**SUPPLEMENTARY DATA**

***for***

# Highly Chemoselective Ligation of Thiol- and Amino-Peptides on a Bromomaleimide Core

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## Materials and methods

Fmoc-Rink amide MBHA resin, 2-CTC resin, Fmoc-*L*-amino acids and coupling reagents were purchased from GL Biochem Pvt. Ltd., China or Luxembourg Industries Ltd., Tel Aviv, Israel. ChemMatrix resin was purchased from PCAS BioMatrix Inc (USA). Side chain functional groups of the amino acids were either protected as *t*Bu (Asn, Thr, Tyr), Trt (Cys) and Boc (Lys). The other chemicals/ reagents like DIEA, piperidine, TFA, $Ac_2O$, ACN, DMF, $SOCl_2$ and DCM were procured from Sigma Aldrich, Germany. Analytical high performance liquid chromatography (HPLC) was performed using Agilent 1100 model, a reversed-phase $C_{18}$ Phenomenex column (3 μm x 4.6 x 50 mm) and a UV detector with wavelength at 220 nm were used. The system was run at a flow rate of 1.0 mL/min over 15 min at room temperature using water (0.1% TFA v/v) and acetonitrile (0.1% TFA v/v) as solvents. Semi-preparative HPLC purification was conducted on a Shimadzu LC-6A instrument with a dual wavelength absorbance detector using a reversed-phase Phenomenex $C_{18}$ (10 μm x 10 x 250 mm) column at a flow rate of 15 mL/min for 30 min using 0.1% TFA

in water (v/v) as solvent A and 0.1% TFA in ACN (v/v) as solvent B. Mass spectra were recorded on Shimadzu 2020 UFLC-MS system with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in ACN as solvent B using XBridge BEH130 $C_{18}$ column (4.6 mm x 100 mm, 3.5 mm). Proton NMR data were recorded on a Bruker AVANCE III 600 MHz NMR spectrometer at 30 °C. The spectrometer was equipped with a 5 mm BBO probe with z gradient. The data were recorded and analyzed with Topspin 2.1 (Bruker, Karlsruhe, Germany). Standard Bruker pulse sequences were used.

**Peptide synthesis**

All the peptides were manually synthesized by SPPS protocol following Fmoc/*t*Bu strategy using Fmoc-Rink amide MBHA resin (0.6 mmol/g loading) or H-Rink amide ChemMatrix resin (0.48 mmol/g loading) or 2-CTC resin (0.6 mmol/g loading) as solid support. 2-CTC resin was pre-activated using $SOCl_2$/DCM (1:9, v/v) overnight before use. When 2-CTC resin was used, first amino acid (5 equiv.) was added in presence of *N*,*N*′-diisopropylethylamine (DIEA – 25 equiv.), reacted for 45 min, washed the resin with excess DCM/DMF after the completion of the reaction and capped the unreacted points using methanol. Subsequent steps were carried out as explained below. When Rink amide resin was used, Fmoc group was removed using piperidine/ DMF (20:80, v/v) and coupling reactions were carried out with Fmoc-*L*-amino acids (5 equiv.), aminium *N*-[(*1H*-benzotriazol-1-yl)(dimethylamino) methylene]-*N*-methylmethanaminium hexafluoro phosphate *N*-oxide (HBTU) (5 equiv.) and DIEA (10 equiv.) in DMF for 45 min at room temperature. The efficiency of each reaction was monitored using ninhydrin test. If positive result was observed, then recoupling was done as above and if the test was negative the reaction was proceeded. Thiol- and amino-peptides were acetylated on-resin using $Ac_2O$/ DIEA (1:2, v/v) in DMF for 30 min and cleaved from the solid support with concomitant deprotection of the side chain protecting groups. It was performed by washing the resin with DCM, dried and treated with TFA/TIS/$H_2O$ (95:2.5:2.5,

v/v/v) for 1.5h. Peptides were then precipitated with cold diethyl ether, centrifuged and washed twice with diethyl ether. The crude peptides were finally dissolved in acetic acid/$H_2O$ (10:90, v/v) and lyophilized. Purification of the peptides was achieved by semi-preparative HPLC using linear gradient of solvent B into A. The purified peptides were characterized by analytical HPLC and LC-MS.

**Conjugation of thiol-peptide2 to BMHA-peptide 1 in-solution**

BMHA-peptide **1** (4 mg, 0.0048 mmol) was dissolved in 1 mL of phosphate buffer of pH 6.5 and added 200 µL ACN to enhance the solubility. To this was added thiol-peptide **2** (2.4 mg, 0.0048 mmol) in portions and the reaction was monitored by analytical HPLC (10-60% of solvent B into A).

HPLC $t_R$: 9.517 min; LC-MS: Expected: 1247.5903; Found: 1248.6371 [M+1], 625.1095 [M+2]/2

**Conjugation of amino-peptide 3 to BMHA-peptide 1 in-solution: General procedure**

BMHA-peptide **1** (4 mg, 0.0048 mmol) was dissolved in 1 mL of 0.1% DIEA, dry DMF and added 200 µL ACN to enhance the solubility. To this was added amino-peptide **3** (2.5 mg, 0.0048 mmol) dissolved in DMF and the reaction was monitored by HPLC (5-60% of solvent B into A).

HPLC $t_R$: 9.230 min; LC-MS: Expected: 1354.1048; Found: 1355.0934 [M+1], 678.1095 [M+2]/2

**Solid-phase conjugation of thiol-peptide 2 to BMHA-peptide1-®**

Thiol-peptide **2** (3 mg, 6 µmol) was dissolved in dry DMF (1 mL) and to this was added DIEA (1 µL, 6 µmol) and OxymaPure (0.85 mg, 6 µmol). This solution was added to BMHA-peptide **1**-® (10 mg, 6 µmol) and shaken till the completion of the reaction as monitored by analytical HPLC. Global deprotection and cleavage from the resin was performed as explained above and submitted to HPLC and LC-MS.

**Solid-phase conjugation of amino-peptide 3 to BMHA-peptide 1-®**

Amino-peptide 3 (3 mg, 6 µmol for 1 equiv.) dissolved in dry DMF (1 mL) and added DIEA (1 µL, 6 µmol for 1 equiv.). This solution was added either to BMHA-peptide **1**-CM resin (10 mg, 6 µmol) or -PS resin (3.6 mg, 6 µmol) and shaken till the completion of the reaction as monitored by analytical HPLC. Global deprotection and cleavage from the resin was performed as explained above and submitted to HPLC and LC-MS.

**Thiol-peptide 2 (Ac-CLAGV-$NH_2$) conjugation:**

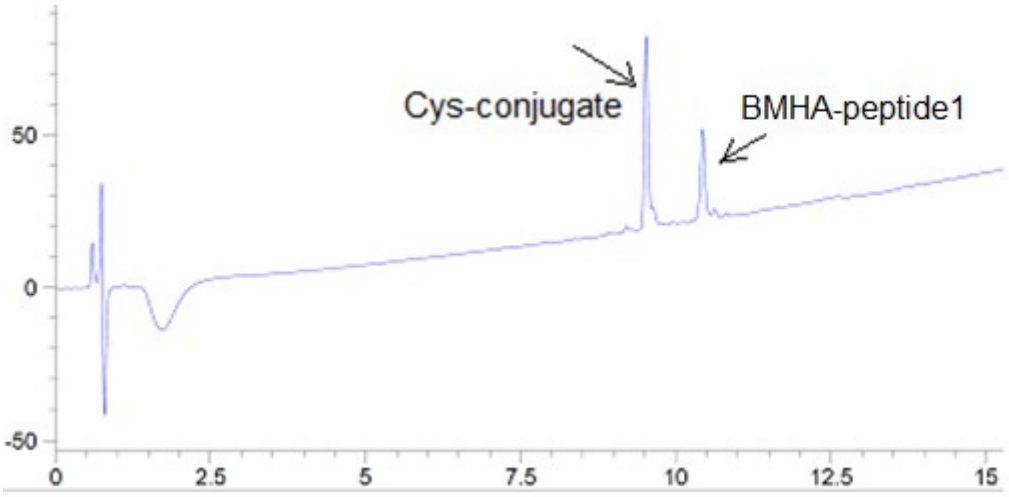


**Fig. S1** HPLC chromatogram of crude thiol-peptide **2**-conjugation

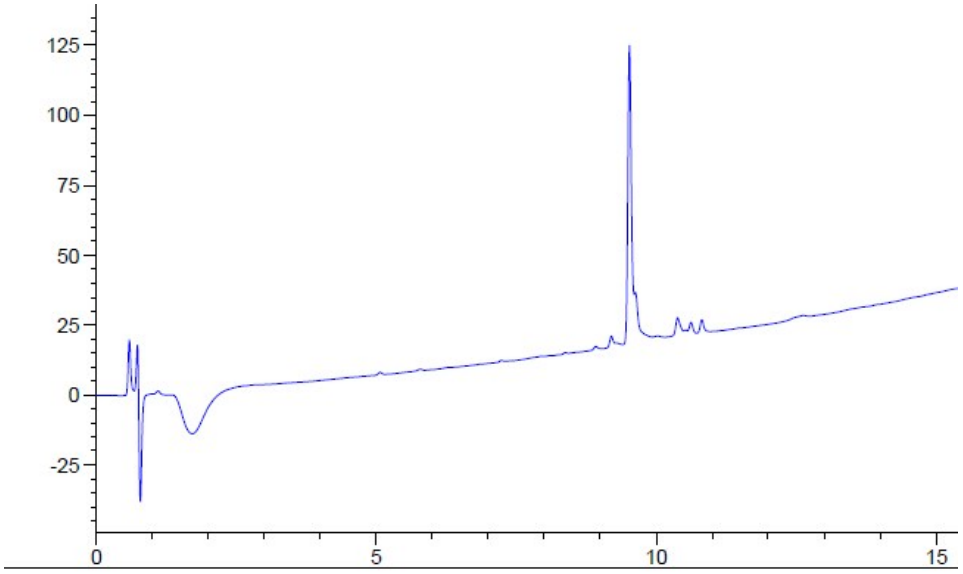


**Fig. S2** HPLC chromatogram of pure thiol-peptide **2**-conjugate

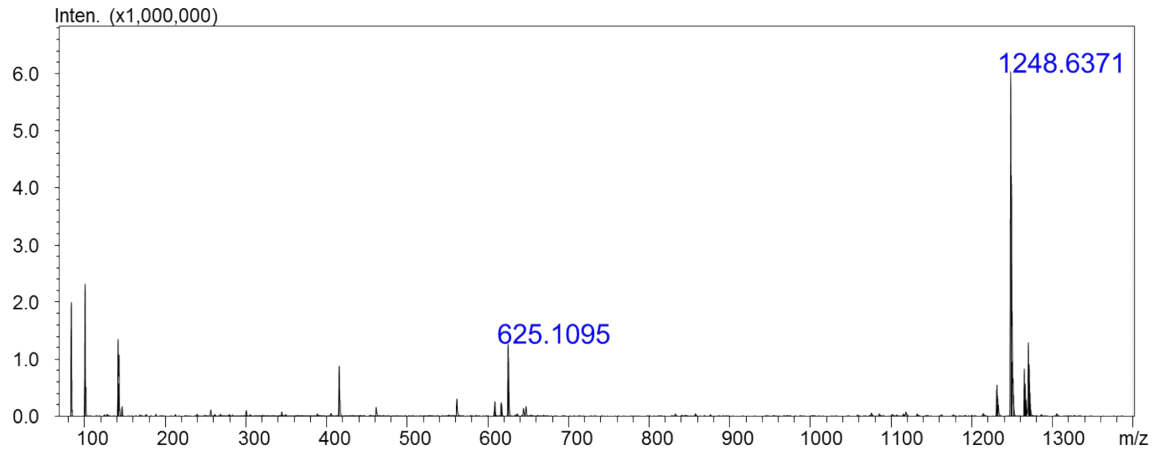


**Fig. S3** LC-MS spectrum of thiol-peptide **2**-conjugate, m/z 1248.6371 [M+1], 625.1095 [M+2]/2

**Amino-peptide 3 (Ac-KLAGV-$NH_2$) conjugation:**

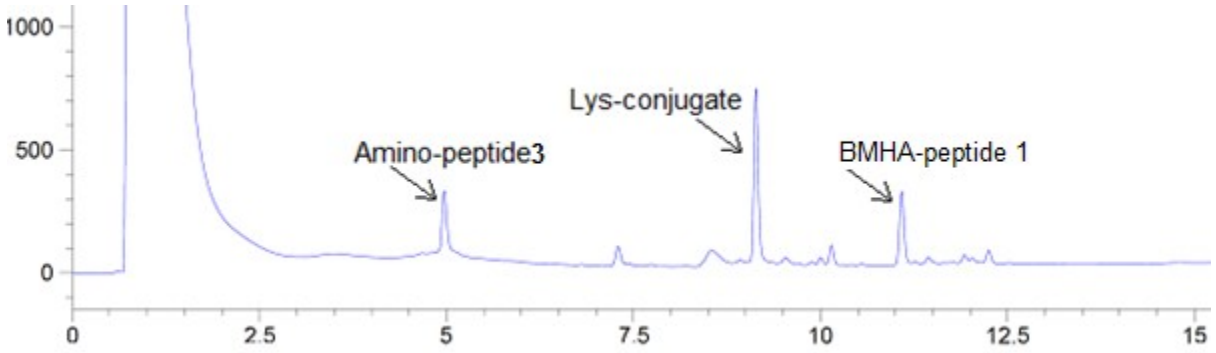


**Fig. S4** HPLC chromatogram of crude amino-peptide **3** conjugation

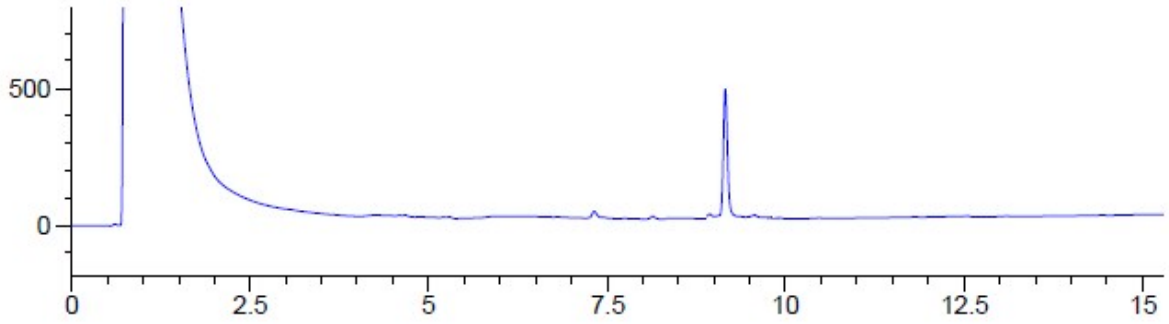


**Fig. S5** HPLC chromatogram of pure amino-peptide **3** conjugate

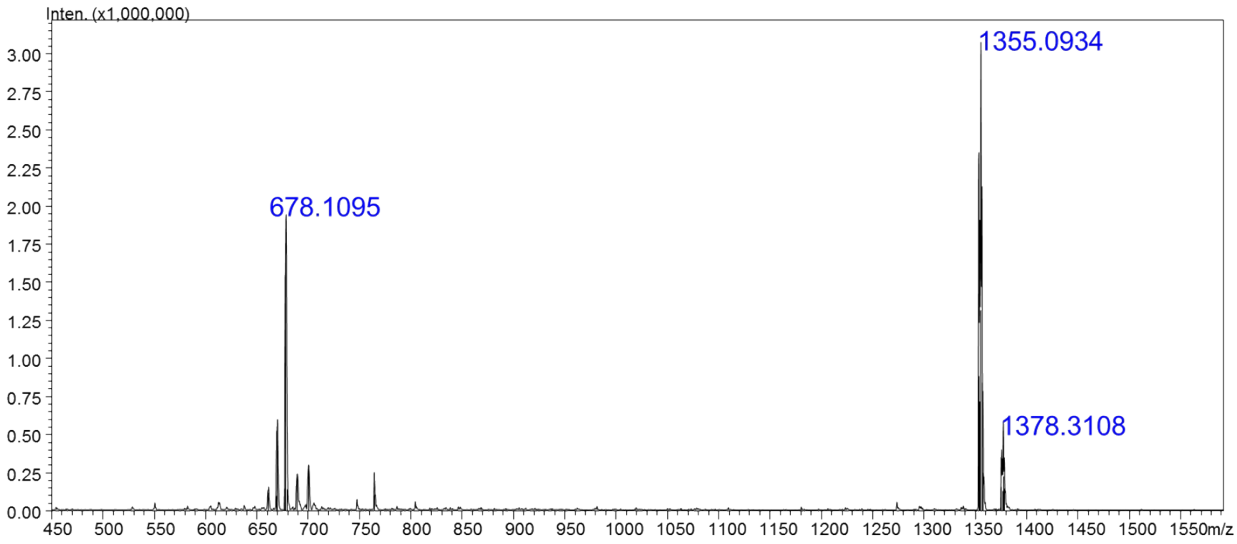


**Fig. S6** LC-MS spectrum of amino-peptide **3**-conjugate, 1355.0934 [M+1], 678.1095 [M+2]/2

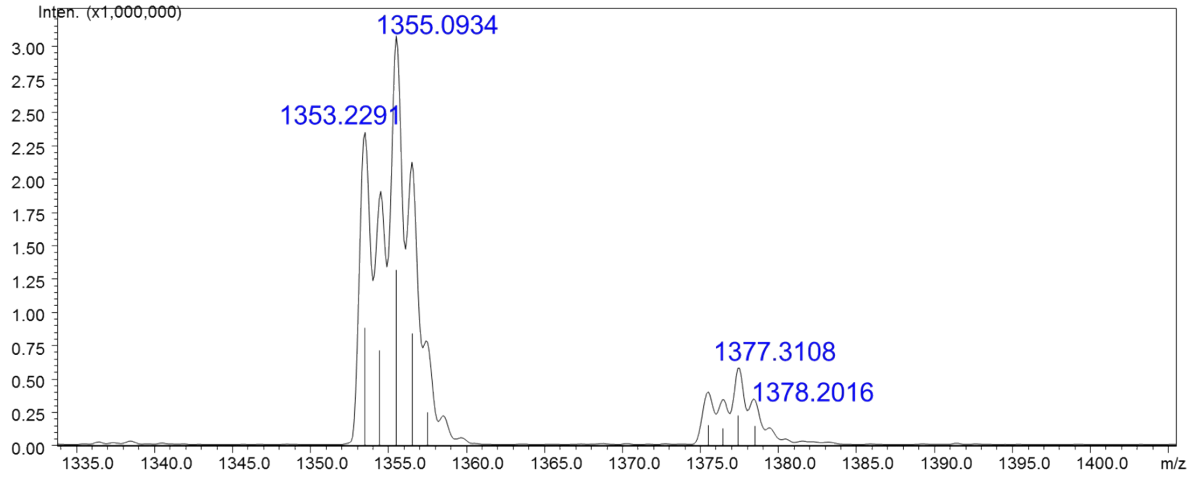


**Fig. S7** Zoomed LC-MS spectrum of amino-peptide **3**-conjugate showing bromide pattern

**Double conjugation**

**Example 1 (using simple amino acids):**

Here Fmoc-Cys-$NH_2$ and Fmoc-Lys-OH were used as thiol- and amino-moieties respectively. Initially, Fmoc-Cys-$NH_2$ (2 mg, 5.8 µmol, 1 equiv.) was reacted at the Br-position of BMHA (1.7 mg, 5.8 µmol, 1 equiv.) using the above described condition. After the completion of the

reaction monitored by HPLC, it was subjected to semi-preparative HPLC purification and dried. To this conjugate (1 mg, 1.81 μmol, 1 equiv.) was added a solution of Fmoc-Lys-OH.HCl (0.7 mg, 1.81 μmol, 1 equiv.)/ DIEA (1.5 μL, 9.05 μmol, 5 equiv.) dissolved in dry DMF (0.5 mL), reaction was monitored by HPLC and LC-MS.

HPLC $t_R$: 10.281 min (15-60% of B into A); LC-MS: Expected: 1323.7065; Found: 1324.6913 [M+1]

**Example 2 (using model peptides):**

Here YGGFL-$NH_2$ was used as the model peptide-**1** and Ac-XLAGV-$NH_2$ (X = C or K) were used as model thiol-peptide **2** and amino-peptide **3** respectively.

To the HPLC purified thiol-peptide-conjugate (1 mg, 0.8 μmol, 1 equiv.) constructed as explained above and lyophilized was added a solution of amino-peptide **3** (0.4 mg, 0.8 μmol, 1 equiv.)/ DIEA (0.68 μL, 4 μmol, 5 equiv.) dissolved in dry DMF (0.5 mL), reaction was monitored by HPLC and LC-MS.

HPLC $t_R$: 7.050 min (10-60% of B into A); LC-MS: Expected: 1775.7335; Found: 1776.7168 [M+1], 889.0873 [M+2]/2

**Example 3 (using specific peptides):**

Here HIV protease inhibitor, TLNF-OH and Ac-XTLNF-OH (X = C or K) were used as specific examples.

BMHA (52.2 mg, 3 equiv.) was reacted with H-TLNF-® (2-CTC resin) as described above, cleaved, purified and dried. On the other hand, Ac-CTLNF-OH and Ac-KTLNF-OH were synthesized on-resin, cleaved, purified and dried. BMH-TLNF (1 mg, 1.31 μmol, 1 equiv.) was reacted with Ac-CTLNF-OH (0.83 mg, 1.31 μmol, 1 equiv.) as explained above, monitored the reaction by HPLC/ LC-MS, purified and dried. To this conjugate (1 mg, 0.75 μmol, 1 equiv.) was added a solution of Ac-KTLNF-OH (0.5 mg, 0.75 μmol, 1 equiv.)/ DIEA

(0.64 µL, 6.55 µmol, 5 equiv.) dissolved in dry DMF (0.5 mL), reaction was monitored by HPLC and LC-MS.

HPLC $t_R$: 7.455 min (20-80% of B into A); LC-MS: Expected: 1985.9445; Found: 994.1279 [M+2]/2

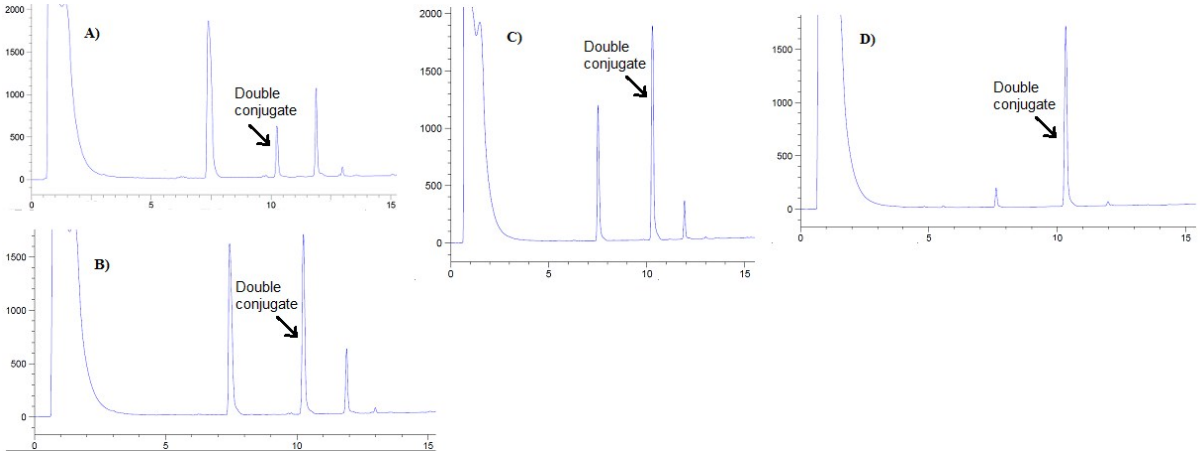


**Fig. S8** HPLC traces of double conjugation of Example 1 [using amino acids, Fmoc-Cys-$NH_2$ and Fmoc-Lys-OH] at A) 1h B) 3h C) 7h and D) overnight

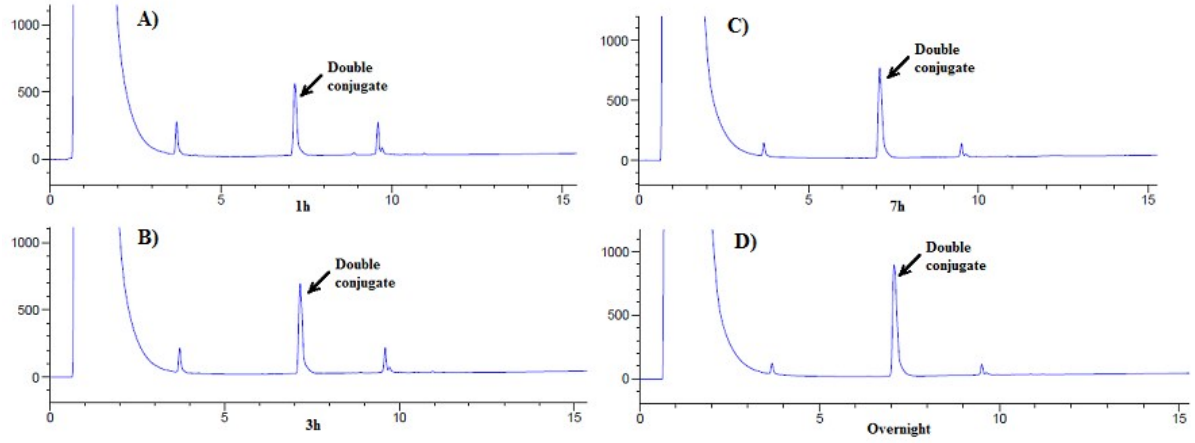


**Fig. S9** HPLC traces of double conjugation of Example 2 [using Model peptides, Ac-XLAGV-$NH_2$, X = C/K] at A) 1h B) 3h C) 7h and D) overnight

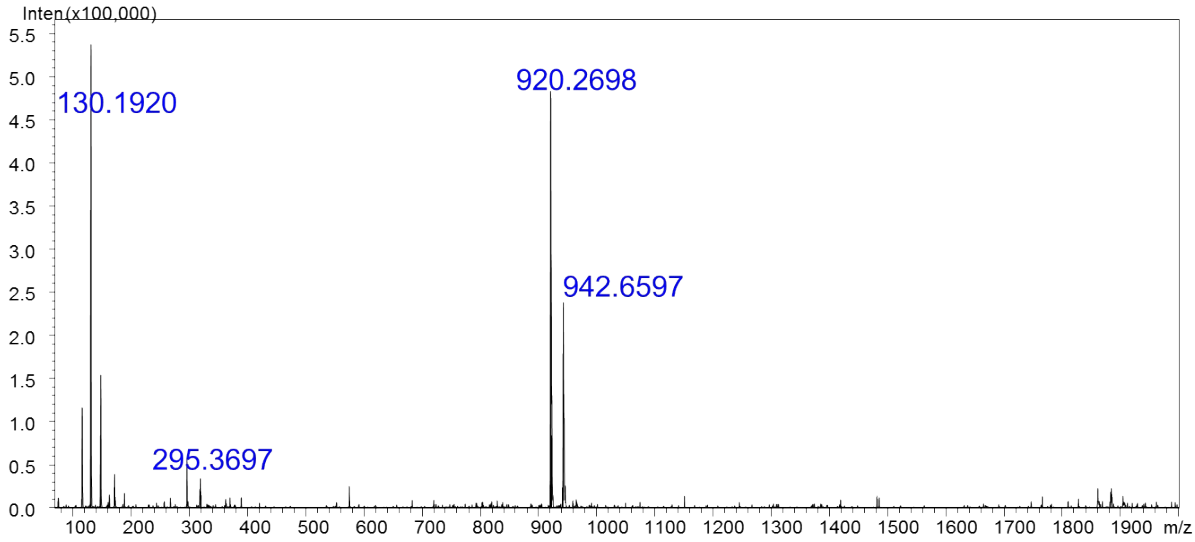


**Fig. S10** LC-MS spectrum of the double conjugate of Example 1; m/z 920.2698 [M+1], 942.6597 [M+Na]

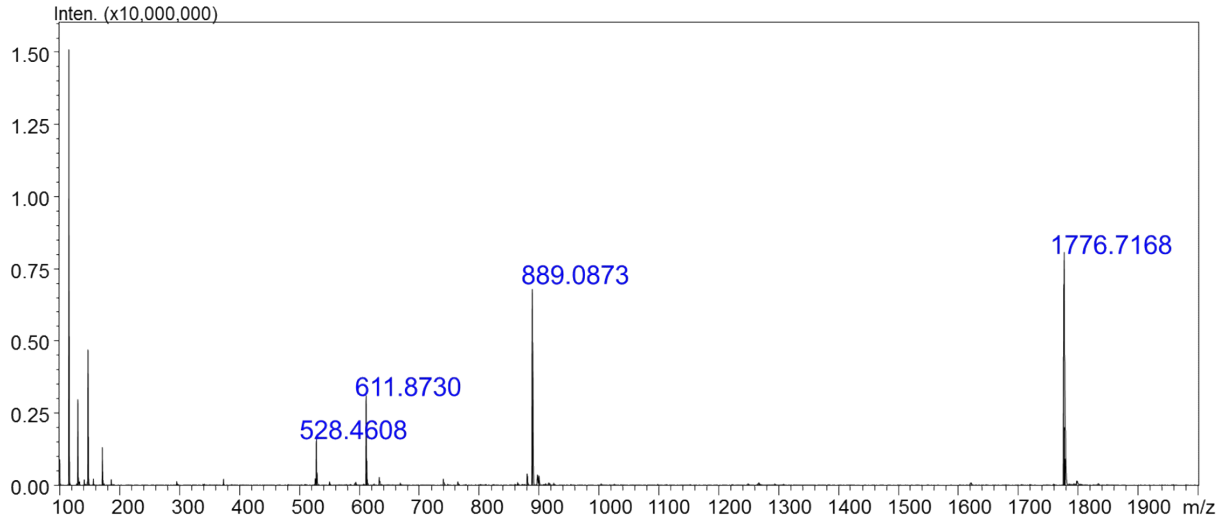


**Fig S11.** LC-MS spectrum of the double conjugate of Example 2, m/z 1776.7168 [M+1], 889.0873 [M+2]/2

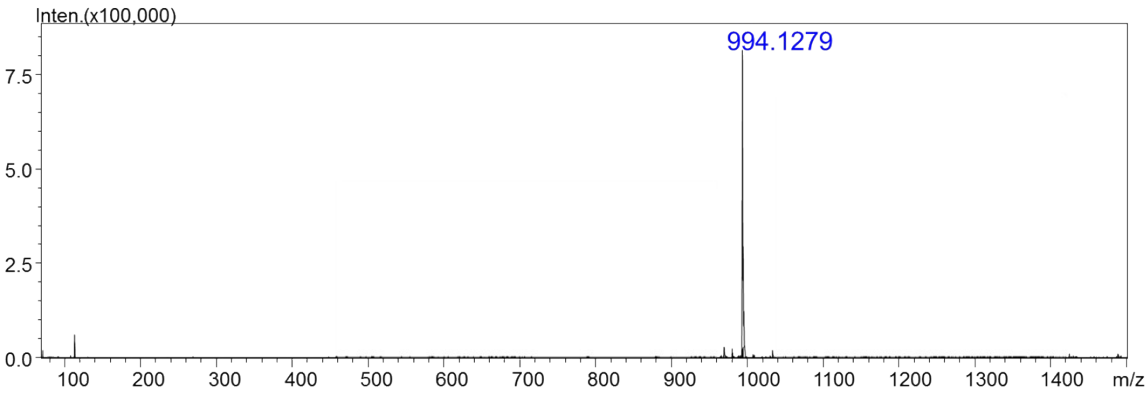


**Fig. S12** LC-MS spectrum of the double conjugate of Example 3, m/z 994.1279 [M+2]/2