Supporting Information

Switching Substitution Groups on the In-tether Chiral Center Influences the Backbone Peptide' Permeability and Target Binding Affinity

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General Information

1. Abbreviations

Fmoc, 9-fluorenylmethyloxycarbonyl; HCTU, 2-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyl uranium hexafluorophosphate; DIPEA, diisopropylethylamine; DMF, dimethylformamide; ESI-MS, electrospray ionization mass spectrometry; RP-HPLC, reserved-phase high performance liquid chromatography; RT, room temperature; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TIS, triisopropylsilane; Trt, triphenylmethyl; Et2O, diethyl ether; LC-MS, liquid chromatography–mass spectrometry;

HPLC, high-performance liquid chromatography; DMPA, 2, 2-dimethoxy-2-phenylacetophenone; MMP, 4-Methoxyacetophenone; MAP, 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone.

2. Materials

All solvents and reagents used for solid phase peptide synthesis were purchased from commercial suppliers including GL Biochem (Shanghai) Ltd., Shanghai Hanhong Chemical Co., J&K Co. Ltd., Shenzhen Tenglong Logistics Co. or Energy Chemical Co. and were used without further purification unless otherwise stated.

3. HPLC and Mass spectrometry

Peptides were analyzed and purified by HPLC (SHIMAZU Prominence LC-20AT) using a C18 analytic column (Agilent ZORBAX SB-Aq, 4.6 × 250 mm, 5 μm, flow rate 1.0 mL/min) and a C18 semi-preparative column (Agilent Eclipse XDB-C18, 9.4 × 250 mm, 5 μm, flow rate 5 mL/min). H2O (containing 0.1% TFA) and pure acetonitrile (containing 0.1% TFA) were used as solvents in linear gradient mixtures. LC-MS spectra were carried out on SHIMAZU LC-MS 8030 (ESI-MS).

4. Peptide Synthesis and Characterization

Peptide synthesis was performed manually on Rink Amide MBHA resin (loading capacity: 0.54 mmol/g) (GL Biochem Ltd.) by standard Fmoc-based solid-phase peptide synthesis. Generally, Rink amide AM resin was pre-swelled with DCM/NMP (1/1) for 30 min. Fmoc deprotection was performed with morpholine (50% in NMP) for 30min × 2. Then the resin was washed with NMP (5 times), DCM (5 times) and NMP (5 times). Fmoc-protected amino acids (6.0 equiv according to initial loading of the resin) and HCTU (5.9 equiv) were dissolved in NMP, followed by DIPEA (12.0 equiv). The mixture was pre-activated for 1 min and added to the resin for 1-2 h, then the resin was washed with NMP (5 times), DCM (5 times) and NMP (5 times). Upon completion of peptide assembly, peptides were N-terminally acetylated with a solution of acetic anhydride and DIPEA in NMP (1:1: 8 in volume) for 1h. Peptides were cleaved from the resin with a mixture of TFA/H2O/EDT/TIS (94:2.5:2.5:1) for 2 h and concentrated under a stream of nitrogen. The crude peptides were then precipitated with Hexane/Et2O (1:1 in volume) at -20°C, isolated by centrifugation then dissolved in water/acetonitrile, purified by semi-preparative HPLC and analyzed by LC-MS.

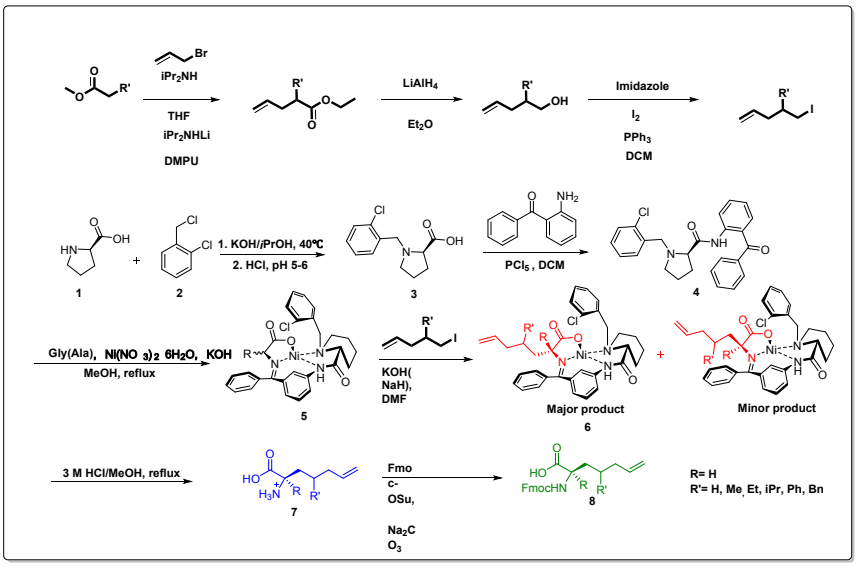
5. NMR Spectroscopy

NMR spectra were recorded on Bruker Avance-III 400MHz spectrometer in CDCl3 or DMSO-d6 or Bruker Avance-III 600 MHz spectrometer with a TXI probe in phosphate buffer (pH 5.0) with 10% D2O at 298K. Data are reported in the following order: chemical shifts are given (δ); multiplicities are indicated as br (broadened), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and app (apparent).

Experimental section

1. Unnatural amino acids synthesis

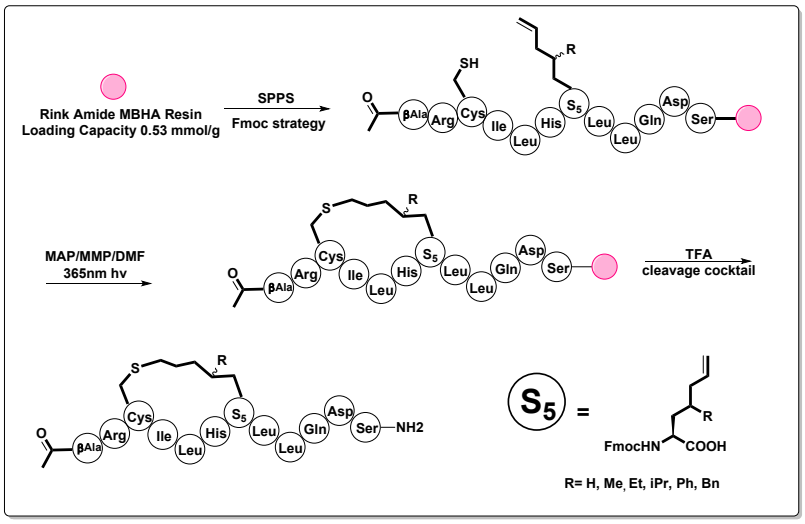
All the unnatural amino acids were synthesized based on the previous literatures.[1-5]



Scheme 1. Flow chart for unnatural amino acids synthesis.

2. Solid phase peptide synthesis

All peptides were synthesized by manual Fmoc-based solid-phase synthesis. Intermolecular thiol-ene reactions were used for constructing target cyclic peptides. Thiol-ene reaction is conducted at ultraviolet light (365nm) with 1eq MAP/MMP (1:1) catalysis in DMF for 2 hr. The photoreaction efficiency is generally >90% for pentapeptides, and >70% for longer peptides. Macrocyclization: Then the resins were added HOBT (1 equiv) and Pypop (1 equiv) in anhydrous DMF for 2 hours. The resulted cyclic diastereomers were separated by HPLC. The linear peptide diastereoisomers were inseparable. The purified peptides were tested by ESI/LC-MS and the pure fractions were combined and then lyophilized.



Scheme 2. The representative synthesis procedures for stapled peptide. (MAP: 4-ethoxyacetophenone, MMP: 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone, DIEA: N, N-diisopropylethylamine), TIS: Triisopropylsilane.

3. Peptide purification and characterization

Linear peptides were synthesized, and then characterized by HPLC and LC-MS. The photoreaction products were purified on the HPLC. In general, for peptides 1-5a/b the a/b epimers were separable with more than 1min

retention time differences. The purified peptides were then characterized by LC-MS. Mass spectra was obtained by ESI in positive ion mode.

4. Circular Dichroism Spectroscopy (CD)

CD spectra were detected by Chirascan Plus Circular Dichroism Spectrometer (Applied photophysics) at 25 ^{o}C. Peptides were dissolved in 300 μl ddH_2O at a final concentration of 0.14 mM. Parameters used in the experiment are as followed: wavelengths from 250 to 190 nm were measured with resolution of 0.5 nm at a scan speed of 20 nm/sec. Each sample was scanned twice and the averaged spectrum was smoothed using Pro-Data Viewer by Applied Photophysics with smooth window of 10. CD data are presented as mean residual elipticity [θ] in deg•cm^2 •dmol^{-1}. Concentration of each sample: absorption (OD) at 280 nm × dilution factor ÷1 (cm) ÷ 1490 (1cm^{-1}M^{-1}) ×1000 (mMM^{-1}) = concentration (mM). Percent helicity was calculated based on the equation described by Fairlie[6] : Helicity% = ($[\theta]_{obs222}$-$[\theta]_c$)/ ($[\theta]_{\infty 222}$-$[\theta]_c$), where $[\theta]_{\infty 222}$= (−44 000 + 250T) (1 − k/n), $[\theta]_c$= 2220-53T for k = 4.0 and n = number of amino acid residues in the peptide (including linker), T = 20 ˚C.

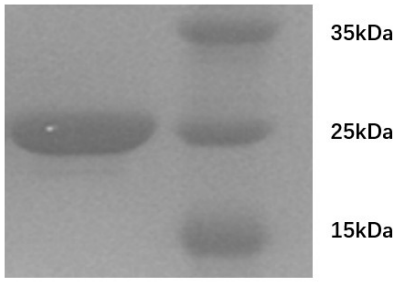
5. Protein Expression and Purification

Plasmids were constructed by cloning DNA encoding human ER-α LBD (301-553) into pET23b via NdeI and XhoI and overexpressed by high-density culture of E. coli BL 21 (DE3) host cell without IPTG. Cultures were grown in 2YT medium and incubated at 37 ˚C till an OD600 of 0.8 and cultures were transferred to 20 ˚C for another 18 hr. Cells were harvested by centrifugation and lysed by sonication in a buffer containing 100 mM Tris-Cl pH 8.1, 300 mM KCl, 5 mM EDTA, 4 mM DTT and 1 mmol/L PMSF. Cell debris was removed by centrifugation and the supernatant was purified by flowing through a pre-equilibrated 1 mL estradiol affinity column (PDI technology) and washed with 100 μM estradiol in 20 mM Tris pH 8.1, 0.25 M NaSCN. High molecular weight species and excess salts were removed on a Superdex 200 column equilibrated in 50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM DTT. Finally, the eluted ERα LBD (301-553) were concentrated to 1-5 mg/ml.

Primer sequence:

ER-alpha-NdeI-301: GTGTACACATATGtctaagaagaacagcctggccttgt

ER-alpha-XhoI-553: Ccctcgagttaagtgggcgcatgtaggcggt



6. Fluorescence Polarization Assay (FP)

Fluorescence polarization experiments were performed in 96-well plates on plate reader (Perkin Elmer, Envision, 2104 multilabel reader) at 25 ˚C with excitation at 485 nm and emission at 520 nm. Concentrations of the peptides were determined by 495 nm absorption of FITC. Purified ER-α LBD (301-553) LBD (at increasing concentrations, 20 μL) and fluorescein-labeled peptides (10 nM, 80 μL) in assay buffer (10 μM 17-β-estradiol, 20 mM Tris-HCl pH 8.0, 25 mM NaCl, 10% glycerol, 10 μM beta-estradiol and 1 mM TCEP) were mixed and incubated at 4 ˚C for 1 hr in the dark. The fluorescence polarization of the labeled peptides was measured at 16 ˚C with excitation at 485

nm and emission at 520 nm and then plotted against the concentrations of the ER-α LBD. The data points were fitted by Origin pro 9.0.

7. Confocal Microscopy Imaging

Hela cells were cultured with DMEM with 10% FBS (v/v) in imaging dishes (50000 cells/well) in 37°C, 5% CO2 incubator for one day until they were about 80% adherent. Peptide were first dissolved in DMSO to make a 1 mM stock and then added to cells to a final concentration of 5 μM. The cells were incubated with peptides for 1 hour at 37°C. After incubation, cells were washed 3 times with PBS and then fixed with 4% formaldehyde (Alfa Aesar, MA) in PBS for 10 minutes. They were then washed 3 times with PBS and stained with 1μg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Invitrogen, CA) in PBS for 5 minutes. Images of peptide localization in cells were taken on PerkinElmer confocal microscopy. Image processing was done using Volocity software package (Zeiss Imaging).

8. Flow cytometry

Hela cells were grown in DMEM medium with 10% FBS (v/v) in imaging dishes (50000 cells/well) in 37°C, 5% CO2 incubator for two days (50,000 cells per well). Cells were treated with fluoresceinated peptides (5 μM) for up to 2 hours at 37°C. After washing with media, the cells were exposed to trypsin (0.25%; Gibco) digestion (5 min, 37°C), washed with PBS, and resuspended in PBS. Cellular fluorescence was analyzed using a BD FACSCalibur flow cytometer (Becton Dickinson). The identical experiment was performed with 30 min pre-incubation of cells at 4°C followed by 4 hours' incubation with fluoresceinated peptides at 4°C to assess temperature-dependence of fluorescent labeling.

9. Serum stability

Standard solution of ER-α-linear (FITC-βARCILHS$_5$(2-Me)LLQDS-NH2), FITC-βAR[cyclo-CILHS$_5$(2-Me)]LLQDS-NH2 (a/b), FITC-βAR[cyclo-CILHS$_5$(2-Et)]LLQDS-NH2 (a/b), FITC-βAR[cyclo-CILHS$_5$(2-iPr)]LLQDS-NH2 (a/b), FITC-βAR[cyclo-CILHS$_5$(2-Ph)]LLQDS-NH2 (a/b), FITC-βAR[cyclo-CILHS$_5$(2-Bn)]LLQDS-NH2 (a/b), FITC-βAR[cyclo-CILHS$_5$(2-H)]LLQDS-NH2 were prepared at a concentration of 100 μM in water. Each peptide was added to the 25% human serum(800uL) and incubated at 37°C. Acetonitrile/water 3:1 was added to aliquots of serum at 0, 2h, 4h, 8h, 16h, 18h, 20h, 22h and 24h to precipitate serum proteins, which are removed by centrifugation. The standard supernatant was analyzed by LC/MS with a grace smart C18 250× 4.6mm column, using a 3% per minute linear gradient from 20% to 80% acetonitrile over 20min. The amount of starting material left in each sample was quantified by LC/MS-based peak detection at 220nm.

Supplementary Figures and Tables

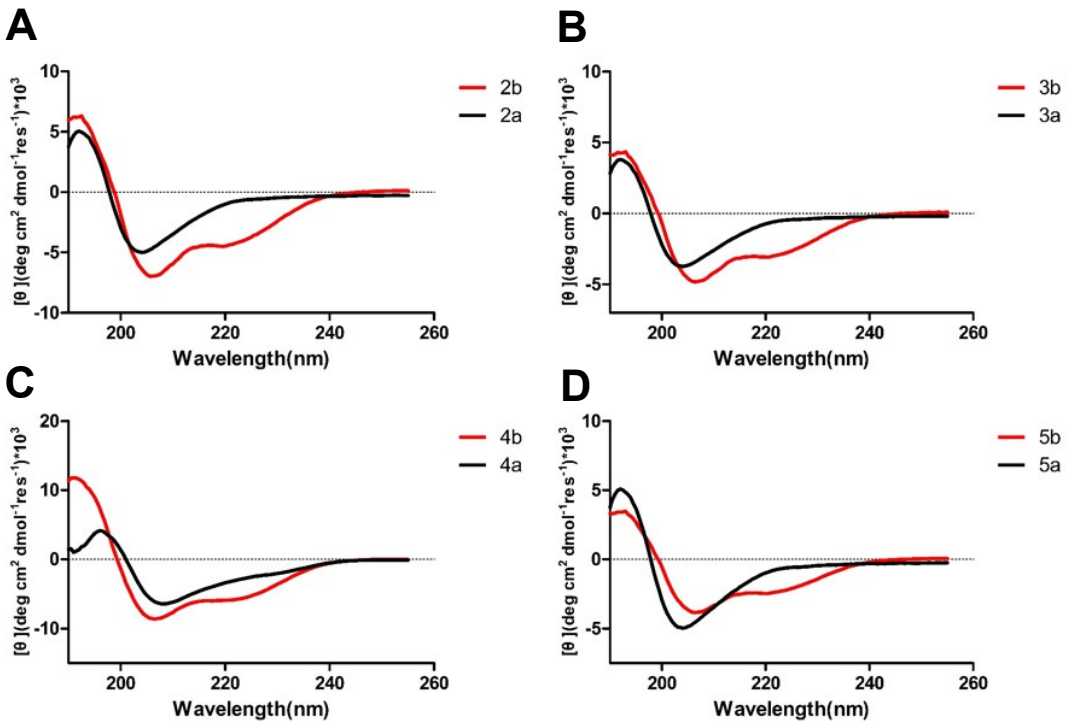


Figure S1. (A-D) CD spectroscopy of peptide of 2, 3, 4, 5.

Peptide	$[\theta]_{222}$	$[\theta]_{208}$	$[\theta]_{190}$	$[\theta]_{222}$ / $[\theta]_{208}$
1a	-3440.17	-10382.29	7865.83	0.33
2a	-2029.46	-4385.83	-4385.83	0.46
3a	-1510.29	-3275.84	2828.63	0.46
4a	-4591.35	-6266.16	1449.46	0.73
5a	-2013.72	-4367.79	3771.51	0.46

Table S1. Molar elipticities $[\theta]_{222}$, $[\theta]_{208}$, $[\theta]_{190}$, $[\theta]_{222}$ / $[\theta]_{208}$ of **1a-5a**.

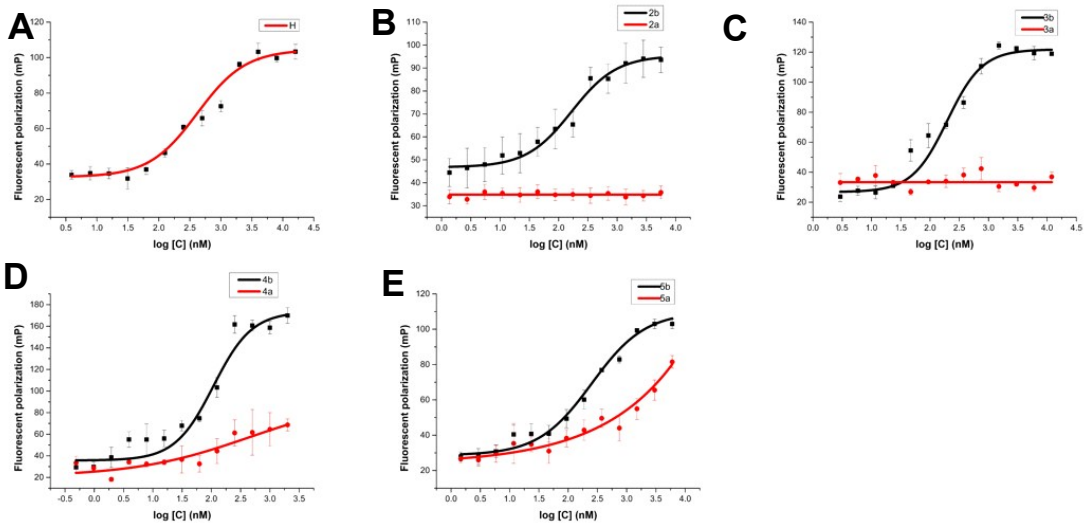


Figure S2. (A-F) Fluorescence Polarization Assay (FP). Binding affinity of FITC-labeled peptide derivatives H, Me, Et, iPr, Ph, Bn with ER-α, respectively. mP, mean ± s. d., n=3. Measurements were performed in triplicates.

Peptide	Sequence	Kd for ER-α（nM）
1a	Ac-R[cyclo-CILHS$_5$(2-Me)]LLQDS-NH$_2$	ND
1b	Ac-R[cyclo-CILHS$_5$(2-Me)]LLQDS-NH$_2$	42±7.7
2a	Ac-R[cyclo-CILHS$_5$(2-Et)]LLQDS-NH$_2$	ND
2b	Ac-R[cyclo-CILHS$_5$(2-Et)]LLQDS-NH$_2$	167±37.2
3a	Ac-R[cyclo-CILHS$_5$(2-iPr)]LLQDS-NH$_2$	ND
3b	Ac-R[cyclo-CILHS$_5$(2-iPr)]LLQDS-NH$_2$	196±27.1
4a	Ac-R[cyclo-CILHS$_5$(2-Ph)]LLQDS-NH$_2$	>500
4b	Ac-R[cyclo-CILHS$_5$(2-Ph)]LLQDS-NH$_2$	75±19.3
5a	Ac-R[cyclo-CILHS$_5$(2-Bn)]LLQDS-NH$_2$	>1000
5b	Ac-R[cyclo-CILHS$_5$(2-Bn)]LLQDS-NH$_2$	252±25.6
6	Ac-R[cyclo-CILHS$_5$(2-H)]LLQDS-NH$_2$	415±81.9
Linear-6	Ac-RCILHS$_5$(2-H)LLQDS-NH$_2$	ND

Table S2. The binding affinity parameters of peptides toward proteins (mean ± standard deviation, n=3). ND means not detected.

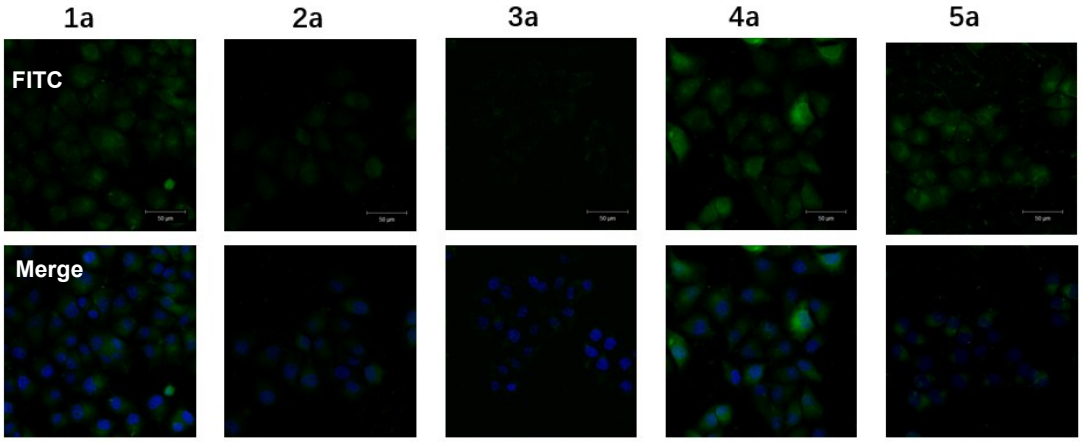


Figure S3. Fluorescent confocal microscopy images of Hela cells incubated with 5 μM FITC labeled peptides **1a-5a** and **6** at 37˚C.

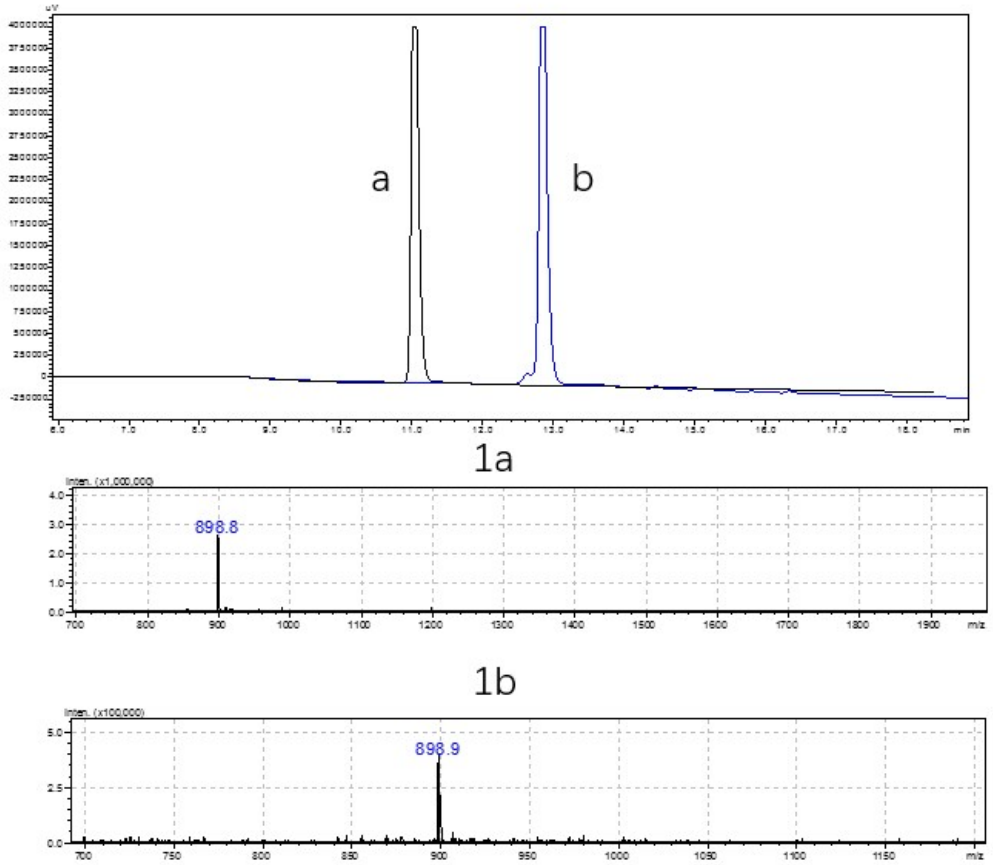
Appendix

Mass spectrometry data for peptides

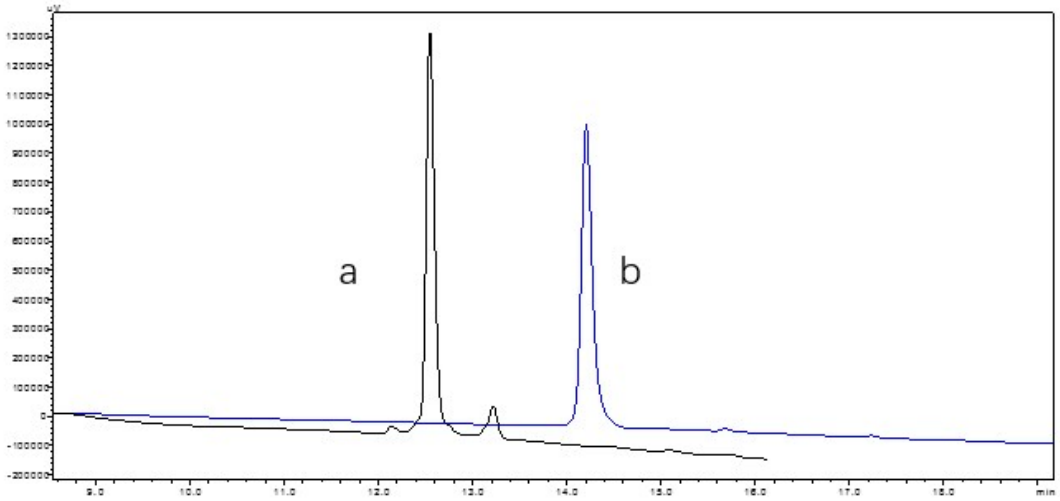
Peptide	Sequence	Calculated mass	Found mass
1a	FITC-βAR[cyclo-CILHS$_5$(2-Me)]LLQDS-NH$_2$	1795.1664	898.8 [M+2H]/2
1b	FITC-βAR[cyclo-CILHS$_5$(2-Me)]LLQDS-NH$_2$		898.9 [M+2H]/2
2a	FITC-βAR[cyclo-CILHS$_5$(2-Et)]LLQDS-NH$_2$	1809.1820	905.7 [M+2H]/2
2b	FITC-βAR[cyclo-CILHS$_5$(2-Et)]LLQDS-NH$_2$		905.8 [M+2H]/2
3a	FITC-βAR[cyclo-CILHS$_5$(2-iPr)]LLQDS-NH$_2$	1823.1977	912.9 [M+2H]/2
3b	FITC-βAR[cyclo-CILHS$_5$(2-iPr)]LLQDS-NH$_2$		912.8 [M+2H]/2
4a	FITC-βAR[cyclo-CILHS$_5$(2-Ph)]LLQDS-NH$_2$	1857.1820	930.3 [M+2H]/2
4b	FITC-βAR[cyclo-CILHS$_5$(2-Ph)]LLQDS-NH$_2$		929.9 [M+2H]/2
5a	FITC-βAR[cyclo-CILHS$_5$(2-Bn)]LLQDS-NH$_2$	1871.1977	936.4 [M+2H]/2
5b	FITC-βAR[cyclo-CILHS$_5$(2-Bn)]LLQDS-NH$_2$		936.8 [M+2H]/2
6	FITC-βAR[cyclo-CILHS$_5$(2-H)]LLQDS-NH$_2$	1781.1507	891.8 [M+2H]/2

HPLC traces and MS spectra

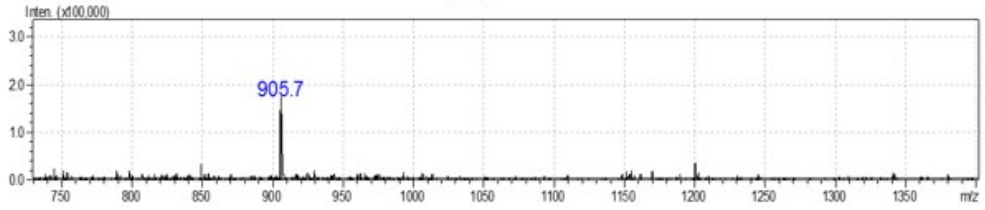
FITC-βAR[cyclo-CILHS5(2-Me)]LLQDS-NH2



FITC-βAR[cyclo-CILHS5(2-Et)]LLQDS-NH2



2a



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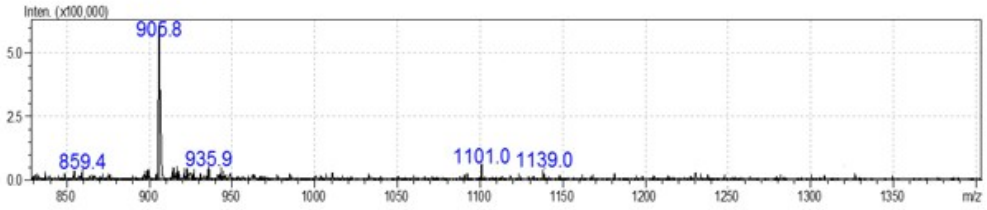
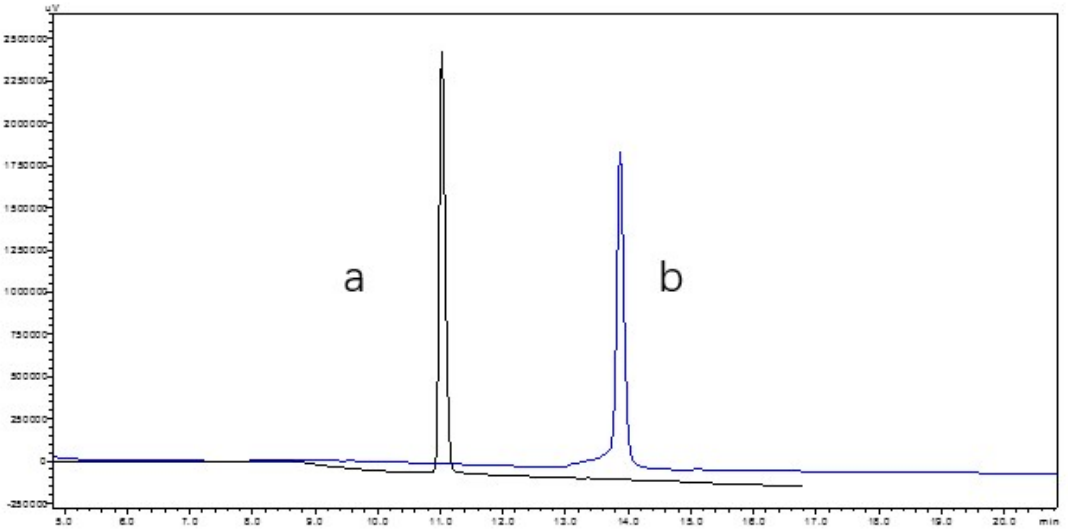


FITC-βAR[cyclo-CILHS5(2-iPr)]LLQDS-NH2



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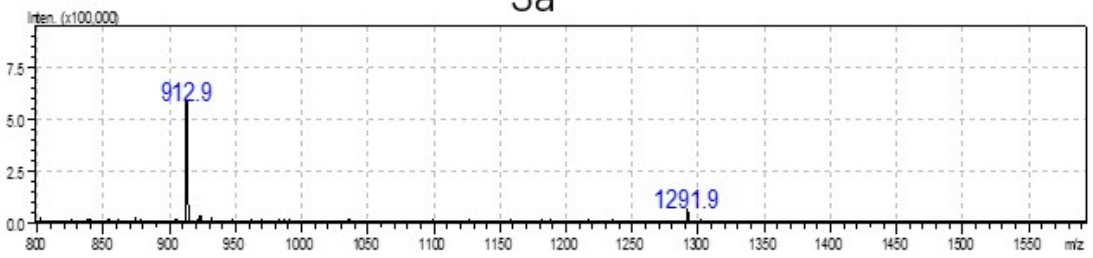

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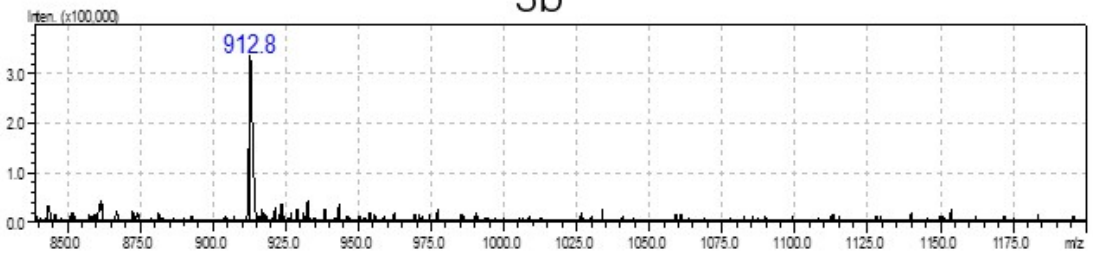

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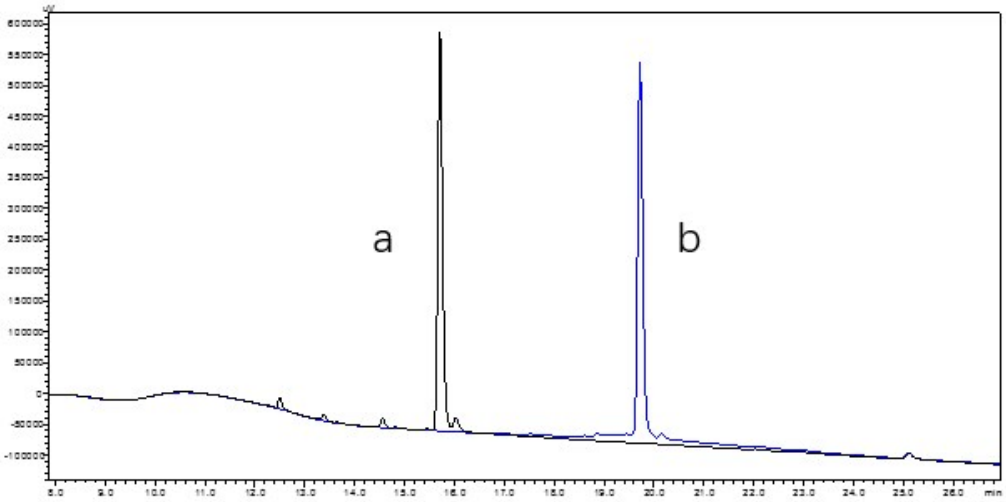

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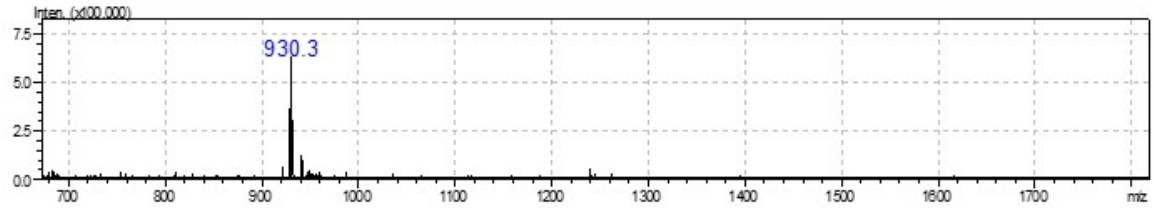

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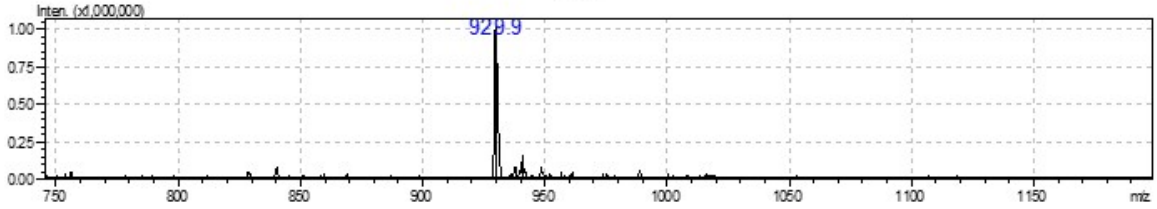

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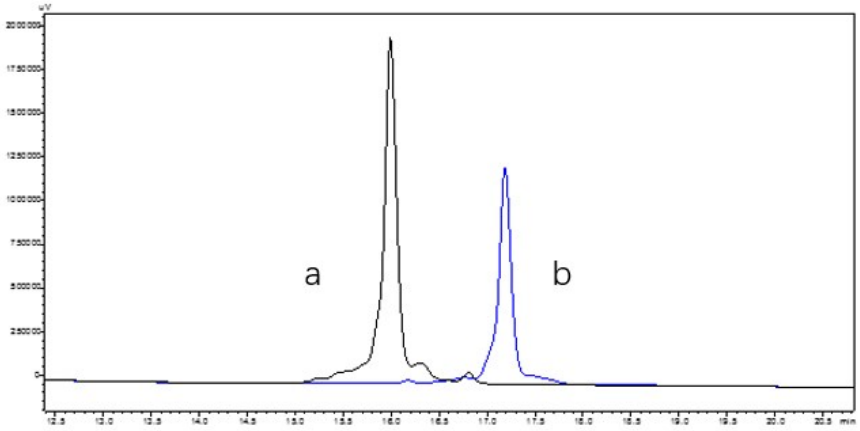

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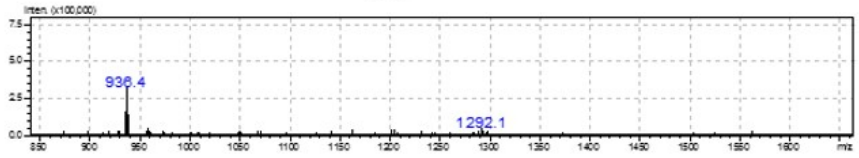

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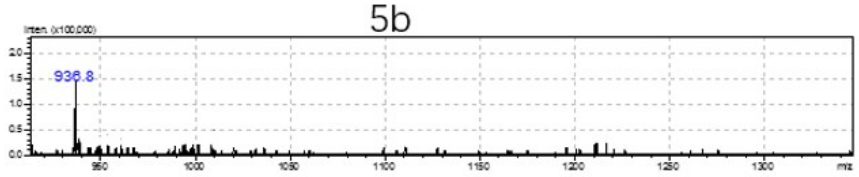

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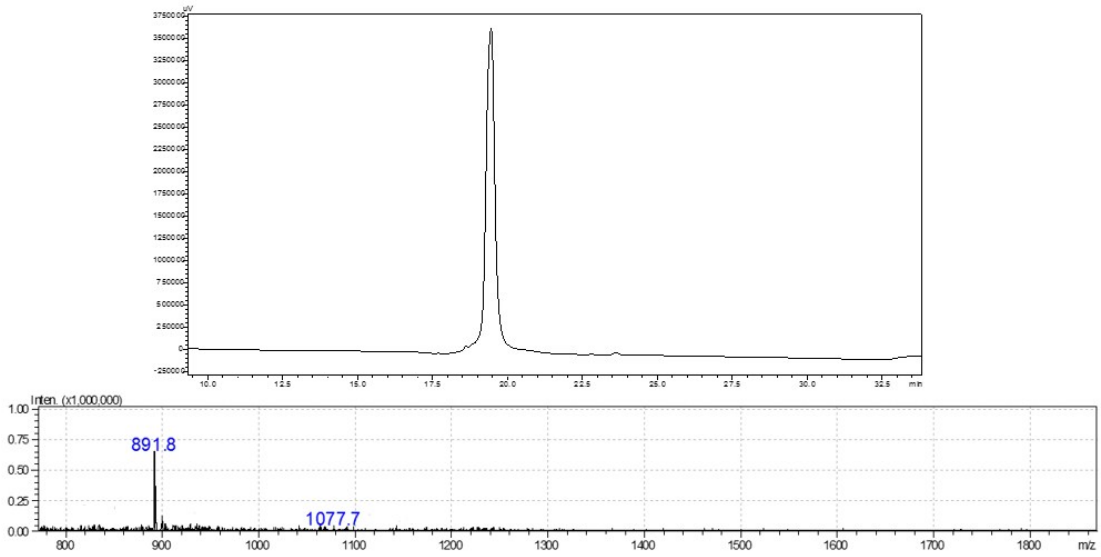
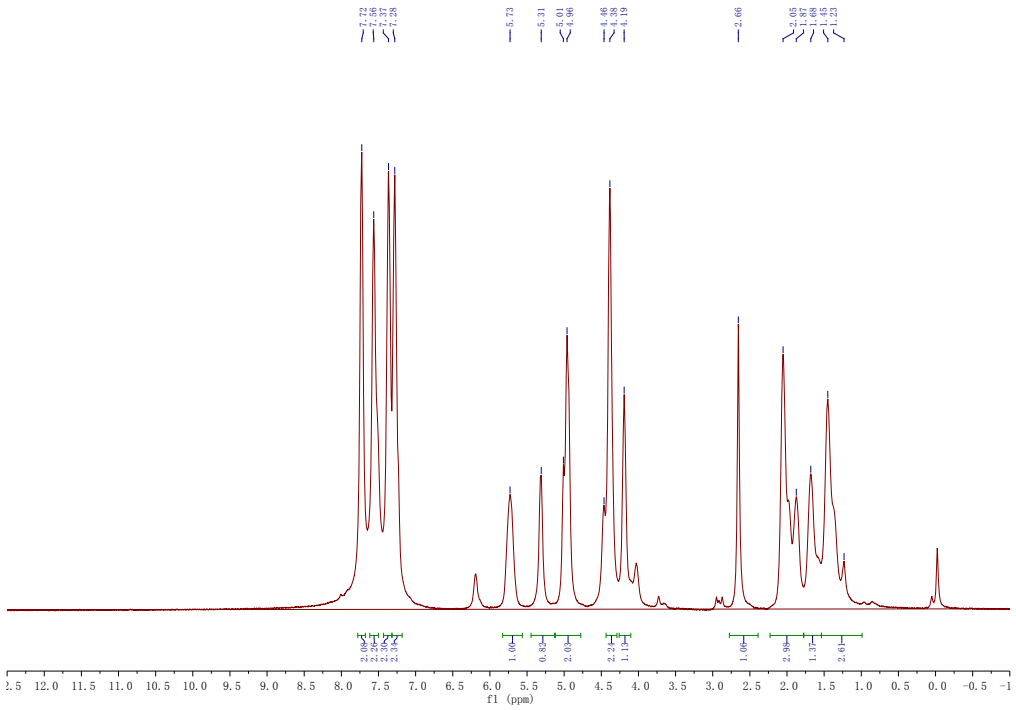


Characterization of unnatural amino acid

^{1}H of Fmoc-S_5(2-H)-OH



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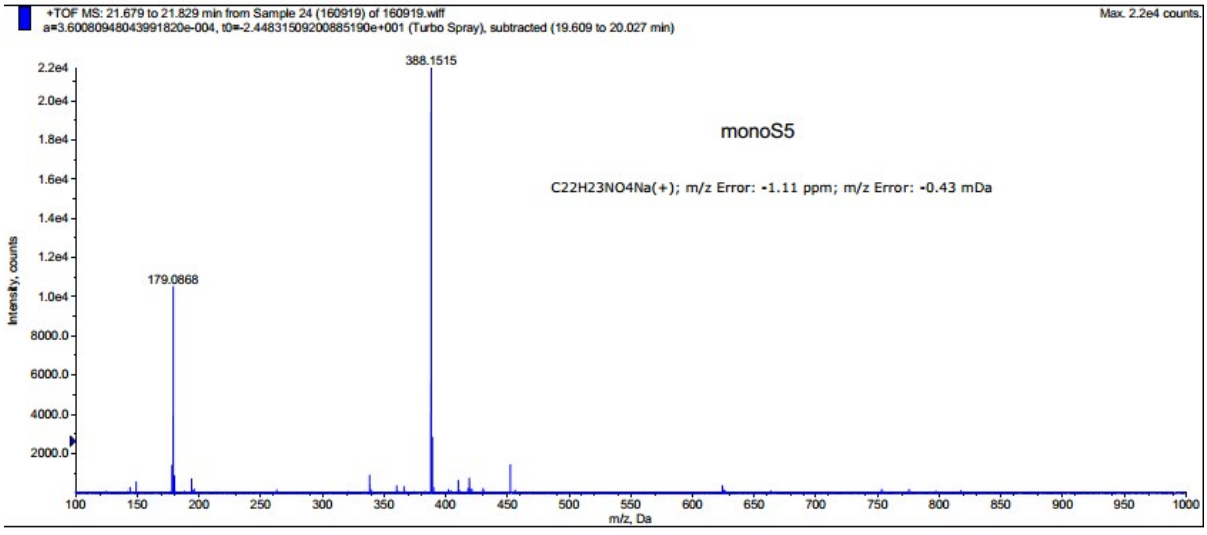

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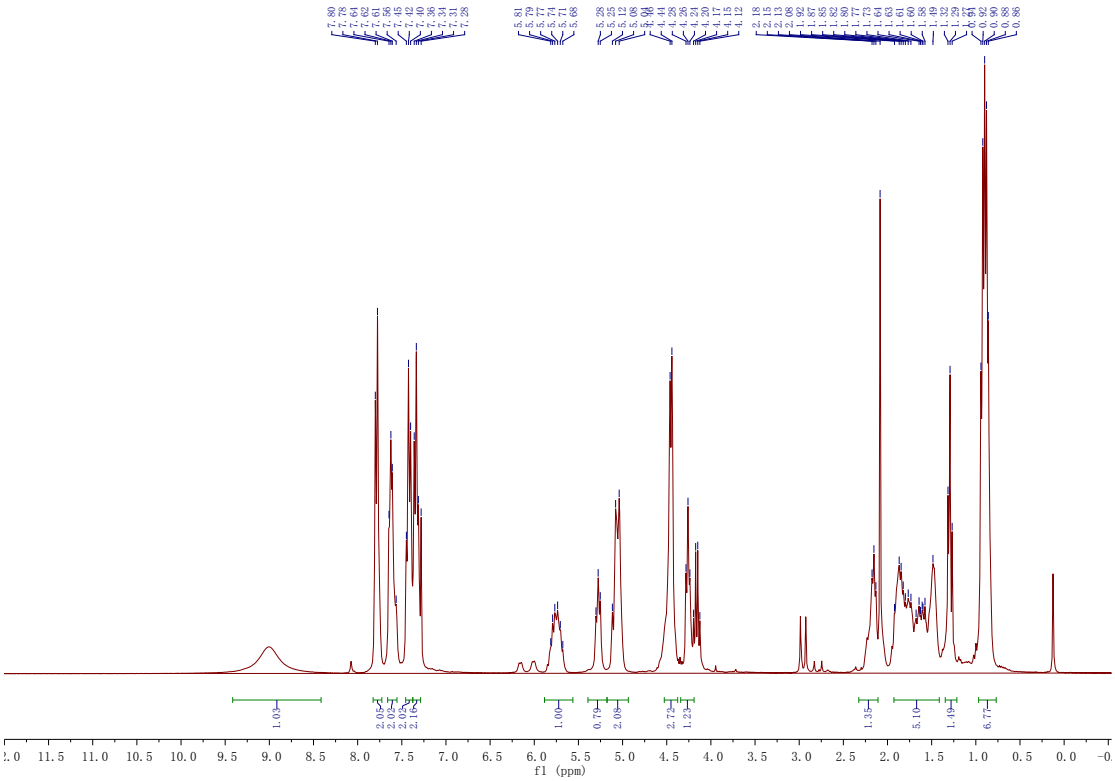

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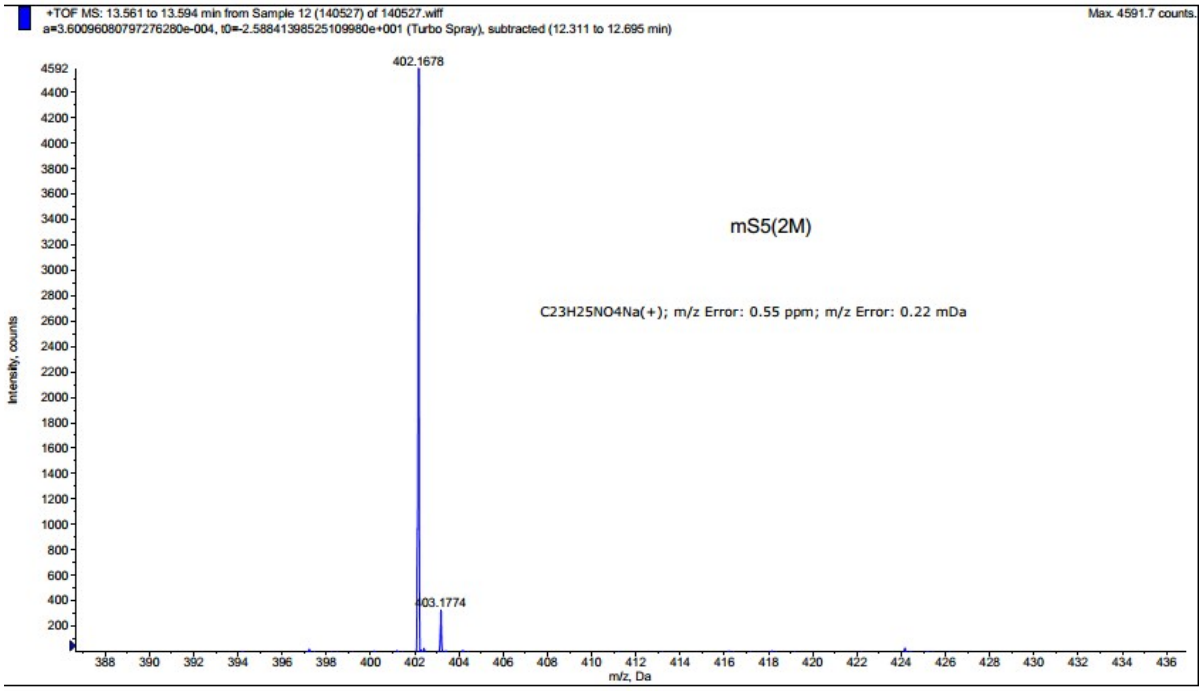
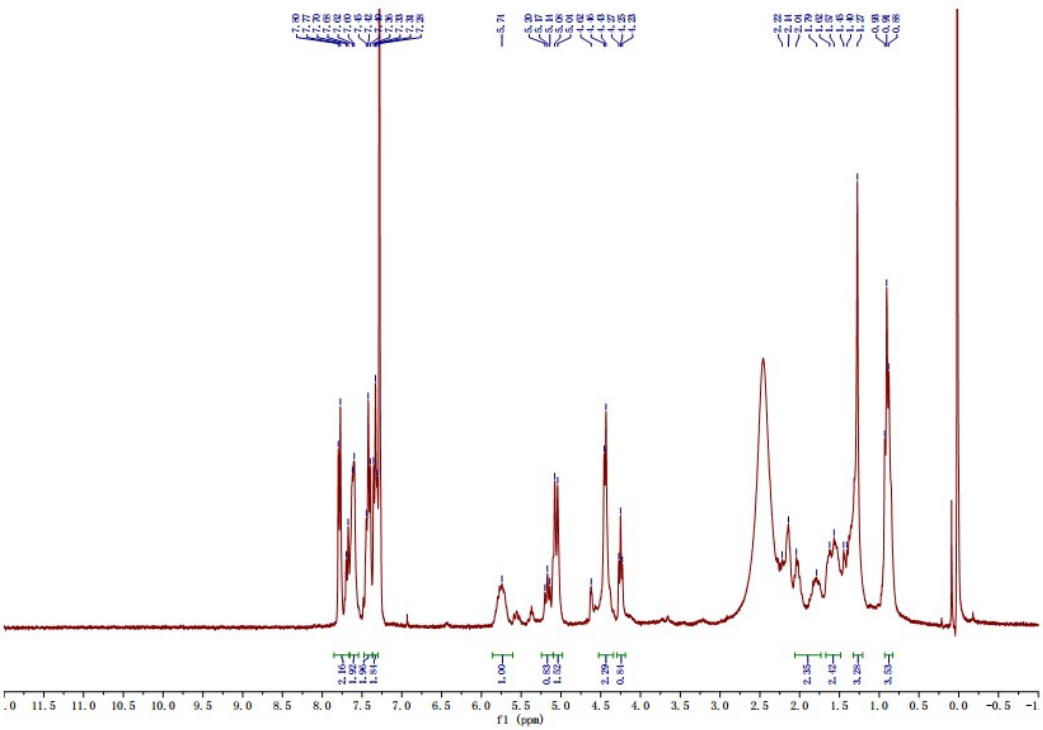
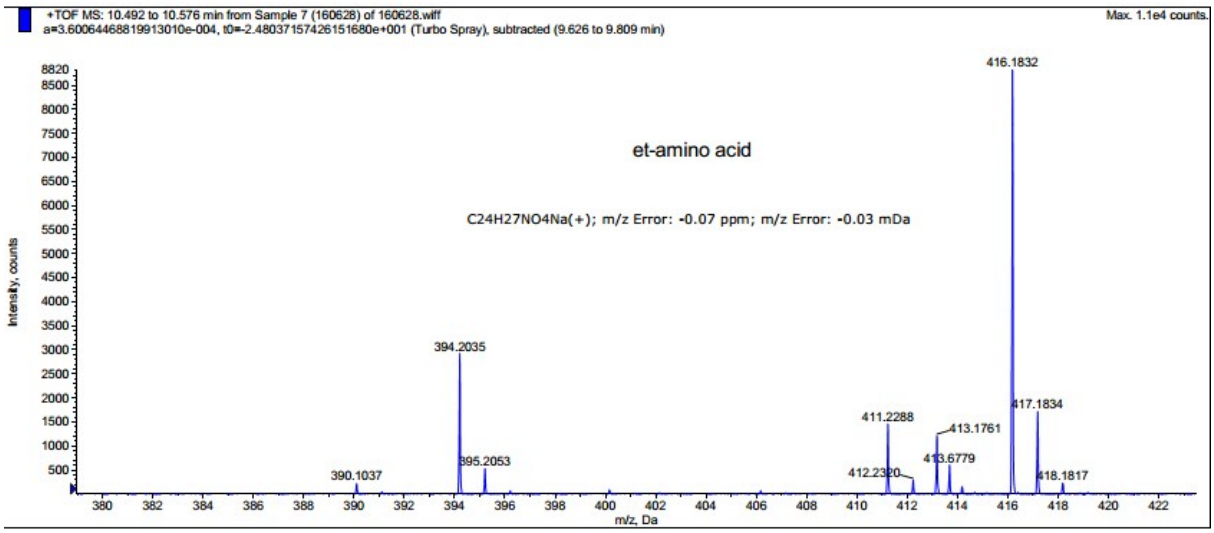


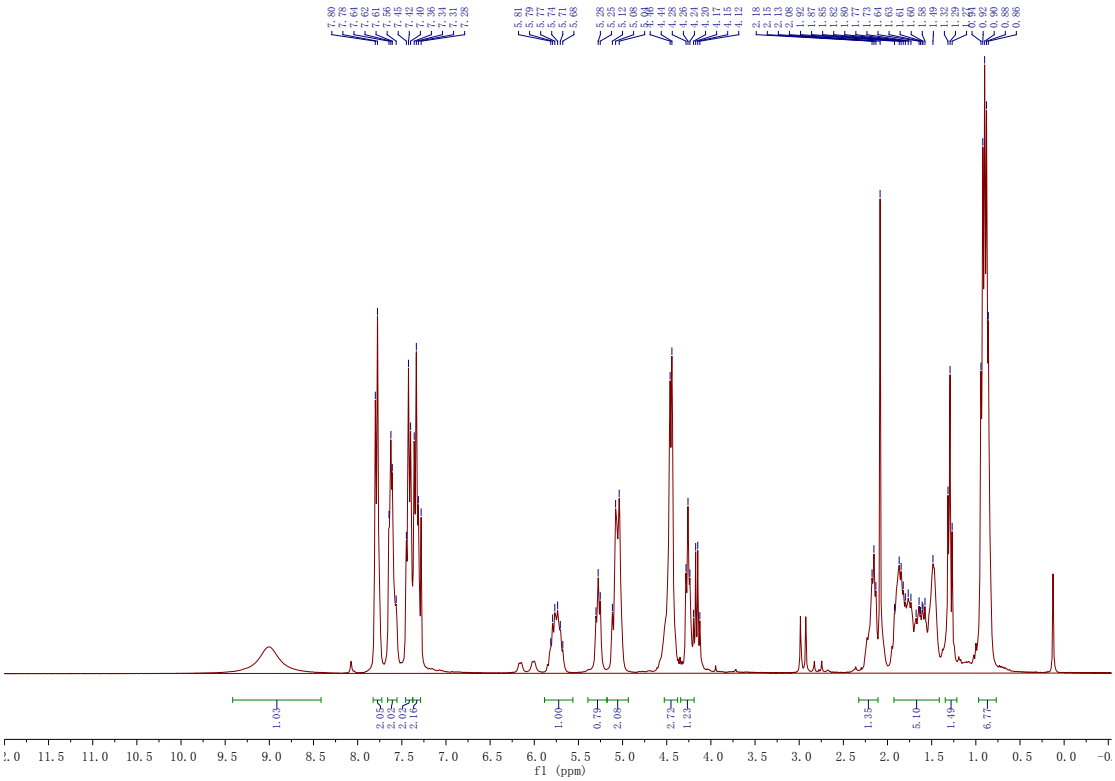
^{1}H of Fmoc-S5(2-Et)-OH



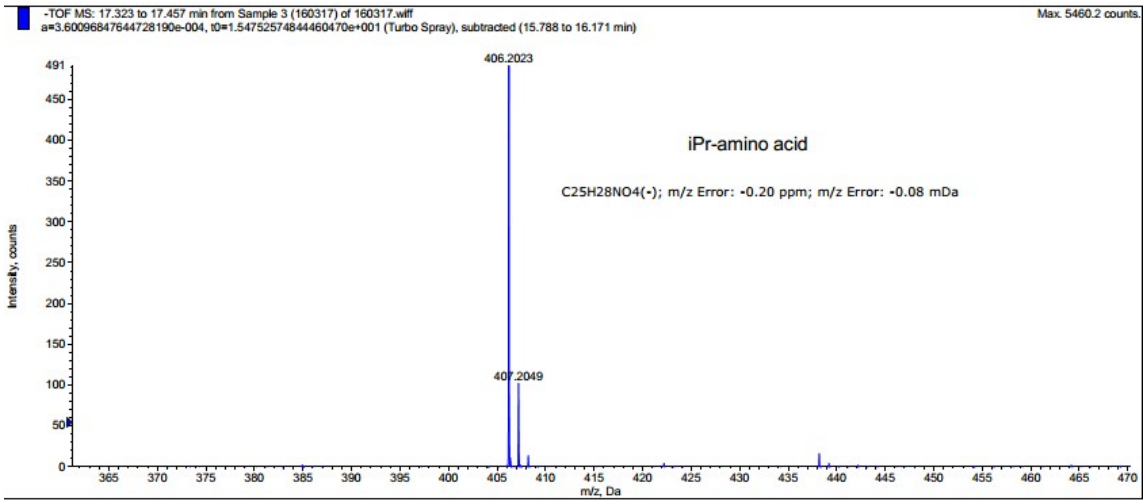
HRMS



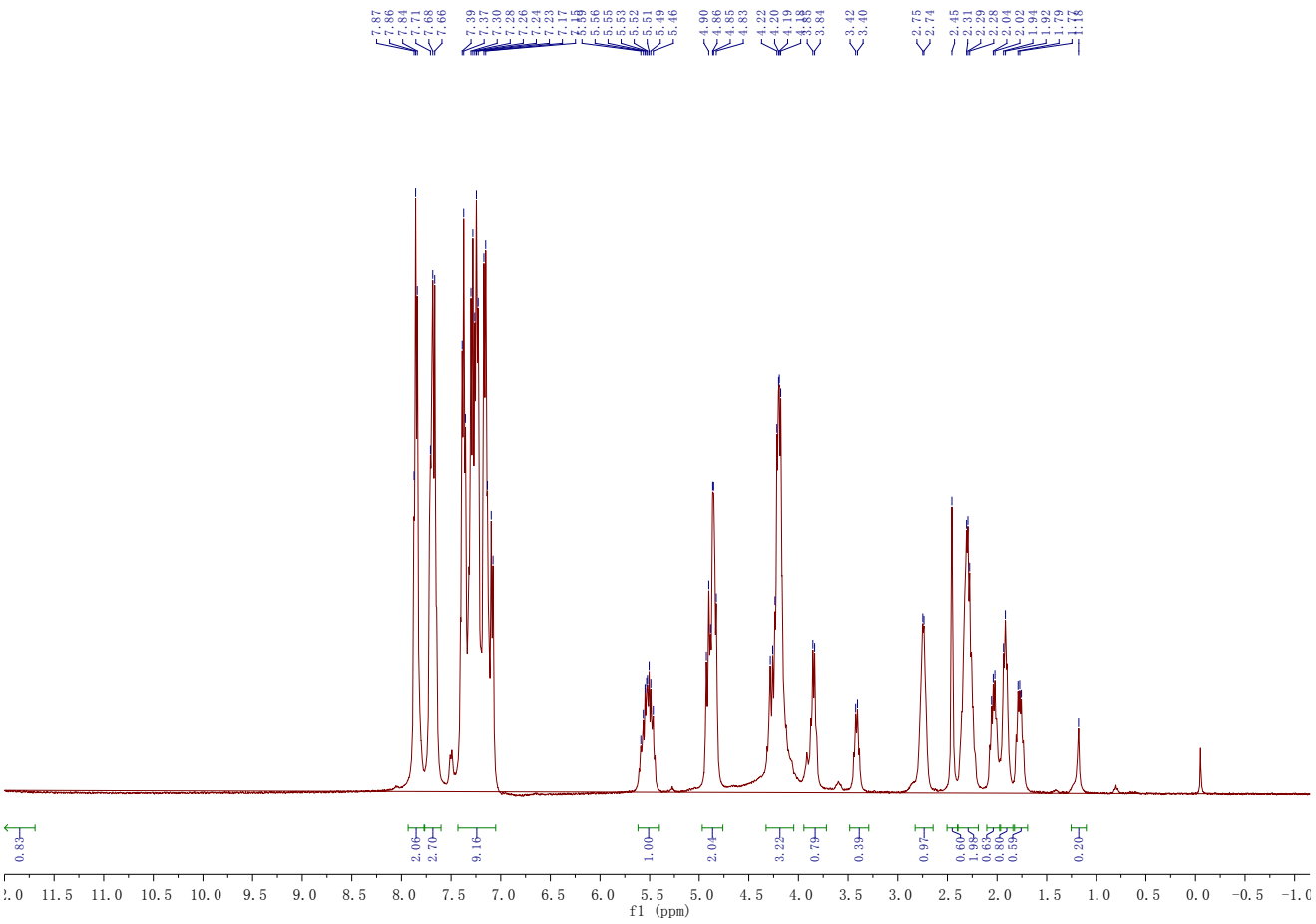
^{1}H of Fmoc-S5(2-iPr)-OH



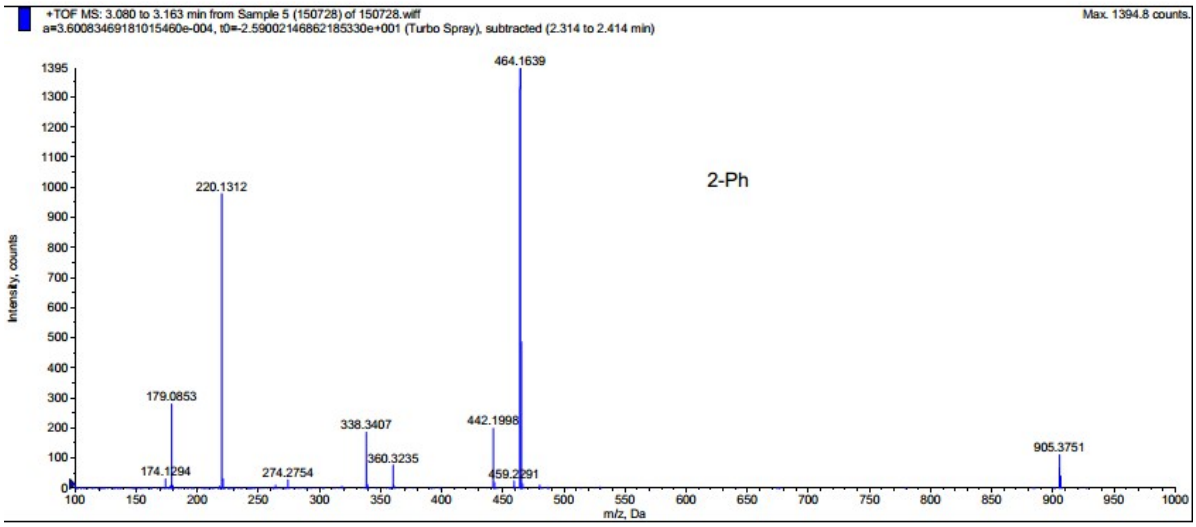
HRMS



^{1}H of Fmoc-S5(2-Ph)-OH

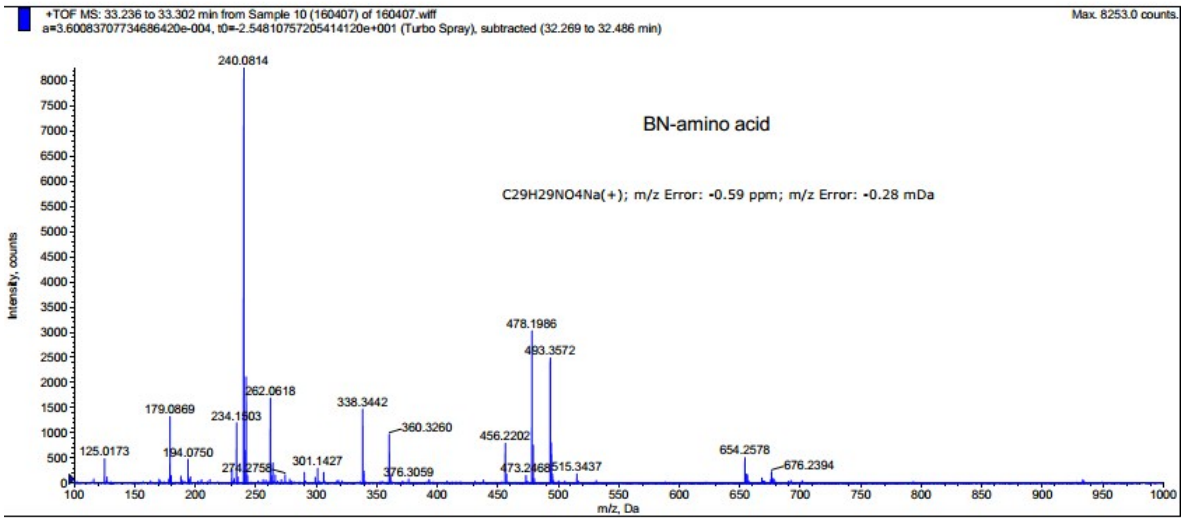

f1 (ppm)

HRMS


+TOF MS: 3.080 to 3.163 min from Sample 5 (150728) of 150728.wiff
a=3.60083469181015460e-004, t0=-2.59002146862185330e+001 (Turbo Spray), subtracted (2.314 to 2.414 min)
Max. 1394.8 counts.
2-Ph
Intensity, counts
m/z, Da
464.1639
220.1312
179.0853
338.3407
442.1998
360.3235
905.3751
174.1294
274.2754
459.2291

^{1}H of Fmoc-S5(2-Bn)-OH

HRMS



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