Supporting Information

Activation of cell-penetrating peptides by disulfide bridge formation of truncated precursors

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1. Experimental Section

Reagents

NovaPEG Rink amide resin and polystyrene Rink amide resin were purchased from Novabiochem and Fmoc-L-amino acids from Bachem (Bubendorf, Switzerland) or Novabiochem (EMD Chemicals, Gibbstown, USA). Boc-Cys(Npys) was obtained from Bachem (Bubendorf, Switzerland). Fmoc-L-γ-azidohomoalanine was purchased from Chiralix (Nijmegen, The Netherlands). Fmoc-ε-aminohexanoic acid was purchased from Novabiochem and Fmoc-8-aminooctanoic acid from Sigma Aldrich. Dulbecco's modified Eagle medium, fetal bovine serum, and RPMI were obtained from Invitrogen, Eugene, U.S.A.). The cell counting kit (CCK-8) was obtained from Dojindo Molecular Technologies (Rockville, U.S.A.). Carboxyfluorescein-labelled nona-arginine was purchased from EMC microcollections (Tübingen, Germany). All other chemicals were purchased from Baker, Fluka or Sigma Aldrich and used as received.

Peptide synthesis

General peptide Synthesis

Peptides were synthesised on NovaPEG Rink amide or polystyrene Rink amide resins using a Labortec640 peptide synthesizer (Labortec, Bubendorf, Schwitzerland), employing a standard Fmoc solid-phase peptide synthesis (SPPS) protocol. In brief, the resin was swollen in DMF for 30 minutes prior to use. The Fmoc protecting groups were removed by washing the resin with piperidine in DMF (20%, v/v) and then shaking it 3 times for 6 minutes with another portion of piperidine in DMF. The desired sequence of amino acids was coupled to the resin using Fmoc-L-amino acids (3.0 equiv), diisopropylcarbodiimide (DIPCDI, 3.3 equiv) and N-hydroxy benzotriazole (HOBt, 3.6 equiv). Peptide couplings were monitored using the Kaiser test. After the final Fmoc removal the resin was washed with DMF, DCM, i-PrOH, DCM, i-PrOH and air-dried for at least 2 h.

Synthesis of fluorescein-labeled peptides

The following peptides were synthesised using standard SPPS as described above: R3 (FITC-AhxArgArgArgCys-NH_2), R4 ((FITC-AhxArgArgArgArgCys-NH_2), R5 ((FITC-AhxArgArgArgArgArgCys-NH_2),R8 ((FITC-AhxArgArgArgArgArgArgArgArg-NH_2), R4-Aoc-R5 (FITC-AhxArgArgArgArgAocArgArgArgArgArg-NH_2) and R5-Aoc-R4 (FITC-AhxArgArgArgAocArgArgArgArgArgArg-NH_2) For these peptides, the last coupling step was performed using fluorescein isothiocyanate (FITC, 2 equiv.) and *N,N*-diisopropylethylamine (DIEA, (3 equiv.) in DMF/DCM (1:1 v/v). The reaction was monitored by using the Kaiser test.[1] The peptides were cleaved from the resin by suspension in a mixture of trifluoroacetic acid/water/triisopropyl-silane/thioanisole (90:5:2.5:2.5 v/v/v/v) for 6 h. The free peptides were precipitated in Et_2O, redissolved in water and lyophilised yielding the crude peptides. The crude peptides were purified by reversed phase HPLC and subsequently lyophilised yielding a yellow powder. Purity was evaluated by analytical reversed-phase HPLC and identity confirmed by mass spectrometry.

Synthesis of activating peptides
The following peptides were synthesised using standard SPPS as described above:
*CR3 (C(Npys)ArgArgArg-NH_2); *CR4 (C(Npys)ArgArgArg-NH_2)) and *CR5 (C(Npys)ArgArgArg-NH_2). The last coupling was performed using 3 equiv. Boc-Cys(Npys), 3.3 equiv DIPCDI and 3.6 equiv HOBt in DMF. The peptides were cleaved from the resin by suspension in a mixture of trifluoroacetic acid/water/triisopropyl-silane(92.5:5:2.5 v/v/v) for 6 h. The free peptides were precipitated in Et_2O, redissolved in water and lyophilised yielding the crude peptides. The crude peptides were purified by reversed phase HPLC yielding a yellow powder after lyophilisation. Purity was evaluated by analytical reversed phase HPLC and identity confirmed by mass spectrometry.

Preparation of extended peptides
Disulfide linkage between the activating and truncated peptides was achieved by dissolving the truncated peptide in a 0.1 M degassed sodium phosphate buffer (pH 7.4) and subsequently mixing it with 1.1 equiv. activating peptide. During this reaction, the final peptide concentration was 1 to 5 mM. The reaction was monitored by reversed-phase HPLC. At the end of the reaction, the resulting peptides were purified by reversed-phase HPLC on a C18 column, using a linear gradient of acetonitrile in an aqueous solution containing 0.1% (v/v) TFA. After lyophilisation, the peptides were obtained as yellow powders. All peptides were obtained with a purity > 95%, as assessed by analytical reversed phase HPLC.

Synthesis of triazole-linked peptide R4-t-R4
R4-Ppg and Azh-R4 were synthesised using standard SPPS as described above. The copper-medicated click-reaction between the two peptides was performed by dissolving 0.99 µmol of both peptides together with 24 µmol $CuSO_3 \cdot 5H_2O$ in water (1 mL). Lutidine (49.4 µmol) was suspended in the reaction mixture and stirred under Ar over night. Sodium ascorbate (20.0 µmol) was added and the reaction was stirred for another night. The crude peptide was purified by reversed-phase HPLC on a C18 column, using a linear gradient of acetonitrile in an aqueous solution containing 0.1% (v/v) TFA. After lyophilisation, the peptide was obtained as a yellow powder with a purity of > 95% as assessed by analytical reversed phase HPLC.

Peptide Characterisation
Mass spectrometry
Mass spectra were acquired on a Thermo Finnigan LCQ-Advantage MAX ESI-ion trap.
R3 ESI-ion trap m/z: $[M+H]^+$ 1105.2 (calcd. 1104.7)
R4 ESI-ion trap m/z: $[M+H]^+$ 1260.3 (calcd. 1260.8)
R5 ESI-ion trap m/z: $[M+H]^+$ 1417.4 (calcd. 1416.9)
R8 ESI-ion trap m/z: $[M+H]^+$ 1782.3 (calcd. 1781.9); $[M+2H]^{2+}$ 891.6 (calcd. 891.5)
R4-Aoc-R5 ESI-ion trap m/z: $[M+2H]^{2+}$ 969.7 (calcd. 969.5); $[M+3H]^{3+}$ 646.7 (calcd. 646.4)
R5-Aoc-R4 ESI-ion trap m/z: $[M+2H]^{2+}$ 969.8 (calcd. 969.5); $[M+3H]^{3+}$ 646.7 (calcd. 646.4)
R4-t-R4 ESI-ion trap m/z: $[M+H]^+$ 1504.3 (calcd. 1503.9); $[M+2H]^{2+}$ 752.8 (calcd. 752.5)
***R3** ESI-ion trap m/z: $[M+H]^+$ 741.8 (calcd. 742.3)
***R4** ESI-ion trap m/z: $[M+H]^+$ 897.9 (calcd. 898.4)
***R5** ESI-ion trap m/z: $[M+H]^+$ 1417.4 (calcd. 1416.9)
R4-R3 ESI-ion trap m/z: $[M+2H]^{2+}$ 923.9 (calcd. 924.7); $[M+3H]^{3+}$ 616.5 (calcd. 616.7)
R3-R5 ESI-ion trap m/z: $[M+2H]^{2+}$ 1002.8 (calcd. 1002.5); $[M+3H]^{3+}$ 668.5 (calcd. 668.7)
R4-R4 ESI-ion trap m/z: $[M+2H]^{2+}$ 1002.7 (calcd. 1002.5); $[M+3H]^{3+}$ 668.5 (calcd. 668.7)
R4-R5 ESI-ion trap m/z: $[M+2H]^{2+}$ 1080.4 (calcd. 1080.6); $[M+3H]^{3+}$ 720.5 (calcd. 720.7)
R5-R3 ESI-ion trap m/z: $[M+2H]^{2+}$ 1002.6 (calcd. 1002.5); $[M+3H]^{3+}$ 668.6 (calcd. 668.7)
R5-R4 ESI-ion trap m/z: $[M+2H]^{2+}$ 1080.4 (calcd. 1080.6); $[M+3H]^{3+}$ 720.6 (calcd. 720.7)
R5-R5 ESI-ion trap m/z: $[M+2H]^{2+}$ 1158.3 (calcd. 1158.7); $[M+3H]^{3+}$ 772.6 (calcd. 772.7)

Reversed phase HPLC data
Analytical HPLC was performed on a Shimadzu LC-20A Prominence system (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a C18 ReproSil column, 150x3 mm, particle size 3 µm (Screening Devices, Amersfoort, The Netherlands). Elution of the peptides was achieved with acetonitrile/water gradient containing 0.1% trifluoroacetic acid (5-100%, 1-40 min, flow 0.4 mL/min). HPLC traces can be found in part 3 of this ESI.

Cell culture and cell viability assays

Cell culture

HeLa cells were maintained in sterile conditions in DMEM or RPMI (Gibco, Invitrogen, Eugene, U.S.A.) supplemented with 10 % heat-inactivated fetal calf serum (FCS; PAN Biotech). All cells were incubated at 37 ºC in a humidified atmosphere of 5 % CO_2. Cells were passaged every 2 to 3 days.

Cell viability assay

Cell viability was evaluated using Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, U.S.A). HeLa cells were seeded in 96-well plates to obtain 40,000 cells/well the day of the experiment. On the day of the experiment, cells were incubated with 100 µL peptide solution in DMEM for 30 minutes at 37°C. Cells were washed 3 times with DMEM + 10% FCS and maintained in this solution after the last washing step. After 4 hours of incubation, the supernatant was removed and a solution of CCK-8 (10 % in DMEM + 10% FCS) was added. After 3 hours of incubation at 37 °C, the absorbance was measured at 450 nm using a microplate reader (Wallac Victor 1420 multilabel counter, Perkin Elmer). The experiments were performed in triplicates and repeated twice independently. A value of 100% survival was obtained by measuring cells that were not incubated with peptides and viability of cells treated with the peptides was calculated from this. Mean values +/- SEM are given in figure S4.

Cellular uptake experiments

Flow cytometry

HeLa cells were seeded in 24-well plates (Sarstedt, Numbrecht, Germany) one (80,000 cells/well) or two (40,000 cells/well) days prior to the experiment. On the day of the experiment, cells were incubated with the peptide solutions (5 µM or 20 µM) for 30 min at 37°C in RPMI + 10% FCS. After washing the cells with HBS buffer pH 7.4 (10 mM HEPES, 135 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1.8 mM $CaCl_2$), cells were detached by trypsinisation for 5 minutes, spun down and resuspended in 200 µL RPMI + 10% FCS. The fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences, Erembodegem, Belgium) and subsequently data was analyzed with the Summit software (Fort Collins, USA). Results were based on 10,000 gated cells.

Confocal laser scanning microscopy

HeLa cells were seeded in chambered coverslips (Nunc, Wiesbaden, Germany) at a density of 40,000 cells (one day) or 20,000 cells per well (two days prior) to the experiment. Cells were incubated with the indicated peptide concentrations for 30 min at 37 ºC. Cells were washed twice after incubation with RPMI + 10 % FCS and living cells were analysed immediately by confocal microscopy using a TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an HCX PL APO 63 x N.A. 1.2 water immersion lens. Cells were maintained at 37 ºC on a temperature-controlled microscope stage. Fluorescein was excited by an argon laser at 488 nm and emission was collected between 500 and 550 nm. The images obtained during these experiments are shown in figures 2, 3, S6 and S7.

Stability of the peptides

Fluorescence correlation spectroscopy

Peptide samples (5 µM) were prepared in HBS supplemented with 10% FCS and heated to 37ºC using a heat block. Aliquots (5 µL) were collected at the indicated time points, diluted 20 times with protease inhibitor cocktail and were directly frozen (Roche, Mannheim, Germany). After 8h proteinase K (2 mg/mL) (Roche, Mannheim, Germany) was added to the remaining peptide solution and incubated for 5 h at 37ºC. After this incubation, the sample was treated as the previous samples. For FCS measurements, 384 well plates were coated with 0.1% BSA (Roche, Mannheim, Germany) for 30 min and washed twice with PBS. Samples were thawed on ice and diluted 200 times with Tris buffer pH 8.8. 20 µL of this diluted solution was taken for FCS measurement. Measurements (per samples 5 measurements of 20 s) were performed on a TCS SP5 confocal microscope (Leica Microsystems) equipped with a dual channel FCS unit using an Argon laser for the excitation of fluorescein at 488 nm. Data was analyzed with a 2 component fit using the ISS VISTA software and is shown in figure S5

2. Supporting Figures

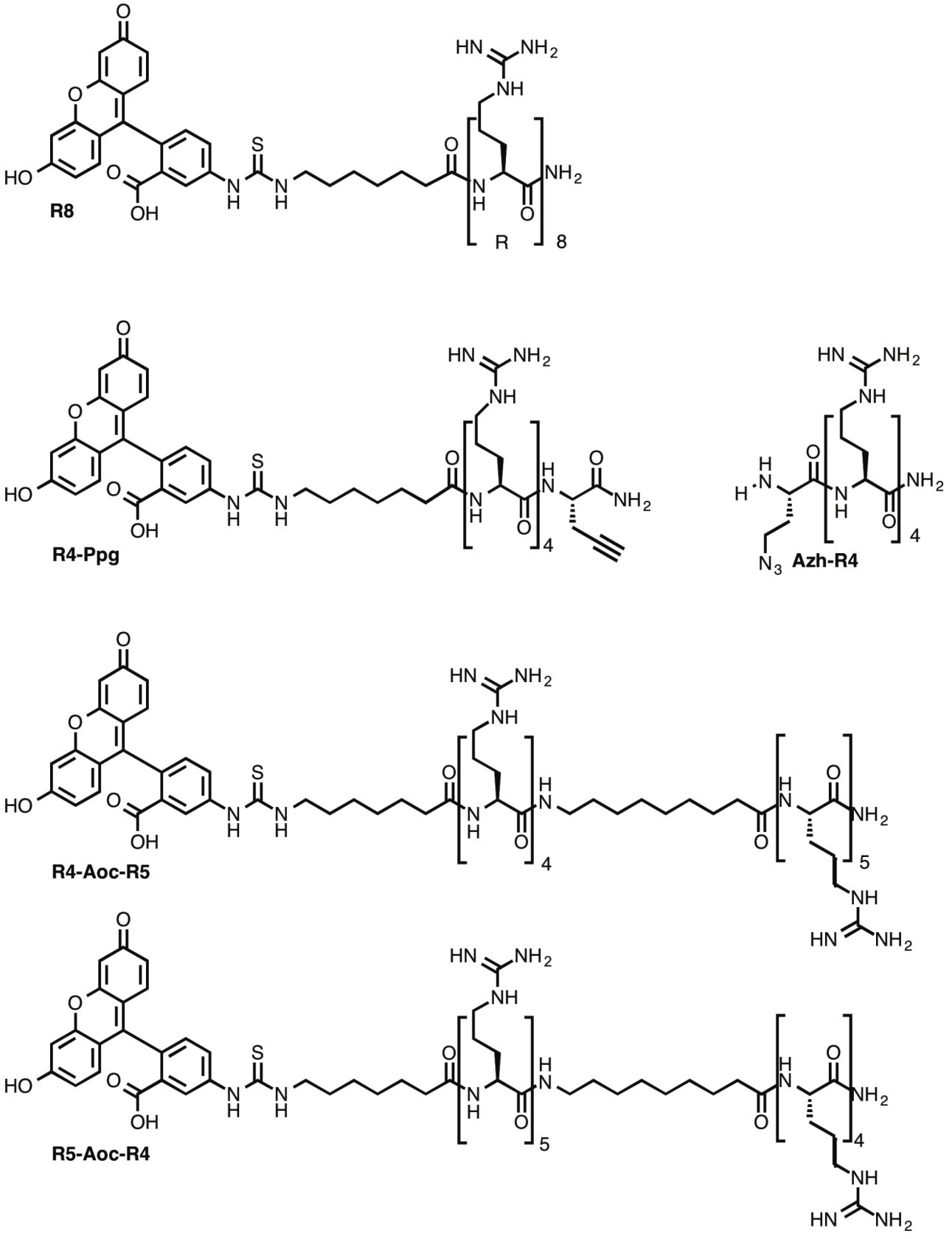


Figure S1 - Structures for the peptides that were not displayed in the main text, octa-arginine (R8), the two precursors from which R4-t-R4 was synthesised (R4-Ppg and Azh-R4) and the amino-octanoic acid inked analogues for R4-R5 and R5-R4, namely R4-Aoc-R5 and R5-Aoc-R4.

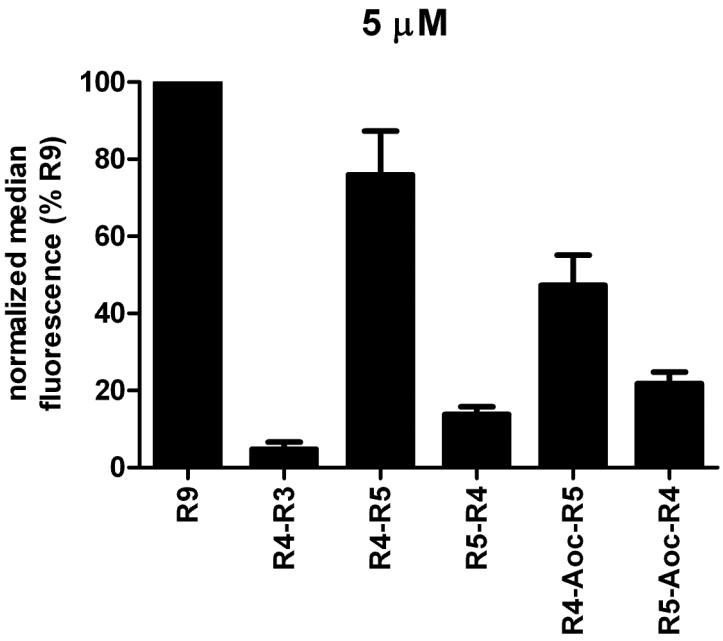


Figure S2 - Additional flow cytometry data of the uptake experiments at 5 μM, 30 minutes incubation at 37ºC.

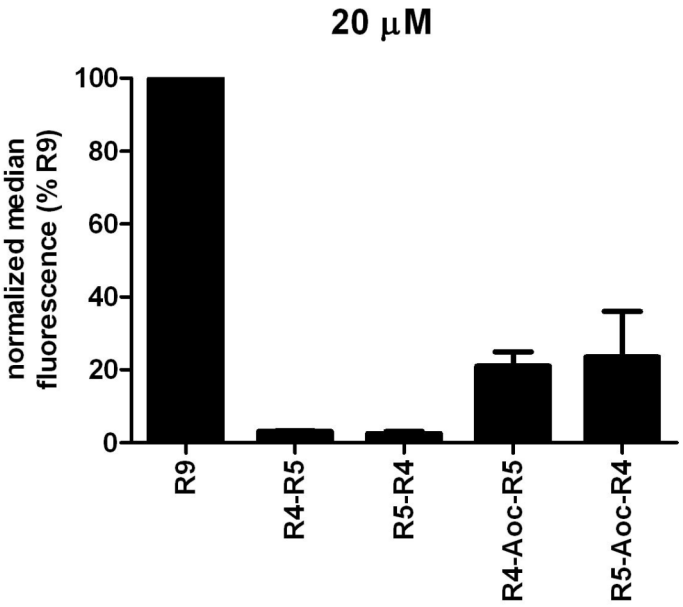


Figure S3 - Additional flow cytometry data of the uptake experiments at 20 μM, 30 minutes of incubation at 37ºC.

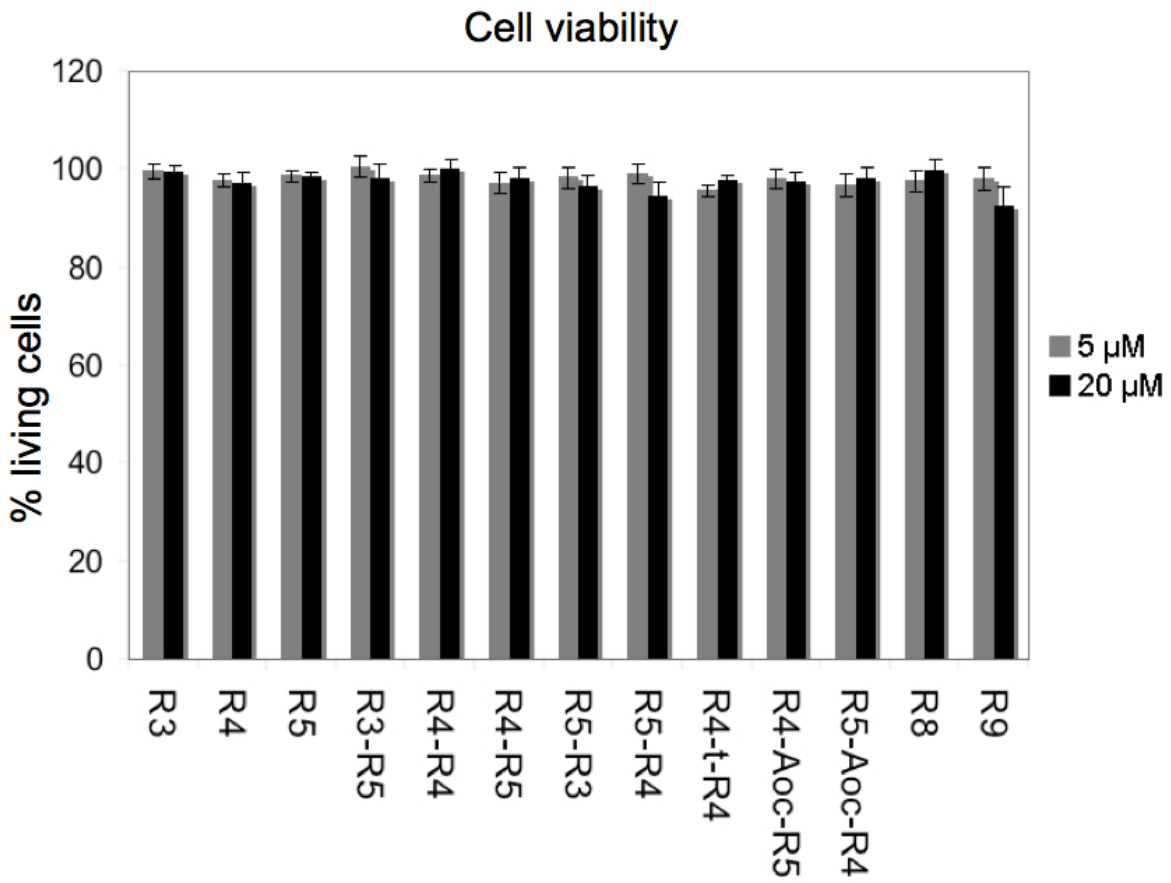


Figure S4 – Results of cell viability studies at 5 and 20 *µ*M.

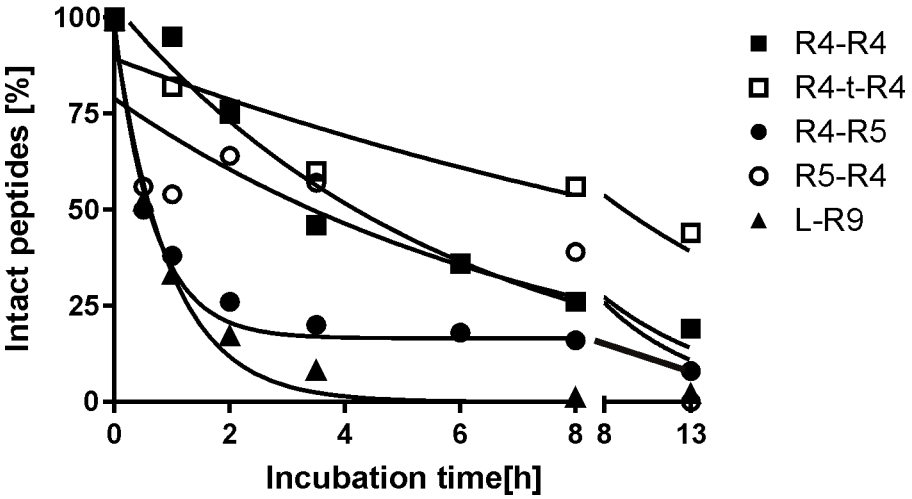


Figure S5 – Results for the FCS experiment to test the stability of peptides R4-R4, R4-t-R4, R4-R5, R5-R4 and R9 in serum. This data indicates that the length of the two arginine modules may have an impact on proteolytic stability. However, it can not explain the observed differences in uptake among the extended peptides, as all of these peptides are equally or more stable in serum than control peptide R9.

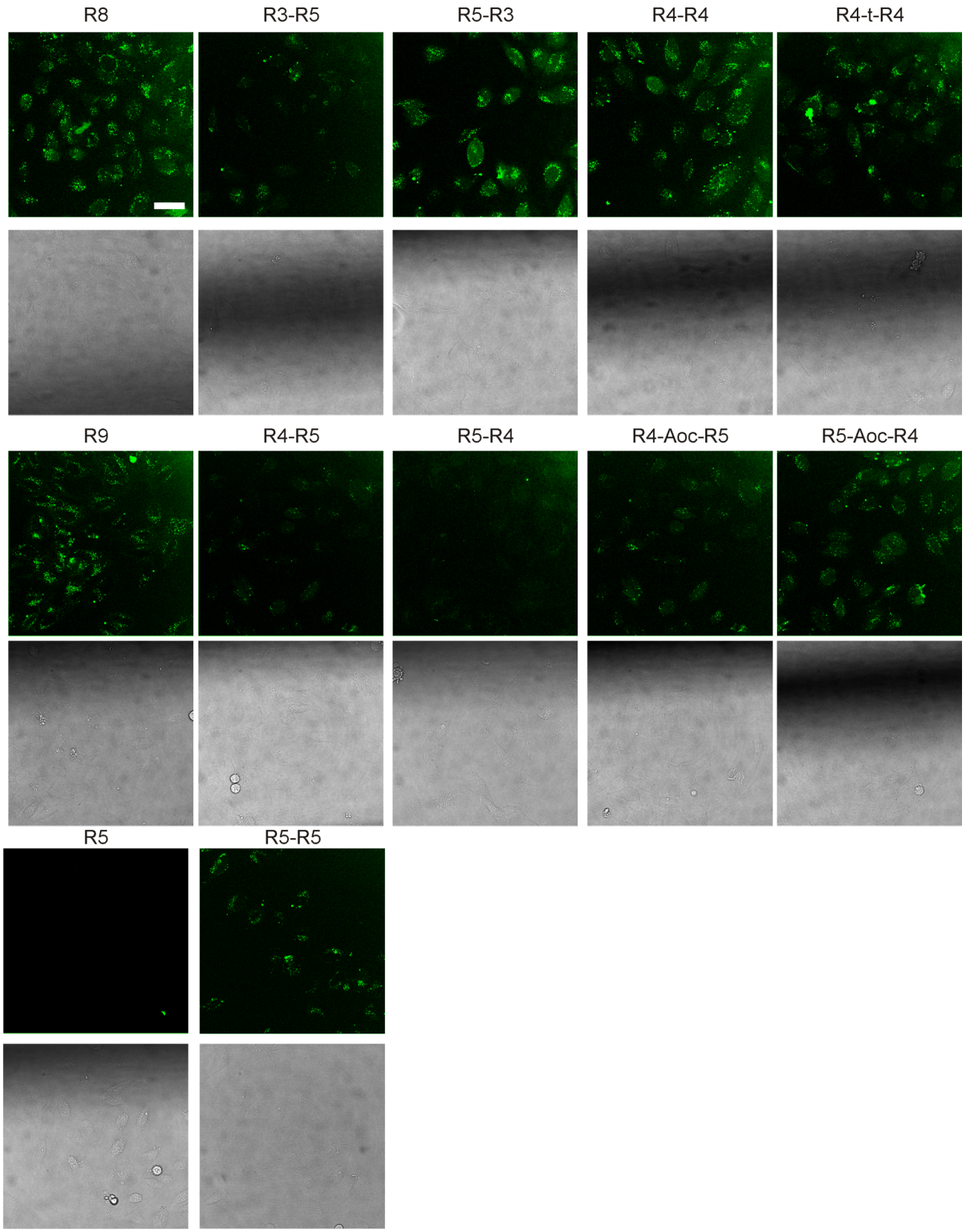


Figure S6 – Uptake experiment at 5 μM. Scale bar represents 40 μm

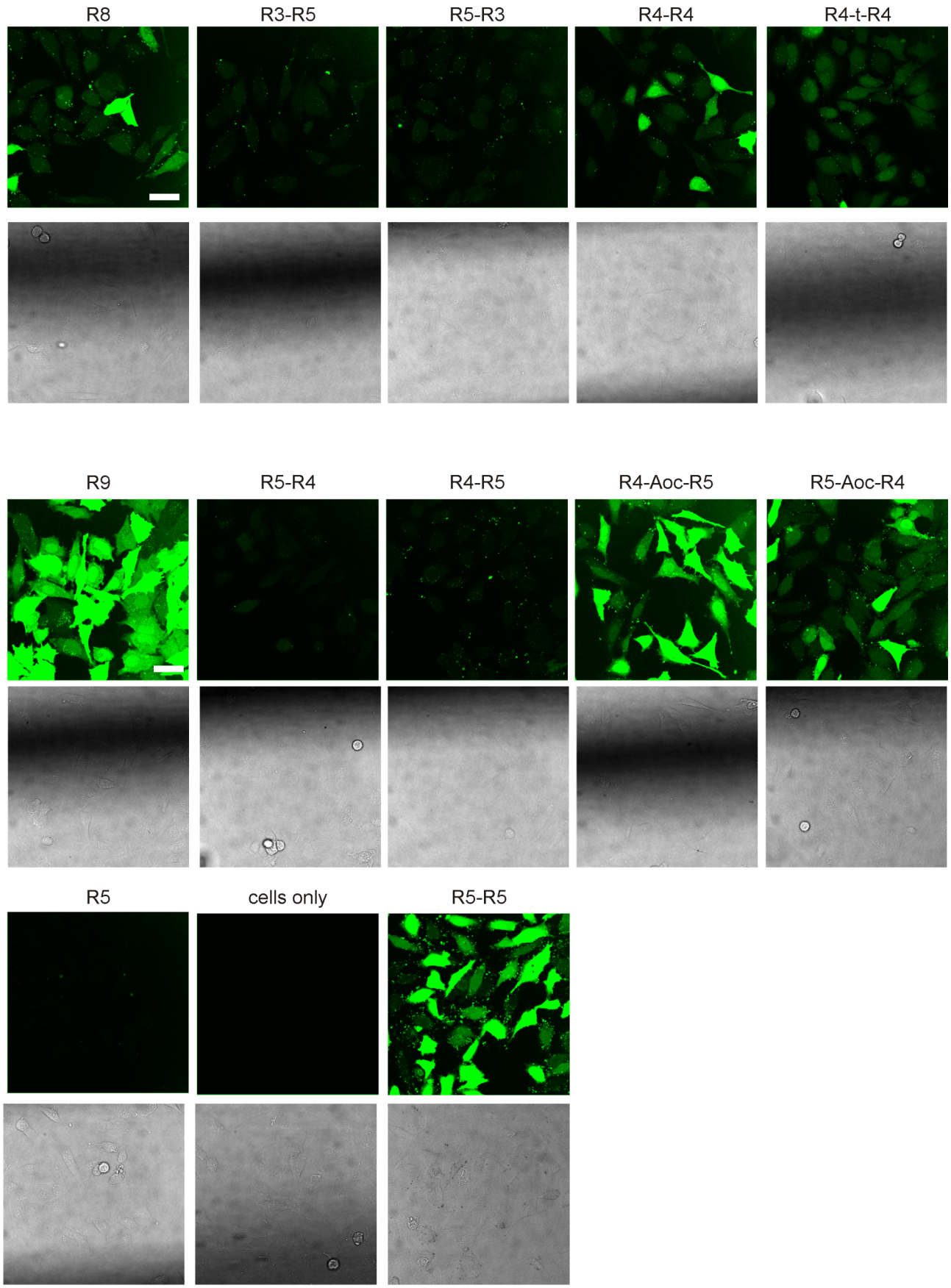
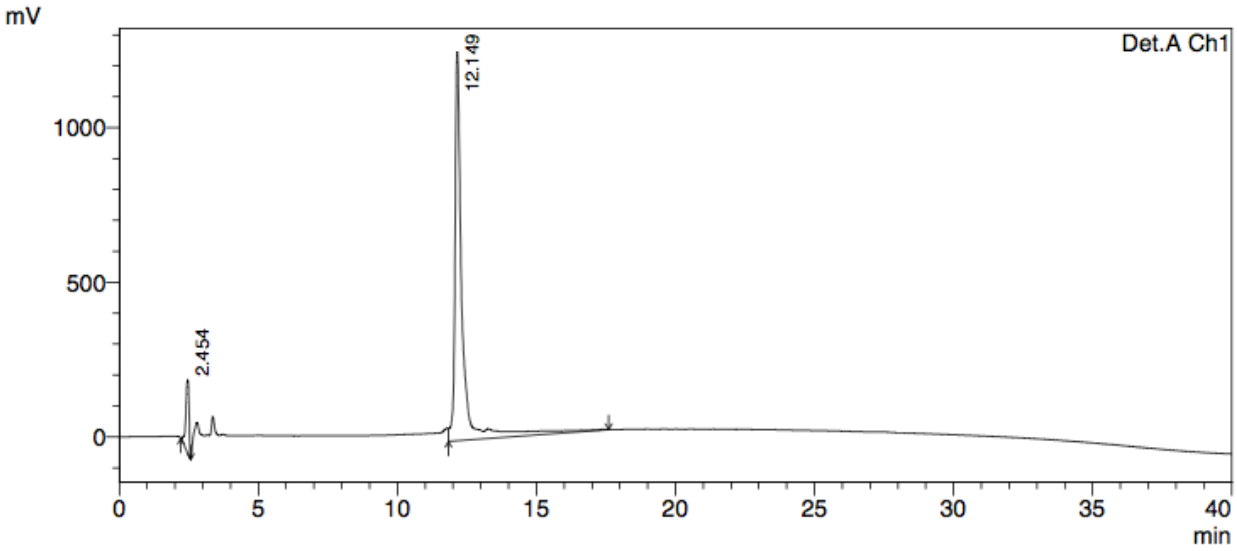


Figure S7 – Uptake experiment at 20 μM. Scale bar represents 40 μm

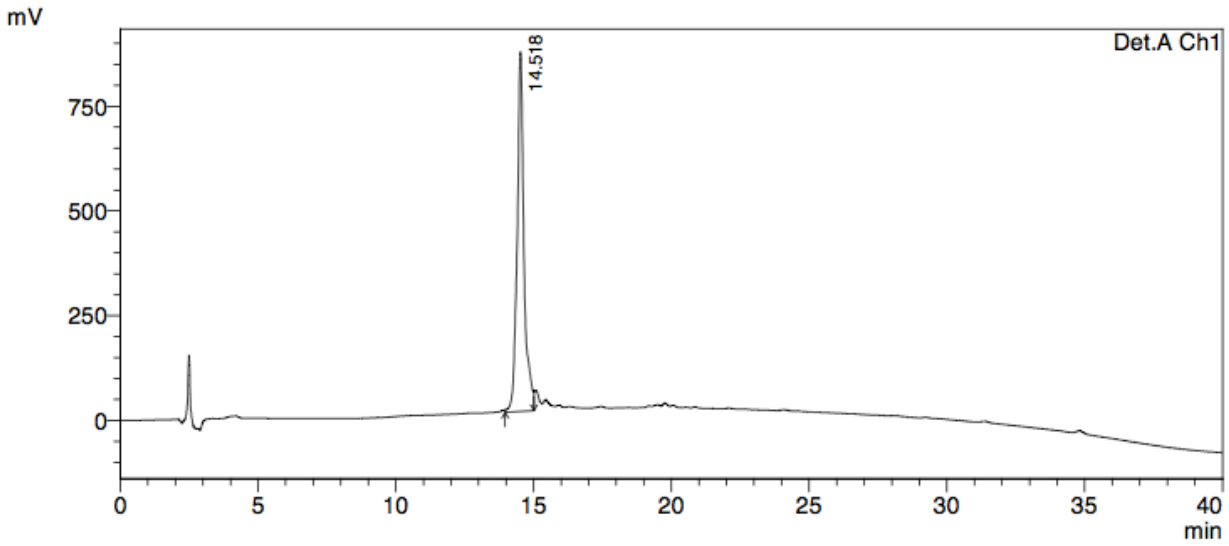
3. Analytical HPLC traces

Det. A. Ch1 corresponds to UV detection at 214 nm, Det. A Ch2 to UV detection at 254 nm. All samples were analyzed using a C-18 column (150 x 3 mm, particle size 3 µm, flow 0.4 mL/min) and a MeCN/water gradient containing 0.1% trifluoroacetic acid (5-100%, 1-40 min).

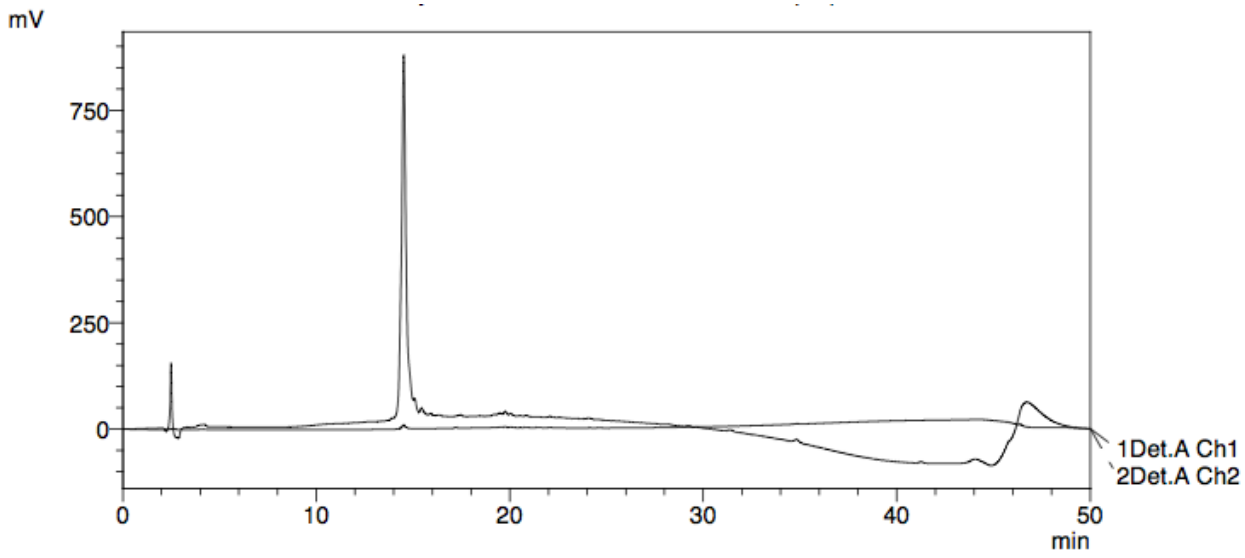
R3



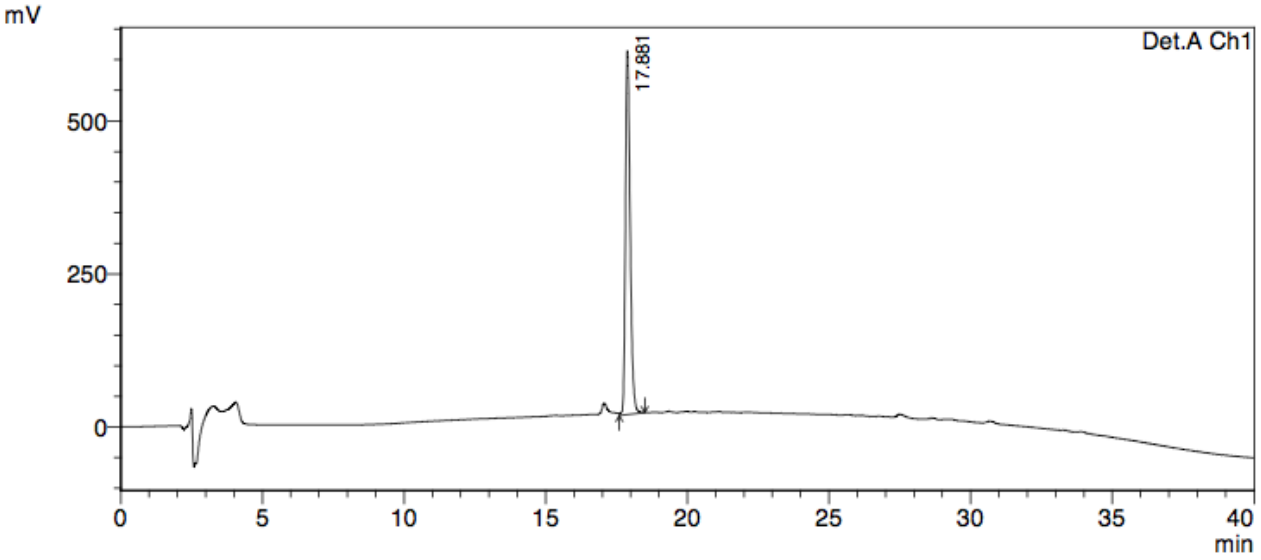
R4



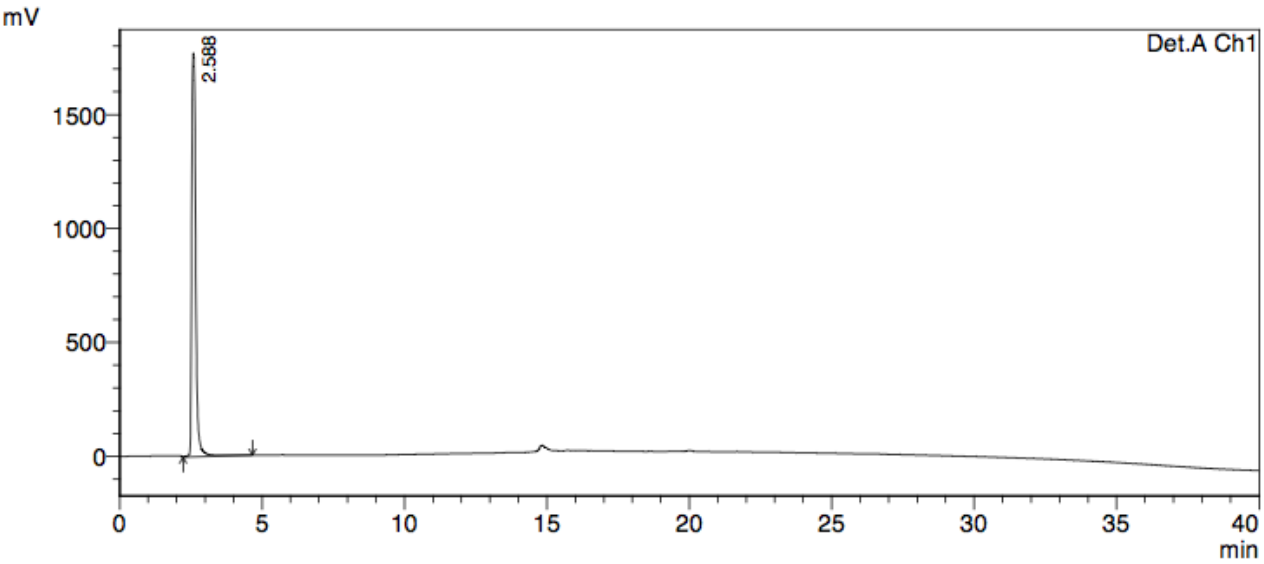
R5


mV
750
500
250
0
0
10
20
30
40
50
min
1Det.A Ch1
2Det.A Ch2

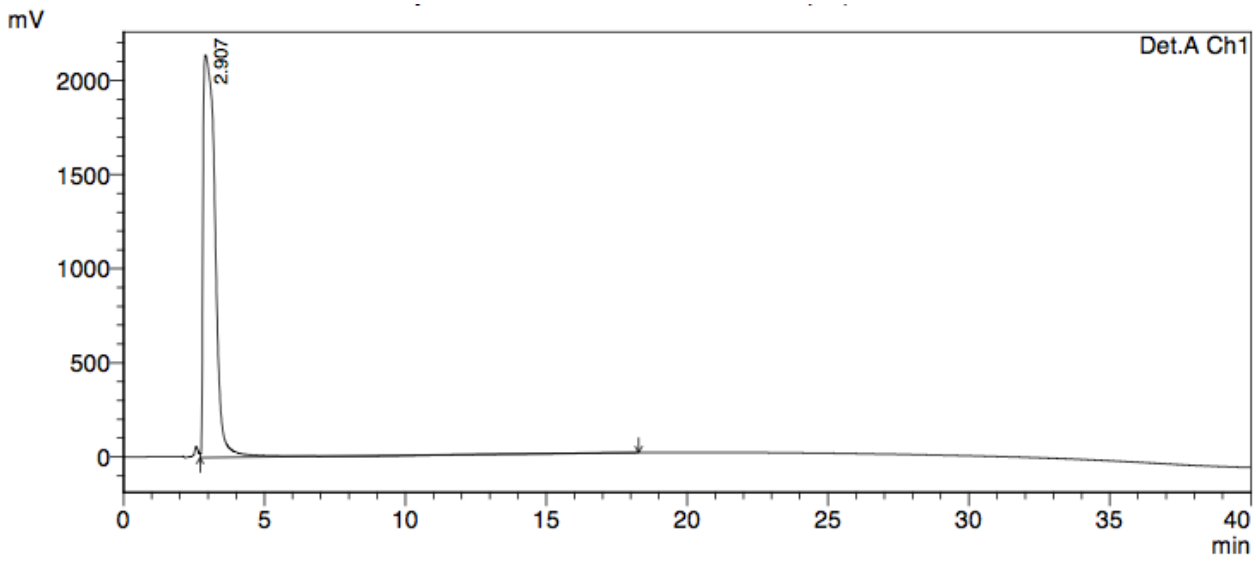
R8


mV
500
250
0
17.881
Det.A Ch1
0
5
10
15
20
25
30
35
40
min

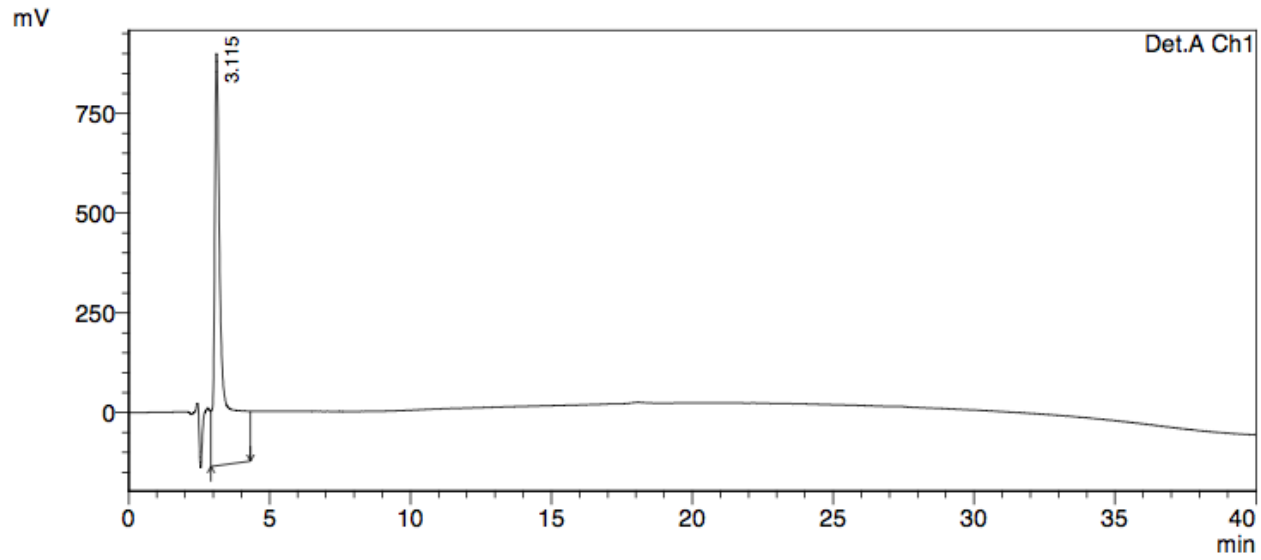
***R3**


mV
1500
1000
500
0
2.588
Det.A Ch1
0
5
10
15
20
25
30
35
40
min

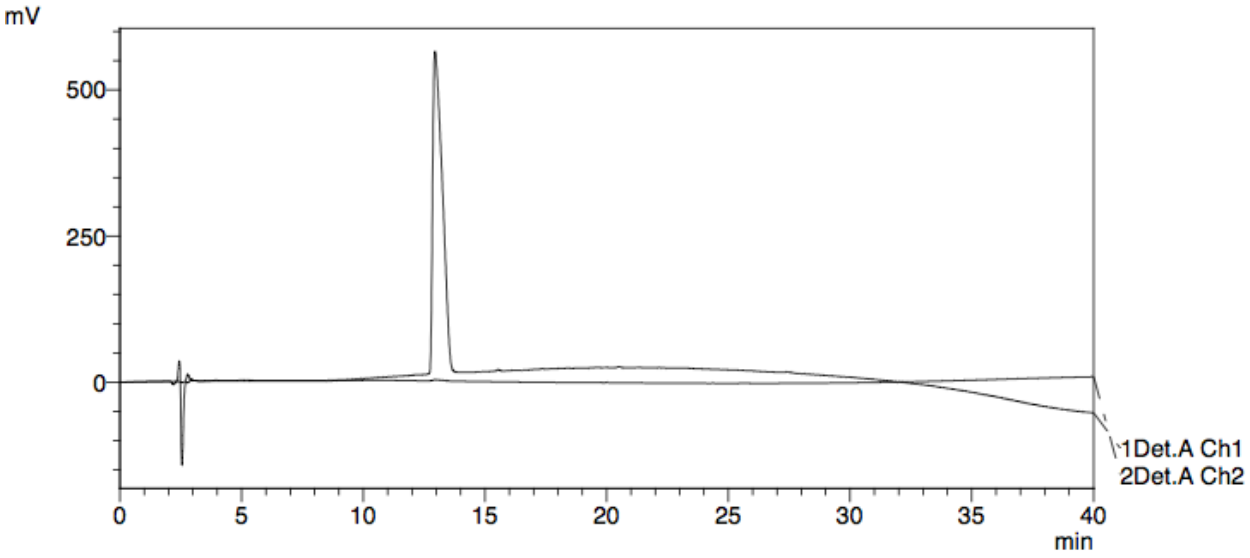
***R4**


mV
Det.A Ch1
2.907
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
40
min

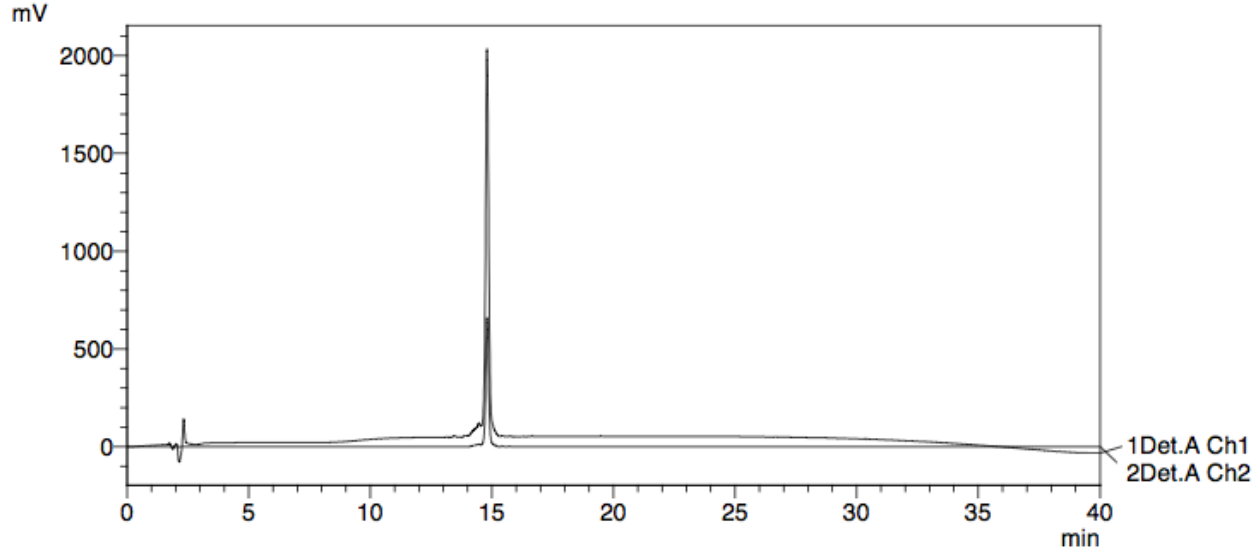
***R5**


mV
Det.A Ch1
3.115
750
500
250
0
0
5
10
15
20
25
30
35
40
min

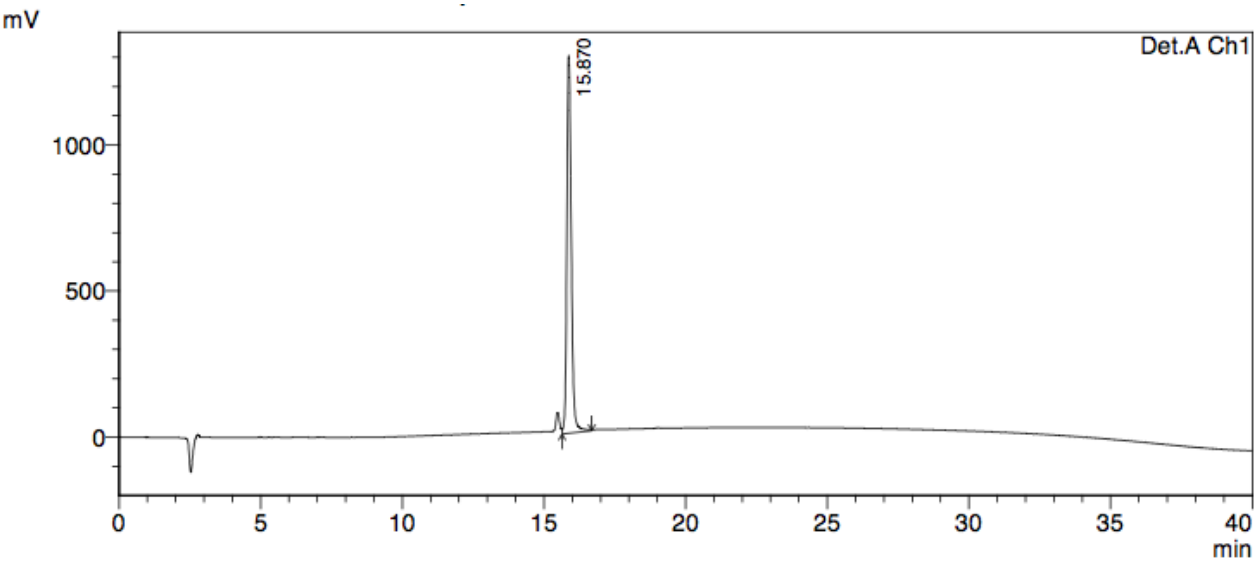
R3-R5


mV
500
250
0
0
5
10
15
20
25
30
35
40
min
1Det.A Ch1
2Det.A Ch2

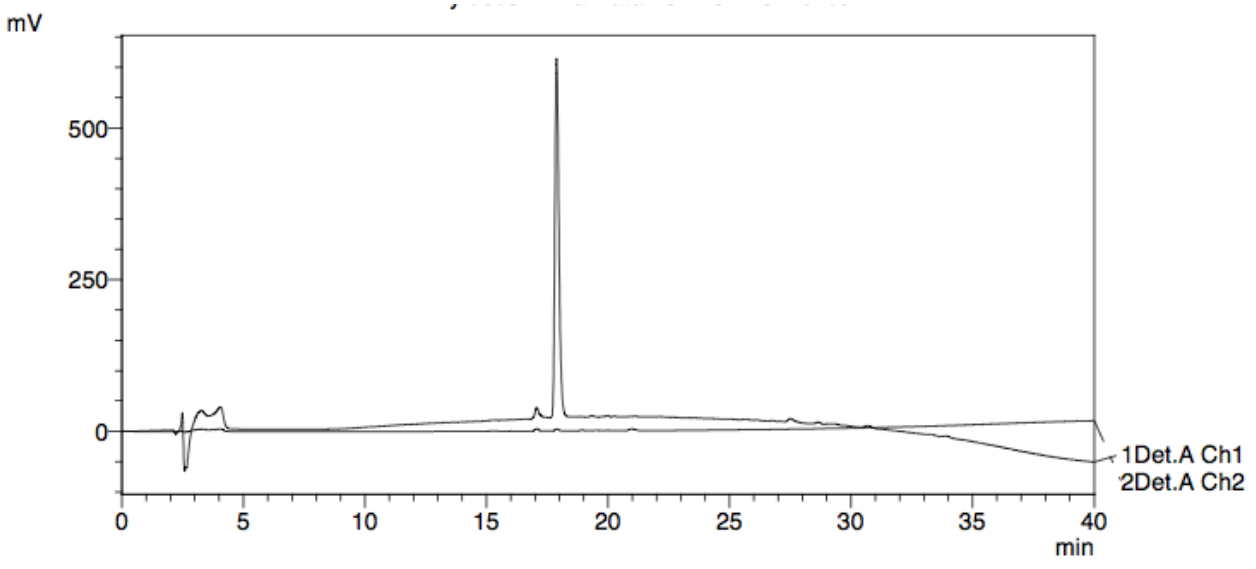
R4-R3



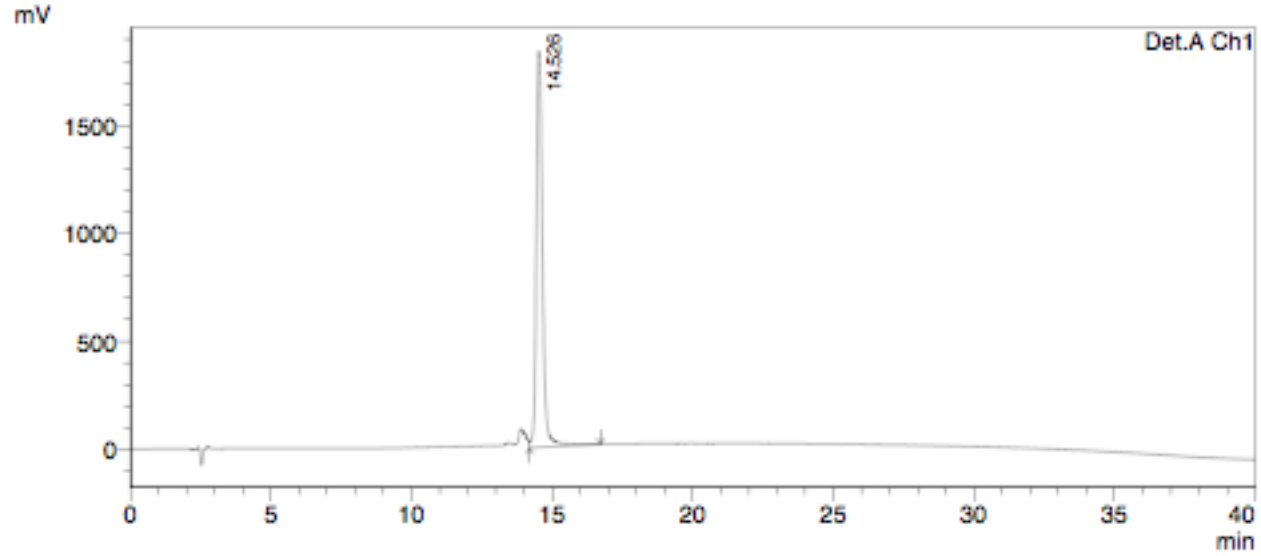
R4-R4



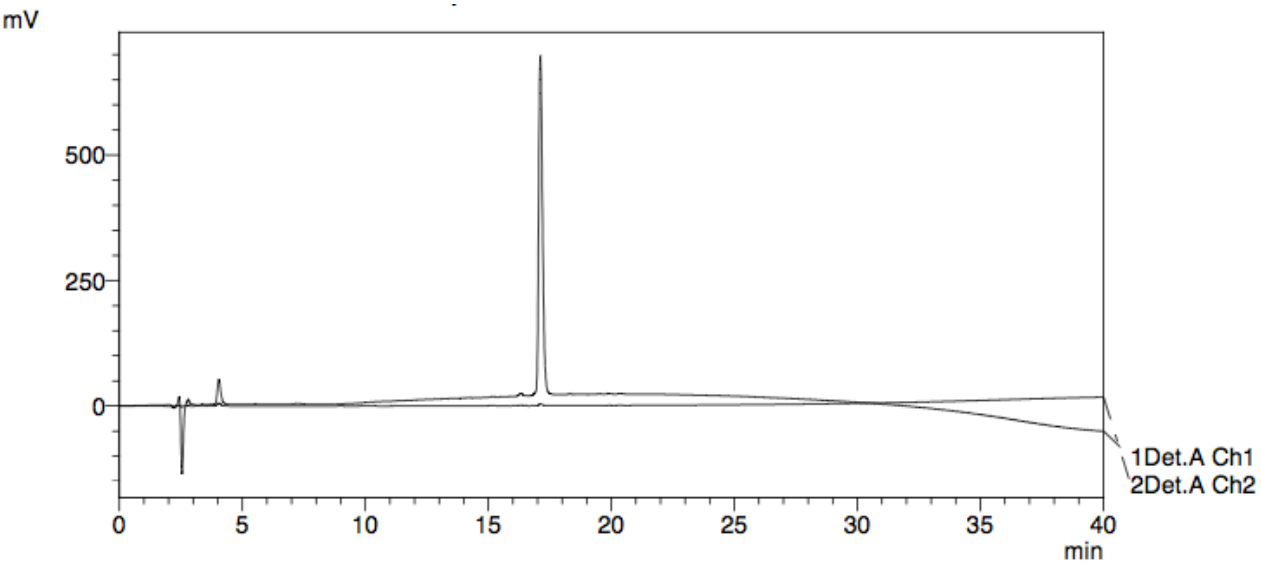
R4-R5



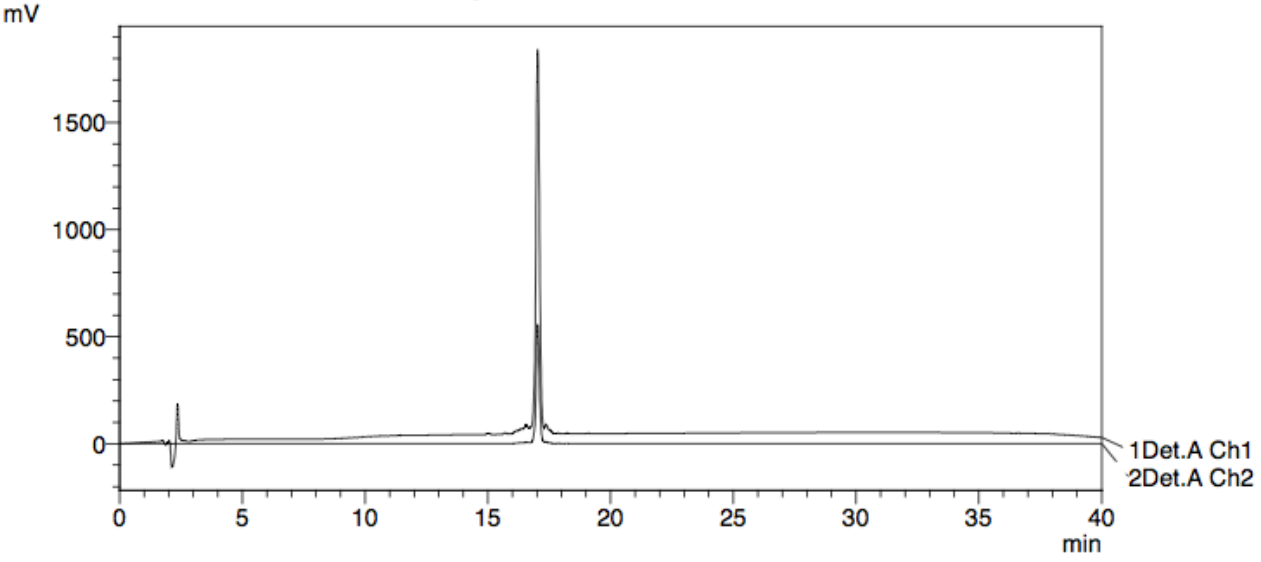
R5-R3



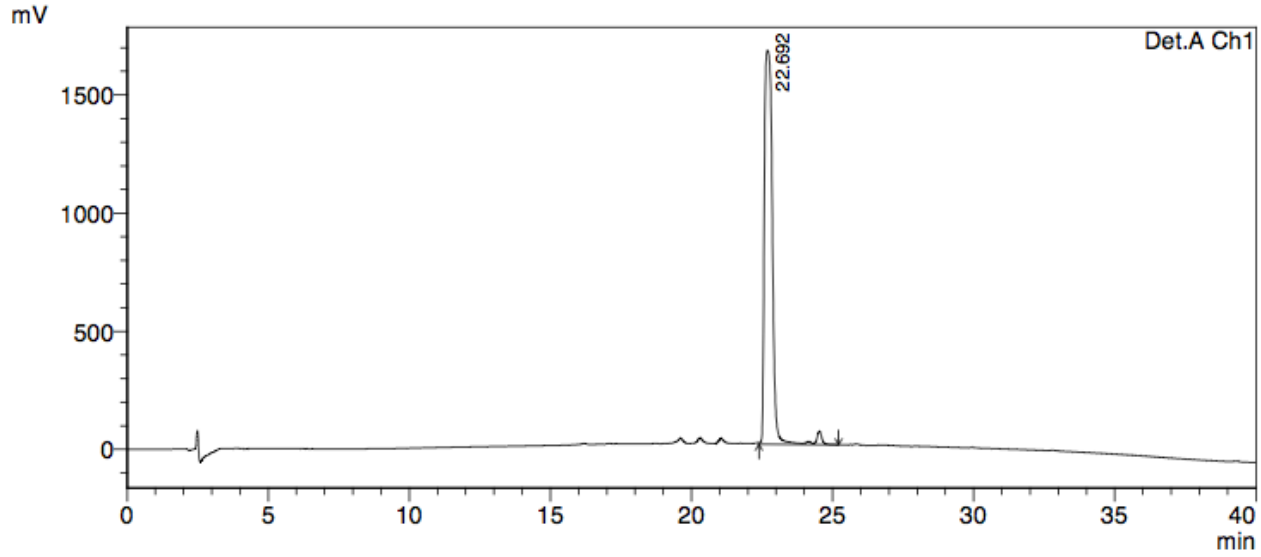
R5-R4



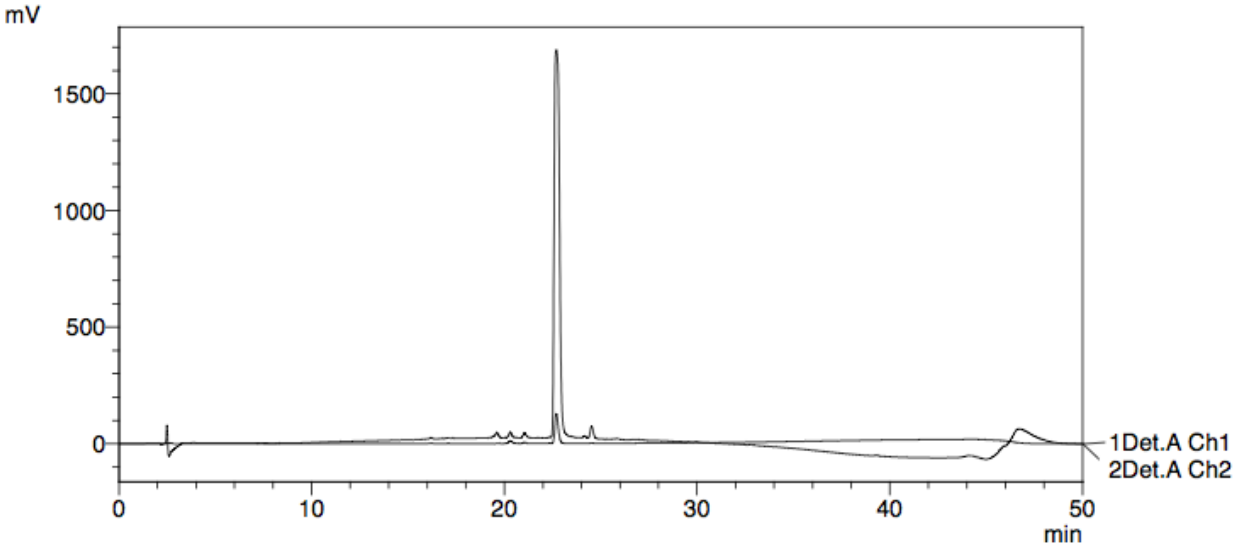
R5-R5



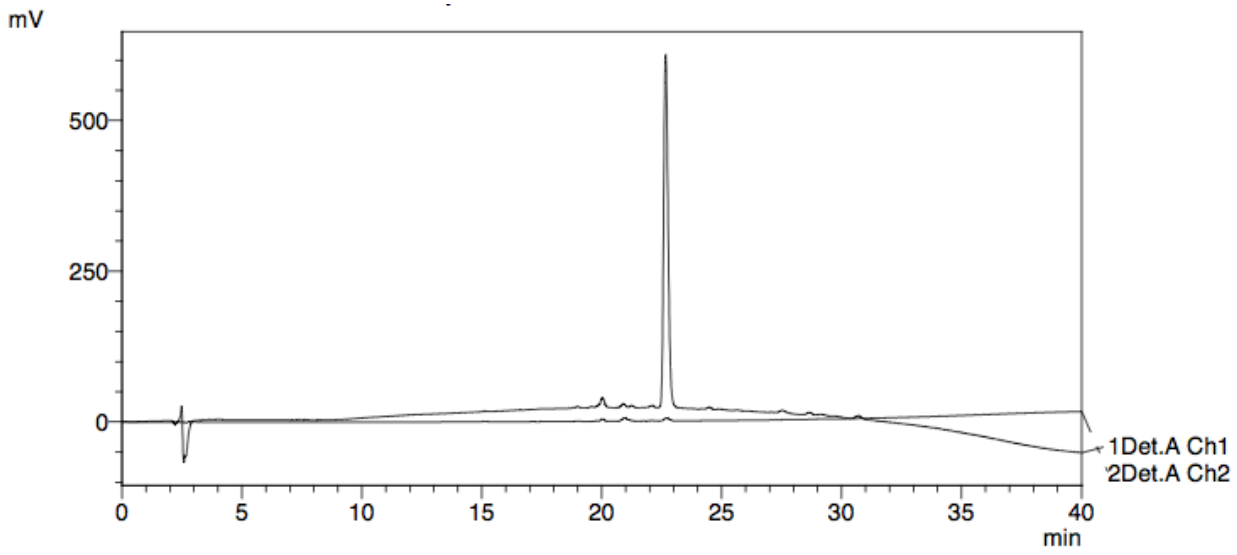
R4-t-R4



R4-Aoc-R5

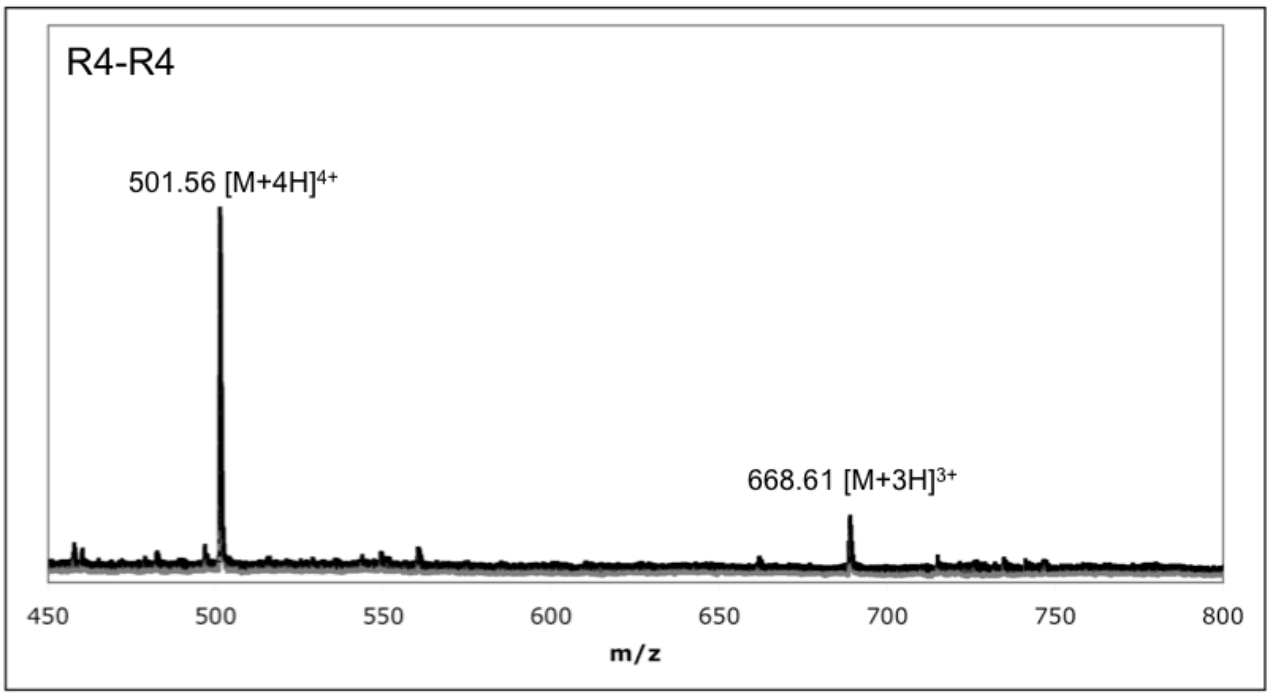


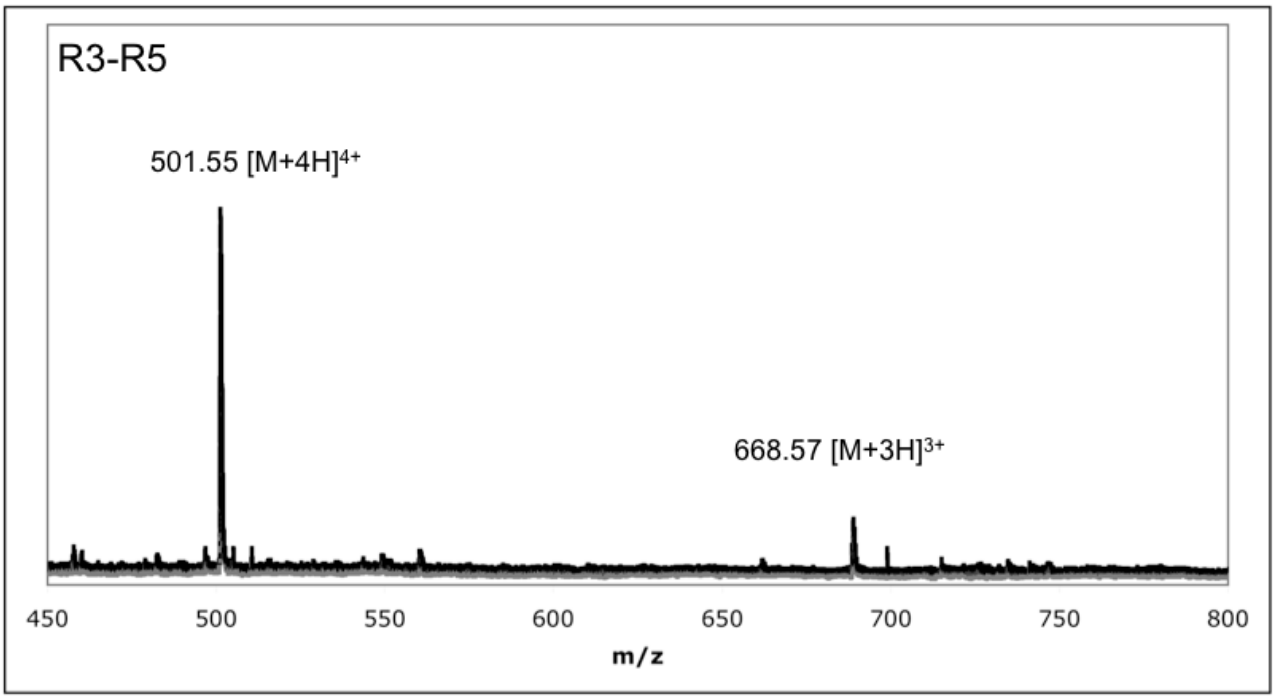
R5-Aoc-R4

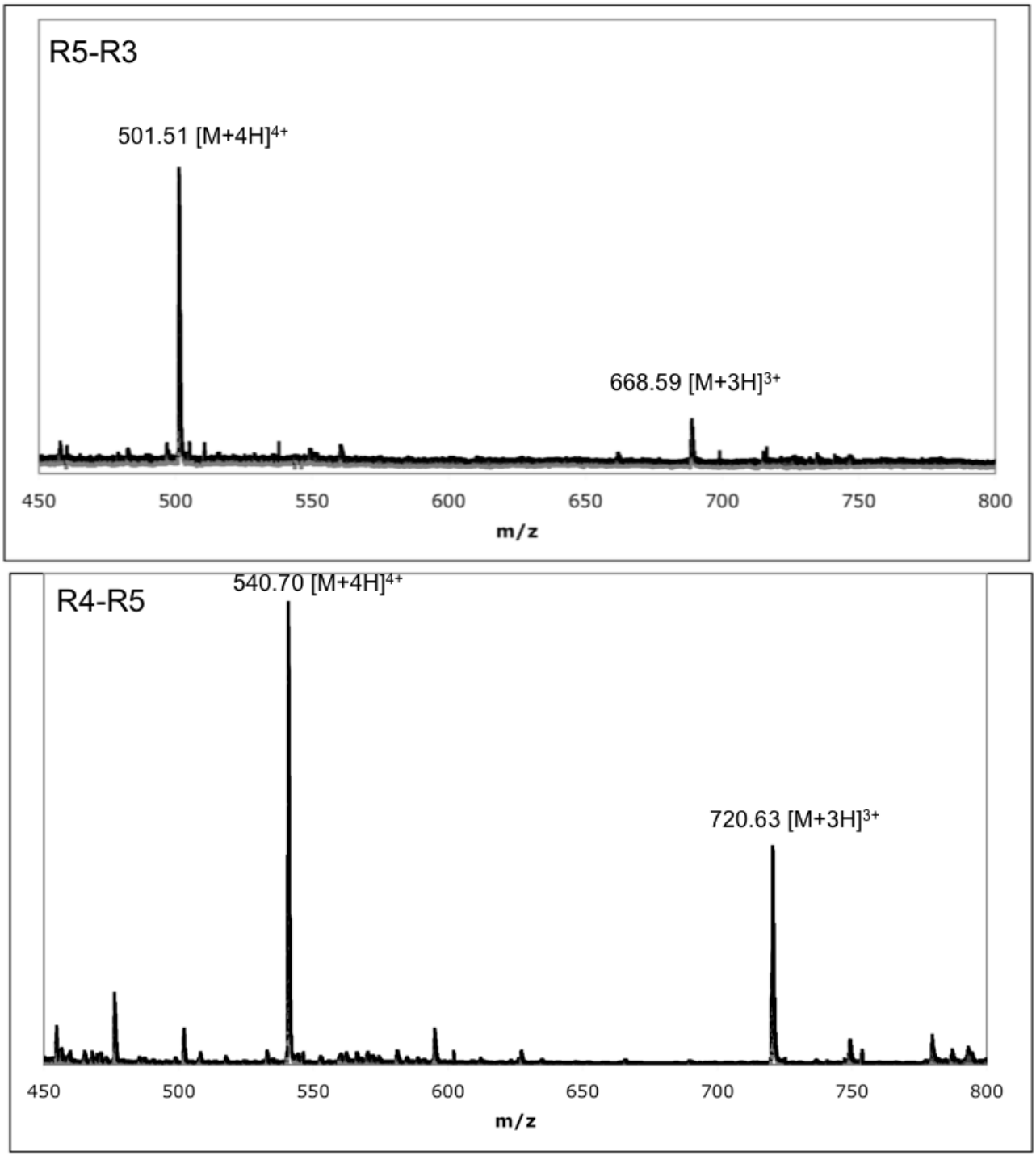


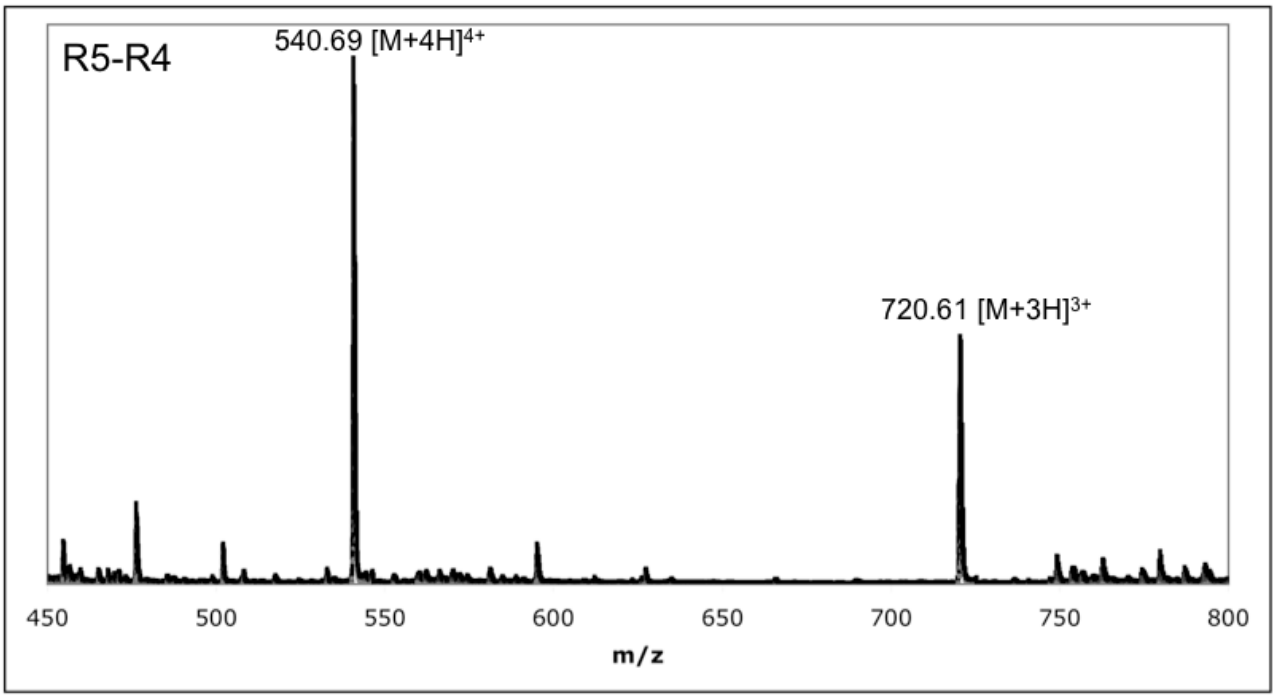
4. Mass spectra for extended peptides

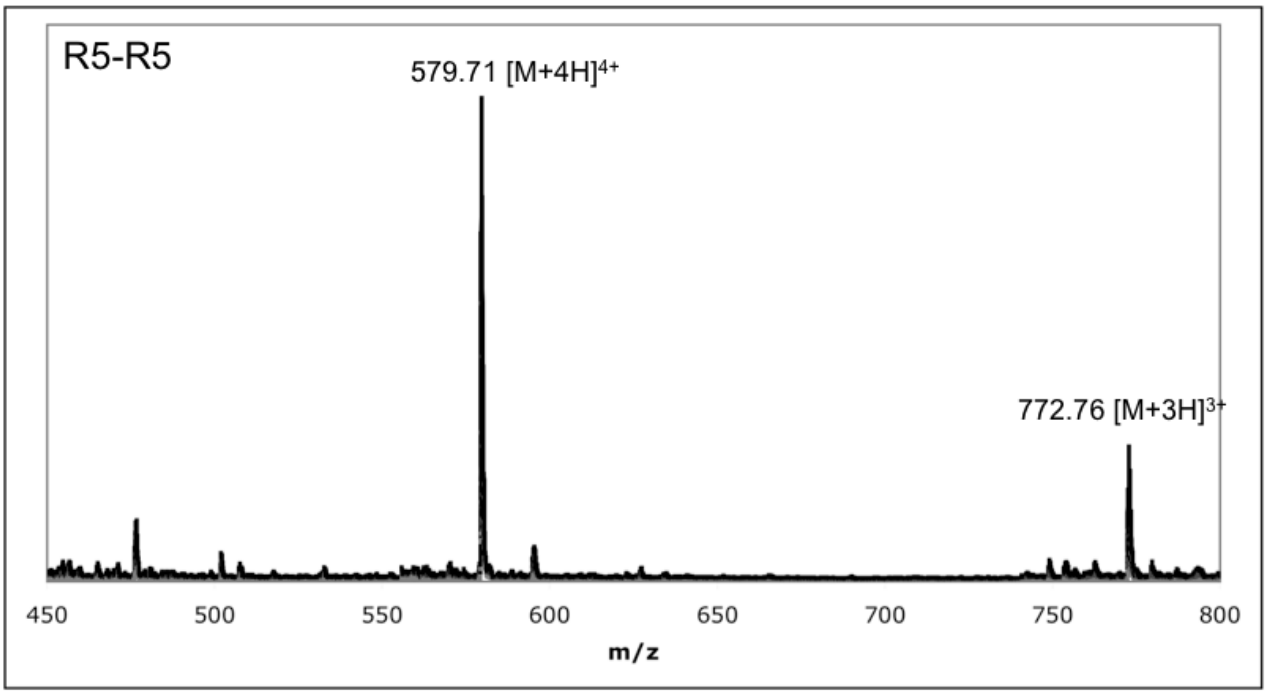
After purification, identity of the extended peptides was confirmed by ESI-TOF MS on a JEOL Accu-TOF.











5. References

1. E. Kaiser, R.L. Colescott, C.D. Bossinger, P.I. Cook, *Anal. Biochem.,* 1970, **34**, 595-598