Salicylaldehyde ester-mediated protein semi-synthesis enables studies on the tetra-acetylation of HMGB1

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Supplementary information

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

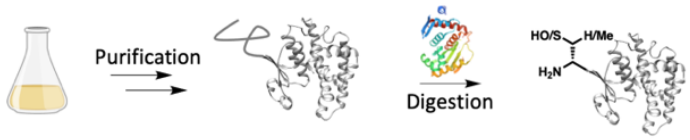


Figure S1. Method to generate recombinant Ser/Thr/Cys N-terminal protein.

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 the indicated enzymes, and the affinity tags can be removed simultaneously.

Note: If the truncated protein is not soluble, we can purify the inclusion body in the denatured buffer (8 M Urea). Then, the protein can be diluted with 2 M Urea. In this concentration of Urea, protein does not precipitate, and enzyme (e.g. TEV) still can cleave the recognition site. After digestion, the protein solution can be further purified by HPLC.

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%)

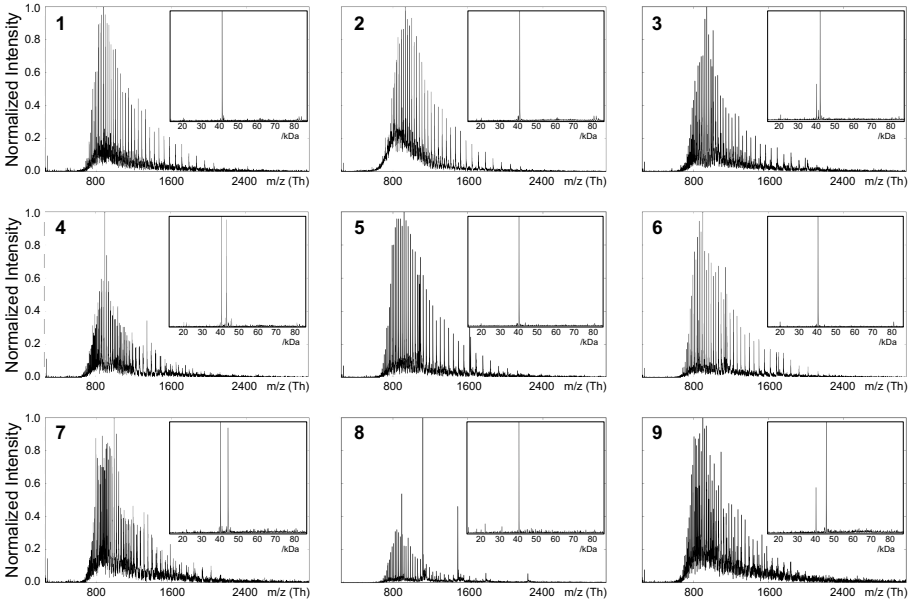


Figure S2. Mass spectra of ligation products obtained from peptide SAL esters and Ser-MBP. The conversion rates were calculated according to the deconvolution results.

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%)

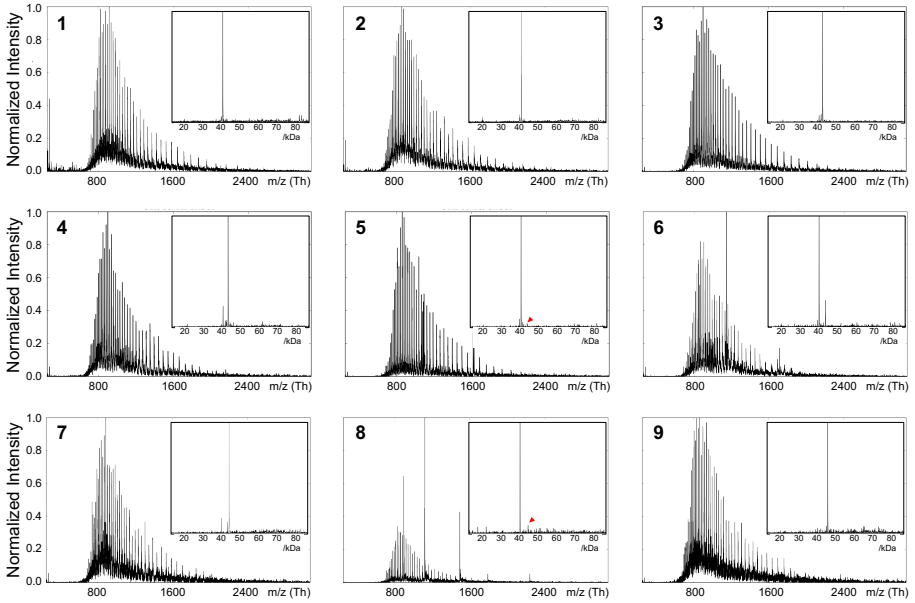


Figure S3. Mass spectra of ligation products obtained from peptide SAL esters and Thr-MBP. The conversions were calculated according to the deconvolution results.

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%)

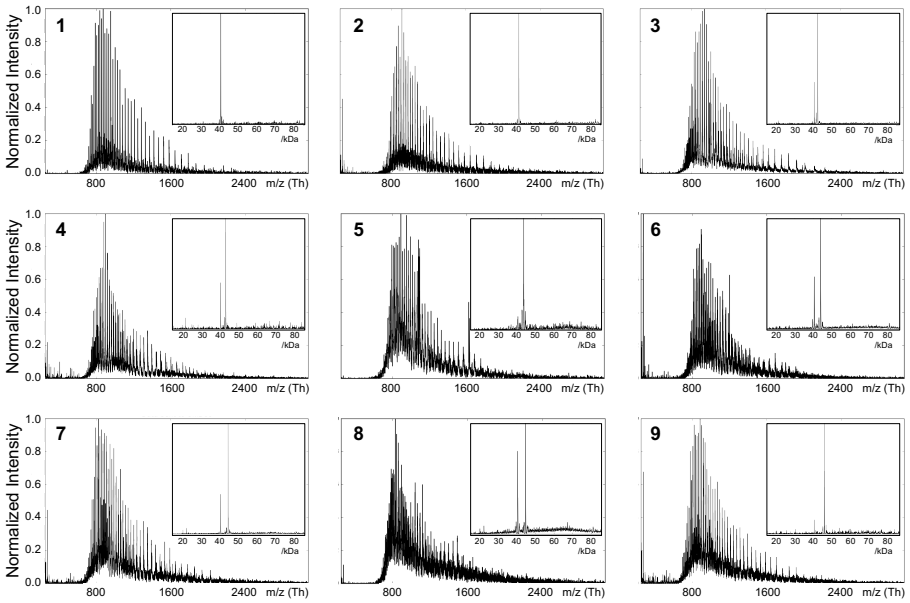


Figure S4. Mass spectra of ligation products obtained from peptide SAL esters and Cys-MBP. The conversions were calculated according to the deconvolution results.

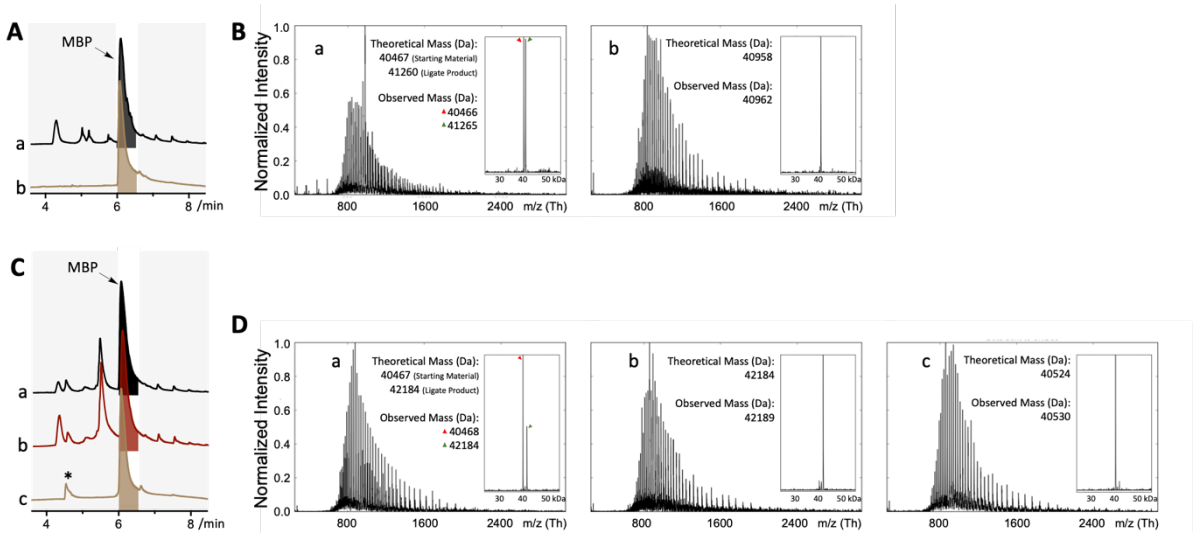


Figure S5. Purification strategy for STL/CPL mediated protein semi-synthesis.

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.

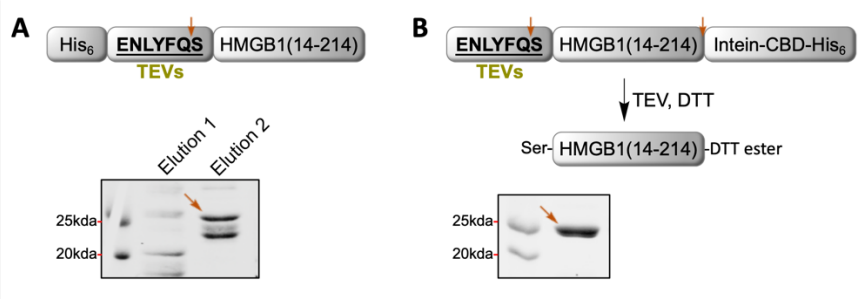


Figure S6. Purification strategy for HMGB1(13-214).

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

B. Intein tag-based purification strategy. Note: The DTT ester will hydrolyze slowly.

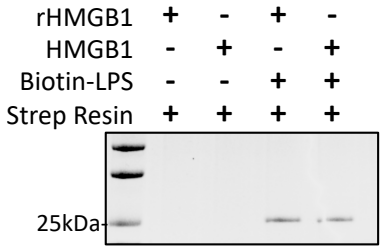


Figure S7. Pull-down experiment using biotin-LPS. Recombinant full-length HMGB1 and synthetic HMGB1 showed comparable affinity with LPS, proving that the refolding step could generate native conformation. rHMGB1: recombinant HMGB1, which was purified under native condition; HMGB1: synthetic HMGB1 after refolding.

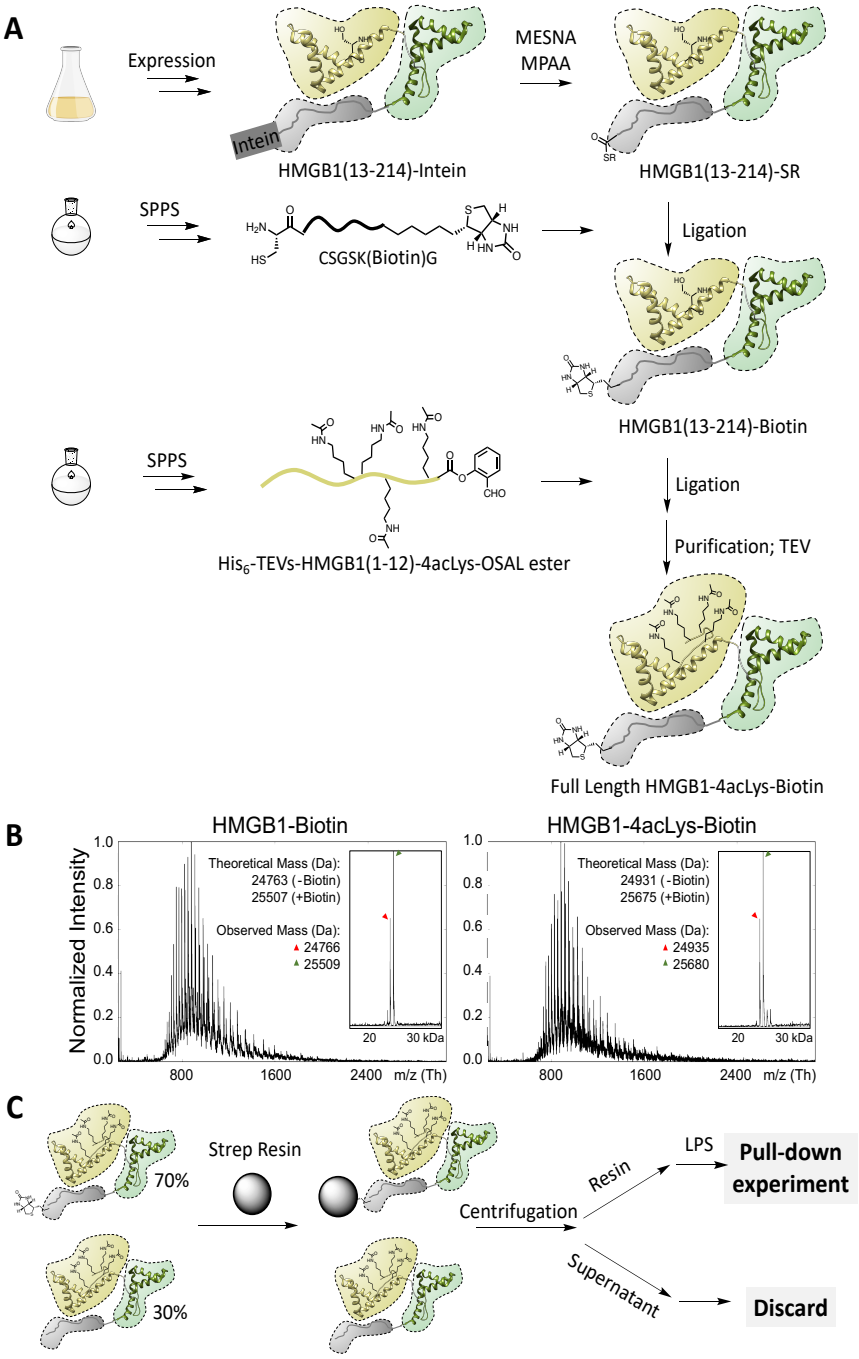


Figure S8. Semi-synthesis of HMGB1-4acLys-Biotin.

A. Synthetic route of HMGB1-4acLys-Biotin.

B. Mass spectra of HMGB1-Biotin and HMGB1-4acLys-Biotin. As ~30% HMGB1-SR was hydrolyzed, the final product contained 70% biotin conjugated HMGB1 and 30% non-biotin conjugated HMGB1.

C. The workflow of the pull-down experiment of biotin-HMGB1 proteins. The non-biotin conjugated HMGB1 proteins were removed via pre-incubation with strep resin.

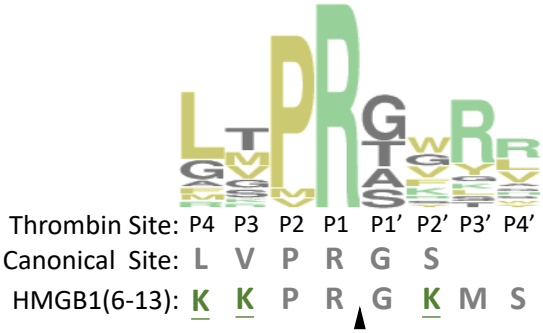


Figure S9. Position weight matrix for substrate preference of thrombin.

The raw data was adopted from Gallwitz M et al, in which the recognition preference of thrombin was profiled by phage display technology. They showed the aliphatic residues (e.g. Leu and Gly) at P4, Pro at P2, Arg at P1, and small residues at P1' were preferred by thrombin. Positively charged amino acid were coloured as green. Acetylation sites were underlined. The cleavage site is P1^P1'. Canonical site means the thrombin recognition site for recombinant protein production.

A

Pyridine/ acetic acid	Remark
1:3	Can not dissolve proteins at 1 mM.
1:6	
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	Could dissolve all tested proteins. Some salicylaldehyde ester may hydrolyzed quickly. If so, we recommend to use DMSO. Side rection was observed with salicylaldehyde ester(the salicylaldehyde esters were displaced by HFIP).
DMSO	Could dissolve all tested proteins. The solution may become viscous during ligation, which may lead the slightly lower ligation conversion than in HFIP. No side rection was observed with salicylaldehyde ester.

D

Temperature	Remark
25℃	We always did the the ligation at room temperature.

E

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

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

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

Table S2. Recommendation for disconnected sites for STL/CPL mediated protein semi-synthesis. This data was original from Liu et al. and Tan et al.[1,2]

I 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(13-214), HMGB1(13-78), and HMGB1(13-51) DNA was inserted into PET28a with an N-terminal TEV recognition sequence and C-terminal intein-CBD-His tag to generate pET28a-TEV-HMGB1s-intein-CBD-His plasmid for HMGB1 expression. Likewise, the full length HMGB1 was constructed as HMGB1-intein-CBD-His.

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 ℃ 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-teminal 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.

For HMGB1 purification, initially, the His-TEV-HMGB1 constructions produced several truncated species probably due to the highly negative charged acidic tail (Figure S6). Therefore, the His 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 tag. The expression was induced by including 0.2 mM IPTG when OD_{600} reached 0.8, and the culture was grown at 16 ℃ 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 was 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 the next section.

Chemical ligation

All ligations were set up at room temperature. HFIP or DMSO was used as the cosolvent to dissolve proteins firstly.

The 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)-4acLys or 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 related 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 the selection of cosolvent, DMSO was usable for all tested peptide SAL esters. HFIP, which could give higher conversion in most cases, could cause hydrolysis of peptide SAL esters in some cases.

For intein-mediated ligation (IPL), after removal of recognition sequence, HMGB1(13-214)-intein-CBD-His was exchanged into IPL buffer (100 mM Tris, pH 8.5, 500 mM NaCl, 100 mM MESNA, 50 mM MPAA) by PD-10 column, 50 equivalents of Cys-biotin was added. After 24 h, the solution was directly injected into HPLC for HMGB1(13-214)-biotin purification (10-40% CH_3CN/H_2O over 40 min). The products were checked by LC-MS and shown as the mixture of HMGB1(13-214)-biotin (~70%) and unreacted HMGB1(~30%), which was combined and lyophilized.

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 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 final HMGB1-4acLys (-biotin) and HMGB1 (-biotin) proteins.

Thrombin digestion assay

HMGB1 proteins were diluted in PBS at 100 μg/mL, then treated with thrombin (20 U/mL) at 37 ℃. The digestion was monitored at indicated time points.

Pull-down experiment

45 μg biotin-polysaccharide or biotin-LPS was incubated with 50 μL streptavidin 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 washing, the resin was divided into several aliquots and incubated with 30 μg N-terminal deleted HMGB1, HMGB1, or HMGB1-4acLys respectively for 3 h at 4 ℃ in binding buffer. After twice washing, the resin was resuspended in 40 μL 1x loading buffer for SDS-PAGE resolving and Coomassie blue staining.

30 μg HMGB1-biotin or HMGB1-4acLys-biotin was incubated with 15 μL streptavidin resin slurry in binding buffer for 1 h, respectively. After washing, the resin was resuspended with binding buffer containing 100 μg LPS (extracted from *E. coli* BL21) for 3 h. After washing twice, the resin was resuspended in 40 μL 1x loading buffer for SDS-PAGE resolving and silver staining.

LPS extraction

Bacteria suspension was centrifuged at 12000 rpm for 10 min. The pellet was washed twice in PBS and the sample was treated with Proteinase K, RNase (40 μg/mL) and DNase (20 μg/mL) in presence of 1 μL/mL of 20% $MgSO_4$ solution and 4 μL/mL chloroform. In the next step, an equal volume of hot (65−70 °C) 90% phenol was added to the mixture, followed by vigorous shaking at 65−70 °C for 30 min. The suspension was then cooled on ice and centrifuged at 6000 rpm for 15 min at 10 ºC. The supernatant was transferred to a sterile tube and the phenol phase was re-extracted three times with 1 mL of distilled water. The upper layers from both extractions were combined together and dialyzed against 1 L of water using a 3000 MWCO dialysis membrane for two days. The dialysate was centrifuged at 12000 rpm for 10 min, and the supernatant was lyophilized. Finally, purified LPS product was dissolved in water with a final concentration of 4 mg/mL and stored at −20 °C. The yield of the extraction was around 600−800 μg of pure LPS from per 2 mL culture.

Delipidation of lipopolysaccharide

Dissolved the biotin-LPS (invivoGen) in water and add acetic acid to a concentration of 1%, then followed by heating at 100 ℃ for 1 h. After centrifugation at 13000g for 10 min. the supernatant was collected (biotin-polysaccharide).

II 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_3CN, ACN); Solvent B: 0.1% TFA (v/v) in water. Mass analysis were performed with a Waters 3100 mass spectrometer.

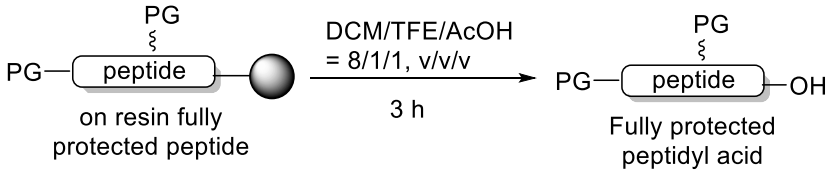
2. General experimental procedures

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_2Cl_2 for 30 min and then it washed with CH_2Cl_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_2Cl_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_2Cl_2 (5 mL × 3), and subsequently treated with a solution of CH_2Cl_2/CH_3OH/DIEA (17:2:1, v/v/v, 5 mL) for 1 h for capping. The resin was washed with DMF (5 mL × 3), CH_2Cl_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_2Cl_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_2Cl_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.

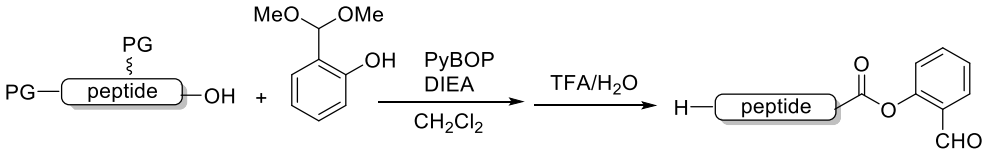
2.2 Cleavage fully protected peptide from 2-chloro-trityl chloride resin



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

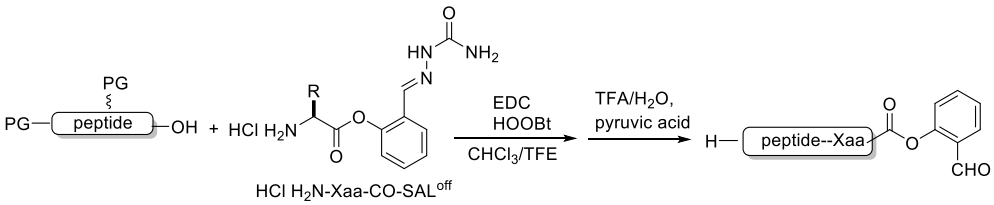
2.3.1 Direct coupling for preparation of C-terminal Gly and Pro peptide SAL esters:



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.

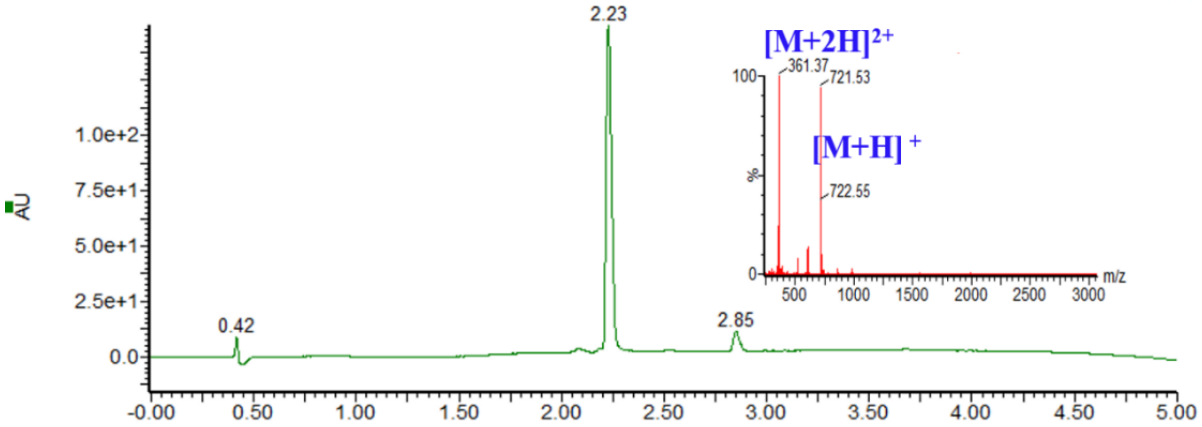
2.3.2 "N+1" strategy for the preparation of C-terminal Ser, Met, Ala, Phe, Val, Leu, Ile, and Thr peptide SAL esters:



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 $HCl{\cdot}H_2N$-Xaa-CO-SAL^{off} (6.0 equiv.), synthesized according to the procedure[3] 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

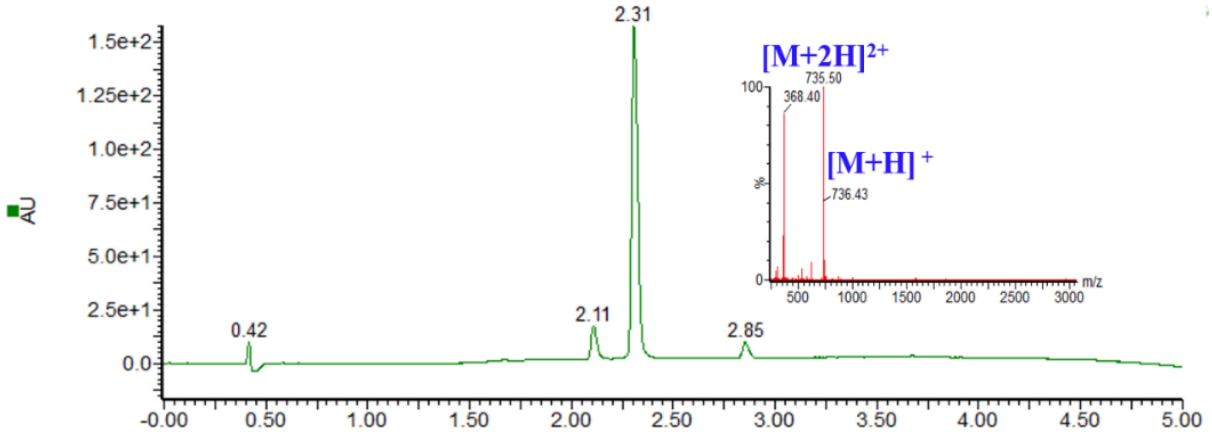
The H-LSQRGG-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1**.



UV trace and corresponding MS from LC-MS analysis of purified H-LSQRGG-CO-SAL. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. 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

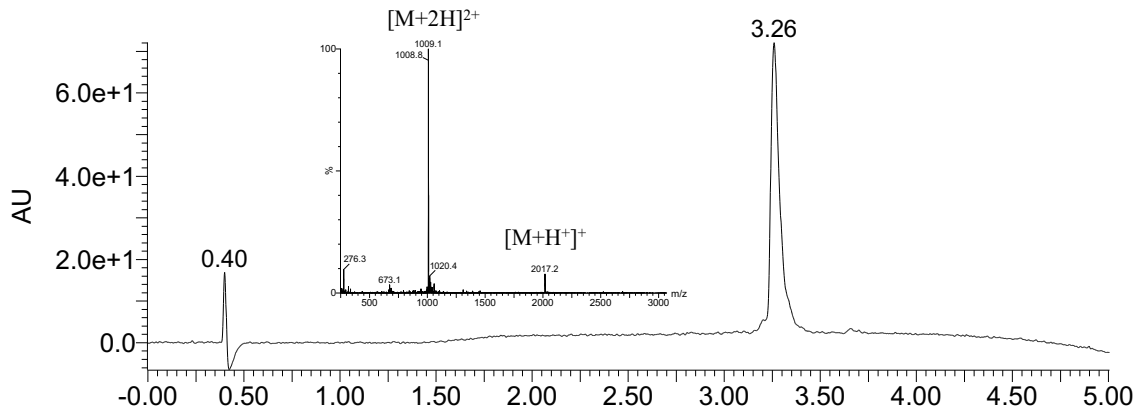
The H-LSQRGA-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2**.



UV trace and corresponding MS from LC-MS analysis of purified H-LSQRGA-CO-SAL. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. 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

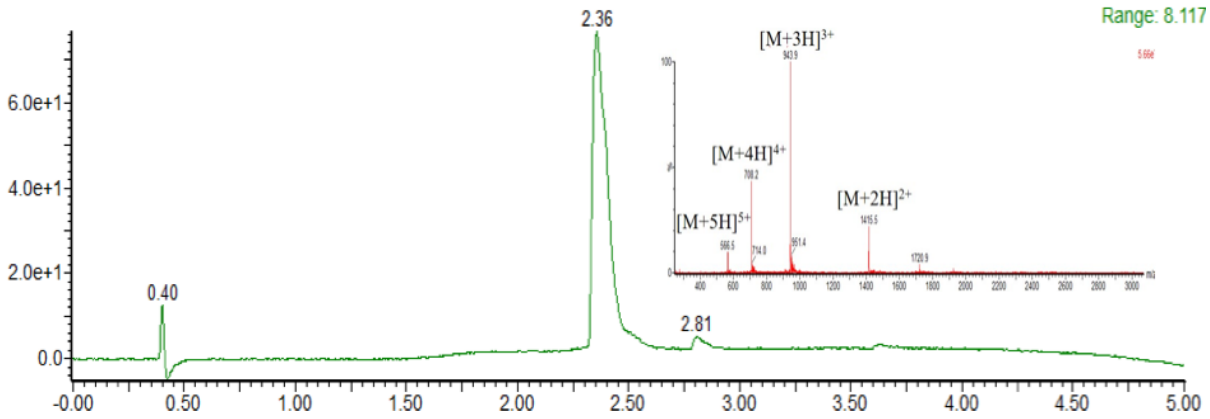
The Ac-ET TTQGPGVLLP LPKGAC(StBu)-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2**.



UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. 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.**

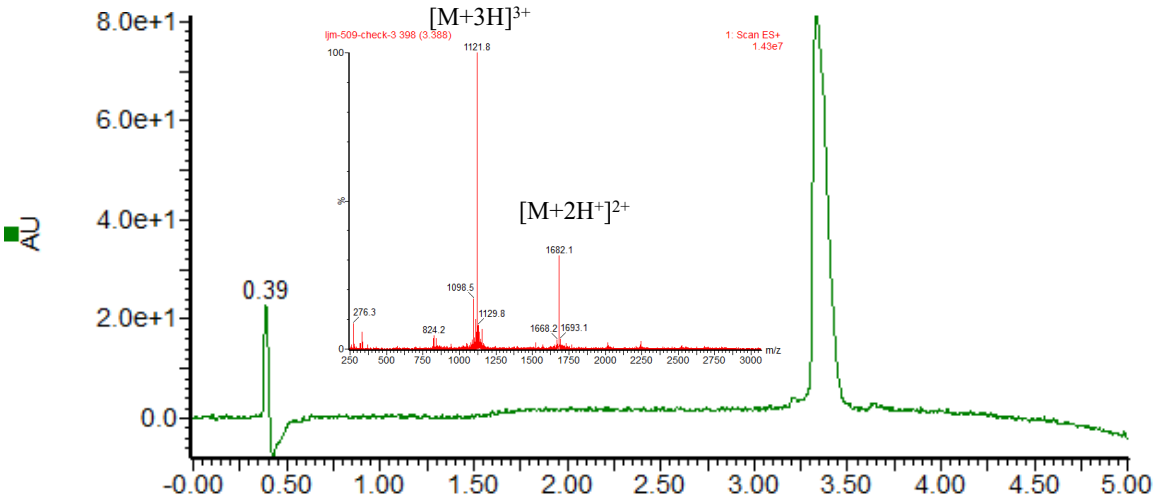


UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. molecular weight: 2828.21. $[M+2H]^{2+}$ m/z = 1415.6, $[M+3H]^{3+}$ m/z = 944.0, $[M+4H]^{4+}$ m/z = 708.3, $[M+5H]^{5+}$ m/z = 566.8, found 1415.5, 943.9, 708.2, 566.5.

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

SAL ester

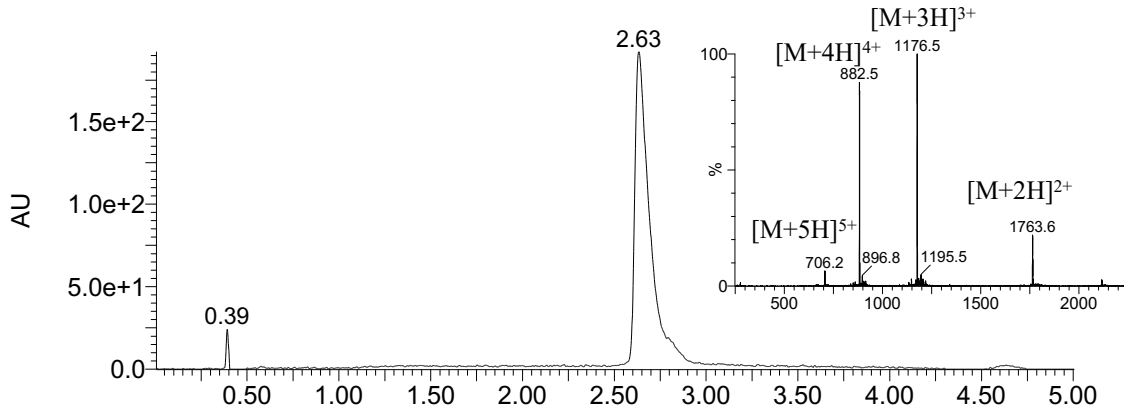
The Fmoc-HN-TLAEAQTETC(Acm)TVAPRERQNC(StBu)GFPGVTP-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**



UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. 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.**

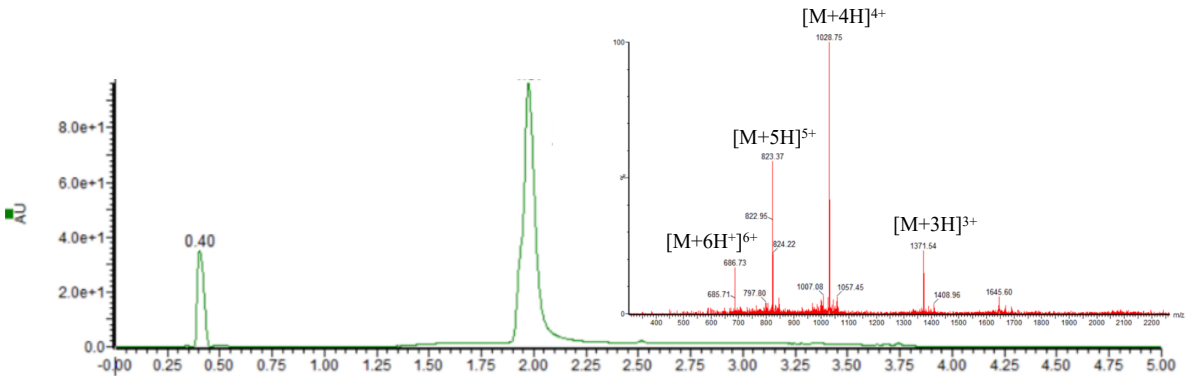


UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 25-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. molecular

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

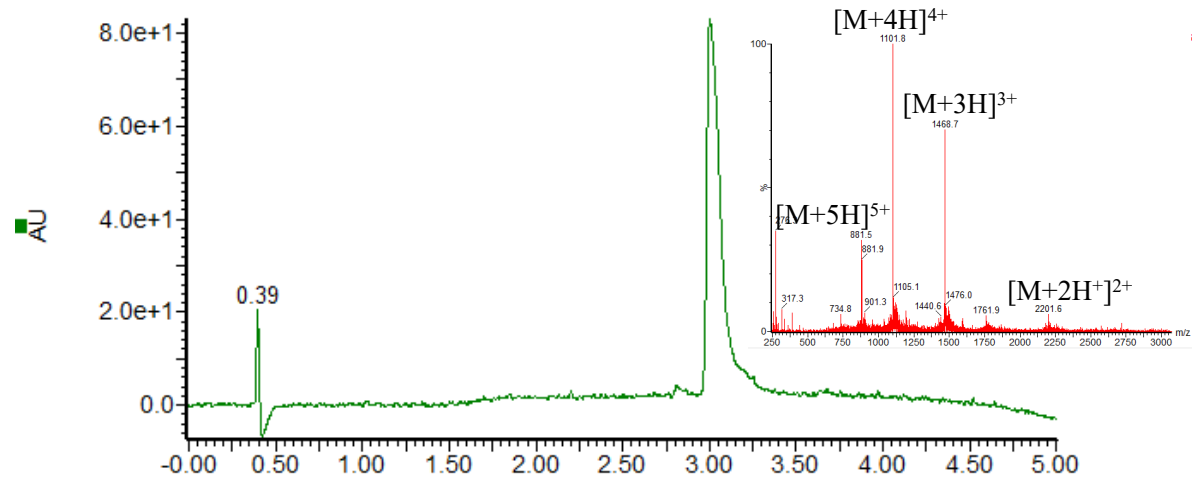
The Ac-SESSSKSSQPLASKQEKDGTEKRGRGRPRKQPPVSPG-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**



UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 10-90% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. 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

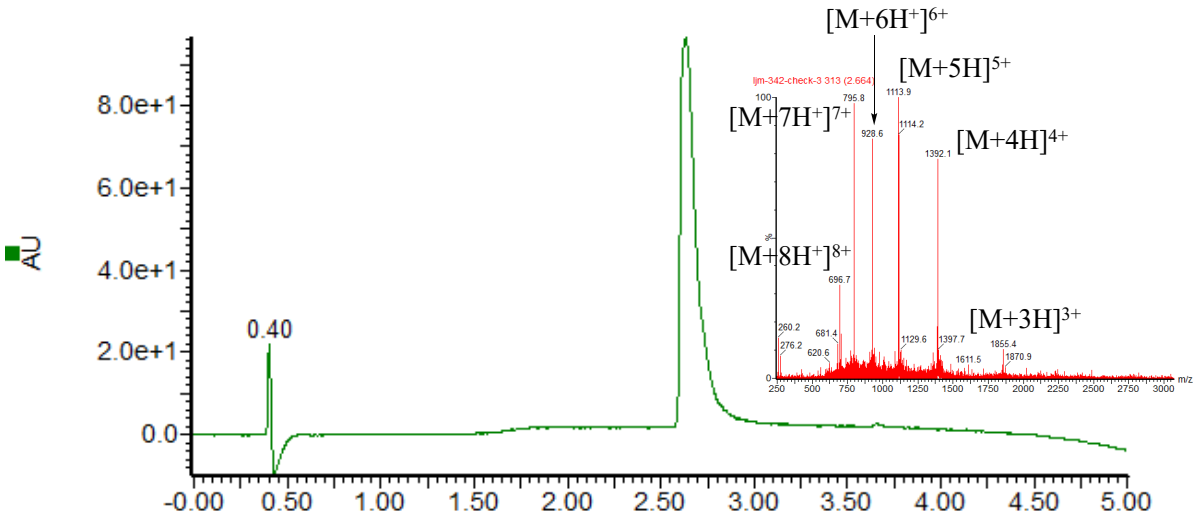
The Fmoc-HN-TGANRDLELPWLEQQGPASHHRRQLGPQGPPHLVADP-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**



UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. 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.

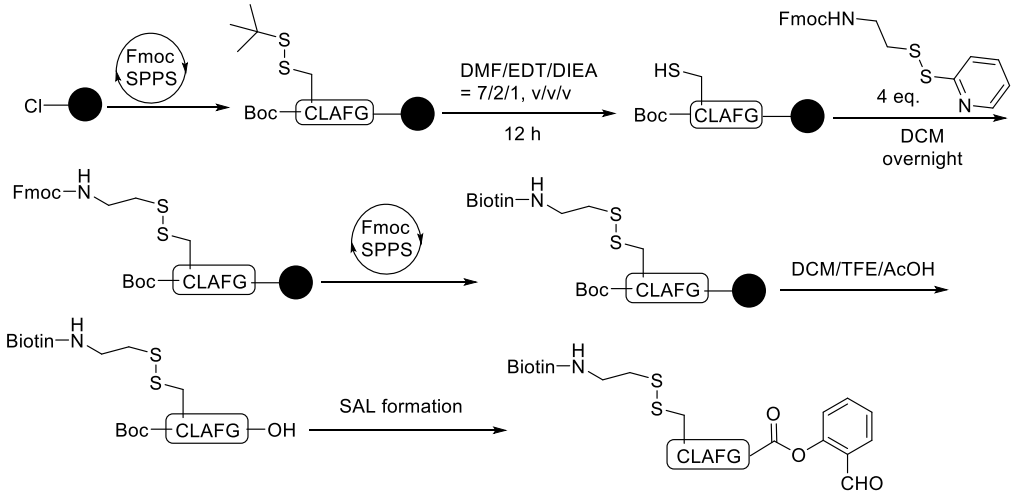
11. Synthesis of Biotin-WRRKRKEKQSETSPKEFLTIYEDVKDLKTRRNHEQEQTF PGGG-CO-SAL ester

The Biotin-WRRKRKEKQSETSPKEFLTIYEDVKDLKTRRNHEQEQTFPGGG-OSAL was synthesized according to the **General Experimental Procedures 2.3.1.**

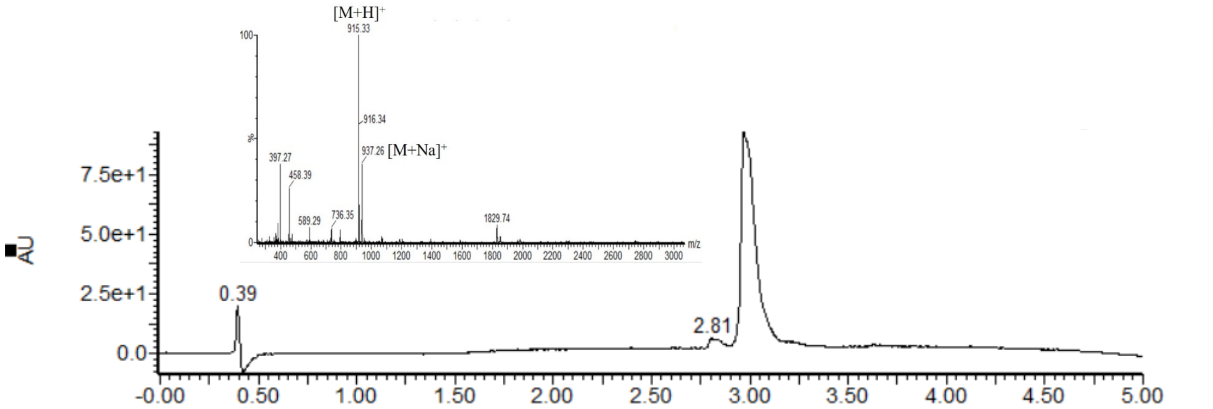


UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. molecular weight: 5565.68. $[M+3H]^{3+}$ m/z = 1856.23, $[M+4H]^{4+}$ m/z = 1392.42, $[M+5H]^{5+}$ m/z = 1114.14, $[M+6H]^{6+}$ m/z = 928.61, $[M+7H]^{7+}$ 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



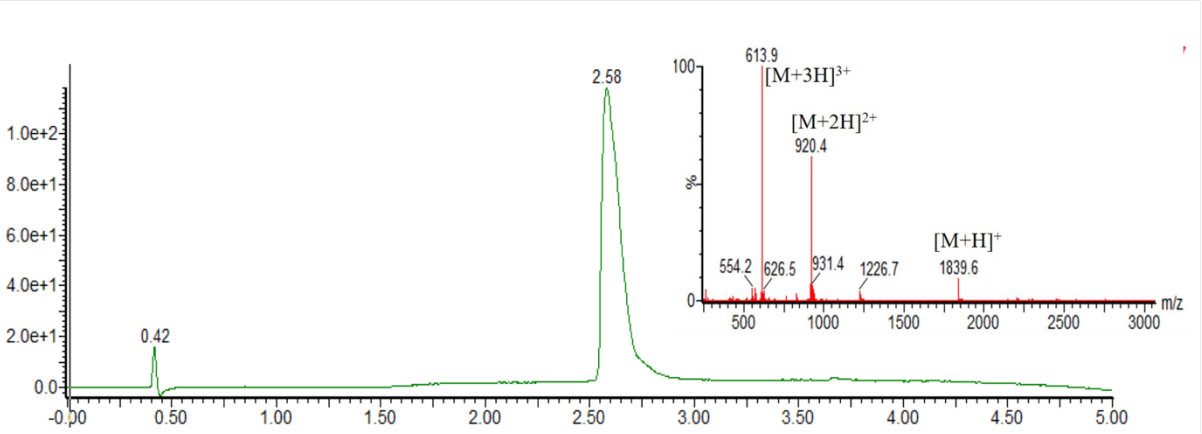
The on resin fully protected peptide obtained using **General Experimental Procedures 2.1** was treated with 5 mL EDT/DIEA/DMF (2/1/7, v/v/v) and shaken for 12 h at room temperature to selectively remove the *tert*-butylthio (StBu) protecting group. Then, the resin was washed with DMF (5 mL × 3) and CH_2Cl_2 (5 mL × 3), and subsequently treated with a solution of (9H-fluoren-9-yl)methyl 2-(pyridin-2-yldisulfanyl)ethylcarbamate (synthesized according to reported procedures)[4] (4.0 equiv. relative to resin loading) in DCM for 12 h. After that, the resin was washed with DMF (5 mL × 3), CH_2Cl_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_2Cl_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_3CN/H_2O 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. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. 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

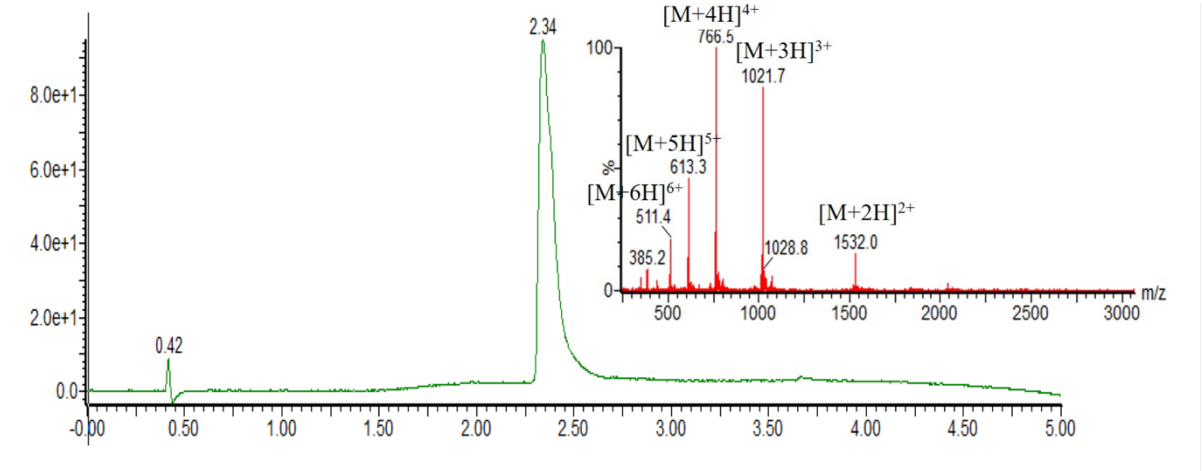
The Ac-HHHHHHENLYFQG-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**



UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. 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.**

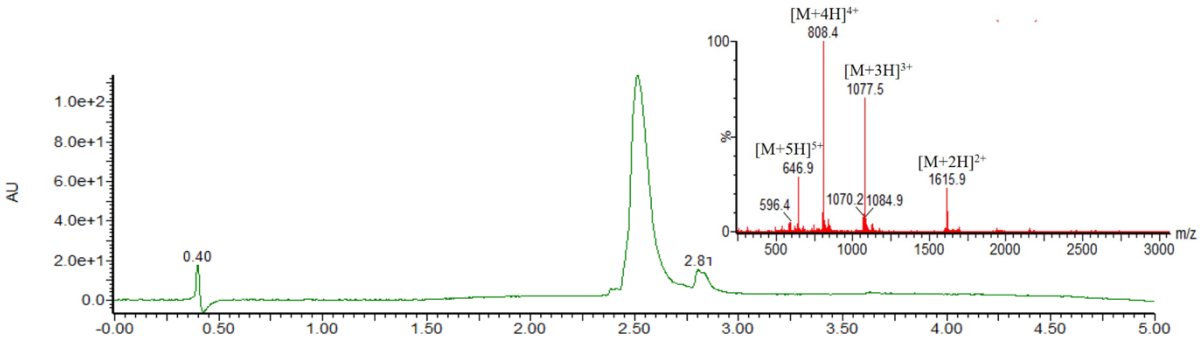


UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 5-95%

ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. 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.

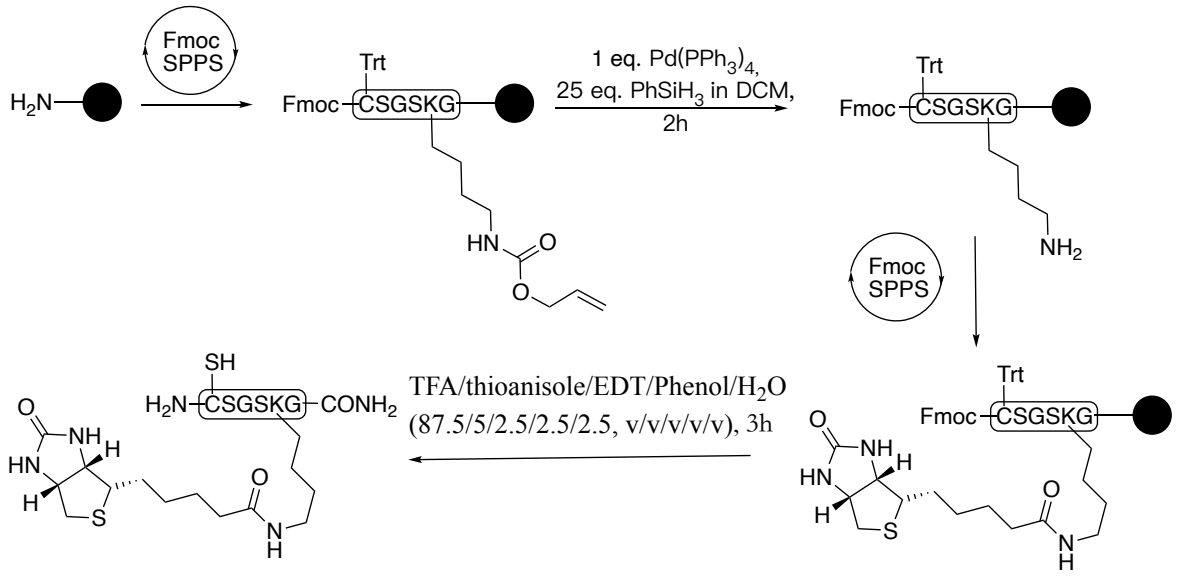
15. Synthesis of Ac-HHHHHHENLYFQGK(ac)GDPK(ac)K(ac)PRGK(ac) M-CO-SAL ester

The Fmoc-Lys(Ac)-OH was synthesized according to the reported procedure.[5] Ac-HHHHHHENLYFQGK(ac)GDPK(ac)K(ac)PRGK(ac)M-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**

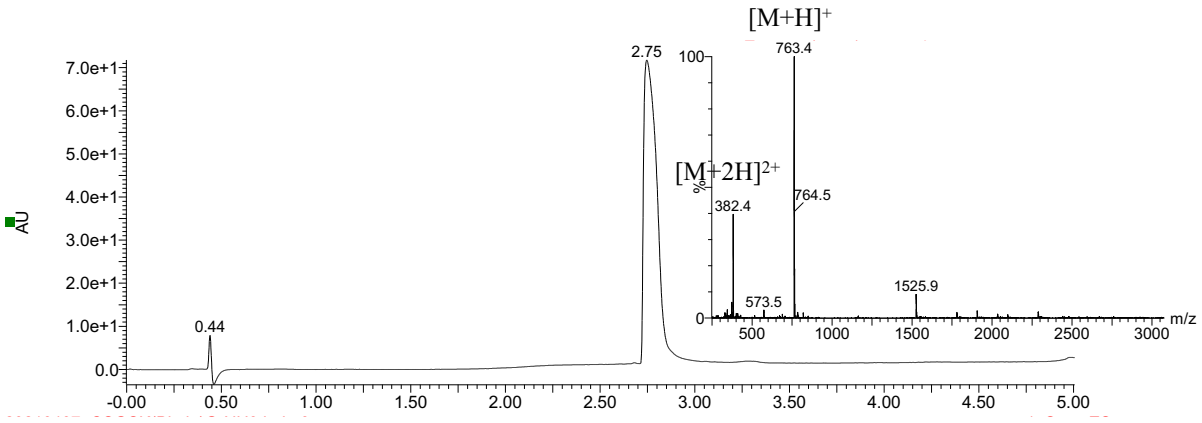


UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. molecular weight: 3230.43. $[M+2H]^{2+}$ m/z = 1616.2, $[M+3H]^{3+}$ m/z = 1077.8, $[M+4H]^{4+}$ m/z = 808.6, $[M+5H]^{5+}$ m/z = 647.1, found 1615.9, 1077.5, 808.4, 646.9.

16. Synthesis of H-CSGSK(biotin)G-$CONH_2$



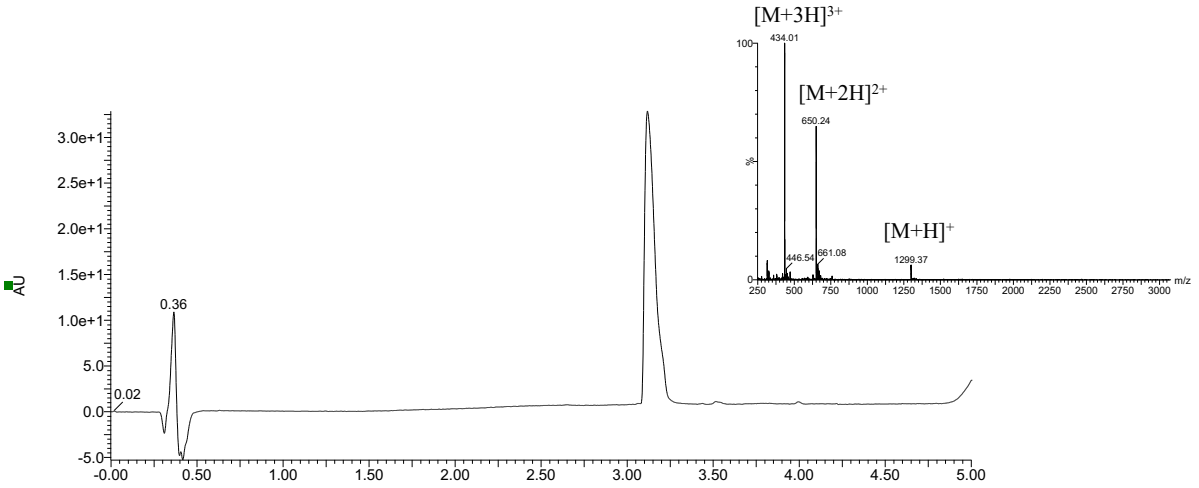
The C(Trt)SGSK(alloc)G peptide was synthesized by CEM synthesizer using rink amide resin. The alloc group was deprotected by treating the resin with 1 equiv. of $Pd(Ph_3P)_4$ and 25 equiv. of $PhSiH_3$ in DCM under argon atmosphere for 2 h. The resin was wash by DCM (5 mL × 6) and DMF (5 mL × 3), and the biotin group was subsequently incorporated by adding D-biotin (4 equiv.), HATU (4 equiv.) and DIEA (8 equiv.). After the synthesis, the resin was subjected to the cleavage solution of TFA/thioanisole/EDT/Phenol/H_2O(87.5/5/2.5/2.5/2.5, v/v/v/v/v) for 3 h. Finally, the crude peptide was purified by preparative reverse-phase HPLC (1%-30% CH_3CN/H_2O over 40 min) and lyophilized.



UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 1-40% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. molecular weight: 762.90. $[M+H]^+$ m/z = 763.90, $[M+2H]^{2+}$ m/z = 382.45, found 763.4, 382.4.

17. Synthesis of HMGB1(1-12): H-GKGDPKKPRGKM-OH

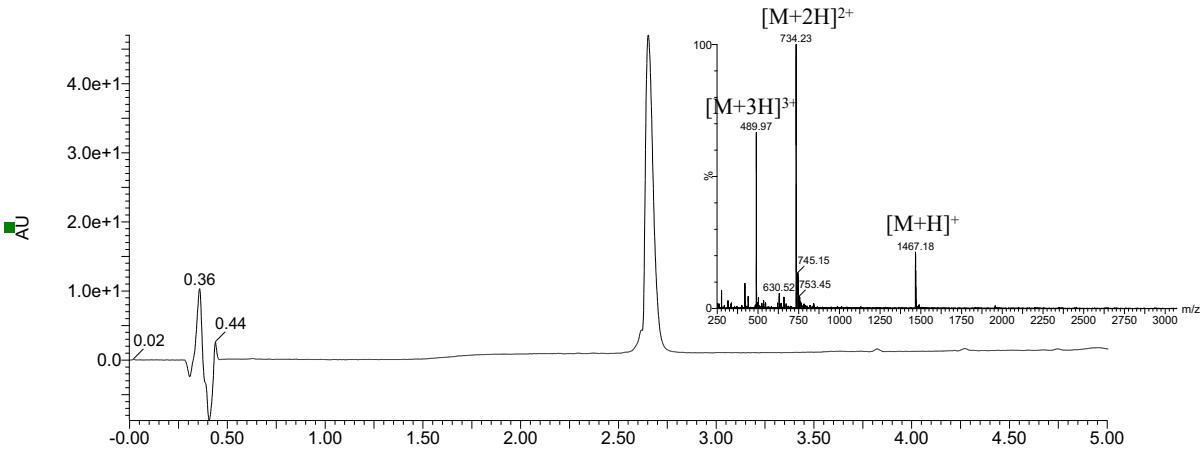
The peptide was synthesized following standard SPPS protocol.



UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 1-15% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. molecular weight: 1298.58. $[M+H]^+$ m/z = 1298.58, $[M+2H]^{2+}$ m/z = 650.29, $[M+3H]^{3+}$ m/z = 433.86, found 1299.37, 650.24, 434.01.

18. Synthesis of HMGB1(1-12): H-GK(ac)GDPK(ac)K(ac)PRGK(ac)M-OH

The peptide was synthesized following standared SPPS protocol.



UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 1-50% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. molecular weight: 1466.72. $[M+H]^+$ m/z = 1467.72, $[M+2H]^{2+}$ m/z = 734.36, $[M+3H]^{3+}$ m/z = 489.91, found 1467.18, 734.23, 489.97.

19. References

1. Liu, H., Li, X. Serine/threonine ligation: origin, mechanistic aspects, and applications. Acc. Chem. Res. **2018**, 51, 1643-1655.

2. Tan, Y.; Li, J.; Jin, K.; Liu, J.; Chen, Z.; Yang, J.; Li, X. Cysteine/Penicillamine Ligation Independent of Terminal Steric Demands for Chemical Protein Synthesis. Angew. Chem., Int. Ed. **2020**, 132, 12841-12845.

3. Lee, C. L.; Liu, H.; Wong, C. T. T.; Chow, H. Y.; Li, X., Enabling N-to-C Ser/Thr Ligation for Convergent Protein Synthesis via Combining Chemical Ligation Approaches. *Journal of the American Chemical Society* **2016,** *138*, 10477-10484.

4. Volmer, A. A.; Carreira, E. M., Active Amphotericin B Derivatives Position the Mycosamine in Two Radial Orientations. *ChemBioChem* **2010,** *11*, 778-781.

5. Altamore, T. M.; Fernández-García, C.; Gordon, A. H.; Hübscher, T.; Promsawan, N.; Ryadnov, M. G.; Doig, A. J.; Woolfson, D. N.; Gallagher, T., Random-Coil:α-Helix Equilibria as a Reporter for the LewisX–LewisX Interaction. *Angewandte Chemie International Edition* **2011,** *50*, 11167-11171.