Supporting Information

A Cysteine-specific Solubilizing Tag Strategy Enables Efficient Chemical Protein Synthesis of Difficult Targets

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Abbreviations

2,2'-dithiodipyridine	DTDP
2-Chlorotrityl chloride resin	2-CTC resin
3-mercaptopropionic acid	MPAA
4-methoxybenzyl	Mob
Acetamidomethyl	Acm
Acetyl acetone	acac
Ammonium bicarbonate	NH_4HCO_3
Circular dichroism	CD
Cysteine	Cys
Dichloromethane	DCM
Diethyldithiocarbamate	DTC
Dimethylformamide	DMF
Dithiothreitol	DTT
Glutamine	Gln
Guanidinium chloride	GnHCl
Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium	HATU
High-performance liquid chromatography	HPLC
Interleukin-2	IL-2
Liquid chromatography–mass spectrometry	LC-MS
Magnesium chloride	$MgCl_2$
Maleimide	Mal
Maleimide-Lysinen	Mal-K_n
Methionine	Met
N,N-Diisopropylethylamine	DIPEA
Native chemical ligation	NCL
Palladium(II) chloride	$PdCl_2$
Recombinant IL-2	rIL-2
Reversed-phase High Performance Liquid Chromatography	RP-HPLC
Segement	Seg
Serine/Threonine Ligation	STL
Signal transducer and activator of transcription	STAT
Sodium nitrite	$NaNO_2$
Solid-phase peptide synthesis	SPPS
Succinimide	Su
Synthetic IL-2	sIL-2
T8P insulin	T8P
Thiazolidine	Thz
Trifluoroacetic acid	TFA
Triisopropylsilane	TIS
Tris(hydroxymethyl)aminomethane	tris
Trityl	Trt
α-Ketoacid-Hydroxylamine Amide-Forming Ligation	KAHA ligation
(Glutamic acid, Lysine)n	(EK)n

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I. General Information

1.1 General reagents and materials

All commercial chemical reagents (Fisher, ChemPep, PurePep, CHEM-IMPEX, Novabiochem, Oakwood chemical, Sigma Aldrich, Acros, TCI, Adamas, *etc*.) were used directly without purification. All solvents were HPLC grade or reagent grade (Fisher chemical, Sigma Aldrich). Ultrapure Milli-Q water (deionized) was generated from the Milli-Q IQ 7000 water purification system (Merck, Darmstadt, Germany). All NCL and solubilizing tag-removal reactions were performed under nitrogen. 2-Chlorotrityl Chloride Resin (2-CTC) and Rink Amide Resin were purchased from ChemPep. 4-Maleimidobutyric acid was purchased from Chemscene (Monmouth Junction, New Jersey, USA)**.** Recombinant Human IL-2 (Mammalian-expressed and Carrier Free) protein was purchased from R&D Systems, Bio-Techne (Minneapolis, Minnesota, USA). Native insulin was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Crude peptides were filtered with a Basix™ Syringe 13 mm filter with 0.45 μm pore size prior to HPLC purification.

1.2 HPLC and LC-MS

HPLC

All crude peptides and proteins were purified with a mobile phase of 0.1% (*v/v*) TFA in water (solvent A) and 0.1% (*v/v*) TFA in acetonitrile (solvent B) on an Agilent 1260 HPLC system. This system consisted of Agilent 1260 Infinity Quaternary pumps and a 1260 Infinity II UV detector. The wavelengths of UV detector were set to 220 nm and 280 nm. Preparative HPLC separations were performed using Phenomenex C5 column (Luna® 250 × 21.2 mm, 10 μm, 100 Å) and Phenomenex C18 column (Luna® 250 × 21.2 mm, 5 μm, 100 Å) at a flow rate of 5 mL/min. Semi-preparative HPLC separations were performed using Phenomenex C4 column (Jupiter® 250 × 10 mm, 5 μm, 300 Å) and Phenomenex C18 column (Jupiter® 250 × 10 mm, 5 μm, 300 Å) at a flow rate of 3 mL/min. Analytical HPLC separations were performed using Phenomenex C4 column (Jupiter® 250 × 4.6 mm, 5 μm, 300 Å) and Phenomenex C18 column (Jupiter® 100 × 4.6 mm, 5 μm, 300 Å) at a flow rate of 1 mL/min and column temperature of 30 °C.

LC-MS

LC-MS analyses were performed using an Agilent 6120 Quadrupole LC/MS system equipped with a Phenomenex C4 column (Jupiter® 50 × 2 mm, 5 μm, 300 Å) at a flow rate of 0.4 mL/min with a mobile phase of 0.1% (*v/v*) formic acid in water and 0.1% (*v/v*) formic acid in acetonitrile. The wavelengths of UV detector were set to 220 nm and 280 nm. The column temperature was set to 40 °C.

II. General Procedures

2.1 Preparation of pre-loaded resins for solid phase peptide synthesis

Loading Fmoc-hydrazine on 2-CTC Resin

2-CTC resin (250 mg) was washed with DCM (3 × 5 mL) and then swelled with DCM (3 mL) in a 10 mL reactor vial before the reaction. A solution of Fmoc-hydrazine (0.05 mmol) in DMF was combined with DIPEA (1 mL) and then was transferred to the reactor vial. The vial was rotated for 2 hours at room temperature. Without draining the reaction vial, MeOH (160 µL) was added to the mixture and the vial was rotated for > 10 min. Finally, after washing with DMF (3 × 5 mL), the peptide resin was immediately transferred to the synthesizer for the subsequent synthesis. If storing resin: wash with DCM (3 × 5 mL), dry for 20 minutes, and store at 4 °C.

Loading the first Fmoc-Xaa on 2-CTC Resin

2-CTC resin (250 mg) was washed with DMF (3 × 5 mL) and then DCM (3 × 5 mL) in a 10 mL reactor vial before the reaction. The first C-terminal amino acid (0.1 mmol) of the target sequence and DIPEA (87.1 µL, 0.5 mmol) were dissolved in DMF/DCM (1:1, 2.5 mL). This solution was mixed with 2-CTC resin (250 mg) and rotated for 2 hours at room temperature. The resin was washed with DMF (3 × 5 mL) and DCM (3 × 5 mL) and then capped with DCM/MeOH/DIPEA (17/2/1, 5 mL) for 5 seconds 5 times. Finally, after washing with DCM (3 × 5 mL) and DMF (3 × 5 mL), it was transferred to the synthesizer for the subsequent synthesis.

2.2 9-Fluorenylmethoxycarbonyl solid-phase peptide synthesis (Fmoc SPPS)

Peptides were synthesized using Fmoc SPPS on a Multiple Synthesizer SYRO I (MultiSynTech GmbH, Witten, Germany) with a vortex stirring system. Fmoc SPPS was performed using the following general protocol. Fmoc was deprotected with 20% 4-methylpiperidine in DMF (1.2 mL) twice at room temperature for 10 minutes. Then, a DMF washing step was performed to wash the resin (1.3 mL × 5). Fmoc amino acids (5 equiv.), HATU (4.8 equiv.), DIPEA (10 equiv.), and DMF (2.5 mL) were mixed with resin for 10 minutes at 50 °C (Cys and His) or at 70 °C (all other amino acids). After the reaction, the resin was washed with DMF (1.3 mL × 3) between amino acid coupling and Fmoc deprotection cycles.

2.3 Peptide Cleavage

General procedure of resin cleavage and global deprotection:

Resin was washed with DCM (5 mL × 3) before cleavage and placed under vacuum for one hour to ensure that it was dry. Resin cleavage and global deprotection were

performed using TFA cleavage cocktails (see below) for 2.5 hours. Next, peptide solutions were filtered away from the resin, and the peptides were precipitated by adding to cold diethyl ether (40 mL). After centrifuging at 3000 × g for 3 minutes, the supernatant was discarded, and the precipitates were washed with diethyl ether (40 mL × 2). Then crude peptides were dried under vacuum for >5 min, dissolved in CH_3CN/H_2O (0.1% TFA), analyzed by ESI-MS, and purified by preparative RP-HPLC.

TFA cleavage cocktails used for different types of peptides:

TFA cleavage cocktails for peptides that do not contain Met or Cys:

- 3 mL TFA, 75 µL TIS, 75 µL water;

for peptides that contain Cys, but not Met:

- 3 mL TFA, 75 µL TIS, 75 µL water, 75 µL EDT;

for peptides that contain Met:

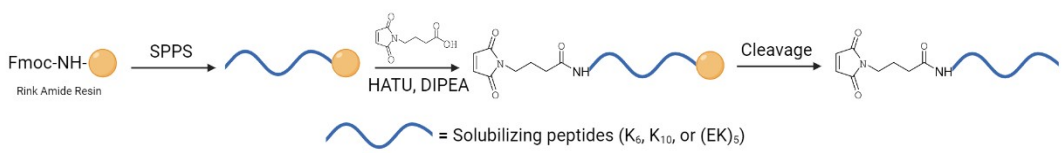
- 3 mL TFA, 75 µL TIS, 75 µL water, 75 µL EDT, 45 mg NH_4I.

When using NH_4I, the powder is directly placed on top of the peptide resin prior to adding the remaining cleavage cocktail.

2.4 Hydrazide-based native chemical ligation

The peptide hydrazide (2 mM, 1 equiv.) was dissolved in activation buffer (6 M GuHCl, 0.1 M phosphate, pH 2.3). The solution was then pre-cooled at -20 ℃ for 15 min. Freshly prepared $NaNO_2$ (0.5 M, dissolved in 6 M GuHCl, 0.1 M phosphate, pH 2.3, 10 equiv.) was added and the resulting mixture was incubated at -20 ℃ for 30 min. Following activation, freshly prepared MPAA (0.2 M, dissolved in 6 M GuHCl, 0.2 M phosphate, pH 6.8, 100 equiv.) was added and the mixture was stirred at room temperature for 30 min. When the thioesterification was completed, peptide (0.8 equiv.) with an N-terminal Cys was added to the reaction mixture and the pH was adjusted to 6.8. The reaction was stirred at room temperature or 37 ℃ and progress was monitored by LC-MS. To monitor the reaction progress, micro-samples were reduced with an equal volume of reducing buffer (6 M GuHCl, 0.2 M phosphate, 0.2 M TCEP HCl, pH = 6.8) before injection into the LC-MS. Upon completion, reactions were treated with reducing buffer for 10 min, and spun at 3000 × g to separate pellet and supernatant. After analysis to determine which part (either supernatant or pellet) contains the product, the compound was purified by preparative or semi-preparative HPLC. See below for further discussion on dealing with challenging, poorly soluble intermediates.

2.5 Synthesis of Mal-based solubilizing tag



Scheme S1: Synthetic route of Mal-based solubilizing tags.

The resin-bound peptide was prepared according to General Procedure 2.1 and 2.2 using Rink Amide Resin (0.56 mmol/g, 0.1 mmol). After SPPS of the solubilizing sequence, a solution of 4-maleimidobutyric acid (55 mg, 0.3 mmol), HATU (114 mg, 0.3 mmol), and DIPEA (52 µL, 0.3 mmol) in DMF (4 mL) was added to the resin. This reaction was performed for 4 hours at room temperature. After the reaction, the resin was washed with DMF (5 mL× 3) and DCM (5 mL× 3). Then, the target peptide was cleaved from resin by General Procedure 2.3. Coupling reaction of 4-maleimidobutyric acid and peptide on resin was monitored by LC–MS with microcleavage of the resin. Finally, the crude peptide was dissolved in 10 mL of CH_3CN/H_2O (10/90, 0.1% TFA, v/v) and purified by preparative HPLC (Phenomenex C18 column, Luna® 250 × 21.2 mm, 5 µm, 100 Å, linear gradient **10-50%** solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide target product.

2.6 Conjugation of Cys with maleimidated solubilizing tag

The target peptide (2 mM, 1 equiv.) was dissolved in 6 M GuHCl, 0.2 M phosphate buffer, pH 6.8 and placed in -20 °C freezer for 10 min. A solution of maleimidated solubilizing tag (10 mM, 1.2 equiv.) in 6 M GuHCl, 0.2 M phosphate buffer, pH 6.8 was added to the peptide solution and allowed to warm to room temperature for 30 min. The reaction was monitored by LC-MS. Upon completion of the reaction, the solution was centrifuged to remove the pellet, and the supernatant was purified by semi-preparative HPLC.

2.7 Removal of solubilizing tag

$PdCl_2$ (5.3 mg, 0.03 mmol) was dissolved in 300 µL of degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8), and the mixture was stirred to complete dissolution under sonication. $MgCl_2$ (30 mg, 0.315 mmol) was dissolved in 315 µL of degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8). The target peptide was dissolved in degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8). Then $PdCl_2$ and $MgCl_2$ were added to the solution, and the resulting mixture was stirred at 37 °C. The reaction was monitored by LC-MS. Upon completion of the reaction, an equal volume of DTT (1 M, dissolved in 6 M GuHCl, 0.2 M phosphate, pH 6.8) or DTC (0.2 M dissolved in water) was added, and the resulting mixture was further incubated for 15 min.

2.8 Determining peptide concentration using NanoDrop

NanoDrop spectrophotometer (NanoDrop Technologies) was utilized for the precise determination of peptide/protein concentration. To establish a baseline, a blank measurement is conducted with solution buffer. Then, a small volume (~ 3 µL) of the protein sample is pipetted onto designated testing point. The absorbance of the sample is measured at the characteristic protein wavelength of 280 nm. The protein concentration is subsequently calculated using the recorded absorbance and the specific molar extinction coefficient for the given peptide/protein. The resulting

concentration data is then available for further analysis and experimental conclusion.

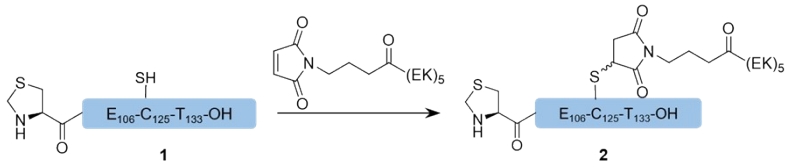
III. Conjugation and Removal of Solubilizing Tag on the Model Peptide

IL(105-133) ***(1)***

1

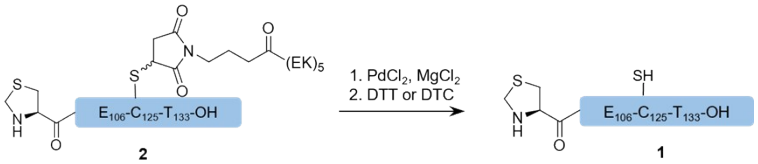
Crude peptide **1** was prepared according to General Procedure **2.1**, **2.2** and **2.3** using 2-CTC resin (0.89 mmol/g, 0.1 mmol), Fmoc-Gln(Trt)-Ser($\psi^{Me,Me}$pro)-OH, Fmoc-L-thiazoline-4-carboxylic acid and other standard amino acids.

IL(105-133)-Su-EK$_5$ ***(2)***



1 2

After global deprotection, crude peptide **1** (3.4 mg) and maleimide-$(EK)_5$ (1.76 mg, 1.2 µmol) were subjected to the conjugation conditions following General Procedure **2.6**. After centrifuging at 3000 × g for 3 minutes, the supernatant was purified by preparative HPLC (Phenomenex C18 column, Luna® 250 × 21.2 mm, 5 µm, 300 Å, linear gradient 25-65% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **2** (0.51 mg, 15%, two steps) as a fluffy white solid.

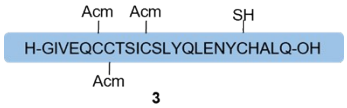


2 1

$PdCl_2$ (5.3 mg, 0.03 mmol) was dissolved in 300 µL of degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8), and the mixture was stirred for complete dissolution under sonication. $MgCl_2$ (30 mg, 0.315 mmol) was dissolved in 315 µL of degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8). Peptide **2** was dissolved in a degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8). Then $PdCl_2$ and $MgCl_2$ were added to the solution, and the resulting mixture was stirred at 37 °C for 2 hours. The reaction was monitored by LC-MS. After two hours, an equal volume of DTT (1 M, dissolved in 6 M GuHCl, 0.2 M phosphate, pH 6.8) or DTC (0.2 M dissolved in water) was added, and the resulting mixture was further incubated for 15 min. The reaction solution was purified by semi-preparative HPLC (Phenomenex C4 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 35-85% solvent B over 50 min). All fractions were analyzed by LC-MS, then

collected and lyophilized to provide **1** as a fluffy white solid.

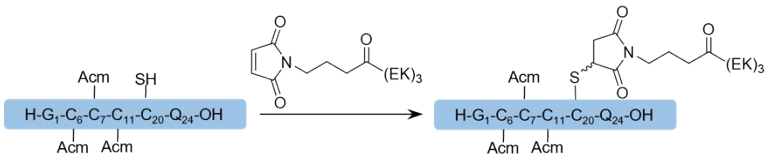
Mini insulin A chain (3)



3

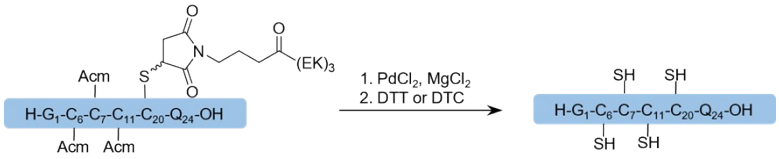
Peptide **3** was prepared according to General Procedure **2.1**, **2.2** and **2.3** using 2-CTC resin (0.89 mmol/g, 0.1 mmol), Fmoc-Cys(Acm)-OH, and other standard amino acids.

Mini insulin A chain-Su-$(EK)_3$ (4)



3 **4**

After global deprotection, crude peptide **3** (13 mg) and maleimide-$(EK)_3$ (5 mg, 5.3 µmol) were subjected to the conjugation conditions following General Procedure **2.6**. After centrifuging at 3000 × g for 3 minutes, the supernatant was purified by preparative HPLC (Phenomenex C18 column, Luna® 250 × 21.2 mm, 5 µm, 300 Å, linear gradient 20-60% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **4** (3.1 mg, 18%, two steps) as a fluffy white solid.



4 **5**

$PdCl_2$ (5.3 mg, 0.03 mmol) was dissolved in 300 µL of degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8), and the mixture was stirred for complete dissolution under sonication. $MgCl_2$ (30 mg, 0.315 mmol) was dissolved in 315 µL of degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8). Peptide **4** (0.39 mg, 0.1 µmol) was dissolved in degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8, 50 µL). Then $PdCl_2$ and $MgCl_2$ were added to the solution, and the resulting mixture was stirred at 37 °. The reaction was monitored by LC-MS. After reaction, an equal volume of DTT (1 M, dissolved in 6 M GuHCl, 0.2 M phosphate, pH 6.8) or DTC (0.2 M dissolved in water) was added, and the resulting mixture was further incubated for 15 min. The reaction solution was centrifuged to remove the pellet and the supernatant was purified by semi-preparative HPLC (Phenomenex C4 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 20-60% solvent B over 40 min). All the fractions were analyzed by LC-MS, then collected and lyophilized to provide **5** as a fluffy white solid.

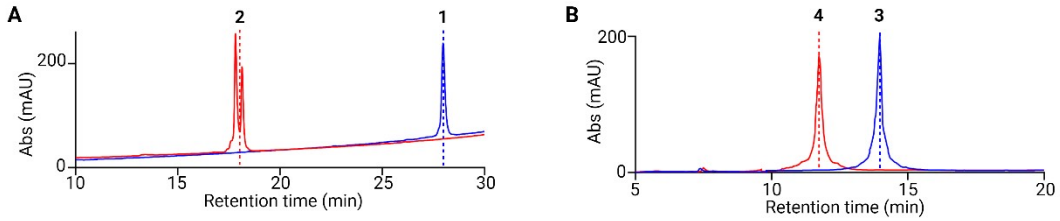


Figure S1: Analytical HPLC traces of peptides **1**, **2** and **3**, **4**. (Liner gradient of 10-90% solvent B over 30 min, 1 mL/min, Jupiter® 250 × 4.6 mm, C4 column)

IV. Preparation and Assembly of Peptide Segments for IL-2

IL(1-57)–NHNH$_2$ (6)

H-APTSSSTK**KT**QLQLEHLLLDLQMILNGINNYKNPKLTRM**LT**FKFYMPKKATELKHLQ-NHNH$_2$

6

Peptide **6** was prepared according to General Procedure **2.1**, **2.2** and **2.3** using 2-CTC resin (0.89 mmol/g, 0.05 mmol), Fmoc-Lys(Boc)-Thr(ψ^{Me,Me}pro)-OH, Fmoc-Leu-Thr(ψ^{Me,Me}pro)-OH and other standard amino acids. After global deprotection, the crude peptide was dissolved in 15 mL of CH_3CN/H_2O (25/75, 0.1% TFA, v/v) and purified by preparative HPLC (Phenomenex C5 column, Luna® 250 × 21.2 mm, 10 µm, 100 Å, linear gradient 25-65% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **6** (72 mg, 21.6%) as a fluffy white solid.

IL(1-57)–MPAA (6-MPAA)

H-APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQ-MPAA

6-MPAA

Crude peptide **6** (320 mg, 48 µmol) and MPAA (161 mg, 20 equiv., 960 µmol) were dissolved in 24 mL buffer (6 M GuHCl, 0.1 M phosphate, pH 2.3), and the final pH was adjusted to 3. Then acetyl acetone (acac, 12.25 µL, 2.5 equiv.) was added to the reaction solution, and the mixture was stirred overnight at room temperature under nitrogen atmosphere. After centrifuging at 3000 × g for 3 minutes, the supernatant was purified by preparative HPLC (Phenomenex C5 column, Luna® 250 × 21.2 mm, 10 µm, 100 Å, linear gradient 30-70% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **6-MPAA** (106 mg, 15.6%, two steps) as a fluffy white solid.

IL(58-104)-NHNH$_2$ (7)

H-CLEEELKPLEEVLNLAQSKNFHLRPRDL**IS**NINVIVLELK**GS**ETTFM-NHNH$_2$

7

Peptide **7** was prepared according to General Procedure **2.1**, **2.2** and **2.3** using 2-CTC resin (0.89 mmol/g, 0.05 mmol), Fmoc-Ile-Ser(ψ^{Me,Me}pro)-OH, Fmoc-Gly-Ser(ψ^{Me,Me}pro)-OH, and other standard amino acids. After global deprotection, the crude

peptide was dissolved in 15 mL of CH_3CN/H_2O (30/70, 0.1% TFA, v/v) and purified by preparative HPLC (Phenomenex C5 column, Luna® 250 × 21.2 mm, 10 µm, 100 Å, linear gradient 30-70% solvent B over 40 min). All the fractions were analyzed by LC-MS, then collected and lyophilized to provide **7** (85 mg, 31.3%) as a fluffy white solid.

IL(1-104)-NHNH$_2$ (8)

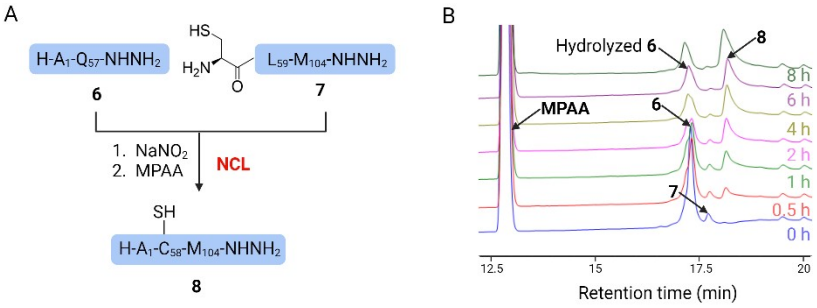
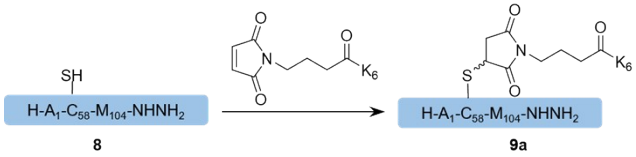


Figure S2: The NCL of **6** and **7**.

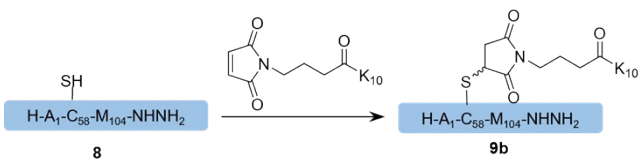
Peptide hydrazides **6** (20 mg, 3.00 µmol) and **7** (13 mg, 2.40 µmol) were subjected to the ligation conditions following General Procedure **2.4**. The reaction mixture was stirred at room temperature for 8 hours, followed by addition of reducing buffer. During the reaction, a significant amount of pellet formation was observed. Analysis of both the supernatant and the pellet fractions revealed the presence of the product primarily in the pellet. After centrifuging at 3000 × g for 3 minutes, the pellet was dissolved in $H_2O/CH_3CN/AcOH$ = 65/30/5 and purified by semi-preparative HPLC (Phenomenex C4 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 30-70% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **8** (16 mg, 55%) as a fluffy white solid.

IL(1-104)-NHNH$_2$-Su-K$_6$ (9a)



Peptide **8** (6 mg, 0.50 µmol) and maleimide-K_6 (0.57 mg, 0.60 µmol) were subjected to the conjugation conditions following General Procedure 2.6. After centrifuging at 3000 × g for 3 minutes, the supernatant and the pellet (dissolved in $H_2O/CH_3CN/AcOH$ = 65/30/5) were purified by semi-preparative HPLC (Phenomenex C4 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 30-70% solvent B over 40 min). All the fractions were analyzed by LC-MS, then collected and lyophilized to provide 9a (5.3 mg, 82%) as a fluffy white solid.

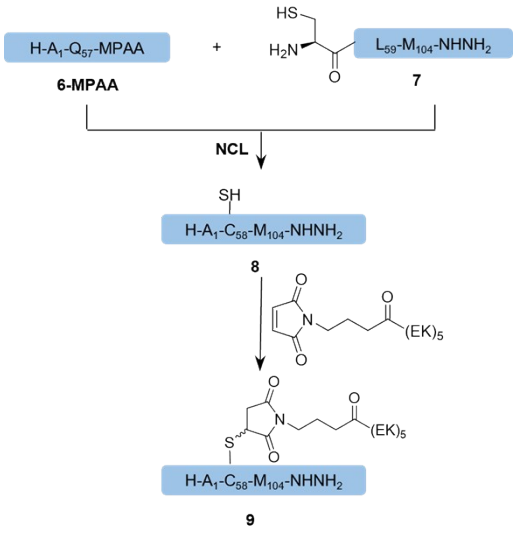
IL(1-104)-NHNH$_2$-Su-K$_{10}$ (9b)



Peptide **8** (6 mg, 0.50 µmol) and maleimide-K_{10} (0.88 mg, 0.60 µmol) were subjected

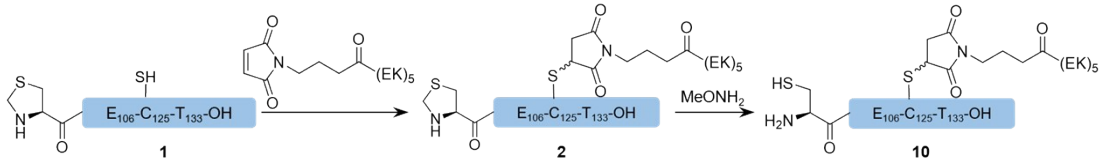
to the conjugation conditions following General Procedure **2.6**. After centrifuging at 3000 × g for 3 minutes, the supernatant and the pellet (dissolved in $H_2O/CH_3CN/AcOH$ = 65/30/5) were purified by semi-preparative HPLC (Phenomenex C4 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 30-70% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **9b** (5.8 mg, 86%) as a fluffy white solid.

IL(1-104)-NHNH$_2$-Su-(EK)$_5$ (9)



Peptide **6-MPAA** (62 mg, 9.1 µmol) and **7** (49.5 mg, 9.1 µmol) were dissolved in 6 M GuHCl, 0.2 M phosphate buffer (9 mL, pH 6.8) and stirred at room temperature under N_2. After 30 minutes of the reaction, tris(2-carboxyethyl)phosphine (TCEP, 0.9 mL, 0.2 M in 6 M GuHCl, 0.2 M phosphate buffer, pH 6.8) was introduced into the reaction mixture, and the reaction continued for 5 hours until complete. Then, the reaction mixture was placed in a -20°C refrigerator for 10 minutes. Subsequently, the Mal-$(EK)_5$ (20 mg, 13.65 µmol) dissolved in 6 M GuHCl, 0.2 M phosphate buffer (1 mL, pH 6.8) was mixed with the reaction mixture and allowed to react at -20°C for 30 minutes. The mixture was purified by semi-preparative HPLC (Phenomenex C4 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 30-55% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **9** (65 mg, 53%, two steps) as a fluffy white solid.

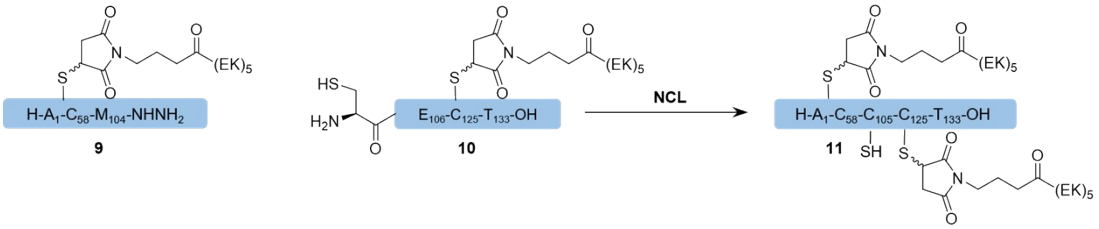
IL-(105-133)-Su-EK$_5$ (10)



After global deprotection, crude peptide **1** (200 mg) and maleimide-$(EK)_5$ (174 mg, ~ 0.12 mmol) were dissolved in 6 M GuHCl, 0.2 M phosphate buffer (30 mL, pH 6.8), and subjected to the conjugation conditions following General Procedure **2.6**. Due to the large scale, the reaction time was extended to 2 hours. Then, methoxylamine (30

mmol, 1.4 g) was added to the mixture, and the pH was adjusted to 4. The mixture was stirred at room temperature for 4 hours. After centrifuging at 3000 × g for 3 minutes, the supernatant was purified by preparative HPLC (Phenomenex C18 column, Luna® 250 × 21.2 mm, 5 µm, 300 Å, linear gradient 25-65% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **10** (63 mg, 13%, three steps) as a fluffy white solid.

Linear IL-2-(Su-(EK)$_5$)$_2$ (11)



Peptides **9** (15.6 mg, 1.15 µmol) and **10** (4.4 mg, 0.92 µmol) were subjected to the ligation conditions following General Procedure **2.4**. The reaction mixture was stirred at 37 °C for 24 hours, followed by addition of reducing buffer. After centrifuging at 3000 × g for 3 minutes, the supernatant was purified by semi-preparative HPLC (Phenomenex C4 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 10-30% solvent B over 10 min and 30-70% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **11** (8.8 mg, 52%) as a fluffy white solid.

Removal of solubilizing tags on linear IL-2

$PdCl_2$ (5.3 mg, 0.03 mmol) was dissolved in 300 µL of degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8), and the mixture was stirred for complete dissolution under sonication. $MgCl_2$ (30 mg, 0.315 mmol) was dissolved in 315 µL of degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8). Solubilizing tags protected linear IL-2 **11** (2.6 mg, 0.14 µmol) was dissolved in degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 6.8, 140 µL). Then $PdCl_2$ (28 µL, 20 equiv.) and $MgCl_2$ (14 µL, 100 equiv.) were added to the solution, and the resulting mixture was stirred at 37 °C for 5 hours. The reaction was monitored by LC-MS. Upon completion of the reaction, an equal volume of DTT (1 M, dissolved in 6 M GuHCl, 0.2 M phosphate, pH 6.8) was added, and the resulting mixture was further incubated for 15 min. The reaction solution was centrifuged to remove the pellet and the supernatant was purified by semi-preparative HPLC (Phenomenex C4 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 10-35% solvent B over 10 min and 35-85% solvent B over 40 min). All fractions were analyzed

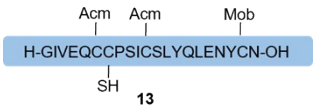
by LC-MS, then collected and lyophilized to provide **12** (1.7 mg, 80%) as a fluffy white solid. The protein was quantified with a NanoDrop spectrophotometer (NanoDrop Technologies).

Protein refolding

The linear IL-2 peptide **12** (0.8 mg, 0.052 µmol) was solubilized in 6 M GuHCl, 0.1 M Tris and 30 mM reduced glutathione solution (pH 8.0, 3 mL) and incubated at room temperature for 30 min until completely dissolved. The mixture was diluted with a buffer (0.1 M Tris and 1.5 mM oxidized glutathione pH 8.0, 6 mL) to obtain a final protein concentration of 0.089 mg/mL and GuHCl concentration of 2 M. The resulting mixture was kept for 24 hours at room temperature to slow the refolding of IL-2. The reaction progress was monitored by LC-MS and analytical HPLC. After centrifuging 3000 × g for 5 minutes, the supernatant was purified by semi-preparative HPLC (Phenomenex C4 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 30-85% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide folded IL-2 (0.264 mg, 33%) as a fluffy white solid. The protein was quantified with a NanoDrop spectrophotometer (NanoDrop Technologies).

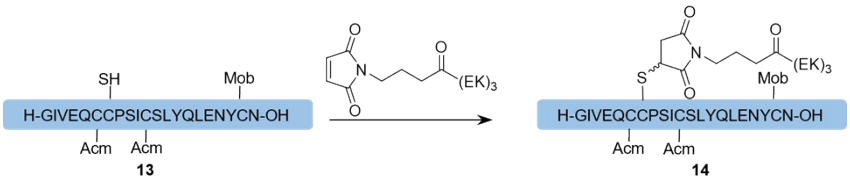
V. Preparation and Assembly of Peptide Segments for T8P Insulin

T8P-A Chain-(Acm)$_2$-SH-Mob (13)



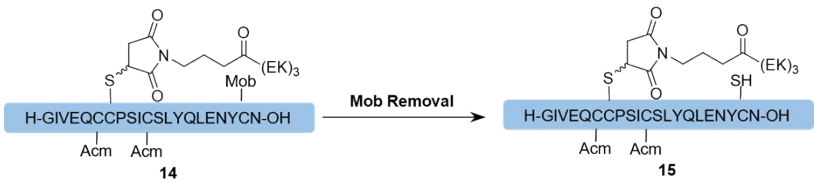
Crude peptide **13** was prepared according to General Procedure **2.1**, **2.2** and **2.3** using 2-CTC resin (0.89 mmol/g, 0.1 mmol), Fmoc-Cys(Acm)-OH, Fmoc-Cys(Mob)-OH, and other standard amino acids.

T8P-A Chain-(Acm)$_2$-Su-(EK)$_3$-Mob (14)



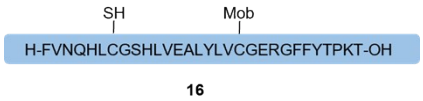
After global deprotection, crude peptide **13** (40 mg) and maleimide-$(EK)_3$ (14.4mg, 0.015 mmol) were subjected to the conjugation conditions following General Procedure **2.6**. After centrifuging at 3000 × g for 3 minutes, the supernatant was purified by preparative HPLC (Phenomenex C18 column, Luna® 250 × 21.2 mm, 5 µm, 300 Å, linear gradient 5-55% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **14** (15 mg, 27%, two steps) as a fluffy white solid.

T8P-A Chain-(Acm)$_2$-Su-(EK)$_3$-SH (15)



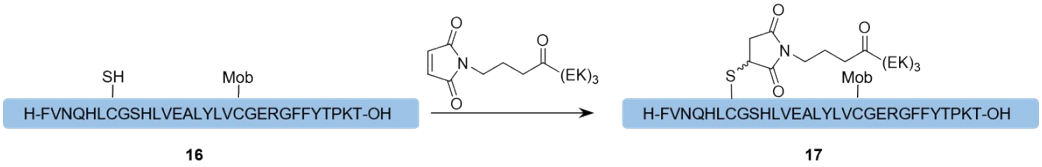
Peptide **14** (15 mg, 4.17 µmol) was added to cleavage buffer (TFA: TIS: H_20 95 %: 2.5 %: 2.5 %, 0.5 mL) and stirred at 45 °C for 2.5 h. Next, the peptides were precipitated by adding to cold diethyl ether (5 mL). After centrifuging at 3000 × g for 3 minutes, the supernatant was discarded, and the precipitates were washed with diethyl ether (5 mL × 2). Then crude peptides were dried under vacuum for >5 min, dissolved in CH_3CN/H_2O (0.1% TFA), and purified by semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 5-55% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **15** (8 mg, 55%) as a fluffy white solid.

T8P-B Chain-SH-Mob (16)



Crude peptide **16** was prepared according to General Procedure **2.1**, **2.2** and **2.3** using 2-CTC resin (0.89 mmol/g, 0.1 mmol), Fmoc-Cys(Acm)-OH, Fmoc-Cys(Mob)-OH, and other standard amino acids.

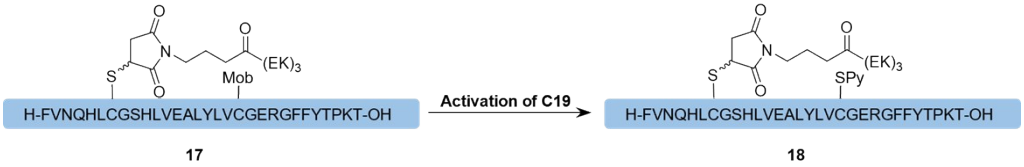
T8P-B Chain-Su-(EK)$_3$-Mob (17)



After global deprotection, crude peptide **16** (22 mg) and maleimide-$(EK)_3$ (5.9 mg, 6.2 µmol) were subjected to the conjugation conditions following General Procedure **2.6**. After centrifuging at 3000 × g for 3 minutes, the supernatant was purified by preparative HPLC (Phenomenex C18 column, Luna® 250 × 21.2 mm, 5 µm, 300 Å, linear gradient 20-60% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **17** (6 mg,

21%, two steps) as a fluffy white solid.

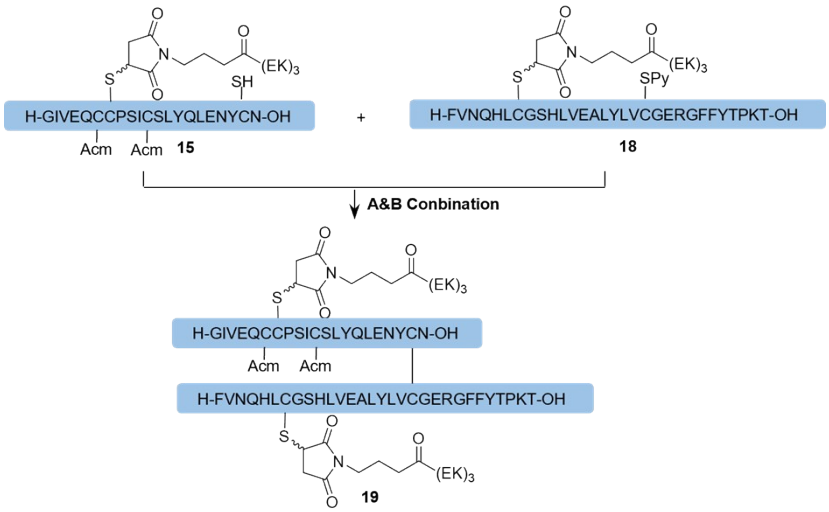
T8P-B Chain-Su-(EK)$_3$-SPy (18)



17 18

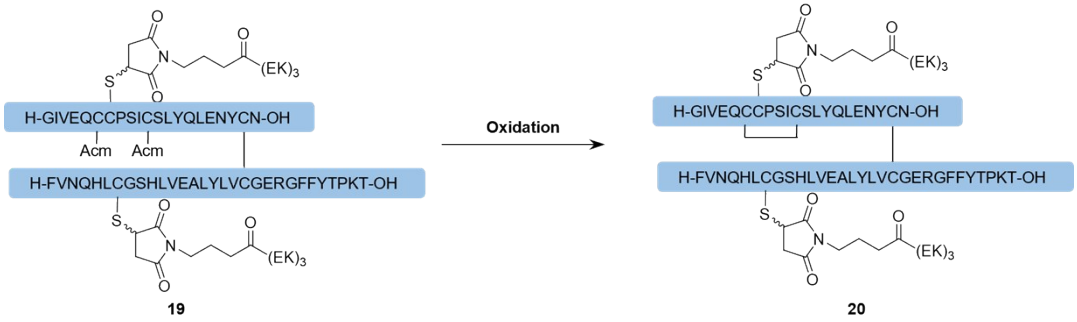
Peptide **17** (6 mg, 1.37 μmol) was added to the cleavage buffer (TFA: TIS: H_20/95%: 2.5%: 2.5%, 0.3 mL) and stirred at 45 ℃ for 4 h. After the mixture was cooled to room temperature, 10 equivalents of 2,2'-Dithiodipyridine (DTDP) (3 mg, 13.7 μmol) were added and stirred for half an hour. Next, the peptides were precipitated by adding to cold diethyl ether (5 mL). After centrifuging at 3000 × g for 3 minutes, the supernatant was discarded, and the precipitates were washed with diethyl ether (5 mL × 2). Then crude peptides were dried under vacuum for >5 min, dissolved in CH_3CN/H_2O (0.1% TFA), and purified by semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 μm, 300 Å, linear gradient 20-65% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **18** (4 mg, 65%) as a fluffy white solid.

A-B Dimer-(Acm)$_2$-(Su-(EK)$_3$)$_2$ (19)



Peptides **15** (3.8 mg, 1.07 μmol) and **18** (4 mg, 0.89 μmol) were dissolved in a 6 M urea buffer containing 0.2 M NH_4HCO_3 (890 μL, pH 8.0) and vigorously stirred for 20 min at room temperature. The reaction was monitored by LC-MS. When **18** was completely consumed, the reaction was diluted with H_2O to 1 mL and the mixture was purified by semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 μm, 300 Å, linear gradient 20-50% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **19** (2.5 mg, 36%) as a fluffy white solid.

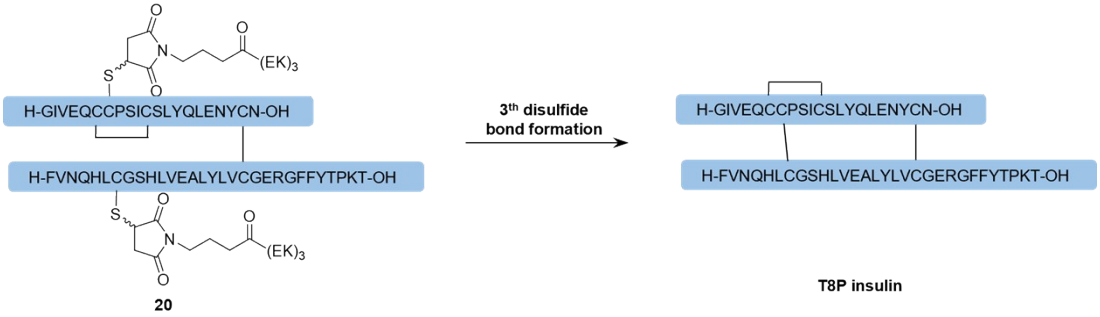
A-B Dimer-(Su-(EK)$_3$)$_2$ (20)



19 **20**

Freshly prepared I_2 solution in MeOH (8 equiv, 28.4 µL, 39.4 µmol/mL) was added drop wise to a stirred solution of **19** (1.1 mg, 0.14 µmol) in AcOH/H_2O = 1: 2 (140 µL), and the mixture was allowed to stir at room temperature for 5 min. The oxidation was quenched with the addition of 1 M ascorbic acid until the dark-red solution turned colorless. The reaction mixture was diluted with H_2O to 1 mL and purified by semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 20-50% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **20** (0.8 mg, 74%) as a fluffy white solid. The protein was quantified with a NanoDrop spectrophotometer (NanoDrop Technologies).

T8P-Insulin



20 **T8P insulin**

$PdCl_2$ (5.3 mg, 0.03 mmol) was dissolved in 300 µL of degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 1), and the mixture was stirred for complete dissolution under sonication. $MgCl_2$ (30 mg, 0.315 mmol) was dissolved in 315 µL of degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 1). Solubilizing tags protected **20** (0.8 mg, 0.1 µmol) was dissolved in degassed buffer (6 M GuHCl, 0.2 M phosphate, pH 1, 200 µL). Then $PdCl_2$ (20 µL, 20 equiv.) and $MgCl_2$ (10 µL, 100 equiv.) were added to the solution, and the resulting mixture was stirred at 37 °C for 2 hours. The reaction was monitored by LC-MS. Upon completion of the reaction, an equal volume of sodium dithiocarbamate (DTC) (0.2 M, dissolved in water, pH 1) was added, and the resulting mixture was further incubated for 30 min. The reaction mixture was diluted with H_2O to 1 mL and purified by semi-preparative HPLC (Phenomenex C18 column, Luna® 250 × 10 mm, 5 µm, 300 Å, linear gradient 20-50% solvent B over 40 min). All fractions were analyzed by LC-MS, then collected and lyophilized to provide **T8P-Insulin** (0.08 mg, 14%) as a fluffy white solid. The protein was quantified with a NanoDrop spectrophotometer (NanoDrop Technologies).

VI. Activity Profiling of Synthesized Protein

Cell-based IL-2 Reporter Assays

The bioactivities of the analogs were evaluated by monitoring secreted embryonic alkaline phosphatase (SEAP) levels induced by the JAK/STAT5 pathway in HEK-Blue™ IL-2 cells derived from HEK293 cells transfected with human CD25 (IL-2Rα), CD122(IL-2Rβ), CD132(IL-2Rγ), JAK3, and STAT5 genes (InvivoGen). Cells were cultured in DMEM (4.5 g/L glucose, 2 mM L-glutamine, Gibco) with 10% v/v fetal bovine serum (FBS, Gibco), 100 U/mL penicillin-streptomycin (Gibco), 100 μg/mL normocin (InvivoGen), 1 μg/mL puromycin (InvivoGen), and 1X HEK-Blue™ CLR Selection (InvivoGen) at 37 °C under 5% CO_2. For the assay, 20 µL of varying concentrations of analogs dissolved in 100 mM AcOH was added per well of a flat-bottom 96-well plate along with 180 µL of 50,000 cells per well suspended in test medium of DMEM supplemented with 10% heat-inactivated FBS (30 min at 56°C, Thermo Fisher) and 100 U/mL penicillin-streptomycin. After 20 hours of incubation at 37°C, 20 µL of IL-2 cell supernatant was added per well of a 96-well plate and 180 μL of QUANTI-Blue™ solution (prepared according to the manufacturer's protocol) was pipetted to each well. The plate was incubated at 37°C for 1 hour and read at 620-655 nm in a SpectraMax iD5 plate reader (Molecular Devices)[1].

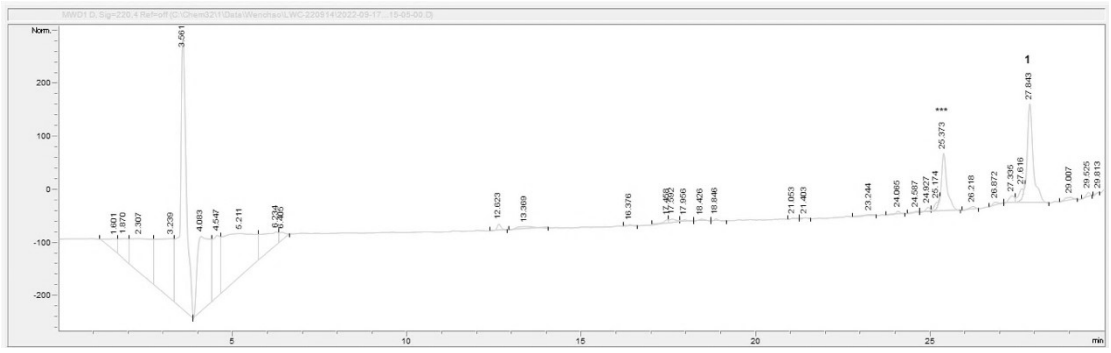
Phospho-AKT (Ser 473) Cell-Based Assay

We employed a standard phosphorylation assay, previously reported[2], to evaluate the pAkt Ser473 levels in a mouse fibroblast cell line NIH 3T3 overexpressing human insulin receptor isoform B (IR-B). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin, and 2.0 mg/mL puromycin. For each assay, 100 µL (∼40,000 cells) per well were plated in 96-well plates with culture media containing 1% FBS. After 20 hours, the original media were removed, and 50 µL of T8P insulin or native insulin series dilution (ranging from 0.003 to 860 nM) was added to each well, followed by a 30-minute incubation period. Subsequently, the insulin solution was removed, and the intracellular pAkt Ser473 level was determined using the HTRF pAkt Ser473 kit (Cisbio, Massachusetts, USA). The cells were lysed by incubation with 50 µL of cell lysis buffer per well for 1 hour under mild shaking. Next, 16 µL of the resulting cell lysate was mixed with 4 µL of detecting reagent in a white 384-well plate. After 4 hours of incubation, the plate was read using a Synergy Neo plate reader, and the data were analyzed following the manufacturer's protocol.

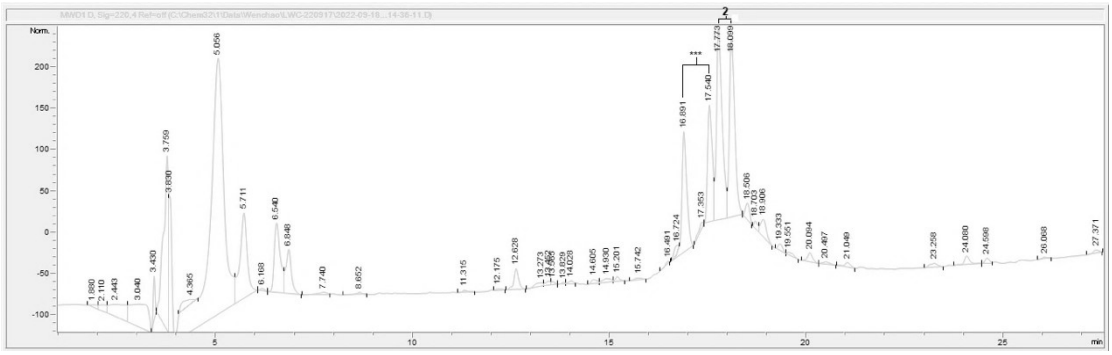
VII. Circular dichroism (CD) spectroscopy

CD spectra were acquired using an OLIS rapid scanning monochromator (RSM) at 25°C with a 1 mm path-length cell. The insulins were dissolved in phosphate-buffered saline (PBS) solution (pH 7.4). The concentration of both insulins was 50 µM. Three spectra were collected from 200 to 250 nm in 0.5 nm increments and subsequently averaged. Data were averaged at each wavelength for a time that varied as a function of voltage applied to the photomultiplier tube[3].

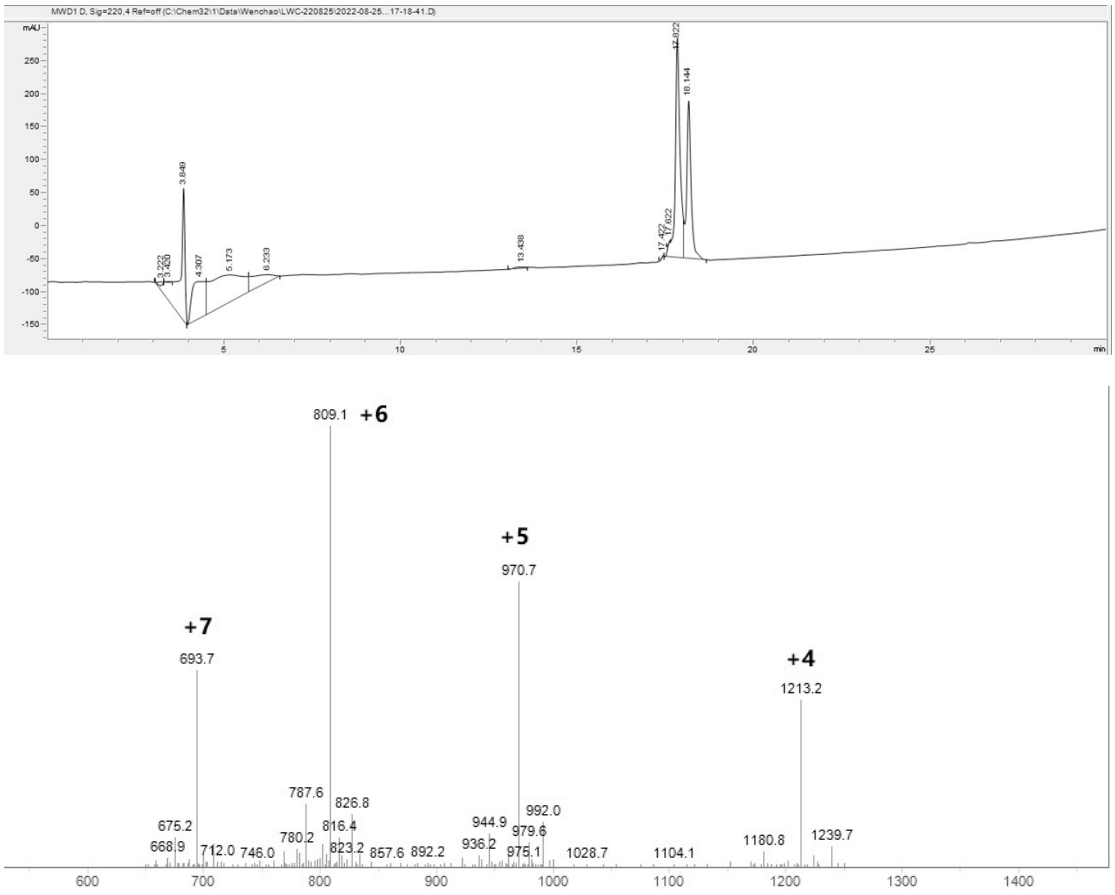
VIII. Analytical HPLC trace and MS



Analytical HPLC trace of crude **1**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 27.843 min; ***The carboxylic acid from the Boc protecting group of Tryptophan was not removed completely).

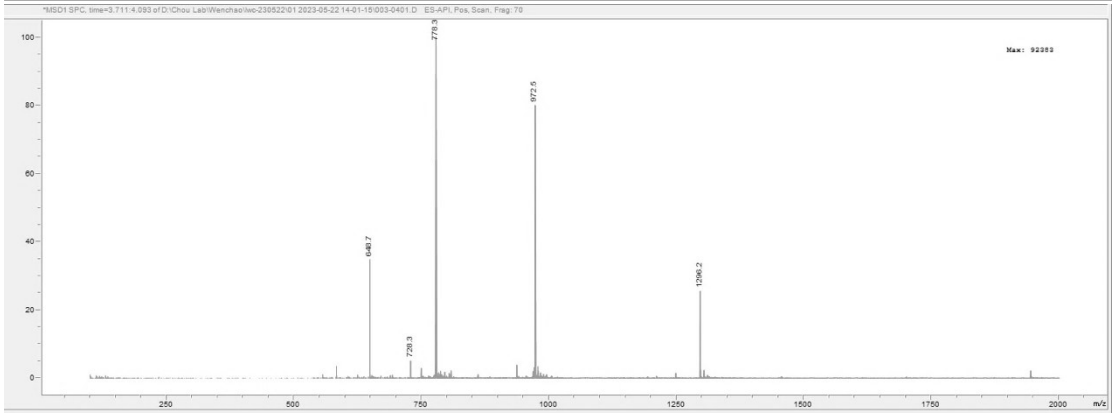


Analytical HPLC trace of reaction mixture (4 hours). (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 17.773, 18.099 min, ***The carboxylic acid from the Boc protecting group of Tryptophan was not removed completely).



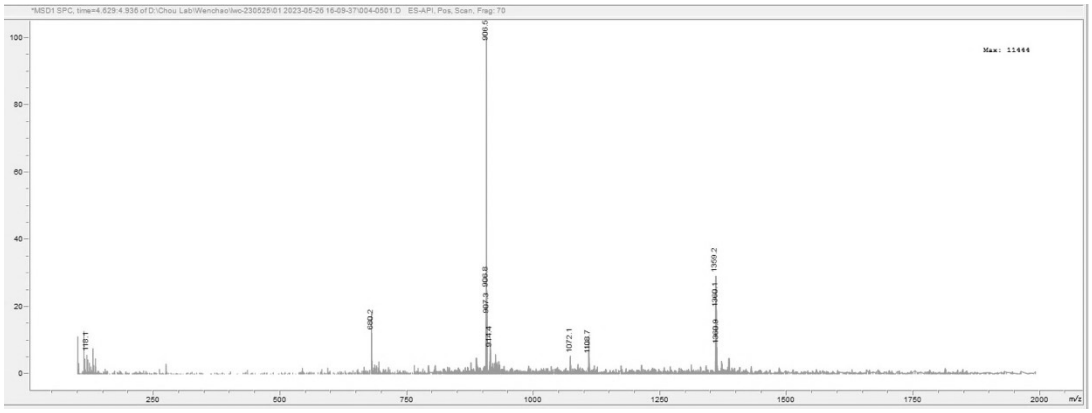
Analytical HPLC trace and ESI-MS of **2**: Upper: analytical HPLC trace of purified **2**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 17.822 min and 18.144 min); Lower: ESI-MS of **2**. Calcd mass: 4849.27 Da (average isotopes), $[M+4H]^{4+}$ m/z = 1213.3, $[M+5H]^{5+}$ m/z = 970.8, $[M+6H]^{6+}$ m/z = 809.2, $[M+7H]^{7+}$ m/z = 693.7; Observed mass: 1213.2, 970.7, 809.1, 693.7.

Ion	Calculated	Observed
+4	1213.3	1213.2
+5	970.8	970.7
+6	809.2	809.1
+7	693.7	693.7



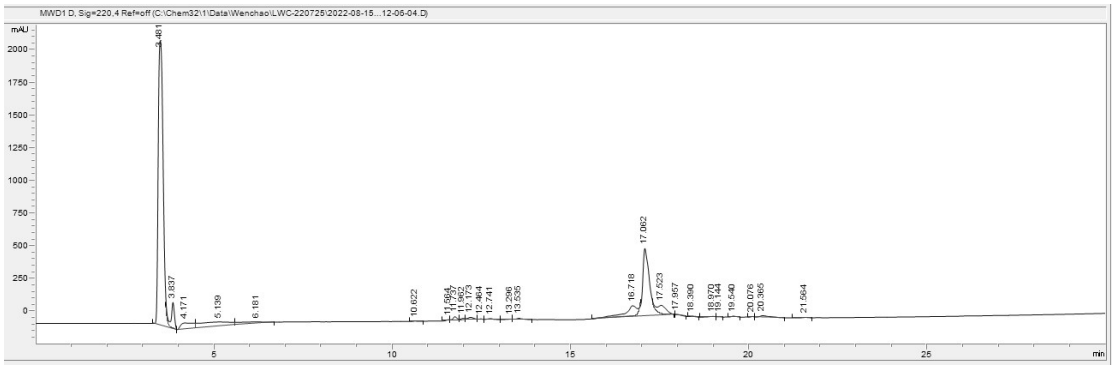
ESI-MS of **4**: Calcd mass: 3886.40 Da (average isotopes), $[M+3H]^{3+}$ m/z = 1296.5, $[M+4H]^{4+}$ m/z = 972.6, $[M+5H]^{5+}$ m/z = 778.3, $[M+6H]^{6+}$ m/z = 648.7; Observed mass: 1296.2, 972.5, 778.3, 648.7.

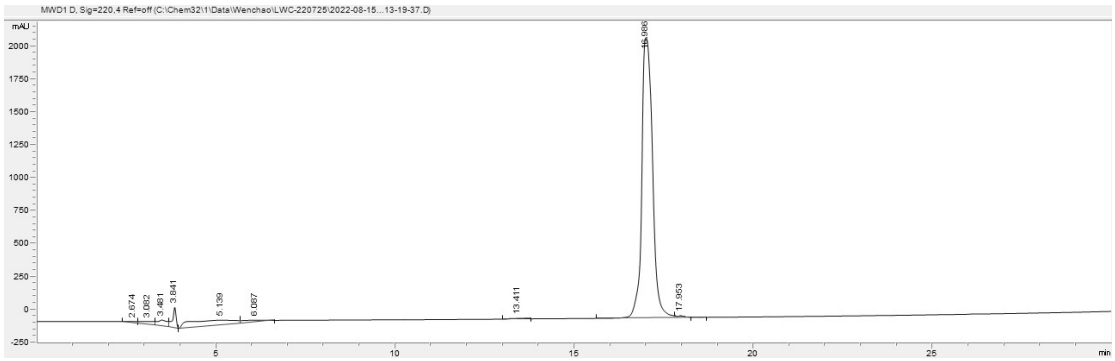
Ion	Calculated	Observed
+3	1296.5	1296.2
+4	972.6	972.5
+5	778.3	778.3
+6	648.7	648.7

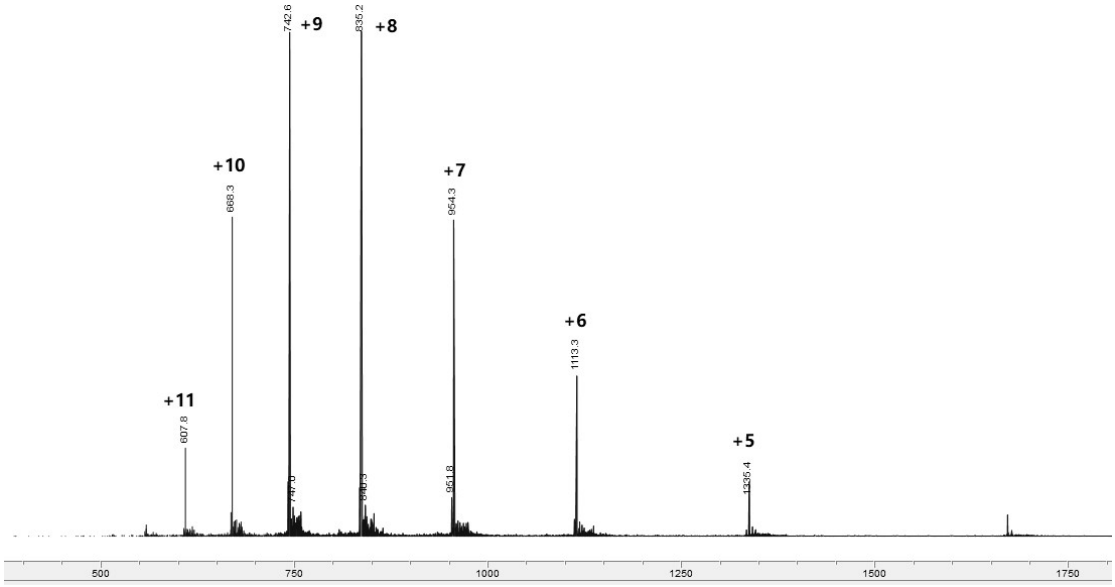


ESI-MS of **5**: Calcd mass: 2719.12 Da (average isotopes), $[M+2H]^{2+}$ m/z = 1360.6, $[M+3H]^{3+}$ m/z = 907.4, $[M+4H]^{4+}$ m/z = 680.8; Observed mass: 1359.2, 906.5, 680.2.

Ion	Calculated	Observed
+2	1360.6	1359.2
+3	907.4	906.5
+4	680.8	680.2

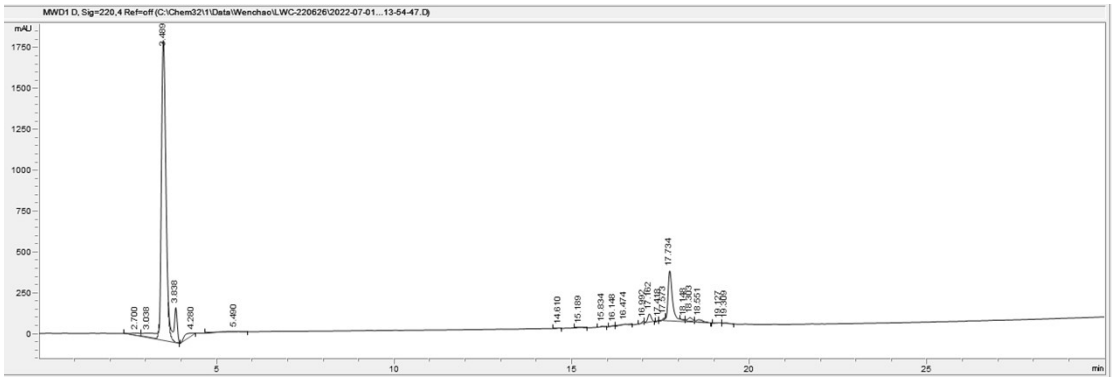


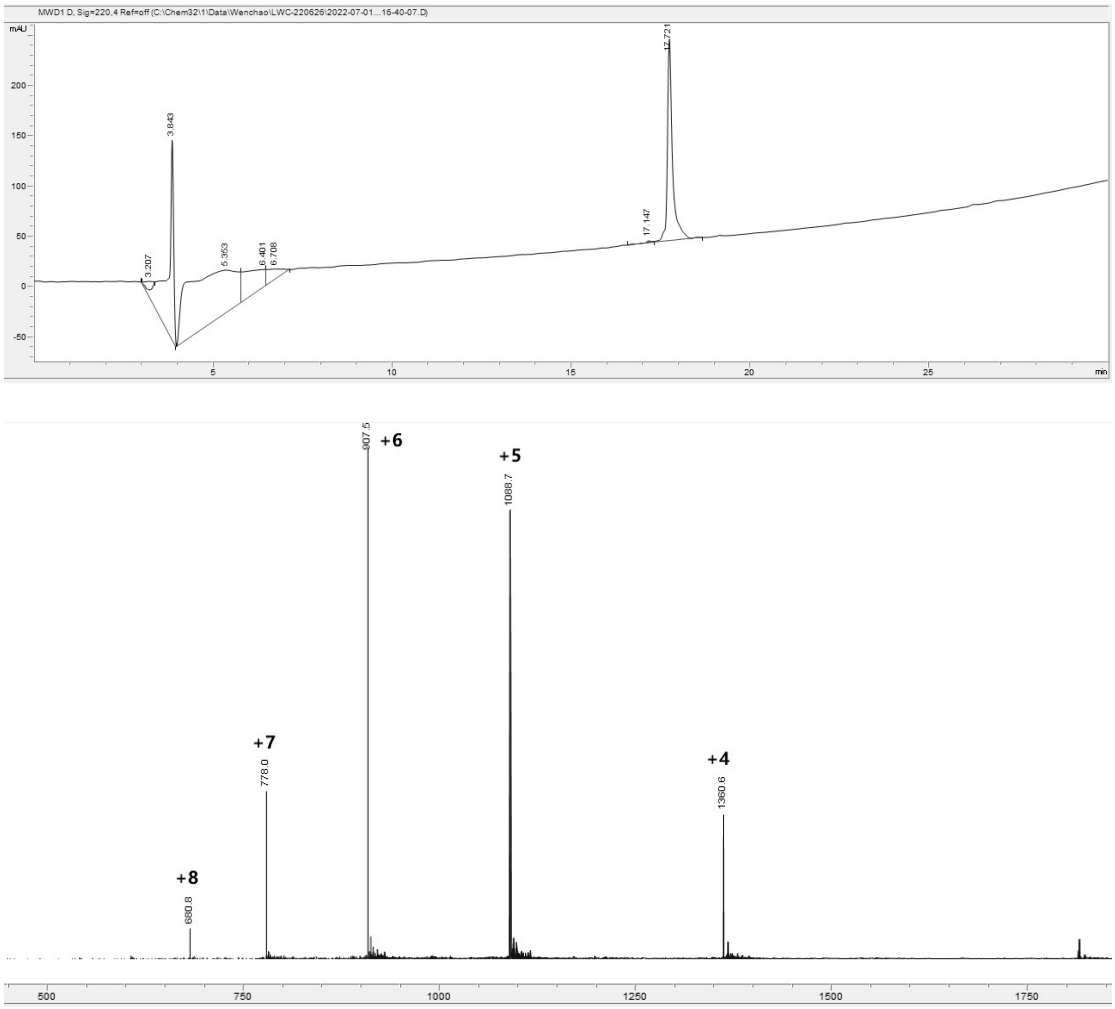




Analytical HPLC trace and ESI-MS of **6**. Upper: analytical HPLC traces of crude and purified **6**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 16.986 min); Lower: ESI-MS of **6**. Calcd mass: 6673.95 Da (average isotopes), $[M+5H]^{5+}$ *m/z* = 1335.8, $[M+6H]^{6+}$ *m/z* = 1113.3, $[M+7H]^{7+}$ *m/z* = 954.4, $[M+8H]^{8+}$ *m/z* = 835.2, $[M+9H]^{9+}$ *m/z* = 742.6, $[M+10H]^{10+}$ *m/z* = 668.4, $[M+11H]^{11+}$ *m/z* = 607.7; Observed mass: 1335.4, 1113.3, 954.3, 835.2, 742.6, 668.3, 607.8.

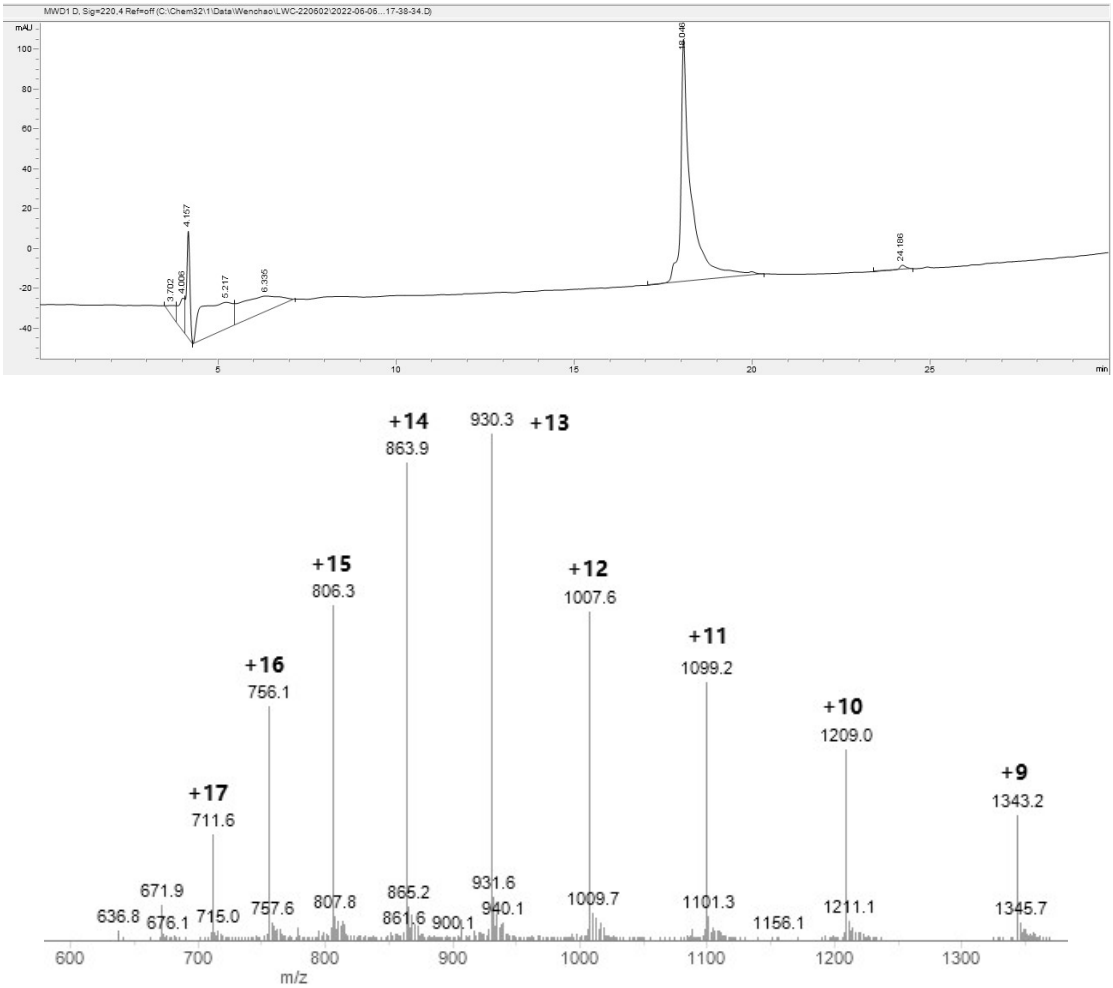
Ion	Calculated	Observed
+5	1335.8	1335.4
+6	1113.3	1113.3
+7	954.4	954.3
+8	835.2	835.2
+9	742.6	742.6
+10	668.4	668.3
+11	607.7	607.8





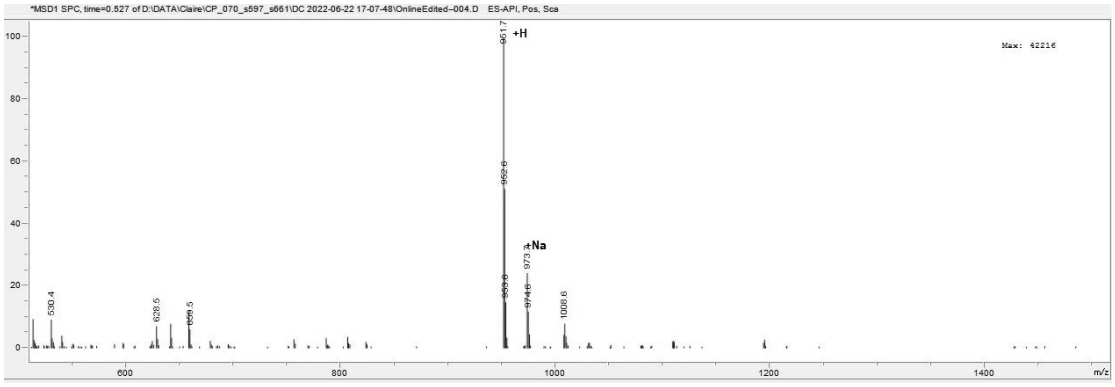
Analytical HPLC trace and ESI-MS of **7**. Upper: analytical HPLC traces of crude and purified **7**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 17.721 min); Lower: ESI-MS of **7**. Calcd mass: 5435.9 Da (average isotopes), $[M+4H]^{4+}$ *m/z* = 1360.0, $[M+5H]^{5+}$ *m/z* = 1088.2, $[M+6H]^{6+}$ *m/z* = 907.0, $[M+7H]^{7+}$ *m/z* = 777.6, $[M+8H]^{8+}$ *m/z* = 680.5; Observed mass: 1360.6, 1088.7, 907.5, 778.0, 680.8.

Ion	Calculated	Observed
+4	1360.0	1360.6
+5	1088.2	1088.7
+6	907.0	907.5
+7	777.6	778.0
+8	680.5	680.8



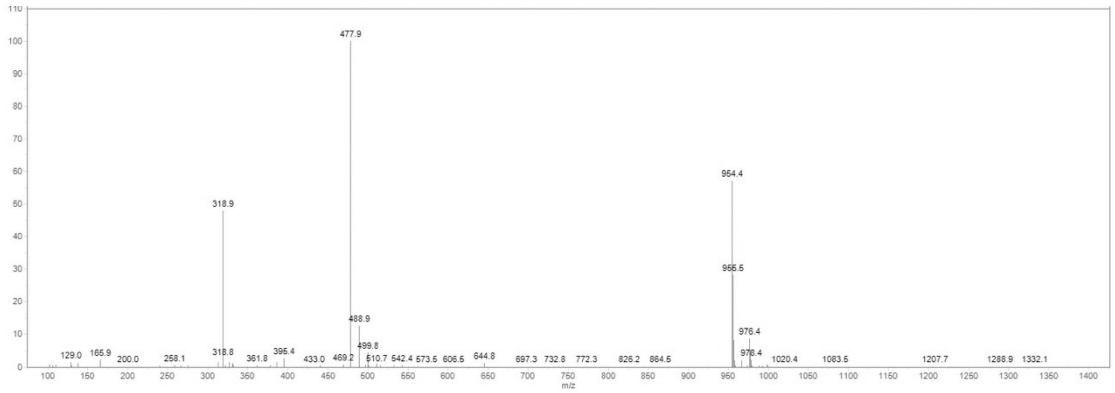
Analytical HPLC trace and ESI-MS of **8**. Upper: analytical HPLC trace of purified **8**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 18.046 min); Lower: ESI-MS of **8**. Calcd mass: 12081.23 Da (average isotopes), $[M+9H]^{9+}$ *m/z* = 1343.3, $[M+10H]^{10+}$ *m/z* = 1209.1, $[M+11H]^{11+}$ *m/z* = 1099.3; $[M+12H]^{12+}$ *m/z* = 1007.8; $[M+13H]^{13}$ *m/z* = 930.3; $[M+14H]^{14+}$ *m/z* = 863.9; $[M+15H]^{15+}$ *m/z* = 806.4; $[M+16H]^{16+}$ *m/z* = 756.1; $[M+17H]^{17+}$ *m/z* = 711.7; Observed mass: 1343.2, 1209.0, 1099.2, 1007.6, 930.3, 863.9, 806.3, 756.1, 711.6.

Ion	Calculated	Observed
+9	1343.3	1343.2
+10	1209.1	1209.0
+11	1099.3	1099.2
+12	1007.8	1007.6
+13	930.3	930.3
+14	863.9	863.9
+15	806.4	806.3
+16	756.1	756.1
+17	711.7	711.6



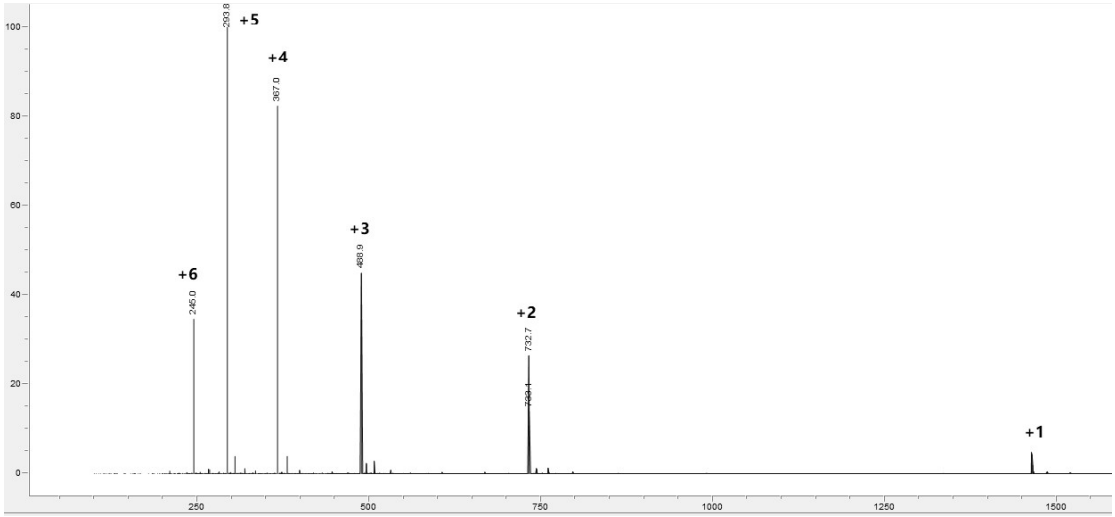
LC-MS analysis of Maleimide-K_6 (**Mal-K_6**). Calcd mass: 951.11 Da (average isotopes), $[M+H]^+$ m/z = 952.11, $[M+Na]^+$ m/z = 974.1; Observed mass: 951.7, 973.7.

Ion	Calculated	Observed
+H	952.11	951.7
+Na	974.1	973.7



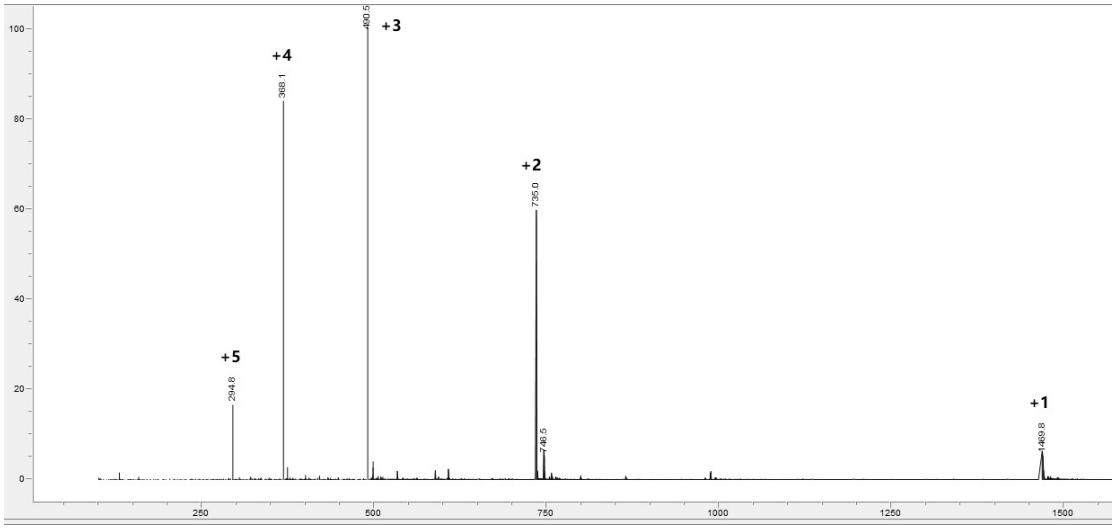
LC-MS analysis of Maleimide-$(EK)_3$ (**Mal-$(EK)_3$**). Calcd mass: 954.05 Da (average isotopes), $[M+H]^+$ m/z = 952.11, $[M+Na]^+$ m/z = 974.1; Observed mass: 951.7, 973.7.

Ion	Calculated	Observed
+H	955.05	954.4
+2H	478.0	477.9
+3H	319.0	318.9



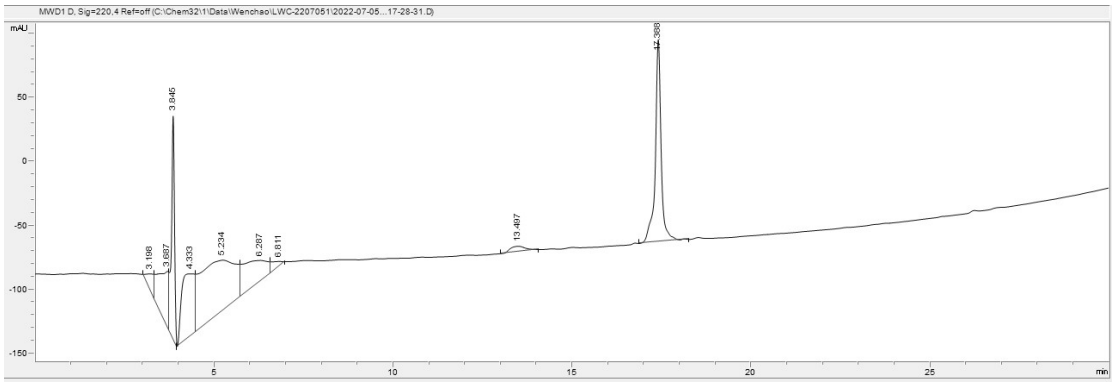
LC-MS analysis of Maleimide-K_{10} (**Mal-K_{10}**). Calcd mass: 1463.9 Da (average isotopes), $[M+2H]^{2+}$ m/z = 732.9, $[M+3H]^{3+}$ m/z = 488.9, $[M+4H]^{4+}$ m/z = 366.9, $[M+5H]^{5+}$ m/z = 293.8, $[M+6H]^{6+}$ m/z = 245.0; Observed mass: 732.7, 488.9, 367.0, 293.8, 245.0.

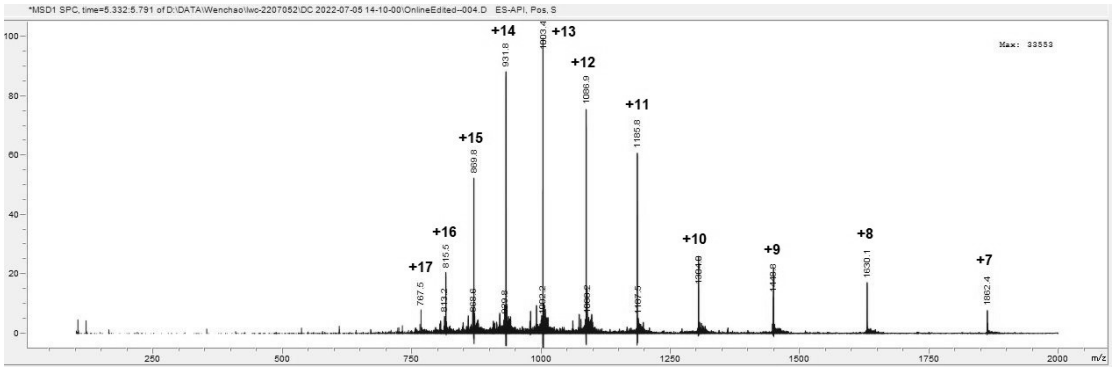
Ion	Calculated	Observed
+2	732.9	732.7
+3	488.9	488.9
+4	366.9	367.0
+5	293.8	293.8
+6	245.0	245.0



LC-MS analysis of Maleimide-$(EK)_5$ (**Mal-$(EK)_5$**). Calcd mass: 1468.6 Da (average isotopes), $[M+H]^{1+}$ m/z = 1469.6, $[M+2H]^{2+}$ m/z = 735.3, $[M+3H]^{3+}$ m/z = 490.5, $[M+4H]^{4+}$ m/z = 368.1, $[M+5H]^{5+}$ m/z = 294.7; Observed mass: 1469.8, 735.0, 490.5, 368.1, 294.8.

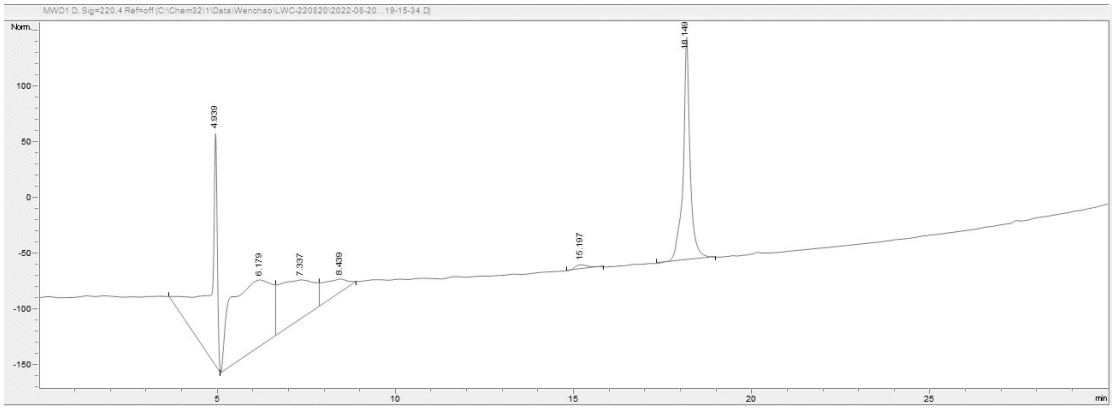
Ion	Calculated	Observed
+1	1469.6	1469.8
+2	735.3	735.0
+3	490.5	490.5
+4	368.1	368.1
+5	294.7	294.8

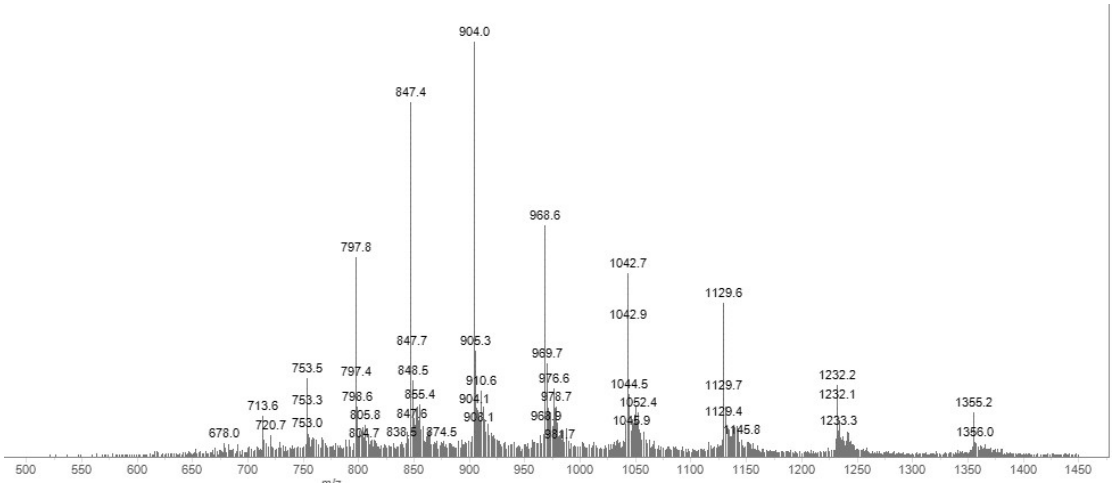




Analytical HPLC trace and ESI-MS of **9a**: Upper: analytical HPLC trace of purified **9a**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 17.388 min); Lower: ESI-MS of **9a**. Calcd mass: 13032.34 Da (average isotopes), $[M+7H]^{7+}$ m/z = 1862.7, $[M+8H]^{8+}$ m/z = 1630.0, $[M+9H]^{9+}$ m/z = 1449.0, $[M+10H]^{10+}$ m/z = 1304.2, $[M+11H]^{11+}$ m/z = 1185.7, $[M+12H]^{12+}$ m/z = 1087.0, $[M+13H]^{13+}$ m/z =1003.5, $[M+14H]^{14+}$ m/z =931.9, $[M+15H]^{15+}$ m/z = 869.8, $[M+16H]^{16+}$ m/z = 815.5, $[M+17H]^{17+}$ m/z = 767.6; Observed mass: 1862.4, 1630.1, 1448.8, 1304.6, 1185.8, 1086.9, 1003.4, 931.8, 869.8, 815.5, 767.5.

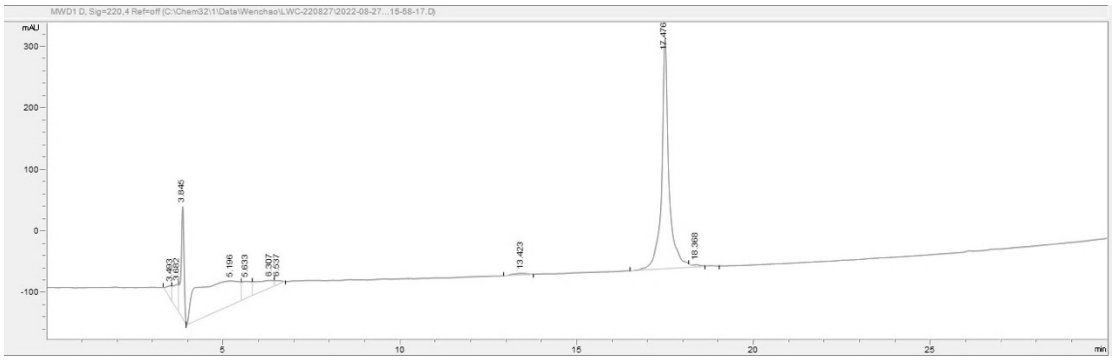
Ion	Calculated	Observed
+7	1862.7	1862.4
+8	1630.0	1630.1
+9	1449.0	1448.8
+10	1304.2	1304.6
+11	1185.7	1185.8
+12	1087.0	1086.9
+13	1003.5	1003.4
+14	931.9	931.8
+15	869.8	869.8
+16	815.5	815.5
+17	767.6	767.5

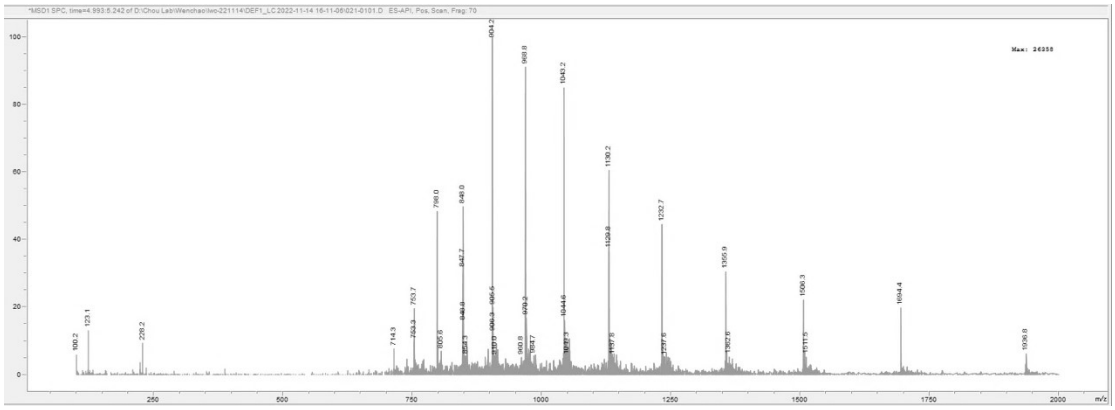




Analytical HPLC trace and ESI-MS of **9b**: Upper: analytical HPLC trace of purified **9b**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 17.388 min); Lower: ESI-MS of **9b**. Calcd mass: 13545.16 Da (average isotopes), $[M+10H]^{10+}$ m/z = 1355.5, $[M+11H]^{11+}$ m/z = 1232.4, $[M+12H]^{12+}$ m/z = 1129.8, $[M+13H]^{13+}$ m/z = 1042.9, $[M+14H]^{14+}$ m/z = 968.5, $[M+15H]^{15+}$ m/z = 904.0, $[M+16H]^{16+}$ m/z = 847.6, $[M+17H]^{17+}$ m/z = 797.8, $[M+18H]^{18+}$ m/z = 753.5. Observed mass: 1355.2, 1232.2, 1129.6, 1042.7, 968.6, 904.0, 847.4, 797.8, 753.5.

Ion	Calculated	Observed
+10	1355.5	1355.2
+11	1232.4	1232.2
+12	1129.8	1129.6
+13	1042.9	1042.7
+14	968.5	968.6
+15	904.0	904.0
+16	847.6	847.4
+17	797.8	797.8
+18	753.5	753.5

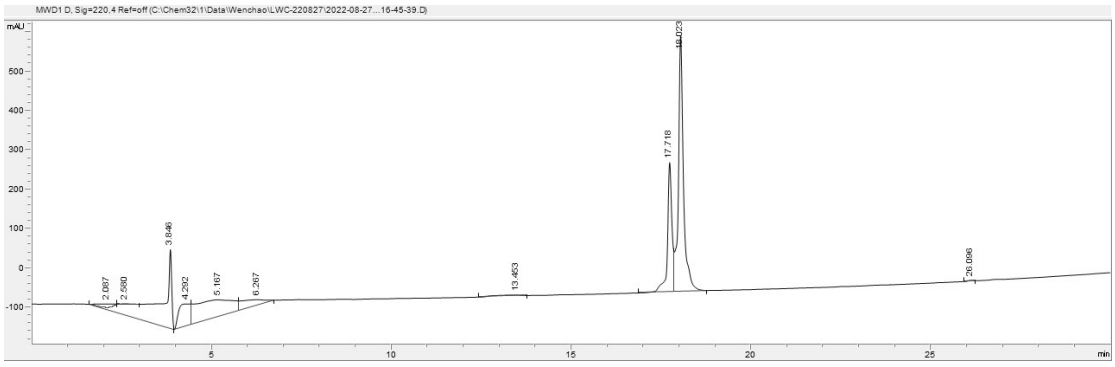


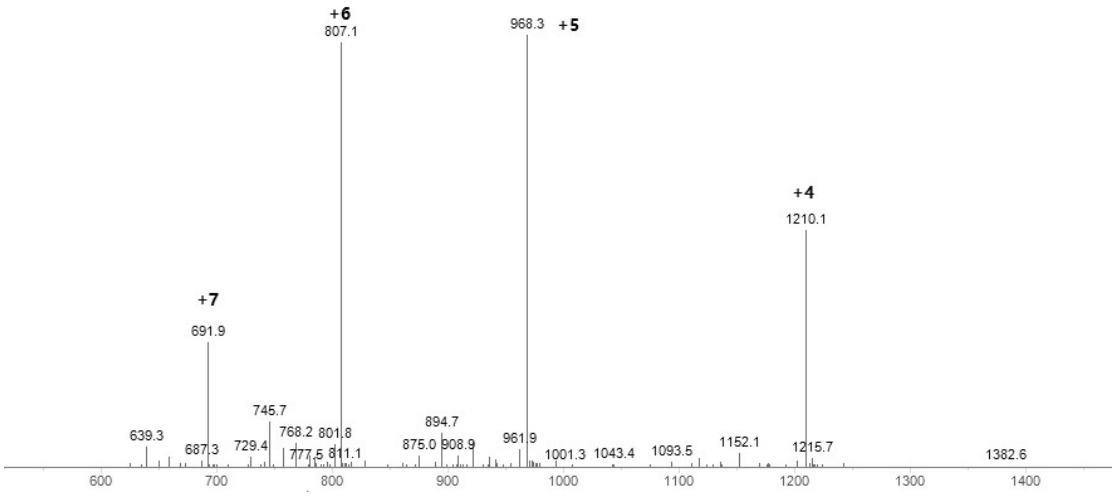


Analytical HPLC trace and ESI-MS of **9**: Upper: analytical HPLC trace of purified **9**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 17.388 min); Lower: ESI-MS of **9**. Calcd mass: 13549.86 Da (average isotopes), $[M+7H]^{7+}$ m/z = 1936.7, $[M+8H]^{8+}$ m/z = 1694.7, $[M+9H]^{9+}$ m/z = 1506.5, $[M+10H]^{10+}$ m/z = 1356.0, $[M+11H]^{11+}$ m/z = 1232.8, $[M+12H]^{12+}$ m/z = 1130.2, $[M+13H]^{13+}$ m/z =1043.3, $[M+14H]^{14+}$ m/z =968.8, $[M+15H]^{15+}$ m/z = 904.3, $[M+16H]^{16+}$ m/z = 847.9, $[M+17H]^{17+}$ m/z = 798.0; Observed mass: 1936.8, 1694.4, 1506.3, 1355.9,

1232.7, 1130.2, 1043.2, 968.8, 904.2, 848.0, 798.0.

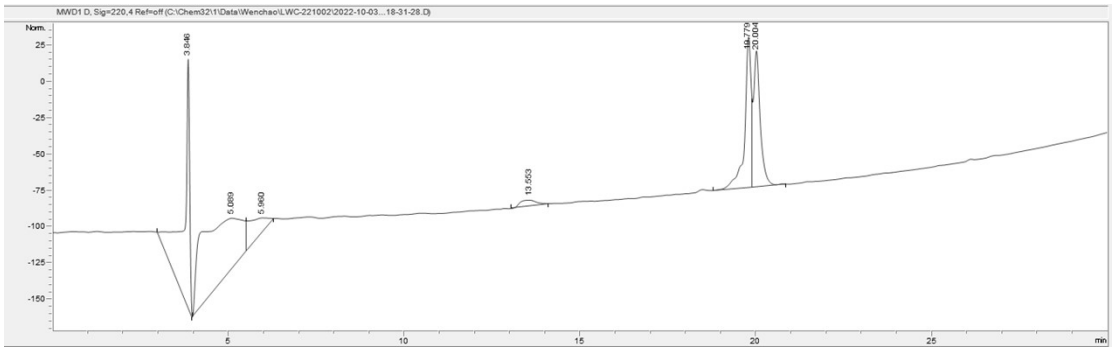
Ion	Calculated	Observed
+7	1936.7	1936.8
+8	1694.7	1694.4
+9	1506.5	1506.3
+10	1356.0	1355.9
+11	1232.8	1232.7
+12	1130.2	1130.2
+13	1043.3	1043.2
+14	968.8	968.8
+15	904.3	904.2
+16	847.9	848.0
+17	798.0	798.0

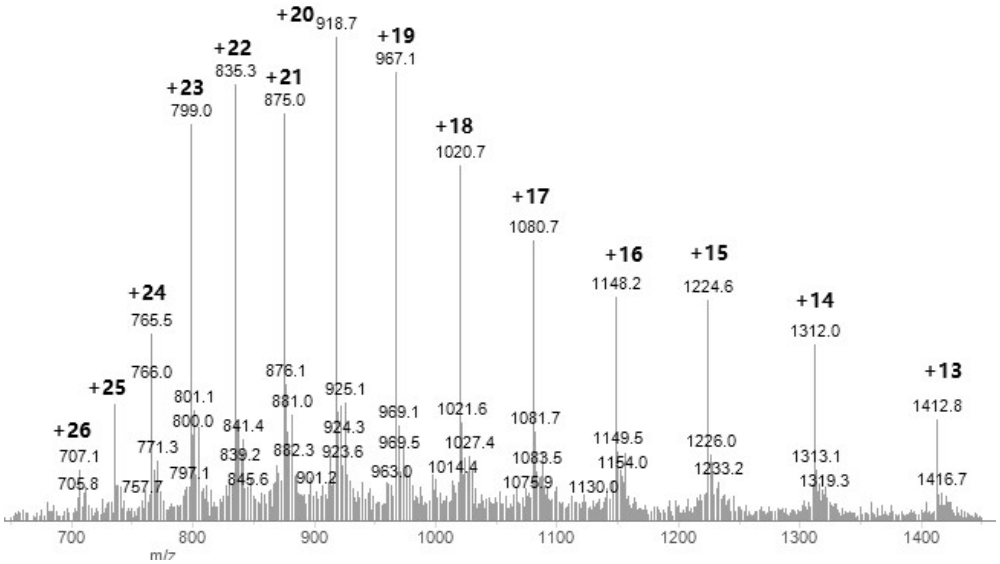




Analytical HPLC trace and ESI-MS of **10**: Upper: analytical HPLC trace of purified **10**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 17.718 and 18.023 min); Lower: ESI-MS of **10**. Calcd mass: 4835.05 Da (average isotopes), $[M+4H]^{4+}$ m/z = 1209.8, $[M+5H]^{5+}$ m/z = 968.0, $[M+6H]^{6+}$ m/z = 806.8, $[M+7H]^{7+}$ m/z = 961.7; Observed mass: 1210.1, 968.3, 807.1, 691.9.

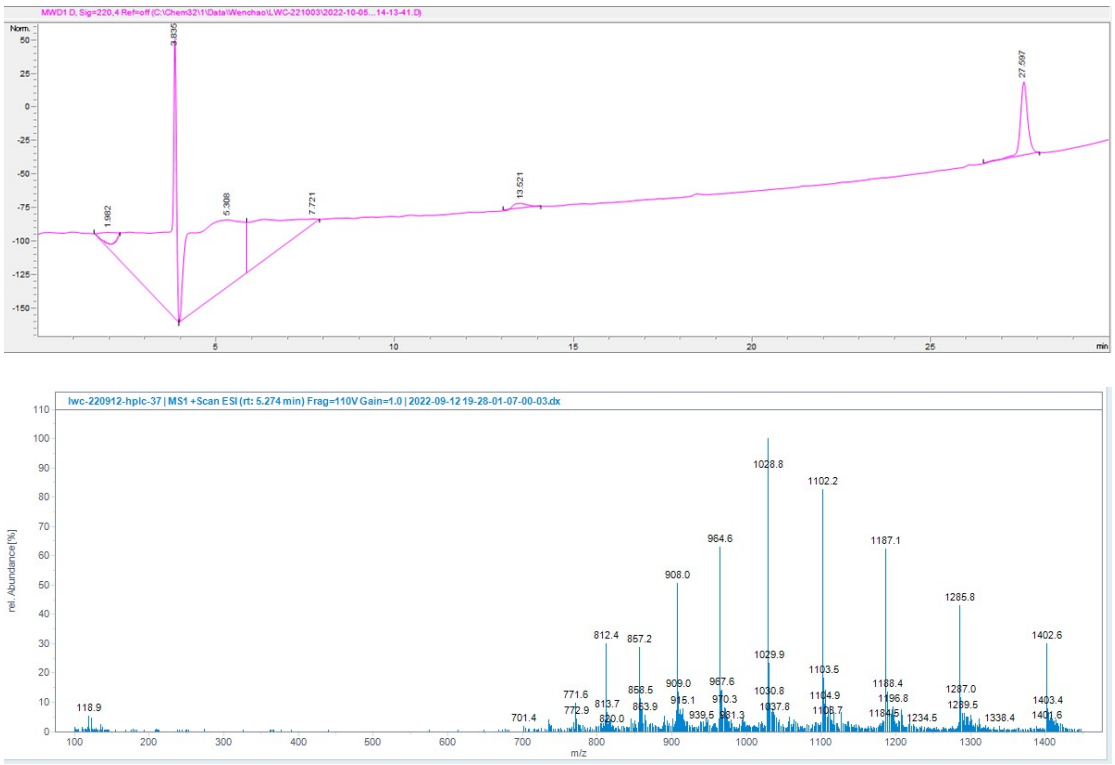
Ion	Calculated	Observed
+4	1209.8	1210.1
+5	968.0	968.3
+6	806.8	807.1
+7	691.7	691.9





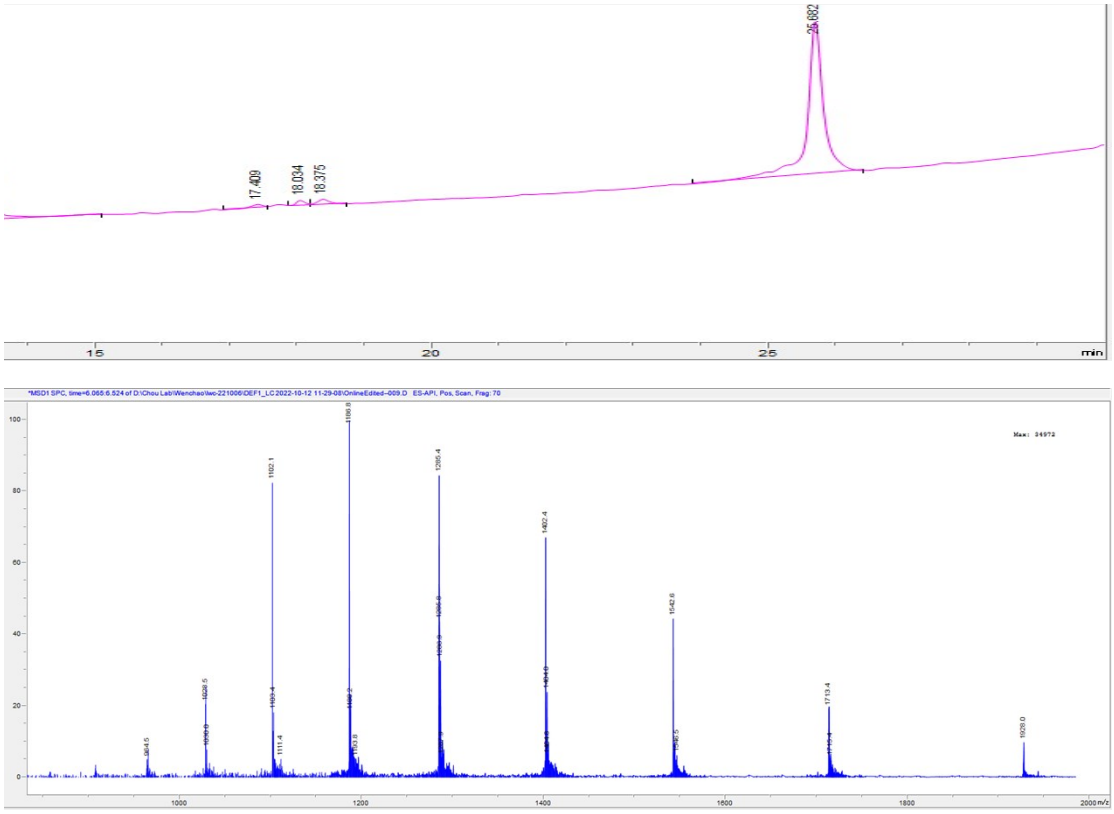
Analytical HPLC trace and ESI-MS of **11**. Upper: analytical HPLC trace of purified **11**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 19.779 and 20.004 min); Lower: ESI-MS of **11**. Calcd mass: 18355.27 Da (average isotopes), $[M+13H]^{13+}$ m/z = 1412.9, $[M+14H]^{14+}$ m/z = 1312.1, $[M+15H]^{15+}$ m/z = 1224.7, $[M+16H]^{16+}$ m/z = 1148.2, $[M+17H]^{17+}$ m/z = 1080.7, $[M+18H]^{18+}$ m/z = 1020.7, $[M+19H]^{19+}$ m/z = 967.1, $[M+20H]^{20+}$ m/z = 918.8, $[M+21H]^{21+}$ m/z = 875.1, $[M+22H]^{22+}$ m/z = 835.3, $[M+23H]^{23+}$ m/z = 799.1, $[M+24H]^{24+}$ m/z = 765.8, $[M+25H]^{25+}$ m/z = 735.2, $[M+26H]^{26+}$ m/z = 707.0; Observed mass: 1412.8, 1312.0, 1224.6, 1148.2, 1080.7, 1020.7, 967.1, 918.7, 875.0, 835.3, 799.0, 765.5, 734.9, 707.1.

Ion	Calculated	Observed
+13	1412.9	1412.8
+14	1312.1	1312.0
+15	1224.7	1224.6
+16	1148.2	1148.2
+17	1080.7	1080.7
+18	1020.7	1020.7
+19	967.1	967.1
+20	918.8	918.7
+21	875.1	875.0
+22	835.3	835.3
+23	799.1	799.0
+24	765.8	765.5
+25	735.2	734.9
+26	707.0	707.1



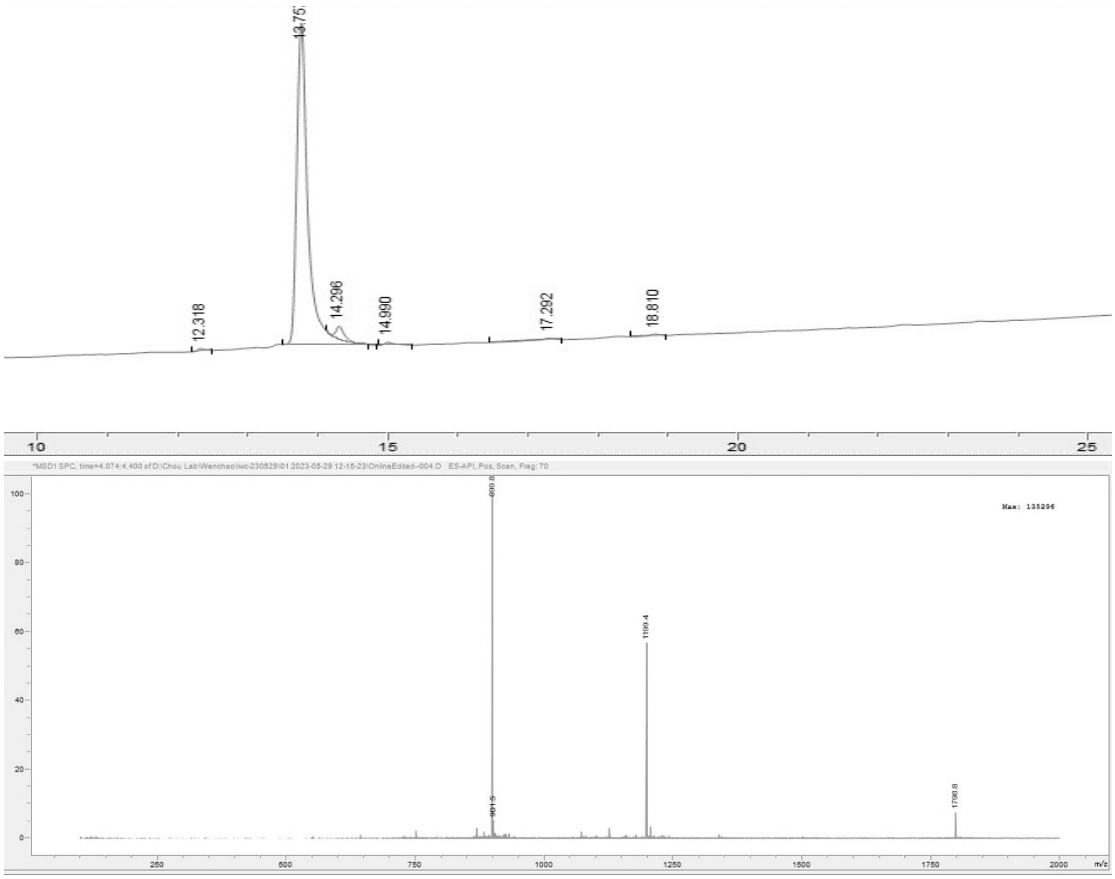
Analytical HPLC trace and ESI-MS of **12**. Upper: analytical HPLC trace of purified **12**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 27.597 min); Lower: ESI-MS of **12**. Calcd mass: 15418.05 Da (average isotopes), $[M+11H]^{11+}$ m/z = 1402.6, $[M+12H]^{12+}$ m/z = 1285.8, $[M+13H]^{13+}$ m/z = 1187.0, $[M+14H]^{14+}$ m/z = 1102.3, $[M+15H]^{15+}$ m/z = 1028.9, $[M+16H]^{16+}$ m/z = 964.6, $[M+17H]^{17+}$ m/z = 907.9, $[M+18H]^{18+}$ m/z = 857.6, $[M+19H]^{19+}$ m/z = 812.5, $[M+20H]^{20+}$ m/z = 771.9; Observed mass: 1402.6, 1285.8, 1187.1, 1102.2, 1028.8, 964.6, 908.0, 857.2, 812.4, 771.6.

Ion	Calculated	Observed
+11	1402.6	1402.6
+12	1285.8	1285.8
+13	1187.0	1187.1
+14	1102.3	1102.2
+15	1028.9	1028.8
+16	964.6	964.6
+17	907.9	908.0
+18	857.6	857.2
+19	812.5	812.4
+20	771.9	771.6



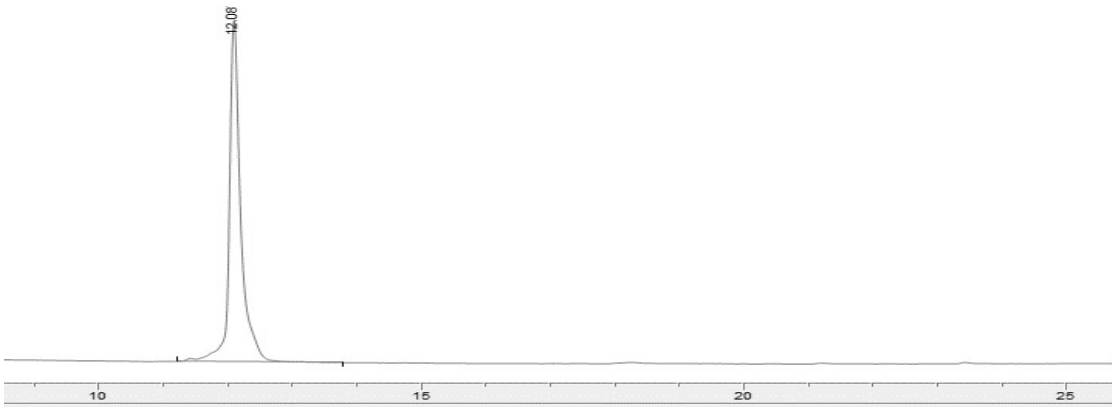
Analytical HPLC trace and ESI-MS of **IL-2**. Upper: analytical HPLC trace of purified **IL-2**. (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 25.682 min); Lower: ESI-MS of **IL-2**. Calcd mass: 15416.05 Da (average isotopes), $[M+8H]^{8+}$ m/z =, $[M+9H]^{9+}$ m/z = 1713.9, $[M+10H]^{10+}$ m/z = 1542.6, $[M+11H]^{11+}$ m/z = 1402.5, $[M+12H]^{12+}$ m/z = 1285.7, $[M+13H]^{13+}$ m/z = 1186.9, $[M+14H]^{14+}$ m/z = 1102.1, $[M+15H]^{15+}$ m/z = 1028.7, $[M+16H]^{16+}$ m/z = 964.5; Observed mass: 1928.0, 1713.4, 1542.6, 1402.4, 1285.4, 1186.8, 1102.1, 1028.5, 964.5.

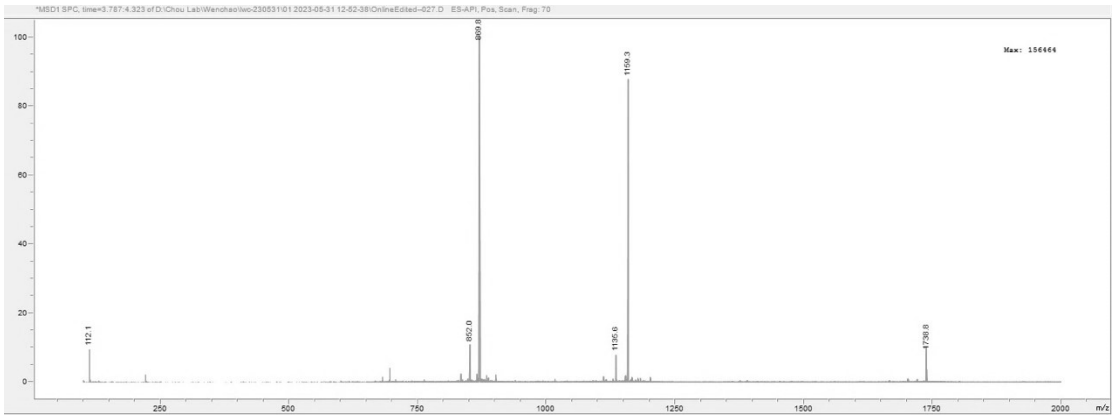
Ion	Calculated	Observed
+8	1928.0	1928.0
+9	1713.9	1713.4
+10	1542.6	1542.6
+11	1402.5	1402.4
+12	1285.7	1285.4
+13	1186.9	1186.8
+14	1102.1	1102.1
+15	1028.7	1028.5
+16	964.5	964.5



Analytical HPLC trace and ESI-MS of **14:** Upper: analytical HPLC traces of crude and purified **14** (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 13.757 min); Lower: ESI-MS of **14.** Calcd mass: 3595.92 Da (average isotopes), $[M+2H]^{2+}$ *m/z* = 1798.9, $[M+3H]^{3+}$ *m/z* = 1199.6, $[M+4H]^{4+}$ *m/z* = 899.9; Observed mass: 1798.8, 1199.4, 899.8.

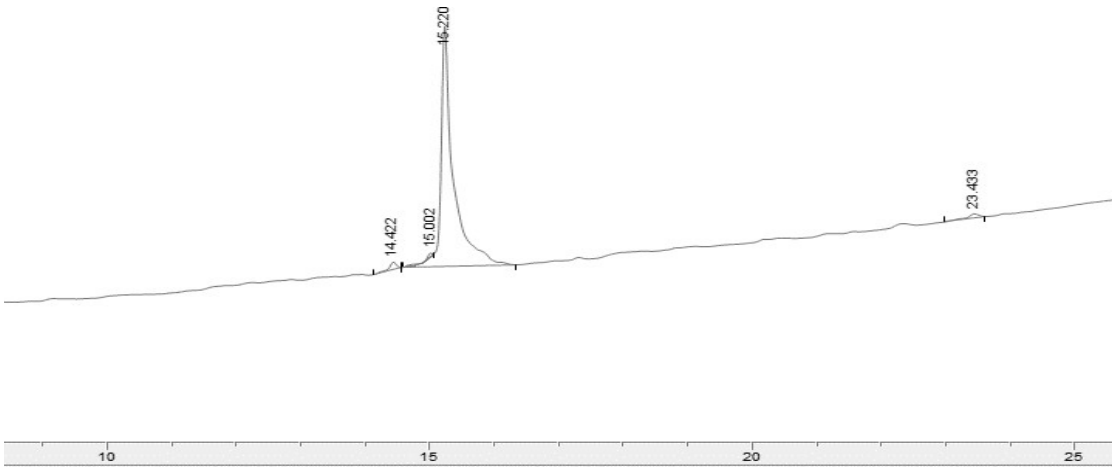
Ion	Calculated	Observed
+2	1798.9	1798.8
+3	1199.6	1199.4
+4	899.9	899.8

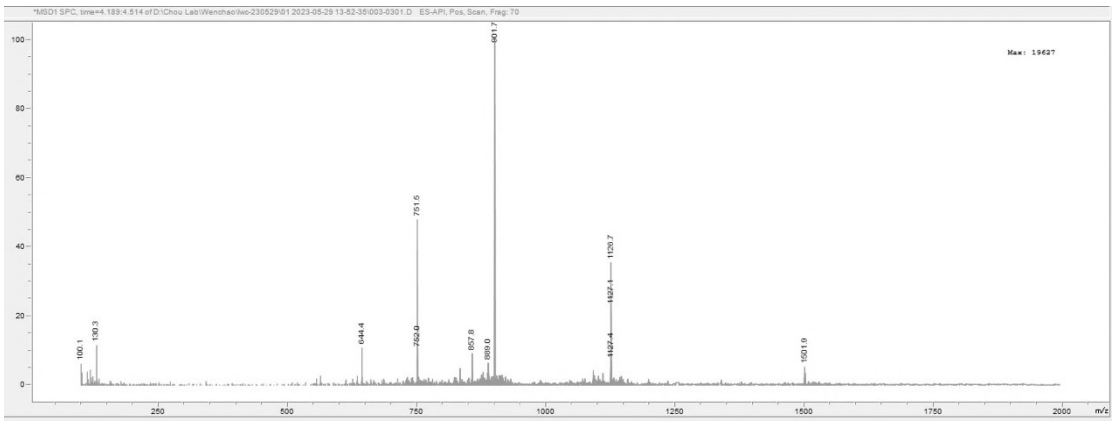




Analytical HPLC trace and ESI-MS of **15:** Upper: analytical HPLC traces of crude and purified **15** (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 12.087 min); Lower: ESI-MS of **15.** Calcd mass: 3475.77 Da (average isotopes), $[M+2H]^{2+}$ *m/z* = 1738.8, $[M+3H]^{3+}$ *m/z* = 1159.6, $[M+4H]^{4+}$ *m/z* = 869.9; Observed mass: 1798.8, 1199.4, 899.8.

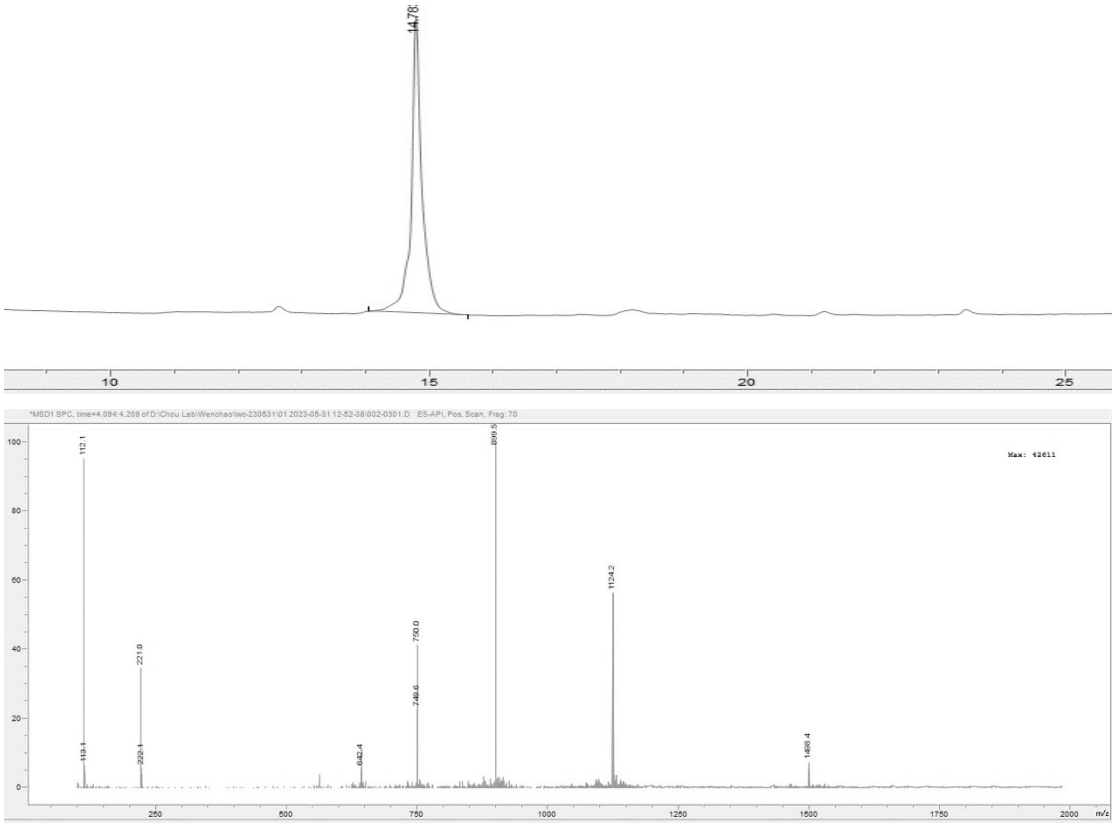
Ion	Calculated	Observed
+2	1738.8	1738.8
+3	1159.6	1159.3
+4	869.9	869.8





Analytical HPLC trace and ESI-MS of **17:** Upper: analytical HPLC traces of crude and purified **17** (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 15.220 min); Lower: ESI-MS of **17.** Calcd mass: 4504.16 Da (average isotopes), $[M+3H]^{3+}$ *m/z* = 1502.4, $[M+4H]^{4+}$ *m/z* = 1127.0, $[M+5H]^{5+}$ *m/z* = 901.8, $[M+6H]^{6+}$ *m/z* = 751.7, $[M+7H]^{7+}$ *m/z* = 644.5; Observed mass: 1501.9, 1126.7, 901.7, 751.5, 644.4.

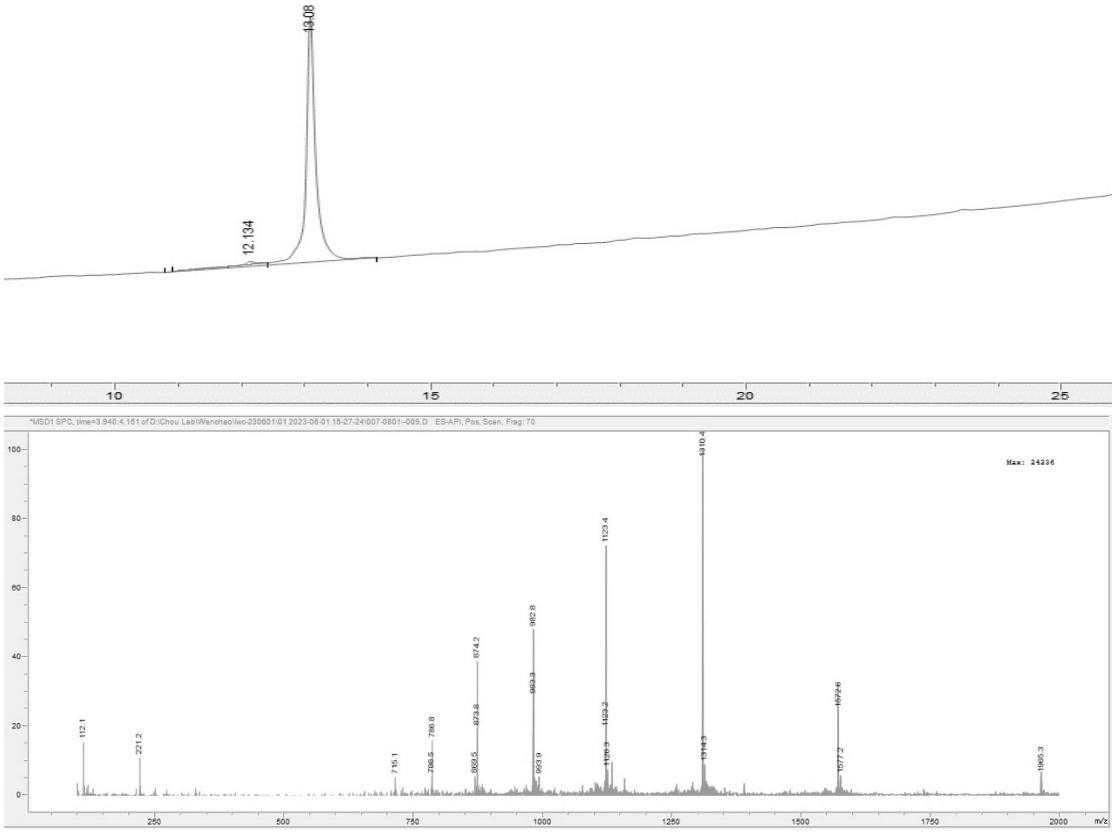
Ion	Calculated	Observed
+3	1502.4	1501.9
+4	1127.0	1126.7
+5	901.8	901.7
+6	751.7	751.5
+7	644.5	644.4



Analytical HPLC trace and ESI-MS of **18:** Upper: analytical HPLC traces of crude and purified **18** (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 15.220 min); Lower: ESI-MS of **18** Calcd mass: 4493.01 Da (average isotopes), $[M+3H]^{3+}$ *m/z* = 1498.67, $[M+4H]^{4+}$ *m/z* = 1124.3, $[M+5H]^{5+}$ *m/z* = 899.6, $[M+6H]^{6+}$ *m/z* = 749.8, $[M+7H]^{7+}$ *m/z* = 642.8; Observed mass: 1498.4, 1124.2, 899.5, 750.0, 642.4.

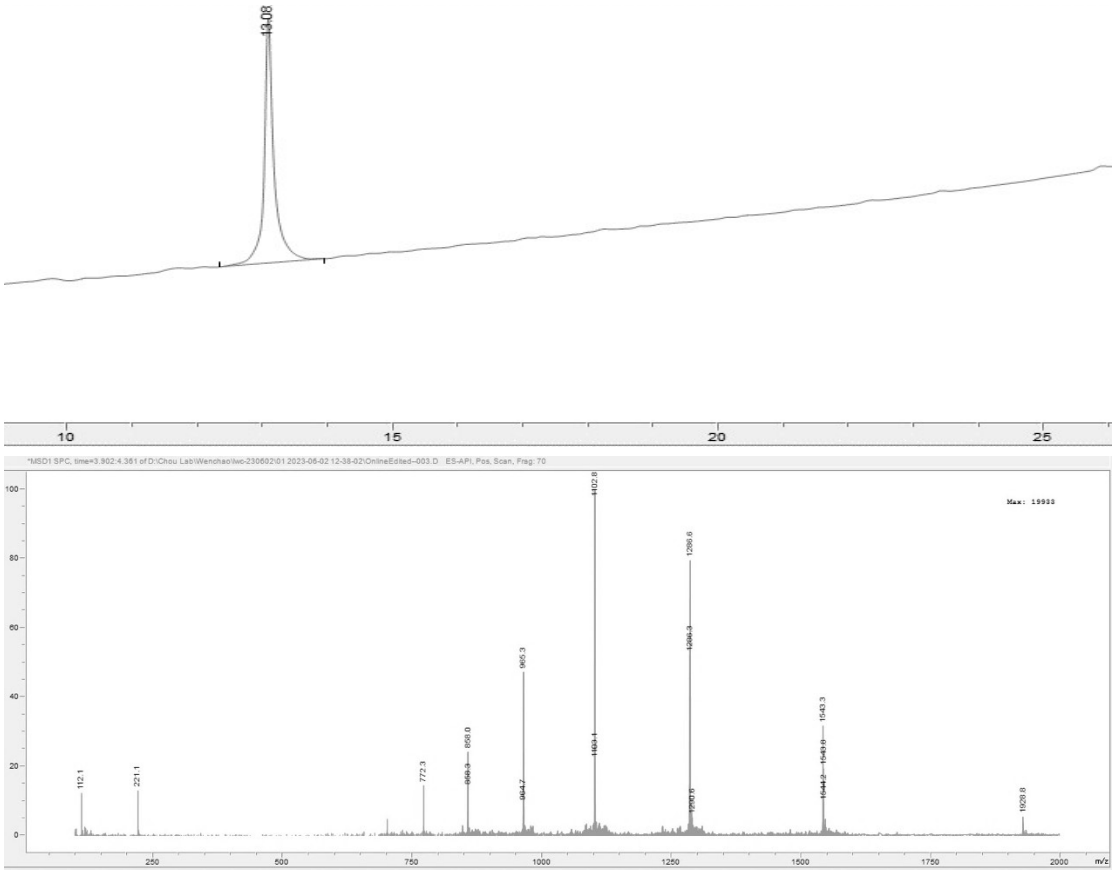
Ion	Calculated	Observed
+3	1498.67	1498.4
+4	1124.3	1124.2
+5	899.6	899.5

+6	749.8	750.0
+7	642.8	642.4



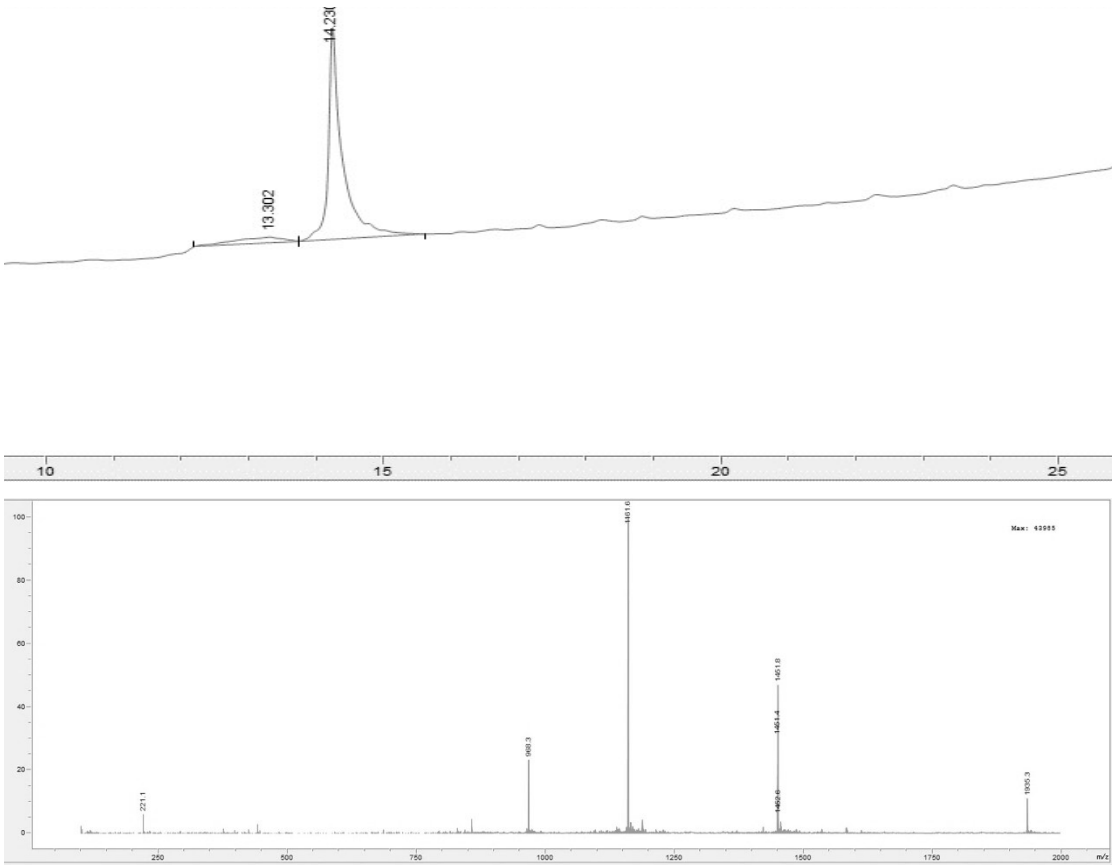
Analytical HPLC trace and ESI-MS of **19:** Upper: analytical HPLC traces of crude and purified **19** (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 13.081 min); Lower: ESI-MS of **19** Calcd mass: 7858.78 Da (average isotopes), $[M+4H]^{4+}$ *m/z* = 1965.7, $[M+5H]^{5+}$ *m/z* = 1572.8, $[M+6H]^{6+}$ *m/z* = 1310.8, $[M+7H]^{7+}$ *m/z* = 1123.7, $[M+8H]^{8+}$ *m/z* = 983.3, $[M+9H]^{9+}$ *m/z* = 874.2, $[M+10H]^{10+}$ *m/z* = 786.9; Observed mass: 1965.3, 1572.6, 1310.4, 1123.4, 982.8, 874.2, 786.8.

Ion	Calculated	Observed
+4	1965.7	1965.3
+5	1572.8	1572.6
+6	1310.8	1310.4
+7	1123.7	1123.4
+8	983.3	982.8
+9	874.2	874.2
+10	786.9	786.8



Analytical HPLC trace and ESI-MS of **20:** Upper: analytical HPLC traces of crude and purified **20** (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 13.082 min); Lower: ESI-MS of **20** Calcd mass: 7858.78 Da (average isotopes), $[M+4H]^{4+}$ *m/z* = 1929.7, $[M+5H]^{5+}$ *m/z* = 1543.9, $[M+6H]^{6+}$ *m/z* = 1286.8, $[M+7H]^{7+}$ *m/z* = 1103.1, $[M+8H]^{8+}$ *m/z* = 965.3, $[M+9H]^{9+}$ *m/z* = 858.2, $[M+10H]^{10+}$ *m/z* = 772.5; Observed mass: 1965.3, 1572.6, 1310.4, 1123.4, 982.8, 874.2, 786.8.

Ion	Calculated	Observed
+4	1929.7	1928.8
+5	1543.9	1543.3
+6	1286.8	1286.6
+7	1103.1	1102.8
+8	965.3	965.3
+9	858.2	858.0
+10	772.5	772.3



Analytical HPLC trace and ESI-MS of **T8P-Insulin:** Upper: analytical HPLC traces of crude and purified **T8P-Insulin** (Linear gradient of 10-90% solvent B over 30 min, Jupiter® 250 × 4.6 mm, C4 column, Rt = 13.082 min); Lower: ESI-MS of **T8P-Insulin** Calcd mass: 5803.68 Da (average isotopes), $[M+3H]^{3+}$ *m/z* = 1935.6, $[M+4H]^{4+}$ *m/z* = 1451.9, $[M+5H]^{5+}$ *m/z* = 1161.7, $[M+6H]^{6+}$ *m/z* = 968.3; Observed mass: 1935.3, 1451.8, 1161.6, 968.3.

Ion	Calculated	Observed
+3	1935.6	1935.3
+4	1451.9	1451.8
+5	1161.7	1161.6
+6	968.3	968.3

VIIII. References

1. Hsu, E. J.; Cao, X.; Moon, B.; Bae, J.; Sun, Z.; Liu, Z.; Fu, Y. X., A cytokine receptor-masked IL2 prodrug selectively activates tumor-infiltrating lymphocytes for potent antitumor therapy. *Nat Commun* **2021,** *12* (1), 2768.

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