Revealing the extracellular function of HMGB1 N-terminal acetylation via a protein semi-synthesis approach

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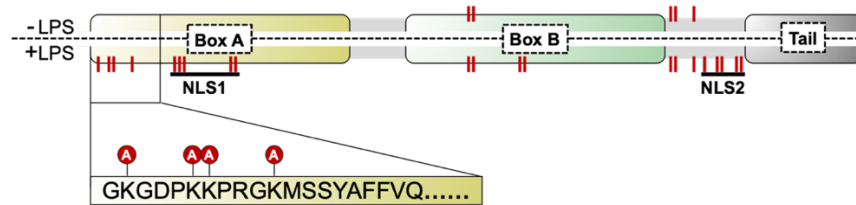
Supplementary Information

Supplementary Figures

Figure S1. HMGB1 was hyper-acetylated after LPS stimulation. The modification sites were highlighted by red lines. Data was adopted from Lu et al.[1]

A

Enzyme	Cleavage site	Used in this study
TEV	Glu-Asn-Leu-Tyr-Phe-Gln↓Gly/Ser/Cys	yes
Ulp1	SUMO↓ C-terminal independent except for Pro	yes
Factor Xa	Ile-Glu/Asp-Gly-Arg↓ C-terminal independent	no
Enterokinase	Asp-Asp-Asp-Asp-Lys↓ C-terminal independent except for Pro	no

B

Purification → → Digestion →

Figure S2. Method to generate recombinant Ser/Thr/Cys N-terminal protein.
(A). List of enzymes which can produce a Ser/Thr/Cys N-terminal protein.
(B). After Purification, N-terminal Ser/Thr/Cys can be released by indicated enzymes.
In cases where the protein is not soluble, it can be purified from the inclusion body using denaturing buffer (8 M Urea). The purified protein can then be diluted with 2 M Urea, at which concentration the protein remains soluble and enzymes (*e.g.,* TEV, Ulp1) can still cleave the recognition site. After digestion, the protein solution can be further purified by HPLC. In addition, methionine aminopeptidase can be used to remove the first Met, producing N-terminal Ser/Thr/Cys *in situ*.

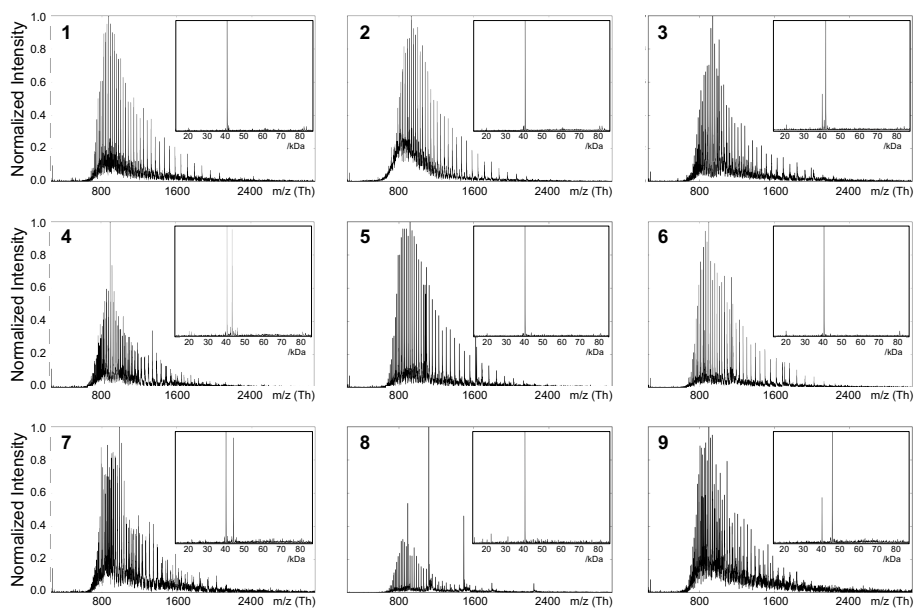| Ser-MBP	1	2	3	4	5	6	7	8	9
Theoretical Mass (Da)	41066	41080	42362	43173	43708	43871	44456	44748	45911
Observed Mass (Da)	41070 (>95%)	41081 (>95%)	42363 (75%)	43178 (50%)	- (<5%)	- (<5%)	44460 (50%)	- (<5%)	45914 (75%)

Figure S3. Mass spectra of ligation products obtained from peptide SAL esters and Ser-MBP. The conversion rates were calculated according to the deconvolution results. Peptide ester 5, 6, and 8 overlap with protein peak on HPLC trace.

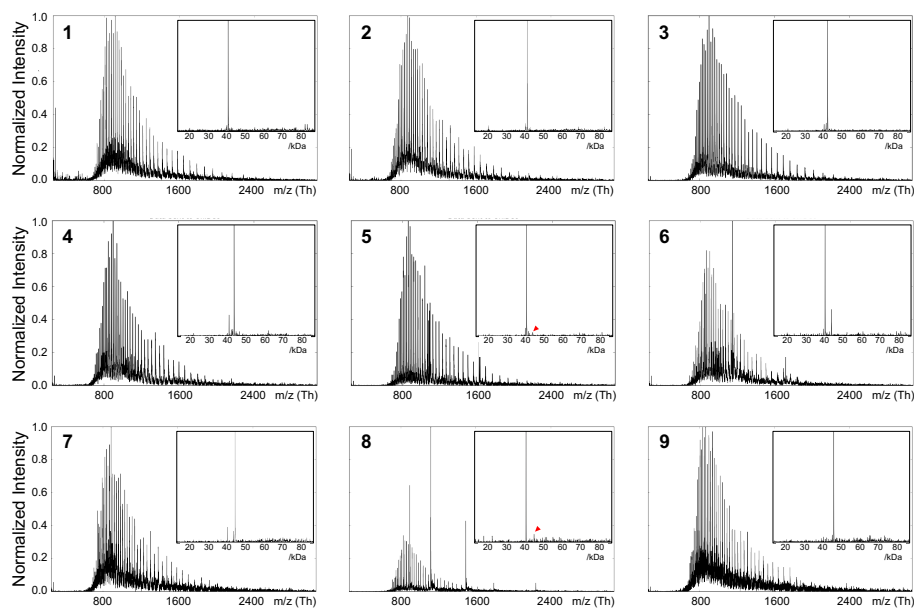| Thr-MBP	1	2	3	4	5	6	7	8	9
Theoretical Mass (Da)	41080	41094	42376	43187	43722	43885	44470	44762	45925
Observed Mass (Da)	41088 (>95%)	41106 (>95%)	42377 (>95%)	43191 (85%)	43719 (<5%)	43910 (20%)	44468 (90%)	44758 (5%)	45922 (>95%)

Figure S4. Mass spectra of ligation products obtained from peptide SAL esters and Thr-MBP. The conversions were calculated according to the deconvolution results. Peptide ester 5, 6, and 8 overlap with protein peak on HPLC trace.

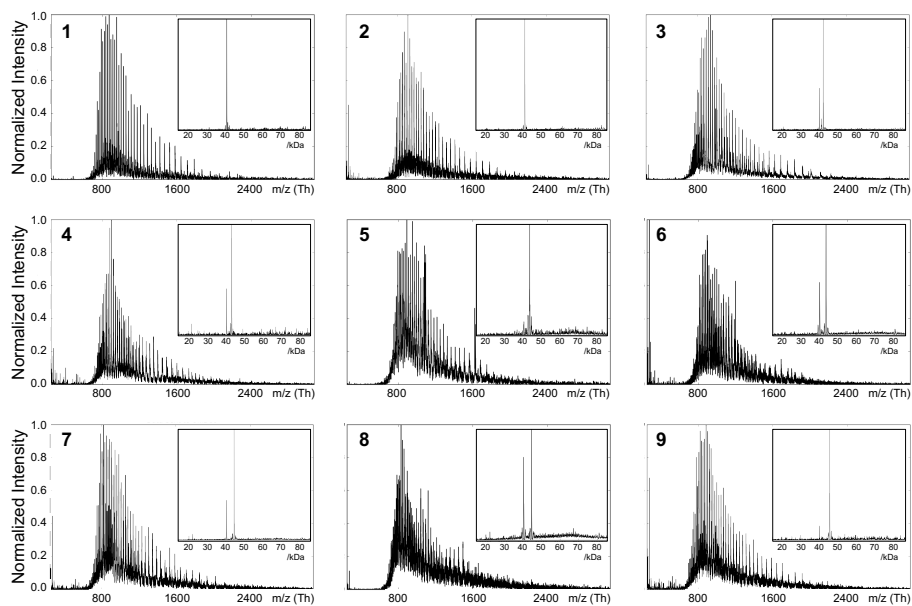| Cys-MBP	1	2	3	4	5	6	7	8	9
Theoretical Mass (Da)	41082	41096	42378	43189	43722	43887	44472	44764	45927
Observed Mass (Da)	41090 (>95%)	41107 (>95%)	42381 (75%)	43194 (70%)	43723 (>90%)	43894 (70%)	44476 (70%)	44771 (60%)	45925 (85%)

Figure S5. Mass spectra of ligation products obtained from peptide SAL esters and Cys-MBP. The conversions were calculated according to the deconvolution results. Peptide ester 5, 6, and 8 overlap with protein peak on HPLC trace.
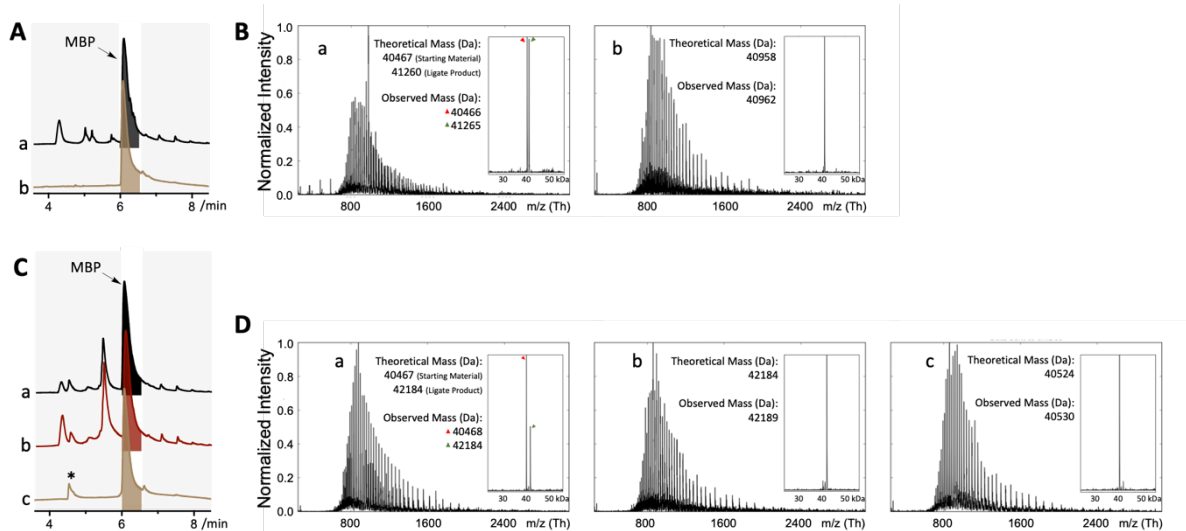
Figure S6. Purification strategy for STL/CPL mediated protein semi-synthesis.

(A). HPLC UV traces of reaction mixtures in Disulfide linker mediated purification strategy. a: before purification; b: purified product.

(B). Mass spectra of the products in a and b.

(C). HPLC UV traces of reaction mixtures in His tag and TEV based purification strategy. a: before purification; b: purified product; c: product after TEV digestion.

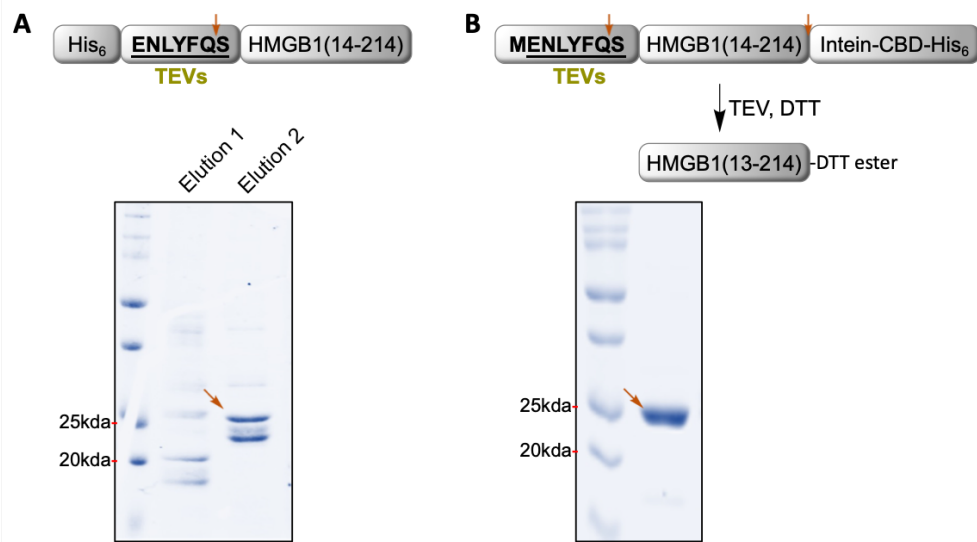(D). Mass spectra of products in a, b, and c. *: the remaining peptide SAL ester.

Figure S7. Purification strategy for HMGB1(13-214).

(A). The N-terminal His tag purification strategy produced several truncated species of HMGB1.

(B). Intein tag-based purification strategy. DTT ester on C-terminus hydrolyzes slowly.
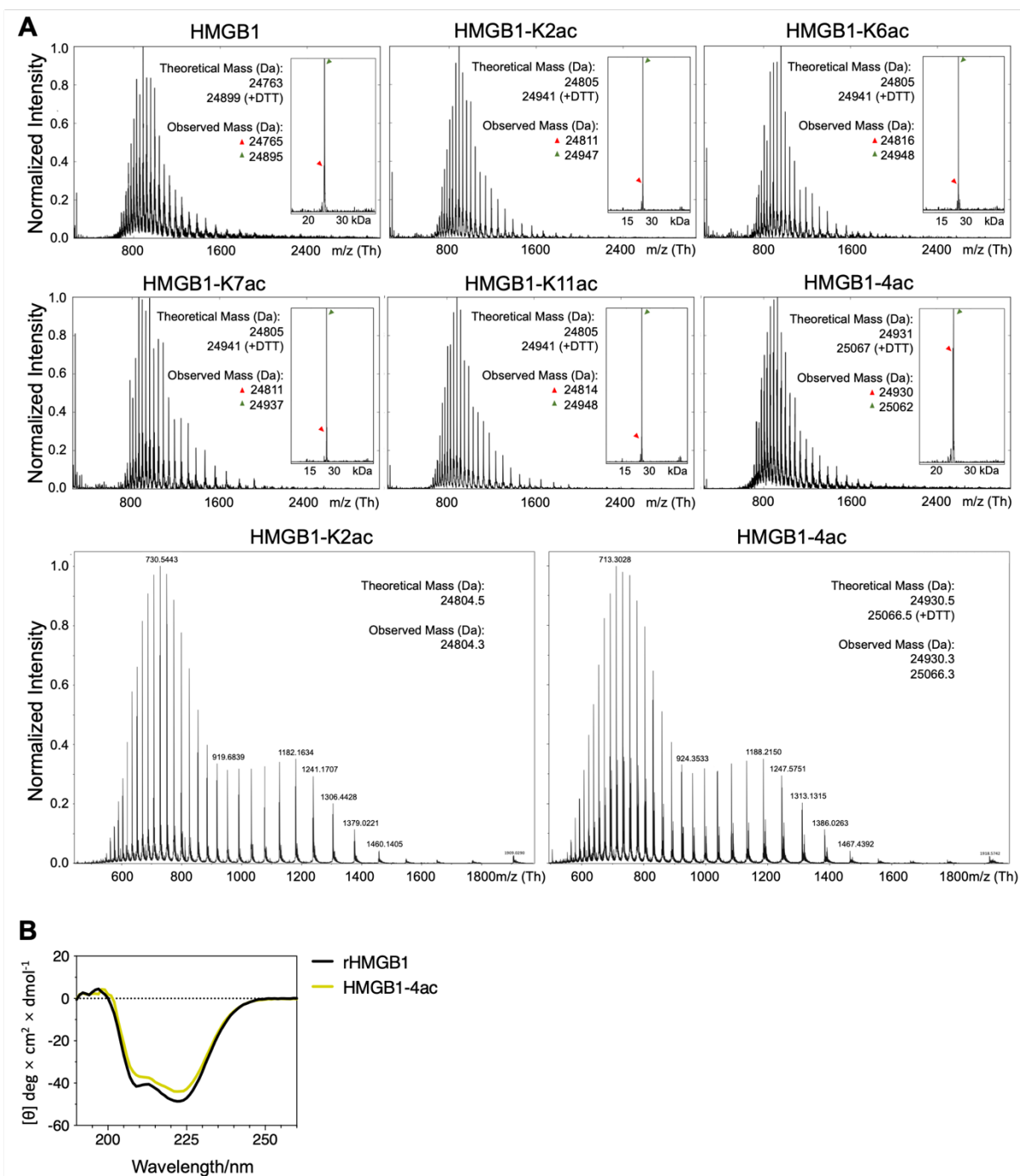
Figure S8. Characteraztion of synthetic HMGB1 proteins.
(A). Mass spectra of synthetic HMGB1 proteins. The high-resolution mass spectra of HMGB1-K2ac and HMGB1-4ac are also showed. DTT ester on C-terminus hydrolyzed slowly.
(B). Circular dichroism (CD) spectrum of synthetic HMGB1-4ac after refolding. Recombinant full length HMGB1 (rHMGB1) purified under native conditions was used as the reference.
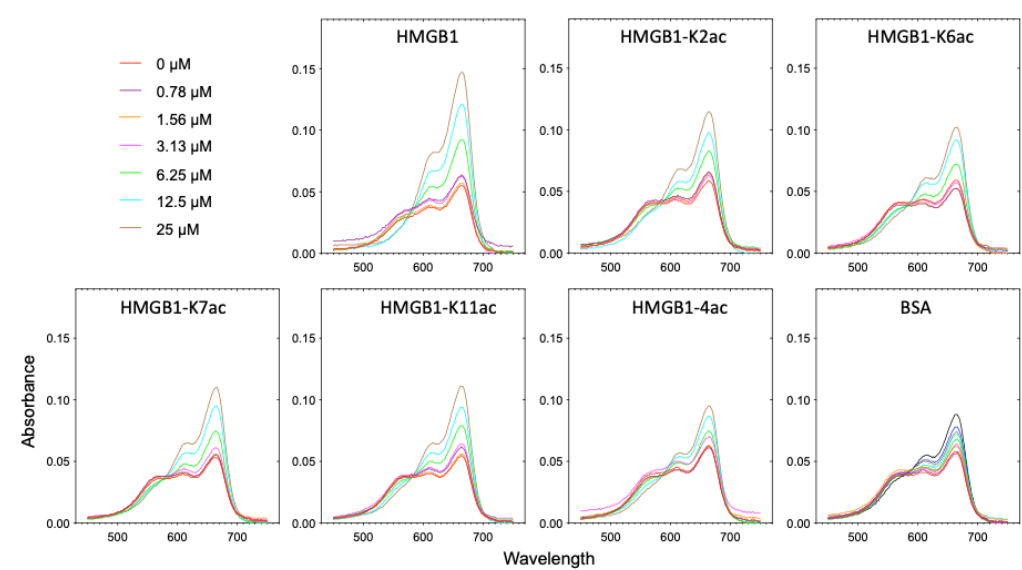
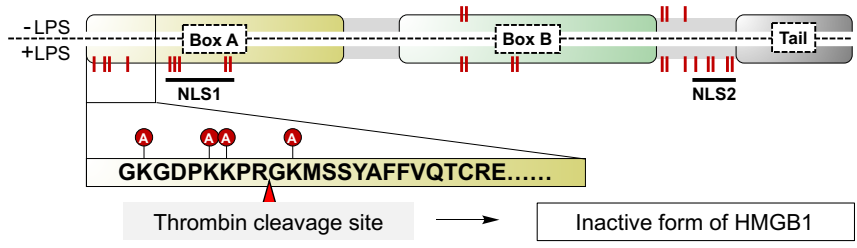**Figure S9. Absorbance spectra of titration of MB-heparin with different synthetic HMGB1 proteins.**

Figure S10. Thrombin degrades HMGB1 to a less proinflammatory form.[2]

Thrombin Site: P4 P3 P2 P1 P1' P2' P3' P4'
Canonical Site: L V P R G S
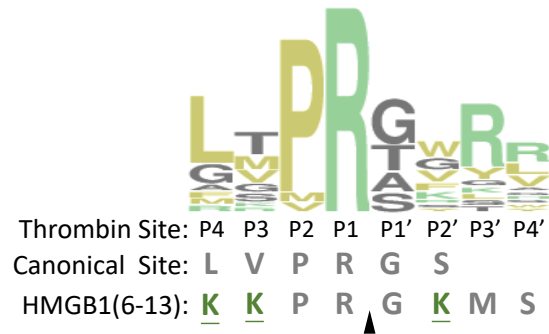HMGB1(6-13): K K P R G K M S

Figure S11. Position weight matrix for substrate preference of thrombin.
Raw data was adopted from Gallwitz M et al, in which the recognition preference of thrombin was profiled by phage display technology.[3] It showed that the aliphatic residues (*e.g.,* Leu) at P4, Pro at P2, Arg at P1, and small residues at P1' are preferred by thrombin. Acetylation sites of HMGB1 are underlined. Cleavage site is at P1^P1'. Canonical site indicates the thrombin recognition site for recombinant protein production.
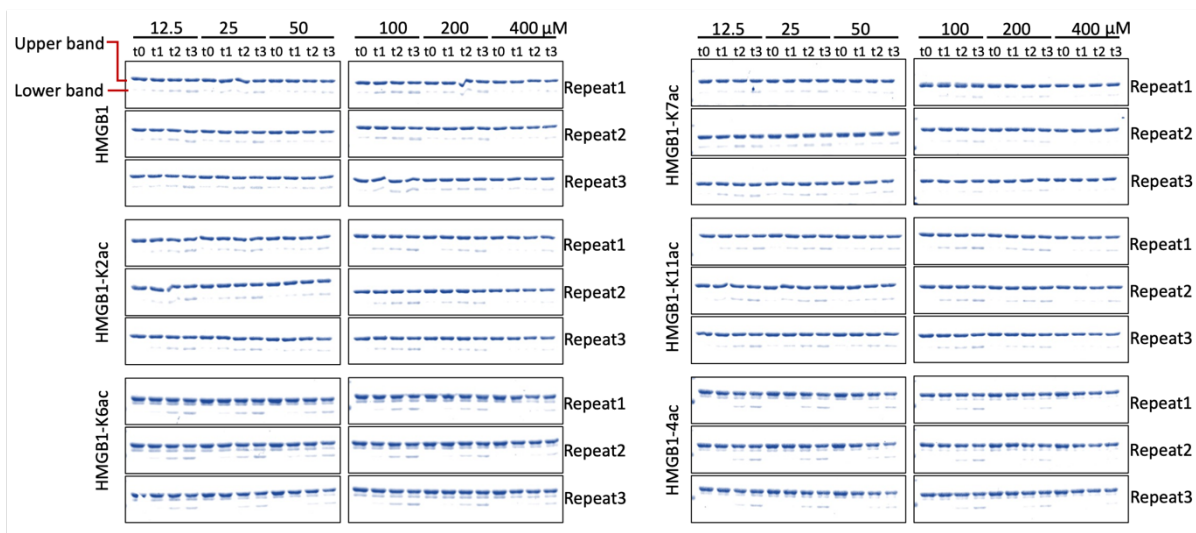
Figure S12. Original data of SDS-PAGE for Michaelis-Menten kinetics analysis. After digestion, equal amounts of protein were loaded for SDS-PAGE analysis. Bands intensities of full length HMGB1 proteins (upper band) and cleaved products (lower bands) were quantified by Image lab software. Consumption = (lower band) / (lower band + upper band) * concentration. Initial reaction velocities were calculated based on the consumption of the substrate at different time points. All the substrate consumption ratios were optimized to be under 25%. Data was fit to a Michaelis–Menten enzyme kinetics model. t0: 0 min; t1, t2, and t3: different time points.
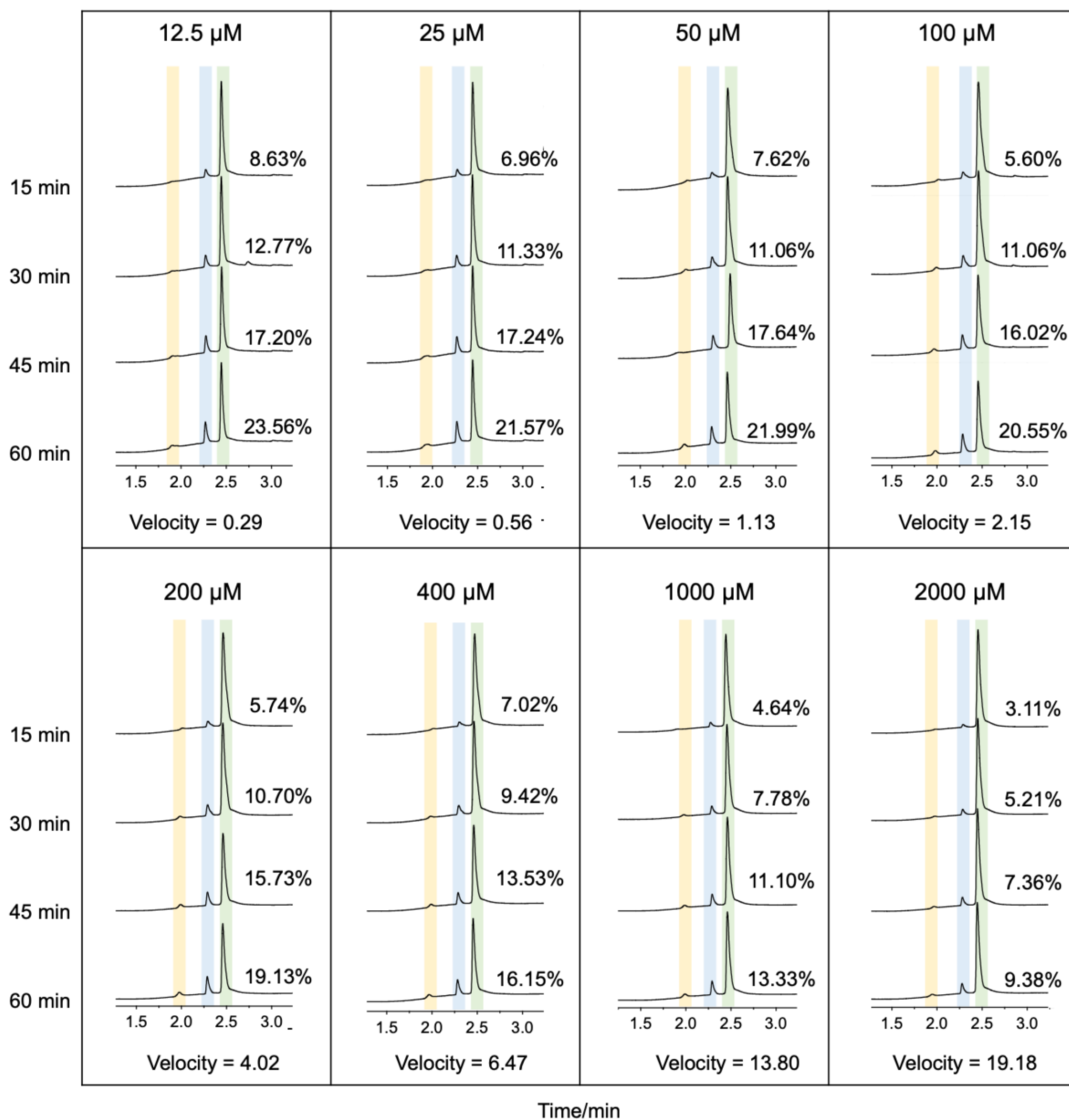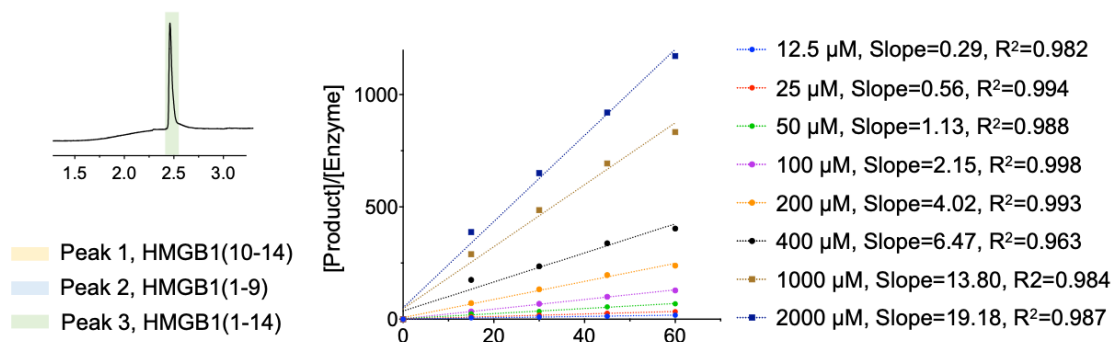
Figure S13. Representative original data of HPLC for Michaelis-Menten kinetics analysis (replicate 1 for HMGB1(1-14)). The peaks areas were integrated for calculation. Consumption = (peak1 + peak2) / (peak1 + peak2 + peak3) * concentration. Initial reaction velocities were calculated based on the consumption of the substrate at different time points. All the substrate consumption ratios were optimized to be under 25%. Data was fit to a Michaelis–Menten enzyme kinetics model.
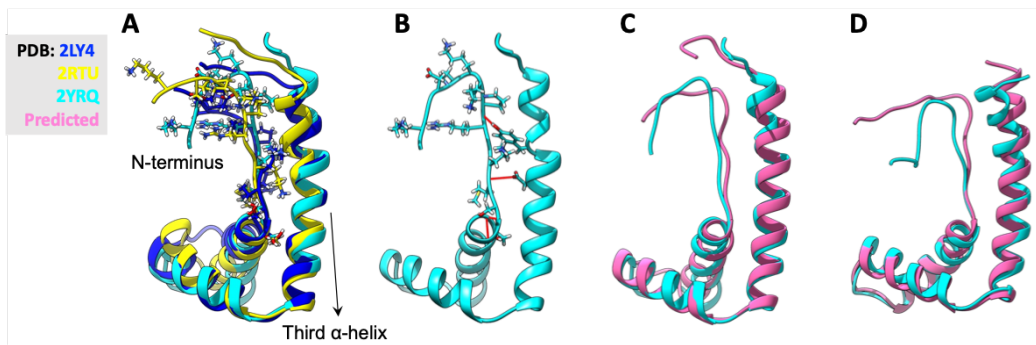
Figure S14. Comparison of HMGB1's structures.

(A). HMGB1 boxA structures which are resolved by independent studies.

(B). Putative hydrogen bonds that stabilize HMGB1 N-terminaus. Hydrogen bonds are highlight by red lines.

(C). HMGB1 boxA structures alignment.

(D). HMGB1 boxB structures alignment. Note: C-terminal tail and the linker between boxA and boxB are highly flexible.
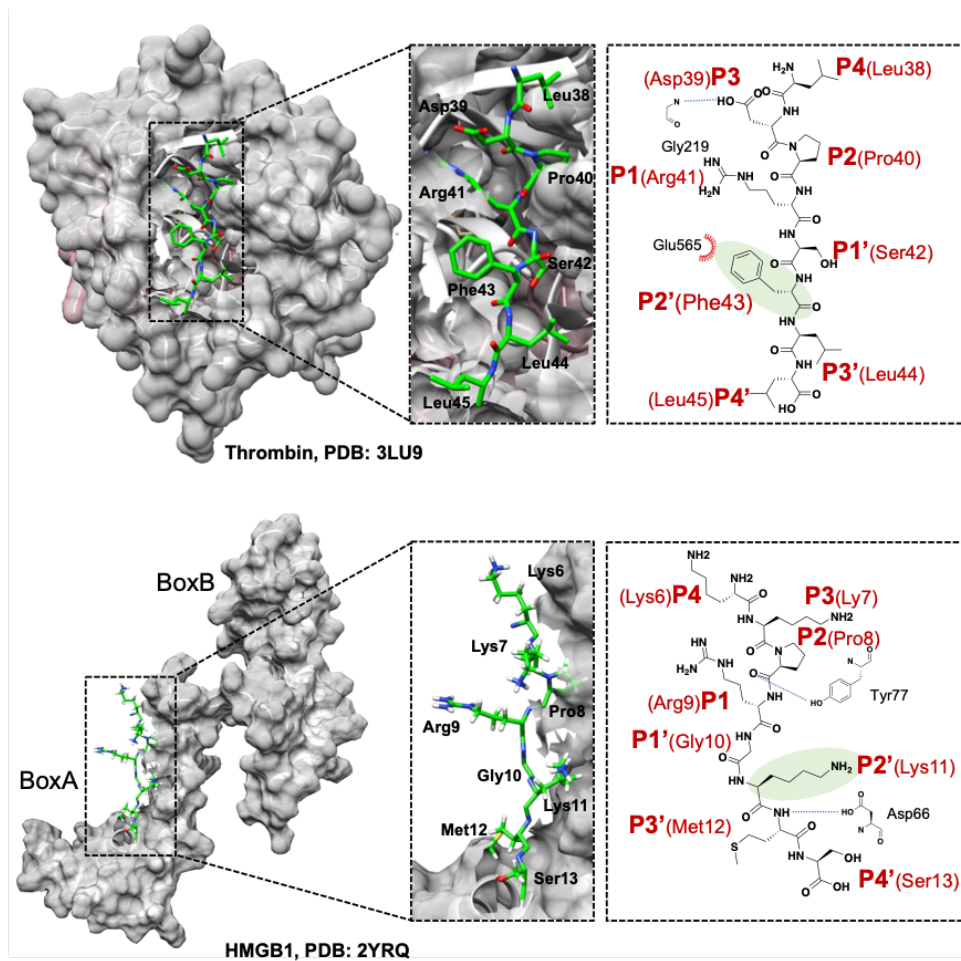
Figure S15. Structure comparison between thrombin substrate and HMGB1 N-terminus.
Phe43 and Lys11 have different directions.
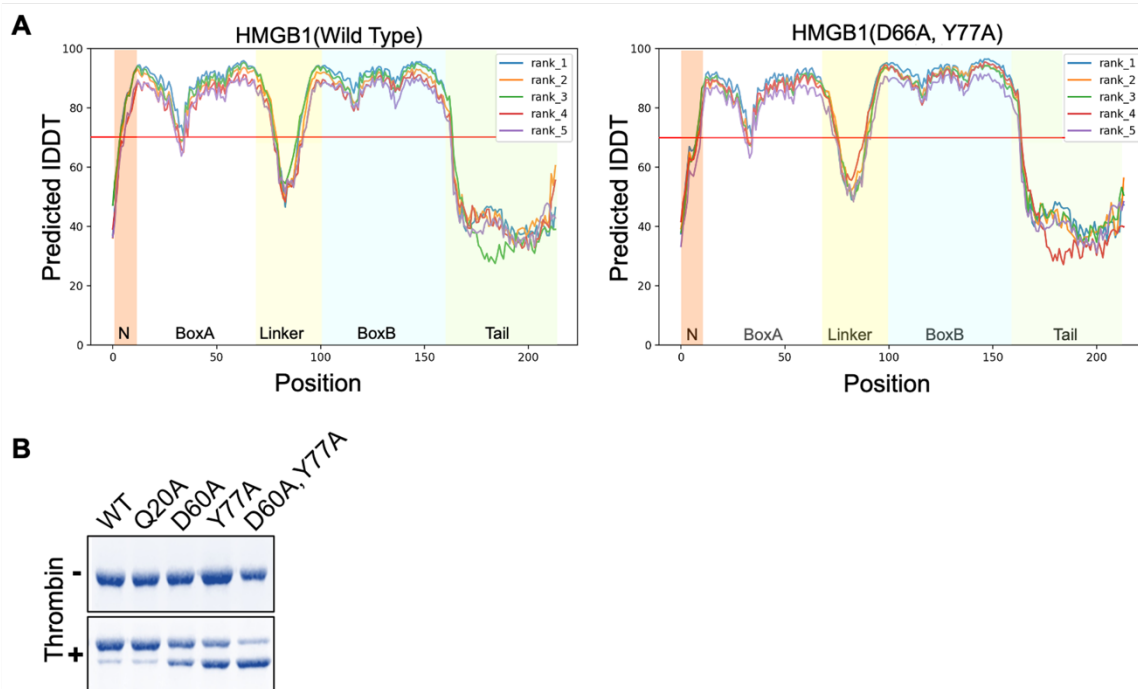
Figure S16. Intramolecular interaction between N-terminus and third α-helix.

(A). pLDDT score per position for HMGB1(wt) and HMGB1(D66A, Y77A). Top5 models were plotted. Regions with pLDDT > 90 are expected to be modelled to high accuracy; Regions with pLDDT between 70 and 90 are expected to be modelled well (a generally good backbone prediction); Regions with pLDDT below 70 are low confidence.

(B). HMGB1 and its mutations were digested by thrombin. D60A, Y77A single site mutation and D60A,Y77A double sites mutation can be digested by thrombin faster.
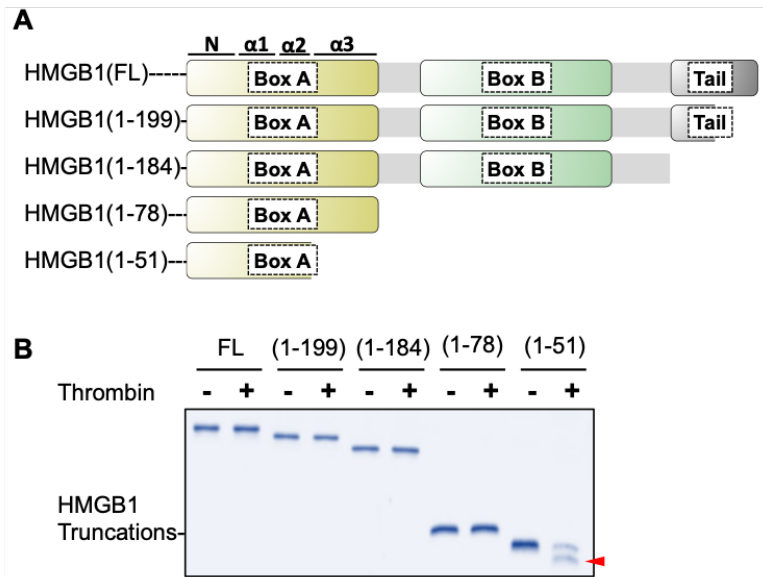
Figure S17. Third α-helix twists N-terminus and inhibit thrombin's activity.
(A). HMGB1 truncations that were used in thrombin digestion assay. N: N-terminus; α1: first α-helix; α2: second α-helix; α3: third α-helix.
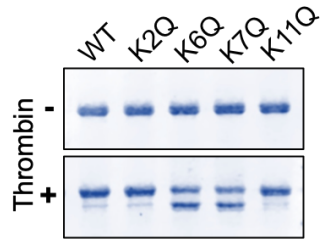(B). HMGB1 truncations were digested by thrombin. The cleavage of HMGB1(1-51) occurred at a faster rate.

Figure S18. HMGB1 site mutations was digested by thrombin. K6Q and K7Q site mutation improve thrombin's activity on HMGB1 proteins.
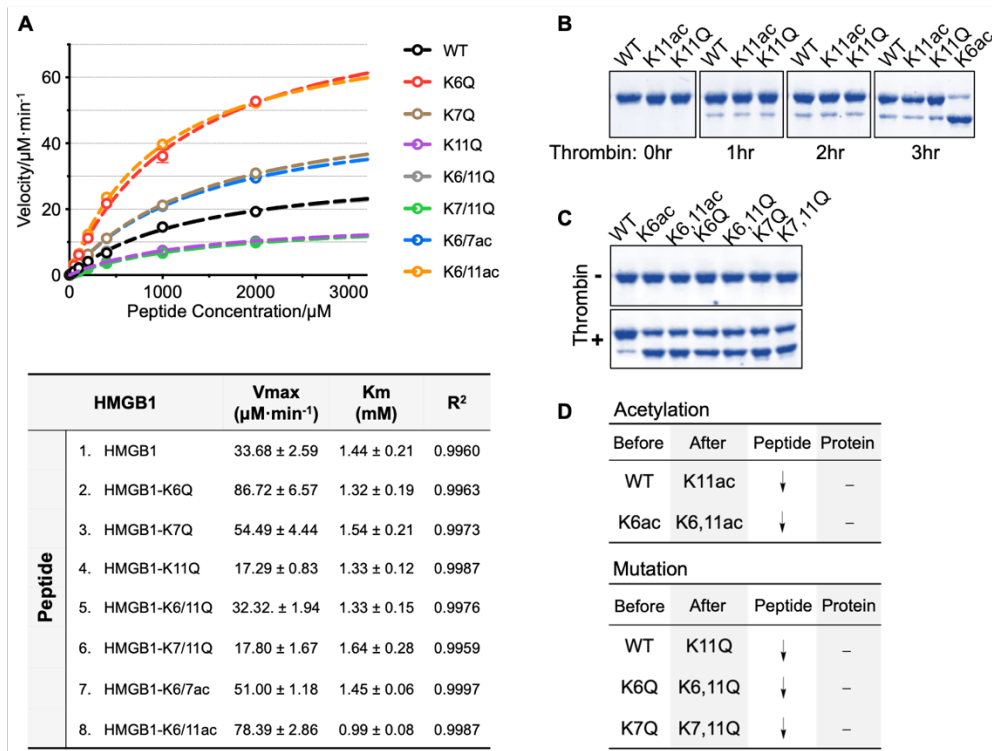
Figure S19. Lys11 is not involved in thrombin digestion.
(A). Michaelis-Menten kinetics analysis of thrombin on HMGB1 N-terminal peptides. Values for Vmax and Km represent 95% profile likelihood.
(B). HMGB1, HMGB1-K11ac, and HMGB1(K11Q) were digested by thrombin for different time. Thrombin showed no difference in activity among these three HMGB1 proteins, which suggests the modification Lys11 does not affect thrombin's activity. HMGB1-K6ac was used as a positive control.
(C). HMGB1, HMGB-K6ac, HMGB1-K6,11ac, HMGB1(K6Q), HMGB1(K6,11Q), HMGB1(K7Q), and HMGB1(K7,11Q) were digested by thrombin. Thrombin showed no difference in activity after Lys11 modification (acetylation or mutation) on HMGB1-6ac, HMGB1(K6Q), and HMGB1(K7Q), which further suggests the modification Lys11 does not affect thrombin's activity.
(D). Results summary of A, B, and C. Acetylation or mutation of Lys11 consistently inhibit thrombin digestion on HMGB1 N-terminal peptides, while have no effect on HMGB1 proteins. ↓ represents decreased thrombin digestion; - represents no change.
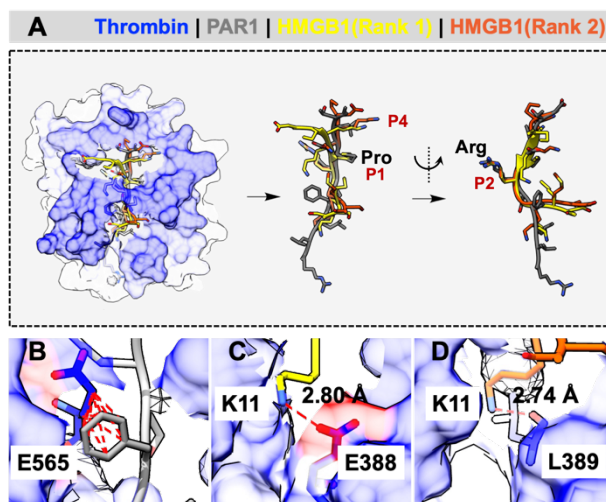
Figure S20. PatchMAN approach for HMGB1 N-terminus and thrombin docking.

(A). Structure overview of docking results of thrombin-HMGB1 N-terminus and HMGB1-PAR1.

(B). Thrombin-PAR1 complex. PDB: 3LU9. Phe43 is in *van der Waals* interaction with Cβ and Cγ of Glu565 of thrombin

(C). Rank 1 model shows ε-amino group of Lys11 interacted with E388 of thrombin by charge.

(D). Rank 2 model shows ε-amino group of Lys11 formed a backbone hydrogen bond with L389 of thrombin.

Thrombin structure from 3LU9 was used for docking.
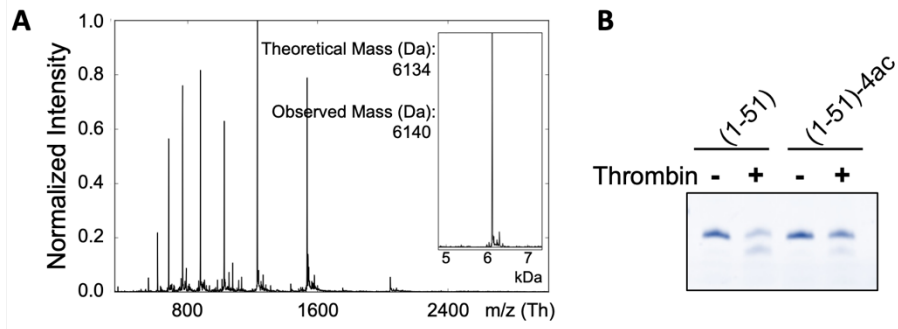
Figure S21. Wild type HMGB1(1-51) was cleaved much faster than its tetra-acetylated form

(A). Mass spectra of the semi-synthetic HMGB1(1-51)-4ac.

(B). HMGB1(1-51) and HMGB1(1-51)-4ac were digested by thrombin. Tricine-SDS-PAGE was used.
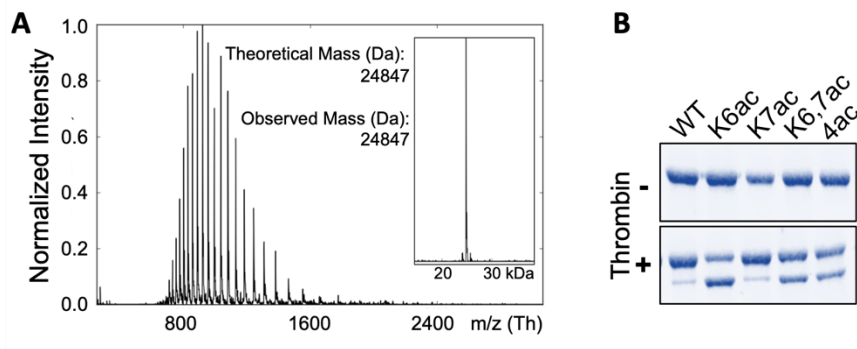
Figure S22. Lys6 acetylation is able to overcome Lys7 acetylation's inhibitory effect on protein.
(A). Mass spectra of the semi-synthetic HMGB1-K6,7ac.
(B). HMGB1 proteins were digested by thrombin. HMGB1-K6,7ac (di-acetylation) exhibited increased digestion.

Supplementary Tables

A

Pyridine/ acetic acid	Remark
1:3	
1:6	Can not dissolve proteins at 1 mM.
1:9	

B

Pyridine derivatives	Remark
Collidine	Can not dissolve proteins at 1 mM.

C

| Co-Solvent	Remark
DMF	Can not dissolve proteins at 2 mM.
DCM	Can not dissolve proteins at 2 mM.
TFE	Can not dissolve proteins at 2 mM.
HFIP	Can dissolve all tested proteins. Some salicylaldehyde esters may hydrolyzed quickly. If so, we recommend to use DMSO. Side rection is observed with salicylaldehyde ester (salicylaldehyde ester is displaced by HFIP).
DMSO	Can dissolve all tested proteins. The solution may become viscous during ligation, which may lead to slightly lower ligation conversion than that in HFIP. No side rection with salicylaldehyde ester is observed.

D

Temperature	Remark
25 °C or R.T.	We always set up the ligation at room temperature.

E

Equivalent of peptide ester	Remark
2	All tested peptide esters are soluble. Low conversion (Most reactions are ~20%).
5	All tested peptide esters are soluble. Medium conversion.
10	All tested peptide esters are soluble. High conversion. Products are pure.
20	Some peptide esters are not soluble. High conversion. Some side reactions occur.

Table S1. Condition screening for STL. To dissolve the protein at 1mM, different ratio of pyridine/acetic acid (A), pyridine derivatives to replace pyridine (B), and co-solvent (C) were screened. We found that protein can be firstly dissolved in HFIP or DMSO at 2 mM, then diluted in pyridine/acetic acid to prepare 1mM concentration of protein for ligation. Besides, the equivalent of peptide salicylaldehyde esters was also screened (E).

N-terminal Amino Acid of POI	C-terminal Amino Acid of Peptide Esters		
	FAST	SLOW	DECOMPOSED
Ser	Ala, Gly, Ser, Gln, Thr, Phe, Cys(SStBu)	Val, Ile, Met, Asn, Tyr, Leu, Trp, Arg, Pro, His	
Thr	Ala, Gly, Ser, Gln, Thr, Phe, Cys(SStBu)	Val, Ile, Met, Asn, Tyr, Leu, Trp, Arg, Pro, His	Lys, Asp, Glu
Cys	ALL except for Lys, Asp, Glu	-	

Table S2. Recommendation for disconnected sites for STL/CPL mediated protein semi-synthesis. Data was original from Liu et al. and Tan et al.[4,5]

Supplementary Notes

Lys11 is not involved in thrombin-mediated degradation on full length HMGB1 proteins

According to thrombin-PAR1(one of thrombin's natural substrates) complex structure, the side chain of Phe at P2' has same direction with the critical Arg at P1 and is in *van der Waals* interaction with Cβ and Cγ of Glu565 of thrombin (Figure S15).[6] However, we found that the sidechain of Lys11 (P2') was stabilized towards to the third α-helix side. Therefore, we inferred that Lys11 sidechain is sequestered by the 3D structure and is unable to actively participate in thrombin recognition and cleavage. This could also explain why the inhibitory effect was not detected on full length HMGB1-K11ac protein. Furthermore, if Lys6 acetylation is capable to counteract Lys7 acetylation's inhibition (the overall effect of di-acetylation on Lys6 and 7 is still accelerative), tetra-acetylation will lead to an increased digestion eventually.

In addition to AlphaFold2 predicted models (Figure 4D), we also recombinantly generated these HMGB1 mutations accordingly and performed thrombin digestion assay (Figure S16B). An increased digestion was observed in D66A and Y77A mutations, indicating that D66 and Y77 indeed contributed to the conformational hindrance or twist by forming hydrogen bonds with the N-terminus. Abrogating the hydrogen bonds allowed the N-terminus to position properly for the thrombin cleavage. This conclusion was further proved by digestion assay on HMGB1 truncations. Several HMGB1 truncations were recombinantly generated and subjected to digestion assay (Figure S17). Consistent with the site mutations, we observed a significant improvement of thrombin activity upon truncating HMGB1 from the third α-helix. Notably, under this theory, thrombin should have higher activity on wild type N-terminus peptide than wild type full length protein, which was not reflected by Michaelis-Menten kinetics analysis (Figure 4A, and B). We considered that this error was down to the different quantification methods (SDS-PAGE for proteins, HPLC for peptides).

We next validated that Lys11 sidechain is not involved in thrombin recognition and cleavage within HMGB1 protein. Herein, in addition to HMGB1-K11ac, a Lys11 mutation, HMGB1-K11Q, was introduced, which was regarded as an acetylation mimic. In short peptide, we could observe a significantly inhibitory effect (Figure S18). Importantly, like acetylation, this site mutation still did not show any effect on full-length protein (Figure S19A, and B). Besides, di-acetylated HMGB1 (HMGB1-K6,11ac) and double mutations (K6,11Q) or (K7,11Q) were generated. Similarly, the inhibitory effect from K11ac or K11Q was undetectable (Figure S19C). It is worth mentioning that K7Q mutation, as the mimic of K7ac, yet showed an opposite effect to K7ac, raising the concern about the widely used Lys acetylation mimic. All these results indicated that on short peptide, Lys11 at P2' is preferred by thrombin, hence acetylation or mutation on Lys11 will impair thrombin digestion; while on protein, Lys11 sidechain is sequestered by HMGB1 3D structure and the cleavage of HMGB1 by thrombin is independent of Lys11 (Figure S19D). To understand the reason why Lys at P2' is preferred, we also employed PatchMAN (Patch-Motif AligNments) to simulate the thrombin-HMGB1 N-terminus interaction.[7] While the abilities of computational method for the *de novo* prediction of ligand-protein interactions are limited, there is a main reason why our case is informative and reliable for explaining the interactions. We found that in top 2 models, HMGB1 N-terminus adopted conformations similar to that of the native substrate peptide (PAR1) and stably bound to the primary substrate-binding pocket of thrombin (Figure S20). In particular, the conserved residues (Pro at P2, Arg at P1) in HMGB1 N-terminus match the positioning of that in PAR1 (Figure S20A, and B). Docking results also showed that Lys6, 7, and 11 all contacted with thrombin. Specifically, the side chains of Lys6 (P4) in HMGB1 in both models and Leu38 (P4)

in PAR1 located in the hydrophobic pocket, proving less hydrophilicity at P4 would benefit thrombin cleavage. Though Lys11 was shown to interact with thrombin in different manners in two models (In rank 1 model, ε-amino group of Lys11 interacted with E388 by charge, Figure S20C; In rank 2 model, ε-amino group of Lys11 formed a backbone hydrogen bond with L389, Figure S20D), acetylation/mutation of Lys11 interrupt both kinds of interactions, which in turn leads to a reduction in thrombin digestion. In addition, we synthesized HMGB1(1-51) with tetra-acetylation (Figure S21A). As there is no intramolecular interaction between N-terminus and the third α-helix, Lys11 is considered to be functional through contacting with thrombin during thrombin digestion. Not surprisingly, the wild type HMGB1(1-51) was cleaved much faster than its tetra-acetylated form, similar to what was observed with peptides (Figure S21B).

Finally, we indeed observed that HMGB1 with Lys6 and 7 di-acetylation still showed increased digestion, which indicated Lys6 acetylation was able to overcome Lys7 acetylation's inhibitory effect both on peptide and protein (Figure S19A, S22). As a result, the predominant promoting effect from Lys6 acetylation, together with the absence of inhibitory effect from Lys11 acetylation ultimately led to the enhanced digestion on tetra-acetylated HMGB1 protein.

General Methods

Plasmids MBP DNA was cloned into pET28a with an N-terminal SUMO tag to generate pET28a-His-SUMO-Ser-MBP, pET28a-His-SUMO-Thr-MBP, and pET28a-His-SUMO-Cys-MBP plasmids for Ser-MBP, Thr-MBP and Cys-MBP expression, respectively. HMGB1 sequence was optimized for *E. coli* codon and the HMGB1 mutations and truncations DNA was inserted into PET28a with an N-terminal TEV recognition sequence and C-terminal intein-CBD-His$_6$ tag to generate pET28a-TEV-HMGB1s-intein-CBD-His plasmid for HMGB1 expression.

Protein expression and purification All pET28a plasmids were transformed into BL21. The expression of MBP proteins was induced by including 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD600 reached 0.8, and the culture was grown at 25 °C for overnight. Bacteria were collected by centrifugation then sonicated in lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 50 mM imidazole, 1mM PMSF). After centrifugation, the supernatant was loaded onto the Histrap HP column, followed by thoroughly washing with 50 mL lysis buffer. The MBP proteins were eluted by 10 mL elution buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 500 mM imidazole). After digestion by Ulp1 to release N-terminal Ser, Thr and Cys, the solution was directly injected into HPLC for Ser/Thr/Cys-MBP purification (10-40% CH_3CN/H_2O over 40 min). Finally, the eluted fractions were checked by LC-MS, and the desired fractions were combined and lyophilized to afford the protein powder. For HMGB1 purification, initially, the His-TEV-HMGB1 constructions produced several truncated species probably due to the highly negative charged acidic tail (Figure S7). Therefore, the His$_6$ tag was inserted at the C-terminus. In addition, to minimize the extra sequence on HMGB1 protein after purification, the intein-CBD tag was inserted between HMGB1 and the His$_6$ tag. The expression was induced by including 0.2 mM IPTG when OD$_{600}$ reached 0.8, and the culture was grown at 16 °C for overnight. The bacteria were collected by centrifugation then sonicated in lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 50 mM imidazole, 1mM PMSF). After centrifugation, the supernatant was loaded onto the Histrap HP column, followed by thoroughly washing with 50 mL lysis buffer. The HMGB1 protein was eluted by 10 mL elution buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 500 mM imidazole). After digestion by TEV to release the N-terminal Ser, 100 mM DTT was included to trigger intein splicing. After 24 h, the solution was directly injected into HPLC for HMGB1(13-214) purification (10-40% CH_3CN/H_2O over 40 min). The eluted fractions were checked by LC-MS. The fractions containing HMGB1-DTT and the hydrolysis product of HMGB1-DTT were combined and lyophilized. The full length HMGB1 and truncations were purified following the same procedure.

Peptide and peptide salicylaldehyde (SAL) esters synthesis Peptides were synthesized under standard Fmoc/tBu SPPS protocols. One equivalent of trityl resin was reacted with four equivalents of amino acids and coupling reagent in DMF. HATU/DIPEA was used for all the peptide coupling steps. The deprotection solution was the mixture of piperidine/DMF (1/4, v/v). The details can be found in synthetic details.

Chemical ligation All ligations were set up at room temperature. HFIP or DMSO was used as the cosolvent to dissolve proteins firstly. For STL, Ser/Thr-MBP powders were dissolved in HFIP at 2 mM, then mixed with equal volume of pyridine/acetic acid solution (1:6, v/v); 10 equivalents of peptide esters were added into the solution. After 5 h, reactions were terminated

by adding 10-fold (volume) of ether to precipitate proteins and peptides. For CPL, Cys-MBP powder was dissolved in 6 M Guanidine in phosphate buffer (pH = 4) at 1 mM and 10 equivalents of peptide esters were added into the solution. After 24 h, the reaction was terminated by adding 4-fold (volume) of acetone to precipitate proteins and peptides. The precipitates were collected by centrifugation. After being dried by the stream of flow air, the STL and CPL intermediates were then subjected to acidolysis with TFA/H_2O/EDT (90%/5%/5%; v/v/v) for 5-30 minutes and precipitated by ether again. The final products were analyzed by LC/MS and SDS-PAGE. The HMGB1 (13-214) powder was dissolved in DMSO at 2 mM, then mixed with equal volume of pyridine/acetic acid solution (1:6, v/v); 5 equivalents of His$_6$-TEV-HMGB1(1-12) esters were added into the solution. After 5 h, the reactions were terminated by adding 10-fold (volume) of ether to precipitate proteins and peptides. The precipitates were collected by centrifugation. After being dried by the stream of flow air, the STL and CPL intermediates were then subjected to acidolysis with TFA/H_2O/EDT (90%/5%/5%, v/v/v) for 15 minutes and precipitated by ether again. The solids were collected. Of note, the HMGB1 N-terminal peptide salicylaldehyde esters were unexpectedly found to undergo hydrolysis quickly in HFIP, resulting in poor yield (less than 10%). Thus, DMSO was used as cosolvent instead. However, the ligation efficiency in DMSO was slightly lower than in HFIP for other peptide SAL esters. In summary, for selection of cosolvent, DMSO was usable for all tested peptide SAL esters. HFIP, which could give higher conversion in most cases, while may cause hydrolysis of peptide SAL esters in some cases.

Disulfide linker mediated purification After chemical ligation, the solids were dissolved in 6 M guanidine, followed by 10 times dilution in refolding buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl). After centrifugation, the supernatant was loaded onto the strep resin. After complete washing, the protein was eluted by directly treating resin with 20 mM TCEP for 1 h. The remining peptides can be removed by following concentration step.

His$_6$ tag and TEV based purification After chemical ligation, the solid was dissolved in 6 M guanidine, followed by 10 times dilution in refolding buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl). After centrifugation, the supernatant was loaded onto nickel resin. After complete washing, the protein was eluted by elution buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 500 mM imidazole). The eluted fraction was desalted and digested by TEV. After digestion, the protein solution was re-loaded onto the nickel resin. The flowthrough was collected. The remining peptides could be removed by following concentration step.

HMGB1 refolding, re-purification After chemical ligation, the solid was dissolved in 6 M guanidine, followed by 10 times dilution in refolding buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl). After centrifugation, the supernatant was loaded onto size-exclusion column. The according HMGB1 monomer fractions were collected and further purified by nickel resin. The eluted fraction was digested by TEV to give the full length HMGB1proteins.

Heparin column elution HMGB1 proteins were exchange in 10 mM sodium phosphate (pH ~7, no NaCl) and loaded on HiTrap Heparin HP (5 mL) column. After washing with 5 mL sodium phosphate (pH ~7), HMGB1 proteins were eluted with 10 mM sodium phosphate (pH ~7) containing 1M NaCl using a continuous gradient from 0%-100% in 25 mL.

Pull-down experiment 20 µg HMGB1 proteins were incubated with 20 µL heparin resin slurry in binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 0.2 mM EDTA, 0.1%

Triton X-100) for 1 h. After twice washing by binding buffer, the resin was resuspended in 40 µL 1x loading buffer for SDS-PAGE resolving and Coomassie blue staining.

Thrombin digestion HMGB1 proteins were diluted in PBS at 5 µM, then treated with thrombin (1 U/mL) at 37 °C. The digestion was monitored at indicated time points (Figure 4A and Figure 5). 20 U/mL thrombin was used for Michaelis-Menten kinetics analysis. HMGB1 N-terminal peptides were diluted in PBS, then treated with thrombin (20 U/mL) at 37 °C. The digestion was monitored by HPLC at indicated time points.

Computational predication Structures of HMGB1 and its mutations/truncations were predicted by AlphaFold2 online server: ColabFold (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb); Thrombin and HMGB1 N-terminus complex structures were predicted by PatchMAN online server (https://furmanlab.cs.huji.ac.il/patchman/).

Synthetic Details

1. General information for reagents and methods

All commercially available amino acids and coupling reagents (purchased from Aldrich and GL Biochem) were used without further purification. All solvents in reagent grade (RCI) or HPLC grade (DUKSAN) were used without purification. Anhydrous dichloromethane (DCM) was freshly distilled from calcium hydride (CaH$_2$) before use. Analytical HPLC was performed on a Waters system equipped with a photodiode array detector (Waters 2996), using a Vydac 218TPTM C18 column (5 µm, 4.6 × 250 mm) at a flow rate of 0.6 mL/min; or on a Waters UPLC H-class system equipped with an ACQUITY UPLC photodiode array detector and a Waters SQ Detector 2 mass spectrometer using a Waters ACQUITY BEH C18 column (1.7 µm, 130 Å, 2.1 × 50 mm) at a flow rate of 0.4 mL/min. Preparative HPLC was performed on a Waters system, using a Vydac 218TPTM C18 column (10 µm, 22 × 250 mm) at a flow rate of 10 mL/min or a Vydac 218TPTM C18 column (10 µm, 30 × 250 mm) at a flow rate of 20 mL/min. Mobile phases of HPLC used are as followed: Solvent A: 0.1% TFA (v/v) in acetonitrile (CH$_3$CN, ACN); Solvent B: 0.1% TFA (v/v) in water. Mass analysis were performed with a Waters 3100 mass spectrometer.

2. General experimental procedures

2.1 Solid-phase peptide synthesis (SPPS)

The solid phase peptide synthesis was carried out manually using 2-chloro-trityl resin (GL Biochem, loading capacity: 0.5 mmol/g). 2-Chloro-trityl chloride resin was swollen in anhydrous CH$_2$Cl$_2$ for 30 min and then it washed with CH$_2$Cl$_2$ (5 mL × 3). After that, a solution of Fmoc-Xaa-OH (4.0 equiv. relative to resin loading capacity) and DIEA (8.0 equiv. relative to resin capacity) in CH$_2$Cl$_2$ was added and the resin was shaken at room temperature for 2 h to load the first amino acid. Then the resin was washed with DMF (5 mL × 3) and CH$_2$Cl$_2$ (5 mL × 3), and subsequently treated with a solution of CH$_2$Cl$_2$/CH$_3$OH/DIEA (17:2:1, v/v/v, 5 mL) for 1 h for capping. The resin was washed with DMF (5 mL × 3), CH$_2$Cl$_2$ (5 mL × 3), and DMF (5 mL × 3). Finally, it was subjected to iterative peptide assembly (Fmoc-SPPS). The deFmoc solution was the mixture of piperidine/DMF 20/80 (v/v). For the deFmoc step, the resin was treated with deFmoc solution at R.T. for 20 min. The deFmoc solution was removed, then the resin was washed with DMF (5 mL × 3), CH$_2$Cl$_2$ (5 mL × 3), and DMF (5 mL × 3). For the coupling step, a solution of Fmoc protected amino acid or Boc protected amino acid (4.0 equiv. according to the resin capacity), HATU (4.0 equiv.) and DIEA (10 equiv.) in DMF was gently agitated with the resin at room temperature for 1h. Double coupling was employed for coupling Histidine. The resin was washed with DMF (5 mL × 3), CH$_2$Cl$_2$ (5 mL × 3), and DMF (5 mL × 3). The following Fmoc amino acids and Boc amino acids from GL Biochem were employed: Fmoc-Ala-OH, Fmoc-Cys(Acm)-OH, Fmoc-Cys(Trt)-OH Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-His(Boc)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-COOH, Fmoc-Thr(tBu)-COOH, Fmoc-Val-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, Boc-Ala-OH, Boc-Met-OH and Boc-Cys(StBu)-OH.

2.2 Cleavage fully protected peptide from 2-chloro-trityl chloride resin
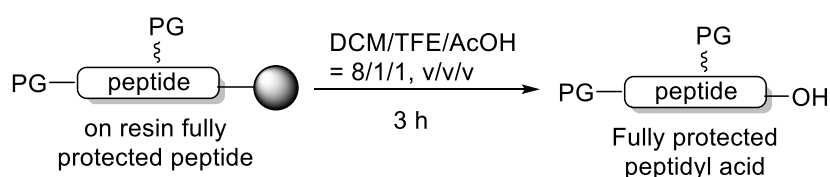
The on-resin fully protected peptide, obtained as described in the **General Experimental Procedures 2.1**, was subjected to the mild acidic cleavage cocktail (5-10 mL) of CH_2Cl_2/AcOH/trifluoroethanol (8/1/1, v/v/v), 3 times for 60 min each. Following filtration, the resulting cleavage solutions were combined and concentrated to afford the crude protected peptide with the free carboxylic acid at the *C*-terminus.

2.3 Synthesis of model C-terminal peptide SAL esters

2.3.1 Direct coupling for preparation of C-terminal Gly and Pro peptide SAL esters:
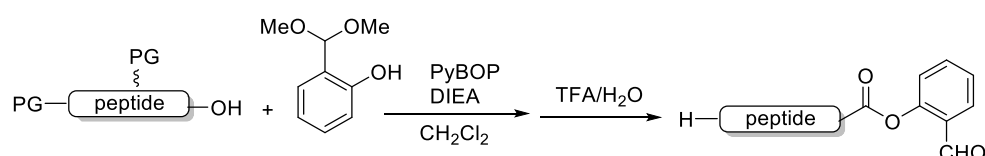
Fully protected crude peptide (1.0 equiv.) obtained from **General Experimental Procedures 2.2** was dissolved in dry DCM at a concentration of 10 mM. DIEA (6.0 equiv.) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (3.0 equiv.) were added, followed by salicylaldehyde dimethyl acetal (30.0 equiv.). The reaction mixture was stirred at room temperature for overnight. After that, the solvent was removed under reduced pressure and the resulting residue was treated with TFA/H_2O (95:5, v/v). After global deprotection for 2 h, TFA was blown off and the oily residue was triturated with diethyl ether and centrifuged. The precipitate was pelleted, and the ether was subsequently decanted. The resulting solid was purified by HPLC and lyophilized to give the peptide SAL esters as a white solid.

2.3.2 "N+1" strategy for the preparation of C-terminal Ser, Met, Ala, Phe, Val, Leu, Ile, and Thr peptide SAL esters:
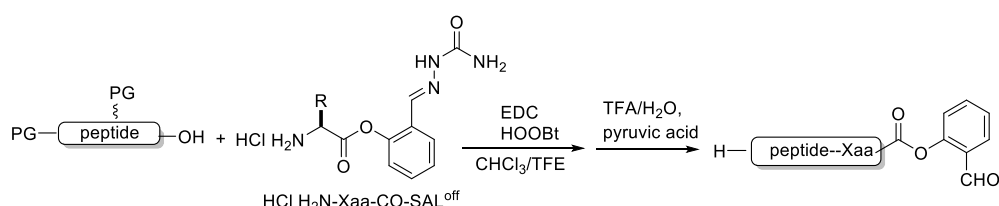
The fully protected peptidyl acid (1.0 equiv.) obtained from **General Experimental Procedures 2.2** was dissolved in $CHCl_3$/trifluoroethanol (10 mM, 3/1, v/v), then the corresponding L-Amino acid derived salicylaldehyde semicarbazone ester hydrochloride HCl·H_2N-Xaa-CO-SAL[off] (6.0 equiv.), synthesized according to the procedure[8] and hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOOBt) (3.0 equiv.) were added. Finally, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) (3.0 equiv.) was added. The reaction mixture was stirred for 3 h to form the crude protected C-terminal peptide SAL[off] ester. After that, the solvent was removed under reduced pressure and the resulting residue was treated with TFA/H_2O (95:5, v/v) containing pyruvic acid (100 equiv.) for 3 h. After that, TFA was blown

off and the oily residue was triturated with diethyl ether and centrifuged. The precipitate was pelleted, and the ether was subsequently decanted. The resulting solid was purified by HPLC and lyophilized to give the peptide SAL esters as a white solid.
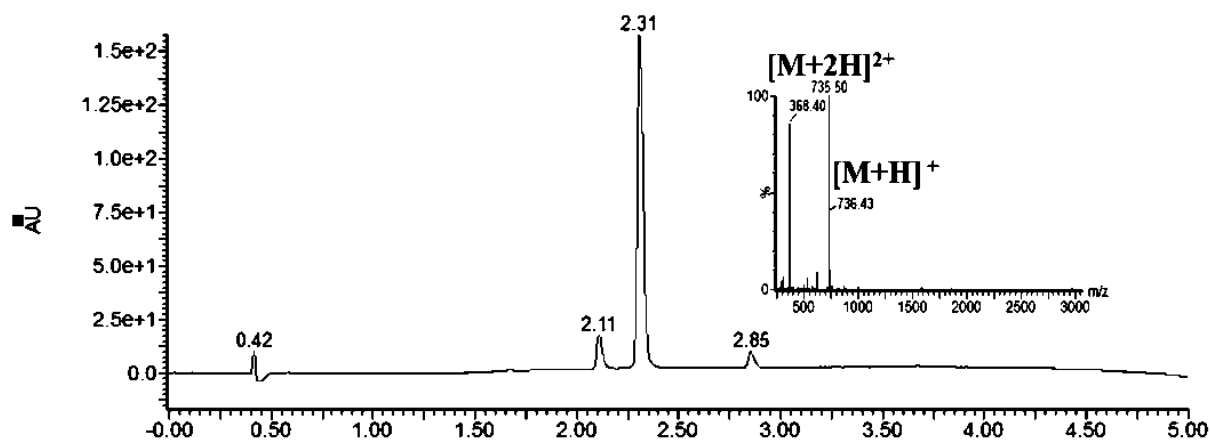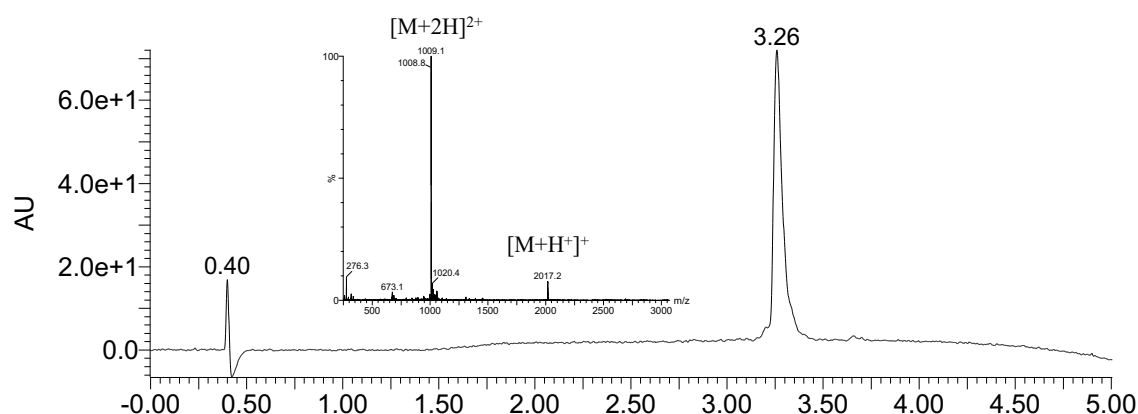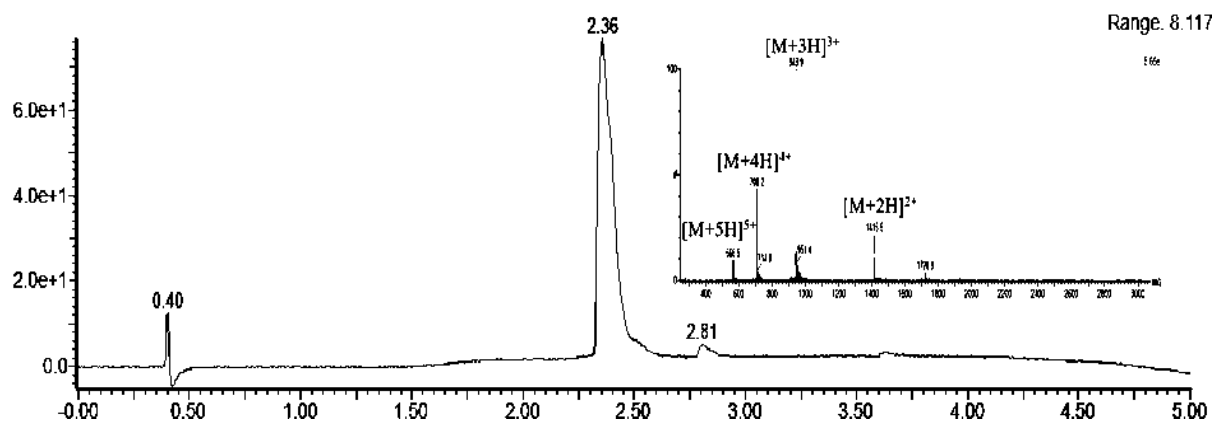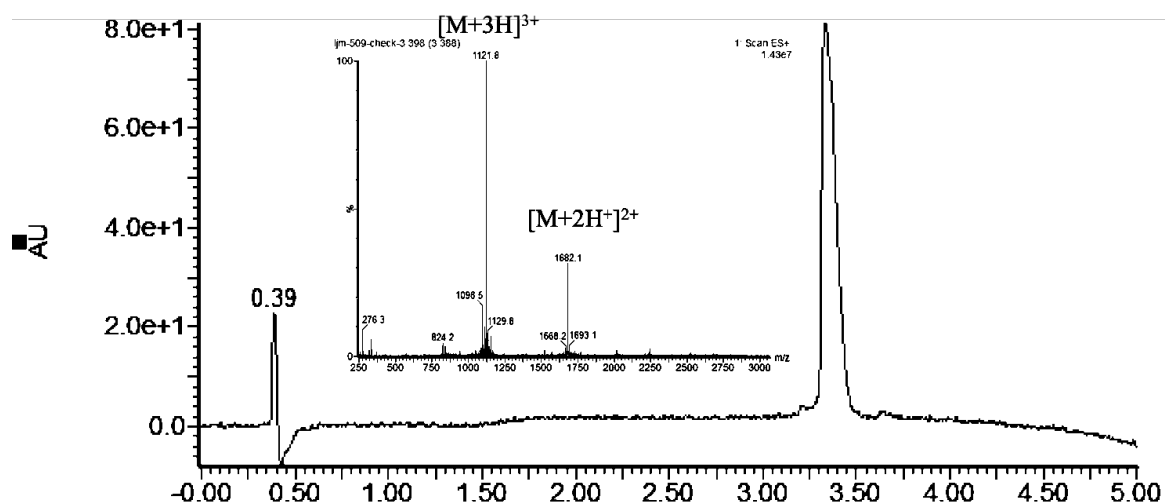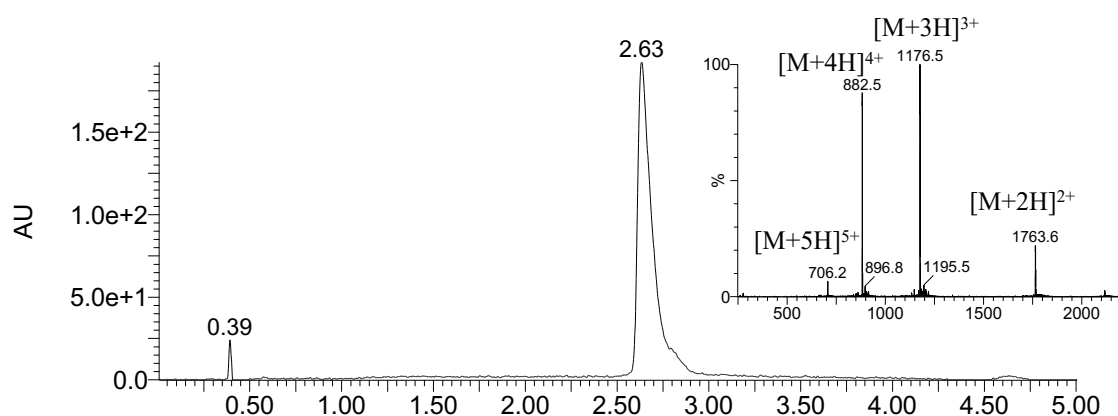
3. Synthesis of LSQRGG-CO-SAL ester

The H-LSQRGG-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1**.
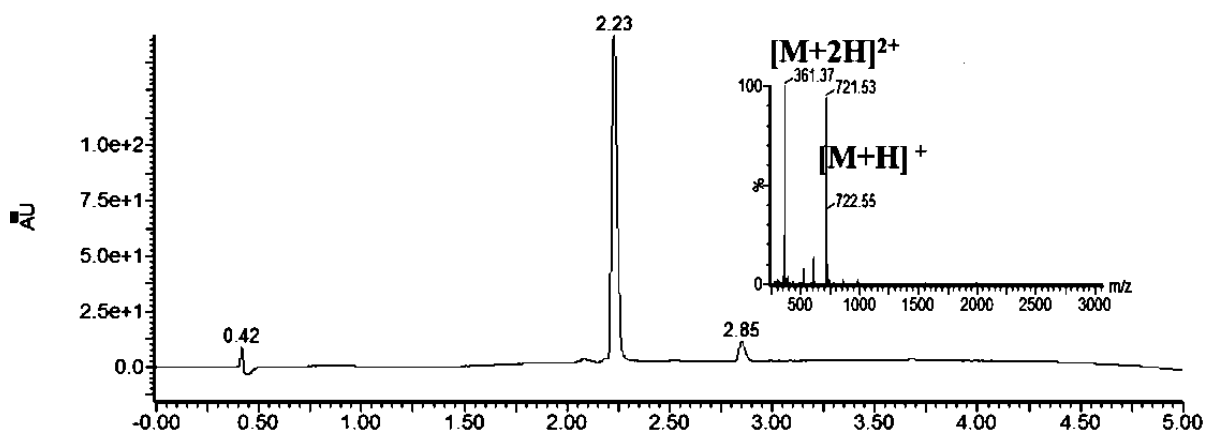
UV trace and corresponding MS from LC-MS analysis of purified H-LSQRGG-CO-SAL. ESI-MS calcd. molecular weight: 720.79. $[M+H]^+$ m/z = 721.79, $[M+2H]^{2+}$ m/z = 361.39, found 721.53, 361.37.

4. Synthesis of H-LSQRGA-CO-SAL ester
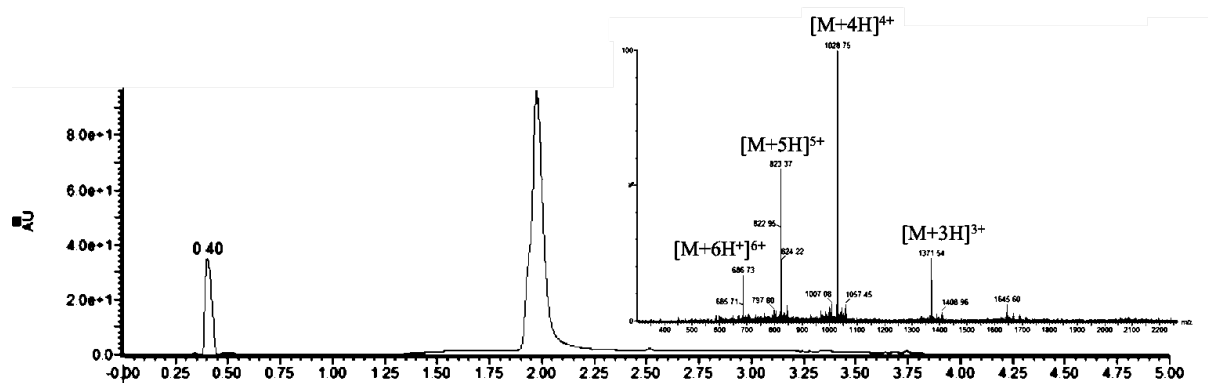
The H-LSQRGA-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2**.

UV trace and corresponding MS from LC-MS analysis of purified H-LSQRGA-CO-SAL. ESI-MS calcd. molecular weight: 734.81. $[M+H]^+$ m/z = 735.81, $[M+2H]^{2+}$ m/z = 368.40, found 735.50, 368.40.

5. Synthesis of Ac-ETTTQGPGVLLPLPKGAC(StBu)-CO-SAL ester

The Ac-ET TTQGPGVLLP LPKGAC(StBu)-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2**.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 2016.40. [M+H]$^+$ m/z = 2017.40, [M+2H]$^{2+}$ m/z = 1009.20, found 2017.20, 1009.10.

6. Synthesis of Biotin-C(Acm)SRAARGTIGARRTGQPLKEDPS-CO-SAL ester

The Biotin-C(Acm)SRAARGTIGARRTGQPLKEDPS-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 2828.21. [M+2H]$^{2+}$ m/z = 1415.6, [M+3H]$^{3+}$ m/z = 944.0, [M+4H]$^{4+}$ m/z = 708.3, [M+5H]$^{5+}$ m/z = 566.8, found 1415.5, 943.9, 708.2, 566.5.

7. Synthesis of Fmoc-HN-TLAEAQTETC(Acm)TVAPRERQNC(StBu)GFPGVTP-CO-SAL ester

The Fmoc-HN-TLAEAQTETC(Acm)TVAPRERQNC(StBu)GFPGVTP-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 3362.80. [M+2H]$^{2+}$ m/z = 1682.4, [M+3H]$^{3+}$ m/z = 1121.93, found 1682.1, 1121.8.

8. Synthesis of Fmoc-SEAVLRGQALLVKSSQPWEPLQLHVDKAV-CO-SAL ester

The Fmoc-SEAVLRGQALLVKSSQPWEPLQLHVDKAV-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 3526.06. [M+2H]$^{2+}$ m/z = 1764.03, [M+3H]$^{3+}$ m/z = 1176.35, [M+4H]$^{4+}$ m/z = 882.51, [M+5H]$^{5+}$ m/z = 706.21, found 1763.6, 1176.5, 882.5, 706.2.

9. Synthesis of Ac-SESSSKSSQPLASKQEKDGTEKRGRGRPRKQPPVSPG-CO-SAL ester

The Ac-SESSSKSSQPLASKQEKDGTEKRGRGRPRKQPPVSPG-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**
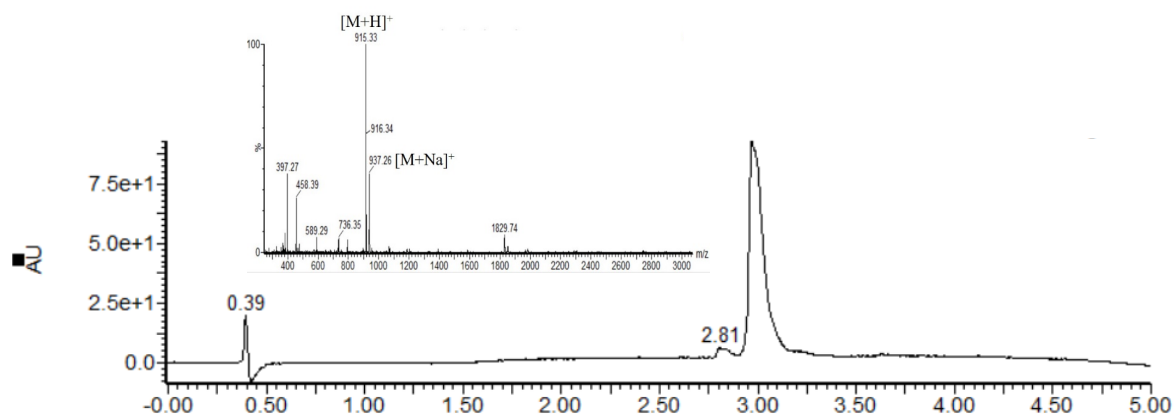
UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 4111.51. $[M+3H]^{3+}$ m/z = 1371.50, $[M+4H]^{4+}$ m/z = 1028.89, $[M+5H]^{5+}$ m/z = 823.30, $[M+6H]^{6+}$ m/z = 686.25, found: 1371.54, 1028.75, 823.37, 686.73.

10. Synthesis of Fmoc-HN-TGANRDLELPWLEQQGPASHHRRQLGPQGPPHLVADP -CO-SAL ester

The Fmoc-HN-TGANRDLELPWLEQQGPASHHRRQLGPQGPPHLVADP-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**
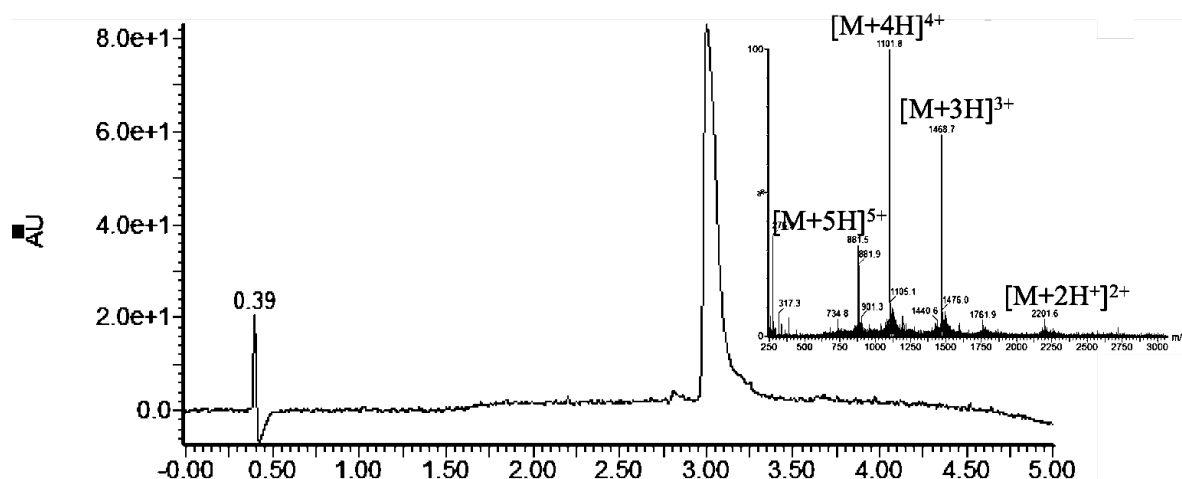
UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 4402.87. $[M+2H]^{2+}$ m/z = 2202.44, $[M+3H]^{3+}$ m/z = 1468.62, $[M+4H]^{4+}$ m/z = 1101.72, $[M+5H]^{5+}$ m/z = 881.57, found 2201.6, 1468.7, 1101.8, 881.5.

11. Synthesis of Biotin-WRRKRKEKQSETSPKEFLTIYEDVKDLKTRRNHEQEQTF PGGG-CO-SAL ester

The Biotin-WRRKRKEKQSETSPKEFLTIYEDVKDLKTRRNHEQEQTFPGGG-OSAL was synthesized according to the **General Experimental Procedures 2.3.1.**
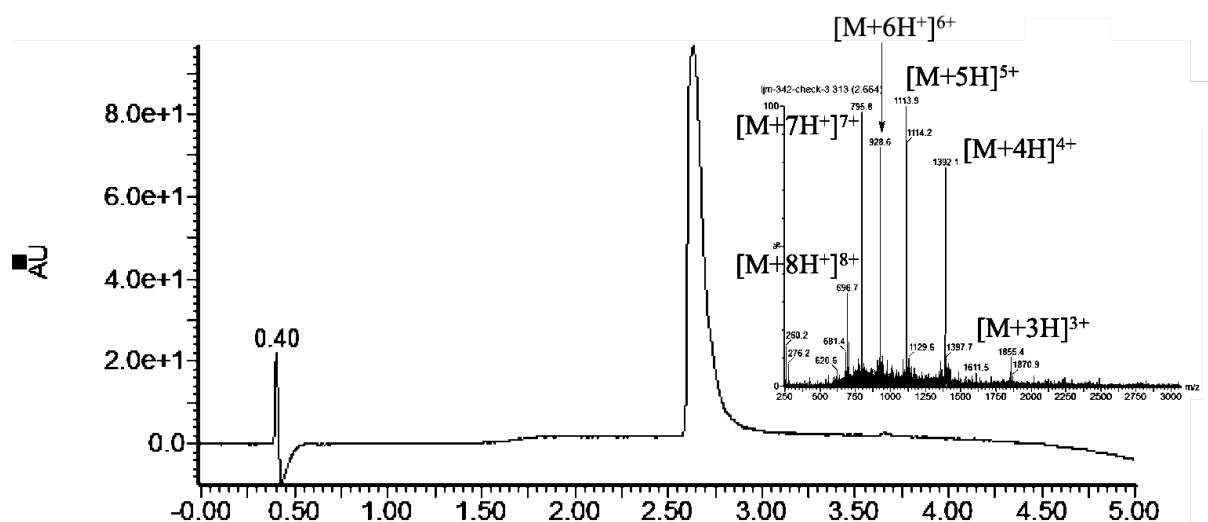
UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 5565.68. $[M+3H]^{3+}$ m/z = 1856.23, $[M+4H]^{4+}$ m/z = 1392.42, $[M+5H]^{5+}$ m/z = 1114.14, $[M+6H]^{6+}$ m/z = 928.61, $[M+7H]^{7+}$ m/z = 796.10, $[M+8H]^{8+}$ m/z = 696.71, found 1855.4, 1392.1, 1113.9, 928.6, 795.8, 696.7.

## 12. Synthesis of disulfide-containing peptide SAL ester
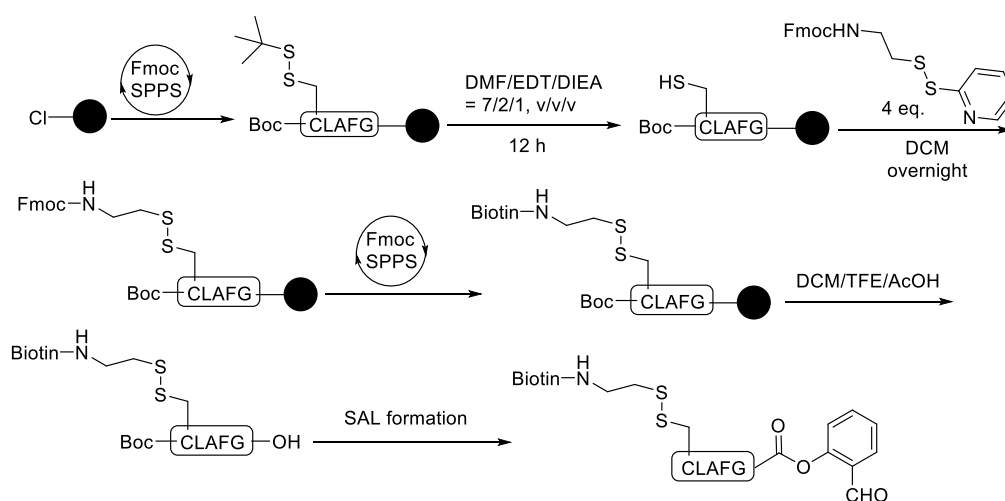
The on resin fully protected peptide obtained using **General Experimental Procedures 2.1** was treated with 5 mL EDT/DIEA/DMF (2/1/7, v/v/v) and shaken for 12 h at room temperature to selectively remove the *tert*-butylthio (StBu) protecting group. Then, the resin was washed with DMF (5 mL × 3) and CH$_2$Cl$_2$ (5 mL × 3), and subsequently treated with a solution of (9H-fluoren-9-yl)methyl 2-(pyridin-2-yldisulfanyl)ethylcarbamate (4.0 equiv. relative to resin loading) in DCM for 12 h.[9] After that, the resin was washed with DMF (5 mL × 3), CH$_2$Cl$_2$ (5 mL × 3), and DMF (5 mL × 3). Subsequently, the Fmoc group on the peptide was removed by treating the resin with a mixture of piperidine/DMF 20/80 (v/v) (4 mL) for 20 min and washed with DMF (5 mL × 3), CH$_2$Cl$_2$ (5 mL × 3), and DMF (5 mL × 3). Biotin was attached by coupling under standard condition in the next step. Finally, the SAL ester product was prepared according to the **General Experimental Procedures 2.3.1.** The crude peptide SAL ester was purified by preparative reverse-phase HPLC (20-60% CH$_3$CN/H$_2$O over 30 min) and lyophilized to afford the desired SAL ester (6 mg, 3.4% yield).

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 915.15. [M+H]$^+$ m/z = 915.34, [M+Na]$^+$ m/z = 937.34, found 915.33, 937.26.

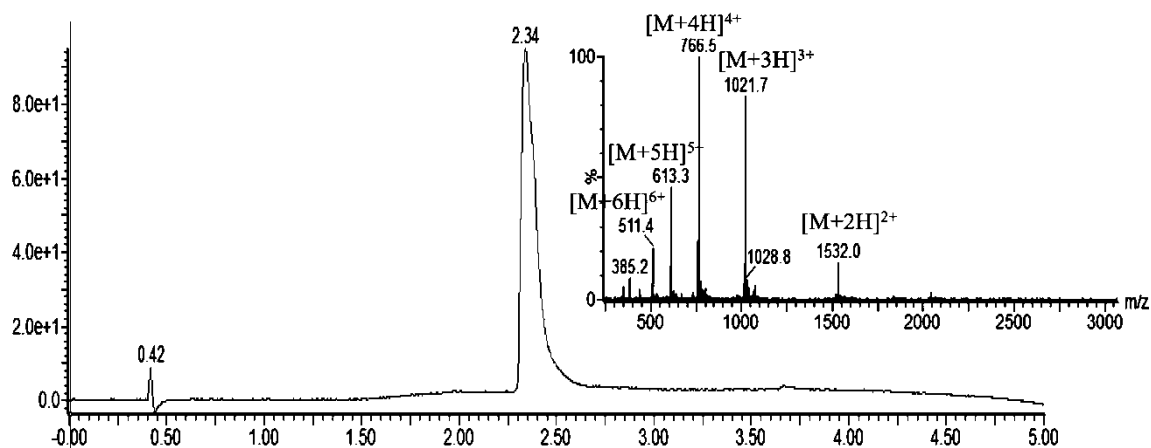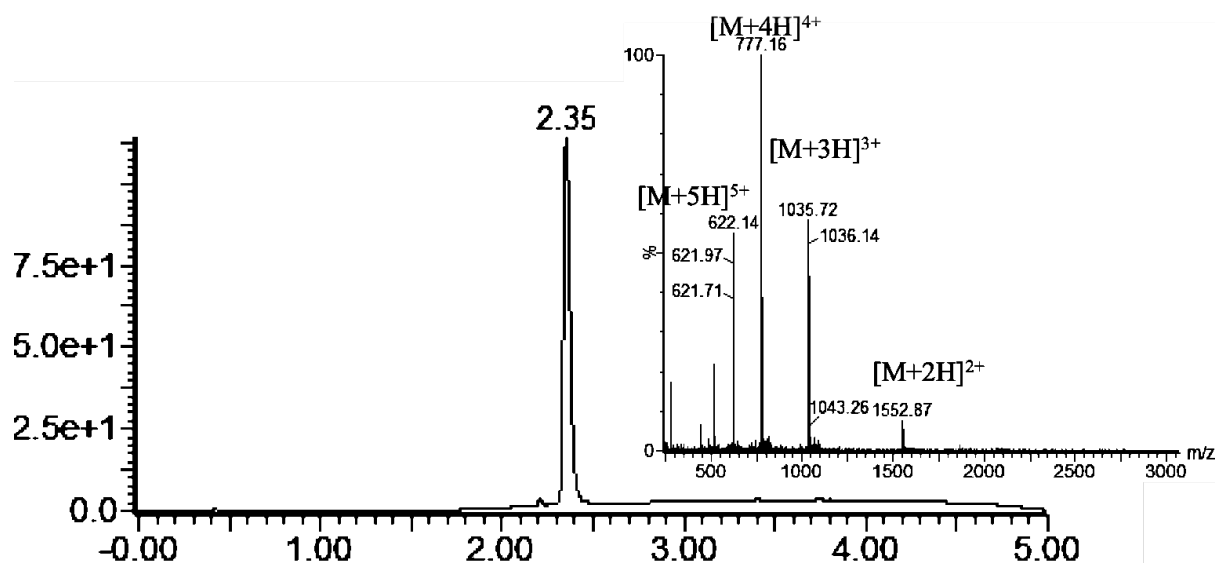## 13. Synthesis of Ac-HHHHHHENLYFQG-CO-SAL ester

The Ac-HHHHHHENLYFQG-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**
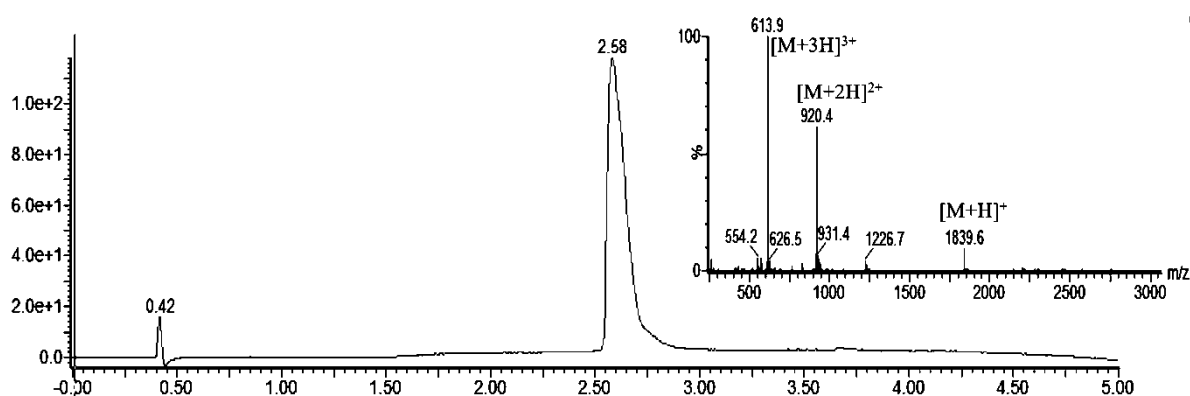
UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1838.93. [M+H]$^+$ m/z = 1839.93 [M+2H]$^{2+}$ m/z = 920.47, [M+3H]$^{3+}$ m/z = 613.98, found 1839.6, 920.4, 613.9.

14. Synthesis of Ac-HHHHHHENLYFQGKGDPKKPRGKM-CO-SAL ester

The Ac-HHHHHHENLYFQGKGDPKKPRGKM-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 3062.43. $[M+2H]^{2+}$ m/z = 1532.2, $[M+3H]^{3+}$ m/z = 1021.8, $[M+4H]^{4+}$ m/z = 766.6, $[M+5H]^{5+}$ m/z = 613.4, $[M+6H]^{6+}$ m/z = 511.4, found 1532.0, 1021.7, 766.5, 613.3, 511.4.

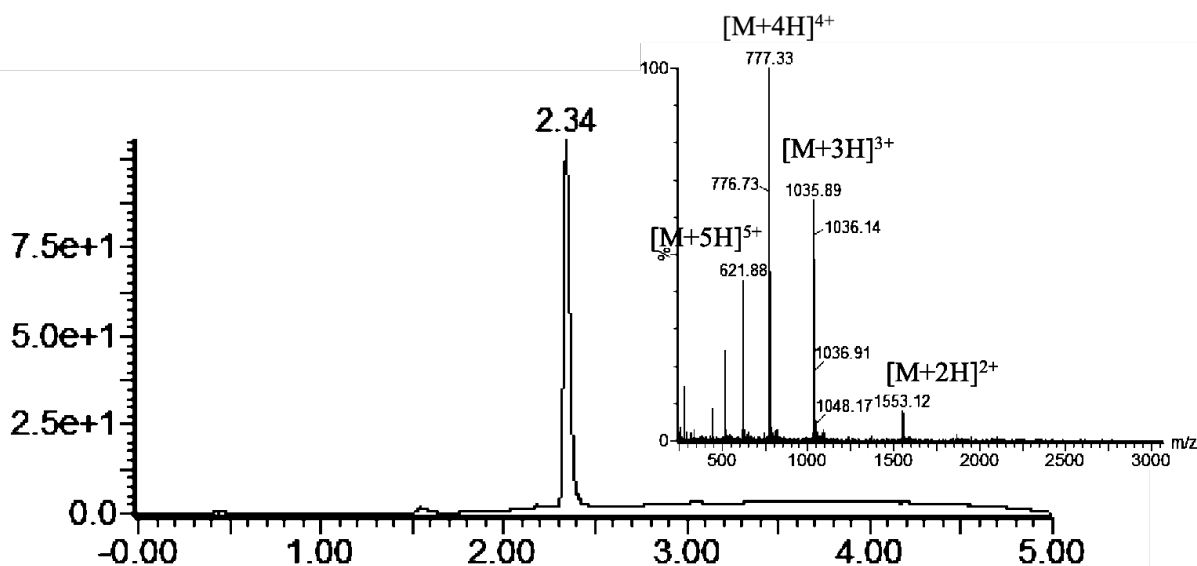## 15. Synthesis of Ac-HHHHHHENLYFQGK(ac)GDPKKPRGKM-CO-SAL ester

The Ac-HHHHHHENLYFQGK(ac)GDPKKPRGKM-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 3104.47. $[M+2H]^{2+}$ m/z = 1553.2, $[M+3H]^{3+}$ m/z = 1035.8, $[M+4H]^{4+}$ m/z = 777.1, $[M+5H]^{5+}$ m/z = 621.9, found 1552.9, 1035.7, 777.2, 622.1.

16. Synthesis of Ac-HHHHHHENLYFQGKGDPK(ac)KPRGKM-CO-SAL ester

The Fmoc-Lys(Ac)-OH was synthesized according to the reported procedure.[10] The Ac-HHHHHHENLYFQGKGDPK(ac)KPRGKM-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 3104.47. $[M+2H]^{2+}$ m/z = 1553.2, $[M+3H]^{3+}$ m/z = 1035.8, $[M+4H]^{4+}$ m/z = 777.1, $[M+5H]^{5+}$ m/z = 621.9, found 1553.1, 1035.9, 777.3, 621.9.

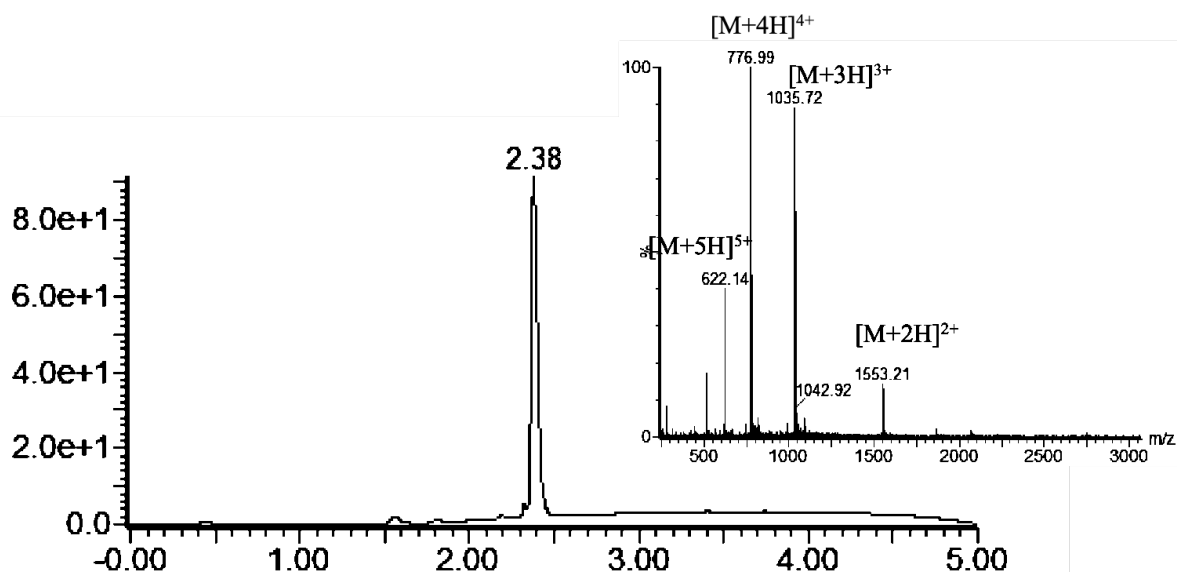### 17. Synthesis of Ac-HHHHHHENLYFQGKGDPKK(ac)PRGKM-CO-SAL ester

The Ac-HHHHHHENLYFQGKGDPKK(ac)PRGKM-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**
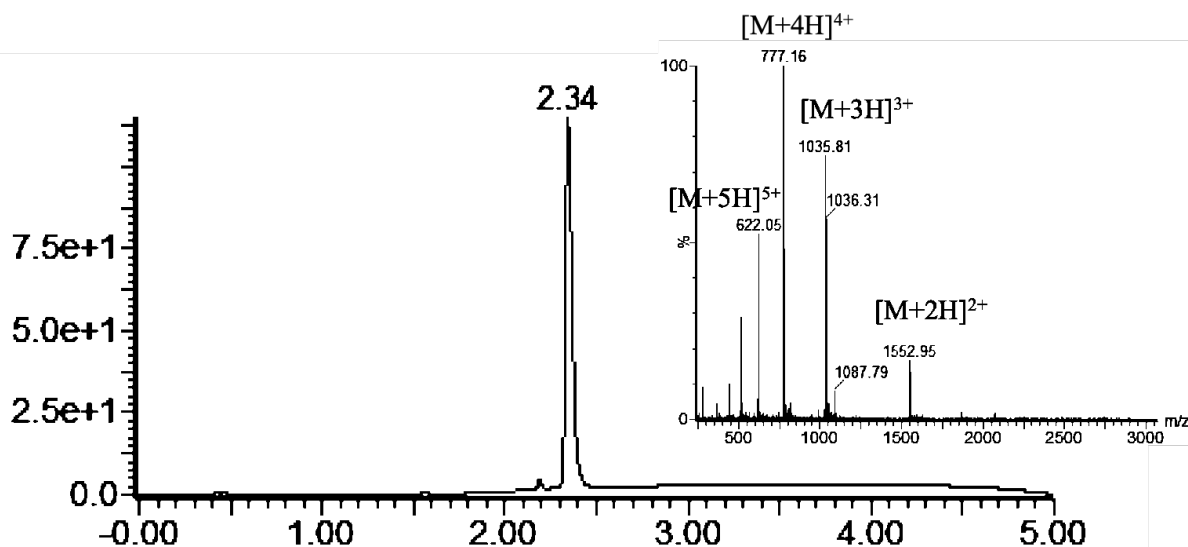
UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 3104.47. $[M+2H]^{2+}$ m/z = 1553.2, $[M+3H]^{3+}$ m/z = 1035.8, $[M+4H]^{4+}$ m/z = 777.1, $[M+5H]^{5+}$ m/z = 621.9, found 1553.0, 1035.8, 777.2, 622.0.

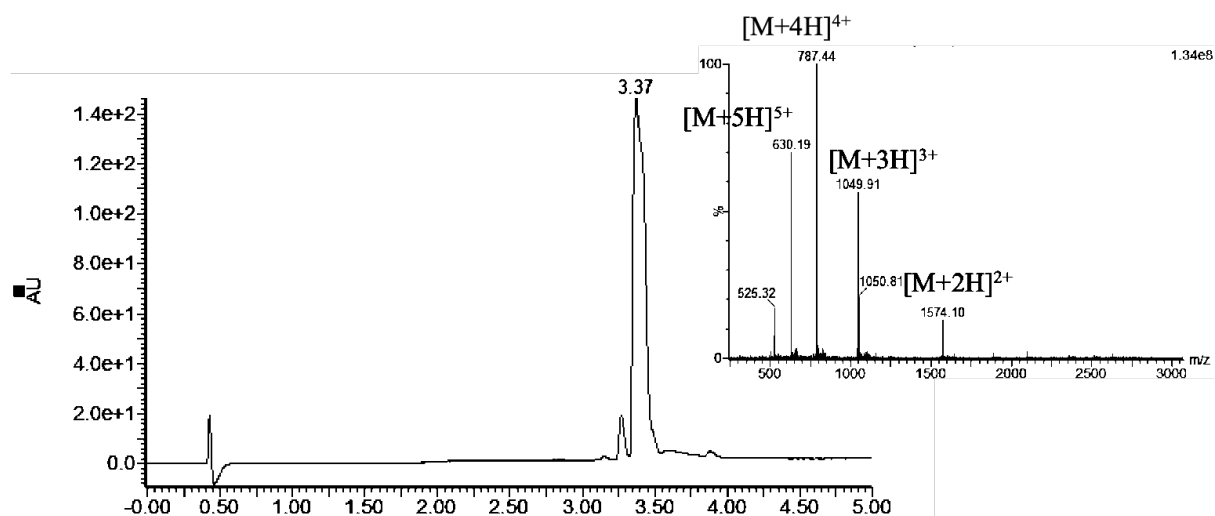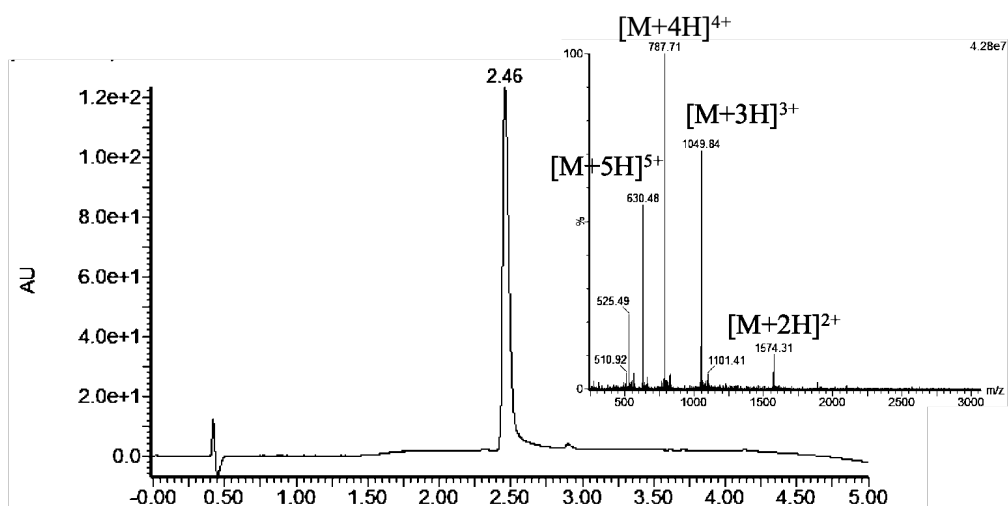### 18. Synthesis of Ac-HHHHHHENLYFQGKGDPKKPRGK(ac)M-CO-SAL ester

The Ac-HHHHHHENLYFQGKGDPKKPRGK(ac)M-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 3104.47. [M+2H]$^{2+}$ m/z = 1553.2, [M+3H]$^{3+}$ m/z = 1035.8, [M+4H]$^{4+}$ m/z = 777.1, [M+5H]$^{5+}$ m/z = 621.9, found 1553.2, 1035.7, 777.0, 622.1.

19. Synthesis of Ac-HHHHHHENLYFQGKGDPK(ac)K(ac)PRGKM-CO-SAL ester

The Ac-HHHHHHENLYFQGKGDPK(ac)K(ac)PRGKM-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 3146.51 [M+2H]$^{2+}$ m/z =1574.3, [M+3H]$^{3+}$ m/z = 1049.8, [M+4H]$^{4+}$ m/z = 787.6, [M+5H]$^{5+}$ m/z = 630.3, found 1574.1, 1049.9, 787.4, 630.2. Should peak does not have MS.

### 20. Synthesis of Ac-HHHHHHENLYFQGKGDPK(ac)K(ac)PRGKM-CO-SAL ester

The Ac-HHHHHHENLYFQGKGDPK(ac)KPRGK(ac)M-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 3146.51 [M+2H]$^{2+}$ m/z =1574.3, [M+3H]$^{3+}$ m/z = 1049.8, [M+4H]$^{4+}$ m/z = 787.6, [M+5H]$^{5+}$ m/z = 630.3, found 1574.3, 1049.9, 787.7, 630.5.

## 21. Synthesis of Ac-HHHHHHENLYFQGK(ac)GDPK(ac)K(ac)PRGK(ac)M-CO-SAL ester

The Ac-HHHHHHENLYFQGK(ac)GDPK(ac)K(ac)PRGK(ac)M-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**
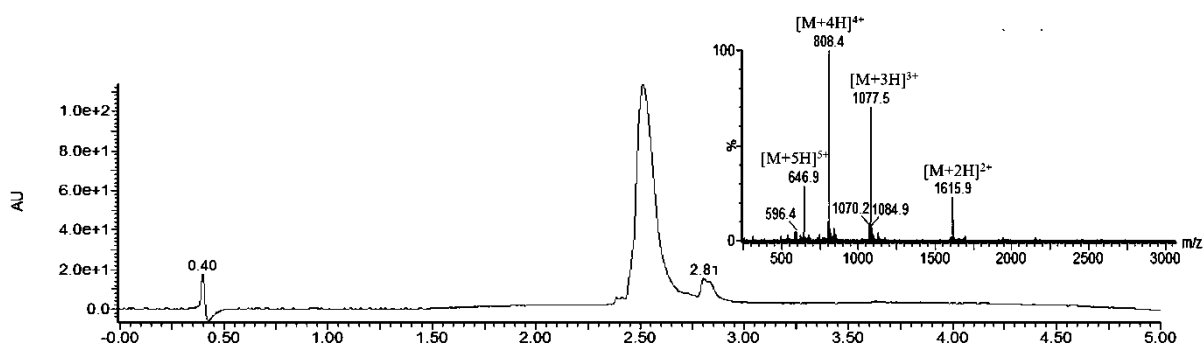
UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 3230.43. [M+2H]$^{2+}$ m/z = 1616.2, [M+3H]$^{3+}$ m/z = 1077.8, [M+4H]$^{4+}$ m/z = 808.6, [M+5H]$^{5+}$ m/z = 647.1, found 1615.9, 1077.5, 808.4, 646.9.
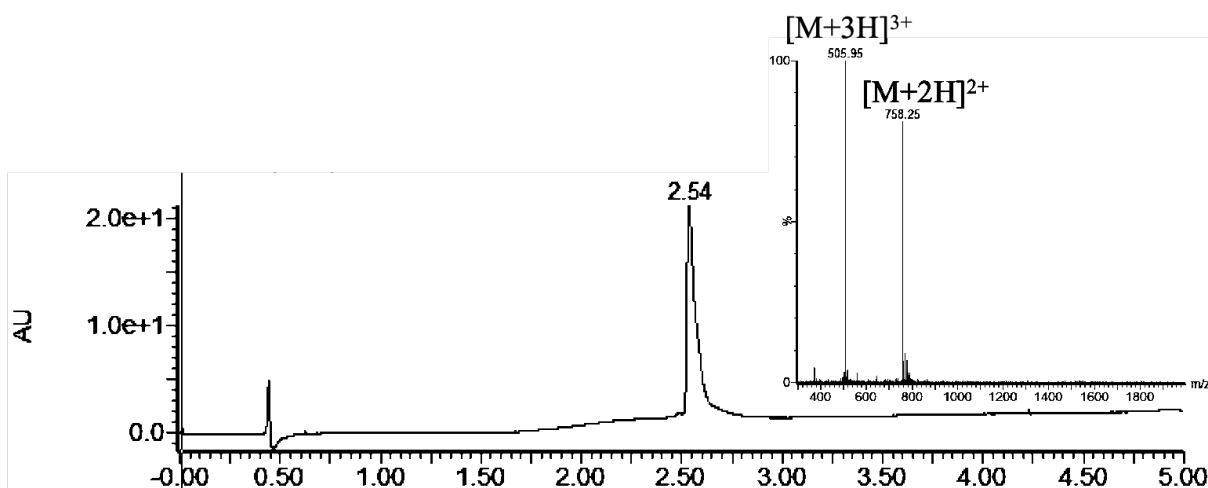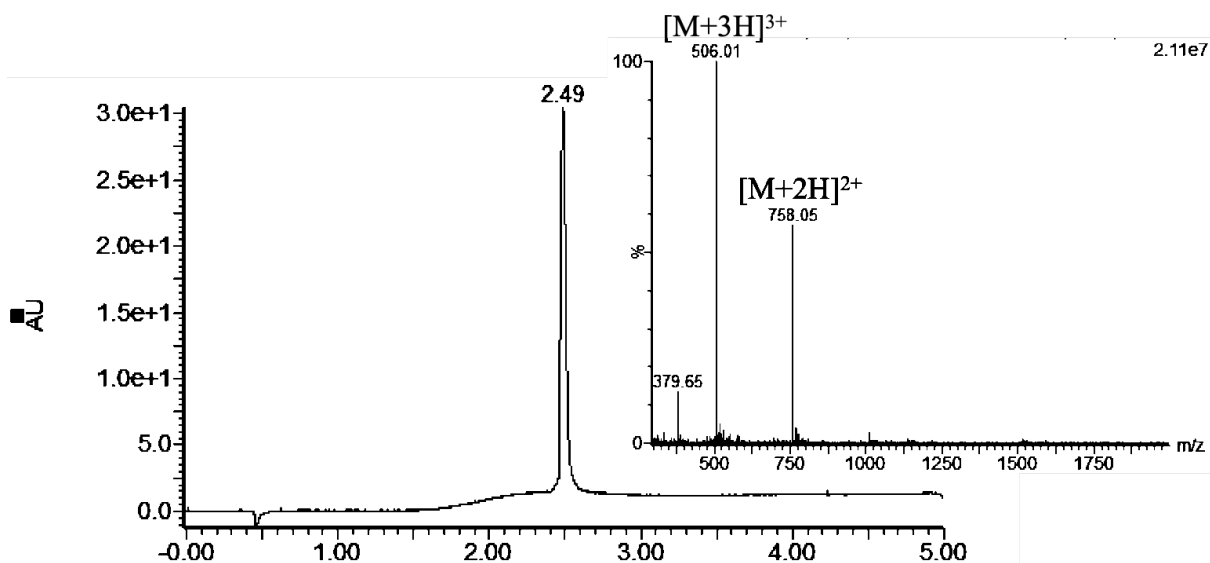
22. Synthesis of H-GKGDPKKPRGKMSS-OH

The peptide was synthesized following standard SPPS protocol.
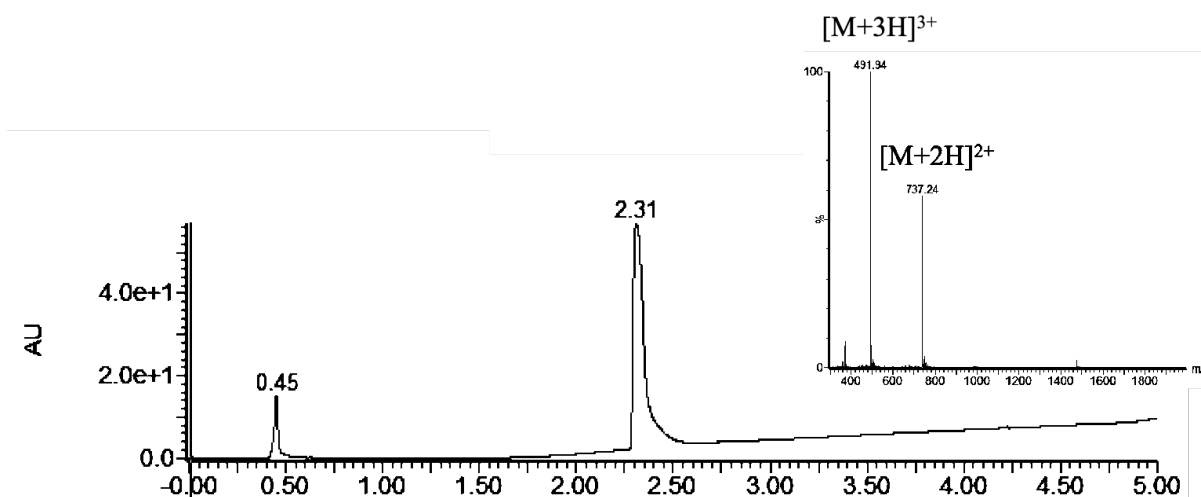
UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1472.73. [M+2H]$^{2+}$ m/z = 737.4, [M+3H]$^{3+}$ m/z = 491.9, found 737.2, 491.9.

23. Synthesis of H-GK(ac)GDPKKPRGKMSS-OH

The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1514.77. [M+2H]$^{2+}$ m/z = 758.4, [M+3H]$^{3+}$ m/z = 505.9, found 758.3, 506.0.
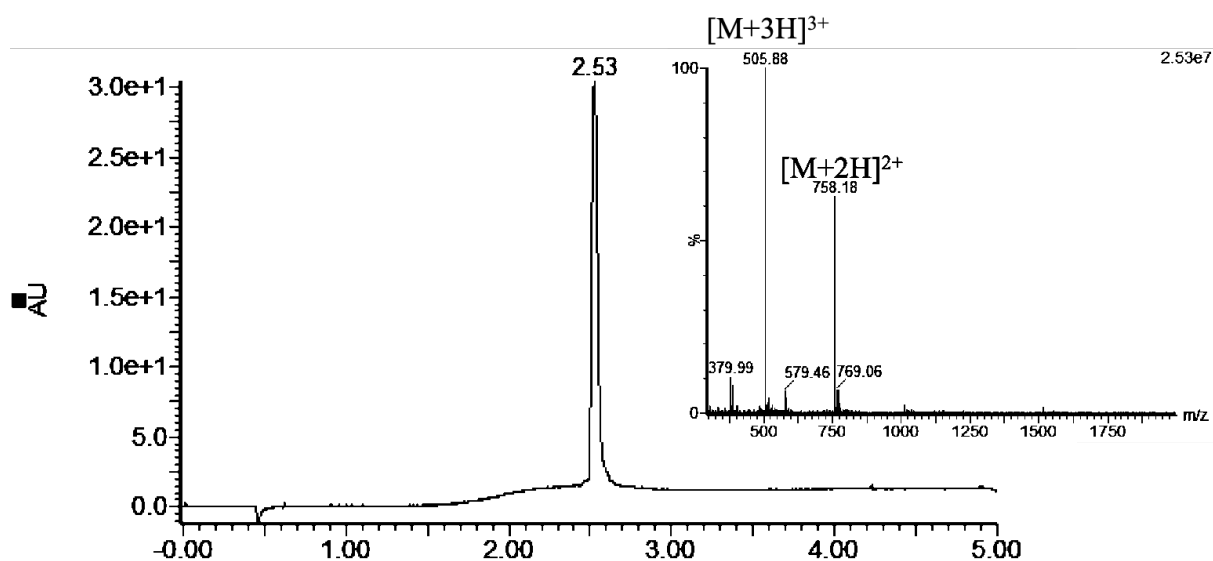
24. Synthesis of H-GKGDPK(ac)KPRGKMSS-OH

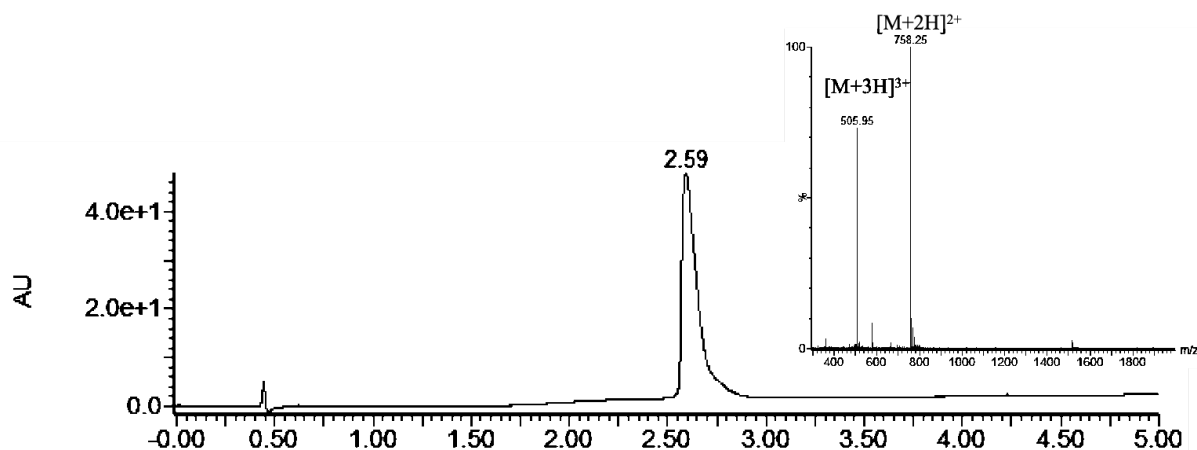The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1514.77. [M+2H]$^{2+}$ m/z = 758.4, [M+3H]$^{3+}$ m/z = 505.9, found 758.1, 506.0.

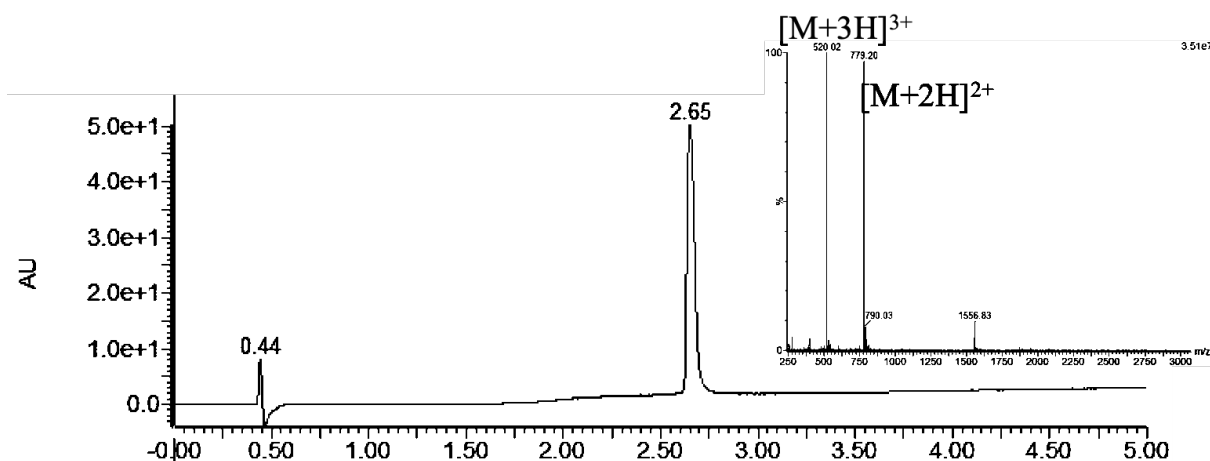## 25. Synthesis of H-GKGDPKK(ac)PRGKMSS-OH

The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1514.77. [M+2H]$^{2+}$ m/z = 758.4, [M+3H]$^{3+}$ m/z = 505.9, found 758.2, 505.9.
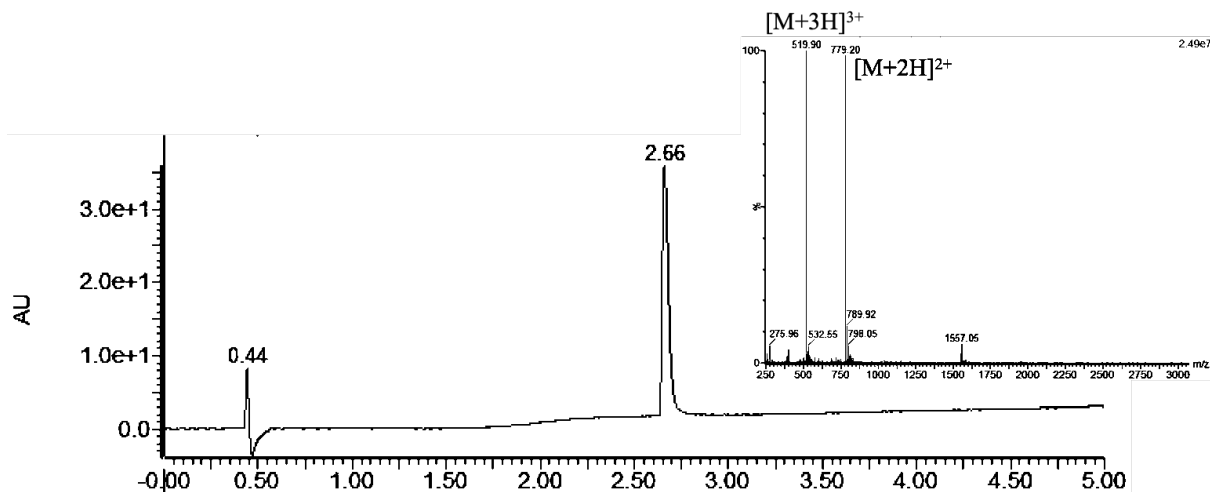
26. Synthesis of H-GKGDPKKPRGK(ac)MSS-OH

The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1514.77. [M+2H]$^{2+}$ m/z = 758.4, [M+3H]$^{3+}$ m/z = 505.9, found 758.3, 506.0.

27. Synthesis of H-GKGDPK(ac)K(ac)PRGKMSS-OH

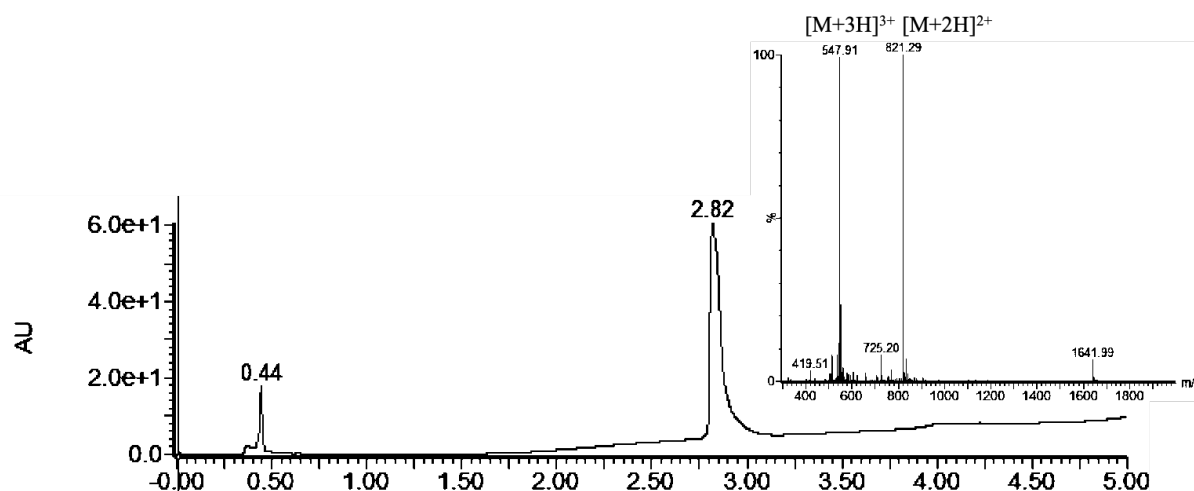The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1556.81. [M+2H]$^{2+}$ m/z = 779.4, [M+3H]$^{3+}$ m/z = 519.9, found 779.2, 520.0.

28. Synthesis of H-GKGDPK(ac)KPRGK(ac)MSS-OH

The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd.

molecular weight: 1556.81. [M+2H]$^{2+}$ m/z = 779.4, [M+3H]$^{3+}$ m/z = 519.9, found 779.2, 520.0.

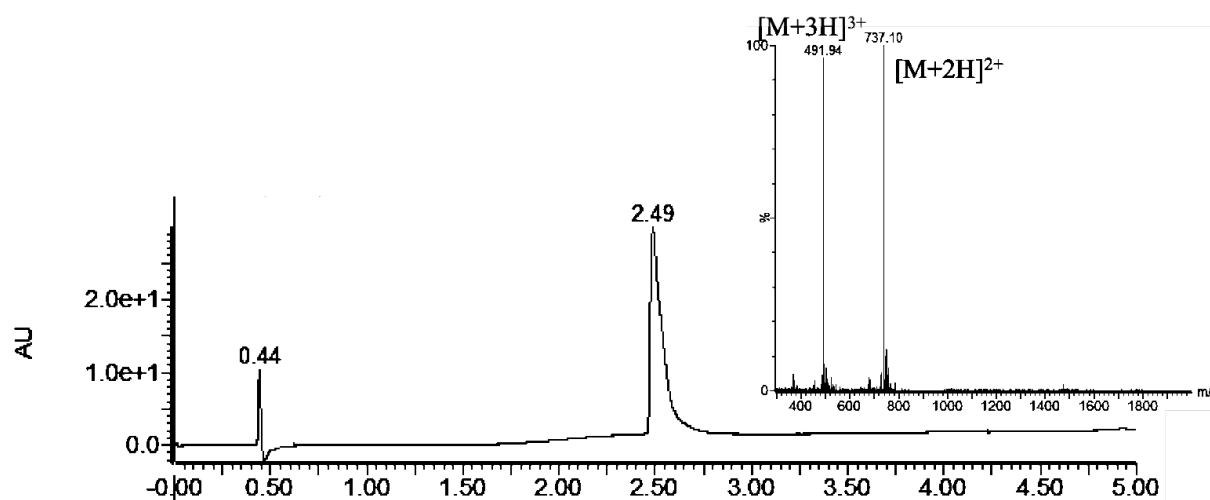### 29. Synthesis of H-GK(ac)GDPK(ac)K(ac)PRGK(ac)MSS-OH

The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1640.88. [M+2H]$^{2+}$ m/z = 821.4, [M+3H]$^{3+}$ m/z = 548.0, found 821.3, 547.9.
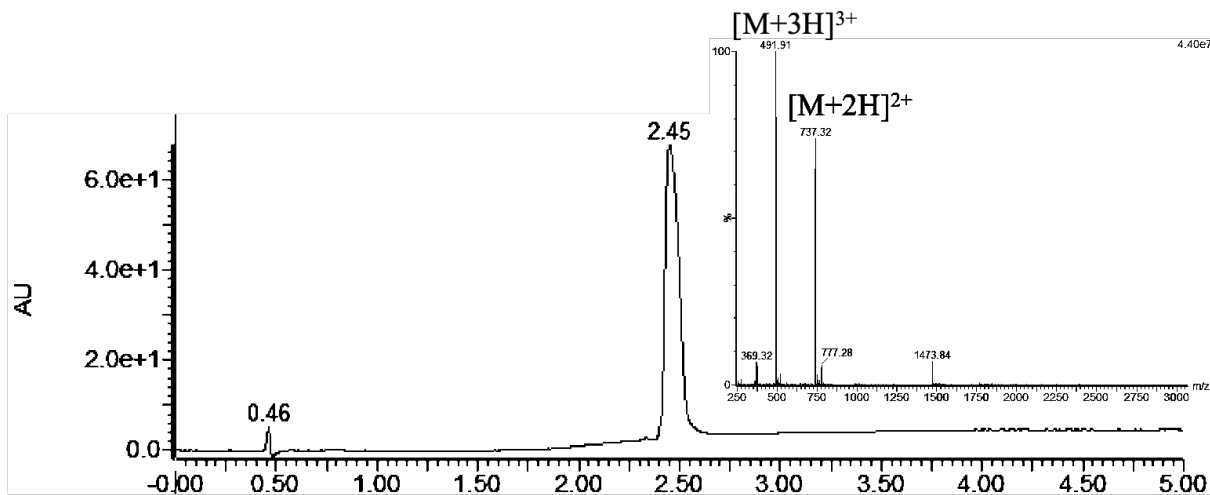
30. Synthesis of H-GKGDPQKPRGKMSS-OH

The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1472.69. [M+2H]$^{2+}$ m/z = 737.3, [M+3H]$^{3+}$ m/z = 491.9, found 737.1, 491.9.

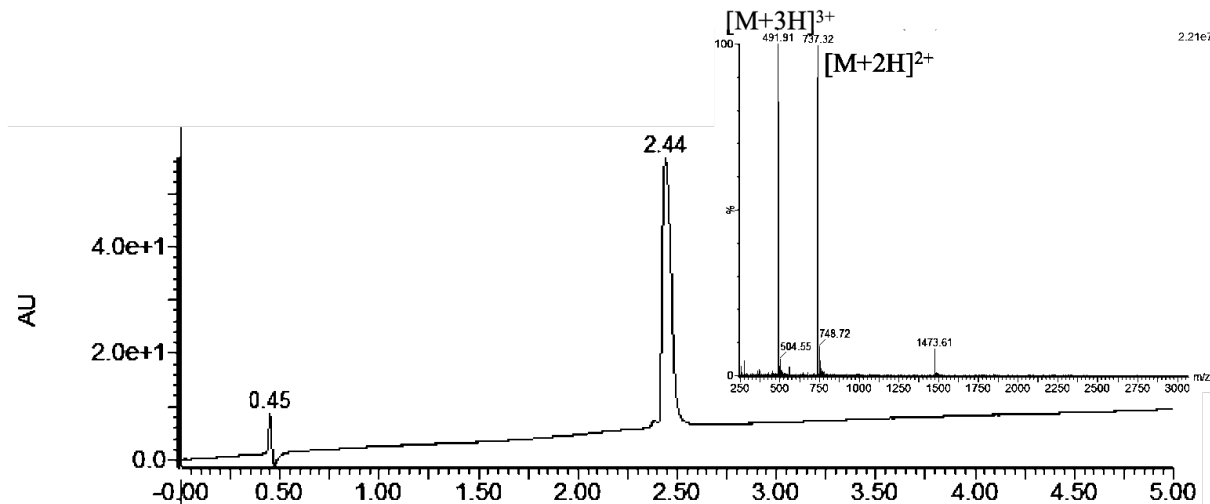### 31. Synthesis of H-GKGDPKQPRGKMSS-OH

The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1472.69. [M+2H]$^{2+}$ m/z = 737.3, [M+3H]$^{3+}$ m/z = 491.9, found 737.3, 491.9.

32. Synthesis of H-GKGDPKKPRGQMSS-OH

The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1472.69. [M+2H]$^{2+}$ m/z = 737.3, [M+3H]$^{3+}$ m/z = 491.9, found 737.3, 491.9.
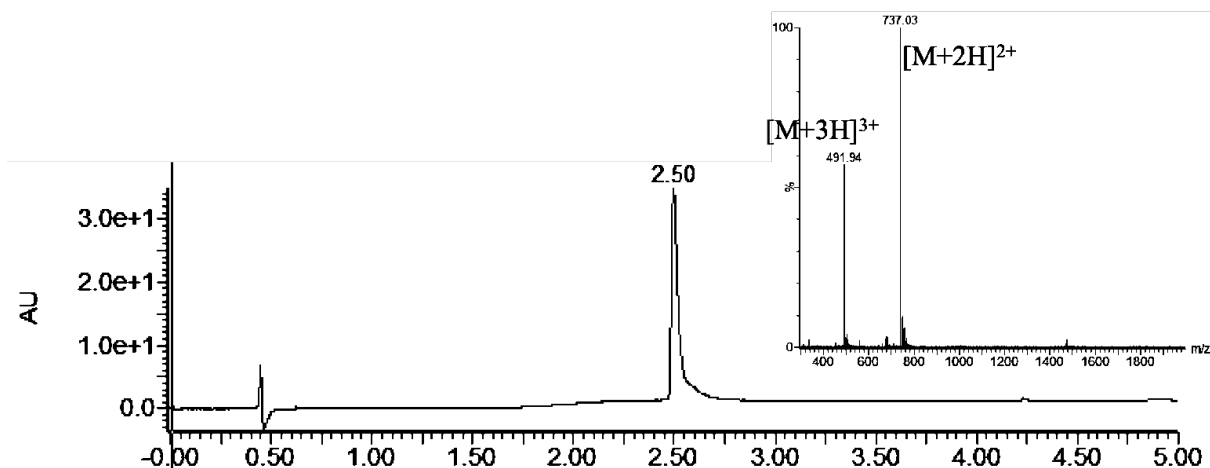
33. Synthesis of H-GKGDPQKPRGQMSS-OH

The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1472.64. $[M+2H]^{2+}$ m/z = 737.3, $[M+3H]^{3+}$ m/z = 491.9, found 737.0, 491.9.

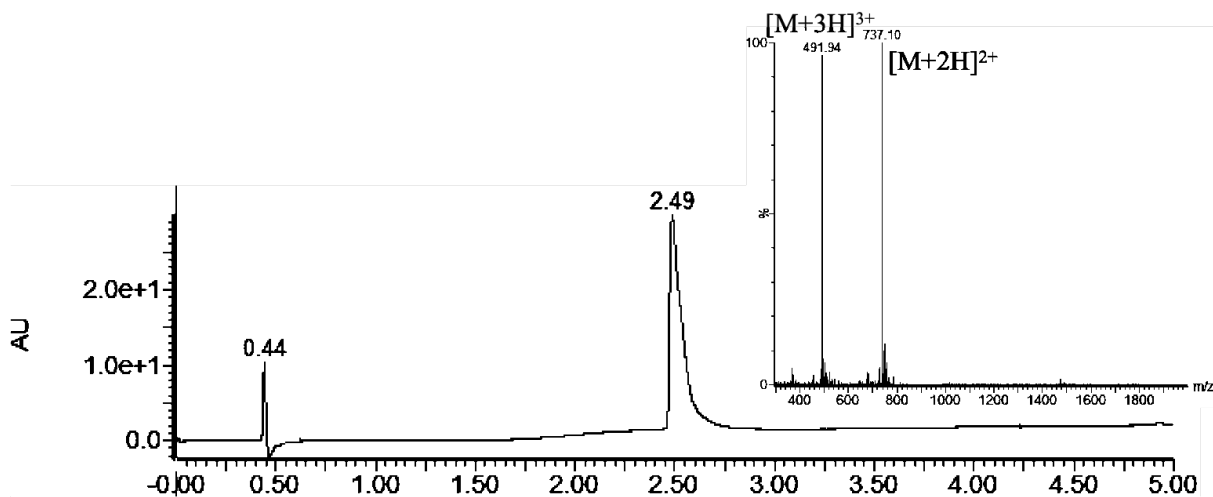### 34. Synthesis of H-GKGDPKQPRGQMSS-OH

The peptide was synthesized following standard SPPS protocol.

UV trace and corresponding MS from LC-MS analysis of purified product. ESI-MS calcd. molecular weight: 1472.64. $[M+2H]^{2+}$ m/z = 737.3, $[M+3H]^{3+}$ m/z = 491.9, found 737.1, 491.9.

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