Electronic Supplementary Material (ESI) for ChemComm.

SUPPORTING INFORMATION

In-tether chiral center modulates the proapoptotic activity of KLA peptide

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Content

Materials

All amino acids and resins were purchased from GL Biochem (Shanghai). The coupling reagents were purchased from Shanghai Hanhong Chemical Co., J&K Scientific or Energy Chemical. Peptides were purified by HPLC (SHIMAZU Prominence LC-20AT) using grace smart C18 250× 4.6mm at flow rate 1ml/min. HPLC fractions containing products were combined and lyophilized. Molecular weights were measured by SHIMAZU-SPD2020.

Peptide synthesis and characterization

Peptide synthesis were performed on Rink Amide MBHA resin (loading capacity: 0.54mmol/g) with standard solid-phase peptide synthesis protocol. Firstly, resin was swelled in DMF for 30min, followed by deprotecting the Fmoc functional group with morpholine (50% in DMF). Wash the resin with DMF and DCM for 6 times, and then amino acid coupling was conducted with addition of 0.4M amino acid solution (5.0 equiv of Fmoc protected amino acid, 4.9 equiv of HCTU and 10.0 equiv of DIPEA). The thiol-ene cyclization was conducted under 365nm UV light with photo initiators MAP/MMP(1:1) for 2h.

2D NMR

2D NMR data were collected on a Bruker Avance III 500MHz spectrometer with a TXI probe. Watergate pulse sequence with gradients were used for water suppression in 1D and 2D ^{1}H spectrum. 2D ^{1}H-^{1}H TOCSY and NOESY spectra were acquired with mixing time of 100ms and 300ms, respectively.

CD measurement

CD spectra were collected with a Chirascan Circular Dichroism Spectrometer with the following measurement parameter: wavelength: 190-250nm, step resolution: 0.5nm, path-length: 0.1cm. Peptides were prepared in ddH_2O at concentration of 100μM. CD data are presented as mean residual elipticity [θ] in deg·cm^{2} ·dmol^{-1}. Each sample was tested for twice and averaged.

Flow cytometry

Hela cells were seeded in 12 well culture plate with DMEM medium(10% FBS) and incubated for 12h under 37℃. Then 3μM FITC-labeled peptides were added to the culture plate. After incubation for 2h, cells were washed with PBS for three times and exposed to trypsin (0.25%) for digestion. Then cells were washed and resuspended with PBS and analyzed by BD FACS Calibur flow cytometer (Becton Dickinson).

MTT

Hela cells were seeded in 96-well plates with 5000 cells per well and incubated under 37℃ for 12h. Serial dilutions of peptides were added to the cells and incubate for another 24h. CCK8 reagent was added to the 96-well plates and incubated for 2h. The absorbance was scanned by a microplate reader (Perkin Elmer, Envision, 2104 Multilabel Reader). Wavelength,450nm.

Mitochondria co-localization

Hela cells were seeded in 12 well culture plate and incubate under 37℃ for 12h. Then 3μM FITC-labeled peptides were added to the culture plate and incubated with Hela cells for 2h. Then wash cells for 3 times with PBS. After staining the cells with 250nM mitotracker for 15min at 37℃, the samples were washed with PBS for three times and examined by a confocal laser scanning microscope without fixation.

Annexin V and PI co-stain

Hela cells were seeded in a 12 well culture plate and incubated at 37℃ for 12h. 5μM peptides were added to the cells and incubated for another 1h. Then cells were collected, washed and stained with anannexin V-FITC kit followed by flow cytometry analysis.

LDH release

Hela cells were seeded in 96-well plate with 5000 cells per well. 100μl Serial dilutions of peptides were added to the cells and incubate for 4h at 37℃. Lysis buffer was added and incubated at 37℃ for 30min as positive control. 100μl working solutions were added to each well and incubated for 30min at room temperature followed by 50μl stop solutions. The absorbance at 490nm was measured by a microplate reader (Perkin Elmer, Envision, 2104 Multilabel Reader).The LDH release activity was calculated by (LDH% $_{samples}$-LDH% $_{blank}$)/ (LDH% $_{total\ death\ cells}$-LDH% $_{blank}$).

Cell penetrating mechanism

Hela cells were seeded in a 12 well culture plate and incubated at 37℃ for 12h. 3mM methyl-β-cyclodextrin, 10mM NaN_3/50mM 2-deoxy-D-glucose, 1mM amiloride, 25μg/ml nystatin and 4℃ were pretreated with cells for 30min. Then 5μM FITC labeled peptides were added to the cells and incubated with cells for 3h with inhibitors. After incubation, cells were washed with PBS for three times and exposed to trypsin (0.25%) for digestion. Then cells were washed and resuspended with PBS and analyzed by BD FACS Calibur flow cytometer (Becton Dickinson).

Protease and trypsin digestion

Peptides(250μM) , mouse serum (25%, v/v) and trypsin (0.1%) mix solution was prepared together and incubated at 37℃ for different time interval. After that, the solution was mixed with 12% trichloroacetic acid (H_2O/CH_3CN: 1:3) to inactivate the protease and trypsin. The peptide remaining was examined by HPLC after 10000rpm centrifuge to remove the inactive protease.

SI figures:

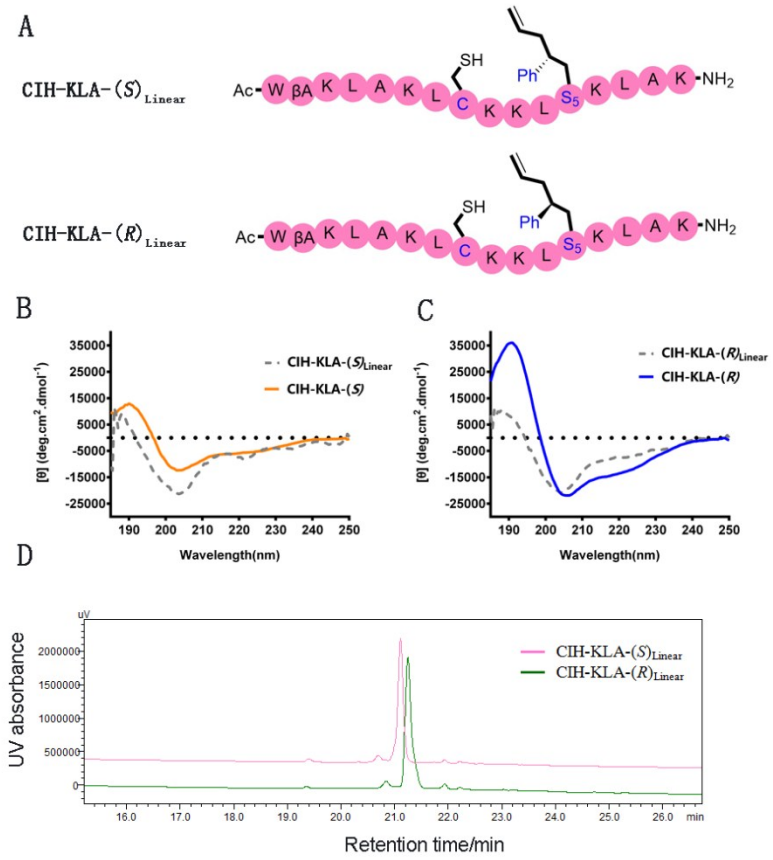
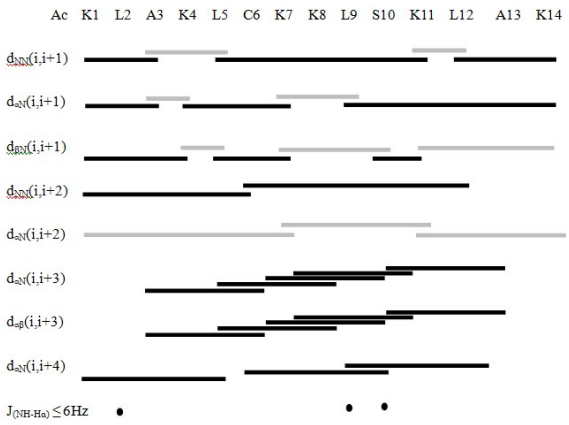


Figure S1. CIH-KLA-$(S)_{Linear}$ and CIH-KLA-$(R)_{Linear}$ for background study in the CD and UV-HPLC experiments. A) The linear peptides' chemical structure. B) The CD spectra of peptide CIH-KLA-(*S*) and its linear counterpart CIH-KLA-$(S)_{Linear}$. C) The CD spectra of peptide CIH-KLA-(*R*) and its linear counterpart CIH-KLA-$(R)_{Linear}$. D) The HPLC spectra of two linear peptides.



*****Grey: ambiguousassignment of peaks in NOESY spectra*

Figure S2.A detailed 1D and 2D 1H-NMR spectroscopy study of the peptide **CIH-KLA-(*R*)** in 20% TFE-d at 298K further confirmed its helical structure with non-sequential medium-range dαN(i, i+3), dαβ(i, i+4) and dαN(i, i+4) NOEs. The peptide used here was the tryptophan and β-alanine depletion form of CIH-KLA-(*R*) in order to avoid the overlap of NH signals in tryptophan and backbone.

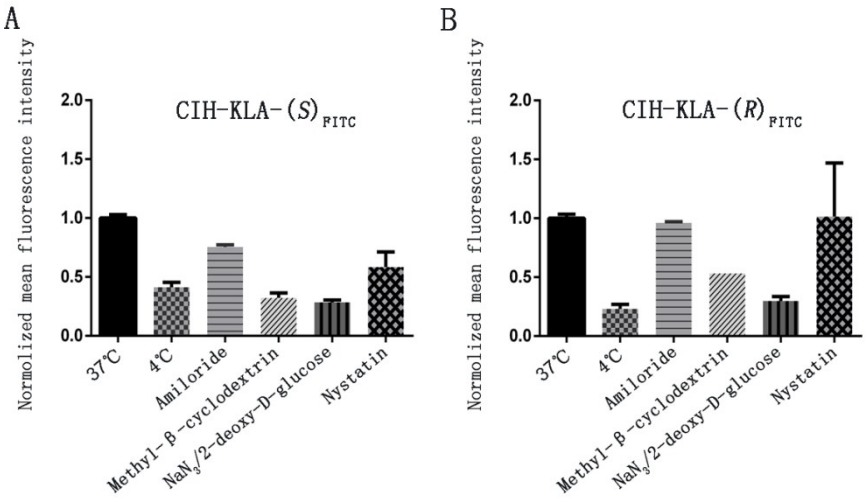


Figure S3. Cell penetrating mechanism of the tested peptides. For the *S* epimer, its normalized mean fluorescence intensity(MFI) was calculated by [$MFI_{different\ inhibitor}$(CIH-KLA-$S_{FITC}$)-$MFI_{DMSO}$]/[$MFI_{37°C}$(CIH-KLA-$S_{FITC}$)-$MFI_{DMSO}$]. For the *R* epimer, its normalized mean fluorescence intensity (MFI) was calculated by [$MFI_{different\ inhibitor}$(CIH-KLA-$R_{FITC}$)-$MFI_{DMSO}$]/[$MFI_{37°C}$(CIH-KLA-$R_{FITC}$)-$MFI_{DMSO}$].

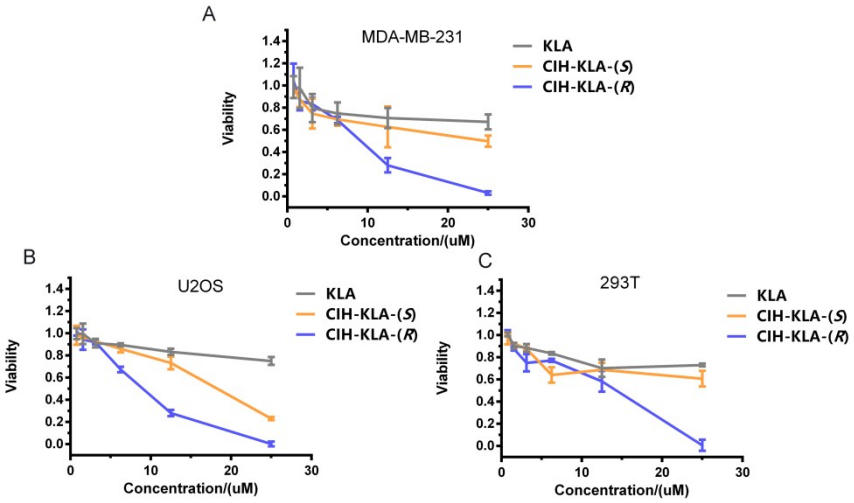


Figure S4 MTT results of peptides in MDA-MB-231, U2OS and 293T.

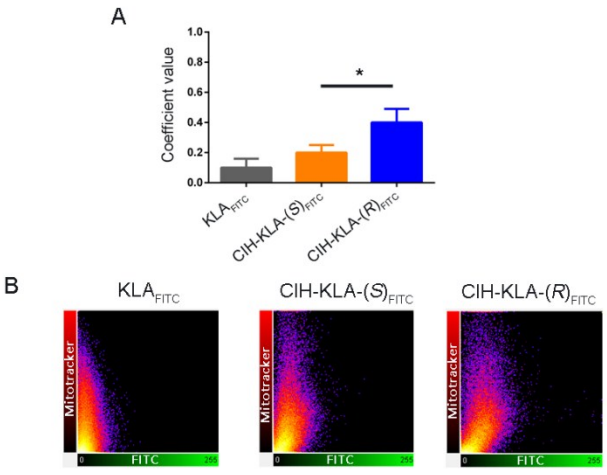


Figure S5. Quantification of the co-localization results. A) Pearson's correlation coefficient was calculated from more than 30 cells for each sample using the Image J software.* ($P<0.05$). B) The scatter-plot pixels of the three peptides which were analyzed using imaris software. For perfect localization, points should fall on a straight line. For the peptide CIH-KLA-$(R)_{FITC}$, the points in its scatter-plot pixel had a stronger tendency to form a straight line comparing with KLA_{FITC} and CIH-KLA-$(S)_{FITC}$.

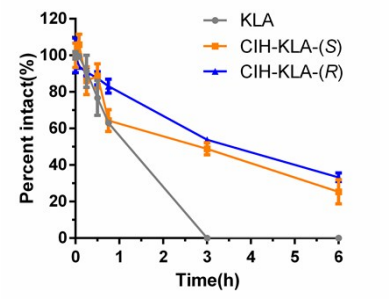
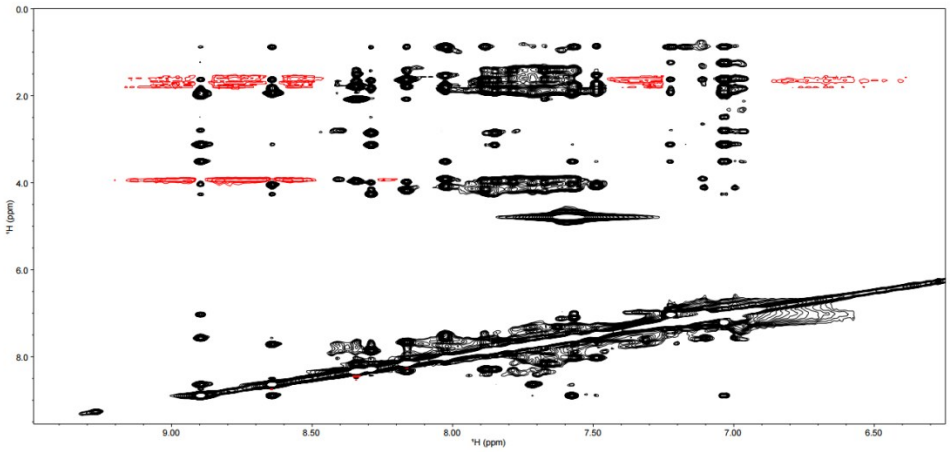


Figure S6. Proteolytic study of the three tested peptides′ stability against proteases and trypsin. Peptides(250μM) were mixed with both mouse serum (25%, v/v) and trypsin (0.1%) together and incubated at 37℃ for different time interval. The peptide remaining was examined by HPLC.

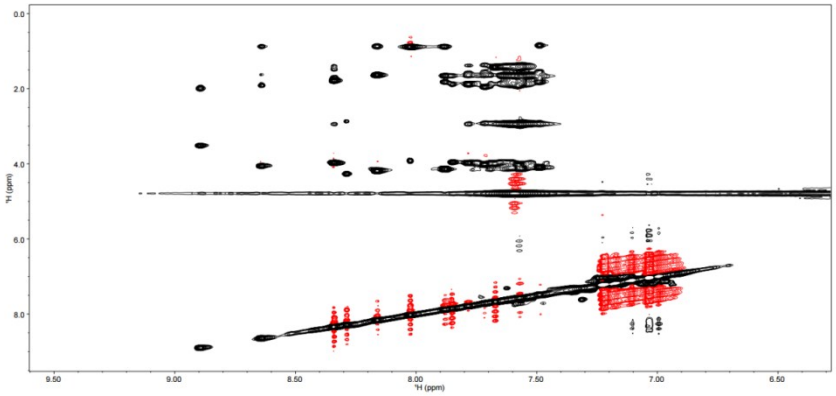
Appendix:selectedpeptide's NMR, HPLC and LC/MS data

NO.	Residue	NH	Ha	Hb	H(sidechain)
1	K	8.34	3.98	1.79	1.79, 1.40/1.49, 1.71, 2.95
2	L	8.16	4.19	1.64	1.64, 0.87
3	A	7.67	4.00	1.42	-
4	K	7.78	3.98	1.88	1.88, 1.38, 1.65/1.59, 2.94
5	L	7.88	4.14	1.82	1.65, 0.88
6	C	8.29	4.27	3.13/2.87	-
7	K	7.85	3.96	1.88	1.45, 1.65, 2.87/3.13
8	K	7.71	4.02	1.95	1.37, 1.56/1.64, 2.92
9	L	8.64	4.04	1.92/1.84	1.63, 0.88
10	S_5	8.89	3.51	3.12	1.99, 7.03/7.22
11	K	7.58	4.02	1.93	1.42, 1.66, 2.94
12	L	7.48	4.10	1.82/1.70	1.55, 0.85
13	A	8.02	3.92	0.88	-
14	K	7.56	4.13	1.87	1.40, 1.75, 2.94

The H chemical shifts assignment of peptide**ClH-KLA-(*R*)**.



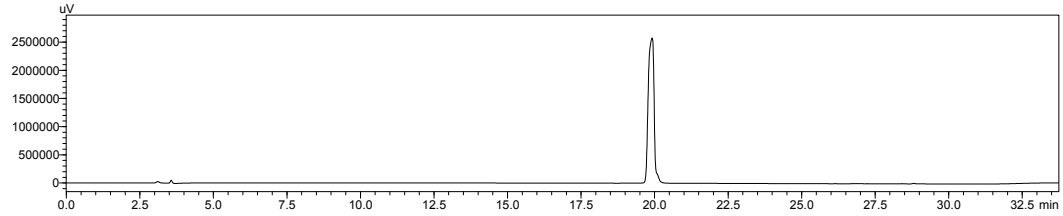
H region of NOESY spectrum of **CIH-KLA-(*R*)** (500MHz in H_2O with 20% TFE-d at 20°C)

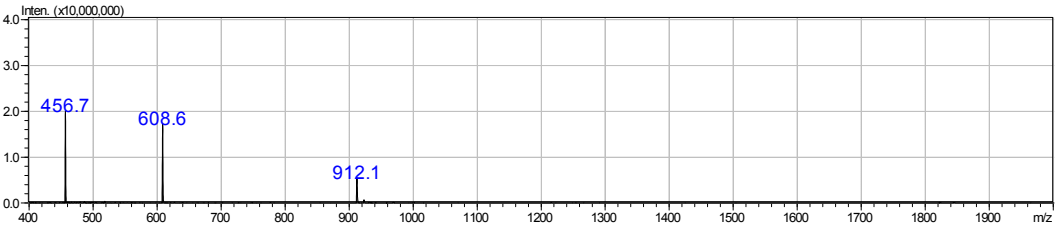


NH region of TOCSY spectrum of **CIH-KLA-(*R*)** (500MHz in H_2O with 20% TFE-d at 20°C)

The mass and HPLC spectra of referenced peptides

KLA:





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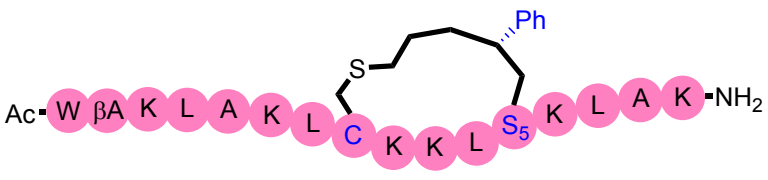

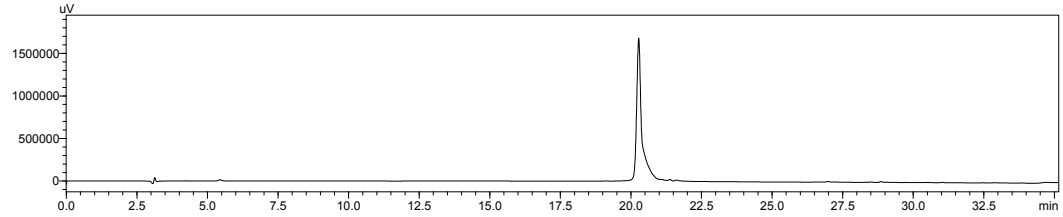

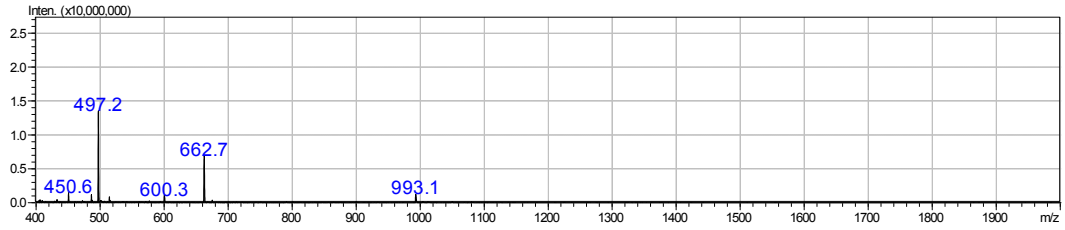

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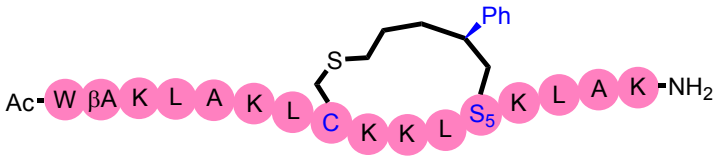
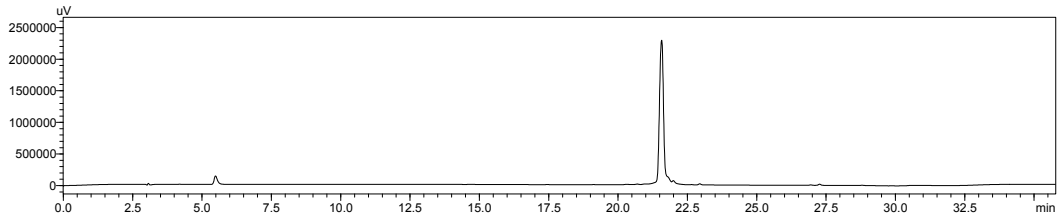
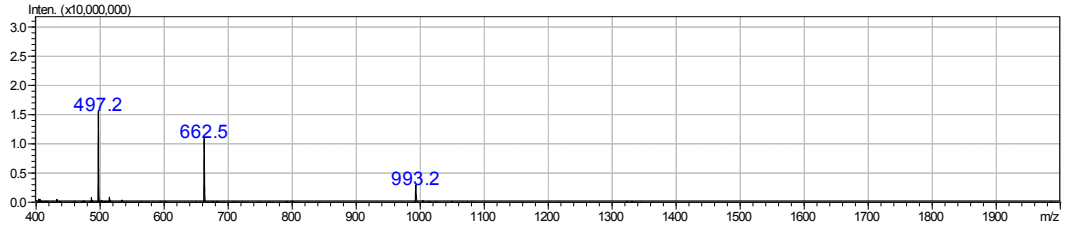
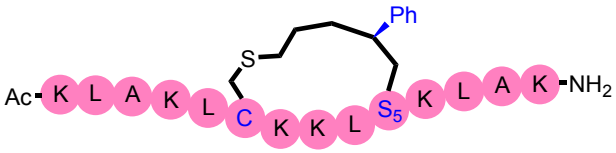
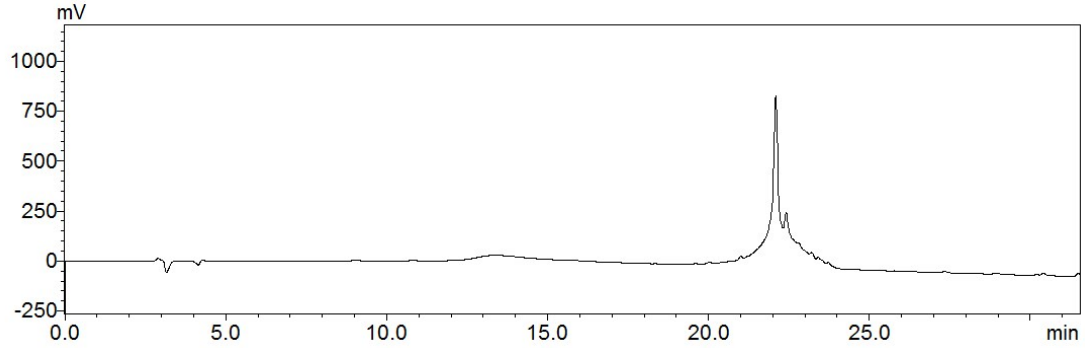


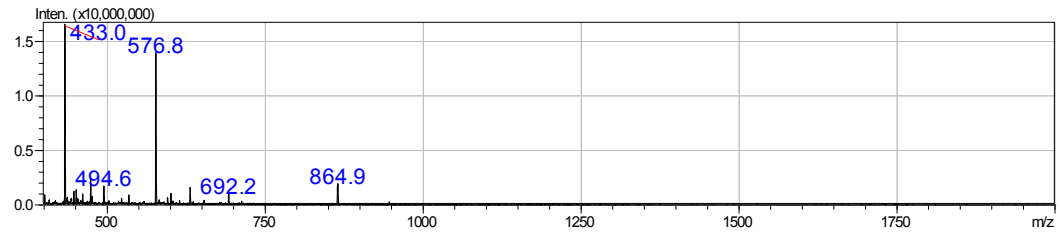




CIH-KLA-(*R*)-no tryptophan







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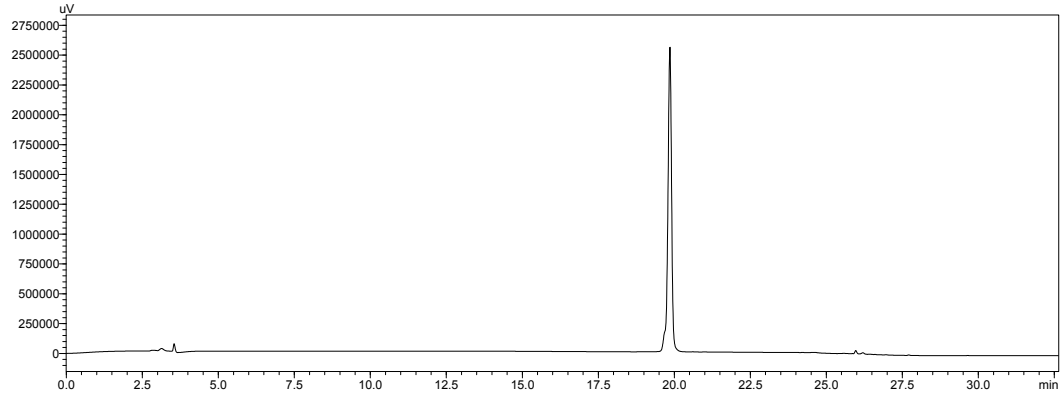

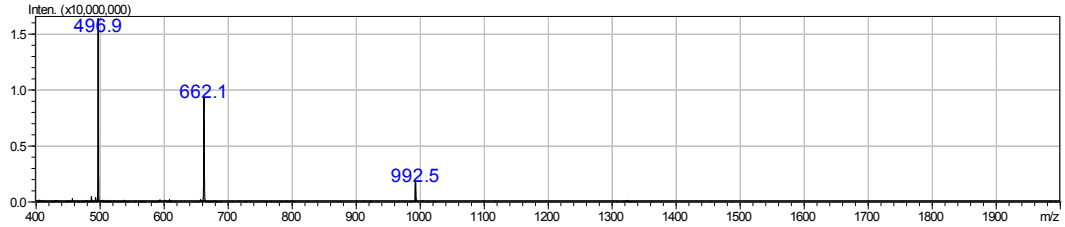

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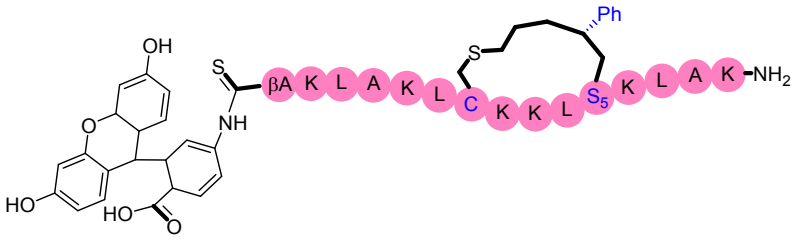

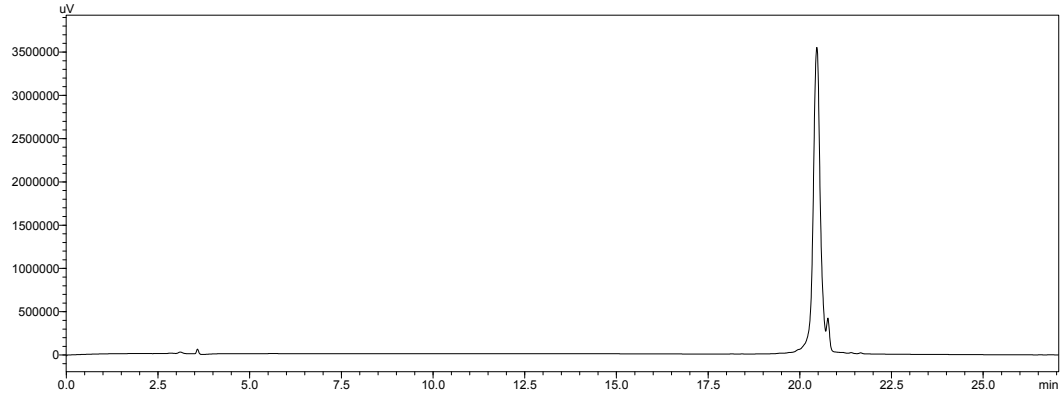

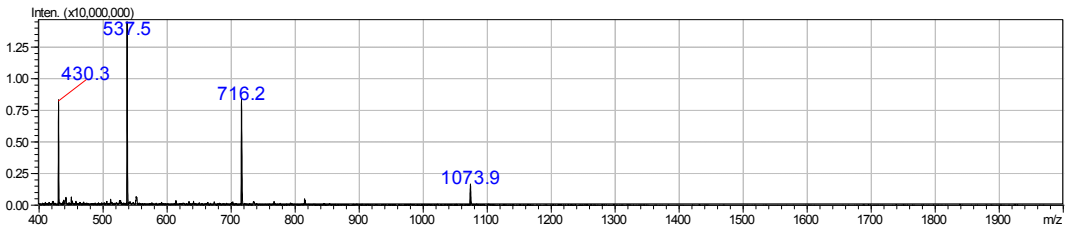

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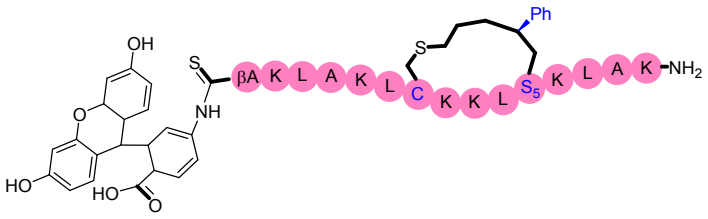
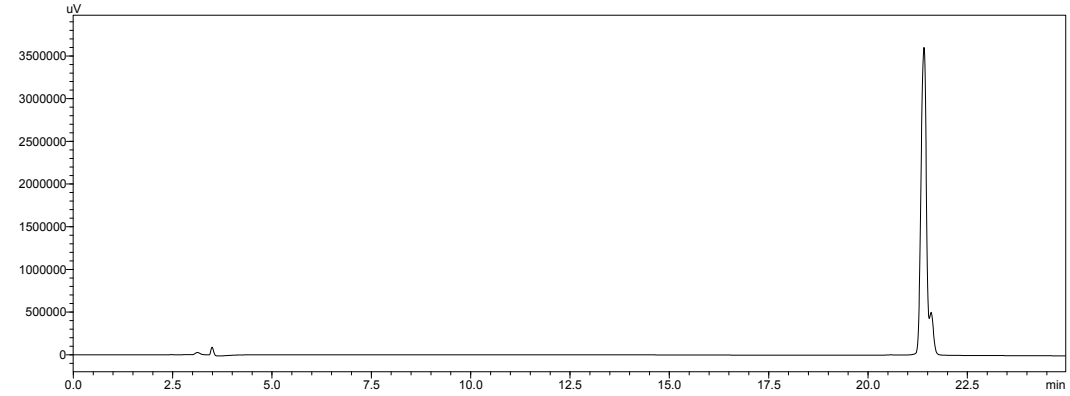
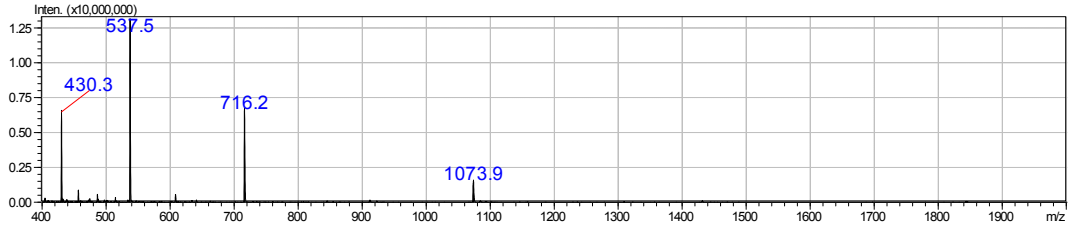
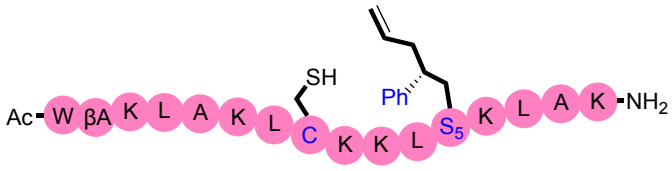
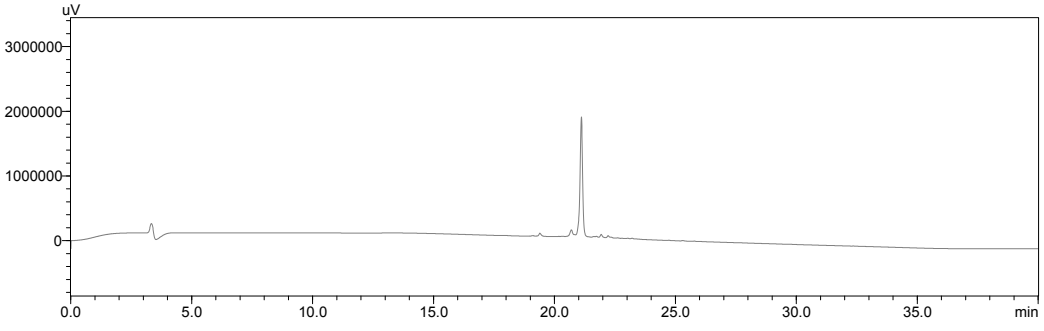


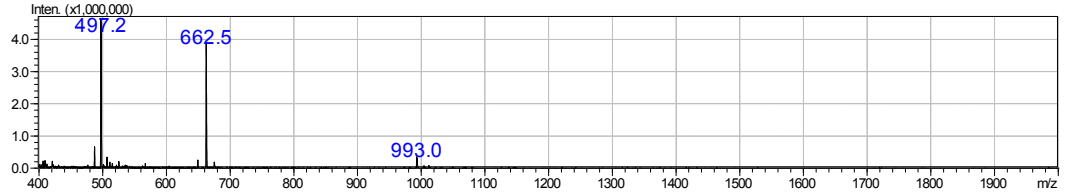




CIH-KLA-(*S*)$_{Linear}$







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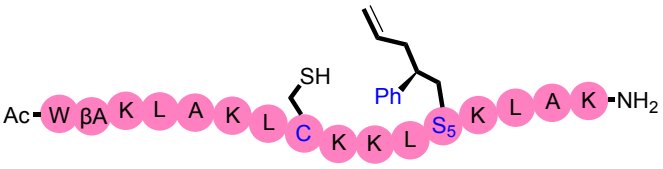
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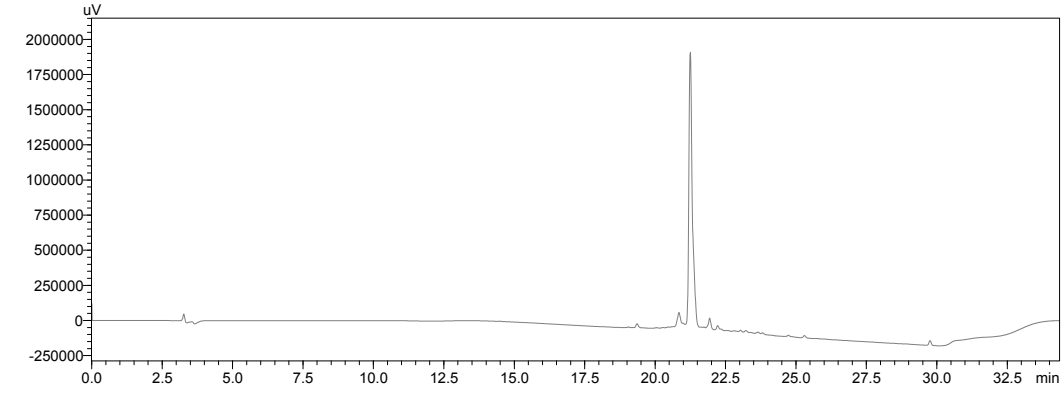
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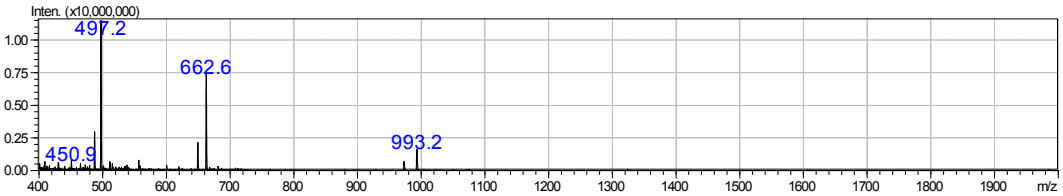
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