Supporting Information

Evaluation of Topologically Distinct Constrained Antimicrobial Peptides with Broad-Spectrum Antimicrobial Activity

Fang Yuan,‡[a,c] Yuan Tian,‡[b,c] Weirong Qin,‡[c] Jingxu Li,[c] Dan Yang,[c, d] Bingchuan Zhao,[c] Feng Yin*[c] and Zigang Li *[c]

[a.] *Institute of Scientific and Technical Information of China, Beijing, 100038, China.*

[b.] *School of Life Science and Engineering, Southwest Jiaotong University, Chengdu, 610031, China.*

[c.] *The State Key Laboratory of Chemical Oncogenomics, School of Chemical Biology and Biotechnology, Peking University, Shenzhen Graduate School, Shenzhen, 518055,China.*

[d.] *Department of Science & technology of Shandong Province, Jinan, 250101, China.*

*Corresponding author. E-mail address: lizg@pkusz.edu.cn, yinfeng@pkusz.edu.cn

‡ These authors contributed equally to the manuscript.

Materials and methods

Peptides synthesis

The peptides used in this study were synthesized by standard Fmoc-solid phase peptide synthesis and were confirmed by LC-MS. Peptide synthesis was performed manually on Rink Amide MBHA resin (loading capacity: 0.53 mmol/g) (GL Biochem Ltd.). Generally, Rink amide AM resin was pre-swelled with DCM/DMF(dichloromethane/N,N-Dimethylformamide) (1/1 in volume) for 0.5-1 h. Fmoc deprotection was performed with morpholine (50% in DMF) for 20 min × 2. Then the resin was washed with DMF (5 times), DCM (5 times) and DMF (5 times). Fmoc-protected amino acids (6.0 equiv according to initial loading of the resin) and HCTU (2-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (5.9 equiv) were dissolved in DMF, followed by DIPEA (diisopropylethylamine) (12.0 equiv). The mixture was pre-activated for 1 min and then added to the resin for 1-2 h, then the resin was washed with DMF (5 times), DCM (5 times) and DMF(5 times). After peptide assembly, the resin-bound peptides were N-terminally acetylated with a solution of acetic anhydride and DIPEA in DCM (1:1:8 in volume) for 45 min. Peptides were then cleaved from the resin with a mixture of TFA (trifluoroacetic acid) /H_2O/EDT (1, 2-ethanedithiol) /TIS (triisopropylsilane) (94:2.5:2.5:1 in volume) for 2 h and concentrated under a stream of nitrogen. The crude peptides were then precipitated with Hexane/Et_2O (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.

Circular dichroism (CD) spectroscopy

CD spectra were obtained using a Chirascan Plus Circular Dichroism Spectrometer at 25˚C. Peptides were dissolved in ddH_2O. Parameters used in the experiment are as followed: wavelengths from 250 to 190 nm were measured with resolution of 0.5 nm, response of 1 s, bandwidth of 1 nm, scanning speed of 20 nm/min. Each spectrum represents the average of two scans and smoothed using Pro-Data Viewer by Applied Photophysics.

Bactericidal micro dilution assays

Minimum inhibitory concentrations (MICs) of the peptides against *Escherichia coli* DH10B (Gram-negative)，*Staphylococcus aureus Newman* (Gram-positive) and *Candida albicans* 5314 (Fungi) were determined using a standardized dilution method according to the Clinical and Laboratory Standards

Institute (CLSI) in the USA and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. A single bacterial colony was inoculated in Mueller-Hinton broth (MHB) and incubated for 12 h at 37 ˚C. Overnight-cultured bacteria were transferred to Mueller-Hinton growth medium and cultured to exponential phase (OD_{600} of ~0.6). The culture was centrifuged and resuspended in sterile MHB and adjusted to a final amount of 1×10^5 colony-forming units (CFU) mL^{-1} by use of the equation CFU mL^{-1} = OD_{600} nm $\times 2.5 \times 10^8$. For MIC determination, peptides were applied at various concentrations (1-200 μM) in MH from a stock solution. Fifty microliters of each concentration of peptide solution was added to each corresponding well of a 96-well plate (Becton & Dickinson, USA) and 50 μL of bacteria (1×10^5) in MH medium was also added. The polypropylene plates (TPP, Switzerland) were incubated at 37 ˚C for 24 h. MICs were determined as the lowest tested concentration that leads to complete inhibition (100%) compared to the negative control group.

Timed killing curve and fractional survival test

E. coli and *S. aureus* were treated at 1×MIC and 2×MIC concentrations with peptide. At various time periods (0, 0.5, 1, 2, 4, 8 and 24h), microbial suspensions were diluted at 3 different dilutions and plated on 1.5% Mueller-Hinton (MH) agar plates. Microbial colonies were formed and counted after 8 h of incubation. Data presented in the results are means from three independent experiments.

AMP-L1 is the linear analogues of AMP-A1 and B1. As control, AMP-L1 was taken to treat *E. coli* and *S. aureus* were treated at 1×MIC and 2×MIC concentrations. AMP-L1 was not presented as effective as AMP-A1 or AMP-B1 in this experiment. In the Fig. S1, we can see bacteria began to grow and proliferate again after several hours.

Hemolytic activity test

Fresh red blood cells drawn from the mouse's eye socket were washed with PBS for three times, and were subjected to 25 times dilution with PBS to reach a concentration of approximately 4% (in volume) of the blood cells. The red blood cell suspension (100 μL) was placed into a 96-well cell culture plate and mixed with 100 μL of the antimicrobial peptide solution in PBS at concentrations of 1, 2, 4, 8, 16, 32, 64 μM. The mixture was then incubated at 37 ˚C for 0.5 h to allow for the hemolysis process to take place. At the end of incubation time, the non-hemolysed red blood cells were separated by centrifugation at 13000 g for 5 min. 100 μL of the supernatant were transferred into a new 96-well plate, and the

hemoglobin release was measured by checking the UV absorbance of the samples at 576 nm using a microplate reader (TECAN, Switzerland). Two controls were provided in this assay: an untreated red blood cell suspension in PBS was used as the negative control, and a solution containing red blood cells lysed with 0.1% Triton-X was used as the positive control. Each assay was performed in 3 replicates, and the data were expressed as means and standard deviations of the 3 replicates. Percentage of hemolysis was calculated using the following formula:

Hemolysis (%) = [(OD_{576} nm of the treated sample - OD_{576} nm of the negative control) / (OD_{576} nm of positive control - OD_{576} nm of negative control)] × 100%.

Antimicrobial mechanism

SYTOX Green uptake

SYTOX Green is a membrane impermeable cationic cyanine dye (~900 Da). When a cell's plasma membrane integrity is compromised, influx of the dye and subsequent binding to DNA causes a large increase in fluorescence. For SYTOX Green assay, bacterial cells were grown overnight and then centrifuged, washed, and resuspended in PBS. Cell suspensions in PBS (~1 × 10^7 cells/ mL) were incubated with peptide A1, B1 at 3×MIC concentration for 1.5 h. After washing three times with PBS, 1 μM SYTOX Green (Invitrogen, Carlsbad, CA) for 10 min was added in the dark prior to the influx assay. And the increase in SYTOX Green fluorescence was measured (excitation wavelength at 485 nm and emission at 520 nm) via laser scanning confocal microscope.

SEM (Scanning electron microscopy) characterization

For SEM sample preparation, bacterial cells were cultured in MH at 37 ˚C under constant shaking at 250 rpm. In this study, *Escherichia coli* DH10B and *Staphylococcus aureus Newman* were cultured to an exponential phase, harvested by centrifugation, washed twice with 20 mM PBS and resuspended. Approximately 1 × 10^5 cells were incubated at 37 ˚C for up to 1 h or 1.5 h with different peptides at a concentration of 1×MIC or 3×MIC. Controls were run without peptides. After incubation, the cells were mixed and centrifuged at 5000 g for 5 min. Bacterial pellets were then washed 3 times with PBS, and subjected to similar centrifugation after each wash. Following that, fixation of bacterial cells was performed with 2.5% (w/v) glutaraldehyde at 4 ˚C overnight, followed by washing with PBS twice. Cells were then dehydrated for 15 min in each of a graded ethanol series (50, 70, 80, 90, 95 and 100%). They

were then transferred to absolute acetone for 20 min. Finally, specimens were lyophilized for 30min. The dehydrated specimens were coated with gold-palladium and observed in a SEM (JSM-7800F, JEOL).

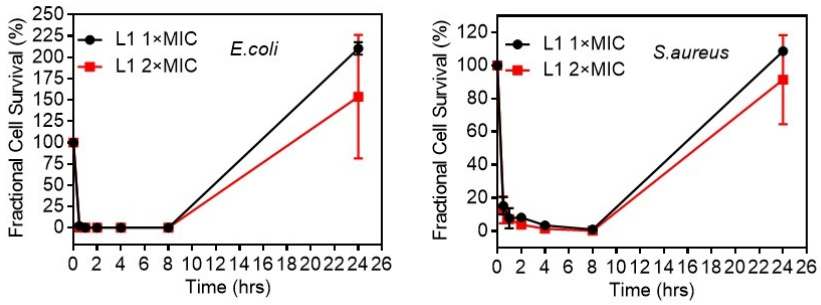


Fig. S1 Fractional microbial survival of *E. coli* and *S. aureus* after 30 min, 1 h, 2 h, 4 h, 8 h and 24h of treatment with AMP-L1 at 1×MIC (black line) and 2×MIC (red line). Bacteria suspensions treated peptides plated on 1.5% MH agar plates to incubate.

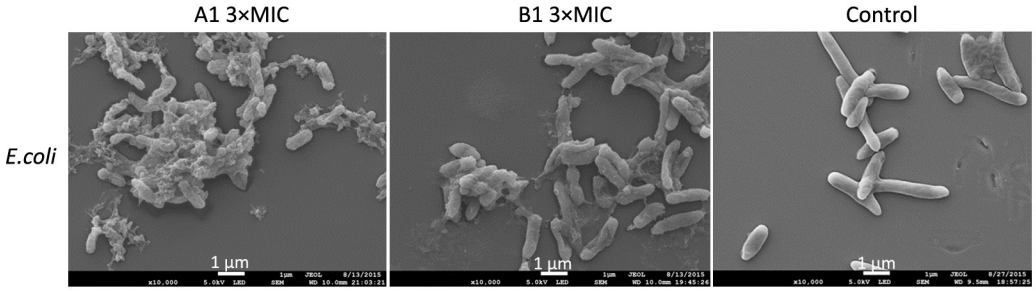
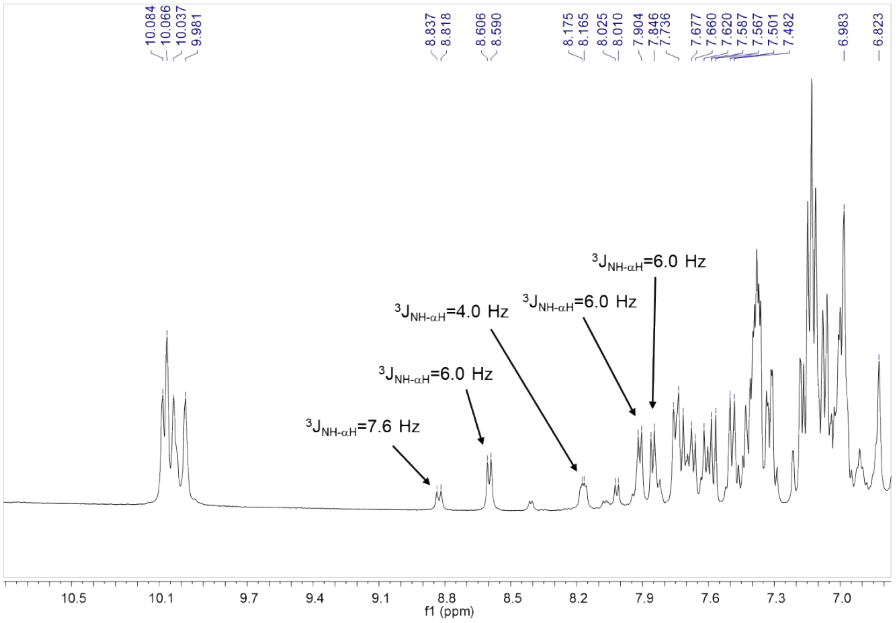


Fig. S2 SEM images of *E.coli* after incubation with AMP-A1, B1 at 3×MIC for 1.5 hours. Controls were run without peptides. After dehydrated treatment, cells were coated with gold-palladium and observed in the SEM.

Peptide A1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10.037
9.981
8.837
8.818
8.606
8.590
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8.165
8.025
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7.904
7.846
7.736
7.677
7.660
7.620
7.587
7.567
7.501
7.482
6.983
6.823
$^3J_{NH-\alpha H}$=7.6 Hz
$^3J_{NH-\alpha H}$=6.0 Hz
$^3J_{NH-\alpha H}$=4.0 Hz
$^3J_{NH-\alpha H}$=6.0 Hz
$^3J_{NH-\alpha H}$=6.0 Hz
10.5
10.1
9.7
9.4
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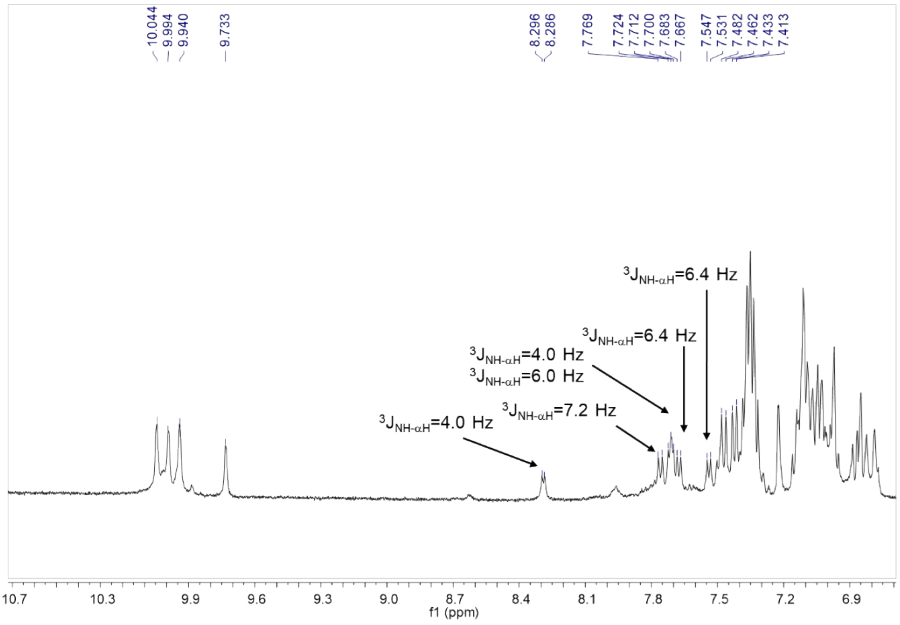
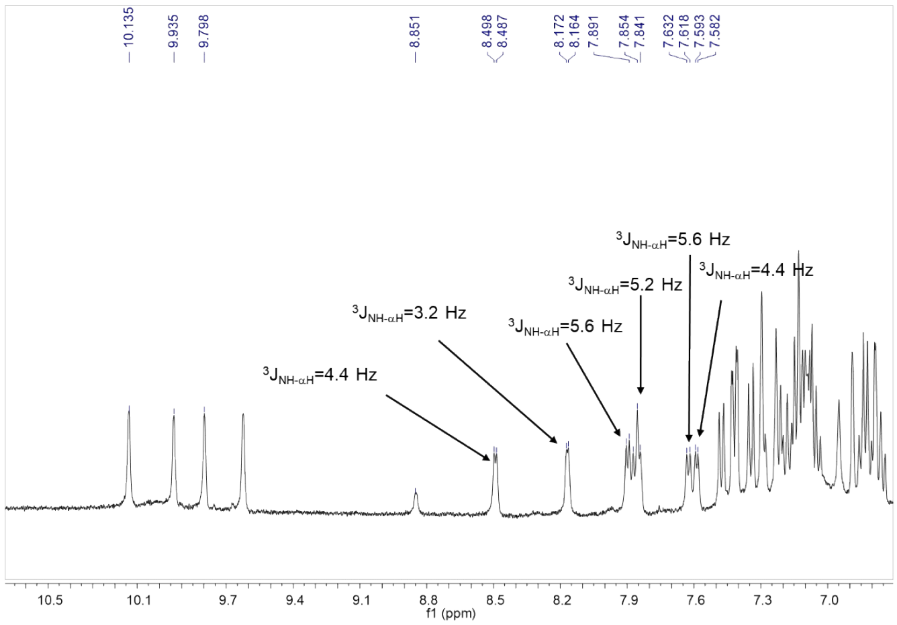
8.8
8.5
8.2
7.9
7.6
7.3
7.0
f1 (ppm)

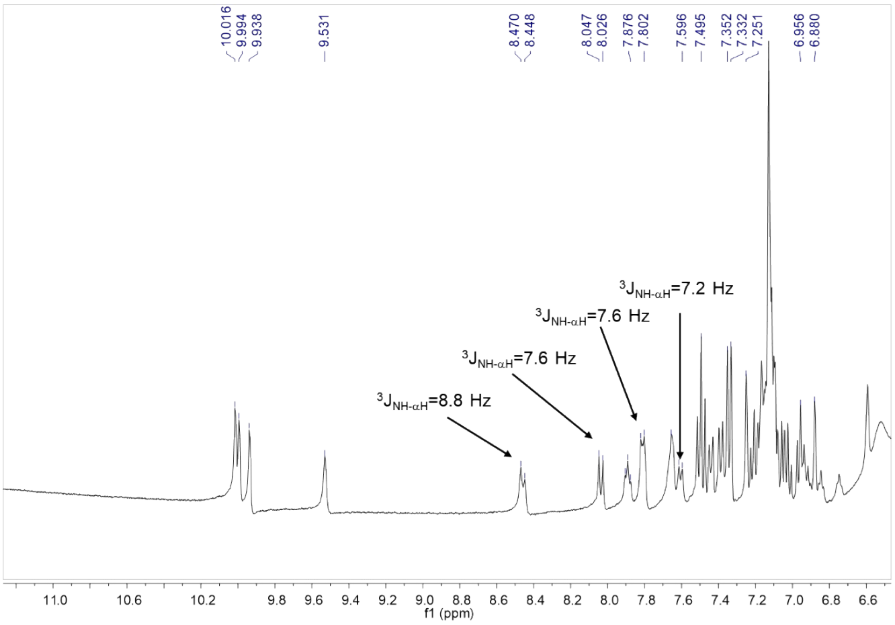
Peptide A2



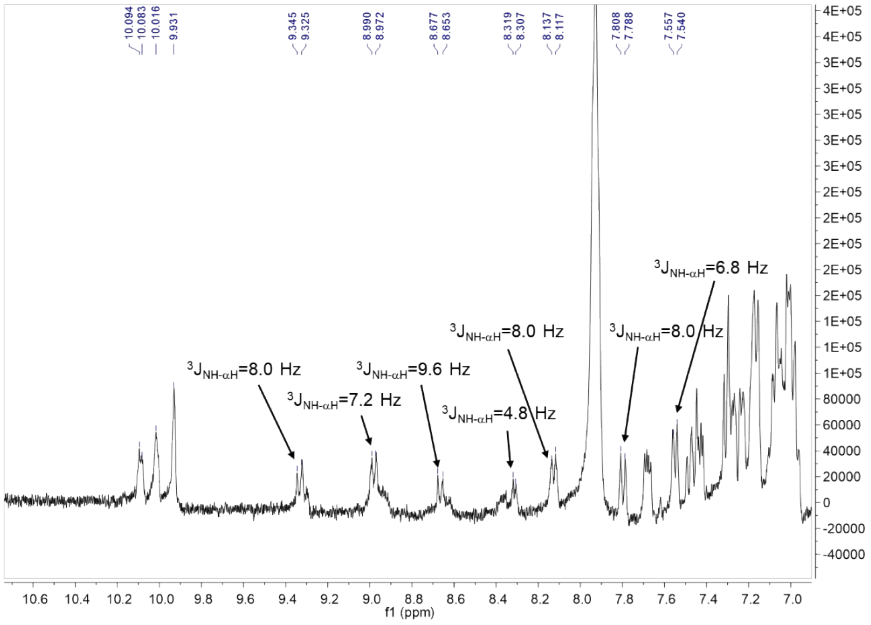
Peptide A3



Peptide B1



Peptide B2



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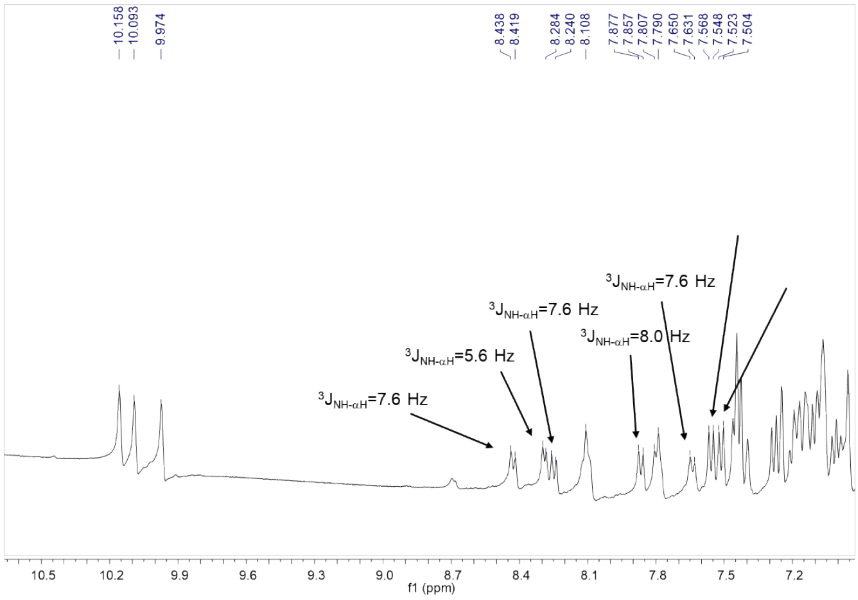


Fig. S3 NH-region of the constrained peptides in 1H-NMR study (at 400 MHz in 10% D_2O in ddH_2O). The A1, A2 and A3 peptides shown a series of low amide coupling constants ($^3J_{NHCH\alpha} \leq 6.0$) in the 1H-NMR study, while the B1，B2 and B3 peptides displayed amide coupling constants above 6.0 Hz.

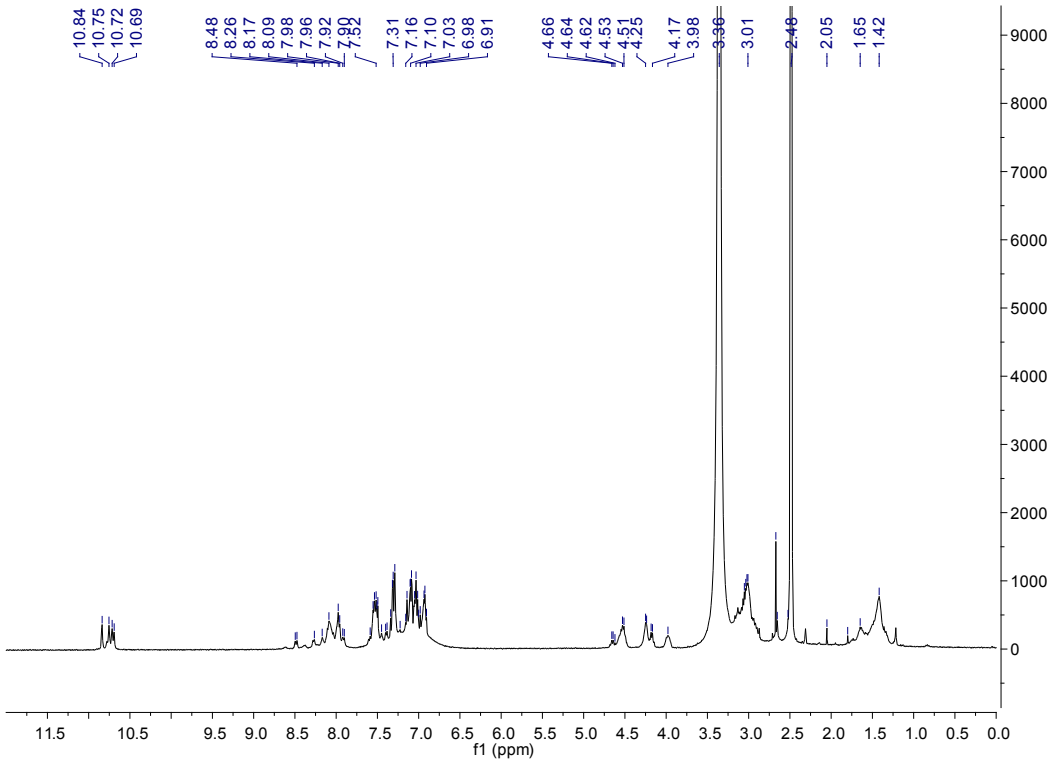
Table S1

Calculated percentages of α-helix and β-strand of each constrained peptides using K2D3 tools. (Prediction of protein secondary structure from circular dichroism using theoretically derived spectra. Proteins, 2012, 80, 374–381)

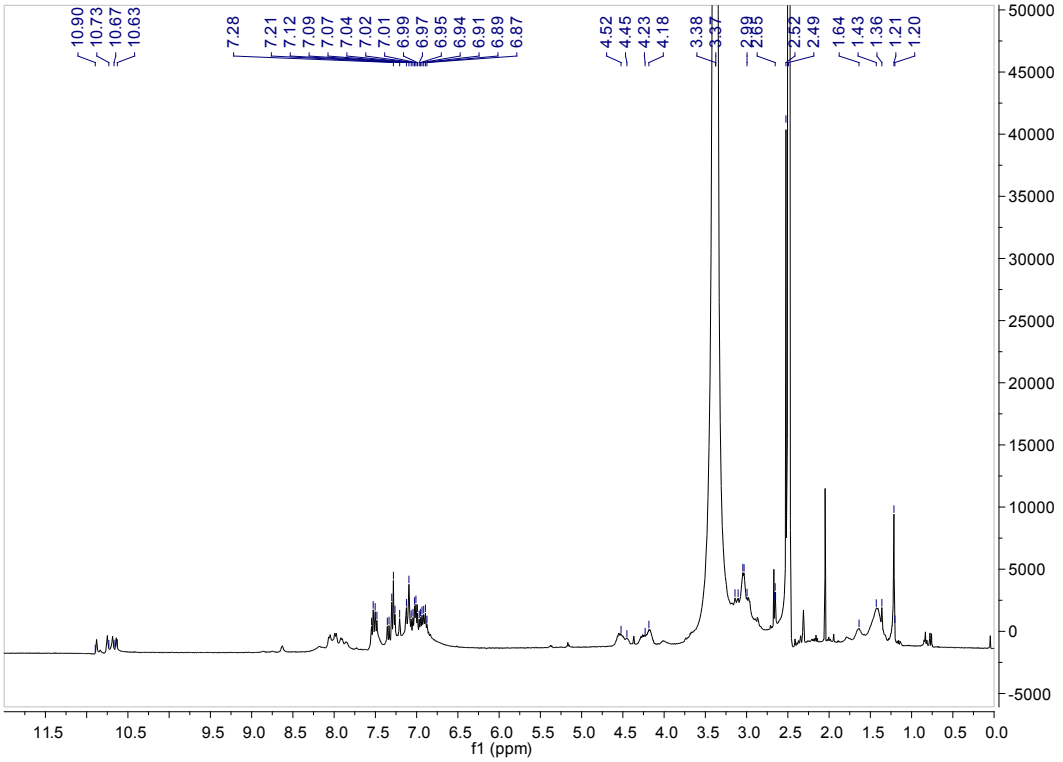
Peptide	**A1**	**A2**	**A3**	**B1**	**B2**	**B3**
α-helix	17.54%	16.02%	20.58%	2.89%	2.96%	3.81%
β-strand	9.02%	11.47%	10.95%	17.95%	16.26%	13.51%

Appendix

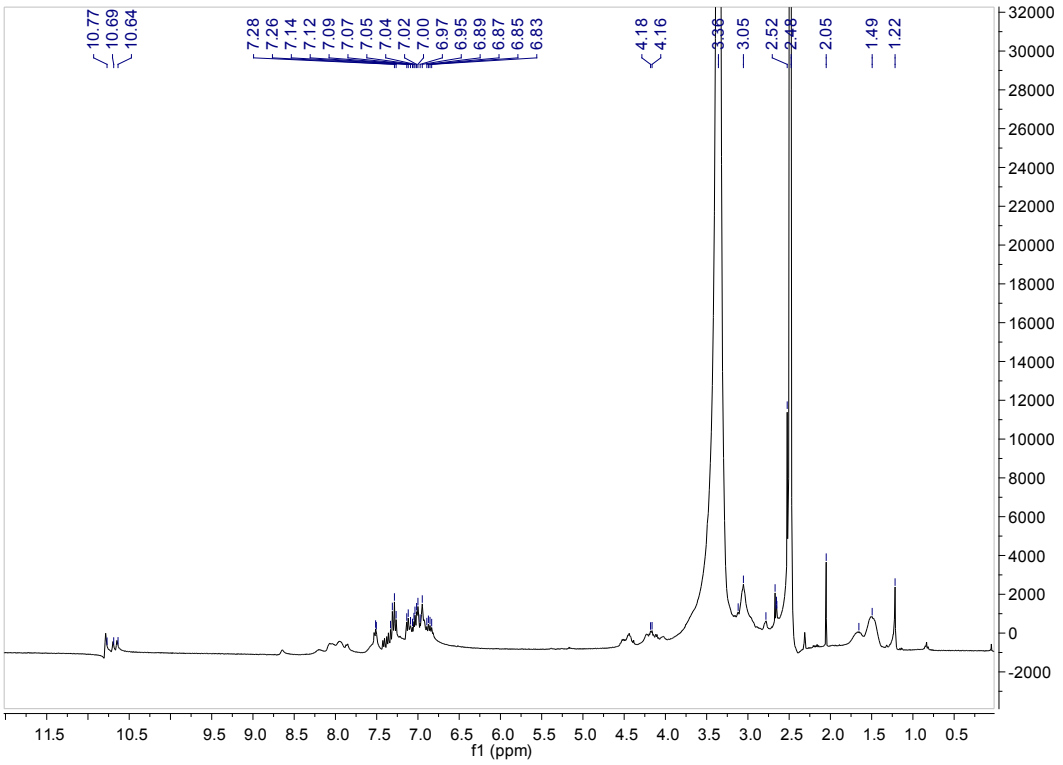
1H-NMR spectra of the constrained peptides at 400 MHz in DMSO-d6



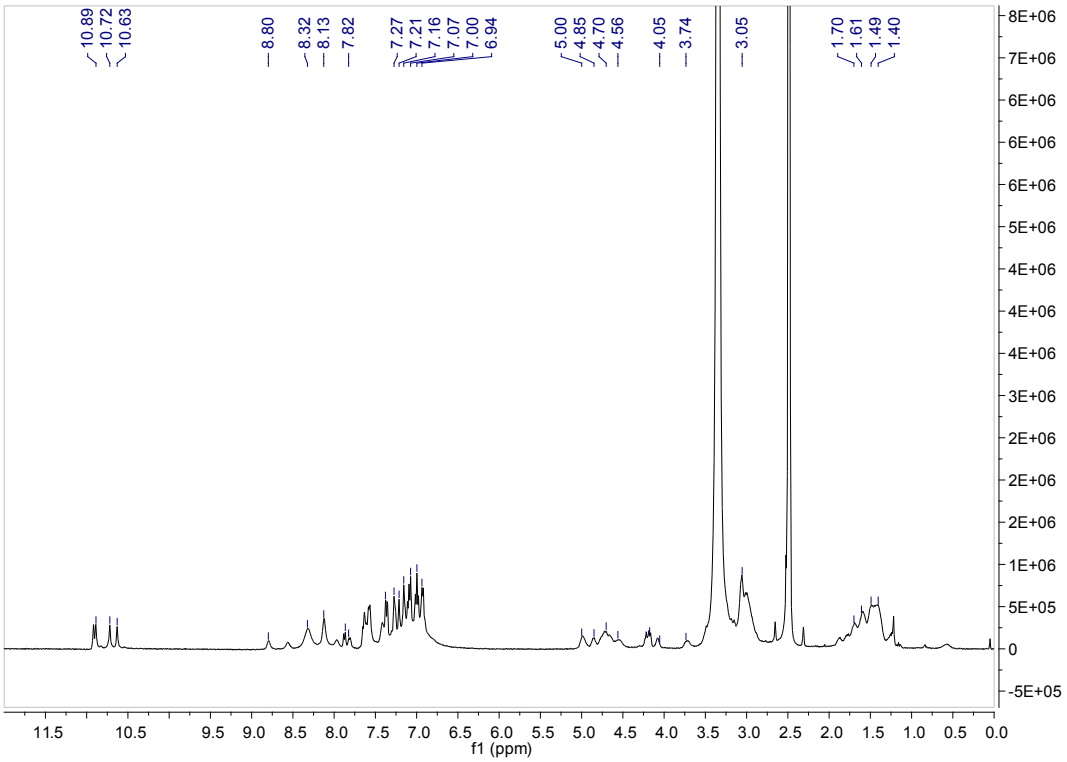
1H-NMR spectrum of A1 (at 400 MHz in DMSO-d6)



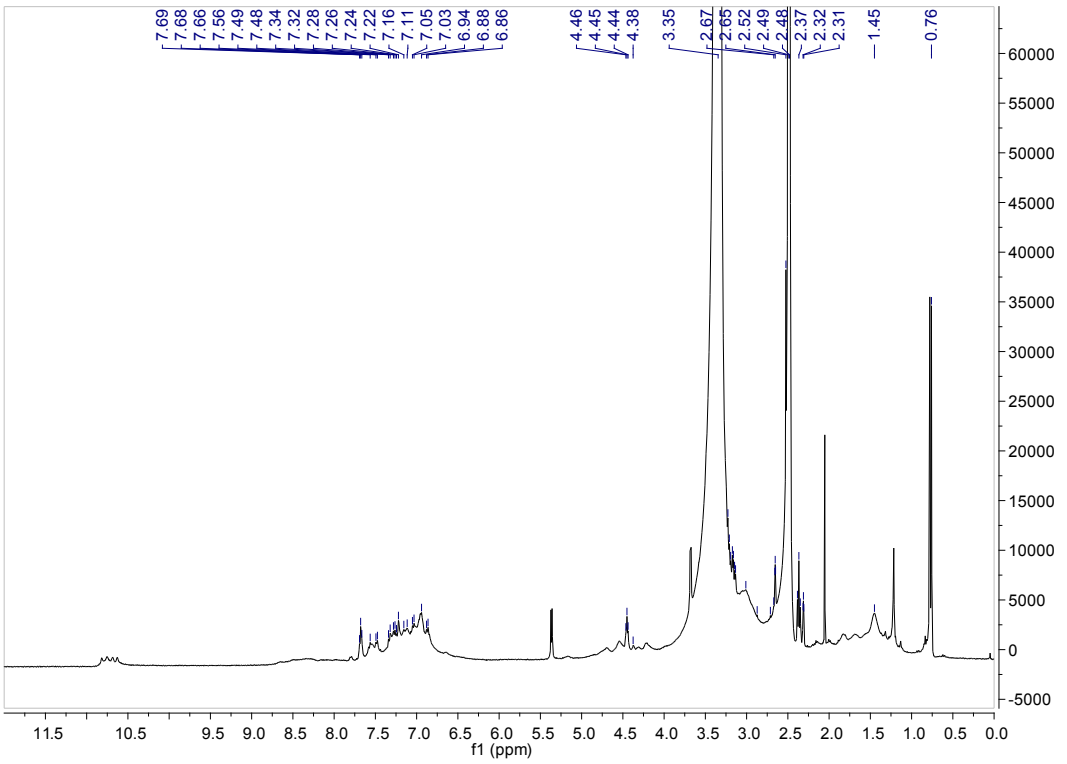
1H-NMR spectrum of A2 (at 400 MHz in DMSO-d6)



1H-NMR spectrum of A3 (at 400 MHz in DMSO-d6)



1H-NMR spectrum of B1 (at 400 MHz in DMSO-d6)



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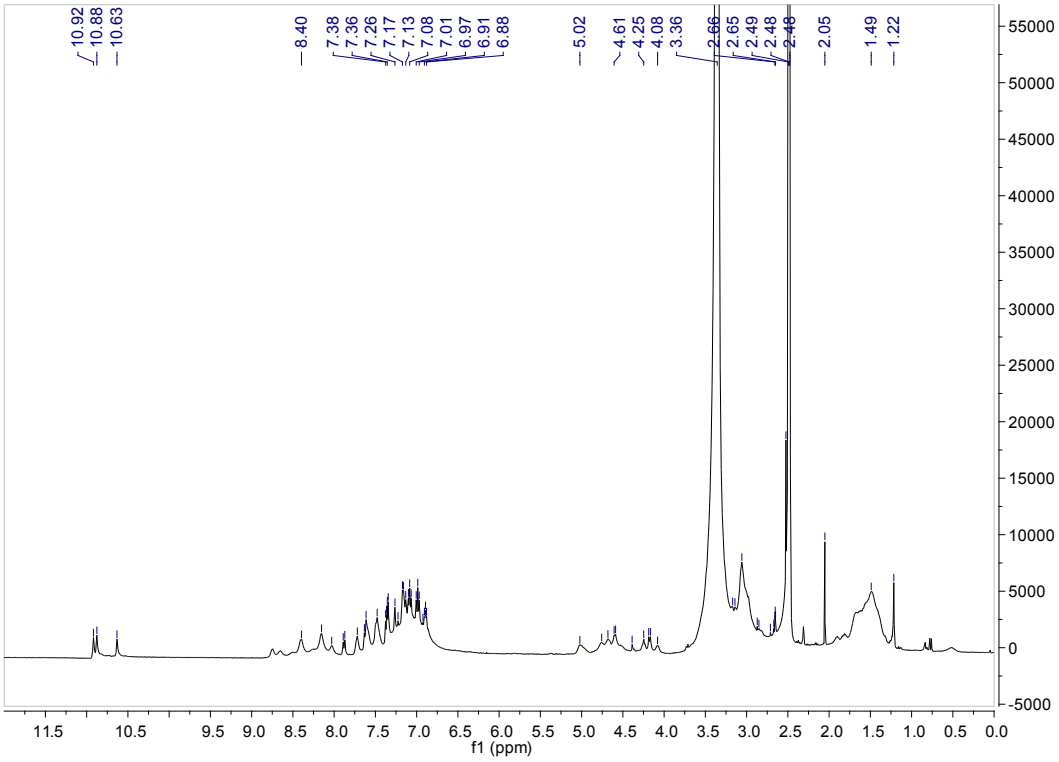
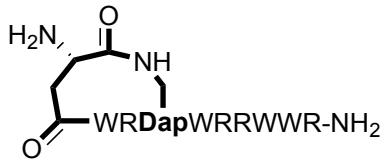
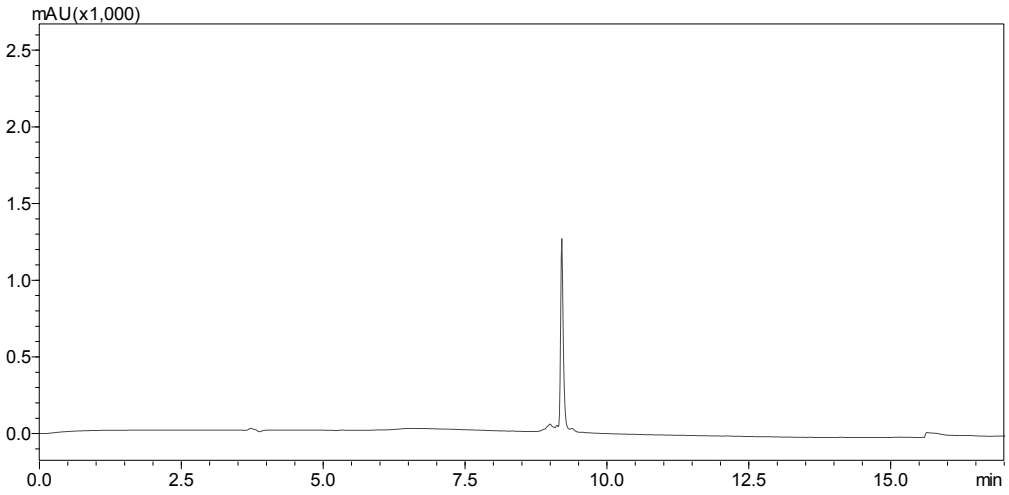


1H-NMR spectrum of B3 (at 400 MHz in DMSO-d6)

HPLC and mass spectra of the constrained peptides



A1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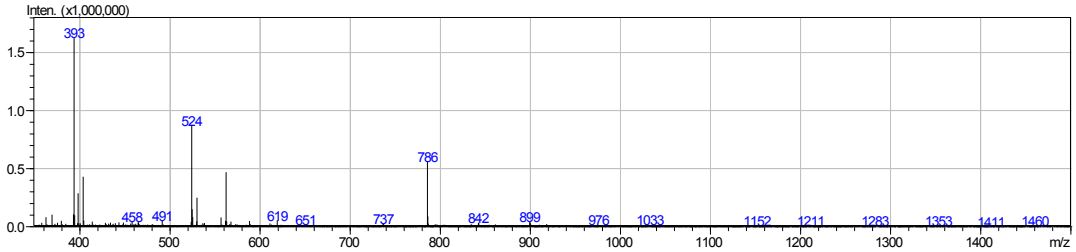
15.0
min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1283
1353
1411
1460
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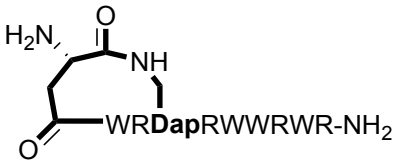
1300
1400
m/z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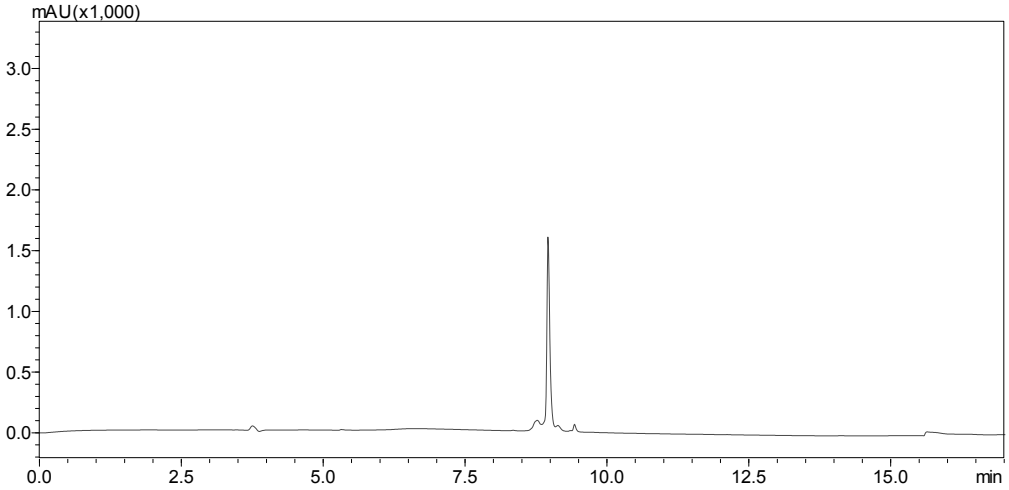
O
NH
O
WRDapRWWRWR-NH2

A2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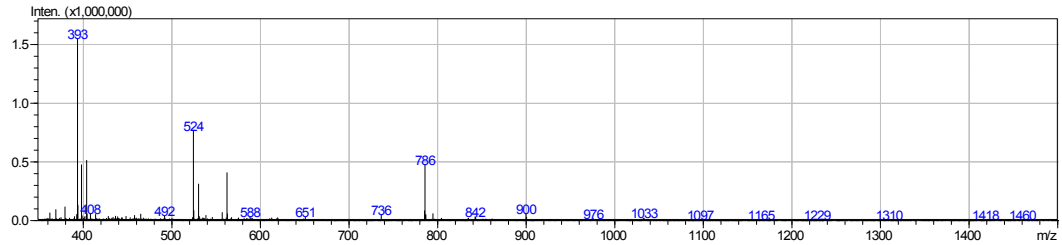
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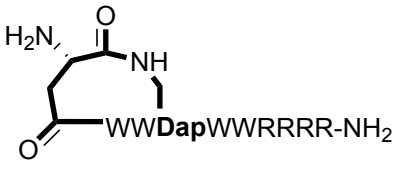
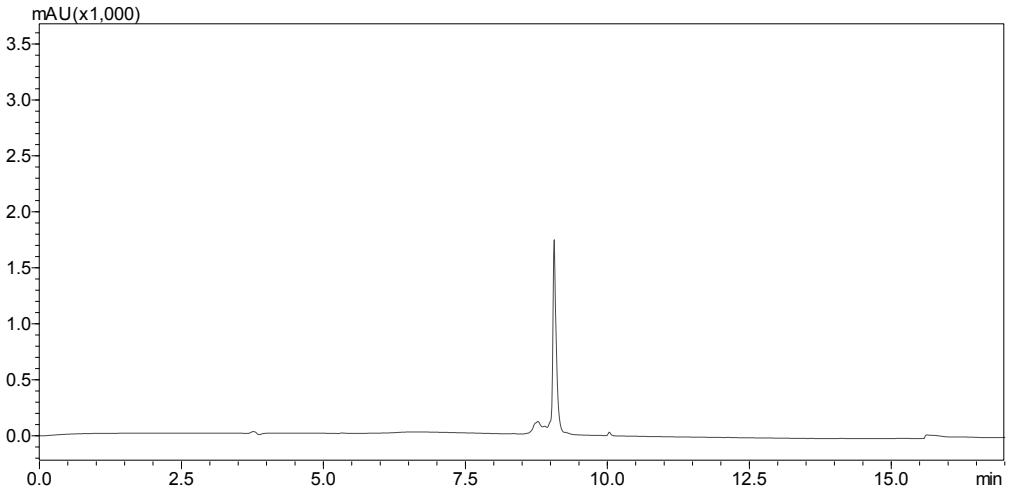

H2N
O
NH
O
WWDapWWRRRR-NH2

A3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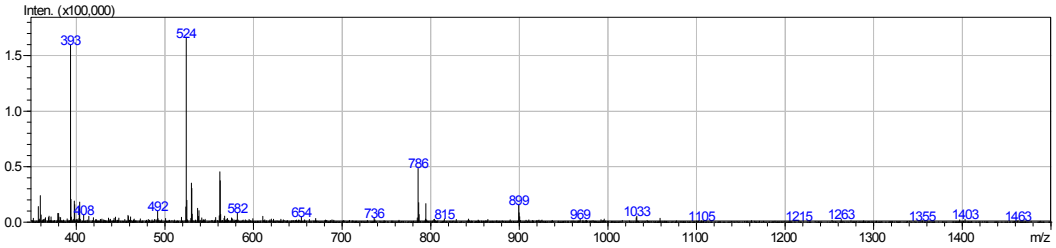
15.0
min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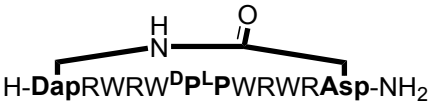
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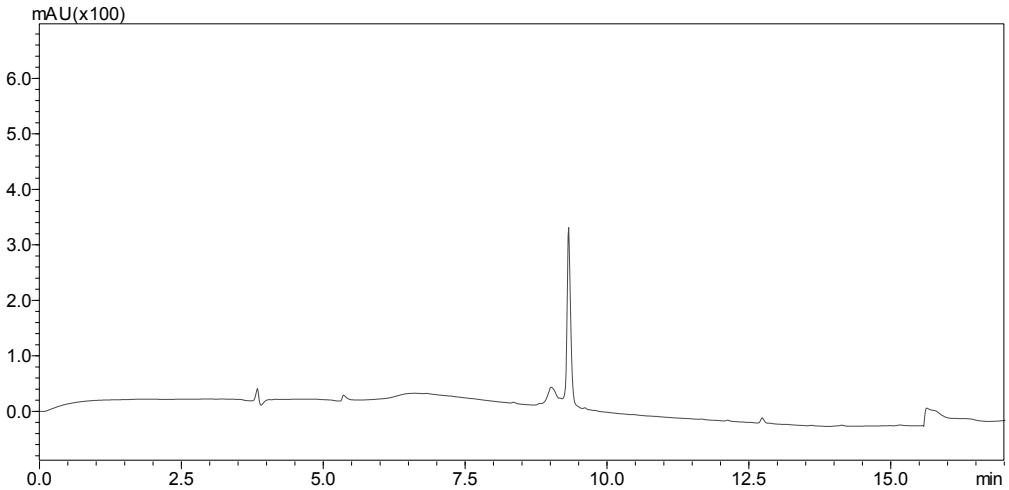
B1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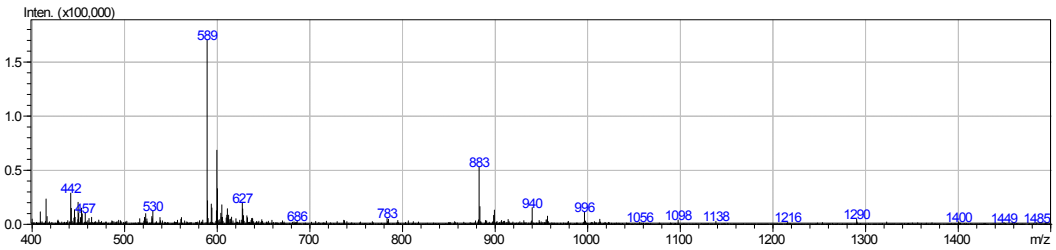
Inten. (x100,000)
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1098
1138
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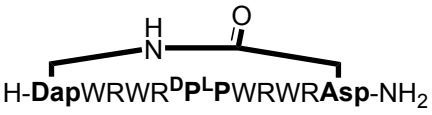
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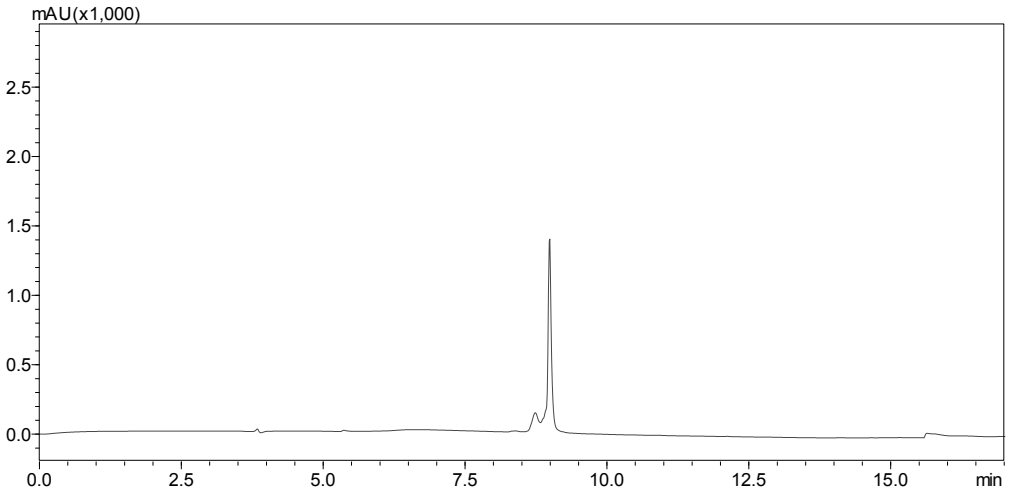
O
H-DapWRWR^{D}P^{L}PWRWRAsp-NH$_2$

B2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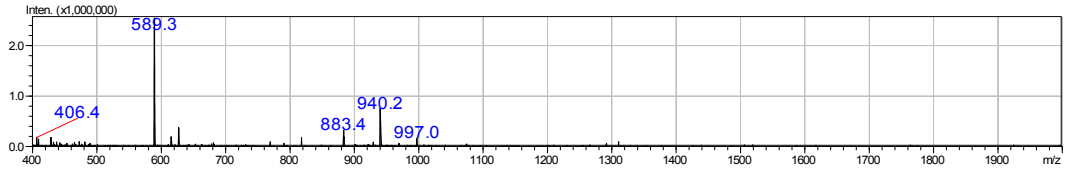
12.5
15.0
min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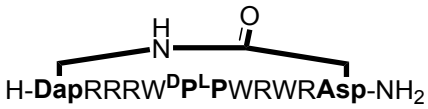
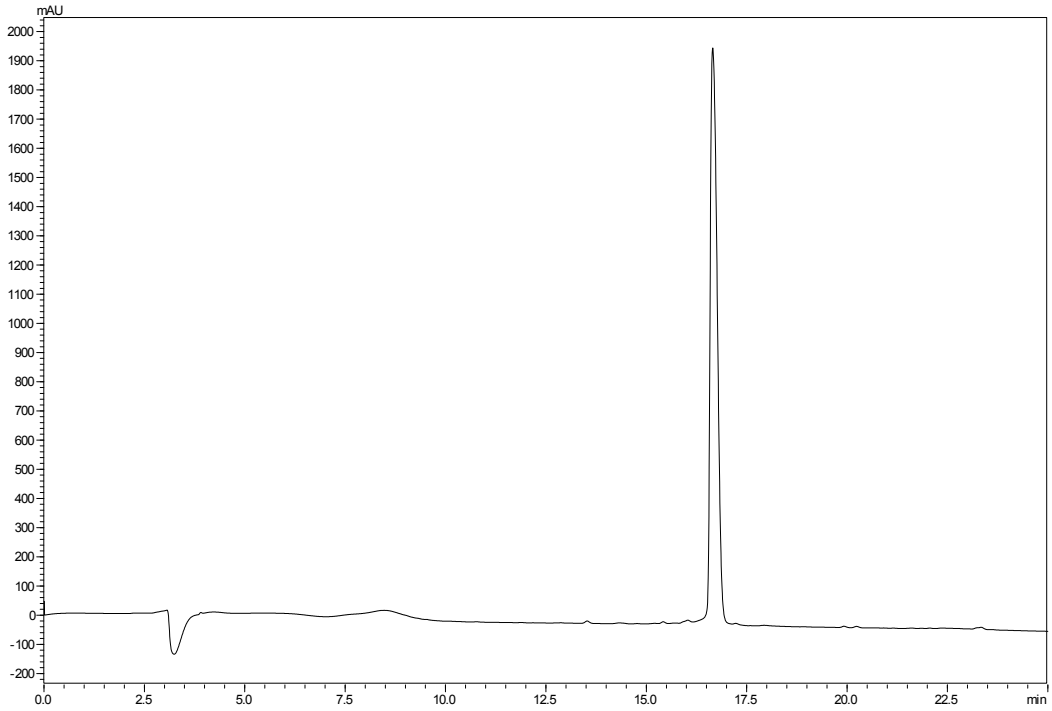
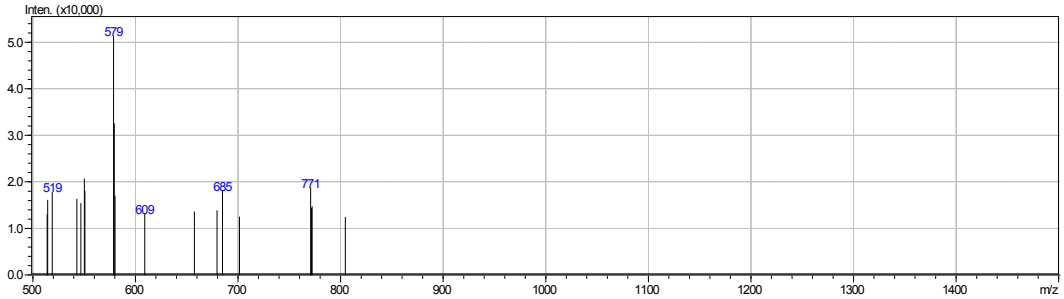
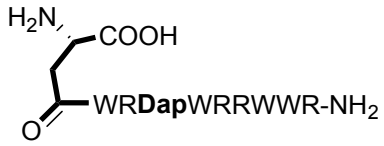
m/z


H
N
O
H-DapRRRW[D]P[L]PWRWRAsp-NH2

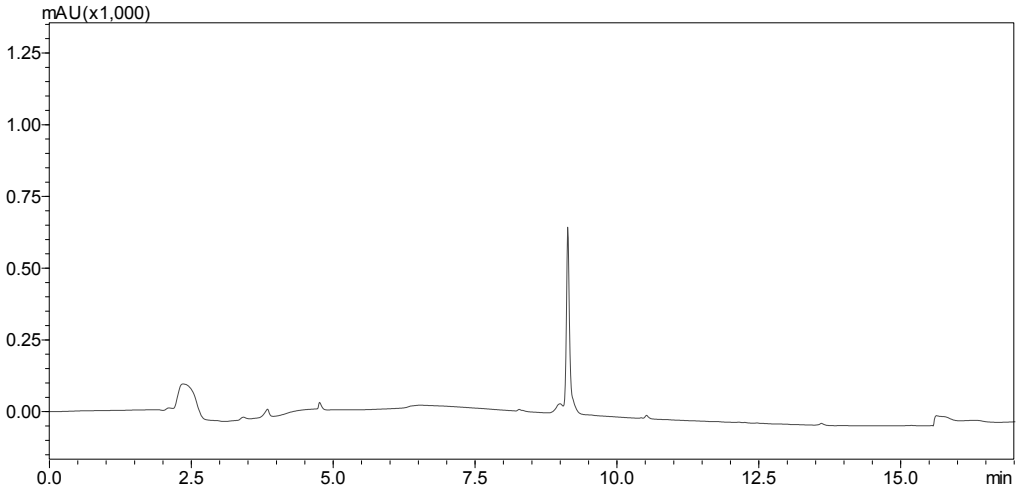
B3

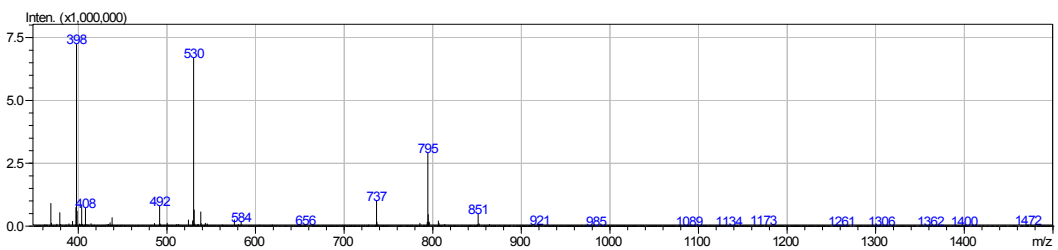

mAU
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
-200
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
min


Inten. (x10,000)
5.0
4.0
3.0
2.0
1.0
0.0
579
519
609
685
771
500
600
700
800
900
1000
1100
1200
1300
1400
m/z


H2N
COOH
WRDapWRRWWR-NH2
O

L1


mAU(x1,000)
1.25
1.00
0.75
0.50
0.25
0.00
0.0
2.5
5.0
7.5
10.0
12.5
15.0
min


Inten. (x1,000,000)
7.5
5.0
2.5
0.0
398
530
795
408
492
584
656
737
851
921
985
1089
1134
1173
1261
1306
1362
1400
1472
400
500
600
700
800
900
1000
1100
1200
1300
1400
m/z