Supporting Information

I. General Reagents and Materials

All commercially available chemical reagents, including those from Fisher, ChemPep, PurePep, CHEM-IMPEX, Novabiochem, Oakwood Chemical, Sigma Aldrich, Acros, TCI, and Adamas, were used without further purification. Solvents used in the experiments were either HPLC or reagent grade, sourced from Fisher Chemical and Sigma Aldrich. Ultrapure deionized water was obtained using the Milli-Q IQ 7000 water purification system (Merck, Darmstadt, Germany). 2-Chlorotrityl chloride (2-CTC) resin and Rink amide resin were purchased from ChemPep. Native human insulin was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Before HPLC purification, crude peptides were pre-filtered using a Basix™ 13 mm syringe filter with a 0.45 µm pore size.

II. General Procedures

2.1 9-Fluorenylmethoxycarbonyl Solid-Phase Peptide Synthesis (Fmoc based-SPPS)

Peptide synthesis was carried out using Fmoc-based solid-phase peptide synthesis (SPPS) on a Multiple Synthesizer SYRO I (MultiSynTech GmbH, Witten, Germany) equipped with a vortex stirring system. The synthesis followed a standard protocol: Fmoc groups were removed by treating the resin twice with 20% 4-methylpiperidine in DMF (1.2 mL) for 10 minutes at room temperature. After deprotection, the resin was thoroughly washed with DMF (1.3 mL × 5). Peptide elongation was achieved by coupling Fmoc-protected amino acids (5 equiv.) with HATU (4.8 equiv.) and DIPEA (10 equiv.) in DMF (2.5 mL). Coupling reactions were conducted at 50 °C for cysteine and histidine, while all other amino acids were coupled at 70 °C for 10 minutes. Between each coupling and deprotection cycle, the resin underwent multiple DMF wash steps (1.3 mL × 3) to ensure efficient removal of unreacted reagents.

2.2 Resin Cleavage and Global Deprotection

Before cleavage, the resin was thoroughly washed with DCM (5 mL × 3) and then placed under vacuum for one hour to ensure complete drying. Peptide cleavage and global deprotection were carried out using TFA-based cleavage cocktails (detailed below) for 2.5 hours. Following cleavage, the peptide-containing solution was separated from the resin by filtration, and the peptides were

precipitated by adding the solution dropwise into cold diethyl ether (40 mL). The precipitate was collected by centrifugation at 3000 × g for 3 minutes, after which the supernatant was discarded. The resulting pellet was further washed with diethyl ether (40 mL × 2) to remove residual impurities. The crude peptides were then dried under vacuum for at least 5 minutes, dissolved in a solution of MeCN/H_2O containing 0.1% TFA, analyzed via ESI-MS, and purified using preparative RP-HPLC.

2.3 TFA cleavage cocktails used for different types of peptides

TFA-based cleavage cocktails were prepared based on the peptide composition. For peptides without methionine or cysteine, the cleavage mixture consisted of 3 mL TFA, 75 µL TIPS, and 75 µL water**.** When cysteine was present, but methionine was absent, the cocktail included an additional 75 µL EDT. For peptides containing methionine, the mixture was further supplemented with 45 mg NH_4I. In this case, NH_4I was first placed directly onto the peptide resin before adding the rest of the cleavage solution.

2.4 High-Performance Liquid Chromatography (HPLC)

Purification of all crude peptides and proteins was carried out using an Agilent 1260 HPLC system with a mobile phase consisting of 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B). The system was equipped with Agilent 1260 Infinity Quaternary pumps and a 1260 Infinity II UV detector, with detection wavelengths set at 220 nm, 240 nm, 260 nm and 280 nm.

For HPLC-MS analysis was conducted on Agilent 6120 Quadrupole LC/MS and Agilent G6160A Quadrupole LC/MS, utilizing a Phenomenex C18 column (Jupiter® 50 × 2 mm, 5 µm, 300 Å) or Phenomenex C4 column (Jupiter® 50 × 2 mm, 5 µm, 300 Å) at a flow rate of 0.4 mL/min and Agilent C18 column (Eslipse Plus 100 × 4.6 mm, 3.5 µm). The mobile phase consisted of 0.1% (*v/v*) formic acid in water (solvent A) and 0.1% (*v/v)* formic acid in acetonitrile (solvent B). UV detection was performed at wavelengths of 220 nm, 240 nm, 260 nm and 280 nm, with the column temperature maintained at 40 °C.

For preparative HPLC, separation was conducted on a Phenomenex C18 column (Luna® 250 × 21.2 mm, 5 µm, 100 Å) at a flow rate of 5 mL/min. Semi-preparative purification was carried out using a Phenomenex C18 column (Jupiter® 250 × 10 mm, 5 µm, 300 Å) or Phenomenex C18 column (Luna® 250 × 10 mm, 5 µm, 300 Å) with a flow rate of 3 mL/min. Analytical HPLC was performed using a Phenomenex C18 column (Jupiter® 50 × 4.6 mm, 5 µm, 300 Å) and a Agilent C18 column (Eslipse Plus 100 × 4.6 mm, 3.5 µm) at a flow rate of 0.4 mL/min, UV detection was performed at wavelengths of 220 nm, 240 nm, 260 nm and 280 nm.

2.5 Recombinant Expression of Sortase A

The Sortase A containing five amino acid mutations P94R, D160N, D165A, K190E and K196T was expressed in E. coli following David R. Liu's reported protocol[1]. Generally, Sortase A with a C-terminal His tag was synthesized and cloned into pET29 plasmid expression vector (Genscript). E. coli BL21(DE3) transformed with pET29 sortase A expression plasmids were cultured at 37 °C in LB with 50 µg/mL kanamycin until OD600 = 0.5-0.8. IPTG was added to a final concentration of 0.4 mM and protein expression was induced for three hours at 30 °C. The cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl supplemented with 1 mM $MgCl_2$, 2 units/mL DNAseI (NEB), 260 nM aprotinin, 1.2 µM leupeptin, and 1 mM PMSF). Cells were lysed by sonication and the clarified supernatant was purified on Ni-NTA agarose following the manufacturer's instructions. Fractions that were >95% purity, as judged by SDS-PAGE, were consolidated and dialyzed against Tris-buffered saline (25 mM Tris pH 7.5, 150 mM NaCl). The concentration of Sortase A was determined by Nanodrop (extinction coefficient of 17,420 M^{-1} cm^{-1}) and mixed with 20% glycerol, then stored at -80 °C freezer.

2.6 Cell-Based pAKT Assays

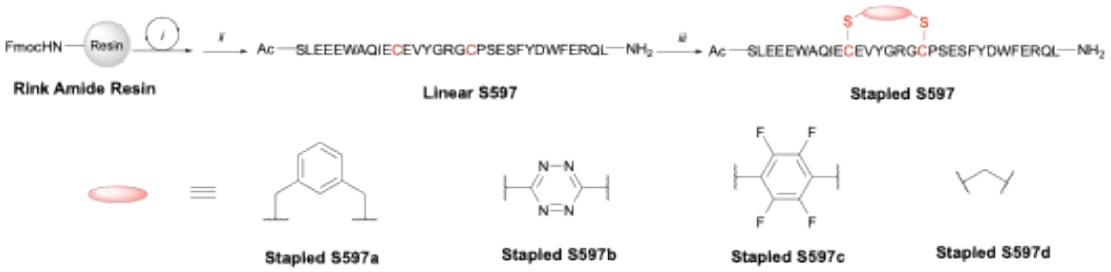
The bioactivities of the insulin derivatives were evaluated through a cell-based pAKT (Ser473) assay. Endogenous levels of pAKT were measured in a human insulin receptor overexpressed NIH 3T3 cell line, derived from IGF-1R knockout mice (a generous gift from A. Morrione, Thomas

[1] I. Chen, B. M. Dorr, D. R. Liu, *Proc. Natl. Acad. Sci.* 2011, **108(28)**, 11399-11404.

Jefferson University). Cells were cultured in DMEM (Sigma) with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin–streptomycin (Gibco), 2 µg/mL puromycin (Gibco), and 0.1 mg/mL normocin (InvivoGen) at 37 °C under 5% CO2. For the assays, 30,000 cells per well and 100 µL per well, were plated in a 96-well plate with culture media containing 1% FBS. 20 h later, the media was removed and 50 µL of culture media with different concentrations of Stapled S597 analogs, Ins-AC derivatives alone (Agonism) or in the presence of 43 nM human insulin (Antagonism) was pipetted into each well. After treatment at 37 °C for 30 min, the solution was removed and the HTRF pAKT Ser473 kit (Revvity) was used to measure the phosphorylation of AKT. Briefly, the cells were first treated with cell lysis buffer (50 µL per well). After mild shaking for 1 h, 16 µL of the cell lysate was added to 4 µL of the detecting reagent in a white 384-well plate. After 4 h of incubation, the plate was read in a SpectraMax iD5 plate reader (Molecular Devices) and the data were processed according to the manufacturer's protocol.

III. Preparation and Characterization of Stapled S597 analogs and Ins-AC and S2 Peptide Conjugates

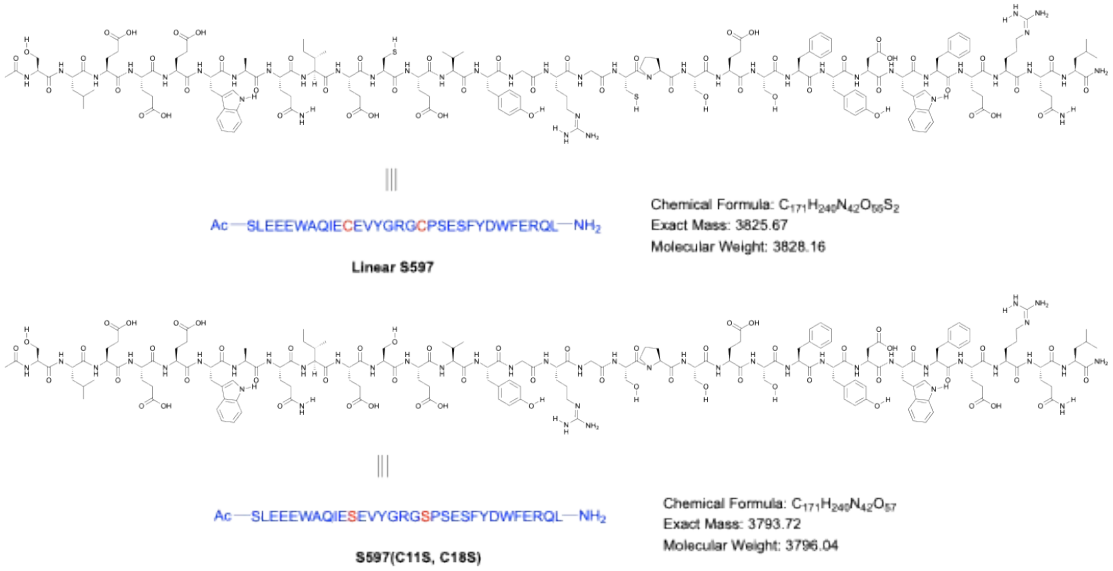
3.1 Synthesis of Stapled S597 Peptides



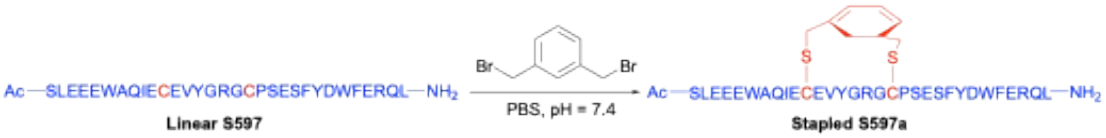
i). Fmoc-based SPPS; ii). 1. Ac_2O: DIPEA: DMF (2: 1: 7, v/v/v); 2. TFA: H_2O: TIPS (95: 2.5: 2.5, v/v/v); iii). Stapling.

Linear S597 and S597(C11S, C18S)

Following the general procedure of automatic Fmoc-based SPPS, linear S597 and S597(C11S, C18S) were synthesized on a 0.1 mmol scale. The crude peptide was cleaved and deprotected under standard condition (TFA:TIPS:H_2O = 95:2.5:2.5, v/v/v), then purified using semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 100 Å, linear gradient 25% to 55% solvent B over 40 min) afforded linear S597 and S597(C11S, C18S) as white powder after lyophilization.

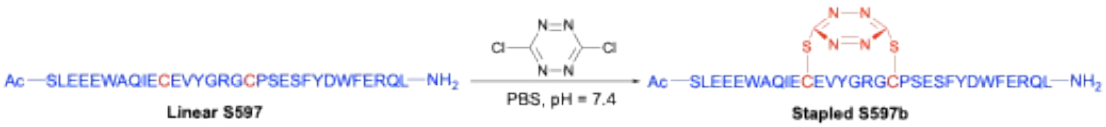


Stapled S597a



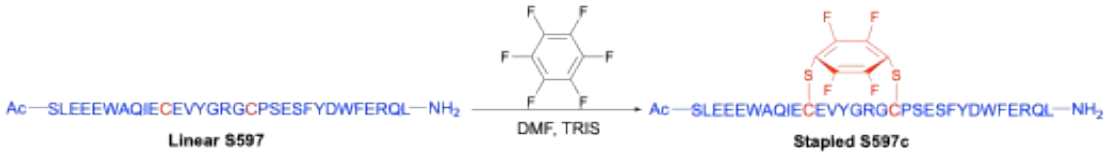
Purified linear S597 (7.6 mg, 2 µmol) was dissolved in PBS at a concentration of 2 mM, while was prepared as a 6.6 mg in 100 µL solution in MeCN. 1,3-Bis(bromomethyl)benzene (24 µL, 3 eq) was then added to the reaction mixture. A TCEP·HCl solution (2 eq) was then added, adjusting the final pH to 7.4. And the reaction was allowed to proceed at room temperature for 1 hour. Finally, the crude peptide purified by semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 100 Å, linear gradient 25-55% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide target product (5.1 mg, 65%).

Stapled S597b



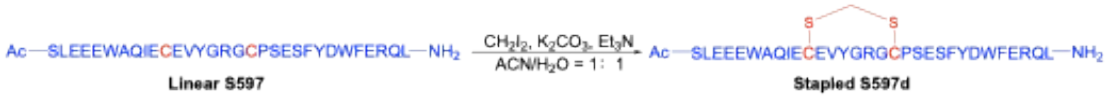
Purified linear S597 (7.6 mg, 2 µmol) was dissolved in PBS at a concentration of 2 mM, while 3,6-Dichloro-1,2,4,5-tetrazine was prepared as a 9 mg in 180 µL solution in MeCN. 3,6-Dichloro-1,2,4,5-tetrazine (60 µL, 10 eq) was then added to the reaction mixture. A TCEP·HCl solution (2 eq) was then added, adjusting the final pH to 7.4. And the reaction was allowed to proceed at room temperature for 1 hour. Finally, the crude peptide purified by semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 100 Å, linear gradient 25-55% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide target product (5.6 mg, 72%).

Stapled S597c



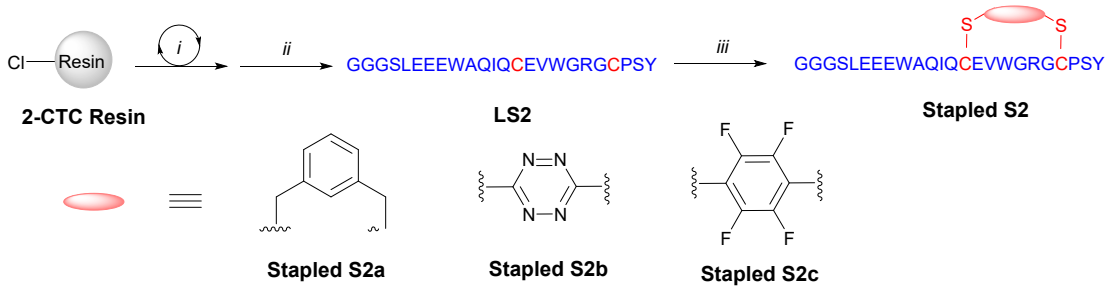
Purified linear S597 (7.6 mg, 2 µmol) was dissolved in DMF at a concentration of 4 mM. while hexafluorobenzene was prepared as a 23 µL in 500 µL DMF. Hexafluorobenzene (125 µL, 25 eq) and 0.4 mL of 50 mM solution of TRIS base in DMF (10 eq) was then added to the reaction mixture. The tube was thoroughly agitated on a shaker for 30 seconds and then allowed to be left at room temperature for 5 hours. Resulting mixture was diluted with 3 mL of 0.1% TFA solution in water and the crude peptide purified by semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 100 Å, linear gradient 25-55% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide target product (2.1 mg, 26%).

Stapled S597d



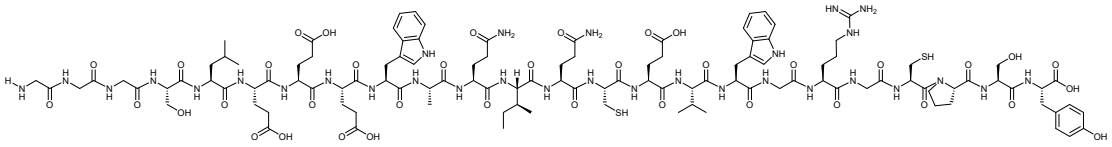
A solution of purified linear S597 (7.6 mg, 2 µmol) in 0.5 mL of H_2O and 0.5 mL of CN was prepared, to which TCEP·HCl (1.1 mg, 2.0 eq) and K_2CO_3 (1.1 mg, 4 eq) were added. The reaction mixture was stirred at room temperature for 30 min. Subsequently, Et_3N (10.0 eq) in THF was introduced, followed by the addition of CH_2I_2 (6 eq) in THF. The reaction was carried out under light protection until the starting material was fully converted, as determined by LC-MS analysis. Finally, the reaction was quenched using 10% AcOH, adjusting the pH to 6.5. Finally, the crude peptide purified by semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 100 Å, linear gradient 25-55% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide target product (2.3 mg, 30%).

3.2 Synthesis of Stapled S2 Peptides



Linear S2 peptide (LS2)

Following the general procedure of automatic Fmoc-based SPPS, linear S2 peptide (**LS2**) was synthesized on a 0.1 mmol scale. The crude peptide was cleaved and deprotected under standard condition (TFA:TIPS:H_2O = 95:2.5:2.5, v/v/v), then purified using semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 100 Å, linear gradient 25% to 38% solvent B over 21 min) afforded linear S2 peptide (**LS2**) as a white powder after lyophilization.

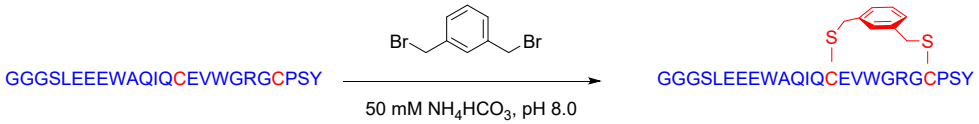


GGGSLEEEWAQIQCEVWGRGCPSY

Chemical Formula: $C_{114}H_{165}N_{31}O_{38}S_2$
Exact Mass: 2640.14
Molecular Weight: 2641.87

Linear S2 (LS2)

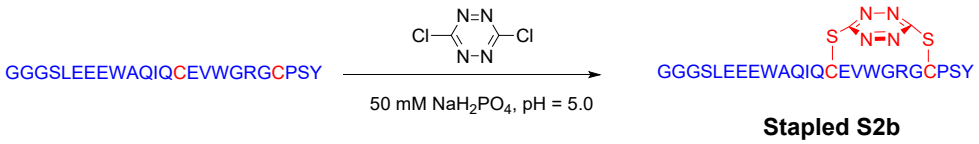
Stapled S2a



Stapled S2a

Linear S2 peptide (**LS2**) was dissolved in 50 mM NH_4HCO_3 (pH 8.0) buffer, at the same time, 1,3-Bis(bromomethyl)benzene was dissolved in MeCN to a solution of 0.1 M, 10 equiv. of 1,3-Bis(bromomethyl)benzene was added to the solution of linear S2 peptide, the resulting mixture was reacted at room temperature and monitored using LC-MS. After 2 hours, the reaction was quenched by a solution of MeCN/H_2O (+ 5% AcOH), then purified using semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 100 Å, linear gradient 26% to 45% solvent B over 18 min) afforded **Stapled S2a** as a white powder after lyophilization.

Stapled S2b

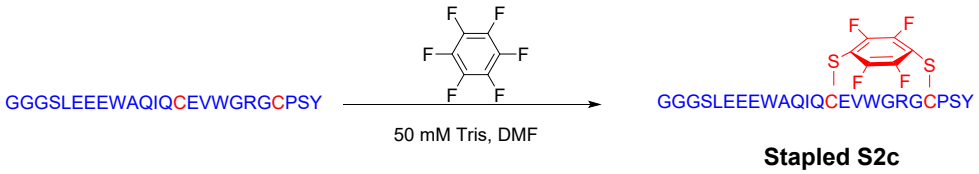


Stapled S2b

Linear S2 peptide (**LS2**) was dissolved in 50 mM NaH_2PO_4 (pH 5.0) buffer, at the same time, Dichloro-1,2,4,5-tetrazine was dissolved in MeCN to a solution of 0.1 M, 1.5 equiv. of Dichloro-1,2,4,5-tetrazine was then added to the solution of linear S2 peptide, the resulting mixture was reacted at room temperature and monitored using LC-MS. After 30 minutes, the reaction was

quenched by a solution of MeCN/H_2O (+ 5% AcOH), then purified using semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 100 Å, linear gradient 25% to 40% solvent B over 27 min) afforded **Stapled S2b** as a white powder after lyophilization.

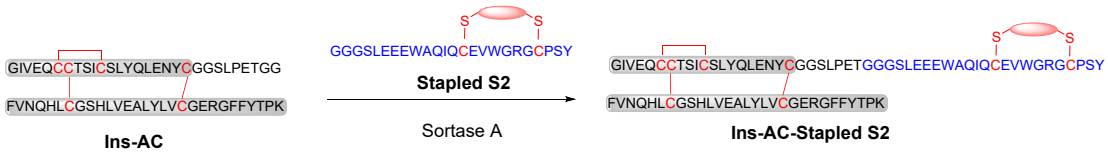
Stapled S2c



Stapled S2c

Linear S2 peptide (**LS2**) was dissolved in 50 mM Tris buffer, at the same time, hexafluorobenzene was dissolved in DMF to a solution of 0.1 M, 10 equiv. of hexafluorobenzene was then added to the solution of linear S2 peptide, the resulting mixture was reacted at room temperature and monitored using LC-MS. After 14 hours, the reaction was quenched by a solution of MeCN/H_2O (+ 5% AcOH), then purified using semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 100 Å, linear gradient 25% to 45% solvent B over 30 min) afforded **Stapled S2c** as a white powder after lyophilization.

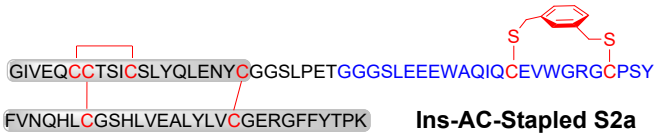
3.3 Sortase A-Mediated Ligation of Ins-AC and Stapled S2 Peptides



Ins-AC **Ins-AC-Stapled S2**

Ins-AC and Stapled S2 were dissolved in reaction buffer (50 mM Tris·HCl, 150 mM NaCl, 5 mM CaCl2, pH 7.5) to a solution of 1 mM independently. Then 2 equiv. of Ins-AC (200 µM) and 1 equiv. of Stapled S2 (100 µM) were mixed in a 15 mL Eppendorf tube, 0.05 equiv. of Sortase A (5 µM) was added and reacted for 5 minutes, the reaction was quenched by a solution of MeCN/H_2O (+ 5% AcOH), and analyzed by LC-MS. The resulting mixture was then purified using semi-preparative HPLC (Proto 300 C4 column, 250 × 10 mm, 10 µm, 100 Å, linear gradient 28% to 40% solvent B over 24 min), the fractions of desired Ins-AC-Stapled S2 was collected and lyophilized to yield the product as a white powder.

Ins-AC-Stapled S2a



Ins-AC-Stapled S2a

Following the general procedure of Sortase A-mediated ligation, 8.2 mg of Ins-AC (1.3 µmol, 2.0 equiv.) and 1.8 mg of Stapled S2a (0.66 µmol, 1.0 equiv.) were mixed and reacted for 5 minutes. The reaction was then quenched by a solution of MeCN/H_2O (+ 5% AcOH), and analyzed by LC-MS. The resulting mixture was purified using semi-preparative HPLC (Proto 300 C4 column, 250 × 10 mm, 10 µm, 100 Å, linear gradient 28% to 40% solvent B over 24 min), the fractions of desired product was collected and lyophilized to yield Ins-AC-Stapled S2a as a white powder (2.3 mg, 39%).

Ins-AC-Stapled S2b

Ins-AC-Stapled S2b

Following the general procedure of Sortase A-mediated ligation, 4.7 mg of Ins-AC (1.3 μmol, 2.0 equiv.) and 1.0 mg of Stapled S2b (0.37 μmol, 1.0 equiv.) were mixed and reacted for 5 minutes. The reaction was then quenched by a solution of MeCN/H_2O (+ 5% AcOH), and analyzed by LC-MS. The resulting mixture was purified using semi-preparative HPLC (Proto 300 C4 column, 250 × 10 mm, 10 μm, 100 Å, linear gradient 28% to 40% solvent B over 24 min), the fractions of desired product was collected and lyophilized to yield Ins-AC-Stapled S2b as a white powder (1.1 mg, 32%).

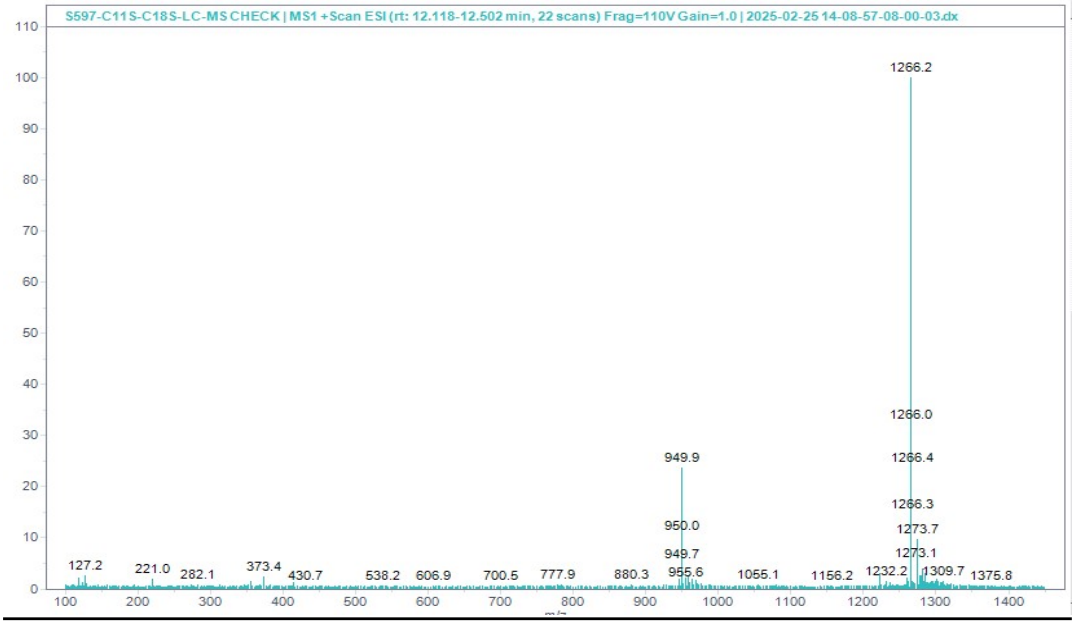
Ins-AC-Stapled S2c

Ins-AC-Stapled S2c

Following the general procedure of Sortase A-mediated ligation, 20 mg of Ins-AC (3.2 μmol, 2.0 equiv.) and 4.4 mg of Stapled S2c (1.6 μmol, 1.0 equiv.) were mixed and reacted for 5 minutes. The reaction was then quenched by a solution of MeCN/H_2O (+ 5% AcOH), and analyzed by LC-MS. The resulting mixture was purified using semi-preparative HPLC (Proto 300 C4 column, 250 × 10 mm, 10 μm, 100 Å, linear gradient 28% to 40% solvent B over 24 min), the fractions of desired product was collected and lyophilized to yield Ins-AC-Stapled S2c as a white powder (6.3 mg, 44%).

IV. LC trace and MS

S597(C11S, C18S)



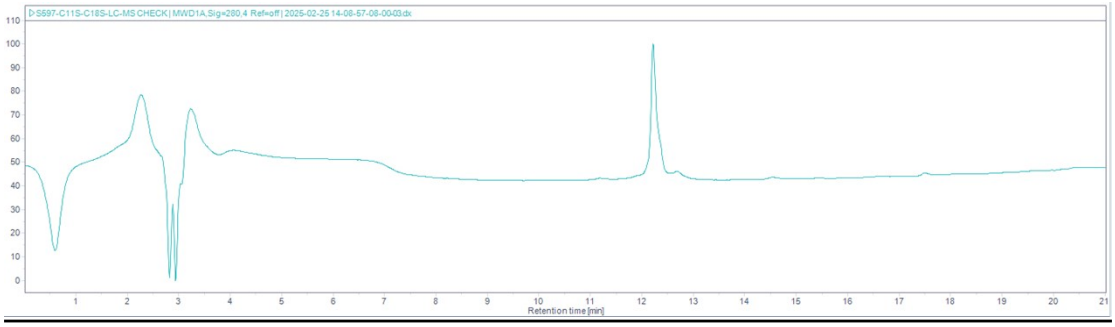


Figure **S1**. Top: UV traces of the purified **S597(C11S, C18S)** at 220 nm using Agilent C18 column (Eslipse Plus 100 × 4.6 mm, 3.5 µm) column; a linear gradient of 5–95% solvent B over 15 min. Bottom: ESI-MS data of the purified **S597(C11S, C18S)**. Calc. for $C_{171}H_{240}N_{42}O_{57}$: 3796.04 Da (average isotopes), (m/z) $[M+3H]^{3+}$:1266.3, $[M+4H]^{2+}$: 950.1; found $[M+3H]^{3+}$:1266.2; $[M+4H]^{4+}$:949.9.

Stapled S597a

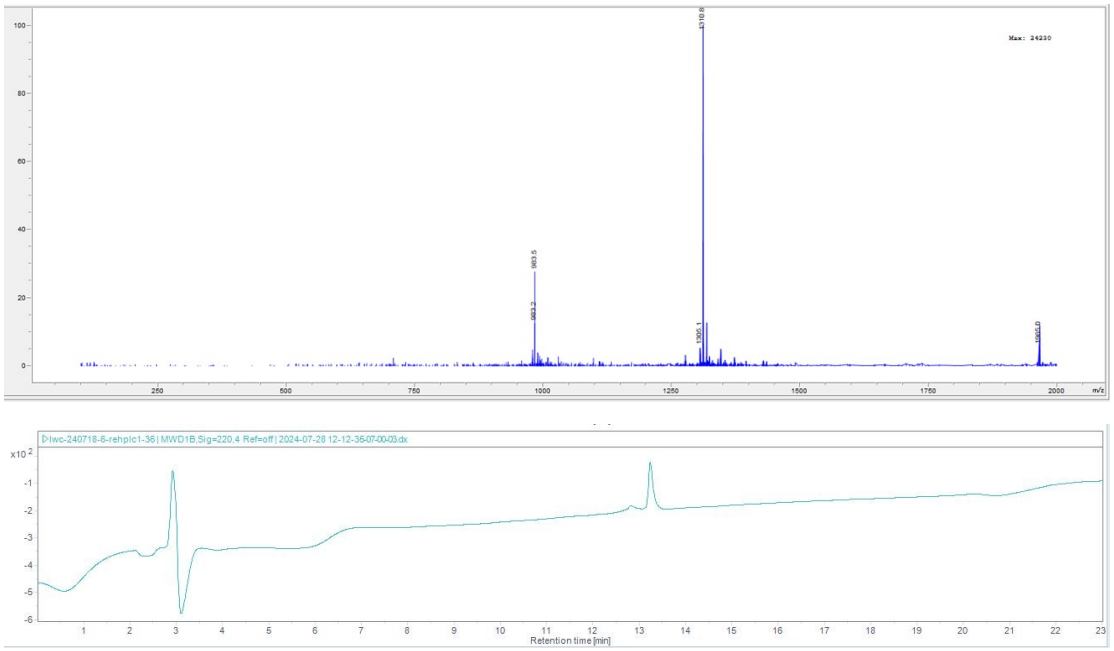


Figure **S2**. Top: UV traces of the purified **Stapled S597a** at 220 nm using Agilent C18 column (Eslipse Plus 100 × 4.6 mm, 3.5 µm) column; a linear gradient of 5–95% solvent B over 15 min. Bottom: ESI-MS data of the purified **Stapled S597a**. Calc. for $C_{179}H_{246}N_{42}O_{55}S_2$: 3930.30 Da (average isotopes), (m/z) $[M+2H]^{2+}$:1966.15, $[M+3H]^{3+}$:1311.1; $[M+4H]^{4+}$:984.6, found $[M+2H]^{2+}$: 1965.0, $[M+3H]^{3+}$:1310.8; $[M+4H]^{4+}$:984.5.

Stapled S597b

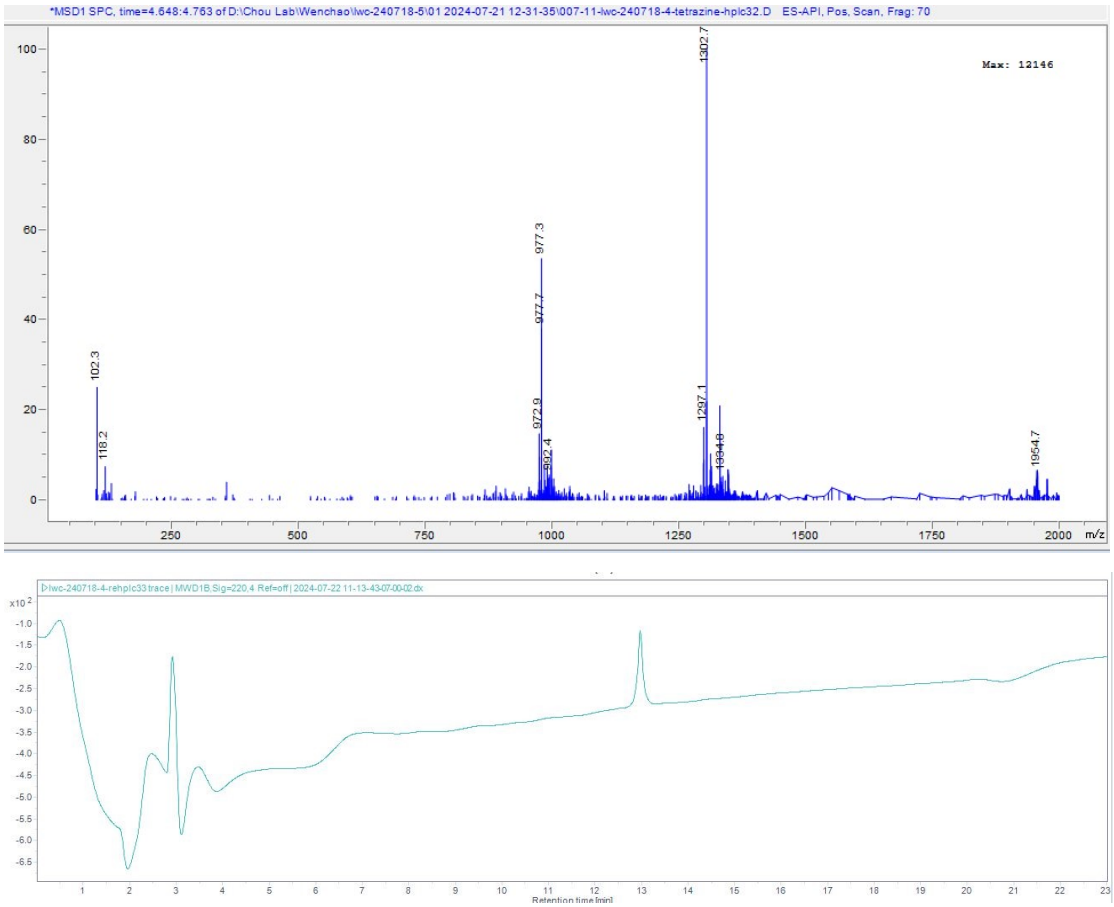
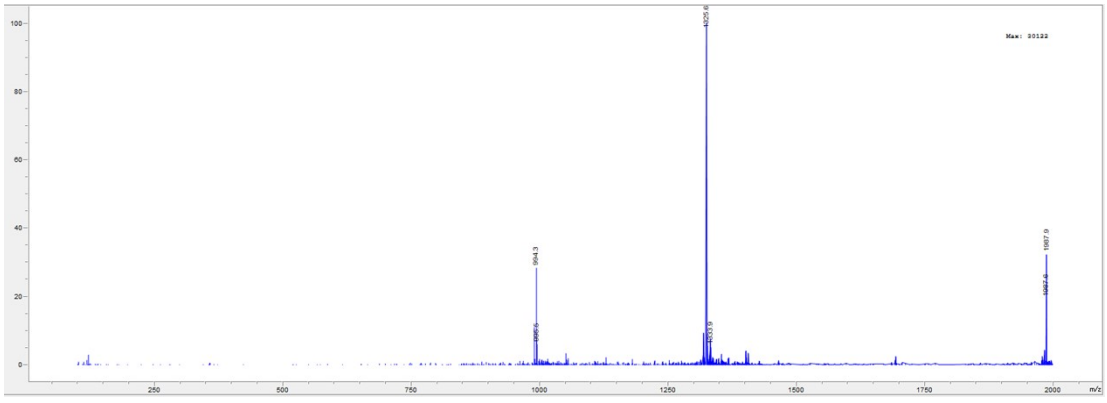


Figure **S3**. Top: UV traces of the purified **Stapled S597b** at 220 nm using Agilent C18 column (Eslipse Plus 100 × 4.6 mm, 3.5 µm) column; a linear gradient of 5–95% solvent B over 15 min. Bottom: ESI-MS data of the purified **Stapled S597b**. Calc. for $C_{173}H_{238}N_{46}O_{55}S_2$: 3906.19 Da (average isotopes), (m/z) $[M+2H]^{2+}$:1954.1, $[M+3H]^{3+}$:1303.1; $[M+4H]^{4+}$:977.5, found $[M+2H]^{2+}$: 1954.7, $[M+3H]^{3+}$:1302.7; $[M+4H]^{4+}$:977.3.

Stapled S597c



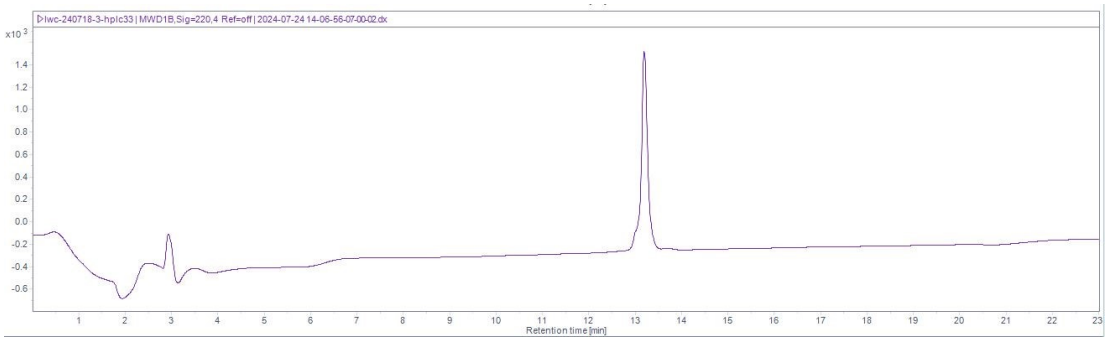


Figure **S4**. Top: UV traces of the purified **Stapled S597c** at 220 nm using Agilent C18 column (Eslipse Plus 100 × 4.6 mm, 3.5 µm) column; a linear gradient of 5–95% solvent B over 15 min. Bottom: ESI-MS data of the purified **Stapled S597c**. Calc. for $C_{177}H_{238}F_4N_{42}O_{55}S_2$: 3974.20 Da (average isotopes), (m/z) $[M+2H]^{2+}$:1988.1, $[M+3H]^{3+}$:1325.7; $[M+4H]^{4+}$:994.6, found $[M+2H]^{2+}$: 1987.9, $[M+3H]^{3+}$:1325.6; $[M+4H]^{4+}$:994.3.

Stapled S597d

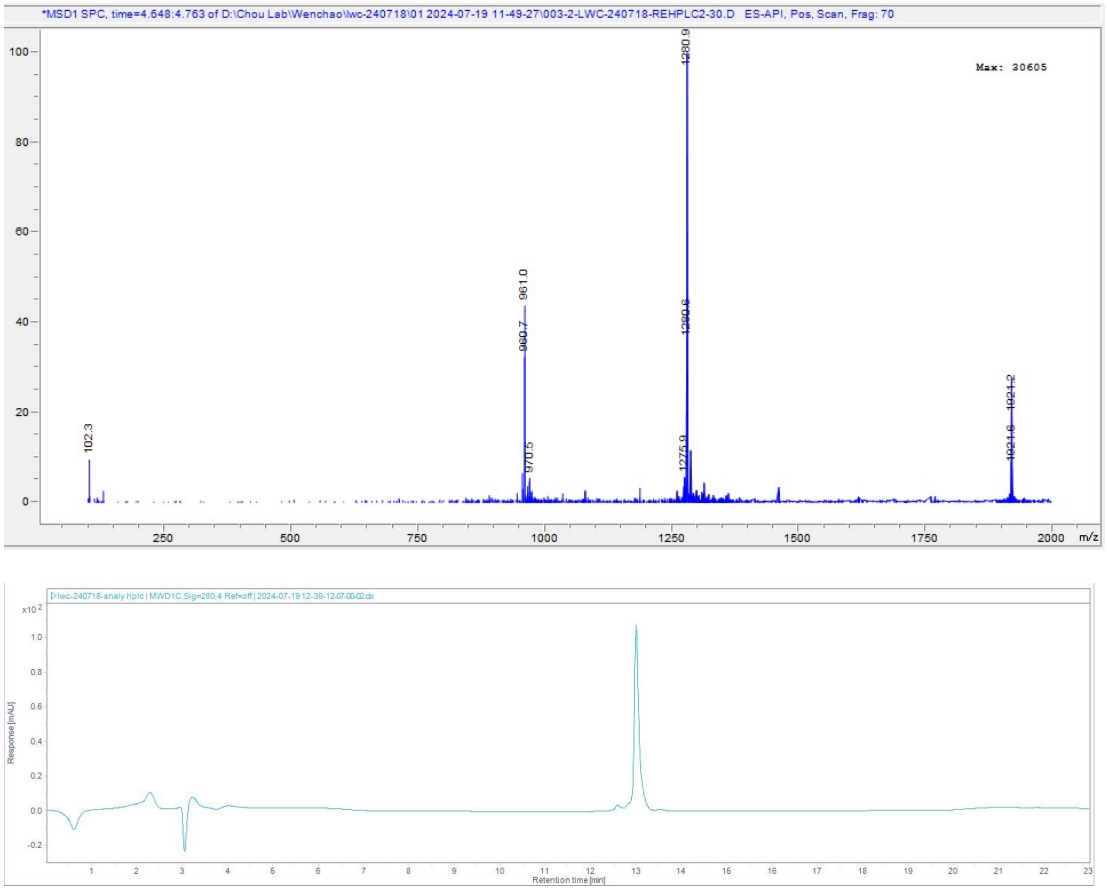


Figure **S5**. Top: UV traces of the purified **Stapled S597d** at 220 nm using Agilent C18 column (Eslipse Plus 100 × 4.6 mm, 3.5 µm) column; a linear gradient of 5–95% solvent B over 15 min. Bottom: ESI-MS data of the purified **Stapled S597d**. Calc. for $C_{172}H_{240}N_{42}O_{55}S_2$: 3840.17 Da (average isotopes), (m/z) $[M+2H]^{2+}$:1921.1, $[M+3H]^{3+}$:1281.1; $[M+4H]^{4+}$:961.0, found $[M+2H]^{2+}$: 1921.2, $[M+3H]^{3+}$:1280.9; $[M+4H]^{4+}$:961.0.

Linear S2 peptide (LS2)

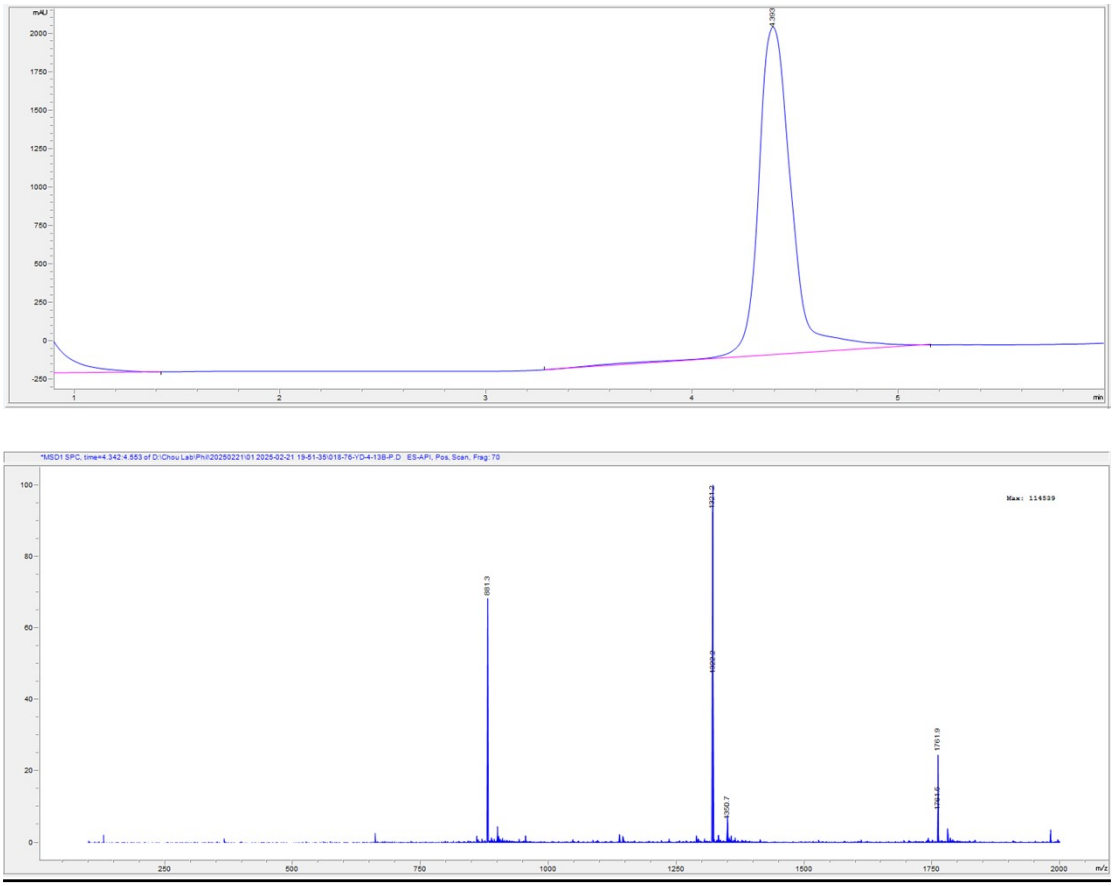
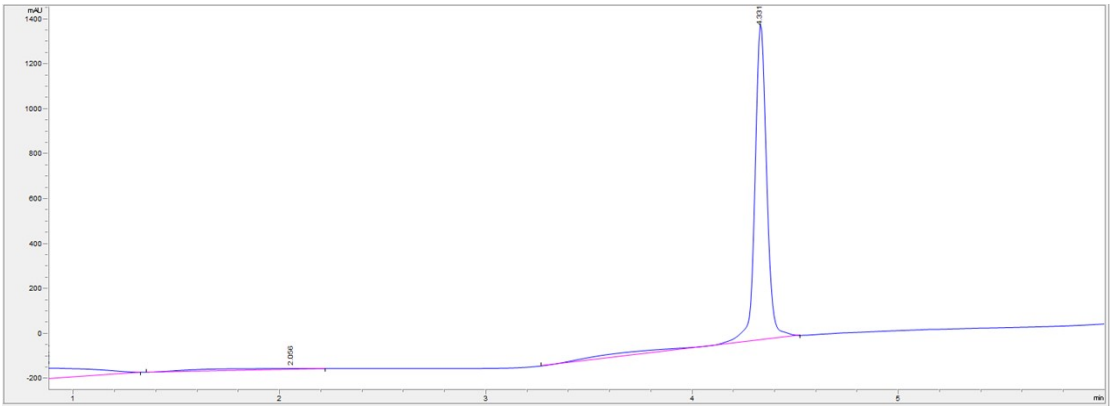


Figure **S6**. Top: UV traces of the purified linear S2 peptide (**LS2**) at 220 nm using Luna® 5 µm C18 100 Å (50 × 2 mm) column; Bottom: ESI-MS data of the purified linear S2 Peptide (**LS2**). Calc. for $C_{114}H_{165}N_{31}O_{38}S_2$: 2641.87 Da (average isotopes), (m/z) $[M+2H]^{2+}$:1321.6, $[M+3H]^{3+}$:881.4; found $[M+2H]^{2+}$: 1321.2, $[M+3H]^{3+}$:881.3.

Stapled S2a



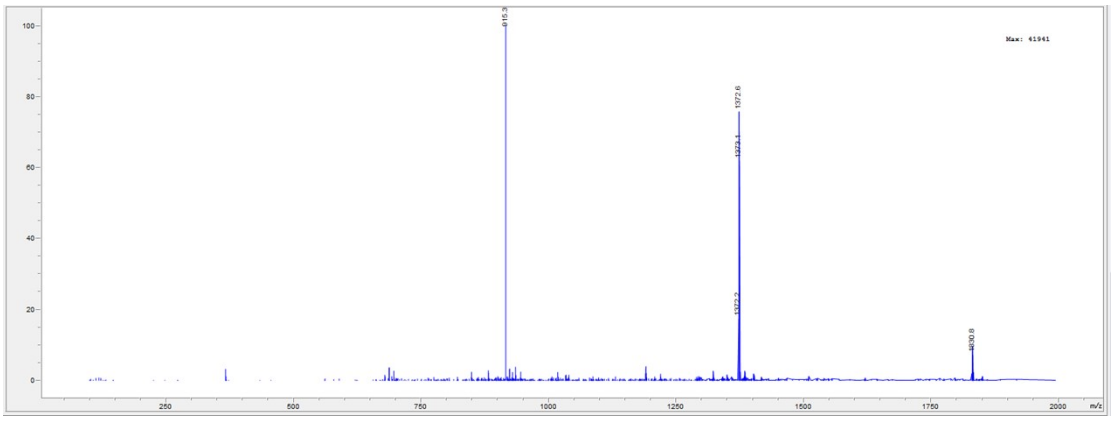


Figure **S7**. Top: UV traces of the purified **Stapled S2a** at 220 nm using Luna® 5 µm C18 100 Å (50 × 2 mm) column; Bottom: ESI-MS data of the purified **Stapled S2a**. Calc. for $C_{122}H_{171}N_{31}O_{38}S_2$: 2744.01 Da (average isotopes), (m/z) $[M+2H]^{2+}$:1372.6, $[M+3H]^{3+}$:915.4; found $[M+2H]^{2+}$: 1372.6, $[M+3H]^{3+}$:915.3.

Stapled S2b

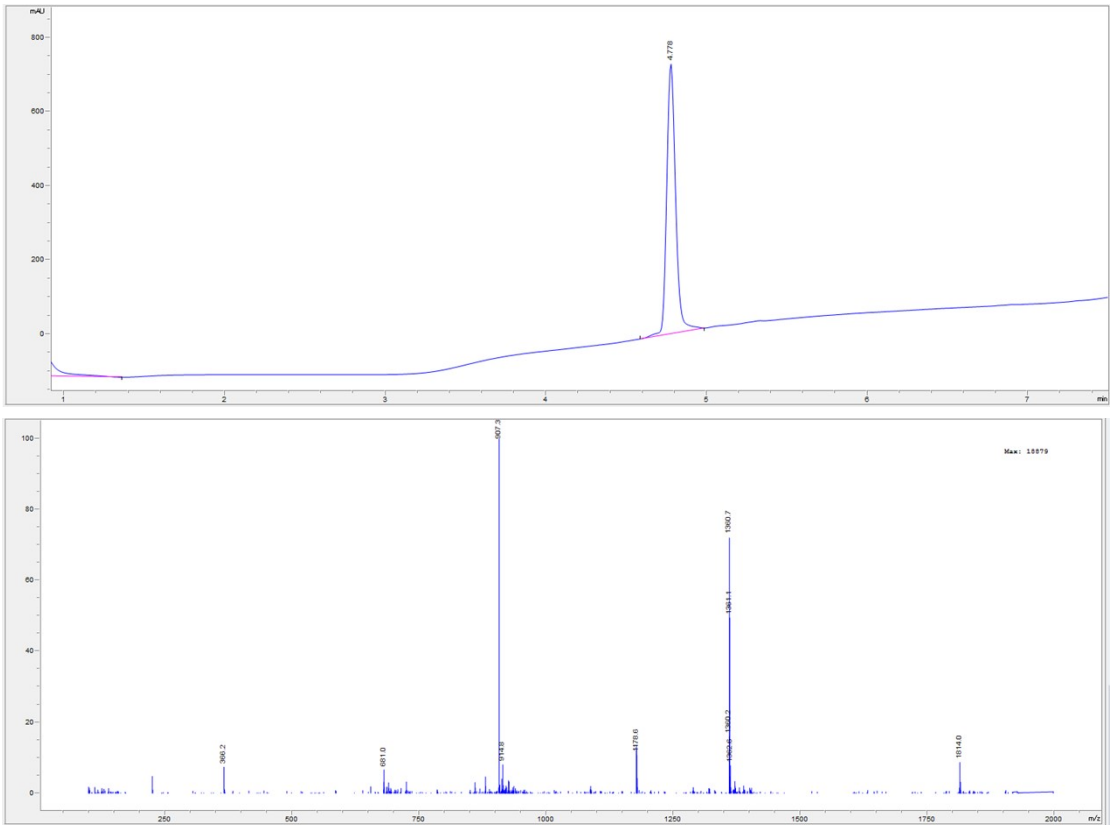


Figure **S8**. Top: UV traces of the purified **Stapled S2b** at 220 nm using Luna® 5 µm C18 100 Å (50 × 2 mm) column; Bottom: ESI-MS data of the purified **Stapled S2b**. Calc. for $C_{116}H_{163}N_{35}O_{38}S_2$: 2719.91 Da (average isotopes), (m/z) $[M+2H]^{2+}$: 1360.6, $[M+3H]^{3+}$: 907.4; found $[M+2H]^{2+}$: 1360.7, $[M+3H]^{3+}$: 907.3.

Stapled S2c

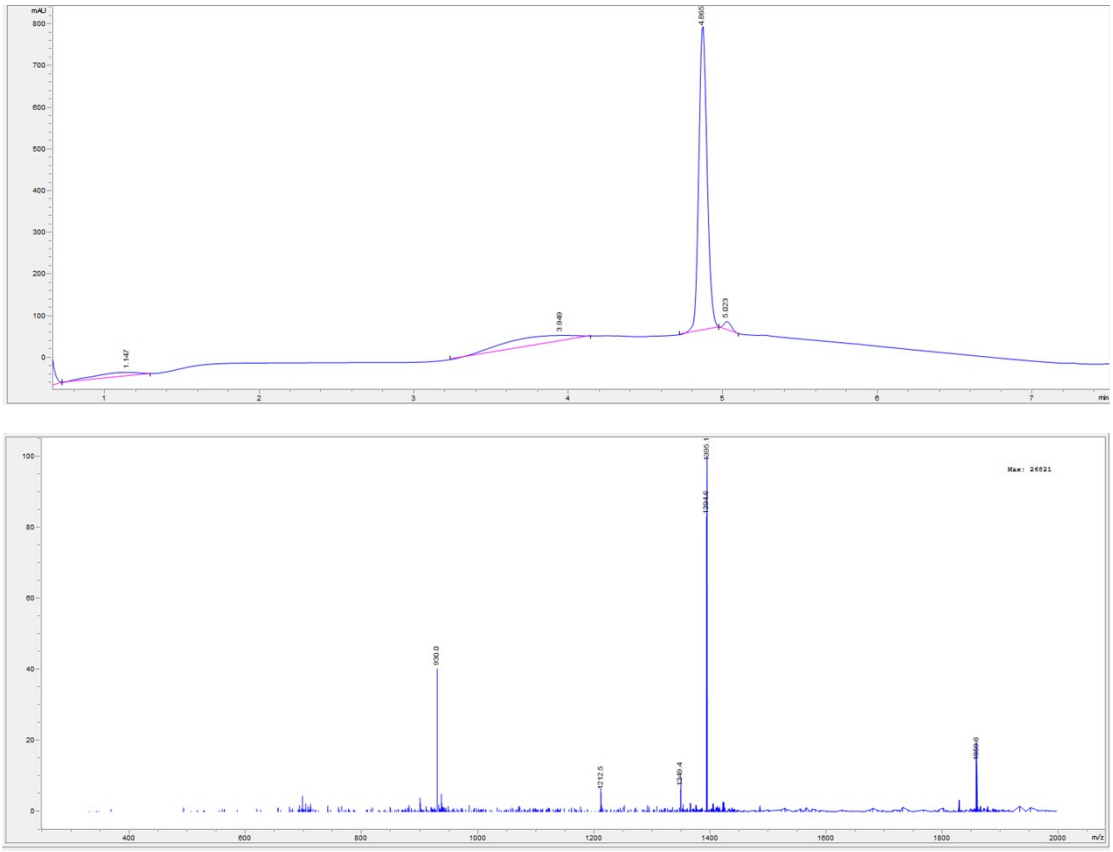
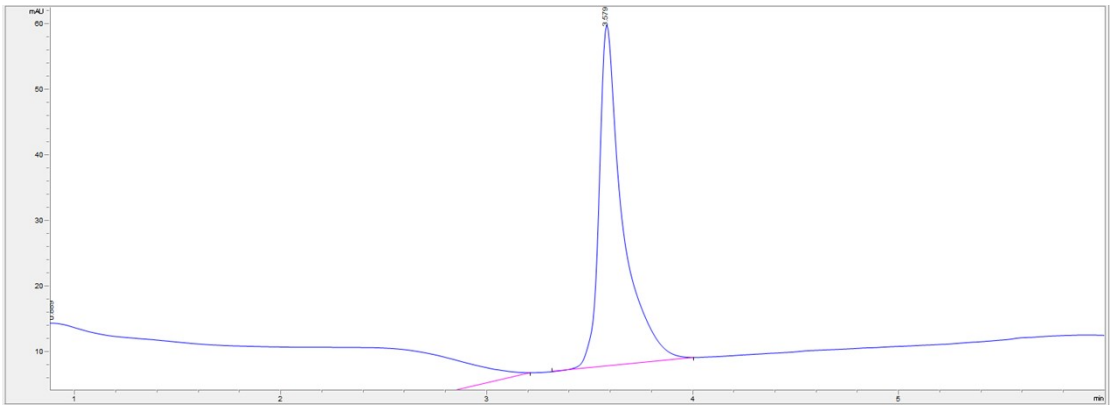


Figure **S9**. Top: UV traces of the purified **Stapled S2c** at 220 nm using Luna® 5 µm C18 100 Å (50 × 2 mm) column; Bottom: ESI-MS data of the purified **Stapled S2c**. Calc. for $C_{120}H_{163}F_4N_{31}O_{38}S_2$: 2787.92 Da (average isotopes), (m/z) $[M+2H]^{2+}$:1394.6, $[M+3H]^{3+}$:930.1; found $[M+2H]^{2+}$: 1395.1, $[M+3H]^{3+}$:930.0.

Ins-AC-Stapled S2a



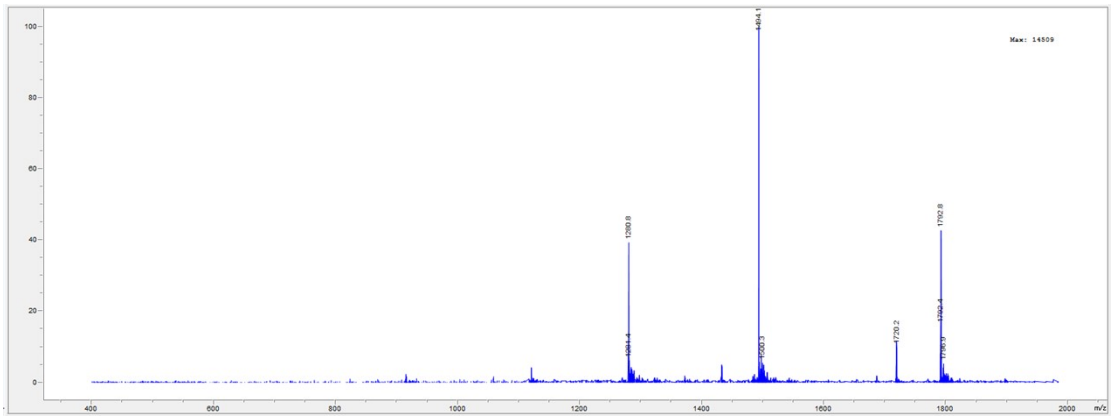


Figure **S10**. Top: UV traces of the purified **Ins-AC-Stapled S2a** at 220 nm using Jupiter®5 µm C4 100 Å (50 × 2 mm) column; Bottom: ESI-MS data of the purified **Ins-AC-Stapled S2a**. Calc. for $C_{398}H_{582}N_{100}O_{121}S_8$: 8960.09 Da (average isotopes), (m/z) $[M+5H]^{5+}$:1792.6, $[M+6H]^{6+}$:1494.0, $[M+7H]^{7+}$:1280.7; found $[M+5H]^{5+}$:1792.8, $[M+6H]^{6+}$:1494.1, $[M+7H]^{7+}$:1280.8.

Ins-AC-Stapled S2b

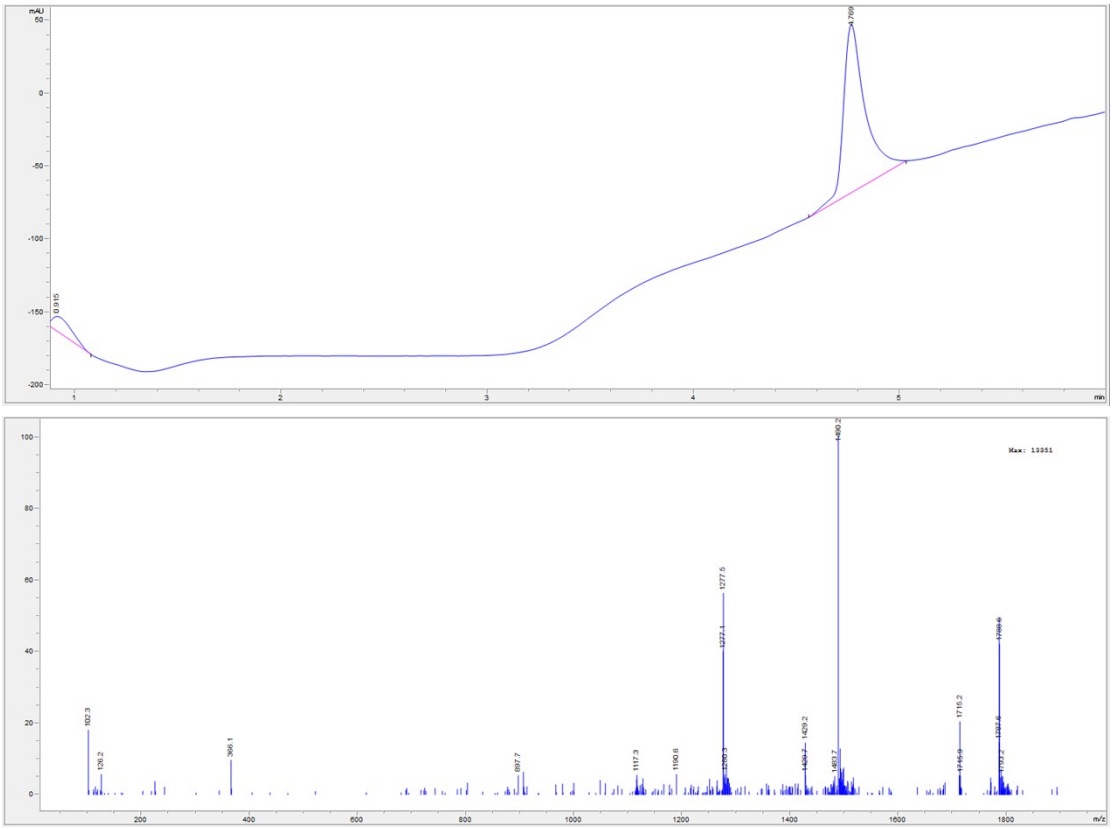


Figure **S11**. Top: UV traces of the purified **Ins-AC-Stapled S2b** at 220 nm using Jupiter® 5 µm C4 100 Å (50 × 2 mm) column; Bottom: ESI-MS data of the purified **Ins-AC-Stapled S2b**. Calc. for $C_{398}H_{582}N_{100}O_{121}S_8$: 8935.99 Da (average isotopes), (m/z) $[M+5H]^{5+}$:1787.8, $[M+6H]^{6+}$:1490.0, $[M+7H]^{7+}$:1277.3; found $[M+5H]^{5+}$:1788.6, $[M+6H]^{6+}$:1490.2, $[M+7H]^{7+}$:1277.5.

Ins-AC-Stapled S2c

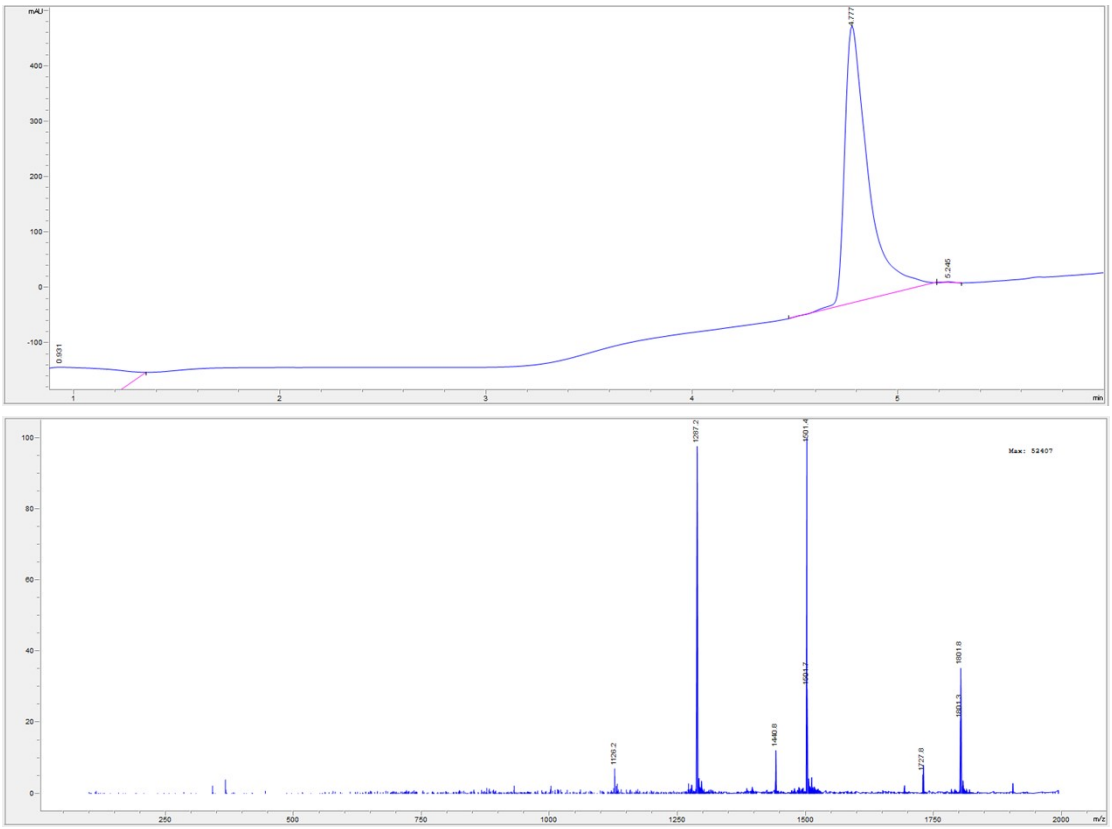


Figure **S12**. Top: UV traces of the purified **Ins-AC-Stapled S2c** at 220 nm using Jupiter® 5 µm C4 100 Å (50 × 2 mm) column; Bottom: ESI-MS data of the purified **Ins-AC-Stapled S2c**. Calc. for $C_{396}H_{574}F_4N_{100}O_{121}S_8$: 9004.00 Da (average isotopes), (m/z) $[M+5H]^{5+}$:1801.4, $[M+6H]^{6+}$:1501.3, $[M+7H]^{7+}$:1287.0; found $[M+5H]^{5+}$:1801.8, $[M+6H]^{6+}$:1501.4, $[M+7H]^{7+}$:1287.2.