Supporting Information

De Novo Design of α-Helical Lipopeptides Targeting Viral Fusion Proteins: A Promising Strategy for Relatively Broad-Spectrum Antiviral Drug Discovery

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Table S1. Inhibitory activity of compounds on oseltamivir-resistant strains[a]

Compound	EC_{50} (μM)[b]	
	LN/1109 (H1N1)	TJ/15 (H1N1)
IIQ	4.36 ± 3.1	3.03 ± 1.1
oseltamivir	21.7 ± 9.6	>100

[a] LN/1109 (H1N1), A/LiaoNing-ZhenXing/1109/2010 (H1N1); TJ/15 (H1N1), A/TianJin-JinNan/15/2009 (H1N1). [b] Data were derived from the results of three independent experiments and are presented as the mean ± standard deviation.

Table S2. Biophysical properties of the IIQ peptide

Compd	Helicity (%) pH 7.4	Helicity (%) pH 5.0	Complex with HR1P		Complex with N66	
			Helicity (%)	*Tm* (°C)	Helicity (%)	*Tm* (°C)
IIQ	79.3	76.8	43.9	>90	74.8	83.1

[a] The final concentration of the tested peptide in PBS (pH 7.4) or a pH 5.0 buffer was 10 μM.

Table S3. Summary of the SVA results of IIQ/HR1P and IIQ/N66 complexes

Complex	Sedimentation coefficient (s)	Observed molecular mass (kDa)	Calculated molecular mass (kDa)
IIQ/HR1P 6-HB[a]	2.78	28.0	28.1
IIQ/N66 6-HB[b]	3.66	36.3	37.8

[a] SVA studies were performed at a concentration of 150 μM in PBS (pH 7.2) and a rotor speed of 60,000 rpm at 25 ºC. [b] The molecular mass was determined at a concentration of 150 μM in a pH 5.0 buffer at a rotor speed of 60,000 rpm at 25 ºC.

Table S4. Biological and biophysical properties of IIQ_{NBD}

Compd	Helicity (%), pH 5.0	EC_{50} (μM)[a]
IIQ_{NBD}	67.6	3.93 ± 0.64
IIQ	76.8	3.19 ± 1.32
oseltamivir	not determined	3.58 ± 2.17

[a] Data were derived from the results of three independent experiments and are presented as the mean ± standard deviation. EC_{50} for inhibiting A/Puerto Rico/8/34 (H1N1) infection.

Table S5. HPLC method used for the purification of peptide compounds[a]

Time (min)	Solvent A (%)	Solvent B (%)
0	70	30
5	40	60
10	20	80
12	15	85
15	10	90
18	0	100
40	0	100
45	70	30
50	70	30

[a] The crude peptide products were purified by preparative reverse phase HPLC with a Waters preparative HPLC system (PrepLC 4000) on a Waters X-bridge C8 column (19.5mm × 250mm, 10μm) at constant flow rate of 16 mL/min. Solvent A: 0.1% trifluoroacetic acid in H_2O; Solvent B: 0.1% trifluoroacetic acid in 70%CH_3CN/H_2O.

Table S6. HPLC method used for the analysis of peptide compounds[a]

Methods	Time (min)	Solvent A (%)	Solvent B (%)
Method A	0	90	10
	5	50	50
	10	0	100
	20	0	100
	23	90	10
	25	90	10
Method B	0	90	10
	4	50	50
	8	0	100
	20	0	100
	23	90	10
	25	90	10

[a] The peptide compounds were analyzed by analytical RP-HPLC was performed on a RP-C8 column (Zorbax Eclipse XDB-C8, 4.6 × 150 mm, 5 μm) using two different solvent systems (Methods A and B), and a flow rate of 1 mL/min with detection at 210 nm. Solvent A: 0.1% trifluoroacetic acid in H_2O; Solvent B: 0.1% trifluoroacetic acid in 70% CH_3CN/H_2O.

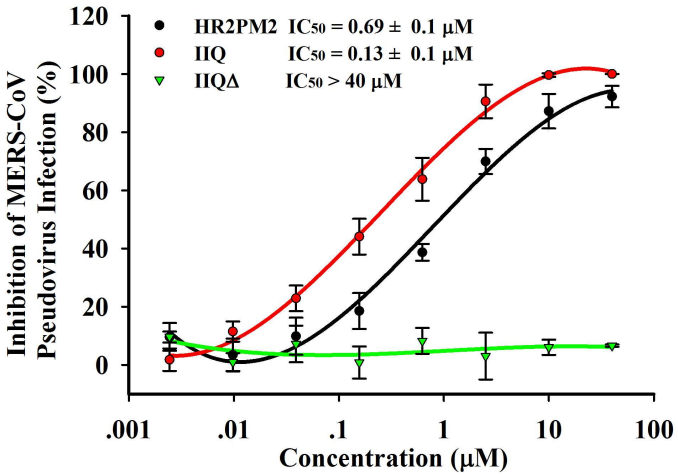


Figure S1. IIQ peptide as inhibitor of MERS-CoV infection. Inhibition of infection by pseudoviruses expressing S protein of MERS-CoV Erasmus Medical Center (EMC)/2012 strain in Huh-7 cells. HR2PM2 and IIQΔ were used as positive and negative controls, respectively. The sequence of IIQΔ is Ac-IEEIQKKIEEIQKKIEEIQKKIEEIQKK-IEEIQKK-βAla-K(Ac)-NH_2. Data were derived from the results of three independent experiments and are presented as the mean with standard deviation.

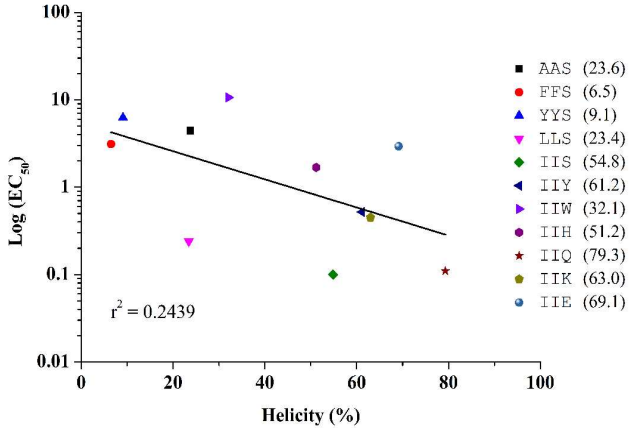


Figure S2. Comparison of observed α-helical contents with observed EC_{50}s for inhibition of MERS-CoV S protein-mediated cell-cell fusion. The EC_{50} data were obtained from Table 1, excluding VVS with EC_{50} > 10 μM. The values in parentheses indicate α-helicity (%) of the respective lipopeptides. A semi-log plot is presented, and a liner fit to data is shown (r^2 = 0.2439).

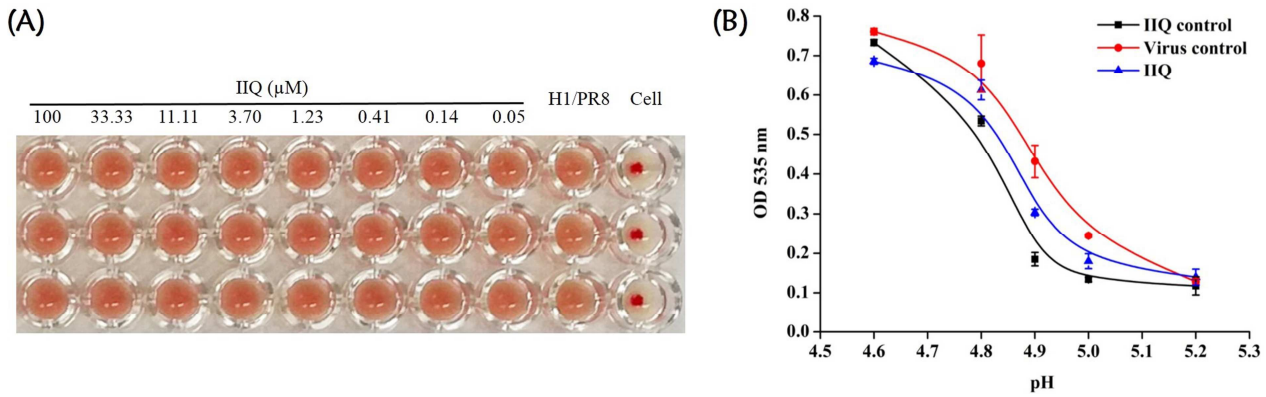


Figure S3. Identification of HA2 subunit as the potential target of IIQ compound. (**A**) The behavior of IIQ in inhibition of H1/PR8-induced aggregation of chicken erythrocytes. PBS without virus was used as a positive control, while H1/PR8 virus alone as a negative control. (**B**) Inhibition of HA2-mediated hemolysis of chicken erythrocytes. The meaning for each group: IIQ control indicates erythrocytes in a pH buffer with the addition of IIQ only; virus control indicates erythrocytes in a pH buffer with the addition of H1/PR8 virus only; IIQ group indicates erythrocytes in a pH buffer with H1/PR8 virus and IIQ peptide.

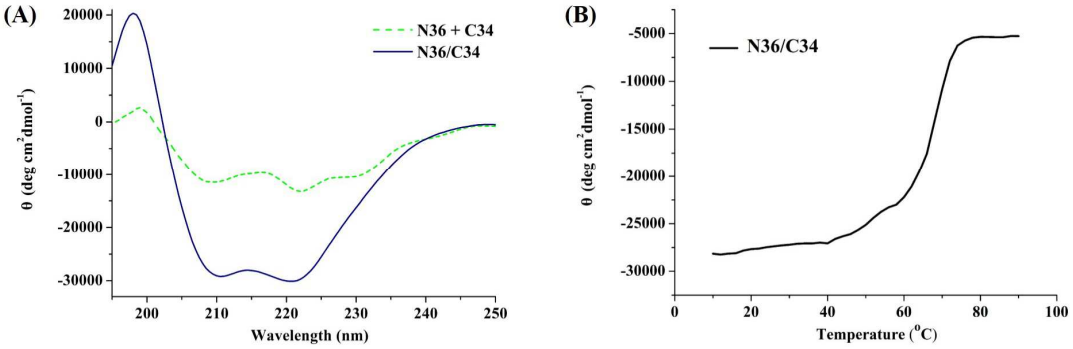


Figure S4. Interaction between C34 and N36. (**A**) 6HB formation between N36 and C34 peptides as characterized by CD spectra. (**B**) Thermal denaturation curves of 6HB formed between N36 and C34. N36/C34 6HB had a *Tm* of 64 °C

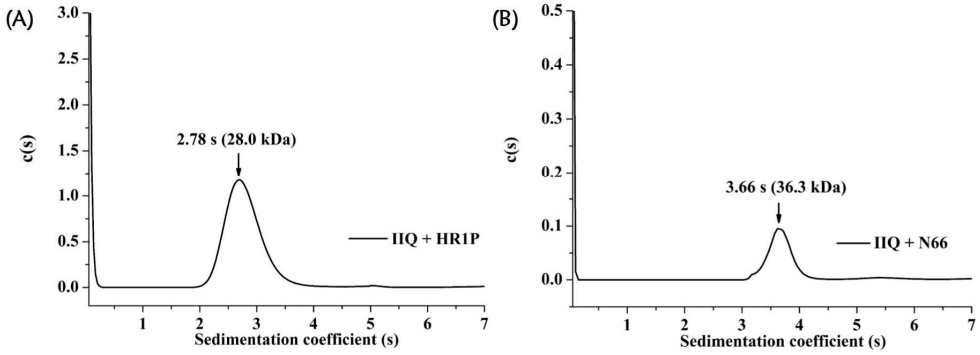


Figure S5. The molecular mass of IIQ/HR1P and IIQ/N66 as determined by SVA. The sedimentation coefficient (s) and observed molecular mass (kilodaltons) of each peak are indicated.

Pharmacokinetic assessments

Qualification assay. Analysis was performed on an LC-MS/MS system consisted of a binary LC-30AD delivery pump, a DUG-20A5R vacuum degasser, a CTO-20A column oven, a SIL-30AC auto-sampler, a CBM-20A system controller (Shimadzu, Japan) and an LCMS-8060 mass spectrometer (Shimadzu, Japan). The mobile phase was composed of solvent A (water containing 0.2 % formic acid and 10 mM ammonium formate) and solvent B (acetonitrile contiaining 5% water, 0.2% formic acid and 10 mM ammonium formate). The chromatographic separation was performed on a CAPCELL PAK C8 column (3 μm, 50 mm × 2.0mm, Shiseido, Japan) at a flow rate of 1.0 mL/min for 4.5 min kept at 40 □, using a gradient method of solvent B from 45% to 55% over 1.5 min, from 55% to 100% over 0.25 min and held for 0.25 min, and back to the initial condition over 2 min to equilibrate the colume. The auto-sampler temperature was maintained at 6 °C, and the injection volume was set to 5 μL. The mass spectrometer was equipped with an electrospray ion (ESI) source working in positive ion multiple reaction monitoring (MRM) mode of the transition *m/z* 969.60→ 129.15 for IIQ. The parameters including nebulizing gas flow, heating gas flow, drying gas flow, interface temperature, desolventizer (DL) temperature, heat block temperature, collision energy, Q1 and Q3 voltage were set at 3.0 L/min, 10 L/min, 10 L/min, 300 °C, 250 °C, 400 °C, -54 eV, -24 V and -14 V, respectively.

Preparation of samples. By use of a simple protein precipitation method, the test compound was extracted from rat plasma. A stock solution of IIQ was prepared in solvent of 50/50 acetonitrile/ 0.5% trifluoroacetic acid, and diluted to working solutions at serial concentraion ranging from 1 to 500 μg/ mL with the same solvent. Plasma samples for standard curves were prepared by spiking 100 μL of rat plasma with 10 μL of 10% trifluoroacetic acid, 10 μL of various working solutions and 120 μL of acetonitrile (containing 0.5% trifluoroacetic acid and 1% ammonium hydroxide). The mixture was vortexed for 2 min and centrifuged at 20,000 g for 20 min, the supernatant was transferred to auto-sampler vials, and injected into the LC/MS/MS system. Calibration curve for IIQ in plasma were linear in the concentration range of 0.1-100 μg/mL, with correlation coefficients of > 0.990. To each tested plasma sample (100 μL), all the preparation steps were the same as above, except that 10 μL of various working solutions was substituted by 10 μL blank solvent.

In vivo animal test. Three male Sprague-Dawley rats (weight 210 ± 10 g) were purchased from Animal Center of Beijing Institute of Pharmacology and Toxicology. Each rat was dosed with IIQ at 5 mg/kg by i.v. administration. Blood samples were collected into heparin tubes at 0, 0.033, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h after administration, and were immediately centrifuged to separate the plasma fractions. The plasma samples obtained were stored at -20 °C until analysis. Concentration-versus-time profiles were obtained for IIQ, and the pharmacokinetic parameters were calculated by DAS version 3.2.8. Data were averaged and reported as mean ± standard deviation.

Aqueous solubility determination

Solubility was measured by using an HPLC-UV method. IIQ peptide (~2 mg) was added to 1.5-mL Eppendorf tubes and double-distilled H_2O (ddH_2O) (100 μL) was added for dissolution with shaking for 24 h at 25 °C, followed by centrifugation of the mixture at 10,000 rpm for 15 min. The saturated supernatant solution was filtered through a 0.45-μm filter membrane and then transferred to other vials for analysis by HPLC with UV detection. Each sample was assayed in triplicate. For quantification, analytical RP-HPLC was used with a Zorbax Eclipse XDB-C8 column (4.6 mm × 150 mm, 5 μm). Solvent A: 0.1% TFA in H_2O; Solvent B: 0.1% TFA in 70% CH_3CN/H_2O; flow rate: 1 mL/min; gradient: 5–100% solvent B in solvent A over 25 min. The aqueous concentration was determined by comparison of the

peak area of the saturated solution with a standard curve plotted for the peak area versus known concentrations, which was prepared by solutions of test compound in PBS or ddH_2O at 20, 10, 5, 2.5, 0.5, and 0.05 mg/mL.

HIV-1 Env-mediated cell-cell fusion assay

Cell-cell fusion assays were performed as described earlier.[1] HL2/3 cells, which stably express HIV Gag, Env, Tat, Rev and Nef proteins, and TZM-bl cells, which stably express large amounts of CD4 and CCR5, were obtained from the NIH AIDS Reference and Reagent Program (contributed by Drs. Barbara Felber and George Pavlakis or Drs. John C. Kappes and Xiaoyun Wu, respectively). TZM-bl cells (2.5×10^4/well) and HL2/3 cells (7.5×10^4/well) were coincubated in 96-well plates (Corning Costar) at 37°C in 5% CO_2 in the presence of different concentrations of inhibitors. After incubation for 6–8 h, the medium was aspirated, the cells were washed and lysed, and luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Inhibition of pseudotyped Ebola virus infection

Lentivirus-based pseudotyped Sudan Ebola virus was produced as described previously.[2] The Sudan ebola virus pseudovirus and serial dilutions of each peptide were premixed and incubated for 30 min before the mixture was added to the target Huh-7 cells (10^4 per well in 96-well plates). 12 h post-infection, culture medium was replaced with fresh medium. After an additional 72 h, the transduced Huh-7 cells were lysed for the detection of luciferase activity, according to the luciferase assay system manual (Promega, USA). The values of relative light units (RLU) were used to assess the inhibitory activity of peptides.

Hemagglutination inhibition assay

Four times of the HA unit (HAU) of influenza A/Puerto Rico/8/34 virus (25 μL) in U-bottomed 96-well plate was prepared, and then an equal volume (25 μL) of IIQ strated from 100 μM concentration of 3-fold serial dilution in PBS was added into the plate. After 15 min incubation at room temperature, 50 μL of freshly prepared chicken erythrocytes (1% v/v in PBS) were added to each well with multichannel pipettes. Subsequently, the mixture was incubated for 30 min at room temperature to allow for hemagglutination to occur.

Hemolysis inhibition assay

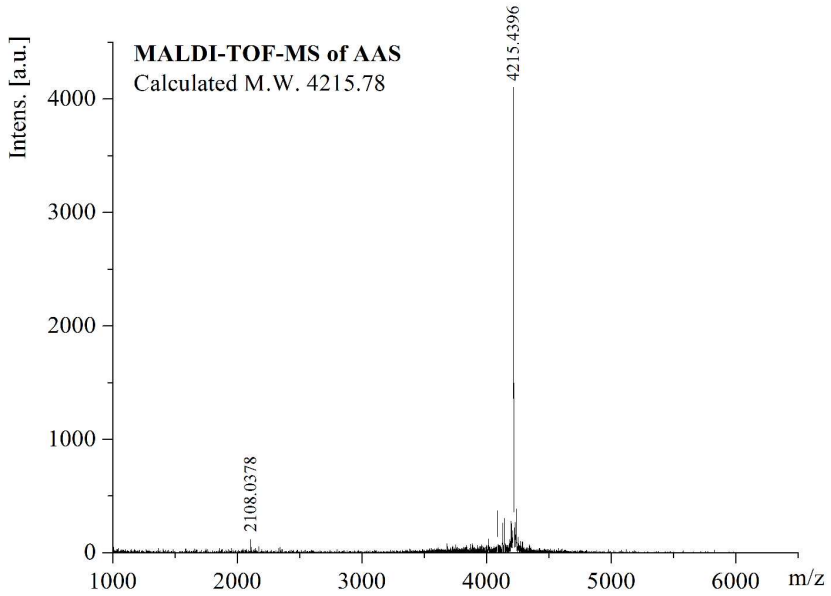
Briefly, the virus stock was diluted with PBS (final titer 10^7 Pfu/mL), and then 100 μL of virus dilution was mix with an equal volume of IIQ solution (20 μM) in a microcentrifuge tube. After 30 min incubation at room temperature, 200 μL of 2% chicken erythrocytes prewarmed at 37 °C was added, followed by incubation for 30 min at 37 °C. 100 μL of sodium acetate (pH 4.6-5.2) was added to above mixture to trigger hemolysis, and then incubation for 30 min at 37 °C. Prior to measure the absorbance of released hemoglobin, the cells were centrifuged at 2000 rpm for 8 min to separate the nonlysed cells. The absorbance of supernatant at OD_{535nm} was measured by SpectraMax M5 (Molecular Devices, USA).

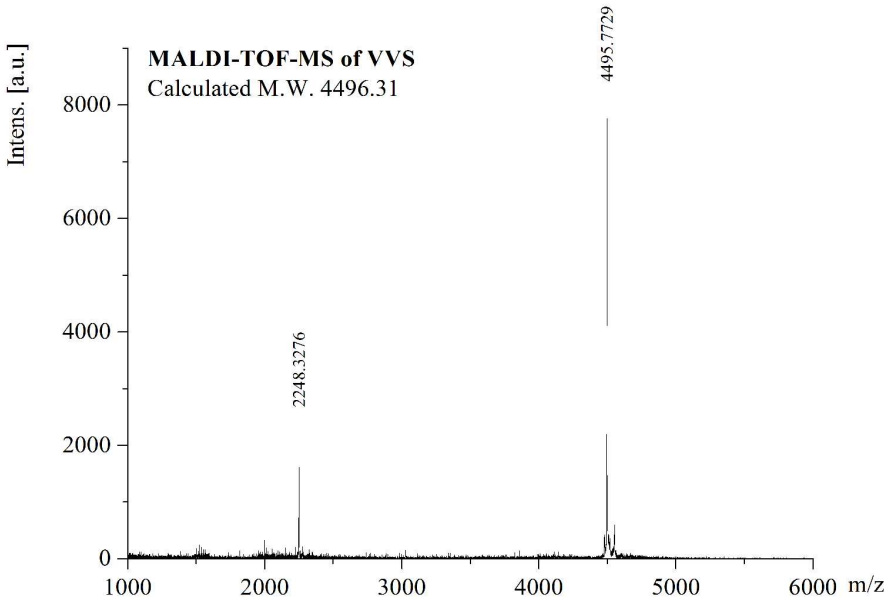
References

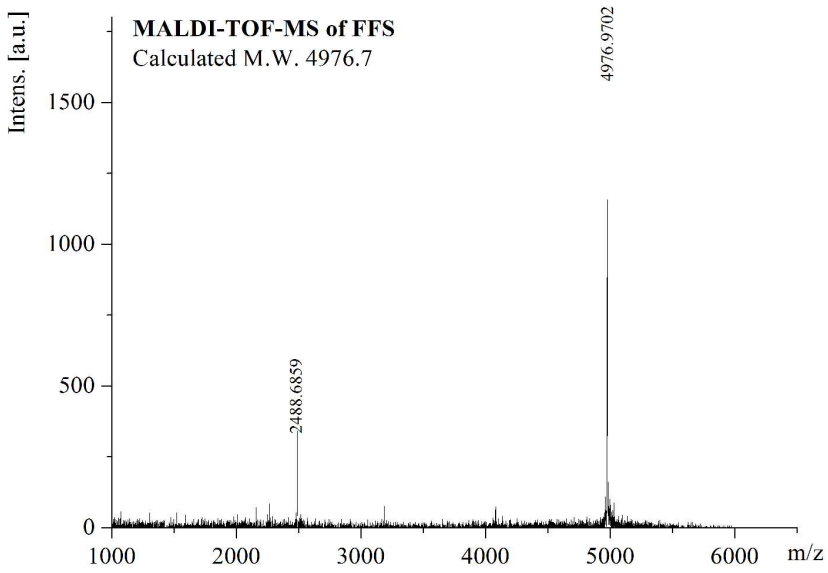
1. Wang, C.; Lu, L.; Na, H.; Li, X.; Wang, Q.; Jiang, X.; Xu, X.; Yu, F.; Zhang, T.; Li, J.; Zhang, Z.; Zheng, B.; Liang, G.; Cai, L.; Jiang, S.; Liu, K. Conjugation of a nonspecific antiviral sapogenin with a specific HIV fusion inhibitor: a promising strategy for discovering new antiviral therapeutics. *J. Med. Chem.* **2014,** 57, 7342-7354.

2. Li, H.; Yu, F.; Xia, S.; Yu, Y.; Wang, Q., Lv, M.; Wang, Y.; Jiang, S.; Lu, L. Chemically modified human serum albumin potently blocks entry of Ebola pseudoviruses and viruslike particles. *Antimicrob. Agents Chemother.* **2017**, *61*, pii: e02168-16.

MALDI-TOF-MS and analytical HPLC of designed peptides



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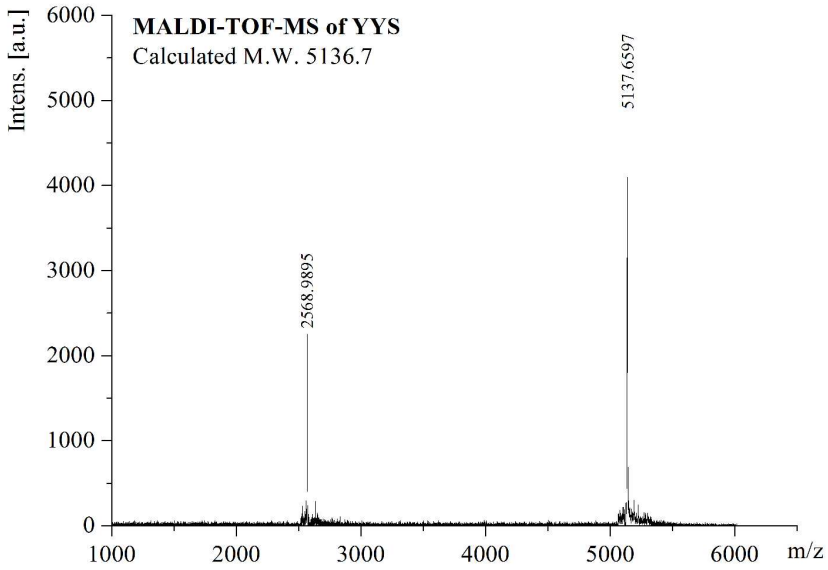
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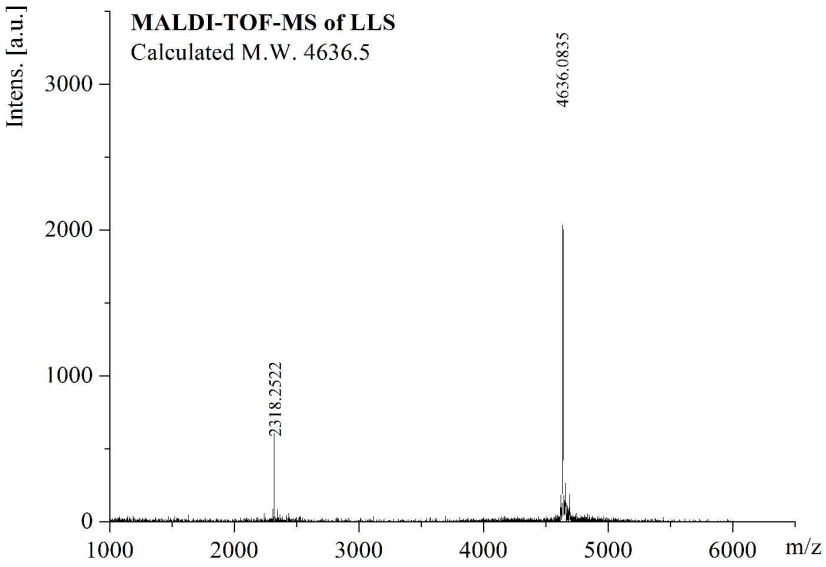
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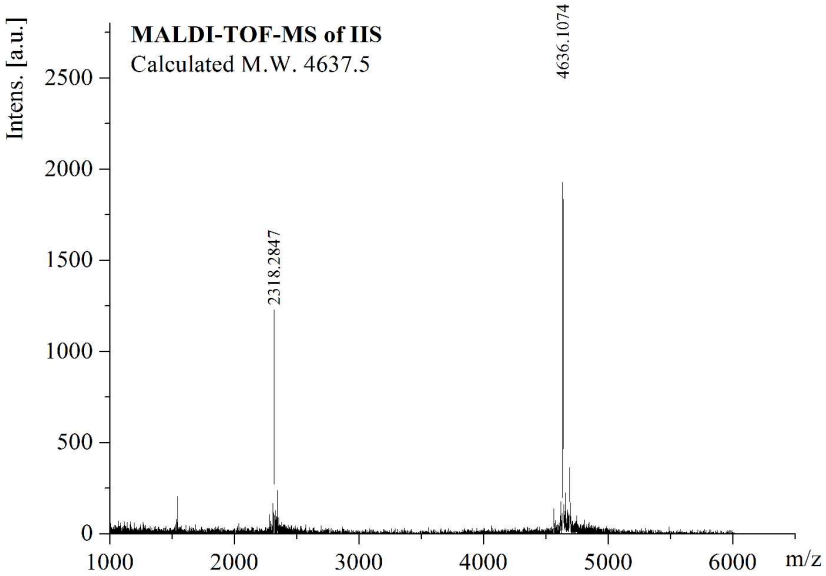
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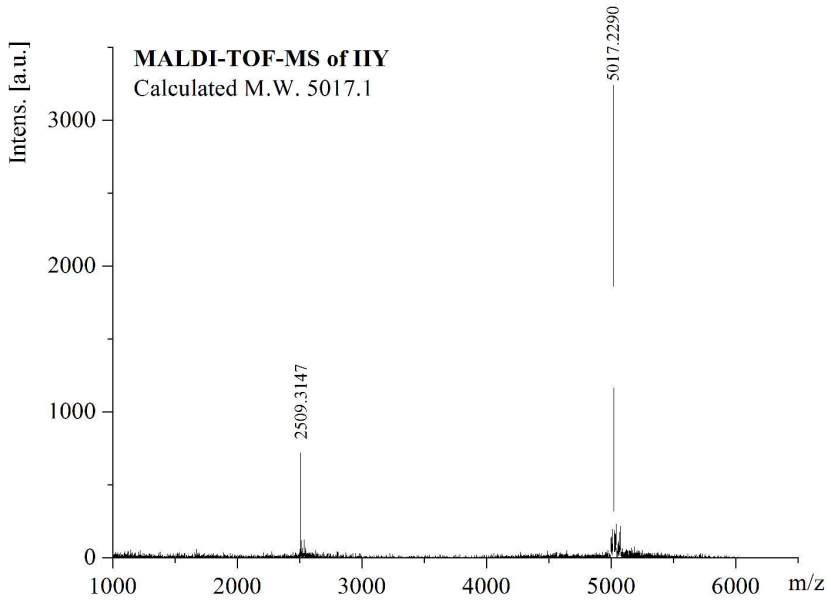
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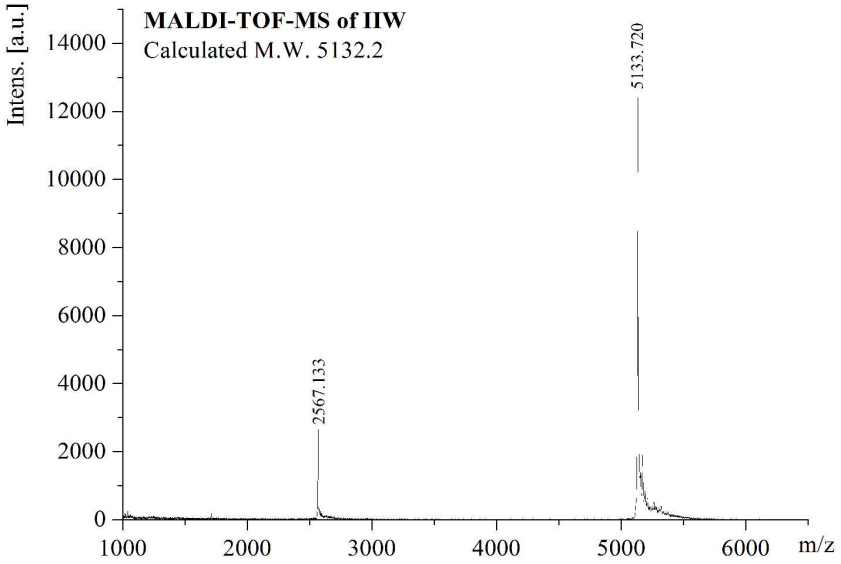
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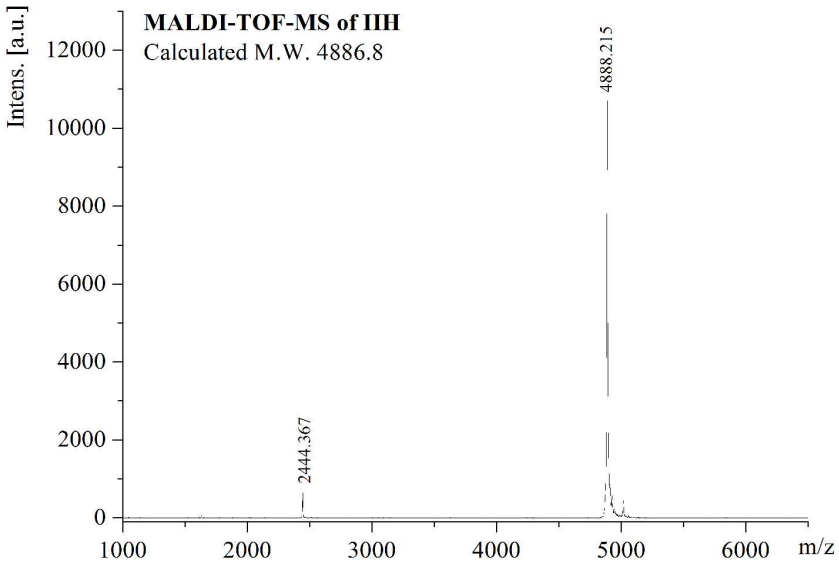
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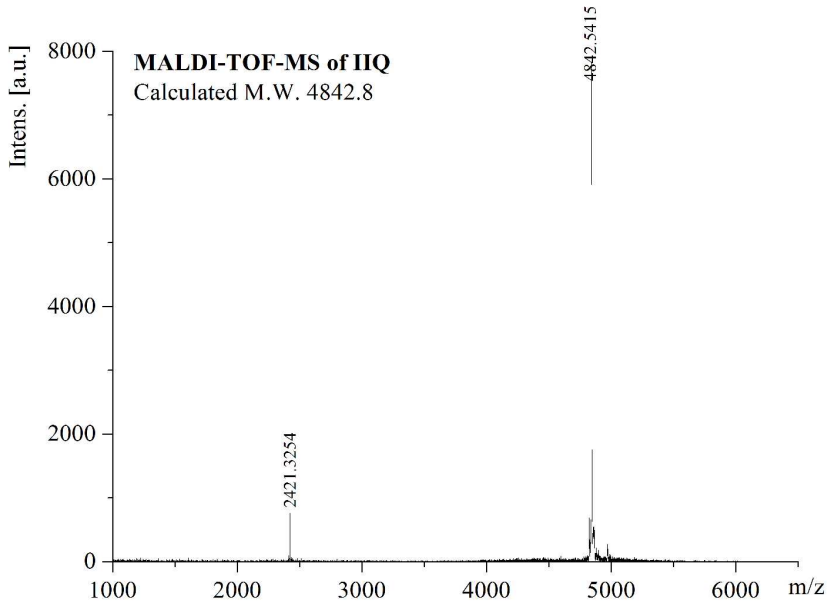
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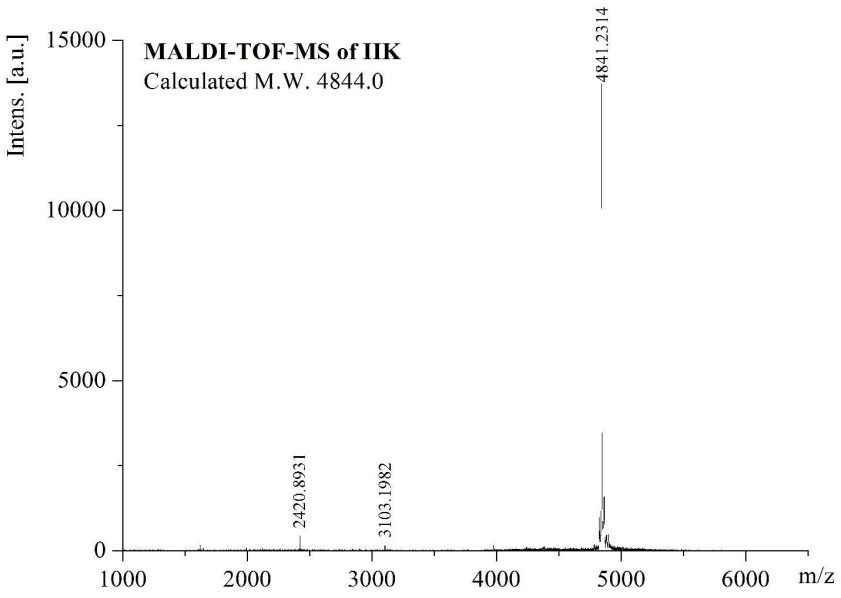
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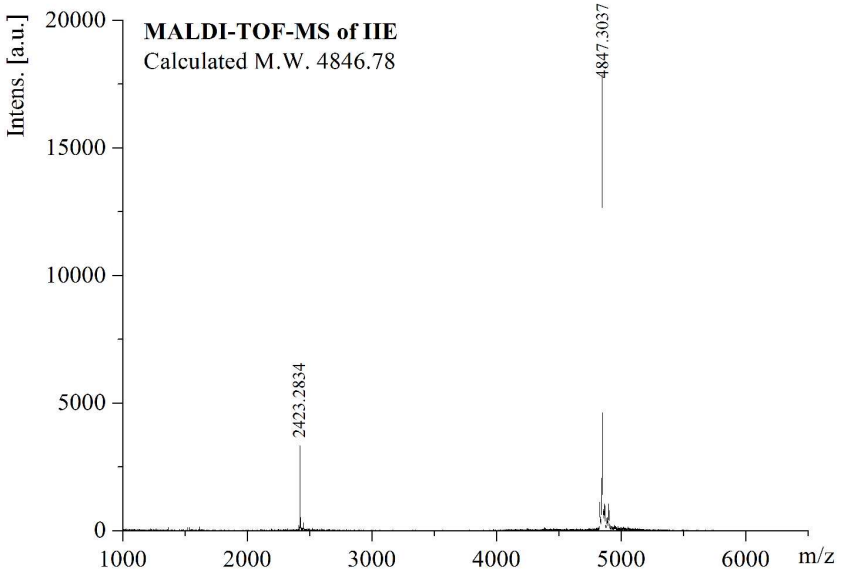
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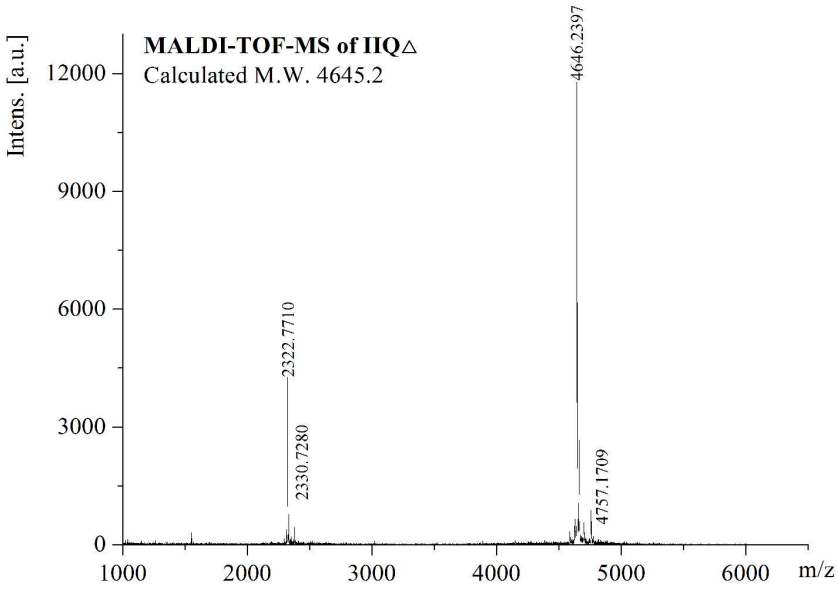
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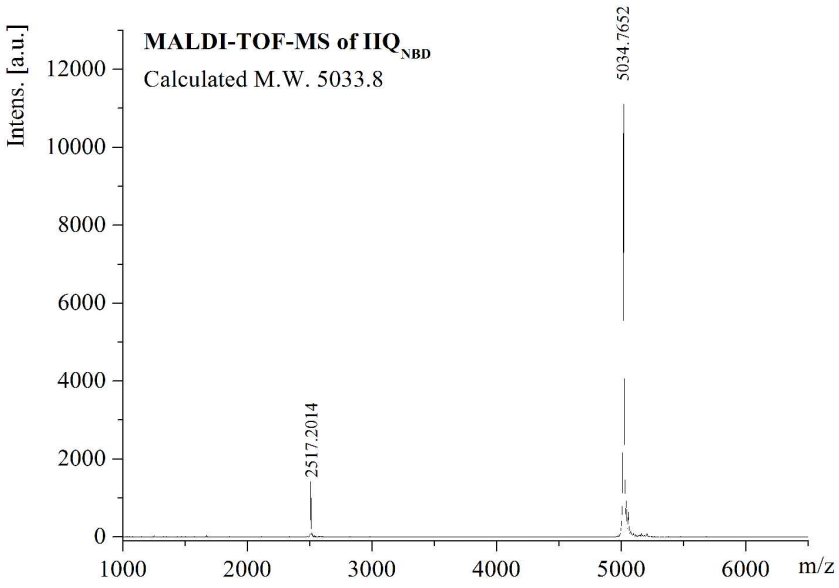
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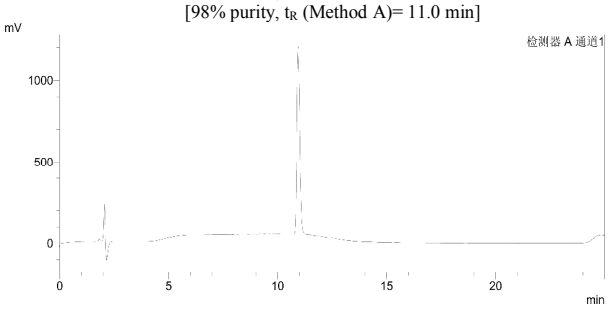
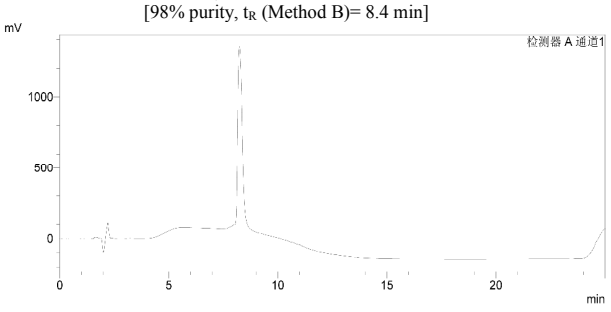
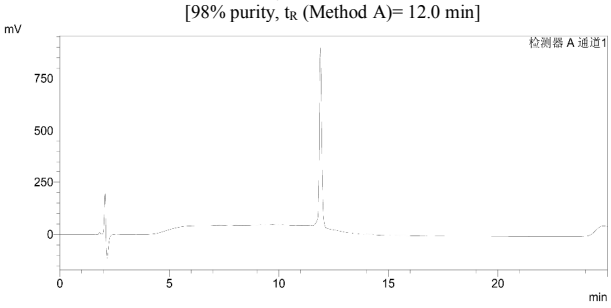
Analytical HPLC of AAS
[98% purity, t_R (Method A)= 11.0 min]



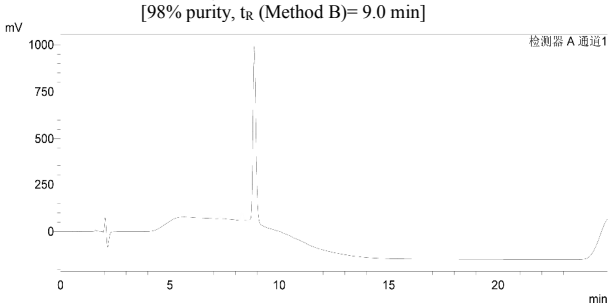
Analytical HPLC of AAS
[98% purity, t_R (Method B)= 8.4 min]



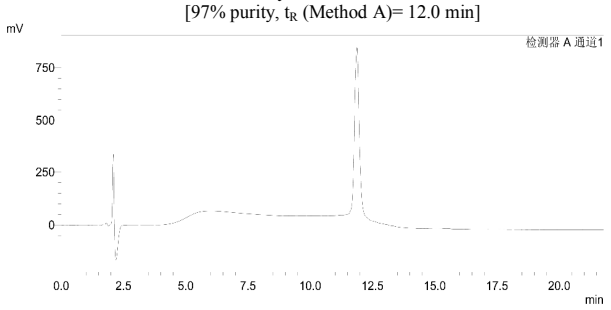
Analytical HPLC of VVS
[98% purity, t_R (Method A)= 12.0 min]



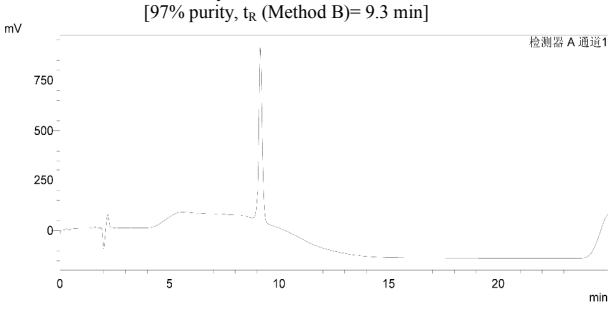
Analytical HPLC of VVS
[98% purity, t_R (Method B)= 9.0 min]



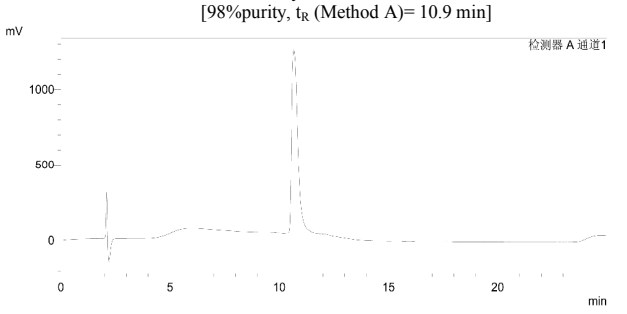
Analytical HPLC of FFS
[97% purity, t_R (Method A)= 12.0 min]



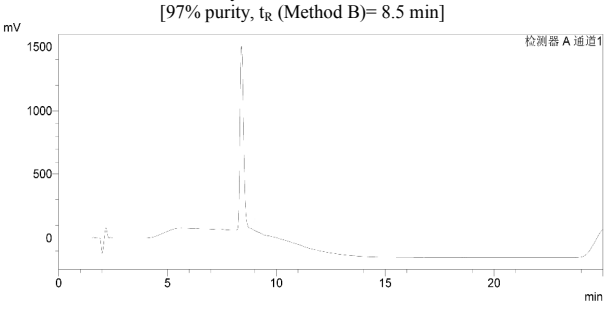
Analytical HPLC of FFS
[97% purity, t_R (Method B)= 9.3 min]



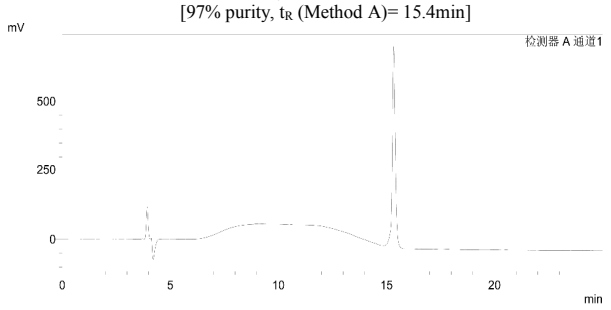
Analytical HPLC of YYS
[98%purity, t_R (Method A)= 10.9 min]



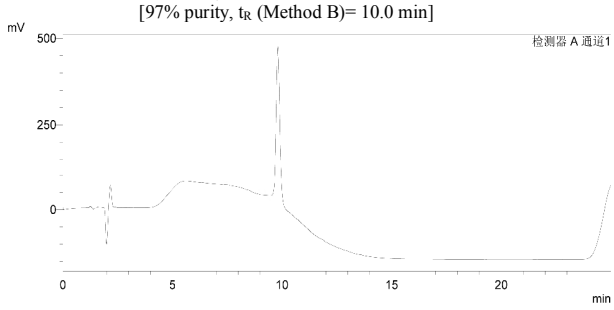
Analytical HPLC of YYS
[97% purity, t_R (Method B)= 8.5 min]



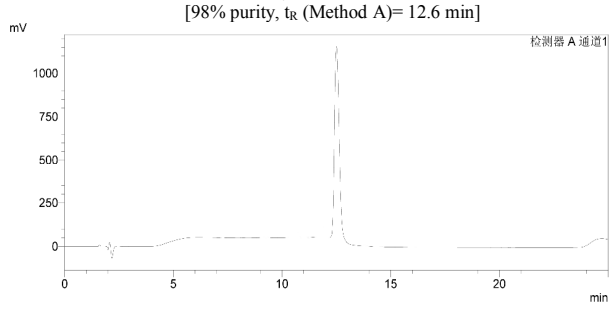
Analytical HPLC of LLS
[97% purity, t_R (Method A)= 15.4min]



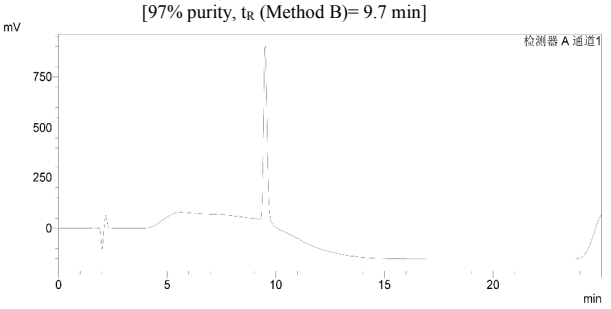
Analytical HPLC of LLS
[97% purity, t_R (Method B)= 10.0 min]



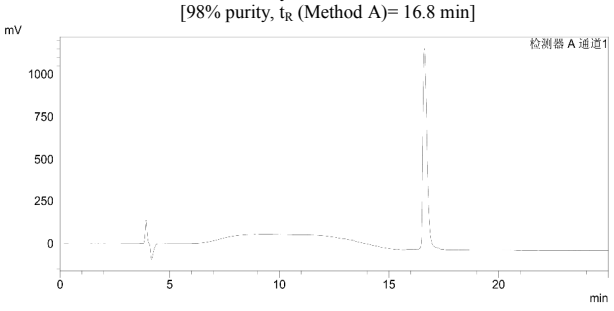
Analytical HPLC of IIS
[98% purity, t_R (Method A)= 12.6 min]



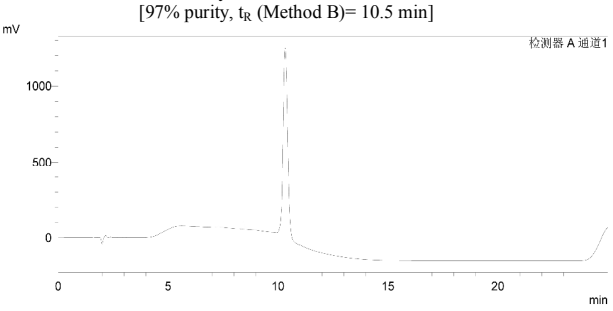
Analytical HPLC of IIS
[97% purity, t_R (Method B)= 9.7 min]



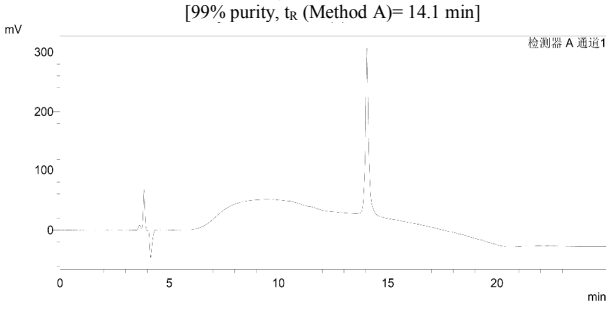
Analytical HPLC of IIY
[98% purity, t_R (Method A)= 16.8 min]



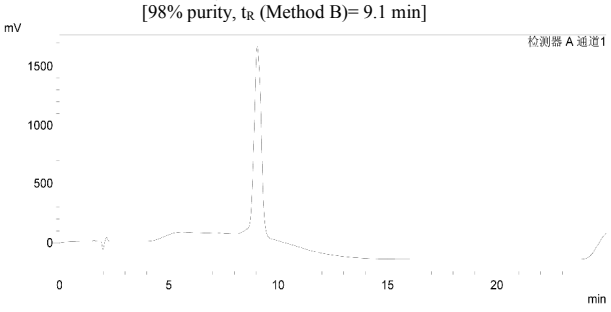
Analytical HPLC of IIY
[97% purity, t_R (Method B)= 10.5 min]



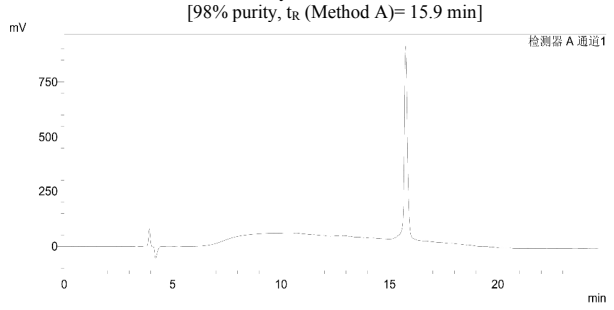
Analytical HPLC of IIW
[99% purity, t_R (Method A)= 14.1 min]



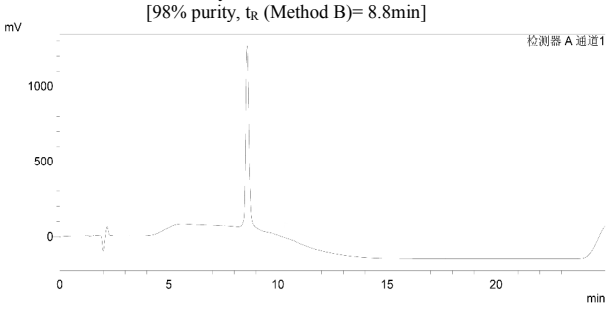
Analytical HPLC of IIW
[98% purity, t_R (Method B)= 9.1 min]



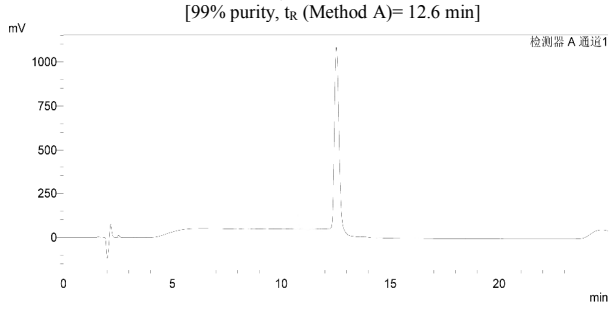
Analytical HPLC of IIH
[98% purity, t_R (Method A)= 15.9 min]



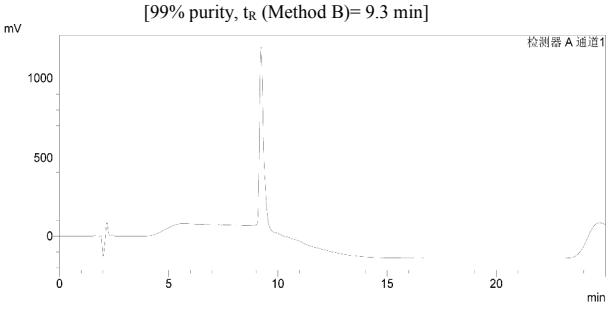
Analytical HPLC of IIH
[98% purity, t_R (Method B)= 8.8min]



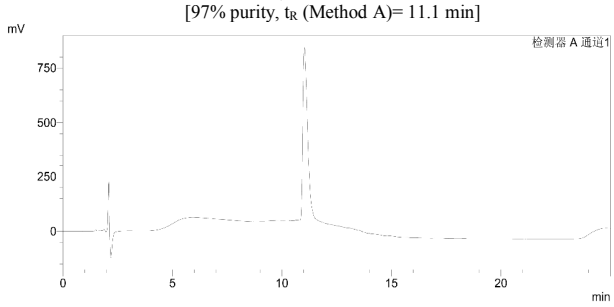
Analytical HPLC of IIQ
[99% purity, t_R (Method A)= 12.6 min]



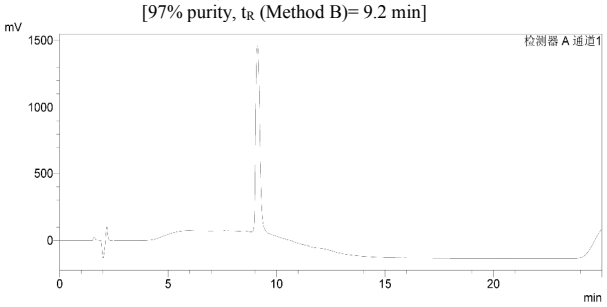
Analytical HPLC of IIQ
[99% purity, t_R (Method B)= 9.3 min]



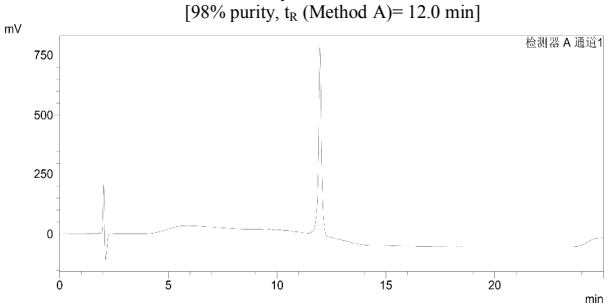
Analytical HPLC of IIK
[97% purity, t_R (Method A)= 11.1 min]



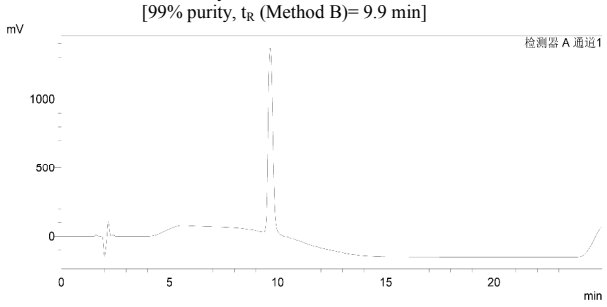
Analytical HPLC of IIK
[97% purity, t_R (Method B)= 9.2 min]



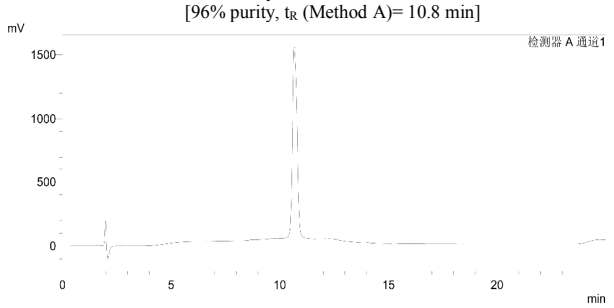
Analytical HPLC of IIE
[98% purity, t_R (Method A)= 12.0 min]



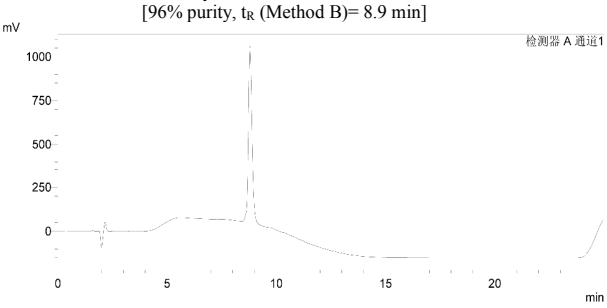
Analytical HPLC of IIE
[99% purity, t_R (Method B)= 9.9 min]



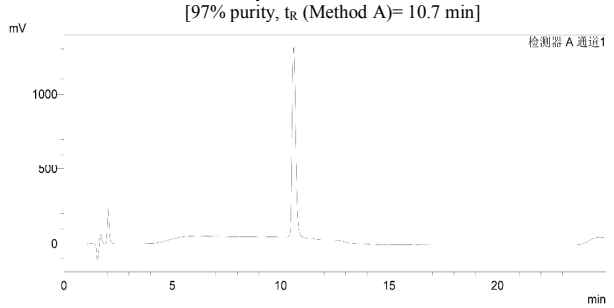
Analytical HPLC of IIQ_{NBD}
[96% purity, t_R (Method A)= 10.8 min]



Analytical HPLC of IIQ_{NBD}
[96% purity, t_R (Method B)= 8.9 min]



Analytical HPLC of IIQΔ
[97% purity, t_R (Method A)= 10.7 min]



Analytical HPLC of IIQΔ
[97% purity, t_R (Method B)= 8.4 min]