Electronic Supporting Information

N terminal N-methylation modulates chiral center induced helical (CIH) peptides' biophysical properties

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1. General information

1.1 Abbreviations

SPPS: solid-phase peptide synthesis; Fmoc: 9-fluorenylmethyloxycarbonyl; DIPEA: diisopropylethylamine; FITC: Fluorescein isothiocyanate, MAP: 4'-Methoxyacetophenone; MNP: 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone; RT: room temperature; HCTU:2-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyl uranium hexafluorophosphate; TFA: trifluoroacetic acid; TFE: 2,2,2-trifluoroethanol; HPLC: high-performance liquid chromatography; LC-MS: liquid chromatography–mass spectrometry; ESI-MS: electrospray ionization mass spectrometry; CD: circular dichroism; DCM: dichloromethane; DMF: dimethylformamide; TIS: triisopropylsilane; Et_2O: diethyl ether; tBu: tert-butyl.

1.2 metarials

All the reagents and solvents used for solid phase peptide synthesis (SPPS) and unnatural amino acid synthesis and others were purchased from commercial suppliers, such as GL Biochem (Shanghai) Ltd. J&K Co. Ltd., Huizhou Deep chemical technology co. LTD, Tianjin Damao Chemical Reagent Factory, Tianjin Yongda Chemical Reagent Company Limited or Shenzhen Tenglong Logistics Co. All of the reagents were used without further purification unless specifically stated.

1.3. HPLC and Mass spectrometry

Peptides were purified by HPLC (SHIMAZU Prominence LC-20AT, UV detection at 220 or 254 nm) equipped with a C18 analytic column (Agilent ZORBAX SB-Aq, 4.6 × 250 mm, 5 μm, flow rate 1.0 mL/min) or a C18 semi-preparative column (Agilent Eclipse XDB-C18, 9.4 × 250 mm, 5 μm, flow rate 4.0 mL/min). Filtered H_2O with 0.1% TFA and pure acetonitrile were used as solvents. Peptides were analyzed by LC-MS (SHIMAZU LC-MS 8030, ESI-MS).

2. Peptides preparation

2.1 Synthesis of unnatural amino acids

The synthesis of R chiral center unnatural amino acid S_5(2-Me) is based on the previous literature showed below.

Reference:

[1] Y. N. Belokon, V. I. Tararov, V. I. Maleev, T. F. Savel'eva, M. G. Ryzhov, *Tetrahedron: Asymmetry*, 1998, 9, 4249.

[2] X. Tang, V. A. Soloshonok, V.J. Hruby, *Tetrahedron: Asymmetry*, 2000, **11**, 2917.

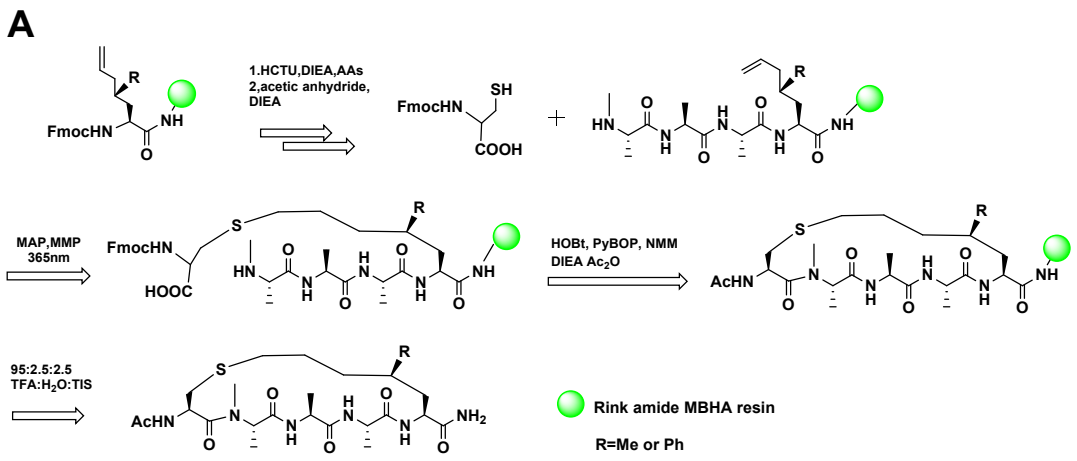
[3] V. A. Soloshonok, X. Tang, V. J. Hruby, *Tetrahedron: Asymmetry*, 2001, **57**, 6375.

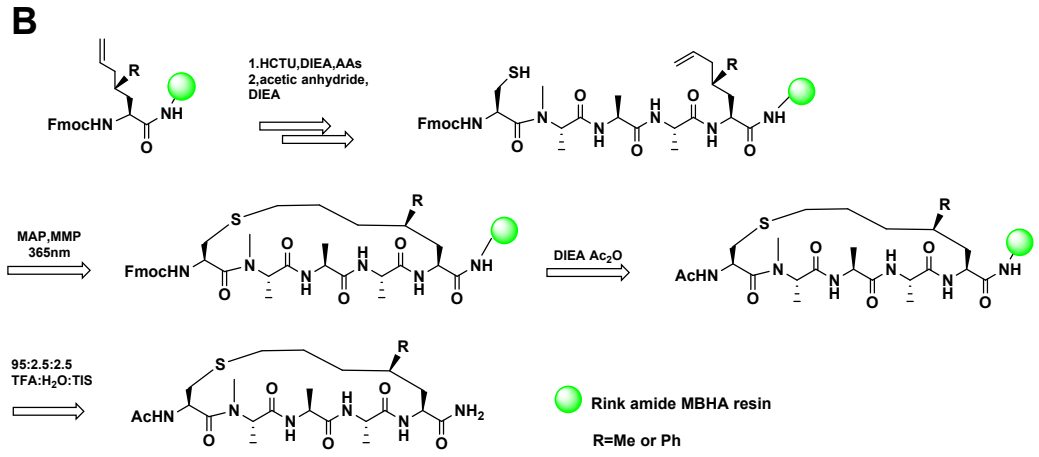
[4] B. Aillard, N. S. Robertson, A. R. Baldwin, S. Robins, A. G. Jamieson, *Org. Biomol. Chem.*, 2014, **12**, 8775.

[5] K. Hu, C. Sun, M. Yu, W. Li, H. Lin, J. Guo, Y. Jiang, C. Lei, Z. Li, *Bioconjug. Chem.* 2017, **28**, 1537.

2.2 Synthesis of pentapeptides.

Peptides were synthesized on MBHA resin (loading capacity: 0.54 mmol/g) by standard Fmoc-based SPPS. Generally, the resin was swelled with NMP for 30 min. Then use the 50% (vol/vol) morpholine in DMF to deprotect the -Fmoc group for 30min × 2. The resin was then washed with DCM and DMF for six times. As for natural amino acids, the Fmoc-protected amino acids (5.0 equiv), HCTU (4.9 equiv), DIPEA (10.0 equiv) were dissolved in DMF and mixed on resin for 2h, followed by washing with DCM and DMF for 6 times. While as for unnatural amino acids (N-methylated Ala and S_5), Fmoc-protected acids (2.5 equiv), HCTU (2.4 equiv) and DIPEA (5.0 equiv)were dissolved in NMP and mixed on resin for 4h, followed by washing with DCM and DMF for 6 times. When capping the amino acid before Cys, after washed with DMF and DCM and MeOH for six time and dried the resins, 1.2e.q of (1:1) MAP/MNP was added with dried DMF as solution and react under ultraviolet light (365nm) for 1.5h× 2 to faciliate the thiol-ene cyclization. Then re-swelled the resins and use a reaction mixture [HOBt (3.0 equiv), PyBOP (3.0 equiv), NMM (7.5 equiv)] with DMF as solvent and react for 3h × 2. Then use 50% (vol/vol) morpholine in DMF to deprotect the -Fmoc group for 30min × 2, and capping the terminal $–NH_2$ with acetylation, mixture (acetic anhydride: DIPEA: NMP=5: 20: 100) was used as reagent to react for 2h× 2. Finally a mixture of TFA/H_2O/TIS (95/2.5/2.5) was added for 2h to cleave the peptide from the resins and dried by blowing nitrogen later. Then the peptides were precipitated with Et_2O at 4°C, isolated by centrifugation and the precipitates was dissolved in about 35% acetonitrile/ water (in volume), purified by semi-preparative HPLC with UV absorbance at 220 nm and identified by LC-MS analysis.





Scheme 2. The synthesis process of peptide 2R. So as the other designed peptides.

Reference:

[1] A. Patgiri, M. Z. Menzenski, A. B. Mahon, P. S. Arora, *Nat. Protoc.,* 2010, **5**, 1857.

[2] K. Hu, C. Sun, Z. Li, *Bioconjug. Chem.* 2017, **28**, 2001.

[3] Y. W. Kim, T. N. Grossmann, G. L. Verdine, *Nat. Protoc.,* 2011, **6**, 761.

2.3 Peptide purification and characterization

The photoreaction products were purified by the HPLC after acetylization or coupling with FITC. The peptide was purified by HPLC with different retardation time. The purified peptides were characterized by LC-MS with ESI as positive ion mode.

3. CD spectroscopy

Peptides were dissolved in deionized water to concentrations of 40-100 μM. The spectra was conducted by a chirascan Circular Dichroism Spectrometer (applied photophysics) at 298.15K (wavelength, 185-250 nm; step resolution, 0.5 nm; speed, 20 nm/sec; accumulations, 10; response, 1 sec; bandwidth, 1 nm; path 3 length, 0.1 cm). Each sample was scanned twice and the final CD spectrum was averaged and smoothed. And the relative α-helical content was calculated as former reporter with peptide 2R as 1 (in figure 2) and peptide 18R as 1 (in figure 5).

Concentration of each sample: absorption (OD) at 280 nm × dilution factor ÷1 (cm) ÷ 1490 ($1cm^{-1}M^{-1}$)×1000 (mMM^{-1}) = concentration (mM). For peptide 15, 16, 17R and 18R, we use UV-Vis to analysis the concentration by the absorption of Tyr at 280nm and for FITC labeled peptides, we used absorption of FITC at 494nm. For peptides without chromophores suitable for UV-VIS quantification, we use their peak integration by the absorption at 220nm in HPLC to measure their concentration, and peptide 2R was sued as control, which was quantified by weight.

Reference:

[1] Chen, Y.-H.; Yang, J. T.; Chaiuu, K. H. *Biochem.* 1974, **13**, 3350.

[2] N. E. Shepherd, H. N. Hoang, G. Abbenante, D. P. Fairlie, *J. Am. Chem. Soc.* 2005, **127**, 2974.

4. NMR spectroscopy

NMR analysis was recorded by Bruker AVANCE III 500 MHz spectrometer in H_2O/D_2O=9:1 at 298.15K. And watergate pulse sequence with gradients were used for water suppression in both 1D and 2D ^1H spectrum. NMR data were processed using Topspin 3.0. And the chemical shifts were calibrated with standard 4,4-dimethyl-4-silapantane-1-sulfonic acid(DSS). We utilized temperature coefficients assay as tools to characterize the propensity for exchangeable protons to form intramolecular hydrogen bonds (IMHBs). In this experiment, peptide was measured in mixture of H_2O/D_2O=9:1 at 298.15K. The protection of the IMHBs decreases with the temperature dependence of the chemical shift of the exchangeable protons. 1H NMR was measured at five different temperature: 298.15K, 303.15K, 313.15K, 323.15K and 333.15K. And at each temperature, the sample was allowed to equilibrate for 15min. In general, the $\Delta\delta/T$ for IMHBs was solvent dependent and can be utilized to identify the hydrogen bond. The slope of each line means the temperature coefficient and values of $\Delta\delta/T$ less than 4.5 ppb/K usually indicate hydrogen bonding.

As for 2D NMR assay, data was collected on a Bruker Avance III 500MHz spectrometer with a TXI probe. 2D ^1H-^1H TOCSY and NOESY spectra were acquired with mixing time of 100ms and 300ms, respectively. The TOCSY and NOESY spectra were acquired with a width of 10ppm and 13C spectra width of 100ppm, and size of 1024×400 complex points. All the 2D NMR spectra were processed by Topspin® to final 2048×1024 complex points, and analyzed by CCPNMFR software. $^3J(NH\text{-}H\alpha)$ couplings were measured from 1D-1H spectrum.

Reference:

[1] Shepherd, N. E.; Hoang, H. N.; Abbenante, G.; Fairlie, D. P. *J. Am. Chem. Soc.* 2005, **127**, 2974.

5. Flow cytometry analysis

HEK-293T (or HeLa) cells were grown in DMEM medium with 10% FBS (v/v) in imaging dishes in 310.15K, 5% CO_2 incubator for one day (~55,000 cells per well). Cells were treated with FITC labelled peptides (5 μM) for up to 3h at 310.15K. After washing with media, the cells were exposed to trypsin (0.25%; Gibco) digestion (5-10 min, 310.15K), then washed with PBS, and resuspended in PBS. Cellular fluorescence was analyzed using a BD FACSCalibur flow cytometer (Becton Dickinson) and CellQuest Pro (or CFlow plus) and the values represent averages of three independent experiments.

6. Confocal microscopy image

HEK-293T cells were cultured with DMEM with 10% FBS (v/v) in imaging dishes (50000 cells/well) in 310.15K, 5% CO_2 incubator for one day until they were about 60% adherent. Peptide were first dissolved in DMSO and then added to cells to a final concentration of 5 μM. The cells were incubated with peptides for 3h at 310.15K. After incubation, cells were washed 3 times with PBS and then incubated with 0.05% Typan Blue for 3-5 minutes, then washed 3 times with PBS and incubated with 4% PFA for 10 minutes. Then after washed with PBS for 3 times, stained with 1μg/ml DAPI (Invitrogen, CA) in PBS for 10 minutes. Images of peptide localization in cells were taken on PerkinElmer confocal microscopy. Image processing was done using Volocity software package (Zeiss Imaging).

7. Serum stability assay

Standard solution of peptides were prepared in ddH_2O. And each peptide was added to mouse serum and then incubated at 310.15K with a final concentration of 250 μM (peptide) in 25% serum. At each time point, mixture (25 μL) was taken periodically from 0 to 24 h, and then add 100 μl 12% trichloroacetic

acid in H_2O/CH_3CN (1:3) and cooled at 277.15K for 30 min to precipitate proteins in serum. Then centrifuge to remove the protein, each sample was analyzed by LC-MS with a 3% per minute linear gradient. The relative amount of each sample was quantified by LC/MS-based peak detection at 220nm.

Reference:
[1] N. E. Shepherd, H. N. Hoang, G. Abbenante, D. P. Fairlie, *J. Am. Chem. Soc.* 2005, **127**, 2974-2983.

8. Supplementary Figures and Tables

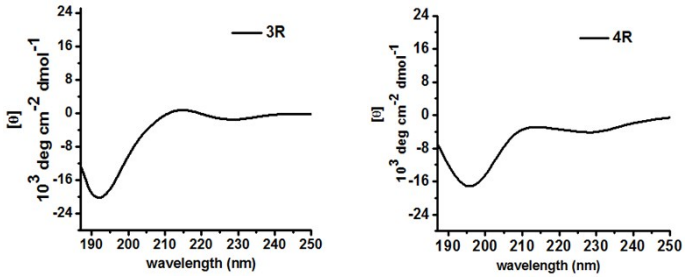


Figure S1. CD spectra of single N-methylated CIH peptide **3R** and **4R** in ddH_2O at 298.15K. The addition of single N-methylation on CIH peptides disturbed its original helical structure.

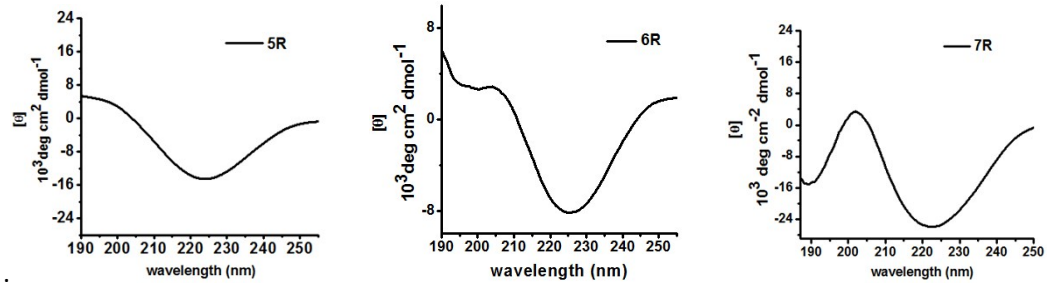


Figure S2. CD spectra of multi-N-methylated CIH peptide **5R-7R** in ddH_2O at 298.15K. The addition of multi-N-methylation on CIH peptides significantly destroyed its original helical structure, indicating that breaking intramolecular hydrogen bond would greatly influence the helical structure of CIH peptides.

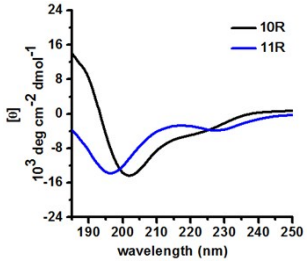


Figure S3. CD spectra of peptide **10R** and **11R** in ddH_2O at 298.15K. Peptide **11R** with addition of extra N-methylation showed a decreased helical content compare to peptide **10R**.

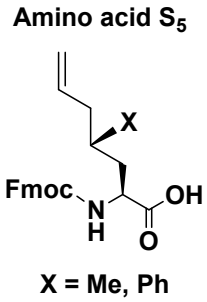


Figure S4. Chemical structure of unnatural amino acid S_5, X is methyl or phenyl.

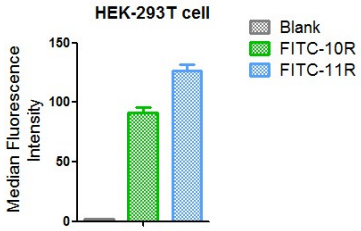


Figure S5. Median fluorescent intensity of peptides **10R** and **11R** in 293T cells. The N-methylated peptide **10R** showed higher fluorescent intensity than peptide **11R**.

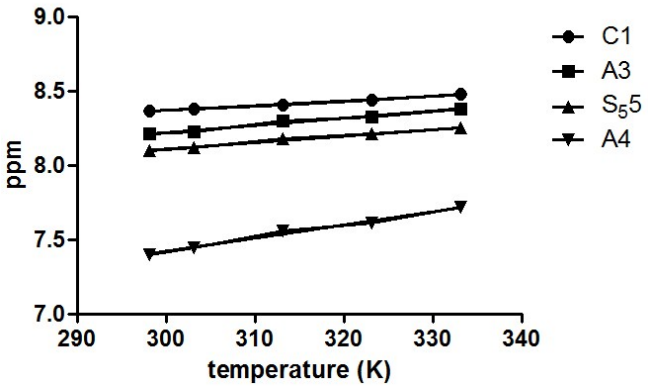


Figure S6. Temperature coefficient (Δδ/T) of peptide **2R**. ^{1}H NMR spectra was used to measure peptide **2R**. The chemical shift of each NH was fit and normalized to linear. The slope of each line means the temperature coefficient. Δδ/T values⩽4 are indicative of hydrogen bonds.

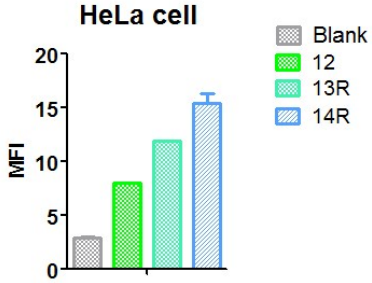


Figure S7. Flow cytometry analysis of N-methyl optimized peptides **12**, **13R** and **14R** in HeLa cells. Peptides (5μM) were incubated with Hela cells for 1.5 h at 310.15K. After washing, digestion, and

resuspension in PBS, cellular fluorescence was analyzed using a BD FACSCalibur flow cytometer. The values represent averages of three independent experiments.

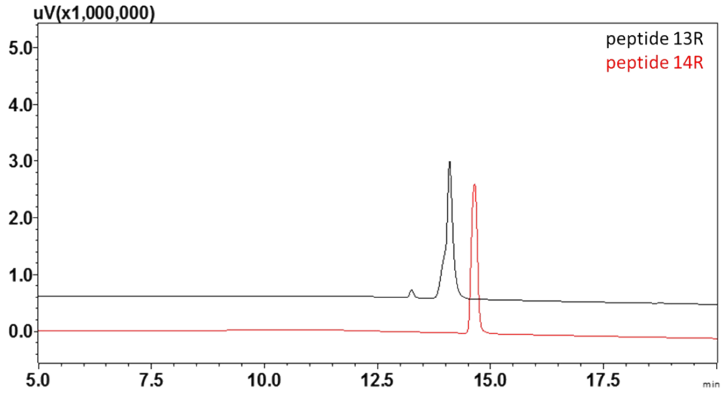


Figure S8. The retention time of peptide **13R** and **14R** by HPLC analysis. The N-methylated peptide **14R** showed more hydrophobicity and rigidity than peptide **13R** as it displayed a clear longer retention time.

No.	Sequence
15-FITC	FTIC-βA-DCEYNA-NH_2
16-FITC	FTIC-βA-CAAAL-DCEYNA-NH_2
19R-FITC	FTIC-βA-CARRS$_5$-DCEYNA-NH_2
20R-FITC	FTIC-βA-CA(Me)RRS$_5$-DCEYNA-NH_2

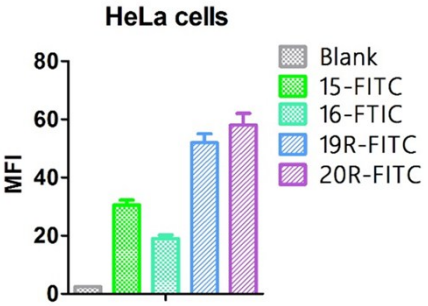
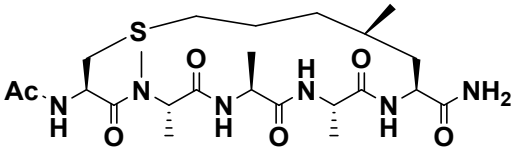


Figure S9. Flow cytometry analysis of FITC labelled peptide**15**, **16**, long peptide **19R** and its N-methylated **20R** in HeLa cells, the N-methylated HIF peptide **20R** showed the highest cellular uptake. The values represent averages of three independent experiments.

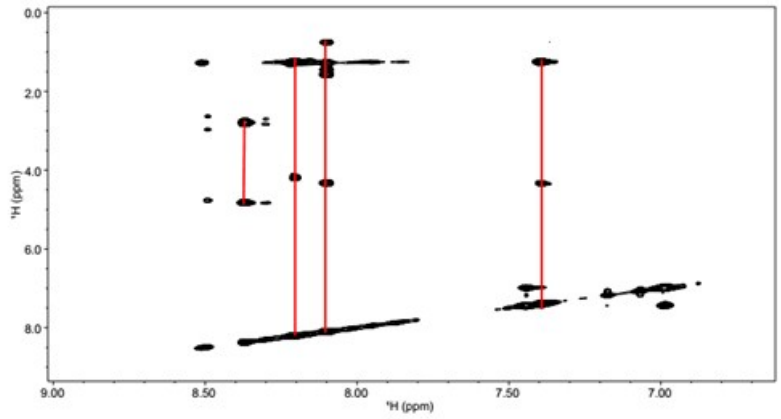
9. 1D and 2D NMR spectra for N-methylated peptide 2R

Peptide 2R

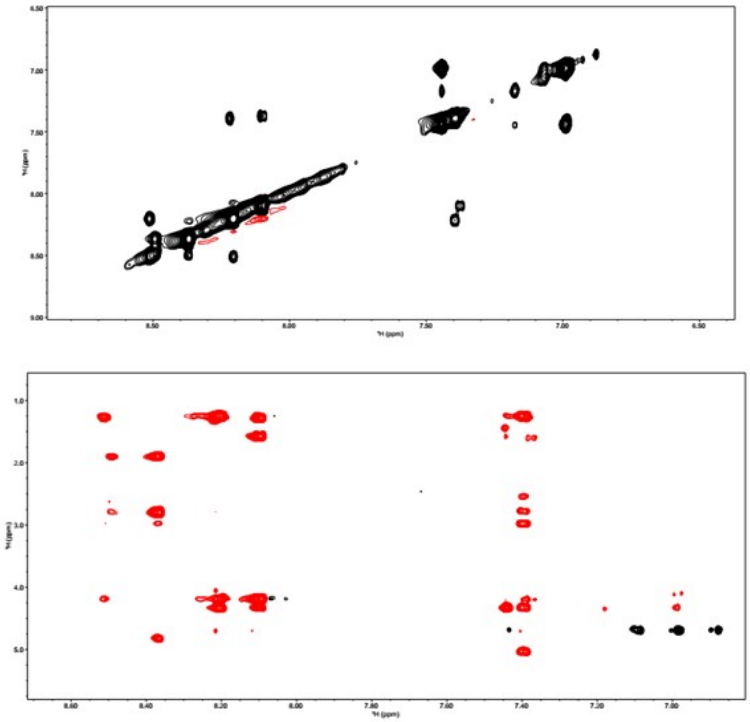


NO.	Residue	NH	Ha	Hb	H(sidechain)
1	C	8.37	4.83	2.81	-
2	A	-	-	1.25	-
3	A	8.21	4.35	1.29	-
4	A	7.39	4.35	1.25	-
5	S5	8.1	4.2	1.62	1.45,1.27,0.77

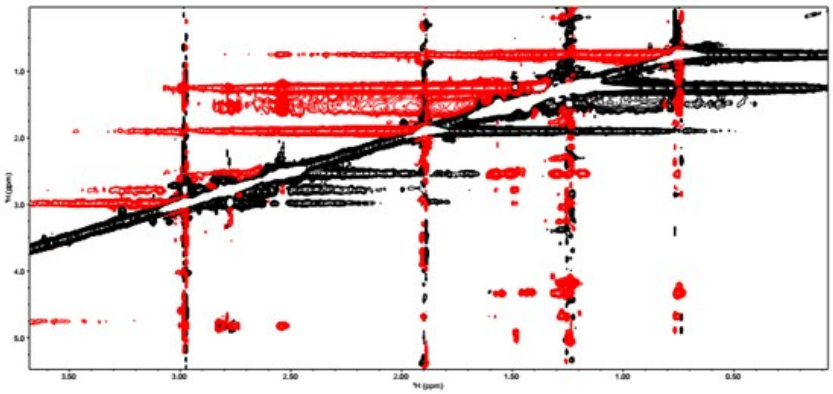
^{1}H-NMR chemical shifts (δ, ppm) for the NH on the backbone of **2R** in solution (H_2O: D_2O=9:1) at 298.15K.



TOCSY spectrum of **2R** [500MHz in mixture (H_2O: D_2O=9:1) at 298.15K]



NH-αCH region of 2D-NOESY spectrum of **2R** [00MHz in mixture (H_2O: D_2O=9:1) at 298.15K]



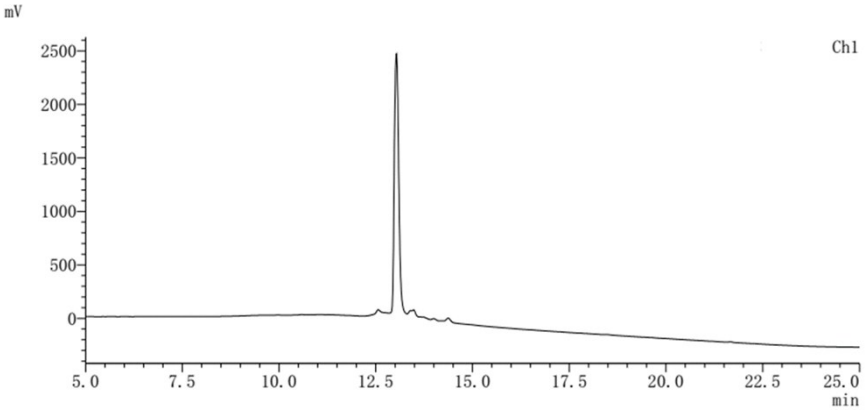
NH region of NOESY spectrum of **2R** [500MHz in mixture (H_2O: D_2O=9:1) at 298.15K].

10. Calculated MS summary

No.	Sequence	Calculated MS Value	Observed MS Value
1R	Ac-CAAAS_5-NH_2	514.3	515.4
2R	Ac-CA(Me)AAS_5-NH_2	528.3	529.4
3R	Ac-CAA(Me)AS_5-NH_2	528.3	529.4
4R	Ac-CAAA(Me)S_5-NH_2	528.3	529.4
5R	Ac-CA(Me)A(Me)AS_5-NH_2	542.3	543.4
6R	Ac-CA(Me)AA(Me)S_5-NH_2	542.3	543.4
7R	Ac-CA(Me)A(Me)A(Me)S_5-NH_2	556.3	557.0
8R	Ac-CAAAS_5(2-Ph)-NH_2	576.3	577.4
9R	Ac-CA(Me)AAS_5(2-Ph)-NH_2	590.3	591.3
10R	Ac-A-CAAAS_5-NH_2	586.3	586.4
11R	Ac-A(Me)-CAAAS_5-NH_2	598.8	599.8
12	FITC-βA-CARRL-NH_2	1075.4	539.3
13R	FITC-βA-CARRS_5-NH_2	1102.5	552.4, 1103.6
14R	FITC-βA-CA(Me)RRS_5-NH_2	1116.5	559.5, 1117.6
15	Ac-DCEYNA-NH_2	754.3	755.3
16	Ac-CAAAL-DCEYNA-NH_2	1183.5	592.9, 1184.5
17R	Ac-CAAAS_5-DCEYNA-NH_2	1209.3	605.9, 1210.4
18R	Ac-CA(Me)AAS_5-DCEYNA-NH_2	1223.3	612.9, 1224.4
15-FITC	FITC-βA-DCEYNA-NH_2	1170.3	586.4, 1171.4
16-FITC	FITC-βA-CARRL-DCEYNA-NH_2	1770.6	591.9, 887.2
19R-FITC	FITC-βA-CARRS_5-DCEYNA-NH_2	1796.0	599.9, 899.0
20R-FITC	FITC-βA-CA(Me)RRS_5-DCEYNA-NH_2	1811.0	604.6, 906.5

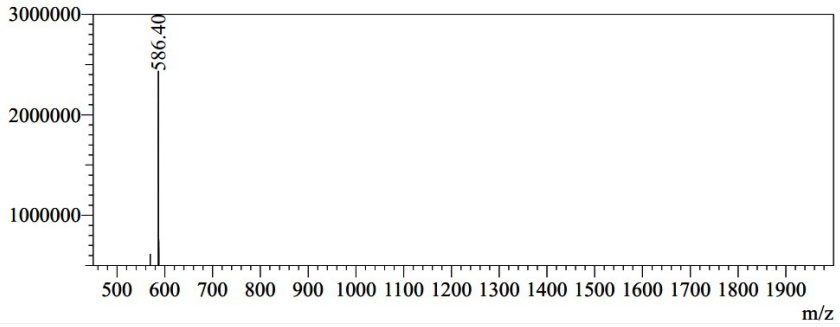
11. LC-MS spectra

Ac-A-(cyclo)-[C-A-A-A-S5(2-Me)]

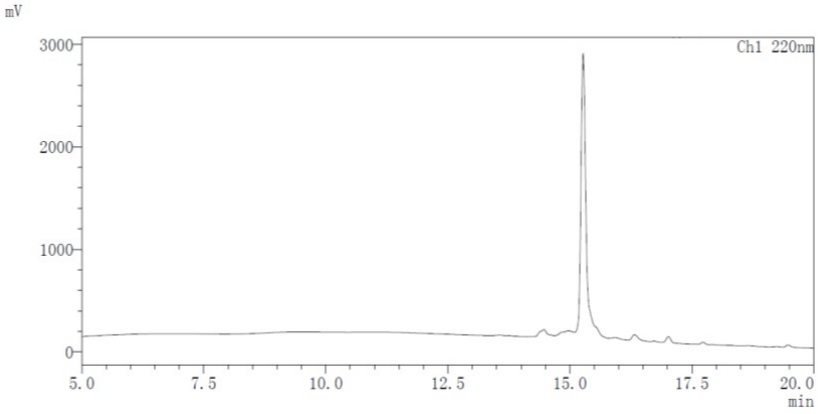


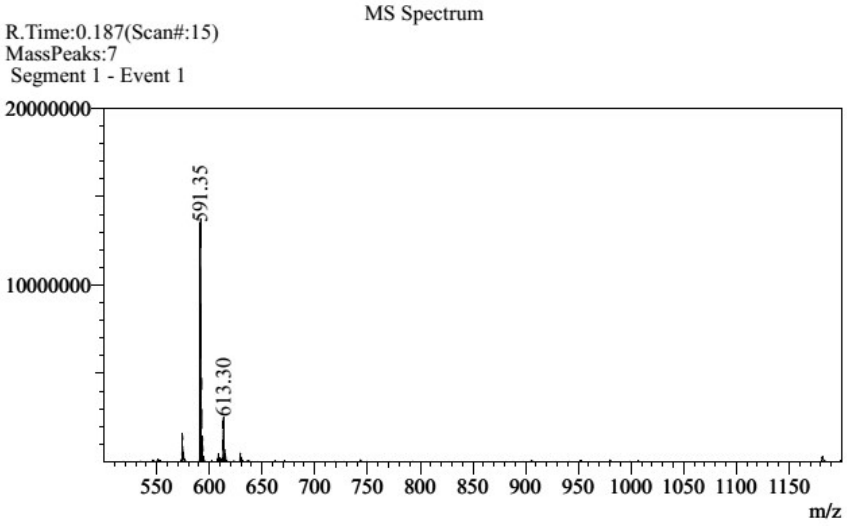
MS Spectrum

R.Time:0.360(Scan#:28)
MassPeaks:6
Segment 1 - Event 1

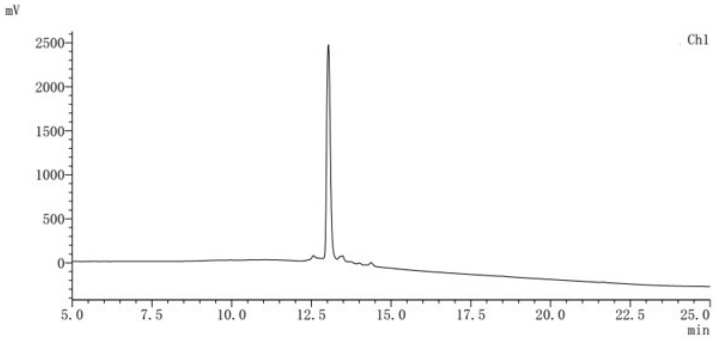


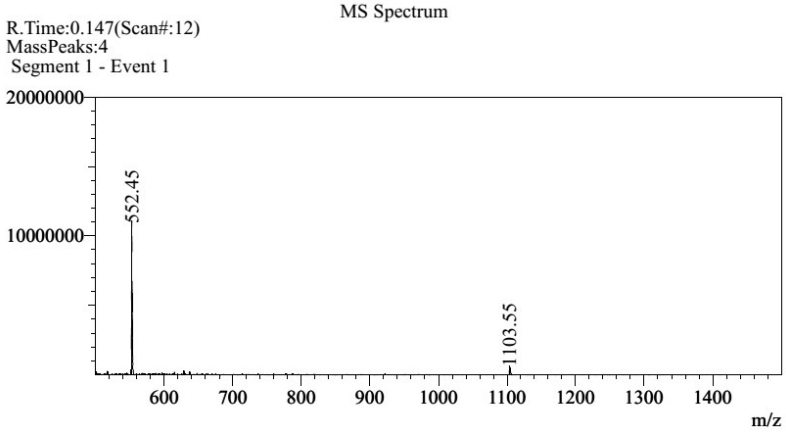
Ac-(cyclo)-[C-A(Me)-A-A-S_5(2-Ph)]



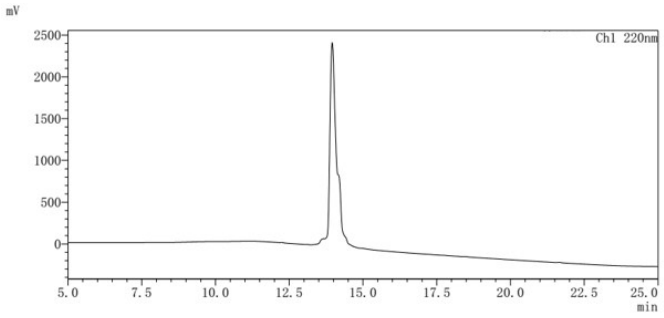


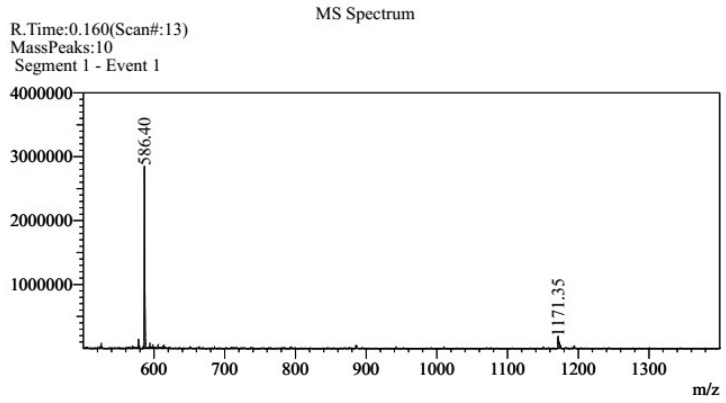
FITC-βA-(cyclo)-[C-A-R-R-S_5(2-Me)]



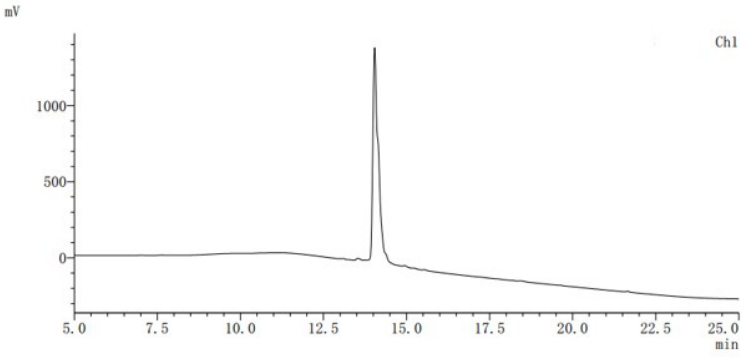


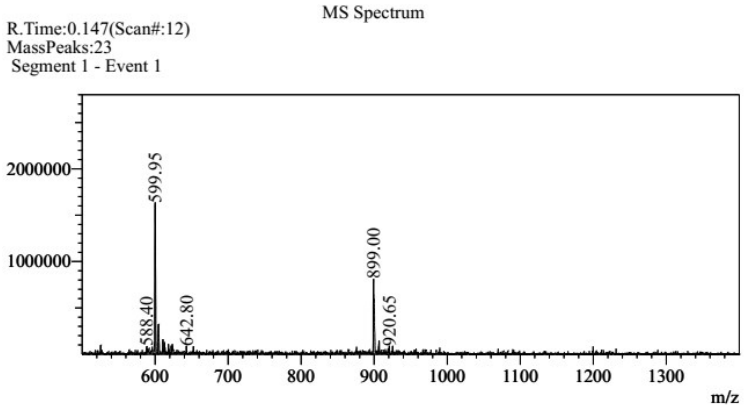
FITC-βA-DCEYNA



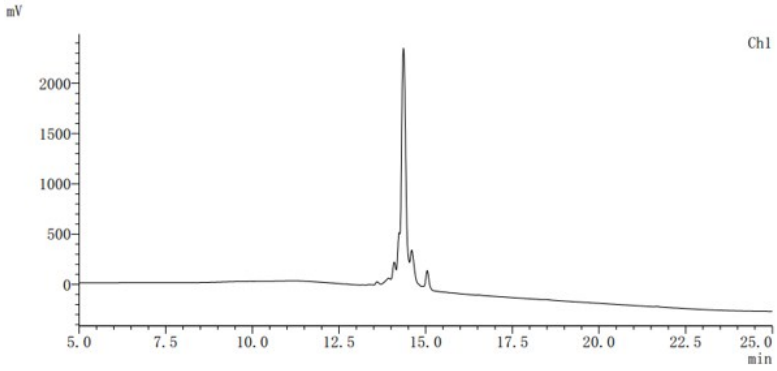


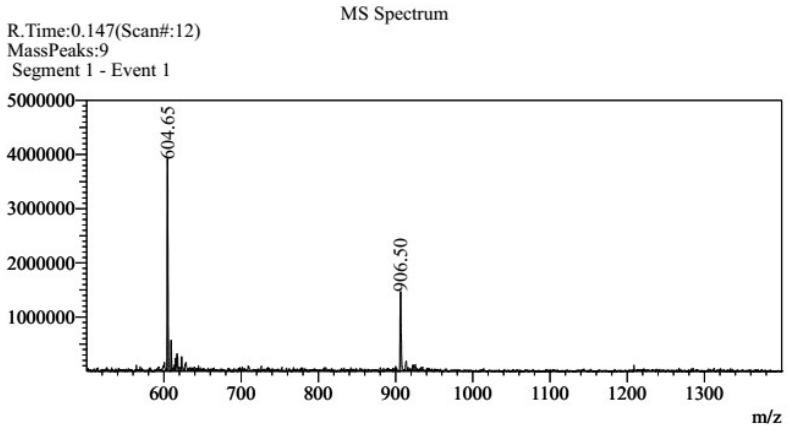
FITC-βA-(cyclo)-[C-A-R-R-S5(2-Me)]-DCEYNA



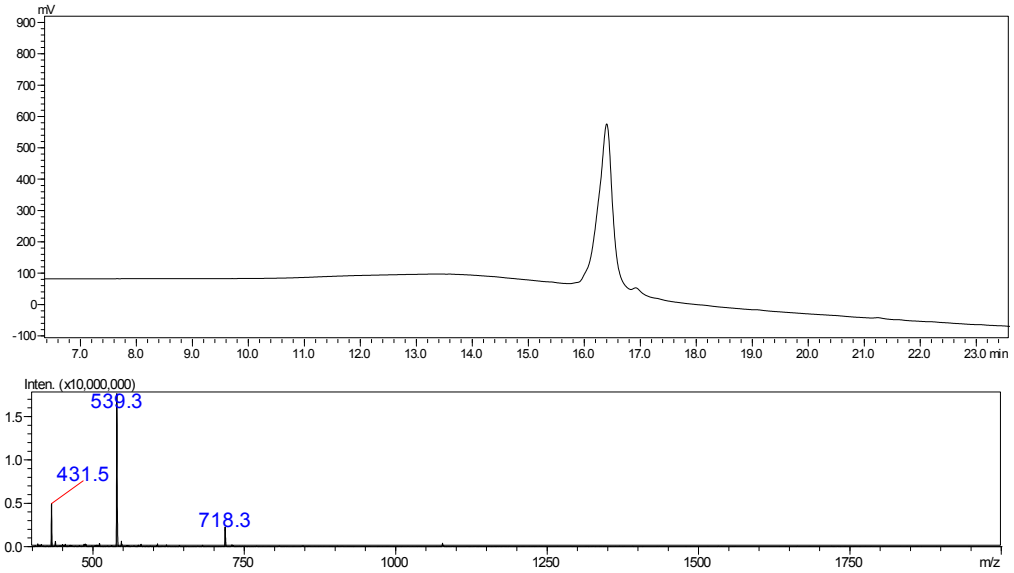


FITC-βA-(cyclo)-[C-A(Me)-R-R-S5(2-Me)]-DCEYNA

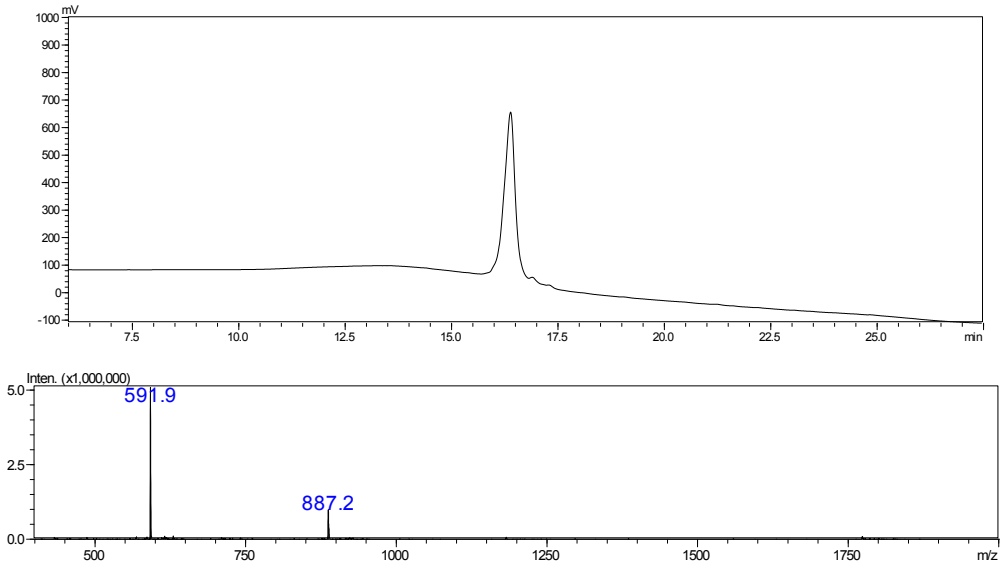




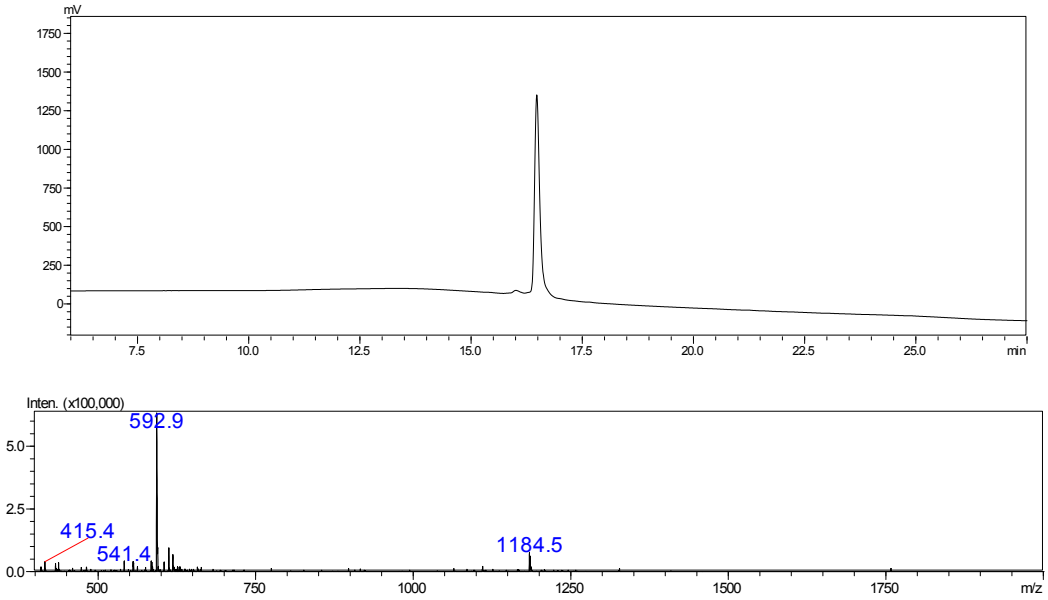
FITC-βA-C-A-R-R-L



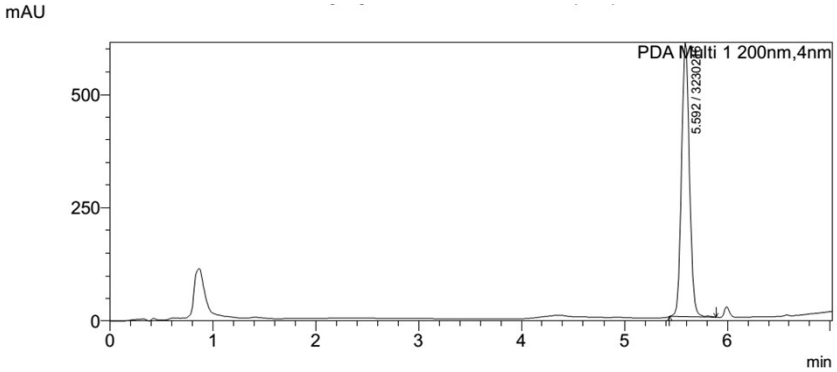
FITC-βA-C-A-R-R-L-DCEYNA



Ac-C-A-R-R-L-DCEYNA

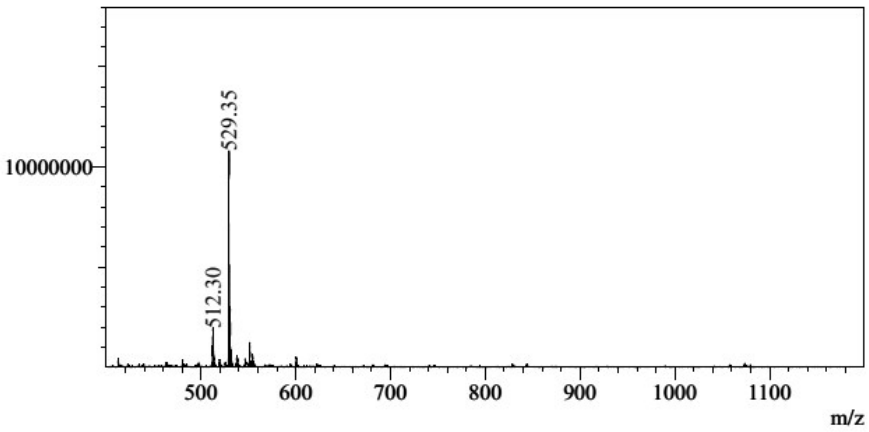


Ac-(cyclo)-[C-A(Me)-A-A-S_5(2-Me)]

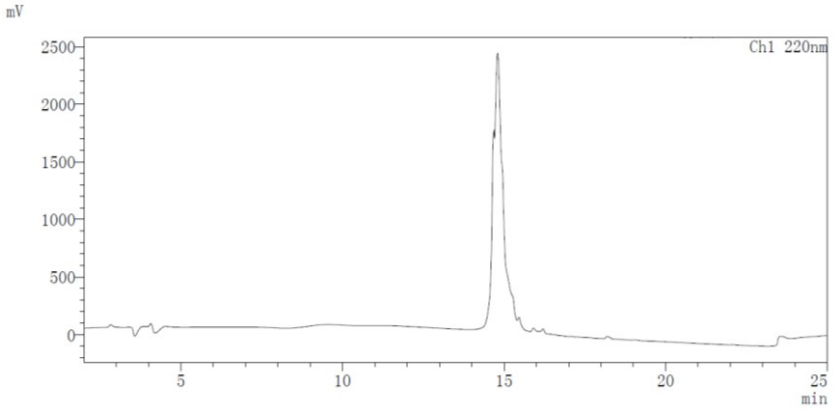


MS Spectrum

R.Time:0.160(Scan#:13)
MassPeaks:8
Segment 1 - Event 1

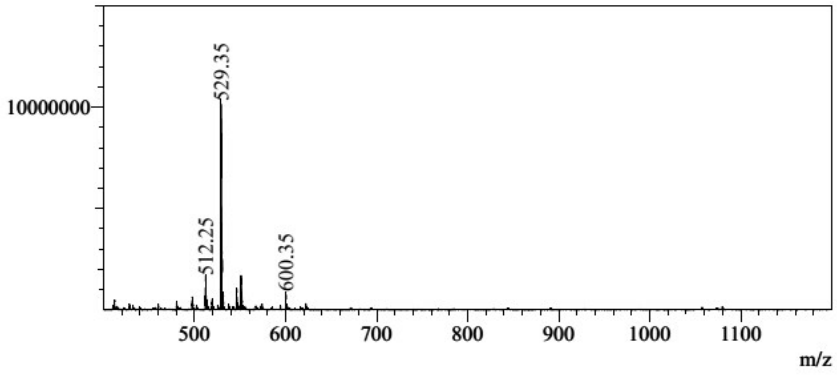


Ac-(cyclo)-[C-A-A(Me)-A-S_5(2-Me)]

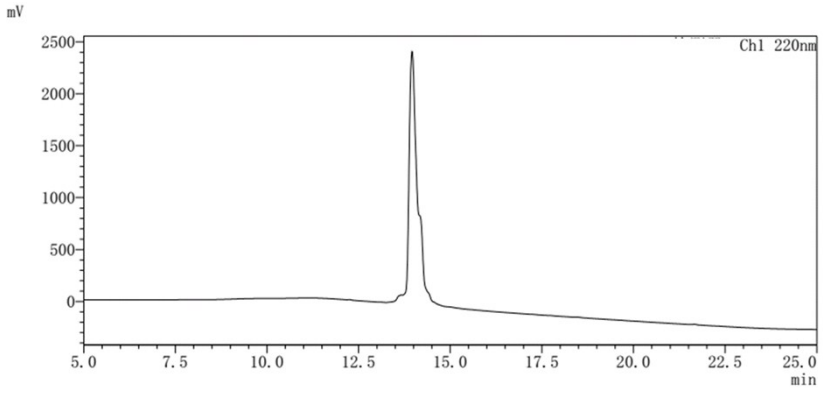


MS Spectrum

R.Time:0.160(Scan#:13)
MassPeaks:11
Segment 1 - Event 1

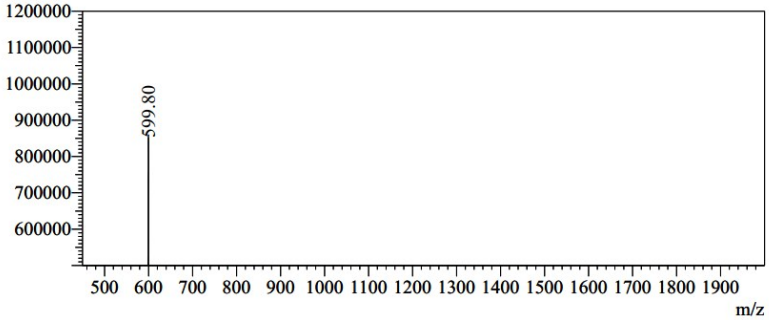


Ac-A(Me)-(cyclo)-[C-A-A-A-S5(2-Me)]

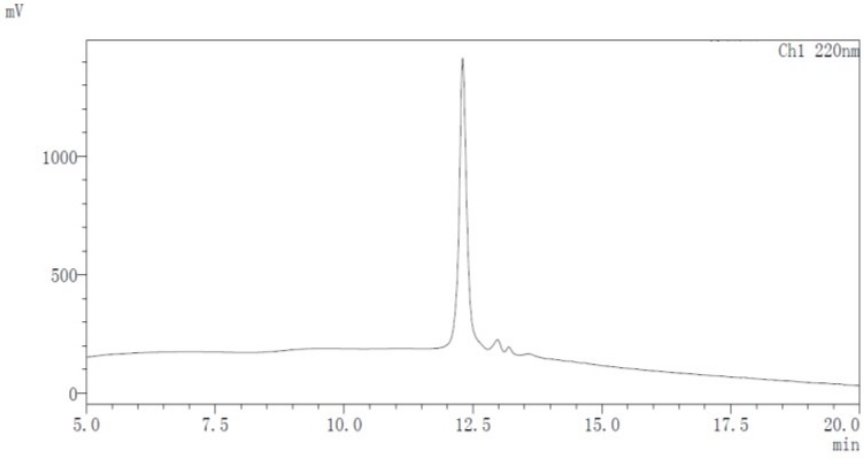


MS Spectrum

R.Time:0.160(Scan#:13)
MassPeaks:30
Segment 1 - Event 1

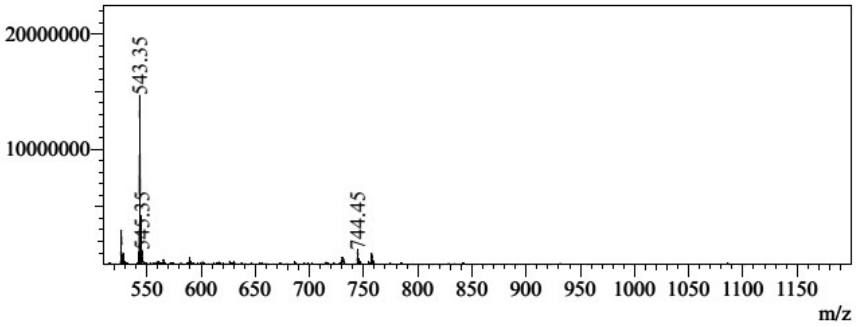


Ac-(cyclo)-[C-A(Me)-A-A(Me)-S_5(2-Me)]

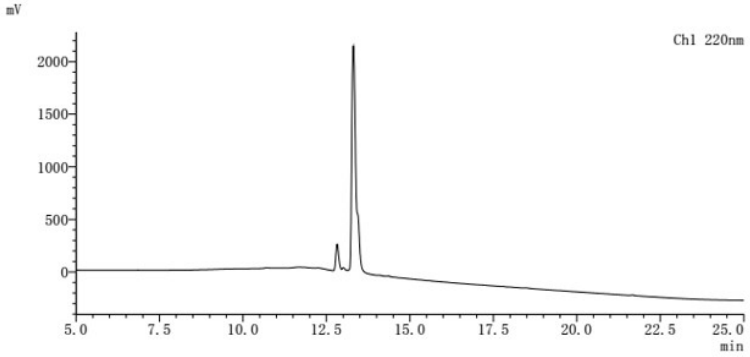


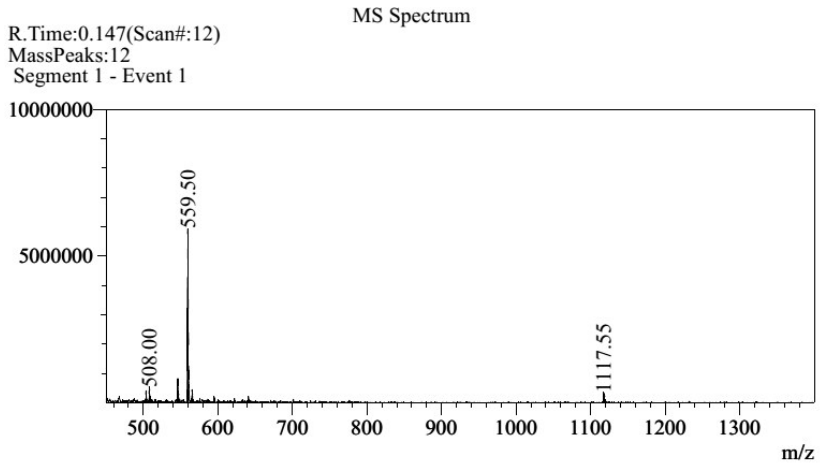
MS Spectrum

R.Time:0.173(Scan#:14)
MassPeaks:10
Segment 1 - Event 1

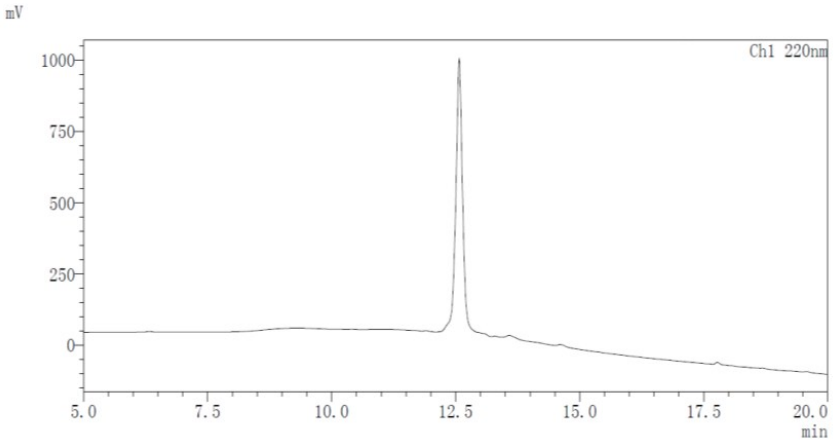


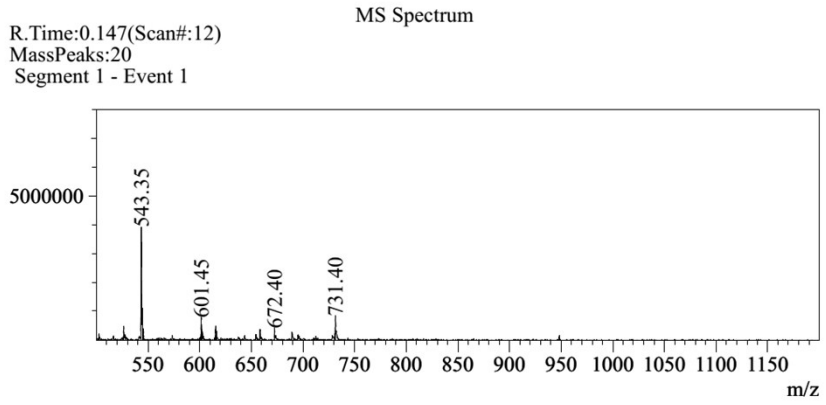
FITC-βA-(cyclo)-[C-A(Me)-R-R-S_5(2-Me)]



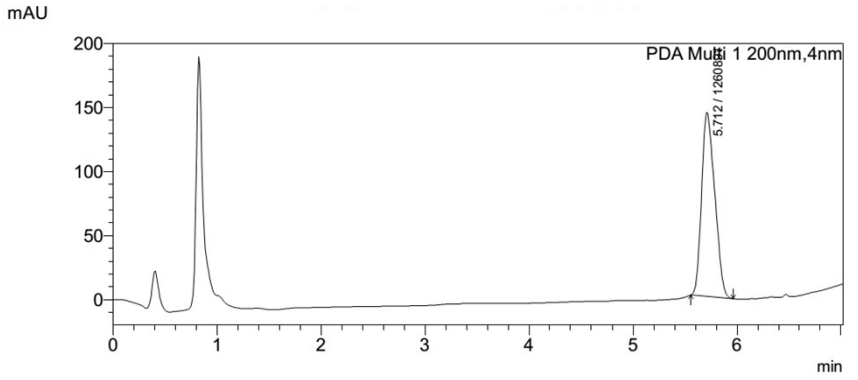


Ac-(cyclo)-[C-A(Me)-A(Me)-A-S_5(2-Me)]



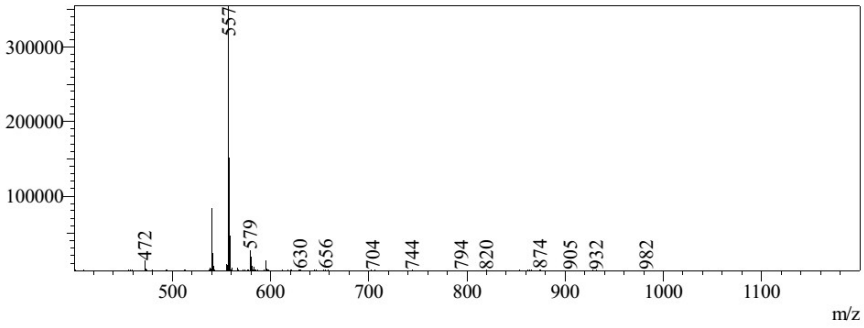


Ac-(cyclo)-[C-A(Me)-A(Me)-A(Me)-S_5(2-Me)]

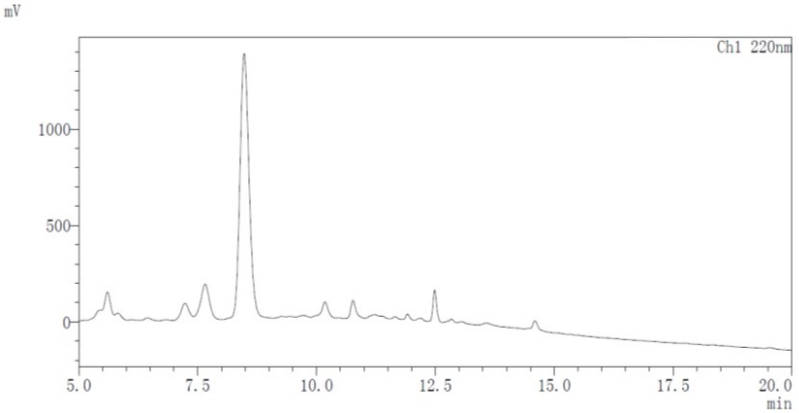


Mass Spectrum

R.Time:5.858(Scan#:?)
MassPeaks:1409
Segment 1 - Event 1

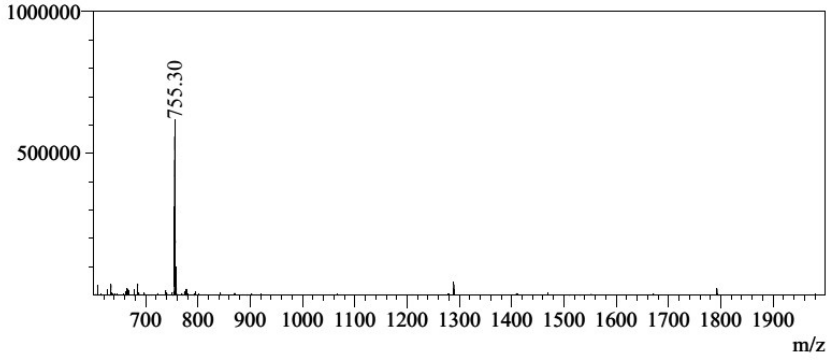


HIF: Ac-DCEYNA

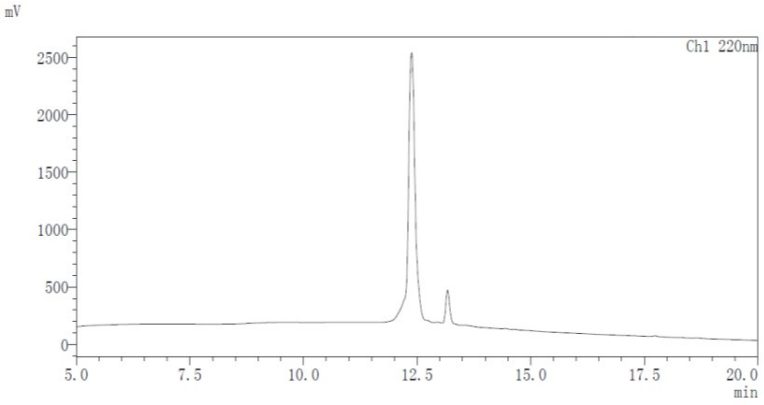


MS Spectrum

R.Time:0.200(Scan#:16)
MassPeaks:10
Segment 1 - Event 1

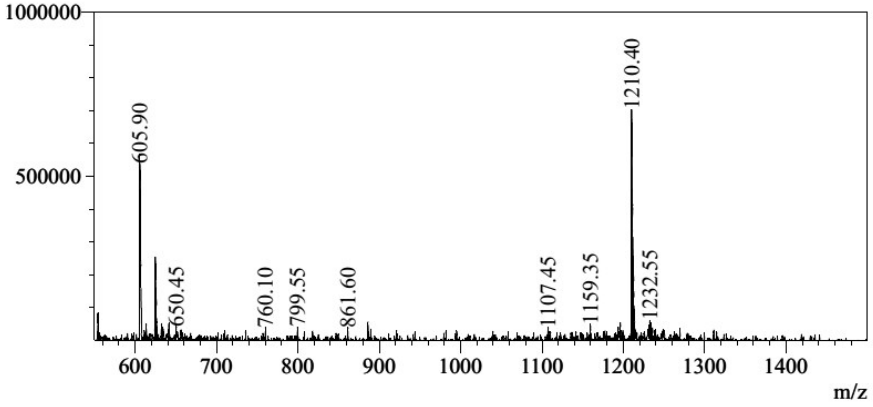


Ac-(cyclo)-[C-A-A-A-S_5(2-Me)]-DCEYNA

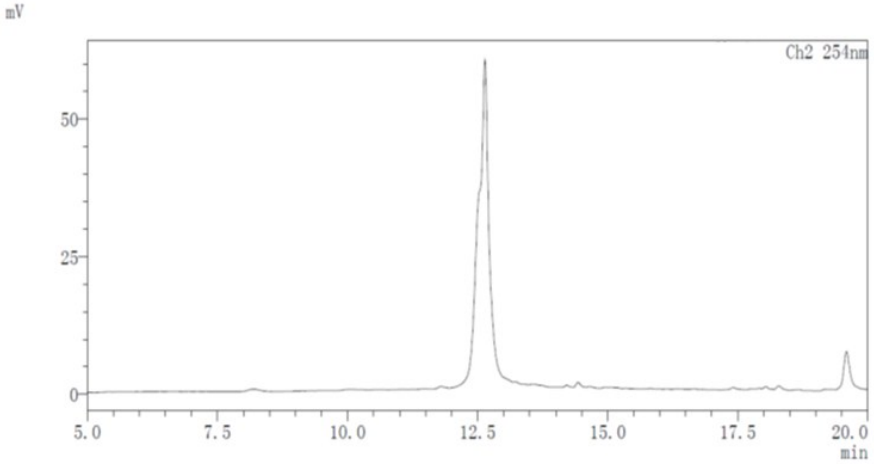


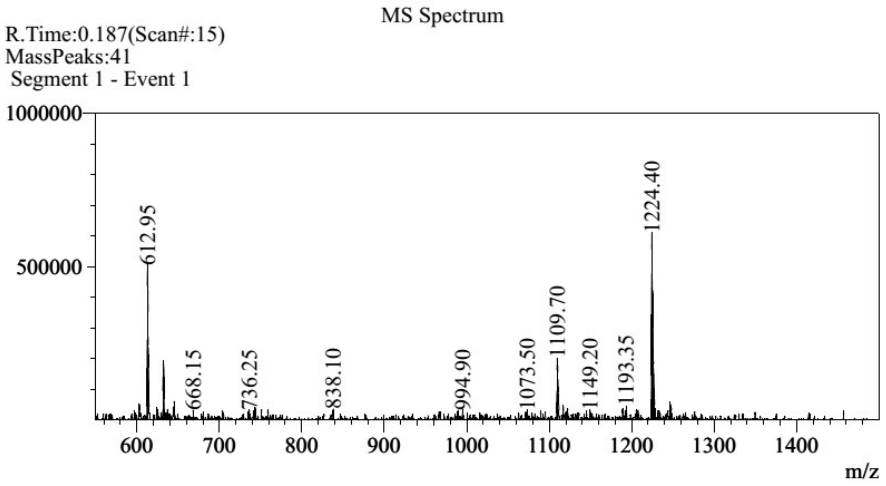
MS Spectrum

R.Time:0.173(Scan#:14)
MassPeaks:37
Segment 1 - Event 1

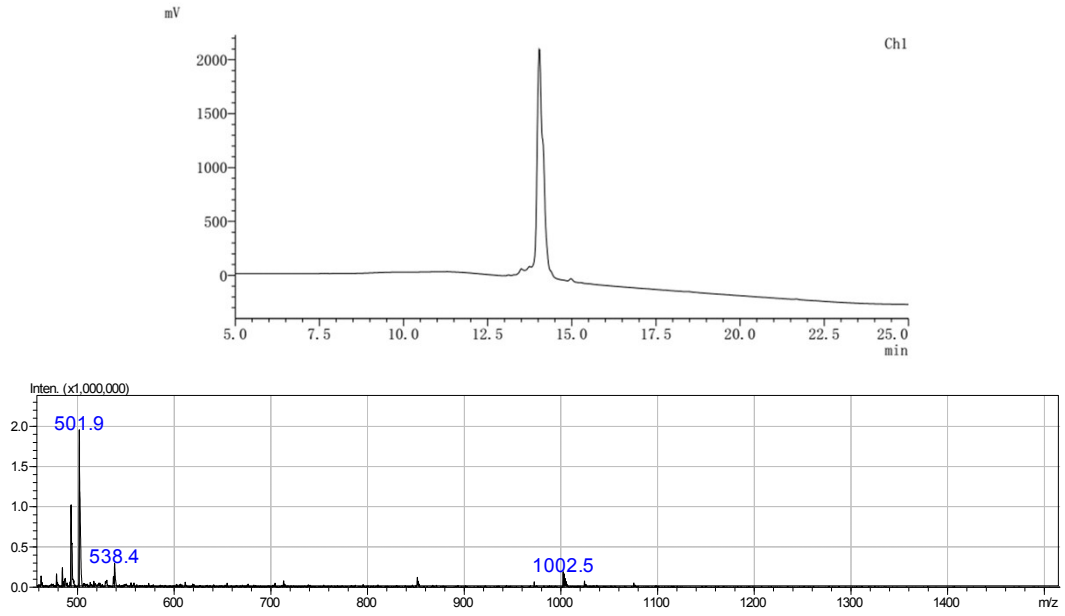


Ac-(cyclo)-[C-A(Me)-A-A-S_5(2-Me)]-DCEYNA

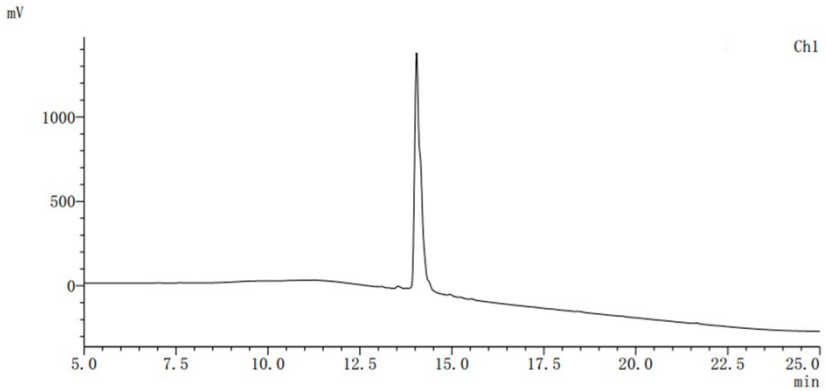




FITC-βA-A-(cyclo)-[C-A-A-A-S5(2-Me)]



FITC-βA-A(Me)-(cyclo)-[C-A-A-A-S5(2-Me)]



MS Spectrum

R.Time:0.260(Scan#:27)
MassPeaks:17
Segment 1 - Event 1