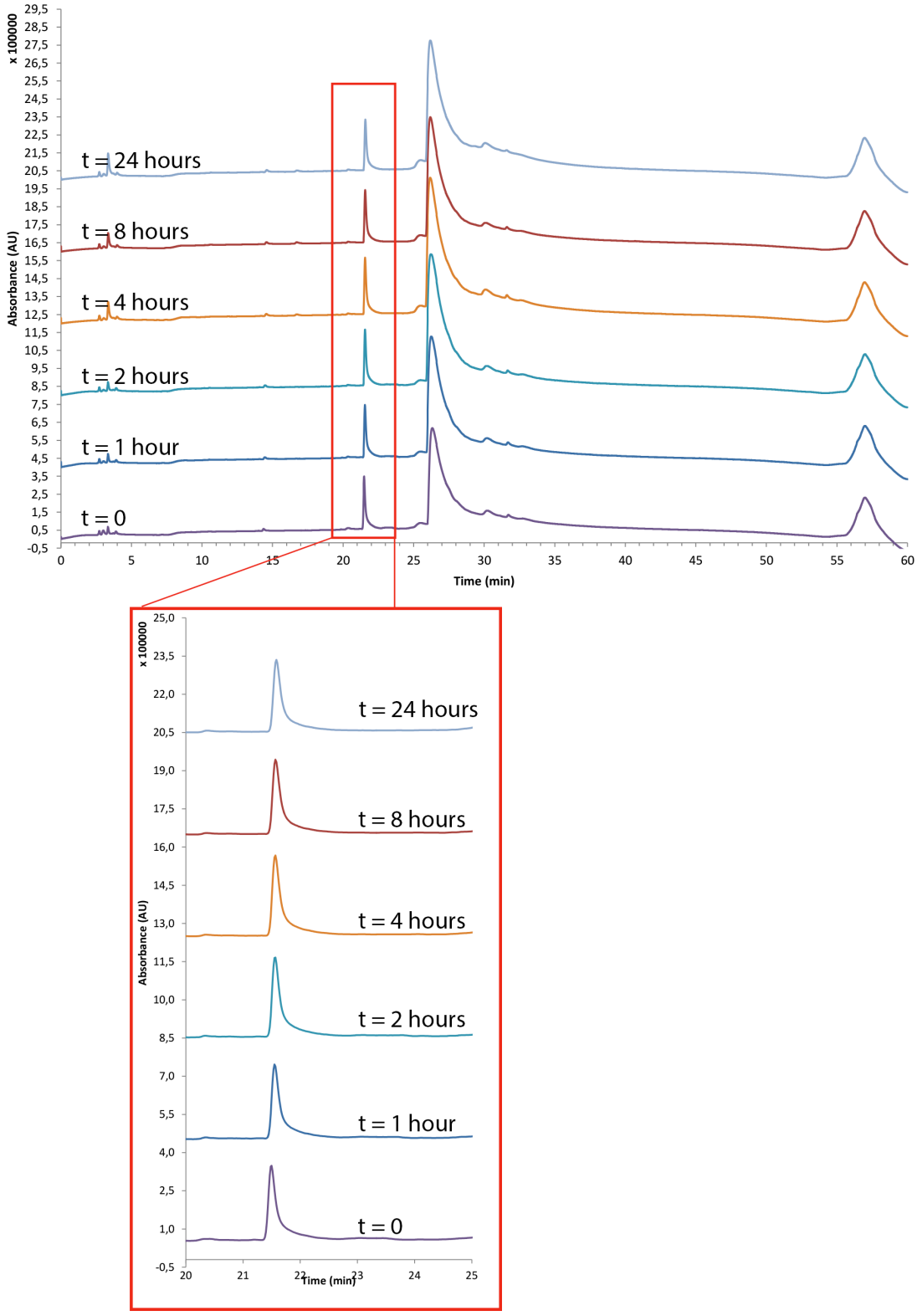


Figure SI 14. Stability of inverso-HHC10 in serum. Combined HPLC chromatograms of HHC10 after incubation in serum for 0, 1, 2, 4, 8 and 24 hours

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