SUPPLMENTARY INFORMATION

Macrocyclic RGD-Peptides with High Selectivity for αvβ3 Integrin in Cancer Imaging and Therapy

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Experimental Procedures

Materials and general methods

Fmoc-protected amino acids and peptide synthesis reagents were purchased from GL Biochem (Shanghai, China). Integrin ligands (fibrinogen and vitronectin) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PEGA resin was purchased from NavoBioChem (0.35 mmol/g, cas: 372109-59-6). Integrin receptor protein ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) was purchased from MERCK. DBCO-Cy5 and DBCO-PEG4-VC-PAB-MMAE were kindly supplied by Professor Huang wei (Shanghai Institute of Materia Medica, Chinese Academy of Sciences). Unless otherwise noted, all chemicals were obtained from commercial sources and used without further purification. Analytical RP-HPLC was performed on Waters E2695 with a C18 column (5 μm, 4.6 mm×250 mm) at 40 ºC. Preparative HPLC was performed on a Waters 1525 with a preparative C18 column (5 μm, 10.0 mm × 250 mm). The column was eluted with a suitable gradient of aqueous acetonitrile containing 0.1% TFA. The enzymatic reaction was monitored using a liquid phase condition as follows: a linear gradient of 20%-60% acetonitrile for 30 min at a flow rate of 1 mL min^{-1}. The product purity was determined using a linear gradient of 10%-90% acetonitrile for 30 min at a flow rate of 1 mL min^{-1}. Solid-phase peptide synthesis was performed on a CEM Liberty Blue peptide synthesizer. Mass spectra were obtained by MALDI-TOF Mass (UltrafleXtreme, Bruker Daltonics; Bremen, Germany) and ESI-MS (Bruker Amazon SL., Germany).

Peptide libraries synthesis

Synthesis of peptides on PEGA resin

For one peptide, 0.05 mmol PEGA resin (loading: 0.35 mmol/g) was swollen and washed with DMF. As the following, Fmoc-protected amino acids were anchored on PEGA resin and protection groups were removed as previously described.[1, 2]

SrtA-mediated on resin peptide cyclization

10 mg dried peptide resin was swollen in 100 μL Srt A reaction buffer (Tris-HCl 0.3 M pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.5 mM mercaptoethanol) for 30 min. SrtA (final concentration: 5 μM) and reaction buffer was added to reach total 200 μL. After the reaction was kept at 37 ºC for 1 h, the reaction mixture was analyzed by HPLC and MALDI-TOF MS (or ESI-MS). The cyclic peptides were purified using preparative HPLC.

Synthesis of 2-c(MMAE)

DBCO-PEG4-VC-PAD-MMAE (20 mg, 0.012 mmol) was dissolved in a mixture of acetonitrile (2 mL) and 50 mM sodium phosphate buffer at pH=7.5 (1 mL) as cyclic RGD peptide 2-c(N_3) (15.5 mg, 0.012 mmol) was added. The mixture was stirred under argon at room temperature for 1 h. the reaction was monitored by HPLC and MALDI-TOF-MS. Finally, the product was separate by semi-preparation column.

SPR

Integrin coating and blocking

A fresh solution of 0.1 M EDC [1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide, EDC] was mixed with an equal volume of 0.4 M NHS (N-Hydroxysuccinimide, NHS). The sensor chip (Series S Sensor Chip CM5, GE Healthcare, USA) was reacted with the mixture for 15 min at flow rate of 30 μL/min. 20 μg/mL $\alpha_v\beta_3$ and 25 μg/mL $\alpha_v\beta_5$ were prepared in 10 mM sodium acetate buffer with pH 4.0 and 4.5, respectively. The $\alpha_v\beta_3$ or $\alpha_v\beta_5$ solution was then injected to the activated surface and left to react for 15 min in 30 μL/min flow. The excess of NHS-esters is deactivated by injecting 1.0 M ethanolamine hydrochloride in pH 8.5 for 15 min. Clean the surface from non-specifically bound molecules with an injection of 2 M NaCl with 0.01 M NaOH solution.

Library screening

A 50 μM macrocyclic RGD peptides and Cilengitide solution were prepared in 1×HBS EP buffer (HEPES buffered saline). The peptides solution was injected to sensor chip to binding at flow rate of 30 μL/min. Reaction conditions: HBS-EP was acted as running buffer and regeneration buffer, and the running contact time and dissociation time was 30 s. Experiments were tested in triplicate. The data was analyzed by Biacore T200 Evaluation Software (Version 2.0，GE Healthcare，USA).

Measurement of Equilibrium Dissociation Constant K_D

Best RGD peptides obtained as described above were measured with K_D. The selected RGD peptides dissolved in 1×HBS EP buffer were diluted into 0.395 μM, 0.78 μM, 1.5625 μM, 3.125 μM, 6.25 μM, 12.5 μM, 25 μM and 50 μM. 200 μL of each

concentration was injected to senor chip and reacted for 15 min at flow rate of 30 μL/min. Each concentration was performed three times. Combination and dissociation curves of each concentration were fitted with a Langmuir model using Biacore T200 Evaluation Software to determine the equilibrium dissociation constant K_D.

Cell adhesion assays

96-well plates were coated with 100 μL integrin solution in coating buffer (5 μg/mL fibrinogen for HEK-293 and SKOV-3, 1 μg/mL vitronectin for HT-29) at 4 ºC overnight. Plates were then blocked with 150 μL/well 1% BSA in PBST (Phosphate Buffered Saline with Tween 20, PBST) buffer at 37 ºC for one hour and followed by 3× washing with 500 μL PBST buffer. Harvested cells were plated to the plates at a given concentration (50000/well for HEK-293 and 30000/well for SKOV-3 and HT-29). A serial dilution of macrocyclic peptides and Cilengitide were added and incubated with cells for two hours at 37 ºC. Non-adhered cells were removed with PBS (Phosphate Buffered Saline, PBS) and followed by adding 20% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT). After incubated for four hours at 37 ºC, 100 μL/well DMSO were added to dissolve the formazan and the optical density was read at 490 nm. Experiments were carried out in triplicate. The adhesion inhibition IC_{50} was calculated using OriginPro 8 software based on the sigmoidal dose-response equation.

Molecular docking

The 3D structure of integrin $\alpha_v\beta3$ was obtained from PDB database (ID: 1L5G).

SWISS-MODEL was applied to build the 3D structure of integrin $\alpha_v\beta_5$ using homology modelling method based on the crystal structure of integrin $\alpha_v\beta_3$. The structure of **2-c** was prepared using the ChemBioDraw software. The geometries of both structures were pre-optimized through molecular mechanics using steepest descent and conjugate gradient. Molecular docking was performed using the Autodock Vina 1.2.0 software. The peptide backbones and integrin were treated as rigid, whereas the peptide side chains were kept flexible using AutoDock Tools. The grid box size was approximately set to 30×40×40 Å to cover the entire binding site and grid center was set to be 20×36×32 Å. In addition, the default values were used for other parameters. The binding free energy of complex was calculated through the Lamarckian genetic algorithm. The best-fitted pose was used for further analysis.

Cellular imaging

SKOV-3 cells were seeded on 14-mm glass-bottom dishes (Solarbio, Beijing, China) in 24-well plates at a density of 2×10^4 cells/well and cultivated for 24 h. The cells were then incubated with 200 μM c-G7RGDLPK(N_3)T and 2-c(N_3) dissolved in growth medium at 37 ºC for 3 h. Subsequently, the glass coverslips were washed two times with DPBS (Dulbecco's Phosphate-Buffered Saline, DPBS, pH=7.5). 20 μM of DBCO-Cy5 were added and allowed to incubate at 37 ºC for one hour. Afterward, the cells were fixed with 4% formaldehyde in DPBS for 20 minutes at room temperature, followed by washing with DPBS two times. The cells were then stained with 2 μg/ml DAPI in DPBS for 10 minutes and finally washed with DPBS five times. Fluorescence

was visualized with an Olympic CKX53 fluorescence microscope (OLYMPUS, Japan). All pictures were acquired using OCULARTM scientific image acquisition software.

Cytotoxicity test

100 μL of SKOV-3 (10000 cells per well) were seeded in 96-well plates for 4 h at 37 ºC/5% CO_2 prior to the experiment. The cells were then incubated with a serial dilution of RGD compounds for 72 h at 37 ºC in a humidified atmosphere of 5% CO_2. Culture medium was used as negative control. After that, 10 μL of CCK-8 (Beyotime, Shanghai, China) solution was added into each well and incubated for another 2 h. The absorbance was recorded at 450 nm using a 1681130 iMark TM Microplate Reader (BIO-RAD) and IC_{50} values were obtained from the dose-response curves using OriginPro 8.0 software. Experiments were carried out in triplicate.

Table S1 Characterization data of the first cyclic peptide library

Compound	Sequence	Theoretical MW	Detected MV	Yield(%)[a]
1-A	c(G_5RGDAVLPET)	1224.277	1225.180 $[M+H]^+$	40
1-F	c(G_5RGDFVLPET)	1300.380	1300.565 $[M+H]^+$	48
1-W	c(G_5RGDWVLPET)	1339.410	1339.576 $[M+H]^+$	56
1-K	c(G_5RGDKVLPET)	1281.372	1281.629 $[M+H]^+$	73
1-Q	c(G_5RGDQVLPET)	1281.328	1281.545$[M+H]^+$	79
1-N	c(G_5RGDNVLPET)	1267.302	1267.374 $[M+H]^+$	59
1-Y	c(G_5RGDYVLPET)	1316.373	1316.774 $[M+H]^+$	69
1-I	c(G_5RGDIVLPET)	1266.357	1266.575 $[M+H]^+$	20
1-H	c(G_5RGDHVLPET)	1290.339	1290.357 $[M+H]^+$	60
1-V	c(G_5RGDVVLPET)	1252.330	1252.144 $[M+H]^+$	27
1-L	c(G_5RGDLVLPET)	1266.357	1267.110 $[M+H]^+$	55
1-T	c(G_5RGDTVLPET)	1254.303	1255.484 $[M+H]^+$	65
1-G	c(G_5RGDGVLPET)	1210.250	1210.901 $[M+H]^+$	74
1-P	c(G_5RGDPVLPET)	1250.314	1250.699 $[M+H]^+$	66
1-E	c(G_5RGDEVLPET)	1282.313	1283.275 $[M+H]^+$	32
1-R	c(G_5RGDRVLPET)	1309.385	1309.564 $[M+H]^+$	42
1-D	c(G_5RGDDVLPET)	1268.286	1268.416 $[M+H]^+$	32
1-C	c(G_5RGDCVLPET)	1256.341	1256.834 $[M+H]^+$	47
1-M	c(G_5RGDMVLPET)	1284.394	1284.785 $[M+H]^+$	56
1-S	c(G_5RGDSVLPET)	1240.276	1240.025 $[M+H]^+$	61
1-k	c(G_5RGDkVLPET)	1281.372	1281.526 $[M+H]^+$	53
1-a	c(G_5RGDaVLPET)	1224.277	1224.636 $[M+H]^+$	50
1-h	c(G_5RGDhVLPET)	1290.339	1291.107 $[M+H]^+$	84
1-y	c(G_5RGDyVLPET)	1316.373	1316.370 $[M+H]^+$	40
1-s	c(G_5RGDsVLPET)	1240.276	1240.759 $[M+H]^+$	47
1-w	c(G_5RGDwVLPET)	1339.410	1339.709 $[M+H]^+$	32
1-e	c(G_5RGDeVLPET)	1282.313	1282.664 $[M+H]^+$	37
1-p	c(G_5RGDpVLPET)	1250.314	1250.654 $[M+H]^+$	20
1-r	c(G_5RGDrVLPET)	1309.385	1309.561 $[M+H]^+$	21
1-n	c(G_5RGDnVLPET)	1267.302	1267.866 $[M+H]^+$	32
1-l	c(G_5RGDlVLPET)	1266.357	1266.185 $[M+H]^+$	34
1-t	c(G_5RGDtVLPET)	1254.303	1254.146 $[M+H]^+$	37
1-m	c(G_5RGDmVLPET)	1284.394	1284.553 $[M+H]^+$	32
1-c	c(G_5RGDcVLPET)	1256.341	1257.197 $[M+H]^+$	45
1-f	c(G_5RGDfVLPET)	1300.380	1300.373 $[M+H]^+$	46
1-q	c(G_5RGDqVLPET)	1281.328	1282.341 $[M+H]^+$	38
1-d	c(G_5RGDdVLPET)	1268.286	1269.844 $[M+H]^+$	37
1-v	c(G_5RGDvVLPET)	1252.330	1253.392 $[M+H]^+$	40
1-i	c(G_5RGDiVLPET)	1266.357	1267.922 $[M+H]^+$	38

Table S2 Characterization data of the second cyclic peptide library

Compound	Sequence	Theoretical MW	Detected MV	Yield(%)[a]
2-S	c(G_5RGDKSLPET)	1269.317	1269.366 $[M+H]^+$	57
2-Q	c(G_5RGDKQLPET)	1310.370	1310.955 $[M+H]^+$	78
2-I	c(G_5RGDKILPET)	1295.399	1295.541 $[M+H]^+$	66
2-L	c(G_5RGDKLLPET)	1295.399	1295.916 $[M+H]^+$	63
2-D	c(G_5RGDKDLPET)	1297.328	1298.696 $[M+H]^+$	77
2-A	c(G_5RGDKALPET)	1253.318	1253.554 $[M+H]^+$	49
2-R	c(G_5RGDKRLPET)	1338.427	1339.856 $[M+H]^+$	63
2-M	c(G_5RGDKMLPET)	1313.436	1313.601 $[M+H]^+$	70
2-T	c(G_5RGDKTLPET)	1283.344	1283.858 $[M+H]^+$	62
2-W	c(G_5RGDKWLPET)	1368.452	1368.931 $[M+H]^+$	56
2-N	c(G_5RGDKNLPET)	1296.343	1296.706 $[M+H]^+$	36
2-H	c(G_5RGDKHLPET)	1319.380	1319.733 $[M+H]^+$	55
2-P	c(G_5RGDKPLPET)	1279.356	1279.459 $[M+H]^+$	65
2-K	c(G_5RGDKKLPET)	1310.413	1310.678 $[M+H]^+$	69
2-F	c(G_5RGDKFLPET)	1329.415	1329.641 $[M+H]^+$	56
2-C	c(G_5RGDKCLPET)	1285.382	1285.859 $[M+H]^+$	47
2-E	c(G_5RGDKELPET)	1311.355	1311.669 $[M+H]^+$	55
2-G	c(G_5RGDKGLPET)	1239.291	1239.578 $[M+H]^+$	37
2-Y	c(G_5RGDKYLPET)	1345.415	1345.747 $[M+H]^+$	66
2-h	c(G_5RGDKhLPET)	1319.380	1319.708 $[M+H]^+$	63
2-k	c(G_5RGDKkLPET)	1310.413	1311.581 $[M+H]^+$	63
2-q	c(G_5RGDKqLPET)	1310.370	1310.651 $[M+H]^+$	55
2-r	c(G_5RGDKrLPET)	1338.427	1338.597 $[M+H]^+$	46
2-s	c(G_5RGDKsLPET)	1269.317	1270.376 $[M+H]^+$	48
2-t	c(G_5RGDKtLPET)	1283.344	1283.676 $[M+H]^+$	45
2-y	c(G_5RGDKyLPET)	1345.415	1345.923 $[M+H]^+$	62
2-a	c(G_5RGDKaLPET)	1253.318	1254.037 $[M+H]^+$	77
2-w	c(G_5RGDKwLPET)	1368.452	1368.449 $[M+H]^+$	30
2-m	c(G_5RGDKmLPET)	1313.436	1313.566 $[M+H]^+$	43
2-e	c(G_5RGDKeLPET)	1311.355	1311.283 $[M+H]^+$	37
2-l	c(G_5RGDKlLPET)	1295.399	1295.745 $[M+H]^+$	74
2-c	c(G_5RGDKcLPET)	1285.382	1286.438 $[M+H]^+$	67
2-n	c(G_5RGDKnLPET)	1296.343	1297.590 $[M+H]^+$	42
2-f	c(G_5RGDKfLPET)	1329.415	1329.686 $[M+H]^+$	55
2-p	c(G_5RGDKpLPET)	1279.356	1280.466 $[M+H]^+$	56
2-d	c(G_5RGDKdLPET)	1297.328	1298.068 $[M+H]^+$	48
2-v	c(G_5RGDKvLPET)	1281.372	1282.324 $[M+H]^+$	38
2-i	c(G_5RGDKiLPET)	1295.399	1295.648 $[M+H]^+$	34

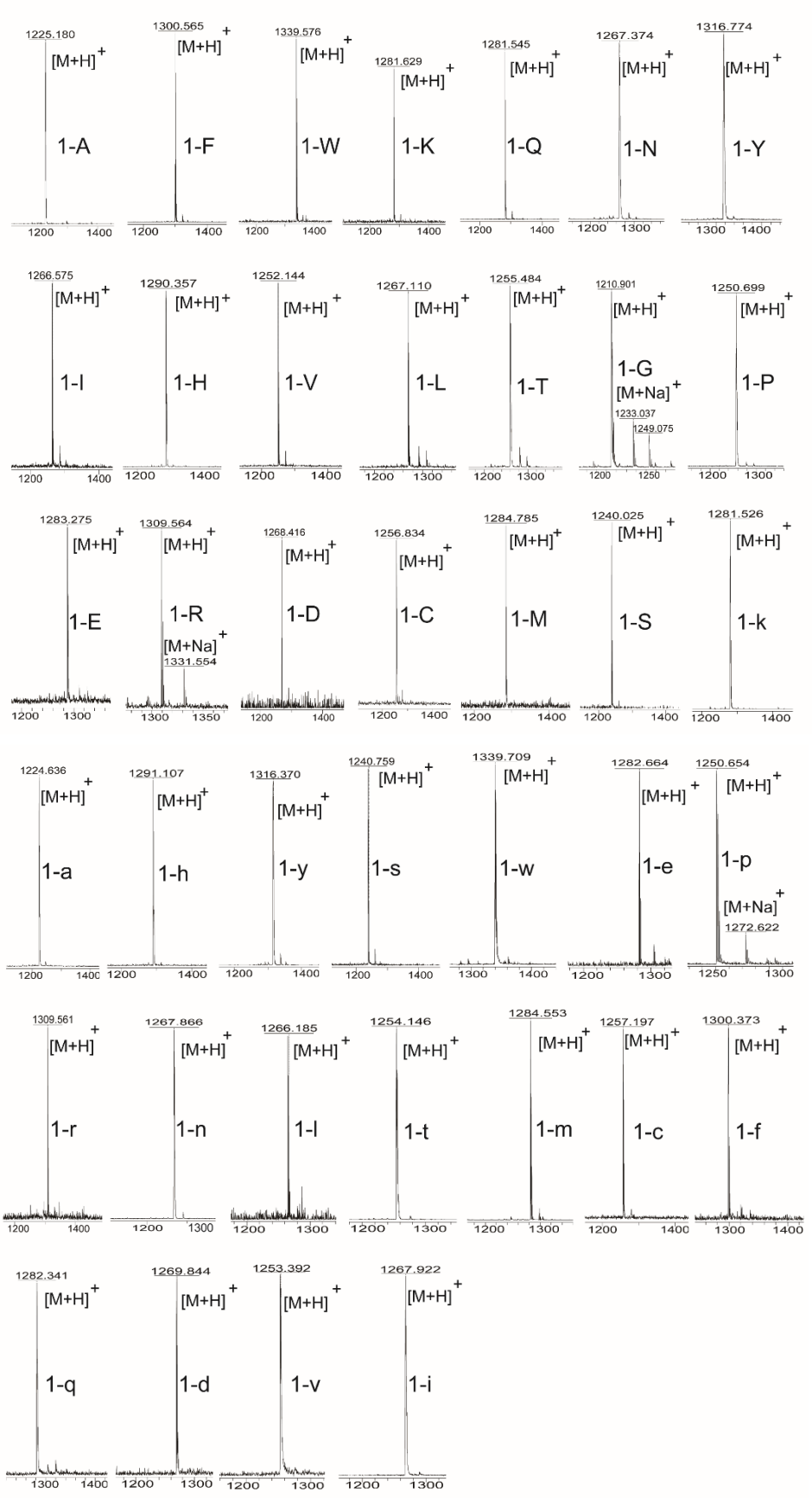

Figure S1. MALDI-TOF-MS spectrum of the first cyclic peptide library.

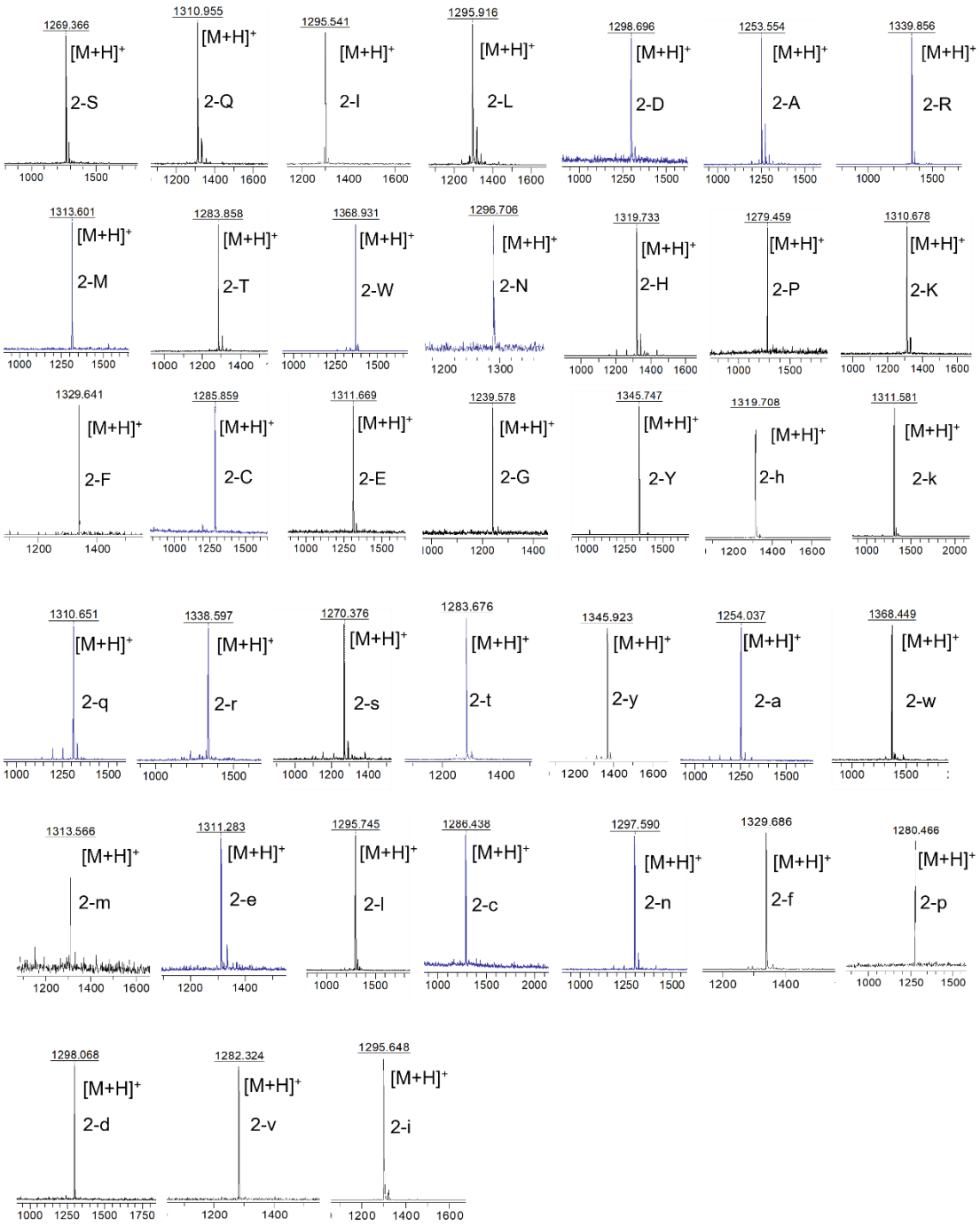

Figure S2. MALDI-TOF-MS spectrum of the second cyclic peptide library.

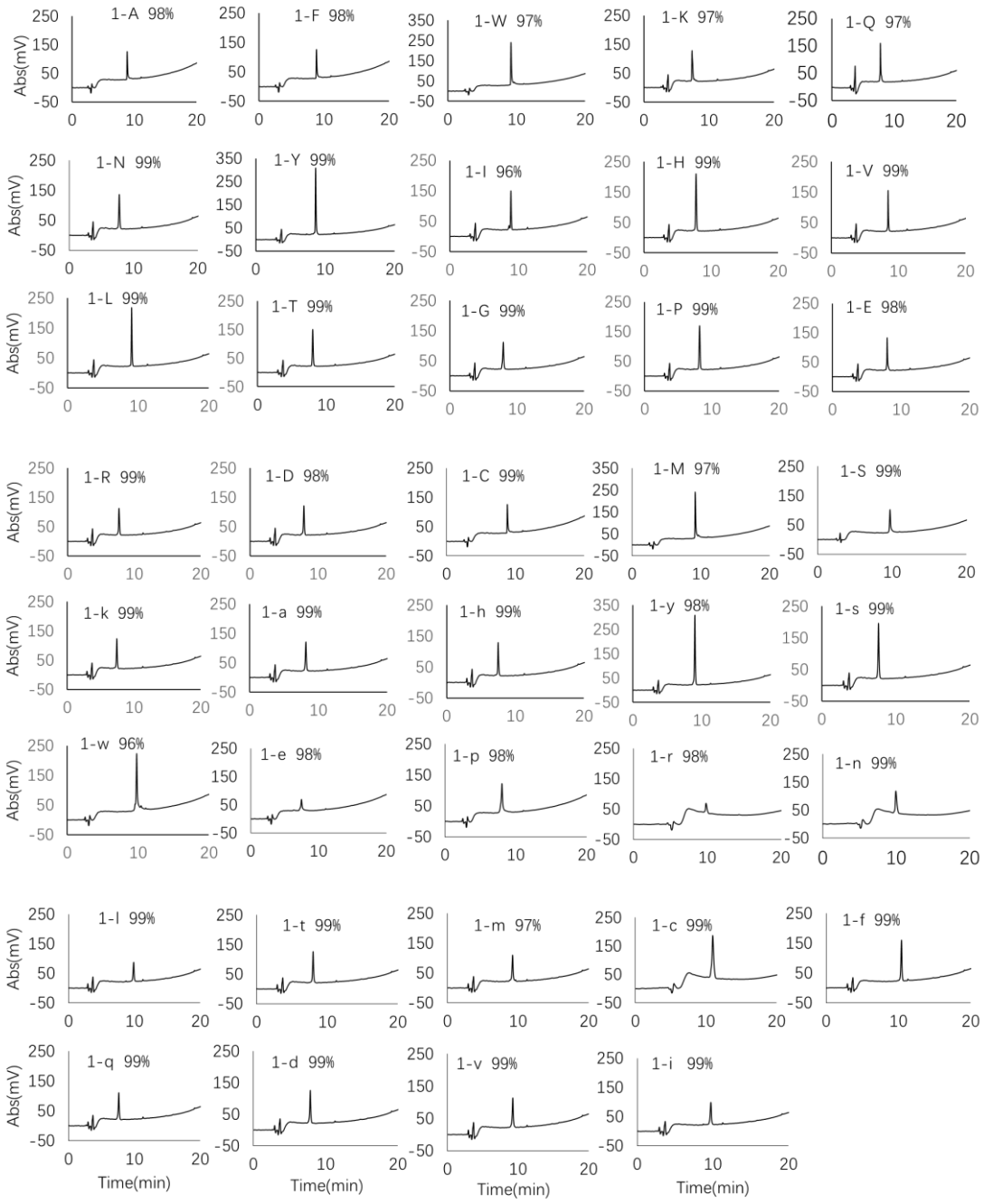

Figure S3. Analytical HPLC traces for the first cyclic peptide library.

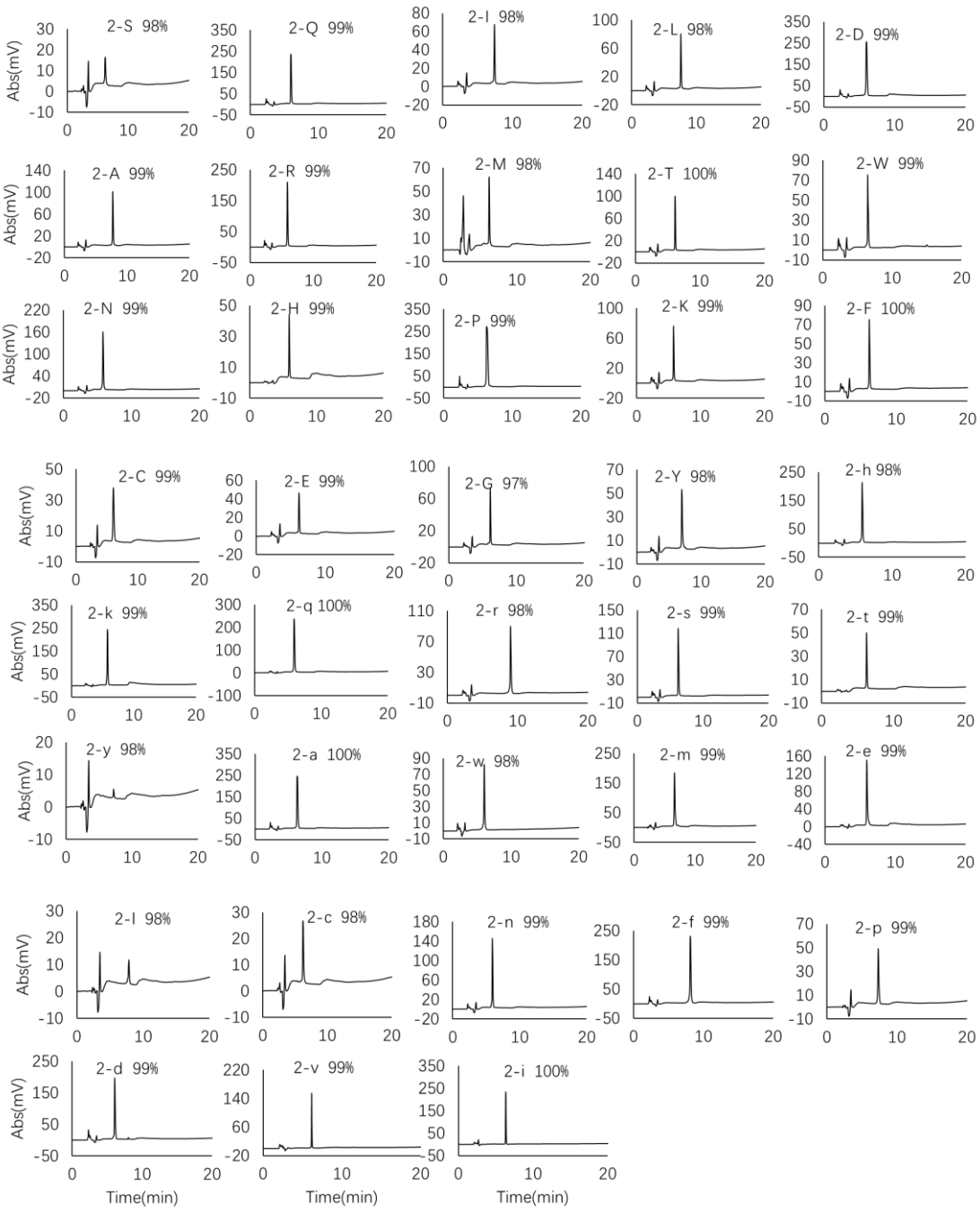

Figure S4. Analytical HPLC traces for the second cyclic peptide library.

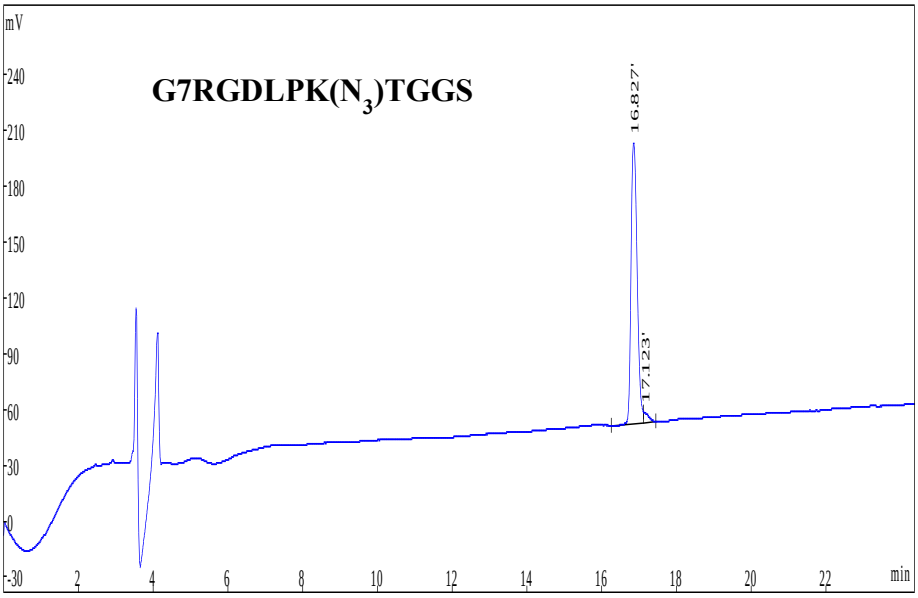

Figure S5. Analytical HPLC traces for compound G7RGDLPK(N_3)TGGS.

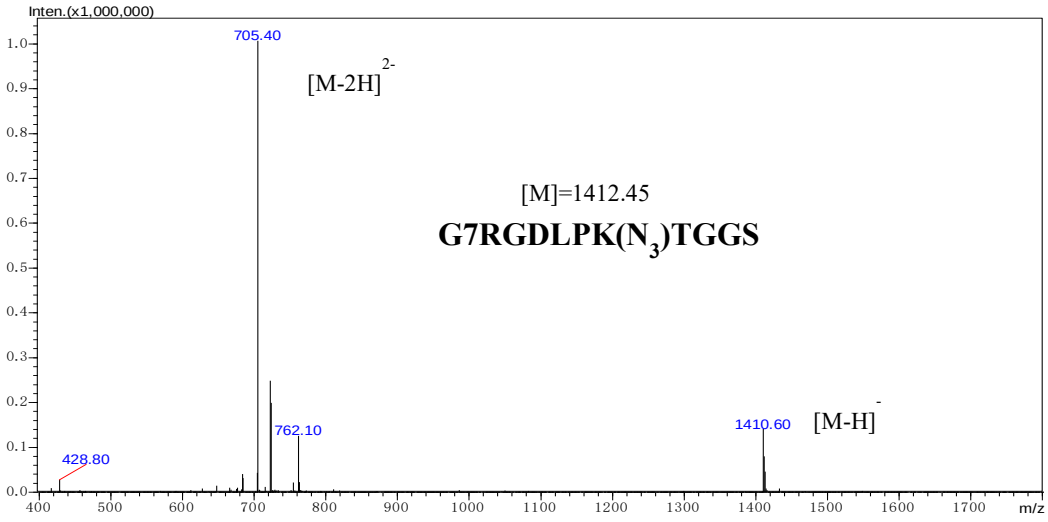

Figure S6. ESI-MS spectrum of compound G7RGDLPK(N_3)TGGS.

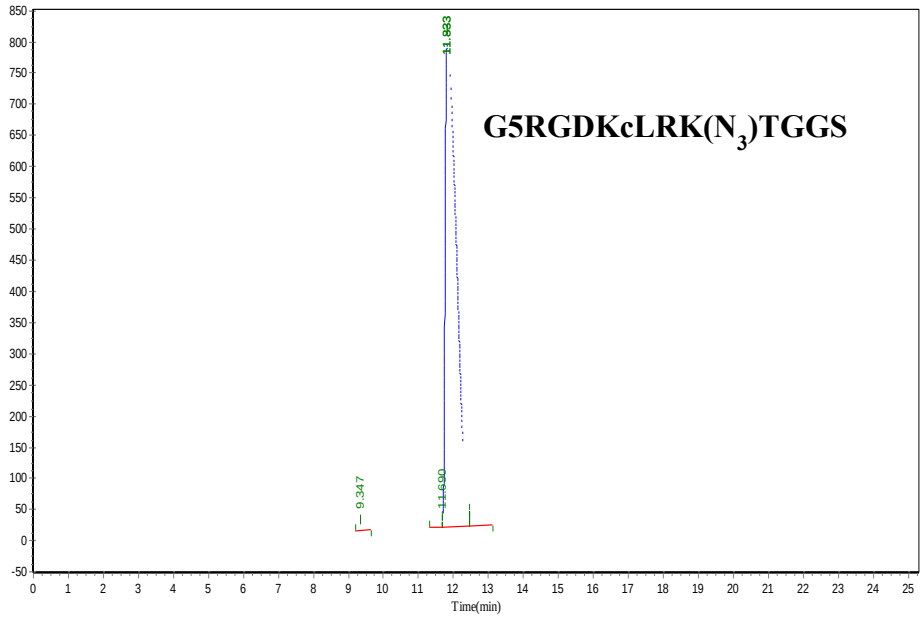

Figure S7. Analytical HPLC traces for compound G5RGDKcLRK(N_3)TGGS.

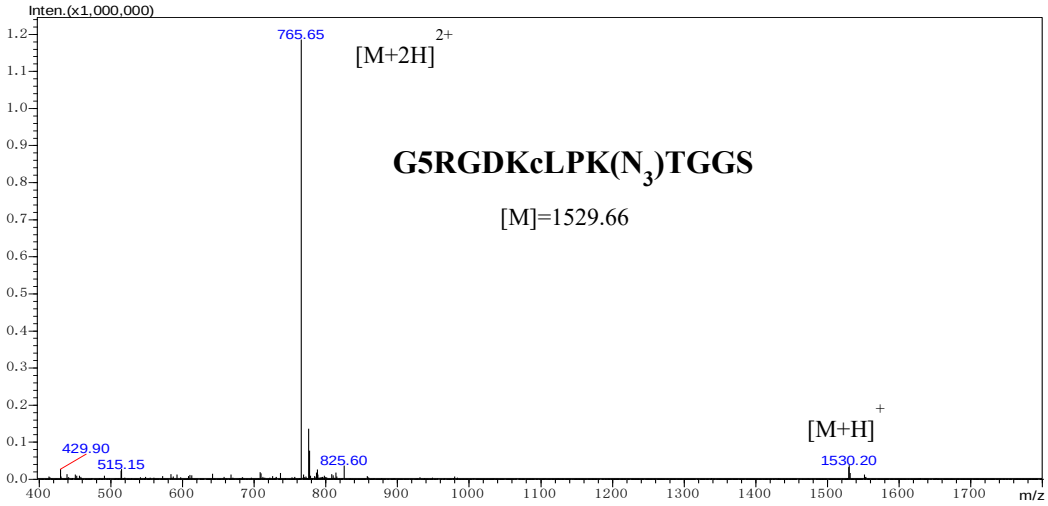

Figure S8. ESI-MS spectrum of compound G5RGDKcLPK(N_3)TGGS.

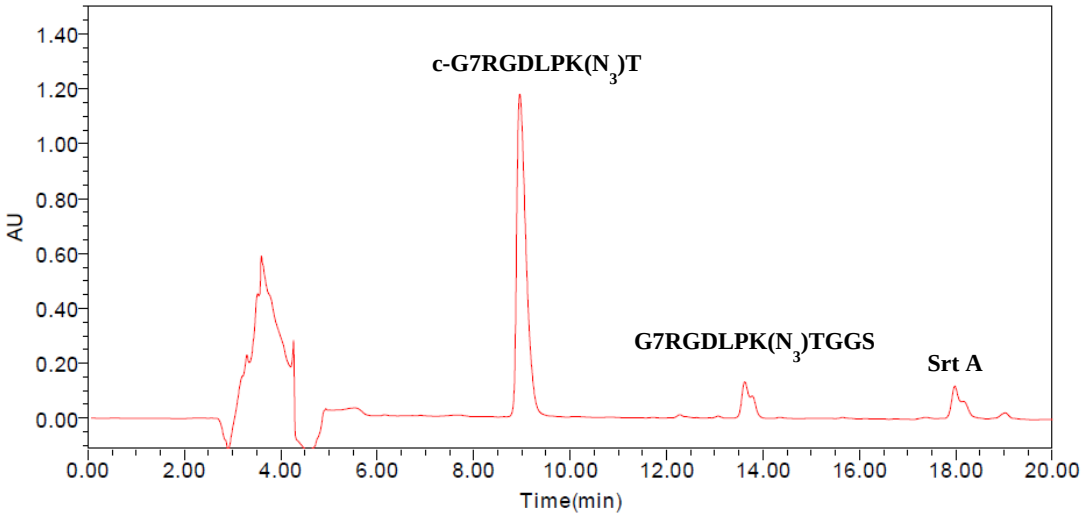

Figure S9. RP-HPLC profile of cyclization of linear G7RGDLPK(N_3)TGGS by Srt A.

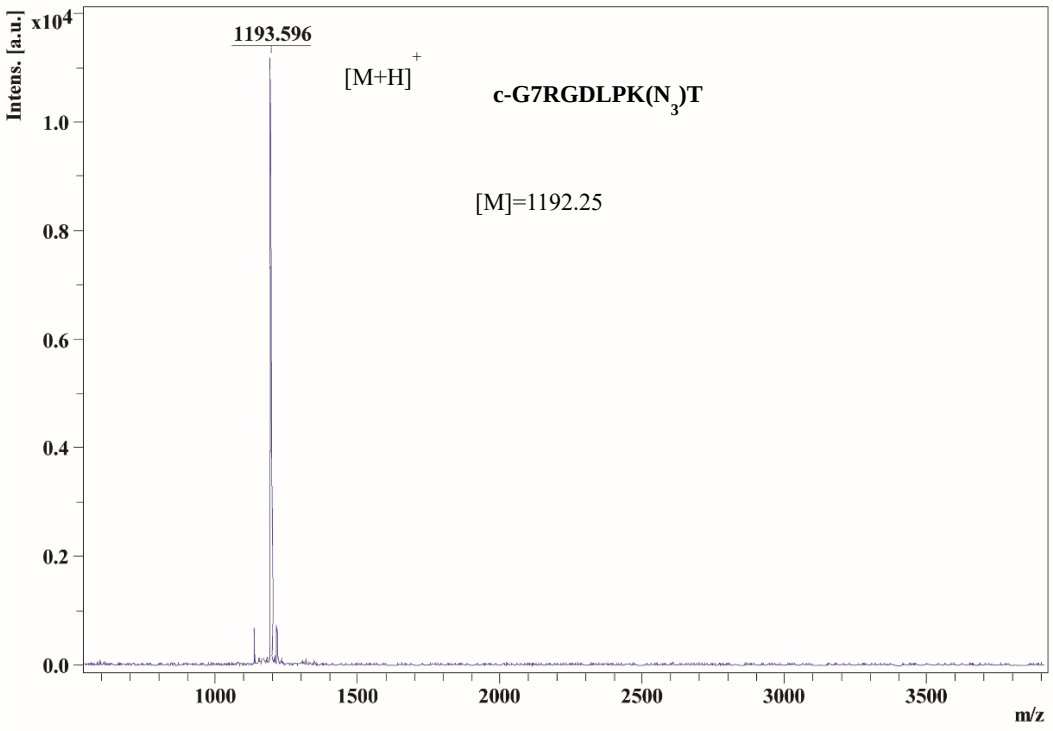

Figure S10. MALDI-TOF analysis of cyclic product of c-G7RGDLPK(N_3)T.

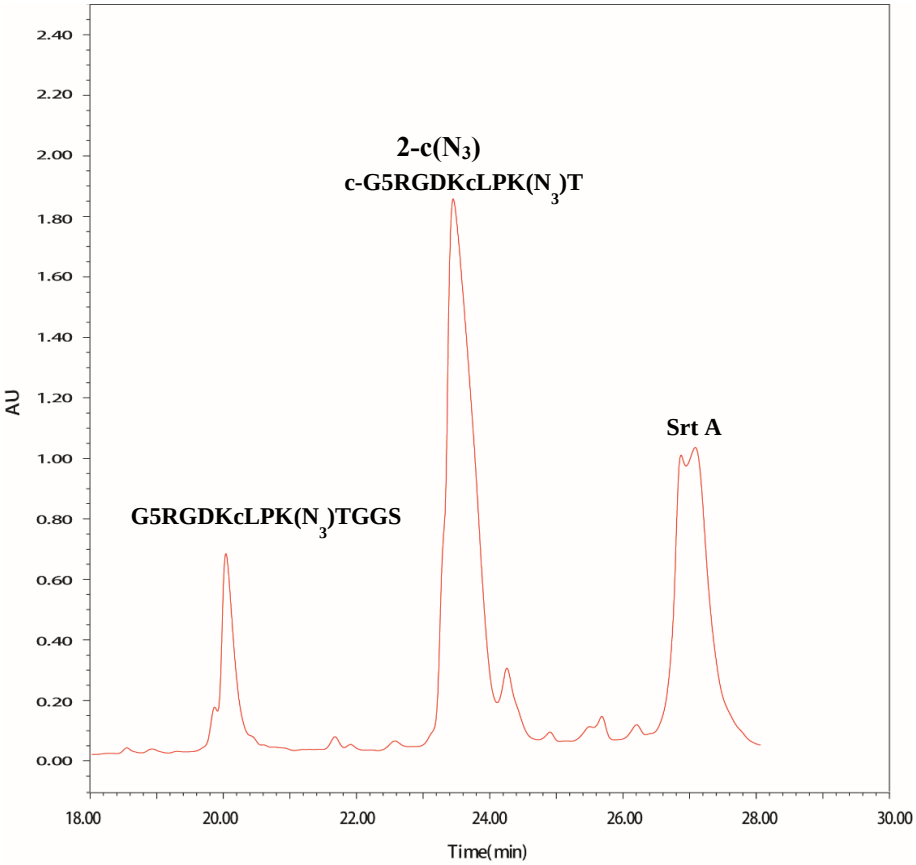

Figure S11. RP-HPLC profile of cyclization of G5RGDKcLRK(N_3)TGGS by Srt A.

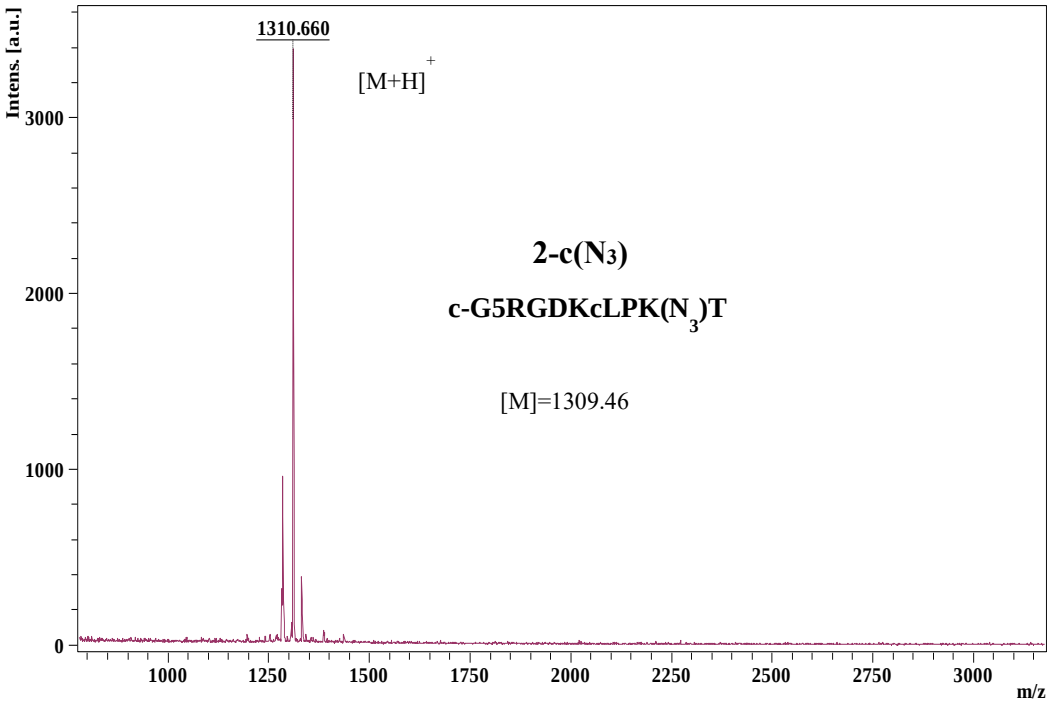

Figure S12. MALDI-TOF analysis of cyclic product of 2-c(N_3).

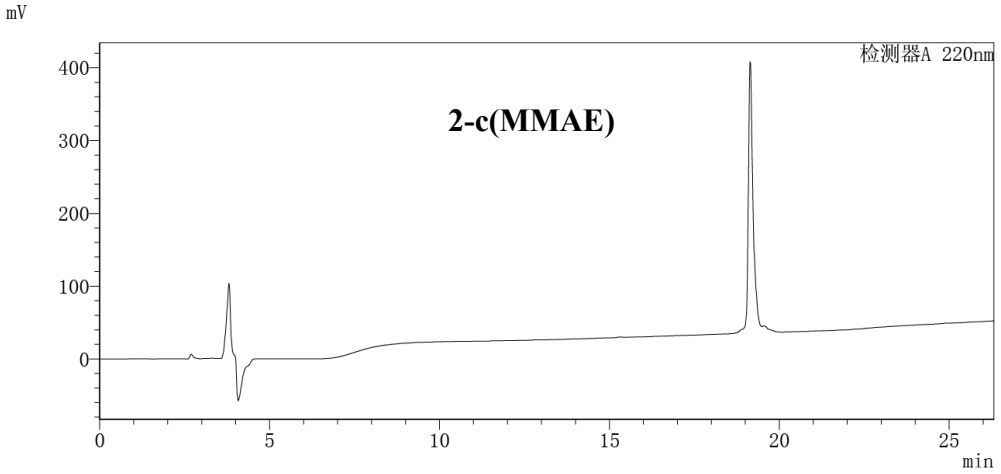

Figure S13. RP-HPLC profile of 2-c(MMAE) .

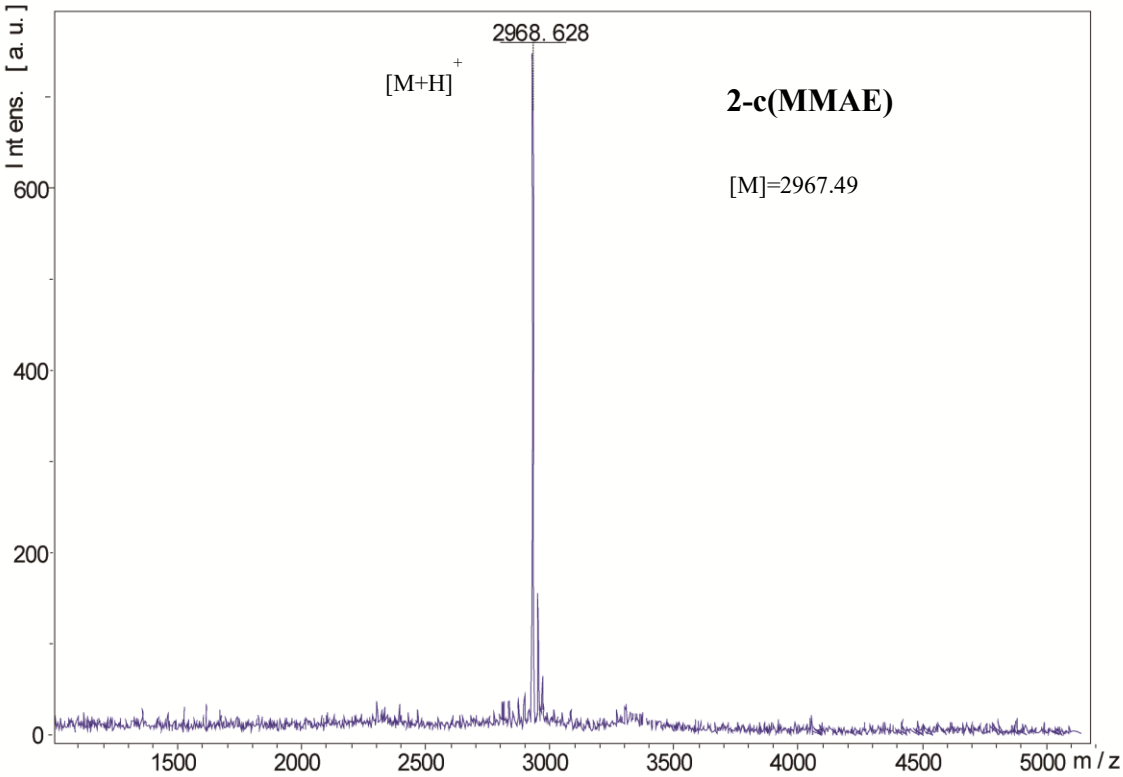

Figure S14. MALDI-TOF analysis of 2-c(MMAE).

references

1. X. Z. Cheng, T. Zhu, H. F. Hong, Z. F. Zhou and Z. M. Wu, *Org. Chem. Front.*, 2017, **4**, 2058-2062.
2. X. Z. Cheng, H. F. Hong, Z. F. Zhou and Z. M. Wu, *J. Org. Chem.*, 2018, **83**, 14078-14083.