Supplementary Information

Acetohydroxamic acid-assisted peptide hydrazide ligation for chemical protein synthesis

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1 Materials and general methods

1.1 Materials

materials and reagents	Company
Rink Amide AM Resin (0.23 mmol/g)	Tianjin Nanka HECHENG
2-Chlorotrityl Resin (0.44 mmol/g)	Tianjin Nankai HECHENG
Wang Resin (0.44 mmol/g)	Tianjin Nankai HECHENG
N, N'-Diisopropylcarbodiimide (DIC)	Energy Chemical
N, N-Diisopropylethylamine (DIEA)	Energy Chemical
Ethyl cyanoglyoxylate-2-oxime (Oxyma)	GL Biochem
1-Hydroxy-7-aza-benzotriazole (HOAT)	GL Biochem
1-Hydroxybenzotriazole (HOBT)	GL Biochem
2-(7-Azabenzotriazol-1-yl)-N, N, N’, N’-tetramethyluronium hexafluorophosphate	GL Biochem
Hexafluoroisopropanol (HFIP)	Energy Chemical
1,2-Dichloroethane	Energy Chemical
N, N-Dimethylformamide (DMF)	Chengdu Kelong Chemical
Dichloromethane (DCM)	Chengdu Kelong Chemical
Piperidine	Sinopharm Chemical Reagent
Trifluoroacetic acid (TFA)	Energy Chemical
1,2-Ethanedithiol (EDT)	Energy Chemical
Phenol	Sinopharm Chemical Reagent
Triisopropylsilane (TIPS)	Energy Chemical
Acetonitrile (HPLC grade)	Energy Chemical

XB-C4 Column Packing Material	Yuexu Technology
XB-C18 Column Packing Material	Yuexu Technology
Guanidine Hydrochloride	Sangon Biotech
Urea	Sangon Biotech
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sangon Biotech
Tris(hydroxymethyl)aminomethane (Tris base)	Sangon Biotech
Sodium Chloride (NaCl)	Sangon Biotech
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Aladdin
Ampicillin	Lablead
Imidazole	Lablead
Tryptone	Sangon Biotech
Yeast Extract	Thermo Fisher
Ni-NTA Resin	Lablead
Sodium Nitrite ($NaNO_2$)	Sinopharm Chemical Reagent
Tris(2-carboxyethyl) phosphine (TCEP)	Energy Chemical
Dithiothreitol (DTT)	Aladdin
Fmoc-Protected Amino Acids	GL Biochem
4-Mercaptophenylacetic Acid (MPAA)	Thermo Scientific
Glutathione (Reduced Form, GSH)	Macklin Reagent
Glutathione Disulfide (Oxidized Form, GSSG)	Macklin Reagent
2,2'-Azobis[2-(2-imidazolin-2-yl)propane] Dihydrochloride (VA-044)	Energy Chemical
Acetohydroxamic Acid	Aladdin
Benzohydroxamic Acid	Aladdin

1.2 List of the protected amino acids for solid-phase peptides synthesis

Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OMpe)-OH, Fmoc-Cys(Trt)-OH, Boc-Cys(StBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Alloc)-OH, Fmoc-Lys(ac)-OH , Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Cys(Acm)-OH, Boc-Ala-OH

2. Peptide synthesis methods

2.1 synthesis of the hydrazine-loaded 2-Cl trityl resin

2-Chlorotrityl resin (0.1 mmol) was soaked in DMF for 15 min to allow complete swelling. After washing with DMF (4 × 8 mL), 5 mL of 5 % (v/v) hydrazine hydrate solution in DMF was added, and the loading reaction was performed at 37 °C for two cycles of 30 min each. The resin was subsequently washed with DMF (4 × 8 mL), followed by the addition of 5 % (v/v) methanol in DMF for capping unreacted active sites (2 × 10 min). Finally, the functionalized resin was thoroughly washed with DMF (6 × 8 mL).

2.2 Fmoc-based solid-phase peptide synthesis

All peptides in this work were obtained based on 9-fluorenyl-methoxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS) using a Liberty Blue™ Automated microwave peptide synthesizer (CEM Corp., North Carolina, USA) with a scale of 0.2 mmol hydrazine-loaded 2-Cl trityl resin (loading: 0.3-0.4 mmol/g), or Rink Amide AM resin (loading: 0.23 mmol/g). In general, 4 eq. Fmoc-protected amino acid building blocks, 4 eq. oxyma, 8 eq. DIC were added to the swelled resin in DMF and microwaved to 90 ℃ for 2 min (the coupling of His were performed at 50 °C for 10 min, and repeated for two times). The Fmoc-group was removed in 20 % piperidine (v/v, containing 0.1 M Oxyma) for 1 min at 90 ℃. After every coupling and Fmoc-deprotection, DMF was added to wash the resin three times. To cleave the peptide from the solid support, the resin was washed three times with DCM and dried in vacuum. A cleavage cocktail of

TFA: thioanisole: H_2O: EDT = 87.5: 5: 5: 2.5 (v/v) was then added to the resin and the reaction mixture was shaken at room temperature for 2 h. The resin was filtered and the combined filtrate was concentrated by nitrogen blowing and then precipitated with cold ether. After centrifugation, the crude peptide was further purified by semi-preparative RP-HPLC.

3. Protein expression and purification

His_6-sumo**-H3(35-135):** the gene was cloned in pET22b vector. Protein gene optimization and synthesis were completed by GenScript Biotech (Nanjing, China).

MHHHHHHHHGSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKV SDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLD MEDNDIIEAHREQIGG**CVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQ RLVREIAQDFKTDLRFQSSAVMALQEAAEAYLVGLFEDTNLAAIHAKRV TIMPKDIQLARRIRGERA**

The expression of His_6-SUMO-H3(35-135) (segment **9'**) in Rosetta cells were induced by 0.6 mM IPTG when OD_{600} reached 0.6. After induction, the cultures were shaken for another 12 h at 37 ℃. Cells were centrifuged and collected at 4000 rpm for 30 min under 4 ℃ and then sonicated to lyse in ice-water bathed lysis buffer (25 mM HEPES, 150 mM NaCl, pH 7.5, 1 mM PMSF, pH = 7.5). After centrifugation (12000 rpm, 30 min, 4 ℃), the insoluble inclusion bodies were collected and dissolved in buffer A (6 M urea, 25 mM HEPES, 150 mM NaCl, pH 7.5). Subsequent centrifugation was carried out under the identical conditions (12000 rpm, 30 min, 4 °C) to collect the soluble fraction (supernatant). The supernatant was loaded onto a Ni-NTA column (GE healthcare). The column was washed with washing buffer (6 M urea, 25 mM HEPES, 150 mM NaCl, 30 mM imidazole, pH 7.5) and then eluted with elution buffer (6 M urea, 25 mM HEPES, 150 mM NaCl, 250 mM imidazole, pH 7.5). The fractions were dialyzed into digestion buffer (500 mM Guanidine-HCl, 25 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.5). Ulp1 was added to the dialysis mixture, followed by overnight incubation at 4 °C to cleave the His_6-SUMO tag. After digestion, Gn-HCl crystals were added to a final concentration of 6 M to re-dissolve precipitated protein. Next, the mixture was purified by semi-preparative reversed-phase high-performance

liquid chromatography (RP-HPLC). After lyophilization, the target histone H3(35-135) (segment **9**) was obtained.

4. Acetohydroxamic acid-assisted peptide hydrazide ligation

4.1 Synthesis of bioactive cyclopeptide Dianthin A via one-pot ligation and desulfurization

The peptide **1** (1.0 mM) was mixed with sodium nitrite (10.0 mM) in reaction buffer (6.0 M Gn-HCl, 0.1 M NaH_2PO_4, pH 3.0) to generate a high-energy acyl azide intermediate. The pH was adjusted to 5.0, followed by the addition of acetohydroxamic acid (100 mM). Subsequently, the pH was re-adjusted to neutrality (pH 6.8), followed by the addition of 10 mM TCEP, with the reaction proceeding for 1 h. HPLC analysis showed that the ligation completed within 1 h to afford the ligation product **2** with a conversion yield of ca. 95%.

Finally, the reaction mixture was diluted with desulfurization buffer (6 M Gn-HCl, 0.2 M NaH_2PO_4, 500 mM TCEP, 40 mM GSH, 100 mM VA044) to adjust the final concentration of peptide **2** to 0.5 mM, and the pH of the reaction was adjusted to 7.0. The desulfurization reaction was stirred at 37 °C for 8 h to afford the bioactive cyclopeptide Dianthin A (**3**) with a conversion of ca. 95%.

MS characterization of peptide 1' (hydrolytic byproduct of 1)

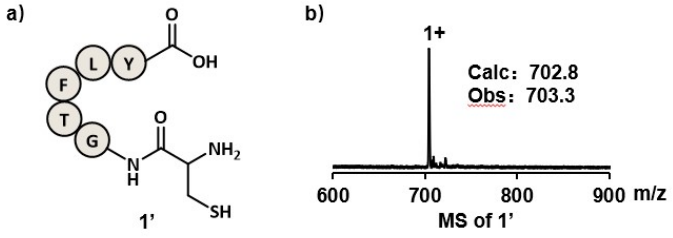


Figure. S1. Characterization of peptide **1'**. **(a)** Structural diagram of the hydrolysis product **1'** corresponding to peptide **1**. **(b)** MS spectrum of **1'**, ESI-MS calculated for **1'** $[M]^+$ m/z=702.8 Da, found: 703.3 Da.

4.2 Peptide ligation reactions of 5a-5h and 7

The peptide **5a-5h** (1.6 mM) was mixed with sodium nitrite (8.0 mM) in reaction buffer (6.0 M Gn-HCl, 0.1 M NaH_2PO_4, pH 3.0) to generate a high-energy acyl azide intermediate. The pH was adjusted to 5.0, followed by the addition of acetohydroxamic acid (160 mM) and peptide **6**（1.0 mM）. Subsequently, the pH of the reaction system was readjusted to neutrality (pH 6.8), followed by the addition of TCEP at a final concentration of 20 mM. For most C-terminal amino acids, the ligation was essentially completed within 3 h, with the coupling efficiency reaching 84–95%. For sterically hindered amino acid residues (e.g., Val), extending the ligation time to 10 h resulted in a coupling efficiency of 71%.

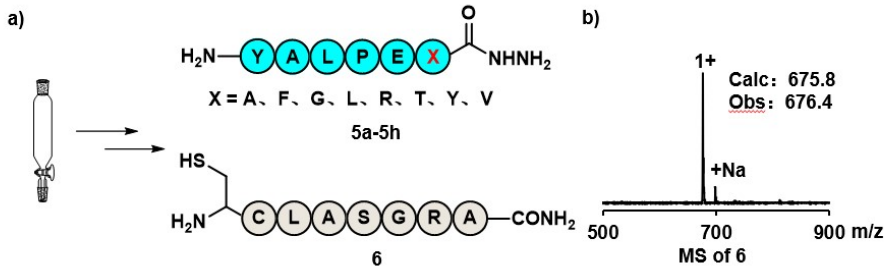


Figure S2. Solid-phase synthesis of peptides 5a–5h and peptide 6. (a) amino acid sequences of peptides **5a–5h** and peptide **6. (b)** MS spectrum of **6**, ESI-MS calculated for **6** $[M]^+$ m/z=675.8 Da, found: 676.4 Da.

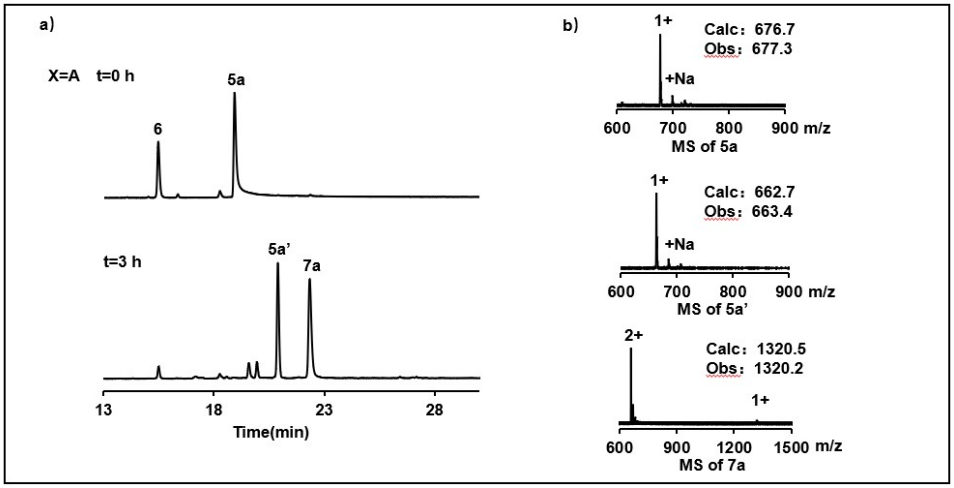


Figure S3. Ligation between peptide 5a and 6 (5a': hydrolytic byproduct of 5a; 7a: ligation product). (a) Analytical RP-HPLC chromatogram (λ = 214 nm) of the reaction mixture at t=0 h and t=3 h on a C18 column, eluted with a linear gradient of 10–70% CH_3CN in water containing 0.1% (v/v) TFA over 30 min.**(b)** MS spectrum of peptides **5a**, **5a'**, and product **7a**. ESI-MS calculated for **5a** $[M]^+$ m/z=676.7 Da, found: 677.3 Da. ESI-MS calculated for **5a'** $[M]^+$ m/z=662.7 Da, found: 663.4 Da. ESI-MS calculated for **7a** $[M]^+$ m/z=1320.5 Da, found: 1320.2 Da.

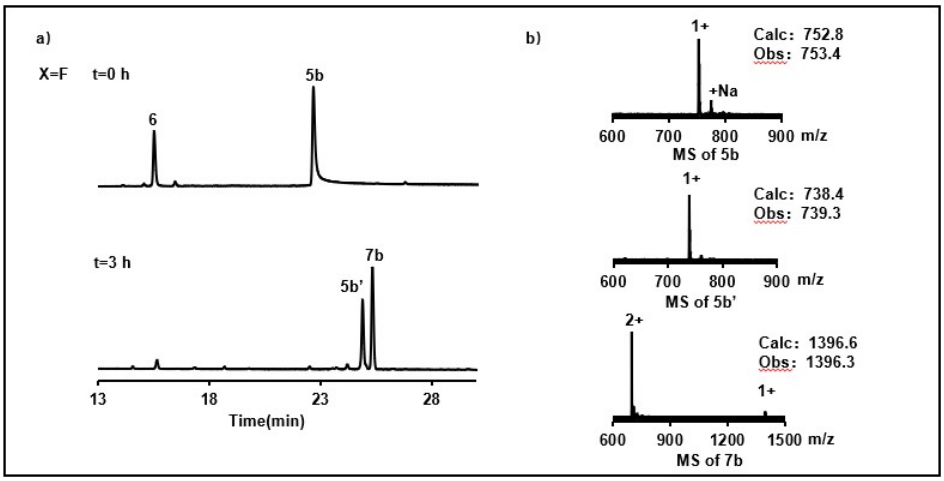


Figure S4. Ligation between peptide 5b and 6 (5b': hydrolytic byproduct of 5b; 7b: ligation product). (a) Analytical RP-HPLC chromatogram (λ = 214 nm) of the reaction mixture at t=0 h and t=3 h on a C18 column, eluted with a linear gradient of 10–70% CH_3CN in water containing 0.1% (v/v) TFA over 30 min.**(b)** MS spectrum of peptides **5b**, **5b'**, and product **7b**. ESI-MS calculated for **5b** $[M]^+$ m/z=752.8 Da, found: 753.4 Da. ESI-MS calculated for **5b'** $[M]^+$ m/z=738.4 Da, found: 739.3 Da. ESI-MS calculated for **7b** $[M]^+$ m/z=1396.6 Da, found: 1396.3 Da.

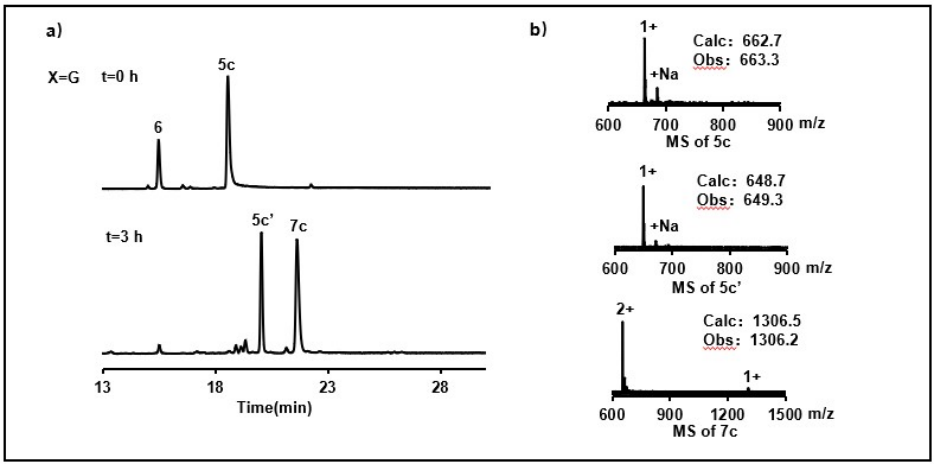


Figure S5. Ligation between peptide 5c and 6 (5c': hydrolytic byproduct of 5c; 7c: ligation product). (a) Analytical RP-HPLC chromatogram (λ = 214 nm) of the reaction mixture at t=0 h and t=3 h on a C18 column, eluted with a linear gradient of 10–70% CH_3CN in water containing 0.1% (v/v) TFA over 30 min.**(b)** MS spectrum of peptides **5c**, **5c'**, and product **7c**. ESI-MS calculated for **5c** $[M]^+$ m/z=662.7 Da, found: 663.3 Da. ESI-MS calculated for **5c'** $[M]^+$ m/z=648.7 Da, found: 649.3 Da. ESI-MS calculated for **7c** $[M]^+$ m/z=1306.5 Da, found: 1306.2 Da.

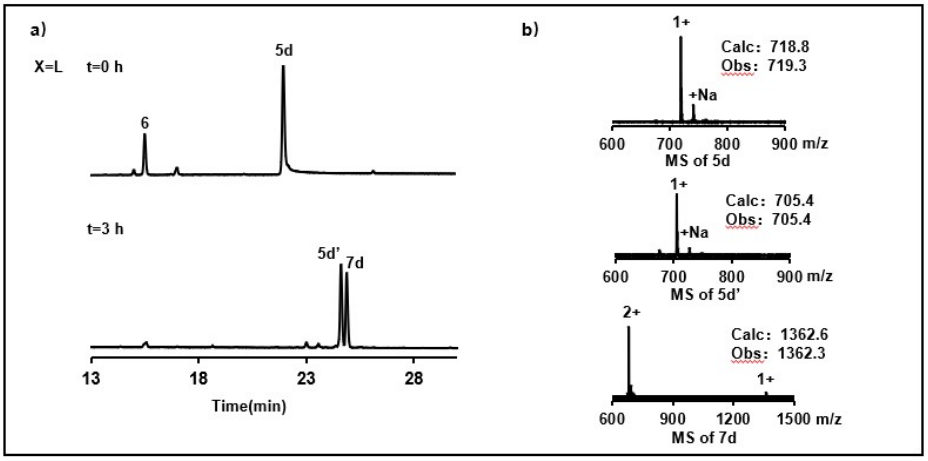


Figure S6. Ligation between peptide 5d and 6 (5d': hydrolytic byproduct of 5d; 7d: ligation product). (a) Analytical RP-HPLC chromatogram (λ = 214 nm) of the reaction mixture at t=0 h and t=3 h on a C18 column, eluted with a linear gradient of 10–70% CH_3CN in water containing 0.1% (v/v) TFA over 30 min.**(b)** MS spectrum of peptides **5d**, **5d'**, and product **7d**. ESI-MS calculated for **5d** $[M]^+$ m/z=718.8 Da, found:

719.3 Da. ESI-MS calculated for **5d'** $[M]^+$ m/z=705.4 Da, found: 705.4 Da. ESI-MS calculated for **7d** $[M]^+$ m/z=1362.6 Da, found: 1362.3 Da.

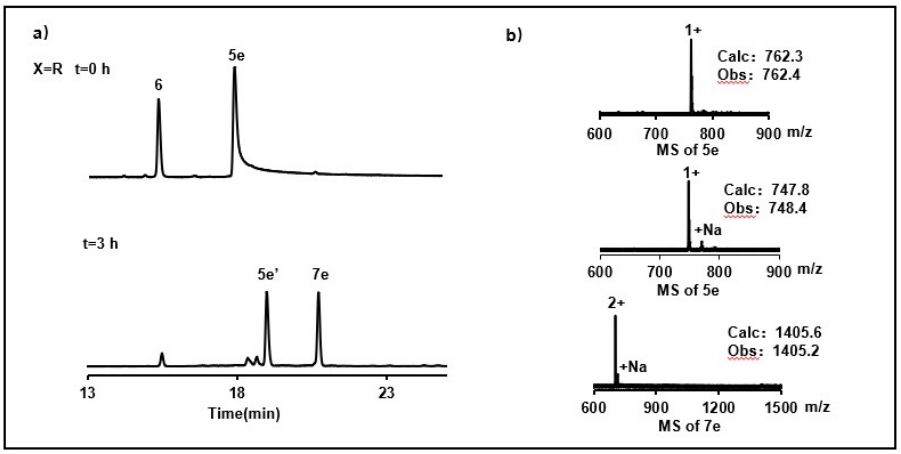


Figure S7. Ligation between peptide 5e and 6 (5e': hydrolytic byproduct of 5e; 7e: ligation product). (a) Analytical RP-HPLC chromatogram (λ = 214 nm) of the reaction mixture at t=0 h and t=3 h on a C18 column, eluted with a linear gradient of 10–70% CH_3CN in water containing 0.1% (v/v) TFA over 30 min.**(b)** MS spectrum of peptides **5e**, **5e'**, and product **7e**. ESI-MS calculated for **5e** $[M]^+$ m/z=762.3 Da, found: 762.4 Da. ESI-MS calculated for **5e'** $[M]^+$ m/z=747.8 Da, found: 748.4 Da. ESI-MS calculated for **7e** $[M]^+$ m/z=1405.6 Da, found: 1405.2 Da.

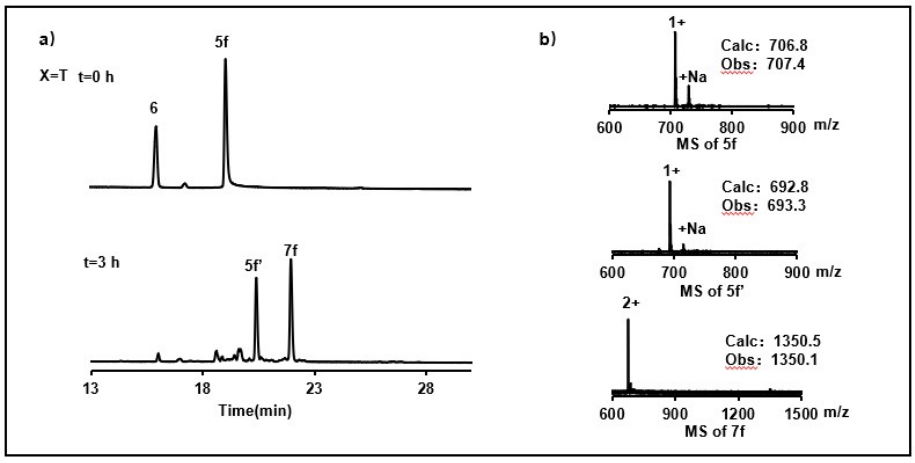


Figure S8. Ligation between peptide 5f and 6 (5f': hydrolytic byproduct of 5f; 7f: ligation product). (a) Analytical RP-HPLC chromatogram (λ = 214 nm) of the reaction mixture at t=0 h and t=3 h on a C18 column, eluted with a linear gradient of 10–70% CH_3CN in water containing 0.1% (v/v) TFA over 30 min.**(b)** MS spectrum of peptides

5f, **5f'**, and product **7f**. ESI-MS calculated for **5f** $[M]^+$ m/z=706.8 Da, found: 707.4 Da. ESI-MS calculated for **5f'** $[M]^+$ m/z=692.8 Da, found: 693.3 Da. ESI-MS calculated for **7f** $[M]^+$ m/z=1350.5 Da, found: 1350.1 Da.

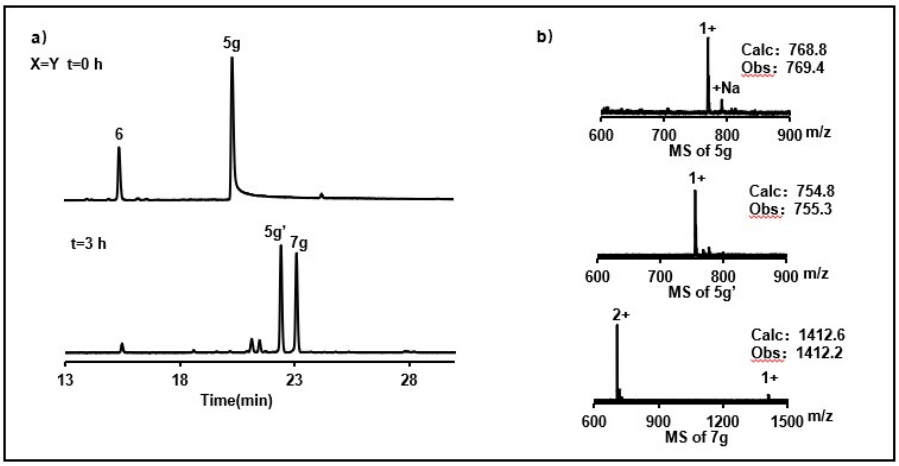


Figure S9. Ligation between peptide 5g and 6 (5g': hydrolytic byproduct of 5g; 7g: ligation product). (a) Analytical RP-HPLC chromatogram (λ = 214 nm) of the reaction mixture at t=0 h and t=3 h on a C18 column, eluted with a linear gradient of 10–70% CH_3CN in water containing 0.1% (v/v) TFA over 30 min.**(b)** MS spectrum of peptides **5g**, **5g'**, and product **7g**. ESI-MS calculated for **5g** $[M]^+$ m/z=768.8 Da, found: 769.4 Da. ESI-MS calculated for **5g'** $[M]^+$ m/z=754.8 Da, found: 755.3 Da. ESI-MS calculated for **7g** $[M]^+$ m/z=1412.6 Da, found: 1412.2 Da.

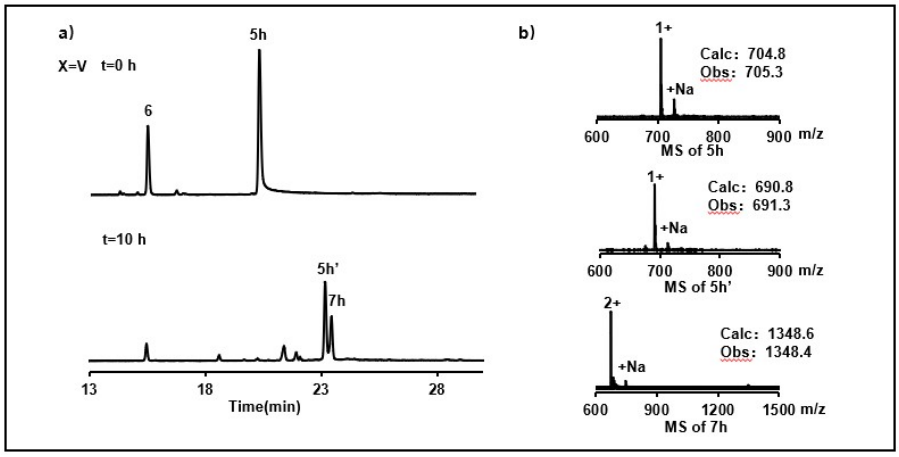


Figure S10. Ligation between peptide 5h and 6 (5h': hydrolytic byproduct of 5h; 7h: ligation product). (a) Analytical RP-HPLC chromatogram (λ = 214 nm) of the

reaction mixture at t=0 h and t=3 h on a C18 column, eluted with a linear gradient of 10–70% CH_3CN in water containing 0.1% (v/v) TFA over 30 min.**(b)** MS spectrum of peptides **5h**, **5h'**, and product **7h**. ESI-MS calculated for **5h** $[M]^+$ m/z=704.8 Da, found:705.3 Da. ESI-MS calculated for **5h'** $[M]^+$ m/z=690.8 Da, found: 691.3 Da. ESI-MS calculated for **7h** $[M]^+$ m/z=1348.6 Da, found: 1348.4 Da.

4.3 Chemical synthesis of H3K18la via one-pot ligation-desulfurization and subsequent Nucleosome assembly

Chemical synthesis of H3K18La

Histone H3K18La (**11**) was divided into two segments (Peptides **8** and **9**). The sequence spanning Gly33 to Ala34 of histone H3 was selected as the ligation site, with H3-Ala34 mutated to Cys. After completion of fragment ligation, the Cys at position 34 was converted back to alanine via a desulfurization reaction. Peptide **8** was synthesized via microwave-assisted SPPS. Peptide **9** was prepared using the previously described method [1].

The peptide **8** (1.5 mM) was mixed with sodium nitrite (15.0 mM) in reaction buffer (6.0 M Gn-HCl, 0.1 M NaH_2PO4, pH 3.0) to generate a high-energy acyl azide intermediate. The pH was adjusted to 5.0, followed by the addition of acetohydroxamic acid (150 mM) and peptide **9**（0.5 mM）. Subsequently, the pH was re-adjusted to neutrality (pH 6.8), and TCEP was added (30 mM), and the reaction allowed to proceed for 8 h. Then, the reaction mixture was diluted with desulfurization buffer (6 M Gn-HCl, 0.2 M NaH_2PO_4, 500 mM TCEP, 40 mM GSH, 100 mM VA044)[2]. The desulfurization reaction was stirred at 37 °C for 8 h to afford H3K18La (**11**) with a conversion of ca. 80%.

Reconstitution of H3K18La Octamer and Nucleosomes

Nucleosomes incorporating the histone variant H3 were assembled using a previously established protocol with minor modifications[2]. Briefly, purified core histones H2A, H2B, H3K18La, and H4 were denatured in 6 M Gn·HCl in 0.1 M PBS. The histones were then combined in a molar ratio of 1.1:1.1:1:1 (H2A:H2B: H3K18La:H4). To facilitate proper folding and octamer formation, the mixture was subjected stepwise dialysis against refolding buffer (10 mM Tris-HCl, pH 7.5, 2 M NaCl, 1 mM EDTA) over a period of 24 hours, with buffer replacement every 6 hours.

Following dialysis, the correctly assembled histone octamers were isolated by size-exclusion chromatography using a Superdex™ 200 Increase 10/300 GL column (GE Healthcare) equilibrated with refolding buffer. The purified octamers were subsequently used for nucleosome reconstitution.

The H3K18La nucleosome was assembled by combining purified octamers and 147 bp Widom 601 DNA at a molar ratio of 1:1.1 in a dialysis cassette. The mixture was dialyzed against refolding buffer (10 mM Tris-HCl, 2 M NaCl, 1 mM DTT, 1 mM EDTA, pH 7.5) and subjected to slow gradient dialysis by continuously pumping HE buffer (10 mM HEPES, 1 mM EDTA, pH 7.5) into the dialysis reservoir until the salt concentration was reduced below 150 mM. The assembled nucleosomes were purified using a DEAE ion-exchange column and verified by native polyacrylamide gel electrophoresis (Native-PAGE).

Sequence of biotinylated Widom 601 DNA

CTGGAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGACAGCTC
TAGCACCGCTTAAACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCCA
AGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGATATATACATCCTGT

Characterization of purified 8-11

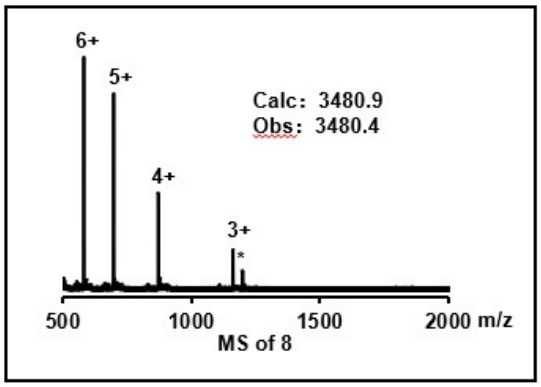


Figure S11. Characterization of peptide 8. MS spectrum of **8**, ESI-MS calculated for **8** $[M]^+$ m/z=3480.9 Da, found: 3480.4 Da.

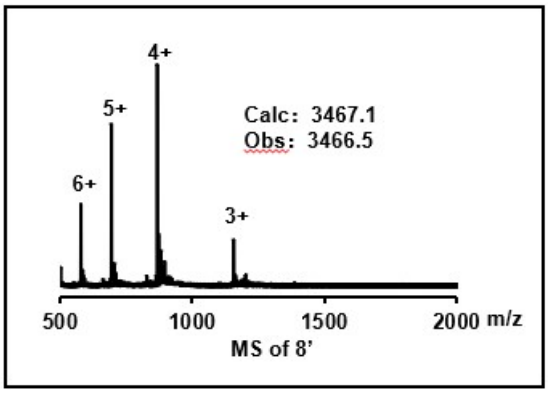


Figure S12. Characterization of peptide 8' (hydrolytic byproduct of 8). MS spectrum of **8'**, ESI-MS calculated for **8'** $[M]^+$ m/z=3467.1 Da, found: 3467.5 Da.

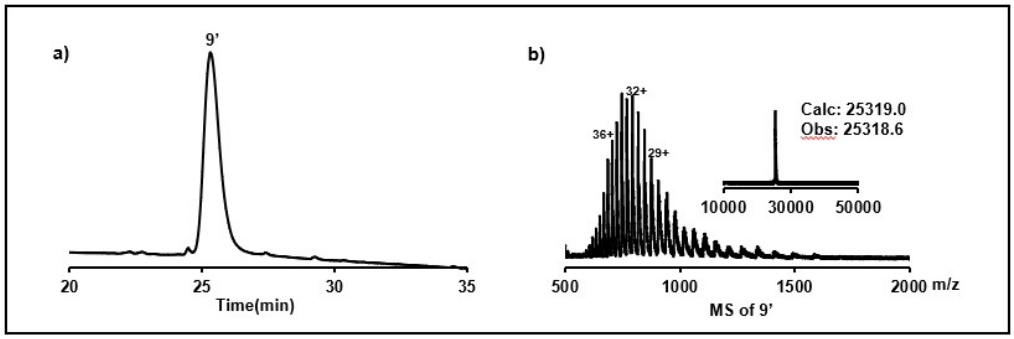


Figure. S13. Characterization of peptide 9'. (a) Analytical RP-HPLC chromatogram (λ = 214 nm) of purified peptide **9'** on a C4 column, eluted with a linear gradient of 20–70% CH3CN in water containing 0.1% (v/v) TFA over 30 min. **(b)** MS spectrum of **9'**, ESI-MS calculated for **9'** $[M]^+$ m/z=25319.0 Da, found: 25318.6 Da.

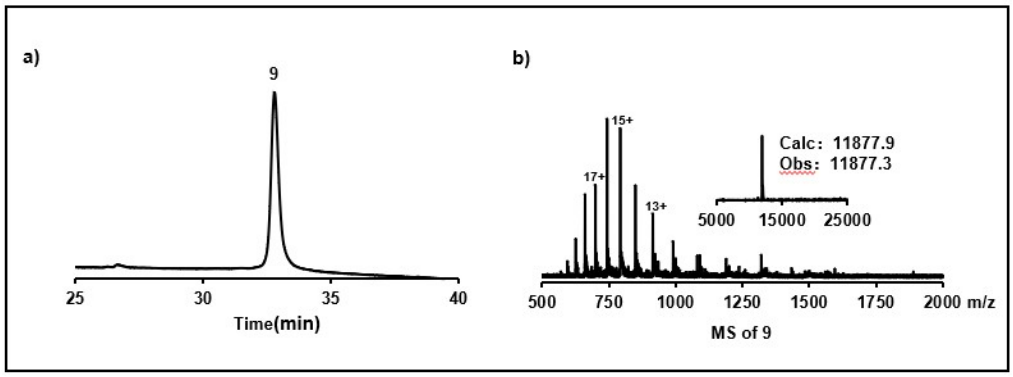


Figure. S14. Characterization of peptide 9. (a) Analytical RP-HPLC chromatogram (λ = 214 nm) of purified peptide **9** on a C4 column, eluted with a linear gradient of 20–70% CH_3CN in water containing 0.1% (v/v) TFA over 30 min. **(b)** MS spectrum of **9**, ESI-MS calculated for **9** [M]+ m/z=11877.9 Da, found: 11877.3 Da.

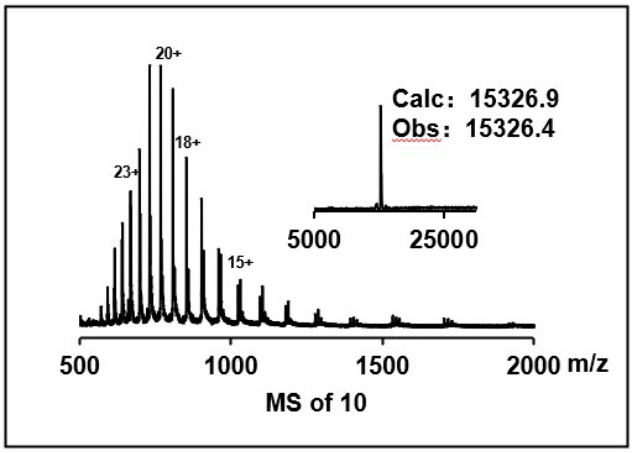


Figure S15. Characterization of peptide 10 (ligation product of 8 and 9). MS spectrum of **10**, ESI-MS calculated for **10** $[M]^+$ m/z=15326.9 Da, found: 15326.4 Da.

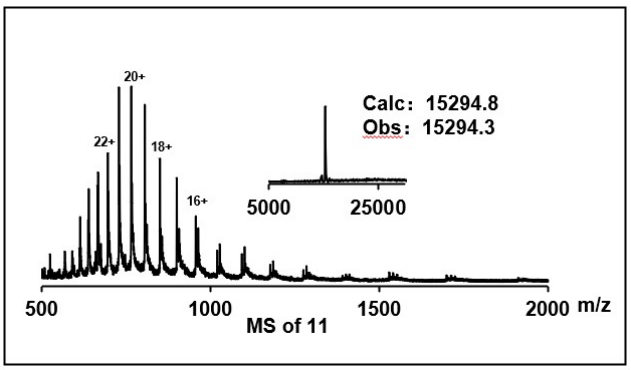


Figure S16. Characterization of peptide 11 (desulfurization product of 10). MS spectrum of **11**, ESI-MS calculated for **11** $[M]^+$ m/z=15294.8 Da, found: 15294.3 Da.

4.4 Chemical synthesis of the neurotoxin Calciseptine via one-pot ligation-refolding

The neurotoxin calciseptine (**15**) was divided into two segments (Peptides **12** and **13**), with Cys39 designated as the ligation site. Both segments were synthesized via microwave-assisted SPPS.

Peptide **12** (1.3 mM) was activated with sodium nitrite (13.0 mM) in ligation buffer (6 M Gn-HCl, 0.2 M Na_2HPO_4, pH 3.0) at -20 °C. The pH was adjusted to 5.0 using 1 M NaOH, followed by the addition of acetohydroxamic acid (130 mM) and peptide **13** (1.0 mM). Subsequently, the pH was re-adjusted to neutrality (pH 6.8), and

TCEP was added (7.5 mM), and the reaction allowed to proceed for 8 h. To enable oxidative folding, the crude reaction mixture was diluted with 25 mM Tris-HCl buffer (pH 7.5) to a final concentration of 0.07 mg/mL and dialyzed against refolding buffer (25 mM Tris-HCl, 1 mM GSH, 0.1 mM GSSG, pH 7.5) at 4 °C for 12 h [3,4]. The folded product (**15**) was monitored and purified by RP-HPLC.

MS characterization of purified 12-15

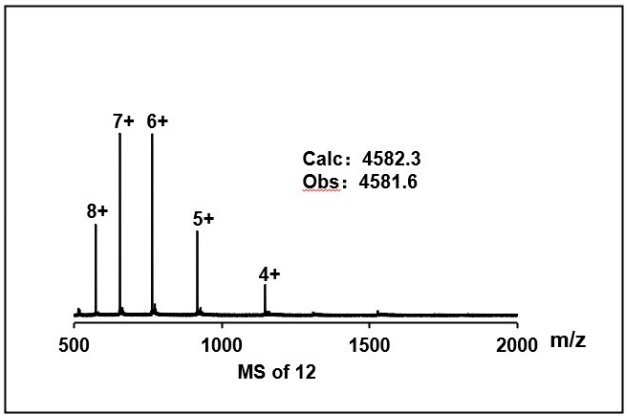


Figure S17 Characterization of peptide 12. MS spectrum of **12**, ESI-MS calculated for **12** $[M]^+$ m/z=4582.3 Da, found: 4581.6 Da.

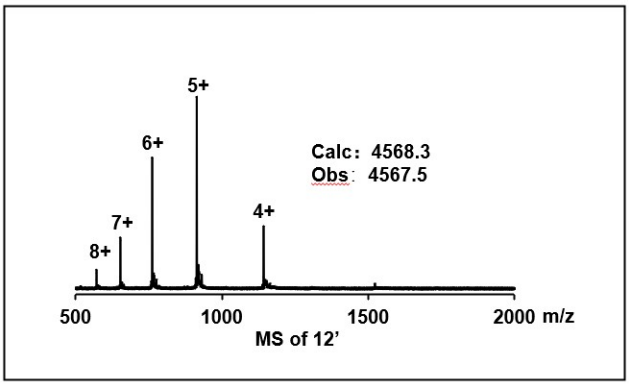


Figure S18. Characterization of peptide 12' (hydrolytic byproduct of 12). MS spectrum of **12'**, ESI-MS calculated for **12'** [M]+ m/z=4568.3 Da, found: 4567.5 Da.

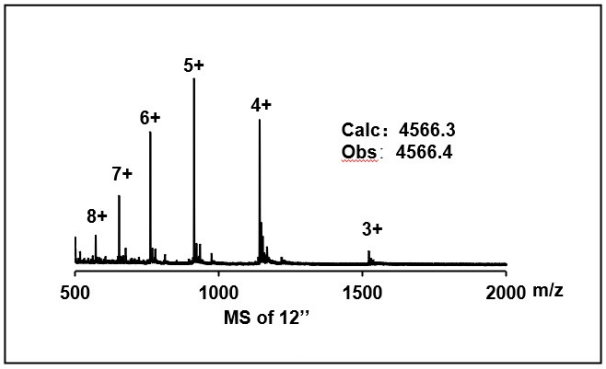


Figure S19. Characterization of peptide 12'' (folded byproduct of 12'). MS spectrum of **12''**, ESI-MS calculated for **12''** $[M]^+$ m/z=4566.3 Da, found: 4566.4 Da.

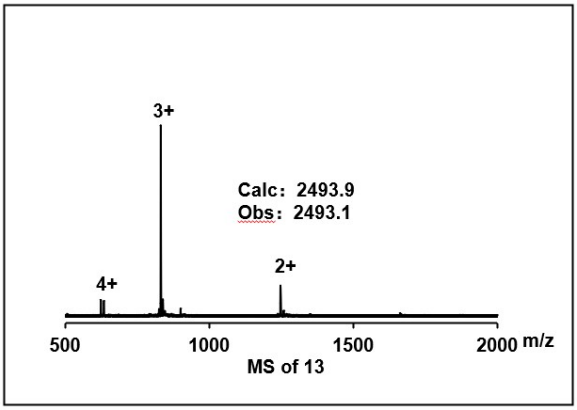


Figure S20. Characterization of peptide 13. MS spectrum of **13**, ESI-MS calculated for **13** $[M]^+$ m/z=2493.9 Da, found: 2493.1 Da.

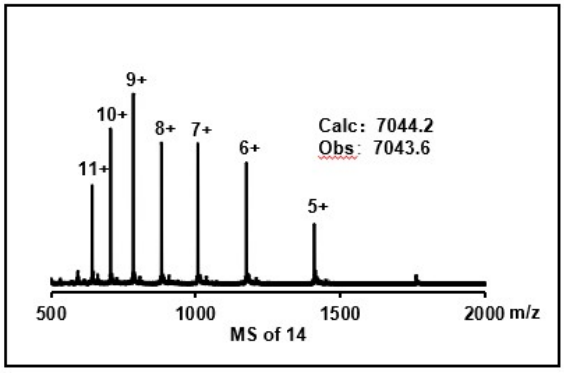


Figure S21. Characterization of peptide 14 (ligation product of 12 and 13). MS spectrum of **14**, ESI-MS calculated for **14** $[M]^+$ m/z=7044.2 Da, found: 7043.6 Da.

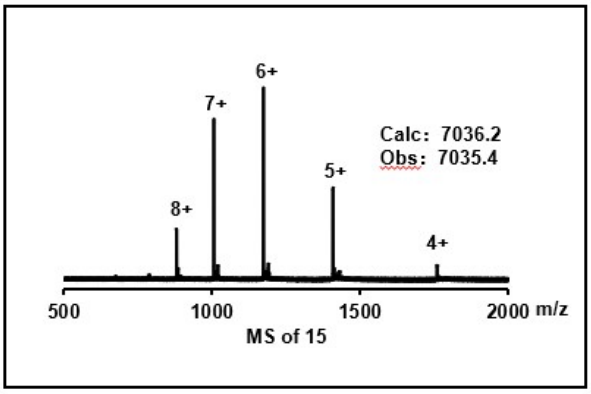
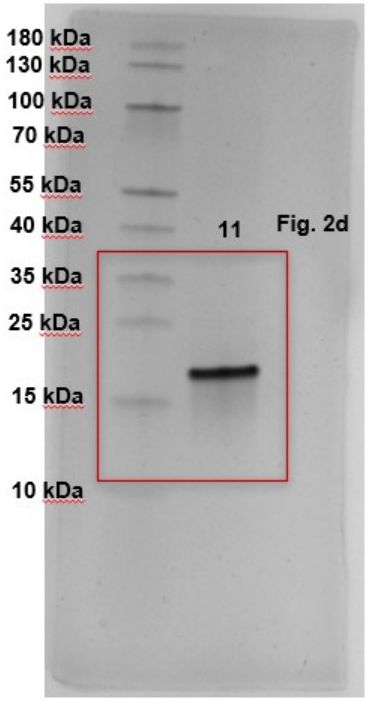
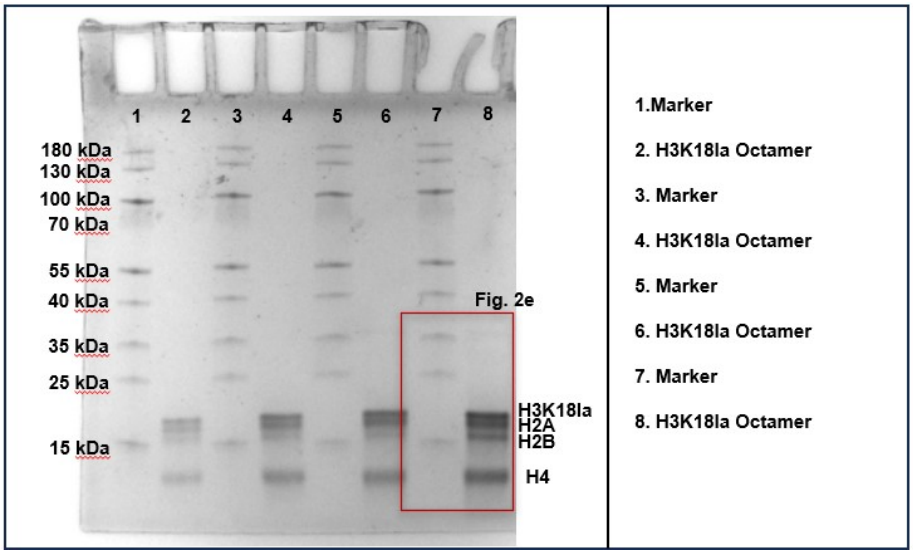


Figure S22. Characterization of peptide 15. MS spectrum of **15**, ESI-MS calculated for **15** $[M]^+$ m/z=7036.2 Da, found: 7035.4 Da.

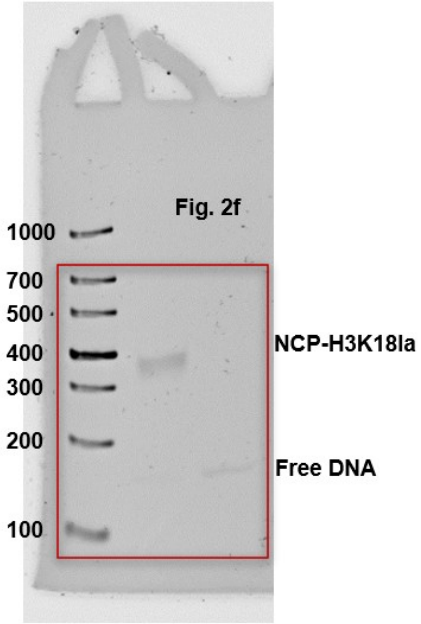
5.Uncropped Gels



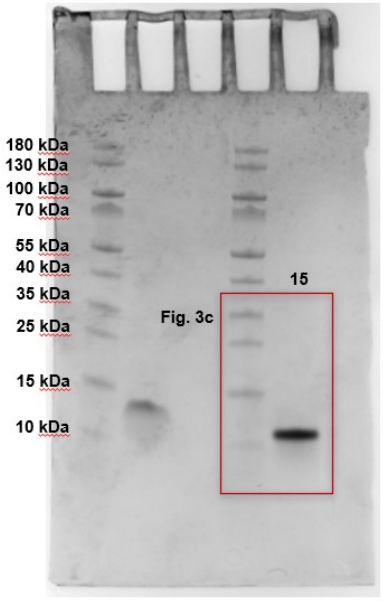
Raw data of Fig. 2d



Raw data of Fig. 2e



Raw data of Fig. 2f



Raw data of Fig. 3c

6. References

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[2] H. Ai, Z. Tong, Z. Deng, et al. *Chem*., 2023, **9**(5), 1221–1240.

[3] C. C. Chen, S. Gao, H. S. Ai, et al. *Sci. Sin. Chim*., 2018, **61**(6), 702-707.

[4] S. Gao, X. Yao, J. Chen, et al. *Cell*., 2023, **186**(24), 5363-5374.