Supplementary Information

Synthesis and Frizzled-receptor binding of a WNT5A hairpin-3 peptide

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1. Solvents and Reagents

Solvents and reagents purchased from commercial sources as follows: Fmoc-His(Boc)-OH (CEM, Charlotte, NC, USA); Fmoc-Glu(OtBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ala-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Trp(Boc)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Val-OH, Fmoc-Thr(tBu)-OH, Oxyma Pure, Fmoc-Osu (Sigma-Aldrich, Novabiochem®, Burlington, MA, USA); 1.4-dioxane, N,N-Dimethylformamide (DMF), Piperidine, N,N'-Diisopropylcarbodiimide (DIC), Diethyl ether, Dichloromethane (DCM), Trifluoroacetic acid (TFA), Acetonitrile, Triisopropylsilane (TIS), Tetraethylthiuram disulfide (DSF), 2,2'-Dithiobis(5-nitropyridine) (DTNP), Iodine, Ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA); L-cysteine hydrochloride (EMD Millipore Corp., Burlington, MA, USA); 2-nitrobenzyl bromide (Combi-Blocks, San Diego, CA, USA).

HPLC solvent A is 0.1% TFA in water and B is 90% acetonitrile and 0.1% TFA in water.

2. Synthesis of *N*-Fmoc-*S*-(2-nitrobenzyl)-*L*-cysteine (Fmoc-Cys(Nbz)-OH)[1]

L-cysteine hydrochloride (Sigma-Aldrich, 8.67g, 55 mmol, 1 eq) was dissolved in 58 mL of 2 M NaOH (2 eq) aqueous solution. 2-nitrobenzyl bromide (Sigma-Aldrich, 12.6 g, 58 mmol, 1.05 eq) was dissolved in 50 mL of 1.4-dioxane, then added dropwise to the L-cysteine hydrochloride aqueous solution in N_2 atmosphere. The reaction mixture was stirred at RT for 1.5 hours, giving rise to large quantities of pale-yellow precipitate. The pH of the reaction mixture was then adjusted with 6 M HCl to 6.0 – 6.5. The precipitate was separated by filtration and washed with water, 2-propanol and diethyl ether twice and then lyophilized to afford H-Cys(Nbz)-OH 12.61 g. Yield 78%. MS (ESI^+, m/z): 257.0 $[M+H]^+$.

H-Cys(Nbz)-OH (12.5 g, 48 mmol, 1 eq) was dissolved in 100 mL 1 M $NaHCO_3$ (2 eq). Fmoc-Osu (Sigma-Aldrich, 17.0 g, 50 mmol, 1.05 eq) was dissolved in 1,4-dioxane and added dropwise to the H-Cys(Nbz)-OH solution on the ice-water bath. After 30 min, the reaction was warmed up to 30°C and stirred for another 3 h. The solvent was removed by a rotary evaporator. To the residue was added dichloromethane (DCM) and water, then acidified with 6M HCl to 1. The aqueous phase was washed three times with DCM. The organic phase was combined, washed with saturated NaCl and dried with anhydrous Na_2SO_4. DCM was removed by a rotary evaporator to obtain Fmoc-Cys(Nbz)-OH 21.94g. light yellow solid. Yield 95%. MS (ESI^+, m/z): 479.1 $[M+H]^+$.

3. Solid-Phase Peptide Synthesis (SPPS)

SPPS was performed on Liberty Blue automated peptide synthesizer at 0.25 mmol scale on H-Rink-Amide-ChemMatrix resin (Biotage, 0.41 mmol/g). *N*-(9-fluorenyl)methoxy-carbonyl (Fmoc)-protected amino acids were coupled using DIC and OxymaPure in DMF at 90°C for 110 s. Fmoc deprotection was carried out with 20% (v/v) piperidine in DMF at 90°C for 70 s. The target sequence was cleaved and deprotected with 6 mL cocktail containing TFA: H_2O: TIS = 95: 2.5: 2.5 at room temperature for 2 hours. Crude peptide was precipitated with 45 mL ice-cold diethyl ether, washed (20 mL x 3) with diethyl ether and dissolved in 20 mL of HPLC solvent (A: B = 1:1) and lyophilized. All peptides were purified using C18 RP-HPLC (Discovery®BIO Wide Pore C18-10, 25 cm x 21.2 mm, 10 μm, Cat# 567212-U). The target peptide sequences were summarized in Table S1.

All reactions were monitored by HPLC Column (Agilent Proshell 120 C18, 50 x 2.1 mm, 2.7 μm, 0.5 mL/min) using a gradient in which percentage of solvent Bincreases from 10% to 40% in 15 min. Eluate was analyzed by MSD (G6125, Agilent) for product identification. Product peptides were quantified by absorbance measurement at 280 nm using extinction coefficient of 5500, 1490

and 125 $M^{-1}cm^{-1}$ for each tryptophan, tyrosine and disulfide [2], respectively. High-resolution mass spectrometry (HRMS) experiments were carried out on SCIEX X500B Q-TOF System.

Table S1 Target peptide sequence

Peptide	Sequence*
WNT5A$^{353-371}$ (**5**)	$_{H-}$ERC(Nbz)HCKFHWC(Acm)C(Acm)YVKCKKC(Nbz)T$_{-NH2}$
N-acetyl WNT5A$^{353-371}$ (R354Q/H356Y/K368A) (**6**)	$_{Ac-}$EQC(Nbz)YCKFHWC(Acm)C(Acm)YVKCKAC(Nbz)T$_{-NH2}$
WNT5A$^{353-371}$ F359A (**11**)	$_{H-}$ERC(Nbz)HCKAHWC(Acm)C(Acm)YVKCKKC(Nbz)T$_{-NH2}$
WNT5A$^{353-371}$ W361A (**12**)	$_{H-}$ERC(Nbz)HCKFHAC(Acm)C(Acm)YVKCKKC(Nbz)T$_{-NH2}$
WNT5A$^{353-371}$ V365A (**13**)	$_{H-}$ERC(Nbz)HCKFHWC(Acm)C(Acm)YAKCKKC(Nbz)T$_{-NH2}$
WNT5A$^{356-358}$ (**14**)	$_{H-}$HCKFHWC(Acm)C(Acm)YVKCK$_{-NH2}$
WNT5A$^{353-371}$ all C-to-A (**15**)	$_{H-}$ERAHAKFHWCAAYVKAKKAT$_{-NH2}$

***** Sequence upon TFA cleavage/deprotection. Mutations are highlighted in red.

4. General procedures of triple-disulfide formation for 5, 6, 11, 12 and 13

One-pot formation of first two disulfides: The lyophilized crude peptide was dissolved at 1 mM in 8 M Gn·HCl containing 50 mM phosphate-Na 7.5 (pH in 1M aqueous stock solution prior to dilution) and treated with 10 mM DSF for 30 min at RT [1]. After completion of the first disulfide formation, the pH of the reaction was adjusted to 3 with HCl. The reaction mixture was exposed to 350-nm UV at RT for 10 min and then immediately diluted with four volumes of ice-cold HPLC buffer A: B = 1: 1 (v/v). DTNP was dissolved in TFA and added to 2 mM final concentration to drive the second disulfide bond formation at RT for 30 min. The resulted two-disulfide intermediate with two Acm-protected cysteines was purified using RP-HPLC.

Formation of the third disulfide: To the HPLC eluate containing the intermediate from the previous step was added 2 mM I_2. After reaction at RT for 30 min, I_2 was reduced with an aqueous solution of excess ascorbic acid and the final product was purified with RP-HPLC, lyophilized as colorless powder.

5. Two-disulfide formation of WNT5A$^{356-358}$, 14

The lyophilized crude peptide was dissolved at 1 mM in 8 M Gn·HCl containing 50 mM phosphate-Na 7.5 (pH in 1M aqueous stock solution prior to dilution) and treated with 10 mM DSF for 30 min at RT. After completion of the first disulfide formation, the intermediate with two Acm-protected cysteines was purified using RP-HPLC and treated with 2 mM I_2 in HPLC eluate at rt for 30 min. Upon quenching with excess ascorbic acid, and the final product was purified with RP-HPLC and lyophilized, yielding in colorless powder.

6. WNT5A$^{353-371}$ all C-to-A, 15

The crude peptide was directly HPLC-purified.

7. Proteolysis of N-acetyl WNT5A$^{353-371}$ (R354Q/H356Y/K368A), 6. See also **Fig.3**.

20 μg **6** was dissolved in 100 μL 100 mM Triethylammonium (TEA) bicarbonate buffer (pH = 8.5) and treated with 2 μg of Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA) at 37°C for 1 h. Both fragments **7** and **8** were HPLC-purified, lyophilized, and then dissolved in 50 μL of 100 mM TEA bicarbonate buffer and treated with 1 μg trypsin and 1 μg Sequencing Grade Chymotrypsin (Promega, Madison, WI, USA) at 37°C for 2 hrs. The more hydrophobic fragment **9** was bound to RP-HPLC and isolated.

8. FZD5^{CRD} labeling with Sulfo-Cyanine5 NHS ester

Expression and Purification of FZD5^{CRD}. The cDNA encoding human FZD5$^{30-159}$ was cloned to pHL-sec vector, which contained an N-terminal signal peptide and C-terminal 6xHis tag. 1.2 mg plasmid was mixed with 3.2 mg PEI Max (Polysciences) in 50 ml FreeStyle™ 293 expression medium for 30 min at 25^{o}C and then added to 1.0 L HEK293S GnTI^{-} cells (cell density: 3x10^{6} cell/mL). The infected cells were cultivated at 130 rpm, 8.0% CO_2, 37^{o}C for 72 hrs. Then, the medium was collected, loaded on Ni-NTA and flowed through by gravity. The Ni-NTA was wash with 20 CV wash buffer (20 mM HEPES, 150 mM NaCl, pH 7.5, 20 mM imidazole) and eluted with 5 CV elution buffer (20 mM HEPES, 150 mM NaCl, pH 7.5, 250 mM imidazole) by gravity. The elution sample was concentrated and further purified by SEC (Superdex 200 Increase, 10/300 GL column, Cytiva).

Fluorescent labeling. To 50 μL of 20 μM CRD5 in 50 mM HEPES, 150 mM NaCl buffer (pH = 7.5) was added 50 μM Sulfo-Cyanine5 NHS ester (Lumiprobe, Hunt Valley, MD, USA). The mixture was let react in ice water bath for 30 min in dark. The unreacted Sulfo-Cyanine5 NHS ester was removed by Zeba™ Spin Desalting Columns 7K MWCO (Thermo Scientific, Rockford, IL, USA). The flowthrough (50 μL) contains 15 μM Sulfo-Cyanine5 labeled CRD5 protein.

9. FZD5^{CRD} binding assay using Microscale Thermophoresis (MST)

Sulfo-Cyanine5-labeled FZD5^{CRD} was diluted to 50 nM with the MST buffer containing 50 mM HEPES, 150 mM NaCl and 0.1 mg/mL BSA (pH = 7.5). Peptides were prepared into 2 mM stock solutions in the MST buffer. For each peptide, a series of sixteen samples were prepared with peptide concentrations diluted down from 1 mM, two-fold at a time down to 60 nM for first 15 samples, while the last sample contained 6 nM peptide. All samples contained 25 nM FZD5^{CRD}. Upon incubation at RT for 30 min in dark, each sample was loaded into the standard MST-glass capillary and detected by the Monolith™ NT.115 instrument. LED power and MST power were set at 20% and 60%, respectively. For each sample, the 30 seconds of laser-on time were flanked by two 5-second laser-off period. The normalized fluorescence in phase II of thermophoresis caused by the temperature dependence of fluorescence (T-jump) was used as the source of binding isotherm. At high concentration (1 mM and 0.5 mM), all cyclic peptides cause a surge of T-jump by the same magnitude likely cause by non-specific interactions. We therefore included samples with peptide concentrations from 0.25mM down in each binding isotherm, which was fitted to a single-site binding model in GraphPad Prism 9.

10. SuperTopFlash HEK293 reporter-cell assays [3].

Wnt-signaling inhibition assays were performed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and SuperTopFlash HEK293 (293STF, ATCC) cells. The pRL-SV40 Renilla luciferase (Promega, Madison, WI, USA) was applied as an internal control for transfection efficiency and gene-expression efficiency in each well. Human WNT3A was cloned to pcDNA3.1 vector with a C-terminal GG linker followed by a Strep tag and HA tag. Human FZD5 was cloned to pcDNA3.1 vector with a C-terminal His tag. Human LRP6 (20-1613) was cloned to the pHL-sec vector. The 293STF cells were setup in 24-well plates and transfected using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA). To provide a Wnt-signaling active background, the cells were co-transfected with expression plasmids for Renilla luciferase (20 ng/well), WNT3A (20 ng/well), FZD5 (60 ng/well) and LRP6 (60 ng/well). Hairpin-3 peptides were added at 0, 10, 25 and 65 μM 24 hours post-transfection. The Dual-Luciferase assays were performed at 48 h post-transfection. Data analysis was performed by using GraphPad Prism 9. Results are shown as mean ± SD from three biologically independent experiments.

Supplementary Figures

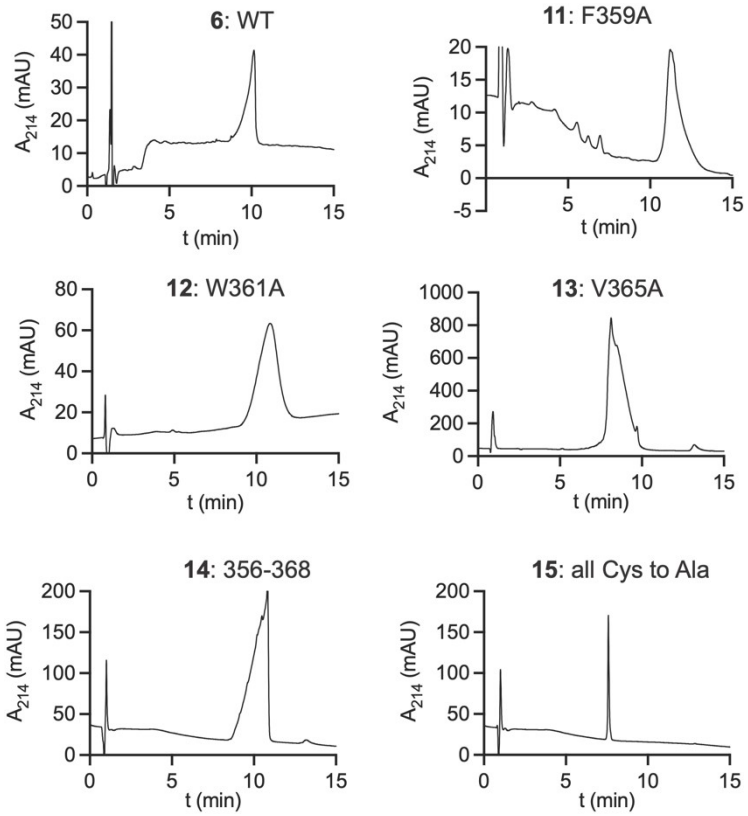


Fig. S1. HPLC-trace of purified **5**, **11**, **12**, **13**, **14** and **15** at 214 nm.

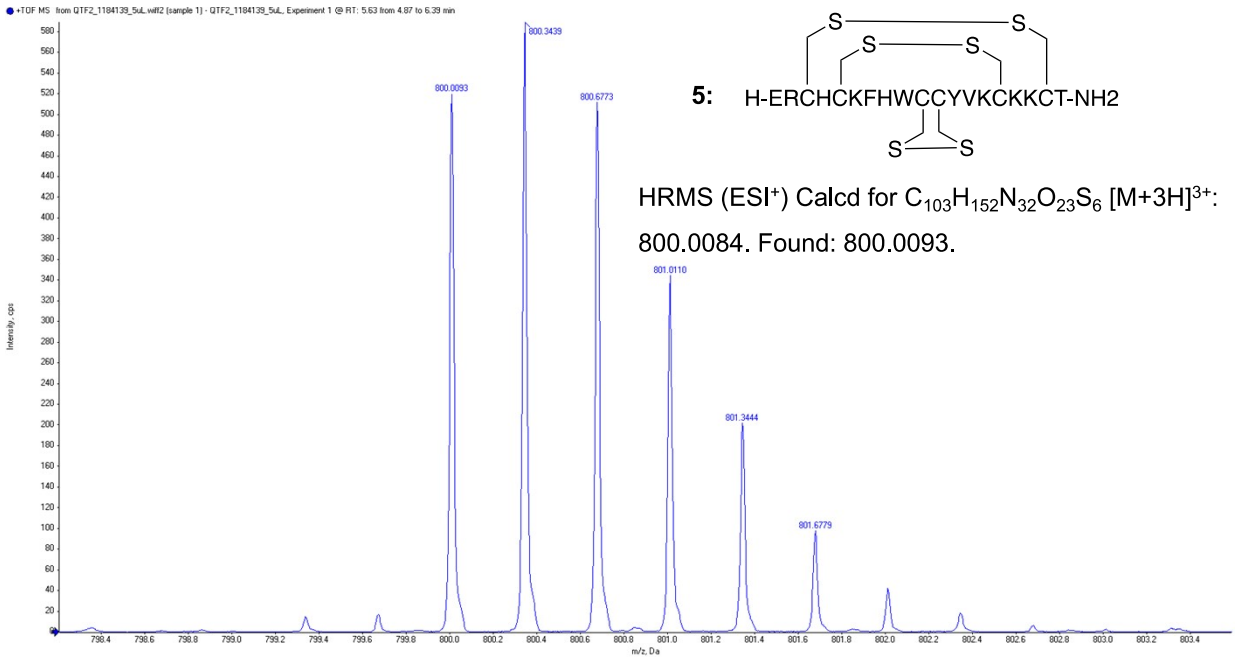


Fig. S2. HRMS spectrum of **5**, zoomed in for a monoisotopic peak at m/z 800.0093.

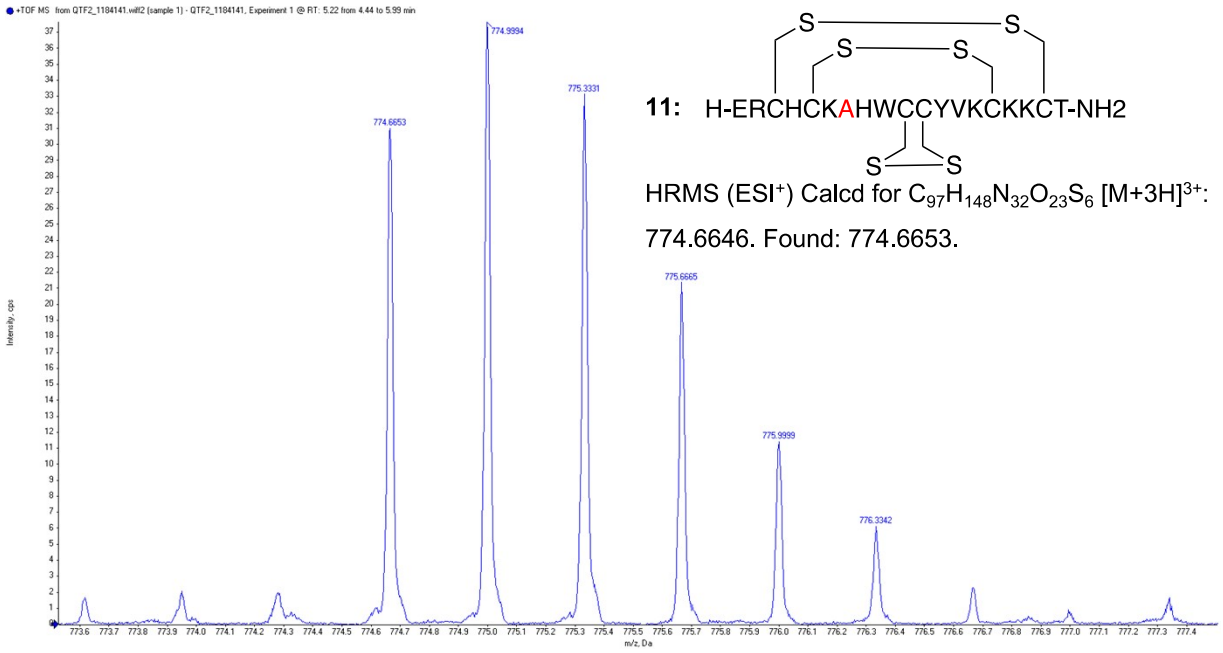


Fig. S3. HRMS spectrum of **11**, zoomed in for a monoisotopic peak at m/z 774.6653.

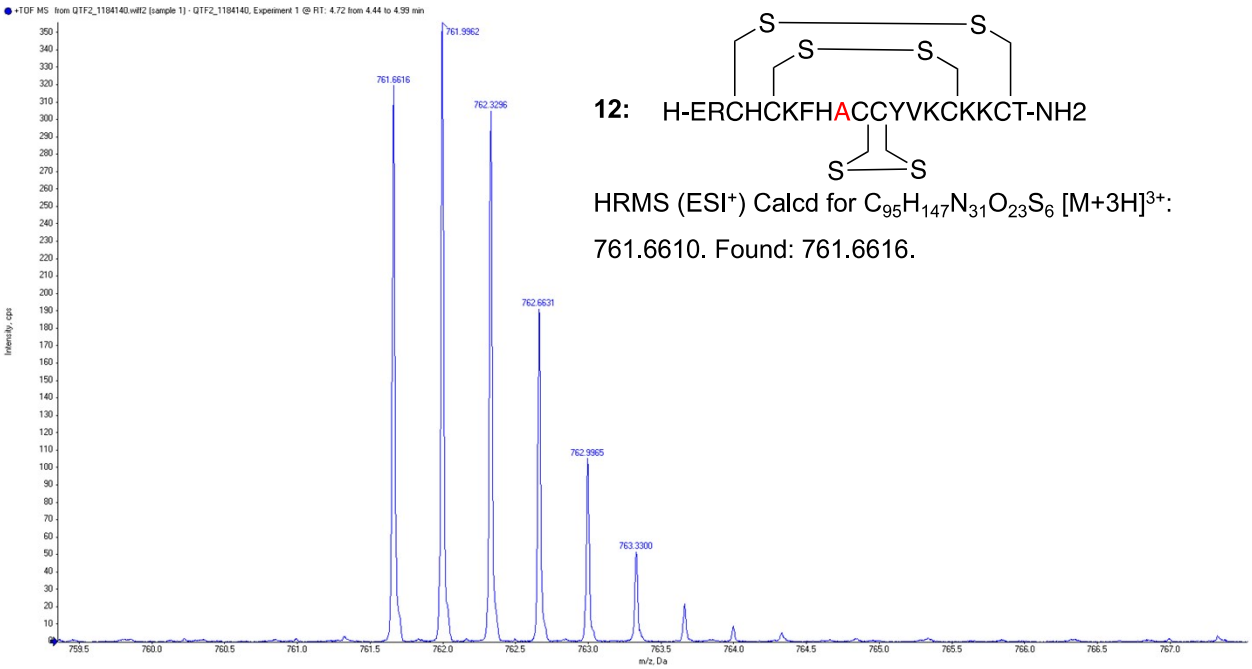


Fig. S4. HRMS spectrum of **12**, zoomed in for a monoisotopic peak at m/z 761.6616.

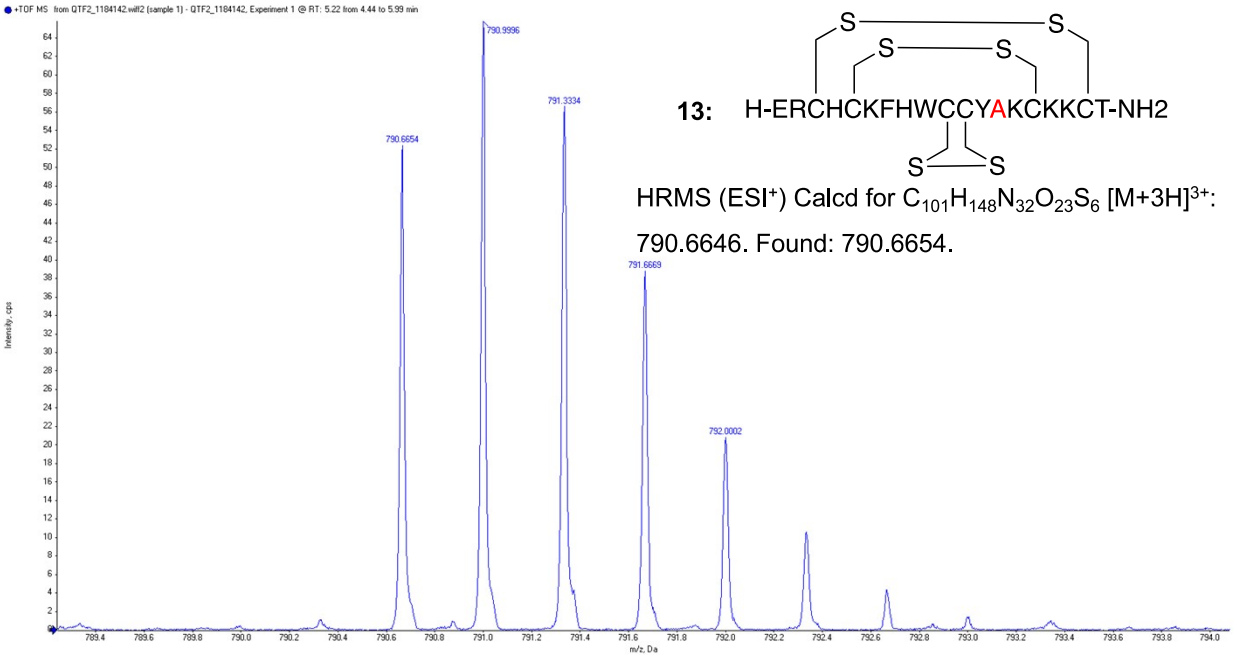


Fig. S5. HRMS spectrum of **13**, zoomed in for a monoisotopic peak at m/z 790.6654.

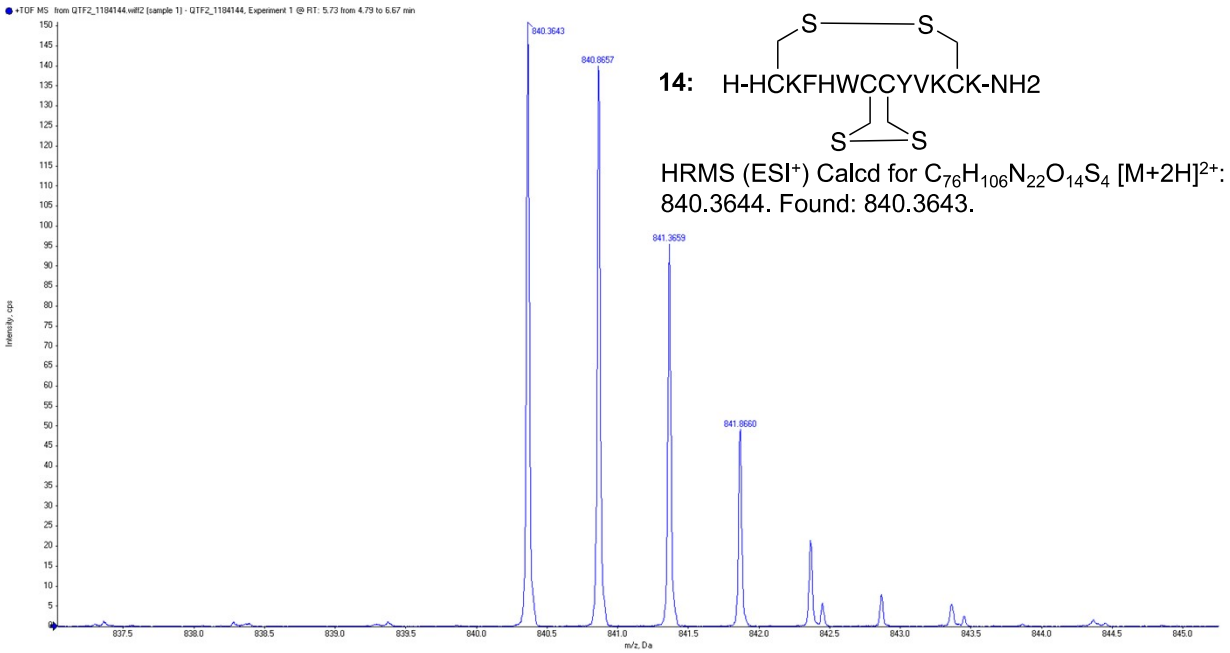


Fig. S6. HRMS spectrum of **14**, zoomed in for a monoisotopic peak at m/z 840.3643.

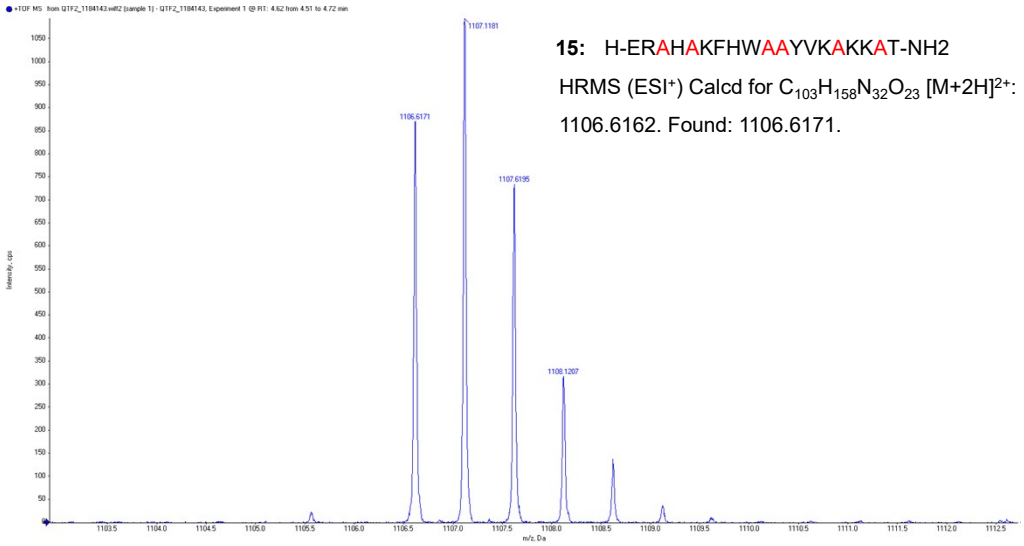
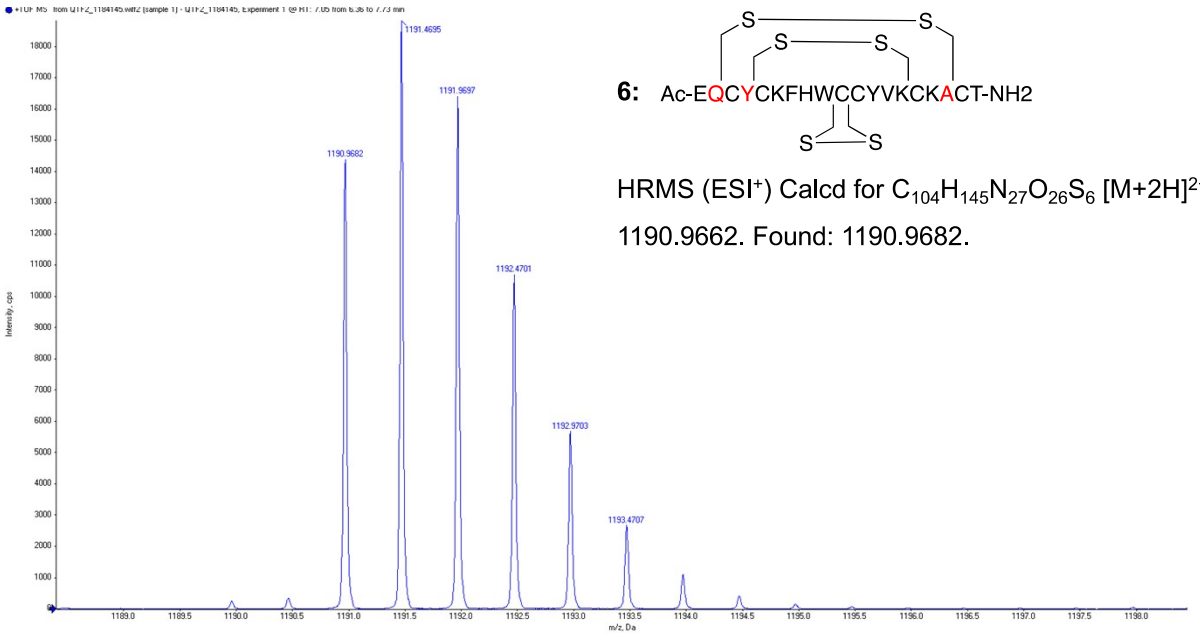


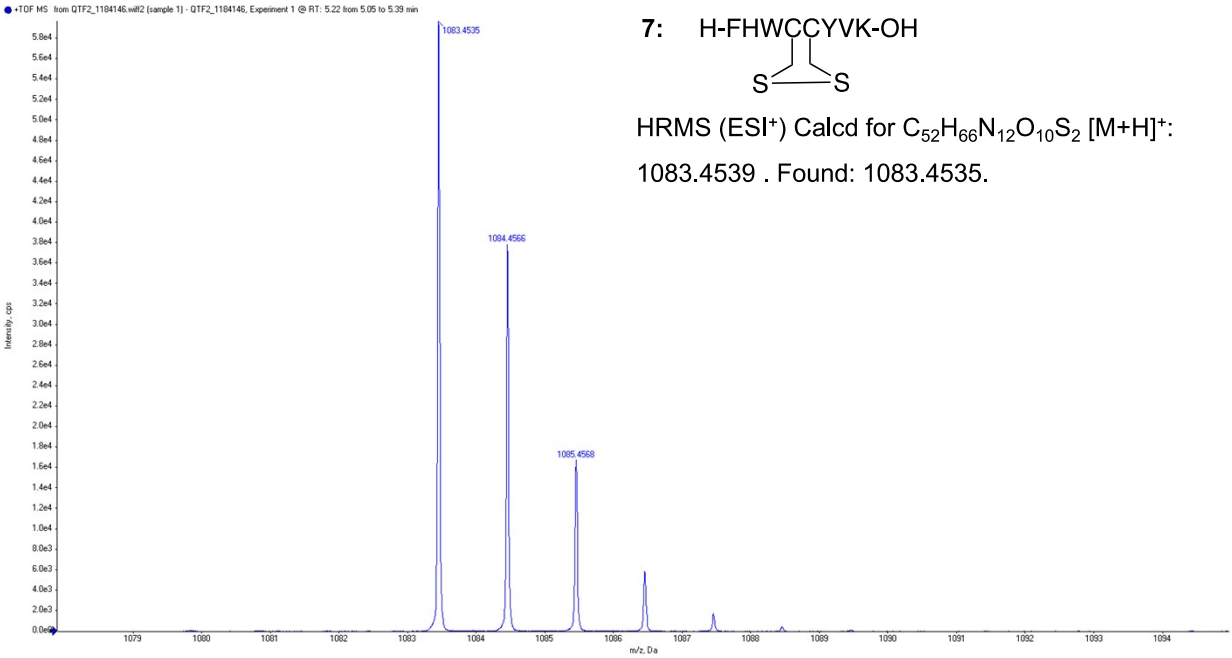
Fig. S7. HRMS spectrum of **15**, zoomed in for a monoisotopic peak at m/z 1106.6171.



6: Ac-EQCYCKFHWCCYVKCKACT-NH2

HRMS (ESI$^+$) Calcd for $C_{104}H_{145}N_{27}O_{26}S_6$ $[M+2H]^{2+}$: 1190.9662. Found: 1190.9682.

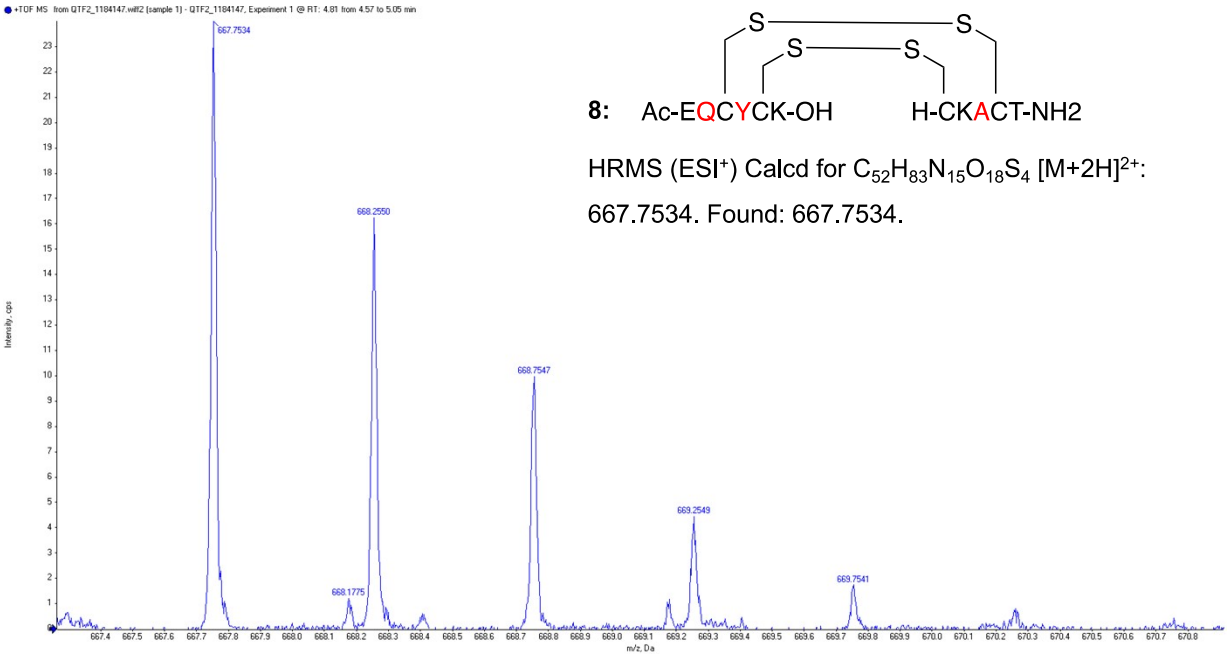
Fig. S8. HRMS spectrum of **6**, zoomed in for a monoisotopic peak at m/z 1190.9682.



7: H-FHWCCYVK-OH

HRMS (ESI$^+$) Calcd for $C_{52}H_{66}N_{12}O_{10}S_2$ $[M+H]^+$: 1083.4539 . Found: 1083.4535.

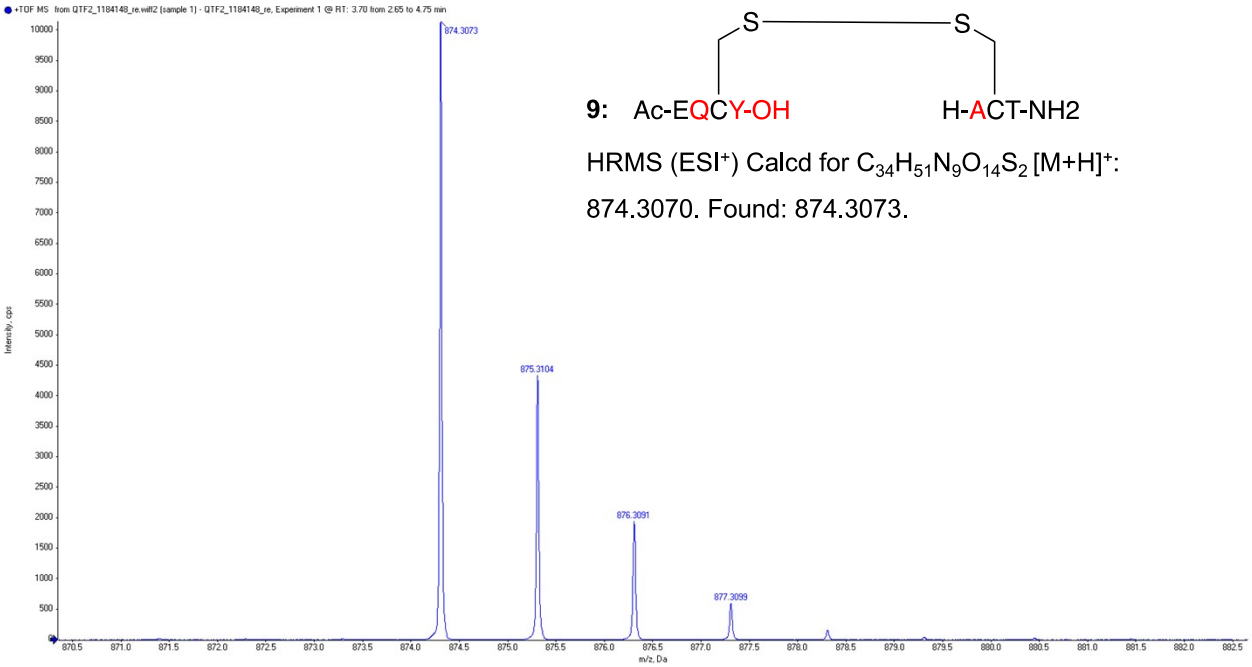
Fig. S9. HRMS spectrum of **7**, zoomed in for a monoisotopic peak at m/z 1083.4535.



8: Ac-EQCYCK-OH H-CKACT-NH2

HRMS (ESI$^+$) Calcd for $C_{52}H_{83}N_{15}O_{18}S_4$ $[M+2H]^{2+}$: 667.7534. Found: 667.7534.

Fig. S10. HRMS spectrum of **8**, zoomed in for a monoisotopic peak at m/z 667.7534.



9: Ac-EQCY-OH H-ACT-NH2

HRMS (ESI$^+$) Calcd for $C_{34}H_{51}N_9O_{14}S_2$ $[M+H]^+$: 874.3070. Found: 874.3073.

Fig. S11. HRMS spectrum of **9**, zoomed in for a monoisotopic peak at m/z 874.3073.

References

1. S. Laps, F. Atamleh, G. Kamnesky, H. Sun and A. Brik, *Nature Communications*, 2021, **12**.
2. M. R. Wilkins, E. Gasteiger, A. Bairoch, J. C. Sanchez, K. L. Williams, R. D. Appel and D. F. Hochstrasser, *Methods Mol Biol*, 1999, **112**, 531-552.
3. Q. Xu, Y. Wang, A. Dabdoub, P. M. Smallwood, J. Williams, C. Woods, M. W. Kelley, L. Jiang, W. Tasman, K. Zhang and J. Nathans, *Cell*, 2004, **116**, 883-895.