Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Serine-Mediated Hydrazone Ligation Displaying Insulin-Like Peptides on M13 Phage pIII

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1. Supporting figures

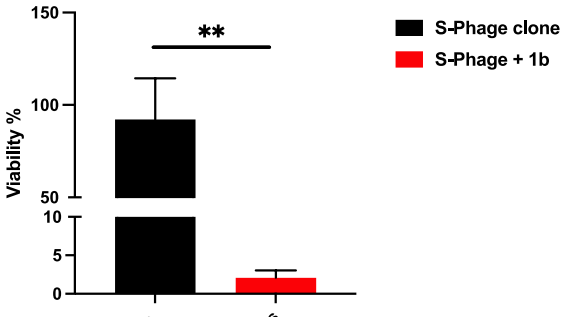


Figure S1. The phage viability change before and after ligation with 1b. Data represent the average of 3 independent measurements. Error bars represent the standard deviation. (**p < 0.01, two-tailed t test)

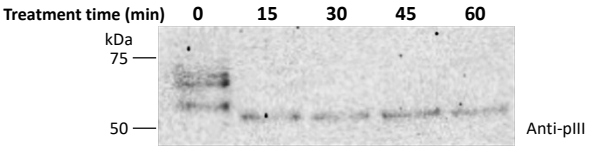


Figure S2. Introduction of a trypsin spacer to improve viability. Western blot showing insulin was removed from phage pIII after trypsin treatment.

2. Phage methods

2.1 General method for M13 phage

S-phage (SRGFFYTPKT) and S-trypsin-phage (SRGFFYTPKTGKDIRG) were constructed by inserting annealed oligonucleotides into a double digested M13KE vector, as previously described[1]. The ligation products were mixed with SS320 cells for electroporation, followed by Sanger Sequencing verification and phage amplification. After amplification, phages were precipitated with PEG 8000/2.5 M NaCl solution overnight at 4 °C and resuspended in 1 mL PBS for subsequent experiments.

Primer sequences for M13 phage clones:

Primer name	Sequence (5'-3')
SRGFFYTPKT_EagI_F	AAAAAAAAGGTACCTTTCTATTCTCATTCTTCTCGTGGTTTCTTC TACACC
SRGFFYTPKT_EagI_R	TTCTTCGGCCGAACCGCCACCGGTTTTCGGGGTGTAGAAGAAAC

	CACGAGA
SRGFFYTPKTGKDIRG_EagI_F	AAAAAAAAGGTACCTTTCTATTCTCATTCTTCTCGTGGTTTCTTCTACACCCCGAAAACC
SRGFFYTPKTGKDIRG_EagI_R	TCTCGTGGTTTCTTCTACACCCCGAAAACCGGTAAAGATATTCGCGGCTCGGCCGAAGAA

2.2 General method for periodate oxidation and insulin ligation on phage

The method was adopted and performed as previously described[2, 3]. 10^{12} pfu/mL phage in GlycoLink Coupling Buffer (100 μL) (Thermo Scientific, #88944) were oxidized by adding 2.6 μL of 4 mM sodium periodate on ice for 5 min in the dark. 1.02 μL glutathione (50 mM) were added into the oxidized phage solution to quench the reaction at room temperature for 10 min. PEG/NaCl-precipitation was conducted to purify phage and exchange the buffer with PBS. 10 μL aliquot of phage was taken for biotin-capture assay to quantify the reaction. Hz-Fmoc-Small insulin (4.27 mg/mL) in 500 μL was incubated with 26 μL 50% piperidine for 35 min and followed by the addition of 58 μL 50% AcOH to quench the reaction. The remaining phage was incubated with 275 μL of prepared small insulin with 3.375 μL aniline as a catalyst at 4°C overnight. After the reaction, 10 μL solution was taken for biotin-capture assay, phage titering and western blot analysis. Two rounds of PEG/NaCl-precipitation were conducted to remove excess small insulin.

2.3 Biotin-capture assay

The biotin-capture assay was followed as previously described[3]. 10 μL 2mM EZ-Link™ Biotin-LC-Hydrazide (Thermo Scientific, #21340) were incubated with 10 μL phage with 0.27 μL aniline at room temperature for 2 h. Phage was diluted to 10^7 pfu/mL in blocking solution (0.1% BSA) and incubated with pre-washed Dynabeads™ M-280 Streptavidin (Invitrogen, #11206D) for 15 min. To remove the unspecific binding, 50 mM biotin was added into the solution to a final concentration of 2 mM for extra 5 min incubation. Beads were captured using magnates, and the supernatant was reserved as the output. The percentage of captured phage was determined as (A-B)/A*100%, where A and B represent phage titers before and after capture, respectively.

2.4 IR binding and pulldown assay

IR binding and pulldown assay was conducted as previously described[1]. Insulin receptor isoform A (R&D systems, #1544-IR) was biotinylated using EZ-Link™ NHS-LC-Biotin (Thermo Scientific, #21336) and purified with Zeba™ Spin Desalting Columns 40K MWCO (Thermo Scientific, #87769). The biotinylated IR was incubated with washed Dynabeads™ M-280 Streptavidin (Invitrogen, #11206D) for 30 minutes to prepare the coated beads. 10^{10} phage was incubated with 30 µL IR pre-coated beads in TBST (0.05% Tween-20) at room temperature for 3 h. After washing the beads five times with 500 µL TBST, phage was eluted using 50 µL 0.2 M Glycine-HCl elution buffer (pH 1.8) for 10 minutes, followed by neutralization with 12.5 µL Tris-HCl (pH 9.5). The eluted phage was titrated by top agar to determine the recovery yield. For the competitive binding assay, different concentrations of human insulin were incubated with phages, followed by the same incubation, wash, and elution.

2.5 Western blot

Western blot was followed as previously described[1]. Briefly, 10^{8} phage was mixed with Pierce™ LDS Sample Buffer, Non-Reducing (4X) (Thermo Scientific, #84788) in total 40 µL and heated at 70 ^{o}C for 10 minutes. The samples were subjected to electrophoresis on Invitrogen™ Bolt™ 8% Bis-Tris gel (ThermoFisher Scientific, #NW00082BOX) in MOPS buffer at 200 V for 30 minutes. Protein bands were transferred to a membrane and analyzed by immunoblot using Anti-M13 PIII Monoclonal Antibody (New England Biolabs, #E8033S) in ChemiDoc XRS+ Gel Imaging System (Bio-Rad Laboratories).

2.6 Trypsin cleavage on phage

Phage was incubated with trypsin (Thermo Scientific, #90057) at a final concentration of 0.1 µg/µL in PBS for varying durations (0 – 60 min). Following trypsin treatment, phage was proceeded for tittering and western blot to analyze phage viability and removal of insulin from pIII.

3. General information for peptide synthesis

3.1 Chemical and protein materials

All Fmoc amino acids, reagents, and solvents were used without purification. Fmoc amino acids and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid

hexafluorophosphate (HATU) were purchase from AAPPTec, Bachem, Chem-Impex, ChemPep, and PurePep. The Rink amide ChemMatrix resin (catalog number: 7-600-1310) was purchased from Biotage. The 2-chlorotrityl chloride (2-CTC) resin (catalog number: 150301) were purchased from ChemPep. *N*,*N*-dimethylformamide (DMF), CH_2Cl_2, MeCN, MeOH, Et_2O, AcOH, EtOAc, hexane, tetrahydrofuran (THF), *N*,*N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), NH_4Cl, NH_4HCO_3, tricine, urea, biotin, and 2-mercaptoethanol were purchased from Fisher Scientific. Piperidine, triisopropylsilane (TIPS), 2,2'-dithiodipyridine (DTDP), 2,2'-dithiobis(5-nitropyridine) (DTNP), I_2, benzyl bromoacetate, hydroxybenzotriazole (HOBt), *N*,*N*'-diisopropylcarbodiimide (DIC), 10% Palladium on active carbon, glycine benzyl ester hydrochloride, and sodium ascorbate were purchased from Sigma Aldrich. H_2O was purified by the Milli-Q® system.

3.2 LC/MS

In Agilent 6120 Quadrupole LC–MS system the method is at 0.4 mL/min with 5% of a H_2O/MeCN + 0.1% TFA solution for 1 minute followed by a linear gradient from 5% to 95% of a H_2O/MeCN + 0.1% TFA solution over 5 minutes. In Agilent 1260 Infinity II LC–MS system, the method is at 0.5 mL/min with 5% of a H_2O/MeCN + 0.1% TFA solution for 1 minute followed by a linear gradient from 5% to 90% of a H_2O/MeCN + 0.1% TFA solution over 5 minutes.

The concentrations of peptides were measured by Thermo Scientific™ NanoDrop 2000c with calculated ε values at 280 nm (ε_{280}): ε_{280} = (number of Trp × 5500) + (number of Tyr × 1490) + (number of disulfide bond × 125)

3.3 General RP-HPLC conditions

Method A: Individual chains were purified by a Preparative C18 (2) Column (Luna®, 5 µm, 250 x 21.2 mm) with a linear gradient from 25% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a 5 $mL.min^{-1}$ flow rate for A chains and from 30% aqueous ACN (0.1% TFA) to 60% aqueous ACN (0.1% TFA) over 45 min at a 5 $mL.min^{-1}$ flow rate for B chains.

Method B: All A-B dimer peptides were purified using a Preparative C18 300 Å Column (Jupiter®, 5 μm, 250 x 10 mm) with a linear gradient from 25% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a 3 $mL.min^{-1}$ flow rate.

Method C: All final products were purified using a Preparative C18 300 Å Column (Jupiter®, 5 μm, 250 x 10 mm) with a linear gradient from 25% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a 3 $mL.min^{-1}$ flow rate.

3.4 Peptide synthesis

3.4.1 Synthesis of Hydrazide-Fmoc-Small insulin A chain

Hydrazide-Fmoc-Small insulin A chain was synthesized with a 0.1 mmol scale by following the general procedure of automated Fmoc/^{t}Bu SPPS with modification by following the reported procedure. Fmoc-Asp-O^{t}Bu was loaded to the H-Rink amide ChemMatrix® resin with the general amide bond formation condition of automated Fmoc/^{t}Bu SPPS. The Asp-O^{t}Bu linked to the Rink amide resin through the β-carboxyl would be converted into Asn of native residue A21 after resin cleavage. Fmoc-Cys(S^{t}Bu)-OH, Fmoc-Cys(Acm)-OH, and Fmoc-Cys(Mmt)-OH were used at A6, A7, and A11 respectively. After the entire sequence was completed on resin, 25% 2-mercaptoethanol in DMF (*v*/*v*, 6 mL) was added to the resin. The reaction mixture was gently agitated at room temperature for 1.5 hours. This step was repeated once. The resulting resin was washed with DMF 3 times and CH_2Cl_2 3 times. A solution of DTNP (310 mg, 1 mmol) in CH_2Cl_2 (6 mL) was added to the resin. The reaction mixture was gently agitated at room temperature for 1.0 hour. The resin was washed with DMF 3 times and CH_2Cl_2 3 times. The resin was and treated with a solution of 1% TFA and 5% TIPS in CH_2Cl_2 (6 mL) for 2 min with 5 repeats. The resin was washed with DMF 3 times and CH_2Cl_2 3 times and gently agitated in CH_2Cl_2 (6 mL) at room temperature for 1 hour. The resin was washed with CH_2Cl_2 3 times. The final peptide was cleaved by following the general procedure of peptide cleavage from resin. The crude was purified on Luna® 5 μm C18 100 Å (250 × 21 mm) column (Phenomenex, California, USA) at 5 mL/min with a linear gradient from 30% to 50% of a H_2O/MeCN + 0.1% TFA

solution over 30 minutes on an Agilent 1260 HPLC system detected at 220, 240, 260, and 280 nm. The fractions containing the products were flash frozen under liquid N_2 and then lyophilized to give the products as a white powder.

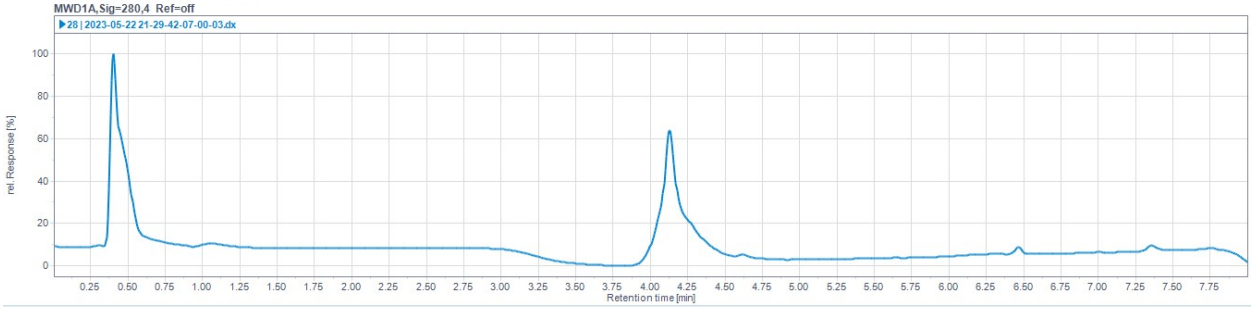
LC and MS data of Hydrazide-Fmoc-Small insulin A chain

MS deconvolution analysis:

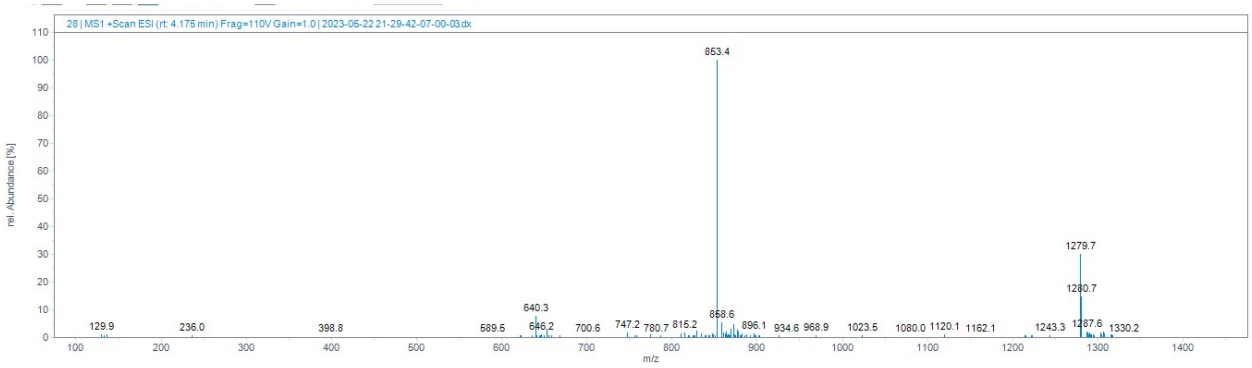
Molecular formula	$C_{107}H_{165}N_{31}O_{34}S_4$			
Calculated monoisotopic mass	Observed m/z		Expected m/z	
	$[M + 3H]^{3+}$	$[M + 2H]^{2+}$	$[M + 3H]^{3+}$	$[M + 2H]^{2+}$
2557.92	853.4	1280.7	853.64	1279.96

Luna® 5 µm C18 100 Å (50 × 2 mm) column (Phenomenex, California, USA) at 0.5 mL/min with 5% of a H_2O/MeCN + 0.1% TFA solution for 1 minute followed by a linear gradient from 5% to 90% of a H_2O/MeCN + 0.1% TFA solution over 5 minutes on an Agilent 1260 Infinity II LC–MS system.

LC chromatogram:



MS spectrum:



3.4.2 Synthesis of Hydrazide-Fmoc-Small insulin B chain

Acm Spy

H-FVNQHLCGSHLVEALYLVCG- NHNH - Fmoc

Hydrazide-Fmoc-Small insulin B chain was synthesized with a 0.1 mmol scale by following the general procedure of automated Fmoc/^{t}Bu SPPS with modification. After the sequence was completed on the resin, the final peptide was cleaved by following the general procedure of peptide cleavage in the presence of additional DTDP (220 mg, 0.1 mmol). The crude peptide was dissolved and mixed with FmocOsu in MeCN/H2O (1:1) at neutral pH for 18 hours, and then purified on Luna® 5 µm C18 100 Å (250 × 21 mm) column (Phenomenex, California, USA) at 5 mL/min with a linear gradient from 25% to 60% of a H_2O/MeCN + 0.1% TFA solution over 35 minutes on an Agilent 1260 HPLC system detected at 220, 240, 260, and 280 nm. The fractions containing product were flash frozen under liquid N_2 and then lyophilized to give the product as a white powder.

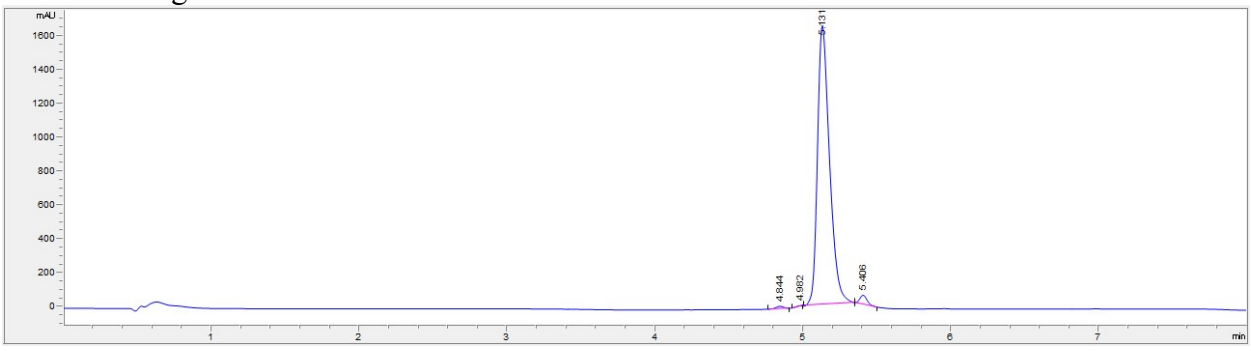
LC and MS data of Hz-Fmoc-Small Ins B chain

MS deconvolution analysis:

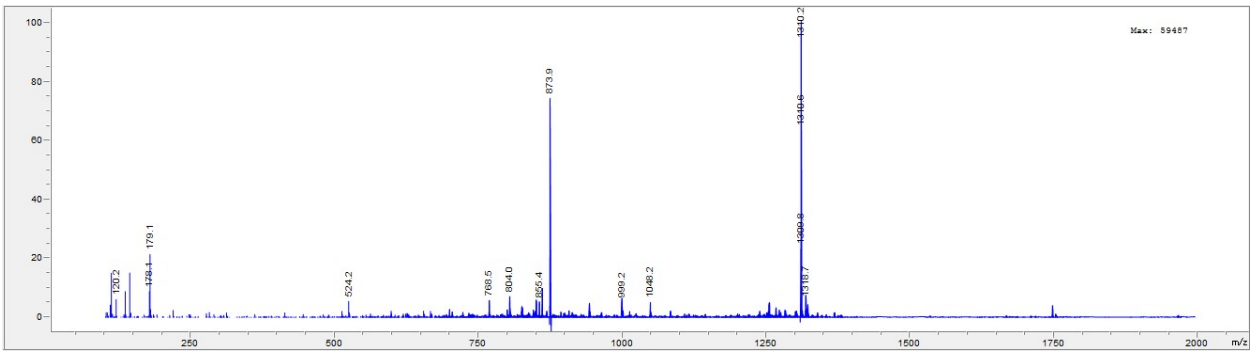
Molecular formula	$C_{122}H_{173}N_{30}O_{29}S_3$			
Calculated monoisotopic mass	Observed m/z		Expected m/z	
	$[M + 3H]^{3+}$	$[M + 2H]^{2+}$	$[M + 3H]^{3+}$	$[M + 2H]^{2+}$
2619.08	873.9	1310.6 1310.2	874.03	1310.54

Luna® 5 µm C18 100 Å (50 × 2 mm) column (Phenomenex, California, USA) at 0.4 mL/min with 5% of a H_2O/MeCN + 0.1% TFA solution for 1 minute followed by a linear gradient from 5% to 95% of a H_2O/MeCN + 0.1% TFA solution over 5 minutes on an Agilent 6120 Quadrupole LC–MS system.

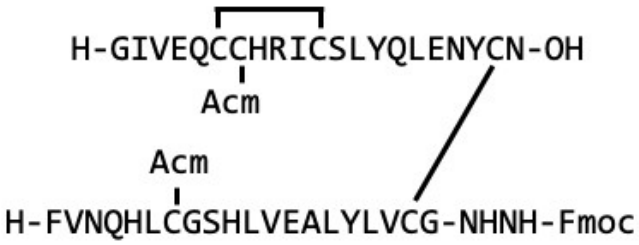
LC chromatogram:



MS spectrum:



3.4.3 Synthesis of Hydrazide-Fmoc-Small insulin A+ B chain combo



Hydrazide-Fmoc-Small insulin A+ B chain combo was synthesized by mixing lyophilized A chain powder and the B chain powder in a solution of 6 M urea and 0.2 M NH_4Cl. The reaction mixture was gently mixed at room temperature for 1 hour. The resulting solution was purified on Jupiter® 5 µm C18 300 Å (250 × 10 mm) column (Phenomenex, California, USA) at 3 mL/min with a linear gradient from 20% to 60% of a H_2O/MeCN + 0.1% TFA solution over 50 minutes on an Agilent 1260 HPLC system detected at 220, 240, 260, and 280 nm. The fractions were flash frozen under liquid N_2 and then lyophilized as a white powder.

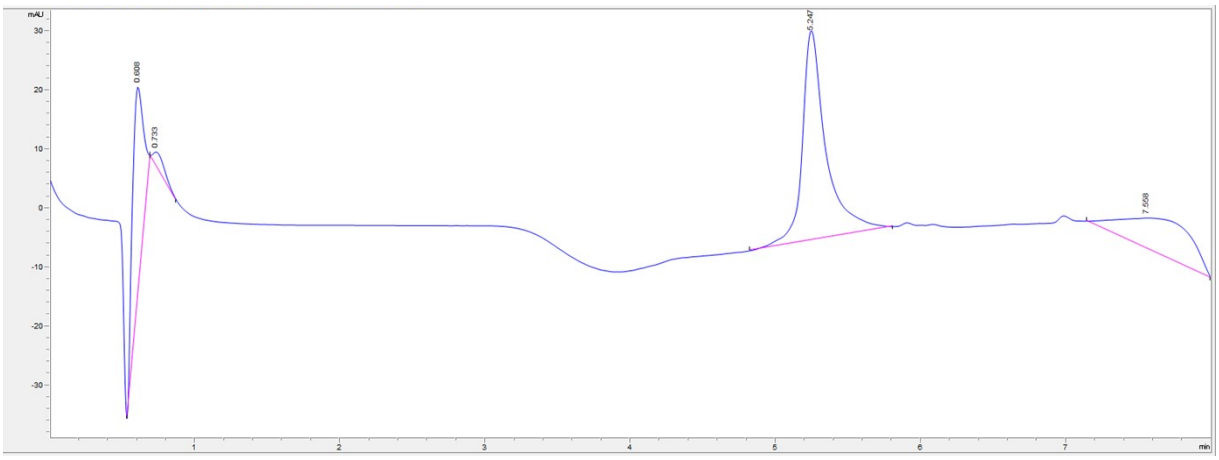
LC and MS data of Hz-Fmoc-Small Ins A+ B chain combo

MS deconvolution analysis:

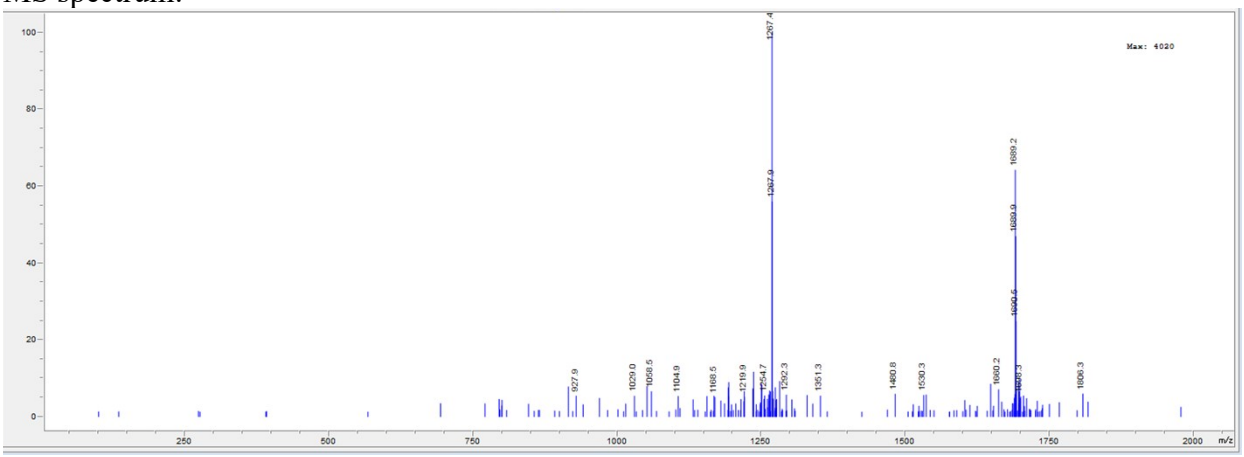
Molecular formula	$C_{224}H_{332}N_{60}O_{63}S_6$			
Calculated monoisotopic mass	Observed m/z		Expected m/z	
	$[M + 4H]^{4+}$	$[M + 3H]^{3+}$	$[M + 4H]^{4+}$	$[M + 3H]^{3+}$
5065.84	1267.4	1689.9	1267.46	1689.61

Luna® 5 µm C18 100 Å (50 × 2 mm) column (Phenomenex, California, USA) at 0.4 mL/min with 5% of a H_2O/MeCN + 0.1% TFA solution for 1 minute followed by a linear gradient from 5% to 95% of a H_2O/MeCN + 0.1% TFA solution over 5 minutes on an Agilent 6120 Quadrupole LC–MS system.

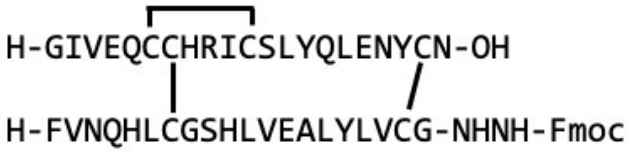
LC chromatogram:



MS spectrum:



3.4.4 Synthesis of Hydrazide-Fmoc-Small insulin (1a)



Hydrazide-Fmoc-Small insulin A+ B chain combo was dissolved in 20% AcOH in H_2O and freshly prepared I_2 in AcOH was added into the solution at room temperature. The reaction mixture was gently agitated at room temperature for 10 minutes. The reaction was monitored by LC–MS to apply the additional amount of I_2 if required. After the reaction was completed, 1 M sodium ascorbate was added to the reaction mixture followed by gentle agitation until the solution color turned to pale yellow. The mixture was purified on Jupiter® 5 µm C18 300 Å (250 × 10 mm) column (Phenomenex, California, USA) at 3 mL/min with a linear gradient from 20% to 60% of a H_2O/MeCN + 0.1% TFA solution over 50 minutes on an Agilent 1260 HPLC system detected at 220, 240, 260, and 280 nm. The solution was flash frozen under liquid N_2 and then

lyophilized to give the product as a white powder. 0.5 mg of Hydrazide-Fmoc-Small insulin was obtained with a yield of 16.7% from Hz-Fmoc-Small Ins A+ B chain combo, and a yield of 5% over two steps based on A chain.

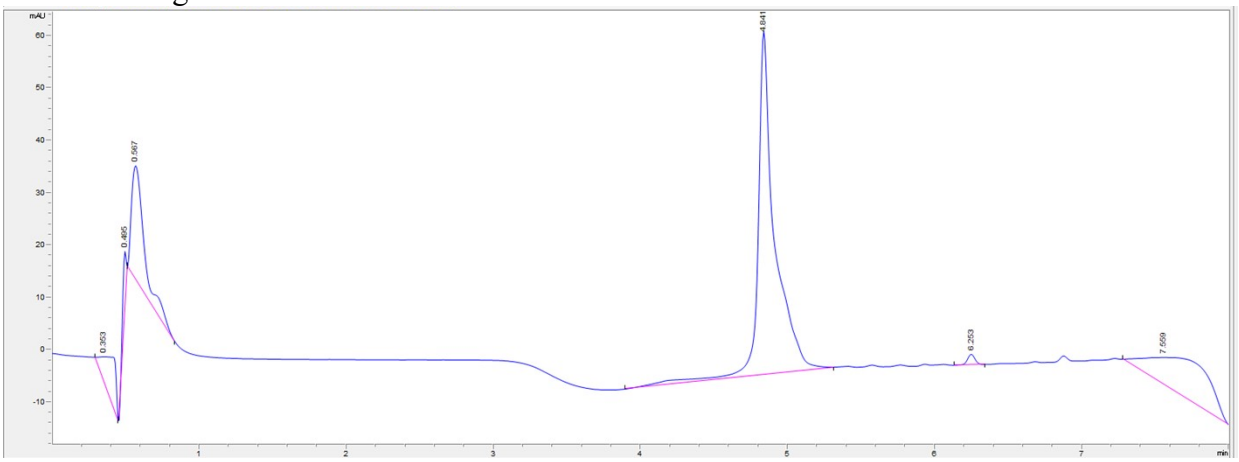
LC and MS data of Hz-Fmoc-Small Ins

MS deconvolution analysis:

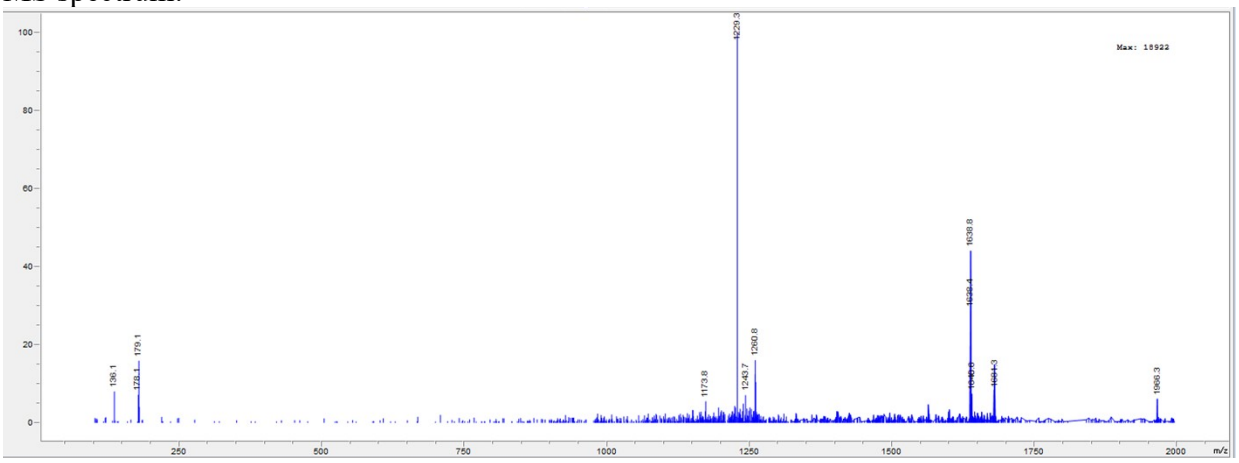
Molecular formula	$C_{218}H_{325}N_{56}O_{62}S_6$			
Calculated monoisotopic mass	Observed m/z		Expected m/z	
	$[M + 4H]^{4+}$	$[M + 3H]^{3+}$	$[M + 4H]^{4+}$	$[M + 3H]^{3+}$
4913.42	1229.3	1638.8	1229.355	1638.807

Luna® 5 µm C18 100 Å (50 × 2 mm) column (Phenomenex, California, USA) at 0.4 mL/min with 5% of a H_2O/MeCN + 0.1% TFA solution for 1 minute followed by a linear gradient from 5% to 95% of a H_2O/MeCN + 0.1% TFA solution over 5 minutes on an Agilent 6120 Quadrupole LC–MS system.

LC chromatogram:



MS spectrum:



3.4.5 Synthesis of SRGFFYTPKT

H-SRGFFYTPKT-OH

Peptide SRGFFYTPKT syntheses were conducted on 2-chlorotrityl chloride resin using a standard Fmoc/HATU/DIEA method. The first amino acids were synthesized manually. The resulting resin-bound A chain (0.1 mmol scale) was treated with 6.0 mL TFA solution containing 2.5% TIS, 2.5% H_2O at room temperature, with gentle shaking for 2.0 hours. The resin was filtered off and the filtrate was precipitated by cold ether (40 mL). The precipitate was collected by centrifugation then washed with cold ether (40 mL × 3), and vacuum dried and purified by preparative C18 column. The fractions containing product were flash frozen under liquid N_2 and then lyophilized to give the product as a white powder.

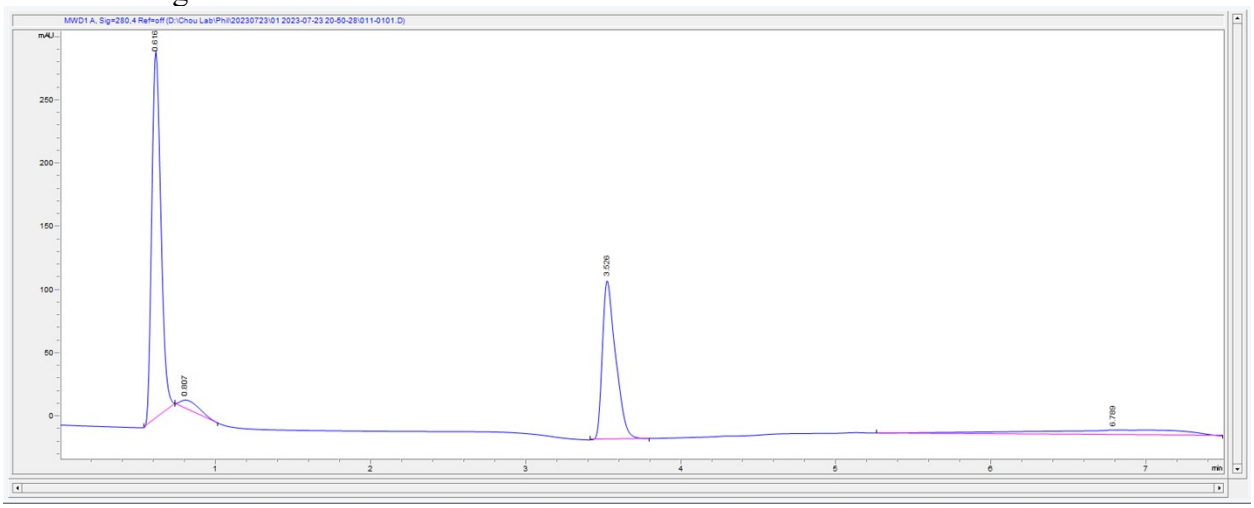
LC and MS data of SRGFFYTPKT

MS deconvolution analysis:

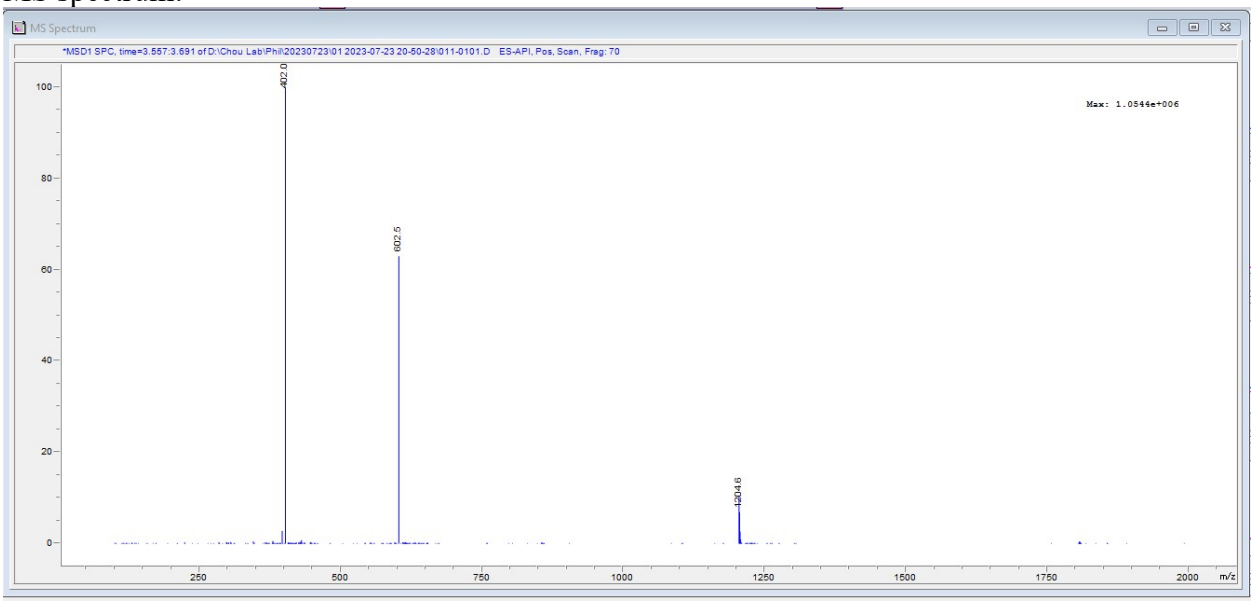
Molecular formula	$C_{57}H_{82}N_{14}O_{15}$					
Calculated monoisotopic mass	Observed m/z			Expected m/z		
	$[M + 3H]^{3+}$	$[M + 2H]^{2+}$	$[M + H]^{+}$	$[M + 3H]^{3+}$	$[M + 2H]^{2+}$	$[M + H]^{+}$
1203.35	402.0	602.5	1204.6	402.116	602.675	1204.35

Luna® 5 µm C18 100 Å (50 × 2 mm) column (Phenomenex, California, USA) at 0.4 mL/min with 5% of a H_2O/MeCN + 0.1% TFA solution for 1 minute followed by a linear gradient from 5% to 95% of a H_2O/MeCN + 0.1% TFA solution over 5 minutes on an Agilent 6120 Quadrupole LC–MS system.

LC chromatogram:



MS spectrum:



3.4.6 Synthesis of Oxidized SRGFFYTPKT

A solution of 1mM Peptide SRGFFYTPKT in PBS (4.5 mL) was treated with 10mM $NaIO_4$ (add 500 uL of 100 mM) at 4°C. After 10 min, 2 mL 200 mM GSH was added to quench the reaction. The solution was purified on a preparative C18 column with a linear gradient from 10% aqueous ACN (0.1% TFA) to 45% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 3 mL/min. The fractions containing product were flash frozen under liquid N_2 and then lyophilized to give the product as a white powder.

LC and MS data of Oxidized SRGFFYTPKT

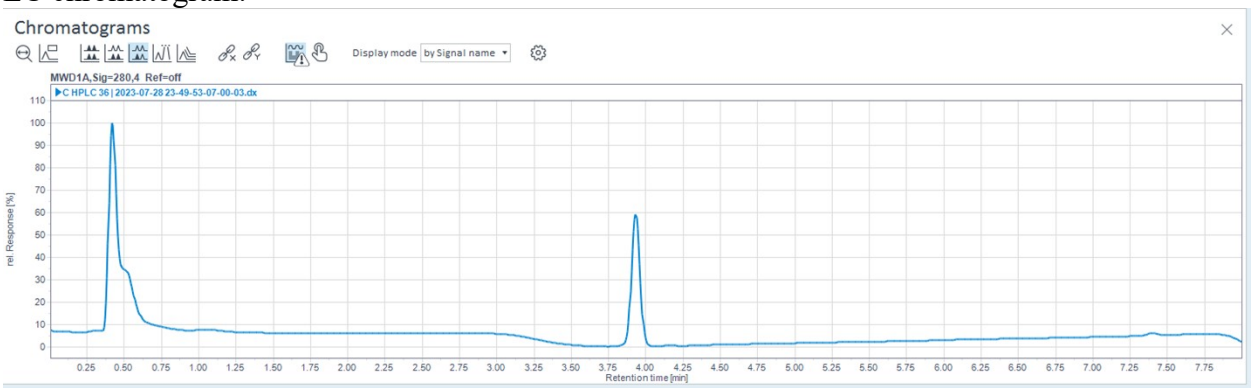
MS deconvolution analysis:

Molecular formula	$C_{56}H_{79}N_{13}O_{16}$					
Calculated monoisotopic mass	Observed m/z			Expected m/z		
	$[M + 2H]^{2+}$			$[M + 2H]^{2+}$		
1190.307	595.9			596.15		

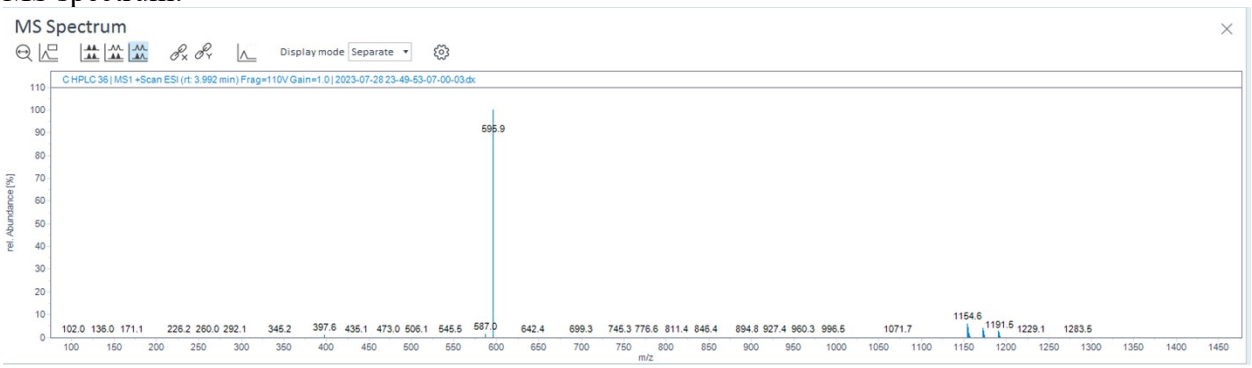
Luna® 5 µm C18 100 Å (50 × 2 mm) column (Phenomenex, California, USA) at 0.5 mL/min with 5% of a H_2O/MeCN + 0.1% TFA solution for 1 minute followed by a linear gradient from 5%

to 90% of a H_2O/MeCN + 0.1% TFA solution over 5 minutes on an Agilent 1260 Infinity II LC–MS system.

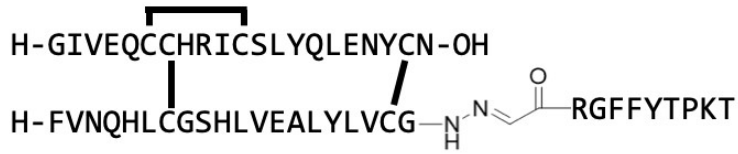
LC chromatogram:



MS spectrum:



3.4.7 Synthesis of Hydrazone-insulin (1c)



Oxidized-SRGFFYTPKT aldehyde (1.2 equiv) was incubated with (1.0 equiv) Hydrazide-Small insulin (1b) in PBS (1.0 mM, pH 4.5) overnight at room temperature. The resulting reaction solution was purified on a preparative C18 column with a linear gradient from 20% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 3 mL/min.

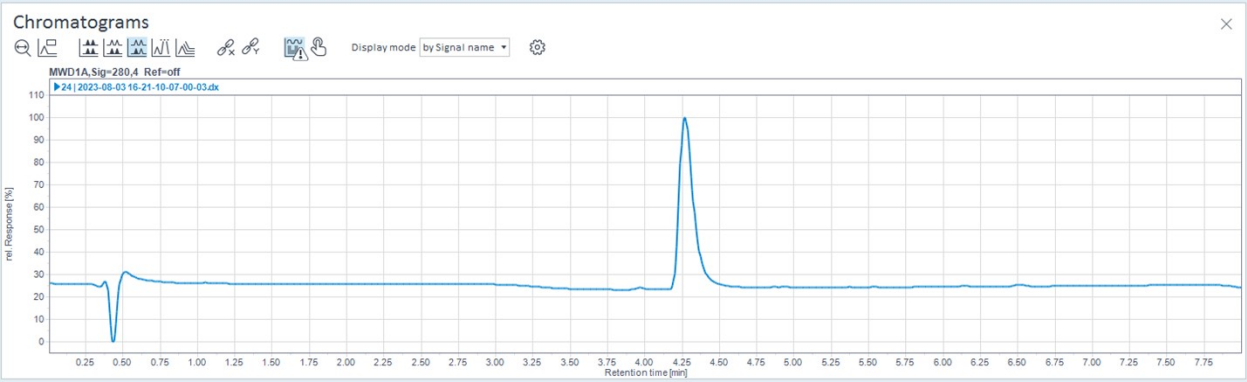
LC and MS data of Hydrazone-insulin

MS deconvolution analysis:

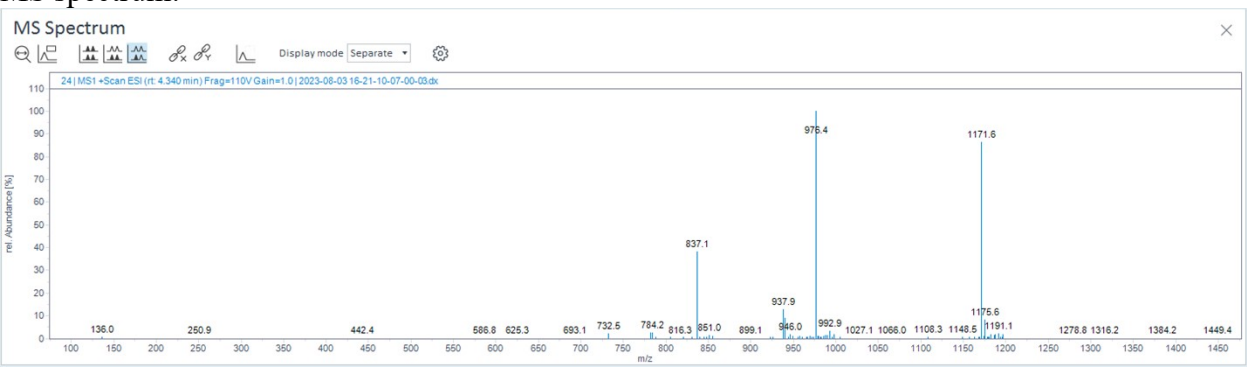
Molecular formula	$C_{259}H_{385}N_{71}O_{73}S_6$					
Calculated monoisotopic mass	Observed m/z			Expected m/z		
	$[M + 5H]^{5+}$	$[M + 6H]^{6+}$	$[M + 7H]^{7+}$	$[M + 5H]^{5+}$	$[M + 6H]^{6+}$	$[M + 7H]^{7+}$
5853.7130	1171.6	976.4	837.1	1171.7	976.6	837.2

Luna® 5 µm C18 100 Å (50 × 2 mm) column (Phenomenex, California, USA) at 0.5 mL/min with 5% of a H_2O/MeCN + 0.1% TFA solution for 1 minute followed by a linear gradient from 5% to 90% of a H_2O/MeCN + 0.1% TFA solution over 5 minutes on an Agilent 1260 Infinity II LC–MS system.

LC chromatogram:



MS spectrum:



4. Insulin activity assay

The assay was conducted as previously described[4]. Briefly, 50 µL of serially diluted insulin samples were added into NIH 3T3 overexpressing human insulin receptor isoform B (IR-B). AKT phosphorylation levels were measured by HTRF pAkt Ser473 kit (Cisbio, Massachusetts, USA) in Synergy Neo plate reader (BioTek, Vermont, USA). Each data point was obtained from four replicates and were processed according to the manufacturer's protocol. EC_{50} was calculated by Prism 9 (GraphPad Software, California, USA) by applying nonlinear regression curve fitting of dose-response asymmetric equation.

Reference

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