Rhodium-catalyzed cysteine modification with diazo reagents

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Supporting Information

Contents

General Considerations.

Solvents and reagents were purchased from Fisher Scientific (unless mentioned otherwise) and used as received. Millipore ultra-purified water (18 MΩ) was used in all cases.

Peptide synthesis. All peptides were manually synthesized using standard solid-phase Fmoc protocols.[1,2] Rink amide MBHA resin (AAPPTEC) to afford C-terminal amides. The peptides were acetylated at the N-terminus prior to cleavage from the resin. The purification was accomplished by reverse-phase HPLC with gradients of water-acetonitrile containing 0.1% trifluoroacetic acid, and peptides were isolated by lyophilization. Analysis and purity assessment was attained by mass spectrometry and analytical HPLC.

HPLC. HPLC was performed on a Shimadzu CBM-20A instrument with Phenomenex Jupiter 4μ Proteo 90A (250 × 15 mm preparative) and Phenomenex Jupiter 4μ Proteo 90A (250 × 4.6 mm analytical) columns. Flow rates of 8 mL/min and 1 mL/min were used for preparative and analytical columns, respectively. Analytical and preparative HPLC were performed with gradient of acetonitrile in water. Both solvents contained 0.1% trifluoroacetic acid (TFA) unless otherwise noted. Data was collected using UV-vis absorption at 220 nm and 300 nm.

Mass Spectrometry.
MALDI-TOF MS and MS/MS analyses were performed on a Bruker Daltonics Autoflex MALDI-TOF/TOF mass spectrometer with α-cyano-4-hydroxycinnamic acid matrix (CHCA, Thermo Scientific, 10 mg/mL) for peptide samples and 2,4-dihydroxyacetophenone (DHAP, Fluka, 10 mg/mL) for protein samples. ESI-MS was performed on a Bruker Daltonics microTOF instrument. Data analysis was performed on mMass program.[3,4]

Experimental

Synthesis of known compounds.

The protein CALP[5] and biotin diazo[6] reagent **1** was prepared and purified according to published procedures.

Modification of CALP (10 μM):

To a 250 μL microcentrifuge tube, 68.7 μL of millipore water was added, along with 10 μL aqueous solution of tert-butyl hydroxylamine hydrochloride (100 mM, pH 6.2). To this 10 μL of CALP (100 μM) was added, followed by the addition of 1 μL aqueous solution of $Rh_2(OAc)_4$ (1 mM). The reaction mixture was vortexed briefly and 1.7 μL t-butanol solution of the biotin-diazo **1** (60 mM) was added in the end. The reaction mixture was centrifuged and then incubated on a laboratory low speed shaker at 4 °C for 5 h. The reaction mixture was quenched using 20 μL of MeCN and then analyzed using MALDI-TOF.
The rhodium catalyst can be removed along with other small molecules like biotin diazo by desalting the reaction mixture using a μC18 ZipTip® pipet tip.

General Procedure for Trypsin Digestion of Modified CALP and MS/MS Analysis of Modified CALP Peptides:

100 μL of the CALP (20 μM) modification reaction mixture was centrifuged and then heated at 65 °C in a water bath for 30 min. This was diluted with 100 μL aqueous solution of NH_4HCO_3 (80 mM, pH 7.8) followed by the addition of 1.1 μL of trypsin solution (Promega, 100 μg reconstituted in 100 μL of 50 mM acetic acid). The digest was incubated at 37 °C for 6 h. The crude digest was desalted using μC18 ZipTip® pipet tip and then analyzed by MALDI-TOF. After analyzing the digestion data using mMass program, MS/MS was performed on the singly modified peptide fragment m/z = 2441 using MALDI-TOF.

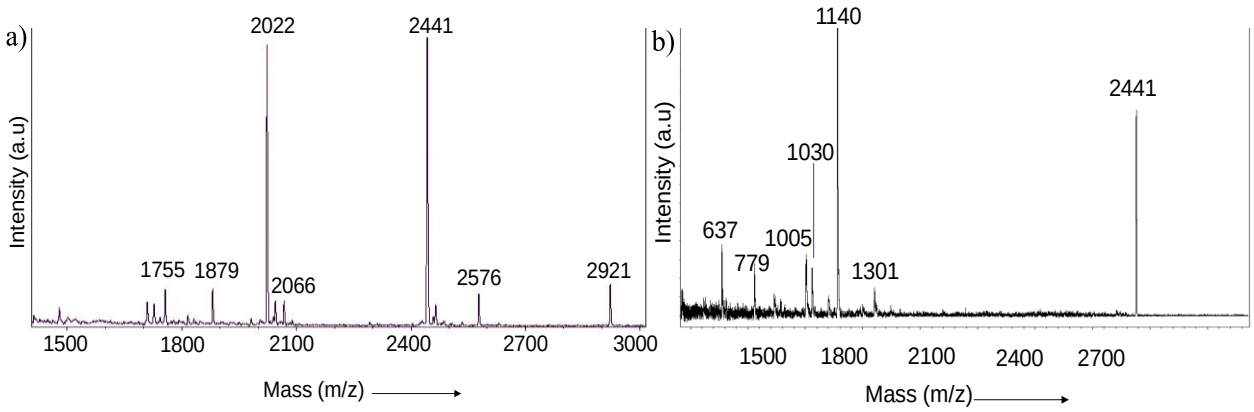


Figure S1. a) Modified CALP trypsin digest. Native peptide fragment containing C44, expected m/z = 1879; singly-modified, expected m/z = 2441. (m/z = 2022 corresponds to native peptide fragment V6-K24 + K^+ adduct) b) MS/MS sequencing of singly modified fragment, m/z = 2441 (Detailed analysis shown in communication).

General Procedure for Blocking Cysteine residue on CALP:[7]

In a 2 mL centrifuge tube, 3 mg of lyophilized CALP was taken and dissolved in 1.5 ml Na_2SO_3 buffer* and incubated at 25 °C for 1 h keeping the tube open. The blocked protein solution is then desalted by dialyzing it in PDZ buffer* (pH 7.4).

*Na_2SO_3 buffer recipe– 0.05 M Na_2SO_3, 0.2 mM cysteine, 6 M guanidine hydrochloride, 0.1 M Tris chloride (pH 8.4).
PDZ buffer recipe – 5% glycerol, 25 mM Na_3PO_4, 150 mM NaCl, 0.02% NaN_3, 0.1 mM TCEP (pH 7.4).

Control Reactions for Modification of CALP:

a) In absence of $Rh_2(OAc)_4$: To a 250 µL microcentrifuge tube, 69.7 µL of millipore water was added, along with 10 µL aqueous solution of tert-butyl hydroxylamine hydrochloride (100 mM, pH 6.2). To this 10 µL of CALP (100 µM) was added. The reaction mixture was vortexed briefly and 1.7 µL t-butanol solution of the biotin-diazo **1** (60 mM) was added in the end. The reaction mixture was centrifuged and then incubated on a laboratory low speed shaker at 4 °C for 5 h. The reaction mixture was quenched using 20 µL of MeCN and then analyzed using MALDI-TOF.

b) In absence of diazo **1**: To a 250 µL microcentrifuge tube, 70.4 µL of millipore water was added, along with 10 µL aqueous solution of tert-butyl hydroxylamine hydrochloride (100 mM, pH 6.2). To this 10 µL of CALP (100 µM) was added, followed by the addition of 1 µL aqueous solution of $Rh_2(OAc)_4$ (1 mM). The reaction mixture was centrifuged and then incubated on a laboratory low speed shaker at 4 °C for 5 h. The reaction mixture was quenched using 20 µL of MeCN and then analyzed using MALDI-TOF.

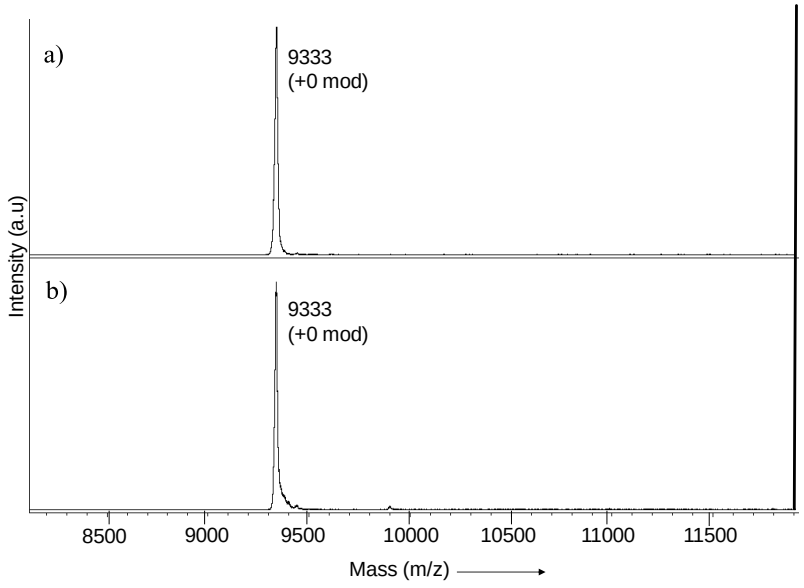
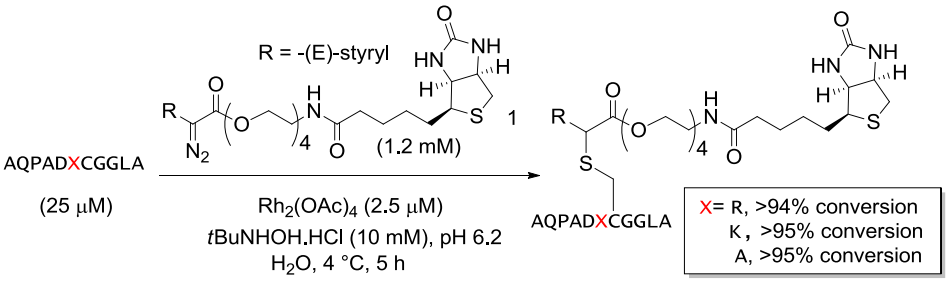


Figure S2. a) Control reaction in absence of the catalyst $Rh_2(OAc)_4$.
b) Control reaction in absence of diazo reagent **1**.

General Protocol for the Modification of Peptides (25 μM) :

To a 250 μL microcentrifuge tube, 85.3 μL of millipore water was added, along with 10 μL aqueous solution of tert-butyl hydroxylamine hydrochloride (100 mM, pH 6.2). To this 2.5 μL of peptide (1 mM) was added, followed by the addition of 0.25 μL aqueous solution of $Rh_2(OAc)_4$ (1 mM). The reaction mixture was vortexed briefly and 2 μL t-butanol solution of the biotin-diazo **1** (60 mM) was added in the end. The reaction mixture was centrifuged and then incubated on a laboratory low speed shaker at 4 °C for 5 h. The reaction mixture was quenched using 20 μL of MeCN and then analyzed using MALDI-TOF.



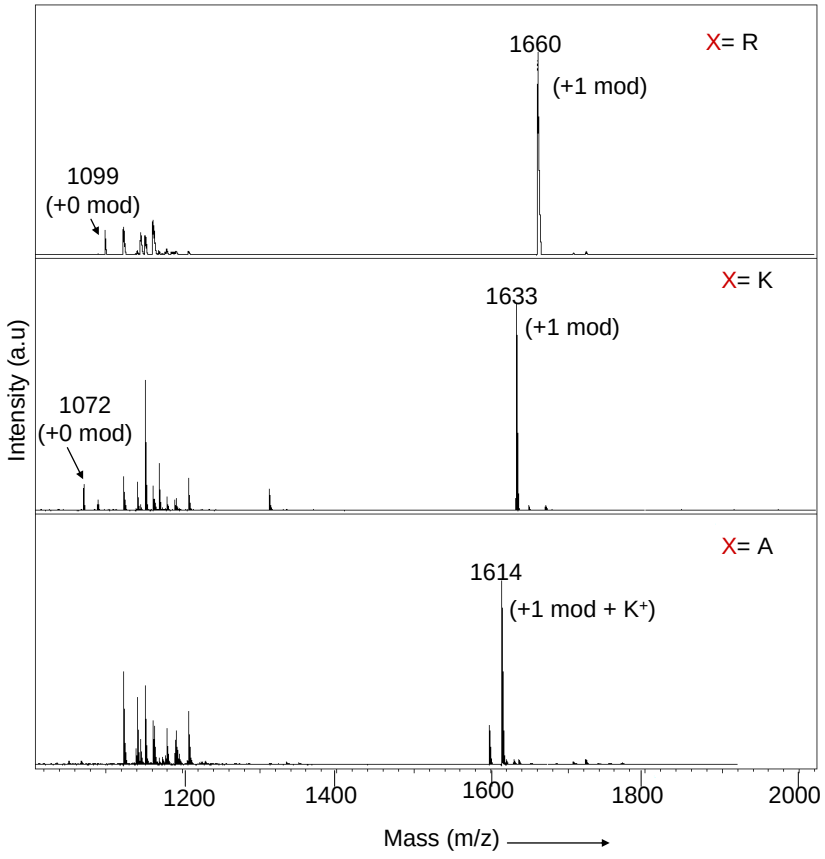


Figure S6. Modification of peptides AQPADXCGGLA (X= R, K, A), catalyzed by $Rh_2(OAc)_4$ using diazo **1**.

General protocol for the Modification of Peptide AQPADRCGGLA using Biotin-Maleimide:
To a 500 μL centrifuge tube, 314 μL of millipore water was added, along with 36.4 μL of buffer*. To this 9.1 μL of AQPADRCGGLA (1 mM) was added, followed by the addition of 4.55 μL of biotin-maleimide (Sigma Aldrich, 1 mM solution in DMF). The reaction mixture was centrifuged and then incubated on a laboratory low speed shaker at 4 °C for 5 h. The reaction mixture was analyzed using MALDI-TOF. This was lyophilized and used in the stability study in human plasma serum.

*Buffer recipe – 100 mM Na_3PO_4, 5 mM EDTA, pH 7.5.

General Protocol for Stability Study of S-C Linkages in Human Plasma Serum:
To a 500 μL centrifuge tube containing the lyophilized modified peptide, 200 μL of human plasma serum (Fischer Scientific) was added, along with 2 μL of phenylmethanesulfonylfluoride (100 mM solution in isopropanol). The reaction mixture was centrifuged and then incubated at 37 °C. At different time intervals, 10 μL aliquots of the reaction mixture were taken out and quenched with 60 μL of MeCN. This was centrifuged and the solution was analyzed using MALDI-TOF.

$Rh_2(OAc)_4$ Catalyzed Modification of CALP in a Mixture of Other Proteins:
To a 250 μL microcentrifuge tube, 67.95 μL of millipore water was added, along with 10 μL aqueous solution of tert-butyl hydroxylamine hydrochloride (100 mM, pH 6.2). To this 15 μL of CALP (100 μM) was added, followed by the addition of 3 μL of BioRad polypeptide standard†. The reaction mixture was vortexed briefly and 1.5 μL aqueous solution of $Rh_2(OAc)_4$ (1 mM) was added to it. Finally 2.55 μL of biotin-diazo **1** (60 mM solution in t-butanol) was added. The reaction mixture was centrifuged and then incubated on a laboratory low speed shaker at 4 °C for 5 h. The reaction mixture was quenched using 20 μL of MeCN and then analyzed using Western blot.

† 3 μL of Biorad polypeptide standard contains 13.5 μg of the proteins aprotinin, α-lactalbumin, myoglobin and triose-phosphate isomerase (TIM).

Biotin-Maleimide Catalyzed Modification of CALP in a Mixture of Other Proteins:
To a 250 μL microcentrifuge tube, 89 μL of buffer* was added, along with 3 μL of CALP (500 μM) and 3 μL of BioRad polypeptide standard†. The reaction mixture was vortexed briefly and 5 μL of biotin-maleimide (Sigma Aldrich, 5 mM solution in DMF) was added to it. The reaction mixture was centrifuged and then incubated on a laboratory low speed shaker at 4 °C for 5 h. The reaction mixture was quenched using 20 μL of MeCN and then analyzed using Western blot.

*Buffer recipe – 100 mM Na_3PO_4, 5 mM EDTA, pH 7.5.
† 3 μL of Biorad polypeptide standard contains 13.5 μg of the proteins aprotinin, α-lactalbumin, myoglobin and triose-phosphate isomerase (TIM).

Western Blot Analysis. A crude reaction sample (0.50 μL) was analyzed directly by SDS-PAGE (10% Tris-Tricine gel, Invitrogen). Proteins were then transferred to a nitrocellulose membrane (GE Healthcare), which was subsequently blocked in 8% milk (8% milk in 100 mL TBS-T*) for 2 h. The membrane was incubated with avidin–horseradish peroxidase conjugate (blotting grade, Bio-Rad) solution (1% in TBS-T) for 1 h, followed by washing with TBS-T (3 × 5 min) and TBS (1×15 min). Blots were developed with chemiluminescent

substrate (RapidStep ECL Reagent, Calbiochem) and images recorded with a Fujifilm LAS-4000 instrument. An estimate of the extent of biotinylation was obtained by comparison to control lanes of authentic HRP-biotin standard (Invitrogen) and quantified using Multi Gauge Version 3.0.

*TBS recipe: 2.4 g tris base, 29.22 g NaCl, was dissolved in 900 mL water, and adjusted to pH 7.5 and then the volume was made up to 1 L.
TBS-T recipe: 1 mL of Tween 20 in 500 mL of TBS solution.

Characterization for AQPADRXCGGLA (X= R, K, A) Peptides:

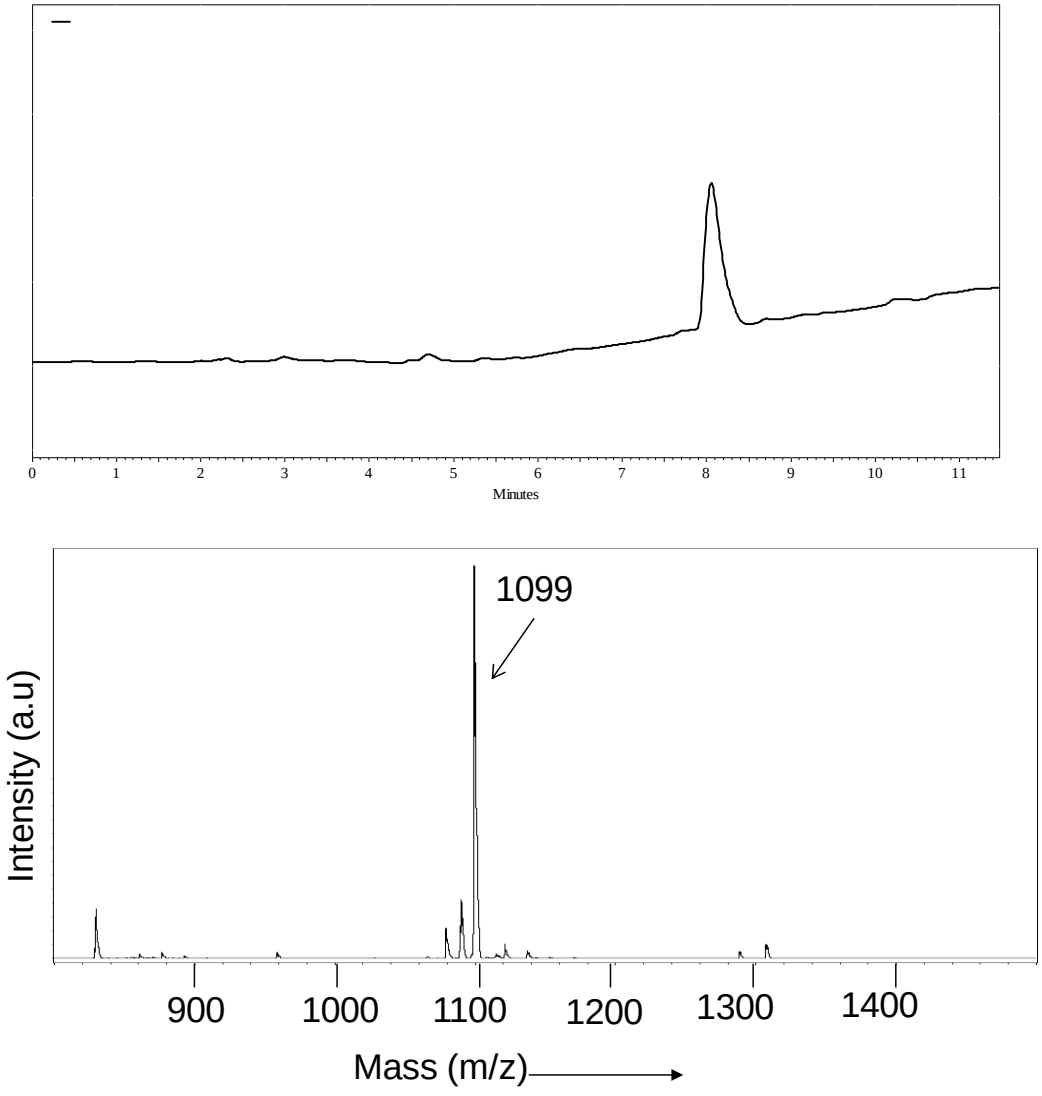


Figure S3. HPLC trace and MALDI-MS of peptide AQPADRCGGLA

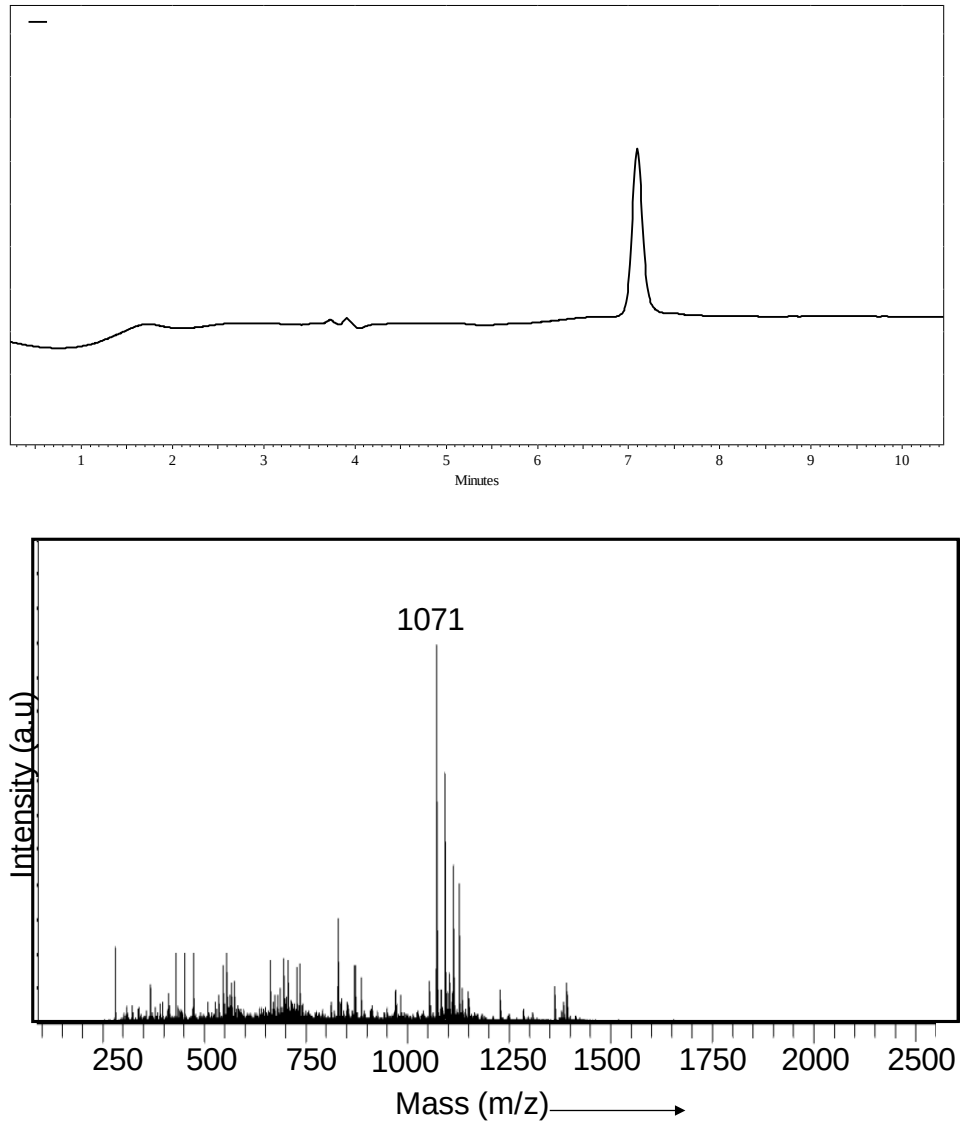


Figure S4. HPLC trace and ESI-MS of peptide AQPADKCGGLA

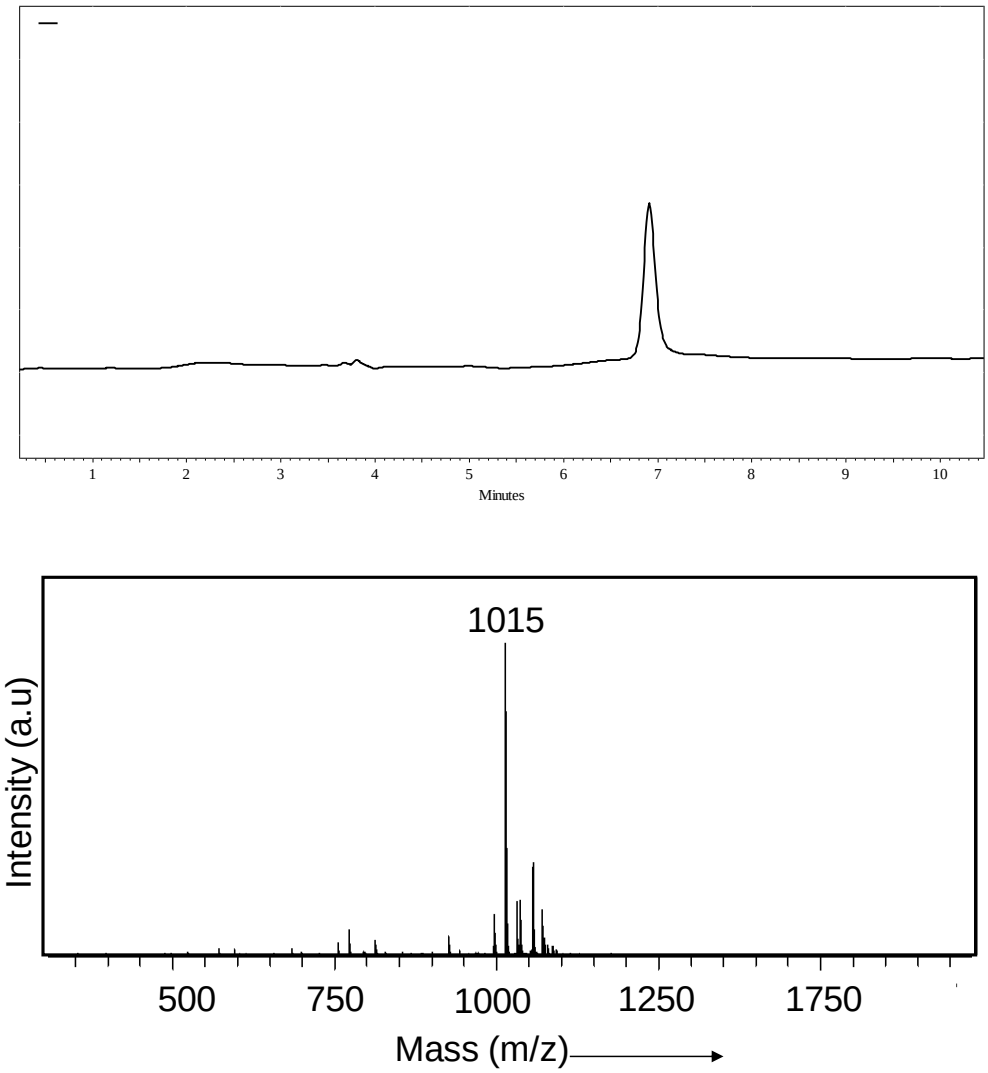


Figure S5. HPLC trace and ESI-MS of peptide AQPADACGGLA

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