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.

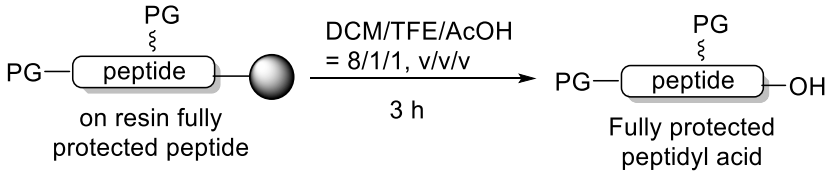
2. General experimental procedures

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.

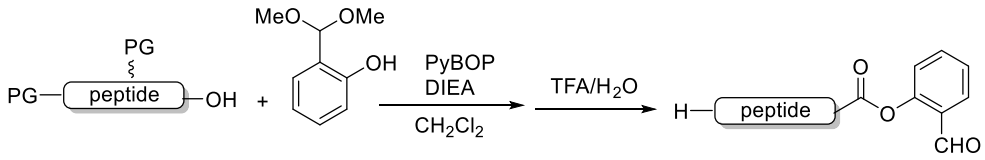
2.2 Cleavage fully protected peptide from 2-chloro-trityl chloride resin



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

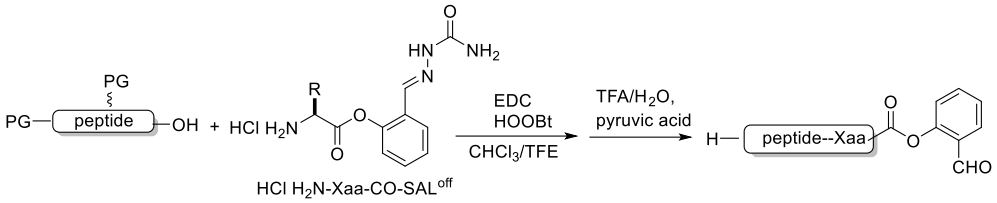
2.3.1 Direct coupling for preparation of C-terminal Gly and Pro peptide SAL esters:



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.

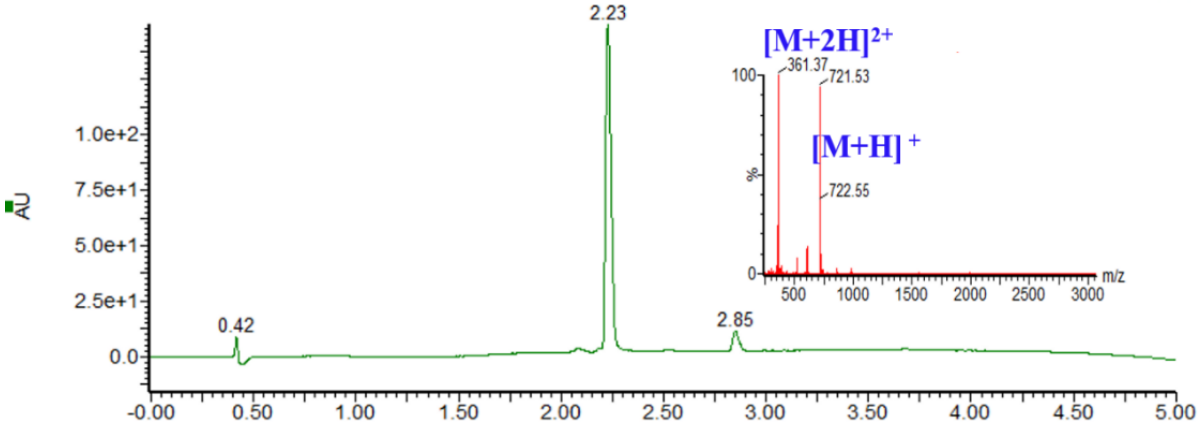
2.3.2 "N+1" strategy for the preparation of C-terminal Ser, Met, Ala, Phe, Val, Leu, Ile, and Thr peptide SAL esters:



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·H_2N-Xaa-CO-SAL^{off} (6.0 equiv.), synthesized according to the procedure[1] 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

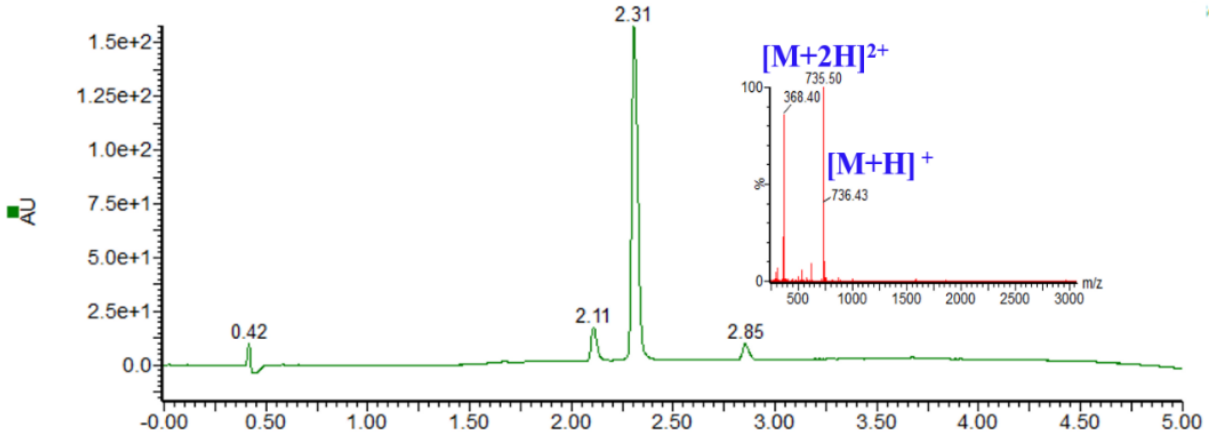
The H-LSQRGG-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1**.



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

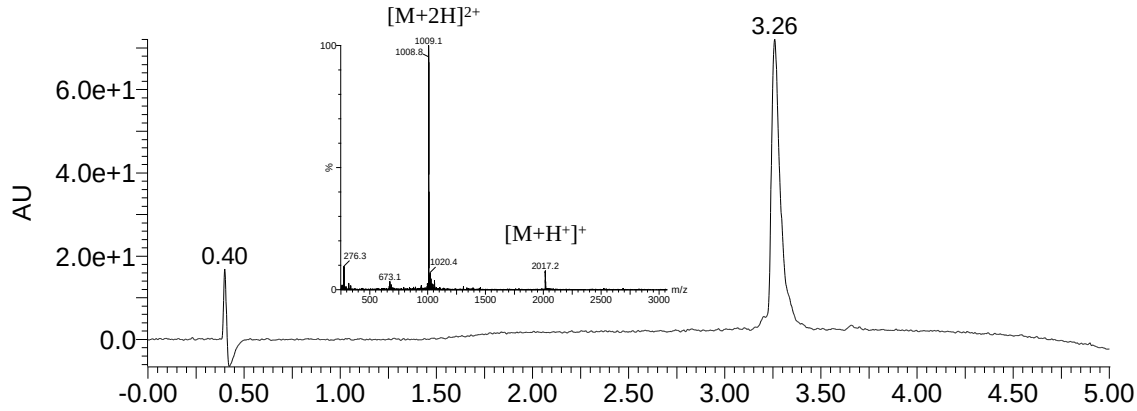
The H-LSQRGA-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2**.



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

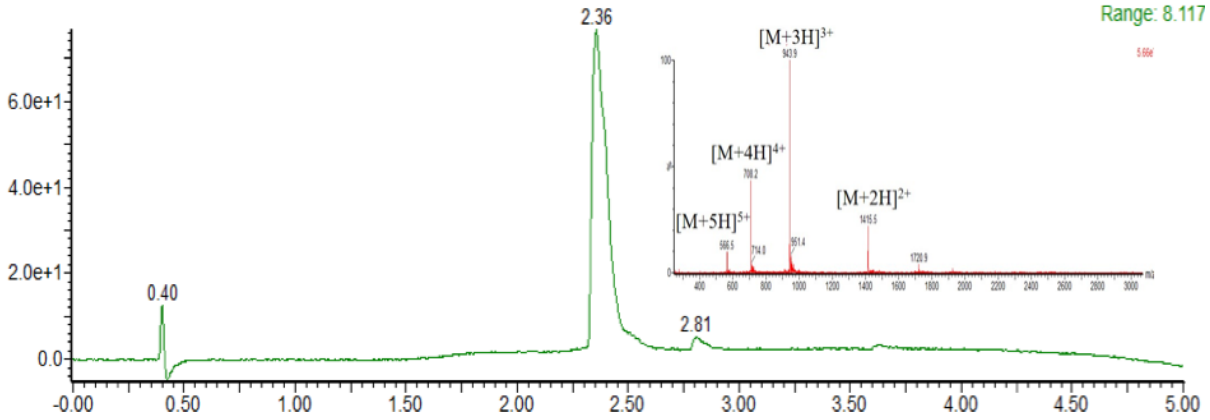
The Ac-ET TTQGPGVLLP LPKGAC(StBu)-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2**.



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.**

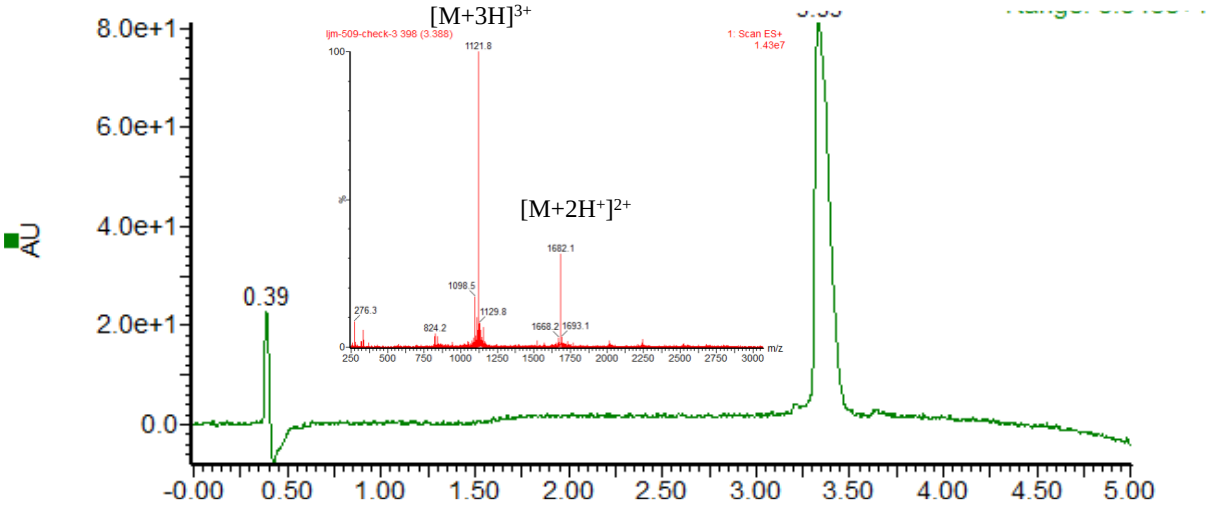


UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. molecular weight: 2828.21. $[M+2H]^{2+}$ m/z = 1415.6, $[M+3H]^{3+}$ m/z = 944.0, $[M+4H]^{4+}$ m/z = 708.3,

$[M+5H]^{5+}$ 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

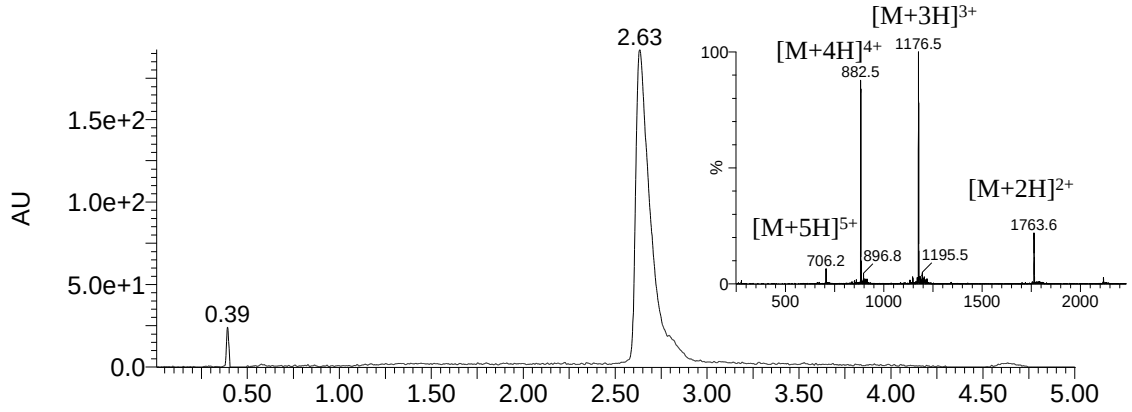
The Fmoc-HN-TLAEAQTETC(Acm)TVAPRERQNC(StBu)GFPGVTP-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**



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

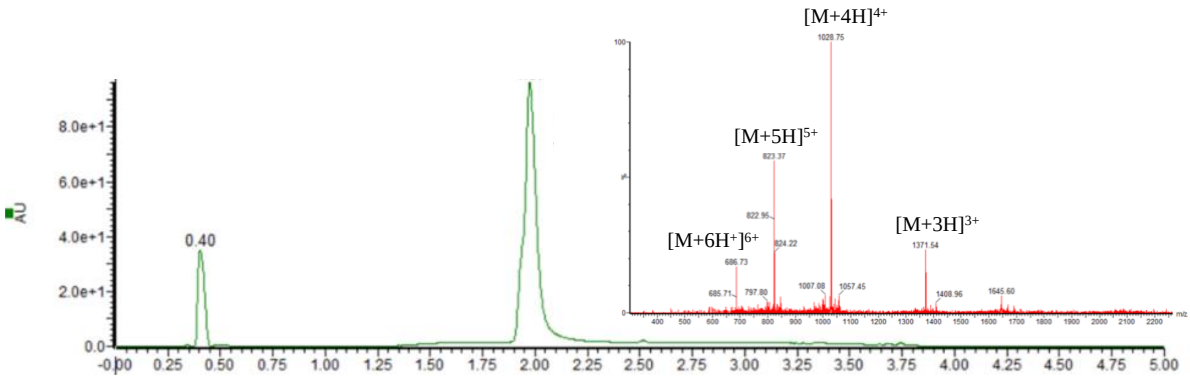
The Fmoc-SEAVLRGQALLVKSSQPWEPLQLHVDKAV-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**



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

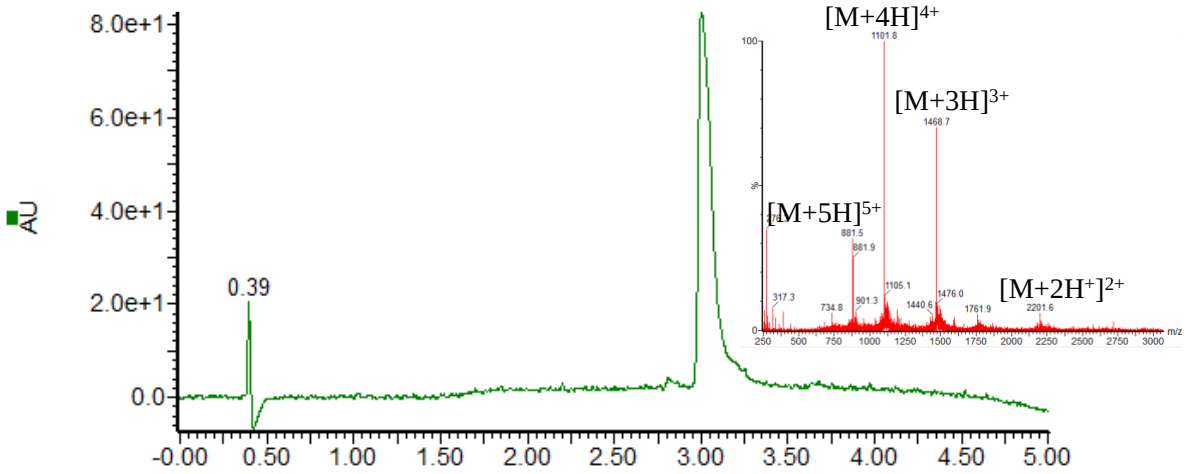
The Ac-SESSSKSSQPLASKQEKDGTEKRGRGRPRKQPPVSPG-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**



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

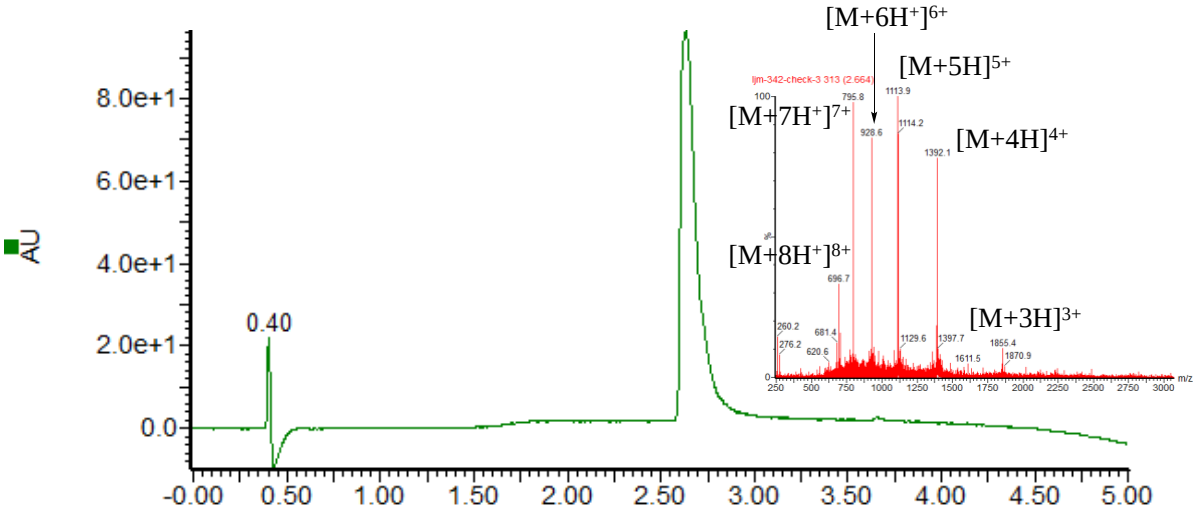
The Fmoc-HN-TGANRDLELPWLEQQGPASHHRRQLGPQGPPHLVADP-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**



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.

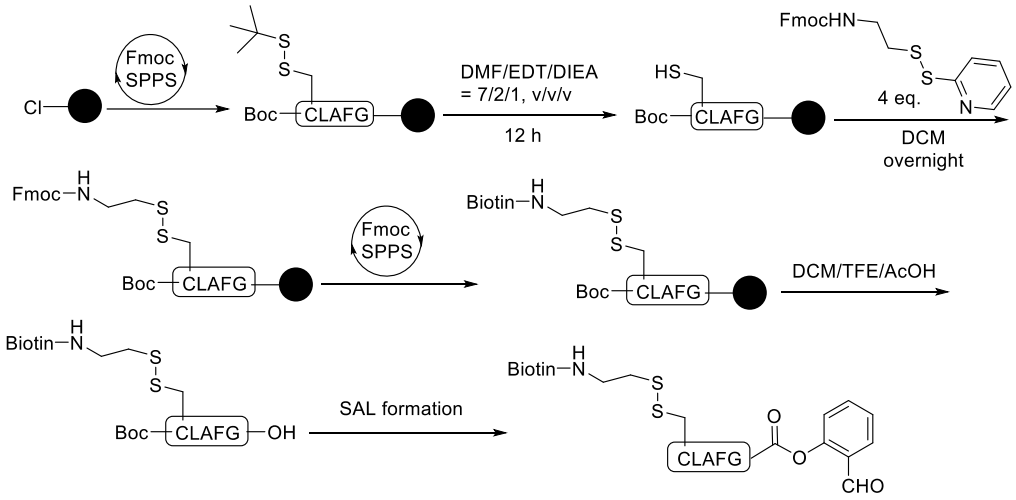
11. Synthesis of Biotin-WRRKRKEKQSETSPKEFLTIYEDVKDLKTRRNHEQEQTF PGGG-CO-SAL ester

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

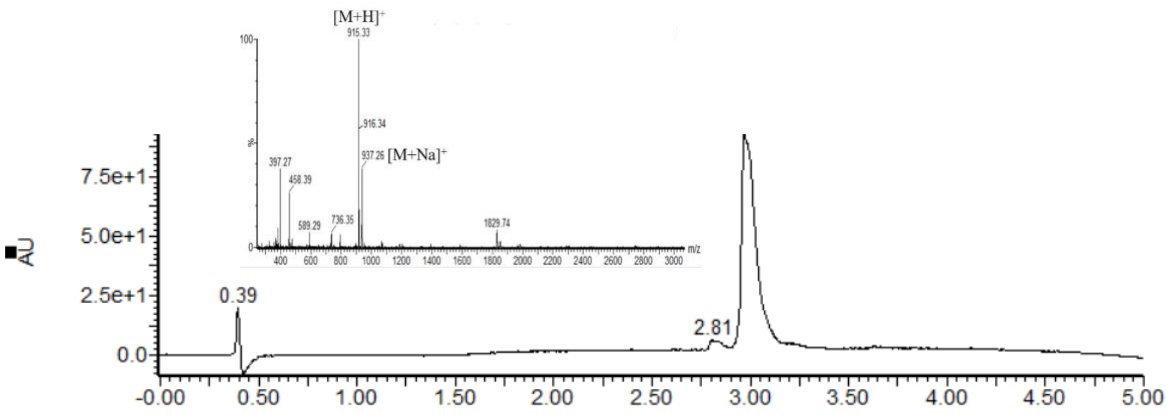


UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. molecular weight: 5565.68. $[M+3H]^{3+}$ m/z = 1856.23, $[M+4H]^{4+}$ m/z = 1392.42, $[M+5H]^{5+}$ m/z = 1114.14, $[M+6H]^{6+}$ m/z = 928.61,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



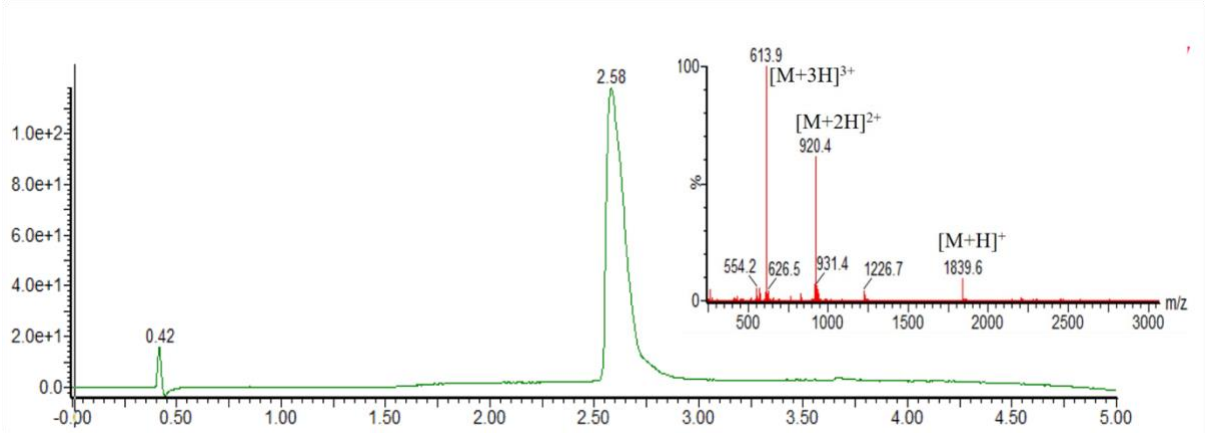
The on resin fully protected peptide obtained using **General Experimental Procedures 2.1** was treated with 5 mL EDT/DIEA/DMF (2/1/7, v/v/v) and shaken for 12 h at room temperature to selectively remove the *tert*-butylthio (StBu) protecting group. Then, the resin was washed with DMF (5 mL × 3) and 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)[2] (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

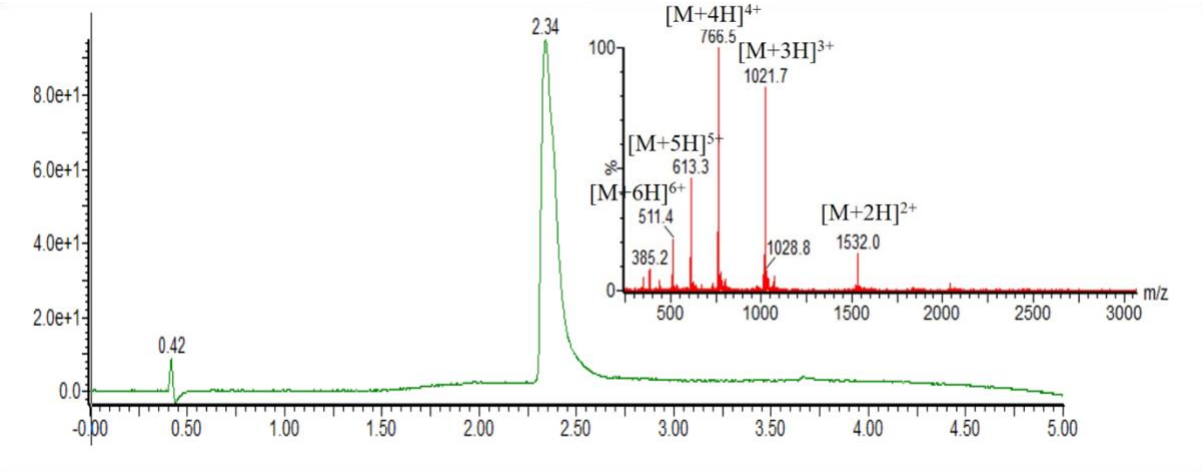
The Ac-HHHHHHENLYFQG-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.1.**



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

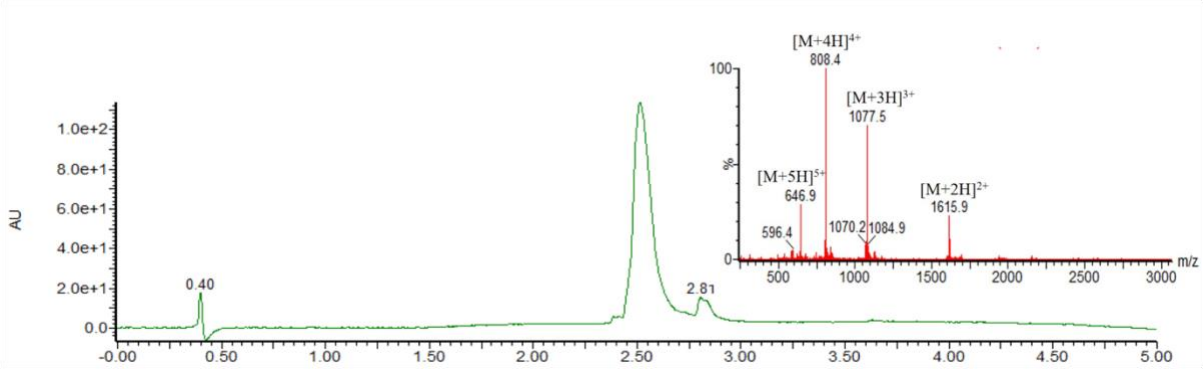
The Ac-HHHHHHENLYFQGKGDPKKPRGKM-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**



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.

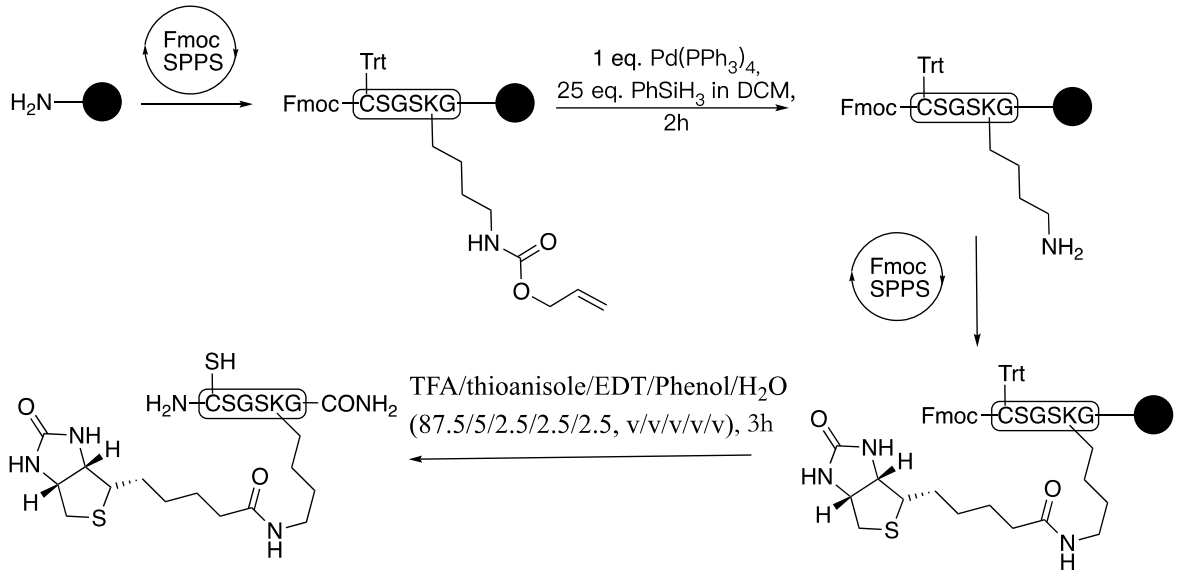
15. Synthesis of Ac-HHHHHHENLYFQGK(ac)GDPK(ac)K(ac)PRGK(ac) M-CO-SAL ester

The Fmoc-Lys(Ac)-OH was synthesized according to the reported procedure.[3] Ac-HHHHHHENLYFQGK(ac)GDPK(ac)K(ac)PRGK(ac)M-CO-SAL was synthesized according to the **General Experimental Procedures 2.3.2.**

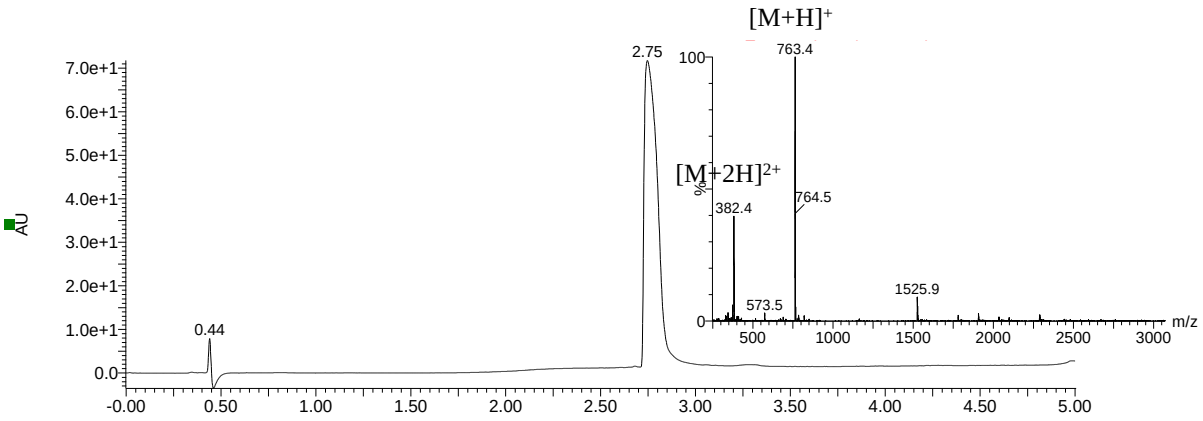


UV trace and corresponding MS from LC-MS analysis of purified product. Gradient: 5-95% ACN/H_2O with 0.1% TFA over 5 min at a flow rate of 0.4 mL/min. ESI-MS calcd. molecular weight: 3230.43. $[M+2H]^{2+}$ m/z = 1616.2, $[M+3H]^{3+}$ m/z = 1077.8, $[M+4H]^{4+}$ m/z = 808.6, $[M+5H]^{5+}$ m/z = 647.1,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

16. Synthesis of H-CSGSK(biotin)G-CONH$_2$



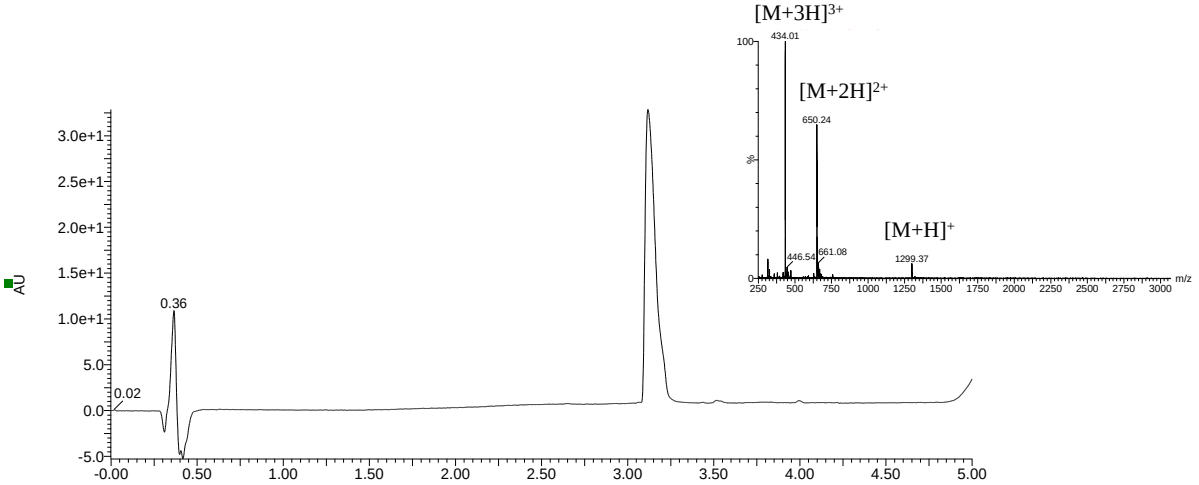
The C(Trt)SGSK(alloc)G peptide was synthesized by CEM synthesizer using rink amide resin. The alloc group was deprotected by treating the resin with 1 equiv. of $Pd(Ph_3P)_4$ and 25 equiv. of $PhSiH_3$ in DCM under argon atmosphere for 2 h. The resin was wash by DCM (5 mL × 6) and DMF (5 mL × 3), and 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

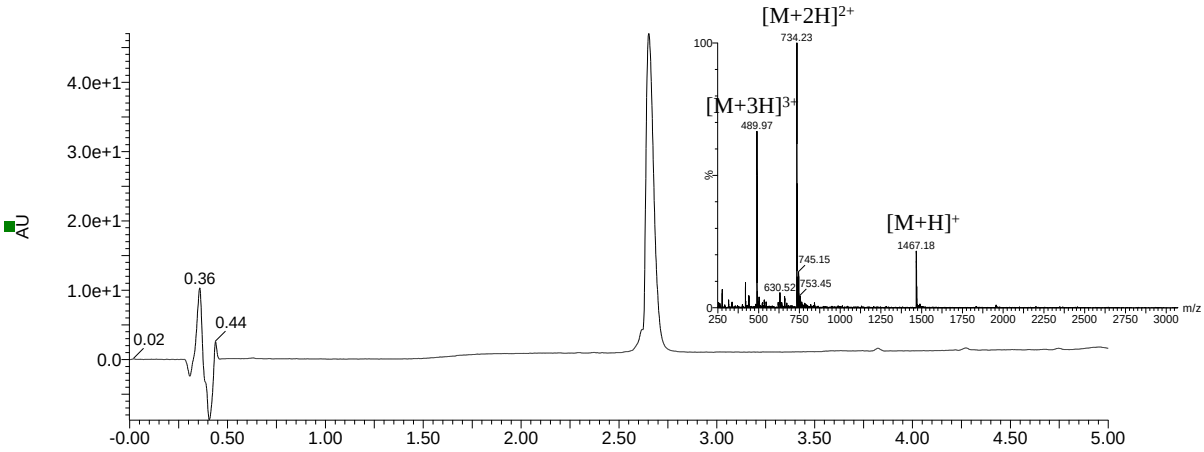
The peptide was synthesized following standard SPPS protocol.



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. 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.

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

3. 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.